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FLAVOR PRODUCTION BY *Acinetobacter soli* USING INDUSTRIAL BYPRODUCTS

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Master in Biotechnology

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Abstract

2-Phenylethanol (2-PE) is a valuable flavor compound recognized for its rose-like scent and extensive application in the cosmetic, perfume, and food industries. While it can be naturally obtained from plants, the majority is currently produced through chemical synthesis, leading to environmental concerns. As an eco-friendly alternative, biotechnological production of 2-PE presents a promising solution, yielding a natural product.

A bacterium, *Acinetobacter soli* ANG344B, isolated from river water, exhibited an exceptional capacity to produce 2-PE using L-phenylalanine (L-Phe) as a precursor—a capability typically observed in yeasts rather than bacteria. Under optimized cultivation conditions, regarding medium composition, pH, temperature and aeration, this strain could produce 2.14 ± 0.18 g/L of 2-PE in a batch cultivation mode.

As an amphiphilic compound, 2-PE presents cytotoxic effects to the producing microorganisms. Concentrations as low as 1 g/L exhibit a detrimental impact on cellular growth of *Acinetobacter soli* ANG344B, with direct implications on aroma production. Improvement of 2-PE production was achieved using an *in situ* product removal (ISPR) approach to avoid product toxicity. Among the different strategies tested, the use of adsorbent resin Amberlite XAD 4 proved to be the most promising approach to improve 2-PE production process. In a batch biotransformation process using 3% (w/v) of Amberlite XAD 4, 2-PE production was improved by 3.3-fold, achieving 6.99 ± 0.06 g/L with a volumetric productivity of 0.17 ± 0.00 g/L.h. Product recovery from the resin, with ethanol, and its further concentration resulted in an extract with 217.56 ± 0.31 g/L of 2-PE, with a chromatographic purity of 98% in a matrix of ethanol.

Agro-industrial wastes were able to support *Acinetobacter soli* ANG344B growth when used in the cultivation media as carbon source. However, the protein content in the feedstock's hydrolysates were not enough to allow L-Phe biotransformation into 2-PE. To ensure 2-PE production, supplementation of the culture media with commercial L-Phe was necessary. Banana peels hydrolysate, composed of glucose and fructose, was the feedstock that allowed better growth and 2-PE production (2.48 ± 0.04 g/L) from L-Phe. The use Amberlite XAD 4 as an ISPR approach led to a substantial improvement in 2-PE production, reaching 7.30 ± 0.01 g/L with a volumetric productivity of 0.27 ± 0.00 g/L.h.

This agro-industrial byproduct show that can be successfully used for 2-PE production, improving the production process when compared to commercial carbon sources. Simultaneously, this approach promotes sustainable management of residues and contributes to a circular economy.

Moreover, it was found that *Acinetobacter soli* ANG344B produces exopolysaccharides (EPS) when cultivated in absence of L-Phe or in the presence of low concentrations of 2-PE, when using ISPR approaches. Under these conditions, the EPS concentrations ranged from 2.4 to 4.7 g/L. The EPS produced are high molecular weight heteropolysaccharides with distinctive compositions depending on the carbon sources used.

Overall, this work highlights the exceptional potential of *Acinetobacter soli* ANG344B for 2-PE production with a production capacity not previously reported for wild-type bacteria. These results can have significant implications for industrial applications also promoting a circular economy. The potential of this strain is not limited to 2-PE production, being able to produce EPS.

Keywords:

Acinetobacter soli ANG344B, 2-Phenylethanol, L-Phenylalanine, *In situ* product removal, Agro-industrial wastes.

Resumo

2-Feniletanol (2-PE) é um composto aromático com valor significativo, reconhecido pelo seu aroma a rosas e amplamente utilizado na indústria cosmética, de perfumes e alimentar. Este aroma pode ser obtido de forma natural, a partir de plantas, mas a maioria é atualmente obtida através de síntese química, que está associada a problemas ambientais. A produção de 2-PE por via biotecnológica, representa uma alternativa promissora e sustentável, resultando num produto natural.

A bactéria *Acinetobacter soli* ANG344B, isolada da água de um rio, demonstrou uma capacidade excecional para produzir 2-PE utilizando L-fenilalanina (L-Phe) como percursor, uma capacidade tipicamente observada em leveduras e não em bactérias. Sob condições ótimas de cultivo, incluindo composição do meio de cultura, pH, temperatura e arejamento, esta estirpe produziu 2.14 ± 0.18 g/L de 2-PE num processo de produção descontinuo.

Sendo um composto anfifílico, o 2-PE apresenta efeitos citotóxicos nos microrganismos produtores. Concentrações de 2-PE tão baixas como 1 g/L apresentam um impacto negativo no crescimento celular de *Acinetobacter soli* ANG344B, com implicações diretas na produção do aroma.

O aumento da produção de 2-PE foi conseguido usando técnicas de remoção do produto *in situ*, de forma a evitar a toxicidade do produto. Entre as diferentes estratégias testadas, a utilização da resina adsorvente Amberlite XAD 4 provou ser a abordagem mais promissora para melhorar o processo de produção de 2-PE. Num processo de biotransformação em modo descontinuo, usando 3% (m/v) de Amberlite XAD 4, a produção de 2-PE foi aumentada 3.3 vezes, atingindo uma concentração de 2-PE de 6.99 ± 0.06 g/L com uma produtividade volumétrica de 0.17 ± 0.00 g/L.h. A recuperação do produto da resina foi realizada com etanol, e a sua posterior concentração, resultou num extrato com 217.56 ± 0.31 g/L de 2-PE, com uma pureza cromatográfica de 98% numa matriz de etanol.

A utilização de resíduos agroindustriais foi capaz de suportar o crescimento de *Acinetobacter soli* ANG344B, quando usados como fonte de carbono. No entanto, o conteúdo em proteína dos resíduos hidrolisados não foi suficiente para a bioconversão de L-Phe em 2-PE. De forma a assegurar produção de 2-PE, foi necessária a suplementação

do meio de cultura com L-Phe comercial. O hidrolisado da casca de banana, composto por glucose e frutose, foi aquele que permitiu um maior crescimento e produção de 2-PE (2.48 ± 0.04 g/L) através da bioconversão de L-Phe. O uso da resina Amberlite XAD 4 no processo de remoção *in situ* permitiu um aumento na produção de 2-PE para 7.30 ± 0.01 g/L com uma produtividade volumétrica de 0.27 ± 0.00 g/L.h. Estes resultados demonstram que este resíduo agroindustrial pode ser usado para a produção de 2-PE com sucesso, melhorando o processo de produção quando comparado com a utilização de fontes de carbono comerciais, promovendo, ao mesmo tempo, uma gestão sustentável de resíduos e uma economia circular.

Na ausência de L-Phe ou na presença de baixas concentrações de 2-PE, quando se usam técnicas de remoção *in situ*, a bactéria *Acinetobacter soli* ANG344B demonstrou capacidade para produzir exopolissacáridos (EPS). Nestas condições, produziu-se EPS numa gama de concentrações entre 2.4 a 4.7 g/L. Os EPS produzidos são heteropolissacáridos com elevado peso molecular e composição distinta dependendo da fonte de carbono usada.

No geral, este trabalho mostra o potencial excecional de *Acinetobacter soli* ANG344B para a produção de 2-PE com uma capacidade de produção que não tinha sido ainda reportada na literatura para bactérias não geneticamente modificadas. Estes resultados podem ter implicações significativas para aplicações industriais, promovendo uma economia circular. O potencial desta estirpe não está apenas limitado à produção de 2-PE, sendo também capaz de produzir EPS.

Palavras-chave:

Acinetobacter soli ANG344B, 2-Feniletanol, L-Fenilalanina, Remoção de produto *in situ*, Resíduos agroindustriais.

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Acronyms

5-HMF	5-Hydroxymethylfurfural
2-PE	2-Phenylethanol
AAAT	Aromatic Aminotransferase
ADH	Alcohol Dehydrogenase
Ac	Acetate
Ara	Arabinose
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSG	Brewer's Spent Grain
BV	Bed volume
CDW	Cell Dry Weight
EPS	Exopolysaccharide
FAME	Fatty Acid Methyl Ester
FID	Flame Ionization Detector
FTIR	Fourier Transform Infra-Red spectroscopy
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
GC	Gas Chromatography
GC-MS	Gas Chromatography – Mass Spectrometry
Glc	Glucose
GRAS	Generally Recognized as Safe
HPLC	High Performance Liquid Chromatography
IL	Ionic Liquid

ISPR	*In Situ* Product Removal
KDC	alpha-Keto acid Decarboxylase
LB	Luria Bertani
L-Phe	L-Phenylalanine
Mn	Molecular number
Mw	Molecular weight
MWCO	Molecular Weight cut-off
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
OD_{600}	Optical density at 600 nm
P	Partition coefficient
PAD	Pulsed Amperometric Detector
PDA	Photodiode Array
PDI	Polydispersity index
POMS	Polyctylmethylsiloxane
PPG	Polypropylene glycol
RI	Refractive Index
ROS	Reactive Oxygen Species
TCA	Tricarboxylic acid
TFA	Trifluoroacetic acid
TGA	Thermogravimetric Analysis
UV	Ultraviolet
vvm	Volume of air per volume of reactor per minute
Xyl	Xylose

Symbols

C_0	Concentration of adsorbate in aqueous phase before adsorption/inlet (g/L)
C_{eq}	Concentration of adsorbate in aqueous phase after the equilibrium (g/L)
C_d	Concentration of adsorbate in the eluent (g/L)
D	Desorption efficiency (%)
E	Adsorption efficiency (%)
E_{24}	Emulsification index (%)
h_e	Height of the emulsion layer (cm)
h_t	Total height of mixture for emulsion test (cm)
k	Consistency index (Pa s^n)
m_0	Mass of dry resin
m_{ads}	Total mass of 2-PE adsorbed on the resin (g)
M_w	Average molecular weight (MDa)
n	Flow behavior index
Q	Adsorption capacity ($g_{adsorbate}/g_{dry\ resin}$)
Q	Flow rate (mL/min)
q_{total}	Maximum capacity of the column ($g_{adsorbate}/g_{dry\ resin}$)
r_p	Volumetric productivity (g/L.h)
t	Time (h)
T_{deg}	Maximal degradation temperature (°C)
t_{sat}	Bed saturation time (min)
V	Volume (L)
V_d	Volume of eluent (L)
x_0	Initial cell concentration (g/L)
x	Cell concentration (g/L)

$Y_{X/S}$ Yield of biomass on substrate ($g_{CWD}/g_{substrate}$)

$Y_{P/S}$ Yield of product on substrate ($g_{product}/g_{substrate}$)

ΔP Product produced (g/L)

ΔS Substrate consumed (g/L)

ΔX Biomass produced (g/L)

$\dot{\gamma}$ Shear rate ($1/s$)

σ Shear stress (Pa)

μ_{max} Maximum specific cell growth rate (1/h)

Motivation and thesis outline

Motivation

2-Phenylethanol (2-PE) stands out as a valuable aroma compound, known for its rose-like scent and widespread use in the cosmetic, perfume, food, beverage, and home care products industries. The production of this aromatic compound traditionally relies on two methods: natural extraction and chemical synthesis, each presenting limitations. Natural extraction often results in low yields and high costs, while chemical synthesis involves extreme operational conditions and the use of toxic chemicals, posing a negative impact on the environment.

The flavor market has witnessed consistent growth, and this trend is expected to persist due to the rising demand for flavored products. Coupled with consumers´ increasing preference for natural products, this demand have led to the development of biotechnological alternatives to address the drawbacks of the conventional production processes.

A novel isolated bacterium, *Acinetobacter soli* ANG344B was found to produce 2-PE, a feature not previously reported for this genus and not common for bacteria. Typically, aroma production of this nature is observed in yeasts. This PhD thesis aims to explore *A. soli* ANG344B as 2-PE producer, and the main objectives were:

1. Assess the 2-PE production potential of *Acinetobacter soli* ANG344B.
2. Optimize the cultivation conditions for 2-PE production, studying the effect of medium composition (regarding nitrogen, carbon source, L-Phe) and environmental conditions (pH, temperature and aeration) aiming at maximizing 2-PE productivity by *A. soli* ANG344B.
3. Improve 2-PE production applying *in* situ product removal approaches to avoid product toxicity that impairs aroma production.
4. Recover 2-PE from the cultivation broth.
5. Use agro-industrial feedstocks in 2-PE production process aiming to reduce the costs, making the process sustainable and contributing to a circular economy.

This research endeavors to shed light on the untapped potential of *Acinetobacter soli* ANG344B, paving the way for innovative and sustainable approaches to 2-PE production.

The outlined objectives aim to not only advance scientific knowledge but also contribute to the development of eco-friendly alternatives in the fragrance industry.

Thesis outline

The work developed in this PhD thesis is organized in six chapters. Chapters 2 to 4 describe the experimental work carried out regarding 2-PE production by *Acinetobacter soli* ANG344B and their findings. Chapter 5 focuses on the experimental exploration of exopolysaccharide production by the same microorganism. Each of these chapters comprises a succinct contextual overview, a detailed description of the methodology used, a discussion of the results, and the presentation of key conclusions.

This work is organized as follows:

Chapter 1 – **General introduction** – consists in a literature review about the 2-PE production process, encompassing the key topics investigated in this PhD thesis. This chapter comprises the biotechnological production regarding producing strains, metabolic pathways and cultivation process, the cytotoxicity of the aroma towards the producing microorganisms, the cultivation using *in situ* product removal approaches and the use of alternative substrates for 2-PE production. A brief overview of the properties and possible applications that are being investigated for 2-PE is also provided. Presents the biotechnological potential of *Acinetobacter*, as along with specific information about the strain in study, *Acinetobacter soli* ANG344B, comprising isolation, strain characterization and genomic analysis, related with 2-PE production.

Chapter 2 – Optimization of 2-phenylethanol production from L-phenylalanine by *Acinetobacter soli* ANG344B – investigates the optimal conditions for 2-PE production aiming to maximize 2-PE concentration and volumetric productivity. Explores factors such as medium composition, operational conditions and L-Phe concentration. Examines the toxicity of 2-PE towards the producing microorganism. Evaluates the scale up potential from 2 to 10 L.

Chapter 3 - Assessment of *in situ* product recovery techniques to enhance 2-phenylethanol production – Explore different approaches for *in situ* product removal to mitigate aroma toxicity and enhance 2-PE production. Different alternatives comprising air stripping, liquid-liquid extraction and resin adsorption based approaches were

evaluated. It was tested product recovery using air stripping supplied by the bioreactor´s air supplied for the cultivation process. Different solvents and polymeric resins were also tested for liquid-liquid extraction and adsorption, respectively. Determines the partition for solvents, attempts cultivation in the presence of oleic acid. Selects the most efficient resin for further characterization using a continuous column procedure. Applies the selected resin in a batch mode during a 2-PE production process in a 2 L bioreactor under optimized conditions described in chapter 2. The 2-PE produced was desorbed from the resin and analyzed by GC-MS.

Chapter 4 - Valorization of agro-industrial feedstocks into 2-phenylethanol by *Acinetobacter soli* ANG344B – presents the carbon sources used by *Acinetobacter soli* ANG344B based on common agro-industrial feedstocks. Based on the carbon sources that can be used by the bacteria, several industrial feedstocks were hydrolyzed and characterized in terms of sugar composition. The hydrolysates were tested for *A. soli* ANG344B cultivation, evaluating their potential as L-Phe and carbon sources. The hydrolysates that allowed better 2-PE production were tested in 2 L bioreactor. Applies the *in situ* product removal approach from the previous chapter using the selected hydrolysate supplemented with L-Phe for a batch 2-PE production in a 2 L bioreactor. The 2-PE produced was desorbed from the resin and analyzed by GC-MS.

Chapter 5 - *Acinetobacter soli* ANG344B exopolysaccharide production and characterization – Investigates the ability of *A. soli* ANG244B to produce exopolysaccharides (EPS) that were identified in previous chapters. Describes EPS production using *in situ* product removal approaches and in the absence of L-Phe in batch cultivation. Evaluates the chemical composition, molecular mass distribution, thermal analysis, Fourier Transform Infra-Red spectroscopy, rheological behavior, and emulsification ability of EPS.

Chapter 6 – **Conclusions and future work** – Highlights the main outputs of this work and propose future research suggestions.

1.

General Introduction

The information regarding *Acinetobacter soli* ANG344B (section 1.3.1) is part of the following manuscript:
Bernardino, A.R.S, Grosso, F., Torres, C. A. V., Reis, M. A. M., Peixe, L. Exploring the biotechnological potential of *Acinetobacter soli* ANG344B: a novel bacterium for 2-phenylethanol production. *Biotechnology Reports.* Submitted

1.1. Bioproduction of 2-Phenylethanol

1.1.1. Background

2-Phenylethanol (2-PE) is an aromatic alcohol with rose-like scent, one of the most popular fragrances, being the major component of rose water. It is applied in a wide range of industries, such as in cosmetic, food and beverage industries. Due to its antimicrobial characteristics is used in laundry and home care products. It is generally recognized as safe (GRAS, 2858) and used in pharmaceuticals (*2-Phenylethanol Market to Hit $350 Million by 2027,Says Global Market Insights Inc.*, 2021; Martínez-Avila et al., 2018; Mitri et al., 2022; Qian et al., 2019; Scognamiglio et al., 2012;).

The global market demand for 2-PE is increasing, reaching USD 255 million in 2021 and is expected to continue growing in the next years (*2-Phenylethanol Market to Hit $350 Million by 2027,Says Global Market Insights Inc.*, 2021). About 93% of the commercialized compound is obtained by chemical synthesis, from chlorobenzene, benzene or styrene oxide. However, the production conditions have serious drawbacks, namely the use of hazardous or corrosive chemicals, harsh conditions of temperature and pressure, strong acid or alkali environments and low selectivity. Additionally, the difficult separation and side products formation affect aroma quality and increase downstream costs (Gen Consulting Company, 2020; Qian et al., 2019; Vorster et al., 2019). Nevertheless, natural 2-PE is extracted mainly from rose essential oils, but due to the complex and costly extraction process, the low concentration obtained from plants and weather dependence production, its costs can be 250 to 300 times higher than the chemically synthesized compound and the production cannot meet the demand for the natural product (*2-Phenylethanol Market to Hit $350 Million by 2027,Says Global Market Insights Inc.*, 2021; Gen Consulting Company, 2020; Qian et al., 2019).

The rising awareness among the consumers regarding environmental and health issues has increased the demand for natural products, leading to the seek and development of biotechnological alternatives for 2-PE production by the suppliers. In fact, the USA and European legislation recognize microbiological produced flavors as natural, making the biotechnological production an effective alternative (European Commission, 2008; *CFR - Code of Federal Regulations Title 21*, n.d.; FDA - U.S Food and Drug Administration and Regulation (EC) No 1334/2008). Compared to the 2-PE extracted from plants,

biotechnological processes have the advantage of not depending on weather conditions, plant diseases and trade restrictions, that strongly influence the price (Akacha and Gargouri, 2015; Hosoglu et al., 2018). Moreover, these biotechnological processes use mild conditions and have high product selectivity and do not generate toxic wastes (Hosoglu et al., 2018). The price of biotechnological produced compounds are still 10 to 100 times higher than the synthetically produced compound due to the low production yields and downstream separation costs. However, the use of cheap feedstocks, such as agro-industrial byproducts can make the process economically attractive and simultaneously contribute to a circular economy (Chreptowicz and Mierzejewska, 2018; Martínez-Avila et al., 2021).

1.1.2. 2-PE biotechnological production

1.1.2.1. Producing strains and metabolic pathways

Several microorganisms, among bacteria, yeast and fungi, have the ability to produce 2-PE through different metabolic pathways. The main pathways for 2-PE production are the Ehrlich and Shikimate pathways. Among these, Ehrlich pathway is considered the most efficient metabolic approach for 2-PE production (Martínez-Avila et al., 2018; Mitri et al., 2022). Figure 1.1. illustrates the principal biosynthetic pathways involved in 2-PE production by microorganisms.

Ehrlich pathway consists in the catabolism of amino acids (valine, leucine, isoleucine, methionine, phenylalanine, tyrosine and tryptophan) to the correspondent aromatic alcohols or acids. 2-PE production by this pathway is achieved in three steps from L-phenylalanine (L-Phe) (Figure 1.1.). L-Phe is transaminated to phenylpyruvate, then decarboxylated, obtaining phenylacetaldehyde and finally reduced to 2-PE, that can be further converted into phenylethyl acetate (Hazelwood et al., 2008; Qian et al., 2019). However, using *Saccharomyces cerevisiae* as a model, the initial transamination reaction is negatively affected by the presence of nitrogen sources that are not involved in the Ehrlich pathway, such as ammonia, proline and asparagine, being benefic to have the amino acid as sole source of nitrogen in the broth to achieve higher 2-PE productions (Hazelwood et al., 2008; Hua and Xu, 2011; Wang, Bai et al., 2017). Additionally, it was found that when the culture is glucose limited, amino acids are converted mainly into

aromatic acids, achieving a low concentration of aromatic alcohols (Hazelwood et al., 2008).

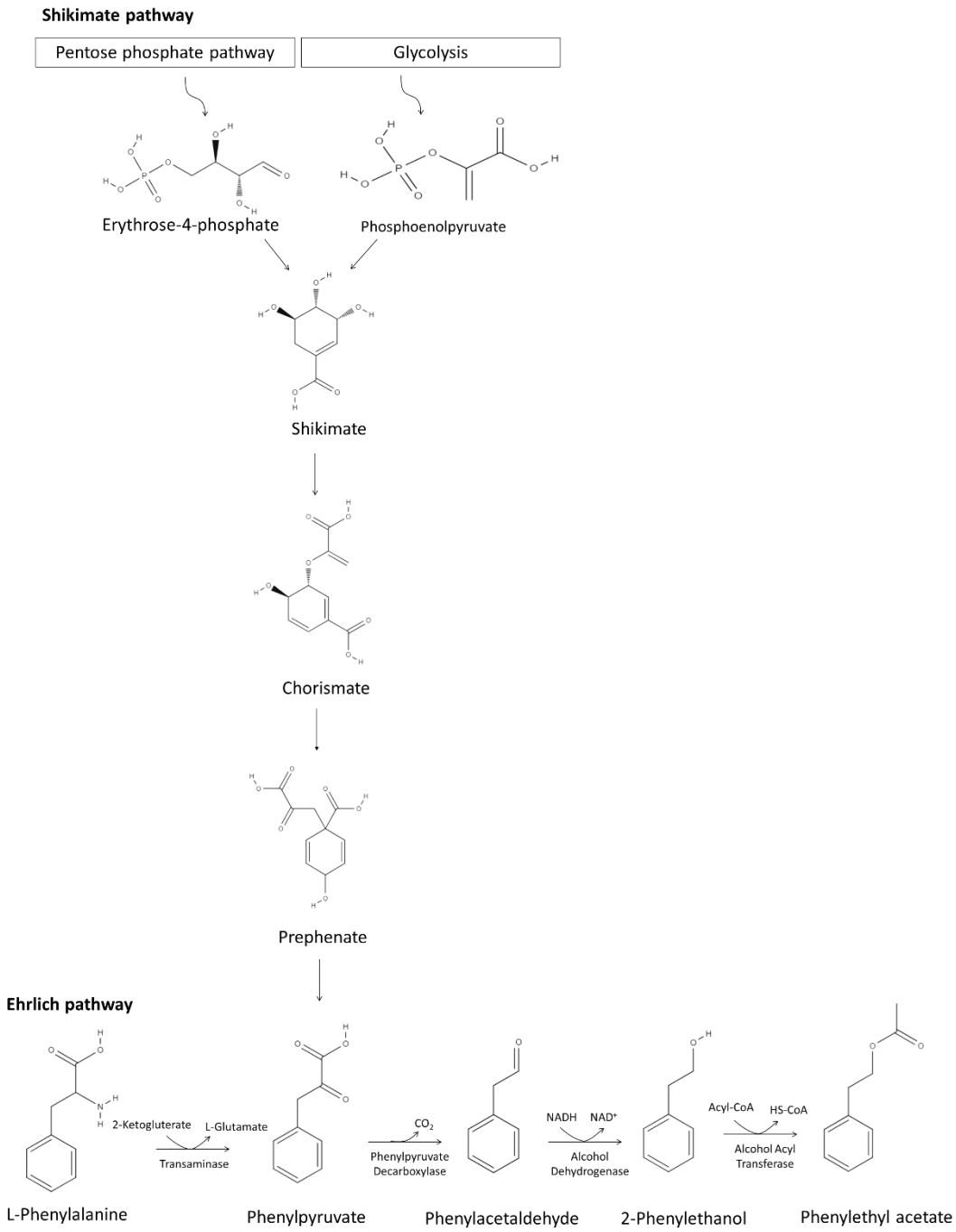

Figure 1.1 Metabolic pathways involved in 2-PE production. Ehrlich pathway for 2-PE production from L-Phe. Shikimate pathway for *de novo* 2-PE biosynthesis from erythrose-4-phosphate produced during glycolysis and phosphoenolpyruvate coming from pentose phosphate pathway, resulting in phenylpyruvate that enter in Ehrlich pathway to be converted in 2-PE (adapted from Mitri et al., 2022).

Microorganisms can also produce 2-PE directly from glucose, by *de novo* Shikimate (or also called Phenylpyruvate) pathway (Figure 1.1.). This pathway consists of condensation in successive steps of phosphoenolpyruvate obtained from glycolysis, and erytrose-4-phosphate from pentose-phosphate pathway. These compounds are converted into phenylpyruvate that enters in Ehrlich pathway to produce 2-PE. However, the availability of phosphoenolpyruvate and erytrose-4-phosphate for Shikimate pathway is low, compromising the productivity of 2-PE production by this way (Zhu et al., 2021). The advantage of this metabolic pathway is the lower price of glucose, compared with the L-Phe (Zhang et al., 2014; Zhu et al., 2021). Therefore, strategies of metabolic engineering may be developed to improve crucial rate-limiting enzymes, reduce feedback inhibition and consequently improve 2-PE production by *de novo* synthesis (Zhang et al., 2014; Zhu et al., 2021). Using a multilevel metabolic engineering strategy, Zhan et al. redirected the carbon flux towards 2-PE production and eliminated the precursor competing pathways of *Bacillus licheniformis*, being able to achieve 6.24 g/L of 2-PE from glucose, in a complex medium (Zhan et al., 2022). Similarly, by increasing phenylpyruvate availability using genetic engineering strategies in *Pichia pastoris*, Kong et al. could accumulate 1.15 g/L of 2-PE from glucose (Kong et al., 2020).

Several microorganisms have the ability to produce 2-PE naturally. The most relevant microorganisms reported for 2-PE production are yeasts. However, this capability can also be found in a few bacteria. For instance, 2-PE production in presence of L-Phe was reported in *Proteus vulgaris, Proteus mirabilis*, *Psychrobacter*, *Brevibacterium linens*, that are bacteria involved in cheese ripening, *Mycobacteria* strains and in *Erwinia carotova* subsp. *atroseptica* (Deetae et al., 2007; Jollivet et al., 1992; Liu et al., 2020; McNerney et al., 2012; Spinnler and Djian, 1991). Nonetheless, it is associated with low aroma production, below 152 $mg_{2\text{-}PE}$/L. Metabolic engineering strategies are being applied to improve 2-PE production in bacterial strains (Liu et al., 2018; Zhang et al., 2014; Zhan et al., 2022). For instance, *Enterobacter* sp. CGMCC 5087 was found to produce 0.1 g/L of 2-PE through Shikimate pathway, using glucose as carbon source and NH_4Cl as nitrogen source. When two genes of rate-limiting enzymes of that metabolic pathway were cloned from *Escherichia coli* and overexpressed, the recombinant bacterium was able to produce 0.33 $g_{2\text{-}PE}$/L (Zhang et al., 2014). Likewise, Liu et al. reported that *Proteus mirabilis* can produce 0.08 $g_{2\text{-}PE}$/L from L-Phe and 3.21 $g_{2\text{-}PE}$/L using the *E. coli* recombinant strain in which they mimic the metabolic pathway and

construct a cofactor regeneration system to improve the aroma production (Liu et al., 2018). *Bacillus licheniformis* DW2 could also produce 5.16 g/L of 2-PE from L-Phe by introduction of Ehrlich pathway, in a fed-batch operational mode (Zhan et al., 2020). The native strain could not produce 2-PE but had alcohol dehydrogenases that were able to convert phenylacetaldehyde into 2-PE (Zhan et al., 2022). When Ehrlich and Phenylpyruvate pathways were engineered to redirect carbon flux towards 2-PE production, the recombinant strain could produce 6.24 g/L of the aroma at 0.13 g/L.h under optimized conditions (Zhan et al., 2022). Similarly, engineered *Bacillus licheniformis* was reported to produce 6.43 g/L of 2-PE using glycerol and molasses. This was achieved by bacterial transformation with a plasmid to allow 2-PE production and replacement of the strain sucrose metabolism to increase phosphoenolpyruvate (important precursor for 2-PE synthesis in shikimate pathway) availability in the cell, followed by optimization of the cultivation process (Xu et al., 2023).

The potential of yeasts to produce 2-PE is widely reported, being the most reported microorganism for 2-PE biotechnological production. *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* are the most effective 2-PE producers, achieving 2-PE productions up to 4.5 and 1.45 g/L, respectively, as can be seen in Table 1.1. (Eshkol et al., 2009; Gao and Daugulis, 2009). However, many other yeasts are being reported for 2-PE production, achieving similar 2-PE production levels to the aforementioned strains, as *Yarrowia lipolytica*, *Wickerhamomyces anomalus* and *Pichia kudriavzevii*, that could produce up to 5 g/L of 2-PE, as it is summarized in Table 1.1 (Celińska et al., 2013; Dai et al., 2020; Drężek et al., 2021; Fan et al., 2020; Huang et al., 2001; Kong et al., 2020; Lu et al., 2016; Tian et al., 2020; Yan et al., 2020).

Even though the ability to produce 2-PE is known by several microorganisms, its production is not as attractive as it needs to be for industrial applications, due to the low titer that can be obtained and the high production costs, compared with the synthetic route. Strategies to improve 2-PE production go through improvement of fermentation conditions, strain engineering and strategies to avoid the impairment that 2-PE has in the production microorganism. The search for alternative and cost-effective substrates for 2-PE production will also improve the attractiveness of the process.

Table 1.1 Overview of 2-PE wild-type production strains, producing method and production parameters. Y(P/S) represents the 2-PE production yield from L-Phe and Rp the volumetric productivity.

Strain	Method	Carbon source	L-Phe (g/L)	2-PE (g/L)	Y(P/S) (g/g)	Rp (g/L.h)	Reference
***Wickerhamomyces anomalus* 1D6**	Single dose Fed-batch 5 L bioreactor	Glucose	5	4.73	-	0.10	Tian et al., 2020
***Yarrowia lipolytica* NCYC3825**	Batch Shake flask	Glucose	7	1.98	0.31	0.02	Celińska et al., 2013
***Kluyveromyces marxianus* and *Debaryomyces hansenii* (co-culture)**	Batch Shake flask	Lactose	4	2.55	0.89	0.03	Castillo et al., 2022
***Zygosaccgaromyces rouxii* M2013310**	Batch Shake flask	Glucose	-	3.58	-	0.05	Dai et al., 2020
***Saccharomyces cerevisiae* Ye9-612**	Fed-batch 3 L Bioreactor	Glucose	10	4.5	0.82	0.065	Eshkol et al., 2009
***Saccharomyces cerevisiae* BCRC 21812**	Batch 250 mL Bioreactor	Glucose	4	2.53	0.69	0.013	Shu et al., 2021
***Pichia kudriavzevii* YF1702**	Batch Shake flask	Glucose	10.7	5.09	-	-	Fan et al., 2020
***Candida Glycerinogenes* WL2002-5**	Batch 5 L Bioreactor	Glucose	7	5	0.71	0.11	Lu et al., 2016
***Saccharomyces cerevisiae* JM2014**	Batch 6.2 L Bioreactor	Glucose	6	3.60	-	0.05	Chreptowicz et al., 2016
***Kluyveromyces marxianus* CBS 600**	Fed-batch ISPR PPG 1200 2.4 L Bioreactor	Glucose	7	10.2	0.61	0.33	Etschmann et al., 2006
***Saccharomyces cerevisiae* JM2014**	Batch ISPR rapeseed oil 4.5 L Bioreactor	Glucose	5	9.79	-	0.14	Chreptowicz and Mierzejewska, 2018

Table 1.1. (Continued).

Strain	Method	Carbon source	L-Phe (g/L)	2-PE (g/L)	Y(P/S) (g/g)	Rp (g/L.h)	Reference
***Saccharomyces cerevisiae* AM1-d**	Batch ISPR [HMPyr][NTf_2] Shake flask	Glucose, saccharose	6	16.46	-	0.34	Okuniewska et al., 2017
***Kluyveromyces marxianus* WUT240W**	Continuous ISPR Ethyl acetate 4.8 L Bioreactor	Whey	5	1.15	1.12	0.057	Drężek et al., 2021
***Kluyveromyces marxianus* ITD0090**	Batch ISPR Oleyl alcohol membrane contactor 5 L Bioreactor	Glucose	8	3.02	-	0.054	Cordero-Soto et al., 2021
***Kluyveromyces marxianus* CBS 600**	Batch ISPR Hollow fiber, Miglyol 2 L Bioreactor	Glucose	9	4.0	-	0.29	Adler et al., 2011
Saccharomyces cerevisiae	Fed-batch ISPR Pertraction – Adsorption, octane Bioreactor	Glucose	11 x 6.5 g	2.69	0.65	0.498	Červeňanský et al., 2020
***Saccharomyces cerevisiae* Ye9-612**	Fed-batch ISPR Microspheres 3 L Bioreactor	Glucose	10	7.05	-	-	Achmon et al., 2011
Saccharomyces cerevisiae	Fed-batch ISPR Hollow fiber + adsorption Macronet MN270 15 L Airlift Bioreactor	Glucose	45 g	11	-	0.15	Mihal' et al., 2021

Table 1.1. (Conclusion)

Strain	Method	Carbon source	L-Phe (g/L)	2-PE (g/L)	Y(P/S) (g/g)	Rp (g/L.h)	Reference
***Kluyveromyces marxianus* CBS 600**	ISPR Pervaporation Bioreactor	Molasses + glucose	9	2.20	0.34	-	Etschmann et al., 2005
Kluyveromyces marxianus	Semi-continuous ISPR Adsorption Hytrel 8206 resin 3 L Bioreactor	Glucose	26 +	20.4	0.67	0.43	Gao and Daugulis, 2009
***Saccharomyces cerevisiae* BD**	Batch ISPR Adsorption D101 resin Shake flask	Sucrose	12	6.17	0.51	0.23	Mei et al., 2009
***Saccharomyces cerevisiae* P-3**	Batch ISPR Adsorption HZ818 resin Shake flask	Molasses	12	6.6	0.55	-	Hua et al., 2010
Kluyveromyces marxianus	Batch 24-well plates	Sweet whey	4.5	1.2	-	0.025	Alonso-Vargas et al., 2022
***Kluyveromyces marxianus* CBS 6556**	Batch 2 L Bioreactor	Grape must	3	0.77	0.62	0.0077	Garavaglia et al., 2007
***Kluyveromyces marxianus* NRRL Y-1109**	Batch Shake flask	Forestry residues	4	1.09	-	-	Pachapur et al., 2022
Yarrowia lipolytica CH 1/5	Fed-batch Bioreactor	Crude glycerol	8 + 4	3.2	0.29	0.0143	Braga et al., 2021
***Pichia fermentans* WUT36**	Batch Shake flask	Corn stover	5	3.67	-	0.05	Mierzejewska et al., 2019

1.1.2.2. Cultivation process optimization

The 2-PE production process is highly influenced by the cultivation medium composition and reactor operational conditions. These factors affect microbial growth and the activation of pathways that lead to 2-PE production, having influence on the process productivity and consequently, on its relevance (Mitri et al., 2022; Mu et al., 2014).

Regarding the cultivation medium composition, nitrogen source, nutrients and carbon sources are key factors for 2-PE production process. The presence of nitrogen sources besides L-Phe are reported to influence the activation of the Ehrlich pathway. In fact, L-Phe is reported to be a non-preferable nitrogen source, for yeasts as *Saccharomyces cerevisiae*, and in the presence of preferable sources of nitrogen, such as ammonium salts, the expression of some enzymes involved in Ehrlich pathway are low or even negligible (Dai et al., 2021; Liu et al., 2021). So, the use of L-Phe as sole nitrogen source leads to an increase in 2-PE production, not only for *Saccharomyces cerevisiae*, but also for nonconventional yeast such as *Wickerhamomyces anomalus, Candida sp.* and *Kluyveromyces marxianus* (Gethins et al., 2015; Liu et al., 2021; Rodríguez-Romero et al., 2020; Seguinot et al., 2020). However, some studies have shown that the presence of moderate concentrations of yeast extract in the cultivation broth have a positive influence in 2-PE production since it is not only a source of nitrogen, but a source of important growth factors, vitamins and microelements needed for cellular metabolism (Gethins et al., 2015; Fan et al., 2020; Lu et al., 2016;). Fan et al. reported an increase in 2-PE production from values under 2 g/L in the absence of yeast extract, to a 2-PE concentration of 2.77 g/L when 6 g/L of yeast extract was present in the cultivation broth of *Pichia kudriavzevii* (Fan et al., 2020).

Carbon source has proven to be an important factor for cellular growth, L-Phe consumption and 2-PE production. L-Phe consumption is reported to rely on ATP or electrochemical gradient generated from the metabolism of the carbon source, namely, glucose (Lima et al., 2018; Yan et al., 2020). The most reported carbon source for 2-PE production is glucose and an increase in this carbon source concentration usually leads to increased 2-PE production until a certain level, that is related to the microorganism in study (Lima et al., 2018; Fan et al., 2020; Tian et al., 2020). Different carbon sources such as glycerol, xylose, fructose and lactose were also studied for 2-PE production by yeasts, even though the ability to use substrates vary with the metabolism of each strain (Braga

et al., 2021; Castillo et al., 2022; Gethins et al., 2015; Mierzejewska et al., 2019; Yan et al., 2020).

Temperature and pH are also key factors for 2-PE production and culture growth, having influence on the expression and activity of enzymes involved in 2-PE production process, and often the best conditions for cell growth are not the same for 2-PE production (Braga et al., 2021; Huang et al., 2001; Mu et al., 2014; Qian et al., 2019). For instance, *Pichia fermentans* L-5 was found to have the highest cellular growth at 25-30 °C, while the 2-PE production was maximized at 30-35 °C and a pH 8.5. For lower pH values, 2-Phenylethyl acetate, a product of the Ehrlich pathway (Figure 1.1.), was not detected in the broth, but when higher pH values were applied, this aromatic compound was detected in different concentrations (Huang et al., 2001). Mu et al. also reported different suitable conditions for cellular growth and 2-PE production, using a mixed microbial culture. In this case, low pH was favorable for cellular growth (pH 4.5) while a pH of 8 was more beneficial for 2-PE production with a temperature of 35 °C (Mu et al., 2014). On the contrary, for *Yarrowia lipolytica* a decrease in the pH from 7.5 to 5.5 improved the 2-PE production but different conditions of oxygen supplementation did not have significant differences in 2-PE productivity (Braga et al., 2021). *Saccharomyces cerevisiae* also show a preferable temperature for cellular growth (25 °C) that is not coincident with the preferable for the aroma production (35 °C), being the oxygen needs different for these two metabolic processes (an aeration rate of 1.3 vvm is optimal for yeast growth while 2 vvm favors the aroma production) (Shu et al., 2021; Shu et al., 2018). Thus, the conditions that assure the best 2-PE production process are dependent on the microorganism.

Furthermore, the production of 2-PE is also affected by the toxicity of the product itself.

1.1.3. Cytotoxicity of 2-PE

One of the most limiting factors for 2-PE production is its cytotoxicity to the producing organism, similar to what happens with other microbial aroma productions, such as vanillin and benzaldehyde (Jain et al., 2010; Ma et al., 2022). The amphipathic nature of 2-PE interferes with the cell membrane, decreasing the lipid order and increasing membrane fluidity by disrupting the phospholipid components, acting as bacteriostatic agent (Jia et al., 2010; Kleinwächter et al., 2021). This interaction disturbs the nutrient and ion transport, energy generation and communication, affecting cellular homeostasis,

leading to growth inhibition and even cell death (Jia et al., 2010; Nicolaou et al., 2010; Segura et al., 2012; Stark, Zala et al., 2003, Zhan et al., 2023).

The inhibitory mechanism of 2-PE for S*accharomyces cerevisiae* was studied at molecular level, revealing inhibition of nitrogen and ions uptake and feedback repression in the synthesis of alcohol dehydrogenase, that is a crucial enzyme in Ehrlich pathway, resulting in 2-PE production limitation. In the presence of 2-PE, yeasts also enhance the mitochondrial activity and initiate their differentiation by spores formation, instead of budding, indicating a lack in yeast nutrition (Jin et al., 2018). *Kluyveromyces marxianus* was not able to grow or consume glucose after an exposure to 3 g/L of 2-PE and an increase in membrane permeability was noticed accompanied with changes in cell membrane composition to counteract the damages caused by 2-PE (Balbino et al., 2021).

Regarding bacteria, the response of *Bacillus licheniformis* to the contact with 2-PE was investigated (Zhan et al., 2023). This strain is a 2-PE tolerant strain that can tolerate up to 5 g/L of the aroma (Zhan et al., 2020). The 2-PE contact with cells induces reactive oxygen species (ROS) formation, causing damage to DNA, RNA, ribosome, cell wall and metabolism. The cell morphology is also changed under 2-PE stress, with increased width. This stress leads to cellular responses, that go through protein synthesis or stimulation of Tricarboxylic acid (TCA) cycle to increase supply of NAD^+ and $NADP^+$, to deal with oxidative stress. The distribution of the head groups of phospholipids is also changed to avoid the access of 2-PE to the cells. The contact of the aroma with this bacterial strain also induces the downregulation of transporters to reduce energy consumption, meeting the increased energy needs for 2-PE resistance (Zhan et al., 2023).

In fact, 2-PE producing microorganisms are inhibited by the product in concentrations between 2 and 5 g/L, depending on the species. For instance, *Saccharomyces cerevisiae* tolerates 2-PE concentrations below 4 g/L, being the cellular growth completely inhibited at that concentration, while no growth inhibition was observed for concentrations bellow 0.6 g/L (Červeňanský et al., 2020; Chreptowicz et al., 2016; Stark, Zala et al., 2003). On the other hand, *Kluyveromyces marxianus* has a lower tolerance to 2-PE, being able to produce the aroma compound in concentrations below 2 g/L, while in the presence of 3 g/L of exogenous 2-PE a decrease in specific growth rate by 80 % was observed (Balbino et al., 2021; Drężek et al., 2021; Gao and Daugulis, 2009). Regarding bacteria, *Bacillus licheniformis* DW2 was reported to tolerate 5 g/L of 2-PE, having a long lag period (16

h) in these conditions, with the growth being completely impaired in the presence of 6 g/L of the aroma compound (Zhan et al., 2020).

To improve 2-PE production, strategies to overcome product toxicity are being developed. These strategies include genetic modifications of the microorganisms to increase the tolerance towards the desired product and find alternatives to remove the product form the cultivation broth, while it is being produced, allowing the microorganism to continue 2-PE production (Qian et al., 2019; Wang et al., 2019; Wang et al., 2020).

1.1.4. *In situ* product removal and product recovery

The cytotoxicity of 2-PE towards the producing microorganism is a bottleneck for flavor production, limiting the viability of the process and therefore its implementation at industrial scale due to the low titer and productivity. To improve 2-PE production process, *in situ* product removal (ISPR) techniques have been extensively studied. These processes consist of removing the product that causes inhibition from the fermentation broth as soon as it is produced. This strategy avoids the exposure to high concentrations of the product with the producing microorganism and consequently allows the continuous production, usually at maximal production rates and for longer periods, resulting in higher productivities (Santos et al., 2020; Teke et al., 2022). The use of ISPR techniques can also minimize product loss by degradation or uncontrolled evaporation and facilitate the downstream processes, thus improving the process feasibility (Santos et al., 2020; Stark and von Stockar, 2003; Teke et al., 2022).

To increase 2-PE production several approaches based on the use of ISPR techniques have been studied, such liquid-liquid extraction and adsorption, membrane-based processes and supercritical fluids (Mitri et al., 2022).

Liquid-liquid extraction is one of the most reported methods and is based on the difference in the solubility of the target product in two immiscible liquids (Teke et al., 2022). The process involves an aqueous phase, where the biotransformation occurs and an organic phase with high affinity for 2-PE, where the aroma is retained. The selection of the solvents is crucial for the process. To be considered a suitable extractant, the solvent must have specific characteristics. The solvent cannot be toxic to the microorganism or reactive with other components of the cultivation media and cannot be available for use

as carbon source by the microorganism. It has to be chemically and thermally stable, non-flammable, low viscous and inexpensive. The solvent has to be water immiscible and provide a good phase separation from the aqueous phase and the target product should have a large partition coefficient in the solvent. The product should be recoverable from the solvent and, regarding the production of flavor compounds, the solvent must be approved for use in food processing (Santos et al., 2020; Stark et al., 2002; Teke et al., 2022). However, hydrophobic solvents can interact with the membrane of the microorganism and may have a harmful effect, but these limitations vary with the microorganisms and their membranes' characteristics (Santos et al., 2020).

The use of liquid-liquid extraction for 2-PE production has been reported to improve the aroma production process, up to 10 g/L, as it can be seen in Table 1.1, using solvents as oleic acid, rapeseed oil, fatty acid methyl ester (FAME), polypropylene glycol, biodiesel, ionic liquids (IL) and ethyl acetate (Chreptowicz and Mierzejewska, 2018; Dręż ek et al., 2021; Etschmann and Shrader, 2006; Hua et al., 2013; Lukito et al., 2019; Stark et al., 2002; Teke et al., 2022; Yan et al., 2020). However, some limitations were identified using these solvents, for instance the presence of oleic acid and FAME were reported to have a negative influence in cellular growth and viability (Stark et al., 2002, Yan et al., 2020). The formation of stable emulsions during bioreactor cultivation in the presence of oleic acid, hindering phase separation and the on-line monitoring of biomass was also described (Stark et al., 2002). Even though IL are commonly reported to be green solvents with unique solubility properties and high stability, their high costs and the potential risk of toxicity during the extractive fermentation is still a weakness in using these solvents, so they are under continuous investigation (Teke et al., 2022).

Adsorption of the product of interest is also reported using a solid material that has high affinity for the product. These materials are non-volatile, non-biodegradable and low cost. The use of these adsorbents has advantages over organic solvents, such as not affecting product organoleptic properties or being toxic to the producing microorganisms (Mitri et al., 2022; Qian et al., 2019). This is a mild technique able to absorb products with both high and low molecular mass. This technique can be performed in columns through which the fermentation broth can contact until the full capacity of the material to adsorb the product is achieved (Teke et al., 2022). However, the capacity of the adsorbents for the target product might decrease due to nonspecific interactions with cells, cellular debris, medium components and byproducts (Santos et al., 2020; Teke et al., 2022).

Several polymeric adsorbents have been reported to enhance 2-PE production process by coupling the product adsorption to the cultivation, as it can be seen in Table 1.1. To be a suitable adsorbent for 2-PE, the polymer should be able to interact with the functional groups of 2-PE, the aromatic ring and/or the hydroxyl group by π or hydrogen interactions, respectively (Gao and Daugulis, 2009). The crystallinity degree is also an important characteristic for product adsorption, since the polymeric material should have space to accommodate the product, and this can be achieved by having an amorphous structure (Gao and Daugulis, 2009).

Non-polar polymeric adsorbents, as the macroporous resins D101 (from Chemical Plant of Nankai University), HZ818 and FD0816 (both from Shanghai Huazhen Science & Technology Co., Ltd.), with a cross-linked polystyrene structure that allows the interaction with the product through the aromatic ring, have been used to improve 2-PE production by different *Saccharomyces cerevisiae* strains (Hua et al., 2010; Mei et al., 2009; Wang, Dong, Guan et al., 2011; Wang, Dong, Meng et al., 2011). Even though these polymers are able to interact with both 2-PE and L-Phe, they have more affinity for the aroma than the precursor. The block copolymer resins Hytrel G3548 and Hytrel 8206 (from DuPont), composed of polybutylene ester and polyether, are able to interact with the hydroxyl group of 2-PE by hydrogen interactions, and were evaluated to improve the aroma production by *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, respectively (Gao and Daugulis, 2009; Shu et al., 2021). The use of these polymers was reported in batch cultivation experiments and the amount of polymer used varied between 2 and 17% w/v, allowing the production of 4 to 14 g/L of 2-PE. Increasing the amount of resin in contact with the cultivation broth also increases the aroma production, so semicontinuous processes have been attempted applying the resin in filter cloth bags inside the bioreactor or in columns through which the cultivation broth passes (Gao and Daugulis, 2009; Wang, Dong, Guan et al., 2011; Wang, Dong, Meng et al., 2011). For instance, Wang et al. increased the production of 2-PE by *Saccharomyces cerevisiae* R-UV3 using the non-polar macroporous resin FD0816, from 4.38 g/L in a fed-batch fermentation process to 13.7 g/L when using 132 g of dry resin in a 3 L bioreactor (Wang, Dong, Guan et al., 2011; Wang, Dong, Meng et al., 2011). By replacing the resin and making the process semicontinuous, the 2-PE production increased to 32.5 g/L with a production rate of 0.54 g/L.h. Passing the cultivation broth through the columns filled with resin, the production rate could even increase to 0.9 g/L.h, being the fouling of the

membrane used to separate the biomass, the limiting step. Likewise, Gao and Daugulis could improve the aroma production by *K. marxianus*, from 1.45 g/L of 2-PE in a single-phase batch run, to 13.7 g/L using 500 g of Hytrel 8206 (Gao and Daugulis, 2009). Further, in a 3 L bioreactor cultivation operated on a fed-batch mode, the increase of resin to 900 g led to a 2-PE production of 20.4 g/L, with an overall productivity of 0.43 g/L.h. Using this strategy, only 1.4 g/L of 2-PE were present in the aqueous phase, avoiding product toxicity.

Different strategies for *in situ* product adsorption were also investigated, namely the entrapment of an organic solvent, dibutylsebacate into a polymeric matrix of polyethylene or alginate to form a stable resin adsorbent, as well as the use of a polydimethylsiloxane sponge or hydrophobic polymethylmetacrylate microspheres (Achmon et al., 2011; Serp et al., 2003; Shu et al., 2018; Stark, Kornmann et al., 2003).

Membrane based processes for 2-PE separation are also being investigated and membrane based solvent extraction is the most reported process, as it is summarized in Table 1.1, allowing 2-PE production up to 21 g/L (Mihal' et al., 2014; Adler et al., 2011; Cordero-Soto et al., 2021). This approach is based on a liquid-liquid extraction process where an organic solvent, that acts as extractant, passes through a hollow fiber membrane module and contacts with the cultivation media, being the product continuously extracted for the organic phase (Cordero-Soto et al., 2021; Etschmann et al., 2005; Mihal' et al., 2012; Mihal' et al., 2014). These hollow fiber contactors can be advantageous by increasing the contact area with the solvent and avoiding the formation of emulsions with the aqueous media. Nevertheless, the membrane pores can be clogged with the biomass produced in the cultivation process, decreasing the efficiency of the process (Etschmann et al., 2005; Qian et al., 2019; Teke et al., 2022). These approaches can be applied as submerged membranes in the cultivation broth (pertraction) or as separated modules in an external loop.

Organophilic pervaporation and supercritical CO_2 were also reported for continuous 2-PE extraction from the production fermenter, but with limited improvement in aroma production due to the high temperatures needed for favoring the process driving force and the toxicity of the CO_2 to the microorganisms, respectively (Fabre et al., 1999; Etschmann et al., 2005).

Alternatives using combinations of these approaches are also being investigated to overcome the toxicity of 2-PE that limits further aroma production, for instance combination of liquid-liquid extraction and adsorption (Červeňanský et al., 2020; Lukito et al., 2019; Mihal' et al., 2021).

The processes for ISPR described above showed improvements of 2-PE production, but there are several ways to do it. The choice of an extraction method to connect to the fermentation process should be based in the product physical, chemical and biochemical characteristics, such as molecular mass, volatility, solubility, charge and hydrophobicity. Moreover, it should meet the requirements of purity, according to the applications (Teke et al., 2022). Due to the low molecular weight of 2-PE and hydrophobicity, ISPR techniques such as gas stripping, solvent extraction, pertraction, vacuum distillation, adsorption, solvent extraction, use of supercritical fluids, evaporation, permeation and immobilization are techniques suitable to be considered in ISPR processes for 2-PE extraction (Teke et al., 2022).

1.1.5. Alternative substrates to produce 2-PE

Agro-industrial wastes are known to be a source of minerals, proteins and sugars with a growing potential to be used in circular economy-based approaches (Yaashikaa et al., 2022). The use of these low value nutrition-rich materials to produce value-added compounds, contribute to promote a sustainable management of the residues, reducing the waste disposal to the environment and decreasing the production costs, due to its high availability and low price (Mitri et al., 2022; Yaashikaa et al., 2022). Though, these raw materials have a variable composition and often need pretreatments to be used (Mitri et al., 2022). The use of agro-industrial wastes as carbon sources in 2-PE production processes are being investigated in submerged fermentation and solid-state fermentation technologies. Examples are summarized in Table 1.1.

Different wastes and byproducts were reported to be used as carbon sources for 2-PE production by yeasts, in submerged fermentation systems (Table 1.1). Some of these residues needs hydrolysis processes to release fermentable sugars from the complex polymers. For instance, corn stover, rich in glucose and xylose, was hydrolyzed in alkaline conditions, while forestry residues, as bark of pine wood, rich in glucose, had to pass through acid hydrolysis and both were supplemented with L-Phe to allow 2-PE

production (Mierzejewska et al., 2019; Pachapur et al., 2022). Grape must, rich in glucose and fructose, whey, rich in lactose and proteins and crude glycerol were also used as part of the cultivation media of different yeasts, in the presence of L-Phe (Alonso-Vargas et al., 2022; Braga et al., 2021; Castillo et al., 2022; Chreptowicz and Mierzejewska, 2018; Garavaglia et al., 2007). These media supported the production of 0.77 to 6.37 g/L of 2-PE as represented in Table 1.1.

Some residues are also being investigated to produce 2-PE by *de novo* synthesis, in absence of L-Phe, using tequila vinasses and cheese whey. Nevertheless, the production by this metabolic pathway leads to lower 2-PE productions (0.065 and 0.96 g/L), comparing with Ehrlich pathway for wild type microorganisms (Conde-Báez et al., 2017; Rodríguez-Romero et al., 2020). Though, metabolically engineered microorganisms are also being investigated to produce 2-PE from low value feedstocks, as *Bacillus licheniformis,* that could produce 6.43 g/L of 2-PE from a combination of molasses and crude glycerol (Xu et al., 2023).

The aforementioned processes are based in submerged fermentation systems, but solid-state fermentation strategies are also being studied, using sugar cane bagasse or red apple pomace supplemented with L-Phe, resulting in 20.1 or 25 mg of 2-PE per gram of used waste (Martínez-Avila et al., 2019; Martínez-Avila et al., 2021).

Despite the promising results obtained with these low value wastes and byproducts, to the best of my knowledge, the use of a feedstock that could work simultaneously as carbon and L-Phe source was not reported. Besides the low L-Phe content in the wastes, the presence of other nitrogen sources, such as different amino acids, influence the Ehrlich pathway efficiency for 2-PE production (Martínez-Avila et al., 2021; Castillo et al., 2022). Thus, the media supplementation with L-Phe is an essential step for an efficient and high titer 2-PE production process.

1.2. 2-PE properties and possible applications

Even though 2-PE is being used in various fields, more applications are being currently investigated mainly in health and food areas. Studies showed that 2-PE can be used as odorant in olfactory training to improve the olfactory function in patients with post-infectious olfactory dysfunction or loss of smell, caused by respiratory tract viruses (Addison et al., 2021). The aroma is also reported to have sedative effects when

administered by inhalation to mice, causing a significant decrease in the amount of spontaneous motor activity in a dose dependent manner (Oshima and Ito, 2021). Other studies in mice, showed that 2-PE can have anti-depressive effects, by acting on central nervous system, suggesting that inhalation of rose oil, that is rich in 2-PE, may be effective against depression and stress-related diseases (Ueno et al., 2019). Similarly, after corticosterone treatment to induce an anxio-depressive-like phenotype in female mice, a prolonged 2-PE inhalation allowed the regulation of the neural circuits' activity, involved in sensory, emotional and feeding behaviors (Ramadan et al., 2022).

Considering the antimicrobial properties, 2-PE can increase antifungal effect when used in combination with Fluconazole and Itraconazole, having potential to be applied in chronic and recurrent candidal vulvovaginitis in animal models (Majdabadi et al., 2018). Rowley et al. evaluated the 2-PE usage in biomaterials, namely polyglobalide-based organogels that showed antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* (Rowley et al., 2020). Together with its rheology modifier properties, these materials are promising candidates to be employed in personal care products for the prolonged release of fragrant molecules and as antimicrobial gels. Zhu et al. found that 2-PE has antityrosinase and antimicrobial activities towards *E. coli* and *Ralstonia solanacearum.* These bacteria are known to cause disease in various food crops such as tomato, banana and ginger and, the enzyme tyrosinase that causes the browning in fruits and vegetables, can be reversibly inhibited by the aroma molecule (Zhu et al., 2011). Still related with its antimicrobial activity, 2-PE could suppress *Botrytis cinerea* growth *in vitro* and in strawberries, reducing the disease without affecting the fruit quality, by inducing reactive oxygen species stress and cell membrane disruption, making it a promising natural alternative to control gray mold in strawberries (Zou et al., 2022). 2-PE was also investigated to protect citrus from infection with *Penicillium* molds and it could inhibit blue and green mold *in vitro* and *in vivo*, not affecting the product quality (Liu et al., 2014).

1.3. Biotechnological potential of *Acinetobacter* genus

Bacteria from *Acinetobacter* genus are ubiquitous, being present in water, soil, human skin, plants, animals, food material, domestic appliances and even in hydrocarbon-contaminated locations (Adewoyin and Okon, 2018; Jung and Park, 2015).

Acinetobacter strains, due to the versatility of their metabolism, have been investigated for the production of valuable compounds as polysaccharides, biosurfactants, bioemulsifiers, lipases and polyesters (Abdel-El-Haleem, 2023; Pirog et al., 2021). Among bioemulsifiers, Alasan and Emulsan are produced by *Acinetobacter* strains, as *A. calcoaceticus and A. radioresistance*, and are known for their commercial applications in microbial enhanced oil recovery and biodegradation of toxic compounds (Mujumdar et al., 2019). These bioemulsifiers are high molecular weight biodegradable compounds consisting of heteropolysaccharides, proteins, lipoproteins and lipopolysaccharides, that due to its amphiphilic nature have affinity for the oil-water interface, increasing the availability of the hydrocarbons and oils, allowing them to be degraded by microorganisms (Al-Hadithi et al.,2017; Mujumdar et al., 2019; Zhao et al., 2016). The production of polyhydroxyalkanoates, biodegradable and plastic-like polymers, have also been identified in *Acinetobacter* strains as *Acinetobacter* sp. AAAID-1.5, *A. calcoaceticus* UMTKB-8 and *Acinetobacter* sp. ASC1, using oils and glycerol as carbon source (Alsaadi et al., 2022; Idris et al., 2023; Muangwong et al., 2016). Lipases, that have a wide range of applications in hydrolysis, esterification, transesterification, acidolysis and aminolysis, can also be produced by *Acinetobacter* strains, as A*cinetobacter* sp. AU07 and *A. haemolyticus* (Al-Limoun et al., 2019; Gururaj et al., 2016; Sarac and Ugur, 2016).

Bacteria from *Acinetobacter* genus are demonstrating a high potential for various environmental and industrial biotechnological applications. Even though the use of aromatic compounds was reported in literature, the production of 2-PE was not previously described for these microorganisms (Abdel-El-Haleem, 2023, Jung and Park, 2015).

1.3.1. *Acinetobacter soli* ANG344B

Acinetobacter soli ANG344B was isolated from river water obtained from the Catumbela river, in Benguela Province (Angola, 2013) as part of a project aiming the detection of antibiotic resistant bacteria or presenting biotechnological potential from environmental sources. During the laboratory screening performed with a river water sample, it was perceived that this strain was able to emit a rose-flavor, prompting its identification and further exploration of genomic characteristics.

Analysis of *A. soli* ANG 344B genomic features revealed that this isolate displayed a limited presence of virulence factors and antimicrobial resistance genes compared to *Acinetobacter* species commonly associated with infections. The presence of genes involved in the catabolism of aromatic compounds was also identified, including salicylate ester, quinate, biphenyl, benzoate and p-hydroxybenzoate degradation, indicating the potential capability of the *A. soli* ANG344B strain to handle and process these types of compounds.

The search for enzymes involved in L-Phe metabolism and potentially responsible for 2-PE production, revealed the presence of the genes coding for the aromatic aminotransferase (AAAT) (EC 2.6.1.57) responsible for transamination of L-Phe to α-ketoglutarate resulting in phenylpyruvate, the alpha-keto acid decarboxylase (KDC) responsible for decarboxylation of phenylpyruvate to phenylacetaldehyde (EC 4.1.1.1) and, alcohol dehydrogenase (ADH) (EC 1.1.1.1) that catalyzes the oxidation of 2-PE to aldehyde and ketone, respectively, and also can catalyze the reverse reaction.

The ability to produce 2-PE was not previously reported for this genus and is not common for bacteria. So, understanding the biosynthetic and metabolic potential of this strain is crucial for exploiting and harnessing its 2-PE production capabilities.

1.4. Overview

The increasing demand for natural products has led to the development of biotechnological alternatives to 2-PE production, namely using microorganisms to produce the desired flavor. Several yeasts can produce 2-PE from L-Phe by Ehrlich pathway, being the most relevant microorganisms reported for this aroma production. Bacteria, especially wild-type bacteria, have a limited ability to produce this aroma and very few reports are found in literature, usually with trace amounts of 2-PE being produced. Even though genetically modified microorganisms can achieve 2-PE production in the same range as yeasts, the concerns about these organisms hinder their widespread adoption. So, improvement of microbial 2-PE production process to become economically and industrially attractive is being investigated, leading to the discovery of more productive species and the development of improved and cost-effective processes. This is being attempted by using techniques to avoid product toxicity as *in situ* product removal approaches and low-cost feedstocks, to reduce the production costs and make

the process more environmentally friendly. At the same time, the study of 2-PE application in various fields, such as in health and food preservation, besides the more conventional and stablished uses, will continue to increase the demand for this compound.

2.

Optimization of 2-phenylethanol production from L-phenylalanine by *Acinetobacter soli* ANG344B

The results presented in this chapter are part of the following published paper:

Bernardino, A.R.S, Torres, C. A. V., Grosso, F., Peixe, L., Reis, M. A. M. (2024). Optimal conditions for 2-phenylethanol production from L-phenylalanine by *Acinetobacter soli* ANG344B. *Journal of Chemical Technology and Biotechnology, 99(3),* 746-754. https://doi.org/10.1002/jctb.7582

2.1. Summary

2-Phenylethanol (2-PE) is a valuable flavor compound renowned for its rose-like scent and widespread application in the cosmetic, perfume, and food industries. While it can be naturally sourced from plants, the majority is currently produced through chemical synthesis, leading to environmental concerns. As an eco-friendly alternative, biotechnological production of 2-PE presents a promising solution, yielding a natural product. In this work, the newly isolated bacterium, *Acinetobacter soli* ANG344B was used to produce 2-PE from L-phenylalanine (L-Phe), using glucose as carbon source, aiming to determine the most favorable conditions for cellular growth and aroma production. The selected conditions for *Acinetobacter soli* ANG344B cultivation and 2-PE production were 30 °C and pH 7. Further, yeast extract proved to be a key element for 2-PE production. The concentration of L-Phe that maximizes 2-PE volumetric productivity was found to be 5 g/L, that should be present in the cultivation media from the beginning of the cultivation. Under these conditions 2.14 ± 0.18 g/L of the aroma compound was produced, which is the highest concentration obtained for a wild-type bacteria. The toxicity of 2-PE was also evaluated, revealing that cellular growth of *A. soli* ANG344B was adversely affected at concentrations above 1 g/L, and beyond 4 g/L no cellular growth was observed. Elucidating the crucial factors affecting 2-PE production by *Acinetobacter soli* ANG344B is a step forward for the development of biotechnological strategies to harness its potential as a natural and sustainable aroma compound.

2.2. Introduction

The ability to produce 2-PE has been reported in several microorganisms, mainly yeasts such as *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Pichia fermentans*, *Yarrowia lipolytica* and *Wickerhamomyces anomalus*, among others. These microorganisms are reported to produce 2-PE in concentrations usually bellow 4 g/L, using L-phenylalanine (L-Phe) as precursor via Ehrlich pathway (Alonso-Vargas et al., 2022; Celińska et al., 2013; Chreptowicz and Mierzejewska, 2018; Dai et al., 2021; Gao and Daugulis, 2009; Huang et al., 2001; Shu et al., 2021; Tian et al., 2020). The bacterial production is less common and reported in concentrations under 152 mg/L for wild-type bacteria, such as *Brevibacterium* sp., *Microbacterium* sp., *Proteus vulgaris*,

Psychrobacter sp., *Erwinia carotovora* and *Proteus mirabilis* (Deetae et al., 2007; Jollivet et al., 1992; Liu et al., 2018; Spinnler and Djian, 1991). Further studies regarding bacterial 2-PE production involve metabolic engineering strategies (Zhang et al., 2014; Zhu et al., 2023).

2-PE production process is dependent on the producing strain, but the medium composition and cultivation conditions also play an important role in aroma production. These factors affect microbial growth, but also the metabolism leading to 2-PE production (Mitri et al., 2022).

The 2-PE production via Ehlich pathway, the one identified for 2-PE production by *Acinetobacter soli* ANG344B here studied, consists in three steps: transamination, decarboxylation and reduction. The use of L-Phe as sole nitrogen source is reported to favor the Ehrlich pathway, thus improving the process yield (Qian et al., 2019). Carbon sources can also influence cellular growth and 2-PE production. The most reported carbon source for 2-PE production is glucose, that usually leads to higher cell growth and 2-PE production when compared to lactose, glycerol, fructose and xylose (Gethins et al., 2015; Yan et al., 2020). However, the ability of the microorganism to use different substrates vary with the strain used. The amount of glucose also impacts on aroma production and usually, an increase in glucose concentration in the cultivation media leads to improved 2-PE productions, until a certain limit, that is strain dependent (Lima et al., 2018). Temperature and pH are also important factors to consider when optimizing the 2-PE production process, since they influence the activity of enzymes involved in the catabolism of L-Phe (Qian et al.,2019). The optimal temperature and pH for cellular growth is often different from those favoring the 2-PE production (Braga et al., 2021; Huang et al., 2001; Mu et al., 2014). These factors not only affect 2-PE concentrations but also the presence of other products of Ehrlich pathway, such as 2-Phenylethyl acetate (Huang et al., 2001). For yeasts, the optimal temperature for growth is reported to be in the range of 25 to 30 °C, while 2-PE production is reported to be enhanced at temperatures in the range of 30 to 35 °C (Huang et al., 2001; Mu et al., 2014).

One of the limiting factors to 2-PE production is the product toxicity towards the producing microorganism (Mitri et al, 2022; Qian et al., 2019). In fact, the amphiphilic nature of 2-PE allows its interaction with the cell membrane, increasing the membrane fluidity and affecting cellular homeostasis (Jia et al., 2010; Kleinwächter et al., 2021). Concentrations of 2-PE between 2 and 4 g/L are reported to inhibit the producing

microorganism. For instance, *Kluyveromyces marxianus* are reported to tolerate 2-PE concentrations below 3 g/L, above this concentration the specific growth rate decreased 80% (Balbino et al., 2021; Drężek et al., 2021; Gao and Daugulis, 2009). *Saccharomyces cerevisiae* present a higher tolerance, but above 4 g/L of 2-PE there is a complete inhibition of growth (Červeňanský et al., 2020; Chreptowicz et al., 2016; Stark, Zala et al., 2003).

Different *Acinetobacter soli* species have already demonstrated additional promising biotechnological potential, as evidenced by its capacity to produce a novel type I l-asparaginase that holds significant promise for applications within the food processing industry, and other *Acinetobacter* strains have been explored for the production of valuable bio-products, including commercially available bioemulsifiers such as emulsan, biodispersan, and alasan (Jiao et al., 2020; Nguyen, 2018).

Acinetobacter soli ANG344B was recently isolated and its capability to produce 2-PE identified, being able to produce this aroma in levels not previously reported for wild-type bacteria and only identified in yeasts. The aim of the present work is to evaluate the impact of different parameters on 2-PE production by *A. soli* ANG344B, from L-Phe biotransformation. A screening of different conditions regarding medium composition, temperature, pH and aeration was performed and the toxicity of the product towards the microorganism was accessed. This study reveals the potential of *A. soli* ANG344B as a promising new platform for 2-PE production.

2.3. Materials and methods

2.3.1. Microorganism and inoculum preparation

Acinetobacter soli ANG344B strain was preserved in cryovials containing glycerol (20 wt.%), at –80 °C.

Reactivation from the stock cultures was performed in Luria-Bertani (LB) agar medium plates (bacto-tryptone, 10 g/L; yeast extract, 5 g/L; sodium chloride, 10 g/L; agar, 18 g/L), sterilized in autoclave at 121 ˚C, 1 bar for 20 minutes.

The inoculum was prepared by growing the bacterial cells in 100 mL of LB medium at 30 °C, 200 rpm, for 20 h in an orbital shaker (until optical density measured at 600 nm (OD_{600}), reaches approximately 3).

2.3.2. Cultivation assays in shake-flasks

Cultivation was carried out in 500 mL baffled shake-flasks with a working volume of 200 mL of the culture medium (composition described below) using 5% (v/v) of inoculum. The culture media was previously sterilized in autoclave at 121 ˚C, 1 bar for 20 minutes. The culture was incubated at controlled temperature (30 °C) in an orbital shaker, 200 rpm, and pH 7 that was corrected periodically by addition of 5 M NaOH or 5 M HCl.

2.3.2.1. Medium composition screening

The best cultivation media for the bacterium cellular growth and 2-PE production was evaluated in shake flask assays. Six different media, from medium M1 to medium M6 listed in Table 2.1, were tested.

Table 2.1 Culture media used in this study.

Medium	Composition (per Liter)
M1	LB medium (tryptone, 10 g; NaCl, 10 g; yeast extract, 5 g); L-Phe, 5 g
M2	beef extract, 3.12 g; peptone, 20.87 g; NaCl, 1.04 g; L-Phe, 5 g
M3	beef extract, 3 g; peptone, 10 g; NaCl, 5 g; L-Phe, 5 g
M4	Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; $MgSO_4$ 1 M, 2 mL; $CaCl_2$ 1M, 0.1 mL; glucose anhydrous, 5 g; L-Phe, 5 g
M5	Na_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCl, 0.2 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2$, 0.05 g; yeast extract 5 g glucose anhydrous, 10 g; L-Phe, 5 g
M6	Na_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCl, 0.2 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2$, 0.05 g; NH_4Cl, 1.91 g; glucose anhydrous, 10 g; L-Phe, 5 g
M7	Na_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCl, 0.2 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2$, 0.05 g; glucose anhydrous, 10 g; L-Phe, 5 g

2.3.2.2. Influence of nitrogen source on 2-PE production

To assess the impact of the source of nitrogen, medium M7 listed in Table 2.1, was tested and a comparison was made between media M5, M6 and M7.

2.3.2.3. Influence of cellular growth phase in 2-PE production

To evaluate if 2-PE production occurs during the stationary growth phase, *A. soli* ANG344B was cultivated in medium M5 in absence of L-Phe for 24 h (shake flask

assays), until reaches the stationary growth phase. At that time, 50 mL of 20 g/L L-Phe solution was added to the cultivation broth to start the 2-PE production.

2.3.2.4. Influence of temperature and pH on cellular growth and 2-PE production

Acinetobacter soli ANG344B was cultivated in medium M5 at 3 different pH, namely pH 5, 7 and 8 in shake flask assays. For each pH, 3 different temperatures were tested, namely 23, 30 and 37 °C.

2.3.2.5. Evaluation of 2-PE concentration effect on the cellular toxicity

2-PE cellular toxicity was evaluated using medium M6 in the absence of L-Phe, by addition of 2-PE in a range of 0 to 5 g/L, in shake flask assays. These experiments were conducted without L-Phe in the cultivation medium to avoid increasing 2-PE concentration in the broth, through the biotransformation of this amino acid.

2.3.3. Bioreactor operation

Bioreactor assays were carried out using the most favorable conditions (standard conditions) for 2-PE production found in the shake flask cultivations, regarding temperature and pH. Therefore, 2 L bioreactors (BioStat B-plus, Sartorius, Germany) (Figure 2.1) were performed using as cultivation medium the medium M5 (Table 2.1) and inoculated with 5% (v/v) of inoculum prepared in LB medium. Temperature was controlled at 30.0 ± 0.1 ºC and pH at 7.00 ± 0.02 by the automatic addition of 5 M NaOH and 5 M HCl. The aeration rate (1 vvm, volume of air per volume of reactor per minute) was kept constant during the experiments. The dissolved oxygen concentration was controlled at 30% saturation by the automatic variation of the stirring rate between 300 and 1000 rpm given by the two six blade impellers. To avoid evaporation losses, the bioreactor condenser was maintained at 3 °C, by cooling recirculation. Foam formation was prevented by automatic addition of silicone antifoaming liquid (AQ).

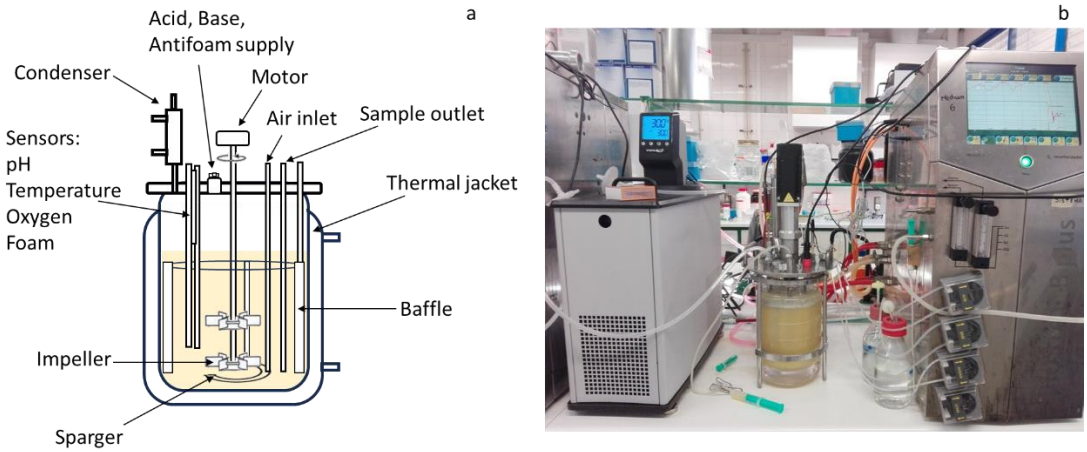

Figure 2.1 Schematic representation of bioreactor (a) and experimental set-up used for 2 L (b) bioreactor cultivation of *Acinetobacter soli* ANG344B.

Variations to the standard conditions described above were evaluated in 2 L bioreactors, increasing the aeration rate to 2 vvm and varying L-Phe concentrations to 2.5 and 7 g/L with an aeration rate of 1 vvm.

The most favorable conditions for 2-PE production determined in 2 L bioreactors (standard conditions) were upscaled to 10 L bioreactor (BioStat B-plus, Sartorius, Germany).

2.3.4. Analytical techniques

Culture broth samples were taken periodically, the optical density was measured at 600 nm and the samples (2 mL for shake flask experiments and 5 mL for bioreactor experiments) were centrifuged at 9056 g, for 15 min at 4 °C, for cell separation. The cell-free supernatant (stored at −20°C) was used for quantification of L-Phe, 2-PE and glucose. Cell pellet was washed twice with deionized water and lyophilized for gravimetrical cell dry weight determination.

2.3.4.1. *L-Phenylalanine and 2-Phenylethanol quantification*

L-Phe and 2-PE concentrations were determined by high performance liquid chromatography (HPLC) (Alliance) using a C18 3.9 mm×150 mm column (particle diameter of 4 mm) (Nova-Pak®) and guard column, coupled to a PDA (Photo Diode Array) detector, set at 215 nm. Samples were diluted in deionized water and filtered

through 0.22 µm centrifuge filters. The injection volume was 20 µL and compounds were separated at 25 ˚C. A gradient method comprising water/acetonitrile was applied as follows: 0-15 min 90/10 (flow 0.5 mL/min), 15-28 min 70/30 (flow 0.5 mL/min); 28-40 min 90/10 (flow 0.5 mL/min). L-Phe (reagent grade, Sigma-Aldrich) and 2-PE (≥99%, Sigma-Aldrich) were used as standards in concentrations between 0.012 and 0.2 g/L.

2.3.4.2. Glucose quantification

For glucose quantification, the cell-free supernatant was filtered through 0.22 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. Samples were separated during 15 min at 30 ˚C with 0.01 N H_2SO_4 as eluent at the flow rate of 0.5 mL/min. D (+) glucose anhydrous (Fisher Scientific) was used as standard, at concentrations between 0.03 and 1.0 g/L.

2.3.5. Kinetic and stoichiometric parameters calculation

The maximum specific cell growth rate μ_{mas} (h^{-1}) was calculated using the following equation:

$$\ln\left(\frac{x}{x_0}\right) = \mu_{max} \times t \qquad (2.1)$$

Where x and x_0 (g/L) represent the cell concentration in the moment t and in the beginning of the run, respectively.

The yield of biomass on substrate ($Y_{X/S}$, $g_{CDW}/g_{substrate}$) was calculated using the following equation:

$$Y_{X/S} = \frac{\Delta X}{\Delta S_1} \qquad (2.2)$$

Where ΔX represents the biomass produced during the process and ΔS_1 represents the substrate consumed for cell growth.

The yield of product (2-PE) on substrate (L-Phe) ($Y_{P/S}$, $g_{2\text{-PE}}/g_{\text{L-Phe}}$) was determined by the following equation:

$$Y_{P/S} = \frac{\Delta P}{\Delta S_2} \qquad (2.3)$$

Where ΔP (g/L) corresponds to the 2-PE produced through the consumption of L-Phe, ΔS_2(g/L).

The volumetric productivity (r_p, $g_{2\text{-PE}}$/L.h) was calculated using the following equation:

$$r_p = \frac{\Delta P}{\Delta t} \quad (2.4)$$

Where Δt (h) corresponds to the time interval considered for the 2-PE production.

2.3.6. Statistical Analysis

Experiments were performed as a triplicate of independent samples. Data are expressed as the mean ± standard deviation. The results were statistically analyzed using one-way ANOVA ($p < 0.05$).

2.4. Results and Discussion

2.4.1. Growth medium composition screening

The *Acinetobacter soli* ANG344B strain was grown in six different media (M1 to M6) in the presence of L-Phe to evaluate the impact of medium composition on cell growth and 2-PE production. The media composition was based on the growth media reported for other *Acinetobacter* species, for other aroma producing bacteria or for soil bacteria (Beshay et al., 2002; Chen et al., 2012; Delneri et al., 1995; Sunitha et al., 2010; Valério et al., 2021; Wang, Sheng et al., 2011).

With the exception of medium M4, all the tested media were able to support cell growth and L-Phe consumption, as represented in Table 2.2. Medium M5 was the one that supported the highest cell growth (cell dry weight (CDW) reached 2.4 ± 0.2 g/L) and 2-PE production (1.77 ± 0.07 g/L).

Table 2.2 Growth media screening: 2-PE production, production yield ($Y_{P/S}$), volumetric productivity (r_p) and cell dry weight (CDW).

Medium	2-PE (g/L)	Y(P/S) (g/g)	r_P (g/L h)	CDW (g/L)
M1	1.40 ± 0.04	0.48 ± 0.02	0.05 ± 0.00	1.3 ± 0.1
M2	0.98 ± 0.07	0.41 ± 0.01	0.03 ± 0.00	1.2 ± 0.1
M3	1.14 ± 0.03	0.45 ± 0.02	0.04 ± 0.00	1.3 ± 0.2
M4	-	-	-	0.6 ± 0.1
M5	1.77 ± 0.07	0.48 ± 0.02	0.06 ± 0.00	2.4 ± 0.2
M6	1.40 ± 0.04	0.42 ± 0.01	0.04 ± 0.00	1.8 ± 0.1
M7	0.87 ± 0.09	0.93 ± 0.03	0.03 ± 0.01	1.5 ± 0.1

Media M4 and M6 have the same components; however, no cellular growth or 2-PE production was observed in medium M4. This lack of activity is likely attributed to the distinct proportions of components in each medium. Medium M4 exhibited higher levels of phosphate, magnesium and sodium and lower content of calcium compared to medium M6. These differences had a detrimental effect on bacterial cellular growth and consequently, on 2-PE production. It's noteworthy that the content of calcium, magnesium, and phosphate in growth media has been reported to influence 2-PE production by other microorganisms, albeit to varying degrees. Elevated concentrations of phosphate (6 g/L KH_2PO_4) and magnesium sulfate (0.5 g/L) were found to be beneficial for yeasts, showcasing the nuanced impact of these components on microbial processes (Cui et al., 2011; Etschmann et al., 2004; Qian et al., 2019).

Media M5 and M6 exhibited the same components, with the only distinction lying in the nitrogen source. Medium M5 has yeast extract, whereas M6 has an inorganic nitrogen source (NH_4Cl), with the nitrogen concentration kept at 0.5 g/L for both media. Notably, the inorganic nitrogen in M6 was not consumed during the experiment, suggesting that the cell growth was supported by the nitrogen supplied by L-Phe.

In yeast, the activation of the Ehrlich pathway is favored when L-Phe is used as sole nitrogen source. In the presence of more easily digestible nitrogen sources, such as ammonium salts, the expression of enzymes involved in that pathway are low or even negligible (Dai et al., 2021; Liu et al., 2021). In order to confirm if this also applies to *A. soli* ANG344B, an experiment with L-Phe as sole nitrogen source was performed (M7)

(Table 2.2). In these conditions *A. soli* ANG344B reached a CDW of 1.5 ± 0.1 g_{CDW}/L and produced 0.87 ± 0.09 g/L of 2-PE. The results obtained using L-Phe as sole nitrogen source resulted in lower 2-PE production than the obtained in M6, in the presence of inorganic nitrogen source (1.40 ± 0.04 $g_{2\text{-}PE}$/L), suggesting that, for this bacterial strain, the activation of Ehrlich pathway is independent of the nitrogen source present in the cultivation media. Conversely, yeast extract seems to play an important role in 2-PE production and in bacterial cellular growth, allowing to achieve higher 2-PE production (1.77 ± 0.07 $g_{2\text{-}PE}$/L) and higher bacterial cellular growth (2.4 ± 0.2 g_{CDW}/L) than in the other tested media (Table 2.2). In fact, yeast extract acts not only as source of nitrogen, but it is also a source of growth factors, such as vitamins and microelements, that are important for cellular metabolism. The presence of moderate concentrations of yeast extract in the cultivation media have also shown beneficial effects on 2-PE production by other microorganisms, namely yeasts (Fan et al., 2020; Gethins et al., 2015; Lu et al., 2016).

Regarding the rich media M1, M2 and M3, they supported similar cellular growth, being M1 the medium that allowed higher 2-PE production (1.40 ± 0.04 g/L), yet lower than the obtained with M5 (Table 2.2). Considering the results discussed above, medium M5 was selected to be used for the following studies.

2.4.2. Influence of cellular growth phase in 2-PE production

The previous experiments were performed with L-Phe present from the onset of the cultivation, resulting in most of 2-PE being produced during the exponential growth phase (Figure 2.2 a, cultivation in M5). To evaluate the aroma production during the stationary growth phase, the culture was grown in the previously selected media (M5) in the absence of L-Phe for the first 24 h, reaching 3.9 ± 0.1 g/L of CDW. At that time, the culture entered in stationary growth phase and a 50 mL L-Phe pulse (20 g/L) was added to the cultivation broth to start the aroma production (Figure 2.2 b). The 2-PE production lasted 24 h, with a production rate of 0.07 ± 0.00 g/L.h reaching an aroma concentration of 1.84 ± 0.11 g/L, resulting in a global volumetric productivity of 0.03 ± 0.00 g/L.h. In comparison, considering the production of 2-PE from the beginning of the cultivation, 1.77 ± 0.07 g/L of 2-PE were produced in 30 h with a volumetric productivity of 0.06 ± 0.00 g/L.h (Table 2.2).

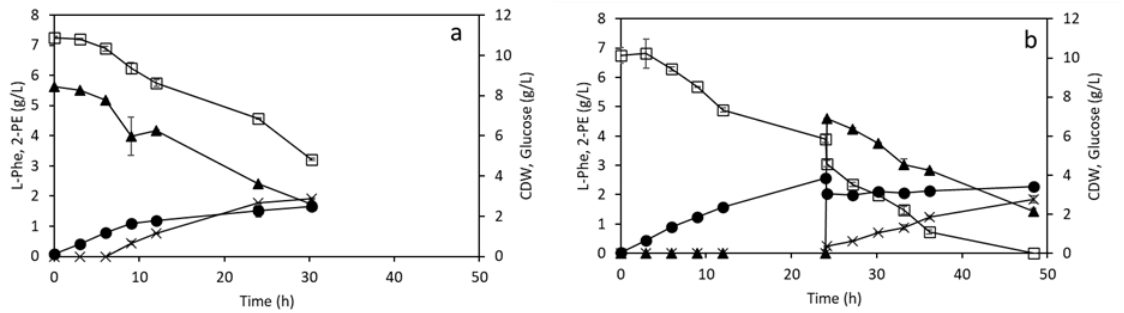

Figure 2.2 Cultivation profile of *Acinetobacter soli* ANG344B in shake flask when L-Phe is part of the cultivation media from the beginning of the experiment (a) and when L-Phe is added to the cultivation broth in stationary growth phase (b). CDW (•), glucose (□), L-Phenylalanine (▲), 2-Phenylethanol (x).

Results indicate that 2-PE can be produced both in exponential and stationary growth phases at similar production rates, leading to similar titers as soon as L-Phe is present in the cultivation broth. However, it is beneficial to have L-Phe present from the beginning of the cultivation to achieve a higher volumetric productivity and make the process more attractive. This approach decreases the total time of the process, once the aroma starts to be produced in the first hours of the process, resulting in an increased volumetric productivity.

For instance, the same behavior is not observed for 2-PE production by yeasts, which is frequently reported to be produced strictly during the exponential growth phase or produced at lower rates when L-Phe addition to the cultivation broth is delayed (Hua et al., 2010; Fan et al., 2020; Martínez-Avila et al., 2018; Stark, Zala, et al., 2003).

2.4.3. Effect of different temperatures and pH on *A. soli* ANG344B performance

Concerning the temperatures and pH values reported for *Acinetobacter* strains and for 2-PE production by other microorganisms, the effect of temperature and pH in cellular growth and 2-PE production was assessed at 23, 30 and 37 °C. For each temperature, pH values of 5, 7 and 8 were tested (Figure 2.3 a and b). The aim was to indentify the conditons that optimize 2-PE production process.

The less favorable conditions were pH 5 at 37 °C, that allowed the production of only 0.2 ± 0.1 g_{CDW}/L. The cell growth seems to be favored by lower temperatures (23 °C) and pH

in the range of 7 to 8, resulting in 3.0 ± 0.0 and 3.2 ± 0.1 g_{CDW}/L, respectively (Figure 2.3 a).

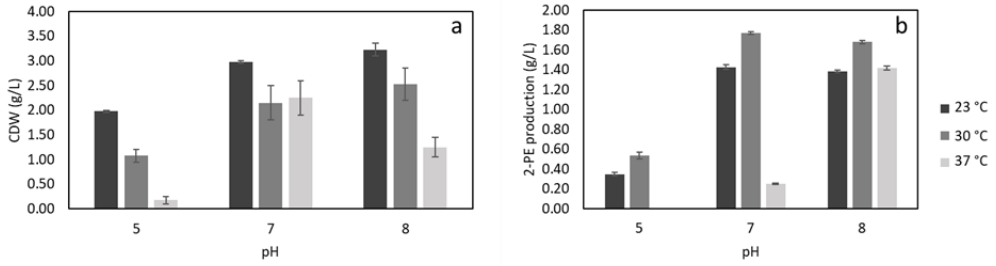

Figure 2.3 Effect of temperature (23, 30, 37 °C) and pH (5, 7, 8) on growth (a) and 2-PE production (b) by *Acinetobacter soli* ANG344B, in shaking flask.

2-PE production was strongly impaired at pH 5, with an aroma production under 0.53 ± 0.04 $g_{2\text{-}PE}$/L and no aroma production at pH 5 at 37 °C, as expected due to the low cellular growth. The best results for 2-PE production were achieved at 30 °C, for pH 7 and 8, with an aroma production of 1.77 ± 0.02 and 1.68 ± 0.02 g/L, respectively (Figure 2.3 b).

Results represented in Figure 2.3 show that the conditions that supported a higher cellular growth are not the same as the conditions that maximize the 2-PE production. Higher cellular growth is obtained at 23 °C, while higher aroma productions are obtained at 30 °C, both for pH 7 and 8. However, the most reported conditions for *Acinetobacter* strains cultivation are reported to be pH 7 and 30 °C (Beshay et al., 2002; Gururaj et al., 2016; Pirog et al., 2009). The different temperatures that favor 2-PE production and cellular growth might be related with the optimal temperature for the expression of the enzymes involved in Ehrlich pathway (Sekar et al., 2019). Indeed, different conditions favoring cellular growth and 2-PE production were also reported for other microorganisms, including engineered *Escherichia coli* and yeasts (Braga et al., 2021; Huang et al., 2001; Mu et al., 2014; Sekar et al., 2019).

Pichia fermentans L-5 was found to have the highest cellular growth at 25-30 °C, but the 2-PE production was maximized at 30-35 °C and at pH 8.5, while for a mixed microbial culture, low pH was favorable for cellular growth (pH 4.5) and a pH of 8 was more beneficial for 2-PE production at 35 °C (Huang et al., 2001; Mu et al., 2014). Even considering only the 2-PE production, the conditions that favor the aroma production vary

with the microorganism, with pH above 8 being better for the above mentioned cultures and pH 5.5 the condition that favored the aroma production by *Yarrowia lipolytica* (Braga et al., 2021).

Considering the results obtained for *A. soli* ANG344B, represented in Figure 2.3, and aiming to determine the conditions that favor a higher aroma production, pH 7 and 30 °C were the conditions selected to continue the process optimization.

2.4.4. Bioreactor production of 2-PE at the selected operational conditions

The most favorable conditions for 2-PE production process found in the previous shake flask tests were scaled-up to a 2 L bioreactor (run 1). Therefore, the assay was conducted using medium M5, pH 7 and 30 °C. The aeration rate was 1 vvm and the dissolved oxygen was controlled at a minimum of 30% (Figure 2.4 a).

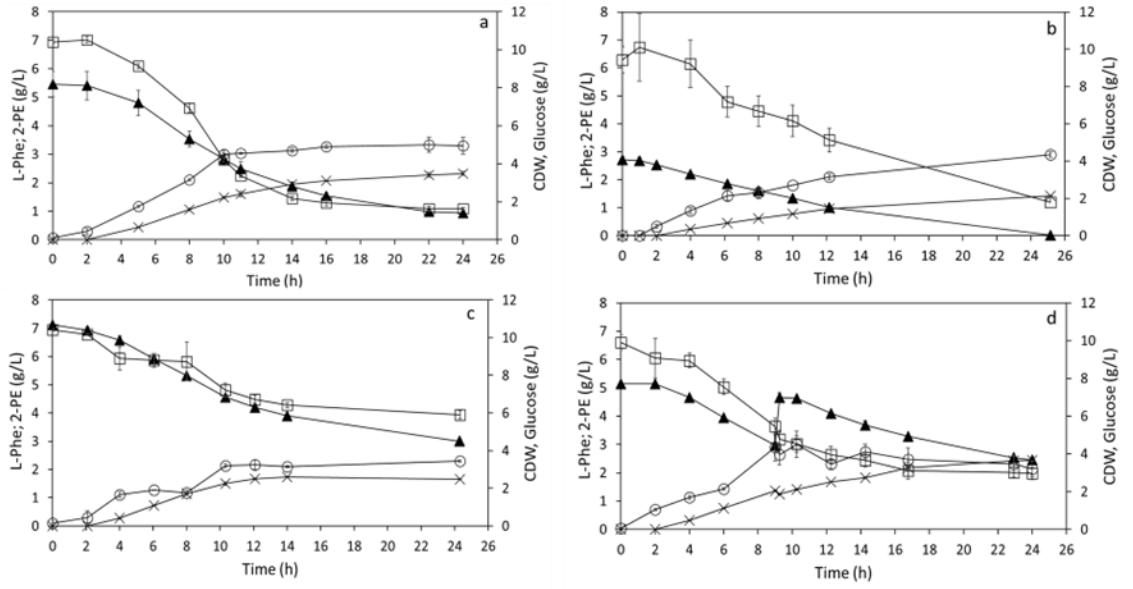

Figure 2.4 Cultivation profile of *Acinetobacter soli* ANG344B using different L-Phe concentrations. Run 1- 5 g/L (a), run 3- 2.5 g/L (b), run 4 - 7 g/L (c), run 5 - 5 g/L and a L-Phe pulse, in a fed-batch operational mode (d). CDW (o), glucose (□), L-Phenylalanine (▲), 2-Phenylethanol (x).

During the first 10 h of cultivation the *A. soli* ANG344B grew exponentially reaching a maximum CDW of 4.5 ± 0.1 g/L, corresponding to a specific growth rate (μ) of 0.33 ± 0.05 h^{-1}. Simultaneously, 2-PE production started with cellular growth, attaining the maximum of 2.14 ± 0.18 g/L at the end of the experiment (at 24 h). This was achieved

through the bioconversion of 4.68 ± 0.67 g/L of L-Phe. The kinetic parameters were computed for the first 16 hours of the run, during the period of increasing 2-PE production. During this timeframe, 1.92 ± 0.13 g/L of 2-PE were produced, resulting in a volumetric productivity of 0.12 ± 0.01 g/L.h and a production yield of 0.49 ± 0.07 $g_{2\text{-}PE}/g_{L\text{-}Phe}$ (Table 2.3).

The cultivation in bioreactor allowed the 2-PE production process to improve when compared to the experiments in shake flasks. The accurate control of the cultivation conditions, namely pH, dissolved oxygen, and temperature, automatically imposed by the bioreactor system, allowed the cultivation to occur in the most favorable conditions through all the process. This mitigated limitations that can affect cellular growth and 2-PE production. Under these controlled conditions, higher cellular growth and 2-PE production were achieved in a shorter period, resulting in higher volumetric productivities compared to the cultivation in shake flasks (as presented in Table 2.2 and 2.3). These results demonstrate a significant improvement in 2-PE production compared to previously reported levels for other wild-type bacteria (typically under 152 mg/L). These titers are comparable with those reported for yeasts, ranging from 0.5 to 5 g/L, without the use of *in situ* product removal techniques. The 2-PE production by *A. soli* ANG344B exhibited a similar production yield from L-Phe as commonly reported for yeasts, usually in the range of 0.1 to 0.75 $g_{2\text{-}PE}/g_{L\text{-}Phe}$ (Deetae et al., 2007; Jollivet et al., 1992; Liu et al., 2018; Martínez-Avila et al., 2018; Qian et al., 2019; Spinnler and Djian, 1991). Despite that, the volumetric productivity achieved by *A. soli* ANG344B (0.12 ± 0.01 g/L.h) stands among the highest reported for 2-PE production by a wild-type microorganism in a batch process without the use of *in situ* product removal techniques, that are usually under 0.1 g/L.h.

The shorter growth time of bacteria compared to yeast, along with the simpler metabolism that avoids the production of ethanol commonly observed in 2-PE production by yeasts amplifying its toxicity, makes *A. soli* ANG344B a valuable platform for this aroma production.

Table 2.3 Kinetic parameters calculated for 2-PE production obtained in different cultivation conditions. Run 1 – 5 g/L of L-Phe (standard conditions), at 16 h. Run 2 – 5 g/L of L-Phe and aeration rate of 2 vvm, at 14 h. Run 3 – 2.5 L-Phe g/L, at 24 h. Run 4 - 7 L-Phe g/L, at 14h. Run 5 – 5 g/L in the batch phase, and a pulse to achieve 5 g/L in the broth, at 17h. Run 6 - optimized operation conditions (standard conditions) scaled up to 10 L, at 15 h. These parameters were calculated for the period before the decrease in the 2-PE productivity.

	Run 1.	**Run 2.**	**Run 3.**	**Run 4.**	**Run 5.**	**Run 6.**
Biomass (g/L)	4.5 ± 0.1	2.8 ± 0.3	4.0 ± 0.3	3.0 ± 0.2	3.5 ± 0.4	4.6 ± 0.1
L-Phe (g/L)	4.00 ± 0.74	2.68 ± 0.04	2.74 ± 0.10	3.23 ± 0.02	3.54 ± 0.14	4.34 ± 0.07
2-PE (g/L)	1.92 ± 0.13	1.66 ± 0.01	1.38 ± 0.05	1.68 ± 0.03	2.18 ± 0.02	2.21 ± 0.01
$Y_{(P/S)}$ (g/g)	0.49 ± 0.07	0.62 ± 0.00	0.50 ± 0.00	0.52 ± 0.01	0.64 ± 0.05	0.51 ± 0.01
r_p (g/L.h)	0.12 ± 0.01	0.12 ± 0.00	0.06 ± 0.00	0.12 ± 0.00	0.13 ± 0.00	0.15 ± 0.00
2-PE – 24h (g)	3.70 ± 0.07	2.75 ± 0.03	2.42±0.26	2.73 ± 0.02	4.05 ± 0.01	24.87 ± 0.03

Under standard conditions, when the dissolved oxygen approached the minimum controlled values (30%), a decrease in the specific growth rate was noticed, being possible calculate two different growth rates (in the first 3 h, 0.70 ± 0.08 h^{-1} and in the following 7 h, 0.18 ± 0.04 h^{-1}) during exponential phase. To investigate whether this effect was due to a limitation in available oxygen for bacterial cellular growth, bioreactor experiments were conducted with an increased aeration rate to 2 vvm (run 2, Table 2.3).

As presented in Table 2.3, the increase in aeration rate led to a decrease in biomass production to 2.8 ± 0.3 g/L, corresponding to a decrease of 1.6-fold when compared to the biomass obtained with an aeration rate of 1 vvm (run 1). Consequently, the 2-PE production was also lower, reaching 1.96 ± 0.02 g/L at the end of the 24 h experiment (1.66 ± 0.01 g/L at 14 h). The increased oxygen supply might have induced stress to the microorganism, adversely affecting its growth. Taking these results into consideration, the observed decrease in the growth rate in the first hours of cultivation does not seem to be linked to oxygen limitation. Instead, it might be attributed to the onset of 2-PE production or the diminishing nutrient concentrations throughout the cultivation process. However, it´s worth noting that the increase in aeration rate to 2 vvm has been demonstrated to be beneficial for 2-PE production for some yeasts strains, namely *Saccharomyces cerevisiae* (Shu et al., 2021).

Therefore, considering these results, an aeration rate of 1 vvm was chosen for the subsequent experiments. This aeration rate is consistent with the reported values for *Acinetobacter* sp. cellular growth and for the 2-PE production by yeast strains (Gao and Daugulis, 2009; Chreptowicz and Mierzejewska, 2018; Han and Eom, 2022).

2.4.5. Effect of L-phenylalanine concentration

The *A. soli* ANG344B cultivation with 5 g/L of L-Phe (run 1, Figure 2.4 a) did not achieve complete precursor conversion into 2-PE, a behavior also reported by many authors for 2-PE production using yeasts (Fan et al., 2020; Shu et al., 2018; Stark, Zala, et al., 2003; Tian et al., 2020). As illustrated in Figure 2.4 a, the initial consumption of L-Phe is faster (0.26 ± 0.05 g/L.h) and gradually slows over the time of the experiment. The L-Phe consumption rate decreases to 0.12 ± 0.01 g/L.h^{-1} when the L-Phe concentration reaches 2.5 g/L and the concentration of 2-PE is approximately 1.6 g/L. Considering these results, different concentrations of L-Phe (2.5 and 7 g/L) were tested to access the impact of L-Phe concentration on aroma production.

Using a lower concentration of L-Phe (run 3, Figure 2.4 b) the culture presented exponential growth for 10 h, achieving a maximum specific growth rate of 0.25 ± 0.05 h^{-1} (a decrease in specific growth rate during the exponential growth phase was also noticed in these conditions), reaching a 2-PE production of 1.38 ± 0.05 g/L at the end of the 24 h experiment.

In these conditions, the production process was slower, but L-Phe was completely consumed by the end of the experiment. The L-Phe consumption rate determined for this run was 0.11 ± 0.00 g/L.h^{-1}, similar to the rate obtained in run 1 from the time that L-Phe reached the concentration of 2.5 g/L in the medium until the end of the experiment (0.12 ± 0.01 g/L.h^{-1}). However, the production yield was comparable in these two runs, as represented in Table 2.3. These results suggest that the presence of low L-Phe concentrations promotes a lower biotransformation rate, and it might be beneficial to have higher precursor concentration in the cultivation broth to have a more competitive process.

Consistent with these results, Shu et al., observed that L-Phe concentration influenced the ability of *Saccharomyces cerevisiae* cells to convert it in 2-PE and reported total L-Phe conversion when initial concentration in the broth was 2 g/L (Su et al., 2018). According

to Castillo et al., that used a co-culture of *Kluyveromyces marxianus* and *Debaryomyces hansenii*, 2-PE production is a function of the initial concentration of L-Phe and carbon source, as yeasts activate the Ehrlich pathway in the presence of a high concentration of complex amino acids (Castillo et al., 2022). Stark et al. also reported, for *Saccharomyces cerevisiae*, a significantly lower 2-PE production when L-Phe concentration was in the range of 0.5 and 1.4 g/L (Stark, Zala et al., 2003). In contrast, Fabre et al. achieved the highest production for *Kluyveromyces marxianus* with 2 g/L of L-Phe (Fabre et al., 1998).

Based on these results, a higher L-Phe concentration was tested to evaluate the impact of higher precursor concentrations on the consumption rate and process productivity. Therefore, *A. soli* ANG344B was cultivated in the presence of 7 g/L of L-Phe (run 4, Figure 2.4 c). In these conditions, the culture reached a maximum CDW of 3.0 ± 0.2 g_{CDW}/L at 10 h of cultivation and produced 1.61 ± 0.02 g/L of 2-PE at the end of the experiment (24 h). As in run 1, the L-Phe consumption rate slowed down across the experiment, decreasing from 0.24 ± 0.00 to 0.11 ± 0.00 g/L.h^{-1} in the final 10 h, despite the L-Phe in the cultivation broth remaining higher than 2.5 g/L. This decline was evident in the production rate of 2-PE, decreasing from 0.12 ± 0.00 g/L.h to 0.01 ± 0.00 g/L.h during that period. At the conclusion of the experiment, the remaining amount of unconverted L-Phe in the broth was 3 g/L. The decrease in the L-Phe consumption rate coincided with the 2-PE concentration reaching around 1.5 g/L, suggesting an inhibitory effect by the product, despite nitrogen and carbon were still available in the broth. However, considering the similar overall kinetic parameters represented in Table 2.3 for run 1 and run 4, the increase in L-Phe concentration from 5 to 7 g/L did not influence the conversion ability of this microorganism. This suggests the existence of a threshold beyond which the increase in precursor concentration does not have affect its consumption rate. Nevertheless, higher L-Phe concentrations were found to be beneficial for 2-PE production by some microorganisms, such as *Meyerozyma* sp. strain YLG18 or *Kluyveromyces marxianus* CBS 600, which achieved better productions with 7 and 9 g/L of L-Phe, respectively (Etschmann et al., 2004; Yan et al., 2020).

Due to the observed low 2-PE production rates in run 3 with a low concentration of L-Phe, a fed-batch approach based on run 1 was implemented in run 5 (Figure 2.4 d). In this approach, a pulse of L-Phe was fed to the bioreactor when its concentration reached 3 g/L, aiming to maintain 5 g/L of L-Phe in the cultivation broth. This strategy helped avoid low L-Phe concentrations, which were hypothesized to decrease precursor consumption

and 2-PE production. Under these conditions, the initial part of the run exhibited behavior comparable to the homologous time of the run 1, with an L-Phe consumption rate of 0.24 ± 0.00 g/L.h^{-1} and a 2-PE production rate of 0.15 ± 0.01 g/L.h^{-1}. After feeding, the L-Phe consumption rate remained similar to the initial part of the experiment but started to decrease by the time 2-PE concentration reached 1.6 g/L (at 12 h), as it happened in run 1, indicating inhibition by product.

The results indicate that a concentration of 5 g/L of L-Phe is optimal as a precursor for cultivating *A. soli* ANG344B in a batch operational mode. This concentration not only results in high production rates but also minimizes the remaining amount of unconverted L-Phe in the cultivation broth.

2.4.6. Scale up of 2-PE production process

The scale up of 2-PE production by *A. soli* ANG344B in the optimized conditions selected from the previous studies (medium M5, pH 7, 30 °C and 5 g/L of L-Phe as part of the cultivation medium from the beginning of the cultivation - standard conditions) were scaled up to 10 L bioreactor (Table 2.3 - run 6, Figure 2.5).

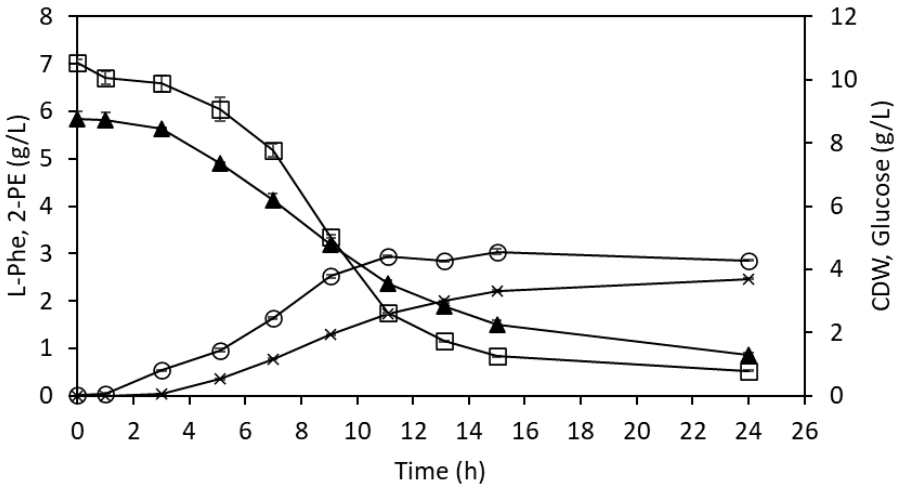

Figure 2.5 Cultivation profile of *Acinetobacter soli* ANG344B in the optimal conditions for 2-PE production upscaled to 10 L bioreactor (Run 6). CDW (o), glucose (□), L-Phe (▲), 2-PE (x).

The culture grew exponentially for 10 h and reached 4.3 ± 0.1 g/L of CDW. The 2-PE production started concomitantly with cellular growth, achieving a maximum production of 2.47 ± 0.00 g/L at the end of the cultivation (at 24 h). Similar to the previous bioreactor cultivations, a decrease in the consumption rate of L-Phe and in 2-PE production was also

noticed. As described for the cultivation in 2 L bioreactor, the kinetic parameters were calculated for the first 15 hours of the process, since after that the production practically stabilizes. At that time, 2.21 ± 0.01 g/L of 2-PE were produced from 4.34 ± 0.07 g/L of L-Phe, with a production yield of 0.51 ± 0.01 $g_{2\text{-PE}}/g_{\text{L-Phe}}$ and a volumetric productivity of 0.15 ± 0.00 $g_{2\text{-PE}}$/L.h (Table 2.3, run 6).

The calculated production and kinetic parameters were similar to the obtained in the same conditions in 2 L cultivations, as represented in Table 2.3, run 1. These results show that it was possible to successfully scale up the 2-PE production process by 5-fold, with no loss of productivity, proving to be an effective process.

2.4.7. Study of the 2-Phenylethanol toxicity concentration

The cultivation profiles indicated limitations in both growth and 2-PE production, despite the continued availability of glucose and L-Phe in the cultivation broth. As an aromatic alcohol, 2-PE is reported to have inhibitory effects on microbial cells (Kleinwächter et al., 2021; Qian et al., 2019; Stark, Zala, et al., 2003). To assess the impact of 2-PE on the viability of *A. soli* ANG344B, the microorganism was cultivated in shake flasks with different 2-PE concentrations (ranging from 0 to 5 g/L) and the resulting bacterial growth was evaluated (Figure 2.6).

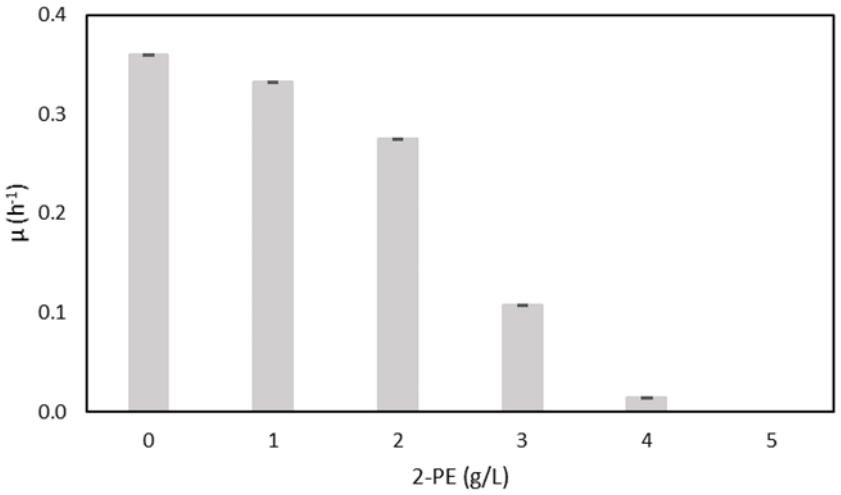

Figure 2.6 2-PE toxicity test. Growth rate calculated for the first 8 h of the cultivation in the presence of 0, 1, 2, 3, 4 and 5 g/L of exogenous 2-PE.

Figure 2.6 revealed a negative influence of 2-PE on bacterial growth, with a noticeable decrease in the growth rate even at low 2-PE concentrations (1 g/L). Specifically, in the presence of 1 g/L of 2-PE in the cultivation broth, the growth rate reaches 92% of the observed in the absence of 2-PE. A stronger inhibitory effect was observed when 2-PE concentrations present in the broth were above 2 g/L (with a growth rate of 76% compared to the absence of 2-PE), and concentrations of 4 g/L completely inhibit growth. Notably, after 24 h of growth in the presence of 1 g/L of 2-PE, the biomass concentration was only 51% of that obtained without the aroma compound in the broth, indicating a substantial inhibitory effect. This inhibition trend aligns with the behavior observed for *A. soli* ANG344B cultivations discussed in previous sections. The reduction in the specific growth rate during the exponential growth phase, evident in all the conditions tested in bioreactor, coincided with the beginning of 2-PE production. This correlation suggests that the presence of 2-PE in the cultivation broth, even at low concentrations, contributed to this growth rate decline. Furthermore, the decrease in L-Phe consumption rate, particularly when 2-PE exceeds 1.6 g/L in the cultivation broth, appears to be linked to product toxicity.

2-PE is noted to have bacteriostatic effect, being used as preservative in the pharmaceutical and cosmetic industry at concentrations up to 12 g/L, despite bacteriostatic activity being noticeable at round 1 g/L (Kleinwächter et al., 2021). Bacteriostatic activity is related with its interaction with the cell membrane, leading to increased membrane fluidity and affecting the structure of transmembrane proteins (Jia et al., 2010; Kleinwächter et al., 2021). However, the extent of 2-PE toxicity varies among microorganisms. For *Saccharomyces cerevisiae*, 4 g/L of exogenous 2-PE is reported to be completely growth inhibiting, similar to the observed effect in *A. soli* ANG344B. In contrast, for *Kluyveromyces marxianus*, concentrations above 0.3 g/L start affecting growth and 3 g/L results in complete growth cessation (Červeňanský et al., 2020; Etschmann et al., 2005; Gao and Daugulis, 2009; Hua et al., 2010; Stark, Zala et al., 2003).

The inhibitory effects of 2-PE on *A. soli* ANG344B impaired further improvement of 2-PE production process. Strategies to mitigate this inhibitory effect and improve the production process, making it more applicable to the industry, may involve the implementation of *in situ* product removal (ISPR) techniques (Qian et al., 2019).

2.5. Conclusion

In this study, the medium composition and operational conditions for 2-PE production by the recently isolated *A. soli* ANG344B were evaluated to optimize the aroma production.

The presence of yeast extract emerged as a crucial element in the medium, playing a pivotal role in promoting both bacterial cellular growth and production of the desired aroma. Furthermore, the favorable operational conditions for maximizing 2-PE production were identified as 30°C and pH 7. These conditions were found to maximize the performance of *A. soli* ANG344B, especially during the exponential growth phase. The 2-PE production during the exponential growth phase was found important to increase the process volumetric productivity. L-Phe concentration of 5 g/L proved to be advantageous for achieving high rates of 2-PE production. This not only promoted higher volumetric productivity but also resulted in the impressive production of 2.14 ± 0.18 g/L of 2-PE. Furthermore, the production process was successfully scaled up by 5-fold without loss in productivity. Remarkably, this achievement rivals the production levels observed in yeasts, demonstrating the potential of *A. soli* ANG344B as a viable bioproduction host.

However, the efforts to further enhance 2-PE production were impeded by its inherent toxic effects. Even at relatively low concentrations (1 g/L), 2-PE exhibited detrimental impacts on cellular growth. To overcome this obstacle, the implementation of *in situ* product removal (ISPR) techniques to avoid loss of cellular viability was investigated in chapter 3.

3.

Assessment of *in situ* product recovery techniques to enhance 2-phenylethanol production

The results presented in this chapter are part of the following manuscript:

Bernardino, A. R. S., Torres C. A. V, Crespo, J G., Reis M. A M , Assessment of *in situ* product recovery techniques to enhance 2-Phenylethanol production by *Acinetobacter soli* ANG344B. In preparation.

3.1. Summary

The 2-phenylethanol (2-PE) production process by *Acinetobacter soli* ANG344B is limited by product toxicity, resulting in low 2-PE concentration and productivity. Hence, to overcome this limitation and improve the 2-PE production process, different alternatives based in *in situ* product removal (ISPR) approaches were evaluated. The gas stripping approach by air supply to the bioreactor resulted in a low aroma recovery due to the slow volatilization of the product, being able to remove 1.2% of the total 2-PE present in the broth. ISPR approach based on liquid-liquid extraction was also evaluated using oleic acid and polypropylene glycol (PPG) 1200 as extractants. The use of oleic acid proved to have a negative influence on bacterial cellular growth, affecting 2-PE production. PPG 1200 showed a higher partition coefficient for 2-PE than oleic acid, but due to the formation of emulsions, it could not be used as extractant. The use of adsorbent resins proved to be a promising approach to increase 2-PE production. Amberlite XAD 4 was chosen from the different adsorbents tested. This resin has high affinity for 2-PE, being able to adsorb 205.8 ± 8.1 $mg_{2\text{-}PE}/g_{dry\ resin}$. In a batch cultivation process, in presence of 3% (dry w/v) of Amberlite XAD 4, *A. soli* ANG344B was able to produce 6.99 ± 0.06 g/L of 2-PE with a volumetric productivity of 0.17 ± 0.00 g/L.h, which represents an improvement of 3.3-fold considering a batch production process without ISPR techniques, a production level not previously reported for a wild-type bacteria. The recovery of the product from the resin was performed by elution with ethanol, obtaining an extract with 217.56 ± 0.31 g/L of 2-PE. These findings highlight the potential of *Acinetobacter soli* ANG344B as 2-PE producer, contributing to the development of natural 2-PE production process.

3.2. Introduction

The attempts to increase 2-PE microbial production through testing different cultivation conditions and different producing strains, have been impaired by the product inhibition for the producing microorganism. Usually, 2-PE concentrations between 2 and 4 g/L are reported to inhibit cellular growth, depending on the specie (Červeňanský et al., 2020; Gao and Daugulis, 2009; section 1.1.3 – chapter 1; section 2.4.7 - chapter 2). In fact, 2-PE is reported as bacteriostatic agent due to its amphiphilic nature, interfering with the cell membrane and affecting cellular homeostasis (Kleinwächter et al., 2021).

To improve 2-PE production process, by reducing product inhibition, several *in situ* product removal (ISPR) techniques have been studied. These approaches consist of continuous removal of 2-PE from the cultivation broth, allowing the microorganism to continue the 2-PE production and cellular growth (Teke et al., 2022). ISPR techniques can also minimize product loss by degradation or evaporation and facilitate the downstream processes (Santos et al., 2020; Teke et al., 2022). The continuous removal of 2-PE can be done using several approaches such as liquid-liquid and solid-liquid extraction or membrane-based processes.

Liquid-liquid extraction is one of the most reported approaches attempted for improvement of 2-PE production process. Solvents such as oleic acid, polypropylene glycol, ethyl acetate and ionic liquids have been tested to recover the 2-PE from the cultivation broth while it is being produced. This can be achieved by mixing the solvents with the cultivation medium or using membrane contactors to avoid the direct contact of the solvents with the cells. The use of these solvents has been reported to successfully improve 2-PE production process in yeasts, improving the aroma production in 1.6 to 5-fold reaching 2-PE concentrations up to 16 g/L in *Saccharomyces cerevisiae* (Chreptowicz and Mierzejewska, 2018; Etschmann and Shrader, 2006; Okuniewska et al., 2017; Stark et al., 2002). However, the use of these solvents presents some drawbacks as formation of stable emulsions, inhibitory effects towards the microorganisms due to the solvent interaction with the cellular membranes, interference with the organoleptic properties of the compound and, in the case of the ionic liquids, the worries about its toxicity.

Organophilic pervaporation has also been reported for process improvement, resulting in a 2-fold increase in 2-PE production to 2.20 g/L by *Kluyveromyces marxianus*, being cell separation and temperature, that influences the fluxes, limiting factors of this process (Etschmann et al., 2005).

Solid-liquid extraction approaches, specially using polymeric adsorbents have been also reported for enhancement of 2-PE production process. These adsorbents are reported to be used in direct contact with the cultivation broth or used in external columns connected to the bioreactor, through which the broth (with or without cells) can be recirculated allowing the contact with the resin. Non-polar polymeric adsorbents, as HZ818, D101 and FD0816, that interact with the aroma through the hydrophobic aromatic ring by π interactions are the most common adsorbents used (Hua et al., 2010; Mei et al., 2009;

Wang, Dong, Gaun et al., 2011; Wang, Dong, Meng et al., 2011). Few polymers can interact by hydrogen bonding with the aroma, being Hytrel 8206 and Hytrel 63548 the reported polymers (Gao et al., 2009; Shu et al., 2021). The amount of adsorbent in contact with the cultivation broth is usually between 2 and 10% (w/v), allowing an increase in 2-PE production by 1 to 3-fold up to 13.7 g/L (Hua et al., 2010; Mei et al., 2009; Wang, Dong, Meng et al., 2011). Higher improvements have been reported when using the adsorbent coupled to the cultivation in an external column, that is replaced for new adsorbent when it reaches its maximum adsorption capacity, in a semicontinuous process (Gao and Daugulis 2009; Wang, Dong, Meng et al., 2011). This strategy has led to the production of 32.5 g/L and 20.4 g/L of 2-PE by *Saccharomyces cerevisiae* R-UV3 and *Kluyveromyces marxianus*, respectively (Gao and Daugulis 2009; Wang, Dong, Meng et al., 2011). Even though this alternative covers some drawbacks of liquid-liquid extraction, as the toxicity or the organoleptic influence, the selectivity of the adsorbent, the product recovery from the polymer and the need for cell separation during production might be bottlenecks of the process.

The present work focuses on the study of different alternatives for 2-PE recovery during the microbiological producing process by *Acinetobacter soli* ANG344B. Alternatives as gas stripping, liquid-liquid extraction and adsorption were evaluated to assess their potential to be used in an effective 2-PE production process.

3.3. Materials and methods

3.3.1. Gas stripping approach

The experimental setup consisted of two condensers-in-series immersed in a water bath at 0 °C for vapor condensation, connected to the gas outlet of the 2 L bioreactor, as represented in Figure 3.1. The air stripping was carried out using the air supplied to the bioreactor cultivation medium, that was maintained constant at 1 vvm (volume of air per volume of reactor per minute) with variation of stirring speed from 300 to 800 rpm. The first experiment was performed using the cultivation medium described in section 2.3.3. (chapter 2) without bacterial inoculation, containing 4 g/L of L-Phe and 1 g/L of 2-PE. The second experiment was accomplished in the presence of bacterial cellular growth under the same conditions described above, with a pulse feeding of 160 mL of a 25 g/L L-Phe solution at 9 h of the experiment.

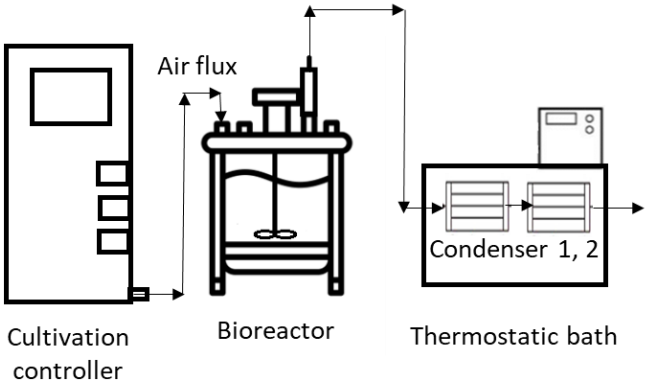

Figure 3.1 Schematic representation of the setup for gas stripping ISPR approach.

3.3.2. Liquid-liquid ISPR approach

3.3.2.1. Partition coefficient determination for liquid-liquid ISPR approach

2-PE partition coefficients in oleic acid and polypropylene glycol (PPG) 1200 were determined by mixing 50% (v/v) of each solvent and solutions with different concentrations of 2-PE, ranging from 0 to 2 g/L, similar to the aroma concentrations achieved during a batch production process by *Acinetobacter soli* ANG344B (0, 0.3, 0.7, 1.0, 1.3, 1.7 and 2.0 $g_{2\text{-PE}}$/L). The vials (10 mL vials) were incubated in an orbital shaker at 30 °C (bacterial cultivation temperature), at 200 rpm, for 24 h, to achieve phase equilibrium. To assure phase separation, a centrifugation step was performed at 13 000 xg, 4 °C, for 15 min. The 2-PE concentration in each phase was analyzed by HPLC, as described below, section 3.3.5.2. The 2-PE partition coefficients towards the solvents were calculated as the slope of 2-PE concentrations in solvent phase versus aqueous phase.

3.3.3. Polymeric adsorption approach

3.3.3.1. Polymeric resins screening

Three different resins, Amberlite XAD 4, Macronet MN 102 and Hytrel 8206, were tested to be used as ISPR adsorbents on the 2-PE production process. Their main characteristics are summarized in Table 3.1.

Table 3.1 Summary of resins' characteristics, tested for 2-PE adsorption in this work.

Resins	**Matrix**	**Functional group**	**Surface area (m^2/g)**	**Porosity**
Amberlite XAD 4	Styrene-divinylbenzene	ND	≥ 750	≥ 0.50
Hytrel 8206	Copolymer polybutylene ester and polyether	ND	ND	ND
Macronet MN 102	Polystyrene-divinylbenzene	Tertiary amine	800	0.40

ND – not disclosed by supplier.

Prior to use, resins were washed with ethanol 96% (v/v) for 24 h under slight agitation (200 rpm in an orbital shaker), and afterwards washed with distilled water and vacuum filtered.

The dry weight of the resins was determined gravimetrically, after drying a 1 g of resin at 100 °C for 24 h.

To determine the best adsorbent ratio for 2-PE adsorption, different adsorbent dosages ranging from 1 to 20% (for Amberlite XAD 4, Macronet MN102) or 30% (w/v) (for Hytrel 8206) in a dry basis were tested using a 2 g/L 2-PE model solution. The different amounts of resin were incubated in 6 mL of the 2-PE solution at 30 °C (the cultivation temperature for the 2-PE production process by *Acinetobacter soli* ANG344B), 200 rpm for 24 h to allow reaching the equilibrium. The 2-PE concentration before the adsorption and after the establishment of the equilibrium was measured by HPLC as described in section 3.3.5.2. The adsorption capacity and the adsorption efficiency were calculated as follows:

Adsorption capacity:

$$Q = \frac{C_0 - C_{eq}}{m_0} \times V \quad (3.1)$$

Adsorption efficiency:

$$E\,(\%) = \frac{(C_0 - C_{eq})}{C_0} \times 100 \quad (3.2)$$

where Q is the adsorption capacity ($g_{adsorbate}/g_{dry\ resin}$), E is the adsorption efficiency (%), C_0 and C_{eq} are the concentrations of adsorbate in aqueous phase before the adsorption and

after the equilibrium was reached, respectively (g/L), m_0 is the mass of dry resin (g) and V is the solution volume (L).

Using the best resin ratio for each adsorbent, the affinity for L-Phe and 2-PE was assessed by incubating the adsorbent with a model solution of 5 g/L of L-Phe and in a binary solution of 2 g/L of L-Phe and 2 g/L of 2-PE in the conditions described above.

3.3.3.2. Desorption of 2-PE and L-Phe

The desorption of both 2-PE and L-Phe adsorbed in Amberlite XAD 4 from model solutions containing 2 g/L of L-Phe and 2-PE, was evaluated. Desorption was performed by incubating the resin containing 2-PE and L-Phe in ethanol 96% (v/v) or in deionized water, overnight at room temperature and 200 rpm. The proportion of resin and desorbing solution was the same used in the adsorption experiments. The desorption process was repeated until the concentration of the compounds measured in the eluent phase was lower than 0.05 g/L. The desorption efficiency was determined as follows:

$$\mathrm{D}\ (\%) = \frac{C_d \times V_d}{(C_0 - C_{eq}) \times V} \times 100 \quad (3.3)$$

where D is the desorption efficiency (%), C_d is the concentration of adsorbate in the eluent (g/L) and V_d is the volume of the eluent (L).

3.3.3.3. Dynamic adsorption and desorption

Dynamic fixed-bed column adsorption and desorption experiments were performed in a glass column with an inner diameter of 2 cm with 13.5 g of dry Amberlite XAD 4, with a fixed bed height of 19.5 cm, corresponding to a bed volume (BV) of 61.3 cm^3, calculated as follows:

$$Bed\ volume\ (cm^3) = bed\ height\ (cm) \times column\ crossectional\ area\ (cm^2) \quad (3.4)$$

The adsorption tests were conducted at 30 °C, maintained constant by a thermostatic water bath, and pH 7 to simulate the 2-PE production process by *Acinetobacter soli* ANG344B. A solution containing only 1 g/L of 2-PE or 1 g/L of 2-PE and 1 g/L of L-Phe was fed to the column from the bottom at 9.79 BV/h, equivalent to 10 mL/min, using a peristaltic pump, for 8 h, as represented in Figure 3.2. Samples were collected periodically at the top of the column and analyzed by HPLC, as described below in the analysis section, to determine the breakthrough curves. The breakthrough curves were expressed

in terms of normalized concentration of the compounds, the ratio of outlet to inlet concentration (C/C_0), as function of time.

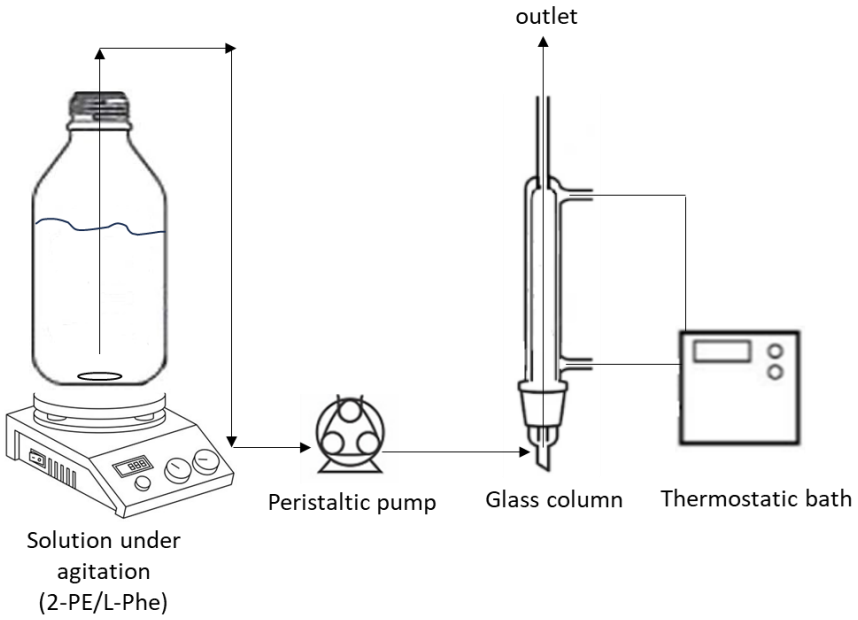

Figure 3.2 Schematic representation of the dynamic fixed-bed column adsorption and desorption experiments setup.

The maximum capacity of the column was calculated from the experimental breakthrough curve using the following equation (Carpiné et al., 2013):

$$q_{total} = \frac{Q \times A}{1000 \times m_0} \tag{3.5}$$

Where q_{total} (g/g) is maximum capacity of the column, A is the area under the breakthrough curve, calculated by integrating the adsorbed 2-PE concentration (C_0 - C_t) as function of time, Q is the flow rate (mL/min) and m_0 is the mass (g) of dry Amberlite XAD 4.

The total mass of the compound adsorbed was calculated using the area under the breakthrough curve as follows (Valério et al., 2022):

$$m_{ads} = \frac{C_0 \times Q}{1000} \int_0^{t_{sat}} \left(1 - \frac{C}{C_0}\right) \tag{3.6}$$

Where the m_{ads} is the total mass of 2-PE adsorbed (g), C_0 is the inlet concentration of 2-PE, Q is the flow rate (mL/min), t_{sat} is the bed saturation time (min).

After adsorption, the compounds were desorbed in the same operating conditions used for adsorption, but at room temperature, with ethanol 96%. Samples were taken periodically for 3 h and analyzed by HPLC, as described below in the analysis section.

3.3.4. Biotransformation experiments with extraction from cultivation broth

3.3.4.1. Microorganism and inoculum preparation

The microorganism preservation, reactivation and the inoculum preparation were performed as described in chapter 2, section 2.3.1.

3.3.4.2. Shake flask cultivation

Different ISPR techniques were tested in cultivations performed in 250 mL baffled shake flasks with a working volume of 100 mL at 30 °C, pH 7 and stirred at 200 rpm, in an orbital shaker. Cultivation media (M5, ensuring 5 g/L of L-Phe in the beginning of the cultivation) and cultivation conditions used were described in chapter 2, section 2.3.2. For cultivation using a liquid-liquid ISPR technique, 20% (v/v) of oleic acid was added to the cultivation media after 3 h of cultivation. For cultivations using a ISPR polymeric adsorption approach, 20% (v/v) of dry Hytrel 8206 and 3% (v/v) of dry Amberlite XAD 4 were added at the beginning of cultivation. When the cultivation was performed in presence of Amberlite XAD 4, 7 g/L of L-Phe was used, taking in consideration the adsorption of that compound, to achieve an initial concentration of 5 g/L Cultivations were performed for 24 or 48 hours, respectively for liquid-liquid or adsorption approaches. Samples were taken periodically for evaluation of cell growth, L-Phe and glucose consumption and 2-PE production.

3.3.4.3. Bioreactor cultivation

Bioreactor cultivations were performed in 2 L bioreactors (BioStat B-plus, Sartorius, Germany), as described in chapter 2, section 2.3.3, modified with 17 g/L of L-Phe to achieve an initial concentration of 10 g/L of the precursor (considering the maximum adsorption of the compound) and 20 g/L of glucose. 3% (w/v) of dry Amberlite XAD 4 was added to the bioreactor cultivation medium for ISPR by adsorption and then sterilized. Samples were taken periodically during the cultivation, and centrifuged at 16000 xg, to measure cell growth, L-Phe and glucose consumption, and 2-PE production

in aqueous phase. The aroma adsorbed to the polymer was quantified at the end of the experiment.

3.3.4.4. Product recovery from the resin

After the bioconversion, the resin was recovered from the cultivation broth, decanted and vacuum filtered. The resin was incubated with ethanol 96% in an orbital shaker at 200 rpm, at room temperature overnight to desorb the 2-PE. The eluent (ethanol) was removed by vacuum filtration. Fresh ethanol was added to the resin for further 3 cycles until the 2-PE concentration in the resin was below 0.01 g/L. The ethanol containing 2-PE was evaporated by vacuum distillation using a rotatory evaporator (Büchi Rotavapor R-210, Switzerland), operating at a pressure of 31 mbar and a temperature of 30 °C, being the ethanol condensed at -11 °C, until the volume of the 2-PE phase was below 50 mL.

3.3.5. Analytical techniques

3.3.5.1. Cell separation and sample processing

Culture broth samples were taken periodically and processed as described in chapter 2, section 2.3.4, for determination of L-Phe, 2-PE, glucose and CDW.

3.3.5.2. L-Phe and 2-PE quantification

L-Phe and 2-PE were quantified by high-performance liquid chromatography (HPLC) (Alliance e2695, Waters, USA) equipped with a C18 3.9 x 150 mm column and a guard column (Nova-Pak®), coupled to a PDA (Photo Diode Array) detector, set at 216 nm. A gradient method comprising water/methanol was applied with a flow rate of 0.5 mL/min, as follows: 0-7 min 70/30; 7-10 min 50/50; 10-16 min 30/70; 16-25 min 70/30. L-Phe and 2-PE were used as standards in concentrations ranging from 0.01 to 0.2 g/L.

3.3.5.3. Glucose quantification

Glucose quantification was performed by HPLC (VWR Hitachi Chromaster), equipped with a Diode Array Detector 5430 and a Refractive Index Detector 5450. The column used was an Aminex HPX-87H (300 x 7.8 mm) coupled to a pre-column Biorad 125-0129 (30 x 4.6 mm), operated at 30 °C. The mobile phase was 0.01 N H_2SO_4 and the flow

rate 0.5 mL/min. D-Glucose was used as standard in concentrations between 0.03 and 1 g/L.

3.3.5.4. 2-PE extract analysis

The recovered extract desorbed from the resin XAD 4 was analyzed by Gas Chromatography (GC) with a Flame Ionization Detector (FID) (Agilent 6890) using a VF5-ms (30 m, 0.25 nmID, 0.25 mm film) column. Helium was used as carrier gas at 1 mL/min. Injector and detector temperatures were 250 °C. The temperature program applied started with 50 °C for 5 min and increased at 20 °C/min until 320 °C. For the identification of compounds present in the extract, samples were analyzed by GC-mass spectrometry (GC-MS). The sample was analyzed by GC-MS (Agilent 7890 GC) with an Agilent HP- MS UI fused silica capillary column (30 mx0.25 mm ID, 0.25 µm df – film thickness), in the same conditions as GC-FID. For GC-MS a Leco Pegasus® BT GC-TOFMS was used. The transfer line was set at 300 °C, source 250 °C. The MS was operated in electron ionization mode (70 eV) using a range of m/z 40-470. Data was processed using software ChromaTof v5.40.12.0 (LECO Corp., Saint Joseph, MI, USA). NIST MS Search Program Version 2.3 was used for spectra matching (NIST, 2015).

3.3.6. Kinetic and stoichiometric parameters calculation

Kinetic and stoichiometric parameters were determined as described in chapter 2, section 2.3.5.

3.3.7. Statistical analysis

Experiments were performed at least in duplicates of independent samples. Data are expressed as the mean ± standard deviation.

3.4. Results and discussion

Acinetobacter soli ANG344B is a strain capable of producing 2-PE in concentrations above 2 g/L, using L-Phe as precursor. However, further 2-PE production is limited by the product toxicity. In chapter 2 it was found that a 2-PE concentration as low as 1 g/L has a negative influence on the cells, causing a decrease in bacterial growth of 50% after 24 h of cultivation, when compared with the cultivation in absence of 2-PE. This effect is stronger as the 2-PE concentration increases, being completely inhibitory for concentrations above 4 g/L (chapter 2). So, in order to improve 2-PE production by this

microorganism and to selectively recover the product of interest from the cultivation broth, several ISPR strategies were attempted.

3.4.1. Gas stripping

During the cultivation process of 2-PE in bioreactor, the aeration supplied to the bioreactor together with the stirring can lead to the loss of the aroma in the off-gas (Lukin et al., 2018). Therefore, in order to assess the potential of using the air supplied to the bioreactor (1 vvm) to remove and recover the 2-PE as it is being produced, experiments using condensers connected to the bioreactor gas outlet and kept at 0 °C to allow for condensation of the evaporated volume were performed. A 2 L bioreactor experiment, mimicking the standard conditions used for *A. soli* ANG344B cultivation (chapter 2), was performed in the presence of 1 g/L of 2-PE for 22 h but without bacterial cultivation. In these conditions, at the end of the experiment, the condensed volume obtained in the traps was 54 mL, with a 2-PE concentration of 0.58 ± 0.04 g/L, representing only 1.2% of the total 2-PE present in the medium at the beginning of the experiment. These results show that the evaporation of 2-PE caused by the air flow and bioreactor stirring was not efficient for 2-PE removal. In fact, similar results were obtained when the bacterium *A. soli* ANG344B was cultivated in similar conditions with a trap connected to the bioreactor. The volume collected from the trap was 60 mL, with a 2-PE concentration of 0.92 ± 0.02 g/L. As expected, the cultivation coupled to the traps did not result in an increase in 2-PE production, being obtained 2.32 ± 0.18 g/L of 2-PE, while experiments performed without the external condensing traps resulted in 2.14 ± 0.18 g/L at 24 h of cultivation (chapter 2).

In fact, gas stripping is a technique used for low molecular weight (lower than 1000 g/mol) and high volatile compounds, such as ethanol and flavors as limonene, that are typically produced at high titers, even though it could be also viable to extract volatile compounds produced at low titers (Teke et al., 2022; Santos et al., 2020; Sun et al., 2020). The 2-PE, with a molecular weight of 122.16 g/mol, has an estimated Henry´s constant of 4.4 x 10^{-7} atm.m^3/mol and vapor pressure at room temperature of 8x10^{-5} atm, being characterized as a semi-volatile compound, with slow volatilization, suggesting that gas stripping approach might have limitations in 2-PE recovery (Lukin et al., 2018). Even though the low vapor pressure of 2-PE, it is important to take into consideration that the activity coefficient of this compound at infinite dilution in aqueous media, at room

temperature, is high (information available in literature indicates that 2-PE has an activity coefficient at infinite dilution at 100 °C of 167 (Hertel and Sommer, 2015)). As the volatility of a solute in solution is calculated by the product of the vapor pressure and the activity coefficient, the recovery of this aroma by air stripping was considered as a potential approach that deserved to be considered assessed. However, it was found that despite the high activity coefficient of this compound in aqueous media, the air stripping approach did not result in an sufficiently expedite aroma recovery, under the cultivation conditions tested.

Martínez and colleagues (Martínez et al., 2018) also reported the use of the continuous air supplied to the system for stripping out the volatile compounds favoring the aroma production in a 2-PE solid-state fermentation process using *Kluyveromyces marxianus*. However, 2-PE stripping was limited, lower than 5%, that is in accordance with the results presented above.

3.4.2. Liquid-liquid extraction

The potential use of oleic acid and polypropylene glycol (PPG) 1200 as extractive solvents was assessed for a liquid-liquid ISPR approach for the 2-PE production process by *A. soli* ANG344B. These solvents are commonly used in cosmetic applications, as skin care products, working as emulsifiers and as moisturizing agents (Franco et al., 2022; Fiume et al., 2012). Therefore, their use as extractive solvents for 2-PE might be an interesting opportunity.

The partition coefficient (P) is a crucial criterion to be considered for the evaluation of suitable extractants, as well as the biocompatibility of the solvents with the microorganism. The partition coefficient calculated for oleic acid for a 2-PE concentration ranging from 0 to 2 g/L was found to be 6.18 ± 0.01, which is in accordance with the reported for this solvent (Chreptowickz and Mierzejewska, 2018; Okuniewska et al., 2017; Sendovski et al., 2010; Stark et al., 2002). After reaching the phase equilibrium and considering the condition in which 2 g/L of 2-PE were used in the aqueous phase, 74% of the aroma compound was removed for the oleic acid phase. For PPG 1200, a partition coefficient of 21.97 ± 1.82 was experimentally determined, a lower value than the reported in literature, that is near 30 (Etschnmann and Schrader, 2006; Gao and Daugulis, 2009). This solvent was able to remove 96% of the 2-PE present in the

aqueous phase, considering the initial aroma concentration of 2 g/L. Despite PPG 1200 presented better results as 2-PE extractant, it formed an emulsion with the aqueous phase upon stirring, making the phase separation an extremely difficult process. For this reason, only oleic acid was tested during *A. soli* ANG344B cultivation for 2-PE production. These experiments were performed in shake flasks with addition of 20% (v/v) of the solvent to the culture broth after 3 h of cultivation, when OD_{600} (Optical density at 600 nm) was above 1. Due to the presence of the oleic acid, pH decreased from 7 to 4.6, a value that negatively influences the bacterial growth and 2-PE production (chapter 2), allowing a 2-PE production lower than 0.3 g/L. However, the addition of NaOH (5 M) to correct the pH to 7 produced a precipitate, caused by a saponification reaction. These results indicate a negative influence of oleic acid towards 2-PE production, despite the availability of L-Phe in the cultivation broth.

The use of oleic acid as 2-PE extractant for ISPR production processes has been reported by many authors, with different results from the obtained in this work. For *Saccharomyces cerevisiae* AM1-d, the biphasic cultivation with 30% of oleic acid, in shake flasks, resulted in an improvement of yeast growth and an improvement in 2-PE production. These authors reported an increase in 2-PE production from 1.65 g/L without oleic acid to 3.68 g/L in the presence of the solvent (Chreptowicz and Mierzejewska, 2018). The same *S. cerevisiae* strain was also evaluated for 2-PE production using 20% of oleic acid, presenting a similar cellular growth in the presence of this solvent and in the absence the solvent, producing 1.3 g/L of the aroma in a biphasic culture (Okuniewska et al., 2017). On other hand, Stark et al. (Stark et al., 2002) showed that oleic acid interfered with *S. cerevisiae* Giv2009 viability, having a synergistic inhibitory effect with the produced 2-PE, limiting aroma production. The authors also noticed the formation of a stable emulsion between the aqueous and the solvent phases and a limiting extractive capacity. Nevertheless, with a continuous addition of oleic acid to the cultivation medium, the authors reported an aroma production of 12.6 g/L, in comparison with the 3.8 g/L that was possible to obtain when no extractant was present (Stark et al., 2002). Similar approaches were attempted by Sendovski et al. and Hua et al. that reported no improvement in 2-PE production using oleic acid as extractant (Hua et al., 2013; Sendovski et al., 2010).

PPG (1200 and 1500) was also reported to be used as extractant solvent for ISPR of 2-PE production processes, being able to support productions of 10.2 and 7.5 g/L for

Kluyveromyces marxianus CBS 600 and *S. cerevisiae* P-3, respectively (Etschmann and Schrader, 2006; Hua et al., 2013). Etschmann and Schrader also reported the formation of an emulsion when using PPG 1200, affecting the biomass quantification during the production process (Etschmann and Schrader, 2006). However, the emulsion formed was not stable and the solvent could be easily separated from the aqueous phase by centrifugation, differently from the results obtained in this work, in which phase separation was not completely achieved.

Different solvents and approaches have also been reported in literature for a successful *in situ* removal of 2-PE during cultivation, improving the aroma production by avoiding product toxicity. Examples are the use of ethyl acetate, fatty acid methyl ester, biodiesel, rapeseed oil or ionic liquids as extractants in direct contact with cultivation broth (Chreptowicz et al., 2016; Domańska et al., 2016; Drężek et al., 2021; Lukito et al., 2019; Okuniewshka et al., 2017; Sendovski et al., 2010; Yan et al., 2020). The use of hollow fiber membrane contactors with solvents as alkanes or oleyl alcohols was also reported as ISPR approach to avoid the direct contact between microorganisms and solvents to avoid the emulsion formation and the toxicity of the solvent to the cells (Cordero-Soto et al., 2021; Mihal' et al., 2014)

3.4.3. Polymeric adsorption

3.4.3.1. Adsorbent screening

A solid-liquid extraction was also studied in order to develop an ISPR process for 2-PE production by *A. soli* ANG344B. Thus, the adsorption capacity of three polymeric resins towards 2-PE was evaluated, namely Amberlite XAD 4, Macronet MN 102 and Hytrel 8206.

Amberlite XAD 4 and Macronet MN 102 have a matrix of styrene-divinylbenzene, being the last one functionalized with a tertiary amine and both have a high surface area (Table 3.1). Although Macronet MN 102 has an ionic matrix, it has a limited ion exchange capacity, being considered as an adsorbent resin. Due to the presence of the aromatic ring and the hydroxyl group, 2-PE can interact through π and/or hydrogen interactions with the matrix of the resin. This aroma compound can interact with the styrene-divinylbenzene matrix through π interactions and the functionalization of the resin with a tertiary amine can also improve the interactions with the hydrophilic group of the

molecule. Hytrel 8206 is a block copolymer of polybutylene ester and polyethylene that offers the possibility of 2-PE interaction (through the hydroxyl group) with the polymer by hydrogen bonding due to the ester and ether linkages (Gao and Daugulis, 2009).

To assess which resin could have a better performance for the recovery of 2-PE from the cultivation broth, different dosages of each resin (from 1 to 20% for Amberlite XAD 4 and Macronet MN 102 and from 1 to 30% for Hytrel 8206) were evaluated, using pure solutions of 2-PE with 2 g/L, the concentration that is usually obtained in a batch production process. The three tested resins have the ability to adsorb 2-PE, being Amberlite XAD 4 and Macronet MN 102 the ones presenting a higher adsorption efficiency (Figure 3.3). These resins are capable of adsorbing above 96% of 2-PE with a resin ratio of 3% (dry w/v), resulting in an adsorption of 65.2 ± 0.2 and 66.5 ± 0.3 $mg_{2\text{-}PE}/g_{dry\ resin}$, for Amberlite XAD4 and Macronet MN102, respectively. Above this ratio the adsorption efficiency was constant at 98% for the 2-PE concentration tested. Regarding the resin Hytrel 8206, the adsorption efficiency is lower and seemed to start stabilizing at around 70% for resin dosages above 25% (dry w/v), resulting in an adsorption of 7.8 ± 0.6 $mg_{2\text{-}PE}/g_{dry\ resin}$, using a resin dosage of 20% (dry w/v). Considering these results, 3% (dry w/v) of Amberlite XAD 4 and Macronet MN 102 and 20% (dry w/v) of Hytrel 8206 were the selected dosages for the next steps of this work.

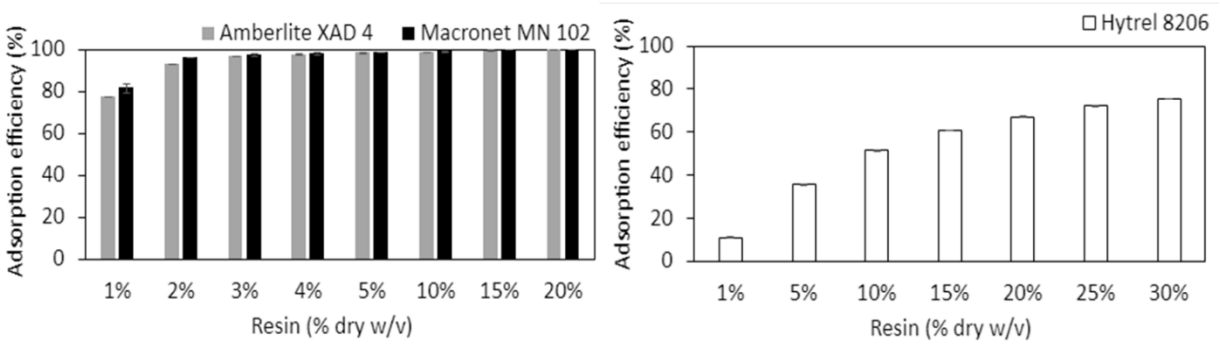

Figure 3.3 Adsorption efficiency calculated from experimental results for 2-PE adsorption by Amberlite XAD 4, Macronet MN 102 and Hytrel 8206 at different resin ratios, from a solution of 2 g/L of 2-PE.

As depicted in Figure 3.3, the lower dosage of resin (1% (dry w/v)) resulted in the lowest adsorption efficiency for all the tested resins. These results indicate that 2-PE was not totally adsorbed into the resin, meaning that there was an excess of the aroma compound for the available amount of resin used. When the available aroma is not limiting it is

possible to calculate the maximum resin adsorption capacity. The adsorption capacity of each resin was, therefore, estimated under resin-limiting conditions (no limitation of aroma available for adsorption). Amberlite XAD 4 could adsorb 155.0 ± 0.7 $mg_{2\text{-}PE}/g_{dry\ resin}$, Macronet MN 102 could adsorb 171.4 ± 5.9 $mg_{2\text{-}PE}/g_{dry\ resin}$ and Hytrel 8206 could adsorb 24.1 ± 0.4 $mg_{2\text{-}PE}/g_{dry\ resin}$. Hytrel 8206 presented the lowest adsorption capacity, and this might be caused by the large particle size of the resin (> 2 mm), that might offer a lower specific surface for adsorption and/or longer diffusion paths to adsorption sites. Further, the absence of regular pores and the type of uptake mechanism (hydrogen bonding) might be the additional cause for the low adsorption capacity. The results obtained are similar to the ones reported by Shu et al. for an analogous polymer, Hytrel G3548, which allows the same type of interactions with the aroma compound (Shu et al., 2021). These authors reported an adsorption capacity of 20.4 $mg_{2\text{-}PE}/g$ with an efficiency of 60.8%, when using 10% (w/v) of the adsorbent, in a solution of 3.4 g/L of 2-PE. Amberlite XAD 4 presented results comparable with other styrene-based resins, such as D101 and HZ818, that are described as having an adsorption capacity of around 140 $mg_{2\text{-}PE}/g_{resin}$ (Hua and Xu, 2011; Mei et al., 2009).

The adsorption efficiency of each resin (in the selected dosage for each resin) towards L-Phe was also evaluated using a pure solution of 5 g/L of L-Phe, the concentration of precursor that is usually used in the beginning of the cultivation process (chapter 2). In these conditions 35.9 ± 0.1, 38.7 ± 0.7 and 3.2 ± 0.2% of L-Phe were adsorbed by Amberlite XAD 4, Macronet MN 102 and Hytrel 8206, with an adsorption capacity of 60.6 ± 0.3, 64.2 ± 1.1 and 0.9 ± 0.0 $mg_{L\text{-}Phe}/g_{dry\ resin}$, respectively. These results show that all the resins tested had a lower affinity for L-Phe than for 2-PE, as expected and desirable.

Moreover, the co-adsorption of L-Phe and 2-PE was also evaluated using a binary solution of 2-PE and L-Phe at 2 g/L of each compound (Figure 3.4). In these conditions, the adsorption efficiency for 2-PE was similar to the obtained for the single component solution for all the adsorbents tested, not being affected by the presence of L-Phe, having obtained similar adsorptions: 66.4 ± 0.1, 61.4 ± 0.4 and 7 ± 0.0 $mg_{2\text{-}PE}/g_{dry\ resin}$, respectively for Amberlite XAD 4, Macronet MN 102 and Hytrel 8206. Regarding L-Phe, the adsorption efficiency was 29.8 ± 0.3% for Amberlite XAD 4, 37.6 ± 0.2% for Macronet MN 102 and 6.1 ± 0.1% to Hytrel 8206, proving a higher selectivity of all the resins towards 2-PE (Figure 3.4). In these conditions 20.0 ± 0.0, 25.4 ± 0.1 and 0.6 ± 0.0 $mg_{L\text{-}Phe}/g_{dry\ resin}$ were adsorbed respectively for Amberlite XAD 4, Macronet MN102 and

Hytrel 8206. All the adsorbents tested show a high specificity for 2-PE but can also adsorb L-Phe. In fact, the majority of the polymeric adsorbent resins reported for 2-PE adsorption have also the ability to adsorb L-Phe in lower amounts. Macronet MN 102 has a hydrophilic functional group, that might contribute to the increase of L-Phe adsorption, without improving the aroma adsorption.

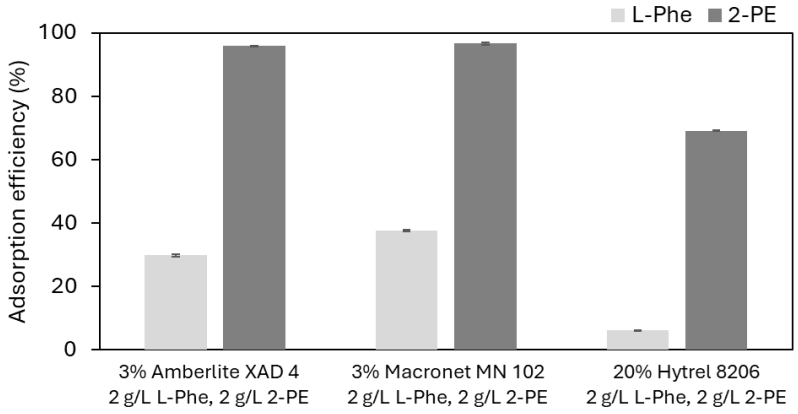

Figure 3.4 Adsorption efficiency calculated from experimental results for 2-PE and L-Phe adsorption by Amberlite XAD 4, Macronet MN 102 and Hytrel 8206, from a solution of 2 g/L of 2-PE and 2 g/L of L-Phe.

Amberlite XAD 4 was reported by Lukito et al., to adsorb 96% of 2-PE and 20% of L-Phe with a resin dosage of 10% (w/v) from solutions with 50 mM of 2-PE or L-Phe. The results reported by these authors allowed the determination of an adsorption of 59 $mg_{2\text{-}PE}/g_{Amberlite\ XAD\ 4}$ and 17 $mg_{L\text{-}Phe}/g_{AmberliteXAD\ 4}$ that are similar to the obtained in the present results. However, it is important to notice that the initial 2-PE and L-Phe concentrations and the resin dosage tested by these authors are different from the used in the present work, as well as the composition of the solution, which can influence product adsorption. Other non-polar resins, HZ818 and D101, were reported to have different capacity for L-Phe adsorption (14 and 80 $mg_{L\text{-}Phe}/g_{resin}$, respectively) despite of similar 2-PE adsorptions (140 $mg_{2\text{-}PE}/g_{resin}$), comparable to the obtained in the present work (60 ± 0.3 $mg_{L\text{-}Phe}/g_{dry\ resin}$ and 155.0 ± 0.7 $mg_{2\text{-}PE}/g_{dry\ resin}$) (Hua et al., 2010; Mei et al., 2009).

Considering an adsorbent functionalized with a tertiary amine, Macronet MN 100 (that is an adsorbent similar to the used in the present work), the maximum adsorption calculated towards 2-PE was 1.5 times higher than for L-Phe, reveling a low 2-PE selectivity (Šimko et al., 2015).

Different from the results obtained in the present work, Gao and Daugulis reported that Hytrel 8206 has no affinity for L-Phe, a difference that can also be related with the environment in which the adsorbent contacts (Gao and Daugulis, 2009).

The adsorption efficiency and adsorption capacity of Amberlite XAD 4 and Macronet MN 102 were similar towards 2-PE. However, Amberlite XAD 4 has a lower adsorption efficiency towards L-Phe, so this adsorbent was selected to perform an ISPR cultivation of *A. soli* ANG344B in shake flasks to access the potential of the adsorbent as *in situ* extractant. Although Hytrel 8206 has a lower adsorption capacity for 2-PE than the other resins, the adsorption of L-Phe is also lower, a feature that can be advantageous for the aroma recovery, thus, this adsorbent was also evaluated on bacterial cultivation.

Bacterial cultivations were performed with the selected dosages of each resin (3% w_{dry}/v for Amberlite XAD 4 and 20% w_{dry}/v for Hytrel 8206) and compared to the production without the use of *in situ* extractant. Standard conditions (without *in situ* product adsorption) allowed the production of 2.00 g/L of 2-PE with 2.9 g_{CDW}/L, consuming 6.80 g/L of glucose and at the end of the cultivation 0.7 g/L of L-Phe was still present in the cultivation broth. In the experiments using the adsorbents, 2-PE overall production increased to 2.80 and 3.86 g/L for Hytrel 8206 and Amberlite XAD 4, respectively, with all the L-Phe being consumed, representing an increase in aroma production of 40 and 93%, respectively. In these conditions, it was possible to achieve a 2-PE production yield from L-Phe of 0.51 and 0.64 $g_{2\text{-}PE}/g_{L\text{-}Phe}$, respectively for Hytrel 8206 and Amberlite XAD 4. Furthermore, in the presence of the resins, 2-PE concentration in aqueous phase decreased to 0.71 and 0.17 g/L, for Hytrel 8206 and Amberlite XAD 4, respectively. The lower 2-PE concentration in the cultivation media led to an increase in cellular growth when using the adsorbent resins (4.8 and 7.4 g_{CDW}/L, respectively for Hytrel 8206 and Amberlite XAD 4) and total glucose consumption in both assays (resins seem to have no affinity for glucose). Similar behavior was reported for *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*, that in the presence of the adsorbents (Hytrel 8206 and D101, respectively), were able to increase cell density, L-Phe consumption and consequently 2-PE production (Gao and Daugulis, 2009; Mei et al., 2009).

Therefore, based on these results Amberlite XAD 4 was selected for further investigation.

3.4.3.2. Dynamic adsorption

In order to determine the adsorption capacity, desorption and operating behavior that allow a scale up, a dynamic adsorption experiment was performed using Amberlite XAD 4, a single component solution with 1 g/L of 2-PE and a binary solution containing L-Phe and 2-PE with 1 g/L of each component.

The breakthrough curve of 2-PE is represented in Figure 3.5 a. It was obtained in a fixed bed column of Amberlite XAD 4 and expressed in terms of normalized concentration of 2-PE (ratio of outlet to inlet 2-PE concentration, C/C_0) as function of time.

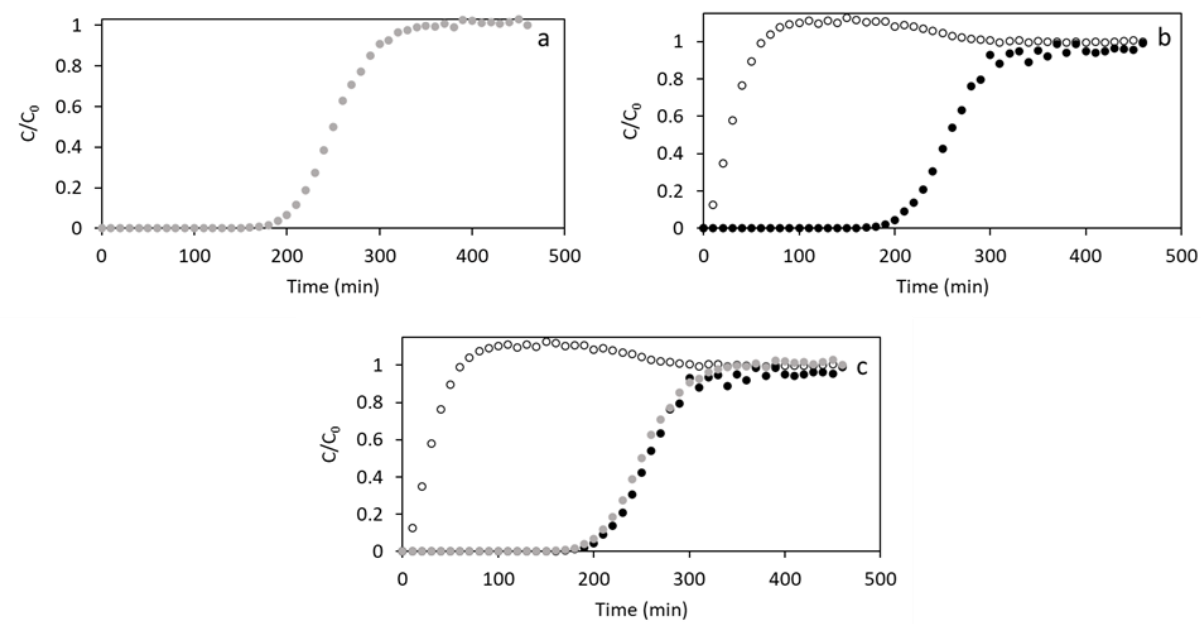

Figure 3.5 Breakthrough curve of 2-PE in a single component solution (a) and 2-PE and L-Phe in a binary solution (b) on Amberlite XAD 4 at 30 °C and the overlay of the curves (c). 2-PE (●), L-Phe (○).

In the conditions tested, the resin saturation ($C/C_0 = 1$) was achieved at 340 min, that corresponds to 55 BV (bed volume, 1 BV = 61.3 mL). At that time 2.7 ± 0.2 g of 2-PE have been adsorbed, corresponding to a maximum adsorption capacity of 205.8 ± 8.1 $mg_{2\text{-}PE}/g_{dry\ resin,}$ higher than the estimated in batch experiments described above (155.0 $mg_{2\text{-}PE}/g_{dry\ reisin}$).

Considering the breakthrough curves for 2-PE and L-Phe in a binary solution, illustrated in Figure 3.5 b., the adsorption of 2-PE does not seem to be affected by the presence of

L-Phe, since the breakthrough curve is similar to the one obtained using the single component solution as clearly shown in Figure 3.5 c by the overlay of both breakthrough curves. L-Phe was detected in the outlet of the column from the beginning of the experiment, reaching the saturation of the resin for this compound after 60 min, corresponding to 10.4 BV. In these conditions, Amberlite XAD 4 adsorbed 21.8 ± 0.0 $mg_{L\text{-}Phe}/g_{dry\ resin}$, that was lower than the estimated in the batch experiments which was limited in the adsorbent available using a single component solution (5 g/L of L-Phe), 60.6 ± 0.3 $mg_{L\text{-}Phe}/g_{dry\ resin}$. The adsorption capacity for 2-PE calculated using a binary solution was 219.2 ± 0.3 $mg_{2\text{-}PE}/g_{dry\ resin}$, similar to the obtained using a single component solution in a dynamic adsorption experiment (205.8 ± 8.1 $mg_{2\text{-}PE}/g_{dry\ resin}$). These results suggest that the adsorption of 2-PE is not affected by the presence of L-Phe, but not the opposite.

Based on these results, Amberlite XAD 4 seems to be a good alternative to enhance 2-PE production process, since it has a high adsorption capacity for the product, higher than the reported for other non-polar macroporous resins or copolymers of polybutylene ester and polyether. In fact, HZ818 was reported to adsorb 140 $mg_{2\text{-}PE}/g_{dry\ resin}$, D101 could adsorb 136.2 $mg_{2\text{-}PE}/g_{dry\ resin}$, FD0816 allowed an adsorption of 190.9 $mg_{2\text{-}PE}/g_{dry\ resin}$, Hytrel 8206 resulted in calculated maximum adsorption capacity of 133 $mg_{2\text{-}PE}/g_{dry\ resin}$ and Hytrel G3548 ensured an adsorption of 20.4 $mg_{2\text{-}PE}/g_{dry\ resin}$ (Gao and Daugulis, 2009; Hua et al., 2010; Mei et al., 2009; Shu et al., 2021; Wang, Dong, Meng et al., 2011).

For the development of a continuous ISPR production process, the determination of the breakthrough point is crucial. The breakthrough point corresponds to the time at which 5% of the initial product concentration is reached in the effluent, coming out from the column. At that moment, the column should be replaced to avoid product loss, that passes through the resin and is not completely adsorbed. Considering this, the maximum adsorbent capacity cannot be reached (Chowdhury, Abd Hamid and Zain, 2015; Fouladi et al., 2020; Valério et al., 2022). Regarding results depicted in Figure 3.5 a, the breakthrough point is achieved at 190 min, corresponding to 31.0 BV. At that time, 2.1 ± 0.0 g of 2-PE had been adsorbed by Amberlite XAD 4, with an adsorption capacity of 160.2 ± 3.9 $mg_{2\text{-}PE}/g_{dry\ resin}$, which corresponds to 78% of the maximum capacity of the adsorbent. It is worth note that, considering the adsorption of L-Phe and 2-PE in a binary solution, at the time the breakthrough point for 2-PE was reached (190 min), a low L-Phe adsorption is achieved (21.8 ± 0.0 $mg_{L\text{-}Phe}/g_{dry\ resin}$). However, the breakthrough curves

are dependent on the flow rate, the bed length, the initial product concentration and the amount of adsorbent used. Thus, to develop a continuous process, these parameters need to be optimized (Carpiné et al., 2013; Chowdhury, Abd Hamid and Zain, 2020), although these results offer a good basis for further development.

3.4.3.3. Product recovery from the resin

Ethanol 96% and deionized water were evaluated as eluents for product recovery from Amberlite XAD 4, in batch experiments. For that, Amberlite XAD 4 contacted with a binary solution with 2 g/L of 2-PE and L-Phe or with a single component solution containing 2 g/L of 2-PE for product adsorption and was further eluted with ethanol 96% or water.

The elution with ethanol 96% could desorb 100.0 ± 0.1% of the adsorbed L-Phe and 88.8 ± 0.4% of the adsorbed 2-PE in only one step of desorption (Figure 3.6). The elution with water could desorb 62.5 ± 0.1% of the adsorbed L-Phe and 2.6 ± 0.0% of the adsorbed 2-PE (Figure 3.6). Similar results were reached for the desorption of 2-PE, adsorbed from a single component solution, that reached a desorption efficiency of 89.6 ± 0.2% using ethanol 96% and 2.3 ± 0.0% using water as eluent. These results show that the presence of L-Phe adsorbed in the resin does not affect the desorption of the aroma. However, using ethanol as eluent, the adsorbed L-Phe will be part of the extract recovered from the resin, since it is completely recovered. Even so, Amberlite XAD 4 has a lower adsorption efficiency for L-Phe and the extract will be mostly composed of 2-PE.

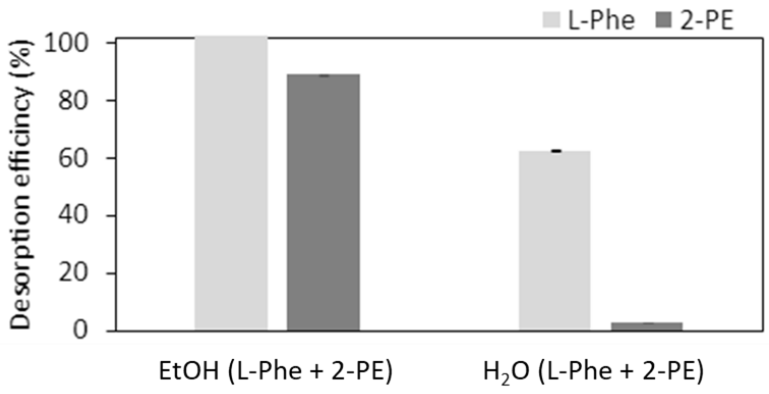

Figure 3.6 Desorption efficiency of L-Phe and 2-PE from Amberlite XAD 4 in one desorption cycle using water and ethanol. A previous adsorption step was performed using a binary solution of L-Phe and 2-PE with 2 g/L of each component.

Considering the moderate cost of ethanol and its acceptance in industrial applications, related with food and cosmetics, it can be considered a suitable elution solvent for 2-PE recovery from the resin. Therefore, this solvent was selected as eluent in the next steps of this work.

After the dynamic adsorption experiments (after reaching the saturation point of the resin towards 2-PE), a desorption procedure was performed, aiming to determine the volume of eluent needed for total recovery of 2-PE from the resin. The desorption was performed with ethanol 96%, at the same flow rate as the adsorption but at room temperature, the results are represented in Figure 3.7. A total of 99% of the adsorbed 2-PE could be recovered from Amberlite XAD 4 after 5 BV (1 BV = 61.3 mL) (Figure 3.7.a) and this behavior did not change when L-Phe was also adsorbed in the resin (Figure 3.7.b), as anticipated from the batch desorption experiments. Regarding L-Phe, 35% of the adsorbed compound was eluted from the resin, during the tested period. The high recovery percentage of 2-PE from the resin further confirm the suitability of this resin to the 2-PE production process, since the aroma is not lost in this recovery step.

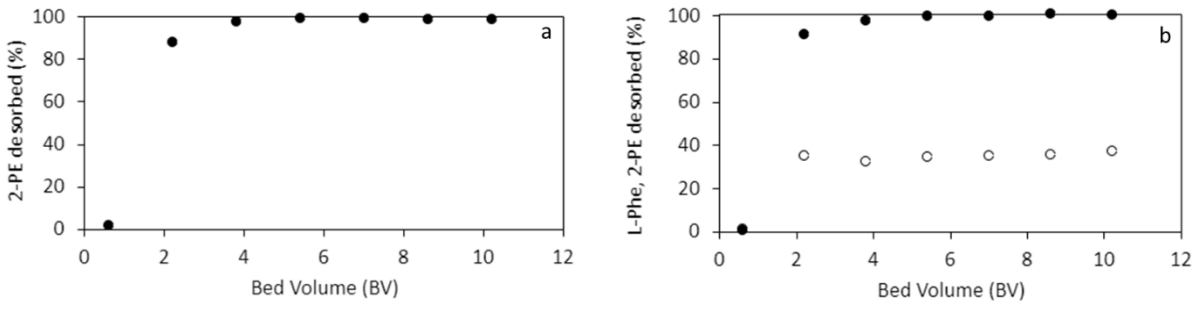

Figure 3.7 Desorption of 2-PE (a) and L-Phe and 2-PE (b) from Amberlite XAD 4 represented as percentage of desorbed compound as function of bed volume (1 BV = 61.3 mL) of ethanol 96% at room temperature. 2-PE (●) and L-Phe (○).

3.4.4. 2-PE bioproduction with ISPR using Amberlite XAD 4

Considering the behavior of the selected resin towards 2-PE and L-Phe, an *A. soli* ANG344B batch cultivation in 2 L bioreactor experiment was performed using 3% (w_{dry}/v) of Amberlite XAD 4 (Figure 3.8). The cultivation was performed using 12 g/L of soluble L-Phe, taking into account the adsorption of that compound, considering the

adsorption capacity of the resin towards 2-PE (205.8 ± 8.1 $mg_{2\text{-}PE}/g_{dry\ resin}$ determined in dynamic adsorption experiments) and the expected production yield (determined in chapter 2), aiming to increase the aroma production.

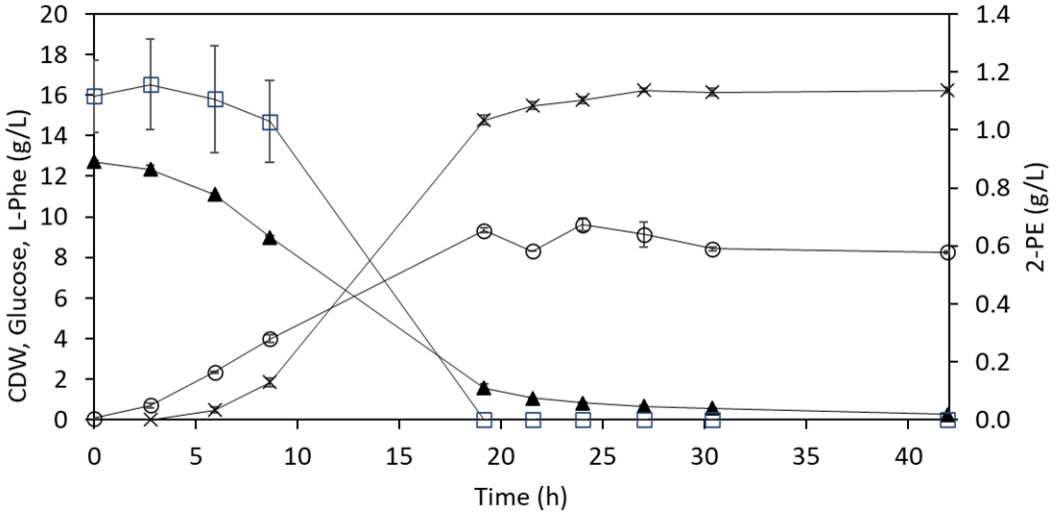

Figure 3.8 Cultivation profile of *Acinetobacter soli* ANG344B in 2 L bioreactor using 3% Amberlite XAD 4 from the beginning of the cultivation, starting with 12 g/L of L-Phe. CDW (○), L-Phe (▲), 2-PE in the broth (x) and glucose (□).

In these conditions, at the end of the experiment, it was possible to achieve 14.33 ± 0.11 g of 2-PE (considering the 2-PE in solution and the amount recovered from the resin), that corresponds to 6.99 ± 0.06 g/L of 2-PE, taking in consideration the total volume of the experiment, with a volumetric productivity of 0.17 ± 0.00 g/L.h. From the total amount of 2-PE produced, 84% was adsorbed into the resin and the concentration in the aqueous phase was kept around 1 g/L though all the cultivation period. The use of Amberlite XAD 4 as *in situ* product adsorbent avoided the accumulation of 2-PE in toxic concentrations, allowing to reach 8.2 ± 0.0 g/L of biomass (dry weight), that is the double of the obtained in standard conditions (without ISPR). In the presence of the resin, L-Phe was totally consumed, which has not happened in standard conditions due to the loss of cell viability by contact with toxic aroma concentrations (chapter 2). The comparison between standard conditions and the use of the selected ISPR technique are presented in Table 3.2. With this ISPR approach, the production yield from L-Phe was 0.58 ± 0.01 $g_{2\text{-}PE}/g_{L\text{-}Phe}$, similar to the obtained in standard conditions, as expected, and the volumetric productivity, increased from to 0.12 $g_{2\text{-}PE}$/L.h to 0.17 ± 0.00 g/L.h. However, the volumetric productivity obtained in standard conditions was calculated at 15 h of assay, by the time that the aroma produced started to stabilize. With ISPR approach, the

volumetric productivity was calculated at the end of the experiment, since the resin was not removed during sample withdrawal. In fact, the use of this ISPR technique resulted in a faster L-Phe consumption. L-Phe consumption rate using this ISPR approach was 0.54 ± 0.01 g/L.h until 22 h by the time that the production process started to slow down, suggesting a much higher volumetric productivity of 2-PE for the same period, as expected since *Acinetobacter soli* ANG344B is reported to achieve a 2-PE volumetric productivity among the highest for a wild-type 2-PE producer. In the standard conditions (without ISPR techniques), the L-Phe was consumed at 0.26 ± 0.05 g/L.h during the first 16 h, half than the rate achieved using the resin. These results suggest that the use of Amberlite XAD 4 might have a higher impact in the process than the suggested by the calculated volumetric productivity. Using this approach, it was possible to increase by 3.3-fold the aroma production when compared to the standard conditions.

Table 3.2 Kinetic parameters calculated for 2-PE production using an ISPR approach with 3% of Amberlite XAD 4 at 42 h and in standard conditions at 16 h. $2\text{-PE}_{overall}$ is calculated having in consideration the total mass of the aroma (2-PE present in solution and recovered from the resin) and the total volume of media used in the experiment.

	3% Amberlite XAD 4	**Standard conditions (chapter 2)**
CDW (g/L)	8.2 ± 0.1	4.5 ± 1.0
L-Phe_{cons} (g/L)	12.12 ± 0.09	4.00 ± 0.74
$\text{2-PE}_{overall}$ (g/L)	6.99 ± 0.06	1.92 ± 0.13
$\text{2-PE}_{\text{in solution}}$ (g/L)	1.14 ± 0.01	1.92 ± 0.13
$\text{2-PE}_{desorbed}$ (g)	11.98 ± 0.10	-
$Y_{(P/S)}$ (g/g)	0.58 ± 0.00	0.49 ± 0.07
R_p (g/L.h)	0.17 ± 0.00	0.12 ± 0.01

During the cultivation process, an increase in the viscosity of the broth was noticed and the presence of an exopolysaccharide (EPS) was investigated. At the end of the experiment 2.4 ± 0.0 g/L of an EPS was quantified in the cultivation broth, a result that was not obtained when *A. soli* ANG344B was cultivated in standard conditions (without ISPR techniques). The production of EPS by *A. soli* ANG344B is discussed in chapter 5.

The use of ISPR techniques was also reported for 2-PE production by different microorganisms, with 2-PE production improvements. *Kluyveromyces marxianus*

cultivation with 100 g of Hytrel 8206, could improve aroma production from 1.45 g/L to 3.82 g/L of 2-PE with a volumetric productivity of 0.10 g/L.h by fed-batch cultivation in a 3 L bioreactor, and this production was further enhanced by increasing the amount of resin in contact with the cultivation broth (Gao and Daugulis, 2009). *Saccharomyces cerevisiae* R-UV3 could produce 13.7 g/L of 2-PE using 300 g of FD0816 in contact with cultivation broth through filter-cloth bags in a 5 L bioreactor with 3 L working volume, resulting in an increase of 213% of aroma production (Wang, Dong, Guan et al., 2011). *Saccharomyces cerevisiae* P-3 cultivated with 7% (w_{dry}/v) of the non-polar macroporous resin HZ818, could increase the aroma production in 66.2% to 6.6 g/L, in a 1 L flask with 100 mL working volume (Hua et al., 2010). The bioconversion in the presence of 2 g of resin D101 allowed the increase in 2-PE production from 4.65 to 6.17 g/L by *Saccharomyces cerevisiae* BD cultivated in a 250 mL flask with 30 mL of working volume (Mei et al., 2009). As reported for other microorganisms, the use of a polymeric adsorbent as an ISPR technique resulted in significant improvements in 2-PE production by *A. soli* ANG344B, that achieved a 2-PE production comparable to the titers obtained by yeasts. Hence, the intrinsic advantages offered by bacteria, in terms of shorter growth time and simple metabolism, that avoid the production of ethanol, make *Acinetobacter soli* ANG344B a strong candidate to advance the 2-PE natural production. To the best of my knowledge, this is the highest 2-PE production reported for a wild-type bacteria.

The 2-PE adsorbed into Amberlite XAD 4 during the production process was 203.3 $mg_{2\text{-}PE}/g_{dry\ resin}$, that is very close to the maximum adsorption capacity of the resin (205.8 ± 8.1 $mg_{2\text{-}PE}/g_{dry\ resin}$), proving that the production environment does not affect the adsorption. These results, besides representing a great improvement in 2-PE production process by *A. soli* ANG344B, suggest that the aroma production and product productivity might be improved by increasing both the L-Phe fed to the cultivation broth and the amount of adsorbent in contact with the broth, paving the way for further improvement in aroma production.

Based on the results described above regarding product desorption, 2-PE produced during the bioconversion process was recovered from the adsorbent using ethanol. Due to the difference in the boiling points of these two components, ethanol could be evaporated to obtain a highly concentrated 2-PE extract. In the conditions tested it was possible to obtain a 44 mL extract with a 2-PE concentration of 217.56 ± 0.31 g/L and 6.16 ± 1.42 g/L of L-Phe. From the total 2-PE desorbed, only a small percentage of 2.3% was lost for the

evaporated phase. The evaporated ethanol was recovered and can be further used in desorption steps of 2-PE production processes.

The GC analysis of this concentrated extract allowed to determine a chromatographic purity of 98% of the compound in a matrix of ethanol. However, it is important to note that the presence of non-volatile compounds in the extract is not being considered in this analysis. The GC-MS analysis suggests the presence of chalcones and propiophenones that are reported to be used as ingredients in cosmetic formulations with pharmacological uses (Dhaliwal et al., 2022; Stompor et al., 2019).

3.5. Conclusion

The use of ISPR techniques aims to improve 2-PE production process by avoiding the contact of the aroma with the producing microorganism. This work studied different approaches for 2-PE recovery from the cultivation broth, based on physical and chemical properties of the product, in cultivation of *Acinetobacter soli* ANG344B. In view of this, gas stripping, liquid-liquid extraction and adsorption approaches were studied, and their implementation attempted in *Acinetobacter soli* ANG344B cultivation conditions, to verify the applicability of each process for enhancement of 2-PE production and its recovery. The gas stripping approach did not improve the process due to the slow volatilization of the product. Liquid-Liquid extraction-based approaches, using oleic acid and PPG 1200 showed that these solvents are not suitable for 2-PE production process by *A. soli* ANG334 due to the negative influence on bacterial growth and the formation of emulsions, respectively. On the other hand, the use of macroporous adsorbents is a promising alternative for improving 2-PE production processes. Amberlite XAD 4 was the adsorbent selected, having high affinity for 2-PE, with an adsorption capacity of 205.8 ± 8.1 $mg_{2\text{-}PE}/g_{dry\ resin.}$ The use of 3% (dry w/v) Amberlite XAD 4 in a batch bioconversion greatly improved the production process, being possible to achieve 6.99 ± 0.06 g/L of 2-PE with a volumetric productivity of 0.17 ± 0.00 g/L.h, compared to the 2.14 ± 0.18 g/L of 2-PE obtained in a conventional process without 2-PE removal, a remarkable result for a wild-type bacteria. During the batch bioconversion process, the maximum adsorption capacity of the resin was achieved with all the L-Phe consumed, suggesting that the process can be further improved by increasing the amount of adsorbent in contact with the cultivation broth and the amount of L-Phe fed to the bioreactor. This study constitutes a useful contribution to the field by assessing three different *in situ* extractive approaches

- air stripping, liquid-liquid extraction and adsorption -, discussing the difficulties encountered in their application to the *Acinetobacter soli* ANG344B cultivation. This work also highlights the potential of *Acinetobacter soli* ANG344B as a viable 2-PE producer, paving the way for the development of a robust 2-PE bioproduction process.

4.

Valorization of agro-industrial feedstocks into 2-phenylethanol by *Acinetobacter soli* ANG344B

The results presented in this chapter are part of the following manuscript:

Bernardino, A. R. S., Torres C. A. V, Reis M. A M. Valorization of agro-industrial feedstocks into 2-PE by *Acinetobacter soli* ANG344B. In preparation.

4.1. Summary

The growing demand for natural products and the interest in health and environment have led to the development of technologies, such as biotechnological processes. The development of these processes can go through the valorization of agro-industrial wastes to generate value-added products, thereby promoting sustainable residue management. The main goal of this study was to cultivate *Acinetobacter soli* ANG344B in waste-based media to produce 2-phenylethanol (2-PE), a highly prized aroma compound known for its rose-like scent. Several commercial carbon sources were tested for bacterial growth, with glucose and fructose identified as the sugars that assured the highest growth and 2-PE production from L-phenylalanine (L-Phe). Based on these results, agro-industrial wastes – specifically banana peels, bread and brewer's spent grains (BSG) - were hydrolyzed and used as carbon sources for the bioconversion of L-Phe into 2-PE. Although the culture was able to grow and produce 2-PE in all tested hydrolysates, the hydrolysate from banana peels, rich in fructose and glucose, supported the highest growth (6.9 ± 0.7 g_{CDW}/L), 2-PE production (2.48 ± 0.04 $g_{2\text{-}PE}$/L) and volumetric productivity (0.17 ± 0.00 $g_{2\text{-}PE}$/L.h). Furthermore, incorporating Amberlite XAD 4 as an *in situ* product adsorbent during 2-PE production using banana peels as carbon source, resulted in an increased aroma production to 7.30 ± 0.01 g/L with a volumetric productivity of 0.27 ± 0.00 g/L.h. The findings highlight the potential of using agro-industrial wastes as sustainable and effective carbon source for enhancing 2-PE production by *A. soli* ANG344B, contributing to the development of eco-friendly bioprocesses.

4.2. Introduction

Microorganisms such as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* have been widely studied for their ability to produce 2-phenylethanol (2-PE), reaching up to 4.5 and 1.45 g/L, respectively, in fed-batch operational mode without *in situ* product removal (ISPR) techniques. With the application of ISPR techniques, these yields can increase to 16.46 and 20.4 g/L, respectively (Eshkol et al.,2009; Gao and Daugulis, 2009; Okuniewska et al., 2017). Despite the substantial production of 2-PE, the price of the biotechnologically produced aroma is still 10 to 100 times higher than the synthetically produced compound. Consequently, there is a need to reduce the production costs by decreasing the costs with the cultivation media, for example, using agro-industrial

byproducts (*2-Phenylethanol Market to Hit $350 Million by 2027, Says Global Market Insights Inc.*, 2021).

Agro-industrial feedstocks are highly available and rich in nutrients, proteins and sugars, making them a suitable alternative to use in microorganisms cultivation media. Through microbial cultivation, these low-cost feedstocks can be utilized to produce high-value products, simultaneously reducing cultivation media costs, minimizing waste disposal, and contributing to a circular economy (Yaashikaa et al., 2022; Karaalioğlu and Yüceer, 2021). Due to the complex structure of the wastes, often a hydrolysis step is needed prior to its utilization, to have available sugars for microbial growth. Different methodologies can be used, but chemical methods, as dilute acid hydrolysis, are the most used, due to its simplicity, rapidity and low-cost, even though it can generate side compounds that could affect cellular growth (Araújo et al., 2021).

For 2-PE production, an ideal substrate is one that is rich in sugars and proteins, providing both the necessary nutrients for microbial growth and L-Phe as precursor for the aroma production. Banana peels, Brewer's spent grains (BSG) and bread waste are examples of such waste materials. Banana is among the most produced fruit in the world, with their peels representing 30 to 40% of the fruit, leading to the generation of large quantities of peels as waste, estimated at around 34.72 to 46.29 million metric tons annually (El Barnossi et al., 2021). Banana peels are reported to have a protein and carbohydrate content of 6.6 and 60%, respectively (Araújo et al., 2021; El Barnossi et al., 2021). BSG, the most abundant byproduct of the brewing industry, has an average global annual production of 39 million tons, with reported 27.5% of protein content and 46.5% of carbohydrates (Araújo et al., 2021; Qin et al., 2018). Bread waste generated predominantly in households and retail, exceeds 100 million tons globally each year (Torabi et al., 2021). Bread is reported to have 13% of protein and 64.1% of carbohydrates in its composition (Adessi et al., 2018; Torabi et al., 2021). Although, these wastes can be used as animal feeds, bio-fertilizers, or for energy production due to their rich nutritional composition, the majority still ends up in landfills (El Barnossi et al., 2021; Yaashikaa et al., 2022).

In view of this, in this study, different commercial carbon sources were tested to understand which sugars could be used by *Acinetobacter soli* ANG344B. Based on these results, different agro-industrial wastes were evaluated to produce 2-PE by the bacteria. Banana peels, BSG, and bread waste were hydrolyzed and used for bacterial cultivation

and 2-PE production from L-Phe. The hydrolysate that assured better 2-PE production proceeded to cultivation using an *in situ* product removal approach.

4.3. Materials and methods

4.3.1. Microorganism and inoculum preparation

The microorganism preservation, reactivation and the inoculum preparation were performed as described on section 2.3.1.

4.3.2. Raw materials

Banana peels were preserved in sealed bags at -20 °C until further use to be mashed. BSG was supplied by Sociedade Central de Cervejas (Vialonga, Portugal), and dried at 70 °C until constant weight, then milled into powder and stored in sealed bags at room temperature until further use. Wheat bread was obtained from a local bakery shop, dried at room temperature for 3 days and milled into powder and stored in sealed bags at -20 °C until further use.

4.3.3. Preparation of hydrolysates

Banana peels were mashed into a puree and subsequently hydrolyzed in an autoclave (121 °C, 1 bar). Milled BSG and bread underwent dilute acid hydrolysis, utilizing sulfuric acid and hydrochloric acid, respectively, in autoclave. The specific hydrolysis conditions are detailed in Table 4.1. Following hydrolysis, the resulting products were centrifuged at 13000 xg and 4 °C for 20 min, with the solid fractions being discarded. The remaining supernatant was filtered using 20 µm paper filter. To optimize the conditions for cultivation, the pH values of the filtered supernatants were adjusted to 7 with NaOH pellets for pH correction of bread and BSG hydrolysates or a solution of NaOH 5 M for banana peels hydrolysate. After, the solutions were centrifuged at 13000 xg and 4 °C for 20 min before being used for cultivation.

Table 4.1 Hydrolysis conditions applied to the different wastes.

Raw material	Acid concentration (% w/w)	Solid load (g/L)	Hydrolysis time (min)	References
Banana peels	-	1204	40	This study
BSG	3	125	20	Araújo et al., 2021
Bread	1	160	20	Torabi et al., 2021

4.3.4. Shake flask cultivations

Cultivations were performed in 250 mL baffled shake flasks with a working volume of 100 mL at 30 °C and pH 7. The medium under investigation, detailed below, was sterilized in an autoclave at 121 °C, 1 bar, during 20 min. A 5 % (v/v) inoculum cultivated as previously described, was used to inoculate the medium. The culture was stirred at 200 rpm in an orbital shaker with temperature control, and pH was periodically adjusted by the addition of 5 M NaOH and 5 M HCl.

The composition of the cultivation medium was Na_2HPO_4, 4 g/L; KH_2PO_4, 1 g/L; NaCl, 0.2 g/L; $MgSO_4.7H_2O$, 0.2 g/L; $CaCl_2$, 0.05 g/L; yeast extract, 5 g/L; L-Phe at concentrations of 5 g/L or 0 g/L. The carbon source involved screening with 10 g/L of glucose, fructose, arabinose, xylose, glycerol or lactose. For cultivation using hydrolysates as carbon sources, 64 mL of banana peels hydrolysate, 59 mL of BGS hydrolysate, or 12 mL of bread hydrolysate were added to the cultivation media. The cultivations were performed for 48 h, and samples were collected periodically for quantification of cell growth, L-Phe, 2-PE and sugars.

4.3.5. Bioreactor cultivation

Batch cultivations were carried in 2 L bioreactors (BioStat B-plus, Sartorius, Germany) as described in chapter 2, section 2.3.3 (standard conditions). The growth medium contains Na_2HPO_4, 4 g/L; KH_2PO_4, 1 g/L; NaCl, 0.2 g/L; $MgSO_4.7H_2O$, 0.2 g/L; $CaCl_2$, 0.05 g/L; yeast extract, 5 g/L; L-Phe, 5 g/L and the carbon source in study.

For experiments with pure carbon sources, 10 g/L of glucose or fructose were used. For experiments using wastes as carbon sources, 830 mL of banana peels or 250 mL of bread hydrolysate were used.

Cultivations using an *in situ* product removal approach were performed under the same temperature, pH and aeration conditions. The cultivation broth was modified to have 16.97 g/L of L-Phe (12 g/L soluble L-Phe) and 1.6 L of banana peels as the carbon source. For ISPR by adsorption, 3% (w/v) of dry Amberlite XAD 4 were added to the bioreactor medium and sterilized with the cultivation media. The 2-PE adsorbed to the polymer was quantified in the end of the experiment.

4.3.6. Product recovery form the resin

After the bioconversion, Amberlite XAD 4 was processed as described in chapter 3, section 3.3.4.4 to recover the produced 2-PE. Ethanol was condensed using a rotatory evaporator until the volume of the 2-PE phase was below 200 mL.

4.3.7. Analytical techniques

4.3.7.1. Characterization of hydrolysates

To characterize the hydrolysates, after neutralization, samples were taken to quantify sugars, furfural, 5-hydroxymethylfurfural (5HMF), acetic acid, lactic acid, levulinic acid and protein present in the hydrolysates.

The sugar composition of the neutralized hydrolysates was determined by HPLC. Samples were filtered through 0.2 µm syringe filters and analyzed using a CarboPac PA10 (4 x 250 mm) and an AminoTrap (4 x 50 mm) columns (Dionex ICS3000). The system was equipped with a pulsed amperometric detector (PAD), and the analysis was conducted at 25 °C with 18 mM NaOH and a flow rate of 1 mL/min. Glucose, fructose, fucose, arabinose, rhamnose, galactose, xylose and mannose were used as standards in a range of 50 to 1 mg/L.

The furfural, 5HMF, acetic acid, lactic acid and levulinic acid present in the neutralized hydrolysates were quantified by HPLC (Waters 600) equipped with a UV-Vis detector, using a Biorad Aminex 87H (350 x 7.7 mm), and H_2SO_4 10 mM as eluent with a flow rate of 0.6 mL/min. Detection was performed at 210 and 280 nm. Furfural, 5HMF, acetic

acid, lactic acid and levulinic acid were used as standards in a range of 650 to 4 mg/L, 400 to 3 mg/L, 1000 to 9 mg/L, 700 to 30 mg/L and 200 to 9 mg/L, respectively.

For protein determination in neutralized hydrolysates, total nitrogen was quantified using a Hach Lange Laton® total nitrogen kit protocol (TN_b). Deionized water was used to dilute the samples to achieve a concentration within the measurable range of the kit. After the protocol, total nitrogen was measured using a DR 2800^{TM} spectrophotometer. The protein content was estimated using the nitrogen-to-protein conversion factor of 6.25 (Mariotti et al., 2008).

4.3.7.2. Cell separation and sample processing

Concerning the biotransformation experiments, samples were periodically taken from the culture broth and centrifuged for cell dry weight quantification (as described in section 2.3.4, chapter 2) and for quantification of L-Phe, sugars and 2-PE.

4.3.7.3. Sugar quantification

Quantification of carbon source (glucose, fructose, arabinose, xylose, glycerol and lactose) was conducted by HPLC (VWR Hitachi Chromaster), equipped with a Diode Array Detector 5430 and a Refractive Index (RI) Detector 5450. The column used was an Aminex HPX-87H (300 x 7.8 mm) coupled to a pre-column Biorad 125-0129 (30 x 4.6 mm), operated at 30 °C. The mobile phase was 0.01 N H_2SO_4 and the flow rate 0.5 mL/min. Glucose, fructose, arabinose, xylose, glycerol and lactose were used as standards in concentrations between 1 and 0.015 g/L.

4.3.7.4. L-Phe and 2-PE quantification

L-Phe consumption and 2-PE production were quantified as described on 3.3.5.2.

4.3.7.5. 2-PE extract analysis

The recovered extract desorbed from the resin Amberlite XAD 4 was analyzed as described on section 3.3.5.4.

4.3.8. Kinetic and stoichiometric parameter calculation

Kinetic and stoichiometric parameters were determined as described on section 2.3.5, chapter 2.

4.3.9. Statistical analysis

Statistical analysis was performed as described on section 3.3.5.6, chapter 3.

4.4. Results and discussion

4.4.1. Selection of carbon sources for 2-PE production

To identify which agro-industrial byproducts could have the potential to support *Acinetobacter soli* ANG344B growth and 2-PE production, several commercial carbon sources were used for bacterial cultivation (Table 4.2). Therefore, glucose, fructose, arabinose, xylose and lactose that are common carbohydrates found in food and lignocellulosic wastes were tested. Additionally, glycerol, as a byproduct of the biodiesel industry, was also tested (Xu et al., 2023; Araújo et al., 2021; Conde-Báez et al., 2017; Braga et al., 2021).

The results presented in Table 4.2 show that *Acinetobacter soli* ANG344B cannot use lactose and glycerol as carbon source, since the there was no consumption of these carbon sources during the experiment. The minimal cellular growth observed (1.05 ± 0.05 g_{CDW}/L for lactose and 0.82 ± 0.03 g_{CDW}/L, for glycerol, in terms of cell dry weight (CDW)) and the associated 2-PE production (1.73 ± 0.01 for lactose and 1.24 ± 0.03 $g_{2\text{-}PE}$/L for glycerol in these conditions) should have been supported by the carbon present in the yeast extract that is part of the medium composition. A similar behavior was observed using xylose, although there was a slight consumption of this medium component (1.03 ± 0.16 g_{xylose}/L), the cellular growth was low (1.45 ± 0.15 g_{CDW}/L). In the case of arabinose, despite complete substrate consumption, there was no improvement in cellular growth (1.43 ± 0.13 g_{CDW}/L) compared to the previous carbon sources, neither 2-PE production (1.75 ± 0.04 $g_{2\text{-}PE}$/L). Fructose and glucose were the carbon sources that supported higher cellular growth and 2-PE production. Specifically, glucose yielded a CDW of 2.78 ± 0.13 g/L and 2.14 ± 0.04 g/L of 2-PE, while the use of fructose resulted in 3.08 ± 0.03 g/L of CDW and 2.82 ± 0.10 g/L of 2-PE (Table 4.2). In the conditions

tested, the production yield from L-Phe was similar for all the evaluated substrates, suggesting that carbon sources do not influence the bioconversion of the amino acid into the aroma, as it is reported for other 2-PE producing microorganisms (Gethins et al., 2015; Braga et al., 2021). However, higher 2-PE production occur using substrates that lead to higher cellular growth.

Table 4.2 Kinetic parameters calculated for 2-PE production by *Acinetobacter soli* ANG344B, using different commercial carbon sources at 48 h of cultivation in shaking flasks.

	Glucose	**Fructose**	**Arabinose**	**Xylose**	**Lactose**	**Glycerol**
Carbon source$_{uptake}$ (g/L)	5.42 ± 0.11	9.00 ± 0.24	10.09 ± 0.04	1.03 ± 0.16	0.00 ± 0.00	0.09 ± 0.09
CDW (g/L)	2.78 ± 0.13	3.08 ± 0.03	1.43 ± 0.13	1.45 ± 0.15	1.05 ± 0.05	0.82 ± 0.03
Y(X/S) (g/g)	0.51 ± 0.01	0.34 ± 0.01	0.14 ± 0.01	1.42 ± 0.07	-	-
L-Phe$_{cons}$ (g/L)	3.80 ± 0.10	4.66 ± 0.09	3.17 ± 0.13	3.23 ± 0.17	3.23 ± 0.00	2.22 ± 0.01
2-PE$_{prod}$ (g/L)	2.14 ± 0.04	2.82 ± 0.10	1.75 ± 0.04	1.88 ± 0.04	1.73 ± 0.01	1.24 ± 0.03
Y(P/S) (g/g)	0.56 ± 0.02	0.61 ± 0.03	0.55± 0.01	0.58 ± 0.02	0.54 ± 0.00	0.56 ± 0.01
Rp (g/L.h)	0.04 ± 0.00	0.06 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00

The best results obtained in shaking flask were upscaled to 2 L bioreactor cultivation. Optimization of 2-PE production conditions for *A. soli* ANG344B was previously described on chapter 2, using glucose as carbon source. This approach yielded 2.14 ± 0.18 g/L of 2-PE at the experiment´s conclusion, after 24 h, with a volumetric productivity of 0.09 ± 0.01 g/L.h from 4.32 ± 0.86 g_{CDW}/L (Figure 4.1 a).

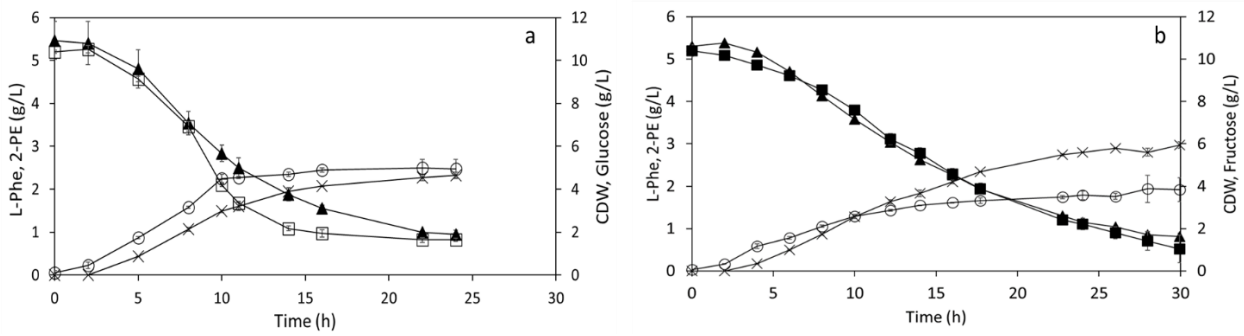

Figure 4.1 Cultivation profile of *Acinetobacter soli* ANG344B using different carbon sources. (a) commercial glucose (chapter 2); (b) commercial fructose. CDW (o), glucose (□), fructose (■), L-Phenylalanine (▲), 2-Phenylethanol (x).

When fructose was used as the carbon source (Figure 4.1 b), the culture displayed rapid growth without a noticeable lag phase, achieving an initial maximum specific growth rate of 0.93 ± 0.1 h^{-1}, similar to the rate observed with glucose (0.70 ± 0.08 h^{-1}). This growth rate declines as 2-PE production started. A CDW of 3.81 ± 0.67 g/L was obtained, comparable to the results achieved with glucose. Notably, a higher 2-PE concentration of 2.97 ± 0.08 g/L was achieved at the end of the assay (30 h), albeit with a volumetric productivity (0.10 ± 0.00 g/L.h) similar to that obtained using glucose as carbon source.

Several 2-PE producing organisms are able to produce the aroma using different carbon sources such as glucose, xylose, fructose, lactose, glycerol and ethanol, depending on the metabolism of each strain (Yan et al., 2020; Gethins et al., 2015; Castillo et al., 2022; Braga et al., 2021; Mierzejewska et al., 2019; Cui et al., 2011; Huang et al., 2000). Nevertheless, glucose is often considered the preferred carbon source leading to higher cell densities and increased 2-PE production. This preference has been reported for *Saccharomyces cerevisiae*, *Meyerozyma* sp., *Yarrowia* sp. and *Kluyveromyces marxianus* (Cui et al., 2011; Yan et al., 2020; Braga et al., 2021; Gethins et al., 2015). However, *A. soli* ANG344B, demonstrate similar performance using either glucose or fructose as carbon source, suggesting the feasibility of efficiently use residues rich in these two sugars for growth during the 2-PE production process.

4.4.2. Characterization of hydrolysates

Considering the previous results on carbon sources used by *A. soli* ANG344B and aiming to have a high protein content to be a potential supplier of L-Phe needed for 2-PE production, three residues - banana peels, brewer's spent grains (BSG), and bread - were hydrolyzed and characterized to be used for bacterial cultivation.

Banana peels were hydrolyzed in autoclave using a solid load of 1204 g/L for 40 min. Different conditions for banana peels processing were also reported in literature, where the authors used dried banana peels for hydrolysis process in acidic conditions or different solid loads, obtaining hydrolysates with glucose concentrations lower than 10 g/L (Araújo et al., 2021; Malakar et al., 2022).

The banana peels hydrolysate achieved was rich in glucose and fructose, with contents of 13.29 ± 0.04 and 16.32 ± 0.05 g/L, respectively. Minimal concentration of acetic acid (0.11 ± 0.00 g/L) and lactic acid (0.55 ± 0.03 g/L) were detected in the hydrolysate.

Although this hydrolysate was estimated to have a low protein content of 1.7 ± 0.0 g/L, its abundance in glucose and fructose, the favored carbon sources for bacterium cultivation, and low concentrations of interfering acids make it a promising feedstock for 2-PE production. Notably, banana peels hydrolysates were previously reported for use as carbon source for microalgae's growth, or for ethanol and xylitol production, for example (Araújo et al., 2021; Malakar et al., 2022; Palacios et al., 2017).

Brewer's spent grain was subjected to an acidic hydrolysis using 3% (w/w) of sulfuric acid, with a solid load of 125 g/L for 20 min in autoclave, as described by Araújo et al (Araújo et al., 2021). The BSG hydrolysate was rich in glucose, arabinose and xylose that were present in concentrations of 17.69 ± 0.05, 10.25 ± 0.04 and 21.97 ± 0.09 g/L, respectively. Degradation products, which can negatively affect the cellular growth, were also obtained during the hydrolysis with sulfuric acid, namely 0.19 ± 0.01 g/L of furfural, 0.13 ± 0.01 g/L of 5HMF, 0.06 ± 0.02 g/L of levulinic acid, 1.48 ± 0.00 g/L of acetic acid and 3.49 ± 0.01 g/L of lactic acid. The protein content of the hydrolysate was 11.5 ± 0.2 g/L. Comparing the hydrolysate composition with results reported by other authors under similar conditions, it was possible to achieve higher sugar compositions, especially for glucose. The reported values for glucose are between 1.2 and 6.4 g/L, 6.2 and 12.8 g/L for arabinose and within 8.9 and 26.7 g/L for xylose, demonstrating the variability of the feedstock (Mussatto and Roberto, 2005; Carvalheiro et al., 2004; Araújo et al., 2021). Regarding the degradation products, the values obtained in these conditions are in line with the reported by other authors (Mussatto and Roberto, 2005; Carvalheiro et al., 2004; Araujo2021). In fact, during the dilute acid hydrolysis procedure, sugars can be degraded to furfural which is formed from pentoses, and 5HMF which is formed from hexoses, and they can be further degraded in formic acid and levulinic acid, respectively, acetic acid can also be liberated (Larsson et al., 1999). The BSG hydrolysate has a high protein content, that might be a valuable source of L-Phe for 2-PE production process. However, the presence of lactic and acetic acid might have a negative impact on cellular growth. BSG hydrolysates are reported for different applications, including xylitol and polyhydroxyalkanoates production and protein extraction (Araújo et al., 2021; Carvalheira et al., 2022; Mussatto and Roberto, 2015; Qin et al., 2018).

Bread hydrolysis was performed in acidic conditions using 1% of hydrochloric acid, with a solid load of 160 g/L in autoclave for 20 minutes, accordingly to Torabi et al. (Torabi et al., 2021). The hydrolysate obtained was rich in glucose (124.11 ± 2.10 g/L), xylose

and arabinose are also present in minor concentrations, 2.10 ± 0.07 and 1.40 ± 0.06 g/L, respectively. The degradation products resulting from hydrolysis with hydrochloric acid were present in concentrations of 0.35 ± 0.00 g/L of 5HMF, 0.03 ± 0.01 g/L of furfural and 1.02 ± 0.03 g/L of lactic acid. The estimated protein content was 10.0 ± 1.1 g/L. Comparable conditions applied by other authors yielded lower concentrations of glucose (57.3 to 67.9 g/L) and higher concentrations of furfural and 5HMF (Narisetty et al., 2022; Cox et al., 2022; Torabi et al., 2021). The high protein and glucose content, makes bread hydrolysate a promising feedstock. The use of bread hydrolysates was reported by some authors in the bioproduction of 2,3-butanediol, lactic acid or xanthan gum (Demirci et al., 2019; Cox et al., 2022; Narisetty et al., 2022).

4.4.3. Cultivation of *Acinetobacter soli* ANG344B in media containing the selected hydrolysates

The hydrolysates were used as carbon source to cultivate *Acinetobacter soli* ANG344B, in presence or absence of commercial L-Phe.

The first experiments were performed without addition of L-Phe since hydrolysates have proteins in their composition. However, the hydrolysates used in these experiments were diluted to achieve the desired sugar concentration, which resulted in a low concentration of proteins in the cultivation medium, and therefore no L-Phe was detected.

The results showed that the hydrolysates could support bacterial growth, with higher biomass being obtained when L-Phe was absent in the cultivation media, except for cultivations with bread hydrolysate, with a biomass production of 6.83 ± 0.43, 5.57 ± 1.18 and 3.95 ± 0.15 g_{CDW}/L, respectively for banana peels, BSG and bread hydrolysates (results obtained in the presence of L-Phe are presented in Table 4.3). In these experiments no 2-PE was detected due to the absence of L-Phe needed for 2-PE production, which led to the higher biomass concentration obtained in these experiments, as the aroma is reported to be cytotoxic to the producing microorganisms (chapter 2; Kleinwächter et al., 2021).

Table 4.3 Kinetic parameters for 2-PE production, using bread, BSG and banana peels hydrolysates as carbon sources as part of the cultivation media supplemented with L-Phe. Experiments performed in shake flasks.

	Bread	**BSG**	**Banana peels**
$Glucose_{cons}$ (g/L)	7.50 ± 1.43	2.78 ± 0.68	5.41 ± 0.07
$Xylose_{cons}$(g/L)	-	3.68 ± 1.64	-
$Arabinose_{cons}$(g/L)	-	3.08 ± 1.16	-
$Fructose_{cons}$(g/L)	-	-	6.19 ± 0.03
CDW (g/L)	5.20 ± 0.50	3.00 ± 0.60	3.60 ± 0.10
L-Phe_{cons} (g/L)	4.63 ± 0.26	2.25 ± 0.14	3.35 ± 0.07
2-PE_{prod} (g/L)	2.17 ± 0.05	0.97 ± 0.14	1.60 ± 0.00
Y(P/S) (g/L)	0.47 ± 0.02	0.43 ± 0.02	0.48 ± 0.01
Rp (g/L.h)	0.05 ± 0.00	0.02 ± 0.00	0.03 ± 0.00

In the presence of L-Phe, *A. soli* ANG344B could produce 2-PE in equivalent amounts to those obtained using commercial carbon sources (Table 4.2 and 4.3), suggesting that the use of hydrolysates do not affect the 2-PE production ability of the bacterium. Although the 2-PE production yield from L-Phe was slightly lower in these conditions, it remained comparable. In the conditions tested, bread hydrolysate emerged as the feedstock that allowed higher bacterial growth (5.2 ± 0.5 g_{CDW}/L) and 2-PE production (2.17 ± 0.05 $g_{2\text{-}PE}$/L). Banana peels hydrolysate allowed the production of 1.60 ± 0.0 $g_{2\text{-}PE}$/L with 3.6 ± 0.1 g_{CDW}/L. As anticipated from results obtained using commercial sugars, BSG hydrolysate was the feedstock that supported lower 2-PE production, with 0.97 ± 0.14 $g_{2\text{-}PE}$/L being produced. The presence of potentially toxic degradation products obtained during the hydrolysis did not affect *A. soli* ANG344B growth, as it was comparable to that obtained using commercial carbon sources, neither 2-PE production, which was produced at similar rates using the hydrolysates.

To the best of my knowledge, this is the first time that these feedstocks are used to support 2-PE production processes in submerged fermentation. Nonetheless, they allowed a 2-PE production in a range similar to that reported for other 2-PE producing microorganisms using agro-industrial feedstocks. For instance, *Pichia fermentans* WUT36 was reported to produce 3.6 g/L of 2-PE from 5 g/L of L-Phe with hydrolyzed corn stover as carbon source. *Yarrowia lipolytica* could produce 1.72 g/L of 2-PE from 4

g/L of L-Phe using crude glycerol as carbon source, and *Kluyveromyces marxianus* produced 1.2 g/L of 2-PE from 4.5 g/L of L-Phe in a whey-based media (Mierzejewska et al., 2019; Braga et al., 2021; Alonso-Vargas et al., 2022). The use of banana peels and BSG were reported by Martínez-Avila et al. via solid state fermentation, in the presence of added L-Phe using *Pichia Kudriavzevii* (Martínez-Avila et al., 2021). The authors reported a 2-PE production yield of 0.1 $g_{2\text{-PE}}/g_{\text{L-Phe}}$ using BSG, lower than the obtained for *A. soli* ANG344B in submerged fermentation in shake flask tests. When using banana peels, the production yield was reported to increase to 0.44 $g_{2\text{-PE}}/g_{\text{L-Phe}}$, comparable to that obtained for *A. soli* ANG344B (Martínez-Avila et al., 2021). Although bread hydrolysate was not previously reported to be used as carbon source for 2-PE production, this aroma has already been identified in bread, being produced during its fermentation process in concentrations in the rage of few $\mu g/g_{\text{bread}}$ (Dueñas-Sánchez et al., 2014).

Considering results obtained, bread and banana peels hydrolysates were selected to be upscaled to 2 L bioreactor cultivations to better assess their potential as carbon sources in the 2-PE production process.

4.4.4. Bioreactor cultivation of *Acinetobacter soli* ANG344B in waste-based media

Bread and banana peels hydrolysates were used as carbon sources for *A. soli* ANG344B cultivation, aiming 2-PE production under controlled bioreactor conditions previously optimized in chapter 2 (Figure 4.2).

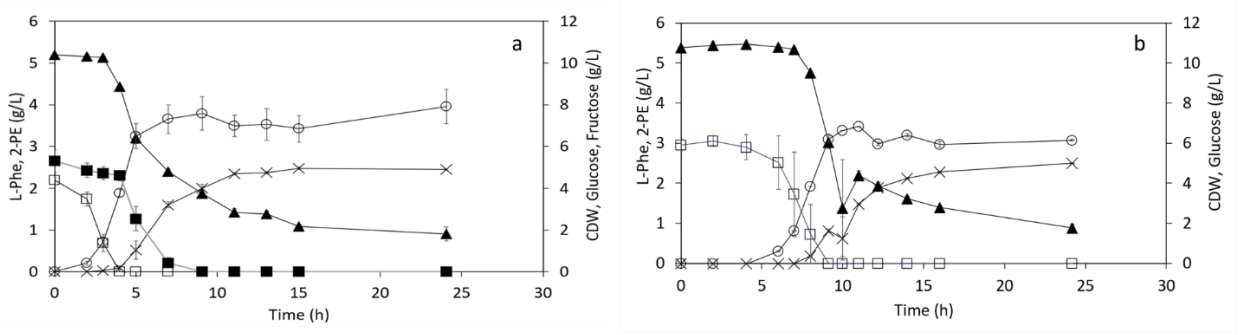

Figure 4.2 Cultivation profile of *Acinetobacter soli* ANG344B using waste-based media. (a) banana peels hydrolysate; (b) bread waste hydrolysate. CDW (o), glucose (□), fructose (■), L-Phenylalanine (▲), 2-Phenylethanol (x).

Using the glucose- and fructose-rich banana peels hydrolysate (Figure 4.2 a), the culture grew without noticeable lag phase, at a maximum specific growth rate of 1.47 ± 0.04 h^{-1},

during a 5 hour exponential growth phase. As depicted in Figure 4.2 a, bacterial growth was firstly supported by glucose consumption, that is completely exhausted during the first 3 hours of cultivation, followed by the consumption of fructose which was totally consumed in the following 4 hours. After depletion of these carbon sources, cellular maintenance likely relied on yeast extract, resulting in 6.9 ± 0.7 g_{CDW}/L after 15 hours. 2-PE production started during the exponential growth phase, with 21% of the aroma being produced in that phase. After 15 hours, 2-PE production stabilized, reaching 2.48 ± 0.04 g/L of 2-PE, yielding a volumetric productivity of 0.17 ± 0.00 g/L.h (Table 4.4).

Table 4.4 Kinetic parameters calculated for 2-PE production in 2 L bioreactor using different carbon sources: glucose, fructose, banana peels and bread hydrolysates, for 15 h of cultivation and using banana peels hydrolysate using an ISPR approach.

	Glucose	**Fructose**	**Banana peels**	**Bread**	**Banana peels ISPR**
$Glucose_{cons}$ (g/L)	8.47 ± 2.26	-	4.40 ± 0.08	6.18 ± 0.57	9.58 ± 0.05
$Fructose_{cons}$ (g/L)	-	5.33 ± 0.00	5.31 ± 0.53	-	11.52 ± 0.06
CDW (g/L)	4.5 ± 1.0	3.3 ± 0.1	6.9 ± 0.7	6.0 ± 0.4	18.7 ± 0.1
L-Phe_{cons} (g/L)	3.75 ± 0.65	2.88 ± 0.14	4.11 ± 0.06	3.25 ± 0.31	11.89 ± 0.31
2-PE_{prod} (g/L)	1.85 ± 0.13	2.00 ± 0.06	2.48 ± 0.04	2.13 ± 0.13	7.30 ± 0.01
Y(P/S) (g/g)	0.50 ± 0.07	0.70 ± 0.01	0.60 ± 0.00	0.66 ± 0.02	0.61 ± 0.02
Rp (g/L.h)	0.12 ± 0.01	0.13 ± 0.00	0.17 ± 0.00	0.14 ± 0.01	0.27 ± 0.00

In the case of bread hydrolysate, rich in glucose, a 5 hour lag phase was noticed, followed by a 4 hours exponential growth phase where the bacterium grew at specific growth rate of 1.17 ± 0.01 h^{-1}. The longer lag phase might be caused by the presence of some trace concentrations of degradation products or preservatives in its constitution, affecting cellular growth. Similar to what happened in the cultivation with banana peels hydrolysate, 21 % of the aroma was produced during the exponential growth phase, with the majority generated during the stationary growth phase. Glucose was not completely consumed by the end of the experiment, resulting in 6.0 ± 0.4 g/L of CDW after 15 hours of the experiment (Table 4.4). At this point, 2-PE production stabilized, reaching 2.13 ± 0.13 g/L of the aroma, with a volumetric productivity of 0.14 ± 0.01 g/L.h (Table 4.4).

The aroma production in bioreactor showed improved 2-PE production compared to the shake flasks results, as expected. This improvement can be attributed to a better control

of conditions, such as pH and oxygen availability, ensuring optimal growth conditions for *A. soli* ANG344B. Both banana peels and bread hydrolysates assured a higher biomass production compared to commercial glucose and fructose (Figure 4.1, Figure 4.2 and Table 4.4), along with a higher specific growth rate, as described above. These hydrolysates have trace elements in its constitution that can be beneficial for bacterial growth and can influence cellular metabolism, acting as cofactors for enzymes (Zheng et al., 2017; Venkateshwar et al., 2010). For instance, banana peels hydrolysate was found to have boron, barium, calcium, potassium, magnesium, manganese, sodium, phosphorous, silicon, strontium and zinc and in bread hydrolysate, beside the above-mentioned elements, aluminum, copper, iron, selenium and tungsten were also found, being its concentration higher in banana peels than in bread hydrolysate (Table 4.5).

Table 4.5 Elements present in bread and banana peels hydrolysates, after neutralization

Element	Bread hydrolysate (mg/L)	Banana peels hydrolysate (mg/L)
Al	0.20	-
B	0.25	0.71
Ba	0.11	0.20
Ca	150	32.0
Cu	0.16	-
Fe	1.00	-
K	579	3479
Mg	62.8	183
Mn	0.76	3.84
Na	6565	246
P	177	129
Se	0.18	-
Si	1.45	42.8
Sr	0.20	0.41
W	0.20	-
Zn	0.37	0.64

The aroma production is reported to be growth-associated for many microorganisms, with aroma being produced in the exponential growth phase (Hazelwood et al., 2008; Etschamnn et al., 2002). However, when these agro-industrial feedstocks were used, most of the aroma was produced during the stationary growth phase (Figure 4.2 a and b). These results are in line with those reported in chapter 2, where 2-PE production was found to be dependent on the availability of L-Phe regardless of the culture growth phase.

While the use of bread hydrolysate did not result in a significant improvement in 2-PE production, when compared to the use of commercial glucose, the aroma production yield from L-Phe was 20% higher (Table 4.4). The most promising results were obtained using banana peels hydrolysate, leading to higher productions and productivities than using pure substrates. Cultivation using banana peels hydrolysate resulted in an improvement of 34% and 24% in 2-PE production and 41% and 30% in volumetric productivity, when compared to the use of commercial glucose and fructose, respectively for the same period (15 h). The enhanced 2-PE production when using wastes was also reported for some microorganisms as *Hanseniaspora opuntiae* WUT19, *Metschnikowia chrysoperlae* WUT25, *Pichia fermentans* WUT36, *Rhodotorula mucilaginosa and Saccharomyces cerevisiae* WUT34 and WUT35, proving that the medium composition has influence in 2-PE production (Chreptowicz et al., 2018).

These results show that banana peels hydrolysate is a promising substrate for 2-PE production. Not only does it contribute to waste reduction and promote a circular economy, but its use also improves the production process, allowing higher 2-PE production at a higher rate. In fact, to the best of my knowledge, this process using banana peels as carbon source for 2-PE production by *A. soli* ANG344B presents the highest volumetric productivity for this aroma production using wastes in a batch operational mode, without the use of ISPR techniques, that typically yield rates below 0.05 $g_{2\text{-}PE}$/L.h. The production titer is in the same range as the obtained for other microorganisms cultivated in waste-based media in bioreactors (Mierzejewska et al., 2019; Braga et al., 2021; Alonso-Vargas et al., 2022; Garavaglia et al., 2007).

4.4.5. 2-Phenylethanol production in waste-based media using ISPR approach

Considering the promising outcomes observed using banana peels hydrolysate as carbon source in the previous experiments, and the improvement of the production process using

an ISPR approach, as described in chapter 3, a batch transformation was conducted using 3% (w_{dry}/v) of Amberlite XAD 4 as *in situ* product adsorbent for *A. soli* ANG344B cultivation in a 2 L bioreactor in waste-based media (Figure 4.3). Similar to the experiments performed in chapter 3, 12 g/L of soluble L-Phe was used for cultivation aiming to increase 2-PE production until the maximum allowed by the adsorption capacity of the resin (205.8 ± 8.1 $mg_{2\text{-}PE}/g_{dry\ resin}$). To avoid limitation by the carbon source, the volume of banana peels hydrolysate was increased to have near 20 g/L of sugars (glucose and fructose) (Figure 4.3).

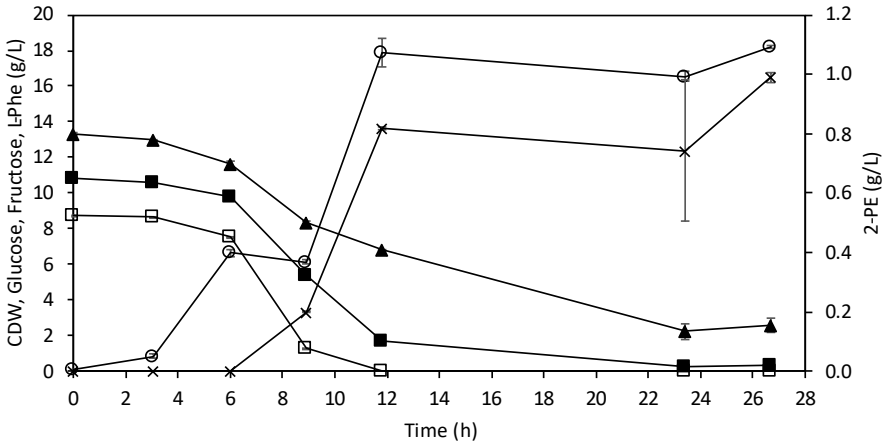

Figure 4.3 Cultivation profile of *A. soli* ANG344B, in 2 L bioreactor, using 3 % Amberlite XAD 4 from the beginning of the cultivation. CDW (○), L-Phe (▲), soluble 2-PE (x), glucose (□) and Fructose (■).

In these conditions, at the end of the experiment, 13.15 ± 0.01 g of 2-PE were produced (taking in consideration the aroma present in solution and recovered from the resin), corresponding to 7.30 ± 0.01 g/L of 2-PE considering the total volume of the experiment (Table 4.4 and Figure 4.3), resulting in a 2.9-fold increase when compared to the process without ISPR approach. From the total amount of 2-PE produced, 86% was adsorbed into the resin, and the concentration in the aqueous phase did not exceed 1 g/L through all the cultivation period. The production yield was 0.61 ± 0.02 $g_{2\text{-}PE}/g_{L\text{-}Phe}$, similar to that obtained in the production without product removal, as expected. The volumetric productivity was 0.27 ± 0.00 $g_{2\text{-}PE}$/L.h, corresponding to an increase of 1.6-fold compared to the experiment without the resin (Table 4.4). The 2-PE adsorbed into Amberlite XAD 4 during the production process corresponded to 190 $mg_{2\text{-}PE}/g_{dry\ resin}$, approaching the

maximum adsorption capacity of the resin. Due to the low concentration of 2-PE in the cultivation broth, it was possible to achieve a biomass concentration of 18.7 ± 0.1 g/L in terms of cell dry weight, that corresponds to a 2.7-fold increase when compared to the cultivation without product adsorption. Glucose and fructose were consumed at the same time, with glucose being exhausted from the cultivation media before fructose. These carbon sources were depleted before the end of the cultivation, indicating that cellular maintenance was supported by the yeast extract.

The use of ISPR approaches has been reported for 2-PE production by yeasts using wastes as carbon source, such as molasses and whey, using polypropylene glycol or continuous extraction with ethyl acetate, achieving 2-PE volumetric productivities in the range of 0.06 to 0.33 g/L.h (Etschmann and Schrader, 2006; Hua et al., 2013; Dręż ek et al., 2021).

The use of Amberlite XAD 4 greatly improved the 2-PE production process in waste-based media, similar to what happened using *in situ* product adsorption in the cultivation media with commercial glucose as carbon source (chapter 3), achieving similar 2-PE productions in both conditions (14.33 ± 0.11 and 13.15 ± 0.01 g of 2-PE, respectively for glucose and banana peels hydrolysate). These results, show the potential of *Acinetobacter soli* ANG344B to produce the valuable rose-like aroma, 2-PE, and potential of banana peels hydrolysate to be used as carbon source, supporting the growth of the bacteria to produce that compound.

The recovery of 2-PE from the adsorbent resin was performed using ethanol, as described in chapter 3. The 2-PE extract obtained from the desorption step was concentrated by vacuum distillation to yield a highly concentrated 2-PE extract. This process allowed the obtention of 193 mL extract containing 41.12 ± 0.11 g/L of 2-PE and a 2.14 ± 0.15 g/L of L-Phe. From the total 2-PE desorbed, only 0.70% was lost to the evaporated ethanol. The evaporated ethanol was recovered and can be further used in the desorption step of 2-PE production processes. GC analysis of this concentrated extract allowed to determine a chromatographic purity of 88% of the compound in an ethanol matrix. As expected, due to the complexity of the hydrolysate used as carbon source, the chromatographic purity calculated for this 2-PE extract is lower than the obtained when commercial glucose was used as carbon source. The GC-MS analysis also suggests the presence of chalcones, propiophenones, benzeneethanamine and propanedioic acid, known ingredients in cosmetic formulations (Dhaliwal et al., 2022; Stompor, et al., 2019).

During the cultivation process, an increase in the viscosity of the broth was noticed and the presence of an exopolysaccharide (EPS) was investigated. At the end of the experiment 4.7 ± 0.2 g/L of EPS was quantified in the cultivation broth. This result was not obtained when *A. soli* ANG344B was cultivated using banana peels hydrolysate without product removal but had already been observed when cultivated using ISPR approach with commercial glucose as carbon source (chapter 3). The production of EPS by *A. soli* ANG344B is discussed in chapter 5.

4.5. Conclusions

Acinetobacter soli ANG344B demonstrated the ability to produce 2-PE by conversion of L-Phe upon cultivation in waste-based medium. Residues, selected based on the bacterium´s ability to utilize the sugars present, were hydrolyzed and used as carbon source to support bacterial growth and aroma production. Among the tested hydrolysates, banana peels hydrolysate, rich in glucose and fructose, yielded the highest 2-PE production (2.48 ± 0.04 g/L) and volumetric productivity (0.17 ± 0.00 g/L.h), surpassing even the results obtained using the corresponding commercial carbon sources. The implementation of an ISPR approach using Amberlite XAD 4 as adsorbent allowed an increase in 2-PE production to 7.30 ± 0.01 g/L with a volumetric productivity of 0.27 ± 0.00 g/L.h.

Further improvement of 2-PE production can be expected by increasing L-Phe feeding and the amount of adsorbent in contact with the cultivation broth. These findings show the potential of this culture to be used for valorization of agro-industrial wastes in the bioproduction of value-added rose-like aroma compound. Despite the promising results achieved for 2-PE production in waste-based media, the concurrent use of residues working simultaneously as carbon and L-Phe sources remains unexplored, being essential the media supplementation with the amino acid to achieve efficient aroma productions.

5.

Exopolysaccharides produced by *Acinetobacter soli* ANG344B: production and characterization

5.1. Summary

During the 2-phenylethanol (2-PE) production process by *Acinetobacter soli* ANG344B the production of exopolysaccharides (EPS) was observed, achieving concentrations ranging from 2.4 to 4.7 g/L. The EPS production occurred when 2-PE concentration in the culture broth was low and in the absence of L-phenylalanine (L-Phe). The produced polymers were high molecular weight heteropolysaccharides with different monomers in its composition, depending on the carbon source used, glucose or banana peels hydrolysate. EPS produced using glucose as carbon source have glucose, xylose and fucose monomers in its composition, while EPS produced using banana peels hydrolysate also have galacturonic acid, arabinose and galactose (these monomers account for up to 28 wt% of the EPS), both with acetyl substituents (ranging from 1.5 to 4.7 wt%). Thermogravimetric analysis demonstrated that EPS have a degradation temperature in the range of 278 to 298 °C. The EPS in study produced viscous solutions and showed a shear-thinning fluid behavior. The capacity for emulsion forming was only observed for EPS produced in absence of L-Phe and 2-PE.

5.2. Introduction

Exopolysaccharides (EPS) are high molecular weight carbohydrate polymers, usually ranging from 10^4 - 10^7 Da, that can be secreted by different microorganisms, namely bacteria (Freitas et al., 2017; Zayed et al., 2021). These bacterial produced polysaccharides can remain as capsular material, being associated with the cell, or as a slime in the surrounding environment (Caccamo et al., 2020; Concórdio-Reis et al., 2021; Torres et al., 2011). These EPS play an important role in cell functions as they are involved in formation of microbial aggregates, cell adhesion to surfaces, biofilm formation, cell-cell recognition and protective barrier (More et al., 2014; Zayed et al., 2021; Freitas et al., 2017).

Even though a wide variety of polysaccharides are found in plants, algae and crustacean, EPS produced by microbial fermentation are more attractive due to independence of climate conditions, higher growth rates, easier manipulation of the production conditions and easier extraction process (Cruz et al., 2011; Zayed et al., 2021; Torres et al., 2011).

Bacterial EPS are mainly composed of carbohydrates, but protein, lipids, nucleic acids, sulphate, acetate, pyruvate, succinate or amines can also be part of its composition (More

et al., 2014; Zayed et al., 2021). The diverse chemical nature of bacterial EPS confers interesting and unique properties with potential to be applied in food, cosmetic, pharmaceutical and biomedical industries (Freitas et al., 2011). For instance, the presence of rare sugars, such as fucose and rhamnose might confer anti-aging, anticarcinogenic and/or anti-inflammatory activity to EPS or these polymers can also be a source of valuable molecules (Freitas et al., 2011; Roca et al., 2015; Zayed et al., 2021). Moreover, bacterial EPS can also show interesting rheological behavior and emulsion, stabilizing and flocculating capacity. Examples of these polymers are Xanthan gum, a natural polysaccharide commercially available; FucoPol, a fucose rich polysaccharide; Sphingans, rhamnose rich polysaccharides; GalactoPol, a galactose rich EPS, among many others (Concórdio-Reis et al., 2020; Gansbiller et al., 2020; Ferreira et al., 2014; Fialho et al., 2008; Freitas et al., 2009; Freitas et al., 2011; Freitas et al., 2017; Li et al., 2016; Torres et al., 2011; Zayed et al., 2021).

Bacteria from *Acinetobacter* genus, as described in section 1.3 (chapter 1), have a versatile metabolism able to produce extra and intracellular valuable products, such as lipases, proteases, bioemulsifiers and biopolymers (Abdel-El-Haleem, 2003; Adewoyin and Okoh, 2018; Jung and Park, 2015). Bioemulsifiers and biosurfactants produced by *Acinetobacter* spp. are reported to have polysaccharide and proteins or lipids in its constitution (Mujumdar et al., 2019). Emulsan, Alasan and Biodispersan are high molecular weight polysaccharides produced by *Acinetobacter* spp. with surfactant or emulsifier properties, known for their industrial applications in bioremediation (Satpute et al., 2010). Emulsan and Alasan are polysaccharide-protein complexes produced by *Acinetobacter calcoaceticus* and *Acinetobacter radioresistens*, respectively. These polymers act as emulsion stabilizers, playing an important role in microbial degradation of emulsified oil, as crude oil, by increasing its bioavailability to the microorganisms, due to their affinity for the oil-water interface (Mujumdar et al., 2019; Satpute et al., 2010). *Acinetobacter calcoaceticus* is also known for Biodispersan production, a polysaccharide that can act as dispersant of limestone and titanium dioxide (Mujumdar et al., 2019; Satpute et al., 2010).

Acinetobacter soli ANG344B was found to produce EPS during 2-PE production process, only in the presence of low aroma concentrations, as described in chapter 3 and 4. In order to access the EPS production by this novel strain, the EPS production kinetics were accessed in absence of L-Phe and the resultant polymer, as well as the polymers produced

in the previous chapters were characterized in terms of chemical composition, molecular mass distribution, thermal properties and rheological behavior. The capacity of the polymer to form emulsions was also assessed.

5.3. Material and methods

5.3.1. EPS production

5.3.1.1. Microorganism and inoculum preparation

The microorganism preservation, reactivation and the inoculum preparation were performed as described on chapter 2, section 2.3.1.

5.3.1.2. Bioreactor operation

Acinetobacter soli ANG344B cultivations were performed in 2 L bioreactors (BioStat B-plus, Sartorius, Germany), as described in chapter 2, section 2.3.3, with no addition of L-Phe.

5.3.1.3. Analytical techniques

5.3.1.3.1. Cell separation and sample processing

Culture broth samples were taken periodically and processed as described in chapter 2, section 2.3.4, for quantification of glucose, nitrogen and phosphate consumption and EPS production.

5.3.1.3.2. Glucose quantification

Glucose quantification was performed as described in chapter 3, section 3.3.5.3.

5.3.1.3.3. 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 have a nitrogen concentration within the range measured by the kit (between 20 and 100 mg/L). After the procedure indicated in the protocol, the total nitrogen was measured in the spectrophotometer DR 2800 TM by recognition of the bar code present in the sample cell.

5.3.1.3.4. Phosphate quantification

Determination of phosphate concentration was accessed by colorimetry, using a flow segmented analyzer (Skalar 5100, Skalar Analytical, The Netherlands) in a range of 20 to 4 mg/L.

5.3.1.3.5. EPS quantification

EPS was quantified by dialysis of the cell free supernatant, using a 12-14 kDa molecular weight cut-off (MWCO) membrane (Spectra/Por® 4 Dialysis membrane) against deionized water at room temperature under constant stirring, until the water conductivity reached a value bellow 10 µS/cm. The purified EPS was gravimetrically quantified after lyophilization.

5.3.2. EPS characterization

Beyond the experiments for EPS production described in this chapter, EPS produced during 2-PE production experiments with an *in* situ product removal approach, described in chapter 3 (section 3.4.4) and chapter 4 (section 4.4.5), were also characterized.

5.3.2.1. Sugar monomers and acyl groups composition

EPS dried samples (5 mg) were dissolved in 5 mL of deionized water and hydrolyzed with 0.1 mL trifluoroacetic acid (TFA, 99%), at 120 °C, for 2 h. These hydrolysates were used for identification and quantification of the constituent monosaccharides and acyl groups by HPLC. Monosaccharides were quantified using a CarboPac PA10 (4 x 250 mm) and a AminoTrap (4 x 50 mm) columns (Thermo Scientific™ Dionex™, Sunnyvale, CA, USA), equipped with a pulsed amperometric detector (PAD). The samples were analyzed at 25 °C with 18 mM NaOH and a flow rate of 1 mL/min. Fucose, arabinose, galactose, glucose, xylose, fructose, ribose and galacturonic acid were used as standards in a range of 50 to 1 mg/L.

Acyl groups were identified by HPLC (Waters 600), using a Biorad Aminex 87H (350 x 7.7 mm) equipped with an UV-Vis detector, with H_2SO_4 10 mM as eluent with a flow rate of 0.6 mL/min at 30 °C. Detection was performed at 210 and 280 nm. Acetate and pyruvate were used as standards in concentrations between 1000 and 15 mg/L.

5.3.2.2. Fourier transform infra-red spectroscopy

Fourier Transform Infra-Red Spectroscopy (FTIR) was carried out using a Perkin-Elmer Spectrum Two™ spectrometer. Dried EPS samples were used. The spectra were obtained in the range of 400−4000 cm^{-1} based on ten scans, at room temperature.

5.3.2.3. Molecular mass distribution

The average molecular weight (Mw), molecular number (Mn) and polydispersity index (PI = Mw/Mn) were determined by Size Exclusion High Performance Liquid Chromatography (SE-HPLC). Analyses were performed on a Knauer Smartline HPLC equipped with a Phenomenex Polysep Linear column (300 x 4.6 mm) and a Water 2414 Refractive Index detector (RI). The analyses were performed at 25 °C, using 0.1 M of $LiNO_3$ as eluent, at a flow rate of 0.5 mL/min with an injection volume of 50 µL. Mn and Mw were calculated using a calibration curve generated with pullulan standards.

5.3.2.4. Thermogravimetry analysis

Thermogravimetric analysis (TGA) was performed using a Thermogravimetric Analyzer Labsys EVO (Setaram, France). Samples were heated at a heating rate of 10 °C/min, from room temperature to 500 °C. The thermal degradation temperature (T_{deg}, °C) corresponds to the temperature value obtained for the maximum decreasing peak of the sample mass.

5.3.2.5. Elemental analysis

The elemental analysis of the EPS was performed using an elemental analyzer (Thermo Finnigan-CE Instruments, Flash EA 1112 CHNS series, Italy). Samples were subjected to a flash combustion in an oxygen environment and the resulting gases (N_2, CO_2, H_2O and SO_2) were separated by gas chromatography. Finally, the content of nitrogen, carbon, hydrogen, and sulfur was calculated using a thermal conductivity detector.

5.3.2.6. Rheological behavior

The apparent viscosity of 1% (w/v) EPS aqueous solutions were determined using a controlled stress rheometer (Anton Paar, Modular Compact Rheometer MCR 92) with a cone-plate geometry (angle 2 °, diameter 35 mm). Flow curves were obtained at 25 °C, at a shear rate ranging from 0.01 to 1000 s^{-1}. Experimental data were fitted into Power Law model, as follows:

$$\sigma = k\,\dot{\gamma}^{n} \quad (5.1)$$

Where σ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (1/s), k is the consistency index (Pa s^n) and n is the flow behavior index (dimensionless).

5.3.2.7. Emulsion forming capacity

The EPS ability to form emulsions was tested using 0.5% (w/v) EPS aqueous solutions that were mixed with sunflower oil at a 2:3 (v/v) ratio (water:oil). Mixtures were stirred under the vortex for 2 minutes and left at room temperature for 24 h. Emulsification index (E_{24}, %) was calculated as follows (Freitas et al., 2014):

$$E_{24} = \frac{h_e}{h_t} \times 100 \quad (5.2)$$

Where h_e (cm) is the height of the emulsion layer and h_t (cm) is the total height of the mixture.

5.4. Results and discussion

5.4.1. EPS production

When *Acinetobacter soli* ANG344B was cultivated in the presence of L-Phe aiming to produce 2-PE, no EPS production was detected (chapter 2). However, when cultivations were performed using an *in situ* product removal (ISPR) approach, an increase in broth viscosity was noticed throughout the time course of the experiment, during the stationary growth phase. These different results are likely related with the toxic effects of 2-PE towards the microorganism. The presence of 2-PE in the cultivation broth might have decreased culture viability before the EPS production started, preventing polymer production.

A. soli ANG344B cultivation aiming to produce 2-PE using glucose or banana peels hydrolysate as carbon sources in the presence of the resin Amberlite XAD 4 to adsorb 2-PE, resulted in an EPS production of 2.4 ± 0.0 g/L and 4.7 ± 0.2 g/L, respectively by the end of the cultivation, as it is described in chapters 3 and 4.

So, to access the potential of *A. soli* ANG344B to produce EPS, this microorganism was cultivated in media similar to the used for 2-PE production but in absence of L-Phe, as represented in Figure 5.1. In these conditions, the culture grew at a maximum specific

cell growth rate of 0.54 ± 0.02 h^{-1}, reaching 4.9 ± 0.8 g_{CDW}/L, at 6h, with a glucose consumption of 8.15 ± 1.40 g/L and a nitrogen consumption of 0.31 ± 0.05 g/L, with a yield of 0.59 ± 0.12 $g_{CDW}/g_{glucose}$. Afterwards, culture entered in stationary growth phase, when EPS production started, at this time nitrogen concentration in the broth must have been growth limiting (0.22 ± 0.12 g/L). After 27 h of cultivation, glucose was exhausted from the cultivation broth, but EPS continued to be produced until 48 h of cultivation, by the time that dissolved oxygen reached high values (80 – 100%). The EPS production after glucose depletion was likely assured by the yeast extract present in the cultivation broth. By the end of the experiment 3.0 ± 0.2 g/L of EPS had been achieved, corresponding to a volumetric productivity of 0.05 ± 0.01 g/L.h.

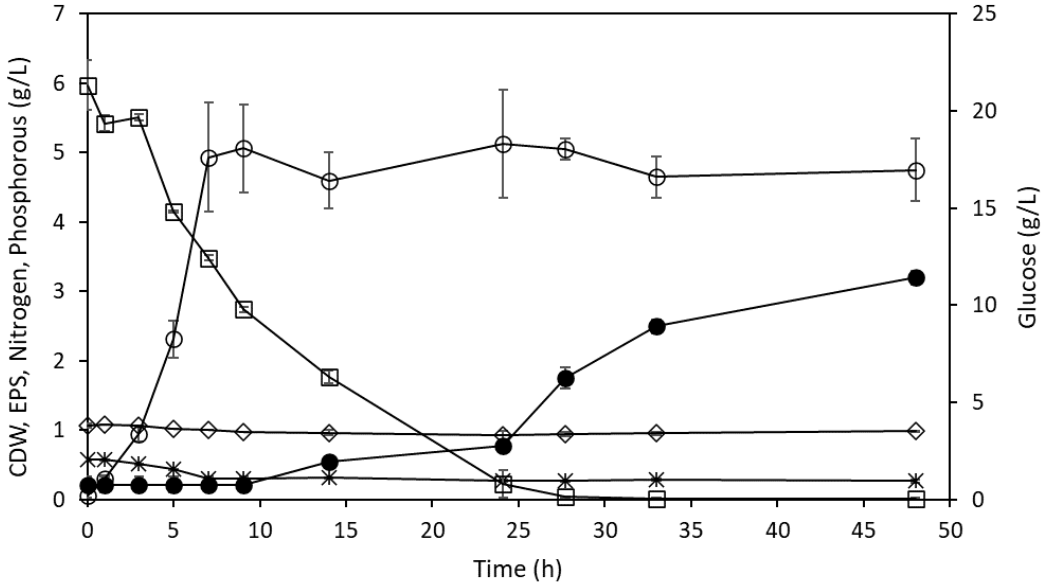

Figure 5.1 Cultivation profile of *Acinetobacter soli* ANG344B in absence of L-Phe. Cell dry weight (○) glucose (□), EPS (●), nitrogen (ж) and phosphorous (◊).

EPS production was already reported for *Acinetobacter* strains, namely *Acinetobacter* sp. 12S and *Acinetobacter calcoaceticus* BD4, using glucose as carbon source, reaching EPS concentrations of 2.25 and 0.61 g/L, respectively (Bryan et al., 1986; Pirog et al., 2002). These concentrations are lower than the one obtained by *Acinetobacter soli* ANG344B in this work (3.0 ± 0.2 g/L), in non-optimized conditions.

5.4.2. EPS characterization

5.4.2.1. Chemical composition

EPS produced by *A. soli* ANG344B were heteropolysaccharides composed of four or seven different monomers, depending on the carbon source used for cultivation, commercial glucose or banana peels hydrolysate (glucose and fructose), respectively, as represented in Table 5.1.

Table 5.1 Characterization of the EPS produced by *A. soli* ANG344B in different conditions.

EPS*	Sugar composition (% mol)					Acyl groups (% wt)	Protein (% wt)	M_w (MDa)	PDI	T_{deg} (°C)
	Fuc	Glc	Xyl	Ara	GalA	Ac				
A	11.5 ± 0.2	55.0 ± 0.6	30.3 ± 0.8	-	-	1.5 ± 0.1	16.3	1.6	1.1	278
B	11.6 ± 0.1	61.6 ± 0.3	26.8 ± 0.1	-	-	4.3 ± 0.1	44.2	4.8	1.6	298
C	4.5 ± 0.3	25.6 ± 0.0	16.4 ± 0.0	16.4 ± 0.2	32.4 ± 0.2	4.7 ± 0.2	20.3	8.3	1.4	288

* EPS A was produced in absence of L-Phe, EPS B and C were obtained in the presence of L-Phe using ISPR approaches and glucose or banana peels hydrolysate as carbon source, respectively. Monosaccharides (Fuc, fucose; Glc, glucose; Xyl, xylose; Ara, arabinose; Gal, galactose; GalA, galacturonic acid) and acyl groups (Ac, acetate) composition, average molecular weight (Mw), polydispersity index (PDI) and maximal degradation temperature (T_{deg}).

The EPS A, produced in the absence of L-Phe assay, was composed of neutral sugars, namely 11.5 ± 0.2 mol% of fucose, 30.3 ± 0.8 mol% of xylose and 55.0 ± 0.6 mol% of glucose, and the acyl group acetate (1.5 ± 0.1 wt%). Similar monomer composition but with different proportions was obtained for EPS B that was produced in presence of L-Phe using glucose as carbon source. This EPS was also richer in glucose (61.6 ± 0.3 mol%) but with a higher content than EPS A, followed by xylose (26.8 ± 0.1 mol%) and fucose (11.6 ± 0.1 mol%) and 4.3 ± 0.1 wt% of acetate. Moreover, in the assay where *A.soli* ANG344B was cultivated in the presence of L-Phe using banana peels hydrolysate as carbon source, the EPS produced was different from EPS A and EPS B (Table 5.1). It is composed by the neutral sugars, namely 4.5 ± 0.3 mol% of fucose, 25.6 ± 0.0 mol% of glucose, 16.4 ± 0.0 mol% of xylose and 16.4 ± 0.2 mol% arabinose, the acidic sugar

galacturonic acid (32.4 ± 0.2 mol%) were also part of the polymer composition as well as the acyl group (acetate, 4.7 ± 0.2 wt%.). The presence of rare sugars in EPS composition, such as fucose and galacturonic acid are reported do confer bioactive properties to the polymers, as anticancer, anti-inflammatory or anti-aging properties, making them attractive to be used in food, pharmaceutical and cosmetic applications (Freitas et al., 2011; Roca et al., 2015; Zayed et al., 2021). The presence of xylose is not common in bacterial EPS (Roca et al., 2015). The presence of acyl groups might confer charge to the polymer, allowing it to interact with other polysaccharides and cations (Freitas et al., 2011; Roca et al., 2015; Zayed et al., 2021). The different EPS were also evaluated about the content on protein, having obtained 16.3, 44.2 and 20.3 wt% for EPS A, B and C, respectively.

EPS produced by *A. soli* ANG344B have a different composition from the exopolysaccharides reported by *Acinetobacter* strains. *A. soli* ANG344B produces EPS with monomers such as fucose, xylose, arabinose, galacturonic acid and acetate, that are not commonly reported in EPS produced by other *Acinetobacter* strains. For instance, *A. calcoacaticus* BD4 was reported to produce an EPS with rhamnose, glucose and mannose it its composition, *A. junii* BB1A was found to produce an EPS composed of mannose, galactose and arabinose and *A. bouvetii* UAM25 was able to produce an EPS with repeated units of rhamnose and galactose (Bryan et al., 1986; Sen et al., 2014; Vázquez-Vázquez et al., 2017). However, the presence of these sugars has been reported in EPS produced by different microorganisms, such as *Paenibacillus elgii*, *Vibrio harveji* VB23, *Klebsiella pneumoniae* and *Enterobacter* A47 (Freitas et al., 2017; More et al., 2014; Zayed et al., 2021). The presence of protein in EPS, as described in this work for *A. soli* ANG344B, are frequently reported in EPS produced by bacteria belonging to *Acinetobacter* genus. For example, Biodispersan, a polymer produced by *A. calcoaceticus* is a polysaccharide rich in protein, having glucosamine, 6-metylaminohexose, galactosamine, uronic acid and an unidentified amino sugar in its composition. Alasan, produced by *Acinetobacter radioresistens* is a protein polysaccharide containing alanine (Ortega-de la Rosa et al., 2018; Satpute et al., 2010).

5.4.2.2. Fourier Transform Infra-Red spectroscopy

Fourier Transform Infra-Red (FTIR) spectra of the EPS produced by *A. soli* ANG344B (in absence of L-Phe; in presence of L-Phe using an ISPR using glucose or banana peels hydrolysate as carbon source) contain bands typically found in carbohydrates (Figure 5.2).

The three EPS in study present similar FTIR spectra with an intense broad band observed in the range of 3000 to 3500 cm^{-1} corresponding to the O-H stretching vibration of the hydroxyl groups and N-H stretching vibration of the amine groups (Adetunji and Olaniran, 2019; Concórdio-Reis et al., 2021). The peak appearing at 2923 cm^{-1} corresponds to the C-H stretching peak of the CH_2 groups (Concórdio-Reis et al., 2021; Freitas et al., 2011). The band at 1724 cm^{-1} might be attributed to C=O stretching of carbonyl in acyl groups, as well as the band at 1259 cm^{-1} that can be attributed to the C-O stretching of the acyl groups and the band at 1544 cm^{-1} that indicates the presence of carboxyl groups by the symmetric stretch peak (Freitas et al., 2011; Mandal et al., 2011). This is in accordance with the EPS composition, that have acetyl groups in its composition, as it is represented in Table 5.1. The increase in the intensity of the bands around 1259 and 1544 cm^{-1} for the EPS B and C might be related with the higher amount of acetyl in both polymers and galacturonic acid in EPS C, respectively, that are present in these polymers, in comparison to EPS A. Bands in the range of 900 to 1200 cm^{-1}, so called fingerprint region, represents the skeletal C-O and C-C vibrations of glycosidic bonds and pyranose ring, as well as the C-N stretching of amine groups (Adetunji and Olaniran 2019; Freitas et al., 2011; Hong et al., 2021). The band that is observed at 1634 cm^{-1} can correspond to N-H bending of amines, from the protein present in the polymer constitution (Table 5.1) (Adetunji and Olaniran 2019). Peaks below 800 cm^{-1} may also be attributed to glycosidic bonds (Hong et al., 2021; Mandal et al., 2011).

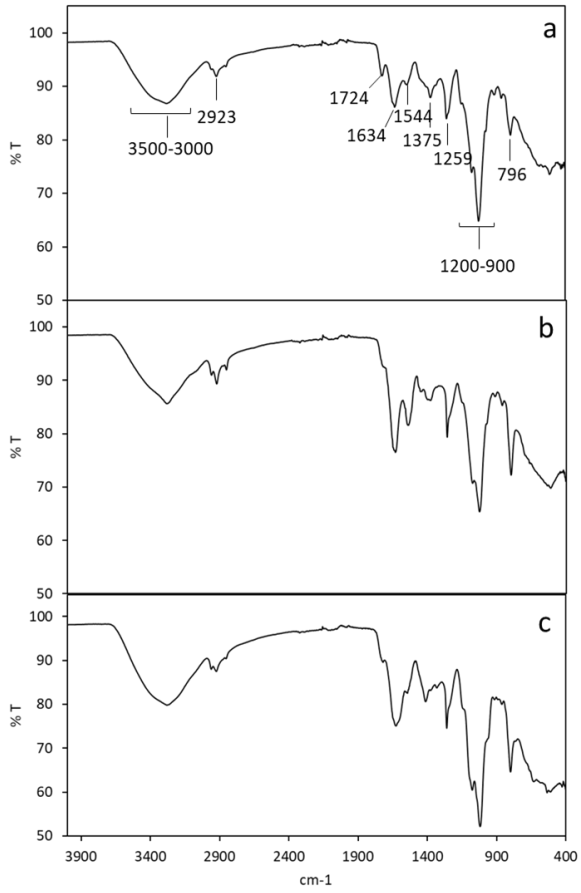

Figure 5.2 FTIR spectra of EPS produced by *Acinetobacter soli* ANG344B. A: EPS produced in absence of L-Phe; B: EPS produced in presence of L-Phe, using an ISPR approach and glucose as carbon source; C: EPS produced in presence of L-Phe using an ISPR approach and using banana peels hydrolysate as carbon source.

5.4.2.3. Molecular mass distribution

EPS produced by *A. soli* ANG344B were characterized in terms of average molecular weight (M_w), as presented in Table 5.1. The EPS produced in all the tested conditions presented high M_w values, 1.6, 4.8 and 8.3 MDa, respectively for EPS A, B and C. These M_w values are within the reported in literature for the most studied bacterial EPS, as Xanthan gum, Gellan, Alginate, Cellulose and Hyaluronic acid, ranging from 0.3 to 50 MDa and for EPS produced by *Acinetobacter* strains, as *A. bouvetii* UAM25, *A. venetianus* RAG-1, *A. calcoacaticus* BD4 and *A. radioresistens* KA53, that ranged from 0.5 to 4.9 MDa (Bryan et al., 1986; Freitas et al., 2017; Navon-Venezia et al., 1995; Ortega-de la Rosa et al., 2018; Su et al., 2009; Vázquez-Vázquez et al.,2017). The degree of heterogenicity of polymer chain length is estimated by the polydispersity index (PI),

being the polymer more homogeneous as the index is closer to 1 (Freitas et al., 2011). The EPS produced by *A. soli* ANG344B presented low PDI values, 1.1, 1.6 and 1.4, respectively for EPS A, B and C (Table 5.1) indicating homogeneity of the polymer's chain length (Freitas et al., 2011).

5.4.2.4. Thermogravimetric analysis

Thermogravimetric analysis (TGA) of the EPS produced by *A. soli* ANG344B showed that polymer degradation occurs in two phases for the three EPS analyzed, as depicted in Figure 5.3. This technique allows to evaluate the thermal stability of EPS that are important for industrial applications that require high temperatures such as sterilization, for example in food industry (Ali et al., 2023; Kim et al., 2023).

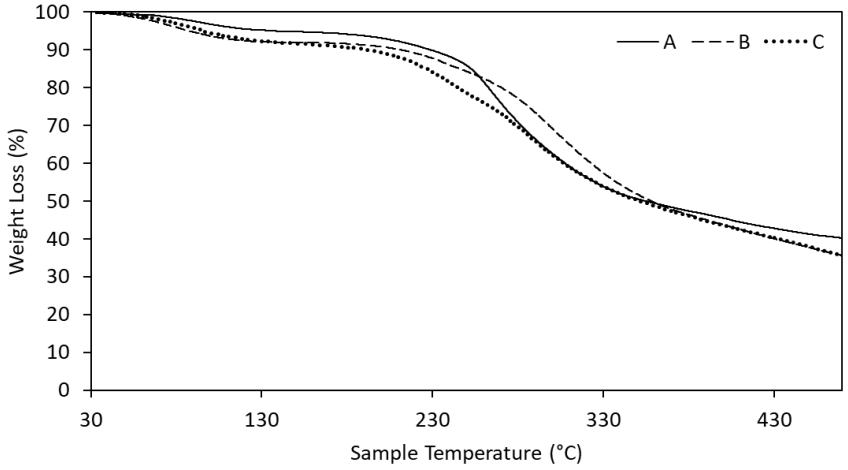

Figure 5.3 Thermogravimetric analysis curves of EPS produced by *Acinetobacter soli* ANG344B in different conditions.

The first degradation phase occurred between 36 - 40 °C and 151 - 160 °C, corresponding to a weight loss of 5.0 to 8.2% and is probably associated with the moisture loss. This moisture loss is related to the elimination of water molecules that are bound to carboxylic groups present in the EPS (Concórdio-Reis et al., 2021; Yadav et al., 2012). In fact, the lower weight loss was observed for EPS A (5.0%) that is the polymer with lower content of acyl groups, thus with lower affinity towards water molecules. The second degradation step occurred between 152 – 160 °C and 452 – 494 °C, accompanied with a significant weight loss of 53.5 to 57.9%, probably related with the decomposition of EPS side chains.

The maximum degradation temperature (T_{deg}) for the tested EPS were 278, 298 and 288 °C, respectively for EPS A, B and C, as represented in Table 5.1. These values are within the reported in literature for other bacterial EPS, such as Xanthan gum (T_{deg} =293 °C) and FucoPol (T_{deg} =266 °C) (Concórdio-Reis et al., 2021; Li et al., 2016; Araújo et al., 2023, Kim et al., 2023; Ali et al., 2023).

5.4.2.5. Rheological behavior

Bacterial EPS can present rheological properties that make them important candidates for several industries, such as cosmetics and food industries (Zayed et al., 2021). For instance, Xanthan gum, Dextran and Pullulan are used as texturizing agents in the referred industries (Rana and Upadhyay, 2020). So, the rheological behavior of the EPS produced by *A. soli* ANG344B were accessed using the Power Law model.

The flow curves obtained for 1 wt% purified EPS aqueous solutions produced by *A. soli* ANG344B are depicted in Figure 5.4.

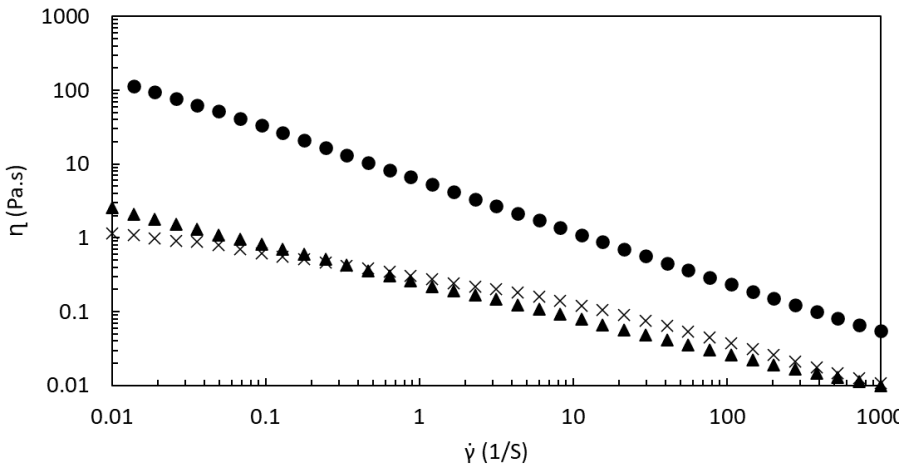

Figure 5.4 Flow curves of aqueous EPS solutions (1 wt%) prepared with EPS A (●), EPS B (X) and EPS C (▲).

Flow curves of the studied EPS presented a shear-thinning behavior with different thickening capacities since they show different values of apparent viscosity for the same shear rate. EPS A is the polymer presenting a higher apparent viscosity for lower shear rates, while EPS B and C presented a similar behavior regardless the difference in its composition. At a shear rate of 0.01 s^{-1}, EPS A presented an apparent viscosity of 131.0 Pa.s, while EPS B and C presented an apparent viscosity of 1.2 and 2.6 Pa.s, respectively

(Figure 5.4). Despite the similar composition of EPS A and EPS B, these polymers have different apparent viscosities, that might be related with the different proportions of substituent groups as the acyl groups or the protein content that can allow different intermolecular interactions. In the range of the shear-rates studied no plateau Newtonian was observed.

The relationship between the shear stress (σ, Pa) and the shear rate ($\dot{\gamma}$, 1/s) could be fitted using Power Law model (Equation 5.1), whose parameters are described in Table 5.2. The Power Law index (n) indicates the degree of non-Newtonian behavior, where for n = 1, the fluid presents Newtonian behavior and for n < 1, the polymer is considered to have a shear-thinning behavior.

Table 5.2 Power Law model parameters estimated for EPS aqueous solutions of 1% (w/v) produced by *Acinetobacter soli* ANG344B in different conditions.

EPS	n (-)	K (Pa. s^n)	R^2
A	0.33 ± 0.00	5.63 ± 0.09	0.999
B	0.45 ± 0.01	0.46 ± 0.01	0.998
C	0.56 ± 0.01	0.20 ±0.01	0.999

The shear-thinning behavior of the three EPS are demonstrated by Power Law indexes of 0.33 ± 0.00, 0.45 ± 0.01 and 0.56 ± 0.01, respectively for EPS A, B and C, meaning that the apparent viscosity decreases with the increase of shear rate, as it is depicted in Figure 5.4. Polymers presenting shear-thinning behavior show potential to be used as thickening agents in paints, cosmetic, pharmaceutical and food products (Concórdio-Reis et al., 2021; Hundschell and Wagemans, 2019). The consistency index (K) increases with the viscosity, as it is represented in Table 5.2. This index indicates the shear strength of the material, being EPS A the polymer with greater resistance from the tested polymers (Oliveira et al., 2018).

5.4.2.6. Emulsion forming capacity

EPS produced by *Acinetobacter* strains are frequently reported to have emulsification properties (Mujumdar et al., 2019). So, the ability of EPS produced by *A. soli* ANG344B to form emulsions were also assessed. A preliminary assessment was performed, using

aqueous solutions of 0.5 wt% of EPS A, B and C and sunflower oil, with a water:oil ratio of 2:3, at room temperature.

Under the conditions tested, EPS A presented an emulsification index (E_{24}) at 24 h of 47 ± 7%, while EPS B and C were not able to stabilize emulsions, that disappeared within a few hours after being prepared. Higher emulsification indexes were reported for polymers produced by other *Acinetobacter* strains, obtaining E_{24} between 60 and 80% using different hydrophobic substances (e.g., mineral oil, edible oils, aromatic or aliphatic hydrocarbons), and different conditions than the used in these experiments for E_{24} determination (Adetunji and Olaniran 2019; Mujumdar et al., 2019; Su et al., 2009).

Emulsion forming and stabilizing capacity of an emulsifier can be assessed by evaluating its ability to maintain at least 50% of the original emulsion after 24 h, being the EPS A in this threshold (Freitas et al., 2014). To act as emulsifier or stabilizer, the polymer must have hydrophilic and hydrophobic groups, such as acetyl groups to allow the interaction of the aqueous phase with the hydrophobic phase. In addition, the protein bound to the polymer may also contribute to its emulsifying capacity (Oliveira et al., 2018). Moreover, the viscosity of the solution can contribute to emulsion stabilization (Oliveira et al., 2018). In fact, EPS A presents higher viscosity than EPS B and C, that allowed the stabilization of the emulsion when using EPS A. Even though the EPS B and C have a higher content of protein and acetate groups than the EPS A, the results suggest that the viscosity has a prevailing role in emulsification capacity of the polymer.

5.5. Conclusions

Acinetobacter soli ANG344B was found to produce EPS during the 2-PE producing process if the aroma concentration in the culture broth is low or in absence of L-Phe, during the stationary growth phase. These EPS were characterized in terms of chemical composition, thermal stability, rheological behavior and emulsion forming capacity. *A. soli* ANG344B culture produced high molecular weight polysaccharides, with a sugar composition that varies with the carbon source used. These EPS showed good thermal stability and presented a shear-thinning behavior that can be interesting for applications in pharmaceutical, food and cosmetic fields. Emulsion forming capacity was only observed for EPS produced in absence of 2-PE. The results obtained show the potential of this culture to produce EPS with interesting properties, so improvement of the

production process as well as further characterization and assessment of functional properties might be interesting.

6.

General conclusions and Future work

6.1. General conclusions

Acinetobacter soli ANG344B, isolated from river water, has been identified and characterized, showcasing an outstanding ability to produce 2-phenylethanol (2-PE). This capability, not commonly reported for bacteria, is particularly noteworthy as bacteria typically yield low titers of 2-PE. Notably, this non-pathogenic bacterium synthesizes 2-PE through L-phenylalanine (L-Phe) via the Ehrlich pathway.

To maximize 2-PE production, this study investigated the optimal medium composition and operational conditions. Yeast extract was found to be a key element in the cultivation media, enhancing both cellular growth and 2-PE production. The presence of L-Phe at concentrations of around 5 g/L in the cultivation media was found to be essential for improving volumetric productivity. The optimal operational conditions that ensured the best performance of *A. soli* ANG344B were determined as 30 °C, pH 7 and an aeration of 1 vvm. Under these conditions, *A. soli* ANG344B achieved a noteworthy 2.14 ± 0.18 g/L of 2-PE in 24 h, a result not previously described for wild-type bacteria. The volumetric productivity of this process, at 0.12 ± 0.01 $g_{2\text{-}PE}$/L.h (for 16 cultivation), was among the highest reported in literature for a batch production process. The use of bacteria for 2-PE production offers advantages over yeasts, due to shorter growth times and simpler metabolic pathways.

Despite its benefits, the aromatic alcohol 2-PE exhibits cytotoxic effects towards the producing microorganisms, hindering further enhancement of its production under the described conditions. *A. soli* ANG344B growth was negatively impaired in the presence of 2-PE concentrations as low as 1 g/L and completely inhibited at 4 g/L.

The improvement of 2-PE production was achieved through *in situ* product removal (ISPR) approaches, with resin adsorption proving to be the most successful. The use of adsorbent resins, particularly Amberlite XAD 4, demonstrated to be promising in enhancing the 2-PE production process. In a batch bioconversion process with 3% (w/v) Amberlite XAD 4, 6.99 ± 0.06 g/L of 2-PE was produced from 12 g/L of L-Phe making a 3.3-fold improvement in 2-PE production. The recovered 2-PE from the resin, desorbed using ethanol, resulted in an extract containing 217.56 ± 0.31 g/L of the aroma, boosting a chromatographic purity of 98% of the compound in a matrix of ethanol.

Exploring carbon sources, different agro-industrial wastes were hydrolyzed and evaluated for bacterial cultivation and 2-PE production. While these low-cost feedstocks supported *A. soli* ANG344B growth as part of the cultivation media, the protein content on their hydrolysates was not enough to supply L-Phe for 2-PE production. Supplementation with L-Phe was necessary, and banana peels hydrolysate, composed of glucose and fructose, emerged as the substrate facilitating optimal growth and 2-PE production, surpassing commercial glucose and fructose. In these conditions, 2.48 ± 0.04 g/L of 2-PE was produced. The ISPR approach, using 3% (w/v) of Amberlite XAD 4, enabled the production of 7.30 ± 0.01 g/L of 2-PE. Recovery of 2-PE from the resin using ethanol resulted in an extract containing 41.12 ± 0.11 g/L of 2-PE, exhibiting a chromatographic purity obtained by GC, of 88 % of the compound in a matrix of ethanol.

Beyond 2-PE production, *A. soli* ANG344B demonstrated the ability to produce exopolysaccharides (EPS) in absence of L-Phe and during 2-PE production using the ISPR approach. EPS concentrations ranged from 2.4 to 4.7 g/L. The strain produced EPS with different sugar monomer compositions depending on the carbon source used for growth. EPS produced using glucose as carbon source have glucose, xylose and fucose monomers in its composition, while EPS produced using banana peels hydrolysate also have galacturonic acid, arabinose and galactose, both EPS with acetyl substituents. The produced EPS had high molecular weight, ranging from 1.6 to 8.3 MDa, presented high degradation temperatures (above 278 °C) and shear-thinning behavior in aqueous solution. EPS produced using commercial glucose as carbon source in absence of L-Phe and 2-PE was able to form emulsions, with an emulsification index at 24 h of 47 ± 7%. These properties might be interesting for EPS application in food, cosmetic and pharmaceutical fields.

Overall, the findings presented in this PhD thesis show the exceptional ability of *Acinetobacter soli* ANG344B to produce 2-PE, with potential implications for industrial applications using both commercial carbon sources or waste-based medium. The production capabilities of this strain are not limited to aroma production but it also includes EPS production.

6.2. Future work

This PhD thesis revealed the potential of *Acinetobacter soli* ANG344B to produce 2-PE and introduces strategies that enhanced aroma production using both commercial carbon sources and waste-based medium. The results obtained in this work may be further improved and extended, so the following suggestions might be considered.

Improvement of 2-PE production should be attempted, increasing the amount of adsorbent resin in contact with the cultivation broth. This might be achieved using external columns filled with resin through which the cultivation broth contacts. Evaluate a cell separation step, prior to circulation through the adsorbent column(s), might be considered to avoid blockage caused by cells passing through the packed column(s). A continuous production process should also be explored, using multiple external columns that can be connected to the bioreactor. In these conditions, nutrients and L-Phe must be fed to the bioreactor to ensure bacterial growth and cellular maintenance.

Further evaluate production conditions, such as decreased aeration or concentration of carbon source supplied to the medium, to avoid EPS production during 2-PE production process. The presence of EPS during the aroma production process can cause difficulties in process operation, especially in cell separation, and circulation of the culture broth through the packed columns and sampling.

The recovery of 2-PE from the adsorbent resin should also be studied aiming to increase the purity of the recovered 2-PE, particularly when using waste-based media. Evaluate different solvents, as ethyl acetate, or the use of a combination of different solvents. For instance, desorption in two phases, using water to desorb contaminants (as L-Phe) from the resin and then desorption using ethanol to recover the 2-PE might result in an extract with higher purity.

Conduct an economical assessment of the integrated process after achieving a continuous or a semi-continuous process to show the feasibility of the process, before scaling up the production process.

Regarding EPS production, since the polymers produced seem to have interesting properties, further characterization studies should be performed. These studies should include rheologic behavior and further functional and biological properties. This strain showed the ability to produce EPS with different composition as result of modification of

culture conditions, as carbon source modification. So, the production of tailored polymers with different properties and applications might also be interesting.

In summary, these recommendations aim to refine and expand the 2-PE production process, optimize recovery methods, and further explore the potential of EPS production by *Acinetobacter soli* ANG344B.

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