UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE PESQUISA EM BIOENERGIA

**PROGRAMA INTEGRADO (UNESP, USP E UNICAMP) DE PÓS-GRADUAÇÃO EM BIOENERGIA**

# DEVELOPMENT OF THE BIOPROCESS OF ETHANOL PRODUCTION FROM STARCH RESIDUES USING AMYLOLYTIC ENZYMES PRODUCED BY *Rhizopus oligosporus* CCT 3762

**BRUNA ESCARAMBONI**

**Rio Claro – SP**

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**BRUNA ESCARAMBONI**

Tese apresentada ao Instituto de Pesquisa em Bioenergia de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências.

Orientador: Dr. Pedro de Oliva Neto

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Rio Claro, 16 de dezembro de 2019

*Inteiramente a Deus, por ser o centro e razão da minha vida;*
*Aos meus pais, Durval e Neide, e ao meu irmão,*
*Gustavo, por serem o meu alicerce;*
*Pelo amor, carinho e apoio*
*em todos os momentos.*
*Dedico.*

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*“Quem faz erra algumas vezes, mas quem nada faz erra continuamente.”*
*Bv. Tiago Alberione*

*“Quando o homem tiver acabado, então estará no começo; e quando cessar a pesquisa, ficará perplexo.”*
*Eclo 18, 6*

## RESUMO

Esta tese propõe algumas rotas biotecnológicas considerando os desafios envolvidos na produção e aplicação de enzimas fúngicas e glicose, bem como novas perspectivas para a produção de etanol utilizando matérias-primas alternativas. Inicialmente, a análise dos dados por mínimos quadrados parciais (PLS) demonstrou a correlação positiva entre a produção de amilase por *Rhizopus oligosporus* e a suplementação do meio de fermentação em estado sólido (FES) com nitrogênio orgânico. O extrato amilolítico apresentou desempenho competitivo e maior produtividade na hidrólise do amido do que uma glucoamilase comercial. A amilase produzida foi aplicada (15 U/g) no bagaço de mandioca (BM) para obtenção do hidrolisado de bagaço de mandioca (HBM) resultado de 42% de conversão do BM em açúcares redutores com uma hidrólise em etapa única de 10 h à 55°C. O HBM concentrado foi utilizado para fermentação alcoólica em batelada alimentada, produzindo 89,2% de rendimento. Além disso, comparando-se apenas o custo das matérias-primas, o HBM demonstrou ser mais econômico para a fermentação alcoólica do que a cana-de-açúcar. Em sequência, foi aplicado um planejamento fatorial em três níveis para otimizar a proporção enzimática de 0-30 U/g na hidrólise. Na condição otimizada 61,67% de BM foi convertido em glicose. Uma mistura sinérgica adequada reduziu 2 vezes a proporção de amilases (15 U/g) e 3 vezes para celulases (5 FPU/g), convertendo 51,79% do BM. Modificações na superfície do BM causadas pela ação enzimática foram evidenciadas por microscopia eletrônica de varredura. A hidrólise de 15% BM em biorreator produziu elevado teor de glicose (90,87 g/L; $Y_{P/S}$ = 0,50 g/g). Além disso, o HBM foi aplicado para produzir 35,25 g/L de etanol (eficiência de 78,3%). Com isso, o bioprocesso proposto produziria 254,1 L de etanol por tonelada de BM seco em 48 h, incluindo hidrólise e fermentação alcoólica. Finalmente, um tratamento biológico de resíduos alimentares via FES foi proposto para a produção de amilases e proteases por *R. oligosporus*. A maior produção ocorreu com: 50% de resíduo alimentar (RA), 10% de bagaço de cana (BC) e 40% de farelo de trigo (FT), suplementado com solução salina, para amilases, e acrescido de 20% (v/m) de água de maceração de milho, para produção de proteases. A suplementação com água de maceração de milho foi adequada como alternativa à solução de sais e o reciclo de biomassa e fibras não hidrolisadas foi capaz de aumentar o rendimento de amilase e protease. O extrato enzimático foi efetivo para hidrolisar o RA, alcançando 36,44% de conversão e um hidrolisado com 47,10 g/L de açúcares redutores.



**Palavras-chave:** Etanol; Amilase; Glicose; Fermentação em estado sólido; *R. oligosporus*.

## ABSTRACT

This thesis proposes some biotechnological routes considering the challenges involved in the production and application of fungal enzymes and glucose, as well as new perspectives for the production of ethanol using alternative raw materials. Initially, partial least square (PLS) data analysis demonstrated the positive correlation between the amylase production by *Rhizopus oligosporus* and the organic nitrogen supplementation of the solid state fermentation (SSF) medium. Crude amylase extract had competitive performance features giving higher productivities in starch hydrolysis than a commercial glucoamylase. The amylase produced was applied (15 U/g) in the cassava bagasse (CB) to obtain cassava bagasse hydrolysate (CBH). More than 42% conversion in reducing sugars was achieved with an efficient 10 h single-step hydrolysis at 55°C. The concentrated CBH was subsequently used in fed-batch process producing 89.2% ethanol yield. Furthermore, comparing just the cost of the raw materials sugarcane and CHB, the latter demonstrated to be a lower-cost feedstock for ethanol fermentation. In sequence, a three-level factorial design was applied to optimize enzymatic proportion from 0-30 U/g in hydrolysis. Optimized enzyme mixture efficiently converted 61.67% CB into glucose. An appropriated synergistic mixture was responsible for decreasing the proportion of amylases 2 times (15 U/g) and cellulases 3 times (5 FPU/g), converting 51.79% of the CB. Scanning electron microscopy image evidenced modifications on CB surface caused by the enzymatic action. Hydrolysis of 15% CB in bioreactor released great level of glucose content (90.87 g/L; $Y_{P/S}$ = 0.50 g/g). In addition, CBH was applied to produce 35.25 g/L ethanol (78.3% efficiency). Therefore, proposed bioprocess would produce 254.1 L of ethanol per ton of dry CB in 48 h, including the hydrolysis and alcoholic fermentation period. Finally, a biological treatment of food waste by SSF culture was proposed for the production of amylases and proteases by *R. oligosporus*. The highest enzymatic production using food waste as the main substrate was using the blend: 50% food waste, 10% sugarcane bagasse and 40% wheat bran supplemented with salt solution for amylases, and added by corn steep liquor 20% (v/w) for proteases production. The SSF supplementation with corn steep liquor was suitable as an alternative to replace the salts solution and the recycle of the biomass and non-hydrolyzed fibers of the wastes was able to increase amylase and protease yields. The enzymatic extract produced was able to hydrolyze food waste achieving 36.44% conversion yield and a hydrolysate with 47.10 g/L reducing sugars.



**Keywords:** Ethanol; Glucose; Amylase; Solid state fermentation; *Rhizopus oligosporus*.

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## GENERAL INTRODUCTION

Bioethanol can be produced from various renewable feedstock such as sugarcane, corn, wheat, cassava, and cellulose biomass. Considering the increasing demand, the better use of low added value materials as a renewable platform in the bioenergy sector for the production of biofuels, especially ethanol, is an economic alternative and a need, since oil and its derivatives are non-renewable, and expansion of agricultural land is limited. Accordingly, residues hydrolysis for ethanol production is a very promising direction that can become extensively adopted once the feedstock cost accounts more than 60% of the production cost of this fuel. Therefore, the total use of the biomass received by the industry, including generated residues, is important for the industrial economy balance.

The need to substitute catalysis for biocatalysis boosts the consumption of enzymes at the international level. In this way, the search for economical bioprocesses for the production of enzymes able to hydrolyze complex carbohydrates, such as the combination of starch and lignocellulosic compounds, has been a current concern. Amylolytic enzymes have received great attention because of their technological importance, economic benefits, and applicability in the commercial production of glucose. Amylases market is well established, however, considering the increasing demand in several industrial sectors, there is a huge interest in the discovery of enzymes with improved properties on starch degradation, as well as developing techniques that reduce the cost of production of amylolytic derivatives and increase the conversion yield.

Alternative feedstock as urban, agricultural and agro-industrial wastes can be efficiently and economically converted in biofuels, but more researches are needed to include these products in the biofuel industry. Additionally, the approach of using enzymes synergistic action with simplified and efficient strategies was possible and, finally, opens new economic, ecological and social perspectives to glucose and bioethanol production. Thus, there are many search possibilities in order to find more efficient enzymes with competitive or higher catalytic performance than of the current available. In addition, it is desirable to minimize the downstream steps of the enzyme extract, such as purification and concentration, followed by robust processes with single step enzymatic hydrolysis. Considering that more than 80% of ethanol production worldwide provide from American continent (USA and Brazil), the proposition of alternative feedstocks in accordance with each region, the climate and main crops and industries around the world, can increase socioeconomics conditions and solve several environmental concerns.

## SCOPE OF THIS THESIS

The objective of this thesis was to propose a strategy for the production of enzymes, glucose and ethanol from alternative raw materials in an integrated bioprocess according to the biorrefinery concept. Strategies to improve amylase production as well as their applications are reviewed in the **Chapter 1**. The challenge involved in the potentially low-cost ethanol from agro-industrial by-products and solid urban wastes, and the difficulties associated in the hydrolysis of these materials is presented in the **Chapter 2**.

A fast and inexpensive bioprocess for amylase production by *Rhizopus oligosporus*, able to hydrolyze complex residues in fermentable sugars to be used for ethanol production was pursued in **Chapter 3**. The goal was to develop a rapid and low cost system using natural, robust, and safe microorganism for the optimized production of glucoamylase from wheat bran supplemented with salts in solid state fermentation using statistical tools. The efficiency of the enzyme produced was demonstrated by comparison with commercial amylases and by the application in the hydrolysis of cassava bagasse. The hydrolyzed with high glucose content obtained was used as only carbon source for more economical ethanol production than sugarcane ethanol. In this chapter a new alternative for the low cost ethanol production intercropped with food production, without any competition between them, was proposed. Additionally, **Chapter 4** describes a fast and cost-effective bioprocess of cassava bagasse hydrolysis treatment for glucose production by a mixture of enzymes from both commercial source (cellulase) and selected microorganism cultivation (amylase by solid state fermentation – **Chapter 3**), in order to increase the residue conversion yield, followed by fermentation to ethanol production.

Faced with the challenge of urban solid wastes disposal and the knowledge that one-third of the food produced in the world is discarded, we have in counterpart an efficient process of obtaining enzymes from agro-industrial waste that opens the possibility for the use of other alternative substrates. Therefore, a new approach of biological treatment of food waste by the culture in solid state fermentation (SSF) for the production of fungal amylases and proteases was demonstrated in **Chapter 5**. A new bioprocess by the culture of *Rhizopus oligosporus* in a culture medium formulated by food waste, others carbon sources and corn steep liquor was applied to produce enzymes with catalytic efficiency, even to hydrolyze food waste. Additionally, a cell recycle system with two steps cultures, in which the second used the non-hydrolyzed substrates and biomass of the first, were able to increase up to several-fold the amylase and protease yields.

## CHAPTER 1 – General review: Microbial Amylases: Production and Applications

ESCARAMBONI, B.; OLIVA NETO, P. Microbial Amylases: Production and Applications. In: ADAM, N. (Ed.). **Amylases: Properties, Functions and Uses**. [s.l.] Nova Science Publishers, 2019. p. 142.

## ABSTRACT

Amylases comprise an enzyme complex with a wide variety of enzymes that act synergistically to break the amylose and amylopectin polysaccharides from the starch into glucose. They can be divided basically into four groups: endoamylases, exoamylases, debranching enzymes, and transferases. The need to substitute catalysis for biocatalysis boosts the consumption of enzymes at the international level. Amylases account for 25 to 33% of the world's enzyme market, acting in food, detergent, paper, textile, baking, chemical and pharmaceutical industries, in which starch hydrolysis is used to produce glucose syrup and ethanol. Amylolytic enzymes have a large occurrence that can be obtained from plants, animals, and microorganisms; however, enzymes of fungal and bacterial origin are predominantly applied in the industrial sectors. The main advantage of the use of microorganisms to obtain amylases is the production capacity in large quantities and ease of handling. Amylolytic enzymes have received great attention because of their technological importance, economic benefits, and applicability in the commercial production of glucose. Currently, the starch hydrolysis processes employ two classes of enzymes, α-amylase, and glucoamylase, at hydrolysis times that typically exceed 24 h. Due to increasing demand, there is a huge interest in the discovery of enzymes with improved properties on starch degradation, as well as developing techniques that reduce the cost of production of amylolytic derivatives. Strategies to improve amylase production as well as their applications are shown in this chapter.

**Keywords**: starch, amylases, microorganisms, solid-state fermentation, hydrolysis

# 1 INTRODUCTION

Considering the environmental and cost issues of conventional chemical processes, biotechnology has expanded rapidly with research and development initiatives that revolutionize the field of industrial enzymes, both in developing new products and in improving existing processes. The global market for industrial enzymes was estimated at $3.3 billion in 2010 (THOMAS; LARROCHE; PANDEY, 2013). A great leap occurred in this period, so that the global enzymes market was valued at $7,082 million in 2017, which is expected to reach $10.5 billion in 2024 with a compound annual growth rate (CAGR) of 5.7% from 2018 to 2024 (RESEARCH AND MARKETS, 2018). North America was the main contributor to the global enzyme market in 2017, with the potential to dominate the market due to the highest growth rate for the use of industrial enzymes in the biofuel production application and the increasing investments and standardizations by the government to increase biofuel use (MORDOR INTELLIGENCE, 2018; RESEARCH AND MARKETS, 2018).

The change of catalysis by biocatalysis boosts the international consumption of enzymes since the enzymatic method has advantages like being more efficient, without generation of toxic residues, and with potential for cost and efficiency improvement (SUKUMARAN et al., 2009). Amylases are enzymes of great importance for industrial applications and represent 25 to 33% of the world market for enzymes (BORGIO, 2011; SAGU et al., 2015; KUMAR; SAHAI; BISARIA, 2012; RAVINDAR; ELANGOVAN, 2013). They belong to the class of hydrolases and catalyze the hydrolysis of starch and its derivatives, releasing various products, such as dextrins and progressively small polymers composed of glucose units (GUPTA et al., 2003; RAVINDAR; ELANGOVAN, 2013).

Starch is the main storage polysaccharide in plant cells, with emphasis on economically important crops such as wheat, rice, maize, cassava, and potatoes. Chemically it is a homopolymer consisting of glucose molecules bound by α-1,4 and α-1,6 bonds. At the end of the polymer chain there is an aldehyde group, which is known as the reducing end. Amylose and amylopectin are the two types of glucose polymers present in starch (VAN DER MAAREL et al., 2002; CASTRO et al., 2010).

Amylases form an enzyme complex comprising a wide variety of enzymes which act synergistically to break the amylose and amylopectin polysaccharides from the starch into glucose. They can be divided basically into four groups: endoamylases, exoamylases, debranching enzymes and transferases (CASTRO et al., 2010; VAN DER MAAREL et al., 2002; SIVARAMAKRISHNAN et al., 2006). They are applied in several areas like food, beer,

cereal for babies, detergents, pulp and paper, leather, textile, bakery, chemical and pharmaceutical industries, liquefaction and saccharification of starch for glucose production, bioenergy industry, animal feed and fermentation industry (GUPTA et al., 2003; FREITAS et al., 2014; XIE et al., 2014; CHOI; HAN; KIM, 2015; EMTENANI; ASOODEH; EMTENANI; 2015; CASTRO et al., 2010; SOUZA; MAGALHÃES, 2010; MITIDIERI et al., 2006; VAN DER MAAREL et al., 2002).

Carbohydrases are estimated to be the largest share, about two-thirds in 2017, among all types of enzymes in the global market for industrial enzymes, which are widely used for converting carbohydrates into small sugars. Among them, amylase is the most common type of carbohydrases (MORDOR INTELLIGENCE, 2018; RESEARCH AND MARKETS, 2018). In 2015, the enzyme sales segment for the bioethanol market was the second largest. In addition, North American manufacturers are investing in new technologies to meet the growing needs of the bioenergy industry (RESEARCH AND MARKETS, 2018; THOMAS; LARROCHE; PANDEY, 2013).

Due to the increasing demand, there is a great interest in the discovery of better enzymes in the degradation of the starch, as well as in the development of techniques that reduce the cost of production of the amylolytic derivatives. The main advantage of the microorganisms use for the amylases production is the economic potential and large-scale production capacity, as well as the relative ease of handling and obtaining enzymes with specific properties (GUPTA et al., 2003; SOUZA; MAGALHÃES, 2010).

Although microorganisms from various groups can be reported as producers of various types of amylases, for commercial purposes α-amylase is mainly derived from the genus *Bacillus, while g*lucoamylase has traditionally been produced employing filamentous fungi, *Aspergillus* and *Rhizopus* spp (SOUZA; MAGALHÃES, 2010; KUMAR; SATYANARAYANA, 2009). The versatility of the microorganisms allows the isolation of specific enzymes when certain characteristics are desired for the bioprocess, such as thermostable enzymes of thermophilic organisms.

Fungi are especially valued for being an excellent source of enzymes of industrial interest. Those of the *Rhizopus* genus, for example, stand out as good amylase producers and have the ability to metabolize complex mixtures of organic compounds present in most agro-industrial wastes (JIN et al., 2002; NOROUZIAN et al., 2006; PEIXOTO-NOGUEIRA et al., 2008). The *Rhizopus oligosporus* species is characterized as safe since it is GRAS (Generally Recognized as Safe) certification that allows and flexibilizes various applications such as food and feed (VATTEM et al., 2004).

Currently, submerged fermentation (SmF) and solid-state fermentation (SSF) are the main technologies for the production of enzymes. The traditional bioprocess of SmF consists of an abundant aqueous phase, in which the microorganisms and nutrients are submerged, and is classically applied by most enzymes companies due to be more easily controlled in parameters such as scaling up, pH and temperature control, aeration, oxygen transfer, and humidity (PRÉVOT et al., 2013). On the other hand, SSF technology occurs with predominant non-aqueous phase in which a microbial culture develops on the surface and in between the particles of a solid matrix in the absence or near absence of dripping water. Nevertheless, the substrate must provide sufficient moisture content to maintain the growth and metabolic activity of the microorganism (THOMAS; LARROCHE; PANDEY, 2013). SSF has gained research attention and credibility among industrial corporations, and could be an interesting alternative, still underexploited, to enzymatic production due to advantages over the submerged conventional process (BARRIOS-GONZÁLEZ, 2012; PRÉVOT et al., 2013). This technology has been recognized to have lower investment capital and lower consumption of water and energy, high yields and product recovery, and is being reported as the most appropriate process for developing countries (SOUZA; MAGALHÃES, 2010).

High value-added compounds, such as enzymes, can be produced using low cost industrial and agricultural wastes. Different agro-industrial residues can be used for the production of amylases by solid-state fermentation, such as wheat bran, soybean meal, cottonseed meal, sugarcane bagasse, rice straw, corncobs and orange residues (CASTRO; SATO, 2013; DJEKRIF-DAKHMOUCHE et al., 2006; HASHEMI et al., 2010; PEIXOTO-NOGUEIRA et al., 2008). Particularly, wheat bran has a relatively high content of proteins and carbohydrates, as well as a small amount of lignin, which is its main differential in comparison to other lignocellulosic biomasses (APPRICH et al., 2014). The starch processing industries employ glucoamylases mainly of *Aspergillus* and *Rhizopus* species in the saccharification of the starch and, although they can be produced by several fermentation methods, the solid-state fermentation system has given the highest enzymatic levels (NOROUZIAN et al., 2006).

## 2 STARCH

Starch is a polysaccharide synthesized by plants and used as an important source of nutrition for other living organisms. It is synthesized in the plastids, which are present in the leaves, and accumulates as insoluble semicrystalline granules with different polymorphic types and degrees of crystallinity. The granules range in size from 2 to 100 μm and exhibit round,

oval and irregular shapes. Starch is the main component of most staple foods and is used in many food and non-food industries, especially in the textile, paper, pharmaceutical, beverage and alcohol industries (SHARMA; SATYANARAYANA, 2013).

Industrial processes allow the starch to be extracted with high purity, which is a renewable, biodegradable and non-toxic raw material, and provides the highest percentage of calories in the human diet. Pure starch is a white, tasteless and odorless powder. The starch granule is inert, structurally stable, condensed and insoluble in water and alcohol, which can be cleaved in the presence of enzymes. In addition, the size and shape of the granules are specific to each plant species (MYERS et al., 2000; PINTO, 2009; VAN DER MAAREL et al., 2002).

Structurally the starch is formed by two high molecular weight compounds, amylose and amylopectin, and both contain α-D-glucose as the sole monomer. Amylose is a linear, long chain, and water-insoluble polymer composed of up to 6000 units of glucose linked by α-1,4-type bonds. Whereas amylopectin is very branched and water-soluble, consisting of short linear chains of 10-60 units of glucose linked by α-1,4 linkages and side chains with 15-45 units of glucose linked by α-1,6 linkages, which give volume to the starch molecule. Starch granules are organized into amorphous and crystalline regions, composed of amylose and amylopectin, respectivelly. Amylose and amylopectin levels vary between starches, but representative percentages of amylose and amylopectin are 15-25% and 75-85%, respectively (ECKERT et al., 2017; KUMAR; SATYANARAYANA, 2009; SHARMA; SATYANARAYANA, 2013; SOUZA; MAGALHÃES, 2010; VAIDYA et al., 2015; VAN DER MAAREL et al., 2002).

## 3 AMYLASES

A variety of different products can be obtained by the enzymatic processing of the starch, such as glucose syrups, fructose, maltodextrin derivatives and cyclodextrins, depending on the type of amylase used. There are basically four groups of enzymes that act on the conversion of starch: endoamylases, exoamylases, debranching enzymes and transferases (CINELLI et al., 2015; SIVARAMAKRISHNAN et al., 2006; VAN DER MAAREL et al., 2002).

Endoamylases are also known as depolymerizing enzymes because they act to liquefy the starch. They are composed mainly by α-amylases (α-1,4-glucan-4-glucanohydrolases - EC 3.2.1.1) which catalyze the random cleavage of the α-D-1,4 glycosidic bonds between the glucose units present in the inner and linear part of the starch, releasing oligosaccharides of various lengths (CASTRO et al., 2010; KANDRA, 2003).

Exoamylases (saccharifying enzymes) exclusively hydrolyze α-1,4-glycosidic bonds such as β-amylase (EC 3.2.1.2) or both α-1,4 and α-1,6 bonds, such as glucoamylase (synonymous with amyloglucosidase; 1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) and α-glucosidase (EC 3.2.1.20). Exoamylases act on the external glucose residues of amylose or amylopectin and thus produce only glucose as the final product (glucoamylase and α-glucosidase), or maltose and dextrins (β-amylase) (AQUINO et al., 2001; PEIXOTO-NOGUEIRA et al., 2008; VAN DER MAAREL et al., 2002). Unlike α-amylase, most glucoamylases are able to hydrolyze α-1,6 bonds at the branching sites of amylopectin, although at a slower rate than α-1,4 linkages (ELLAIAH et al., 2002).

Debranching enzymes, such as the isoamylase (EC 3.2.1.68) and pullulanase type I (EC 3.2.1.41), exclusively hydrolyze α-1,6 type glycosidic bonds. Pullulanases cleave pullulan and amylopectin, whereas isoamylase can hydrolyze only α-1,6 bonds in amylopectin. The transferases cleave the α-1,4 glycosidic linkages of the donor molecule and transfer part of the donor to a glycosidic acceptor with the formation of a novel binding (VAN DER MAAREL et al., 2002).

## 4 AMYLASES APPLICATIONS

Traditionally, the hydrolysis of the starch is carried out using an acid medium under high temperature, however the enzymatic hydrolysis has replaced the acid hydrolysis of the starch in more than 75% of the processes (KANDRA, 2003; WANG; LIANG; LIANG, 2011). Enzymatic hydrolysates are advantageous because the catalytic enzymes are specific for the reaction, consequently there is no formation of undesirable products providing higher yield (BALLESTEROS et al., 2002).

Starch hydrolysis is a simple way to produce carbohydrates with special functional properties (MOORE et al., 2005). Several studies point to glucoamylase and α-amylase as the most important enzymes used in starch processing (CARVALHO et al., 2008; FERREIRA-NOZAWA et al., 2008; HASHEMI et al., 2010; PEIXOTO-NOGUEIRA et al., 2008; SOUZA; MAGALHÃES, 2010).

Amylases are one of the most important enzymes in industrial bioprocesses (WANG; LIANG; LIANG, 2011). Its commercialization is the oldest, being used for the first time in 1984 as a pharmaceutical aid for the treatment of digestive disorders (GUPTA et al., 2003). In the current scenario there is a wide range of applications for amylases including food industries, detergents, paper, textiles, baking, chemical industry, pharmaceuticals, starch hydrolysis and

ethanol manufacturing (CASTRO et al., 2010; GUPTA et al., 2003; MITIDIERI et al., 2006; SOUZA; MAGALHÃES, 2010; VAN DER MAAREL et al., 2002).

An important market for the application of amylases is in the production of starch hydrolysates obtaining glucose and fructose as the final product. Starch is converted to high fructose corn syrups (HFCS or High Fructose Corn Syrups). Due to their high sweetener property, they are used in large quantities as sweeteners in the soft drink industry (GUPTA et al., 2003).

The enzymatic treatment for the starch hydrolysis occurs in several steps and using more than one type of amylase in view of its complete conversion to glucose syrup. Two main groups of enzymes are involved in this process: endoamylases, which act randomly at the interior of starch molecule to release oligosaccharides, and exoamylases, which act in the nonreducing ends to produce glucose (FREITAS et al., 2014; KUMAR; SATYANARAYANA, 2009).

The first step is liquefaction to solubilize the starch in short chain dextrins using thermostable α-amylase, generally from *Bacillus* sp, under the conditions of 90°C and pH 7. The achievement of final product depends on the incubation time and the amount of enzyme added. The next step is saccharification of the hydrolyzed starch syrup to produce a high concentration glucose syrup (more than 95%). This occurs using a glucoamylase, commonly of fungal origin - *Aspergillus niger* or a similar species. This glucoamylase is used in the saccharification process of liquefied hydrolyzed material, acting in pH adjusted to 4.5-5.0 and the step is performed for 12-96 hours at 60-62°C. A third step in the industrial processing of starch is the conversion of a high glucose syrup into a high fructose syrup (BIAŁAS; SZYMANOWSKA; GRAJEK, 2010; CINELLI et al., 2015; ECKERT et al., 2017; VAN DER MAAREL et al., 2002).

Amylases are extensively employed in baking, beverages, preparation of digestion aids, production of cakes, fruit juices and starch syrups (COUTO; SANROMÁN, 2006). In the baking industry, α-amylases have been widely used. They may be added in the bread dough to degrade the flour starch into small dextrins, which are subsequently fermented by the yeast. The addition of the enzyme to the mass results in increased fermentation rate and viscosity reduction, improving the volume and texture of the product. In addition, it generates additional sugar in the dough, which improves the taste, color and texture of the bread (SOUZA; MAGALHÃES, 2010).

In the textile industry, the most commonly used enzyme is amylase, which is normally employed to reshape fabrics by hydrolysis of starch (MARYAN; MONTAZER, 2013). A removable protective layer, consisting of starch gums or derivatives, is applied to the yarns to

give greater strength during the weaving process. Starch is quite attractive for this purpose, since it is cheap, has great availability in several regions of the world and can be easily removed. Amylases are used in the elimination of gums rather than the use of oxidants, acids or bases, which damage the cellulose of the tissue. The enzymes break down the starch molecules into dextrins that are water soluble and can be easily removed by washing (GUANDALINI, 2007; SPIER, 2005).

In the paper industry the amylases are used in the modification of starch which is applied as a paper coating to provide protection against mechanical damage during processing and to improve the quality of the final product, increasing the strength and the erasability. The starch is added in the pressing step, where two cylinders transfer the starch paste to the paper. The temperature of this process is about 45 to 60 °C (GUPTA et al., 2003).

The detergent industries are large consumers of enzymes in terms of volume and value. Expansion in the field of enzyme applications enhances the performance of enzymatic liquid detergents. The proteases class is the first used in detergent formulation and amylases the second type of enzymes employed for this purpose (MITIDIERI et al., 2006).

Ethanol is a biofuel, produced by fermenting sugar extracted from sugar and starch rich plants (ALTHURI; GUJJALA; BANERJEE, 2017; NIGAM; SINGH, 2011). The raw starch is the most common for the production of fuel ethanol in temperate regions (Europe, North America, Central Asia), which undergoes an enzymatic hydrolysis process to be converted to glucose. Alpha-amylase and glucoamylase are also the two enzymes usually employed for starch liquefaction and saccharification, for subsequent ethanol fermentation (ECKERT et al., 2017).

Renewable sources utilization for the production of biofuels has as a key advantage the employment of natural resources, which are geographically more evenly distributed than fossil fuels and ecologically appropriate (NIGAM; SINGH, 2011). In this way, bioethanol can be produced from various types starchy raw materials, such as corn (*Zea mays*), cassava (*Manihot spp.*), wheat (*Triticum spp*.), and rice (*Oryza sativa*) (AZHAR et al., 2017; BAEYENS et al., 2015; ECKERT et al., 2017).

## 5 AMYLASES PRODUCTION

The amylases present a large occurrence and can be obtained from plants, animals and microorganisms. However, enzymes of fungal and bacterial origin are predominantly applied in industrial sectors. Amylolytic enzymes have received great attention because of their

technological importance, economic benefits and applicability in the commercial production of glucose (GUPTA et al., 2003).

There is a large interest in amylases with improved properties on starch degradation in order to reduce the cost of starch derivatives. In this way, both use of agro-industrial wastes as substrate for an economical amylases production by microorganism, as in the case of the increasing solid-state fermentation, and the utilization of amylase with high catalytic efficiency without expensive downstream steps, as crude enzymatic extracts, can contribute to improve the viability of the bioprocesses (ESCARAMBONI et al., 2018; FERNÁNDEZ NUÑEZ et al., 2016).

Industrial production of enzymes is often limited due to the costs of the substrates used to grow the microorganisms. It is estimated that around 30 to 40% of the cost involved in the production of enzymes is related to the culture medium used for the growth of the microorganism, therefore its optimization is of great importance (JOO; CHANG, 2005). The potential of using microorganisms as sources of industrially relevant enzymes has stimulated interests in the exploitation of extracellular enzymes from various microorganisms.

There are several microorganisms producing amylases, including *Rhizopus*, *Aspergillus* and *Bacillus* (Table 1). In the production of amylases by fungi, the culture processes by SSF and SmF are recurrent, however, for bacteria, a predominance of SmF is observed. In addition, the literature shows more frequently the production of glucoamylase by fungi and bacteria producing α-amylase. Table 1 also shows a diversity of substrates used as carbon source, including several wastes that reduce production costs and collaborate to solve environmental concerns. However, for the production of amylase by some bacteria, the starch substrate may undergo previous hydrolysis to release sugars (LOLASI et al., 2018; RAVINDAR; ELANGOVAN, 2013), which is not the case in most studies in which the substrate is not pretreated.

Fungi are especially valued for being an excellent source of enzymes of industrial interest. For specific applications, as in the food industry, some fungal amylases are preferred over other microbial sources due to their more accepted GRAS status (GUPTA et al., 2003). Those belonging to the genus *Rhizopus* are prominent, because they are good amylases producers and have the capacity to metabolize complex mixtures of organic compounds present in most agro-industrial residues (JIN et al., 2002; NOROUZIAN et al., 2006; PEIXOTO-NOGUEIRA et al., 2008). In addition, species of this genus have already been used with efficiency in reducing the levels of contamination by the mycotoxins aflatoxin B1 and ochratoxin A (FURLONG; CACCIAMANI; BUFFIN, 2007). Antimicrobial properties of

phenolic extracts obtained from *Rhizopus oligosporus* in SSF have also been reported (VATTEM et al., 2004).

**Table 1 - Amylases produced by different microorganisms in SSF and SmF.**

| Microorganism | Carbon source | Fermentation | Enzyme | Reference |
|---|---|---|---|---|
| *Fungi* | | | | |
| *Aspergillus awamori* | Babassu cake; Olive oil cake | SSF | Amylases | (CASTRO et al., 2010; KARAM et al., 2017) |
| *Aspergillus fumigatus* | Lignocellulosic substrates | SSF | Amylases | (SINGH et al., 2014) |
| *Aspergillus niger* | Wheat bran; Tapioca | SSF | α-amylase; glucoamylase | (ABDULLAH et al., 2014; VARZAKAS; ROUSSOS; ARVANITOYANNIS, 2008) |
| *Aspergillus oryzae* | Wheat | SmF | α-amylase | (KAMMOUN; NAILI; BEJAR, 2008) |
| *Coprinus comatus* | Starch | SmF | α-amylase; glucoamylase | (PALUDO et al., 2018) |
| *Penicillium chrysogenum* | Linseed oil cake | SmF | α-amylase | (DAR et al., 2015) |
| *Piriformospora indica* | Soluble starch | SmF | Amylases | (KUMAR; SAHAI; BISARIA, 2012) |
| *Rhizopus microsporus* var. *rhizopodiformis* | Wheat bran; Corncob | SSF | a-amylase; Glucoamylase | (PEIXOTO-NOGUEIRA et al., 2008) |
| *Rhizopus oligosporus* | Wheat bran | SSF | Amylases; glucoamylase | (BARCHI et al., 2016; ESCARAMBONI et al., 2018; ITO et al., 2017) |
| *Rhizopus oryzae; Rhizopus oligosporus* | Type II wheat flour | SmF | Glucoamylase | (FREITAS et al., 2014) |
| *Thermomyces lanuginosus* | Wheat bran | SSF | Amylases | (KUNAMNENI; PERMAUL; SINGH, 2005) |
| *Bacteria* | | | | |
| *Bacillus amyloliquefaciens* | Potato starchy waste | SmF | Amylases | (ABD-ELHALEM et al., 2015) |
| *Bacillus methylotrophicus* | Soluble starch | SmF | α-amylase | (XIE et al., 2014) |
| *Bacillus subtilis* | Sago starch; Brewery wastes | SmF | α-amylase; amylases | (BLANCO et al., 2016; RAVINDAR; ELANGOVAN, 2013) |
| *Bacillus subtilis; Bacillus licheniformis* | Strawberry leaves; Watermelon rind | SmF; SSF | α-amylase | (AHMED et al., 2017) |
| *Chryseobacterium sp.; Bacillus sp* | Organic kitchen wastes | SmF | Amylases | (HASAN et al., 2017) |
| *Exiguobacterium sp* | Artocarpus | SmF | α-amylase | (SEN et al., 2016) |
| *Geobacillus thermoleovorans* | Starch | SmF | α-amylase | (SUDAN et al., 2018) |
| *Nesterenkonia sp.* | Sweet sorghum | SmF | α-amylase | (LOLASI et al., 2018) |
| *Paenibacillus chitinolyticus* | Sugar beet pulp | SmF | β-amylase | (MIHAJLOVSKI et al., 2016) |
| *Streptomyces sp* | Soluble starch | SmF | α-amylase | (MANIVASAGAN et al., 2015) |

Although industrial enzymes have traditionally been produced by SmF, SSF processes have been used more frequently in the production of these enzymes (HASHEMI et al., 2010). Enzymes and secondary metabolites, which are products of biotechnological interest, were produced by microorganisms throughout the evolutionary process for use in wet solid substrates, consequently, cultivation in aqueous suspensions may impair their metabolic efficiency (BARRIOS-GONZÁLEZ, 2012). Therefore, SSF provide an environment to the microorganisms close to its natural where they normally exist and have been isolated. This seems to be the main reason for the high yields in this type of bioprocess. At SSF, the solid matrix may be a source of carbon and other nutrients or be an inert material with impregnated nutrient solutions for the microbial growth (THOMAS; LARROCHE; PANDEY, 2013).

Limitations of SmF such as high energy consumption, waste generation and difficult sterility, because many contaminants can compete with the microorganism of the process under conditions of high water availability. On the other hand, SSF has gained significant attention for the development of industrial bioprocesses, particularly due to the lower energy requirement associated with higher yields and less wastewater production as well as a lower risk of bacterial contamination. Therefore, SSF processes do not require a rigorously aseptic bioreactor operation, so design requirements are less stringent, resulting in lower costs representing an economic advantage over SmF. In addition, it is eco-friendly, as mostly utilizes solid agro-industrial wastes as the substrate to generate high added-value products such as enzymes (CASCIATORI et al., 2015; CHEN, 2013; PANDEY et al., 2000; THOMAS; LARROCHE; PANDEY, 2013).

However, the industrial scaling-up of the SSF to enzyme production sufficient to supply demand is still under development. While in the SmF the processes of scale-up and control of operations are well established, the SSF still needs to be further studied for the elaboration of strategies that ensure wide applicability due to the advantages of the technology. Among the types of SSF bioreactor are those of packed-bed, fixed-bed, gas double-dynamic, counter-current, deep-bed, tray, rotary drum and modular (THOMAS; LARROCHE; PANDEY, 2013).

One of the main drawbacks in SSF bioreactors is the reduction in moisture content during the process, once microorganisms are sensitive to a reduction in water concentration and a dependence of enzyme production with the water content of the porous media is known. Moreover, the humidity directly affects gas and solute diffusions in the culture (CASCIATORI et al., 2015). The water content in the solid substrate of SSF generally ranges from 12-80%, mainly around 60% (CHEN, 2013), since the ideal moisture depends on both the solid substrate and the microorganism used.

The porosity of a packed-bed in a bioreactor is another key variable, which directly affects the fluid dynamics and the heat and mass transfer properties of the porous media. The size of the particle also interferes in the utilization of the substrate by the microorganism, so that small particle size harms microbial respiration, while large sizes limit the available surface for the microbial attack (CASCIATORI et al., 2014).

Nevertheless, there are no general rules for guiding scale-up of SSF bioreactors, considering specific characteristics of each microorganism and substrate. Therefore, several studies can be found in the literature in order to define the ideal porous matrix for SSF bioreactors using, not infrequently, modeling and simulation tools to determine selected properties, and scale-up the processes on a case-by-case basis (BÜCK et al., 2015; CASCIATORI et al., 2013, 2014, 2015, 2016; FINKLER et al., 2017; THOMAS; LARROCHE; PANDEY, 2013).

Wheat bran is widely used in several amylase production processes and considered to be an ideal substrate for SSF, due to the correct balance between the carbon, nitrogen and phosphorus sources (CASCIATORI et al., 2015; FERNÁNDEZ NUÑEZ et al., 2016; ITO et al., 2017). The composition of this by-product is approximately 12% water, 13-18% protein, 3.5% fat and 56% carbohydrates (PRÜCKLER et al., 2014). In the literature, several studies carried out with wheat bran and other low cost agro-industrial residues in SSF systems have demonstrated the superiority of wheat bran as a substrate and inducer for the secretion of amylases, mainly from fungi, and obtaining the maximum levels of enzymatic production (ELLAIAH et al., 2002; HASHEMI et al., 2010; PEIXOTO-NOGUEIRA et al., 2008).

The composition of the substrate directly influences the enzymatic activity of the fermented product. In addition to the source of carbon, minerals and nitrogen are essential factors in meeting the nutritional requirements of culture media for most microorganisms. Thus, an additional protein content in the substrate may contribute to the enrichment of the medium and improve the enzymatic production. Wheat bran, for example, is a substrate that provides an important nutritional contribution to the microorganism, providing nitrogen in the form of protein in proportions higher than other substrates, such as cassava meal with only 2.6% protein. However, an increase in nitrogen concentration leads to greater expression of amylase, pointing to the need for an additional nitrogen supply (ESCARAMBONI et al., 2018; FREITAS et al., 2014; KAMMOUN; NAILI; BEJAR, 2008).

A direct correlation between the organic nitrogen content in the SSF medium and the increasing in amylase production by *Rhizopus oligosporus* has already been identified (ESCARAMBONI et al., 2018). In addition, different sources of nitrogen, such as urea and

$(NH_4)_2SO_4$, have a positive effect on amylase production (KAMMOUN; NAILI; BEJAR, 2008). Nevertheless, relatively high concentrations of some nutrients may cause a possible inhibition, affecting the osmotic balance in the microbial cells, and consequently the amylase synthesis (ESCARAMBONI et al., 2018).

Phosphate is another important component, which acts as a regulator in the synthesis of primary and secondary metabolites in microorganisms, affecting the growth and amylase production. Adequate levels of phosphate may promote significant increases in enzyme production; on the other hand, high concentrations appear to be inhibitory in reports of fermentation with bacteria and fungi (ESCARAMBONI et al., 2018; GUPTA et al., 2003).

The influence of temperature on the production of amylase is related to the growth of the organism. However, among mesophilic fungi, it is possible to find enzymes for which optimal temperatures of action are up to 30°C above the maximum growth temperature, so that several known amylases are acting in the range of 50-65°C. This is the case of glucoamylase from *Rhizopus oligosporus*: the fungus is cultivated at 30°C, but the enzyme produced has optimal temperature of 60°C (BARCHI et al., 2016; GOMES et al., 2007; GUPTA et al., 2003; PEIXOTO-NOGUEIRA et al., 2008; WANG; LIANG; LIANG, 2011).

The composition of saccharides obtained after disruption of the starch chains is highly dependent on hydrolysis conditions, temperature, pH dependence, stability, specificity and source of the enzymes. Therefore, the screening and study of different amylase producing microorganisms may facilitate the discovery of amylolytic enzymes with specific biochemical properties and characteristics for a variety of industrial processes requiring efficient saccharification of the starch (WANG; LIANG; LIANG, 2011).

## 6 CONCLUSIONS

Amylases market is well established, however, considering the increasing demand in several industrial sectors, the better use of low added value materials as a renewable platform in biotechnological applications is an economic alternative and a need. The use of alternative substrates for the production of amylases required for the hydrolysis of the same or another biomass using the biorefinery platform concept is a trend (ESCARAMBONI et al., 2018; LOLASI et al., 2018). In this way, projects involved in the development of strategies to the whole utilization of the substrates, since the enzymatic production, until its application in innovative bioprocess, tends to be highlighted in the scientific and business circles.

The fungal amylolytic synthesis using solid-state fermentation, mainly scaled-up for bioreactors, is a challenge to be studied case by case, according to the microbial characteristics and properties of the substrate. Considering the economic issue at each stage of the development of bioprocesses is an important approach in order to narrow the distance between laboratory benches and companies.

Generally, the starch hydrolysis processes use two amylolytic enzymes, α-amylase and glucoamylase, in hydrolysis reactions that normally exceed 24 hours (BIAŁAS; SZYMANOWSKA; GRAJEK, 2010; CINELLI et al., 2015).

Thus, there are many search possibilities in order to find more efficient amylases with competitive or higher catalytic performance than of the current available amylases. In addition, it is desirable to minimize the downstream steps of the enzyme extract, such as purification and concentration, followed by robust processes with single step enzymatic hydrolysis.

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# CHAPTER 2 – General review: Ethanol Production from Alternative Feedstocks: New Perspectives


## ABSTRACT

Bioethanol can be produced from various renewable feedstock such as sugarcane, corn, wheat, cassava, and cellulose biomass. In addition, residues hydrolysis for ethanol production is a very promising direction that can become extensively adopted once the feedstock cost accounts more than 60% of the production cost of fuel ethanol and the need for more agricultural areas today used for food production. Considering the increasing demand, the better use of low added value materials as a renewable platform to produce biofuels is an economic alternative and a need. Therefore, the total use of the biomass received by the industry, including generated residues, is important for the industrial economy balance. The conversion of complex wastes by saccharification and subsequent fermentation process of agroindustry by-products and solid urban wastes could lead to a potentially low-cost ethanol. However, these materials are difficult to be hydrolyzed involving a complex technology needed to be developed and the high cost of enzymes. This challenge is showed in this chapter.


**Keywords:** Bioethanol, wastes, saccharification, enzymatic hydrolysis, feedstock, low-cost, fermentation.

## 1 INTRODUCTION

The concern about the environmental impacts and dependence on petroleum fuels over the years has motivated the worldwide search for new friendly, safe and economical ways of producing biofuels. Alternatively, bioethanol offers an environmentally sound and renewable solution. For this reason, this biofuel has been identified as the most widely used in the world as it contributes significantly to the reduction of crude oil consumption and pollution (AZHAR et al., 2017).

Since the 1980s the interest in using bioethanol has been increasing (AZHAR et al., 2017). Global ethanol production doubled from 13.12 billions of gallons in 2007 to 26.74 billions of gallons in 2017 (RENEWABLE FUELS ASSOCIATION, 2016, 2018). The United States is the largest producer of ethanol in the world, followed by Brazil. Together, they

produced 22.9 billion gallons (86.5 billion liters) in 2017 contributing with 84% world's ethanol, mainly from corn in the U.S., and primarily by sugarcane in Brazil (AZHAR et al., 2017; RENEWABLE FUELS ASSOCIATION, 2018). In European Union countries, responsible for 5% of world ethanol production (RENEWABLE FUELS ASSOCIATION, 2018), beet pulp is used for the production of ethanol (ECKERT et al., 2017). In the US, the use of corn grain must be balanced for food and feed, and ethanol production, as well as other issues such as the economic impacts on food prices, land use and environmental effects (MUMM et al., 2014). Likewise in Brazil, despite only 1.2% of the total Brazilian land being occupied with sugarcane, land resources are limited, and sugarcane expansion can displace other productive sectors and thereby influence socioeconomic impacts (BRINKMAN et al., 2018).

Renewable sources utilization for the production of biofuels has as a key advantage the employment of natural resources, which are geographically more evenly distributed than fossil fuels and ecologically appropriate (NIGAM; SINGH, 2011). Bioethanol can be produced from various types of renewable feedstocks, which contains sucrose or carbohydrate, such as sugarcane (*Saccharum spp*.), corn (*Zea mays*), sugar beet (*Beta vulgaris*), cassava (*Manihot spp.*), wheat (*Triticum spp*.), rice (*Oryza sativa*), sweet sorghum (first generation), lignocellulosic biomass (second generation), and algal biomass (third generation) (AZHAR et al., 2017; BAEYENS et al., 2015; ECKERT et al., 2017). Fermentable sugars (six-carbon) from the raw material are usually converted in bioethanol by enzymes produced from yeast. Depending on raw materials, available sugar content can be directly fermented by the yeast, as in the case of materials saccharins, or a previous hydrolysis process can be necessary to convert polysaccharides (starches and cellulose) into glucose when the raw materials are grains or cellulose biomass (NIGAM; SINGH, 2011). The yeast *Saccharomyces cerevisiae* is the commonly employed in ethanol production due to advantages like high ethanol productivity and tolerance, and ability of fermenting wide range of sugars (AZHAR et al., 2017).

Utilizing wastes as raw materials can minimize the conflict between food and fuel, and a potential of trillions of tons can be used. Therefore, among potential benefits of biofuels are included energy security, economic stability and environmental gains as the better use of wastes, which implies the opening of new industrial dimensions (NIGAM; SINGH, 2011). Accordingly, starchy and cellulose rich residues hydrolysis for ethanol production is a very promising technology that can become extensively adopted at large scale (CINELLI et al., 2015; YE et al., 2018). However, considering the challenges of food-fuel competition, land use

change, and improvement of technology efficiency, this chapter discusses possible alternatives as sustainable bioethanol sources strategically important in the foreseeable future.

## 2 BIOFUELS CLASSIFICATION

Biofuels are the most environment friendly source of energy among strategically important sustainable fuels (NIGAM; SINGH, 2011). In addition, they have emerged as an alternative to meet new energy demands, stimulate agricultural growth and provide better economic conditions in agro-energy (ECKERT et al., 2017). Biofuels are classified as primary and secondary. The primary biofuels are used in an unprocessed form (e.g. fuelwood, wood chips, and pellets), and the secondary biofuels are produced by processing of biomass (e.g. ethanol, biodiesel, and bio-oil) (NIGAM; SINGH, 2011). The secondary biofuels are divided in to first (1G), second (2G) and third (3G) generation according to the feedstock and technology used for their production (NIGAM; SINGH, 2011). Therefore, proper understanding of the energy matrix and the main aspects and characteristics of each resource requires knowledge to adjust and adapt the resource for its better use in the energy industry (ECKERT et al., 2017).

First-generation liquid biofuels are produced from sugars, grains or seeds and are obtained from relatively simple process to get the finished fuel product. Ethanol is the most well-known first-generation biofuel, produced by fermenting sugar extracted from sugary raw materials and starch rich plants (ALTHURI; GUJJALA; BANERJEE, 2017; NIGAM; SINGH, 2011). Sugar cane, sugar beet, sweet sorghum, saccharin, molasses and fruit are among the group of raw materials (ECKERT et al., 2017). For molasses-based ethanol production, sugarcane juice is clarified, concentrated, and crystallized up to obtain sugar and molasses with about 50% of fermentable material, which is fermented by yeasts to obtain high-yield ethanol. Differently, in direct juice-ethanol, the ethanol production from sugarcane juice is processed in autonomous distilleries, which implies the production of only hydrated ethanol at 96% (GARCÍA; MANZINI; ISLAS, 2017). The raw starch is the most common for the production of fuel ethanol in temperate regions (Europe, North America, Central Asia), which undergoes an enzymatic hydrolysis process to be converted to glucose. Alpha-amylase and glucoamylase are the two enzymes usually employed for starch liquefaction and saccharification, respectively, for subsequent ethanol fermentation (ECKERT et al., 2017).

Second-generation biofuels can be classified according to the process used to convert biomass to fuel, biochemicals (e.g. ethanol and butanol) or thermochemical (e.g. methanol and dimethyl ether) (NIGAM; SINGH, 2011). Generally 2G biofuels are produced from lignocellulosic biomass as the energy crops, grasses, agricultural crop residues, forest remains and industrial wastes are foreseen as the prospective raw materials for commercial second generation (2G) ethanol production (ALTHURI; GUJJALA; BANERJEE, 2017). In agreement, feedstock can be bred specifically for energy purposes, enabling higher production per unit land area, and an increase land use efficiency compared to first generation biofuels (NIGAM; SINGH, 2011). The main advantage of producing 2G biofuels is related to the cost-effectiveness of the raw materials and because they are essentially inedible by humans, compared to those used in 1G. In addition, lignocellulosic biomass does not directly interfere with human food reserves, which limits direct competition between food and fuel (ALTHURI; GUJJALA; BANERJEE, 2017; NIGAM; SINGH, 2011). On the other hand, when compared with 1G ethanol production, 2G ethanol production is still not economically feasible, mainly due low ethanol concentration and yield, once processes are not mature enough, requiring a high investment in advanced technologies and facilities to aid the conversion process (JAMBO et al., 2016; YE et al., 2018). Moreover, starch is much easier than cellulose to convert to glucose. (NIGAM; SINGH, 2011).

Third-generation biofuels using algae (microalgae and macroalgae) as a sustainable feedstock whose biomass can be converted directly into energy has directed the searches and gained worldwide attention (JAMBO et al., 2016). Microalgae can produce lipids, proteins and carbohydrates in large amounts over short periods of time, considering that the proportion of each component vary in different species (JAMBO et al., 2016; NIGAM; SINGH, 2011). Thus, the main 3G advantage is the high growth rate, which is a way to make the production more commercially viable. In this case, the land usage for cultivation can be seawater, freshwater and wastewater, besides the positive environment impact contributing to mitigate $CO_2$ (JAMBO et al., 2016). Currently, macroalgae (seaweeds) are reported one as of the best candidates to be employed as the bioethanol feedstock. However, limited investments and difficulties in process design are still a challenge (JAMBO et al., 2016).

## 3 FEEDSTOCKS HYDROLYSIS

Except for sugar-rich raw materials (sugar cane, sugar beet, sweet sorghum and fruits) used for some first-generation bioethanol production, the feedstocks need to be processed in

different ways to release fermentable sugar for a microorganism biotransformation. The choice for each treatment depends on the composition and consequently, recalcitrance of the substrates. Since the first-generation bioethanol from starch (corn, wheat, rice, potato, cassava, sweet potato and barley), second-generation bioethanol from lignocellulosic biomass such as wood, straw and grasses, and third-generation bioethanol derived from algal biomass (AZHAR et al., 2017; NIGAM; SINGH, 2011), hydrolytic treatments are needed.

Although sugarcane is the most economical raw material for ethanol production in the world (BAEYENS et al., 2015), its cost represents 66.7% of the ethanol fuel producing cost (ESCARAMBONI et al., 2018). So, the use of raw materials with a low cost of obtaining or without cost, as is the case of wastes, is important to enable a jump in the world production of ethanol from alternative sources. However, the optimization of the production technology is fundamental so that the processes employed are not more expensive than the acquisition and processing of traditional raw materials.

In this way, acid and enzymatic hydrolysis are the primarily methods employed for biomass hydrolysis with varying efficiencies depending on treatment conditions, type of biomass and the properties of the hydrolytic agents. Acid hydrolysis is a traditional processing with disadvantages as the generation of hazardous acid wastes and difficulties in recovering sugar, however, the enzymatic method is more efficient and proceeds under ambient conditions without generation of toxic waste, which has immense potentials for improvement in cost and efficiency (SUKUMARAN et al., 2009). In order to obtain a wide commercialization of bioethanol from alternative feedstocks, the reduction of the production costs is need, which depends on the pretreatment technologies needed, cost of enzymes as well as the convenient enzymatic hydrolysis (NAIR; PADMAJA; MOORTHY, 2011; ZHAO et al., 2018). In this sense, cellulose, starch and hemicellulose hydrolysis, requires commercial enzymes that are very costly and this is an important limitation in the use of this feedstocks (CRIPWELL et al., 2015).

For starch hydrolysis, an important issue is that a complex group of amylolytic enzymes is needed to convert the polysaccharides into glucose, with action of different enzymes, such as endoamylases, exoamylases and debranching enzymes (CINELLI et al., 2015). Generally, the starch hydrolysis processes use two amylolytic enzymes, α-amylase and glucoamylase, in hydrolysis reactions that normally exceed 24 hours (BIAŁAS; SZYMANOWSKA; GRAJEK, 2010; CINELLI et al., 2015). Amylases market is well established, since has several industrial and biotechnological applications (KUMAR; SAHAI; BISARIA, 2012). There is a large interest in amylases with improved properties on starch degradation in order to reduce the cost

of starch derivatives. In this way, both use of agro-industrial wastes as substrate for an economical amylases production by microorganism, as in the case of the increasing solid-state fermentation, and the utilization of amylase with high catalytic efficiency without expensive downstream steps, as crude enzymatic extracts, can contribute to reduce the cost of starch ethanol (ESCARAMBONI et al., 2018; FERNÁNDEZ NUÑEZ et al., 2016).

Enzymatic hydrolysis of the lignocellulosic feedstocks is more complex than with starch and requires a pretreatment to disrupt the complex structure involving hemicellulose, lignin and cellulose to make it more accessible to enzymes reactions (BRIENZO et al., 2016; PENGILLY et al., 2015). Cellulases (endoglucanases, cellobiohydrolases and β-glucosidases) and hemicellulases (endo-1,4-β-D-xylanases and 1,4-β-D-xylosidases) are enzymes able to hydrolyze lignocellulosic biomass by synergic action (BUSSAMRA; FREITAS; COSTA, 2015). However, the cost from enzyme consumption to produce lignocellulosic ethanol was reported as 6.8 times higher than for the production of corn ethanol (NEWMAN et al., 2013). This is the reason why the commercialization of the lignocellulosic biomass ethanol is hindered (SUKUMARAN et al., 2009). Thereby, the reduction of the cellulases costs need to be achieved in terms of the enzyme production aspects, from the raw material used to microbial strain improvement. The use of cheaper raw materials and cost effective fermentation strategies like solid state fermentation can improve the economics of cellulase production (SUKUMARAN et al., 2009). Enzymatic hydrolysis optimization for specific feedstock and pretreatment conditions is a key to obtain efficient sugar and ethanol yields, but depending on the severity of the pretreatment, the solubilized sugars can be further degraded into furans that impact negatively on the subsequent biological transformations (PENGILLY et al., 2015).

Using agricultural and agro-industrial residues to produce second generation bioethanol (2G) is advantageous because there is no problem of competition with the food chain. However, 2G bioethanol process is challenge due to limitations associated with: lignocellulosic biomass properties, including the slow rate of enzymatic hydrolysis and high enzyme cost, what significantly impact the economic viability (CRIPWELL et al., 2015; NEWMAN et al., 2013). Optimization of the hydrolysis through enzymatic cocktails is able to improve the saccharification of the biomass in several applications since they use adequate proportions enzymes for different substrates (BUSSAMRA; FREITAS; COSTA, 2015), and can be an important alternative, especially for hydrolysis of the feedstocks which are still potential for the production of ethanol, since they are constituted by a mixture of carbohydrates, usually lignocellulosic and starch portions. The decrease in the cost of the technology for biofuels production can be reached by the decrease in the amount of enzymes used increasing the

catalytic activity for example using a synergistic enzymatic action by different classes of enzymes in complex substrates and this way has been much explored in scientific terms. In addition, the bioprocess feasibility depends on getting higher product content, with lower possible expenses, in a shorter time possible. In this way, the enzymatic hydrolysis of different raw materials will be economically viable when lower the enzyme dosage mainly in the case of cellulases which represents the greatest influence on bioethanol cost from substrates with lignocellulosic compounds (CRIPWELL et al., 2015; ZHAO et al., 2018).

# 4 ALTERNATIVE FEEDSTOCKS

## 4.1 Cassava bagasse

Bioethanol can be produced from cassava (*Manihot esculenta*) bagasse, which is mainly discarded as waste, although it is a highly efficient source of fermentation sugars. World cassava production increased 57.6% from 2000 to 2016, while the cassava area harvested increased 38.5% in the same period (FAOSTAT, 2019), which means that cultivation techniques and varieties have been improved. Nigeria is the largest producer worldwide, with 57.1 million tons in 2016 (FAOSTAT, 2019), additionally this root crop constitutes an important source of food in Sub-Saharan Africa, so much that 29 million tons of the annual Nigerian production are demanded as food (ANYANWU et al., 2015). Cassava production across the African continent represents 56.8% of world production, followed by Asia with 32.2% and the Americas with 10.9% (FAOSTAT, 2019). The Brazilian production of cassava was the third largest in 2016 with 21.1 million tons (FAOSTAT, 2019). Cassava shows the highest yield of carbohydrates per hectare after sugarcane and sugar beet (*Beta spp*.), and one of the best water-footprints especially on relatively low fertility soils, in drought conditions, requiring low agrochemical input and adapts well in all ecological zones (OKUDOH et al., 2014). Therefore, cassava has gained prominence as biofuel feedstock (ANYANWU et al., 2015), since it accounts for around 35% of China's total bioethanol production capacity (BAEYENS et al., 2015). The ethanol production by two strains of *Saccharomyces cerevisiae* using enzymatically hydrolyzed cassava starch was reported by an Nigerian study (AJIBOLA; EDEMA; OYEWOLE, 2012). In addition, cassava was reported as a higher annual yield of bioethanol when compared with other crop including sugarcane, sweet sorghum, and maize (OKUDOH et al., 2014).

However, cassava processing in starchy industry can generate up to 90% of wet waste (~15% dry basis) called cassava bagasse, which is rich in carbohydrate (JIANG et al., 2018;

PANDEY et al., 2000). In addition, the large amounts of cassava waste are justified by the great production of roots worldwide. Currently, most of the cassava bagasse is discarded as waste, however, this residue can be considered as an excellent source of fermentation for higher value biochemicals, contributing to the development of countries and supply of food and other compounds to the world (ESCARAMBONI et al., 2018). Furthermore, cassava bagasse consists of approximately 82.6% carbohydrates, with 56-60% starch, 15-18% cellulose and 4-5% hemicellulose (NAIR; PADMAJA; MOORTHY, 2011; VERSINO; LÓPEZ; GARCÍA, 2015). These carbohydrates can be hydrolyzed to produce fermentable sugars, for which must have a thermal hydrolytic treatment with acid or enzyme (WOICIECHOWSKI et al., 2002). In agreement, this waste was successfully evaluated as an economic alternative in the production of glucose and ethanol (ESCARAMBONI et al., 2018), n-butanol (LU et al., 2012), succinic acid (SHI et al., 2014), fatty acid, and neutral lipid (CHEN et al., 2015) demonstrating its fermentability.

Different studies seek to prove the use of this waste as feedstock to produce fermentable sugars. The hydrolysis product is generally referred to as cassava bagasse hydrolysate (CBH). Recently, Escaramboni et al. (ESCARAMBONI et al., 2018) proposed a scaled hydrolysis of cassava bagasse in 3-L bioreactor to obtain 42.82% conversion of the residue dry mass into reducing sugars in 10 h of reaction using only the crude amylolytic extract produced by *Rhizopus oligosporus* at the proportion of 15 U/g dry cassava bagasse. From the initial mass 54.38% remained as solid waste which can be used for cogeneration electricity or other purposes. The applicability of the method proposed in the cited work was supported by the absence of enzymatic extract downstream steps, such as purification and concentration, and process robustness with a single step enzymatic hydrolysis. Subsequently the concentrated CBH was used in fed batch process to produce 89.2% ethanol yield (bases on theoretical ethanol).

Shi et al. (SHI et al., 2014) achieved 45.7% yield of total sugars from the starch and cellulose portions of the cassava bagasse after a two-step enzymatic hydrolysis. They used three commercial enzymes to obtain the conversion reported: 15 U/g α-amylase, 200 U/g glucoamylase, and cellulase totalizing 28 h of reaction. Differently, a cassava bagasse hydrolysis yield of 16.2% was obtained in 4.5 h of reaction using the steps of gelatinization, liquefaction with α-amylase and saccharification with glucoamylase, and yield of 33% after a total of 52.5 h when associated with cellulase (CHEN et al., 2015). Therefore, it is remarkable that hydrolysis yields may be different for each region since the starch content in the substrate can vary, and according to the process applied.

It is known that cassava crop produces cyanogenic glucosides, which are stored in plant vacuoles (GUPTA; BALOMAJUMDER; AGARWAL, 2010) to be used as a defense mechanism against predators (BRADBURY; CLIFF; DENTON, 2011). Some cyanide may remain in the cassava bagasse in small proportions (GLANPRACHA; ANNACHHATRE, 2016). Nevertheless, thermal treatment can remove cyanides from industrial wastes (GUPTA; BALOMAJUMDER; AGARWAL, 2010). Thus, the fermentability without microbial toxic effects of the CBH proved in the works presented, can be explained the temperature of the enzymatic reaction at the rate of 55 °C. Since the steps of CBH heating and evaporating can eliminate the possible presence of cyanide by volatilization (ESCARAMBONI et al., 2018).

The use of cassava bagasse for the production of ethanol can reduce its cost and add value to the cassava processing industry, as well as reduce environmental pollution caused by bagasse disposal (LU et al., 2012), without food competition. Considering that in the tapioca starch industry cassava root accounts to 83% of the costs (CHAVALPARIT; ONGWANDEE, 2009), the whole use of the raw material received by the industry, including generated residues, is important for the industrial economy balance. Comparing the cost sugarcane and cassava bagasse as raw materials, the cassava waste was reported as a lower-cost feedstock for ethanol fermentation. Furthermore, the use of only the Brazilian cassava bagasse amount in 2014 as feedstock for ethanol production was reported as possible to increase 3% the ethanol production in Brazil, the second largest ethanol producer in the world (ESCARAMBONI et al., 2018).

## 4.2 Food waste

About 1.3 billion tons of food, one-third of food produced in the world for human consumption, are wasted every year, according to a study from the Food and Agriculture Organization of the United Nations (FAO, 2019). In addition, high rates of waste are noted for specific food groups, such as 30% for cereals and 40-50% for roots, fruits and vegetables. The amount of waste and food wasted corresponds to approximately US$ 680 billion in industrialized countries and US$ 310 billion in developing countries, since beyond food losses itself, this represents a waste of resources used in production such as land, water, energy and inputs (FAO, 2019). Such waste can be considered valuable if there are adequate biotechnological strategies so that the value of subsequent products exceeds the cost of reprocessing. Municipal organic waste may be a different and alternative source of raw material for the production of biofuels, especially household waste. This waste represents a challenge regarding disposal and can cause severe environmental issues. In addition, there is a tendency

to increase the quantities of this waste from residences, industries, restaurants, buffet services and wholesale, which, according to the European Commission, will increase from 89.3 million tonnes in 2006 to 126.2 million tonnes by 2020 (MATSAKAS et al., 2014).

Food wastes are rich in carbohydrates (both soluble and insoluble), which represent more than 50% of the total solids, so they are good substrates for the production of ethanol (KIM; LEE; PAK, 2011; MATSAKAS et al., 2014). Unlike the cultivation of food (e.g. maize, sugar cane) for the production of biofuels, the use of food waste to produce these bioenergy products does not compete with foods grown for human consumption and therefore the demand for land is much lower (KIBLER et al., 2018). Whereas the remaining food is a complex biomass containing various components that can be used as a suitable feedstock for bioenergy production, such as starch (55–65%), proteins (15–20%), lipid fats (10–15%) and cellulosic materials (5–10%), a treatment to hydrolyze the carbohydrates is necessary for its conversion in fermentable sugars for producing bioethanol (MA; CAI; LIU, 2017). In this context, hydrolytic enzymes can reduced the viscosity of the high solid content substrate and act on the structure of starch and cellulose to release glucose, which can subsequently be fermented by yeast (KIM; LEE; PAK, 2011; MATSAKAS et al., 2014).

Kim et al. (KIM; LEE; PAK, 2011) demonstrated the possibility for the production of bioethanol from food waste in lab-scale reactor. A previous enzymatic hydrolysis using commercial enzymes to produce 0.63 g glucose/g total solid by the action of carbohydrase was applied. The adequate effect of this enzyme class is expected, assuming that carbohydrates are major food waste component. With the sugar produced they achieved higher ethanol yield (0.43 g/g total solids) through *Saccharomyces cerevisiae* using separated hydrolysis and fermentation (SHF) than in the simultaneous process. In agreement, SHF was adequate for ethanol production by waste bread with two-stage hydrolysis using commercial α-amylase, glucoamylase and protease for later *Saccharomyces cerevisiae* fermentation (PIETRZAK; KAWA-RYGIELSKA, 2014). Enzymatic hydrolysis using amylase and pectinase enzymes and fermentation process was applied on pie waste at 20% solids to produce 0.33 g/g of waste in dry basis (MAGYAR et al., 2017). Ma et al. (MA; CAI; LIU, 2017) proposed an integrated system to apply food waste for producing a fungal enzyme mixture, needed for enzymatic hydrolysis resulting in 67.5% of solid reduction, and bioethanol from *Zymomonas mobilis*. Authors also reported other studies in which the ethanol production (g/g glucose) from *Saccharomyces cerevisiae*, *Pichia stipilis* and *Zymomonas mobilis* varied between 0.31 and 0.51. Finally, remaining solids containing the unhydrolyzed fraction can be hydrothermally treated for further fermentation and increase of the overall ethanol production yield

(MATSAKAS et al., 2014). Moreover, if assumed 20% of dry basis in the global food waste and the conversion factor to ethanol into 0.43 g/g of dried residue, is possible to obtain 127.5 billion litters ethanol each year, only from food waste, which means 1.4% increase in world ethanol production in 2017.

### 4.3 Agave

Agave is a succulent genus native to arid and semi-arid regions from the southern USA to northern South America that can be economically used as feedstock in the biorefinery context without food competition, once this crop requires minimal amounts of water and the input required for cultivation is minimum (DÍAZ-BLANCO et al., 2018; ESCAMILLA-TREVIÑO, 2012). Although the composition varies according to species, agave cell wall composition is mainly cellulose (65-84%) and hemicelluloses (5.3-10.3%), that can be hydrolyzed for further ethanol fermentation, and lignin (7.4-16%). Furthermore, agave composition is positive for processability since the cellulose content is higher and the lignin content is lower in relation to other crops (ESCAMILLA-TREVIÑO, 2012). For comparison, the cellulose and lignin content in sugarcane bagasse is 37.5 and 29.7%, respectively (YE et al., 2018). Ethanol production from *Agave lechuguilla* was reported using dilute sulfuric acid pretreatment followed by enzymatic hydrolysis with commercial cellulases to obtain sugars subsequently fermented by *Escherichia coli* (DÍAZ-BLANCO et al., 2018). *Agave tequilana* bagasse also can be used for ethanol production, considering 360 thousand tons in dry bases produced in 2010, based on the production of tequila (RIOS-GONZÁLEZ et al., 2017). Rios-González et al. (RIOS-GONZÁLEZ et al., 2017) pretreated the bagasse with autohydrolysis to be hydrolysated with a commercial cellulase cocktail and fermentated by *Saccharomyces cerevisiae* to obtain ethanol (65.2 g/L, 8.26% v/v). Ethanosolv was also used as pretreatment for subsequent enzymatic hydrolysis with commercial cellulase and beta-glucosidade applied in the proportions of 15 FPU/g and 30 CBU/g of solids, respectively. Then, fermentable sugar content was used by *Saccharomyces cerevisiae* to produce the ethanol yield of 0.25 g/g of dry agave bagasse (CASPETA et al., 2014). Although further research is still needed concerning biomass conversion and bioprocess engineering in this topic, it is possible to assume a positive outlook for an economically competitive agave biofuel industry (ESCAMILLA-TREVIÑO, 2012). Regarding the use of agave bagasse from the tequila industry, there is a prospective production of about 50 million liters of ethanol fuel, plus the potential solution for disposal problems (CASPETA et al., 2014).

## 4.4 Others

Sweet potato can be an alternative feedstock to produce bioethanol. Zhang et al. (ZHANG et al., 2013) obtained 0.14 g of ethanol per gram of fresh sweet potato by *Zymomonas mobilis*. In China, sweet potato has been used for bioethanol fermentation, since the country produces about 85% of the total sweet potato in the world (ZHANG et al., 2013). In Brazil, a pilot scale plant produced satisfactory results with the ethanol yield of 161.4 L/ton applying enzymatic hydrolysis and then fermentation with *Saccharomyces cerevisiae* (VIRGÍNIO E SILVA et al., 2017). These authors reported the ethanol production of 10,598 L/ha as higher than sugarcane (46%) and corn (149%).

The free-floating aquatic plants water hyacinth and water lettuce were indicated by Mishima et al. (MISHIMA et al., 2008) as candidate energy crops for their use in ethanol production, due to advantages as growing without land competition with grains and vegetables, and to solve environmental issues as water purification and extract heavy metals. The carbohydrate polymer composition in raw aquatic plants was higher than 40%, and for these substrates simultaneous saccharification and fermentation mode (SSF) was better than SHF. In addition, the ethanol yield (0.14-0.17 g/g biomass) produced by *Saccharomyces cerevisiae* or *Escherichia coli* (with *Zymomonas mobilis* genes) was comparable to other agricultural biomasses (e.g. sugarcane bagasse).

Sivaramakrishnan and Incharoensakdi (SIVARAMAKRISHNAN; INCHAROENSAKDI, 2018) proposed the *Scenedesmus* sp. as feedstock for the production of biodiesel and bioethanol by an integrated process, since this microalga is able to accumulate both lipid and starch. Acid treatment was used to hydrolyze the carbohydrate (22%) from biomass, followed by *Saccharomyces cerevisiae* fermentation 0.08 g of ethanol per gram of initial biomass.

The co-fermentation using a starch-rich and a lignocellulosic material was appointed to increase ethanol concentration and become a competitive form for the commercialization of cellulosic ethanol (YE et al., 2018). New biotechnologies that could be specifically applied on each continent, especially in poor countries, could serve the new large supplies of the world bioenergy demands, which would also be very valuable for the continent's progress.

## 5 CONCLUSIONS

Considering that more than 80% of ethanol production worldwide provide from American continent (USA and Brazil), the proposition of alternative feedstocks in accordance with each region, the climate and main crops and industries around the world, can increase socioeconomics conditions and solve several environmental concerns. Moreover, the possibility of incorporating new raw materials in the bioenergy sector for the production of biofuels, especially ethanol, requires the development of economic and efficient strategies that allow the expansion of scale. Alternative feedstock as urban, agricultural and agro-industrial wastes can be efficiently and economically converted in biofuels, but more researches are need to include these products in the biofuel industry.

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# CHAPTER 3 – Ethanol biosynthesis by fast hydrolysis of cassava bagasse using fungal amylases produced in optimized conditions

ESCARAMBONI, B. et al. Ethanol biosynthesis by fast hydrolysis of cassava bagasse using fungal amylases produced in optimized conditions. **Industrial Crops and Products**, v. 112, p. 368–377, 2018.

## ABSTRACT

The search for a renewable platform to produce high-value biochemicals and energy that are environmentally correct has been a current concern. A fast and inexpensive bioprocess for amylase production, able to hydrolyze complex residues in fermentable sugars to be used for ethanol production was developed. High titer amylase from *Rhizopus oligosporus* in solid state fermentation (SSF) was obtained by optimizing the medium supplementation using agro-industrial waste as substrate. Statistical experimental design and partial least square (PLS) regression were used to establish a relation between added chemical compounds and enzyme production, showing that urea was the most important nutrient. Crude amylase extract had competitive performance features giving higher productivities in starch hydrolysis than a commercial glucoamylase. The amylase produced was applied in the proportion of 15 U/g dry cassava bagasse to obtain cassava bagasse hydrolysate (CBH). More than 42% conversion in reducing sugars was achieved with an efficient 10 h single-step hydrolysis at 55°C in a bioreactor. The concentrated CBH was subsequently used in fed batch process producing 89.2% ethanol yield. Furthermore, comparing just the cost of the raw materials sugarcane and CHB, the latter demonstrated to be a lower-cost feedstock for ethanol fermentation.

**Keywords:** Amylase. *Rhizopus oligosporus*. Solid state fermentation. Cassava bagasse hydrolysate. Glucose. Ethanol production

# 1 INTRODUCTION

Bio-ethanol can be produced from various renewable feedstock such as sugarcane (*Saccharum spp.*), corn (*Zea mays*), wheat (*Triticum spp.*), cassava (*Manihot spp.*), and cellulose biomass (BAEYENS et al., 2015). In addition, starchy residues hydrolysis for ethanol production is a very promising technology that can become extensively adopted at large scale (CINELLI et al., 2015). In the US, world's largest ethanol producer (USDA, 2015), corn starch has been used as source of biofuel for decades, with the use of corn grain balanced for food and feed, and ethanol production (MUMM et al., 2014).

Cassava production has been increasing annually in developing countries like Nigeria, the largest producer worldwide (FAOSTAT, 2016), and for these countries is an important source of food (ANYANWU et al., 2015). This crop constitutes a staple food in Sub-Saharan Africa, so much that 29 million tons of the annual Nigerian production are demanded as food (ANYANWU et al., 2015). Cassava shows the highest yield of carbohydrates per hectare after sugarcane and sugar beet (*Beta spp.*), and one of the best water-footprints especially on relatively low fertility soils, in drought conditions, requiring low agrochemical input and adapts well in all ecological zones (OKUDOH et al., 2014). Advances in the use of wastes from cassava industry can contribute to the development of these countries and supply of food and other compounds to the world. Between 2000 and 2013, Africa continent was the largest cassava producer with about 54% of the production, followed by Asian with 31% and Americas with 15%. The Brazilian production of cassava was the fourth largest in 2014 with 23.36 million tons (FAOSTAT, 2016), justifying the large amounts of cassava processing waste in the country.

For each 250–300 tons of cassava root processed, about 280 tons of bagasse with 85% moisture is produced (PANDEY et al., 2000). Cassava bagasse is used as feed and has also been successfully assessed in the production of interest biomolecules demonstrating its fermentability as *n*-butanol (LU et al., 2012), succinic acid (SHI et al., 2014), fatty acid, and neutral lipid (CHEN et al., 2015). Currently, the majority of the cassava bagasse is discarded as waste, however, this residue can be considered as an excellent source of fermentation for higher-value biochemicals.

Currently, the starch hydrolysis processes employ two classes of enzymes, α-amylase, and glucoamylase, at hydrolysis times that typically exceed 24 hours (BIAŁAS; SZYMANOWSKA; GRAJEK, 2010; CINELLI et al., 2015). Amylases include an important enzyme class with many industrial and biotechnological applications, representing 25% of the

enzymes market (KUMAR; SAHAI; BISARIA, 2012). They belong to the class of hydrolases and catalyze the hydrolysis of starch and its derivatives, releasing various products, including progressively smaller dextrins and glucose polymers (GUPTA et al., 2003).

There is a wide range of applications for the hydrolysis of starch and ethanol production, such as in the food, detergent, paper, textiles, baking, chemical, and pharmaceutical industries. (BAEYENS et al., 2015; CASTRO et al., 2010; GUPTA et al., 2003). Due to increasing demand, there is a huge interest in the discovery of enzymes with improved properties on starch degradation, as well as developing techniques that reduce the cost of production of amylolytic derivatives. Potential of fungi, such as *Aspergillus* and *Rhizopus* among others, are known to produce enzymes including amylases (VAIDYA et al., 2015). Fungi are especially valued as excellent producers of enzymes of industrial interest. *Rhizopus* has been highlighted for being a good producer of amylases and its ability to metabolize complex mixtures of organic compounds in most organic residues (FREITAS et al., 2014; JIN et al., 2002; PEIXOTO-NOGUEIRA et al., 2008). In addition, *Rhizopus oligosporus* is considered safe, which is traditionally used in the food industry (BOURDICHON et al., 2012).

Solid-state fermentation (SSF) has gained attention over the past 20 years in the development of industrial bioprocesses, due to several advantages over the conventional method of submerged fermentation, such as the particular physiology presented by fungi in this mode of cultivation and particularly due to the lower energy requirement, higher product yields, and less wastewater production with lesser risk of contamination (BARRIOS-GONZÁLEZ, 2012; THOMAS; LARROCHE; PANDEY, 2013).

The search for economical bioprocesses for the production of enzymes able to hydrolyze complex carbohydrates, such as the combination of starch and lignocellulosic compounds, has been a current concern. This fact is due to the need for better use of waste as a renewable platform to produce chemicals, food, and biofuels, considering that oil and its derivatives are non-renewable, and expansion of agricultural land is limited. In this context, the objective of this study was to develop a rapid and low cost bioprocess using natural, robust, and safe microorganism for the production of enzymes able to use agro-industrial residues hydrolysis to produce fermentable sugars which can be used for ethanol production or another bioprocess.

# 2 MATERIALS AND METHODS

## 2.1 Microorganism and inoculum preparation

*Rhizopus oligosporus* (CCT 3762) was obtained from Fundação Tropical de Pesquisas e Tecnologia "André Tosello", Campinas, SP, Brazil. The fungal strain was grown on potato dextrose agar (PDA) (Difco, USA) for 7 days at 30°C. Viable spores were harvested from the culture by washing with 0.01% (v/v) Tween 80 water solution and the spore suspension was used as inoculum for SSF.

## 2.2 Solid state fermentation (SSF)

Tests for SSF were conducted in 250 mL Erlenmeyer flasks containing 10 g of wheat bran (Moinho Nacional, Assis, SP, Brazil). To determine the effect of nutrient supplementation on amylase production was carried out in a preliminary experiment to define the concentration range to be used in the central composite design (CCD). The standard solution comprised: 9.3% w/w $(NH_4)_2SO_4$; 4.75% w/w $KH_2PO_4$; 2.3% w/w urea (SOCCOL et al., 1994)) was compared with trials without supplementation and intermediate concentrations. The substrates were prepared with 60% moisture content, sterilized at 121°C for 20 min, inoculated with $1.10^6$ spores per gram of dry substrate and incubated at 30±0.5°C (Fanem Ltda Mod.002 CB, São Paulo, Brazil) for 120 h (ESCARAMBONI et al., 2018; ESCARAMBONI; OLIVA NETO, 2016). The amylase was extracted from the fermented substrates with 5 mL/g distilled water at 30°C on an orbital shaker (Tecnal TE-421, São Paulo State, Brazil) at 180 rpm for 30 min. The enzymatic extract was separated by filtration and used for amylase activity assay.

## 2.3 Central composite design (CCD)

Response surface methodology (RSM) was used to determine the mutual interactions among the selected variables and their corresponding optimum concentrations for maximizing the amylase production. Three variables ($X_1$: ammonium sulfate; $X_2$: monobasic potassium phosphate; and $X_3$: urea) were analyzed. A CCD having five coded levels (-1.68, -1, 0, +1, +1.68), eight points associated to $2^3$ factorial design, six axial points and three replicates at the center point with a total number of 17 runs was formulated. The experimental range and the levels of each variable are summarized in Table 1.

**Table 1 -** Experimental range and levels (including star points*) of each factor (nutrient concentration) in the CCD.

| Nutrient[a] concentration | Coded levels | | | | |
|---|---|---|---|---|---|
| (%, w/w) | -1.68* | -1 | 0 | 1 | 1.68* |
| $X_1$: ammonium sulfate | 0 | 0.5 | 1.25 | 2 | 2.5 |
| $X_2$: potassium phosphate | 0 | 0.25 | 0.63 | 1 | 1.25 |
| $X_3$: urea | 0 | 0.25 | 0.63 | 1 | 1.25 |

[a]Ammonium sulfate – $(NH_4)_2SO_4$ Pure for analysis -American Chemical Society (P.A.-A.C.S.) (Catalog number: 01S1051.01.AH, Synth, Brazil); Monobasic potassium phosphate – $KH_2PO_4$ P.A. (Catalog number: 01F2002.01.AG, Synth, Brazil); Urea P.A. (Catalog number: 01U1001.01.AH, Synth, Brazil).

The second-order polynomial model equation was fit for response variables, *y* (amylase activity, U/g), was given below:

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where *y* is the predicted response, $\beta_0$ is the offset term, $\beta_i$ the linear coefficients, $\beta_{ii}$ the quadratic coefficients and $\beta ij$ the interaction effect. The optimum levels of variables for maximal amylase production were obtained by analysis the response surface and contour plots.

## 2.4 Statistical analysis

The statistical software STATISTICA 8.0 trial version software (Statsoft Inc.) was used to define CCD experimental design as well as analyze statistically the derived data and fit polynomial model for response variable under consideration (amylase activity). Means multiple comparisons in other experiments were performed by ANOVA and subsequently Tukey test (if necessary) in BioEstat 5.0 free software (Mamirauá Institute, Brazil).

## 2.5 Analytical methods

Amylase activity was determined using 0.5% (w/v) soluble starch (Sigma-Aldrich, USA) as substrate in 0.05 M sodium acetate buffer, pH 5.5 as a described method previously (BERNFELD, 1955) with some modifications. The reducing sugar concentration was estimated

by the 3,5-dinitrosalicylic acid (DNS) method (MILLER, 1959). The reaction mixture containing 100 µL of crude enzyme and 650 µL of substrate was incubated for 10 min at 60±0.1°C (Marconi MA 127, Piracicaba, SP, Brazil). The reaction was stopped by adding 500 µL of DNS solution followed by heating it in a boiling water bath for 5 min, and cooling and diluting it with 3.75 mL of distilled water. The concentration of reducing sugar was measured at 540 nm using glucose as standard. One unit of amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugars per minute and expressed as units per gram of dry substrate (U/g). Detailed steps and equation performed to measure amylase activity were previously described (BARCHI et al., 2016).

## 2.6 PLS data analysis

The PLS technique was used to define correlation levels between the chemical elements added in the fermentation medium, represented by a matrix X and the amylase activity, representing the response variable matrix Y. The independent variables matrix (X) included concentrations of total nitrogen (TN), organic nitrogen (ON), inorganic nitrogen (IN), total phosphorus (P), total potassium (K), and total sulfur (S), which were calculated based on different concentrations of nutrients used in experimental design to optimize the chemical composition of salt solution for wetting wheat bran. The organic nitrogen contribution provided by wheat bran was calculated considering that it contains 14% protein, according to the manufacturer's information (Moinho Nacional, Assis, SP, Brazil). The PLS modeling was done in SIMCA 14 demo version software (Umetrics, Umeå, Sweden).

## 2.7 Enzymatic hydrolysis

Soluble starch was mixed and gelatinized with 0.05 M sodium acetate buffer (pH 5.5) to obtain a 5% (w/v) concentration and amylolytic crude extract from *R. oligosporus* (1.5 U/mL starch solution) was added to the mixture and incubated at 55 or 60±0.1°C (Marconi MA 127, Piracicaba, SP, Brazil). In another experiment, glucoamylase NS22035 from the Novozymes' cellulosic ethanol enzyme kit was reacted with 5% (w/v) soluble starch in 0.05 M sodium acetate buffer (pH 5.5) at 60°C (1.5 U/mL starch solution). In the hydrolysis for obtaining cassava bagasse hydrolysate (CBH), the amylolytic crude extract from *R. oligosporus* obtained by solid fermentation was reacted with 1.5 L of 10% (w/v) dried cassava bagasse in 0.05 M sodium acetate buffer (pH 5.5) at 55±0.1°C (1.5 U/mL starch solution) 3-L bioreactor (TEC-

BIO-Plus, Tecnal, Piracicaba, SP, Brazil) with intermittent agitation in every 30 minutes. The cassava bagasse was previously gelatinized at 80ºC per 30 min. To study the kinetics of reducing sugar production, samples were taken at appropriate time intervals for 12 h. The yield and productivity of glucose were calculated as previously detailed (FREITAS et al., 2014).

### 2.8 Fermentation

For ethanol submerged fermentation, CBH was concentrated at 10.8% (w/v) of reducing sugars and supplemented with following composition of chemicals (Labsynth, Diadema SP, Brazil) (% w/v): 0.5% yeast extract, 0.1% ammonium sulfate, 0.114% $K_2HPO_4.3H_2O$, 0.024% $MgSO_4.7H_2O$, 0.0028% $ZnSO_4.7H_2O$ and 0.0017% $MnSO_4.H_2O$, pH 5.0 and sterilized at 121°C for 15 min (DORTA et al., 2006). The microorganism was a commercial strain of *Saccharomyces cerevisiae* (Fleischmann®, Brazil). To start the fermentation 9 g (dry biomass) of yeasts was suspended in 75 mL distilled water in 500 mL Erlenmeyer flasks. The fed batch process was carried out by adding 15 mL of the must (semi-synthetic media with CBH) at 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 h of fermentation. The final volume of the fermentative cycle was 225 mL. The flasks were shaken at 80 rpm on an orbital shaker (Tecnal-TE421, São Paulo State, Brazil) at 32±0.1°C for 12 h. The yeasts were centrifuged (Excelsa-206 BL, Brazil) after the end of the fermentative cycle at 3000 rpm for 20 min. The concentration of ethanol was determined using distiller and digital densimeter (Anton Paar-DMA 4500 ME) technique. Ethanol yield was calculated using 0.6475 as the w/v conversion factor of consumed sugar to ethanol based on the theoretical maximum yield.

## 3 RESULTS AND DISCUSSION

### 3.1 Optimization of the medium composition for amylase production in SSF

High fermentation cost limits the application of bioprocesses (HUANG et al., 2013) and the potential of wastes for industrialization. About 30–40% of the production cost of the industrial enzymes is due to the growth medium (JOO; CHANG, 2005). The development of an economically viable bioprocess seeks to maximum enzyme production with the lowest substrate cost. The use of agro-industrial residues as a substrate and minimum nutrients supplementation leads to a low cost bioprocess.

The amylase produced from SSF process depended on the level of salts concentration used. The intermediate level determined in this work showed better performances. The preliminary experiment to determine the optimal range of nutrient concentrations to be used in CCD indicated the differences in amylase production among standard solution (treatment 1) and other solutions under consideration (Table 2). The highest amylase production was 367 U/g (treatment 3) for intermediate values of concentration of salts $(NH_4)_2SO_4$, $KH_2PO_4$, and urea. This represents a 100% increase in enzyme activity compared with the tests using only wheat bran (180 U/g) as substrate (treatment 2) and those supplemented with the maximum concentration of salt (181 U/g) from the standard solution (treatment 1). As the treatments 3, 4, and 5 were statistically equal in enzyme activity, the treatment with the lowest concentration of nutrients (4) was selected to define the positive axial point (1.68) for each nutrient concentration in CCD (Table 1).

The experimental design and results are represented in Table 3. The analysis of variance (ANOVA) for the variables indicated that enzyme activity can be well described by a polynomial model with a high coefficient of determination ($r^2 = 0.9571$). This result showed an excellent correlation between independent variables and amylase activity and only 4.29% of the total variations could not be explained by the model. The statistical significance of the regression model was supported by the high F-value (29.01) with very low *p*-value (< 0.0001).

**Table 2 -** Comparison of different chemical compositions of solution for wetting solid substrate on amylase production by *Rhizopus oligosporus* in solid state fermentation.

| Runs | Variables (% w/w) | | | Amylase activity (U/g) |
|---|---|---|---|---|
| | $(NH_4)_2SO_4$ | $KH_2PO_4$ | Urea | |
| 1 – Standard | 9.3 | 4.8 | 2.3 | 180.90 ± 10.07 a[a] |
| 2 | 0.0 | 0.0 | 0.0 | 179.94 ± 11.81 a |
| 3 | 5.0 | 2.5 | 2.5 | 367.61 ± 9.68 b |
| 4 | 2.5 | 1.25 | 1.25 | 353.92 ± 4.26 b |
| 5 | 2.5 | 1.25 | 2.5 | 361.86 ± 2.71 b |

[a]Means followed by the same letter indicate no significant differences ($p > 0.05$).

The significance of regression coefficients, in the equation for amylases production, was defined considering significance level of 90% (α=0.1). As a result, a reduced quadratic model (equation (2)) in terms of coded factors was fitted (ESCARAMBONI; OLIVA NETO, 2016):

$$y = 327.42 + 37.41X_3 - 10.25X_1^2 - 11.61X_2^2 - 13.70X_3^2 - 9.89X_2X_3 \quad (2)$$

where *y* represents amylases activity (U/g), and $X_1$, $X_2$, and $X_3$ were the coded values of the test variables ammonium sulfate, potassium phosphate, and urea concentrations, respectively. The statistical analysis of the model showed that each variable had a significant effect on amylase synthesis: linear term ($X_3$), all the quadratic terms ($X_1^2$, $X_2^2$ and $X_3^2$) ($p<0.05$) and the interaction term ($X_2X_3$) ($p<0.1$).

The amylase enzyme synthesized by *R. oligosporus* ranged from 227.7 U/g to 361.6 U/g in the experimental design. This variation reflects the need of medium optimization to obtain higher yields. In order to gain a better understanding of the effects of the significant factors on amylase production, the three dimensional response surface, and contour graphs were generated to show the interaction of each pair of medium composition, which provided an interpretation of the optimum experimental conditions, according to data previous published by the author (ESCARAMBONI; OLIVA NETO, 2016).

There is a strong influence of urea concentration on the response since the lowest enzymatic activity was obtained without this nutrient, and higher activities were produced with the highest urea concentration (1.25%). Therefore, the axial points of urea are the responses of maximum and minimum activity. These results are in accordance with other reports in which urea, as medium supplementation, was the best nitrogen source for the α-amylase production by *Rhizopus microsporus* on SSF using cassava peel (VIJAYARAGHAVAN; REMYA; VINCENT, 2011) and glucoamylase by *Aspergillus* sp. on SSF with wheat bran (ELLAIAH et al., 2002). In the last studies cited, the authors evaluated the effect of different nitrogen sources including urea, at a fixed concentration (1%), while in the present work was presented a design to model and optimize the concentration of urea for SSF using *R. oligosporus* in which 1.25% (w/w) urea was the appropriate level.

**Table 3 -** Experimental design for CCD with observed and predicted amylase activity for defining nutrient supplementation influence on amylolytic enzyme production by *Rhizopus oligosporus* (CCT 3762) using wheat bran as substrate.

| Runs | Independent variables | | | Amylase activity (U/g) | | |
|---|---|---|---|---|---|---|
| | $(NH_4)_2SO_4$ | $KH_2PO_4$ | Urea | Experimental | Predicted | Absolut error[a] |
| 1 | 0.5 | 0.25 | 0.25 | 244.42 | 244.56 | -0.14 |
| 2 | 2 | 0.25 | 0.25 | 240.58 | 264.34 | -23.76 |
| 3 | 0.5 | 1 | 0.25 | 258.38 | 244.56 | 13.82 |
| 4 | 2 | 1 | 0.25 | 264.67 | 264.34 | 0.33 |
| 5 | 0.5 | 0.25 | 1 | 328.19 | 339.16 | -10.97 |
| 6 | 2 | 0.25 | 1 | 339.14 | 319.38 | 19.76 |
| 7 | 0.5 | 1 | 1 | 312.31 | 339.16 | -26.85 |
| 8 | 2 | 1 | 1 | 313.95 | 319.38 | -5.43 |
| 9 | 0 | 0.63 | 0.63 | 287.94 | 298.49 | -10.55 |
| 10 | 2.5 | 0.63 | 0.63 | 320.80 | 298.49 | 22.30 |
| 11 | 1.25 | 0 | 0.63 | 318.88 | 294.65 | 24.23 |
| 12 | 1.25 | 1.25 | 0.63 | 282.19 | 294.65 | -12.46 |
| 13 | 1.25 | 0.63 | 0 | 227.72 | 225.90 | 1.81 |
| 14 | 1.25 | 0.63 | 1.25 | 361.59 | 351.60 | 9.98 |
| 15 | 1.25 | 0.63 | 0.63 | 323.81 | 327.42 | -3.61 |
| 16 | 1.25 | 0.63 | 0.63 | 324.90 | 327.42 | -2.52 |
| 17 | 1.25 | 0.63 | 0.63 | 331.47 | 327.42 | 4.05 |

[a]Difference between experimental and predicted values by the mathematical model. Uncertainty of amylase measurement was calculated in 2%.

The results showed the concentrations of 1.25% and 0.63% (center point conditions) for ammonium sulfate and monobasic potassium phosphate, respectively, as the best for the production of amylolytic enzymes. In addition, higher levels of potassium phosphate decreased amylase production. The observed decrease in amylase production when a high level of potassium phosphate was used match with previously described. Literature reports show that the production of enzymes may be limited by the high phosphorus concentration in the culture medium, such as the case of the manganese-dependent peroxidase from *Phanerochaete chrysosporium* in a medium of submerged culture (LIANG; GAO; ZENG, 2012). Accordingly, a high phosphate concentration was responsible for inhibition of amylases activity of *B. amyloliquefaciens* (GUPTA et al., 2003).

### 3.2 Validation of optimal chemical composition of nutrient solution

Maximum amylase activity (361.6 U/g) was observed in experimental treatment 14 (0, 0, +1.68) with medium containing in w/w: 1.25% $(NH_4)_2SO_4$, 0.63% $KH_2PO_4$, and 1.25% urea. The adequacy of the model was examined by an additional experiment using the derived optimal conditions. The higher predicted response was 351.6 U/g and the experimental response was 346.8 ± 4 U/g. This revealed more than 98% of the predicted value, which indicated that the generated model gave an adequate prediction. The statistical optimization for amylase production was effective in predicting the fermentation profiles as a function of different salts content with a high coefficient of determination.

As the amylase activity increased with lower concentrations of potassium phosphate, and the bioprocesses requires an economical medium with the lowest concentrations of nutrients, an experiment was conducted comparing two solutions with the following concentrations of ammonium sulfate, potassium phosphate, and urea: (1) 1.25%, 0.63%, and 1.25% – coded values: (0, 0, +1.68) and (2) 1.25%, 0.25%, and 1.25% – coded values: (0, -1, +1.68), in four replications.

The activity of 386.36 ± 4.79 was obtained on the test with the salt solution 1 and 393.00 ± 3.47 with the salt solution 2. The analysis of variance showed there was no difference between the averages of salt solutions ($p$> 0.01), so the salt solution 2 with lower potassium phosphate was selected as standard. This experiment showed that is possible to obtain a large amount of amylase using a concentration range between 0.25 and 0.63% of potassium phosphate, giving versatility to the process.

The combination of 9.3% (w/w) $(NH_4)_2SO_4$, 4.8% $KH_2PO_4$, and 2.3% urea was reported as a nutrient solution to produce 108 U/g glucoamylase using *R. oryzae* 28627 in SSF and raw cassava as substrate (SOCCOL et al., 1994). In this study, reduction in these nutrients' concentration of 86.6, 94.8, and 45.7%, respectively, doubles the enzyme activity of an extract produced by SSF with wheat bran. This fact demonstrates an inhibitory effect on the glucoamylase synthesis by *R. oligosporus* when relatively high concentrations of these salts were used, probably due to the increase in osmotic balance between the fungal cells and culture medium.

The amylase activities of crude aqueous extracts were significantly higher than the values from solid state fermentations (PEIXOTO-NOGUEIRA et al., 2008; VIJAYARAGHAVAN; REMYA; VINCENT, 2011) and submerged fermentations (FREITAS et al., 2014; HASHEMI et al., 2015) using agro-industrial wastes as substrate. These results

demonstrate that the processes proposed in this work were superior to others published using bacteria or fungi as a biological agent.

### 3.3 PLS data analysis

Although partial least square (PLS) regression technique is one of the most published algorithms for multivariate calibration in different fields of knowledge, this is probably the first study which utilizes PLS to establish a relation between chemical elements in culture medium and amylase production in solid state fermentation by fungi. The choice was based on the suitability of using PLS as a tool to analyze multivariate datasets together with multiple targets (PROCOPIO et al., 2013).

Two main components were required to satisfactorily perform modeling with a correlation coefficient of 0.89. Due to the natural variety of biological systems, models for protozoa and fungi allow approximate predictions ($0.8124 < r < 0.8944$) so that is considered suitable when the overall correlation coefficient is in the range of 0.89 (REEVES; MCCARTY; MEISINGER, 2000; ZORNOZA et al., 2008).

The amylase activity was directly influenced by the organic nitrogen content added to the SSF medium as represented in Figure 1. The increased production of amylase shows a positive correlation with the highest amount of organic nitrogen added to the medium as urea, corresponding to the trial of number 14 from experimental design (Table 3).

The statistical importance of independent variables in the model in relation to dependent variables is corroborated by the variable importance in the projection (VIP) values. The effect analysis of each nutrient added in the formulation of culture medium for *R. oligosporus* showed that inorganic nitrogen, phosphorus, and sulfur were indifferent to the expression of amylase by the fungus. Whereas the organic nitrogen content positively influences the production of this enzyme. The amount of total nitrogen was also favorable since in this experiment the sum of the amounts of organic and inorganic nitrogen was regarded (Figure 2).

**Figure 1 -** Biplot graph for Nutrients-Amylase activity PLS model. Definition of model terms. *AA*: amylase activity; *TN*: total nitrogen; *ON*: organic nitrogen; *IN*: inorganic nitrogen; *P*: total phosphorus; *K*: total potassium; *S*: total sulfur. Numbers represent samples included in CCD.

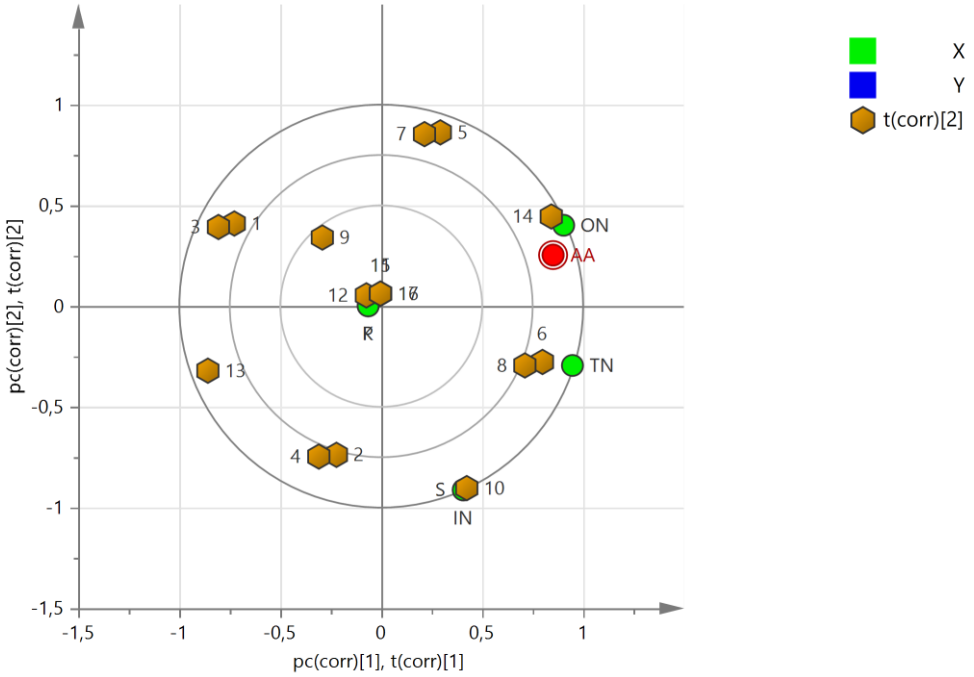
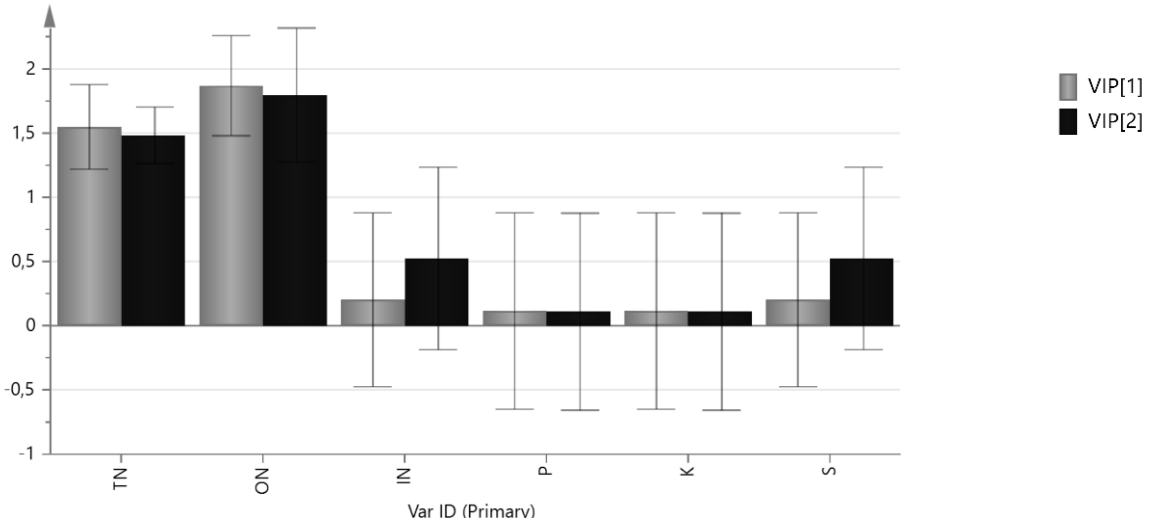



**Figure 2 -** Variable importance in the projection (VIP) values for Nutrients-Amylase activity PLS model in both principal components. Definition of model terms *TN*: total nitrogen; *ON*: organic nitrogen; *IN*: inorganic nitrogen; *P*: total phosphorus; *K*: total potassium; *S*: total sulfur.



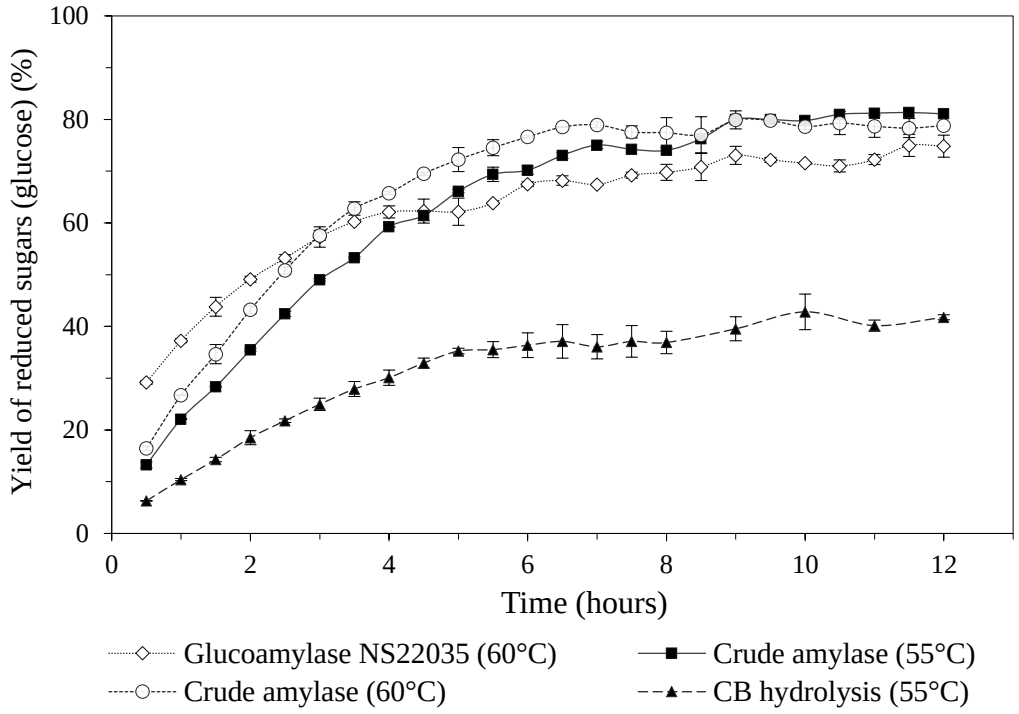
In the present work, urea showed stronger influence, followed by $(NH_4)_2SO_4$ in this enzyme expression. Different nitrogen sources lead to higher amylase expression (KAMMOUN; NAILI; BEJAR, 2008), according to the results obtained. This supports the use of these nitrogen sources to the formulation of SSF culture medium in this work, mainly urea, as mentioned in PLS data analysis, which is an organic nitrogen source.

### 3.4 Enzymatic hydrolysis and glucose production

Hydrolysis profile with crude amylolytic extract from *R. oligosporus* was similar in both tested temperatures. A gradual increase in conversion yield of substrates assessed for reducing sugars up to 7 h was observed, reaching 75.1 and 79.0% of pure starch conversion for hydrolysis at 55 to 60°C, respectively. After 11 h of reaction, the yield was 81.1% at 55°C and remained constant (78.8%) at 60°C. In comparison, only in the first 2.5 h, the hydrolysis profile by commercial glucoamylase at 60°C was higher than amylase from the studied fungi, evaluated at the same temperature. After this period, the commercial enzyme had lower yields than those of the enzyme of this study ($p<0.01$), with a maximum yield of 75.0% after 11 h (Figure 3). In addition, glucose productivity in hydrolysis using different protocols were compared and described in Figure 4. The amylases from *R. oligosporus* showed higher productivities than commercial amylases. This fact suggests the amylases in this study are competitive with commercial amylases. The productivity of commercial glucoamylase (6.2 g $L^{-1}$ $h^{-1}$) was lower than amylase of *R. oligosporus*, which was 6.5 and 7.1 g $L^{-1}$ $h^{-1}$ at 55 and 60°C, respectively, in 6 h of reaction.

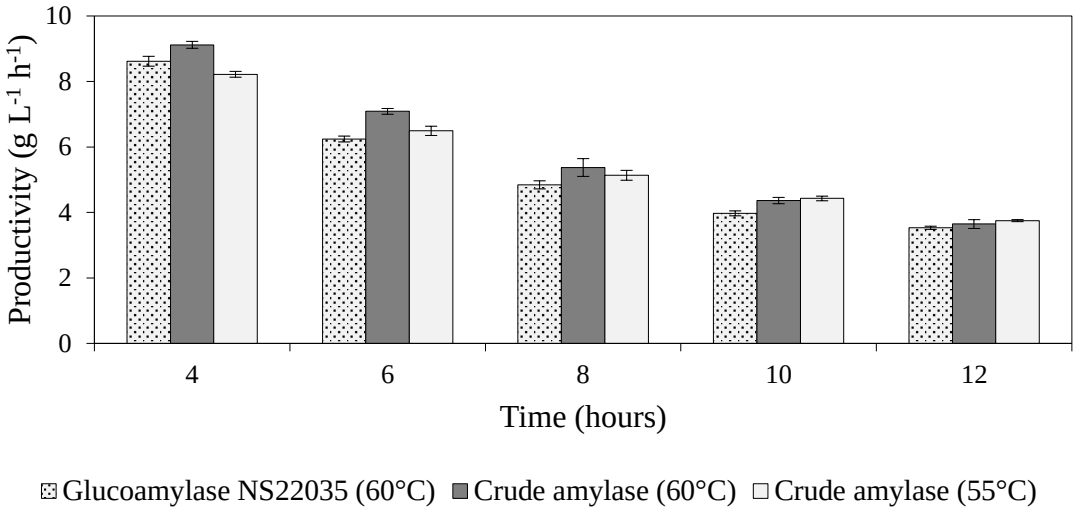
The efficiency of releasing sugars by mixing α-amylase and glucoamylase is known to be much higher than that using only one enzyme (CHEN et al., 2015). *Rhizopus* species are also known to produce α-amylase and glucoamylase (HAN et al., 2003; PEIXOTO-NOGUEIRA et al., 2008; SOCCOL et al., 1994; VIJAYARAGHAVAN; REMYA; VINCENT, 2011). The *R. oligosporus* strain used in this work produces glucoamylase (DEL RE et al., 2003). Freitas et al. (2014) obtained just glucose as a final product during starch hydrolysis with glucoamylase produced by the same microrganism. In accordance, *R. oligosporus* has been reported as good glucoamylase producer (FREITAS et al., 2014; JIN et al., 1999, 2002), which was purified and characterized (KAREEM et al., 2011, 2014).

**Figure 3 -** Starch hydrolysis using commercial and produced amylases. 10% (w/v) cassava bagasse (CB) hydrolysis using crude enzyme extract from *Rhizopus oligosporus* (▲). 5% (w/v) soluble starch hydrolysis: a comparison of crude enzyme extract from *Rhizopus oligosporus* at 55°C (■), 60°C (○) and the commercial glucoamylase NS22035 60°C (◊). The *error bars* represent the standard deviations (n=3).




The application of *R. oligosporus* CCT 3762 amylase from submerged fermentation for the production of glucose from starch due to the high yield and product quality was demonstrated (FREITAS et al., 2014). The authors obtained the productivity of 9.7 g $L^{-1}$ $h^{-1}$ in 4 h of hydrolysis, similar to the one obtained in this work (9.1 g $L^{-1}$ $h^{-1}$) with the same reaction time but with amylase from solid fermentation. In addition, this enzyme showed catalytic performance competitive with commercial glucoamylase. Therefore, the use of amylolytic extract from this study suggests a simple and widely applicable method for the hydrolysis of starch residues such as bagasse cassava. Additionally, the costs of starch-based ethanol production can be reduced if starch hydrolysis is performed at temperatures below its gelatinization temperature (CINELLI et al., 2015). Therefore, in order to reduce energy demand, the temperature selected for 10% cassava bagasse hydrolysis was 55°C since the pure starch conversion was greater than 80% using the *R. oligosporus* amylase at this temperature.

**Figure 4 -** Glucose productivity of starch hydrolysis: a comparison of crude enzyme extract from *Rhizopus oligosporus* at 55°C or 60°C and the commercial glucoamylase NS22035 at 60°C. The *error bars* represent the standard deviations (n=3).




The crude amylolytic extract was used for the cassava bagasse hydrolysis and obtaining CBH. After 6.5 h of reaction, 37% of the dry mass of the residue was converted into reducing sugar, reaching 42.82% after 10 h of reaction. Furthermore, after hydrolysis, 54.38% of the initial mass remained as solid waste from cassava bagasse. Hydrolysis yields may be different for each region since the starch content in the substrate can vary. Cassava bagasse contains about 30-50% of dry weight basis due to the processing techniques and crop varieties (PANDEY et al., 2000). In spite of this, this work proposes a novel method for scaled hydrolysis of cassava bagasse in 3-L bioreactor, with which it is possible to obtain a yield of 42.82% of reducing sugars in 10 h of reaction using only the crude extract of *R. oligosporus* in the proportion of 15 U/g dry cassava bagasse.

The crude enzymes from *R. oligosporus* produced in the present work reached a rapid hydrolysis of cassava bagasse showing high starch conversion efficiency in glucose. The applicability of this method is supported by the absence of the need for investment in downstream steps, such as purification and concentration, and process robustness.

Among about 3000 species of plants, animals, microbes that contain cyanide, cassava is a food crop which produces cyanogenic glucosides that are stored in plant vacuoles (GUPTA; BALOMAJUMDER; AGARWAL, 2010). Plants use cyanide as a defense mechanism to deter predators (BRADBURY; CLIFF; DENTON, 2011). High cyanide content is found in wastes as cassava peels (OGBO, 2010) and wastewater (manipueira) (MARÓSTICA JR.; PASTORE,

2007). Despite the cassava bagasse be considered free cyanide (PANDEY et al., 2000) it is known that some cyanide may still remain in small proportions (GLANPRACHA; ANNACHHATRE, 2016). Cyanides can be removed from industrial wastes by biodegradation, physical, and chemical methods, for example, evaporation, thermal treatment (GUPTA; BALOMAJUMDER; AGARWAL, 2010), and wetting (BRADBURY; CLIFF; DENTON, 2011). The bioprocess proposed assures recovery and fermentability of a cassava residue even with a presence of residual cyanide. The steps of heating and evaporating the broth can eliminate by volatilization the possible presence of cyanide.

The cassava bagasse hydrolysis was studied with three enzymes, α-amylase (15 U/g), glucoamylase (200 U/g), and cellulase, totalizing 28 h of reaction to achieve 45.7% of total sugars coming from the starch and cellulose (SHI et al., 2014). In addition, a hydrolysis yield of only 16.2% expressed in percentage of reducing sugars released was obtained in 4.5 h of reaction using the steps of gelatinization, liquefaction with α-amylase and saccharification with glucoamylase, and yield of 33% when associated with cellulase in a total of 52.5 h (CHEN et al., 2015). According to these results and considering that just one crude extract of amylases from *R. oligosporus* was used in this work, we can conclude these amylases of this strain is very efficient in starch residues hydrolysis.

### 3.5 Ethanol production

After cassava bagasse hydrolysis, CBH was concentrated to 10.8% (w/v) reducing sugars to be used for ethanol fermentation. Fermented must (wine) showed ethanol concentration of 3.95 ± 0.03% (v/v) obtained after 12 h of fermentation using *Saccharomyces cerevisiae* at 32°C. Ethanol efficiency (calculated based on consumed sugars and theoretical yield) were 89.20 ± 0.68%. In this sense, this bioprocess represents a potential production of 247.32 L ethanol per ton of cassava bagasse on a dry basis.

The ethanol efficiency under consideration was significantly higher than the values derived from 72 h of simultaneous saccharification and fermentation process using corn and cassava flour, recently published (BIAŁAS; SZYMANOWSKA; GRAJEK, 2010; NGUYEN; LE; CHU-KY, 2014). In addition, several studies have been reported on ethanol production by starch hydrolysis of raw material from cassava and corn (CINELLI et al., 2015). They obtained a conversion efficiency of 84.6 to 89.6% in more than 48 h. In the present study, there was more than 89% of the ethanol efficiency using a fast method of cassava bagasse hydrolysis in just 12 h fermentation, and a total period of 24 h for cassava bagasse hydrolysis and alcoholic

fermentation. The amylolytic extract optimized in this work enables the rapid hydrolysis of starch with higher productivity than commercial glucoamylase, and conversion of complex wastes to fermentable sugars which can be used for an efficient production of ethanol.

Considering spending on raw materials, Table 4 shows the costs of producing ethanol from cassava bagasse hydrolyzed in comparison with the sugarcane. The sugarcane price (US$ 20.51/ ton) was calculate according to UNICA data (UNICA, 2015), and for cassava bagasse, the price considered in the present work was R$ 100.00 or almost US$ 31.25/ton (dry basis), estimated in accordance with the traded value of the producer's milk in the Paranapanema Valley region (São Paulo State, Brazil). The value of this by-product in dry basis could be cheaper (US$ 9.38/ton) if were not increased by the transportation value (DBO, 2017). Considering just the cost of feedstock, ethanol from cassava bagasse (US$ 0.13/L) is two times cheaper than ethanol from sugarcane (US$ 0.26/L). In studies using only commercial amylolytic enzymes for the cassava bagasse hydrolysis, the cost of ethanol, based on the raw material price and conversion yield, was similar or higher than ethanol from sugarcane. This confirms the superiority of the treatment with amylase produced by *R. oligosporus* compared to previous studies. Similar values to the present study were reported only in treatments with commercial cellulase, α-amylase and glucoamylase (Table 4).

Cassava root represents 83% of the costs in the tapioca starch industry (CHAVALPARIT; ONGWANDEE, 2009). Therefore, the total use of this biomass received by the industry, including generated residues, is important for the industrial economy balance. In addition, the use of this agro-industrial waste as a carbon source for another process helps to solve the pollution problems. Each ton of cassava contain 211.2 Kg of native starch and after industrial process 115.5–132.0 Kg (CHAVALPARIT; ONGWANDEE, 2009) or 150.0 Kg (PANDEY et al., 2000) is generated as solid waste. In addition, if considered the cassava bagasse generated in Brazil in 2014 (about 3.5 million tons, dry basis), it would have the potential to produce 866.6 million liters of additional ethanol, corresponding an increase of 3.04% of total ethanol production in Brazil during the same period, which was 28.48 billion liters, according to UNICA (UNICA, 2014).

**Table 4 -** Comparison of ethanol production costs from cassava bagasse (CB - dry basis) and sugarcane considering spending on raw materials.

| Source | Hydrolysis treatment [a] | Sugar[b] Kg/ton | Ethanol[c] L/ton | Source price US$/ton | Ethanol cost US$/L | Reference |
|---|---|---|---|---|---|---|
| Cassava bagasse | Fungal amylase (crude extract) | 428.20 | 247.32 | 31.25 | 0.13 | This study |
| Cassava bagasse | α-amylase + glucoamylase | 162 | 93.57 | 31.25[d] | 0.33[e] | (CHEN et al., 2015) |
| Cassava bagasse | Cellulase + α-amylase + glucoamylase | 330 | 190.59 | 31.25[d] | 0.16[e] | (CHEN et al., 2015) |
| Cassava bagasse | Cellulase + α-amylase + glucoamylase | 457 | 263.94 | 31.25[d] | 0.12[e] | (SHI et al., 2014) |
| Cassava bagasse | Glucoamylase | 206 | 118.99 | 31.25[d] | 0.26[e] | (RATTANACHOMSRI et al., 2009) |
| Sugarcane | - | 136.58 | 79.59 | 20.51 | 0.26 | (UNICA, 2015) |

[a]Amylase produced by *R. oligosporus* in optimized conditions for this study and commercial enzymes for the other references.

[b]Calculated as the total reducing sugar for CB and total recoverable sugar for sugarcane.

[c]Calculated based on the conversion of sugar into ethanol using the coefficient 0.6475 with 89.2% yield for CB and 90% yield for sugarcane.

[d]The same price of CB used in this study was adopted for the calculation with reference data.

[e]Calculated in this study

Sugarcane is the most economical feedstock for ethanol production in the world (BAEYENS et al., 2015). The ethanol price was US$ 0.39/L in the 2014/2015 harvest in Brazil (UNICA, 2015) and also achieved similar prices in the United States (US$ 0.37-0.45/L – New York and Chicago market) in 2015 (ANP, 2016). This value indicates that cost of feedstock accounts for 66.7% of the production cost of fuel ethanol. Cassava bagasse is a by-product of the agroindustry with low added value. Therefore, the use of this biomass as raw material to produce ethanol can be an economic alternative (Table 4). In addition, cassava was previously reported as a higher annual yield of bioethanol when compared with any other crop including sugarcane, sweet sorghum, and maize (OKUDOH et al., 2014). Additionally, the processing of sugarcane results in 30% of bagasse with 50% moisture, which is used in the generation of electricity (YADAV; SOLOMON, 2006). In the present work, after the hydrolysis of cassava bagasse, the amount of the initial mass remained as solid waste (54,38%), which can be used for electricity or other purposes, was a higher than sugarcane residue.

## 4 CONCLUSIONS

This study reported a fast and economical bioprocess for the optimized production of amylases from a natural and safe microorganism using wheat bran supplemented with 1.25 (% w/w) $(NH_4)_2SO_4$, 0.25 (% w/w) $KH_2PO_4$, and 1.25 (% w/w) urea. The amylolytic complex containing glucoamylase presented competitive performance based on commercial glucoamylase. This crude extract was able to hydrolyze cassava bagasse to fermentable sugars in a fast and efficient hydrolysis. A cassava bagasse hydrolyzed with high glucose content was obtained and was used as only carbon source for more economical ethanol production than sugarcane ethanol. This work proposes a new alternative for the low cost ethanol production intercropped with food production, without any competition between them. Developing countries could work in this direction in order that, besides the starch and their derivatives to meet the demand in the food sector, the solid residue could be used to improve the fuel ethanol production, contributing to supply the demand for renewable fuels in the world.

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# CHAPTER 4 – Improved cassava bagasse enzymatic biocatalysis for alternative low-cost feedstock and bioethanol production

## ABSTRACT

A fast and cost-effective bioprocess able to hydrolyze cassava bagasse (CB) in glucose as an alternative feedstock for bioethanol production was developed. Enzymes were provided from both commercial source (cellulase) and selected microorganism cultivation (amylase by solid state fermentation). Three-level factorial design was performed to optimize enzymatic proportion from 0-30 U/g f substrate in dry basis. Optimized enzyme mixture efficiently converted 61.67% CB into glucose. An appropriated synergistic mixture was responsible for decreasing the proportion of amylases 2 times (15 U/g) and cellulases 3 times (5 FPU/g), converting 51.79% of the CB. Scanning electron microscopy image evidenced modifications on CB surface caused by the enzymatic action. Hydrolysis of 15% CB in bioreactor released great level of glucose content (90.87 g/L; $Y_{P/S}$ = 0.50 g/g). In addition, the hydrolysate was applied in fed-batch fermentation producing 35.25 g/L ethanol (78.3% efficiency). Therefore, proposed bioprocess would produce 254.1 L of ethanol per ton of dry CB in 48 h, including the hydrolysis and alcoholic fermentation period. This approach of using enzymes synergistic action with simplified and efficient strategies was possible and, finally, opens new economic, ecological and social perspectives to glucose and bioethanol production.

**Keywords:** Enzymatic hydrolysis; Cassava bagasse; Glucose; Ethanol; *Saccharomyces cerevisiae*.

# 1 INTRODUCTION

The environmental concern through the years motivates the worldwide search for new friendly, safer and economical ways to produce chemicals, food and biofuels. As an alternative, the bioethanol offers an environmentally correct and renewable solution. Bioethanol can be produced from various feedstock, including cassava (*Manihot esculenta*) bagasse, which is most discarded as waste, even though is a highly efficient source of fermentation sugars. Cassava is an important source of food in developing countries, such as Nigeria (ANYANWU et al., 2015), which is the largest producer worldwide. Cassava production across the African continent represents 56.8% of world production, followed by Asia with 32.2% and the Americas with 10.9% (FAOSTAT, 2019).

Currently, cassava accounts for around 35% of China's total bioethanol production capacity (BAEYENS et al., 2015) and around 440000 ha of cassava are cultivated annually which yields about 9110000 tons of roots, most of which are used to produce starch. In addition, the large amounts of cassava waste in Brazil are justified by the great production of roots in the country, being the third largest producer in 2016 (ESCARAMBONI et al., 2018; FAOSTAT, 2019).

Development of industrial manufacture on starch raised the production of cassava continuously in the last two decades (FAOSTAT, 2019). However, cassava processing can generate up to 90% of wet waste (humidity 85%), which is rich in carbohydrate (JIANG et al., 2018; PANDEY et al., 2000). Cassava bagasse has been reported as a powerful source to produce industrial interesting biomolecules, but there are limits to its use due to low protein concentration. Cassava bagasse consists of approximately 82.6% carbohydrates, being 56-60% starch, 15-18% cellulose and 4-5% hemicellulose (NAIR; PADMAJA; MOORTHY, 2011; VERSINO; LÓPEZ; GARCÍA, 2015). These carbohydrates can be hydrolyzed to produce fermentable sugars, for which it must it must be undergone to a thermal hydrolytic treatment with acid or enzyme (WOICIECHOWSKI et al., 2002).

Acid hydrolysis is a traditional processing with the disadvantages of generation of hazardous acid wastes and difficulties in recovering sugar, however, the enzymatic method is more efficient and proceeds under ambient conditions without generation of toxic waste, which has immense potentials for improvement in cost and efficiency (SUKUMARAN et al., 2009). The best way to optimize an enzymatic hydrolysis is through enzymatic cocktails, which in proportions suitable for different substrates are capable of improving the saccharification of the biomass in various applications (BUSSAMRA; FREITAS; COSTA, 2015), including for the

hydrolysis of cassava bagasse carbohydrates, due to this substrate contain a mixture of LCM (lignocellulose) and non-extracted starch.

The use of cassava bagasse for the production of ethanol can reduce its cost and add value to the cassava processing industry, as well as reduce environmental pollution caused by bagasse disposal (LU et al., 2012). Using agricultural residues to produce second generation bioethanol (2G) is advantageous because there is no problem of competition with the food chain. However, 2G bioethanol process is challenge due to limitations associated with lignocellulosic biomass properties, including the slow rate of enzymatic hydrolysis and high enzyme cost, what significantly impact the economic viability (CRIPWELL et al., 2015; NEWMAN et al., 2013). In order to obtain a wide commercialization, the reduction of the production costs is needed, which depends on the pre-treatment technologies needed, cost of enzymes as well as the convenient enzymatic hydrolysis (NAIR; PADMAJA; MOORTHY, 2011; ZHAO et al., 2018). Cellulose, starch and hemicellulose hydrolysis requires commercial enzymes that are very costly and not feedstock specific (CRIPWELL et al., 2015). An important issue is that a complex group of amylolytic enzymes is needed to convert starch polysaccharides into glucose, with action of different enzymes, such as endoamylases, exoamylases and debranching enzymes (CINELLI et al., 2015).

Reducing the amount of enzymes used by the increase in its catalytic activity, for example using a synergistic enzymatic action by different classes of enzymes in complex substrates, can reduce the cost of the technology for biofuels production and this subject has been much explored in scientific terms. In this sense, fungi are recognized as excellent producers of enzymes of commercial interest, such as *Aspergillus* and *Rhizopus*, that can produce enzymes such as amylases (VAIDYA et al., 2015). Known for being safe and traditionally used in the food industry, *Rhizopus* can be distinguished as a good producer of amylase, and able to metabolize complex mixtures formed by organic residues (FREITAS et al., 2014).

Microbial enzymes are obtained through fermentation processes and the choice of solid-state fermentation (SSF) has been increasing as an important alternative process to the conventional method of submerged fermentation, particularly due to the lower energy requirement, higher product yields and less wastewater production with lesser risk of contamination, and its economic value is more attractive (BARRIOS-GONZÁLEZ, 2012; THOMAS; LARROCHE; PANDEY, 2013). SSF systems show promise in the production of fungal enzymes due to the microbial growth occurring in conditions closer to the natural habitat (SOUZA; MAGALHÃES, 2010). Although there is still limitation in the scale SSF production

of enzymes, a strong advantage of this process is the application of these catalysts in the composition of enzymatic cocktails for the hydrolysis of waste materials, since the high cost of the enzymes have a direct impact on the economic viability of the process.

In this present study a fast and cost-effective bioprocess of cassava bagasse hydrolysis treatment for glucose production by a mixture of amylases and cellulases action, from both commercial source and microorganism cultivation, followed by fermentation to ethanol production, was developed. An amylolytic extract was produced by SSF in a tray system. The crude amylases secreted was applied with a cellulase complex in a three-level factorial design to improve enzymatic treatment of CB hydrolysis and the production of fermentable sugars. Once the optimization of the enzyme parameters was achieved, a high amount of hydrolyzed CB was produced in a pilot reactor and used for ethanol fermentation test. New biotechnologies that could be applied on the African continent, especially in poor countries, could serve the new large supplies of the world food and bioenergy demands, which would also be very valuable for the continent's progress.

## 2 MATERIALS AND METHODS

### 2.1 Amylase production by *Rhizopus oligosporus* in a tray system solid state fermentation (SSF)

The microorganism *Rhizopus oligosporus* (CCT 3762) (Fundação Tropical de Pesquisa e Tecnologia "André Tosello", Campinas - SP, Brazil) was grown on potato dextrose agar (PDA) (Difco, USA) for seven days at 30 °C. Viable spores were harvested from the culture by washing the biomass with a sterile solution of 0.01% (v/v) Tween 80.

The SSF were carried out in circular glass trays with dimensions of 10.5 cm radius and 3 cm height (1.04 L), containing 70 g of the substrate on a dry basis, to scale-up at about 7 times the production of amylase by SSF in Erlenmeyer flasks. The substrate for SSF was wheat bran (Moinho Nacional, Assis, SP, Brazil) adjusted to 55% initial moisture content with the nutrient solution optimized by Escaramboni et al. (ESCARAMBONI et al., 2018) consisting of (% w/w): 1.25% $(NH_4)_2SO_4$ P.A (Biotec, Brazil), 0.25% $KH_2PO_4$ P.A (Impex, Brazil) and 1.25% (w/w) urea P.A (Impex, Brazil). SSF medium was sterilized at 121 °C for 20 min, inoculated with $1.10^6$ spores per gram of dry substrate and incubated at 30°C for 120 h. The tray was wrapped by an autoclavable laboratory bag in which the opening was sealed in a nozzle containing a hydrophobic cotton plug to simulate the Erlenmeyer system.

Crude amylase was extracted by adding 10 mL/g of distilled water at 28 °C on an orbital shaker (Tecnal TE-421, Piracicaba, SP Brazil) at 180 rpm for 30 min. The enzymatic extract was filtered and the amylolytic activity was determined. One unit was One unit was defined as the amount of enzyme that releases 1 μmol of reducing sugar per minute, estimated by the 3,5-dinitrosalicylic acid (DNS) method according to Miller (MILLER, 1959).

## 2.2 Viscosity reduction

A preliminary test to determine CB viscosity reduction was performed. The hydrolysis was performed in a reactor of 400 mL work volume with 10% CB (m/v) in water, gelatinized and adjusted to 50 °C. Cellulase complex (NS-22086, Novozymes) plus β-glucosidase (NS22118, Novozymes) in the ratio of 10 FPU/g dry mass CB was used. For the synergistic effect of the cellulolytic enzymes, the volume of β-glucosidase was 4% of the volume of cellulase in all assays, as recommended by the manufacturer. The viscosity of the samples was monitored over time in a coaxial roller viscometer (Brookfield LV DV II + spindle 34). The rotation of the sensor ran between 45 and 200 rpm with the sample temperature of 37 °C.

## 2.3 Enzymatic hydrolysis of cassava bagasse

The substrate for enzymatic hydrolysis was 10% (w/v) CB in dry basis previously gelatinized in water at 80 °C per 40 min. The hydrolysis was performed in 125 mL Erlenmeyer flasks with 50 mL work volume, using hydrolytic enzymes provided from both commercial source (cellulase) and microorganism cultivation (amylase). Cellulase complex (NS22086, Novozymes) plus β-glucosidase (NS22118, Novozymes) from the Novozymes' cellulosic ethanol enzyme kit was applied at start of the reaction. Crude amylolytic extract produced by *Rhizopus oligosporus* was added after 10 h. A three-level factorial design ($3^3$) was performed in order to determine the best combination ratio of amylase (A: 0-30 U/g) and commercial cellulase (C: 0-30 FPU/g) for CB hydrolysis (Table 1). Enzymatic reaction occurred at 50 °C for 36 h and samples were collected in 0, 10, 24 and 36 h for measurement of reducing sugar and glucose. The product yield ($Y_{P/S}$, g/g) was calculated as the ratio between glucose produced (P, g) and CB (dry mass) offered for the hydrolysis reaction (S, g). The conversion yield was calculated considering the conversion factor (0.9) of glucose in cellulose/starch. Yield of the substrate CB conversion in glucose was expressed in percentage (%, w/w) and calculated by Eq. (1):

$$Yield\ (\%) = \frac{C_g\ .\ V_h\ .\ 0.9}{m_{ds}}\ .\ 100 \qquad (1)$$

where $C_g$ is the concentration of glucose in the hydrolysate (g/L), $V_h$ is the hydrolysate volume (L), 0.9 is the conversion factor of starch and cellulose in glucose, and $m_{ds}$ is the mass of the substrate in dry basis.

## 2.4 Scale up of Cassava Bagasse Hydrolysate (CBH) production

The hydrolysis in a scale-up production of CBH occurred with two substrate concentrations in dry basis: 10 and 15% (w/v). The reaction medium was CB, previously gelatinized in water at 80 °C per 40 min, added by 5 FPU/g cellulase complex (NS-22086, Novozymes®) at the start of the hydrolysis time and 15 U/g amylase produced by *Rhizopus oligosporus* in 10 h reaction. The 1 L working volume of the reaction was set to 50 ± 0.1 °C in a 3-L bioreactor (TECBIO- Plus, Tecnal, Piracicaba, SP, Brazil) with intermittent agitation in every hour.

## 2.5 Scanning electron microscopy (SEM)

For SEM images, the samples were the residues from the hydrolysis assays in which the CB was enzymatically treated with the different proportions of amylase (A - U/g) and cellulase (C - FPU/g): 15A:5C, 30A:30C, 15A:15C, 0A:30C, 30A:0C, 0A:15C, and 15A:0C. The negative control was with water replacing the enzymes (0A:0C). For the assays with both enzymes, cellulase was added at time zero and amylase at time 10 h, whereas in the tests with the separate enzymes they were applied at time zero. After 24 h hydrolysis the enzymes were inactivated in a boiling bath for 10 min, cooled and the liquid and solid fractions were separated by centrifugation.

Samples of the CB non-hydrolyzed solid fraction were washed with distilled water to remove the soluble material following the steps: addition of water, stirring for resuspension and centrifugation. The procedure was repeated 5 times. Subsequently, samples were dried at 40 °C and fragmented manually. SEM analyses were carried mounting samples onto stubs using carbon double-sided tape and examined in a FEI Quanta FEG 450 scanning electron microscope, operating at an accelerating voltage of 1 kV.

## 2.6 Ethanol production

The CBH was separated by centrifugation and the supernatant phase was filtered and concentrated by evaporation to 88.07 g/L glucose, composing the wort. Concentrated CBH was supplemented with chemicals (Labsynth, Diadema SP, Brazil) (% w/v): 0.05% yeast extract, 0.1% ammonium sulfate, 0.114% dipotassium phosphate, 0.024% magnesium sulfate, 0.00104% manganese sulfate and 0.0028% zinc sulfate, pH 5.0 and sterilized at 121 °C for 15 min. The microorganism used for ethanol production was *Saccharomyces cerevisiae* from Levapan® commercial yeast. A suspension of 9 g (dry biomass) of yeasts was suspended in 75 mL sterile distilled water followed by the addition of 15 mL of the must at the start and each hour until 10 h, according to Dorta et al. (DORTA et al., 2006). The fed batch process was performed in 500 mL Erlenmeyer flasks at 30 °C and 80 rpm on an orbital shaker (Tecnal-TE421, São Paulo State, Brazil) for 24 h. The fermentation occurred in triplicate. Cell viability was determined at 0, 4, 8, 12 and 24 h of fermentation, by sampling and counting in the Neubauer chamber with methylene blue dye. The liquid fraction in the alcoholic fermentation was separated by centrifugation in a refrigerated centrifuge (Heraeus, Germany) at 4 °C, 3500 x *g* for 25 min. Samples were taken for analysis at the end. Ethanol yield was calculated using 0.6475 and 0.511 as the w/v and w/w, respectively, conversion factors of theoretical maximum yield finding an effective amount produced per gram of sugar.

## 2.7 Analytical methods

For amylase activity quantification the reaction mixture containing 100 μL of enzyme solution and 650 μL of 0.5% (w/v) starch (Sigma-Aldrich, USA) in 0.05 M sodium acetate buffer (pH 5.5) was incubated for 10 min at 60 ± 0.1 °C (Marconi MA 127, Piracicaba, SP, Brazil). The reducing sugar released was determined by DNS method (3,5-dinitrosalicylic acid) (MILLER, 1959). One unit of amylase activity was defined as the amount of enzyme that releases 1 μmol of glucose per minute and expressed in U/g of dry substrate. Detailed steps and equation performed to measure amylase activity are described by Barchi et al. (BARCHI et al., 2016).

Cellulase activity was determined according to Ghose (GHOSE, 1987). Substrate was filter paper Whatman n° 1 strip (1 cm x 6 cm) with 0.05 M sodium citrate buffer (pH 4.8) and the reaction mixture was incubated at 50 °C for 1 h. The reducing sugar dosage released during

the enzymatic reaction was performed by the DNS method. The result was expressed in filter paper units, FPU/mL of enzyme.

Ethanol and glucose concentration were measured by High Performance Liquid Chromatography (HPLC) in Aminex HPX-87H column (300 × 7.8 mm, BioRad, Richmond, CA, EUA) using refractive index detector. The mobile phase was 5 mM $H_2SO_4$ at 0.6 mL/min flowrate and 45 °C. The sample injection volume was 20 µL. The samples were filtered through cellulose acetate membranes of 0.2 micrometers. The total time for each run was 25 min.

### 2.8 Data analysis

Three-level factorial design was set and analyzed using Design-Expert software (Version 11, Stat-Ease, Minneapolis). Statistical decisions were taken considering a 95% confidence level ($p < 0.05$). The CB conversion yield using amylolytic and cellulolytic complex in three-level factorial design was optimized using a desirability function. The optimization criterion was to maximize yield based on glucose concentration according to the fitted model. This analysis was performed in Design-Expert software (Version 11, Stat-Ease, Minneapolis). Graphical data treatments from the monitoring of glucose concentration, yield, and viscosity at different hydrolysis times were performed using Excel 2016 (Microsoft Corporation, Redmond, WA).

## 3 RESULTS AND DISCUSSION

### 3.1 Amylase production by *Rhizopus oligosporus* in a tray system SSF

The choice to use a tray system for fungal amylase production was based on the similarity of this with the lab-scale model in Erlenmeyer flasks. This fact is caused by the aeration is over the bed in a tray bioreactor, different from the packed bed bioreactor aeration over and under the bed (FIGUEROA-MONTERO et al., 2011).

The tray system was suitable for amylolytic enzymes production (305.62 ± 4.53 U/g) by *Rhizopus oligosporus* using wheat bran as substrate. The amylase activity obtained was 77.8% of the values derived from lab-scale SSF using the same microorganism and substrate, recently published for Erlenmeyer flasks (BARCHI et al., 2016; ESCARAMBONI et al., 2018; ITO et al., 2017). Amylase production at large scale in order to add value to agro-industrial

wastes was pointed out as desirable (FERNÁNDEZ NUÑEZ et al., 2016), thereby this bioprocess can demonstrate applicability and environmental importance.

### 3.2 Optimization of cassava bagasse enzymatic hydrolysis and glucose production

The bioprocess feasibility depends on getting higher product content, with lower possible expenses, in a shorter time possible. In this way, the enzymatic hydrolysis of cassava bagasse will be appropriate when lower the enzyme dosage mainly in the case of cellulases which represents the greatest influence on bioethanol cost from substrates with lignocellulosic compounds (CRIPWELL et al., 2015; ZHAO et al., 2018).

The increase of glucose concentration on CBH was monitored from 0 to 36 h of reaction according to different proportions of amylases and cellulases separately and in mixtures defined by experimental matrix from three-level factorial design (Figure 1). Similar profiles with glucose above 50 g/L were identified after 24 h of reaction in the mixtures of amylases and cellulases assays, or with only cellulases.

Glucose concentration remained constant up to 36 h, indicating 24 h of reaction as long enough. The highest glucose levels obtained were 68.86 and 61.22 g/L by 15 U/g amylases and 30 FPU/g cellulases (15A:30C), and 30 U/g amylases and 15 FPU/g cellulases (30A:15C), respectively, in 24 h. These results with cassava bagasse showed a faster process than the enzymatic hydrolysis of wheat straw by the same cellulase applied in this work where the highest glucose concentrations were obtained over a 30 h period (PAULOVÁ et al., 2014).

In trials with only amylase, gelatinized CB was incubated without enzyme until 10 h, when the addition of amylase was performed. Thus, 24 and 36 h of the hydrolysis for these experiments corresponded to 14 and 26 h of effective reaction. Maximum glucose amount was obtained in 26 h of hydrolysis, around 38.5 g/L, for both 15 and 30 U/g amylase dosages. The performance of the amylolytic fungus extract on the CB substrate was nearly twice higher than previously reported for commercial amylases in a shorter time (CHEN et al., 2015).

A fast reduction of the viscosity was observed in the CB reaction medium from 1220 mPa.s in 2 h of reaction to 223 mPa.s in 10 h was verified by the action of cellulase in the proportion of 10 FPU/g of CB. This was expected, since the capacity of cellulase complex (NS22086 – Novozymes) to reduce the viscosity is reported by the company. In 0-2 h period was not possible to measure the viscosity since the 10% (w/v) CB presented texture of a sticky porridge. Currently, the cellulase treatment is applied to decrease cellulose viscosity and precisely adjust this parameter y in the pulp (DUAN et al., 2015). For the CB, although α-

amylase was used for liquefaction (SHI et al., 2014), the cellulase complex used in this work was efficient to reduce the initial viscosity of this agro-industrial residue.

**Figure 1** - Viscosity decrease and profile of cassava bagasse enzymatic hydrolysis using amylolytic and cellulolytic complex from three-level factorial design experiments (Table 1). A: Amylase produced by *Rhizopus oligosporus* added in 10 hours reaction and used in the proportions 0, 15 and 30 U/g of cassava bagasse (dry basis). C: Cellulase complex (NS-22086, Novozymes®) added at the start of the reaction and used in the proportions 0, 15 and 30 FPU/g. (--●--) Dashed lines mean the individual enzyme effects for amylase and cellulase. (—▲—) Filled lines mean the effects of the associated enzymes. The decrease in viscosity was evaluated with the 0A:10C condition. Error bars represent standard deviation, and each point represents the average of replicates (n=5).

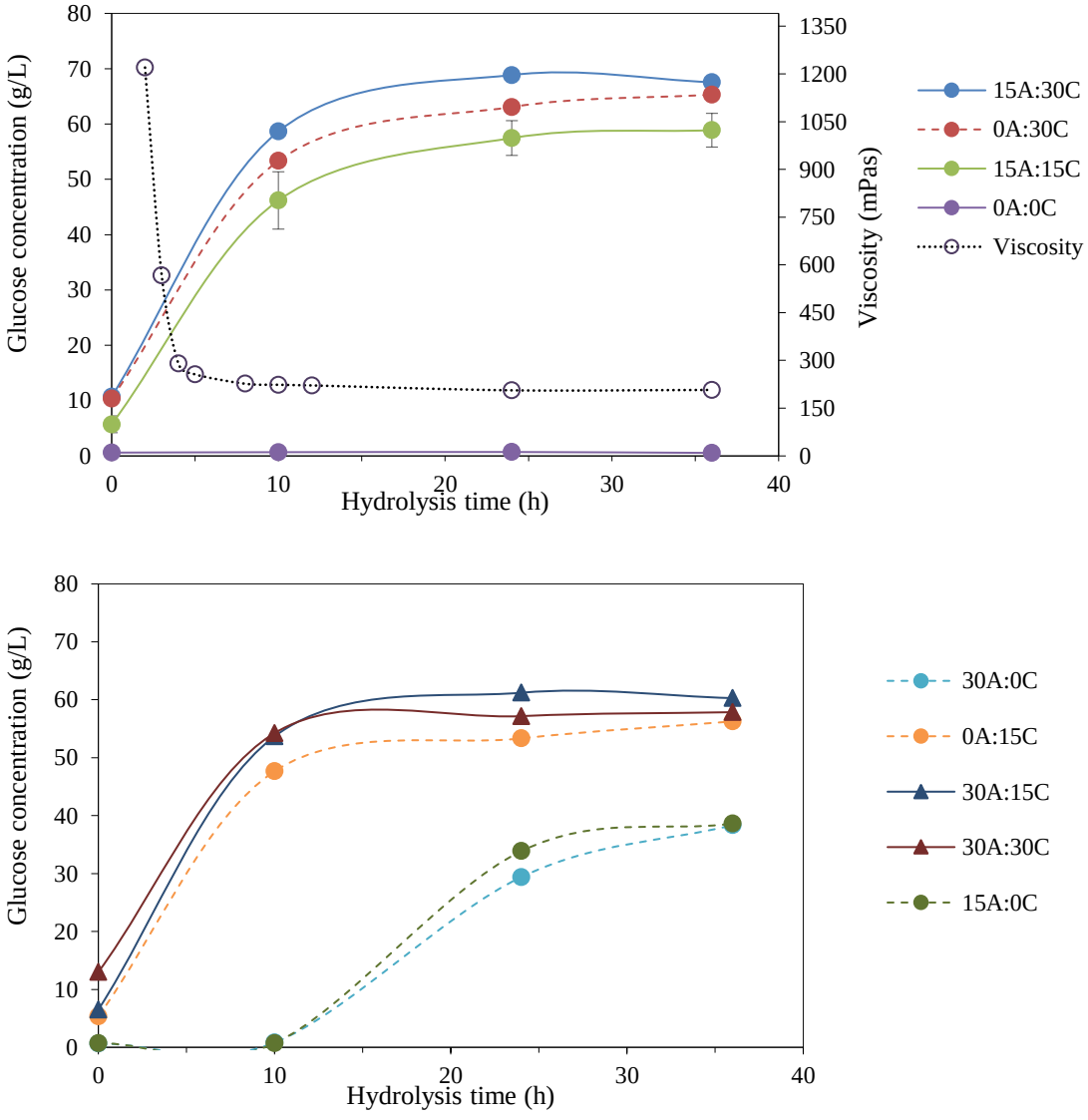


**Table 1 -** Three-level factorial design for defining the effect of enzymatic hydrolysis on cassava bagasse conversion into reducing sugars using amylase produced by *Rhizopus oligosporus* and commercial cellulase complex (NS-22086, Novozymes®) separately and associated. Cellulase complex was added at the start of the reaction and the amylase was added after 10 hours.

| Hydrolysis runs | Cellulase (FPU/g at 0 h) | Amylase (U/g at 10 h) | Yield (%, w/w) |
|---|---|---|---|
| 1 | 30 | 15 | 53.22 |
| 2 | 30 | 0 | 56.63 |
| 3 | 15 | 15 | 56.49 |
| 4 | 0 | 0 | 1.06 |
| 5 | 0 | 30 | 34.58 |
| 6 | 15 | 15 | 60.01 |
| 7 | 15 | 0 | 50.23 |
| 8 | 15 | 30 | 57.51 |
| 9 | 15 | 15 | 51.33 |
| 10 | 15 | 15 | 59.00 |
| 11 | 30 | 30 | 49.95 |
| 12 | 15 | 15 | 59.53 |
| 13 | 0 | 15 | 28.88 |

Statistical experimental design was previously identified as a tool to optimize the enzymatic proportion and provide a specific treatment for each substrate (BANERJEE et al., 2010; BUSSAMRA; FREITAS; COSTA, 2015; PENGILLY et al., 2015). Three-level full factorial design ($3^3$) assuming the conversion yield of CB into glucose as the response variable resulted in a model, which was used to optimize the proportion of amylases and cellulases complexes for CB hydrolysis. Experimental simplex lattice mixture design was performed to optimize the enzymatic proportion. The primary data obtained from the experimental design was performed at lab scale (Table 1).

The analysis of variance (ANOVA) for the variables indicated that conversion yield of CB in glucose can be satisfactorily fitted to the reduced quadratic model with R-Squared of 0.9527 as reported in Tables 2 and 3, and that observed and predicted data were adequately fitted (Figure 2B). The F-value of 40.32 implies the model is significant (significance tests, $p < 0.0001$ and lack of fit tests, $p > 0.05$). The confidence level was 95% ($p < 0.05$) of significance

of regression coefficients, in equation for amylases production. The mathematical model (Equation 2) presents the significant coefficients in the coded factors amylase (A) and cellulase (C). Quadratic term for amylase was not detected as significant ($p > 0.05$) for the yield.

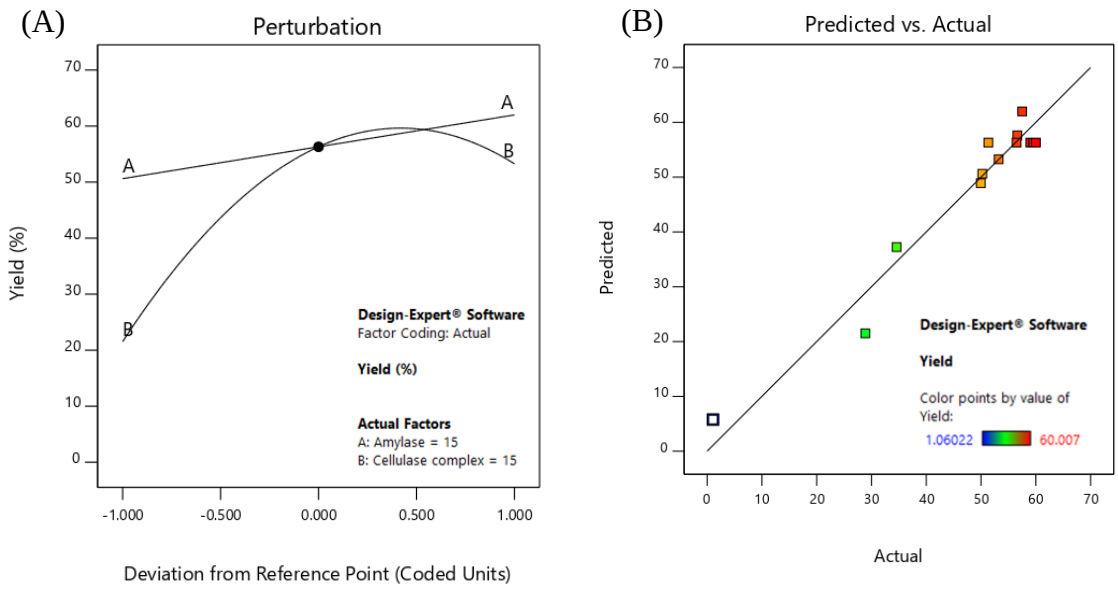
$$Yield\ (\%\ w/w) = 56.30 + 5.69A + 15.88C - 10.05AC - 18.91C^2 \quad (2)$$

The minimum (1.06%) and the maximum (60.01%) experimental conversion yield data varied from the run without enzymatic treatment and the runs treated with amylase 15 U/g and cellulase 15 FPU/g (Table 1). The equation in conjunction with trace graphs (Figure 2A) showed cellulase as the most significant positive term in the model, giving the major contribution for CB conversion, followed by amylase. However, antagonistic effects were confirmed between both enzymes (Equation 2, Figure 2C), since the interaction of them resulted in a negative effect, which can be attributed to the end-product (glucose) inhibitory effect on cellulase activity, leading to a decrease in glucose yields (CAO et al., 2015; HSIEH et al., 2014).

This is confirmed by the fact that CB pre-treatment with amylase does not benefit the production of sugar in a subsequent hydrolysis with cellulase (CHEN et al., 2015). However, cellulases contribute to exposing the raw starch to amylases improving the conversion and the feasibility of granular starch hydrolysis (CASTRO et al., 2011). This effect can be explained by the fact that starch granules are involved in the remaining cell walls of parenchymal tissue in the CB (VERSINO; LÓPEZ; GARCÍA, 2015). Therefore, although the application of amylase for CB liquefaction and afterwards cellulase (CHEN et al., 2015; SHI et al., 2014) is recurrent, the innovation of this work is the use of a cellulase at the beginning of the reaction capable of reducing the viscosity of the reaction medium and also initiating the hydrolysis process of the fibers, favoring the performance of the amylase that is fed in the system after 10 h.

The maximum CB conversion yield, as expected for the model using the desirability function, was the association of 30 U/g amylase (at 10 h) with 14.4 FPU/g (at 0 h) cellulase in order to obtain the predicted yield of 61.72%. The adequacy of the model and the higher predicted response was evaluated by an additional validation experiment using the optimal condition and its variations.

**Figure 2 -** Perturbation graph (A), Predicted vs. Actual (B), and 3-D graph (C) corresponding to model fitted for cassava bagasse hydrolysis conversion yield using amylolytic and cellulolytic complex in a three-level factorial design.



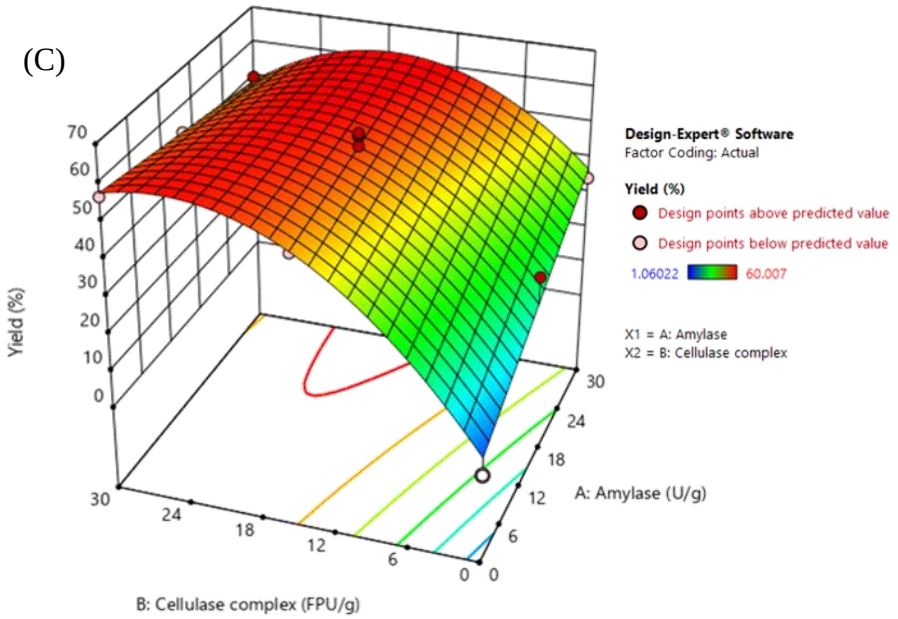



**Table 2 -** ANOVA for Reduced Quadratic model for conversion yield of cassava bagasse hydrolysis.

| Source | Sum of squares | Degrees of freedom | Mean square | F-value | p-value |
|---|---|---|---|---|---|
| Model | 3266.89 | 4 | 816.72 | 40.32 | < 0.0001 |
| A-Amylase | 194.02 | 1 | 194.02 | 9.58 | 0.0148 |
| B-Cellulase complex | 1513.14 | 1 | 1513.14 | 74.69 | < 0.0001 |
| AB | 404.04 | 1 | 404.04 | 19.94 | 0.0021 |
| $B^2$ | 1155.68 | 1 | 1155.68 | 57.05 | < 0.0001 |
| Residual | 162.07 | 8 | 20.26 | | |
| Lack of Fit | 110.59 | 4 | 27.65 | 2.15 | 0.2386 |
| Total | 3428.95 | 12 | | | |

**Table 3 -** Coefficients and statistical parameters for conversion yield of cassava bagasse hydrolysis using coded values.

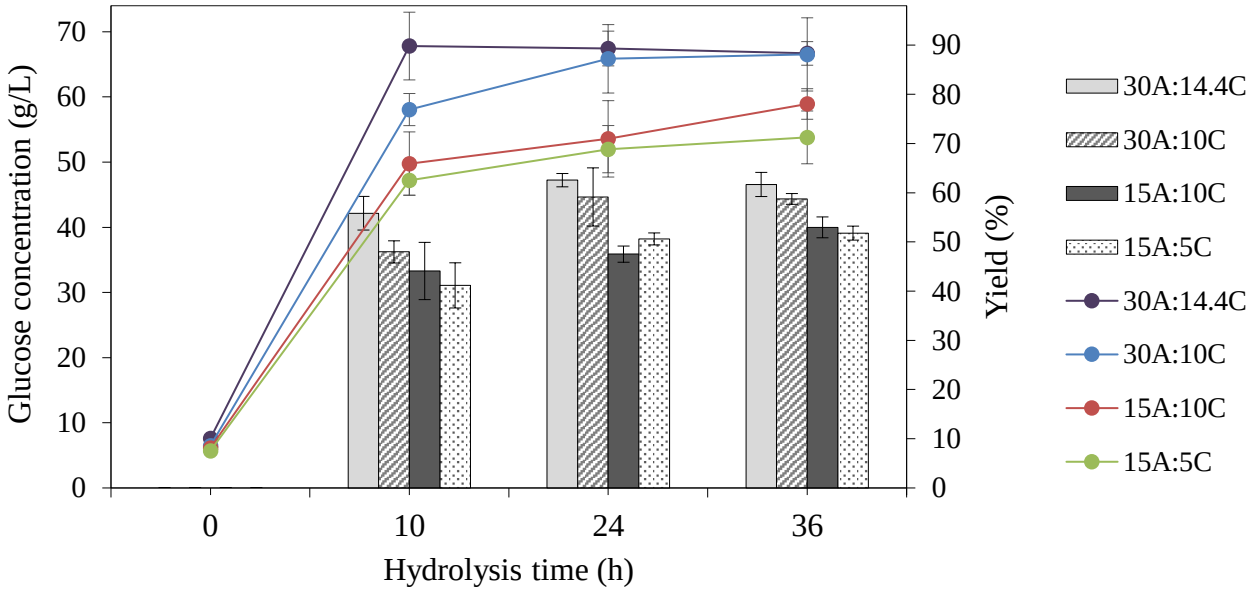
| Term | Coefficient | Standard error | Statistics | |
|---|---|---|---|---|
| Intercept | 56.30 | 1.70 | Std. Dev. | 4.50 |
| A-Amylase | 5.69 | 1.84 | Mean | 47.57 |
| B-Cellulase complex | 15.88 | 1.84 | C.V. % | 9.46 |
| AB | -10.05 | 2.25 | R-Squared | 0.9527 |
| $B^2$ | -18.91 | 2.50 | Adj R-Squared | 0.9291 |

### 3.3 Validation of optimal dosage of amylases and cellulases for CB hydrolysis

Validation of optimized condition of CB enzymatic hydrolysis was performed using four different proportions for amylases and cellulases to obtain high yield and glucose concentration with the minimum enzymes' consumption (Figure 3). The optimized enzyme proportion of fungal amylase and cellulase (30A:14.4C), converted 61.67 ± 2.45% of cassava bagasse into fermentable sugars in 36 h of hydrolysis. This result totally confirms the predicted value, indicating the prediction adequacy of the generated model using three-level factorial design tool. Therefore, the statistical optimization for CB hydrolysis was effective in predicting the conversion yield as a function of different hydrolytic enzymes content with a high coefficient of determination.

When the proportion of amylases and cellulases were reduced to a half and 3 times (15A:5C), respectively, compared to the original optimized amount (30A:14.4C), the yield of conversion was 51.79 ± 1.42% ($Y_{P/S}$ = 0.58 g/g) at 36 h. This result represents 0.84 of the yield obtained experimentally from the validation condition. Furthermore, the economic mixture with 15 U/g crude fungal amylase and only 5 FPU/g cellulase was indicated as more advantageous than the conversion yield obtained from enzymes separately in experimental design, 28.88% for 15 U/g amylase and 50.23% for the lower dosage of only cellulases used in experimental design (15 FPU/g) cellulase after 36 h (Table 1).

**Figure 3 -** Validation of optimized condition of CB hydrolysis and its variations to obtain high yield and glucose concentration with low enzymes spending. A: Amylase from *R. oligosporus* added in 10 h. C: Cellulase complex (NS-22086, Novozymes®) added at the start of the reaction. Glucose concentrations (Lines) and conversion yield (Bars). Error bars represent standard deviation, and each point represents the average of three repetitions (n=3).




Regarding the need for reduction time of enzymatic reaction, similar conversion yield was found in 24 h of hydrolysis confirming this time as suitable to make the bioprocess faster, in order to increase the productivity. In agreement, equivalent yields were obtained after 24 and 36 h of reaction with the different proportions of the enzymatic mixtures (Figure 3). The advantage of 24 h was also confirmed by the similarity in the glucose concentration measured in these times in both the validation and experimental design assays in lab scale, as shown in Figure 1 and Figure 3. Then, for the total glucose production from 10 % (w/v) CB, applying the

mixture of 15 U/g amylase and 5 FPU/g cellulase (15A:5C – 50.61% conversion yield in 24 h), the yield of 0.562 g/g is possible to obtain, which means the potential of 0.56 ton/ton of processed CB (dry basis) after 24 h of hydrolysis.

In the bioreactor hydrolysis (1 L working volume) for obtaining cassava bagasse hydrolysate (CBH) from 10% (w/v) CB, the glucose production of 61.93 g/L ($Y_{P/S}$ = 0.52 g/g) was obtained, corresponding to 47% conversion yield. However, raising the concentration of CB (dry basis) to 15% (w/v) resulted in a significant increase in the glucose level of 90.87 g/L ($Y_{P/S}$ = 0.50 g/g) on the CBH, corresponding to a conversion yield of 45.09% after the same reaction period (24 h), which means a glucose productivity of 3.79 g/L.h.

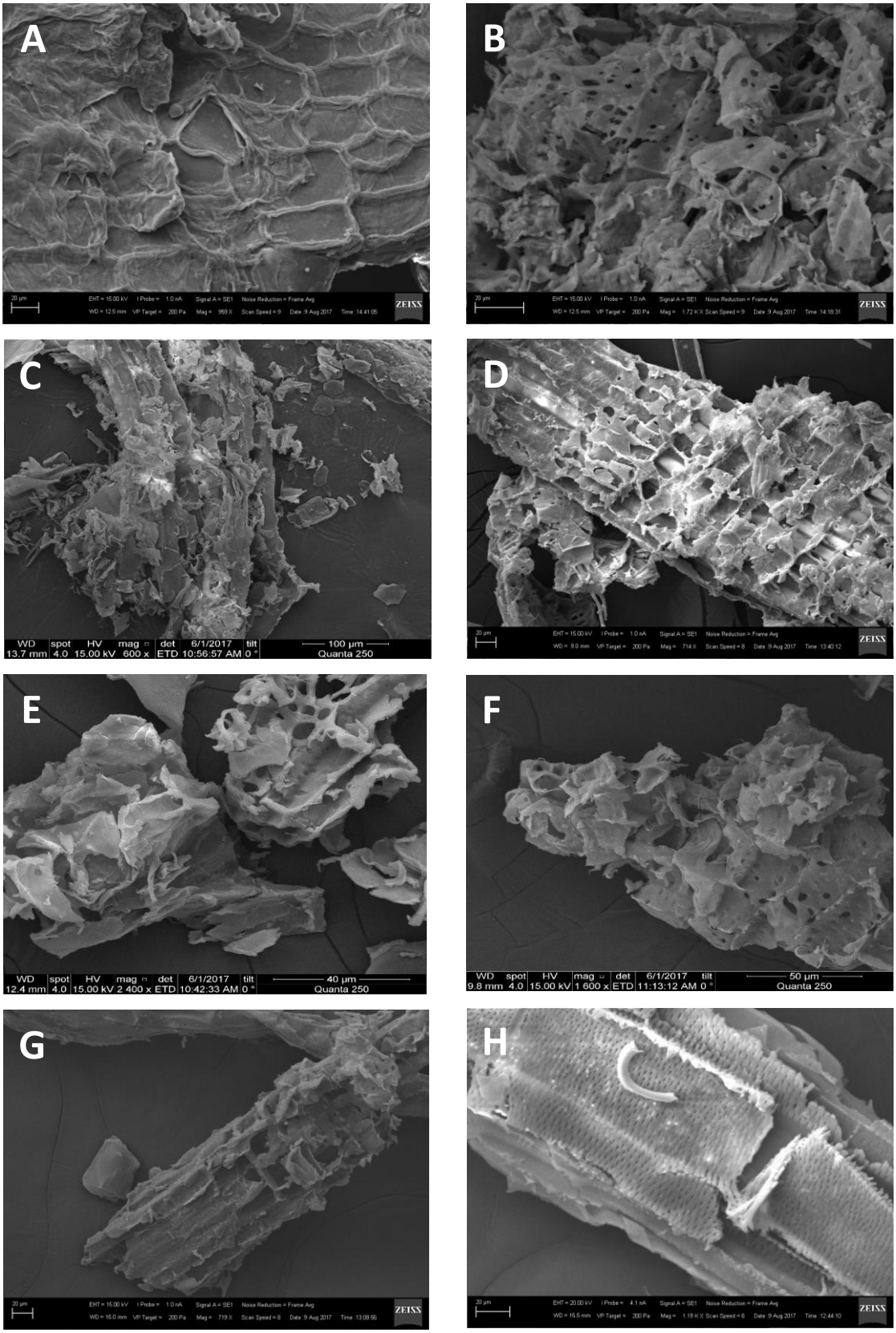
The increase in the amount of glucose released increasing substrate concentration was observed by Paulová et al. (PAULOVÁ et al., 2014). Glucose concentration in 24 h CBH in the present study was 6.19 times higher than that previously reported using an enzyme mixture of cellulase and beta-glucanase for 120 h cassava peel hydrolysis (BAYITSE et al., 2015).

The hydrolysis of 10% (w/v) CB by amylases and cellulases to obtain glucose, with yields between 33.0 and 45.7% is reported for the production of butanol, succinic acid, fatty acid and neutral lipids (CHEN et al., 2015; LU et al., 2012; SHI et al., 2014). These results were lower than that obtained in this work. In addition, a reaction time of 28 to over 50 h were found to be necessary for the enzymatic reaction in these reports. Therefore, when compared these reported data with the proposed method in the present work is possible to conclude a faster and competitive method was developed. The use of a *R. oligosporus* amylases in the proportion of 15 U/g of substrate, associated with only 5 FPU/g of commercial cellulases in only 24 h was responsible for higher glucose yields than those reported in the literature using high levels of three commercial, purified and concentrated enzymes, α-amylase, glucoamylase and cellulases (CHEN et al., 2015; LU et al., 2012; SHI et al., 2014). In addition, the increase of 10 to 15% in cassava bagasse concentration as substrate resulted in more than two times increase in sugar concentration in CBH compared to previous literature data, decreasing the cost of downstream process.

### 3.4 SEM

Modifications on the surface of CB samples caused by the enzymatic treatment were observed using SEM (Figure 4).

**Figure 4 -** Scanning electron microscopy (SEM) images of cassava bagasse after 24 h (A) without enzymatic treatment (0A:0C) and enzymatically treated with different proportions of amylase and cellulase: (B) 15A:5C; (C) 30A:30C; (D) 15A:15C; (E) 0A:30C; (F) 30A:0C; (G) 0A:15C; (H) 15A:0C.



A closed and non-porous structure of intact cassava cell wall was exhibited by CB without enzymatic treatment, when the cell wall was intact with the presence of cassava starch granule (Figure 4A). After enzymatic treatment, all samples showed a more disrupted structure. Several perforations appeared on the CB surface by the action of the economical enzyme mixture (15A: 5C), derived from the optimized composition (Figure 4B) proving its physical disruption. Enzymes progressing along the polysaccharide chains form cavities on the surface by the centripetal degradation from the surface to the center (CINELLI et al., 2014). SEM analysis revealed a more deconstructed structure for CB under treatments with both enzymes (Figure 4B, C and D) than in those with amylase and cellulase separately (Figure 4E, F, G and H) demonstrating a synergistic effect between two different classes of enzymes on cassava cells. Cripwell et al. (CRIPWELL et al., 2015) also observed the effectiveness of starch and cellulose depolymerization of the wheat bran structure. In fact, cellulases cocktail was successful in hydrolyzing the cellulose and simultaneously exposing the starch to the amylases.

### 3.5 Ethanol production

The CBH was used for the preparation of the must for 24 h alcoholic fermentation using *Saccharomyces cerevisiae* at 30 °C. The hydrolyzed concentrated up to 88.07 g/L glucose, determined by HPLC, was subsequently applied in fed batch process producing 35.25 ± 0.75 g/L ethanol with 78.33 ± 1.66% efficiency and 1.47 g/L.h productivity. In addition, cell viability of yeast remained high throughout the process (96.30 ± 1.34%), confirming that CBH is suitable for alcoholic fermentation. Therefore, from 15% CB hydrolysis, the proposed bioprocess would have the potential to produce 254.1 L of ethanol per ton of dried cassava bagasse in a total period of 48 h for a single step of hydrolysis and alcoholic fermentation.

The ethanol content in the fermented broth was higher than that reported in previous studies of ethanol production from fungal enzyme-hydrolyzed starch (PRANAMUDA et al., 1994). The efficiency of the conversion of sugar into ethanol was two times higher than that obtained by Ajibola et al. (AJIBOLA; EDEMA; OYEWOLE, 2012) using enzymatically hydrolyzed cassava starch as a substrate for fermentation. The ethanolic productivity obtained was superior to other values previously described from Babassu flour (1.12 g/L.h) and Corn flour (1.32 g/L.h) in two to three times longer processes (BIAŁAS; SZYMANOWSKA; GRAJEK, 2010; CINELLI et al., 2014).

Recently, an economic consideration was approached about ethanol production from cassava bagasse compared to ethanol from sugarcane (ESCARAMBONI et al., 2018).

Regarding spending on raw materials, 66.7% of the fuel ethanol production cost, the ethanol cost from cassava bagasse using fungal amylase for hydrolysis (0.13 US$/L) was twice as cheaper as ethanol from sugarcane (US$ 0.26/L). Therefore, this work presents the technical feasibility of this substrate as a new potential alternative for the production of bioethanol.

## 4 CONCLUSIONS

This study reports a new perspective for obtaining a low-cost alternative raw material from the hydrolysis of cassava bagasse, a solid agro-industrial residue, considering its fermentability by *S. cerevisiae*, and for the production of bioethanol. An efficient and simple bioprocess using a relatively small amount of amylases obtained from *R. oligosporus* (15 U/g) associated with commercial cellulase (5 FPU/g) was developed to obtain predominantly glucose in hydrolyzed cassava bagasse with 90.9 g/L of fermentable sugars in 24 h. A high efficiency ethanol production from this substrate was shown to be possible with a potential of 254.1 L of ethanol per ton of cassava bagasse (dry basis). Thus, the approach of different enzymes used in the waste biomass hydrolysis with simplified and efficient strategies can open new economic, ecological, and social perspectives to glucose and bioethanol production.

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# CHAPTER 5 – The potential of the food waste as feedstock for fungal amylases and proteases biosynthesis

## ABSTRACT

The research of new alternatives as feedstock for biotechnological process is a global concern due to the cost of substrate and the need of more economical bioprocesses. On the other hand, one third of the food produced in the world is wasted. In the present study, a new approach of biological treatment of food waste by the culture in solid state fermentation (SSF) for the production of fungal amylases and proteases was evaluated. A new bioprocess by the culture of *Rhizopus oligosporus* in a culture medium formulated by food waste, others carbon sources and corn steep liquor in a cell recycle system was studied. In the first evaluation, the kinetic assays for enzymes production showed 120 h as the best time of incubation. In the I-optimal design method for SSF modelling, mixtures containing at least 50% food waste (FW) associated with sugarcane bagasse (SCB) (10-20%) and wheat bran (WB) (20-40%) resulted in high levels of amylase and protease. The SSF supplementation with 20% (v/m) corn steep liquor was suitable as an alternative to replace the salts solution and the recycle of the biomass and non-hydrolyzed fibers of the wastes was able to increase amylase and protease yields. The better enzymatic production was performed with *Rhizopus oligosporus* in SSF using food waste as the main substrate and the two steps of culture as strategy to improve the yield. The best condition for amylase production (260.9 U/g substrate) was the waste blend FW 50%, SCB 10%, WB 40% supplemented with the salt solution. The highest protease level (665 U/g substrate) were obtained by the same mixture added by 20% (v/m) corn steep liquor. This proposed bioprocess approach showed to be economic, simple and rustic to produce these enzymes and should be scaled up for the industrial purpose.

**Keywords:** simple and rustic bioprocess, enzyme, corn steep liquor, cell recycle, *Rhizopus oligosporus*

# 1 INTRODUCTION

About 1.3 billion tons of food, one-third of food produced in the world for human consumption, are wasted every year which also represents a waste of the resources used in production, such as land, water, energy, and funds (FAO, 2019). This waste represents a challenge regarding disposal and can cause severe environmental issues (MATSAKAS et al., 2014). In contrast, they can be considered valuable if there are adequate biotechnological strategies so that the value of subsequent products exceeds the cost of reprocessing.

On the other hand, the global enzymes market was valued at $7.1 billion in 2017, which is expected to reach $10.5 billion in 2024 with a compound annual growth rate (CAGR) of 5.7% from 2018 to 2024 (RESEARCH AND MARKETS, 2018). Among all types of industrial enzymes in the global market, carbohydrases are estimated to be the largest share, about two-thirds in 2017, with amylases being the most common type. Furthermore, proteases are the second largest revenue generator, since they accounted for one-fourth share of the market in the same period. Thus, the prediction is that up to 2024 carbohydrases and proteases will maintain market dominance due to commercial applications in food products and detergents (MORDOR INTELLIGENCE, 2018; RESEARCH AND MARKETS, 2018).

Although the enzyme market is well established, considering the increasing demand, the better use of low added value materials as a renewable platform in biotechnological applications is an economic alternative and a need. The use of substitute substrates for the production of enzymes required for the hydrolysis of different substrates, especially vegetable biomass using the biorefinery platform concept, is a trend (ESCARAMBONI et al., 2018; LOLASI et al., 2018).

The enzymatic production through microorganisms has advantages as the process viability, high rate of yield, possibility of the use of economical substrates, ease of handling of this kind of biological entities and also the obtaining of enzymes with specific characteristics (GUPTA et al., 2003; SOUZA; MAGALHÃES, 2010). Even though microbial enzymatic synthesis may occur through submerged fermentation (SmF), the solid state fermentation (SSF) process is also possible, and in some cases as the use of residues as a substrate, may be more feasible and economical (FERNÁNDEZ NUÑEZ et al., 2016). Both amylases and proteases can be produced concomitantly by fungi of the genus *Rhizopus* and *Aspergillus* under SSF. However, *Rhizopus oligosporus*, besides being a good enzyme producer, has GRAS (Generally Recognized as Safe) certification because of its safety (VATTEM et al., 2004), which represents an important characteristic for commercial applications.

High added value compounds, such as enzymes, can be produced using low cost wastes. Remaining food is a complex biomass containing various components that can be used as a suitable feedstock, such as starch (55–65%), proteins (15–20%), lipid fats (10–15%) and cellulosic materials (5–10%) (MA; CAI; LIU, 2017). The composition of the food residues suggests this biomass as a potential substrate for microbial bioconversion in fermentative processes, either alone or in composition with other materials. Thus, in addition to food residues, other residues, such as wheat bran, sugarcane bagasse and corn steep liquor, may constitute a culture medium for SSF, in view of a nutritional balance for metabolism targeting microorganisms for the synthesis of products of interest.

Wheat bran accounts for about 25% of the total wheat mass (PRÜCKLER et al., 2014) and comprises 56% carbohydrate, including starch, 13-18% protein and 3.5% fat (APPRICH et al., 2014). It can be used as a source of C and N in SSF for the production of fungal enzymes (ESCARAMBONI et al., 2018). Sugarcane bagasse is one of the most abundant residues due to the high world production of sugarcane (1.84 billion tons worldwide in 2017) (FAOSTAT, 2019). Its composition is mainly holocellulose (74.6%) and lignin (23.85%) (ÁVILA; FORTE; GOLDBECK, 2018). However, despite its low nutritional value, sugarcane bagasse can be used in small amounts in SSF media for the production of enzymes by non-cellulolytic microorganisms (BARCHI et al., 2016; FERNÁNDEZ NUÑEZ et al., 2016).

Corn steep liquor is a by-product of corn milling and has the soluble proteins as its main component. This waste is used in the fermentation industry due to the high nutrient content, acting as low-price source of nitrogen for the microorganisms (HOFER et al., 2018; TAIWO; MADZIMBAMUTO; OJUMU, 2018). In this sense, corn steep liquor is an alternative source as an economical raw material for the synthesis of biomolecules, since it has the potential to substitute more expensive components of the fermentative processes. Nutrients as yeast extract, beef extract, and peptone, widely used in microbial cultivations, are expensive and estimated to cost more than 30% of the total cost of production (SEESURIYACHAN et al., 2011). On the other hand, corn steep liquor proved to be a lower-cost nutrient to replace yeast extract (TAIWO; MADZIMBAMUTO; OJUMU, 2018).

Mixture experimental designs are suggested for optimizing substrates composition in solid-state fermentation and improving process efficiency when complex waste blends are necessary to support the microorganism growth and enzyme productions (FERNÁNDEZ NUÑEZ et al., 2016). Specifically, if the proportion of at least one of the mixture components should be explored within a more narrow range than 0-100 %, the designs are classified as a mixture problem with constraints (NÚÑEZ et al., 2013).

The aim of this work was to define a bioprocess for enzymes production based on solid-state fermentation by *Rhizopus oligosporus* using food waste as the main carbon source. Statistical optimization and modelling of the amylolytic and proteolytic activity in aqueous extracts as a function of remaining food and other supplementary agro-industrial wastes' proportions were used as tools to achieve this goal.

## 2 MATERIALS AND METHODS

### 2.1 Microorganism and inoculum preparation

The fungus used in the present work, *Rhizopus oligosporus* (CCT 3762) kindly given by Fundação Tropical de Pesquisas e Tecnologia "André Tosello", Campinas, SP, Brazil, was grown on potato dextrose agar (PDA) (Difco, USA), in 250 mL Erlenmeyer flasks, for 7 days at 30°C. The biomass was washed with 0.01% (v/v) Tween 80 water solution under gentle magnetic stirring to obtain the spore suspension to be used as inoculum for SSF.

### 2.2 Wastes used in SSF

The food waste was collected during a month (From May 1$^{st}$ to June 1$^{st}$, 2016) at the University Restaurant of the São Paulo State University (UNESP), Faculdade de Ciências e Letras, Campus Assis. The material collected was dried at 50ºC, followed by grinding in industrial blender and storage at -10ºC. Wheat bran (WB) (Moinho Nacional, Assis, SP, Brazil) and sugarcane bagasse (SCB) (Raízen Energia S/A Group, Tarumã, SP, Brazil) were used associated with food waste (FW) as SSF substrate. The corn steep liquor (Tereos Starch & Sweeteners, Palmital, SP, Brazil) was applied as SSF nutrient supplementation.

### 2.3 Chemical composition of the wastes

For the chemical composition analysis of wastes, the mass percentages (w/w) of moisture (gravimetric method: oven drying at 105°C until weight constancy), proteins (the Kjeldhal method and conversion factor 6.25), lipids (ethylic ether extraction in Soxhlet apparatus) and ashes (incineration at 550 °C for 4 h) were determined in triplicate by classical

methods (ZENEBON; PASCUET; TIGLEA, 2008). The carbohydrate content was determined by difference between 100% (w/w) and the sum of the components previously determined.

## 2.4 General procedure for solid-state fermentations

Experimental runs for SSF were performed in 250 mL Erlenmeyer flasks containing 10 g substrate (dry basis). The food waste or ternary mixtures of wastes were wetted with optimized nutrient aqueous solution for the microorganism under study, composed of 1.25% (w/w) ammonium sulphate, 0.25% (w/w) potassium phosphate and 1.25% (w/w) urea (ESCARAMBONI et al., 2018; ESCARAMBONI; OLIVA NETO; INDUSTRIAL, 2016). The substrates moisture was adjusted to 55% for this fermentation, followed by sterilization (121 ºC, 1 atm, 20 min) and aseptic inoculation with $10^6$ spores per gram of dry substrate. The temperature for bioconversions was 30°C. The fermentation times were changed according to the specific experiment. When fermentations were completed, the enzymes in fermented substrates were extracted by addition of 10 mL/g distilled water. The mixture was incubated in an orbital shaker (Tecnal, TE-421, Piracicaba, Brazil) at 180 rpm and 28 °C for 30 min. The extracts were filtered and used to quantify the amylase and protease activities.

## 2.5 Kinetic study and Mixture experimental design

Firstly, the fermentations were conducted as kinetic studies for a single substrate (food waste). The enzymes production using food waste substrate was monitored for 9 days (216 h). Each 24 h from inoculation, the amylolytic and proteolytic activities of corresponding aqueous extracts were quantified. The final aqueous extract pH and the consumed food waste percentage, calculated according to the dry mass of the residual material obtained after extraction, was also measured. The enzymatic activities, consumed substrate and final pH associated with each assessed fermentation time were determined in quadruplicate (four individual fermentations were performed for each time).

In order to evaluate the effect of the sugarcane bagasse (0-20%, w/w) and wheat bran (0-50%, w/w) added to SSF medium with food waste (50-100%, w/w), an I-Optimal experimental design for mixture problems with constraints was performed. The total of 14 experimental points was sufficient to fit a quadratic model. The food waste was adopted as the main substrate (minimum of 50% w/w) in the medium composition. The ternary mixtures are

listed in Table 2. After the analysis of the results, the best combinations of substrates that ensure the maximization of enzyme production were applied in validation tests processed in quadruplicates, as well as variations of these combinations in order to guarantee flexibility to the bioprocess.

### 2.6 Corn steep liquor as a nutritional supplement of SSF

Two sets of waste blends confirmed by the I-Optimal design validation experiment (FW 50%, SCB 10%, WB 40% and FW 70%, SCB 10%, WB 20%) were submitted to SSF tests, replacing the salt solution (1.25% (w/w) ammonium sulphate, 0.25% (w/w) potassium phosphate and 1.25% (w/w) urea) with corn steep liquor. Different amounts (5, 10, 20 and 40% v/m) of corn steep liquor were added to 10 grams of dry substrate and supplemented with distilled water until reaching 55% moisture. The control trials were elaborated with the salt solution as supplement.

### 2.7 Recycle of fungal biomass and non-hydrolyzed fiber in two steps culture

After the extraction filtration step, the liquid fraction containing the enzymes was destined for the analytical tests and the solid portion (fungal biomass and non-hydrolyzed fibers from carbon sources) was subjected to a new culture. For the improvement of enzyme/substrate yields, the biosystem was performed in two steps. The first one with the original culture medium and spores' inoculations, and the second step was performed by cells recycle concept, in which the filtration occurred aseptically and the solid residue, containing the fungal biomass and non-hydrolyzed substrate, was immediately incubated in Erlenmeyer flasks at 30 °C for 120 h. After this period, a new extraction with distilled water (10 mL/g of initial dry substrate) was carried out and the liquid fraction was quantified for the amylase and protease activities.

### 2.8 Amylolytic activity

The amylolytic activity was measured in triplicate, using 0.5% (w/v) soluble starch (Sigma-Aldrich, USA) as substrate in 0.05 M sodium acetate buffer, pH 5.5, by the quantification of released sugar with reducing groups, using the 3,5-dinitrosalicylic acid (DNS)

method (MILLER, 1959). The reaction mixture containing 100 µL of the enzymatic extract and 650 µL of substrate was incubated for 10 min at 60±0.1°C. After the reaction time, 500 µL of DNS solution was added, followed by its heating in a boiling water bath for 5 min, and its dilution with 3.75 mL of distilled water. The absorbance was measured at 540 nm using glucose as standard. One unit of amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugars per minute and expressed as units per gram of dry substrate (U/g). Detailed steps and equation performed to measure amylase activity was previous described (BARCHI et al., 2016).

## 2.9 Proteolytic activity

The proteolytic activity was measured according to Leighton's method (LEIGHTON et al., 1973) in triplicate using 1% (w/v) azocasein (Sigma-Aldrich, USA) solution in 0.05 M sodium acetate buffer, pH = 5.5 as substrate. The reaction mixture containing 150 µL of the enzymatic extract and 250 µL of substrate was incubated for 30 min at 50±0.1°C. Then, 1 mL of 10% (m/v) trichloroacetic acid (TCA) was added, followed by centrifugation to precipitate proteins. Next, 1 mL supernatant was mixed with 1 mL of 1 M NaOH solution and the absorbance was measured at 440 nm. One unit (U) of proteolytic enzyme activity was defined as the amount of enzyme needed to change the absorbance by 0.01 per minute and expressed as units per gram of dry substrate (U/g). Detailed steps and equation performed to measure protease activity was previous described (BARCHI et al., 2016).

## 2.10 Enzymatic hydrolysis

In the food waste enzymatic hydrolysis, the amylases produced in this work also from food waste in SSF were used. The substrate was prepared at 10% (w/v) in 0.05 M sodium acetate buffer (pH 4.5) and gelatinized at 90°C for 30 minutes. Then, the temperature was reduced to 50°C for subsequent addition of 15 U/g amylase, and the catalytic mixture incubated at 24 and 36 h. After the reaction time, the hydrolysate was boiled for enzyme inactivation and centrifuged at 3000g for 15 minutes. The supernatant was removed and the reducing sugar was quantified (MILLER, 1959).

### 2.11 Data analysis

The I-optimal experimental design, the generation of experimental combinations as well as polynomial statistical modeling were performed in Design-Expert software (Version 11, Stat-Ease, Minneapolis). The significance level of 0.05 was considered for statistical decisions. The amylolytic and proteolytic activities in ternary mixtures of wastes in I-optimal experimental design were optimized using the desirability function implemented in Design-Expert software. Means multiple comparisons in other experiments were performed by ANOVA and subsequently Tukey test (if necessary) in BioEstat 5.0 free software (Mamirauá Institute, Brazil), and graphical data treatments were performed using Excel 2016 (Microsoft Corporation, Redmond, WA).

## 3 RESULTS AND DISCUSSION

### 3.1 Kinetic study of *Rhizopus oligosporus* culture in food waste as solid substrate

For the food waste evaluated individually as a carbon source in the process SSF, the maximum amylolytic expression was reached in 96 h, remaining stable up to 192 h after inoculation ($p>0.05$), with activities around 83.3 U/g of substrate in dry basis (Figure 1A). A marked increase in amylase synthesis occurred between 48-96 h, tripling the yield (25 to 75 U/g). This can be explained by the fact that in the first two days after inoculation by spores, the fungus mycelium was growing, so that, in sequence, the enzymatic synthesis became more pronounced and evident. The proteolytic synthesis gradually increased over time, reaching the highest production of 170 U/g in 168 h, stabilizing after this period.

The substrate consumption (Figure 1B) presented a similar profile than amylase production. This equivalence is related to the major carbohydrate composition in food waste (Table 1). In addition, a rapid reduction of enzymatic extract final pH (up to pH 4.5) in the first 72 hours, associated with the biomass growth period and lower enzyme synthesis, occurred. Which gradually increased to values close to neutrality (pH 6.4) throughout the incubation period.

The yield of amylases and proteases, and the incubation time to achieve the maximum expression depend on the agro-industrial waste used as substrate in SSF bioprocess (BARCHI et al., 2016). In accordance with the results obtained, the kinetic profiles of amylase previously

published for wheat bran, type II wheat flour, soybean meal and sugarcane bagasse showed the fermentation time of 120 h as the peak of production or plateau region containing the highest values of amylolytic enzyme activity (FERNÁNDEZ NUÑEZ et al., 2016).

The amylase obtained in the present work was similar or higher than that obtained in similar processes using cassava root (108 U/g) (SOCCOL et al., 1994) ), soybean meal and sugarcane bagasse (lower than 70 U/g) (FERNÁNDEZ NUÑEZ et al., 2016) as substrate. In addition, the amylolytic activity of the aqueous extract was significantly higher than the values of submerged fermentation with the same fungus (FREITAS et al., 2014). However, the values were lower than those obtained with wheat bran (ESCARAMBONI et al., 2018), due to the physical and chemical properties of this substrate (FERNÁNDEZ NUÑEZ et al., 2016). In agreement, in the chemical composition of both food residues and wheat bran, the protein content is higher than 18% (Table 1). However, the first contains proteins of vegetable and animal origin and the second only vegetable. In addition, the carbon-nitrogen ratio of the food waste is lower than the wheat bran ratio, considering that it has a carbohydrate content 20% (dry basis) higher than the food waste.

Protease obtained in the present work was superior to that obtained with *R. oligosporus* in SSF using the residues of soybean meal and sugarcane bagasse. On the other hand, the protease synthesis from other agro-industrial residues such as wheat flour type II and wheat bran were higher than that obtained in the present work (BARCHI et al., 2016). However, the cost-benefit ratio and the socio-environmental impact make the food waste feasible and justifiable to use.

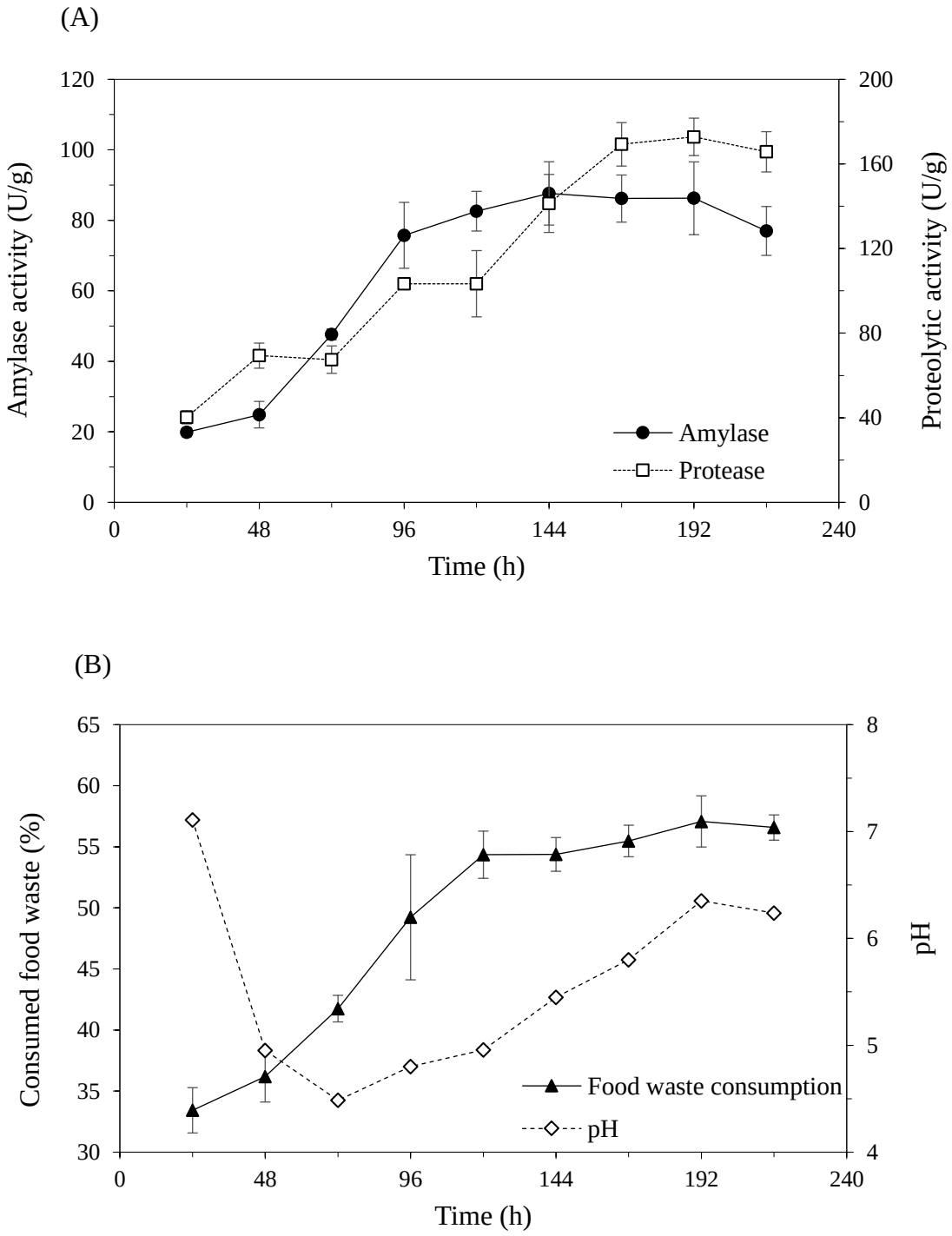
**Table 1 -** Centesimal composition (%, w/w) of the wastes used for amylase and protease production in the present work.

| Chemical components | Food waste | Wheat bran[a] | Sugarcane bagasse[a] | Corn steep liquor[b] |
|---|---|---|---|---|
| Moisture (%, w/w) | 7.83 ±0.17 | 15.20±0.15 | 8.01±0.27 | 50.00±5.00 |
| Protein (%, w/w) | 24.70±1.17 | 18.54±0.69 | 2.52±0.20 | 45.00±5.00 |
| Lipids (%, w/w) | 15.94±0.043 | 1.85±0.24 | 0.70±0.29 | nd |
| Ashes (%, w/w) | 3.28±0.33 | 2.95±0.01 | 3.00±0.32 | max 20 |
| Carbohydrates (%, w/w) | 48.24 | 61.46 | 85.77 | nd |

[a]Data previous published by the authors (FERNÁNDEZ NUÑEZ et al., 2016). The material used in this work was from the same batch previously characterized by the authors.

[b]Data provided by the supplier (General product specification).

**Figure 1 -** Kinetic study of (A) amylolytic and proteolytic enzyme production by *Rhizopus oligosporus* (CCT 3762) using food waste as substrate; (B) consumed food waste and final aqueous extract pH. Error bars represent standard deviation, and each point represent the average of four repetitions (n= 4).




Therefore, the application of exclusive or majority of food residues as substrate for the production of amylase and protease by SSF indicated to be an interesting alternative route due

to its low economic value, high availability and environmentally feasible. Additionally, the consumption of more than 50% of food residues in 120 h associated with the synthesis of amylases and proteases represents an innovative waste treatment favorable to recurring environmental issues, linked to the production of molecules of biotechnological interest.

After these results, 120 h of the fermentation time and the minimum of 50% of food waste were defined as conditions to be used for further studies with ternary mixtures of substrates.

### 3.2 Mixture experimental design

The values corresponding to response variables of the primary data obtained from the mixture experimental design performed at lab scale are reported in Table 2. The amylase (AA) and protease (PA) activities synthesized by *R. oligosporus* ranged from 50.9-168.8 U/g and 143.9-264.8 U/g, respectively, for the aqueous extract obtained from ternary mixtures composed of food waste (FW), sugarcane bagasse (SCB) and wheat bran (WB). The results were satisfactorily fitted to a quadratic model for AA and cubic model for PA (model significance tests, $p<0.05$ and lack of fit tests, $p> 0.05$).

For amylolytic activity, the equation (Equation (1)) in terms of coded factors in conjunction with trace graph (Figure 2 A) showed the major contribution of wheat bran for higher values of activities. The fitted models revealed a synergism of food waste with sugarcane bagasse, and wheat bran with sugarcane bagasse. However, for higher values of proteolytic activity, both wheat bran and sugarcane bagasse showed the greatest contributions, as verified by Equation (2), in terms of coded factors, and trace graph (Figure 2 B).

**Table 2 -** I-optimal experimental design for defining substrate influence on amylolytic and proteolytic enzyme production. The ternary mixtures of wastes were composed of food waste, sugarcane bagasse and wheat bran. Response variables derived from I-optimal experimental design were amylolytic and proteolytic activity.

| Run | Wastes in culture medium | | | Response variables | |
|---|---|---|---|---|---|
| | Food waste (%) | Sugarcane bagasse (%) | Wheat bran (%) | Amylase activity (U/g) | Protease activity (U/g) |
| 1 | 85.0 | 5.0 | 10.0 | 74.83 | 169.26 |
| 2 | 50.0 | 10.0 | 40.0 | 168.79 | 225.19 |
| 3 | 75.0 | 0.0 | 25.0 | 85.39 | 207.00 |
| 4 | 65.0 | 20.0 | 15.0 | 123.18 | 221.11 |
| 5 | 80.0 | 20.0 | 0.0 | 85.39 | 202.14 |
| 6 | 50.0 | 20.0 | 30.0 | 162.00 | 235.25 |
| 7 | 50.0 | 0.0 | 50.0 | 130.86 | 211.10 |
| 8 | 100.0 | 0.0 | 0.0 | 50.91 | 151.29 |
| 9 | 75.0 | 0.0 | 25.0 | 80.22 | 233.00 |
| 10 | 100.0 | 0.0 | 0.0 | 53.06 | 143.89 |
| 11 | 60.0 | 10.0 | 30.0 | 150.69 | 264.75 |
| 12 | 50.0 | 0.0 | 50.0 | 125.00 | 215.22 |
| 13 | 80.0 | 20.0 | 0.0 | 75.91 | 204.15 |
| 14 | 90.0 | 10.0 | 0.0 | 68.15 | 194.01 |

A synergistic effect was also observed between food waste and wheat bran on the proteolytic expression by the fungus *R. oligosporus* at binary mixture compositions of 66.5% of food waste and 32.5% of wheat bran. The correlation coefficients for the models between the predicted and experimental values associated with Equations (1) and (2) were 0.982 and 0.930, respectively (Figure 3).

$$AA = 47.06 \cdot FW - 41.35 \cdot SCB + 127.10 \cdot WB + 277.65 \cdot FW \cdot SCB + 450.10 \cdot SCB \cdot WB \quad (1)$$

$$PA = 154.51 \cdot FW + 247.41 \cdot SCB + 212.98 \cdot WB + 150.72 \cdot FW \cdot WB - 512.33 \cdot FW \cdot WB \cdot (FW - WB) \quad (2)$$

**Figure 2 -** Cox trace graphs corresponding to the models adjusted for amylase (A) and protease (B) activities of the I-optimal experimental design. A: Food waste; B: Sugarcane bagasse and C: Wheat bran.

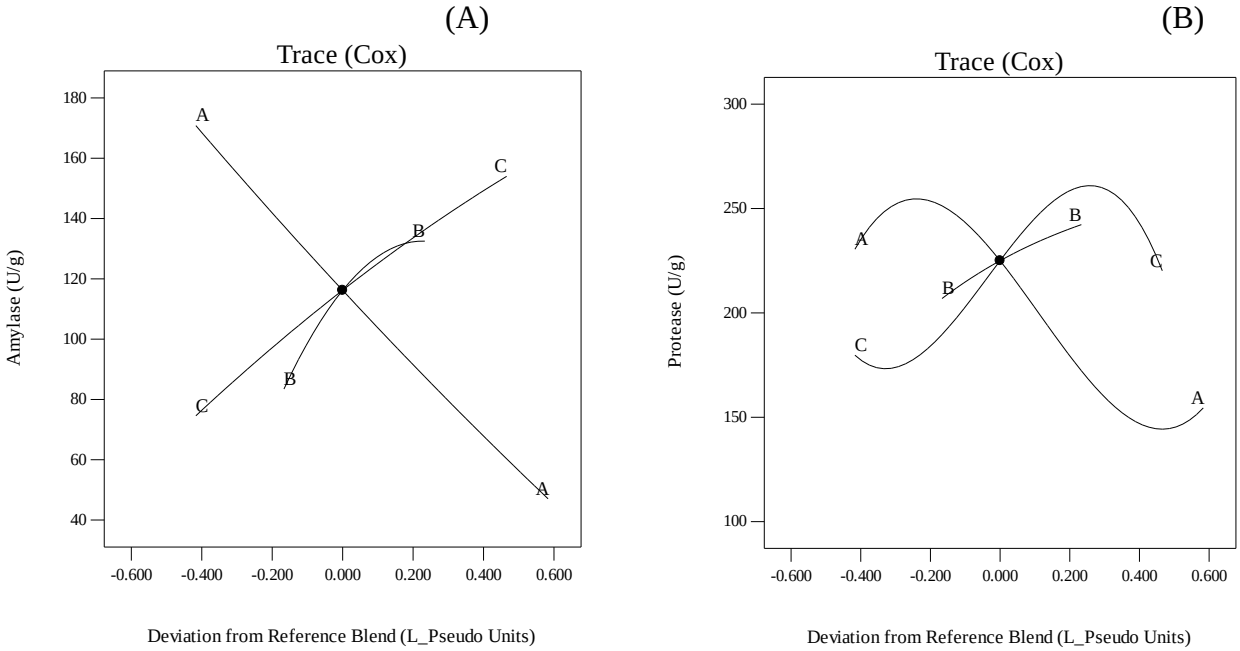
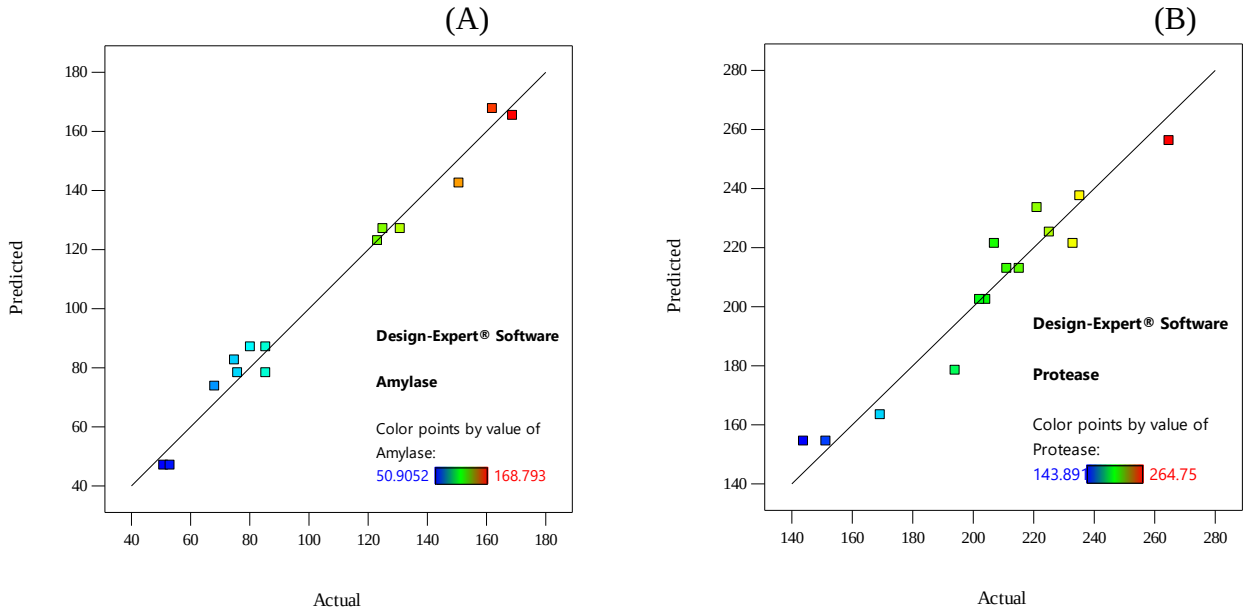



**Figure 3 -** Predicted and experimental values for the amylase (A) and protease (B) activities of the I-optimal design.




The improvement on enzymatic activities obtained for wheat bran can be explained by the favorable composition of this substrate for the growth and metabolism of *R. oligosporus* (Table 1). Wheat bran is widely used in several amylase production processes and considered

to be an ideal substrate for SSF, due to the correct balance between the carbon, nitrogen and phosphorus sources (CASCIATORI et al., 2015; FERNÁNDEZ NUÑEZ et al., 2016; ITO et al., 2017). The carbohydrates content around 56%, most of them being starch, and 15% protein (PRÜCKLER et al., 2014) justify this substrate as an inducer for the secretion of amylases, mainly from fungi in SSF systems (ELLAIAH et al., 2002; HASHEMI et al., 2010; PEIXOTO-NOGUEIRA et al., 2008). Moreover, the protein content can induce the protease secretion by the microorganism (CASTRO; SATO, 2013).

On the other hand, sugarcane bagasse is poor in nutritional values since it contains mainly cellulose, hemicellulose and lignin (ZANATTA et al., 2016). However, since very small particles of the solid-state fermentation medium result in the reduction of the respiratory activities of the microorganism (VASEGHI et al., 2013), this fibrous substrate together with the food residue and wheat bran was favorable to enzymatic production due to its ability to distance the particles from the fermentation medium favoring the aeration that is fundamental to fungus metabolism. The negative influence of sugarcane bagasse individually in the model (Eq. 1) can also be explained by the fact that when the particle size of the medium exceeds a certain value it can affect the enzymatic production by reducing the contact surface between the particle's substrate and fungi (CASTRO; SATO, 2013).

The maximum production of amylases and proteases in the present work from mixtures containing at least 50% of food waste was similar to data previously reported with a mixture of soybean meal (40%), wheat bran (35%) and sugarcane bagasse (25%) for amylolytic activity (198 U/g) and proteolytic activity (257 U/g) (FERNÁNDEZ NUÑEZ et al., 2016). In addition, protease synthesis was significantly higher than that obtained by *Aspergillus oryzae* in SSF with wheat bran (60 U/g) (CASTRO; SATO, 2013).

This is probably the first study that uses food wastes for the simultaneous production of two of the most widely consumed enzymes in the world: amylases and proteases. Furthermore, food wastes demonstrated efficacy for the synthesis of these enzymes at low cost by SSF and, when supplemented with other substrates, such as the agro-industrial residues wheat bran and sugarcane bagasse, resulted in an enhanced enzymatic expression.

### 3.3 Validation of optimal substrate mixtures

Validation tests were performed in order to confirm empirically the predicted best compositions using the desirability function, the proportion of at least 50% of food residue in

the mixtures was adopted as an initial requirement, aiming at the greater use of food residues as SSF substrate. Five optimization criteria were assessed, all of them seek to maximize: (1) amylase and protease activities and food waste concentration; (2) amylase and protease activities; (3) amylase activity. The criteria (4) and (5) were variations of the previous ones in order to increase the wheat bran content to 40% and the food waste to 70%, respectively, both maintaining the cane bagasse concentration at 10% (Table 3).

All the experimental data were within the tolerance range, confirming the data predicted by the model obtained from the I-optimal experimental design. As a result, only the mixture (5) containing 70% FW showed an amylase synthesis slightly below the other mixtures, however, the protease production was similar for all compositions.

**Table 3 -** Results derived from the validation experiments for ternary mixtures of food waste (FW), sugarcane bagasse (SCB) and wheat bran (WB).

| Run | Wastes (%) | | | Response variables | | | |
|---|---|---|---|---|---|---|---|
| | FW | SCB | WB | Amylase activity (U/g) | | Protease activity (U/g) | |
| | | | | Experimental | Predicted | Experimental | Predicted |
| 1 | 60 | 10 | 30 | 154.50 ± 4.79 a* | 142.52 | 282.41 ± 2.51 a* | 256.25 |
| 2 | 50 | 20 | 30 | 145.30 ± 17.60 ab | 167.74 | 252.78 ± 13.36 a | 237.56 |
| 3 | 50 | 15 | 35 | 159.67 ± 6.97 a | 169.00 | 269.09 ± 42.80 a | 231.41 |
| 4 | 50 | 10 | 40 | 169.78 ± 7.90 a | 165.43 | 268.93 ± 3.19 a | 225.27 |
| 5 | 70 | 10 | 20 | 120.50 ± 9.20 b | 119.62 | 260.03 ± 6.39 a | 226.00 |

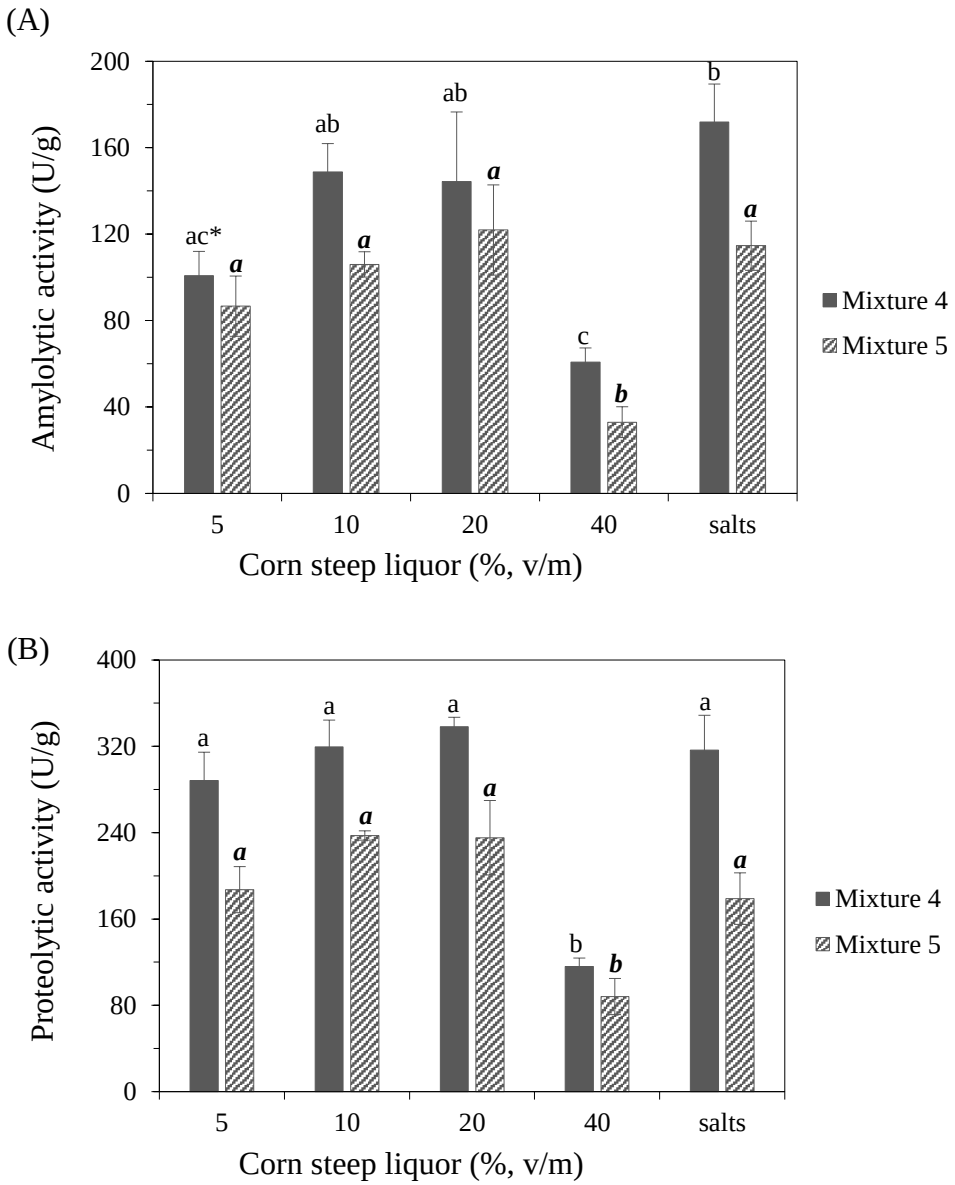
*Means followed by the same letter indicate no significant differences by Tuckey's test ($p > 0.05$).

### 3.4 Corn steep liquor as a nutritional supplement of SSF

Mixtures (4) and (5) were used as substrate to verify the effect of different amounts of corn steep liquor as SSF supplementation replacing the salt solution (Figure 4). For the mixture 4 (more efficient) the amount of 10 and 20% of corn steep liquor showed no statistical difference in relation to the control salts for amylases, showing the salts can be replaced by this ingredient. For the proteolytic activity and mixtures 4 and 5, there is no difference between the corn steep liquor in 5 to 20% and the salts in the control. These results reveal the possibility of using corn by-product as an economical alternative for the SSF medium enrichment to fungal growth and enzymatic production. Proportions of 10 and 20% (v/m) of corn steep liquor related

to the substrate (dry basis) of SSF was suitable. This can be explained by the high protein content that contributes to improve the nutritional quality of the SSF medium (Table 1).

**Figure 4 -** Influence of corn steep water supplementation on SSF medium compared to the salt solution in amylase (A) and protease (B) production by *R. oligosporus*. Mixtures (4) and (5) of the validation experiment were used (4): FW 50%, SCB 10%, WB 40% and (5): FW 70%, SCB 10%, WB 20%. *Means followed by the same bold and non-bold letters indicate no significant differences ($p > 0.05$). Statistical analysis was performed for each group individually.




However, 40% of corn steep liquor was excessive for the synthesis of both enzymes by the fungus *Rhizopus oligosporus*, using food waste as the main substrate during SSF (≥

50%). A reduction of 2-3.5-fold in the amylolytic and proteolytic activities was found when compared to the control. Additionally, complementation of the mixture (4) with 5% corn steep liquor resulted in an amylase expression similar to the medium supplemented with 40% ($p>0.05$), both inferior to the control.

Corn steep liquor has been reported as a low-cost nutrient source and an alternative to the expensive ingredients since it can provide nitrogen, carbon, vitamin or trace element to fermentative bioprocess (HOFER et al., 2018; TAIWO et al., 2018). Furthermore, it has been reported as a nitrogen source for submerged (DESSIE et al., 2018) and solid state process (AKHTAR et al., 2018; OLIVEIRA et al., 2019), and ethanol fermentation (TAIWO; MADZIMBAMUTO; OJUMU, 2018). Regarding enzymes production, although this nutrient can improve enzyme production (WANG et al., 2014), high concentrations decreased the enzymatic synthesis (KONA; QURESHI; PAI, 2001), as observed in the present work.

### 3.5 Two steps of culture for improvement of enzymes yield

The solid residue obtained after the enzyme's extraction from the culture in SSF represented the non-hydrolyzed substrate (not consumed during the first cultivation) and the biomass (mycelium) grown.

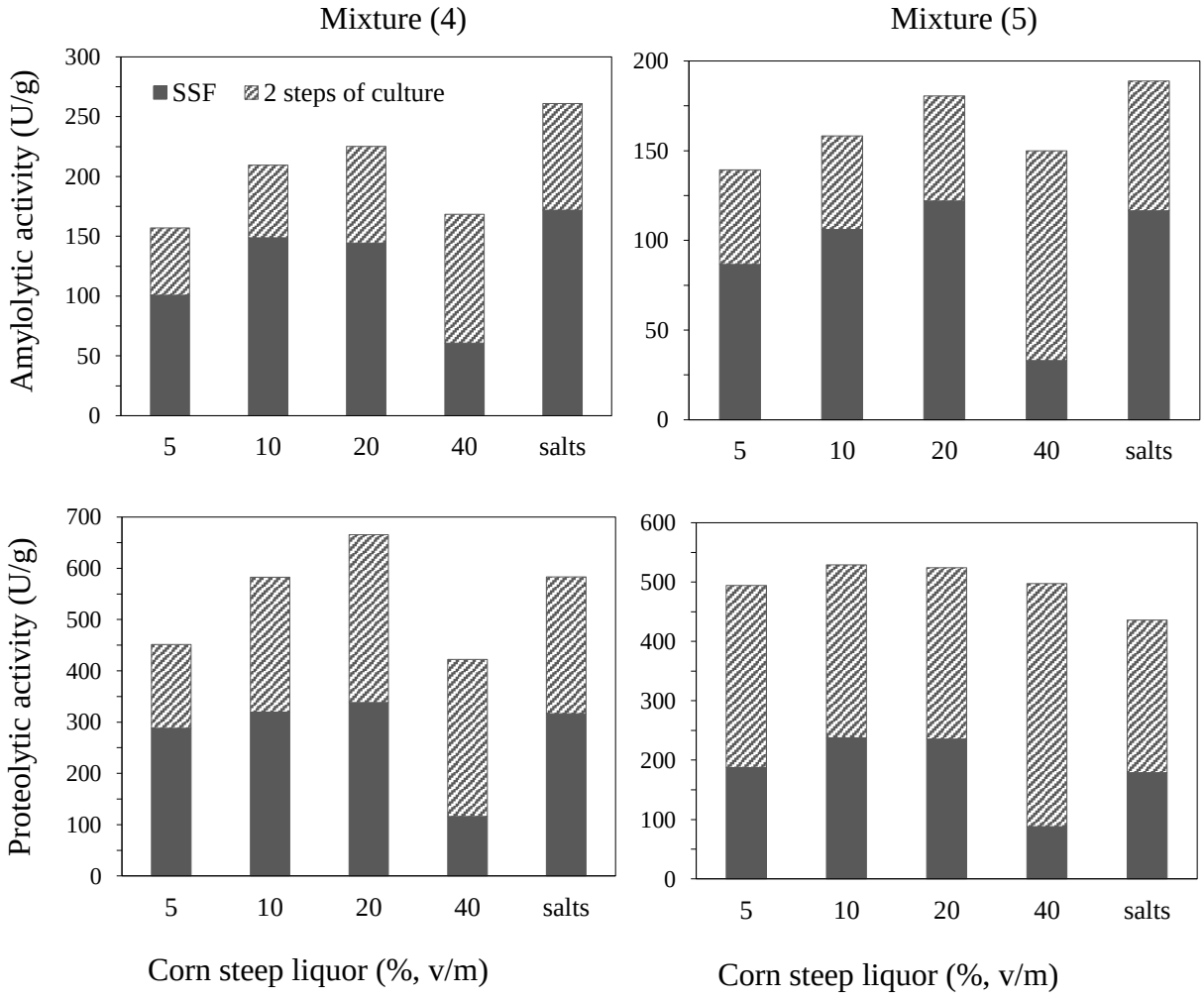
In this concept of two steps of fermentation aiming an increase in the enzymatic production, the sum of mycelium and non-hydrolyzed substrate was cultured by the second time, without any new addition of inoculum or new culture medium. The solid residues from the extraction of the Mixtures (4) and (5) (corn steep liquor tests) were evaluated in these two steps of fermentation system (Figure 5).

The highest amylolytic synthesis for both mixtures occurred in the first fermentation, so that the production of the second fermentation was a complement of the first, except for the mixtures supplemented with 40% corn steep liquor which inhibited the first stage of cultivation. The increase from 41 to 63% was achieved in media supplemented with 10 and 20% corn steep liquor or with salts, considering the amylase obtained in two cultures ($1^{st}$ and $2^{nd}$) compared to the amount of enzyme produced only in the first culture step.

In contrast, for the tests with 40% corn steep liquor, higher amylase yields occurred during the second culture than the first. The increase was about 2.8 times for the mixture (4) and 4.6 times for the mixture (5). These results can be explained by the incomplete bioconversion during the first culture, maybe due to some chemical inhibition of the enzymes

biosynthesis. Similar to the unfavorable effect of excess salts for enzymatic production, due to changes in the osmotic balance (ESCARAMBONI et al., 2018), excess of corn steep liquor was also detrimental to the synthesis of amylases and proteases by *R. oligosporus*. However, at the end of the first cultivation, washing for extraction of the enzymes also removes the excess of corn steep liquor. Thus, in the second cultivation, the residual mass had more favorable conditions than the previous ones and, therefore, the increase was so expressive, since the production of the first stage was very small.

**Figure 5 -** Amylase and protease biosynthesis by two subsequent cultures. Mixtures (4) and (5) of the validation experiment were used: (4): FW 50%, SCB 10%, WB 40% e (5): FW 70%, SCB 10%, WB 20%. Filled columns mean the enzymatic production by the first SSF process. Dashed columns mean the enzymatic production by two subsequent cultures using non-hydrolyzed substrate from and biomass grew in the 1st culture



In view of the protease yield, for the mixture (4), the increase of 83 to 97% was achieved with 10 and 20% corn steep liquor or salts supplementation in two steps culture (Figure 5). In addition, these compositions resulted in the best proteolytic syntheses (from 583 to 665 U/g). On the other hand, smaller yields were obtained with the mixture (5) and, unlike proteases in mixture (4) and amylases, the production was higher in the second fermentation than in the first.

Thus, 10 and 20% (v/m) corn steep liquor concentrations may be indicated as an alternative to the use of the salts for SSF with *Rhizopus oligosporus* to produce enzymes, because of their low cost and the nutritional benefit provided in the mixtures (4) and (5). In addition, the two steps of culture process represent an effective alternative to increase the utilization of the substrate and the yield of amylolytic and proteolytic enzyme production in SSF.

Although the concept of two step cultures using the non-hydrolysed substrate and biomass of the first step in the second one has been reported for silage fungal growth (BRITT; HUBER, 1975), and more recently for submerged fermentation processes, in the manufacture of beverages such as beer (ROSSI et al., 2018), thereby the bioprocess considered in this work shows for the first time to be possible in Solid State Fermentation process, as well. In fact, this bioprocess in two steps for SSF was: a) more efficient in proteases and amylases production, since a higher levels of yields were obtained, b) simpler, since a cells recycle and non-hydrolyzed substrate was used, c) economical, since only a food waste and low-cost agro-industrial wastes were used d) innovative, since it has been reported by the first time for SSF.

### 3.6 Food waste hydrolysis

The enzymatic extract produced in this work was able to hydrolyze food waste achieving 36.44% conversion yield and a hydrolysate with 47.10 g/L reducing sugars. No yield differences ($p>0.01$) were observed between 24 and 36 h (37.44%) of reaction time. These results demonstrate the effectiveness of the enzymes produced by *R. oligosporus* in SSF with >50% food waste. In agreement, the glucoamylase from the same fungus, but in SSF with only wheat bran, resulted in 42% cassava bagasse conversion in reducing sugars (ESCARAMBONI et al., 2018). The hydrolysis of municipal solid waste achieved similar results using amylases and cellulases (BALLESTEROS et al., 2010) and lower glucose concentration by applying the acid treatment (MAHMOODI; KARIMI; TAHERZADEH, 2018).

## 4 CONCLUSIONS

This study reports the use of food wastes for the production of fungal amylases and proteases in SSF or Solid State Fermentation. In kinetics studies for these enzymes production the best time of the cultures of *R. oligosporus* was 120 h. The I-optimal design method used in SSF modelling using food waste mixed with sugarcane bagasse and wheat bran in the cultures showed to be efficient in the definition of the best conditions for higher amylase and protease production. The supplementation of the process with 20% (v/m) corn steep liquor was suitable as an alternative of salts solution. The two steps cultures, in which the second used the non-hydrolysed substrates and biomass of the first, were responsible for a great increase in the total amylase and protease produced by SSF. Under the best conditions, the highest enzymatic production with *Rhizopus oligosporus* in SSF using food waste as the main substrate was the blend: 50% food waste, 10% sugarcane bagasse and 40% wheat bran supplemented with salt solution for amylases, and added by salts or 20% (v/w) corn steep liquor for proteases.

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## GENERAL CONCLUSIONS

This thesis proposes some biotechnological routes in order to obtain fungal enzymes, glucose, and ethanol from wastes as an alternative for the low cost production intercropped with food production, without any competition between them. Solid state fermentation was a suitable method to obtain fungal enzymes using residues as substrate. The influence of the medium composition and supplementation was demonstrated, so that, adequate mixtures of wastes can lead to high enzymes production with equivalent quality to current enzymes.

The agro-industrial wastes, wheat bran, sugarcane bagasse and corn steep liquor, and food waste as the urban residue, were able to supply the nutrients for *Rhizopus oligosporus* to produce amylases and proteases in solid state fermentation. Furthermore, while the cell recycling is a technique more specifically employed for submerged fermentation processes, this work proposed the two steps cultures in solid state fermentation, in which the second used the non-hydrolysed substrates and biomass of the first, to increase the amylase and protease yields. Besides the contributions given, the need to deepen these techniques for industrial application is evident, considering that the application of the enzymatic extract produced was demonstrated.

The use of the amylase produced for the hydrolysis of pure starch as well as of cassava bagasse was presented. In addition, the approach of different enzymes used in the waste biomass hydrolysis can be a strategy adopted to increase the conversion yields. In agreement, the fermentability of the cassava bagasse hydrolysate allowed to propose this residue as a substrate, until then unexplored for this purpose, to increase the productivity of bioethanol.

These strategies can open new economic, ecological, and social perspectives to glucose and bioethanol production, mainly in developing countries, contributing to supply the demand for renewable fuels in the world.

**CURRICULUM VITAE**

Bruna Escaramboni was born on October 3, 1989 in Assis, São Paulo, Brazil. Before university, Bruna took secondary education classes together with a Technical in Agroindustry (2005 to 2006) and Technical in Sugar and Alcohol (2006 to 2008) course at the Centro Estadual de Educação Tecnológica Paula Souza (CEETEPS) in Cândido Mota, São Paulo, Brazil. In 2008, Bruna entered the São Paulo State University (UNESP), campus Assis, to study Biological Sciences. From 2009 to 2011, Bruna conducted research on amylase production by *Rhizopus oligosporus* and *Rhizopus oryzae* in submerged fermentation under the supervision of Prof. Dr. Pedro de Oliva Neto. In 2012, Bruna started his master's course in Biological Sciences – Applied Microbiology at the São Paulo State University (UNESP), campus Rio Claro, under the supervision of Prof. Dr. Pedro de Oliva Neto. In this period, Bruna research about amylase production by *Rhizopus oligosporus* in solid state fermentation and filed a patent on that. In 2015, Bruna started his PhD course in the PhD Program in Bioenergy (USP, UNESP, UNICAMP) associated with the Bioenergy Research Institute (IPBEN) at UNESP campus Rio Claro, and developed research at the IPBEN, Bioprocess Unit, UNESP campus Assis, under the supervision of Prof. Dr. Pedro de Oliva Neto.

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