Screening of fungi with potential for producing fructooligosaccharides with enhanced bioactivity

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Abstract

Fructooligosaccharides (FOS) are present in plants and fruits at low concentrations and with varying individual relative proportions. So, for the industrial application, fructooligosaccharides extraction from natural sources may not be economically viable. Recently, several works have been published within the field of research usually aiming to establish the best experimental conditions to maximizing fructooligosaccharides yields/synthesis. However, although it is known that these compounds may have a great health impact, it is also reported that beneficial health effects may depend on the relative fructoligosaccharisdes composition, seeming that nystose-rich diet is preferable compared to a kestose-rich preparation, exhibiting a higher anti-hydroxyl radical activity. So, in this work, a screening study was firstly carried out aiming to evaluate the bioactive activity of the fermentation broths obtained using different fungi (*A. aculeatus*, *A. japonicus*, *P. corylophilum*, *P. spinulosum* and *P. thomii*) with reported potential for producing fructooligosaccharides. Based on the screening antioxidant results (DPPH free radical scavenging activity and iron chelating activity), *A. aculeatus* was selected as the fungi with the highest antioxidant potential. After, batch fermentations were carried out aiming to establish, based on an experimental design, the initial sucrose concentration (from 88 to 265 g/L) and fermentation temperature (from 22 to 32ºC), which enhance the broths bioactivities. Among the two evaluated experimental conditions, the initial sucrose concentration significantly influenced biomass growth (a maximum value of 16± 1.5 g was achieved) although it did not significantly affect the maximum FOS yield obtained, which varied between 51 to 59 g of FOS/g of initial sucrose. Finally, the preliminary results obtained did not allowed establishing straightforward correlation betwwen antioxidant activities of the fermentation broths and the individual or total FOS concentrations.

Keywords: Fructooligosaccharide, Oligosaccharide, Carbohydrate,
Fructofuranosylnystose (GF4), Nystose (GF3), 1-Kestose (GF2)

Resumo

Fruto-oligossacáridos (FOS) podem ser encontrados em plantas e frutos, embora em concentrações reduzidas e com diferentes proporções relativas de cada FOS individual. Assim, de um ponto de vista industrial, a extração de fruto-oligossacáridosa partir de fontes naturais pode não ser economicamente viável. Recentemente, vários trabalhos foram publicados com o objetivo de estabelecer as melhores condições experimentais para maximizar os rendimentos / síntese de fruto-oligossacáridos. No entanto, embora se saiba que estes compostos podem ter um grande impacto na saúde, também tem sido reportado que os efeitos benéficos para a saúde podem depender da composição relativa de fruto-oligossacáridos. Aparentemente, uma dieta rica em nistose é preferível em comparação com uma preparação rica em questose, exibindo uma maior atividade antioxidante. Assim, neste trabalho, realizou-se um estudo inicial para avaliar a bioatividade de meios fermentativos obtidos usando diferentes fungos (*A. aculeatus, A. japonicus, P. corylophilum, P. spinulosum* e *P. thomii*), os quais são conhecidos produtores de FOS. Com base nos resultados preliminares da actividade antioxidante de (atividade de eliminação de radicais livres DPPH e atividade quelante de ferro), o fugo *A. aculeatus* foi selecionado como sendo aquele que apresentava maior poder antioxidante. Posteriormente, foram realizadas fermentações com o objetivo de estabelecer, com base num desenho experimental, a concentração inicial de sacarose (de 88 a 265 g / L) e a temperatura de fermentação (de 22 a 32ºC), que maximizava o poder antiooxidante dos meios fermentativos. De entre as duas condições experimentais avaliadas, verificou-se que a concentração inicial de sacarose influenciou significativamente o crescimento da biomassa (valor máximo de 16 ± 2 g), embora não tenha afetado significativamente o rendimento de FOS obtido, que variou entre 51 a 59 g de FOS/g de sacarose inicial. Finalmente, os resultados preliminares obtidos não permitiram estabelecer uma correlação direta entre as atividades antioxidantes dos caldos de fermentação e as concentrações de FOS individuais ou totais.

Palavras-chave: Fruto-oligossacáridos, Oligossacáridos, Hidratos de carbono, Frutofuranosilnistose (GF4), Nistose (GF3), 1-Questose (GF2)

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LIST OF OF ABBREVIATIONS

FOS -Fructooligosaccharides

DP -Degree of polymerization

ScFOS -Short chain Fructooligosaccharides

Ftase -Fructosyltransferase

SmF -Subermerged Fermentation

SSF -Solid State fermentation

DPPH -1,1 diphenyl-2-picrylhydrazyl

HPLC -High performance liquid chromatography

GF_2 -1-Kestose

GF_3 -Nystose

GF_4 - Fructofuranosyl nystose

GOS -Galactooligosaccharides

IMO -Isomalto-oligosaccharides

MOS -Malto-oligosaccharides

SOS -Soybean oligosaccharides

CHAPTER 1

Context, aims and thesis outline

1.1. Context and motivation

Dietary carbohydrates are a diverse group of compounds with a range of chemical, physical and physiological properties. They include sugars, complex carbohydrates or starch, and fiber. Usually they are obtained from the starch of plant foods and from the small molecules that contain glucose, fructose, galactose, maltose, lactose or sucrose found in many fruits and milk.

The analysis of dietary sugars is a crucial aspect of carbohydrate chemistry, namely because they play a crucial role in human diet being consumed in large amounts in Western countries. In addition, dietary carbohydrates are at the core of the debate regarding healthy diets that promote weight loss and decrease heart disease and metabolic syndrome, although some of them are incompletely absorbed in the normal small intestine and so, may act as dietary triggers for several clinical symptoms. Also, some oligosaccharides (*e.g.*, fructo-, gluco-, galacto-oligosaccharides), lactulose, lactosucrose, among others, are of major importance namely due to the ability to stimulate the specific growth/activity of a restricted number of beneficial colonic bacteria. Therefore, these sugars have a huge economic importance in the food industry, as well as a great health impact. Among the diversity of dietary carbohydrates this work was focused in a specific sub-group of oligossacharides, the fructooligossacharides (FOS). FOS are naturally present in several natural matrices, namely, vegetables and fruits. However, the total and individual FOS contents in these natural sources are not constant and so, their use for obtaining FOS commercial formulations with constant contents relative proportions is not feasible. Taking into account these issues and considering different individual FOS may possess different biological activities, in this work, it was envisaged to produce FOS via fermentation. The antioxidant activities of the fermentation broths collected at different fermentation times were evaluated aiming to find a possible correlation with the individual or total FOS contents. Thus, first, FOS production via sucrose fermentation with different fungi (*P. corylophilum*, *P. spinulosum*, *P. thomii*, *A. japonicus* and *A. aculeatus*) was evaluated aiming to identify the fungus that would allow obtaining fermentation broths with the highest antioxidant potential, which was assessed by the DPPH free radical scavenging activities and the iron chelating activities. Then, based on the preliminary screening study carried out, *A. aculeatus* was chosen as the fungus with the highest biological potential. Finally, an

experimental design was used to establish the best experimental fermentation conditions (i.e., initial sucrose concentration and fermentation temperature) that would enhance obtaining fermentation broths with the highest antioxidant activities.

1.2. Research Aims

The objective of this work was to select the fungi strain that allowed the production of a FOS mixture with the highest antioxidant activity. *A. aculeatus* was selected as the fungus with greatest potential to get a FOS profile with higher antioxidant activities. The aim of work is to study the FOS production through sucrose fermentation using A. aculeatus in order to obtained a product not only with high amount of FOS but also with a known profile of individual FOS.

1.3. Thesis outline

This work has been structured in five chapters, including this one and other four, that cover the research aims stated above:

- Chapter 2: Presents the literature review that supports the thesis work.
- Chapter 3: Lists the materials and methods used in the experimental work.
- Chapter 4: Combines the result section and discussion part into a single chapter. This chapter sets out the experimental results including the statistical analysis, interprets and explains the obtained results and answers the research question.
- Chapter 5: Involves conclusive comments, observations and perspectives for further research on this research subject.

CHAPTER 2

Introduction

2.1 Prebiotics

Prebiotics are defined as non-digestible substances that provide a beneficial physiological effect to the host through the growth stimulation and/or activity of a specific endogenous microflora, that confer benefits which are beneficial for the host´s health (Flores-Maltos *et al*., 2014). These compounds are not absorbed or broken down by the body, so they enter intact into colon and can be fermented only by a group of intestinal bacteria (*e.g.*, lactobacilli and bifidobacteria). Some specific dietary carbohydrates are prebiotics since they selectively stimulate the growth of individual groups or species of beneficial bacteria (Roberfroid, 2005). Several benefits have been attributed to prebiotics (Macfarlane *et al*., 2008; Rafter, 2007; Szajewska *et al*., 2006), ranging from acting as adhesion particles for pathogenic bacteria, and thus reducing the risk of gastrointestinal infection (Gibson *et al*., 2006), improving laxation (Szajewska *et al*., 2006), increasing calcium absorption, maintaining a functional gut mucosal barrier (Kleessen & Blaut, 2005) and stimulating the gastrointestinal-immune system (Gibson *et al*., 2006). Prebiotics include fructans (inulin and fructo-oligosaccharides (FOS)), galacto-oligosaccharides (GOS), isomalto-oligosaccharides (IMO), malto-oligosaccharides (MOS), xylo-oligosaccharides (XOS), soybean oligosaccharides (SOS) (raffinose and stachyose), lactoscrose (LS) and lactulose (Macfarlane *et al*., 2008; Roberfroid, 2005), although some of them only recognized as potential prebiotics. Within this work, a special focus will be given to FOS. FOS helps to improved cardio metabolic risk factors preventing diabetes by the reduction of development of fat and overweight. It helps in maintaining glucose homeostasis and decreasing inflammation and hepatic steatosis (Olveira and González, 2016; Vega and Zuniga-Hansena, 2016).

2.2 Probiotics

Probiotics are live microorganisms that when administrated in adequate amounts confer health benefits on the host. Probiotics microorganism can be found in some fermented foods and beverages such as yogurt and kefir. Regularly consumption of probiotics, namely some strains of *Bifidobacteria* and *Lactobacilli*, has a favourable effect in intestinal tract flora, stimulating the growth of beneficial gut microflora and simultaneously inhibiting the growth of pathogenically bacteria preventing certain

disease such as acute diarrhea, and constipation. Some probiotics strains have been shown to have a favourable effect on, bone mineralization, risk reduction of colon cancer, chrononic digestive disorder and inflammatory disease etc. (Scholz-Ahrens *et al.*, 2016). Probiotics microorganisms can also modulate the immune system, especially in children, probiotics can be effective in the prevention and treatment of allergic disease (Yasuda *et al*., 2012).

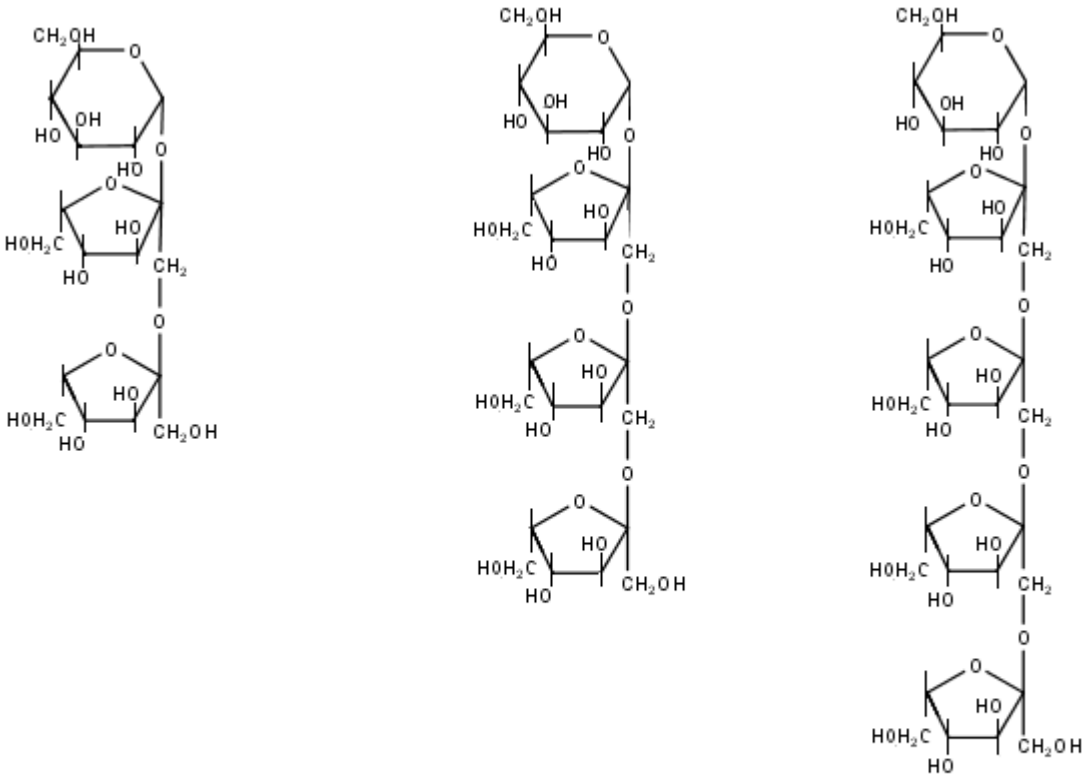
2.3 Synbiotics

Synbiotic is the combination of prebiotic and probiotics. In this combination, prebiotic enhance the survival and activity of beneficial microbes (Scholz-Ahrens *et al*., 2016). Valdés-Varela *et al*. (2016) reported that the combination of bifidobacterial with FOS are the best synbiotic food supplements.

2.4 Fructooligossacharides

Fructooligosaccharides (FOS) are dietary fibers composed of fructose units linked by glysocidic linkage. They commonly are 1-kestose, 1-nystose and 1-β-fructofuranosylnystose (Muniz-Marquez *et al*., 2016). FOS are short-chain dietary carbohydrates (oligosaccharides) with relevance in human nutrition. They have a degree of polymerization (DP) between 3 and 9, being classified as non-α-glucans (Roberfroid, 2005). Usually, chemical approaches involving separation, identification and quantification of the different FOS present in food, contribute to a better understanding of the physiological and health effects of these macronutrients. FOS is also known as fructans, oligofructans, glucofructans, inulins or oligosaccharides. FOSs are considered as prebiotics which activate the mucosal immunity with beneficial effects in gastrointestinal tract (Yasuda *et al*., 2012). The composition of FOS is more consistent and glucose terminated in all chain. FOS are indigestible carbohydrates fermented by microorganism present in gastrointestinal tract, resulting in the production of organic acids (Moharib, 2016). Due to the structure of C2 units oligo fructose are non-digestible carbohydrates, thus cannot hydrolysis by digestive enzymes (Flores-Maltos *et al*., 2014). Fructans are a part of diet that serves storage and protective function in many plants. FOS are present in edible plant like onion, asparagus, leek, garlic, wheat, yacon, tomatoes, banana. The concentration of FOS that are present in natural foods are as banana (0,3%), barley (0,15%), garlic (0,6%), honey (0,75%), onion (0,23%), brown sugar (0,3%) and tomato (0,15%) (Sangeetha, 2003). FOS are present in plants and

fruits at low concentrations and with varying individual relative proportions. So, for industrial application, FOS extraction from natural sources may not be economically viable. Recently, several works have been published within this field of research usually aiming to establish the best experimental conditions to maximizing FOS yield/synthesis. However, although it is known that these compounds may have a great health impact, it is also reported that beneficial health effects may depend on the relative FOS composition, seeming that nystose-rich diet is preferable compared to a kestose-rich preparation, exhibiting a higher anti-hydroxyl radical activity (Moharib, 2016; Correia *et al*., 2014; Flores-Maltos *et al*., 2014). Oligofructose is a linear non-digestible carbohydrate obtained from the enzymatic hydrolysis of inulin. Short chain FOS (scFOS) are a mixture of oligosaccharides that formed fructooligosaccharides. (1-kestose, nystose,1-fructofuranosyl nystose). scFOS is linear chain with 3 to 5 sugar units and consists low molecular weight. The bond between scFOS monomers are not digested in small intestine, as the small intestine does not secret enzymes capable to hydrolysed β-(1→2) fructosyl-fructose linkages. Therefore, they fermented in large intestine to stimulate the growth of beneficial bacteria and produce short-chain fatty acids (SCFAs), mainly acetate, propionate, glucogenic and butyrate. FOS are linked by β-(1→2) fructosyl-fructose linkages in sucrose Inulin is a linear fructose polymer of β-(2-1) linked fructosyl fructose terminated by glucose residue via sucrose. Mainly two types of enzymes are responsible for the hydrolysis of inulin named as endo and exoinulinase. Endoinulinase release oligosaccharides in the inulin while exoinulinases remove terminal glucose residue. Kestose is composed of glucose with the addition of fructose molecule stimulating the activity of bifidobacteria. Later on, Nystose is formed by the addition of a fructose molecule while the formation of fructofuranosyl nystose take place by the addition of another fructose molecule (Flores-Maltos *et al*., 2014). Figure 2.1 shows the chemical structure of fructooligosaccharides.



i) DP (Kestose) = 3 monomers

ii) DP (Nystose) = 4 monomers

iii) DP (Fructofuranosyl nystose) = 5 monomers

Figure 2.1-Chemical structure of Fructooligosaccharides

2.4.1 Properties of FOS

FOS possess interesting functional properties in the food ingredients. The nutritional and health benefits of FOS have been extensively reviewed in the recent years FOS possess several properties including a low calorie, non-cariogenic and are water soluble. FOS are considered as soluble dietary fibre. FOS are odourless, white in powder with small size particle. As the pH ranges from 4-7 it is highly stable, even the food can be stored for longer period of time in refrigerator. The viscosity and thermal stability of FOS is higher than sucrose. FOS forms fine white crystals. The physiological effects of FOS help to improved mineral absorption and decrease

cholesterol level, phospholipids and triacylglycerol. Due to the prebiotics effects FOS are possessively included in food products to stimulate the growth of non-pathogenic gut microflora Furthermore having the functional proper ties, FOS achieved commercial importance in large scale industries. The β- configuration in C2 of fructose monomers makes FOS not hydrolysed by digestive enzymes of human and are not utilized as source of energy by the body. Thus, they enter the large intestine where it undergoes fermentation in colon and promote the growth of beneficial bacteria. FOS is suitable to make low calorie diet foods, artificial sweeteners such as aspartame, phenylalanine or sucralose that are safe for consumption by diabetes persons (Sangeetha, 2003). FOS are used as chewing gums, yoghurts and drinks as non-cariogenic sugars. FOS produce large amounts of short chain fatty acids. Beneficial bacteria such as *Bifidobacterium sp* and *Lactobacillus sp* that are present in lumen of large intestine are resistant to the acidic pH of short chain fatty acids while other *Clostridium* sp are sensitive to acidic condition. In colonic fermentation of FOS leads to decrease the pH in the colon and enhanced the mineral ions absorption from the intestine which significantly increase bone mineralization. Some experiments were carried out on the effect of FOS in rats where FOS fed group results with significant decrease in weight of the body and lower faecal excretion with compared to other groups fed with cellulose. Some research work has been reported formation of Ca and Mg salts due to the acidification of colon in fermentation and production of short chain carboxylic acids. Prebiotics properties of FOS enhances the immunity of the cells, cell wall components and extracellular components of bifidobacterial which has an indirect effect on curing of cancer in human beings High bifidobacterial count in colon leads in the production of nutrients such as vitamin, nicotine acid and folic acid (Sangeetha *et al*., 2005; Alméciga-Díaz *et al*., 2011; Bhalla *et al*., 2016).

2.4.2 Importance of FOS

Fructooligosaccharides (FOS) are dietary sugars quite used as food ingredients and are usually incorporated as dietary fibers in many food products. FOS as dietary fibres improves the gut microbiota by increasing stool frequency, and the blood lipid parameters by decreasing serum triglycerides and controlling blood cholesterol levels (Park *et al*., 2016). FOS resist digestion and absorption in the small intestine of humans. Ingestion of FOS decrease clostridium present in lumen, reducing the production of

flatulence. Thus, creating the acidic condition for the solubility of mineral FOS are prebiotics with some specific physiological functions that produce short chain fatty acids in the intestinal lumen. Short chain fatty acids help in control of mucosal proliferation, improving the gastrointestinal physiology and immune function, prevention of colonic carcinogenesis, availability of minerals, elimination of nitrogenous compound, metabolism of lipid, increase faecal bolus (Dominguez *et al*., 2012; Flores-Maltos *et al*., 2014). FOS are non-toxic, biodegradable and water soluble. Due to physiological and pathological conditions, FOS are used in pharmaceutical and biomedical purposes. FOS prevent tooth decay (Moharib, 2016). They show protective effects against some diseases, development of anticancer drugs, antioxidant enzymes activity (Sangeetha *et al.,* 2004). Purification of FOS by removing glucose and sucrose leads to development of food products hence aim to control diabetes. Consumption of FOS like polysaccharides are used for the protection against colon cancer (Sangeetha *et al*., 2004; Moharib, 2016). The β-configuration of anomeric carbon C2 makes FOS non-digestible carbohydrates that cannot be hydrolysed by the microorganism present in intestine. Although they can be metabolized in the gut with number of beneficial bacteria (Lopes *et al*., 2016). Ingestion of FOS, increased the uptake of calcium and mineral thus preventing from osteoporosis (Sheraji *et al*., 2013). scFOS produces SCFAS which lower the ph inhibiting growth of pathogenic bacteria. Furthermore, scFOS reduces the production of phenols. Several authors have studied the digestibility of FOS in different ways Animal fed scFOS have found reduction in pathogenic levels, toxic level, disease and symptoms. while they are exposed to antibiotics. Compared with sucrose and maltose the scFOS was not digested by human salivary enzymes in mouth. Some bacteria such as *Escherichia coli. Candida albicans, and Clostridium difficile* does not utilize scFOS. Ingestion of FOS decrease the faecal ph level and increase the faecal cholesterol concentrations. Piglets were give milk with scFOS, Out of 8 only 1 piglets developed diarrhea when they passed through *E.coli* challenge. scFOS reduces severity of the infection.

FOS being calorie-free are nondigestible by the digestive enzyme and cannot be utilized in the body as energy source, thus are safe for diabetics. FOS are non-carcinogenic sweeteners, used as replacement of fat and sugar in low calorie food (Lopes *et al*., 2016). FOS are used as bio-preservatives from both plants and microorganism which increase the quality of food product. Sc-FOS are used in food

products like dairy products to reduce the growth of pathogenic microorganism, which provides freshness to the low-fat cookies and increase shelf life of food products (Flores-Maltos *et al*., 2014; Vega and Zuniga-Hansena, 2015). FOS has a wide range of application in pharmaceutical industry for tablets, cosmetics (dermatological purposes) and food industry such as breaker (dairy products, bread, frozen desserts, baked food, yoghurts, gum), beverage products, chocolates (Belghitha *et al*., 2012; Bhalla *et al*., 2016; Park *et al*., 2016). FOS have been applied for the manufacture of raw material such as production of water-soluble resins, water -soluble films (Alméciga-Díaz *et al*., 2011). Yacon syrup helps to decrease the cholesterol of body, body weight and obesity. (Campos *et al*., 2012). FOS can be used as drugs in the form of medical tea against cardiovascular disease (Pejin *et al*., 2013).

2.4.3 Production of FOS

FOS can be obtained by the transfructosylation activity of fructosyltransferases (FTase) enzymes. The enzymatic synthesis of FOS consists plants, bacteria and fungi. Some yeast and fungi like *Neurospora crassa, Candida utilis, Fusarium oxysporum, Aspergillus niger, Aspergillus oryzae, Saccharomyces cerevisiae, Schizosaccharomyces pombe and Schwanniomyces occidentallis* are used in the production of FOS. In fermentation process, sucrose from sugar beer or sugar cane are used for the manufactured of FOS (Alméciga-Díaz *et al*., 2011). Although the yield of FOS production is low, for the industrial production of FOS, β-fructofuranosides (FFase) (E.E.3.2.1.2.6) and fruxtosyltransferases (FTase) (E.C.2.4.1.9) enzymes are used (Dominguez *et al*., 2012; Lorenzoni *et al*., 2013).

FOS can be produced by the action of enzyme found in both plant (asparagus, sugar beet, onion, Lactuca sativa etc.) and other microorganism (bacteria and fungi) such *as Arthrobacter sp., Fusarium sp., Aspergillus sp, Bacillus macerans, Penicillium frequentans,* etc. FOS produced from the plant is low and has limited production of enzyme (Dominguez *et al*., 2012). The first methods used in the production of FOS is microbial fermentation and the other by transfructosylation activity of enzymes (Zeng *et al*., 2015). Immobilization prevents denaturation of enzyme. The use of immobilized enzymes is an effective and economic method for large-scale production of FOS (Flores-Maltos *et al*., 2014). This method is cost effective and accurate (Correia *et al*., 2014). Another advantage of immobilized cell system is to facilitates the reuse of

biomass, easily separation of immobilized enzymes, low mass transfer and development of effective and economic methods for the large-scale production of FOS. Immobilization can be performed by binding to a solid carrier, entrapment or microencapsulation and cross-linking of enzyme aggregates, resulting in carrier-free macromolecules for the large-scale production of FOS (Flores-Maltos *et al*., 2014; Mussatto *et al.,* 2012). Enzymes such as lipase, chitosanase, D-amino acid oxidase, and β-galactosidase are used for the immobilization of enzymes. Different oligosaccharides, small saccharides along with residue from fermentation can be easily distinguished by applying magnetic field (Chen *et al*., 2013). Correia *et al.* (2014) observed the production of FOS approximately 60 (g FOS/g initial sucrose) through initial sucrose concertation 50 g/l using *A. aculeatus* in fermentation hours 60 at 27˚C at 150 rpm. Mussatto *et al*. (2009) evaluated higher annual productivity of FOS by using immobilized cells in corn cobs, and solid-state fermentation (SSF) using coffee silver skin as support material and nutrient source. Park *et al*. (2016) observed that addition of fructooligosaccharides (FOS) and isomalto-oligosaccharides(IMO), improved quality of baking resulting in increased volume, moisture content as well as hardness of bread.

FOS are synthesis through the microbial action of β-fructosyltransferases or by β-fructofuranosidases (Bhalla *et al*., 2016). In the fermentative process, FOS are produced by mixing significant amount of salt with other non-prebiotics sugars. Many researchers have studied the impact of the continuous removal of glucose and sucrose residue from the fermentation during the production of FOS (Nobre *et al.,* 2014).

Different fermentative methods have been used for the production of FOS. Submerged fermentation (SmF) and Solid State fermentation (SSF) can be used to produce FOS. Solid state fermentation utilizes various agroindustry products for the production of FOS. Solid state fermentation (SSF) is used for the microbial production of enzyme in industries as the production cost and energy consumption is low with minimum risk of contamination, requires low amount of water. SSF is environmentally more favourable than the other processes. SSF has a great impact on the industrial economy process as the size of fermentation is small, low cost in sterilization, reduced stirring and reduction in downstream process. The main drawbacks for SSF are fermentation parameters such as temperature, pH, moisture and normally low yields of products (Mussatto *et al*., 2012; Dominguez *et al*., 2012). Experimental conditions for FOS and β-fructofuranosidase production are mainly based on submerged fermentation (SmF)

experiments (Mussatto *et al*., 2010). The enzyme obtained from submerged fermentation is 6 times more than that of batch fermentation which is beneficial for the industrial production of FOS (Vega and Zuniga-Hansena, 2014).

2.5 Antioxidant

Antioxidants are natural or synthetic substances that may prevent or delay oxidative cell damage caused by physiological oxidants. Traditionally the antioxidants are divided in two groups: the antioxidants that act directly by scavenging reactive species of oxygen (ROS) or nitrogen (RNS) and the antioxidants that inhibit their generation, usually by transition metal chelation (Madhavi *et al.,* 1996).

2.5.1 DPPH free radical scavenging activity

The DPPH radical scavenging method is widely used to evaluate the free radical scavenging ability of natural antioxidants in food and beverage (Shekhar and Anju, 2014). DPPH (1,1 diphenyl-2-picrylhydrazyl) is a free radical which has a violet color that changes to yellow after reduction by either the process of hydrogen or electron transfer. The scavenging reaction between (DPPH) and an antioxidant (H-AO) can be written as:

$DPPH^{\bullet} + H\text{-}AO \leftrightarrow DPPH_2 + AO^{\bullet}$

where DPPH• is a stable chromogen radical with λmax =515 nm. (Apak *et al*., 2016).

Antioxidants react with DPPH and reduce it to $DPPH_2$ and as consequence the absorbance decreases. The degree of decolourization (yellow colour) indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (Dehpour *et al*., 2009).

The DPPH method is simple, rapid, accurate and inexpensive assay and in this work, was used for the measurement of free radical scavenging ability of FOS produced by fungi. throughout the fermentation process.

2.5.2 Iron chelating activity

Iron ions plays an important role as catalyst in oxidative process forming hydroxyl radicals by decomposing hydrogen peroxide. The iron-chelating activities of the fermentation broth samples were evaluated following the method of Dinis *et al.* (1994) with minor changes. Ferrozine can quantitatively produce complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted resulting in decreased red color of the complex compound. Fe^{2+} has ability to move single electron which allow the formation and propagation of many radical reactions.

CHAPTER 3

Materials and methods

3.1 Fungi growth and inoculum preparation

Many microorganisms produce FOS, particularly bacteria and fungi from sucrose, starting with the synthesis of 1-kestose (GF2), followed by nystose (GF3), and lastly 1^F-fructofuranosyl nystose (GF4) (Sangeetha *et al.*, 2005). The yield and ratio of GF2, GF3 and GF4 change throughout the fermentation and at the same time depends on microorganism.

Three strains belonging to *Penicillium* genus and two strains belonging to *Aspergillus* genus were used: *A. aculeatus, A. japonicus, P. corylophilum, P. spinulosum and P. thomii* provided by University of Minho. Fungi were inoculated in Potato Dextrose Agar medium (PDA) manufactured by HiMedia Laboratories Pvt. Ltd. containing potatoes infusion from 200 g/L, dextrose 20 g/L, agar 15 g/L and incubated for 15 days at 25˚C in the dark. After incubation, 4 mL of sterilized water with 0.1% Tween80 (v/v) were added to the culture and spores were scrubbed to obtain a suspension. The concentration of spores in this suspension was determined by counting spores with the aid of a Neubauer counting chamber. The fermentation media was inoculated with 10^5 spores/mL.

3.2 Fermentations conditions

Firstly, a fermentation assay was performed in order to selected the fungi with maximum FOS yield and/or whose FOS extract presented the highest anti-oxidant activity. For the assays, 400 ml Czapek liquid medium (modified) OXOIDE CM0095 (sodium nitrate 2.0 g/L, potassium chloride 0.5 g/L, magnesium glycerophosphate, 0.5g/L, ferrous sulphate 0.01g/L, potassium sulphate 0.35 g/L and sucrose 30.0g/L) previously sterilized was used as culture medium. The fermentation medium was inoculated with 10^5 spores/mL of each fungus. Fermentations were carried out on a rotary shaker at 27°C for 96 h under agitation (100 rpm). Fermentation broth samples were taken at regular time intervals (0, 24, 48, 72 and 96 h of fermentation). These samples were filtered through a 0.25 μm nylon membrane filter (Millipore) and frozen at -20ºC for further determination of FOS, residual sugars and antioxidant activity. At

the end of fermentation, final fungus biomass was collected by filtration with Whatman filter paper (Sigma- Aldrich**).** All fermentations were carried out in duplicate.

3.3 Biomass determination

At the end of fermentation, the mycelium was removed from the medium through filtration and dried in a forced air incubator at 50ºC for 48h. The dry biomass was estimated as the difference between the weight of samples and the filtration paper and expressed in grams (g).

3.4 DPPH free radical scavenging activity

The free radical scavenging activities of the FOS in broth medium was estimated using DPPH according to the method described by Marinova and Batchvarov (2011). The DPPH solution (0.1 mM) was prepared by dissolving 4 mg of DPPH (Sigma-Aldrich) in 100ml of methanol. In brief, 50 µL of DPPH (0.1 mM solution in methanol) was added to 50 µL of fermentation samples. The reaction mixture was shaken thoroughly and incubated in dark at room temperature for 20 min. After 20 min, the absorbance of the mixture was read at 515 nm in the spectrophotometer (thermo scientific type 1510) against control solution of 50 µL of water and 50 µL of DPPH. Measurement was performed at least in triplicate. The percentage of the DPPH free radical was calculated using the following equation:

DPPH scavenging effect (%) = ((A0- A1)/ A0) x 100,

being A0 the absorbance of the control and A1 the samples absorbance.

The free radical scavenging activity of samples are calculated by calibration curves, prepared with Ascorbic acid (Vitamin C) and expressed as equivalent vitamin C antioxidant activity (EVCAA). Calibration curves were obtained using ascorbic acid (Vitamin C) as the chemical standard. The stock solutions were prepared by dissolving a 100 mg of VIT in a total volume of 100 mL of water to give the concentration of 1 mg/L. The calibration curve was made using the following concentrations: 1µg/ml; 5µg/ml; 10µg/ml; 20µg/ml; 25µg/ml; 50µg/ml.

3.5 Iron-chelating activity

The iron-chelating activities of the samples were evaluated following the method of Dinis *et al.* (1994) with minor changes. Briefly, 15 µL of each sample was added to a 225 µL H_2O. Thereafter, 20 µL of 2 mM FeCl2 were added and the reaction was initiated by the addition of 5 mM ferrozine (40 µL). The solution was mixed thoroughly and incubated in dark at room temperature for 10 min. The absorbance was read at 562 nm. The ability of samples to chelate ferrous ion was calculated using the formula:

Chelating activity (%) = ((A0- A1)/ A0) x 100, being A0 the absorbance of the control and A1 the samples absorbance.

Calibration curves were obtained using EDTA as the chemical standard.

3.6 Optimization of fermentation conditions for maximizing biological activity of fermentation broths

3.6.1 Factorial design on the production of FOS

Factorial design is widely used in experiments to study the effects of the factors. The K factors is the most important as it comprise only two levels i.e. may be qualitative (temperature, pressure or time) or may be quantitative such as two machines, operators in the terms of high, low and presence or absence of factors. The 2^K design is useful in the premier stage of experimental work when many factors are to be investigated. These designs are widely used in factor screening experimental as it provides the smallest number of run with which K factor can be studied in the complete factorial design (Montgomery, 2001).

The production of FOS was optimized by *Aspergillus aculeatus* with greatest antioxidant activity (from screening of FOS production). Inoculation was determined by counting the spores in a Neubauer chamber after inoculating them in 4 ml of 0.1% (w/v) Tween 80.A 4 ml volume of spore suspension (10^5 spores /ml) will be transferred to a 1000 L shaken flask containing 250 ml of inoculum medium with following composition (%w/v): sucrose 30, $NaNO_3$ 2.0, KCL 0.5, $FeSO_4$ 0.01, K_2SO_4 0.35, $C_3H_7MgO_6P$ 0.5. Two successive full factorial designs (2^k) [where k = 2 being one

factor temperature and the other sucrose concentration] with three central points was carried out to study the effects of temperature and sucrose concentration on the production of FOS. Experimental assays were evaluated within interval range 22-32˚C for the temperature and 100g-300g concentration of sucrose. Fermentation was performed under temperature and sucrose concentration condition previously optimized for the production of FOS. Samples was collected at 0, 48, 72, 96, 120 and 144h hours of fermentation. At the end of fermentation samples were withdrawn and filtered with Watman number 1 filtration paper.

DPPH was measured as described previously (3.4). This time iron-chelating activities of the samples were evaluated with minor changes in concentration of water (190 µL) and sample. (50 µL) along with same procedure as mentioned previously in (3.5).

3.7 FOS by HPLC analysis

For the FOS quantification, the HPLC method described by Correia *et al.* (2014) was followed. Briefly, a RI detector (RI knauer smarlline 2300) was used with a mass alinjector of 20 µl loop. Isocratic elution was obtained using a mixture of acetonitrile and (0.04%) ammonium hydroxide in (70:30 v/v) water at a flow rate of 1.25 ml/min. Each sample was analyzed in triplicate. A standard calibration methodology was applied to identify and quantify the sugar and FOS derivatives (fructose, glucose, sucrose, 1-kestose, nystose and 1 ^{F} fructofuranosylnystose).

HPLC method was used to detect and quantify mono and disaccharides FOS. This assays was performed to evaluate the linearity, detection and quantification limits for repeatability and intermediate precision.

CHAPTER 4

Results and discussion

4.1. Fungi screening

Many microorganisms produce FOS, particularly bacteria and fungi. The microorganisms produce FOS from sucrose, starting with the synthesis of 1-kestose (GF2), followed by nystose (GF3), and lastly 1^F-fructofuranosyl nystose (GF4). The strain and the substrate used as well the time and temperature of fermentation influence not only the yield of total FOS produced but also the ratio between the different FOS (Kurakake *et al.*, 2008; Aziani *et al.*, 2012*).*

Additionally, biological activity and its physiological effect may depend not only from total FOS concentration but also due to the FOS profile, which can influence the biological activity. Zduńczyk *et al.* (2005) reported that, when compared with a kestose-rich preparation, the administration of a nystose-rich diet increased the concentration of volatile fatty acids in rats. Pejin *et al.* (2013) observed that nystose exhibit a higher anti-hydroxyl radical activity then 1-kestose, showing that the nystose can be a more active natural product. Zary-Sikorska and Juskiewicz (2008) observed that FOS-rich diet reduced the concentrations of TBARS (thiobarbituric acid-reactive substances), in the liver tissue and in the serum of rats. Reactive oxygen species (ROS) and free radicals play an important role in many degenerative diseases like cancer, atherosclerosis and diabetes. These radicals are produced in cells during the aerobic respiration; they are very unstable and react with organic compounds leading to cell or tissue injury. To reduce the damage caused by free radicals antioxidants may be used. Reports about antioxidant activity of FOS in literature are so far very scarce.

The aim of this task was to select the fungi strain that allowed the production of a FOS mixture with the highest antioxidant activity. Thus, in this study the production of FOS by sucrose fermentation via free cells has been performed using Czapek liquid medium. The fungi strains of *P. corylophilum, P. spinulosum, P. thomii, A. aculeatus,* and *A. japonicus* were used aiming to evaluate the possible effect of the fungi strain on the FOS production and antioxidant activity of the fermentation broths. The *in vitro* antioxidant activities of the different fermentation broths, collected during the fermentation assays, were evaluated using the DPPH radical scavenging and iron-chelating assay.

4.1.1. DPPH Radical Scavenging Activity Assay

The antioxidant DPPH free radical scavenging activity of fermentation broths, collected during 96 h of fermentation with the different fungi evaluated, are shown in Figure 4.1. The results pointed out that regardless the strain used, the fermentation broths (after filtration through a Nylon filter) exhibited low radical scavenging activity. The measured antioxidant activities differed according to the strain and the maximum inhibition rates observed (compared to the initial fermentation time) varied from 4% (*P. corylophilum)* to 8%. (*P. spinulosum).* However, the different strains showed different behavior regarding the antioxidant capacity of the fermentation broths through the fermentation. In the case of *P. thomii, A. japonicus* and *P. corylophilum* it was observed that the antioxidant activity increased with fermentation time, while *P. spinulosum* and *A. aculeatus* showed an irregular behavior throughout the fermentation.

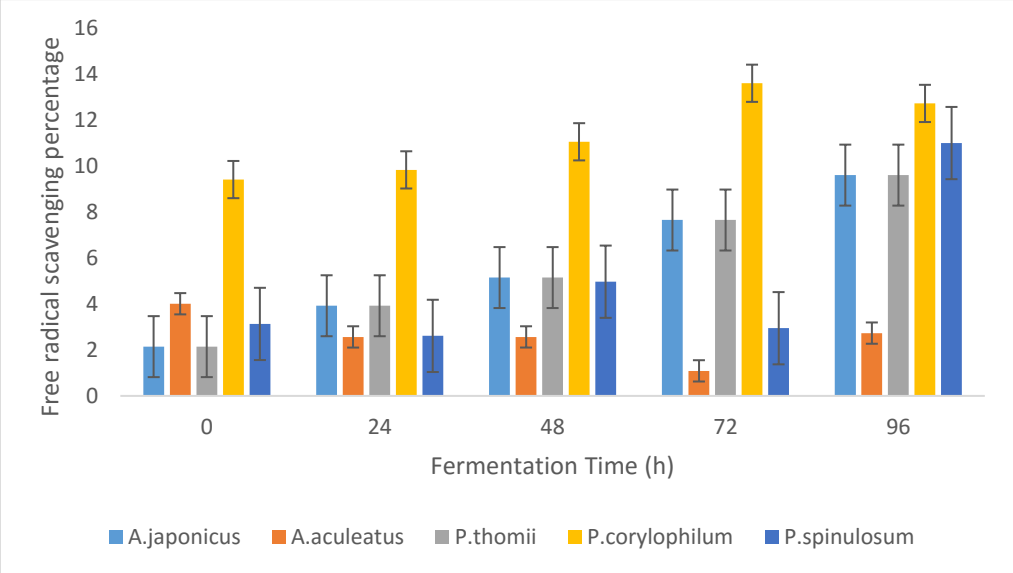


Figure 4.1-Radical scavenging activity (%) measured during fermentation of sucrose by *A. japonicus, A. aculeatus, P. thomii, P. corylophilum* and *P. spinulosum***.**

Initial concentration of sucrose 30 g/L, temperature 27˚C and agitation 100rpm.

Calibration curves: Calibration curves were obtained using ascorbic acid (Vitamin C) as the chemical standard (Figure 4.2)

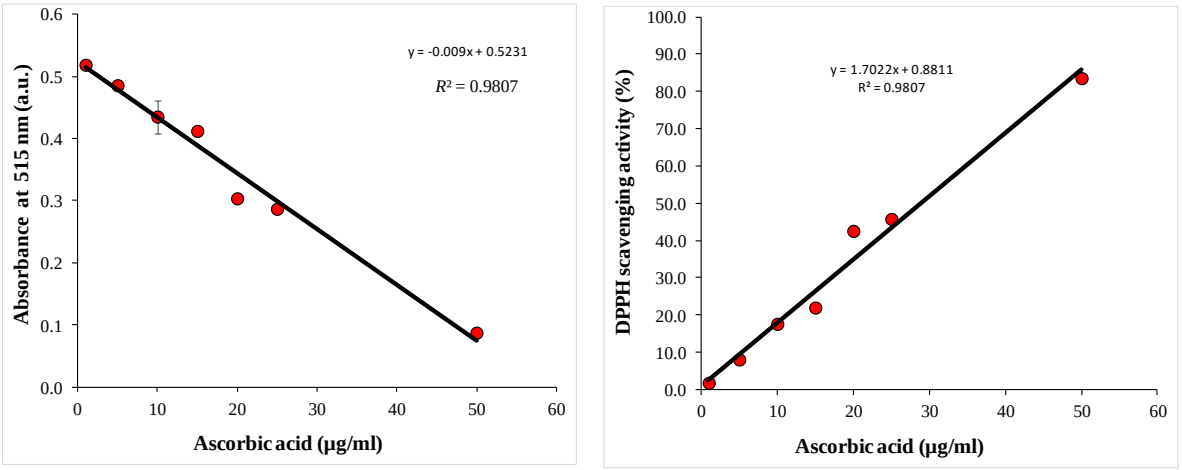


Figure 4.2- Calibration curves using ascorbic acid.

4.1.2. Iron chelating activity

The Fe^{2+} chelating activities of the filtered fermentation broths along the fermentation time, were estimated following the method of Dinis et al. (YEAR). In this assays, it was also verified that the iron chelating activity depends on the strain used and all strains showed moderate capacity to chelate ferrous ion. The fungus *P. spinulosum* exhibited the lowest iron chelating activity (10.7% maximum increase regarding the value for the initial fermentation time) and *A. aculeatus* the highest activity (36.7%), as can be visualized from Figure 4.3. The Fe^{2+} chelating activity increased during the fermentation period for *A. japonicus, A. aculeatus, P. corylophilum and P. spinulosum*, while the fungus *P. thommi* reached the maximum iron chelating activity after 48h of fermentation, being observed a decrease (approximately of 23.5%) during the following days, remaining the fermentation broth with iron chelating activity similar to that of the culture medium.

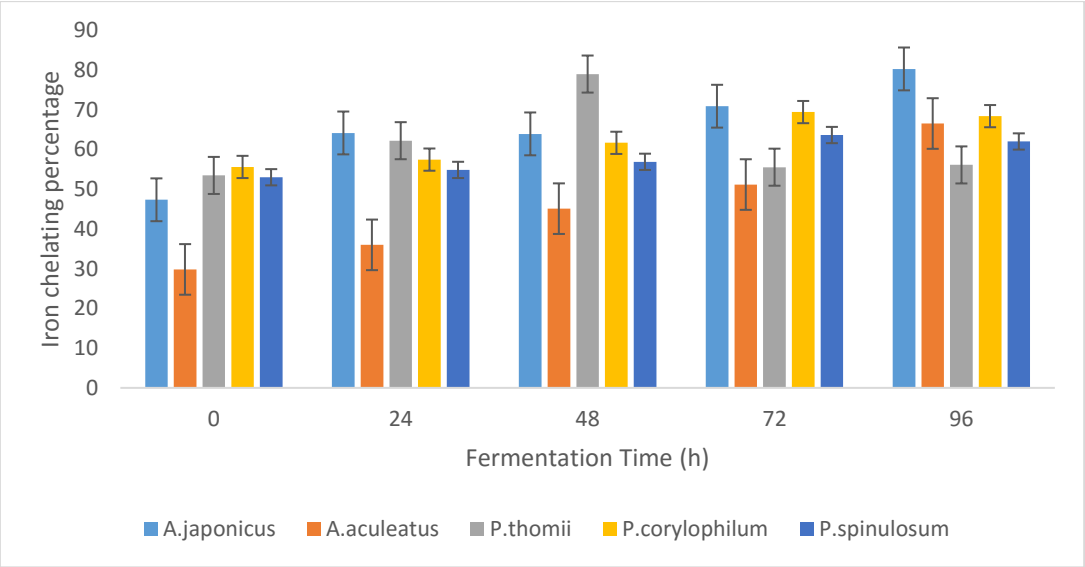


Figure 4.3-Iron chelating activity (%) measured during fermentation of sucrose by *A. japonicus*, *A. aculeatus*, *P. thomii*, *P. corylophilum* and *P. spinulosum*. Initial concentration of sucrose 30 g/L, temperature 27˚C and agitation 100rpm.

Calibration curves: Calibration curves were obatained using EDTA as the chemical standard (Figure 4.4)

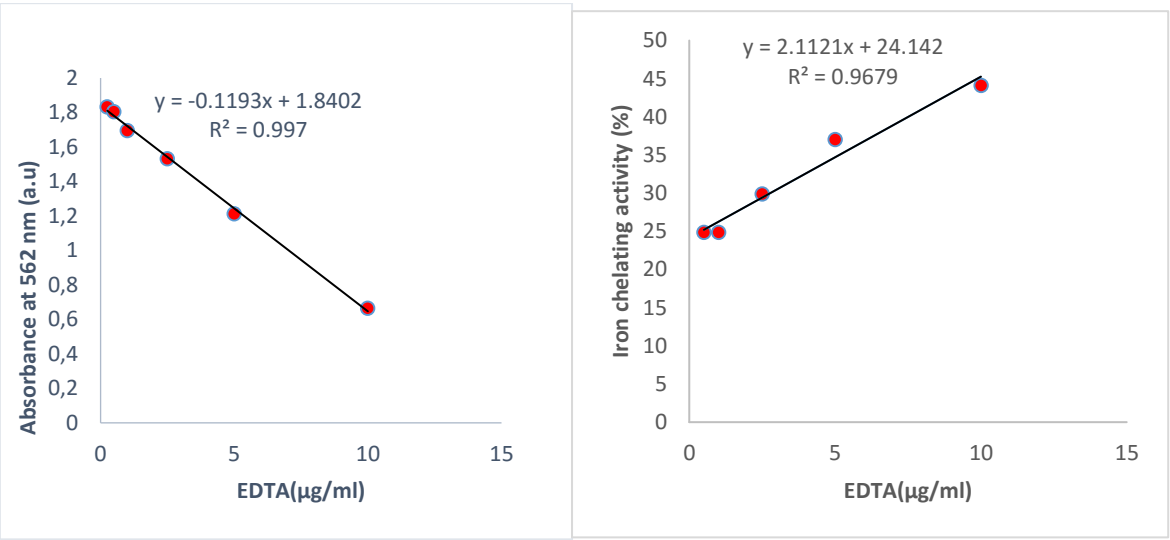


Figure 4.4- Calibration curves using EDTA.

Based on the results obtained for the Fe^{2+} chelating activity, the fermentation broths for *A. aculeatus* showed the highest chelating activity (36.7%) followed by *A. japonicus* (33%), which showed the second highest DPPH free radical scavenging activity (7.5%). So, aiming to select the microorganism with the best potential for

allowing obtaining a broth media with a specific FOS profile with high antioxidant activity, the FOS concentration profiles of the fermentation broths of both fungi were assessed using a HPLC method developed at University of Minho. The concentration of 1-kestose, nystose and 1^F-fructofuranosylnystose, as well the by-products glucose and fructose are showed in Tables 4.1 and 4.2, for the two abovementioned fungi. Concerning the FOS yields (g FOS produced/g of initial sucrose), the maximum total FOS yield, taking into account the three individual FOS produced by the extracellular enzymes during the sucrose fermentation, was obtained at 24h for both fungi. However, the results obtained do not allow to establish a straightforward relation between antioxidant activities of the fermentation broths (DPPH and iron chelating activities) and the individual or total FOS concentrations, although some differences could be found between the FOS profiles for both fungi.

Table 4.1-Total FOS and individual FOS yields (1-kestose, nystose and fructofuranosyl nystose (FN); g FOS produced/g initial sucrose, regarding the production via sucrose fermentation in Czapek liquid medium with *A. aculeatus*, at 100 rpm and 27ºC

Sampling time	FOS yield (%)			
	%GF2	%GF3	%GF4	%total FOS=
0h	0	0	0	0
24h	45.1	4.9	0.0	50.0
48h	29.0	16.0	2.6	47.5
72h	7.4	9.9	2.8	20.4
96h	3.1	5.6	2.8	11.7

Table 4.2-Total FOS and individual FOS yields (1-kestose, nystose and fructofuranosyl nystose (FN); g FOS produced/g initial sucrose, regarding the production via sucrose fermentation in Czapek liquid medium with *A. japonicus*, at 100 rpm and 27ºC

Sampling time	FOS yield (%)			
	%GF2	%GF3	%GF4	%total FOS
0h	0	0	0	0
24h	42.2	7.4	1.8	51.5
48h	19.1	19.1	2.6	40.7
72h	2.9	5.9	2.4	11.3
96h	1.5	2.4	2.4	6.4

According to the results obtained, the antioxidant activity was dependent on the strain used and in general increased during fermentation but was not directly connected to fermentation time. All fermentation broths showed higher iron chelating activity than radical scavenging activity. Concerning the FOS yield were not found significantly differences between both fungi. So, based on the results obtained with chelation of Fe^{2+}, *A. aculeatus* was selected as the fungus with greatest potential to get a FOS profile with higher biological value.

4.2. Influence of sucrose concentration and temperature on FOS profile and antioxidant activity

As referred to above the time and temperature of fermentation as well initial sucrose concentration can affect the yield of total FOS produced and the profile of specific FOS (Kurakake *et al.*, 2008; Aziani *et al.*, 2012*).* In order to obtain, not only different FOS concentrations but also different FOS profiles, the FOS production via sucrose fermentation using *A. aculeatus* was performed using two factors: sucrose concentration (100, 200 and 300 g/L; which corresponded to real concentrations of 88, 176.5 and 265 g/L respectively) and temperature (22, 27 and 32 ºC). Since in the previous work was observed that the antioxidant activity still increased after 96h of fermentation, the fermentation time was extended.

The antioxidant DPPH free radical scavenging activity of fermentation broths, collected during 144 h of fermentation with *A. aculeatus* at different temperature and initial sucrose concentration are shown in Figure 4.5. The results showed that the fermentation broths (after filtration through a Nylon filter), except in one case, exhibited

moderate radical scavenging activity and the antioxidant activity increased with fermentation time. The measured antioxidant activities differed according to the fermentation temperature and initial sucrose concentration and the maximum inhibition rates observed (compared to the initial fermentation time) varied from 11% (265g/L sucrose at 22ºC) to 35.8% (88g/L sucrose at 32ºC). The fermentation performed at 22ºC with 88g/L of sucrose showed a different behavior since exhibited low radical scavenging activity (4%) until the 96h of fermentation and after this time was observed a slight decrease in antioxidant activity. The results of radical scavenging activity pointed out that low temperatures (22ºC) and high initial concentration of sucrose (265g/L) are not suitable for obtaining fermentations broths with a high radical scavenging activity.

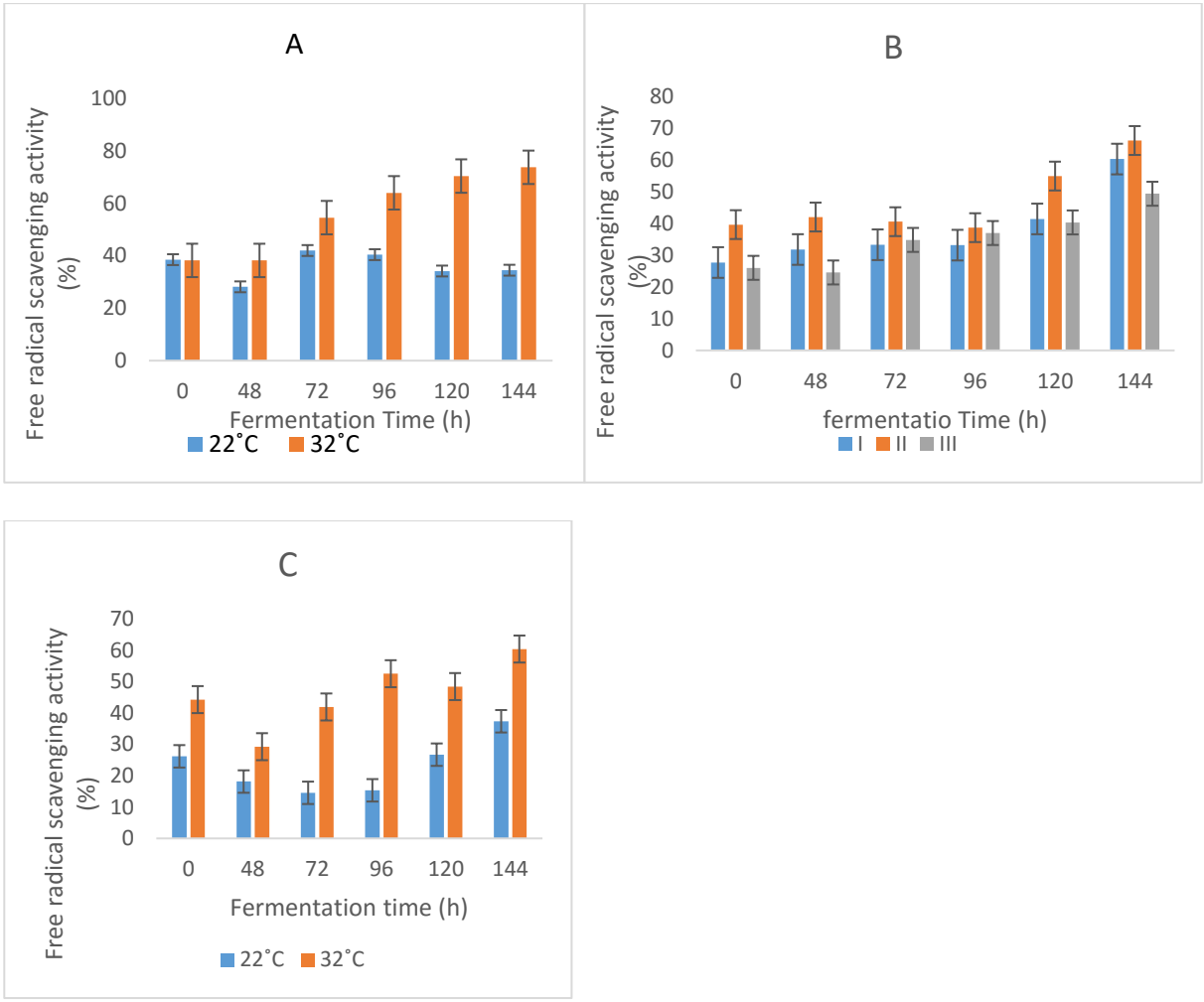
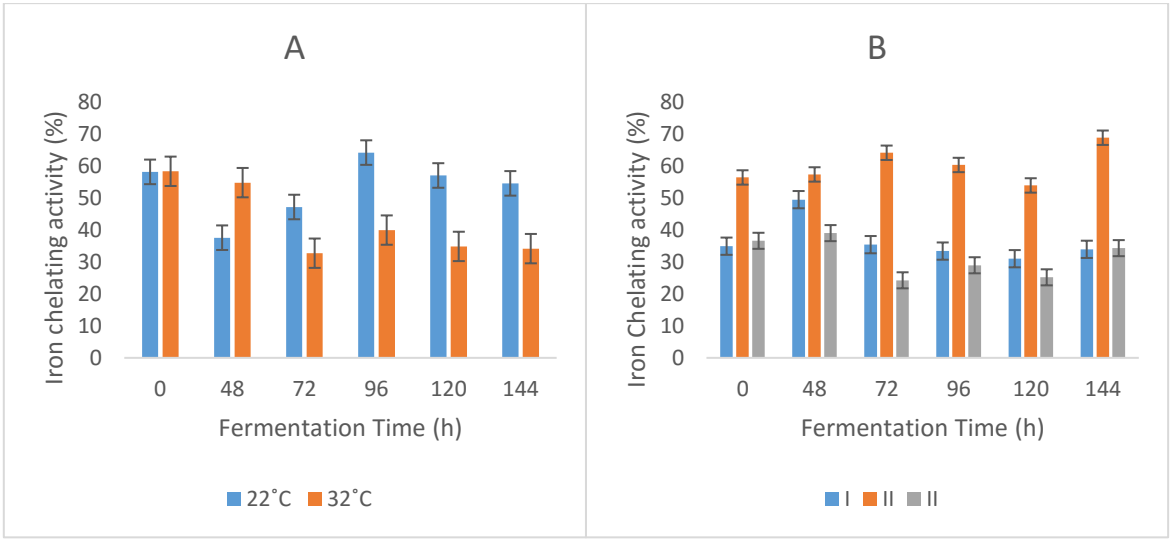


Figure 4.5-Radical scavenging activity (%) measured during fermentation of sucrose by A. aculeatus. A=intial sucrose 88 g/L at temperature 22ºC and 32ºC, B= intial sucrose 176.5 g/L with different fermentation I, II and III at temperature 27ºC and C= intial sucrose 265 g/L at temperature 22ºC and 32ºC.

Nevertheless, regarding the capacity of fermentation broths to chelate ferrous ion, was found that the high temperature (32ºC) had a negative effect on iron chelating activity and the initial sucrose concentration does not appear to have any influence as can be visualized from Figure 4.6. Moreover, the fermentation broths obtained from fermentation performed at 22ºC and 27ºC exhibited low iron chelating activity (3% to 12%) and only in the first days of fermentation.



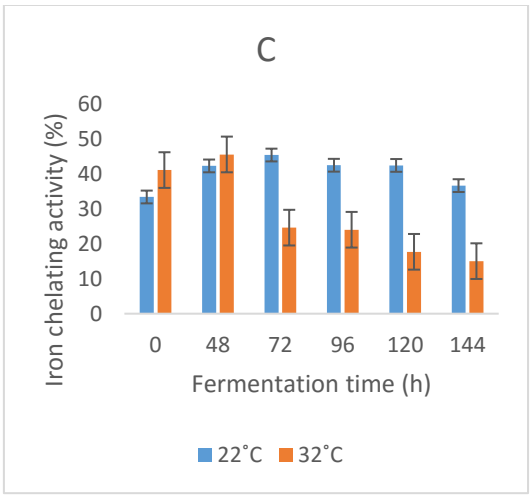


Figure 4.6-Iron chelating activity (%) measured during fermentation of sucrose by A. aculeatus. A=intial sucrose 88 g/L at temperature 22ºC and 32ºC, B= intial sucrose 176.5 g/L with different fermentation I, II and III at temperature 27ºC and C= intial sucrose 265 g/L at temperature 22ºC and 32ºC.

Based on the results obtained was not possible established a correlation between antioxidant activities of the fermentation broths (DPPH and iron chelating activities) and the individual or total FOS concentrations, although some differences could be found between the FOS profiles for both fungi (Table 4.3). For example, the higher yield of FOS as well as nystose were obtained in the fermentation carried out with 265 g/L of sucrose, however the fermentation broths from these fermentations exhibited the lowest free radical scavenging activity. Moreover, the highest free radical scavenging activity on obtained in last 3 days of the fermentation performed with 88 g/L sucrose at

32ºC, when was not quantified any FOS. Although some authors reported that the FOS and essentially nystose have antioxidant activities ((Zduńczyk *et al.*, 2005; Pejin *et al*., 2013; Zary-Sikorska and Juskiewicz, 2008), the results obtained in this work do not allow to establish a straightforward correlation between antioxidant activities of the fermentation broths (DPPH and iron chelating activities) and the individual or total FOS concentrations.

Table 4.3-Total FOS and individual FOS yields (1-kestose, nystose and fructofuranosyl nystose (FN); with initial sucrose 88 g/L with different temperature 32˚C and 22 ˚C.

	FOS yield 32˚C				FOS yield 22˚C			
Time	Total	GF2	GF3	GF4	Total	GF2	GF3	GF4
0h	0	0	0	0	0	0	0	0
48h	6.40	0	2.54	3.85	7.19	0	2.86	4.33
72h	4.55	0	2.01	2.54	5.12	0	2.25	2.87
96h	0	0	0	0	0	0	0	0
120h	0	0	0	0	0	0	0	0
144h	0	0	0	0	0	0	0	0

4.2.1 FOS production using A. aculeatus: biomass and FOS profile

It is known that the total FOS concentration as well as of the individual oligomers contents and proportions, namely 1-kestose (GF2), nystose (GF3) and 1^F-fructofuranosylnystose (GF4), are highly dependent on the source (e.g., fruits or vegetables) and extraction/production conditions (Correia *et al*., 2014). So, although feasible, the use of natural sources aiming to obtain FOS commercial preparations may be conditioned by the intrinsic composition variability of the natural products used, which would be dependent of their year availability. However, due to the reported positive effects of FOS, which are widely used in the food industry as prebiotics, together with their known biological activity, which is related to their molecular structure (Fabrik *et al.*, 2012), the possible FOS production by fermentation using raw materials is of utmost relevance. FOS may be produced either using an enzymatic based approach or by using either free or immobilized whole cells in bioreactors, which has the advantage of obviating purification of FOS-producing enzymes from the cell extracts (Dominguez *et al*., 2012). It should be remarked that nowadays, the current

strategies for improving FOS yield still rely on production process development, although some works have been focused on the genetic improvement of common used industrial fungus (Zhang *et al.*, 2017). So, in this work the production of these oligosaccharides by sucrose fermentation via free cells has been studied aiming to establish the best fermentation conditions that would allow obtaining a broth medium with a specific final FOS composition with known proportions of the individual FOS that could have high biological potential. Indeed, as previously discussed, it has been described that nystose can exhibit higher biological activity than 1-kestose (Pejin *et al*., 2013; Zduńczyk *et al.*, 2005). Thus, it is essential to optimize not only the FOS yield, but also the content of each individual oligomer, in order to obtain a product with higher market value. In this context, the aim of this work was to study FOS production, via sucrose fermentation, using *A. aculeatus*, in order to obtain a product not only with a high amount of FOS but also with a known profile of individual oligomers. The concentration profiles were assessed using a HPLC method developed at University of Minho. The analytical chromatographic method allowed quantifying 1-kestose, nystose and 1^F-fructofuranosylnystose, the by-products glucose and fructose, as well as the remaining amount of sucrose. The fungi *A. aculeatus* was selected based on the results obtained during screening assays. The preliminary results pointed out that among the five fungi studied (i.e., *A. aculeatus, A. japonicus, P. corylophilum, P. spinulosum, and P. thomii*), *A. aculeatus* seemed to be the most promising one taking into account the biological activities (DPPH free radical scavenging activity and iron-chelating activity) of the fermentation broths. The FOS production via sucrose fermentation using *A. aculeatus* was evaluated considering the possible effects of two key factors: sucrose concentration (88, 176.5 and 265 g/L) and temperature (22, 27 and 32 ºC). For that, a 2^k factorial design was implemented (k = 2, representing the 2 factors under study), totalizing 7 assays, which included triplicates at the central point (Table 4.4 and 4.5). Other effects, like agitation speed or medium composition, were fixed (100 rpm and Czapek liquid medium OXOIDE CM0095, respectively).

Table 4.4-Total FOS and individual FOS yields (1-kestose, nystose and fructofuranosyl nystose (FN); with initial sucrose 176.5 g/L with different fermentation I, II and III at 27˚C.

	FOS yield I				FOS yield II				FOS yield III			
Time	Total	GF2	GF3	GF4	Total	GF2	GF3	GF4	total	GF2	GF3	GF4
0h	0	0	0	0	0	0	0	0	0	0	0	0
48h	28.13	5.51	15.01	7.60	29.57	5.69	15.65	8.23	28.27	5.47	15.23	7.57
72h	10.98	1.55	3.48	6.01	11.81	1.56	3.65	6.60	10.37	1.35	3.27	5.75
96h	5.43	0	1.66	3.77	5.77	0	1.78	3.99	4.67	0	1.69	3.06
120h	3.75	0	1.32	2.42	3.93	0	1.38	2.58	3.09	0	1.28	1.78
144h	2.75	0	1.10	1.65	2.84	0	1.14	1.69	2.41	0	1.08	1.34

Table 4.5-Total FOS and individual FOS yields (1-kestose, nystose and fructofuranosyl nystose (FN); with initial sucrose 265 g/L with different temperature 32˚C and 22 ˚C.

	FOS yield 32˚C				FOS yield 22˚C			
Time	Total	GF2	GF3	GF4	Total	GF2	GF3	GF4
0h	0	0	0	0	0	0	0	0
48h	30.20	5.78	14.28	10.14	22.35	20.19	2.15	0
72h	15.69	2.13	4.98	8.58	52.49	38.99	13.49	0
96h	9.69	1.06	2.65	5.99	49.45	18.89	27.38	3.17
120h	5.98	0	1.99	3.99	39.74	9.16	25.22	5.35
144h	4.46	0	1.71	2.74	30.42	5.43	14.48	10.50

4.2.2 Biomass

The biomass or cell density was quantified using the dry weight method, as the mass of dry cell weight per sample volume, which although being a time-consuming task usually gives consistent results. However, it should be kept in mind that this methodology may lead in some cases to unreliable results namely when the broth contains other insoluble particulate matter, which was not expected (or visualized) in the present study. Thus, at the end of the fermentations (time equal to 144 h), the reaming broth (approximately 300 mL) was filtered (using dried weighted filters from

Whatman nº 1) and then the recovered mycelium was dried in an oven with forced air at 50 ºC during 48 h. The dry mass (in grams) obtained for each fermentation, are given in Table 4.6, as well as the related experimental conditions used.

Table 4.6-Experimental design ranges (coded values in brackets) and experimental biomass data concerning the 2^2-full factorial design used FOS production via sucrose fermentation using *A. aculeatus*.

	Factor 1 (A)	**Factor 2 (B)**	**Response**
Assay number	**Sucrose (g/L)**	**Temperature (ºC)**	**Biomass Concentration (g)**
1	88.0 (“-1”)	22 (“-1”)	9.80
2	265.0 (“+1”)	22 (“-1”)	15.17
3	265.0 (“+1”)	32 (“+1”)	15.77
4	88.0 (“-1”)	32 (“+1”)	10.40
5	176.5 (“0”)	27 (“0”)	16.82
6	176.5 (“0”)	27 (“0”)	17.66
7	176.5 (“0”)	27 (“0”)	14.77

Although the experimental design was not set to optimize the final biomass (i.e., the biomass growth), the design output (ANOVA results) pointed out that the only the sucrose concentration had a significant and positive statistical effect over the final biomass content (*P*-value = 0.0214), being the temperature and the interaction [sucrose concentration] × [temperature] not statistically significant at a 5% significance level (*P*-value ≥ 0.6550). Thus, a hierarchical reduced significant regression model could be established (*P*-value = 0.0477) considering that: the effects are only disregarded from the analysis if the hierarchy of the model is not affected; the effects are not removed if the final model is a ridge system; and the final model has a satisfactory prediction performance (Mandenius and Brundin, 2008; Montgomery, 1997):

$$Biomass\ (g) = 5.81 + 0.03 \times \left[Sucrose, \frac{g}{L}\right] + 0.06 \times [Temperature, ºC] \quad [4.1]$$

The above-mentioned model (eq. [1]) showed satisfactory R^2 (0.8685), adjusted R^2 (0.7809) and predicted R^2 values (0.7042). Indeed, according to the literature (Mandenius and Brundin, 2008), R^2 and predicted R^2 values higher than 0.75 and 0.60, respectively, are an indicative that the model is good, i.e., that the mathematical model can be used not only to satisfactorily reproduce the current runs but also showing its prediction potential to describe new experiments. Unfortunately, as canbe easily visualized in Figure 4.7, the mathematical model showed a statistical significant

curvature (*P*-value = 0.0296, which could not be attributed to noise effects), which would require to augment the initial design through a rotatable design to be able to established the optimal conditions that would maximize the *A. aculeatus* biomass growth.

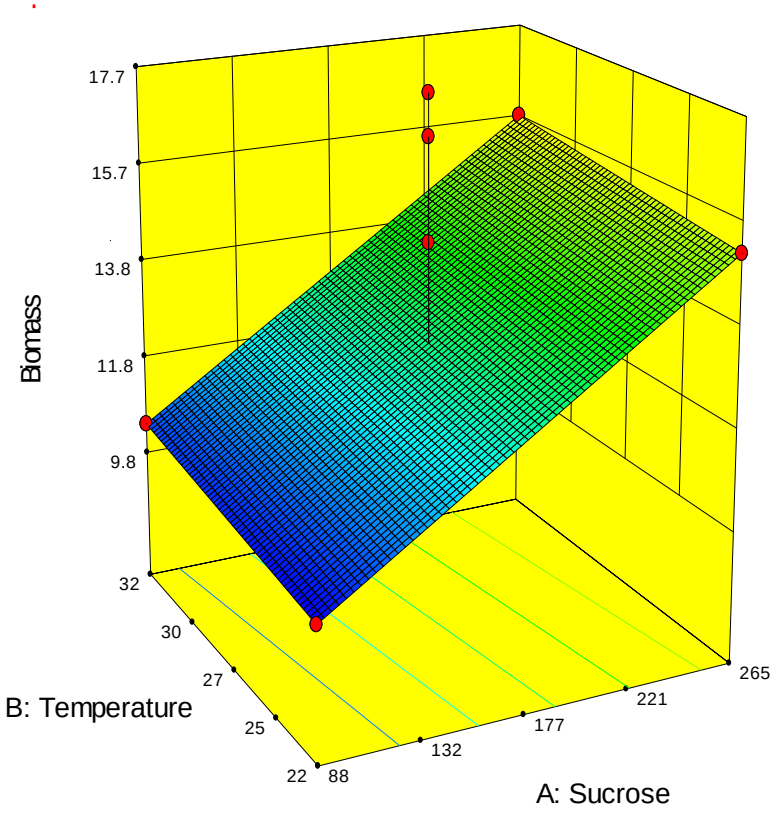


Figure 4.7-Response surface (3D surface) described by the model for an agitation equal to 100 rpm, in the region (initial sucrose concentration and temperature) explored experimentally.

Nevertheless, the reported preliminary results concerning the biomass growth clearly indicate that, among the two evaluated factors (initial sucrose concentration and fermentation temperature) and within the studied experimental ranges, the initial sucrose concentration would be the most important factor to be taken into account when a maximum biomass growth is envisaged. Finally, maximum fungal biomass (16.4±1.5 g corresponding to a biomass concentration of 55±5 g/L) could be expected for an initial sucrose concentration of 176.5 g/L, 27ºC and 100 rpm. The biomass obtained is in agreement with the levels reported by other researchers, which may vary between 10 and 25 g depending on the initial sucrose concentration and/or microorganism used (Fialho *et al.*, 2013).

4.2.3 FOS concentration profiles

A. aculeatus is a FOS producing microorganism, and several studies have reported high total FOS production yields using the enzyme β-fructofuranosidase produced by this fungus, either as extracellular FOS-producing enzymes or immobilized cells (Lorenzoni *et al*., 2014; Huang *et al.,* 2016). Average yields (grams of FOS per grams of initial sucrose), ranging from 55% up to 68%, have been reported with *A. aculeatus* using sucrose at different concentrations (varying from 200 up to 700 g/L) as a carbon source (Correia *et al*., 2014; Flores-Maltos *et al*., 2016; Huang *et al.,* 2016; Lorenzoni *et al.*, 2014), which are similar to the yields achieved when different microorganisms were used (varying from 2.3% up to 69%), such as *Aureobasidium pullulans*, *Penicillium expansum*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus japonicus* among others (Dominguez *et al*, 2012; Flores-Maltos *et al*., 2016; Guo *et al*., 2016; Kashyap *et al*., 2015; Sangeetha *et al*., 2004, 2005; Sheu *et al.,* 2013; Yoshikawa *et* al., 2008; Zeng *et al.*, 2016).

In the present work, as previously mentioned, a 2^k experimental design was used aiming to evaluate the possible effect of the initial sucrose concentration (varying from 88 to 265 g/L) and the temperature (22, 27 and 32ºC) on the FOS production and expected biological activity of the fermentation broths, by using *A. aculeatus* as the microorganism of interest. Unfortunately, due to experimental constrains and based on FOS production data from preliminary fermentation assays, the first sampling time was set at 48 h of fermentation, being then verified that at this fermentation time the maximum FOS yield had already been exceeded in 6 of the 7 assays established for the experimental design (*data not shown*), being already evident the consumption of 1-kestose (GF2) as an alternative carbon source since sucrose availability had already decreased in the medium to very low concentrations (in the order of 2.9 to 10.5 g/L). Thus, it was not possible to establish the optimal operation conditions for maximizing total or individual FOS production. Nevertheless, among the several fermentation assays carried out during the work, it was possible to group a total of 6 assays within the temperature and initial sucrose concentration of the experimental design (2 fermentations at 27 ºC and 165 g/L of sucrose; and, 4 fermentations at 22 ºC and initial sucrose concentrations varying from 205 to 265 g/L), for which the maximum

production yields of FOS (total and individual) could still be observed (Figures 4.8 and 4.9).

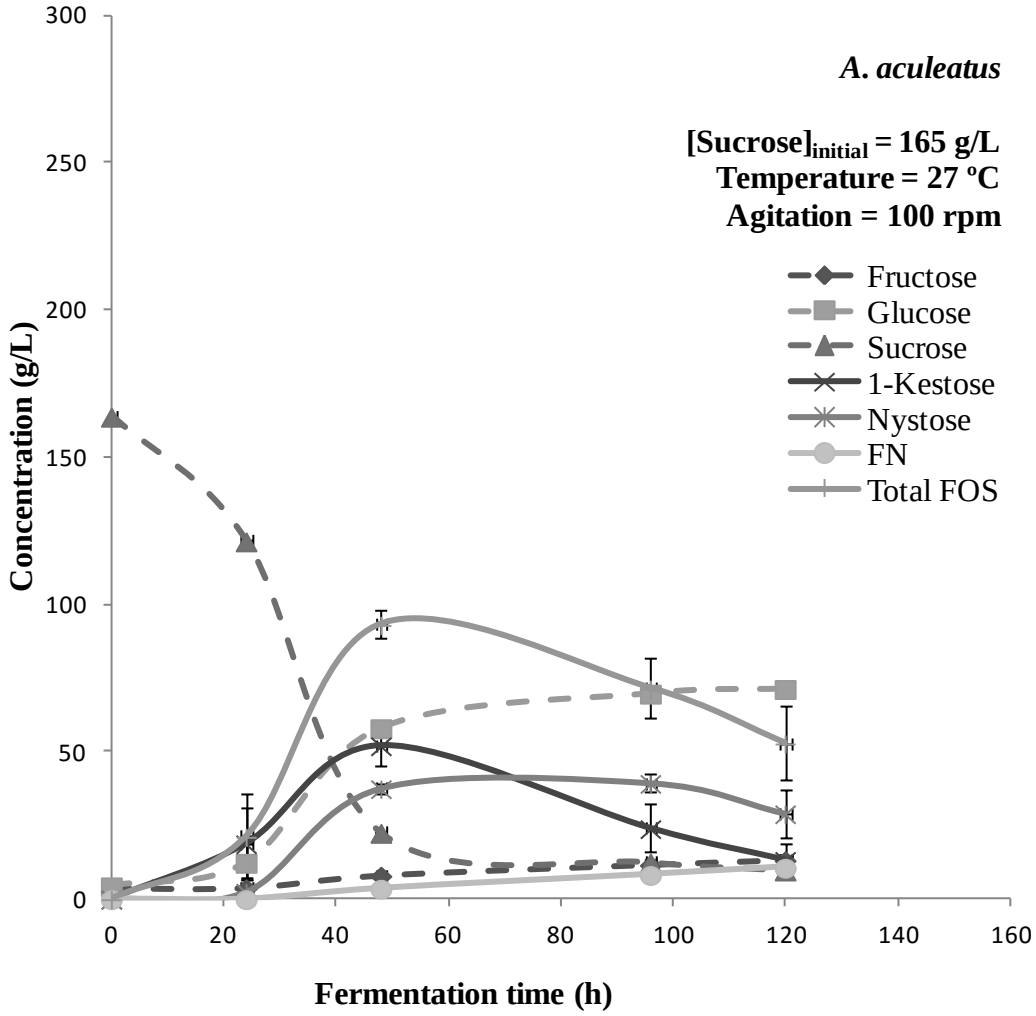


Figure 4.8-Concentration profiles (average ± standard deviation) of substrate (sucrose) and fermentation products (glucose, fructose, 1-kestose, nystose, fructofuranosyl nystose and total FOS) determined by chromatography for sucrose fermentations with *A. aculeatus* at 27ºC, 100 rpm and an initial sucrose concentration of 165 g/L (dot lines for fructose, glucose and sucrose; full lines for total and individual FOS).

Figure 4.8 shows that the methodology used allowed to obtain sugar profiles with satisfactory repeatability (low standard deviations). Moreover, as expected, the results showed an initial high rate of sucrose hydrolysis and the consequent release of the by-products glucose and fructose as well as 1-kestose and nystose, being the highest production of total FOS (93±5 g/L) achieved after 48 h of fermentation. Then a

reduction of total FOS content (mainly due to the consumption of 1-kestose) coincided with the sucrose depletion in the medium and with the accumulation of glucose in the medium, which as reported in the literature might have inhibited the transfructosylating activity and increased the hydrolytic activity (Fialho *et al.,* 2013). Indeed, as described in the literature for initial sucrose concentration higher than 100 g/L sucrose is preferably hydrolyzed and used for mycelial growth, but, the sucrose excess is generally converted into FOS (Maiorano *et al.,* 2008). This may justify that for the fermentation assays carried out at higher sucrose concentrations (between 205 and 265 g/L) slightly different overall FOS production profiles were obtained (Figure 4.9). However, the main trends were similar, being initially observed high sucrose consumption with the production of 1-kestose and nystose, in the first 24-48 h of fermentation, followed by a decrease of the total FOS content, which also coincide with sucrose depletion and glucose possible inhibition. Furthermore, as would be expected, from Figure 4.9 it is obvious that a slight increase of the initial sucrose concentration (205 g/L → 235 g/L → 260-265 g/L) would result in a slight increase of the maximum total FOS concentration (from 115 g/L → 145 g/L).

Concerning the FOS yield (g FOS produced/g of initial sucrose), the maximum yields FOS produced by the extracellular enzymes during the sucrose fermentation with *A. aculeatus* were in the order of 51 to 59% (which are in agreement with yields found in the literature), showing low variability, which could possible demonstrate the low influence of sucrose concentration (in the range of 165-265 g/L) and/or temperature (22 to 27 ºC). The low impact of the initial sucrose concentration on the FOS productivity using *A. aculeatus* was previously described by Huang *et al*. (2016). These authors reported that total FOS production increased to the same extent (65–67%) by increasing the initial sucrose concentration, and no significant differences were observed in the FOS yields at sucrose concentrations ranging from 400 to 600 g/L.

The results (Figures 4.8 and 4.9 and Table 4.7) clearly pointed out that FOS production and yields were mostly influenced by the time of fermentation and maximum yields were obtained after slightly different inoculation times, showing that, in general, fermentations with low initial sucrose concentrations would require more time to reach similar maximum FOS yields. Also, globally, the yields achieved for each individual FOS or for total FOS seem to be independent of the initial sucrose concentration or temperature, although narrow ranges were evaluated.

Table 4.7-Total FOS and individual FOS (1-kestose, nystose and fructofuranosyl nystose (FN)) yields (g FOS produced/g initial sucrose) achieved during the sucrose fermentation with A. aculeatus, at 100 rpm and for different initial sucrose concentrations (from 165 to 265 g/L) and temperatures (22 and 27 ºC). In brackets, it is given the fermentation time for the achieving the highest FOS yield.

FOS compound	**Highest fermentation yields (fermentation time in brackets)**					
	165 g/L; 27 ºC (I)	**165 g/L; 27 ºC (II)**	**205 g/L; 22 ºC**	**235 g/L; 22ºC**	**260 g/L; 22 ºC**	**265 g/L; 22 ºC**
1-kestose	35% (48 h)	29% (48 h)	36% (48 h)	30% (48 h)	35% (48 h)	39% (72 h)
Nystose	23% (96 h)	25% (96 h)	17% (48 h)	19% (48 h)	21% (48 h)	27% (96 h)
FN	6% (120 h)	7% (120 h)	8% (72 h)	10% (72 h)	9% (72 h)	11% (144 h)
Total FOS	59% (48 h)	55% (48 h)	55% (48 h)	51% (48 h)	58% (48 h)	52% (72 h)

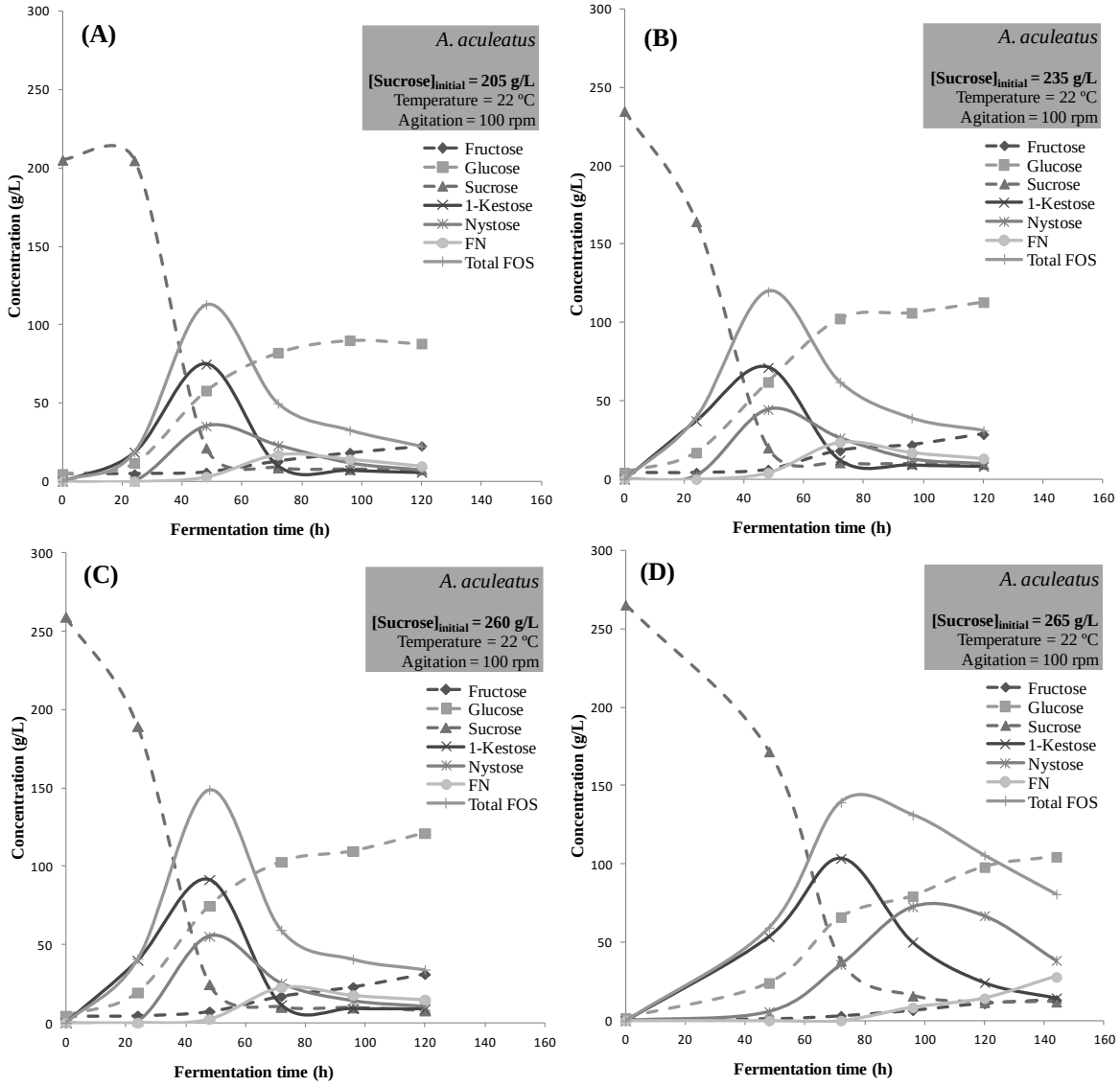


Figure 4.9-Concentration profiles of substrate (sucrose) and fermentation products (glucose, fructose, 1-kestose, nystose, fructofuranosyl nystose and total FOS) determined by chromatography for sucrose fermentations with A. aculeatus at 22ºC, 100 rpm but at slight different initial sucrose concentrations (dot lines for fructose, glucose and sucrose; full lines for total and individual FOS): (A) 205 g/L; (B) 235 g/L; (C) 260 g/L); and, (D) 265 g/L.

CHAPTER 5

Conclusions and future perspectives

In this work, comprehensible study was performed, in order to screen a fungus with potential for producing fructooligosaccharides that has been receiving much attention recently in application in food, pharmaceutical, and veterinary studies, being increasingly important in food and nutrition sciences.

A lot of attention has been given to the initial process of selecting the best molds screening methodology. Using DPPH and ferrozine, the different strains showed different behavoiur regarding the antioxidant capacity of the fermentation broths through the fermentation time. *A. aculeatus* was selected as a potential fungus for FOS production. The selected fungus was then optimized with fermentation conditions for maximizing biological activity of fermentation broths. Initial sucrose, time and temperature of fermentation were selected parameters for the total FOS yield production and the profile of individual FOS.The result point to this work do not allow to establish a correlation between antioxidant activities of fermentation broths (DPPH and iron chelating activities) and individual FOS profile or total FOS concentration.

This analysis provides evidence that FOS production and yields were mostly influenced by the time of fermentation, in general fermentation with low initial sucrose concentration would require more time to reach maximum FOS yield. In other condition, for example with 24h of fermentation the maximum value of yield the results should more consistent and this can be a future investigation.

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