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Process optimization of cell-wall polysaccharides production by *Komagataella pastoris*

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“Se tiveres asas e raízes, tu possuis o futuro. Precisas das asas para voar, sonhar e acreditar. Mas precisas das raízes para receberes a sabedoria dos mais velhos”

Papa Francisco

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Abstract

This PhD thesis had the objective of optimizing the process developed by Pharma73 S.A. for cultivation of the yeast *Komagataella pastoris* to produce the cell-wall polysaccharides, chitin-glucan complex (CGC) and mannans.

A repeated fed-batch strategy was developed that resulted in the stable and reproducible production of biomass and CGC. Seven consecutive fed-batch cycles were maintained, with a daily biomass production of 119-124 g/L and a CGC volumetric productivity of 13-20 g/L.day. Reducing the dissolved oxygen (DO) level from 50 to 15% was demonstrated to result in an overall volumetric productivity of 8.67 and 10.69 g/L.day, for CGC and mannans, respectively. The CGC and mannans composition was not significantly affected by the reduction of the DO level. Medium K was developed to avoid the operational problems associated with the use of Basal Salts Medium (BSM). Medium K was successfully implemented in the repeated fed-batch process, where the CGC and mannans productivities were improved to 17.5-26 and 19.2-26.4 g/L.day, respectively, without compromising the composition of the two polysaccharides.

The downstream was optimized to obtain both polysaccharides with high extraction efficiency and purity. The optimized extraction conditions included treating the biomass with NaOH 4 M, at 84 ºC, during 4 h. The structural analysis of the polysaccharides revealed the presence of β-glucans and α-chitin, in the CGC, and α-mannans. Both polysaccharides degraded at high temperatures: above 300 ºC for CGC and between 233-253 ºC for mannans.

The work performed in this thesis resulted in a 3.4-fold increase of the CGC productivity and the extraction of mannans from *K. pastoris* was demonstrated for the first time. The optimization of this production process made the process simpler and resulted in considerable savings in terms of overall production costs.

Key-words: Chitin-glucan complex (CGC), Mannans, *Komagataella pastoris*, high cell density cultivation, bioprocess optimization, polysaccharides characterization

Resumo

Esta tese de doutoramento teve como objetivo a otimização do processo desenvolvido pela empresa Pharma73 S.A. para o cultivo da levedura *Komagataella pastoris*, para a produção de polissacáridos da parede celular, complexo quitina-glucanos (CQG) e mananos.

Foi desenvolvido um processo de produção cíclico semi-contínuo, que resultou numa produção de biomassa e de CQG estável e reprodutível. Sete ciclos semi-contínuos consecutivos foram mantidos, com uma produção diária de biomassa de 119-124 g/L e uma produtividade volumétrica de CQG de 13-20 g/L.dia. Reduzir o nível de oxigénio dissolvido (OD) de 50 para 15% resultou numa produtividade volumétrica global de 8.67 e 10.69 g/L.dia, para o CQG e mananos, respetivamente. A composição de CQG e mananos não foi afetada significativamente pela redução do nível de OD. O Meio K foi desenvolvido de forma a evitar os problemas operacionais associados ao uso de Meio Básico Salino (MBS). O Meio K foi implementado com sucesso num processo cíclico semi-contínuo, onde foram melhoradas as produtividades de CQG e mananos para 17.5-26 e 19.2-26.4 g/L.dia, respetivamente, sem comprometer a composição dos dois polissacáridos.

A extração e purificação foram otimizadas para se obter os dois polissacáridos com elevada eficiência de extração e pureza. As condições de extração otimizadas incluiu o tratamento da biomassa com NaOH 4M, a 84 ºC, durante 4 h. A análise estrutural dos polissacáridos revelou a presença de β-glucanos e α-quitina, no CQG, e α-mananos. Os dois polissacáridos degradam a elevadas temperaturas: acima dos 300 ºC para o CQG e entre 233-253 ºC para as mananos.

O trabalho desenvolvido nesta tese resultou num aumento de 3.4 vezes da produtividade em CQG e a extracção de mananos a partir da *K. pastoris* foi demonstrada pela primeira vez. A otimização deste processo de produção tornou o processo mais simples e dela resultaram poupanças consideráveis ao nível dos seus custos de produção.

Palavras-chave: Complexo quitina-glucanos (CQG), Mananos, *Komagataella pastoris*, cultivo de elevada densidade celular, otimização de bioprocesso, caracterização de polissacáridos

Nomenclature

Abreviations

^{13}C-NMR – ^{13}C Solid-state Nuclear Magnetic Resonance

AIM – Alkaline Insoluble Material

ANOVA – Analysis of Variance

ASM – Alkaline Soluble Material

BSM – Basal Salts Medium

CCRD – Central Composite Rotatable Design

CGC – Chitin-glucan Complex

CHS - Chitin Synthases

CI – Crystallinity Index

CP – Cross-Polarization

CSD – Corn Steep Dextrose

CWI – Cell-Wall Integrity

DCW – Dry cell Weight

DO – Dissolved Oxygen

DSC – Differential Scanning Calorimetry

FTIR - Fourier Transform Infrared Spectroscopy

GPI – Glycosylphosphatidyl Inositol

GRAS – General Recognized as Safe

HPLC – High Performance Liquid Chromatography

MALS – Multi-Angle Light Scattering

MAS – Magic-Angle Spinning

MBSM – Modified Basal Salts Medium

MLR – Multiple Linear Regression

MM – Minimal Medium

MSM – Minimal Salt Medium

Mw – Molecular Weight

PBS – Phosphate-Buffered Saline solution

PDI -Polydispersity Index

PTM – *Pichia* Trace Mineral solution

RI – Refractive Index

RSM – Response Surface Methodology

SEC – Size Exclusion Chromatography

SLPM – Standard Liters Per Minute

TGA – Thermogravimetric Analysis

XRD – X-ray Diffraction

Variables

CGC_{PBS} – Chitin-glucan Complex treated with PBS

CGC_{pure} – Pure Chitin-glucan Complex

DCW – Dry Cell Weight (g/L)

I_{110} – Maximum intensity of the diffraction of the (110) lattice peak at $2\theta \approx 19^{o}$

I_{am} – Intensity of the amorphous material at $2\theta \approx 16^{o}$

Mw – Molecular weight (Da)

p-value – Regression parameter significance

q_S – Specific substrate consumption rate ($g_s/g_x.h$)

R^2 – Multiple correlation coefficient

r_C – CGC volumetric productivity (g/L.day)

r_M – Mannans volumetric productivity (g/L.day)

r_X – Biomass volumetric productivity (g/L.day)

t - time

T_{deg} – Degradation temperature (ºC)

X – Coded value for independent variable at CCRD

X – DCW at time t (g/L)

X_0 – DCW at time = 0 (g/L)

$Y_{C/S}$ – CGC yield on substrate basis (g_c/g_s)

$Y_{M/S}$ – Mannans yield on substrate basis (g_m/g_s)

Y_p – Predicted response of CCRD

$Y_{X/S}$ – Biomass yield on substrate basis (g_x/g_s)

Greek letters

α – axial level in RSM

β – regression coefficient of CCRD

δ – chemical shift (ppm)

μ_{max} – maximum specific cell growth rate (h^{-1})

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Chapter 1

Motivation and thesis outline

1.1. Motivation

In the last years, the interest in yeast cell-wall polysaccharides, namely chitin-glucan complex (CGC) and mannans, has been increasing, mostly due to their bioactive properties and high range of potential applications. Nowadays, CGC is already commercially produced as a by-product of citric acid production by the fungi *Aspergillus niger* (Kytozyme©'s KioTransine®, http://kitozyme.com). Mannans from *Saccharomyces cerevisiae* (Elicityl's mannans, http://www.elicityl-oligotech.com) can also be found in the market.

In view of the valuable properties of CGC and mannans, the patented bioprocess proposed by Pharma73 S.A. (http://www.pharma73.com) has high interest and potential for industrial development (Reis *et al.*, 2010; Freitas, Roca, *et al.*, 2013; Freitas *et al.*, 2015). The higher biomass concentration achieved using a simple culture medium with a low-cost carbon source, such as glycerol, combined with high products productivities, makes Pharma73 S.A. process more competitive, comparing with other fungal cell-wall polysaccharides production processes (Feofilova *et al.*, 2006; Zlotnikov *et al.*, 2007; Smirnou, Krcmar and Prochazkova, 2011). However, the Pharma73 S.A. process (also described in Roca *et al.* (2012)), resulted in a highly impure CGC, mannans were not recovered and the CGC volumetric productivity was still low. Therefore, there was a need to improve these products productivities and optimize the downstream procedures to reach pure polysaccharides, in order to make this bioprocess more economically viable.

The main objective of this PhD thesis was the optimization of the *K. pastoris* bioprocess developed by Pharma73 S.A. to obtain CGC and mannans, by increasing the products productivities and reducing the production costs, at the same time. In this way, this thesis included some fermentation and downstream optimization studies. For optimization of the fermentation, a repeated fed-batch process was established, the dissolved oxygen level was optimized, and a new culture medium was developed, to increase the polysaccharides productivities. The downstream (polysaccharides extraction and purification) was optimized to obtain pure CGC and mannans, by developing a simple purification process and evaluating the impact of the extraction conditions on final products properties.

1.2. Thesis outline

This thesis is structured considering the main purposes of this PhD project. Chapters 3, 4 and 5 includes the fermentation optimization studies, while Chapters 6 and 7 is related with the downstream optimization and biopolymers characterization. This work resulted in two

scientific papers published in international peer reviewed journals and another two manuscripts were already submitted. Part of this work was also included in the international patent applications WO2013140222 and WO2015177622, owned by Pharma73 S.A. (Freitas, Roca, *et al.*, 2013; Freitas *et al.*, 2015).

This PhD thesis is organized according to the following chapters:

- Chapter 2 – describes the state of the art
- Chapter 3 – includes the experiments performed for the implementation of a repeated fed-batch process to improve the CGC productivity
- Chapter 4 – study of the dissolved oxygen level influence on cell-wall polysaccharides production
- Chapter 5 – culture medium design to improve the *K. pastoris* cell-wall polysaccharides production
- Chapter 6 – downstream process development to obtain a pure CGC and its physico-chemical properties
- Chapter 7 – study of the influence of basic extraction conditions on *K. pastoris* polysaccharides composition and physico-chemical properties.
- Chapter 8 – conclusions and future work

Chapter 2

State of the art

Polysaccharides are biomolecules composed of sugar units covalently linked to form polymeric chains. They are one of the most abundant materials available in nature from wide and diverse origins. Polysaccharides can be found in plants (e.g., glucomannans, cellulose), animals (e.g., chitin), algae (e.g., alginates, carrageenan), bacteria (e.g., xanthan, gellan) and yeasts/fungi (e.g., chitin, glucans) (Rinaudo, 2008). These biomaterials are being the subject of increasing interest due to their renewable, biodegradable, non-toxic and biocompatible properties. Besides that, many possess biological activity (e.g., immunologic, anti-inflammatory, antioxidant and antiviral activities) that makes them interesting for use in the medical and pharmaceutical fields (Liu, Willför and Xu, 2015; Shi, 2016). Moreover, polysaccharides can be used in food and cosmetic products and processes, as well as in wastewater treatment, oil recovery and many other industrial applications (Blackburn, 2004; Freitas *et al.*, 2014; Hamed, Özogul and Regenstein, 2016).

This PhD thesis was focused on chitin-glucan complex (CGC) and mannans extracted from the cell-wall polysaccharides of the yeast *Komagataella pastoris*, including bioprocess development and optimization, as well as polysaccharides characterization.

2.1. Yeast cell-wall composition

The yeast cell-wall is a dynamic structure that ensures the cell's viability and morphogenesis (Gow, Latge and Munro, 2017). In most yeast cultures, the cell-wall represents up to 25% of the cells dry weight, being a double layer structure mainly composed of polysaccharides: β-glucans (around 60%), mannans (around 40%) and chitin (up to 2%) (Figure 2.1) (Lipke and Ovalle, 1998; Bowman and Free, 2006; Klis, Boorsma and De Groot, 2006; Kogani *et al.*, 2008; Brown *et al.*, 2014).

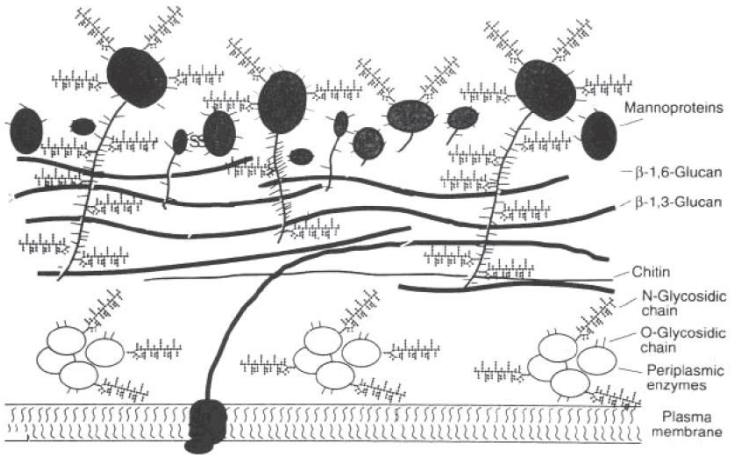

Figure 2.1 – Composition and structure of a yeast cell-wall (Kogan *et al.*, 2008)

The major polysaccharide component of the cell-wall is β-(1,3)-glucans, helix structures composed of glucose units. Combined with chitin, these glucans contribute to the elasticity and mechanic strength of the cell-wall (Klis *et al.*, 2002). Chitin is a linear polysaccharide composed of N-acetylglucosamine units, mostly present in the bud scar and in lateral wall of the yeast cells (Klis *et al.*, 2002). This crystalline polysaccharide plays an important role in cell division cycle and contributes to the rigidity and strength of the cell-wall (Bowman and Free, 2006; Arroyo *et al.*, 2016). The inner cell-wall includes β-(1,3)-glucans covalently linked to chitin, both synthesized in the plasma membrane (Arroyo *et al.*, 2016). Beyond β-(1,3)-glucans, cell-wall composition also includes another type of glucans, β-(1,6)-glucans, which are responsible for the linkage between the two cell-wall layers, connecting the β-(1,3)-glucans to glycosylphosphatidyl inositol(GPI)-dependent cell-wall proteins of the outer cell-wall (Gow, Latge and Munro, 2017).

The outer cell-wall is mainly composed of highly glycosylated mannans (50-95% of carbohydrates) (Lipke and Ovalle, 1998) that are synthesized in the endoplasmic reticulum (Arroyo *et al.*, 2016). They include linear α-(1,6)-mannans highly branched with α-(1,2) and α-(1,3) side chains (Gow, Latge and Munro, 2017). These mannans have several functions in the yeast cell-wall, including the maintenance of cell integrity, the protection against external agents and the transmission of intracellular signals in response to external stimuli (Bowman and Free, 2006).

The composition of the cell-wall can be affected by several environmental factors, including oxidative stress, medium composition, temperature, external pH and oxygen levels (Aguilar-Uscanga and Francois, 2003; Pillet *et al.*, 2014). Under certain conditions, the yeast cells activate the cell-wall integrity (CWI) pathway to avoid loss of viability. The activation of the CWI can result in increased production of some components, such as chitin and mannans, and/or in the reorganization of the linkages in other components (Smits, van den Ende and Klis, 2001; Klis, Boorsma and De Groot, 2006; Arroyo *et al.*, 2016).

2.2. Chitin-glucan complex (CGC)

Chitin-glucan complex (CGC) is a co-polymer of covalently linked chitin and β-(1,3)-glucans (Arroyo *et al.*, 2016). It is a natural cell-wall component of most yeast and fungi cultures, conferring protection and stability to the cells (Aguilar-Uscanga and Francois, 2003; Klis, Boorsma and De Groot, 2006). Depending on the yeast/fungal strain and the production conditions used, the CGC content in the biomass/mycelium can range between 4 and 30% of the microbial dry mass (Table 2.1).

Table 2.1 – CGC content in the biomass of several yeast and fungi strains.

Microorganism	CGC content (%)	Refs.
Cephaliophora tropica	12-27	Zlotnikov *et al.* (2007)
Armillariella mellea	4.5	Ivshin *et al.* (2007); Ivshina *et al.* (2009)
Morchella esculenta	9.6	Ivshina *et al.* (2009)
Schizophyllum commune	15-30	Smirnou, Krcmar and Prochazkova (2011)
Aspergillus niger	15-18	Feofilova *et al.* (2006)
Pichia pastoris	11-20	Roca *et al.* (2012); Chagas *et al.* (2014)

CGC is insoluble in water and in most organic solvents, being a dimethlyacetamide (DMAc)/lithium chloride solution the best option to solubilize it (Pillai, Paul and Sharma, 2009; Hamed, Özogul and Regenstein, 2016). However, CGC is hygroscopic and has high swelling capacity. It combines the antimicrobial, antioxidant and hemostatic effects of chitin (Younes and Rinaudo, 2015; Hamed, Özogul and Regenstein, 2016; Singh, Shitiz and Singh, 2017) with the anti-inflammatory, anti-tumor, hypocholesterol and immunomodulating activities of β-glucans (Dalonso, Goldman and Gern, 2015; Kagimura *et al.*, 2015; De Oliva-Neto *et al.*, 2016; Zhu, Du and Xu, 2016). Since CGC is composed of two bioactive polysaccharide moieties, there has been a growing interest on this biopolymer, especially for use in the pharmaceutical and medical fields (Giavasis, 2014; Freitas, Roca and Reis, 2015). For example, CGC can be used for the treatment of obesity and diabetes (Neyrinck *et al.*, 2012; Marzorati, Maquet and Possemiers, 2017), for wound healing (Abdel-Mohsen *et al.*, 2016) or surgical adhesives (Pravata, 2012). Moreover, CGC can be used in other applications fields, such as cosmetic formulations (Gautier *et al.*, 2008; Gautier, Bruyere and Maquet, 2010), food additives (Kulev and Negrutsa, 2015), in wine clarification (Bornet and Teissedre, 2008) or as a metal absorbent (Skorik, Pestov and Yatluk, 2010). The use of fungal CGC as food supplement was already authorized by Food and Drug Administration (FDA) (GRAS Notice No. 412; http://www.fda.gov). Recently, Pharma73 S.A. also proposed the use of CGC as a pharmaceutical excipient, due to its high bulk density and compression capacity, important issues for processing of pharmaceutical formulations into solid dosage forms (Freitas, Roca, *et al.*, 2013; Freitas *et al.*, 2015).

In addition, CGC produced by yeast/fungi is an alternative source of non-animal chitin or chitinous derivatives. In contrast with crustacean chitinous materials, yeast/fungal CGC is an allergen-free polysaccharide, there is no variability on polymer's composition and properties, as its production is not affected by the seasonality of the raw materials (Smirnou, Krcmar and Prochazkova, 2011; Roca *et al.*, 2012).

2.3. Mannans

Mannans are homo- or heteropolymers mainly composed of mannose units that can be covalently linked to cell-wall proteins, in the form of mannoproteins. This polysaccharide is also a yeast cell-wall component with the main function of protecting the cell from the osmotic pressure and keep the cell's integrity, especially during the cell cycle (Liu *et al.*, 2015). Mannans can be extracted from several yeast strains, representing between 4 and 13% of the cell dry weight content (Table 2.2).

Table 2.2 – Mannans content in the biomass of several yeast strains.

Microorganism	Mannans content (%)	Refs.
Kluyveromyces marxianus	13.3	Galinari et al. (2017)
Saccharomyces cerevisiae	5.6	Liu et al. (2015)
Saccharomyces uvarum	4.2	Araújo et al. (2014)
Kluyveromyces marxianus	7.0	Lukondeh, Ashbolt and Rogers (2003)

Mannans are soluble polysaccharides, with emulsifying and stabilizer properties (Lukondeh, Ashbolt and Rogers, 2003; Araújo *et al.*, 2014). Because of this, mannans can be used in cosmetic formulations (Paufique, 2008) and food products, such as mayonnaise (Araújo *et al.*, 2014). Like CGC, mannans are also interesting materials for the pharmaceutical and medical fields, due to their reported antioxidant and antimutagenic properties (Križková *et al.*, 2001; Machová and Bystrický, 2013; Liu and Huang, 2018). Mannans were recently proposed for use in allergy vaccines and immunotherapy (Ueno *et al.*, 2013; Manzano *et al.*, 2016; Yuba *et al.*, 2017).

2.4. *Komagataella pastoris*

Komagataella pastoris, formerly known as *Pichia pastoris*, is a methylotrophic and anaerobic facultative yeast culture (Yamada *et al.*, 1995; Kurtzman, 2009; Morales *et al.*, 2014). *K. pastoris* was introduced in the 1970s by Phillips Petroleum to produce single cell proteins and, nowadays, is one of the most used cultures for the production of recombinant proteins (Cereghino and Cregg, 2000; Çalık *et al.*, 2015; Looser *et al.*, 2015; Spohner *et al.*, 2015; Schmideder *et al.*, 2016). The increasing interest on this GRAS (General Recognized as Safe)

culture to be used as an expression vector for the production of recombinant proteins is mainly due to its simplicity of molecular genetic manipulation and the capacity of achieving high biomass concentrations (over 100 g/L), using simple mineral media with low-cost carbon sources, such as glycerol, glucose and methanol (Cos *et al.*, 2006; Looser *et al.*, 2015; Spohner *et al.*, 2015; Pais-Chanfrau and Trujillo-Toledo, 2016; Schmideder *et al.*, 2016) (Table 2.3).

From the several culture media already used in *K. pastoris* fermentations, the Basal Salts Medium (BSM) proposed by Invitrogen Co. (Carlsbad, CA, USA) is the most frequently used (Table 2.3). However, the use of this medium includes some operational problems, due to its turbidity with high ionic strengths and the formation of precipitates, especially at pH values above 5 and after sterilization, generating an unbalanced nutrients composition (Zhang, Inan and Meagher, 2000; Cos et al., 2006; Ghosalkar, Sahai and Srivastava, 2008; Pais-Chanfrau and Trujillo-Toledo, 2016).

Several modes of operation have been tested for *K. pastoris* cultivation, including batch, fed-batch and continuous modes (Potvin, Ahmad and Zhang, 2012) (Table 2.3). The most common operation mode is the fed-batch strategy (Çalık *et al.*, 2015). A typical fed-batch process for *K. pastoris* recombinant proteins production includes: a batch phase using glucose or glycerol as carbon sources, to obtain high biomass concentration; a glucose/glycerol fed-batch phase with the addition of a feeding solution (usually with an exponential feeding rate), to maximize the biomass concentration; and, a fed-batch induction phase with methanol to induce proteins production (Zhang, Inan and Meagher, 2000; Cos *et al.*, 2006; Gao and Shi, 2013; Looser *et al.*, 2015).

Table 2.3 – *K. pastoris* high cell density bioproduction processes. Its products, biomass production (dry cell weight, DCW) and fermentation characteristics: carbon source, cultivation media (Basal Salts Medium, BSM; Minimal Medium, MM; Corn Steep Dextrose medium, CSD; FM22; Minimum Salt Medium MSM) and operation modes

***K. pastoris* strain**	**Product**	**Carbon source**	**Cultivation medium**	**Operation mode**	**DCW (g/L)**	**Refs.**
X-33	Lipase B	Glycerol	BSM / MM	Batch with pulses	n.a.	Robert *et al.* (2017)
X-33	Fructose-releasing exo-levanase	Glycerol and Methanol	Minimal medium	Fed-batch	115	Menéndez *et al.* (2004)
X-33	Human antigen-binding fragment	Glycerol and Glucose	Mineral medium	Fed-batch	95-102	Garcia-Ortega *et al.* (2013)
X-33	β-mananase	Glucose	CSD	Fed-batch	110	Zheng *et al.* (2012)
GS115	Chymotrypsinogen B	Glycerol and Methanol	Mineral medium	Fed-batch	170	Curvers *et al.* (2001)
GS115	Polygalacturonate lyase	Glycerol and Methanol	BSM	Fed-batch	155	Wang *et al.* (2012)
GS115	α-amylase	Glycerol and Methanol	BSM	Fed-batch	97.2	Lee *et al.* (2003)
GS115	Human leukotactin-1	Glycerol and Methanol	BSM	Fed-batch	123-142	Woo *et al.* (2005)
GS115	CD83 glycoprotein	Glycerol and Methanol	BSM	Fed-batch	186.6	Guo *et al.* (2014)
GS115	Salmosin	Glycerol and Methanol	BSM	Fed-batch	116	Seo *et al.* (2012)
GS115	Bovine chymosin B	Glycerol and Methanol	BSM	Fed-batch	160-240	Noseda *et al.* (2013)
GS115	10-deacetyltaxol	Glycerol and Methanol	FM22	Fed-batch	75-108	Liu *et al.* (2016)
SMD1168H	β-aminopeptidase 3-2W4 BapA	Glucose	Mineral medium	Fed-batch	218	Heyland *et al.* (2010)
n.a.	Phytase	Glycerol	MSM	Fed-batch	146	Tang *et al.* (2009)
KM71	Porcine interferon-α	Glycerol / Methanol	Mineral medium	Fed-batch	132-148	Ding *et al.* (2014)
X-33	Lipase A	Glycerol / Methanol	BSM	Repeated Fed-batch	120-160	Pfeffer *et al.* (2006)
GS115	rGuamerin	Glycerol / Methanol	BSM	Repeated Fed-batch	140	Lim *et al.* (2003)
X-33	Recombinant human chitinase	Glucose / Methanol	BSM	Continuous	100	Goodrick *et al.* (2001)
GS115	Polygalacturonate lyase	Glycerol / Methanol	BSM	Continuous	105	Wang *et al.* (2012)

n.a. – data not available

Recently, the use of *K. pastoris* as a source of yeast cell-wall polysaccharides, CGC and mannans, was proposed by Pharma73 S.A., in a patented bioprocess (Reis *et al.*, 2010; Freitas, Roca, *et al.*, 2013; Freitas *et al.*, 2015). Since cell-wall polysaccharides production is growth associated, *K. pastoris* became an interesting culture for this bioprocess due to its capacity to achieved high biomass concentration (above 100 g/L) with high specific cell growth rates (Roca *et al.*, 2012). A typical CGC production process is shown in Figure 2.2, based on the bioproduction process described by Roca *et al.* (2012). Briefly, this process includes a fed-batch fermentation with BSM supplemented with glycerol as the sole carbon source, to produce around 100 g/L of dry biomass concentration and 25 g/L of CGC. The biomass and CGC productivities of the process were 58.5 g/L.day and 14.6 g/L.day, respectively.

In that study, recovery of CGC involves several steps as depicted in Figure 2.2. CGC extraction from the biomass involved a hot alkaline treatment with NaOH 1 M, at 65 ºC, during 2 h. The CGC present in the alkaline insoluble material (AIM) was washed with phosphate buffer solution (PBS), ethanol and water, resulting in a rather impure polymer, with 18 wt% of inorganic salts, 9.5 wt% of protein and 28 wt% of mannose (Roca *et al.*, 2012). Although mannans could be recovered from the alkaline soluble material (ASM), this fraction was disregarded in the described process.

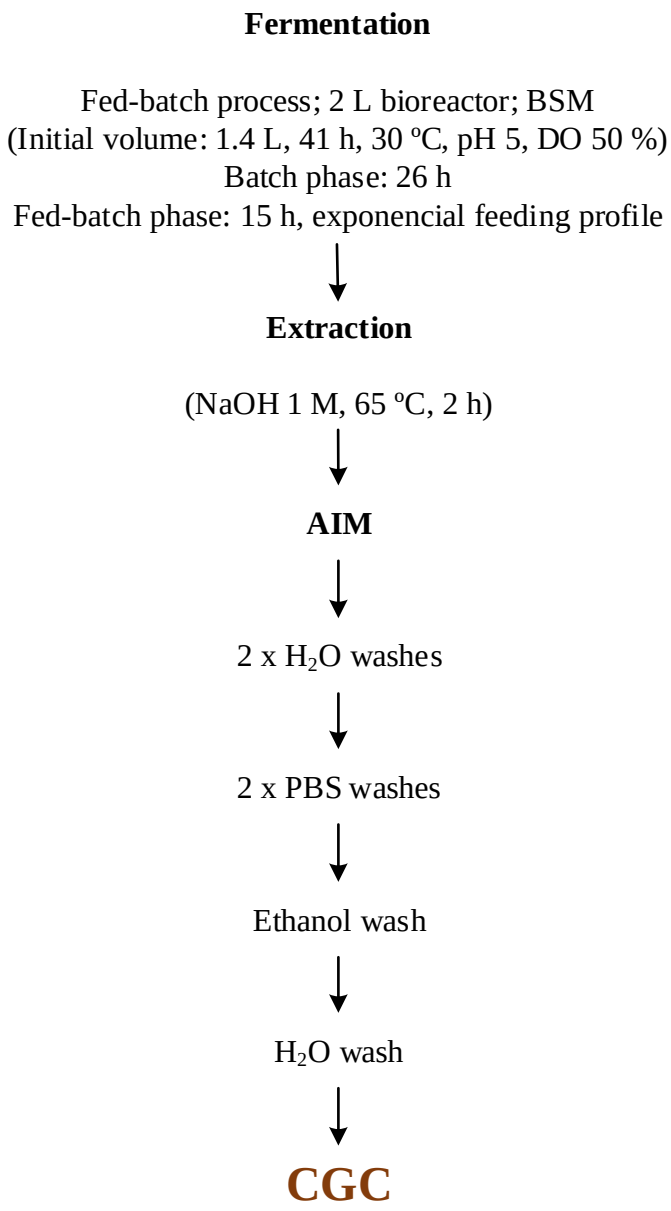

Figure 2.2 – CGC production process from *K. pastoris* biomass as described by Roca *et al.* (2012).

Chapter 3

Implementation of a repeated fed-batch process for the production of chitin-glucan complex by *Komagataella pastoris*

The results presented in this chapter were published in the peer reviewed paper:
Farinha, I., Freitas, F. and Reis, M. A. M. (2017) 'Implementation of a repeated fed-batch process for the production of chitin-glucan complex by *Komagataella pastoris*', *New Biotechnology*, 37, pp. 123–128. doi: 10.1016/j.nbt.2016.06.1460.

3.1. Abstract

The yeast *Komagataella pastoris* was cultivated under different fed-batch strategies for the production of chitin-glucan complex (CGC), a co-polymer of chitin and β-glucan. The tested fed-batch strategies included dissolved oxygen (DO)-stat mode, predefined feeding profile and repeated fed-batch operation. Although high cell dry mass and high CGC production were obtained under the tested DO-stat strategy in a 94 h cultivation (159 and 29 g/L, respectively), the overall biomass and CGC productivities were low (41 and 7.4 g/L.day, respectively). Cultivation with a predefined profile significantly improved both biomass and CGC volumetric productivities (87 and 10.8 g/L.day, respectively). Hence, this strategy was used to implement a repeated fed-batch process comprising 7 consecutive cycles. A daily production of 119-124 g/L of biomass with a CGC content of 11-16 wt% was obtained, thus proving this cultivation strategy is adequate to reach a high CGC productivity that ranged between 13 and 20 g/L.day. The process was stable and reproducible in terms of CGC productivity and polymer composition, making it a promising strategy for further process development.

3.2. Introduction

Chitin-glucan complex (CGC) is a cell-wall component of most yeasts and fungi, contributing to the stability and protection of the cell (Aguilar-Uscanga and Francois, 2003; Klis, Boorsma and De Groot, 2006). Composed of chitin and β-glucans, this nontoxic and biologically active copolymer is a valuable biomaterial for use in pharmaceutical, cosmetic and food applications (Gautier *et al.*, 2008; Freitas, Alves, *et al.*, 2013; Freitas, Roca, *et al.*, 2013; Mogoşanu and Grumezescu, 2014). CGC has also the advantage of being a non-animal chitin source (Roca *et al.*, 2012).

Komagataella pastoris, formerly known as *Pichia pastoris* (Yamada *et al.*, 1995), is a methylotrophic yeast that was recently proposed as a source of CGC in a patented bioprocess (Reis *et al.*, 2010; Freitas, Roca, *et al.*, 2013) based on the use of glycerol (Roca *et al.*, 2012; Chagas *et al.*, 2014). *K. pastoris* is widely used for the production of recombinant proteins due to its capacity in achieving high cell densities and high product productivities (Spohner *et al.*, 2015). Most of *K. pastoris* production processes are performed under fed-batch mode, using generally glucose/glycerol and methanol as carbon sources, for the growth and heterologous protein induction phases, respectively (Zhang, Inan and Meagher, 2000; Potvin, Ahmad and Zhang, 2012; Looser *et al.*, 2015). The fed-batch mode is usually preferred over batch operation, since it allows for higher cell densities, while easily ensuring the culture's stability

during the extended cultivation periods (Potvin, Ahmad and Zhang, 2012; Croughan, Konstantinov and Cooney, 2015).

Different feeding strategies have been tested for the fed-batch cultivation of *K. pastoris*, including the use of exponential feeding profiles (Oliveira *et al.*, 2005; Martens *et al.*, 2011; Roca *et al.*, 2012), the dissolved oxygen (DO)-stat mode (Lim *et al.*, 2003; Yamawaki *et al.*, 2007; Hu *et al.*, 2008; Ding *et al.*, 2014), sequential pulse feeding (Pfeffer *et al.*, 2006; Hu *et al.*, 2008), constant feeding flow (Curvers *et al.*, 2001; Bhatacharya, Pandey and Mukherjee, 2007) and repeated fed-batch operation (Lim *et al.*, 2003; Ohya, Ohyama and Kobayashi, 2005; Pfeffer *et al.*, 2006; Martens *et al.*, 2011). Most of these substrate feeding strategies were developed to maximize the production of heterologous proteins by different *P. pastoris* strains (Cos *et al.*, 2006). Since different target products were envisaged in each of those processes, it is difficult to compare the performance of the tested fed-batch strategies. Moreover, many of them were based on monitoring and controlling methanol concentration to avoid its accumulation to inhibitory values in the broth (Cos *et al.*, 2006).

Among the proposed fed-batch strategies, the repeated fed-batch presents several advantages. This type of fermentation process consists in a cyclic fed-batch operation, where, at the end of each cycle, a defined volume of the fermentation broth is purged from the reactor and replaced by fresh medium. The broth left inside the reactor serves as inoculum for the following cycle (Ohya, Ohyama and Kobayashi, 2005; Lim and Shin, 2013). Operation under a repeated fed-batch mode joins in the same fermentation process the advantages of the conventional fed-batch with those of continuous fermentation. It allows for achieving higher product productivities by keeping the culture's viability for longer operation periods, so that high cell density and high product production is maintained along this multi-cycle operation (Potvin, Ahmad and Zhang, 2012; Lim and Shin, 2013). Moreover, it avoids the need for reactor cleaning and sterilization steps between fed-batch cycles (Ohya, Ohyama and Kobayashi, 2005). This cultivation strategy was successfully used for the production of Lipase A (Pfeffer *et al.*, 2006) and rGuamerin (Lim *et al.*, 2003) by *P. pastoris* strains X33 and GS115, respectively, wherein high cell densities were achieved (120-160 g/L) during 10-11 consecutive fed-batch cycles.

In previous work, *K. pastoris* was cultivated under a fed-batch mode with and exponential feeding profile for the production of CGC (Roca *et al.*, 2012). In this work, alternative fed-batch strategies were tested for the first time aiming to improve the process in terms of CGC production and productivity. The tested cultivation strategies included the DO-stat mode, feeding with a predefined profile and the repeated fed-batch operation. The produced co-polymers were extracted and characterized to evaluate the impact of each cultivation strategy on CGC content in the biomass and its composition.

3.3. Materials and methods

3.3.1. Yeast strain and culture medium

In all experiments, *Komagataella pastoris* strain DSM 70877 was cultivated in standard Basal Salts Medium (BSM) (Pichia Fermentation Process Guidelines, Invitrogen) containing per liter of solution: H_3PO_4 (85%), 26.7 mL; $CaSO_4.2H_2O$, 1.176 g; K_2SO_4, 18.2 g; $MgSO_4.7H_2O$, 14.9 g; KOH, 4.13 g; glycerol (86-88 wt%, Scharlau), 50 g and 4.35 mL of *Pichia* trace mineral (PTM) solution. PTM solution had the following composition (per liter): $CuSO_4.5H_2O$, 6 g; NaI, 0.08 g; $MnSO_4.H_2O$, 3 g; $Na_2MoO_4.2H_2O$, 0.2 g; H_3BO_3, 0.02 g; $CoCl_2.6H_2O$, 0.5 g; $ZnCl_2$, 20 g; $FeSO_4.7H_2O$, 65 g; Biotin, 0.2 g and H_2SO_4, 5 mL. The PTM solution was filter sterilized separately and added to the BSM after its sterilization at 120 ºC, during 20 min. Ammonium hydroxide (25%, v/v) was used to set the medium's pH to 5.0, serving also as the nitrogen source.

3.3.2. Fed-batch bioreactor cultivation

For the bioreactor experiments, inocula were prepared by inoculating 2 × 1 mL of the cryopreserved culture in 2 × 150 mL BSM supplemented with 50 g/L glycerol (in 500 mL baffled shake-flasks) and incubating during 40 h, at 30 ºC and 200 rpm, to ensure that the culture is at exponential growth phase at the moment of the broth transfer to the bioreactor.

The broth thus obtained was used to inoculate a 5 L bioreactor (BioStat B-plus, Sartorius) with an initial working volume of 3 L. The 5 L bioreactor used in these experiments had an internal height:internal diameter ratio of 2.2 (160:345 mm), two Rushton impellers with 6 blades in each one (blade dimension: 15 × 13 mm), 4 buffles with 204 mm of height and a ring sparger with 14 holes and 58.5 mm diameter.

All experiments were performed with controlled temperature at 30 ºC. The pH was controlled at 5.0 by the automatic addition of a 25% (v/v) ammonium hydroxide solution. The air flow rate was kept constant at 3 SLPM (standard liters per minute). The dissolved oxygen (DO) level was controlled at 50% of the air saturation by an automatic cascade comprising the variation of the stirring rate (300-2000 rpm) and supplementation of the air stream with pure oxygen that was triggered when the maximum stirring rate was not enough to maintain the DO level at the set point.

All experiments comprised an initial 24 h batch phase, followed by the fed-batch phase wherein the feeding solution was supplied to the culture. The feeding solution was composed of

glycerol (86-88 wt%) supplemented with PTM solution (24 mL per liter of glycerol). Online data acquisition was performed using BioCTR software developed by Eusébio (2006). Samples (≈ 25 mL) were periodically withdrawn from the bioreactor for determination of the dry cell weight (DCW), glycerol and ammonia concentrations, CGC content in the biomass and polymer composition.

In experiment A (DO-stat mode), the feeding flow rate was controlled as a function of DO level (under a constant stirring of 1300 rpm), i.e., when the DO level rose above 50%, the substrate was automatically fed to the bioreactor until the DO reached the set point again. In experiment B (predefined feeding profile), the feeding profile was set at the Biostat B-plus control unit, which automatically fed the reactor with substrate at a rate that gradually increased from 9.3 to 24 g/L.h (considering the reactor's starting volume) over the 22.5 h of the fed-batch phase, giving an overall glycerol feeding of 1178 g.

3.3.3. Implementation of CGC production under repeated fed-batch mode

The repeated fed-batch cultivation of *K. pastoris* for CGC production comprised 7 cycles. The experiment was initiated by inoculating the bioreactor as described above for experiments A and B. The first cycle comprised a 24 h batch phase and a 23 h fed-batch phase wherein the feeding rate increased from 8 to 12 g/L.h to give an overall glycerol feeding of 722 g. The subsequent 6 cycles took 23 h, with a 6 h batch phase and a 17 h fed-batch phase, with the feeding rate increasing from 14 to 16 g/L.h (overall glycerol feed of 764-799 g). At the end of each cycle, 90% of the culture broth (≈ 3.5 L) were withdrawn from the reactor under aseptic conditions. The remaining broth (≈ 380 mL) was kept in the reactor, serving as inoculum for the following cycle. The new cycle was initiated by filling the bioreactor with 2.6 L of fresh BSM medium.

In all cycles, the reactor was operated as described above for experiments A and B in terms of temperature (30 ºC), pH (5.0) and DO level (50%) control. Online data was acquired by the BioCTR software (Eusébio, 2006). Samples (≈ 25 mL) were periodically withdrawn from the bioreactor for determination of the DCW, glycerol and ammonia concentrations, CGC content in the biomass and polymer composition.

3.3.4. CGC Extraction

For extraction of CGC from *K. pastoris*, dried biomass samples (≈ 100 mg) were treated with NaOH 5 M (30 mL), at 65 ºC, for 2 h. After cooling, the suspension was centrifuged (8,000 × *g*, 10 min) and the alkaline insoluble material (AIM) was resuspended in deionized water (30 mL), neutralized with HCl 1 M and centrifuged as described above. The AIM was further

washed twice with deionized water (30 mL, for each wash) and, finally, the CGC samples were freeze dried. All extractions were performed in duplicate.

3.3.5. Analytical Techniques

For the determination of the DCW, 5 mL culture broth samples were centrifuged at 8,000 × *g* for 10 min. The cell-free supernatant was used for glycerol and ammonia quantification, while the pellet was used for the gravimetric quantification of biomass concentration. The pellet was washed twice (resuspension in 5 mL deionized water and centrifuged at 8,000 × *g* for 10 min) and freeze-dried. Three replicas were used for the quantification of DCW.

For glycerol quantification, the cell-free supernatant was analyzed by High Performance Liquid Chromatography (HPLC), in a Dionex ICS 3000 equipment with a Shodex RI-101 detector. The analysis was performed using an Aminex 87H Biorad column, at 30 ºC with H_2SO_4 10 mN as eluent, at a flow rate of 0.6 mL/min. Glycerol (Scharlau) was used as standard, at concentrations of 0.005-1.0 g/L. The samples were diluted in order to have their concentration in glycerol below 1.0 g/L. Ammonia was quantified in the cell-free supernatant by segmented flow analysis (Skalar san++, Skalar Analytical, The Netherlands). An ammonia chloride solution was used as standard, at a concentration range of 4.0-20.0 ppm. The samples were diluted to have their concentration below 20 ppm.

For the compositional analysis, dried CGC samples were subjected to two acid hydrolysis. The first one relies upon trifluoroacetic acid (TFA) treatment to hydrolyse the glucan fraction of the polymer. A stronger acid (HCl) was imposed for the quantification of the chitin fraction. In practice, TFA hydrolysis was conducted resuspending dried CGC samples (≈ 5 mg) in 5 mL deionised water followed by addition of 0.1 mL TFA 99%. The hydrolysis was performed at 120 ºC, for 2 h. For HCl hydrolysis, the samples (≈ 5 mg) were resuspended in 5 mL HCl 4 N, and the hydrolysis was carried out at 120 ºC, for 5 h. Both hydrolysates were used for quantification of glucose and glucosamine by HPLC. The hydrolysates were analyzed using a Dionex ICS 3000 equipment with an amperometric detector. These analyses were performed using a CarboPac PA10 column (Dionex), at 30 ºC with NaOH 4 mN as eluent, at a flow rate of 0.9 mL/min. Glucose and glucosamine (Sigma) were used as standards, at concentrations between 0.006 and 0.1 g/L, being subjected to the same hydrolysis procedures as the samples.

3.4. Results and discussion

3.4.1. Fed-batch cultivation under DO-stat mode

The results obtained in experiment A, wherein the bioreactor was operated under a DO-stat mode, are presented in Figure 3.1 and Table 3.1. During the batch phase, the culture grew at a maximum specific cell growth rate of 0.15 h^{-1}, reaching a DCW of 20 g/L within 24 h of cultivation (Figure 3.1A). Concomitant with cell growth, the DO level decreased during the first hours of cultivation, being controlled at 50% of the air saturation by automatically increasing the stirring rate (Figure 3.1B). The fed-batch phase was initiated when a sudden increase of the DO level above 50%, accompanied by a reduction of the stirring rate, was noticed (Figure 3.1B), which was indicative of carbon source exhaustion (Figure 3.1A). This sharp increase of the DO level is commonly used as an online signal for initiating the fed-batch phase of *Pichia pastoris* processes (Cos *et al.*, 2006). From that point on, the stirring rate was kept constant and the DO level was controlled at 50% by automatically supplying the feeding solution (glycerol supplemented with trace elements) to the bioreactor. This strategy allowed the culture to continue growing. During the first 8 h of fed-batch phase, a feeding flow rate of 8.3 g/L.h provided enough glycerol to maintain a high specific cell growth rate (0.14 h^{-1}). However, afterwards, the automatic feed flow rate gradually decreased to 4.5 g/L.h and the culture's cell growth was significantly decreased (Figure 3.1A). This decrease was not due to any nitrogen source limitation since ammonium concentration was high throughout the entire cultivation run, varying from an initial value of 6.3 g/L to 5.0 g/L at the end of the run.

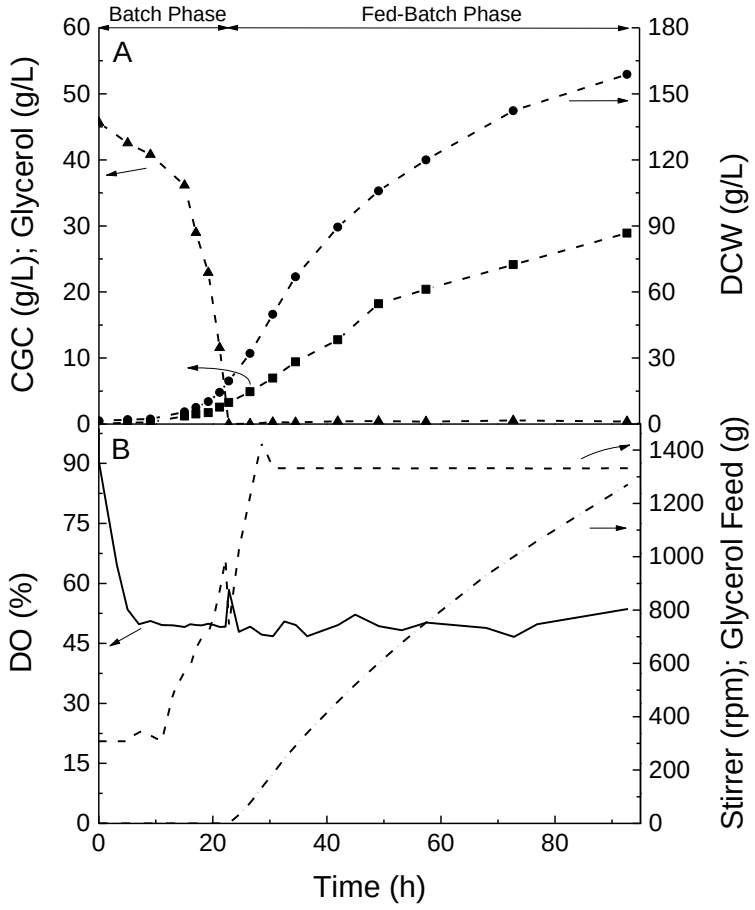

Figure 3.1 – Cultivation profiles obtained in Experiment A for the cultivation of *Komagataella pastoris* DSM 70877 under a DO-stat mode: (A) offline data (▲, glycerol concentration; ■, CGC; ●, DCW); (B) online data (––, DO concentration; – –, Stirring speed; – · –, Glycerol Feed). The arrows are pointing to the respective axis of each parameter.

Table 3.1 – Results obtained for the cultivation of *K. pastoris* DSM 70877 in glycerol under different operation modes (dry cell weight, DCW; overall biomass, r_X, and CGC, r_C, volumetric productivities; yield of biomass, $Y_{X/S}$, and CGC, $Y_{C/S}$, on a substrate basis).

Experiment	Cultivation mode	DCW (g/L)	r_X (g/L.day)	$Y_{X/S}$ (g_x/g_s)	CGC content (wt%)	CGC (g/L)	r_C (g/L.day)	$Y_{C/S}$ (g_c/g_s)
A	DO-stat mode	159	41	0.41	18	29	7.4	0.08
B	Predefined profile feeding	170	87	0.46	12	21	10.8	0.06
C	Repeated fed-batch	119–124	99–111*	0.39–0.44	11-16	13-20	13–20*	0.05–0.08

* considering only cycles 2-7

A final DCW of 159 g/L was attained at the end of the run (94 h), corresponding to an overall biomass volumetric productivity of 41 g/L.day (Table 3.1). Although the biomass concentration obtained in this study was considerably higher than that reported by Roca *et al.* (2012) for cultivation of *K. pastoris* DSM 70877 in glycerol (104 g/L), the biomass volumetric productivity was not improved. Lower biomass concentration (132-151 g/L) but higher volumetric productivity values (59-118 g/L.day) were also reported for the cultivation of other strains with glycerol using the DO-stat strategy. Hu *et al.* (2008) reported a DCW of 147 g/L and a biomass productivity of 59 g/L.day for the cultivation of *P. pastoris* GS115 for the biosynthesis of S-adenosyl-L-methionine. Another example is the production of phytase by a *P. pastoris* strain, in which 146 g/L of biomass were produced with an overall biomass productivity of 66 g/L.day (Tang *et al.*, 2009). Ding *et al.* (2014) optimized a DO-stat strategy for cultivation of *P. pastoris* KM71 for production of porcine interferon-α that reached biomass concentration values between 132 and 151 g/L within 27-32 h of cultivation.

All the glycerol fed to the bioreactor was consumed by the culture, giving a total substrate consumption of 1429 g (Figure 3.1B). A biomass yield of 0.41 g_x/g_s was obtained in Experiment A (Table 3.1). Although this yield is lower than that reported by Roca *et al.* (2012) for cultivation of *K. pastoris* DSM 70877 in glycerol (0.55 g_x/g_s), it is within the range reported for *P. pastoris* GS115, 0.34-0.61 g_x/g_s (Ren, Yuan and Bellgardt, 2003; Bhatacharya, Pandey and Mukherjee, 2007; Jungo, Marison and von Stockar, 2007).

The specific glycerol consumption rate was not constant throughout the run, reaching values as high as 0.40 $g_S/g_X.h$ until around 36 h of cultivation, but gradually decreasing thereafter (< 0.1 $g_S/g_X.h$) (Figure 3.2). This result suggests that the amount of substrate that was automatically fed to the culture as a function of DO level might have been limiting or some of the medium components was depleted, thus restricting cell growth. On the other hand, the observed reduction of the specific glycerol consumption rate and, also, the specific growth rate, may be due to cell aging and senescence. In the tested fed-batch DO-stat strategy, all the cells were kept in the bioreactor for a rather extended period of time, during which senescent cells have accumulated. Yeast cells are only able to complete a certain number of cell cycles before entering the resting state (Austriaco, 1996). Moreover, the cell cycle length increases as the cells get older and the aged cells of many yeasts tend to accumulate intracellular lipid granules (Austriaco, 1996). Therefore, under certain cultivation conditions, yeast cells may show signs of aging and senescence that include a continuous decrease of the metabolism and cell cycle, which will have a significantly influence on the process productivity and yield (Curvers *et al.*, 2001).

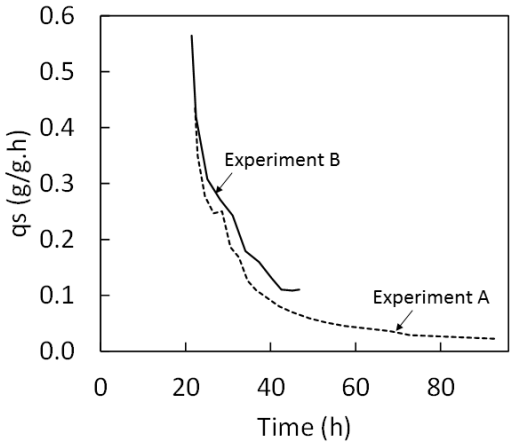

Figure 3.2 – Specific glycerol consumption rate in Experiments A (DO-stat mode) and B (cultivation with a predefined profile) during the fed-batch phase of the cultivation runs.

At the end of Experiment A, 29 g/L CGC were produced, corresponding to an overall volumetric productivity of 7.4 g/L.day (Table 3.1). The CGC content in the biomass achieved in Experiment A (18 wt%) was higher than that reported previously for *K. pastoris* DSM 70877 cells (11-14%) (Chagas *et al.*, 2014). This result may be related to the carbon limiting conditions the culture was subjected to during operation under the DO-stat strategy tested. Moreover, the CGC obtained in Experiment A had a chitin:glucan molar ratio of 22:78, which is higher than that reported in previous studies (4:96-16:84) for CGC extracted from *K. pastoris* DSM 70877 biomass (Roca *et al.*, 2012; Chagas *et al.*, 2014). It is known that imposing stress conditions to yeasts and fungi can trigger metabolic changes that involve the cell-wall structure, especially the polysaccharides synthesis pathways (Aguilar-Uscanga and Francois, 2003; Klis, Boorsma and De Groot, 2006; Hall, 2015). In particular, nutrient starvation has been reported to lead to increased chitin and CGC content (Feofilova, 2010). On the other hand, it may also be related to the accumulation of aged cell in the bioreactor, as discussed above. In fact, yeast cell that have been through several budding divisions will present more budding scars, whose main component is chitin (Holan *et al.*, 1981; Sinclair, Mills and Guarente, 1998). Hence, the higher chitin content may be indicative of cell senescence.

The results obtained with Experiment A have shown that the DO-stat cultivation strategy tested was not the most appropriate for CGC production since although high DCW and CGC production were reached, the overall productivities were not improved, probably due to the generation of a senescent culture and/or to the substrate limiting conditions it was subjected to during the run.

3.4.2. Fed-batch cultivation with predefined profile

Based on the results of Experiment A, a predefined feeding profile was set for the fed-batch phase of Experiment B aiming to guaranty enough glycerol was made available to the culture and substrate limitation was avoided, as well as to improve the biomass and product volumetric productivities by shortening cultivation time. Similarly to Experiment A, in Experiment B the culture grew with a maximum specific cell growth rate of 0.15 h^{-1} and a DCW of 20 g/L was reached within 24 h of cultivation (Figure 3.3A).

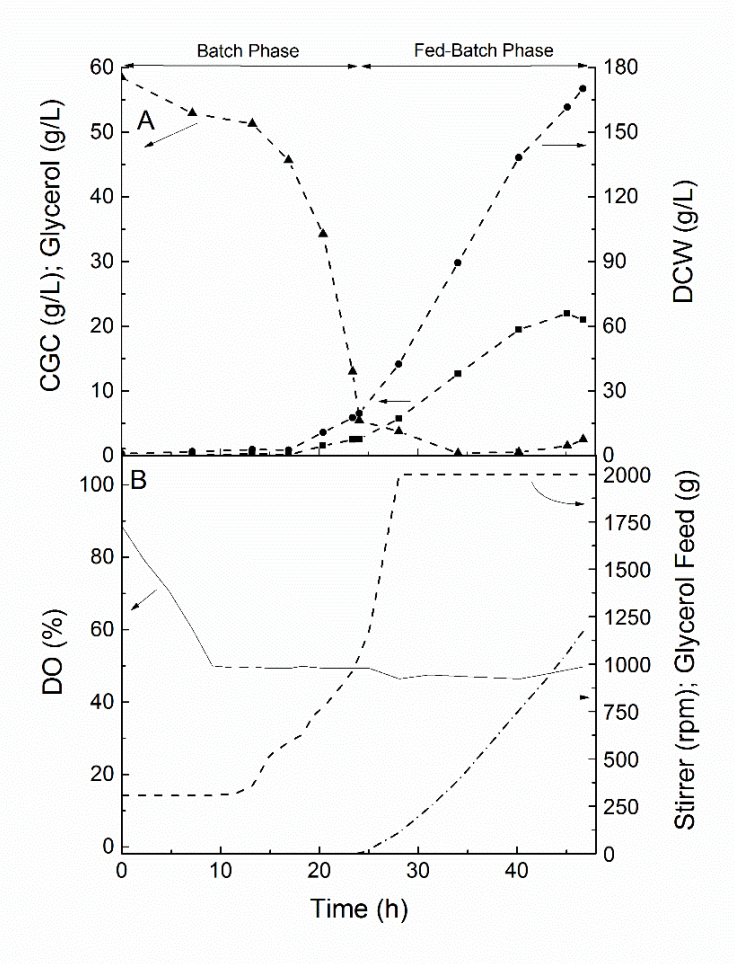

Figure 3.3 – Cultivation profiles obtained in Experiment B for the cultivation of *Komagataella pastoris* DSM 70877 with a predefined feeding profile: (A) offline data (▲, glycerol concentration; ■, CGC; ●, DCW); (B) online data (––, DO concentration; – –, Stirring speed; – · –, Glycerol Feed). The arrows are pointing to the respective axis of each parameter.

During the initial 8 h of the fed-batch phase of Experiment A, the feed flow rate of 8.3 g/L.h guaranteed a high cell growth rate. Hence, in Experiment B, the fed-batch phase was initiated at 24 h with a flow rate of 9.3 g/L.h to guaranty enough glycerol was made available to

the culture and substrate limitation was avoided. Afterwards, the feed flow rate was gradually increased to 22.5 g/L.h over the following 23 h, to match the culture's needs as it grew to higher cell density. This predefined profile apparently provided an adequate glycerol feeding, since the culture maintained a specific cell growth rate of 0.13 h^{-1} for most of the fed-batch phase. Similar to what was observed in Experiment A, in Experiment B, the ammonium concentration ranged between 5.8 and 7.1 g/L, confirming that there was not no limitation of the nitrogen source in the reactor during the entire process.

At around 37 h of cultivation (Figure 3.3A), there was a reduction of the culture's specific growth rate, which was related to the increasing temperature in the bioreactor. In fact, as the cell density became higher, it was not possible to control the temperature at the intended setpoint (30 ºC) since a glass bioreactor was used in this study. Hence, during the fed-batch phase, it was difficult to control the temperature and, during the last hours of the experiment, it rose above 35 ºC, which probably impaired cell growth. The cell growth of several *K. pastoris* strains was reported to be optimal for temperatures between 28 and 34 ºC (Cos *et al.*, 2006; Gasser *et al.*, 2007; Eda Çelik *et al.*, 2008; Chagas *et al.*, 2014). Chagas *et al.* (2014) reported decreased cell growth rates for *K. pastoris* DSM 70877 cultivation at temperatures above 34 ºC. To overcome such temperature control problems, a compromise between the specific growth rate and the glycerol feed rate must be made, so the cultivation of *K. pastoris* is possible within the technical limitations of the available equipment (Looser *et al.*, 2015).

Despite the temperature control problems experienced during the run, a final DCW of 170 g/L was achieved (Table 3.1). This value is similar to those reported by Curvers *et al.* (2001) and Jungo *et al.* (2007), 150-170 g/L, in mixed substrates (glycerol and methanol) cultivations of *P. pastoris* GS115. The overall biomass volumetric productivity obtained in Experiment B, 87 g/L.day, was considerably improved when compared with Experiment A, 41 g/L.day (Table 3.1), and also higher than the values reported by Curvers *et al.* (2001) and Jungo *et al.* (2007), 44-66 g/L.day.

A total of 1355 g of glycerol were consumed during the 47 h cultivation run (Figure 3.3B), giving a biomass yield of 0.46 g_x/g_s (Table 3.1). This value is higher than that obtained in Experiment A (0.41 g_x/g_s), thus showing the culture used the substrate more efficiently for cell growth. Similarly to Experiment A, the specific substrate consumption rate was above 0.40 $g_S/g_X.h$ up to around 30 h of cultivation but it decreased thereafter to below 0.20 $g_S/g_X.h$ (Figure 3.2), which was probably due the higher temperature the culture was exposed to.

The biomass recovered at the end of Experiment B had a CGC content of 12 wt% (Table 3.1). Despite the lower CGC production observed in Experiment B, 21 g/L, an overall volumetric productivity of 10.8 g/L.day was attained (Table 3.1). This value is considerably higher than that obtained in Experiment A, 7.4 g/L.day (Table 3.1). In experiment B, the CGC had a chitin:glucan molar ratio of 21:79, which is similar to the co-polymer obtained in

experiment A, 22:78, and also higher than the values reported in previous studies, ≤ 16:84 (Roca *et al.*, 2012; Chagas *et al.*, 2014). This higher chitin:glucan molar ratio might have been due to the higher temperature the culture was exposed during the last hours of the run. Indeed, exposing yeast cells to high temperature has been reported to lead to increased chitin and CGC content (Feofilova, 2010).

3.4.3. Repeated fed-batch cultivation

The cultivation strategy tested in Experiment B proved to be successful since the process was significantly improved in terms of both biomass and CGC volumetric productivity. Hence, in Experiment C (Figure 3.4), a repeated fed-batch process was implemented for the production of CGC, using a feeding profile based on that tested in Experiment B. However, considering *K. pastoris* cell growth to reach high densities was compromised by the inability to control the temperature in the glass bioreactor, in Experiment C, the feeding flow rate was reduced to avoid such problems and make it possible to continue the study in the available equipment. The temperature control problems encountered in this study are not likely to occur at larger production scales, wherein steel fermenters equipped with efficient temperature control systems are used.

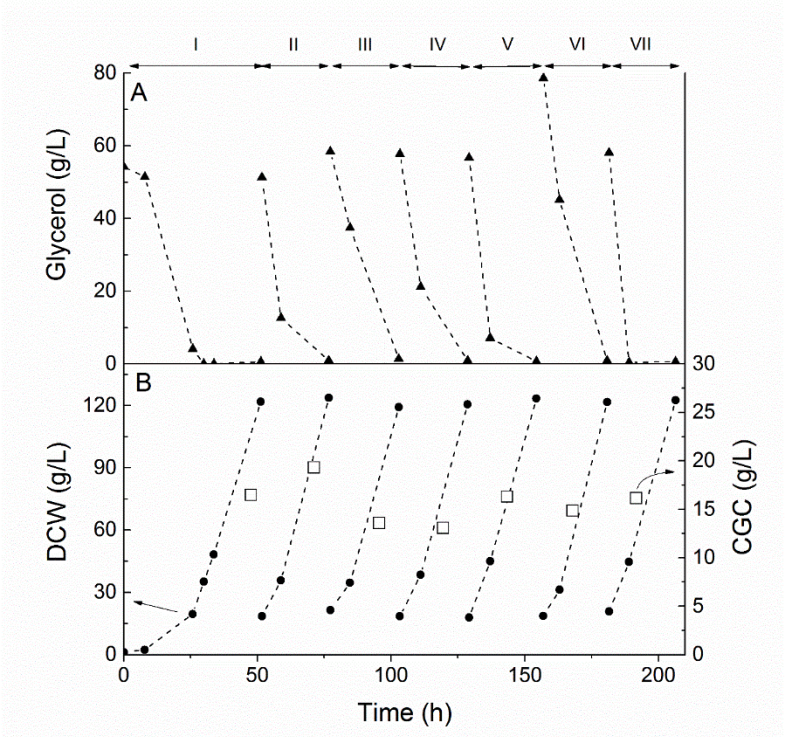

Figure 3.4 – Cultivation profiles obtained in Experiment C for the repeated fed-batch cultivation of *Komagataella pastoris* DSM 70877: (A) ▲, glycerol concentration; (B) ●, DCW; □, CGC. The arrows are pointing to the respective axis of each parameter.

The first cycle of Experiment C was similar to Experiment B: it comprised a 24 h batch phase, during which glycerol was consumed (Figure 3.4A) and the culture grew (Figure 3.4B), followed by a 23 h fed-batch phase with a predefined feeding profile. The fed-batch phase was initiated with a feed flow rate of 8.0 g/L.h that was subsequently increased up to 12.0 g/L.h. The following 6 cycles comprised a batch phase of 6 h, followed by a 17 h feeding phase, wherein the feeding profile started with a flow rate of 13.7 g/L.h that was increased to 15.7 g/L.h. In each cycle, on average, 774 g of glycerol were fed to the bioreactor. No nitrogen limitation occurred during the repeated cycles operation since the ammonium concentration ranged between 6.5 and 8.2 g/L during the entire experiment.

Although a lower DCW was obtained at the end of each cycle, 119-124 g/L, the biomass volumetric productivity in cycles 2-7 was significantly improved, 99-111 g/L.day, compared to Experiment B, 87 g/L.day (Table 3.1). This high biomass volumetric productivity was close to the range of the values reported for *P. pastoris* strains X33 (Pfeffer *et al.*, 2006) and GS115 (Lim *et al.*, 2003) grown on glycerol and methanol, 120-180 g/L.day, in repeated fed-batch processes. The biomass yield on glycerol averaged between 0.39 and 0.43 g_x/g_s (Table 3.1), which is comparable to the values reported for *P. pastoris* strains GS115 and X33, 0.34-0.62 g_x/g_s (Ren, Yuan and Bellgardt, 2003; Cos *et al.*, 2005; Bhatacharya, Pandey and Mukherjee, 2007; Jungo, Marison and von Stockar, 2007).

A daily CGC production of 13-20 g/L was attained in cycles 2-7 (Table 3.1). Despite the lower CGC production obtained in each repeated cycle, the volumetric productivity was significantly higher, 13-20 g/L.day, than in experiment B, due to the shorter production time (23 h instead of 47 h). The CGC content in biomass ranged between 11 and 16 wt% during the repeated cycles of Experiment C, which was similar to the value obtained in Experiment B and to the range reported in previous studies for *K. pastoris* DSM 70877 cells (11-19%) (Chagas *et al.*, 2014; Farinha *et al.*, 2015). Moreover, the co-polymers extracted from the biomass at the end of each repeated cycle had a chitin:glucan molar ratio between 11:89 and 19:81 (Figure 3.5). These values are comparable with the values reported in previous *K. pastoris* studies, ≤ 16:84 (Roca *et al.*, 2012; Chagas *et al.*, 2014).

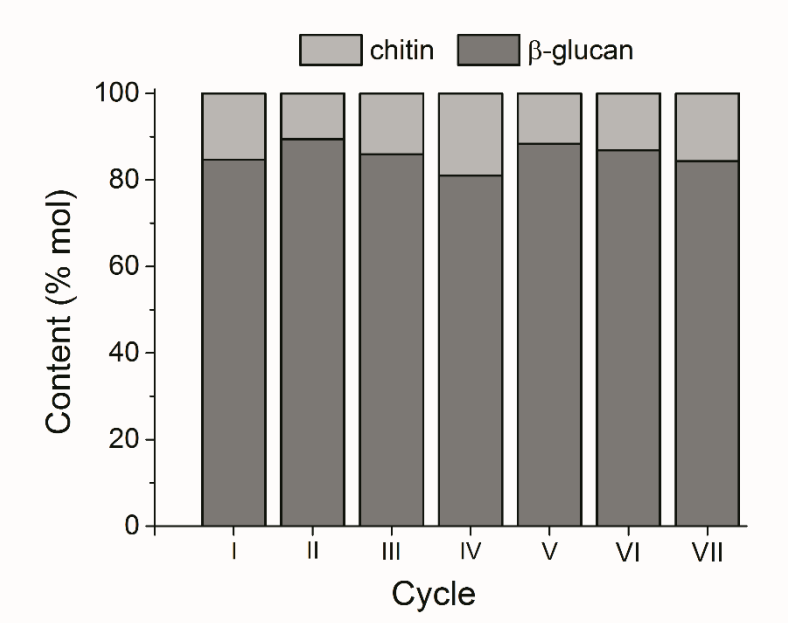

Figure 3.5 – Chitin:glucan molar ratio of the CGC produced in each cycle of the repeated fed-batch Experiment C.

The results obtained in Experiment C show that cultivation of *K. pastoris* DSM 70877 under the repeated fed-batch mode was successful in improving the overall biomass and CGC volumetric productivity beyond any previous study. The cycles were stable and reproducible and can easily be implemented at larger scale. Moreover, there was no significant impact on the biomass content in CGC neither on the co-polymer's composition that was maintained relatively constant throughout the repeated cycles.

3.5. Conclusions

A repeated fed-batch strategy was implemented for the first time for the production of the polysaccharide CGC. *K. pastoris* DSM 70877 was cultivated with daily repeated cycles, resulting in a high production of biomass and, hence, CGC. The cycles were reproducible and stable, in terms of CGC production and polymer composition, thus demonstrating the suitability of this cultivation strategy for the production of CGC. This repeated fed-batch strategy enabled the improvement of both biomass and CGC productivities compared to the other tested fed-batch feeding strategies, namely, DO-stat, exponential feeding rate and constant feeding rate.

Thus, the repeated fed-batch strategy is a promising process for CGC production by *K. pastoris*.

Chapter 4

Influence of dissolved oxygen concentration on cell-wall polysaccharides production by *Komagataella pastoris*

4.1. Abstract

The yeast *Komagataella pastoris* was cultivated under different dissolved oxygen (DO) levels (5, 10, 15, 30 and 50%) to evaluate the impact of this operational parameter on cell-wall polysaccharides, chitin-glucan complex (CGC) and mannans, production. Decreasing the DO levels from 50% to 15% resulted in high biomass concentration, 95.1 g/L, in a 47 h fed-batch bioreactor cultivation with glycerol as sole carbon source. Substrate conversion into biomass was more efficient at 15% than at higher DO levels and the CGC content in biomass was improved to 18 wt%, compared to 15 wt% obtained at 50% DO. Mannans were recovered from *K. pastoris* biomass for the first time and it was obtained 22 wt% of mannans content in biomass. This corresponds to an overall CGC volumetric productivity of 8.67 g/L.day, representing an improvement of around 13 %, and a mannans volumetric productivity of 10.69 g/L.day. On the other hand, the polymers composition was not significantly affected by decreasing the DO level. These results demonstrated that considerable energy savings can be made in the polysaccharides production process by reducing the DO level during cultivation of *K. pastoris*, without compromising the overall polymers productivity. This has also impact in the polysaccharides production costs, which is of considerable relevance for process scale-up and products' commercialization.

4.2. Introduction

Chitin-glucan complex (CGC) is a copolymer composed of chitin and β-glucans. The combination of the two bioactive polysaccharides in the same macromolecule makes CGC a promising biomaterial for several medical applications, due to its immunomodulator, antitumor, antioxidant and antimicrobial properties (Kim *et al.*, 2006; Dubey *et al.*, 2014; Giavasis, 2014; Freitas, Roca and Reis, 2015).

Mannans are polysaccharides mainly composed of mannose units. Similar with CGC, mannans is also an interesting material in the medical field, due to its immunological, antimutagenic and antioxidant properties (Križková *et al.*, 2001; Pinto *et al.*, 2015). Moreover, mannans can also be used in food formulations due to their emulsifying and prebiotics effects (Araújo *et al.*, 2014; Domizio *et al.*, 2014; Pinto *et al.*, 2015).

CGC and mannans can be extracted from the cell-wall of several yeasts and fungi, representing 12-36% and 5-29% of their cell-wall content, respectively (Nguyen, Fleet and Rogers, 1998; Feofilova *et al.*, 2006; Zlotnikov *et al.*, 2007; Smirnou, Krcmar and Prochazkova,

2011; Bzducha-Wróbel, Kieliszek and Błażejak, 2013; Liu *et al.*, 2015; Galinari *et al.*, 2017). The yeast cell-wall is a dynamic structure mainly composed of polysaccharides (chitin, glucans and mannans) and proteins that confer rigidity and stability to the cells in response of several environmental stress factors (Lipke and Ovalle, 1998; Klis *et al.*, 2002; Aguilar-Uscanga and Francois, 2003). The yeasts cell-wall composition can be affected by several factors, including the culture medium, temperature, external pH, oxygen levels and hypo-osmotic stress (Smits, van den Ende and Klis, 2001; Aguilar-Uscanga and Francois, 2003; Klis, Boorsma and De Groot, 2006). To avoid the loss of cell viability in some situations, yeast cells activate the cell-wall integrity (CWI) pathway that results, for example, in increased production of some cell-wall components, such as chitin and mannans, and/or in the reorganization of certain covalent linkages between the cell-wall components (Smits, van den Ende and Klis, 2001; Klis, Boorsma and De Groot, 2006; Arroyo *et al.*, 2016).

Komagataella pastoris (formerly known as *Pichia pastoris*) is a methylotrophic yeast usually used to produce recombinant proteins (Çalık *et al.*, 2015; Spohner *et al.*, 2015). Due to its capacity to achieve high cell densities and high cell growth rates, *K. pastoris* is also an interesting source of CGC and mannans (Reis *et al.*, 2010; Roca *et al.*, 2012; Freitas, Roca, *et al.*, 2013; Freitas *et al.*, 2015). Moreover, *K. pastoris* has the ability to grow over a wide range of pH values (3.0-7.0), temperatures (20-30 ºC) and dissolved oxygen (DO) levels (10-50%) (Cos *et al.*, 2006; Tang *et al.*, 2009; Gao and Shi, 2013; Chagas *et al.*, 2014; Çalık *et al.*, 2015; Gmeiner *et al.*, 2015). Despite the high biomass concentration (above 100 g/L) and high production of proteins achieved in most fermentation processes, little is known about the impact of the fermentation conditions on the yeast cell-wall composition and, specifically, on CGC and mannans contents. According to Chagas *et al.* (2014), the chitin content in CGC is reduced to below 14 %mol by cultivating *K. pastoris* outside pH and temperature ranges of 4.5-5.8 and 26-33 ºC, respectively. Gmeiner *et al.* (2015) also noticed slight changes on recombinant *P. pastoris* CBS7435 cells morphology by changing the pH, temperature and DO level. Nevertheless, the impact of DO level on yeast cell-wall composition remains unrevealed, especially for *K. pastoris* strains.

In this work, the influence of the DO level on CGC and mannans content in *K. pastoris* cells and the polymers' composition were evaluated. Batch bioreactor cultivations under different DO levels were conducted. Afterward, a fed-batch experiment was performed under the DO level that led to the highest biomass and polymers production to validate such conditions in a high cell density cultivation.

4.3. Materials and methods

4.3.1. Yeast strain and culture medium

Komagataella pastoris strain DSM 70877 was used in all the experiments. *K. pastoris* was cultivated in standard Basal Salts Medium (BSM) (Pichia Fermentation Process Guidelines, Invitrogen), as described in 3.3.1 section of Chapter 3.

4.3.2. Batch bioreactor cultivations

The inoculum for each bioreactor cultivations was prepared by inoculating 1 mL of the cryopreserved (at -80 ºC) culture in 140 mL BSM (in 500 mL baffled shake-flasks) and incubation during 40 h, at 30 ºC and 200 rpm. The inoculum thus obtained was used to inoculate a 2 L bioreactor (BioStat B-Plus, Sartorius), with an initial working volume of 1.4 L. The 2 L bioreactor used in these experiments had an internal height:internal diameter ratio of 1.8 (130:240 mm), two Rushton impellers with 6 blades in each one (blade dimension: 13.5 × 10.6 mm), 4 buffles with 130 mm of height and a ring sparger with 14 holes and 49 mm diameter. All the fermentations were performed with controlled temperature (30 ± 0.1 ºC) and pH (5.0 ± 0.02). The pH was automatically controlled by the addition of 25 % (v/v) ammonia hydroxide solution that also served as the nitrogen source, and a HCl 2 M solution. The air flow rate was kept constant at 1.4 SLPM (standards liters per minute). The DO level was controlled by the automatic variation of stirrer rate (300-2000 rpm). Batch experiments were performed with DO levels of 5, 10, 15, 30 and 50% of the air saturation, during 42 h. Samples (12 mL) were periodically withdrawn from the bioreactor, for the determination of dry cell weight (DCW), CGC and mannans content and glycerol and ammonia concentrations.

4.3.3. Fed-batch bioreactor cultivation

For the fed-batch bioreactor experiment, an inoculum was prepared by inoculating 4 × 1 mL of the cryopreserved (at -80 ºC) culture in 4 × 200 mL of BSM (in 500 mL baffled shake-flasks) and incubation during 40 h, at 30 ºC and 200 rpm. This inoculum was used to inoculate a 10 L bioreactor (BioStat B-Plus, Sartorius), with an initial working volume of 8 L. The 10 L bioreactor used in this experiment had an internal height:internal diameter ratio of 2.5 (190:470 mm), two Rushton impellers with 6 blades in each one (blade dimension: 19 × 15 mm), 1 buffle with 280 mm of height and a ring sparger with 16 holes and 88 mm diameter. This fed-batch fermentation occurred under the same operational conditions as the previous batch

fermentations, except for the DO level that was controlled at 15% of the air saturation, by an automatic cascade comprising the variation of the stirring rate (300-1000 rpm) and supplementation of the air stream with oxygen that was triggered when the maximum stirring rate was not enough to maintain the DO level at the set point.

The experiment started with a 24 h batch phase, followed by a 23 h fed-batch phase, wherein a feed solution was added, as described in 3.3.2 (Experiment B) of Chapter 3. The feeding solution was composed of glycerol (86-88 wt%) supplemented with PTM solution in a proportion of 24 mL of PTM solution per liter of glycerol. The feeding profile was set in the control unit of the bioreactor, which automatically fed the reactor with substrate at a rate that gradually increased from 6.6 to 7.6 g/L.h (considering the initial working volume of the reactor), giving an overall feeding of 1359 g of glycerol. Samples (20 mL) were periodically withdrawn from the bioreactor, for determination of the DCW, CGC and mannans content and glycerol and ammonia concentrations.

4.3.4. Analytical techniques

For the determination of DCW, 4 mL fermentation broth samples were centrifuged (8000 × *g*, 10 min). The pellet was used for the gravimetric quantification of biomass, while the cell-free supernatant was used for glycerol and ammonia quantification. The gravimetric quantification of DCW was made as described in 3.3.5 section of Chapter 3.

For glycerol quantification, the cell-free supernatant was analyzed by High Performance Liquid Chromatography (HPLC). The analysis was performed with a Metacarb 87H column (Varian) and a differential refractometer RI-71 detector (Merck). The samples were analysed at 50 ºC, with H_2SO_4 0.01 N as eluent, at a flow rate of 0.6 mL/min. Glycerol 86-88 wt% (Scharlau) was used as standard, at a concentration range of 0.065-1.0 g/L. The samples were diluted to have their concentration below 1.0 g/L.

For the ammonia quantification, the cell-free supernatant was analysed as described in 3.3.5 section of Chapter 3. An ammonia chloride solution was used as standard, at a concentration range of 4.0-20.0 ppm. The samples were diluted to have their concentration below 20 ppm.

4.3.5. Polymers extraction

For polymers extraction from *K. pastoris* biomass, 100 mg of dried biomass samples were resuspended in 30 mL NaOH 5 M. The biomass extraction was performed at 65 ºC, during 2 h. After the chemical treatment, the extraction broth was centrifuged (8,000 × *g*, 10 min), for the separation of alkaline insoluble material (AIM) and alkaline soluble material (ASM). To

obtain the CGC samples from AIM, the CGC purification was performed as described in 3.3.4 section of Chapter 3.

To obtain the mannans samples from ASM, the mannans were dialysed with a 12,000 MWCO membrane (Nadir® - dialysis tubing, Carl Roth) against deionized water, during 48 h. The deionized water was changed twice a day, until a conductivity below 10 μS/cm was achieved. The dialysed samples were freeze dried, for gravimetric quantification of mannans.

4.3.6. Polymers characterization

For CGC sugar compositional analysis, dried samples were subjected to two acid hydrolysis, with TFA 99% and HCl 4 M respectively, as described in 3.3.5 section of Chapter 3. The mannans sugar composition was also performed with the same TFA hydrolysis procedure to hydrolyse the mannans into their mannose monomers. The hydrolysates were analyzed by HPLC, as described in 3.3.5 section of Chapter 3. Glucose, mannose and glucosamine (Sigma) were used as standards, at concentration ranging between 0.005 and 0.1 g/L and were subjected to the same hydrolysis procedures as the polysaccharides samples.

For determination of the protein content, dried polysaccharide samples (7 mg) were treated with NaOH 2 M (1 mL) in sealed vials, at 120 °C, for 15 min. The supernatant obtained by centrifugation (10,000 × *g*, 10 min) was used for the protein assay, according to the modified Lowry method (Stoscheck, 1990). An alkaline sulphate copper solution was prepared by mixing 1 volume of a 50 mL solution composed of $C_4H_4KNaO_6 \cdot 4H_2O$ (0.5 g) and $CuSO_4 \cdot 5H_2O$ (0.250 g), with 10 volumes of a solution of Na_2CO_3 (10 g) in 100 mL of NaOH 0.5 N. A 1 mL aliquot of this copper sulphate solution was mixed with 1 mL of the supernatant and the mixture was allowed to stand for 10 min at room temperature (in the dark). Diluted (1:10) Folin-Ciocalteu reagent (3 mL, Panreac) was added and the mixture was incubated for 30 min, at room temperature. The absorbance was read at 750 nm. Bovine serum albumin (BSA, Sigma) was used as standard, at concentrations of 0-6.0 mg/mL.

The inorganic salts content was evaluated by pyrolysis decomposition of the samples (≈ 50 mg) at a temperature of 550 ºC, for 24 h.

4.3.7. Kinetic parameters

The maximum specific cell growth rate (μ_{max}, h^{-1}) was determined by using the following equation (Equation 4.1):

$$\ln\left(\frac{X}{X_0}\right) = \mu_{max}\, t \qquad (4.1)$$

where X and the X_0 are the DCW (g/L) at time t and at time t=0, respectively. In practice, μ_{max} is the slope of the line of the ln (X/X_0) vs time graph.

The biomass, CGC and mannans yields, $Y_{X/S}$ (g_x/g_s), $Y_{C/S}$ (g_c/g_s) and $Y_{M/S}$, (g_m/g_s) were determined by Equations 4.2, 4.3 and 4.4, respectively:

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \quad (4.2)$$

$$Y_{C/S} = \frac{\Delta CGC}{\Delta S} \quad (4.3)$$

$$Y_{M/S} = \frac{\Delta Mannans}{\Delta S} \quad (4.4)$$

where ΔX is the biomass (g) produced during the experiment, ΔCGC and ΔMannans are the CGC and mannans produced (g), respectively, and ΔS is the total glycerol consumed during the same time interval (g).

The CGC and mannans volumetric productivity, r_C (g/L.day) and r_M (g/L.day), respectively, were determined by using the Equation 4.5 and 4.6:

$$r_C = \frac{\Delta CGC}{t} \quad (4.5)$$

$$r_M = \frac{\Delta Mannans}{t} \quad (4.6)$$

where ΔCGC and ΔMannans are the CGC and mannans produced (g/L) at time t (day).

4.4. Results and discussion

4.4.1. Effect of the DO level on *K. pastoris* cultivation

4.4.1.1. Biomass production

Batch bioreactor fermentations were performed with the DO level controlled at 5, 10, 15, 30 or 50% of the air saturation, to evaluate the impact on both *K. pastoris* cell growth and polymers production and composition. The specific cell growth rate was similar (0.15-0.17 h^{-1}) for all assays, except for the lowest DO level tested (5%), in which the culture grew at a lower specific cell growth rate, 0.12 h^{-1} (Table 4.1). Similar values, 0.15-0.18 h^{-1} (Chapter 3; Roca *et al.*, 2012; Chagas *et al.*, 2014) have been reported for *K. pastoris* DSM 70877 cultivation at a DO level of 50%, under batch conditions. Similar or higher specific cell growth rates (0.17-0.27 h^{-1}) were reported for several *Pichia pastoris* strains, using DO levels of 20-30%. For example, Garcia-Ortega *et al.* (2013) and Noseda *et al.* (2013) obtained specific cell growth rates of 0.17-0.19 h^{-1} for *P. pastoris* X-33 and GS115 batch processes, respectively. Higher cell specific

growth rate, 0.26-0.27 h^{-1}, was observed for *P. pastoris* GS115 productions with a DO level of 30% (D'Anjou and Daugulis, 2000; Tang *et al.*, 2009).

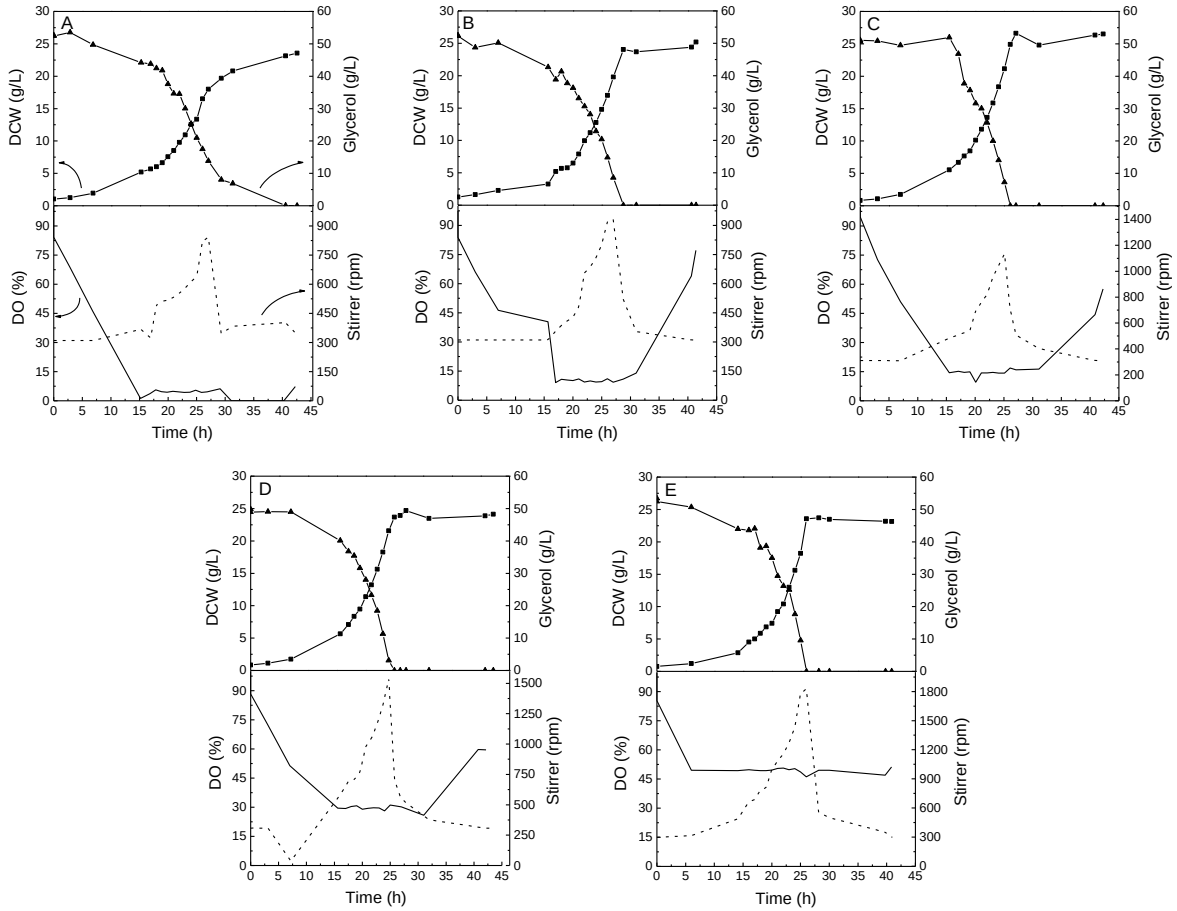

Figure 4.1 – Cultivation profiles obtained in DO batch experiments for the cultivation of *Komagataella pastoris* DSM 70877: (A) 5%, (B) 10%, (C) 15%, (D) 30% and (E) 50%. Offline data: ▲, glycerol concentration; ■, DCW. Online data: ––, DO concentration; – –, Stirring speed. The arrows are pointing to the respective axis of each parameter.

Table 4.1 – Results obtained in the DO batch experiments for *K. pastoris* growth and polymers production: maximum specific cell growth rate (μ_{max}); dry cell weight (DCW); overall CGC (r_C) and mannans (r_M) volumetric productivities; yields of CGC ($Y_{C/S}$) and mannans ($Y_{M/S}$) on a substrate basis.

DO (%)	μ_{max} (h^{-1})	DCW (g/L)	CGC content (wt%)	CGC (g/L)	Mannans content (wt%)	Mannans (g/L)	r_C (g/L.day)	r_M (g/L.day)	$Y_{X/S}$ (g_x/g_s)	$Y_{C/S}$ (g_c/g_s)	$Y_{M/S}$ (g_m/g_s)
5	0.12	22.51	18	4.16	17	3.92	2.36	2.23	0.38	0.07	0.07
10	0.16	23.91	19	4.74	21	5.21	2.75	3.02	0.39	0.08	0.09
15	0.15	25.71	17	4.42	20	5.32	2.51	3.02	0.45	0.08	0.09
30	0.16	23.29	17	4.16	16	3.80	2.37	2.16	0.41	0.07	0.07
50	0.17	22.37	15	3.55	15	3.47	2.09	2.04	0.36	0.06	0.06

An overall biomass production of 25.71 g/L was obtained by cultivation with a DO level of 15%, while lower DCW values (22.51-23.91 g/L) were achieved for higher or lower DO levels (Figure 4.1 and Table 4.1). Glycerol was completely consumed in all experiments, taking around 25-29 h (Figure 4.1). However, a lower glycerol consumption rate was observed for the experiment performed at the lowest DO level of 5%, in which substrate exhaustion took around 40 h (Figure 4.1). This result is in accordance with the lower specific cell growth rate observed for that experiment (Table 4.1). In all experiments, no ammonia limitation was observed. The highest biomass yield, 0.45 g_x/g_s, was also achieved in the experiment performed with a DO level of 15% (Table 4.1). In opposition, fermentations with DO levels of 5% and 50% resulted in lower biomass yields (0.36-0.41 g_x/g_s). Cultivation at a DO level of 50% resulted in a less efficient conversion of glycerol into biomass, probably due to substrate oxidation (in www.aciscience.org/docs/chemical_properties_and_derivatives_of_glycerol.pdf). The lower biomass yield observed at the DO level of 5%, might be related with the production of by-products (such as organic acids or ethanol) that were detected at low levels. These results suggest that cultivation of *K. pastoris* under a DO level of 15% provides a more efficient conversion of the substrate into biomass.

A similar trend was reported for cultivation of *P. pastoris* at DO levels between 10 and 30%. For example, Bhatacharya, Pandey and Mukherjee (2007) and Garcia-Ortega *et al.* (2013) reported a lower biomass production of around 19 g/L, but with similar biomass yields (0.40-0.49 g_x/g_s) in fermentation processes with a DO level of 20%, with *P. pastoris* strains GS115 and X-33, respectively. A higher biomass concentration of 28.1 g/L and biomass yield of 0.7 g_x/g_s was already reported in a batch fermentation with *P. pastoris* X-33 at a DO level of 20% (Irani *et al.*, 2015).

4.4.1.2. CGC production

As shown in Table 4.1, the CGC content in *K. pastoris* biomass was enhanced by decreasing the DO level. In fact, a CGC content of 18-19 wt% was obtained for the experiment performed at a DO of 5-10%, while at high DO levels (50%), the CGC content was 15 wt%. Chagas *et al.* (2014) and in Chapter 3 studies were obtained CGC contents in the biomass of 13 wt% in cultivation of *K. pastoris* under similar conditions, with DO levels of 50%. These results suggest that oxygen limitation might have been a stress factor to the yeast cells, influencing cell-wall polysaccharides synthesis. It was reported that stress factors, such as environmental (pH, DO level or temperature) or chemical induced factors (medium composition), can trigger cell-wall compensatory mechanisms to protect the yeast cells, keeping their integrity (Aguilar-Uscanga and Francois, 2003). Those response mechanisms is the so-called cell-wall integrity

(CWI) pathway that is characterized by alterations of cell-wall composition and thickness (Smits, van den Ende and Klis, 2001). However, the CGC content in the biomass achieved in all experiments (15-19 wt%) is within the range of values reported for the cell-wall polysaccharides content of several yeast and fungi strains (5-30 wt%) (Lipke and Ovalle, 1998; Nguyen, Fleet and Rogers, 1998; Feofilova *et al.*, 2006; Zlotnikov *et al.*, 2007; Smirnou, Krcmar and Prochazkova, 2011; Chagas *et al.*, 2014). For example, Feofilova *et al.* (2006) reported a CGC content between 15 and 18 wt% for *Aspergillus niger* and similar values (10-20 wt%) were also obtained for *Saccharomyces cerevisiae* biomass (Lipke and Ovalle, 1998; Nguyen, Fleet and Rogers, 1998).

The highest CGC production, 4.74 g/L, was achieved in the experiment performed at a DO level of 10%, which also resulted in the highest volumetric productivity, 2.75 g/L.day (Table 4.1). Compared with cultivation at a DO of 50% that resulted in a productivity of 2.09 g/L.day (Table 4.1), this represents an improvement of around 30%. The CGC yield on a substrate basis was also higher for the experiment performed with a DO of 10-15% (0.08 g_c/g_s), compared to the other DO levels tested.

On the other hand, the CGC composition does not seem to suffer any impact by lowering the oxygen level, as shown by the similar chitin:β-glucan molar ratio, between 11:89 and 13:87, for the co-polymers obtained in all experiments. These ratios are within the values reported for *K. pastoris* CGC, between 11:89 and 22:78 (Chapter 3 and Roca *et al.*, 2012).

4.4.1.3. Mannans production

Mannans production was also higher for the experiments performed at a lower DO level of 15% (5.32 g/L), coincident with one of the highest mannans content in the biomass of 20 wt%, among the tested conditions (Table 4.1). Lower values (4-20 wt%) have been reported for mannans production by other yeasts and fungi (Aguilar-Uscanga and Francois, 2003; Araújo *et al.*, 2014; Liu *et al.*, 2015; Galinari *et al.*, 2017). Galinari *et al.* (2017), for example, obtained 13 wt% of mannans content from *Kluyveromyces marxianus* biomass. A mannans content of 4-5 wt% was also reported by Araújo *et al.* (2014) and Liu *et al.* (2015) for *Saccharomyces uvarum* and *S. cerevisiae*, respectively.

The batch experiments with the DO level of 10-15% was also the one with the highest mannans yield and volumetric productivity, 0.09 g_m/g_s and 3.02 g/L.day, respectively. Another interesting point is the fact that, with the DO level of 10-15%, mannans production was higher than CGC, with an overall cell-wall polysaccharides content in the biomass of 37-40 wt% (Table 4.1).

The mannans composition was also not significantly affected by the DO levels tested. The samples were mainly composed of mannose (82-87 wt%), with traces of glucose (2.1-5.3

wt%) and glucosamine (3.0-4.4 wt%) that may have arisen from remnants of CGC resulting from inefficient separation of the AIM and ASM fractions. The mannans samples obtained in this study also contained an inorganic salts content of 0.3-1.5 wt%. The protein content of these mannans were 6.8-9.0 wt%. The presence of protein in mannans fractions extracted from yeasts is common. For example, the mannans extracted from *S. cerevisiae* had a protein content up to 20 wt%, in a crude form (Liu *et al.*, 2015; Maru *et al.*, 2015). The mannans fraction extracted from *S. cerevisiae* also revealed the presence of 1.6-6.0 wt% of glucans (Liu *et al.*, 2015).

Given these findings, cultivation of *K. pastoris* at a DO level of 15% seems to be the most adequate condition for the production of both biomass and cell-wall polysaccharides, with high productivities and without any impact on the polymers' composition. With this DO level, it was obtained the highest biomass production, concomitant with one of the highest cell-wall polysaccharides productions.

4.4.2. Fed-batch bioreactor cultivation

A fed-batch bioreactor experiment was performed as described in 3.4.2 section of Chapter 3, except for the use of a lower DO level (15%). This cultivation included a batch phase that lasted 24 h, followed by a 23 h fed-batch phase (Figure 4.2). During the batch phase, the culture grew at a maximum specific cell growth rate of 0.14 h^{-1}, similarly to the value obtained in the batch experiment performed with the same DO level, 0.15 h^{-1} (Table 4.1). A cell concentration of 12.2 g/L was reached within 24 h of cultivation, while at the end of the run (47 h), 95.1 g/L were obtained (Figure 4.2 and Table 4.2).

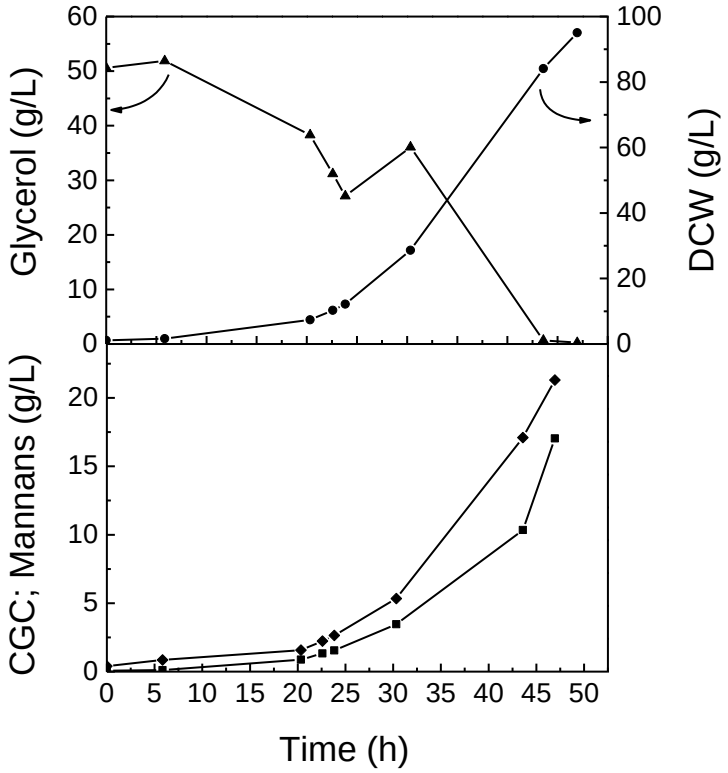

Figure 4.2 – Cultivation profiles obtained in fed-batch experiment with *Komagataella pastoris* DSM 70877: ▲, glycerol concentration; ●, DCW; ■, CGC; ♦, Mannans. The arrows are pointing to the respective axis of each parameter.

Table 4.2 – Results obtained in the fed-batch experiment for K. pastoris biomass and polymers production and comparison with the literature: dry cell weight (DCW); overall CGC (r_C) and mannans (r_M), volumetric productivities; yields of biomass ($Y_{X/S}$), CGC ($Y_{C/S}$) and mannans ($Y_{M/S}$) on glycerol basis.

K. pastoris strain	DO (%)	DCW (g/L)	CGC content (wt%)	CGC (g/L)	Mannans content (wt%)	Mannans (g/L)	r_C (g/L.day)	r_M (g/L.day)	$Y_{X/S}$ (g_x/g_s)	$Y_{C/S}$ (g_c/g_s)	$Y_{M/S}$ (g_m/g_s)	Refs.
DSM 70877	15	95.1	18	17.04	22	21.30	8.67	10.69	0.51	0.09	0.11	This work
DSM 70877	50	104	13	14	n.a.	n.a.	7.7	n.a.	0.55	0.08	n.a.	Roca *et al.* (2012)
DSM 70877	50	121.9	13.5	16.49	n.a.	n.a.	8.32	n.a.	0.49	0.07	n.a.	Chapter 3
GS115	10	97.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.49	n.a.	n.a.	Lee *et al.* (2003)
X-33	20	101.7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.50	n.a.	n.a.	Garcia-Ortega *et al.* (2013)

n.a. – data not available

All the glycerol supplied to the culture was completely consumed during the run, with an average specific consumption rate of 0.21 g_s/g_x.h. Lower values were reported for strains GS115 and X-33, which had specific glycerol consumption rates of 0.09-0.18 g_s/g_x.h for cultivations under a similar DO level (Lee *et al.*, 2003; Garcia-Ortega *et al.*, 2013). There was an overall glycerol consumption of 1771.6 g, corresponding to a biomass yield of 0.51 g_x/g_s (Table 4.2). These results represent an improvement on biomass yield compared to the value reported in 3.4.3 section of Chapter 3, 0.49 g_x/g_s, for cultivation under a DO level of 50% (Table 4.2). Similar results were also obtained for the cultivation of strains X-33 and GS115 that achieved DCW values of 101.7 g/L and 97.2 g/L, respectively, and biomass yields of 0.50 g_x/g_s and 0.49 g_x/g_s, respectively (Lee *et al.*, 2003; Garcia-Ortega *et al.*, 2013) (Table 4.2).

At the end of the run, the biomass had a CGC contents of 18 wt% (Table 4.2). The corresponding overall production and volumetric productivity values were 17.04 g/L and 8.71 g/L.day, respectively (Table 4.2). The co-polymer's yield (0.09 g_c/g_s) was also improved in the fed-batch experiment compared to previous reports for cultivation of *K. pastoris* with DO level of 50%. In fact, the CGC volumetric productivity was improved by around 13%, from 7.70 g/L.day (Roca *et al.*, 2012) to 8.67 g/L.day. Similarly to the batch experiment performed with a DO level of 15%, the mannans production (21.3 g/L) was higher than that of CGC (17.04 g/L). The mannans content in the biomass was 22 wt%, slightly higher than the value obtained in the batch experiment at 15% DO level (Table 4.1 and Table 4.2). The mannans yield on a substrate basis was also increased to 0.11 g_m/g_s (Table 4.2), compared to 0.09 g_m/g_s obtained in the batch experiment (Table 4.1).

The chitin:β-glucan molar ration of the CGC produced in the fed-batch experiment (16:84) was higher than the one obtained in the batch studies (11:89-13:87), but still within the range of values previously reported, 11:89-22:78 (Chapter 3 and Roca *et al.*, 2012). The mannans produced in this experiment had a similar composition compared with the batch studies. The polymers were also mainly composed of mannose (82 wt%), with traces of glucose and glucosamine (2.0 and 2.9 wt%, respectively%). The sample also contained low inorganic salts content (1.3 wt%) and a protein content of 12.3 wt%.

The results obtained in the fed-batch experiment at a DO level of 15% demonstrated that the lower DO level influenced *K. pastoris* cell-wall composition, by enhancing the CGC and mannans contents, and by increasing the chitin fraction of the CGC co-polymer and the protein content in mannans. On the other hand, lowering the DO concentration during operation also enabled considerable energy savings in this bioprocess, without compromising the overall product productivity and polymers composition.

4.5. Conclusions

This work demonstrated that reducing the DO level from 50 to 15% during cultivation of *K. pastoris* provided a more efficient conversion of glycerol into biomass, as demonstrated by the higher growth yield obtained. More importantly, the production of the cell-wall polysaccharides CGC and mannans was also improved. The contents of both polymers' in the biomass were increased, which resulted in higher production and volumetric productivities. In particular, CGC productivity was improved by 13 % without any significant impact on its composition. Moreover, lowering the DO level from 50 to 15%, also enabled considerable bioprocess energy and cost savings regarding the oxygen demand.

Chapter 5

Medium design for improved polysaccharides production by *Komagataella pastoris*

5.1. Abstract

Basal salts medium (BSM) is one of the most commonly used medium for cultivation of the yeast *Komagataella pastoris* (formerly known as *Pichia pastoris*). *K. pastoris* was recently proposed as a source of valuable polysaccharides, namely, the co-polymer chitin-glucan complex (CGC) and mannose-containing polymers (mannans), that are extracted from its cell-wall. To overcome the problems associated with the use of BSM, namely excessive salts content and precipitation during medium preparation or sterilization, a novel cultivation medium, Medium K, was designed. The use of Medium K for the cultivation of *K. pastoris* improved the biomass volumetric productivity (90.3-136.7 g/L.day), compared to BSM (59.0-86.9 g/L.day), under similar bioreactor cultivation conditions. More importantly, the contents of CGC and mannans in the biomass were increased (18-19 and 21 wt%, respectively), resulting in significantly higher products' volumetric productivities (17.5-26.0 and 19.2-26.4g/L.day, respectively). These results demonstrate that the designed medium was suitable for cultivation of *K. pastoris* and the production of both CGC and mannans. It comprised fewer components and considerably reduced the salts content, thus representing a significant cost saving for the bioprocess and avoiding the precipitation problems of BSM, without impacting on the polymers' composition.

5.2. Introduction

Komagataella pastoris (formerly known as *Pichia pastoris*) is a methylotrophic yeast, extensively used for production of recombinant proteins (Çalık *et al.*, 2015; Spohner *et al.*, 2015) due to its capacity to reach high cell densities and high product productivities (Looser *et al.*, 2015). *K. pastoris* was recently proposed as a source of valuable cell-wall polysaccharides, namely, the co-polymer chitin-glucan complex (CGC) and mannose-containing polymers (mannans), in a patented bioprocess (Reis *et al.*, 2010; Freitas, Roca, *et al.*, 2013; Freitas *et al.*, 2015).

Depending on the cultivation conditions, such as the pH, temperature or medium composition, the content of CGC and mannans in yeast and fungal biomass ranges within 10-30 wt% and 7-13 wt%, respectively (Nguyen, Fleet and Rogers, 1998; Zlotnikov *et al.*, 2007; Smirnou, Krcmar and Prochazkova, 2011; Liu *et al.*, 2015; Galinari *et al.*, 2017). More specifically for *K. pastoris*, the CGC content in the biomass varies between 11 and 20 wt%, depending on the cultivation conditions and the extraction procedures (Roca *et al.*, 2012;

Chagas *et al.*, 2014). Despite not being so well studied as CGC, *K. pastoris* was also reported as a source of mannans by Freitas, Roca, *et al.* (2013) and Reis *et al.* (2010).

Basal salts medium (BSM), developed by Invitrogen Co. (Carlsbad, CA, USA), is the most commonly used cultivation media for *K. pastoris* processes (Cos *et al.*, 2006; Wang *et al.*, 2012; Zheng *et al.*, 2012; Guo *et al.*, 2014; Schmideder *et al.*, 2016). However, the utilization of BSM suffers from some operational problems. It is a white turbid medium with high ionic strength that often forms precipitates, especially at pH values above 5 and after sterilization, generating an unbalanced nutrients composition (Cos *et al.*, 2006; Ghosalkar, Sahai and Srivastava, 2008). Moreover, the *Pichia* trace mineral (PTM) solution comprises some toxic compounds, such as boric acid and sodium iodide (Hubbard, 1998). In order to minimize or eliminate those problems, several alternative cultivation media have been proposed, with the purpose of improving heterologous proteins production (Wegner, 1983; Tang *et al.*, 2009; Ding *et al.*, 2014; Irani *et al.*, 2015; Pais-Chanfrau and Trujillo-Toledo, 2016). For example, a modified version of BSM (MBSM) was proposed by Pais-Chanfrau and Trujillo-Toledo (2016), wherein phosphoric acid was replaced by KH_2PO_4 and the culture medium had an overall lower salts concentration, compared to BSM. Another example was the minimal salts medium (MSM) used by Tang *et al.* (2009), that had lower concentrations of some mineral components, such as copper, iron and zinc, and used KH_2PO_4 instead of phosphoric acid as the phosphorus source.

In this work, different media were tested for cultivation of *K. pastoris* and production of the cell-wall polysaccharides CGC and mannans. The study was initiated with a set of shake flask experiments to evaluate the culture's performance. Based on those experiments, a novel medium was designed, Medium K, and tested in both shake flask and bioreactor cultivation experiments. Medium K had considerably lower overall salts contents and some of the toxic compounds present in BSM were eliminated, which translated into significant cost savings and avoidance of the problems commonly associated with the use of BSM.

5.3. Materials and methods

5.3.1. Yeast strain

Komagataella pastoris DSM 70877 was used in this study. The culture was cryopreserved at -80 ºC, in 20% (v/v) glycerol, in 1 mL aliquots.

5.3.2. Shake flask experiments

For the shake flasks experiments, the inoculum was prepared by inoculating the cryopreserved culture (1 mL) in 100 mL standard Basal Salt Medium (BSM) (Pichia Fermentation Process Guidelines, Invitrogen) (Table 5.1) supplemented with 40 g/L glycerol (86-88 wt%, Scharlau), in 250 mL buffled shake flasks. The inoculum was incubated at 30 ºC and 200 rpm, during 41 h.

Based on literature reports, nine different cultivation media were selected for the shake flask experiments (Exp. 1-9) (Table 5.1) to evaluate their suitability to support *K. pastoris* cell growth and polysaccharides' production. Medium K (Exp. 10) was designed based on the results obtained with the first set of shake flask experiments.

Table 5.1 – Culture media composition (per liter) used for cultivation of *Komagataella pastoris*.

Component	Exp. 1 (BSM)	Exp. 2	Exp. 3	Exp. 4 (MSM)	Exp. 5	Exp. 6 (MBSM)	Exp. 7	Exp. 8	Exp. 9 (Dox liquid medium)	Exp. 10 (Medium K)
Glycerol	40 g	40 g	40 g	40 g	40 g	40 g	40 g	40 g	40 g	40 g
$(NH_4)_2SO_4$	-	-	-	13.55 g	13.55 g	13.55 g	13.55 g	13.55 g	13.55 g	13.55 g
H_3PO_4	26.7 mL	14 mL	20 mL	-	-	-	-	-	-	-
KH_2PO_4	-	-	-	42.9 g	13 g	10 g	10 g	10 g	1 g	28 g
K_2HPO_4	-	-	-	-	2.7 g	-	-	-	-	-
K_2SO_4	18.2 g	9.5 g	1 g	14.33 g	2 g	-	-	-	-	-
KOH	4.13 g	2.13 g	-	-	-	-	-	-	-	-
KCl	-	-	-	-	-	-	-	-	0.5 g	-
NaCl	-	-	-	-	0.1 g	-	-	-	-	-
$NaNO_3$	-	-	-	-	-	-	-	-	2 g	-
$CaSO_4$	0.93 g	0.47 g	0.1 g	0.79 g	-	-	-	-	-	0.099 g
$CaCl_2$	-	-	-	-	0.2 g	0.26 g	-	-	-	-
$MgSO_4$	7.28 g	3.8 g	1 g	5.7 g	0.98 g	1.56 g	2.4 g	2.4 g	0.24 g	0.98 g
Trace mineral solution	4.35 mL	5.2 mL	10 mL	2 mL	2 mL	4.3 mL	-	-	-	2 mL
Trace mineral solution composition (per liter)										
Biotin	0.2 g	0.32 g	0.3 g	0.2 g	0.2 g	0.2 g	-	0.2 g	-	0.2 g
H_3BO_3	0.02 g	-	0.02 g	0.02 g	0.02 g	0.1 g	-	-	-	-
NaI	0.08 g	-	0.08 g	0.08 g	-	-	-	-	-	-
KI	-	-	-	-	0.09 g	0.42 g	0.002 g	0.002 g	-	-
$Na_2MoO_4.2H_2O$	0.2 g	-	0.2 g	0.2 g	0.24 g	1 g	0.08 g	0.08 g	-	-
$CuSO_4.5H_2O$	6 g	4.68 g	6 g	2 g	6 g	6 g	0.006 g	0.006 g	0.2 g	2 g
$CoCl_2$	0.27 g	-	0.5 g	0.5 g	0.5 g	-	0.003 g	0.003 g	-	-
$MnSO_4.H_2O$	3 g	2.34 g	3 g	3 g	3 g	3 g	-	-	-	3 g
$MnCl_2.2H_2O$	-	-	-	-	-	-	0.02 g	0.02 g	-	-
$ZnCl_2$	20 g	-	20 g	7 g	10 g	-	-	-	-	7 g
$ZnSO_4.7H_2O$	-	18.72 g	-	-	-	20 g	0.09 g	0.09 g	-	-
$FeSO_4.7H_2O$	65 g	50.7 g	65 g	22 g	20 g	65 g	0.06 g	0.06 g	0.003 g	22 g
H_2SO_4	5 mL	3.9 mL	5 mL	-	5 mL	10 mL	-	-	-	5 mL
Refs.	Roca et al. (2012)	Wegner (1983)	Ding et al. (2014)	Tang et al. (2009)	Irani et al. (2015)	Pais-Chanfrau and Trujillo-Toledo (2016)	Castrillo et al. (1996)	Castrillo et al. (1996)	Hefnawy and Razak (1998)	This study

All experiments were performed in 500 mL buffled shake flasks, with 200 mL of culture medium and 10 % (v/v) of inoculum prepared as described above. The initial pH was set to 5.0 by addition of ammonia hydroxide 25 % (v/v) (Exp. 1-4), NaOH 2 M (Exp. 5, 7-10) or HCl 1 M (Exp. 6). The trace mineral solutions of Exp. 1-6 and 10 and biotin (in Exp. 8) were filter sterilized separately and added to the media after their sterilization at 121 ºC for 20 min. Cultivation was performed at 30 ºC and 200 rpm, during around 96 h. At the end of the experiments, the cultivation broth was collected and used for the quantification of the dry cell weight (DCW), glycerol and ammonia concentration, CGC and mannans content in the biomass and the polymers' composition.

5.3.3. Bioreactor cultivation

For the bioreactor experiment, the inoculum was prepared by inoculating 1 mL of the cryopreserved culture in 140 mL Medium K (in 500 mL baffled shake-flasks) (Table 5.1), supplemented with 40 g/L of glycerol. The inoculum was incubated at 30 ºC and 200 rpm, during 40 h. The inoculum obtained was used to inoculate a 2 L bioreactor (BioStat B-plus, Sartorius), with 1.4 L Medium K as the initial working volume. The 2 L bioreactor used in this experiment had the same geometry as the bioreactor described in 4.3.2 section of Chapter 4.

The bioreactor experiment included two sequential fed-batch cycles, similar with the repeated fed-batch experiment described in 3.3.3 section of Chapter 3. The first cycle comprised a 24 h batch phase, followed by a 24 h fed-batch phase, while the second cycle had a 3 h batch phase and a 20 h fed-batch phase. During the fed-batch phase of the first cycle, a feeding solution was automatically fed to the reactor at a constant feed rate of 19.5 g/L.h (considering the reactor's starting volume). The feeding solution was composed of glycerol (86-88 wt%) supplemented with the mineral solution, at a proportion of 24 mL of mineral solution per liter of glycerol. At the end of the first cycle, around 80% of the cultivation broth (≈ 1.5 L) was withdraw from the bioreactor under aseptic conditions. The remaining broth (300 mL) was kept in the bioreactor, serving as inoculum for the second fed-batch cycle, which was initiated by filling the bioreactor with 1.2 L of fresh Medium K. After 3 h, the fed-batch phase was initiated and a feeding solution was automatically fed to the reactor at a constant feed rate of 22.3 g/L.h.

The bioreactor was operated with controlled temperature and pH at 30 ± 2 ºC and 5.0 ± 0.02, respectively. The pH was controlled by the automatic addition of ammonium hydroxide 25 % (v/v) that also served as the nitrogen source, and HCl 1 M. The air flow rate was kept constant at 1.4 SLPM (standard liters per minute). The dissolved oxygen (DO) level was controlled at 15% of the air saturation, by an automatic cascade comprising the variation of the stirring rate (300-1500 rpm) and the supplementation of the air stream with oxygen, when the maximum stirring rate was reached and it was not sufficient to keep the DO level at the set

point. During the bioreactor experiment, 10 mL samples were periodically withdrawn for determination of the DCW, glycerol and ammonia concentration, CGC and mannans content in the biomass and polymers composition.

5.3.4. Analytical techniques

For the determination of the DCW, culture broth samples (4 mL) were centrifuged (8000 × *g*, 10 min). The pellet was used for the gravimetric quantification of biomass, while the cell-free supernatant was used for glycerol and ammonia quantification. The pellet was washed twice (by resuspension in 4 mL of deionized water and centrifugation at 8000 × *g* during 10 min) and freeze-dried. Two replicas were used for the gravimetric quantification of the DCW.

For glycerol quantification, the cell-free supernatant was analysed by high performance liquid chromatography (HPLC), as described in 4.3.4 section of Chapter 4. Glycerol 86-88 wt% (Scharlau) was used as standard, at a concentration range of 0.03-1.0 g/L. The samples were diluted to have their concentration below 1.0 g/L.

For ammonia quantification, the cell-free supernatant was analysed as described in 3.3.5 section of Chapter 3. An ammonia chloride solution was used as standard, at a concentration range of 4.0-20.0 ppm. The samples were diluted to have their concentration below 20 ppm.

5.3.5. Cell-wall polysaccharides extraction

Dried biomass samples (100-200 mg) were treated with 30 mL NaOH 5 M at 65 ºC, during 2 h. After cooling, the mixture was centrifuged (8000 × *g*, 10 min) to separate the alkali insoluble material (AIM) from the alkali soluble material (ASM). The AIM was resuspended in 30 mL of deionized water, neutralized with HCl 1 M and centrifuged again. The AIM was further washed twice with deionized water (30 mL, for each wash), and freeze dried for the gravimetric quantification of the extracted CGC.

The ASM was dialysed with a 12,000 MWCO membrane (Nadir® – dialysis tubing, Carl Roth) against deionized water, at room temperature. The dialysis water was changed until a conductivity below 20 μS/cm was achieved. The dialysed samples were freeze dried, for the gravimetric quantification of the extracted mannans.

5.3.6. Polysaccharides characterization

The CGC and mannans sugar compositional analysis was performed as described in 4.3.5 section of Chapter 4. Glucose, mannose and glucosamine (Sigma) were used as standards,

at concentrations between 0.005 and 0.1 g/L, being subjected to the same hydrolysis procedures as the samples.

For determination of the polymers protein content, dried samples (7 mg) were treated with NaOH 2 M (1 mL) in sealed vials, at 120 °C, for 15 min. The supernatant obtained by centrifugation (10,000 × *g*, 10 min) was used for the protein assay, according with the modified Lowry method (Stoscheck, 1990) as described in 4.3.6 section of Chapter 4. Bovine serum albumin (BSA, Sigma) was used as standard, at concentrations of 0-6.0 mg/mL.

5.4. Results and discussion

5.4.1. Media screening experiments

Eight cultivation media (Exp. 2-9) were selected from the literature for testing (Table 5.1) and compared with BSM (Exp. 1) in terms of cell growth and polysaccharides production.

In Exp. 2-6, media described in the literature for the cultivation of different *P. pastoris* strains were tested. All media had most of the components found in BSM, but at lower concentrations (Table 5.1). The medium used in Exp. 2 avoided the use of boric acid and sodium iodide that are toxic compounds. In Exp. 4-9, KH_2PO_4 was used as the phosphorous source instead of H_3PO_4 (Table 5.1). The use of KH_2PO_4 as the phosphate source represents an advantage since it also served as the source of potassium, replacing KOH and K_2SO_4 that are present in BSM. In Exp. 7 and 8, the medium described by Castrillo *et al.* (1996) for cultivation of the yeast *Candida utilis* CBS 621 was tested. The media used in both experiments was identical, except for the absence of biotin in Exp. 7. Finally, in Exp. 9, the cultivation medium was based on that used for cultivation of *Fusarium oxysporum* by Hefnawy and Razak (1998). Comparing to BSM, this medium enabled a considerable reduction of the salts concentration (Table 5.1).

The use of BSM in Exp. 1 resulted in a DCW of 14.82 g/L with CGC and mannans contents of 16 and 14 wt%, respectively. This corresponds to an overall production of 2.38 g/L of CGC and 2.04 g/L of mannans (Table 5.2). Similar DCW values were achieved in Exp. 2, 3 and 4 (13.68-14.63 g/L), which suggests that reducing the concentration of the medium components had no significant impact on *K. pastoris* cell growth. Moreover, the use of KH_2PO_4 instead of H_3PO_4 (Exp. 4) and the elimination of H_3BO_3 and KI from the trace mineral solution (Exp. 2) also had no significant impact. The mannans content in the biomass was also similar for Exp. 2-4 (14-17 wt%) and Exp. 1 (14 wt%), but the CGC content was higher (20-22 wt%) (Table 5.2).

Table 5.2 – Results obtained in the shake flasks experiments for *K. pastoris* growth and cell wall polysaccharides production: dry cell weight (DCW); CGC and mannans content in biomass (CGC and mannans content, respectively); CGC and mannans concentration (CGC and Mannans, respectively).

Exp.	DCW (g/L)	CGC content (wt%)	Mannans content (wt%)	CGC (g/L)	Mannans (g/L)
1	14.82	16	14	2.38	2.04
2	13.68	22	17	3.00	2.37
3	14.40	20	15	2.90	2.17
4	14.63	21	14	3.13	2.06
5	10.48	25	25	2.63	2.63
6	10.08	26	18	2.63	1.85
7	8.57	29	21	2.46	1.79
8	7.83	25	30	1.92	2.33
9	6.51	24	36	1.56	2.31
10	11.30	23	24	2.61	2.73

On the other hand, lower DCW values were obtained in Exp. 5-9 (6.51-10.48 g/L) (Table 5.2), which suggests that the reduction or withdrawal of some components of those media was detrimental for cell growth. The low DCW values obtained in Exp. 7 and 9 may have been due to the lack of biotin, besides the extremely low concentration of several trace mineral components in the media tested in both experiments. Biotin, or vitamin H, is an essential component for growth of several yeast cultures such as *K. pastoris*, playing an important role in many metabolic reactions (Jungo *et al.*, 2007; Kumar and Rangarajan, 2011). Interestingly, despite the lower biomass production, the contents of CGC and mannans in the biomass were increased (24-29 wt% and 18-36 wt%, respectively). Nevertheless, the overall polysaccharides production was not significantly improved due to the lower biomass concentration (Table 5.2).

As can be observed in Figure 5.1 where the normalized DCW, CGC and mannans concentrations are presented, all the tested media resulted in a considerable reduction of the DCW, except for Exp. 2, 3 and 4. Nevertheless, most of the tested media favoured CGC and/or mannans production. In fact, CGC production was increased by over 20% in Exp. 2, 3 and 4, while mannans production was particularly improved in Exp. 5. Therefore, the results suggest that the overall production of cell-wall polysaccharides was favoured by the media tested in Exp. 2, 3, 4 and 5.

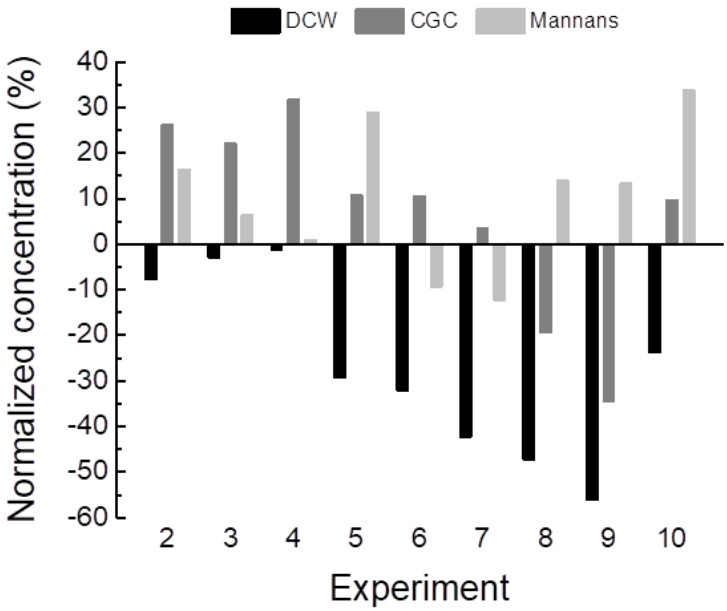

Figure 5.1 – Normalized concentration results obtained in the shake flasks experiments for *K. pastoris* growth and polymers production.

4.4.2. Medium K design

Based on the results obtained in the shake flask experiments, a novel cultivation medium, Medium K, was designed (Table 5.1). Considering that in Exp. 4 biomass production and mannans concentration were not affected by replacing H_3PO_4 by KH_2PO_4, with an increased CGC production (Figure 5.1), the later was selected as a component for Medium K. Since KH_2PO_4 also served as the source of potassium, K_2SO_4 and KOH were not included in the medium's composition. A concentration of 28 g/L was selected for the concentration of KH_2PO_4 in Medium K, which corresponded to concentrations of PO_4^{3-} and K^+ of 19.5 and 8.0 g/L, respectively. These values were selected because higher concentrations were shown not to lead to further improvement of the DCW, as shown by Figure 5.2. Ca^{2+} and Mg^{2+} were present in the media tested in Exp. 2, 3, 4 and 5 in concentrations between 0.03-0.23 g/L and 0.2-1.14 g/L, respectively. Hence, the lowest concentration of each cation (i.e., 0.03 g/L for Ca^{2+}, and 0.2 g/L for Mg^{2+}) was considered as sufficient and they were incorporated in Medium K as sulphate salts (i.e., $CaSO_4$ and $MgSO_4$) at concentrations of 0.099 g/L and 0.98 g/L, respectively (Table 5.1).

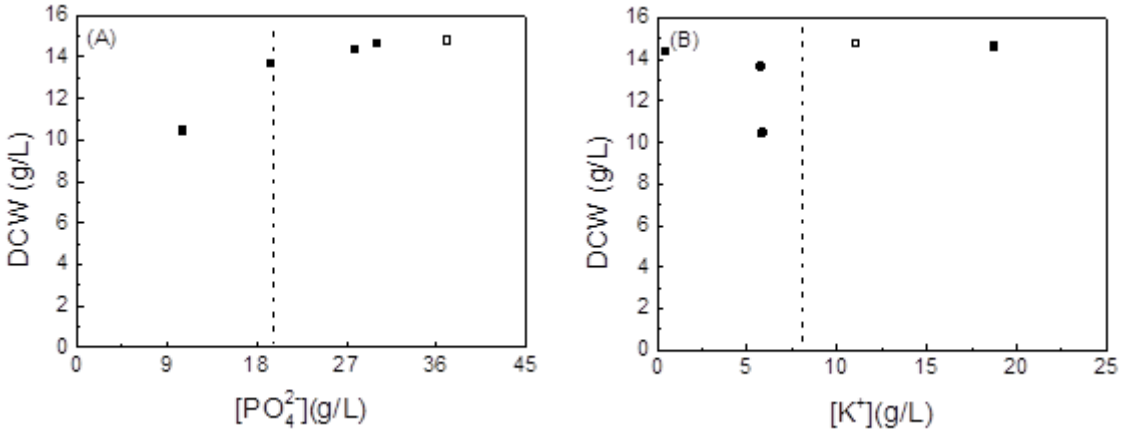

Figure 5.2 – DCW results obtained for the *K. pastoris* shake flasks experiments for (A) PO_4^{2-} and (B) K^+ ions, used for the Medium K design: □, Exp. 1; ■, Exp. 2-5.

As far as the trace mineral solution is concerned, the elimination of BO_3^-, I^-, MoO_4^{2-} and Co^{2+} in Exp. 2 showed that those ions were not essential for *K. pastoris* since cell growth was not significantly affected and polysaccharides' production was improved in that experience (Figure 5.1). Therefore, only Cu^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+} salts were included in the composition of the trace mineral solution. $CuSO_4$, $FeSO_4$, $MnSO_4$ and $ZnCl_2$ were used in the lower range of their concentrations in the media tested in Exp. 2, 3, 4 and 5 (Table 5.1). Isidro *et al.* (2016) and Plantz *et al.* (2007) also reported that Co^{2+}, I^-, MoO_4^{2-} and BO_3^- had no impact on yeast cell growth, while Fe^{2+}, Mn^{2+} and Zn^{2+} had significant impact on biomass production. Moreover, several trace metals, such as Mg^{2+}, Ca^{2+}, Mn^{2+} or Zn^{2+}, were demonstrated to play an important structural and functional role on yeast cells and influence polysaccharides production by yeasts, namely chitin synthesis in the cell-wall by activating the chitin synthases (CHS) genes promotors (Munro *et al.*, 2007; Venkateshwar *et al.*, 2010).

Biotin was also included in the trace mineral solution composition as it was shown to be essential, as its absence in Exp. 7 and 9 leads to considerable reduction of both the DCW and polysaccharides' production.

5.4.3. Validation of Medium K in shake flask cultivation

The designed Medium K was tested in a shake flask experiment (Exp. 10) to evaluate the culture's performance in terms of cell growth and polysaccharides production. A DCW of 11.30 g/L was reached, with CGC and mannans contents of 23 wt% and 24 wt%, respectively (Table 5.2). This resulted in a production of 2.61 g/L of CGC and 2.73 g/L of mannans (Table 5.2), corresponding to an increase of 10% and 34%, on CGC and mannans production,

respectively. Therefore, despite the lower DCW compared to Exp. 1, in which BSM was used, the overall polysaccharides production was improved (Table 5.2).

The results observed in Exp. 10 for the designed *K. pastoris* medium revealed that it is a promising medium for the production of CGC and mannans by *K. pastoris*.

5.4.4. Validation of Medium K in fed-batch bioreactor cultivation

Medium K was further tested in a 2 L fed-batch bioreactor cultivation. This experiment took 71 h and included two fed-batch sequential cycles. The first cycle comprised a 24 h batch phase followed by a 24 h fed-batch phase, with a feed flow rate of 19.5 g/L.h, while the second cycle was shorter, comprising a 3 h batch phase and a 20 h fed-batch phase, with a feed flow rate of 22.3 g/L.h.

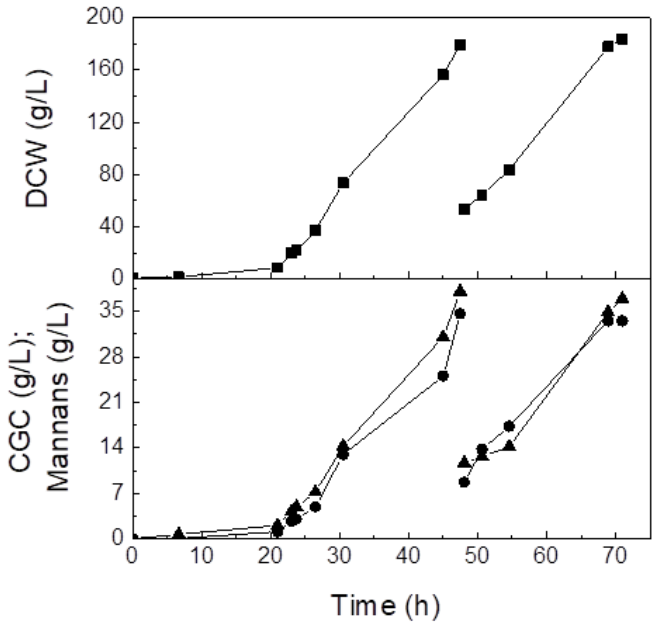

Figure 5.3 – Results obtained for the *K. pastoris* bioreactor cultivation with Medium K: ■, DCW; ●, CGC; ▲, Mannans.

Table 5.3 – Overall biomass and polysaccharides production obtained in fed-batch bioreactor cultivation of *Komagataella pastoris* DSM 70877 (dry cell weight (DCW), overall biomass (r_x), CGC (r_C) and mannans (r_M) volumetric productivities; yield of biomass ($Y_{X/S}$) CGC, ($Y_{CGC/S}$) and mannans ($Y_{M/S}$) on a substrate basis).

Cultivation time (h)	DCW (g/L)	CGC (wt%)	Mannans (wt%)	CGC (g/L)	Mannans (g/L)	r_x (g/L.day)	r_C (g/L.day)	r_M (g/L.day)	$Y_{X/S}$ (g_x/g_s)	$Y_{CGC/S}$ (g_c/g_s)	$Y_{M/S}$ (g_m/g_s)	Ref.
48 (1st cycle)	179.4	19	21	34.6	38.0	90.3	17.5	19.2	0.50	0.10	0.11	This work
23 (2nd cycle)	183.8	18	21	33.5	36.9	136.7	26.0	26.4	0.53	0.10	0.11	
41	104.0	13	n.a.	14.0	n.a.	59.0	7.7	n.a.	0.55	0.08	n.a.	Roca *et al.* (2012)
47	170.2	12	n.a.	21.0	n.a.	86.9	10.8	n.a.	0.47	0.06	n.a.	Chapter 3
47	95.1	18	22	17.0	21.3	48.0	8.7	10.7	0.51	0.09	0.11	Chapter 4

n.a. – data not available

As shown in Figure 5.3, at the end of the first cycle, a DCW of 179.4 g/L was achieved, which corresponds to a biomass volumetric productivity of 90.3 g/L.day (Table 5.3). The second fed-batch cycle was initiated with a DCW of 53.3 g/L that increased to 183.8 g/L within 23 h (Figure 5.3). This corresponds to a biomass productivity of 136.7 g/L.day, for the second cycle of the experiment (Table 5.3). These results show that Medium K resulted in improved biomass production and productivity compared to BSM used in previous studies (Chapter 3 and Roca *et al.*, 2012). In a 41 h fed-batch cultivation, Roca *et al.* (2012) achieved a DCW of 104 g/L and a biomass volumetric productivity of 59.0 g/L.day. Considerably higher biomass concentration (170.2 g/L) and volumetric productivity (86.9 g/L.day) were reported in 3.4.2 section of Chapter 3 (Table 5.3), in a 47 h fed-batch cultivation. In the current study, the DCW was further improved (179.4-183.8 g/L), but the most relevant result was that the CGC content in the biomass was considerably improved. In fact, the CGC content obtained with Medium K (18-19 wt%) was almost 50% higher than that obtained with BSM (12-13 wt%) (Table 5.3).

Given the higher DCW and CGC content in the biomass, the CGC production and volumetric productivity was also significantly improved: 34.6 and 33.5 g/L of CGC were obtained in the first and second cycles, respectively, corresponding to volumetric productivity values of 17.5 g/L.day for the first cycle and 26.0 g/L.day for the second one (Table 5.3). This represents a 2- to 3-fold increase in the overall productivity of CGC as compared to previous studies, 7.7-10.8 g/L.day (Chapters 3 and 4 and Roca *et al.*, 2012).

The mannans content in the biomass achieved 21 wt%, resulting in a mannans production of 36.9-38.0 g/L and an overall mannans productivity of 19.2 g/L.day and 26.4 g/L.day, in the first and second cycles, respectively (Table 5.3). Despite the similar mannans content in biomass (22 wt%), the mannans production with Medium K was considerably higher compared with the DO 15% fed-batch experiment performed with BSM in 4.4.2 section of Chapter 4 (10.69 g/L.day of mannans productivity). Comparing with others yeasts cultures, the mannans content in biomass obtained with *K. pastoris* in this study represents one of the highest values achieved. Some authors have reported mannans contents in the biomass ranging between 4 and 13 wt% for other yeasts, including *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* (Nguyen, Fleet and Rogers, 1998; Liu *et al.*, 2015; Galinari *et al.*, 2017). The mannans content in *K. pastoris* biomass observed in this study is considerably above the reported range.

During this experiment, 671.3 g and 687.7 g of glycerol were consumed in the first and second cycles, respectively. A biomass yield of 0.50-0.53 g_x/g_s was achieved, which is similar to the values reported for cultivation of *K. pastoris* in BSM (0.51-0.55 g_x/g_s) (Chapter 4 and Roca *et al.*, 2012). It is also in accordance with the values attained for other *P. pastoris* strains (0.50-0.51 g_x/g_s) (Tang *et al.*, 2009; Garcia-Ortega *et al.*, 2013). CGC and mannans yields of 0.10 g_c/g_s and 0.11 g_m/g_s, respectively, were also attained (Table 5.3). Regarding the CGC, Medium

K enabled a higher yield, over 20% higher than the values attained by Roca *et al.* (2012) and in section 3.4.2 of Chapter 3 (0.06-0.08 g_c/g_s). The CGC and mannans yields where similar to the values obtained in 4.4.2 section of Chapter 4, performed with BSM and with the same DO level of 15% (0.09 g_c/g_s and 0.11 g_m/g_s) (Table 5.3).

The results obtained in this study clearly demonstrated that the developed Medium K was suitable for cultivation of *K. pastoris*, allowing for high cell densities and improved volumetric productivity of cell-wall polysaccharides.

5.4.5. Polysaccharides characterization

The polysaccharides extracted from the biomass produced during the bioreactor cultivation of *K. pastoris* in the novel designed Medium K were characterized in terms of their composition. The two fed-batch cycles performed in bioreactor cultivation resulted in CGC with a chitin:β-glucan molar ratio of 12:88, which is within the range reported for *K. pastoris* CGC (11:89-19:81) produced with BSM medium under similar cultivation conditions (Chapters 3 and 4). This shows that there was no significant impact on the co-polymer's composition by altering the cultivation medium composition.

The mannans produced in the bioreactor experiment were mainly composed of mannose units. Nevertheless, traces of glucose (3.2 wt%) and glucosamine (3.7 wt%) were also detected in the samples. Liu *et al.* (2015) also reported a similar glucans content (1.6-6.0 wt%) for mannans extracted from *S. cerevisiae* biomass. The mannans samples also included a total proteins content of 10 wt%. This value is within the range reported for several yeast mannans (3-20 wt%) (Liu *et al.*, 2015; Maru *et al.*, 2015; Galinari *et al.*, 2017).

5.5. Conclusions

A novel cultivation medium, Medium K, was developed to improve the production of the cell-wall polysaccharides CGC and mannans by *K. pastoris*. Medium K enabled improved biomass and polysaccharides volumetric productivities. The designed medium had a simpler composition, with less components, and with considerably lower salts concentration than BSM. Therefore, Medium K not only avoided the commonly encountered BSM problems of salts precipitation, but also allowed for the reduction of production costs, without compromising cell growth nor polysaccharides production. In fact, high volumetric productivities were achieved for both products, which demonstrated the suitability of the designed cultivation medium for this bioprocess.

Chapter 6

Chitin-glucan complex production by *Komagataella pastoris*: downstream optimization and product characterization

The results presented in this chapter were published in the peer reviewed paper:
Farinha, I. *et al.* (2015) 'Chitin-glucan complex production by *Komagataella pastoris*: Downstream optimization and product characterization', *Carbohydrate Polymers*, 130, pp. 455–464. doi: 10.1016/j.carbpol.2015.05.034.

6.1. Abstract

Purified chitin-glucan complex (CGC_{pure}) was extracted from *Komagataella pastoris* biomass using a hot alkaline treatment, followed by neutralization and repeated washing with deionised water. The co-polymer thus obtained had a chitin:β-glucan molar ratio of 25:75 and low protein and inorganic salts contents (3.0 and 0.9 wt%, respectively). CGC_{pure} had an average molecular weight of 4.9×10^5 Da with a polydispersity index of 1.7, and a crystallinity index of 50%. Solid-state NMR provided structural insight at the co-polymer. X-ray diffraction suggests that CGC_{pure} has α-chitin in its structure. CGC_{pure} presented an endothermic decomposition peak at 315 ºC, assigned to the degradation of the saccharide structures. This study revealed that *K. pastoris* CGC has properties similar to other chitinous biopolymers and may represent an attractive alternative to crustacean chitin derived-products, being a reliable raw material for the development of new/improved pharmaceutical, cosmetic or food products.

6.2. Introduction

Chitin-glucan complex (CGC) is a co-polymer made from chitin (N-acetyl-D-glucosamine polymer) and β-glucan (glucose homopolymer) (Gautier *et al.*, 2008). It is a natural component of most fungi and yeast cell wall, such as, for example, *Aspergillus niger* (Feofilova *et al.*, 2006), *Schizophyllum commune* (Smirnou, Krcmar and Prochazkova, 2011), *Saccharomyces cerevisiae* (Lipke and Ovalle, 1998) and *Komagataella pastoris* (formerly known as *Pichia pastoris*) (Chagas *et al.*, 2014), conferring rigidity and stability to the cells (Bowman and Free, 2006).

CGC is insoluble in water and in most organic solvents, but it is hygroscopic and has a high swelling capacity. It is a biocompatible and biodegradable biopolymer that has demonstrated to combine antioxidant, antibacterial and anti-inflammatory properties. This natural polymer is also a non-animal alternative source of chitin to avoid any allergen risk of crustacean sources (Smirnou, Krcmar and Prochazkova, 2011).

Most of the methods proposed so far for extraction of fungal cell wall polysaccharides are tedious and time consuming. They include cell disruption by physical, chemical or enzymatic methods, followed by fractionation of the carbohydrates by solubilisation/precipitation steps using different solvent systems, such as, for example, water, buffers (Dallies, Francois and Paquet, 1998) and/or alkali/acid solutions (Aguilar-Uscanga and Francois, 2003), or using specific enzymatic treatments (Baker *et al.*, 2007). Cell wall disruption is usually performed under hot alkaline conditions, using different alkali solutions,

temperatures and reaction times. The end products of these purifications are an alkali-soluble fraction (containing mainly mannans and alkali-soluble β-glucans) and an alkali-insoluble fraction (containing mainly chitin/chitosan, alkali-insoluble β-glucans and/or chitin-glucan complex) (Kim and Yun, 2006; Bzducha-Wróbel, Kieliszek and Błażejak, 2013). Contaminant proteins, inorganic salts and lipids have been removed adopting different media, such as PBS (phosphate-buffered saline) solution, ethanol (Baker *et al.*, 2007; Roca *et al.*, 2012), hydrochloric acid (Amorim *et al.*, 2001) and extensive water washes (Smirnou, Krcmar and Prochazkova, 2011).

In a previous work (Roca *et al.*, 2012) a low purity CGC biopolymer was obtained from *K. pastoris* biomass. To improve the polysaccharide's purity and obtain a more detailed knowledge of its macromolecular features, envisaging its use in high-value applications, in this work, several downstream procedures were tested to extract CGC from *K. pastoris* biomass. The extraction efficiency of the procedures was evaluated first on the basis of the extraction yield and purity of the polymer. In a second step, the physico-chemical properties of the resulting polymer with the lowest contaminants content, coded "CGC_{pure}", were then compared to "CGC_{PBS}" produced by the standard procedure reported by Roca *et al.* (2012). Commercial chitin and/or β-glucan containing polymers, namely, crab-shell chitin, laminarin (a β-glucan extracted from algae *Laminaria digitata*) and KiOsmetine® (CGC extracted from *A. niger* biomass) were also adopted as reference materials.

6.3. Materials and methods

6.3.1. CGC production

Komagataella pastoris DSM 70877 (formerly known as *Pichia pastoris*) was cultivated in a 10 L bioreactor (BioStat B-plus, Sartorius) in standard basal salts medium (BSM) (Pichia Fermentation Process Guidelines, Invitrogen), supplemented with glycerol (40 g/L), as described by Roca *et al.* (2012).

6.3.2. CGC extraction procedures

Different procedures were tested for extraction of CGC from *K. pastoris* biomass. All methods involved two main steps: (1) cell disruption by alkaline treatment and (2) solvent washing of the resulting alkali insoluble material (AIM) (Figure 6.1).

For each test, 40 mL culture broth samples were centrifuged (8,000 × *g*, 10 min), the biomass was collected and subjected to different alkaline treatments. Methods I-V involved alkaline treatment of the biomass with 40 mL NaOH 1 M, at 65 ºC, for 2 h. In method VI, the treatment was extended for 5 h, while in method VII 40 mL NaOH 5 M was used.

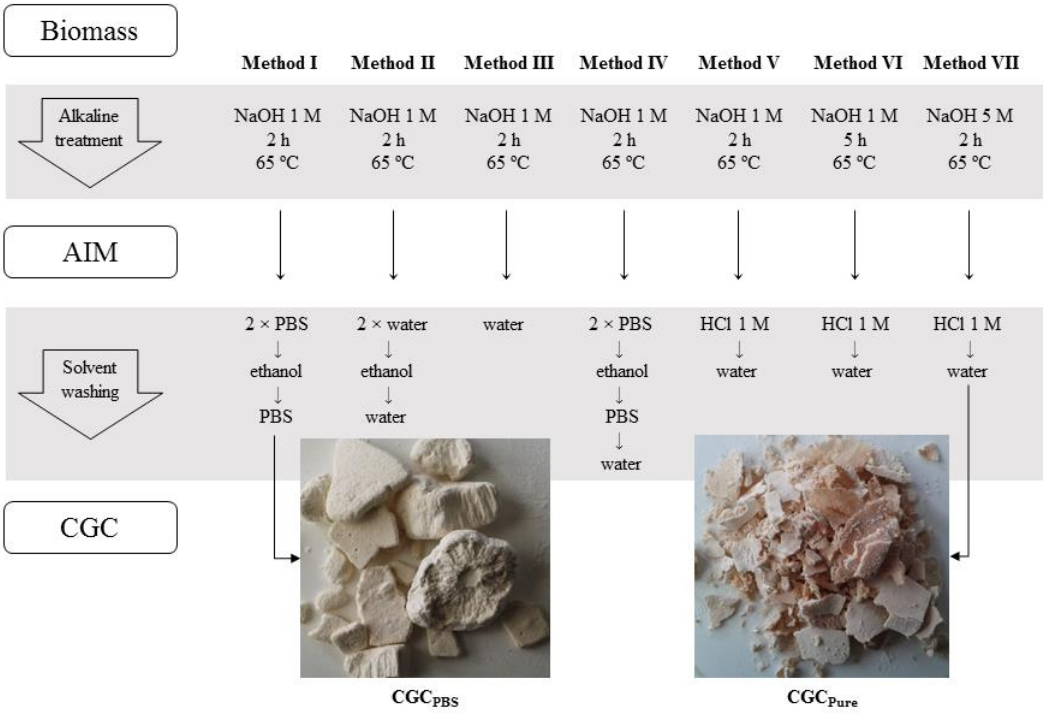

Figure 6.1 – Schematic representation of the procedures used for extraction and purification of CGC from *K. pastoris* biomass.

After the alkaline treatment, the AIM was washed, at room temperature, with different solvent systems (Figure 6.1):

- Method I: Sequential washing with 40 mL phosphate-buffered saline (PBS) solution (20.45 g/L NaCl; 0.46 g/L KCl; 10.14 g/L $Na_2HPO_4 \cdot 7H_2O$; 0.54 g/L KH_2PO_4, pH 7.2) (twice), 40 mL ethanol and 40 mL PBS.
- Method II: Sequential washing with 40 mL deionized water (twice), 40 mL ethanol and 40 mL deionized water.
- Method III: Repeated washing with 40 mL deionized water, until constant pH and conductivity values were reached.
- Method IV: Sequential washing with 40 mL PBS (twice), 40 mL ethanol and 40 mL PBS, followed by repeated washing with deionized water, until constant pH and conductivity values were reached.

- Methods V, VI and VII: Neutralization with HCl 1 M, followed by repeated washing with deionized water, until constant conductivity values were reached and keeping a neutral pH.

All samples obtained with each method were freeze dried. This drying was performed in two steps: a first phase occurred at -110 ºC, during 24 h, and was followed by a secondary drying imposed at 25 ºC, during 24 h. All methods were tested in duplicate experiments.

6.3.3. CGC characterization

All CGC samples obtained in previous experiment were characterized in terms of sugars composition and protein, water and inorganic salts content. CGC_{pure} and CGC_{PBS} were then structurally characterized by Fourier transform infrared spectroscopy (FTIR), ^{13}C Solid-state Nuclear Magnetic Resonance (^{13}C-NMR) and X-ray diffraction (XRD). The thermal properties and the molecular weight of these biopolymers were also evaluated. For comparison, commercial chitin- and β-glucan-containing polymers, namely, crab-shell chitin (Fluka), laminarin (Sigma) and KiOsmetine® (Kitozyme SA) were also analysed by FTIR, XRD and thermal analysis.

6.3.3.1. Composition

The polymers' sugar composition was assessed after the two acid hydrolysis modes, with TFA 99% and HCl 4 M, as it is described in 3.3.5 section of Chapter 3. Glucose and glucosamine (Sigma) were used as standards, at concentrations between 0.006 and 0.1 g/L, being subjected to the same hydrolysis procedures as the samples.

For determination of the protein content, dried CGC samples (7 mg) were treated with NaOH 2 M (1 mL) in sealed vials, at 120 °C, for 15 min. The supernatant obtained by centrifugation (10,000 × *g*, 10 min) was used for the protein assay, according to the modified Lowry method (Stoscheck, 1990) and described in 4.3.6 section of Chapter 4.

The water content of the samples was evaluated by gravimetric analysis after subjecting them to a temperature of 100 ºC, overnight. The inorganic salts content was evaluated by pyrolysis decomposition of the samples (≈ 50 mg) at a temperature of 550 ºC, for 24 h.

6.3.3.2. Infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) was carried out using a Thermo-Nicolet 6700 spectrophotometer from Thermo Electron Corporation. The samples were prepared in KBr

pellets (2 wt%). The spectra were recorded based on 64 scans performed in the mid-infrared range (400-4000 cm^{-1}) adopting a resolution of 4 cm^{-1}.

6.3.3.3. ^{13}C Solid-state nuclear magnetic resonance

^{13}C Cross-Polarization (CP) Magic-Angle Spinning (MAS) NMR spectra were recorded on a 9.4 T WB (400 MHz, ^{1}H Larmor frequency) Bruker Avance III spectrometer. A 4 mm double-resonance MAS probe was employed at 400.1 (^{1}H) and 100.6 MHz (^{13}C) Larmor frequencies. Samples were spun in ZrO_2 rotors using a spinning rate of 12 kHz. ^{13}C CPMAS NMR experiments were employed a ramp CP step (varying from 100% to 50% in amplitude using 100 points); contact time: 2.0 ms; ^{1}H 90º excitation pulse: 3.0 μs; ^{1}H and ^{13}C RF field strength for CP was set to 87 kHz and 68 kHz, respectively; number of scans: 46 k; recycle delay: 5 s. ^{13}C Chemical shifts are quoted in parts per million (ppm) and calibrated with respect to, the external reference, glycine (C=O, 176.03 ppm).

6.3.3.4. X-ray diffraction

The structural analysis of the samples was performed by X-ray diffraction (XRD) using a X´Pert Pro X-ray diffractometer from PANalytical, equipped with an X'Celerator detector, in a Bragg-Brentano geometry with Cu Kα line radiation (λ = 1.5406 Å). The 2θ scans were performed from 3 to 40º, with a step size of 0.03º. The crystallinity index (CI) was determined using the empirical method proposed by Segal *et al.* (1959) and applied by the first time to chitosan by Struszczyk (1987) by using Equation 6.1:

$$CI(\%) = \frac{I_{110} - I_{am}}{I_{110}} \times 100 \qquad (6.1)$$

where I_{110} is the maximum intensity (arbitrary units) of the diffraction of the (110) lattice peak at 2θ ≈ 19° and I_{am} is the intensity of the amorphous material, which is taken at a 2θ angle around 16° where the intensity is at a minimum. It should be noted that CI is only valid at a comparison basis since it is used to indicate the order of crystallinity rather than the precise value of crystalline regions.

6.3.3.5. Thermal properties

Thermogravimetric measurements were carried out with a Simultaneous Thermal Analyser (TGA-DSC-STA 449 F3 Jupiter), under dynamic nitrogen atmosphere (50 mL/min)

and loading 5 mg of each material into a covered aluminium crucible. Two heating cycles were performed according to the following thermal procedure: ramp 5 ºC/min from room temperature to 110 ºC (run 1), isotherm at 110 ºC for 15 min, cooling to room temperature and ramp 5 ºC/min from room temperature to 500 ºC (run 2).

6.3.3.6. Average molecular weight and polydispersity index

The polymers' average molecular weight (Mw) and polydispersity index (PDI) were determined by Size Exclusion Chromatography (SEC). CGC samples were dissolved in DMAc/LiCl (5 wt/v%) according to a procedure adapted from the protocol for cellulose dissolution described by Dupont (2003). In brief, 5 mg of CGC polymer, pre-activated by drying overnight at 100 °C under vacuum (10^{-2} mm Hg) were dissolved within 2 mL of DMAc/LiCl (5 wt/v%) under magnetic agitation for 5 days at 60 °C in a silicone oil bath. The resulting solutions were filtered at room temperature through a 0.3 µm glass fiber filter (Whatman) and transferred into HPLC vials for SEC analysis. SEC analysis was conducted using DMAC/LiCl (0.5 wt/v%) as the mobile phase and a set of Polymer Laboratories columns made from two nonlinear columns: PLgel 5 µm 10^4 Å (300 × 7.5 mm) and a PLgel 5 µm 500 Å (300 × 7.5 mm) This set of analytical columns was protected with a 5 µm guard column (50 × 7.5 mm). SEC analysis has been conducted at a flow-rate of 0.5 mL/min, at 50°C, with a refractive index detector (HP 1047A). Relative Mw of CGC polymers were determined using polystyrene standards with Mw ranging between 800 and 504 kDa. Polymer Laboratories software relying upon the universal calibration method has been adopted to calculate relative molecular weight of CGC polymers.

6.4. Results and discussion

6.4.1. CGC extraction from *K. pastoris* biomass

In previous studies, CGC was extracted from *K. pastoris* biomass by subjecting it to alkaline treatment with NaOH 1 M, at 65 ºC for 2 h, followed by washing with PBS, ethanol and water (Roca *et al.*, 2012). The resulting polymer, CGC_{PBS}, was a rather impure biomaterial, with inorganic salts and protein contents of 15 wt% and 9.5 wt%, respectively. Aiming at obtaining pure CGC, which is important to study the polymer's macromolecular properties and for the development of pharmaceutical or medical applications, several downstream procedures were tested for extracting CGC from *K. pastoris* biomass.

In all methods assessed, the biomass was exposed to alkaline conditions (NaOH 1 M or 5 M), at 65 ºC, for a period of 2 or 5 h, followed by sequential washing of the alkali insoluble material (AIM) according to the different solvent systems listed on Figure 6.1.

Table 6.1 – Extraction yield, chitin:β-glucan molar ratio and content in protein, inorganic salts and water of the CGC polymer samples obtained with the different procedures tested.

Method	Extraction yield (wt%)	Chitin:β-glucan molar ratio (mol%)	Protein (wt%)	Inorganic salts (wt%)	Water (wt%)
I	21.1 ± 1.1	21:79	9.8 ± 0.6	18.1 ± 1.5	5.9 ± 0.9
II	16.8 ± 0.8	22:78	9.7 ± 0.1	5.6 ± 0.02	7.2 ± 0.7
III	19.3 ± 0.2	20:80	11.3 ± 0.8	4.6 ± 0.3	7.5 ± 0.7
IV	14.2 ± 0.1	13:87	12.7 ± 0.1	3.5 ± 0.1	7.6 ± 1.1
V	18.1 ± 0.8	16:84	7.2 ± 0.6	1.5 ± 0.003	6.7 ± 0.6
VI	15.9 ± 0.7	25:75	3.0 ± 0.001	1.8 ± 0.01	5.2 ± 0.6
VII	13.4 ± 1.3	25:75	3.0 ± 0.04	0.9 ± 0.5	6.6 ± 0.1

* repeated washing with deionized water, until constant pH and conductivity values were reached.

Table 6.1 shows the main results obtained with each method tested. The standard method for CGC_{PBS} purification included washings with PBS, ethanol and deionized water (Roca *et al.*, 2012). Method I, which was similar to the standard method, except for the absence of deionized water washes, gave the highest extraction yield (21 wt%), similar to the standard method (18-26 wt%). However, the polymers obtained with both methods had inorganic salts contents of 15-18 wt% and a protein content of 9.5-9.8 wt%.

Considering that the high inorganic salts content of the polymers was most likely related to the use of PBS for washing the AIM, PBS was replaced by deionized water (Method II). For comparison, in Method III, the AIM was washed several times only with deionized water (Figure 6.1). To evaluate the efficiency of this washing procedure, the pH and the conductivity were monitored until constant values were reached. In Method III, the conductivity dropped from an initial value of 15 mS/cm to below 35 µS/cm, after a total of 14 water-washing cycles. This reduction of the sample's conductivity was indicative of the procedure's efficiency for salts removal, which was confirmed by the low inorganic salts content in the polymer (4.6 wt%). The final pH was 9.92. Adopting water and ethanol (Method II) or only water (Method III) for washing, a similar inorganic salts content was noticed, 5.6 and 4.6 wt%, respectively. The comparison of the final protein content arising from the application of Method I or standard method (9.7 and 11.3 wt%) than those obtained in Methods II or III clearly highlight that PBS was more efficient to remove protein than either ethanol/water or water alone (Table 6.1). These

differences support that ionic interactions (PBS versus water) and solvent quality (water versus water/ethanol) significantly control the protein extraction from the AIM. The lower extraction yield obtained with Methods II and III (16.8 and 19.3 wt%, respectively) can readily be explained by the lower contaminants content (especially inorganic salts) of the samples (Table 6.1).

Following these results, Methods IV and V were future tested in order to achieve simultaneously higher protein and inorganic salts removals. In Method IV, PBS and ethanol were used (similarly to Method I) to guaranty good protein removal, but the AIM was afterwards extensively washed with deionized water to efficiently remove inorganic salts. In this method, pH and conductivity were monitored and constant values of 9.71 and 60 µS/cm, respectively, were reached after 11 water-washing cycles. In Method V, the AIM was first neutralized with HCl 1M and then also extensively washed with deionized water (Figure 6.1). Acid treatment with HCl has been reported to improve protein removal (Ferrer *et al.*, 1996). Method V resulted in considerably lower pH and conductivity values (7.7 and 17.6 µS/cm, respectively), after 8 water-washing cycles. Both methods allowed to reduce significantly the polymer's content in inorganic salts (3.5 and 1.5 wt%, respectively) (Table 6.1). Even though higher protein content was achieved with Method IV (12.7 wt%), lower protein content was achieved in Method V (7.2 wt%) comparing to Method I (9.5 wt%). Despite the lower protein content observed in Method V, protein still represents a significant contaminant of the polymer. From this comparative analysis, Method V is advantageous over Method IV thanks to reagent savings (no PBS and no ethanol, and less water were used), while achieving lower protein and inorganic salts residues.

As a final attempt to further purify the polymer, two additional strategies were assessed: increasing the reaction time of the alkaline treatment to 5 h (Method VI) and increasing the NaOH concentration to 5 M (Method VII) (Figure 6.1). In both methods, the AIM obtained after the alkaline treatment was neutralized with HCl 1 M and extensively washed with deionized water, similarly to Method V (Figure 6.1). The harsher alkaline treatments were intended to improve the solubility of alkaline-soluble components of the yeast cell wall and increase their removal efficiency during washing. Both methods achieved the anticipated results, with the lowest protein content of this study (3.0 wt%) and a residual inorganic salts content (1.8 and 0.9 wt%, for Methods VI and VII respectively) (Table 6.1).

Considering all extraction procedures tested, Method VII represents the most appropriate technique to achieve the highest purity of CGC_{pure} with a final yield of 13.1 % (Table 6.1). Consisting in three main steps: alkaline extraction with NaOH 5 M, at 65 ºC for 2 h, neutralization with HCl 1 M, and washings against deionized water until a conductivity value below 20 µS/cm (keeping the pH between 6-8), this procedure is simple and avoids the use of organic solvents and/or saline solutions (ethanol and PBS). The polymer obtained with the

standard method, CGC_{PBS}, was a slightly grey powder, forming large and dense granules, while CGC_{pure}, arising from method VII, was a brownish powder, composed by low density sheets (Figure 6.1). CGC_{pure} obtained from *K. pastoris* biomass using Method VII had purity similar to KiOsmetine®, the CGC extracted from *Aspergillus niger* mycelium that has an inorganic salts content below 2 wt% and a protein content below 10 wt% (GRAS notice No. 412, 2012; http://www.fda.gov).

Higher reaction time or higher alkalinity, in methods VI and VII, respectively, resulted in polymers with a higher chitin content, as shown by the chitin:β-glucan molar ratio of 25:75 (Table 6.1). This chitin enrichment of the co-polymer can be accounted by an improvement of the removal of alkali soluble β-glucans provided by the alkaline treatment of the methods. The chitinous content of the polymers in the cell wall of fungi is strain dependent and is well-known to be affected by the cultivation conditions (Nguyen, Fleet and Rogers, 1998). Moreover, as shown by this study, the extraction yield of such biopolymers is highly dependent on the procedure used. Values within the range 12-36 wt% have been reported for chitinous polymers extracted from the biomass of different fungi, including *A. niger* (Feofilova *et al.*, 2006), *Saccharomyces cerevisiae* (Nguyen, Fleet and Rogers, 1998) and *Schizophyllum commune* (Smirnou, Krcmar and Prochazkova, 2011).

6.4.2. Polymer Characterization

The CGC_{pure} biopolymer purified according to the optimized extraction (Method VII), as well as CGC_{PBS} obtained with the standard method described by Roca *et al.* (2012), were characterized in terms of their physical-chemical properties. For comparison, commercial biopolymers were also subjected to the same analyses, namely: commercial chitin extracted from crab shell, laminarin, a β-glucan extracted from the algae *Laminaria digitata*, and KiOsmetine®, the CGC extracted from *A. niger* mycelium.

6.4.2.1. Infrared spectroscopy

Figure 6.2 presents the FTIR spectra of CGC_{PBS} and CGC_{pure} samples extracted from *K. pastoris* biomass. For comparison, spectra of crab shell chitin, laminarin and KiOsmetine® were

also included. All spectra are very similar, presenting the characteristic bands of chitin and β-glucans, which are summarized in Table 6.2.

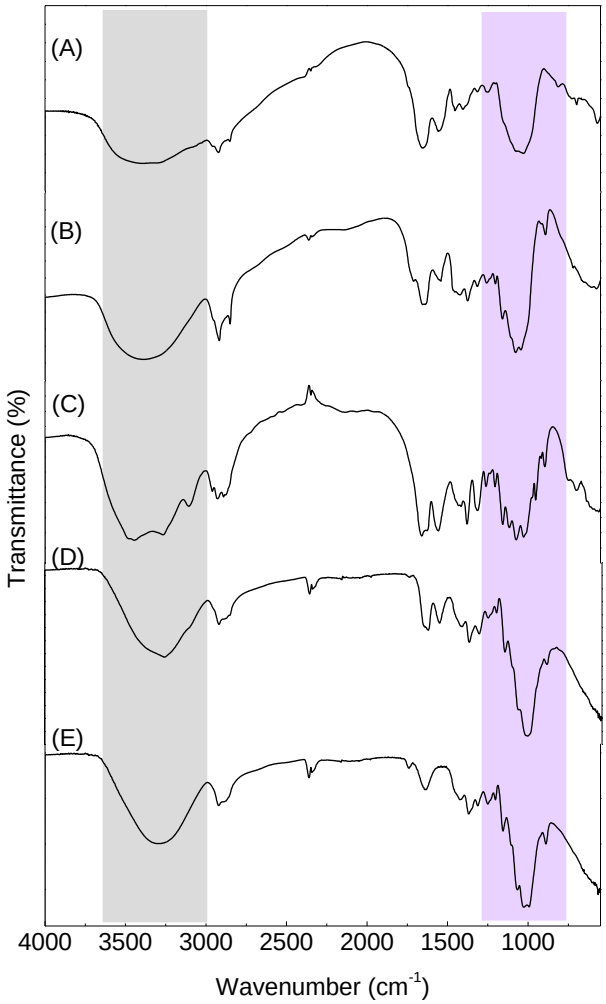

Figure 6.2 – FTIR spectra of (A) CGC_{PBS}, (B) CGC_{pure}, (C) crab shell chitin, (D) KiOsmetine® and (E) Laminarin.

Table 6.2 – FTIR spectral values of the main bands for the polymer samples: crab shell chitin, Laminarin, KiOsmetine®, CGC_{PBS} and CGC_{pure}.

Vibration Modes	Chitin (cm^{-1})	Laminarin (cm^{-1})	KiOsmetine® (cm^{-1})	CGC_{PBS} (cm^{-1})	CGC_{pure} (cm^{-1})
C-H deformation (β-glycosidic bond) [1, 2, 3]	893	890	891	-	893
C-OH stretching [1, 2, 3]	1024	1022	1020	1024	1041
C-OH stretching [1, 2, 3]	1074	1047	1068	1079	1076
C-O-C stretching (asymmetric) [1, 3]	1158	1155	1151	1150	1159
O-H in-plane bending and C-O stretch (symmetric) [3]	1205	1173	1205	1203	1203
N-H deformation (Amide IV) [1, 3]	1261	1250	1253	1257	1258
Amide III band and CH_2 wagging [1, 2, 3]	1315	1311	1311	1313	1313
CH bending and symmetric CH_3 deformation [1, 2, 3]	1379	1367	1375	1370	1376
CH_2 bending and CH_3 deformation [1, 2, 3]	1415	1421	1429	1407	1418
Amide II band [1, 2, 3]	1557	-	1558	1554	1542
Amide I band [1, 2, 3]	1660 1623	-	1654 1623	1657	1657
C-H stretching [1, 3]	2875 2890	2881	2854	2849	2848
CH_2 stretching (symmetric) [1, 2, 3]	2929	2922	2923	2919	2916
CH_3 stretching (asymmetric) [1, 3]	2962	-	2958	2961	2958
N-H stretching (symmetric) [1, 2, 3]	3107	Band	Band	Band	Band
N-H stretching (asymmetric) and O-H···O stretching (intermolecular hydrogen-bonded) [1, 3]	3263	Band	Band	Band	Band
OH stretching [1, 2, 3]	3447	Band	Band	Band	Band

* 1 – Cardenas *et al.* (2004); 2 – Jalal *et al.* (2012); 3 – Lu *et al.* (2013)

CGC_{PBS}, CGC_{pure} and KiOsmetine® spectra (Figure 6.2A, B and D, respectively) show a broad and intense band around 3400 cm^{-1}, common to all polysaccharides, which represents O-H stretching of hydroxyls groups (Jalal, Risheed and Ibrahim, 2012; Lu *et al.*, 2013; Synytsya and Novak, 2014). Also in all CGCs samples, this band overlaps with the asymmetric and symmetric N-H stretching peaks appearing at 3107 and 3263 cm^{-1} in the crab shell chitin spectra. The peaks appearing between 2848 and 2958 cm^{-1} can be assigned to the C-H stretching of CH_2 and CH_3 groups. They are also present in the spectra of the other studied polysaccharides (Synytsya and Novak, 2014).

The more obvious structural differences between the studied polymers are associated with the frequency of the vibration modes of amide I, in the region 1660-1620 cm^{-1} (Figure 6.2). In the chitin and KiOsmetine® spectra, two small and broad bands are observed (at 1660-1623 and 1654-1623 cm^{-1}, respectively), while for CGC_{PBS} and CGC_{pure} polymers a large single band is seen in the same region. As proposed by Cárdenas *et al.* (2004), those peaks can be attributed

to the different polymorphic forms of chitin present in each polymer. However, the lower resolution of those peaks, especially in *K. pastoris* CGCs samples, makes it difficult assessing chitin polymorphism. Laminarin, a commercial β-glucan, also shows a peak at 1631 cm^{-1} (Figure 6.2E), probably due to the bending vibration of water molecules, present in this sample. Cardenas *et al.* (2004) also found distinctive patterns for α and β-chitin in the 3500-3400 cm^{-1} region, relative to O-H and N-H stretch. For α-chitin, two peaks are present at 3479 and 3448 cm^{-1}, while for β-chitin only one peak appears at 3426 cm^{-1}. However, in the CGC_{pure}, CGC_{PBS} and KiOsmetine® spectra only one single broad band appears in this region masking any other additional characteristic peaks.

FTIR is also informative on the nature of the linkages existing between β-glucan units (Thanardkit *et al.*, 2002; Corradi da Silva *et al.*, 2008). The small peaks noticed at 890, 1156 and 1370 cm^{-1} for CGC_{pure}, KiOsmetine® and laminarin should be assigned to β-(1,3)-glucans linkage. However chitin spectra (Figure 6.2C) also disclosed these small peaks, probably because within this bandwidth region (950-1150 cm^{-1}) the signal of C-OH, C-O-C and C-C linkages of the pyranose ring, common to glucose and acetylglucosamine units are also present. Peaks around 920, 1045 e 1730 cm^{-1} are associated to the β-(1,6) linkage, but their presence in spectra of CGCs samples is doubtful (Thanardkit *et al.*, 2002). An observation which could be explained by a higher content of β-(1,3)-glucans in *K. pastoris* CGC samples compared to β-(1,6)-glucans.

6.4.2.2. ^{13}C solid-state NMR

Figure 6.3A shows the ^{13}C CPMAS spectrum of CGC_{pure}. The ^{13}C resonances centred at the chemical shifts (δ) of 103.5, 85.7, 74.2, 68.5 and 62.0 ppm correspond, respectively, to the anomeric C1, C3, C2,5, C4,6$^{\#}$ and C6 chemical environments of the β-glucan monomers (Thanardkit *et al.*, 2002; Roca *et al.*, 2012; Synytsya and Novak, 2014). Many of these ^{13}C resonances are overlapped with the resonances arising from the chitin monomer. Figure 6.3B shows which of those peaks may potentially overlap. The resonances at δ = 103.5 ppm (C1) shows that the linkage between glucan monomers is of β type, confirming the presence of β-glucans in CGC_{pure} (Synytsya and Novak, 2014). The chemical shift at 85.7 ppm (C3) confirms the presence of β-(1-3) linkages in C3 of β-glucan monomers and indicates a triple helix conformation of β-glucan chains (Fričová and Koval'aková, 2013). These assignments are in agreement with FTIR results, where the peaks associated to this type of glycosylic linkage were also observed. Lipke and Ovalle (1998) also reported the existence of β-(1-6) linkage in yeast cell wall and, for this reason, C6 might be substituted (C6$^{\#}$) or not (C6). The ^{13}C CPMAS spectrum, shows two peaks at 62.0 and 68.5 ppm which coincide with typical chemical shifts

for free and substituted C6#, respectively (Corradi da Silva *et al.*, 2008). However, in CGC_{pure} and CGC_{PBS}, reported in Roca *et al.* (2012), the C6# resonance (arising from β-(1-6) linkages) is not very clear (δ ≈ 68.7 ppm), probably due to signal masking induced by a higher content of β-(1-3) linkages in the β-glucan fraction (Thanardkit *et al.*, 2002; Roca *et al.*, 2012).

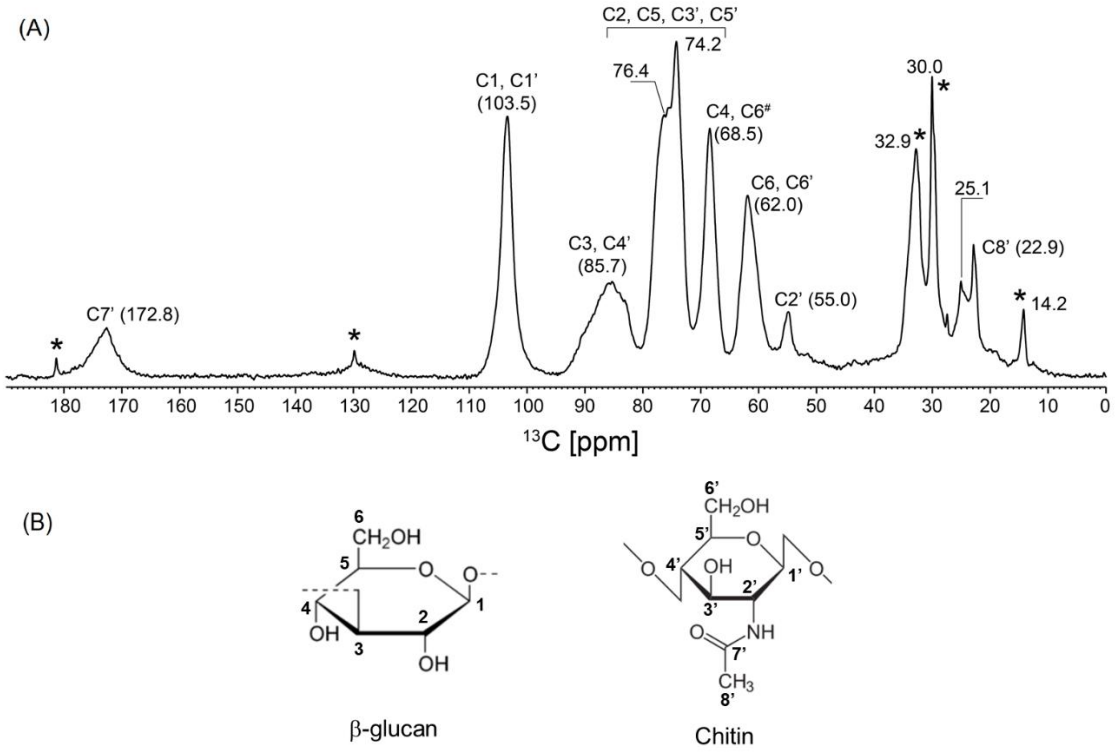

Figure 6.3 – ^{13}C CPMAS-NMR spectrum of CGC_{pure} (A). Resonance assignment is shown in agreement with the atom labelling shown (B) in the β-glucan and chitin molecular structures. # represents the carbon resonances involved in β-(1-6)-glycosilic bonds. Apostrophe represents the carbon resonances of chitin. Asterisks depict impurities.

The ^{13}C resonances from the chitin monomers (Figure 6.3A) are also clearly observed at δ ≈ 172.8, 55.0 and 22.9 ppm, which are assigned, respectively, to the carbonyl (C7') from acetyl groups, the carbon (C2') involved in the C-N bond, and the methyl group (C8'), from acetyl groups (Cárdenas *et al.*, 2004; Kumirska *et al.*, 2010) . The resonances of C1', C3', C4', C5' and C6' from chitin overlap to the ones from the β-glucan residues (Figure 6.3A). The resonance at δ ≈ 85.7 ppm is broad and shows contributions from C3 (β-glucan) and C4' (chitin) carbons, both related with glycosylation linkages (Kumirska *et al.*, 2010; Fričová and Koval'aková, 2013). It is also worth to point out the similarity of the CGC_{pure} spectrum (Figure 6.3A) to the CGC_{PBS} spectrum reported earlier by Roca *et al.* (2012) where the same ^{13}C

resonances have been observed. Comparing the CGC_{pure} with the chitin and laminarin spectra, also reported in Roca *et al.* (2012), NMR analysis confirms the higher β-glucan molar content of CGC_{pure} obtained in HPLC sugar composition analysis, due the similarity between the ^{13}C CPMAS spectrum presented in our work and laminarin, especially in the region of $\delta \approx$ 50-120 ppm (Roca *et al.*, 2012).

Besides the similarities between CGC_{pure} and commercial β-glucan NMR spectra, the polymorphic form of *K. pastoris* CGC_{pure} chitin cannot be unambiguously confirmed due to the masking effect of the β-glucans resonances of C2 and C5 ($\delta \approx$ 74.4 ppm) on the spectral region characteristic of α and β-chitin. Indeed, as reported by Cárdenas *et al.* (2004), α-chitin spectra has two peaks at 73 and 75 ppm, while the β-chitin has a single peak at 74 ppm.

Several peaks, identified with asterisks in Figure 6.3A, are observed at $\delta \approx$ 181.5, 128.7, 32.9, 30.0 and 14.2 ppm. Their presence is likely to be associated to minor quantities of lipids or proteins residues in this biopolymer (Synytsya and Novak, 2014).

6.4.2.3. X-ray diffraction

Despite its low resolution, the X-ray powder diffractogram of CGC_{pure} (Figure 6.4B), exhibited the main characteristic diffraction peaks of chitin, at 2θ angle of 9.4º, 12.8º, 19.3º and 20.9º, corresponding to (020), (101), (110) and (120) planes, respectively (Cárdenas *et al.*, 2004). CGC_{pure} analysis also revealed a reflection at $2\theta = 6º$, which can be related to the presence of β-glucans, since it is present in laminarin analysis and is absent in chitin sample (Figure 6.4B, C and E). Similar diffraction pattern have been observed for KiOsmetine® (Figure 6.4D) and was also reported for CGC extracted from *S. cerevisiae* (Beran, Holan and Baldrián, 1972) or *Ganoderma lucidum* (Ospina Álvarez *et al.*, 2014). For CGC_{PBS} it was not possible to identify the characteristic peaks of chitin, due to the masking action given by the crystalline salts contaminating this sample.

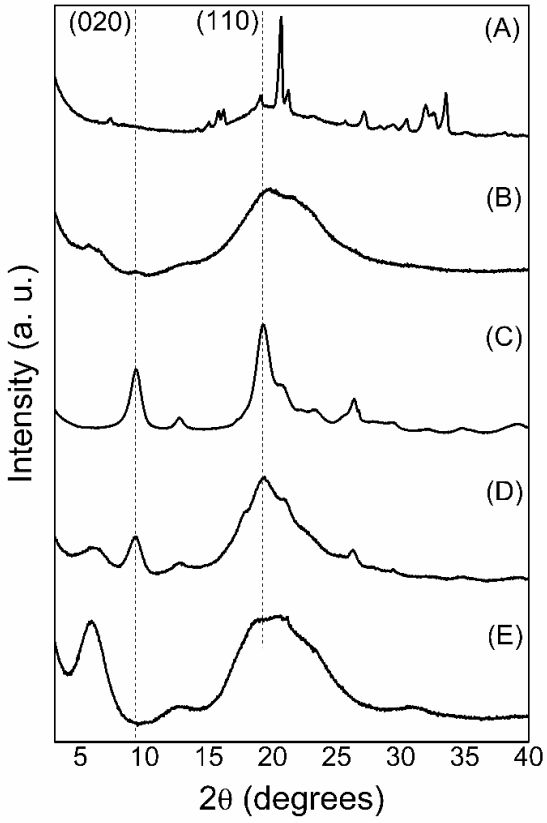

Figure 6.4 – X-ray powder diffractograms of (A) CGC_{PBS}, (B) CGC_{pure}, (C) crab shell chitin, (D) KiOsmetine® and (E) Laminarin.

As suggested by Kumirska *et al.* (2010), a small shift to higher angles is noticeable in the CGC_{pure} and KiOsmetine® X-ray diffractograms; a difference which can be assigned to a lower acetylation degree. The larger peaks observed at the (110) plane might be indicative of a reduction in crystallinity index (CI) of CGC_{pure} and KiOsmetine® compared with crab shell chitin (Figure 6.4C). Laminarin, an amorphous β-glucan, also exhibits a large peak at this plane similar to CGC samples, which confirms the amorphous nature of these fungal CGCs, probably due to the higher β-glucan content in CGC. This result is in accordance with Novák *et al.* (2012), where no crystalline peak was observed in *S. cerevisiae* β-glucan XRD spectra.

From the XRD analysis, it is also possible to evaluate the chitin polymorphs present in CGCs samples. Kumirska *et al.* (2010) reported the X-ray pattern differences between α and β-chitin. For α-chitin the X-ray pattern includes four crystalline peaks at 9.6, 19.6, 21.1 and 23.7°, while for β-chitin it includes only two peaks at 9.1 and 20.3° (Kumirska *et al.*, 2010). Due to the poor resolution in CGC_{pure} sample (Figure 6.4B), in the (110) plan, the type of linkage in chitin chain cannot be discriminated. However, the broad peak at $2\theta \approx 19\text{-}23^{o}$, is indicative that most

contributions characteristic of α-chitin are present. In Figure 6.4D, the KiOsmetine® X-ray pattern is better-resolved compared to CGC_{pure}, allowing the identification of three peaks, 19.3 º, 20.9º and 26.3º, supporting that *A. niger* CGC has an α-chitin in its structure. At 2θ = 9.4º, both CGC_{pure}, KiOsmetine® and crab shell chitin exhibit a peak revealing that the chitin polymorphic form in these three samples is the same.

From the intensity ratio of the peaks at (110) plane (at 2θ = 19º) to the amorphous region (at 2θ = 16º), a crystallinity index (CI) of 50 % and 62 % have been calculated for CGC_{pure} and KiOsmetine® respectively (Table 6.3). These results confirm the lower crystallinity of the fungal CGC co-polymers compared with crab shell chitin characterized by a CI of 85%. The CI obtained for crab shell chitin is in accordance with the CI values reported for crustacean α-chitin, 76.2-82.7 % (Cárdenas *et al.*, 2004). The lower crystallinity of the fungal CGC can be anticipated from the introduction of the β-glucans in the co-polymers structure. Ivshin *et al.* (2007) reported a CI of 60 % for *Armillariella mellea* CGC, with a chitin content of 80 wt%, a value similar to *A.* niger CGC, i.e. 62 % as measured for KiOsmetine®, whose chitin content ranges between 25-60 mol% (GRAS notice No. 412, 2012; http://www.fda.gov). *K. pastoris* CGC_{pure} has a lower CI probably due to its lower chitin content, 24.6 mol%.

Table 6.3 – Physico-chemical properties comparison of *K. pastoris* CGCs with chitin and/or β-glucan containing polymers from other strains and commercial polymers (CI: crystallinity index; Mw: molecular weight; T_{deg}: degradation temperature).

Polymer	Microorganism	CI (%)	Mw (Da)	T_{deg} (ºC)	Refs.
CGC_{PBS}	*Komagataella pastoris*	(*)	4.8×10^5	312	This study
CGC_{pure}	*Komagataella pastoris*	50	4.9×10^5	315	This study
CGC	*Aspergillus Niger* (KiOsmetine®)	62	n.a.	292	This study
	Armillariella mellea	60	n.a.	n.a.	Ivshin *et al.* (2007)
Chitin	Crab shells	85	n.a.	365	This study
	Pleuroncodes monodon	76	n.a.	n.a.	Cárdenas *et al.* (2004)
	Ganoderma lucidum	n.a.	n.a.	314	Ospina Álvarez *et al.* (2014)
	Aspergillus niger	n.a.	$1.3\text{-}1.6\times10^5$	n.a.	Teng *et al.* (2001)
β-glucan	*Laminaria digitata* (Laminarin)	n.a.	n.a.	275	This study
	Saccharomyces cerevisiae	n.a.	n.a.	267	Novák *et al.* (2012)
	Ganoderma lucidum	n.a.	3.75×10^6	n.a.	Liu *et al.* (2014)

(*) It was not possible to determine the CI of CGC_{PBS} due to its high inorganic salts content; n.a. – data not available

6.4.2.4. Thermal properties

The thermal degradation curve of CGC_{pure} (Figure 6.5B) is indicative of its thermal resistance up to 200 °C (Δm = 2% at this temperature). A single weight loss phase is noticed with a maximum degradation temperature (T_{deg}) at 315 °C, with a char yield of 17.3%, at 500 ºC. Crab sell chitin showed a similar behaviour, except made of a T_{deg} occurring at a higher temperature (365 ºC), with a char yield of 28.4%, at 500 ºC (Figure 6.5C). The lower thermal stability of CGC_{pure} came from the reduction in crystallinity imposed by the β-glucan moieties compared to pure chitin. As can be seen in laminarin thermogravimetric analysis (TGA) (Figure 6.5E), the T_{deg} of this β-glucan is the lowest of all analysed samples (275 ºC). The degradation temperature of crustacean chitin has been reported to be 381-385 ºC (Kaya *et al.*, 2015), while lower degradation temperatures have been reported for yeast β-glucans, as *S. cerevisiae* β-glucan, where the onset temperature of the decomposition is 267 ºC (Novák *et al.*, 2012) (Table 6.3).

The CGC_{PBS} decomposed at a similar temperature compared to CGC_{pure}, showing a T_{deg} at 312 ºC. From Figure 6.5A, it is visible that CGC_{PBS} is a less pure polymer, compared to CGC_{pure}, since a higher char yield was obtained at 500 ºC (43.3%). Interestingly enough, the thermogravimetric behavior of KiOsmetine® was composed of two steps, with a weight loss between 250 and 350 °C and T_{deg} at 292 ºC. These two decomposition steps presented in KiOsmetine® are shown at 287 ºC and 350 ºC (Figure 6.5D). The region at 287 ºC can be related to the degradation of the saccharide structure of this sample, including dehydration of saccharide rings and decomposition of acetylated and deacetylated units of chitin, and the region at 350 ºC can be related with the complete degradation of the polymer (Ospina Álvarez *et al.*, 2014).

The CGC_{pure} differential scanning calorimetry (DSC) curve (Figure 6.5B) also showed an endothermic peak corresponding to the polymer's decomposition, with a maximum at 315 ºC. This decomposition temperature is similar to that reported by Ospina Álvarez *et al.* (2014), 313.6 ºC, for the decomposition of chitin extracted from *G. Lucidum* biomass (Table 6.3). CGC_{pure} also showed a similar behaviour than chitin. The DSC curve of chitin also presented an endothermic decomposition at a higher degradation temperature (365 ºC). By comparing DSC with TGA analysis of CGC_{pure} and chitin (Figure 6.5B and C), it was confirmed that the endothermic decomposition peaks (at 315 ºC and 365 ºC, respectively) are related to polymers degradation.

DSC analysis of laminarin showed a completely different behaviour confirming that β-glucans have different thermal properties when compared with chitinous polymers (Figure 6.5E). Laminarin presented an endothermic and exothermic decompositions related to polymer

degradation at 266 ºC and 277 ºC, respectively. This different behaviour in β-glucan thermal analysis is in accordance with *S. cerevisiae* β-glucan thermal properties reported earlier by Novák *et al.* (2012).

Also in accordance with TGA analysis, DSC spectra of CGC_{PBS} and KiOsmetine® show two decomposition peaks, which are both exothermic for CGC_{PBS} at 207 and 312 ºC (Figure 6.5A). In contrast KiOsmetine® DSC spectra are made from an exothermic peak at 250 ºC and an endothermic at 292 ºC (Figure 6.5D). The exothermic decomposition event observed for both polymers can be ascribed to contaminants, inorganic salts and/or proteins, well-known to influence the melting event of polysaccharides in this range of temperatures (Lee, Thomas and Schmidt, 2011). The decomposition peaks near or above 300 ºC in all the analysed samples is typically observed during the degradation of saccharide structure and may involve dehydration of saccharide rings and decomposition of acetylated and deacetylated units of chitin (Ospina Álvarez *et al.*, 2014).

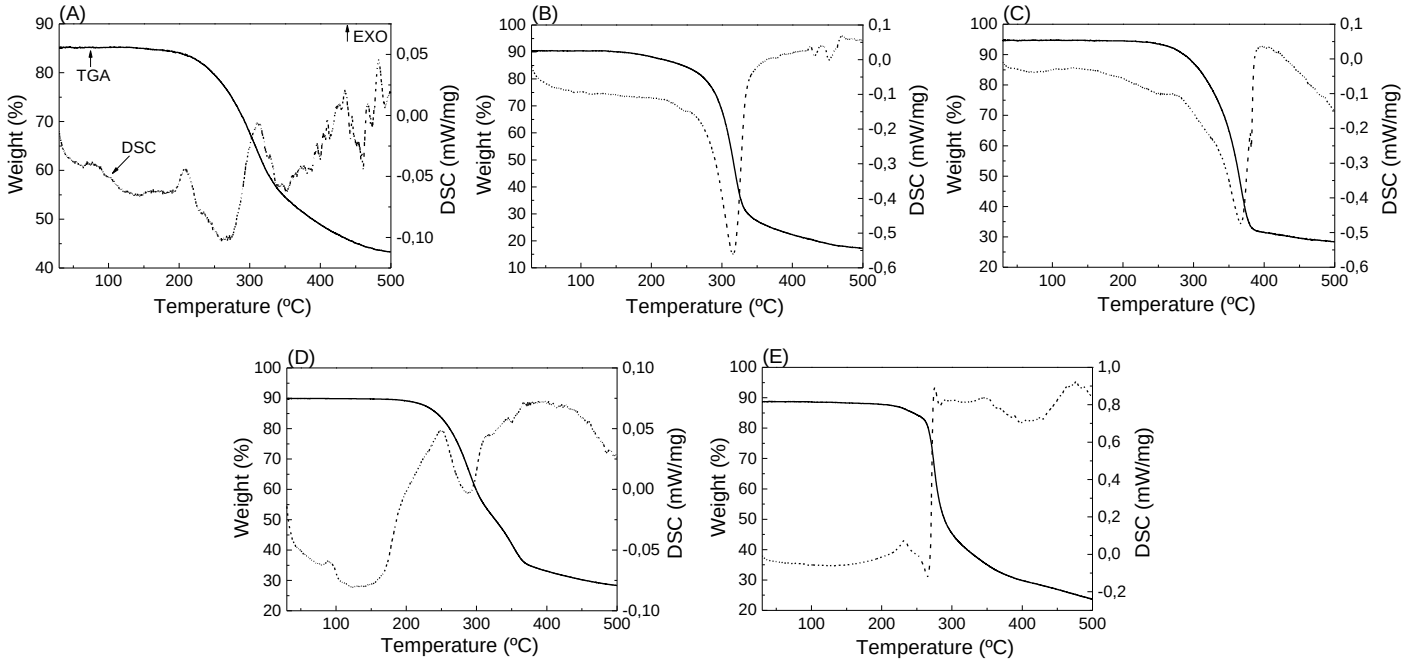

Figure 6.5 – Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) curves of (A) CGC_{PBS}, (B) CGC_{pure}, (C) crab shell chitin, (D) KiOsmetine® and (E) Laminarin.

6.4.2.5. Molecular weight analysis by Size Exclusion Chromatography (SEC)

Molecular weight is an important parameter in polymers characterization, since it can influence various physical properties such as, viscosity or tensile strength. For chitinous polymers, like CGC, the determination of the molecular weight is particularly difficult because of its low solubility in the majority of organic solvents. In this work, the average molecular weight (M_w) was determined for *K. pastoris* CGC_{PBS} and CGC_{pure}, by dissolving those biopolymers in a solvent system composed of dimethylacetamide and lithium chloride (DMAc/LiCl). This solvent strategy is commonly reported for chitin solubilisation (Pillai, Paul and Sharma, 2009). For CGC_{PBS} and CGC_{pure}, identical M_w values were obtained, of 4.8×10^5 Da and 4.9×10^5 Da, respectively (Table 6.3). The polymers polydispersity index (PDI) values were also similar, 1.5 and 1.7, respectively. These results show that the downstream procedures performed for polymer extraction and purification influenced neither the molecular weight nor the samples homogeneity. Samples with a PDI below 2, as both CGCs samples, have a relatively narrow molecular weight distribution and, consequently, a more homogeneous structure.

The M_w of *K. pastoris* CGC polymers is of the same order of magnitude of that of crustacean chitin (1.9×10^5 Da) and also of chitin extracted from *A. niger* mycelium ($1.3\text{-}1.6\times10^5$ Da) (Teng *et al.*, 2001). On the other hand, higher M_w values were reported for yeast β-glucans, such as those extracted from *G. lucidum* (3.75×10^6 Da) (Liu *et al.*, 2014) (Table 6.3).

6.5. Conclusions

A simple organic solvent free procedure was developed for extraction and purification of chitin-glucan complex from *Komagataella pastoris* biomass. Our optimised methodology avoids the use of large volumes of PBS solution and ethanol, used in previous studies. Hence, besides being more environmental friendly, this new bioprocessing approach also contributes to cut down the overall production costs of *K. pastoris* CGC. The resulting polymer, CGC_{pure}, has low contents of inorganic salts and proteins, key properties to envisage high added value applications, including cosmetics and pharmaceuticals.

Chapter 7

Extraction and characterization of cell-wall polysaccharides from *Komagataella pastoris*

7.1. Abstract

Optimized conditions for the hot alkaline extraction of the cell-wall polysaccharides chitin-glucan complex (CGC) and mannans from the yeast *Komagataella pastoris* were defined by response surface methodology. Extraction with NaOH 4 M, at 85 ºC, for 4 h, yielded both polysaccharides with high purity degrees (95 and 82% for CGC and mannans, respectively). The CGC thus obtained had a chitin:β-glucan molar ratio of 13:87 and decomposed at 330 ºC. The mannans, on the other hand, contained three fractions with different molecular weight, mainly composed of mannose, with a glucose content of 4.3 wt%. The use of harsher extraction conditions leads to the reduction of the polymer's protein content from 24.6 to only 8.6 wt%, while the decomposition temperature was lowered from 312 to 253 ºC. This study resulted in a simple procedure to simultaneously obtain two valuable polysaccharides with purity, which can find use in pharmaceutical, cosmetic or food applications.

7.2. Introduction

The yeast cell-wall represents up to 25% of the dry cell weight and consists of about 80-90% of polysaccharides and 10-20% of proteins (Nguyen, Fleet and Rogers, 1998; Liu *et al.*, 2016). The polysaccharides fraction includes β-glucans (up to 60%), α-mannans (up to 40%) and chitin (about 1-2%) (Aguilar-Uscanga and Francois, 2003; Bzducha-Wróbel, Kieliszek and Błażejak, 2013). Mannans often appear covalently linked to proteins, forming mannoproteins (Bzducha-Wróbel *et al.*, 2014).

The fractionation of the yeast cell-wall for extraction of its components, namely polysaccharides, can be performed by mechanical (e.g., bead mill, ultrasonication) or non-mechanical methods (e.g., enzymatic, chemical), or a combination of both (Thammakiti *et al.*, 2004; Bzducha-Wróbel *et al.*, 2014; Pinto *et al.*, 2015). Mechanical methods are less expensive but are non-selective and require a higher energy consumption, while the non-mechanical methods, although being costlier, are more product selective and have lower energy requirements (Liu *et al.*, 2016).

The most commonly used methods for extraction of cell-wall polysaccharides include the hot alkaline treatment (NaOH or KOH, 0.25-5.0 M; 60-130 ºC) of the biomass (Thammakiti *et al.*, 2004; Kim and Yun, 2006; Maru *et al.*, 2015). That treatment results in the fractionation of the biomass into alkali-insoluble material (AIM) and alkali-soluble material (ASM) fractions

(Bzducha-Wróbel, Kieliszek and Błażejak, 2013). The AIM contains chitin and β-glucans that are usually covalently linked in the form of the co-polymer chitin-glucan complex (CGC) (Feofilova *et al.*, 2006; Smirnou, Krcmar and Prochazkova, 2011). Mannose homo- or heteropolysaccharides, as well as mannans, can be recovered from the ASM (Araújo *et al.*, 2014; Liu *et al.*, 2015; Galinari *et al.*, 2017).

Komagataella pastoris (formerly known as *Pichia pastoris*), a methylotrophic yeast widely used for the production of recombinant proteins (Çalık *et al.*, 2015; Spohner *et al.*, 2015), was recently proposed as a source of CGC and mannans in a patented bioprocess (Reis *et al.*, 2010; Freitas, Roca, *et al.*, 2013; Freitas *et al.*, 2015). The hot alkaline treatment of *K. pastoris* biomass with NaOH (1-5 M), at 65 ºC, for 2-5 h, yielded a CGC content in the biomass of 13-19 wt% (Chapter 3 and 6 and Roca *et al.*, 2012). The extraction yield, polymer purity and composition were demonstrated to be affected by the extraction procedure. *K. pastoris* mannans fraction was not previously characterized.

Therefore, this study was focused on the development of an optimized procedure for extraction of both CGC and mannans from *K. pastoris* biomass. Response surface methodology (RSM) based on the central composite rotatable design (CCRD) model was used to evaluate the impact of temperature, reaction time and alkali concentration on polymers' extraction efficiency (yield and purity). The impact of the tested conditions on CGC and mannans composition were also assessed. Moreover, the CGC and mannans fractions were characterized in terms of their physical and chemical properties.

7.3. Materials and methods

7.3.1. Cell-wall polysaccharides extraction

Yeast biomass was obtained by cultivation of *Komagataella pastoris* DSM 70877 as described in 3.4.2 section of Chapter 3. For each extraction experiment, culture broth samples (30 mL) were diluted with deionized water (1:2, v/v) and centrifuged at 8,000 × *g*, during 10 min. The biomass pellet was resuspended in 60 mL of a NaOH aqueous solution and submitted to a thermal treatment according to the experimental design (Table 7.1). After the hot alkaline treatment, the suspension was centrifuged (8,000 × *g*, 10 min), to separate the alkaline insoluble material (AIM) from the alkaline soluble material (ASM). The AIM fraction was resuspended in 60 mL of deionized water and its purification was performed as described in 3.3.4 section of Chapter 3.

The ASM fraction was neutralized with HCl 4 M and dialyzed with a 12,000 MWCO membrane (Nadir®- dialysis tubing, Carl Roth) against deionized water. The deionized water was changed twice a day, until a conductivity below 20 μS/cm was achieved. The dialyzed samples were freeze dried, for the gravimetric quantification of the mannans fraction.

7.3.2. Experimental design

The experimental design was based on a central composite rotatable design (CCRD) that included three independent variables: temperature, T (ºC, X_1), reaction time, t (h, X_2) and NaOH concentration, [NaOH] (M, X_3). The observed responses were: CGC extraction yield (wt%, Y_1), mannans extraction yield (wt%, Y_2), CGC purity (%, Y_3) and mannans purity (%, Y_4). It included seventeen experiments (Lundstedt *et al.*, 1998), performed randomly: eight factorial design points at levels ± 1 (Exp. 1-8), six axial points at levels $\alpha = \pm 1.682$ (Exp. 9-14), and a central point with three replicas (Exp. 15-17) (Table 7.1).

The independent variables of the CCRD were expressed as a quadratic model (Equation 7.1):

$$Y_p = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (7.1)$$

where Y_p corresponds to the predicted responses, X_1, X_2 and X_3 are the coded values for the three independent variables and β_0, β_1, β_2 and β_3 are the regression coefficients, where β_0 is the interception, β_1, β_2 and β_3 are the linear terms, β_{11}, β_{22} and β_{33} are the quadratic terms and β_{12}, β_{13} and β_{23} are the interaction terms.

The statistical analysis was performed using the software Statistica 7.0 (StatSoft Inc., Tulsan, UK). The model was fitted to experimental data through the analysis of variance (ANOVA) and by multiple linear regression (MLR). The fitted model was evaluated for each observed response based upon the multiple correlation coefficients (R^2), the regression parameter significance (*p*-value) and the tested lack of fit. All the factors and their interactions were also evaluated by the *p*-value at a 95% confidence level. The effect of the three independent variables on the observed responses was given by statistical analysis and surface plot analysis.

7.3.3. Validation experiments

For the validation of the experimental design model, three experiments were performed, namely, Exp. A with NaOH 1 M and Exp. B with NaOH 5 M, both at a temperature of 65 ºC, during 2 h, as described in Methods 5 and 7 of 6.3.2 section of Chapter 6, and Exp. C with NaOH 4 M, at 84 ºC during 4 h.

7.3.4. Polysaccharides characterization

For the compositional analysis, the polysaccharides were hydrolyzed as described in 4.3.5 section of Chapter 4. The total protein content was determined as described in 4.3.6 section of Chapter 4. The inorganic salts content was evaluated by pyrolysis decomposition of the samples (≈ 50 mg) at a temperature of 550 ºC, for 24 h. The mannans average molecular weight (Mw) was determined by Size Exclusion Chromatography with Multi-Angle Light Scattering (SEC-MALS) as described in 6.3.3.6 section of Chapter 6. Fourier transform infrared spectroscopy (FTIR) was carried and thermogravimetric measurements (DSC/TGA) were carried out as described in sections 6.3.3.2 and 6.3.3.5, respectively, of Chapter 6.

7.4. Results and discussion

7.4.1. CGC and mannans extraction

The conditions used for the hot alkaline extraction of CGC from *K. pastoris* biomass, namely the NaOH concentration and the reaction time, were demonstrated to impact on the extraction yield and the co-polymer's purity (Chapter 6). In fact, increasing the alkali concentration or extending the reaction time resulted in CGC with higher purity degree. Moreover, the co-polymer had a higher chitin:β-glucan molar ration (25:75). In previous studies, the ASM was discarded and *K. pastoris* mannans fraction was not recovered nor characterized. In this study, response surface methodology (RSM) and the central composite rotatable design (CCRD) were used to develop an optimized method for extraction of both CGC and mannans from *K. pastoris* biomass.

Table 7.1 – Central composite rotatable design (CCRD) with three independent variables, X_1 (temperature, T), X_2 (reaction time, t) and X_3 (NaOH concentration, [NaOH]), and the observed responses studied: Y_1 and Y_2 (CGC and Mannans extraction yields, respectively); Y_3 and Y_4 (CGC and Mannans purity, respectively).

Experiment	T (ºC) X_1	t (h) X_2	[NaOH] (M) X_3	Extraction yield (wt%)		Purity (%)	
				CGC Y_1	Mannans Y_2	CGC Y_3	Mannans Y_4
1	66.1	1.8	1.4	16	33	90	59
2	83.9	1.8	1.4	14	28	93	65
3	66.1	4.2	1.4	15	30	93	63
4	83.9	4.2	1.4	13	25	93	68
5	66.1	1.8	4.1	16	23	91	74
6	83.9	1.8	4.1	15	19	93	83
7	66.1	4.2	4.1	15	21	91	76
8	83.9	4.2	4.1	15	18	93	80
9	60.0	3.0	2.75	15	26	92	72
10	90.0	3.0	2.75	14	20	94	74
11	75.0	1.0	2.75	15	23	93	67
12	75.0	5.0	2.75	13	23	94	72
13	75.0	3.0	0.5	16	31	93	65
14	75.0	3.0	5.0	15	22	92	78
15	75.0	3.0	2.75	14	30	93	72
16	75.0	3.0	2.75	13	23	93	75
17	75.0	3.0	2.75	14	26	93	70

7.4.1.1. Experimental analysis

CGC and mannans were extracted from *K. pastoris* biomass using different conditions of temperature (60-90 ºC), reaction time (1-5 h) and NaOH concentration (0.5-5 M) (Table 7.1). Each parameter range was selected considering that in previous studies CGC was extracted from *K. pastoris* biomass using hot alkaline procedures involving the use of 1 or 5 M of NaOH, at 65ºC, during 2 or 5 h (Chapter 6 and Roca *et al.*, 2012). Moreover, several studies described the extraction of yeast and fungal cell-wall polysaccharides using temperatures ranging from 80 to 100 ºC, alkali concentrations between 0.25 and 4.2 M, and reaction times of 2-6 h (Thammakiti *et al.*, 2004; Kim and Yun, 2006; Smirnou, Krcmar and Prochazkova, 2011; Maru *et al.*, 2015; Galinari *et al.*, 2017).

As shown in Table 7.1, the CGC extraction yield ranged between 13 and 16 wt%. These values are within the range of the ones reported for CGC extraction from *K. pastoris*, 13-19

wt% (Chapters 3 and 6 and Roca *et al.*, 2012), and from other yeasts and fungi, 15-30 wt% (Nguyen, Fleet and Rogers, 1998; Feofilova *et al.*, 2006; Zlotnikov *et al.*, 2007; Smirnou, Krcmar and Prochazkova, 2011), using alkaline conditions. The highest values were obtained for Exp. 1 and 5, in which the extraction was performed at a temperature of 66.1 ºC for 1.8 h. The resulting co-polymer had a purity of 90-91 %. On the other hand, the lowest extraction yields were obtained for Exp. 4 and 12 that were performed at higher temperatures (83.9 and 75 ºC, respectively), for longer reaction times (4.2 and 5 h, respectively). Such conditions also lead to higher co-polymer purity (93-94 %) (Table 7.1). These results suggest that higher temperatures and longer reaction times lead to lower CGC extraction yields, but such conditions also lead to a more pure co-polymer. In fact, the results show that higher temperatures and longer reaction times have apparently been more efficient in solubilizing the cell components, namely, proteins and alkali-soluble polysaccharides. This is confirmed by the lower protein and mannose contents of the samples, both below 3 wt%.

In the case of mannans, the tested conditions had a more significant impact on the extraction yield and polymer purity, as shown by the wider range of values obtained for the extraction yield, 18-33 wt%, and polymer purity, 59-83 % (Table 7.1). These extraction yield values were within the range of the ones (7-30 wt%) reported for several yeasts and fungal mannans (Nguyen, Fleet and Rogers, 1998; Maru *et al.*, 2015; Galinari *et al.*, 2017). The highest extraction yields (≥ 30 wt%) were obtained in Exp. 1, 3 and 13 that were performed at 66.1 or 75 ºC, and low alkali concentration (0.5-1.4 M) (Table 7.1). Despite the higher yield, the polymer was rather impure (59-65% of polymer's purity). Increasing the temperature to 83.9 and 90 ºC, concomitant with the use of harsher alkaline conditions (NaOH 2.75 or 4.1 M), in Exp. 6, 8 and 10, resulted in a considerable increase of the polymers' purity (74-83 %), although reducing the extraction yields (18-20 wt%) (Table 7.1). This mannans purity was also reflected on polymers proteins content that was less than 10 wt% in these experiments. These results seem to indicate that mannans extraction from *K. pastoris* cell-wall was mainly influenced by temperature and NaOH concentration.

7.4.1.2. RSM model

The response surface methodology (RSM) analysis, namely the ANOVA results, is presented in Table 7.2. All the responses studied in this second order model showed an acceptable fit ($R^2 > 0.7$), according with Lundstedt *et al.* (1998) (Table 7.2 and Figure 7.1). The ANOVA *p*-values showed that this model had significance (*p*-value < 0.05) for all responses and there is no evidence of lack of fit (*p*-value > 0.05) for any of the model responses (Table

7.2). The absence of lack of fit observed in this model means that the model error is in the same range of the pure error.

Table 7.2 – Analysis of variance (ANOVA) of CGC and mannans central composite design: model and lack of fit significance levels (*p*-values) and correlation values (R^2) for the responses studied: CGC and Mannans Extraction Yields, CGC and Mannans Purity.

	Model	Lack of fit	R^2
	p-value	*p*-value	
CGC Extraction Yield (Y_1)	0.0000	0.603	0.824
Mannans Extraction Yield (Y_2)	0.0047	0.879	0.888
CGC Purity (Y_3)	0.0000	0.108	0.777
Mannans Purity (Y_4)	0.0004	0.390	0.889

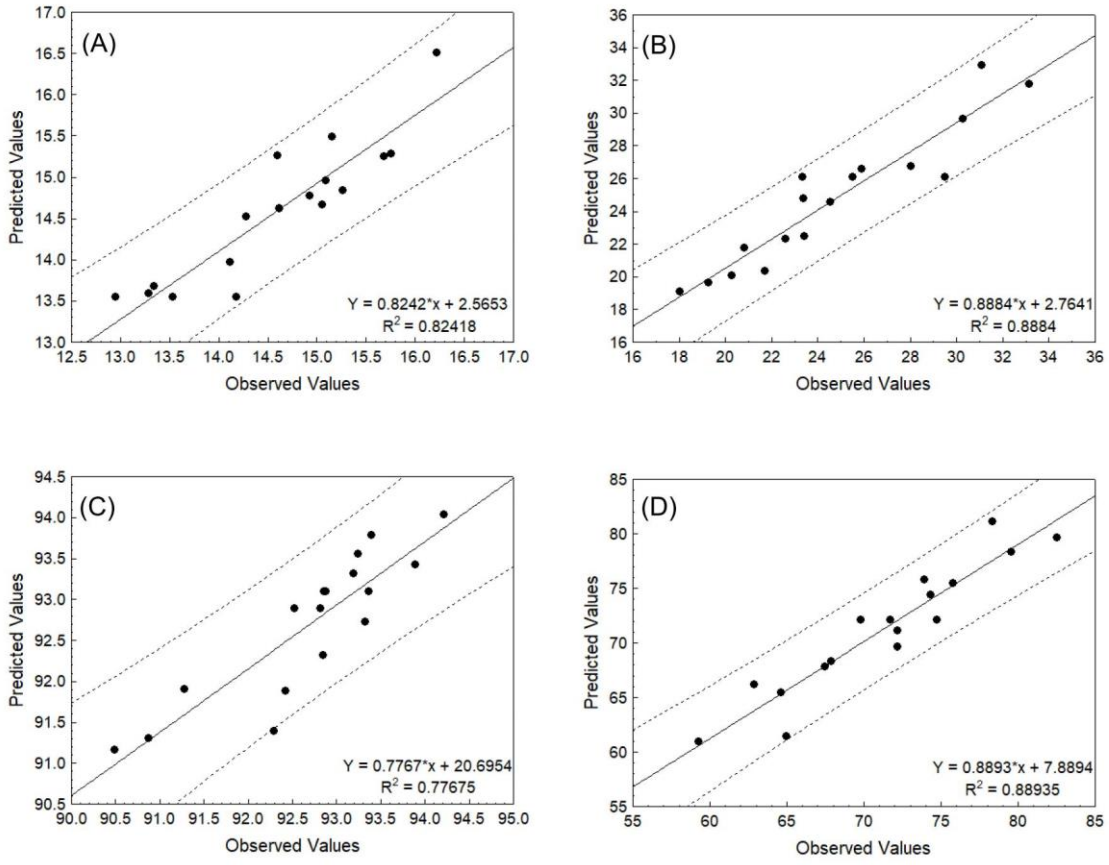

Figure 7.1 – Parity plots for observed and predicted values for the responses studied in the RSM model: CGC Extraction Efficiency (A), Mannoproteins Extraction Efficiency (B), CGC Purity (C) and Mannans Purity (D).

The MLR and the ANOVA also give information about the linear, quadratic and interception effects of the three variables on each response studied, for a significance level of 5% (Table 7.3). The CGC extraction yield was significantly affected by the linear term of temperature and by the quadratic term of NaOH concentration (p-value < 0.05), while the CGC purity was only affected by the linear term of temperature. Mannans extraction yield and purity were both significantly influenced by the linear term of NaOH concentration.

Table 7.3 – Multiple linear regression (MLR) analysis of the polynomial model: constants and *p*-values for linear, quadratic and interaction effects of temperature (T), reaction time (t) and NaOH concentration ([NaOH]) for the responses studied: CGC Extraction Yield (wt%), Mannans Extraction Yield (wt%), CGC Purity (%) and Mannans Purity (%).

Effect	Constant	Linear				Quadratic				Interaction		
		T (X_1)	t (X_2)	[NaOH] (X_3)		T × T (X_1^2)	t × t (X_2^2)	[NaOH] × [NaOH] (X_3^2)		T × t ($X_1 \times X_2$)	T × [NaOH] ($X_1 \times X_3$)	t × [NaOH] ($X_2 \times X_3$)
CGC Extraction Yield (Y_1)	13.545	-0.454	-0.381	-0.004		0.419	0.273	0.610		0.199	0.340	0.287
p-value	0.000	0.026	0.050	0.981		0.050	0.167	0.010		0.377	0.150	0.214
Mannans Extraction Yield (Y_2)	26.106	-1.936	-0.685	-3.743		-0.982	-0.871	0.188		-0.010	0.592	-0.409
p-value	0.005	0.149	0.503	0.047		0.402	0.448	0.859		0.993	0.646	0.747
CGC Purity (Y_3)	93.093	0.785	0.210	-0.131		-0.134	-0.006	-0.350		-0.371	0.080	-0.283
p-value	0.000	0.009	0.112	0.231		0.256	0.950	0.054		0.066	0.509	0.107
Mannans Purity (Y_4)	72.118	1.840	0.978	5.854		0.208	-0.936	-0.291		-0.594	0.209	-1.051
p-value	0.000	0.112	0.283	0.013		0.805	0.333	0.732		0.569	0.834	0.354

The 3D surface plots presented in Figure 7.2 demonstrate more clearly the interaction effects arising from the MLR analysis. Low CGC extraction yields, below 14 wt% are obtained for extraction conditions between 75-85 ºC, during 3.5-4.5 h and with NaOH 2-3 M (Figure 7.2A, B and C). The parabolic form of Figure 7.2B and C confirmed the influence of the NaOH concentration quadratic term, revealed by the MLR analysis. Nevertheless, concomitant with this low extraction yields, higher CGC purity (> 93 %) is obtained (Figure 7.2G, H and I). On the other hand, high extraction yields (> 18 wt%) but lower purity (< 91 %) are obtained with temperatures below 60 ºC, reaction times of less than 1.5 h and/or with a NaOH concentration below 1 M or above 5.5 M (Figure 7.2A-C and G-I).

The 3D surface plots for mannans confirm the influence of the linear terms of NaOH concentration, due to the plane surface observed in NaOH concentration axis of the surface plots (Figure 7.2E-F and K-L). Low mannans extraction yields (< 16 wt%) are achieved with temperatures above 80 ºC, reaction times of over 4 h or of less than 1 h, and with a NaOH concentration above 4 M (Figure 7.2D-F). Similarly, the mannans purity was also higher than 84 % for temperatures above 80 ºC, reaction times of 2-3 h and NaOH concentration higher than 4 M (Figure 7.2J-L). Higher mannans extraction yields (> 30 wt%) can be obtained for extractions conditions with a temperature between 60-70 ºC, during 1-3 h and with less than 1 M of NaOH concentration (Figure 7.2D-F). As Figure 7.2J-L shows, mannans purities lower than 60 wt% are only dependent of the NaOH concentration (less than 1 M) and don´t depends of the time and temperature.

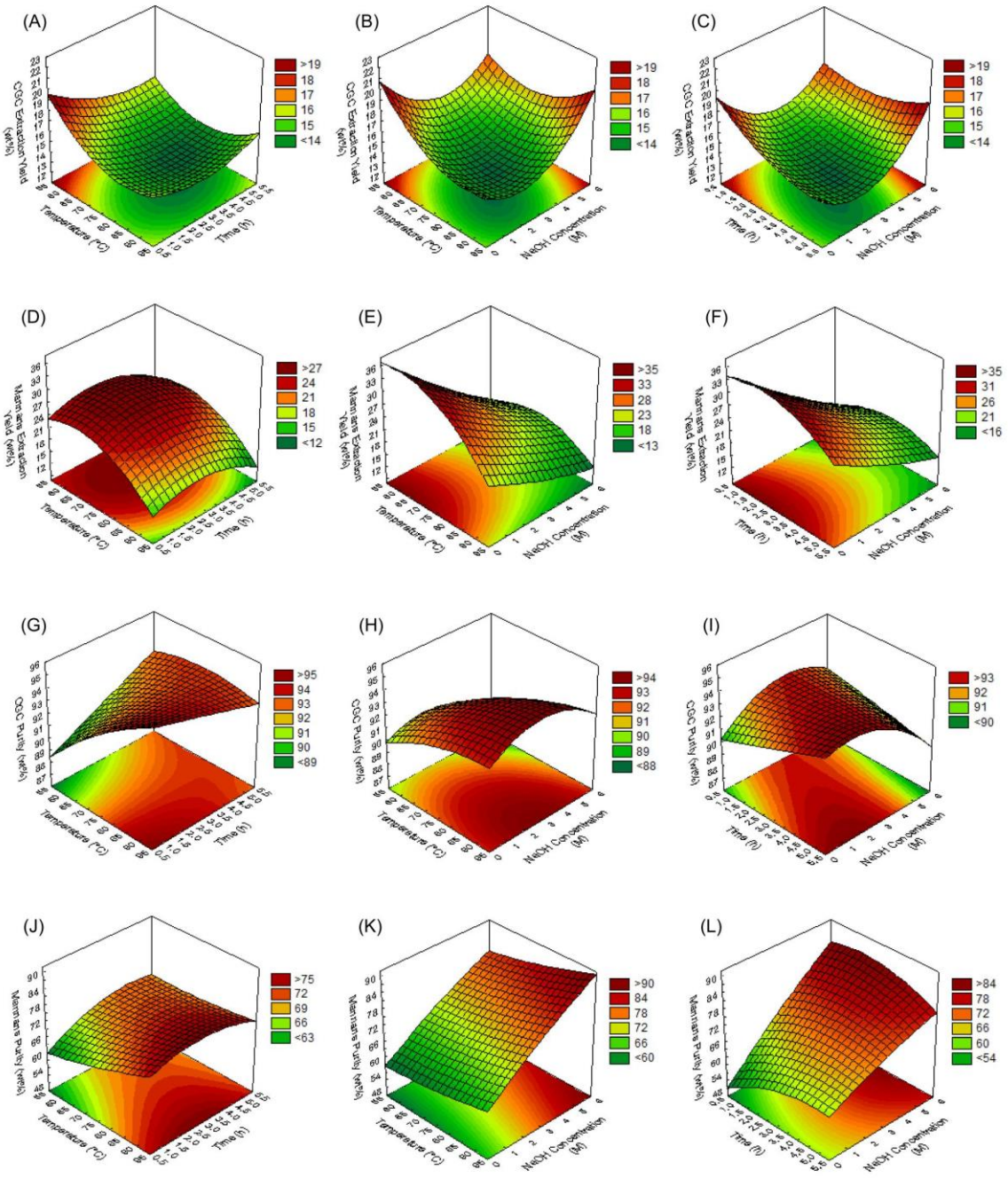

Figure 7.2 – Response surface plots for experimental design model for the CGC Extraction Yield (A-C), Mannans Extraction Yield (D-F), CGC Purity (G-I) and Mannans Purity (J-L), as a function of temperature, reaction time and NaOH concentration.

The developed model allows to predict the extraction conditions that enable obtaining both cell-wall polysaccharides, CGC and mannans, with high purity degrees. Those conditions are subjecting *K. pastoris* biomass to temperatures above 80 ºC, in the presence of NaOH at concentrations higher than 3 M, during a period of time of at least 3.5 h.

7.4.1.3. Model validation

For model validation, three experiments were performed, where the extraction conditions were 65 ºC, during 2 h with NaOH 1 M (Exp. A) or NaOH 5 M (Exp. B), and 84 ºC, during 4 h with NaOH 4 M (Exp. C). The conditions used in Exp. A and B were similar to those described by Roca *et al.* (2012) and in section 6.3.2 of Chapter 6, which resulted in different extraction yields and CGC purity. Mannans were not recovered in either study. In Exp. C, the best conditions, as defined by the RSM model, were used, which corresponded to the ones of Exp. 8 of the experimental design.

As shown in Table 7.4, there was an overall good correlation between the observed and the predicted results. However, there was some discrepancy between the observed and predicted values for mannans purity for Exp. A and C, and also for mannans extraction yield for Exp. B. Nevertheless, these validation results confirmed the good fit of the model to the experimental results, being valid to predict and control the yield of extraction and the polymers purity. As predicted by the model, CGC and mannans with the highest purity were obtained in Exp. C.

Table 7.4 – Experiment A (65 ºC, 2 h, NaOH 1 M), B (65 ºC, 2 h, NaOH 5 M) and C (84 ºC, 4 h, NaOH 4 M) results of model validation: comparison between observed and predicted results for CGC Extraction Yield (wt%), Mannans Extraction Yield (wt%), CGC Purity (%) and Mannans Purity (%).

	Exp. A		Exp. B		Exp. C	
	observed	predicted	observed	predicted	observed	predicted
CGC Extraction Yield (Y_1)	17	17	16	16	16	15
Mannans Extraction Yield (Y_2)	36	34	26	20	19	20
CGC Purity (Y_3)	90	91	92	91	95	93
Mannans Purity (Y_4)	66	59	76	78	82	78

7.4.2. CGC characterization

7.4.2.1. Composition

The CGC obtained in the model validation experiments (CGC_a, CGC_b and CGC_c) had similar chitin:β-glucan molar ratios of 12:88, 14:86 and 13:87, respectively. These values are within the range reported for *K. pastoris* CGC (11:89-25:75) (Chapters 3-6 and Roca *et al.*, 2012) and confirm the harsher extraction conditions used in Exp. C had no significant impact on the co-polymer's composition.

CGC_c had a low protein content (4.1 wt%), similarly to CGC_a (4.1 wt%) and CGC_b (5.1 wt%). Moreover, only minor traces of mannose (<1 wt%) were detected in CGC_c, while CGC_a and CGC_b had 5.0 and 2.3 wt%, respectively. This confirms the higher purity of the CGC obtained from *K. pastoris* using the optimal extraction conditions predicted by the model.

7.4.2.2. Infrared spectroscopy

A structural analysis of CGC_c by FTIR (Figure 7.3) revealed its spectrum was quite similar to that reported in 6.4.2.1 section of Chapter 6, although the peaks were better defined. This confirms the higher purity of the co-polymer obtained with the optimized extraction conditions. The characteristic peaks of glucans and chitin observed in Figure 7.3 are summarized in Table 7.5. A broad band is shown around 3400 cm^{-1}, representing the O-H stretching of hydroxyls groups overlapped with the N-H stretching peaks of chitin that appear in the same region (3107 and 3269 cm^{-1}) (Lu *et al.*, 2013). The stretching related with the C-H of CH_2 and CH_3 groups appear between 2850 and 2954 cm^{-1} (Synytsya and Novak, 2014). As observed in 6.4.2.1 section of Chapter 6, the peaks appearing at 889, 1155 and 1369-1370 cm^{-1} are assigned to β-1,3-glucans linkages and to the linkages associated with the pyranose ring (C-OH, C-O-C and C-C linkages), common to glucose and acetylglucosamine units. The peak at 1708 cm^{-1} (Figure 7.3) is probably related to the presence of β-(1,6)-glucans linkages. As reported by Thanardkit *et al.* (2002), the peaks around 920, 1045 and 1730 cm^{-1} are associated with β-(1,6)-glucans linkages, but their presence on CGC_c spectra is usually doubtful due to the higher content of β-(1,3)-glucans.

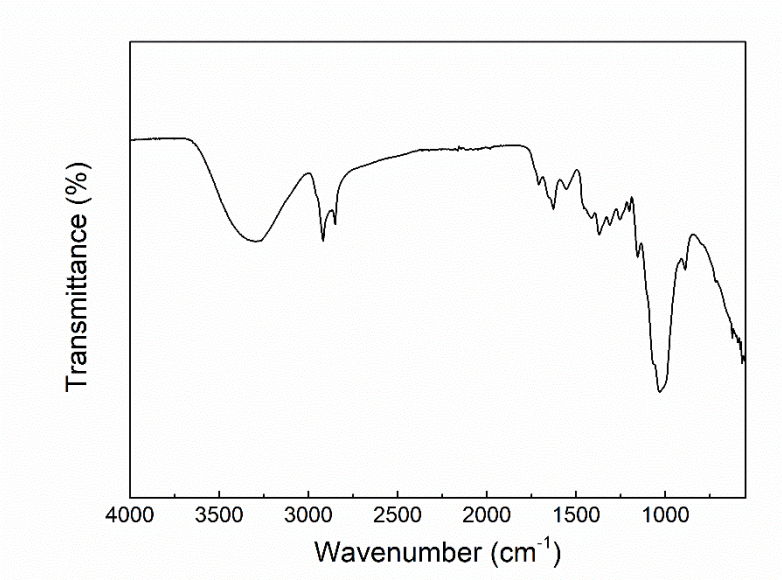

Figure 7.3 – FTIR spectra of CGC_c obtained in the model validation experiment C.

Table 7.5 – FTIR spectral values of the main bands for the CGC and mannans samples obtained in experimental design model validation.

Vibration Modes	Exp. A	Exp. B	Exp. C	
	Mannans (cm^{-1})	Mannans (cm^{-1})	CGC (cm^{-1})	Mannans (cm^{-1})
C-H deformation (α-glycosidic bond of mannans) [d, e]	810	812	-	802
C-H deformation (β-glycosidic bond of glucans) [a, b]	-	-	889	-
C-OH stretching [a, b, e]	1026	1022	1032	1020
C-OH stretching [a, b]	1057	1051	1068	1051
C-O-C stretching (asymmetric) [a, b, e]	1124	1126	1155	1130
O-H in-plane bending and C-O stretching (symmetric) [a, b, e]	1240	1216	1201	1217
N-H deformation (Amide IV) [a, b]	-	-	1254	1259
C-N stretching, N-H deformation (Amide III) and CH_2 wagging [a, b]	1309	1304	1311	1301
CH bending and symmetric CH_3 deformation [a, b, d]	1394	1377	1369	1371
CH_2 bending and CH_3 deformation [a, b, c]	1452	1452	1414	1408
C-N stretching and N-H deformation in the CONH plane (Amide II) [a, b]	1537	1535	1554	1533
C=O stretching (Amide I) [a, b, e]	1637	1637	1626 1653	1635
C-H stretching [a, b, e]	2852	2854	2850	2852
CH_2 stretching (symmetric) [a, b, c]	2924	2924	2918	2919
CH_3 stretching (asymmetric) [a, b]	2953	-	2953	-
N-H stretching (symmetric) [a, b]	Band	Band	Band	Band
N-H stretching (asymmetric) and O-H···O stretching (intermolecular hydrogen-bonded) [a, b]	Band	Band	Band	Band
OH stretching [a, b, d]	Band	Band	Band	Band

[a] Farinha *et al.* (2015)
[b] Lu *et al.* (2013)
[c] Galinari *et al.* (2017)
[d] Liu and Huang (2018)
[e] Liu *et al.* (2015)

7.4.2.3. Thermal properties

The thermal degradation curve of CGC_c (Figure 7.4) is indicative of the polymer's thermal stability up to a temperature of 200 ºC (Δm of 2.5% at this temperature). A single weight loss phase of 73% is noticed at a maximum degradation temperature (T_{deg}) of 330 ºC, with a char yield at 500 ºC of 16%. These results showed that the higher temperature imposed during the hot alkaline treatment of the biomass had some impact on the thermal stability of the co-polymer. In fact, the T_{deg} increased from 312-315 ºC for the CGC extracted at 65 ºC (6.4.2.4 section of Chapter 6) to 330 ºC, for the CGC_c polymer obtained at 85 ºC.

The differential scanning calorimetry (DSC) curve shows an exothermic peak at 330 ºC, corresponding to the co-polymer's thermal degradation. It is related with the degradation of the saccharide structure by dehydration of the saccharide rings and decomposition of the chitin units (Ospina Álvarez *et al.*, 2014). In 6.4.2.4 section of Chapter 6 is observed a different type of degradation transition for CGC samples where a single endothermic decomposition peak was observed. This difference can be related with the higher β-glucans content of the samples obtained in this study, since β-glucans commonly show an exothermic decomposition at 277 ºC (6.4.2.4 section of Chapter 6).

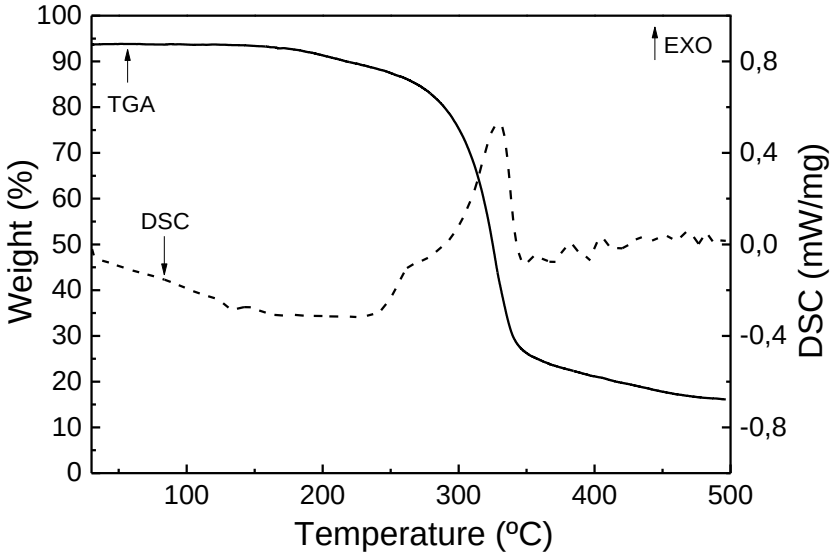

Figure 7.4 – Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) curves of CGC_c.

7.4.3. Mannans characterization

7.4.3.1. Composition

The mannans obtained in the model validation experiments (MN_a, MN_b and MN_c) were mainly composed of mannose residues. All samples had minor contents of glucose (3.1-5.1 wt%) that may be indicative of the presence of alkali soluble glucans (Ruszova *et al.*, 2008). MN_a and MN_b had glucosamine contents of 2.3-3.5 wt%, which were probably remnants of CGC due to inefficient separation of the AIM and ASM fractions upon centrifugation. Only traces of glucosamine (<1.0 wt%) were detected in MN_c, thus confirming the higher purity of the polymer. MN_c also had a lower protein content (8.6 wt%) than MN_a (24.6 wt%) and MN_b (14.4 wt%). The reduction of the polymer's protein content was probably related with the harsher extraction conditions used. The use of a high alkali concentration (4 M), together with a higher temperature (85 ºC) and extended reaction time (4 h) in Exp. C resulted in the lowest protein content of MN_c, while the milder extraction conditions used in Exp. A (extraction with NaOH 1 M, at 65 ºC, for 2 h) resulted in the high protein content observed for MN_a.

Several deproteinization methods are available that include the use of enzymes (proteases) or organic solvents, such as trichloroacetic acid (TCA) or chloroform/isoamyl alcohol (Sevage method), for protein removal from mannans (Huang, Yang and Wang, 2010; Liu *et al.*, 2018). Liu *et al.* (2018) reported that the use of papain and pronase (proteases) allowed to reduce the proteins content of *S. cerevisiae* mannans from 25.9 wt% to 6.7 wt%. A reduction from 21.0% to 5.2% for *S. cerevisiae* mannans using a treatment with TCA and gel filtration chromatography was also reported by Liu *et al.* (2015). The optimized mannans extraction conditions of this study has the considerable advantage of not being based on the use of enzymes and/or other chemicals, which add to the overall downstream costs.

7.4.3.2. Molecular mass distribution

The SEC-MALS analysis revealed the mannans recovered from *K. pastoris* biomass were heterogenous polymer samples (Figure 7.5). The heterogeneity of the samples was revealed by the multimodal distribution of the SEC chromatograms: a high Mw fraction (Mw1) with average molecular weights above 10^6 Da, and two lower Mw fractions (Mw2 and Mw3), with average molecular weights of 10^4 - 10^5 Da (Table 7.6).

MN_b and MN_c had similar peaks profile, with higher refractive index (RI) signals for fractions Mw1 and Mw3, and a very low RI signal for fraction Mw2 (Figure 7.5). MN_a, on the contrary, had a significant RI signal for fraction Mw2, which was similar to that of fraction Mw1. The harsher extraction conditions used in Exp. B and C apparently lead to the reduction

of fraction Mw2. This result suggests that the protein contained in MN_a might be included in that fraction and its reduction in fraction Mw2 of MN_b and MN_c was related to those polymers' lower protein contents.

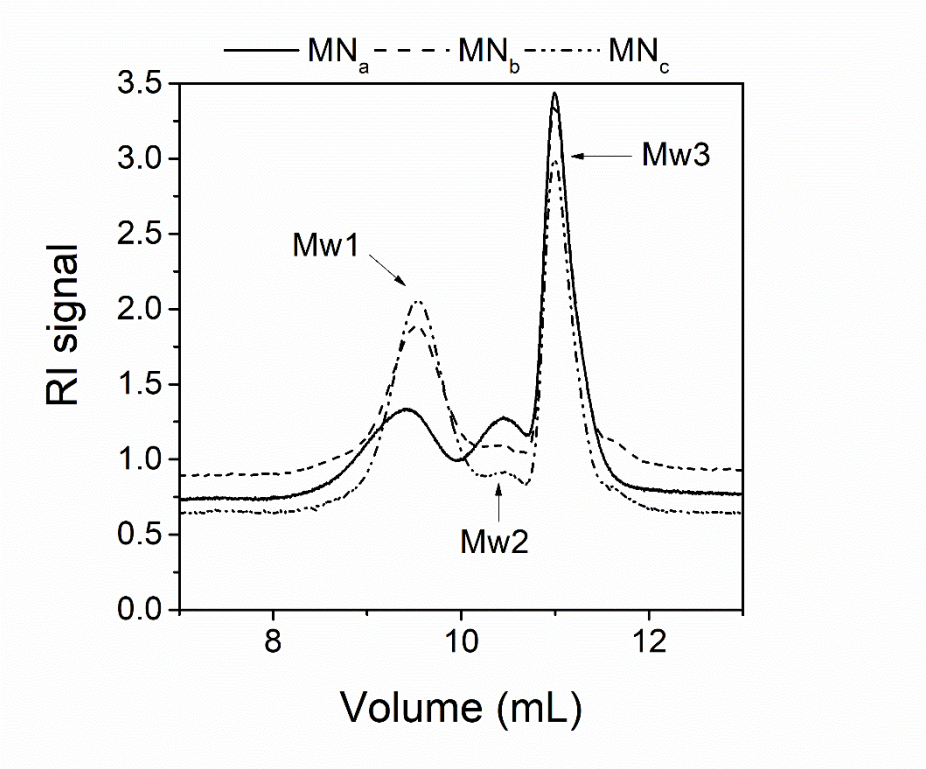

Figure 7.5 – Size exclusion chromatograms (SEC) of MN_a, MN_b and MN_c.

Table 7.6 – Average molecular weight of the mannans obtained in the model validation experiments.

Sample	Mw1 (Da)	Mw2 (Da)	Mw3 (Da)
MN_a	5.2×10^6	8.2×10^4	1.7×10^4
MN_b	3.2×10^6	7.3×10^4	4.9×10^4
MN_c	3.6×10^6	1.6×10^5	6.0×10^4

Due to the usual heterogenicity of the extracted yeast mannans, the average molecular weight values reported for other yeast cell-wall mannans are widely variable. For example, the mannans extracted from *S. cerevisiae* and *Kluyveromyces marxianus* biomass have average molecular weights of 1.0-2.0 x 10^5 Da (Liu *et al.*, 2015; Galinari *et al.*, 2017), while those extracted from Sake yeast strain, Kyokai No.7, and from *Candida albicans* presented distinct fractions with molecular weight values between 2×10^4 and 5×10^5 Da (Kumagai, Nunokawa and Akiyama, 1981; Elorza, Marcilla and Sentandreu, 1988). On the other hand, higher values have

been reported for fungal mannans, such as, for example, the mannans extracted from *Ceratocystis fagacearum*, that presented molecular weight fraction of 0.12-1.32 x 10^6 Da (McWain and Gregory, 1972).

7.4.3.3. Infrared spectroscopy

The structural analysis of the mannans performed by FTIR is presented in Figure 7.6. The spectra of MN_a, MN_b and MN_c are quite similar, except for the peaks in the region between 1540 cm^{-1} and 1640 cm^{-1}. The two absorption peaks that appears in this region show a higher intensity for MN_a than for MN_b or MN_c (Figure 7.6). This region of the spectra is related with the N-H absorption peaks that can be assigned to the presence of glycoproteins (Liu *et al.*, 2015) since the extraction conditions used in Exp. A resulted in mannans with high protein content (24.6 wt%). The higher peaks definition observed in Figure 7.6 for MN_c is also indicative of the polymer's higher purity.

The characteristic peaks of polysaccharides observed in Figure 7.6 are summarized in Table 7.5. Similarly to CGC spectra, there is a broad band around 3000-3500 cm^{-1} (representing the O-H stretching of hydroxyls groups), peaks between 2850 and 2950 cm^{-1} (corresponding to the stretching of the C-H of CH_2 and CH_3 groups) and peaks at 900-1200 cm^{-1} and 1369-1370 cm^{-1} (assigned to C-H of CH_2 and CH_3 groups and to the C-OH, C-O-C and C-C linkages associated with the pyranose ring) (Liu *et al.*, 2015; Galinari *et al.*, 2017). The absorption peak appearing at 802-812 cm^{-1} can be attributed to the presence of α-mannans chains (Liu and Huang, 2018).

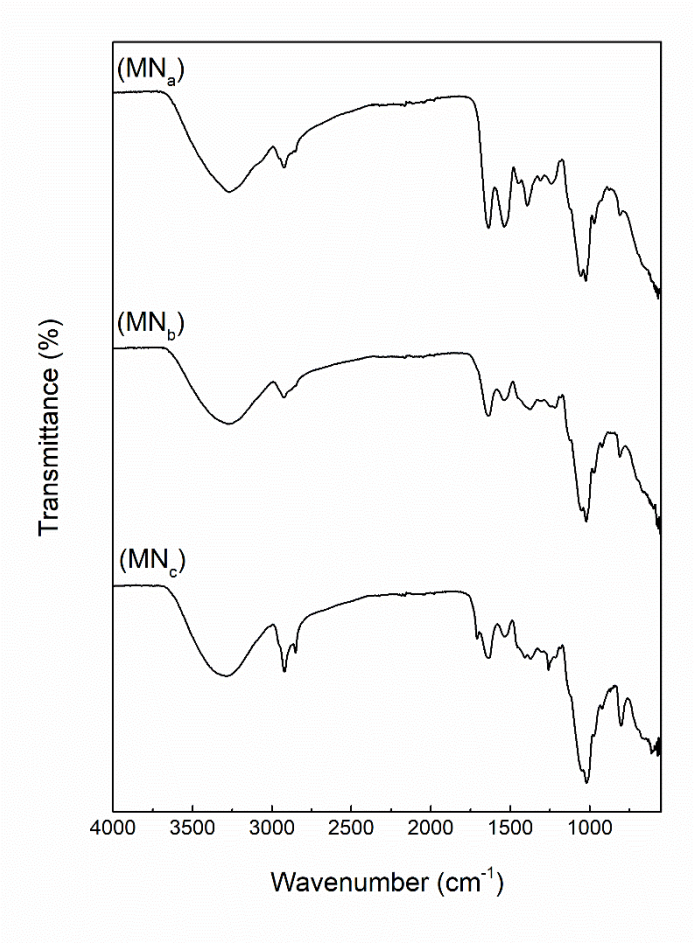

Figure 7.6 – FTIR spectra of MN_a, MN_b and MN_c.

7.4.3.4. Thermal properties

The thermal properties of the mannans obtained in the model validation experiments were determined by DSC and TGA (Figure 7.7). A single weight loss phase was noticed for all samples but it occurred at different temperatures. For MN_a, a weight loss of 47.3% was observed between 255 and 345 ºC, with a maximum T_{deg} of 312 ºC (Figure 7.7), while for MN_b and MN_c the weight loss (61 and 60 %, respectively) occurred faster and at lower temperatures (between 225 and 274 ºC) (Figure 7.7). The lower maximum T_{deg} observed for MN_b and MN_c (266 ºC and 253 ºC, respectively) suggest that the conditions imposed to the biomass during the hot alkaline treatment impacted the thermal stability of the extracted mannans. Moreover, after the fast weight loss observed for MN_b and MN_c, the weight loss continued more slowly until around 370 ºC. This weight loss behavior can be related to the heterogenicity of the mannans samples revealed by the SEC analysis. The char yields at 500 ºC were 43, 25 and 29%, respectively, confirming the higher purity level of MN_b and MN_c compared with MN_a.

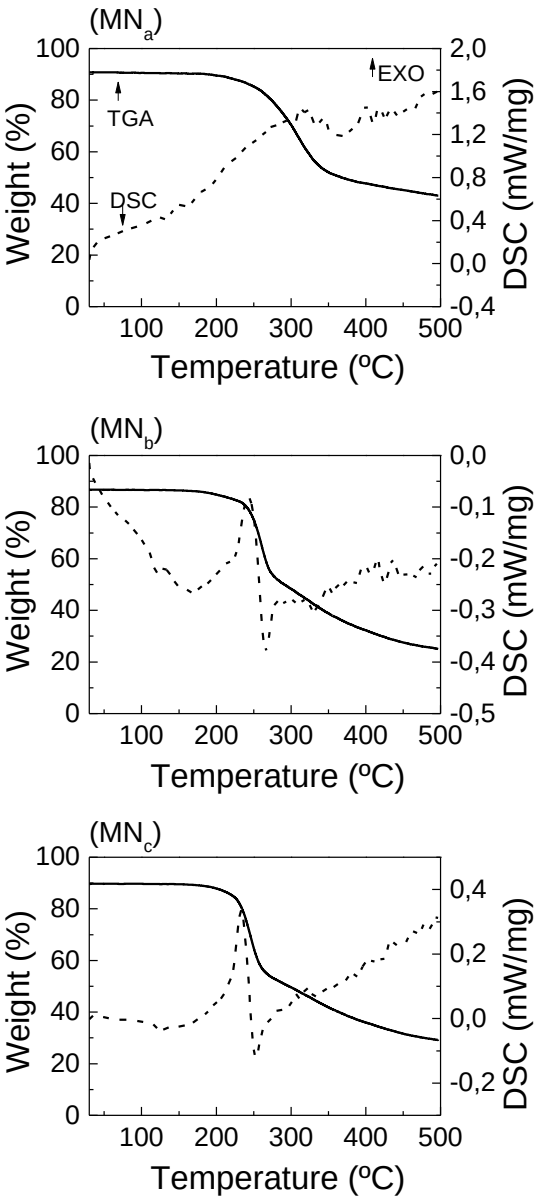

Figure 7.7– Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) curves of MN_a, MN_b and MN_c.

The DSC curves also revealed different behavior for the mannans extracted from *K. pastoris* (Figure 7.7). The DSC spectra of MN_a shows a single exothermic peak at 312 ºC that corresponds to the thermal degradation of the polysaccharide, as confirmed by the TGA curve (Figure 7.7). The DSC spectra of MN_b and MN_c, on the other hand, displayed an exothermic peak at 243 and 233 ºC, respectively, followed by an endothermic peak at 266 and 253 ºC, respectively (Figure 7.7). These peaks correspond to the thermal degradation of the polymers, as shown by the TGA analysis. A similar behavior was reported for laminarin, a β-glucan extracted from *Laminaria digitate* (6.4.2.4 section of Chapter 6). Laminarin DSC curve presented an

endothermic peak at 266 ºC followed by an exothermic peak at 277 ºC, also associated with the polysaccharide's decomposition.

The observed differences in terms of thermal properties of the mannans extracted from *K. pastoris* might be related to the lower purity of MN_a (66%), namely its higher protein content (24 wt%) compared with 76% and 82% of mannans purity in MN_b and MN_c, respectively. The three mannans DSC spectra also revealed a small endothermic transition at 120-130 ºC (Figure 7.7). Since this thermal transition was not associated with any significant weight loss, it might be related with a melting transformation or some structural rearrangement of the polymeric chains.

7.5. Conclusions

RSM was used to optimize the hot alkaline conditions to extract polysaccharides, CGC and mannans, from the cell-wall of the yeast *K. pastoris*. The developed model allowed to define the conditions that yielded both polymers with high purity degree. Despite lowering the extraction efficiency, the use of harsher conditions in terms of temperature, alkali concentration and reaction time, resulted in CGC and mannans with purity values of 95 and 82%, respectively. The polysaccharides' properties confirmed their potential for use in high-value pharmaceutical, cosmetic or food applications.

Chapter 8

Conclusions and future work

8.1 Conclusions

In this PhD thesis, *K. pastoris* CGC and mannans production bioprocess developed by Pharma73 S.A. was optimized and considerable improvements were made, both at the level of biopolymers production and the downstream, as demonstrated in Figure 8.1.

Firstly, a repeated fed-batch strategy was implemented, which resulted in improved CGC production. The implementation of consecutive daily cycles, resulted in higher biomass and CGC productivities, which increased by 1.8 and 2.1-fold, respectively, compared to the previous exponential feed strategy used during the fed-batch. Moreover, the fed-batch cycles were stable and reproducible, demonstrating the robustness of the process.

Although *K. pastoris* processes is highly demanding in terms of oxygen supply in order to reach high cell density, low DO levels (15%) were demonstrated to guaranty high cell growth rates and contributed to enhance CGC and mannans production, compared to the operation with a DO level of 50%. This reduction of the DO level from 50 % to 15 % enabled considerable savings regarding the oxygen demand, which is of the outmost relevance at higher production scales.

Polysaccharides productivity was further improved by using Medium K, a culture medium designed to overcome the operational problems associated with the used of BSM. Medium K was simpler, had a considerably lower salts content compared with BSM and enabled a 2.3 and 3.4-fold increase of biomass and CGC productivities.

Regarding the downstream optimization, firstly, a simplified process was developed to purify the CGC obtained from *K. pastoris* biomass. This new process is more environmentally friendly and contributed to reducing the downstream costs. The resulting CGC had low proteins and inorganic salts content, important features for cosmetic, pharmaceutical or medical applications.

Subsequently, the impact of the extraction conditions on CGC and mannans extraction yields and properties was studied. By using an experimental design and response surface methodology, it was observed that CGC extraction yield and purity were not much affected by the extraction conditions used, despite having some impact on chitin:β-glucans molar ratio. In opposition, mannans were more affected by the extraction conditions, being achieved lower extraction yields and purity levels.

The characterization studies performed revealed that CGC is composed of α-chitin and β-glucans, while mannans are α-mannans. Both polysaccharides had an amorphous structure, with high thermal resistance. The degradation temperature of these polysaccharides is above 300 ºC for CGC and 233-253ºC for mannans.

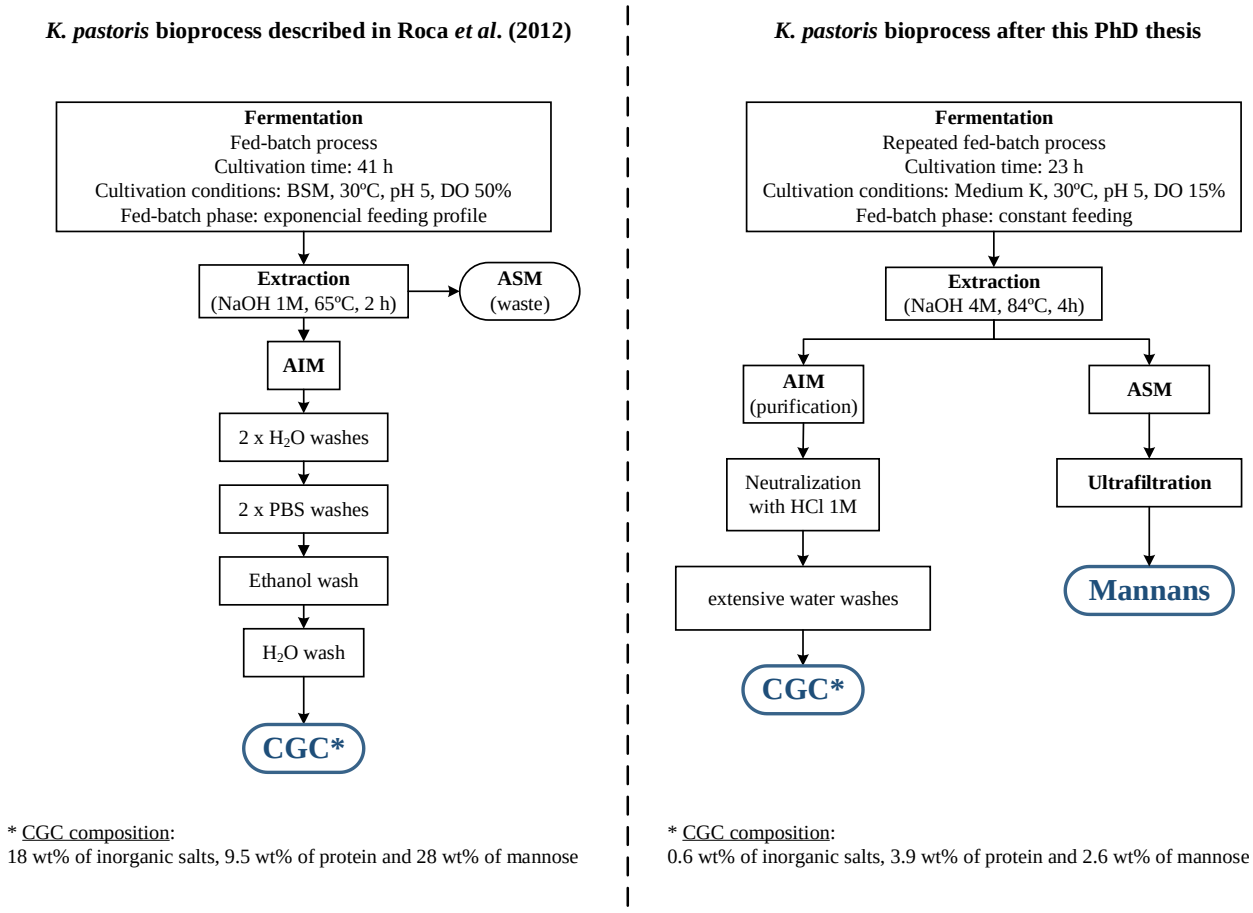

Figure 8.1 – Comparison between the *K. pastoris* bioprocess described by Roca *et al.* (2012) and the optimized process developed in this thesis.

8.2 Future work

Despite the significant developments achieved with this thesis, some aspects should be considered to improve even more the polysaccharides productivities and evaluate their applicability at an industrial scale.

With the development of Medium K, the operational conditions (pH, temperature and DO level) might need to be reviewed, to improve the process productivity. With the same purpose, efforts can be put into further increasing the biomass concentration, to above 200 g/L, by increasing the feed flow rate of the fed-batch phase. Also, increasing CGC and/or mannans content, should be attempted by applying stress factors to yeast cells (i.e., the addiction of other compounds). The use of feed solution only with glycerol and without mineral solution could also be tested, to simplify and reduce the feed solution costs.

Another important point in this process is related with the use of pure oxygen to supplement the air during the fermentation. Since industrial processes use pressure instead of

pure oxygen supplementation, it is necessary to study the impact of pressure on *K. pastoris* production and cell-wall composition.

Some attention must also be made to the downstream process, especially on mannans processing. Mannans purification at lab-scale is performed with dialysis membranes. With an outlook to the scale-up, filtration processes, such as ultrafiltration, should be tested to purify the mannans fraction.

To produce mannans (enriched in mannose), harsher extraction conditions need to be applied in the downstream process. In case of using milder extraction conditions, the resulting mannans will contain a high protein content (above 20 wt%). In this way, alternative methods to mannans purification can be tested, such as, the use of organic solvents (i.e., trichloroacetic acid) to precipitate these proteins.

Beyond the production process, several studies can be done to increase the knowledge about the CGC and mannans properties and evaluation of potential applications.

For CGC and mannans, the following studies are suggested:

- Elucidate the polysaccharides chemical structure, namely the linkages types between monomers, by performing two-dimensional NMR analyses (i.e. COSY, HSQC)
- Search for most appropriate and friendly solvents to dissolve CGC or develop water soluble CGC derivatives.
- Development of suspensions (in case of CGC) and gels, for example, for cosmetic or food formulations. Study of gels properties.
- Development of CGC and/or mannans films and study their mechanical properties and morphology.
- Evaluate the flocculation potential of *K. pastoris* CGC and mannans.
- Study the CGC and mannans electrostatic potential and the possibility of charge accumulation, properties that can be interesting for electronic applications.
- Study the swelling capacity of CGC in several environments, such as solutions with different pH values and ionic strengths, that could be useful for drug-delivery systems, for example.
- Study the CGC and mannans absorption capacity for metals or hydrocarbons, that could be interesting for metal removal applications and waste water treatment. Particularly, the evaluation of CGC and mannans absorption capacity for fat could be interesting for anti-fat or anti-cholesterol food supplements and its interaction with other sugars can also be interesting for pharmaceutical application such the diabetes treatment.
- Evaluate the biocompatibility, bioactivity and immunologic properties of CGC and mannans.

- Evaluate the impact of chitin:glucan molar ratio of CGC and the influence of proteins content in mannans for some of the proposed studies.

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