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Valorisation of corn fibre: optimisation of an integrated process to produce bio- vanillin

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ABSTRACT

The work presented in this PhD thesis aims at developing an integrated process that maximises the potential corn fibre by-products to be used as bio-vanillin production. The development of this process compromised in first step, recovery ferulic acid (substrate for bio-vanillin production) from corn fibre. A mild alkaline extraction to recovery ferulic acid from wet corn fibre was optimised, resulting an extract with a concentration of $2.10 \pm 0.09 \text{ g.L}^{-1}$ and a recovery yield of $11.14 \pm 1.00 \text{ Kg ferulic acid.Ton}^{-1} \text{ dry corn fibre}$ (in pilot scale). The optimised seven-hour process uses a low concentration of sodium hydroxide (0.25 M), a mild temperature (30° C) and a liquid-to-solid ratio of $10 \text{ g solvent.g}^{-1} \text{ dry fibre}$. Alternatively, an enzymatic hydrolysis using available commercial enzyme preparations was studied. However, even with a hydrothermal pre-treatment before the enzymatic hydrolysis, the yield of ferulic acid was lower than in mild alkaline extraction.

The ferulic acid released by alkaline extraction was successfully separated from the arabinoxylans (add-value compounds, with high viscosity), by ultrafiltration using the membrane Nadir® UP150. Through a mathematical modelling, involving the response surface methodology (RMS), it was possible to determine that the conditions of ultrafiltration (temperature, initial cross-flow velocity and transmembrane pressure) that led to the maximisation of ultrafiltration performance, with values of minimised of ferulic acid rejection and with maximised values of arabinoxylans rejection and permeabilities. The adsorption process with the Macronet® MN102 resin allowed the elimination/reduction of compounds with antimicrobial effect from ultrafiltrate alkaline extract. These compounds were having a negative effect on the bioconversion of ferulic acid into vanillin by the *Amycolatopsis* sp. ATCC 39116. The study of the adsorption process in columns continuous allowed to determinate the parameters for scale up the process. The bio-vanillin production by *Amycolatopsis* sp. ATCC 39116 was studied using synthetic ferulic acid as a precursor, aiming at achieving maximised overall vanillin productivities. It is also compared the bio-vanillin production using commercial and ferulic acid extracted from corn fibre after the purification. Similar synthetic parameters were obtained using both types of ferulic acid, except vanillin yield, that was inferior using ferulic acid extracted from corn fibre, when compared with synthetic ferulic acid.

To obtain purified vanillin from the bioconversion broth, organophilic pervaporation and vacuum distillation processes were studied and compared. In both process was possible to recover vanillin, without the presence of undesirable contaminants. Vacuum distillation with consecutive pulses was preferred over pervaporation due to the higher vanillin fluxes obtained: $2.7 \pm 0.1 \text{ g.m}^{-2}\text{h}^{-1}$ for vacuum distillation compared to $1.19 \pm 0.01 \text{ g.m}^{-2}\text{h}^{-1}$ for pervaporation, although the highest energy consumption in vacuum distillation. Concerning the economic assessment of the bio-vanillin plant (20 ton/ano), the payback period calculates was 0.9 years.

Keywords: Corn fibre, Ferulic acid, *Amycolatopsis* sp. ATCC 39116, Vanillin

RESUMO

O trabalho apresentado nesta tese de doutoramento visa desenvolver um processo integrado para a produção de bio-vanilina a partir da fibra de milho, subproduto da indústria do amido, representando uma valorização deste subproduto. A primeira etapa do processo envolveu a recuperação de ácido ferúlico (substrato na produção de bio-vanilina) da fibra de milho. Através de uma extração alcalina da fibra de milho, com condições operacionais suaves, à escala piloto, foi possível obter um rendimento maximizado de $11.14 \pm 1.00 \text{ kg}_{\text{ácido ferúlico}} \cdot \text{Ton}_{\text{fibra seca}}^{-1}$. Este valor de rendimento foi obtido através da seleção das condições operacionais da extração alcalina: tempo de extração de 7 horas, uma solução aquosa de hidróxido de sódio (0.25 M), uma temperatura de 30° C e uma razão solido-líquido de 10 g solvente.g⁻¹ fibra seca. Alternativamente, foi estudada uma hidrólise enzimática usando preparações enzimáticas comerciais. Contudo, mesmo com a aplicação de um pré-tratamento hidrotérmico, o rendimento de ácido ferúlico foi inferior quando comparado com a extração alcalina.

O processo de ultrafiltração utilizando a membrana Nadir® UP150, permitiu a eficaz separação do ácido ferúlico dos arabinoxilanos (compostos de valor acrescentado, com grande viscosidade), presentes no extrato alcalino. A partir de um modelo matemático, envolvendo a metodologia de superfície de resposta (RSM), foi possível determinar que as condições da ultrafiltração (temperatura, velocidade de fluxo cruzado inicial e pressão transmembranar) que levaram a uma maximização da performance da ultrafiltração, com valores minimizados de rejeição de ácido ferúlico, valores maximizados de rejeição de arabinoxilanos e permeabilidades. O processo de adsorção com a resina Macronet® MN102 permitiu a eliminação/redução de compostos com efeito antimicrobiano, cuja presença influencia negativamente a bioconversão de ácido ferúlico em vanilina pela *Amycolatopsis* sp. ATCC 39116. O estudo do processo de adsorção em contínuo em colunas de adsorção, permitiu determinar os parâmetros de dimensionamento do processo. A produção de bio-vanilina pela bactéria *Amycolatopsis* sp. ATCC 39116 foi estudada utilizando ácido ferúlico sintético como precursor, com o objetivo de maximizar a produtividade da vanilina. Foi também comparada a produção de bio-vanilina utilizando o ácido ferúlico sintético e ácido ferúlico extraído da fibra de milho. Os parâmetros cinéticos foram semelhantes para os dois tipos de ácido ferúlico, exceto o rendimento de vanilina que foi inferior a partir de ácido ferúlico extraído da fibra de milho, quando comparado ao ácido ferúlico sintético.

Por fim, foi estudada a recuperação de bio-vanilina do caldo de bioconversão por pervaporação e por destilação em vácuo. Em ambos os processos, foi possível recuperar vanilina sem contaminantes indesejáveis. A destilação em vácuo com pulsos consecutivos foi selecionada devido a apresentar maiores fluxos de vanilina: $2.7 \pm 0.1 \text{ g} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ em comparação com $1.19 \pm 0.01 \text{ g} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ na pervaporação, apesar de a destilação em vacuum apresentar um maior consumo de energia. Em relação ao estudo económico do processo de produção de bio-vanilina (20 ton/ano) obteve-se um retorno de 0.9 anos.

Palavras-chave: Fibra de milho, Ácido ferúlico, *Amycolatopsis* sp. ATCC 39116, Vanilina

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LIST OF ACRONYMS

Abbreviation

AAPH - 2, 2'-azobis (2-amidino-propane) dihydrochloride

DMEM -Dulbecco's modified Eagle medium

BV - bed volumes

CA – p-coumaric acid

Capex - capital expenditure

CAR/DVB/PDMS - carboxen/divinylbenzene/polydimethylsiloxane

CDW - cell dry weight

CF - concentration factor

COD - chemical oxygen demand

DoE - design of experiments

FA - ferulic acid

FBS - fetal bovine serum

FL - disodium fluorescein

GAX - (glucurono)arabinoxylans

GC-MS - gas chromatography/mass spectrometry

HMF - 5-hydroxymethylfurfural

HPLC - high-performance liquid chromatography

HS-SPME - headspace solid-phase microextraction extraction

ICP-MS - inductively coupled plasma mass spectrometry

L-DOPA - l-3,4-dihydroxyphenylalanine

LRI - linear retention index

LRI* - linear retention index in the literature

NEAA - non-essential amino acids

NMR- nuclear magnetic resonance

PCA - protocatechuate 4,5 – cleavage

PBS - phosphate buffer saline

PDMS - polydimethyl siloxane

PEBA - polyether block amine

PES - polyethersulfone

PESH - hydrophilic polyethersulfone

PSH - hydrophilic polysulfone

RI - refractive index

RC - regenerated cellulose

RSM - response surface methodology

SD - standard deviation

UF - ultrafiltration

TCA - tricarboxylic acid

TEAC - trolox equivalent antioxidant capacity

TFA - trifluoroacetic acid

UV - ultraviolet

Variables

A - area (m^2)

C - concentration (g.L^{-1}) or (g.kg)

D - desorption percentage (%)

E - efficiency (%)

EC_{50} – Half maximal effective concentration (mg. mL^{-1})

$\text{Log } R_o$ – combined severity factor (-)

J - mass flux ($\text{g.m}^{-2}\text{h}^{-1}$)

m - mass (kg or g)

MWCO - Molecular weight cut-off (kDa)

ORAC -oxygen radical absorbance capacity ($\mu\text{mol}_{\text{TEAC}} \cdot \text{mg}^{-1}$)

p - partial pressure (Pa)

P- permeance ($\text{L} \cdot \text{m}^{-2} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$)

q - adsorption capacity ($\text{mg} \cdot \text{g}_{\text{resin}}^{-1}$)

q_P - specific vanillin productivity ($\text{g}_{\text{product produced}} \cdot \text{g}_{\text{biomass}}^{-1} \cdot \text{h}^{-1}$).

Q - volumetric flow rate ($\text{L} \cdot \text{h}^{-1}$ or $\text{mL} \cdot \text{min}^{-1}$)

R - global rejection of each compound (%)

RT - retention time (min)

r_p - volumetric productivity

r_v - rate of vanillin production ($\text{g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$)

t - time (s, min or h)

T - temperature ($^{\circ}\text{C}$ or K)

V - volume (L)

v - velocity ($\text{m}^2 \cdot \text{s}^{-1}$)

w - weight (g or kg)

x - the molar fraction in feed side

y - the molar fraction in permeate side

Y - yield ($\text{g} \cdot \text{g}^{-1}$ or %)

Greek symbols

Δp - transmembrane pressure (bar)

δ - thickness (m)

μ - viscosity ($\text{mPa} \cdot \text{s}$)

Subscripts

0 - initial

ads - adsorption

b - breakthrough

condens - condenser

D - dry

e - equilibrium

feed - feed side

i - compound i

perm - permeate side

P/S - product/ subtract

retentate - retentate side

van - vanillin

sat - saturated

w - water

W - wet

1. Background and Motivation

1.1. Background and Motivation

Natural vanilla is a complex mixture of compounds (more than 200 [1]) extracted from the cured beans of two different species of vanilla orchids: *Vanilla planifolia* and *Vanilla tahitensis* [2]. The major aromatic components of vanilla flavour are vanillin, vanillic acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, *p*-hydroxybenzyl alcohol and vanillyl alcohol. Of these, the most important contributor to its characteristic aroma is vanillin [3,4], with a concentration of about 2% w/w in cured vanilla pods, see Table 1.1.

Table 1.1. Major aromatic components of cured Vanilla pod [2].

Aromatic Components	g.kg ⁻¹ (dry weight basis)
Vanillin	20
Vanillic acid	1
<i>p</i>-hydroxybenzaldehyde	2
<i>p</i>-hydroxybenzoic acid	0.2

Vanillin is the world's most popular flavour and is used in a wide range of products, especially in the food and beverage industries (45 %) but also in perfumes and cosmetics (38 %) [5]. Moreover, 17 % of vanillin produced is used as a product intermediate in synthesis processes in chemical and pharmaceutical industries [5] for the production of antifoaming agents, herbicides and even medications such as aldomet, L-DOPA, dopamine and papaverine. Moreover, its antimicrobial and antioxidant properties find use in food preservative [6,7].

Market prices for natural vanillin extracted from vanilla orchids are high and varies considerably ranging, it can reach to values between 10,000-25,000 €/kg [9,10]. Many factors contribute to this fluctuation. The plant has extremely low yields [1] and unpredictable availability resulting from weather conditions, crop dependence and political instability. What is more, production is slow and laborious. First of all, the flowers must be hand pollinated and then green vanilla pods must be cured for several months [2]. Natural vanillin is, therefore, a luxury product that accounts for less than 1 % of the vanillin flavour market [1,9].

In order to satisfy the increasingly high demand, a synthetic vanillin is produced. Approximately 85 % of synthetic vanillin is produced using the petrochemical substrate guaiacol, and most of the rest comes from lignin [11]. There are several methods for synthesising vanillin from guaiacol, the most significant of which is that developed by Rhodia (now Solvay), a two-step process in use since the 1970's [1,12], which has the advantage of avoiding side-products and thus costly separations [12]. The disadvantage of this process is that it is dependent on petroleum-derived compounds.

Chapter 1 – Background and Motivation

Vanillin production from lignin uses nitrobenzene or copper oxide as a catalyst to degrade lignin mixed with sodium or calcium hydroxide at elevated temperatures and pressures [2,10,12], after which intense purification from accompanying contaminants is required [2]. Although lignin is a natural raw material, the vanillin produced is considered synthetic, due to the extensive use of organic solvents and the elevated temperature and pressure required [9]. Synthetic vanillin is rather cheap, with prices ranging between 9 – 20 €/Kg [5].

Nowadays consumer interest and market demand is growing for healthy, natural food products, with giant food companies such as General Mills, Hershey's, Kellogg's, and Nestlé promising to eliminate artificial flavours from many food products [9]. However, global production of vanillin from vanilla orchids cannot meet this increasing demand with acceptable quantity at an affordable price. This has created a need for alternative sources to natural vanillin that might offer quality levels comparable to those of botanically sourced compounds [1,9].

Biotechnological processes have aroused the interest of the chemical industry, due to their promise to replace synthetic products with bio-based alternatives that might be labelled as natural, according to US and the European food legislation (U.S Food and Drug Administration and Regulation (EC) No 1334/2008, respectively). According to the European Union, a substance can be labelled as natural if it is “obtained by appropriate physical, enzymatic or microbiological processes from a material of vegetable or animal origin” [13]. In the United States, natural flavour “means the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating or enzymolysis, which contains the flavouring constituents derived from a spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof, whose significant function in food is flavouring rather than nutritional” [14].

Biotechnological products have several advantages, such as, carrying the natural label, not depending on agricultural conditions (climate and seasonality), their capacity to be produced in controlled and sustainable industrial processes, which may be highly specific to the substrate or to the product and are easily performed using safe and renewable feedstocks [15,16].

Table 1.2 summarises the main characteristics of the three types of commercially available vanillin: the natural one extracted from orchids, the synthetic, and the biotechnological, or bio-based one, legally defined as natural.

Chapter 1 – Background and Motivation

Table 1.2. Main characteristics of the three types of vanillin commercially available, (adapted from Eswaran 2017) [5].

Type of vanillin	Feedstock	Process time	Yield Rate (%)	Natural Label	Price /kg (€)
Natural	Vanilla beans	Months	<2	√	10,000-25,000 €/kg [9,10]
Synthetic	Guaiacol, Lignin	Hours	92-95	X	9 – 20 [5]
Bio-based (Biotechnological)	Rice bran, clove oil, Sugars	Days	55-60	√	450-900 [10,17,18]

Bio-vanillin is more expensive than the synthetic form but cheaper than the natural product, while it is well accepted by consumers due to its excellent flavouring properties. Therefore, the bio-vanillin industry is expected to grow [19]. It was valued at US\$ 150 million in 2018 and is expected to reach US\$ 400 million by the end of 2025 [20]. This explosion in demand has led to the growth in production capacity. In late 2019, it was announced that Solvay would increase production capacity to 120 tonnes, after a rise from 20 to 60 tonnes in 2017 [21].

A wide range of bio-vanillin's are currently available on the market produced by a variety of processes and which already offer reliable, better-quality alternatives to synthetic vanillin. The first bio-vanillin was launched on the commercial market in the early 2000's by Solvay under the name Rhovanil® Natural CW. It was produced from the bioconversion of ferulic acid extracted from rice bran by an unmodified strain [9,22], preferably by *Amycolatopsis* sp. ATCC 39116, according to Rhodia's (Solvay) patent [23]. The initial trading price of approximately 640 €/kg [10,24] then rose at least 15 % in 2017 [10,25]. Today, Evolva® and International Flavours and Fragrances are two key players in the global bio vanillin market [19] selling vanillin synthesised from glucose using genetically modified microorganisms [9]. These microorganisms, however, led some environmental groups to claim that this type of vanillin is artificial [19,26]. Another process by De Monchy Aromatics uses the bioconversion of ferulic acid from rice bran oil [27] to produce bio-vanillin, while Mane obtains bio-vanillin by the bioconversion of eugenol from clove oil [9].

The best-explored precursor for bio-vanillin production is ferulic acid [1,28]. It is a naturally occurring compound found in plant cell walls and is a common bio-product of wheat/rice/corn bran, oat hulls and corn fibre. Tremendous economic potential exists, therefore, to combine bio-vanillin production with the valorisation of these cereal by-products (through the purification and/or enrichment of the ferulic acid present in these feedstocks). This valorisation will result not only from current low-value feed applications but also as low-value sources of ferulic acid for bio-vanillin production, in line with a

Chapter 1 – Background and Motivation

circular economy approach. The ready availability and relatively stable composition of these by-products make them high potential feedstocks for large-scale biorefineries [29].

Figure 1.1 illustrates the different fractions of the corn kernel obtained during wet milling process to produce starch, where 8.3-11.9 % dry basis is corn fibre [30].

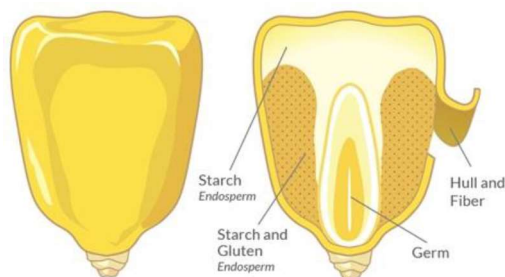


Figure 1.1. Constituents of corn kernel [31].

Corn fibre is normally used for animal feed, however it is one of the most abundant natural sources of ferulic acid [32], as it is composed of approximately 1.5-3 % of ferulic acid [33–36]. The significantly greater content of ferulic acid in corn fibre means it has great potential as profitable feedstock for bio-vanillin production and also promises great enhancement of sustainability and competitiveness for the starch industry [29].

This research project aims to contribute to the development of a sustainable, integrated process for maximising the potential of using ferulic acid sourced from corn fibre as a precursor in bio-vanillin production. The motivation was: 1) to use corn fibre due to its greater ferulic acid content than the feedstocks currently in industrial use; 2) the valorisation of corn fibre obtained as a co-product in starch industry, 3) to optimise each step and integrate them, assuring an economic and sustainable process. The extraction of ferulic acid from agro-industrial by-products can be performed either by enzymatic [35,39–41] or chemical (acid or alkaline) [42–45] extraction and/or hydrolysis. Enzymatic hydrolyses provides an eco-friendly alternative to chemical treatment [35]. However, due to the complex structure of lignocellulosic materials in general, and the specific structure of corn fibre [34,46,47], a synergy between several enzymes such as ferulic acid esterase, xylanase, arabinoxylanase and xylosidases is required to release ferulic acid [35,39,41,48]. Information about ferulic acid release from corn fibre by available commercial enzyme preparations is lacking. Moreover, low ferulic acid yields are reported for other corn constituents using commercial enzymes preparations [41,49,50]. In order to increase the accessibility of enzymes, and consequently boost the yield of ferulic acid released from lignocellulosic materials, several pre-treatment techniques were studied, including hydrothermal [50–52], steam explosion [53], acidic [54] and alkaline processes [55] carried out prior to enzymatic hydrolysis. Hydrothermal pre-treatment is relatively simple and environmentally friendly.

Alkaline extraction is the most commonly reported technique for releasing ferulic acid from lignocellulosic materials [43–45,56,57], since high percentages of ferulic acid yields have been reported

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even under mild conditions (low temperature and low alkaline solution concentrations) [43,44], making it a more economic and eco-friendly process. There are no results to date, however, about the use of mild alkaline extraction to release ferulic acid from corn fibre. Enzymatic extraction using several commercial prepared enzymes with and without hydrothermal treatment has to be evaluated as well the comparison with that of an optimised mild alkaline extraction.

Purifying the ferulic acid released by alkaline extraction is challenging, since several compounds are released. For example, the added-value abundant arabinoxylans [58–60] and other compounds which may have a negative impact on vanillin bio-production, due to toxic and/or inhibiting effects. These compounds include, for example, organic acids [61–64], mainly acetic and lactic ones, present in corn fibre [46,65,66], metals and aldehydes. They must be removed prior to bio-vanillin production. The purification of ferulic acid has been reported using a variety of processes, such as membrane processes [56,67], liquid-liquid extraction [68,69], weak anion-exchange with a resin with a amine group [70], and adsorption [71–73]. Liquid-liquid extraction is expensive, time-consuming and low yield and is only suitable at small scales. Weak anion-exchange, mainly Amberlyst A21 has been used to purify ferulic acid in enzymatic hydrolysates [48,70]. However the organic acids (potential inhibitors/toxic compounds for the microorganisms) are also retained by this type of resin [74,75]. The retention of ferulic acid and the organic acids occurs by the acid-base neutralization reaction between the carboxylic group and the tertiary amine of resin [48,70]. Adsorption is an easy process to scale up, implement and operate [71], but the arabinoxylans in the alkaline extract are highly viscous polysaccharides which can adhere to resins and influence ferulic acid adsorption of [56]. They are added-value compounds with many health benefits [76] that can be used either to produce edible films [77] or as emulsifiers in oil-in-water emulsion systems [78]. Therefore, a previous step separating ferulic acid from arabinoxylans is required to valorise both compounds. Ferulic acid can be separated from arabinoxylans using ultrafiltration [56,59,79] due to the significant difference in their molecular masses, 194 Da and 244–491 kDa [80], respectively. However, the solution's viscosity leads to external mass transfer limitations and a gel layer forms on the membrane surface [81] compromising its permeance. Ultrafiltration operating conditions require careful investigation in order to come up with a separation mechanism for these two compounds. After the separation of arabinoxylans from ferulic acid, an efficient adsorption of ferulic acid to remove the bio-toxic compounds that might inhibit bio-vanillin production might be required.

Another challenge of this process is the biological production of bio-vanillin from the ferulic acid into vanillin, since vanillin is toxic for most microorganisms, decreasing the yield and productivity of the process [1,82,83]. One candidate is the bacteria *Amycolatopsis* sp. ATCC 39116, which not only appears to be particularly efficient in using ferulic acid but it also exhibits higher tolerance to vanillin and produces this compound with higher yields [83]. However, even this strain begins to suffer vanillin toxicity when the latter reaches concentrations above 7g.L⁻¹ [84]. Strategies reported to address this

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issue and improve the vanillin production yield and overall vanillin productivity include: the optimisation of medium composition and reactor operating conditions [3,85,86]; the reduction of vanillin-microorganism contact, by way of a vanillin-adsorbing resin [87] in conjunction with a solid-liquid two-phase partitioning bioreactor system for *in situ* product removal [86]; and, using genetically engineered *Amycolatopsis* sp. ATCC 39116. However, sufficient information is yet lacking to optimise the medium composition in the bioreactor by adjusting operational parameters. The use of adsorbent resins or polymers for *in situ* removal of vanillin from the broth, has the disadvantage of vanillin recovery from these solid matrices being associated with losses and use of organic solvents. The use of genetically modified microorganisms in vanillin production is controversial, as environmental groups claim that the vanillin produced is artificial [19,26]. It is essential, therefore, that we achieve a full understanding of the behaviour of the bacterium *Amycolatopsis* sp. ATCC 39116, how it influences carbon source (glucose), vanillin and ferulic acid concentrations, as well as how it responds to multiple pulse feeding strategies with product removal, so as to improve both vanillin yield and productivity.

Following bio-production, vanillin must be purified. Crystallisation is one of the most commonly used techniques for recovering bio-products from bioconversion broths [88]. However, in the case of vanillin, it will only become a competitive technique, when the concentration of vanillin is much higher than 10 g.L⁻¹ [88], (minimum concentration to allow its crystallization at 20° C [89]). This concentration of vanillin is toxic for the microorganisms, including the *Amycolatopsis* sp. ATCC 39116 bacteria, thus it is not possible to obtain bioconversion broth with higher concentration, that allows a crystallisation. An additional concentration step of the bioconversion broth is required to turn the crystallisation possible. The use of resins or activated charcoals to recover vanillin from broths has been reported [87,90,91]. Activated charcoal and some resins are not selective. The absorption of other key compounds such as bioconversion metabolites (vanillic acid, vanillyl alcohol and guaiacol) and ferulic acid [90,92–94] make necessary an additional step to purify vanillin. Moreover, adsorbent contaminant traces are difficult to avoid, which requires the use of strict quality control processes [95]. Other reported techniques like membrane-based solvent extraction [96–98] and two-phase systems [99] have been reported, but they use (non-sustainable) organic solvents, namely ethyl acetate, n-butyl acetate, tributyl phosphate and acetonitrile. Pervaporation has many advantages over other recovery processes as organic solvents are not required, and it yields pure vanillin, free of other broth constituents [95,100,101]. The use of reduced downstream pressure enables vanillin capture by condensation at mild temperatures [95]. Vacuum distillation is still another potential vanillin purification technique. The performance parameters of these last two procedures requires evaluation.

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1.2. Research Strategy and Objectives

This PhD project was carried out in conjunction with a partnership between the Faculty of Science and Technology - Universidade Nova de Lisboa and Copam- Companhia Portuguesa de Amidos, S.A., a producer of corn-based starch. The goal was to design an integrated process that maximises the potential corn fibre by-products to be used as feedstock for bio-vanillin production by way of the study and optimisation of the following four steps (Figure 1.2).

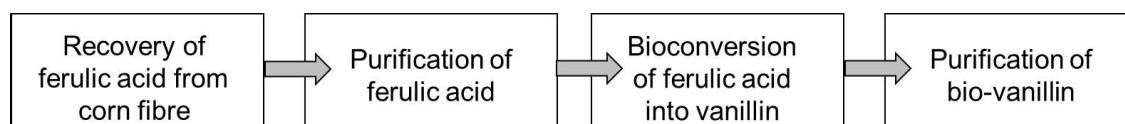


Figure 1.2. Four processing steps required for the production of pure bio-vanillin from corn fibre.

The research strategy used in this work was: 1) to employ corn fibre in the process, due to its greater ferulic acid content than the feedstocks presently commercially used; and 2) to individually optimise each step in the bio-vanillin production from corn fibre considering their subsequent integration into corn gluten feed production. Figure 1.3 displays the main processing steps studied. This research started by studying and comparing recoveries of ferulic acid from corn fibre, using a mild alkaline extraction and an enzymatic hydrolyses using commercial enzyme preparations. In order to improve ferulic acid extraction performance obtained after enzymatic hydrolyses, hydrothermal pre-treatments with different conditions were applied to fibre prior to the enzymatic hydrolyses. This procedure led to improved ferulic acid although of lesser concentrations and yields than that obtained by optimised mild alkaline extraction. The ferulic acid required purification before its use in the bioconversion. Therefore, ultrafiltration membranes were evaluated to separate ferulic acid from large arabinoxylans. An adsorption process was then applied to purify the ferulic acid and separate it from contaminants that might further compromise bio-vanillin production from the fraction enriched in ferulic acid. Bio-vanillin production (using *Amycolatopsis* sp. ATCC 39116 bacteria) was optimised to maximize vanillin yield and productivity. Subsequently, bio-vanillin production (bioconversion) using commercial ferulic acid was compared with bio-vanillin production using ferulic acid extracted and purified from corn fibre, under previously optimised conditions. Vanillin from the bioconversion broths was recovered and purified by pervaporation and compared with that obtained by standard vacuum distillation. Finally, the technical and economic viability of the integrated process to produce bio-vanillin from corn fibre was assessed.

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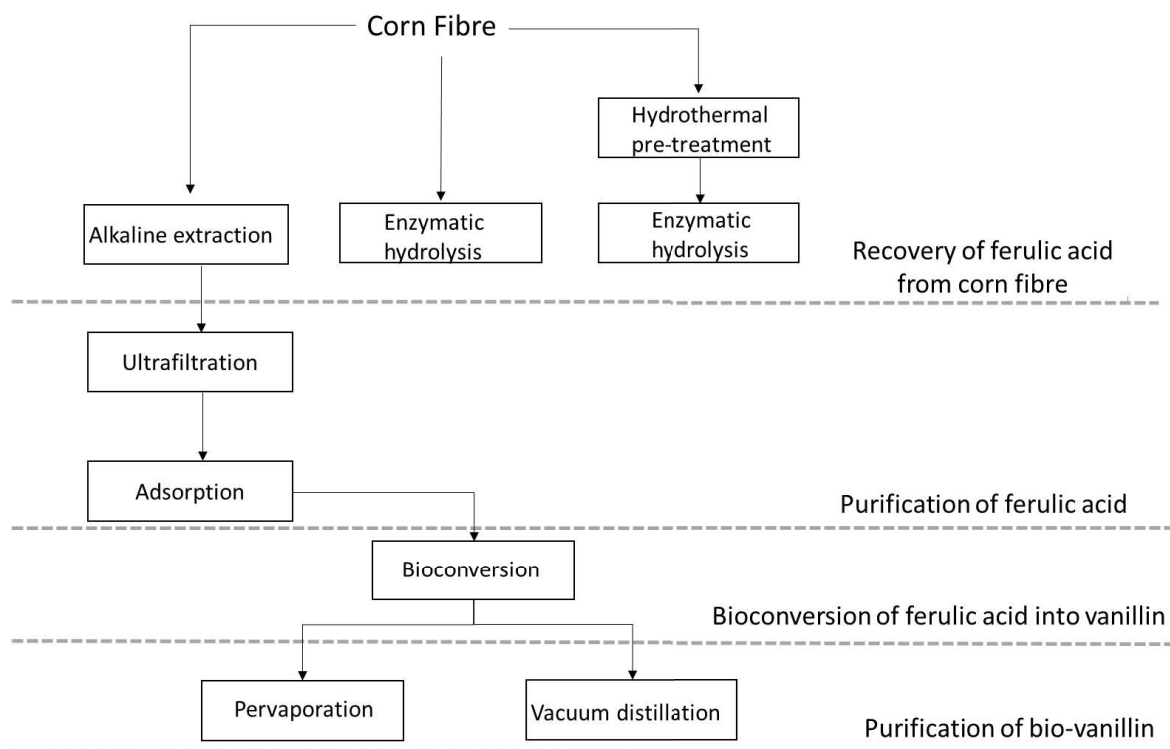


Figure 1.3. Schematic representation of work performed.

The main objectives of this work were to:

- I. Compare the recovery of ferulic acid from corn fibre by enzymatic hydrolyses using commercial enzymatic preparations (with hydrothermal pre-treatments) with that of mild alkaline extraction;
- II. Purify ferulic acid extracted;
- III. Study and optimise bio-vanillin production from ferulic acid using the bacteria *Amycolatopsis* sp. ATCC 39116 and compare it with bioconversion using commercial ferulic acid and ferulic acid extracted from corn fibre;
- IV. Compare vanillin purification techniques; and
- V. Evaluate economic feasibility of bio-vanillin production from corn fibre, through a technical-economic analysis.

1.3. Thesis outline

The present work comprises seven chapters, starting with the motivation and outline. The subsequent four chapters describe the experimental work carried out and their findings. Each chapter corresponds to one of the four stages of the bio-vanillin production process.

Chapter six brings all the steps together and undertakes an economic analysis of the global process. A chapter with conclusions and suggestions for future research finish off the thesis.

This work is organised as follows.

Chapter 1 – Background and Motivation

Chapter 1 lays out the background and motivation for project and outlines its main objectives.

Chapter 2 deals with the recovery of ferulic acid from corn fibre. **Chapter 2.1** describes how ferulic acid was recovered by alkaline extraction. The operating parameters were optimised, specifically the temperature, time of contact, liquid-to-solid ratio ($\text{g}_{\text{extracting solution}} \cdot \text{g}^{-1}_{\text{dry fibre}}$), NaOH concentration and type of fibre (with or without grinding and drying). This optimisation aimed at obtaining an extract with a high recovery of ferulic acid, using conditions as mild as possible, as well as obtaining a low viscosity extract. **Chapter 2.2** describes the recovery of ferulic acid using a commercial preparation of enzymes. A hydrothermal pre-treatment was carried out to improve the ferulic acid yield. The level of contaminants and ferulic acid yield were assessed and results with and without pre-treatment were compared with the mild alkaline extraction optimised in Chapter 2.1. The enzymatic hydrolysates were assessed for their antioxidant and antiproliferative properties.

Chapter 3 describes the procedure to purify ferulic acid obtained by alkaline extraction. **Chapter 3.1** reports on the use of ultrafiltration to separate the acid from arabinoxylans and outlines the relevant operating parameters assessed in order to identify the optimal operating conditions (**chapter 3.1**). The second part of this chapter (**chapter 3.2**) demonstrates how adsorption served as a strategy to eliminate toxic/ inhibitor compounds for the bio-vanillin strain. In order to identify the best operational conditions, the adsorption process was studied in batch mode. Research included which polymer type was more effective for ferulic acid adsorption, what the ideal pH was, and which was the most effective desorbent of ferulic acid from the resin in order to collect it. Scale-up parameters (adsorption capacity, desorption and operating behaviour of the selected resin) were determined using a continuous column procedure.

Chapter 4 details the study of the biotechnological vanillin production by *Amycolatopsis* sp. ATCC 39116, using commercial ferulic acid as a precursor, namely the influence of the concentration of glucose, ferulic acid, vanillin and the use of a multiple pulse feeding strategy for the maximized use of biomass, avoiding the accumulation of toxic levels of vanillin to the bacterial strain. It also compares production using commercial ferulic acid from that based on corn fibre ferulic acid.

Chapter 5 reports on the use of single step pervaporation and vacuum distillation for recovery and purification of vanillin comparing their performance and purity of vanillin obtained.

Chapter 6 analyses the economic viability of the integrated bio-vanillin process.

Chapter 7 comprises the conclusions and suggestions for future research on this bio-vanillin production process.

2. Recovery of ferulic acid from corn fibre

2.1. Extracting ferulic acid from corn fibre using mild alkaline extraction: a pilot scale study

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The author was involved in planning and performing the experiments of semi-pilot and pilot scales, as well as on the discussion and interpretation of the results and preparation of the manuscript.

2.1.1. Summary

There is an increasing demand from the cosmetic, pharmaceutical and food industries for naturally sourced ferulic acid. This study used alkaline extraction under mild operating conditions to recover ferulic acid from corn fibre, with the aim of developing a cost-effective process. The aim was to develop an efficient process of mild alkaline extraction, with an efficient balance between the maximum concentration of ferulic acid and its recovery yield, while producing low-viscous extracts. Methods: Alkaline extraction experiments were performed at different scales (laboratory, semi-pilot and pilot scales) to select various operating conditions (type of raw material, concentration of NaOH, liquid-to-solid ratio, time and temperature of extraction) involved in this process. Results: The optimised mild alkaline extraction conditions obtained were: wet corn fibre, without milling and drying, as a starting material followed by extraction with an alkaline solution of NaOH with a concentration of 0.25 M, use of a liquid-to-solid ratio of $10.0 \text{ g}_{\text{extracting solution}} \text{g}^{-1} \text{ dry fibre}$, during a period of 7 hours at a temperature of 30° C. A low-cost, efficient extraction process of ferulic acid from corn fibre (without any pre-treatment) was developed at pilot scale. The resultant extract had ferulic acid concentration and recovery yield, respectively of $2.10 \pm 0.09 \text{ g}_{\text{ferulic acid}} \cdot \text{L}^{-1}$ and $11.14 \pm 1.00 \text{ Kg}_{\text{ferulic acid}} \cdot \text{Ton}^{-1} \text{ dry corn fibre}$.

2.1.2. Introduction

Low-cost corn fibre (by-product of the starch industry) may be used as a feedstock for high-value compounds, such as ferulic acid since it is one of the most abundant natural sources of ferulic acid [32], containing about 1.5-3 % of this compound [33–36].

Ferulic acid (4-hydroxy-3-methoxycinnamic) is the most important phenolic compound in corn fibre and it can be used in cosmetic, pharmaceutical and food products. Due to the anti-ageing properties of ferulic acid [102,103], its main growth area is in the cosmetics and cosmeceuticals (cosmetics with health claims) industries [104]. Pharmaceutical use of ferulic acid is also expected to grow due to its health benefits [104] and therapeutic potential, as it has anti-inflammatory, antiatherogenic, antidiabetic, neuroprotective, radioprotective, hepatoprotective, and antiproliferative properties [102,105,106]. In some countries ferulic acid is used as a natural antioxidant additive in food and beverages [105]. Besides

Chapter 2 – Recovery of ferulic acid from corn fibre

these applications, ferulic acid may also be used as a precursor to the production of bio-vanillin [86] or as a crosslinking agent for polymeric films [107,108].

Presently, ferulic acid from natural sources, particularly agro-industrial (cereal) by-products, is preferred over synthetic ferulic acid, for which demand is decreasing [104,109]. Natural ferulic acid is rarely found in its free form. Instead, it is covalently linked to polysaccharides (arabinoxylans) by ester bonds and it is covalently linked to lignin by both ester and ether bonds [39,110,111]. In corn fibre, about 90 % of the total ferulic acid is linked to these polysaccharides (arabinoxylans) [36]. Ferulic acid may be extracted by enzymatic hydrolysis [41,50] or by chemical extraction (alkaline or acidic extraction [42,43])

Enzymatic hydrolysis is an eco-friendly process, but the yields of ferulic acid released by commercially available enzyme preparations are still lower than those obtained by chemical extraction [41,49,50]. Other disadvantages of enzymatic hydrolysis are the relatively high cost of enzymes, slower reaction times compared to chemical extraction and the difficulty of recovery and reuse [22].

In chemical extraction, the alkaline process is generally considered to be better than acidic extraction as it causes less degradation of the phenolic acids that are released [114,115]. Alkaline extraction under extremely severe conditions (e.g. 170° C, 4 M of NaOH, over 2 hours) breaks the ester and ether bonds that connect phenolic acids to the other cell wall components (polysaccharides and lignin) [36,116,117] releasing all the ferulic acid from lignocellulosic materials. Severe alkaline extraction has been extensively reported to release ferulic acid from several agro-industrial by-products [39,45,118–122].

Alkaline extraction under mild conditions (concentration of NaOH lower than 1 M, close to room temperature and in less than 12 hours) only breaks the ester bonds in lignocellulosic materials, releasing esterified ferulic acid [36,43,44,123]. When compared to severe alkaline extraction, mild alkaline extraction has the advantages of making the process more cost-effective and the resultant product less hazardous.

Various agro-industrial by-products have been tested using mild alkaline extraction. An extract from rice straw was produced by mild alkaline extraction (with low concentrations of NaOH, 0.2–0.8 M, at 30° C, using a liquid-to-solid ratio of 50 g_{extracting solution}·g⁻¹_{dry raw material}, in less than 10 hours), with a ferulic acid recovery yield of 3.7 ± 0.1 mg_{ferulic acid}·g⁻¹_{dry rice straw} [44]. An extract from corn cobs was produced using mild alkaline extraction (with 0.5 M of NaOH, at room temperature, using a liquid-to-solid ratio of 12 g_{extracting solution}·g⁻¹_{dry raw material}, in 6 hours), with a ferulic acid concentration of 1.171 g·L⁻¹ [43]. Also, an extract from corn bran was produced by relatively mild alkaline extraction (with 0.25 M of NaOH, at 75° C, using a liquid- to-solid mass ratio of 10, in 2 hours), with a ferulic acid yield of 16.9 mg_{ferulic acid}·g⁻¹_{dry corn bran} [56]. When using corn fibre as a feedstock, an alkaline extract was obtained at laboratory scale, using a mild temperature of 20° C, high concentration of NaOH 1 M, in 96 hours, with a ferulic acid yield of 14 mg_{ferulic acid}·g⁻¹_{dry corn fibre} [118]. To the best of our knowledge, there is no study on ferulic

Chapter 2 – Recovery of ferulic acid from corn fibre

acid recovery from corn fibre using alkaline extraction under mild conditions (defined as a concentration of NaOH lower than 1 M, close to room temperature and in less than 12 hours).

Alkaline extraction, even under mild conditions, also releases other compounds from corn fibre, such as arabinoxylans [32,56,58,60]. Arabinoxylans' (polysaccharides) solutions exhibit high viscosity, which makes more difficult to perform additional steps to purify or increase the concentration of ferulic acid [56]. Increases in extraction yields of arabinoxylans are reported when longer extraction times and higher temperatures are applied [58,124]. However, the viscosity of the arabinoxylan solutions is not only directly correlated with the concentration of arabinoxylans, but also with the molecular properties of the extracted arabinoxylans (related with their molecular conformation, molecular mass and molecular mass distribution) [125], which are influenced by the alkaline conditions used [58]. Therefore, the viscosity of the extract is related to the concentration and different types of arabinoxylans obtained in different types of alkaline extraction and it is an important parameter to be studied when performing an alkaline extraction.

The pilot scale study performed in this work has two goals: evaluate the alkaline extraction of ferulic acid from corn fibre under mild conditions, that emulate an industrial operation; obtain an extract reaching a maximum recovery of ferulic acid, assuring the best compromise between concentration and yield of extraction, while producing extracts with low viscosity. To attain these goals, the performance of ferulic acid recovery was first assessed in laboratory using erlenmeyer flasks and semi-pilot studies using a 5 L extractor to examine the influence of the main alkaline extraction conditions (concentration of NaOH, extraction time, type of fibre, liquid-to-solid ratio and temperature). The optimal operational conditions were then validated with a pilot equipment using a 50 L extractor.

2.1.3. Materials and Methods

2.1.3.1. Materials

Samples of corn fibre were collected from different stages of the starch process. Three types of fibres were studied: first, corn gluten feed (produced by mixing wet corn fibre with steepwater and broken corn and drying the mixture down to 8 %–11 % humidity); second, wet corn fibre (withdrawn after the wet milling process); and third, dry milled corn fibre. The dry milled corn fibre was the wet corn fibre which had been dried at room temperature for several days. Then it was milled to a fine powder using a grinding mill to increase the mass transfer surface between the extracting solution and the solid material. The humidity content of each type of corn fibre was measured in order to determine its dry weight (Erro! A origem da referência não foi encontrada.).

Chapter 2 – Recovery of ferulic acid from corn fibre

Table 2.1. Humidity content of each type of fibre used in this work.

Type of fibre	Humidity content
Corn gluten feed	9.1 %
Wet corn fibre	61.5 %
Dry milled corn fibre	5.8 %

The reagents used were sodium hydroxide (Akzo Nobel, Sweden), Folin–Ciocalteu’s reagent (Panreac, Folin–Ciocalteu’s DC), and ferulic acid (Sigma Aldrich, ≥ 99 %).

2.1.3.2. Methods

2.1.3.2.1. Extraction Procedures

Different alkaline extraction experiments were performed at different scales to assess each of the various operating conditions involved in this process. Figure 2.1 shows a summary of the experiments.

Recovery of ferulic acid at lab scale: selection of NaOH concentration, extraction time and pre-selection of temperature and liquid-to-solid ratio (extracting solution/corn fibre).

The optimization of NaOH concentration and temperature, $T_{\text{extraction}}$ ($^{\circ}\text{C}$), in alkaline extraction experiments was carried out by setting a liquid-to-solid ratio of $15.0 \text{ g extracting solution} \cdot \text{g}^{-1} \text{ dry fibre}$ and an extraction time of 24 hours. Corn gluten feed (6.7 g) was mixed in 250 ml of NaOH, where NaOH concentration and temperature were varied in different tests (see Figure 2.1). After 24 hours of magnetic stirring at 200 rpm, the extract was centrifuged at $9174 \times g$ for 15 min. The ferulic acid and total phenolic compounds concentrations were measured. Then, the kinetic study of the extraction was performed at a constant temperature and NaOH concentration (previously optimised, keeping the liquid-to-solid ratio of $15.0 \text{ g extracting solution} \cdot \text{g}^{-1} \text{ dry fibre}$). Eight samples were collected over a 24-hour period and the content in phenolic compounds in each sample was analysed. Finally, the liquid-to-solid ratio was varied (see Figure 2.1) and the other parameters (NaOH concentration, temperature and extraction time) were set to the previously optimised values.

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2.1.3.2.2. Recovery of ferulic acid at semi-pilot scale: selection of type of fibre and final selection of liquid-to-solid ratio (extracting solution/corn fibre) and temperature of extraction

The assays were performed in a 5 L extractor with mechanical stirring provided by two six-blade impellers, using the wet corn fibre and the dry milled corn fibre. Each type of fibre was added to 1.2 L of extracting solution according to the optimal conditions noted above (see Figure 2.1). After each assay, the extracts were centrifuged at $9174\times g$ for 15 minutes and the supernatant was analysed. The liquid-to-solid ratio in these assays was considered the mass of distilled water (g) plus mass of water (g) present in corn fibre per mass of dry corn fibre.

2.1.3.2.3. Recovery of ferulic acid at pilot scale: validation of the selected operating conditions

The assays were performed with different batches of wet corn fibre provided by COPAM, Companhia Portuguesa de Amidos, S.A. at different times of the year. The extraction was performed using the conditions selected at lab and semi-pilot scales (see Figure 2.1) in a 50 L extractor (JUCHHEIM Laborgeräte GmbH, Germany) with controlled mechanical stirring provided by two six-blade impellers at 300 rpm. The total mass (mass of fibre and mass of extracting solution) in the extractor ranged from 22 to 44 kg. At the end of each experiment, the extracts were centrifuged twice at $9174\times g$ for 15 minutes. The liquid-to-solid ratio in these assays was considered the mass of distilled water (g) plus mass of water (g) present in corn fibre per mass of dry corn fibre.

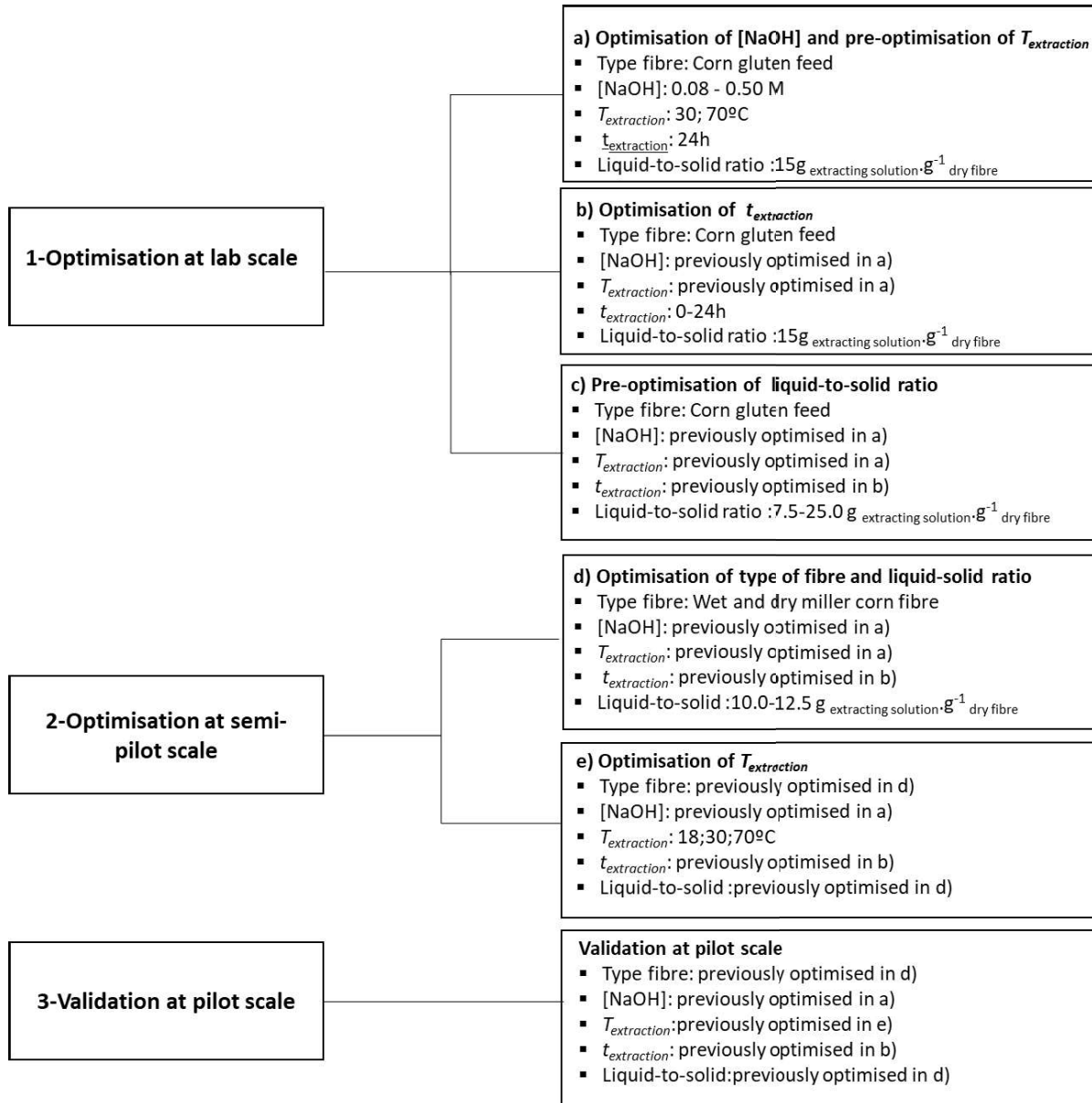


Figure 2.1. Alkaline extraction experiments for the selection of most adequate operating conditions: NaOH concentration, extraction time ($t_{\text{extraction}}$), temperature ($T_{\text{extraction}}$) and liquid-to-solid ratio (extracting solution / type of fibre) at laboratory, semi-pilot and pilot scales.

2.1.3.3. Analytical Methods

The humidity content was determined by drying 20 g of each sample in the oven at 100° C for 24 hours. The humidity was calculated using the following equation:

$$\%humidity = \frac{W_W - W_D}{W_W} \times 10 \quad (1)$$

where, W_W (g) and W_D (g) are the weight of the wet and dry corn samples, respectively.

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The content of the extracts in total phenolic compounds was determined by the Folin–Ciocalteu's method [126].

The content in ferulic acid was determined by HPLC (Alliance e2695, Waters, USA) coupled with a UV detector at 280 nm and equipped with a Nova-Pak® C18 3.9 mm×150mm column (particle diameter of 4mm) and guard column. The gradient elution program was as follows: 0 % B (0–10 min), from 0 to 15 % B (10–25min), from 15 to 50 % B (25–35 min) and 50 to 0 % B (35–38 min) at a flow rate of 0.5 mL.min⁻¹ at 30 °C, where eluent A was made up of 10 % (v/v) methanol, 2 % (v/v) acetic acid in Milli-Q water and eluent B was made up of 90 % methanol, 2 % acetic acid in Milli-Q water. The ferulic acid (≥99 %) (Sigma Aldrich) was used as standards at concentrations between 0.006 – 0.2g.L⁻¹.

The apparent viscosity of extracts was measured at room temperature using a digital viscometer (Brookfield, Model DV_II, Canada).

2.1.4. Results

2.1.4.1. Recovery of ferulic acid at lab scale: selection of NaOH concentration, temperature, extraction time and pre-selection of the liquid-to-solid ratio of extracting solution to corn fibre

The release of ferulic acid from corn fibre has already been studied for high concentrations of NaOH (above 1 M) at room temperature [118] but there is no study on the release of ferulic acid under milder alkaline conditions (concentration of NaOH lower than 1 M, close to room temperature, in a few hours). Corn gluten feed was the type of fibre used in this study. The concentration of NaOH and the temperature were assessed. Several extraction experiments were performed using corn gluten feed at a liquid-to-solid ratio of 15.0 g extracting solution.g⁻¹ dry fibre, over the course of 24 hours, at different temperatures (30° C and 70° C) and different NaOH concentrations (between 0.08 and 0.50 M of NaOH). Figure 2.2 shows the evolution of the concentration of total phenolic compounds and ferulic acid extracted against the concentration of NaOH, at 30° C and 70° C (Figure 2.2 a), and the evolution of the total phenolic compounds and ferulic acid recovery yields against the concentration of NaOH, at 30° C and 70° C (Figure 2.2 b).

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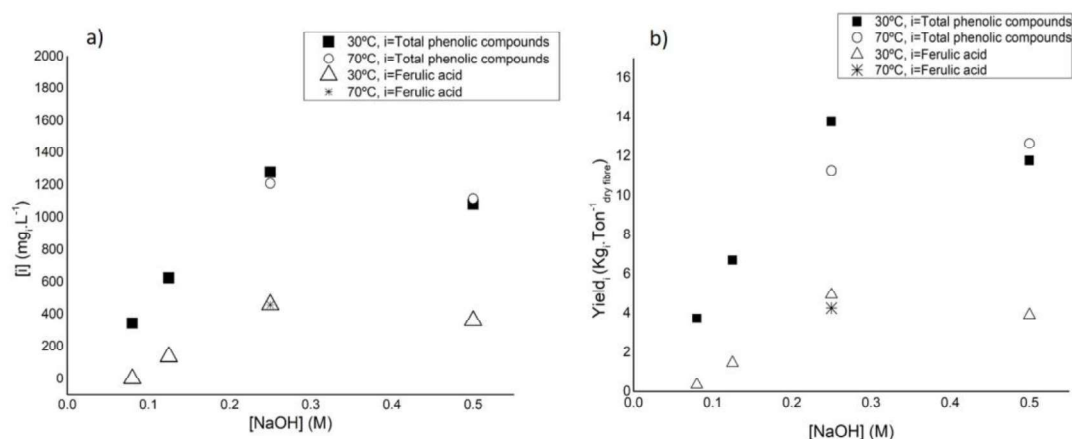


Figure 2.2. Mild alkaline extraction experiments with corn gluten feed at the liquid-to-solid ratio of 15.0g extracting solution·g⁻¹ dry fibre, at 30° C and at 70° C: a) Concentrations of total phenolic compounds and ferulic acid (mg.L⁻¹) and b) Recovery yields of total phenolic compounds and ferulic acid (Kg. Ton⁻¹ dry fibre), plotted against the NaOH concentration.

At 30° C, the increase of NaOH concentration to 0.25 M had a significant impact on the concentration of total phenolic compounds and on the concentration and yield of ferulic acid (see Figure 2.2 a) and b)). At a higher concentration of NaOH (0.50 M), the concentrations of total phenolic compounds and ferulic acid, and the recovery yields remained constant. Therefore, the NaOH concentration is a relevant parameter when its value varies below 0.25 M. For NaOH concentrations at 0.25 M, the increase of temperature (from 30° C to 70° C) had no impact on the concentration of total phenolic compounds neither on the concentration and recovery yield of ferulic acid (see Figure 2.2 a) and b)). The same trend was also observed for 0.50 M of NaOH, regarding the total phenolic compounds. Therefore, above a concentration of 0.25 of NaOH temperature does not seem to be a relevant parameter. From the results obtained, the selected parameters for the mild extraction of the type of corn fibre used (corn gluten feed) are: 0.25 M of NaOH, 30 °C, (with an extraction time of 24 h and a liquid-to solid ratio of 15.0 g_{extracting solution}·g⁻¹ dry fibre). The resultant extract reached a ferulic acid concentration of 456.7 mg.L⁻¹ and a recovery yield of 4.9 Kg_{ferulic acid}·Ton⁻¹ dry fibre.

In order to optimise the extraction time, the extraction kinetics were studied using corn gluten feed at the liquid-to-solid ratio of 15.0 g_{extracting solution}·g⁻¹ dry fibre and using the previously optimised parameters (0.25 M of NaOH, 30° C). Several samples were taken during the 24-hour timespan of the experiment. The results are shown for the concentration of the total phenolic compounds (mg.L⁻¹), since the profile of the total phenolic compounds and ferulic acid concentration were similar, as shown in Figure 2.2. Figure 2.3 shows the evolution of the total phenolic compounds against the extraction time.

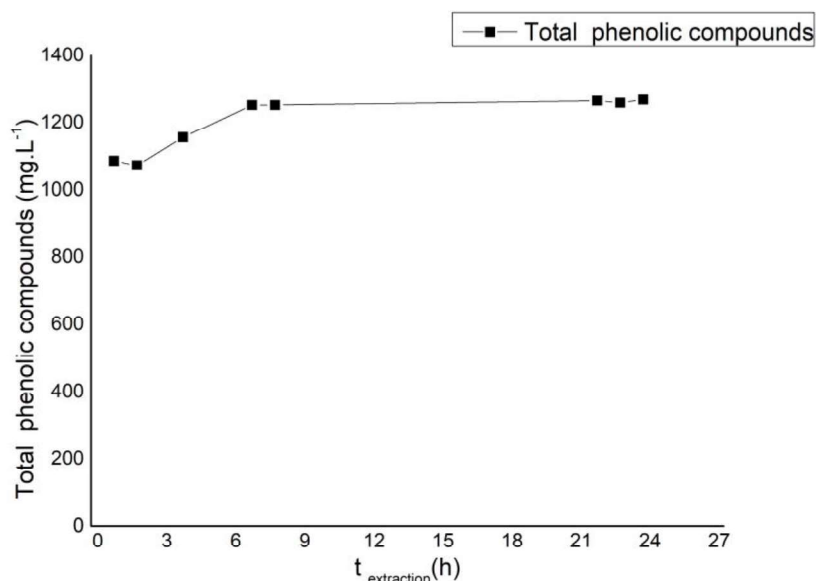


Figure 2.3. Extraction kinetics experiments with corn gluten feed, at the liquid-to-solid ratio of $15.0 \text{ g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{ dry fibre}$, at 30°C , with 0.25 M NaOH : concentration of the total phenolic compounds against the extraction time ($t_{\text{extraction}}$).

As shown in Figure 2.3, the concentration of total phenolic compounds increased until it reached a plateau at 1252 mg.L^{-1} after 7 hours of hydrolysis. Therefore, the value of 7 hours was selected as the extraction time.

The effect of the liquid-to-solid ratio was also evaluated by performing mild alkaline experiments using corn gluten feed and the previously optimised parameters: 0.25 M of NaOH , at 30°C , during 7 hours. Several liquid-to-solid ratios were tested, between 7.5 and $25.0 \text{ g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{ dry fibre}$. Figure 2.4 presents the evolution of the ferulic acid concentration against the liquid-to-solid ratio (Figure 2.4 a)) and the ferulic acid recovery yield against the liquid-to-solid ratio (Figure 2.4b)). Due to the formation of a slurry difficult to manage, the minimum liquid-to-solid ratio that could be tested was $10.0 \text{ g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{ dry fibre}$.

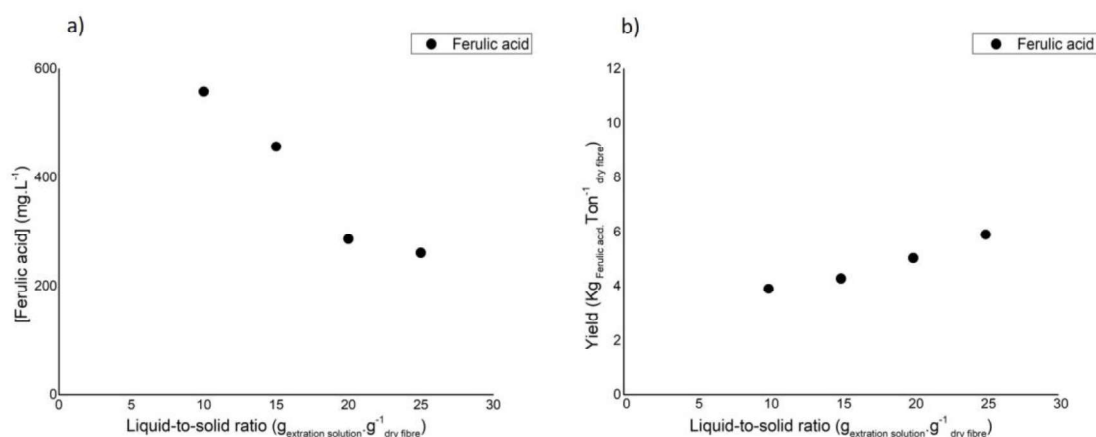


Figure 2.4. Mild alkaline extraction experiments with corn gluten feed at 30° C, with 0.25 M NaOH: a) ferulic acid concentration (mg.L⁻¹) and b) ferulic acid recovery yield (Kg_{ferulic acid}.Ton⁻¹ dry fibre) against the liquid-to-solid ratio (g_{extracting solution}.g⁻¹ dry fibre).

As expected, the concentration of ferulic acid decreased as the liquid-to-solid ratio increased (as the ferulic acid released became more diluted), achieving the maximum of 553.4 g.L⁻¹ at a ratio of 10.0 g_{extracting solution}.g⁻¹ dry fibre (Figure 2.4 a). The ferulic acid yield increases with the increase of the liquid-to-solid, achieving the maximum of 5.9 Kg ferulic acid.ton⁻¹ dry fibre at a ratio of 25.0 g_{extracting solution}.g⁻¹ dry fibre (Figure 2.4 b). However, operation with a lower liquid-to-solid ratio requires the use of less solvent and, consequently, reduced costs for solvent recovery for reuse at industrial scale. Following these experiments, a value of 10.0 g_{extracting solution}.g⁻¹ dry fibre was selected for the liquid-to solid ratio because it led to the extract with the highest concentration of ferulic acid.

2.1.4.2. Recovery of ferulic acid at semi-pilot scale: selection of the type of fibre, final selection of the liquid-to-solid ratio (extracting solution to corn fibre) and extraction temperature

Although it has been reported in the literature that corn fibre contains about 1.5-3 % ferulic acid [33–36], the maximum recovery yield of ferulic acid from corn gluten feed was only 4.6 kg_{ferulic acid}.Ton⁻¹ dry corn fibre corresponding to 0.46 % (w_{ferulic acid}/ w_{dry sample}). Therefore, two other types of feed (wet corn fibre and dry milled corn fibre) were evaluated. Milling lignocellulosic materials, such as corn fibre, increases the mass transfer surface between the extracting solution and the solid material during extraction, improving its yield [44,127]. In a first series of extraction experiments, the type of fibre (wet and dry milled corn fibre with dried fibre to facilitate the milling) and the liquid-to-solid ratio (10.0 and 12.5 g_{extracting solution}.g⁻¹ dry fibre) were evaluated, using the previously optimised parameters: temperature of extraction of 30°C, extracting solution of 0.25 M of NaOH and extraction time of 7 h. These experiments were performed at a larger semi-pilot scale, using a 5 L extractor with improved stirring conditions (mechanical stirring provided by two six-blade impellers. Table 2.2 shows the ferulic acid concentration

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and recovery yield and the apparent viscosity of the extracts, for each extraction performed at liquid-to-solid ratios of 10.0 and 12.5 $\text{g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{ dry fibre}$, using the wet and the dry milled corn fibre.

Table 2.2. Type of fibre and liquid-to-solid optimisation in a 5 L extractor.

	Wet corn fibre		Dry milled corn fibre	
Liquid-to-solid ratio ($\text{g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{ dry fibre}$)	10.0	12.5	10.0	12.5
[Ferulic acid] ($\text{g} \cdot \text{L}^{-1}$)	2.51	2.25	2.92	2.64
Yield _{ferulic acid} ($\text{Kg}_{\text{ferulic acid}} \cdot \text{Ton}^{-1} \text{ dry fibre}$)	13.54	15.52	16.10	18.38
Apparent viscosity (mPa.s)	4.53	4.20	8.90	6.70

Average analytical error of ferulic acid determination of 2.6 %

As shown in Table 2.2 the ferulic acid recovery yield increased with the increase of the liquid-to-solid ratio for each type of fibre under study, as expected, like the previous lab study. In any case, the ferulic acid content obtained in the semi-pilot experiments (Table 2.2), between 13.54 and 18.38 $\text{Kg}_{\text{ferulic acid}} \cdot \text{Ton}^{-1} \text{ dry fibre}$, corresponds to 1.4-1.8 % ($\text{w}_{\text{ferulic acid}} / \text{w}_{\text{dry sample}}$) which are close to the values of ferulic acid content reported in the literature (1.5-3 % of ferulic acid in corn fibre) [33–36]. These values are much higher than the values obtained when using corn gluten feed, which cannot probably be solely explained by the improved stirring conditions. The types of fibre used in these later studies, shown in Table 2.2, seem to be much more suitable raw materials than corn gluten feed for alkaline extraction of ferulic acid.

Generally, when comparing different extractions (Table 2.2), the extract with a higher concentration of ferulic acid (which is desired) is also the extract with higher viscosity (which is undesired). Highly viscous extracts are disadvantageous since they make difficult the subsequent purification process and other procedures aiming the concentration of ferulic acid. As the experiments represented in Table 2.2 were performed at the same temperature and NaOH concentration, this increase of viscosity seems to be related to the increase of the concentration of arabinoxylans (it is unlikely that extracts produced at the same temperature and NaOH concentration lead to different physical sizes of arabinoxylans, contributing to viscosity in different ways). Therefore, a compromise had to be reached between extracts with high ferulic acid concentration and manageable viscosity.

In regard to the results obtained with the types of fibre, namely when comparing dry milled fibre with wet fibre, ferulic acid concentration and yield increased by about 16-19 % (with similar behaviour at 10.0 and 12.5 $\text{g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{ dry fibre}$), but the viscosity increased (97 % at 10.0 $\text{g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{ dry fibre}$ and 60 % at 12.5 $\text{g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{ dry fibre}$), which is much more than the increase in the concentration of ferulic acid. Furthermore, when using the dry milled fibre, a 16-19 % increase of ferulic acid yield and concentration does not justify the energy expenditure of drying and milling the fibre. Therefore, wet fibre was selected as a working raw material.

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Concerning the results obtained with the liquid-to-solid ratios using the wet corn fibre, namely when comparing a liquid-to-solid ratio of 10.0 with 12.5 $\text{g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{dry fibre}$, the ferulic acid increased 12 % when the lower ratio is used (following the same trend as at lab scale) and the viscosity increased 8 %, which is less than the increase in ferulic acid concentration. Therefore, the liquid-to-solid ratio of 10.0 $\text{g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{dry fibre}$ was selected, confirming the previous selection at lab scale.

A final series of extraction experiments at semi-pilot scale were conducted to confirm the temperature using the previously optimised parameters: extracting solution of 0.25 M of NaOH, extraction time of 7 hours and liquid-to-solid ratio of 10.0 $\text{g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{dry fibre}$. Table 2.3 shows the ferulic acid concentration and recovery yield and the apparent viscosity, for each extraction performed.

Table 2.3. Temperature optimisation in the 5 L extractor.

Temperature	18° C	30° C	70° C
[Ferulic acid] ($\text{g} \cdot \text{L}^{-1}$)	1.58	2.51	2.65
Yield _{ferulic acid} ($\text{Kg}_{\text{ferulic acid}} \cdot \text{Ton}^{-1} \text{dry fibre}$)	4.07	13.54	14.44
Apparent viscosity (mPa.s)	4.41	4.53	9.92

Average analytical error of ferulic acid determination of 4.2%

The results shown in Table 2.3 indicate that alkaline extraction at a temperature of 18° C was not efficient for releasing ferulic acid from corn fibre. Both the yield and the concentration of ferulic acid were very low (4.07 $\text{Kg}_{\text{ferulic acid}} \cdot \text{Ton}^{-1} \text{dry fibre}$ and 1.58 $\text{g}_{\text{ferulic acid}} \cdot \text{L}^{-1}$) when compared to a temperature of 30° C (13.54 $\text{Kg}_{\text{ferulic acid}} \cdot \text{Ton}^{-1} \text{dry fibre}$ and 2.51 $\text{g}_{\text{ferulic acid}} \cdot \text{L}^{-1}$). When comparing extractions at 70° C to extractions at 30° C (Table 2.3), the results should be discussed in relation to the results shown in Table 2.3. The ferulic acid concentration and yield increased, but only by 6-7 %, and the viscosity also increased significantly (by 2.2 times), which counteracts the gains in ferulic acid concentration. Additionally, the energy consumption of extractions at 70° C is much higher than at 30° C. For these reasons, 30° C was selected as the extraction temperature.

Based on the results at semi-pilot scale, the following operating conditions were selected for the next stage of the research: use wet fibre as a starting material, perform extraction with an alkaline solution of NaOH with a concentration of 0.25 M, use of a liquid-to-solid ratio of 10.0 $\text{g}_{\text{solvent}} \cdot \text{g}^{-1} \text{dry fibre}$, during a period of 7 hours at a temperature of 30° C.

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2.1.4.3. Recovery of ferulic acid at pilot scale: validation of the selected operating conditions

To obtain a ferulic acid concentration and yield for further industrial implementation of the mild alkaline extraction of ferulic acid, extraction experiments were performed at pilot scale (with a 50 L extractor) using the extraction conditions selected at laboratory and semi-pilot scales. At the end of each experiment, the extracts were centrifuged twice. Five assays were carried out using different batches of wet corn fibre, produced at different times of the year, in order to take into account the seasonal variation of the feedstock. Table 2.4 presents the ferulic acid concentration and extraction yield of each extract obtained, leading to an overall ferulic acid concentration of $2.10 \pm 0.09 \text{ g.L}^{-1}$ and an overall ferulic acid yield of $11.14 \pm 1.00 \text{ Kg}_{\text{ferulic acid}} \cdot \text{Ton}^{-1} \text{ dry corn fibre}$

Table 2.4. Ferulic acid concentration and yield of extracts at pilot scale using the extraction conditions selected at laboratory and semi-pilot scale (wet corn fibre as a starting material, with 0.25 M of NaOH, a liquid-to-solid ratio of $10.0 \text{ g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{ dry fibre}$, during 7 h at 30° C).

Assay	[Ferulic acid] (g.L^{-1})	Yield _{ferulic acid} ($\text{Kg}_{\text{ferulic acid}} \cdot \text{Ton}^{-1} \text{ dry fibre}$)
1	2.09	11.13
2	2.20	12.37
3	2.02	10.47
4	2.02	9.89
5	2.19	11.85

The extracts obtained yielded, on average, a slightly lower concentration of ferulic acid ($11.14 \pm 1.00 \text{ Kg}_{\text{ferulic acid}} \cdot \text{Ton}^{-1} \text{ dry corn fibre}$) compared with the extract obtained at semi-pilot scale under the same extraction conditions ($13.4 \text{ Kg}_{\text{ferulic acid}} \cdot \text{Ton}^{-1} \text{ dry corn fibre}$). This difference may be attributed to differences in the variety of corn fibre due to variable corn cultivation conditions, and/or the difficulties of controlling the operational factors in a larger scale study.

2.1.5. Conclusions

It was possible to produce an extract rich in ferulic acid with relatively low viscosity, using an alkaline extraction under mild operating conditions. Regarding optimum parameters, it is possible to conclude that the increase of NaOH concentration above 0.25 M had no effect on the ferulic acid extraction yield. At a temperature above 30° C , at a concentration of 0.25 M of NaOH, an insignificant increase of ferulic acid yield was detected, which does not justify the energy cost of a higher-temperature extraction. In extractions with liquid-to solid ratios below $10.0 \text{ g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{ dry fibre}$, the extraction media (extraction

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solution and raw material) was impossible to stir. The optimum liquid-to solid ratio was found at $10.0 \text{ g}_{\text{extracting solution}} \cdot \text{g}^{-1} \text{ dry fibre}$ as it corresponded to the maximum ferulic acid concentration.

The smaller particle size of milled corn fibre increased the ferulic acid recovery yield, but this increase does not justify the energy cost of drying and milling. Furthermore, the extracts obtained from milled dry fibre had a higher viscosity, making more difficult to subsequently purify and concentrate the ferulic acid using membrane and adsorption processes [56].

Thereby, the mild alkaline extraction conditions that result in the best balance between a high concentration and recovery yield of ferulic acid and relatively low viscosity were: wet corn fibre, without milling and drying, as a starting material followed by extraction with an alkaline solution of NaOH with a concentration of 0.25 M, use of a liquid-to-solid ratio of $10.0 \text{ g}_{\text{solvent}} \cdot \text{g}^{-1} \text{ dry fibre}$, during a period of 7 hours at a temperature of 30°C . These operating conditions were validated at pilot scale, resulting in an extract with a ferulic acid concentration of $2.10 \pm 0.09 \text{ g} \cdot \text{L}^{-1}$ and a recovery yield of $11.14 \pm 1.00 \text{ Kg}_{\text{ferulic acid}} \cdot \text{Ton}^{-1} \text{ dry corn fibre}$. These results are promising, but a detailed economic analysis of the process would be required prior to industrial implementation.

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2.2. Combined hydrothermal pre-treatment and enzymatic hydrolysis of corn fibre: production of ferulic acid extracts and assessment of their antioxidant and antiproliferative properties

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The author was involved in planning and performing the extractions and the hydrothermal pre-treatments experiments, as well as on the discussion and interpretation of all the results and preparation of the manuscript.

2.2.1. Summary

Ferulic acid may be used as a nutraceutical ingredient or as a substrate to produce bio-vanillin. There is an increasing market demand for ferulic acid obtained from natural sources such as low-cost agro-industrial by-products, due to its potential applications as nutraceutical ingredient and as a substrate to produce bio-vanillin. This work aims to study ferulic acid recovery from corn fibre (one of the most abundant natural sources of ferulic acid), involving an integrated process of hydrothermal pre-treatment followed by enzymatic hydrolysis. The objective is to produce natural extracts with a maximum ferulic acid recovery yield and to assess their antioxidant and antiproliferative properties.

Different commercial enzyme preparations were tested for release of ferulic acid from corn fibre. The best results were obtained for Ultraflo®XL in a concentration of 2 % ($w_{\text{enzyme preparation}} \cdot w^{-1}_{\text{dry corn fibre}}$) at a pH of 5 and at 55° C, presenting a recovery yield of esterified ferulic acid of $7.83 \pm 1.35\%$ ($w_{\text{recovered ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$), which corresponds to $0.13 \pm 0.02\%$ ($w_{\text{ferulic acid}} \cdot w^{-1}_{\text{dry corn fibre}}$). When using a hydrothermal pre-treatment at a temperature of 140° C for 40 minutes, prior to the use of the same enzymatic hydrolysis procedure, the recovery yield of esterified ferulic acid increased to $28.94 \pm 2.40\%$ ($w_{\text{recovered ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$), which corresponds to $0.49 \pm 0.03\%$ ($w_{\text{ferulic acid}} \cdot w^{-1}_{\text{dry corn fibre}}$). The use of this pre-treatment leads not only to the highest yield of ferulic acid, but also to the lowest concentration of furfural and 5-hydroxymethylfurfural, without the formation of formic and levulinic acid (not detected). All pre-treatments tested led to an improved quality of the extract in terms of bioactivity.

2.2.2. Introduction

Ferulic acid is an added-value compound that can be used as a nutraceutical ingredient due to its reported therapeutic properties (antioxidant, anti-inflammatory, antiatherogenic, antidiabetic, antiaging, neuroprotective, radioprotective, hepatoprotective, antiproliferative [102,105,106]), and as a substrate/precursor for bio-vanillin production [86]. Ferulic acid may also be used as a crosslinking agent of polymeric films [107,108]. The market for ferulic acid from natural sources is increasing, along

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with the global nutraceuticals' market (which is expected to increase from \$184.092 million in 2015 to \$302.306 million in 2022 [128]) and the bio-vanillin production (for example, the production of Solvay doubled from 2017 to 2019 [10,129]).

Ferulic acid is present in various plant cell walls, in a complex lignocellulosic structure [34,46,47], where ferulic acid is linked to xylans mainly by ester bonds [110]. Ferulic acid is particularly predominant in low-cost agro-industrial (cereal) by-products, such as wheat/rice/corn bran, oat hulls and corn fibre, typically used in low-value feed applications. The use of this type of agro-industrial by-product for the production of added-value compounds may contribute to improve sustainability and, specifically, the economic competitiveness of agro-food industries [29].

Corn fibre is one of the most abundant natural sources of ferulic acid compared to various other agricultural products [32]: it contains 1.5-3% (w/w) of total ferulic acid [33–36]. Corn fibre, a by-product of the starch industry, is obtained by the wet milling of corn and is also a very abundant feedstock, since corn is the most widely produced grain in the world, with 1.099 billion metric tons produced globally in 2018/2019 [130].

Corn fibre is mainly constituted by hemicelluloses (35 % ww) [33,34]. The main hemicelluloses in corn fibre are xylans, specifically (glucurono)arabinoxylans (GAX), which consist of chains of D-xylopyranose residues branched by β -[1,4] bonds, and can be ramified by L-arabinofuranose, D-glucuronic and acetic acid. GAX can be substituted with acetyl and feruloyl esters [46]. Corn fibre has one of the most complex structures of agro-industrial by-products [34,46,47]. Besides the common substitution of the backbone with single unit side chains as in other cereals, corn fibre xylan also contains oligomeric side chains, consisting of arabinosyl, xylosyl, galactosyl, and feruloyl residues [47,116].

Alkaline extraction is the easiest, most economic and most commonly studied way to release ferulic acid from agro-industrial by-products [43–45,56,57], still NaOH, used in alkaline extraction, is an aggressive compound. In order to propose a more sustainable process, with no aggressive chemicals, this work studies the enzymatic hydrolysis of corn fibre with different pre-treatments. An in-depth comparison is performed between enzymatic hydrolysis with pretreatments and alkaline extracts, primarily for maximum ferulic acid recovery, but also in terms of 1- potential inhibitor compounds present, 2- antioxidant and antiproliferative properties and 3-cytotoxicity. The use of enzymatic hydrolysis/extraction from biomass (using enzymes produced by fungi or bacteria) that has been studied evaluated by several studies [35,41,48,50,131,132], since it presents with additional several advantages over alkaline extraction: 1. it is more environmentally friendly; 2. it does not use an aggressive chemical such as NaOH; 3. 1. it uses biodegradable compounds (enzymes); 4. 2. it is more energy-efficient, using mild operating conditions; and 5. 3. it is usually more selective, and therefore releases specific products without damaging other valuable chemicals [40,133].

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Feruloyl esterase is the most important enzyme in the release of ferulic acid from agro-industrial by-products. However, due to the complex structure of the lignocellulosic materials, a synergy between feruloyl esterase and other hemicellulases (particularly xylanases, arabinofuranosidases and xylosidases) is needed [35,39,41,48] to break the bonds of the hemicellulose-ferulic acid and, consequently, release ferulic acid. Enzymatic hydrolysis for ferulic acid recovery has already been studied with different feedstocks, using both enzymes extracted from fungi or bacteria at small laboratory scale [35,48,134] and commercial enzymatic preparations [41,49,50,131]. To the best of our knowledge, only one study has reported the use of corn fibre feedstock for ferulic acid recovery. In this case, an enzyme preparation, extracted from the filamentous fungus *Neosartorya spinose* NRRL185 and concentrated 10-50 times by ultrafiltration at laboratory scale resulted in the recovery of 99.5 % of ferulic acid ($1.85 \text{ \% (w}_{\text{ferulic acid}} \cdot \text{W}^{-1}_{\text{dry corn fibre}})$) from corn fibre [35].

Commercial enzyme preparations should be considered in industrial extraction processes rather than enzymes prepared at laboratory scale, as they are more economical, and their production has already been scaled-up and tested in terms of safety [135]. However, enzymatic hydrolysis using commercial enzyme preparations for ferulic acid recovery results in a low yield of ferulic acid recovery from corn by-products (e.g. corn cob and bran [41,49,50]) and other agro-industrial by-products (e.g. sugar beet pulp [41] and wheat bran [131]).

In order to increase the concentration and recovery yield of ferulic acid from agro-industrial by-products, several pre-treatments prior to enzymatic hydrolysis have been explored. Hydrothermal pre-treatment [50–52], steam explosion [53], and acidic [54] or alkaline pre-treatment [55] are commonly used in agro-industrial by-products to increase the degradability of the feedstocks, and consequently, increase the accessibility of enzymes. Hydrothermal pre-treatment is considered to be relatively simple and environmentally friendly since no chemical compounds are added besides water, and it is reported to be effective in increasing the yield of ferulic acid released from biomass. Using a hydrothermal pre-treatment, Saulnier et al. (2001) increased the ferulic acid extraction yield from maize bran from 30% to 70% using the Novozym 342 commercial enzyme preparation [50]. Jiang et al. (2018) also obtained a high percentage of ferulic acid recovered (74.13 %) from corn bran, using autohydrolysis at 165° C for 40 min and sequent hydrolysis with an enzyme blend from *Aspergillus oryzae* and *Eupenicillium parvum* [52].

In general, the main disadvantage of the pre-treatments is the degradation of sugars, which causes the formation of toxic and fermentation inhibitors such as furfural and hydroxymethylfurfural (HMF) [51,52,136,137] and organic acids, such as formic and levulinic acid [52]. These compounds are known as strong microbial inhibitors and their removal or reduction is important in the development of processes for bio-based products [138]. These compounds must also be minimised for nutraceutical application since they are toxic. Other compounds that inhibit fermentation, like acetic acid, cannot be avoided since they are part of the cell wall structure [33,34,116].

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The objective of this work is to understand the impact of operating conditions (reaction time and temperature) of the hydrothermal pre-treatment and the effect of commercial enzyme preparations for enzymatic hydrolysis aiming ferulic acid recovery from corn fibre. Other objectives of this work are the identification of which operating conditions lead to the maximum ferulic acid recovery yield and to the minimum concentration of undesirable products (like furfural, hydroxymethylfurfural and organic acids from the pre-treatment step) and the assessment of the antioxidant and antiproliferative properties of the extracts.

2.2.3. Materials and Methods

2.2.3.1. Materials

The feedstock corn fibre was supplied by COPAM, Companhia Portuguesa de Amidos, S.A. (São João da Talha, Portugal) after the wet milling process. The corn fibre was used without any pre-treatment (drying or grinding) and all the assays were performed with the same lot of corn fibre. The Viscozyme® L, Ultraflo® L, Ultraflo® XL, Ultraflo® Core and Ultraflo® Max commercial food-grade enzyme preparations were provided by Novozymes A/S (Bagsværd, Denmark). These commercial enzyme preparations are specially designed to break down cell wall materials in cereals [139]. The main characteristics of each commercial enzyme preparation are summarised in Table 2.5.

Table 2.5. Main characteristics of commercial enzyme preparations used in this work.

Enzyme preparations ^a	Enzyme class	Optimal Conditions ^b	
		pH	Temperature (°C)
Viscozyme® L	β -glucanase	4.5	55
Ultraflo® L	β -glucanase	4.5-5.0	65
Ultraflo® XL	β -glucanase	4.5-5.0	55
Ultraflo® Core	Cellulase	5.0	65
Ultraflo® Max	β -glucanase, Xylanase	5.0	65

^a Commercial product of Novozymes A/S

^b Information supplied by the producer

Enzymatic hydrolysis was performed with a 50 mM sodium acetate buffer pH 4.5 and pH 5 (prepared with sodium acetate anhydrous, reagent grade (Scharlab, Spain) and acetic acid glacial (Carlo Erba reagents, Spain). Sodium hydroxide (Akzo Nobel, Sweden) was used in the alkaline extraction.

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2.2.3.2. Experimental procedure

2.2.3.2.1. Enzymatic hydrolysis from corn fibre - screening of commercial enzyme preparations

Wet corn fibre (12.14 g) was suspended into 100 ml of 50 mM sodium acetate buffer at optimal pH of each commercial enzyme preparations (Table 2.5). In the case of Viscozyme® L commercial enzyme preparation at pH 4.5 and for the other commercial enzyme preparations under study at pH 5. The solution was autoclaved at 1 bar and 121° C for 20 minutes. The liquid-to-solid ratio was $25 \text{ g}_{\text{solvent}} \cdot \text{g}_{\text{dry fibre}}^{-1}$, taking into account the water present in corn fibre (the liquid-to-solid ratio is the mass of buffer (g) plus the mass of water (g) present in corn fibre per mass of dry corn fibre). According to Novozymes A/S, the recommended ratio is 0.010-2.50 % (w/w) enzyme preparation per dry feedstock [140]. In this work, the selected ratio was 2 % ($\text{W}_{\text{enzyme preparation}} \cdot \text{W}_{\text{dry corn fibre}}^{-1}$) for each enzymatic hydrolysis performed under sterile conditions. The hydrolysis was conducted for 72 hours at optimal temperature (Table 2.5) for each commercial enzyme preparation, stirring at 200 rpm (with a magnetic bar). Samples were withdrawn during hydrolysis and centrifuged at $13680 \times g$ for 5 minutes. The supernatant was collected and boiled at 100° C for 10 minutes for the thermal inactivation of enzymes. Then it was centrifuged again at $13680 \times g$ for 5 minutes. The supernatant was used for HPLC determination of ferulic acid. A commercial enzyme preparation was selected based on the results of the enzymatic hydrolysis. The results were also compared with a mild alkaline extraction previously optimized in chapter 2.1, at 30° C, with 0.25 M NaOH, during 7 h and at 200 rpm for the liquid-to-solid ratio of $25 \text{ g}_{\text{solvent}} \cdot \text{g}_{\text{dry fibre}}^{-1}$. After 7 h, the extract was centrifuged at $9174 \times g$ during the 15 min. The supernatant was used for HPLC determination of ferulic acid.

2.2.3.2.2. Hydrothermal treatment of corn fibre

The hydrothermal treatment of corn fibre was performed in a stainless-steel reactor (Parr Instruments Company, USA). The temperature was controlled with a PID controller (model 4842, Parr Instruments Company, USA). The wet fibre (307g) was mixed with distilled water (893 ml) in the reactor to reach a liquid-to-solid ratio of $10 \text{ g}_{\text{water}} \cdot \text{g}_{\text{dry fibre}}^{-1}$, taking into account the water present in corn fibre (the liquid-to-solid ratio is the mass of water added (g) plus the mass of water (g) present in corn fibre per mass of dry corn fibre).

Four different treatment procedures were performed at 150 rpm (mechanical stirring), using temperatures of 165 and 140° C and times of 40 minutes and 2 hours (excluding the heating and cooling periods). After pre-treatment, the liquid and solid phases were separated by filtration. The liquid phase was characterised in terms of the concentration of sugars, 5-hydroxymethylfurfural (HMF), furfural and

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organic acids by HPLC. Both phases were subjected to enzymatic hydrolysis and an alkaline extraction for comparison.

The pre-treatment severity was characterised by the combined severity factor ($\text{Log } R_O$). This factor combines the effects of temperature and residence time of the biomass in the reactor, the two most influential effects on optimising biomass fractionation. The $\text{Log } R_O$ factor was calculated by the following equation [141] .

$$\text{Log } R_O = \text{Log} (t \times \text{Exp} \left(\frac{(T_H - T_R)}{14.75} \right)) \quad (2)$$

where t is reaction time (min); T_H is the target temperature ($^{\circ}\text{C}$); T_R is a reference temperature (100°C), and the value of 14.75 is presented as an empirical parameter related to the activation energy (pseudo first-order kinetics).

2.2.3.2.3. Mild alkaline extractions from the solid and liquid fractions of each hydrothermal pre-treatment

The mild alkaline extraction of the fractions resulting from hydrothermal pre-treatment was performed in the same conditions as the mild alkaline extraction without pre-treatment (30°C , with 0.25 M NaOH, during 7 h, at 200 rpm). The resulting solid from each hydrothermal treatment was added to 100ml of 0.25 M NaOH solution to reach a liquid-to-solid ratio of $25 \text{ g}_{\text{solvent}} \cdot \text{g}^{-1}_{\text{dry pre-treatment fibre}}$ and NaOH was added directly to the 100 ml of liquid that resulted to reach a concentration of 0.25M.

2.2.3.2.4. Enzymatic hydrolyses of the solid and liquid fractions of each hydrothermal pre-treatment

The solid and liquid phases that resulted from each hydrothermal treatment were autoclaved separately at 1 bar, 121°C for 20 minutes. The enzymatic hydrolysis of the fractions resulting from hydrothermal pre-treatment was performed under the same conditions as the enzymatic hydrolysis without pre-treatment, using the 2 % mass ratio ($w_{\text{enzyme preparation}} \cdot w^{-1}_{\text{dry corn fibre}}$) of the commercial preparation enzymes selected in the previous sub-section (enzyme preparation that released more ferulic acid from corn fibre) in 50 mM sodium acetate buffer pH 5 for 50h (the time optimised in the previous sub-section). The resulting solid from each hydrothermal treatment was added to 100ml of 50 mM sodium acetate to reach a liquid-to-solid ratio of $25 (\text{g}_{\text{solvent}} \cdot \text{g}^{-1}_{\text{dry pre-treatment fibre}})$ and sodium acetate was directly added to the resulting 100 ml of liquid to reach a concentration of 50 mM and 0.2 g of enzyme preparation.

Samples were taken during hydrolysis to confirm that the ferulic acid concentration was stabilizing. Afterwards, the samples were centrifuged at $13680 \times g$ for 5 minutes. The supernatant was collected and boiled at 100°C for 10 minutes to thermally inactivate the enzymes. Then it was centrifuged again at $13680 \times g$ for 5 minutes. The supernatant was used for HPLC determination of ferulic acid.

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2.2.3.3. Analytical Methods

2.2.3.3.1. Water content in corn fibre

The water content (humidity) of corn fibre was determined by drying a small quantity of corn fibre at 100° C in the oven for 24 hours. The humidity was calculated by the following equation:

$$\%humidity = \frac{W_W - W_D}{W_W} \times 100 \quad (3)$$

where w_w and w_D correspond to mass of the wet and dry corn fibre, respectively.

The humidity was measured in triplicate.

2.2.3.3.2. Esterified ferulic acid content in corn fibre

Wet corn fibre (12.14 g) was mixed in 100 ml of 2M NaOH in a liquid-to-solid ratio of 25 g_{solvent}·g⁻¹_{dry fibre} at 200 rpm and 50° C [36,123]. After 24 h, the extract was centrifuged at 9174 x g, at room temperature, during 15 min. The total esterified ferulic acid content of the supernatant was determined by HPLC.

2.2.3.3.3. HPLC analyses

The concentration of ferulic acid was determined by high performance liquid chromatography. The analytical HPLC system (Alliance e2695, Waters, USA) was equipped with a UV detector at 280 nm. Ferulic acid was separated on a Nova-Pak® C18 3.9 mm×150 mm column (particle diameter of 4 mm), preceded by a guard column, at 30° C with a gradient elution program at the flow rate of 0.5 mL.min⁻¹. The mobile phase was a mixture of two eluents, one of them (A) consisting of 10 % (v/v) methanol, 2 % (v/v) acetic acid in Milli-Q water and the other (B) consisting of 90 % methanol, 2 % acetic acid in Milli-Q water. The gradient elution program was 0 % B (0–10 min), from 0 to 15 % B (10–25min), from 15 to 50 % B (25–35 min) and 50 to 0 % B (35–38 min). The ferulic acid (≥99 %) (Sigma Aldrich) was used as standards at concentrations between 0.006 – 0.2g.L⁻¹.

The concentration of sugars (arabinose, glucose, xylose, rhamnose, galactose) was analysed by HPLC (Dionex, Thermofisher Scientific, USA) equipped with an amperometric detector and CarboPac PA10 column. The analysis was performed at 30° C, with sodium hydroxide (NaOH 18mM) as the eluent, at a flow rate of 1 mL min⁻¹.

The concentration of furfural and HMF was also determined by HPLC (Waters 600, Waters, USA) equipped with a Biorad Aminex HPX 87H 300 x 7.8 mm, coupled to a UV/ Vis detector, set at 280 nm. The compounds were separated at 30° C at the flow rate of 0.6 mL.min⁻¹, with 10 mN H₂SO₄ as the mobile phase.

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Organic acids were also analysed by HPLC (VWR Hitachi Organizer, VWR, USA) equipped with an RI Detector 5450 and using an Aminex HPX-87H 300x7.8 mm column. The analysis was performed at 30° C, using 0.010 mM H₂SO₄ as the eluent at a flow rate of 0.5 ml. min⁻¹.

2.2.3.3.4. Antioxidant activity assay - Oxygen radical absorbance capacity (*ORAC*) assay

The *ORAC* assay method measures the capacity of the antioxidants species, present in the extracts, to protect the disodium fluorescein (FL) from oxidation by peroxy radicals. The assay was performed using the method described in Serra et al 2011 [142]. Briefly, in a black 96-well microplate, 25 µL of the appropriate corn extracts and phenolic acid sample dilutions and 150 µL of disodium fluorescein (2×10⁻⁷ mM) were added to each well. After an incubation period of 10 min, the fluorescence of each well was read in an FLx800 fluorescence microplate reader (FL800 Bio-Tek Instruments, Winooski, VT, USA) during a period of 40 minutes at 37° C. The reaction was started with 25 µL of 2,2'-azobisisobutyramidium chloride (AAPH) (153 mM) at the same temperature. AAPH and FL solutions were prepared with phosphate buffer saline (PBS), 75 mM, pH=7.4, which was used as a blank. The control standards were prepared using concentrations of 5, 10, 20, 30, 40 µM of Trolox using the same PBS solution. All the samples were analysed in triplicate, using up to three different dilutions. The results were expressed as Trolox equivalents per gram of dry hydrolysate (µmol TEAC.g⁻¹ dry hydrolysate).

2.2.3.3.5. Cell-based assays

Cell culture

Human colon cancer cell lines, HT29 and Caco-2, were obtained from American Type Culture Collection (ATCC, USA) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), respectively. Caco-2 cells were cultured in a standard Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 % non-essential amino acids (NEAA), 10 % fetal bovine serum (FBS; Biowest, Riverside, USA) and 1 % (v/v) penicillin-streptomycin (PenStrep; Gibco, Carlsbad, USA). HT29 cells were grown in Roswell Park Memorial Institute medium (RPMI) supplemented with 10 % FBS. The cells were routinely maintained as monolayers in 75 cm² culture flasks and incubated at 37° C with 5 % CO₂ in a humidified atmosphere.

Cytotoxicity assays

Cytotoxicity assays were performed using confluent and non-differentiated Caco-2 cells. This cell model is widely used as it shares some characteristics with crypt enterocytes so is considered as an acceptable

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intestinal model [143,144]. Briefly, cells were seeded at a density of 2×10^4 cells/well in 96-well culture plates and allowed to grow for 7 days, with medium renewal every 48 h [145].

After 7 days, cells were incubated with different concentrations of the sample (the range was 0.078 mg.mL^{-1} to 10 mg.mL^{-1}) diluted in culture medium (DMEM medium containing 0.5 % FBS). After 96 h of incubation, the cell viability was evaluated using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) containing MTS reagent. Absorbance was measured at 490 nm using an EPOCH2 microplate reader (Biotech Instruments, Winooski, USA) and cell viability was calculated relative to the control with the culture medium. Three independent experiments were performed in triplicate. All data is expressed as mean $\pm$ standard deviation (SD).

Antiproliferative assays

Corn fibre samples and phenolic acids were evaluated for their cell growth inhibition potential in HT29 cells, which are a widely used model for *in vitro* colorectal cancer studies [142]. Briefly, cells were cultured in 96-well culture plates at a density of 1×10^4 cells/well [142] in an RPMI medium supplemented with 10 % FBS. After 24 hours at 37°C in 5 % CO_2 , cells were incubated with different concentrations of the samples diluted in the culture medium (RPMI medium containing 0.5 % FBS) (concentration range: 0.78 mg.mL^{-1} to 10 mg.mL^{-1} for extracts, $1.2 \times 10^{-3} \text{ mg.mL}^{-1}$ to 0.25 mg.mL^{-1} for *p*-coumaric acid and $1.5 \times 10^{-3} \text{ mg.mL}^{-1}$ to 0.19 mg.mL^{-1} for ferulic acid mg.mL^{-1}) for 96 hours. After that, the cell viability was evaluated using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) containing MTS reagent, as described above and cell viability was calculated relative to the control with the culture medium. Three independent experiments were performed in triplicate. All data is expressed as mean $\pm$ standard deviation (SD) and EC_{50} values (the concentration of the sample that is required to decrease 50 % of the cell population after 96 h of proliferation) was calculated using GraphPad Prism version 8 software (GraphPad Software, Inc., La Jolla, CA, USA).

Statistical Analysis

The antioxidant and antiproliferative activity, as well as the content in ferulic acid and *p*-coumaric acid, are expressed as the mean value $\pm$ standard deviation (SD), obtained from at least three independent experiments. Statistical analysis of the results was performed using GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA). When homogeneous variance was confirmed, the results were analysed by one-way analysis of variance (ANOVA), followed by the Tukey test for multiple comparisons. In the case of heterogeneous variances, an appropriate unpaired student t-test was performed in order to determine whether the means were significantly different. A *p*-value ≤ 0.05 was

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accepted as statistically significant in all cases. All outliers were identified and properly eliminated from the analysis.

2.2.4. Results and discussion

The enzymatic hydrolysis of ferulic acid from corn fibre was studied, using five different commercially available food-grade enzyme preparations, designed to break down cell wall materials in cereals [139]. The release of ferulic acid from this abundant feedstock using commercial enzyme preparations has not been reported before. The results were compared with a mild alkaline extraction that was previously optimised by this research group (chapter 2.1). The extraction of ferulic acid from corn fibre was studied in an integrated process of hydrothermal pre-treatment and enzymatic hydrolysis. The solid and liquid fractions from each pre-treatment were subjected to enzymatic hydrolysis and, additionally, to mild alkaline extraction.

2.2.4.1. Characterisation of corn fibre

The feedstock corn fibre was characterised in terms of humidity content, in order to calculate the dry weight of corn fibre, and in terms of the total esterified ferulic acid content. The humidity of corn fibre was $64.5\% \pm 1.2\%$. The typical value of ferulic acid in corn fibre is 1.5-3 % (w/w) of total ferulic acid [33–36]. The total content of esterified ferulic acid in dry corn fibre measured in this work was $1.70 \pm 0.10\%$ ($W_{\text{ferulic acid}} \cdot W^{-1}_{\text{dry corn fibre}}$). This result is in line with the literature. Shin et al. (2006) reported a total content of ferulic acid in corn fibre of 1.85 % ($W_{\text{ferulic acid}} \cdot W^{-1}_{\text{dry corn fibre}}$) [35] and Akin and Rigsby (2007) reported a total content of 1.67 % ($W_{\text{ferulic acid}} \cdot W^{-1}_{\text{dry corn fibre}}$) and an esterified ferulic acid content of 1.48 % ($W_{\text{ferulic acid}} \cdot W^{-1}_{\text{dry corn fibre}}$) [36].

2.2.4.2. Direct enzymatic hydrolysis from corn fibre - screening of commercial enzyme preparations and comparison with alkaline extraction

Five commercial enzyme preparations, Ultraflo® XL, Ultraflo® L, Viscozyme® L, Ultraflo® Max, and Ultraflo® Core were used to release ferulic acid from corn fibre. The evolution of the concentration of the ferulic acid released from corn fibre along time by enzymatic hydrolysis, using different commercial enzyme preparations: , Ultraflo® XL, Ultraflo® L, Viscozyme® L, Ultraflo® Max, and Ultraflo® Core, is shown in Figure 2.5 a). The recovery yields (%) of ferulic acid from corn fibre in the enzymatic hydrolysis and in the mild alkaline extraction are shown in Figure 2.5 b).

The recovery yields (%) of ferulic acid from corn fibre for all experiments were calculated using the following equation:

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$$\text{Recovery yield} = \frac{\text{Ferulic acid recovered} \left(\frac{\text{g ferulic acid}}{\text{g dry corn fibre}} \right)}{\text{Total esterified ferulic acid in corn fibre} \left(\frac{\text{g total esterified ferulic acid}}{\text{g dry corn fibre}} \right)} \times 100 \quad (4)$$

where the total esterified ferulic acid in corn fiber is 1.70 ± 0.10 % ($w_{\text{ferulic acid}} \cdot w^{-1}_{\text{dry corn fibre}}$) as described in section 2.2.4.1.

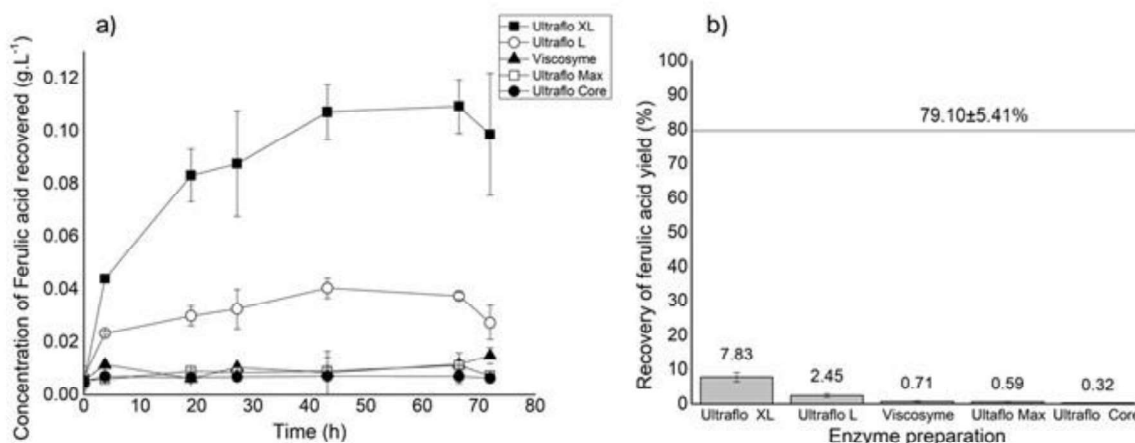


Figure 2.5. Results of enzymatic hydrolysis using commercial enzyme preparations (Ultraflo® XL, Ultraflo® L Viscosyme® L Ultraflo® Max and Ultraflo® Core) incubated in a 2 % enzyme concentration ($w_{\text{enzyme preparation}} \cdot w^{-1}_{\text{dry corn fibre}}$) and of a mild alkaline extraction: a) concentration of the ferulic acid released from corn fibre along time by enzymatic hydrolysis using the different commercial enzyme preparations; b) recovery yield (%) of ferulic acid from corn fibre by the enzymatic hydrolysis and the mild alkaline extraction (line at 79.10 ± 5.41 % recovery yield when using corn fibre without pre-treatment).

As shown in Figure 2.5 a) and b), the highest ferulic acid concentration and the highest ferulic acid recovery yield was obtained in the hydrolysates using the commercial enzyme preparation Ultraflo® XL, when comparing the various commercial enzyme preparations. The concentration of ferulic acid obtained in 43 hours (0.107 ± 0.011 g.L⁻¹) was very similar to that obtained after 72 hours (0.099 ± 0.023 g.L⁻¹), indicating that under the conditions used, Ultraflo® XL can release all the ferulic acid possible in less than 50 hours. The concentration of ferulic acid in these hydrolysates corresponds to a recovery yield of 7.83 ± 1.35 % ($w_{\text{recovery ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$), which corresponds to 0.13 ± 0.02 % ($w_{\text{ferulic acid}} \cdot w^{-1}_{\text{dry corn fibre}}$). However, this recovery is 10 times lower than the recovery yield achieved when using a mild alkaline extraction, performed under optimised conditions, which is 79.10 ± 5.41 % ($w_{\text{recovery ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$) which corresponds to 1.35 ± 0.05 % ($w_{\text{ferulic acid}} \cdot w^{-1}_{\text{dry corn fibre}}$).

According to Novoyes A/S, Ultraflo® XL is a combination of Ultraflo® L and the alpha-amylase BAN 480 L. Ultraflo® L is a commercial enzyme preparation that has already been shown to release ferulic acid from lignocellulosic materials [49,131,146], and exhibits notable enzymes activities on the degradation of lignocellulosic materials such as endo-xylanase, xylosidase, arabinofuranosidase, ferulic acid esterase, cellulose [49,131,147]. Cellulase breaks down the cellulose of plant cell walls, endo-

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xylanase cleaves the xylan backbone internally, xylosidase liberates xylose monomers from the end of xylo-oligosaccharides produced by endo-xylanases, arabinofuranosidase liberates arabinose substituents from the xylan backbone, and ferulic acid esterases release ferulic acid groups from arabinose substituents [148,149]. The combination of Ultraflo® L and the alpha-amylase BAN 480 L had positive effects in the degradation of corn fibre since, with Ultraflo® XL, it is obtained a recovery yield three times higher than with Ultraflo® L, 7.85 ± 1.35 % ($W_{\text{recovery ferulic acid}} \cdot W_{\text{total esterified ferulic acid}}^{-1}$), 2.45 ± 0.5 % ($W_{\text{recovery ferulic acid}} \cdot W_{\text{total esterified ferulic acid}}^{-1}$), respectively).

In the literature, to the best of our knowledge, there are no studies reporting the release of ferulic acid from corn fibre using commercial enzyme preparations or the use of Ultraflo® XL for the release of ferulic acid from lignocellulosic materials. Nevertheless, the commercial enzyme preparation Ultraflo® L was used in enzymatic hydrolysis for ferulic acid recovery from corn cob (another constituent of corn) with a ferulic acid recovery yield of 16.85 % ($W_{\text{recovery ferulic acid}} \cdot W_{\text{total esterified ferulic acid in corn cob}}^{-1}$), which corresponds to 0.19 % ($W_{\text{ferulic acid}} \cdot W_{\text{corn cob}}^{-1}$), with a liquid-to-solid ratio of 10 [49]. Comparing that reported ferulic acid (recovery) yield value to the values obtained in the present work, that is higher than the ferulic acid yield obtained with Ultraflo® L (0.042 ± 0.008 % ($W_{\text{ferulic acid}} \cdot W_{\text{dry corn fibre}}^{-1}$)), but it is similar to the ferulic acid yield obtained in this work with Ultraflo® XL (0.13 ± 0.02 % $W_{\text{ferulic acid}} \cdot W_{\text{dry corn fibre}}^{-1}$). The difference in ferulic acid yields obtained in enzymatic hydrolysis with the same commercial enzyme preparation (Ultraflo® L), although using different feedstocks, may be explained by the more complex structure of corn fibre compared to corn cob [34,46,47].

As the direct enzymatic hydrolysis from corn fibre using the optimised enzyme (Ultraflo® XL) still exhibited a low value of ferulic acid recovery yield (see Figure 2.5), in order to improve this recovery output, the effect of the concentration of the optimised enzyme was evaluated. Specifically, the evolution of the concentration of ferulic acid when using Ultraflo® XL with two different concentrations, 2 % and 40 % ($W_{\text{enzyme preparation}} \cdot W_{\text{dry corn fibre}}^{-1}$) is plotted against time in Figure 2.6 a). The recovery yields (%) of ferulic acid from corn fibre in the two enzymatic hydrolysis are shown in Figure 2.6 b).

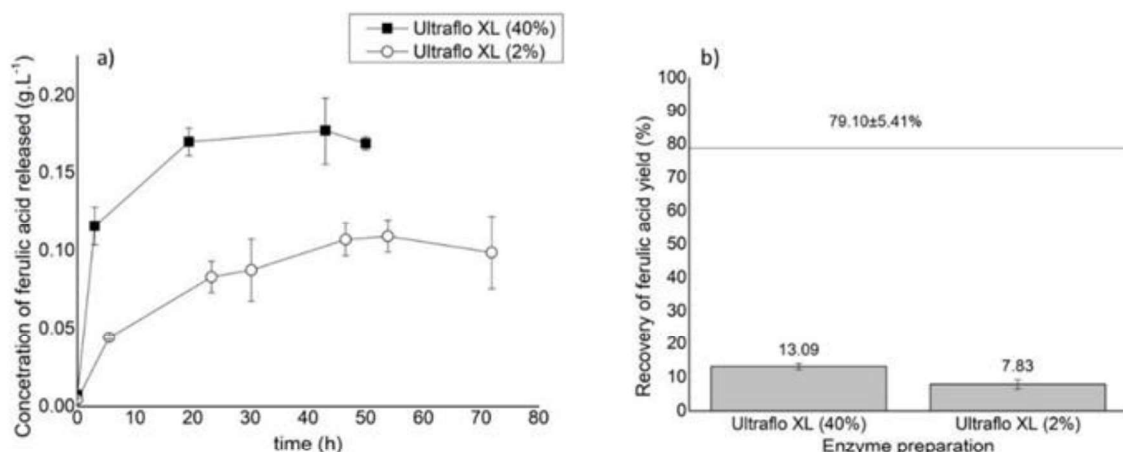


Figure 2.6. Experiments of enzymatic hydrolysis using the optimised commercial enzyme preparation Ultraflo® XL, at 2 % and 40 % ($w_{\text{enzyme preparation}} \cdot w^{-1}_{\text{dry corn fibre}}$): a) concentration of ferulic acid released from corn fibre along time by enzymatic hydrolysis; b) recovery yield (%) of ferulic acid from corn fibre. The line represents the percentage of ferulic acid released under the mild alkaline extraction.

As shown in Figure 2.6 a) and b), the concentration of commercial enzyme preparations did not have a significant effect on the ferulic acid recovery yield. In fact, even though the commercial enzyme preparations were able to reach steady-state in a shorter time (20 hours), increasing the concentration of the commercial enzyme preparation by 20 times, from 2 % to 40 % ($w_{\text{enzyme preparation}} \cdot w^{-1}_{\text{dry corn fibre}}$) led only to an increase of ferulic acid recovery yield from 7.83 ± 1.35 % to 13.09 ± 0.88 %, ($w_{\text{recovery ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$). Consequently, it seems necessary to perform a pre-treatment before enzymatic hydrolysis to improve the ferulic acid recovery yield.

2.2.4.3. Integrated process of the hydrothermal pre-treatment of corn fibre and the enzymatic hydrolysis

In order to improve the recovery yield of ferulic acid, several hydrothermal pre-treatments were applied to corn fibre. The hydrothermal pre-treatment was performed by adding distilled water to the reactor to reach a liquid-to-solid ratio of 10 ($\text{g}_{\text{water}} \cdot \text{g}^{-1}_{\text{dry fibre}}$), using several severity factors ($\text{Log } R_o$). The conditions were selected according to the work by Jiang et al. (2018) that reported a temperature of 165° C and 40 minutes (that corresponds to a severity of 3.5) as optimal conditions for the hydrothermal pre-treatment for releasing ferulic acid from corn bran by enzymatic hydrolysis. In this work, four hydrothermal pre-treatments were tested, at a temperature of 165° C, during 40 minutes and 2 hours ($\text{Log } R_o$ of 3.5 and 4, respectively) and at a temperature of 140° C for 40 minutes and 2 hours ($\text{Log } R_o$ of 2.8 and 3.3, respectively).

The hydrothermal pre-treatment aims to promote the degradation of solid raw material, making it more accessible to enzymes [50–52]. Nevertheless, the exposure of sugars, mainly pentoses (arabinose and xylose) and hexoses (glucose, mannose, galactose and rhamnose) to high temperatures during long

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periods of time leads to their degradation into furfural and hydroxy-methyl-furfural (HMF), respectively [51,52,136,137]. These two compounds may also be degraded into organic acids, such as formic and levulinic acids [52,150]. Therefore, hydrothermal pre-treatments should be designed in order to minimize the formation of these compounds.

Figure 2.7 illustrates the concentration of sugars (Figure 2.7 a), and furfural and HMF (Figure 2.7 b), organic acids (Figure 2.7 c) in the liquid fraction, of each pre-treatment performed.

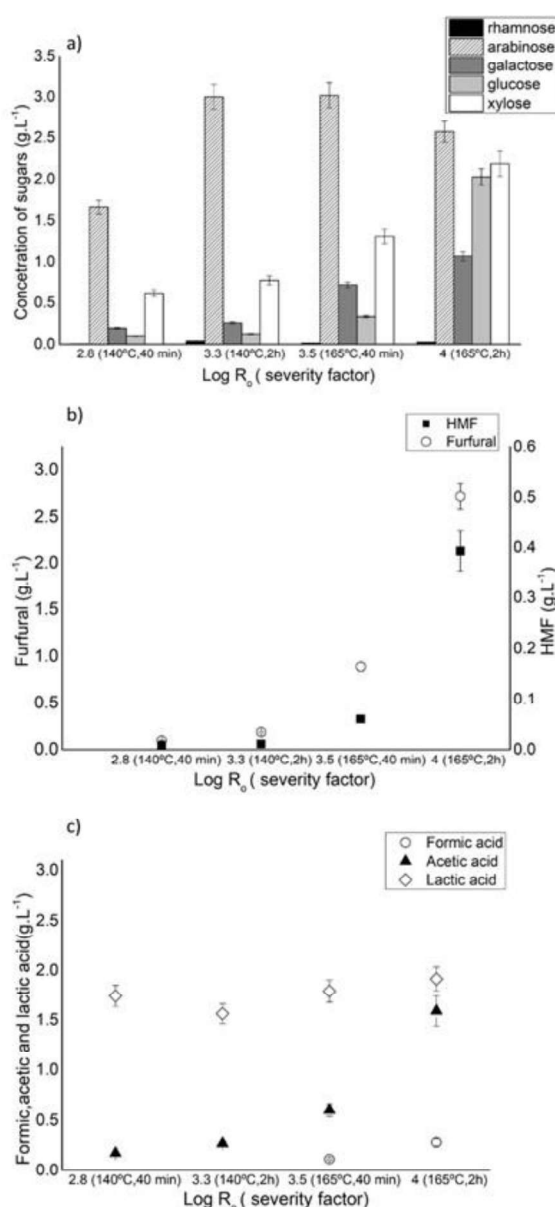


Figure 2.7. Hydrothermal treatments results for corn fibre subjected to four different hydrothermal pre-treatments (140° C for 40 min, 140° C for 2h, 165° C for 40 min and 165° C for 2 h). Concentrations of different compounds after each pre-treatment in the liquid fraction: a) concentration of sugars (rhamnose, arabinose, galactose, glucose, xylose); b) concentration of furfural and hydroxy-methyl-furfural (HMF); c) concentration of organic acids (formic, acetic and lactic acids).

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The more severe the pre-treatment, the greater the breaking of corn fibre structure, leading to an increase in the concentration of sugars (Figure 2.7 a). However, the concentration of arabinose stabilised at a *Log R_o* of 3.3 and decreased at a *Log R_o* of 4. This is because arabinose is released faster than the other sugars [151] so the maximal concentration is always obtained at a lower *Log R_o*. The decrease in arabinose concentration, due to its degradation into furfural, was detected before the other sugars. A similar behaviour is described in Moniz et al. 2014 for the autohydrolysis of rice straw [51]. The concentration of arabinose started to decrease before a *Log R_o* of 4, while the concentration of xylose started to decrease after a *Log R_o* of 4.

As expected, as the severity factor increased, the concentration of furfural and HMF (Figure 2.7 b) also increased due to the higher degradation of the sugars for longer times of pre-treatment and at higher temperatures, achieving $2.71 \pm 0.14 \text{ g.L}^{-1}$ of furfural and $0.39 \pm 0.28 \text{ g.L}^{-1}$ of HMF in the most severe pre-treatment (*Log R_o* of 4). In the less severe pre-treatment (*Log R_o* of 2.8), there was a slight formation of HMF and furfural, $0.0072 \pm 0.0004 \text{ g.L}^{-1}$ and $0.097 \pm 0.005 \text{ g.L}^{-1}$ respectively.

The concentration of furfural obtained in each pre-treatment was higher than that of HMF because furfural is formed from the decomposition of pentoses (arabinose and xylose), which are more highly concentrated in the structure of xylan in corn fibre [33,152] than the others sugars (hexoses), that lead to HMF.

The concentration of organic acids also increased with the *Log R_o* (Figure 2.7 c), except for lactic acid. Formic acid was only present in the most severe pre-treatments (*Log R_o* of 3.5 and 4) because formic acid is produced during the degradation of furfural and HMF [150], which are in high concentration in the most severe pre-treatments (Figure 2.7 b), thus there is a high possibility for these compounds to be degraded, forming formic acid. Acetic acid, which is formed by the removal of the acetyl group from the hemicellulose [46], increased at several combined severity factors, due to the increasing corn fibre degradation.

Lactic acid is produced in the corn wet-milling process by fermentation [65,66], which explains why a similar concentration of lactic acid was detected in the liquid phase of all the pre-treatments that were performed. Lactic acid is not produced during corn fibre degradation though it is present on the surface of corn fibre. The acid levulinic was not detected in any pre-treatment.

Acetic and lactic acid are also present in the samples from alkaline extraction and enzymatic hydrolysis that were performed without pre-treatment (results not shown). These acids are unavoidable since acetic acid is part of the corn fibre structure [33,34,116] and lactic acid is present on the surface of corn fibre due to wet-milling processing, as mentioned before.

Furfural, HMF and formic acid were not detected in alkaline and enzymatic extracts, meaning they are products formed during pre-treatment.

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The milder pre-treatment with a *Log Ro* of 2.8 (140° C during 40 min) led to a lower degradation of sugars and, consequently, lower formation of toxic compounds (furfural and HMF) and formic acid was not detected. These results, however, do not allow the estimation of the recovery yield of ferulic acid in the next step (enzymatic hydrolysis or alkaline extraction).

2.2.4.4. Enzymatic hydrolysis and alkaline extraction from liquid and solid fractions of each hydrothermal pre-treatment

The liquid and solid fractions resulting from the pre-treatment were subjected to enzymatic hydrolysis and a mild alkaline extraction. Although the liquid fraction did not present a significant amount of free ferulic acid (data not shown), this fraction is rich in complex xylan based oligosaccharides [33], so an enzymatic hydrolysis or an alkaline extraction were necessary to release the ferulic acid from the oligosaccharides. Figure 2.8 illustrates the yield of ferulic acid released through a) mild alkaline extraction and b) enzymatic hydrolysis from the solid and liquid fractions of each hydrothermal pre-treatment. The ferulic acid yield of solid and liquid fractions was calculated based on the total content of esterified ferulic acid in the initial corn fibre (1.70 ± 0.10 % ($w_{\text{ferulic acid}} \cdot w^{-1}_{\text{dry corn fibre}}$), it was not calculated based on total content of esterified ferulic acid in solid and liquid fractions.

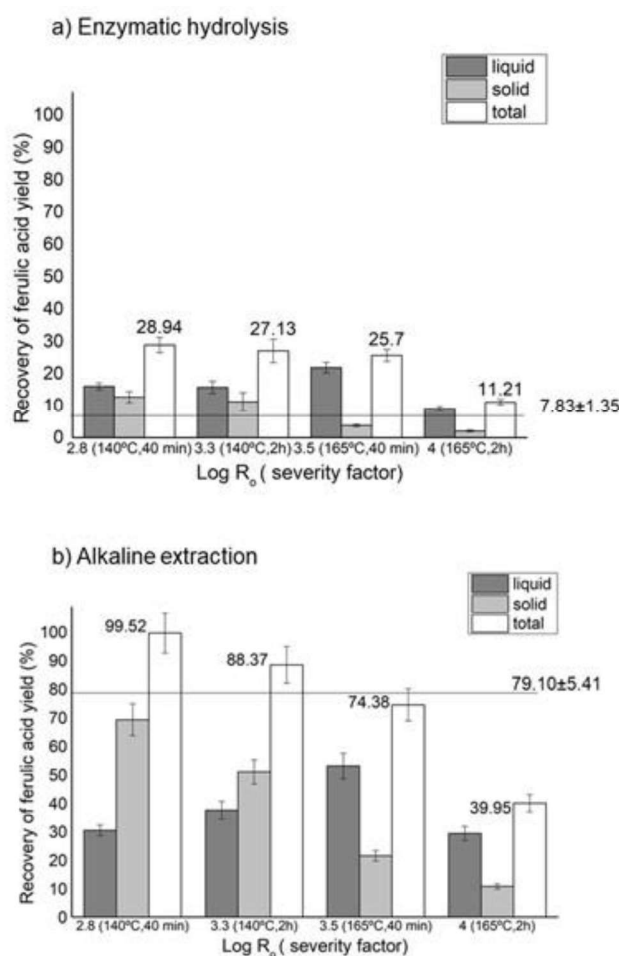


Figure 2.8. Yield of ferulic acid released from corn fibre after hydrothermal pre-treatment through a) enzymatic hydrolysis and b) alkaline extraction. The line represents the total percentage of ferulic acid released in mild alkaline extraction (at 30° C, with 0.25 M NaOH, during 7 h and at 200 rpm for the liquid-to-solid ratio of 25 g_{solvent}·g_{dry fibre}⁻¹) without pre-treatment and in enzymatic hydrolysis (using the commercial enzyme preparation Ultraflo® XL, at 2 %) without pre-treatment.

As shown in Figure 2.8 a) all the enzymatic hydrolysis were improved when the pre-treatment is performed, because ferulic acid becomes more accessible. Pre-treatments with a $Log R_o$ of 3.5, 3.3, and 2.8 presented a similar ferulic acid total yield and showed a slight improvement as the severity factor decreased. The best result was obtained with the least severe pre-treatment ($Log R_o$ of 2.8, 140° C during 40 min), achieving a recovery yield of 28.94 ± 2.4 % ($w_{\text{recovery ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$), which corresponds to 0.49 ± 0.03 % ($w_{\text{ferulic acid}} \cdot w^{-1}_{\text{dry corn fibre}}$), an 3.7 fold increase compared to the result of enzymatic hydrolysis without pre-treatment. A similar improvement was obtained for alkaline extraction performed after pre-treatment, where the best result was found for a $Log R_o$ of 2.8, leading to a recovery yield of ferulic acid of 99.5 ± 7.01 % ($w_{\text{recovery ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$), which corresponds to 1.69 ± 0.07 % ($w_{\text{ferulic acid}} \cdot w^{-1}_{\text{dry corn fibre}}$), as shown in Figure 2.8 b). The most severe pre-treatment (165° C, 2h) with a $Log R_o$ of 4 presented the worst result for both extractions (alkaline and enzymatic), releasing only 40.0 ± 3.0 % $w_{\text{recovery ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$, which corresponds to 0.68 ± 0.03 % ($w_{\text{ferulic acid}} \cdot w^{-1}_{\text{dry corn fibre}}$) of esterified ferulic acid in alkaline extraction, substantially inferior to the yield

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released in alkaline extraction without pre-treatment (79.10 ± 5.41 % ($w_{\text{recovery ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$) corresponding to 1.35 ± 0.05 % ($w_{\text{enzyme preparation}} \cdot w^{-1}_{\text{dry corn fibre}}$)). In enzymatic hydrolysis, the most severe pre-treatment presented a ferulic acid recovery yield of 11.21 ± 0.86 % ($w_{\text{recovery ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$), which corresponds to 0.191 ± 0.09 % ($w_{\text{ferulic acid}} \cdot w^{-1}_{\text{dry corn fibre}}$), only a slight increase compared with the enzymatic hydrolysis without pre-treatment (7.83 ± 1.35 % ($w_{\text{recovery ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$), which corresponds to 0.13 ± 0.02 % ($w_{\text{ferulic acid}} \cdot w^{-1}_{\text{dry corn fibre}}$)). These results, from the worst pre-treatments, probably resulted from the degradation of ferulic acid due to exposure during an excessive time at high temperatures. Previous studies have reported the degradation of this compound between 170-200° C [153,154]. Jing et al. (2018) reported similar results at 175° C for 20 minutes, corresponding to a *Log R_O* of 3.5, with a decrease in the ferulic acid released by the selected enzymes when compared with a lower *Log R_O* [52].

As expected, when the *Log R_O* was increased, the liquid fraction contributed more to the total yield of ferulic acid, while the solid fraction contributed less due to the dissolution of hemicellulose constituents in the liquid fraction.

A *Log R_O* of 2.8 produced the best result in terms of ferulic acid yield and was also the best pre-treatment in terms of formation of fewer undesirable, potentially toxic products, presenting a lower concentration of furfural, HMF, formic acid and acetic acid (Figure 2.7 b).

Despite undergoing a pre-treatment, the ferulic acid recovery yield using enzymatic hydrolysis with the best pre-treatment (*Log R_O* of 2.8) was still 2.7 times lower than a mild alkaline extraction without pre-treatment, 28.94 ± 2.4 % and 79.10 ± 5.41 % ($w_{\text{recovery ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$), respectively.

2.2.4.5. Evaluation of the bioactivity of enzymatic hydrolysates: antioxidant and antiproliferative effect

2.2.4.5.1. Antioxidant activity

In order to evaluate the bioactivity of enzymatic hydrolysates, the liquid and solid dry fractions that resulted from the pre-treatments, followed by enzymatic hydrolysis, were analysed for antioxidant activity and antiproliferative effect using human colorectal cancer cells (HT29 cell line). In parallel, cytotoxicity assays were also performed in confluent Caco-2 cells, to evaluate the gastrointestinal safety of the samples. Since the most severe pre-treatment (165° C, 2 h) with a *Log R_O* of 4 showed the lowest extraction yields of ferulic acid and a higher concentration of compounds resulting from thermal degradation, the liquid and solid phases resulting from this pre-treatment were not evaluated in terms of bioactivity.

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Table 2.6 shows the results of antioxidant activity, measured by oxygen radical absorbance capacity (*ORAC*) expressed as Trolox equivalents per gram of dry hydrolysate ($\mu\text{mol}_{\text{TEAC}} \cdot \text{g}^{-1}$ dry hydrolysate) of each hydrolysate, as well as the ferulic acid and p-coumaric content per gram of dry hydrolysate ([FA] and [CA], respectively). The concentrations of these two phenolic compounds were analyzed, since ferulic acid is the most abundant phenolic compound followed by p-coumaric acid [32,33,36].

Table 2.6. Characterisation of the selected dry hydrolysates in terms of antioxidant (*ORAC*) and antiproliferative activities (*EC*₅₀), and in terms of ferulic acid and p-coumaric acid content.

Enzymatic hydrolysate	<i>ORAC</i> ($\mu\text{mol}_{\text{TEAC}} \cdot \text{mg}^{-1}$ dry hydrolysate)	<i>EC</i> ₅₀ (mg dry hydrolysate, mL ⁻¹)	[FA] (mg ferulic acid·g ⁻¹ dry hydrolysate)	[CA] (mg p-coumaric acid·g ⁻¹ dry hydrolysate)
Without Pre-treatment	0.27±0.04 ^a	>10	3.36±0.02 ^a	0.36±0.03 ^a
Solid of CS 2.8	0.82±0.10 ^{b,c}	2.25±0.35 ^a	4.98±0.03 ^b	1.02±0.07 ^b
Solid of CS 3.3	1.17±0.14 ^d	3.32±0.56 ^a	4.60±0.03 ^c	1.06±0.07 ^c
Solid of CS 3.5	1.05±0.14 ^{d,e}	2.47±0.52 ^a	4.00±0.03 ^d	0.82±0.06 ^d
Liquid of CS 2.8	0.68±0.10 ^{b,f}	8.54±0.45 ^b	4.97±0.03 ^a	0.35±0.02 ^a
Liquid of CS 3.3	0.58±0.10 ^f	7.29±0.10 ^b	5.21±0.04 ^e	0.41±0.03 ^{a,e}
Liquid of CS 3.5	0.89±0.10 ^{c,e}	3.19±0.36 ^a	4.76±0.03 ^f	0.52±0.04 ^e
Ferulic acid	15.6±1.10	0.19±0.03	-	-
p-coumaric	23.0±2.7	0.22±0.01	-	-

Observation: *Log R_o* values of 2.8, 3.3, 3.5 correspond to pre-treatments at 140° C and 40 min, 140° C and 2h, 165° C and 40 min, respectively. Statistical differences between extracts are identified by different letters (p<0.05).

As can be seen, all hydrolysates resulting from the pre-treatments (*Log R_o* of 2.8, 3.3, 3.5) presented higher antioxidant activity (higher *ORAC* value) than the hydrolysates without pre-treatment, confirming that all pre-treatments improved the bioactivity of the hydrolysate. The highest *ORAC* value (> 1 $\mu\text{mol}_{\text{TEAC}} \cdot \text{mg}^{-1}$) was obtained for the solid extract obtained from the pre-treatment with *Log R_o* factors of 3.3 and 3.5 (Table 2.6). The difference in antioxidant activity between the samples can be related to their composition in phenolic compounds. In corn fibre, the most abundant phenolic compound is ferulic acid followed by p-coumaric acid [32,33,36] and both compounds are recognised as having antioxidant properties [155,156]. Although ferulic acid is the most abundant phenolic compound in corn fibre, a direct correlation was not observed ($r^2=0.08$) between the ferulic acid content (mg ferulic acid·g⁻¹ dry hydrolysate) and the antioxidant activity of the samples. This result is in accordance with Boonma et al. 2018, who also observed that the content of ferulic acid in corn hydrolysates was not related to the antioxidant activities, suggesting that antioxidant activity may be attributed to the presence of other compounds in corn [157]. In our work, a stronger correlation was found between the *ORAC* value and

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the concentration of p-coumaric acid ($\text{mg}_{\text{p-coumaric acid}} \cdot \text{g}^{-1} \text{ dry hydrolysate}$) ($r^2=0.58$) suggesting that this phenolic acid could be one of the major contributors to the antioxidant activity of the samples. In fact, when comparing the *ORAC* value of both phenolic acids, p-coumaric acid exhibits a higher antioxidant activity (23.0 ± 2.7 and $15.6 \pm 1.1 \mu\text{mol}_{\text{TEAC}} \cdot \text{mg}^{-1}$ for p-coumaric acid and ferulic acid respectively) (Table 2.6). The values of ferulic acid obtained in Table 2.6 seems have less variation than the values obtained in Figure 2.8. This is explained by the use of different concentration units, the concentration of ferulic acid in Table 2.6 is presented per mass (g) of dry hydrolysate, while in Figure 2.8 the concentration is presented per volume (L) of liquid hydrolysate.

2.2.4.5.2. Antiproliferative activity

Figure 2.9 presents the results of the antiproliferative activity of all extracts in a colorectal cancer cell line (HT29), together with the cytotoxicity effect in a non-differentiated and confluent Caco-2 cell model that mimics the human intestinal epithelium. Because this cell model shares some characteristics with crypt enterocytes, it is considered to be an accepted intestinal model and has been widely implemented to assess the effect of chemical and food compounds on intestinal function [143,144].

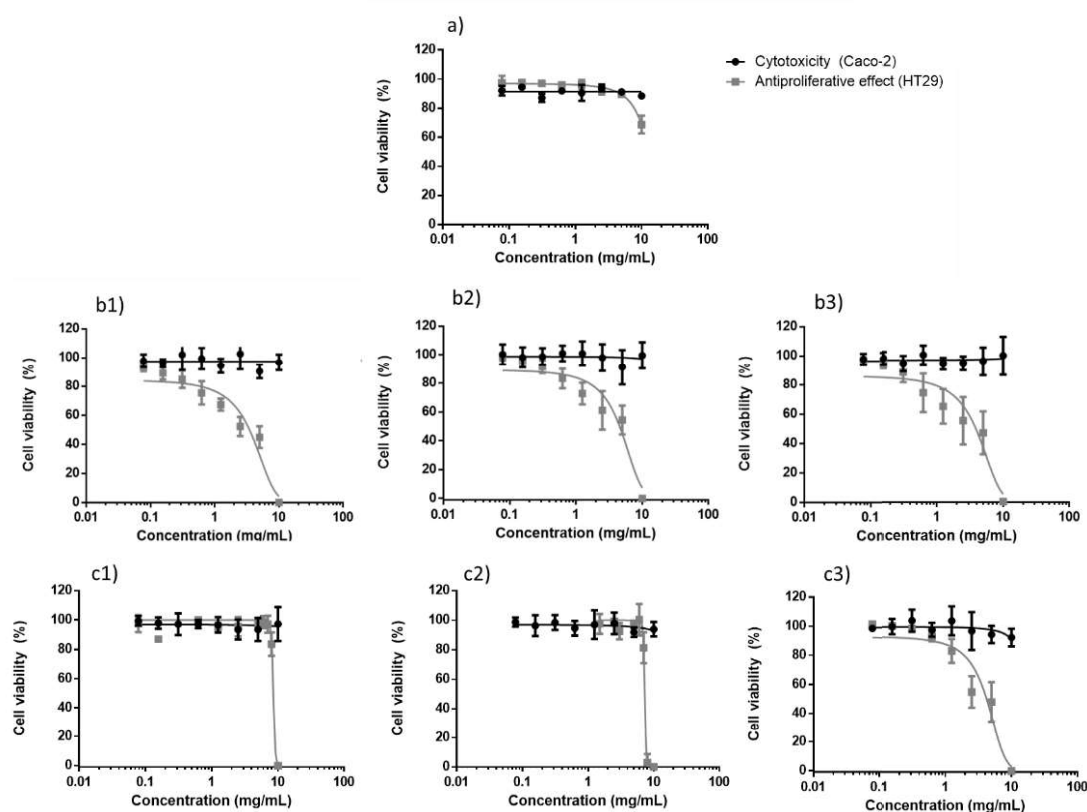


Figure 2.9. Cytotoxicity (●) and antiproliferative effect (■) of dry hydrolysates in Caco-2 and HT29 cells, respectively, after 96 h of incubation a) hydrolysate produced by 2 % (w/w) Ultraflo® XL enzyme preparation without pre-treatment b) c) solid and liquid fraction, respectively, obtained from pre-treatment followed by enzymatic hydrolysis (notations 1, 2 and 3 correspond to pre-treatment at a *Log Ro* of 2.8 (140° C, 40 min), 3.3 (140° C, 2h) and 3.5 (165° C, 40 min), respectively).

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As shown in Figure 2.9, none of the samples reduced the Caco-2 cell viability indicating that they are not cytotoxic up to the maximum concentration that was tested (10 mg.ml^{-1}). Regarding the antiproliferative effect, all samples inhibited the proliferation of HT29 cells after 96 h of incubation. In order to compare the antiproliferative effect of the samples, the value of EC_{50} was calculated (Table 2.6), which measures how concentrated a sample needs to be to decrease cellular viability by 50 % after 96 h of incubation. Table 2.6 shows that all pre-treatments ($\text{Log } R_O$ of 2.8, 3.3 and 3.5) lead to higher antiproliferative activity (lower EC_{50} values) than the hydrolysate produced without the pre-treatment. The highest antiproliferative activity (EC_{50} value $< 3.4 \text{ mg/ml}$) was detected in several samples (the solid fractions resulting from pre-treatments with $\text{Log } R_O$ factors of 2.8, 3.3 and 3.5 and the liquid fraction resulting from pre-treatment with a $\text{Log } R_O$ factor of 3.5) (Table 2.6), which suggests that these samples presented a higher concentration of bioactive compounds with the capacity to inhibit HT29 cell proliferation. For the two phenolic acids studied, besides presenting a similar antiproliferative effect (with similar values of EC_{50} , Table 2.6) [158], a fair correlation was only observed between the value of EC_{50} and the *p*-coumaric acid content (0.68). Therefore, the antiproliferative effect of the samples can be explained by the complexity of the corn fibre hydrolysates and could be attributed to the presence of other compounds such as xylooligosaccharides, which have already been reported as antiproliferative compounds in several cancer cell lines, such as hepatic carcinoma cells (HepG2) [159] and also in human colorectal cancer cells (HT29) (the same cells used in this work) [160]. In general, after undergoing pre-treatment followed by enzymatic hydrolysis, the solid fraction presented the best results in terms of antiproliferative and antioxidant activity, indicating that in pre-treated corn fibre there are still many bioactive compounds that are not solubilised in the liquid fraction. The pre-treatment at a $\text{Log } R_O$ of 3.5 led to an overall production (liquid plus solid fractions) of dry enzymatic hydrolysates with the highest bioactivity (Table 2.6).

2.2.5. Conclusions

None of the commercial enzyme preparations tested was able to completely release ferulic acid from corn fibre cell walls due to the very complex structure of corn fibre. Under the best conditions tested, using the commercial enzyme preparation (2 % ($\text{W}_{\text{Ultraflo}}^{\text{® XL}} \cdot \text{W}^{-1} \text{ dry corn fibre}$)), it was only-possible to release about 7.83 ± 1.35 % of ferulic acid ($\text{W}_{\text{recovery ferulic acid}} \cdot \text{W}^{-1} \text{ total esterified ferulic acid}$) corresponding to 0.13 ± 0.02 % ($\text{W}_{\text{ferulic acid}} \cdot \text{W}^{-1} \text{ dry corn fibre}$) of esterified ferulic acid from corn fibre. It was therefore necessary to apply a hydrothermal pre-treatment to the corn fibre, in order to improve the recovery of ferulic acid.

The hydrothermal pre-treatment conditions had a great influence on the amount of ferulic acid that was released but they also determined the concentration of undesirable products released. The increase of the severity factor (used to evaluate the combined impact of the temperature and the time used in the pretreatments to hydrolyse hemicellulose) increased the concentration of furfural,

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hydroxymethylfurfural and formic acid and had no benefit on the release of ferulic acid from corn fibre. Severe conditions (a severity factor of 4) led to ferulic acid degradation.

Hydro-thermal pre-treatment at a temperature of 140° C for 40 minutes improved access of the Ultraflo® XL enzyme preparation and consequently improved the yield of recovery of ferulic acid from 7.83 ± 1.35 % ($w_{\text{recovery ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$), corresponding to 0.13 ± 0.02 % ($w_{\text{ferulic acid}} \cdot w^{-1}_{\text{dry corn fibre}}$), to 28.94 ± 2.4 % ($w_{\text{recovery ferulic acid}} \cdot w^{-1}_{\text{total esterified ferulic acid}}$), which corresponds to 0.49 ± 0.03 % ($w_{\text{ferulic acid}} \cdot w^{-1}_{\text{dry corn fibre}}$), with a minimum concentration of undesirable compounds (furfural, hydroxymethylfurfural, organic acids). Although the hydrothermal pre-treatment made the corn fibre more accessible to enzymes, and improved the yield of ferulic acid released, the results achieved by enzymatic hydrolysis using commercial enzyme preparations were inferior to the results achieved in mild alkaline extraction (at a low concentration of NaOH and low temperature). It is important to note that all pre-treatments (with *Log Ro* factors of 2.8, 3.3, 3.5) followed by enzymatic hydrolysis (with 2 % (w/w) of Ultraflo® XL enzyme preparation per dry corn fibre) improved the quality of the hydrolysate in terms of antioxidant and antiproliferative effects. This bioactive effect can be attributed not only to ferulic acid but also to other compounds that are present in this type of matrix, such as *p*-coumaric acid and probably xylooligosaccharides. The pre-treatment with a *Log Ro* factor of 3.5 followed by enzymatic hydrolysis led to overall production of dry enzymatic hydrolysates with the highest bioactivity.

3. Purification of ferulic acid

3.1. Effect of ultrafiltration operating conditions for separation of ferulic acid from arabinoxylans in a corn fibre alkaline extract

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The author was involved in planning and performing all the experiments, as well as on the discussion and interpretation of all the results and preparation of the manuscript.

3.1.1. Summary

Corn fibre, a co-product of starch industry, is rich in compounds with high added-value such as ferulic acid and arabinoxylans, which are released during alkaline extraction. This work aims to optimise an efficient separation method for the recovery of these two compounds (ferulic acid and arabinoxylans) from a corn fibre alkaline extract, allowing an efficient valorisation of this co-product. Ultrafiltration was selected as separation method due to its potential to fractionate these compounds. In order to minimise the loss of membrane permeance, due to mass transfer limitations caused by the high arabinoxylan viscosity, the impact of relevant ultrafiltration operating parameters (membrane molecular weight cut-off, fluid dynamics conditions, transmembrane pressure and operating temperature) were evaluated. A Nadir UP 150 membrane was found to be an adequate choice, allowing for an efficient separation of ferulic acid from arabinoxylans, with null rejection of ferulic acid, a high estimated rejection of arabinoxylans 98.0 ± 1.7 % and the highest permeance of all tested membranes. A response surface methodology (RSM) was used to infer the effect of ultrafiltration conditions (cross-flow velocity, transmembrane pressure and operating temperature) on the rejection of ferulic acid, retention of arabinoxylans (assessed through apparent viscosity of the retentate stream) and permeance. Through mathematical modelling it was possible to determine that the best conditions are the highest operating temperature and initial cross-flow velocity tested (66°C and 1.06 m.s^{-1} , respectively) and the lowest transmembrane pressure tested (0.7 bar).

3.1.2. Introduction

The most abundant low-value co-product (used for animal feed) of the corn wet milling process is corn fibre [161], which contains a significant fraction of valuable phenolic compounds, mainly ferulic acid. Corn fibre contains between 1.5-3 % of ferulic acid ($w_{\text{total ferulic acid}} \cdot w_{\text{dry corn fibre}}^{-1}$) [33–36]. Ferulic acid presents several recognised benefits, namely antioxidant, anti-inflammatory, antiatherogenic, antidiabetic, antiaging, neuroprotective, radioprotective, hepatoprotective, and antiproliferative properties [102,105,106], which make this compound widely used in the food, cosmetics and

Chapter 3 – Purification of ferulic acid

pharmaceutical industries. It can also be used as a crosslinking agent for the preparation of films [107,108] and as precursor of bio-vanillin production [1,28]. Ferulic acid in corn fibre is mainly covalently linked by ester bonds to arabinoxylans. Arabinoxylans, also designated as corn fibre gum, are valuable non-starch cereal polysaccharides [110], with many recognized health benefits, such as a prebiotic, for the control of diabetes, immunomodulatory activity and a cholesterol lowering effect [76,162,163]. They also have a functional application in edible film forming [77,164] and as an emulsifier for oil-in-water emulsion systems [78].

Alkaline extraction is the most commonly reported method for releasing ferulic acid [43–45,56,57] from agro-industrial by-products. However, alkaline extraction also releases arabinoxylans [58–60]. It is not possible to use a sequential two-step extraction process, using different operating conditions, for the release of ferulic acid and then of arabinoxylans. Hence, in order to achieve an efficient recovery of these two compounds, for their use in different applications, an efficient separation is required. This separation is quite challenging due to the high viscosity of the alkaline extract caused by the presence of arabinoxylans [56]. In fact, the high viscosity of arabinoxylans makes difficult to use adsorption to purify ferulic acid in an alkaline extract [56].

Therefore, the development of a process able to efficiently separate these two compounds is very important for the valorisation of corn fibre extracts and the future use of both compounds. Ultrafiltration seems to be a suitable option for the separation of ferulic acid from arabinoxylans due to the high molecular mass difference between the two compounds. Although the molecular mass of arabinoxylans is not constant (it fluctuates depending on the raw material and on the alkaline extraction conditions [58]), high molecular masses in the range of 244 to 491 kDa have been reported for arabinoxylans extracted from corn fibre [80]. This range of masses makes possible the use of ultrafiltration to separate arabinoxylans from ferulic acid, which has a molecular mass of 194 Da. Ultrafiltration has already been reported as a method to separate arabinoxylans from ferulic acid and other compounds present in different extracts (e.g. alkaline-alcohol extract from corn bran [56], alkaline-alcohol extract from wheat bran [81] and alkaline extract from wheat bran [59,79,165]).

However, the high viscosity of arabinoxylans can also influence the ultrafiltration process, leading to poor fluid dynamic conditions and, ultimately, causing membrane fouling due to the formation of a gel layer on the membrane surface [81]. In order to overcome the viscosity problem of arabinoxylans, strategies have been reported involving the addition of ethanol during alkaline extraction, which decreases the viscosity of the extracts [56,81], and the use of dia-ultrafiltration [59]. However, the use of ethanol during the alkaline extraction decreases the ferulic acid released from the biomass [56] so the use of this organic solvent should be avoided. Dia-ultrafiltration allows to maintain a constant viscosity in the retentate stream by addition of a solvent (at the same flow-rate as the permeate is removed) but a diluted permeate stream is obtained requiring further concentration of ferulic acid, which is expensive (a reverse osmosis or thermal process will be required for that purpose). Aiming at recovering not only the arabinoxylans, but also the ferulic acid, and since the ferulic acid is not expected to be rejected by

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the ultrafiltration membrane, an ultrafiltration process was selected instead of dia-ultrafiltration. The loss of ferulic acid in the retentate by processing the corn fibre extract by ultrafiltration is minimised by operating until achieving a high concentration factor. Dia-ultrafiltration has only been reported for the purification of arabinoxylans, without recovery of other valuable compounds, such ferulic acid from the permeate [59].

The purification of ferulic acid by ultrafiltration from alkaline extracts was reported, not using alkaline extracts from corn fibre, but from wheat bran [81] and from corn bran [56]. Also, in the reported cases, ethanol was used to decrease the viscosity of the extracts. The purification of arabinoxylans from alkaline extracts (or a steam explosion extract [166] was reported, using wheat bran [59], wheat bran and wheat straw [165] and barley husks [166], as feedstocks. The purification of arabinoxylans was performed by ultrafiltration [165,167] by ultrafiltration operated in a diafiltration mode (dia-ultrafiltration) [59] or by ultrafiltration followed by dia-ultrafiltration [166]. The main innovation of this work relies of the purification of both ferulic acid and of arabinoxylans using corn fibre alkaline extract, where no solvent, such ethanol, was added (in order to decrease the viscosity of the extracts) and where the optimisation of the process involved the use of a design of experiments approach considering main operating conditions of ultrafiltration (operating temperature, initial cross-flow velocity and transmembrane pressure). Consequently, increasing the overall sustainability of the corn wet milling process through the efficient, and also sustainable, valorisation of corn fibre.

Therefore, this work evaluates the use of ultrafiltration for the separation of ferulic acid from arabinoxylans in alkaline extracts from corn fibre rich in ferulic acid. In order to minimise the loss of membrane permeance induced by the high arabinoxylan viscosity, the most relevant ultrafiltration operating parameters were studied: membrane molecular weight cut-off, cross-flow velocity of the feed stream, transmembrane pressure and operating temperature. First, the type of membrane was evaluated by analysing the behaviour of each membrane in a dead-end configuration cell in terms of ferulic acid rejection, arabinoxylans retention (assessed through the apparent viscosity of the retentate and rejection of the sugar constituents of arabinoxylans), and permeance.

The most suitable membrane was selected and was then used to set the other operating parameters (cross-flow velocity, transmembrane pressure and operating temperature) using a response surface methodology (RSM). Response surface methodology (RSM) is a multivariate regression tool based on statistical analysis, which allows the factors and response variables to be correlated using regression models that are optimised for each response studied [168,169]. RSM is combined with design of experiments (DoE) to find an optimal response. DoE introduces variation in the independent factors that directly affect the variation of responses and allows a reduction in the number of experiments required to develop a model [169]. In this work, this statistical method was used to establish the correlation between the factors (operating temperature, cross-flow velocity, and transmembrane pressure), and the responses (rejection of ferulic acid, retention of arabinoxylans, measured through the viscosity of

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retentate stream, and permeance), in order to define the best ultrafiltration conditions for an efficient separation of ferulic acid from arabinoxylans while maintaining a good permeance.

3.1.3. Materials and Methods

3.1.3.1. Materials

The extracts were obtained by a mild alkaline extraction under the following optimised conditions: 0.25 M NaOH, at 30° C, for 7 hours, using a liquid-to-solid ratio of 10 g_{solvent}.g_{dry fibre}⁻¹, using wet corn supplied by COPAM, Companhia Portuguesa De Amidos, S.A. (São João da Talha, Portugal) after the wet milling process (chapter 2.1). The alkaline extracts were then centrifuged at 9174× g for 15 minutes.

Four membranes with different molecular weight cut-offs and materials were selected. Since corn fibre arabinoxylans have a molecular weight ranging from 244 to 491 kDa [80], membranes with a molecular weight cut-off ranging from 50 to 150 kDa were selected to ensure the retention of these compounds. Membranes with good stability at high operating temperature and pH were chosen. High operating temperature allows for reducing the viscosity of the streams containing arabinoxylans during membrane processing, increasing permeance. Regarding the material, it was decided to test membranes made of different materials to cover a good range of hydrophobicity/hydrophilicity.

Their main characteristics are summarised in

Table 3.1.

Table 3.1. Summary of membrane properties tested in this work.

Membrane	Manufacturer	Material	MWCO (kDa)	Max. Temperature (°C)	pH
UP150	Microdyn-Nadir	PES ^a	150	95	0 - 14
US100	Microdyn-Nadir	PSH ^b	100	95	1 - 14
UH50	Microdyn-Nadir	PESH ^c	50	95	0 - 14
PL series	Merk-Millipore	RC ^d	100	50	2 -13

^a Polyethersulfone

^b Hydrophilic Polysulfone

^c Hydrophilic Polyethersulfone

^d Regenerated Cellulose

3.1.3.2. Methods

3.1.3.2.1. Screening of ultrafiltration membranes

Ultrafiltration experiments for membrane selection were performed in dead-end mode under controlled transmembrane pressure conditions. The dead-end module used was METCell (Membrane Extraction

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Technology, United Kingdom) (Figure 3.1), using flat sheet membranes with an effective membrane area of 51.4 cm². The feed reservoir (ultrafiltration cell) was filled with 230 g of alkaline extract (the same lot for all membrane experiments) with an apparent viscosity of 3.13 ±0.05 mPa.s.

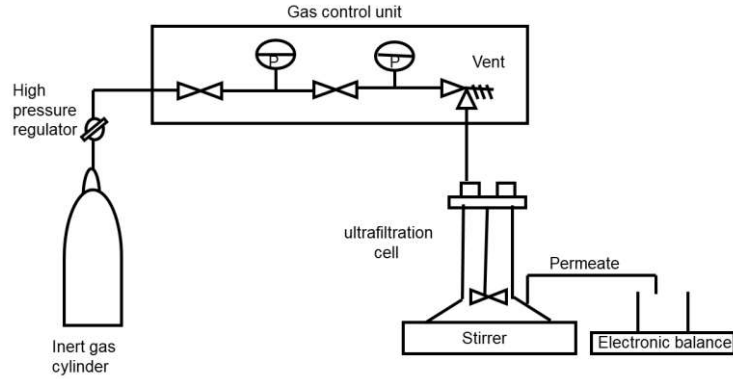


Figure 3.1. Experimental ultrafiltration (UF) set-up in dead-end module.

Membrane filtrations were carried out at constant transmembrane pressure (the difference between the pressure at the feed compartment, 0.7 bar relative pressure, obtained using a gas pressurizing unit, and the pressure at the permeate compartment, 0.0 bar relative pressure). Stirring was done with a cross-head magnetic bar, set at 250 rpm. The control of operating temperature at 50° C (maximum operating temperature for Millipore membranes) in the cell was carried out by immersing the membrane module in a controlled temperature bath. The permeate was collected and its mass was recorded continuously with an electronic balance (Kern 572, Kern, Germany) connected to a computer for data acquisition. The concentration factor, CF, was calculated for each membrane ultrafiltration experiment, using the following equation:

$$CF = \frac{m_0}{m_0 - m_{permeate}} \quad (5)$$

Where, m_0 (g) is the mass in the feed vessel at the beginning of the experiment and $m_{permeate}$ (g) is the mass of permeate recovered up to a given instant. The concentration factor at the end of each experiment, $CF_{t=final}$, was 7. In each experiment, the initial and final feed (final retentate) solutions and the final cumulative permeate solution were characterised in terms of content of ferulic acid and arabinoxylans. The content of the arabinoxylans in the samples was analysed through the quantification of the four main neutral sugars that make up the structure of arabinoxylans (arabinose, xylose, glucose, galactose) [59,170], which were obtained by acid hydrolysis.

The global rejection of each compound i , R_i (%), was calculated using the following equation:

$$Ri(\%) = \left(1 - \frac{C_{perm,overall,i}(t=t(final))}{C_{feed,i}(t=t(final))} \right) \times 100 \quad (6)$$

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Where $C_{perm,overall,i(t=tfinal)}$ (g.kg⁻¹ of extract) and $C_{feed,i(t=tfinal)}$ (g.kg⁻¹ of extract), are respectively the final cumulative concentration in the permeate and the final feed (final retentate) concentration of compound i .

In order to assess membrane fouling phenomena, the adsorption of each compound i (%) in each experiment was calculated using the following equation:

$$Adsorption, i (\%) = 1 - \frac{m_{feed,i(t=tfinal)} + m_{perm,i(t=tfinal)}}{m_{feed,i(t=0)}} \times 100 \quad (7)$$

Where $m_{feed,i(t=tfinal)}$ (g) is the mass of the compound in the retentate, m_{perm} (g) is the mass of permeate at the end of each experiment and (g) is the mass in the initial feed stream of compound i .

Where $m_{feed,i(t=tfinal)}$ (g) is the mass of the compound in the final retentate, m_{perm} (g) is the cumulative mass of permeate at the end of each experiment and (g) is the mass of compound i in the initial feed stream.

The rejection and adsorption of all arabinoxylans were estimated by, respectively, the average of rejections (equation 6) and of adsorptions (equation 7) of the neutral sugars that constitute the structure of arabinoxylans (arabinose, xylose, glucose, and galactose).

The apparent viscosity was measured at 20° C at the end of each ultrafiltration experiment. It was measured in the retentate for all experiments. Since arabinoxylans may be considered the only compounds in corn fibre extracts/fractions relevant to contribute for the viscosity of the solutions, the viscosity of the retentate and the permeate solutions in these experiments were related to the concentration of the arabinoxylans in these streams [56,60]. High viscosity of the retentate and low viscosity in permeate mean that the membrane is efficiently retaining the arabinoxylans.

The permeance P (L.m⁻².h⁻¹.bar⁻¹) was calculated using the following equation:

$$P = \frac{V_{perm}}{A \times t \times \Delta p} \quad (8)$$

where V_{perm} (L) is the permeate volume, t (h) the time of permeation, A (m²) the membrane effective area and Δp (bar) the transmembrane pressure.

The average permeance $\bar{P}$ (L.m⁻².h⁻¹.bar⁻¹) was calculated through equation 8 considering V_{perm} (L) as the total permeated volume and t (h) as the total time of permeation.

Membrane selection was based on rejection of ferulic acid, retention of arabinoxylans (measured by the viscosity of the retentate and by the rejection of the sugars constituents of arabinoxylans), and permeance.

3.1.3.2.2. Evaluation of the effect of operating temperature, cross-flow velocity and transmembrane pressure in ultrafiltration using design of experiments (DoE)

In order to study the effect of the operating temperature, the initial cross-flow velocity (as cross-flow velocity varies during each experiment because the retentate viscosity increases during each experiment) and transmembrane pressure, the ultrafiltration experiments were performed in a GE-Sepa CF cross-flow module (GE Osmonics, USA) connected to a feed pump (Hydra-cell model G13, Wanner Engineering Inc., USA). The membrane had an effective membrane area of 140 cm². The permeate mass was measured continuously with a Kern 572 balance connected to a computer to record the data. The Figure 3.2 illustrates the experimental set-up in cross-flow module.

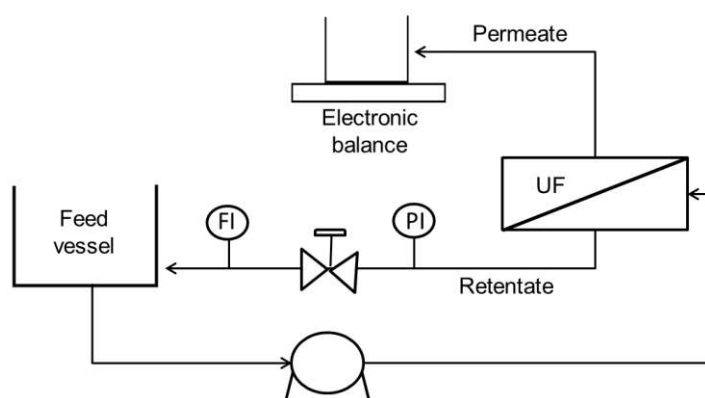


Figure 3.2. Experimental ultrafiltration (UF) set-up of the cross-flow. PI and FI refer to pressure transducer and flow-rate sensor, respectively.

Each experiment started with 2 L of alkaline extract (the same lot was used for all cross-flow experiments) with an apparent viscosity of 6.97 ± 0.133 mPa.s at 20° C. The extract was then concentrated to the minimum volume possible (the dead volume of the membrane unit), achieving a final concentration factor of 3.5.

For the experimental design, three independent factors were considered: the operating temperature (°C), the initial cross-flow velocity (m.s⁻¹) and the transmembrane pressure (bar). A design of experiments methodology was applied using a full factorial design with 2 replicas of the central point. The Table 3.2 shown the experimental design used in this work, which allowed the 3 factors to be correlated at two levels (minimum and maximum values of the 3 factors) using 10 experiments. The minimum and the maximum values of the 3 factors selected were: operating temperature of 30 and 70° C, initial cross-flow velocity of 0.60 m.s⁻¹ and 1.07 m.s⁻¹ and transmembrane pressure of 0.7 and 3 bar. The minimum temperature was selected taking in account the operating temperature used during the previous step of the process, alkaline extraction at 30° C. Although the membranes selected allowed a maximum operating temperature higher than 70° C, this value was selected due to possible constraints at real scale, because large membrane modules comprising these membranes refer commonly 80° C as the maximum operating temperature.

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The initial cross-flow velocity and the transmembrane pressure are set independently but, when using a high cross-flow velocity, the minimum achievable transmembrane pressure (with the retentate valve totally open) was higher than 0.7 bar. Therefore, in these experiments it was selected a maximum velocity of 1.07 m.s⁻¹, to avoid the increase of the transmembrane pressure. Using this rational, the minimum transmembrane pressure was set at 0.7 bar. Above a transmembrane pressure of 3 bar we anticipate a fast and significant fouling of the membrane.

When required, the transmembrane pressure was adjusted on the retentate side with a needle valve and measured with a pressure gauge. The operating temperature of the feed was controlled with a temperature-controlled bath (FP50, Julabo, USA) connected to the jacketed feed vessel. The initial cross-flow velocity in the feed compartment of the membrane element was controlled by adjusting the pump speed and measuring the flow rate.

Table 3.2. Design of experiments (DoE) – full factorial design matrix, T (°C) is operating temperature, $v_{initial}$ is the initial cross-flow velocity (m.s⁻¹) and Δp (bar) the transmembrane pressure.

Exp.	T (°C)	$v_{initial}$ (m.s ⁻¹)	Δp (bar)
1	30	0.60	0.7
2	70	0.60	0.7
3	30	1.07	0.7
4	70	1.07	0.7
5	30	0.60	3.0
6	70	0.60	3.0
7	30	1.07	3.0
8	70	1.07	3.0
9	50	0.80	1.9
10	50	0.80	1.9

In order to ascertain the optimal conditions, three responses were analysed: the rejection of ferulic acid, the viscosity of the retentate solution (used as measure of the concentration of arabinoxylans) and membrane permeance. For the response surface methodology (RSM), a partial least squares regression (PLS) model was used to assess each factor individually as well as analysing the linear interactions between factors. The DoE and data analysis were performed using the MODDE Go 12.1 software. The statistical parameters used to evaluate the quality of the model (R^2 , Q^2 , Model validity and reproducibility) were calculated using the software, as defined in the User Guide to MODDE [171].

3.1.3.3. Analytical methods

The concentration of ferulic acid was determined using high performance liquid chromatography (Alliance e2695, Waters, USA) with a Nova-Pak® C18 3.9 mm×150 mm column (particle diameter of 4 mm) and a guard column, using a UV detector at 280 nm. The injection volume of each sample was 20 µL, and the column temperature was 30° C. Ferulic acid was analysed with a gradient elution program

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at the flow rate of 0.5 mL.min⁻¹. The mobile phase used a mixture of two eluents: eluent (A) contained 10 % (v/v) methanol and 2 % (v/v) acetic acid in Milli-Q water; eluent (B) contained 90 % methanol and 2% acetic acid in Milli-Q water. The gradient elution program was 0 % B (0–10 min), from 0 % to 15 % B (10–25min), from 15 % to 50 % B (25–35 min) and from 50 % to 0 % B (35-38 min).

The neutral sugar content in the arabinoxylans was determined based on the acid hydrolyses of polysaccharides as described in Carvajal-Millan et al. 2005 [170]. Briefly, the hydrolysis was performed by using 5 mg of dry sample suspended in 5 mL of deionised water and adding 0.1 mL of TFA (trifluoroacetic acid) at 120°C for 2 hours. The reaction was stopped by submerging the sample in ice and the neutral sugars were analysed using a HPLC (Dionex, Thermofisher scientific, USA) equipped with an amperometric detector and a CarboPac PA10 column. The analysis was performed at 30° C with an aqueous sodium hydroxide (NaOH 18mM) as the eluent, at a flow rate of 1 mL min⁻¹.

The apparent viscosity of the extracts was measured at 20° C using a digital viscometer (FungiLab Alpha Series, Spain) at different rotational speeds in the interval of 100 to 0.3 rpm, with 16 mL of each sample. All samples exhibited a Newtonian behaviour.

3.1.4. Results and discussion

3.1.4.1. Screening of ultrafiltration membranes

The aim of fractionation is to obtain a permeate rich in ferulic acid and a retentate with the maximum concentration of arabinoxylans. Membrane selection was accomplished with a first set of experiments, performed in a dead-end cell. Since corn fibre arabinoxylans have a molecular weight from 244 to 491 kDa [80], membranes of different materials with a molecular weight cut-off ranging from 50 to 150 kDa were evaluated in order to characterise their behaviour concerning the rejection of ferulic acid (targeting as low as possible values) and their permeance for ferulic acid (targeting high values). Regarding the arabinoxylans, the viscosity of the final retentate and the estimated rejection of arabinoxylans needed to be high, as the main objective was the effective separation between arabinoxylans and ferulic acid.

The first set of membrane experiments was performed at 0.7 bar and 50° C using 230 mL of alkaline extract with an apparent viscosity of 3.13 ± 0.05 mPa.s at 20° C. The main results are shown in Table 3.3,

Figure 3.3, Figure 3.4. Table 3.3 shows the rejection of ferulic acid and of the four sugars that compose arabinoxylans and the estimated rejection of arabinoxylans; it also shows the viscosity of the permeate and the retentate at the end of each membrane experiment.

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Table 3.3. Rejection of ferulic acid and of the four sugars present in arabinoxylans, estimated rejection of arabinoxylans and apparent viscosity of permeate and retentate, at the end of each experiment performed by dead-end ultrafiltration at 50° C, pressure of 0.7 bar until a concentration factor of 7.

Membrane	$R_{\text{ferulic acid}} (\%)$	$R_{\text{glucose}} (\%)$	$R_{\text{xylose}} (\%)$	$R_{\text{arabinose}} (\%)$	$R_{\text{gactose}} (\%)$	$R_{\text{average}}^a (\%)$	$\mu_{\text{retentate}} (\text{mPa.s})$	$\mu_{\text{permeate}} (\text{mPa.s})$
UH50	0	97.1	99.4	99.4	98.9	98.7±1.1	158.54	1.37
UP150	0	95.6	99.2	99.1	98.1	98.0±1.7	126.5	1.35
US 100	0	89.5	96.4	96.7	94.9	94.4±3.3	115.26	1.30
PL	0	87.3	97.0	96.5	94.7	93.9±4.5	85.54	1.34

^a estimated rejection of arabinoxylans calculated by the averages of the rejections of the neutral sugars that constitute arabinoxylans

As shown in Table 3.3, all membranes proved to be efficient in separating ferulic acid from arabinoxylans. For all membranes tested, no rejection of ferulic acid was observed (represented as 0 % in this work), meaning that ferulic acid concentration in the final permeate and in final retentate is the same (equation 6). Ferulic acid passes freely through the membrane due to its low molecular weight (194 Da). The rejection of arabinoxylans measured as sugar monomers and the estimated rejection of arabinoxylans (based on the average of the global rejection of neutral sugars) was higher than 94 % and the viscosity of the retentates (85.54-158.54 mPa.s) was much higher than the permeates (1.30-1.37 mPa.s), indicating the efficient separation of the arabinoxylans from ferulic acid. The viscosity of the retentate (PL <Nadir US 100 <Nadir UP 150 <Nadir UH 50) follows the same order as the rejection of the 4 sugar monomers, indicating that high viscosity in the retentate means a high rejection of arabinoxylans, as expected.

More hydrophilic membranes, such as US (made of polysulfone with a hydrophilic treatment) and PL (made of regenerated cellulose), exhibited slightly lower rejection of arabinoxylans (which can be seen from the results obtained for the sugar monomers) and their retentates presented lower viscosities, as expected. On the other hand, the more hydrophobic polyethersulfone-based membranes, either with hydrophilic treatment (UH 50) or without (UP 150), presented a slightly better performance as they rejected the arabinoxylans, presenting an average rejection of these compounds of 98.7± 1.1 % and 98.0± 1.7 %, respectively.

Figure 3.3 shows the permeance in relation to the concentration factor for each membrane as well as the average permeance. Figure 3.4 shows membrane UP150 after the experiments (the other membranes exhibited the same behaviour, data not shown). Figure 3.5 represents the adsorption of ferulic acid and arabinoxylans on the membrane.

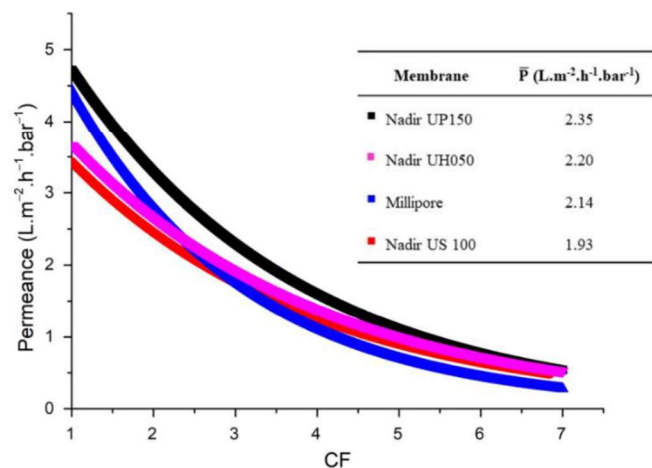


Figure 3.3. Evolution of permeance versus concentration factor (CF) for four different ultrafiltration membranes at 50° C, pressure of 0.7 bar until a concentration factor of 7.

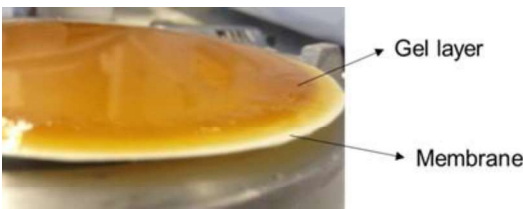


Figure 3.4. Image of the UP150 membrane at the end of the experiment at 50° C, pressure of 0.7 bar until a concentration factor of 7 (all membranes presented a similar gel layer).

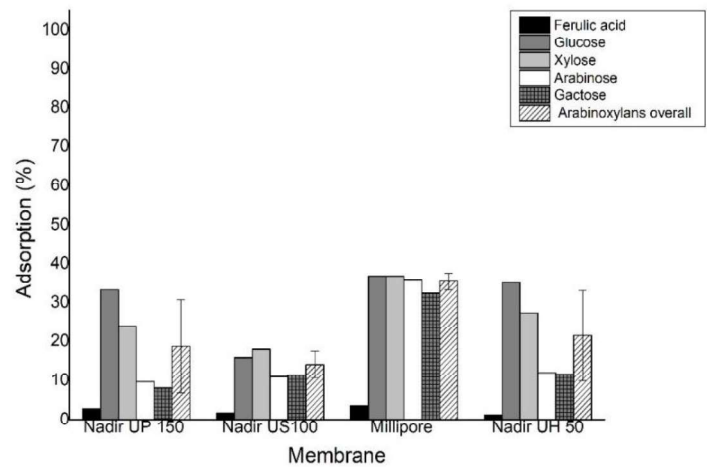


Figure 3.5. Adsorption of ferulic acid and the main sugars (glucose, xylose, arabinose, galactose) that constitute the structure of arabinoxylans to four different ultrafiltration membranes at 50° C, pressure of 0.7 bar until a concentration factor of 7.

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As shown in Figure 3.3, it was possible to achieve a concentration factor of 7. The achievement of such high concentration factor is important to minimise the loss of ferulic acid in the retentate. Particularly, a maximum concentration factor of 7 was achieved due to the increase in retentate viscosity, and it corresponds to the reasonable value of 85 % of recovery of ferulic acid. The average permeance obtained for all membranes was modest for an ultrafiltration process. This can be explained by the formation of a gel layer on the membrane surface, induced by the relatively poor mass transfer conditions near the membrane surface, due to the high viscosity of the media containing arabinoxylan, which was observed at the end of each experiment. The formation of a gel layer was more pronounced due to the use of a dead-end cell, where a process of frontal filtration takes place. The accumulation of the rejected arabinoxylans occurred near the membrane surface, promoting membrane fouling and affecting permeance. Relatively high values of arabinoxylan adsorption were detected for all membranes (Figure 3.5), indicating the presence of membrane fouling phenomena. Nevertheless, the formation of a gel layer did not affect the rejection of ferulic acid, since all membranes presented no rejection of ferulic acid (0 %) (Table 3.3, Figure 3.4) and only a slight adsorption of ferulic acid was detected (Figure 3.5).

All membranes allowed to achieve the main objective of this work, an efficient separation of ferulic acid from arabinoxylans (low ferulic acid rejection and high arabinoxylan rejection). The UP 150 membrane was selected as the best performing membrane, because it exhibits the second highest arabinoxylan rejection (98.0 ± 1.7 %) and the highest average permeance ($2.35 \text{ L.m}^2.\text{h}^{-1}.\text{bar}^{-1}$).

3.1.4.2. Evaluation of the effect of operating temperature, initial cross-flow velocity and transmembrane pressure using design of experiments (DoE)

After selecting the membrane, the effect of the operating conditions (operating temperature, initial cross-flow velocity and transmembrane pressure) in the filtration performance was analysed in a cross-flow cell. The 10 DoE experiments (Table 3.2) were carried out, though with slight deviations between the values imposed and the ones selected to be set, due to practical operating circumstances. Temperature deviations were mainly due to differences between the set-point in the control and the temperature actually measured inside the feed vessel. The initial cross-flow velocity and the transmembrane pressure were independent factors but when using a high cross-flow velocity, the minimum achievable transmembrane pressure (with the valve totally open) was higher than 0.7 bar. Therefore, some adjustments were required to operate the equipment for the conditions selected. Table 3.4 shows the value of the parameters actually used in the DoE, as well as that of the responses obtained, global rejection of ferulic acid [%], retention of arabinoxylans measured by retentate viscosity [mPa.s] and the average permeance [$\text{L.m}^2.\text{h}^{-1}.\text{bar}^{-1}$]. As discussed above, the retention of arabinoxylans was related with the retentates' apparent viscosity, which was measured at the end of each experiment at 20°C .

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Table 3.4. Design of experiments (DoE) – full factorial design matrix and the responses: global rejection of ferulic acid ($R_{\text{ferulic acid}}$ (%)), retention of arabinoxylans measured by apparent viscosity of retentate ($\mu_{\text{retentate}}$ (mPa.s)) and average permeance ($\bar{P}$ (L.m⁻². h⁻¹.bar⁻¹)).

Design of experiments				Responses		
Exp	T (°C)	v_{initial} (m.s ⁻¹)	Δp (bar)	$R_{\text{ferulic acid}}$ (%)	$\mu_{\text{retentate}}$ (mPa.s)	$\bar{P}$ (L.m ⁻² . h ⁻¹ .bar ⁻¹)
1	30	0.69	0.7	0	115.7	33.5
2	66	0.65	0.7	0	105.5	52.8
3	30	1.11	1.3	0	164.3	17.2
4	66	0.98	1.0	0	131.3	49.5
5	30	0.69	3.0	0	113.9	8.8
6	66	0.65	3.0	0	94.6	14.8
7	30	1.02	3.0	0	98.4	10.3
8	66	1.02	3.0	0	140.3	17.0
9	50	0.89	1.9	0	153.5	19.9
10	50	0.89	1.9	0	130.7	22.7

From the analysis of the responses obtained (Table 3.4), it was found that there was no rejection of ferulic acid (0 %) for all cases, indicating that the conditions tested had no influence (in the range of study) on the rejection of ferulic acid. Therefore, RSM was only applied to the other two responses, retention of arabinoxylans measured by apparent viscosity of retentate, and average permeance, in order to find the models capable of correlating each of them with the factors.

An increase of the feed operating temperature and initial cross-flow velocity, although with some negative impact on the energy needed for the separation process, had a positive and significant impact on the performance of the process, reflected on an increase of the average permeance. Particularly, an increase of the operating temperature from 30 to 66° C, led to an increase of the average permeance of 58 % in experiments 1 and 2, and to an increase of the average permeance by 65 % in experiments 7 and 8. Also, an increase of the initial cross-flow velocity from 0.69 to 1.02 m/s, led to an increase of the average permeance by 17 % in experiments 5 and 7.

The multilinear models were optimised removing the factors and/or the interaction between factors that were insignificant for each response. The effect of the significant factors (and interactions between factors) on retention of arabinoxylans (assessed through the apparent viscosity of the retentate) and on permeance can be shown in Figure 3.6 where the coefficients are normalised in order to compare the input weights.

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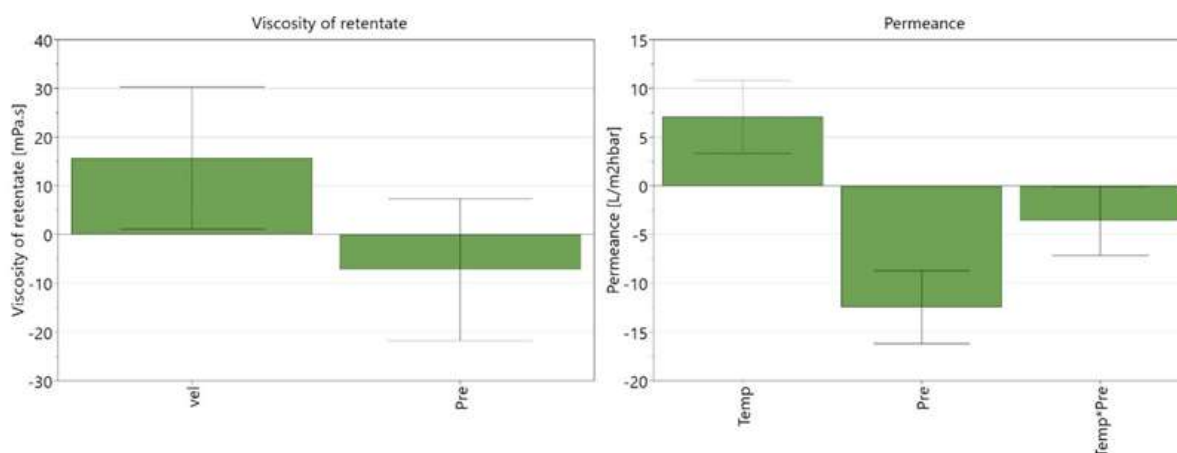


Figure 3.6. Regression coefficients (scaled and centred) of the model for a) retention of arabinoxylans measured by the apparent viscosity of retentate and b) average permeance ($\bar{P}$), where the factors are operating temperature ($Temp$), Pressure (Pre) and cross-flow velocity (vel).

The optimised models show that retention of arabinoxylans (measured by the viscosity of the retentate at 20° C in the final of each experiment), is only affected by the initial cross-flow velocity and by the transmembrane pressure. The operating temperature of the ultrafiltration (in the range tested) did not effluence the retention of the arabinoxylans.

The initial cross-flow velocity is the most significant term as it had a positive impact on fluid dynamics in the feed/retentate circuit, leading to better mass transfer conditions that improve the retention of arabinoxylans, which translates into a higher apparent viscosity of the retentate (high cross-flow velocity increases the retentate's apparent viscosity). On the opposite direction, operating with higher transmembrane pressure, increases the convective transport of solutes towards the membrane surface and, ultimately, facilitating fouling with a direct negative impact on the arabinoxylans present in solution (Figure 3.6 a).

Regarding the permeance, the most significant term is the transmembrane pressure applied, which has a large negative impact, followed by operating temperature, which has a positive impact on permeance, and by the interaction between pressure and operating temperature (Figure 3.6 b). The negative impact of pressure has been reported by Lui and Wang (2002) in a study on the purification of ferulic acid from wheat bran alkaline-alcohol extract by ultrafiltration [81] where an increase of transmembrane pressure led to the quicker formation of a gel layer, resulting in a faster membrane flux decline. The operating temperature, as expected, has a positive impact, since the viscosity of the retentate solution decreased during the experiment as the operating temperature increased and consequently increased the permeance.

Figure 3.7 a) exhibits the statistical parameters of the model (Q^2 , R^2 , model validity and reproducibility) provided by the software (MODDE Go 12.1). Figure 3.7 b) represents the observed values for permeance

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and rejection of arabinoxylans measured by the apparent viscosity of retentate versus the values predicted by the model. These two figures allow the model to be evaluated.

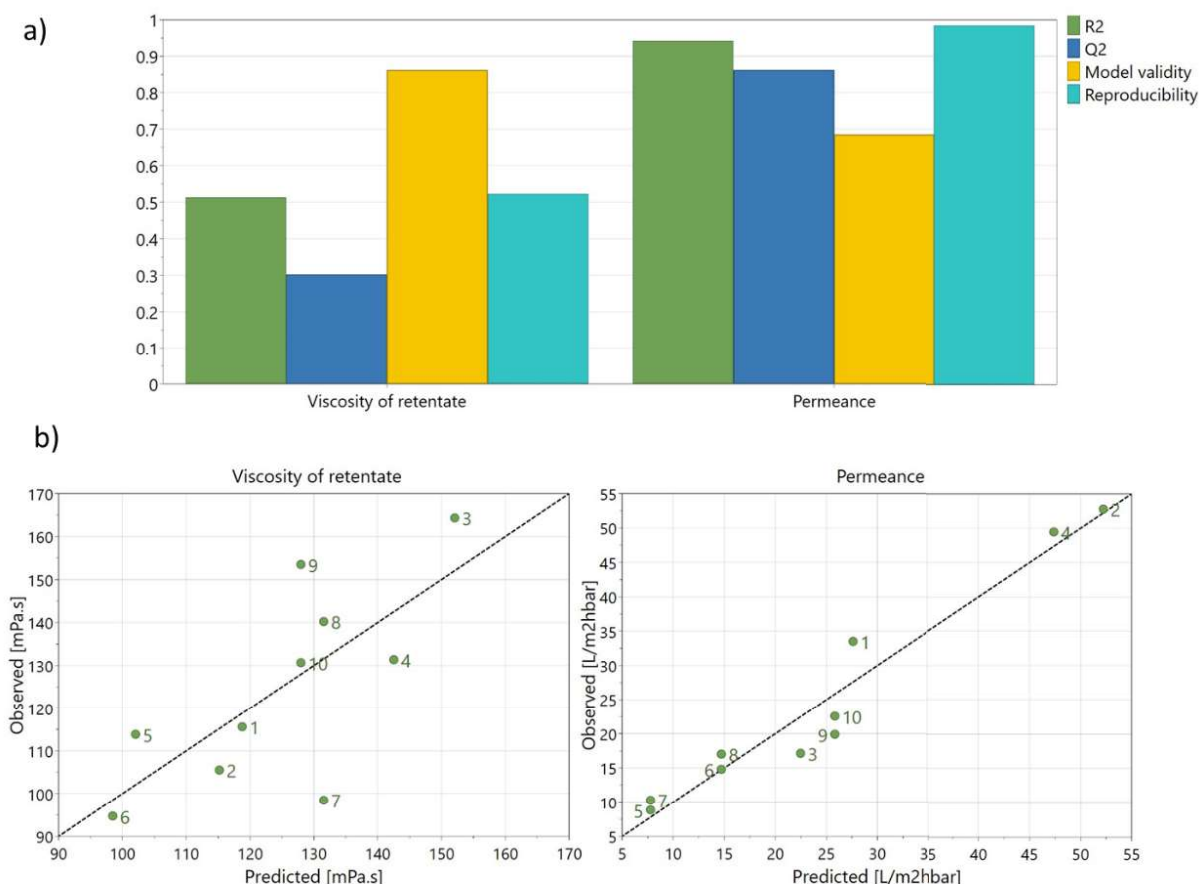


Figure 3.7.- Model evaluation: a) Summary statistics presented as four parameters (R^2 , Q^2 , Model validity, and Reproducibility) for the model of retention of arabinoxylans, measured by the apparent viscosity, and for permeance b) Observed data versus Predicted values of apparent viscosity and permeance.

According to the MODDE software, parameter R^2 shows how the model fits the data and should be higher than 0.5 to be significant (showing a true correlation), Q^2 estimates the prediction precision, and should be higher than 0.1 for a significant model and higher than 0.5 for a good model [171]. Model validity should be higher than 0.25, to exclude statistically significant problems, such as the high presence of outliers, an incorrect model or a transformation problem [171]. Reproducibility, defined as the variation of the replicated experience compared to overall variability, should be above 0.5 [171].

Thus, by analysing the statistical parameters of each model individually (Figure 3.7 a), we can see that the permeance model can be considered a very good model ($R^2 = 0.93$, $Q^2 = 0.86$, reproducibility = 0.99 and model validity of 0.69). Furthermore, the fitting between predicted and observed values for the permeance model is good (Figure 3.7 b), also indicating a good model.

By analysing the statistical parameters for the viscosity model (Figure 3.7 a), we can see that the values of R^2 , Q^2 and reproducibility were low (0.51, 0.32 and 0.52, respectively). However, the model can be

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considered a significant model since R^2 was higher than 0.5, Q^2 was higher than 0.1 and the reproducibility was higher than 0.5. Additionally, the model validity was much higher at 0.86, indicating there are no statistically significant problems. In Figure 3.7 b, showing predicted and observed values for the viscosity, it is possible to observe some data dispersion. The exclusion of experiment 7 from the model was assessed, and it resulted in a better model, with R^2 increasing from 0.51 to 0.80, Q^2 from 0.32 to 0.72, and model validity from 0.86 to 0.95. However, as it is not recommended to exclude experiments [171], the initial model with all data was used.

Therefore, the final equations that translate the models achieved and that can be used for estimation of viscosity ($\mu_{retentate}$) and permeance ($\bar{P}$) when varying the cross-flow velocity (v), transmembrane pressure (Δp) and temperature (T), within the range studied, are:

$$\mu_{retentate}(mPa.s) = 89.592v - 7.2752\Delta p + 62.042 \quad (9)$$

$$\bar{P}(L.m^{-2}.h^{-1}.bar^{-1}) = 0.88338T - 2.2063\Delta p \times T + 8.6267 \quad (10)$$

3.1.4.3. Optimal operating conditions

A visual evaluation of the model equations shows which ultrafiltration conditions lead to a higher permeance and a higher retentate viscosity (related to the concentration of arabinoxylans). Figure 3.8 shows contour plots for each model for different pressures. The contour plots show the interaction between the factors in each model and the predicted response.

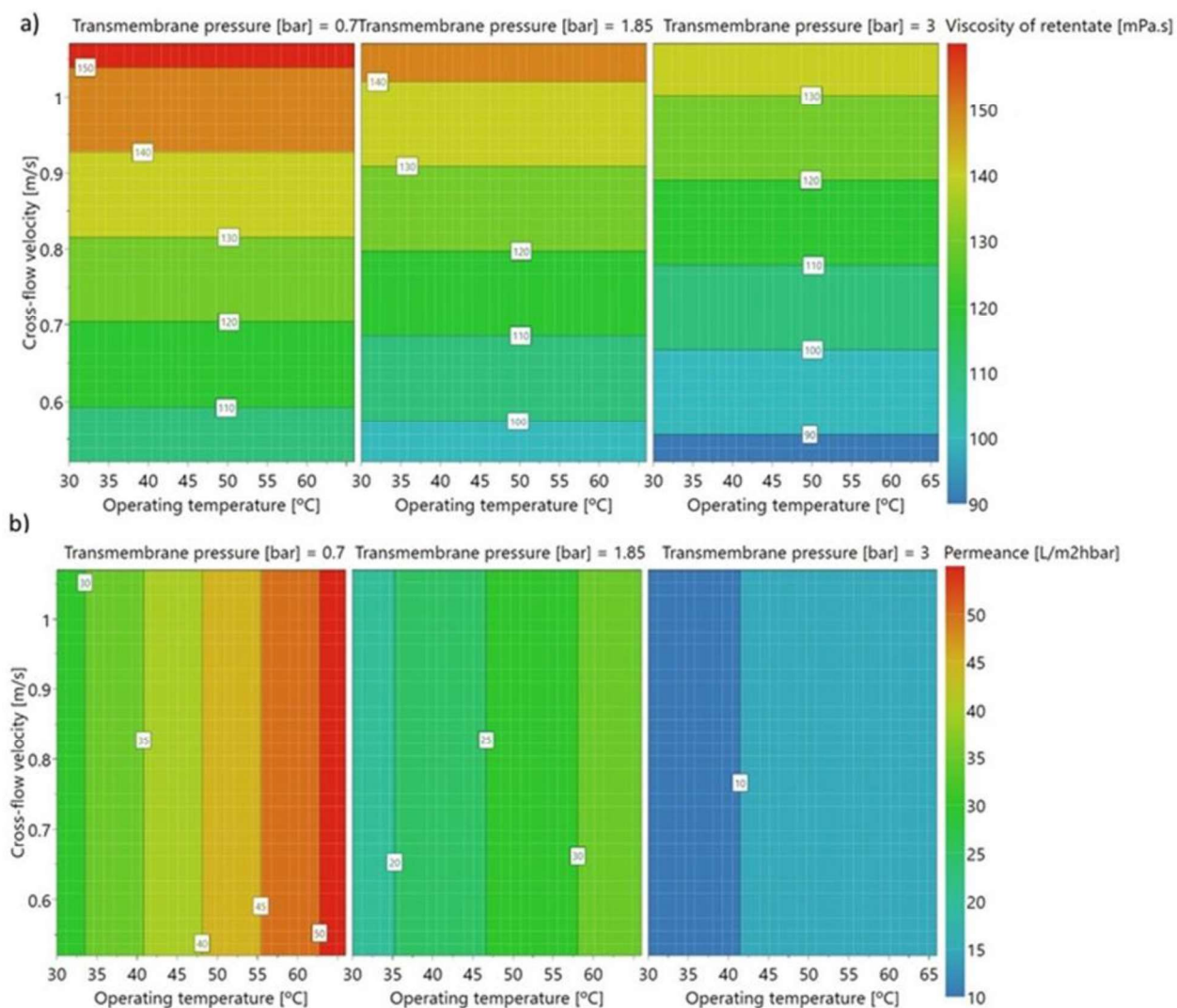


Figure 3.8. Response contour plots of the model for a) apparent retentate viscosity (as a measure of retention of arabinoxylans) b) average permeance (in the range of 0.7 to 3 bar).

As shown in the contour plots for apparent viscosity (in Figure 3.8 a), a high final viscosity of the retentate (measured in the final of each experiment at 20° C, and used as a measurement of the retention of arabinoxylans) is achieved with low transmembrane pressure and high initial cross-flow velocity, independently of the operating temperature used during ultrafiltration (within the range of study), as also shown in Figure 3.6 a). The contour plot for permeance shows that high permeance is achieved with high operating temperature and low transmembrane pressure, and it is independent of velocity (in the study range). These graphs allow us to conclude that the best conditions (high permeance and high retentate viscosity) are: high operating temperature (66° C), high initial cross-flow velocity (1.06 m.s^{-1}) and low transmembrane pressure (0.7 bar). This result was confirmed by using the optimiser function of the MODDE software, set up to maximise these two factors simultaneously. With this function it was possible to conclude that the transmembrane pressure is the main factor in achieving best conditions (46.3 %), followed by the cross-flow velocity (30.0 %) and operating temperature (23.7 %). As mentioned before, in this ultrafiltration system it was not possible to work in best conditions as at high

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velocity the transmembrane pressure is higher than 0.7 bar. In the model, the transmembrane pressure had more impact than velocity, so a compromise has to be made between both the parameters, working at high velocity without much increase to the transmembrane pressure.

Furthermore, the same best conditions were shown using the two models assessed for viscosity (with and without experiment 7), showing that despite the dispersion of data obtained (which resulted in a lower quality model), the tendency is well described by the models, even when an experiment is excluded.

3.1.4.4. Model validation

In order to validate the final model and ensure that its predictions were accurate, an additional experiment was performed at a temperature of 52° C, an initial cross-flow rate of 1.06 m.s⁻¹ and a transmembrane pressure of 1.4 bar. Table 3.5 shows the model predictions, which were generated using the prediction function of the MODDE software, and values obtained for this experiment.

Table 3.5. Model predictions (with estimation error) and values obtained experimentally for 52° C, 1.06 m.s⁻¹ and 1.4 bar.

	$\mu_{retentate}$ (mPa.s)	$\bar{P}$ (L.m ⁻² .h ⁻¹ .bar ⁻¹)
Model prediction	146.8 ± 23.6	33.3 ± 4.2
Obtained experimentally	123.8	29.7

The models predict an apparent viscosity of retentate ($\mu_{retentate}$) of 146.8 mPa.s and an average permeance of 33.3 m⁻².h⁻¹.bar⁻¹ for the conditions used in this experiment. However, due to the errors associated with the experiments (shown through duplicates, experiments 9 and 10) plus the error associated with models fitting, the MODDE software estimates each value within an interval (corresponding to an estimation error for each model), where the experimental observation should be (Table 3.5). Therefore, the results obtained experimentally are within the range of values predicted by the model in Table 3.5, proving the accuracy of the model.

3.1.5. Conclusions

With the aim of optimising the conditions needed to fractionate an alkaline extract from corn fibre, we assessed the most relevant operating parameters of an ultrafiltration process - membrane molecular weight cut-off, operating temperature, cross-flow velocity and transmembrane pressure. Out of the membranes tested, Nadir UP 150 (polyethersulfone with MWCO of 150 kDa) was found to be the best in terms of permeance, presenting no rejection of ferulic acid and a high estimated rejection of

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arabinoxylans (98.0 ± 1.7 %). A response surface methodology (RSM) was used to infer the effect of temperature, cross-flow velocity and transmembrane pressure on the rejection of ferulic acid, retention of arabinoxylans (assessed through the retentate viscosity) and permeance. It was found that the ultrafiltration conditions (in the range tested) do not influence the global rejection of ferulic acid. The increase of transmembrane pressure showed a negative impact on the permeance and on the retention of arabinoxylans, while the temperature and cross-flow velocity showed a positive impact on the permeance and on the retention of arabinoxylans retention, respectively.

Analysis of the two mathematical models developed to describe the retention of arabinoxylans (measured by apparent viscosity of retentate in the final of ultrafiltration) and permeance shows that it is possible to achieve the best conditions for ultrafiltration using the membrane Nadir UP 150, with an operating temperature of 66°C , an initial cross-flow velocity of 1.06 m.s^{-1} and a transmembrane pressure of 0.7 bar. This study allowed us to determine the best conditions for an efficient separation of ferulic acid from arabinoxylans and obtain a fraction rich in ferulic acid with low viscosity, due to the removal of the arabinoxylans, facilitating a further step for purification and concentration of ferulic acid through adsorption or by nanofiltration, for use in bio-vanillin production. A fraction with a high concentration of arabinoxylans was also produced, which could be used, for example, as a material for edible films [77,164] and emulsifier for oil-in-waters emulsion systems [78].

3.2. Purification of ferulic acid from corn fibre alkaline extract for bio-vanillin production using an adsorption process

To be submitted as: Rita Valério, Cristiana A.V. Torres, João G. Crespo, Isabel M. Coelho, Carla Brazinha “Purification of ferulic acid from corn fibre alkaline extract for bio-vanillin production using an adsorption process”.

The author was involved in planning and performing all the experiments, as well as on the discussion and interpretation of all the results and preparation of the manuscript.

3.2.1. Summary

Ferulic acid is the most widely studied precursor for bio-vanillin production [1,28]. This work assessed the use of a concentrated alkaline extract from corn fibre, with 10 g.L⁻¹ of ferulic acid, for bio-vanillin production. The results show that an additional step is needed to purify ferulic acid to remove toxic/inhibitor compounds from extracts. An adsorption process was selected to purify the ferulic acid because of its relative ease of operation and scale-up, its high capacity and its ease of resin regeneration [71]. The performance of four macroporous resins with different matrix materials was evaluated. Macronet® MN102 (whose use in ferulic acid purification has not been previously reported) offered the highest ferulic acid adsorption capacity. A column packed with Macronet® MN102 was used to perform dynamic adsorption and desorption tests, which showed a maximum ferulic acid adsorption capacity of 176 mg_{ferulic acid}·g⁻¹ dry resin at pH 4.5 at a flow rate of 5.23 ml.min⁻¹. The breakthrough point was at 115 minutes obtaining an adsorption capacity of 85 mg_{ferulic acid}·g⁻¹ dry resin. In the desorption step, 90.9 % of ferulic acid was recovered using as eluent ethanol absolute (≥ 99.8 %) and maintaining the flow rate at 5.23 ml.min⁻¹. This procedure also confirmed the elimination/reduction of compounds with an antimicrobial effect, such as organic acids, metals and some aldehydes. Using an adsorption process to purify ferulic acid allowed the production of bio-vanillin with about 10 g.L⁻¹ of ferulic acid, without using any further ferulic acid purification steps such as crystallisation.

3.2.2. Introduction

The biorefinery concept aims to efficiently utilize biomass resources (e.g. microalgae, agro-industrial by-products, grasses) for the production of value-added compounds, chemicals and biofuels. Ferulic acid is a hydroxycinnamic acid found in agro-industrial by-products such as corn fibre [32], which is a low value by-product of the starch industry that is often incorporated into animal feed. However, corn fibre enriched with ferulic acid may have added value. Specifically, ferulic acid from corn fibre may be used as a nutraceutical constituent [102,105], as a crosslinking agent in polymeric films [107,108] and as a precursor for bio-vanillin production. In fact, ferulic acid is the most widely studied precursor for bio-vanillin production [1,28], it was the precursor of the first commercially available fermentation-derived bio-vanillin, which was produced by Solvay [1,9]. Vanillin is the world's most popular flavour

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compound and is used in several industries, mainly in the food industry. Natural vanillin is extracted from vanilla orchids but the process is very laborious, costly and has a low yield, making natural botanical vanillin a luxury product, representing only 1 % of the global market for vanillin flavour [1,9]. The increasing demand for natural products has led to greater interest in vanillin production using biotechnology [3], particularly as biotech-grade vanillin is considered a natural product according to the US and European food legislation (US Food and Drug Administration and Regulation (EC) No 1334/2008, respectively). Ferulic acid can be extracted from the cell wall of lignocellulosic materials by enzymatic [35,39] or chemical treatments [42,172].

Alkaline extraction has been reported as an efficient process to release ferulic acid from lignocellulosic mater [43,45,56]. However, it produces a complex alkaline extract consisting of several compounds, some of which may be toxins/inhibitors that affect the production of bio-based compounds such as bio-vanillin [67]. Organic acids, particularly acetic and lactic acids, are examples of toxic/inhibitor compounds that are known for their antimicrobial potential [61–64]. Acetic acid is formed by the removal of the acetyl group from the hemicellulose during alkaline extraction [46]. Lactic acid is produced by fermentation during counter-current steeping in the corn wet-milling process [65,66] and is present on the surface of corn fibre. Other examples of toxins/inhibitors are metals and some aldehydes which may be present in lignocellulosic extracts and have an antimicrobial effect [173,174]. In addition, the NaCl that forms as the pH of the alkaline extract decreases can also have a negative impact on the microorganisms [175,176]. Therefore, it is essential, albeit challenging, to purify the ferulic acid that is recovered from alkaline extracts.

The purification of ferulic acid from agro-industrial extracts is usually achieved using membrane processes [56,67], liquid-liquid extraction [68,69], weak anion-exchange with a resin with a amine group [70] or adsorption (using activated carbon or polymeric adsorbents) [71,72,177]. Liquid-liquid extraction has several disadvantages, since it is expensive, time-consuming and lower recovery of the products [178,179]. Weak anion-exchange, mainly Amberlyst A21 has been used to purify ferulic acid in enzymatic hydrolysates [48,70]. However the organic acids (potential inhibitors/toxic compounds for the microorganisms) are also retained by this type of resin [74,75]. The retention of ferulic acid and the organic acids occurs by the acid-base neutralization reaction between the carboxylic group and the tertiary amine of resin [48,70].

Adsorption is an attractive process for its relative ease of implementation, operation and scale-up, along with its high capacity and ease of regeneration [71]. The process comprises three steps: the adsorption of the target compound, the rinse of the resin and the desorption of the target compound using a desorption eluent. Adsorption with polymeric macroporous synthetic resins especially with polystyrene-divinylbenzene matrix are known to have an excellent selectivity towards aromatic solutes, including ferulic acid, as a result of the hydrophobic interaction between its aromatic ring and the phenyl rings of these resins and specific π - π interaction [75,180,181]. They have been reported to recover ferulic acid

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from several agro-industrial alkaline and enzymatic extracts [45,71,178,182,183]. The acrylic resins are also known for their potential retention of the aromatic and/ or low-polarity compounds, and they have been reported to recover ferulic acid from adlay bran aqueous extract [178]. Although this polymer seems to have less adsorption capacity than polystyrene-divinylbenzene material [178,181]), due to the fact of acrylic polymer is a more hydrophilic structure than polystyrene-divinylbenzene polymer [75]. These types of resins (acrylic and polystyrene-divinylbenzene) normally retain ferulic acid and exclude polar compounds [70,75] such as the case of acetic acid and lactic acid allowing the separation of these toxic/inhibitors for the microorganism. Fargues et al. 2010 reported the exclusion of acetic acid by the XAD 4 resin (polystyrene-divinylbenzene polymer) and XAD 7 (acrylic) [75].

The aim of this work is the study of the purification of ferulic acid from an ultrafiltrated corn fibre alkaline extract by an adsorption process, to allow it to be used as a precursor for bio-vanillin production by the *Amycolatopsis* sp. ATCC 39116. This microorganism is one of the best performing microorganisms for this purpose and has already been widely studied for vanillin production from ferulic acid [3,85,87]. This work evaluated the possibility of producing bio-vanillin by alkaline extraction using concentrated ferulic acid extracted from corn fibre. In preliminary studies the ultrafiltrate permeate of the corn fibre alkaline extract was concentrated until was achieved 10 g.L⁻¹ of ferulic acid (above this concentration, ferulic acid have a toxic effect for the bacterium [1,3,87,184,185]) and then fed directly to the microorganism at pH of 8.2 [185]. However, in these preliminary studies, neither production of bio-vanillin nor consumption of ferulic acid was detected, unlike when using pure ferulic acid, proving the existence of toxic/inhibitor compounds in the alkaline extract fed to the microorganism.

To purify ferulic acid, four different macroporous resins were compared for their capacity to adsorb ferulic acid, one with an acrylic matrix (Amberlite XAD 7 HP), one with a polydivinylbenzene matrix (Purosorb PAD 900) and two with Polystyrene-divinylbenzene matrix (Macronet MN102 and Amberlite XAD-16 (the most reported for ferulic acid recovery). To the best of our knowledge, there have been no other studies using the Macronet MN102 and Purosorb PAD 900 resins for ferulic acid adsorption. The pH of the adsorption step and the type of eluent used in the desorption step were optimised in batch experiments using the resin with the highest ferulic acid adsorption capacity. The three-step adsorption process (comprised of adsorption, rinse of the resin and desorption) using the selected resin was studied in fixed-bed adsorption columns to determine the operating behaviour of the resin and the parameters that allow the process to be scaled up namely, the maximum ferulic acid adsorption capacity of the resin and the time and the adsorption capacity at the breakthrough point. The different fractions obtained in the column experiment (from the column inlet, the column outlet and the fraction desorbed from the selected resin) were analysed and compared, in order to confirm the elimination of the compounds that can be potential inhibitors of the microorganism. (organic acids and some metals). Finally, the ferulic acid that was obtained under the selected adsorption operating conditions was tested, at a concentration of 10 g.L⁻¹, in vanillin bioconversion using the *Amycolatopsis* sp. ATCC 39116 strain, in order to confirm

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the elimination/reduction of toxic/inhibitor compounds. To the best of our knowledge, there are no other reports of the complete study of ferulic acid adsorption from a corn fibre alkaline extract for the efficient bioproduction of vanillin using such a high concentration of ferulic acid.

3.2.3. Material and Methods

3.2.3.1. Materials

The feed solution for this work was produced through the following steps: an alkaline extraction was performed using corn fibre supplied by Copam (São João da Talha, Portugal), under the mild conditions that had been previously optimised (see chapter 2.1) at 30° C, with 0.25 M NaOH, for 7h, using a liquid-to-solid ($\text{g}_{\text{extracting mixture}} \text{g}^{-1} \text{dry fibre}$) ratio of 10 (data not shown). The resulting alkaline extracts were centrifuged at 9174x g during 15 minutes to remove the solids. The supernatant underwent ultrafiltration to separate the ferulic acid from high molecular weight polysaccharides (arabinoxylans) using an ultrafiltration Nadir® UP150 membrane, as optimised previously (see chapter 3.1). These polysaccharides are very viscous and can adhere to the resin and greatly influence the separation so, they must be removed before the adsorption purification step [56]. The fraction that was rich in ferulic acid (permeate) was used in the adsorption process experiments.

Four resins were selected to purify the ferulic acid (permeate) fraction. Purosorb™ PAD900 and Macronet™ MN102 were recommended by Purolite as potential resins for ferulic acid purification. Amberlite XAD 16N and XAD 7HP have already been studied for this purpose [45,71,178,182] and their main characteristics are summarised in Table 3.6. All the resins are non-ionic adsorbents, however, Macronet™ MN102 has a weak base anion functionality but with low ion exchange capacity (only 0.15 eq.L⁻¹), so its main function is also as an adsorbent [186,187].

Table 3.6. Summary of resin characteristics tested in this work.

Resin	Manufacturer	Matrix	Functional Group
Macronet™ MN102	Purolite ®	Polystyrene- divinylbenzene (non-polar)	Tertiary amine ¹
Purosorb™ PAD900	Purolite ®	Polydivinylbenzene (non-polar)	Non ionic
Amberlite XAD 16N	Rohm and Hass	Polystyrene- divinylbenzene (non-polar)	Non ionic
Amberlite XAD 7HP	Rohm and Hass	Acrylic (moderately polar)	Non ionic

¹technically functionalized, but with limited ion exchange capability (only 0.15 eq/L) and function primarily as adsorbent [186,187].

Analytical-grade standard ferulic acid (>99 % purity), vanillin (99 % purity) and DL-Lactic acid (~90 %) were purchased from Sigma-Aldrich (Germany). Analytical-grade standard Acetic acid glacial was purchased from Fisher Scientific (USA). Ethanol absolute (≥99.8 % purity) was obtained from VWR

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(Germany). Methanol was purchased from Sigma-Aldrich (Germany). Sodium hydroxide was obtained from Akzo Nobel (Sweden) and chloride hydroxide (37 %) from Fisher Scientific (US).

3.2.3.2. Methods

3.2.3.2.1. Resins preparation

Before running the experiments, the resin samples were washed to remove the preservatives and to be conditioned to ensure full swelling and hydration of the polymer. By recommendation of Purolite [188], the Macronet™ MN102 and Purosorb™ PAD900 resins were submerged in a (50:50 (v/v)) solution of water and ethanol, for 24h with slight agitation. Afterwards, the resin was washed in deionized water and vacuum filtrated. The XAD 16N and XAD 7HP Amberlite resins were washed and conditioned according to a previously optimised procedure (results not shown). The optimised procedure consisted of successive washes with different solutions. Between washes, the resin was rinsed with water. The washing solutions were as follows: 96 % of ethanol solution; 4% of HCl, 5% of NaOH to remove salts and impurities. After the washes, the resins were submerged in methanol for 12h to activate them, then washed again in distilled water before being vacuum filtrated. All adsorption experiments were performed using wet resins because the removal of moisture can change the resin adsorption capacity [188,189]. In order to consider the dry weight of resin in the calculations, the moisture content was determined by drying 2g of each wet resin overnight at 70°C. This measurement was performed in duplicate.

3.2.3.2.2. Resin Screening in batch experiments

The adsorption experiments were performed in batch mode by adding 1 g of each resin to 50 ml of feed solution with $2.16 \pm 0.06 \text{ g.L}^{-1}$ of ferulic acid at pH of 12.5 (the pH of the extract) and 7.3. Each resin solution was mildly stirred in an orbital Mistral Multi-mixer (model 4600, Lab-line, India) for 24 h at room temperature (20° C). Each experiment was performed in duplicate. During the experiments, several samples were withdrawn to determine ferulic acid concentration over time. The ferulic acid adsorption capacity of each resin (q) was calculated with the following equation:

$$q = \frac{V(C_0 - C_t)}{W} \quad (11)$$

Where q (mg.g⁻¹) is the adsorption capacity of the dry resin; V (L) is the volume of the alkaline extract; W (mg) is the resin dry weight; C_0 and C_e are the initial and equilibrium concentrations of ferulic acid in solution (mg.L⁻¹).

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3.2.3.2.3. Effect of pH on the adsorption capacity in batch experiments

After the selection of the best resin in terms of its capacity for ferulic acid adsorption from the alkaline fraction, several initial values of pH were tested under the same conditions as the resin screening experiments, without adjusting the pH during the experiment. 1 g of the selected wet resin (0.43 g of dry resin) was added to 50ml of feed solution with $2.16 \pm 0.06 \text{ g.L}^{-1}$ of ferulic acid. The initial pH settings were 6.55, 5.75, 5.00, 4.50 and were adjusted using HCl. Each experiment was performed in duplicate, during 8 hours at room temperature (20° C), stirred with a Mistral Multi-mixer (model 4600, Lab-line, India). To compare the experiments, the adsorption efficiency, E (%), was calculated with the following equations:

$$E(\%) = \frac{m_0 - m_e}{m_0} \times 100 = \frac{C_0 - C_e}{C_0} \times 100 \quad (12)$$

Where m_0 , m_e , C_0 and C_e are the initial and equilibrium mass (g) and concentrations of ferulic acid in solution (g.L^{-1}), respectively. Since the volume is constant, the adsorption efficiency can be determined using the concentrations measured.

3.2.3.2.4. Adjustment of ethanol concentration in desorption eluent using batch experiments

In order to maximise the mass of the desorbed ferulic acid, different solutions of ethanol in water were tested (10 % (v/v), 20 % (v/v), 50 % (v/v), 70 % (v/v) and ethanol absolute). The recovery of adsorbed ferulic acid was studied in desorption experiments carried out after adsorption using 1.3 g (0.56 g dry) selected resin in 50ml of feed solution with $2.16 \pm 0.06 \text{ g.L}^{-1}$ of ferulic acid at the selected pH for 6 h, stirred in a Mistral Multi-mixer (model 4600, Lab-line, India). After adsorption, the resin was separated from the extract by vacuum filtration using qualitative filter paper (Filter-lab, Canada). The concentration of ferulic acid was measured in the filtered samples, for the determination of the adsorbed ferulic acid mass. The resin was submerged in 50 ml of desorption solution and stirred in the Mistral Multi-mixer overnight at room temperature (20° C). Each experiment was performed in duplicate. After desorption, the resin was vacuum filtrated again and ferulic acid was determined from the supernatant. The percentage of desorption, D (%), was calculated with the following equation:

$$D(\%) = \frac{C_d \times V_d}{(C_0 - C_e) \times V} \quad (13)$$

where C_d (mg.L^{-1}) is the final concentration of ferulic acid in solvent and V_d (L) the employed solvent volume.

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3.2.3.2.5. Dynamic adsorption and desorption experiments

The adsorption capacity, desorption and operating behaviour of a specific installed polymer (parameters that allow the work to be scaled up) can only be determined by dynamic adsorption and desorption experiments [188]. The dynamic adsorption and desorption experiments were performed in a glass column with an inner diameter of 2.5 cm with 28.6 g (12.3 g dry weight) of selected resin, corresponding to a fixed bed height of 17.1 cm. The bed volume of 83.93 cm³ was calculated with the following equation:

$$\text{bed volume (cm}^3\text{)} = \text{bed height (cm)} \times \text{column crosssectional area (cm}^2\text{)} \quad (14)$$

The pH of the extract was adjusted to the selected the acidification caused precipitation of some compounds that were removed by centrifugation at 9174× g for 15 minutes. The column was fed from the bottom with feed solution, containing 1.91 g.L⁻¹ of ferulic acid, at 3.7 BV (bed volumes).h⁻¹ equivalent at 5.23 ml.min⁻¹ during 8.5 h. The experiment was performed at room temperature (20° C). In order to ascertain the breakthrough curve, several samples were taken from the top of the column during the experiment. The breakthrough curve was expressed in terms of normalized concentration defined as the ratio of outlet to inlet ferulic acid concentration (C/C_0) as a function of time. The area under the breakthrough curve was used to calculate the total mass of adsorbed ferulic acid (g) with the following equation [190]:

$$m_{ads} = \frac{C_0 \times Q}{1000} \int_0^{t_{sat}} \left(1 - \frac{C}{C_0}\right) \quad (15)$$

where C_0 is the inlet ferulic acid concentration (1.91 g.L⁻¹), Q is the volumetric flow rate (5.23 ml.min⁻¹), t_{sat} is the bed saturation time (min), the mass of ferulic acid adsorbed at the breakthrough point (the time when 5 % of the initial concentration is reached in the effluent) is calculated using the equation above for the breakthrough time (t_b).

After adsorption, the column was rinsed with distilled water and the ferulic acid was desorbed using the same conditions of adsorption (flow rate of 3.7 BV [bed volume].h⁻¹ and room temperature), and 45 ml samples were taken. The ferulic acid in the desorption samples and the organic acids in the rinsing water were analysed.

3.2.3.2.6. Bioconversion tests of the ferulic acid recovered from the resin to vanillin

The bioconversion tests were performed in shake flasks without control for pH or dissolved oxygen. The tests were carried out only to analyse whether vanillin had been produced, not the quantity produced.

A colony of *Amycolatopsis* sp. ATCC 39116 bacteria was transferred to 200 ml of growth medium containing: Na₂HPO₄, 4 g.L⁻¹; KH₂PO₄, 1 g.L⁻¹; NaCl, 0.2 g.L⁻¹; MgSO₄.7H₂O, 0.2 g.L⁻¹; CaCl₂.H₂O,

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0.05 g.L⁻¹; glucose anhydrous, 20 g.L⁻¹ and yeast extract, 5 g.L⁻¹, previously sterilized at 1 bar, at 121° C for 30 minutes in an autoclave. The culture was incubated in an orbital shaker at 45° C at 200 rpm. After 24 h of growth, 25 ml of cells were centrifuged for 10 minutes and suspended in 25 ml of ferulic acid solution.

The ferulic acid solution was prepared from the ferulic acid desorbed from the selected resin. The eluent was evaporated and the desorbed ferulic acid was dissolved in a solution of 0.05 M of NaOH with 1g.L⁻¹ of yeast extract and 8 g.L⁻¹ of glucose. The pH was adjusted to 8.2 (the pH of bioproduction) with a 2M NaOH solution. The bioproduction was performed in an orbital shaker at 45° C. After 16 h the amount of ferulic acid and vanillin was analysed by HPLC. All the experiments were performed with a control using commercial ferulic acid in the same concentration as the ferulic acid from corn fibre.

3.2.3.3. Analytical Methods

The water content of each resin was calculated with the following equation:

$$\% \text{ water content} = \frac{W_W - W_D}{W_W} \times 100 \quad (16)$$

where W_W and W_D correspond to the wet and dry weight of the resin (g), respectively.

The elemental analyses were performed by inductively coupled plasma mass spectrometry (ICP-MS).

Vanillin and ferulic acid were quantified at 30° C with a gradient elution program at the flow rate of 0.5 mL.min⁻¹ by high-performance liquid chromatography (Alliance e2695, Waters, USA) equipped with a C18 (Nova-Pak®) 3.9 mm×150 mm column (particle diameter of 4 mm) and guard column, coupled to a PDA detector, set at 280 nm. The mobile phase was a mixture of two eluents: (A) 10 % (v/v) methanol, 2 % (v/v) acetic acid in Milli-Q water and (B) 90 % (v/v) methanol, 2 % (v/v) acetic acid in Milli-Q water. The gradient elution program was 0 % B (0–10 min), from 0 to 15 % B (10–25min), from 15 to 50 % B (25–35 min) and from 50 to 100 % B (35–38 min). The ferulic acid and vanillin were used as standards at concentrations between 0.006 – 0.2g.L⁻¹. Organic acids were analysed by HLPC (VWR Hitachi Organizer, VWR, USA) equipped with an RI Detector 5450 and using an Aminex HPX-87H 300x7.8 mm column. The analysis was performed at 30° C, using 0.01 H₂SO₄ as the eluent at a flow rate of 0.5 mL.min⁻¹.

The identification of volatile organic compounds was performed by gas chromatography/mass spectrometry analysis (GC-MS). The samples for GC-MS were prepared by Headspace Solid-Phase Microextraction Extraction (HS-SPME). The HS SPME extractions were performed by exposing 1cm of CAR/DVB/PDMS fibre, 50/30 µm film thickness (df) (Supelco, Bellefonte, PA, USA). Prior to use, the fibre was conditioned according to the manufacturer's recommendations. Fibre blanks were run regularly to guarantee the absence of contaminants and/or carryover. The samples, each with 4.0 mL

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volume, were introduced in a 22.0 mL vial, sealed with a Teflon-lined rubber septum/magnetic screw cap. The vial was equilibrated for 5 min at 45° C and then extracted at the same temperature for 20 min. The thermal desorption of the analytes was performed by exposing the fibre to the GC injection port at 260° C for 3 min in splitless mode. The samples were analysed in duplicate.

The gas chromatography/mass spectrometry analysis (GC-MS) was performed on a Bruker GC 456 with a Bruker mass selective detector Scion TQ coupled to an automatic sampler injector (CTC Analysis autosampler CombiPAL). Data were acquired with MSWS 8.2 Bruker and analysed with Bruker MS Data Review 8.0. The chromatographic separation was performed in a ZB5-MS, 30 m x 0.25 mm i.d., 0.25 μm d_f , (Phenomenex, Torrance, CA, USA). The oven temperature program began with a 40° C hold for 2 min, was raised at 4° C min^{-1} up to 250° C and held for 5 min. Helium was used as the carrier gas at a constant flux of 1.2 $\text{mL}\cdot\text{min}^{-1}$. The MS transfer line and source temperatures were set at 240° C and 220° C, respectively. The spectra were matched by NIST MS Search Program, Version 2.0g. The recorded spectrum was adjusted between 40 and 450 Da and the electronic ionization 8 (EI) was adjusted to 70 EV, in order to detect the mass fragments. A solution of linear hydrocarbons from C8 to C20 was used to calculate the LRI (linear retention index). The samples were analysed in duplicate.

3.2.4. Results and Discussion

3.2.4.1. Batch adsorption experiments

3.2.4.1.1. Resin Screening

In order to adsorb ferulic acid from the feed solution, containing $2.16 \pm 0.06 \text{ g}\cdot\text{L}^{-1}$ of ferulic acid, the ferulic acid adsorption capacity of four different resins was tested. Three were non-ionic resins with different matrices. The fourth, MacronetTM MN102, was a resin with an ionic matrix but limited ion exchange capability (of only $0.15 \text{ eq}\cdot\text{L}^{-1}$), so, due to its low ionic exchange capacity, this resin is considered as an adsorbent. The adsorption experiments were performed by adding 1 g of each resin in 50 ml of alkaline extract at a pH of 12.5 (pH of the extract ultrafiltration process) and at a lower pH of 7.3. Figure 3.9 shows the ferulic acid adsorption capacity ($\text{mg}_{\text{ferulic acid}}\cdot\text{g}^{-1}_{\text{dry resin}}$) in equilibrium of each resin at pH 12.5 and 7.3.

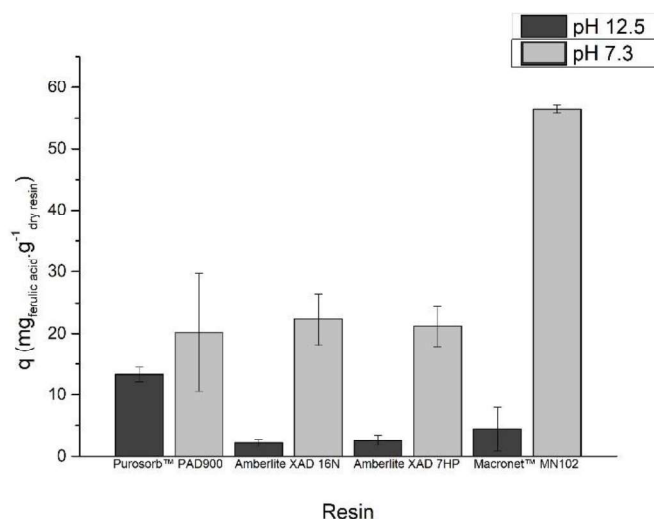


Figure 3.9. Ferulic acid adsorption capacity in equilibrium at 20° C of each resin at pH of 12.5 and 7.3.

As shown in Figure 3.9, at pH 12.5 none of the resins was able to adsorb a significant amount of ferulic acid. At a pH of 7.3 an increase in the adsorption of ferulic acid was detected, for all resins. The increase in adsorption efficiency as the pH decreases has been reported previously, and it relates to the degree of ionization of the ferulic acid. In a non-polar matrix as the case of polydivinylbenzene, matrix of Purosorb™ PAD900 and Polystyrene- divinylbenzene, matrix of Macronet™ MN102 and Amberlite XAD 16N adsorb better non-ionized compounds. As the pH decreases to values close to the pK_a (4.58 [191]) less ionized is ferulic acid increasing the ferulic adsorption, because diminishes the repulsion of ferulic acid and the adsorbent [192]. Although the Amberlite XAD 7HP has a moderately polar matrix, the same behaviour is observed [181].

The most reported resin for ferulic acid adsorption, Amberlite XAD 16N, presented at pH 7.3 an adsorption capacity of $22.3 \pm 4.13 \text{ mg}_{\text{ferulic acid}} \cdot \text{g}^{-1} \text{ dry resin}$, this value is within to obtained by Dávila-Guzman of $21.4 \text{ mg}_{\text{ferulic acid}} \cdot \text{g}^{-1} \text{ dry resin}$ for recovery ferulic acid from a model solution, with the same resin at pH 7. However the best results were obtained with Macronet™ MN102, which retained $56.46 \pm 0.65 \text{ mg}_{\text{ferulic acid}} \cdot \text{g}^{-1} \text{ dry resin}$ at pH 7.3, although this resin has the same matrix of Amberlite XAD 16N the ferulic acid adsorption capacity of Macronet™ MN102 at pH 7.3 was double of obtained with Amberlite XAD 16N. This result may be explained by the fact of the Macronet™ MN102 presents a hyper-crosslinked polystyrene structure that provides macro and microporosity at the same time. This means to have control over the pores size and relatively high surface areas [193]. Their unique manufacturing technology allows their swollen-state porosity and, hence, show little or no change in swelling with the change of the permeating liquid, unlike the conventional polymeric sorbents [193,194]. These types of resins are known for their high surface area and high adsorption capacity [194,195]. Pan et al. 2007 reported the highest adsorption of 4-Nitrophenol with hyper-crosslinked polystyrene resin than with a normally crosslinked polystyrene[194]. Other reason for the highest ferulic acid adsorption of the

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MacronetTM MN102 can be the presence of a tertiary amine. The most promising resin was MacronetTM MN102 so this resin was selected to perform the subsequent adsorption studies.

3.2.4.1.2. Effect of pH on the adsorption capacity

According to the results obtained in the resin screening, and knowing that the pH of the solution and the degree of ionization of the ferulic acid can influence the resin retention capacity [70,71,192,196], the effect of pH was analysed for the MacronetTM MN102 resin. Figure 3.10 shows the retention efficiency of ferulic acid by the MacronetTM MN102 resin at different pHs.

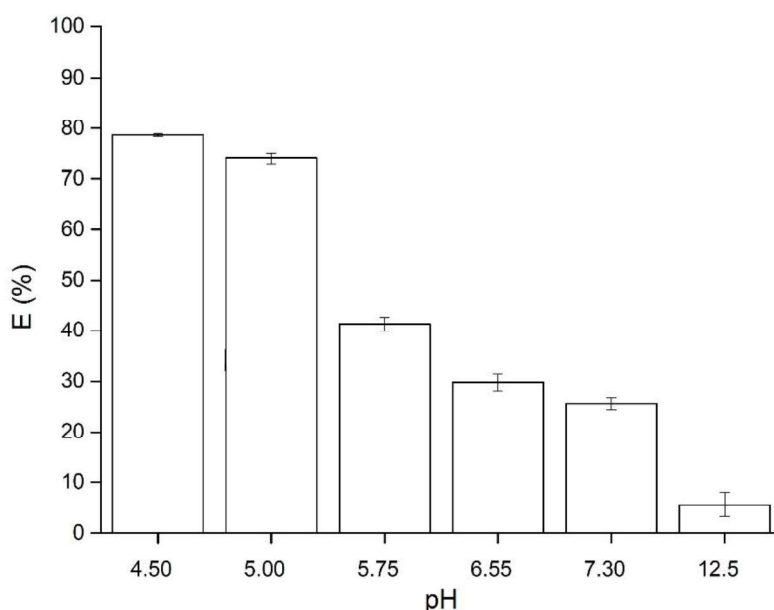


Figure 3.10. Adsorption efficiency (%) of ferulic acid at different pHs using the MacronetTM MN102 resin at 20° C.

As illustrated in Figure 3.10, the adsorption efficiency increased with the decrease of pH values, as expected. As mentioned before the matrix of MacronetTM MN102 (Polystyrene- divinylbenzene) is a non-polar matrix and adsorbed better non-ionized compounds. At $\text{pH} < \text{p}K_{a1} = 4.58$ [191] the ferulic acid is non-ionized and is better adsorbed in this type of matrix [192]. However, it was observed that the retention capacity of MacronetTM MN102 only increased slightly from pH 5 to pH 4.5, $74 \pm 1.2\%$ to 78.7% , respectively. Still, to achieve the maximum adsorption of ferulic acid, a pH of 4.5 was selected.

3.2.4.1.3. Adjustment of ethanol concentration in desorption eluent

As mentioned before MacronetTM MN102 is a resin with an ionic matrix but limited ion exchange capability (of only 0.15 eq.L^{-1}), so, due to its low ionic exchange capacity, this resin is considered as an adsorbent. So, Purolite ® the recommended eluent to recover the phenolic compounds is a mixture of

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water and alcohol, according to (the manufacturer of MN102). Therefore, the desorption of ferulic acid in MN102 was performed with different water-ethanol mixtures in batch experiments. Figure 3.11 shows the percentage of ferulic acid desorbed at different water-ethanol mixtures.

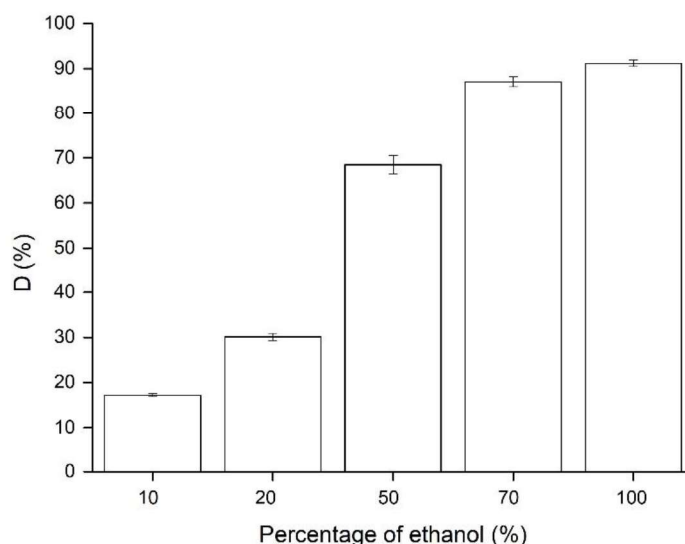


Figure 3.11. Percentage of ferulic acid recovery from Macronet™ MN102 resin at different percentages of ethanol at 20° C.

Figure 3.11 illustrates the increase of ferulic acid desorption with the increase of the percentage of ethanol in the desorption water-ethanol solution, reaching 91.2 ± 0.7 % of ferulic acid recovery with absolute ethanol (≥ 99.8 %). This trend has been also observed in other studies. Simon et al. 2015 reported a significant increase in the adsorption of ferulic acid from Amberlite XAD-16 N (which has the same polymer matrix as MN102) by increasing the percentage of ethanol from 70 % to 96 %; with 96 % of ethanol it was possible to recover almost all the ferulic acid [71].

The percentage of ferulic acid desorbed with ethanol absolute (≥ 99.8 %) was very high (91.2 ± 0.7 %), which proves that ferulic acid adsorption was a result of hydrophobic interaction between its aromatic ring and the phenyl rings of resin and specific π - π interaction. To recover the highest percentage of ferulic acid, ethanol absolute was selected as desorption eluent.

3.2.4.2. Dynamic adsorption and desorption experiments

3.2.4.2.1. Ferulic acid adsorption

The batch method only allows a choice of which type of adsorbent is more effective in terms of pure adsorption for a particular process. Adsorption capacity, desorption and operating behaviour of a specific adsorbent (parameters that allow scale-up) are only determined by a continuous column technique [188]. Therefore, a dynamic adsorption experiment was performed using a feed solution at

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pH 4.5 with 1.91 g.L⁻¹ of ferulic acid. The feed solution was fed to the column, after removing the precipitate (formed after the acidification) by centrifugation, at a flow rate of 5.23 ml/min. Figure 3.12 illustrates the breakthrough curve expressed in terms of normalized concentration defined as the ratio of outlet to inlet ferulic acid concentration (C/C_0) as a function of time.

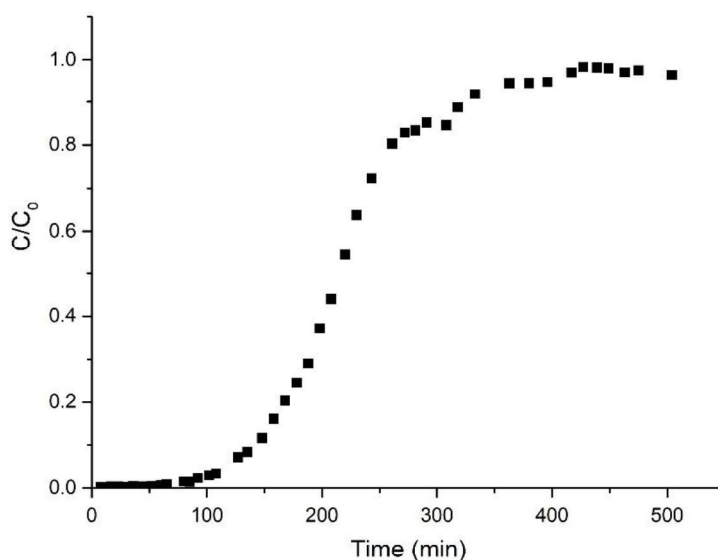


Figure 3.12. Breakthrough curve for ferulic acid in alkaline extract from corn fibre at pH of 4.5 on MacronetTM MN102 resin at 20° C.

As shown in Figure 3.12 resin saturation ($t=0.95 C/C_0$) was reached at 333 minutes corresponding to 20.6 BV (bed volume) of extract (1BV=83.93ml), the mass of ferulic acid adsorbed was 2.17 g corresponding to 176 mg_{ferulic acid} g⁻¹ dry resin. The industrial adsorption process stops before a significant leakage of the target compound has occurred at the column outlet. Consequently, only part of the adsorbed capacity was fully used. The leakage is defined as a breakthrough point and it is normally when 5% of the initial concentration is reached in the effluent.

The breakthrough point was reached at 115 minutes, corresponding to 7.2 BV of extract, and the mass of ferulic acid adsorbed was 1.06 g giving a resin capacity of 85 mg_{ferulic acid}·g⁻¹ dry resin.

3.2.4.2.2. Rinsing the resin and desorption of ferulic acid

After adsorption and before proceeding to desorption, the resin should be rinsed with water to remove any residual extract/ compounds from the resin [70,178,188,197]. The resin was rinsed at a flowrate of 3.7 BV/h, the outlet water was collected in samples of 45 ml (for 8.6 minutes) and the conductivity was measured. After rinsing the resin, the desorption process was examined in detail, since this step involves most of the costs of this process (the cost of ethanol, the cost of its separation from ferulic acid by evaporation, and disposal or recirculation of the ethanol). Therefore, it was important to study the

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sorbent volume required for the quantitative recovery of the adsorbed ferulic acid. Desorption of ferulic acid was performed with ethanol absolute ($\geq 99.8\%$), as optimized in section 3.2.4.1.3, maintaining the flow rate at 3.7 BV/h (5.23 ml/min). The outlet ethanol was collected in samples of 45 ml and the ferulic acid concentration was measured. Figure 3.13 a) shows the profile of rinse water conductivity against the bed volume and Figure 3.13 b) shows the percentage of ferulic acid desorbed in relation to the total ferulic acid adsorbed by the resin (2.17 g) against the bed volume of ethanol.

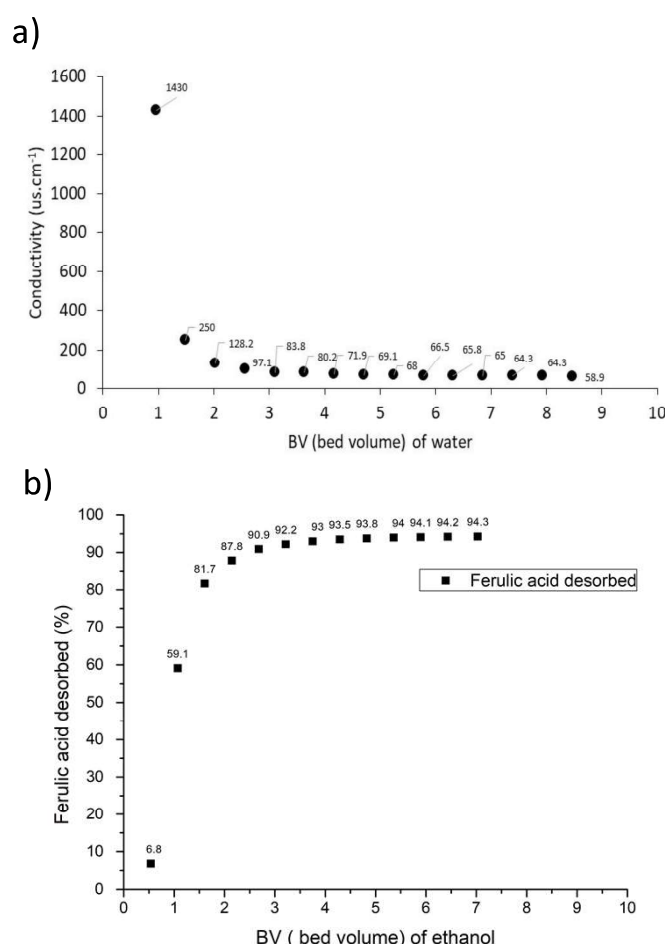


Figure 3.13. a) Conductivity of rinse water of Macronet™ MN102 resin, (after adsorption of ferulic acid) evolution with bed volume (1BV= 83.93 ml) b) percentage of ferulic acid desorbed from the Macronet™ MN102, evolution with bed volume of ethanol absolute at 20° C.

Figure 3.13 a) shows that the conductivity of the rinsed water started to stabilize at 4 BV, however, the conductivity of the rinse water did not reach the conductivity of distilled water, about 8 $\mu\text{S}.\text{cm}^{-1}$, even at 8.5 BV. As shown in Figure 3.13 b) after 7 BV, 94.3 % of ferulic acid was desorbed from the resin. This result is higher than that obtained in previous batch experiments, where it was possible to desorb $91.2 \pm 0.7\%$ (Figure 3.11). Although 94.3 % of ferulic acid was adsorbed at 7 BV, the mass of ferulic acid recovered from 2.6 BV upward was not significant and did not compensate the cost of ethanol necessary. Thus, it was possible to release 90.9 % (2.048 g) of the ferulic acid with 2.6 BV of ethanol.

3.2.4.3. Assessment of the need to purify the ferulic acid extract

3.2.4.3.1. Analyses of the potential toxic/inhibitor compounds in the fractions obtained in dynamic adsorption and desorption experiments

The presence of compounds with antimicrobial properties, that can be toxins/inhibitor for the microorganism, was assessed in the different fractions used in the dynamic adsorption and desorption experiments. The presence of organic acids, metals and volatile organic compounds was analysed in the initial extract (in the column inlet), in the column outlet and in the samples of ferulic acid desorbed with ethanol. Table 3.7 A) shows the concentration of organic acids and metals in the initial extract (column inlet) and in the second sample of ferulic acid desorbed with ethanol (mg.L^{-1}). Ferulic acid is concentrated 13x in the second sample ethanol. Table 3.7 (B) shows the results of GC-MS in the initial extract, in the column outlet and in the samples of ferulic acid desorbed with ethanol. Figure 3.14 illustrates the concentration of organic acid in the column outlet during the dynamic adsorption experiment.

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Table 3.7. A) Concentration of organic acids and elements in the extract in the column inlet and in the second sample of ferulic acid desorbed with ethanol; B) GC-MS results from the feed solution in the column inlet, in the column outlet and in the sample of resin desorbed with ethanol (RT = retention time; LRI = linear retention index; LRI* = linear retention index in the literature).

(A)

Compounds	Initial Concentration (mg.L ⁻¹)	Concentration in the 2 nd sample of desorption with ethanol (mg.L ⁻¹)
Ferulic acid	1910	25230
Organic acid		
Acetic acid	2590	<4
Lactic acid	1510	<4
<i>Elements</i>		
Al	0.07	0.04
B	0.13	0.02
Ca	6.83	80.56
Cu	0.93	0.05
Fe	0.11	0.02
K	102.8	13.24
Mg	2.58	0.10
P	64.13	0.42
Si	6.19	2.58
Zn	1.16	0.14

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(B)

Compounds	RT (min)	LRI	LRI*	Area (AU)		
				Initial	2 nd sample of desorption with ethanol	Column outlet ^a
Heptanal	7.31	904	902	5.10x10 ⁷	-	7.77x10 ⁷
Benzaldehyde	9.31	965	961	3.10x10 ⁹	1.31x10 ⁹	9.35x10 ⁸
Nonanal	14.64	1105	1108	1.10x10 ⁸	-	1.68x10 ⁸
Decanal	18.30	1206	1188	9.07 x10 ⁷	-	7.17x10 ⁷
Dodecanal	25.11	1411	1409	2.44x10 ⁸	-	2.863x10 ⁷
Ethanone 1-(2-aminophenyl)	21.40	1297	1285	4.75 x10 ⁹	2.63x10 ⁹	1.18x10 ⁸
2-Methoxy-4-vinylphenol	21.79	1310	1993	5.17x10 ⁸	6.93x10 ⁷	2.74x10 ⁷
Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	30.40	1585	1581	2.63x10⁸	1.67x10⁷	3.16x10⁷

^asample up until the breakthrough point

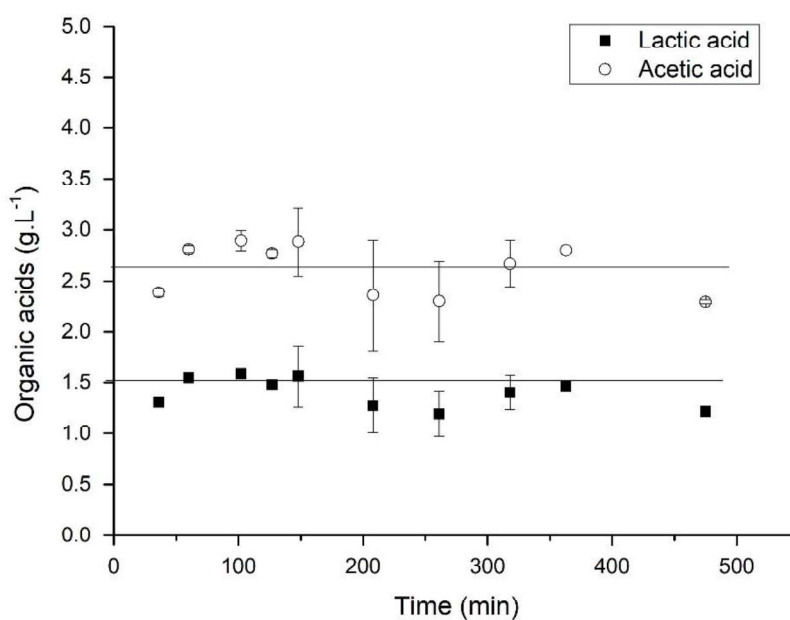


Figure 3.14. Concentration of organic acids in the column outlet during the dynamic adsorption experiment.

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As shown in Table 3.7 (A), a high concentration of organic acids (lactic and acetic acid) was detected in the initial feed solution (column inlet). These organic acids are known for their antimicrobial properties [61–64]. In order to achieve a concentration of 10 g.L⁻¹ of ferulic acid in the bioconversion broth, the initial extract, with about 2.0 g.L⁻¹ of ferulic acid, needs to be concentrated 5 times, achieving a concentration of acetic acid and lactic acid of 13.0 g.L⁻¹ and 7.5 g.L⁻¹, respectively. These high concentrations of organic acids are probably toxic for *Amycolatopsis* sp. ATCC 39116 so they do not allow the bioproduction of vanillin. As shown in Table 3.7 (A), no trace of these organic acids was detected in the samples of ethanol (the eluent used), indicating that organic acids were not adsorbed by the resin. In fact, as shown in Figure 3.14, the concentration of organic acids in the extract at the column outlet was constant and similar to the concentration of each organic acid in the extract at the column inlet (2.6 g.L⁻¹ and 1.5 g.L⁻¹ of acetic acid and lactic acid, respectively), during the ferulic acid adsorption. Thus, it is possible to separate ferulic acid from organic acids by adsorption with this resin. As explained before this resin has a polystyrene-divinylbenzene matrix that adsorbs ferulic acid by result of the hydrophobic interaction between its aromatic ring and the phenyl rings of these resins and specific π - π interaction [75,180,181] and excludes polar compounds as acetic acid and lactic acid. However, these compounds could have been retained by the amine group of the resin, but this was not observed, possibly by the fact of the low presence of this groups in the this resin [186,187].

The only metals detected in the initial extract at the column inlet were aluminium, calcium, copper, iron, potassium, magnesium and zinc in low concentration values. However, these compounds have a high antimicrobial effect even in small quantities [173].

Analysing the concentration of these compounds in the second desorption sample, (Table 3.7 A), where ferulic acid is concentrated 13x, the concentration of all the elements decreased to a residual concentration range, indicating that the compounds were not significantly adsorbed by the resin. The exception is calcium, which is concentrated 12x in the desorption sample, indicating that this compound is retained by the resin.

The volatile organic compounds were identified by comparing the resulting spectra with MS NIST version 2.0 mass spectra database library by matching the mass spectra and by comparing the linear retention indices (LRIs) with those found in the literature (Table 3.7 B). The LRIs were calculated with the Van den Dool and Kratz equation [198] and the retention indices in the literature [23]. As shown in Table 3.7 B, some volatile organic compounds (heptanal, nonanal, decanal, dodecanal) that were detected in the initial (column inlet), were not detected in the ethanol sample (where ferulic acid was desorbed) and were detected in the samples from the column outlet, indicating that these compounds were not adsorbed to the resin. These compounds (aldehydes) have an antimicrobial effect [174] and their presence could interfere with the bioproduction of vanillin. Other compounds, such as Benzaldehyde, Ethanone 1-(2-aminophenyl), 2-Methoxy-4-vinyl phenol, pentanoic acid 2,2,4-trimethyl-3-carboxyisopropyl, and isobutyl ester, although present in the second desorption sample

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(containing ethanol) are also present in the column outlet samples, indicating a reduction of these compounds in the microorganism feeding media. The results show that the corn fibre extract with ferulic acid was purified and that compounds that are toxins/inhibitors of the microorganism were eliminated or reduced.

3.2.4.3.2. Bioconversion of the ferulic acid recovered from the resin to vanillin

Ferulic acid desorbed from the resin was tested as a potential material for vanillin production. *Amycolatopsis* sp. ATCC 39116 was grown for 24 h, the cells were centrifuged and ferulic acid was added. In this test a maximum concentration of ferulic acid of about 9 g.L⁻¹ was tested, this concentration having been reported as optimal for vanillin production, [1,3,87,184]. Table 3.8 represents the results of bioconversion using ferulic acid recovered from the Macronet™ MN102 resin with ethanol absolute ($\geq 99.8\%$) (based on the results obtained previously), with and without the step of rinsing the resin with water between the adsorption and desorption steps.

Table 3.8. Bioconversion of the ferulic acid recovered from the resin to vanillin, with different concentrations of ferulic acid and different strategies of desorption.

Concentration of ferulic acid (g.L ⁻¹)	Strategy of desorption	Consumption of ferulic acid	Production of vanillin
4.6	Ethanol absolute - without rinsing the resin after adsorption	All ferulic acid was consumed	+
8.7	Ethanol absolute - without rinsing the resin after adsorption	A small amount of ferulic acid was consumed	-
9.0	Ethanol absolute- rinsing the resin after adsorption	All ferulic acid was consumed	+
10.0 ^a	-	All ferulic acid was consumed	+

^a Commercial ferulic acid

As shown in Table 3.8 *Amycolatopsis* sp. ATCC 39116 could produce vanillin when it was fed with 9 g.L⁻¹ of desorbed ferulic acid. However, at a concentration of 9.0 g.L⁻¹ ferulic acid, it was only possible to produce vanillin when the resin was rinsed with water between adsorption and desorption. The procedure of rinsing the resin with water to remove any residual solution is always used in adsorption processes [70,178,188,197]. In this case, however, the rinsing procedure has particular significance since it compromises vanillin production.

3.2.5. Conclusions

This work demonstrates that it is possible to purify ferulic acid extracted from corn fibre and consequently produce biotechnological-vanillin with *Amycolatopsis* sp. ATCC 39116, using 10 g.L⁻¹ of purified ferulic acid. An efficient adsorption of ferulic acid through the MacronetTM MN102 resin was achieved. The parameters for scaling up the process were determined by studying the adsorption process with MacronetTM MN102 (adsorption, desorption, rinse) in a fixed-bed column. A maximum adsorption capacity of 176 mg ferulic acid.g⁻¹ dry resin was achieved at pH 4.5 at a flow rate of 5.23 ml.min⁻¹ and 90.9 % of ferulic acid was recovered when using ethanol absolute (≥99.8 %) as an eluent and maintaining the flow rate at 5.23 ml.min⁻¹. The purification of ferulic acid from the corn fibre alkaline extract allowed the removal or reduction of the potential toxic/inhibitor compounds for the *Amycolatopsis* sp. ATCC 39116 bacteria, such as organic acids, metals, aldehydes and phenols. The step of rinsing the resin between adsorption and desorption turned out to be extremely important to assure the biological production of vanillin.

4. Bioconversion of ferulic acid into vanillin

This chapter was partially adapted from: Rita Valério, Ana R. S. Bernardino, Maria L. Tavares, Carla Brazinha, João G. Crespo, Cristiana A.V. Torres, Maria A. M. Reis, “Feeding strategies to optimize vanillin production by Amycolatopsis sp. ATCC 39116”, Bioprocess and Biosystems Engineering (2021), <https://doi.org/10.1007/s00449-020-02482-7>. The chapter was complemented with the biotechnological-vanillin production using ferulic acid from corn fibre, according to the optimization of ferulic acid extraction and purification process previously reported in chapters 2.1,3.1 and 3.2 of this thesis.

The author was involved in planning and performing the experiments, as well as on the discussion and interpretation of the results and preparation of the manuscript.

4.1. Summary

The growing consumer demand for natural products led to an increasing interest in vanillin production by biotechnological routes. In this work, the biotechnological vanillin production by *Amycolatopsis* sp. ATCC 39116 is studied using ferulic acid as precursor, aiming to achieve maximized vanillin productivities. It is also compared the bio-vanillin production using commercial and ferulic acid extracted from corn fibre.

During biotech-vanillin production, the effects of glucose, vanillin and ferulic acid concentrations in the broth proved to be relevant for vanillin productivity. Concerning glucose, its presence in the broth during the production phase avoids vanillin conversion to vanillic acid and, consequently, increases vanillin production. To avoid the accumulation of vanillin up to a toxic concentration level, a multiple pulse feeding strategy was implemented, with vanillin removal from the broth and biomass recovery. This strategy turned out fruitful, leading to $0.46 \text{ g.L}^{-1}.\text{h}^{-1}$ volumetric productivity of vanillin of and a production yield of $0.69 \text{ g}_{\text{vanillin}}.\text{g}_{\text{ferulic acid}}^{-1}$, which are among the highest values reported in literature for non-modified bacteria. Finally, the biotechnological-vanillin production by *Amycolatopsis* sp. ATCC 39116, using a single pulse of around 10 g.L^{-1} of ferulic acid extracted from corn fibre, was successful, leading to an overall volumetric productivity of vanillin of $0.29 \text{ g.L}^{-1}.\text{h}^{-1}$, a maximum concentration of vanillin of 5.00 g.L^{-1} and a vanillin yield of $0.52 \text{ g}_{\text{vanillin}}.\text{g}_{\text{ferulic acid}}^{-1}$, values similar to those obtained in a single pulse around 10 g.L^{-1} of commercial ferulic acid.

4.2. Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the main constituent of natural vanilla flavour responsible for its characteristic aroma [3]. It is the world’s most used flavour compound, applied widely in the food, beverage, pharmaceutical and perfumery industries. Natural botanical vanilla is extracted from the cured beans of two species of vanilla orchids. However, to satisfy the increasing market needs, vanillin is mostly synthesized through chemical routes. About 85 % of vanillin from synthetic origin is produced using the petrochemical precursor guaiacol and most

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remaining vanillin is produced from lignin, more precisely, from lignosulfonates [11]. Nevertheless, there is a rising consumer interest and market demand for healthy, natural food products. Therefore, producers use both the synthetic route and natural vanillin sources, with a trend towards the substitution of synthetic by natural-like vanillin, in order to label their products as all-naturals [9]. However, the global production of botanical vanillin cannot keep up with this increasing demand in terms of quantity and affordable price, leading to the need of flavour companies to find alternative sources of natural vanillin, with a similar quality presented by the botanical product [1,9]. Biotechnological processes are a good option for producing vanillin, since the vanillin produced by this way is considered natural, according to both US and European food legislation. Indeed, the USA legislation considers as natural “an essence or extractive derived from fermentation products” and the EU considers as natural a substance “obtained by microbiological processes from a material of vegetable or animal origin” (U.S Food and Drug Administration and, Regulation (EC) No 1334/2008, respectively) [13]. Compared to the botanical aroma, the vanillin produced by biotechnological processes has the advantage of not depending on agriculture conditions, which has an enormous impact on the production cost and market price. The possibility of scaling-up the production as a controlled process is also an important advantage over the botanical product [3,11,15].

Ferulic acid is the most reported substrate for biotech vanillin production due its natural abundance, particularly in the plant cell wall, and its high structural similarity to vanillin [1,28]. Ferulic acid is a hydroxycinnamic acid commonly found in agricultural by-products (e.g. corn and wheat bran [68,199], sugar beet pulp [41] and rice bran [37], which can be released by enzymatic [39,40] or chemical treatment (acid or alkaline) [42,43]. These agro-industrial by-products are low-cost and sustainable resources for the production of value-added compounds and their utilization can contribute to reducing environmental impact, lowering production costs and helping to tackle the problem of waste disposal. Corn fibre is one of these agro-industrial byproducts from starch industry, a particularly interesting source for the production of vanillin, due to its high content of ferulic acid (about 3% 1.5-3% (w/w) of total ferulic [33–36].

Several species of microorganisms have been studied for biotech-vanillin production using ferulic acid as a precursor [200–207]. However, vanillin has a toxic effect on most microorganisms [1,82,83], which can cause the bacterial strain to degrade vanillin to compounds less harmful, such as vanillic acid, vanillyl alcohol and guaiacol. As a consequence, vanillin concentration does not reach high values and the production yield remains rather low [16,82]. The bacteria *Amycolatopsis* sp. ATCC 39116 (previously known as *Streptomyces setonii* ATCC 39116) is one of the most promising microorganisms for vanillin accumulation as it has the ability to produce and tolerate higher levels of vanillin [82], making this strain particularly interesting for industrial applications [83,208]. However, it has been reported that concentrations of vanillin above 7 g.L⁻¹

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¹ cause cell death after a few hours, and concentrations above 10 g.L⁻¹ have an even stronger effect in a shorter period of time [84].

In order to overcome the toxic effect of vanillin and improve the vanillin production yield and the overall vanillin productivity, several strategies have been reported, including the following: (1) optimizing the medium composition and reactor operating conditions[3,85,86]; (2) reducing the contact of vanillin with the microorganism by using resins to adsorb vanillin[87] in a solid-liquid two-phase partitioning bioreactor system for *in situ* product removal [86] and (3) using genetically engineered *Amycolatopsis* sp. ATCC 39116 to avoid vanillin conversion to vanillic acid [84,209].

However, most of the experiments to optimize the composition of the medium were performed in shake flasks without a suitable control for pH or dissolved oxygen. Moreover, the yields were low as there was a high conversion of vanillin to other by-products product [3,85]. The use of adsorbent resins or polymers for *in situ* removal of vanillin from the broth (with a consequent decrease of its contact with the microbial culture) shows some advantages in vanillin production processes: a higher quantity of ferulic acid can be added to the broth and a higher quantity of vanillin can be produced without reaching toxic concentrations [86,87]. The disadvantage of these processes is that they take several steps to purify the vanillin, making them relatively inefficient. The resin or polymer adsorbs the vanillin so its recovery from these solid matrices is associated with losses and involves the use of organic solvents. The production of vanillin by genetically modified microorganisms may improve vanillin productivity but the use of these type of organisms is a concern to consumers so they may not be readily accepted.

In this work, different operating conditions were studied in order to improve the yield and productivity of vanillin production by the bacterium *Amycolatopsis* sp. ATCC 39116, namely the control of the concentration of glucose, ferulic acid and vanillin in the fermentation broth. To the best of our knowledge, the study of the effect of glucose during the production phase, has not been extensively studied by other authors in a bioreactor under controlled conditions. Furthermore, vanillin production by fed-batch is a common strategy reported by other authors. However, the use of a multiple pulse feeding strategy that maximizes the use of the biomass and avoids the accumulation of levels of vanillin that are toxic to the bacterial strain, have not been reported before.

In this work it was also compared the bio-vanillin production using commercial and ferulic acid extracted from corn fibre.

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4.3. Materials and Methods

4.3.1. Microorganism and medium

The strain used in all experiments was *Amycolatopsis* sp. ATCC 39116. It was conserved in cryovials containing glycerol (20 %, v/v), as a cryoprotectant agent, at -80 °C.

The culture was grown in a basal medium, containing: Na₂HPO₄, 4 g.L⁻¹; KH₂PO₄, 1 g.L⁻¹; NaCl, 0.2 g.L⁻¹, MgSO₄.7H₂O, 0.2 g.L⁻¹; CaCl₂.H₂O, 0.05 g.L⁻¹; glucose anhydrous, 20 g.L⁻¹ and yeast extract, 5 g.L⁻¹. The medium was sterilized at 1 bar, 121 °C for 30 minutes in autoclave.

Reactivation from the stock cultures was performed in the growth medium, which was also used to prepare *inocula* for the assays. Each pre-*inoculum* was prepared by transferring a colony to 200 mL of growth medium and incubating the culture in an orbital shaker for 36 h (45 °C, 200 rpm).

4.3.2. Fed-batch bioreactor operation

Experiments were performed, at lab scale, in 2 L bioreactor (BioStat B-plus, Sartorius, Germany) with automatic control of pH, temperature, aeration, dissolved oxygen concentration and an efficient mechanical stirring provided by two six-blade impellers and 4 baffles. This study is a proof of concept performed at lab scale, which has not been reported before. All assays were performed at 45 °C and, to minimize evaporation losses, the bioreactor condenser was maintained at 3 °C with a recirculating coolant. The bioreactor experiments consisted of two phases: an initial batch phase (growth phase) controlled at pH 7.2 ± 0.02, followed by a fed-batch phase (production phase) controlled at pH 8.2 ± 0.02 (best pH value for vanillin production according to Gunnarsson and Palmqvist (2006)) [210]. In the growth phase, the bioreactors, containing 1.6 L of growth medium, were inoculated with about 10 % (v/v) of inoculum, prepared as described above, making up an initial volume of about 1.8 L. The experiments started with an initial cell mass concentration of 0.71 ± 0.26 g_{CDW}.L⁻¹. The production phase started at the end of growth phase, with the addition of 200-220 mL of a ferulic acid solution (90 g.L⁻¹ of ferulic acid in 0.5 M of NaOH) in a single pulse, resulting in a concentration of 8.20 ± 0.63 g.L⁻¹ of ferulic acid in the culture broth. The aeration rate was kept constant during the experiments at 1 vvm, (volume of air per volume of reactor per minute). The dissolved oxygen concentration was controlled at 30 % saturation by the automatic variation of the stirring rate (500-1000 rpm).

The different pulse feeding strategy experiments were performed at lab scale. Depending on the desired ferulic acid concentration in the bioreactor, multiple pulses of 200-220 ml or 400 ml of a ferulic acid solution (90 g.L⁻¹) were fed to the bioreactor leading to a concentration of 8.20 ± 0.63 g.L⁻¹ or 14.91 ± 0.29 g.L⁻¹ of ferulic acid in the culture broth. The rationale to feed the ferulic acid pulses to the bioreactor was the raise of the dissolved oxygen level that, from previous experiments, indicates the ferulic acid exhaustion. For the experiments with biomass recovery

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before each pulse (including the first one), the culture broth was centrifuged at $4424 \times g$ for 20 minutes. The biomass was resuspended in a solution with 8 g.L^{-1} of glucose and 0.18 g.L^{-1} of yeast extract and immediately reintroduced in the bioreactor.

For the bioreactors with single pulse addition of ferulic acid, duplicates were performed. Standard deviation was always lower than 13 % of the average values for each parameter evaluated. Therefore, we concluded that there was no need to do replicas for all the assays.

4.3.3. Fed-batch bioreactor with ferulic acid from corn fibre operation

The experiment was performed as describe in Fed-batch operation section. After the growth phase, the culture broth was centrifuged. The biomass was resuspended in 9.64 g.L^{-1} ferulic acid solution from corn fibre supplemented with 12 g.L^{-1} of glucose and 1 g.L^{-1} of yeast extract and immediately reintroduced in the bioreactor. The ferulic acid solution from corn fibre was prepared according to the optimization of ferulic acid extraction and purification process previously reported in chapters 2.1, 3.1 and 3.2 of this thesis. Ferulic acid was extracted from corn fibre by alkaline extraction optimized at 30°C , with 0.25 M NaOH , during 7 h, using a liquid-to-solid ($\text{g}_{\text{solvent}}/\text{g}_{\text{dry fibre}}$) ratio of 10. The alkaline extract obtained was centrifuged at $9174 \times g$ during 15 minutes to remove the solids. The supernatant was subjected to an ultrafiltration with the UP150 membrane at the optimized conditions, according to the chapter 3.1, in order to separate the ferulic acid from high molecular weight polysaccharides (arabinoxylans). Ultrafiltration permeate was passed through the Macronet[™] MN102 resin for ferulic acid adsorption. The ferulic acid was desorbed from resin with absolute ethanol, after the resin was rinsed. The ethanol was evaporated and ferulic acid was resuspended in 1.8 L of water, resulting in a concentration of 9.64 g.L^{-1} of ferulic acid. The pH was adjusted to 8.2 (pH of production phase).

4.3.4. Analytical techniques

The culture broth samples were centrifuged at $2000 \times g$, for 10 min, for cell separation. The cell-free supernatant (stored at -20°C) was used to measure the phenolic compounds, glucose and total nitrogen. The cell pellet was used for the gravimetric determination of the biomass concentration. After washing twice with deionized water (resuspended in water, centrifuged at $2000 \times g$ for 10 minutes) the pellets were lyophilized and weighed.

4.3.4.1. Quantification of phenolic compounds

The concentration of ferulic acid, vanillin and vanillic acid was determined by high performance liquid chromatography (HPLC; Alliance) equipped with a C18 (Nova-Pak®) $3.9 \text{ mm} \times 150 \text{ mm}$ column (particle diameter of 4 mm) and guard column, coupled to a PDA detector, set at 280 nm.

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The compounds were separated at 30° C with a gradient elution program at the flow rate of 0.5 mL.min⁻¹. The mobile phase was a mixture of two eluents, (A) 10 % (v/v) methanol, 2 % (v/v) acetic acid in Milli-Q water and (B) 90 % (v/v) methanol, 2 % (v/v) acetic acid in Milli-Q water. The gradient elution program was 0 % B (0–10 min), from 0 to 15 % B (10–25min), from 15 to 50 % B (25–35 min) and from 50 to 100 % B (35–38 min). The ferulic acid (≥99 %), vanillin (99 %) and vanillic acid (≥97 %) (Sigma Aldrich) were used as standards at concentrations between 0.006 – 0.2g.L⁻¹.

4.3.4.2. Glucose quantification

For glucose quantification, the cell-free supernatant was filtered through 0.2 µm centrifuge filters and analyzed by HPLC (VWR Hitachi Organizer) with a detector Merck Differential Refractometer RI-71. The pre-column and the column were Metacarb 87H (VARIAN) and Aminex HPX - 42A 125-0129 (Bio-Rad), respectively. The samples were processed during 15 min at 30 °C with 0.01 N H₂SO₄ as eluent at a flow rate of 0.5 mL.min⁻¹. D (+) glucose anhydrous (Scharlau) was used as standard, at concentrations between 0.006 – 1.0 g.L⁻¹.

4.3.4.3. Nitrogen quantification

Total nitrogen was quantified using the Hach Lange Laton® total nitrogen (TN_b) kit protocol. The samples were diluted in deionized water to assure a nitrogen concentration within the range measured by the kit (between 20 and 100 mgL⁻¹). After the procedure indicated in the protocol, the total nitrogen was measured in a spectrophotometer DR 2800™ by recognition of the bar code present in the sample cell.

4.4. Results and discussion

4.4.1. Vanillin production in a single pulse fed-batch

Vanillin production from ferulic acid by *Amycolatopsis* sp. ATCC 39116 was performed in a 2 L bioreactor, using yeast extract (5 g.L⁻¹) as nitrogen source and glucose (20 g.L⁻¹) as carbon source, in a fed-batch mode. Due to the reported toxicity of vanillin for cell growth, the process was divided in two consecutive phases, a growth phase in which bacteria were cultivated in the growth medium and a production phase, starting in the beginning of the stationary growth phase, with the addition of ferulic acid [86].

Figure 4.1 illustrates the process of vanillin production in a single pulse fed-batch operating mode (run 1), showing the profiles of cellular mass, glucose consumption and production of vanillin and vanillic acid from ferulic acid. The cell mass concentration reached 4.4 g_{CDW}.L⁻¹ by the end

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of the batch phase, with a biomass / substrate yield of $0.96 \text{ g}_{\text{CDW}} \cdot \text{g}_{\text{glucose}}^{-1}$, and a nitrogen consumption of $0.33 \text{ g}_{\text{N}} \cdot \text{L}^{-1}$

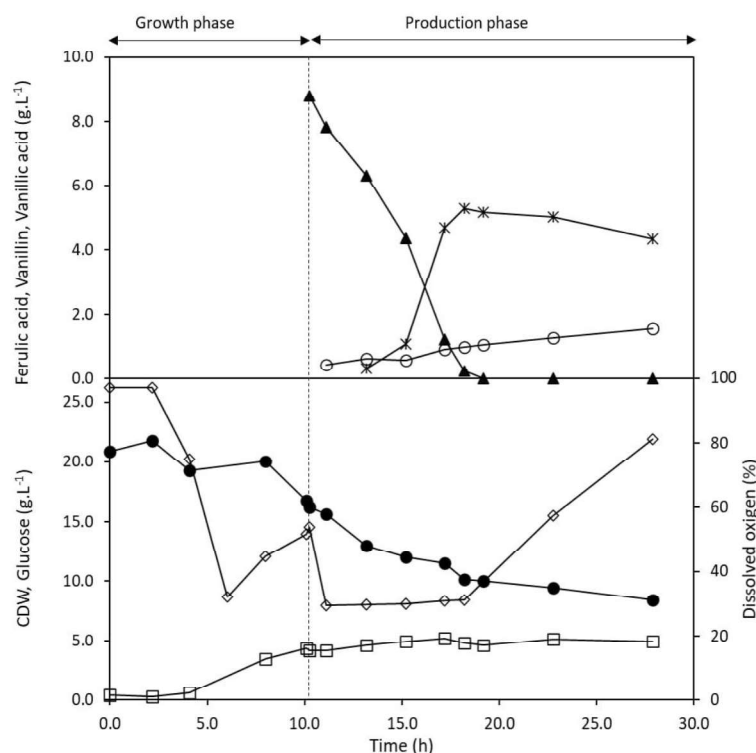


Figure 4.1. Cultivation of *Amycolatopsis* sp. ATCC 39116 in a single pulse fed-batch operational mode with glucose concentration of 20 g.L^{-1} , during the run time (run 1). Cell Dry Weight (CDW) (□), glucose (●), ferulic acid (▲), vanillin (⋈), vanillic acid (o) and dissolved oxygen (◇). The dashed line represents the moment of addition of ferulic acid.

After 10 h of cultivation, the nitrogen concentration becomes limiting for cellular growth, and the culture entered the stationary phase, which was indicated by the increase in the dissolved oxygen level (data not shown). Then, a single pulse of ferulic acid (8.79 g.L^{-1}) was added to the culture broth.

Following the addition of ferulic acid, there was an adaptation phase of around 3 h during which vanillic acid accumulated (0.63 g.L^{-1}) in the broth before vanillin production began. Muheim and Lerch (1999) and Fleige et al. (2013) also reported vanillin production after a period during which vanillic acid accumulated [82,83]. Fleige et al. (2013). This suggests that there is a regulatory mechanism by which vanillic acid influences the gene expression of the enzyme that catabolizes vanillin or even the enzymatic activity itself, thus allowing for the accumulation of vanillin [82]. During the production phase, an increase in the concentration of vanillic acid was observed, due to the oxidation of vanillin, reaching a concentration of 1.57 g.L^{-1} . This accumulation of vanillic acid is defined as a detoxification process since vanillin is an aromatic aldehyde toxic to the cells [1,28,83,86].

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A maximum vanillin concentration of 5.28 g.L⁻¹ was reached 18 h into the cultivation process, corresponding to the production of a total mass of 10.37 g vanillin, with a vanillin / ferulic acid yield ($Y_{P/S}$) of 0.62 g.g⁻¹ (molar yield of 78.8 %) and a volumetric productivity (r_P) of 0.29 g.L⁻¹.h⁻¹ (calculated from the beginning of the run to the point of maximum production of vanillin) and a rate of vanillin production (r_v) of 0.66 g.L⁻¹.h⁻¹ (calculated from when the ferulic acid was fed in to the point of maximum production of vanillin). These results are within those reported in the literature for vanillin production in bioreactors, albeit under different operating conditions, using glucose as carbon source. Ma and Daugulis (2013) reported a molar yield of 89.2 % and a r_P of 0.27 g.L⁻¹.h⁻¹, while Fleige *et al.* (2016) reported a molar yield of 73.9%, using glucose as carbon source for *Amycolatopsis* sp [84,86].

At the end of the production phase addition of acid was required to keep the pH at 8.2. The increasing rate of addition of acid for pH control and the rise in the dissolved oxygen concentration, indicates a decrease in cellular activity, which is coincident with the maximum vanillin production. Such behaviour could be used as a good indicator to stop the process, in order to avoid the conversion vanillin into vanillic acid, and therefore achieve higher vanillin production yields. Ma and Daugulis (2014) and Pérez-Rodríguez *et al.* (2016) also reported an alkalization by the end of the vanillin production phase [3,85].

4.4.1.1. Impact of glucose in the production of vanillin

Taking into account the initial glucose concentration reported by Muheim *et al.* (1988) in the European patent 885968 [211] a previous experiment was carried out using a glucose concentration of 20 g.L⁻¹, being consumed a total of 11.62 g.L⁻¹, resulting in a significant amount of residual glucose (8.38 g.L⁻¹) at the end of the run (Figure 4.1). Therefore, in order to reduce the waste of glucose, an experiment with an initial glucose concentration similar to the total consumed in the previous run (12 g.L⁻¹), was performed (Figure 4.2, run 2).

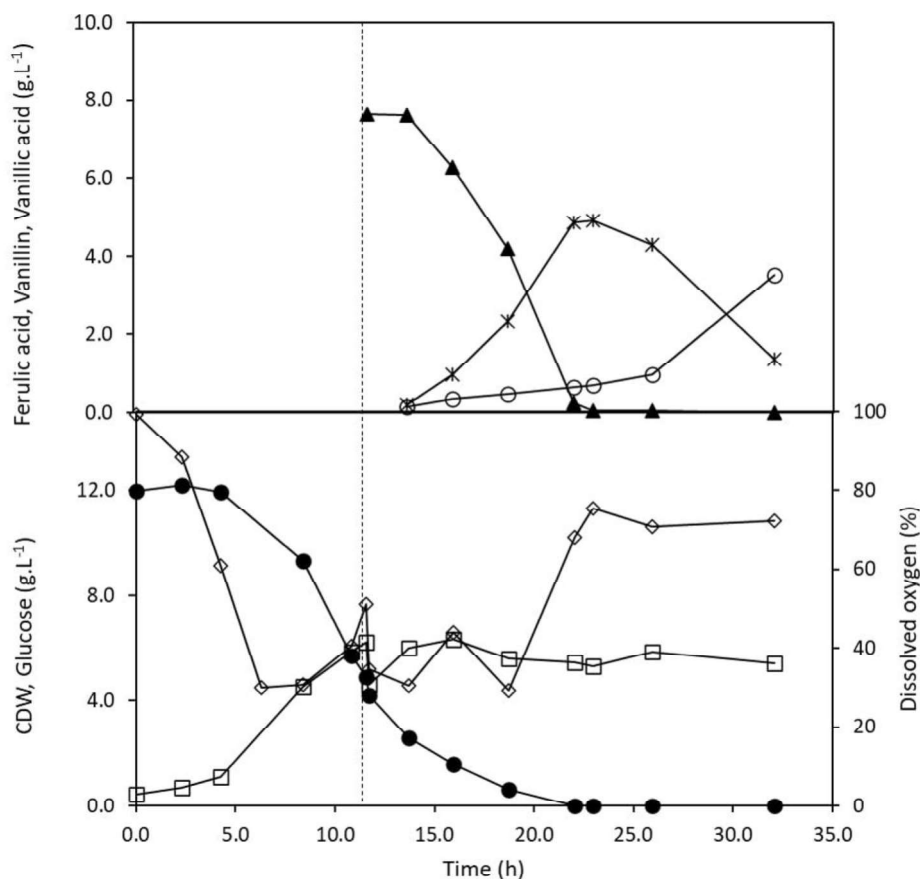


Figure 4.2. Cultivation of *Amycolatopsis* sp. ATCC 39116 in a single pulse fed-batch operational mode, with glucose a concentration of 12 g.L⁻¹ (run 2). CDW (□), glucose (●), ferulic acid (▲), vanillin (✱), vanillic acid (○) and dissolved oxygen (◇). The dashed line represents the moment of addition of ferulic acid.

During the growth phase, which lasted for 11.6 h, 6.2 g.L⁻¹ of biomass was produced by consuming 5.7 g.L⁻¹ of glucose, resulting in a similar cellular growth per unit of glucose consumed ($Y_{X/S} = 0.92 \text{ g}_{\text{CDW}} \cdot \text{g}_{\text{glucose}}^{-1}$) when compared against the first run. After, the ferulic acid was added (7.64 g.L⁻¹). There was then a short adaptation period of 2 h before the start of the production phase, during which the vanillin reached a maximum concentration of 4.92 g.L⁻¹, 23 h, equivalent to the production of 9.51 g of vanillin, with a 0.65 g.g⁻¹ vanillin yield on ferulic acid and a volumetric productivity of 0.21 g.L⁻¹.h⁻¹ (calculated from the beginning of the run to the point of maximum production of vanillin) and a rate of vanillin production (r_v) of 0.44 g.L⁻¹.h⁻¹ (calculated from when the ferulic acid was added to the point of maximum production of vanillin).

The concentration of glucose at the beginning of the production phase was lower than in the previous run (16.20 g.L⁻¹ and 4.20 g.L⁻¹ in run 1 and run 2, respectively). In the second run all glucose was consumed by the point when the maximum vanillin concentration was achieved (after 23 h).

The comparison between runs 1 and 2 (Figure 4.1 and Figure 4.2) indicates that in the absence of glucose, vanillin is metabolized to vanillic acid at a higher rate than when glucose is present in

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the fermentation broth. In run 1, with glucose available throughout the process, vanillin was metabolized at a rate of $0.097 \text{ g.L}^{-1}.\text{h}^{-1}$ once it had reached its maximum concentration, resulting in a vanillic acid concentration of 1.57 g.L^{-1} at the end of the run. In run 2, glucose depletion occurred after 23 h, which coincided with the achievement of the maximum vanillin concentration. After that, vanillin was metabolized into vanillic acid (3.52 g.L^{-1}) at a rate of $0.39 \text{ g.L}^{-1}.\text{h}^{-1}$, a value four times higher than observed in run 1.

Since vanillin is a reactive aldehyde, it is degraded by the microbial culture in order to avoid toxic concentration levels. Further, in the absence of glucose vanillin is catabolized into vanillic acid working as an energy source [82,212]. In fact, it has been reported that vanillin can be converted to pyruvate and oxaloacetate, which enter into the tricarboxylic acid (TCA) cycle to produce energy for the microorganism through the protocatechuate 4,5–cleavage (PCA) pathway [155]. However, a microorganism can obtain more energy from the consumption of glucose than from the degradation of vanillin, so when glucose is present in the bioreactor, the microorganism consumes the glucose instead of degrading the vanillin [82,212].

In run 2, after glucose depletion, vanillin appears to have been used as substrate for cellular maintenance, which would explain the increased consumption rate indicated by the rising values of dissolved oxygen).

4.4.1.2. Multiple pulse feeding strategy

The amount of vanillin production depends on the ferulic acid available in the medium: a higher concentration of ferulic acid produces more vanillin [83]. It was found that when the ferulic acid concentration was below 4 g.L^{-1} , vanillin was rapidly converted to vanillic acid. However, since ferulic acid has an antimicrobial effect [1,155], a compromise between productivity and toxicity has to be found. Therefore, a fed-batch operating mode has advantages over a batch process [85].

In order to increase vanillin production a multiple pulse feeding strategy was implemented, keeping the growth conditions the same as in run 1, but adding three consecutive ferulic acid pulses with a concentration of around $8.20 \pm 0.63 \text{ g.L}^{-1}$ each (run 3).

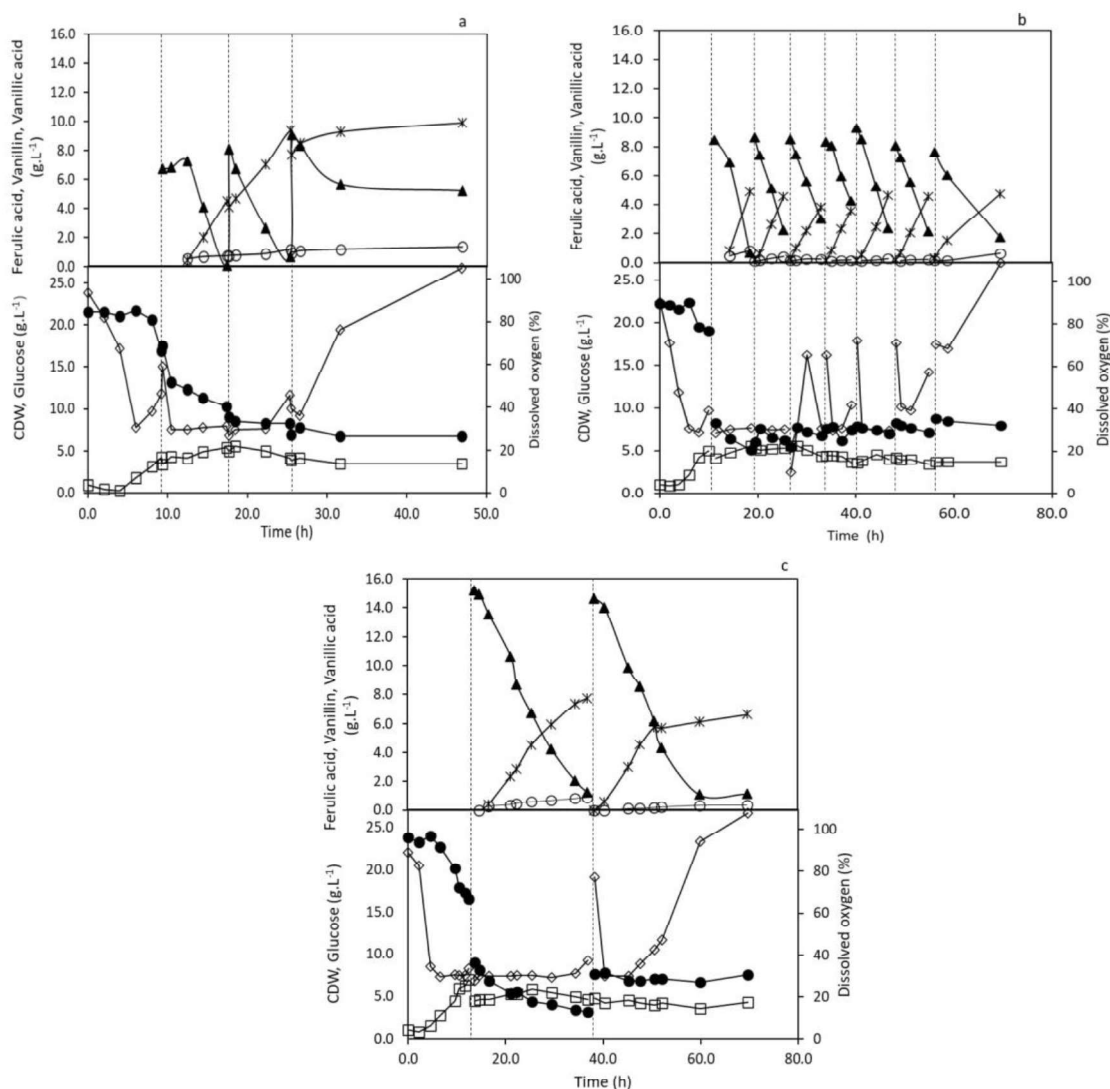


Figure 4.3. Cultivation of *Amycolatopsis* sp. ATCC 39116 in fed-batch operational mode. CDW (□), glucose (●), ferulic acid (▲), vanillin (✕), vanillic acid (○) and dissolved oxygen (◇).
a. Pulse feeding with three given pulses of ferulic acid (8.20 ± 0.63 g.L⁻¹) (run 3). b. Pulse feeding with seven given pulses of ferulic acid (8.20 ± 0.63 g.L⁻¹) and biomass recovery (run 4).
c. Pulse feeding with two pulses of ferulic acid (14.91 ± 0.29 g.L⁻¹) and biomass recovery (run 5). The dashed lines represent the moments of addition of ferulic acid.

During the growth phase (around 9 h), the biomass concentration reached 4.2 g.L⁻¹, which is similar to that observed in run 1.

During the first pulse, 4.47 g.L⁻¹ of vanillin was produced, with a $Y_{P/S}$ of 0.67 g_{vanillin}·g_{ferulic acid}⁻¹ (Table 4.1). When the second ferulic acid pulse (7.97 g.L⁻¹) was given, the vanillin production continued without an adaptation phase and gave a vanillin / ferulic acid yield of 0.71 g_{vanillin}·g_{ferulic acid}⁻¹, reaching a total concentration of vanillin of 9.34 g.L⁻¹ by the end of the pulse (Figure 4.3a).

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Table 4.1. Kinetic parameters of vanillin production obtained with different ferulic acid pulse feeding strategies (FA is the concentration of ferulic acid fed to the bioreactor (g.L^{-1}), $Y_{P/S}$ is the yield of vanillin generated per glucose consumed ($\text{g}_{\text{vanillin produced}} \cdot \text{g}_{\text{ferulic acid consumed}}^{-1}$), r_v is the volumetric rate of vanillin production ($\text{g}_{\text{vanillin produced}} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$), q_P is the specific vanillin productivity ($\text{g}_{\text{vanillin produced}} \cdot \text{g}_{\text{biomass}}^{-1} \cdot \text{h}^{-1}$).

	Run 3					Run 4					Run 5				
Pulse	[FA] (g.L^{-1})	$Y_{P/S}$ (g.g^{-1})	r_v ($\text{g.L}^{-1} \cdot \text{h}^{-1}$)	q_P ($\text{g.g}_X^{-1} \cdot \text{h}^{-1}$)	[Vanillin] (g.L^{-1})	FA (g.L^{-1})	$Y_{P/S}$ (g.g^{-1})	r_v ($\text{g.L}^{-1} \cdot \text{h}^{-1}$)	q_P ($\text{g.g}_X^{-1} \cdot \text{h}^{-1}$)	[Vanillin] (g.L^{-1})	FA (g.L^{-1})	$Y_{P/S}$ (g.g^{-1})	r_v ($\text{g.L}^{-1} \cdot \text{h}^{-1}$)	q_P ($\text{g.g}_X^{-1} \cdot \text{h}^{-1}$)	[Vanillin] (g.L^{-1})
1	6.73	0.67	0.55 ^a	0.10	4.47	8.45	0.63	0.68 ^a	0.12	4.92	15.20	0.56	0.33 ^a	0.07	7.76
2	8.07	0.71	0.68 ^a	0.17	5.24	8.64	0.68	0.74 ^a	0.14	4.37	14.62	0.56	0.41 ^a	0.10	5.72
3	9.06	0.57	0.25 ^a	0.03	2.19	8.50	0.65	0.58 ^a	0.13	3.58	—	—	—	—	—
4	—	—	—	—	—	8.31	0.85	0.67 ^a	0.18	3.45	—	—	—	—	—
5	—	—	—	—	—	9.27	0.65	0.69 ^a	0.18	4.47	—	—	—	—	—
6	—	—	—	—	—	8.05	0.74	0.66 ^a	0.2	4.39	—	—	—	—	—
7	—	—	—	—	—	7.61	0.75	0.33 ^a	0.09	4.4	—	—	—	—	—
Overall	14.01 ^b	0.69	0.38 ^c	—	4.85±0.39 ^c	36.54 ^b	0.69	0.46 ^c	—	4.20±0.52 ^d	24.23 ^b	0.56	0.26 ^c	—	6.74±1.02 ^d

^a Calculated from the addition of ferulic acid to the point of maximum production of vanillin for each pulse.

^b Overall FA was calculated taking into consideration all the FA consumed in the different pulses.

^c Overall volumetric productivity (r_v) calculated from the beginning of the run until the decrease in the cellular activity.

^d Average value of the vanillin produced in the ferulic acid pulses considered in the calculations.

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When a third ferulic acid pulse (9.06 g.L⁻¹) was added, the dissolved oxygen continued increasing until 100%, indicating a loss of cellular activity. In fact, the rate of vanillin production calculated for this pulse (timed from the addition of ferulic acid at 26 h up until 32 h) (Table 4.1), decreased (to 0.25 g.L⁻¹.h⁻¹), which is lower than the amount obtained from the previous pulses (0.55 and 0.68 g.L⁻¹.h⁻¹, respectively). During the time considered for this pulse (6.2 h), only a small amount of ferulic acid was consumed (3.40 g.L⁻¹).

The loss of cellular activity was probably related to the high vanillin concentration (9.34 g.L⁻¹) in the fermentation broth since, as reported in the literature, bacteria shows a reduction of cell viability for vanillin concentrations above 4 g.L⁻¹ and a strong inhibitory effect above 7 g.L⁻¹ [84].

The overall parameters of production in run 3 are presented in Table 4.1. These parameters were calculated considering the first two pulses, since, during the third pulse, a strong production inhibition affected the cellular activity. The total mass of vanillin produced was 17.78 g and, at the end of the run, 2.17 g of vanillic acid had accumulated. The overall vanillin volumetric productivity was 0.38 g.L⁻¹.h⁻¹, which is higher than that obtained in the run with a single ferulic acid pulse (0.29 g.L⁻¹.h⁻¹). However, the yield of vanillin production (0.69 g_{vanillin}.g_{ferulic acid}⁻¹) was similar to that of run 1, indicating that a strategy of various ferulic acid pulses is advantageous for the vanillin production process. Nevertheless, this feeding strategy still needs to overcome the product toxicity problem caused by vanillin accumulation in the cultivation broth.

To overcome its toxic effect, vanillin should not reach a high concentration in the broth. To test this, a run with multiple pulses, with broth removal before each pulse, was performed (Figure 4.3 b). A centrifugation step was carried out to remove the broth containing vanillin and recover the biomass to continue the production. In this new operation strategy, the growth phase of this run (run 4) took 10 h and produced 5.0 g.L⁻¹ of biomass.

As in the previous experiments, the production phase started by feeding the first ferulic acid pulse at a concentration of 8.45 g.L⁻¹ and the vanillin production started after an adaptation phase of 2 h, reaching a concentration of 4.92 g.L⁻¹ at the end of the pulse, with a yield of 0.63 g_{vanillin}.g_{ferulic acid}⁻¹. After the ferulic acid depletion (indicated by the increasing acid addition rate needed for pH control and by the pO₂ rising) the broth was removed by centrifugation and the biomass recovered; a second pulse of ferulic acid was then given. This process was repeated for the following six pulses of ferulic acid. For each pulse, the concentration of substrate, yield, volumetric and specific productivity (q_p) of vanillin, and product concentration are presented in Table 4.1. The first six pulses had almost identical kinetic parameters ($Y_{P/S}$, r_v and q_p) and had no significant differences from the parameters of the single pulse fed-batch (run 1, with a $Y_{P/S}$ of 0.62 g_{vanillin}.g_{ferulic acid}⁻¹, a r_v of 0.66 g.L⁻¹.h⁻¹ and a q_p of 0.14 g.gx⁻¹.h⁻¹, with the pulse until timed up to the point where the maximum vanillin concentration had been achieved). However, in the seventh pulse the level of dissolved oxygen reached 100 % and the rate of vanillin

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production and specific productivity were lower ($0.33 \text{ g.L}^{-1}.\text{h}^{-1}$ and $0.09 \text{ g.g}_x^{-1}.\text{h}^{-1}$), indicating a decrease of bacterial activity, probably due to the long duration of the experiment and the lack of micronutrients (e.g. phosphate) in the broth.

During the first six pulses, a global vanillin production of 46.49 g was achieved, with an overall yield of vanillin / ferulic acid of $0.69 \text{ g}_{\text{vanillin}}.\text{g}_{\text{ferulic acid}}^{-1}$ and an overall vanillin volumetric productivity (r_p) of $0.46 \text{ g.L}^{-1}.\text{h}^{-1}$, as reported in Table 4.1. The volumetric productivity achieved with this approach increased by 64% relative to that obtained in the single pulse approach and by 21% relative to the multiple pulse strategy without broth removal.

The multiple pulse strategy shows that broth removal by centrifugation is advantageous for the vanillin production process. This approach allows a higher amount of ferulic acid to be fed in while avoiding the accumulation of vanillin above toxic concentrations, keeping the process running for a little over 50 h. As a consequence, a higher overall volumetric productivity was achieved. In addition, the successive vanillin removal seems to lead to a lower rate of conversion to vanillic acid.

At the start of the production phase, and when vanillin started to be produced, there was already some vanillic acid present in the broth (in this run, 0.47 g.L^{-1}), as in the previous experiments. As suggested by Muheim and Lerch (1999) and Fleige et al. (2013), the vanillin accumulation can be metabolically triggered by the presence of low levels of vanillic acid [82,83]. In fact, in the first pulse of run 4 a higher vanillic acid production was observed when compared with the pulses that followed (0.86 g.L^{-1} in the first pulse and $0.17 \pm 0.06 \text{ g.L}^{-1}$ in the pulses that followed) and this higher initial vanillic acid level may have triggered the accumulation of vanillin.

Several strategies to avoid the accumulation of vanillin in the broth have been reported in the literature [87]. Ma and Daugulis (2013) added polymer beads (Hytrel® G4078W) to the bioreactor decreasing the contact of vanillin with cells and, consequently, its conversion to vanillic acid [86]. Their approach led to a vanillin volumetric productivity of $0.45 \text{ g.L}^{-1}.\text{h}^{-1}$ [86], similar to the value achieved in the present work. To the best of our knowledge, regarding non-modified strains, these two strategies are the ones that present better results for vanillin production.

4.4.1.3. Impact of using an increased ferulic acid concentration

Considering the promising results obtained with the strategy applied in run 4, an additional experiment was performed under the same operating conditions but with a ferulic acid concentration of $14.91 \pm 0.29 \text{ g.L}^{-1}$ in each pulse (run 5, Figure 4.3). This experiment aimed to establish whether a higher concentration of ferulic acid can be added to increase the productivity of vanillin without reaching toxic levels, and also aimed to test a simpler process with fewer centrifugation steps.

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In this run, 6.9 g.L⁻¹ of biomass was achieved during the growth phase (about 12 h). The production phase started with a ferulic acid pulse of 15.20 g.L⁻¹ and, after an adaptation phase of around 2 h, vanillin production began to reach a concentration of 7.76 g.L⁻¹ at the end of the pulse with a $Y_{P/S}$ of 0.56 g_{vanillin}·g_{ferulic acid}⁻¹. Even though the vanillin / ferulic acid yield was not significantly different from those obtained previously, the rate of vanillin production in this pulse (0.33 g.L⁻¹·h⁻¹) was lower (Table 4.1). This behaviour can be related with ferulic acid, which may inhibit bacterial activity. After feeding the second pulse to the bioreactor, the production proceeded without an adaptation phase as in runs 3 and 4. However, near to the 50th hour of operation, the dissolved oxygen level in the broth started to rise (data not shown) and the rate of vanillin production decreased from 0.41 to 0.052 g.L⁻¹·h⁻¹. This even higher reduction of biotransformation efficiency can be attributed to the long duration of the experiment as was the case in run 4 and in preliminary tests (data not shown), along with the use of more concentrated ferulic acid solution.

The overall kinetic parameters (Table 4.1) were calculated for the first 52 h of the process, during which time 26.57 g of vanillin was produced with a vanillin yield from ferulic acid of 0.56 g·g⁻¹ and a volumetric productivity of 0.26 g.L⁻¹·h⁻¹, much lower than the yield obtained in run 4 (0.46 g.L⁻¹·h⁻¹), and similar to the experiment that had a single pulse of ferulic acid.

These results show that a higher ferulic acid concentration does not favor the vanillin production process, since the rate of production is significantly lower than that obtained when it is fed with around 8.20 ± 0.63 g.L⁻¹ of ferulic acid. In fact, ferulic acid is reported to have an antimicrobial effect and some authors have reported a decrease in the bioconversion rate of ferulic acid for concentrations above 9 g.L⁻¹ [1,3,87,184]. Therefore, the ferulic acid concentration used in run 5 probably affected the metabolism needed to produce vanillin, making the process slower.

4.4.1.4. Vanillin production in a single pulse fed-batch using the corn fibre ferulic acid extract

After extensively study the vanillin production by the bacterium *Amycolatopsis* sp. ATCC 39116, using a ferulic acid model solution, best operation conditions were used to assay bio-vanillin production using an alkaline ferulic acid extract from corn fibre. In this experiment, due to the corn fibre ferulic acid extract concentration obtained, only one pulse of ferulic acid was fed to the bioreactor. Figure 4.4 illustrates the process of vanillin production in a single pulse fed-batch operating mode showing the production of vanillin and vanillic acid from ferulic acid extract and the consumption of *p*-coumaric, other hydroxycinnamic acid like ferulic acid, present in the walls of corn fibre [36], which was extracted during the alkaline extraction and absorbed by the resin MN102.

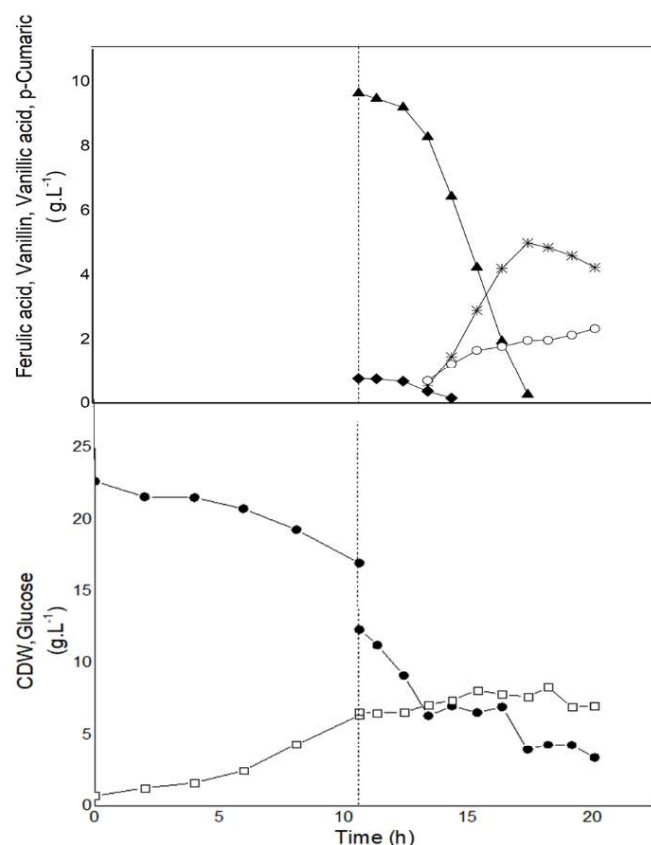


Figure 4.4. Cultivation of *Amycolatopsis* sp. ATCC 39116 in single fed-batch operational mode with extract from corn fibre. ferulic acid (▲), vanillin (⌘) and vanillic acid (○) *p*-coumaric (◆), CDW (□), glucose (●), The dashed lines represent the moments of addition of ferulic acid

The growth phase lasted for 10.6 h producing $5.67 \text{ g}_{\text{CDW}}.\text{L}^{-1}$ of biomass with a biomass/substrate yield of $1.00 \text{ g}_{\text{CDW}}.\text{g}_{\text{glucose}}^{-1}$, and a nitrogen consumption of $0.46 \text{ g}_{\text{N}}.\text{L}^{-1}$. After the end of the exponential growth phase the cells were centrifuged and resuspended in a solution with 12 g.L^{-1} of glucose, 1 g.L^{-1} of yeast extract and 9.64 g.L^{-1} of ferulic acid extracted from corn fibre and immediately reintroduced in the bioreactor. Vanillin production reached a maximum concentration of 5.00 g.L^{-1} , 17.6 h after the beginning of the process, equivalent to 8.79 g of vanillin produced, with a 0.52 g.g^{-1} vanillin yield on ferulic acid (molar yield of 66.15 %) and a volumetric productivity (r_p) of $0.28 \text{ g.L}^{-1}.\text{h}^{-1}$ (calculated from the beginning of the run to the maximum production of vanillin) and a rate of vanillin production (r_v) of $0.74 \text{ g.L}^{-1}.\text{h}^{-1}$ (calculated from ferulic acid feed to the maximum production of vanillin). In the extract, it was detected a small concentration of *p*-coumaric acid (0.76 g.L^{-1}), which was also metabolized by the *Amycolatopsis* sp. ATCC 39116 as shown in Figure 4.4. In fact, in literature is reported that *Amycolatopsis* sp. ATCC 39116 can metabolize *p*-coumaric into *p*-hydroxybenzaldehyde (not analysed in this work) [213], a component present in natural vanillin [214].

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Table 4.2. Kinetic parameters of vanillin production obtained with different ferulic acid sources (commercial ferulic acid and ferulic acid from corn fibre).

		Ferulic acid from corn fibre	Commercial ferulic acid (Synthetic)
$Y_{P/S}$	(g.g ⁻¹)	0.52	0.67±0.03 ^a
Molar $Y_{P/S}$	%	66.15	81.83 ±0.04 ^a
r_v	(g.L ⁻¹ .h ⁻¹)	0.74	0.65±0.06 ^a
r_p	(g.L ⁻¹ .h ⁻¹)	0.29	0.28±0.02 ^b
q_p	(g.gx ⁻¹ .h ⁻¹)	0.10	0.15±0.03 ^a
[Vanillin máx]	(g.L ⁻¹)	5.00	5.28
[Vanillic Ac.]	(g.L ⁻¹)	1.96	0.99
r_d	(g.L ⁻¹ .h ⁻¹)	0.284	0.097 ^c 0.392 ^d

^a average of all pulses from run 1,3, and 4

^b average of volumetric productivity from run 1, 3,and 4 calculated from the beginning of the run to the maximum production of vanillin in first pulse

^c rate of vanillin degradation in the presence of glucose

^d rate of vanillin degradation in the absent of glucose

The comparison between the bioconversion using commercial ferulic acid and ferulic acid extracted from corn fibre (Table 4.2), showed that the production of vanillin was very similar in both experiments, 5.28 and 5.00, respectively. However in the bioconversion using commercial ferulic acid, it was consumed (until the maximum production of vanillin) 8.55 g.L⁻¹ of ferulic acid corresponding at a yield of $0.67 \pm 0.03 \text{ g}_{\text{vanillin}} \cdot \text{g}_{\text{ferulic acid}}^{-1}$ (molar yield of $81.83 \pm 0.04 \%$), while in experiment using extracted from corn fibre, it was consumed 9.38 g.L⁻¹ of ferulic acid corresponding at a $0.52 \text{ g}_{\text{vanillin}} \cdot \text{g}_{\text{ferulic acid}}^{-1}$ (molar yield of 66.15 %). The results also shown a higher production of vanillic acid, until the maximum production of vanillin, in the bioreactor with ferulic acid from corn fibre, than in the bioreactor with commercial ferulic acid, 1.96 g.L⁻¹ and 0.99 g.L⁻¹ respectively, this fact can explain the lower yield of vanillin production (until the maximum production of vanillin) in the bioreactor with ferulic acid from corn, the vanillin produced may be converted in vanillic acid more quickly for a detoxification of the cells.

Despite the vanillin yield being lower in experiments using ferulic acid from corn fibre than with commercial ferulic acid solution, the molar yield achieved (66 %) was one of the highest yields reported for vanillin production from ferulic acid recovered from agro-industrial by-products. In fact, there are very few studies in literature for vanillin production using agro-industrial by-products and the existing studies were performed using low concentrations of ferulic acid. Shin et al. (2006) [35] reported a molar yield of 43 % using 200 ppm ferulic acid extracted from corn fibre by enzymes and without purification, the bioconversion was performed by *Amycolatopsis* sp. ATCC 39116 without a suitable control of pH or dissolved oxygen. Torres et al. (2009) [215] reported a molar yield of 66 % for vanillin production

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from corn cob alkaline hydrolysate with 700 mg.L⁻¹ of ferulic acid, used directly without ferulic acid purification, using the engineered strain *Escherichia coli* JM109/pBB1. Although the yield of vanillin obtained by Torres et al. (2009) was the same obtained in this work, the vanillin volumetric productivity obtained in this work was much higher than that obtained by Torres et al. (2009), 0.290 g.L⁻¹.h⁻¹ and 0.011 g.L⁻¹.h⁻¹ respectively.

The volumetric productivity (r_p) and the rate of vanillin production (r_v) obtained using ferulic acid from corn fibre as well as commercial ferulic acid were very similar, r_p of 0.29 g.L⁻¹.h⁻¹ and 0.28 ± 0.02 g.L⁻¹.h⁻¹, respectively and r_v of 0.74 g.L⁻¹.h⁻¹ and 0.65 ± 0.06 g.L⁻¹.h⁻¹, respectively (Table 4.2). The use of ferulic acid from corn fibre did not affect the rate of vanillin production velocity.

The detailed analysis of this assay Figure 4.4 showed that even with the presence of glucose in the broth, vanillin was metabolized to vanillic acid, after reaching its maximum concentration, at higher rate (0.284 g.L⁻¹.h⁻¹) than in the case of experiment using commercial ferulic acid, (run 1 - 0.097 g.L⁻¹.h⁻¹). However, as expected vanillin was also metabolized at a lowest rate that when glucose is not available in the broth (run 2 - 0.39 g.L⁻¹.h⁻¹). As previously shown (run 1), with the presence of glucose in higher concentration (10.12 g.L⁻¹) than vanillin concentration (5.28 g.L⁻¹) in the bioreactor, the microorganism consumed glucose instead of degraded vanillin to obtain energy. In the current experiment (Figure 4.4), glucose was present in the broth at a concentration around 4 g.L⁻¹, after vanillin reaching the maximum concentration (5g.L⁻¹). The lower glucose concentration than vanillin concentration (5 g.L⁻¹) may explain the consumption of vanillin instead of glucose and the higher rate of vanillin degradation. *Amycolatopsis* sp. ATCC 39116 seems to require a glucose concentration higher than vanillin concentration in order to not metabolize vanillin as happen in the run 1, where the glucose presence in the broth was higher than the maximum of vanillin. Nevertheless, as in the previous experiments, it is possible to stop the process when the maximum vanillin production is reached, based on the increasing rate of acid for pH control concomitant with the rise in the dissolved oxygen concentration (results do not show), indicating a cellular activity decrease.

4.5. Conclusions

Biotech-vanillin production by *Amycolatopsis* sp. ATCC 39116, using of ferulic acid as a precursor, was studied in a bioreactor under controlled temperature, pH and dissolved oxygen, aiming to achieve the maximum vanillin productivity.

During the production phase, the presence of glucose was proven to have the beneficial effect of avoiding the conversion of vanillin to vanillic acid. A multiple-pulse-feeding approach was proposed, with the addition of 8.20 ± 0.63 g.L⁻¹ of ferulic acid), removal of the broth and reuse of the biomass for further bioconversion. This strategy made it possible to increase the vanillin volumetric productivity of the process in 64 % (0.46 g.L⁻¹.h⁻¹), compared with a single pulse feed (0.29 g.L⁻¹.h⁻¹). Removing the broth

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and reusing the biomass allows vanillin to be recovered in one single step by pervaporation [95] or vacuum evaporation. This is advantageous when compared with the use of solid matrices to avoid the vanillin coming in contact with the biomass because it reduces the complexity of processing later on. Further work could include the use of a separating device to remove the broth containing vanillin, recycling the biomass to proceed with the production process, so avoiding the need for centrifugation. Regarding the ferulic acid concentration, high ferulic acid concentrations ($14.91 \pm 0.29 \text{ g.L}^{-1}$) were shown to affect the vanillin production process, making the process slower.

Finally, the biotechnological-vanillin production by *Amycolatopsis* sp. ATCC 39116, using 10 g.L^{-1} of ferulic acid extracted from corn fibre was possible, achieving one of the highest vanillin production yields reported for the bioconversion of ferulic acid extracted from agro-industrial by-products. The vanillin production using the corn fibre ferulic acid extract was very similar to the experiment using the model solution of ferulic acid. The other kinetic parameters, namely volumetric productivity (r_p), rate of vanillin production (r_v) and the maximum vanillin concentration obtained were also very similar, except for the yield of vanillin production which was lower when using ferulic acid extracted from corn fibre than using commercial ferulic acid solution.

5. Comparative analysis of bio-vanillin purification using pervaporation and vacuum distillation

To be submitted in Separation and Purification Technology as: Rita Valério, João G. Crespo and Carla Brazinha, "Comparative analysis of bio-vanillin purification production using pervaporation and vacuum distillation"

The author was involved in planning and performing all the experiments, as well as on the discussion and interpretation of all the results and preparation of the manuscript.

5.1. Summary

The increased demand for natural products has led to the development of biotechnological vanillin production, which requires the recovery of vanillin (and vanillyl alcohol at trace concentrations, as in botanical vanillin) from the bioconversion broth, free from potential contaminants: the substrate (ferulic acid) and metabolites of bioconversion (vanillic acid, and guaiacol). This work discusses the recovery of bio-vanillin by pervaporation and by vacuum distillation from a complex bioconversion broth (the initial feed), aiming to recover vanillin free of the potential contaminants. In pervaporation experiments, the polydimethyl siloxane (PDMS) membrane selected presented a higher vanillin flux, higher permeability to vanillin and selectivity for vanillin in comparison with water than another commonly used membrane, with an active layer made of polyether block amine (PEBA). In vacuum distillation experiments, the addition of several consecutive water pulses to the feed during the process increased the percentage of recovered vanillin. Vacuum distillation experiments presented higher vanillin fluxes than pervaporation experiments, $2.7 \pm 0.1 \text{ g} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ and $1.19 \pm 0.01 \text{ g} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$, respectively, however vacuum distillation requires a higher energy consumption per mass of vanillin recovered, when comparing with pervaporation, and $9.75 \text{ kJ} \cdot \text{kg}_{\text{VAN}}^{-1}$ at 85°C and $4.91 \text{ kJ} \cdot \text{kg}_{\text{VAN}}^{-1}$ at 75°C , respectively. In both pervaporation with fractionated condensation and vacuum distillation processes, it was possible to recover vanillin and traces of vanillyl alcohol without the presence of the potential contaminants (ferulic acid, vanillic acid and guaiacol).

5.2. Introduction

Vanillin is the world's most popular flavour and is commonly used in food, beverages, perfume, cosmetics and pharmaceutical products. Most vanillin is chemically produced, mainly from guaiacol and lignin [11]. Nowadays, there is increasing consumer interest and market demand for healthy, natural food products. However, natural vanillin extracted from the beans of vanilla flowers cannot keep up with this increasing demand in terms of quantity and affordable price and makes up less than 1 % of the overall vanillin market [1,9]. Bio-vanillin is a good alternative to the vanillin extracted from vanilla flowers, since it is considered natural, according to US and European food legislation (U.S Food and Drug Administration [14] and Regulation (EC) No 1334/2008 [13], respectively).

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The global bio-vanillin market is increasing, due to bio-vanillin's excellent flavouring properties. In 2018, it was valued at 150 million US\$ and it is expected to reach 400 million US\$ by the end of 2025 [20], leading bio-vanillin companies to increase their production capacity [21]. Bio-vanillin can be obtained from the biotransformation of natural substrates by fungi, bacteria, genetically modified microorganisms and plant cells[1]. Ferulic acid is the most widely studied precursor for bio-vanillin production [1,28], mainly using the bacteria *Amycolatopsis* sp. ATCC 39116 due to its ability to tolerate higher levels of vanillin [82].

One of the challenges of vanillin bio-production is the recovery/purification of vanillin from the complex bioconversion broth. Crystallisation is, perhaps, the most extensively employed technique for the recovery/purification of bio-products from bioconversion broths [88]; however, in the case of vanillin, it only becomes a competitive technique when the concentration of vanillin is higher than 10 g.L⁻¹ (minimum concentration to allow its crystallization at 20° C) [89]. Adsorption is another extensively studied process for vanillin recovery [87,90,91,216]. However, adsorption exhibits some disadvantages: 1) some resins and, in particular, activated charcoal are not very selective to vanillin and other compounds present in the bioconversion broth are also adsorbed, especially metabolites of vanillin bioconversion (vanillic acid, vanillyl alcohol and guaiacol) and the substrate, ferulic acid [90,92–94]; 2) traces of adsorbent contaminants are difficult to avoid, requiring strict quality control of the recovered product [95] and 3) organic solvents are normally required for desorption [90].

Other processes have been reported, such as liquid-liquid extraction [217], but these processes use (non-sustainable) organic solvents such as ethyl acetate, propyl acetate, isopropyl acetate, methyl ethyl ketone, cyclohexane and dichloromethane. Membrane-based solvent extraction has been also proposed but the solvents used are typically the same [96–98]. Alternatively, evaporative processes have been studied and offer the main advantage of not using organic solvents.

Pervaporation was also proposed, mainly using PEBA (polyether block amine) membranes [100,218,219] and POMS (polyoctylmethylsiloxane) membranes [95]. Camera et al. [218,219] studied pervaporation with a PEBA membrane, coupled with photocatalysis for the green synthesis of vanillin from ferulic acid, to recover vanillin from the reactor with the minimum possible concentration of by-products. The PEBA membrane had a high rejection of all compounds presents in the photocatalysis reactor, except vanillin and 4-vinyl guaiacol [218,219]. Camera et al. 2019 compared pervaporation with dialysis in the recovery of vanillin from a photocatalysis reactor and the results showed that pervaporation is much more selective than dialysis. In fact, using pervaporation only vanillin and 4-vinyl guaiacol permeated the membrane whereas, when using dialysis, ferulic acid and caffeic acid were also detected in the permeate [218]. However, the vanillin flux of dialysis is much higher than in pervaporation [218]. Brazinha et al. 2011 reported the recovery of pure vanillin from a bioreactor broth containing ferulic acid (used as a precursor for bio-vanillin production), using pervaporation (POMS

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membrane). The vanillin was captured by condensation at mild temperatures [95]. Bøddeker et al. [100] reported pervaporation using a PEBA membrane to purify vanillin from a model bioconversion broth.

Vacuum distillation has been also reported to purify vanillin from agro-industrial hydrolysates [220–222]. Rhodia (Solvay) [223] used vacuum distillation to purify vanillin from the bioconversion broth. The Rhodia patent includes a vanillin purification process with one or more distillation columns and purification in an evaporator followed by condensation.

The main objective of this work is to evaluate and compare the use of pervaporation and vacuum distillation for the recovery of vanillin from a complex bioconversion broth, with the aim of recovering vanillin free of contaminants and, if possible, with a minimum content of water. The impact of the membrane polymeric material used (polydimethyl siloxane [PDMS] and polyether block amine [PEBA]) and of operating conditions, such as the feed temperature, were assessed with the aim of obtaining a high vanillin permeability, absence of media contaminants and a high selectivity of vanillin in relation to water. To the best of our knowledge the transport of vanillin through a PDMS membrane, optimising the most relevant operating conditions, from a complex bioconversion broth, has not yet been reported and it is quite useful from an upscaling process point of view.

A pervaporation-fractionated condensation process has shown to recover pure vanillin from a bioconversion broth [95], but no vanillin fluxes were reported. In this work, the impact of the feed temperature on the performance of vanillin recovery by pervaporation-fractionated condensation was assessed, aiming to obtain maximised vanillin fluxes and minimised membrane adsorption phenomena, simultaneously assuring the purity of the recovered vanillin. Considering that in purely evaporative processes (such as vacuum distillation), the selectivity of vanillin against water is low, but the selectivity of water in relation to vanillin is high, vacuum distillation was considered in this work as an alternative to pervaporation in a vacuum distillation-fractionated condensation configuration.

5.3. Materials and Methods

5.3.1. Materials

The two pre-selected membranes used in the pervaporation experiments were commercial hydrophobic (organophilic) pervaporation membranes: PervapTM 4060 and Pebax. Their main characteristics are summarised in Table 5.1.

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Table 5.1. Main characteristics of the pervaporation membranes used in this work (information provided by the manufacturer).

Membrane	Manufacturer	Material	$T_{maximum}^a$ (°C)	pH (-)	$\delta_{active\ layer}^b$ (µm)
PervapTM 4060	Sulzer ChemTech, Switzerland	Poly dimethyl siloxane (PDMS)	80	5 - 8	~ 2
PEBA	Pervatech, The Netherlands	Polyether block amine (PEBA)	70	3-8	~ 4

Legend: ^a $T_{maximum}$, maximum temperature; ^b $\delta_{active\ layer}$, thickness of the active layer.

The feed used in this work was the vanillin bioconversion broth produced in two bioreactors operating under the same conditions, obtained according to the biochemical pathway of ferulic acid conversion (in this case commercial ferulic acid) into vanillin by the strain *Amycolatopsis* sp. ATCC 39116 [211], which is the microorganism most commonly used for vanillin bioconversion [3,85,87]). The production of bio-vanillin was carried out in 2 L bioreactors (BioStat B-plus, Sartorius, Germany) according to chapter 4. At the end of production (set for maximum vanillin production), the bioconversion broth was centrifuged at 4424 g for 20 minutes at 7° C to remove the cells. The pH of the supernatant of the bioconversion broth was adjusted with HCl to a pH value to be defined in section 5.4.1. Table 5.2 shows the concentration of phenolic compounds that could have been present in the vanillin bioconversion broth (vanillin, ferulic acid, vanillic acid, vanillyl alcohol and guaiacol), and their saturation vapour pressures (p_{vi}) and activity coefficients (γ_i) at 25° C. Based on HPLC analyses, no ferulic acid, vanillyl alcohol or guaiacol were detected in the broth only vanillin and vanillic acid were detected in the broth, within the limit of detection of each compound in the HPLC method used.

Table 5.2. - Concentration of phenolic compounds present in the vanillin bioconversion broth (the feed for this work) and their saturation vapour pressures (p_{vi}) and activity coefficients(γ_i), at 25° C.

Compound	Concentration (g.L ⁻¹)	p_{vi} (mbar) (at 25° C)	γ_i (-) (at 25° C)
Vanillin	3.99±0.02 ⁽¹⁾ , 4.81±0.41 ⁽²⁾	3.1x10 ⁻⁴ ⁽⁴⁾	813.0 ⁽⁴⁾
Ferulic acid	< 0.08 ^(1,2)	3.59 x10 ⁻⁶ [224]	1.3 [225]
Vanillic acid	1.03±0.04 ⁽¹⁾ , 1.48±0.1 ⁽²⁾	(6.50±0.98)x10 ⁻⁷ [226]	2.8 [225]
Vanillyl alcohol	< 0.12 ⁽¹⁻³⁾	7.04x10 ⁻⁶ [227]	523.76 ⁽⁴⁾
Guaiacol	<0.10 ⁽¹⁻³⁾	0.33 ⁽⁴⁾	273.7 [228]

⁽¹⁾ Bioconversion broth 1

⁽²⁾ Bioconversion broth 2

⁽³⁾ Each value corresponds to the limit of detection for each compound in these samples (HPLC limit of detection)

⁽⁴⁾ See estimation of these parameters explained in section 5.3.3 by equation (20'')

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The standards for HPLC analyses were vanillin (99 %, Sigma Aldrich, USA), ferulic acid (≥ 99 %, Sigma Aldrich, USA), vanillic acid (≥ 97 % Sigma Aldrich, USA), vanillyl alcohol, (≥ 97 % Sigma Aldrich, USA), guaiacol (98 %, Sigma Aldrich, USA).

5.3.2. Experiments

5.3.2.1. Pervaporation-fractionated experiments

The pervaporation experimental set-up (Table 5.1) consists of a closed feed vessel, a flat circular pervaporation (GKSS, Germany) module with an effective membrane area of 0.01 m^2 , two condensers-in-series, and a single-stage rotary vane vacuum pump (Pfeiffer Duo 2.5, Pfeiffer Vacuum, Germany).

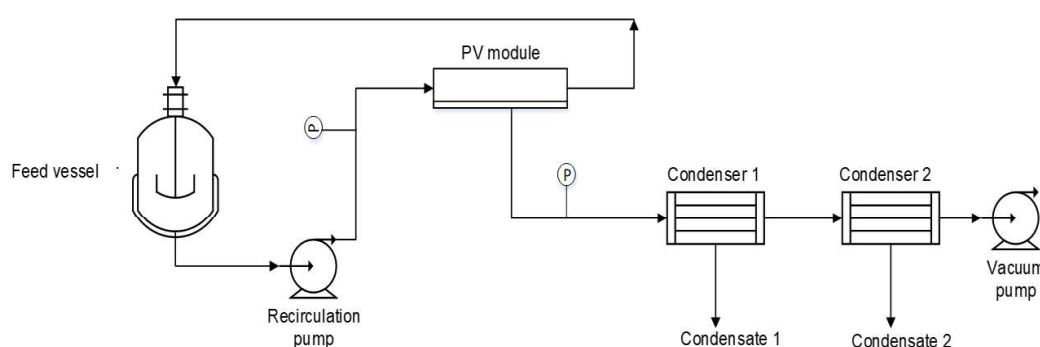


Figure 5.1. Experimental pervaporation laboratory set-up similar to [95]. P is the pressure transducer.

About 300 g of feed (Bioconversion broth 1, Table 5.2) was used in each pervaporation experiment. The feed was placed in a closed jacket feed vessel with a small headspace, to minimise aroma losses by evaporation. The jacket feed vessel was connected to a heating bath (E100, LAUDA, USA), which kept constant the temperature of the feed, T_{feed} (°C), at a defined value during each experiment. A recirculation pump (IMS 901B, Ismatec, Germany) also pumped the feed at a feed flow rate of 80 L.h^{-1} (measured at room temperature) into the pervaporation module, corresponding to a Reynolds number in the feed compartment at room temperature of 430 [95,229]. The temperature of the feed stream, T_{feed} (°C), was firstly defined as 45 ± 3 °C, the value of the temperature of the bioconversion broth, where bio-vanillin was produced according to chapter 4. An additional pervaporation experiment, using the PDMS membrane, was performed at 75° C.

The condensers used in the downstream side of the pervaporation set-up were glass U-shaped traps. The first condenser was immersed in a refrigerated bath (Corio CP-300F, Julabo, Germany) and the second condenser in liquid nitrogen (-196° C). The temperature of the first condenser was set at pre-defined values.

In order to avoid vanillin condensation in the circuit between the membrane module and the first condenser, this circuit was heated using an electric heating tape connected to a temperature controller (CB100, from RKC Instruments Inc., Japan), at 60° C, as in Brazinha et al. [95]. The permeate pressure

was defined around 1.5 mbar, the value optimised by Brazinha et al. [95] in order to obtain maximised vanillin fluxes. Each experiment was performed for 4 hours. Before each experiment, the membranes were conditioned with water during one hour.

5.3.2.2. Vacuum distillation experiments

The vacuum distillation set-up (Figure 5.2) consists of a closed feed vessel, three condensers-in-series, and a single-stage rotary vane vacuum pump (Pfeiffer Duo 2.5, Pfeiffer Vacuum, Germany).

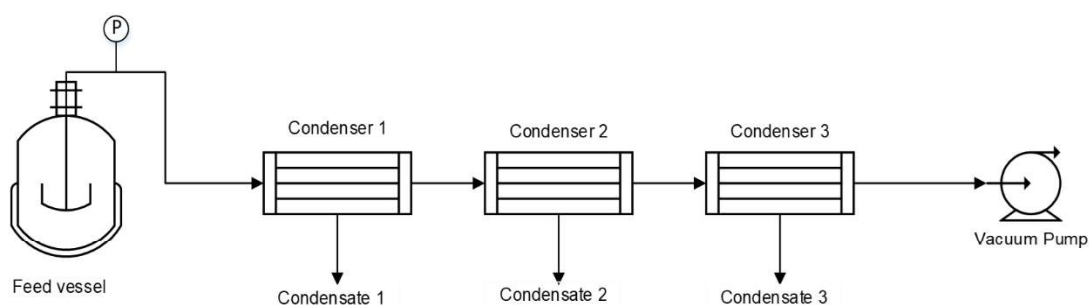


Figure 5.2. Experimental vacuum distillation laboratory set-up. P is the pressure indicator.

An amount of 5.53 ± 0.14 g of feed (Bioconversion broths 1 and 2, Table 5.2, was used in the vacuum evaporation experiments. The feed was stirred at 100 rpm and heated at different defined feed temperatures using a heating and stirring plate. Apart from the closed feed vessel, the vacuum distillation circuit was similar to the downstream side of the pervaporation set-up. The difference was the use of an additional condenser; the first two condensers were set at the same temperature, controlled by a refrigerated bath (Corio CP-300F, Julabo, Germany). The last condenser was identical to the pervaporation set-up (a trap immersed in liquid nitrogen). The vacuum for the whole set-up decreased very rapidly (within around 5 min) to pressure values below 1 mbar and, at 10 minutes, reached a value of around 0.15 mbar. A summary of the operating conditions, which were varied during the vacuum distillation experiments performed in this work, are presented in Table 5.3. Water was added to the feed in the last 3 vacuum distillation experiments, in order to promote vanillin evaporation, keeping constant its high activity coefficient and, hence, its volatility.

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Table 5.3. Operating conditions showing the operating values used during the vacuum distillation experiments.

Experiments	Feed Temperature T_{feed} (°C)	Addition of water	Time of experiment $t_{experiment}$ (min)
1 ⁽¹⁾	45	No	60
2 ⁽¹⁾	85	No	15
3 ⁽¹⁾	85	No	70
4 ⁽¹⁾	85	Yes (4 pulses) ⁽³⁾	15 x4 = 60
5 ⁽¹⁾	85	Yes (10 pulses) ⁽³⁾	15 x10 =150
6 ⁽¹⁾	85	Yes(18 pulses) ⁽³⁾	15 x18 = 270
7 ⁽²⁾	85	Yes(18 pulses) ⁽³⁾	15 x18 = 270

⁽¹⁾ where the feed was the bioconversion broth 2 (Table 5.2)

⁽²⁾ where the feed was the bioconversion broth 1 (Table 5.2)

⁽³⁾ Several consecutive water pulses were added, each with the same mass as the evaporated water (keeping constant the volume in the feed vessel)

5.3.3. Methods for estimating parameters

The saturation vapour pressure of water was estimated at 45° C (feed temperature used in the pervaporation and vacuum distillation experiments), 75° C (the feed temperature used in one pervaporation experiment) and 85° C (feed temperature used in vacuum distillation experiments), as shown in Table 5.4. The estimated saturation vapour pressure of water at different temperature values was calculated using the Antoine equation, for a temperature range between 0° C and 100° C.

$$\log_{10}(p) = 8.0731 - \frac{1730.63}{233.426+T} \quad (p \text{ in mmHg, } T \text{ in } ^\circ\text{C}) \quad (17)$$

The saturation vapour pressures and the activity coefficients of vanillin (the most abundant phenolic compound present in the vanillin bioconversion broth) were estimated at 25° C (room temperature), as shown in Table 5.2, and at 45, 75 and 85° C, as shown in Table 5.4. The saturated vapour pressures of vanillin at 25 and 45° C were estimated through the following equation [230,231], for a temperature range between 24 and 55° C.

$$\log_{10}(p) = 10.93562 - \frac{4535.023}{T} \quad (p \text{ in kPa, } T \text{ in K}) \quad (18)$$

The saturated vapour pressures of vanillin at 75 and 85° C were estimated through the following equation [232], for a temperature range between 82 and 504° C.

$$\log_{10}(p) = 7.81755 - \frac{2260.06}{233.426+T} \quad (p \text{ in mmHg, } T \text{ in } ^\circ\text{C}) \quad (19)$$

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The activity coefficients (in aqueous solutions) of vanillin were estimated using the mutual solubility method [233]. This method works only for very hydrophobic compounds, such as vanillin, and it is based on the measurement of the concentration of the aroma compound in an aqueous phase, equilibrated with the organic compound phase (vanillin). Indeed, if the thermodynamic equilibrium is established, the aroma compound activity in the organic phase and in the aqueous phase can be related as [233]:

$$a_i^{aqu} = a_i^{org} \quad (20)$$

$$\gamma_i^{aqu} x_i^{aqu} = \gamma_i^{org} x_i^{org} \quad (20')$$

Assuming that the organic phase is almost pure, then, $\gamma_i^{org} \cong 1$ and $x_i^{org} \cong 1$

$$\gamma_i^{\infty} = \frac{1}{x_i^{aqu}} \quad (20'')$$

Where a_i is the chemical activity of compound i, x_i is the molar fraction of compound i in a liquid phase and γ_i is the activity coefficient.

Where x_i^{aqu} of vanillin at 25 and 45° C are 1.23×10^{-3} and 2.56×10^{-3} , respectively [234] and at 75° C it was calculated based on the concentration of vanillin in a saturated aqueous phase at this temperature [235]. The x_i^{aqu} of vanillyl alcohol at 25° C was calculated based on the concentration of vanillyl alcohol in a saturated aqueous phase, which is 16.12 g.L^{-1} [236].

Table 5.4. Values of the estimated saturation vapour pressure p_{vi} (mbar) (A) and of the estimated activity coefficient (in aqueous solutions) γ_i^{∞} (-) (B) of vanillin and of water at 25, 45, 75 and 85° C.

(A)	Vanillin	Water
p_{vi} (mbar) (at 45°C)	4.8×10^{-3}	95.93
p_{vi} (mbar) (at 75°C)	6.7×10^{-2}	385.98
p_{vi} (mbar) (at 85°C)	0.15	579.10
(B)	Vanillin	Water
γ_i^{∞} (-) (at 45°C)	390.60	1.00
γ_i^{∞} (-) (at 75°C)	161.60	1.00
γ_i^{∞} (-) (at 85°C)	161.60 ^a	1.00

^a the γ_i^{∞} at 85° C was considered the same of at 75° C

In the pervaporation experiments, when the temperature increased from 45 to 75° C, the saturation vapour pressure of vanillin increased more significantly (14x) than the saturation vapour pressure of

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water (4x). In vacuum distillation experiments when the temperature increased from 45 to 85° C, the saturation vapour pressure of vanillin increased more significantly (31x) than the saturation vapour pressure of water (6x). The activity coefficient of vanillin decreased with the increase of temperature (as vanillin became more stable in water in a system with higher energy, related to the temperature increase) and the activity coefficient remained constant for water.

The estimated saturated vapour pressure of guaiacol at 25° C was calculated through the following [232], for a temperature range between 31°C and 424° C.

$$\log_{10}(p) = 7.899 - \frac{2204.06}{233.426 + T} \quad (p \text{ in mmHg, } T \text{ in } ^\circ\text{C}) \quad (21)$$

5.3.4. Analytical methods

At the end of each pervaporation and vacuum distillation experiment, each condensate was recovered from the traps and was chemically characterised in terms of the concentration of phenolic compound. When the condensates only contained solid vanillin, the compound was recovered by adding hot water.

The concentration of the phenolic compounds (vanillin, vanillic acid, ferulic acid, vanillyl alcohol and guaiacol) was measured using HPLC, High-Performance Liquid Chromatography. A high-performance liquid chromatograph (Alliance e2695, Waters, USA) was used, equipped with a UV detector at 280 nm and a Nova-Pak® C18 3.9 mm×150 mm column (particle diameter of 4 mm), preceded by a guard column. A gradient elution program at the flow rate of 0.5 mL.min⁻¹ was used, at 30° C. The mobile phase was a mixture of two eluents, one of them (A) containing 10 % (v/v) methanol, 2 % (v/v) acetic acid in Milli-Q water and the other (B) containing 90 % methanol, 2 % acetic acid in Milli-Q water. The gradient elution program was 0 % B (0–10 min), from 0 to 15 % B (10–25 min), from 15 to 50 % B (25–35 min) and 50 to 0 % B (35–38 min).

A Nuclear Magnetic Resonance (NMR) spectroscopy analysis was also performed. ¹H NMR spectra were obtained by dissolving the sample in deuterium oxide (D₂O, 99.9 %) and subjecting the samples to 3 freeze-drying cycles, with samples resuspended in deuterium oxide between those cycles. The samples were analysed using an Avance III 400 NMR spectrophotometer (Bruker, Switzerland), at 25° C, with an NMR magnet operating at 400.13 MHz for ¹H.

5.4. Results and Discussion

5.4.1. Optimization of feed pH used in the pervaporation and vacuum distillation processes

The volatility of a certain solute *i* in a solution where the solvent is water, or the relative volatility of solute *i* in relation to water, is quantified by the Henry constant of compound *i*, *H_i* (mbar), calculated by

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the product of its saturation vapour pressure, p_{vi} (mbar) and its activity coefficient, γ_i (-), at a certain temperature. Table 5.5 indicates the values of the Henry constant, H_i (mbar), and of pK_{a_i} of the compounds commonly present in a bioconversion broth for the production of vanillin, at 25° C.

Table 5.5. Henry constants and pK_a of compounds commonly present in a vanillin bioconversion broth at 25° C.

Compound <i>i</i>	H_i (mbar)	pK_{a_i} (-)
Vanillin	0.25	7.4 [191]
Ferulic acid	3.7×10^{-4}	4.58 [191]
Vanillic acid	1.8×10^{-6}	4.16 [237]
Vanillyl alcohol	3.69×10^{-3}	9.92 [237]
Guaiacol	90.3	9.98 [237]

Volatile compounds with high Henry constants, H_i (mbar), and in non-ionic form (when the pH value of the solution is clearly lower than the pK_a of the compound) may be recovered in the downstream side of the membrane in a pervaporation process and are evaporated by distillation. On the other hand, non-volatile compounds, with extremely low Henry constant, H_i (mbar), or/and compounds that are in ionic form at the pH of the solution, [95] are not recovered by evaporative processes.

A complex vanillin bioconversion broth comprises various non-volatile compounds (glucose and salts, yeast extract [95]) that remain in the bioconversion broth. Other contaminants reported to be present in the vanillin bioconversion broth are noted in Table 5.2.

To assure the separation of vanillin from ferulic acid and vanillic acid, the pH value of the bioconversion broth has to be adjusted, in order to assure that vanillin is protonated (HVan) (which is the second most volatile compound, with the second highest Henry constant, see Table 5.5) and both ferulic acid (HFer⁻ and Fer²⁻) and vanillic acid (HVac⁻ and Vac²⁻) remain non-protonated. Camera-Roda et al 2014 [219] proved that the performance of pervaporation for recovering/permeating vanillin is pH-dependent: permeation decreases at pH 7.9 when vanillin is partially dissociated and declines significantly at pH 10.3 when vanillin is completely dissociated [219].

Table 5.6 illustrates the relative fraction of vanillin (HVan), ferulic acid (H₂Fer), vanillic acid (H₂Vac), alcohol vanillyl (Halc) and guaiacol (HGuai), all in protonated form, at different pH values, including at pH 8.2 (the pH value at the end of the vanillin bioconversion in Chapter 4) and at pH 7.2 (the pH value reported in [95] as optimal for vanillin bioconversion).

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Table 5.6. Fractions of vanillin (HVan), ferulic acid (H₂Fer), vanillic acid (H₂Vac), vanillyl alcohol (Halc) and guaiacol (HGuai) in protonated form, at different bioconversion broth pH values.

pH	HVan	H ₂ Fer	H ₂ Vac	Halc	HGuai
6.5	88.5%	1.1%	0.6%	100.0%	100.0%
6.8	81.7%	0.6%	0.4%	100.0%	100.0%
7.2	61.3%	0.2%	0.1%	100.0%	100.0%
8.2	13.7%	0.0%	0.0%	98.1%	98.4%
10.3	0.0%	0.0%	0.0%	29.4%	32.4%

Table 5.6 shows that at pH 8.2, only 13.7 % of vanillin is in protonated form, which has a negative effect on permeation [219]; the ferulic acid and vanillic acid that remain in the bioconversion broth are in their non-volatile, non-protonated (ionic) form. Therefore, a pH of 6.8 was selected as at this pH 81.7 % of vanillin is in the protonated form and the ferulic acid and vanillic acid are still, mostly, in their non-protonated form (>99 %) (Table 5.6), which makes vanillin recovery possible and significantly prevents the evaporation of ferulic acid and vanillic acid. Neither ferulic acid nor vanillic acid are expected to be recovered, so they will not contaminate the vanillin aimed product.

Vanillyl alcohol is more volatile than vanillic acid and guaiacol is the most volatile of the compounds present (see Table 5.5), but none of these compounds was detected by HPLC (Table 5.2). So, although both guaiacol and vanillyl alcohol are protonated at the same pH values as vanillin, they are not will be recovered using an evaporative process, unlike vanillin.

Although it is expected that vanillin will be recovered free from contaminants, an NMR analysis should be performed for the selected condensate to confirm whether or not the recovered vanillin is free from contaminants, particularly if vanillic acid, which is present in the bioconversion broth, is recovered.

5.4.2. Pervaporation experiments

5.4.2.1. Pervaporation

PEBA membranes have been used in prior works reported in the literature for the recovery of vanillin by pervaporation [100,218,219] and PDMS membranes have been applied for removing organics from aqueous mixtures (e.g. ethanol [238,239], methanol [238], phenol [240]), exhibiting high selectivity and permeability to organic substances. The PMDS membrane was also used to recovery apple juice aroma compounds [241]. However, to the best of our knowledge, no studies have reported the recovery of vanillin by pervaporation using PDMS membranes. Performing pervaporation for vanillin recovery from a bioconversion broth at a temperature of 45° C makes sense from a process point of view. It is also

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interesting to test a feed temperature higher than 45° C, e.g. 75° C, because the permeability to vanillin is expected to increase, although the selectivity of vanillin in relation to water is expected to decrease.

This work studies the influence of the membrane material (PEBA, PDMS) and of the feed temperature (T_{feed} of 45 and 75° C) on the performance of vanillin recovery by pervaporation, aiming to find a membrane with high permeability to vanillin and high selectivity of vanillin in relation to water.

In the pervaporation process for vanillin recovery, from the liquid feed to the permeate vapor (before fractioned condensation), the parameters of permeability to vanillin, P_{van} (mol.(m.s.Pa)⁻¹) and selectivity of vanillin in relation to water (-), allow a better understanding of the intrinsic performance of the membrane, and these parameters are adequate for comparing the transport properties of the different membranes [242–244]. On the other hand, the separation factor of vanillin in relation to water compares the initial feed concentration with the permeate concentrations. The permeability, P_i , to compound i (vanillin or water) is calculated by following equations:

$$J_i = \frac{P_i}{\delta} (p_{i,feed} - p_{i,perm}) \quad (22)$$

$$J_i = \frac{P_i}{\delta} (x_{i,feed} H_i - y_i p_{perm}) \quad (22')$$

where in relation to compound i , J_i (mol.m⁻².s⁻¹) is the molar flux, p_i (Pa) is the partial pressure of compound i in the feed (feed refers to the feed side and perm refers to the permeate side), x_i (-) is the molar fraction in the feed side, y_i (-) is the molar fraction of compound i in the permeate side, δ (m) is the thickness of the dense layer of the composite membrane and p_{perm} (Pa) is the permeate total pressure.

The selectivity of vanillin in relation to water and the separation factor of vanillin-water are calculated by the following equations:

$$Selectivity_{van-water,pervaporation} = \frac{P_{van}}{P_{water}} \quad (23)$$

$$Separation\ factor = \frac{y_{van}/y_{water}}{x_{van}/x_{water}} \quad (24)$$

According to the recommendation given in Brazinha et al.[95] for maximised vanillin fluxes, a permeate pressure of around 1.5 mbar should be employed for maximising the driving force.

Therefore, the following permeate pressure values were used: 1.33±0.19 mbar in the experiment using a PEBA membrane, 2.11±0.09 mbar in the experiment using a PDMS membrane at 45° C and 1.75±0.37 mbar in the experiment using a PDMS membrane at 75° C. The permeate pressure values were similar to the target value of 1.5 mbar. Table 5.7 shows the Henry constants of compound i (vanillin and water) at 45 and 75° C.

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Table 5.7. Values of the calculated Henry constants of vanillin and water at 45 and 75° C.

Compound	H_i (mbar) (45° C)	H_i (mbar) (75° C)
Vanillin	1.88	10.89
Water	95.60	384.63

With the increase of the feed temperature from 45 to 75° C, the Henry constants of water increased (4x). The Henry constants of vanillin increased slightly more (~6x), explained by a much larger increase of saturation vapour pressure (14x, see section 5.3.3) than for water (4x). The different parameters related to the pervaporation processes are shown in Table 5.8.

Table 5.8. Pervaporation operating and performance parameters of the two membranes tested (PDMS (PervapTM4060) and PEBA(Pebax), where $J_{i,T}$ is the total flux of the compound i and P_i is the permeability of compound i.

Membrane	PDMS	PEBA	PDMS
Temperature (° C)	45	45	75
$J_{\text{vanillin}, T}$ (g.m ⁻² .h ⁻¹)	0.35±0.01	0.17±0.005	1.19±0.02
$J_{\text{water}, T}$ (Kg.m ⁻² .h ⁻¹)	1.06	1.40	2.38
P_{van} (mol.(m.s.Pa) ⁻¹)	(1.61±0.06)x10 ⁻¹¹	(1.49±0.06)x10 ⁻¹¹	(8.71±0.31)x10 ⁻¹²
P_{water} (mol.(m.s.Pa) ⁻¹)	3.50 x10 ⁻¹²	9.16x10 ⁻¹²	1.92 x10 ⁻¹²
$\alpha_{\text{van-w, pervaporation}}$ (-)	4.6	1.6	4.5
Separation factor (van-w) (-)	0.08	0.03	0.13

At a feed temperature of 45° C, when comparing the use of a PDMS membrane (PervapTM4060) with a PEBA (Pebax) membrane, the flux of vanillin doubled when using the PDMS membrane and its permeability for vanillin is slightly higher (10 %). The flux of water is lower for the PDMS membrane and its permeability for water decreased, which favours the separation process aimed. The permeability values take into consideration the fact that the PDMS and the PEBA membranes have different top layer

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thicknesses, about 2 and 4 μm , respectively. When comparing the PDMS membrane to the PEBA membrane, the selectivity for vanillin in relation to water and the separation factor increased ($\sim 3\times$). Therefore, the PDMS membrane was selected. With a PDMS membrane, when the feed temperature increased from 45 to 75° C, vanillin and water fluxes increased, as expected. The vanillin fluxes increase more than the water fluxes, probably due to the fact the Henry's constant (Table 5.7) and, consequently, the feed partial pressure increased more in the case of vanillin. This increase in feed temperature also led to the unexpected decrease of individual permeabilities, which is related to the fact that the partial fluxes did not increase as expected in comparison with the increase of the driving force. This behaviour is most probably explained by the presence of a feed-side polarization of concentration of the permeating compounds. This phenomenon occurs when the transport of the permeating compounds in the feed boundary layer towards the membrane is not fast enough to compensate the sorption of the permeating compounds at the feed-side of the membrane, decreasing their concentration near the membrane surface. The increase of temperature enhances this concentration polarisation phenomena, as reported in [245]. Guo et al. 2004 reached a similar conclusion about unexpected permeability trends when studying the dehydration of aqueous butanol solutions, where the water and butanol fluxes increased with the increasing temperature, while their corresponding water permeance (the ratio of permeability and membrane thickness) decreased and butanol permeance showed relatively insignificant variation with temperature [244]. Baker et al. 2010 [242] reported also the similar unexpected permeability trends when Ray and Ray et al. 2006 studied the separation of toluene from toluene/methanol mixtures [246] where the toluene fluxes increased with the increasing temperature, while the permeability decreased.

With the aim of achieving the highest values of permeability and vanillin selectivity in relation to water, a temperature of 45° C was selected as the feed temperature when using a PDMS membrane, even though there was a fairly moderate value of selectivity of vanillin in relation to water (4.6).

5.4.3. Pervaporation with Fractionated Condensation

Bio-vanillin recovery from a bioconversion broth using a pervaporation-fractionated condensation process, with two condensers-in-series, has been reported to lead to a separation factor of vanillin in relation to water of an infinite value (the recovery of pure vanillin from a bioconversion broth) [95]. In this work, the aim is to maximise the vanillin flux and provide conditions that assure the vanillin recovered from a bioconversion broth is free from contaminants. Two pervaporation-fractionated condensation experiments, using a PDMS membrane (at 45 and at 75° C), were performed, using the pervaporation operating conditions defined in section 5.3.3.

In these experiments, the prediction of the condensation behaviour of the mixture vanillin and water in the fractionated condensation step was based on theoretical thermodynamic condensation estimations of pure vanillin and pure water, for a defined value of permeate pressure, since vanillin and water have

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very different condensation behaviours. Indeed, water is much more volatile than vanillin (the Henry constant of water is twice the order of magnitude of vanillin at the same temperature, Table 5.7). Figure 5.3 shows the percentage of condensation of vanillin and of water in the first condenser, for a defined value of permeate pressure, in a pervaporation-fractionated condensation experiment with two condensers-in-series. In particular, for the first condenser, it is possible to estimate the range of temperature values, $T_{1,condens}$ ($^{\circ}\text{C}$), at a certain value of permeate pressure, where pure solid vanillin is obtained. In this work, a permeate pressure of 2 mbar was used (average value of permeate pressure for all experiments) and assuming the absence of inert gases.

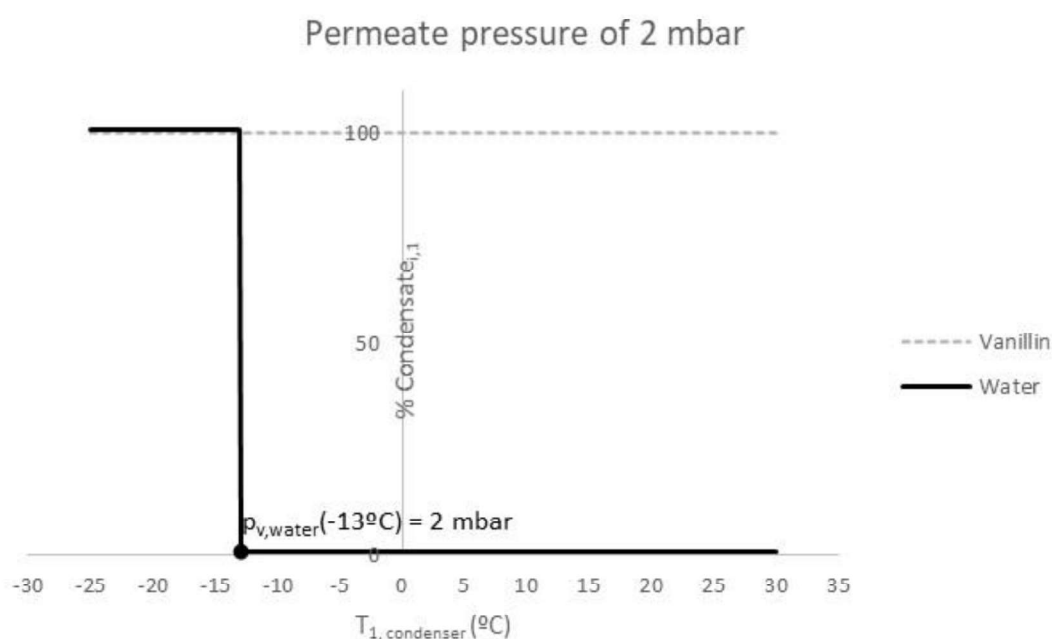


Figure 5.3. Simulation of the first condenser in a pervaporation-fractionated experiment: the theoretical estimation of the % of condensation of compound i in the first condenser, % Condensate _{$i,1$} , where compound i is vanillin or water, against the temperature of the first condenser, $T_{1,condens}$ ($^{\circ}\text{C}$) at an operating downstream pressure of 2 mbar. Observations: at 2 mbar, water condensates only at temperatures below -13°C , where the saturation vapour pressure is equal to the operating pressure (2 mbar).

Figure 5.3 estimates that at a pressure of 2 mbar, and at temperature values higher than -13°C , no water condensation occurs in the first condenser, only in the 2nd (total vapour) condenser. Regarding vanillin, due to its extremely low values of saturation vapour pressure, vanillin will condensate (even at 35°C) in the first condenser, for this total pressure in the downstream circuit. In a previous work, it was experimentally confirmed that a complete condensation of vanillin occurs for a temperature as high as 0°C in the first condenser [95]. From Figure 5.3 it can be anticipated that the temperature of 5°C , selected for operation of the first condenser, is sufficient to assure a complete condensation of vanillin. The results of the pervaporation-fractionated condensation experiments are presented in shows the HPLC chromatograms obtained, referring to samples taken during the experiment using a PDMS membrane at 75°C (a similar behaviour was observed for all experiments).

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Table 5.9 where it is shown the total fluxes of vanillin and the percentage of the total permeated vanillin compounds, recovered in the different sections of the permeate circuit (first and second condensers and permeate tubes) at the end of each experiment. In the first condenser, vanillin was in solid form without water so, to recover vanillin for its quantification, hot water was added. The permeate tubes were also rinsed with hot water, to be sure that all vanillin attached to the tubes was recovered. The % of vanillin recovered in each section was performed by calculating the mass of vanillin that permeated and comparing it with the total mass of compound recovered in the different sections of the downstream circuit (the two condensers and the permeate tubing). Table 5.9 also presents the mass balance deviation for vanillin. The mass balance deviation was calculated by the mass of vanillin in the initial feed and comparing it with the sum of the total mass of vanillin recovered in the different sections of the downstream circuit and remaining in the feed at the end of each experiment. Figure 5.4 shows the HPLC chromatograms obtained, referring to samples taken during the experiment using a PDMS membrane at 75° C (a similar behaviour was observed for all experiments).

Table 5.9. Pervaporation-fractionated condensation experiments: mass permeation fluxes and mass balance deviations for pervaporated vanillin and their in each section of the permeate circuit, in relation to the total mass of each compound permeated. First condenser at 5° C and second condenser (full condenser) at -196° C.

Compound	Exp.	$J_{i,T}$ (g.m ⁻² .h ⁻¹)	1 st Cond. (%)	2 nd Cond. (%)	Permeate tubes (%)	Mass Balance deviation (%)
Vanillin	45° C	0.35±0.01	47.7±1.7	50.3±1.8	2.0±0.05	-5.7
	75° C	1.19±0.01	44.5 ±1.6	51.9±1.8	3.5±0.10	-3.0

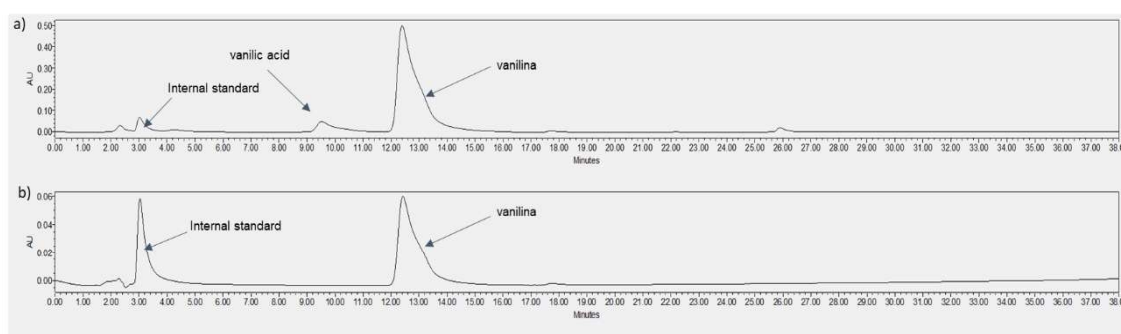


Figure 5.4. Pervaporation-fractionated condensation experiment using a PDMS membrane at a feed temperature of 75° C and a first condenser at 5° C. HPLC chromatograms refer to different samples taken during the experiment: a) final feed solution; b) condensate in the first condenser.

Vanillin is the only compound recovered in the permeate circuit and vanillic acid was not detected in any sample from the permeate circuit (the chromatograms are similar for all permeate samples). Therefore, the vanillic acid fluxes can be considered to be null or close to zero, considering the detection

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limit of the HPLC method used. The HPLC analyses show that vanillic acid and the other potential contaminants (Table 5.2) were not detected in the feed stream nor in the condensers.

The vanillin recovered in the first condenser was in solid form without water, so the separation factor of vanillin in relation to water had an infinite value. However only about 46 % of the permeated vanillin was recovered in the first condenser (Table 5.9). The remaining permeated vanillin was recovered in the second condenser with water (Table 5.9). As explained before, at 5° C, all vanillin should be (thermodynamically) retained in solid form in the first condenser. This deviation can be explained by the fact that the flux of water is much higher than the flux of vanillin (see Table 5.8), which causes the vanillin to be dragged together to the second condenser. In order to increase the amount of solid vanillin in the first condenser, the residence time/area of the first condenser should be increased and/or the temperature of the first condenser should be decreased. In the case of a temperature decrease in the first condenser, the values could be lowered to between -5 and -10° C (higher than -13 °C), as explained above. But the more economically advantageous strategy of increasing the area of the first condenser should be implemented first, preferably in a pilot-scale study.

The pervaporation-fractionated condensation experiment at 75° C was selected as it leads to the highest vanillin flux.

5.4.4. Vacuum distillation experiments

The pervaporation experiments, without fractionated condensation, have shown a low selectivity for the recovery of vanillin, mostly probably due to polarization of concentration phenomena. The selectivity of vanillin in relation to water was 4.6 and 4.5 when using a PDMS membrane at feed temperatures of 45 and 75° C, respectively. Therefore, the selectivity of vacuum distillation (-), a purely evaporative process, was calculated for the separation of vanillin from water, using the following equation:

$$\text{selectivity}_{\text{van-water, evaporative}} = \frac{H_{\text{van}}}{H_{\text{water}}} \quad (25)$$

The values of selectivity of vanillin in relation to water (-) obtained by vacuum distillation are 0.02 and 0.03, at the feed temperatures of 45 and 75° C, respectively, which are very low values, lower than in the case of pervaporation, which is to be expected due to the presence of a dense hydrophobic membrane with an affinity to vanillin. However, the selectivity of water in relation to vanillin (-) has the values of 50.99 and 35.31 at 45 and 75° C, respectively, meaning that water is much more volatile than vanillin in this process. In order to recover vanillin, vacuum distillation may be combined with fractionated condensation.

A set of experiments was performed to recover vanillin through vacuum distillation with fractionated condensation (using three condensers-in-series, see Figure 5.2), in order to increase the condensation area, aiming at condensing as much as possible the evaporated vanillin) The objective was to achieve

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the evaporation of both water and vanillin (avoiding non-volatile potential contaminants) and then promote the condensation of vanillin in the first condenser(s). As water is much more volatile than vanillin - the Henry constant of water is twice the order of magnitude of vanillin (Table 5.7) - it is relatively easy to separate vanillin from water in the first two condensers. Table 5.10 shows the results of the evaporation of vanillin during the vacuum distillation experiments.

Table 5.10. Vacuum distillation with fractionated condensation experiments: percentages of evaporated vanillin, mass balance deviations of vanillin and total vanillin fluxes.

Exp.	Temperature (°C)	Time (min)	Vanillin evaporated ^a (%)	Vanillin balance mass deviation (%)	$J_{\text{vanillin}, T}$ (g.m ⁻² .h ⁻¹)
1 ⁽¹⁾	45	65	4.2±0.2	-1.7	0.4±0.03
2 ⁽¹⁾	85	15	18.6±1.3	-10.6	12.9±0.6
3 ⁽¹⁾	85	70	16.7±1.3	-9.9	4.5±0.1
4 ⁽¹⁾	85	15 (x4)	42.5±7.9	-9.3	6.7±0.3
5 ⁽¹⁾	85	15(x10)	68.2±4.5	-1.4	3.7±0.2
6 ⁽¹⁾	85	15(x18)	87.8±5.8	-5.4	2.7±0.1
7 ⁽²⁾	85	15(x18)	84.4±0.46	-4.0	2.3±0.0

^a Vanillin recovery in the 3 condensers, vanillin evaporated from the feed vessel

⁽¹⁾ where the feed was the bioconversion broth 2 (Table 5.2)

⁽²⁾ where the feed was the bioconversion broth 1 (Table 5.2)

As shown in Table 5.10, at 45° C (temperature of the bioconversion) only 4 % of vanillin was evaporated in 60 min (Exp.1) and the flux of vanillin evaporation was extremely low. In experiments 2-6, the increase of the temperature from 45 to 85° C, led to an increase in Henry constants and consequently an increase in vanillin volatility. This increase of temperature increased the evaporation of vanillin, although only about 18 % of vanillin was evaporated (Exp 2). The duration of the experiment from 15 minutes to 70 minutes (Exp.2 and Exp.3), did not lead to an increase of vanillin evaporation, meaning that vanillin evaporated during the first few minutes of each experiment. Experiments 1-3 show a low percentage of evaporated vanillin. This behaviour can be explained by the fact that water evaporates much faster than vanillin. After evaporation of water, vanillin becomes more concentrated (and consequently with a lower activity coefficient in the concentrated aqueous solution), which lowers its volatility in solution. Therefore, in order to increase the rate of evaporation of vanillin without increasing the temperature, a strategy of multiple feeding water pulses was implemented. This approach consists of adding several consecutive water pulses, each one with the same mass of the evaporated water, keeping constant the mass of the feed solution. As shown in Table 5.10, in experiments 4, 5, and 6, adding more pulses of water to the remaining broth increased the recovery of vanillin, making possible to achieve a total vanillin recovery of 88 % (experiment 6) with the addition of 18 water pulses, with

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the same mass each. In each experiment the mass balance of vanillin, closed within a negative value, most probably due to vanillin losses in the vacuum distillation circuit, but the deviations were small and reasonable ($\leq 10\%$). Regarding the flux of vanillin in the vacuum distillation process, although the mass of evaporated vanillin increased with increasing number of water pulses, the flux of vanillin decreased slightly within the range $3.7 \text{ g.m}^{-2}\text{h}^{-1}$ with 10 water pulses steps and $2.7 \text{ g.m}^{-2}\text{h}^{-1}$ with 18 water pulses.

From the set of vacuum distillation experiments performed with the same bioconversion broth 2 (see Table 5.10), experiment 6 was selected as it corresponds to the highest percentage of recovered vanillin, $87.8 \pm 5.8\%$, as well as a low mass balance deviation of vanillin (-5.6%) and no vanillic acid in the condensates.

An additional vacuum distillation experiment, experiment 7, was performed with the same operating conditions as the selected vacuum distillation experiment (experiment 6) but with the bioconversion broth 1, which was used in the pervaporation experiments. As expected, experiments 6 and 7 performed similarly (see Table 5.10).

Figure 5.5 shows the HPLC chromatograms obtained. The chromatograms were taken at the end of experiment 6 (the same as in experiments 1-5, where the bioconversion broth 2 was processed) and at the end of experiment 7 (where the bioconversion broth 1 was processed), see Table 5.10.

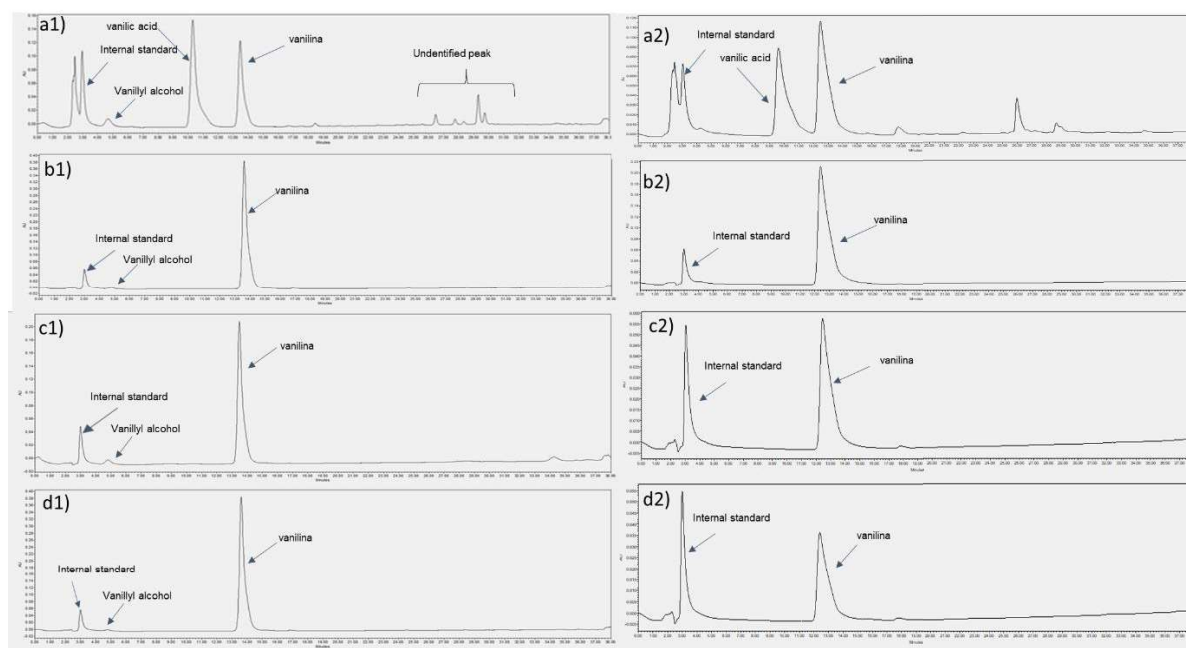


Figure 5.5. Vacuum distillation experiments: HPLC chromatograms obtained at the end of experiment 6 (Chromatogram 1) and at the end of experiment 7 (Chromatogram 2): (i a) final feed; (i b) 1st condenser; (i c) 2nd condenser and (i d) 3rd condenser, where i is the number of the chromatogram.

As, in pervaporation experiments, vanillin and vanillic acid were present in the feed and no vanillic acid was detected in the condensers (see Figure 5.5b2, c2, d2). In the first and second condensers, vanillin was obtained pure in solid form, without water. However, as shown in Figure 5.5 d2), vanillin is still present in the third condenser indicating that vanillin was dragged with water, as it seems to happen in

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the pervaporation experiments. In order to decrease the amount of vanillin lost to the total vapour condenser (together with water), the area of the first condenser should be increased. This strategy should be implemented first, preferably as a pilot-scale study, as it is more cost-effective. If it is still necessary, the strategy of decreasing the temperature of the first two condensers should then be evaluated.

The chromatograms from experiment 6 are similar to the ones from experiment 7, except for vanillyl alcohol. Indeed, a peak of vanillyl alcohol was detected by HPLC in the final feed (see Figure 5.5 a1), indicating the existence of vanillyl alcohol in the feed. Consequently, a very small peak of vanillyl alcohol was also detected in the condensers in experiment 6 (Figure 5.5 1b, 1c, 1d), due to the presence of a minor amount of vanillyl alcohol in the initial feed.

The condensate from vacuum distillation experiment 6 (condensate obtained in the first condenser), the final product of the experiment, was qualitatively characterised by ^1H -NMR in D_2O to confirm the presence of vanillyl alcohol and to assess the presence of other contaminants. The sample was completely saturated in order to observe lower concentrations of compounds. Figure 5.6 represents the ^1H -NMR spectrum, the first condensate of vacuum distillation experiment 6.

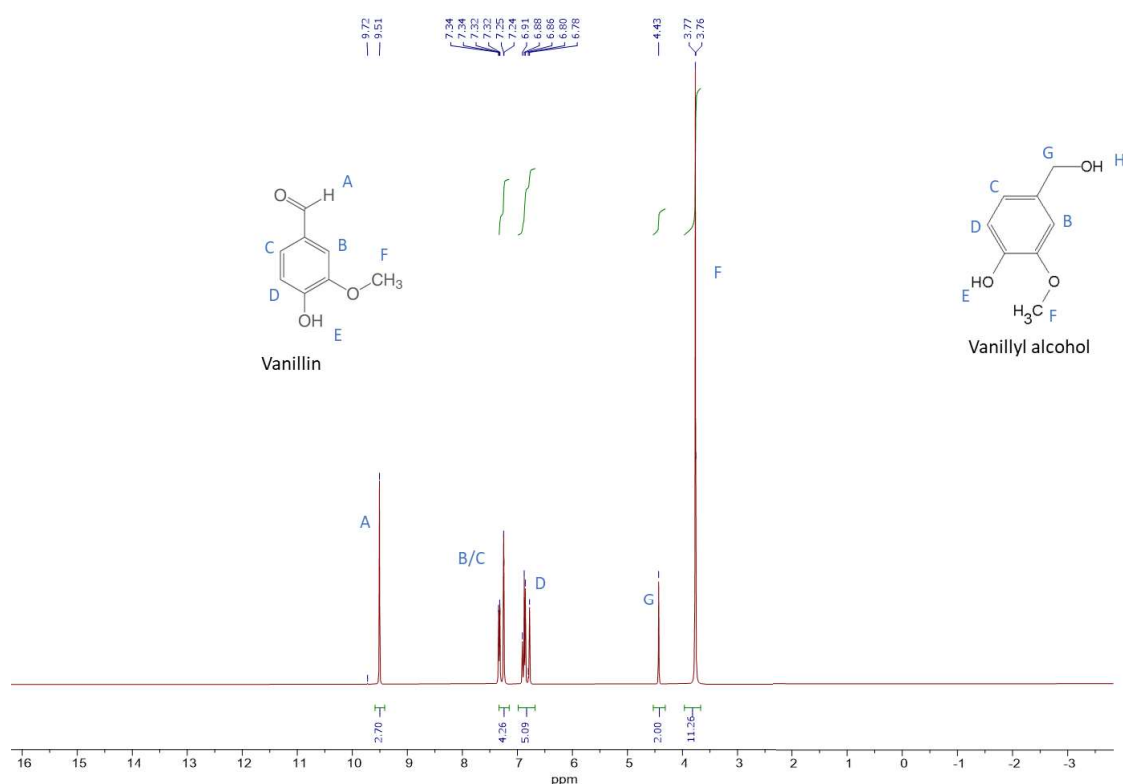


Figure 5.6. ^1H - NMR spectrum of the first condenser of vacuum distillation experiment 6

As shown in Figure 5.6, vanillin is present in the sample, since the proton characteristic of the aldehyde linkage, $-\text{CHO}$ (linkage A in the Figure 5.6), is represented at 9.51 ppm. This linkage is not present in the other contaminants (ferulic acid, vanillic acid, alcohol vanillyl and guaiacol). On the ^1H - NMR spectrum, a peak was also observed at 4.43 ppm, characterised by the protons on the linkage $-\text{CH}_2-$ (linkage G in the Figure 5.6). This linkage is only present in alcohol vanillyl, it is not present in ferulic

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acid, vanillin, vanillic acid or guaiacol, confirming the presence of alcohol vanillyl in the sample. The proton of the linkage -OH in both compounds (linkage E in Figure 5.6) is not detected on the ^1H NMR spectrum using the D_2O solvent [247]. Through the integration of the peaks, it was possible to confirm that only vanillin and vanillyl alcohol were present in the sample, since there were no remaining protons.

In terms of the total composition of the different streams at the end of each experiment, both experiments 6 and 7 performed similarly (see Figure 5.5) because the initial feed contained vanillyl alcohol in minor quantities, which was not detected by HPLC but was confirmed by NMR analysis in the condensate. However, vanillyl alcohol is also present in botanical vanillin [2]. Rhodia (Solvay) produces a bio-vanillin with 3 % by weight of impurities, where the most abundant impurity is vanillyl alcohol [223]. When comparing the selected pervaporation-fractionated condensation experiment (with a PDMS membrane at 75°C) to the selected vacuum distillation experiment, at 85°C , with the addition of several consecutive water pulses (experiments 6-7), we may conclude that both processes allow for recovering pure vanillin free from undesirable contaminants. Both processes were also compared in terms of the process productivity, where the selected pervaporation-fractionated and vacuum distillation condensation processes exhibited vanillin fluxes, J_{van} ($\text{g}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$), respectively of 1.19 ± 0.01 $\text{g}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$ (Table 5.8) and 2.7 ± 0.1 $\text{g}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$ (Table 5.10). Both processes were also compared in terms of energy consumption per mass of vanillin recovered ($\text{kJ}\cdot\text{kg}_{\text{VAN}}^{-1}$), which is calculated by equation (26), considering that in the sake of simplicity, water was considered the only specie in the process, since vanillin is in minor compound in these processes, compared to water:

$$E (\text{kJ}\cdot\text{kg}_{\text{VAN}}^{-1}) = \frac{m_{\text{water}} [(C_{p\text{water}} \times (T_{\text{final}} - T_{\text{initial}}) + \Delta H_{\text{vaporization}} (T_{\text{final}})]}{m_{\text{VAN}}} \quad (26)$$

Where m_{water} (kg) and m_{VAN} (kg) are respectively the mass of water and the mass of vanillin permeated (in the pervaporation-fractionated condensation experiment) or evaporated (in the vacuum distillation experiment) with the same numerical values of the total flux in each experiment (2.38 $\text{Kg}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$ and 10.76 $\text{Kg}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$, respectively) and of the vanillin flux in each experiment (1.19×10^{-3} $\text{Kg}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$ and 2.7×10^{-3} $\text{Kg}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$, respectively); $C_{p\text{water}}$ ($\text{kJ}\cdot\text{kg}^{-1}\cdot\text{K}^{-1}$) is the specific heat capacity of water; T_{initial} is the temperature of bioconversion of 45°C ; T_{final} is the operating temperatures of pervaporation and vacuum distillation 75 and 85°C , respectively, and $\Delta H_{\text{vaporization, water}} (T_{\text{final}})$ is the enthalpy of vaporization of pervaporation and vacuum distillation, respectively 4.91 $\text{kJ}\cdot\text{kg}_{\text{VAN}}^{-1}$ at 75°C and 9.75 $\text{kJ}\cdot\text{kg}_{\text{VAN}}^{-1}$ at 85°C .

5.5. Conclusions

The selected pervaporation-fractionated condensation experiment was performed with a PDMS membrane at 75°C , with the highest vanillin flux of the pervaporation experiments performed. The selected vacuum distillation experiment was performed at 85°C (with 18 pulses of water, followed by

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15 minutes of operation), giving the highest percentage value of evaporated vanillin, 87.8 ± 5.8 %. Both selected processes recovered vanillin without undesirable contaminants and were compared in terms of vanillin flux, where the selected pervaporation-fractionated and vacuum distillation condensation processes exhibited vanillin fluxes, J_{van} ($\text{g} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$), respectively of 1.19 ± 0.01 $\text{g} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ and 2.7 ± 0.1 $\text{g} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$, the latter 2.3 times higher. On the other hand, the vacuum distillation process presented an energy consumption per mass of vanillin recovered 2.0 times higher than pervaporation-fractionated, respectively $9.75 \text{ kJ} \cdot \text{kg}_{\text{VAN}}^{-1}$ at 85°C and $4.91 \text{ kJ} \cdot \text{kg}_{\text{VAN}}^{-1}$ at 75°C . For future work, using either pervaporation-fractionated or vacuum distillation condensation processes, the complete separation of vanillin from water may be achieved by increasing the area of the first condenser (the more economically advantageous strategy), preferably in a pilot-scale study. The strategy of decreasing the temperature of the first condenser should only be studied if strictly necessary.

6. The economic assessment of the integrated bio-vanillin process

6.1. Summary

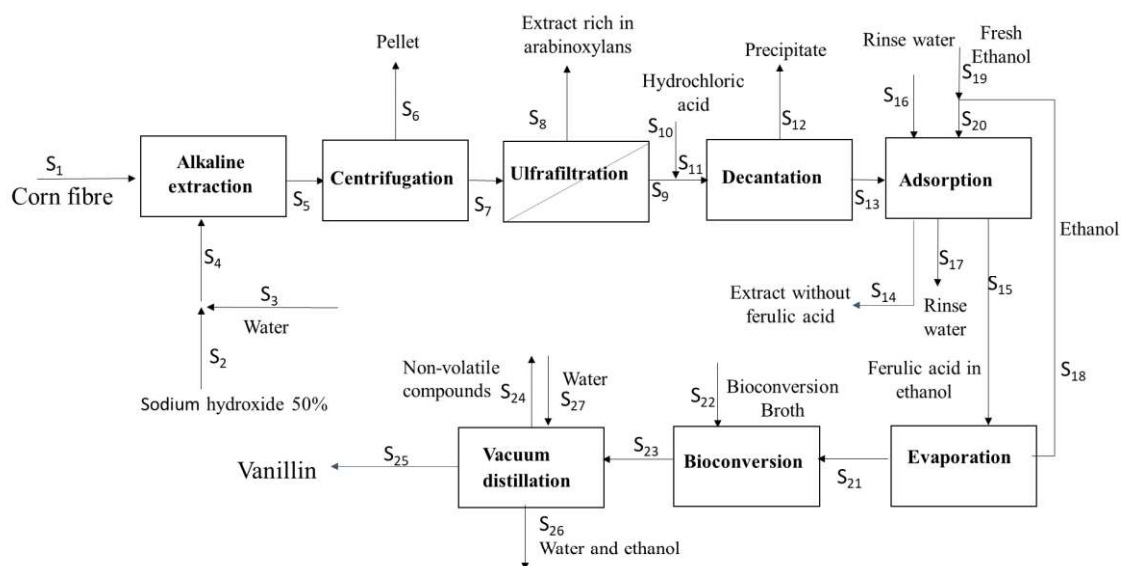
This chapter presents and discusses the proposed economic assessment of an integrated process to obtain bio-vanillin from corn fibre, comprising the main processing steps optimised: 1-recovery of ferulic acid from corn fibre, 2-purification of ferulic acid, 3- bioconversion of ferulic acid into vanillin, and 4- purification of vanillin. The economic assessment shows that the proposed process is feasible, based on experimental data obtained at laboratory scale.

6.2. Integrated process for the production of bio-vanillin

The proposed integrated process to obtain bio-vanillin from corn fibre comprises different processing steps divided by the following main operating units with the following purposes:

- 1- Alkaline solid-liquid extraction, to recover ferulic acid from corn fibre, optimised in chapter 2.1;
- 2- Centrifugation, to separate the solid residue from the liquid extract;
- 3- Ultrafiltration, to separate the arabinoxylans present in the extract from ferulic acid, as described in chapter 3.1;
- 4- Decantation, to remove the precipitated proteins formed by acidification until pH 4.5 (this mild acidic pH value allows for a better adsorption of the ferulic acid by the Macronet® MN102 resin, in the next processing step), as described in chapter 3.2;
- 5- Adsorption, comprising the steps of adsorption of ferulic acid, the rinse of the resin and the desorption of ferulic acid with ethanol, as described in chapter 3.2;
- 6- Evaporation of the ethanol used as ferulic acid desorbent;
- 7- Bioconversion of ferulic acid into vanillin by the stain *Amycolatopsis* sp. ATCC 39116, optimised in chapter 4;
- 8- Vacuum distillation for vanillin purification, as studied in chapter 5.

The global flow chart of the integrated process is presented in Table 6.1.



Streams	Main compounds/components	Streams	Main compounds/components
S ₁	(Untreated) wet corn fibre	S ₁₄	Extract without ferulic acid
S ₂	NaOH 50%	S ₁₅	Ferulic acid Extract (desorbed in ethanol)
S ₃	Water	S ₁₆	Rinse water
S ₄	Solution of 1% NaOH	S ₁₇	Rinse water
S ₅	Wet corn fibre in 1% NaOH	S ₁₈	Recirculated ethanol
S ₆	Pellet (wet solids)	S ₁₉	Fresh ethanol
S ₇	Ferulic acid extract	S ₂₀	Total Ethanol
S ₈	Ferulic acid extract rich in arabinoxylans	S ₂₁	Concentrated Ferulic acid
S ₉	Feulic acid extract	S ₂₂	Bioconversion Broth
S ₁₀	HCl 33%	S ₂₃	Bioconversion Broth
S ₁₁	Ferulic acid Extract (pH of 4.5)	S ₂₄	Non-volatile compounds
S ₁₂	Precipitated	S ₂₅	Vanillin
S ₁₃	Ferulic acid extract (without precipitated)	S ₂₆	Water and ethanol
		S ₂₇	Water

Figure 6.1. Scheme of the proposed process to obtain bio-vanillin from ferulic acid, which is extracted from corn fibre. S_i corresponds to stream *i* of the process.

The optimised values of the operating conditions and the main results obtained in each processing step, in a study performed at laboratory scale, except in the alkaline extraction performed at pilot scale, are resumed in Table 6.1. A resume of mass balance to each operating unit is shown in appendix A1.

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Table 6.1. Optimised values of the operating conditions and main results obtained in each processing step

Processing step (divided by main operating units)	Optimised operating parameters	Main results
Alkaline extraction	• Solvent of extraction: 1% NaOH (0.25M) • Liquid-to-solid ratio: 10 (w/w) • Residence time: 7 h	• Concentration of Ferulic acid in extract: 2.0 g.kg⁻¹ (minimum obtained)
Centrifugation		• Centrifugation efficiency: 49% (% of extract recovery without solids)
Ultrafiltration	• Concentration factor: 7 • Permeance: 32 L.m⁻² h⁻¹ bar⁻¹ (65°C with a compromise between high velocity and low pressure) • Effective transmembrane pressure: 1.4 bar	• Ferulic acid rejection: 0 %
Decantation	• Acid to adjust the pH to 4.5: 19.1g 37%HCL .kg⁻¹ extract • Residence time: 1h	• Solids: 2%
Adsorption	• Losses of ferulic acid: 5% (breakthrough point) • Ethanol recycles: 95%	• Desorption: 11.1 g ferulic acid kg⁻¹ ethanol • Rise water: 0.20Kg water/kg extract • Desorbent of resin : 72.4% of ferulic acid • T_{br} : 115.41min • Adsorption: 0.0125 g ferulic acid adsorbed / cm³ resin
	• Cells growing: 11 h • Ferulic acid pulses: 6 pulses of 10. g.L⁻¹, each one 7 hours	• Vanillin yield : 0.52 g vanillin. g⁻¹ ferulic acid • Vanillic acid yield: 0.20 g vanillic acid. g⁻¹ ferulic acid

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Bioconversion	• Production medium: 12g.L⁻¹ glucose and 0.18g.L⁻¹ yeast extract • Rate of vanillin production (r_v): 0.67 g.L⁻¹.h⁻¹	• Consumption by the microorganism: 8 g.L⁻¹ glucose and 0.18g. L⁻¹ yeast extract • Rate of vanillin production (r_v): 0.67 g.L⁻¹.h⁻¹
Vacuum distillation	• It was considered the addition of water of 3 times de volume of the bioreactor	• Vanillin recovery: 88 % • Flux: 2.7±0.1 g.m⁻²h⁻¹

6.3. Economic evaluation

The overall bio-vanillin production from corn fibre was simulated considering a bio-vanillin production of 20 ton/ year (2.48kg/h) from 5081ton/ year of dry corn fibre (13731 ton of wet fibre). A continuous operating regime was chosen with a working period of 336 day/year (with a break of 4 weeks a year) and 24h/day, corresponding to 8064 h/year. Mass balances were accomplished for each main operating unit (processing step), considering the optimised results obtained at laboratory scale (see Table 6.1). The economic evaluation was performed considering that the unit of bio-vanillin production is integrated in a starch industrial plant, in this case, to the production plant of Copam - Companhia Portuguesa de Amidos, S.A, allowing the proximity with the raw material, the easy reintegration of the corn fibre (extraction) in the starch process, and the use of the process auxiliar services (natural gas boiler, refrigerated tower).

6.3.1. Equipment

In the processing steps of the overall biovanillin process, the main equipment was included:

1. Alkaline extraction: 2 mixing tanks and 1 decanter centrifuge (centrifuge which can process liquids/suspensions with high solid material contents);
2. Ultrafiltration: 1 ultrafiltration membrane unit, with 4 modules of membrane UP150 Microdyn-Nadir (3 in continuous operation and 1 in cleaning), each one with 25 m² [248]; 1 shell and tube floating heat exchanger, 1 settler, 2 adsorption columns (1 in adsorption step, 1 in the rinse and desorption steps) and 1 falling film evaporator;
3. Bioconversion of ferulic acid into vanillin: 1 autoclavable inoculum bioreactor, 1 autoclavable growth bioreactor and 2 production bioreactors;
4. Vanillin purification: 1 vacuum evaporator and 1 chiller.

Table 6.2 shows the type of equipment, total sizing, number of equipment, material and equipment cost.

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Table 6.2. Main characteristics of each processing step required for the dimensioning of the equipment for the overall biovanillin process.

Operating Unit	Equipment	Inlet rate	Additional information
Alkaline extraction	Mixing tank	$6.93 \text{ m}^3 \cdot \text{h}^{-1}$ (stream5) ^a	• Useful volume: 80 %
Centrifugation	Centrifuge	$6.93 \text{ m}^3 \cdot \text{h}^{-1}$ (stream5) ^a	-
Ultrafiltration	Membrane module	$3.40 \text{ m}^3 \cdot \text{h}^{-1}$ (stream7) ^a	• Membrane module: 25 m ² each [248]
	Heat exchanger	$3.40 \text{ m}^3 \cdot \text{h}^{-1}$ (stream7) ^a	• Estimated overall heat transfer coefficient: 350 W/m ² °C [249] • Useful volume :85 %
Decantation	Settler	$2.97 \text{ m}^3 \cdot \text{h}^{-1}$ (stream11) ^a	• Useful volume:80 %
Adsorption	Adsorption column	$2.91 \text{ m}^3 \cdot \text{h}^{-1}$ (stream13) ^a	• Useful volume :80 %
Evaporation	Evaporator	$0.59 \text{ m}^3 \cdot \text{h}^{-1}$ (stream18)	• Estimated overall heat transfer coefficient: 600W/m ² °C [249] • Useful volume: 85%
Bioconversion	Bioreactor	$0.58 \text{ m}^3 \cdot \text{h}^{-1}$ (stream 22+Stream21)	• Vanillin production: 2.8 kg.h ⁻¹ (stream 23) • Inoculum: 3 % • Useful volume: 75 %
Vacuum distillation	Vacuum distillation	$2.8 \text{ kg}_{\text{vanillin}} \cdot \text{h}^{-1}$ (stream 23)	• Vanillin flux:3x of the flux obtained (8.1 g.m ⁻² h ⁻¹) ^b

^a It was considered a density of 1 kg.L⁻¹

^b considering the vacuum distillation operating conditions (operation time, the evaporation area and the residence time) optimized

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Table 6.2. Equipment used in the overall biovanillin process: type of equipment, total sizing, number of equipment, material and equipment cost.

Operating unit	Equipment	Total Sizing Required	N° of equipment	Material	Capex (k€)
Alkaline extraction	Mixing tank	60 m ³	2x 30m ³	316 stainless steel	90 ^a
Centrifugation	Decanter centrifuge	-	1 of 8 m ³ ·h ⁻¹	-	70 ^b
	Ultrafiltration unit	76.6 m ²	4x 25m ²	-	50 ^c
Ultrafiltration	Heat exchanger	5.6 m ²	5.6m ²	Tubes in 316 stainless steel	16 ^d
Decantation	Settler	3.6 m ³	3.6 m ³	316 stainless steel	15 ^a
Adsorption	Column	1 m ³	2x 1m ³		35 ^a
Evaporation	Falling film evaporator	3.0 m ²	3.0 m ²	316 stainless steel	65 ^e
	Inoculum bioreactor	84 L	90 L	316 stainless steel	25 ^f
Bioconversion	Growth bioreactor	2800 L	3000 L	316 stainless steel	336 ^f
	Production bioreactor	5600 L	2x 3000 L	316 stainless steel	600 ^f
	Vacuum evaporator	348 m ²	348 m ²	Tubes in 304 stainless steel	311 ^e
Vacuum distillation	Chiller	-	-	-	68 ^e
Total					1681

^aProvided by Arsopi

^bbased on <http://www.fedequip.com>

^cConsidering 500 €/m² of installed membrane unit

^dprovided by Purolite

^e<http://www.matche.com>

^fProvided Solaris Biotech, with automation and instrumentation include and 316 stainless steel in the parts in contact with the culture.

6.3.2. Reagents and raw material costs

Table 6.4 shows the reagents cost. Since the corn fibre, after ferulic acid extraction, return to the COPAM process (after adjust of pH), the cost of the raw material was considered 0 €/ton (the cost of pH adjustment is calculated in section 6.3.4). The cost of the other constituents of the basal medium in the bioreactor (described in chapter 4) where the culture grows, was assumed as 20% of the cost of glucose and yeast extract (the main constituents of the basal medium).

Table 6.3. Reagents and raw material costs

Reagents/ Raw materials	Unit price (€/ton)	Consumption (Ton/year)	Costs (k€/year)
Corn Fibre	0	13732	0
NaOH (50%)	320 ^a	843	270
Ethanol (99%)	700 ^b	392	192
HCl (37%)	150 ^a	448	67
Water	1.91 ^c	51215	123
Basal medium of bioreactor			
Glucose	800 ^a	45	53
Yeast extract	2500 ^b	5.1	13
Other compounds ^d	-	-	13
Total			731

^a according to the current market price of the main traders

^b <https://www.chembid.com>

^c <https://www.epal.pt>

^d considering 20% of total cost glucose and yeast extract

6.3.3. Energy

Table 6.5 indicates the electrical energy cost of the main types of equipment, considering that the electrical energy cost was 0.138 €/kwh [250]. Table 6.5 shows the utility costs. The steam was considered at 4 bar (absolute pressure) and at 143° C with a latent heat of 509.55 Mcal/ton and at a cost of 30 €/ton (based on the production cost in a conventional natural gas boiler). In the ultrafiltration, the steam was used to heat the extract from 25° C to 65° C, to reduce the viscosity of the extract, according to the optimisation of ultrafiltration in chapter 3.1. In the bioreactors, the steam was used to heat from 25° C to 45° C (bioconversion temperature). Regarding the cooling water, it was considered that water has an inlet temperature of 25° C and an outlet temperature of 35° C to condensate the ethanol in stream S₁₈, with a cost of 0.20 €/ton (based on the production of cooling water in a cooling tower). For vanillin sublimation at 5° C in vacuum distillation step (stream, S₂₃), it was chosen a mixture of 20 % propylene glycol and 80 % water, with an inlet temperature of 5° C and an outlet temperature of 15° C of the condenser, with a cost of 2.5 €/ton (based on cooling this mixture in a chiller).

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Table 6.4. Energy costs of the equipment used in bio-vanillin process calculated for 336 day/year, 7 days/ week and 24h/day.

Equipment	Power (kw)	(kwh/year)	Cost (k€/year)
Mixing tank (stirring)	75.9 ^a	5.3x10 ⁵	85
Decanter Centrifuge	14.0 ^b	1.1x10 ⁵	16
Bioreactors (stirring)	79.6 ^c	6.4 x10 ⁵	89
Vacuum pump (vanillin purification)	15.0 ^d	1.5 x10 ⁵	17
Total without feed and recirculation pumps			206
Feed and recirculation pumps	-	-	62 ^e
Total			268

^a calculated considered an anchor agitator

^b provided by Alfa Laval

^c provided by Solaris

^d [https:// www.in-eco.eu/vacuum-pumps/](https://www.in-eco.eu/vacuum-pumps/)

^e 30% of Energy total cost

Table 6.5. Cost of producing utilities in the overall bio-vanillin process calculated for 336 day/year, 7 days/ week and 24h/day.

Utilities	Stream	Vapour steam (ton/year)	Cost (k€/year)
Heat the inlet stream of ultrafiltration	S ₇	2203	66
Evaporation of ethanol from the desorbed ferulic acid from the resin	S ₁₈	1843	55
Heat the Bioreactors	-	1046	31
Evaporation of vanillin and of water in vacuum distillation	S ₂₃	21985	605
Utilities		Cooling Liquid(ton/year)	Cost (k€/year)
Ethanol condensation	S ₁₈	93939	19
Vanillin sublimation in vacuum distillation S ₂₃	S ₂₃	67063	168
Total			945

6.3.4. Co-products and residual effluents

Table 6.7 presents the costs of the co-products and residues produced in the biovanillin production process.

Table 6.7. Cost of the co-products and residual effluents of the bio-vanillin production.

Co-products and residual effluents	Stream	Production (Ton/year)	Characteristics	Costs (k€/year)	Destination
Pellet of Centrifugation	S ₆	28 502	pH 13 Humidity:81%	76	Return the starch industry process
Extract rich in arabinoxylans	S ₈	3 912	pH 13	0	Co-product
Precipitate of decantation	S ₁₂	478	pH 4.5	1	Return the starch industry process
Extract without ferulic acid	S ₁₄	23 399	pH 5.5 COD:3.5 gO ₂ .L ⁻¹ ; total suspend solid 4.82 g.L ⁻¹	82	-
Rinse water	S ₁₇	4 688		16	-
Total cost				175	

The co-product of stream 6, (the pellet of centrifugation after an alkaline extraction) can be combined with the co-product of stream 12 (precipitate of decantation) and return to the starch industry process. However, it is necessary to assess the need of using a pre-treatment (pH adjustment), since the co-products pellet of centrifugation (stream 6) and precipitate of decantation (stream 12) have pH values of 13 and 4.5, respectively. The cost considered to adjust the pH after the combination of these streams is 2.7 €/Ton_{co-product} using HCL 37 %. The ultrafiltration concentrate is rich in arabinoxylans (stream 8), a compound with many health benefits [76,162,163], film-forming properties [77,164] and it may work as an emulsifier for oil-in-water emulsion systems [78]. Although arabinoxylans have the potential to be valorised, no value [76,77,162–164] was given to the co-product arabinoxylans in S₈, since arabinoxylans may need a pre-treatment before being sold. The streams S₁₄ and S₁₇ are considered residues. These residual effluents cannot be discharged in a public water treatment plant, since the stream S₁₄ has a chemical oxygen demand (COD) and a total suspend solids of about 3300 mgO₂.L⁻¹ and 4800 mg.L⁻¹, when the maximum limit allowed to a water treatment plant is 1500 mgO₂.L⁻¹ and 1000 mg.L⁻¹, according to the Portuguese Legislation (Decreto-Lei N° 238/98). Therefore, a process to treat these residues is required [251], which has to be efficient, economical and under the Portuguese Legislation; this study was considered out of the scope of this thesis. Nevertheless, in this economic analysis, a cost of 3.5 €/Ton of residual effluents, for both streams S₁₄ and S₁₇ was assumed (value based on the pre-treatment of a water treatment plant).

6.4. Economic assessment

The economic study for the production of biovanillin is shown in Table 6.6. Taking into account the continuous operation of the process, in what concerns the personnel cost, it was considered 3 shifts of 8

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hours, with 1 worker per shift. Considering two days off per worker per week and a supplementary worker, a total of 5 workers will be required. The worker cost per year per worker (26 800 €), includes an illiquid salary of 1300 € per month per worker for 14 months plus 1.5 % of the illiquid salary for accidents insurance, 23.5 % of the illiquid salary for social security and 22 % of the illiquid salary for allowance shift. It was also considered an engineer with a total cost of 42084 €/ year (illiquid salary of 2400€, 1.5% for accidents insurance of the illiquid salary, 23.5 % for social security of illiquid salary). The selling price of bio-vanillin was considered to be 733 €/Kg (the initial trading price of vanillin from Rhodia, 640 €/kg [10,24] plus 15 % of increase by Rhodia 2017 [10,129]). The vanillin packing cost was considered 1 €/kg (packaging and labelling prices).

Table 6.6. Economic assessment of the overall biovanillin process calculated for 336 day/year, 7 days/ week and 24h/day considering a vanillin production of 20 ton/year (2.5 kg.h⁻¹).

Investment costs	Cost (k€)
Total base equipment	1681
Auxiliary equipment (20 % of total base equipment cost) ^a	336
Equipment assembly (45 % of total base equipment cost [252])	756
Piping (65 % of total base equipment cost [252])	1093
Automation (15 % of total base equipment cost)	252
Instrumentation (30 % of the base equipment cost [252])	504
Thermal insulation (10 % of total base equipment cost [252])	168
Electrical installation (15 % of total base equipment cost [252])	252
Buildings (20 % of total base equipment cost [252])	336
Engineering, procurement, construction and project management	600
Licensing	50
Total fixed investment cost	6030
Unexpected cost (15 % of the total fixed investment costs)	904
Total fixed investment cost + Unexpected costs	6934
Capital costs^b (k€/year)	898
Operating costs	
Maintenance (k€/year) (5% of total fixed investment + unexpected costs)	347
Consumables (k€/year) ^c (5% of total fixed equipment + unexpected costs)	347
Solvents (k€/year)	731
Energy (k€/year) (utilities and electrical energy)	1212
Manpower (k€/year)	176
Residues (k€/year)	175
Package (k€/year)	20
Insurance (k€/year) (1% of fixed investment cost + unexpected costs)	69
Total Operating costs	3077
Total costs (capital + total operational) (€/year)	3975
Revenues (k€/year)	14651
Return without taxes (Revenues-Total costs) (k€/year)	10676
Return with taxes	7847
Pay back (years)	0.9

^a equalisation tanks, holding tanks, pumps

^b based on a depreciation over 10 years and 5% annual taxes

^c including replacement of resins and membranes

The base equipment cost plays an important role in the total fixed investment cost, representing 24 % of the total fixed investment. From all equipment required to this overall biovanillin process, the equipment used in the bioconversion step (bioreactors) is the most important, representing 57% of the total equipment base followed by the vacuum distillation equipment (23 %) (see Table 5.3). Therefore, the production and purification of vanillin represents most of the investment cost. The piping also represents a significant portion of the total investment cost (16 %). On the other hand, the equipment necessary for the production of ferulic acid is just 20 % of the total equipment base (or 341 k€).

The parameters with a higher impact in the operating cost are the energy cost followed by the solvents cost which represent 39 % and 24 % of the total operating costs, respectively. The vacuum distillation is the processing step with the higher energy cost from all the processing steps representing 65 % of the total energy cost.

6.4.1. Sensitivity analyses

A sensitivity analyses study was performed to evaluate the impact that the most relevant parameters have on the payback period. In this work, the selected key parameters of this process were identified as being the market-price of bio-vanillin, the energy cost and the total fixed investment cost (fixed investment cost plus unexpected costs). The market price of biovanillin was chosen due to its high importance in the economic evaluation and to its high oscillation in the market, between 450 € and 900 € [10,17,18]. The energy cost was also considered as it contributes significantly to the total operating cost (39 %) and due to the oscillation of the energy market. Finally, the total investment cost was also chosen due to its importance in the economic assessment to the uncertainty of the prices of the various equipment considered in this work. Figure 6.2 shows the influence of these three parameters on the payback of the overall bio-vanillin process.

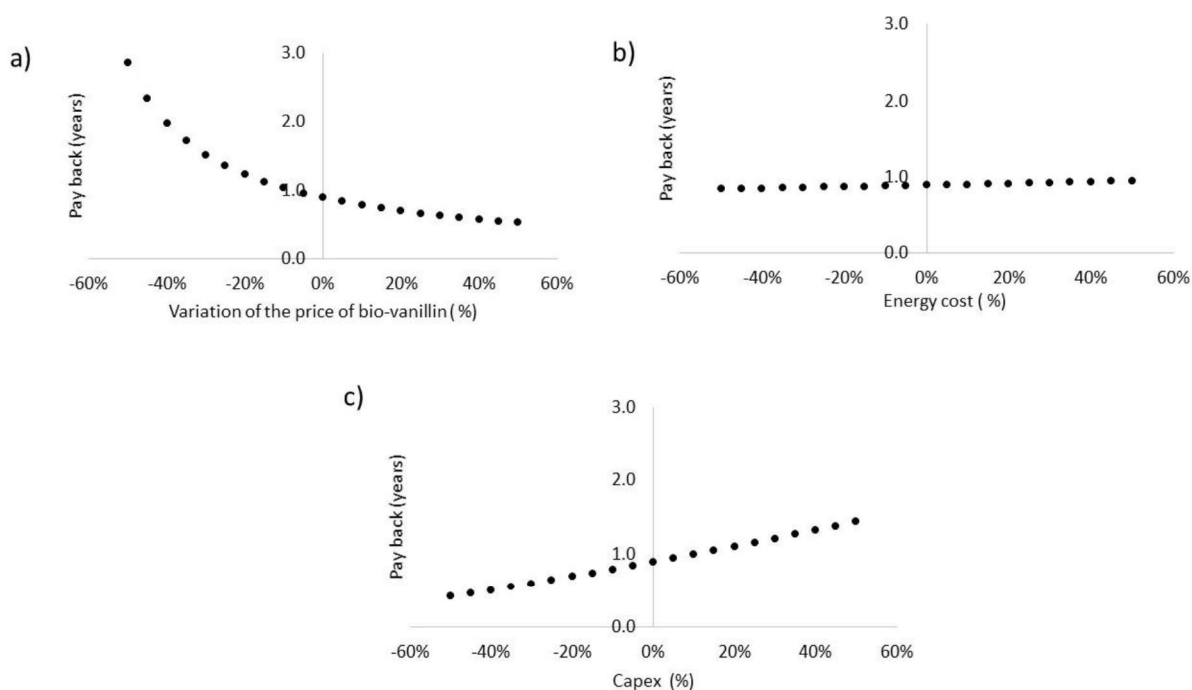


Figure 6.2. The impacts of the variations of a) the market price of bio-vanillin b) Energy cost and c) Total investment cost on the payback period for the production plant of bio-vanillin.

As shown in Figure 6.2, the payback was much more sensitive to the selling price of vanillin than to the total investment cost. The energy cost had very small influence on the payback. The payback period, for the ranges of values considered, never exceeds 3 years, which is a good result. Considering the worst scenario for the ranges considered, in which energy cost increases 50 %, the selling price of biovanillin decreases 50% and the total investment cost increases 50 %, the payback value increases to 7.4 years.

6.5. Conclusions

This work proposes a global process, which comprises several operating units, to produce bio-vanillin from ferulic acid, which is extracted from corn fibre. The economic evaluation for a continuous vanillin production of 20 ton/years carried out shows that the process is economically viable with a very low payback period lower than 1 year. The market price of the bio-vanillin is the parameter with the highest effect on the payback period.

7. Conclusions and Future Work

7.1. General Conclusions

This thesis is focused on the development of an integrated process to produce bio-vanillin from ferulic acid extracted from corn fibre. The research started by optimising the extraction of ferulic acid from corn fibre under alkaline operating conditions. The aim was to develop an alkaline extraction process with an efficient balance between the maximum concentration and the recovery yield of ferulic acid while producing low-viscous extracts. This balance was achieved using wet corn fibre, without milling and drying, as a feedstock followed by extraction with an alkaline solution of NaOH at a concentration of 0.25 M, with a liquid-to-solid ratio of $10.0 \text{ g}_{\text{solvent}} \cdot \text{g}^{-1}_{\text{dry fibre}}$, for 7 hours at a temperature of 30° C. These operating conditions were validated at pilot scale, resulting in an extract with a ferulic acid concentration of $2.10 \pm 0.09 \text{ g} \cdot \text{L}^{-1}$ and a recovery yield of $11.14 \pm 1.00 \text{ Kg}_{\text{ferulic acid}} \cdot \text{Ton}^{-1}_{\text{dry corn fibre}}$.

A study was also carried out on enzymatic hydrolysis using several available commercial enzyme preparations. The best commercial enzyme preparation, Ultraflo® XL (2 % (w Ultraflo® XL · w⁻¹ dry corn fibre)) was only able to release about $7.83 \pm 1.35 \%$ (w_{recovery ferulic acid} · w⁻¹ total esterified ferulic acid). Hydro-thermal pre-treatments were applied to improve the ferulic acid recovery yield. Pre-treatment at a temperature of 140° C for 40 minutes improved access for the Ultraflo® XL enzyme preparation and consequently improved the recovery yield of ferulic acid from $7.83 \pm 1.35 \%$ (w_{recovery ferulic acid} · w⁻¹ total esterified ferulic acid) to $28.94 \pm 2.4 \%$ (w_{recovery ferulic acid} · w⁻¹ total esterified ferulic acid), with the minimum concentration of undesirable compounds (furfural, hydroxymethylfurfural, organic acids). Although the hydrothermal pre-treatment made the corn fibre more accessible to enzymes and improved the yield of ferulic acid released, the results achieved by enzymatic hydrolysis using commercial enzyme preparations were inferior to the results achieved by mild alkaline extraction ($79.10 \pm 5.41 \%$ (w_{recovery ferulic acid} · w⁻¹ total esterified ferulic acid)).

The ferulic acid released by alkaline extraction was successfully separated from the arabinoxylans (added-value compounds with high viscosity) using the ultrafiltration Nadir® UP150 membrane. This membrane exhibited no rejection to ferulic acid, a high estimated rejection of arabinoxylans of $98.0 \pm 1.7 \%$ and the highest permeability among the membranes tested. A Response Surface Methodology (RSM) was used to infer the effect of ultrafiltration operating parameters (temperature, initial cross-flow velocity and transmembrane pressure) on the rejection of ferulic acid, the viscosity of retentate solution (related to the concentration of arabinoxylans) and permeability. It was concluded that the ultrafiltration conditions (in the range tested) do not influence the global rejection of ferulic acid. However, both the rejection of arabinoxylans and the permeability were influenced by the ultrafiltration conditions. The analysis of the mathematical models developed, designed to describe the apparent viscosity (retention of arabinoxylans) and the permeability behaviour, showed that it is possible to achieve optimal

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conditions for ultrafiltration using the Nadir® UP150 membrane at a temperature of 66° C, initial cross-flow velocity of 1.06 m.s⁻¹ and transmembrane pressure of 0.7 bar.

An adsorption process using the Macronet® MN102 resin allowed the elimination/reduction of compounds with an antimicrobial effect from the ultrafiltrated alkaline extract (the permeate of the ultrafiltration process). A study of the adsorption process (comprising the steps of adsorption of ferulic acid, rinse of contaminants from the resin and desorption of ferulic acid) in continuous columns allowed for determining the parameters for scaling up the process. The maximum ferulic acid adsorption capacity was 176 mg_{ferulic acid.g⁻¹ dry resin} at pH 4.5 and a flow rate of 5.23 ml.min⁻¹. The breakthrough point was at 115 minutes, at which point an adsorption capacity of 85 mg_{ferulic acid.g⁻¹ dry resin} was obtained. After the purification of ferulic acid, it was possible to produce bio-vanillin with the *Amycolatopsis* sp. ATCC 39116 bacteria using 10 g.L⁻¹ ferulic acid that had been extracted from corn fibre and then purified, confirming the elimination/reduction of compounds that are toxic to *Amycolatopsis* sp. ATCC 39116.

Bio-vanillin production with *Amycolatopsis* sp. ATCC 39116, using a model solution of ferulic acid as a precursor, was studied in first place with the aim of maximising vanillin productivity. It was concluded that the presence of glucose in the broth during the production phase avoids vanillin being converted to vanillic acid and, consequently, increases the overall vanillin productivity. A concentration of vanillin above 9-10 g.L⁻¹ was confirmed to be toxic to the bacteria. The use of a multiple pulse feeding strategy increased the vanillin volumetric productivity of the process by 64 % (0.46 g.L.h⁻¹), compared with a single pulse strategy (0.29 g.L.h⁻¹). Regarding the ferulic acid concentration, high concentrations (14.91 ± 0.29 g.L⁻¹) were shown to affect the vanillin production process, making the process slower. To sum up, this work demonstrated that it is possible to produce biotechnological-vanillin with *Amycolatopsis* sp. ATCC 39116, using a medium with 10 g.L⁻¹ of ferulic acid extracted from corn fibre, and through this process it was achieved one of the highest vanillin production yields yet reported for the bioconversion of ferulic acid extracted from agro-industrial by-products.

To obtain purified vanillin from the bioconversion broth, organophilic pervaporation and vacuum distillation processes were studied and compared. It was possible to obtain solid vanillin free from undesirable contaminants using either a pervaporation-fractionated condensation or a vacuum distillation-fractionated condensation configuration. Vacuum distillation with 18 consecutive pulses was preferred over pervaporation due to the higher vanillin fluxes obtained: 2.7±0.1 g.m⁻²h⁻¹ for vacuum distillation compared to 1.19±0.01 g.m⁻²h⁻¹ for pervaporation although the highest energy consumption 9.75 kJ.kg_{VAN}⁻¹ at 85° C and 4.91 kJ.kg_{VAN}⁻¹ at 75° C, respectively.

Concerning the economic assessment of the bio-vanillin plant, the payback period calculates is 0.9 years. The equipment base cost plays an important role in the total fixed investment cost, representing 24 % of the total fixed investment. From all equipment necessary to this overall bio-vanillin process, the equipment used in the bioconversion step (bioreactors) is the most important, representing 57 % of the

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total equipment base, followed by the equipment for vacuum distillation, representing 23 % of the equipment base. The parameters with a higher impact in the operating costs are the energy costs, which represent 39 % of the total operating costs. Through a sensitive analysis it was possible to conclude that the payback period is mostly sensitive to the selling price of vanillin.

7.2. Future Work

This PhD thesis proposes a process to produce bio-vanillin from ferulic acid, extracted from corn fibre. A mild alkaline extraction study was accomplished to recover ferulic acid from corn fibre. In this step, the extraction time should be optimised for raw corn fibre and controlled stirring conditions should be applied. This last parameter was studied with corn gluten feed under magnetic stirring, which did not allow the use of an extraction time shorter than 7 hours. The decrease of the extraction time will allow for a reduction of the equipment size.

Other processes or the combination of processes to recover ferulic acid from corn fibre should be studied, namely using enzymatic hydrolysis with other commercial preparations combined with other pre-treatments (such as mild alkaline extraction).

Regarding the purification step, further work is needed to optimise the membrane cleaning protocol in the process of separation of ferulic acid from arabinoxylans, by ultrafiltration. Membrane cleaning is an important procedure in membrane plants, aiming to find the best balance between flux recovery and extended life-time of the membranes. Specifically, membrane autopsy studies are required, in order to assess which compounds cause membrane fouling. The identification of the nature and amount of compounds that contribute to membrane fouling will help for the rationale design of efficient membrane cleaning protocols to restore membrane permeability, without compromising its life-time. The membranes could be cleaned *in-situ* by physical methods (forward flushing, reverse flushing, backwashing, vibrations, air sparge and CO₂ back permeation) and/or chemical methods (acid cleaners for removal of inorganic and/or ions compounds and alkaline cleaners for the removal bio/organic compounds).

In the purification of ferulic acid by adsorption, through a breakthrough curve, it was possible to calculate the adsorption capacity of the adsorbent. However, it is important to determine if the behaviour of the adsorbent is consistent in subsequent cycles. For this reason, when using several repeated cycles of adsorption, resin rinsing and desorption is recommended. These assays should be performed at pilot scale and only until the breakthrough point since in industrial applications the adsorption process stops at a breakthrough point. The effect of a pH below the *pKa* (4.58 [191]) of ferulic acid in the MacronetTM MN102 retention capacity should be evaluated as this work only tested down to pH 4.5.

In the bioconversion process the impact of decreasing the bioconversion temperature in the kinetic parameters and by-products production should be evaluated, since the use of a lower temperature turns

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the process more energetically sustainable. Other authors used temperatures below 45° C for vanillin bioproduction [3,85,86,211], however, in different operating conditions from those used in this work (e.g. different pH, ferulic acid concentration, basal medium, in shake flasks assays). Although using a different condition Ma and Daugulis (2014) [3] and Fleige et al. (2016) [84] obtained at 28° C, similar results of those obtained in this work.

In this work, the production of the by-product vanillic acid was studied since, at pH 8.2 (the pH used in this work), the microorganism has a higher tendency to oxidise vanillin into vanillic acid than to reduce vanillin into vanillyl alcohol [210]. The presence of vanillyl alcohol detected in the vanillin after purification can be desirable in low concentrations, since vanillyl alcohol is also present in botanical vanillin [2] and the company Rhodia (Solvay) produces a bio-vanillin with 3 % by weight of impurities, where the most abundant impurity is vanillyl alcohol [223]. However, if it is necessary to reduce the vanillyl alcohol formation from the vanillin the pH of the bioconversion should be increased to 8.5. Gunnarsson N, Palmqvist EA reported that at a pH of 8.5 the production of vanillyl alcohol is even lower than at a pH of 8.2 [210]. The bioconversion process was optimised in batch mode.

The final step of purification of vanillin by vacuum distillation with fractionated condensation was performed with a bioconversion broth using the commercial synthetic ferulic acid feed. Future work should consider this step with ferulic acid extracted from corn fibre, in order to validate this step of the proposed process. Although the ethanolic solution of ferulic acid that was desorbed from the resin was free from compounds that are toxins/inhibitors to *Amycolatopsis* sp. ATCC 39116, the solution may contain other compounds that may interfere with the vanillin purification process. The validation studies must be performed at pilot scale, optimising the effect of the vacuum distillation operating conditions (operation time, the evaporation area and the condensation area and residence time). As the purification of vanillin step represents 22 % of the total base equipment cost, an alternative, other more economic process to obtain pure vanillin should be considered. Alternative processes may include common pressure-driven membrane processes such as reverse osmosis, followed by crystallization of vanillin through the addition of sustainable solvents (according to REACH). One problem in the proposed bioconversion process is the residual effluents that are generated, specially from the adsorption column. One possible way to treat these effluents is to use reverse osmosis to reduce their volume. The permeate obtained (clean water) could be considered for reuse in the alkaline extraction, reducing the water footprint of the process. However, the concentrate, obtained from reverse osmosis, remains a problem, since this concentrate could not be sent to a water treatment unit due to its high COD, about 3.3 g_{O₂}.L⁻¹, when the maximum allowed COD is 1.5 g_{O₂}.L⁻¹.

This concentrate might be flocculated or evaporated to obtain a solid residue. Biological treatment is another possibility for dealing with these residual effluents, namely in anaerobic processes with the production of bio-gas from this waste which is rich in organic acids [251,253].

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Finally, the proposed bio-vanillin production process should be implemented at pilot scale to test and validate the operational conditions parameters and address any problems that may affect productivity and cost -effectiveness.

BIBLIOGRAPHY

BIBLIOGRAPHY

- [1] N.J. Gallage, B.L. Møller, Vanillin–Bioconversion and Bioengineering of the Most Popular Plant Flavor and Its De Novo Biosynthesis in the Vanilla Orchid, *Mol. Plant.* 8 (2015) 40–57. <https://doi.org/10.1016/j.molp.2014.11.008>.
- [2] S. Ramachandra Rao, G. a. Ravishankar, Vanilla flavour: production by conventional and biotechnological routes, *J. Sci. Food Agric.* 80 (2000) 289–304. [https://doi.org/10.1002/1097-0010\(200002\)80:3<289::AID-JSFA543>3.0.CO;2-2](https://doi.org/10.1002/1097-0010(200002)80:3<289::AID-JSFA543>3.0.CO;2-2).
- [3] X.K. Ma, A.J. Daugulis, Effect of bioconversion conditions on vanillin production by *Amycolatopsis* sp. ATCC 39116 through an analysis of competing by-product formation, *Bioprocess Biosyst. Eng.* 37 (2014) 891–899. <https://doi.org/10.1007/s00449-013-1060-x>.
- [4] E. Adam, S. Gaglione, A. Miiller, Oxidation behaviour of vanillin in dairy products, *Food Chem.* 60 (1997) 43–51. [https://doi.org/10.1016/S0308-8146\(96\)00299-3](https://doi.org/10.1016/S0308-8146(96)00299-3).
- [5] A.K. Eswaran, Fermentation-derived vanillin could be a sustainable natural alternative to synthetic vanillin, *Beroe.* (2017) 2–5. <https://www.beroeinc.com/article/fermentation-derived-vanillin/> (accessed February 5, 2020).
- [6] N.J. Walton, M.J. Mayer, A. Narbad, Vanillin, *Phytochemistry.* 63 (2003) 505–515. [https://doi.org/10.1016/S0031-9422\(03\)00149-3](https://doi.org/10.1016/S0031-9422(03)00149-3).
- [7] A.K. Sinha, U.K. Sharma, N. Sharma, A comprehensive review on vanilla flavor: Extraction, isolation and quantification of vanillin and others constituents, *Int. J. Food Sci. Nutr.* 59 (2008) 299–326. <https://doi.org/10.1080/09687630701539350>.
- [8] N.A. Zamzuri, S. Abd-Aziz, Biovanillin from agro wastes as an alternative food flavour, *J. Sci. Food Agric.* 93 (2013) 429–438. <https://doi.org/10.1002/jsfa.5962>.
- [9] M.M. Bomgardner, The problem with vanilla - After vowing to go natural, food brands face a shortage of the favored flavor, *Chem. Engineering News*, Vol. 94 Issue 36. (2016) pp.38-42.
- [10] R. Ciriminna, A. Fidalgo, F. Meneguzzo, F. Parrino, L.M. Ilharco, M. Pagliaro, Vanillin: The Case for Greener Production Driven by Sustainability Megatrend, *ChemistryOpen.* 8 (2019) 660–667. <https://doi.org/10.1002/open.201900083>.
- [11] M. Fache, B. Boutevin, S. Caillol, Vanillin Production from Lignin and Its Use as a Renewable Chemical, *ACS Sustain. Chem. Eng.* 4 (2016) 35–46. <https://doi.org/10.1021/acssuschemeng.5b01344>.
- [12] R. Kumar, P.K. Sharma, P.S. Mishra, A Review on the Vanillin derivatives showing various Biological activities, 4 (2012) 266–279. <https://doi.org/10.1002/chin.201228263>.
- [13] EUROPEAN_COMMISSION, Regulation (EC) No 1334/2008 of European Parliament and of the council, *Off. J. Eur. Union.* (2008) 34–50. <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32008R1334&qid=1552047523627&from=EN>.
- [14] FDA - U.S. Food & Drug Administration, Food Additive Status List, (n.d.). <http://www.fda.gov/food/ingredientpackaginglabeling/foodadditivesingredients/ucm091048.htm#ftnE> (accessed February 5, 2020).
- [15] U. Krings, R.G. Berger, Biotechnological production of flavours and fragrances, *Appl. Microbiol. Biotechnol.* 49 (1998) 1–8. <https://doi.org/10.1007/s002530051129>.
- [16] B. Kaur, D. Chakraborty, Biotechnological and Molecular Approaches for Vanillin Production : a Review, *Appl Biochem Biotechnol.* 169 (2013) 1353–1372. <https://doi.org/10.1007/s12010-012-0066-1>.
- [17] A. Braga, C. Guerreiro, I. Belo, Generation of Flavors and Fragrances Through Biotransformation and De Novo Synthesis, *Food Bioprocess Technol.* 11 (2018) 2217–2228. <https://doi.org/10.1007/s11947-018-2180-8>.

BIBLIOGRAPHY

-
- [18] D. Paterson, Vanilla: natural or not?, ChemEd NZ. (2010) 2–6. http://nzic.org.nz/chemed-nz/issue-archive/ChemEdNZ_Jan10_Paterson1.pdf.
 - [19] G.V. Research, Bio Vanillin Market Analysis, By Application and Segment Forecasts to 2024, (2016) 76. <https://www.grandviewresearch.com/industry-analysis/bio-vanillin-market> (accessed February 6, 2020).
 - [20] Marketwach, Bio Vanillin Market by Type , Top Key Players , Size , Share , Growth , Trends - Opportunity , Analysis , Forecast, (2019). <https://www.marketwatch.com/press-release/bio-vanillin-market-will-grow-at-130-cagr-to-hit-400-mn-by-2025-2019-10-09> (accessed February 7, 2020).
 - [21] F. Southey, Solvay triples, then doubles, natural vanillin capacity in France: “FMCGs are driving the push to natural,” News Anal. Food Beverage Dev. (2019). <https://www.foodnavigator.com/Article/2019/12/06/Solvay-doubles-natural-vanillin-capacity-in-France> (accessed February 7, 2020).
 - [22] Solvay, Rhovanil® Natural, 2014. https://www.solvay.jp/ja/binaries/RhovanilNatural_RegulationsFocus2014-179507.pdf.
 - [23] M. Chauve, S. Foucher, S. Galinant, G. Pireau, Improved Production of Vanillin by Fermentation, WO 2017/025339 A1, 2017. <https://patentimages.storage.googleapis.com/e5/42/ac/6cc9ac8f7dce9e/WO2017025339A1.pdf>.
 - [24] A.M. Rouhi, C. Washinton, Fine chemicals firms enable flavor and fragrance industry, Chem. Eng. News. 81 (2003) 54. <http://pubs.acs.org/cen/coverstory/8128/8128finechemicals2b.html>.
 - [25] Solvay, Press release Solvay announces price increase for vanillin and ethyl-vanillin across North America, (2017). https://www.solvay.us/en/binaries/Solvay_Aroma_price_increase_01.12.2017.Final-325702.pdf (accessed February 6, 2020).
 - [26] F. of the E. U.S, Synthetic Biology Vanillin: not natural, not sustainable, not likely to be labeled, and coming to an ice-cream cone near you, (2012) 1–4.
 - [27] D.M. Aromatics, Vanillin Natural, [http://www.demonchyaromatics.com/en/products/vanillin-natural-\(ex-ferulic-acid\)/](http://www.demonchyaromatics.com/en/products/vanillin-natural-(ex-ferulic-acid)/) (accessed May 2, 2020).
 - [28] H. Priefert, J. Rabenshorst, A. Steinbuchel, Biotechnological production of vanillin, Appl Microbiol Biotechnol. 56 (2001) 296–314. <https://doi.org/10.1007/s002530100687>.
 - [29] Q. Jin, L. Yang, N. Poe, H. Huang, Trends in Food Science & Technology Integrated processing of plant-derived waste to produce value-added products based on the biorefinery concept, Trends Food Sci. Technol. 74 (2018) 119–131. <https://doi.org/10.1016/j.tifs.2018.02.014>.
 - [30] American Association of cereal Chemistry, Corn Chemistry and Technology, 1987.
 - [31] Appreciate Goods, Corn (Maize): Health Benefits, Side Effects, Fun Facts, Nutrition Facts and History, (2017). <https://appreciategoods.com/corn-maize/%0D> (accessed February 10, 2020).
 - [32] D.J. Rose, G.E. Inglett, S.X. Liu, Utilisation of corn (Zea mays) bran and corn fiber in the production of food components, J. Sci. Food Agric. 90 (2010) 915–924. <https://doi.org/10.1002/jsfa.3915>.
 - [33] B.C. Saha, Hemicellulose bioconversion, J. Ind. Microbiol. Biotechnol. 30 (2003) 279–291. <https://doi.org/10.1007/s10295-003-0049-x>.
 - [34] D. Van Eylen, F. van Dongen, M. Kabel, J. de Bont, Corn fiber, cobs and stover: Enzyme-aided saccharification and co-fermentation after dilute acid pretreatment, Bioresour. Technol. 102 (2011) 5995–6004. <https://doi.org/10.1016/j.biortech.2011.02.049>.
 - [35] H.-D. Shin, S. McClendon, T. Le, F. Taylor, R.R. Chen, A complete enzymatic recovery of ferulic acid from corn residues with extracellular enzymes from *Neosartorya spinosa* NRRL185., Biotechnol. Bioeng. 95 (2006) 1108–15. <https://doi.org/10.1002/bit.21056>.

BIBLIOGRAPHY

-
- [36] D.E. Akin, L.L. Rigsby, Corn fiber: Structure, composition, and response to enzymes for fermentable sugars and coproducts, *Appl. Biochem. Biotechnol.* 144 (2008) 59–68. <https://doi.org/10.1007/s12010-007-0053-0>.
 - [37] S.M. Gadalkar, V.K. Rathod, Pre-treatment of ferulic acid esterases immobilized on MNPs to enhance the extraction of ferulic acid from defatted rice bran in presence of ultrasound, *Biocatal. Agric. Biotechnol.* 10 (2017) 342–351. <https://doi.org/10.1016/j.bcab.2017.03.016>.
 - [38] N. Shibuya, Phenolic acids and their carbohydrate esters in rice endosperm cell walls, *Phytochemistry*. 23 (1984) 2233–2237. [https://doi.org/10.1016/S0031-9422\(00\)80526-9](https://doi.org/10.1016/S0031-9422(00)80526-9).
 - [39] P. Yu, D.D. Maenz, J.J. McKinnon, V.J. Racz, D.A. Christensen, Release of Ferulic Acid from Oat Hulls by *Aspergillus* Ferulic Acid Esterase and *Trichoderma* Xylanase, *J. Agric. Food Chem.* 50 (2002) 1625–1630. <https://doi.org/10.1021/jf010984r>.
 - [40] L. Long, D. Ding, Z. Han, H. Zhao, Q. Lin, S. Ding, Thermotolerant hemicellulolytic and cellulolytic enzymes from *Eupenicillium parvum* 4-14 display high efficiency upon release of ferulic acid from wheat bran, *J. Appl. Microbiol.* 121 (2016) 422–434. <https://doi.org/10.1111/jam.13177>.
 - [41] E. Bonnin, L. Saulnier, M. Brunel, C. Marot, L. Lesage-meessen, M. Asther, J. Thibault, Release of ferulic acid from agroindustrial by-products by the cell wall-degrading enzymes produced by *Aspergillus niger* I-1472, *Enzyme Microb. Technol.* 31 (2002) 1000–1005. [https://doi.org/10.1016/S0141-0229\(02\)00236-3](https://doi.org/10.1016/S0141-0229(02)00236-3).
 - [42] F. Xu, R.C. Sun, J.X. Sun, C.F. Liu, B.H. He, J.S. Fan, Determination of cell wall ferulic and p-coumaric acids in sugarcane bagasse, *Anal. Chim. Acta.* 552 (2005) 207–217. <https://doi.org/10.1016/j.aca.2005.07.037>.
 - [43] P. Torre, B. Aliakbarian, B. Rivas, J.M. Domínguez, A. Converti, Release of ferulic acid from corn cobs by alkaline hydrolysis, *Biochem. Eng. J.* 40 (2008) 500–506. <https://doi.org/10.1016/j.bej.2008.02.005>.
 - [44] T.N. Linh, H. Fujita, A. Sakoda, Release kinetics of esterified p-coumaric acid and ferulic acid from rice straw in mild alkaline solution, *Bioresour. Technol.* 232 (2017) 192–203. <https://doi.org/10.1016/j.biortech.2017.02.009>.
 - [45] A. Tilay, M. Bule, J. Kishenkumar, U. Annature, Preparation of ferulic acid from agricultural wastes: Its improved extraction and purification, *J. Agric. Food Chem.* 56 (2008) 7644–7648. <https://doi.org/10.1021/jf801536t>.
 - [46] M.M. Appeldoorn, M.A. Kabel, D. Van Eylen, H. Gruppen, H.A. Schols, Characterization of oligomeric xylan structures from corn fiber resistant to pretreatment and simultaneous saccharification and fermentation, *J. Agric. Food Chem.* 58 (2010) 11294–11301. <https://doi.org/10.1021/jf102849x>.
 - [47] M.M. Appeldoorn, P. De Waard, M.A. Kabel, H. Gruppen, H.A. Schols, Enzyme resistant feruloylated xylooligomer analogues from thermochemically treated corn fiber contain large side chains, ethyl glycosides and novel sites of acetylation, *Carbohydr. Res.* 381 (2013) 33–42. <https://doi.org/10.1016/j.carres.2013.08.024>.
 - [48] S. Dupoirion, M.L. Lameloise, M. Pomet, O. Bennaceur, R. Lewandowski, F. Allais, A.R.S. Teixeira, C. Rémond, H. Rakotoarivonina, A novel and integrative process: From enzymatic fractionation of wheat bran with a hemicellulase cocktail to the recovery of ferulic acid by weak anion exchange resin, *Ind. Crops Prod.* 105 (2017) 148–155. <https://doi.org/10.1016/j.indcrop.2017.05.004>.
 - [49] N. Pérez-Rodríguez, C.D. Moreira, A. Torrado Agrasar, J.M. Domínguez, Feruloyl esterase production by *Aspergillus terreus* CECT 2808 and subsequent application to enzymatic hydrolysis, *Enzyme Microb. Technol.* 91 (2016) 52–58. <https://doi.org/10.1016/j.enzmictec.2016.05.011>.

BIBLIOGRAPHY

-
- [50] L. Saulnier, C. Marot, M. Elgorriaga, E. Bonnin, J. Thibault, Thermal and enzymatic treatments for the release of free ferulic acid from maize bran, *Carbohydr. Polym.* 45 (2001) 269–275. [https://doi.org/10.1016/S0144-8617\(00\)00259-9](https://doi.org/10.1016/S0144-8617(00)00259-9).
 - [51] P. Moniz, H. Pereira, L.C. Duarte, F. Carvalheiro, Hydrothermal production and gel filtration purification of xylo-oligosaccharides from rice straw, *Ind. Crop. Prod.* 62 (2014) 460–465. <https://doi.org/10.1016/j.indcrop.2014.09.020>.
 - [52] K. Jiang, L. Li, L. Long, S. Ding, Comprehensive evaluation of combining hydrothermal pretreatment (autohydrolysis) with enzymatic hydrolysis for efficient release of monosaccharides and ferulic acid from corn bran, *Ind. Crops Prod.* 113 (2018) 348–357. <https://doi.org/10.1016/j.indcrop.2018.01.047>.
 - [53] L. Matsakas, V. Raghavendran, O. Yakimenko, G. Persson, E. Olsson, U. Rova, L. Olsson, P. Christakopoulos, Lignin-first biomass fractionation using a hybrid organosolv – Steam explosion pretreatment technology improves the saccharification and fermentability of spruce biomass, *Bioresour. Technol.* 273 (2019) 521–528. <https://doi.org/10.1016/j.biortech.2018.11.055>.
 - [54] H. Yu, W. Xiao, L. Han, G. Huang, Characterization of mechanical pulverization/phosphoric acid pretreatment of corn stover for enzymatic hydrolysis, *Bioresour. Technol.* 282 (2019) 69–74. <https://doi.org/10.1016/j.biortech.2019.02.104>.
 - [55] T.H. Kim, Y.. Lee, Pretreatment of Corn Stover by Soaking in Aqueous Ammonia at Moderate Temperatures, *Appl. Biochem. Biotechnol.* 137 (2007) 81–92. <https://doi.org/10.1007/s12010-007-9041-7>.
 - [56] S. Zhao, S. Yao, S. Ou, J. Lin, Y. Wang, X. Peng, A. Li, B. Yu, Preparation of ferulic acid from corn bran: Its improved extraction and purification by membrane separation, *Food Bioprod. Process.* 92 (2014) 309–313. <https://doi.org/10.1016/j.fbp.2013.09.004>.
 - [57] J.L. Bauer, B. Harbaum-Piayda, K. Schwarz, Phenolic compounds from hydrolyzed and extracted fiber-rich by-products, *LWT - Food Sci. Technol.* 47 (2012) 246–254. <https://doi.org/10.1016/j.lwt.2012.01.012>.
 - [58] F.E. Ayala-Soto, S.O. Serna-Saldívar, J. Welti-Chanes, Effect of processing time, temperature and alkali concentration on yield extraction, structure and gelling properties of corn fiber arabinoxylans, *Food Hydrocoll.* 60 (2016) 21–28. <https://doi.org/10.1016/j.foodhyd.2016.03.014>.
 - [59] M. Aguedo, C. Fougnyes, M. Dermience, A. Richel, Extraction by three processes of arabinoxylans from wheat bran and characterization of the fractions obtained, *Carbohydr. Polym.* 105 (2014) 317–324. <https://doi.org/10.1016/j.carbpol.2014.01.096>.
 - [60] M.S. Kale, B.R. Hamaker, O.H. Campanella, Alkaline extraction conditions determine gelling properties of corn bran arabinoxylans, *Food Hydrocoll.* 31 (2013) 121–126. <https://doi.org/10.1016/j.foodhyd.2012.09.011>.
 - [61] J. Trček, N.P. Mira, L.R. Jarboe, Adaptation and tolerance of bacteria against acetic acid, *Appl. Microbiol. Biotechnol.* 99 (2015) 6215–6229. <https://doi.org/10.1007/s00253-015-6762-3>.
 - [62] A.J. Roe, C. O’Byrne, D. McLaggan, I.R. Booth, Inhibition of *Escherichia coli* growth by acetic acid: A problem with methionine biosynthesis and homocysteine toxicity, *Microbiology.* 148 (2002) 2215–2222. <https://doi.org/10.1099/00221287-148-7-2215>.
 - [63] A. El Baaboua, M. El Maadoudi, A. Bouyahya, O. Belmehdi, A. Kounnoun, R. Zahli, J. Abrini, Evaluation of Antimicrobial Activity of Four Organic Acids Used in Chicks Feed to Control *Salmonella typhimurium*: Suggestion of Amendment in the Search Standard, *Int. J. Microbiol.* 2018 (2018). <https://doi.org/10.1155/2018/7352593>.
 - [64] C.E. östling, S.E. Lindgren, Inhibition of enterobacteria and *Listeria* growth by lactic, acetic and formic acids, *J. Appl. Bacteriol.* 75 (1993) 18–24. <https://doi.org/10.1111/j.1365-2672.1993.tb03402.x>.

BIBLIOGRAPHY

- [65] L. Du, B. Li, J.F. Lopes-Filho, C.R. Daniels, S.R. Eckhoff, Effect of selected organic and inorganic acids on corn wet-milling yields, *Cereal Chem.* 73 (1996) 96–98.
- [66] S.R. Eckhoff, S.A. Watson, Corn and Sorghum Starches: Production, in: *Starch Chem. Technol.*, 3rd Editio, 2009: pp. 373–439. <https://doi.org/10.1016/B978-0-12-746275-2.00009-4>.
- [67] Y. Li, B. Qi, J. Luo, R. Khan, Y. Wan, Separation and concentration of hydroxycinnamic acids in alkaline hydrolyzate from rice straw by nanofiltration, *Sep. Purif. Technol.* 149 (2015) 315–321. <https://doi.org/10.1016/j.seppur.2015.06.006>.
- [68] A.U. Buranov, G. Mazza, Extraction and purification of ferulic acid from flax shives, wheat and corn bran by alkaline hydrolysis and pressurised solvents, *Food Chem.* 115 (2009) 1542–1548. <https://doi.org/10.1016/j.foodchem.2009.01.059>.
- [69] T. Llano, M. Alexandri, A. Koutinas, C. Gardeli, H. Papapostolou, A. Coz, N. Quijorna, A. Andres, M. Komaitis, Liquid–Liquid Extraction of Phenolic Compounds from Spent Sulphite Liquor, *Waste and Biomass Valorization*. 6 (2015) 1149–1159. <https://doi.org/10.1007/s12649-015-9425-9>.
- [70] S. Dupoirion, M.L. Lameloise, M. Bedu, R. Lewandowski, C. Fargues, F. Allais, A.R.S. Teixeira, H. Rakotoarivonina, C. Rémond, Recovering ferulic acid from wheat bran enzymatic hydrolysate by a novel and non-thermal process associating weak anion-exchange and electrodialysis, *Sep. Purif. Technol.* 200 (2018) 75–83. <https://doi.org/10.1016/j.seppur.2018.02.031>.
- [71] V. Simon, A. Thuret, L. Candy, S. Bassil, S. Duthen, C. Raynaud, A. Masseron, Recovery of hydroxycinnamic acids from renewable resources by adsorption on zeolites, *Chem. Eng. J.* 280 (2015) 748–754. <https://doi.org/10.1016/j.cej.2015.06.009>.
- [72] D. Couteau, P. Mathaly, Fixed-bed purification of ferulic acid from sugar-beet pulp using activated carbon: Optimization studies, *Bioresour. Technol.* 64 (1998) 17–25. [https://doi.org/10.1016/S0960-8524\(97\)00152-1](https://doi.org/10.1016/S0960-8524(97)00152-1).
- [73] J. Hou, S. Zhang, X. Zhang, S. Liu, Q. Zhang, Adsorption of ferulic acid from an alkali-pretreated hydrolysate using a new effective adsorbent prepared by a thermal processing method, *J. Hazard. Mater.* (2020). <https://doi.org/10.1016/j.jhazmat.2020.122281>.
- [74] P. Gluszczyk, T. Jamroz, B. Sencio, S. Ledakowicz, Equilibrium and dynamic investigations of organic acids adsorption onto ion-exchange resins, *Bioprocess Biosyst. Eng.* 26 (2004) 185–190. <https://doi.org/10.1007/s00449-003-0348-7>.
- [75] C. Fargues, R. Lewandowski, M.L. Lameloise, Evaluation of ion-exchange and adsorbent resins for the detoxification of beet distillery effluents, *Ind. Eng. Chem. Res.* 49 (2010) 9248–9257. <https://doi.org/10.1021/ie100330y>.
- [76] M. Mendis, S. Simsek, Arabinoxylans and human health, *Food Hydrocoll.* 42 (2014) 239–243. <https://doi.org/10.1016/j.foodhyd.2013.07.022>.
- [77] C. Péroval, F. Debeaufort, D. Despré, A. Voilley, Edible arabinoxylan-based films. 1. Effects of lipid type on water vapor permeability, film structure, and other physical characteristics, *J. Agric. Food Chem.* 50 (2002) 3977–3983. <https://doi.org/10.1021/jf0116449>.
- [78] J. Cirre, S. Al-Assaf, G.O. Phillips, M.P. Yadav, K.B. Hicks, Improved emulsification performance of corn fibre gum following maturation treatment, *Food Hydrocoll.* 35 (2014) 122–128. <https://doi.org/10.1016/j.foodhyd.2013.05.002>.
- [79] L. Jacquemin, A. Mogni, R. Zeitoun, C. Guinot, C. Sablayrolles, L. Saulnier, P.Y. Pontalier, Performance evaluation of a semi-industrial production process of arabinoxylans from wheat bran, *Process Biochem.* 50 (2015) 605–613. <https://doi.org/10.1016/j.procbio.2015.01.015>.
- [80] M.P. Yadav, M.L. Fishman, H.K. Chau, D.B. Johnston, K.B. Hicks, Molecular characteristics of corn fiber gum and their influence on CFG emulsifying properties, *Cereal Chem.* 84 (2007) 175–180. <https://doi.org/10.1094/CCHEM-84-2-0175>.

BIBLIOGRAPHY

- [81] S. Liu, B. Wang, Purification of ferulic acid from wheat by ultrafiltration technology, *Adv. Mater. Res.* 527 (2012) 2294–2297. <https://doi.org/10.4028/www.scientific.net/AMR.524-527.2294>.
- [82] C. Fleige, G. Hansen, J. Kroll, A. Steinbüchel, Investigation of the *Amycolatopsis* sp. strain ATCC 39116 vanillin dehydrogenase and its impact on the biotechnical production of vanillin., *Appl. Environ. Microbiol.* 79 (2013) 81–90. <https://doi.org/10.1128/AEM.02358-12>.
- [83] A. Muheim, K. Lerch, Towards a high-yield bioconversion of ferulic acid to vanillin, *Appl. Microbiol. Biotechnol.* 51 (1999) 456–461. <https://doi.org/10.1007/s002530051416>.
- [84] C. Fleige, F. Meyer, Metabolic Engineering of the Actinomycete *Amycolatopsis* sp. Strain ATCC 39116 towards Enhanced Production of Natural Vanillin, *Appl. Environ. Microbiol.* 82 (2016) 3410–3419. <https://doi.org/10.1128/AEM.00802-16>.
- [85] N. Pérez-Rodríguez, R. Pinheiro de Souza Oliveira, A.M. Torrado Agrasar, J.M. Domínguez, Ferulic acid transformation into the main vanilla aroma compounds by *Amycolatopsis* sp. ATCC 39116, *Appl. Microbiol. Biotechnol.* 100 (2016) 1677–1689. <https://doi.org/10.1007/s00253-015-7005-3>.
- [86] X.K. Ma, A.J. Daugulis, Transformation of ferulic acid to vanillin using a fed-batch solid-liquid two-phase partitioning bioreactor, *Biotechnol. Prog.* 30 (2014) 207–214. <https://doi.org/10.1002/btpr.1830>.
- [87] D. Hua, C. Ma, L. Song, S. Lin, Z. Zhang, Z. Deng, P. Xu, Enhanced vanillin production from ferulic acid using adsorbent resin, *Appl. Microbiol. Biotechnol.* 74 (2007) 783–790. <https://doi.org/10.1007/s00253-006-0735-5>.
- [88] A. Converti, B. Aliakbarian, J. Domínguez, G.B. Vázquez, P. Perego, Microbial production of biovanillin, *Brazilian J. Microbiol.* 41 (2010) 519–530. <https://doi.org/10.1590/S1517-83822010000300001>.
- [89] G. Clark, Vanillin, *Perfum Flavor.* 15 (1990) 45–54.
- [90] C. Stentelaire, L. Lesage-Meessen, M. Delattre, M. Haon, J.C. Sigoillot, B. Colonna Ceccaldi, M. Asther, By-passing of unwanted vanillyl alcohol formation using selective adsorbents to improve vanillin production with *Phanerochaete chrysosporium*, *World J. Microbiol. Biotechnol.* 14 (1997) 285–287. <https://doi.org/10.1023/A:1008811019693>.
- [91] Q.-F. Zhang, Z.-T. Jiang, H.-J. Gao, R. Li, Recovery of vanillin from aqueous solutions using macroporous adsorption resins, *Eur. Food Res. Technol.* 226 (2008) 377–383. <https://doi.org/10.1007/s00217-006-0548-x>.
- [92] C. Michailof, G.G. Stavropoulos, C. Panayiotou, Enhanced adsorption of phenolic compounds, commonly encountered in olive mill wastewaters, on olive husk derived activated carbons, *Bioresour. Technol.* 99 (2008) 6400–6408. <https://doi.org/10.1016/j.biortech.2007.11.057>.
- [93] D. Richard, M. de L. Delgado Núñez, D. Schweich, Adsorption of complex phenolic compounds on active charcoal: Adsorption capacity and isotherms, *Chem. Eng. J.* 148 (2009) 1–7. <https://doi.org/10.1016/j.cej.2008.07.023>.
- [94] S. Ou, Y. Luo, F. Xue, C. Huang, N. Zhang, Z. Liu, Separation and purification of ferulic acid in alkaline-hydrolysate from sugarcane bagasse by activated charcoal adsorption / anion macroporous resin exchange chromatography, 78 (2007) 1298–1304. <https://doi.org/10.1016/j.jfoodeng.2005.12.037>.
- [95] C. Brazinha, D.S. Barbosa, J.G. Crespo, Sustainable recovery of pure natural vanillin from fermentation media in a single pervaporation step, *Green Chem.* 13 (2011) 2197–2203. <https://doi.org/10.1039/c1gc15308k>.
- [96] L. Sciubba, D. Di Gioia, F. Fava, C. Gostoli, Membrane-based solvent extraction of vanillin in hollow fiber contactors, *Desalination.* 241 (2009) 357–364. <https://doi.org/10.1016/j.desal.2007.10.104>.

BIBLIOGRAPHY

-
- [97] C. Zidi, R. Tayeb, N. Boukhili, M. Dhahbi, A supported liquid membrane system for efficient extraction of vanillin from aqueous solutions, *Sep. Purif. Technol.* 82 (2011) 36–42. <https://doi.org/10.1016/j.seppur.2011.08.013>.
 - [98] Y.T. Wu, M. Feng, W.W. Ding, X.Y. Tang, Y.H. Zhong, Z.Y. Xiao, Preparation of vanillin by bioconversion in a silicon rubber membrane bioreactor, *Biochem. Eng. J.* 41 (2008) 193–197. <https://doi.org/10.1016/j.bej.2008.05.002>.
 - [99] G. De Brito Cardoso, T. Mourão, F.M. Pereira, M.G. Freire, A.T. Fricks, C.M.F. Soares, Á.S. Lima, Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin, *Sep. Purif. Technol.* 104 (2013) 106–113. <https://doi.org/10.1016/j.seppur.2012.11.001>.
 - [100] K.W. Böddeker, I.L. Gatfield, J. Jähnig, C. Schorm, Pervaporation at the vapor pressure limit: Vanillin, *J. Memb. Sci.* 137 (1997) 155–158. [https://doi.org/10.1016/S0376-7388\(97\)00187-7](https://doi.org/10.1016/S0376-7388(97)00187-7).
 - [101] G. Camera-Roda, V. Augugliaro, A. Cardillo, V. Loddo, G. Palmisano, L. Palmisano, A pervaporation photocatalytic reactor for the green synthesis of vanillin, *Chem. Eng. J.* 224 (2013) 136–143. <https://doi.org/10.1016/j.cej.2012.10.037>.
 - [102] M. Srinivasan, A.R. Sudheer, V.P. Menon, Ferulic Acid: Therapeutic Potential Through Its Antioxidant Property, *J. Clin. Biochem. Nutr.* 40 (2007) 92–100. <https://doi.org/10.3164/jcbtn.40.92>.
 - [103] V. Staniforth, W.C. Huang, K. Aravindaram, N.S. Yang, Ferulic acid, a phenolic phytochemical, inhibits UVB-induced matrix metalloproteinases in mouse skin via posttranslational mechanisms, *J. Nutr. Biochem.* 23 (2012) 443–451. <https://doi.org/10.1016/j.jnutbio.2011.01.009>.
 - [104] Ferulic Acid Market: Global Industry Analysis and Opportunity Assessment 2016-2026, (n.d.). <https://www.futuremarketinsights.com/reports/ferulic-acid-market> (accessed March 3, 2020).
 - [105] S. Ou, K.C. Kwok, Ferulic acid: Pharmaceutical functions, preparation and applications in foods, *J. Sci. Food Agric.* 84 (2004) 1261–1269. <https://doi.org/10.1002/jsfa.1873>.
 - [106] M. ElKhazendar, J. Chalak, W. El-Huneidi, A. Vinod, W.M. Abdel-Rahman, E. Abu-Gharbieh, Antiproliferative and proapoptotic activities of ferulic acid in breast and liver cancer cell lines, *Trop. J. Pharm. Res.* 18 (2019) 2571–2576. <https://doi.org/10.4314/tjpr.v18i12.16>.
 - [107] S. Mathew, T.E. Abraham, Characterisation of ferulic acid incorporated starch–chitosan blend films, *Food Hydrocoll.* 22 (2008) 826–835. <https://doi.org/10.1016/j.foodhyd.2007.03.012>.
 - [108] N. Cao, Y. Fu, J. He, Mechanical properties of gelatin films cross-linked, respectively, by ferulic acid and tannin acid, *Food Hydrocoll.* 21 (2007) 575–584. <https://doi.org/10.1016/j.foodhyd.2006.07.001>.
 - [109] A.R. and Consulting, Ferulic Acid Market Global Worth US \$ 26 Million By 2025 : Acumen Research and Consulting, (2019). <https://www.globenewswire.com/news-release/2019/01/14/1690865/0/en/Ferulic-Acid-Market-Global-Worth-US-26-Million-By-2025-Acumen-Research-and-Consulting.html> (accessed March 3, 2020).
 - [110] A.R.L. Damásio, C. Márcio, P. Braga, F. Mandelli, J. Cota, R.F. De Almeida, V.H. Salvador, D. Antonio, A. Paixao, F. Segato, A.Z. Mercadante, M.D.O. Neto, Biomass-to-bio-products application of feruloyl esterase from *Aspergillus clavatus*, *Biotechnologically Relev. Enzym. Proteins.* 97 (2013) 6759–6767. <https://doi.org/10.1007/s00253-012-4548-4>.
 - [111] M. Tenkanen, J. Schuseil, J. Puls, K. Poutanen, Production, purification and characterization of an esterase liberating phenolic acids from lignocellulosics, *J. Biotechnol.* 18 (1991) 69–83. [https://doi.org/10.1016/0168-1656\(91\)90236-O](https://doi.org/10.1016/0168-1656(91)90236-O).
 - [112] M. Uraji, M. Kimura, Y. Inoue, K. Kawakami, Y. Kumagai, K. Harazono, T. Hatanaka, Enzymatic production of ferulic acid from defatted rice bran by using a combination of bacterial enzymes, *Appl. Biochem. Biotechnol.* 171 (2013) 1085–1093. <https://doi.org/10.1007/s12010->

BIBLIOGRAPHY

- 013-0190-6.
- [113] F. He, S. Zhang, X. Liu, Immobilization of feruloyl esterases on magnetic nanoparticles and its potential in production of ferulic acid, *J. Biosci. Bioeng.* 120 (2015) 330–334. <https://doi.org/10.1016/j.jbiosc.2015.01.006>.
 - [114] K. Kim, R. Tsao, R. Yang, S.W. Cui, Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions, *Food Chem.* 95 (2006) 466–473. <https://doi.org/10.1016/j.foodchem.2005.01.032>.
 - [115] B.A. Acosta-estrada, J.A. Gutiérrez-uribe, S.O. Serna-saldívar, Bound phenolics in foods , a review, *FOOD Chem.* 152 (2014) 46–55. <https://doi.org/10.1016/j.foodchem.2013.11.093>.
 - [116] L. Saulnier, C. Marot, E. Chanliaud, J.F. Thibault, Cell wall polysaccharide interactions in maize bran, *Carbohydr. Polym.* 26 (1995) 279–287. [https://doi.org/10.1016/0144-8617\(95\)00020-8](https://doi.org/10.1016/0144-8617(95)00020-8).
 - [117] K. Iiyama, T.B.T. Lam, B.A. Stone, Phenolic acid bridges between polysaccharides and lignin in wheat internodes, *Phytochemistry.* 29 (1990) 733–737. [https://doi.org/10.1016/0031-9422\(90\)80009-6](https://doi.org/10.1016/0031-9422(90)80009-6).
 - [118] J.L. Bauer, B. Harbaum-piayda, K. Schwarz, Phenolic compounds from hydrolyzed and extracted fiber-rich by-products, *LWT - Food Sci. Technol.* 47 (2012) 246–254. <https://doi.org/10.1016/j.lwt.2012.01.012>.
 - [119] A. Aarabi, M. Honarvar, M. Mizani, F. Faghihian, A. Gerami, Extraction and Purification of ferulic acid as an antioxidant from sugar beet pulp by alkaline hydrolysis, *Ital. J. Food Sci.* 28 (2016) 362–375. <https://doi.org/10.14674/1120-1770/IJFS.V143>.
 - [120] P. Ideia, I. Sousa-Ferreira, P. C. Castilho, A Novel and Simpler Alkaline Hydrolysis Methodology for Extraction of Ferulic Acid from Brewer's Spent Grain and its (Partial) Purification through Adsorption in a Synthetic Resin, *Foods.* 9 (2020) 600. <https://doi.org/10.3390/foods9050600>.
 - [121] E.P.A. Cantillo-Pérez, N., Villarraga-Palencia, F., González Delgado, A., Ojeda Delgado, K, Sánchez Tuiran, Ferulic Acid Production from Agroindustrial Waste of Barley Malting Process, *Int. J. ChemTech Res.* 10 (2017) 226–232.
 - [122] S.I. Mussatto, G. Dragone, I.C. Roberto, Ferulic and p-coumaric acids extraction by alkaline hydrolysis of brewer's spent grain, *Ind. Crops Prod.* 25 (2007) 231–237. <https://doi.org/10.1016/j.indcrop.2006.11.001>.
 - [123] K. Jiang, L. Li, L. Long, S. Ding, Comparison of alkali treatments for efficient release of p-coumaric acid and enzymatic saccharification of sorghum pith, *Bioresour. Technol.* 207 (2016) 1–10. <https://doi.org/10.1016/j.biortech.2016.01.116>.
 - [124] D. Bender, M. Schmatz, S. Novalin, R. Nemeth, F. Chrysanthopoulou, S. Tömösközi, K. Török, R. Schoenlechner, S. D'Amico, Chemical and rheological characterization of arabinoxylan isolates from rye bran, *Chem. Biol. Technol. Agric.* 4 (2017) 1–8. <https://doi.org/10.1186/s40538-017-0096-6>.
 - [125] L. Saulnier, P.-E. Sado, G. Branlard, G. Charmet, F. Guillon, Wheat arabinoxylans: Exploiting variation in amount and composition to develop enhanced varieties, *J. Cereal Sci.* 46 (2007) 261–281. <https://doi.org/10.1016/j.jcs.2007.06.014>.
 - [126] K. Slinkard, V.L. Singleton, Total Phenol Analysis: Automation and Comparison with Manual Methods, *Am J Enol Vitic.* 28 (1977) 49–55.
 - [127] S. Rezazi, S. Abdelmalek, S. Hanini, Kinetic Study and Optimization of Extraction Process Conditions, *Energy Procedia.* 139 (2017) 98–104. <https://doi.org/10.1016/j.egypro.2017.11.180>.
 - [128] E. Prasad, *Nutraceuticals Market*, (2016). <https://www.alliedmarketresearch.com/nutraceuticals-market> (accessed May 20, 2020).
 - [129] Solvay, Solvay announces price increase for vanillin and ethyl-vanillin across North America,

BIBLIOGRAPHY

- (2017). https://www.solvay.us/en/binaries/Solvay_Aroma_price_increase_01.12.2017.Final-325702.pdf (accessed February 6, 2020).
- [130] Statista, Grain production worldwide by type, Shahbandeh,M. (2019). <https://www.statista.com/statistics/263977/world-grain-production-by-type/> (accessed February 10, 2020).
- [131] M. Jeffrey, Z. Cheng, L. Su, L. Yu, Effects of Solid-State Enzymatic Treatments on the Antioxidant Properties of Wheat Bran, *J. Agric. Food Chem.* 54 (2006) 9032–9045. <https://doi.org/10.1021/jf0616715>.
- [132] P. Leite, C. Silva, J.M. Salgado, I. Belo, Simultaneous production of lignocellulolytic enzymes and extraction of antioxidant compounds by solid-state fermentation of agro-industrial wastes, *Ind. Crops Prod.* 137 (2019) 315–322. <https://doi.org/10.1016/j.indcrop.2019.04.044>.
- [133] M. Ferri, A. Happel, G. Zanaroli, M. Bertolini, S. Chiesa, M. Commisso, F. Guzzo, A. Tassoni, Advances in combined enzymatic extraction of ferulic acid from wheat bran, *N. Biotechnol.* 56 (2020) 38–45. <https://doi.org/10.1016/j.nbt.2019.10.010>.
- [134] P.A. Kroon, M.T. Garcia-Conesa, I.J. Fillingham, G.P. Hazlewood, G. Williamson, Release of ferulic acid dehydrodimers from plant cell walls by feruloyl esterases, *J. Sci. Food Agric.* 79 (1999) 428–434. [https://doi.org/10.1002/\(SICI\)1097-0010\(19990301\)79:3<428::AID-JSFA275>3.0.CO;2-J](https://doi.org/10.1002/(SICI)1097-0010(19990301)79:3<428::AID-JSFA275>3.0.CO;2-J).
- [135] H.R. Sørensen, S. Pedersen, A. Viksø-Nielsen, A.S. Meyer, Efficiencies of designed enzyme combinations in releasing arabinose and xylose from wheat arabinoxylan in an industrial ethanol fermentation residue, *Enzyme Microb. Technol.* 36 (2005) 773–784. <https://doi.org/10.1016/j.enzmtec.2005.01.007>.
- [136] N. Azahanim Khamis, S. Shamsudin, N. Siha Abd Rahman, K. Farihan Kasim, Effects of autohydrolysis on rice biomass for reducing sugars production, *Mater. Today Proc.* 16 (2019) 2078–2087. <https://doi.org/10.1016/j.matpr.2019.06.095>.
- [137] B.E.L. Baêta, D.R.S. Lima, O.F.H. Adarme, L.V.A. Gurgel, S.F. de Aquino, Optimization of sugarcane bagasse autohydrolysis for methane production from hemicellulose hydrolyzates in a biorefinery concept, *Bioresour. Technol.* 200 (2016) 137–146. <https://doi.org/10.1016/j.biortech.2015.10.003>.
- [138] W. Dessie, F. Xin, W. Zhang, J. Zhou, H. Wu, J. Ma, M. Jiang, Inhibitory effects of lignocellulose pretreatment degradation products (hydroxymethylfurfural and furfural) on succinic acid producing *Actinobacillus succinogenes*, *Biochem. Eng. J.* 150 (2019). <https://doi.org/10.1016/j.bej.2019.107263>.
- [139] Novozyme A/S, Faster throughput and more extract, (2003). <https://shop.brewcraftusa.com/img/product/description/Ultraflo.pdf>.
- [140] Novozymes, Efficient wort separation and beer filtration, 2017.
- [141] R.P. Overend, E. Chornet, J.A. Gascoigne, Fractionation of Lignocellulosics by Steam-Aqueous Pretreatments [and Discussion], *Philos. Trans. R. Soc.* 321 (1987) 523–536. <https://doi.org/10.1098/rsta.1987.0029>.
- [142] A.T. Serra, R.O. Duarte, M.R. Bronze, C.M.M. Duarte, Identification of bioactive response in traditional cherries from Portugal, *Food Chem.* 125 (2011) 318–325. <https://doi.org/10.1016/j.foodchem.2010.07.088>.
- [143] G. Cano-Sancho, C.A. González-Arias, A.J. Ramos, V. Sanchis, M.L. Fernández-Cruz, Cytotoxicity of the mycotoxins deoxynivalenol and ochratoxin A on Caco-2 cell line in presence of resveratrol, *Toxicol. Vitro.* 29 (2015) 1639–1646. <https://doi.org/10.1016/j.tiv.2015.06.020>.
- [144] L. Rodrigues, I. Silva, J. Poejo, A.T. Serra, A.A. Matias, A.L. Simplício, M.R. Bronze, C.M.M. Duarte, Recovery of antioxidant and antiproliferative compounds from watercress using pressurized fluid extraction, *RSC Adv.* 6 (2016) 30905–30918.

BIBLIOGRAPHY

- <https://doi.org/10.1039/c5ra28068k>.
- [145] L.P. Pereira, P. Silva, M. Duarte, L. Rodrigues, C.M.M. Duarte, C. Albuquerque, A.T. Serra, Targeting colorectal cancer proliferation, stemness and metastatic potential using *Brassicaceae* extracts enriched in isothiocyanates: A 3D cell model-based study, *Nutrients*. 9 (2017) 1–26. <https://doi.org/10.3390/nu9040368>.
- [146] N. Pérez-Rodríguez, A. Torrado Agrasar, J.M. Domínguez, High hydrostatic pressure as pretreatment and adjuvant for the enzymatic release of ferulic acid from corn cob, *Process Biochem.* 58 (2017) 204–210. <https://doi.org/10.1016/j.procbio.2017.04.011>.
- [147] D. Szwajgier, J. Pielecki, Z. Targoński, The Release of Ferulic Acid and Feruloylated Oligosaccharides During Wort and Beer Production, *J. Inst. Brew.* 111 (2005) 372–379. <https://doi.org/10.1002/j.2050-0416.2005.tb00222.x>.
- [148] E. Dornez, K. Gebruers, J.A. Delcour, C.M. Courtin, Grain-associated xylanases: occurrence, variability, and implications for cereal processing, *Trends Food Sci. Technol.* 20 (2009) 495–510. <https://doi.org/10.1016/j.tifs.2009.05.004>.
- [149] D. Dodd, I.O. Cann, Enzymatic deconstruction of xylan for biofuel production, *GCB Bioenergy*. 1 (2009) 2–17. <https://doi.org/10.1111/j.1757-1707.2009.01004.x>.
- [150] E. Palmqvist, B. Hahn-Hägerdal, Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition, *Bioresour. Technol.* 74 (2000) 25–33. [https://doi.org/10.1016/S0960-8524\(99\)00161-3](https://doi.org/10.1016/S0960-8524(99)00161-3).
- [151] F. Carvalheiro, M.P. Esteves, J. Parajó, H. Pereira, F. Gírio, Production of oligosaccharides by autohydrolysis of brewery's spent grain, *Bioresour. Technol.* (2004) 93–100. [https://doi.org/10.1016/S0960-8524\(03\)00148-2](https://doi.org/10.1016/S0960-8524(03)00148-2).
- [152] L.W. Doner, K.B. Hicks, Isolation of Hemicellulose from Corn Fiber by Alkaline Hydrogen Peroxide Extraction, *Cereal Chem.* 74 (1997) 176–181. [https://doi.org/10.1016/S0034-6267\(97\)00016-1](https://doi.org/10.1016/S0034-6267(97)00016-1).
- [153] W. Fiddler, W.E. Parker, A.E. Wasserman, R.C. Doerr, Thermal Decomposition of Ferulic Acid, *J. Agric. Food Chem.* 15 (1967) 757–761. <https://doi.org/10.1021/jf60153a003>.
- [154] C. Fabian, N.Y. Tran-Thi, N.S. Kasim, Y.-H. Ju, Release of phenolic acids from defatted rice bran by subcritical water treatment, *J. Sci. Food Agric.* 90 (2010) 2576–2581. <https://doi.org/10.1002/jsfa.4123>.
- [155] N. Kumar, V. Pruthi, Potential applications of ferulic acid from natural sources, *Biotechnol. Reports*. 4 (2014) 86–93. <https://doi.org/10.1016/j.btre.2014.09.002>.
- [156] I. Kiliç, Y. Yeşiloğlu, Spectroscopic studies on the antioxidant activity of p-coumaric acid, *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* 115 (2013) 719–724. <https://doi.org/10.1016/j.saa.2013.06.110>.
- [157] S. Boonma, W. Rangsee, S. Chaiklangmuang, Effect of hydrothermal pre-treatment on ferulic acid content and antioxidant activities of corn hydrolysate, *Japan J. Food Eng.* 19 (2018) 27–34. <https://doi.org/10.11301/jsfe.17500>.
- [158] L. De Souza Rosa, N.A. Jordão, N. Da Costa Pereira Soares, J.F. De Mesquita, M. Monteiro, A.J. Teodoro, Pharmacokinetic, antiproliferative and apoptotic effects of phenolic acids in human colon adenocarcinoma cells using in vitro and in silico approaches, *Molecules*. 23 (2018). <https://doi.org/10.3390/molecules23102569>.
- [159] S. Qian, J. Zhou, X. Chen, W. Ji, L. Zhang, W. Hu, Z. Lu, Evaluation of an efficient fed-batch enzymatic hydrolysis strategy to improve production of functional xylooligosaccharides from maize straws, *Ind. Crops Prod.* 157 (2020) 112920. <https://doi.org/10.1016/j.indcrop.2020.112920>.
- [160] K. Sharma, S. Morla, K.C. Khaire, A. Thakur, V.S. Moholkar, S. Kumar, A. Goyal, Extraction,

BIBLIOGRAPHY

- characterization of xylan from *Azadirachta indica* (neem) sawdust and production of antiproliferative xylooligosaccharides, *Int. J. Biol. Macromol.* 163 (2020) 1897–1907. <https://doi.org/10.1016/j.ijbiomac.2020.09.086>.
- [161] M.P. Yadav, R. a. Moreau, K.B. Hicks, Phenolic acids, lipids, and proteins associated with purified corn fiber arabinoxylans, *J. Agric. Food Chem.* 55 (2007) 943–947. <https://doi.org/10.1021/jf0624493>.
- [162] Z.X. Lu, K.Z. Walker, J.G. Muir, K.O. Dea, Arabinoxylan fibre improves metabolic control in people with Type II diabetes, *Eur. J. Clin. Nutr.* 4 (2004) 621–628. <https://doi.org/10.1038/sj.ejcn.1601857>.
- [163] D.J. Rose, J.A. Patterson, B.R. Hamaker, Structural differences among alkali-soluble arabinoxylans from Maize (*Zea mays*), Rice (*Oryza sativa*), and Wheat (*Triticum aestivum*) brans influence human fecal fermentation profiles, *J. Agric. Food Chem.* 58 (2010) 493–499. <https://doi.org/10.1021/jf9020416>.
- [164] M. Serra, V. Weng, I.M. Coelho, V.D. Alves, C. Brazinha, Purification of arabinoxylans from corn fiber and preparation of bioactive films for food packaging, *Membranes (Basel)*. 10 (2020) 1–22. <https://doi.org/10.3390/membranes10050095>.
- [165] J. Jorda, P. Marechal, L. Rigal, P.Y. Pontalier, Biopolymer purification by ultrafiltration, *Desalination*. 148 (2002) 187–191. [https://doi.org/10.1016/S0011-9164\(02\)00696-3](https://doi.org/10.1016/S0011-9164(02)00696-3).
- [166] H. Krawczyk, T. Persson, A. Andersson, A.S. Jönsson, Isolation of hemicelluloses from barley husks, *Food Bioprod. Process.* 86 (2008) 31–36. <https://doi.org/10.1016/j.fbp.2007.10.018>.
- [167] L. Jacquemin, R. Zeitoun, C. Sablayrolles, P.Y. Pontalier, L. Rigal, Evaluation of the technical and environmental performances of extraction and purification processes of arabinoxylans from wheat straw and bran, *Process Biochem.* 47 (2012) 373–380. <https://doi.org/10.1016/j.procbio.2011.10.025>.
- [168] T. Kikhavani, S.N. Ashrafzadeh, B. Van Der Bruggen, Identification of optimum synthesis conditions for a novel anion exchange membrane by response surface methodology, *J. Appl. Polym. Sci.* 131 (2014) 1–16. <https://doi.org/10.1002/app.39888>.
- [169] M. Pessoa Lopes, F.C. Galinha, J.G. Crespo, S. Velizarov, Optimisation of arsenate removal from water by an integrated ion-exchange membrane process coupled with Fe co-precipitation, *Sep. Purif. Technol.* 246 (2020). <https://doi.org/https://doi.org/10.1016/j.seppur.2020.116894>.
- [170] E. Carvajal-Millan, V. Landillon, M.H. Morel, X. Rouau, J.L. Doublier, V. Micard, Arabinoxylan gels: Impact of the feruloylation degree on their structure and properties, *Biomacromolecules*. 6 (2005) 309–317. <https://doi.org/10.1021/bm049629a>.
- [171] S.D. Analytics, User guide to MODDE, (2017). [https://blog.umetrics.com/hubfs/DownloadFiles/MODDE 12.0.1 User Guide.pdf](https://blog.umetrics.com/hubfs/DownloadFiles/MODDE%2012.0.1%20User%20Guide.pdf).
- [172] V. V. Lozovaya, T.A. Gorshkova, E. V. Yablokova, N.I. Rumyantseva, A. Valieva, A. Ulanov, J.M. Widholm, Cold alkali can extract phenolic acids that are ether linked to cell wall components in dicotyledonous plants (buckwheat, soybean and flax), *Phytochemistry*. 50 (1998) 395–400. [https://doi.org/10.1016/S0031-9422\(98\)00575-5](https://doi.org/10.1016/S0031-9422(98)00575-5).
- [173] J.A. Lemire, J.J. Harrison, R.J. Turner, Antimicrobial activity of metals: Mechanisms, molecular targets and applications, *Nat. Rev. Microbiol.* 11 (2013) 371–384. <https://doi.org/10.1038/nrmicro3028>.
- [174] G. Bisignano, M.G. Laganà, D. Trombetta, S. Arena, A. Nostro, N. Uccella, G. Mazzanti, A. Saija, In vitro antibacterial activity of some aliphatic aldehydes from *Olea europaea* L., *FEMS Microbiol. Lett.* 198 (2001) 9–13. [https://doi.org/10.1016/S0378-1097\(01\)00089-1](https://doi.org/10.1016/S0378-1097(01)00089-1).
- [175] N. Chikthimmah, R.C. Anantheswaran, R.F. Roberts, E.W. Mills, S.J. Knabel, Influence of Sodium Chloride on Growth of Lactic Acid Bacteria and Subsequent Destruction of *Escherichia coli* O157: H7 during Processing of Lebanon Bologna, *J. Food Prot.* 64 (2001) 1145–1150.

BIBLIOGRAPHY

- <https://doi.org/10.4315/0362-028x-64.8.1145>.
- [176] K.M. Rath, A. Maheshwari, P. Bengtson, J. Rousk, Comparative Toxicities of Salts on Microbial Processes in Soil, *Appl. Environ. Microbiol.* 82 (2016) 2012–2020. <https://doi.org/10.1128/AEM.04052-15.Editor>.
 - [177] J. Hou, S. Zhang, X. Zhang, S. Liu, Q. Zhang, Adsorption of ferulic acid from an alkali-pretreated hydrolysate using a new effective adsorbent prepared by a thermal processing method, *J. Hazard. Mater.* 392 (2020) 122281. <https://doi.org/10.1016/j.jhazmat.2020.122281>.
 - [178] Q. Yang, M. Zhao, L. Lin, Adsorption and desorption characteristics of adlay bran free phenolics on macroporous resins, *Food Chem.* 194 (2016) 900–907. <https://doi.org/10.1016/j.foodchem.2015.08.070>.
 - [179] G. Jia, X. Lu, Enrichment and purification of madecassoside and asiaticoside from *Centella asiatica* extracts with macroporous resins, *J. Chromatogr. A.* 1193 (2008) 136–141. <https://doi.org/10.1016/j.chroma.2008.04.024>.
 - [180] M.D. Saikia, Revisiting adsorption of biomolecules on polymeric resins, *Colloids Surfaces A Physicochem. Eng. Asp.* 315 (2008) 196–204. <https://doi.org/10.1016/j.colsurfa.2007.07.026>.
 - [181] D.R. Pompeu, F.G. Moura, E.M. Silva, H. Rogez, Equilibria, kinetics, and mechanisms for the adsorption of four classes of phenolic compounds onto synthetic resins, *Sep. Sci. Technol.* 45 (2010) 700–709. <https://doi.org/10.1080/01496390903562274>.
 - [182] D. Couteau, P. Mathaly, Purification of ferulic acid by adsorption after enzymic release from a sugar-beet pulp extract, *Ind. Crops Prod.* 6 (1997) 237–252. [https://doi.org/https://doi.org/10.1016/S0926-6690\(97\)00014-9](https://doi.org/https://doi.org/10.1016/S0926-6690(97)00014-9).
 - [183] N. Gopalan, K.M. Nampoothiri, Biorefining of wheat bran for the purification of ferulic acid, *Biocatal. Agric. Biotechnol.* 15 (2018) 304–310. <https://doi.org/10.1016/j.bcab.2018.07.004>.
 - [184] M. Sova, Antioxidant and antimicrobial activities of cinnamic acid derivatives., *Mini Rev. Med. Chem.* 12 (2012) 749–67. <https://doi.org/10.2174/138955712801264792>.
 - [185] R. Valério, A.R.S. Bernardino, C.A. V Torres, C. Brazinha, M.L. Tavares, J.G. Crespo, M.A.M. Reis, Feeding strategies to optimize vanillin production by *Amycolatopsis* sp. ATCC 39116, *Bioprocess Biosyst. Eng.* (2021). <https://doi.org/10.1007/s00449-020-02482-7>.
 - [186] Purolite, Industrial Adsorbent, *Polym. Adsorbent Resins Ind. Appl.* (2018). https://www.purolite.com/dam/jcr:6aac5b61-4f9b-4d70-870f-8ee092cba83b/puro-adsorbents-brochure_V16_DIGITAL.pdf.
 - [187] Purolite, Product data sheet Macronet MN102, (2018). <https://www.purolite.com/product-pdf/MN102.pdf>.
 - [188] Purolite, Laboratory guide, Polymeric Adsorbents, 2012.
 - [189] M. Scordino, A. Di Mauro, A. Passerini, E. Maccarone, Adsorption of Flavonoids on Resins: Hesperidin, *J. Agric. Food Chem.* 51 (2003) 6998–7004. <https://doi.org/10.1021/jf034496q>.
 - [190] N.E. Davila-Guzman, F.J. Cerino-Córdova, E. Soto-Regalado, M. Loredó-Cancino, J.A. Loredó-Medrano, R.B. García-Reyes, A mass transfer model for the fixed-bed adsorption of ferulic acid onto a polymeric resin: axial dispersion and intraparticle diffusion, *Environ. Technol. (United Kingdom)*. 37 (2016) 1914–1922. <https://doi.org/10.1080/09593330.2015.1135993>.
 - [191] E.. Serjeant, B.. Dempsey, Ionisation constants of organic acids in aqueous solution, 1979.
 - [192] N.E. Dávila-guzman, F.J. Cerino-córdova, P.E. Díaz-flores, J.R. Rangel-mendez, Equilibrium and kinetic studies of ferulic acid adsorption by Amberlite XAD-16, *Chem. Eng. J.* 183 (2012) 112–116. <https://doi.org/10.1016/j.cej.2011.12.037>.
 - [193] C. Valderrama, J.L. Cortina, A. Farran, X. Gamisans, F.X. de las Heras, Evaluation of hyper-cross-linked polymeric sorbents (Macronet MN200 and MN300) on dye (Acid red 14) removal

BIBLIOGRAPHY

- process, React. Funct. Polym. 68 (2008) 679–691. <https://doi.org/10.1016/j.reactfunctpolym.2007.11.005>.
- [194] B. Pan, W. Du, W. Zhang, X. Zhang, Q. Zhang, B. Pan, L. Lv, Q. Zhang, J. Chen, Improved adsorption of 4-nitrophenol onto a novel hyper-cross-linked polymer, *Environ. Sci. Technol.* 41 (2007) 5057–5062. <https://doi.org/10.1021/es070134d>.
- [195] R. Castaldo, G. Gentile, M. Avella, C. Carfagna, V. Ambrogio, Microporous hyper-crosslinked polystyrenes and nanocomposites with high adsorption properties: A review, *Polymers (Basel)*. 9 (2017). <https://doi.org/10.3390/polym9120651>.
- [196] N. Du, S. Cao, Y. Yu, Research on the adsorption property of supported ionic liquids for ferulic acid, caffeic acid and salicylic acid, 879 (2011) 1697–1703. <https://doi.org/10.1016/j.jchromb.2011.04.013>.
- [197] C. Lv, J. Yang, R. Liu, Q. Lu, Y. Ding, J. Zhang, J. Deng, A comparative study on the adsorption and desorption characteristics of flavonoids from honey by six resins, *Food Chem.* 268 (2018) 424–430. <https://doi.org/10.1016/j.foodchem.2018.06.100>.
- [198] H. Van Den Dool, P.D. Kratz, A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography, *J. Chromatogr. A*. 11 (1963) 463–471. https://doi.org/10.1007/978-3-319-70262-9_7.
- [199] Y.Y. Gong, X. Yin, H.M. Zhang, M.C. Wu, C.D. Tang, J.Q. Wang, Q.F. Pang, Effect of bioconversion conditions on vanillin production by *Amycolatopsis* sp. ATCC 39116 through an analysis of competing by-product formation, *J. Ind. Microbiol. Biotechnol.* 40 (2013) 1433–1441. <https://doi.org/10.1007/s10295-013-1339-6>.
- [200] R. Plaggenborg, J. Overhage, A. Steinbüchel, H. Priefert, Functional analyses of genes involved in the metabolism of ferulic acid in *Pseudomonas putida* KT2440., *Appl. Microbiol. Biotechnol.* 61 (2003) 528–535. <https://doi.org/10.1007/s00253-003-1260-4>.
- [201] S. Ghosh, A. Sachan, S.K. Sen, A. Mitra, Microbial transformation of ferulic acid to vanillic acid by *Streptomyces sannanensis* MTCC 6637, *J. Ind. Microbiol. Biotechnol.* 34 (2007) 131–138. <https://doi.org/10.1007/s10295-006-0177-1>.
- [202] R. Plaggenborg, J. Overhage, A. Loos, J.A.C. Archer, P. Lessard, A.J. Sinskey, A. Steinbüchel, H. Priefert, Potential of *Rhodococcus* strains for biotechnological vanillin production from ferulic acid and eugenol, *Appl. Microbiol. Biotechnol.* 72 (2006) 745–755. <https://doi.org/10.1007/s00253-005-0302-5>.
- [203] A.M. Falconnier B, Lapierre C, Lesage-Meessen L, Yonnet G, Brunerie P, Colonna-Ceccaldi B, Corrieu G, Vanillin as a product of ferulic acid biotransformation by the white-rot fungus *Pycnoporus cinnabarinus* I-937: Identification of metabolic pathways, *J. Biotechnol.* 37 (1994) 123–132. [https://doi.org/10.1016/0168-1656\(94\)90003-5](https://doi.org/10.1016/0168-1656(94)90003-5).
- [204] A. Paz, I. Costa-Trigo, F. Tugores, M. Míguez, J. de la Montaña, J.M. Domínguez, Biotransformation of phenolic compounds by *Bacillus aryabhattai*, *Bioprocess Biosyst. Eng.* 42 (2019) 1671–1679. <https://doi.org/10.1007/s00449-019-02163-0>.
- [205] P. Chen, L. Yan, S. Zhang, Z. Wu, S. Li, X. Yan, N. Wang, N. Liang, H. Li, Optimizing bioconversion of ferulic acid to vanillin by *Bacillus subtilis* in the stirred packed reactor using Box-Behnken design and desirability function, *Food Sci. Biotechnol.* 26 (2017) 143–152. <https://doi.org/10.1007/s10068-017-0019-0>.
- [206] M. Nazila, B.T.I. Maznah, N. Forough, Bioconversion of ferulic acid to vanillin by combined action of *Aspergillus niger* K8 and *Phanerochaete chrysosporium* ATCC 24725, *African J. Biotechnol.* 12 (2013) 6618–6624. <https://doi.org/10.5897/AJB2013.12416>.
- [207] J. Rosazza, Z. Huang, L. Dostal, T. Volm, B. Rousseau, Review: Biocatalytic transformations of ferulic acid: an abundant aromatic natural product, *J. Ind. Microbiol.* 15 (1995) 457–471. <https://doi.org/10.1007/BF01570016>.

BIBLIOGRAPHY

- [208] M. Brunati, F. Marinelli, C. Bertolini, R. Gandolfi, D. Daffonchio, F. Molinari, Biotransformations of cinnamic and ferulic acid with actinomycetes, *Enzyme Microb. Technol.* 34 (2004) 3–9. <https://doi.org/10.1016/j.enzmictec.2003.04.001>.
- [209] C. Fleige, A. Steinbüchel, Construction of expression vectors for metabolic engineering of the vanillin-producing actinomycete *Amycolatopsis* sp. ATCC 39116, *Appl Microbiol Biotechnol.* (2014) 6387–6395. <https://doi.org/10.1007/s00253-014-5724-5>.
- [210] N. Gunnarsson, E.A. Palmqvist, Influence of pH and carbon source on the production of vanillin from ferulic acid by *Streptomyces setonii* ATCC 39116, *Dev. Food Sci.* 43 (2006) 73–76. [https://doi.org/10.1016/S0167-4501\(06\)80018-X](https://doi.org/10.1016/S0167-4501(06)80018-X).
- [211] A. Muheim, B. Muller, T. Munch, Process for the production of vanillin, EP 0 885 968 B1, 1998.
- [212] R. Kumar, P.K. Sharma, P.S. Mishra, A Review on the Vanillin derivatives showing various Biological activities, *Int. J. PharmTech Res.* 4 (2012) 266–279. <https://doi.org/10.1002/chin.201228263>.
- [213] J.B. Sutherland, D.L. Crawford, A.L. Pometto, Metabolism of cinnamic, p-coumaric, and ferulic acids by *Streptomyces setonii*, *Can. J. Microbiol.* 29 (1983) 1253–1257. <https://doi.org/10.1139/m83-195>.
- [214] A. Longares-Patrón, M.P. Cañizares-Macías, Focused microwaves-assisted extraction and simultaneous spectrophotometric determination of vanillin and p-hydroxybenzaldehyde from vanilla fragans, *Talanta*. 69 (2006) 882–887. <https://doi.org/10.1016/j.talanta.2005.11.030>.
- [215] B.R. Torres, B. Aliakbarian, P. Torre, P. Perego, J.M. Domínguez, M. Zilli, A. Converti, Vanillin bioproduction from alkaline hydrolyzate of corn cob by *Escherichia coli* JM109/pBB1, *Enzyme Microb. Technol.* 44 (2009) 154–158. <https://doi.org/10.1016/j.enzmictec.2008.10.003>.
- [216] I.F. Mota, P.R. Pinto, J.M. Loureiro, A.E. Rodrigues, Purification of syringaldehyde and vanillin from an oxidized industrial kraft liquor by chromatographic processes, *Sep. Purif. Technol.* 234 (2020) 116083. <https://doi.org/10.1016/j.seppur.2019.116083>.
- [217] L. Extraction, (12) Patent Application Publication (10) Pub. No.: US 2014/0316165 A1, 1 (2014).
- [218] G. Camera-Roda, V. Augugliaro, A. Cardillo, V. Loddo, G. Palmisano, L. Palmisano, A pervaporation photocatalytic reactor for the green synthesis of vanillin, *Chem. Eng. J.* 224 (2013) 136–143. <https://doi.org/10.1016/j.cej.2012.10.037>.
- [219] G. Camera-Roda, A. Cardillo, V. Loddo, L. Palmisano, F. Parrino, Improvement of membrane performances to enhance the yield of vanillin in a pervaporation reactor, *Membranes (Basel)*. 4 (2014) 96–112. <https://doi.org/10.3390/membranes4010096>.
- [220] C. c. Bryan, Vanillin Purification by distillation, 1950.
- [221] M.I.F. Mota, P.C.R. Pinto, J.M. Loureiro, A.E. Rodrigues, Recovery of Vanillin and Syringaldehyde from Lignin Oxidation: A Review of Separation and Purification Processes, *Sep. Purif. Rev.* 45 (2016) 227–259. <https://doi.org/10.1080/15422119.2015.1070178>.
- [222] N. Khwanjaisakun, S. Amornraksa, L. Simasatitkul, P. Charoensuppanimit, S. Assabumrungrat, Techno-economic analysis of vanillin production from Kraft lignin: Feasibility study of lignin valorization, *Bioresour. Technol.* 299 (2020) 122559. <https://doi.org/10.1016/j.biortech.2019.122559>.
- [223] Hubert Gayet, Denis Revelant, Martine Vibert, Method for the purification of natural vanillin, US 9,617,198 B2, 2017. <https://patents.google.com/patent/US9617198>.
- [224] D.G. Barceloux, Medical Toxicology of Natural Substances: Food, Fungi, Medicinal Herbs and venomous animals, 2008. 10.1002/9780470330319.
- [225] C.M. Galanakis, V. Goulas, S. Tsakona, G.A. Manganaris, V. Gekas, A knowledge base for the recovery of natural phenols with different solvents, *Int. J. Food Prop.* 16 (2013) 382–396.

BIBLIOGRAPHY

- <https://doi.org/10.1080/10942912.2010.522750>.
- [226] A.M. Booth, T. Bannan, M.R. McGillen, M.H. Barley, D.O. Topping, G. McFiggans, C.J. Percival, The role of ortho, meta, para isomerism in measured solid state and derived sub-cooled liquid vapour pressures of substituted benzoic acids, *RSC Adv.* 2 (2012) 4430–4443. <https://doi.org/10.1039/c2ra01004f>.
- [227] V.L.S. Freitas, A.C.M.O. Lima, E. Sapei, M.D.M.C. Ribeiro da Silva, Comprehensive thermophysical and thermochemical studies of vanillyl alcohol, *J. Chem. Thermodyn.* 102 (2016) 287–292. <https://doi.org/10.1016/j.jct.2016.07.015>.
- [228] S.C. Lee, H. Hung, W.Y. Shiu, D. Mackay, Estimations of vapor pressure and activity coefficients in water and octanol for selected aromatic chemicals at 25°C, *Environ. Toxicol. Chem.* 19 (2000) 2623–2630. <https://doi.org/10.1002/etc.5620191102>.
- [229] C. Brazinha, J.G. Crespo, Aroma recovery from hydro alcoholic solutions by organophilic pervaporation: Modelling of fractionation by condensation, *J. Memb. Sci.* 341 (2009) 109–121. <https://doi.org/10.1016/j.memsci.2009.05.045>.
- [230] R.. Stephenson, S. Malanowski, *Handbook of the Thermodynamics of Organic Compounds*, 1987.
- [231] A.R.R.P. Almeida, V.L.S. Freitas, J.I.S. Campos, M.D.M.C. Ribeiro da Silva, M.J.S. Monte, Volatility and thermodynamic stability of vanillin, *J. Chem. Thermodyn.* 128 (2019) 45–54. <https://doi.org/10.1016/j.jct.2018.07.023>.
- [232] Irinian Chemical Engineers website, Antoine coefficient for vapor pressure, (n.d.). www.IrChem.com (accessed October 29, 2020).
- [233] A. Baudot, M. Marin, Pervaporation of aroma compounds: Comparison of Membrane Performances with Vapour-Liquid Equilibria and Engineering Aspects of Process Improvement, *Trans IchemE.* 75 (Part (1997) 117–142. <https://doi.org/10.1205/096030897531432>.
- [234] F. Shakeel, N. Haq, N.A. Siddiqui, Solubility and thermodynamic function of vanillin in ten different environmentally benign solvents, *Food Chem.* 180 (2015) 244–248. <https://doi.org/10.1016/j.foodchem.2015.01.102>.
- [235] K. Bournot, Mitteilung: Menthol und Vanillin, *Pharmazie.* 54 (1960) 73–75.
- [236] J.A. Méndez-Líter, I. Tundidor, M. Nieto-Domínguez, B.F. De Toro, A. González Santana, L.I. De Eugenio, A. Prieto, J.L. Asensio, F.J. Cañada, C. Sánchez, M.J. Martínez, Transglycosylation products generated by *Talaromyces amestolkiae* GH3 β -glucosidases: Effect of hydroxytyrosol, vanillin and its glucosides on breast cancer cells, *Microb. Cell Fact.* 18 (2019) 1–12. <https://doi.org/10.1186/s12934-019-1147-4>.
- [237] ChemAxon, (n.d.). www.chemaxon.com (accessed October 30, 2020).
- [238] T. Mohammadi, A. Aroujalian, A. Bakhshi, Pervaporation of dilute alcoholic mixtures using PDMS membrane, 60 (2005) 1875–1880. <https://doi.org/10.1016/j.ces.2004.11.039>.
- [239] A. Dobrak, A. Figoli, S. Chovau, F. Galiano, S. Simone, I.F.J. Vankelecom, E. Drioli, B. Van der Bruggen, Performance of PDMS membranes in pervaporation: Effect of silicalite fillers and comparison with SBS membranes, *J. Colloid Interface Sci.* 346 (2010) 254–264. <https://doi.org/10.1016/j.jcis.2010.02.023>.
- [240] A. Bashshi, T. Mohammadi, A. Aroujalian, Pervaporation Separation of Binary and Ternary Mixtures with Polydimethylsiloxane Membranes, *J. Appl. Polym. Sci.* 107 (2008) 1777–1782. <https://doi.org/10.1002/app>.
- [241] S. Álvarez, F.A. Riera, R. Álvarez, J. Coca, F.P. Cuperus, S. Th Bouwer, G. Boswinkel, R.W. Van Gemert, J.W. Veldsink, L. Giorno, L. Donato, S. Todisco, E. Drioli, J. Olsson, G. Trägårdh, S.N. Gaeta, L. Panyor, New integrated membrane process for producing clarified apple juice and apple juice aroma concentrate, *J. Food Eng.* 46 (2000) 109–125. <https://doi.org/10.1016/S0308->

BIBLIOGRAPHY

- 8146(00)00139-4.
- [242] R.W. Baker, J.G. Wijmans, Y. Huang, Permeability, permeance and selectivity: A preferred way of reporting pervaporation performance data, *J. Memb. Sci.* 348 (2010) 346–352. <https://doi.org/10.1016/j.memsci.2009.11.022>.
- [243] I.T. Meireles, C. Brazinha, I.M. Coelho, J.G. Crespo, Membranes for ethanol dehydration, in: A. Figoli, A. Cassano, A. Basile (Eds.), *Membr. Technol. Biorefining*, 2016: pp. 241–263.
- [244] W.F. Guo, T. Chung, T. Matsuura, Pervaporation study on the dehydration of aqueous butanol solutions : a comparison of flux vs . permeance , separation factor vs . selectivity, 245 (2004) 199–210. <https://doi.org/10.1016/j.memsci.2004.07.025>.
- [245] I.L. Borisov, A. Kujawska, K. Knozowska, V. V. Volkov, W. Kujawski, Influence of feed flow rate, temperature and feed concentration on concentration polarization effects during separation of water-methyl acetate solutions with high permeable hydrophobic pervaporation PDMS membrane, *J. Memb. Sci.* 564 (2018) 1–9. <https://doi.org/10.1016/j.memsci.2018.07.001>.
- [246] S. Ray, S.K. Ray, Separation of organic mixtures by pervaporation using crosslinked and filled rubber membranes, *J. Memb. Sci.* 285 (2006) 108–119. <https://doi.org/10.1016/j.memsci.2006.08.009>.
- [247] M. Bogdan, C.G. Floare, A. Pirnău, 1H NMR investigation of self-association of vanillin in aqueous solution, *J. Phys. Conf. Ser.* 182 (2009). <https://doi.org/10.1088/1742-6596/182/1/012002>.
- [248] Microdyn Nadir, Nadir Catalogue, (2014). <https://www.lenntech.com/Data-sheets/Microdyn-Product-catalogue-L.pdf>.
- [249] K. Sinnott, R. Coulson and Richardson's Chemical Engineering- Chemical Engineering Design, Fourth, 2005.
- [250] Pordata, [www.pordata](http://www.pordata.com) (accessed September 1, 2020).
- [251] M. Carvalheira, J. Cassidy, J.M. Ribeiro, B.A. Oliveira, E.B. Freitas, C. Roca, G. Carvalho, A. Oehmen, M.A.M. Reis, Performance of a two-stage anaerobic digestion system treating fruit pulp waste : The impact of substrate shift and operational conditions, 78 (2018) 434–445. <https://doi.org/10.1016/j.wasman.2018.06.013>.
- [252] M. Peters, K. Timmerhaus, R. West, *Plant Design and Economics for Chemical Engineers*, 2003.
- [253] C.E. De Farias Silva, G.N.S.B. Gois, A.K.S. Abud, N.C.S. Amorim, F. Girotto, G. Markou, C.M. Carvalho, J. Tonholo, E.L. Amorim, Anaerobic Digestion: Biogas Production from Agro-industrial Wastewater, Food Waste, and Biomass, in: A.A. Rastegari, A.N. Yadav, A. Gupta (Eds.), *Prospect. Of Renewable Bioprocess. Futur. Energy Syst. Biofuel Biorefinery Technol.* 10, 2019: pp. 431–470. https://doi.org/10.1007/978-3-030-14463-0_16.

APPENDIX

A.1. Supplementary Material for the biorefinery of economic viability of the integrated bio-vanillin process

Table A.1 - Mass balance of the bio-vanillin production process in kg.h⁻¹ of each stream (Si), considering a vanillin production of 20 ton/year (2.5kg.h⁻¹).

Kg.h ⁻¹	S ₁	S ₂	S ₃	S ₄	S ₅	S ₆	S ₇	S ₈	S ₉	S ₁₀	S ₁₁	S ₁₂	S ₁₃	S ₁₄
Dry Corn fibre	630.0				630.0									
Water in corn	1072.8				1072.8									
Water			5123.1	5123.1	5123.1									
NaOH 50%		104.6		104.6	104.6									
Ferulic acid							6.8	1.0	5.8		5.8	0.1	5.7	0.3
HCl 33%										55.6	55.6			
Pellet						3534.5								
Others							3389.2	484.2	2905.0		2905.0	59.2	2901.4	2899.2
Total	1702.8	104.6	5123.1	5227.7	6930.5	3534.5	3396.0	485.2	2910.8	55.6	2966.4	59.3	2907.1	2900.1
Kg.h ⁻¹	S ₁₅	S ₁₆	S ₁₇	S ₁₈	S ₁₉	S ₂₀	S ₂₁	S ₂₂	S ₂₃	S ₂₄	S ₂₅	S ₂₆	S ₂₇	
Water		581.4						542.0	542.0			1268.0	1626.0	
Ferulic acid	5.4						5.4							
Ethanol	486			461.7	24.3	486.0	24.3		24.3			24.3	24.3	
Vanillin									2.8	0.3	2.5			
Glucose								6.5	2.2	2.2				
Yeast extract								0.1						
Vanillic acid									1.1	1.1				
Dirty water			581.4											
Others	1.5						1.5		3.0	3.0				
Total	492.9	581.4	581.4	461.7	24.3	486.0	31.2	548.6	575.4	6.6	2.5	2192.3	1650.3	