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BIOPROSPECÇÃO DE MICRORGANISMOS DE FRUTAS BRASILEIRAS PARA A PRODUÇÃO DE EXOPOLISSACARÍDEOS

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Dissertação apresentada ao programa de Pós-Graduação em Ciência e Tecnologia de Alimentos da Universidade Federal dos Vales do Jequitinhonha e Mucuri, como requisito parcial para obtenção do título de Mestre.

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Bioprospecção de microrganismos de frutas brasileiras para a produção de exopolissacarídeos

Dissertação apresentada ao MESTRADO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS, nível de MESTRADO como parte dos requisitos para obtenção do título de MAGISTER SCIENTIAE EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

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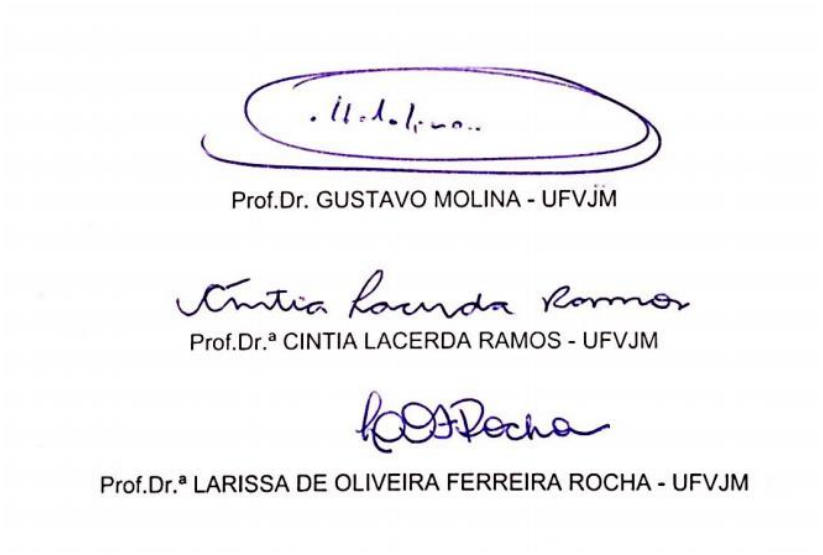

DIAMANTINA

RESUMO

A bioprospecção é tida como a principal forma de descobrimento de linhagens microbianas e bioprodutos de interesse industrial. Devido à grande biodiversidade encontrada em frutas brasileiras, estas oferecem potencial ilimitado para descoberta de biocatalisadores com características e propriedades únicas. Polissacarídeos microbianos, especialmente exopolissacarídeos (EPS) constituem um importante grupo de moléculas industrialmente relevantes com variadas estruturas e propriedades, sendo utilizados em diversos setores industriais como indústria de alimentos, química, médica e farmacêutica. A grande diversidade e facilidade de alteração da composição e propriedade dos EPS tornam essas moléculas ainda mais interessantes, já que a descoberta de novas linhagens produtoras e consequentemente, novos compostos, levam ao desenvolvimento de novos bioprocessos capazes de suprir demandas industrias como baixo custo, alto rendimento e ampla faixa de aplicação em diversos setores. Neste trabalho, 25 linhagens de microrganismos foram isoladas das frutas brasileiras jatoba (*Hymenaea courbaril*), jabuticaba (*Plinia cauliflora*), lobeira (*Solanum lycocarpum*), morango (*Fragaria × ananassa*) e manga (*Mangifera indica*). Dentre essas, uma linhagem foi capaz de produzir EPS. Esta linhagem, isolada da lobeira, foi submetida a um processo fermentativo a 30°C, 150 rpm e meio YM durante 48 horas, alcançando 0,95 g/L de EPS após 32 horas de processo. O comportamento e habilidade de produção de EPS desta linhagem foi ainda avaliado utilizando três diferentes fontes de carbono (glicose, frutose e lactose) nas mesmas condições de incubação. O microrganismo isolado apresentou curvas de crescimento semelhantes para todas as fontes de carbono testadas, porém diferenças significativas para a produção de EPS foram observadas, indicando diferentes capacidades metabólicas quando diferentes fontes de carbono são utilizadas. Crescimento variando de 2,81 a 4,49 g/L de biomassa seca, e produção de EPS em torno de 1 g/L para todas as fontes de carbono testadas foram observadas. A linhagem isolada foi caracterizada por análise sequencial e filogenética do gene 16s do RNA ribossomal, sendo identificada como *Kosakonia cowanii*, anteriormente conhecida como *Enterobacter cowanii*, uma bactéria gram-negativa. Com o objetivo de obter incrementos na produção e analisar como a composição de meio e as condições de processo interferem na produção de EPS, uma otimização foi conduzida utilizando ferramentas estatísticas. Dentre as variáveis analisadas, temperatura, agitação e glicose foram as mais influentes, tendo como ótimas as condições de 28°C, agitação acima de 250 rpm, e concentração de glicose de 38%, alcançando uma produção de 17,37 g/L. Até o presente momento, não existem relatos da *Kosakonia cowanii* como linhagem produtora de EPS. Pesquisas futuras ainda são necessárias quanto a caracterização do EPS produzido (composição, propriedades

reológicas e toxicologia) e quanto a outros componentes do meio de cultivo para entendimento total do processo de produção de EPS pela bactéria *Kosakonia cowanii*.

Palavras-chave: Polissacarídeos microbianos. *Kosakonia cowanii*. Otimização. Bioprocesso.

ABSTRACT

Bioprospecting is considered the main way for the discovery of microbial strains and bioproducts of industrial interest. Due to the great biodiversity found in Brazilian fruits, they offer unlimited potential for the discovery of new biocatalysts with unique characteristics and properties. Microbial polysaccharides, especially exopolysaccharides (EPS) constitute an important group of industrially relevant molecules with varied structures and properties, being used in various industrial sectors such as food, chemical, medical and pharmaceutical. The great diversity and ease of alteration of EPS composition and properties make these molecules even more interesting, since the discovery of new production lines and consequently, new compounds lead to the development of new bioprocesses capable of meeting industrial demands such as low cost, high yield and wide range of application in several sectors. In this work, 25 microorganisms were isolated from Brazilian fruits jatoba (*Hymenaea courbaril*), jabuticaba (*Plinia cauliflora*), lobeira (*Solanum lycocarpum*), strawberry (*Fragaria* × *ananassa*) and mango (*Mangifera indica*). Among these, one strain was able to produce EPS. The strain, isolated from lobeira fruit, was submitted to a fermentation process at 30°C, 150 rpm and YM medium for 48 hours, reaching 0.95 g/L of EPS after 32 hours of process. The behavior and ability of EPS production of the strain was further evaluated using three different carbon sources (glucose, fructose and lactose) at same incubation conditions. The isolated microorganism presented similar growth curves for all the carbon sources tested, but significant differences for EPS production were observed, indicating different metabolic capacities when different carbon sources are used. Growth ranging from 2.81 to 4.49 g/L of dry biomass, and EPS production around 1 g/L for all carbon sources tested were verified. The isolated strain was characterized by sequential and phylogenetic analysis of the 16s gene of ribosomal RNA, being identified as *Kosakonia cowanii*, formerly known as *Enterobacter cowanii*, a gram-negative bacteria. In order to obtain increases in production and to analyze how the media composition and process conditions interfere in EPS production, an optimization was conducted using statistical tools. Among the analyzed variables, temperature, agitation and glucose were considered the more influential, with optimum conditions of 28°C, agitation above 250 rpm, and glucose concentration of 38%, reaching a production of 17.37 g/L. Until the present moment, there are no reports of *Kosakonia cowanii* as an EPS-producing strain. Further research is still needed regarding the characterization of EPS produced (composition, rheological properties and toxicology) and other components of the culture medium to fully understand the EPS production by the bacteria *Kosakonia cowanii*.

Keywords: Microbial polysaccharides. *Kosakonia cowanii*. Optimization. Bioprocess.

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INTRODUÇÃO GERAL

Polissacarídeos microbianos são carboidratos de alto peso molecular produzidos por uma grande variedade de microrganismos, como bactérias, algas e fungos, sendo normalmente chamados de biopolímeros (FREITAS; ALVES; REIS, 2011).

De acordo com a sua posição na célula microbiana, estes compostos são divididos em polissacarídeos estruturais, intracelulares (IPS) e extracelulares, também chamados de exopolissacarídeos (EPS) (BOELS et al., 2001; LAHAYE, 2006). Devido a facilidade de recuperação e consequente redução de custos em etapas *downstream*, os EPS são considerados os polissacarídeos microbianos de maior importância comercial (BERGMAIER, 2002; RÜHMANN; SCHMID; SIEBER, 2015a). Considerados moléculas potencialmente bioativas, alguns EPS possuem atividades antitumoral, imunomoduladora, antioxidante e antiulcera, além de serem capazes de modificar as propriedades reológicas de diversos produtos dos setores alimentício, químico, farmacêutico e médico (MADHURI; VIDYA PRABHAKAR, 2014; MOSCOVICI, 2015).

As diversas diferenças em estrutura observadas nos EPS, e consequentemente suas diferentes propriedades, estão diretamente relacionadas aos diferentes microrganismos utilizados para sua síntese, bem como as condições de processo e composição do meio de cultivo, incluindo fontes de carbono e nitrogênio utilizadas, pH, temperatura e taxas de agitação (CHUG et al., 2016).

Devido à crescente demanda do mercado por moléculas com propriedades e estruturas únicas, capazes de serem aplicadas em diversos setores industrias, especialmente aqueles com alto valor agregado, a busca por novas linhagens capazes de sintetizar EPS oferece a possibilidade de desenvolvimento de novos bioprocessos, contribuindo para futuros desenvolvimento de processos e aplicações industriais (BUSHRA et al., 2017; CHANDRAN; SHARMA, 2015; FREITAS; ALVES; REIS, 2011).

Neste cenário, a bioprospecção de novas linhagens capazes de sintetizar EPS é uma ferramenta promissora, visando reconhecer o potencial da biodiversidade microbiana de fontes naturais.

Os diferentes biomas e condições ambientais presentes no Brasil propiciam uma grande variedade de frutas de climas temperado e tropical em todo o país, oferecendo grande biodiversidade microbiana, ainda a serem exploradas. Atualmente, o estado de Minas Gerais é o terceiro maior produtor de frutas do país, possuindo em seu território três diferentes biomas

(cerrado, mata atlântica e caatinga), dispondo de plantas e frutas únicas, facilitando o isolamento de microrganismos distintos (CARVALHO et al., 2017; IEF, 2017).

Neste trabalho, a biospospecção de microrganismos das frutas brasileiras jatobá (*Hymenaea courbaril*), jabuticaba (*Plinia cauliflora*), lobeira (*Solanum lycocarpum*), morango (*Fragaria* × *ananassa*) e manga (*Mangifera indica*) foi realizada para a obtenção de linhagens com potencial para a produção de EPS, visando o desenvolvimento e otimização do processo fermentativo.

OBJETIVOS

1.1 Objetivo geral

Isolamento e seleção de microrganismos de frutas brasileiras visando o conhecimento do potencial da biodiversidade microbiana para a produção de exopolissacarídeos (EPS), com o intuito de desenvolver e otimizar o processo fermentativo.

1.2 Objetivos específicos

- ✓ Isolamento de microrganismos de fontes naturais;
- ✓ Seleção de linhagens com potencial para a produção de EPS;
- ✓ Avaliação do comportamento e da cinética de crescimento das linhagens em diferentes fontes de carbono;
- ✓ Cálculo de cinética dos processos fermentativos;
- ✓ Quantificação dos produtos gerados nos processos fermentativos;
- ✓ Otimização da composição do meio de cultivo e das condições de processo visando incremento na produção de EPS.

CHAPTER 1 - CURRENT STATUS OF BIOTECHNOLOGICAL PRODUCTION AND APPLICATIONS OF MICROBIAL EXOPOLYSACCHARIDES

1 INTRODUCTION

Polysaccharides are macromolecules belonging to the carbohydrate group, constituting the most important and abundant group of compounds, normally stored as starch by higher plants and being synthetized by bacteria, fungi and algae (BARBOSA et al., 2004; SOUZA; GARCIA-CRUZ, 2004).

Microbial polysaccharides are classified depending on their position inside the microbial cell: (i) polysaccharides constituting the cell wall (structural polysaccharides), such as techoid acids, lipopolysaccharides (LPS) and peptidoglycans; (ii) polysaccharides which provide energy and act as carbon source for the cell, called cytosolic polysaccharides or intracellular polysaccharides (IPS); and (iii) exopolysaccharides (EPS), polysaccharides that are secreted in the form of biofilm or capsules to the extracellular medium (BERGMAIER, 2002; BOELS et al., 2001; KUMAR; MODY; JHA, 2007; LAHAYE, 2006; RUAS-MADIEDO; DE LOS REYES-GAVILÁN, 2005).

Polysaccharides obtained through biotechnological process presents itself as an available market, especially EPS, which can be produced by various classes of microorganisms, such as bacteria, molds and yeasts, due to its water solubility with constant physical and chemical properties (ARANDA-SELVERIO et al., 2010; BANIK; KANARI; UPADHYAY, 2000; SOUZA; GARCIA-CRUZ, 2004; SUTHERLAND, 2001). The microbial polysaccharides' market is expanding due to the use of renewable sources and the various desirable characteristics, such as polymers substitute for improvement of rheological properties of several products in food and pharmaceutical industry, and antitumor and immunostimulatory properties (BERGMAIER, 2002; NWODO; GREEN; OKOH, 2012; RÜHMANN; SCHMID; SIEBER, 2015b).

One of the main exopolysaccharides produced nowadays is xanthan gum with a production of 50,000 tons per year with worldwide market evaluated between U$600 and U$800 million per year, by the bacteria *Xanthomonas campestris* (SUBHASH; JADHAV; JANA, 2015). Xanthan gum accounts for 6% of the polysaccharides market being mainly used by food industries in sauces, frozen food, juices, and desserts. Another relevant EPS's are gellan, dextran and pullulan gums used especially to improve rheological properties of a range of food and pharmaceutical products (BUENO; GARCIA-CRUZ, 2006; FREITAS; ALVES; REIS, 2011; WELMAN; MADDOX, 2003).

Normally EPS are divided into two groups: homopolysaccharides (*e.g.* dextran) and heteropolysaccharides (*e.g.* xanthan and gellan). Homopolysaccharides are composed by only one type of monosaccharide while heteropolysaccharides are constituted of several monosaccharides, which synthesis takes place inside the cell generating complex structures. The majority of bacterial EPS can be classified as heteropolysaccharides (ARANDA-SELVERIO et al., 2010; BERGMAIER, 2002; CZACZYK; MYSZKA, 2007; DE BAETS et al., 2002; LAHAYE, 2006).

In addition, the various functions that EPS plays for the cell, such as protection against biotic and abiotic stress (DE BAETS et al., 2002; LIMOLI et al., 2015; NWODO; GREEN; OKOH, 2012), its production ensures greater resistance, affects the way microorganism interacts with the environment, and allows its use as a substrate for microorganism growth (CZACZYK; MYSZKA, 2007; OZTURK; ASLIM; SULUDERE, 2010).

The wide range of EPS applications, based on its physical and chemical properties, demonstrates the potential of these substances for industrial approach and the importance of further studies to better understand their production. In this sense, this review aims to present the biotechnological production of microbial EPS, in a general way, exploring all the production steps, optimization processes and current applications of these important commercial products.

2 BIOTECHNOLOGICAL PRODUCTION OF EXOPOLYSACCHARIDES

The biotechnological production of EPS offers advantages over chemical and plant-derived production such as energy efficient (when produced by algae), quick production in a matter of days independently of location and season, and the chance to use industrial waste as substrate. However, some limitations such as cost of production may hinder the process due to the requirements needed (GONZÁLEZ LÓPEZ et al., 2009; RUTERING et al., 2016; THOMPSON; HE, 2006).

Currently, many EPS are produced through biotechnology. Each EPS has its own biosynthetic pathway and, consequently, factors that can influence these pathways. The biosynthesis and accumulation of polysaccharides normally occur after the growth phase of the microorganism, and the synthesis of EPS can be divided within 4 different steps: (i) assimilation of the carbon source, (ii) synthesis of the oligosaccharide repeat units or direct synthesis by successive or progressive activity of glycosyltransferases (iii) assembly of the polysaccharide from the repeat units, and (iv) exportation of the EPS to the extracellular medium (BECKER, 2015).

Meanwhile, four general mechanisms describe the production of polysaccharides by bacteria: (i) the so called Wzx/Wzy-dependent pathway where individual repeating units are assembled by several glycosyltransferases, which gives heteropolymers; (ii) the ATP-binding cassette (ABC) transporter-dependent pathway, which are classified as capsular polysaccharides; (iii) the synthase-dependent pathway, which are mostly homopolymers; and (iv) the extracellular synthesis by the use of a single sucrase protein (SCHMID; SIEBER; REHM, 2015).

In all the processes of EPS biosynthesis, enzymes constitute an important role being responsible for almost all steps during the process. These enzymes are located at different parts of the microbial cell and can be divided into four groups: (i) intracellular enzymes involved in other metabolic processes, such as hexokinase; (ii) enzymes that are believed to be intracellular which are the source of monosaccharides residues; (iii) enzymes located at the cell periplasmic membrane, such as glycosyltransferases; and (iv) enzymes situated outside the cell membrane responsible for the polymerization of macromolecules (KUMAR; MODY; JHA, 2007).

Thus, as EPS biosynthesis is dependable of several biological variables, improvements in their production are still necessary and desirable for industrial applications. For this, an increase in the rate of synthesis, enzyme management, and genetic modifications are needed.

2.1 Process conditions and medium composition influence on EPS production

The amount of polysaccharides produced is directly linked to the process conditions and medium composition (KUMAR; MODY; JHA, 2007). Availability of carbon and nitrogen sources, temperature, pH, growth phase, agitation, and aeration play major influence on EPS production by microorganism, especially bacteria (BARBOSA et al., 2004; CZACZYK; MYSZKA, 2007). Is noteworthy that how these variables influence the EPS production is related to the microorganism used, as shown in the sequence.

The most influent conditions for EPS production is the availability of carbon (*e.g.* glucose, sucrose) and nitrogen (*e.g.* ammonium sulfate, peptone, sodium nitrate, yeast extract). These conditions have contradictory effects, since the greater the amount of carbon, better yields are achieved, with the opposite occurring with the nitrogen, since large amounts of carbon can extend the growth phase of the microorganism and large amounts of nitrogen can increase the production of degrading enzymes (BARBOSA et al., 2004; CZACZYK; MYSZKA, 2007; KUMAR; MODY; JHA, 2007). The relation between carbon and nitrogen sources is called C/N ratio and it can be used as a production parameter, where it is observed

that the greater the C/N ratio, maximal production of EPS can be achieved, with 10:1 considered the most favorable (DE SOUZA; SUTHERLAND, 1994).

The EPS production is dependent on growth phase and microorganism. Many reports have described that the major production rate is associated to the stationary phase, normally 24 hours after de initial of the incubation (PETRY et al., 2000; RAGUENES et al., 1996; SKALIY; EAGON, 1972). However, it also dependent on the microorganism used, being reported at different growth phase, such as stationary phase for *Rhodopseudomonas acidophila*, late log phase for *Bacillus megaterium*, and late log phase to initial stationary phase for *Saccharophagus degradans* and *Vibrio harveyi strain VB23* (CHUG et al., 2016).

Regarding incubation temperature, those below to the optimal for growth of the biocatalyst used can provide higher production of EPS. This is supported by the presence of enzymes involved at the synthesis, which have different optimum temperatures (CERNING et al., 1992; KUMAR; MODY; JHA, 2007).

A constant pH is desirable for maximum production of EPS, with some studies suggesting that the pathway for its production may be directly dependent on pH values (GORRET et al., 2001; KUMAR; MODY; JHA, 2007). It is known that the enzyme glycosyl-hydrolase is capable of degrading EPS after long fermentations, which reduce the molar mass. In this way, the optimum pH would be capable to balance the effects of production and degradation (LAWS; GU; MARSHALL, 2001).

According to some authors, high aeration and agitation can give better results to EPS production since almost all EPS's producers are aerobic (LEE et al., 2001; YANG; LIAU, 1998). This impact may be explained using molecular oxygen as a primary source of energy metabolism, which lead to an increase of EPS production. Since the production of EPS in submerged medium can lead to higher viscosity, this can result in an irregular distribution of oxygen, which can negatively affect the EPS synthase and quality. These problems can be avoided with agitation rates depending on the microorganism used (KUMAR; MODY; JHA, 2007).

Using *Lactobacillus delbrueckii* subsp. *bulgaricus*, Petry *et al.* (2000) defined the factors that affect the EPS production using a chemically defined medium. Four main factors were observed: oxygen, pH, temperature, and medium constituents. The controlled pH at 6.0 was the most important one, since it led to an increase in EPS production. However, changes oxygen, temperature and medium constituents did not affect the EPS composition (PETRY et al., 2000).

Rutering and coworkers (2016) described the EPS production by *Paenibacillus* sp. The influence of carbon and nitrogen sources on EPS composition and biosynthesis were investigated using different carbon (sucrose, glucose and glycerol) and nitrogen (peptone and $NaNO_3$) sources with concentrations of 30 g/L and 0.05 mol/L respectively, incubated at 30°C, 150 rpm, for 48 hours with initial pH of 7.0. The authors observed that different carbon and nitrogen sources induces to different biosynthesis pathways of EPS for *Paenibacillus*, obtaining a levan-type polymer and a heteropolysaccharide when using sucrose and peptone, and levan when using sucrose and $NaNO_3$ (RUTERING et al., 2016).

A culture of the algae *Cylindrotheca closterium* was tested at temperatures of 4°C, 10°C, 15°C, 25°C and 35°C, chosen based on the normal environmental temperature of the microorganism, for EPS production. The highest results, 9 μg/mL and 11 μg/mL, were observed during the stationary phase (after 13 days of process) at 15° and 25°C respectively, with a higher influence of temperature occurring during the growth phase (WOLFSTEIN; STAL, 2002).

The production of EPS by the fungal strain *Ganoderma lucidum* was evaluated under different initial pH, varying from 3.5 to 7.0. It was observed that the increase in pH value led to an increase in biomass production. However, the reduction in pH, with value close to 3.5, resulted in higher EPS production, which indicates a prolonged phase of production of EPS at lower pH (FANG; ZHONG, 2002). A similar result was observed for *Lactobacillus casei* CRL 87, where the maximum cell viability occurred at pH 6.0, but the maximum yield for EPS was close to pH 4.0 (MOZZI et al., 1996).

Wisbeck, Furlan and Ninow (2005) studied the effect of initial pH (ranging from 4.0 to 6.0) and concentration of glucose (from 20 to 40 g/L) for the production of EPS by the fungal *Pleurotus ostreatus* DSM 1833. It was observed that the maximum productivity suffers significant influence of the interaction between glucose and pH, especially when fixing the pH at 6.0 and changing the glucose concentration from 20 g/L to 40 g/L, achieving maximum productivity, with values four times higher (WISBECK; FURLAN; NINOW, 2005).

The effect of the C/N ratio was studied for the EPS production by a bacterial strain under semi anaerobic and anaerobic conditions. Three different C/N ratio were used: 13.6 g carbon/g nitrogen, 6.8 g carbon/g nitrogen and 3.4 g carbon/g nitrogen. For all the experiments it was noted that the production of cellulose, the main EPS produced, was dependent on the C/N ratio, except the ones with strictly anaerobic conditions. The best result was achieved with a C/N ratio of 13.6 g carbon/g nitrogen, reaching 23.3 g/g carbon, which confirms that a high C/N ratio benefits EPS production (MIQUELETO et al., 2010).

Some studies also show the influence of heavy metals in the EPS production and its capacity of further metal removal. analyzed the effects of different concentrations of heavy metals such as lithium, copper, lead and cadmium, on *Cyanothece* sp. CCY 0110 growth and EPS production. It was observed that different heavy metals influence the cells at different manners triggering different responses such as tune down the metabolic rate for detoxification mechanisms with remarkable recovery; nevertheless, the EPS production continued to follow the growth pattern (MOTA et al., 2015).

Using chromium, cadmium and a metal mixed (chromium + cadmium), it was investigated the removal ability and EPS production by *Synechocystis* sp. BASO671. It was noted that the removal ability was direct linked to the exposure metal and EPS production increased when the metal concentration was changed from 15 ppm to 35 ppm, altering the EPS' monomers composition (OZTURK et al., 2014).

Thus, it is possible to observe the importance of process conditions and medium composition on EPS production, which can lead to higher yields and even tailor-made polymers. An approach to study all of these variables is the use of optimization tools in search of process increments.

2.2 Optimization of process conditions and medium composition

The biotechnological production of EPS is a promising alternative for the production of new and different biopolymers. Optimization procedures are indispensable for the achievement of maximum production, aiming to obtain the better medium, parameters and conditions of the process, especially when it comes to fermentations (SIRAJUNNISA et al., 2016). Thus, the optimization of process and medium conditions represents an alternative for faster production with higher yields. Furthermore, it may have the ability of improve EPS mass and consequently change its rheological properties (CHUG et al., 2016; LI; GUO; ZHU, 2016). Some of the statistical tools used for this purpose are One-Factor-at-a-Time, Response Surface Methodology (RSM), Orthogonal Matrix, Full Factorial design, Central Composite Design (CCD), among others.

One of the first works describing the optimization for EPS production was carried out by Lee *et al.* (1997), using the One-Factor-at-a-Time optimization approach. This work described the production of EPS by *Bacillus polymyxa* using different carbon and nitrogen sources, with satisfactory results occurring with sucrose and potassium nitrate, respectively. The production of EPS was dependent on the growth of the biocatalyst with highest production occurring between pH 7 and 8 reaching 54 g/L (LEE et al., 1997). Afterwards, the same

methodology was combined with factional factorial design for the production of EPS by *Paenibacillus polymyxa* SQR-21. The most favorable results were collected by using iron (242 µM), calcium (441 µM) and galactose (48.5 g/L) obtaining a maximum yield of 3.44 g/L of EPS (RAZA et al., 2011).

The production of pullulan, one of the main EPS currently used, by *Rhodotorula bacarum* was optimized using One-Factor-at-a-Time, altering the concentrations of glucose, soybean cake (nitrogen source), initial pH (varying from 4.0 to 7.0), temperature, and agitation. The optimal conditions were described as 8% glucose, 2% soybean cake, pH 7.0, 28°C and 180 rpm, given a yield of 5.9% (w/v), presenting great potential among the yeasts (CHI; ZHAO, 2003).

It is known that EPS can contribute to the texture and mouthfeel of various products, especially dairy products and some baked goods. For this reason, Kaditzky and Vogel (2008) studied the optimization of EPS production in fermented sourdough by *Lactobacillus reuteri* TMW 1.106 using One-Factor-at-a-Time methodology. They analyzed dough yield (DY), pH, sucrose content and fermentation substrate. It was possible to conclude that EPS production was higher at softer dough, with an optimum pH of 4.7, sucrose content up to 350 mM and when rye bran was used as substrate (KADITZKY; VOGEL, 2008).

In order to have the most favorable EPS production from *Armillaria mellea*, Lung and Huang (2010) used One-Factor-at-a-Time methodology with different types and concentrations of carbon (maltose, sucrose, xylose, corn powder, galactose, lactose and fructose at concentrations varying from 10 to 60 g/L), and nitrogen sources (peptone, soybean powder, malt extract, glycine, meat extract, and glutamine acid at concentrations, varying from 1 to 8 g/L), temperature within the range of 19° to 34°C, and pH varying from 3 to 8. The best results were obtained with a glucose concentration of 40 g/L, concentration of yeast extract at 3 g/L, KH_2PO_4 4 g/L and $MgSO_4$ 2 g/L at an optimal temperature of 22°C and pH 4.0 with an EPS yield of 0.27 g/L (LUNG; HUANG, 2010).

Razack *et al.* (2013) evaluated the EPS production by *Bacillus subtilis*, using synthetic sources and agro-industrial wastes (cane molasses and rice bran) through the One-Factor-at-a-Time methodology. The medium with sucrose as carbon source was composed of sucrose (20 g/L), yeast extract (5 g/L), NaCl (7 g/L), $CaCl_2$ (0.5 g/L), L-asparagine (0.05 g/L), and ascorbic acid (0.05 g/L), yielding 2.98 g/L of EPS. In the same conditions, when the sucrose was substituted by cane molasses and rice bran, it was obtained a maximum yield of 4.86 g/L and 2.14 g/L of EPS, respectively (RAZACK; VELAYUTHAM; THANGAVELU, 2013). The use of industrial residues as substrates is an interest, natural and low cost alternative for the

development of cost-effective process, reducing the cost of the fermentation medium (HABIBI; KHOSRAVI-DARANI, 2017).

Klebsiella sp. H-207, isolated from activated sludge, was applied for the optimization of fermentation medium using One-Factor-at-Time, for the determination of main factors, followed by RSM. The optimized medium was composed by 31.93 g/L of sucrose, 2.17 g/L of KNO_3 and 5.47 g/L of K_2HPO_4; obtaining 15.05 g/L of EPS with an incubation temperature of 28.9°C (QIANG et al., 2013).

EPS producer *Chlamydomonas reinhardtii* strain RAC was optimized by the classical One-Factor-at-a-Time approach and Plackett–Burman design, followed by RSM. After this sequential approach, the medium consisting of 74 mg/L of $CaCl_2$, 422 mg/L of $NaNO_3$, 10 mg/L of K_2HPO_4 and 200 mg/L of $MgSO_4$ with a pH 7.0, led to the maximum production of 0.63 g/L of EPS (BAFANA, 2013).

The EPS production by *Lactobacillus delbrueckii* subsp. *bulgaricus* was evaluated in a 2.5 liters fermenter using RSM. The experiments consisted at analyze temperature (35 to 45°C), pH (4 to 6), and bacto-casitone (nitrogen source) concentration (10 to 30 g/L), with the maximum EPS yield of 0.23 g/L under 38°C, pH of 5.0, and 30 g/L of bacto-casitone (KIMMEL; ROBERTS; ZIEGLER, 1998).

Using RSM, Duta *et al.* (2006) optimized the production of EPS by *Rhizobium* sp. in a 20-liters fermenter with fixed temperature and pH, at 30°C and 7.0 respectively. Calcium carbonate concentration, aeration, and agitation were assessed, and the ideal conditions were found to be 1.1 g/L, 1.3 vvm and 800 rpm, respectively, yielding 0.35 g/g of the EPS (DUTA; DE FRANCA; LOPES, 2006).

Using an Orthogonal Matrix method with three levels, four factors, and nine experiments, Lim *et al.* (2004) described the medium optimization for EPS production using *Collybia maculata*. Variations of carbon, nitrogen, and mineral sources were made into the culture media, leading to a maximum yield of 2.4 g/L of EPS, using 30 g/L of glucose, 20 g/L of Martone A-1, 1 g/L of K_2HPO_4 and 1 g/L of $CaCl_2$ (LIM et al., 2004). This research group also studied the production of EPS by *Agrocybe cylindracea* using the same optimization approach. The most promising culture media was composed of maltose 60 g/L, Martone A-1 6 g/L, $MgSO_4$ $7H_2O$ 0.9 g/L, and $CaCl_2$ 1.1 g/L, yielding 3.0 g/L of EPS (KIM et al., 2005).

EPS production by the medicinal mushroom *Fomes fomentarius,* isolated from the fruiting body of a wild *F. fomentarius*, was improved using Orthogonal Matrix method. In this experiment, 3.64 g/L of EPS was attained under the optimized conditions with 50 g/L glucose,

15 g/L silkworm chrysalis, 3 g/L yeast extract, 0.5 g/L $CaCl_2$, and 0.8 g/L $MgSO_4$ (CHEN et al., 2008).

Full Factorial design was applied for the optimization of the culture medium using *Coriolus (Trametes) versicolor*, evaluating glucose concentration (5, 15 and 25 g/L) and initial pH (4.0, 5.5, and 7.0). The other conditions were fixed at 28°C at 180 rpm for 9 days. The culture media containing 15 g/L of glucose and pH of 5.5 promoted the best production and productivity of EPS (0.64 g/L and 80 mg/(L.day)) and cell growth (4.2 g/L) (TAVARES et al., 2005).

Meng *et al.* (2010) used *Morchella esculenta* SO-02 in submerged culture to enhance EPS production aiming the antioxidant potential of these products. Medium optimization was made using bran as basic substrate with the addition of different carbon sources (fructose, glucose, lactose, sucrose, xylose) and nitrogen sources (peptone, yeast extract, NH_4NO_3, $(NH_4)2SO_4$, $NaNO_3$, NH_4Cl) initially selected using One-Factor-at-a-Time followed by a Five-Factor-Three-Level Orthogonal test. For the cultivation optimization, the best results of medium composition were submitted at different cultivation times, temperature, initial pH, rotary speed and volume of medium in 250-mL flasks. The best medium constituents were composed of 200 g/L of bran, 30 g/L of glucose and 1 g/L of yeast extract, while the optimum parameters for cultivation were achieved under 25°C of temperature, 100 mL of medium, 200 rpm and initial pH 6.5, within 4 days of cultivation. Combining these conditions, the EPS yield achieved was 2.9 ± 0.2 g/L (MENG et al., 2010a).

The endophytic bacterium *Paenibacillus polymyxa* EJS-3 was used for the EPS production using Factorial Fractional Design, for the selection of the most influential variables in the process (sucrose, yeast extract and $CaCl_2$) followed by a Central Composite Design (CCD), to optimize the process. The ideal composition for culture media was 188.2 g/L of sucrose, 25.8 g/L of yeast extract, 5 g/L of K_2HPO_4 and 0.34 g/L of $CaCl_2$, with a corresponding yield of 35.26 g/L (LIU et al., 2010).

Cui and Jia (2010) determined the influence of glucose and peptone on EPS production by *Cordyceps militaris* using a Plackett-Buman design. Factors were then optimized using CCD obtaining the most suitable composition of 48.67 g/L glucose, 12.56 g/L peptone, 1 g/L KH_2PO_4, 10 g/L yeast extract, and 0.5 g/L $MgSO_4 \cdot 7H_2O$. Optimization led to a 2.5-fold increase in yield when compared to basic medium, obtaining 1.96 g/L of EPS (CUI; JIA, 2010).

More recently, studying the fungal strain *Bionectria ochroleuca* M21, Li, Guo and ZHU (2016) optimized the culture medium using CCD for glucose (40 g/L), yeast extract (5 g/L), $MgSO_4$ (0,75 g/L) and Tween80 0,3% (v/v). The optimized medium composed of 55.7

g/L of glucose, 6.04 g/L of yeast extract, 0,25 g/L of $MgSO_4$ and 0,1% (v/v) of Tween80 was tested at a 5 liter bioreactor with best results achieved after 4 days of fermentation with a concentration of 2.65 g/L of EPS (LI; GUO; ZHU, 2016).

Padmanaban and coworkers (2015) studied the effect of different medium composition with the addition of sweet potato for the production of EPS by *Aureobasidium pullulans*. For that, four variables were evaluated with 12 experiments in a Plackett–Burman design: sweet potato (5 to 15%), yeast extract (0.5 to 1.0%), pH (3.5 to 7.5), and time of fermentation (60 to 140 hours). Maximum EPS production achieved was 9.3 g/L, obtained with 10% of sweet potato, 0.75% of yeast extract 0.75% and pH 5.5, after 100 hours of process. These results showed the potential use of sweet potato as a low-cost and effective substrate for the production of pullulan (PADMANABAN et al., 2015).

De Baets *et al.* (2002) were able to optimize the EPS production by *Tremella mesenterica* NRRL Y-6158 using fed-batch fermentation with controlled addition of the carbon source and comparing with shake flask fermentation. Process was conducted under 25°C of temperature, aeration of 1.6 L/min, agitation of 150 rpm and without controlled pH. It was observed that the fed-batch fermentation obtained a yield of 9.9 g/L, 2.2 times higher compared to shake flask fermentation, 4.5 g/L (DE BAETS et al., 2002).

Chung and coworkers (2016) studied the effects of pH, temperature and growth phase on the production of EPS by two bacteria: *Azotobacter beijreinckii* and *Bacillus subtilis* on nutrient broth medium. Both bacteria had optimum pH at 7, with maximum production of EPS within 24 hours and 30°C for *Azotobacter beijreinckii* (21.33 mg of dry mass), and 96 hours and 37°C for *Bacillus subtilis* (35.33 mg of dry mass). The EPS produced was further used at tests with chromium removal, using Fourier-transform infrared spectroscopy, with best results being found for *B. subtilis* achieving 48% of chromium removal (CHUG et al., 2016).

Table 1 presents in a comprehensive way the main examples of microorganisms, process and culture media optimized conditions and yields of EPS obtained.

Table 1 – Microorganisms, optimum conditions for EPS production and yields obtained

Microorganism	Optimum conditions for EPS production	Yield	Ref.
Bacteria			
Bacillus polymyxa	Sucrose and potassium nitrate as carbon and nitrogen sources, respectively. EPS production growth associated and the best pH was 7-8	54 g/L	(LEE et al., 1997)
Paenibacillus polymyxa SQR-21	Iron (242µM), calcium (441µM) and galactose (48.5 g/L)	3.44 g/L	(RAZA et al., 2011)
Paenibacillus polymyxa EJS-3	188.2 g/L of sucrose, 25.8 g/L of yeast extract, 5 g/L of K_2HPO_4 and 0.34 g/L of $CaCl_2$	35.26 g/L	(LIU et al., 2010)
Lactobacillus delbrueckii subsp. *bulgaricus*	38°C, pH of 5.0, and 30 g/L of bacto-casitone	2.25 g/L	(KIMMEL; ROBERTS; ZIEGLER, 1998)
Bacillus subtilis	Sucrose (20 g/L), yeast extract (5 g/L), NaCl (7 g/L), $CaCl_2$ (0.5 g/L), L-asparagine (0.05 g/L), and ascorbic acid (0.05 g/L)	Sucrose: 2.98 g/L. Cane molasses: 4.86 g/L, Rice bran : 2.14 g/L	(RAZACK; VELAYUTHAM; THANGAVELU, 2013)
Bacillus subtilis	Nutrient broth medium with pH of 7.0, 96 hours and 37°C	35.33 mg of dry mass	(CHUG et al., 2016)
Klebsiella sp.	31.93 g/L of sucrose, 2.17 g/L of KNO_3 and 5.47 g/L of K_2HPO_4 and 28,9°C	15.05 g/L	(QIANG et al., 2013)

Azotobacter beijreinckii	Nutrient broth medium with pH of 7.0, 24 hours and 30°C	21.33 mg of dry mass	(CHUG et al., 2016)
Fungi			
Tremella mesenterica NRRL Y-6158	25°C, aeration of 1.6 L/min, agitation at 150 rpm and without controlled pH.	Yield 2.2 times higher	(DE BAETS et al., 2002)
Rhodotorula bacarum	8% glucose, 2% soybean cake, pH 7.0, 28°C and 180 rpm	5,9% (w/v)	(CHI; ZHAO, 2003)
Collybia maculate	30 g/L of glucose, 20 g/L of Martone A-1, 1 g/L of K_2HPO_4, and 1 g/L of $CaCl_2$	2.4 g/L	(LIM et al., 2004)
Agrocybe cylindracea	Maltose 60g/L, Martone A-1 6 g/L, $MgSO_4$ $7H_2O$ 0.9g/L, and $CaCl_2$ 1.1g/L	3.0 g/L	(KIM et al., 2005)
Coriolus (Trametes) versicolor	Incubation for 9 days at 28°C at 180 rpm and media with 15 g/L of glucose and pH of 5.5	0.64 g/L	(TAVARES et al., 2005)
Rhizobium sp.	Temperature and pH constant (30°C and 7.0) with 1.1 g/L of calcium carbonate, aeration of 1.3 vvm and 800 rpm	0.35 g/g	(DUTA; DE FRANCA; LOPES, 2006)
Fomes fomentarius	50 g/L glucose, 15 g/L silkworm chrysalis, 3 g/L yeast extract, 0.5 g/L $CaCl_2$, and 0.8 g/L $MgSO_4$	3.64 g/L	(CHEN et al., 2008)
Morchella esculenta SO-02	Bran 200 g/L, glucose 30 g/L, yeast extract 1 g/L, and the optimum parameters for cultivation were: temperature 25°C, cultivation time 4 days, volume of medium 100 ml, rotary speed 200 rpm and initial pH 6.5	2.9 ± 0.2 g/L	(MENG et al., 2010a)

Armillaria mellea	Glucose 40 g/L, yeast extract 3 g/L, KH_2PO_4 4 g/L and $MgSO_4$ 2 g/L at an optimal temperature of 22°C and pH 4.0	0.27 g/L	(LUNG; HUANG, 2010)
Aureobasidium pullulans	Sweet potato 10%, yeast extract 0.75%, pH of 5.5, and 100 hours	9.3 g/L	(PADMANABAN et al., 2015)
Cordyceps militaris	48.67 g/L glucose, 12.56 g/L peptone, 1 g/L KH_2PO_4, 10 g/L yeast extract, and 0.5 g/L $MgSO_4 \cdot 7H_2O$	1.96 g/L	(CUI; JIA, 2010)
Bionectria ochroleuca M21	4 days of fermentation in a 5 liter bioreactor, using 55.7 g/L of glucose, 6.04 g/L of yeast extract, 0,25 g/L of $MgSO_4$ and 0,1% (v/v) of Tween80	2.65 g/L	(LI; GUO; ZHU, 2016)
Algae			
Chlamydomonas reinhardtii	74 mg/L of $CaCl_2$, 422 mg/L of $NaNO_3$, 10 mg/L of K_2HPO_4 and 200 mg/L of $MgSO_4$ with a pH 7.0	0.63 g/L	(BAFANA, 2013)

2.3 Engineered exopolysaccharide production

The use of genetic engineering for the biotechnological production of microbial polysaccharides is a promising alternative and an ongoing process focused on improve the commercial scale production and increase the possibilities of applications in several fields . The most viable genetic tools to achieve these goals are the transfer of the genetic determinants of microbial polysaccharides to more efficient hosts, enhancing the production, or the change of the repeat unit of the polysaccharide (FREITAS; ALVES; REIS, 2014; VAN KRANENBURG et al., 1999).

Attractive options such as increase the number of glycosyltransferases, the enzyme responsible for the synthesis of the repeated unit presented at the polysaccharide; introducing new or exchanging the existing enzymes in the EPS microorganism-producer; and the use of different hosts, can lead to a reduction at production costs, increasing production yields, and allow the use of cheaper substrates or other cost-effective fermentation conditions (VAN KRANENBURG et al., 1999).

Stingele and coworkers (1999) employed *Streptococcus thermophilus* Sfi6, a specie capable of producing EPS composed of glucose, galactose and N-acetylgalactosamine, isolated and transferred its gene cluster to *Lactococcus lactis* MG1363, a non-producer EPS host. The authors were able to produce a new EPS with high molecular weight and different structure. This indicates that glycosyltransferases can have multiple specificities for the donor and acceptor, and the polymerase is able to polymerize the repeated unit in different ways (STINGELE et al., 1999).

A homologous overexpression of the gene *gal*U, which code for enzymes in the central metabolism, was made by *Streptococcus thermophilus* LY03 leading to an increase in EPS yield when used in combination with *pgmA*, showing that EPS production can be improved by altering the level of certain enzymes (LEVANDER; SVENSSON, 2002).

In another studied, EPS production was increased by elevating the expression of the gene cluster pNZ4000 in *Lactococcus lactis* NIZO B40, a possible strain for polysaccharide production. This finding suggests that the EPS production is limited by the activity level of the expression of the pNZ4000 gene cluster rather than by the level of EPS precursors (BOELS et al., 2003).

The use of genetic and metabolic engineering for production of EPS is still little used due to the lack of genetic tools (SHIN et al., 2016). The development and obtainment of

engineered tailored polymers can lead to higher performance products, opening their potential application in several industrial segments (SCHMID; SIEBER; REHM, 2015).

3 EXTRACTION AND PURIFICATION METHODS

Extraction methods of EPS can be divided into chemical and physical methods. Chemical methods include the use of alkaline, ethylenediamine tetraacetic acid (EDTA) and cation exchange resin, which can contaminate the extracted products (COMTE; GUIBAUD; BAUDU, 2006). Meanwhile, physical methods include centrifugation, ultrasonication, and heating, which normally present lower efficiencies when compared to chemical methods (LIANG et al., 2010; LIU; FANG, 2002).

A large number of methods for the extraction of microbial EPS has been developed and optimized in the last years. Freitas, Alves and Reis (2011) described three fundamental steps, which include chemical and physical methods, for extraction from culture broth: cell removal, polymer precipitation, and drying. The cell removal is normally obtained using centrifugation, with speed and time depending on the polysaccharide, while the precipitation is done using a precipitating agent which is a water miscible solvent (*e.g*. methanol, ethanol, isopropanol, or acetone). When the polysaccharide is thermally stable, heat can be applied for the destruction of enzymes that can degrade the EPS (FREITAS; ALVES; REIS, 2011; SINGHA, 2012).

When a high level of purity is desirable, the polysaccharide can be submitted to one or more additional methods, such as re-precipitation, deproteinization by physical or chemical methods, and membrane processes combined (ROCA et al., 2015). In this way, the best extraction method must be a procedure that causes minimum cell lysis, does not disrupt the exopolysaccharide (GEHR; HENRY, 1983), and avoids a large amount of DNA and protein released with the EPS (LIU; FANG, 2002). Zhang and coworkers (2014) tested the selectivity and influence of process parameters to release polysaccharides and proteins from yeast cells using ultrasound. It was observed that selected-release is possible by only manipulating the sonication temperature, in which high temperatures (65 to 85°C) favors the release of polysaccharides. In general, elevated temperatures can lead to denaturation and coagulation of proteins (ZHANG et al., 2014).

Some studies have compared the different chemical and physical extraction methods. One of the first was made using aerobic, acidogenic, and methanogenic sludges and different conditions of extraction: only centrifugation, extraction with different chemicals (EDTA 2%; at 4°C for 3h; cation exchange resin Dowex 50×8, Fluka, USA; at 4°C for 1 h;

formaldehyde at 4 °C for 1 hour, formaldehyde plus NaOH 1 N; at 4 °C for 3 h; and formaldehyde plus ultrasonication 60 W for 2.5 min) followed by centrifugation. In these sludges, formaldehyde and NaOH were the most effective for the extraction, although they have guaranteed the extraction of a limited portion of the EPS (LIU; FANG, 2002).

Liang *et al.* (2010) compared four extraction methods divided in 3 chemical (EDTA, NaOH, cationic exchange resin) and one physical (ultrasound), also on sludges. Authors observed that EDTA and ultrasound were more effective for EPS extraction since they led to a higher activity of glucose-6-phosphate dehydrogenase from cell lysis. In this study, the presence of DNA and glucose-6-phosphate dehydrogenase were used to determine the degree of rupture of the cells (LIANG et al., 2010).

Another work compared six different methods, including ultrasonication, heating, formaldehyde + NaOH, H_2SO_4, glutaraldehyde, and EDTA, for the extraction of EPS from *Bacillus megaterium* TF10, isolated from a soil sample with high EPS-producing capacity. The methods were compared based on EPS yields and compositions, cell lysis, flocculation activities, and spectrum characteristics of extracted EPS, which led to the follow results: heating, formaldehyde + NaOH and H_2SO_4 led to a high EPS yield compared to ultrasonication or EDTA, while the ultrasonication and H_2SO_4 caused much more cell lysis than the formaldehyde + NaOH. They also concluded that the extraction method could affect the structure and composition of the EPS (SUN et al., 2012).

Meng and coworkers (2010) optimized the extraction of EPS from *Morchella esculenta* SO-01 and analyzed by Plackett–Burman (PB) experimental factors that affected the extraction (concentration temperature, varying from 70 to 90°C; precipitation time, from 12 to 24 hours; and pH, from 7.0 to 9.0), and to optimize the extraction conditions using RSM. The best extraction conditions found were at 84.07°C, precipitation with 3 volumes of cold (4°C) ethanol for 22.19 h and pH 8.44, yielding 5.45 g/L of EPS (MENG et al., 2010b).

The extraction procedure of EPS is not simple and cannot guarantee the extraction of all the microbial polysaccharide produced. Thus, the optimization of a method is required for each case, having to take into account the characteristics of the EPS and final aim of the extraction (qualitatively or quantitatively) (DONOT et al., 2012).

Additionally, a purification step is needed after the extraction, since residues of DNA, proteins, and even chemicals may be present. The polymer can be subjected to re-precipitation from an aqueous solution (<1.0 g/L); chemical deproteinization (*e.g.* salting out or protein precipitation with trichloroacetic acid, or enzymatic methods (*e.g.* proteases)); and membrane processes (*e.g.* ultrafiltration and diafiltration) (AYALA-HERNÁNDEZ et al.,

2008; BAHL et al., 2010; FREITAS et al., 2011; KUMAR; MODY; JHA, 2007; WANG et al., 2007).

Kanmani and coworkers (2011) described the use of gel filtration for purification of the EPS produced by *Streptococcus phocae* through a phenyl Sepharose column with the sample eluted by phosphate buffer with 2 mL/min of flow rate (KANMANI et al., 2011). Meanwhile, Kumar *et al.* (2004) showed the purification of EPS produced by *Bacillus* sp. I-450 by treating with cetylpyridinium chloride (CPC), which precipitate the anionic cell wall of the polymer (KUMAR et al., 2004).

For the suitable choice of EPS purification method, it is necessary to analyze its impact on the polymer properties, product recovery, and purity since some methods can decrease the product recovery or have negative impact on its properties (FREITAS; ALVES; REIS, 2011). It is noteworthy that the development of processes which integrated production and recovery, with higher EPS yield and quality are suitable choices for reduction of costs (ANTUNES et al., 2017).

4 APPLICATIONS OF MICROBIAL EXOPOLYSACCHARIDES

Considering the great potential of microbial EPS, their application has very broad range from the food industry to medical applications, mainly since EPS can exhibit a wide variability in molecular structure, resulting in a wide range of properties and potential applications (FREITAS; ALVES; REIS, 2014; SINGHA, 2012).

One of the oldest applications of microbial polysaccharides, especially EPS, is the change or improvement of rheological properties within the food industry (NWODO; GREEN; OKOH, 2012) by chemical or enzymatic means (SINGHA, 2012). The microbial polysaccharides can be used as a viscosifying agent, stabilizers, emulsifiers, gelling agents, or water-binding agents (DONOT et al., 2012), mainly used in dairy industries (DUBOC; MOLLET, 2001; MADHURI; VIDYA PRABHAKAR, 2014). The main example is gellan gum, considered the most used and known EPS in the food industry. This gum has been able to provide adequate controlled flavor release in a wide range of pH, while also improving texture and physical stability of food products (BANIK; KANARI; UPADHYAY, 2000).

An important role at medical industry is also observed. Microbial EPS are becoming promising material for drug release systems, due to the ability of retain a large amount of water and still remaining insoluble, and drug-targeting carriers, based on the particular binding and penetrating features to cellular receptors (MOSCOVICI, 2015).

Another highly relevant property of microbial EPS is the ability to create films, also called polysaccharide-based membranes. Within this classification, some of the most used polysaccharides are pullulan, gellan gum, levan, curdlan, hyaluronan, bacterial alginates and bacterial cellulose (FREITAS; ALVES; REIS, 2014).The main uses of this type of microbial polysaccharides include:

i. Medical and pharmaceutical biomaterials for tissue regeneration, drug delivery agents, adhesives, surgical sealants, and coating of medical devices, specially pullulan (CHENG; DEMIRCI; CATCHMARK, 2011; COSTA et al., 2013; MISHRA; VUPPU; RATH, 2011);
ii. Food applications, as edible and/or biodegradable membranes. Polysaccharides such as gellan, pullalan, xanthan, curdlan, and Galactose-Pol have been already applied as films with effective gas barrier properties (ALVES et al., 2011; FLORES et al., 2010; SHIH; DAIGLE; CHAMPAGNE, 2011; TROVATTI et al., 2012a, 2012b; YANG; PAULSON; NICKERSON, 2010; YANG; PAULSON, 2000); and
iii. Solvent dehydration and wastewater treatment, due to the hydrophilic character and charge, with a high adsorption capacity for dyes, heavy metal ions and aromatic compounds (FREITAS; ALVES; REIS, 2014).

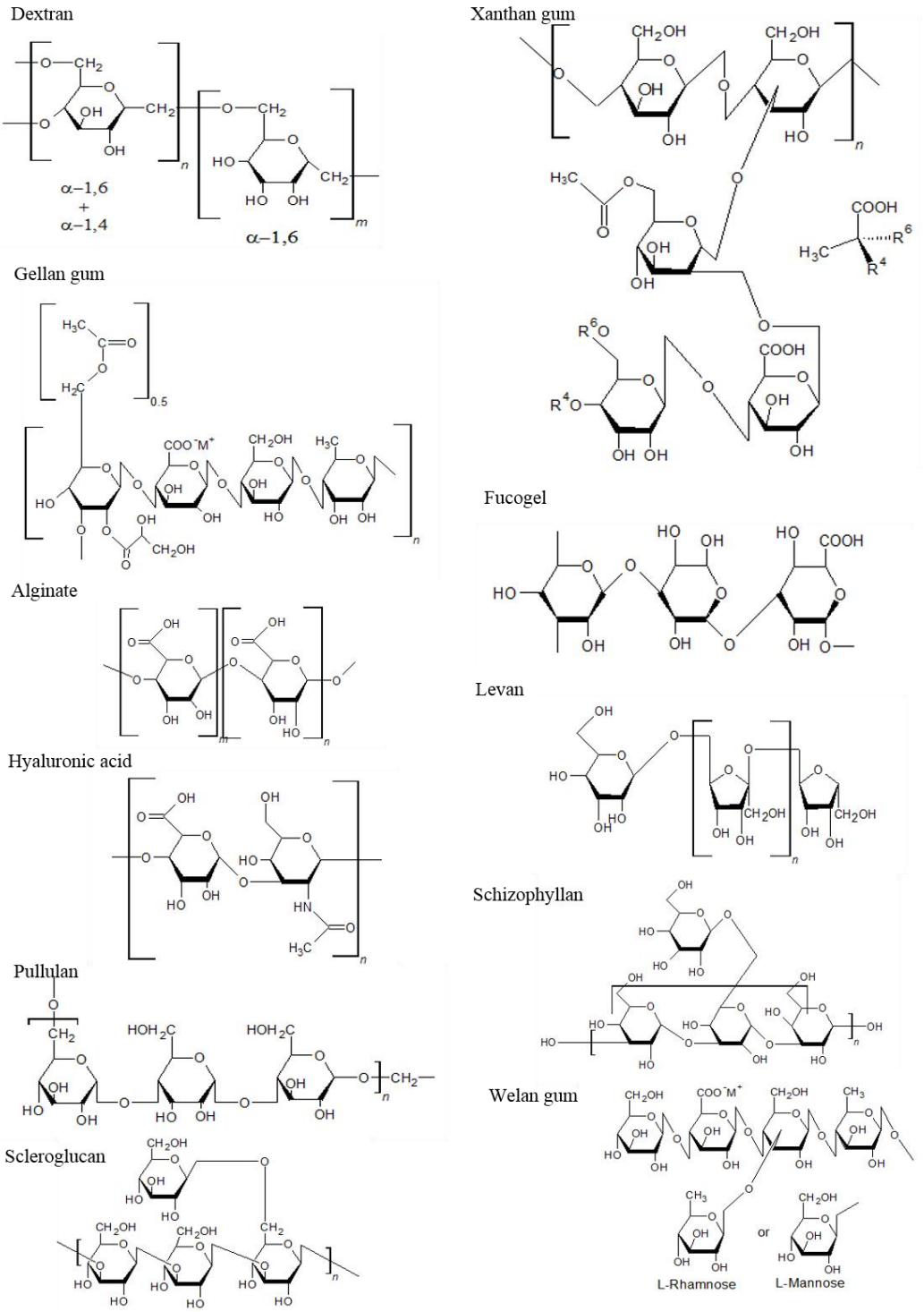
Polysaccharides obtained from fungal strains (*e.g.* lentinan, schizophyllan and glucan) are most known for their medical properties, being often described as 'Biological Response Modifiers'. These compounds are able to trigger a non-specific reaction against tumor cells, viral and bacterial infections, and inflammation (GIAVASIS, 2014). Limin and coworkers (2016) proved the antioxidant activity of EPS and IPS from *Fomitopsis pinicola* with strong scavenging abilities of DPPH, hydroxyl radical and protective effects on yeast cells by UV and H_2O_2 induced oxidative damage (LIMIN et al., 2016). The EPS and IPS from *Cordyceps gracilis* also had their antioxidant activity studied, proving a significant DPPH and ABTS radical scavenging activities, iron chelating activity and reducing power (SHARMA; NANDINI; ATRI, 2015). Also, the EPS and IPS of *Ganoderma neojaponicum* showed potential to be used as immunomodulating agents stimulating the immune system to fight infectious diseases (UBAIDILLAH; ABDULLAH; SABARATNAMA, 2015).

EPS obtained from lactic acid bacteria are widely used in the food industry to improve the rheological properties of several fermented dairy products in terms of stability, mouthfeel, firmess and texture. Some studies have also shown prebiotic, antigastritis, antiulcer and cholesterol lowering effects (JAISWAL et al., 2014; PATEL; PRAJAPAT, 2013).

5 MAIN EXOPOLYSACCHARIDES OF INDUSTRIAL INTEREST

As shown, EPS are molecules of great interest, both at laboratory and industrial scale. Despite a lot of the laboratory research, some polymers are already recognized by the industry and widely applied, as can be seen below and at Figure 1.

Figure 1 – Main exopolysaccharides of industrial interest



5.1 Dextran

Dextran is a homopolysaccharide of glucose with α-(1-6)-linkages and molecular weight varying from 10 to 2000 kDa. This compound can be synthesized by *Leuconostoc*, *Streptococcus, Weissella* and *Lactobacillus* species, outside the microbial cell from sucrose by dextransucrase enzymes, with optimum conditions of temperature between 25° and 30°C, and pH of 6 – 6.9. The commercial dextran are produced by *L. mesenteroides* and *L. dextranicum* (AHMED et al., 2012; ANDHARE et al., 2014; VU et al., 2009).

The dextransucrase uses two pathways for the production of dextran: (i) hydrolyzing the sucrose and binding the glycosyl moiety; (ii) building up the dextran by an insertion mechanism (MOOSAVI-NASAB; ALAHDAD; NAZEMI, 2009). The dextran size and structure are synthesized based on the dextransucrase produced by the microbial strain, which justifies the differences in size and molecular weight of the various dextran (PURAMA et al., 2009).

Dextran, can be considered the most important polysaccharide obtained by fermentation, first reported in 1874, starting the study and development of microbial exopolysaccharides in industrial scale (BARBOSA et al., 2004).

Dextran presents many industrial uses due to its good stability and non-ionic character, being used, mostly, as an indirect food additive, as a stabilizer and viscosity agent. Additionally, this polysaccharide can be also applied in the medical and pharmaceutical industries, being used as a plasm expander and as a blood flow adjuvant (BILIADERIS; IZYDORCZYK, 2007). Derivatives of dextran such as Sephadex®, a crossed linked dextran gel launched by Pharmacia in 1959, are used in gel filtration for separation and purification of products such as proteins (MOOSAVI-NASAB; ALAHDAD; NAZEMI, 2009; SUTHERLAND, 1998). The potential of the dextran produced by *Lactobacillus sakei* MN1 as an antiviral agent and as a probiotic in aquaculture was recently reported (NÁCHER-VÁZQUEZ et al., 2015, 2017).

Low-cost substrates for the production of dextran are reported, including molasses and cheese whey. The influence of such substrates, in the isolated form or in mixtures, were studied for the polysaccharide. The highest yield of dextran was obtained when a mixture of molasses and 10% cheese whey was used, and the lowest using a 2% mixture. No dextran produced at only whey medium (MOOSAVI-NASAB et al., 2010a).

Nowadays, companies that produce and commercialize dextran are: Pharmacosmos, Sigma-Aldrich, Pharmachem Corporation and Amersham Biosciences

(OLIVEIRA, 2013). Among those, Pharmacosmos is the number one company for the production and distribution of dextran for pharmaceutical uses, being an expert at fractionation techniques, possessing a great number of different dextran in their catalog (PHARMACOSMOS, 2017).

5.2 Xanthan gum

The xanthan gum is a heteropolysaccharide produced by the pathogenic, gram-negative bacteria *Xanthomonas* sp., mainly by *Xanthomonas campestris, Xanthomonas pelargonii, Xanthomonas phaseoli* and *Xanthomonas malvacearum*. This polymer is composed of repeated units of *D*-glucose, *D*-mannose, *D*-glucoronic acid, acetal linked pyruvic acid, and *D*-acetyl groups with high molecular weight, varying from $2x10^6$ and $20x10^6$ Da (HABIBI; KHOSRAVI-DARANI, 2017; RUAS-MADIEDO; HUGENHOLTZ; ZOON, 2002; SCHMID; SIEBER; REHM, 2015).

Discovered in 1950, its commercialization started in 1960 by Kelco, being the first natural polymer produced in industrial scale. The consumption of this polysaccharide is estimated at US$23 million per year, with an annual worldwide growth of 6 – 7%. This is one of the most expensive microbial polysaccharides due to the use of only glucose and sucrose (expensive substrates costing US$4,000 – 5,000 per ton) as carbon sources, and the cost of the downstream process (approximately 50% of the final cost), since a high purity level is required when it is used in the food industry (LI et al., 2016; SUBHASH; JADHAV; JANA, 2015).

The production of xanthan gum is a non-continuous process operating at pH 7.0, 28°C and high agitation rates (400 to 800 rpm) (FREITAS; ALVES; REIS, 2011; HABIBI; KHOSRAVI-DARANI, 2017; SUBHASH; JADHAV; JANA, 2015). When a high average molecular mass is desirable, the fermentation must occur at temperatures below 25°C, in which the use of surfactants, such as Tween-40 and Tween-80, can improve the production (HABIBI; KHOSRAVI-DARANI, 2017; KUMAR; MODY; JHA, 2007). Sucrose and glucose are the main carbon sources used at the industrial production, being the production enhanced by the carbon concentration but negatively affected by nitrogen concentration enhancement. Nitrogen is only needed for the growth of the bacteria, and it is not necessary for the production of xanthan gum, which occurs during the stationary phase. In this way, a two-step fermentation is proposed, firstly conducted with a low C/N ratio (for the bacteria growth), followed by a high C/N ratio (for the xanthan gum production) (BUENO; GARCIA-CRUZ, 2006; HABIBI; KHOSRAVI-DARANI, 2017).

The downstream process of xanthan gum production can be made with precipitation and recovery using acetone or alcohols. Heat treatment is necessary due to the pathological nature of the bacteria, and when heat is applied, an increase in the viscosity of the product is observed, despite being pH dependent (KUMAR; MODY; JHA, 2007; RÜHMANN; SCHMID; SIEBER, 2015b).

The main properties of xanthan gum are the high viscosity (even at low concentrations), water solubility, good stability at ample pH range (1 to 12), and resistance to degradation at high temperatures (HABIBI; KHOSRAVI-DARANI, 2017; PATEL; PRAJAPAT, 2013). Due to these particular characteristics, this gum can be widely used as a stabilizing, thickening and suspending agent (VELU; VELAYUTHAM; MANICKKAM, 2016) at the food, pharmaceutical and cosmetic industries (BILIADERIS; IZYDORCZYK, 2007). It can also be used for enhanced oil recovery (GAO, 2016; JANG et al., 2015), as fat replacer (RATHER et al., 2015), mucoadhesive polymer (BHATIA; AHUJA; MEHTA, 2015), treating osteoarthritis (HAN et al., 2017), soil strengthening (CHANG et al., 2015), and controlled drug release carrier (BENNY; GUNASEKAR; PONNUSAMI, 2014; MOSCOVICI, 2015). Its use with other polymers, *e.g.* starch, chitosan and zein protein, is reported for the development of biodegradable films with potential use in the food and pharmaceutical industries (FREITAS; ALVES; REIS, 2014; LIMA et al., 2017).

The biotechnological production of xanthan gum can still be extensively explored and developed from the use of new producer strains, with high efficiency, and the use of cheap substrates. Some of the alternative substrates reported include the use of kitchen waste (LI et al., 2016), milk whey (NITSCHKE; RODRIGUES; SCHINATTO, 2001), cheese whey (NIKNEZHAD et al., 2015), sugar cane broth (FARIA et al., 2011), glycerol (WANG et al., 2017), green coconut shells (NERY; CRUZ; DRUZIAN, 2013), rice straw (JAZINI; FEREYDOUNI; KARIMI, 2017), and sugar cane molasses (ZAKERI; PAZOUKI; VOSSOUGI, 2015), for the low cost production of xanthan gum.

In the recent years, the biggest companies which commercialized xanthan gum are Merck and Pfizer (USA), Rhone Poulenc, Mero Rousselot-Santia and Sanofi – EIF (France), Saidy Chemica (China), and Jungbunzlauer (Austria) (HABIBI; KHOSRAVI-DARANI, 2017).

5.3 Gellan gum

The gellan gum was discovered in 1978 and had its first use in food products ten years later, in Japan. Only in 1990, the FDA approved its use in the USA and Europe as a

stabilizer and thickener, being patented and first commercialized by Kelco. This gum is an anionic linear heteropolysaccharide with repeating units of α-rhamnose, two residues of β-*D*-glucose and β-*D*-glucuronate possessing high viscosity and thermal stability (BANIK; KANARI; UPADHYAY, 2000; GIDLEY; REID, 2006; MOSCOVICI, 2015).

Gellan belongs to the sphingan family (SCHMID; SIEBER; REHM, 2015), and it is mainly produced by aerobic submerged fermentation by *Sphingmonas paucimobilis*, formerly known as *Pseudomonas elodea*, a gram-negative bacterium, in batch culture (GIDLEY; REID, 2006).

The fermentation process is conducted at 30°C with a pH range of 6.0 – 7.0, during 30 to 60 hours with agitation of 250 rpm, and precipitation made using alcohol, in which the extraction conditions can be varied to obtain a range of gellan gums with different degrees of esterification. Gellan gum production is direct related to microbial growth, which means that factors affecting negatively microbial growth can reduce its production. It is known that the use of yeast extract as nitrogen source and sucrose as carbon source gives the maximum production of gellan gum (PRAJAPATI et al., 2013; ZHANG et al., 2015).

Other properties and uses for gellan gum include film formation with mechanical and water vapor barrier properties (YANG; PAULSON; NICKERSON, 2010; YANG; PAULSON, 2000) *e.g.* application in paper cups for hot drinks (ZHANG et al., 2017) and the combined use with purple sweet potato for pH monitoring (WEI et al., 2017); vehicle for ophthalmic drugs and drug release (D'ARRIGO et al., 2014; MAHDI; CONWAY; SMITH, 2014, 2015; OSMAŁEK; FROELICH; TASAREK, 2014; PREZOTTI; CURY; EVANGELISTA, 2014); gelling agent in dental and personal care (BANIK; KANARI; UPADHYAY, 2000); wound healing and tissue engineering (BECKER, 2015; BONIFACIO et al., 2017; DOUGLAS et al., 2016; HADJIZADEH; DOILLON, 2010); inhibition of acid corrosion of iron cast (RAJESWARI et al., 2013); and the use of gellan hydrogel for paper cleaning (MAZZUCA et al., 2014).

The gellan gum is one of the most used exopolysaccharides due to its ability to form a transparent gel in the presence of multivalent cations, with good thermal and acid stability, being also thermo-reversible. Some commercial gellan products include Gelrite® and Kelcogel® being a potential replacement for agar and gelatin (BANIK; KANARI; UPADHYAY, 2000; MADHURI; VIDYA PRABHAKAR, 2014; PRAJAPATI et al., 2013).

5.4 Alginates

The alginates represent a family of polysaccharides composed of mannuric acid and guluronic acid firstly extracted from brown algae being patented at Oxford in 1881. These are linear polysaccharides with units of β-D-mannuronic acid and α-L-guluronic acid linked by 1-4 bonds (ERTESVÅG; VALLA, 1998; GACESA, 1988). Normally, they are obtained from marine brown algae but can also be produced by soil bacteria such as *Azotobacter* and *Pseudomonas*, being quite abundant in nature (GIDLEY; REID, 2006; GOH; HENG; CHAN, 2012).

The production of alginates is estimated in 30,000 tons per year (GIDLEY; REID, 2006), with most commercial available alginates from brown algae, due to the economic unfeasibility of those produced by bacteria for commercial applications, being still confined to small scale studies (GOH; HENG; CHAN, 2012) using bacteria such as *Pseudomonas aeruginosa* (SCHÜRKS et al., 2002; STAPPER et al., 2004), *Pseudomonas putida* (CHANG et al., 2007) and *Pseudomonas syringae* (LAUE et al., 2006).

The alginates have an important role at the food, pharmaceutical, textile, and paper industries, being used as stabilizers and thickeners for foods and for color pigments, adhesive agents and fillers in the paper industry, and encapsulation material in the pharmaceutical industry (GIDLEY; REID, 2006; GOH; HENG; CHAN, 2012). The biomedical applications study their use for therapeutical cell entrapment and immunologic properties (DRAGET; TAYLOR, 2011). All these uses are based on the physiochemical properties of the alginates, such as high viscosity, sol-gel transformation, thermo-stability and potential for controlled drug release (GOH; HENG; CHAN, 2012).

5.5 Fucose containing EPS

Fucose is a rare sugar, not commonly found in nature, presenting interesting physical and bioactive properties, being already used in the pharmaceutical, cosmetics and food industry. The potential applications of these polysaccharides include its use in anticarcinogenic and anti-inflammatory drugs, justifying the interest in EPS containing fucose, which includes Fucogel, Clavan and FucoPol (ROCA et al., 2015).

Fucogel is a linear anionic polymer commercialized by Solabia (France) and mainly used in the formulation of skin care cosmetics due to its moisturizing properties and action as an anti-aging agent. This polymer can be produced by *Klebsiella pneumoniae* with repeating units of galacturonic acid, *L*-fucose, and *D*-galactose (ROBERT; ROBERT; ROBERT, 2003; ROBERT et al., 2004; ROCA et al., 2015).

Clavan, produced by *Clavibacter* strains, especially *C.michiganensis*, is composed of units of *L*-fucose, *D*-glucose, and *D*-galactose, being able to form high viscosity solutions (VANHOOREN; VANDAMME, 2000).

FucoPol is synthesized by *Enterobacter* A47 presenting high molecular weight, composed mainly by fucose (32–36 mol%), galactose (25–26 mol%), glucose (28–37 mol%) and glucuronic acid (9–10 mol%) (TORRES et al., 2015). The film-form ability of FucoPol makes possible its use as an inner layer at multilayer packaging material, featuring ductile mechanical properties, hydrophilic character and good barrier properties to gases when low water content is used (FERREIRA et al., 2014). These properties were enhanced using chitosan, which provides the use for a broader ranging of food (FERREIRA et al., 2016). Other uses of FucoPol are based on its adhesive properties (ARAÚJO et al., 2016), emulsion stabilizing and flocculating capacities (ROCA et al., 2015). Recently, the production of FucoPol using tomato-paste by-product was presented, showing the possibility of using low-cost substrates to produce this high-value product (ANTUNES et al., 2017).

5.6 Hyaluronic acid or hyaluronan

The hyaluronic acid (HA), also called hyaluronan, is a linear heteropolysaccharide, extremely hydrophilic with units of β-D-glucuronic acid and β-D-N-acetyl-glucosamine residues linked via β-(1-4) and β-(1-3)-glycosidic bonds produced by *Streptococcus* strains, belonging to group C. The main properties of these polysaccharides are due to their ability of high water retention, biocompatibility, and viscous behavior, being used mostly in the medical and cosmetics industries (RUFFING; CHEN, 2006; SCHMID; SIEBER; REHM, 2015; SUTHERLAND, 1998).

The HA was discovered in 1934 and first obtained from animal tissues. Nowadays, the production is strictly from recombinant bacteria (MOSCOVICI, 2015). The use of HA at high-priced segments, with a world market of US$500 million and a selling price of US$100,000.00 per kg, motivates the use of metabolic engineering approaches, which leads to the enhanced HA synthesis (SARANRAJ; NAIDU, 2013; SCHMID; SIEBER; REHM, 2015).

For most of the biomedical uses, HA is processed in the form of hydrogels being increasingly versatile, as they can be used as cell-based and regenerative therapies (HIGHLEY; PRESTWICH; BURDICK, 2016); tissue-engineering cartilage constructor (COLLINS; BIRKINSHAW, 2013; LEVETT et al., 2014); and drug delivery (DOSIO et al., 2016; MERO; CAMPISI, 2014). Other uses for HA are: wound healing; osteoarthritis treatment (intra-articular injection) (AHRQ, 2015; FAKHARI; BERKLAND, 2013); and eye surgery

(commercial names: Healon® from Abbott, and Hylan GF-20® from Genzyme) (LIANG; JIANG; NOBLE, 2016; MOSCOVICI, 2015).

5.7 Levan

Levan is a high molecular mass homopolysaccharide synthesized from sucrose by levansucrose outside the cell (similar mechanism to dextran), formed by groups of β-(2−6)-D-fructans with some β-(2−1)-branches. This compound is produced mainly by bacteria such as *Bacillus, Zymomonas, Halomonas, Pseudomonas, Rahnella, Aerobacter, Erwinia, Streptococcus, Microbacterium*, but also by some yeasts and fungal strains (ANDHARE et al., 2014; BILIADERIS; IZYDORCZYK, 2007; KUMAR; MODY; JHA, 2007; LAUE et al., 2006; MOSCOVICI, 2015; POLI et al., 2009; SUTHERLAND, 2001). Is noteworthy that levan produced by different organisms have different molecular weight and degree of branching which impacts on their possible applications (ABDEL-FATTAH et al., 2012).

Some studies reported the use of levansucrose by different microorganisms for the efficient biosynthesis of levan, *e.g. Brenneria goodwinii* (LIU et al., 2017a), *Pseudomonas syringae* (BONDARENKO et al., 2015), *Bacillus subtilis* (ESAWY et al., 2013). The enzyme can be used in crude, recombinant, purified or immobilized forms and achieved satisfactory yields which showed potential for industrial use (ESAWY et al., 2013; LIU et al., 2017a). The use of immobilized cells can also offer advantages for industrial production, facilitating the product isolation and biocatalyst reuse (SHIH; CHEN; WU, 2010).

Some of the main properties of levan include neutral charge, low viscosity, high water solubility but non-swelling, biological activity: anti-tumor, antioxidant and anti-inflammatory and cholesterol lowering effect (ABDEL-FATTAH et al., 2012; MOOSAVI-NASAB et al., 2010b), adhesive strength, film-forming capacity, and it is also used as thickener and binder in pet feed (ANDHARE et al., 2014; FREITAS; ALVES; REIS, 2014; NEDRA et al., 2012). In the food industry, levan may have potential application as prebiotic, since upon acid hydrolysis is converted into small fructo-oligosaccharides (FOS) (SRIKANTH et al., 2015). Levan from *Lactobacillus sanfranciscensis* had also demonstrated its influence on bread texture and volume (KADITZKY; VOGEL, 2008).

Low-cost substrates are being tested for the production of levan. One example includes the use of molasses and sugar cane syrup by the bacteria *Zymomonas mobilis*. The product obtained was 2.53 g/L and 15.46 g/L with molasses and sugar cane syrup, respectively, and although these values are still lower than the production using commercial sucrose (21.69 g/L), there is a great potential in the use of alternative substrates considering the cost and

sustainability aspects (DE OLIVEIRA et al., 2007). Date syrup was also tested with *Microbacterium laevaniformans* obtaining a yield of 10.48 g/L (MOOSAVI-NASAB et al., 2010b).

Although levan is a remarkable polysaccharide for the industry, the scale up process presents technical issues due to the difficulty to handle large amounts of alcohol (used for precipitation of the polymer), unavailability of ultrafiltration equipment for multi-ton operations, impracticality of dialysis and the necessity of enzyme inhibition at the end of the fermentation (ONER; HERNÁNDEZ; COMBIE, 2016).

Nowadays, commercialization of levan is made by several companies with Rahn® selling the most finished products (Proteolea® and Slimexir®) containing this polysaccharide. Natural Polymers Inc., Bainbridge, GA, USA using *Bacillus subtilis*, Real Biotech Co. (Chungnam, Korea), using *Zymomonasmobilis* and Advance Co. (Tokyo, Japan), using *Streptococcus salivarius*, are the main companies that produce and commercialize levan (ONER; HERNÁNDEZ; COMBIE, 2016).

5.8 Pullulan

The pullulan is a linear homopolysaccharide consisting of maltotriose units connected by α-(1-4) glycoside bonds whereas consecutive maltotrioses are connected by α-(1-6) glycoside bonds. This polysaccharide is produced industrially by the yeast-like fungi *Aureobasidium pullulans* during late exponential and early stationary phase with average molecular weight of 362–480 kDa (MISHRA; VUPPU; RATH, 2011; SINGH; SAINI; KENNEDY, 2008; WELMAN; MADDOX, 2003). The application of pullulan as a food ingredient has been approved since 2002 in the USA and Europe (MOSCOVICI, 2015), mainly as a dietary fiber or a prebiotic, due to the partial degradation by human amylases (GIAVASIS, 2014).

Pullulan has been widely used in the food, medical and pharmaceutical industries due to properties such as water solubility, capacity of mimic petrochemical-derived polymers, pH stability at a broad range, low viscosity, excellent adhesive properties, good oxygen barrier, and film-forming properties (FREITAS; ALVES; REIS, 2014; PADMANABAN et al., 2015; PRAJAPATI; JANI; KHANDA, 2013; RAVELLA et al., 2010; SINGH; SAINI; KENNEDY, 2008). Some of the uses consist of partial replacement of starch, edible films and packaging material in the food industry (ANDHARE et al., 2014; FARRIS et al., 2014; SHIH; DAIGLE; CHAMPAGNE, 2011; XIAO et al., 2017); for granulation and coating of tablets (Plantcaps-Capsugel, Inc) (JAHANSHAHI-ANBUHI et al., 2014), drug-carrier for cancer treatment

(BALASSO et al., 2017; HUANG et al., 2017), oral (RAVI et al., 2014) and transmucosal (DIONÍSIO et al., 2013) drug delivery and wound care products such as Listerine® in the pharmaceutical industry (RAM et al., 2017); and engineering tissue (MOSCOVICI, 2015; SARUP et al., 2016), and RNA protection (HSIEH et al., 2017) in the biomedical area.

The production of pullulan is performed by an aerobic fermentation, conducted in batch or fed-batch with high aeration rates, optimum pH of 4.5 and temperature ranging from 24 to 30°C, during 100 hours. It is possible to use several different carbon sources, *e.g*. sucrose, glucose, fructose, maltose, starch, or malto-oligosaccharides, with the best nitrogen sources being ammonium and complexes nitrogen sources (SINGH; SAINI; KENNEDY, 2008). During the production of pullulan, the total consumption of the nitrogen source can be observed (KUMAR; MODY; JHA, 2007), with an increase of pullulan production under nitrogen-limitation conditions (WANG et al., 2015). The positive influence of Tween 80, a surfactant, during the production of pullulan was reported, favoring pullulan release and uptake of nutrients (SHENG et al., 2016).

One of the main obstacles found in pullulan fermentation by *A. pullulans* is melanin pigmentation that turns the broth to dark green or black color (CHI; ZHAO, 2003; SINGH; SAINI; KENNEDY, 2008). This color requires the use of special solvents for the polysaccharide precipitation, being preferred solvents with relatively low hydrophilicity, like propyl alcohol, isopropyl alcohol, tetrahydrofuran, dioxane, with further purification made with ultrafiltration or ion exchanges resins (SINGH; SAINI; KENNEDY, 2008). The production of pullulan by non-pigment strains of *Rhodotorula bacarum* had been reported (CHI; ZHAO, 2003), preventing this major obstacle. Another study reported the use of light-emitted diode to produce low melanin pullulan from sugarcane bagasse by a wild strain of *A. pullulans*, obtaining satisfactory results with higher yield (15.77 g/L) and low melanin content (4.46 UA_{540nm}/g) when compared to the untreated strain (11.75 g/L and 45.70 $UA5_{40nm}$/g) (TERÁN et al., 2017).

The pullulan was one of the most expensive polysaccharides, with its market value being three times higher than dextran and xanthan (SINGH; SAINI; KENNEDY, 2008). Optimization of process conditions and medium composition, the use of genetic tools and low-cost substrates, such as sweet potato (PADMANABAN et al., 2015), coconut and palm kernel (SUGUMARAN et al., 2013), potato starch (AN et al., 2016; WU et al., 2016), cassava bagasse (SUGUMARAN; JOTHI; PONNUSAMI, 2014), and corn steep liquor (MEHTA; PRASAD; CHOUDHURY, 2014), could contribute to a more competitive overall process cost (YOON et al., 2012).

5.9 Schizophyllan (SPG) or sizofiran

Schizophyllan is a fungal beta-glucan polysaccharide composed of β-(1−3)-D-glucopyranose backbone which is branched with a single β-(1−6)-D-glucopyranose residue at every third glucose unit, produced by the edible mushroom *Schizophyllum commune* with triple helical conformation and molecular weight varying from 100,000 to 200,000 Da (GIAVASIS, 2014; MOSCOVICI, 2015).

This glucan is a water soluble and non-ionic polysaccharide produced by submerged fermentation with pH of 4.8 in a glucose rich medium, precipitated after 4 to 8 days by adding water-miscible organic solvents (*e.g.* methanol) (ZHANG et al., 2013). Some low-cost substrates have been used for the production of schizophyllan, such as date syrup (JAMSHIDIAN et al., 2016) and corn fiber with satisfactory results suggesting the potential industrial use (LEATHERS et al., 2016).

Most of the applications of schizophyllan are based on its immunostimulatory activity probably due to its triple helical conformation, being a high-value polysaccharide, used for the treatment of various types of cancer, such as breast, gastric, lung, cervical and colorectal (KUMAR; AUROSHREE; MISHRA, 2016; SMIRNOU et al., 2017; ZHONG et al., 2015; ZHOU et al., 2015). It also contributes to the prevention of metastasis and reduces the side effects of chemotherapy (GIAVASIS, 2014). The schizophyllan is also used in skin care products as an anti-aging and healing agent, having a suggested potential to enhance oil recovery (GAO, 2016).

5.10 Scleroglucan

The scleroglucan is a fungal beta-glucan with the similar structure (β-D-glucan (1→3 and branch at 1→6)) as schizophyllan, produced mainly by *Sclerotium glucanicum* with the triple helical conformation and, consequently, anti-tumor activity (ANDHARE et al., 2014; BARBOSA et al., 2004).

The growth of *Sclerotium glucanicum* occurs as pallets surrounded by a layer of scleroglucan, which reduces the polysaccharide production due to the reduction in the mass transfer rate to/from cells (KUMAR; MODY; JHA, 2007). The production of oxalic acid, an undesirable by-product of the process, can be observed below 28°C. Optimum conditions for scleroglucan production by *Sclerotium glucanicum* are described as 20-37°C, pH of 4.0 to 5.5, with vigorous agitation and high supply of oxygen (CASTILLO; VALDEZ; FARIÑA, 2015; SURVASE; SAUDAGAR, 2007; WANG; MCNEIL, 1995). During the production of this

compound, the total consumption of nitrogen from the medium can be noted (KUMAR; MODY; JHA, 2007). When it concerns the downstream process, the method chosen can affect stability and flow behavior of the polysaccharide, originating different scleroglucan variants (VIÑARTA et al., 2013a). The polymer can be precipitated by ethanol or isopropanol, with high levels of purity obtained when isopropanol is used (VIÑARTA et al., 2013b).

Different substrates with economic and ecological benefits are being tested for the production of scleroglucan, such as coconut water, sugar cane molasses, sugar cane juice (SURVASE; SAUDAGAR; SINGHAL, 2007), and condensed corn solubles, a by-product of corn-based ethanol production (FOSMER; GIBBONS; HEISEL, 2010).

The production of scleroglucan by other *Sclerotium* strains is also reported, with *Sclerotium rolfsii* being the most studied. The use of different strains makes the process more attractive industrially providing an option for the production of scleroglucan with similar properties of the one produced by *Sclerotium glucanicum*, once different *Sclerotium* strains can produce polysaccharides with different molecular weight, number and length of side chains, degree of polymerization and rheological characteristics (FARIÑA et al., 2001; SURVASE; SAUDAGAR; SINGHAL, 2006; VIÑARTA et al., 2006, 2007).

Scleroglucan has a potential industrial interest due to its high molecular weight, water solubility, high viscosity, stability in the presence of salts, high temperature and extreme pH, biocompatibility, and non-ionic character (VIÑARTA et al., 2006, 2007). Due to these features, this compound is reported for potential oil recovery, with anti-microbial and immunostimulatory activity (BARBOSA et al., 2004; WANG; MCNEIL, 1995), syneresis prevent agent (VIÑARTA et al., 2006), drug delivery vehicle (COVIELLO et al., 2005, 2007), suspension stabilizer and emulsifier (VIÑARTA et al., 2007), film forming ability (FRANOIS et al., 2011), and compatibility and synergism with industrial thickeners (VIÑARTA et al., 2013b).

This polysaccharide was first introduced to the market under the name Polytran® and commercialized by CECA S.E. (France) with the name Biopolymer CS®. After, Satia, a division of Mero-Rousselot (France), sold scleroglucan with the trade name Acti-gum CS6®. Sanofi Bio-Industries (Carentan, France), obtained the rights from Satia and CECA, and became the biggest producer and distributor of scleroglucan, being sold to Degussa Food Ingredients (Germany) in 1995. In 2006, Cargil (Germany) acquired Degussa, selling the polysaccharide as Actigum™ CS (SURVASE; SAUDAGAR, 2007).

5.11 Welan gum

Welan gum, another polysaccharide from the sphingans class, is an anionic, non-gelling heteropolysaccharide produced by *Sphingomonas* sp. and a mutant strain of *Alcaligenes*, with the follow structure [→3)-β-Glcp-(1→4)-β-D-GlcpA-(1→4)-β-D-Glcp-(1→4)-α-L-Rha-(1→], where Glcp is glucose, GlcpA is glucuronic acid, Rha is rhamnose, with singular side chains containing either L-rhammose or L-mannose substituted on C3 of every 1,4-linked glucose repeating units (BILIADERIS; IZYDORCZYK, 2007; FREITAS; ALVES; REIS, 2011; KAUR et al., 2014).

The welan gum can be produced by submerged fermentation with optimum temperature ranging between 28 – 35°C, pH of 6.5 –7.5, and high agitation rates (800 rpm), since its synthesis is limited by dissolved oxygen levels (KAUR et al., 2014; LI et al., 2011; LIU et al., 2017b). In addition, the use of surfactants, such as Tween-40, showed positive potential in the production of this compound (LI et al., 2012).

Welan gum is easily precipitated with alcohols and acetone (RÜHMANN; SCHMID; SIEBER, 2015a) and its purified form can be obtained by 3 different ways: (i) dissolved in NaCl, precipitated with isopropanol and vacuum dried; (ii) use of dialysis; and (iii) dissolved in aqueous solution of NaOH, neutralized with HCl followed by centrifugation and filtration (KAUR et al., 2014).

The uses of welan gum are based on its strong temperature (20 – 120°C) and pH (2 – 12) tolerance (LIU et al., 2017b), being used as thermostable thickener for oilfield application (XU et al., 2014); suspending, stabilizing, emulsifying and thickening agent in the food industry; coating materials; concrete additive (LACHEMI et al., 2004); mud thickener (GAO, 2015a), and enhance oil recovery (GAO, 2015b). This polysaccharide presents higher viscoelasticity than xanthan gum even with a lower molecular weight, resisting a higher shear, temperature, and salinity (XU et al., 2013).

This gum is one of the most valuable polysaccharides due to its use as an enhancing oil recovery, and at high-value products. It is commercialized by CP Kelco, but still remains as one of the most expensive bio-gums due to its inefficient conversion from carbon sources and low yields (KAUR et al., 2014). To reduce fermentation costs and for a better commercial exploitation, efficient and cost-effective substrates are being studied, such as cane molasses (AI et al., 2015).

6 CONCLUSION

Microbial EPS are renewable, biodegradable and biocompatible substances with a wide range of applications, such as improve or change of rheological properties of various

products. In this sense, these compounds can be used in competitive markets within new industrial applications in a diversity of sectors, such as food, medical and pharmaceutical, besides being used to control environmental issues.

However, despite their potential, there are still some difficulties in their production and purification processes that may hinder their scale of production and, consequently, the extension of commercial applications, due to the high cost and lower yields obtained. In this way, several researches aim to circumvent some of these problems for the development of a viable process. Some of these activities include the isolation of new strains and the use of low-cost substrates. Additionally, many studies are taking place to optimize the production of EPS and to better understand how process conditions may affect their biosynthesis, followed by the best methods for extraction and purification.

Thus, considering their properties and broad fields of application, the production of microbial EPS aiming to ally the scale of production, specific properties, purity and quality are necessary to meet the industrial demands.

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CHAPTER 2 – BIOPROSPECTING OF POLYSACCHARIDE-PRODUCER MICROORGANISMS FROM BRAZILIAN FRUITS

ABSTRACT

Bioprospecting, nowadays, is the key for the discovery of new microbial strains and bioproducts of industrial interest with unique characteristics and properties. The use of Brazilian fruits for this purpose presents unlimited potential due to their great biodiversity influenced by different climates and soils found in the country. Endophytic and epiphytic microorganisms are able to synthesize industrially important compounds, *e.g.* antibiotics, organic acids, polysaccharides and enzymes, among others. These microorganisms can be found living within plant tissues or only on the surface of plants and fruits, respectively. One of the most industrially important compounds are polysaccharides, which can be synthetized by microorganisms offering advantages over plant-production, where the exopolysaccharides (EPS) group is the most relevant. The isolation of new strains for EPS production represents a crucial step for the development of novel bioprocess and discovery of new molecules. In this work, 25 strains were isolated from the follow Brazilian fruits: jatoba (*Hymenaea courbaril*), jabuticaba (*Plinia cauliflora*), lobeira (*Solanum lycocarpum*), strawberry (*Fragaria* × *ananassa*) and mango (*Mangifera indica*). Among them, one strain was able to produce EPS. The strain was isolated from lobeira fruit, and further submitted to fermentative process at 30°C, 150 rpm and using YM medium, presenting satisfactory production results reaching 0.95 g/L after 32 hours of process. Further research is still needed for comprehensive understanding of the microorganism kinetics, and development of a bioprocess with process and medium conditions defined to increase yields of EPS production.

CAPÍTULO 2 – BIOPROSPECÇÃO DE MICRORGANISMOS PRODUTORES DE POLISSACARIDEOS A PARTIR DE FRUTAS BRASILEIRAS

RESUMO

Bioprospecção, atualmente, é a chave para a descoberta de novas linhagens microbianas e bioprodutos de interesse industrial com características e propriedades únicas. O uso de frutas brasileiras para este propósito apresenta potencial ilimitado devido à sua grande biodiversidade influenciada por diferentes climas e solos encontrados no país. Microrganismos endofíticos e epífitos são capazes de sintetizar compostos de importância industrial, como antibióticos, ácidos orgânicos, polissacarídeos e enzimas. Estes microrganismos podem ser encontrados nos tecidos vivos de plantas ou apenas na superfície de plantas e frutas, respectivamente. Um dos compostos mais importantes industrialmente são os polissacarídeos, que podem ser sintetizados por microrganismos, oferecendo vantagens em relação à produção vegetal, onde o grupo dos exopolissacarídeos (EPS) é o mais relevante. O isolamento de novas linhagens para a produção de EPS representa um passo crucial para o desenvolvimento de novos bioprocessos e descoberta de novas moléculas. Neste trabalho, foram isoladas 25 linhagens a partir das seguintes frutas brasileiras: jatobá (*Hymenaea courbaril*), jabuticaba (*Plinia cauliflora*), lobeira (*Solanum lycocarpum*), morango (*Fragaria × ananassa*) e manga (*Mangifera indica*). Dentre essas uma linhagem foi capaz de produzir EPS. A linhagem foi isolada a partir da lobeira, e posteriormente submetida a um processo fermentativo a 30°C, 150 rpm, e usando meio YM, apresentando resultados de produção satisfatórios obtendo 0,95 g/L após 32 horas de processo. Pesquisas ainda são necessárias para uma compreensão abrangente da cinética do microrganismo e desenvolvimento de um bioprocesso com condições de meio e processo bem definidas, buscando incremento da produção.

1 INTRODUCTION

The bioprospecting of microorganisms, nowadays, represents the main alternative to obtain new biocatalyst as the demand for novel products and process continues to increase (LIU et al., 2012). It is a term usually used for screening of biological resources in order to extract commercially important compounds, relying on the biodiversity of natural sources (BUSHRA et al., 2017).

The Brazilian fruits presents great biodiversity, due to the different environmental conditions and biomes in which they can be found, offering tropical and temperate fruits. Brazil is the third largest fruit producer in the world, responsible for 4.8% of all harvest fruits, accounting for 40.2 mil tons (ANDRADE, 2017). In this panorama, Minas Gerais is the third largest producer in Brazil (CARVALHO et al., 2017).

The state of Minas Gerais possess great assortment of climes and soils, with characteristic vegetation, insert at three different Brazilian biomes: Cerrado, the Atlantic Forest and Caatinga, being the Cerrado the predominate biome at the state (57%), followed by the Atlantic Forest (41%) and Caatinga (2%). Cerrado vegetation is composed of grasses, shrubs and trees, while Atlantic Forest has trees with large and smooth leaves, and the Caatinga has a unique vegetation, not found at anywhere else in the word (IEF, 2017). Thus, plants and fruits with different and specific characteristics can be found easily at the state, facilitating the isolation of unique microorganisms.

Plants microorganisms can be divided into two different groups, depending on their location at fruits and plants: within or on the surface of plant tissues. Endophytic microorganisms are those resident at living plant tissues, ranging from symbiotic to pathogenic, producing substances which provide protection for the plant (STROBEL; DAISY, 2003; STROBEL et al., 2004). The epiphytic microorganisms do not penetrate the plants tissues, remaining restricted to the plant surface (ZAMBELL; WHITE, 2015). Endophytic and epiphytic microorganisms are responsible for synthesize numerous industrially important compounds, such as vitamins, antibiotics, organic acids, polysaccharides and enzymes, being functional components of the plant microbiome (BACON; WHITE, 2016; DIAS et al., 2015).

Due to the industrial potential of microbial polysaccharides, their exploration and utilization are increasing, especially of the exopolysaccharides (EPS) group, due to the ease of recovery (DONOT et al., 2012; LIU et al., 2017). Microbial polysaccharides also offers advantages over plant-derived polysaccharides, such as high yields and quality, besides reproducible production parameters (MOSCOVICI, 2015). Possessing a wide range of

application, being used at food, chemical, pharmaceutical, and medical industries, the discovery of new strains able to synthetize EPS represents an important step for the development of novel bioprocesses, in pursuit of new molecules with unique properties, enabling the increase of commercial applications. Thus, the main purpose of this work was to isolate and identify producer-polysaccharide strains from different Brazilian fruits, allowing the development of new fermentation process.

2 MATERIAL AND METHODS

2.1 Microbial isolation

Several microorganisms were isolated from commonly Brazilian fruits: jatoba (*Hymenaea courbaril*), jabuticaba (*Plinia cauliflora*), lobeira (*Solanum lycocarpum*), strawberry (*Fragaria × ananassa*) and mango (*Mangifera indica*) at the Laboratory of Food Biotechnology, located at Campus JK of the Universidade dos Vales do Jequitinhonha e Mucuri – UFVJM, located at Diamantina – Minas Gerais.

For the isolation of endophytic microorganisms, the fruits were previously sterilized externally and then fragments (husk, pulp, seeds and stem) were removed and deposited in Petri dishes containing Yeast Medium (YM), consisting in w/v of glucose 1%, yeast extract 0,3%, bacteriological peptone 0,5% and agar 2%, pH was not adjusted. For the isolation of epiphytes, fruit fragments were directly removed and deposited on the Petri dishes containing YM. After 48 hours of incubation at 30°C, different colonies were transferred to another plate containing YM, until obtain pure colonies. To certify the sterility of the process to avoid contamination, a Petri dish containing only YM was used as process control, in which no colony should develop (MOLINA, 2010).

2.2 Screening of exopolysaccharides-producer strains

Exopolysaccharides-producer strains were identified visually, due to the mucoid growth presented at solid medium at 30°C using Petri dishes, and increase in viscosity in the YM fermentation broth at 30°C and 150 rpm, using 250 mL conical flasks (RÜHMANN; SCHMID; SIEBER, 2015).

2.3 Fermentation process

Two full loops of a 24 hours-old culture on a Petri dish were transferred to a 250 mL conical flask containing 100 mL of YM broth. The flasks were incubated at 30°C and 150

rpm during 48 hours. It could be noted the visual increased of viscosity in the fermentation broth, as the EPS was secreted to the extracellular medium (RÜHMANN; SCHMID; SIEBER, 2015).

2.4 Microscopic identification of the strain

Strains able to produce exopolysaccharides were identify as gram-negative or gram-positive by the Gram stain technique.

The Gram stain differentiates bacteria into two different groups, based on the cell wall structure, leaving the gram-negative bacteria stained in red and gram-positive bacteria stained in purple (BEVERIDGE, 2001).

2.5 Cell growth and exopolysaccharide production

The cell growth during the fermentation process was measured by absorbance at 600 nm using a spectrophotometer Tecnal UV 5100, at 0, 2, 4, 8, 24, 28, 32 and 48 hours of process. An aliquot of 3 mL was centrifuged at 2000 g during 12 minutes for biomass removal, which was resuspended at 3 mL of distilled water. The supernatant was reserved for EPS quantification. The cell growth results were expressed in Units of Absorbance (UA) (AYALA-HERNÁNDEZ et al., 2008).

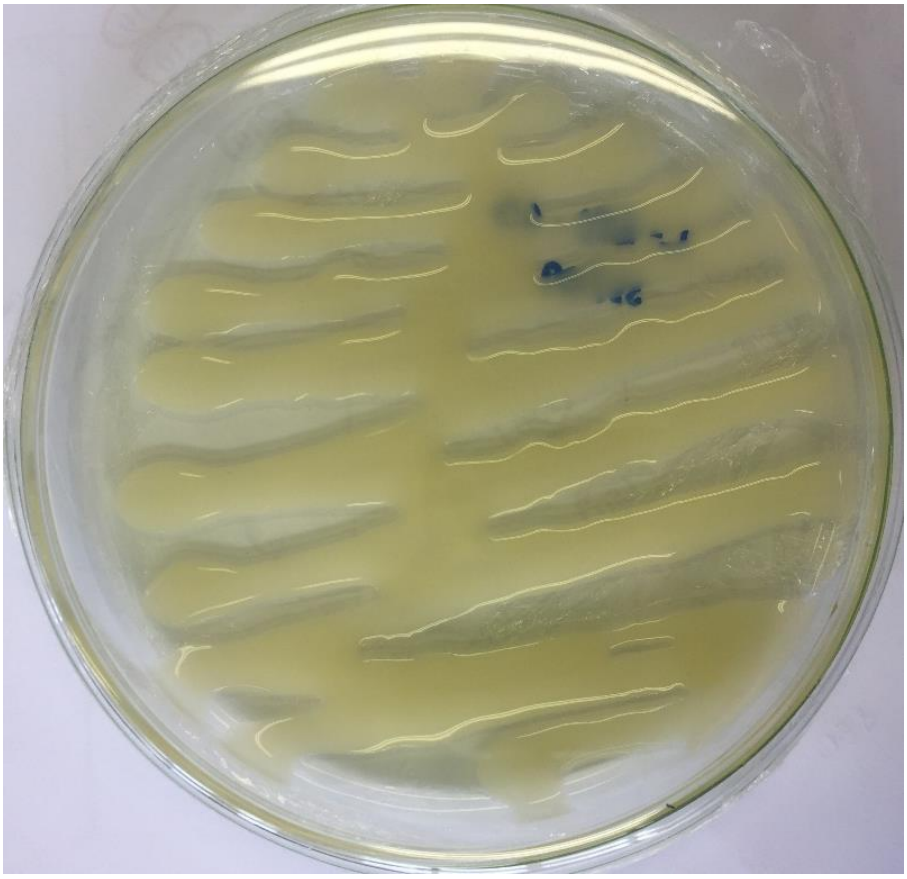
The EPS production was quantified by dry weight, where the supernatant previously saved was precipitated using three volumes of cold ethanol (4°C) and stored overnight at 4°C. The precipitates were collected by centrifugation at 2000 g and 12 minutes and resuspended at one volume of demineralized water, followed by drying at 70°C until dry weight. The results of EPS production were expressed in g/L. All analyzes were performed in triplicate (COMTE; GUIBAUD; BAUDU, 2006).

3 RESULTS AND DISCUSSION

Twenty-five colonies of bacteria and yeasts were isolated from the brazilian fruits jatoba, jabuticaba, lobeira, strawberry and mango. The colonies were named using a three-digit code, where the first digit indicates the fruit from which the microorganism were isolated (jatoba = 5; jabuticaba = 6; lobeira = 7; strawberry = 8; mango = 9); the second digit indicates if the microorganism is endophytic (1) or epiphyte (2); and the third digit, the colony number. Among the isolated microorganisms, only one showed potential for EPS production, presenting increased viscosity of fermentation broth during submerged fermentation. This microorganism

is an epiphyte isolated from lobeira fruit (*Solanum lycocarpum*), coded as LBA725, as show in Figure 1.

Figure 1 – Mucoid growth of microorganism LBA725 isolated from lobeira fruit



The lobeira fruit (*Solanum lycocarpum*) (Figure 2), also known as “fruta-do-lobo”, is native from the Brazilian Cerrado, described as a tropical savannah, the second-largest biome in Brazil, growing and developing under unfavorable environmental conditions, such as acid and nutrient-poor soils (OLIVEIRA JUNIOR et al., 2003). The fruit presents green-yellowish coloring, reaching 15 cm of diameter, being possible to harvest between the months of June and January (DALL’AGNOL; LINO VON POSER, 2000). It is considered one of the most important fruits of the Brazilian Cerrado, economically and scientifically, together with Pequi (*Caryocar brasiliense*), Cagaita (*Eugenia dysenterica*) and Araçá (*Psidium cattleyanum*) (DIAS et al., 2015).

Figure 2 – Green lobeira fruit (*Solanum lycocarpum*)

The green lobeira fruit is used in traditional medicine as a dried white powder, rich at complex polysaccharides, to fight diabetes and epilepsy, possessing, also, solamargine and solasonine, bioactives compounds which have antitrypanosomal, schistosomicidal, antiherpetic, antifungal, immunomodulatory, and anticancer activities; and the crude ethanol extract is been prove to have anti-inflammatory effects (ALVES et al., 2016; BAILÃO et al., 2015; DALL'AGNOL; LINO VON POSER, 2000; ROESLER et al., 2007; VIEIRA et al., 2003).

The epiphytic microbiota is composed of bacteria, yeasts and filamentous fungi, which may be pathogenic to humans. The microbiota diversity is dependable of regional environmental factors, in which the surface of fruits presents a rich niche when it comes to nutrients for the growth and development of microorganisms (DIAS et al., 2015). Until the closure of this material, there were no papers describing the lobeira fruit epiphytic microbiota.

The culture isolated from lobeira fruit was identified microscopically as a gram-negative bacteria. Gram-negative bacteria are commonly known for the ability to produce EPS, being two of the most commercialized EPS originated from these type of bacteria: Xanthan gum, from *Xanthomonas campestris*, and Gellan gum, from *Sphingomonas paucimobilis* (SUTHERLAND, 2001; VELU; VELAYUTHAM; MANICKKAM, 2016).

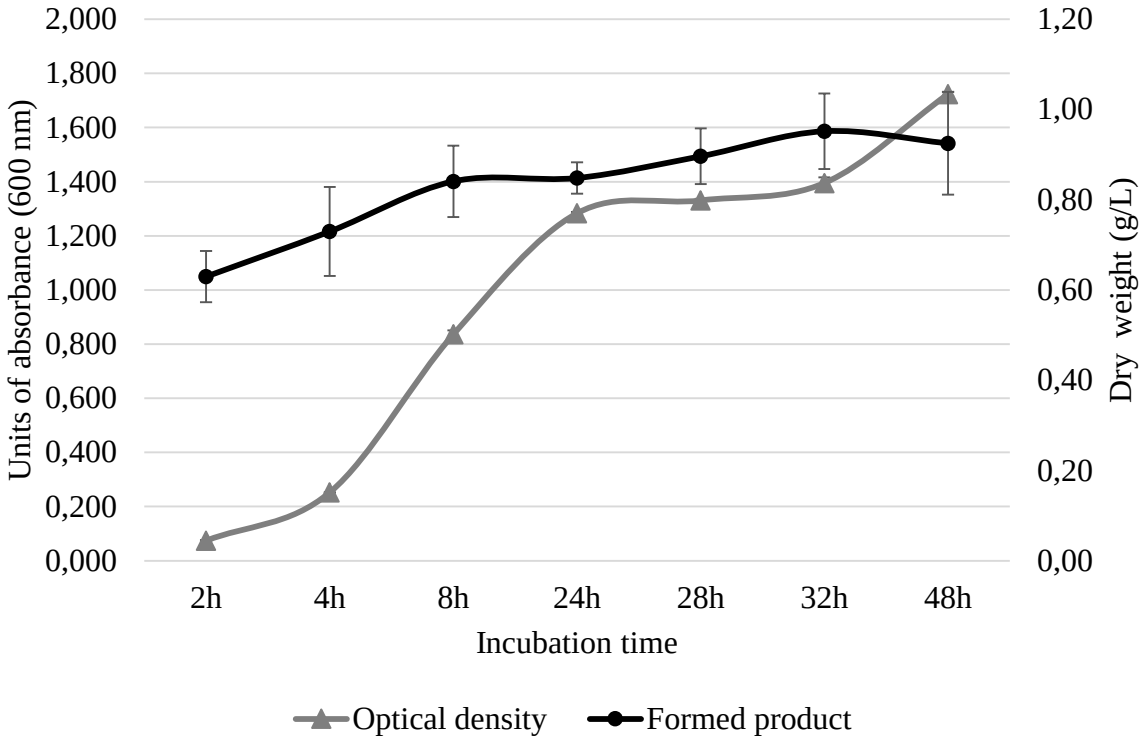
The EPS production process by microorganisms is individual, varying with process conditions and biocatalyst used. However, its synthesis can be presented in a general way within four steps: (i) assimilation of the carbon source, (ii) synthesis of the oligosaccharide repeat units or direct synthesis by successive or progressive activity of glycosyltransferases (iii) assembly of the polysaccharide from the repeat units, and (iv) exportation of the EPS to the extracellular medium (BECKER, 2015).

Enzymes are responsible for most all steps at EPS production, being present at different parts of the microbial cell, where intracellular enzymes are involved in other metabolic processes, such as hexokinase; enzymes which are believed to be intracellular, are the source of monosaccharides residues; enzymes located at the cell periplasmic membrane, such as glycosyltransferases, responsible for the EPS synthesis; and extracellular enzymes which are responsible for the polymerization of macromolecules (KUMAR; MODY; JHA, 2007).

The EPS of gram-negative bacteria presents some peculiarities, being normally assembly by undecaprenyl phosphate, a lipid carrier. The assembly of the repeating units involves sequential transfer of the sugar nucleotide diphosphates precursors to an isoprenoid lipid carrier, in this case, undecaprenyl phosphate, occurring at the cytoplasmic membrane (BECKER, 2015; SUTHERLAND, 2001).

The kinetics of growth of the bacteria LBA725, and the quantification of EPS produced during the 48 hours fermentation is shown in Figure 3.

Figure 3 - Growing kinetics and EPS produced by the bacteria LBA725, isolated from lobeira fruit



The biosynthesis of EPS may occur at different growth phases, depending on environmental conditions and microorganism used (MADHURI; VIDYA PRABHAKAR, 2014). When using glucose as carbon source, most of EPS are produced during *log* phase, being synthetized throughout bacterial growth (LOOIJESTEIJN et al., 1999; SUTHERLAND, 2001), thus, EPS production is growth associated which can be perceived in the process described for the bacteria LBA725. The growth associated EPS production is also observed when using *Propionibacterium acidi-propionici* (GORRET et al., 2001), *Lactobacillus pentosus* (SÁNCHEZ et al., 2006), *Halomonas* sp. (POLI et al., 2009), *Lactobacillus plantarum* (YOUSUFF et al., 2016), *Xanthomonas campestris* (ZAKERI; PAZOUKI; VOSSOUGI, 2015) and microorganisms able to synthesized levan, such as the genera *Acetobacter, Bacillus, Erwinia, Gluconobacter, Halomonas,Microbacterium, Pseudomonas, Streptococcus* and *Zymomonas* (ONER; HERNÁNDEZ; COMBIE, 2016).

The relation between the kinetics of growth and EPS production of this bacteria can be noted since as the logarithmic phase begin (as the incubation time begin), it is observed an increase in the amount of EPS produced, reaching 0.95 g/L after 32 hours of process. Later on, it was observed a decrease of product concentration at 48 hours, finishing the process with a yield of 0.93 g/L, as it can be observed at FIG. 2.

The decrease of yield at EPS production can be justified by the presence of degrading enzymes, called polysaccharases or polysaccharide lyases also produced by bacteria. These enzymes are normally intracellular however, if the cells lyse during culture, the enzymes may be released, with a reduction of EPS mass being observed (SUTHERLAND, 2001). EPS mass reduction is also reported at the biotechnological production of Hyaluronic acid (MERO; CAMPISI, 2014), and Xanthan gum (BENNY; GUNASEKAR; PONNUSAMI, 2014) after long fermentation periods.

The screening of novel polysaccharides-producer strains is promising due to the wide range of polysaccharides yet to the be explored, characterized and developed for commercial applications (CHANDRAN; SHARMA, 2015). Previous work has similarities regarding the production of EPS by gram-negative bacteria isolated from different sources.

Fifty-one strains were isolated from deep-sea hydrothermal vents, selected by a preliminary screening presenting mucoid appearance. Strains were growth at ZHPUF medium (aquarium salts 33.3 g/L, ultra-filtered yeast extract 1.0 g/L, Tryptone N1 4.0 g/L, glucose, saccharose or mannitol 30 g/L), at 25°C or 37°C (depending on the bacteria) and 150 rpm for 48 hours. Twenty-seven strains produced EPS, identify as *Vibrio*, *Alteromonas*, or *Pseudoalteromonas*, all gram-negative bacteria, reaching yields varying from 0.1 g/L to 0.58 g/L (DELBARRE-LADRAT et al., 2017).

Using effluents from Indian sweets shops, Chandran and Sharma (2015) isolated 15 strains with mucoid appearance, obtaining one strain able to produce significant amount of EPS, forming viscous solutions. The strain identify as *Agrobacter*, is a gram-negative bacteria reaching maximum EPS production after 6 days (16.21 g/L), having its production decreased afterwards, the same was observed for cell growth. The EPS production and cell growth were determined during growth in medium containing (g/L) 40 brown sugar; 0.2 $MgSO_4$.$7H_2O$; 9 K_2H PO_4; 3 KH_2PO_4; 2 yeast extract; 2 peptone; 2 NaCl; and 15 agar. The isolate was incubated at 28°C, 150 rpm for 10 days (CHANDRAN; SHARMA, 2015).

Thus, the EPS production by the isolated strain from lobeira fruit (*Solanum lycocarpum*) is an important step towards the development of new bioprocess and discovery of new molecules with potential industrial application.

4 FINAL CONSIDERATIONS

In this study, twenty-five microorganisms were isolated from the Brazilian fruits jatoba (*Hymenaea courbaril*), jabuticaba (*Plinia cauliflora*), lobeira (*Solanum lycocarpum*),

strawberry (*Fragaria* × *ananassa*) and mango (*Mangifera indica*) presenting one positive result for polysaccharide-producer strain, isolated from lobeira fruit.

The isolated microorganism was coded as LBA725, a gram-negative bacteria, with satisfactory results concerning growth and production of EPS, achieving 1.6 UA and 0.95 g/L, respectively. Additionally, EPS production was growth associated, being a potential biocatalyst for the development of new bioprocesses regarding EPS production.

Further research is still needed in order to determine the optimum process parameters and medium composition to increase EPS production, making the process cost-effective and more feasible, besides the biochemical and genetic characterization of the isolated microorganism.

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CHAPTER 3 – GROWTH KINETICS OF A NOVEL EXOPOLYSACCHARIDE-PRODUCER STRAIN ISOLATED FROM LOBEIRA FRUIT USING DIFFERENT CARBON SOURCES AND CHARACTERIZATION

ABSTRACT

The growing demand of industries for new and affordable molecules with wide range of applications and specific physicochemical properties leads to the search of novel biocatalysts capable to supply such demands. The growth behavior and ability of exopolysaccharide (EPS) production by a novel microorganism isolated from lobeira fruit (*Solanum lycocarpum*) was evaluated using three different carbon sources (glucose, fructose and lactose), and standard conditions (30°C, 150 rpm and 48 hours of incubation). This microorganism presented similar growth curves and some significant differences in EPS production, indicating different metabolic capacity when different carbon sources were used. Growth ranging from 2.81 to 4.49 g/L of dry biomass and production around 1 g/L were observed using the three carbon sources used. The isolated strain was then characterized by sequencing and phylogenetic analysis of RNA ribosomal 16s gene, being identify as *Kosakonia cowanii* (formerly known as *Enterobacter cowanii*). This is the first report of *K. cowanii* as an EPS-producer strain.

CAPÍTULO 3 – CINÉTICA DE CRESCIMENTO DE UMA NOVA LINHAGEM PRODUTORA DE EXOPOLISSACARÍDEOS ISOLADA A PARTIR DE LOBEIRA UTILIZANDO DIFERENTES FONTES DE CARBONO E CARACTERIZAÇÃO

RESUMO

A crescente demanda de indústrias para moléculas novas e acessíveis com ampla gama de aplicações e propriedades físico-químicas específicas leva à busca de novos biocatalisadores capazes de suprir a tais demandas. A cinética de crescimento e a capacidade de produção de exopolissacarídeo (EPS) de um novo microrganismo isolado de frutos de lobeira (*Solanum lycocarpum*) foram avaliados utilizando três diferentes fontes de carbono (glicose, frutose e lactose) e condições padrão (30°C, 150 rpm e 48 horas de incubação). O microrganismo apresentou curvas de crescimento semelhantes e algumas diferenças significativas na produção de EPS, indicando diferentes capacidades metabólicas da linhagem quando são utilizadas diferentes fontes de carbono. Crescimento variando de 2,81 a 4,49 g/L de biomassa seca, e produção em torno de 1 g/L foram observados nas três fontes de carbono utilizadas. A linhagem isolada foi então caracterizada por sequenciamento e análise filogenética do gene RNA ribossomal 16s, identificando-se como *Kosakonia cowanii* (anteriormente conhecida como *Enterobacter cowanii*). Essa é a primeira vez que *K. cowanii* é relatada como uma linhagem produtora de EPS.

1 INTRODUCTION

Microbial exopolysaccharides (EPS) are widely used at a variety of industries, such as food, pharmaceutical, and medical, due to specific and variety of physicochemical properties, as well as biocompatibility, biodegradability, and both environmental and human compatibility, with potential to replace petro based polymers, registering an increased at worldwide demand over recent years (ATES, 2015; LEROY; VUYST, 2016; REHM, 2010). Since the biopolymer is secreted to extracellular medium, its harvest is facilitated and efficient, favoring a continuous and cost-effective process (SCHMID; SIEBER; REHM, 2015). Besides that, microbial EPS are preferred by industry due to several advantages inherent to microbial cultures, such as high yield and productivity, high growth rates, and the possibility to control polymer properties (MARQUES et al., 2017).

Due to the growing market demand, the search of new strains capable of synthesizing EPS offers new possibilities, enabling the development of new bioprocess and discovery of novel biopolymers, with unique properties (FREITAS; ALVES; REIS, 2011). Thus, the exploring of the biodiversity of biological resources and discovery of new molecules facilitates the proper characterization and exploration of EPS, followed by the development of industrial processes and commercial applications, achieving its full potential (BUSHRA et al., 2017; CHANDRAN; SHARMA, 2015).

A great number of EPS varieties is known, distinguishing itself by its complex structures, based on the monosaccharides presented in the molecule and the way these are connected, providing different properties (RÜHMANN; SCHMID; SIEBER, 2015).

Several factors may affect the EPS production and composition, such as pH, agitation rates and medium composition, among others. The must influent condition is medium composition, where appropriate amounts of carbon source and minimum amounts of nitrogen sources can lead to adequate secretion of polysaccharides (CZACZYK; MYSZKA, 2007; PRAJAPATI et al., 2013). Glucose, fructose and lactose are often cited as suitable carbon sources for the production of EPS by a wide spectrum of microorganisms due to their abundance and cost (ANTUNES et al., 2017; KUMAR et al., 2004; LI et al., 2016; LOOIJESTEIJN et al., 1999; PETRY et al., 2000).

In this context, the characterization and growth kinetics of the previously isolated microorganism from lobeira fruit (*Solanum lycocarpum*) characterize as EPS producer, can help in the better understanding of the strain behavior and its production of EPS. Thus, the goal of

this work is to evaluate the growth kinetics and EPS production using different carbon sources and identify the isolated microorganism specie.

2 MATERIAL AND METHODS

2.1 Microorganism cultivation

The microbial strain employed in this study was previously isolated from lobeira fruit (*Solanum lycocarpum*), and characterized as EPS producer, coded as LBA725. The bacterial strain was maintained on Yeast Medium (YM) Agar (in g/L: agar = 20; glucose = 10; peptone = 5; yeast extract = 3; pH ~ 6.7) and stored at 4ºC.

2.2 Fermentation process conditions

Two full loops of a 24 hours-old culture on a Petri dish were transferred to a 250 mL conical flask containing 100 mL of Yeast Medium broth, consisting in w/v of the carbon source (glucose, fructose or lactose) 1%, yeast extract 0.3%, bacteriological peptone 0.5%. The flasks were incubated at 30°C and 150 rpm for 48 hours (RÜHMANN; SCHMID; SIEBER, 2015).

2.3 Growth kinetics and exopolysaccharide production

The growth kinetics of the microorganism at different carbon sources (glucose, fructose or lactose) were evaluated by absorbance measured at 600 nm, at 0, 2, 4, 8, 24, 28, 32 and 48 hours of process. An aliquot of 3 mL from the fermentation process was centrifuged at 2000 g during 12 minutes for biomass removal. The biomass was resuspended at 3 mL of distilled water and the supernatant was then used for EPS quantification. The results of bacterial growth were expressed in Units of Absorbance (UA) (AYALA-HERNÁNDEZ et al., 2008). The maximum specific growth rate (μ_{max}) was experimentally determined in the exponential growth phase using the equation:

$$\mu_{max} = \frac{ln\frac{X_0}{X_1}}{t_1 - t_0}$$

where X_0 and X_1 are the biomass dry weight and t_0 and t_1 are the times along the exponential phase, respectively (SCHMIDELL et al., 2001).

The EPS production was quantified by dry weight, where the supernatant previously saved was precipitated using three volumes of cold ethanol (4°C) and stored overnight at 4°C. The precipitates were collected by centrifugation at 2000 g and 12 minutes,

resuspended at one volume of distilled water, being dry at 70°C until constant weight. The results were expressed in g/L (COMTE; GUIBAUD; BAUDU, 2006). Volumetric productivities were graphically estimated by mean of differentiation of EPS produced (g/L) per unit time (1/h) (SCHMIDELL et al., 2001). All analyzes were performed in triplicate.

2.4 Microorganism characterization

The microorganism characterization was made by sequencing and phylogenetic analysis of RNA ribosomal 16s gene.

The genomic DNA was extracted using one loop of the culture diluted at 5 µL of deionized water and heated to 95°C for 3 minutes in thermocycler. The amplification of the ribosomal RNA gene was done by the PCR technique using as genetic template the genomic DNA extracted from culture, using primers p10f and p1100r, complementary to conserved regions of the 16s ribosomal RNA gene of the bacteria.

The amplification product was column-purified (GFX PCR DNA and Gel Band Purification Kit, GE Healthcare) and subjected to sequencing using the automatic AB13500XL Series sequencer (Applied Biosystems). The used primers were p10f and p1100r.

The partial sequences of the 16s ribosomal RNA gene obtained with the different primers were assembled in a consensus (single consensus sequence combining the different fragments obtained) and compared to sequences of organisms represented in the GenBank (https://www.ncbi.nlm.nih.gov/) and RDP (https://rdp.cme.msu.edu/) databases. Sequences of microorganisms related to the unknown microorganism were then selected for construction of the phylogenetic analysis. The DNA sequences were aligned using the CLUSTAL X program and the phylogenetic analyzes were conducted using the MEGA program version 6.0. The evolutionary distance matrix was calculated using the Kimura model (KIMURA, 1980) and the construction of the phylogenetic tree from evolutionary distances was done by the neighbor-joining method (SAITOU; NEI, 1987), with bootstrap values calculated from 1000 re-samples using the software included in the MEGA 6.0 program.

2.5 Data analysis

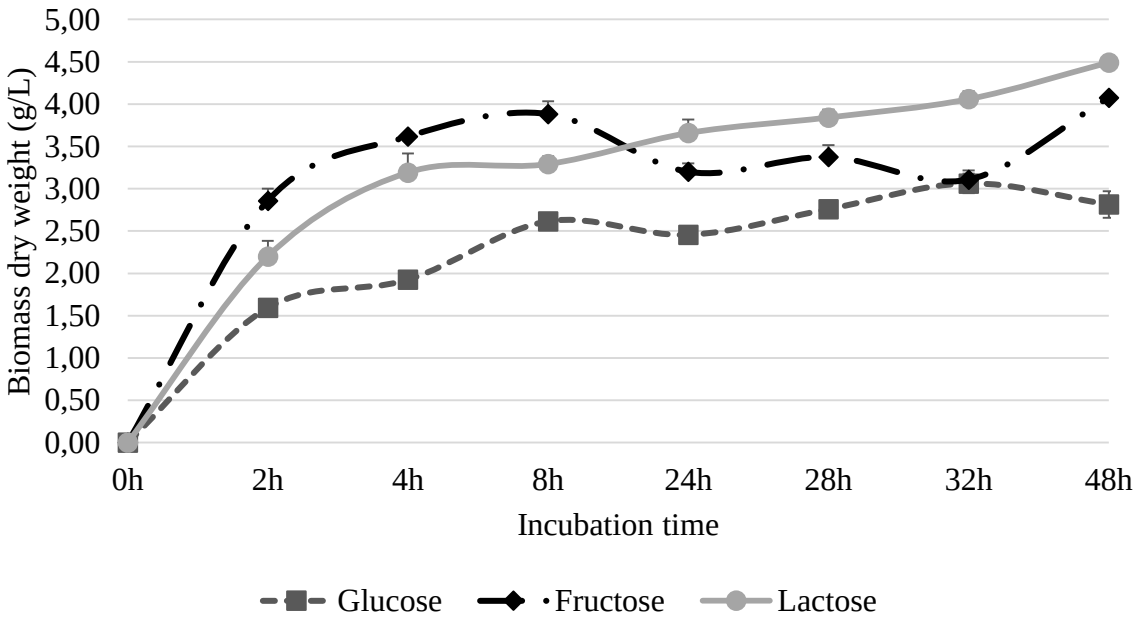
Statistical analysis of the experimental data were performed using Tukey test and the Statistica 12.0 software (StatSoft Inc, Oklahoma, USA).

3 RESULTS AND DISCUSSION

The strain used in this work was previously isolated from lobeira fruit (*Solanum lycocarpum*) at the Laboratory of Food Biotechnology, located at Campus JK of the Universidade dos Vales do Jequitinhonha e Mucuri – UFVJM, located at Diamantina – Minas Gerais. The microorganism was identified as a gram-negative bacteria and coded as LBA725, with the capacity to produce EPS using glucose as only carbon source, achieving 0.95 g/L. Thus, the growth kinetics and ability to produce EPS is now analyzed using glucose, fructose and lactose.

Cell growth and product formation were examined with experimental data. The microorganism kinetics of growth, monitored by dry biomass weight, are shown in Figure 1, and product formation using different carbon sources (glucose, fructose and lactose) can be observed at Figure 2 and Table 1.

Figure 1 – Dry biomass of the novel EPS-producer microorganism isolated from lobeira fruit (*Solanum lycocarpum*) using different carbon sources

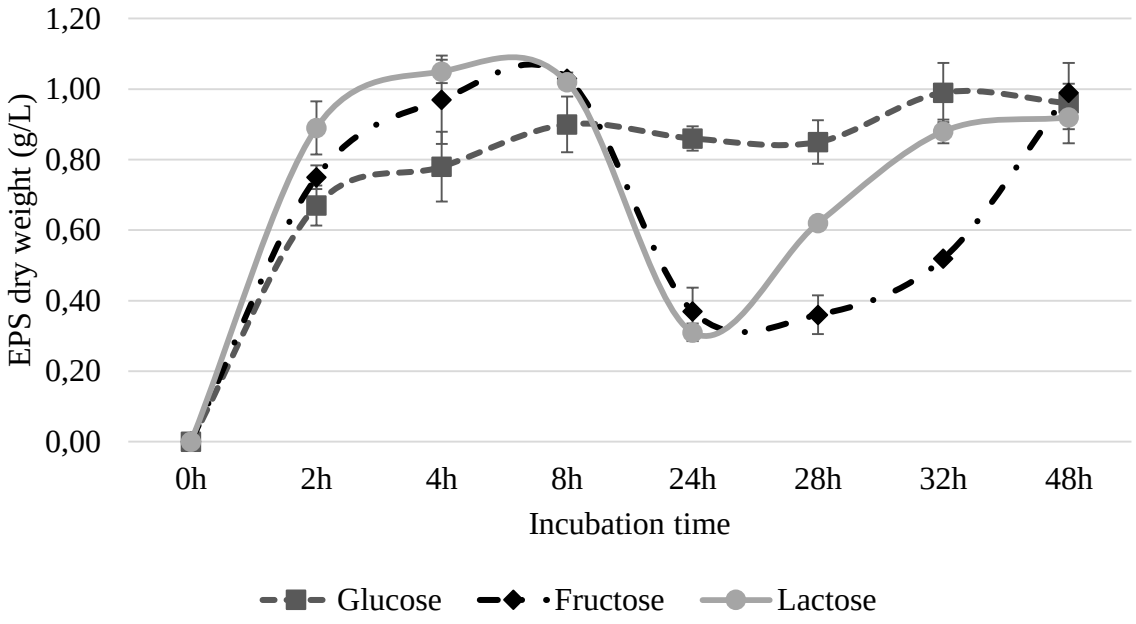


The dry biomass of the microorganism at the different carbon sources observed in FIG. 1 presents no apparent *lag* phase, starting directly at logarithmic phase, which ends approximately within 24 hours, indicating that the culture is well adapted to all of the different carbon sources (in the range tested). The increase dry biomass observed within 48 hours can indicate diauxic growth of the microorganism; however, previous tests (data not shown) showed the decrease of formed product after 48 hours of process. Then, subsequent incubation times

were not evaluated. The maximum specific growth rates (μ_{max}) of the microorganism are 0.018 h^{-1}, 0.005 h^{-1} and 0.021 h^{-1} for glucose, fructose and lactose, respectively.

According to Sánchez and coworkers (2006), culture conditions, carbon source used and growth rate of the microorganism can influenced the yield and composition of the EPS (SÁNCHEZ et al., 2006). Thus, despite the fact that the microorganism is well adapted to the different carbon sources, the difference among the growth rates and carbon sources used may lead to different structural organizations, leading to different EPS.

Figure 2 – EPS dry weight of the novel EPS-producer microorganism isolated from lobeira fruit (*Solanum lycocarpum*) using different carbon sources



Even though the same growth pattern is observed for the different carbon sources, the EPS production curves presents some significant differences between incubation times and carbon sources tested, as it can be observed in FIG. 2 and TAB. 1. The decrease observed in EPS production at the end of logarithmic phase (24 hours), especially for fructose and lactose, may be due to the presence of degrading enzymes released by the microorganism, followed by the reutilization of monosaccharides released by the enzymes as building blocks to overcome nutrient starvation (LAUE et al., 2006; ONER; HERNÁNDEZ; COMBIE, 2016; SINGHA, 2012).

Enzymes possess great relevance at EPS production. Genes responsible for EPS biosynthesis are organized at clusters which encode enzymes responsible for the formation of the polysaccharide, through the sequential addition of sugars to repeating unit, followed by

export and polymerization (BOELS et al., 2001; SCHMID; SIEBER; REHM, 2015). Likewise, the microorganism can synthesize and release degrading enzymes among the biosynthetic enzymes (DE SOUZA; SUTHERLAND, 1994; FREITAS; ALVES; REIS, 2011). Such enzymes are normally intracellular, but if the cells lyse, they might be released (SUTHERLAND, 2001). Overtime, the culture medium may be depleted of essential nutrients, such as carbon and nitrogen sources, thus the cells resort to their reserve material, storing in the form of EPS and others, hydrolyzing and using them as carbon and nitrogen sources for proper cell growth and maintenance (MORE et al., 2014).

It is also possible to note that the EPS production is growth associated independent of the carbon source used. The formed product is directly linked to reactions of catabolism and substrate degradation and microorganism growth, in which the EPS concentration increased with biomass concentration.

Table 1 – EPS production during 48 hours of incubation using different carbon sources from novel microorganism isolated from lobeira fruit

Incubation time	EPS production (g/L)		
	Glucose	**Fructose**	**Lactose**
2 hours	$0.67\pm0.06^{a, A}$	$0.75\pm0.03^{a, AC}$	$0.89\pm0.07^{a, A}$
4 hours	$0.78\pm0.10^{b, A}$	$0.97\pm0.13^{b, A}$	$1.05\pm0.03^{b, A}$
8 hours	$0.90\pm0.08^{c, A}$	$1.03\pm0.02^{c, A}$	$1.02\pm0.01^{c, A}$
24 hours	$0.86\pm0.03^{d, A}$	$0.37\pm0.07^{e, B}$	$0.31\pm0.02^{e, B}$
28 hours	$0.85\pm0.06^{f, A}$	$0.36\pm0.05^{g, B}$	$0.62\pm0.02^{f, C}$
32 hours	$0.99\pm0.08^{h, A}$	$0.52\pm0.00^{i, BC}$	$0.88\pm0.03^{h, A}$
48 hours	$0.96\pm0.11^{j, A}$	$0.99\pm0.02^{j, A}$	$0.92\pm0.03^{j, A}$

Mean values followed by the same lowercase letter in rows and uppercase in columns do not differ by Tukey test (5% probability).

Process conditions: 30°C, 150 rpm and ~6.7 pH not adjusted.

EPS production using glucose, fructose and lactose presents few or none significant difference among the incubation times analyzed, especially glucose. The final EPS values at 48 hours from the different carbon sources also do not differ significantly. This behavior is contrary to the observed at literature, where the carbon source used is direct linked to the amount of EPS produced at defined incubation times (MIQUELETO et al., 2010; RAVELLA et al., 2010; ZHANG et al., 2015), proving the proving the ability of the lineage to adapt at different carbon sources.

The use of different carbon sources can lead to the synthesis of different EPS, based on its composition, since the monosaccharides used at the EPS assembly are derived from the carbon sources present in the incubation medium (BOELS et al., 2001; CHUG et al., 2016).

Despite the fact of higher values of EPS production can be observed at 4 and 8 hours within the process for fructose and lactose, there is no significant difference from the final value at 48 hours. The volumetric productivity of EPS at the final hour of incubation are 0.02 g/L·h for all carbon sources tested.

The differences between specific growth rates and volumetric productivity of EPS (Table 2) indicates different metabolic capacity of the strain when different carbon sources are used, as it was already discussed above.

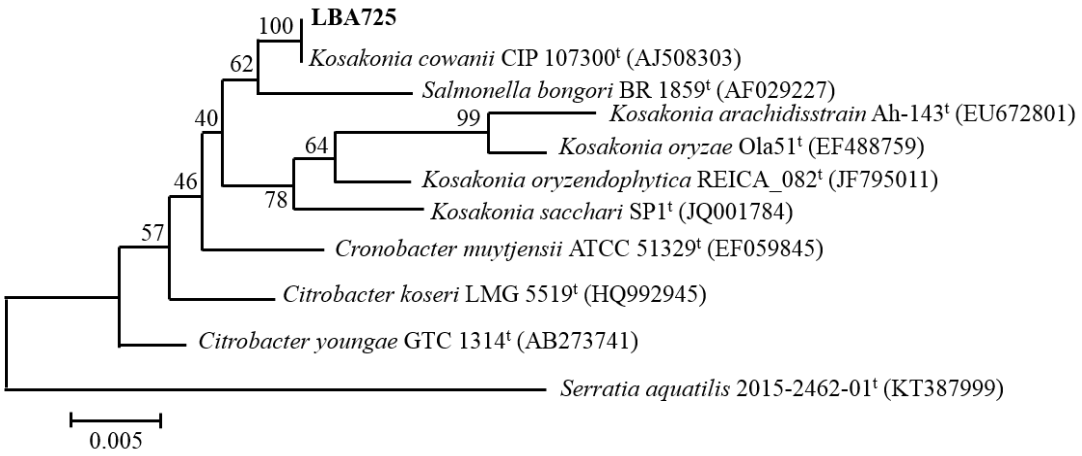
Table 2 – Specific growth rates and volumetric productivity of EPS for the different carbon sources used

Carbon source	Specific growth rates (μ_{max})	Volumetric productivity of EPS
Glucose	0.018 h^{-1}	0.02 g/L·h
Fructose	0.005 h^{-1}	0.02 g/L·h
Lactose	0.021 h^{-1}	0.02 g/L·h

The specific growth rates found in this study are lower than others gram-negative bacteria reported for the production of EPS. For *Halomonas sp.*, μ_{max} = 0.31 h^{-1} when using sucrose as carbon source, achieving 1.03 g/L of the EPS levan (POLI et al., 2009), and for *Enterobacter* A47, μ_{max} = 0.29 h^{-1} when using tomate waste, a by-product rich in glucose and fructose, as carbon source for the production of FucoPol, achieving 1.64 g/L of EPS with volumetric productivity of 0.02 g/L·h (ANTUNES et al., 2017). The high ability of the isolated microorganism for synthesized EPS is perceived since low values for growth rates lead to similar volumetric productivity of EPS as those with higher growth rates.

Concerning the strain characterization, sequencing and phylogenetic analysis of RNA ribosomal 16s gene was made. The phylogenetic tree demonstrating the relationship between the isolated microorganism (coded as LBA725) and known strains of GenBank and RDP databases is showed at Figure 3.

Figure 3 – Phylogenetic tree demonstrating the evolutionary relationships between the partial sequence of the 16s ribosomal RNA gene of the sample **LBA725** and sequences of strains of related microorganisms present in the GenBank and RDP databases



The sample LBA725, isolated from lobeira fruit, was identify as *Kosakonia cowanii,* formerly known as *Enterobacter cowanii* (BRADY et al., 2013). The *Kosakonia cowanni* is a gram negative, facultative anaerobic, motile rod bacteria with yellow pigmentation able to fermented glucose, lactose, sucrose, raffinose, sorbitol, dulcitol, and salicin, and reduce nitrate to nitrite (INOUE et al., 2000).

This strain was first isolated from human blood culture, urine and pus, the *Kosakonia cowanii* is considered an opportunist pathogen (MARDANEH; SOLTAN-DALLAL, 2014) with clinical significance still unknown (INOUE et al., 2000), having pathogenesis suggested by the presence of 262 genes predicted to be virulence genes, but further research is still needed (YANG et al., 2017).

The specie may also be isolated from plants (INOUE et al., 2000) such as eucalyptus (BRADY et al., 2009), tomatoes (SHI; SUN, 2017; VICALVI et al., 2013), where presents antagonistic effect having potential biocontrol effect against fungi (SHI; SUN, 2017), and sugar cane, where presents diazotrophic behavior (ANTONIO et al., 2016). The production of extracellular chitinase, a chitinolytic enzyme, by *K. cowanii* CH-02 is also reported (YOO; PARK, 2015).

However, there is still no report of the production of EPS by *Kosakonia cowanii*. This work could supply evidence for new perspectives and applications of this novel EPS-producer strain, proved to be biotechnologically important, providing necessary information for the development of novel bioprocess and EPS characterization.

4 FINAL CONSIDERATIONS

The epiphytic bacteria isolated from lobeira fruit identify as *Kosakonia cowanii* was able to grow and produce EPS using different carbon sources: glucose, fructose and lactose, even though different metabolic capacity is perceived. This is the first report that *K. cowanii* as an EPS-producer strain.

Achieving values around 1 g/L of EPS production, the optimization of process and medium conditions become valid in order to attain greater specific growth rates and values of production. The knowledge of the isolated microorganism favors the definition of process parameters and medium composition based on microorganism characteristics, which may facilitate the increase of EPS production.

Further EPS characterization is appropriate in order to distinguish the possible differences in composition related to the carbon source used, due to the metabolic flexibility of *K. cowanii* allowing the production of tailor-made polysaccharides.

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CHAPTER 4 – OPTIMIZATION OF EXOPOLYSACCHARIDE PRODUCTION BY NEWLY ISOLATED *Kosakonia cowanii*

ABSTRACT

Microbial exopolysaccharides (EPS) possess great commercial potential due to its numerous uses at different industrial segments, such as food, pharmaceutical, chemical and medical industries. Since its composition and properties can be altered and due to the great diversity of EPS still to be explored, the discovery of new strains capable of synthesize such molecules and novel molecules represents an important step towards the development of new bioprocess and products. A gram-negative bacterium isolated from lobeira fruit (*Solanum lycocarpum*) identify as *Kosakonia cowanii* proved to be good EPS-producer strain. Optimization of medium composition and process conditions was conducted to achieve production increments evaluating the influence of temperature, pH, agitation rates and glucose concentration using Central Composite Design (CCD) and Response Surface Methodology (RSM). Temperature of 28°C, agitation rates above 250 rpm, and glucose concentration of 38% were the most suitable and influential values for EPS production achieving 17.37 g/L. Until now, it is the first time that *K. cowanii* is reported as an EPS-producer strain. The high values of EPS production demonstrate the strain potential for further scale up process and the potential for commercial applications. Further EPS characterization, based on composition, rheological properties and toxicology, besides research on others medium components is still needed for total understanding of the EPS-production process by *K. cowanii*.

CAPÍTULO 4 – OTIMIZAÇÃO DA PRODUÇÃO DE EXOPOLISSACARIDEO UTILIZANDO *Kosakonia cowanii*

RESUMO

Os exopolissacarídeos microbianos (EPS) possuem grande potencial comercial devido aos seus inúmeros usos em diferentes segmentos industriais, como indústrias alimentícia, farmacêutica, química e médica. Uma vez que a sua composição e propriedades podem ser alteradas, e devido à grande diversidade de EPS ainda a ser explorada, a descoberta de novas linhagens capazes de sintetizar tais moléculas, e novas moléculas representa um passo importante para o desenvolvimento de novos bioprocessos e produtos. Uma bactéria gram-negativa isolada de frutos da lobeira (*Solanum lycocarpum*) identificada como *Kosakonia cowanii* provou ser uma boa linhagem produtora de EPS. Otimização da composição do meio de cultivo e das condições do processo foi realizada de forma a obter incrementos de produção, avaliando a influência da temperatura, pH, taxas de agitação e concentração de glicose usando Delineamento Composto Central Rotacional (DCCR) e Metodologia de Superfície de Resposta (RSM). A temperatura de 28°C, taxa de agitação acima de 250 rpm e a concentração de glicose de 38% foram os valores mais adequados e influentes para a produção de EPS atingindo 17,37 g/L. Até o presente momento, é a primeira vez que *K. cowanii* é relatada como uma linhagem produtora de EPS. Os altos valores de produção de EPS demonstram o potencial para futura *scale up* do processo e aplicações comerciais. É ainda necessário a caracterização do EPS, baseada em composição, propriedades reológicas e toxicologia, além da pesquisa com outros componentes do meio de cultivo, para uma compreensão total do processo de produção de EPS por *K. cowanii*.

1 INTRODUCTION

The production of microbial exopolysaccharides (EPS) has been gaining space due to the extensive properties and functions presented (FREITAS; ALVES; REIS, 2011). Widely used at food, chemical, pharmaceutical and medical sectors, EPS can altered rheological properties of diverse products, acting as thickening, emulsifying, chelating, or stabilizing agents, having, also, antioxidant, antibacterial and antitumor activities reported, applied at in wound healing, tissue engineering and drug delivery (RIBEIRO; BURKERT, 2016). The different properties and applications of EPS are due to the variations in monosaccharide composition, molecular weight, condensation linkages, and non-sugar decorations, given rise to distinct structures (BECKER, 2015).

Several factors which control microorganisms metabolism have impact on quantity, quality and structure of the EPS produced, such as pH, incubation temperature and agitation rates, and composition of growth medium, especially regarding the amount and sources of carbon and nitrogen, direct related to the microorganism used (CHUG et al., 2016).

The *Kosakonia cowanii*, formerly known as *Enterobacter cowanii,* is a gram-negative bacteria able to fermented glucose, lactose, sucrose, raffinose, sorbitol, dulcitol, and salicin, and reduce nitrate to nitrite. It is a facultative anaerobic, motile rod bacteria with yellow pigmentation (BRADY et al., 2013; INOUE et al., 2000). The *K. cowanii* used in this work was previously isolated from lobeira fruit (*Solanum lycocarpum*), having its ability to produced EPS proved.

The optimization of process conditions and medium composition is an essential tool for cost-effective production of EPS, aiming higher production yields and reduction of overall process time and energy (YOON et al., 2012). The main strategy for optimization of bioprocess is the One-factor-at-a-time methodology, which assess one variable at a time, however, interaction between factors cannot be analyzed (YOUSUFF et al., 2016).

The use of statistical tools such as Central Composite Design (CCD) and Response Surface Methodology (RSM) are efficient and viable for planning experiments and predicting the optimal conditions of parameters involved in the process, assessing, also, their interactions (FINORE et al., 2014; MOGHANNEM et al., 2018). In these tools, a kinetic model is responsible for describe the variables behavior, elucidating how the bioprocess works, searching optimum values for the variables and desirable response (SIRAJUNNISA et al., 2016). The adequacy of the proposed model is analyzed using analysis of variance (ANOVA) (JAMSHIDIAN et al., 2016).

The EPS production of *K. cowanii*, as far as the authors know, is not reported yet. The manipulation of process and medium conditions enhance the chances of better understanding the mechanisms of EPS production (CRISTINA et al., 2017) by this new EPS-producer strain, achieving higher yields, and providing a new source of microbial polysaccharides. Thus, the aim of this work is to optimize the production of EPS by *Kosakonia cowanii* using statistical tools, varying process and medium conditions.

2 MATERIAL AND METHODS

5.1 Microorganism cultivation

The microbial strain employed in this study was isolated from lobeira fruit (*Solanum lycocarpum*), and characterized by sequencing and phylogenetic analysis of RNA ribosomal 16s gene being identified as *Kosakonia cowanii*. The bacterial strain was maintained on Yeast Medium (YM) Agar (in g/L: agar = 20; glucose = 10; peptone = 5; yeast extract = 3; pH ~ 6.7) and stored at 4ºC.

5.2 Fermentation process conditions

Two full loops of a 24 hours-old culture on a Petri dish were transferred to a 250 mL conical flask containing 100 mL of Yeast Medium broth, consisting in w/v: yeast extract 0.3%, bacteriological peptone 0.5%, and glucose according to the optimization conditions (see item 2.3). The flasks were incubated for 48 hours, according to optimization design conditions (item 2.3) (RÜHMANN; SCHMID; SIEBER, 2015).

5.3 Optimization of exopolysaccharide production

The strategy for the optimization of EPS production was initially based on a 2^3 Central Composite Design (CCD), carried out to evaluate the effects of process variables on the response EPS production. The variables selected were: i) initial pH (4 to 8), ii) temperature (24 to 36°C), and iii) agitation rate (50 to 250 rpm), fixing the glucose percentage at 3.5% (35 g/L). The initial pH values were achieved using HCl and NaOH 1M. The three independent variables were evaluated at five levels (−1.68, −1, 0, 1, +1.68) with 20 experimental runs and 5 central points. Table 1 presents the coded and real values used in the screening of process variables for EPS production.

Table 1 – Coded and real values used in the screening of process variables for EPS production

Variables	Unit	Levels				
		-1.68	-1	0	+1	+1.68
pH	-	4	4,8	6	7,2	8
Temperature	^{o}C	24	26	30	34	36
Agitation	rpm	50	90	150	210	250

After the selection of most influent process variables, the influence of glucose concentration was tested. A second 2^3 CCD was carried out using the follow variables: i) temperature (24 to 36°C), ii) agitation (50 to 250 rpm), and iii) perceptual of glucose (5 to 27%). The independent variables were evaluated at five levels (−1.68, −1, 0, 1, +1.68) with 20 experimental runs and 5 central points. Coded and real values used in the optimization of process and medium variables can be seen at Table 2.

Table 2 – Final coded and real values used in the optimization of process and medium variables for EPS production

Variables	Unit	Levels				
		-1.68	-1	0	+1	+1.68
Glucose	%	5	9	16	23	27
Temperature	^{o}C	24	26	30	34	36
Agitation	rpm	50	90	150	210	250

The study ranges were defined in preliminary tests and all the experiments were carried out in a randomized way. In the best conditions, the cultivations were performed in triplicate to validate the mathematical model for EPS production at 48 hours of process.

5.4 Exopolysaccharide quantification

The EPS production was quantified by dry weight, where the supernatant previously saved was precipitated using three volumes of cold ethanol (4°C) and stored overnight at 4°C. The precipitates were collected by centrifugation at 2000 g, 12 minutes and 4°C, resuspended at one volume of demineralized water being dry at 70°C until constant weight. The results were expressed in g/L (COMTE; GUIBAUD; BAUDU, 2006).

5.5 Cell viability

The cell viability of *Kosakonia cowanii* was measured using the serial dilution methodology (BLODGETT, 2009), in which an aliquot of 3 mL was centrifuged at 2000 g during 12 minutes for biomass removal. The biomass dilutions were transferred to Petri dishes containing Plate Count Agar (PCA) and viable cells were counted after incubation at 28°C and 24 hours.

5.6 Data analysis

Statistical analysis of the experimental data, screening of variables and the response surface methodology were performed using the Statistica 12.0 software (StatSoft Inc, Oklahoma, USA).

6 RESULTS AND DISCUSSION

For wide industrial use and commercialization, bioprocess costs must be effective (PADMANABAN et al., 2015). The optimization of process and medium conditions for EPS production is largely used for different microorganisms including *Phellinus igniarius, Chlamydomonas reinhardtii, Collybia maculate, Bionectria ochroleuca, Alcaligenes sp.*, among others, in order to achieve higher yields and reduction at production costs concerning time and energy, obtaining EPS with desirable properties and applications (BAFANA, 2013; BISWAS; PAUL, 2017; LI et al., 2012; LI; GUO; ZHU, 2016; LIM et al., 2004; LUNG; HUANG, 2010; MAO; MAO; MENG, 2013; TAVARES et al., 2005; WANG; QUAN; ZHOU, 2014; ZHANG et al., 2015).

The *Kosakonia cowanii* used in this studied was isolated from lobeira fruit (*Solanum lycocarpum*), after an extensive screening of the Brazilian fruits jatoba (*Hymenaea courbaril*), jabuticaba (*Plinia cauliflora*), lobeira (*Solanum lycocarpum*), strawberry (*Fragaria × ananassa*) and mango (*Mangifera indica*), at the laboratory of Biotecnologia de Alimentos, from the Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM), campus Diamantina – MG. The strain ability of EPS production was then tested using three different carbon sources (glucose, fructose and lactose), with satisfactory results achieving approximately 1 g/L of EPS production for all tested carbon sources. Thus, the screening of most influential variables and optimization of EPS production using *Kosakonia cowanii* isolated from lobeira fruit (*Solanum lycocarpum*) represents an important step towards the development of novel bioprocess using a newly EPS-producer strain, initiating its metabolic and behavior understanding.

For all experimental runs, both in screening of variables and optimization of the response EPS production, glucose was chosen as sole carbon source. Previously data, (see Chapter 3) do not show significant difference at EPS production among others tested carbon sources (fructose and lactose), besides, glucose has higher abundance and lower cost when compared to the others, assisting the cost-effective production of EPS by *K. cowanii*.

In order to evaluate only process parameters, such as pH, temperature and agitation, identifying the best fit for the EPS production process, a 2^3 CCD was conducted. The first CCD aimed to recognize the significant important process parameters for further analysis with the carbon source, the most influent medium component. Table 3 represents the responses obtained from the screening of processes variables concerning EPS production at different experimental runs.

Table 3 – 2^3 CCD matrix with real values and responses obtained at the evaluation of processes variables for EPS production

	pH	Temperature (°C)	Agitation (rpm)	EPS production (g/L)
1	4.8	26	90	4.17
2	7.2	26	90	4.37
3	4.8	34	90	1.92
4	7.2	34	90	1.89
5	4.8	26	210	6.69
6	7.2	26	210	5.57
7	4.8	34	210	4.26
8	7.2	34	210	4.31
9	4	30	150	3.01
10	8	30	150	4.08
11	6	24	150	4.88
12	6	36	150	0.54
13	6	30	50	0.83
14	6	30	250	3.61
15	6	30	150	4.06
16	6	30	150	3.77
17	6	30	150	4.77
18	6	30	150	4.53
19	6	30	150	3.65
20	6	30	150	3.96

In summary, depending on the conditions applied, the values for EPS production varied from 0.54 g/L to 6.69 g/L after 48 hours of fermentative process. Experiments carried out with temperatures below 30°C and higher agitation rates (150 to 200 rpm) achieved the best results. At all range tested for pH values (4 to 8), is not possible to perceive a direct influence into EPS production, which achieved similar results when only pH was altered (experiments 9 and 10). It can be verified the small variation among the central points experiments (15 to 20), which indicates good repeatability of the process. The data was treated with statistical software STATISTICA 12.0, generating regression coefficients and respective statistical analysis of the parameters considered as it can be observed at Table 4.

Table 4 – Regression coefficients and statistical analysis of process parameters for 48 hours of process

	Regression coefficient	Standard error	t(15)	*p-value*
Mean	**4.07467**	**0.374787**	**10.87196**	**0.000001**
pH (L)	0.06537	0.248790	0.26276	0.798067
pH (Q)	0.09231	0.242470	0.38071	0.711379
Temperature (L)	**-1.14997**	**0.248790**	**-4.62225**	**0.000947**
Temperature (Q)	-0.20295	0.242470	-0.83699	0.422147
Agitation (L)	**0.96333**	**0.248790**	**3.87206**	**0.003099**
Agitation (Q)	-0.37616	0.242470	-1.55138	0.151856
pH (L) x T (L)	0.11806	0.324917	0.36334	0.723913
pH (L) x A (L)	-0.15417	0.324917	-0.47448	0.645349
T (L) x A (L)	0.12917	0.324917	0.39754	0.699324

T temperature, A agitation, L linear, Q quadratic.
Parameters in bold are statistically significant for the model ($P < 0.05$).

The significance of each coefficient was determined by *t*-Student e *p*-value, where the greater the magnitude of the *t*-value and lower the *p*-value, more significant is the corresponding coefficient. The negative sign (-) before the *t*-student value represents a negative interaction of the variable with the response, in which a reduction at the variable imply an increase at the response. In this way, only temperature and agitation were statistically significant, being possible to assemble the model including only significant terms, with a significance level of 0.05, as it can be seen below:

$$Y = 4.07467 - 1.14997x_2 + 0.96333x_3$$

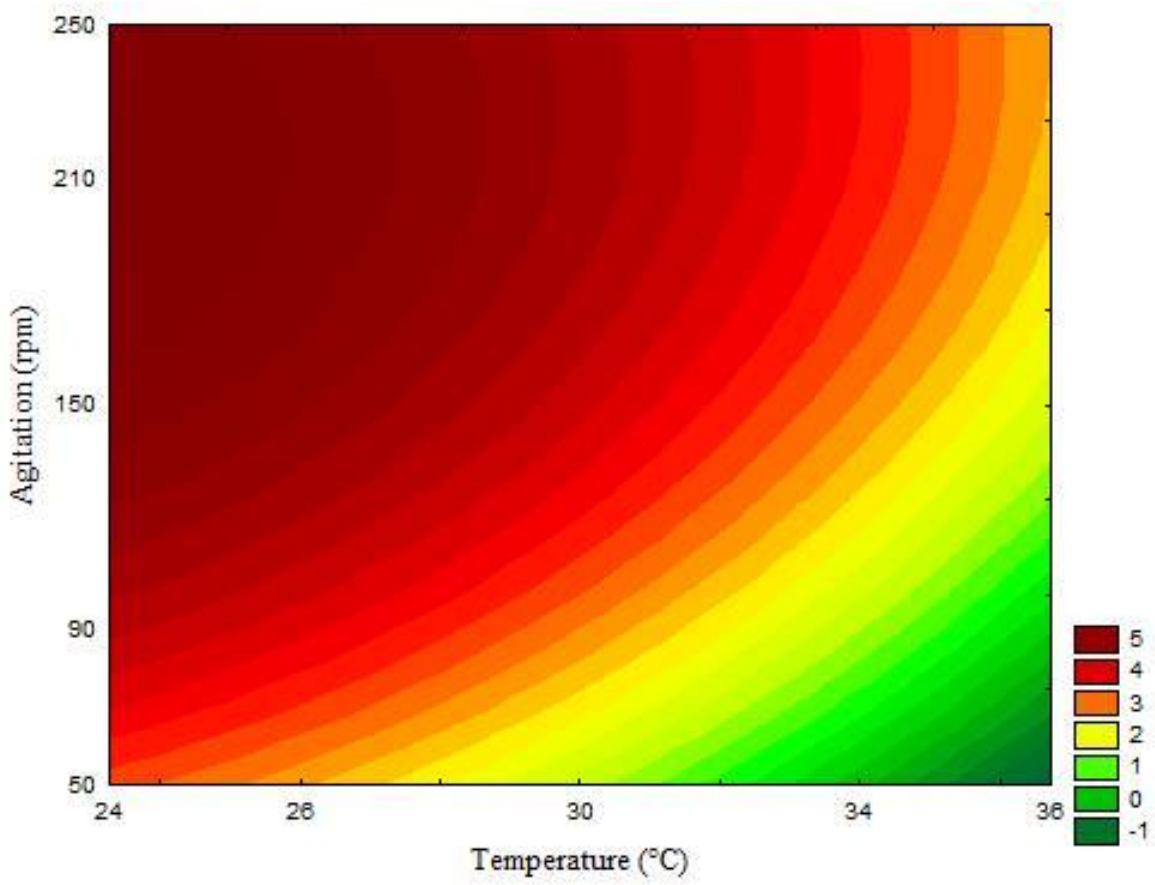
In order to verify the validity of the model, an analysis of variance (ANOVA) was performed (Table 5).

Table 5 – ANOVA for the model of EPS production using process variables

Variation source	Sum of squares	Degrees of freedom	Mean square	*F* value	*P* value
Regression	33.21	5	6.64	11.01	0.0002
Residues	8.45	14	0.60		
Total	41.66	19			
R^2 = 0.80				$F_{0.95(5,14)}$ = 2.96	

TAB. 5 demonstrates the satisfactory model fit for the processes. The value calculated for *F* was greater than the respective listed value, proving the model validity for these variables, and *P* value was lower than 0.05. Thus, response surface of the significant variables can be built to illustrate the behavior of each variable at the model (Figure 1).

Figure 1 – Contour plot of the production of EPS after 48 hours of process as a function of temperature and agitation. PH was fixed at central point value, 6.0



The optimal conditions could be determined using mathematical methods (derivation of model´s equation). The surface response also allows to verify the optimal conditions graphically and define the best conditions to each variable applied in the experiment. In this case, an increase in agitation and temperatures below 30°C, favors EPS production. Values of pH show no influence on EPS production.

Due to practical reasons, it was decided that 26°C, 250 rpm, normal medium pH (~ 6.7), and glucose 3.5% were the best conditions for the model, achieving values of 4.46±0.02 for EPS production. The predicted values were not reached possibly due to equipment limitation, in which agitation values greater than 250 rpm can not be achieved.

As the carbon source, in this case glucose, is essential for the assembling of the EPS (BARBOSA et al., 2004; CZACZYK; MYSZKA, 2007), tests to evaluate the influence of the amount of glucose at the medium were conducted, in order to define test ranges for the second CCD. Using the conditions defined at the previously optimization (26°C and 250 rpm), five different glucose concentrations were tested (3.5, 6, 10, 20 and 30%) and at the end of incubation time (48 hours), the EPS produced was quantify by dry weight. The results obtained can be observed at Table 6.

Table 6 – EPS production after 48 hours using different amounts of glucose, 26°C and 250 rpm

Glucose concentration	**EPS production (g/L)**
3.50%	3.56±0.04a
6%	3.17±0.05a
10%	4.86±0.08a
20%	9.13±0.68b
30%	12.62±0.27c

Mean values followed by the same lowercase letter in columns do not differ by Tukey test (5% probability).

From TAB. 6 it is possible to observe that as the amount of glucose increases, production values also increase, with significant differences being noted at values of glucose greater than 10%. In this way, the percentage of glucose used at medium preparation directly influences the amount of EPS produced by the bacteria *Kosakoniia cowanii*. Similar results were obtained when evaluated the EPS production by *Phellinus nigricans* (WANG; QUAN; ZHOU, 2014), *Cordyceps militaris* (CUI; JIA, 2010), *Pseudomonas fluorescens* (SIRAJUNNISA et al., 2016), where an increase at glucose concentration have positive effect at EPS production.

The final optimization was performed using glucose, temperature and agitation as variables based on the results obtained above, in which these were the most significant variables for EPS production by *K. cowanii*. The responses and values used at the experimental run can be seen at Table 7.

Table 7 – 2^3 Central Composite Design matrix with real values and responses obtained from variables optimization for EPS production

	Glucose (%)	Temperature (°C)	Agitation (rpm)	EPS production (g/L)
1	9	26	90	3.34
2	23	26	90	8.04
3	9	34	90	1.96
4	23	34	90	8.66
5	9	26	210	3.60
6	23	26	210	10.00
7	9	34	210	2.36
8	23	34	210	6.78
9	5	30	150	2.71
10	27	30	150	9.26
11	16	24	150	5.99
12	16	36	150	4.74
13	16	30	50	5.19
14	16	30	250	7.00
15	16	30	150	6.41
16	16	30	150	6.86
17	16	30	150	6.77
18	16	30	150	6.78
19	16	30	150	6.81
20	16	30	150	7.09

Values varying from 1.96 g/L to 10.00 g/L were observed for EPS production when using glucose, temperature and agitation as variables. There was an increase at EPS production values when compared to the first CCD (from 6.69 g/L to 10.00 g/L). High values were achieved for the experiments with amounts of glucose above 23%, in which the best results experiments (6 and 10) possess glucose of 23 and 27%, temperature of 26 and 30°C, and agitation rates of 210 and 150 rpm, respectively. These results were in agreement with the previously results found in this

work. Few variations among the central points experiments (25 to 30) are observed, which indicates the good repeatability of the process. The treated date can be observed at Table 8.

Table 8 – Regression coefficients and statistical analysis of process and medium parameters for 48 hours of process

	Regression coefficient	Standard error	t(15)	*p-value*
Mean	**6.790600**	**0.302758**	**22.42916**	**0.000000**
Glucose (L)	**2.434399**	**0.200976**	**12.11289**	**0.000000**
Glucose (Q)	**-0.318426**	**0.195870**	**-1.62570**	**0.013507**
Temperature (L)	**-0.477576**	**0.200976**	**-2.67483**	**0.023299**
Temperature (Q)	**-0.536916**	**0.195870**	**-2.74118**	**0.020790**
Agitation (L)	0.276735	0.200976	1.37696	0.198566
Agitation (Q)	-0.279058	0.195870	-1.42471	0.184693
G (L) x T (L)	0.002778	0.262472	0.01058	0.991764
G (L) x A (L)	-0.072222	0.262472	-0.27516	0.788794
T (L) x A (L)	-0.461111	0.262472	-1.75680	0.109469

G glucose, T temperature, A agitation, L linear, Q quadratic.
Parameters in bold are statistically significant for the model ($P < 0.05$).

The significant variables for EPS production by *K. cowanii* after 48 hours of process were glucose and temperature (both linear and quadratic), with linear glucose the most significant one with positive effect at EPS production, while temperature as negative effect. The model assembling with only the statistically significant variables was:

$$Y = 6.790600 + 2.434399x_1 - 0.318426x_1^2 - 0.477576x_2 - 0.536916x_2^2$$

The validity of the model assembled was verify using an analysis of variance (ANOVA) (Table 9).

Table 9 – ANOVA for the model of EPS production using process and medium variables

Variation source	Sum of squares	Degrees of freedom	Mean square	*F* value	*P* value
Regression	93.36	9	10.37	18.82	0.0000
Residues	5.51	10	0.55		
Total	98.87	19			
$R^2 = 0.94$				$F_{0.95(9,10)} = 3.02$	

The value calculated for *F* was approximately six times greater than the respective listed value, and *P* value was lower than 0.05, which indicates the satisfactory of fitted model for this process. The value of R^2 was 0.94, indicating 94.00% of the variability in the response could be explained by the model. The contour plot of significant variables and its behavior can be observed at Figure 2 and Figure 3.

Figure 22 – Contour plot of the production of EPS after 48 hours of process as a function of glucose and temperature. Agitation was fixed at central point value, 150 rpm

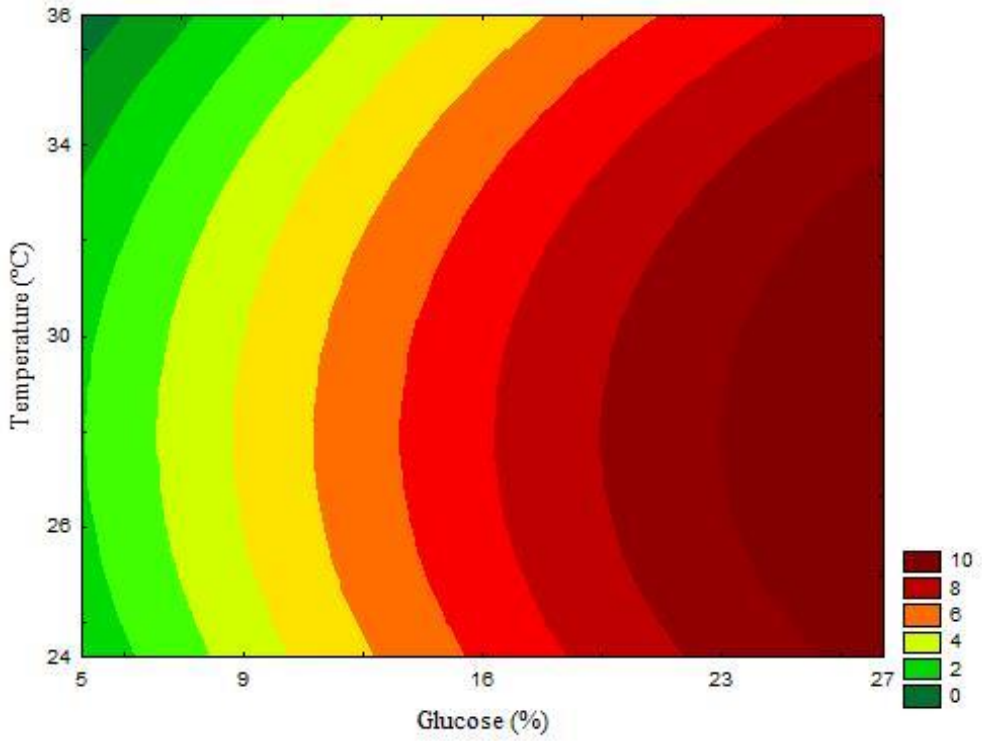
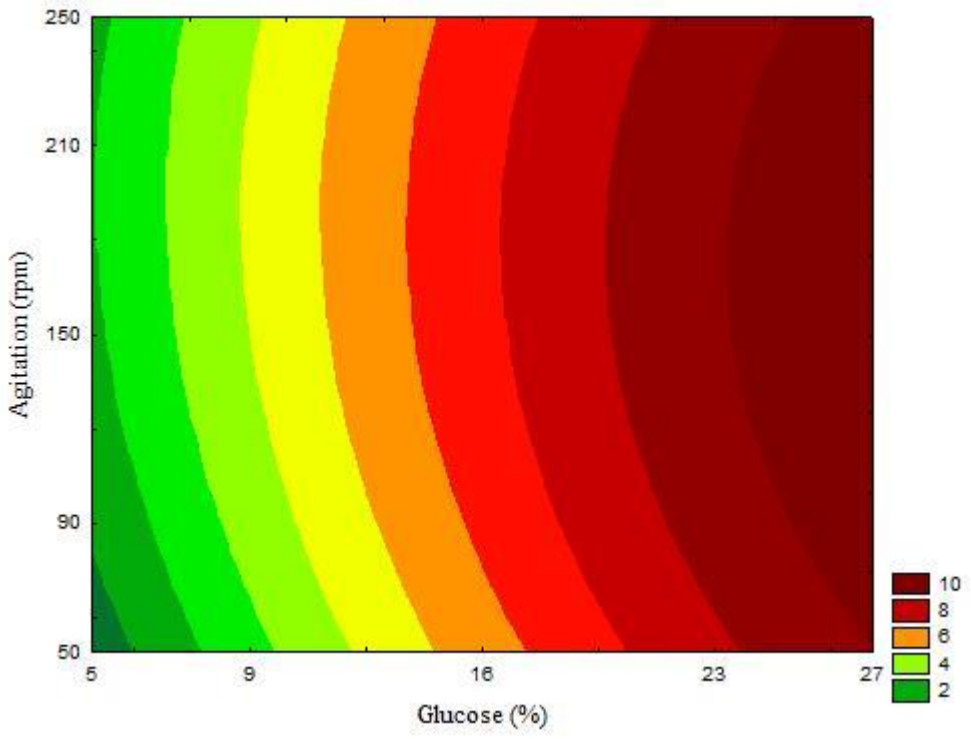


Figure 3 – Contour plot of the production of EPS after 48 hours of process as a function of glucose and agitation. Temperature was fixed at central point value, 30°C



The profiles obtained at the contour plote is close to the ideal. Both FIG. 2 and FIG. 3 presented the predicted optimal regions within the levels studied.

FIG. 2 shows the effect of interaction between glucose and temperature. An increase at the amount of glucose used favors the increase of EPS production when temperatures between 26 and 30°C are used. FIG. 3 demonstrated the effect between glucose and agitation, where a similar pattern is observed, but an increase at glucose amount together with an increase at agitation rates leaves to greater levels of EPS production.

The suitable temperatures and agitations for EPS production vary according to the microorganism used. Incubations temperatures below optimum temperature of microorganism growth can lead to better yields for EPS production (CERNING et al., 1992; KUMAR; MODY; JHA, 2007), while high agitation rates favors the use of molecular oxygen as a primary source of energy metabolism and prevents poorly distribution of oxygen as the viscosity of the medium at submerged fermentation increases due to the production of EPS (KUMAR; MODY; JHA, 2007). According to the Leibniz Institute DSMZ, a German catalogue of microorganisms and cell cultures, *Kosakonia cowanii* is a facultative anaerobic mesophilic bacteria with growth temperature of 30°C (DSMZ, 2018). The results found at the contour plot suggests that the metabolic pathway for the production of EPS by *K. cowanii* is an aerobic process benefited by temperatures below 30°C, with no pH influence.

Both glucose amount and agitation rates propitiate higher values of EPS production at levels higher than those studied. An indication of ideal conditions can be obtained by the derivation of the model's equation, obtaining levels value of 3.70 for glucose and -0.444 for temperature. These levels correspond to 59.46% of glucose, and 28.24°C respectively.

For the validation of the process model, processes conditions were as the predicted by the model: 28°C for temperature and agitation of 250 rpm, since greater agitation rates are not possible due to the equipment limitations. For glucose concentration, values of 27% (the highest level tested), 38% (a concentration determined for 24 hours of incubation – data not show), and 60% were tested. In addition, as the concentrations of glucose were higher than the expected, a serial dilution methodology (BLODGETT, 2009) was execute to evaluate the cell viability at all three conditions tested for validation. Validation results and cell viability can be seen at Table 10.

Table 10 – EPS production and cell viability of *Kosakonia cowanii* after 48 hours using different amounts of glucose, 28°C and 250 rpm

Glucose concentration	EPS production (g/L)s	Cell viability (log UFC/mL)
27%	13.85±1.03a	5.71±0.07a
38%	17.37±1.47a	5.56±0.02b
60%	15.15±0.82a	5.52±0.01b

Mean values followed by the same lowercase letter in columns do not differ by Tukey test (5% probability).

A maximum production of EPS was achieved using 38% (380 g/L) of glucose during 48 hours, followed by 60% (600 g/L) and 27% (270 g/L), at 28°C and 250 rpm. Despite there was no significant difference between the results, increases of approximately 14, 18 and 16 times for 27%, 38% and 60% respectively, can be observed when compared to the production using 1% (10 g/L) glucose, which achieve 0.96 g/L (see Chapter 3). The results achieved were in good agreement with the predict response.

The optimum glucose concentration used in this work was higher than most concentrations used for EPS production by other microorganisms. The optimum level of glucose for *Ganoderma lucidum* was 60 g/L for EPS production (YUAN; CHI; ZHANG, 2012), 51.67 g/L for *Phellinus nigricans* (WANG; QUAN; ZHOU, 2014), 30 g/L for *Morchella esculenta* (MENG et al., 2010), 100 g/L for *Lactobacillus paracasei* (XU et al., 2010) and 20 g/L for *Xanthomonas campestris* (HABIBI; KHOSRAVI-DARANI, 2017).

The cell viability of *Kosakonia cowanii* for the different glucose concentrations , presents satisfactory results, showing the strain ability of adapt at extreme conditions, indicating

that its EPS production may be a strategy to endure adverse conditions, which is common observed for EPS-producers strains from deep sea bacteria (FINORE et al., 2014). The glucose concentration also affects the metabolic path of EPS production, in which the best production results were achieved (17.37 g/L) when intermediate values for viable cells (5.56 log UFC/mL) were detected and an intermediate concentration of glucose was used (380 g/L), indicating the use of carbon source for the production of EPS. When lower (270 g/L) values of glucose were used, the strain presents more viable cells. The bacteria *Lactobacillus paracasei* presented the same pattern when glucose concentrations varying from 50 g/L to 250 g/L were used. Is was observed the decreased at cell viability and EPS production as the glucose concentration was increased, being the optimum concentration 100 g/L (XU et al., 2010).

Thus, optimization of EPS production by *Kosakonia cowanii* shows the novel EPS-producer strain as a viable option for the development of bioprocess, aiming its potential uses and further industrial application.

7 FINAL CONSIDERATIONS

This work studied the effect of agitation, temperature and glucose concentration on EPS production using *Kosakonia cowanii* through Response Surface Methodology (RSM). The optimal levels for temperature (28°C), agitation (above 250 rpm) and glucose concentration (above 27% or 270 g/L) were determined to enhance EPS yield by 18 times when compared with the yield obtained before the optimization (from 0.95 to 17.37 g/L). Validation experiments were also carried out to verify the adequacy and the accuracy of the model, and the results showed that the predicted value agreed with the experimental values well, using 38% of glucose as carbon source.

The optimum conditions obtained in this experiment gives a basis for further study with large-scale batch fermentation in a bioreactor for the production of EPS. As far as the authors know, this was the first time that EPS production from *Kosakonia cowanii* was optimized. The ability of the strain to adapt to extreme conditions was also observed when high concentrations of glucose were used (from 270 g/L to 600 g/L). Further research of medium composition and characterization of the EPS is still necessary for total understanding of EPS production by this strain.

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CONSIDERAÇÕES FINAIS

A presente dissertação apresentou os resultados obtidos utilizando a bioprospecção de microrganismos de frutas brasileiras para a produção biotecnológica de exopolissacarídeos (EPS), visando o desenvolvimento e otimização do processo fermentativo.

O grande interesse industrial por EPS é respaldado pela literatura científica, devido a seu grande potencial de aplicação e variedades de propriedades, sendo utilizado pelas industrias alimentícia, química, farmacêutica e médica.

A busca por novas linhagens produtoras de EPS torna-se essencial para a descoberta de novas moléculas e desenvolvimento de novos bioprocessos, além de oferecer vantagens em relação a obtenção química de tais compostos.

Nesse estudo 25 linhagens provenientes de frutas brasileiras foram analisadas quanto a sua capacidade de produção de EPS, apresentando um resultado positivo. A linhagem capaz de produzir EPS foi isolada a partir de frutos de lobeira e identificada como *Kosakonia cowanii,* uma bactéria gram-negativa.

A habilidade de secreção de EPS pela *K. cowanii* foi testada em diferentes fontes de carbono e seu processo fermentativo otimizado em relação a condições de processo (pH, temperatura e agitação) e concentração de fonte de carbono (glicose) presente no meio, visando incremento na produção.

Até onde é de conhecimento dos autores, é a primeira vez que a produção de EPS por *K. cowanii* é descrita, seguida de sua otimização.

O presente trabalho possibilita o entendimento inicial do processo metabólico de produção pela bactéria isolada, abrindo precedentes para pesquisas futuras quanto a outras variáveis influentes na produção de EPS, além da caracterização do EPS produzido e suas possíveis aplicações.