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**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
2019**

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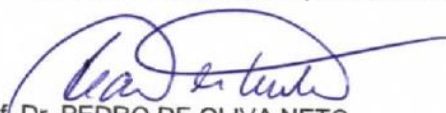
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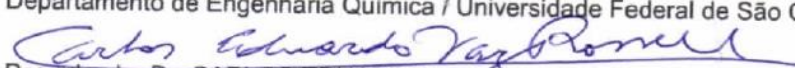
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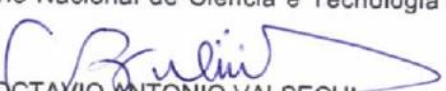
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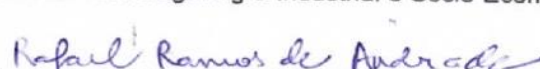
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*Inteiramente a Deus, por ser o centro e razão da minha vida;
Aos meus pais, Durval e Neide, e ao meu irmão,
Gustavo, por serem o meu alicerce;
Pelo amor, carinho e apoio
em todos os momentos.
Dedico.*

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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.”*

Eccl 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 celulasas (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.

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.

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