Diana Filipa Vieira Araújo

Degree in Biochemistry

Production of chitin-glucan complex by *Pichia pastoris*

Dissertation for the Degree of Master in Biotechnology

Supervisor: Dr.ª Maria Filomena Andrade de Freitas, Post-Doctoral Researcher, Faculdade de Ciências e Tecnologia, UNL

Co-supervisor: Professor Maria da Ascensão Carvalho Fernandes Miranda Reis, Full professor, Faculdade de Ciências e Tecnologia, UNL

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Acknowledgments

I wish to express my sincere gratitude to my supervisor Dr.ª Filomena Freitas for all kinds of help, friendship, continuous support and guidance during my research on this project.

I am grateful to my co-supervisor Prof. Dr. Maria Ascensão Reis for giving me the opportunity to use the laboratories of Biochemical and Process Engineering Group (FCT/UNL) and for her valuable advices and critics.

I would like to thank to Bárbara Chagas, for her help and important suggestions through this work.

I am also thankful to all members of BPEG group, especially to Inês Farinha, Sílvia Antunes and Margarida Carvalho for their help, patience and advices that give to me.

I am grateful to my friends for their friendship and continuous support for years, even in sleepless days and nights.

The most importantly, I would to like specially thank to my dear parents, sisters, grandparents and my boyfriend for their love, support, understanding and encouragement me all through my life. I wish especially thankful to my mum for her strength and support in my formation and education.

Abstract

The yeast *Pichia pastoris* produces chitin-glucan complex (CGC), as a cell wall component. CGC is composed of two types of biopolymers, chitin and β-glucans, which confer it great potential for use in the food, cosmetic and pharmaceutical industries. CGC hydrolysis, allows obtaining chitin/chitosan and glucans individually. The chitin and chitosan obtained from CGC have the considerable advantage of being of non-animal origin, which further extends their applications. In last year's, the production of CGC by *Pichia pastoris* was realized with glycerol as sole carbon source, achieving high cell density.

In this study, different substrates were tested for cultivation of *P. pastoris* and CGC production. In the first part, mixtures of glucose and xylose, in varying proportions, were tested. Since glucose and xylose are two of the main sugar components of lignocellulosic wastes, the ability of *P. pastoris* to use them as carbon sources would allow their valorization into value-added products. In the second part, several wastes and byproducts generated by different industries were tested for their suitability as substrates for *P. pastoris* cultivation.

In study of the glucose/xylose mixtures, the best performance was achieved in batch bioreactor experiments with 25% xylose in the medium (20 gL^{-1} of xylose and 60 gL^{-1} of glucose), where 35.25 gL^{-1} biomass was obtained in 64 hours of cultivation. CGC content in the cell wall reached 15% with a volumetric productivity of 0.085 $gL^{-1}.h^{-1}$. The molar ratio of chitin:β-glucan in the extracted biopolymer was 47:53, higher than obtained with crude glycerol (16:84).

In the second part of study, several wastes and byproducts (used cooking oil, sugarcane molasses, cheese whey, waste paper and spent coffee grounds) were tested. The results show that *P. pastoris* presented low biomass concentration using any of these substrates. Nevertheless, in batch bioreactor experiments the best results were achieved with sugarcane molasses, where 17.78 gL^{-1} biomass were obtained with a CGC content of 17%.

Among the tested substrates, the mixtures of glucose/xylose appear to be the most promising due the good CGC production obtained and the high glucosamine molar fraction in produced polymer. This study opens the hypothesis of utilization of lignocellulosic materials with xylose percentages up to 50%.

Keywords: Chitin, Chitin-glucan complex, *Pichia pastoris*, Carbon source, Residues, Xylose

Resumo

A levedura *Pichia Pastoris* produz, como componente da parede celular, o complexo quitina-glucanos (CQG). CQG é composto por dois tipos de biopolímeros, quitina e β-glucanos, que lhe conferem grande potencial para utilização nas indústrias alimentar, cosmética e farmacêutica. A hidrólise de CQG permite obter individualmente quitina/quitosano e glucanos. A quitina/quitosano obtida a partir de CQG possue uma vantagem considerável por ser de origem não animal, alargando a gama de aplicações.

Neste estudo, foram testados diferentes substratos para o cultivo de *P. pastoris* e produção de CQG. Na primeira parte, misturas de glucose e xilose, em proporções variáveis, foram estudadas. Uma vez que glucose e xilose são dois dos principais açúcares constituintes de resíduos lignocelulósicos, a capacidade de *P. pastoris* para usá-los como fonte de carbono permitirá a sua valorização em produtos de valor acrescentado. Na segunda parte do estudo, vários resíduos e subprodutos gerados por diferentes indústrias foram testados quanto à sua potencialidade como substratos para o cultivo de *P. pastoris*.

No estudo de misturas de glucose e xilose, o melhor desempenho foi alcançado pela mistura com 25% de xilose no meio (20 gL^{-1} de xilose e 60 gL^{-1} de glucose), onde 35.25 gL^{-1} de biomassa foi obtida em 64 horas de cultivo. A percentagem de CQG atingiu os 15% com uma produtividade volumetrica de 0.085 $gL^{-1}.h^{-1}$. O ratio molar de quitina:glucanos no biopolímero extraído foi de 47:53, maior que o obtido com glicerol da indústria do biodiesel (16:84).

Na segunda parte do estudo, vários resíduos e subprodutos como melaços de cana-de-açúcar, soro de leite e borra de café, foram testados. Os resultados demonstram que utilizando estes substratos, *P. pastoris* atinge uma baixa concentração de biomassa. No entanto, em ensaios batch em bioreactor os melhores resultados foram atingidos com melaço de cana de açúcar onde 17.78 gL^{-1} de biomassa foram obtidos com um teor de 17% em CQG.

De entre os substratos testados, as misturas de glucose/xilose aparentam ser as mais promissoras devido à boa produção de CQG obtida e à elevada fracção molar de glucosamina no polímero produzido. Este estudo permite a hipótese de utilização de materiais lignocelulósicos com percentagem de xilose não superior a 50%.

Keywords: Quitina, Complexo Quitina-glucanos, *Pichia pastoris*, Fonte de carbono, Resíduos, Xilose

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Abbreviations

AOX – Peroxisome Alcohol Oxidase

CDW – Dry Cell Weight (gL^{-1})

CGC – Chitin-Glucan Complex

DOC – Dissolved Oxygen Concentration (pO_2 (%))

EPS – Exopolysaccharides

Glc – Glucose

GlcN – Glucosamine

GlcNAc – N-acetyl-D-glucosamine

HCl – Hydrochloric acid

HPLC – High Performance Liquid Chromatography

OD_{600nm} – Optical density at 600 nm

PHA – Polyhydroxyalkanoate

r_p – Overall volumetric productivity ($gL^{-1}.h^{-1}$)

rpm – rotation per minute

R_S – Substrate consumption rate

SCG – Spent Coffee Grounds

SCP – Single-Cell Protein

TFA – Trifluoroacetic acid

vvm – gas volume per liquid volume per time

Xyl – Xylose

$Y_{P/S}$ – Production yield ($g\ g^{-1}$)

$Y_{X/S}$ – Growth yield ($g\ g^{-1}$)

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

1. Motivation

In the last decade, the advancement of technology has allowed industries to have a remarkable development, which led to an exponential demand of their processes. On the other hand, the high demand for industrial products has led to an increased generation of wastes and/or byproducts that often pose economical and environmental concerns (Pipatti et al., 2006).The growing demand for raw materials, coupled to the exponential increase of industrial wastes/byproducts generated, has led to the emergence of the valorization concept, which aims at the production of value-added products from industrial wastes/byproducts (sustainable development) (FitzPatrick et al., 2010; Laufenberg et al., 2003).

Industrial wastes/byproducts have a very varied composition, depending on the type of industry that generates them. In particular, many agro-food industry wastes/byproducts are essentially composed of lignocellulosic material, which gives them great advantages for their use in biotechnological processes. Agro-industrial wastes and byproducts, such as wheat straw, corn stover, winter rye, corn cobs, beet pulp, bran and waste wood are examples of lignocellulosic-rich raw materials (Nigam and Pandey, 2009). The lignocellulosic materials are composed essentially of cellulose, hemicellulose and lignin. Cellulose and hemicellulose, the major constituents of lignocellulosic materials, are macromolecules of different sugars, whereas lignin is an aromatic polymer (Sánchez, 2009).

In most cases, agro-industrial residues are wasted in landfills or used in low-valued applications, such as burning and composting. However, in the last decade, the growing environmental awareness increased the interest in using those materials, due to their eco-friendly properties: they are renewable, recyclable, sustainable and biodegradable materials (Satyanarayana et al., 2009). Consequently, the value of such materials has increased due to the development of new chemical and physical techniques that allowed their conversion into value-added products, such as, for example, reducing sugars, ethanol, furfural and many other chemical products (Sun and Cheng, 2002; Demirbas, 2008).

Currently, there is great need to develop novel bio-based products and other innovative technologies that can decrease dependence on fossil fuel. In recent years, biopolymers production from renewable “feedstock” has attracted the public attention due to environmental and economical concerns: preservation of fossil-based raw materials, complete biological degradability, the reduction in the volume of garbage and compostability in the natural cycle, protection of the climate through the reduction of carbon dioxide released, as well as the application possibilities of agricultural resources for the production of bio/green materials (Mohanty et al., 2000; Mohanty et al., 2002).

An alternative for the use of these residues is their biological valorization, using those carbon rich wastes/byproducts as substrates for microbial cultivation and production of different value added products, namely microbial biopolymers, such as polyhydroxyalkanoate (PHAs) (Koller et al., 2010) and exopolysaccharides (EPS) (Öner, 2013).

Another example of microbial biopolymer is chitin-glucan complex (CGC) produced by yeasts, including *Pichia pastoris* (*P. pastoris*) (Roca et al., 2012). As any other yeast, *P. pastoris* produces CGC as a component of its cell wall that confers rigidity and stability to the cells. Hydrolysis of this complex allows obtaining chitin/chitosan and glucans individually, two bioactive biopolymers, which gives CGC great potential for use in pharmaceutical, cosmetics and food applications (Roca et al., 2012). Recently, a patented process based on the cultivation of *P. pastoris* to produce CGC was developed, based on the use of crude glycerol from biodiesel industry as carbon source (Reis et al., 2010; Roca et al., 2012).

2. Introduction

2.1 *Pichia pastoris*

Pichia pastoris was initially developed in 1970 by a North American oil company, named Philips Petroleum Company, for production of single-cell protein (SCP) as high-protein animal feed (Cereghino and Cregg, 2000; Cos et al., 2006). The increased interest of this company for the development of tools and protocols for the growth of this yeast, was based on the attractive cell densities achieved on methanol (>130 gL^{-1} dry weight) (Cereghino and Cregg, 2000; Cregg, 1998). However, the production of SCP from this source has become unattractive, due to the rising cost of methane, caused by the oil crisis and also to the fall in soybean prices. In the following decade, *P. pastoris* has emerged as an important organism in biotechnology, when Phillips Petroleum Company together with the Salk Institute Biotechnology / Industrial Associates, Inc. (Sibia, La Jolla, CA) made this yeast an organism for heterologous protein expression (Cos et al., 2006). At present, this ability still remains active, being *P. pastoris* widely applied in various industries, mainly pharmaceutical, for the production of heterologous proteins (Cereghino and Cregg, 2000).

Taxonomically, *P. pastoris* was classified as belonging to the Kingdom Fungi, Phylum *Ascomycota*, Class *Saccharomycetes*, Order *Saccharomycetales*, Family *Saccharomycetaceae* and Genus *Pichia*. Recently, a few species belonging the Genus *Pichia* were reclassified as *Komagataella*, namely *K. pastoris*, *K. phaffii* and *K. pseudopastoris* (Kurtzman, 2009; Kurtzman, 2005).

In accordance with the structural organization, the cells are divided into prokaryotes and eukaryotes. Figure 2.1 allows visualizing the yeast morphology, a eukaryotic cell.

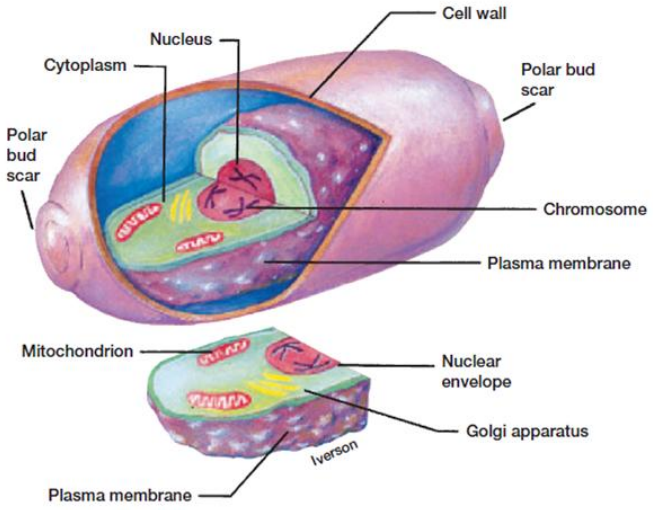

Figure 2.1 Schematic drawing of a yeast cell showing its typical morphology (Prescott and Klein, 2002)**.**

Belonging to the Phylum *Ascomycota*, *P. pastoris* exists preferentially in the haploid vegetative state, with asexually reproduction by multilateral budding. In this type of reproduction, a dilation of the external surface of the parent body occurs, formed by mitosis. This expansion, called the bud, remains attached to the mother cell until it is ripened. After that, the bud can separate and give rise to a new individual cell, which is genetically identical to the parent organism. Alternatively, sexual reproduction may occur, where the union between two diploid cells creates the diploid phase (Figure 2.2) (Prescott and Klein, 2002).

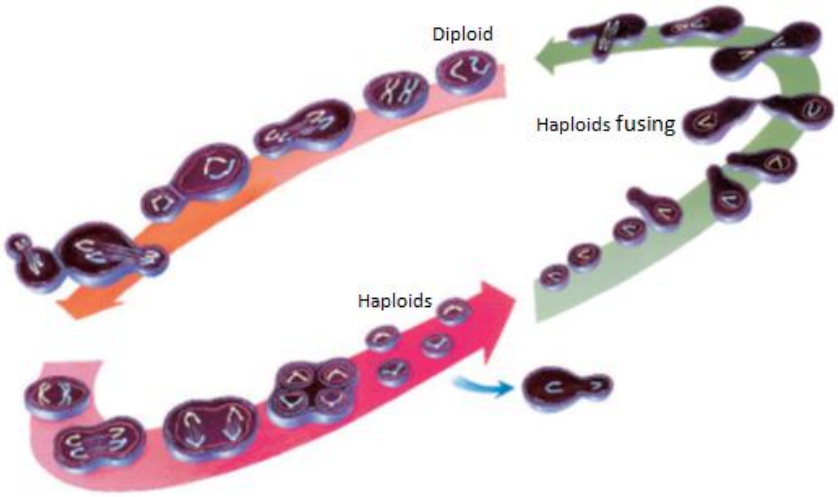

Figure 2.2 Diagrammatic life cycle of yeast (adapted from Prescott and Klein, 2002).

P. pastoris is a unicellular eukaryotic organism, being its cells generally oval visible under optical microscopy. Figure 2.3 shows *P. pastoris* cells visualized under electron microscope at different growth conditions.

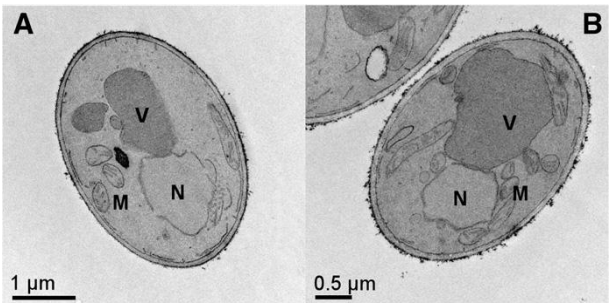

Figure 2.3 Electron micrographs of *P. pastoris* grown on different carbon sources: glucose (A) and glycerol (B). The organelles are identified: mitochondria, M; Vacuole, V; Nucleus, N (bars indicate the size of cells and organelles) (adapted from Wriessnegger et al., 2007)**.**

Yeast growth is conditioned by many factors, including temperature and nutrition. For the temperature, *P. pastoris* is known as a mesophilic organism, with an optimum growth temperature of 30 ºC. As for nutrition, yeasts are chemoorganoheterotrophic organisms that require the supply of organic compounds as carbon and energy sources (Prescott and Klein, 2002).

The peculiarity that most characterizes the yeast *P. pastoris*, is its ability to metabolize reduced one-carbon compounds, such as methanol, using them as the sole carbon and energy sources for growth (methylotrophic yeast). This ability is possible due to overexpression of the enzyme peroxisome alcohol oxidase (AOX), which can reach about 30% of the intracellular protein in the presence of methanol (Couderc, 1980). In addition to this feature, the widespread use of *P. pastoris* in biotechnology is based on two major advantages. One advantage consists in achieving high cell densities during fermentation, over 130 gL^{-1} dry weight, in a controlled environment (Cregg, 1998; Cereghino and Cregg, 2000). Another advantage of *P. pastoris* is its capacity to efficiently use a wide range of substrates, including low-cost raw materials (e.g. biodiesel waste glycerol and methanol) (Bai et al., 2009; Cui and Ellison, 2012), making the production process economically viable and sustainable (Roca et al., 2012).

2.2 Yeast cell wall

The cell wall of yeast is about 100 to 200 nm thick and represents from 15% to 25%, by weight, of the dry cell weight (Waites et al., 2001). Chemically, the yeast cell wall is composed mainly of polysaccharides (85 - 90%) and protein (10 - 15%) (Nguyen and Fleet, 1998). The polysaccharides have a structural function, while the proteins are important for the permeability of the wall. The polysaccharides present in the yeast cell wall are essentially divided into three classes: glucans, mannans and chitin.

Mannose molecules are attached to large proteins, located on the periphery of the cell wall, are named mannoproteins. The constituents of the cell membrane and cell wall of yeast are represented in Figure 2.4.

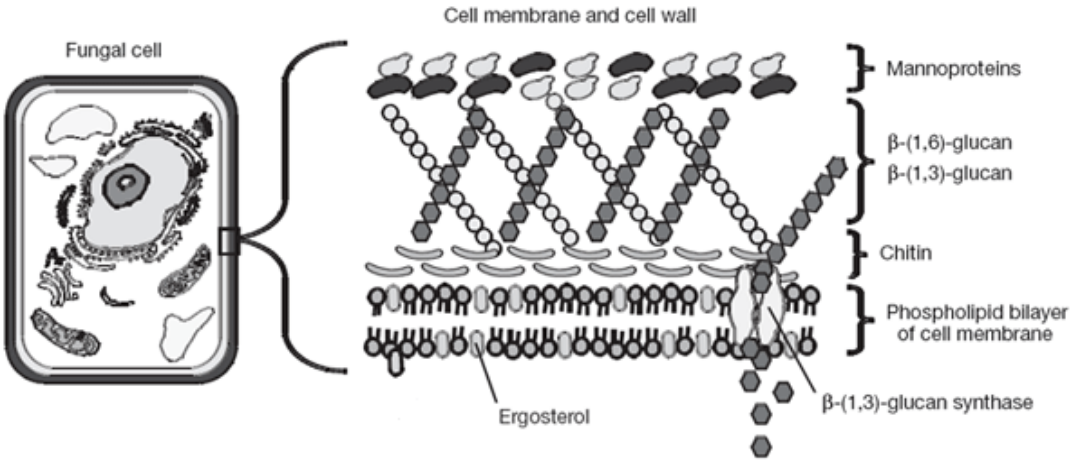

Figure 2.4 Schematic representation of the constituents of the cell membrane and cell wall of a yeast (adapted from Ashley et al., 2006).

The cell wall structure is relatively uniform, except the septal region. The septal region is the area where a scar is formed after budding. Figure 2.5 shows the formation of the bud scar.

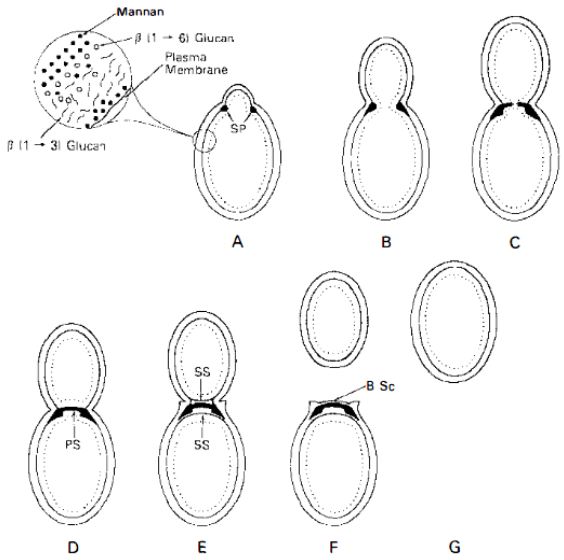

Figure 2.5 Scheme of the cell cycle of budding yeast. In A to G, the old wall has been represented as empty and the new one is stippled; chitin is filled in black and the plasma membrane is shown as a dotted line. SP, septal primordia; PS, primary septum; SS, secondary septa; BSc, bud scar (Cabib and Roberts, 1982).

In the vegetative cycle of budding yeast, the bud creation occurs by phases. At beginning, a polysaccharide (chitin) appears on the inner surface of the cell wall and, with as cell division progresses, the bud and its wall, grow to a size slightly smaller than that of the mother cell (Figure 2.5 A, B). By invaginations of the plasma membrane, the primary septum is formed (Figure 2.5 C, D). Following this stage, occurs the emergence of secondary septa (consisting of

glucans and mannans) on both sides of the primary septum (Figure 2.5 E). Finally, the cells separate, with bud scar formation (Figure 2.5 F) (Cabib and Roberts, 1982).

2.2.1 Glucans and Mannans

Among the various polysaccharides cell wall constituents of ascomycetes yeast, β-glucans are major structural components. β-glucans are polysaccharides of D-glucose monomers linked by β-glycosidic linkages. It is possible to find a huge variety of attachment position of glucose molecules. There is often a linear chain of glucose molecules repetitions, defining a main chain and from that, there are branches that produce smaller side chains (Cabib and Roberts, 1982; Lipke and Ovalle, 1998).

In the case of yeast cell wall, the main chains are formed by β-(1,3) linkages, while the branching are β-(1,6) linked (Figure 2.6). Thus, the polysaccharide main structural support of the wall is the β-(1,3)-glucan, forming a fibrous network located on the inner surface of the cell wall. Due to the presence of the side chains, the three-dimensional network is formed by the association of β-(1,3)-glucan molecules, through hydrogen bonds (Klis et al., 2006).

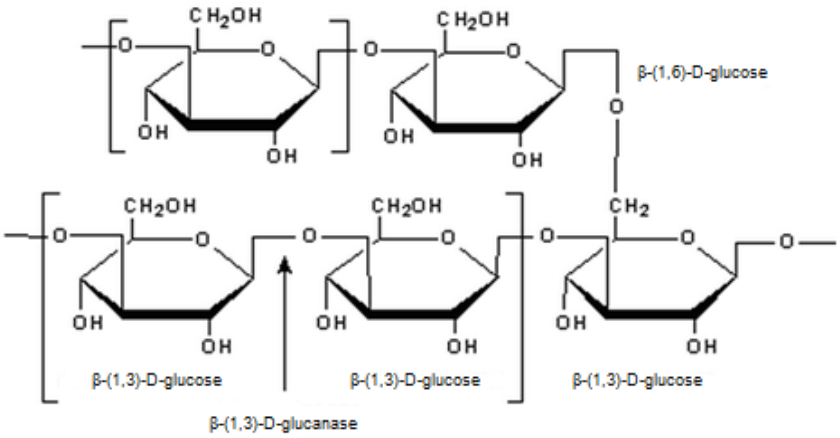

Figure 2.6 Schematic representation of β-(1,3) and β-(1,6) linkages of a β-glucan polysaccharide. (www.sigmaaldrich.com)

One of the characteristics that this network prints in the cell wall is the high flexibility allowed by the helical structure of β-(1,3)-glucan molecules. Structurally, the β-(1,3)-glucan helix may exist in the singular form, that means, be composed of only a single chain, or present a complex of three chains (triple helix) stabilized by hydrogen bonds. Through electron micrographs, it is estimated that the helix has a diameter between 10 nm and 30 nm, depending on various combinations of side chains, having 0.5 nm to 1 nm in diameter each one (Lipke and Ovalle, 1998).

On the outer side of the network β-(1,3)-glucan, arise β (1,6)-glucan chains which is characterized by being highly branched and allow for linking to other cell wall components, including mannoproteins (Lipke and Ovalle, 1998).

Yeast wall mannoproteins polypeptides are extensively glycosylated and their main function is to limit cell wall permeability to solutes. In yeast, these polypeptides are composed of 50 to 200 mannose monomers, linked to a long chain molecules of β-(1,6)-glucan. Phosphorylation of the mannosyl side chains confers a negative charge on the surface of the yeast cell wall (Lipke and Ovalle, 1998).

With regard to solubility, there is a cell wall fraction soluble in alkaline medium, constituted by β-(1,3)-glucans with significant number of links β(1,6) in the branches, a soluble fraction in acidic medium, constituted by β-(1,6)-glucans, and also a fraction insoluble in acidic and basic media, constituted by β-(1,3) glucan linked to chitin (Phaff, 2001).

2.2.2 Chitin / Chitosan

With the exception of cellulose, chitin is the most abundant polymer in nature. This polymer is synthesized by various organisms, being a common component of exoskeletons of invertebrates and cell wall of fungi and yeasts (Synowiecki and Al-Khateeb, 2003; Dutta et al., 2004).

Currently, the main sources of chitin for industrial processing are shellfish, namely crustacean. The synthesis of this polymer in annual freshwater and marine ecosystems is about 600 and 1600 million ton, respectively. The chitin content in crustaceans varies from 2 to 12%, and this number varies between different crustacean (Synowiecki and Al-Khateeb, 2003). Belonging to the order *Decapoda*, the crab and shrimp are species that have a higher content of chitin. Table 2.1presents the amount of chitin present in different crustacean species.

Table 2.1 Proximate quantification of chitin, on a percent (%), in different species of Crustacean (Synowiecki and Al-Khateeb, 2003).

Chitin Source		Chitin content (%)
Species		
Crab	*Callinectes sapidus*	13.5
	Chionoecetes opilio	26.6
Shrimp	*Pandalus borealis*	17.0
	Crangon crangon	17.8
	Penaeus monodon	40.4
Crawfish	*Procamborus clarkii*	13.2
Krill	*Euphausia superba*	24.0
Prawn		33.0

Although chitin is a product with high added-value in many application areas (pharmaceutical and cosmetic), its use is limited when it comes from crustacean due to the potential allergen. Other limiting factors are the seasonal nature of the producing species, environmental pollution produced by processing of crustaceans, the variability of the composition of the raw material and the limited supply of shell waste in some countries. These limitations make the extraction process less economical and with low reproducibility (Synowiecki and Al-Khateeb, 2003).

An alternative source of chitin, surpassing the limitations described above, is its production by microbial organisms, such as fungi and yeast (Synowiecki and Al-Khateeb, 2003).

The utilization of fungi and yeasts to obtain chitin has numerous advantages over the chitin produced from crustacean wastes. Yeasts reach high growth rates, associated with large cell densities (which increases polymer productivity), decrease the cost of demineralization (because the cell wall does not contain high amounts of calcium carbonate), allows for greater control of product quality by fermentation conditions, composition and properties stable, use of culture media of low cost, the production does not have a seasonal nature and there is the possibility of use in biomedical applications, since it is not a product derived from animal sources. All these advantages make the process economically feasible (Synowiecki and Al-Khateeb, 2003).

The chitin content differs among different species of yeast and fungi. The following table (Table 2.2) shows the wide range of chitin content in different species of microorganisms.

Table 2.2 Comparison of chitin content in different species of microorganisms (Feofilova et al., 2006; Nguyen and Fleet, 1998; Roca et al., 2012).

Microorganism species	Chitin content (%)
Aspergillus niger	38 - 41
Saccharomyces cerevisiae	3.11 - 3.61
Pichia pastoris	7

Chitin is the minor component present in the yeast cell wall and is mainly located around bud scars (see section 2.2). It is a white biomaterial, highly hydrophobic, corneal and with no elasticity. Chemically, chitin is a linear polymer consisting of monomers of N-acetyl-D-glucosamine (GlcNAc) linked by glycoside bonds the type β-(1,4). It is structurally identical to cellulose, but is has acetamide groups (-$NHCOCH_3$) at the C-2 positions (Figure 2.7 A). The length of the chitin molecule ranges from 5 000 to 8 000 residues of N-acetyl-glucosamine in crustaceans, and in yeast it may be composed of as little as 100 residues (Synowiecki and Al-Khateeb, 2003).

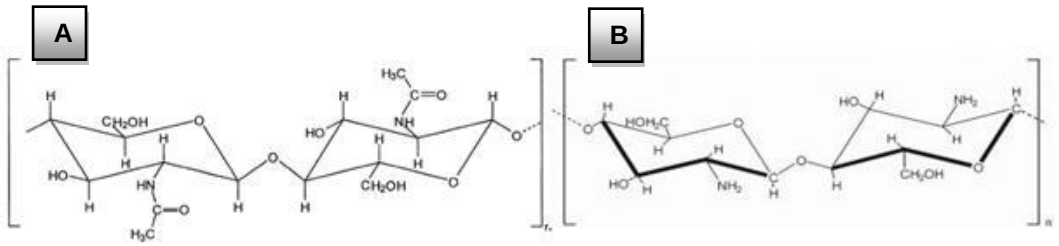

Figure 2.7 Structure of chitin (A) and chitosan (B) molecules (Synowiecki and Al-Khateeb, 2003).

Chitin molecules are joined together by hydrogen bonds and these linkages are responsible for chitin's low solubility in water and in most organic solvents, and allow fibril formation occurring in polymorphic forms, designated α- and β-chitin. In the yeast cell wall, the existing crystalline form is α-chitin, in which the chains are antiparallel in alignment and oriented so that hydrogen bonding is maximal. As a result, chitin is highly resistant to chemical extraction (Lipke and Ovalle, 1998).

In yeast, the chitin chains are located on the inside of the three-dimensional network formed by β-(1,3)-glucan (Figure 2.4). Chitin has the ability to link to both β-(1,3)-glucan and β-(1,6)-glucan.

Recently, the commercial value of chitin has increased due to the beneficial properties of their soluble derivatives that are used in chemistry, biotechnology, agriculture, cosmetics, papermaking or textile. Chitosan is the main derivative of chitin, obtained by N-deacetylation reaction. For chitin to be transformed into chitosan, the degree of deacetylation must be greater than 50% (Synowiecki and Al-Khateeb, 2003). The deacetylation reaction is obtained by impregnation of chitin in concentrated solutions of NaOH at high temperatures (thermal alkaline deacetylation).

Chitosan is a linear polymer of α (1, 4)-linked 2-amino-2-deoxy-β-D-glucopyranose (Figure 2.7 B), more polar and, consequently, more soluble than chitin. Chitin and chitosan are highly basic polysaccharides which have excellent properties such as biodegradability, biocompatibility, non-toxic, chelation ability and adsorption ability. The table below (Table 2.3) shows some of the applications of chitin and chitosan.

Table 2.3 Industrial applications of chitin and chitosan (Synowiecki & Al-Khateeb, 2003; Dutta et al., 2004).

Specific area	Main applications
Cosmetics	• Tooth-paste • Hand and body creams • Hair-care products
Agriculture / Food preservation	• Inhibitory effect on the growth of phytopathogenic fungi and bacteria • Induce resistance of plant to fungal, viral or viroid infections
Textile Industry	• Production of manmade fibers • Impart antistatic and soil repellent characteristics • Remove dyes from dye processing effluents
Water Engineering	• Flocculating agent • Chelating agent • Heavy metals trapper
Paper Industry	• Strengthen recycled paper • Increase the environmental friendliness of packaging and other products
Sorbents and Enzyme supports	• High affinity for heavy metals ions • Sorption capacity
Biomedical Applications	• Burn treatment • Tissue Engineering • Ophthalmology

With the development of new technologies, chitin and chitosan are two polysaccharides that play an important role in the creation of new industrial applications allowing generating a more promising future.

2.2.3 CGC

Chitin-glucan complex (CGC) is a copolymer of fungal origin, composed of chitin and glucan, two bioactive biopolymers, covalently linked through glycoside linkages the type β-(1,4). This complex is the main component of the inner cell wall of yeast and contributes for the stiffness and stability of the cells (Roca et al., 2012). Studies in *Saccharomyces cerevisiae* (*S. cerevisiae*) suggests that high mechanical strength of the cell wall of yeasts is due to the strong covalent bonds leading to the formation of rigid microfibers (Klis et al., 2006).

Traditionally, CGC is recovered as an insoluble residue after successive acidic and alkaline treatments. CGC hydrolysis allows obtaining chitin / chitosan and β-glucans individually, which have attracted great interest due to their wide applications in agriculture, wastewater treatment, cosmetics, tissue engineering and biomedical (See section 2.2.2). Fungal CGC is considered as an alternative source of chitin/chitosan, as well as a potent agent for application in medicine for wound-healing management or process improvement of desquamation and

xerosis reduction in diabetic patients, for reduction of aortic fatty streak accumulation (Smirnou et al., 2011). Currently, chitin-glucan complex is obtained mainly from waste biomass from the production of citric acid by *Aspergillus niger* (Ul-Haq et al., 2002).

CGC can be extracted from the cell wall of *P. pastoris* grown on glycerol from the biodiesel industry as the sole carbon source (Roca et al., 2012). A CGC content of 16% in *P. pastoris* cell wall, corresponding a ratio of 16:84 (mol%) of chitin:β-glucan were reported (Roca et al., 2012). These results are similar to the CGC content reported for other fungi, such as *Aspergillus niger* (15-25%), and yeast, such as *S. cerevisiae* (25%), using cane molasses and glucose as carbon sources, respectively (Feofilova et al., 2006). The biomass concentration obtained with *P. pastoris* (104 gL^{-1}) was much higher than for *Aspergillus niger* (20 gL^{-1}) and similar to *S. cerevisiae* (120 gL^{-1}). The high cellular density achieved, the acceptable amount of CGC and utilization of wastes as substrate, make *P. pastoris* an organism favorable for CGC production.

2.3 Wastes / byproducts

In fermentation processes, the use of a culture medium based on low cost substrates allows increasing the economic viability of biopolymers production. As any other biopolymer, production of CGC has a great interest in use renewable materials. In previous studies, glycerol from biodiesel industry was used as carbon source to produce CGC, minimizing production costs (Roca et al., 2012). The search for new carbon sources for this bioprocess, allows making the process more versatile, eliminating the dependence on a single substrate (pure or crude glycerol) and may increase productivity and / or have an impact on the CGC composition.

Alternatively to the biodiesel byproduct, several wastes/byproducts can be used as substrates for microbial growth, such as, for example, sugarcane molasses, cheese whey and spent coffee grounds.

2.3.1 Sugarcane Molasses

Sugarcane molasses are a byproduct resulting from the processing of sugarcane (*Saccharum L*.) for the production of refined sugar. Currently, Brazil is the largest producer of sugarcane and consequently, of molasses from this plant. About 17.9 million tons of sugarcane molasses are produced annually (In FAOSTAT 2011) that, in addition to being used for cultivation of microorganisms, is a major feed ingredient, used as an energy source and as a binder in compound feeds. Sugarcane molasses is a viscous, dark and sugar-rich byproduct of sugar extraction from the sugarcane. The composition of sugarcane molasses is divergent due to the peculiarity of sugarcane and the manufacturing conditions (Olbrich, 2006). Average composition values are represented in Table 2.4.

Table 2.4 Average composition of sugarcane molasses (Olbrich, 2006).

Sugarcane molasses (%)					
Sugars			Ash	Water	Nonsugars
Sucrose	Glucose	Fructose			
32	14	16	8	20	10

Due to high concentration of sugars (62%), sugarcane molasses is a potential substrate for microorganism cultivation, namely yeasts. Previous studies, such as Shahidan et al. (2011), report the utilization of this substrate to produce lipase by *P. pastoris* and Bhosale and Gadre (2001) reported the utilization of sugarcane molasses to produce β-carotene by *Rhodotorula glutinis*.

2.3.2 Cheese Whey

Cheese whey is a green-yellowish liquid resulting from the precipitation and removal of milk casein in cheese making processes, representing 85-95% of the milk volume it retains 55% of milk nutrients. According to the procedure used for casein precipitation, the cheese whey produced can be acid (pH <5) or sweet (pH 6-7). Cheese whey contains about 93-94% of water and the following nutrients from original milk: lactose (4.5-5% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5% w/v), minerals (0.8-10% w/v) and lactic acid (0.05% w/v) (Siso, 1996; Guimarães et al., 2010).

Presently, cheese whey is considered a agro-industrial byproduct and for 1kg of cheese produced 9kg of cheese whey are generated (Siso, 1996; Guimarães et al., 2010). Applying the valorization concept, this byproduct rich in lactose and protein, is widely used in biotechnology, being incorporated into the culture media for production of value-added products, such as biopolymers (e.g. polyhydroxyalkanoates, polysaccharides), single cell proteins (SCP), and bioethanol (Siso, 1996).

In opposition to sugarcane molasses, the yeasts that ferment lactose are rather rare, including *Kluyveromyces lactis*, *Kluyveromyces marxianus*, and *Candida pseudotropicalis* that ferment lactose to produce ethanol (Guimarães et al., 2010). The low yield of the fermentation of lactose by *P. pastoris* has been reported by Shahidan et al. (2011).

2.3.3 Spent Coffee Grounds

Coffee is one of the most important beverages of the world, has been consumed for over 1000 years and about one million tons are produced yearly, in more than 50 countries (Leifa et al., 2000). At different stages of processing, several residues are generated, in more than two million tons, as coffee husk and spent coffee grounds (Leifa et al., 2000).

Spent coffee grounds (SCG) is the main insoluble residue generated during the production of soluble coffee by thermal water extraction from roasted coffee beans. SCG is an acid residue, with 80-85% of humidity and constituted by fine particle size. For 1 ton of green coffee 650 Kg of SCG was generated and about 2 Kg of wet SCG are obtained for each 1 Kg of soluble coffee produced (Mussato et al., 2011a). The chemical composition of this residue reveals a high sugar content, particularly mannose and galactose (Table 2.5).

Table 2.5 Chemical composition (g $100g^{-1}$) of spent coffee grounds (Mussato et al., 2011b).

Spent Coffee Grounds hydrolysate (%)						
Sugars				Ash	Water	Nonsugars
Arabinose	Glucose	Mannose	Galactose			
1.7	8.6	21.2	13.8	1.6	36.7	15.8

Due to the toxic character conferred by caffeine, tannins and polyphenols present in SCG, this is considered a dangerous residue to the environment. Nevertheless, SCG has some applications in different areas, as fuel in industrial boilers of the same industry, as potential source for biodiesel production and other value-added products, such as hydrogen and ethanol (Mussato et al., 2011a). The high content in sugar allows integration of SCG in cultivation medium for microorganisms, such as *S. cerevisiae*. The hydrolysate resulting from the acid hydrolysis of SCG has been used in the fermentation medium by this yeast for ethanol production, with a 50.1% efficiency (Mussato et al., 2011a).

2.3.4 Other wastes/byproducts

In last decade, wastes from the paper and pulp industries have being used as substrates for yeast growth. The valorization of recycled paper sludge have been reported for cultivation of yeasts such as *Pichia stipitis* for production of ethanol (Marques et al., 2008). The composition of waste was determinate do be (on a dry weight basis): 34.1% cellulose, 29.3% ash, 20.4% lignin, 7.9% xylan, 4.8% protein and 3.5% fat (Marques et al., 2008).

Other wastes with potential for use as substrates for microbial growth are wastes rich in fatty acids. Previous studies indicate that *P. pastoris* mutant strains were able to grow in medium with 0.2% oleic acid with a specific growth rate of 0.35 h^{-1} (Wriessnegger et al., 2009). Liu et al. (1992) reported that wild type of *P. pastoris* grew poorly in medium supplemented with 0.1% of oleic acid.

This study aimed to assess the suitability of different substrates for the cultivation of the yeast *P. pastoris* and production of CGC. The first part of the work consisted in investigating the

ability of the culture to use glucose and xylose mixtures for growth and their impact on CGC production. In the second part of the study, the potential of different industrial wastes and / or byproducts as substrates for this yeast was evaluated, to finding substrates alternative to glycerol. The utilization of alternative substrates allows increase the process versatility, extinguishing dependence on a single substrate, and decrease the production cost, since the price of glycerol (even from biodiesel industry) can vary widely and also lead to a variation of cost of CGC production.

3. Materials and methods

3.1 Yeast strain and growth media

All experiments were performed with *Pichia pastoris* strain DSM 70877. The culture was cultivated in standard basal salts medium (BSM) (Pichia Fermentation Process Guidelines, Invitrogen), with the following composition: H_3PO_4 85%, 26.70 mL L^{-1}; $CaSO_4$, 0.93 gL^{-1}; K_2SO_4, 18.20 gL^{-1}; $MgSO_4.7H_2O$, 14.90 gL^{-1}; KOH, 4.13 gL^{-1} and 4.35 mL L^{-1} of a trace elements solution (PTM) comprising: $CuSO_4.5H_2O$, 6 gL^{-1}; NaI, 0.08 gL^{-1}; $MnSO_4.H_2O$, 3 gL^{-1}; $Na_2MoO_4.2H_2O$, 0.2 gL^{-1}; H_3BO_3, 0.02 gL^{-1}; $CoCl_2.6H_2O$, 0.5 gL^{-1}; $ZnCl_2$, 20 gL^{-1}; $Fe_2SO_4.7H_2O$, 65 gL^{-1}; biotin, 0.2 gL^{-1} and H_2SO_4, 5.0 mL L^{-1}. The BSM medium was sterilized at 121 ºC for 20 minutes and the PTM solution was filter-sterilized (0.2 µm, Sartorius stedim Minisart). The PTM solution was added after cooling. The pH was adjusted to 5.0 with a 25% (v/v) ammonium hydroxide solution (Scharlau).

BSM was supplemented with the different substrates used in this study, which were prepared separately. For the study of the use of glucose/xylose mixtures, the sugar solutions were prepared individually 100 gL^{-1} monohydrate glucose (Fragon); 100 gL^{-1} D-xylose (Sigma-aldrich) and autoclaved at 121 ºC for 20 minutes. Each solution was added to BSM medium at the time of inoculation to give the appropriate glucose and xylose percentages for a total sugar concentration of 80 gL^{-1} (Table 3.1).

Table 3.1 Percentages of glucose and xylose used in each trial.

Trial No.	Percentage (%)	
	Glucose	Xylose
1	100	0
2	85	15
3	70	30
4	60	40
5	50	50
6	40	60
7	25	75
8	10	90
9	0	100

For the study of the wastes/byproducts, several materials were used: cheese whey, sugarcane molasses, used cooking oil, waste fats, waste paper and spent coffee grounds.

The cheese whey used in this study was supplied by Lactogal (Portugal). For the experiments, the cheese whey powder was dissolved in deionised water to obtain a

concentration of 40 gL^{-1}. The solution was autoclaved at 121 ºC for 20 minutes and centrifuged (17 418 *g* for 15 minutes) to remove precipitated protein aggregates.

The sugarcane molasses used in this study was supplied by RAR – Refinarias de Açúcar Reunidas. The sugarcane molasses were diluted in deionised water to obtain a pretended concentration and the solution was sterilized at 121 ºC for 20 minutes.

The used cooking oil for this study came from the snack bar of the Chemistry Department, at FCT-UNL. For the experiments, the used cooking oil was sterilized by autoclaving (121 ºC for 20 minutes).

The waste fat used in this study was supplied by FIMA – Produtos Alimentares, SA (Grupo Unilever, Jerónimo Martins). The solid waste fat sample was sterilized by autoclaving (121 ºC for 20 minutes) and stored at room temperature until used in the tests. Prior to its use in the experiments, the solid waste fat was melted at 70 ºC, for 20 minutes and added to the cultivation media while still hot (~50 ºC).

The paper and spent coffee grounds were both subjected to acid hydrolysis prior to their use in the experiments. The waste paper used in this study was a mixture of magazines and used office paper. Spent coffee grounds were supplied by the snack bar of the Chemistry Department, at FCT-UNL. Before hydrolysis, the waste paper was ground into a powder to increase the efficiency of the process, while the spent coffee grounds were subjected to drying (70 ºC for 3 hours). To prepare the solutions for hydrolysis, 10 g of spent coffee grounds were mixed with 100 mL of deionised water and 4 g of powdered paper were mixed with 200 mL of deionised water. Two acid hydrolysis procedures were performed with different acids, namely, orto-phosphoric acid 85% (H_3PO_4) and sulfuric acid 98% (H_2SO_4), at different concentrations (26.7 mL L^{-1} and 100 mL L^{-1}). To select the best hydrolysis conditions, hydrolysis at different temperatures and reaction time were performed (Table 3.2).

Table 3.2 Conditions of acid hydrolysis of spent coffee grounds.

Trial No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Temperature (ºC)	30			60			90			100			121 [1]		
Time (min)	20	45	60	20	45	60	20	45	60	20	45	60	20	45	60

After cooling to room temperature, the hydrolysates were centrifuged (9820 *g*, for 30 minutes) and two fractions were obtained: a solid residue and a sugar solution. The solid residues were discarded and the sugar solutions were used for the experiments. Before their use, the pH of the hydrolysates was adjusted to 5.0 by the addition of a 25% (v/v) ammonium hydroxide solution. Table 3.3 summarizes the substrates and treatments applied to each one.

[1] Autoclaving at 121 ºC and 1 bar.

Table 3.3 Initial substrates screening and their treatment applied.

Substrate	Treatment
Cheese whey	Sterilization by autoclaving of an aqueous solution and centrifugation
Sugarcane molasses	Dilution in deionised water and sterilization by autoclaving
Used cooking oil	Sterilization by autoclaving
Waste fat	Sterilization by autoclaving
Waste paper	Acid hydrolysis
Spent coffee grounds	Acid hydrolysis

3.2 Inocula preparation

Pre-inocula for the experiments were prepared in 100 mL shake flasks by inoculating 1 mL of the cryopreserved culture (stored at -80 ºC) in 40 mL of BSM medium, containing glycerol (40 gL^{-1}). The pre-inocula were incubated for 72 hours at 30 ºC and 200 rpm, in an orbital shaker (IKA KS 260 basic).

Inocula for the experiments were prepared by inoculating 10% (v/v) of pre-inoculum in BSM medium, supplemented with the appropriate substrate, in 500 mL shake flasks, and incubating for 72 hours at 30 ºC, in an orbital shaker (200 rpm).

The inoculums prepared for all experiments, were performed with glycerol as substrate obtaining high cell density and the same cell concentration at the beginning of all trials.

3.3 Shake Flask Screening

The experiments were performed in 500 mL shake flasks with 150 mL of BSM medium, supplemented with the appropriate substrate. The medium was inoculated with 10% (v/v) of inoculums, prepared as described above, and growth occurred at 30 ºC and 200 rpm.

The assays were run for 50-175 hours, depending on the substrate tested, and 3 mL samples were periodically taken for measurement of the optical density at 600nm (OD_{600nm}) and pH, and determination of biomass and substrate concentrations. At the end of the experiments, 20 mL samples were also collected for CGC quantification and polymer composition analysis.

3.4 Batch bioreactor experiments

Experiments were carried out in BioStat® B-plus bioreactors (Sartorius) with 2 L and 3 L of working volumes. The bioreactors, containing BSM medium, were inoculated with 10% (v/v) of inoculums, prepared as described above, and operated with controlled temperature (30±0.1 ºC) and pH (5.0±0.02), for all runs. pH was controlled by the automatic addition of 25% (v/v) ammonium hydroxide and HCl 2M solutions. The ammonium hydroxide solution also served as the nitrogen source. The dissolved oxygen concentration (DOC, pO_2) was controlled at 50% air saturation by the automatic variation of the stirring rate, from 300 to 2000 rpm, while the airflow rate was kept constant during all experiments (1 vvm). When the stirring rate reached its maximum value (2000 rpm), the DOC was controlled by the automatic supplementation of the air stream with pure oxygen (> 99.995%). During the assay, foam formation was suppressed by the automatic addition of Antifoam A (Sigma-aldrich). All experiments were performed in a batch mode. 25 mL samples were periodically taken for biomass, substrate and CGC quantification.

3.5 Analytical techniques

3.5.1 Cell growth

Cell growth was monitored during the experiments by measurement of the optical density at 600 nm (OD_{600nm}) in UV-Vis spectrophotometer (Thermo Spectronic, Heλios α) and pH (Crison, Basic 20). Broth samples were diluted with deionised water so that the measured OD_{600nm} value was below 0.3. The measurements were done in duplicate.

3.5.2 Biomass quantification

Cell dry weight (CDW), defined as the dry weight of cells per litre of fermentation broth, was determined gravimetrically. 20 mL broth samples were centrifuged at 17 418 *g* for 15 minutes at +4 ºC. The cell-free supernatant was stored at -20 ºC for substrate quantification.

The cell pellet was washed twice with deionised water (re-suspension in water and centrifugation at 17 418 *g,* for 15 minutes at +4 ºC). After washing, the pellets were frozen in liquid nitrogen and lyophilized (Telstar, Cryodos) for 48 hours at -40 ºC and bellow 0.3 mbar. The CDW was determined as the weight of the lyophilized cell pellets. This analysis was done in quadruplicate.

3.5.3 Substrate concentration

For quantification of sugars, cell-free supernatant samples collected during the experiments were diluted. After dilution, the samples were filtered with 0.20 µm centrifuge filters (9 600 *g* for 5 minutes; VWR) prior to the analysis.

The concentration of glucose and xylose was determined by high performance liquid chromatography (HPLC), using an ion exchange column (Metacarb 87H; 300 mm x 7.8 mm, 9 mm; Varian) coupled to a refractive index detector (RI-71, Merck). The mobile phase was 0.001 M H_2SO_4 solution at a flow rate of 0.5 mL min^{-1} and the column was operated at 30 ºC. For this method, samples were dilutes with 0.001 M H_2SO_4 solution. Standard solutions of glucose (Fragon) and xylose (Sigma-aldrich, 99%) with 0.156 – 5 gL^{-1} were used to generate the calibration curves (Appendix).

The sugar monomers composition of the wastes/byproducts substrates (Lactose, galactose, glucose, fructose, sucrose, mannose and arabinose) were determined by HPLC using a CarboPac PA10 column (Dionex), equipped with an amperometric detector. The analysis was performed with sodium hydroxide (NaOH 18 mM) as eluent, at a flow rate of 0.8 mL min^{-1} and carried out at 30 ºC. For this method, samples were dilutes with deionised water. Lactose, galactose, glucose, fructose, sucrose, mannose and arabinose (Sigma-aldrich) with 0.006 - 0.2 gL^{-1}, were used as standards.

For the concentration of the total sugars in the waste paper and spent coffee grounds hydrolysate and sugarcane molasses samples, Dubois method was used. Dubois is a colorimetric method where 2.5 mL of H_2SO_4 was added to the 0.5 mL of 5% (v/v) phenol solution and 0.5 mL of sample. The solution was conserved in dark place for 30 minutes and its optical density was measured at 490 nm. In this method, glucose solutions with concentrations of 0.003 - 0.1 gL^{-1} were used as standards. The calibration curves are given in Appendix.

3.5.4 CGC Extraction

For extraction of CGC from the yeast biomass, 100-300 mg of the lyophilized cells were treated with 30 mL NaOH 5M at 65 ºC, for 2 hours, under constant stirring (800 rpm), for solubilisation of cell wall components. The alkali-insoluble material (containing the CGC) obtained by centrifugation of the mixture (17 418 *g*, for 15 min) was re-suspended in deionised water (30 mL) and neutralised with HCl 6M. After centrifugation (17 418 *g*, for 15 min), the polymer was washed twice with deionised water to remove alkali soluble components and, finally, it was freeze dried for the gravimetric quantification of the polymer content in the biomass.

3.5.5 CGC composition

The determination of CGC sugar composition was performed by acid hydrolysis, using trifluoroacetic acid (TFA) and hydrochloric acid (HCl) to hydrolyse the glucan and the chitin fractions of the polymer, respectively. To increase the hydrolysis efficiency, both hydrolysis were performed with a constant stirring (800 rpm), keeping the solution homogenous. To quantify the glucan content in the polymer, about 5 mg of lyophilized polymer samples were re-suspended in 5 mL of deionised water and 100 µL TFA 99% was added. The hydrolysis was performed at

120 ºC for 2 hours. For the HCl hydrolysis, about 5 mg of dry polymer samples were re-suspended in 5 mL of HCl 12M and hydrolysis was performed at 120 ºC for 5 hours. After HCl hydrolysis, 1 mL of the hydrolysates was evaporated to dryness and re-suspended in 1 mL of deionised water.

Both hydrolysates were used for the quantification of the constituent monosaccharides by HPLC using a CarboPac PA10 column (Dionex), as described in section 3.5.3. Glucose, mannose and glucosamine with 0.006 - 0.2 gL^{-1} (Sigma-aldrich) were used as standards, being subjected to the same hydrolysis procedures as the polymer samples. The calibration curves are given in Appendix.

3.5.6 Kinetic parameters

The specific cell growth rate (μ_{max}, h^{-1}) was determined using the follow equation:

$$\ln (x/x_0) = \mu\, t \qquad (1)$$

where x_0 (gL^{-1}) is the CDW at the beginning of the assay and x (gL^{-1}) is the CDW at time t (h).

The CGC volumetric productivity (r_p, $gL^{-1}h^{-1}$) was determined as follows:

$$r_p = \Delta p / \Delta t \qquad (2)$$

$$\Delta p = \text{CDW} \times \%\text{CGC} \qquad (3)$$

where Δp corresponds to the product, CGC (gL^{-1}), produced at time t (h), and Δt is the time interval between the beginning of the assay and time t (h).

The biomass yield ($Y_{X/S,}$ g_{CDW} $g_{substrate}^{-1}$) was determined using the follow equation:

$$Y_{X/S} = dX / dS \qquad (4)$$

where dX (gL^{-1}) is the CDW at time and dS (gL^{-1}) is the substrate consumed at time t (h).

The product yield ($Y_{P/S,}$ g_{CGC} $g_{substrate}^{-1}$) was determined as follows:

$$Y_{P/S} = dP / dS \qquad (5)$$

where dP (gL^{-1}) is the product at the time and dS (gL^{-1}) is the substrate consumed at time t (h).

4. Results and Discussion

4.1 Use of glucose and xylose mixtures for cultivation of *Pichia pastoris* and chitin-glucan complex production

Glucose and xylose are two of the main sugar components present in lignocellulosic wastes (e.g. corn stover, wheat straw, winter rye), in varying proportions. The content in each of those sugars depends on the type of material, but, in most of cases, glucose is present in higher amounts than xylose. For example, corn stover, an abundant agricultural byproduct, which consists on the stalk, cob and leaves, is constituted mostly by glucose (39.0% (w/w)) and xylose (14.8% (w/w)) (Aristidou and Penttilä, 2000). Similar glucose/xylose contents are found in wheat straw: 36.6% (w/w)% glucose and 19.2% (w/w)) xylose (Aristidou and Penttilä, 2000; Kootstra et al., 2009). Winter rye has higher glucan and xylan contents, reaching 40.8% (w/w) and 22.3% (w/w), respectively (Petersson et al., 2007). More rarely, the xylose content can be higher than that of glucose, as is the case of: corn cobs, with 68.2% (w/w) of xylose and 8.4% (w/w) of glucose (Kim et al., 1999) and birch wood, with 55.4% (w/w) of xylose and 7.8% (w/w) of glucose (Morris, 1992). The aim of this study was to assess the ability of *P. pastoris* for utilization of mixtures of those sugars, xylose and glucose, as carbon sources and, hence, evaluate the suitability of lignocellulosic materials rich in glucose and/or xylose for production of CGC. Commercial glucose and xylose were chosen for this study as model substrates, considering that the results could be extrapolated for lignocellulosic materials.

4.1.1 Shake flask screening

In order to determine the most appropriate glucose and xylose mixtures for *P. pastoris* cultivation, a preliminary shake flask screening was performed, wherein different percentages of xylose were tested (Table 3.1). Figure 4.1 shows the results obtained in each assay in terms of CDW and CGC production.

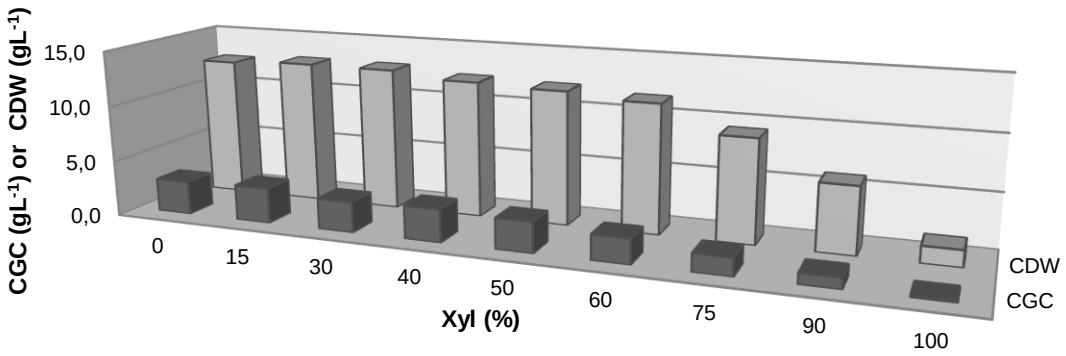

Figure 4.1 CDW and CGC obtained by cultivation of *P. pastoris* in the shake flask assays with different glucose/xylose mixtures.

The culture was able to grow in all assays, but cell growth was reduced as the xylose percentage was increased (Figure 4.1). High CDW values were obtained (12.08 – 12.90 gL^{-1}) for the assays performed with xylose percentages between 0 and 50%, but the CDW was gradually reduced as the xylose percentage was further increased. In the assay performed with xylose as the sole carbon source, the CDW was considerably reduced (1.50 gL^{-1}).

Along the experiments, glucose and xylose consumption were measured and the results have shown that glucose was depleted in the first hours of all assays (~24 h). Xylose was completely consumed in the assays performed with xylose percentages up to 40%, but its consumption rate was slower than that of glucose. In those assays, xylose and glucose consumption occurred simultaneously, but xylose was consumed faster after the depletion of glucose. In opposition, in the assays with higher xylose concentration (75 – 100%), xylose was not exhausted within 175 hours of the experiments, suggesting a slower consumption rate, justifying the reduced CDW obtained.

These results are in accordance with Inan and Meagi-ier (2001) that reported the inability of *P. pastoris* to use xylose as sole substrate. On the other hand, the preference of glucose over xylose may be due to catabolite repression: in the presence of glucose, the enzymes required for xylose metabolism are synthesized at low rates or are not synthesized at all. This phenomenon has been reported for several yeast, including *Pachysolen tannophilus* and *Pichia stipitis* (Bicho et al., 1988).

The results obtained showed that CGC was produced in all nine experiments (Figure 4.1). However, the CGC content in the biomass was similar for the experiments performed with xylose percentages up to and 50% (22– 25%, w/w), but it decreased for higher xylose percentages (12.95 - 19.44%, w/w). Concomitantly, CGC production was higher (2.75 – 3.17 gL^{-1}) in the assays with xylose percentages up to and 50%, wherein higher biomass concentration was also reached. In the experiments with the highest xylose percentages tested (75 and 100%), concomitantly with the low cell growth observed, CGC production was also very low: 0.19 – 1.61 gL^{-1}. So, it appears that with the increase in the percentage of xylose in the medium, a decrease in both biomass and CGC production occurs.

The shake flask screening allowed verifying that *P. pastoris* was able to consume glucose and xylose, but for the higher xylose percentages tested, cell growth was reduced and xylose consumption was very low. Based on these results, the best conditions for production of CGC by *P. pastoris* seem to be using xylose/glucose mixtures with xylose percentages up to 40%.

4.1.2 Batch bioreactor experiments

Glucose/xylose mixtures with different xylose percentages (0, 12.5, 25 and 50%) were selected to perform batch bioreactor experiments due to the high cell growth and CGC production observed within that range in the preliminary shake flask assays. A xylose percentage of 75% was also tested to evaluate in the bioreactor a higher percentage of xylose that can be present in several lignocelullosic wastes (e. g., corn cob (Kim et al., 1999), birch wood (Morris, 1992) and oat hulls (Garleb et al., 1991)). Hence, these experiments aimed to evaluate the ability of the culture to grow and produce CGC in a large range of xylose concentrations.

4.1.2.1 CGC production

The first experiment (0%Xyl) was performed (Figure 4.2 A) using glucose as the sole substrate. In this trial, after a lag phase of 15 hours, *P. pastoris* grew at a specific cell growth rate of 0.20 h^{-1} and reached a final CDW of 33.90 gL^{-1} within 37 hours of cultivation (Table 4.1). As expected, after 20 hours, the growth stabilized when the carbon source was depleted. Along the experiment, the culture consumed all the initial glucose (74.75 gL^{-1}), with a consumption rate of 8.59 $gL^{-1}.h^{-1}$ (Table 4.1). The CGC concentration reached the highest value (5.57 gL^{-1}) in the stationary phase. This value represents a 16.44 % content of CGC in the biomass.

The specific cell growth rate achieved in this experiment was slightly higher than the value obtained with glycerol as sole carbon source (0.17 h^{-1}) by Chagas et al. (2013). Despite this difference, the CGC content in the biomass was similarly in both assays with either carbon source (16%). These results suggest that *P. pastoris* grew faster with glucose than with glycerol but this fact did not significantly affect the CGC content.

The overall volumetric productivity of the assay (0.149 $gL^{-1}.h^{-1}$) was within the range of values obtained with glycerol as sole carbon source (0.105 – 0.168 g_{CGC} $L^{-1}.h^{-1}$) reported by Chagas et al. (2013).

The culture reached a growth yield of 0.45 $g_{biomass}$ $g_{glucose}^{-1}$ demonstrating the high conversion of glucose into biomass. This value was slightly lower than that obtained from crude glycerol (0.55 $g_{biomass}$ $g_{glycerol}^{-1}$) in Roca et al. (2012), indicating that glycerol was more efficiently converted into biomass. Similarly, the product yield (0.08 g_{CGC} $g_{glucose}^{-1}$) was also lower than that obtained for crude glycerol (0.15 g_{CGC} $g_{glycerol}^{-1}$) (Roca et al., 2012).

The results obtained in this experiment were lower than the results obtained by Heyland et al. (2010), where *P. pastoris* SMD1168H grew on glucose at a specific grow rate of 0.28 h^{-1}, reaching a final CDW of 68 gL^{-1}, in a batch bioreactor experiment, for recombinant protein production. The biomass yield achieved (0.55 $g_{biomass}$ $g_{glucose}^{-1}$) was also higher than that reached in the 0%Xyl experiment (Table 4.1).

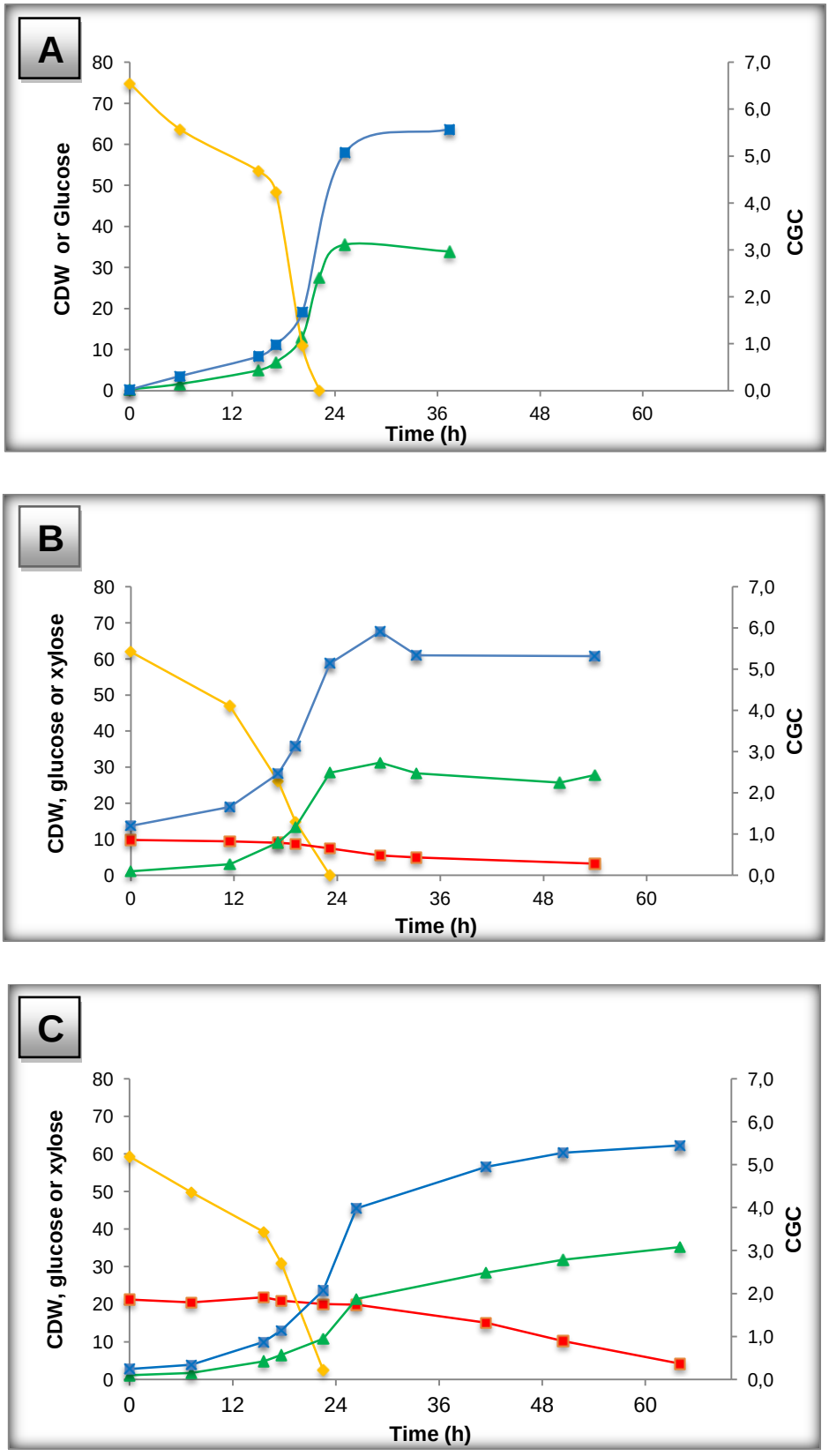

Figure 4.2 Cultivation profiles of the batch bioreactor experiments: 0%Xyl (A), 12.5%Xyl (B) and 25%Xyl (C). Dry cell weight (▲, gL^{-1}), glucose (◆, gL^{-1}), xylose (■, gL^{-1}) and CGC concentrations (●, gL^{-1}).

The addition of xylose to the medium, at percentages of 12.5% (Figure 4.2 B) and 25% (Figure 4.2 C) led to the decrease of the maximum specific growth rate to 0.17 h^{-1} and 0.14 h^{-1}, respectively, comparing to the 0%Xyl experiment (Table 4.1). This decrease may suggest that the presence of xylose affected the cellular division of the culture, causing an attenuation of exponential phase, leading to a specific growth rate reduction. These results are in agreement with studies for *Pichia stipitis* (Agbogbo et al., 2006), in which a maximum specific growth rate of 0.10 h^{-1} was obtained for cultivation in glucose, while the addition of xylose (25%Xyl) led to a reduction of the maximum specific growth rate to 0.075 h^{-1}.

Nevertheless, similar maximum CDW were obtained in all three assays, 0%Xyl, 12.5%Xyl and 25%Xyl (27.80 - 35.25 gL^{-1}), as well as similar CGC content in the biomass (15.47 – 19.13%) and CGC production (5.32 – 5.57 gL^{-1}) (Table 4.1). In opposition, the addition of xylose negatively affected the overall volumetric productivity, reducing the values to 0.099 and 0.085 $gL^{-1}h^{-1}$ for assays with 12.5% and 25% of xylose, respectively, comparing to the 0%Xyl (Table 4.1).

In all three experiments (0, 12.5 and 25% of xylose), glucose was completely consumed during the first 23 hours (Figure 4.2). Although the xylose consumption rate in the presence of glucose was much lower, Figure 4.2 shows that it was initiated before glucose was depleted, indicating that *P. pastoris* was able to simultaneously metabolize both sugars. Similar results were reported for *P. stipitis*, with glucose being the preferred substrate when glucose/xylose mixtures were used as substrates. This is due to repression of xylose uptake by glucose (Bicho et al., 1988). However, for *P. stipitis*, xylose fermentation started only after glucose depletion.

As Figure 4.2 shows, the CDW obtained during the consumption of glucose was higher than that achieved with xylose, after glucose depletion. After glucose depletion, cell growth was considerably reduced, indicating that the observed xylose consumption was probably for cell maintenance or production of secondary metabolites.

As for CDW values, the biomass (0.36 - 0.45 g g^{-1}) and production yields (0.07 g g^{-1}) obtained for experiments 12.5%Xyl and 25%Xyl were similar to the values obtained for 0%Xyl (0.45 g g^{-1} and 0.08 g g^{-1}, respectively) (Table 4.1). These results show that it is possible to use glucose/xylose mixtures with xylose percentages up to 25% with no significant impact on cell growth or CGC production.

In order to determine the culture's ability to grow in medium with higher concentrations of xylose, experiments with 50 and 75% xylose were performed (Figure 4.3). The results show that specific cell growth rate was not significantly affected by increasing xylose percentage in the medium. The specific cell growth was similar for all assays performed with xylose percentages between 12.5 and 75% (Table 4.1), which is probably related to the fact that the initial cell growth during the exponential phase was due to the available glucose.

In the 50%Xyl experiment (Figure 4.3 A), after a 14 hours lag phase, the culture grew at a maximum specific growth rate of 0.17 h^{-1} and reached a maximum CDW of 16.99 gL^{-1} within 91 hours (Table 4.1). In the 75% xylose experiment (Figure 4.3 B), the maximum specific growth rate was slightly lower (0.14 h^{-1}) and a lower biomass concentration (13.64 gL^{-1}) was attained at the end of the cultivation (66 hours) (Table 4.1). In these two assays, the CDW achieved was low comparatively to experiments with xylose percentages between 0 and 25%. These results are probably due to lower glucose concentration available in medium in assays 50%Xyl and 75%Xyl: cell growth was mainly based on xylose consumption that resulted in lower production of biomass.

At the end of the 50%Xyl experiment, the CGC content in the biomass reached 12.28%, whereas in 75%Xyl it achieved 15.07%. Regarding the CGC production, similar values were reached in both experiments: 2.09 and 2.06 gL^{-1}, respectively, being these values much lower than that obtained for the assays with 0 - 25% of xylose (Table 4.1). Hence, lower volumetric productivity values were also obtained: 0.023 and 0.031 $gL^{-1}.h^{-1}$, in the 50%Xyl and 75%Xyl experiments, respectively. These results show that with the high percentage of xylose in the medium used in these two experiments, cell growth was impaired and, concomitantly, CGC production was also reduced.

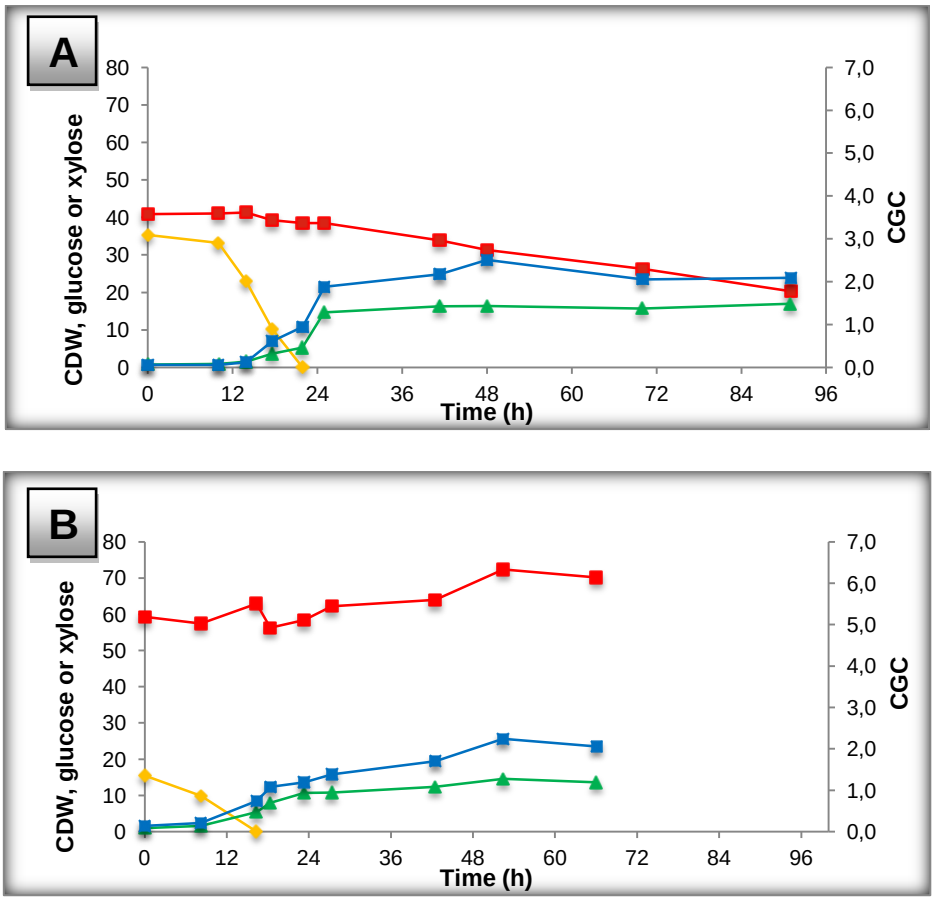

Figure 4.3 Cultivation profiles of the batch bioreactor experiments: 50%Xyl (A) and 75%Xyl (B). Dry cell weight (▲, gL^{-1}), glucose (●, gL^{-1}), xylose (■, gL^{-1}) and CGC concentrations (◆, gL^{-1}).

As shown in Figure 4.3, unlike the first experiments (0%Xyl, 12.5%Xyl and 25%Xyl), in both experiments, the low glucose concentration available was exhausted during the first 20 hours, while xylose was not completely consumed. In fact, in the 75%Xyl experiment, there was no consumption of xylose (Figure 4.3 B). Hence, a lower biomass concentration was achieved since cell growth was only due to the glucose available in the medium, which was low (15.42 gL^{-1}). These results corroborate the results obtained in the shake flasks assays, which suggested that the presence of high concentrations of xylose (above 75 gL^{-1}), may cause substrate inhibition. The explanation for this may be that *P. pastoris* requires that a specific glucose concentration is reached before significant xylose can be metabolized, analogously to *P. stipitis* , for which a similar sequential fermentation profile has been reported (Agbogbo et al., 2006). Studies performed with *P. stipitis* showed that glucose concentration should be below 2% (w/v) before significant xylose consumption occurred (Panchal, 1988). This behavior has still not been reported for *P. pastoris*, but the results of this study suggest that a similar behavior might have occurred.

In accordance with the reduced CDW obtained in the two assays, the growth yields in the 50%Xyl and 75%Xyl experiments were also lower, reaching only 0.271 and 0.194 g g^{-1}, respectively, compared with the previous assays. The production yields were also lower (0.033 and 0.029 g g^{-1}) (Table 4.1).

Table 4.1 Comparison of parameters obtained for glucose and xylose mixtures in batch bioreactor experiments. μ_{max}, maximum specific growth rate; CDW, maximum cell dry weight; % CGC, % of CGC in dry cell biomass; CGC, maximum active concentration; r_p, overall volumetric productivity; $Y_{X/S}$, growth yield; $Y_{P/S}$ production yield; R_{Glu}, glucose consumption rate; R_{Xyl}, xylose consumption rate.

Xyl (%)	0	12.5	25	50	75
Cultivation time (h)	37	54	64	91	66
μ_{max} (h^{-1})	0.20	0.17	0.14	0.17	0.14
CDW (gL^{-1})	33.90	27.80	35.25	16.99	13.64
% CGC	16.44	19.13	15.47	12.28	15.07
CGC (gL^{-1})	5.57	5.32	5.45	2.09	2.06
r_p (g_{CGC} $L^{-1}.h^{-1}$)	0.149	0.099	0.085	0.023	0.031
$Y_{X/S}$ (g g^{-1})	0.45	0.36	0.45	0.27	0.19
$Y_{P/S}$ (g g^{-1})	0.08	0.07	0.07	0.03	0.03
R_{Glc}	8.59	6.54	5.81	3.13	1.19
R_{Xyl}	-	0.13	0.35	0.26	-

Based on these results, it may be concluded that cell growth (27.80 – 35.25 gL^{-1}) and CGC (5.32 – 5.45 gL^{-1}) production were similar for xylose percentages until 25%, but the volumetric productivity (0.085 – 0.099 $gL^{-1}.h^{-1}$) and production yield (0.07 g g^{-1}) were lower in the presence of xylose, comparing with 0%Xyl experiment (0.149 $gL^{-1}.h^{-1}$ and 0.08 g g^{-1}, respectively) (Table 4.1). For the highest percentages of xylose (50 and 75%), both CDW and CGC production were greatly reduced (16.99 and 13.64 gL^{-1}, and 2.09 and 2.06 gL^{-1}, respectively) compared to experiments with lower xylose concentration. As mentioned above, the presence of xylose affected negatively the kinetic parameters probably due to repression of xylose by glucose, preventing the use of the former. Similar results have been reported for other yeast, such as *S. cerevisiae* (Krahulec et al., 2010; Kuyper et al., 2005) and *Pichia stipitis* (Agbogbo et al., 2006). Such strains have been genetically modified to allow improvement of xylose fermentation, which may also be attempted for the strain used in the present study.

4.1.2.2 CGC composition

In order to determine the composition of CGC, the complex was submitted an acid hydrolysis performed with the TFA, to determine the glucose fraction, and with HCl for chitin fraction quantification, as described in section 3.5.6. The TFA and HCl hydrolysates were analyzed for CGC characterization.

The polymers produced in three of the experiments with different xylose percentage (0%Xyl, 25%Xyl and 75%Xyl) were selected for CGC characterization (Figure 4.4).

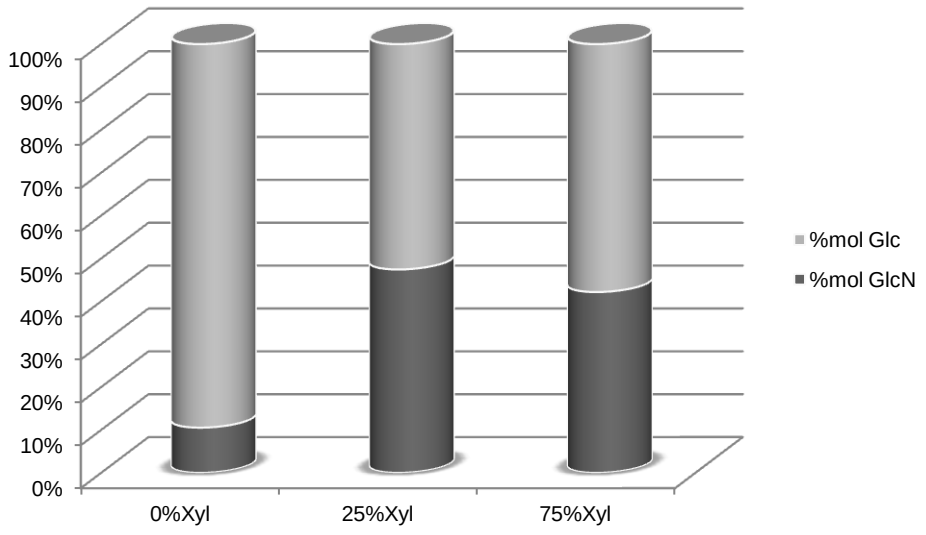

Figure 4.4 CGC composition for experiments with 0%, 25% and 75% of xylose. %mol Glc, % molar of glucose; %mol GclN, % molar of glucosamine.

The CGC biopolymer produced from glucose as carbon source (0%Xyl experiment) had glucosamine and glucose molar contents of 5 and 39%, respectively, corresponding to a chitin:β-glucan ratio of 11:89 (%mol). This ratio is similar to the values reported by Roca et al. (2012) for CGC production with crude glycerol (16:84 %mol). These results are in accordance with the typical composition of yeast cell wall (section 2.2.1), where β-glucans are the major structural component.

CGC polymers obtained in experiments with addition of xylose presented considerably higher chitin contents, namely 26 and 23%, for the 25%Xyl and 75%Xyl assays, respectively. These values correspond to a chitin:β-glucan ratio of 47:53 (mol%) and 42:58 (mol%) for 25%Xyl and 75%Xyl experiments, respectively, suggesting that the presence of xylose in the medium was advantageous for the process, since it led to increased chitin content in the produced biopolymer (Figure 4.4). This highest fraction of chitin in CGC may confer new properties to the polymer and also can allow to obtain pure chitin in highest quantity than usual CGC, produced in other cultivation conditions. These results were much higher than those obtained from crude glycerol, where the glucose and glucosamine contents was 35 and 7 wt%, respectively, corresponding to a chitin:β-glucan ratio of 16:84 (mol%) (Roca et al., 2012).

As mentioned above, currently CGC is extracted from the biomass of *Aspergillus niger* from the citric acid production process (section 2.2.3). The CGC composition of *Aspergillus niger* is mainly composed of glucan, presenting a chitin:β-glucan ratio of 40:60 (mol%) (Feofilova et al., 2006). This CGC composition is similar to that obtained in this study for *P. pastoris* grown on glucose/xylose mixtures, namely the 25%Xyl and 75%Xyl experiments (47:53 (mol%) and 42:58 (mol%), respectively).

4.1.3 Conclusions

In the present study, glucose and xylose mixtures were used as substrates for cultivation of *P. pastoris* and production of CGC. The results indicate that not all glucose/xylose mixtures are suitable for this bioprocess, namely the mixtures with highest percentages of xylose (above 75%). Nevertheless, this study has shown that it is feasible to valorize glucose and xylose containing lignocellulosic materials for the production of the value-added CGC biopolymer, as long as the percentage of xylose is not above 50%. According to the results, *P. pastoris* might be able to use a large of range of lignocellulosic residues (e.g. corn stover, rice straw and wheat straw) that are mostly composed by glucose, with lower xylose contents (Aristidou and Penttilä, 2000).

The specific cell growth rate and biomass yield obtained using glucose/xylose mixtures, with xylose percentages up to 50%, namely 12.5 and 25% (0.17 – 0.14 h^{-1} and 0.36 – 0.45 g g^{-1}, respectively), were similar to the results obtained with glycerol biodiesel byproduct (0.17 h^{-1} and 0.55 $g_{biomass}$ $g_{glycerol}^{-1}$, respectively) (Roca et al., 2012). In terms of overall volumetric productivity, the results for glucose/xylose mixtures (0.023 – 0.059 $gL^{-1}.h^{-1}$) was very lower comparatively to

obtained with glycerol (0.105-0.168 $gL^{-1}.h^{-1}$) (Chagas et al., 2013). This reduction of productivity decreases the expectation to industrial *scale-up*, however the process based on glucose/xylose containing substrates can be improved to achieve this aim.

Despite the lower overall volumetric productivity, the use of glucose/xylose mixtures increased the content of chitin in CGC from 5% with glucose as sole carbon source to 23 - 26% with xylose in the medium (25%Xyl and 75%, respectively). The maximum chitin content was achieved with 25% of xylose, corresponding to a chitin:β-glucan ratio of 47:53 (mol%), which is considerably higher than the chitin:β-glucan ratio (16:84 mol%) reported for growth in crude glycerol (Roca et al., 2012).

According to the results, lignocellulosic wastes with percentages of xylose not above 50% could be an alternative to glycerol, thus avoiding the dependence on this substrate and making the process more versatile. Another advantage of using lignocellullsic materials is the decrease of the overall production costs, since the price of glycerol (even from biodiesel industry) can vary widely and also lead to a variation of cost of CGC production. The availability of raw material is increased with the use of these wastes due to weak utilization of such materials, in opposition to glycerol which is used in several applications.

Process improvements may be attained with implementation of fed-batch or continuous systems, which can enable considerable increases of productivity. Genetically modified *P. pastoris* strains, able to efficiently grow on higher xylose percentages, might also be an alternative to improve the process. These variations on procedure can contribute to the increase of the CGC content and also impact on the chemical composition of the polymer.

4.2 Valorization of food and industry byproducts for the production of chitin-glucan complex

Agro-industrial residues are derived from the processing of a particular crop or animal product (Nigam and Pandey, 2009). Examples of those residues (cheese whey, sugarcane molasses, used cooking oil, waste fat, waste paper and spent coffee sounds) were used in this study for cultivation of *P. pastoris* and CGC production. As mentioned above, the wastes used in this study were supplied by several agro-industrial companies, namely Lactogal (cheese whey supplier), RAR (sugarcane molasses supplier) and FIMA (waste fat supplier). The used cooking oil for this study came from the snack bar of the Chemistry Department, at FCT-UNL, and the waste paper was a mixture of magazines and used office paper collected in the lab.

4.2.1 Substrates preparation

In order to enable the accessibility of the nutrients constituents of the wastes by *P. pastoris*, the substrates were subjected to specific pre-treatment procedures (Table 3.3).

The aqueous solutions of cheese whey and sugarcane molasses were simply sterilized by autoclaving prior to their addition to the cultivation media. After sterilization in autoclave, used cooking oil and waste fat were added directly to the BSM medium.

Waste paper and spent coffee grounds are complex substrates and it was necessary to hydrolyze them to obtain simple sugars more easily metabolized by the yeast. Acid hydrolysis of waste paper and spent coffee grounds were performed with two different acids: 1) orto-phosphoric acid (H_3PO_4) was used in the amounts usually present in BSM medium; and 2) sulfuric acid (H_2SO_4) was used as an attempt to increase the efficiency of the hydrolysis since it is a stronger acid.

Prior to hydrolysis, both wastes were characterized by elemental analysis and their ash contents were determined. The elemental analysis showed that the spent coffee grounds contained 1.37% of nitrogen, 8.14% of hydrogen and 30.64% of carbon, whereas waste paper contained 0.10% of nitrogen, 5.11% of hydrogen and 38.75% of carbon. The spent coffee grounds had an ash content of 0.42% (w/w), while a considerably higher value was obtained for the waste paper (32.43%, w/w), which is in accordance with literature values for recycled paper sludge (29.3% ash, see section 2.3.4).

Figure 4.5 shows the results of total sugars concentration obtained for each acid hydrolysis for different temperatures (30 – 121 ºC) and reaction times (20 – 60 minutes).

Figure 4.5 shows that the hydrolysis performance was affected by temperature and reaction time: an increase of the hydrolysis performance was observed by the increase of both variables. The results illustrated that for both substrates, the highest total concentration of

sugars was found at the highest levels of temperature (121 ºC) and reaction time (60 minutes) assayed.

As expected, the results obtained show that the hydrolysis with sulfuric acid was more efficient than with orto-phosphoric acid (Figure 4.5). This fact was justified by difference in strength of the used acids and their concentration. The H_3PO_4 concentration used was lower (26.7 mL L^{-1}) to maintain the concentration used in normal BSM medium (see section 3.1), while the H_2SO_4 concentration used (100 mL L^{-1}) was based in values reported in the literature for hydrolysis of olive tree pruning residues (Romero et al., 2010) and spent coffee grounds (Mussatto et al., 2011b). Thus, for spent coffee grounds hydrolyzed with H_3PO_4 and H_2SO_4 the maximum total sugar concentrations achieved were 12.54 gL^{-1} and 35.83 gL^{-1}, respectively. For waste paper, the maximum total sugar concentrations achieved were considerably lower in both hydrolysates: 1.15 gL^{-1}, for H_3PO_4, and 6.20 gL^{-1}, for sulfuric acid. These values was measured by Dubois method, where all soluble carbohydrates present in mixture are accounted, including monosaccharides, oligosaccharides and polysaccharides, as well as methyl ester with free or potentially free reducing groups (DuBois et al., 1956).

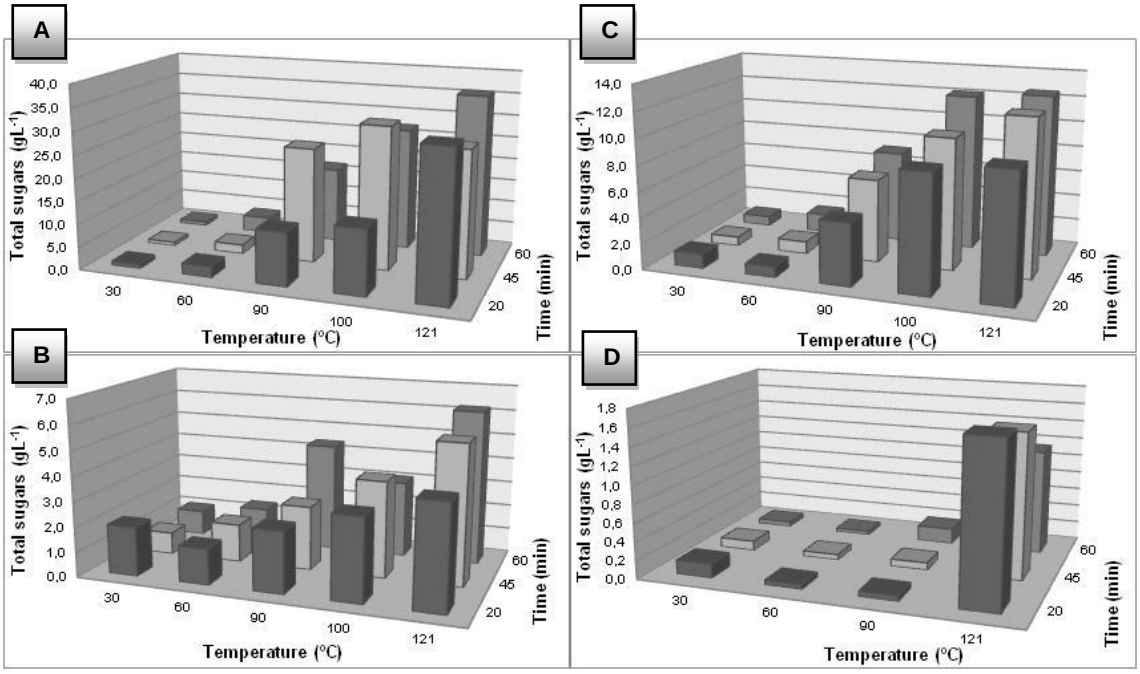

Figure 4.5 Efficiency of acid hydrolysis, in terms of total sugar concentration in the hydrolysates, with sulfuric acid applied to spent coffee grounds (A) and waste paper (B), and with orto-phosphoric acid applied to spent coffee grounds (C) and waste paper (D).

In order to corroborate the results of acid hydrolysis, the monosaccharides from spent coffee grounds hydrolysate were quantified by HPLC. The results show that spent coffee grounds hydrolysate contained 35.66 gL^{-1} of monosaccharides, being constituted by 48.93% mannose, 39.40% galactose, 8.61% arabinose and 3.06% glucose. These values are a bit different from those reported by Mussatto et al. (2011b) where are reported the presence of mannose (46.8%), galactose (30.4%), glucose (19%) and arabinose (3.8%). The higher glucose concentration obtained by Mussatto et al. (2011b) might be due to the acid hydrolysis conditions used, namely higher temperature and longer reaction time (163 ºC for 45 minutes), which were necessary for complete hydrolysis of the cellulose fraction.

Based on lower total concentration of sugars achieved and on highest content in ashes, waste paper was not selected to be tested as substrate for *P. pastoris* growth. Although the best acid hydrolysis performance for spent coffee grounds in terms of total sugar concentration (35.83 gL^{-1}) was obtained for sulfuric acid by autoclaving at 121 ºC for 60 minutes, similar total sugar concentration was obtained for milder conditions, namely 100 ºC for 45 min (31.38 gL^{-1}) (Figure 4.5). This hydrolysis condition was chosen for testing the cultivation of *P. pastoris* in shake flasks in order to reduce the energetic costs associated to autoclaving.

4.2.2 Shake flask screening

As for assays with glucose/xylose mixtures, in this study a shake flask screening was performed with the aim to select the best residues for *P. pastoris* cultivation and CGC production. The screening was performed with cheese whey, sugarcane molasses, used cooking oil, waste fat and spent coffee grounds hydrolysate with both sulfuric and orto-phosphoric acids. The results obtained regarding biomass growth are shown in Figure 4.6.

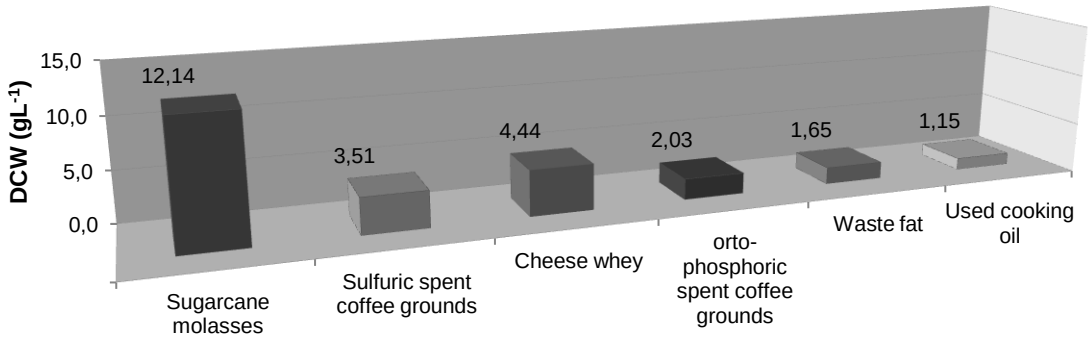

Figure 4.6 Biomass growth (CDW) obtained by cultivation of *P. pastoris* in the shake flask assays with different wastes.

The highest biomass concentration was achieved with sugarcane molasses (12.14 gL^{-1}) as carbon source and this result is similar to studies for the yeast *Rhodotorula glutinis* (6.6 –

12.0 gL^{-1}), where sugarcane molasses were used to produce β-carotene, in shake flask experiments (Bhosale and Gadre, 2001). In previous studies (Shahidan et al., 2011), the utilization of molasses by *P. pastoris* strain GS115 has already been reported but biomass concentration achieved with this residue was not reported.

The lower biomass concentration was attained with used cooking oil (1.15 gL^{-1}) being identical to that obtained with fat waste (1.65 gL^{-1}). These results suggest that *P. pastoris* can use residues rich in fatty acids to grow, although the values achieved are extremely low. The results obtained in this study are in accordance with previous studies, where *P. pastoris* X33 grew in medium with 0.2% of oleic acid but cell growth was much lower than in medium with glucose (Wriessnegger et al., 2007). Liu et al. (1992) also reported the lower growth of wild type *P. pastoris* strain JC100 on medium supplemented with 0.1% of oleic acid.

For the assays performed with cheese whey as carbon source, the culture achieved a biomass concentration of 4.44 gL^{-1}, a value higher than with used cooking oil and fat waste but lower than for growth with sugarcane molasses (Figure 4.6). These results are consistent with studies for *P. pastoris* strain GS115 growing in medium containing lactose (1%), where biomass growth was lower than that obtained with 1% of sugarcane molasses (Shahidan et al., 2011).

In the experiments with spent coffee grounds hydrolysates as carbon sources, the biomass concentration achieved was different for the two types of acids. As expected, due to the different sugar concentration in each hydrolysate, in the medium supplemented with sulfuric acid hydrolysate, a higher biomass concentration (3.51 gL^{-1}) was reached compared to the medium with orto-phosphoric acid hydrolysate (2.03 gL^{-1}) (Figure 4.6). The result obtained for sulfuric acid is slightly lower than previous studies (5.5 gL^{-1}), where sulfuric acid was used for acid hydrolysis of spent coffee grounds and cultivation of *S. cerevisiae* (Mussatto et al., 2012). However, growth of *P. pastoris* in spent coffee grounds hydrolysates has still not been reported.

The shake flask screening allowed verifying that *P. pastoris* was able to consume the different tested residues, being the composition of those residues a determinant factor to yeast growth. The yeast demonstrated preference to residues with glucose in its constitution and this fact favored the sugarcane molasses, while used cooking oil and waste fat were not suitable substrates. Based on these results, sugarcane molasses and sulfuric acid spent coffee grounds hydrolysate were selected, among the tested residues, for further experiments with *P. pastoris* cultivation in bioreactor.

4.2.3 Batch bioreactor experiments

Sugarcane molasses and spent coffee grounds hydrolysate with sulfuric acid were selected to perform batch bioreactor experiments due to results observed in the preliminary shake flask trials. An experiment using glycerol from biodiesel industry was also tested in bioreactor to serve of comparison between other residues. Glycerol from biodiesel industry (supplied by SGC Energia, SGPS, SA, Portugal), with a glycerol content of 86%, was used as

standard residue due to high cellular density achieved and interesting amount of CGC production achieved previously (Roca et al., 2012).

The experiment using crude glycerol as sole carbon source was the first experiment to be performed (Figure 4.7). In this trial, after 24 hours of lag phase, *P. pastoris* grew at a specific cell growth rate of 0.15 h^{-1} and achieved a final CDW of 37.94 gL^{-1} within 42 hours of cultivation (Table 4.2). The specific cell growth rate achieved in this experiment was slightly lower than that obtained by Chagas et al. (2013) (0.17 h^{-1}) with pure glycerol as carbon source and this difference could be due to lower initial concentration of glycerol used (40 gL^{-1}) in contrast with concentration used in this study (~70 gL^{-1}).

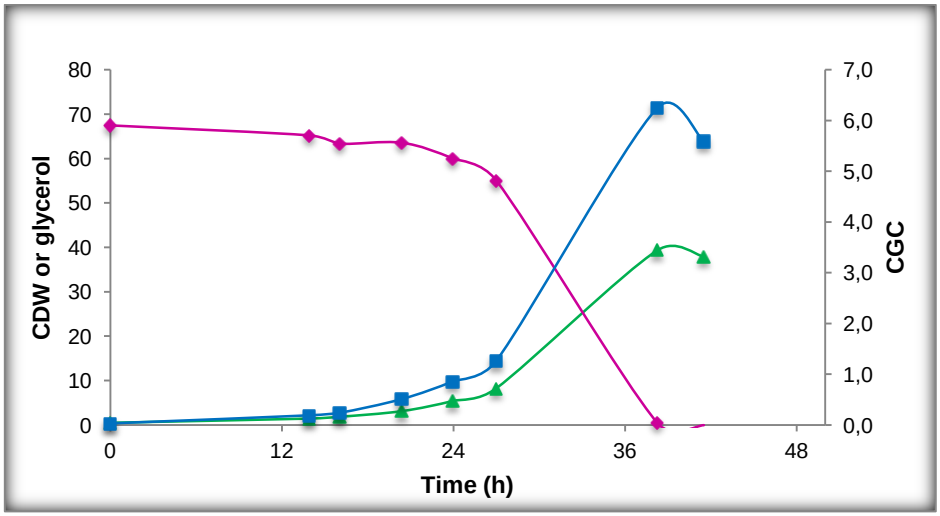

Figure 4.7 Cultivation profile of the batch bioreactor with biodiesel byproduct crude glycerol as sole carbon source. Dry cell weight (, gL^{-1}), glycerol (, gL^{-1}) and CGC concentrations (, gL^{-1}).

Along the experiment, the culture consumed all initial glycerol concentration (67.50gL^{-1}), with a consumption rate of 3.98 $gL^{-1}.h^{-1}$ and, as expected, after 38 hours, the growth stabilized when the carbon source was depleted (Figure 4.7). The CGC concentration reached the highest value (6.25 gL^{-1}) at the end of the exponential phase. This value represented a 15.88 % content of CGC in the biomass (Table 4.2). The difference in specific cell growth rate seems not to have affected the CGC content since its value was even higher than that obtained in identical conditions (13%) by Chagas et al. (2013).

The overall volumetric productivity obtained in this experiment (0.135 $gL^{-1}.h^{-1}$) was in accordance with studies for pure glycerol (0.105 – 0.168 gCGC $L^{-1}.h^{-1}$) reported by Chagas et al. (2013), suggesting that this parameter was not affected by purity/concentration of crude glycerol.

In terms of yields, in this study, the culture achieved lower biomass (0.493 $g_{biomass}\ g_{glycerol}^{-1}$) and a product yields (0.073 $g_{CGC}\ g_{glycerol}^{-1}$) than the values obtained by

Roca et al. (2012) (0.55 $g_{biomass}$ $g_{glycerol}^{-1}$ and 0.15 g_{CGC} $g_{glycerol}^{-1}$, respectively), where crude glycerol was used as sole carbon source.

In order to determine the culture´s ability to grow in medium with the other residues selected in this study, under controlled bioreactor conditions, experiments with sugarcane molasses (Figure 4.8) and spent coffee grounds hydrolysate (Figure 4.9) were performed in duplicate experiments.

In the experiments with sugarcane molasses (Figure 4.8), after a 7 hours lag phase, the culture grew at a specific cell growth rate of 0.11 h^{-1} reaching a maximum CDW of 17.78 gL^{-1}, within 71 hours of cultivation (Table 4.2). The maximum of CDW reached in end of trial was higher than that obtained for *Rhodotorula glutinis* (10 gL^{-1}) cultivated on molasses for β-carotene production in batch fermentation, reported by Bhosale and Gadre (2001). For *P. pastoris* wild type strains, there are no reported studies about its growth in sugarcane molasses.

At the end of experiment, the CGC concentration reached 3.12 gL^{-1} that corresponds to a CGC content of 17.53% in the biomass. This value reflected in an overall volumetric productivity of 0.044 $gL^{-1}h^{-1}$ (Table 4.2).

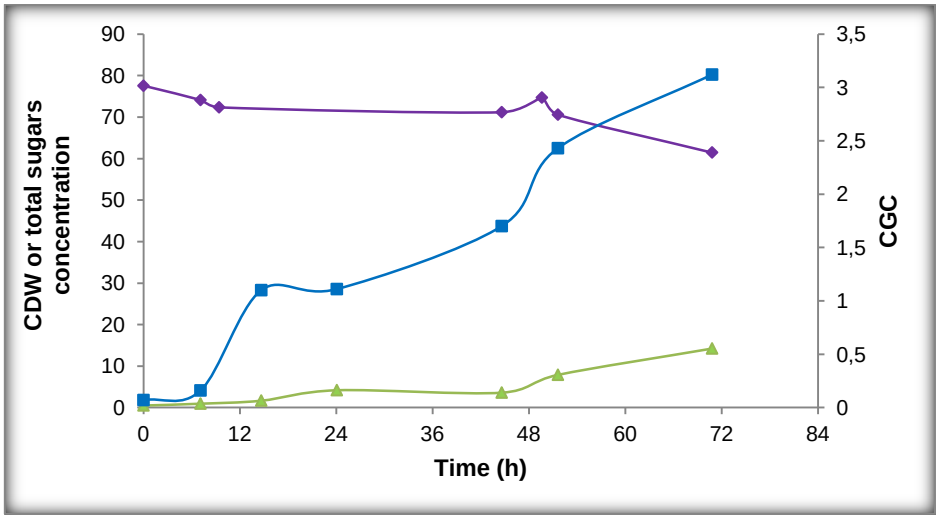

Figure 4.8 Cultivation profiles of the batch bioreactor experiments with sugarcane molasses. Dry cell weight (▲, gL^{-1}) and CGC (■, gL^{-1}) and total sugars concentration (◆, gL^{-1}).

The experiment with spent coffee grounds hydrolysate (Figure 4.9) had a longer cultivation time of 92 hours. In this assay, the maximum biomass concentration (3.46 gL^{-1}) was much lower than that obtained for growth with sugarcane molasses as carbon source (17.78 gL^{-1}).

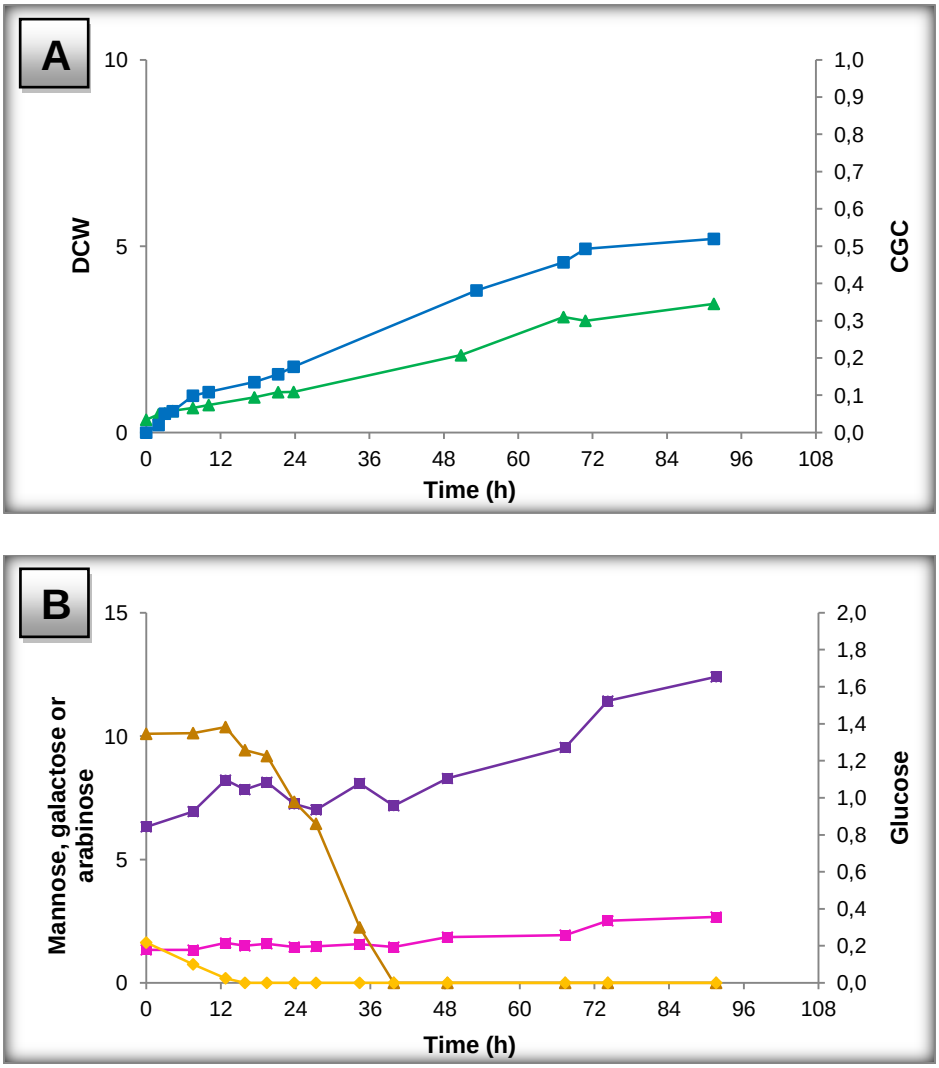

Figure 4.9 Cultivation profiles of the batch bioreactor experiments with spent coffee grounds hydrolysate: A - Dry cell weight (▲, gL^{-1}) and CGC concentration (■, gL^{-1}); B - galactose (■, gL^{-1}), glucose (◆, gL^{-1}), mannose (▲, gL^{-1}) and arabinose concentration (■, gL^{-1}).

As mentioned above, cultivation of *P. pastoris* in spent coffee grounds hydrolysates has still not been reported. However, this residue has been studied for other yeast, such as *S. cerevisiae* (Mussatto et al., 2012). The results, namely biomass concentration, obtained in this study for *P. pastoris* were lower than reported by Mussatto et al. (2012) for *S. cerevisiae* (5.5 gL^{-1}) for ethanol production, in shake flasks. This difference could be due to higher temperature used in the acid hydrolysis (163 ºC, for 45 minutes), reaching a higher total sugar concentration (50 gL^{-1}, approximately) that allowed higher cell growth.

As for CDW values, the CGC concentration obtained with spent coffee grounds hydrolysate (0.52 gL^{-1}), that represented 15.04% of biomass, was lower than that achieved with sugarcane molasses. This difference could be justified by the lower biomass concentration reached with spent coffee grounds hydrolysate due to lower sugar concentration available (only

18 gL^{-1} of monosaccharides were detected in the hydrolysate). The overall volumetric productivity was also lower, reaching only 0.0057 $gL^{-1}h^{-1}$ (Table 4.2).

The consumption of monosaccharides present in spent coffee grounds hydrolysate (glucose, mannose, arabinose and galactose) was measured along the experiment (Figure 4.9 B). The first sugar to be consumed was glucose and its depletion occurred within the initial 16 hours of the experiment, allowing the intense initial growth observed (specific growth rate of 0.051 h^{-1}) (Figure 4.9). After glucose exhaustion, mannose started to be consumed and the culture continued to grow but at a lower specific growth rate (0.033 h^{-1}). As Figure 4.9 B illustrates, the concentration of galactose and arabinose increased during the experiment. Such an increase of concentration could be due to the enzymatic hydrolysis of the oligo- and polysaccharides present in the hydrolysate. Their hydrolysis generated more glucose and mannose that the culture assimilated promptly, while galactose and arabinose were left, thus accumulating in the broth. Since the culture continued to grow after mannose depletion, it must only be based on the consumption of such oligo- and polysaccharides.

Table 4.2 Comparison of parameters obtained for different resiudes in batch bioreactor experiments. μ_{max}, maximum specific growth rate; CDW, maximum cell dry weight; % CGC, % of CGC in dry cell biomass; CGC, maximum active concentration; r_p, overall volumetric productivity; $Y_{X/S}$, growth yield; $Y_{P/S}$ production yield; R_{Gly}, glycerol consumption rate.

Residue	Biodiesel byproduct crude glycerol	Sugarcane molasses	Spent coffee grounds hydrolysate
Cultivation time (h)	42	71	92
μ_{max} (h^{-1})	0.15	0.11	0.051
CDW (gL^{-1})	39.36	17.78	3.46
% CGC	15.18	17.53	15.04
CGC (gL^{-1})	6.25	3.12	0.52
r_p (g_{CGC} $L^{-1}.h^{-1}$)	0.135	0.044	0.006

In comparison with the results obtained with glycerol (Figure 4.7), the use of sugarcane molasses and spent coffee grounds hydrolysate as carbon sources significantly decreased *P. pastoris* cell growth and low biomass concentration values were obtained at the end of both experiments: 17.78 and 3.46 gL^{-1}, for sugarcane molasses and spent coffee grounds hydrolysate, respectively. Since the maximum CDW was lower, the possibility to produce CGC was concomitantly lower too and, thus, the final CGC concentration obtained for sugarcane molasses and spent coffee grounds hydrolysate (3.12 and 0.52 gL^{-1}, respectively) was much lower than obtained with crude glycerol. Despite those lower values, the CGC content in the biomass obtained for sugarcane molasses (17.53%) was higher than that obtained with glycerol, suggesting that the lower growth could have caused stress for the cells and increased the production of CGC, as suggested by Ketela, Green, and Bussey (1999). For spent coffee grounds hydrolysate, the CGC content in the biomass (15.04%) was slightly lower. In opposition, the value of the overall volumetric productivity suffered a significant decrease to

0.044 and 0.0057 $gL^{-1}.h^{-1}$ for sugarcane molasses and spent coffee grounds hydrolysate, respectively, comparatively to that obtained with crude glycerol (0.135 $gL^{-1}.h^{-1}$) (Table 4.2).

The presence of caffeine in SCG confers a potential advantage for CGC production, since for *S. cerevisiae* it has been reported that although caffeine inhibits the cellular growth by 50% (Kuranda et al., 2006), it induces the increase of the chitin content in the cell wall as a response to stress (Ketela et al., 1999). Thus, one of the factors that may have contributed to lower biomass growth in this study was the presence of caffeine, but it was not possible to analyze its content over time in the samples. This analysis will be carried out in future work.

Due to the lower biomass concentration achieved in the experiments with sugarcane molasses and spent coffee grounds hydrolysate, it was not possible to analyze the CGC polymers for their composition in glucans and chitin.

4.2.4 Conclusions

In the present study, several residues, namely sugarcane molasses, cheese whey, waste fat, used cooking oil and spent coffee grounds, were used as substrates for cultivation of *P. pastoris* and production of CGC. The results indicate that not all wastes are suitable for this bioprocess, namely waste paper, used cooking oil and waste fat. Nevertheless, this study has shown that sugarcane molasses, cheese whey and spent coffee grounds can be used as substrates for *P. pastoris* cultivation, although cell growth was not efficient for all of them.

The experiment performed with glycerol biodiesel byproduct was used as a standard for comparison, since this waste was already reported for CGC production by *P. pastoris* (Roca et al., 2012). The specific cell growth rate and biomass yield obtained in this study (0.15h^{-1} and 0.49 $g_{biomass}\ g_{glycerol}^{-1}$, respectively) are similar to reported by Roca et al. (2012) (0.17h^{-1} and 0.55 $g_{biomass}\ g_{glycerol}^{-1}$, respectively).

The biomass concentration obtained using sugarcane molasses and spent coffee ground hydrolysate (3.46 - 17.78 gL^{-1}, respectively) were much lower than the results obtained with glycerol biodiesel byproduct (0.15 and 39.36 gL^{-1}, respectively). In terms of overall volumetric productivity, the results for sugarcane molasses and spent coffee ground hydrolysate (0.044 and 0.006 $gL^{-1}.h^{-1}$) were very low comparatively to that obtained with glycerol biodiesel byproduct (0.135 $gL^{-1}.h^{-1}$). This reduction of volumetric productivity is a consequence of lower biomass concentration achieved with those wastes and indicates that they are not suitable for *P. pastoris* cultivation nor for CGC production.

According to the results, sugarcane molasses is the best substrate among the tested of wastes. Nevertheless, the process needs improvements to achieve the proposed aim. The process improvement may be attained with implementation of fed-batch or continuous system to increase the biomass concentration and, consequently, CGC production.

5. General conclusions and future work

5.1 General conclusions

This study aimed to assess the suitability of different substrates for the cultivation of the yeast *P. pastoris* and production of CGC. The first part of the work consisted in investigating the ability of the culture to use glucose and xylose mixtures for growth and their impact on CGC production. In the second part of the study, the potential of different industrial wastes and / or byproducts as substrates for this yeast was evaluated, to search for alternative substrates to glycerol. This strategy is advantageous for industries generating those materials, which can valorize them by producing value-added products (CGC, chitin, chitosan, glucans) with applications in many areas.

Firstly, glucose and xylose mixtures were used as substrates for cultivation of *P. pastoris* and production of CGC. This study has shown that it is feasible to valorize glucose and xylose containing materials, such as lignocellulosic wastes and byproducts, for the production of the value-added CGC biopolymer, as long as the percentage of xylose is not above 50%. The best performance was achieved in batch bioreactor experiment with addition of 25% of xylose in the medium, where 35.25 gL^{-1} biomass was obtained in 64 hours of cultivation. The CGC content in the cell wall reached 15% with a volumetric productivity of 0.085 $gL^{-1}.h^{-1}$ and the molar ratio of chitin:β-glucan in the extracted biopolymer was 47:53, which is a considerably higher chitin content than the values obtained with crude glycerol (16:84 mol%) by Roca et al. (2012). According to the results, lignocellulosic wastes with percentage of xylose below 50% could be an alternative to glycerol, thus eliminating the dependency of the bioprocess on a single substrate (glycerol) and making it more versatile. The availability of raw material is increased with use of these wastes due to weak utilization of these materials, in opposition to glycerol which is used to several applications.

In the second part of the study, several wastes and /or byproducts (used cooking oil, waste fats, sugarcane molasses, cheese whey and spent coffee grounds) were tested. The results show that *P. pastoris* reached low biomass concentration using these substrates. In batch bioreactor experiments the best results were achieved with sugarcane molasses, where 17.78 gL^{-1} biomass obtained contains 17% of CGC, a value higher than that obtained with crude glycerol (16%).

5.2 Future work

Based on the results, it is clear the need to improve the process and to reach this aim the following improvements should be implemented.

5.2.1 Testing lignocellulosic residues

In the first part of this study, it was possible to demonstrate that *P. pastoris* grows efficiently in glucose and xylose containing materials where the percentage of xylose is not above 50%. This fact allows valorizing several wastes containing those sugars, particularly those that have this preferred percentage of xylose. In this way, lignocellulosic wastes that are constituted mostly by glucose, with lower xylose contents, such as corn stover, wheat straw and winter rye are potentially suitable for use as substrates for CGC production by *P. pastoris* (see section 4.1). The utilization of one of these wastes could increase the growth of *P. pastoris* and CGC production.

Another waste that could be interested tested was the waste paper due to composition in glucose and xylose (see section 2.3.4). As mentioned above, for use of this waste as substrate, the conditions of acid hydrolysis should be optimized, such as increasing the temperature, the reaction time and the concentration of sulfuric acid used. Moreover, the high inorganic content in this waste should be reduced to improve the hydrolysate's sugar content. This optimization will allow obtaining high sugar concentration that would work as substrate for yeast cultivation.

5.2.2 Bioreactor operating mode

As described above, *P. pastoris* was able to grow in many substrates including industrial wastes and/or byproducts. However, in the wastes studied in this work the cellular growth was not very high and this affects the overall productivity of the process. In order to increase biomass growth and, consequently the CGC productivity, a fed-batch mode or a continuous system should be applied. In this way it could be possible to achieve the high cell concentration and a higher maximum of CGC might be reached.

5.2.3 Impact of residues in CGC composition

In this study, the residues tested as substrates for *P. pastoris* growth did not allow to achieve high cellular density and consequently the determination of CGC composition was limited. Applying the two strategies submitted earlier, the biomass concentration could be attained by modifying the bioreactor operating mode and CGC with variable chitin:glucan ratios could be achieved with variability of the wastes composition. Such CGC polymers with different composition might possess distinct properties that could be useful for different applications.

In case of spent coffee grounds the insignificant growth can be justify by presence of caffeine. As mentioned above, caffeine provokes a negative effect in growth but can induce an increase of chitin fraction in CGC. To explore this hypothesis, an increase of CGC production was necessary to allow analyzing the CGC composition in chitin and glucans. To overcome the eventual caffeine growth inhibition, mixed substrates could be used, for example, by supplying the culture with a glucose-rich substrate during the initial phase of the run to attain high cell-

density, followed by feeding it with SCG hydrolysate afterwards to obtain a glucosamine enriched polymer.

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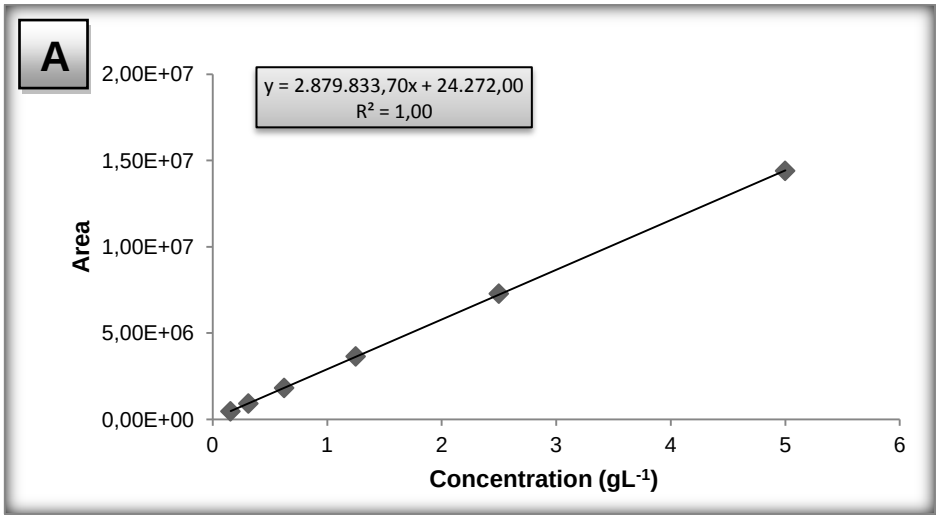
7. Appendix

Calibration curves

Calibration curve for determination of glucose and xylose concentration in shake flask experiments



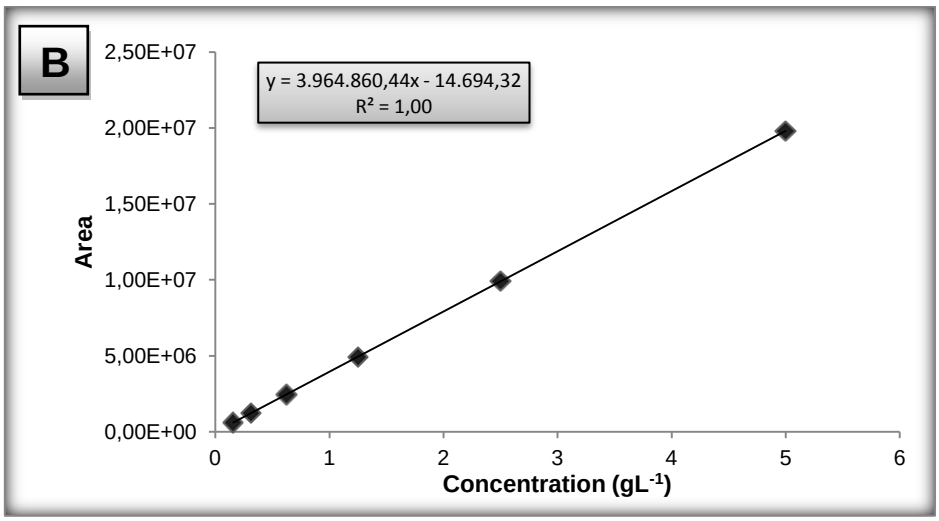
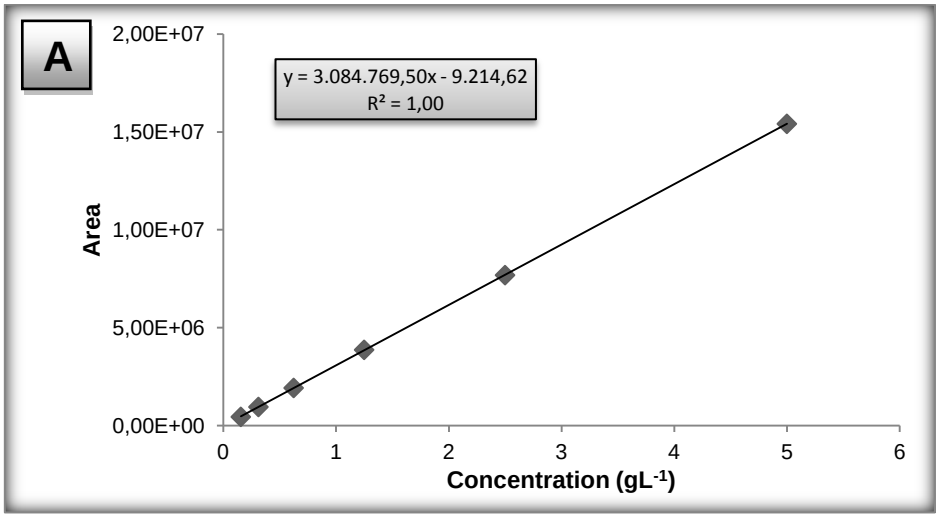


Figure 7.1 Calibration curves obtained for glucose (A) and xylose (B) concentration; analysis was performed by HPLC.

Calibration curve for determination of glucose and xylose concentration in batch bioreactor experiments



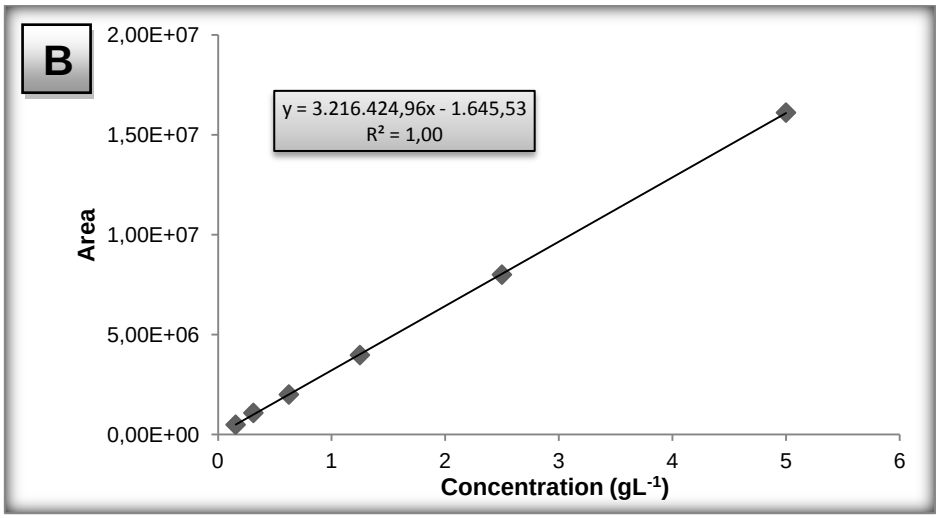


Figure 7.2 Calibration curves obtained for glucose (A) and xylose (B) concentration; analysis was performed by HPLC.

Calibration curve for Dubois assay (acid hydrolysis)

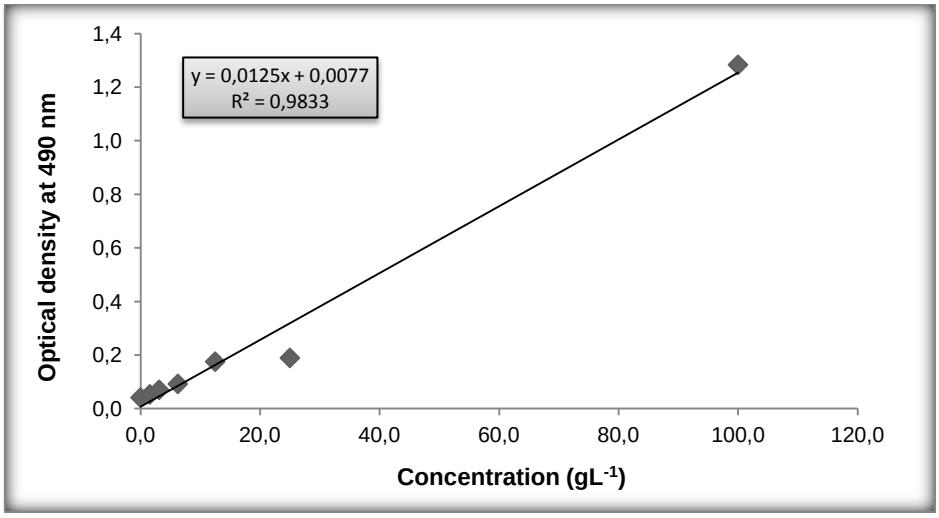


Figure 7.3 Standard curves for Dubois assay.

Calibration curve for CGC composition in Dionex

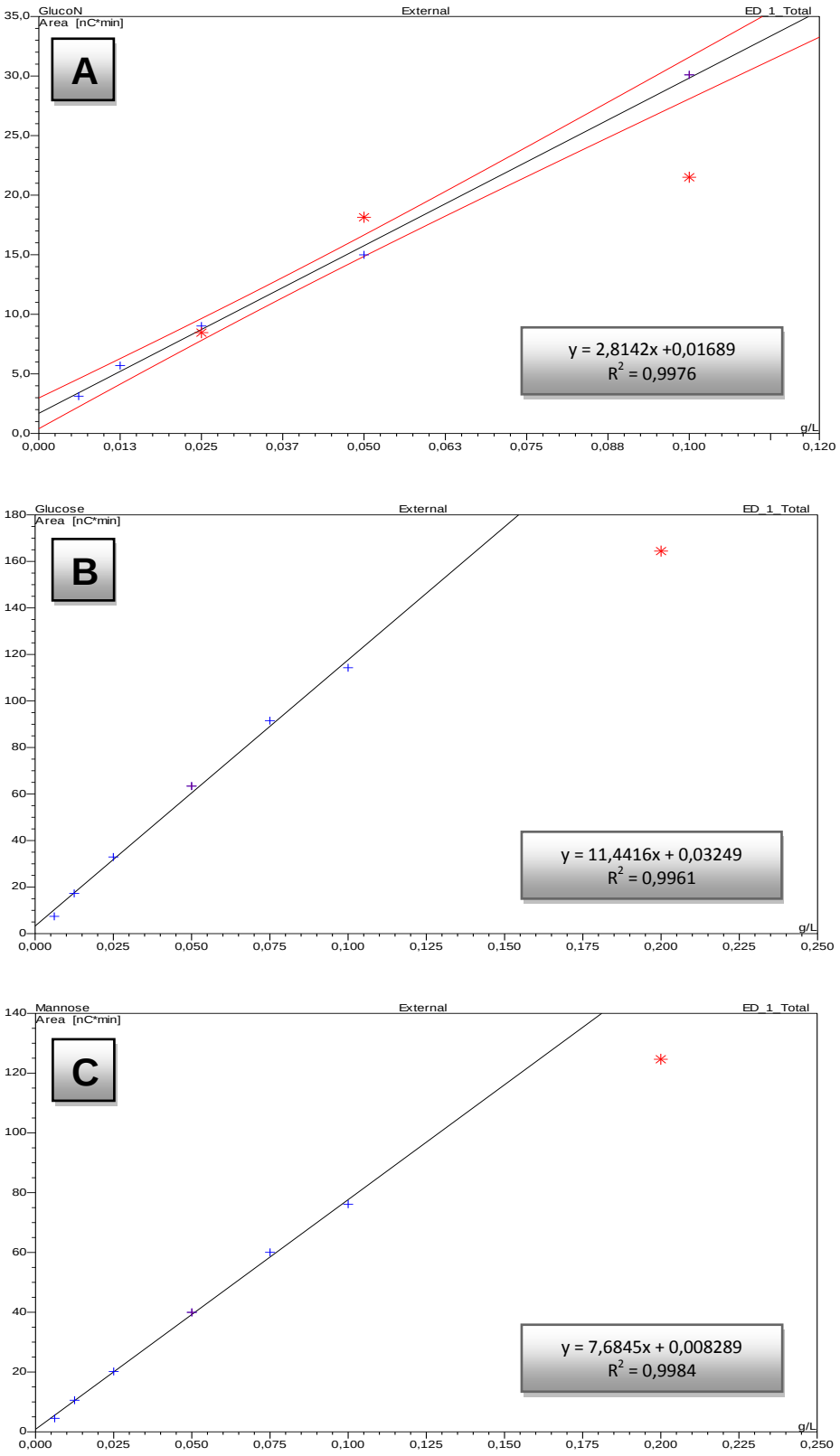


Figure 7.4 Calibration curves obtained for glucosamine (A), glucose (B) and mannose (C) concentration; analysis was performed by HPLC (Dionex).