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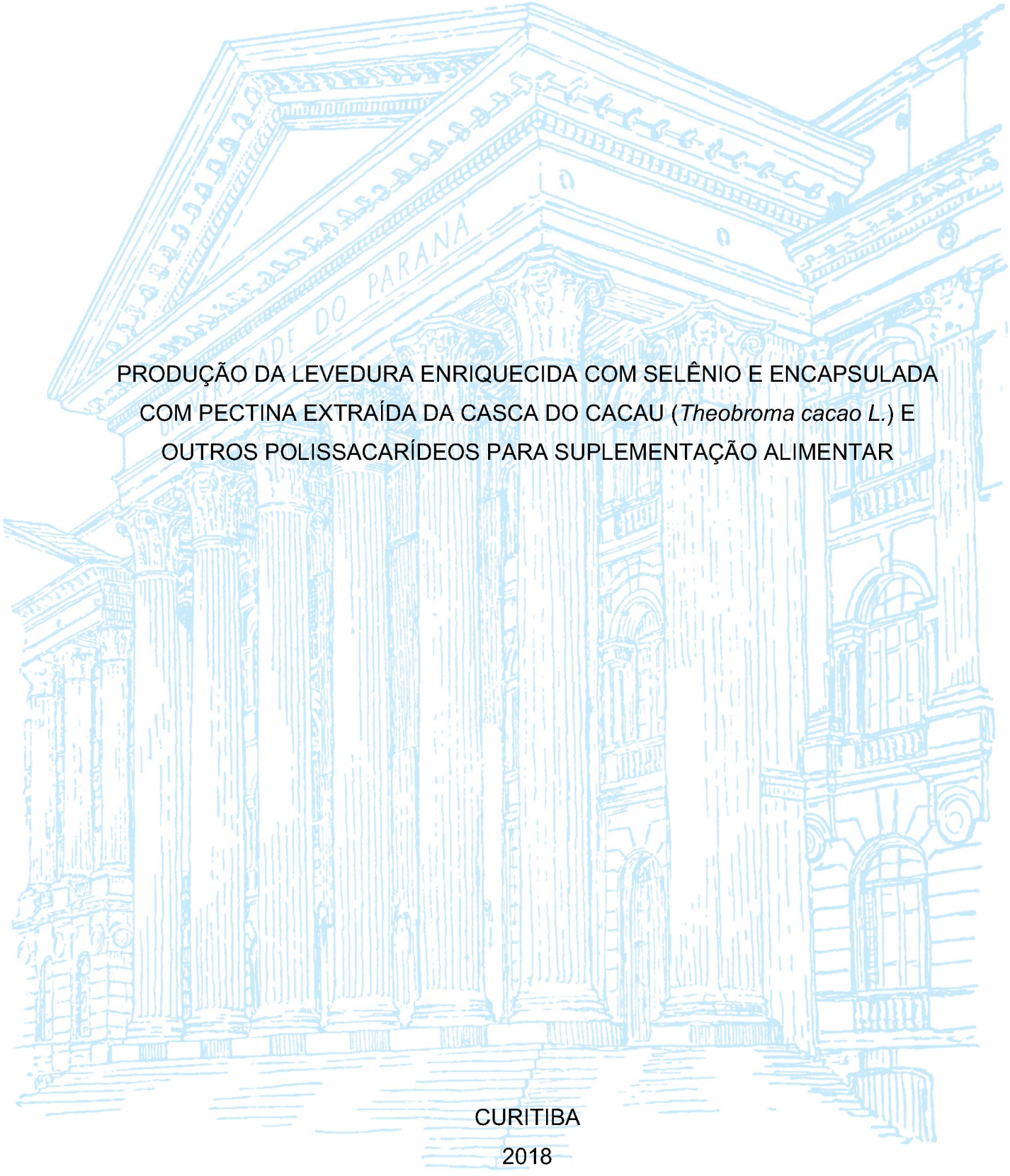
NATÂNIA MARTINS SABATH



PRODUÇÃO DA LEVEDURA ENRIQUECIDA COM SELÊNIO E ENCAPSULADA COM PECTINA EXTRAÍDA DA CASCA DO CACAU (*Theobroma cacao L.*) E OUTROS POLISSACARÍDEOS PARA SUPLEMENTAÇÃO ALIMENTAR

CURITIBA

2018

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Dissertação apresentada ao curso de Pós-Graduação em engenharia de bioprocessos e biotecnologia, Setor de tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em engenharia de bioprocessos e biotecnologia.

Orientadora: Profa. Dra. Luciana Porto De Souza Vandenbergh

Co-orientadora: Profa. Dra. Cristine Rodrigues e Prof. Dr. Carlos Ricardo Soccol

CURITIBA

2018

Catalogação na Fonte: Sistema de Bibliotecas, UFPR
Biblioteca de Ciência e Tecnologia

S113p Sabath, Natânia Martins
Produção da levedura enriquecida com selênio e encapsulada com pectina extraída da casca do cacau (Theobroma cacao L.) e outros polissacarídeos para suplementação alimentar / Natânia Martins Sabath. – Curitiba, 2018.

Dissertação - Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-graduação em Engenharia de Bioprocessos e Biotecnologia, 2018.

Orientador: Luciana Porto de Souza Vandenbergh – Coorientador: Cristine Rodrigues – Coorientador: Carlos Ricardo Soccol.

1. Levedos. 2. Pectina. 3. Polissacarídeos. 4. Suplementos dietéticos. 5. Melaço. 6. Cana-de-açúcar - Derivados. I. Universidade Federal do Paraná. II.Vandenbergh, Luciana Porto de Souza. III. Rodrigues, Cristine. IV. Soccol, Carlos Ricardo. V. Título.

CDD: 579.563

Bibliotecário: Elias Barbosa da Silva CRB-9/1894

MINISTÉRIO DA EDUCAÇÃO
SETOR SETOR DE TECNOLOGIA
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA - 40001016036P8

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **NATÂNIA MARTINS SABATH** intitulada: **Produção da levedura enriquecida com selênio e encapsulada com pectina extraída da casca do Cacau (Theobroma cacao L.) e outros polissacarídeos para suplementação alimentar**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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A todos que sempre me inspiraram a dar o melhor
de mim e em especial à minha família.

AGRADECIMENTOS

Agradeço primeiramente a Deus, pelo amor incondicional, pelas oportunidades dada e pelas pessoas maravilhosas que ele colocou em minha vida durante o mestrado.

Agradeço à professora Luciana Vandenbergh pela disposição em me orientar, pelas conversas, ensinamentos, pela preocupação e também por todo suporte dado durante todos os momentos.

Agradeço à professora Cristine Rodrigues pela co-orientação, conversas, ensinamentos e por sempre me desafiar a encontrar a solução para os meus problemas. À Val Tanobe por estar sempre disposta a ajudar em análises externas, por todo apoio e ensinamento. Aos professores Ciro Ribeiro e Júlio Carvalho, por disponibilizar equipamentos e pelo acompanhamento na análise das amostras. Ao Centro de Microscopia Eletrônica (CME) e ao Departamento de Química por me autorizar a usar equipamentos e por me auxiliar. Agradeço também a CAPES e a rede integrada BIOCAU e seus discentes, pelo financiamento da pesquisa.

Agradeço também aos meus amigos, Adonay, Ana Caroline, Anderson, Carol, Débora, Jéssica e Matheus por sempre estarmos juntos desde o inicio. Obrigada pelo apoio durante as provas, durantes os momentos de dúvida e por sempre estarmos juntos em todas experiências que tivemos. Obrigada também Carol e Alexander pelas noites no laboratório e por todo apoio emocional que sempre me deram. Amo vocês. Obrigada por todo apoio e por acreditar em mim!

Aos meus pais, meus tios, primos, a minha irmã e meus amigos por sonharem meus sonhos e sempre acreditarem em mim e por entenderem minha ausência nas datas comemorativas. Em especial agradeço às minhas amadas avós por suas orações. Obrigada pelo apoio e por estarem comigo todo tempo, amo vocês incondicionalmente.

Por fim, ao Laboratório de Engenharia de Bioprocessos e Biotecnologia, pela oportunidade de desenvolver este projeto e me propiciar meios pelos quais eu pude realizá-lo. Obrigada a Mitiyo pelo auxílio, pela força e também pelas broncas que me fizeram crescer e aprender. E especialmente aos meus companheiros de bancada Antônio, Marcela e Luiz por toda parceria e ensinamento. Obrigada pela ajuda, pela paciência e principalmente pela amizade de você!

Success consists of going from failure to failure
without loss of enthusiasm
(Winston Churchill)

RESUMO

Diversos microrganismos são capazes de bioacumular e biotransformar elementos traço do ambiente em formas biodisponíveis. Dentre estes o elemento selênio (Se) vem sendo relatado por seus benefícios à saúde humana e animal. As leveduras enriquecidas em Se podem ser uma possível fonte deste mineral, sendo utilizada para suplementação alimentar humana e de animais. A levedura *Wickerhamomyces anomala* LPB-CPB5 classificada como um micro-organismo GRAS (Geralmente Reconhecida como Segura) apresenta capacidade de bioacumular diferentes minerais, o que demostra o seu potencial para suplementação dietética do elemento traço selênio. Durante a produção de levedura enriquecida, é importante atentar para a manutenção da viabilidade e estabilidade. As técnicas de liofilização e *spray-drying* são conhecidas para a conservação de leveduras à longo prazo. Ambas podem usar agentes crioprotetores/adjuvantes que auxiliam a manutenção da levedura. Polissacarídeos, como a pectina e o Kefiran, possuem ação protetora e propriedades promotoras de saúde, sendo polímeros adequados para o encapsulamento de leveduras enriquecidas. A pectina é um polissacarídeo que pode ser extraído de diversas frutas e vegetais. A casca de cacau (CH) é o principal produto residual da cadeia de produção de chocolate, sendo relatada com uma boa fonte de pectina. O objetivo deste trabalho é revisar a capacidade de leveduras em bioacumular oligoelementos, apresentando o seu potencial em acumular selênio para suplementação alimentar, discutindo a importância da microencapsulação utilizando polissacarídeos naturais. Também visou-se a otimização da extração de pectina da CH avaliando a pectina extraída e seu uso como adjuvante para o microencapsulamento da levedura enriquecida em Se individual ou conjuntamente com o amido de milho e Kefiran. O meio de cultivo otimizado foi em meio contendo melaço de cana-de-açúcar, ureia, extrato de levedura, KH_2PO_4 e $(NH_4)_2PO_4$, com cultivo em "fed-batch" de 10 h de alimentação e duração de 48 h. O uso do melaço torna o meio nutritivo e barato por ser uma rica fonte de carbono e de micronutrientes. A presença de selênio ocasionou uma inibição no crescimento da levedura, quando em altas quantidades de selênio. O maior bioacúmulo de selênio foi de 9,51 mg/g de levedura, em "fed-batch" de 48 h, quando alimentado com 200 mg/L de Se. A alimentação de 50 mg/L foi escolhida para os testes de microencapsulação por alcançar 12,70 g/L de biomassa e uma concentração de selênio bioacumulado de 3,94 mg de Se/g de levedura, na análise com Azure B, e 2,25 mg of Se/g na análise em ICP-OES. Um planejamento experimental fatorial completa 3^2 com 3 pontos centrais foi realizado para a otimização das condições de extração de pectina. A extração de pectina alcançou melhor rendimento (15,66%) com uma concentração de CH de 10% (m/v), durante 120 min. A pectina extraída foi analisada por FT-IR obtendo-se um baixo grau de metil-esterificação, classificando-a como pectina LM. A matriz de maior eficácia para o encapsulamento de leveduras (EE) foi a T1 (amido) com EE = 88,75%, enquanto as de maior conservação foram as matrizes T3 (amido e Kefiran) e T2 (amido e pectina de cacau) com taxa de sobrevivência, respectivamente, de 100,00% e 95,66%. Este trabalho mostra a capacidade da levedura LPB-CPB5 de bioacumular selênio e as vantagens da utilização da pectina de cacau e do Kefiran como matrizes crioprotetoras, mostrando-se uma metodologia simples e de baixo custo.

Palavras-chave: *Wickerhamomyces anomala* 1. Bioacúmulo 2. Extração de pectina 3. Polissacarídeos 4. Suplementação alimentar 5. Melaço de cana 6.

ABSTRACT

Many microorganisms are capable of bioaccumulate and biotransform trace elements from the environment in bioavailable forms. Those elements have functional and structural roles in the organism. From those the element selenium (Se) has been reported for its health benefits. Se-enriched yeasts can be a possible source of this mineral, been used in human and animals' dietary supplementation. The mineral bioaccumulation capability of *Wickerhamomyces anomalus* LPB-CPB5 and its GRAS (Generally Recognized as Safe) classification show the potential of this yeast to be used in dietary supplementation of the trace element selenium. During the enriched yeast production, it is important to beware about its maintain. The freezer-dryer and spray-dryer are known techniques to yeast conservation at long term. Both techniques can use cryoprotective/adjuvants to help yeast maintenance. Polyssacharides, such as pectin and Kefiran has protective action and health promoting properties, been suitable for enriched yeasts encapsulation. Pectin is a hydrocolloid polyssacharides that can be extracted from many fruits and vegetables. Cocoa husk (CH), main waste product of chocolate production chain, been reported as a good source of pectin. The aim of this work is to review the capacity of yeasts to bioaccumulate trace elements, presenting the potential of Se-enriched yeasts, and discussing the importance of microencapsulation using nature polysaccharides. Also, it is to optimize pectin extraction from CH, characterizing it, and to evaluate its use as adjuvant in the microencapsulation of the Se-enriched-yeast together with corn starch and also Kefiran. The optimum culture medium contained sugar cane molasses, urea, yeast extract, KH2PO4 and (NH4)2PO4, used in a fed-batch culture with fed at 10 h and duration of 48 h. The use of molasses makes the medium nutritious, because it is a rich source of carbon and micronutrients, and cheap, been a by-product of the sugarcane industry. The presence of selenium caused a small inhibition in the growth of yeast, when in high amounts of selenium. The highest bioaccumulation of selenium for the Azure B analysis was 9.51 mg / g of yeast, in fed-batch of 48 h, when fed with 200 mg/L of selenium after 24 h of culture. The 50 mg/L feed was chosen for the microencapsulation tests to reach a high biomass (12.70 g/L) and a bioaccumulated selenium concentration of 3.94 mg Se / g yeast for the Azure B analysis, and 2.25 mg Se / g yeast for the ICP-OES analysis. A complete factorial experimental design 32 with 3 central points was carried out to optimize pectin extraction conditions. Pectin extraction achieved a better yield (15.66%) at CH concentration of 10 % (w/v) for 120 min. The extracted pectin was analyzed by FT-IR obtaining a low degree of methyl esterification, classifying it as pectin LM. The most effective matrix for the encapsulation of yeasts (EE) was T1 (starch) with EE = 88.75%, while the ones with the highest conservation were the T3 (starch and Kefiran) and T2 (starch and cocoa pectin) with survival rate, respectively, of 100.00% and 95.66%. This work shows the ability of LPB-CPB5 to bioaccumulate selenium and the advantages of using cocoa pectin and Kefiran as cryoprotective matrices, proving to be a simple and low-cost methodology.

Keywords: *Wickerhamomyces anomalus* 1. Bioaccumulation 2. Pectin extraction 3. Polyssacharydes 4. Dietary supplemantation 5. Sugarcane molasses 6.

FIGURE LIST

TABLE LIST

LIST OF ABBREVIATIONS

CFU	- Colony-forming unit
DE	- Degree of Esterification
EFSA	- European Food Safety Authority
FDA	- U S Food and Drug Administration
FT-IR	- Fourier transform infrared spectroscopy
GalA	- Galacturonic acid
HG	- Homogalactorunan
HM	- High Methoxyl
ICCO	- International
LC-MS	- Liquid chromatography - mass spectrometry
LM	- Low Methoxyl
GRAS	- Generally recognized as safe
SEM	- Scanning Electron Microscopy
WCF	- World Cocoa Fundation
CH	- Cocoa husk powder

LIST OF SYMBOLS

α - Alfa

β - Beta

® - Trademark

SUMMARY

CHAPTER 1 - OVERVIEW ABOUT ENRICHED YEAST

1.1 INTRODUCTION

All living organisms can accumulate different substances from the environment. Some microorganisms, such as yeasts, can accumulate and biotransform those substances in organic forms, by the bioaccumulation process (YIN et al., 2009). This process is frequently mistaken with the biosorption process, however the main difference is related to the metabolism, been the first one active and the second passive. The path, through which a bioaccumulant enters an organism, can be the same as it takes up nutrients; in unicellular organisms, for example, metals can enter through the transport channels (CHOJNACKA, 2009). Many yeast can naturally bioaccumulate trace elements, been enriched yeasts a promising tool for diet supplementary (CHOJNACKA, 2009; KIELISZEK et al., 2015).

Trace elements, which are also called microelements, are chemical particles required in small amounts by all living organisms. They are constituents of numerous proteins, enzymes and hormones, showing functional and structural roles in the microorganism (KIELISZEK et al., 2015; RYCEWICZ-BORECKI et al., 2016). The microelements have a slight limit between benefit and toxicity to the cell; its deficiency can be related to pathological conditions (GÖNEN; AKSU, 2008), while high concentrations can form toxic compounds (CHEN et al., 2005; GÖNEN; AKSU, 2008). Some trace elements, such as copper, iron, magnesium, zinc, and selenium show functional and structural properties, from those selenium has been defined as an essential micronutrient endowed with significant health benefits (ALLMANG et al. 2009).

The element selenium (Se) can be found in the environment in organic and inorganic forms. Inorganic selenium forms are often selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}), while the organic forms are selenoamino acids (selenomethionine - SeMet and selenocysteine - SeCys), selenopeptides (e.g. plasma selenoprotein P), and selenoenzymes (e.g. glutathione peroxidase) (MÉPLAN, 2011; KIELISZEK et al., 2015; TIE et al., 2017). The selenium functions are related to the selenoproteins applications, such as antioxidant activity, protection against infection, and reduction of peroxides slowing the aging process (KIELISZEK et al., 2015; PORTO et al.,2015; GILBERT-LÓPEZ et al., 2017; XIAO et al., 2017). Selenium naturally appears in a

series of animal and plants based-foods, been dietary supplementation another possible font. The Se dietary supplementation can be done with preparations of inorganic selenium or by Se-enriched yeast, which biotransform the inorganic Se into a bioavailable Se, such as SeMet and SeCys (YIN et al., 2009). The benefits of Se dietary supplementation are related to the regulation of inflammatory pathway, to anticarcinogenic properties, to the reduction of cardiovascular disease, and to slowing aging process (MÉPLAN, 2011; EL-BAYOUMY et al., 2012; XIAO et al., 2017).

The most used techniques for yeasts' conservation, aiming its commercial application, are the freeze-drying and spray-drying techniques. During the enriched cells obtainment and conservation, it is important to beware about the storage of these yeasts, since it must have a protection, such as a matrix, to guarantee that the cell will be viable to consumption. Both techniques can use polyssacharides, such as maltodextrin, starch, Arabic gum, pectin and Kefiran, as cryoprotective agents. Some of these polyssacharides can present health benefits beside its protective action.

Kefiran is a water soluble molecule composed of many units of glucose and galactose (KOOIMAN, 1968; BABAEI-GHAZVINI et al., 2018) it has the functional property of a transparent shape, homogeneous, edible films or gels (PIERMARIA et al., 2015), which are an adequate barrier been a promising biomaterial for drug delivery (BLANDÓN et al., 2016). Its biological effects, such as antioxidant activity (SABAGHI et al., 2015), anti-microbial and healing activity (RODRIGUES et al., 2005) and health promoting properties, make it suitable for the development of functional foods (PIERMARIA et al., 2008; GHASEMLOU et al., 2011; MOTEDAYEN et al., 2013).

The hydrocolloid pectin, in its turn, is a major cell wall polysaccharide formed by polymers of homogalacturonan (HG), xylogalacturonan (XGA), and rhamnogalacturonan type I and type II (RGI and RGII) (SCHELLER et al., 2007; VORAGEN et al., 2009; SCHOLS et al., 2009; MUZZARELLI et al., 2012). This polymer can be methyl-esterified and acetylated, and this characteristic can be used to classify the pectin and to determine its function (VINCKEN et al., 2003; MOHNEN, 2008; NGOUÉMAZONG et al., 2012; EINHORN-STOLL, 2017). Pectins have health effects, applications in biodegradable films and paper substitutes, and can be used in many industries, such as food, cosmetic, pharmacology, and manufacture industry

(LÖFGREN; HERMANSSON, 2007; MOHNEN, 2008; SCHOLS et al., 2009; ADETUNJI et al., 2017).

The gelling characteristic is one of the most important function of this polymer. This propriety is related to the methyl-esterification degree: If higher than 50% it is called high-methoxy pectin (HM), and If lower it is classified as low-methoxy (LM) (GIACOMAZZA et al., 2016; ADETUNJI et al., 2017). The HM pectin forms gel in the presence of high amounts of sugars, as co-solute, and low pH (SHARMA et al., 2006; FISHMAN; COOKE, 2009; MORRIS et al., 2010). In the food industry, they are used in products with high-sugar content such as preserves, jellies and jams (VRIESMANN; PETKOWICZ, 2013). On the other hand, LM pectin requires the presence of calcium ions (Ca^{+2}) under high pH conditions to the gel formation (Morris et al., 2010). Since it does not require the presence of sugar as co-solute, in the food industry, it can be used in dietetic foods and low-calorie products (LOPES DA SILVA; RAO, 2006; NGOUEMAZONG et al., 2012; VRIESMANN; PETKOWICZ, 2013).

However, commercial pectin are traditionally extracted from citrus peels and apple pomace, other plants have been reported as good sources; from those sugar beet, potato pulp, pumpkin, okra and tropical fruits, such as watermelon and cocoa husk can be quoted (ADETUNJI et al., 2017; EINHORN-STOLL, 2017). The cocoa pod (*Theobroma cacao* L.) is a tropical fruit that is native of the Amazon, which is used in the chocolate production. The cocoa husks are the first discarded by-product, and it is a rich source of pectin. The extracted cocoa pectin has been reported as able to form gels (VRIESMANN et al. 2012; VRIESMANN; PETKOWICZ, 2013) and it can be employed as emulsifier agent (YAPO; KOFFI, 2013), which are good characteristics for polymers that are used as a cryoprotective agent.

In literature, there are many examples of microorganisms, which are capable of bio accumulating trace elements, such as selenium, among them there are the genus *Saccharomyces*, *Candida* and *Pichia* (BEKATOROU et al., 2006; KIELISZEK; BŁAZEJAK, 2013).

Arakaki (2010), isolated the strain identified as *Wickerhamomyces anomalus LPB-CPB5* yeast, and tested its capability to grow in different culture mediums. This strain showed the potential to bioaccumulate copper and zinc. The aim of this work was to test the potential of *Wickerhamomyces anomalus LPB-CPB5* yeast to bioaccumulate selenium. The selenium-rich strain will then be encapsulated in different materials including pectin (from cocoa husk) and Kefiran polymers through

spray-drying technique. Stability tests of the encapsulated cells and freeze dried cells will also be carried out as an alternative method.

1.2 JUSTIFICATION

Yeasts have efficient assimilation mechanisms of different minerals from the environment, incorporating them into its cellular structures (KIELISZEK; BŁAZEJAK, 2013). Many of those minerals are naturally bioaccumulated by yeast, been enriched yeasts a promising tool for diet supplementary, especially as food and feed additives (CHOJNACKA, 2009; KIELISZEK et al., 2015).

The yeasts bioaccumulate the substances during the growth stage making the process cheaper since it can be carried out in a short cultivation period (YIN et al., 2009). The yeast *Wickerhamomyces anomalus LPB-CPB5*, isolated by Arakaki (2010) have already shown the capability of bioaccumulating zinc and copper, which indicates its great potential. Also, it has been classified as a Generally Recognized as Safe (GRAS), which makes it adaptable to be used as dietary supplement. The mineral selenium was chosen because of its health benefits, which are related to its active organic forms. Se-enriched yeast must be than produced and stabilized to be used as food or feed supplement. In this way, encapsulation processes must be conducted with the use of protective agents such as pectin (from cocoa husk), Kefiran and others polymers with their health properties, which are suitable to be used as matrixes.

1.3 OBJECTIVES

1.3.1 General objective

The objective of this work was to produce stable selenium enriched *Wickerhamomyces anomalus LPB-CPB5* cells encapsulated using pectin from cocoa husk (*Theobroma cacao* L.), Kefiran and other materials.

1.3.2 Specific objectives

- To optimize *Wickerhamomyces anomalus LPB-CPB5* growth conditions;
- To test the best operational conditions for yeast cultivation (batch or fed-batch);
- To evaluate *Wickerhamomyces anomalus LPB-CPB5*'s selenium tolerance;
- To evaluate *Wickerhamomyces anomalus LPB-CPB5*'s selenium tolerance and bioaccumulation potential through different analytical tools;
- To optimize the extraction of pectin from cocoa husk (*Theobroma cacao* L.) and to characterize it;
- To evaluate the use of pectin, Kefiran, and modified starch as microencapsulation matrixes;
- To evaluate Se-enriched *Wickerhamomyces anomalus LPB-CPB5* cells' stability.

CHAPTER 2 - SE-ENRICHED YEAST: PRODUCTION AND MICROENCAPSULATION FOR DIETARY SUPPLEMENTATION – A REVIEW

ABSTRACT

Trace elements are chemical components, which show functional and structural roles in the microorganism. There are many examples of microorganisms capable of bioaccumulating and biotransform trace elements from the environment into bioavailable forms. Some elements show functional and structural properties, and, among them, selenium is reported for its health benefits. This trace element can be obtained from different foods and by dietary supplementation with Se-enriched yeasts, cultivated through fermentation techniques. It is important to beware about its conservation that can be reached by, freezer-drying or spray-drying techniques. Both methods use cryoprotective agents or adjuvants to increase cells' stability, such as some polysaccharides like pectin and Kefiran with their protective action and health promoting properties. The aim of this review is to show the capacity of yeasts cells to bioaccumulate trace elements, presenting the potential of Se-enriched yeasts. Besides, the importance of yeast cells microencapsulation using polysaccharides to enhance stability will be discussed.

Keywords: *Wickerhamomyces anomalus* 1. Bioaccumulation 2. Microencapsulation 3. Polyssacharydes 4. Dietary supplemantation 5.

2.1 INTRODUCTION

Some microorganisms, such as yeasts, can accumulate and biotransform substances from the environment in bioavailable forms, through the bioaccumulation process (YIN et al., 2009). Trace elements, are chemical particles constituents of numerous proteins, required by all living organisms, in a small amount, which show functional and structural roles in the microorganisms (KIELISZEK et al., 2015; RYCEWICZ-BORECKI et al., 2016). Many yeast can naturally bioaccumulate trace elements, and these enriched yeasts are certainly a promising tool for diet supplementary (CHOJNACKA, 2009; KIELISZEK et al., 2015). In literature there are many examples of microorganisms, capable of bioaccumulating trace elements,

where *Saccharomyces*, *Candida* and *Pichia*, are the most reported genus (BEKATOROU et al., 2006; KIELISZEK & BŁAZEJAK, 2013).

Some trace elements, such as copper, zinc, and selenium show functional and structural properties. Selenium has been defined as an essential micronutrient with significant health benefits (ALLMANG et al. 2009). The selenium functions are related to its proteins application such as slowing the aging process, and antioxidant activity (KIELISZEK et al., 2015; PORTO et al.,2015; GILBERT-LÓPEZ et al., 2017; XIAO et al., 2017). This trace element can be obtained from different foods; however, for some regions where selenium is scarce, the dietary supplementation is a possible solution.

During Se-enriched yeasts production, it is important to beware about its conservation, keeping its viability and stability. For commercial applications, the most suitable conservation techniques for yeasts are freezer-drying and spray-drying. Both techniques can use polyssacharides, as cryoprotective agents and adjuvants, respectively, which present complementary health benefits other than their protective action. Kefiran, for example, is a water soluble molecule with functional properties, which are able to form edible films or gels, with biological and health promoting properties (PIERMARIA et al., 2015; SABAGHI et al., 2015; BLANDÓN et al., 2016). Pectin is another possible fit. This polysaccharide that is known by its gelling characteristics, have health effects, applications in biodegradable films and paper substitutes. It can be used in many industries, such as food, cosmetic, pharmacology, and manufacture industry (LÖFGREN; HERMANSSON, 2007; MOHNEN, 2008; SCHOLS et al., 2009; ADETUNJI et al., 2017).

The gelling properties of pectins are related to its methyl-esterification degree. If it is higher than 50%, it is called as high-methoxy pectin (HM). If it is lower, it is named low-methoxy (LM) (GIACOMAZZA et al., 2016; ADETUNJI et al., 2017). So, the HM pectins form gel in the presence of high amounts of sugars and low pH (SHARMA et al., 2006; FISHMAN; COOKE, 2009; MORRIS et al., 2010), while the LMs require the presence of calcium ions (Ca^{+2}) under high pH conditions (MORRIS et al., 2010). Commercial pectins are normally extracted from citrus peels and apple pomace, however other plants have been reported as good sources. From those the cocoa pod (*Theobroma cacao* L.), a tropical fruit used in the chocolate production, can be quote as a good source of pectin. The extracted cocoa pectin has been reported as able to form gels and to work as emulsifier agent (VRIESMANN ET AL.,

2012; VRIESMANN; PETKOWICZ, 2013; YAPO; KOFFI, 2013), which are good characteristics for its application as a cryoprotective or adjuvant matrix.

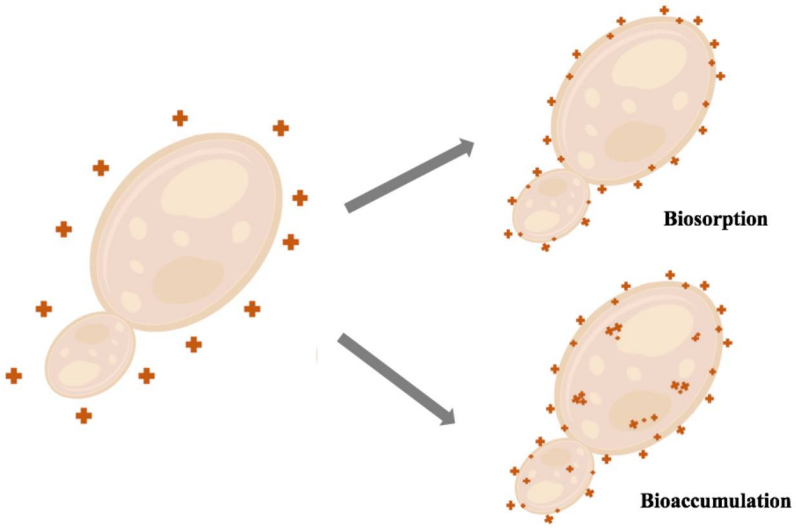
The aim of this review is to show the capacity of yeast to bioaccumulate trace elements, presenting the potential of Se-enriched yeasts to be employed as a dietary supplement for humans, animals and even for plants. It will also be discussed the importance and advantages of yeast cells' microencapsulation using polysaccharides, such as Kefiran and Pectin.

2.2 BIBLIOGRAPHIC REVIEW

2.2.1 Bioaccumulation by yeasts

The bioaccumulation process is frequently mistaken with the biosorption process, so it is important to define the difference between these two mechanisms. The main difference is related to the metabolism while biosorption is a passive process, bioaccumulation is an active. As shown in Figure 1, in the biosorption process the mineral stays in the surface of the yeast, while in the bioaccumulation it gets into the yeast.

FIGURE 1 - BIOSORPTION AND BIOACCUMULATION PROCESS IN YEAST CELLS



SOURCE: THE AUTHOR.

Biosorption is defined as the ability of the organism to bind substances to its cellular structure. This process occurs naturally by the interaction between metal ions and functional groups that are present on the cell surface, following a variety of

mechanisms including adsorption, ion exchange, surface precipitation and complexation (VIRARAGHAVAN; SRINIVASAN, 2011). Since the biosorption does not require an active metabolism, this process occurs in both living and dead biological cells; which differs this process to the bioaccumulation, since on the last one the biomass has to be alive (CHOJNACKA, 2009).

Bioaccumulation is the process that involves the accumulation of substances by an organism. It occurs when it absorbs and retains the bioaccumulant faster than losing it by catabolism and excretion. Bioaccumulants are substances, which are accumulated by living organisms. Generally, the bioaccumulation process has two distinctive steps. In the first one, metal ions are bound to the surface of the cells, by a passive process, which is similar to the biosorption process. The second step is the transportation of the metal ions into the cell, by an active process (CHOJNACKA, 2009; VIRARAGHAVAN; SRINIVASAN, 2011; PANKIEWICZ et al., 2017)

It is known that all living organisms has the capability of accumulating essential, non-essential and toxic substances (CHOJNACKA, 2009). The mineral assimilation process involves its incorporation into cellular structures, and then its conversion with biotransformation of these substances in organic forms. These organic forms are then easily assimilated by living cells (YIN et al., 2009; KIELISZEK; BŁAZEJAK, 2013).

Yeasts are eukaryotic, unicellular, non-filamentous fungi. They are typically spherical or oval, and, in nature, they appear as a white powdery, that coat fruits and leaves (TORTORA et al., 2018). Even though their form and size are well defined, they may vary according to the physiological stage, growing conditions, and the presence of nutrients. Thus, most yeasts that are used in the industry has an average size, from 4 to 8 μm wide and 7 to 12 μm length (ROEPCKE, 2007).

These microorganisms show the capability of growing in alternative cultivation mediums, under facultative anaerobic conditions, which allow them to survive in different environments. In the presence of oxygen, yeasts perform aerobic respiration by metabolizing carbohydrates and liberating carbon dioxide and water. They also ferment carbohydrates producing ethanol and carbon dioxide (TORTORA et al., 2018). Yeasts are part of a group of microorganisms that present asexual reproduction by budding, which is an important characteristic since it leads to rapid proliferation and intense increase of cell biomass (KIELISZEK et al., 2015). In this type of reproduction, the parent cell forms a projection (bud) on its membrane

surface. The nucleus of the parent cell divides, and one sample migrates into the projection, as the bud elongates (ROEPCKE, 2007; TORTORA et al., 2018).

Yeast has a rapid metabolism, which gives it some advantages over other microorganisms, since they have a high level of interaction with the extracellular environment (KIELISZEK et al., 2015). They can assimilate a variety of substrates and bind to different metals. This process occurs when yeast cells are cultivated in soluble sugars and organic acids based medium to produce biomass with high protein content (YIN et al., 2010). The metal-binding property is determined by the chemical structure in the surface of the cell, which differs depending on the cell type (CHOJNACKA, 2009).

Many trace elements are naturally bioaccumulated by yeast. These elements are constituents of numerous proteins, enzymes and hormones showing functional and structural roles (KIELISZEK et al., 2015; RYCEWICZ-BORECKI et al., 2016). The bioaccumulation process has different pathways, depending on the organism. These pathways can protect them from an excessive bioaccumulation, by blocking the entrance or excretion of the bioaccumulant. The path through which a bioaccumulant enters an organism can be the same as it takes up nutrients, so they bioaccumulate during the growth stage. In unicellular organisms, for example, metals can enter through the transport channels (CHOJNACKA, 2009).

The bioaccumulation capability makes some yeasts a target for many applications in fermentative processes resulting in diverse products. These diverse products can be used in waste bioremediation, in the production of metal-enriched food and feed and in the biotechnology and pharmaceutical industries as direct diet supplementary (KIELISZEK et al., 2015).

Microelement-enriched yeasts are a promising tool for diet supplementary, especially in the production of food and feed (CHOJNACKA, 2009; KIELISZEK et al., 2015). In literature there are many examples of microorganisms that have been used in mineral bioaccumulation; generally, they are represented by the genus *Saccharomyces*, *Candida*, *Pichia* (BEKATOROU et al., 2006; ROEPCKE et al., 2011; KIELISZEK; BŁAZEJAK, 2013). They are cultivated in industrial wastes, or by-products, as growth media, which make their cultivation cheaper. Some of these by-products are sugarcane, soybean, and corn molasses, that can be used, as they are rich carbon and nutrient sources (CHOJNACKA, 2009). Table 1 exemplify different microorganism, minerals bioaccumulated and cultivation medium.

Candida utilis is generally used in food and feed industries due to its high protein and amino acids content, and for its capacity of easy adaptation to different substrates. Gönen; Aksu (2008) reported capability of *C. utilis* to bioaccumulate copper showing a yield of 34.2 % from a copper 50 mg/L fed. Kieliszek et al. (2017) prepared a good review about the biotechnological uses of *Candida* genus yeast in food industry.

The most used species of *Saccharomyces* is the *Saccharomyces cerevisiae*, due to of its well-consolidated application in bread and fermented drinks production, which gives new possibilities for enriched foods consumption (KIELISZEK et al., 2016). There are many studies to understand the trace elements bioaccumulation process using *S. cerevisiae* as a model organism (KAMRA AZAD et al., 2014). Generally, in *S. cerevisiae*, this process happens in two stages: the first is a passive biosorption, and the second the active accumulation where the metal ions are stored primarily in the yeast vacuole (PANKIEWICZ et al., 2017). Jach; Serefko (2018) reviews the use of yeasts by reporting some nutritional aspects, giving a special attention to enriched and non-enriched *S. cerevisiae*, and demonstrating its commercial interest as supplement for humans and animals.

TABLE 1 – DATA OF ENICHED YEASTS

Strain	Mineral	Bioaccumulated Amount	Cultivation Medium	Reference
Candida pelliculosa BARU05	Copper	1.738 mg/g	Sugarcane molasses 5 °Brix $CuSO_4.5H_2O$ 0.01 % YE 1.0 % $(NH_4)_2SO_4$, 0.5 % KH_2PO_4 0.5 % $MgSO_4$ 0.05 %	Arakakiet al. (2011)
Saccharomyces cerevisiae	Iron	8 mg/g	Glucose 1.0 % Peptone 0.5 % YE 0.3 % Malt extract 0.3 %	Gaensly et al. (2011)
Pichia guilliermondii	Zin	0.075 mg/g	Soybean molasses 5 °Brix $(NH_4)_2SO_4$, 0.5 % YE 0.5 % KH_2PO_4 0.5 % $MgSO_4$ 0.05 % $ZnSO_4$ 1.0 % $Fe_2(SO_4)_3$ 0.01 %	Roepcke et al. (2011)
Saccharomyces cerevisiae	Copper	1.8 mg/g	Glucose 8.0 % Sodium citrate 1.0 % Cysteine 0.4 % Glycine 0.4 % $(NH_4)_2SO_4$ 0.7 % KH_2PO_4 0.35 % $MgSO_4$ 0.05 %	Rollini et al. (2011)
Saccharomyces cerevisiae LN-17	Zinc and Iron	4.976 mg/g and 7.854 mg/g	Molasses 4 % $(NH_4)_2SO_4$ 0.05 % H_3PO_4 1 mL	Wang et al. (2011)
Saccharomyces cerevisiae	Zinc	4.132 mg/g	Peptone 1 % Dextrose 2 %	Kamran et al. (2014)
Saccharomyces cerevisiae	Iron	15 mg/g	Glucose 2 % YE 0.05 % $(NH_4)_2SO_4$, 0.5 % KH_2PO_4 0.1 % $MgSO_4$ 0.05 %	Kyyaly et al. (2015)
Saccharomyces cerevisiae	Zinc	14.48 mg/g	Peptone (1 %) YE (0.5 %) Glucose (1 %)	Pankiewicz et al. (2017)

SOURCES: THE AUTHOR.

2.2.1.1 Candida pelliculosa

Since the shearch for new yeasts that bioaccumulate is important, Arakaki (2010) isolated, from Baru (*Dipteryx alata*) fruit, eleven yeasts strains. They were tested according to their capability to grow in sugarcane molasses and their potential

in bioaccumulating copper and zinc. One of them, identified and named as *Candida pelliculosa* BARU05, showed very good bioaccumulation capacity and biomass production. The *C. pelliculosa* was identified, by the API kit, as *Candida lusitaniae* or, in a 100% molecular identification, as *Candida pelliculosa*. Its strains have been classified as a "GRAS" microorganism, meaning that *C. pelliculosa* BARU05 can be consummed by animals and humans. The strain BARU05 was deposed in the *Coleção de Culturas Tropicais – CCT*, Brazil, and registered under the number CCT 7734 as *Pichia anomala*.

The results presented by Arakaki (2010) showed the capability of *C. pelliculosa* BARU05 in bioaccumulating different types of minerals, such as zinc and copper, and its flexibility of growing in different culture mediums. The use of agro-industrial residues such as soybean molasses, sugarcane molasses, among others, in fermentation, helps the development of an environmentally-friendly process. Cells were cultivated in Erlenmeyer flasks with sugarcane molasses reaching 11.99 g/L in fed-batch operation. Biomass concentration reached exceptionally 57.54 g/L of biomass in a 10 L bioreactor also in fed-batch cultivation. For the accumulation of copper, it was reported that 100 % of the mineral were absorbed in 24 h. The strain was also capable to highly bioaccumulate zinc (96.17 %). The performant mineral bioaccumulation of *C. pelliculosa* BARU05 showed that this microorganism has potential to be used in dietary supplementation for replacement of some trace elements such as zinc, copper and other such as selenium.

The strain *C. pelliculosa* BARU05, can be also called of *Pichia anomala* and *Hansenula anomala*. Kurtzman (2011) discussed the phylogeny of the *Ascomycetous* yeasts, showing that most of the 20 genus related to *P. anomala* were from the genus *Wickerhamomyces*. *P. anomala,* and its assigned species were reclassifyed to the genus *Wickerhamomyces*, for these reason *C. pelliculosa* BARU5 can also be called *W. anomalus* LPB-CPB5.

2.2.2 Minerals Bioaccumulation - Trace Elements

Trace elements, also called microelements, are chemical substances that are required in small amounts by all living organisms. Some trace elements, such as Ca, Cu, Cr, Fe, K, Mg, Mn, Se, and Zn, show functional and structural properties in the composition of some metalloproteins, enzymes, and hormones (KIELISZEK et al.,

2015; RYCEWICZ-BORECKI et al., 2016). These elements have a hold limit between been beneficial or toxic to the organism. Their deficiency is associated with a range of pathological conditions (GÖNEN; AKSU, 2008), while in high concentrations it reacts to form toxic compounds (CHEN et al., 2005; GÖNEN; AKSU, 2008).

Many trace elements are naturally bioaccumulated by yeasts, which has two important applications: bioremediation and enriched-yeast for dietary supplementary. The first application is the use of yeasts that bioaccumulate as an alternative to the classical methods of heavy metal removal, aiming the control of liquid and solid wastes, which can have serious consequences for the environmental and human health (CHEN et al., 2005). The second one, the use of yeasts for dietary supplementary, is a promising tool for the production of enriched food and feed, being indicated for the nutrition of humans, animals, and even plants (CHOJNACKA, 2009; ARAKAKI et al., 2011; KIELISZEK et al., 2015).

The interaction between the microelements has benefits and consequences for human and animal health. For this reason, it is important to know the interaction and the amounts of each element that are required. For example, high concentrations of Zn, Cd or Cu can have negative effect in the iron utilization and storage. The relationship between copper and iron is important too, since together they act as cofactors of some proteins, they can participate in the production of reactive oxygen species (ROS), and in the cleaving of DNA and RNA molecules (TAPIERO; TEW, 2003). Copper and zinc ions, in its turn, can be inhibitors of the HIV-1 protease, reducing the proliferation of the virus (ROLLINI et al., 2011).

There are many studies in literature about the interactions of trace elements showing their benefits and consequences, which enhance the dietary supplementation importance. Rashed (2011) reviewed the role of Fe, Cu, Co, Mn, and Zn on hepatitis virus infection, showing the participation of Cd, Cu and Mn in hepatitis C virus (HCV), and Co and Pb in the hepatitis B. Méplan (2011) reported the influence of trace elements in the aging process and in the processes related to the aging, using selenium as an example to illustrate it. Finally, Biswas et al. (2014) investigated the effect of Cr supplementation on semen and carcass traits of adult male turkey observing its physical and biochemical characteristics and showing the importance of dietary supplementation for animals too.

2.2.3 Selenium

The element selenium (Se), belonging to the group 16 of the chalcogen, was discovered in 1817 in a Sweden factory. The chemist Jöns Jacob Berzelius, who believed that an impurity in its chambers of sulfuric acid production was the element Tellurium, realized, after analysis, that it was an unknown element (ALLMANG et al., 2009). Today it is well known that selenium, atomic mass is 78.971 g and its density is 4.809 g/cm^3; at 24º C it is in a solid state, while its melting point is 220.8°C and boiling at 685°C. This mineral can be found in the environment in organic and inorganic forms.

Inorganic selenium forms are often selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) (TIE et al., 2017). Naturally, it does not occur in a pure form of the element and there are no mineral deposits having selenium as a major constituent. Selenium can appear in some rocks, such as coal, black shale, limestone and phosphate rocks, as a trace element; and be found in rare minerals as Clausthalite (PbSe), Crooksite (CuThSe) and Eucairite (CuAgSe). However, it can be obtained as a by-product of other base metals (KHAMKHASH et al., 2017).

Even so, selenium was defined as an essential micronutrient endowed with significant health benefits in the mid of 1950 (ALLMANG et al. 2009). Its greatest biological significance could be attributed to the discovered of selenoproteins, in 1973, one of its organic forms (KIELISZEK; BŁAZEJAK, 2013, RYCEWICZ-BORECKI et al., 2016). Selenium is a trace element required in small amounts by humans and animals. There is a tiny level between toxicity and benefit effects, been the recommended level, for humans, between 40-60 µg/day (PORTO et al., 2015; XIAO et al., 2017). The organic forms are mostly the selenoamino acids (selenomethionine and selenocysteine), selenopeptides (e.g. plasma selenoprotein P), and selenoenzymes (e.g. glutathione peroxidase) (MÉPLAN, 2011; KIELISZEK et al., 2015; TIE et al., 2017).

The most known Se-amino acids are selenomethionine (SeMet) and selenocysteine (SeCys) (Porto et al. (2015). For the formation of selenomethionine and selenocysteine the sulfur (S), which belongs to the same chemical family as selenium, is present in the methionine (Met) and cysteine (Cys) composition switched to Se. The SeMet and Met has the same sites in the peptide chain and the same transport mechanism. SeMet can randomly be inserted into peptide chains, instead

of methionine, forming Se-proteins. This substitution can happen in humans and animal cells (TIE et al., 2017). The SeCys mechanism is different. This amino acid has its own codon and tRNA molecule. The SeCys translation codon is the UGA sequence, the same sequence of the stop point. The UGA is reprogrammed to mean SeCys instead of stop. When the ribosome translates UGA codon the t-RNA^{Se} brings SeCys to the composition of the peptide (HONDAL; RAINES, 2002; ALLMANG et al., 2009; TIE et al., 2017).

Most physiological functions of selenium are related to the selenoproteins applications, such as antioxidant activity, metabolism of some hormones, DNA synthesis and protection against infection (GILBERT-LÓPEZ et al., 2017). About 25 Se-proteins are translated in mamals, using SeMet and SeCys. Most of them are involved in reactions of oxidation and reduction (ALLMANG et al. 2009). From those proteins, several Se-enzymes, the plasma Se-transport protein, selenoprotein P (SePP) and selenoprotein S (SELS) can be quoted, where SePP and SELS are associated with colorectal cancer risk (MÉPLAN et al., 2010; MÉPLAN, 2011). Selenium's antioxidant activity is represented by the Se-enzyme, glutathione peroxidase (GPx). This enzyme protects the cells against the effects of the free radicals, resulted from the oxidation process, by controlling the reduction of peroxides and slowing the aging process (KIELISZEK et al., 2015; PORTO et al.,2015; GILBERT-LÓPEZ et al., 2017; XIAO et al., 2017). The Se-enzymes can be represented by the GPx, iodothyronine deiodinase (DIO), and thioredoxin reductase (TRxR).

The first selenium deficiency was related in 1935, in a mountain area in China. The disease was named Keshan in the endemic area. The low content of Se in the soil was reflected in the rice and maize cultivation and caused the selenium dietary deficiency, which resulted in muscular dystrophy, cardiac arrhythmia, and heart failure (RATTANACHAIWONG; SINGER , 2018). The treatment with sodium selenite fail to reverse the heart condition of the disease, which led to other supplementation methods (KIELISZEK et al., 2016). Another Se-disease described is the Kashin-Beck disease, which causes osteoarthritis, cartilage abnormalities and deformity (KIELISZEK et al., 2016; RATTANACHAIWONG; SINGER, 2018). Xiao et al. (2017) reported that around 0.5 - 1 billion people all over the world may present Se dietary deficiencies.

2.2.3.1 Sources

Selenium naturally appears in a series of foods, such as, milk, muscle meat, seafood, vegetables, mushrooms, cereals, grains, and nuts (e.g. Brazil nuts). The plant sources has the amount of Se varying among regions and soil nutrients, for these reason animal-based sources are better sources of selenium, as they retain Se in theirs cells (RATTANACHAIWONG; SINGER, 2018). The ability of plants to accumulate and biotransform inorganics forms of Se has been studied to evaluate the production of Se-enriched plants. The Se-enriched plants has been produced in regions such as in China with low levels of selenium, using enriched fertilizers containing Se salts (TIE et al., 2017).

The ingested selenium is absorbed from the duodenum, been a minute amount absorbed in the jejunum and ileum. In some mammals, a little amount of selenium can be absorbed in the stomach and/or in the rumen. The digestion pathway of selenium is dictated by its chemical form. The selenites are absorbed into the brush-border membranes making a passive diffusion, while selenates, which has a slight affinity for this region, are incorporated through the sodium cotransport chain. The Se-amino acids are absorbed through active amino acid transport (HALL, 2018). Once absorbed the selenium metabolites are processed in the liver and kidney and then excreted in the urine (RATTANACHAIWONG; SINGER, 2018).

Another possible font of selenium is by dietary supplementation. The supplementation can occur by preparations with inorganic selenium, using sodium selenite (whose toxicity and pollute potential can be a limiting factor) or organic Se, using selenium-enriched yeast, or free Se-proteins and Se-amino acids, in a pharmaceutic preparation (KIELISZEK et al., 2016). Se-enriched yeast has been widely used due to the excellent capability of yeasts to accumulate selenium from inorganic sources and biotransform it into a bioavailable Se, such as SeMet and SeCys (YIN et al., 2009). Yeasts are generally chosen for being economic and easy to cultivate, and for their tolerance to high levels of selenium in the medium, which certainly increases its bioaccumulation rate (YIN et al., 2010). Table 2 show se-enriched yeasts and the quantities bioaccumulated.

Kieliszek et al. (2016) tested the selenium bioaccumulation efficiency of *S. cerevisiae* MYA-2200 and *C. utilis* ATCC 9950. These yeasts were chosen since they are classified as GRAS and are able to be employed in the feed industry, and provide

high biomass yield. It was concluded that the selenium source, sodium selenite (IV), had negative effect on the growth of both yeasts at the lowest concentration (10 mg/L). For this study the lowest concentration of selenium (10 - 30 mg/L) and the cultivation in short time seemed to be the most effective method.

TABLE 2 - DATA OF SELENIUM BIOACCUMULATION BY MICROORGANISMS

Strain	Bioaccumulated Selenium	Reference
Saccharomyces cerevisiae	1200 -1400 µg/g	Suhajda et al. (2000)
Se-enriched yeast	2064.6 µg/g	McSheehy et al. (2005)
Saccharomyces cerevisiae	0.116 mg/g	Pankiewicz; Jamroz (2008)
Saccharomyces cerevisiae	150 µg/g	Stabnikova et al. (2008)
Saccharomyces cerevisiae	3,53 mg/L	Yin et al. (2009)
Saccharomyces cerevisiae	5,9 mg/L	Yin et al. (2010)
Candida utilis	90,2%	Xiaoguang et al. (2011)
Saccharomyces cerevisiae	107.9 – 287.65 mg/Kg	Esmaeili et al. (2012)
Candida utilis	905,3 µg/g	Yang et al. (2013)
Saccharomyces cerevisiae ATCC MYA-2200 *Candida utilis ATCC 9950*	5,64 mg/g 5,47mg/g	Kieliszek et al. (2016)
Saccharomyces cerevisiae	43.07 mg/g	Pankiewicz et al. (2017)

SOURCES: THE AUTHOR.

2.2.3.2 Benefits and toxicity

The benefits of Se dietary supplementation are related to regulation of inflammatory pathway, anticarcinogenic properties, reduction of cardiovascular disease, and slowing aging process (MÉPLAN, 2011; EL-BAYOUMY et al., 2012; XIAO et al., 2017). Aiming the demonstration if selenium alters the levels of dietary-proteins that may play a critical role in cancer prevention, El-Bayoumy et al. (2012) used Se-enriched baker's yeast to see its effects on these proteins. Authors showed that the supplementation with Se-enriched yeast was able to up-regulate some proteins, which is relevant for the carcinogenesis process, and down-regulate others, giving an important view of the Selenium role in cancer prevention.

The aging process is gradual and accumulated over the years from cells and tissues damage in the molecular level. This process is related to lifestyle, free radicals, and environmental conditions. As a result, there is the increase of diseases, loss of functions, and death. Méplan (2011) brings a good review about the participation of trace elements in the modulation of inflammatory and oxidative processes, which slow down the aging process. This article gives special attention to

the selenium participation in this process, showing that Se role in the aging process depends on its bioavailability and regulation of metabolic pathways. Authors conclude that the use of dietary supplementation must be addressed carefully to avoid potentially harmful effects.

The labels of supplementary products usually present the total Se content, but not about the composition including other compounds. There is a narrow line between toxicity and benefit effects, that is why it is important to be careful to the toxic dose (XIAO et al., 2017). It has been reported intoxication with selenium from formulated dietary supplements, which accentuate the importance of correct label. Some symptoms were reported such as fatigue, nausea, hair loss, and nail changes (RATTANACHAIWONG; SINGER, 2018). The toxicity of organic selenium is lower than that of inorganic selenium, what makes important to specify which is source of selenium (KIELISZEK; BŁAZEJAK, 2013; TIE et al., 2017).

2.2.3.3 Application and Existing Products

The hidden hunger is a deficiency of microelements in the diet of human, animals, and plants (CHOJNACKA, 2009). This deficiency has been a global problem and its eradication has been listed as a goal to the FAO (Food and Agriculture Organization of the United Nations - http://www.fao.org/about/what-we-do/so1/en/). The price increase of protein supplements and cereal highlights the interest in the addition of microorganisms' biomass in foods and feeds industry (ROEPCKE et al., 2011). Yeast enriched with microelements is a promising tool for diet supplementary, for been accepted by costumers and rarely toxic or pathogenic (KIELISZEK et al., 2015). The biomass works as an additional source of carbon and nitrogen, amino acids, vitamins of the B complex, and proteins (ROEPCKE, 2007).

The bioaccumulation process is active and happens during the yeast growth. There are some reported techniques that are applied for yeast cultivation to improve the metal bioaccumulation, such as the pulsed electric fields (PEF), which the bioaccumulation of selenium in 65 % and zinc in 100 % (PANKIEWICZ et al.,2017). The production of metal enriched yeast is a fast and cheap process, where the metal salt is dissolved in the cultivation medium (CHOJNACKA, 2009). These yeasts, especially the selenium enriched ones, that can be used as a supplement for humans, animals, and plants, to fill the mineral deficiencies.

Some selenium-yeast supplement products are presented in Table 3.

TABLE 3 - SELENIUM-YEAST SUPPLEMENT PRODUCTS

Product	Selenium Source	Brand	Website
DiaMune Se Dairy Formula	*Saccharomyces cerevisiae*	Diamondv	http://www.diamondv.com/
DiaMune Se Poultry Formula	*Saccharomyces cerevisiae*	Diamondv	http://www.diamondv.com/
DiaMune Se Swine Formula	*Saccharomyces cerevisiae*	Diamondv	http://www.diamondv.com/
Prolaject® B12 1000 +Selenium	Sodium Selenite	Bayer	https://www.bayeranimal.co.nz/en/products/ products-details.php?id=946
Sprayfo Violet Sprayfo Yellow Sprayfo Vitesse	Uninformed	Sprayfo	https://www.sprayfo.com/en/
Selemax®	*Saccharomyces cerevisiae*	Biorigin	http://www.biorigin.net/biorigin/selemax/index.html
Sel-Plex®	Selenomethionine	Altech	https://www.alltech.com/sel-plex

SOURCES: THE AUTHOR.

Se-enriched yeast is already an established product with potential to the feed of fishes, poultries, pigs and cows. The search for new bioaccumulating microorganisms with others benefits such as probiotics is a promising field. Besides, the definition of powerful cells stabilizing matrices is a good approach to increase their viability and stability.

2.2.4 Microencapsulation

After yeasts' biomass production and separation, it is imperative to find solutions to maintain cells' viability and prolong their stability. Some methods for cell maintaining are constantly reported, which include some techniques of drying such as freeze-drying and spray-drying with cell encapsulation (NAZZARO et al., 2012). These techniques aim yeasts' commercial application (ABADIAS et al., 2001).

Freeze-drying, also known as lyophilization, is a long term method for conservation of microorganisms. Since there is no addition of chemicals, it is a very good process for the preservation of products from different industrial sectors: as food (fruits, vegetables, meats), the conservation of different cells (viruses, yeasts, algae) (MIYAMOTO-SHINOHARA et al., 2000). This method is based on the water sublimation, which results in a porous structure free of moisture and capable of being

reconstituted by simple addition of water. When the technique is well performed and a good matrix for cryopreservation is used, the sample can be stable even at room temperature (COSTA et al., 2000). Some cryoprotective agents can be skim milk, glycerol or some polyssacharides.

The spray-drying is a process widely used in the food industry. The process consists of spraying the sample at a controlled speed in a heated chamber, at temperatures up to 200 °C, occurring a fast drying of the sample to obtain a powder of different particle sizes. the spray-drying technique can be a challenge, since the high temperatures used in the process can kill the microorganism (CHAMPAGNE; FUSTIER, 2007). In this case, some adjuvants may be employed for cell preservation such as maltodextrin, modified starch, Arabic gum, pectin, and Kefiran, which have some properties that make them suitable for use in food and pharmaceutical industries (GOPINATH et al., 2018). Noello et al. (2016) presented the use of pectin and whey protein combined to form a suitable matrix with maltodextrin 10 DE showing an encapsulation efficiency of 99 %.

TABLE 4 - YEAST MICORENCAPSULATION AND ADJUVANT MATERIALS

Strain	Microencapsulation technique	Matrices	Reference
Saccharomyces cerevisiae	Freeze-drying	Skimmed milk 10 % Sodium Glutamate 1 %	Miyamoto-Shinohara et al. (2000)
Candida sake	Freeze-drying	Skimmed milk 10 % Lactose 10 %	Abadias et al. (2001)
Candida sake	Spray-dryer	Skimmed milk 10 %	Cañamás et al. (2008)
Saccharomyces boulardii	Spray-dryer	Gum Arabic 10 % β-cyclodextrin (9:1)	Arslan-Tontul; Erbas (2017)
Saccharomyces sp.	Spray-dryer	Whey protein 10 % Maltodextrin 10-20 %	Cruz-Gavia et al. (2018)
Sacharomyces cerevisiae	Freeze-drying	Oak chips 15 % or Cellulose 15 %	Berbegal et al. (2019)

SOURCES: THE AUTHOR.

2.2.4.1 Kefiran

Kefir is a fermented beverage that has a unique smell and taste, which is popular in many countries. It was originated in the Caucasian mountains (IRIGOYEN et al., 2005). It is produced by a mixed microflora named Kefir grains, which are, generally, composed of lactic acid bacteria, acetic acid bacteria, and yeasts (PIERMARIA ET AL., 2008). The kefir grains are irregular gelatinous granules that

vary in size from 0.3 to 3.5 cm, which resembled tiny florets of cauliflower (GUZEL-SEYDIM et al., 2005). In its composition there are proteins and polysaccharides enclosing the microflora, where the Kefiran is the main polymer (IRIGOYEN et al., 2005; GARCÍA, 2016).

Kefiran is a water soluble molecule produced under anaerobic conditions by *Lactobacillus kefiranofaciens* and *Lb. kefir*, which are displayed in the center of the grain (IRIGOYEN et al., 2005; GARCÍA, 2016). This polysaccharide is composed of many units of glucose and galactose (KOOIMAN, 1968), and it has the functional property of form transparent, homogeneous, edible films or gels, which are an adequate barrier (PIERMARIA et al., 2015). In industry, the use of Kefiran is related to its biological effects, such as in vitro growth of probiotics (SERAFINI et al., 2014), antioxidant activity (SABAGHI et al., 2015), anti-microbial, and healing activity (RODRIGUES et al., 2005) and hydrophilic characteristic. It is a promising biomaterial for drug delivery (BLANDÓN et al., 2016) and, as it has properties of heath promoting, to the developmental of functional foods (PIERMARIA et al., 2008; GHASEMLOU et al., 2011; MOTEDAYEN et al., 2013; PIERMARIA et al., 2015). Piermaria et al. (2008) showed that Kefiran was able to form gels in cryogenic treatment, such as freezing and can be storage frozen. The gel demonstrated a high water-holding capacity and its behavior at body temperature determines its ability to melt in mouth, in case of been used to encapsulate drugs or diet supplements, for example.

2.2.4.2 Modified starch

Starch is a polysaccharide wildly found in various plants, fruits, and algae (GOPINATH et al., 2018). This substance has a strong interaction with water, forming complexes with hydrophobic compounds to help its dispersal (BEMILLER, 2019). In the food industry, this material can be used to cover fresh or processed foods extending its shelf life, by forming a barrier against oxygen and though preventing deterioration (MOTEDAYEN et al., 2013). This ability to form films and its protective effect make starch suitable to be applied as a matrix for protection of microorganisms, such as enriched yeast. Modified starches have the same general characteristics of starch, forming stronger films, which also indicates it as a good barrier. Some of modified polymers can play a role in lowering the fat content of

foods, demonstrating its applications as functional food (BEMILLER, 2019). Motedayen et al. (2013) tested corn starch and Kefiran mixed together, concluding that both polymers are compatible and can combine forming a new edible flexible film that could be used as matrix for enriched yeasts microencapsulation.

2.2.5 Pectin

In 1825, the French scientist Henri Braconnot described an acid that is present in different vegetables. This acid was detected in apples, carrots, onions and other vegetables. In all tested materials Braconnot noted a gelling characteristic of this acid and gave the term pectic acid to nominate it. Today it is known that this acid is the polysaccharide pectin (MUZZARELLI et al., 2012).

The hydrocolloid pectin is a major cell wall polysaccharide that is present in different land-growing plants, fruits and vegetables (NGOUÉMAZONG et al., 2012; ADETUNJI et al., 2017; EINHORN-STOLL, 2017). It can appear in the cell wall, middle lamella, cell corners, and on the junctions zone between cells with secondary walls (MOHNEN, 2008). This polysaccharide plays diverse roles in the plant constitution. It lends strength and support, activates plant defense responses, stimulates lignification and influences various physical properties that help the ion transport (VORAGEN et al., 2009).

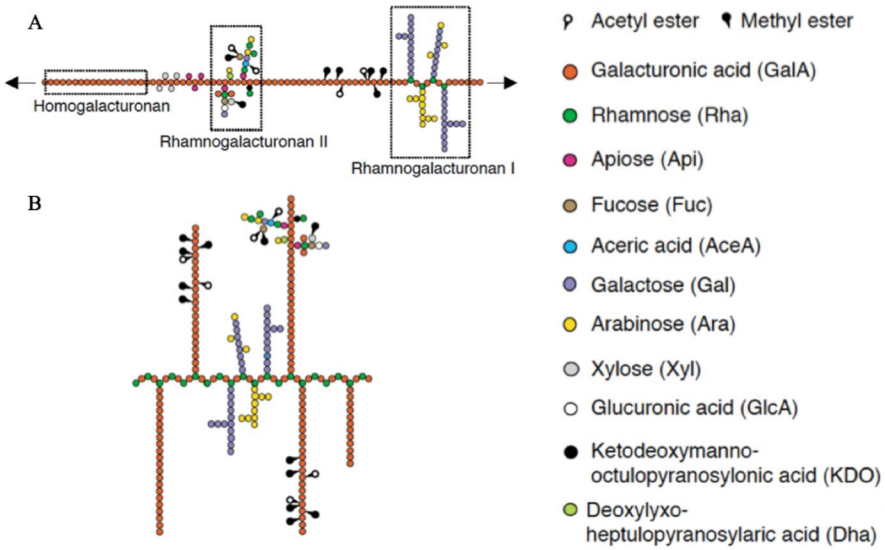
This polysaccharide has a complex chemical structure with a micromolecular organization, the exact architecture is still being explored (FRAEYE et al., 2010; AMORIM et al., 2016). The structure of pectin is still not fully known because it has a complex molecular arrangement, which can vary according to the plant species, development stage, cellular tissues, and between cells wall (MOHNEN, 2008; FRAEYE; DUVETTER et al., 2010; MUZZARELLI et al., 2012).

Pectin can be fractionated into the main monosaccharides of D-galacturonic acid (GalA), D-xylose, L-rhamnose, L-arabinose and D-galactose, forming the polymers such as homogalacturonan (HG), xylogalacturonan (XGA), and rhamnogalacturonan type I and type II (RGI and RGII) (SCHELLER et al., 2007; VORAGEN et al., 2009; SCHOLS et al., 2009; MUZZARELLI et al., 2012). These polymers are covalently linked to assemble the pectin, where it is possible to combine more than 20 different linkages. However, there is no agreement about how they are linked (MOHNEN, 2008; VORAGEN et al., 2009). At present, there are two

models that are most accepted, which describe the pectin structure: the smooth and hairy region model (SCHOLS; VORAGEN, 1996) and the rhamnogalacturonan (RG) backbone model (VINCKEN et al., 2003).

The difference between the two pectin structure models is presented in Figure 2 where it is shown how the polymers are linked together. In the first model, pectin has smooth regions of methyl esterified HG interspersing hairy regions that has a backbone of RG and neutral sugar side-chains. The RG models suggests that HG polymers are in the side chain of a RGI backbone attached to the RG residues (WILLATS et al., 2006, SCHOLS et al., 2009; MUZZARELLI et al., 2012). Even if these are the most used models, there are others models reported in literature. Voragen et al. (2009) have reviewed and described some models concluding that the base compounds of pectin and its linkages are changeable according to different developmental stages and different sources of pectin.

FIGURE 2 - SCHEME OF THE TWO PECTIN STRUCTURE MODELS



SOURCE: WILLATS et al. (2006) ADAPTED

Generally, the HG, in the pectin structure, consists of a linear homopolymer 1,4-linked α-D-GalA residues. This polymer can be methyl-esterified at C-6 and the percentage of carbonyl groups methylated defines the degree of methyl-esterification (DE). Pectin also may be acetylated at O-2/O-3 positions defining the degree of acetylation (DA) (VINCKEN et al., 2003; MOHNEN, 2008; FRAEYE; DUVETTER et al., 2010; NGOUÉMAZONG et al., 2012). The DE and DA are physicochemical

characteristics that can be used to classify the pectin and to determine its function (EINHORN-STOLL, 2017).

2.2.5.1 Gelation mechanism

Pectins have been used by the food industry for their gelling characteristics. The degree of methyl-esterification (DE) defines the gelling properties and the gelling mechanism of the pectin (SHARMA et al., 2006; FRAEYE et al., 2010; NGOUÉMAZONG et al., 2012). If it is higher than 50% the polymer is classified as high-methoxy pectin (HM), and if it is lower than 50% it is called low-methoxy (LM) (GIACOMAZZA et al., 2016; ADETUNJI et al., 2017). In conclusion, HM pectin gelifies by adding sucrose and reducing its pH, while LM pectin requires a light level of sucrose and a high content of Calcium to polymerize (VRIESMANN; PETKOWICZ, 2013; PETKOWICZ et al., 2017).

Most biopolymer gels are structured by weak interactions, such as hydrogen bond, hydrophobic interactions, and electrostatic linkage (WILLATS et al., 2006). The polymer–polymer interactions can create prolonged junction zones by side-by-side associations from physical nature. Some structural properties are important to avoid having too large polymers gels. In pectins, these attribute is provided by the rhamnose residues into smooth regions For the hairy regions, there are neutral sugar side chains (LOPES DA SILVA; RAO, 2006; FISHMAN; COOKE, 2009).

Gelation is the formation of a continuous chain of polymer molecules, which are resistant to external stress. The gel formed by pectins are influenced by the DE, however, there are extrinsic factors that may affect, such as the molecular weight, the pH, the temperature, the ionic strength, the presence of sugars, the distribution of charged groups along the pectin chain, and the DA content (LOPES DA SILVA; RAO, 2006; MORRIS et al., 2010)

The pectin molecular weight can vary depending on the plant source and extraction conditions, and it interferes in the polymer interactions (ADETUNJI et al., 2017). HM pectin forms gel in the presence of high amounts of sugars, as co-solute, and low pH. The sugar is a dehydrating agent, which create a low water activity surface, and the low pH helps increasing the dehydration rate. On this surface, the pectin polymers are immobilized by hydrogen bonds and hydrophobic interactions

forming the junction zones and building the 3-D network (SHARMA et al., 2006; FISHMAN & COOKE, 2009; MORRIS et al., 2010).

The maximum jelly strength is reached when the system reaches an equilibrium. However, any component added to the system as salts can cause a change in the jelly strength because it affects the equilibrium of the system (OWENS et al.,1954; SHARMA et al., 2006). In the food industry HM pectins are used in processed fruit products with high-sugar content such as preserves, jellies and jams (VRIESMANN; PETKOWICZ, 2013).

The LM pectin in its turn requires the presence of calcium ions (Ca^{+2}) under high pH conditions to the non-covalent ionic interactions take place (MORRIS et al., 2010). The free carboxylic acid groups in the polymer can interact with the Ca+2 forming a continuous network, this interaction is explained as the "egg-box" model, in which two pectin chains involve the Ca^{+2} ions (FRAEYE et al., 2010; GIACOMAZZA et al., 2016). These pectins can have three domains of gelation properties: a sol phase in which the gel does not gelifies at any concentration of pectin and calcium or pH; a phase with gel formation; and syneresis phase, heterogeneous system with precipitate pectin or gel formation (LOPES DA SILVA; RAO, 2006).

The pH and the calcium content has effects in the rheological behavior of LM pectin gels (LOPES DA SILVA; RAO, 2006). The decrease in pH leads to the need for more calcium to induce gelation (LOPES DA SILVA; RAO, 2006; LÖFGREN; HERMANSSON, 2007). The presence of salts can affect the quantity of calcium required for the gelation, in salt-free solutions less of calcium is (LOPES DA SILVA; RAO 2006).The presence of co-solutes, in its turn, such as sucrose is not required, but the gel can be formed in the presence and absence of sugars. Since it does not require sugar as co-solute the LM pectin gels, in the food industry, can be used in dietetic foods and low-calorie products (LOPES DA SILVA; RAO, 2006; NGOUÉMAZONG et al., 2012; VRIESMANN; PETKOWICZ, 2013)

2.2.5.2 Function and extraction methods

In nature, the presence of pectin in a plant has an important role in its development, with structural and functional properties. Pectin acts in cell wall structure, cell-cell adhesion, growth, defense, fruit development, seed maintaining, and many other functions (MOHNEN, 2008). When extracted, the pectin applications

are still related with its structural and functional properties. Pectins can be extracted from plants, fruits, and vegetables by different procedures, been described as classical or complexes methods. For the classical ones, the extraction occurs using an acid or water as a solvent with the variation of temperature and time of extraction. The complex methodologies involve the use of pulsed electric field, enzymes, ultrasounds, microwave heating, enter other that require more investment, which in turn makes the technique difficult to scale-up to industries (ADETUNJI et al., 2017; EINHORN-STOLL, 2017).

The pectin extraction method has effects in its chemical structure, DE classification, and consequently in its function (SCHOLS et al., 2009). It has been reported that some methodologies lead to LM pectins extraction; in other cases, the HM pectins can be modified by purification process (acidic, alkaline or enzymatic treatments) to reduce the methoxylations (FRAEYE; DUVETTER et al., 2010; EINHORN-STOLL, 2017; VRIESMANN; PETKOWICZ, 2017). After the extraction, the pectins are precipitated, dried and milled to facilitate its application, transportation and preservation. Pectins are commercially traded as dry powder and it requires dispersion in water before being used (ADETUNJI et al., 2017).

The use of extracted pectin have a huge range of applications; going from the food industry to health applications, passing by manufacture industry (ADETUNJI et al., 2017). The most important functions of pectin are its gelling or thickening properties, which are related to texturizing applications such as viscosity enhancement, and colloid stabilizing (LOPES DA SILVA; RAO, 2006; FISHMAN; COOKE, 2009; EINHORN-STOLL, 2017). Those qualities can be applied in the food industry (e.g. jam, jellies, preserves, dietetics products) in the cosmetic industries (e.g. gelling, thickening and stabilizing). They can also have health effects (e.g. stimulating immune responses, lowering the levels of cholesterol and glucose, reducing cancer, benefits cardiovascular health), used in pharmaceutics (e.g. biomaterials, drug delivery, microencapsulation), applications in biodegradable films and paper substitutes (LÖFGREN; HERMANSSON, 2007; MOHNEN, 2008; SCHOLS et al., 2009; ADETUNJI et al., 2017).

2.2.5.3 Cocoa husk (*Theobroma cacao* L.) as a Pectin sources

All plants have pectin in their composition. Commercial pectin are traditionally extracted from citrus peels and apple pomace because of its industrial application (FRAEYE; DUVETTER et al., 2010). Other plants have been reported as good sources of pectin for industrial applications; among them sugar beet (DRUSCH, 2007) , potato pulp (YANG et al., 2018), pumpkin (DE SOUZA et al., 2013), okra (KPODO et al., 2017) and tropical fruits, such as watermelon rind (PETKOWICZ et al., 2017), cupuassu pulp (VRIESMANN et al., 2010) and cocoa husk (MOLLEA et al., 2008), can be cited (ADETUNJI et al., 2017; EINHORN-STOLL, 2017).

The cocoa pod (*Theobroma cacao* L.) is a tropical fruit native of the Amazon region widely known because of its seed that originates the chocolate. It is a perennial crop that grows around the Equator line (BEG et al., 2017). The fruit composition varies between 74-76% of husk, and 20-50 of seeds (VRIESMANN; PETKOWICZ, 2017). During cocoa beans processing it is estimated that around 700.000 tonnes of cocoa husks has been produced per year (The International Cocoa Organization - ICCO, 2016).

Cocoa husks are the first discarded by-product of the cocoa production chain; it is extremely important to find uses for all this material. ICCO and World Cocoa Foundation (WCF) are global organizations that are involved in the cocoa production and consumption. Both organizations keep on track of the cocoa production in all over the world. The ICCO supervised a project in Ghana to use the by-products of cocoa chain: the cocoa beans that were discarded from the chocolate production were used to produce cocoa butter, cocoa butter soap and pomade; the cocoa husk was destined to the production of animal feed and soft soap; other by-products were destined to the production of alcohol to process into gin and pectin to use in jellies. The World Cocoa Foundation (WCF) disclosed, in July 2018, that the biggest cocoa bean productor Côte d'Ivoire, was intended to use the by-products to generate electricity from the biomass, aiming the generation of 434 megawatts by 2030.

Researchers have been looking for refined uses for the re-use of by-products of cocoa beans processing. It has been reported that pectin extracted from cacao husk were able to form gels (VRIESMANN et al., 2012; VRIESMANN; PETKOWICZ, 2013), to modulate immune molecules (AMORIM et al. (2016), to work as emulsifier

agent (YAPO; KOFFI, 2013), and the use of cocoa pod as a source of antioxidants (BOUNGO TEBOUKEU et al., 2018). These applications demonstrate how to add value to the main by-product of cocoa beans production (VRIESMANN; PETKOWICZ, 2017).

2.3 CONCLUSIONS

Yeasts and biotechnology together has many economic benefits such as the production of different enzymes, chemicals, polymers, antibiotics (ARAKAKI et al., 2011). They can work in the bioremediation, solving environmental pollutions, industrial wastes (DÖNMEZ; AKSU, 1999; CHEN et al., 2005; GÖNEN; AKSU, 2008), having health benefits too, been used directly as probiotics and mineral dietary supplements (MÉPLAN, 2011). Se-enriched yeast can provide selenium in a organic form, more avaliable to the organism, avoiding selenium deficiency and the deseases related to it. The use of cryoprotective and adjunvant matrices increase the stability of the Se-enriched cells during storage. The use of polysscaharides as powerful matrices can also have nutritions benefitis against some deseases, making the combo, microencapsulated Se-enriched yeast, a suitable and promising product for mineral dietary supplementation of humans and animals.

CHAPTER 3 - EXTRACTION AND CHARACTERIZATION OF WATER SOLUBLE PECTIN FROM COCOA HUSKS (*Theobroma cacao* L.)

ABSTRACT

Based on the annual production of cocoa beans it can be estimated that about 17.0 million tonnes of cocoa husks (CH) were produced per year in the past five years (2012 – 2016). Great part of these by-product is discarded as waste, showing the importance of finding alternative applications for this material. CH has between 5.3 - 7.1% of pectin in its composition, been a rich source of it. Pectins are present in the plants cell wall and play roles related to its strength, support and defense responses. This polysaccharide is main composed by homogalacturonan (HG) and rhamnogalacturonan (RGI and RGII) polymers. The degree of methyl-esterification (DE) of HG chains determines the gelation properties of the pectin. The aim of this work was to optimize an water base pectin extraction and to characterize the extracted pectin. To optimize CH's pectin extraction a complete 3^2 experimental design, testing temperature and time, was carried out. The best conditions for pectin extraction were defined as 10% (w/v) of CH during 120 min reaching a yield of 15.66%. The extracted pectin (SP) was analyzed through FT-IR to confirm its structure. The interaction and classification of the SP were compared to those of a commercial pectin (CP). The degree of methyl-esterification (DE) obtained was 28.48% and 54.70%, respectively, which classifies the SP as LM and CP as HM. The LM classification increases SP value, since it can be used in the food industry acting in the conservation and gelation of dietetic products and other bioactive molecules.

Keywords: Pectin extraction 1. Cocoa Husk 2. LM Pectin 3. Polyssacharydes 4. *Theobroma cacao* L. 5.

3.1 INTRODUCTION

The hydrocolloid pectin was discovered in 1825 by the French scientist Henri Braconnot. Pectin is present in different land-growing plants, fruits and vegetables (NGOUÉMAZONG et al., 2012; ADETUNJI et al., 2017; EINHORN-STOLL, 2017). This polysaccharide can be found in the cell wall, cell corners, junctions zone and

middle lamella (MOHNEN, 2008). Pectin plays diverse roles in the plant constitution as strength, support and defense responses (VORAGEN et al., 2009).

The polysaccharide pectin has a complex chemical structure that is still being explored (FRAEYE et al., 2010; AMORIM et al., 2016). In general, it is formed by polymers of homogalacturonan (HG), xylogalacturonan (XGA), and rhamnogalacturonan Type I and Type II (RGI and RGII) (SCHELLER et al., 2007; VORAGEN et al., 2009; SCHOLS et al., 2009; MUZZARELLI et al., 2012). These polymers can covalently combine in more than 20 different linkages. However, the mode that they are linked is unclear (MOHNEN, 2008; VORAGEN et al., 2009).

The HG consists of a linear homopolymer of 1,4-linked α-D-GalA (D-galacturonic acid) residues that can be methyl-esterified at C-6. The percentage of carbonyl groups that are methylated defines the degree of methyl-esterification (DE). The DE can be used to classify the pectin, determining its gelation properties (VINCKEN et al., 2003; MOHNEN, 2008; NGOUÉMAZONG et al., 2012; EINHORN-STOLL, 2017). Pectins with DEs higher than 50% are classified as high-methoxy (HM) pectins, those who have DEs lower than 50% are classified as low-methoxy (LM) pectins (GIACOMAZZA et al., 2016; ADETUNJI et al., 2017).

HM pectin requires high amounts of sugars and low pH to form gel (SHARMA et al., 2006; FISHMAN; COOKE, 2009; MORRIS et al., 2010). In the food industry, HM pectins are used in high-sugar content products, such as preserves, jellies and jams (VRIESMANN; PETKOWICZ, 2013). Otherwise, LM pectin forms gel in the presence of calcium ions (Ca^{+2}) under high pH (MORRIS et al., 2010). Since does not require sugar as co-solute, LM pectins can be used in dietetic foods and low-calorie products, which increase its value (LOPES DA SILVA; Rao, 2006; NGOUÉMAZONG et al., 2012; VRIESMANN; PETKOWICZ, 2013).

Traditionally, commercial pectins are extracted from citrus peels and apple pomace. However, there are other plants that are reported as good sources, such as sugar beet, pumpkin, okra, watermelon, and cocoa husks (DRUSCH, 2007; MOLLEA et al., 2008; DE SOUZA et al., 2013; KPODO et al., 2017; PETKOWICZ et al., 2017; ADETUNJI et al., 2017; EINHORN-STOLL, 2017). Cocoa pod (*Theobroma cacao* L.) is a tropical fruit from Amazon region. Cocoa beans are widely known due its chocolate production. The cocoa pod composition varies presenting around 67-76 % of husk, 8.7-9.9 % pulp, and 21-23 % of beans (VRIESMANN; PETKOWICZ, 2017; CAMPOS-VEGA et al., 2018).

The data for cocoa pod production and its by-products can be estimated from the cocoa beans production reports. The world's average cocoa beans production in the past five years (2012 – 2016) was 4.5 millions of tonnes (FAOSTAT 2018). The first by-product of chocolate chain production is the cocoa husk. It is possible to estimate that all over the world around 15.7 millions of tonnes of cocoa husks are produced per year. Researchers have been looking for potential re-use of these by-products of cocoa beans production. Part of the generated cocoa husks is destined to feed production. However, a great part is discarded as waste, showing the importance of find alternative applications for this material.

Cocoa husk is a rich source of pectin and its extraction has been reported using different techniques. The type of extraction technique has effects on pectin chemical structure and on its function (SCHOLS et al., 2009). There are acid and aqueous extraction methods that were reported with yield and DE ranging between 3 and 18% (BARAZARTE et al., 2008; MARSIGLIA et al., 2016) and 6 - 58% (HUTOMO et al., 2016; SARAH et al., 2018), respectively. After extraction, pectins are precipitated, dried and milled to facilitate its application, transportation and preservation (ADETUNJI et al., 2017). The extracted pectin is able to form gels (VRIESMANN et al. 2012; VRIESMANN; PETKOWICZ, 2013) and act as emulsifier agent (YAPO; KOFFI, 2013). Cocoa husk pectin applications surely add value to this subproduct avoiding its discard (VRIESMANN; PETKOWICZ, 2017).

The aim of this work was to optimize pectin extraction conditions and to characterize and analyze the extracted pectin according to its DE and FT-IR profile.

3.2 MATERIAL AND METHODOS

3.2.1 Raw material and chemicals

Cocoa husks (*Theobroma cacao* L.), Forastero variety, were obtained from Fazenda Konagano, Tomé Açu - PA Brazil. The fruits were washed, with water and neutral soap, and dried. Then the pulp and husks were separated. The husks were cut in small pieces and dried in an oven at 60ºC, for 48 h. Finally, the dried husks were milled.

3.2.2 Cocoa husks classification

The milled cocoa husks were classified according to the following particle sizes: $x > 2.8$ mm; $2.8 \text{ mm} \geq x > 2.0$ mm; $2.0 \text{ mm} \geq x > 1.18$ mm; $1.18 \text{ mm} \geq x > 0.84$ mm; < 0.84 mm. Samples with particle size < 0.84 mm were chosen and referred as cacao husk powder (CH).

3.2.3 Cocoa husks analysis

Cocoa husks were analyzed, in triplicate, for ashes, pH, water-soluble ions, total reducing sugars and soluble proteins determination.

3.2.3.1 Determination of pH and ash

The CH where dried in a muffle oven until remain the ashes, which were calculated by weight difference from the initial CH. For the pH and total water-soluble ions analysis, the CH were suspended in water. The pH measurement was carried out using a pH meter (Tecnopon, model LUCA-210). Essays were carried out in triplicate.

3.2.3.2 Determination of total sugars and total proteins

The amount of CH's total sugars was measured using the colorimetric method of DNS (3.5-dinitrosalicylic acid) according to Miller (1959), using glucose as the standard curve. The absorbances of the samples were measured at 540 nm. The total soluble proteins were determined by the Bradford (1976) method, using bovine serum as standard and reagents of the QuickStart Bradford kit (Biorad – USA). The absorbances of the samples were measured at 595 nm. Both analyses were done using a ELISA plate reader (Power Waver XS – BioTeck). Essays were carried out in triplicate.

3.2.4 Optimization of cocoa husks' pectin extraction

CH's pectin extraction was conducted using hot-water-soluble extraction described by Vriesmann et al. (2011), using boiling water at the same pH as that of CH's aqueous extract. A complete experimental factorial design 3^2, with triplicate of the center point, was employed to optimize the time of boiling and concentration of CH. Coded and uncoded levels of studied factors are presented in Table 5.

TABLE 5 - CODED AND UNCODED LEVELS OF STUDIED FACTORS – EXPERIMENTAL DESIGN 3^2 AND 3 CENTRAL POINTS

Coded Value	Time (min)	CH Concentration % (w/v)
-1	80	2
0	100	6
+1	120	10

SOURCE: THE AUTHOR

After extraction procedure, samples were cooled and filtered. Pectin was then precipitated using cold ethanol 1:3 (sample/ethanol) overnight. The samples were centrifuged at 3000 rpm for 15 min at 10ºC and re-precipitate with cold ethanol (1:3) to remove residual sugars from the precipitate. Results were analyzed by Statistica version 7.0. This extracted pectin was freeze-dried and macerated. Pectin extraction yield was determined as described by Chan; Choo (2013), with modification (Equation 1)

$$\text{Extracted Pectin Yield (\%)} = \frac{\text{Extracted Pectin (g)}}{\text{Initial CH (g)}} \text{ x } 100 \quad (1)$$

where: "Extracted Pectin" corresponds to the dry weight of pectin. The dry weight of extracted pectin was determined by drying the samples in an oven at 100 ºC until constant weight.

3.2.5 FT-IR spectroscopy

The dried extracted pectin was analyzed using the Fourier transform infrared spectroscopy (FT-IR) methodology, in triplicate. The analyzes used 200 mg of the sample without KBr The FT-IR spectra was collected using a Vertex 70 (Brucker) spectrophotometer, at the reflectance mode. 256 scans were obtained at a resolution

of 4 cm^{-1}, in the frequency range of 4000-400 cm^{-1}. The FT-IR spectra of SP was compared to commercial pectin (CP). The P9135 - Pectin from citrus peel/Sigma-Aldrich was used as control. The interactions were evaluated according to Pretsch et al. (2009).

3.2.6 Determination of degrees of O-methyl esterification (DE)

In DE classification the bands corresponding to esterified carboxyl groups (1745 cm^{-1} COO-R) and the non-esterified carboxylic groups (1630 cm^{-1} -COO-) were integrated using the equipment software (OPUS). The areas obtained were used to measure the DE, as described by Vriesmann; Petkowicz (2009), using Equation 2:

$$DE(\%) = \frac{A\,(1745)}{A(1745) + A(1630)}\ x\ 100 \quad (2)$$

where: A (1745) is the area of esterified carboxyl groups and A (1630) is the area of non-esterified carboxylic groups.

The DE values of the extracted pectin were compared to the commercial pectin.

3.3 RESULTS AND DISCUSSION

Cocoa husks were dried to avoid material's deterioration. After drying, CHs were milled and classified by particle size, < 0.84 mm, to obtain the cocoa husks powder (CH). CHs were then analyzed and characterized to observe some characteristics of the raw material. Focusing the composition CHs' in pectin, which has many industrial applications, its extraction conditions were optimized. The extracted pectin was classified according to its degree of methyl-esterification (DE).

3.3.1 Physical-chemical analysis of cocoa husks

Physical-chemical composition of CH's aqueous extract was determined (Table 6). The results showed that CH's pH was 5.44, and total reducing sugars

content of 50.73 mg/g The ash content (7.86 %) corresponds to previous reports, which ranges from 6.4 to 8.4 % (VRIESMANN et al., 2011; LU et al., 2018). Total water-soluble proteins were determined as 7.08 mg/g (0.7%). In the literature the protein content appears in the range of 7-10 % (VRIESMANN et al., 2011; GU et al., 2013; LU et al., 2018). Similar results (1.46 % of protein) were reported by Marsiglia et al. (2016).

TABLE 6 - PHYSICAL-CHEMICAL COMPOSITION OF COCOA HUSK

Physic-chemical parameters	CH (<0.84 mm)
pH	5.44 ± 0.05
Ash (%)	7.86 ± 0.11
Total reducing sugar (mg/g)	50.73 ± 3.90
Total proteins (mg/g)	7.08 ± 0.05

SOURCE: THE AUTHOR

Different data of cocoa' composition and protein amount can be observed in the literature. This fact could be explained by the differences in cocoa varieties, sources and, certainly, the different soil compositions. Another factor to be consider would be the different analytical techniques used (VRIESMANN et al., 2011).

3.3.2 Optimization of cocoa husks' pectin extraction

Sobamiwa; Longe (1994) reported that there is between 5.3 - 7.1 % of pectin in CHs. Pectins can be extracted using different classical and complexes procedures. The classical extraction involves the use of acids or water as a solvent, varying pH, temperature, and time of extraction. Between the complex techniques there are: use of a pulsed electric field, enzymes, microwave heating, and others that require more investment, which turns difficult its scale-up to industries (ADETUNJI et al., 2017; EINHORN-STOLL, 2017; SARAH et al., 2018). So, it is important to find simple, low cost and environmentally friendly techniques for pectin extraction.

The study of CH's pectin extraction was carried out using a 3^2 complete factorial design. The results of extracted pectin (g/L) and the yield of extraction (%) are presented in Table 7. The highest yield was 15.66 %, which was obtained with 120 min of extraction and using 10 % (w/v) of CH. Vriesmann et al. (2011) in their

work with CH extraction, also using water as solvent, reached a yield of 12.6 %. This means that an increase of 23.8 % was obtained.

TABLE 7 - COMPLETE FACTORIAL EXPERIMENTAL DESIGN 3^2 WITH 3 CENTER POINTS FOR THE OPTIMIZATION OF PECTIN EXTRACTION FROM COCOA HUSKS

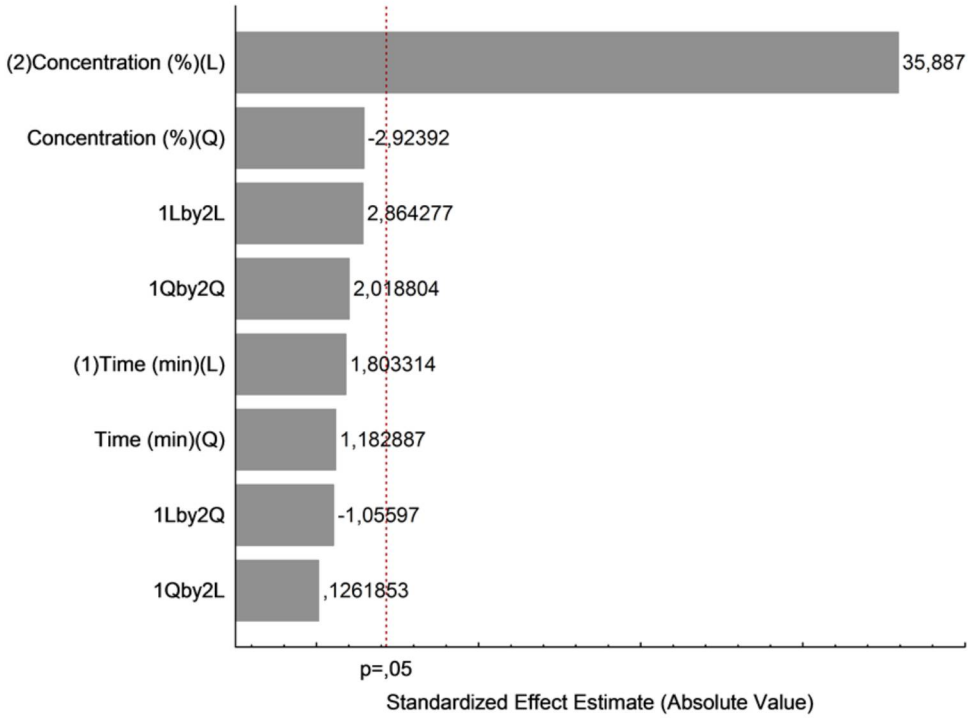
Conditions	Time (min)	CH Concentration % (w/v)	Extracted Pectin (g/L)	Yield % (g Pectin/ 100 g CH)
1	80	2	2.05	10.25
2	80	6	6.98	11.63
3	80	10	13.51	13.51
4	100	2	1.80	9.00
5	100	10	14.6	14.60
6	120	2	1.71	8.55
7	120	6	7.09	11.82
8	120	10	15.66	15.66
9	100	6	7.94	13.23
10	100	6	7.78	12.97
11	100	6	8.60	14.33

The yield was expressed in “g of extracted pectin/ 100g of CH” as described in equation 01.
SOURCE: THE AUTHOR

The Pareto chart of the standard effects of the studied factors (time in minutes and concentration of CH) over pectin extraction is shown Figure 3. It is possible to see that CH’s concentration is the most significant factor at $p \leq 0.05$. The determination coefficient (R^2) was 0.998. On the other hand, the time of treatment was not significant for pectin extraction, which means that it would be possible to work with shorter times.

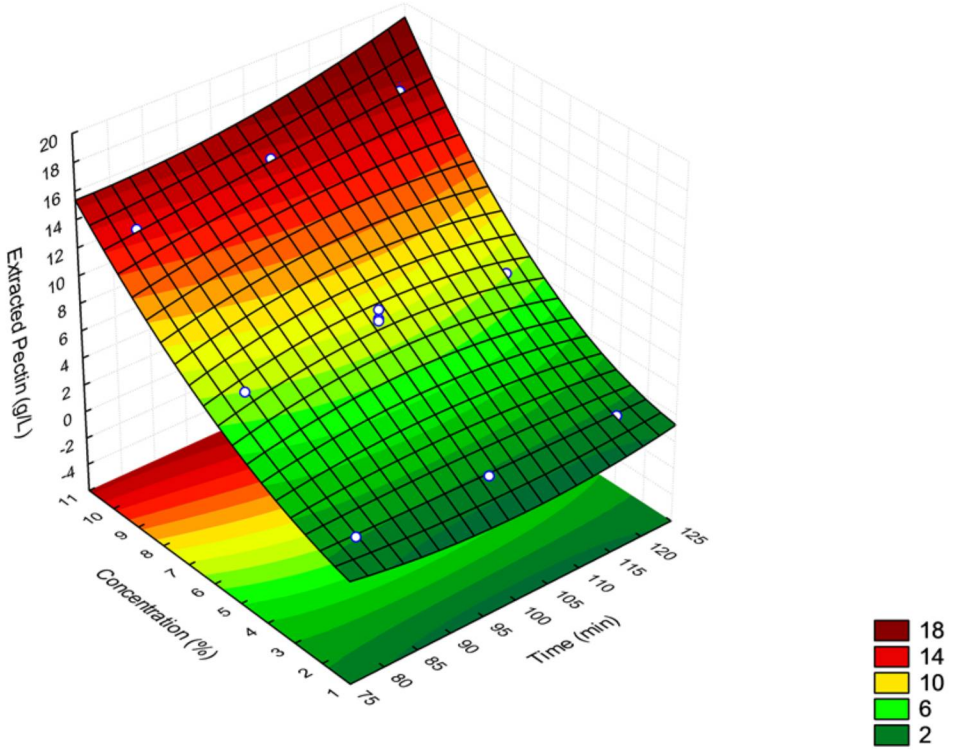
Statistically, there is low interaction between the studied factors. The response surface, in Figure 4, shows that pectin concentration is higher for higher concentrations of CH (over 8-10 %) and any extraction time (between 80 to 120 min). However, the interaction of extraction time (120 min) with cocoa husks concentration (10% w/v) showed the higher yield (15.66 %). Thus, the extraction process was defined with 120 minutes and 10% (w/v) CH.

FIGURE 3 - PARETO CHART REPRESENTING THE INFLUENCE OF EXTRACTION TIME AND COCOA HUSK POWDER CONCENTRATION ON PECTIN EXTRACTION



SOURCE: THE AUTHOR

FIGURE 4 - RESPONSE SURFACE OBTAINED FOR 3^2 COMPLETE FACTORIAL DESIGN SHOWING THE EFFECT OF PECTIN EXTRACTION TIME AND CONCENTRATION OF COCOA HUSK POWDER ON PECTIN EXTRACTION



SOURCE: THE AUTHOR

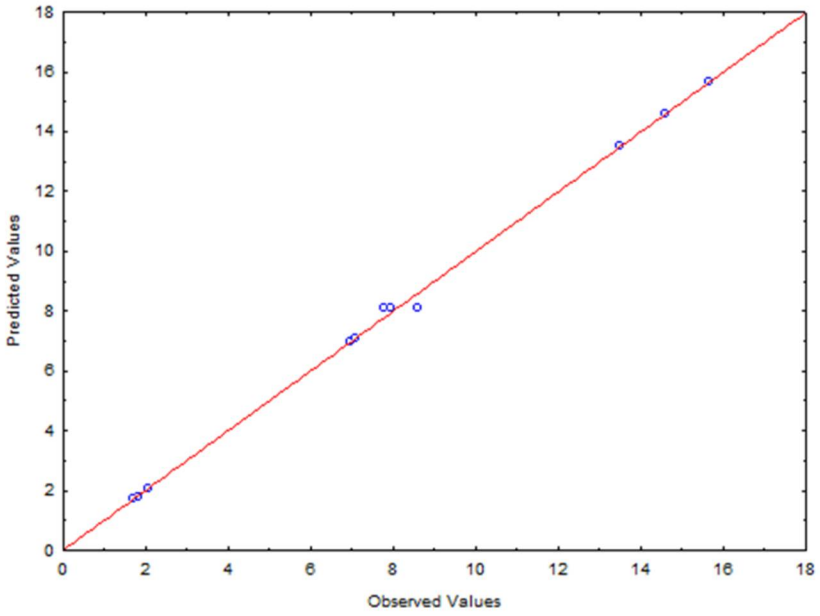
It was obtained a mathematical model (Equation 3), with a reliability of R^2=0.998, which correlates the extracted pectin with the time of extraction and CH concentration.

$$\text{Extracted pectin (g/L)} = 35.7375 - 0.7412X + 0.0037\,X^2 - 18.7575\,Y + 1.6069\,Y^2 + 0.4129\,XY - 0.0333XY^2 - 0.0021\,X^2Y + 0.0002\,X^2Y^2 \quad (3)$$

where: X is time (min) and Y the concentration of CH in % (w/v).

The model was validated using the plot of observed vs. predicted values that are represented in Figure 5.

FIGURE 5 - PREDICTED VS. OBSERVED VALUES PLOT OF A COMPLETE FACTORIAL EXPERIMENTAL DESIGN 3^2 DEMONSTRATING THE EXPECTED AND THE OBTAINED VALUES OF EXTRACTED PECTIN (R^2= 0.998)



SOURCE: THE AUTHOR

The pectin extraction technique, described by Vriesmann et al. (2011), demonstrated high efficiencies when compared to acid extraction. The obtained extraction yield can be compared to pectin extraction from CH using different techniques (Table 8). The use of citric acid in pectin extraction has been widely reported (VRIESMANN et al. 2012). In this case, authors obtained a yield of 10 % (w/w) using pH 3.0, 95 ºC and 95 min. Marsiglia et al. (2016) obtained a yield of

18.12 % (w/vw) using citric acid as solvent, low pH (2), time of extraction (90 min) and temperature (90 ºC). Hydrochloric acid was employed by Hutomo et al. (2016) reaching a yield of 11.70% and obtaining HM pectins.

TABLE 8 - DIFFERENT TECHNIQUES AND RESULTS OF PECTIN EXTRACTION AND DE FROM COCOA HUSKS

Extraction Technique	Yield	DE	Reference
Chloric Acid (pH 4.0 at 90 ºC)	3.89%	48.26%	Barazarte et al. (2008)
Boiling Water	12.60%	42.30%	Vriesmann; Amboni; et al. (2011)
50ºC Water	7.50%	37.00%	Vriesmann; Amboni; et al. (2011)
Nitric acid (pH 1.5, 100 ºC and 30 min)	9.00%	56.60%	Vriesmann; Teófilo; et al. (2011)
Citric acid (pH 3.0, 95 ºC and 95 min)	10.00%	40.30%	Vriesmann et al. (2012)
Hydrochloric acid (1.58 M, 62.28 ºC, 4,8 h)	11.70%	58.45%	Hutomo et al. (2016)
Citric acid (pH 2.0, 90 ºC and 90 min)	18.12%	15.50%	Marsiglia et al. (2016)
Ascorbic acid (pH 2.5, 95 ºC and 45 min)	4.20%	8.10%	Priyangini et al. (2018)
Citric Acid (pH1.5 and microwaved 180 W at 30 min)	42.30%	6.51%	Sarah et al. (2018)

SOURCE: THE AUTHOR

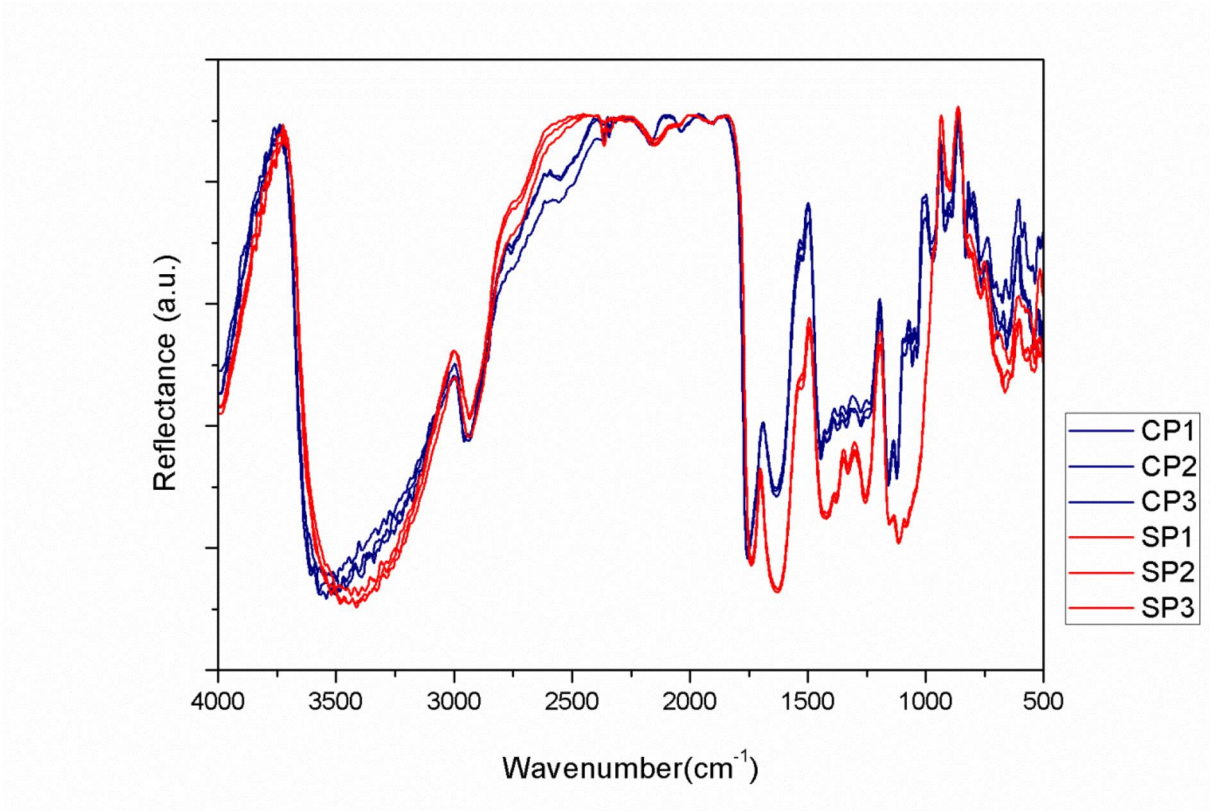
Sarah et al. (2018), described an acid extraction of pectin from cocoa husk using microwave as a heater system. The yield obtained reached 42.30%. However, at industrial scale, the use of complexes techniques could be a challenge and not economically viable. That is why the use of boiling water as solvent in pectin extraction could be advantageous. In this work it was shown that the aqueous extraction is a suitable and simple method to obtain pectin from CH with low DE pectins and similar extraction yields than those techniques that use acids as solvent.

3.3.3 FT-IR spectroscopy

Copikova; Cerna (2001) used FTIR analysis to check the presence of different polymers in food. The same idea was applied to analyze the extracted pectin. FT-IR analysis was then carried for the extracted pectin (SP) and also for a commercial pectin (CP). Figure 6 shows that SP spectra had similar behavior to CP's confirming that the extracted SP has the same spectra profile as commercial pectins,

such as that presented by Copikova; Cerna (2001). Similar studies were used to understand the spectra obtained in the FT-IR.

FIGURE 6 - FT-IR SPECTRA OF EXTRACTED PECTIN (SP) AND CONTROL PECTIN (CP) SHOWING THE WAVENUMBER (cm^{-1})



SOURCE: THE AUTHOR

Thakur et al. (1997) reported pectin's chemical composition. In general, there are the following interactions: C=O; C-OH; O-H; C-H_3; COO^-; $COOCH_3$. Assignments of the interactions in the extracted pectin (SP) and in commercial pectin (CP) samples are presented in Table 9. The spectra values are the average of each spectrum that were analyzed in triplicate. The interactions were evaluated using literature data, through interpretation of FT-IR's Handbook by Pretsch et al. (2009) for elucidation.

The bands 2170/2150 cm^{-1} and 2034/2046 cm^{-1} represent CO_2 interactions, which can be related to the environment during the analysis. An additional band, which was obtained at 970 cm^{-1}, generally appears in highly esterified samples (Figure 6). The appearance of this band only in CP sample demonstrate that the commercial pectin is highly esterified than SP (FELLAH et al., 2009; BAUM et al., 2017).

TABLE 9 - FT-IR SPECTRA AND CORRESPONDING INTERACTIONS FOR EXTRACTED PECTIN (SP) AND CONTROL PECTIN (CP)

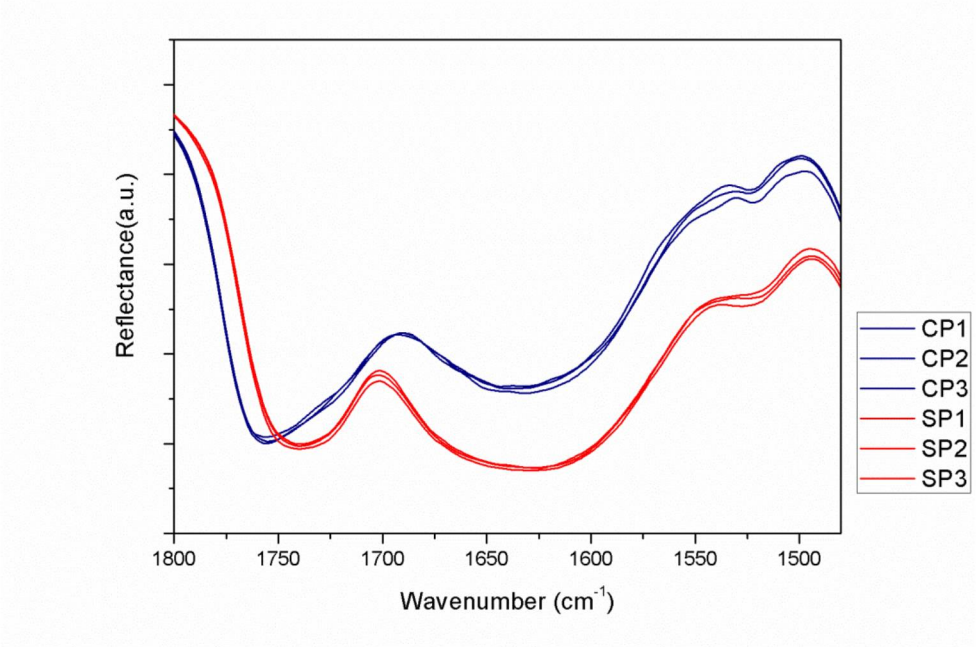
Wavenumber (cm^{-1})		Assignment	Reference
Extracted Pectin	Control Pectin		
3433	3513	Stretching vibration of O-H group	Marsiglia et al. (2016)
2930	2943	Stretching vibration of C-H group	Marsiglia et al. (2016); Sato et al. (2011)
2365	2366	Stretching vibration of C≡C group	Marsiglia et al. (2016)
1740	1752	Stretching vibration C=O in ester	Fracasso et al. (2018); Fellah et al. (2009); Sato et al. (2011)
1630	1637	Stretching vibration of COO^- group	Fracasso et al. (2018); Fellah et al. (2009); Sato et al. (2011)
1423	1448	Stretching vibration of O-C-O in ester	Copikova; Cerna (2001)
1328	1336	Stretching vibration of C-H ring (waging)	Copikova; Cerna (2001)
1255	1275	Stretching vibration of C-O-C in ester	Copikova; Cerna (2001)
1152	1160	Stretching vibration of O-C-O ring	Copikova; Cerna (2001)
1117	1120	Stretching vibration of C-O and C-C ring	Copikova; Cerna (2001)
1081	1062	Stretching vibration of C-O-C	Marsiglia et al. (2016)
-	970	Stretching vibration of $C-H_3$ in ester	Copikova; Cerna (2001)
900	913	Stretching vibration of CH_3 (rocking) in ester	Copikova; Cerna (2001); Baum et al. (2017)
821	830	Stretching vibration of OH (ring)	Copikova; Cerna (2001)
768	760	Stretching vibration of CH_3 (rocking); Breathing ring	Copikova; Cerna (2001)

SOURCE: THE AUTHOR

3.3.4 Determination of degrees of methyl-esterification (DE)

The area of the bands 1745 and 1630 (Figure 7) was quantified for DE calculation, as shown in the Equation 2. Table 10 brings the area of each band and the DE obtained. The average of each triplicate was calculated to obtain the standard deviation. SP samples were classified as LM pectin with a DE of 28.48 %.

FIGURE 7 - FT-IR SPECTRA OF EXTRACTED PECTIN (SP) AND CONTROL PECTIN (CP) AT 1740 cm^{-1} AND 1630 cm^{-1} BANDS



SOURCE: THE AUTHOR

LM pectin from cocoa husk has been tested as its gelation capacity by Vriesmann; Petkowicz (2017). However, under LM gel formation environment (high pH and presence of Ca) this polysaccharide was not able to form gel. These results demonstrate the importance of understanding of gel formation of cocoa pectins.

TABLE 10 - PECTIN FT-IR SPECTRA, DEGREES OF METHYL-ESTERIFICATION DETERMINATION AND PECTIN CLASSIFICATION

Sample	Band (1745 cm^{-1})	Band (1630 cm^{-1})	DE (%)	Classification
SP 1	10.985	27.409	28.61	LM
SP 2	11.269	28.317	28.47	LM
SP 3	11.202	28.277	28.37	LM
CP 1	14.675	12.009	55.00	HM
CP 2	15.325	12.813	54.46	HM
CP 3	15.829	13.14	54.64	HM
SP	-	-	28.48 ± 0.12	LM
CP	-	-	54.70 ± 0.27	HM

THE DE CALCULATION WAS PERFORMED AS DESCRIBED IN EQUATION 2
SOURCE: THE AUTHOR

The pectin extraction technique affects pectin chemical structure, the DE classification and, consequently, its function (SCHOLS et al., 2009). Some

methodologies have been reported where pectins were characterized as LM (VRIESMANN et al., 2012; MARSIGLIA et al., 2016; PRIYANGINI et al., 2018; SARAH et al., 2018).

The DE classification of cocoa husk pectin's obtained through different extraction techniques showed a range of 58.45 % to 6.51 % (Table 8). Another way to obtain LM pectins is the modification of HM pectins by acidic, alkaline or enzymatic processes so as to reduce the methylations (FRAEYE; DUVETTER et al., 2010; VRIESMANN; PETKOWICZ, 2017; EINHORN-STOLL, 2017). The capability of changing from HM to LM explains why some acid techniques lead to HM pectin whiles other lead to LM. Generally, commercial pectins are classified as HM, explaining the value of CP's DE (54.70%) (VRIESMANN et al., 2012).

Pectin has application in different industrial branches, showing also some health benefits. It can stimulate immune responses, reduce cholesterol levels and cancer (LÖFGREN; HERMANSSON, 2007; MOHNEN, 2008; SCHOLS et al., 2009; ADETUNJI et al., 2017). These applications punctuate the importance to study this polysaccharide and to find new applications. Besides, the results certainly bring a new way to add value to CH and stimulate its study for different applications.

3.4 CONCLUSIONS

The optimization of pectin extraction, from cocoa husks powder using boiling water, was efficient, increasing pectin extraction in 23.80 %. The best condition was defined as: CH concentration of 10% and time of process of 120 min, reaching a yield of 15.66 %. The extracted pectin was analyzed by FT-IR and compared to a commercial pectin product demonstrating similar behavior. The DE of the extracted pectin was calculated (28.48 %) classifying it as a LM pectin. The FT-IR spectra analysis was a good approach to evaluate CH's pectin behavior. Further investigations are certainly required to purify the polymer and to test possible applications in food and feed industrial products or as adjuvant agent for biomolecules and living cells encapsulation.

CHAPTER 4 - PRODUCTION AND MICROENCAPSULATION OF SELENIUM ENRICHED *Wickerhamomyces anomalus* LPB-CPB5

ABSTRACT

Selenium (Se) is a trace element known for its health benefits, its dietary supplementation through Se-enriched yeasts is a possible source of this mineral. For these reason, it is important to promote and increase the viability and stability of yeast cells, using freeze-drying and spray-drying techniques. In both cases some natural polymers can be employed as cryoprotective/adjuvant agents. The mineral bioaccumulation capability of *Wickerhamomyces anomalus* LPB-CPB5 and its GRAS (Generally Recognized as Safe) status show its potential in dietary supplementation. The aim of this work was to study the production of Se-enriched LPB-CPB5 cells using sugarcane molasses and to evaluate the potential of corn starch, cocoa husks' pectin and Kefiran matrices as microencapsulation agents. The bioaccumulation of selenium achieved the highest amount of 9.51 mg of Se/g of yeast cells, with a biomass of 11.58 g/L, when the medium was supplemented with 200 mg/L of Se in fed-batch. However, the 50 mg/L was chosen for microencapsulation tests due to the higher accumulation yield (100.00%), biomass (12.70 g/L), and Se bioaccumulation (3.94 mg of Se/g of yeast). Cell stability tests were conducted after 30 and 60 days to test the efficacy of the different microencapsulation systems. The best encapsulation efficiency (EE) matrix was the T1 (modified starch) with EE= 88.75%, while best matrices for cell conservation were T2 (starch and cocoa pectin) and T3 (starch and Kefiran) with, respectively, 95.66 % and 100.00% of survival rate. Great perspectives were observed for the developed product, which could be used in different applications for human and animal Se-supplements.

Keywords: Pectin 1. *Wickerhamomyces anomalus* BARU05 2. Cocoa husk 3. Selenium 4. Kefiran 5.

4.1 INTRODUCTION

The strain *Candida pelliculosa* BARU05 (LPB-CPB5) was one of the eleven strains isolated by Arakaki (2010), from Baru (*Dipteryx alata*) fruit. The author

showed the capability of *C. pelliculosa* LPB-CPB5 in bioaccumulating different trace elements, such as zinc and copper, and its flexibility to growth in alternative culture mediums, such as sugarcane molasses, by-product of the bioethanol production chain, rich in sugars and nutrients. This strain is classified as a GRAS (Generally Recognized as Safe) microorganism. The GRAS classification is given for any substance approved by FDA (U.S. Food & Drug Administration) or for microorganisms classify as level 1.

C. pelliculosa yeast can be called as *Pichia anomala and Hansenula anomala*. Kurtzman (2011) showed that most of the 20 genus related to *P. anomala* were from the genus *Wickerhamomyces*. Explaining the reason for reclassification of *P. anomala,* and its assigned species, in the genus *Wickerhamomyces*. In this work the *C. pelliculosa* BARU05 yeast will be called *W. anomalus* LPB-CPB5. The mineral bioaccumulation capability of *W. anomalus* LPB-CPB5 and its GRAS classification show that this microorganism has potential to be used in dietary supplementation of some trace elements such as selenium (Se).

The element Se was discovered in 1817 in a Sweden factory and, since then, it has been studied for its potential applications (KIELISZEK; BŁAZEJAK, 2013). It was defined as an essential micronutrient in mid-1950's (ALLMANG et al., 2009). Now, it is classified as a trace element, which is constituent of numerous proteins, enzymes and hormones, showing functional and structural roles (RYCEWICZ-BORECKI et al., 2016). Selenium can be found in the environment in organic and inorganic forms. Inorganic forms are often selenite (SeO32−) and selenate (SeO42−). While, the organic forms are mostly selenoproteins, Se-lipids, Se-peptides and Se-amino acids (TIE et al., 2017). Se can be found in different foods such as the Brazilian nut. Another possible source is through dietary supplementation. Selenium inorganic forms can be biotransformed by yeasts to bioavailable forms, organic forms. The Se-enriched yeasts can be produced for food and feed, with free proteins and amino acids in the medium.

To increase the viability and stability of the Se-enriched-yeasts it is important to encapsulate the cells. Different materials can be used as microencapsulation agents, such as corn starch, kefiran and pectin. Modified starches are a polysaccharide with strong interaction with water (BEMILLER, 2019), they can extend fresh or processed foods' shelf life by forming strong film which are a barrier against oxygen (MOTEDAYEN et al., 2013). The Kefiran is a water soluble polysaccharide

enclosing the microflora of the Kefir grains (IRIGOYEN et al., 2005; GARCÍA, 2016). It has biological effects, such as antioxidant activity, anti-microbial, and healing activity (RODRIGUES et al., 2005; SABAGHI et al., 2015; BLANDÓN et al., 2016). Kefiran shows hydrophilic characteristic, been a promising biomaterial for drug delivery.

The hydrocolloid pectins is a polysaccharide, with a complex chemical structure, present in different land-growing plants, and can be found in different parts of the vegetal cell (MOHNEN, 2008; FRAEYE et al., 2010; AMORIM et al., 2016). It can form gels and act as emulsifier agent (YAPO; KOFFI, 2013), which are good characteristics for yeast microencapsulation (VRIESMANN et al. 2012; VRIESMANN; PETKOWICZ, 2013). Commercial pectins are, traditionally, extracted from apple pomace and citrus peels. The literature has reported pectin extraction from other sources, such as sugar beet, okra and tropical fruits, such as cocoa husk (DRUSCH, 2007; MOLLEA et al., 2008; DE SOUZA et al., 2013; KPODO et al., 2017; PETKOWICZ et al., 2017; ADETUNJI et al., 2017; EINHORN-STOLL, 2017).

The aim of this work was to produce selenium-enriched *W. anomalus* LPB-CPB5 cells by fermentation using molasses and their microencapsulation using different materials such as a corn starch, cocoa husks' pectin, and kefiran.

4.2 MATERIAL AND METHODOS

4.2.1 Production of selenium-enriched yeast cells

4.2.1.1 Strain

The yeast *Wickerhamomyces anomalus* LPB-CPB5 was chosen based on the work of Arakaki (2010), who studied the bioaccumulation of zinc and copper. The strain was cultured and maintained in YM (Yeast Malt) culture medium, and stored in 50 % glycerol at -80 °C.

4.2.1.2 Optimization of biomass production

The production of *W. anomalus* LPB-CPB5 strain was based on the medium described by Arakaki (2010), which was composed of sugarcane molasses at 10

ºBrix (75 g/L of total sugar) and 3 g/L of urea, optimized. Three steps of optimization were carried out using a Plackett-Burman 2^{7-4} with 7 factor, an incomplete factorial experimental design 3^{3-1}, and a validation. In each test the salts tested were added into the base medium, composed by sugarcane molasses (75 g/L) and urea (3 g/L), which was used as control in all the steps.

The Plackett-Burman 2^{7-4} (Table 11) was used to test the effect of presence or absence of seven factors (yeast extract, (NH4)2HPO4, MgSO4.7H_2O, K_2HPO_4, KH_2PO_4, $Fe_2(SO_4)_3$, $MnSO_4.H_2O$) in the biomass growth. Essays were conducted according to details described in Table 11, where coded and uncoded levels of factors are presented. Biomass production was conducted in 250 mL Erlenmeyer flasks at 28ºC at 120 rpm during 48 h, and evaluated by dry weight analyses. An aliquot of 10 mL from the fermented broth was centrifuged at 4500 rpm for 10 min, and washed two times with distilled water. Then it was placed in a drying oven for 24 h (100 ºC) till constant weight.

TABLE 11 - PLACKETT-BURMAN EXPERIMENTAL DESIGN 2^{7-4} -CODED AND UNCODED LEVELS OF STUDIED FACTORS

	Uncoded value						
Coded Value	Yeast Extract (g/L)	$(NH_4)_2HPO_4$ (g/L)	$MgSO_4.7H_2O$ (g/L)	KH_2PO_4 (g/L)	K_2HPO_4 (g/L)	$Fe_2(SO_4)$ (g/L)	$MnSO_4.H_2O$ (g/L)
-1	0	0	0	0	0	0	0
0	6	0.5	0.7	0.5	0.065	0.05	0.000438
+1	12	1	1.4	1	0.13	0.1	0.000876

SOURCE: THE AUTHOR

The second step of biomass production optimization was carried out using a 3^{3-1} experimental design (Table 12) with three factors (yeast extract, dibasic $(NH_4)_2HPO_4$) and KH_2PO_4 and 3 central points. Validation procedures was are presented in Table 13. Biomass production was conducted in 250 mL Erlenmeyer flasks at 28 ºC at 120 rpm during 48 h.

TABLE 12 - 3^{3-1} EXPERIMENTAL DESIGN - CODED AND UNCODED

Coded Value	Uncoded value		
	Yeast Extract (g/L)	$(NH_4)_2HPO_4$ (g/L)	KH_2PO_4 (g/L)
-1	12	1	1
0	21	1.5	1.5
+1	30	2	2

SOURCE: THE AUTHOR

The validation essays were carried out with the same factors in different concentrations as described in Table 13.

TABLE 13 - VALIDATION CONDITIONS

Condition	Yeast Extract (g/L)	$(NH_4)_2HPO_4$ (g/L)	KH_2PO_4 (g/L)
1	12	1.5	2
2	12	1.5	1.5
3	12	2	2
4	0	0	0

SOURCE: THE AUTHOR

4.2.1.3 Biomass production kinetics in batch and fed-batch

Biomass growth kinetics was conducted using optimal conditions during 96 h in 250 mL Erlenmeyer flasks at 28 ºC at 120 rpm. Kinetics of biomass production was carried out in both batch and fed-batch operations. The fed-batch culture was conducted with feeding of sugarcane molasses at 30 g/L of total sugar in the fermentation volume at feeding time of 10 and 12 h. The concentration of total reducing sugars was determined using the DNS (3.5-dinitrosalicylic acid) colorimetric method described by Miller (1959). Biomass dry weight was determined as described in section 4.2.1.2. The productivity was determined by Equation 4:

$$\text{Productivity}\ (\%) = \frac{\text{Biomass}\ (\text{g/L})}{\text{Time}\ (\text{h})} \tag{4}$$

4.2.1.4 Selenium tolerance and bioaccumulation by *Wickerhamomyces anomalus* LPB-CPB5

The capacity of *Wickerhamomyces anomalus* LPB-CPB5 to bioaccumulate selenium without causing significant stress was studied. Selenium tolerance by *W. anomalus* LPB-CPB5 was tested with 6 different concentrations of selenium: 0 mg/L, 25 mg/L, 50 mg/L, 100 mg/L, 200 mg/L and 400 mg/L (mg/L of Se^{3+}). Selenium added to the medium as the salt Na_2SeO_3. Se-feeding time was chosen to be in the beginning of stationary phase, in which the bioaccumulation minerals started and to reduce the inhibition of yeast growth by the presence selenium. Thus, the Se-feeding

was determined at 24 h of cultivation. After fermentation, biomass dry weight and selenium concentration in the supernatant were measured.

Biomass dry weight was determined as described in section 4.3.1.2. The bioaccumulation analysis was done using the Azure B methodology described by Mathew and Narayana (2006) with adaptations. Bioaccumulated selenium was determined by the difference between the selenium concentration Selenium-feeding solution and the Selenium concentration of the supernatant. The produced biomass dry weight was used to calculate the bioaccumulation yield, which was obtained by the ratio of bioaccumulated selenium and the total selenium.

4.2.1.5 Yeas cell disruption procedure

For LC-MS analysis, the intracellular yeast fraction was required. For that, the disruption of the yeast membrane was tested. The tested procedures were sonic bath, glass beads and French press with slow freezing. For the sonic bath, 0.01g/mL of the sample were maintained during 4 cycles of 480 s using a SCHUSTER ultrasonic washer (L-100). Glass beads of 450-600 µm (0.1 g/mL) were used for 0.01 g/mL of yeast suspension during 4 cycles of 8 min under vortex agitation. The French press analysis was performed in 5 cycles of 20000 psi, max gauge 1000 (Thermo Electron Corporation, French press cell disruptor) Samples were then frozen, at -6 ºC overnight, with glass beads (0.1 g/mL). The frozen samples were thawed for 8 min under vortex agitation. The disrupted cells were analyzed in a Neubauer chamber, for cells' counting.

4.2.1.6 Optical emission spectrometry inductively coupled plasma (ICP-OES) for determination of Bioaccumulated selenium

The ICP-OES analysis was carried out to analyze the selenium present on the Se-rich, fed with 50 mg/L of Se, and non-Se-rich yeast. One gram of the dry samples was digested in 5 mL of nitric acid overnight at room temperature. Then, 4 mL of hydrogen peroxide 30% were add and the system let in reflux digestion for 1h. After cooling, the samples were transferred to 50 mL volumetric flasks and the volume completed with deionized water. Then, the samples were determined by ICP-OES (VARIAN 720-ES), under the following conditions: power (1.00 kW), plasma flow (15.0 L/min), auxiliary flow (0.75 L/min), nebulizer pressure (180 kPa), replicate read

time (10 s), pump rate (50 rpm), rinse time (15 s). During the analysis the sample were diluted 10X.

4.2.2 Microencapsulation of yeast cells

After biomass production, yeast cells were dried for conservation and stability maintaining. In this way, different materials were tested for both spray-drying and freeze-drying,

4.2.2.1 Encapsulation matrices

Pectin was extracted from cocoa husks as described in the section 3.3.2.

Modified corn starch (Loremalt 2015M) was kindly provided by Lorenz® (Maringá, PR, Brazil).

The kefir grains were cultivated in whole milk at 28 ºC, which was renewed every day. Kefiran extraction was done as described by García (2016b). After precipitation with ethanol (3:1) the Kefiran wass freeze-dried and macerated.

4.2.2.2 Yeast biomass spray-drying and freeze-drying

Biomass production

Cells were actived in liquid YM medium for 24 h, to be used as inoculum (10% v/v). Biomass production was carried out for 48 h using previously optimized medium during was carried in a fed-batch operation with feeding (30 g/L of total sugar) after 10 h cultivation. For selenium-rich yeast cells, sodium selenite was added to the medium after 24 h of fermentation to guarantee the viability of cells, in an equivalent amount of 50 mg/L of Se^{3+}.

Yeast cells spray-drying

After yeast's cultivation different mixtures of polysaccharides were prepared with Se-rich yeasts' cells to study their conservation and stabilization according to Table 14. The material was previously sterilized at 121 ºC during 15 min and cooled before being added to yeasts samples. The matrices T1 and T3 were then dried at laboratory scale in a spray dryer Lab Plant SD-05 (Huddersfield, England). Spray-

drying was conducted at 150ºC, pressure of 0.06 MPa, air flow 73 m^3/h and air flow of 600 mL/h with exhausting temperature between 81 - 83 ºC.

Yeast cells lyophilization

Se-rich yeasts were mixed with different polysaccharides according to Table 14. Lyophilization was carried out in a freeze-dryer (RVT4104 - Thermo Scientific), at -45ºC, during 48 h, till a pressure of 50 mbar according to the conditions T1 to T4.

TABLE 14 - TREATMENTS COMPOSITION FOR SPRAY AND FREEZE-DRYING OF *W. anomalus* LPB-CPB5 YEAST CELLS

Components % (w/v)	T1	T2	T3	T4
Corn Starch	18%	18%	18%	18%
Cocoa Pectin	-	1%	-	1%
Kefiran	-	-	0.1%	0.1%
LPB-CPB5	0.85%	0.85%	0.85%	0.85%
Total Solid Contents	18.85%	19.85%	18.95%	19.95%

THE MATRICES T1 TO T4 WERE FREEZE-DRIED AND T1 AND T3 WERE SPRAY-DRIED.
SOURCE: THE AUTHOR

The total solid content of 20% was chosen based on the spray-dried conditions described in the literature to ensure high viability (NUNES et al., 2018).

4.2.2.3 Yeast cells' viability

LPB CB05 cells' viability and stability were tested according to Nunes et al. (2018) with adaptations, after 30 and 60 days of the drying processes. The samples were stored in a vacuum desiccator at room temperature. For the analyzes, 0.1 g of sample were diluted in 900 µL of peptone water (0.1% w/v), from seriated dilutions. Three dilutions were then transferred to sterile Petri plates containing YM agar, in triplicate, and were incubated at 30 °C for 48 h. The results were shown as log of colony forming units per gram of sample (log CFU/g). For comparison, the amount of cells in the different samples, were based on the theoretical concentration of cells in 0.1 g of samples after polysaccharides' addition.

4.2.2.4 Encapsulation efficiency

The efficiency of encapsulation (EE) is the survival rate of the microorganisms during the microencapsulation process. It was calculated according to Equation 5:

$$EE(\%) = \frac{N}{No}\ x\ 100 \quad (5)$$

where: N is the number of viable cells (log CFU/g) released from the microparticles after 30 days of drying process and N_0 is the number of viable cells (log CFU/g) that are in the free-cells suspension before the drying process (NUNES et al., 2018).

4.2.2.5 Microparticle morphology

The analyses of microparticle morphology and particle size determination were performed through scanning electron microscopy (SEM). For SEM analyses the samples were placed on aluminum stubs and covered with gold using a Balzers Sputtering SCD 030. For SEM-EDS analyses the samples were not covered with gold and it was analyzed in the EDS apparatus of the Balzers Sputtering SCD 030 microscope. The SEM images were captured with a TESCAN VEGA LMU microscope, operated at 10 kV and 5-8 mm working distance.

4.2.2.6 Statistical analysis

The results were analyzed using one-way analysis (ANOVA) and Ducan's test showed where was significantly different ($p \leq .05$). The analysis were performed using Statistic 7.0 software.

4.3 RESULTS AND DISCUSSION

4.3.1 Production of selenium rich *Wickerhamomyces anomalus LPB-CPB5* cells

4.3.1.1 Optimization of medium composition for *W. anomalus LPB-CPB5* biomass production

The use of agro-industrial residues such as soybean and sugarcane molasses, helps the development of a nutritive environment for microorganism's growth (Arakaki et al., 2011). This substrate is a rich source of sugars, macro and micro minerals. Aiming to increase the *W. anomalus* LPB-CPB5 biomass production, the effect of six salts and yeast extract on biomass growth was studied in a Plackett-Burman experimental design with 7 factors (yeast extract, (NH4)2HPO4, MgSO4.7H_2O, K_2HPO_4, KH_2PO_4, $Fe_2(SO_4)_3$, $MnSO_4.H_2O$) with the addition of 3 central point's (Table 15).

TABLE 15 - EFFECT OF DIFFERENT SALTS ON BIOMASS PRODUCTION - PLACKETT-BURMAN 2^{7-4} WITH 3 CENTER POINTS

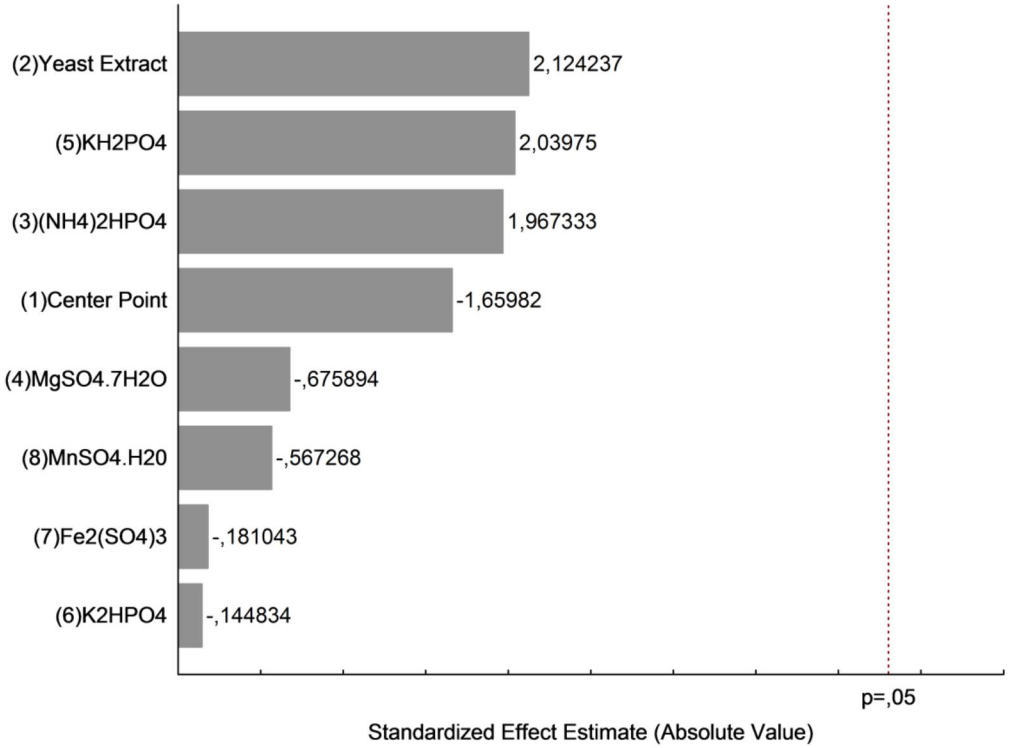
Yeast Extract (g/L)	$(NH_4)_2HPO_4$ (g/L)	$MgSO_4.7H_2O$ (g/L)	KH_2PO_4 (g/L)	K_2HPO_4 (g/L)	$Fe_2(SO_4)$ (g/L)	$MnSO_4.H_2O$ (g/L)	Biomass (g/L)
0	0	0	1	0.13	0.1	0	11.49
12	0	0	0	0	0.1	0.000876	11.35
0	1	0	0	0.13	0	0.000876	11.30
12	1	0	1	0	0	0	13.32
0	0	1.4	1	0	0	0.000876	11.11
12	0	1.4	0	0.13	0	0	11.32
0	1	1.4	0	0	0.1	0	11.24
12	1	1.4	1	0.13	0.1	0.000876	12.67
6	0.5	0.7	0.5	0.065	0.05	0.000438	12.83
6	0.5	0.7	0.5	0.065	0.05	0.000438	12.60
6	0.5	0.7	0.5	0.065	0.05	0.000438	11.72

SOURCE: THE AUTHOR

Biomass production reached a maximum of 13.32 g/L with the presence of yeast extract, $(NH_4)_2HPO_4$ and KH_2PO_4 in the medium. The Pareto chart, in Figure 8, shows that there are no significant factor significant factors ($p < 0.5$) on biomass production (R^2= 0.889). The control essay led to a biomass production of 8.76 g/L, which means that an increase of 52.05% on biomass concentration was reached after this step of optimization.

Since the objective was to increase the biomass production, another step of optimization of medium composition was carried out using a 3^{3-1} factorial experimental design where different levels of KH_2PO_4, $(NH_4)_2HPO_4$ and Yeast Extract were tested.

FIGURE 8 - PARETO CHART AND STANDARD EFFECTS OF A PLACKETT-BURMAN 2^{7-4} INFLUENEC OF MEDIUM COMPONENTS (R^2=0.88969)



SOURCE: THE AUTHOR

Biomass production was evaluated according to the 3^{3-1} incomplete factorial experimental design with 2 center points (Table 16). An increase of 36 % of biomass production (14.24 g/L) was reached for essay number 2 using yeast extract (12 g/L) KH_2PO_4 (2 g/L), $(NH_4)_2HPO_4$ (1.5 g/L) comparing to control (10.47 g/L).

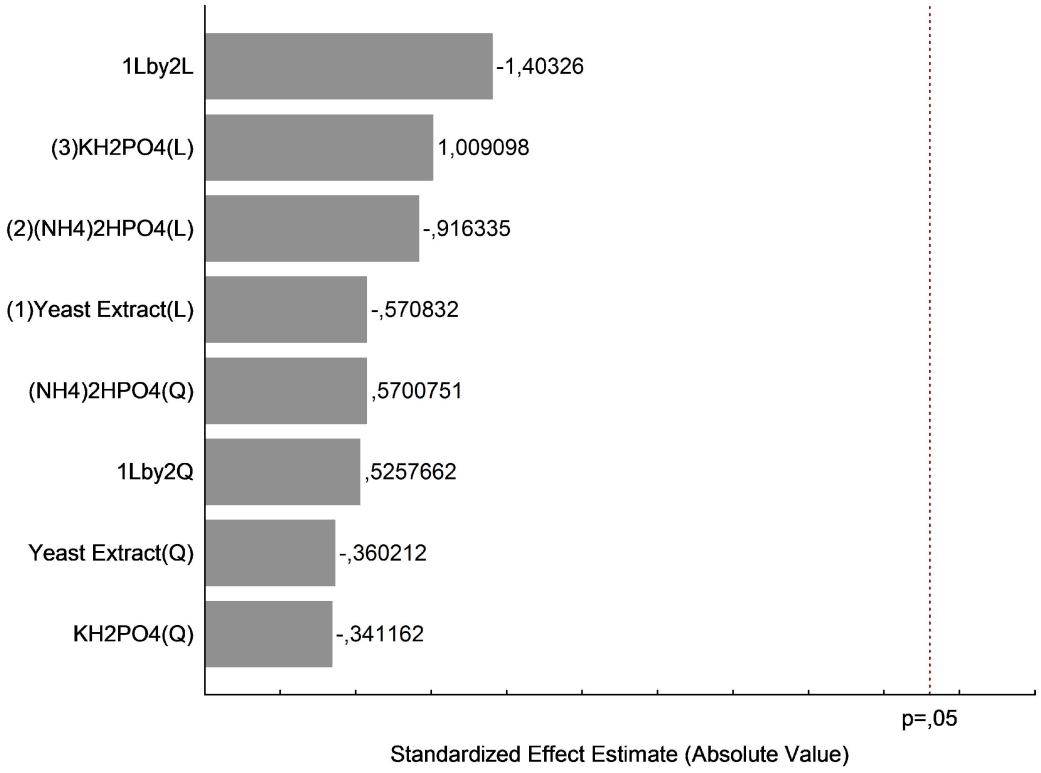
TABLE 16 - EFFECT OF MEDIUM COMPOSITION ON BIOMASS PRODUCTION – EXPERIMENTAL DESIGN 3^{3-1} INCOMPLETE FACTORIAL EXPERIMENTAL DESIGN WITH 2 CENTER POINTS

Essays	Yeast Extract (g/L)	$(NH_4)_2HPO_4$ (g/L)	K_2HPO_4 (g/L)	Biomass (g/L)
1	12	1	1	13.46
2	12	1.5	2	14.24
3	12	2	1.5	14.21
4	21	1	2	14.22
5	21	1.5	1.5	13.12
6	21	2	1	13.18
7	30	1	1.5	14.13
8	30	1.5	1	13.32
9	30	2	2	13.20
10	21	1.5	1.5	14.09
11	21	1.5	1.5	14.03

SOURCE: THE AUTHOR

Analyzing the Pareto chart in Figure 9, it is possible to observe that the studied factors are not significant at $p < 0.5$. Even so, KH_2PO_4 had positive effect. The interaction between KH_2PO_4 and yeast extract and also between KH_2PO_4 and $(NH_4)_2HPO_4$ was analyzed (Figure 9). The determination coefficient (R^2) was 0.719.

FIGURE 9 - PARETO CHART AND STANDARD EFFECTS OF YEAST EXTRACT, $(NH_4)_2HPO_4$ AND KH_2PO_4 ON BIOMASS PRODUCTION - 3^{3-1} INCOMPLETE FACTORIAL EXPERIMENTAL DESIGN

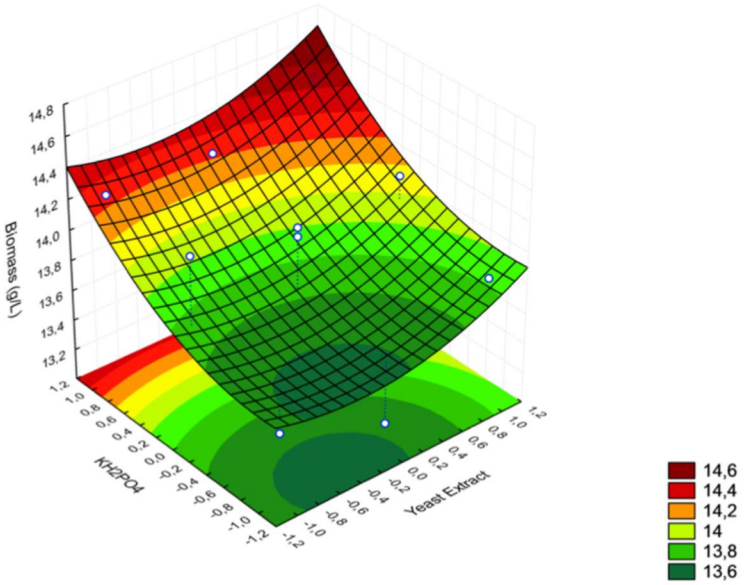


SOURCE: THE AUTHOR

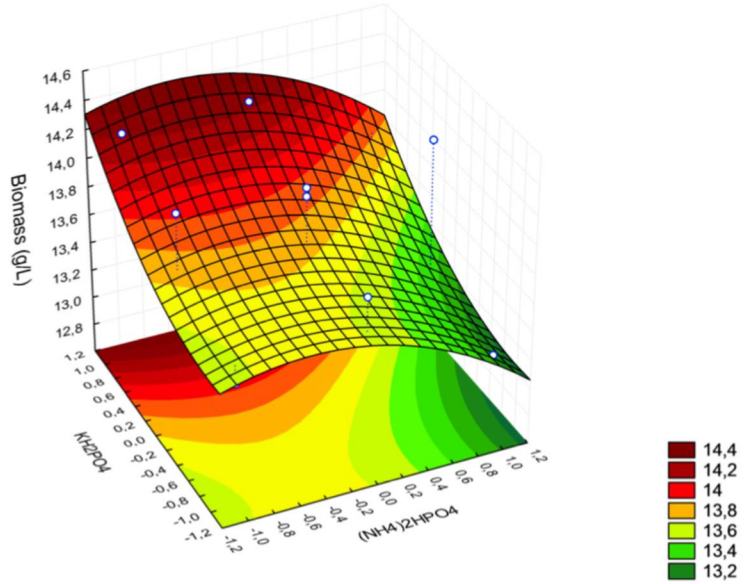
The analysis of the response surface, in Figure 10, shows that yeast extract could be used in any of the studied levels. The interaction of yeast extract with $(NH_4)_2HPO_4$ could be used in any level. The concentration of XX (g/L) of Yeast Extract and XX g/L and XX g/L of KH_2PO_4 and $(NH_4)_2HPO_4$, respectively were chosen for experiments'. A third experiment was carried out in triplicate to validate these conditions.

FIGURE 10 - FITTED SURFACE, OF A COMPLETE FACTORIAL 3^{3-1} (R^2= 0.719), SHOWING THE INTERACTIONS BETWEEN KH_2PO_4 AND YEAST EXTRACT (A), AND BETWEEN KH_2PO_4 AND $(NH_4)_2HPO_4$ (B) IN THE BIOMASS PRODUCTION

a)



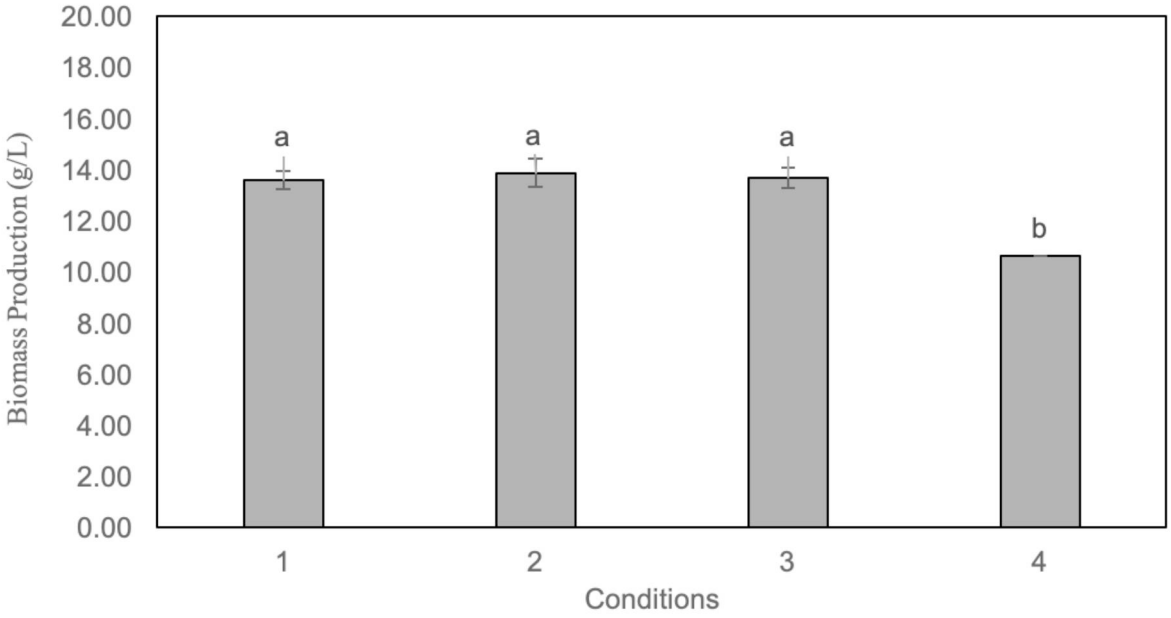
b)



SOURCE: THE AUTHOR

Validation essays were carried out and are shown in Figure 11. The higher biomass obtained was (13.87 g/L) 30.76 % higher than the control medium (10.61 g/L). Since Ducan's test showed that there was no significant difference between the conditions, the condition 2 was chosen because it used the lowest concentration of each component. At end the medium with yeast extract (12 g/L), $(NH_4)_2HPO_4$ (1.5 g/L) and KH_2PO_4 (1.5 g/L) besides cane molasses (75 g of total sugar/L) and urea (3 g/L), was defined as the culture medium for the fermentation.

FIGURE 11 - VALIDATION ESSAYS FOR *W. anomalus* LPB-CPB5 BIOMASS PRODUCTION USING OPTIMIZED CONDITIONS



DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P≤ .05) BY DUCAN`S TEST.
SOURCE: THE AUTHOR

The obtained results are in agreement with the literature, where there are reports showing the positive effect of phosphate on yeasts's growth (Schulze, 1956; Markham; Byrne, 1967). Yeast extract is a source of nitrogen that favors yeasts' biomass growth (Li et al., 2009).

4.3.1.2 Growth kinetics of *W. anomalus* LPB-CPB5 in batch and fed-batch cultivation

The growth kinetics of *W. anomalus* LPB-CPB5 was evaluated in batch and fed-batch fermentation. Biomass growth and carbon source (total sugars) evolution was followed during 96 hours, which is presented in Figure 12: batch (a); fed-batch with feeding after 10 h (b), and fed-batch with feeding after 12 h.

Batch kinetics of *W. anomalus* LPB-CPB5 showed a *lag* phase between 0 – 12 h of cultivation and the exponential phase starting from this point, which goes till 24 h, when the stationary phase starts. The highest biomass obtained was 16.20 g/L at 96 h of cultivation, and 0.169 g/L.h of productivity. However, the productivity at 72 h was 0.219 g/L.h, meaning that the process could end at 72 h.

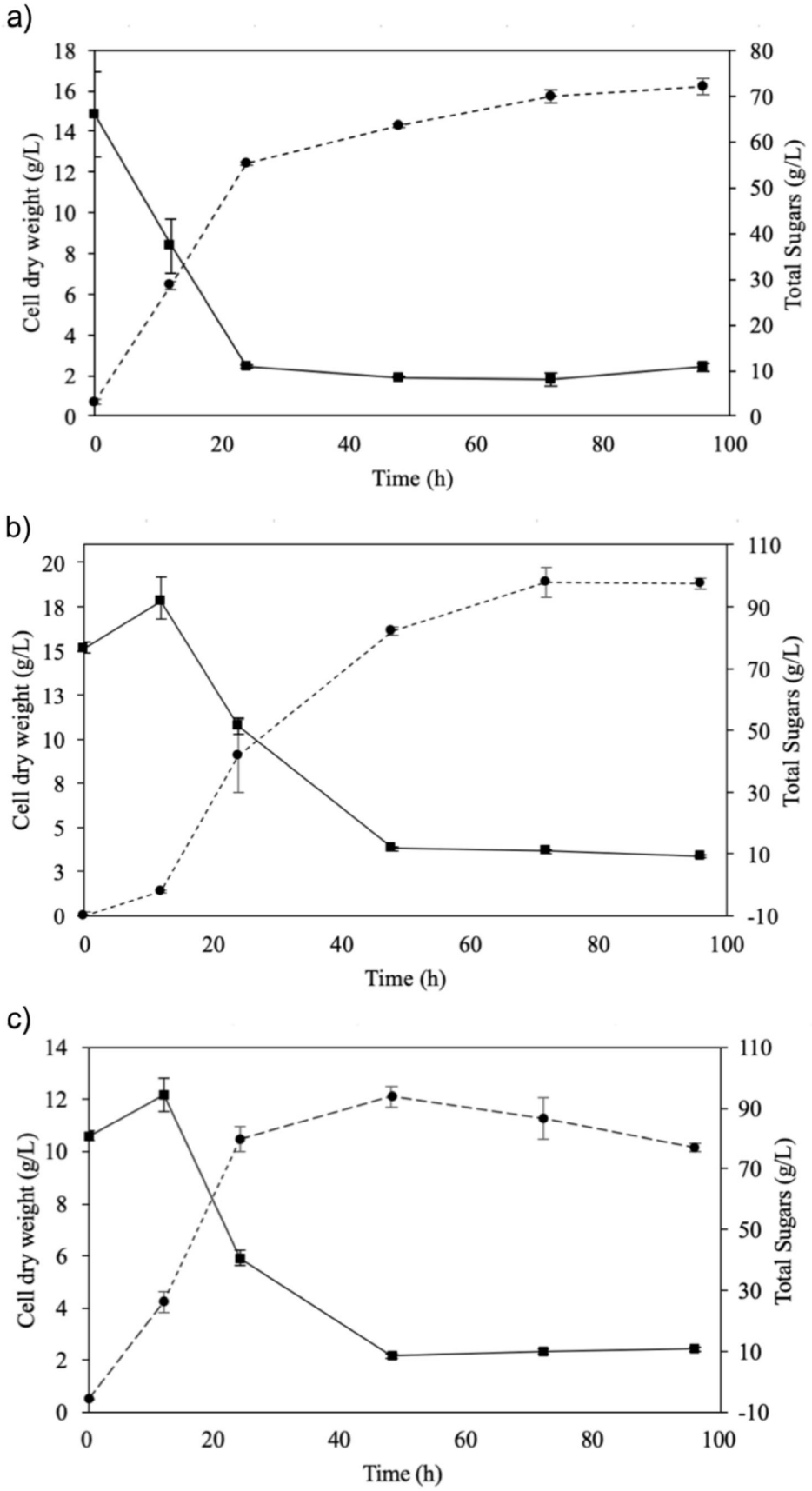
Fed-batches were also performed to study the effect of molasses' feeding after 10 and 12 h on biomass growth. The starting sugars concentration was 75 g/L

and a feeding of 30 g/L performed. When molasses feeding was performed at 10 h, a maximal biomass production of 18.91 g/L was reached, which corresponds to a productivity of 0.263 g/L.h at 72 h. At 48 h the biomass was 16.10 g/L and the productivity was 0.3354 g/L.h, showing that the process could end early.

Molasses's feeding after 12 h led to a production of 12.11g/L in 48 h, which corresponds to a productivity of 0.252 g/L.h. The 10 h feeding conditions for *W. anomalus* LPB-CPB5 biomass production in fed-batch operation was then chosen for further bioaccumulation experiments.

Xu; Xu (2014) compared the cultivation of *Bacillus coagulans* in sugarcane molasses medium, to obtained latic acid, in batch and fed-batch culture using a 5 L bioreactor. It was showed a low increase of the biomass content (1.56 %) from the batch cultivation of to a fed-batch. Besides, the productivity increased 31.25%, decreasing the time of the cultivation process from 99 h to 78 h. Similar results were showed by Lee; Kim (2001), in which *Candida utilis* was cultivated in molasses for ethanol production. It was showed that in fed-batch cultivation the productivity increased 4 times compared to batch cultivation. These results indicated that fed-batch cultivation improves the biomass production reducing the time of the process. The advantages of using fed-batch for yeast cultivation have been reported in several articles for the production of different bioproducts (CHANG et al., 2012; TAN et al., 2016; ONODERA et al., 2017; BLAGA et al., 2018).

FIGURE 12 - GROWTH KINETICS OF *W. anomalus* LPB-CPB5 YEAST IN BATCH (A), FED-BATCH WITH FEEDING AFTER 10 H (B) AND FED-BATCH WITH FEEDING AFTER 12 H (C)



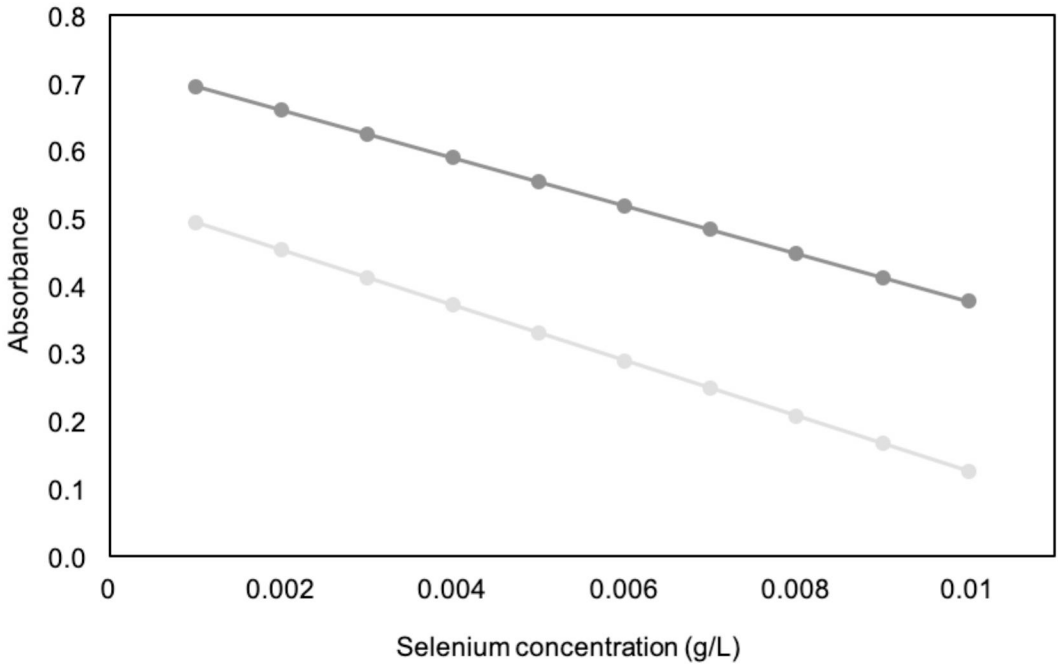
SOURCE: THE AUTHOR

4.3.1.3 Selenium bioaccumulation of *W. anomalus* LPB-CPB5

Several tests were carried out for the adequacy of the method proposed by developed by Mathew & Narayana (2006) to the samples of this work, which to some modifications of procedures. The samples where in cane molasses medium, for these reason cane molasses was used as solvent of the azure B curve reaction. There was a decrease of the added Azure B solution (0.1 %), from 0.5 mL to 0.15 mL.

The curve that was obtained using sugarcane molasses diluted 5X as solvent showed a R^2 of 0.976. The curve with water as solvent, in its turn, showed a R^2= 0.971. Both curves were compared using the predicted equation of each curve, with the same sample concentration value, to observe the predicted absorbance. Calibration curves suing sugarcane molasses and water as solvents are presented in Figure 13.

FIGURE 13 - COMPARISON OF CALIBRATION CURVES WITH WATER (LIGHT GRAY) AND SUGARCANE MOLASSES (DARK GRAY) AS SOLVENTS IN AZURE B ANALYSIS



SOURCE: THE AUTHOR

The obtained profile of both curves were comparable. Similar proportion of absorbance increase for sugarcane molasses curve (dark gray) was shown for all

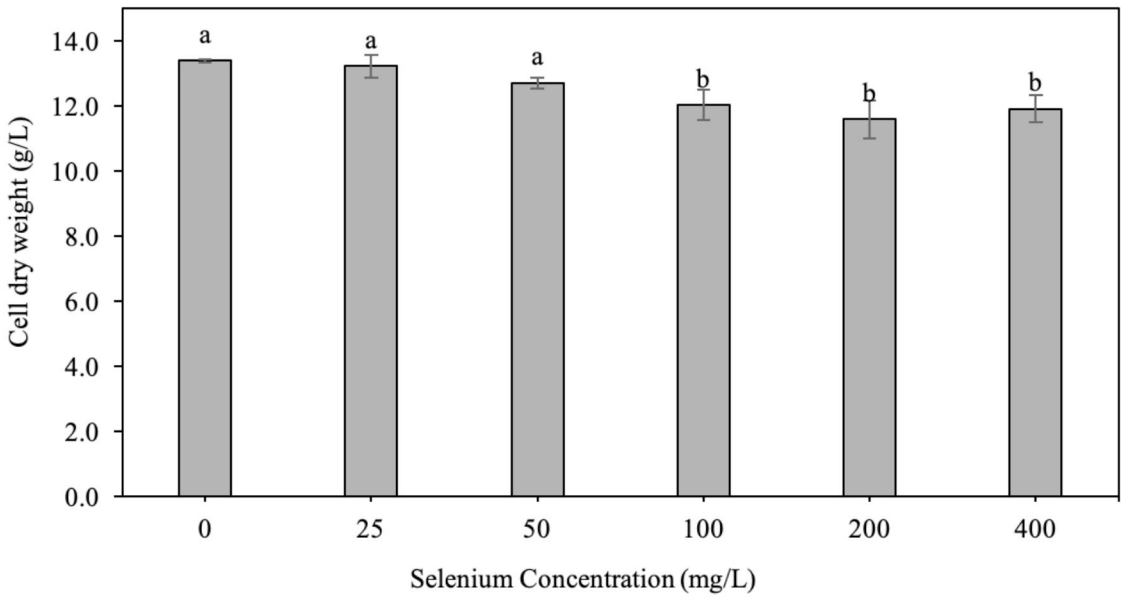
experimental points. Since both curves similar behavior, the sugarcane molasses curve was then employed for selenium analyses of fermentation supernatant.

4.3.1.4 Selenium tolerance and bioaccumulation of *W. anomalus* LPB-CPB5

A first evaluation of selenium tolerance and bioaccumulation of *W. anomalus* LPB-CPB5 was carried out with the analysis of the residual selenium of the supernatant obtained through fermentation. Essays were carried out with the addition of different concentrations of selenium to biomass production medium 25 to 400 mg/L. Biomass production (dry weight), which was obtained with different Se-concentrations in the medium is present in Figure 14.

A light effect of the presence of Selenium in the medium was observed. The control (0 mg/L) presented a biomass growth of 13.37 g/L, while the biomass production in the presence of selenium varied from 13.21 to 11.58 g/L showing a maximal decrease of 13.38 % from 200 mg/L (11.58 g/L). These results demonstrate that the growth of the yeast *W. anomalus* LPB-CPB5 is significantly affected by the presence of selenium in the medium after 100 mg/L as showed in Figure 14. This fact, shows that the studied yeast can be a promising selenium bioaccumulator.

FIGURE 14 - SELENIUM TOLERANCE AND BIOACCUMULATION OF *W. anomalus* LPB-CPB5 IN THE PRESENCE OFIN DIFFERENT CONCENTRATIONS OF SELENIUM IN THE GROWTH MEDIUM



DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P≤ .05) BY DUCAN`S TEST.
SOURCE: THE AUTHOR

According to Table 17, selenium bioaccumulation by the strain *W. anomalus* LPB-CPB5 occurs between 25 – 200 mg/L. When 400 mg/L were added to the medium, biomass was still produced (11.90 g/L), but it was stressful for Selenium bioaccumulation. The best bioaccumulation condition (9.51 mg/ g of yeast) was obtained with a selenium addition of 200 mg/L. However, in this condition the bioaccumulation yield was 55.1 % that means that, even though the selenium bioaccumulation per gram of yeast was higher in 200 mg/L the amount of selenium wasted in the supernatant was higher too. In the 200 mg/L, 89.8 mg/L of Se would be wasted.

TABLE 17 - SELENIUM TOLERANCE AND BIOACCUMULATION OF *W. anomalus* LPB-CPB5 IN THE PRESENCE OFF DIFFERENT CONCENTRATIONS OF SELENIUM IN THE GROWTH MEDIUM

Se-feed Conditions	Supernatant selenium (mg/ L) [a]	Bioaccumulated Selenium (mg/ L) [b]	Bioaccumulated Selenium (mg/ g of yeast) [c]	Yield %[d]
25 mg/L	0.0 ± 0.0	25.0 ± 0.0	1.89 ± 0.04	100.0
50 mg/L	0.0 ± 0.0	50.0 ± 0.0	3.94 ± 0.05	100.0
100 mg/L	46.5 ± 17.3	53.5 ± 17.3	4.43 ± 1.2	53.5
200 mg/L	89.8 ± 15.1	110.2 ± 15.1	9.51 ± 1.06	55.1
400 mg/L	400 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0

Calculus were performed from the results obtained by Azure B analysis
a: Supernatant selenium obtained by azure B analysis in mg/L
b: Estimated selenium bioaccumulated calculated by the difference between the added selenium and the supernatants selenium.
c: Estimated bioaccumulated selenium *per* gram of dried yeast.
e: Grams of bioaccumulated selenium *per* gram of added selenium in %.
SOURCE: THE AUTHOR

The highest selenium bioaccumulation yield was 100% for both 25 and 50 mg/L selenium concentrations in the medium, which corresponded, respectively, to 1.89 and 3.94 mg of Se / g of yeast. Low rates of selenium bioaccumulation (11.06 % and 58.56 %) were obtained, respectively, for 100 mg/L and 200 mg/L. However, the results obtained were comparable to those of literature (Table 18).

Yang et al. (2013) used the genus *Candida* in their study aiming selenium-enriched yeast production and study of its antioxidant effects in rats. Xiaoguang et al. (2011) used amino acids supplementation to increase selenium enrichment and yeast growth. Authors reported that 8.85 g/g of yeast bioaccumulated selenium,

which encourages the searching for new methods to improve selenium bioaccumulation.

TABLE 18 - DATA OF SE BIOACCUMULATED IN MG OF SELENIUM PER GRAM OF YEAST AND QUANTIFICATION METHODOLOGY

Reference	Microorganism	Bioaccumulated Se mg/g of yeast	Se methodology
Yin et al. (2010)	*Saccharomyces cerevisiae*	0.639	Atomic absorption spectroscopy
Stabnikova et al. (2008)	*Saccharomyces cerevisiae*	0.15	Mini-fluorometer with diaminonaphthalene reagent
Yang et al. (2013)	*Candida utilis*	0.9053	ICP-MS
Kieliszek et al. (2016)	*Saccharomyces cerevisiae* ATCC MYA-2200 *and Candida utilis* ATCC 9950	5.64 and 5.47	Colorimetric with VB reagent
Yin et al. (2009)	*Saccharomyces cerevisiae*	0.42	Atomic absorption spectroscopy
Pankiewicz; Jamroz (2008)	*Saccharomyces cerevisiae*	0.116	Atomic absorption spectroscopy
Xiaoguang et al. (2011)	*Candida utilis*	8.85	Atomic absorption spectroscopy
	Obtained Results		
25 mg/L fed	*W. anomalus* LPB-CPB5	1.89	Colorimetric with Azure B spectroscopy
50 mg/L fed	*W. anomalus* LPB-CPB5	3.94	Colorimetric with Azure B spectroscopy
100 mg/L fed	*W. anomalus* LPB-CPB5	4.43	Colorimetric with Azure B spectroscopy
200 mg/L fed	*W. anomalus* LPB-CPB5	9.51	Colorimetric with Azure B spectroscopy

SOURCE: THE AUTHOR

Yang et al. (2013) obtained 0.905 mg of selenium per gram of yeast and demonstrated optimal dietary supplementation enhancing with antioxidant capacity for rats. The trace element selenium is required by humans and animals in small amounts. It has been reported that the recommended daily level, for humans, is between 40-60 µg/day (PORTO et al., 2015; XIAO et al., 2017). Evaluating the presented points, the concentration of 50 mg/L was chosen to be added in the medium for Selenium-enriched yeasts production. This condition was defined then carried out due to the combination of a good biomass production, that did not present

significantly difference from the control (0 mg/L), and amount of the trace element that was bioaccumulated.

4.3.1.5 Selenium bioaccumulation of *W. anomalus* LPB-CPB5 by ICP-OES analysis

The ICP-OES analyses of Se-enriched microencapsulated yeasts were performed, in triplicate, testes. In this case, the medium was fed with 50 mg/L of selenium reaching a final concentration of 3.94 mg of Se bioaccumulated/ g of dried yeast cells. The ICP analysis showed a concentration of 2.25 ± 0.05 mg of Se / g of dried yeast, which is the concentration to be assumed. As showed before in Table 18 the amount of Se that was bioaccumulated was higher than most values showed in the literature.

Pieniz et al. (2017) determined the bioaccumulated selenium in *Enterococcus durans* using the ICP-OES analysis. It was showed that 1.43 mg of Se were bioaccumulated/g of microorganism. The results were obtained after feeding with 120 mg/L of Selenium, which shows the advantage of the bioaccumulation performed by *W. anomalus* LPB-CPB5, since 50 mg/L of Se were added to the medium with a corresponding bioaccumulation of 2.25 mg of Se/g of yeast.

Intracellular Selenium concentration in the yeast without selenium addition. The pure *W. anomalus* LPB-CPB5 presented 0.82 ± 0.05 mg of Se/ g of dried yeast. This amount of selenium could be explained by the composition of the cultivation medium that was employed, which was sugarcane molasses that could be the source of selenium.

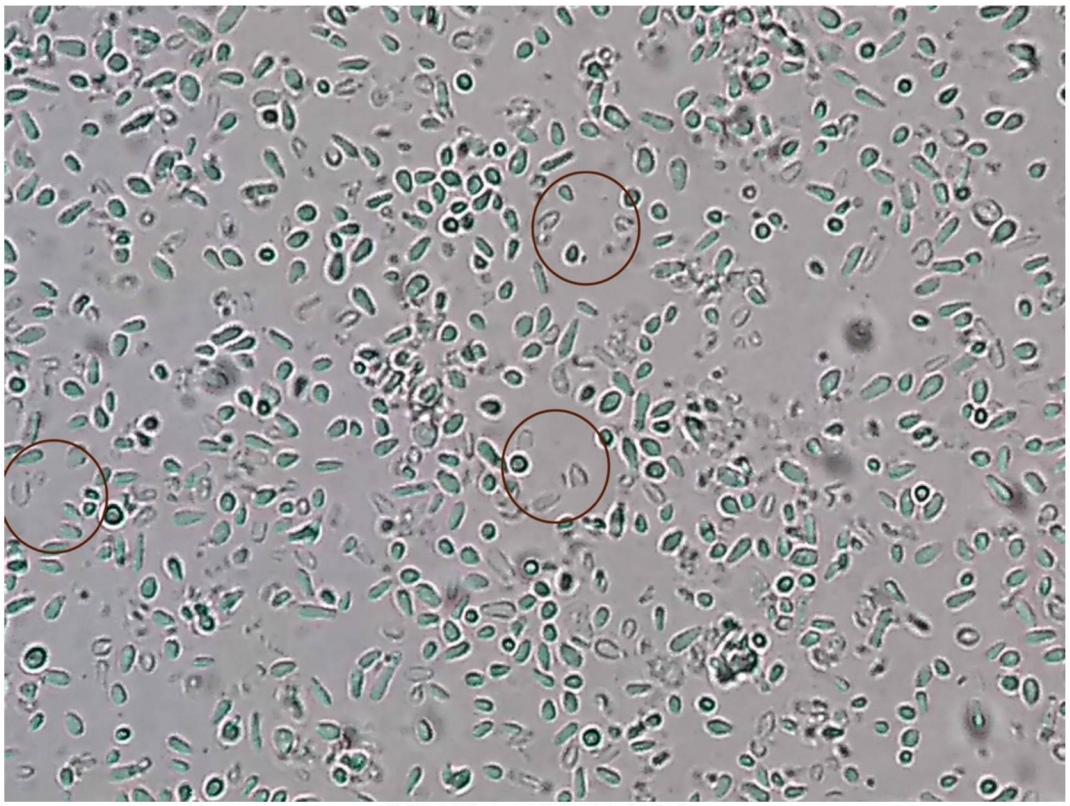
4.3.1.6 Cell disruption procedure for intracellular selenium determination

It is important to study the selenium-enriched yeasts membrane and to find the best disruption technique for recovering *W. anomalus* LPB-CPB5's intracellular selenium (Se-Cysteine and Se-Methionine). Among the different cells' disruption techniques that were tested, only the French press followed by a cycle of freezing was effective for disrupting cells. Some broken cells were observed in an optical microscope (Figure 15). The intact cells, before and after disruption procedures, were counted. It was observed $9.33x10^{+8}$ and $5.92x10^{+8}$ cells, respectively, before and

after the treatment, representing a loss of viability of 36.54 %. Even if, the number of disrupted cells was low, the intracellular material was liberated.

Kitamura (2013) analyzed the organic selenium present in the Se-enriched *Saccharomyces boulardii*. Some difficulties were also observed to disrupt the yeast membrane, even when the disruption tests were performed after the digestion with methanesulfonic acid.

FIGURE 15 - YEAST CELLS AFTER DISRUPTION IN FRENCH PRESS AND A CYCLE OF FREENZING AT -18ºC - 40X MICROSCOPY PLUS 1.2X OF CAMERA ZOOM



SOURCE: THE AUTHOR

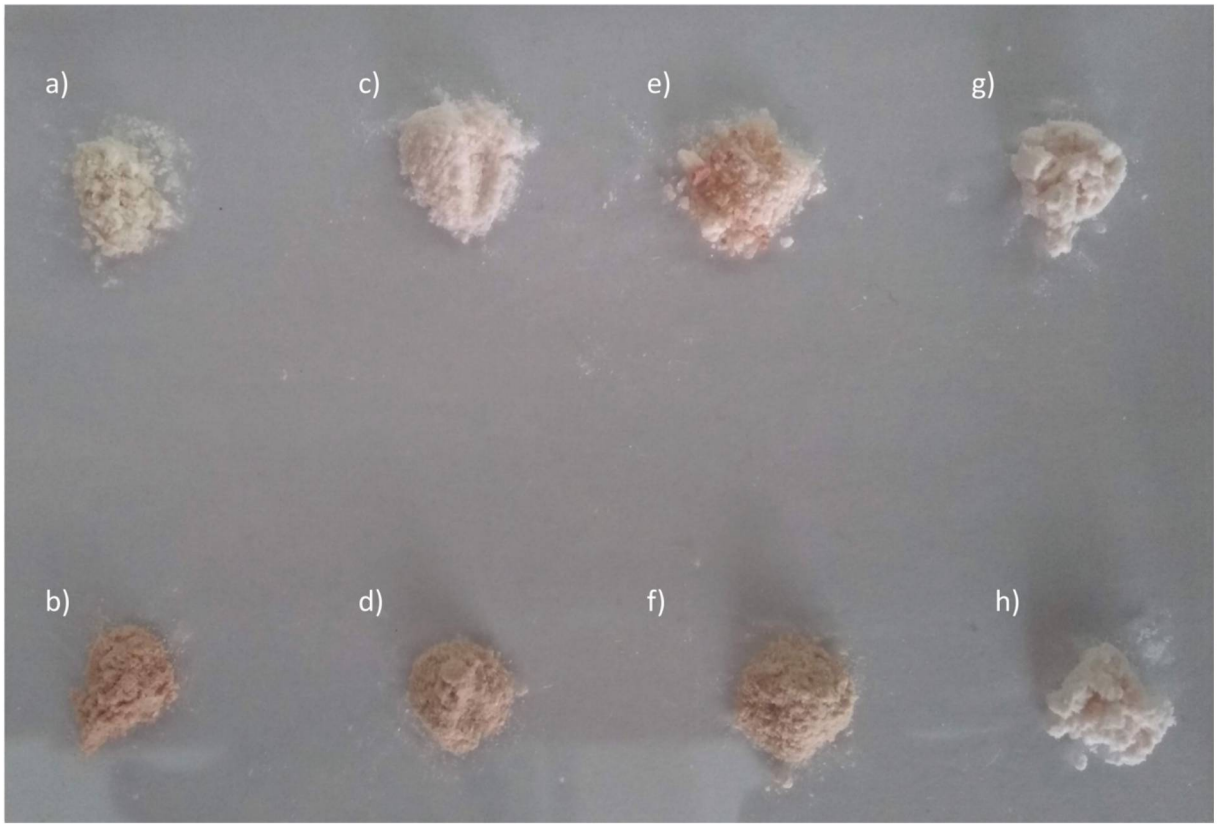
4.3.2 Study of *W. anomalus* LPB-CPB5 biomass drying by freeze and spray drying

After *W. anomalus* LPB-CPB5 biomass production, samples passed through freeze and spray-drying. Dried samples are shown in Figure 16. The lyophilized pure and Selenium-enriched yeast samples are presented in Figure 16a and Figure 16b. It is important to observe the change of color related to the selenium bioaccumulation, the yeast pass from a white powder to a pink-orange powder. This difference of color proves the presence of selenium qualifying the bioaccumulation process. The yeast

change of color has been reported in the literature exemplifying the selenium bioaccumulation process (ESMAEILI et al., 2012; KIELISZEK et al., 2015).

Samples T1 and T3 have a change of color, from white to a light rose tone, due to the presence of the Selenium-enriched yeast biomass. Samples T2 and T4 is in a light brown color because of the presence of cocoa husks' pectin, which was extracted from the cocoa husks powder (CHP), as described in section 3.4.2. The spray-dried samples T1 and T3 (Figure 16g and 16h, respectively) were characterized as a thin powder of slight different colors.

FIGURE 16 - FREEZE-DRIED SAMPLESOF *W. anomalus* LPB-CPB5 WITHOUT ADJUVANTS (a); - SELENIUM-ENRICHED WITH WITHOUT ADJUVANTS 50 mg/L (b); ADDED OF MODIFIED STARCH T1 (c); ADDED OF MODIFIED STARCH AND COCOA HUSKS' PECTINT2 (d); ADDED OF MODIFIED STARCH AND KEFIRANT3 (e); ADDED OF MODIFIED STARCH, COCOA HUSKS' PECTIN AND KEFIRANT4 (f). SPRAY-DRIED SAMPLES: T1 (g) T3 (h)



SOURCE: THE AUTHOR

4.3.2.1 Biomass encapsulation efficiency and stability

Biomass encapsulation efficiency and stability were calculated to show the efficiency of the drying process and effect of the different encapsulation matrices on the stability (Table 19). The composition and the encapsulation efficiency (EE) of

each drying condition, comparing to the viability of the free cells obtained after 30 days of encapsulation. The best EE was obtained for the freeze-drying samples, where sample T1 which showed the highest EE (88.75 %) followed by T3 (88.00%), T2 (81.48%), and T4 (79.19%).

TABLE 19 - ENCAPSULATION EFFICIENCY AND STABILITY OF SELENIUM-ENRICHED CELLS OF *W. anomalus* LPB-CPB5 AFTER 30 DAYS OF FREEZE DRYING

Sample	Matrix Composition	Post-encapsulation Stability (log CFU/g)	Encapsulation Efficiency (EE%)
Free enriched yeast	-	7.92 ± 0.06	-
T1	Starch	7.03 ± 0.03[a]	88.75
T2	Starch + Pectin	6.45 ± 0.00[b]	81.48
T3	Starch + Kefiran	6.97 ± 0.15[a]	88.00
T4	Starch + Pectin + Kefiran	6.27 ± 0.05[b]	79.19

DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P≤ .05) BY DUCAN`S TEST
SOURCE: THE AUTHOR

Encapsulating efficiency (EE) of biomass produced strains with different freeze-drying matrices are presented in Table 20. From the showed values it is possible to conclude that some encapsulation processes were efficient. The highest EE were reported that by Chen et al. (2017), and Nunes et al. (2018), respectively 96.35 %, and 94.36 %, respectively, which were obtained using Alginate for *Lactobacillus bulgaricus* and a Mixture of Arabic gum, Glycerol, Tween and Maltodextrin, adding inulin or H-imaze for *Lactobacillus acidophilus*.

TABLE 20 - ENCAPSULATION EFFICIENCY (EE) OF FREEZE-DRYING OF MICROORGANISM CELLS USING DIFFERENT POLYMER MATRICES

Microorganism	Freeze-dried Matrix	EE (%)	Reference
Lactobacillus acidophilus	Konjac glucomannan hydrogel	62.50%	Mu et al. (2018)
Honokiol *drug delivery*	Pectin nanoparticles	52.89%	Zhang et al. (2015)
Lactobacillus plantarum	Whey milk in $CaCl_2$ or $CaCO_3$ solution	58.4% e 75.6%	Tian et al. (2015)
Lactobacillus bulgaricus	Alginate	96.35% and 95.28%	Chen et al. (2017)
Lactobacillus acidophilus	Mixture of Arabic gum, Glycerol, Tween and Maltodextrin, adding inulin or H-imaze	93.12% and 94.26%	Nunes et al. (2018)

SOURCE: THE AUTHOR

The results do not discourage the use of pectin as adjuvant in freeze-drying of biomass, showing the importance of optimizing the matrix composition and freeze-drying conditions. Zhang et al. (2015) used pectin as encapsulation matrix for drug delivery, showing a EE of 52.89 %. The presented results showed that pectin has potential as drug delivery system, and other applications such as se-enriched yeasts cells encapsulation. Table 21 shows the viability of the encapsulated yeasts after 30 and 60 days of encapsulation increasing the value of the tested matrices.

TABLE 21 - FREEZE-DRIED *W. anomalus* LPB-CPB5 CELLS WITH AND WITHOUT ENCAPSULATION MATRICES VIABILITY AND STABILITY AFTER 30 AND 60 DAYS STORAGE

Sample	Viability (log CFU/g of sample)		Stability (%)
	30 days	60 days	
Control Se-enriched yeast	6.18 ± 0.16^{b}	4.77 ± 0.18^{a}	77.18
T1	7.03 ± 0.03^{a}	6.67 ± 0.02^{b}	94.88
T2	6.45 ± 0.00^{c}	6.17 ± 0.03^{c}	95.66
T3	6.97 ± 0.15^{a}*	6.97 ± 0.06^{d}*	100.00
T4	6.27 ± 0.05^{bc}	5.58 ± 0.15^{e}	88.85

DIFFERENT LETTERS, IN EACH COLUMN, ARE SIGNIFICANTLY DIFFERENT (P≤ .05) BY DUCAN`S TEST. * SUGGEST THAT T3 WAS THE ONLY MATRIX THAT DID NOT SHOWED SIGNIFICANTLY DIFFERENCE (P> .05) BETWEEN 30 AND 60 DAYS, BY DUCAN`S TEST.
SOURCE: THE AUTHOR

The survival rate or stability of *W. anomalus* LPB-CPB5 cells was determined by the calculated viability obtained after 30 and 60 days of sample's storage, in a

vacuum desiccator at room temperature. Samples, which were added polymeric matrices, presented higher stability due to the protective effect over cells' maintenance. The best results were obtained with the matrices T3, T2 and T1, with a stability of 100 %, 95.66 % and 94.88 %. Pectin from CHP and Kefiran showed their potential as encapsulation agents. N'Guessan et al. (2016), presented the use of sucrose and cassava flour, which is another source of starch, for the protection of *Saccharomyces cerevisiae and Candida tropicalis mix cells,* showing a survival rate of 53.5 % at 4 ºC after similar periods. Authors also demonstrated the importance of using cheap raw materials (Table 22).

TABLE 22 - STABILITY OF MICROORGANISM CELLS USING DIFFERENT POLYMER MATRICES AFTER FREEZE-DRYING

Microorganism	Freeze-dried Matrix	Survival Rate (%)	Reference
Lactobacillus plantarum	Whey milk in $CaCl_2$ or $CaCO_3$ solution	64.73% and 63.86%	Tian et al. (2015)
Lactobacillus acidophilus	Mix of de Arabic Gum, Glycerol, Tween and Maltodextrin, adding inulin or H-imaze	90.96% and 89.18%	Nunes et al. (2018)
Pantoea agglomerans	Trehalose at 5% and Sucrose 10%	83% e 75%	Costa et al. (2000)
Streptococcus thermophilus	Glycerol, Sodium glutamate and Skim Milk	93.58%	Lu et al. (2017)
Sourdough yeasts	Trehalose 15%	81%	Stefanello et al. (2018)
Saccharomyces cerevisiae and Candida tropicalis	Sucrose and Cassava flour	53.5%	N'Guessan et al. (2016)

SOURCE: THE AUTHOR

Spray-dried samples, using T1 and T3 matrices, did not present viability after 30 and 60 days, showing contamination. This fact means that this drying operation was not very well adapted, probably due to the spray-drying operational condition and/or equipment conception The negative results can also be explained by the high temperature that are used in the spray-drying process and the difficulty to maintain the sterility of the material. The employed polymeric matrices, modified starch and modified starch-kefiran, promoted the formation of spherical particles, as shown in SEM microscopy (Figure 18e, 18f, 19 e and 19 f). Another problem of spray-drying process was the low mass recovery. From 100 mL of sample the expected dried value would be 18.85 g (T1) and 18.95 g (T2). However, the obtained weight was of 0.85 g and 0.76 g, only 4.50 % and 4.01 %, respectively, of the expected value,

meaning that the recovery of dried cells was so low that it could not be detected in the viability analysis.

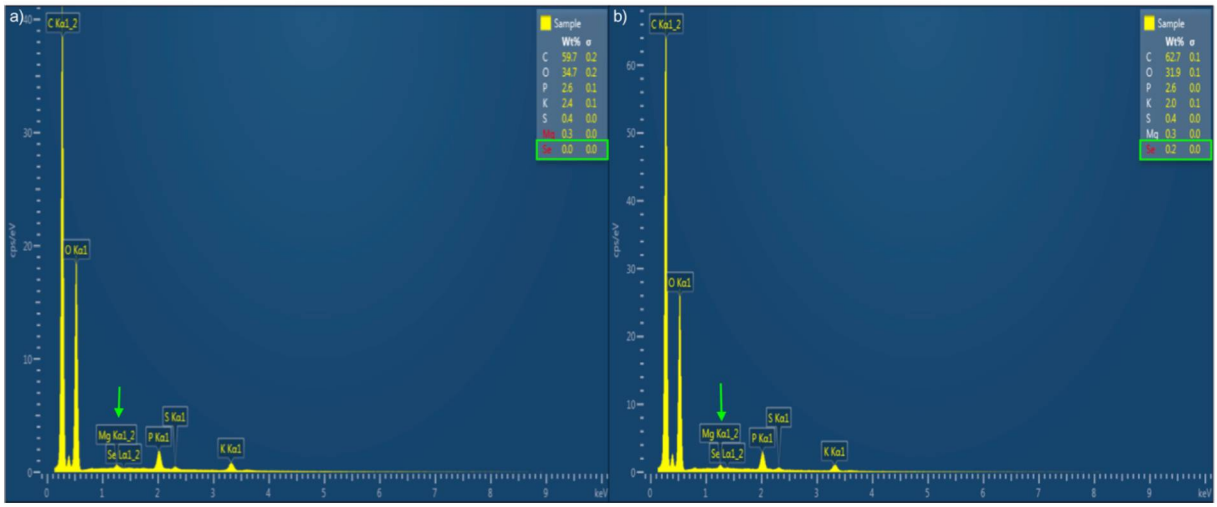
The obtained results demonstrate that all used materials have positive effect on the protection of *W. anomalus* LPB-CPB5 Se-enriched cells. Future studies with the variation of polymers concentration could be a good strategy to determine the best matrix composition. Additionally, Kefiran and pectin from CH also present health benefits, which could increase the yeast benefits and aggregate value to the final product.

The use of a commercial pectin, from citrus peel, in the immobilization of yeast cells has been reported by Navarro et al. (1983), for ethanol production, showing the possibility of the reuse of encapsulated cells. No other reports were found for the use of pectin or Kefiran, extracted from CH, as Se-enriched cells' encapsulate agents, showing the innovation of this work.

4.3.2.2 Scanning electron microscopy of *W. anomalus* LPB-CPB5 dried cells

The scanning electron microscopy (SEM) analysis of *W. anomalus* LPB-CPB5 dried cells (with Se and without Se) was performed in two conditions: the EDS analysis and the contrast using a bath of gold. The EDS analysis allowed to observe the minerals present in the sample. Figure 17 shows the EDS analyses for cells without selenium bioaccumulation (a) and Selenium-enriched cells (b). This technique can be used as a quantitative analysis if the quantity of the trace element in the sample is into the detection limit, which should be higher than 30 mg. In this case, there was not enough material to be quantified, but it was enough to be identified. These results attested the presence of selenium in the sample.

FIGURE 17 - EDS ANALYSIS OF *W. anomalus* LPB-CPB5 DRIED CELLS WITHOUT SE (a) AND SE-ENRICHED CELLS (b)

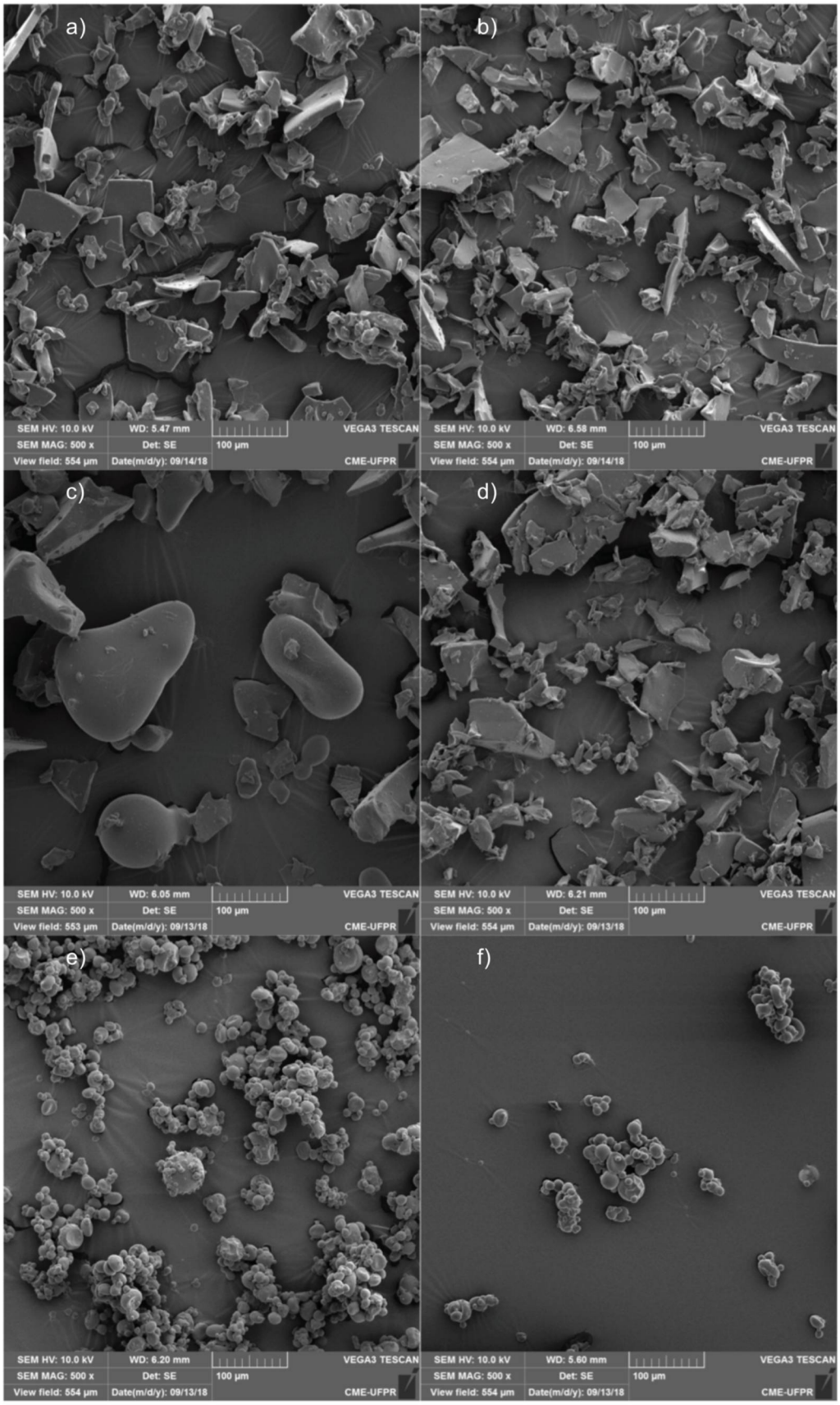


SOURCE: THE AUTHOR

The SEM images are presented in Figures 18-20 showing some general characteristics of the lyophilized samples. The general profile of the dried samples at 500X approximation is shown in Figure 18. Spherical shape of the microencapsulated samples (Figure 18e, 18f, 19e and 19 f) were also reported by Nunes et al. (2018) as being a striking feature of the spray-dried encapsulation process. Although, the encapsulation process was not efficient. Sample homogeneity can be viewed in Figure 19, showing that even different particles sizes have the same pattern. In Figure 20 it is possible to observe that *W. anomalus* LPB-CPB5 lyophilized particle size has a diameter of 3-5 µm.

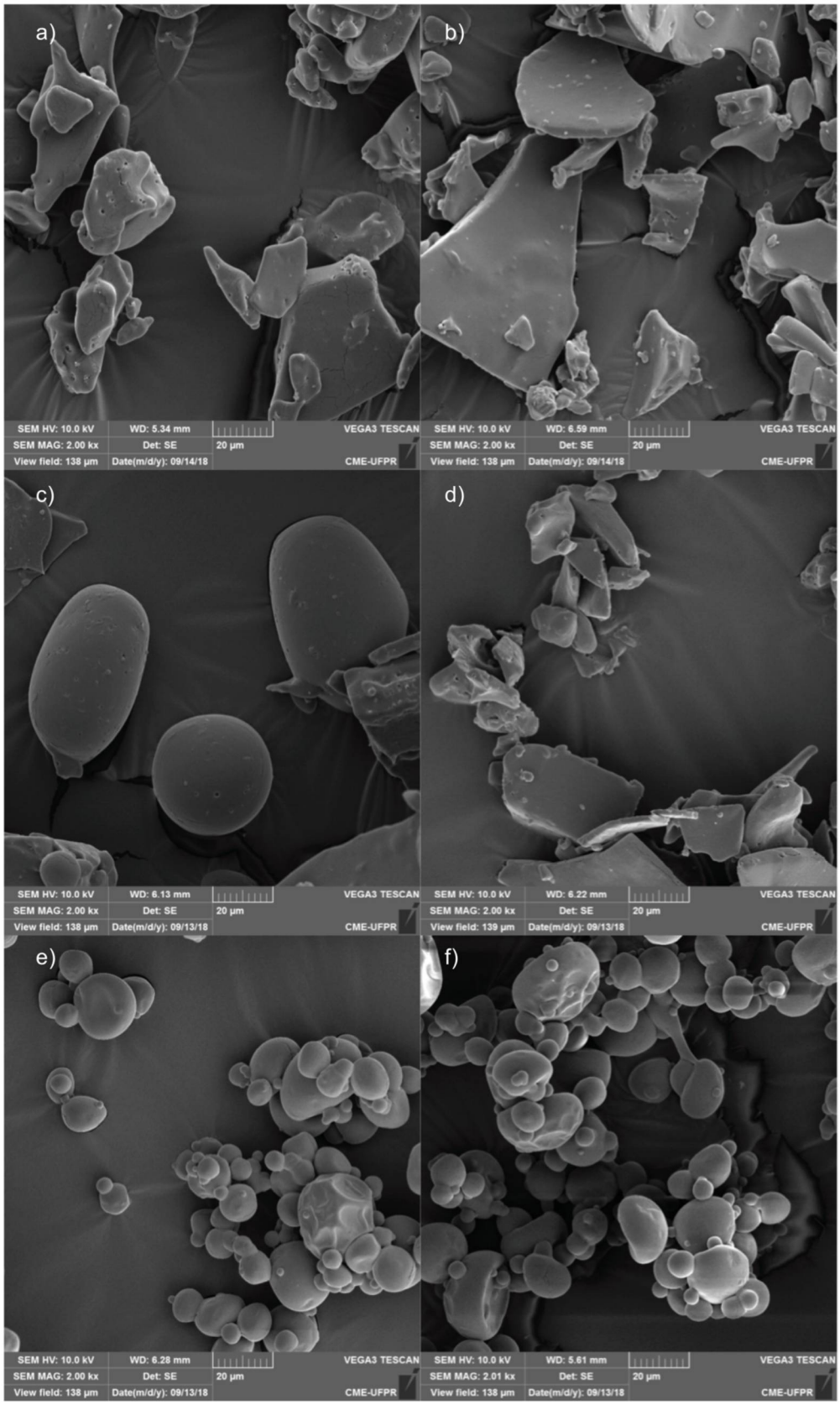
Polymeric matrices certainly promote a more protective barrier against environmental agents, temperature and other degradation factors, comparing to the exposure of yeasts cells without matrix. These results validate corn starch, cocoa husk pectin and Kefiran as suitable cryoprotective matrix components. From ICP-OES analysis the final product showed a concentration of 19.10 µg of Se/ g of microencapsulated yeast. Since the human and animals require small amounts of Se per day, it is possible to estimate that 1 g of the final product could be administrated in a human dietary supplementation. This value was estimated based on the recommended Se level for humans, which is between 40-60 µg/day (PORTO et al., 2015; XIAO et al., 2017)..

FIGURE 18 - MICROSCOPY AT 500X SHOWING THE GENERAL PATTERN OF *W. anomalus* LPB-CPB5 CELLS AFTER DRYING. FREEZE-DRIED CELLS : MATRIX T1 (a); MATRIX T2 (b); MATRIX T3 (c); MATRIX T4 (d). SPRAY-DRIED CELLS: MATRIX T1 (e) MATRIX T3 (f)



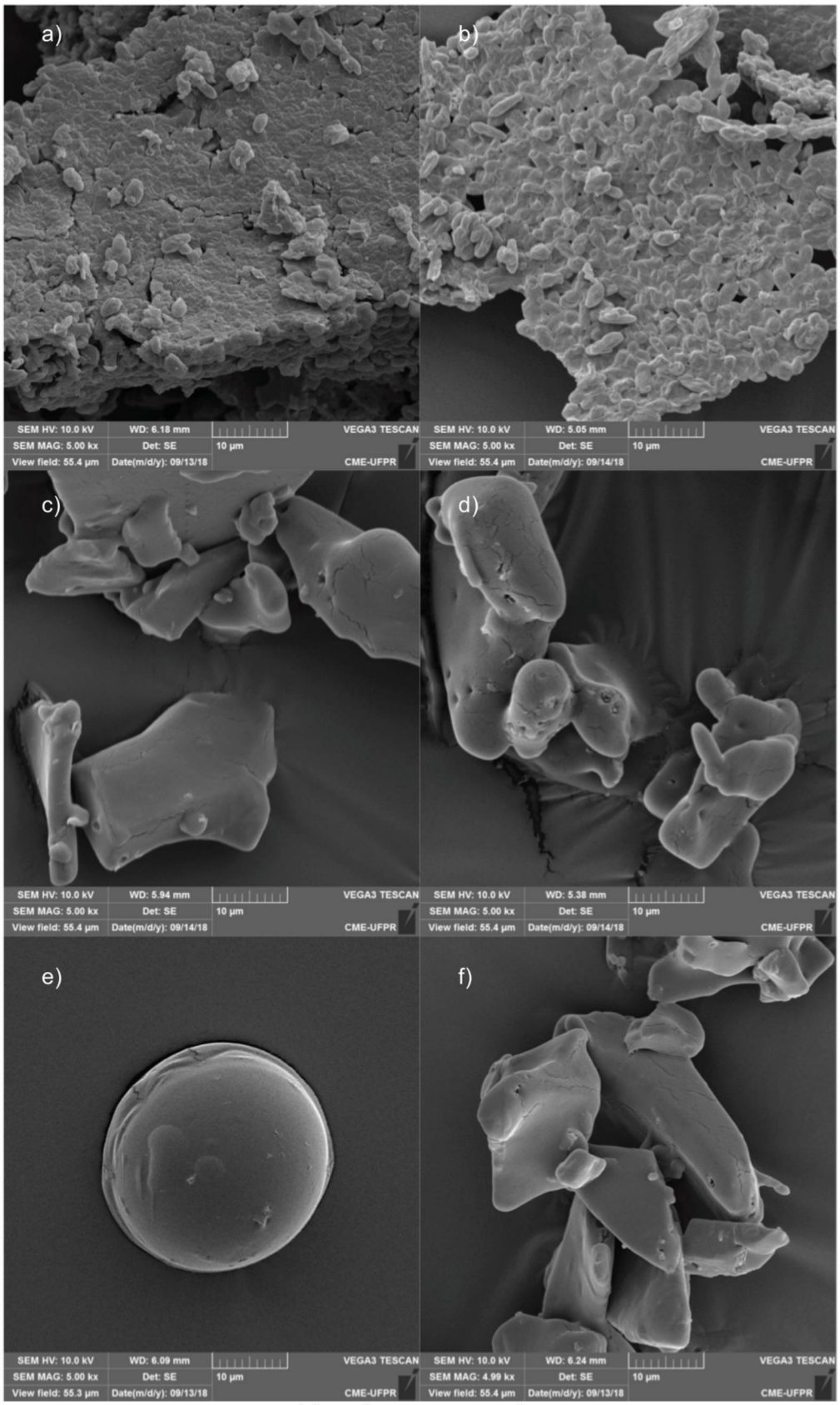
SOURCE: THE AUTHOR

FIGURE 19 - SEM MICROSCOPY AT 2000X SHOWING THE GENERAL PATTERN AND HOMOGENEITY OF THE *W. anomalus* LPB-CPB5 DRIED CELLS. FREEZE-DRIED SAMPLES : MATRIX T1 (a); MATRIX T2 (b); MATRIX T3 (c); MATRIX T4 (d). SPRAY-DRIED SAMPLES: MATRIX T1 (e) MATRIX T3 (f)



SOURCE: THE AUTHOR

FIGURE 20 - SEM MICROSCOPY AT 5000X SHOWING THE PATTERN OF THE *W. anomalus* LPB-CPB5LYOPHILIZED CELLS WITHOUT MATRIX, WITH (a) AND WITHOUT SELENIUM (b); AND THE PROTECTION AND HOMOGENEITY OF THE FREEZE-DRIED MATRICES; MATRIX T1 (c); MATRIX T2 (d); MATRIX T3 (e); MATRIX T4 (f)



SOURCE: THE AUTHOR

4.4 CONCLUSIONS

In this work the growth medium composition for *Wickerhamomyces anomalus LPB-CPB5* cultivation was defined as: yeast extract (12 g/L), $(NH_4)_2HPO_4$ (1.5 g/L), KH_2PO_4 (1.5 g/L), sugarcane molasses (75 g of total sugar/L) and urea (3 g/L). A fed-batch process, with feeding at 10h, was chosen as the best growth condition. The capability of *W. anomalus* LPB-CPB5 to bioaccumulate selenium was demonstrated. The azure B analysis showed that selenium enrichment reached a maximum of 9.51 mg of Se/g of yeast, when 200 mg/L of Se were added to the medium. However, the addition of 50 mg/L of selenium, presented the highest biomass growth without significant difference from the control showing a yield of 100.00% and a bioaccumulation of 3.94 mg of Se/g of yeast. ICP analysis showed a concentration of2.25 mg of Se/g of yeast. The most effective microencapsulation matrices was the modified starch (88.75 %), with the highest survival rate or stability promoted by starch + Kefiran (100.00%), and starch + coca pectin (95.66%). These results showed that *W. anomalus* LPB-CPB5 can bioaccumulate Se, and that Kefiran and cocoa husks pectin has potential as adjuvant agents and/or cryoprotective matrices.

GENERAL CONCLUSIONS

This work presented a review about selenium bioaccumulation by yeasts and their potential in dietary supplementation. *Wickerhamomyces anomalus LPB-CPB5* was studied as a potential selenium bioaccumulator. Culture conditions were studies where the optimal composition was determined as yeast extract (12 g/L), $(NH_4)_2HPO_4$ (1.5 g/L), KH_2PO_4 (1.5 g/L), sugarcane molasses (75 g of total sugar/L) and urea (3 g/L). Yeast cells were grown in a fed-batch process, with sugarcane molasses feeding after 10h of cultivation. W. *anomalus LPB-CPB5* showed its very good capacity to bioaccumulate selenium (2.25 mg of Se/g of yeast) after feeding with 50 mg/L of selenium, which did not cause significant stress. Cocoa husks pectin was extracted at optimized conditions to be employed in yeast encapsulation together with other polysaccharides.,. The most effective microencapsulation matrices were defined as modified starch (88.75 %) while the matrices with starch + Kefiran (100.00%), and starch + coca pectin (95.66%), promoted the highest survival rate or cell stability. Thus, *W. anomalus* LPB-CPB5 can be used as Se bioaccumulation agent, and cocoa husks pectin and Kefiran have potential to be employed as adjuvant agents and/or cryoprotective matrices.

FUTURE PERSPECTIVES

The bioaccumulation process can be improved by testing the best selenium feeding time and scaling-up the process. These tests could be performed and evaluated using atomic absorption to best calculate the amount of bioaccumulated Selenium. The analysis of organic selenium by LC-MC is very important to identify and quantify the organic forms of selenium. For this reason, it is important to find a technique of membrane disruption and digestion system to effectively liberate the organic selenium from the yeast. A study of the *W. anomalus* LPB-CPB5 is important to understand its membrane to find a suitable disruption system. Knowing this yeast, the best fixation process can be determined allowing its study through the transmission electronic microscopy (TEM) and the observation of possible structural changes related to the mineral bioaccumulation process. The microencapsulation study can be improved by varying the concentration of the polymers, doing long term tests to see the cellular viability and the concentration of selenium present in the sample. With the best conditions set the developed product could be tested in feed supplementation and human health.

REFERENCE

ABADIAS, M.; BENABARRE, A.; TEIXIDÓ, N.; USALL, J.; VIAS, I. Effect of freeze drying and protectants on viability of the biocontrol yeast Candida sake. **International Journal of Food Microbiology**, v. 65, p. 173–182, 2001.

ADETUNJI, L. R.; ADEKUNLE, A.; ORSAT, V.; RAGHAVAN, V. Advances in the pectin production process using novel extraction techniques: A review. **Food Hydrocolloids**, v. 62, p. 239–250, 2017.

ALLMANG, C.; WURTH, L.; KROL, A. The selenium to selenoprotein pathway in eukaryotes: More molecular partners than anticipated. **Biochimica et Biophysica Acta - General Subjects**, v. 1790, n. 11, p. 1415–1423, 2009.

AMORIM, J. C.; VRIESMANN, L. C.; PETKOWICZ, C. L. O.; MARTINEZ, G. R.; NOLETO, G. R. Modified pectin from Theobroma cacao induces potent pro-inflammatory activity in murine peritoneal macrophage. **International Journal of Biological Macromolecules**, v. 92, p. 1040–1048, 2016.

ARAKAKI, A. **Desenvolvimento de bioprocesso para produção de biomassa rica em cobre e zinco por fermentação submersa utilizando leveduras isoladas do Baru (Dipteryx alata Vog.) E testados em resíduos da agroindústria**, 2010. Federal University of Paraná.

ARAKAKI, A. H.; VANDENBERGHE, L. P. DE S.; SOCCOL, V. T.; et al. Optimization of biomass production with copper bioaccumulation by yeasts in submerged fermentation. **Brazilian Archives of Biology and Technology**, v. 54, n. 5, p. 1027–1034, 2011.

ARSLAN-TONTUL, S.; ERBAS, M. Single and double layered microencapsulation of probiotics by spray drying and spray chilling. **LWT - Food Science and Technology**, v. 81, p. 160–169, 2017.

BABAEI-GHAZVINI, A.; SHAHABI-GHAHFARROKHI, I.; GOUDARZI, V. Preparation of UV-protective starch / ke fi ran / ZnO nanocomposite as a packaging fi lm : Characterization. , v. 16, n. December 2017, p. 103–111, 2018.

BARAZARTE, H.; SANGRONIS, E.; UNAI, E. La cáscara de cacao (Theobroma cacao L.): Una posible fuente comercial de pectinas. **Archivos Latinoamericanos de Nutricion**, v. 58, n. 1, p. 64–70, 2008.

BAUM, A.; DOMINIAK, M.; VIDAL-MELGOSA, S.; et al. Prediction of Pectin Yield and Quality by FTIR and Carbohydrate Microarray Analysis. **Food and Bioprocess Technology**, v. 10, n. 1, p. 143–154, 2017. Food and Bioprocess Technology. Disponível em: <http://dx.doi.org/10.1007/s11947-016-1802-2>. Acesso em: 28 novembro 2018.

BEG, M. S.; AHMAD, S.; JAN, K.; BASHIR, K. Status, supply chain and processing of cocoa - A review. **Trends in Food Science and Technology**, v. 66, p. 108–116, 2017.

BEMILLER, J. N. Starches: Molecular and Granular structures and properties. **Carbohydrate Chemistry for Food Scientists**. p.159–189, 2019. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/B9780128120699000066>. Acesso em: 12 novembro 2018 .

BERBEGAL, C.; POLO, L.; GARCÍA-ESPARZA, M. J.; et al. Immobilisation of yeasts on oak chips or cellulose powder for use in bottle-fermented sparkling wine. **Food Microbiology**, v. 78, n. December 2017, p. 25–37, 2019.

BISWAS, A.; DIVYA, S.; MANDAL, A. B.; MAJUMDAR, S.; SINGH, R. Effects of dietary supplementation of organic chromium (picolinate) on physical and biochemical characteristics of semen and carcass traits of male turkeys. **Animal Reproduction Science**, v. 151, n. 3–4, p. 237–243, 2014.

BLAGA, A. C.; CIOBANU, C.; CAŞCAVAL, D.; GALACTION, A. I. Enhancement of ergosterol production by Saccharomyces cerevisiae in batch and fed-batch fermentation processes using n-dodecane as oxygen-vector. **Biochemical Engineering Journal**, v. 131, p. 70–76, 2018.

BLANDÓN, L. M.; ISLAN, G. A.; CASTRO, G. R.; et al. Kefiran-alginate gel microspheres for oral delivery of ciprofloxacin. **Colloids and Surfaces B: Biointerfaces**, v. 145, p. 706–715, 2016.

BOUNGO TEBOUKEU, G.; TONFACK DJIKENG, F.; KLANG, M. J.; et al. Polyphenol antioxidants from cocoa pods: Extraction optimization, effect of the optimized extract, and storage time on the stability of palm olein during thermoxidation. **Journal of Food Processing and Preservation**, v. 42, n. 5, p. 1–11, 2018.

CAMPOS-VEGA, R.; NIETO-FIGUEROA, K. H.; OOMAH, B. D. Cocoa (Theobroma cacao L.) pod husk: Renewable source of bioactive compounds. **Trends in Food Science & Technology**, v. 81, p. 172–184, 2018.

CAÑAMÁS, T. P.; VIÑAS, I.; USALL, J.; et al. Impact of mild heat treatments on induction of thermotolerance in the biocontrol yeast Candida sake CPA-1 and viability after spray-drying. **Journal of Applied Microbiology**, v. 104, n. 2003, p. 767–775, 2008.

CHAMPAGNE, C. P.; FUSTIER, P. Microencapsulation for the improved delivery of bioactive compounds into foods. **Current Opinion in Biotechnology**, v. 18, n. 2, p. 184–190, 2007.

CHAN, S. Y.; CHOO, W. S. Effect of extraction conditions on the yield and chemical properties of pectin from cocoa husks. **Food Chemistry**, v. 141, n. 4, p. 3752–3758, 2013.

CHANG, Y. H.; CHANG, K. S.; HUANG, C. W.; HSU, C. L.; JANG, H. DER. Comparison of batch and fed-batch fermentations using corncob hydrolysate for bioethanol production. **Fuel**, v. 97, p. 166–173, 2012.

CHEN, H. Y.; LI, X. Y.; LIU, B. J.; MENG, X. H. Microencapsulation of Lactobacillus bulgaricus and survival assays under simulated gastrointestinal conditions. **Journal of Functional Foods**, v. 29, p. 248–255, 2017.

CHEN, X. C.; WANG, Y. P.; LIN, Q.; et al. Biosorption of copper(II) and zinc(II) from aqueous solution by Pseudomonas putida CZ1. **Colloids and Surfaces B: Biointerfaces**, 2005.

CHOJNACKA, K. **Biosorption and Bioaccumulation in Practive**. New York: Nova Science Publishers, Inc, 2009.

COPIKOVA, J.; CERNA, M. Application of FT-IR spectroscopy in detection of food hydrocolloids confectionery jellies and in food supplements. **Journal of Food**, v. 19, n. 2, p. 51–56, 2001.

COSTA, E.; USALL, J.; TEIXIDO, N.; GARCIA, N.; VIN, I. Effect of protective agents , rehydration media and initial cell concentration on viability of Pantoea agglomerans strain CPA-2 subjected to freeze-drying., n. Rhodes 1993, p. 793–800, 2000.

CRUZ-GAVIA, A.; PEREZ-ALONSO, C.; BARRERA-DÍAZ, C. E.; et al. Survival of Saccharomyces cerevisiae microencapsulated with complex coacervate after freezing process. **Food Hydrocolloids**, v. 82, p. 45–52, 2018.

DÖNMEZ, G.; AKSU, Z. The effect of copper(II) ions on the growth and bioaccumulation properties of some yeasts. **Process Biochemistry**, v. 35, n. 1–2, p. 135–142, 1999.

DRUSCH, S. Sugar beet pectin: A novel emulsifying wall component for microencapsulation of lipophilic food ingredients by spray-drying. **Food Hydrocolloids**, v. 21, n. 7, p. 1223–1228, 2007.

EINHORN-STOLL, U. Pectin-water interactions in foods - From powder to gel. **Food Hydrocolloids**, v. 30, p. 1–11, 2017.

EL-BAYOUMY, K.; DAS, A.; RUSSELL, S.; et al. The effect of selenium enrichment on baker's yeast proteome. **Journal of Proteomics**, v. 75, n. 3, p. 1018–1030, 2012.

ESMAEILI, S.; KHOSRAVI-DARANI, K.; POURAHMAD, R.; KOMEILI, R. An experimental design for production of selenium-enriched yeast. **World Applied Sciences Journal**, v. 19, n. 1, p. 31–37, 2012.

FELLAH, A.; ANJUKANDI, P.; WATERLAND, M. R.; WILLIAMS, M. A. K. Determining the degree of methylesterification of pectin by ATR/FT-IR: Methodology optimisation and comparison with theoretical calculations. **Carbohydrate Polymers**, v. 78, n. 4, p. 847–853, 2009.

FISHMAN, M. L.; COOKE, P. H. The structure of high-methoxyl sugar acid gels of citrus pectin as determined by AFM. **Carbohydrate Research**, 2009.

FRACASSO, A. F.; PERUSSELLO, C. A.; CARPINÉ, D.; PETKOWICZ, C. L. DE O.; HAMINIUK, C. W. I. Chemical modification of citrus pectin: Structural, physical and rheologial implications. **International Journal of Biological Macromolecules**, v. 109, p. 784–792, 2018.

FRAEYE, I.; COLLE, I.; VANDEVENNE, E.; et al. Influence of pectin structure on texture of pectin-calcium gels. **Innovative Food Science and Emerging Technologies**, v. 11, n. 2, p. 401–409, 2010.

FRAEYE, I.; DUVETTER, T.; DOUNGLA, E.; VAN LOEY, A.; HENDRICKX, M. Fine-tuning the properties of pectin-calcium gels by control of pectin fine structure, gel composition and environmental conditions. **Trends in Food Science and Technology**, 2010.

GAENSLY, F.; WILLE, G. M. F. DE C.; BRAND, D.; BONFIM, T. M. B. Iron enriched Saccharomyces cerevisiae maintains its fermenting power and bakery properties. **Ciência e Tecnologia de Alimentos**, v. 31, n. 4, p. 980–983, 2011.

GARCÍA, L. M. B. **Development of a System For Carrying or Release Bioactive Molecules Based on a Composite Matrix of Alginate and Kefiran**, 2016. Universidade Federal do Paraná.

GHASEMLOU, M.; KHODAIYAN, F.; OROMIEHIE, A. Rheological and structural characterisation of film-forming solutions and biodegradable edible film made from kefiran as affected by various plasticizer types. **International Journal of Biological Macromolecules**, v. 49, n. 4, p. 814–821, 2011.

GIACOMAZZA, D.; BULONE, D.; SAN BIAGIO, P. L.; LAPASIN, R. The complex mechanism of HM pectin self-assembly: A rheological investigation. **Carbohydrate Polymers**, v. 146, p. 181–186, 2016.

GILBERT-LÓPEZ, B.; DERNOVICS, M.; MORENO-GONZÁLEZ, D.; MOLINA-DÍAZ, A.; GARCÍA-REYES, J. F. Detection of over 100 selenium metabolites in selenized yeast by liquid chromatography electrospray time-of-flight mass spectrometry. **Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences**, v. 1060, n. June, p. 84–90, 2017.

GÖNEN, F.; AKSU, Z. Use of response surface methodology (RSM) in the evaluation of growth and copper(II) bioaccumulation properties of Candida utilis in molasses medium. **Journal of Hazardous Materials**, v. 154, n. 1–3, p. 731–738, 2008.

GOPINATH, V.; SARAVANAN, S.; AL-MALEKI, A. R.; RAMESH, M.; VADIVELU, J. A review of natural polysaccharides for drug delivery applications: Special focus on cellulose, starch and glycogen. **Biomedicine and Pharmacotherapy**, v. 107, n. April, p. 96–108, 2018.

GU, F.; TAN, L.; WU, H.; et al. Comparison of Cocoa Beans from China, Indonesia and Papua New Guinea. **Foods**, v. 2, n. 2, p. 183–197, 2013.

GUZEL-SEYDIM, Z.; WYFFELS, J. T.; SEYDIM, A. C.; GREENE, A. K. Turkish kefir and kefir grains: Microbial enumeration and electron microscobic observation. **International Journal of Dairy Technology**, v. 58, n. 1, p. 25–29, 2005.

HALL, J. O. Selenium. **Veterinary Toxicology: Basic and Clinical Principles**. Third Edit ed., v. 2, p.469–477, 2018.

HONDAL, R. J.; RAINES, R. T. Semisynthesis of proteins containing selenocysteine. **Methods in Enzymology**, v. 347, n. 1993, p. 70–83, 2002.

HUTOMO, G. S.; RAHIM, A.; KADIR, S. Pectin Isolation from Dry Pod Husk Cocoa with Hydrochloride Acid. **Int.J.Curr.Microbiol.App.Sci**, v. 5, n. 11, p. 751–756, 2016.

ICCO. The International Cocoa Organization. Disponível em: <https://www.icco.org>.

IRIGOYEN, A.; ARANA, I.; CASTIELLA, M.; TORRE, P.; IBÁÑEZ, F. C. Microbiological, physicochemical, and sensory characteristics of kefir during storage. **Food Chemistry**, 2005.

JACH, M. E.; SEREFKO, A. **Chapter 9 - Nutritional Yeast Biomass: Characterization and Application**. Elsevier Inc., 2018.

KAMRAN; AZAD, S.; SHARIATMADARI, F.; KARIMI TORSHIZI, M. A. Production of zinc-enriched biomass of saccharomyces cerevisiae. **Journal of Elementology**, v. 19, n. 2, p. 313–326, 2014.

KHAMKHASH, A.; SRIVASTAVA, V.; GHOSH, T.; et al. Mining-Related Selenium Contamination in Alaska, and the State of Current Knowledge. **Minerals**, v. 7, n. 3, p. 46, 2017. Disponível em: <http://www.mdpi.com/2075-163X/7/3/46>. Acesso em: 10 novembro 2018.

KIELISZEK, M.; BŁAZEJAK, S. Selenium: Significance, and outlook for supplementation. **Nutrition**, v. 29, n. 5, p. 713–718, 2013.

KIELISZEK, M.; BŁAŻEJAK, S.; GIENTKA, I.; BZDUCHA-WRÓBEL, A. Accumulation and metabolism of selenium by yeast cells. **Applied Microbiology and Biotechnology**, v. 99, p. 5373–5382, 2015.

KIELISZEK, M.; BŁAZEJAK, S.; PŁACZEK, M. Spectrophotometric evaluation of selenium binding by Saccharomyces cerevisiae ATCC MYA-2200 and Candida utilis ATCC 9950 yeast. **Journal of Trace Elements in Medicine and Biology**, v. 35, p. 90–96, 2016.

KIELISZEK, M.; KOT, A. M.; BZDUCHA-WRÓBEL, A.; et al. Biotechnological use of Candida yeasts in the food industry: A review. **Fungal Biology Reviews**, v. 31, p. 185–198, 2017.

KITAMURA, D. H. **Produção de Biomassa Probiótica e Enriquecida com Selênio de Saccharomyces Boulardii Utilizando Melaço De Cana-De-Açúcar**, 2013. Universidade Federal do Paraná.

KOOIMAN, P. The chemical structure of kefiran, the water-soluble polysaccharide of the kefir. **Carbohydrate Research**, v. 7, p. 200–211, 1968.

KPODO, F. M.; AGBENORHEVI, J. K.; ALBA, K.; et al. Pectin isolation and characterization from six okra genotypes. **Food Hydrocolloids**, v. 72, p. 323–330, 2017.

KURTZMAN, C. P. Phylogeny of the ascomycetous yeasts and the renaming of Pichia anomala to Wickerhamomyces anomalus. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, v. 99, n. 1, p. 13–23, 2011.

KYYALY, M. A.; POWELL, C.; RAMADAN, E. Preparation of iron-enriched baker's yeast and its efficiency in recovery of rats from dietary iron deficiency. **Nutrition**, v. 31, n. 9, p. 1155–1164, 2015.

LEE, B.-K.; KIM, J. K. Production of Candida utilis biomass on molasses in different culture types. **Aquacultural Engineering**, v. 25, p. 111–124, 2001. Disponível em: <www.elsevier.nl/locate/aqua-online>. Acesso em: 15 agosto 2017.

LI, X.; XU, J. O. Y.; CHEN, M.; et al. Optimization of culture medium for lincomycin production by response surface methodology. **Bioresource Technology**, v. 100, p. 3613–3617, 2009.

LÖFGREN, C.; HERMANSSON, A. M. Synergistic rheological behaviour of mixed HM/LM pectin gels. **Food Hydrocolloids**, v. 21, n. 3, p. 480–486, 2007.

LOPES DA SILVA, J. A.; RAO, M. A. Pectins: Structure, Functionality, and Uses. In: A. M. Stephen; G. O. Phillips; P. A. Williams (Orgs.); **Food Polysaccharides and Their Applications**. 2nd ed, p.353–412, 2006. Boca Raton: Taylor & Francis Group.

LU, F.; RODRIGUEZ-GARCIA, J.; VAN DAMME, I.; et al. Valorisation strategies for cocoa pod husk and its fractions. **Current Opinion in Green and Sustainable Chemistry**, v. 14, p. 80–88, 2018.

LU, Y.; HUANG, L.; YANG, T.; LV, F.; LU, Z. Optimization of a cryoprotective medium to increase the viability of freeze-dried Streptococcus thermophilus by response surface methodology. **LWT - Food Science and Technology**, v. 80, p. 92–97, 2017.

MARKHAM, E.; BYRNE, W. J. Uptake, Storage And Utilization Of Phosphate By Yeast. , v. 73, p. 271–273, 1967.

MARSIGLIA, D. E.; OJEDA, K. A.; RAMÍREZ, M. C.; SÁNCHEZ, E. Pectin extraction from cocoa pod husk (Theobroma cacao L.) by hydrolysis with citric and acetic acid. **International Journal of ChemTech Research**, v. 9, n. 07, p. 497–507, 2016.

MATHEW, M.; NARAYANA, B. An easy spectrophotometric determination of selenium using azure B as a chromogenic reagent. **Indian Journal of Chemical Technology**, v. 13, p. 455–458, 2006.

MCSHEEHY, S.; YANG, L.; STURGEON, R.; MESTER, Z. Determination of methionine and selenomethionine in selenium-enriched yeast by species-specific isotope dilution with liquid chromatography-mass spectrometry and inductively coupled plasma mass spectrometry detection. **Analytical Chemistry**, v. 77, n. 1, p. 344–349, 2005.

MÉPLAN, C. Trace elements and ageing, a genomic perspective using selenium as an example. **Journal of Trace Elements in Medicine and Biology**, v. 25, n. SUPPL. 1, p. 11–16, 2011.

MÉPLAN, C.; HUGHES, D. J.; PARDINI, B.; et al. Genetic variants in selenoprotein genes increase risk of colorectal cancer. **Carcinogenesis**, v. 31, n. 6, p. 1074–1079, 2010.

MILLER, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. **Analytical Chemistry**, v. 31, n. 3, p. 426–428, 1959.

MIYAMOTO-SHINOHARA, Y.; IMAIZUMI, T.; SUKENOBE, J.; et al. Survival rate of microbes after freeze-drying and long-term storage. **Cryobiology**, v. 41, n. 3, p. 251–255, 2000.

MOHNEN, D. Pectin structure and biosynthesis. **Current Opinion in Plant Biology**, v. 11, p. 266–277, 2008.

MOLLEA, C.; CHIAMPO, F.; CONTI, R. Extraction and characterization of pectins from cocoa husks: A preliminary study. **Food Chemistry**, v. 107, n. 3, p. 1353–1356, 2008.

MORRIS, G. A.; KÖK, S. M.; HARDING, S. E.; ADAMS, G. G. Polysaccharide drug delivery systems based on pectin and chitosan. **Biotechnology and Genetic Engineering Reviews**, v. 27, n. 1, p. 257–284, 2010.

MOTEDAYEN, A. A.; KHODAIYAN, F.; SALEHI, E. A. Development and characterisation of composite films made of kefiran and starch. **Food Chemistry**, v. 136, n. 3–4, p. 1231–1238, 2013.

MU, R.-J. J.; YUAN, Y.; WANG, L.; et al. Microencapsulation of Lactobacillus acidophilus with konjac glucomannan hydrogel. **Food Hydrocolloids**, v. 76, p. 42–48, 2018.

MUZZARELLI, R. A. A.; BOUDRANT, J.; MEYER, D.; et al. Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. **Carbohydrate Polymers**, v. 87, n. 2, p. 995–1012, 2012.

N'GUESSAN, F. K.; COULIBALY, H. W.; ALLOUE-BORAUD, M. W. A.; COT, M.; DJÈ, K. M. Production of freeze-dried yeast culture for the brewing of traditional sorghum beer, tchapalo. **Food Science and Nutrition**, v. 4, n. 1, p. 34–41, 2016.

NAVARRO, A. R.; RUBIO, M. C.; CALLIERI, D. A. S. Production of Ethanol by Yeasts Immobilized in Pectin. **European Journal Of Applied Microbiology and Biotechnology**, v. 17, p. 148–151, 1983.

NAZZARO, F.; ORLANDO, P.; FRATIANNI, F.; COPPOLA, R. Microencapsulation in food science and biotechnology. **Current Opinion in Biotechnology**, v. 23, n. 2, p. 182–186, 2012.

NGOUÉMAZONG, D. E.; TENGWEH, F. F.; FRAEYE, I.; et al. Effect of de-methylesterification on network development and nature of Ca 2+-pectin gels: Towards understanding structure-function relations of pectin. **Food Hydrocolloids**, 2012.

NOELLO, C.; CARVALHO, A. G. S.; SILVA, V. M.; HUBINGER, M. D. Spray dried microparticles of chia oil using emulsion stabilized by whey protein concentrate and pectin by electrostatic deposition. **FRIN**, v. 89, p. 549–557, 2016.

NUNES, G. L.; ETCHEPARE, M. DE A.; CICHOSKI, A. J.; et al. Inulin, hi-maize, and trehalose as thermal protectants for increasing viability of Lactobacillus acidophilus encapsulated by spray drying. **LWT - Food Science and Technology**, v. 89, n. May 2017, p. 128–133, 2018.

ONODERA, K.; HAMA, S.; YOSHIDA, A.; NODA, H.; KONDO, A. Development of fed-batch process for high-yielding B-glucosidase displayed on cell surface of industrial yeast Saccharomyces cerevisiae. **Biochemical Engineering Journal**, v. 128, p. 195–200, 2017.

OWENS, H. S.; SWENSON, H. A.; SCHULTZ, T. H. Factors Influencing Gelation with Pectin. **Natural Plant Hydrocolloids**, p. 10–15, 1954. Disponível em: <http://pubs.acs.org/doi/abs/10.1021/ba-1954-0011.ch003> Acesso em: 26 novembro 2017 .

PANKIEWICZ, U.; JAMROZ, J. Accumulation of selenium and catalase activity changes in the cells of Saccharomyces cerevisiae on pulsed electric field (PEF) treatment. **Annals of Microbiology**, v. 58, n. 2, p. 239–243, 2008.

PANKIEWICZ, U.; SUJKA, M.; KOWALSKI, R.; et al. Effect of pulsed electric fields (PEF) on accumulation of selenium and zinc ions in Saccharomyces cerevisiae cells. **Food Chemistry**, 2017.

PETKOWICZ, C. L. O.; VRIESMANN, L. C.; WILLIAMS, P. A. Pectins from food waste: Extraction, characterization and properties of watermelon rind pectin. **Food Hydrocolloids**, v. 65, p. 57–67, 2017.

PIENIZ, S.; ANDREAZZA, R.; MANN, M. B.; CAMARGO, F.; BRANDELLI, A. Bioaccumulation and distribution of selenium in Enterococcus durans. **Journal of Trace Elements in Medicine and Biology**, v. 40, p. 37–45, 2017.

PIERMARIA, J. A.; CANAL, M. L. DE LA; ABRAHAM, A. G. Gelling properties of kefiran , a food-grade polysaccharide obtained from kefir grain. **Food Hydrocolloids**,

v. 22, p. 1520–1527, 2008.

PIERMARIA, J.; DIOSMA, G.; AQUINO, C.; GARROTE, G.; ABRAHAM, A. Edible kefiran films as vehicle for probiotic microorganisms. **Innovative Food Science and Emerging Technologies**, v. 32, p. 193–199, 2015.

PORTO, B. A. A.; MANGIAPANE, E.; PESSIONE, A.; et al. Evaluation of sodium selenite effects on the potential probiotic Saccharomyces cerevisiae UFMG A-905: A physiological and proteomic analysis. **Journal of Functional Foods**, 2015.

PRETSCH, E.; BÜHLMANN, P.; BADERTSCHER, M. **Structure Determination of Organic Compounds. Tables of Spectral Data**. Fourth ed. Springer-Verlag Berlin Heidelberg, 2009.

PRIYANGINI, F.; WALDE, S. G.; CHIDAMBARAM, R. Extraction optimization of pectin from cocoa pod husks (Theobroma cacao L.) with ascorbic acid using response surface methodology. **Carbohydrate Polymers**, v. 202, p. 497–503, 2018.

RASHED, M. N. The role of trace elements on hepatitis virus infections: A review. **Journal of Trace Elements in Medicine and Biology**, v. 25, n. 3, p. 181–187, 2011. Elsevier GmbH. Disponível em: <http://dx.doi.org/10.1016/j.jtemb.2011.07.001>. Acesso em: 10 novembro 2018.

RATTANACHAIWONG, S.; SINGER, P. **Diets and Diet Therapy: Enteral Nutrition**. Elsevier, 2018. Disponível em: <http://www.sciencedirect.com/science/article/pii/B9780081005965219367>. Acesso em 10 novembro 2018.

RODRIGUES, K. L.; GAUDINO CAPUTO, L. R.; TAVARES CARVALHO, J. C.; EVANGELISTA, J.; SCHNEEDORF, J. M. Antimicrobial and healing activity of kefir and kefiran extract. **International Journal of Antimicrobial Agents**, v. 25, n. 5, p. 404–408, 2005.

ROEPCKE, C. B. S. **Desenvolvimento de bioprocesso para produção de biomassa de levedura rica em zinco orgâncio**, 2007. Disponível em: <http://www.bioprocessos.net.br/docs/Dissertacao-2007-Clarisse-Roepcke.pdf> Acesso em: 15 agosto 2018.

ROEPCKE, C. B. S.; VANDENBERGHE, L. P. S.; SOCCOL, C. R. Optimized production of Pichia guilliermondii biomass with zinc accumulation by fermentation. **Animal Feed Science and Technology**, 2011.

ROLLINI, M.; MUSATTI, A.; ERBA, D.; et al. Process for obtaining copper-enriched cells of Saccharomyces cerevisiae. **Process Biochemistry**, v. 46, n. 7, p. 1417–1422, 2011.

RYCEWICZ-BORECKI, M.; MCLEAN, J. E.; DUPONT, R. R. Bioaccumulation of copper, lead, and zinc in six macrophyte species grown in simulated stormwater bioretention systems. **Journal of Environmental Management**, 2016.

SABAGHI, M.; MAGHSOUDLOU, Y.; HABIBI, P. Enhancing structural properties and antioxidant activity of kefiran films by chitosan addition. **Food Structure**, v. 5, p. 66–71, 2015.

SARAH, M.; HANUM, F.; RIZKY, M.; HISHAM, M. F. Microwave-assisted extraction of pectin from cocoa peel. **IOP Conference Series: Earth and Environmental Science**, v. 122, n. 1, 2018.

SATO, M. DE F.; RIGONI, D. C.; CANTERI, M. H. G.; et al. Caracterização química e instrumental de pectinas isoladas de bagaço de 11 cultivares de maçã. **Acta Scientiarum - Agronomy**, v. 33, n. 3, p. 383–389, 2011.

SCHELLER, H. V.; JENSEN, J. K.; SØRENSEN, S. O.; HARHOLT, J.; GESHI, N. Biosynthesis of pectin. **Physiologia Plantarum**, v. 129, n. 2, p. 283–295, 2007.

SCHOLS, H. A.; COENEN, G.-J.; VORAGEN, A. G. J. Revealing pectin's structure. In: H. A. Schols; R. G. F. Visser; A. G. J. Voragen (Orgs.); **Pectins and Pectinases**. 1° ed, p.19–31, 2009. Wageningen, the Netherlands, the Netherlands: Wogeningen Academic Publishers.

SCHOLS, H. A.; VORAGEN, A. G. J. Complex Pectins: Structure elucidation using enzymes. **Progress in Biotechnology**, v. 14, n. C, p. 3–19, 1996.

SCHULZE, K. L. **The Effect of Phosphate Supply on the Rate of Growth and Fat Formation in Yeasts**, v. 4, p. 207–210, 1956.

SERAFINI, F.; TURRONI, F.; RUAS-MADIEDO, P.; et al. Kefir fermented milk and kefiran promote growth of Bifidobacterium bifidum PRL2010 and modulate its gene expression. **International Journal of Food Microbiology**, v. 178, p. 50–59, 2014.

SHARMA, B.; DHULDHOYA, N.; MERCHANT, S.; MERCHANT, U. An Overview on Pectins. **Times Food Processing Journal**, , n. June-July, p. 44–51, 2006.

SOBAMIWA, O.; LONGE, O. G. Utilization of cocoa-pod pericarp fractions in broiler chick diets. **Animal Feed Science and Technology**, v. 47, n. 3–4, p. 237–244, 1994.

DE SOUZA, J. R. R.; FEITOSA, J. P. A.; RICARDO, N. M. P. S.; et al. Spray-drying encapsulation of mangiferin using natural polymers. **Food Hydrocolloids**, v. 33, n. 1, p. 10–18, 2013.

STABNIKOVA, O.; IVANOV, V.; LARIONOVA, I.; et al. Ukrainian dietary bakery product with selenium-enriched yeast. **LWT - Food Science and Technology**, v. 41, p. 890–895, 2008.

STEFANELLO, R. F.; MACHADO, A. A. R.; PASQUALIN CAVALHEIRO, C.; et al. Trehalose as a cryoprotectant in freeze-dried wheat sourdough production. **LWT - Food Science and Technology**, v. 89, n. May 2017, p. 510–517, 2018.

STEPHEN, A. M.; PHILLIPS, G. O.; WILLIAMS, P. A. (ORGS.). **Food Polysaccharides and Their Applications**. 2nd ed. Boca Raton: Taylor & Francis Group, 2006.

SUHAJDA, Á.; HEGÓCZKI, J.; JANZSÓ, B.; PAIS, I.; VERECZKEY, G. Preparation of selenium yeasts I. Preparation of selenium-enriched Saccharomyces cerevisiae. **Journal of Trace Elements in Medicine and Biology**, v. 14, n. 1, p. 43–47, 2000.

TAN, Y.; LEONHARD, M.; MA, S.; SCHNEIDER-STICKLER, B. Influence of culture conditions for clinically isolated non-albicans Candida biofilm formation. **Journal of Microbiological Methods**, v. 130, p. 123–128, 2016.

TAPIERO, H.; TEW, K. D. Trace elements in human physiology and pathology: Zinc and metallothioneins. **Biomedicine and Pharmacotherapy**, v. 57, n. 9, p. 399–411, 2003.

THAKUR, B. R.; SINGH, R. K.; HANDA, A. K. Chemistry and Uses of Pectin - A Review. **Critical Reviews in Food Science and Nutrition**, v. 37, n. 1, p. 47–73, 1997.

TIAN, W.; SONG, J.; WANG, Y.; et al. Effect of different calcium salts and methods for triggering gelation on the characteristics of microencapsulated Lactobacillus plantarum LIP-1. **RSC Advances**, v. 5, n. 90, p. 73352–73362, 2015. Royal Society of Chemistry. Disponível em: <http://dx.doi.org/10.1039/C5RA13354H>. Acesso em: 27 novembro 2018.

TIE, M.; LI, B.; SUN, T.; et al. HPLC-ICP-MS speciation of selenium in Se-cultivated Flammulina velutipes. **Arabian Journal of Chemistry**, 2017. King Saud University. Disponível em: <http://dx.doi.org/10.1016/j.arabjc.2017.05.012>. Acesso em: 03 dezembro 2018.

TORTORA, G. J.; FUNKE, B. R.; CASE, C. L. **Microbioloy: an Introduction**. 13º ed. Boston: Pearson, 2018.

VINCKEN, J.-P.; SCHOLS, H. A.; OOMEN, R. J. F. J.; et al. If Homogalacturonan Were a Side Chain of Rhamnogalacturonan I. Implications for Cell Wall Architecture. **Plant Physiology**, v. 132, n. 4, p. 1781–1789, 2003. Disponível em: <http://www.plantphysiol.org/cgi/doi/10.1104/pp.103.022350>. Acesso em: 26 novembro 2017 .

VIRARAGHAVAN, T.; SRINIVASAN, A. **Microbial Biosorption of Metals**. Prague, Czech Republic, Czech Republic: Springer, 2011.

VORAGEN, A. G. J.; COENEN, G. J.; VERHOEF, R. P.; SCHOLS, H. A. Pectin, a versatile polysaccharide present in plant cell walls. **Structural Chemistry**, v. 20, n. 2, p. 263–275, 2009.

VRIESMANN, L. C.; AMBONI, R. D. DE M. C.; PETKOWICZ, C. L. DE O. Cacao pod husks (Theobroma cacao L.): Composition and hot-water-soluble pectins. **Industrial Crops and Products**, v. 34, n. 1, p. 1173–1181, 2011.

VRIESMANN, L. C.; PETKOWICZ, C. L. DE O. Polysaccharides from the pulp of cupuassu (Theobroma grandiflorum): Structural characterization of a pectic fraction. **Carbohydrate Polymers**, v. 77, n. 1, p. 72–79, 2009.

VRIESMANN, L. C.; PETKOWICZ, C. L. DE O. Cacao pod husks as a source of low-methoxyl, highly acetylated pectins able to gel in acidic media. **International Journal of Biological Macromolecules**, v. 101, p. 146–152, 2017.

VRIESMANN, L. C.; PETKOWICZ, C. L. O. Highly acetylated pectin from cacao pod husks (Theobroma cacao L.) forms gel. **Food Hydrocolloids**, v. 33, n. 1, p. 58–65, 2013.

VRIESMANN, L. C.; SILVEIRA, J. L. M.; PETKOWICZ, C. L. DE O. Rheological behavior of a pectic fraction from the pulp of cupuassu (Theobroma grandiflorum). **Carbohydrate Polymers**, 2010.

VRIESMANN, L. C.; TEÓFILO, R. F.; LÚCIA DE OLIVEIRA PETKOWICZ, C. Extraction and characterization of pectin from cacao pod husks (Theobroma cacao L.) with citric acid. **LWT - Food Science and Technology**, v. 49, n. 1, p. 108–116, 2012.

VRIESMANN, L. C.; TEÓFILO, R. F.; PETKOWICZ, C. L. D. O. Optimization of nitric acid-mediated extraction of pectin from cacao pod husks (Theobroma cacao L.) using response surface methodology. **Carbohydrate Polymers**, v. 84, n. 4, p. 1230–1236, 2011.

WANG, Z.; ZHANG, J.; SU, T.; GUAN, Z.; JI, M. Screening of iron- and zinc-enriched yeast strain and optimization of cultivation conditions. **Preparative Biochemistry and Biotechnology**, v. 41, n. 3, p. 278–286, 2011.

WCF. CocoaAction - World Cocoa Foundation. Disponível em: <http://worldcocoafoundation.org/about-wcf/cocoaaction/>. .

WILLATS, W. G. T.; KNOX, J. P.; MIKKELSEN, J. D. Pectin: New insights into an old polymer are starting to gel. **Trends in Food Science and Technology**, v. 17, p. 97–104, 2006.

XIAO, Z.; LI, S.; GUO, J.; et al. Application of enzymatic probe sonication for selenium speciation in animal feeds. **Journal of Chromatography A**, v. 1530, p. 51–58, 2017.

XIAOGUANG, G.; DAHUI, W.; GONGYUAN, W.; MIN, N.; NA, S. Improvement of Physiological Characteristic of Selenium-Enriched Candida utilis with Amino Acids Addition. **Biotechnology research international**, v. 2011, n. June 2000, p. 1–7, 2011.

XU, K.; XU, P. Efficient production of l-lactic acid using co-feeding strategy based on cane molasses/glucose carbon sources. **Bioresource Technology**, v. 153, p. 23–29, 2014.

YANG, B.; WANG, D.; WEI, G.; LIU, Z.; GE, X. Selenium-enriched Candida utilis: Efficient preparation with l-methionine and antioxidant capacity in rats. **Journal of Trace Elements in Medicine and Biology**, v. 27, n. 1, p. 7–11, 2013.

YANG, J. S.; MU, T. H.; MA, M. M. Extraction, structure, and emulsifying properties of pectin from potato pulp. **Food Chemistry**, v. 244, p. 197–205, 2018.

YAPO, B. M.; KOFFI, K. L. Extraction and Characterization of Gelling and Emulsifying Pectin Fractions from Cacao Pod Husk. **Journal of Food and Nutrition Research**, v. 1, n. 4, p. 46–51, 2013. Disponível em: <http://pubs.sciepub.com/jfnr/1/4/3>. Acesso em: 13 novembro 2018 .

YIN, H.; CHEN, Z.; GU, Z.; HAN, Y. Optimization of natural fermentative medium for selenium-enriched yeast by d-optimal mixture design. **LWT - Food Science and Technology**, v. 42, n. 1, p. 327–331, 2009.

YIN, H.; FAN, G.; GU, Z. Optimization of culture parameters of selenium-enriched yeast (Saccharomyces cerevisiae) by response surface methodology (RSM). **LWT - Food Science and Technology**, v. 43, p. 666–669, 2010.

ZHANG, Y.; CHEN, T.; YUAN, P.; et al. Encapsulation of honokiol into self-assembled pectin nanoparticles for drug delivery to HepG2 cells. **Carbohydrate Polymers**, v. 133, p. 31–38, 2015.