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**BIOPROCESSOS PARA PRODUÇÃO DE BIOMASSA DE LEVEDURA E
ENRIQUECIMENTO NUTRICIONAL DE RESÍDUOS AGROINDUSTRIAIS**

THAÍS YUMI SHINYA

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Área de Microbiologia Aplicada).

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Orientador: Prof Dr Pedro de Oliva Neto

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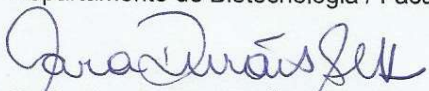
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*Para os meus pais, por todo esforço realizado
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Resumo

O aumento da população mundial levou à busca por novas fontes de proteína e maior produção animal. Nesse sentido, a biomassa microbiana produzida em larga escala demonstra ser uma saída viável para amenizar tais problemas, e, aliada ao uso de resíduos agroindustriais como substrato, representa uma solução para entraves ambientais, sociais e econômicos. O resíduo final tem seus valores nutricionais incrementados, sendo um interessante alimento para os animais. O objetivo desse estudo foi a seleção de leveduras com capacidade de crescer em bagaço de cana e de mandioca, pré-tratados ou não, para produção de biomassa microbiana e incremento do valor nutricional dos resíduos para uso como ração. A capacidade em crescer em anaerobiose, em ausência pontual de aminoácidos e vitaminas, assimilação de alguns carboidratos e avaliação da composição centesimal das espécies foram avaliados. A pré-seleção de nove leveduras isoladas do meio ambiente ocorreu em agitador orbital em aerobiose contendo bagaço de cana sem ou pré-tratado com CaO, H₂SO₄ e NaOH. Quatro linhagens foram selecionadas e cultivadas em fermentador com bagaço de cana e de mandioca hidrolisado com amilases ou não, em batelada simples ou alimentada. Uma linhagem de *Saccharomyces cerevisiae* M26 serviu como comparativo para os testes em fermentador. O pré-tratamento do bagaço de cana não foi benéfico para o crescimento celular. As maiores atividades celulolíticas foram atingidas para o meio contendo soro de leite, variando até 0,18 FPU/mL e 0,90 U/mL (CMCase). De acordo com as maiores concentrações celulares, quatro espécies foram selecionadas e identificadas como *Sporobolomyces japonicus* Sia 70a, *Sporidiobolus pararoseus* Sia 33.1, *Wickerhamomyces onychis* LABI2 e *Rhodotorula mucilaginosa* LABI1. Todas produziram xilanases em cultivo com bagaço de cana em fermentador (0,25 U/mL para *S. pararoseus* Sia 33.1, 0,31 U/mL da *R. mucilaginosa* LABI1, 0,34 U/mL da *W. onychis* LABI2 Sia 70a, 0,52 U/mL da *S. japonicus* Sia 70a), sendo as amilases produzidas por *S. japonicus* Sia 70a (0,2 U/mL), *S. pararoseus* Sia 33.1 (0,26 U/mL) e *R. mucilaginosa* LABI1 (0,33 U/mL) em cultivo com bagaço de mandioca. As maiores concentrações de biomassa foram atingidas com o uso do bagaço de mandioca como substrato. O bagaço de mandioca hidrolisado resultou na produção de 5,2 g/L de *S. pararoseus* Sia 33.1, 8,5 g/L de *R. mucilaginosa* LABI1 e 10,9 g/L de *W. onychis* LABI2, sendo muito superior ao obtido por *S. cerevisiae* M26 no mesmo meio (3,1 g/L). *S. japonicus* Sia 70a alcançou 8,1 g/L utilizando a mandioca sem tratamento, valor duas vezes maior que *S. cerevisiae* M26 nas mesmas condições. Após os cultivos em fermentador, ambos resíduos tiveram seu conteúdo proteico incrementado em todos tratamentos, sendo o máximo atingido de 1,8% para 8,3% (*S. pararoseus* Sia 33.1 em mandioca hidrolisada). As quatro cepas demonstraram capacidade em crescer em anaerobiose, produzir seus próprios aminoácidos e grande versatilidade no uso dos carboidratos testados. A biomassa unicelular obtida possui altas concentrações de carbono, nitrogênio, fósforo, com concentração proteica de 20,2% para *W. onychis* LABI2, 25,1% para *R. mucilaginosa* LABI1, 35,6% para *S. japonicus* Sia 70a e 43,3% para *S. pararoseus* Sia 33.1. Além do crescimento favorável em resíduos agroindustriais e incremento do valor nutricional desses resíduos, as leveduras possuem características desejáveis aos micro-organismos probióticos com possibilidade de uso na alimentação animal.

Palavras-chave: Proteína unicelular, leveduras probióticas, enriquecimento proteico de resíduos, biomassa microbiana.

Abstract

The increase of world population led to the search for new sources of protein and higher animal production. In this sense, microbial biomass produced on large scale demonstrate to be a viable way to soften such problems and combined with the use of agro-industrial residues represent a solution to environmental, social and economic concerns. The final residue has its nutritional values increased, being an interesting food for animals. The objective of this study was to select yeasts with growth capacity in sugarcane bagasse and cassava residue, pre-treated or not, for the production of microbial biomass as well as increase the residue nutritional value to use as feed. The ability to grow in anaerobiosis, absence of amino acids and vitamins, assimilation of some carbohydrates and evaluation of the chemical composition of the species were evaluated. The preselection of nine yeasts isolated from the environment occurred in orbital shaker incubator in aerobiosis with sugarcane bagasse with or without treatment (CaO, H₂SO₄ and NaOH). Four strains were selected and cultivated in bioreactor with sugarcane bagasse and cassava residue, hydrolysed with amylases or not, in single-batch or fed-batch process. An industrial strain of *Saccharomyces cerevisiae* M26 served as a comparison for bioreactor tests. Pretreatment of sugarcane bagasse was not beneficial for cell growth. Higher cellulolytic activities were measured in medium containing whey, ranging up to 0.18 FPU/mL and 0.90 U/mL (CMCase). According to the highest cellular concentrations, four yeasts were selected and identified as *Sporobolomyces japonicus* Sia 70a, *Sporidiobolus pararoseus* Sia 33.1, *Wickerhamomyces onychis* LABI2 and *Rhodotorula mucilaginosa* LABI1. All produced xylanase in bioreactor with sugarcane bagasse (0.25 U/mL for *S. pararoseus* Sia 33.1, 0.31 U/mL *R. mucilaginosa* LABI1, 0.34 U/mL *W. onychis* LABI2 and 0.52 U/mL *S. japonicus* Sia 70a), and amylases were produced by *S. pararoseus* Sia 33.1 (0.26 U/mL), *R. mucilaginosa* LABI1 (0.33 U/mL) and *S. japonicus* Sia 70a (0.2 U/mL) in cultures with cassava residue. The highest biomass concentrations were obtained using cassava residue as substrate. The hydrolyzed cassava residue produced 5.2 g/L of *S. pararoseus* Sia 33.1, 8.5 g/L of *R. mucilaginosa* LABI1 and 10.9 g/L of *W. onychis* LABI2, being much higher than obtained by *S. cerevisiae* M26 in the same medium formulated (3.1 g/L). *S. japonicus* Sia 70a produced 8.1 g/L after culture with cassava residue without treatment 2 times more than *S. cerevisiae* M26 under the same conditions. After fermentation, both residues had their protein content increased in all treatments, and the maximum reached from 1.8% to 8.3% was obtained by *S. pararoseus* Sia 33.1 in hydrolyzed cassava residue. The four strains demonstrated ability to grow in anaerobiosis, producing amino acids by its owns and great versatility in the use of the carbohydrates tested. The yeast biomass obtained had high concentrations of carbon, nitrogen and phosphorus, with a protein concentration of 20.3% for *W. onychis* LABI2, 25.1% for *R. mucilaginosa* LABI1, 35.6% for *S. japonicus* Sia 70a and 43.3% for *S. pararoseus* Sia 33.1. In addition to the favorable growth in agro-industrial residues and increase the the nutritional value of these residues, these yeasts have desirable characteristics as probiotic microorganisms with possible application in animal feed.

Key words: Single cell protein, probiotic yeasts, proteic enrichment of residues, microbial biomass.

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1 INTRODUÇÃO

Diversas razões sociais, geográficas e ambientais têm levado a procura pela produção sustentável de combustíveis e alimentos. O aumento da população mundial consequentemente criou uma pressão pela busca por novas fontes de alimentos e/ou aumento do desempenho e produtividade animal. Nesse sentido, a produção de proteína unicelular microbiana se mostra uma alternativa pela grande concentração proteica dessas células. Tal composto pode ser utilizado tanto na alimentação humana quanto animal, o que pode diminuir a necessidade de grandes áreas de pastagens, caso utilizada como alimentos para gado e caprinos por exemplo, que podem ser utilizadas para outros fins. Além disso, a obtenção dessa proteína pode ser realizada através do cultivo em resíduos agroindustriais, enriquecendo seu valor nutricional para uso como ração animal sem a necessidade de adição de químicos ou realização de pré-tratamentos físico-químicos custosos para tal fim. Outra vantagem são os possíveis efeitos probióticos causados por certos micro-organismos, levando a um efeito benéfico para o animal. A principal espécie de levedura utilizada como fonte de proteína unicelular e probiótico é a *Saccharomyces cerevisiae* podendo também expressar atividade probiótica. Devido à alta biodiversidade no Brasil, é de grande interesse científico e tecnológico o melhor aproveitamento das características fisiológicas que outras diferentes espécies podem fornecer, aumentando a gama das possibilidades além daquelas existentes em *S. cerevisiae*.

O uso de substratos baratos como os resíduos possui destaque tendo em vista a grande quantidade que geralmente é lançada no meio ambiente e causa sérios problemas ambientais. Tais resíduos são formados por substâncias que podem ser aproveitadas para obtenção de produtos de alto valor agregado, sendo uma alternativa para os entraves ambientais e econômicos. A bioconversão destes resíduos representa uma maneira viável de produção pela facilidade de escalonamento e de cultivo, sem a necessidade de uma grande área para sua realização. Ainda assim, o desenvolvimento de bioprocessos para obtenção de tais produtos ainda está em progresso.

2 REFERENCIAL TEÓRICO

2.1 Resíduos agroindustriais

A intensa atividade agroindustrial mundial gera toneladas de resíduos e subprodutos da indústria. Apesar do grande apelo ambiental, uma grande parte é descartada na natureza, o que causa sérios problemas ambientais, resultantes do acúmulo no meio ambiente (PINTO et al., 2005; YI et al., 2009; FERREIRA-LEITÃO et al., 2010). Projeções sobre a escassez de recursos naturais aumentou ainda a demanda pela busca de atividades sustentáveis que, aliado a leis que

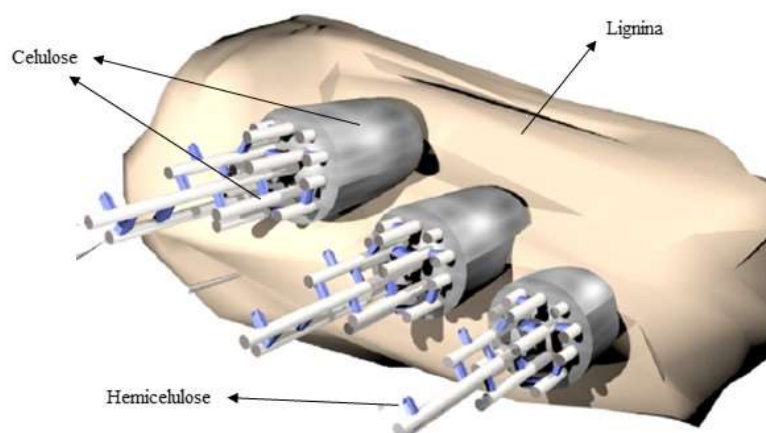
prevêm essa redução da geração de resíduos, incentiva a geração de pesquisas visando o aproveitamento tecnológico destes subprodutos (PNUMA, 2005). Apesar de alguns resíduos gerados possuírem certo valor nutritivo, sendo utilizados diretamente como alimento ou componente de dietas formuladas industrialmente para gado, a maioria *a priori* não gera grandes ganhos econômicos para a indústria (YANG et al., 2001; YI et al., 2009).

A grande quantidade de substâncias aproveitáveis nestes resíduos, que podem ser utilizados como substrato para produção de outros produtos com maior valor agregado, tais como o etanol, proteínas unicelulares, cogumelos, enzimas, óleos essenciais, ácidos orgânicos, aminoácidos (PANDEY et al., 2000b; SOCCOL e VANDENBERGHE, 2003; CHANDEL et al., 2012). Com isso, uma crescente atenção fosse voltada às pesquisas para o reaproveitamento de resíduos e minimização do impacto causado pelo acúmulo destes no meio ambiente (COELHO et al., 2001). Estas pesquisas vêm se tornando mais necessárias dadas as limitações de terras agriculturáveis, aumento populacional e os problemas ambientais que vêm se agravando.

2.1.1 Bagaço de cana-de-açúcar

As matérias-primas lignocelulósicas são as fontes renováveis mais abundantemente encontradas na natureza, sendo compreendidas, majoritariamente, pelos materiais agroindustriais e pelos resíduos urbanos (CASTRO e PEREIRA JR, 2010). Esses materiais lignocelulósicos são formados por uma estrutura complexa composta da interação entre celulose, lignina e hemicelulose, que são unidas entre si por ligações covalentes (entre hemicelulose e lignina) e pontes de hidrogênio (entre hemicelulose e celulose), formando uma rede estável e resistente a ataques microbianos (GARROTE et al., 2002; FERREIRA-LEITÃO et al., 2010; HAMZEH et al., 2013). Por meio de uma despolimerização, essas estruturas complexas podem se tornar simples monômeros que podem, por sua vez, serem convertidos em bioetanol e outros produtos de alto valor agregado (SOCCOL et al., 2010; CHANDEL et al., 2012). Internamente, as fibrilas da fração celulósica encontram-se dispostas como espirais, de forma a conferir força e flexibilidade ao material, sendo envolvida pela lignina, que aumenta a resistência da estrutura a ataques químicos e enzimáticos (ALCADE, 2015; BEZERRA e RAGAUSKAS, 2016). Já a hemicelulose atua como um elo químico entre as outras duas frações (ALCADE, 2015; BEZERRA e RAGAUSKAS, 2016) (Figura 1). Assim, as fibras lignocelulósicas podem ser consideradas compósitos de fibrilas de celulose mantidas juntas por uma matriz que possui lignina e hemicelulose (SILVA et al., 2009).

Figura 1 – Esquema da parede celular elucidando o complexo lignocelulolítico (modificado de ALCADE, 2015).



A cana-de-açúcar (*Saccharum* sp.) é uma das mais abundantes matérias-primas mundiais, consistindo em seis espécies: *S. officinarum*, *S. spontaneum*, *S. robustum*, *S. sinense*, *S. barberi* e *S. edule* (BEZERRA e RAGAUSKAS, 2016). Assim sendo, um dos maiores subprodutos lignocelulósicos da agroindústria mundial é o bagaço de cana-de-açúcar. Este resíduo é o material orgânico fibroso que sobra depois do processo de moagem, após extração do caldo, para produzir açúcar e álcool (PANDEY et al., 2000a; SILVA et al., 2007; MULINARI et al., 2009). Cerca de 90% do bagaço produzido é utilizado pelas próprias usinas na combustão em caldeiras e geração de energia elétrica, porém de forma ineficiente. Caso fosse feito um aproveitamento mais eficiente, utilizando-se metade do bagaço de cana produzido seria suficiente para suprir a demanda elétrica da usina, sendo o restante deste resíduo comercializado (PANDEY et al., 2000a; SILVA et al., 2007; FERREIRA-LEITÃO et al., 2010; KARP et al., 2013; ROCHA et al., 2011). Somente no Brasil a quantidade de cana-de-açúcar obtida na safra de 2015-2016 ultrapassou 666 milhões de toneladas (UNICA, 2016), sendo que para cada tonelada de cana são gerados cerca de 280 kg de bagaço úmido (50% de umidade) ou 140 kg em massa seca (SOCCOL et al., 2010).

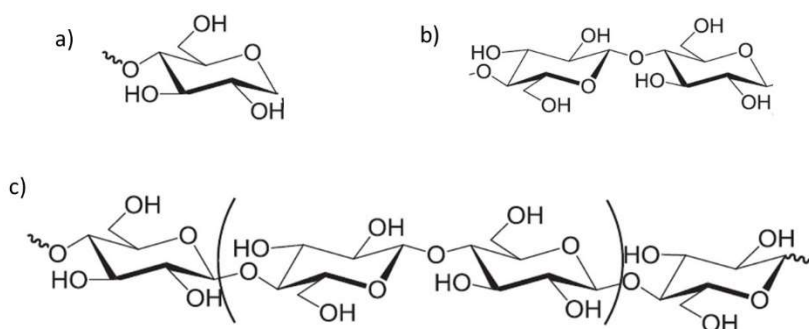
A caracterização do bagaço de cana é dificultada por diversos fatores como as condições climáticas, localização, variedades de espécie, além do tipo de análise realizada, sendo composto por porcentagens de aproximadamente 45% de celulose, 27% de hemicelulose e de 21,1% de lignina, além de 2,2% de cinzas e 4,6% de extrativos (WALFORD, 2008; CARDONA et al., 2010; CHANDEL et al., 2012; ROCHA et al., 2011). Tal caracterização dificilmente alcançará um balanço de 100% de massa, e sendo assim a composição do bagaço de cana não é um padrão observado dentre os autores (ROCHA et al., 2011). A composição complexa do

bagaço de cana e seu baixo valor nutricional limitam o seu uso como ração animal em comparação a outros resíduos, como farelo de trigo e de arroz. Entretanto, um enriquecimento com proteína microbiana através da fermentação pode torná-lo mais atrativo para esse fim (BRAVO et al., 1994; BEZERRA e RAGAUSKAS, 2016). Além dessa finalidade, tal substrato pode servir para produção de enzimas, como celulases, pectinases e xilanases, etanol de segunda geração e outros (DUEÑAS et al., 1995; SOLIS-PEREYRA et al., 1996; MULINARI et al., 2009; SILVA et al., 2009; BEZERRA e RAGAUSKAS, 2016).

2.1.1.1 Celulose

A celulose é o polímero orgânico mais abundante do planeta e o maior componente da biomassa vegetal (SANCHÉZ, 2009; SILVA et al., 2009). Pode ser encontrada na forma pura com alta cristalinidade, como no algodão, mas é comumente encontrada associada à hemicelulose e lignina compondo a parede celular das plantas (LYND et al., 2002; SILVA et al., 2009). É um polímero linear de glicose (FERREIRA-LEITÃO et al., 2010; BEZERRA e RAGAUSKAS, 2016) e possui uma estrutura relativamente simples, sendo formada de monômeros de D-glicose unidos através de ligações glicosídicas β -(1,4), sendo composta por unidades monoméricas de celobiose, a qual é formada pela junção de duas moléculas de glicose seguida da eliminação da água através das hidroxilas ligadas ao carbono 1 e 4 (FENGEL e WEGENER, 1989; BEZERRA e RAGAUSKAS, 2016) (Figura 2).

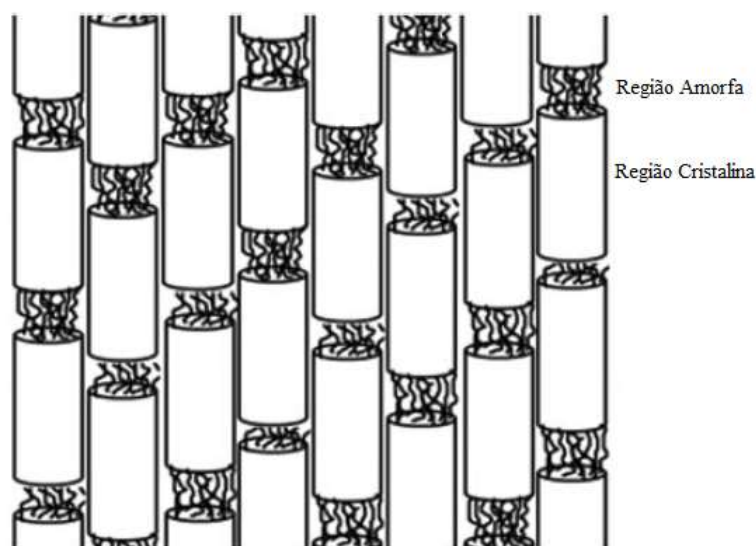
Figura 2 - a) unidade de D-glicose; b) celobiose; c) estrutura linear de celulose (modificado de BEZERRA e RAGAUSKAS, 2016).



A massa molecular da celulose do bagaço de cana varia de 157.800 a 168.400 g/mol, possuindo fibras de 1,0 a 1,5 mm (BIAN et al., 2014). A celulose apresenta estrutura cristalina, em que as fibrilas são unidas por ligações de hidrogênio tanto intra quanto inter cadeias de celulose (SUN e CHENG, 2002; PU et al., 2008; BEZERRA e RAGAUSKAS, 2016). Como

cada camada adjacente de celulose é unida por forças de van der Waals, a fibra é densamente compacta, o que impede o acesso de enzimas e moléculas pequenas (FENGEL e WEGENER, 1989). As regiões cristalinas da celulose (2/3 do homopolímero) são intermediadas por regiões menos compactas, chamadas amorfas em que as fibras apresentam maior distância uma das outras (FENGEL e WEGENER, 1989) (Figura 3). Irregularidades dentre as fibras de celulose, como microporos e falhas, também facilitam o acesso das moléculas e enzimas celulolíticas (FENGEL e WEGENER, 1989; LYND et al., 2002). Essa característica semicristalina faz com que a degradação da celulose seja considerada um problema, e raramente ocorre por meio de um processo isolado. Além disso, a matriz de hemicelulose aumenta mais a resistência à degradação por químicos e enzimas (BEZERRA e RAGAUSKAS, 2016).

Figura 3 - Estrutura esquemática de uma fibrila de celulose mostrando regiões cristalinas e amorfas (MARIANO et al., 2014).

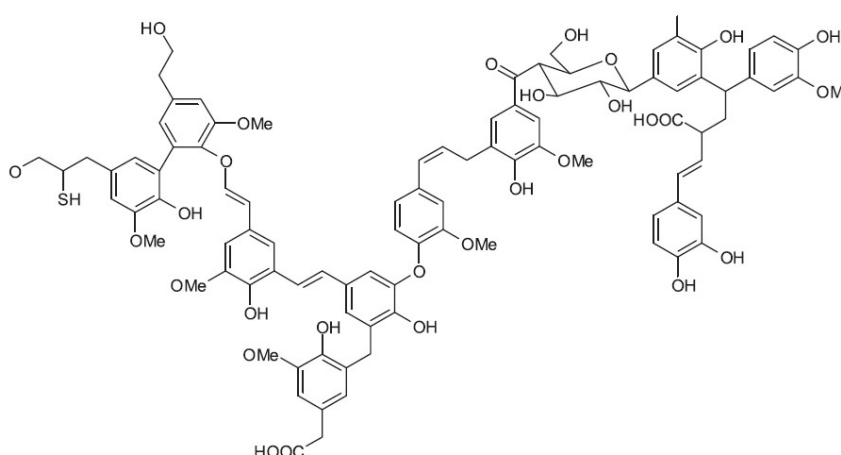


2.1.1.2 Lignina

A lignina é uma macromolécula aromática com ligações cruzadas e de alta massa molecular (FERREIRA-LEITÃO et al., 2010), sendo uma molécula complexa responsável por manter unidas as fibras dos materiais lignocelulósicos (WAGNER e WOLF, 1999; BEZERRA e RAGAUSKAS, 2016). É constituída por unidades derivadas de fenilpropano, como unidades de álcool trans-p-cumarílico, álcool trans-coniferílico e álcool trans-sinapílico (FENGEL e WEGENER, 1989), com uma conformação tridimensional amorfa e ligações carbono-oxigênio-carbono que unem pequenas cadeias de hidrocarbonos, criando uma proteção física rígida que protege a planta de ações enzimáticas (SILVA et al., 2009; MOGHADDAM et al., 2014)

(Figura 4). A hidrólise da lignina dá-se pela ruptura das ligações C-O-C, liberando os hidrocarbonos menores, que podem ser tratados para produção de álcool (RABELO, 2007). A massa molecular da lignina do bagaço de cana varia de 507 a 3.97 g/mol (ZENG et al., 2014), e sua fração na cana e no bagaço varia aproximadamente de 4,0% a 25,0% correspondente a fração não-polissacarídica dos materiais lignocelulósicos (ANDRADE et al., 2004; CRUZ et al., 2014).

Figura 4 - Estrutura química da lignina da lignocelulose (WILSON e LEE, 2016).



As funções da lignina são: fornecer suporte estrutural à parede secundária de plantas vasculares, tornar a parede celular vegetal hidrofóbica, permitindo o desenvolvimento eficiente dos tecidos para transporte de água em plantas vasculares e conferir resistência contra ataques microbianos (KUHAD et al., 1997a; LEE e MOON, 2003). Como a lignina apresenta um dos principais entraves para os processos industriais, como polpação da madeira para fabricação de papel e produção de açúcares solúveis a partir de biomassa vegetal, é interessante que a lignina seja removida, podendo ser utilizada em aplicações específicas como produção de quelantes através de oxidação enzimática (GONÇALVES e OVIEDO, 2002; ZHAO et al., 2009), produção de adesivos para madeira (EL MANSOURI e SALVADÓ, 2006), vanilina e ácido gálico (HOWARD et al., 2003; PRIEFERT et al., 2001).

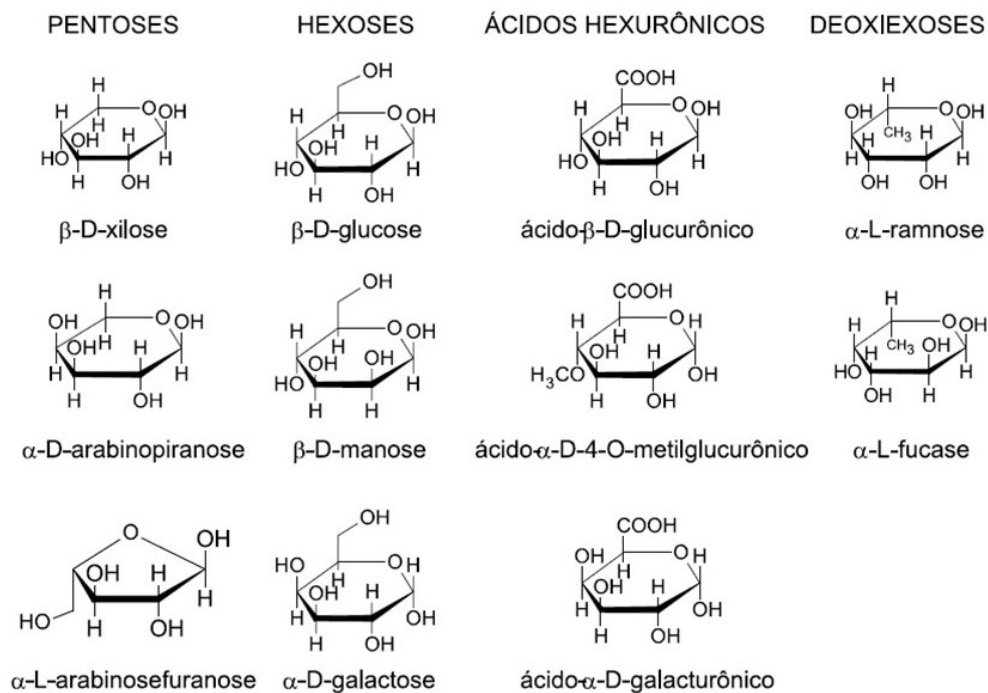
2.1.1.3 Hemicelulose

A hemicelulose é um heteropolímero altamente ramificado, formado por uma estrutura complexa de carboidratos poliméricos, incluindo a xilana, xiloglicana (heteropolímero de D-xilose e D-glicose), glicomanana (heteropolímero de D-glicose e D-manose), galactoglicomanana (heteropolímero de D-galactose, D-glicose e D-manose) e

arabinogalactana (heteropolímero de D-galactose e arabinose) (SHALLOM e SHOHAM, 2003; FERREIRA-LEITÃO et al., 2010) (Figura 5). A xilana é o principal componente da fração hemicelulósica e o segundo polissacarídeo mais abundante na natureza (COLLINS et al., 2006; SILVA et al., 2009). A massa molecular da hemicelulose no bagaço de cana é de aproximadamente 7.380 g/mol (SUN et al., 2004), e segundo Peng et al. (2010), os principais constituintes da hemicelulose são galactose (11,5% a 39,9%), xilose (15,5% a 28,9%), glicose (17,5% a 50,5%), arabinose (5,35% a 14,31%), manose (até 14,0%), raminose e ácidos urônicos em menores quantidades. Aproximadamente 19,9% da biomassa da cana é composta por hemicelulose (ANDRADE et al., 2004), enquanto o bagaço de cana possui entre 30 a 35% dessa fração (CARDONA et al., 2010). Diversos tratamentos visam a remoção da hemicelulose (BEZERRA e RAGAUSKAS, 2016).

Devido à estrutura complexa da fração hemicelulósica, torna-se necessário uma variedade de enzimas para sua degradação ou modificação. As duas principais enzimas capazes de despolimerizar a cadeia de hemicelulose são a endo- β -1,4-D-xilanase e a endo- β -1,4-D-mananase, ambas glicosilhidrolases (SINGH et al., 2003). No processo de hidrólise ácida, a hemicelulose é decomposta em xilose, arabinose, ácido urônico e furfural (PATURAU, 1989).

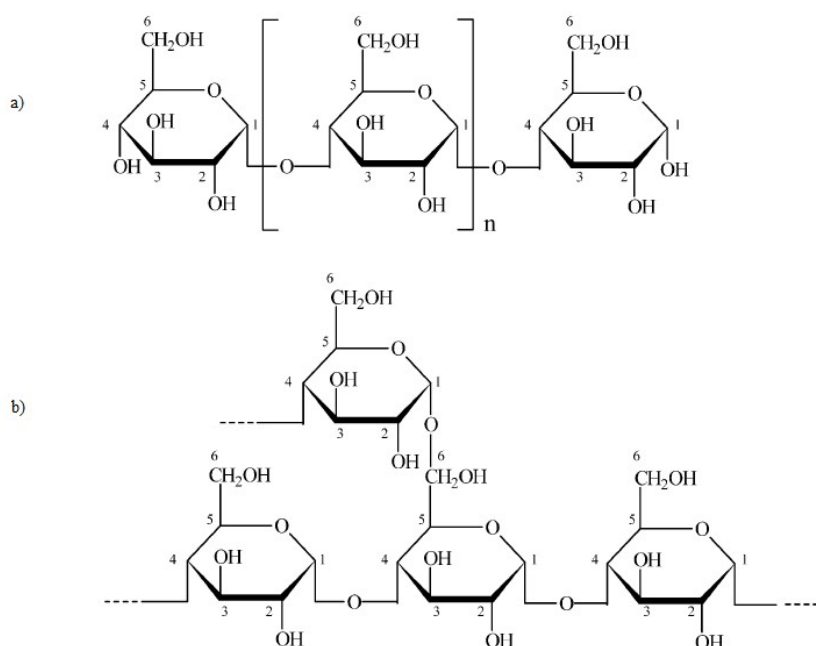
Figura 5 – Monômeros que compõem a hemicelulose (MORAIS et al., 2005).



2.1.2 Bagaço de mandioca

O amido é um carboidrato de reserva presente em diversas espécies vegetais, composto por dois polímeros: a amilose e a amilopectina (LAJOLO e MENEZES, 2006; PINTO, 2009). O primeiro possui cadeias lineares de unidades de D-glicose unidas por ligações α -1,4, e o segundo é ramificado, com ligações α -1,4 lineares e α -1,6 nas ramificações (NELSON e COX, 2006; PINTO, 2009) (Figura 6).

Figura 6 – Estrutura da: a) amilose; e b) amilopectina (HERRERO-MARTÍNEZ et al., 2004).



A mandioca (*Manihot esculenta*) é uma das principais fontes alimentícias nos continentes africano, asiático e latino-americano, sendo considerada um dos seis alimentos mais importantes no mundo (PANDEY et al., 2000b). É consumida diretamente pelos humanos na forma natural ou fermentada, representando 60% do total produzido mundialmente, sendo o restante direcionado à alimentação animal (33%) e indústria (7%) (PANDEY et al., 2000b). O Brasil figura entre os principais produtores de mandioca, estando entre Nigéria, Tailândia e Indonésia, sendo que em 2016 foram produzidos cerca de 23 milhões de toneladas de mandioca somente no Brasil destes (FAO, 2016), cerca de 1,6 milhões de toneladas processadas na indústria. Tal processamento da mandioca para produção de farinha e amido gera resíduos sólido e líquido, sendo que para cada 300 toneladas de mandioca processada, aproximadamente 280 toneladas de bagaço de mandioca (com 85% de umidade) são gerados (PANDEY et al., 2000b). Os resíduos sólidos e líquidos geralmente são descartados no ambiente sem tratamento,

gerando sérios problemas ambientais como mudança da microbiota, e principalmente produção de compostos tóxicos e gases nocivos (PANDEY et al., 2000b).

O bagaço de mandioca é um resíduo fibroso, com aproximadamente 50% de amido (base seca) em sua composição, considerado o carboidrato principal, e em torno de 43% de fibras (LEONEL e CEREDA, 2000; FERREIRA-LEITÃO et al., 2010). Sua alta capacidade em reter água aumenta custos com processos de secagem e transporte desse resíduo, sendo em grande parte depositado no meio ambiente, gerando problemas ambientais e de ocupação de áreas que poderiam ser melhor aproveitadas (CURTO, 1998). Dentre os resíduos amiláceos, o bagaço de mandioca destaca-se como substrato de culturas microbianas por sua baixa quantidade de cinzas (entre 0,66% a 1,5%) sendo um resíduo que poderia ser melhor reaproveitado devido à sua composição (CEREDA, 1994; STERTZ, 1997; VANDENBERGHE, 1998; PANDEY et al., 2000b; FERREIRA-LEITÃO et al., 2010). Estudos demonstram a utilização do bagaço de mandioca para produção de diferentes compostos aromáticos (BRAMORSKI et al., 1998; MEDEIROS, 1998), cogumelos (BARBOSA et al., 1995; BEUX et al., 1995), ácidos orgânicos (CARTA et al., 1999; VANDENBERGHE, 1998) e enzimas (GONÇALVES, 2016). A grande maioria dos estudos é realizada por fermentação em estado sólido, utilizando principalmente fungos filamentosos como agentes (PANDEY et al., 2000b; FERREIRA-LEITÃO et al., 2010).

Além da facilidade em utilizar o bagaço de mandioca sem tratamento, outra alternativa para conversão dos carboidratos presente no resíduo em bioprodutos é o cultivo após hidrólise enzimática com enzimas do complexo amilolítico ou utilizando micro-organismos que produzam amilases possibilitando assim fermentação com sacarificação simultânea (PANDEY et al., 2000b). A obtenção de xarope de glicose a partir do resíduo após ação enzimática é um atrativo principalmente para produção de etanol de segunda geração (FERREIRA-LEITÃO et al., 2010). Seu baixo teor proteico faz com que seu uso não seja adequado para alimentação animal direta, embora a adição de suplementos torna-o viável para uso como ração (PANDEY et al., 2000b). Outra alternativa é a realização de uma simples fermentação, que aumentaria seu valor nutricional, podendo assim ser utilizado como alimento animal, diminuindo seu acúmulo no meio ambiente, e com possibilidade de trazer ganhos econômicos para a indústria (BRAVO et al., 1994).

2.1.3 Água de maceração de milho

O processamento do milho resulta em subprodutos sólidos e líquidos que, se depositados no meio ambiente sem tratamento, podem se tornar fontes de contaminação e poluição ambiental (LOSS, 2009). Em 2009 cerca de 42 milhões de toneladas de milho foram

produzidos, gerando resíduos como a palha, sabugo e milhocina – água de maceração de milho – que podem ser reaproveitados para produção de produtos de alto valor agregado (LOSS, 2009). Dentre eles, a água de maceração de milho vem sendo usada na formulação de meios de cultura, sendo uma excelente fonte de nitrogênio orgânico, podendo suprir também carboidratos, peptídeos, minerais, metais e vitaminas, muitas vezes podendo ser utilizada como um substituto ao uso do extrato de levedura, que pode baratear custos com o meio de cultura (AMARTEY e LEUNG, 2000; RIVAS et al., 2004).

2.2 Enzimas

Enzimas são proteínas que catalisam reações bioquímicas, estando envolvidas em todos processos bioquímicos que ocorrem nas células (OLIVEIRA et al., 2004). Além da sua importância biológica, as enzimas atuam em diversos processos industriais, sendo altamente importantes na indústria de alimentos, têxtil, sucroalcooleira, área da saúde, área ambiental, entre outras (OLIVEIRA et al., 2004). Elas são responsáveis também pela decomposição de moléculas complexas em unidades menores, sendo altamente específicas (OLIVEIRA et al., 2004). O mercado enzimático mobiliza bilhões de dólares anualmente, estimado em 3,3 bilhões de dólares em 2010, com projeções anuais de 6% (BINOD et al., 1981). De acordo com Pandey et al. (2005) há seis grupos em que as enzimas podem ser alocadas (Tabela 1).

Tabela 1 – Classificação internacional das enzimas de acordo com a International Union of Biochemistry and Molecular Biology (IUBMB) (PANDEY et al., 2005; CARVALHO, 2015).

Grupo de Enzimas	Tipo de Reação	Quantidade listada até 2015 no IUBMB
EC 1 Oxirredutases	Catalisam reações de oxi-redução, envolvendo oxigenação ou remoção de hidrogênio	1011
EC 2 Transferases	Mediam a transferência de grupos acil, açúcares, fosforil e aldeído ou porções de cetonas de uma molécula para outra	1057
EC 3 Hidrolases	Promovem hidrólise e formação de ésteres, glicosídeos, amidas, éteres, peptídeos e outros grupos que contenham C-N	1041
EC 4 Liasas	Catalisam reações de adição, usualmente de HX, as duplasligações como C=C, C=N e C=O, e processos reversos	312
EC 5 Isomerases	Efetua, várias isomerizações, incluindo migração da ligação C=C, isomerização cis-trans e racemização	157
EC 6 Ligases	Mediam a formação ou clivagem de C-O, C-S, C-N, C-C e ligações éteres fosfato, por meio de reações acopladas à quebra de ATP	129

Diversos micro-organismos produzem enzimas usadas comercialmente, dentre elas: amilases, lipases, proteases, pectinases, glicose-oxidases (PANDEY et al., 2005). As duas principais formas de produção de enzimas por micro-organismos são a fermentação no estado sólido (FES), com fase não-aquosa predominante, e a fermentação submersa (FSm), com fase aquosa abundante (PANDEY e RADHAKRISHNAN, 1992; CASTRO e PEREIRA Jr, 2010).

A FES, comparada com a FSm, possui vantagens como: fácil manejo e manutenção, menor custo de operação, maior concentração de produtos formados, facilidade na extração do produto com a utilização de solventes apropriados, menor chance de contaminação e possibilidade de se usar resíduos agroindustriais como bagaços de cana-de-açúcar e de laranja, cascas de frutas, farelos de trigo e de mandioca, entre outros, como substratos para obtenção de produtos com alto valor agregado (MITCHELL et al., 2000; PANDEY et al., 2000a; SINGHANIA et al., 2010). Entretanto, as dificuldades no controle do processo fermentativo, necessidade de um grande inóculo são desvantagens comparada à FSm (MITCHELL et al., 2000). Na FSm, há um melhor controle do processo, recuperação facilitada de enzimas extracelulares e a determinação de biomassa, sendo realizadas por filtração simples ou centrifugação para a remoção das células, sendo o sobrenadante da cultura utilizado para os estudos enzimáticos, e o crescimento microbiano quantificado após secagem da biomassa, por

gravimetria, podendo ainda ser realizado por densidade ótica (LIMA et al., 2003; SOUZA e MAGALHÃES, 2010).

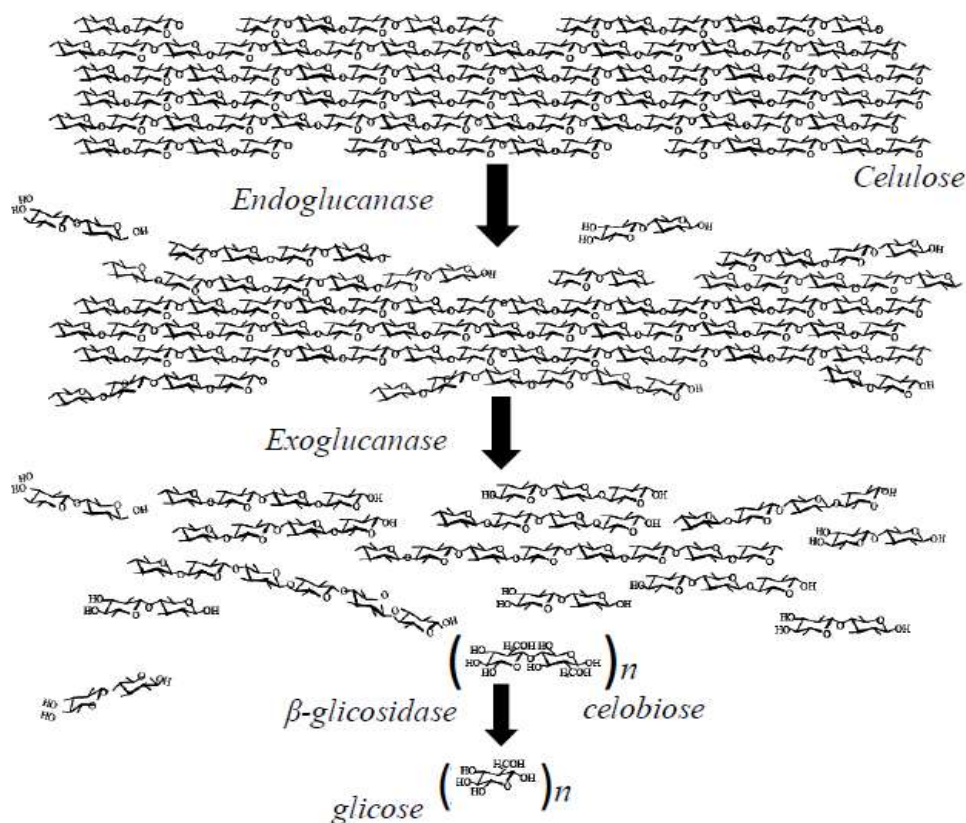
Diversos substratos são utilizados com o objetivo de induzir a produção de todas as enzimas, entre eles o papel de filtro, carboximetilcelulose (CMC) e resíduos agroindustriais (AGUIAR e MENEZES, 2000; GONÇALVES, 2010). Os resíduos agrícolas podem ser moídos ou triturados para servir de fonte de nutrientes, ou ainda enriquecidos com fontes extras de nitrogênio, minerais e/ou vitaminas tornando-se fonte de produção elevada de atividade enzimática (BISARIA e GHOSE, 1981). O poder de indução da síntese de enzimas desses materiais varia de acordo com a concentração em que se encontram e podem, também, servir como fonte de carbono para o crescimento celular (GONG e TSAO, 1975).

2.2.1 Celulases

Uma grande variedade de fungos e bactérias consegue degradar o material lignocelulósico usando um conjunto de enzimas hidrolíticas e oxidativas (VITTI, 1988). As celulases são enzimas que formam um complexo com capacidade de agir sobre materiais celulósicos. São biocatalisadores altamente específicos que atuam em sinergia para a liberação de açúcares hidrolisando a celulose e liberando a glicose, despertando um grande interesse industrial, devido à possibilidade de sua conversão em etanol, xilitol, xilooligossacarídeos (XOS) (OLSSON e HAHN-HAGERDAL, 1996). Os fungos filamentosos são os mais utilizados industrialmente para produção de celulases, principalmente representantes dos gêneros *Aspergillus*, *Trichoderma*, *Hemicella*, *Penicillium*, *Fusarium*, *Phanerochaete* (SINGHANIA et al., 2010).

As principais enzimas responsáveis pela quebra das ligações β -1,4 da celulose são as endoglucanases (β -1,4-D-glucanohidrolase), as exoglucanases (β -1,4-D-glucanocelobiohidrolase) e as β -glucosidases, todas encontradas em certas espécies de fungos (LYND et al., 2002). As endoglucanases são responsáveis pela quebra das ligações β -1,4 da celulose, deixando, assim, as pontas das regiões amorfas abertas para hidrólise pelas celobiohidrolases, formando fragmentos menores. Finalmente, as β -glucosidases hidrolisam celobiose de baixa massa molecular liberando os monômeros de glicose (LESCHINE, 1995; VAN RENSBURNG et al., 1996; BOISSET et al., 2000; SUN e CHENG, 2002). As enzimas do complexo celulolítico atacam o substrato sinergicamente, o que resulta num aumento significativo da velocidade de formação de produtos solúveis quando comparado à velocidade de ação das enzimas isoladamente (MARTINS et al., 2005; SANDGREEN et al., 2005) (Figura 7).

Figura 7 – Esquema da ação das celulasas em materiais lignocelulósicos (MARTINS, 2005).

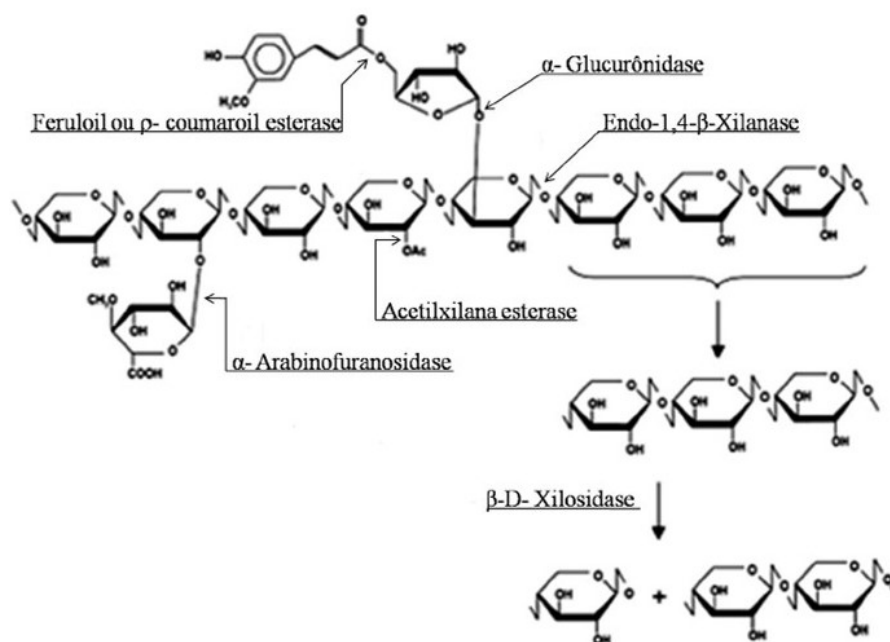


2.2.2 Xilanases

As enzimas xilanolíticas estão no grupo das glicosidasas (hidrolases) que catalisam a hidrólise das ligações β -1,4 dos resíduos de β -xilopiranosse na xilana, principal constituinte da hemicelulose dos resíduos lignocelulósicos (JUTURU e WU, 2012; AHMAD et al., 2013). A hidrólise da xilana requer um consórcio de enzimas hidrolíticas que atuam de forma conjunta na despolimerização da hemicelulose convertendo a xilana em xilooligossacarídeos e xilose (BEG et al., 2001; JUTURU e WU, 2012; AHMAD et al., 2013). Entre elas estão as β -1,4-endoxilanase, β -xilosidase, acetil xilana esterase, α -arabinofuranosidade, α -glucuronidase, ácido ρ -coumárico esterase e ácido ferúlico esterase (BEG et al., 2001; POLIZELI, 2009) (Figura 8).

A principal forma de obtenção das xilanases é a produção por micro-organismos, principalmente fungos dos gêneros *Aspergillus* e *Trichoderma* (FENGXIA et al., 2008; LIU et al., 2011; AHMAD et al., 2013). Suas aplicações são diversas, incluindo o branqueamento de papel e polpa, indústria farmacêutica, de alimentos e rações (DERVILLY et al., 2002; COLLINS et al., 2006; SOLEIMANI et al., 2006; BAJPAI, 2012).

Figura 8 – Enzimas que atuam na despolimerização da xilana (COSTA, 2016).



2.2.3 Amilases

As amilases pertencem à classe das hidrolases, catalisando a hidrólise do amido e seus derivados, e representam 25% do mercado mundial de enzimas (GUANDALINI, 2007; KUMAR et al., 2012). São responsáveis pela hidrólise do amido acumulado nas raízes, digestão do amido contido nos alimentos e fonte de carbono em muitos micro-organismos (PEIXOTO et al., 2003). Os principais fungos produtores de amilases são do gênero *Aspergillus*, *Rhizopus* e *Fusarium* (PANDEY et al., 2005; TORRADO et al., 2013).

A classificação das amilases compreende os grupos endoamilases, exoamilases, de desramificação e transferases (VAN DER MAAREL et al., 2002; GOESAERT et al., 2009). O primeiro grupo (α -amilases) são aquelas que catalisam a hidrólise das ligações glicosídicas α -1,4 na parte interna da molécula de amido, liberando oligossacarídeos. O segundo grupo, das β -amilases e glicoamilases, hidrolisam, respectivamente, as ligações α -1,4 da amilose e amilopectina a partir da extremidade não redutora, liberando principalmente maltose, e as ligações α -1,4 e α -1,6 da extremidade não redutora da amilose e amilopectina, liberando glicose. No terceiro grupo encontram-se as enzimas desramificadoras pululanases e isoamilases, que hidrolisam ligações glicosídicas α -1,6 do pululano e amilopectina. Por fim, as transferases agem na ligação glicosídica α -1,4 de uma molécula doadora, transferindo parte do doador para um aceptor glicosídico, formando uma nova ligação (VAN DER MAAREL et al., 2002).

2.3 A biomassa microbiana

Fungos, bactérias e algas quando cultivados em larga escala, podem ser utilizados como aditivos alimentares, sendo as leveduras e extratos fúngicos os mais utilizados na dieta dos ruminantes (GOES et al., 2005). Estes micro-organismos são alimentos muito atraentes, já que podem ser cultivados em resíduos agroindustriais, com produção de grandes quantidades de células ricas em proteínas que normalmente contêm todos os aminoácidos essenciais, além de composição alta de vitamina e minerais (PELCZAR Jr et al., 1996; KUHAD et al., 1997b; BRUM et al., 1999). Além disso, o crescimento de micro-organismos em resíduos lignocelulósicos é capaz de fornecer todas as enzimas hidrolíticas uma vez que eles próprios podem produzir tais enzimas, não sendo necessária a adição destas na preparação dos alimentos. Tais enzimas ajudam na hidrólise dos alimentos fazendo com que os minerais estejam mais disponíveis para absorção pelo animal (VILLAS-BÔAS et al., 2002).

A principal espécie de levedura utilizada como aditivo alimentar animal é a *Saccharomyces cerevisiae*, sendo que seu uso passou de 16% para cerca de 51% dos produtores de rebanhos leiteiros nos Estados Unidos da América de 2000 para 2009 (FRANÇA e RIGO, 2011). Além da qualidade do produto e segurança para uso, o objetivo dos aditivos é contribuir com a produtividade animal com redução do risco de contaminação do consumidor e reduzir excreção de poluentes, como o metano (FRANÇA e RIGO, 2011).

A obtenção de bioprodutos ocorre por meio de fermentações. Tais cultivos podem ser classificados de acordo com o tipo de alimentação nas dornas, sendo principalmente em batelada simples, alimentada ou contínua. No processo em batelada simples, um volume fixo de nutrientes é disponível para o micro-organismo, finalizando a fermentação conforme os nutrientes cessam e a atividade biológica é finalizada (CARVALHO e SATO, 2001; PORTO 2005). Tal fermentação pode levar a baixos rendimentos e produtividades uma vez que o substrato torna-se limitante, além da possibilidade de causar efeitos como inibição, repressão, ou desvio de rotas metabólicas para outros bioprodutos que não os de interesse. Dentre as vantagens desse cultivo, podem ser citados as boas condições de assepsia, flexibilidade de operação, bem como boa estabilidade genética da unidade biológica (CARVALHO e SATO, 2001; SHULER e KARGI, 2002; BORGES, 2008). É importante salientar que o conhecimento do processo em batelada simples gera conhecimento básico da cinética do bioprocessamento, sendo assim de extremo interesse como base para comparações (CARVALHO e SATO, 2001).

A batelada alimentada caracteriza-se pela adição contínua ou intermitente de nutrientes a longo da fermentação, sem retirada de líquido e bioprodutos durante a fermentação (CARVALHO e SATO, 2001). Tal processo supera problemas como inibição por substrato ou

produto, sendo possível controlar a via metabólica microbiana levando ao acúmulo de um produto específico. A batelada alimentada leva a um aumento da concentração celular ao longo do tempo, podendo atingir um perfil decrescente correspondente ao enchimento da dorna por ser dependente da massa microbiana e da variação de volume decorrente da adição do mosto (CARVALHO e SATO, 2001; SHULER e KARGI, 2002). Dessa maneira, a batelada alimentada possui como principais vantagens maiores rendimentos e produtividades, possuindo também a possibilidade do reciclo celular, com o retorno do fermento ao início de uma nova fermentação após um tratamento adequado e separação do caldo recém fermentado para recuperação do produto (BORGES, 2008).

Outro modo de fermentação, é a alimentação contínua, com um fluxo de entrada de nutrientes ao longo da fermentação junto a uma vazão de saída de mosto de fermentação de forma a manter o volume no biorreator (FACCIOTTI, 2001). Apesar desse modelo aumenta a produtividade dos bioprocessos, com ampla possibilidade de automação, as desvantagens são as maiores chances de contaminação, mutação genética espontâneas dos micro-organismos e seleção dos mutantes menos produtivos, maior investimento fixo na planta e dificuldade de homogeneidade no reator (FACCIOTTI, 2001; BORGES, 2008; PACHECO, 2010).

Com a grande quantidade de resíduos agroindustriais gerados e o elevado potencial de aproveitamento desses resíduos por meio de bioprocessos para enriquecimento nutricional para uso como ração animal, produção de enzimas, pigmentos e proteína unicelular, diversos estudos têm surgido para estas finalidades.

2.3.1 Proteína unicelular

O crescimento exponencial da população levou ao aumento da demanda por alimentos sendo alarmante a distância entre demanda e suprimento. Visando diminuir essa lacuna, novas fontes de proteína e/ou aumento do rendimento das fontes conhecidas vem sendo estudadas. Uma possibilidade é a produção de proteína unicelular (*single cell protein* – SCP), que é a proteína extraída da biomassa de micro-organismos (ANUPAMA e RAVINDRA, 2000; DHANASEKARAN et al., 2011; NASSERI et al., 2011), podendo ser obtida por meio do cultivo em resíduos agroindustriais em FES ou FSm. Ao final do processo, a biomassa é separada do resíduo podendo ser tratada com lavagem, lise celular, extração de proteína e purificação antes de ser utilizada (ANUPAMA e RAVINDRA, 2000).

Tal fonte proteica serve também como suplemento dietário e pode substituir o uso convencional de carnes vermelhas e brancas, aliviando o problema da falta de alimentos (ANUPAMA e RAVINDRA, 2000; GHORAI et al., 2011). Os micro-organismos são

excelentes fontes de proteínas (em torno de 35%), possuem baixo índice de gordura, e ainda mais, suplementam aminoácidos, vitaminas, carboidratos, gorduras e minerais essenciais ao crescimento, podendo aumentar também a performance e produtividade dos animais (PELCZAR Jr et al., 1996; KUHAD et al., 1997b; BRUM et al., 1999; GHORAI et al., 2011; DHANASEKARAN et al., 2011). Além disso, sua produção é simples, podendo ser obtida de substratos muitas vezes considerados descartes, como sisal, palma, coco, algodão, arroz, bagaço de cana, bagaço de mandioca (PANDEY et al., 2000a; VILLAS-BÔAS et al., 2002; GHORAI et al., 2011; DHANASEKARAN et al., 2011; NASSERI et al., 2011). Outra vantagem no uso de proteína unicelular é aliviar a pressão por áreas de pastagem e plantações para aumentar produção de alimentos, já que sua obtenção não necessita de grandes áreas (ANUPAMA e RAVINDRA, 2000), e a independência da sazonalidade para sua obtenção (NASSERI et al., 2011).

Algas, bactérias, fungos filamentosos e leveduras podem ser utilizadas como SCP, embora bactérias e fungos sejam os mais convenientes pela sua ampla faixa de características como maior concentração de proteínas, vitaminas e minerais, além da capacidade de crescer em resíduos, rápido crescimento e superior qualidade nutricional (ANUPAMA e RAVINDRA, 2000; NASSERI et al., 2011). Dentre as leveduras, as mais comuns são dos gêneros *Candida*, *Hansenula*, *Pichia*, *Torulopsis* e *Saccharomyces* (ANUPAMA e RAVINDRA, 2000; NASSERI et al., 2011) (Tabela 2).

Apesar dos principais parâmetros na avaliação de produção de SCP serem o rendimento de biomassa, concentração de proteína e de ácidos nucleicos, a seleção de novas cepas isoladas do meio ambiente faz-se necessária (NASSERI et al., 2011). Diferentes espécies possuem características distintas, como a capacidade de crescer em substratos baratos, o que diminui custos com meio de cultivo, ajudando assim a tornar a produção de proteína unicelular mais viável (NASSERI et al., 2011).

Tabela 2 – Principais micro-organismos usados como fonte de proteína unicelular (NASSERI et al., 2011).

Micro-organismo	Substrato
Bactérias	
<i>Aeromonas hydrophyla</i>	Lactose
<i>Acromobacter delvacvate</i>	n-alcanos
<i>Acinetobacter calcoaenticus</i>	Compostos nitrogenados não proteicos
<i>Bacillus subtilis</i> , <i>Cellulomonas</i> sp., <i>Flavobacterium</i> sp., <i>Thermomonospora fusca</i>	Celulose e hemicelulose
<i>Lactobacillus</i> sp.	Glicose, amilose, maltose
<i>Methylomonas methylotrophus</i>	Metanol
<i>Pseudomonas fluorescens</i>	Ácido úrico e outros compostos nitrogenados não proteicos
<i>Rhodopseudomonas capsulata</i>	Glicose
Fungos	
<i>Aspergillus fumigatus</i>	Maltose, glicose
<i>Aspergillus niger</i> , <i>A. oryzae</i> , <i>Cephalosporium eichhorniae</i> , <i>Chaetonium cellulolyticum</i>	Cellulose, hemicelulose
<i>Penicillium cyclopium</i>	Glucose, lactose, galactose
<i>Rhizopus chinensis</i>	Glucose, maltose
<i>Scytalidium aciduphlium</i> , <i>Thricoderma viridae</i> , <i>T. alba</i>	Cellulose, pentose
Leveduras	
<i>Amoco torula</i>	Etanol
<i>Candida tropicalis</i>	Maltose, glicose
<i>Candida utilis</i>	Glicose
<i>Candida novas</i>	n-alcanos
<i>Candida intermedia</i>	Lactose
<i>Saccharomyces cerevisiae</i>	Lactose, maltose
Algas	
<i>Chlorella pyenoidosa</i> , <i>C. sorokiana</i> , <i>Chondus crispus</i> , <i>Scenedesmus</i> sp., <i>Spirulina</i> sp., <i>Porphyrium</i> sp.	Dióxido de carbono (fotossíntese)

Alguns estudos visam a produção de biomassa microbiana através de sub-produtos da agroindústria. Utilizando resíduos sem tratamento, a palha de trigo produziu 4,53 g/L de *Chaetomium cellulolyticum* em FES (ABDULLAH et al., 1985) e a farinha de milho rendeu 0,6 g/g de *Rhizopus oligosporus* (SARGANTANIS et al., 1993). O uso de pré-tratamentos para liberação de açúcares fermentescíveis demonstra ser uma saída para obtenção de biomassa microbiana. O hidrolisado de palha de arroz produziu 6,56 g/L de *Candida utilis* e 6,58 g/L de *Saccharomyces cerevisiae* (ARAÚJO e D'SOUZA, 1986) e o hidrolisado da hemicelulose de eucalipto alcançou 6,35 g/L de *Paecilomyces variotii* (SILVA et al., 1995). A partir de resíduos

hidrolisados provenientes da indústria de abacaxi resultaram em 5,71 g/L de *S. cerevisiae* e 4,92 g/L de *Candida tropicalis* após 7 dias em FSm (DHANASEKARAN et al., 2011). O hidrolisado da hemicelulose do bagaço de cana produziu 15 g/L e 19,5 g/L de *Candida utilis* e *Geotrichum candidum* (HOLDER et al., 1989), 26,67 g/L de *Candida langeronii* (NIGAM, 2000), 10,1 g/L de *Candida tropicalis* (PESSOA JR et al., 1996) e 0,28 g/g de *Candida utilis* (MEYER et al., 1992). Rodriguez-Vazquez et al. (1992) obtiveram concentrações de 4,5 g/L de *Cellulomonas flavigena* em fermentação mista com *Xanthomonas* sp. utilizando o bagaço de cana pré-tratado.

Outros resíduos agroindustriais utilizados para o crescimento de micro-organismos são: glicerol, água de maceração de milho, água de parboilização de arroz e melação de cana. Valduga et al. (2014) obtiveram 5,1 g/L de *Sporidiobolus pararoseus* e Machado e Burkert (2014) produziram 7,18 g/L da mesma levedura, ambos em combinações desses quatro resíduos.

Há algumas limitações para o uso de determinadas espécies como fonte de proteína unicelular, como produção de micotoxinas por fungos, acúmulos de compostos tóxicos devido ao substrato utilizado, metais pesados e alto nível de ácidos nucleicos – no caso de uso para humanos (ANUPAMA e RAVINDRA, 2000). Dessa maneira, antes de ser utilizado como alimento, principalmente para humanos, a proteína unicelular deve ser descontaminada de tais compostos e purificada. Alguns testes como imunogênicos e de mutagenicidade devem ser realizados para determinar os possíveis efeitos da proteína unicelular no organismo hospedeiro, bem como uma profunda análise de possíveis contaminantes durante a fermentação (ANUPAMA e RAVINDRA, 2000). Antes de virar uma realidade comercial, a proteína unicelular deve ser caracterizada em termos de porcentagem de proteína, aminoácidos, ácidos nucleicos, lipídios, gorduras, toxinas e vitaminas, além de propriedades como densidade, tamanho de partículas, textura, cor e estocagem devem ser informadas para empacotamento e revenda (ANUPAMA e RAVINDRA, 2000).

2.3.2 Probióticos

O processo de fermentação por micro-organismos aumenta a durabilidade do alimento e pode aumentar também sua digestibilidade, além de contribuir para textura, sabor e aroma dos alimentos (BOURDICHON et al., 2012). Além disso, algumas espécies possuem efeitos benéficos em mamíferos e, conseqüentemente, na performance do animal com uma maior assimilação da comida e ganho de peso, apesar de não se saber exatamente suas causas (APÁS et al., 2008). Tal efeito classifica tais micro-organismos como probióticos que são definidos

como “preparos de células microbianas ou componentes das células que possuem um efeito benéfico na saúde e bem-estar do hospedeiro” (SALMINEN et al., 1999).

Aditivos microbianos utilizados para aumentar a performance de animais também são definidos pelo *Foods and Drugs Administration* (FDA) como *Direc-fed Microbials* (DFM), ou “fonte natural de micro-organismos viáveis”, substituindo o termo probiótico pelas empresas produtoras desses aditivos (FRANÇA e RIGO, 2011). Estudos acerca de aditivos microbianos têm crescido levando-se em conta principalmente a natureza dos alimentos atualmente utilizados para os animais, que em sua maioria alteram o ambiente gastrointestinal, causando risco de problemas de saúde e diminuição de sua performance (FRANÇA e RIGO, 2011).

Entre os efeitos positivos dos probióticos podem ser citados a atuação como moduladora da imunidade, aprimoramento da flora intestinal, competição com micro-organismos maléficos no trato gastrointestinal, produção de enzimas que ajudam na degradação e digestibilidade da fibra dos alimentos, controle do pH e fornecimento de compostos essenciais ao hospedeiro (NEWBOLD et al., 1996; VILLAS-BÔAS et al., 2002; KUMURA et al., 2004; APÁS et al., 2008).

Os probióticos utilizados no mercado farmacêutico são em sua maioria bactérias, e duas espécies principais de leveduras: *Saccharomyces boulardii* e *Saccharomyces cerevisiae* (MARTINS et al., 2005). Como aditivo animal, *S. cerevisiae* é a espécie mais utilizada, sendo oriunda principalmente da indústria de panificação e de cerveja (FRANÇA e RIGO, 2011). Por isso há estudos em busca de novos micro-organismos que possam atuar como probióticos tanto em humanos quanto em animais. O *screening* por tais micro-organismos geralmente é realizado ao isolar cepas provenientes do trato gastrointestinal ou de alimentos (KUMURA et al., 2004).

A inibição de outros micro-organismos maléficos ao trato gastrointestinal também é um fator interessante ao se considerarem um micro-organismo probiótico (MARTINS et al., 2005). É importante ressaltar que o uso de Antibióticos Promotores do Crescimento (AGPs) na alimentação animal, a fim de controlar bactérias patogênicas que podem ser transmitidas ao longo da cadeia alimentar, chegando a causar doenças em humanos, é uma prática abolida desde Janeiro de 2006 na União Européia tendo em vista a emergência de micro-organismos resistentes a antibióticos, sendo necessário assim outras alternativas para o controle dessas populações (GAGGIÀ et al., 2010). Nesse sentido, a atividade antagonista dos probióticos surge como alternativa para o controle desses micro-organismos patogênicos aliado ao aumento da eficiência e produção dos animais.

Outros fatores a serem considerados para o uso de uma nova cepa como probiótico é sua capacidade de tolerar o ambiente gástrico e de adesão na mucosa intestinal a fim de excluir

patógenos e bactérias indesejáveis (KUMURA et al., 2004). No caso dos ruminantes, cuja fermentação ocorre em pH >6, a produção de lactato leva a um aumento da acidez, o que diminui a eficiência da digestão ruminal. Assim, um dos efeitos causados pelos aditivos é a diminuição dessa produção, promovendo o crescimento de micro-organismos que utilizam esse lactato (FRANÇA e RIGO, 2011).

Apesar de diversos estudos demonstrarem o efeito benéfico do uso de culturas de *S. cerevisiae* (NEWBOLD et al., 1995; SINGH et al., 1995; WOHLT et al., 1998; STELLA et al., 2007; DESNOYERS et al., 2009), existem alguns estudos em que a relação entre aumento da performance e uso de probióticos não é conclusiva (ARCOS-GARCÍA et al., 2000). Dessa maneira, entender melhor os mecanismos de ação exato dos probióticos é de grande interesse (FRANÇA e RIGO, 2011). Além do mais, os animais reagem de forma diferente dependendo da cepa utilizada, o que torna mais difícil a determinação desses mecanismos (KUMURA et al., 2004; GAGGIÀ et al., 2010; FRANÇA e RIGO, 2011).

2.3.3 Enriquecimento nutricional de resíduos pelo uso de micro-organismos

Com o intuito de minimizar custos com alimentação animal, muitos estudos utilizando substratos não convencionais vêm sendo realizados. O investimento no processamento de subprodutos gerados nas indústrias alimentícias, como os resíduos, mostra-se uma saída para a demanda de produtividade e performance do animal (VILLAS-BÔAS et al., 2002). O uso do bagaço de cana-de-açúcar na alimentação animal é restrito e associado a tratamentos físicos (pressão e vapor) ou químico (Ca(OH)_2 e amônia), que aumentam o custo do alimento e limitam as propriedades produtoras (LIMA JR et al., 2010).

Segundo Lima Jr et al. (2010), alimentos ofertados aos ruminantes devem possuir pelo menos 7% de proteína bruta ou 1% de nitrogênio total, pois a baixa porcentagem desestimula o crescimento microbiano, conseqüentemente diminuindo o aporte de proteínas para o intestino delgado. Um enriquecimento com proteína microbiana torna-o mais nutritivo, com uma maior porcentagem proteica (BRAVO et al., 1994; BEZERRA e RAGAUSKAS, 2016). Alguns estudos quantificaram o incremento proteico de resíduos após fermentação com diversos micro-organismos. El-Sayed et al. (1994) usaram o fungo *Pleurotus ostreatus* em FSm com bagaço de cana. O fungo foi incubado por 14 dias, 28 °C, 150 rpm, com bagaço sem tratamento e tratado com cloreto de sódio sendo obtida uma maior degradação para o bagaço sem tratamento e aumento no conteúdo de proteína no resíduo para 22,6% para o bagaço sem tratamento e 18,0% para o tratado. Quanto maior o período de incubação, maior foi o aumento proteico obtido pelos autores. Outras fermentações utilizando o bagaço de cana foram realizadas com a

alga *Spirulina platensis* em FES por sete dias, o qual incrementou o conteúdo proteico do resíduo de 2,3% para 7,2% (PELIZER et al., 2007), com o fungo *Trichoderma reesei* em cultura mista com *Rhizopus* em FSm, o qual aumentou de 1,58% para 7,70% (MONTEIRO et al., 1991) e com o fungo *Chaetomium cellulolyticum* em FES, aumentando o conteúdo de proteína de 2% para 8,65% (BRAVO et al., 1994).

Outros resíduos agroindustriais, além do bagaço de cana, foram estudados como substrato para inoculação com micro-organismos. *Trichoderma viride* incrementou o teor proteico da polpa de beterraba de 10,8% para 26,6% (DURAND e CHEREAU, 1988), *Rhizopus oligosporus* enriqueceu o teor proteico do sagu em 20,4 vezes (GUMBIRA-SA'ID et al., 1992) e *Saccharomyces* e o *Rhizopus* sp. aumentaram de 3,2% para 8,4 e 18,5%, respectivamente, a concentração proteica no resíduo (YANG et al., 1993). A palha de trigo inoculada com *Chaetomium cellulolyticum* em FES aumentou o teor proteico de 2,87% para 16,4%, (ABDULLAH et al., 1985) e fermentação da polpa de maçã por *Saccharomyces cerevisiae*, *Candida utilis* e *Torula utilis* resultou em um aumento de 5,80% para 16,80%, 18,50% e 15,57%, respectivamente (JOSHI e SANDHU, 1996). Shojaosadati et al. (1999) enriqueceram a polpa de beterraba, o farelo de trigo e resíduos da indústria cítrica em FES utilizando *Neurospora sitophila*, alcançando valores de proteína de 15,0%, 13,0% e 7,0% para 30,0%, 30,0% e 18,2%, respectivamente, e Vandenberg et al. (2000) enriqueceu o bagaço de mandioca de 13,3% para 23,1% após 120 h de cultivo com *Aspergillus niger*.

O uso de resíduos da indústria sucro-alcooleira para produção de probióticos pode ser encontrada na literatura. O resíduo *sugarcane blunting*, foi utilizado como meio de cultivo para inoculação de bactérias probióticas da flora de ruminantes, e em conjunto com bactérias ácido lácticas de cabra ou duas bactérias ácido lácticas isoladas da cana-de-açúcar (APÁS et al., 2008). Os autores demonstraram que o resíduo poderia ser utilizado como suplemento da alimentação ruminante após inoculação do silo com bactérias, obtendo valores considerados bons de matéria seca (32,10%), carboidratos (14,22%) e fibras (11,57%), além da possibilidade do crescimento de bactérias lácticas probióticas para o rúmen. A porcentagem de proteína bruta do resíduo foi de 1,0% para 1,4%, e porcentagens de cinzas e lipídeos de 3,6% e 0,47% respectivamente, após 60 dias de fermentação. Os autores afirmam que as concentrações atingidas de matéria seca, cinzas, lipídeos e fibra bruta foram valores ideais para que o resíduo possa ser utilizado na alimentação animal.

2.4 Leveduras

2.4.1 *Sporobolomyces japonicus*

Domínio: Eukarya;

Reino: Fungi;

Filo: Basidiomycota;

Sub filo: Pucciniomycotina;

Classe: Microbotryomycetes;

Ordem: Sporidiobolales;

Gênero: *Sporobolomyces* (NCBI, 2016).

Iizuka e Goto (1965), ao investigarem campos de petróleo de Yabase e Nishiyama (Japão), encontraram em salmouras de petróleo uma pequena quantidade de *Rhodotorula* juntos a uma nova espécie, identificada pelos autores como *Sporobolomyces japonicus*. Suas colônias possuem uma coloração vermelha e enrugada, com células ovóides ou elipsoidais, únicas, em pares ou em cadeias, ocasionalmente apresentando esterigma. Tal levedura é capaz de assimilar fontes de carbono como: glicose, sacarose, rafinose, galactose, maltose, amido solúvel, celobiose, etanol, manitol, glucitol, citrato, entre outros, sendo capaz de crescer em meio livre de vitaminas (HAMAMOTO et al., 2011). Seu crescimento é estimulado na presença de pantotenato de cálcio e a presença de tiamina é um requisito essencial para seu crescimento (HAMAMOTO et al., 2011). A espécie já foi listada como sinônimo de *Sporidiobolus pararoseus*, sendo reclassificada como espécie distinta por Bai et al. em 2002 (HAMAMOTO et al., 2011).

Há uma escassez de informações na literatura sobre demais características da espécie. Khanh et al. (2012) observaram a produção de uma fitase por *S. japonicus*, isolada do Parque Nacional Cat Tien. Devido à sua coloração a espécie provavelmente produza algum pigmento. Ao considerar que *Sporobolomyces roseus* seja reconhecida como *S. japonicus*, segundo Mata-Gómez et al. (2014), a espécie produz toruleno, torularholdin, γ -caroteno e β -caroteno (MATA-GÓMEZ et al., 2014). Tais carotenoides possuem um apelo pelo seu potencial antioxidante, pro-vitamínico e aumento do sistema imune (MALDONADE et al., 2012).

2.4.2 *Sporidiobolus pararoseus*

Domínio: Eukarya;

Reino: Fungi;

Filo: Basidiomycota;

Sub filo: Pucciniomycotina;

Classe: Microbotryomycetes;

Ordem: Sporidiobolales;

Gênero: *Sporidiobolus* (NCBI, 2016).

Sporidiobolus pararoseus é uma levedura basidiomicota encontrada no filoplano, superfície de frutas e água do mar. Suas células são ovóides e se apresentam individualmente, em pares ou como cadeias curtas, com brotamento predominantemente polar (SAMPAIO, 2011b). As colônias possuem uma coloração avermelhada (coral, pêssego, romã), brilhante, lisa ou, menos frequentemente, rugosa, com margens bem definidas. Pode produzir pseudo-hifa e hifas verdadeiras dependendo das condições, sendo capaz de crescer utilizando glicose, sacarose, rafinose, galactose, maltose, amido solúvel, celobiose, etanol, glicerol, lactato, citrato, entre outros e meio sem vitaminas. O crescimento em xilose e arabinose varia de cepa para cepa (SAMPAIO, 2011b). *S. pararoseus* possui teliósporos elipsoidais, sendo a incapacidade de crescer em ausência de nitrato e utilizar D-ribose as características fisiológicas que permitem distingui-la de outras espécies de seu clado (SAMPAIO, 2011b).

Tal espécie vem sendo associada à comunidade de leveduras em uvas de regiões produtoras de vinho sendo primeiro reportada como parte da microbiota de uvas na China, isolada de uvas Chardonnay na região vinícola de Wuwei por Li et al. (2010). Baffi et al. (2010) isolaram a levedura em uma região de vinhos no Brasil, e na Alemanha *S. pararoseus* também foi encontrada como parte da comunidade de leveduras de uvas (BRYSCH-HERZBERG e SIEDEL, 2015).

O isolamento da espécie também já foi reportado em folhas de cana-de-açúcar, no Rio de Janeiro (AZEREDO et al., 1998), de tangerina (SHARMA et al., 2008), de folhas de *Hibiscus rosa-sinensis* (BUSSAMARA et al., 2010) e de morango (HUANG et al., 2012).

Dentre as enzimas que a espécie produz podem ser citadas β -glucosidases (1,7 U/mL) (BAFFI et al., 2010), lipases (BUSSAMARA et al., 2010; SMANIOTTO et al., 2012; QIAO et al., 2013), amilase e celulase (QIAO et al., 2013).

Há estudos sobre o potencial uso como agente de biocontrole. A espécie suprimiu o crescimento de *Penicillium italicum*, *P. digitatum*, *Botryodiplodia thepbromae*, *Geotrichum candidum* e *Alternaria alternata* (SHARMA et al., 2008), *Monilinia fructicola*, causador da podridão marrom em nectarina (JANISIEWICZ et al., 2010), *Botrytis cinérea*, *Mucor* sp., *Penicillium* sp. e *Rhizopus* sp. (HUANG et al., 2012), *Fusarium fujikuroi* (MATIĆ et al., 2014). Segundo Huang et al. (2012), a levedura deve ser um competidor por espaço e nutrientes, além

de produzir compostos orgânicos voláteis, que agem antagonisticamente a algumas espécies de fungos. Os testes com os compostos produzidos por *S. pararoseus* inibiram eficazmente a germinação e crescimento micelial de fungos em placas de Petri, bem como a doença do mofo cinza em morangos (HUANG et al., 2012). Laconi e Pompei (2007) isolaram a espécie do intestino de peixes, e testaram sua atividade antagônica frente a bactérias, sendo que duas cepas das quatro isoladas apresentaram antagonismo contra *Staphylococcus aureus* e uma inibiu consideravelmente *Escherichia coli*.

S. pararoseus produz carotenoides, em meio complexo (CABRAL et al., 2011), ou em resíduos como glicerol, água de maceração de milho e água de parboilização de arroz (VALDUGA et al., 2014). Han et al. (2012) afirmam que os carotenos produzidos pela espécie são principalmente β -caroteno, tolureno e torularhodin, podendo ser obtidos de glicose, sacarose, manose, galactose, glicerol ou álcool como fontes de carboidratos.

2.4.3 *Wickerhamomyces onychis*

Domínio: Eukarya;

Reino: Fungi;

Filo: Ascomycota;

Sub filo: Saccharomycotina;

Classe: Saccharomycetes;

Ordem: Saccharomycetales;

Família: Phaffomycetaceae;

Gênero: *Wickerhamomyces* (NCBI, 2016).

Wickerhamomyces (Pichia) onychis foi primeiro descrita em 1965 por Yarrow, sendo identificada como *Pichia onychis*. Em 2008 a espécie foi alocada ao novo gênero *Wickerhamomyces*. Possui células ovoides, ocorrendo individualmente ou em pares, com colônias de coloração creme, cintilante, sendo capaz de fermentar glicose, sacarose e rafinose, e crescer em glicose, sacarose, maltose, celobiose, trealose, rafinose, xilose, etanol, glicerol, entre outro, e em alguns casos arabinose (KURTZMAN, 2011). *W. onychis* é uma das poucas espécies de leveduras capazes de assimilar 2,3-butaenodiol (KURTZMAN, 2011).

É comumente encontrada no meio ambiente, e associada a alimentos fermentados, como *Kimchi*, uma comida coreana consistente de vegetais fermentados (OH e HAN, 2003) e *Lafun*, um fermentado de mandioca consumido em partes do Oeste da África, sendo parte de um consórcio de micro-organismos responsáveis por sua fermentação (HAHN, 1988).

Alguns estudos reportaram a produção de enzimas pela espécie. Oh e Han (2003) produziram, purificaram e caracterizaram uma gulonolactona oxidase, enzima relacionada à síntese de vitamina C, sintetizada pela levedura em altas concentrações de ácido galacturônico. Hou (1993) testou a atividade de esterase da hidrólise do 2-etilhexil butirato, e dentre os 757 fungos e bactérias testados apenas 62 foram capazes de hidrolisar o composto, sendo duas linhagens de *P. onychis*. A espécie foi ainda capaz de crescer em meio contendo 0,5% de 2,3-butanodiol como fonte de carbono, e produziu pequenas quantidades de álcool desidrogenase e acetona redutase (VERDUYN et al., 1988).

O impacto de pesticidas na saúde humana e no meio ambiente fez com que fosse estudado o desenvolvimento de biopesticidas (KURTZMAN, 2014). Um modo de ação é por meio da inoculação de uma suspensão microbiana que possui efeito antagonista ao patógeno do vegetal (KURTZMAN, 2014). Para essa suspensão é comum o uso de leveduras, que possuem como habitat natural esses mesmos ambientes a serem controlados, e que não causem doenças aos humanos, animais e plantas (KURTZMAN, 2014). Cotes et al. (2011) estudaram o potencial biocontrolador de leveduras na doença mofo cinzento, causada por *Botrytis cinerea* em pétalas de rosa, e dentre as leveduras que demonstraram maior potencial de aderência e atividade biocontroladora estavam *Rhodotorula glutinis*, *Rhodotorula mucilaginosa* e *P. onychis*. Dando continuidade a tal estudo, Zapata et al. (2014) estudaram as condições de crescimento e tolerância de *R. glutinis* e *P. onychis*, sendo que esta última demonstrou uma inibição completa à radiação ultra violeta B (UVB) e uma alta tolerância a diferentes temperaturas (5-37 °C) e pHs (3-9). Outro estudo que demonstrou potencial para uso como agente de biocontrole da espécie foi contra *Botrytis cinerea* em cebolas e *Rhizopus stolonifer* em tomates (GARCÍA et al., 2002). Tais estudos revelam a espécie como possível agente biocontrolador.

2.4.4 *Rhodotorula mucilaginosa*

Domínio: Eukarya;

Reino: Fungi;

Filo: Basidiomycota;

Sub filo: Pucciniomycotina;

Classe: Microbotryomycetes;

Ordem: Sporidiobolales;

Gênero: *Rhodotorula* (NCBI, 2016).

A levedura *Rhodotorula mucilaginosa* possui células elípticas a ovoides, isoladas ou em pares, com brotamento predominantemente polar (SAMPAIO, 2011a). Suas colônias possuem coloração rosa (laranja a coral), cintilante, mucosa, normalmente lisa e ocasionalmente rugosa. É encontrada em todo mundo, tanto em ambientes aquáticos como terrestres, e de uma variedade de substratos incluindo ambientes extremos como lagos altiplânicos, fundo dos oceanos, Antártica e lagos hipersalinos, serapilheira, águas ácidas, sendo considerada uma das espécies mais ubíquas de basidiomicetos (SAMPAIO, 2011a; WIRTH e GOLDANI, 2012). É capaz de crescer utilizando diversos carboidratos, como glicose, sacarose, rafinose, galactose, trealose, maltose, arabinose, ribose, entre outros, e, em alguns casos, assimila também sorbose, manitol, glucitol e lactato (SAMPAIO, 2011a). Possui importância biotecnológica que vai da produção de pigmentos a bioremediação (SAMPAIO, 2011a).

Sua elevada flexibilidade em colonizar ambientes a tornou um oportunista emergente, com cerca de 50 casos de fungemia reportados em humanos entre os anos 2000 e 2012 (WIRTH e GOLDANI, 2012). Os pacientes acometidos com a fungemia apresentavam como condições, dentre outras: linfoma, aids, câncer de ovário, múltiplas cirurgias abdominais, leucemia aguda, comprometendo o sistema imune do indivíduo e deixando-o susceptível a outras infecções (WIRTH e GOLDANI, 2012). O isolamento da espécie em fezes de humanos saudáveis demonstra ainda a alta capacidade de tolerância de *R. mucilaginosa* ao trato gastrointestinal (SILVA et al., 2004). Apesar do isolamento da espécie de alimentos, não há indícios dessa relação com os casos de fungemia (WIRTH e GOLDANI, 2012).

A espécie já foi isolada de mosto de uva no Brasil, e é associada ao filoplano (PONZZES-GOMES et al., 2014), além de já ter sido isolada de linguiças artesanais (MENDONÇA et al., 2013), folhas, haste e rizosfera de cana-de-açúcar (AZEREDO et al., 1998), acerola, pitanga e umbu (TRINDADE et al., 2002).

Lhomme et al. (2016) isolando micro-organismos de padarias na França encontraram uma presença majoritária de *R. mucilaginosa* ao final do processo da fabricação de pães, sugerindo que a espécie esteja relacionada à textura externa do produto, sabor e aroma. Tais diferenças organolépticas nos alimentos causados pela presença de leveduras deve-se, em geral, à produção de compostos como etanol, acetato de etila, acetaldeído, e outros compostos, dependendo do açúcar utilizado e da espécie (MENDONÇA et al., 2013), o que contribui na digestibilidade dos alimentos e palatabilidade.

A capacidade fermentativa da espécie demonstrou a produção de xilitol de xilose, etanol de hexoses e arabitol de arabinose, sendo capaz de crescer em altas concentrações de açúcares (150 g/L), além de demonstrar a possibilidade da utilização da bioconversão de hidrolisados

lignocelulolíticos em bioquímicos (BURA et al., 2012). O metabolismo de assimilação de D-xilose em xilitol e etanol também foi confirmado por Xu et al. (2011). A versatilidade da espécie também reside na capacidade de degradar nitrobenzeno, um resíduo descartado com água industrial (ZHENG et al., 2009), e na biotransformação de betulina em betulona (MAO et al., 2012).

A espécie já foi reportada como produtora de diversas enzimas. Estudos de Li et al. (2014) detectaram atividade celulolítica de endoglucana em duas cepas de *R. mucilaginosa* isoladas de ambiente marinho (PKU Y1 e PKU Y7), sendo que em uma delas a atividade lipolítica também foi mensurada. Lee et al. (1987) detectaram a produção de uma acetilxilana esterase extracelular por *R. mucilaginosa*, responsável pela degradação de constituintes como arabinosil, glucunoril ou grupos acetil presentes na fração hemicelulósica da lignocelulose. *R. mucilaginosa* isolada de alga marinha da Antártica demonstrou produção de uma protease extracelular (LARIO et al., 2015). A espécie também já foi reportada como produtora de proteases (TRINDADE et al., 2002; YANG et al., 2013), lipases (MENDONÇA et al., 2013; YANG et al., 2013; ARCURI et al., 2014), pectinases (LUH e PHAFF, 1951; ARCURI et al., 2014), celulases (ARCURI et al., 2014), além de uma atividade de manganês-peroxidase (YANG et al., 2013), enzima esta considerada ligninolítica já que oxida porções fenólicas da lignina (AGUIAR e FERRAZ, 2012).

Por sua coloração rosa, há diversos estudos sobre a produção de carotenoides pela espécie. Yoo et al. (2016) isolaram uma cepa de *R. mucilaginosa* do solo na Coreia do Sul e determinaram as melhores condições para produção do composto, obtendo 161 a 195 µg de carotenoide/g de biomassa seca de levedura. Tais pigmentos demonstraram capacidade antioxidante e antibacteriana contra cepas de Bactérias Resistentes a Antibióticos (ARB). Maldonado et al. (2012) obtiveram 152 µg/g de carotenoides produzidos pela espécie. Sabe-se que carotenoides como os β-carotenos possuem potencial antioxidante, servem com pro-vitaminas, elevam o sistema imune e previnem alguns tipos de câncer, sendo compostos de grande interesse industrial (MALDONADO et al., 2012).

Em outro estudo, a cepa CRUB 0138 de *R. mucilaginosa* demonstrou produzir carotenoides, além de observarem o efeito *killer* em células de *Rhodospiridium kratochvilovae* CRUB 0121 e de *R. mucilaginosa* CRUB 0137 (LIBKIND et al., 2004). O efeito *killer* também pôde ser observado em uma cepa isolada de pitanga (TRINDADE et al., 2002).

Visando a diminuição dos custos com meio de cultivo para produção de carotenoides por *R. mucilaginosa*, Aksu e Eren (2005) utilizaram melaço de beterraba e a lactose do soro de leite. De maneira geral, o aumento na concentração dos carboidratos aumentou a produção de

biomassa e pigmentos. Em outro estudo, o uso de melaço de cana também demonstrou a possibilidade de produção de carotenoides por *R. mucilaginosa*, sendo obtidos 268,6 µg/g de carotenoides totais numa concentração inicial de 4,26 g/L do melaço, sendo o pigmento composto por 23,8% de β-caroteno, 67,5% de tolureno e 8,7% de torularhodin (CHENG e YANG, 2016).

Há estudos sobre o uso da levedura como agente de biocontrole em doenças de pós-colheita como mofo azul causado por *Penicillium expansum* e *Rhizopus stolonifer* em pêssego (ZHANG et al., 2013), mofo cinza e o azul em maçãs causado por *Botrytis cinerea* e *Penicillium expansum*, respectivamente (LI et al., 2011), e mofo cinza (*B. cinerea*) em morangos (ZHANG et al., 2013).

3 OBJETIVOS

3.1 Objetivo geral

Produção de proteína unicelular através de bioprocessos utilizando resíduos agroindustriais e enriquecimento nutricional dos resíduos para uso como ração.

3.2 Objetivos específicos

- Seleção de leveduras capazes de crescer e produzir celulases em bagaço de cana-de-açúcar pré-tratado ou não;
- Determinação da necessidade ou não de um pré-tratamento do bagaço de cana;
- Identificação das leveduras selecionadas;
- Definição da necessidade do uso de extrato de levedura em combinação com bagaço de cana, água de maceração de milho e (NH₄)₃PO₄ para duas leveduras através de crescimento celular;
- Determinação da melhor combinação de bagaço de mandioca ou bagaço de mandioca pré-tratado com amilase, combinado com água de maceração de milho, por meio de crescimento celular;
- Cultivo das leveduras selecionadas em bioreator utilizando bagaço de cana e bagaço de mandioca (hidrolisado com amilase ou não), em batelada simples ou alimentada;
- Avaliação da produção de xilanases e amilases e alguns parâmetros cinéticos de crescimento em bioreator;
- Comparação entre os rendimentos de biomassa em bioreator com a levedura industrial *Saccharomyces cerevisiae* M26;
- Caracterização da composição visando o potencial nutricional dos bagaços residuais e da biomassa das leveduras ao final do cultivo em bioreator;

- Estudos fisiológicos com as leveduras selecionadas:
 - Capacidade de crescer em anaerobiose;
 - Assimilação de alguns carboidratos;
 - Necessidade de aminoácidos e vitaminas para o crescimento;
 - Determinação da composição centesimal da biomassa das leveduras.

4 CAPÍTULO 1

Seleção de leveduras com capacidade de crescer em resíduos agroindustriais

4.1 Resumo

O objetivo deste estudo foi selecionar leveduras com capacidade de crescer em bagaço de cana-de-açúcar ou de mandioca, para serem utilizadas como possível aditivo microbiano. Nove cepas foram isoladas do meio ambiente e cultivadas em cultivo submerso aeróbio em frascos agitados por 96 h a 28 °C e 180 rpm, com bagaço de cana sem tratamento ou pré-tratado, sendo quantificadas as concentrações celulares (cél/mL) e enzimas celulolíticas ao final do cultivo. Seis cepas foram selecionadas para identificação e, numa segunda etapa, duas foram cultivadas em bagaço de cana sem tratamento ou bagaço de mandioca em combinações com extrato de levedura, $(\text{NH}_4)_3\text{PO}_4$ e água de maceração de milho. O pré-tratamento do bagaço de cana com CaO, H_2SO_4 ou NaOH, em diferentes condições, não foi benéfico para as leveduras, sendo obtidas as menores concentrações celulares bem como pouca produção de celulases. O uso de 0,5% de extrato de levedura foi excessivo para a maioria, e o uso de $(\text{NH}_4)_3\text{PO}_4$ e K_2SO_4 ao invés de K_2HPO_4 também foi benéfico para o crescimento celular. As maiores atividades de celulases totais e endoglucanases foram obtidas em meio contendo soro de leite, com valores de FPase e CMCase de até 0,18 FPU/mL e 0,9 U/mL, respectivamente, para a levedura *Sporidiobolus pararoseus* Sia 33.1. As maiores concentrações celulares atingidas foram de $4,2 \times 10^8$ células/mL (*Rhodotorula mucilaginosa* LABI1), $3,9 \times 10^8$ células/mL (*Sporobolomyces japonicus* Sia 70a) e $3,8 \times 10^8$ células/mL (*Sporidiobolus pararoseus* Sia 33.1) e $2,1 \times 10^8$ células/mL (*Wickerhamomyces onychis* LABI2). *R. mucilaginosa* LABI1 e *S. japonicus* Sia 70a foram cultivadas na segunda etapa, revelando a preferência pelos meios constituídos de extrato de levedura pela primeira espécie (máximo de $3,1 \times 10^8$ células/mL), e pelo bagaço de mandioca pela segunda (máximo de $1,0 \times 10^9$ células/mL). A água de maceração de milho ajudou no crescimento celular demonstrando ser um substituto ao extrato de levedura barateando custos com meio de cultura. Além da capacidade em produzir biomassa a partir de resíduos, fornecendo proteína, minerais, vitaminas e aminoácidos, algumas características especiais as tornam potenciais leveduras probióticas. O uso de substratos baratos para sua produção diminuem custos do processo, sendo uma possível solução para problemas ambientais, sociais e econômicos.

Palavras-chave: Biomassa microbiana, probióticos, proteína unicelular, leveduras celulolíticas.

4.2 Introdução

A atividade agroindustrial gera toneladas de resíduos que, em sua maioria, são descartados no meio ambiente, causando prejuízos ambientais (COELHO et al., 2001; PINTO et al., 2005). Tais sub-produtos são compostos por substâncias aproveitáveis que podem ser utilizadas como substrato para produção de produtos químicos e produtos finos de alto valor agregado, como o etanol, proteínas unicelulares, cogumelos, enzimas, óleos essenciais, ácidos orgânicos, aminoácidos (PANDEY et al., 2000b; SOCCOL e VANDENBERGHE, 2003; CHANDEL et al., 2012).

O bagaço de cana é um co-produto lignocelulósico da indústria de açúcar e álcool, composto por celulose, hemicelulose e lignina (MULINARI et al., 2009). Já o bagaço de mandioca é uma consequência das indústrias de processamento da mandioca, composto por 50% de amido (PANDEY et al., 2000b). Ambos podem ser utilizados como ração animal mas, devido ao seu baixo valor nutricional, muitas vezes é necessária a complementação com proteínas, vitaminas e minerais, o que encarece o processo (VILLAS-BÔAS et al., 2002; LIMA JR et al., 2010). Uma maneira alternativa de enriquecer os valores nutricionais de tais resíduos é a fermentação microbiana, o que aumenta também sua digestibilidade e palatabilidade (APÁS et al., 2008). Tal cultivo é uma alternativa viável aos tratamentos físicos e químicos que muitas vezes processam esses resíduos antes de seu destino como ração e encarecem o processo (VILLAS-BÔAS et al., 2002; LIMA JR et al., 2010).

O cultivo de micro-organismos em resíduos gera uma biomassa microbiana proteica, conhecida como proteína unicelular (*Single Cell Protein* – SCP), que serve como fonte de proteína para humanos e animais (ANUPAMA e RAVINDRA, 2000; GHORAI et al., 2011). Além de ser uma alternativa viável para obtenção de proteína, o bioprocesso diminui a pressão por grandes áreas de pastagens, de plantações e problemas ambientais associados ao descarte de resíduos no meio ambiente além da possibilidade de trazer ganhos econômicos para a indústria (ANUPAMA e RAVINDRA, 2000). Os micro-organismos mais utilizados como SCP são os fungos por seu valor nutricional e ampla combinação de características das espécies como alto teor de proteína, vitaminas e composição de minerais e aminoácidos (ANUPAMA e RAVINDRA, 2000; GHORAI et al., 2011).

Algumas espécies de micro-organismos promovem também efeitos benéficos ao trato gastrointestinal e consequente aumento de produtividade e performance animal (APÁS et al., 2008). Tais organismos são conhecidos como probióticos, ou *Direct-Fed Microbials*, que contribuem com a produção de enzimas hidrolíticas que podem aumentar a absorção dos nutrientes dos alimentos, com o controle de populações maléficas, a regulação do pH, além de

serem fonte de proteínas, vitaminas e minerais (SALMINEM et al., 1999; FRANÇA e RIGO, 2011).

Tendo em vista a baixa quantidade de leveduras utilizadas como probióticos, sendo principalmente das espécies *Saccharomyces cerevisiae* e *Saccharomyces boulardii* (MARTINS et al., 2005), o objetivo deste estudo foi a seleção de espécies de leveduras com potencial probiótico capazes de crescer em cultivo submerso com bagaço de cana ou bagaço de mandioca.

4.3 Material e Métodos

4.3.1 Isolamento e preservação das leveduras

Sete cepas de leveduras gentilmente cedidas pelo Prof Dr Fernando Carlos Pagnocca, responsável pelo Laboratório de Microbiologia (LM) do Centro de Estudo de Insetos Sociais (CEIS) da Universidade Estadual Paulista (UNESP) – Rio Claro –, foram isoladas de serapilheira e denominadas Sia 23.1a, Sia 24.1, Sia 33.1, Sia 42.2b, Sia 70a, Sia 76.1 e Sia 83. Além destas, outras duas foram isoladas de farelo de arroz no Laboratório de Biotecnologia Industrial (LABI) da UNESP – Assis –, e denominadas LABI1 e LABI2. O isolamento foi realizado diluindo o resíduo em água destilada, sendo coletados 1,0 mL com posterior plaqueamento *spread plate* em meio *Potato Dextrose Agar* (PDA), 3,9% (m/v). As placas foram incubadas em estufa de cultura (002CB, Fanem, São Paulo/SP, Brasil) a 28 °C por 120 h. O isolamento foi realizado após sucessivos repiques utilizando a técnica de estrias de esgotamento (MURO e LUCHI, 1989).

A manutenção das leveduras foi realizada por meio de repiques mensais em tubo contendo meio PDA inclinado (3,9%, m/v) incubados a 28 °C por 72 h em estufa de cultura (002CB, Fanem, São Paulo/SP, Brasil) com posterior armazenamento em refrigerador (Ecoplus 430, Bosch, Joinville/SC, Brasil).

A preservação foi realizada utilizando três métodos distintos: criopreservação, preservação em óleo mineral e liofilização. A criopreservação foi realizada em armazenamento em tubos *Eppendorf* em nitrogênio líquido (Volta20, Nitropec, Garça/SP, Brasil). Para tanto, foi utilizada uma suspensão contendo 1,5 mL do micro-organismo concentrado crescido em meio *Yeast Medium* (YM) (OLIVA NETO et al., 2004) líquido com 2,0% de glicose, 0,5% de extrato de levedura, 0,13% de $(\text{NH}_4)_3\text{PO}_4$, 0,10% de $(\text{NH}_4)_2\text{SO}_4$, 0,031% de K_2SO_4 , 0,0028% de ZnSO_4 , 0,0012% de MnSO_4 e 0,024% de MgSO_4 (m/v), pH 5,5, e 15,0% (v/v) de glicerol estéril.

A preservação em óleo mineral foi realizada por meio de repique em meio PDA inclinado, com adição de óleo mineral estéril, e armazenadas em refrigerador (Ecoplus 430,

Bosch, Joinville/SC, Brasil). Por fim, a liofilização das culturas foi realizada utilizando uma cultura concentrada dos micro-organismos em meio YM contendo 10,0% de glicerol (v/v) em equipamento liofilizador (Liotop L101, Liobras, São Carlos/SP, Brasil).

Os procedimentos de esterilização de meios e equipamentos compreenderam autoclavagem por 20-40 minutos em 1 atm de pressão e temperatura de 121 °C (SP215-26, Phoenix, Araraquara/SP, Brasil), filtração em sistema *Millipore* utilizando uma membrana de 0,22 µm ou esterilização a seco em estufa a 105 °C por 4 h (TE394/2, Tecnal, Piracicaba/SP, Brazil).

4.3.2 Seleção de leveduras com capacidade de crescer em bagaço de cana-de-açúcar

As nove leveduras foram cultivadas em meios contendo ou não bagaço de cana-de-açúcar moído com ou sem pré-tratamento a fim de selecionar quatro linhagens mais promissoras baseadas em seu crescimento e produção de enzimas do complexo celulolítico. O bagaço de cana foi colhido na Destilaria Água Bonita (Tarumã - SP) em Julho de 2013, e passou pelos procedimentos de lavagem em água corrente durante três minutos para remoção de eventuais sujeiras e açúcares residuais, com posterior secagem em estufa de circulação forçada a 50 °C por 48 h e moagem em moinho de facas, mesh 14 para obtenção de partículas de aproximadamente 1,4 mm (CE430, Cienlab, Campinas/SP, Brazil). O bagaço moído foi estocado em temperatura ambiente após tratamentos.

Os pré-tratamentos do bagaço de cana consistiram no uso de óxido de cálcio, ácido sulfúrico (YANG et al., 2005) ou hidróxido de sódio (PIRES et al., 2006). O bagaço moído foi adicionado a frascos *Erlenmeyer* sendo adicionados os diferentes tratamentos descritos segundo a Tabela 3. Após tratamento, o mesmo foi lavado em água corrente por três minutos (1:20) a fim de retirar quaisquer resquícios da solução, sendo balanceado pH do bagaço em água ao final do processo. Para o pré-tratamento com 0,1% de ácido (v/v), a solução contendo bagaço ficou em agitação por 12 h a 60 °C, 180 rpm (TE421, Tecnal, Piracicaba/SP, Brazil), sendo lavado com água (1:20) e tratado em autoclave (SP215-26, Phoenix, Araraquara/SP, Brasil) de acordo com as condições da Tabela 3.

Tabela 3 - Descrição dos diferentes tratamentos de hidrólise do bagaço de cana com CaO, NaOH e H₂SO₄.

Parâmetro	Tratamento							
	1	2	3	4	5	6	7	8
CaO (% m/v)	1,0	0,1	---	---	---	---	---	---
H ₂ SO ₄ (% v/v)	---	---	0,1	0,1	0,1	---	---	---
NaOH (% m/v)	---	---	---	---	---	4,5	1,0	6,0
Proporção*	10:1	10:1	10:1	10:1	10:1	10:1	10:1	20:1
Temperatura (°C)	30	30	121	121	121	75	30	120
Tempo	7 dias	7 dias	30 min	60 min	90 min	45 min	7 dias	40 min

*Volume de solução/massa seca de bagaço de cana moído

Ao todo, quatorze diferentes meios foram elaborados, variando-se o tipo de tratamento do bagaço de cana, concentração de extrato de levedura, presença ou ausência de (NH₄)₃PO₄ e fonte de potássio (K₂SO₄ ou K₂HPO₄) de acordo com a Tabela 4. Os sais fixos utilizados em todos meios testados foram (m/v): (NH₄)₂SO₄ (0,1%), ZnSO₄ (0,0028%), MnSO₄ (0,0012%) e MgSO₄ (0,024%).

Tabela 4 - Formulação dos meios de cultura para seleção de leveduras em agitador orbital a 180 rpm, 28 °C e 96 h.

Meio	Sacarose (%)	Soro de leite (%)	Bagaço de cana (5% m/v)				Extrato de levedura (%)	(NH ₄) ₃ PO ₄ (%)	K ₂ SO ₄ (%)	K ₂ HPO ₄ (%)
			Sem tratamento	Tratado com CaO*	Tratado com H ₂ SO ₄ *	Tratado com NaOH*				
1	2,0	-	-	-	-	-	0,5	-	-	0,087
2	-	-	+	-	-	-	0,5	-	-	0,087
3	-	1,0	+	-	-	-	0,2	-	-	0,087
4	-	-	+	-	-	-	0,2	-	-	0,087
5	-	-	+	-	-	-	0,2	0,13	0,031	-
6	1,0	-	+	-	-	-	0,2	0,13	0,031	-
7	-	-	-	Trat 1	-	-	0,2	0,13	0,031	-
8	-	-	-	Trat 2	-	-	0,2	0,13	0,031	-
9	-	-	-	-	Trat 3	-	0,2	0,13	0,031	-
10	-	-	-	-	Trat 4	-	0,2	0,13	0,031	-
11	-	-	-	-	Trat 5	-	0,2	0,13	0,031	-
12	-	-	-	-	-	Trat 6	0,2	0,13	0,031	-
13	-	-	-	-	-	Trat 7	0,2	0,13	0,031	-
14	-	-	-	-	-	Trat 8	0,2	0,13	0,031	-

*Tratamento realizado de acordo com a Tabela 3.

O inóculo foi realizado após suspensão das células crescidas em meio PDA inclinado em água estéril. Após inoculação (1,0x10⁶ cél/mL), os frascos foram incubados em condições

aeróbicas em agitador orbital (TE421, Tecnal, Piracicaba/SP, Brazil) a 28 °C, 180 rpm por 96 h em pH 5,5, ajustado com adição de NaOH (1 M) ou HCl (1 M). Ao final do cultivo, o meio foi filtrado manualmente com gaze, sendo o sobrenadante mensurado quanto à concentração celular (cél/mL) e atividade celulolítica segundo item 4.3.5.2. Duas cepas foram selecionadas para a segunda etapa, de acordo com as maiores concentrações celulares atingidas.

4.3.3 Identificação das cepas selecionadas

A identificação das leveduras foi realizada por profissionais do Laboratório de Microbiologia (LM) - Centro de Estudo de Insetos Sociais (CEIS), da Universidade Estadual Paulista (UNESP) – IB/Rio Claro -, sob supervisão do Prof Dr Fernando Carlos Pagnocca e auxílio da Dra Weilan Gomes da Paixão Melo. A extração de DNA genômico foi realizada de acordo com o protocolo de Sampaio et al. (2001) e a reação de amplificação seguiu o protocolo elaborado por Pagnocca et al. (2008). O DNA foi extraído por meio de lise celular com microesferas de vidro (420-600 µm de diâmetro) e 500 µL solução de lise em agitação em vortex por 4 minutos, sendo incubados em banho a 65 °C por 60 min e centrifugados posteriormente a 13.000 rpm por 15 min. As leveduras foram identificadas pelo sequenciamento da região D1/D2 da subunidade maior do rRNA, amplificados com os *primers* NL1 e NL4 (KURTZMAN e ROBNETT, 1998). A PCR continha: 4,0 µL de dNTPs (1,25 mM cada); 2,5 µL de buffer 10X; 1,0 µL de MgCl₂ (50 mM); 0,2 µL de Taq-polimerase (5,0 U/µL); 2,0 µL de cada primer (10 µM), 10,3 µL de água ultrapura e 5,0 µL de DNA template (1:750). Foi realizada uma eletroforese em gel agarose 1% (m/v) a fim de checar os produtos da amplificação, corados com Loading Dye acrescido de GelRed, sendo visualizados em transluminador UV. Os produtos foram purificados usando NucleoSpin Gel e PCR Clean-up (Macherey-Nagel, MN). Sequências *forward* e *reverse* foram geradas em sequenciador ABI 3130 Genetic Analyser (Life Technologies), usando BigDye Terminator v3.1. As sequências foram editadas e usadas para montar os contigs em BioEdit v7.1.3 (HALL, 1999). *Contigs* foram usados para pesquisar por sequencias homólogas nas bases de dados do Centraalbureau voor Schimmelcultures (CBS) (www.cbs.knaw.nl) e NCBI – GenBank (www.ncbi.nlm.nih.gov).

4.3.4 Determinação dos meios de cultivo com bagaço de cana e bagaço de mandioca com água de maceração de milho, extrato de levedura e/ou (NH₄)₃PO₄

A fim de determinar a necessidade do uso de extrato de levedura, do (NH₄)₃PO₄ e capacidade de crescer utilizando água de maceração de milho como fonte de nitrogênio e

bagaço de mandioca como substrato, duas leveduras foram selecionadas e inoculadas em frascos contendo 100 mL dos meios formulados de acordo com a Tabela 5. Ao todo 28 combinações foram testadas para cada levedura, sendo designadas com número do meio mais a letra “b” para melhor diferenciação dos meios formulados da primeira etapa. Uma solução de sais contendo (m/v) $(\text{NH}_4)_2\text{SO}_4$ (0,1%), ZnSO_4 (0,0028%), MnSO_4 (0,0012%) e MgSO_4 (0,024%) foi utilizada para todos os meios, com pH ajustado para 5,5 com auxílio de NaOH (1 M) ou HCl (1 M). As leveduras foram incubadas em agitador orbital a 28 °C, 180 rpm por 96 h em aerobiose (TE421, Tecnal, Piracicaba/SP, Brazil). Durante o cultivo amostras foram retiradas para a construção das curvas de crescimento das leveduras, sendo considerada a melhor combinação aquela em que foi observada maior concentração celular mensurada (cél/mL).

Tabela 5 – Formulação de meios de cultivo com bagaço de cana ou de mandioca combinado com outros nutrientes para crescimento celular de duas leveduras em agitador orbital a 180 rpm, 28 °C e 96 h.

Meio	Bagaço de cana (%)	Bagaço de mandioca (%)	Extrato de levedura (%)	(NH ₄) ₃ PO ₄ (%)	Água de maceração de milho (%)
1b	5,00	-	-	0,13	-
2b	5,00	-	0,20	0,13	-
3b	5,00	-	-	-	-
4b	5,00	-	0,20	-	-
5b	5,00	-	-	0,26	-
6b	5,00	-	0,05	-	-
7b	5,00	-	0,05	0,13	-
8b	5,00	-	0,05	0,26	-
9b	-	-	-	-	-
10b	5,00	-	-	0,13	0,30
11b	5,00	-	0,05	0,13	0,30
12b	5,00	-	-	-	0,30
13b	5,00	-	0,20	-	0,30
14b	5,00	-	0,20	0,13	0,30
15b	-	-	-	-	0,30
16b	-	5,00	-	0,13	-
17b	-	5,00	0,20	0,13	-
18b	-	5,00	-	-	-
19b	-	5,00	0,20	-	-
20b	-	5,00	-	0,26	-
21b	-	5,00	0,05	-	-
22b	-	5,00	0,05	0,13	-
23b	-	5,00	0,05	0,26	-
24b	-	5,00	-	0,13	0,30
25b	-	5,00	0,05	0,13	0,30
26b	-	5,00	-	-	0,30
27b	-	5,00	0,20	-	0,30
28b	-	5,00	0,20	0,13	0,30

4.3.5 Procedimentos analíticos

4.3.5.1 Quantificação da concentração celular

A concentração celular foi quantificada de maneira direta por meio de contagem em câmara Neubauer (7301-1B, New Optics) de acordo com Lee et al. (1981). A determinação da quantidade foi realizada segundo a equação:

$$n^{\circ} \text{ células/mL} = \sum \text{ dos quadrantes} \times 5 \times 10^4 \times \text{FD}$$

Onde: \sum dos quadrantes $\times 5$ = somatória do número de células contadas nos cinco quadrantes vezes o número de quadrantes totais na câmara;

10^4 = ajuste de volume na câmara;

FD = fator de diluição da amostra.

A determinação da velocidade específica máxima de crescimento ($\mu_{\text{máx}}$) foi calculada de acordo com Shuler e Kargi (2002) levando-se em conta a fase exponencial de crescimento através da representação, sendo o $\mu_{\text{máx}}$ o maior coeficiente angular da reta:

$$\ln X = f(t)$$

4.3.5.2 *Quantificação enzimática*

4.3.5.2.1 *FPase*

A atividade de celulase total do extrato foi medida através da técnica de FPase descrita por Mandels et al. (1976) com algumas modificações segundo Silva (2016). Um total de 0,5 mL do sobrenadante após o cultivo foi adicionado a 1,0 mL de solução tampão acetato de sódio (0,05 M, pH 5,6) e uma tira de papel Whatman n° 01 de 0,5 x 3,0 cm. Os tubos foram deixados por uma hora a 50 °C em banho-maria (MA 127, Marconi, Piracicaba/SP, Brasil) para reação de hidrólise do papel e liberação de açúcares redutores (AR). Após a reação, os açúcares foram quantificados após adição de ácido 3,5 dinitrosalicílico e leitura em espectrofotômetro conforme o método de Miller (1959) listado de acordo com item 4.3.5.3. Uma unidade de atividade enzimática (Filter Paper Unit - FPU) foi definida com a liberação de 2 mg de glicose (equivalente μmol : 2,0/0,18016) por mL de enzima por minuto, e expresso como FPU/mL (GHOSE, 1987). Como controle, foram calculadas as concentrações de AR presente no substrato e no extrato, subtraindo-se essa concentração da reação enzimática (extrato + substrato).

4.3.5.2.2 *Endoglucanase*

A atividade de endoglucanase foi mensurada após hidrólise do substrato carboximetilcelulose (CMC) de acordo com Silva (2016) modificado de Ghose (1987). Em tubos de ensaio foram adicionados 0,1 mL do sobrenadante após cultivo 0,65 mL de uma suspensão de CMC a 0,5% (m/v) em tampão acetato (0,05 M, pH 5,6). A reação ocorreu por 10

minutos em banho-maria a 50 °C (MA 127, Marconi, Piracicaba/SP, Brasil), seguido de resfriamento imediato a fim de interromper a reação, e leitura de AR segundo o método do ácido 3,5-dinitrosalicílico (MILLER, 1959) (item 4.3.5.3). Uma unidade enzimática foi definida como a liberação de 1 μmol de AR por minuto por mL de enzima. Os controles quantificaram as concentrações de AR presente no substrato e no extrato, subtraindo-se essa concentração da reação enzimática (extrato + substrato).

4.3.5.3 Açúcares redutores totais e açúcares redutores

Para determinação de açúcares redutores totais (ART) no caldo fermentado foi realizado uma uma hidrólise a 100 °C por 10 minutos (MA 127, Marconi, Piracicaba/SP, Brasil) utilizando H_2SO_4 (2M) na proporção de 1:1. A neutralização ocorreu após hidrólise adicionando-se NaOH (2M), na mesma proporção de ácido. Após neutralização, os ARTs liberados foram quantificados de acordo com o método de Miller (1959).

A quantificação de AR ou ART do caldo fermentado foi realizada através da reação do ácido 3,5-dinitrosalicílico (MILLER, 1959). Em tubos contendo 0,75 mL do extrato/caldo foram adicionados 0,5 mL de solução de ácido dinitrosalicílico (1%), sendo incubados para reação a 100 °C por 10 minutos (MA 127, Marconi, Piracicaba/SP, Brasil), seguido de resfriamento imediato por 5 minutos em banho de gelo a fim de interromper a reação. Foram adicionados 3,75 mL de água destilada, sendo realizada a leitura de açúcares em espectrofotômetro (UV-M51, Bel Engineering, Monza, Italy) ao comprimento de onda 540 nm. A concentração de açúcares foi ajustada à uma curva padrão de concentrações de glicose (0,05 – 0,5 mg/mL) relacionado à densidade óptica, previamente construída.

4.3.6 Análises estatísticas

Os experimentos foram realizados em triplicatas, sendo o resultado exposto a média das leituras. As comparações das médias foram feitas pelo teste de comparações ANOVA e Tukey, a 5% de probabilidade, gerados por meio do programa GaphPad Prism 5.01.

4.4 Resultados e Discussão

A pré-seleção das leveduras foi realizada utilizando bagaço de cana sem tratamento ou pré-tratado. Ao cultivar as leveduras com o meio 1, um meio controle formulado com 2,0% de sacarose ao invés de bagaço de cana, todas as leveduras atingiram concentrações acima de $1,0 \times 10^8$ cél/mL (Tabela 6). Tal concentração celular também foi atingida para quase todas leveduras na formulação 6, contendo 1,0% de sacarose e 5,0% de bagaço de cana sem

tratamento. Nesse caso, somente Sia 42.2b e Sia 83 não atingiram esse patamar ($7,7 \times 10^7$ e $4,8 \times 10^7$ cél/mL respectivamente). Entretanto, vale ressaltar que Sia 33.1, Sia 70a e LABI1, ultrapassaram o valor da concentração celular do meio controle 1, atingindo $3,1 \times 10^8$, $3,9 \times 10^8$ e $4,2 \times 10^8$ cél/mL respectivamente ($P < 0,05$). Além do mais, a concentração de extrato de levedura foi maior para o meio controle – meio 1 – (0,5% contra 0,2%) e a redução de sacarose (meio 6) de 2,0% para 1,0% reduz os custos com o meio de cultivo. As concentrações celulares observadas para Sia 42.2b foram estatisticamente iguais para todos os meios ($P > 0,05$), exceto o meio controle (Meio 1).

Tabela 6 – Concentrações celulares (cél/mL) de nove leveduras em meios contendo bagaço de cana sem tratamento ou pré-tratado com CaO, H₂SO₄ ou NaOH ao final de cultivo em agitador orbital a 180 rpm, 28 °C e 96 h.

Meios de cultivo*	Micro-organismos/Concentração celular (cél/mL)								
	Sia 23.1a	Sia 24.1	Sia 33.1	Sia 42.2b	Sia 70a	Sia 76.1	Sia 83	LABI2	LABI1
1	2,4E+08	3,6E+08	1,7E+08	4,0E+08	3,1E+08	2,5E+08	1,5E+08	7,9E+08	3,6E+08
2	3,7E+07	1,4E+07	4,3E+07	4,4E+07	2,0E+07	7,0E+07	4,6E+06	1,2E+08	2,5E+08
3	7,4E+07	7,3E+07	7,5E+07	5,3E+07	8,3E+07	1,4E+08	3,1E+07	3,2E+07	9,2E+07
4	2,5E+07	7,8E+07	4,2E+07	7,9E+07	7,4E+07	6,1E+07	1,0E+07	1,2E+07	2,7E+07
5	5,9E+07	4,3E+07	7,1E+07	7,0E+07	7,0E+07	5,3E+07	1,5E+07	7,8E+07	8,7E+07
6	1,0E+08	1,8E+08	3,1E+08	7,7E+07	3,9E+08	1,9E+08	4,8E+07	2,1E+08	4,2E+08
7	2,9E+07	6,7E+06	2,6E+07	1,9E+07	2,3E+07	9,8E+06	1,0E+07	2,1E+07	5,2E+07
8	2,4E+07	1,3E+07	1,1E+07	8,0E+06	9,8E+06	3,0E+06	1,5E+06	3,0E+07	7,5E+07
9	9,6E+06	1,0E+07	2,6E+07	2,0E+07	4,3E+07	4,5E+06	1,1E+07	2,4E+07	2,8E+07
10	7,3E+06	8,3E+06	1,3E+07	1,0E+07	7,9E+06	9,5E+06	4,0E+06	3,9E+07	4,1E+07
11	1,3E+07	1,4E+07	1,4E+07	1,4E+07	3,2E+07	6,1E+06	4,9E+06	4,8E+07	5,0E+07
12	1,7E+07	6,3E+06	3,1E+07	2,0E+07	2,1E+07	6,1E+07	4,9E+06	3,8E+07	9,0E+07
13	2,8E+07	7,4E+06	4,9E+07	3,9E+07	1,7E+07	1,0E+07	4,1E+06	3,4E+07	9,0E+07
14	1,2E+07	4,5E+06	1,4E+07	7,5E+06	1,6E+07	2,1E+07	4,1E+07	1,1E+07	7,8E+07

*Meios de cultivo formulados de acordo com a Tabela 4

A concentração de extrato de levedura de 0,5% no meio 2 mostrou ser excessiva para Sia 24.1 e Sia 70a ($P < 0,05$), tendo em vista que uma taxa maior de crescimento foi atingida com a menor concentração do composto no meio (meio 4). Embora as leveduras Sia 23.1a, Sia 83, LABI1 e LABI2 tenham crescido mais com uma maior concentração de extrato de levedura, o resultado não foi significativo para Sia 23.1a e Sia 83 ($P > 0,05$). Os maiores gastos das fermentações estão ligados à formulação do meio de cultivo (WAITES et al., 2001). A diminuição ou ausência do extrato de levedura, por se tratar de um composto caro, mostra-se

uma saída para baratear tais custos, muitas vezes ajudando a tornar os bioprocessos economicamente viáveis.

O meio formulado com adição de soro de leite (meio 3), comparado ao meio sem adição desse composto e igual formulação restante (meio 4), obteve maiores concentrações celulares para Sia 23.1, Sia 76.1, Sia 83 e LABI1 ($P < 0,05$), sendo os resultados não significativos para o restante dos micro-organismos. Segundo a concentração celular ao final do cultivo das leveduras também foi possível observar que a substituição de K_2HPO_4 por K_2SO_4 e $(NH_4)_3PO_4$ (meio 4 comparado ao meio 5) foi benéfica para LABI1 e LABI2 ($P < 0,05$) sendo esses sais mantidos nas outras formulações (meios 6 ao 14).

Comparando ambos os tratamentos com CaO, de maneira geral, o meio com o bagaço tratado com 1,0% de cal (meio 8) resultou em menores concentrações celulares que o meio com bagaço tratado com 0,1% de CaO (meio 7), embora para nenhuma levedura essa diferença tenha sido significativa ($P > 0,05$). Os tratamentos com H_2SO_4 (meios 9, 10 e 11) em diferentes tempos também não foram significativos para o crescimento celular dos micro-organismos testados ($P > 0,05$). De modo semelhante, entre os diferentes tratamentos com NaOH não foi observada uma diferença entre os crescimentos ($P > 0,05$) exceto para a levedura Sia 76.1, com uma maior concentração celular ($6,1 \times 10^7$ cél/mL) para o meio 12 (4,5% NaOH) e para Sia 83 no meio 14 (6% de NaOH) alcançando $4,1 \times 10^7$ cél/mL. Comparando todos meios com bagaço pré-tratado (7 ao 14), as leveduras Sia 23.1a, Sia 24.1, Sia 33.1, Sia 42.2b, Sia 70a e LABI2 não demonstraram preferência a nenhum pré-tratamento ($P > 0,05$). LABI1 atingiu maiores concentrações ao utilizar os pré-tratamentos com NaOH ($7,8-9,0 \times 10^7$ cél/mL) comparado aos pré-tratamentos com CaO e H_2SO_4 ($P < 0,05$). Sia 76.1 apresentou uma preferência ao meio 12 (pré-tratamento do bagaço de cana com 4,5% de NaOH) em comparação aos outros meios, e Sia 83 ao meio 14, bagaço pré-tratado com 6% de NaOH ($4,1 \times 10^7$ cél/mL), sendo a concentração celular para os demais meios com bagaço pré-tratado estatisticamente iguais ($P > 0,05$).

Ao comparar os meios com bagaço de cana tratados (7 ao 14) com o meio formulado com 5% de bagaço de cana sem tratamento (meio 5), e as mesmas concentrações dos demais sais, ficou um pouco mais evidente os menores crescimentos observados para todos meios com bagaço tratado. Embora os meios 12 e 13 para a levedura LABI1 e meio 12 para Sia 76.1, foram atingidas maiores concentrações celulares, essa diferença não foi significativa ($P > 0,05$). Todas concentrações celulares atingidas foram maiores para o meio com bagaço de cana sem tratamento em comparação ao bagaço de cana pré-tratado (meios 7 e 8 com CaO, meios 9, 10 e 11 com H_2SO_4 e meios 12, 13 e 14 com NaOH). Estatisticamente, as maiores concentrações

do meio 5 comparados com os meios 7 a 14 foram todas significativas ($P < 0,05$) para Sia 24.1. Sia 76.1 e LABI2 tiveram duas concentrações celulares estatisticamente iguais ($P > 0,05$) entre o meio 5 e meios 12 e 11, respectivamente, demonstrando preferência pelo bagaço sem tratamento. Para a levedura Sia 70a a diferença não foi significativa entre o meio 5 e os meios 9 e 11, com bagaço pré-tratado com H_2SO_4 (30 e 90 minutos respectivamente) e para LABI1 entre o meio 5 e os meios 8 (CaO 1%) e 12 (NaOH 4,5%). Para Sia 83, quase todos crescimentos entre o meio 5 e 7-14 (exceto meios 8 e 14) não foram estatisticamente significativos. Por fim, Sia 23.1a e Sia 33.1 tiveram maiores concentrações no meio 5 comparados aos meios 10, 11 e 14, e para o meio 8 para a Sia 33.1 e meio 9 para Sia 23.1a ($P < 0,05$), e crescimentos estatisticamente iguais entre o meio 5 e os meios 7, 12 e 13 para ambas ($P > 0,05$), e meios 8 e 9 para Sia 23.1a e Sia 33.1 respectivamente.

Essa diferença entre os meios que utilizaram o bagaço de cana sem tratamento prévio para os que utilizaram pode ser devido à produção de subprodutos tóxicos durante o processo de pré-tratamento. A pré-hidrólise ácida de substratos lignocelulósicos, apesar de liberar os monômeros da hemicelulose, também gera alguns inibidores de fermentação, como furfural e ácidos fenólicos, que devem ser eliminados antes de se iniciar a fermentação (CHANDEL et al., 2012; PARAWIRA e TEKERE, 2011). Talvez o baixo crescimento das leveduras em bagaço pré-tratado com ácido tenha sido causado justamente pelos compostos inibidores descritos. Outra explicação para o baixo crescimento observado pode ter sido devido a lavagem do bagaço de cana após tratamento ácido. Como tal pré-tratamento hidrolisa a fração hemicelulósica do resíduo, liberando os carboidratos que o compõe, principalmente glicose, galactose, xilose e arabinose (PENG et al., 2010), esses açúcares foram removidos após a lavagem em água corrente não ficando disponíveis para as células utilizarem como fonte de carbono.

A levedura Sia 83 apresentou os menores valores de concentração celular em todos os meios, exceto 7, 9 e 14, embora somente tenha sido uma diferença significativa ($P < 0,05$) para o meio 14. Como o bagaço de cana é um substrato complexo, um consórcio de enzimas é necessário para sua quebra e consequente liberação de açúcares fermentescíveis, que são usados para manutenção e crescimento celular. A baixa ou nula produção de celulases pela Sia 83 em partes ajuda a explicar o baixo crescimento celular observado (Tabelas 7 e 8).

Tabela 7 - Atividade celulolítica (FPU/mL) de nove leveduras em meio contendo bagaço de cana sem tratamento ou pré-tratado com CaO, H₂SO₄ ou NaOH, após cultivo em agitador orbital a 180 rpm, 28 °C e 96 h.

Micro-organismo	Meio de cultivo*/Atividade FPase (FPU/mL)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sia 23.1a	0	0,01 ^b	0,16 ^a	0	0	0	0	0	0	0	0	0,01 ^b	0	0
<i>S. japonicus</i> Sia 24.1	0	0	0,15 ^a	0,02 ^b	0	0	0	0	0	0	0	0	0	0
<i>S. pararoseus</i> Sia 33.1	0	0	0,18 ^a	0	0	0	0	0	0	0	0	0,01 ^b	0	0,01 ^b
<i>S. pararoseus</i> Sia 42.2b	0	0,01 ^b	0,14 ^a	0	0	0	0	0	0	0	0	0,01 ^b	0	0,01 ^b
<i>S. japonicus</i> Sia 70a	0	0,01 ^b	0	0,01 ^b	0,01 ^b	0	0	0,01 ^b	0	0	0	0	0	0
Sia 76.1	0	0,01 ^b	0,02	0,01 ^b	0	0	0	0	0	0	0	0,01 ^b	0,01 ^b	0
Sia 83	0	0,01 ^b	0,13 ^a	0,01 ^b	0	0,01 ^b	0	0	0	0	0	0,01 ^b	0,01 ^b	0
<i>W. onychis</i> LABI2	0	0	0	0,01 ^b	0,01 ^b	0,01 ^b	0	0	0	0	0	0,01 ^b	0,01 ^b	0,01 ^b
<i>R. mucilaginosa</i> LABI1	0	0	0	0,01 ^b	0,01 ^b	0	0	0,01 ^b	0	0	0	0,01 ^b	0,01 ^b	0

^{a, b} = Letras diferentes representam médias estatisticamente diferentes (P<0,05)

*Meios de cultivo formulados de acordo com a Tabela 4

Tabela 8 - Atividade celulolítica de endoglucanases (U/mL) de nove leveduras em meio contendo bagaço de cana sem tratamento ou pré-tratado com CaO, H₂SO₄ ou NaOH, após cultivo em agitador orbital a 180 rpm, 28 °C e 96 h.

Micro-organismo	Meio de cultivo*/Atividade CMCase (U/mL)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sia 23.1a	0	0,3 ^b	0,4 ^b	0	0,2 ^b	0,2 ^b	0,2 ^b	0,1 ^b	0	0	0,2 ^b	0	0,1 ^b	0,1 ^b
<i>S. japonicus</i> Sia 24.1	0	0,1 ^b	0,6 ^a	0,2 ^b	0	0	0	0,2 ^b	0,2 ^b	0	0	0,1 ^b	0,1 ^b	0
<i>S. pararoseus</i> Sia 33.1	0	0	0,9 ^a	0	0	0,3 ^b	0	0,2 ^b	0	0,2 ^b	0,2 ^b	0	0,1 ^b	0,1 ^b
<i>S. pararoseus</i> Sia 42.2b	0	0	0	0,2 ^b	0,2 ^b	0,2 ^b	0	0,2 ^b	0	0	0,2 ^b	0	0,1 ^b	0,1 ^b
<i>S. japonicus</i> Sia 70a	0	0	0	0	0	0	0	0,2 ^b	0	0	0,2 ^b	0	0,1 ^b	0,1 ^b
Sia 76.1	0	0	0,2 ^b	0,2 ^b	0,3 ^b	0,3 ^b	0,3 ^b	0	0	0	0,2 ^b	0,1 ^b	0,1 ^b	0,1 ^b
Sia 83	0	0	0	0	0	0	0,2 ^b	0	0	0	0,2 ^b	0,2 ^b	0,2 ^b	0,1 ^b
<i>W. onychis</i> LABI2	0	0	0,5 ^a	0,2 ^b	0	0,2 ^b	0,2 ^b	0,2 ^b	0	0	0,2 ^b	0,2 ^b	0,1 ^b	0,2 ^b
<i>R. mucilaginosa</i> LABI1	0	0,1 ^b	0	0	0	0	0,2 ^b	0,2 ^b	0	0	0,2 ^b	0,1 ^b	0,1 ^b	0,1 ^b

^{a, b} = Letras diferentes representam médias estatisticamente diferentes (P<0,05)

*Meios de cultivo formulados de acordo com a Tabela 4

Utilizando como base as maiores concentrações celulares obtidas após 96 h de cultivo em agitador orbital, foram escolhidas as leveduras Sia 24.1, Sia 33.1, Sia 42.2b, Sia 70a, LABI1 e LABI2 para identificação taxonômica. As sequências foram submetidas à caracterização taxonômica e comparação com a base de dados, confirmando similaridades com *Sporobolomyces japonicus* (Sia 70a e Sia 24.1), *Sporidiobolus pararoseus* (Sia 33.1 e Sia 42.2b), *Rhodotorula mucilaginosa* (LABI1) e *Wickerhamomyces onychis* (LABI2) (Tabela 9).

Tabela 9 – Leveduras selecionadas para identificação molecular.

Código	Espécie	pb	D1/D2		
			Id%	GeneBank Closest relative	CBS Nº de acesso
Sia 24.1	<i>Sporobolomyces japonicus</i>	571	99	<i>S. japonicus</i> AY158640.1	CBS 5744
Sia 33.1	<i>Sporidiobolus pararoseus</i>	571	100	<i>S. pararoseus</i> EY003452.1	CBS7716
Sia 42.2b	<i>Sporidiobolus pararoseus</i>	571	99	<i>S. pararoseus</i> KP346960	CBS 13667
Sia 70a	<i>Sporobolomyces japonicus</i>	571	100	<i>S. japonicus</i> AY070009.1	CBS 5744
LABI2	<i>Wickerhamomyces onychis</i>	597	98	<i>W. onychis</i> EF550279	CBS 5587
LABI1	<i>Rhodotorula mucilaginosa</i>	570	100	<i>R. mucilaginosa</i> KF850509.1	-

Sporobolomyces japonicus Sia 24.1 e Sia 70a apresentaram colônias com coloração coral e superfície levemente rugosa, além de células elipsoidais em cadeias. *Sporidiobolus pararoseus* Sia 33.1 e Sia 42.2b apresentaram coloração avermelhada/coral e brilhante, com células elipsoidais individuais. *Wickerhamomyces onychis* LABI2 apresentou-se com colônias de cor creme e brilhante, e células ovóides individuais, e *Rhodotorula mucilaginosa* LABI1 com colônias rosa/coral, brilhante, com células ovóides individuais.

As leveduras Sia 24.1 e Sia 70a, identificadas como sendo da espécie *Sporobolomyces japonicus* (Tabela 9), demonstraram crescimentos semelhantes em todos meios cultivados com bagaço de cana sem tratamento (Tabela 6) ($P < 0,05$). Entretanto, para os tratamentos realizados com ácido ou base, a linhagem Sia 70a demonstrou maior versatilidade, apresentando maiores concentrações celulares nos 7, 9 e 11 ($P < 0,05$). O tratamento afetou o crescimento de ambas

linhagens, com maiores concentrações celulares ($P < 0,05$) nos meios com bagaço sem tratamento (3, 4, 5 e 6) em relação aos meios com bagaço de cana pré-tratado (7 ao 14).

Sia 33.1 e Sia 42.2b, ambas pertencentes à espécie *Sporidiobolus pararoseus*, demonstraram poucas diferenças entre suas concentrações celulares sendo que a linhagem Sia 33.1 atingiu maior concentração significativa somente no meio 6 ($P < 0,05$). Na quantificação enzimática de endoglucanase a linhagem de *S. pararoseus* Sia 33.1 obteve 0,9 U/mL enquanto Sia 42.2b não produziu esta enzima. A quantificação de celulase total ambas produziram 0,14-0,18 FPU/mL (Tabelas 7 e 8).

Algumas diferenças observadas entre as concentrações celulares atingidas podem ser explicadas pelo tempo de fermentação. Caso a curva de crescimento tenha atingido a fase de declínio e morte celular antes do final do cultivo de 96 h, a concentração de células é menor que ao final da fase log de crescimento. Da mesma maneira, se a fase log não finalizou até as 96 h de cultivo, uma maior concentração celular poderia ser obtida.

Os maiores valores de celulases totais foram obtidos na formulação com soro de leite (meio 3), com produções de 0,13-0,18 FPU/mL ($P > 0,05$) pelas leveduras Sia 23.1a, Sia 24.1, Sia 33.1, Sia 42.2b e Sia 83 (Tabelas 7 e 8). De certa maneira, os meios formulados com bagaço de cana sem tratamento estimularam mais a produção dessas enzimas. No caso da CMC_{Case}, as maiores concentrações da enzima também foram obtidas para o meio com soro de leite, atingindo 0,5-0,9 U/mL para Sia 24.1, Sia 33.1 e LABI2. O soro de leite é conhecido como indutor de celulases (MANDELS e WEBER, 1969), fato que foi observado neste estudo. A maior concentração de enzimas celulolíticas produzidas no meio 3 pode ter estimulado uma maior quebra de celulase e liberação de monômeros de glicose, que poderia ter sido utilizado para crescimento celular e conseqüentemente a uma maior concentração celular para leveduras que produziram mais dessas enzimas. A quebra dos resíduos lignocelulósicos requer um complexo enzimático (SANDGREEN et al., 2005; AHMAD et al., 2013), e, dessa maneira, a produção de outras enzimas além de celulases totais e endoglucanases também pode ter sido responsável por uma liberação de monômeros que compõe esses resíduos e que podem ser utilizados para crescimento celular, embora não tenham sido quantificados neste estudo.

Comparando-se a produção de FPase nos meios contendo bagaço de cana pré-tratado (7 ao 14), a produção não foi expressiva em nenhum tratamento para nenhuma levedura, sendo abaixo de 0,01 FPU/mL. Já a quantificação de CMC_{Case} foi detectada para a maioria desses meios citados, com destaque para os meios 8, 11, 13 e 14, nos quais pelo menos sete dos nove micro-organismos testados produziram concentrações detectáveis da enzima (exceto Sia 42.2b e Sia 70a).

No meio controle (1) não foi possível detectar a produção de celulases totais ou endoglucanases, e isso se deve à ausência de substrato indutor. Vale ressaltar que os meios contendo bagaço de cana pré-tratado com H_2SO_4 também não estimularam a produção de celulases totais, não sendo detectada nenhuma atividade em nenhum meio de cultivo com as leveduras testadas. A dificuldade em crescer nesses meios pré-tratados com ácido pode ter sido causada pela produção de compostos tóxicos que inviabilizaram as leveduras. Entretanto, no meio 11, formulado com bagaço de cana pré-tratado com H_2SO_4 por 90 minutos, quase todas leveduras produziram endoglucanases, com atividades variando até 0,2 U/mL. Uma possível explicação para tal fato talvez seja o maior tempo de exposição ao tratamento, onde maiores quantidades da hemicelulose foram hidrolisadas consequentemente deixando a celulose mais susceptível à produção e ataque de enzimas fibrolíticas (CHANDEL et al., 2012; PARAWIRA e TEKERE, 2011).

Embora a produção de FPase nos meios contendo bagaço pré-tratado com CaO demonstraram uma mínima produção pelas leveduras com o tratamento de 1,0% de CaO (meio 8), não sendo detectadas tais enzimas no meio 7 (bagaço tratado com 0,1% de CaO), endoglucanases foram produzidas no meio 7 para cinco das leveduras. De maneira similar, quase todas leveduras produziram endoglucanases no meio 8. O tratamento com maior concentração de CaO resultou numa maior produção de enzimas celulolíticas, embora a diferença entre concentrações celulares entre os tratamentos não tenha sido expressiva.

O tratamento básico ajuda a remover a lignina, sendo que a fração hemicelulósica e celulósica remanescente necessita do ataque de um conjunto de enzimas como exo e endoglucanases, β -glucosidases e xilanases para liberação dos açúcares fermentescíveis (CHANDEL et al., 2012). Por esse motivo, a atividade celulolítica pôde ser detectada na maioria dos meios com bagaço tratado basicamente, com destaque para produção de FPase no meio 8, tratado com 1,0% de CaO e nos meios 12, 13 e 14, correspondentes ao tratamento do bagaço com NaOH. De maneira geral, a maior liberação de açúcares fermentescíveis pode ter levado a maiores concentrações celulares.

As leveduras que produziram as maiores concentrações mensuradas de FPase foram para o meio 3, formulado com soro de leite, para Sia 33.1 (0,18 FPU/mL no meio 3), Sia 23.1a (0,16 FPU/mL no meio 3), Sia 24.1 (0,15 FPU/mL no meio 3), Sia 42.2b (0,14 U/mL) e Sia 83 (0,13U/mL) ($P>0,05$). Já a quantificação de CMC_{Case} foi maior para a levedura Sia 33.1 (0,9 U/mL no meio 3), seguida pela Sia 24.1 (0,6 U/mL no meio 3), LABI2 (0,5 U/mL no meio 3), embora para as três as diferenças não sejam significativas ($P>0,05$) e pela Sia 23.1a (0,4 U/mL no meio 3). A ausência de enzimas celulolíticas ao final da fermentação pode ser explicada pela

correlação entre produção de enzimas e fase exponencial do crescimento celular. Caso a fase log de crescimento tenha finalizado antes do final da fermentação, a concentração de enzimas será conseqüentemente menor, pois muitas enzimas são produzidas durante a fase exponencial de crescimento, sendo produzidas em menor quantidade após essa fase (ALTHEERTUM, 2001). Possivelmente a concentração de enzimas poderia ser maior se fosse quantificada durante a fase exponencial.

Não foram encontrados registros de produção de celulases por *S. japonicus*. Dentre os poucos estudos com a levedura, Khanh et al. (2012) demonstraram a produção de uma fitase pela espécie, com uma atividade de 2,7 U/mL.

A espécie *S. pararoseus* foi reportada com produtora de β -glucosidases (1,7 U/mL) (BAFFI et al., 2010), lipases (BUSSAMARA et al., 2010; SMANIOTTO et al., 2012; QIAO et al., 2013), amilases e celulases (QIAO et al., 2013), confirmando a capacidade da espécie em produzir outras enzimas fibrolíticas.

Apesar de não terem sido encontradas muitas informações sobre a síntese de enzimas por *W. onychis*, Oh e Han (2003) produziram, purificaram e caracterizaram uma gulonolactona oxidase, enzima relacionada à síntese de vitamina C, sintetizada pela levedura e Hou (1993) demonstrou a capacidade de duas cepas da espécie em hidrolisar 2-ethylhexil butirato através da produção de esterases. Essa espécie também produziu pequenas quantidades de álcool desidrogenase e acetona redutase (VERDUYN et al., 1988).

A versatilidade fisiológica da *R. mucilaginosa* é conhecida. Uma cepa da espécie isolada em ambiente marinho na Antártica demonstrou capacidade para produzir protease extracelular (LARIO et al., 2015), já tendo sido reportada como produtora de proteases (TRINDADE et al., 2012; YANG et al., 2013), lipases (MENDONÇA et al., 2013; YANG et al., 2013; ARCURI et al., 2014), pectinases (LUH e PHAFF, 1951; ARCURI et al., 2014) e celulases (ARCURI et al., 2014). A atividade de manganês-peroxidase já foi confirmada (YANG et al., 2013), enzima esta considerada ligninolítica já que oxida porções fenólicas da lignina (AGUIAR e FERRAZ, 2012). Além desses estudos confirmando a habilidade da espécie em produzir enzimas fibrolíticas, Li et al. (2014) detectaram atividade celulolítica em duas cepas de *R. mucilaginosa* isoladas de ambiente marinho (PKU Y1 e PKU Y7), sendo que em uma delas a atividade lipolítica também foi mensurada. Em 1987, Lee et al. detectaram a produção de uma acetilxilana esterase extracelular por *R. mucilaginosa*, responsável pela degradação de constituintes como arabinosil, glucunoril ou grupos acetil presentes na fração hemicelulósica da lignocelulose.

Os fungos filamentosos são os mais utilizados industrialmente na produção de celulases, principalmente os representantes dos gêneros *Aspergillus*, *Trichoderma*, *Humicola*,

Penicillium, *Fusarium*, *Phanerochaete* (SINGHANIA et al., 2010). Dentre os fungos produtores de celulases, *Trichoderma reesei* produziu 0,71 FPU/mL (SUN et al., 2008), 0,5 U/mL (CMCase) e 0,2 FPU/mL (BASSO et al., 2010), 0,09 FPU/mL (AIELLO et al., 1996), 0,07 FPU/mL e 1,33 U/mL (CMCase) (RIBEIRO, 2010). Outra espécie de *Trichoderma*, *T. harzianum* produziu 1,64 U/mL de CMCase (RUEGGER e TAUK-TORNISIELO, 2004) e 1,21 FPU/mL (DELABONA et al., 2012), e *Penicillium simplicissimum* e *Penicillium decumbens* produziram 0,021 FPU/mL (RUEGGER e TAUK-TORNISIELO, 2004). Basso et al. (2010) também testaram o cultivo de *Peacilomyces variotti*, *Moniliophthora pernicioso* e *Penicillium verruculosum* em bagaço de cana, obtendo FPase e CMCase que variaram de 0,02 a 0,06 U/mL e 0,15 a 0,25 U/mL respectivamente. Dessa maneira, as atividades de celulases das leveduras deste estudo, principalmente FPase, encontram-se entre as produções de alguns fungos filamentosos. Alguns valores estão acima dos obtidos pelas leveduras deste estudo, embora com espécies de fungos conhecidas por produzir celulases. A determinação das condições ideais de crescimento e produção de enzimas poderia aumentar as produções enzimática pelas leveduras deste estudo.

Alguns autores reportaram a presença de enzimas celulolíticas produzidas naturalmente por outras espécies de leveduras. *Pichia pastoris* produziu uma endoglucanase (COUTURIER et al., 2011), e nos estudos de Rai et al. (2012) endo e exoglucanases foram produzidas por *Candida* sp. cultivada em bagaço de cana. *Trichosporon cutaneum*, *Trichosporon pullulans*, *Cryptococcus macerans* e *Cryptococcus albidus* var *albidus* foram reportados também como leveduras celulolíticas (DENNIS, 1972). Nos estudos de Souza (2011), *Cryptococcus laurentii* isolado do solo foi caracterizado como produtor de β -glicosidases. Tais estudos demonstram que as leveduras podem ser promissoras na produção de enzimas de valor biotecnológico com a vantagem do fácil cultivo.

Levando-se em conta principalmente as concentrações celulares obtidas no meio formulado 6 da primeira etapa, as linhagens Sia 70a (*S. japonicus*) e LABI1 (*R. mucilaginosa*) foram escolhidas para testes posteriores, variando-se concentrações de extrato de levedura, $(\text{NH}_4)_3\text{PO}_4$ e água de maceração de milho, e capacidade de usar bagaço de mandioca sem tratamento para crescimento, a fim de baratear os custos com meio de cultivo. Os resultados desse experimento estão expressos na Tabela 10. As respectivas curvas de crescimento estão anexadas nos Anexos 1 e 2.

Tabela 10 – Concentração celular máxima de *Rhodotorula mucilaginosa* LABII e *Sporobolomyces japonicus* Sia 70a em meios contendo bagaço de cana ou bagaço de mandioca em diferentes combinações com outros nutrientes agitador orbital a 180 rpm, 28 °C e 96 h.

Meio de cultivo*	<i>Rhodotorula mucilaginosa</i> LABII			<i>Sporobolomyces japonicus</i> Sia 70a		
	Final fase log (h)	$\mu_{\text{máx}}$ (h ⁻¹)	Concentração celular máx (cél/mL)	Final fase log (h)	$\mu_{\text{máx}}$ (h ⁻¹)	Concentração celular máx (cél/mL)
1b	24	0,13	7,2E+07	28	0,08	4,6E+07
2b	24	0,19	2,9E+08	48	0,15	9,5E+07
3b	28	0,10	4,9E+07	28	0,13	5,3E+07
4b	28	0,17	2,0E+08	24	0,15	5,2E+07
5b	28	0,09	5,3E+07	28	0,16	7,3E+07
6b	28	0,17	7,2E+07	24	0,16	5,0E+07
7b	24	0,12	7,3E+07	48	0,09	1,9E+08
8b	28	0,07	6,5E+07	24	0,17	1,8E+08
9b	12	0,10	4,4E+06	12	0,30	1,5E+07
10b	48	0,05	1,4E+08	28	0,08	9,2E+07
11b	28	0,10	1,2E+08	28	0,05	1,1E+08
12b	24	0,11	1,0E+08	28	0,06	1,3E+08
13b	28	0,11	1,7E+08	48	0,10	2,7E+08
14b	28	0,09	1,6E+08	48	0,10	2,8E+08
15b	24	0,17	1,3E+08	24	0,17	7,2E+07
16b	12	0,27	4,6E+07	48	0,08	5,4E+08
17b	28	0,05	1,5E+08	48	0,08	3,7E+08
18b	24	0,08	4,2E+07	48	0,08	2,8E+08
19b	28	0,11	1,5E+08	48	0,07	2,4E+08
20b	28	0,09	4,9E+07	48	0,08	2,7E+08
21b	24	0,07	5,4E+07	48	0,07	2,4E+08
22b	24	0,12	5,4E+07	48	0,09	3,0E+08
23b	48	0,09	3,1E+08	96	0,04	3,6E+08
24b	48	0,07	1,7E+08	72	0,08	6,3E+08
25b	24	0,17	8,2E+07	72	0,07	5,3E+08
26b	28	0,11	6,8E+07	72	0,08	1,0E+09
27b	72	0,14	1,5E+08	28	0,14	1,1E+08
28b	24	0,17	1,1E+08	24	0,17	1,1E+08

*Meio de cultivo formulado de acordo com a Tabela 5

De maneira geral a fase exponencial de crescimento para *R. mucilaginosa* LABII terminou entre 24 h e 28 h de crescimento. Os valores de velocidades específicas máxima de crescimento da levedura foram semelhantes, variando-se de 0,05 até 0,27 h⁻¹ (meio 16b). Já

para leveduras *S. japonicus* Sia 70a o final da fase log nos meios formulados com bagaço de cana se deu entre 24 h e 28 h (exceto para os meios 13b e 14b) e para aqueles formulados com bagaço de mandioca essa fase se estendeu para 48 h até 72 h, com exceção dos meios 23, em que a fase log terminou com 96 h de cultivo, e 27 e 28, com final da fase log em 28 h e 24 h, respectivamente. O aumento da fase log conseqüentemente levou à diminuição das velocidades específicas máxima de crescimento para esses meios. Apesar da maior demora na adaptação de *S. japonicus* Sia 70a nos meios formulados com bagaço de mandioca (meios 16b ao 28b), maiores concentrações celulares foram mensuradas em todos esses meios em relação à *R. mucilaginosa* LAB11, tornando-o o substrato mais adequado para a espécie crescer.

Para a levedura *R. mucilaginosa* LAB11 pode-se observar uma pequena preferência pelo bagaço de cana ao invés do de mandioca, embora o fator mais discrepante seja a concentração de extrato de levedura adicionado. De modo geral, as maiores concentrações celulares foram obtidas com os meios contendo maiores concentrações de extrato de levedura.

A água de maceração de milho também demonstrou ser essencial como indutor de crescimento celular, sendo que nos meios ausentes desse resíduo as menores concentrações celulares foram obtidas, com exceção de alguns meios formulados com 0,2% de extrato de levedura (meios 2b, 4b, 17b e 19b). Um crescimento de $1,3 \times 10^8$ cél/mL no meio formulado apenas com água de maceração de milho (meio 15b) demonstra-se que para algumas formulações apenas esse resíduo já é suficiente para a duplicação celular.

As maiores concentrações celulares de *R. mucilaginosa* LAB11 foram de $3,1 \times 10^8$ cél/mL, no meio 23b (5,0% de bagaço de mandioca, 0,05% de extrato de levedura e 0,26% de $(\text{NH}_4)_3\text{PO}_4$), $2,9 \times 10^8$ cél/mL cultivada no meio 2 (5,0% de bagaço de cana, 0,2% de extrato de levedura e 0,13% de $(\text{NH}_4)_3\text{PO}_4$), embora a diferença não seja estatisticamente diferente ($P > 0,05$), e no meio 4 (5,0% de bagaço de cana e 0,2% de extrato de levedura), com $2,0 \times 10^8$ cél/mL no meio

Uma maior afinidade de *S. japonicus* Sia 70a pelo bagaço de mandioca pode ser observada, onde as maiores concentrações celulares foram obtidas com o uso desse substrato ao invés do bagaço de cana. Em todos os meios formulados com bagaço de mandioca a levedura apresentou concentrações celulares acima de $1,1 \times 10^8$ cél/mL. Apesar de não terem sido mensuradas, a linhagem talvez produza amilases, responsáveis por quebrar o resíduo, liberando os açúcares que o compõe. Outra vantagem desses meios foi a adaptação da levedura nos meios sem ou com baixa concentração de extrato de levedura. A espécie atingiu concentrações altas com o uso de água de maceração de milho, demonstrando ser um bom substituto para o extrato

de levedura como fonte de nitrogênio orgânico para esses meios. Além disso, a concentração de 0,13% de $(\text{NH}_4)_3\text{PO}_4$ foi positiva no crescimento.

Considerando os meios formulados com bagaço de cana, qualquer concentração de extrato de levedura resultou em maiores concentrações celulares de *S. japonicus* Sia 70a, bem como a combinação com água de maceração de milho resultou em maiores crescimentos de forma geral. Nos meios ausentes de $(\text{NH}_4)_3\text{PO}_4$ obtiveram os menores crescimentos registrados da espécie demonstrando sua necessidade pelo sal em meios com bagaço de cana. Apesar desta levedura ter atingido até $2,8 \times 10^8$ cél/mL com o meio 14 (5,0% de bagaço de cana, 0,2% de extrato de levedura, 0,13% de $(\text{NH}_4)_3\text{PO}_4$ e 0,3% de água de maceração de milho), o uso do bagaço de mandioca mostrou-se mais versátil para produção de biomassa microbiana. Além disso, com um meio mais econômico do que o 14, contendo 5,0% de bagaço de mandioca e 0,3% de água de maceração de milho (meio 26b) a concentração celular foi de $1,0 \times 10^9$ cél/mL ($P < 0,05$), sendo este mais adequado para a levedura considerando a produção de biomassa.

A água de maceração de milho é um resíduo da manufatura do milho, e contém elevadas concentrações de aminoácidos, vitaminas e minerais, sendo uma fonte de nitrogênio excelente (WAITES et al., 2001). Por se tratar de um resíduo, seu uso na formulação de meios de cultivo pode baratear os custos da fermentação ao utilizá-lo como substituto à fonte de nitrogênio como o extrato de levedura, um composto muitas vezes mais caro o que torna inviável sua utilização para obtenção de determinados produtos microbianos.

Em todas as formulações com bagaço de mandioca, *S. japonicus* Sia 70a apresentou maiores concentrações celulares quando comparada à *R. mucilaginosa* LABI1, com significância ($P < 0,05$) para 9 meios dos 13 formulados com o bagaço de mandioca, e embora para o meio 27b a maior concentração foi para *R. mucilaginosa* LABI1, a diferença não foi significativa ($1,1$ e $1,5 \times 10^8$ cél/mL). Para os meios formulados com bagaço de cana (13 ao todo), os crescimentos foram estatisticamente iguais, diferindo somente nos meios 2, 4, com maiores concentrações celulares de *R. mucilaginosa* LABI1, e nos meios 7 e 8, com maiores para *S. japonicus* Sia 70a. Dessa maneira, o uso do bagaço de cana como principal fonte de carbono foi semelhante para ambas as leveduras testadas. Apesar dos rendimentos dos cultivos não terem sido gravimetricamente determinados, a concentração celular é um bom indicativo da produção de biomassa microbiana.

A produção de proteína unicelular a partir de resíduos é uma alternativa para o aumento da demanda por alimentos e uma solução para melhor aproveitamento dos resíduos agroindustriais, sem a necessidade de grandes áreas para sua produção (ANUPAMA e RAVINDRA, 2000). Alguns estudos demonstram a possibilidade do uso de diversos resíduos

e micro-organismos para atingir essa meta, como os hidrolisados de palha de arroz, que produziu 6,56 g/L de *Candida utilis* e 6,58 g/L de *Saccharomyces cerevisiae* (ARAÚJO e D'SOUZA, 1986), e de eucalipto, produzindo 6,35 g/L de *Paecilomyces variotii* (SILVA et al., 1995). Valduga et al. (2014) obtiveram 5,1 g/L de *Sporidiobolus pararoseus* e Machado e Burkert (2014) 7,18 g/L da mesma levedura, em combinações de glicerol, água de maceração de milho, água de parboilização de arroz e melão de cana. O uso do bagaço de mandioca com água de maceração de milho para produção de *S. japonicus* demonstrou ser viável, de acordo com as altas concentrações celulares atingidas, sendo este um bioprocesso interessante para futuros estudos.

Por serem uma atraente fonte de proteína, vitaminas, aminoácidos e minerais, os micro-organismos melhoram a qualidade nutricional dos resíduos agroindustriais. Dessa forma, após o cultivo, tais resíduos podem ser utilizados como ração animal, além de diminuir gastos com adição desses compostos na ração (PELCZAR Jr et al., 1996; KUHAD et al., 1997b; BRUM et al., 1999; LIMA JR et al., 2010; BEZERRA e RAGAUSKAS, 2016).

Vale ressaltar que algumas cepas possuem efeitos probióticos nos animais, como a produção de enzimas hidrolíticas que ajudam na quebra e absorção dos nutrientes e que muitas vezes são adicionadas na preparação de alimentos (VILLAS-BÔAS et al., 2002). A produção de celulasas pelas leveduras *S. pararoseus* Sia 33.1, *S. japonicus* Sia 70a, *W. onychis* LABI2 e *R. mucilaginosa* LABI1 quantificadas no presente estudo em cultivo submerso com resíduos agroindustriais demonstra ser benéfica para o trato gastrointestinal dos animais, sendo uma vantagem na escolha de novos micro-organismos com potencial probiótico.

Outros estudos apontam ainda a capacidade de produção de outras enzimas que auxiliam na hidrólise e absorção de nutrientes, como a fitase de *S. japonicus* (KHANH et al., 2012), enzima que possui uma utilização reconhecida para animais monogástricos, agindo na diminuição do efeito do fitato, aumentando assim a digestibilidade de fósforo, cálcio, aminoácidos e outras fontes de energia (DERSJANT-LI et al., 2015). *S. pararoseus* já foi reportada como produtora de β -glucosidases (BAFFI et al., 2010), lipases (BUSSAMARA et al., 2010; SMANIOTTO et al., 2012; QIAO et al., 2013), amilases e celulasas (QIAO et al., 2013). *R. mucilaginosa* produz proteases (TRINDADE et al., 2012; YANG et al., 2013; LARIO et al., 2015), lipases (MENDONÇA et al., 2013; YANG et al., 2013; ARCURI et al., 2014; LI et al., 2014), pectinases (LUH e PHAFF, 1951; ARCURI et al., 2014), celulasas (ARCURI et al., 2014; LI et al., 2014), manganês-peroxidase (YANG et al., 2013) e acetilxilana esterase (LEE et al., 1987).

Outro efeito desejado nos probióticos é a inibição de micro-organismos maléficos ao trato gastrointestinal (MARTINS et al., 2005). As espécies *S. pararoseus*, *W. onychis* e *R. mucilaginosa* possuem algum efeito antagônico contra micro-organismos reportado na literatura. *S. pararoseus* inibiu as bactérias *Staphylococcus aureus* e *Escherichia coli* (LACONI e POMPEI, 2007), além de *Penicillium italicum*, *P. digitatum*, *Botryodiplodia thepbromae*, *Geotrichum candidum*, *Alternaria alternata* (SHARMA et al., 2008), *Monilinia fructicola* (JANISIEWICZ et al., 2010), *Botrytis cinerea*, *Mucor* sp., *Penicillium* sp., *Rhizopus* sp. (HUANG et al., 2012) e *Fusarium fujikuroi* (MATIĆ et al., 2014). Já *W. onychis* possui capacidade biocontroladora contra *Botrytis cinerea* (COTES et al., 2011) e *Rhizopus stolonifer* (GARCÍA et al., 2002) e *R. mucilaginosa* demonstraram efeito antagônico contra *Rhodosporidium kratochvilovae* (LIBKIND et al., 2004) além da inibição causada pelos pigmentos da espécie contra algumas bactérias resistentes a antibióticos (YOO et al., 2016).

Outros compostos possuem efeito prebiótico como os pigmentos. Carotenoides são conhecidos por aumentar o sistema imune, possuir efeito antioxidante e prevenir algumas doenças (MALDONADE et al., 2012). É relatada na literatura a produção de tais compostos por *S. pararoseus* (HAN et al., 2012) e *R. mucilaginosa* (LIBKIND et al., 2004; AKSU e EREN, 2005; HAINAL et al., 2012; MALDONADE et al., 2012; CHENG e YANG, 2016; YOO et al., 2016). Ao considerar que o gênero *Sporobolomyces* é conhecido por produzir tais pigmentos (MANIMALA e MURUGESAN, 2014; MATA-GÓMEZ et al., 2014) e a coloração rosada de *S. japonicus* é característica da espécie (HAMAMOTO et al., 2011), possivelmente ela seja capaz de produzir esses compostos. Tais pigmentos demonstram ser uma vantagem prebiótica dessas espécies para considerar a busca por novos micro-organismos com potencial benéfico ao hospedeiro.

Por fim, outra característica desejável aos probióticos é a capacidade de crescer em anaerobiose. Apesar de não terem sido encontrados relatos sobre *S. japonicus*, a espécie *R. mucilaginosa* já foi reportada como sendo facultativa (BRAD et al., 2008; VILLEGAS et al., 2009; OLLIVIER et al., 2011) e *W. onychis* demonstra capacidade de crescer em ausência de oxigênio devido ao seu isolamento em comidas fermentadas (HAHN, 1988; OH e HAN, 2003) e dorna de produção alcoólica (CAMPBELL, 2003; IBARRA, 2015). Segundo Cobban et al. (2016), *Sporobolomyces roseus* (*S. pararoseus*) já foi reportada com capacidade de crescimento em ambientes micro-óxidos e em anaerobiose (ALI e HIPKIN, 1989; COBBAN et al., 2016).

Além da capacidade de crescer em resíduos agroindustriais para obtenção de biomassa microbiana, as leveduras selecionadas possuem características que as tornam especiais ao considerar a procura por novos micro-organismos probióticos. O uso de resíduos

agroindustriais inoculados com leveduras demonstra ser uma saída interessante para diminuição do acúmulo prejudicial de resíduos no meio ambiente, aumento do desempenho do animal além de barateamento com custos de alimentação através do bioprocesso proposto, embora novos estudos sejam necessários para comprovar seus efeitos benéficos na produtividade animal.

4.5 Conclusões

Dentre as nove cepas de leveduras cultivadas em agitador orbital com bagaço de cana-de-açúcar sem tratamento ou pré-tratado, seis demonstraram maior potencial em utilizar o bagaço e produzir celulases. As espécies *Sporidiobolus pararoseus* Sia 33.1, *Sporobolomyces japonicus* Sia 70a, *Wickerhamomyces onychis* LABI2 e *Rhodotorula mucilaginosa* LABI1 foram selecionadas como mais versáteis no uso do bagaço de cana. Dessas, *S. japonicus* Sia 70a e *R. mucilaginosa* LABI1 foram cultivadas em bagaço de cana ou de mandioca em diferentes combinações com extrato de levedura, $(\text{NH}_4)_3\text{PO}_4$ e água de maceração de milho. O bagaço de mandioca demonstrou um aumento na concentração celular de *S. japonicus* Sia 70a, atingindo concentrações de 1×10^9 cél/mL e a água de maceração de milho um substituto para o extrato de levedura e fosfato de amônio ao utilizar tal substrato. Algumas características interessantes tornam as quatro espécies selecionadas especiais, com características probióticas desejáveis além de servir como fonte proteica para alimentação animal. O bioprocesso utilizando resíduos agroindustriais pode baratear custos com a produção de biomassa microbiana e se mostra uma alternativa para o acúmulo prejudicial desses resíduos no meio ambiente.

5 CAPÍTULO 2

Use of agro-industrial residues for yeast biomass production and some physiological characteristics of *Sporobolomyces japonicus* Sia 70a

5.1 Abstract

The aim of this study was to characterize some physiological and nutritional aspects of *Sporobolomyces japonicus* Sia 70a as well as to produce yeast biomass under aerobic submerged fermentation using agro-industrial residues. This little-known yeast was characterized regarding essential amino acids and vitamins and carbohydrates assimilation in homogeneous liquid culture media. In addition, submerged culture of *S. japonicus* Sia 70a was studied in bioreactor with sugarcane bagasse or cassava residue as complex carbon source. This yeast produced all amino acids by its own although its metabolism depended on the vitamins: folic acid, ascorbic acid, pyridoxine, pantothenic acid and nicotinic acid. Besides, the strain showed affinity to glucose, fructose, sucrose, galactose, and high assimilation of maltose and starch confirming the capacity to grow using cassava residue. While single batch process using sugarcane bagasse as carbon source produced only 1.4 g/L yeast biomass in culture with *S. japonicus* and 0.4 g/L of *Saccharomyces cerevisiae* M26 biomass the highest concentration was achieved with fed-batch submerged culture with cassava residue. *S. japonicus* Sia 70a culture in the last medium produced 8.1 g/L with 35.0% protein, two times more than *S. cerevisiae* M26 under the same conditions. The concentration of xylanases (up to 0.5 U/mL) and amylases (maximum 0.2 U/mL) produced by *S. japonicus* Sia 70a helps to explain the higher biomass concentration. The protein content of cassava residue increased from 1.8% to 6.7% after culture with this yeast. The highest consumption of the residues by *S. japonicus* 70a compared to *S. cerevisiae* M26 and similar chemical and nutritional composition between these yeasts cells suggest *S. japonicus* Sia 70a is more interesting to obtain protein and yeast biomass from agro-industrial residues and possible use in feed with probiotics characteristics.

Key words: Single cell protein, probiotic yeast, animal feed, direct-fed microbes.

5.2 Introduction

The exponential growth of human population led to the new researches for increasing the productivity of animal performance as well as to find new sources of protein. To supply such demand, microorganisms biomass production is an interesting biotechnological method proposed to provide sufficient protein, amino acids, vitamins, carbohydrates, fats and essential minerals to the animal diet (Anupama and Ravindra, 2000; Ghorai et al., 2011). This technological alternative demonstrates to be a way to reduce the pressure for areas for livestock or plantation since its production requires less space for manufacturing and also can serve as a supplement in feed enhancing animal productivity (Anupama and Ravindra, 2000; Ghorai et al., 2011). Algae, bacteria, and fungi are sources of protein, although fungae and bacteriae cultures are more convenient due to its wide range of characteristics such as protein concentration, amino acids and vitamins, and rapid growth. The term single cell protein (SCP) refers to the production of microbial biomass with possibilities to be obtained from submerged or solid state fermentation, and *Saccharomyces cerevisiae* is the main specie used for this purpose (Anupama and Ravindra, 2000).

Agro-industrial wastes are used as carbon source, in raw or pre-treated form, to SCP production, which decreases the quantity of waste in the environment. Although agro-industrial wastes are used in animal feed, sometimes physical and/or chemical treatment (sugarcane bagasse) and the addition of additives such as minerals, vitamins and amino acids are necessary in order to improve its nutritional parameters (Lima Jr et al., 2010; Villas-Bôas et al., 2002; Yang et al., 2001). Biological pretreatment demonstrates to be an interesting way to enrich nutritional values of the waste in comparison to physical and chemical methods mainly for its low cost and possible probiotics effects with specific microorganisms (Villas-Bôas et al., 2002). In addition, the fermentation process increases food durability and digestibility, generating positive impacts on animal performance as nutrients assimilation and weight gain (Apás et al., 2008). Moreover, specific microorganisms can act as probiotics in the gastro-intestinal tract, causing a health benefit on the host (França and Rigo, 2011; Salminen et al., 1999).

Agro-industrial wastes are often rich in complex carbohydrates that can be used as substrate or carbon source for the production of other compounds such as, enzymes, acids, ethanol, oils and mushrooms (Chandel et al., 2012; Lima Jr et al., 2010; Pandey et al., 2000a, 2000b; Silva et al., 2007; Soccol and Vandenberghe, 2003). Sugarcane bagasse and cassava residue are the two most important wastes from food industries generated in Brazil. Although they are used as feed both show low protein content (Lima Jr et al., 2010; Pandey et al., 2000a; Silva et al., 2007). Some researchers found a protein content enrichment of these residues after

they had been cultured with microorganisms. This is an alternative to solve the problem of low protein concentration in order to avoid other compounds complementation as additives increasing the feed cost (Bravo et al., 1994; El-Sayed et al., 1994; Pelizer et al., 2007; Monteiro et al., 1991).

The search for new microorganisms, especially yeasts with probiotic effects is an interesting way to enhance animal productivity since in general, these microorganisms are rich in protein, vitamins, and minerals (Martins et al., 2005). These microorganisms with remarkable characteristics may be produced in cheaper ways considering the use of residues as substrates. *Sporobolomyces japonicus* is a yeast belonging to the Order Sporidiobolales (NCBI, 2016), capable of assimilating glucose, sucrose, raffinose, galactose, trehalose, maltose, starch, cellobiose, ethanol, among others, being very versatile and capable of growing in vitamin-free medium (Hamamoto et al., 2011).

In view of the high quantity of agro-industrial wastes poor of nutrients and use of mainly *S. cerevisiae* as SCP, the aim of this work was to improve the nutritional parameters of some residues through aerobic submerged culture with the not so well known yeast *Sporobolomyces japonicus* Sia 70a as well as to obtain microbial biomass with potential probiotic effect and to characterize some physiological aspects of this yeast.

5.3 Materials and Methods

5.3.1 Microorganisms and anaerobiosis test

The strain of *Sporobolomyces japonicus* Sia 70a was isolated from soil and one strain of *Saccharomyces cerevisiae* M26 isolated by Oliva Neto et al. (2004) from sugar mill was used for comparison in submerged culture in bioreactor using agro-industrial residues.

S. japonicus Sia 70a was inoculated in Petri dishes and cultivated at 33 °C for 72 h in an anaerobic jar (Anaerocult, Merck, Darmstadt, Germany) with an anaerobic generator (Anaerobac, Probac do Brasil, São Paulo, Brazil). Capacity to grow in anaerobic condition was visually evaluated through comparison with control Petri dishes incubated in oxygen presence.

5.3.2 Amino acids and vitamins single-omission test

Single-omission tests occurred through *S. japonicus* Sia 70a tubes culture in medium containing (w/v): 2.00% glucose, 0.13% (NH₄)₃PO₄, 0.10% (NH₄)₂SO₄, 0.031% K₂SO₄, 0.0028% ZnSO₄, 0.0012% MnSO₄, 0.024% MgSO₄ modified from Oliva Neto et al. (2004), eight vitamins and 20 pure amino acids (0.03%) (listed on Table 11). The yeast ability to grow in absence of one of each pure amino acid was tested without its addition in tube culture.

Vitamins and corresponding concentrations used to perform the single-omission teste were thiamin (1.0 µg/mL), riboflavin (2.0 µg/mL), folic acid (0.02 µg/mL), ascorbic acid (2.0 µg/mL), pyridoxine (2.0 µg/mL), panthotenic acid (1.0 µg/mL), nicotinic acid (2.0 µg/mL) and aminobenzoic acid (0.2 µg/mL).

Vitamins single-omission test occurred in tubes culture of *S. japonicus* Sia 70a containing the same medium described above (glucose, salts and amino acids) with the absence of one of the vitamins. Positive and negative controls were performed, respectively with all amino acids and vitamins or without them. Evaluation of cells growth was indirectly determined by turbidity measurements at 600 nm using a spectrophotometer (UV-M51, Bel Engineering, Monza, Italy) after 120 h of incubation at 28 °C (Oliva Neto and Yokoya, 1997).

5.3.3 Carbohydrates assimilation

S. japonicus Sia 70a ability to grow using different carbohydrates: lactose, maltose, fructose, glucose, sucrose, xylose, galactose, arabinose and starch, was performed through its inoculation (1.0×10^6 cells/mL) in flasks containing (w/v): 0.2% carbohydrate, 0.2% yeast extract, 0.13% $(\text{NH}_4)_3\text{PO}_4$, 0.10% $(\text{NH}_4)_2\text{SO}_4$, 0.031% K_2SO_4 , 0.0028% ZnSO_4 , 0.0012% MnSO_4 and 0.024% MgSO_4 (Oliva Neto et al., 2004). Incubation occurred for 96 h, 28 °C, 180 rpm in orbital shaker incubator (TE421, Tecnal, Piracicaba/SP, Brazil). Control was composed by a culture with medium without carbohydrate. Growth curve was constructed through direct cell quantification in Neubauer counting chamber (Lee et al., 1981). Kinect parameters such as maximum specific growth rate (μ_{max}), yeast biomass yield ($Y_{x/s}$) and yeast biomass productivity were defined according to Shuler and Kargi (2002).

5.3.4 Submerged culture using agro-industrial residues

The concentration of cassava residue and the need of a pretreatment with amylases were performed after inoculation of *S. japonicus* Sia 70a in flasks with 3.0% or 5.0% (w/v) of solid cassava residue non-treated (CR) or hydrolyzed (HCR) combined with 0.3% or 0.5% of corn steep liquor. The medium also contained (w/v): 0.031% K_2SO_4 , 0.0028% ZnSO_4 , 0.0012% MnSO_4 and 0.024% MgSO_4 (Oliva Neto et al., 2004). Flasks stayed on orbital shaker incubator for 96 h at 180 rpm and 28 °C and aerobic condition (TE421, Tecnal, Piracicaba/SP, Brazil).

Pretreatment of CR occurred through enzymatic hydrolysis using 1.2 U/mL of amylase from *Rhizopus oligosporus* with solid concentration of 10% (w/v) in sodium acetate buffer (0.05 M, pH 5.5) for 24 h at 50 °C (Gonçalves, 2016). Final residue was dried in oven for 72 h, 50 °C (TE394/2, Tecnal, Piracicaba/SP, Brazil) after hydrolysis.

Raw sugarcane bagasse (SB) and cassava residue non-treated (CR) were evaluated as carbon sources in submerged fermentation (SmF) in bioreactor (Bioflo 115, New Brunswick, New Jersey, USA) with *S. japonicus* Sia 70a and *S. cerevisiae* M26 yeasts. Each inoculum was prepared by culture of the respective yeast with 50 mL of the same medium of bioreactor culture added by 1.0% (w/v) glucose without residue and incubated in orbital shaker incubator (TE394/2, Tecnal, Piracicaba/SP, Brazil) for 24 h, 28 °C and 180 rpm. Bioreactor's culture conditions were 28 °C, 1 vvm of air supply and pH 5.5. Dissolved oxygen was measured with a polarographic oxygen electrode (Mettler Toledo AG, Greifensee, Switzerland) and pH was measured with a glass electrode (Mettler Toledo AG, Greifensee, Switzerland) and controlled with addition of 1.0% (v/v) H₂SO₄ and 1.0 M NaOH.

Medium for cultivation with SB in single batch culture was composed by (w/v) (modified from Oliva Neto et al. (2004)): 0.10% (NH₄)₂SO₄, 0.031% K₂SO₄, 0.0028% ZnSO₄, 0.0012% MnSO₄, 0.024% MgSO₄ (w/v), 2.0% SB, 0.2% yeast extract, 0.05% glucose and 0.13% (NH₄)₃PO₄. Batch culture occurred for 72 h and 200 rpm with 1,7 L of culture medium.

Submerged culture using CR were also performed in fed-batch process in bioreactor for both yeasts during 96 h. Culture medium contained 0.5% corn steep liquor and initial 5.0% CR (w/v) besides minerals mentioned above without yeast extract, (NH₄)₂SO₄ and (NH₄)₃PO₄, and initial 500 mL. During fed-batch, three feeds of 300 mL were added at 12, 36 and 72 h of fermentation. The supply of medium during the fed-batch was performed according to maintaining the same level of initial CR concentration (5.0%) at the beginning of culture in three different times measured with humidity balance (BTS110, Scientific Industries, Poland). Mechanical agitation of the culture was initially 400 rpm, and increased to 500, 600 and 700 respectively after feeds due to the increase of medium viscosity.

The separation of liquid medium and residual SB or CR after SmF occurred through manual filtration using polymer fabric. The liquid was centrifuged at 5000 x g for 30 minutes, 4 °C (Megafuge 16R, Heraeus, Thermo Fisher, Massachusetts, USA) for cell separation. Both yeast and residue were dried in oven (TE394/2, Tecnal, Piracicaba/SP, Brazil) for 72 h at 50 °C before nutritional tests.

5.3.5 Analytical procedures

Samples collected during SmF were evaluated according to cell concentration, enzymes and reducing sugars (RS). Growth curve was performed through direct cell concentration using Neubauer counting chamber (7301-1B, New Optics) and cells viability was assessed in a second moment using methylene blue (Lee et al., 1981). Dried biomass was performed according to

Chen et al. (2012). Reducing sugars (RS), xylanase and amylase enzymatic activities were quantified according to Miller (1959), Carvalho et al. (2015) and Gonçalves (2016) respectively. One enzymatic unit means the releasing of 1 μmol of RS per minute per mL of enzymatic extract. Total reducing sugars (TRS) quantification occurred after acid hydrolysis with H_2SO_4 (2.0 M), at the proportion 1:1, for 10 minutes at 100 °C followed by neutralization with NaOH (2.0 M) (1:1:1) and quantification through the Miller (1959) method.

The kinetics parameters maximum specific growth rate (μ_{max}), biomass yield ($Y_{x/s}$) and biomass productivity were determined according to Shuler and Kargi (2002). Specific rate of O_2 consumption of batch culture using SB was calculated according to Núñez et al. (2014) considering the concentration of saturated O_2 at 28 °C equals to 0.244 mMol/L.

Nutritional analysis of dry matter, ashes, fat content, crude fiber and crude protein of yeasts biomass as well as the residues, before and after SmF, were quantified according to the Association of Official Analytical Chemists (AOAC) methods (Helrich, 1990). Tests comprehended respectively: constant weight at 105 °C using humidity balance (BTS110, Scientific Industries, Poland); burning at 700 °C for 6 h using muffle oven (SP2707-20, Spencer, São Paulo/SP, Brazil); ethereal extraction using petroleum ether for 6 h using Soxhlet; acid (H_2SO_4) and basic (NaOH) – 1.25% – hydrolysis at 400 °C for 40 min and drying at 105 °C for 24 h; Kjeldahl method (digestor TE040125 and nitrogen distiller TE036/1, Tecnal, Piracicaba/SP, Brazil). Non-nitrogenous extracts was calculated subtracting the percentage of ashes, fat content, crude fiber and crude protein and Total Digestible Nutrients calculated according to Kears (1982). Yeast's centesimal composition was also evaluated in accordance with the AOAC: carbon according to Walkley (1947), sulfur and phosphorus using visible light spectrum, calcium, potassium, magnesium and zinc with digestion and atomic absorption and nitrogen through Kjeldahl method (Helrich, 1990).

Tests were performed in triplicates, and statistical analysis occurred through ANOVA and Tukey ($P < 0.05$) comparison between replicates using the software GraphPad Prism 5.01.

5.4 Results and Discussion

5.4.1 Anaerobic test

The ability of *S. japonicus* Sia 70a to grow under anaerobic conditions demonstrated that such environment caused a small inhibition of yeast growth, which demonstrated lesser and smaller colonies than control Petri dishes. These colonies have a pink characteristic in aerobic conditions which was not noticed under anaerobic environment, although more studies are necessary to affirm that the yeast produced less pink pigment quantity in this environment.

There are few reports about this species on literature. Ten *Sporobolomyces* strains belonging to the same clade of *S. japonicus* were isolated from leaves in the USA. These species successfully grew in microaerophilic environment although in total anoxia (90% of nitrogen) no significant growth was observed for all isolates (Cobban et al., 2016).

5.4.2 Amino acids and vitamins single-omission test

Amino acids and vitamins single-omission tests with this yeast demonstrated its capacity to produce its own amino acids although some vitamins were essential to its growth (Table 11). The absence of each amino acid did not affect completely this yeast growth. Culture tubes without proline (Pro), isoleucine (Ile), lysine (Lys), aspartate (Asp), phenylalanine (Phe), glutamic acid (Glu) and asparagine (Asn) showed a slightly less growth than positive control tubes. Perhaps growth metabolism was only retarded by the absence of such amino acids and more incubation days would be necessary to *S. japonicus* Sia 70a reaches equal cell concentration of positive control tubes.

Despite the characteristic to produce its own amino acids, the absence of vitamins such as folic acid, ascorbic acid, pyridoxine, pantothenic acid and nicotinic acid affected the yeast metabolism, which led to growth absence. In addition, although *S. japonicus* Sia 70a was capable of growing in culture tube without riboflavin, the yeast was visually less colorful than control tubes. Riboflavin can be related to the yeast's pink pigment production. Another observation was the normal growth in vitamin-free medium but the non-capacity to grow in a certain pool of vitamins. Further studies are necessary to deduce the metabolic paths that this species uses when in presence or absence of some vitamins, but maybe the transport of these nutrients in an imbalance way could explain these results (Oliva Neto and Yokoya, 1997).

Table 11 – Amino acids and vitamins single-omission tests of *Sporobolomyces japonicus* Sia 70a in tubes culture for 72 h and 28 °C.

Amino acid		Vitamin	
Nutrient	Growth	Nutrient	Growth
Trp	+++	Thiamin	+++
Leu	+++	Riboflavin	++
Pro	++	Folic Acid	-
Val	+++	Ascorbic Acid	-
Tyr	+++	Pyridoxine	-
Ala	+++	Pantothenic Acid	-
Ser	+++	Nicotinic Acid	-
Ile	++	Aminobenzoic Acid	+++
Cys	+++		
Gln	+++		
Thr	+++		
Lys	++		
Asp	++		
Phe	++		
Glu	++		
His	+++		
Arg	+++		
Met	+++		
Gly	+++		
Asn	++		

- = Absent; + = Little growth; ++ = Partial growth; +++ = Full growth

P<0.05 for all replicates

According to Hamamoto et al. (2011), this species is capable of growing in vitamins-free media being in accordance with results found by this research. In contrast, Iizuka and Goto (1965) related the necessity of thiamin to grow and the growth stimulation in presence of pantothenic acid. These differences can be explained by strain variations. In the present work, *S. japonicus* grew normally in absence of thiamin, and pantothenic acid did not stimulate this yeast, contradicting Iizuka and Goto (1965). The addition of essential elements and omission of non-essential is important due to the fact that higher productivities can be achieved with the presence of certain compounds and the omission of non-essential nutrients reduces cost with the medium.

5.4.3 Carbohydrates assimilation test

Assimilation tests with some carbohydrates showed that *S. japonicus* Sia 70a possess high affinity to maltose, followed by glucose, fructose, sucrose, starch and galactose (Table 12). Maltose cell concentration reached almost 10 times more (3.4×10^8 cells/mL) compared to control (3.9×10^7 cells/mL) ($P < 0.05$). Fructose had the second highest cell concentration, with 1.8×10^8 cells/mL, followed by glucose (1.5×10^8 cells/mL), sucrose (1.3×10^8 cells/mL), starch (9.2×10^7 cells/mL) and galactose (7.8×10^7 cells/mL). Maximum specific growth rate (μ_{\max}) also confirms the affinity of the yeast to those carbohydrates, with the best values of: 0.32 h^{-1} (maltose), 0.18 h^{-1} , 0.20 h^{-1} (glucose), 0.16 h^{-1} (sucrose), 0.16 h^{-1} (starch) and 0.19 h^{-1} (galactose). Biomass yield ($Y_{x/s}$) are in accordance with maximum cell concentration, highlighting starch assimilation, with 0.98 g/g of yield, being the highest of all carbohydrates tested. Lactose was not assimilated by this yeast.

The highest biomass was obtained with the medium formulated with maltose, followed by sucrose, glucose, fructose, galactose, starch, arabinose and xylose, although the highest productivity was verified with the use of maltose (0.02 g/L.h), while those related to the other carbohydrates were 0.01 g/L.h . Perhaps productivities would be enhanced if culture ended after log phase final than at 96 h of culture (decline phase).

Exponential phases started between 4 and 12 h of culture, ending between 24 to 28 h (Figure 8). Although xylose growth curve was similar to the control, this carbohydrate yielded 0.44 g/g , confirming *S. japonicus* Sia 70a ability to assimilate xylose. The weak growth curve for lactose was probably due to basal culture medium rather than carbohydrate assimilation. Stationary phases of growth curves for maltose, glucose, sucrose and fructose could not be recognized, declining fast after the end of log phase.

Table 12 – Kinect parameters of *Sporobolomyces japonicus* Sia 70a in submerged culture in aerobiosis in orbital shaker incubator (180 rpm, 28 °C, 96 h) with different carbohydrates.

Carbohydrate	Maximum cell concentration (cells/mL)	μ_{\max} (h ⁻¹)	Biomass concentration (g/L)	Biomass yield (g/g)	Biomass productivity (g/L.h)
Arabinose	6.3E+07	0.20	0.93 ^{c,*}	0.49*	0.01**
Fructose	1.8E+08	0.18	1.28 ^{b,*}	0.72*	0.01**
Galactose	7.8E+07	0.19	1.03 ^{d,*}	0.55*	0.01**
Glucose	1.5E+08	0.20	1.30 ^{a,b,*}	0.70*	0.01**
Lactose	6.6E+07	0.13	0.49*	0	0
Maltose	3.4E+08	0.32	1.62*	0.88*	0.02**
Starch	9.2E+07	0.16	0.98 ^{c,d,*}	0.98*	0.01**
Sucrose	1.3E+08	0.16	1.37 ^{a,*}	0.67*	0.01**
Xylose	4.4E+07	0.13	0.72*	0.44*	0.01**
Control	3.9E+07	0.13	0.47*	-	-

a, b, c, d = Different letters correspond to statistically different averages (P<0.05)

Standard deviation: * = <0.04; ** = <0.004

Assimilation results obtained by this research are almost in total accordance to those stated by Hamamoto et al. (2011). *S japonicus* is capable of assimilating glucose, sucrose, galactose, soluble starch and galactose but not xylose, arabinose and lactose. Differences between strains would be responsible for the ability to assimilate different carbohydrates such as xylose and arabinose. According to Iizuka and Goto (1965) the assimilation of galactose by this specie is weak being in accordance to results found in the present study.

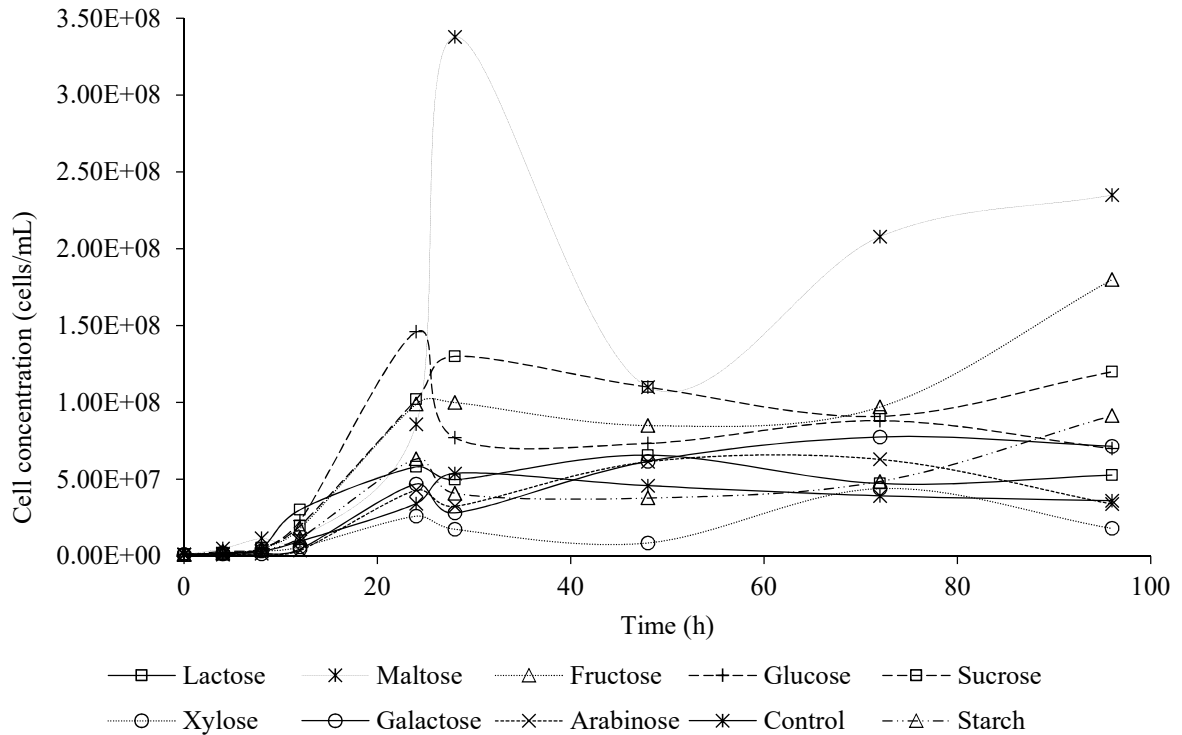


Figure 8 – Growth curve of *Sporobolomyces japonicus* Sia 70a with different carbohydrates cultivated in orbital shaker incubator and aerobiosis (180 rpm, 28 °C).

5.4.4 Submerged culture of *S. japonicus* Sia 70a in flasks with cassava residue

The hydrolysis of cassava residue with *Rhizopus oligosporus* amylases produced 35.44 g/L of reducing sugars (RS) after 24 h. The content of RS is similar (39.9 g/L) to that obtained by Gonçalves (2016), and 18% lower (43.3 g/L) than that one obtained by Shi et al. (2014).

Submerged culture in flasks using cassava residue hydrolyzed (HCR) or not (CR), with 0.3% or 0.5% of corn steep liquor (CSL), demonstrated higher biomass concentrations with the use of raw cassava (Table 13).

Table 13 – Kinect and nutritional composition of *Sporobolomyces japonicus* Sia 70a in medium formulated with solid cassava residue hydrolyzed (HCR) or not (CR) and corn steep liquor (CSL) in aerobiosis orbital shaker agitator (180 rpm, 28 °C).

Conditions of medium composition			After submerged culture								
CR (%)	HCR (%)*	CSL (g)	Log phase	μ_{\max} (h ⁻¹)	Max cell concentration (cells/mL)	Biomass concentration (g/L)	Yeast protein (%)	Biomass productivity (g/L.h)	Final residue (g/L)	Final residue protein (%)**	Y _{x/s} (g/g)***
3.0	-	0.3	8-96 h	0.030	3.7E+08	3.30±0.22 ^a	33.4±1.25 ^a	0.035	1.5	7.4±0.70 ^a	0.17
3.0	-	0.5	8-96 h	0.033	2.9E+08	2.90±0.20 ^a	33.4±0.75 ^{a,b}	0.030	1.6	4.8±0.18 ^{a,b}	0.16
5.0	-	0.3	8-48 h	0.025	3.0E+08	4.36±0.27 ^{a,b}	27.6±0.71 ^{a,c}	0.045	2.5	6.2±0.32 ^{a,b}	0.12
5.0	-	0.5	8-96 h	0.030	5.1E+08	8.61±0.83 ^e	22.2±1.22 ^{b,d,e,f}	0.089	2.4	7.6±1.91 ^a	0.23
-	3.0	0.3	8-96 h	0.027	5.4E+08	4.00±0.08 ^{a,c}	35.6±0.65 ^{a,d}	0.041	1.3	3.5±0.60 ^b	0.19
-	3.0	0.5	8-96 h	0.029	6.7E+08	4.00±0.33 ^{a,d}	40.0±1.30 ^{a,c,e,g}	0.041	1.3	5.7±0.19 ^{a,b}	0.18
-	5.0	0.3	8-48 h	0.083	5.7E+08	4.85±1.14 ^{b,c,d}	30.9±2.23 ^{a,g}	0.051	2.4	5.5±0.46 ^{a,b}	0.14
-	5.0	0.5	8-96 h	0.031	8.3E+08	6.98±0.30 ^f	34.1±0.63 ^{a,f}	0.073	2.1	6.3±0.70 ^{a,b}	0.17

*Enzymatic hydrolysis of cassava; **Protein in CR and HCR were 1.8% and 1.9% respectively; ***Calculated according to the amount of residue;

a, b, c, d, e, f, g = Different letters correspond to statistically different averages (P<0.05)

Flasks cultures of *S. japonicus* Sia 70a with cassava residue treated or not reached higher biomass concentration with 0.5% (v/v) CSL mixed with 5.0% (w/v) CR (8.6 g/L yeast biomass and 0.23 g/g $Y_{x/s}$) and with 5.0% HCR (7.0 g/L of yeast biomass and 0.17 g/g of $Y_{x/s}$).

The concentration of the CR in the culture was important for biomass production since a higher yeast biomass (4.4 g/L – 8.6 g/L) was obtained with 5.0% while only 2.9 – 4.0 g/L were obtained in the cultures with 3.0% of cassava hydrolyzed or not. However, the maximum cell concentration was higher for HCR. Cultures with CR obtained maximum of 5.1×10^8 cells/mL while all cultures with HCR reached from 5.4×10^8 cells/mL to 8.3×10^8 cells/mL.

The log phases started in general after 8 h and ended at 48 h in flasks with 5.0% of cassava residue hydrolyzed or not and 0.3% CSL. For all other cultures, log phase started at 8 h and was noticed the end until 96 h. Perhaps, if cultures continued until log phase ended higher biomass concentration could be obtained. Extended log phase decreased the maximum specific growth rate with values below 0.083 h^{-1} .

The protein concentration of *S. japonicus* Sia 70a biomass was higher for cultures with HCR (30.9-40.0%) than those corresponding to CR (22.2-33.4%). The combination of 3.0% HCR and 0.5% CSL in the culture medium presented higher protein concentration (40.0%) but the final residue protein reached 5.7%. Although the lowest protein concentration of yeast biomass (22.2%) was obtained after cultivation with 5.0% CR and 0.5% CSL this mixture showed the highest protein improvement of final residue from 1.8% to 7.6%.

On the other hand, the best concentration for biomass production and the improvement of protein in the final residue were obtained with the use of 5.0% raw cassava residue and 0.5% corn steep liquor. This combination was chosen for fed-batch culture in bioreactor. Another advantage in the use of non-treated residue is the lower cost of culture medium without an enzymatic treatment with amylases.

5.4.5 Kinect of *S. japonicus* Sia 70a and *S. cerevisiae* M26 in submerged culture using sugarcane bagasse and cassava residue in bioreactor

According to *S. japonicus* Sia 70a and *S. cerevisiae* M26 growth curve in aerobic submerged culture (SmF) with agro-industrial residues in bioreactor, a higher affinity of cassava residue (CR) over sugarcane bagasse (SB) was observed for both yeasts. *S. japonicus* Sia 70a reached higher cell concentrations than *S. cerevisiae* M26 in both residues (Figure 9). SB batch cultures of *S. japonicus* Sia 70a and *S. cerevisiae* M26 demonstrated the maximum cell concentrations were reached at the end of log phase (24 h for both yeasts) respectively

5.7×10^7 cells/mL and 3.1×10^7 cells/mL. These results showed *S. japonicus* Sia 70a grown almost 2 times more than *S. cerevisiae* M26 at the same conditions using SB medium. The growth curve of fed-batch culture with CR demonstrated a log phase initiated at the beginning of culture for both yeasts, reaching a maximum cell concentration at 72 h for *S. japonicus* Sia 70a (9.6×10^8 cells/mL) with a decrease to 5.9×10^8 cells/mL at 96 h. For *S. cerevisiae* M26, log phase end was at 12 h with a slower growth remained until the end of culture, achieving up to 1.5×10^8 cells/mL at 96 h (Figure 9). *S. japonicus* Sia 70a obtained 6.4 times more cell concentration than *S. cerevisiae* M26 at the same conditions. Therefore, in both cases, *S. japonicus* Sia 70a was more versatile to use those agro-industrial residues than *S. cerevisiae* M26 with higher biomass concentration. The use of CR as substrate was more appropriated to single cell protein (SCP) production over SB, since the differences of yeast cell concentration between these residues are 17 (*S. japonicus* Sia 70a) and 3.5 times (*S. cerevisiae* M26) higher.

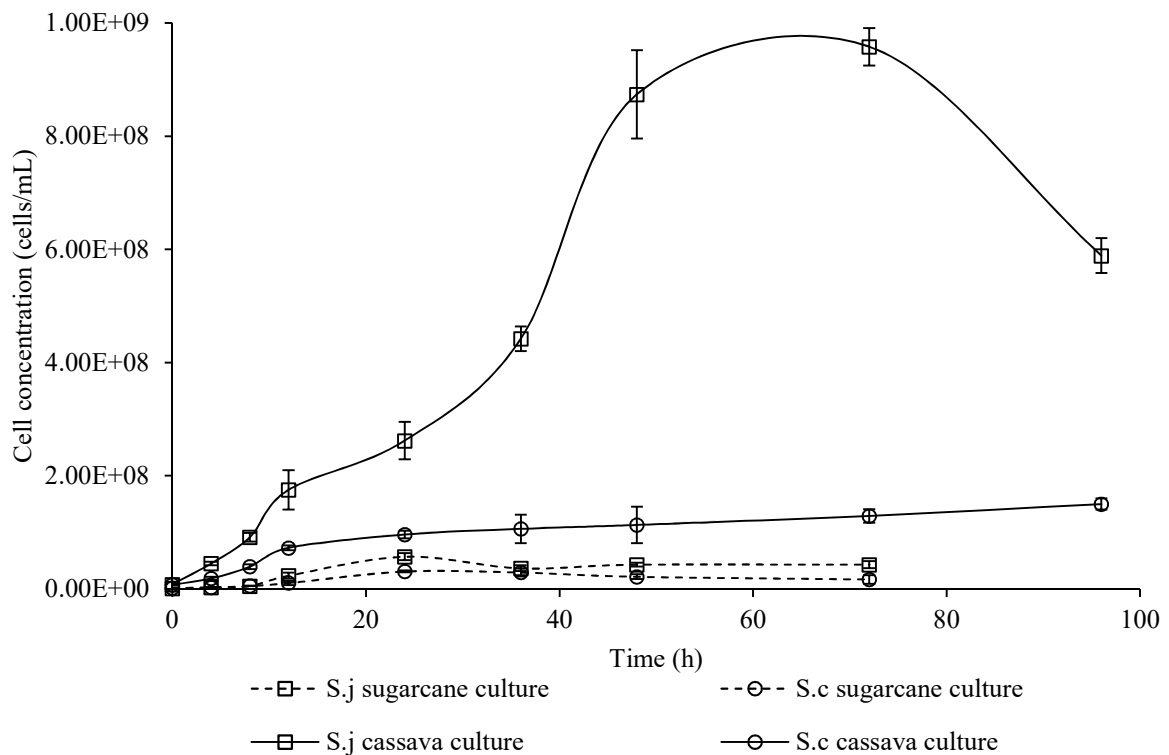


Figure 9 – Growth curve of *Sporobolomyces japonicus* Sia 70a (S.j) and *Saccharomyces cerevisiae* M26 (S.c) single-batch culture using sugarcane bagasse and fed-batch culture with cassava residue in bioreactor at 28 °C, pH 5.5, 1 vvm.

The biomass concentration in fed-batch culture of *S. japonicus* Sia 70a in bioreactor using CR as substrate reached 8.1 g/L (Table 14). This production was 1.8 times higher than *S.*

cerevisiae M26 culture at the same conditions and respectively, 5.9 and 19.4 times higher than batch culture using SB with *S. japonicus* Sia 70a and *S. cerevisiae* M26 (Tables 14 and 15). The productivity of both yeasts in fed-batch using CR was also 4.5 and 5 times higher than batch culture with SB for *S. japonicus* Sia 70a and *S. cerevisiae* M26 respectively. Therefore, CR is more interesting to produce yeast biomass (SCP) preferably by *S. japonicus* Sia 70a. Moreover, in accordance with biomass concentration, *S. japonicus* Sia 70a productivity in both cultures (sugarcane or cassava) was 1.8 to 2.0 times higher than *S. cerevisiae* M26.

Table 14 – Kinect and nutritional parameters of *Sporobolomyces japonicus* Sia 70a (S.j) and *Saccharomyces cerevisiae* M26 (S.c) in batch culture using sugarcane bagasse in bioreactor at 28 °C, pH 5.5, 1 vvm.

Parameters	Supplied bagasse before culture	After submerged culture				P < 0.05
		Final residue		Yeast biomass		
		<i>S.j</i>	<i>S.c</i>	<i>S.j</i>	<i>S.c</i>	
Residue (g)	34.00	27.87	27.61	2.36	0.71	No
Crude protein (%)	1.56±0.19	4.71±0.78	3.71±0.32	26.19±0.89	28.21±0.43	No
Max cell concentration (cell/mL)	-	-	-	5.90x10 ⁷	3.09x10 ⁷	Yes
Yeast biomass (g/L)	-	-	-	1.39	0.42	Yes
Biomass productivity (g/L.h)	-	-	-	0.02	0.01	Yes
Y _{x/s} (g/g)	-	-	-	0.37	0.10	Yes
μ _{max} (h ⁻¹)	-	-	-	0.15	0.09	Yes
Yeast cells viability at the end of culture (%)	-	-	-	91.49	80.95	Yes

Table 15 – Kinect and nutritional parameters of *Sporobolomyces japonicus* Sia 70a (S.j) and *Saccharomyces cerevisiae* M26 (S.c) in fed-batch culture using solid raw cassava residue in bioreactor at 28 °C, pH 5.5, 1 vvm.

Parameters	Supplied residue		After submerged culture				P<0.05
	before culture		Final residue		Yeast biomass		
	<i>S.j</i>	<i>S.c</i>	<i>S.j</i>	<i>S.c</i>	<i>S.j</i>	<i>S.c</i>	
Residue (g)	89.0	80.0	41.2	36.2	8.93	5.08	-
Yeast biomass (g/L)	-	-	-	-	8.12	4.63	Yes
Max cell concentration (cells/mL)	-	-	-	-	9.58x10 ⁸	1.50x10 ⁸	Yes
Biomass productivity (g/L.h)	-	-	-	-	0.09	0.05	Yes
Y _{x/s} (g/g)	-	-	-	-	0.19	0.12	Yes
μ _{max} (h ⁻¹)	-	-	-	-	0.09	0.06	Yes
Yeast cells viability at the end of culture (%)	-	-	-	-	96.7	92.7	No
Moisture (%)	8.72±0.20 ^a		5.63±0.90 ^b	6.90±0.64 ^b	3.59±1.25 ^c	5.32±0.08 ^c	-
Dry matter (%)	91.28±0.20 ^a		94.37±0.90 ^b	93.10±0.64 ^b	96.41±1.25 ^c	94.68±0.08 ^c	-
Ashes (%)	2.06±0.14 ^a		4.08±0.23 ^b	3.91±0.08 ^b	6.29±0.17 ^c	5.21±0.10 ^d	-
Ethereal extract (%)	0.49±0.21 ^{a,b}		1.93±0.96 ^a	0.33±0.36 ^b	0.91±0.53 ^c	2.10±1.20 ^c	-
Crude protein (%)	1.80±0.12 ^a		6.65±0.63 ^b	7.00±0.50 ^b	35.61±0.69 ^c	41.95±1.65 ^d	-
Crude fiber (%)	19.50±0.14 ^a		34.34±0.75 ^b	36.81±1.02 ^b	3.49±1.13 ^c	2.16±0.47 ^d	-
Non-nitrogenous extracts (%)	76.15±0.12 ^a		53.00±1.49 ^b	51.95±6.16 ^b	53.70±4.46 ^c	48.58±4.97 ^c	-
Total digestible nutrients (%)	58.45±0.46 ^a		55.18±1.90 ^b	51.89±3.15 ^b	82.28±4.89 ^c	86.05±2.48 ^c	-

a, b, c, d = Different letters correspond to statistically different averages (P<0.05)

On the other hand, *S. japonicus* Sia 70a biomass yield ($Y_{x/s}$) was higher for SB culture than CR (0.37 g/g versus 0.19 g/g). Medium formulated for batch culture with SB contained yeast extract, which was not considered in the $Y_{x/s}$ calculation. In addition, this nutrient is economically unfeasible due to its high cost (US\$ 18/kg, Biorigin, Quatá/SP, Brazil). Another advantage of the use of CR over SB, besides higher biomass concentration, was the use of another residue (corn steep liquor) as nitrogen source, which can reduce culture medium costs and contribute to solve the ambiental problems.

Approximately 18.0% of the total SB supplied in batch culture was consumed by the yeasts, from 34.0 g to 27.9 g (*S. japonicus* Sia 70a) and to 27.6 g (*S. cerevisiae* M26). Meanwhile, for cassava fed-batch culture, a total of 53.7% and 54.7% of the supplied bagasse were consumed by *S. japonicus* Sia 70a and *S. cerevisiae* M26 respectively. Since *S. japonicus* Sia 70a produced more biomass concentration with SB or CR than *S. cerevisiae*, the first yeast demonstrated more versatility to use both carbon sources to produce biomass. The yeast cells viability at the end of the culture was above 80.0% demonstrating the conditions for these cultures were healthy for the yeasts.

The maximum specific growth rate in bioreactor cultures using CR (0.09 h⁻¹ for *S. japonicus* Sia 70a and 0.06 h⁻¹ for *S. cerevisiae* M26) were lower than SB culture (0.15 h⁻¹ for *S. japonicus* and 0.09 h⁻¹ for *S. cerevisiae*). The extended fed-batch growth phase with CR may explain the lower maximum specific growth rate. While culture with cassava ended at 72-96 h, sugarcane culture ended earlier, at 24 h. Results of biomass concentration, productivity and yield demonstrated higher affinity to *S. japonicus* Sia 70a than *S. cerevisiae* M26 indicating that *S. japonicus* Sia 70a is more robust and convenient strain for SCP in CR.

The high affinity of *S. japonicus* Sia 70a to maltose and starch and low assimilation of xylose (Table 12) helps to explain explain the higher preference of this yeast to CR than SB. The inability of *S. cerevisiae* to consume xylose and starch could explain the lower growth capacity of using both residues (Vaughan-Martini and Martini, 2011).

Xylanases production from SB culture was higher for *S. japonicus* Sia 70a than *S. cerevisiae* M26 (Figure 10). While in *S. japonicus* Sia 70a culture xylanases started with 0.32 U/mL and reached up to 0.52 U/mL after 36 h of fermentation, *S. cerevisiae* M26 culture started with 0.14 U/mL and decreased along the culture time. Xylanases detected in the beginning of *S. cerevisiae* M26 culture may be due to the own SB or other microorganisms (up to 1×10^5 cells/mL) in the broth since this yeast does not have the apparatus to produce xylanases by itself (La Grange et al., 2001). These enzymes are responsible for the hydrolysis of the xylan component of hemicellulose, releasing fermentable sugars that can be used for cell growth and

maintenance (Alponti et al., 2016). Others enzymes may be produced to hydrolyze lignocellulolytic complexes such as cellulases and β -glucosidases although they were not quantified in this study. Reducing sugars (RS) in the beginning of SmF with SB was approximately 0.84 g/L for both yeasts and decreased along the culture, reaching 0.24 g/L for *S. japonicus* Sia 70a and 0.38 g/L for *S. cerevisiae* M26, demonstrating the use of the RS for growth.

During the fed-batch culture with CR *S. japonicus* Sia 70a, amylases were produced, which were quantified at 12 h (0.20 U/mL), 36 h (0.13 U/mL) and 72 h (0.18 U/mL). Such production was detected in culture medium before the introduction of new fed medium when nutrients were scarce and the yeast needed to produce enzymes to release fermentable sugars from the residue (Alponti et al., 2016). Since *S. cerevisiae* cannot produce amylases by itself (Van Zyl et al., 2012) they were not detected. High amounts of RS in culture medium were observed after each fed, more evident for *S. japonicus* Sia 70a culture. Amylase production by *S. japonicus* Sia 70a can also have contributed to the sugar release detected in the next sample. Both cultures stabilized after 72 h, not being noticed the additional use of the sugars available in the medium to cell growth.

The production of amylases and xylanases by *S. japonicus* Sia 70a can help to explain higher biomass concentration found at the end of the culture. The production of these enzymes by *S. japonicus* was not found in the literature for comparison, demonstrating the need to further study this special yeast. In addition, *S. japonicus* produces phytases (Khanh et al., 2012), an important enzyme widely used in monogastric animals to decrease the phytate effect and improvement of digestibility of phosphorus, calcium, amino acids and other energy sources (Dersjant-Li et al., 2015).

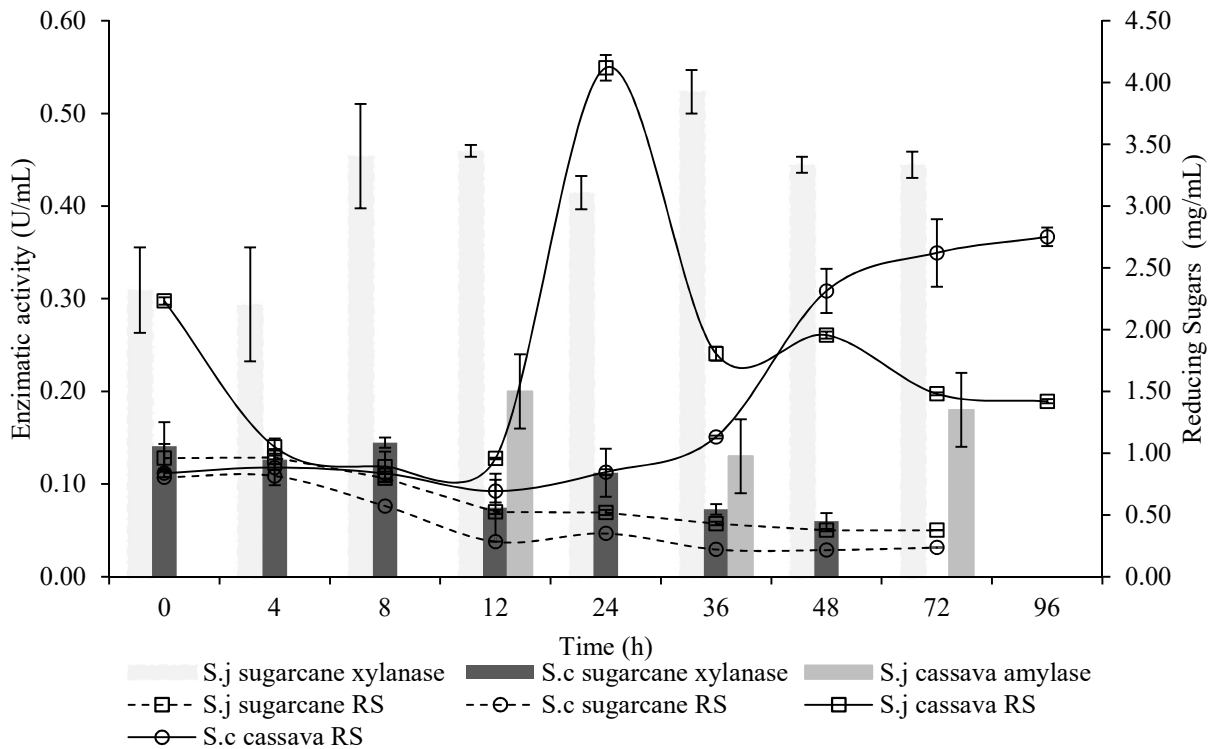


Figure 10 – Reducing sugars (RS), xylanase and amylase produced during submerged batch culture in bioreactor (28 °C, pH 5.5, 1 vvm) with sugarcane bagasse or fed-batch culture with solid cassava residue with *Sporobolomyces japonicus* Sia 70a (S.j) and *Saccharomyces cerevisiae* M26 (S.c).

The specific rate of oxygen consumption for both yeasts in batch culture using SB showed a direct correlation respect to cell concentration, which indicates an intense metabolic activity by these yeasts (Figure 11). At the end of log phase there was an abrupt fall to almost zero, which can be related to nutrient availability in the culture medium and also a less reproduction and metabolism by the cells. Cell concentration, nutrients available, oxygen solubility, as well as how fast oxygen is consumed are important parameters to indicate yeast metabolism (Gonçalves et al., 2001). With the stoppage at 24 h (stationary phase), these parameters were also stabilized. *S. japonicus* Sia 70a had a higher cell growth in the same conditions than *S. cerevisiae* M26, but the last yeast had a higher specific rate of oxygen consumption during log phase, indicating a greater need for dissolved oxygen for *S. cerevisiae* M26 under the same conditions (1 vvm). Oxygen consumption rate is a good indicator of biological activity and provides a rapid response method to substrate consumption (Campos et al., 2006).

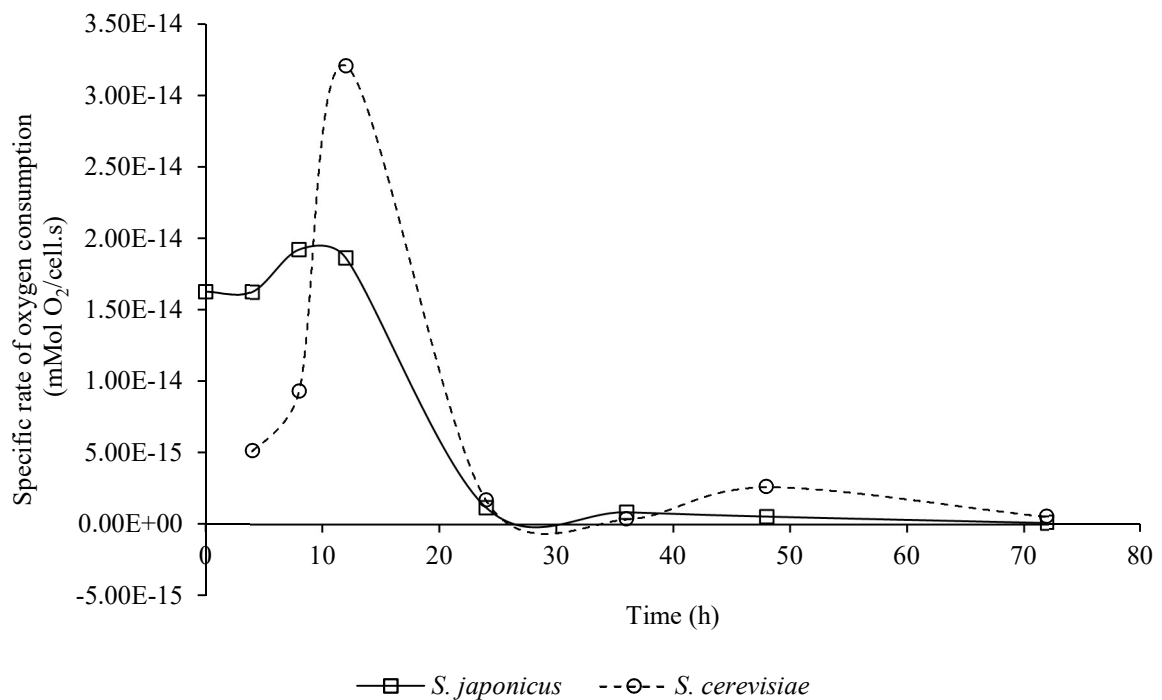


Figure 11 – Specific rate of oxygen consumption of *Sporobolomyces japonicus* Sia 70a and *Saccharomyces cerevisiae* M26 culture with sugarcane bagasse in bioreactor at 28 °C, pH 5.5, 1 vvm.

5.4.6 Nutritional parameters of residues and *S. japonicus* Sia 70a and *S. cerevisiae* M26 biomass after submerged culture in bioreactor

Crude protein of the yeasts biomass was higher for CR culture (35.6% for *S. japonicus* Sia 70a and 41.9% for *S. cerevisiae* M26) than SB culture (26.2% and 28.2% respectively), reaffirming cassava as a more interesting residue to apply in SCP production (Tables 14 and 15). Different moistures between the yeasts biomass (3.6% for *S. japonicus* Sia 70a and 5.3% for *S. cerevisiae* M26) led to a different dry matter concentration. Ashes (6.3%) and crude fiber (3.5%) were higher for *S. japonicus* Sia 70a than *S. cerevisiae* M26 biomass. However was lower for *S. japonicus* Sia 70a respectively 0.9% and 82.3% than for *S. cerevisiae* M26 (2.1% and 86.1%) (Table 15).

Submerged culture with the yeasts changed the nutritional parameters of cassava residue (Table 15). Ashes were increased almost 2 times for both yeasts and crude fiber enhanced from 19.5% to 34.3% (*S. japonicus* Sia 70a) and to 36.8% (*S. cerevisiae* M26). Higher crude fiber obtained after submerged culture decreased the percentage of no-structural carbohydrates from 76.2% to 53.0% (*S. japonicus* Sia 70a) and 51.9% (*S. cerevisiae* M26). The total digestible nutrients also decreased after the cultures of *S. japonicus* Sia 70a and *S. cerevisiae* M26 from

58.5% to 55.2% and 51.9% respectively. Probably these decreases were caused by the consumption of glucose from starch, which increased the percentage of crude fibers and ashes and decreased total digestible nutrients, since the lignin was not consumed by the yeast. Ethereal extracts increased to 1.9% with *S. japonicus* Sia 70a but decreased to 0.3% with *S. cerevisiae* M26 culture.

Regarding the crude protein from both residues, this component increased at least 2.4 times (Tables 14 and 15). SB enhanced from 1.6% (before culture) to 4.7% and 3.7% after the *S. japonicus* Sia 70a and *S. cerevisiae* M26 cultures respectively. The protein of raw CR obtained after bioreactor culture was higher than SB, from 1.8% to 6.7% (*S. japonicus* Sia 70a) and 7.0% (*S. cerevisiae* M26), an improvement of 269% and 289% respectively. In addition, these protein concentrations do not include protein from yeast biomass since there was a filtration and separation of the final residue from the yeast biomass. This combination with yeast cells and protein from residue would enhance up to 11.8% for CR since *S. japonicus* Sia 70a biomass contain 35.6% of protein. The highest CR protein improvement over SB is another advantage to the use of such residue as a substrate for SCP production.

Although protein content of yeast biomass and CR after submerged culture were higher for *S. cerevisiae* M26 than *S. japonicus* Sia 70a, the fed-batch process produced 1.8 more biomass concentration for the last yeast, also confirming its importance to SCP production.

There is weak scientific information about *S. japonicus* biomass production was found. Some studies with other microorganisms, process and residues are reported. *Penicillium janthinellum*, a cellulolytic fungus was cultivated under SmF with cassava peel and pretreated sugarcane bagasse with acid achieved 1.3 g/L (0.013 h^{-1}) and 0.5 g/L (0.030 h^{-1}) respectively (Oliveira et al., 2006). The culture of *Candida utilis* and *S. cerevisiae* achieved 6.56 g/L and 6.48 g/L of biomass production respectively, in a culture medium formulated with hydrolyzed rice straw (Araújo and D'Souza, 1986). Another study using eucalyptus hydrolysate obtained 6.35 g/L of *Paecilomyces variotii* (Silva et al., 1995). *Sporidiobolus pararoseus* culture for carotenoids production reached 3.86 g/L of biomass cultivated in complex medium (Cabral et al., 2011). In another study, the same yeast produced 5.1 g/L of biomass using glycerol, corn steep liquor and parboiled rice water (Valduga et al., 2014). *S. pararoseus* biomass was obtained using sugarcane molasses and corn steep liquor achieving 8.27 g/L (Machado and Burkert, 2014). Except the last one, all other studies showed lower biomass than *S. japonicus* Sia 70a in the culture with cassava residue (8.1 g/L and 0.09 h^{-1}), highlighting the biotechnological potential of this yeast. However, other studies are need, for example, studies of optimization of the ideal conditions for biomass production would enhance such productivity.

According to Neumann et al. (2016) sugarcane bagasse contain 1.81% of crude protein, in accordance with this research (1.6%), confirming the low protein content of this residue (Pandey et al., 2000a). Cassava residue contains a range from 0.53% to 1.06% ethereal extract, 14.88% to 50.55% crude fiber, 0.32% to 1.61% crude protein, 0.66% to 1.5% ashes, 5.02% to 11.2% moisture and 40.50% to 63.84% carbohydrates (Pandey et al., 2000b). In the present results was obtained similar compositions for cassava, being slightly lower for ethereal extract (0.5%), and higher for ashes (2.1%) and crude protein (1.8%).

Agro-industrial residues used as feed often requires some pretreatments and the addition of compounds to enrich nutrients composition and turns the process more expensive and less viable (Lima Jr et al., 2010; Pandey et al., 2000a; Silva et al., 2007; Yang et al., 2001). Biological treatment through culture helps to enhance wastes nutritional parameters and are a valuable source of protein, vitamins, acids and minerals, raising digestibility and palatability, often providing probiotic effects on host tract (Apás et al., 2008; Lima Jr et al., 2010). The use of agro-industrial residues as feed also help to solve the problem of its accumulation into the environment (Pinto et al., 2005).

Some studies aimed the protein enrichment of wastes. El-Sayed et al. (1994) studied the submerged fermentation (SmF) of *Pleurotus ostreatus* in raw sugarcane bagasse with an increase to 22.6% after 14 days of incubation. SmF mixed culture of *Trichoderma reesei* and *Rhizopus* sp. obtained 7.7% SB crude protein (Monteiro et al., 1991). Culture with *S. japonicus* Sia 70a in this study increased SB protein concentration from 1.6% to 4.7% after only 3 days of culture.

Solid-state fermentation (SSF) of the algae *Spirulina platensis* and *Chaetomium cellulolyticum* increased SB protein content to 7.2% (Pelizer et al., 2007) and to 8.65% (Bravo et al., 1994), respectively. Vandenberghe et al. (2000), Soccol et al. (1995a), and Soccol et al. (1995b) improved protein concentration of cassava residue from 13.1% to 23.1% using *Aspergillus niger* and 12.0% and 13.5% (scaling-up) using *Rhizopus oryzae*, in SSF. Such values for SB are higher than *S. japonicus* Sia 70a obtained in SmF with the residue but similar to CR cultivation (6.7%). The results are for SSF process while the present work was with SmF. Different conditions could explain the difference of results within the literature. In addition, protein content would be enhanced if all *S. japonicus* Sia 70a cells were dried with the residue achieving up to 11.8% for CR protein.

Apás et al. (2008) enhanced nutritional parameters of sugarcane blunting after 60 days of silo fermentation with probiotic bacteria, obtaining 14.22% of carbohydrates, 11.57% of fiber, 1.4% of protein, 3.6% of ashes and 0.47% of lipids. *S. japonicus* Sia 70a SmF of 72 h

using SB and 96 h using CR achieved higher concentrations of all parameters mentioned, highlights carbohydrates (53.0% for cassava) and crude protein (4.7% for sugarcane and 6.7% for cassava). Comparing such parameters, both residues could be used for feed after submerged cultivation.

5.4.7 Chemical composition of *S. japonicus* and *S. cerevisiae* cells

Cellular composition of *S. japonicus* Sia 70a and *S. cerevisiae* yeast biomass demonstrated similar concentrations of phosphorus (15.16-15.47 g/kg) and sulfur (3.89-4.00 g/kg) (Table 16). When comparing other chemical components *S. japonicus* Sia 70a was superior to *S. cerevisiae* in magnesium (11.9%), copper (69.4%), manganese (300%) and zinc (48%) and inferior in carbon (8.4%), nitrogen (7.9%) and sulfur (2.7%).

Levels of potassium and calcium of *S. japonicus* Sia 70a biomass were lower than *S. cerevisiae* in 25.3% and 95.2%, respectively. Considering the chemical composition of both species allied to the search of new sources of SCP, *S. japonicus* Sia 70a has the advantage of growing better in cheap substrates as agro-industrial residues. In addition, this microorganism provides desirable quantities of mineral and protein (35.0%). Since minerals cannot be synthesized, and yeast cells are a valuable source of such compounds. The relationship with metabolism involves, among others, energy for nucleic acids production, cofactors, synthesis of vitamins and amino acids and synthesis of pigments (Alterthum, 2001; Cooper and Hausman, 2004).

Table 16 – Chemical composition of *Sporobolomyces japonicus* Sia 70a and *Saccharomyces cerevisiae* biomass after submerged culture in aerobiosis in orbital shaker agitator at 180 rpm, 28 °C for 96 h.

Element	<i>S. japonicus</i> (g/kg)	<i>S. cerevisiae</i> (g/kg)*
Carbon	421.50±3.44	460.00±10.0
Nitrogen	74.96±2.70	82.50±7.50
Phosphorus	15.47±0.15	15.16±0.2
Potassium	15.20±0.10	20.35±0.005
Calcium	0.07±0.002	1.47±0.014
Magnesium	1.60±0.02	1.43±0.003
Sulfur	3.89±0.003	4.00±1.00
Copper	0.083±0.0001	0.049±0.0003
Manganese	0.042±0.0002	0.014±0.0001
Zinc	0.188±0.001	0.127±0.001

*Belluco (2001)

Besides a higher biomass enriched in protein and amino acids to the host animal, another advantage of *S. japonicus* Sia 70a compared to *S. cerevisiae* is the possible supply of carotenoids. Some *Sporobolomyces* species such as *S. roseus* (now *S. pararoseus*) and *S. salmonicolor* (now *Sporidiobolus salmonicolor*) and *S. patagonicus* produce torulene, torularodine, γ -carotene and β -carotene, which are carotenoids with antioxidant activity and pro-vitamin effects (Manimala and Murugesan, 2014; Mata-Gómez et al., 2014). Although *S. japonicus* forms colonies with pink pigmentation more studies are necessary to prove the chemical nature of this pigment. Antioxidant effect, cancer prevention and enhancing immune system are desirable effects caused by the ingestion of such pigments (Maldonado et al., 2012). Thus, the use of new probiotics microbes as additives in animal or human nutrition is interesting. Moreover, since the production of pigments are expensive which limit its industrial obtaining, the use of low-costs substrates as agro-industrial residues provides the possibility to diminish such value (Maldonado et al., 2012).

Harsh residues as sugarcane and cassava need the presence of fibrolytic enzymes as well as amylases to degrade the starch components, and these enzymes help on the digestion process (Newbold et al., 1996). Some probiotics microorganisms, mainly bacteria, are present in the rumen flora, and produce extracellular enzymes (Newbold et al., 1996).

Some specific characteristics of *S. japonicus* are advantageous over *S. cerevisiae* for SCP production. Higher biomass concentration, high protein content and the production of enzymes and other nutritional enrichment of the agro-industrial residues are some important

advantages of *S. japonicus*. This species emerges as an alternative yeast for single cell protein with potential probiotic effects, although more studies are necessary to prove the positive effects on animal nutrition.

5.5 Conclusions

The yeast *Sporobolomyces japonicus* Sia 70a presents interesting nutritional and metabolic characteristics, which makes it a special species for biotechnological application. Capacity to produce its own amino acids, high affinity to maltose, starch assimilation, enzymes production (xylanases and amylases) and high biomass productivity with non-treated residues make *S. japonicus* an alternative as food or feed additive with possible probiotics effect. The use of low-cost substrates as sugarcane bagasse and cassava solid residue in submerged culture may reduce bioprocess costs, increase the added value of the residue and improve its nutritional parameters that can be used for feed. This biological treatment could be used as an alternative to physical-chemical treatments for several applications of wastes. However, more studies are necessary to confirm probiotic effects of *S. japonicus* and the safety and quality of its nutrients to use as feed.

6 CAPÍTULO 3

Protein biomass production from agro-industrial residues and physiological aspects of the yeast *Sporidiobolus pararoseus* Sia 33.1

6.1 Abstract

In order to supply the demand for food due to the increase of world population, new and economic nutrient sources are necessary to be developed. Currently, the research for new bioprocesses using agro-industrial wastes and some special microorganisms consist in a strategic alternative for this purpose. The aim of this research was to obtain yeast biomass from *Sporidiobolus pararoseus* Sia 33.1 in aerobic submerged fermentation using agro-industrial residues as well as to improve the protein content of the residues to use as feed. This yeast was characterized regarding chemical and nutritional composition, assimilation of different carbohydrates and the growth in absence of amino acids and vitamins. Submerged culture in bioreactor occurred in single-batch with medium formulated with sugarcane bagasse and fed-batch with solid hydrolyzed cassava residue. Kinetics parameters demonstrated that *S. pararoseus* Sia 33.1 showed a higher affinity to agro-industrial residues than *Saccharomyces cerevisiae* M26. The highest biomass production in a bioreactor was reached by *S. pararoseus* Sia 33.1 with hydrolyzed cassava residue as carbon source. This yeast produced 5.2 g/L of biomass with 43.3% of protein (biomass yield of 0.09 g/g and productivity of 0.05 g/L.h), while *S. cerevisiae* M26 produced only 3.1 g/L of biomass under the same conditions. Production of amylases (0.26 U/mL) and xylanases (0.25 U/mL) by *S. pararoseus* Sia 33.1 helps to elucidate its ability to grow in sugarcane bagasse or hydrolyzed cassava residue. Nutritional parameters of these residues improved after the culture with *S. pararoseus* Sia 33.1. The protein content increased from 1.6% to 4.6% (sugarcane bagasse) and 1.9% to 8.3% (solid cassava residue). *S. pararoseus* Sia 33.1 showed high affinity to maltose, fructose, glucose, sucrose, galactose and xylose, produced all amino acids by its own although was sensitive to the absence of riboflavin, folic acid, ascorbic acid, pyridoxine and pantothenic acid. The desirable chemical and nutritional composition of *S. pararoseus* Sia 33.1 biomass, higher affinity to grow in agro-industrial wastes and some specific enzymes production make this special yeast more interesting to use as protein source than *S. cerevisiae* M26 with desirable probiotic characteristics and application as feed and food additive. However, studies of animal nutrition with this yeast biomass are necessary to prove the safety and nutritional advantages of this species for animal production.

Key words: Single cell protein; Special yeast; Direct-fed microbials; Animal feed.

6.2 Introduction

The exponential growth of population in the last decades led to a higher demand for food, and the distance between the demand and the supply is alarming (Anupama and Ravindra, 2000). In order to supply such demand, new protein sources as well as new methods to enhance animal performance and productivity demonstrated to be a viable way to solve the problem (Anupama and Ravindra, 2000; Ghorai et al., 2011). The use of low-cost substrates as agro-industrial residues and technologies using small areas for the food production are important to reduce the pressure for pasture and new plantation areas (Anupama and Ravindra, 2000). Single cell protein (SCP) production, which is the protein biomass from microorganisms, is a biotechnological method which can help to solve the problem of food sources (Anupama and Ravindra, 2000). Algae, fungi and bacteria are rich on protein, amino acids, carbohydrates, vitamins, fats and essential minerals, which can serve as nutrients for humans or animals (Anupama and Ravindra, 2000; Ghorai et al., 2011). The wide range of characteristics of fungi and bacteria cultures make them more convenient for SCP sources (Anupama and Ravindra, 2000). Between the yeasts used for SCP production the mainly genus used are *Candida*, *Hansenula*, *Pichia*, *Torulopsis* and *Saccharomyces* (Anupama and Ravindra, 2000).

The use of microorganisms that provide beneficial effects on the animal's gastrointestinal tract is another alternative to supply food demand, enhancing animal performance and productivity (Apás et al., 2008). Such microorganisms are classified as probiotics, or direct-fed microbial (DFM), a term recognized by the Food and Drug Administration (FDA) (Salminen et al., 1999; França and Rigo, 2011). Although the exact causes for enhancing animal performance are not entirely known, microorganisms can contribute to flavor, texture and aroma of the food (França and Rigo, 2011). Probiotics also control maleficent microorganism's population, stimulate cellulolytic bacteria growth, maintain the rumen pH and supply essential compounds for the animal (Newbold et al., 1996; França and Rigo, 2011). Currently, the most feed used can modify gastrointestinal environment of the animal causing some health problems and decrease of the animal productivity (França and Rigo, 2011).

The use of non-conventional substrates as agro-industrial residues decreases the costs with feed. These substrates demonstrate to be a viable solution to the pollution caused by its accumulation in the environment (Yang et al., 2001; Villas-Bôas et al., 2002; Pinto et al., 2005). Unfortunately, besides some agro-industrial wastes are used as feed, they are poor nutrients, especially protein, and/or these nutrients are not available (low digestibility). For these reasons, they often require physical or chemical treatments and nutrient complementation in order to

increase the feed content which also increases process costs (Villas-Bôas et al., 2002; Lima Jr et al., 2010). Culture with microorganisms could be an alternative to these problems, since they enhance food durability, digestibility and are a valuable source of protein, vitamins and other beneficial compounds to the animal (Apás et al., 2008).

Sugarcane bagasse and cassava residue are two of the most important wastes in Brazil, produced by ethanol and food industries (Pandey et al., 2000a; Silva et al., 2007). Both contains low protein content, and are used as feed with or without pretreatments and minerals complementation (Yang et al., 2001; Villas-Bôas et al., 2002). Protein enrichment of such residues after microorganism inoculation would help to solve the low protein content (Monteiro et al., 1991; Bravo et al., 1994; El-Sayed et al., 1994; Pelizer et al., 2007).

The yeast *Sporidiobolus pararoseus* belongs to the Phylum Basidiomycota, Class Microbotryomycetes and Order Sporidiobolales (NCBI, 2016). This species is naturally found in the environment (Sampaio, 2011), commonly associated with grapes surface (Baffi et al., 2010; Li et al., 2010; Brysch-Herzberg and Siedel, 2015). Literature reports the production of lipase (Bussamara et al., 2010; Smaniotto et al., 2012; Qiao et al., 2013), β -glucosidase (Baffi et al., 2010; Baffi et al., 2011), amylase and cellulase (Qiao et al., 2013) by this species. Moreover, *S. pararoseus* is a potential biocontrol agent, with antagonistic effect on *Penicillium italicum*, *Penicillium digitatum*, *Botryodiplodia thepbromae*, *Geotrichum candidum*, *Alternaria alternata* (Sharma et al., 2008), *Monilinia fructicola* (Janisiewicz et al., 2010), *Botrytis cinerea*, *Mucor* sp., *Penicillium* sp., *Rhizopus* sp. (Huang et al., 2012), *Fusarium fujikuroi* (Matić et al., 2014), *Escherichia coli* and *Staphylococcus aureus* (Laconi and Pompei, 2007). This species also produces carotenoids (Cabral et al., 2011; Han et al., 2012; Valduga et al., 2014), mainly β -carotene, γ -carotene, toluene and torularhodin (Han et al., 2012).

In view of the few species currently used as SCP and DFM, as well as the strategic to use nutritionally enriched wastes as feed, the aim of this work was to study some physiological aspects of *Sporidiobolus pararoseus* Sia 33.1 culture, using cassava residue and sugarcane bagasse as carbon source, to produce SCP and nutritionally enrich final residues.

6.3 Materials and Methods

6.3.1 Microorganisms and culture medium

The strain of *Sporidiobolus pararoseus* Sia 33.1 used in the present study was isolated from soil. *Saccharomyces cerevisiae* M26 used was isolated from sugar mills by Oliva Neto et al. (2004) was used as comparison in submerged culture tests in bioreactor.

Basal culture medium mentioned was composed by (w/v): 0.13% $(\text{NH}_4)_3\text{PO}_4$, 0.10% $(\text{NH}_4)_2\text{SO}_4$, 0.031% K_2SO_4 , 0.0028% ZnSO_4 , 0.0012% MnSO_4 and 0.024% MgSO_4 , modified from Oliva Neto et al. (2004).

6.3.2 Anaerobiosis test

The target yeast was cultivated in Petri dishes with 3.9% (w/v) of Potato Dextrose Agar (PDA) and incubated in anaerobic jar (Anaerocult, Merck, Darmstadt, Germany) in presence of an anaerobic generator (Anaerobac, Probac do Brasil, São Paulo, Brazil) for 72 h at 33 °C. Control Petri dishes were incubated at the same conditions in oven (002 CB, Fanem, São Paulo/SP, Brazil). Visual comparison between Petri dishes evaluated the yeast ability to grow in absence of oxygen.

6.3.3 Carbohydrates growth test

S. pararoseus Sia 33.1 ability to grow using different carbohydrates (arabinose, fructose, galactose, glucose, lactose, maltose, sucrose or xylose) was performed through inoculation of 1.0×10^6 cells/mL in flasks and incubated at 28 °C, 180 rpm for 96 h in orbital shaker incubator (TE421, Tecnal, Piracicaba/SP, Brazil). Medium composition contained basal culture medium, (w/v), 0.2% of carbohydrate and 0.2% of yeast extract. Negative control flask was absent of carbohydrate.

6.3.4 Amino acid and vitamins single-omission growth tests

In order to evaluate *S. pararoseus* Sia 33.1 capacity to grow in absence of specific amino acids in aerobic conditions, tubes cultures containing basal culture medium, 2.0% (w/v) glucose, 8 vitamins (aminobenzoic acid (0.2 µg/mL), ascorbic acid (2 µg/mL), folic acid (0.02 µg/mL), nicotinic acid (2 µg/mL), panthotenic acid (1 µg/mL), pyridoxine (2 µg/mL), riboflavin (2 µg/mL), thiamin (1 µg/mL) and 19 pure amino acids at 0.03% (w/v) out of 20 available (Table 18).

Vitamins single-omission test occurred in tubes culture with basal culture medium, 2.0% (w/v) glucose, 20 amino acids (0.03%) and 7 out of the 8 vitamins. *S. pararoseus* Sia 33.1 ability to grow in absence of one vitamin was evaluated. Positive and negative control were performed respectively, with tubes containing all nutrients and without amino acids/vitamins.

Cell growth was measured indirectly through turbidity (600 nm) (UV-M51, Bel Engineering, Monza, Italy) after 120 h of incubation at 28 °C (Oliva Neto and Yokoya, 1997).

6.3.5 Submerged culture using agro-industrial residues in bioreactor

Sugarcane bagasse (SB) and solid hydrolyzed cassava residue (HCR) were evaluated as main carbon sources in aerobic submerged fermentation (SmF) by *S. pararoseus* Sia 33.1 or *S. cerevisiae* M26 cultures in bioreactor (Bioflo 115, New Brunswick, New Jersey, USA). Cultures conditions of 28 °C, pH 5.5 and 1 vvm of air supply were equal for both residues fermentation while time of the culture and mechanical agitation of culture medium were different. Dissolved oxygen was measured with a polarographic electrode (Mettler Toledo AG, Greifensee, Switzerland) and pH was measured with a glass electrode (Mettler Toledo AG, Greifensee, Switzerland) and controlled with 1.0% (v/v) H₂SO₄ and 1.0% (w/v) NaOH. Each inoculum was prepared by the yeasts culture in flasks with 50 mL of basal culture medium, 1.0% (w/v) glucose and 0.5% (w/v) of yeast extract incubated in orbital shaker incubator (TE421, Tecnal, Piracicaba/SP, Brazil) for 24 h, 28 °C and 180 rpm.

Medium used for single-batch with SB was composed by (w/v): 2.0% SB, 0.2% yeast extract, 0.05% glucose and basal culture medium in 1.5 L for *S. pararoseus* Sia 33.1 and 1.7 L for *S. cerevisiae* M26. Mechanical agitation of culture was 200 rpm for 72 h.

Pretreatment of solid cassava residue (CR) occurred by hydrolysis of 10% (w/v) of concentration in sodium acetate buffer (0.05 M, pH 5.5) for 24 h, 50 °C and 1.2 U/mL of *Rhizopus oligosporus* amylase (Gonçalves, 2016). The final residue was dried in oven for 72 h, 50 °C (TE394/2, Tecnal, Piracicaba/SP, Brazil) after hydrolysis.

SmF using HCR occurred in fed-batch process. Medium used was composed by (w/v) 5.0% HCR, 0.5% corn steep liquor and basal culture medium without (NH₄)₃PO₄ and 500 mL at the beginning of culture. Medium supply was performed according to maintain the level of initial soluble solids concentration (Brix) (Portable Refractometer, Biosystems) at the beginning of culture in three different times for both strains. Feds occurred at 12, 24 and 36 h for *S. pararoseus* Sia 33.1 culture and at 12, 36 and 72 h for *S. cerevisiae* M26 culture. Culture ended at 96 h for *S. pararoseus* Sia 33.1 and at 168 h for *S. cerevisiae* M26, with initial mechanical agitation of 350 rpm, and increased to 450, 550 and 650 respectively after feds due to increase of medium viscosity.

The separation of liquid medium and residual SB or HCR after submerged culture occurred through manual filtration. The liquid was centrifuged at 5000 x g for 30 minutes and 4 °C (Megafuge 16R, Heraeus, Thermo Fisher, Massachusetts, USA) for cell separation. Both yeast and residue were dried in oven (TE394/2, Tecnal, Piracicaba/SP, Brazil) for 72 h at 50 °C before nutritional tests.

6.3.6 Analytical procedures

Growth curve was constructed through direct cell concentration with Neubauer counting chamber (7301-1B, New Optics) and yeasts cells viability was assessed according to Lee et al. (1981). Dried yeast biomass was performed according to Chen et al. (2012). Quantification of reducing sugars (RS), xylanase and amylase were performed according to Miller (1959), Carvalho et al. (2015) and Gonçalves (2016) respectively. One enzymatic unit means the releasing of 1 μmol of RS per minute per mL of enzymatic extract. When necessary, total reducing sugars (TRS) quantification occurred after acid hydrolysis using H_2SO_4 (2.0 M), at the proportion 1:1, for 10 minutes at 100 °C followed by neutralization with NaOH (2.0 M) (1:1:1) and quantification through the Miller (1959) method.

Kinetics parameters maximum specific growth rate (μ_{max}), yield coefficient of cell growth ($Y_{x/s}$) and biomass productivity were determined according to Shuler and Kargi (2002). Specific rate of O_2 consumption of the culture using sugarcane bagasse was calculated according to Núñez et al. (2014) considering the concentration of saturated O_2 at 28 °C equals to 0.244 mMol/L.

Nutritional analysis of dry matter, ashes, fat content, crude fiber and crude protein of yeast's biomass and residues, before or after culture, as well as yeast's chemical composition were quantified according to the Association of Official Analytical Chemists (AOAC) methods (Helrich, 1990). Dry matter was quantified drying at constant weight at 105 °C using humidity balance (BTS110, Scientific Industries, Poland), ashes burning at 700 °C for 6 h using muffle oven (SP2707-20, Spence, São Paulo/SP, Brazil) and ethereal extraction with petroleum ether for 6 h with a Soxhlet equipment. Crude fiber was determined after acid (H_2SO_4) and basic (NaOH) – 1.25% – hydrolysis at 400 °C for 40 min and drying at 105 °C for 24 h and crude protein through the Kjeldahl method (digestor TE040125 and nitrogen distiller TE036/1, Tecnal, Piracicaba/SP, Brazil). Non-nitrogenous extracts was determined subtracting the percentage of ashes, fat content, crude fiber and crude protein. Total Digestible Nutrients was calculated according to Karl (1982). Yeast's centesimal composition of carbon was determined according to Walkley (1947), sulfur and phosphorus through “visible light spectrum”, calcium, potassium, magnesium and zinc with digestion and atomic absorption and nitrogen through Kjeldahl method.

All tests were performed in triplicates, and statistical analysis occurred through ANOVA and Tukey ($P < 0.05$) comparison between replicates using the software GraphPad Prism 5.01.

6.4 Results and Discussion

6.4.1 Anaerobiosis test

Tests using anaerobic jar demonstrated yeast's capacity to grow in environment absent of oxygen, although with little less colonies than control Petri dishes. Colonies also seemed less pink under anaerobic conditions, although more studies are necessary to affirm that *S. pararoseus* produced less pink pigment quantity in this environment. Growth tests of *S. pararoseus* under anaerobic condition were not found for comparison. According to Cobban et al. (2016), *Sporobolomyces roseus* is now recognized as *Sporidiobolus pararoseus*, a species capable of grown in anaerobiosis conditions (Ali and Hipkin, 1985; Cobban et al., 2016) and possibly confirm the facultative metabolism of this yeast.

6.4.2 Carbohydrates assimilation test

S. pararoseus Sia 33.1 demonstrated high affinity to grow using maltose, followed by fructose, glucose, sucrose, galactose and xylose as carbon source (Table 17). Greater cells concentrations were achieved with maltose (1.8×10^8 cells/mL), fructose (1.6×10^8 cells/mL), glucose (1.4×10^8 cells/mL), sucrose (1.3×10^8 cells/mL) and galactose (9.8×10^7 cells/mL). The use of xylose obtained intermediary cells concentrations (6.3×10^7 cells/mL) while arabinose and lactose demonstrated to be unviable to *S. pararoseus* Sia 33.1 with a slightly growth compared to the control flask.

Table 17 – Kinect parameters of *Sporidiobolus pararoseus* Sia 33.1 cultivated in flasks (180 rpm, 28 °C for 96 h) using different carbohydrates.

Carbohydrate	μ_{\max} (h ⁻¹)	Maximum cell concentration (cells/mL)	Log phase (h)	Biomass concentration (g/L)	Biomass yield (g/g)	Biomass productivity (g/L.h)
Arabinose	0.18	5.6E+07	12 - 28	0.57 ^{a*}	0	0
Fructose	0.17	1.6E+08	12 - 48	1.52 ^{b*}	0.87*	0.02**
Galactose	0.03	9.8E+07	12 - 96	1.32 ^{c*}	0.69*	0.01**
Glucose	0.19	1.4E+08	12 - 28	1.43 ^{b*}	0.78*	0.01**
Lactose	0.13	5.5E+07	8 - 28	0.53 ^{a*}	0	0
Maltose	0.18	1.8E+08	12 - 28	1.65 ^{d*}	0.90*	0.02**
Sucrose	0.19	1.3E+08	12 - 28	1.43 ^{b*}	0.71*	0.01**
Xylose	0.04	6.3E+07	12 - 72	0.76 ^{e*}	0.39*	0.01**
Control	0.17	4.5E+07	12 - 24	0.38 ^{a*}	-	-

a, b, c, d, e = Different letters correspond to statistically different averages (P<0.05)

Standard deviation: * = <0.03; ** = <0.004

Log phases started at 8-12 h, except for lactose culture and ended at different times (Figure 12). Flasks with maltose, glucose, sucrose, arabinose and lactose, log phase ended at 28 h, while for fructose, xylose and galactose, end of log phase were at 48, 72 and 96 h respectively. μ_{\max} were higher for the assimilation of maltose (0.18 h^{-1}), glucose (0.19 h^{-1}) and sucrose (0.19 h^{-1}), and the lowest values were achieved for galactose and xylose (0.03 h^{-1} and 0.04 h^{-1} respectively). Extended log phases and the low affinity to carbohydrates are parameters that can decrease the μ_{\max} .

Biomass concentrations were in accordance to maximum cells concentrations achieved for each carbohydrate, with 1.65g/L, 1.52 g/L, 1.43 g/L, 1.43 g/L, 1.32 g/L and 0.76 g/L for maltose, fructose, glucose, sucrose, galactose and xylose respectively. Biomass yield coefficient ($Y_{x/s}$) achieved values of 0.90 g/g, 0.87 g/g, 0.78 g/g, 0.71 g/g, 0.69 g/g and 0.39 g/g for maltose, fructose, glucose, sucrose, galactose and xylose respectively. Possibly, a longer cultivation with galactose and xylose would lead to a higher biomass concentration with the use of all sugars available. In arabinose and lactose culture was the absence of growth since sugars concentration was equal in the beginning and end of the culture ($Y_{x/s} = 0$).

Productivities of 0.02 g/L.h for maltose and fructose, and 0.01 g/L.h for glucose, sucrose, galactose and xylose were achieved. Time cultivation is one parameter responsible to increase or decrease productivity, and higher values could be achieved if culture ended at the end of log phase.

Literature reports are in accordance to results found by the present research. Sampaio (2011) stated that *S. pararoseus* is capable of assimilating glucose, sucrose, galactose, maltose, among others, but not lactose. Assimilation of arabinose and xylose varies depending on the strain (Sampaio, 2011b). *S. pararoseus* strain used in this research was capable to assimilate xylose, but not arabinose. Han et al. (2012) also confirmed the capacity of the yeast to grow using glucose, sucrose, mannose and galactose as carbon sources.

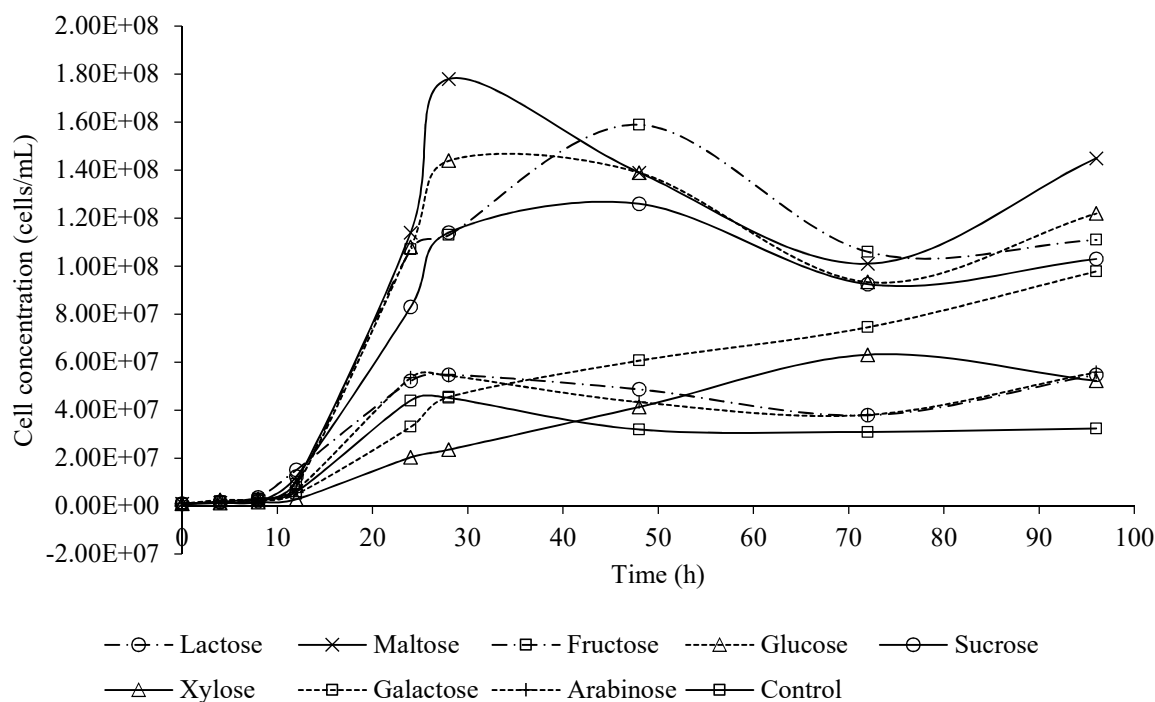


Figure 12 – Growth curves of *Sporidiobolus pararoseus* Sia 33.1 cultivated in flasks (180 rpm, 28 °C) with different carbohydrates.

6.4.3 *S. pararoseus* Sia 33.1 amino acids and vitamins single-omission test

The yeast *S. pararoseus* Sia 33.1 demonstrated to produce all amino acids by itself, although some vitamins were important for its growth (Table 18). Full growth of the yeast was achieved with the privation of mostly amino acids, and partial growth was observed in absence of tyrosine (Tyr) and glutamic acid (Glu). Perhaps yeast metabolism was only retarded by the absence of Tyr and Glu and more incubation days would be necessary to *S. pararoseus* Sia 33.1 achieve equal cell concentration of positive control tubes.

The absence of vitamins: riboflavin, folic acid, ascorbic acid, pyridoxine and pantothenic acid lead to cell growth absence (Table 18). In privation of nicotinic acid this species had a partial growth, but a full growth was observed in depletion of thiamin and aminobenzoic acid. Tubes cultures without any vitamins obtained partial growth of the yeast. Perhaps, the presence of certain vitamins stimulates this specie to use a different metabolic route, thus being capable to grow or not although more studies are necessary to elucidate this species metabolic routes. Capacity of *S. pararoseus* to grow in vitamin-free medium is in accordance to Sampaio (2011b) statements.

Table 18 – Tests of amino acids and vitamins single-omission in tube culture of *Sporidiobolus pararoseus* Sia 33.1 at 28 °C for 72 h.

Amino acid		Vitamin	
Nutrient	Growth	Nutrient	Growth
Trp	+++	Thiamin	+++
Leu	+++	Riboflavin	-
Pro	+++	Folic Acid	-
Val	+++	Ascorbic Acid	-
Tyr	++	Pyridoxine	-
Ala	+++	Panhotenic Acid	-
Ser	+++	Nicotinic Acid	++
Ile	+++	Aminobenzoic Acid	+++
Cys	+++		
Gln	+++		
Thr	+++		
Lys	+++		
Asp	+++		
Phe	+++		
Glu	++		
His	+++		
Arg	+++		
Met	+++		
Gly	+++		
Asn	+++		

- = Absence; + = Poor; ++ = Partial; +++ = Full
P<0.05

6.4.4 Kinect of submerged culture with *S. pararoseus* Sia 33.1 and *S. cerevisiae* M26 in bioreactor using sugarcane bagasse or hydrolyzed cassava residue

Cassava residue hydrolysis with *Rhizopus oligosporus* amylases produced 35.44 g/L of reducing sugars (RS) after 24 h. The content of RS are similar to Gonçalves (2016) obtained but lower than Shi et al. (2014) achieved, respectively 39.9 g/L and 43.3 g/L.

According to the growth curves of *S. pararoseus* Sia 33.1 and *S. cerevisiae* M26 in submerged culture (SmF) in bioreactor with agro-industrial residues, the use of solid hydrolyzed cassava (HCR) as carbon source achieved higher cells concentrations for both yeasts in comparison to sugarcane bagasse (SB) in SmF. Thus, pretreated cassava is more interesting to single cell protein (SCP) production from low-cost substrates than SB (Figure 13).

The yeast *S. pararoseus* Sia 33.1 demonstrated higher affinity to the agro-industrial residues than *S. cerevisiae* M26. Maximum cells concentrations were achieved at the end of log phase at 72 h using HCR as carbon source, with 6.6×10^8 cells/mL for *S. pararoseus* Sia 33.1

and 3.2×10^8 cells/mL for *S. cerevisiae* M26. In comparison, SmF with SB reached up to 1.6×10^8 cells/mL (*S. pararoseus* Sia 33.1) and 3.1×10^7 cells/mL (*S. cerevisiae* M26) at 24 h and 48 h, respectively.

Maximum specific growth rate (μ_{\max}) was higher for *S. pararoseus* Sia 33.1 than *S. cerevisiae* M26 in all cultures. While *S. cerevisiae* M26 culture with SB and HCR obtained 0.04 h^{-1} - 0.09 h^{-1} , *S. pararoseus* μ_{\max} were 0.19 h^{-1} and 0.13 h^{-1} respectively, confirming the higher affinity to agro-industrial residues for the last yeast. The close values of this parameter between cultures with SB and HCR can be explained by the extended log phase of fed-batch process with HCR which may led to a decrease of μ_{\max} .

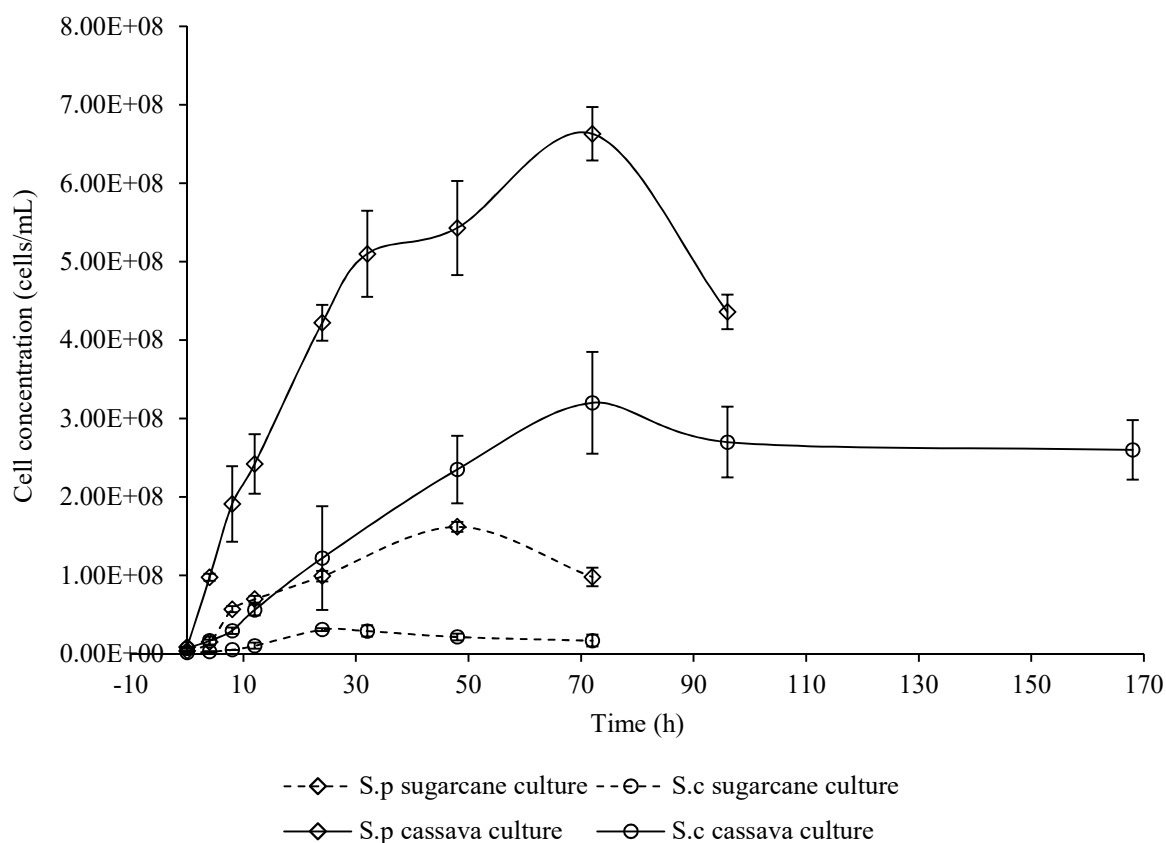


Figure 13 – Growth curves of *Sporidiobolus pararoseus* Sia 33.1 (S.p) and *Saccharomyces cerevisiae* M26 (S.c) during batch submerged culture with sugarcane bagasse or fed-batch submerged culture with hydrolyzed cassava residue in bioreactor at 28 °C, pH 5.5 and 1 vvm

S. pararoseus Sia 33.1 culture obtained a higher biomass concentration than *S. cerevisiae* M26 in both cultures (Tables 19 and 20). While *S. cerevisiae* M26 achieved 0.4 g/L and 3.1 g/L of biomass concentration in SmF with SB and HCR, respectively; *S. pararoseus* Sia 33.1 showed 3.8 and 1.7 times more biomass, with 1.6 g/L and 5.2 g/L respectively. The

use HCR was more appropriate to single cell protein (SCP) production over SB with 7.4 times more biomass from *S. cerevisiae* M26 and 3.2 times more from *S. pararoseus* Sia 33.1. High affinity of the last yeast to maltose and low affinity to xylose (Table 17) also helps to elucidate the greater biomass yielded by *S. pararoseus* Sia 33.1 with HCR compared to SB. According to the literature, *S. cerevisiae* is capable to assimilate maltose, but not capable to assimilate xylose (Vaughan-Martini and Martini, 2011) and also elucidates higher biomass concentration obtained in medium formulated with HCR than SB.

Biomass yield coefficient of *S. pararoseus* Sia 33.1 in SmF with HCR achieved 0.09 g/g, while *S. cerevisiae* M26 achieved 0.05 g/g. Although $Y_{x/s}$ for both cultures with SB were higher than HCR (0.42 g/g for *S. pararoseus* Sia 33.1 and 0.10 g/g for *S. cerevisiae* M26), the medium formulated for batch culture with SB contained yeast extract which was not considered for $Y_{x/s}$ calculation. Such extract possess a high cost (US\$ 18/kg, Biorigin, Quatá/SP, Brazil), which may be economically unfeasible depending on the industry. The use of HCR resulted in higher biomass concentrations, another advantage that is the use of another residue (corn steep liquor) as nitrogen source replacing yeast extract, can be an alternative to reduce culture medium costs.

Table 19 – Kinect and nutritional parameters of *Sporidiobolus pararoseus* Sia 33.1 (S.p) and *Saccharomyces cerevisiae* M26 (S.c) in batch culture using sugarcane bagasse in bioreactor (28 °C, pH 5.5, 1 vvm, 200 rpm for 72 h).

Parameters	Supplied bagasse before culture		After submerged culture			
			Final residue		Yeast biomass	
	S.p.	S.c.	S.p	S.c	S.p	S.c
Residue (g)	30.0	34.0	24.45	27.61	2.4	0.7
Crude protein (%)	1.6±0.19 ^a		4.6±0.7 ^b	3.7±0.3 ^b	39.2±0.3 ^c	28.2±0.4 ^d
Yeast biomass (g/L)	-	-	-	-	1.6	0.4
Max cell concentration (cell/mL)	-	-	-	-	1.6x10 ⁸	3.1x10 ⁷
Biomass productivity (g/L.h)	-	-	-	-	0.02	0.01
$Y_{x/s}$ (g/g)	-	-	-	-	0.42 ^a	0.10 ^b
μ_{max} (h ⁻¹)	-	-	-	-	0.19	0.09
Yeast cells viability at the end of culture (%)	-	-	-	-	94.9	81.0

^{a, b, c, d} = Different letters correspond to statistically different averages (P<0.05)

Table 20 – Kinect and nutritional parameters of *Sporidiobolus pararoseus* Sia 33.1 (S.p) and *Saccharomyces cerevisiae* M26 (S.c) in fed-batch culture using hydrolyzed cassava residue in bioreactor (28 °C, pH 5.5, 1 vvm).

Parameters	Supplied residue		After submerged culture			
	before culture		Final residue		Yeast biomass	
	<i>S.j</i>	<i>S.c</i>	<i>S.j</i>	<i>S.c</i>	<i>S.j</i>	<i>S.c</i>
Residue (g)	107.0	130.0	49.9	51.2	5.2	3.6
Yeast biomass (g/L)	-	-	-	-	5.2 ^a	3.1 ^b
Max cell concentration (cells/mL)	-	-	-	-	6.6x10 ⁸	3.2x10 ⁸
Biomass productivity (g/L.h)	-	-	-	-	0.05	0.03
Y _{x/s} (g/g)	-	-	-	-	0.09 ^a	0.05 ^b
μ _{max} (h ⁻¹)	-	-	-	-	0.13 ^a	0.04 ^b
Yeast cells viability at the end of culture (%)	-	-	-	-	95.4 ^a	96.0 ^a
Moisture (%)	11.6±0.5 ^a		4.3±0.3 ^b	6.9±0.7 ^c	5.5±1.2 ^d	3.4±0.1 ^e
Dry matter (%)	88.4±0.5 ^a		95.7±0.3 ^b	93.1±0.7 ^c	94.5±1.2 ^d	96.6±0.1 ^e
Ashes (%)	3.4±0.8 ^a		4.0±0.1 ^a	5.6±0.2 ^b	5.8±0.2 ^d	7.4±0.4 ^e
Ethereal extract (%)	1.0±0.6 ^a		0.1±0.1 ^b	0.8±1.3 ^a	1.0±0.5 ^d	1.1±0.7 ^d
Crude protein (%)	1.9±0.2 ^a		8.3±0.1 ^b	6.6±0.3 ^c	43.3±0.6 ^d	33.8±0.8 ^e
Crude fiber (%)	22.8±2.2 ^a		44.6±1.1 ^b	35.9±1.3 ^c	2.6±1.4 ^d	1.9±0.4 ^d
Non-nitrogenous extracts (%)	70.9±3.7 ^a		42.9±1.1 ^b	51.2±0.8 ^c	47.4±1.6 ^d	55.8±1.5 ^e
Total digestible nutrients (%)	56.9±2.5 ^a		48.9±0.3 ^b	51.4±1.0 ^c	84.4±1.7 ^d	83.6±0.7 ^d

a, b, c, d = Different letters correspond to statistically different averages (P<0.05)

While fed-batch cultures used 107 g and 130 g of HCR (dry weight) for *S. pararoseus* Sia 33.1 and *S. cerevisiae* M26 respectively, batch culture with SB used only 30 g – 34 g. A total of 18.6% of SB supplied was consumed by both yeasts. Meanwhile, the yeasts consumed a total of 53.4% (*S. pararoseus* Sia 33.1) and 60.6% (*S. cerevisiae* M26) of HCR supplied. Since *S. pararoseus* Sia 33.1 produced more biomass concentration, this yeast demonstrated to be more versatile to SCP production at this substrate.

Biomass productivities were in accordance to biomass concentration, with 0.02 g/L.h for *S. pararoseus* Sia 33.1 with SB and 0.05 g/L.h with HCR and approximately 2 times less for *S. cerevisiae* M26 (0.01 g/L.h and 0.03 g/L.h respectively). Yeasts productivities in SmF with HCR would be higher if cultivation ended at the end of log phase at 72 h. Yeasts cells viability were greater than 80% at the end SmF demonstrating the conditions for these cultures

were healthy for the yeasts. Besides higher affinity that *S. pararoseus* Sia 33.1 demonstrated with agro-industrial residues, less influence for any kind of inhibitor indicates this yeast is more robust and convenient strain for SCP with cassava residue.

Small quantities of xylanases enzymes were detected during both SB cultures in bioreactor, being higher for *S. pararoseus* Sia 33.1 than *S. cerevisiae* M26, while amylases were produced only by *S. pararoseus* Sia 33.1 (Figure 14). Xylanases concentrations increased after the beginning of *S. pararoseus* Sia 33.1 culture, achieving the maximum activity (0.25 U/mL) at 32 h, while *S. cerevisiae* M26 culture started with 0.14 U/mL and decreased along the culture. Xylanases detected in the culture of *S. cerevisiae* M26 culture may be due to the own SB or some contaminant bacterium (up to 1×10^3 cells/mL) since this yeast does not have the apparatus to produce xylanases itself (La Grange et al., 2001).

Reducing sugars (RS) of batch cultures with SB for both yeasts decreased along the cultivation demonstrating the use of the RS to grow. A high RS concentration at 32 h (0.78 mg/mL) after a high xylanase production by *S. pararoseus* Sia 33.1 (0.25 U/mL) demonstrate the break and release of some fermentable sugars from hemicellulose by the enzyme. High RS measured at the beginning of the cultures (approximately 0.81 mg/mL) may be explained by the soft hydrolysis caused by the autoclave conditions which released some fermentable sugars from the lignocelulosic substrate. Higher cell concentration of *S. pararoseus* Sia 33.1 than *S. cerevisiae* M26 (Figure 13) may be due to xylanases secreted (Figure 14), which are responsible for the hydrolysis of the xylan component of hemicellulose, releasing fermentable sugars that can be used for cell growth (Alponti et al., 2016). Other enzymes such as cellulases could also be responsible for higher cell concentration of *S. pararoseus* Sia 33.1 although they were not quantified in this study.

The yeast *S. pararoseus* Sia 33.1 produced amylases in SmF with HCR during all cultivation time with a maximum production at 96 h (0.26 U/mL). Since *S. cerevisiae* is not capable to produce this enzyme by its own (Van Zyl et al., 2012) amylases were not detected during *S. cerevisiae* M26 culture. RS during *S. pararoseus* Sia 33.1 culture decreased along cultivation, which demonstrates the use for cell growth, while the concentration of RS in *S. cerevisiae* M26 culture increased. One explanation for this increase is the low affinity of *S. cerevisiae* to the fermentable sugars released by starch, which led to less cell growth and use of available RS. In addition, high concentrations of RS observed for the last yeast coincide with the feds medium added, after 12, 24 and 36 h of culture.

Representativos of *S. pararoseus* are able to produce β -glucosidases (Baffi et al., 2011, 2010) which are enzymes related to lignocelulosic materials and are responsible for the

hydrolysis of cellobiose and oligosaccharides with the release of glucose (Ng et al., 2010). Qiao et al. (2013) reported the production of cellulases by this species, another fibrolytic enzyme. These enzymes as well as xylanases confirm the ability of *S. pararoseus* to grow using sugarcane as substrate. Previous studies demonstrated this species produced cellulases (data not shown) confirming reports by Qiao et al. (2013). One strain of *S. pararoseus* produced amylases in the studies of Qiao et al. (2013), also confirming the yeast metabolism for the production of this enzyme.

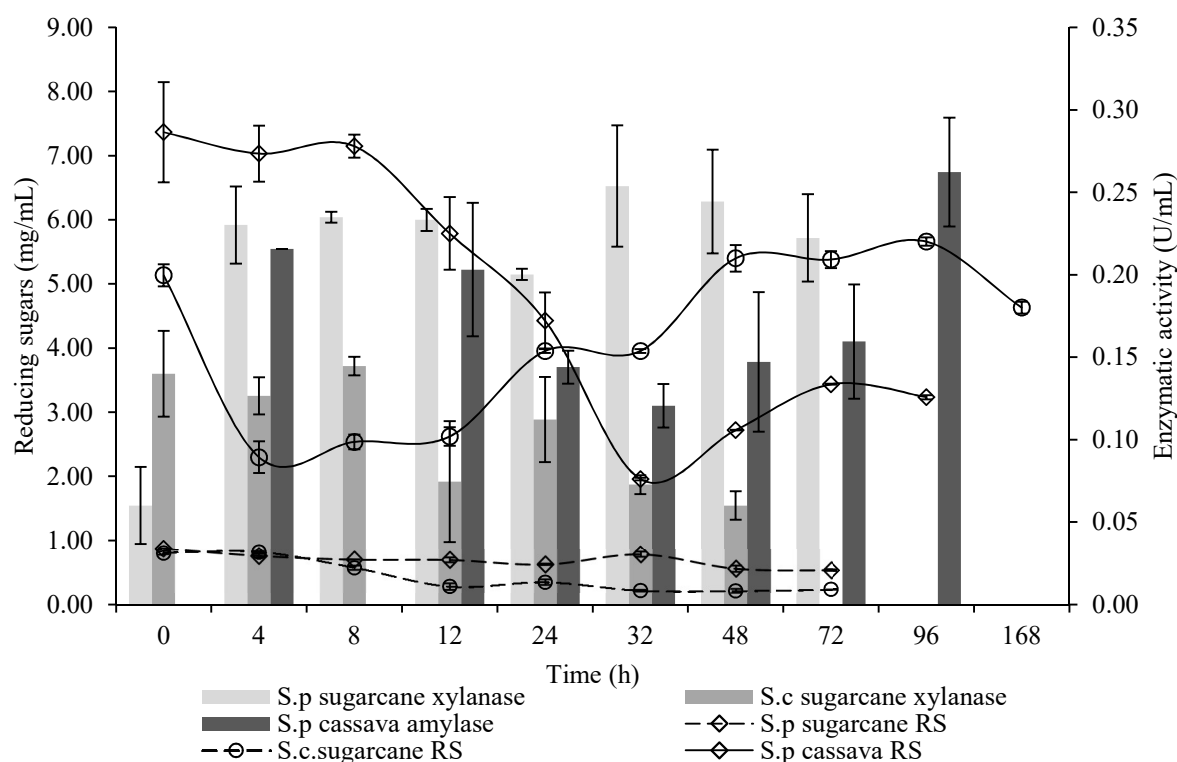


Figure 14 – Reducing sugars and amylases/xylanases produced during *Sporidiobolus pararoseus* Sia 33.1 (S.p) and *Saccharomyces cerevisiae* M26 (S.c) with single-batch culture with sugarcane bagasse and fed-batch with hydrolyzed cassava residue in bioreactor (28 °C, pH 5.5, 1 vvm).

Intense metabolic activity by both yeasts during the log phase of single-batch culture with SB enhanced specific rate of oxygen consumption (Figure 15). At the end of log phase, there was an abrupt fall of this parameter reaching almost zero. Nutrients availability, cell concentration, oxygen solubility and oxygen transfer rate (K_{La}) contributes to specific rate of oxygen consumption (Gonçalves et al., 2001). With cell growth establishment (approximately at 24 h) the necessity for dissolved oxygen was also stabilized until the end of the cultures. The

similar curve of oxygen consumption by the yeasts indicate a greater need for oxygen for *S. cerevisiae* M26 than *S. pararoseus* Sia 33.1 due to the higher yeasts cells concentration at the same aeration rate (1 vvm). Specific rate of oxygen consumption provides reliable indications of biological activity (Campos et al., 2006).

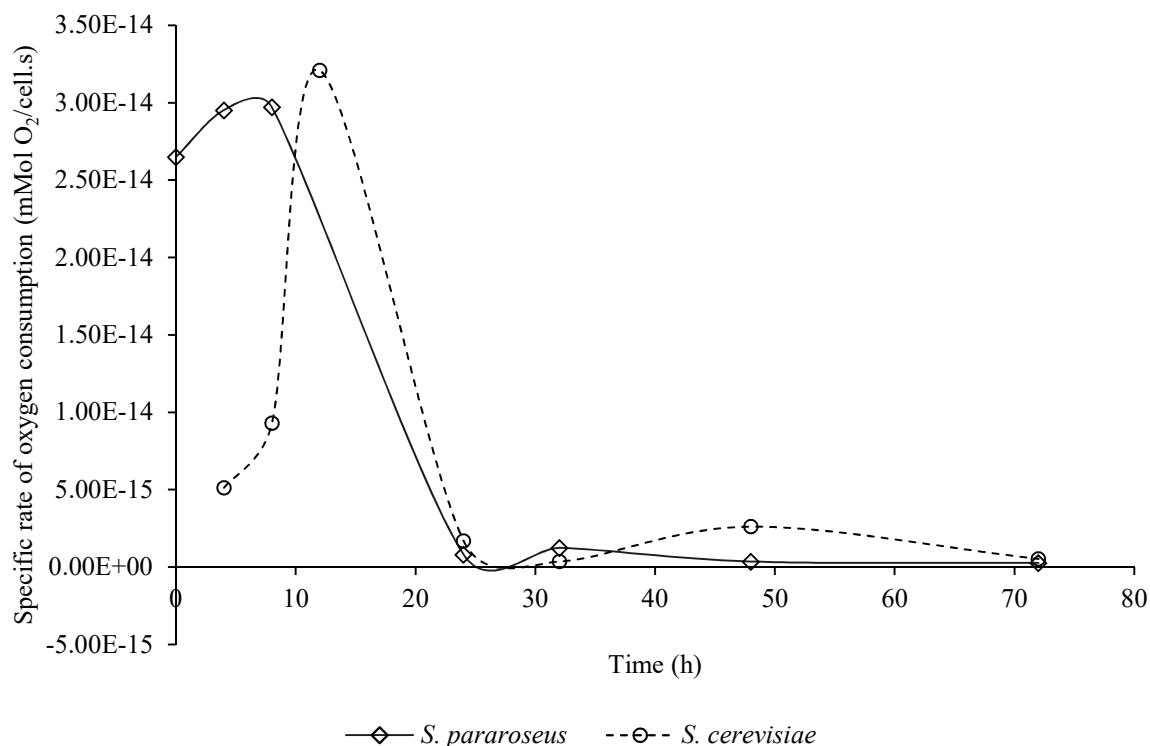


Figure 15 – Specific rate of oxygen consumption of *Sporidiobolus pararoseus* Sia 33.1 and *Saccharomyces cerevisiae* M26 during single-batch culture in bioreactor using sugarcane bagasse at 28 °C, pH 5.5, 1 vvm for 72 h.

Submerged fermentation of *S. pararoseus* in shaker for carotenoids production obtained up to 3.86 g/L of yeasts biomass (dry weight) in defined medium with different concentrations of malt extract, peptone, glucose and yeast extract (Cabral et al., 2011). In another study, Valduga et al. (2014) obtained 5.1 g/L of this yeast biomass cultivated in medium with 40 g/L of glycerol, 40 g/L of corn steep liquor and 20 g/L of parboiled rice water. Maldonado et al. (2007) obtained 3.3 g/L of *Sporobolomyces roseus* using complex medium (Yeast Medium), a species that is now recognized as *S. pararoseus* (Cobban et al., 2016). Based on these results, HCR is more interesting for biomass production according to higher concentration obtained (5.2 g/L). In addition, the use of yeast extract, malt extract, peptone and glucose in the reported studies enhances the cost with culture medium. Then, the use of HCR to cultivate this

microorganism is more efficient and economical. Machado and Burkert (2014) achieved higher biomass concentration (7.18 g/L) of *S. pararoseus* aiming carotenoids production with medium formulated with 7.5 g/L of glycerol and 25 g/L of corn steep liquor, and produced 8.27 g/L of this yeast with medium formulated with 30 g/L of sugarcane molasses and 6.5 g/L corn steep liquor. The determination of ideal conditions of production of *S. pararoseus* biomass would lead to higher biomass concentrations.

Medium formulated with hydrolyzed sugarcane bagasse was used as substrate to obtain SCP from *Candida langeronii* in SmF (Nigam, 2000). The yeast produced 26.67 g/L of biomass (μ_{\max} 0.43 h⁻¹) composed by 48.2% of protein and *Candida utilis* and *Geotrichum candidum* produced 15.0 g/L and 19.5 g/L respectively, with the same medium (Holder et al., 1989). Although these results are higher than those found in the present study, the cost with physical-chemical pretreatment and cellulases to lignocellulosic residues hydrolysis is higher than a simple amylase hydrolysis. Probably if these treatments were used in the present work, a greater conversion of biomass would be obtained but the cost of the process also would be higher. Another advantage of the present research is the use of residual cassava in feed, with protein content improved which diminishes its accumulation in the environment. On the other hand, in the present study was not performed an optimization of the ideal conditions of the production of *S. pararoseus* biomass, therefore if done probably it could increase the yeast biomass concentrations.

Another suitable substrate to yeast biomass production is hydrolysate rice straw (Araújo and D'Souza, 1986). *Candida utilis* and *Saccharomyces cerevisiae* displayed biomass concentrations of 6.56 and 6.48 g/L after 48 h of SmF with the residue pretreated with cellulase. Eucalyptus hydrolyzate was also used for microorganisms productions as *Paecilomyces variotii*, which produced 6.35 g/L, 0.44 g/g biomass yield, 0.10 h⁻¹ of maximum specific growth rate and a productivity of 0.26 g/L.h (Silva et al., 1995). Based on these results, hydrolyzed cassava residue in submerged culture with *S. pararoseus* are close to the range obtained by the authors, with 5.15 g/L, 0.09 g/g yield, 0.054 g/L.h and $\mu_{\max} = 0.134$ h⁻¹. The use of amylase to hydrolysis is advantageous compared to hydrolysis with cellulases since the process is easier and less expensive.

6.4.5 Nutritional parameters of yeast biomass and residues obtained from submerged culture in bioreactor

Nutritional parameters of both yeast's biomass revealed greater differences in crude protein and non-nitrogenous extracts (Table 20). Crude protein contents were higher (two times

more) for HCR culture than SB culture for both yeasts (Tables 19 and 20). In addition, *S. pararoseus* Sia 33.1 biomass contained higher protein concentration than *S. cerevisiae* M26. While *S. pararoseus* Sia 33.1 possess from 39% - 43% crude protein and 48% non-nitrogenous extracts, *S. cerevisiae* M26 is composed by 28% - 33% protein and almost 56% non-nitrogenous extracts. Moisture, crude fiber and total digestible nutrients are slightly higher for *S. pararoseus* Sia 33.1 cells than *S. cerevisiae* M26 biomass, while dry matter and ashes are smaller. Ethereal extracts content was equal for both species.

Submerged culture with yeasts modified all nutritional parameters of HCR (Table 19). *S. pararoseus* Sia 33.1 improved dry matter (from 88.4% to 95.7%), ashes (from 3.4% to 4.0%) and crude fiber (from 22.8% to 44.6%). Since dry matter and moisture are correlated parameters, dry matter enhance lead to the decrease of the moisture, from 11.6% to 4.3%. Ethereal extract decreased from 1.0% to 0.1% after SmF and non-nitrogenous extracts and total digestible nutrients passed from 70.9% and 56.9% to 42.9% and 48.9% respectively. Probably these decreases were caused by the consumption of glucose from starch hydrolysis, which increased percentage of crude fibers and ashes. *S. cerevisiae* M26 SmF improved and reduced the same parameters of HCR compared to *S. pararoseus* Sia 33.1 culture, with 93.0% dry matter, 5.6% ashes and 35.9% crude fiber, while moisture, ethereal extract, non-nitrogenous extracts and total digestible nutrients decreased to 6.9%, 0.8%, 51.2% and 51.4% respectively.

SmF improved crude protein content of both residues (Table 19 and 20). SB improved from 1.6 % to 4.6% (or 194%) and 3.7% (or 138%), being the highest for *S. pararoseus* Sia 33.1 culture. This yeast was also responsible for the higher amount of protein of HCR residue than *S. cerevisiae* M26 after culture, from 1.9% to 8.3% versus 6.6% achieved by the last yeast, values 340% and 247% higher than before cultures. Besides higher protein content of *S. pararoseus* Sia 33.1 biomass, this yeast improved more protein content of the residue than *S. cerevisiae* M26. These results indicates *S. pararoseus* Sia 33.1 is more suitable for SCP production from HCR with a more protein biomass. In addition, protein concentration of residues do not include protein from yeast biomass since yeast cells were separated from residue after filtration. Therefore, the mixture of residual HCR and yeast cells would enhance the protein parameters up to 11.6% since the protein in *S. pararoseus* biomass is 43.3% (Table 20).

The chemical composition of these residues in literature is established. Quantities of 1.6% of crude protein of sugarcane bagasse determined by this research is similar to 1.8% proposed by Neumann et al. (2016). The concentration of nutrients present in cassava residue are within the range found in literature: 0.5% to 1.1% of ethereal extracts, 14.9% to 50.6% of crude fiber, 0.3% to 1.6% of crude protein, 0.7% to 1.6% of ashes, 5.0% to 11.2% of moisture

and 40.5% to 63.8% of carbohydrates (Pandey et al., 2000b). Although crude protein and ashes are slightly higher from the range found for non-hydrolyzed cassava residue, the level of nutrients determined in the present study was for hydrolyzed cassava residue (Table 20).

The use of agro-industrial residues in animal feed (Lima Jr et al., 2010; Yang et al., 2001) often requires chemical-physical pretreatment and addition of some compounds to enrich protein, minerals and amino acids (Pandey et al., 2000a; Silva et al., 2007). SmF with micro-organisms demonstrate to be an interesting way to enhance these parameters and serve as source of protein, vitamins, acids and minerals (Apás et al., 2008; Lima Jr et al., 2010). Moreover, some micro-organisms can also provide probiotic effect into the animal gastrointestinal tract (Lima Jr et al., 2010).

The feed offered to ruminants must have at least 7% crude protein or 1% of total nitrogen (Bravo et al., 1994). Fed-batch culture of *S. pararoseus* Sia 33.1 with HCR achieved such percentage, with 8.3%. Moreover, this concentration would be up to 11.6% if included all yeast biomass. The use of this special yeast after the bioprocess besides improve the nutritional parameters of the residue, are a valuable source of protein, minerals and amino acids which serve to the animal. The yeast produced amino acids by itself without relying on the addition of them in the culture medium.

Others microorganisms were studied for residues improvement. Submerged culture with both *Trichoderma reesei* and *Rhizopus* sp. achieved 7.7% of crude protein from sugarcane bagasse (Monteiro et al., 1991). *S. pararoseus* Sia 33.1 submerged culture in the present work improved SB protein content to 4.59% and up to 8.31% of HCR, which demonstrate that the last residue is more advantageous for production of SCP with more protein content. *Pleurotus ostreatus* increased the protein content of non-treated sugarcane bagasse after SmF to 22.6% after 14 days of incubation (El-Sayed et al., 1994). Protein content of sugarcane and treated cassava cultured with *S. pararoseus* Sia 33.1 increased to 4.6% and 8.3% respectively, after only 3 and 4 days, 4 times earlier than *P. ostreatus*. This protein content would be higher if all yeast biomass were dried together with the residue.

Using cassava residue used as substrate, Vandenberghe et al. (2000) reported the improvement of protein concentration from 13.1% to 23.1% after state-solid fermentation (SSF) with *Aspergillus niger*. *Rhizopus oryzae* improved protein content of cassava residue during SSF to 12.0% in laboratorial scale, and to 13.5% in scale-up (Soccol et al., 1995a; Soccol et al., 1995b). *S. pararoseus* Sia 33.1 improved to 8.31% but in submerged fermentation, a value that would be higher if all yeast biomass were dried together with the residue since yeast cells contains 43.3% of crude protein.

Fermentation using probiotic bacteria with another residue from sugar factory, the sugarcane blunting, achieved, after 60 days of inoculation, 14.22% of carbohydrates, 11.57% of fiber, 1.4% protein, 3.6% of ashes and 0.47% of lipids (Apás et al., 2008). Submerged fed-batch culture of *S. pararoseus* Sia 33.1 using HCR obtained higher parameters than the authors did, except lipids, with a 6 times more protein concentration (8.3%) after only 4 days. According to these results, pretreated cassava could be assessed for animal feed after submerged cultivation of this yeast taken into account its nutritional parameters.

6.4.6 Chemical composition of yeasts cells

Comparison between *S. pararoseus* Sia 33.1 and *S. cerevisiae* chemical composition demonstrated greater difference in nitrogen, potassium, calcium and manganese quantities (Table 21). *S. pararoseus* Sia 33.1 reveals greater amounts of carbon (469.5 g/kg), copper (0.085 g/kg), manganese (0.31 g/kg) and zinc (0.16 g/kg) compared to *S. cerevisiae* cells. However, nitrogen (50.9 g/kg), potassium (1.0 g/kg), calcium (0.1 g/kg), magnesium (1.1 g/kg) and sulfur (2.4 g/kg) were lower. Phosphorus was the only parameter measured that are almost equal for both yeasts (around 15.1 g/kg).

Table 21 – Centesimal composition of *Sporidiobolus pararoseus* Sia 33.1 and *Saccharomyces cerevisiae* biomass after submerged culture in shaker (180 rpm, 28 °C, 96 h).

	<i>S. pararoseus</i> (g/kg)	<i>S. cerevisiae</i> (g/kg)*
Carbon	469.5±4.30	460.0±10.0
Nitrogen	50.9±4.22	82.5±7.50
Phosphorus	15.1±0.08	15.2±0.2
Potassium	1.0±0.003	20.4±0.005
Calcium	0.1±0.02	1.5±0.014
Magnesium	1.1±0.003	1.4±0.003
Sulfur	2.4±0.06	4.0±1.00
Copper	0.085±0.004	0.049±0.0003
Manganese	0.3±0.01	0.02±0.0001
Zinc	0.16±0.003	0.13±0.001

*Belluco (2001)

Therefore, considering cell composition, *S. pararoseus* Sia 33.1 could be used as feed additive once *S. cerevisiae* serves for the same purpose. High protein content of *S. pararoseus* Sia 33.1 turns this species into a valuable SCP source and high affinity to residues makes it more interesting to biomass production as well as improvement of the waste protein content.

As minerals cannot be synthesized by organisms, yeast cells are a valuable source of such compounds. The relationship with metabolism involves energy for nucleic acids, cofactors, synthesis of vitamins and amino acids, synthesis of pigments (Alterthum, 2001; Cooper and Hausman, 2004).

Yeasts nitrogen and ashes higher content than fungi, algae and bacteria turns them more suitable to the use as food and feed (Nigam, 2000). The assimilation of arabinose may be one advantage considering biomass production since arabinose may be released during hydrolysis of agro-industrial residues and serve as carbon source to the growth of microorganisms that can assimilate such carbohydrate (Nigam, 2000). Capacity to grow in temperatures of 40 °C may cheapen the bioprocess for biomass production since it can decrease the energy necessities (Nigam, 2000). Although *S. pararoseus* is not capable to grow in temperatures higher than 35 °C (Sampaio, 2011b) and this specific strain cannot assimilate arabinose, this characteristic varies on the strain (Sampaio, 2011b). These features are not determinative to any species be used as food and feed additive.

The production of enzymes such as amylases and fibrolytic enzymes is believed to be important to probiotic microorganisms since they would increase food digestibility (Laconi and Pompei, 2007). Such enzymes degrade the residues components helping on digestion and digestibility in the rumen (Newbold et al., 1996). In this view, *S. pararoseus* demonstrate to possess the apparatus to produce xylanases, cellulases, lipases and amylases (Baffi et al., 2010; Bussamara et al., 2010; Qiao et al., 2013; Smaniotto et al., 2012) which are benefic to animals.

Another probiotic features to consider a new probiotic microorganism are the tolerance to different pHs and production of antimicrobials (Martins et al., 2005). Although the sensitivity of *S. pararoseus* of pHs below 3 and above 9 (Han et al., 2012), some studies demonstrate antagonistic effect against *Staphylococcus aureus* and *Escherichia coli* (Laconi and Pompei, 2007). *Penicillium italicum*, *P. digitatum*, *Botryodiplodia thepbromae*, *Geotrichum candidum*, *Alternaria alternata* (Sharma et al., 2008), *Monilinia fructicola* (Janisiewicz et al., 2010), *Botrytis cinerea*, *Mucor* sp., *Penicillium* sp., *Rhizopus* sp. (Huang et al., 2012) and *Fusarium fujikuroi* are other species sensitive to *S. pararoseus* cells.

Besides the SCP production using hydrolyzed cassava residue and improvement of nutritional composition of the residue to be used as feed, such technology could be used for carotenoids production. The biotechnological production of carotenoids has advantages over other natural carotenoids obtaining because of the use of cheap substrates such as agro-industrial residues and climate independence as well as the possibility to control the conditions (Silva et al., 2004). Moreover, carotenoids are known to enhance immune response, possess

antioxidant effects and prevent some diseases, being antioxidants agents providing desirable effects even for humans or animals (Maldonado et al., 2012). *S. pararoseus* produce β - carotene, γ - carotene, tolurene and torularhodin (Han et al., 2012) therefore, beyond serving as protein, vitamins, amino acids and minerals supply, would provide carotenoids beneficial to the host. The use of residues minimize environmental problems related to its disposal (Mata-Gómez et al., 2014). Higher biomass concentration, protein content of yeast cells, enzymes and carotenoids, and a suitable composition of minerals and nutritional parameters turns *S. pararoseus* Sia 33.1 more advantageous to SCP from hydrolyzed cassava residue than *S. cerevisiae* M26. However, further studies are need to prove the advantages of this new biomass on animal nutrition.

6.5 Conclusion

The yeast *Sporidiobolus pararoseus* Sia 33.1 possess various characteristics interesting when considering the development of new probiotic microorganism. Capacity to grow in harsh residues and improving its protein content after submerged culture, allied to the enzymes production, high versatility in assimilating different carbohydrates, high protein content of biomass and suitable nutritional and minerals composition, emerges the species as an alternative source of single cell protein from economic residues and, moreover, as a potential probiotic yeast. More studies are necessary to confirm the advanges of this biomass for animal nutrition.

7 CAPÍTULO 4

Single cell protein production from agro-industrial residues and some physiological aspects of *Wickerhamomyces onychis* LABI2

7.1 Abstract

The objective of this study was to evaluate the production of single cell protein from *Wickerhamomyces onychis* LABI2 using two residues as well as to characterize some physiological aspects of this not so well known yeast. The microorganism was cultivated in bioreactor in medium formulated with sugarcane bagasse or hydrolyzed cassava residue and aerobic condition. Nutritional parameters of yeast and residual bagasse as well as yeasts chemical composition, ability to grow in absence of some amino acids or vitamins and different carbohydrates assimilation were analyzed. *W. onychis* LABI2 produced xylanases in medium with sugarcane bagasse and demonstrated higher growth in both residues when compared to *Saccharomyces cerevisiae* M26. A higher biomass and productivity (10.9 g/L with 20.3% of protein, at 0.07 g/L.h) was obtained through fed-batch culture of *W. onychis* LABI2 with hydrolyzed cassava but higher protein of yeast biomass was obtained in batch culture with the same residue (40.7%). However, *S. cerevisiae* M26 achieved up to 3.1 g/L with 0.02 g/L.h using the same residue in fed-batch culture. These results confirms higher performance of *W. onychis* LABI2 in medium formulated with solid cassava residue. Crude protein content of cassava improved at least 200% after all cultures, when compared to before culture. *W. onychis* LABI2 showed to be dependent on histidine, arginine, asparagine, and substantially all tested vitamins for its normal grow, and assimilated mainly maltose, xylose, glucose, sucrose and fructose. A similar chemical composition of *W. onychis* LABI2 in comparison to *S. cerevisiae* demonstrates the possibility to use this special yeast as single cell protein source. The presence of enzymes like xylanases, the optimal performance in degradation of agro-industrial residues and anaerobic growth are desirable characteristics for *W. onychis* LABI2 become a potential new probiotic yeast for ruminants.

Key words: Single cell protein, cassava residue, probiotic yeast, animal feed.

7.2 Introduction

Worldwide population increase brought the need for more sources of protein and higher productivities of animal cattle (Anupama and Ravindra, 2000). Enhance animal performance with alternatives feed is an interesting target on research once it can be the solution for residues accumulation concern besides lower feed costs (Villas-Bôas et al., 2002). Agro-industrial segment generates tons of liquid and solid wastes and either can be used for some purposes, or treated and discarded back into the environment, causing a serious pollution problem (Pinto et al., 2005). One finality for solid wastes lies in the use as animal feed with or without chemical and physical pretreatment (Yang et al., 2001; Lima Jr et al., 2010). This application is mostly complemented with the addition of protein, vitamins, amino acids and minerals, due to the lack of such compounds, therefore increasing its costs (Villas-Bôas et al., 2002). However, agro-industrial residues are rich in complex carbohydrates which can be used as substrates for production of high added value compounds like enzymes, acids, oils, ethanol (Pandey et al., 2000a; Pandey et al., 2000b; Soccol and Vandenberghe, 2003; Chandel et al., 2012).

Sugarcane bagasse and cassava residue are wastes generated from ethanol and cassava industries, respectively, known for its low protein content (Pandey et al., 2000a; Silva et al., 2007). Currently, these products are used as feed after pretreatment (cane bagasse) and minerals complementation. Some researches aimed the protein enrichment of these residues after inoculation with microorganisms to improve protein concentration (Monteiro et al., 1991; Bravo et al., 1994; El-Sayed et al., 1994; Pelizer et al., 2007).

Fermentation using microorganisms is important to increase protein content of feed and to enhance digestibility, palatability and other beneficial responses in which specific microorganisms can cause on the gastrointestinal tract of the host (Apás et al., 2008). These microorganisms can be considered as probiotics, or direct-fed microbial, term defined as “microbial cell preparations or components of cells which have a beneficial effect on the health and well-being of the host” (Salminen et al., 1999; França and Rigo, 2011). Raise on performance of animal production includes better assimilation of feed, weight gain and higher productivity although its causes are not exactly known (Apás et al., 2008; França and Rigo, 2011). Using biological instead chemical or physical treatments lower the cost of the process and provides important compounds found in the microorganism cells (Villas-Bôas et al., 2002; Lima Jr et al., 2010).

Besides beneficial action of probiotics yeasts on digester tract, yeasts can be used as single cell protein. Algae, filamentous fungi, yeasts and bacterium are a valuable source of protein used as food additive for human and animals due to its fast growth and wide range of

characteristics (Anupama and Ravindra, 2000; Ghorai et al., 2011). Fungae and bacteriae cultures demonstrate to be more attractive than algae production because of their versatility in using agro-industrial wastes, consequently increasing the added value of the residue in addition to decrease their disposal into the environment (Anupama and Ravindra, 2000).

Wickerhamomyces (Pichia) onychis is a yeast belonging to the Family Phaffomycetaceae (NCBI, 2016). This specie is commonly found in the environment such as water, air and soil. There are some reports of its growth on human foods as “kimchi”, a Korean fermented vegetables food (Oh and Han, 2003) and “lafun”, a kind of fermented cassava consumed in some parts of the west of Africa (Hahn, 1988). In both cases, *W. onychis* is part of a consortium of microorganisms responsible for fermentation process, which may include production of enzymes and building-up aromas compounds along the process (Oyewole, 2001).

The aim of this study was to obtain single cell protein from agro-industrial residues as well as to characterize some nutritional parameters of residual biomass, both yeast and waste and finally to study some physiological aspects of target yeast *Wickerhamomyces onychis* LABI2.

7.3 Materials and Methods

7.3.1 Microorganisms and identification

The *Wickerhamomyces onychis* LABI2 used in the tests was isolated from rice bran at Laboratory of Industrial Biotechnology of Universidade Estadual Paulista (UNESP/Assis) and *Saccharomyces cerevisiae* M26 was isolated from sugar industry (Oliva Neto et al., 2004) was used as comparison in submerged cultures tests in bioreactor.

The ability of *W. onychis* LABI2 grow in absence of oxygen was verified by cultivation in Petri dishes incubated under anaerobic jar (Anaerocult, Merck, Darmstadt, Germany) with an anaerobic generator (Anaerobac, Probac do Brasil, São Paulo, Brazil) at 33 °C for 72 h. Control Petri dishes were incubated at the same conditions in oven (002 CB, Fanem, São Paulo/SP, Brazil). Evaluation occurred through visual differences between colonies.

All tests were performed in aseptically environment and culture media was sterilized either in autoclave (AV 137, Phoenix, Araraquara/SP, Brazil) or through filtration using 0.22 µm membrane (Millipore, Merck, Darmstadt, Germany).

7.3.2 Submerged culture using agro-industrial residues as substrate

The concentration of solid cassava residue and the need of a pretreatment with amylase were performed after inoculation of *W. onychis* LABI2 in flasks with 3.0% or 5.0% (w/v) of

solid cassava residue (CR) or hydrolyzed solid cassava residue (HCR) mixed with 0.3% or 0.5% of corn steep liquor (CSL) (w/v). Culture medium also contained (w/v) 0.031% K₂SO₄, 0.0028% ZnSO₄, 0.0012% MnSO₄ and 0.024% MgSO₄ modified from Oliva Neto et al. (2004). Flasks were incubated in orbital shaker incubator for 96 h at 180 rpm and 28 °C and aerobiosis (TE421, Tecnal, Piracicaba/SP, Brazil).

Pretreatment of solid cassava residue occurred by hydrolysis at 10% (w/v) in sodium acetate buffer (0.05 M, pH 5.5) for 24 h at 50 °C with 1.2 U/mL of amylase from *Rhizopus oligosporus* (Gonçalves, 2016). Final residue was dried in oven for 72 h, 50 °C (TE394/2, Tecnal, Piracicaba/SP, Brazil) after hydrolysis.

Sugarcane bagasse (SB) and HCR were evaluated as carbon sources in submerged aerobic fermentation by culture in bioreactor (Bioflo 115, New Brunswick, New Jersey, USA). Inoculums were prepared by yeasts culture with 50 mL of same bioreactor medium with 1.0% glucose and without the residue, and incubation in orbital shaker agitator (TE421, Tecnal, Piracicaba/SP, Brazil) for 24 h, 28 °C and 180 rpm.

The medium used for single batch in bioreactor with SB was composed by (w/v): 2.0% of SB, 0.2% of yeast extract, 0.05% glucose, 0.13% (NH₄)₃PO₄, 0.10% (NH₄)₂SO₄, 0.031% K₂SO₄, 0.0028% ZnSO₄, 0.0012% MnSO₄ and 0.024% MgSO₄. The cultivation occurred for 72 h, 28 °C, pH 5.5, 200 rpm and 1 vvm of aeration with 1.7 L of culture medium. Dissolved oxygen was measured with a polarographic oxygen electrode (Mettler Toledo AG, Greifensee, Switzerland) and pH was measured with a glass electrode (Mettler Toledo AG, Greifensee, Switzerland) and controlled with 1.0% (v/v) H₂SO₄ and 1.0 M NaOH.

Submerged fermentation using HCR was done in batch and fed-batch processes in bioreactor. Medium was composed by (w/v): 5.0% of HCR, 0.5% corn steep liquor, 0.031% K₂SO₄, 0.0028% ZnSO₄, 0.0012% MnSO₄ and 0.024% MgSO₄. The medium supply during the fed-batch was performed according to maintain same level of initial soluble solids concentration (Brix) (Portable Refractometer, Biosystems) at the beginning of the culture in three different times for both strains. Culture started with 500 mL of culture medium, and feds occurred at 28, 76 and 120 h for *W. onychis* LABI2 culture and at 12, 36 and 72 h for *S. cerevisiae* M26 culture. The parameters of the culture were: 168 h, 28 °C, pH 5.5 and 1 vvm of air supply. The mechanical agitation of the culture was initially 100 rpm for *W. onychis* LABI2 and increased to 150, 250 and 500 respectively, and initially 350 rpm and increased to 450, 550 and 650 for *S. cerevisiae* M26 respectively after feds. pH and dissolved oxygen were measured with assistance of electrodes (Mettler Toledo AG, Greifensee, Switzerland). The same conditions of

temperature, air supply and pH were maintained for batch culture for *W. onychis* LABI2 in bioreactor for 96 h, 100 rpm and 1 L of culture medium.

The separation of liquid medium and residual SB, CR or HCR after submerged culture occurred through manual filtration using polymer fabric. The liquid was centrifuged at 5000 x g for 30 minutes and 4 °C (Megafuge 16R, Heraeus, Thermo Fisher, Massachusetts, USA) for cell separation. Both yeast and residue were dried in oven (TE394/2, Tecnal, Piracicaba/SP, Brazil) for 72 h at 50 °C before nutritional tests.

Nutritional analysis analysis of biomass (residue and yeast) were quantified according to the Association of Official Analytical Chemists (AOAC) methods (Helrich, 1990). Dry matter was determined through constant weight at 105 °C (humidity balance BTS110, Scientific Industries, Poland) and ashes after burning in muffle oven at 700 °C for 6 h (SP2707-20, Spencer, São Paulo/SP, Brazil). Fat content was extracted using Soxleht equipment and petroleum ether for 6 h. Crude fiber was determined after acid (H₂SO₄) and basic (NaOH) – 1.25% – hydrolysis at 400 °C for 40 min and drying at 105 °C for 24 h. Crude protein was quantified using Kjeldhal method. Total digestible nutrients was calculated according to Kearn (1982) and non-nitrogenous extracts by subtracting ashes, ethereal extract, crude fiber and crude protein percentages.

Samples were taken along the culture to quantify the cell growth and viability using Neubauer counting chamber (7301-1B, New Optics) and methylene blue (Lee et al., 1981), dried biomass (constant weight at 105 °C) at the end of culture (Chen et al., 2012), reducing sugars (RS) (Miller, 1959) and xylanase (Carvalho et al., 2015) or amylase (Gonçalves, 2016) production. One enzymatic unit means the releasing of 1 µmol of RS per minute per mL of enzymatic extract. When necessary, total reducing sugars (TRS) quantification occurred after acid hydrolysis using H₂SO₄ (2.0 M) (1:1) for 10 minutes at 100 °C followed by neutralization with NaOH (2.0 M) (1:1:1) and quantification through the Miller method (Miller, 1959). Maximum specific growth rate (μ_{max}), yield coefficient of cell growth ($Y_{x/s}$) and biomass productivity were determined according to Shuler and Kargi (2002). Specific rate of O₂ consumption of the culture using sugarcane bagasse was calculated considering saturated O₂ concentration at 28 °C (0.244 mMol/L) (Núñez et al., 2014).

7.3.3. Chemical composition of *Wickerhamomyces onychis* LABI2

The determination of the minerals carbon, nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, copper, manganese and zinc of *W. onychis* cells was performed according to the AOAC (Helrich, 1990) parameters. Carbon content of yeast biomass was determined

according to Walkley (1947), calcium, potassium, magnesium and zinc after digestion and atomic absorption, sulfur and phosphorus through visible light spectrum and nitrogen through the Kjeldahl method.

7.3.4 Amino acids and vitamins single-omission growth tests

Amino acids single-omission test was performed through yeast culture in tubes containing (w/v): 2.00% glucose, 0.13% $(\text{NH}_4)_3\text{PO}_4$, 0.10% $(\text{NH}_4)_2\text{SO}_4$, 0.031% K_2SO_4 , 0.0028% ZnSO_4 , 0.0012% MnSO_4 , 0.024% MgSO_4 modified from Oliva Neto et al. (2004), vitamins (thiamin, riboflavin, folic acid, ascorbic acid, pyridoxine, panthotenic acid, nicotinic acid and aminobenzoic acid at 1.0, 2.0, 0.02, 2.0, 2, 1, 2 and 0.2 $\mu\text{g}/\text{mL}$ respectively) and 19 out of 20 amino acids tested (0.03% w/v). Ability of *W. onychis* LABI2 to grow in the absence of each amino acid was tested. Positive control was evaluated using a medium containing all amino acids, and as negative control the absence of them.

Vitamins single-omission test was performed in tubes culture containing same basal solution and concentrations mentioned (glucose and salts), including 20 amino acids (0.03% w/v) and 7 of 8 vitamins in the same concentrations mentioned above. The list of 20 amino acids is described on Table 26. Thus, the *W. onychis* LABI2 ability to grow in absence of one vitamin tested was evaluated. Positive and negative control were performed with all vitamins and without vitamins respectively (Oliva Neto and Yokoya, 1997).

Evaluation of cell growth was indirectly determined by turbidity measurements at 600 nm using a spectrophotometer (UV-M51, Bel Engineering, Monza, Italy) after 120 h of incubation at 28 °C.

7.3.5 Carbohydrates assimilation test

The growth ability of *W. onychis* LABI2 using different carbohydrates (lactose, maltose, fructose, glucose, sucrose, xylose, galactose or arabinose) was performed through inoculation of 1.0×10^6 yeast cells/mL in flasks containing 100 mL of culture medium with the respective carbohydrate and incubated in orbital shaker incubator at 28 °C, 180 rpm for 96 h (TE421, Tecnal, Piracicaba/SP, Brazil). Samples were taken along cultivation to determine cell concentration through direct counting in Neubauer chamber (Lee et al., 1981). Culture medium was composed by (w/v) (modified from Oliva Neto et al. (2004)): 0.2% carbohydrate, 0.2% yeast extract, 0.13% $(\text{NH}_4)_3\text{PO}_4$, 0.10% $(\text{NH}_4)_2\text{SO}_4$, 0.031% K_2SO_4 , 0.0028% ZnSO_4 , 0.0012% MnSO_4 and 0.024% MgSO_4 . As negative control, a medium without carbohydrate was cultivated.

All tests were performed in triplicates, and statistical analysis occurred through ANOVA and Tukey ($P < 0.05$) comparison between replicates using the software GraphPad Prism 5.01.

7.4 Results and Discussion

7.4.1 Anaerobic test

The *W. onychis* LABI2 ability to grow under anaerobic conditions in tests through anaerobiosis jar demonstrated a facultative metabolism of this strain, being capable of growing in absence or presence of oxygen. The presence of this yeast in fermented foods (Hahn et al., 1992; Oh and Han, 2003) and alcohol production (Campbell, 2003; Ibarra, 2015) also confirm its ability to grow in anaerobic conditions.

7.4.2 Batch cultures with cassava residue with or without treatment

The hydrolysis of solid cassava residue (CR) with *Rhizopus oligosporus* amylases produced 35.4 g/L of reducing sugars (RS) after 24 h a similar production than obtained by Gonçalves (2016) but lower than Shi et al. (2014) respectively, 39.9 g/L and 43.3 g/L.

W. onychis LABI2 preference for hydrolyzed cassava residue (HCR) over non-treated cassava residue (CR) can be verified by higher biomass yields, cells concentrations and biomass productivity in flasks cultivations. The medium formulated with CR achieved only up to 2.6×10^8 cells/mL or 2.9 g/L of yeast biomass, while all medium formulated with HCR reached concentrations above 1.0×10^9 cells/mL or 6.1-7.7 g/L of yeast biomass (Table 22).

Protein of *W. onychis* LABI2 biomass ranged from 26% to 34%. Higher protein content of final residue as well as higher biomass productivities and yield ($Y_{x/s}$) were achieved for HCR over CR. The best combination for biomass production was 5.0% of HCR with 0.5% of corn steep liquor (CSL) and this mixture was defined to the bioreactor culture.

Table 22 – Kinect and nutritional composition of *Wickerhamomyces onychis* LABI2 batch culture in medium formulated with solid cassava residue, hydrolyzed (HCR) or not (CR) and corn steep liquor (CSL) in aerobiosis in orbital shaker incubator (180 rpm, 28 °C, 96 h).

Conditions of medium composition			After submerged culture									
CR (g)	HCR* (g)	CSL (g)	Log phase	μ_{\max} (h ⁻¹)	Max cell concentration (cell/mL)	Biomass concentration ^a (g/L)	Yeast protein (%)	Biomass productivity ^a (g/L.h)	Residual bagasse ^a (g)	Residual bagasse protein **(%))	Y _{x/s} (g/g)***	
3.0	-	0.3	8-28 h	0.13	1.5E+08	2.1±1.3 ^a	33.9±2.4 ^a	0.02	1.41	4.1±0.5 ^a	0.10	
3.0	-	0.5	8-24 h	0.19	1.6E+08	2.1±0.6 ^a	31.9±2.2 ^{a,b}	0.02	1.48	4.6±0.1 ^{a,c}	0.11	
5.0	-	0.3	8-28 h	0.11	1.4E+08	1.9±0.0 ^a	27.4±3.3 ^{a,b}	0.02	2.64	4.0±0.6 ^a	0.06	
5.0	-	0.5	8-28 h	0.15	2.6E+08	3.0±1.1 ^a	32.2±3.1 ^a	0.03	2.61	4.1±0.6 ^a	0.09	
-	3.0	0.3	8-96 h	0.03	1.4E+09	6.1±0.4 ^b	32.7±1.6 ^a	0.06	1.10	4.9±0.3 ^a	0.26	
-	3.0	0.5	8-72 h	0.05	1.2E+09	6.1±0.3 ^b	33.9±2.0 ^a	0.06	1.13	5.6±0.4 ^{b,c}	0.27	
-	5.0	0.3	8-72 h	0.05	1.6E+09	7.6±0.2 ^c	25.9±1.6 ^b	0.08	1.93	5.3±0.3 ^{b,c}	0.19	
-	5.0	0.5	12-96 h	0.04	1.4E+09	7.7±0.5 ^c	27.3±1.2 ^b	0.08	1.99	6.1±0.3 ^b	0.21	

^{a, b, c} = Different letters correspond to statistically different averages (P<0.05)

*Enzymatic hydrolysis of cassava; **Protein in CR and treated cassava were 1.8% and 1.9% respectively; ***Calculated according to the amount of bagasse

7.4.3 Kinect of submerged culture of *W. onychis* LABI2 and *S. cerevisiae* M26 in bioreactor with sugarcane bagasse (batch) or hydrolyzed cassava residue (batch and fed-batch)

According to yeasts growth curve in submerged culture using SB and HCR in bioreactor, *W. onychis* LABI2 demonstrated a higher growth in comparison to *S. cerevisiae* M26, mainly with cassava (Figure 16). The SB culture reached maximum cell concentration at the end of log phase for both yeasts, being 1.1×10^8 cells/mL for *W. onychis* LABI2 ($\mu_{\max} = 0.26 \text{ h}^{-1}$) and 3.1×10^7 cells/mL ($\mu_{\max} = 0.09 \text{ h}^{-1}$) for *S. cerevisiae* M26 (Figure 16a).

Growth curve for HCR in fed-batch culture showed that log phase started at 4 h for both yeasts, but while *S. cerevisiae* M26 culture ended at 72 h, *W. onychis* LABI2 remained until 168 h. Longer log phase for *W. onychis* LABI2 may be responsible for higher cell concentration of 3.2×10^9 cells/mL than *S. cerevisiae* (3.2×10^8 cells/mL) (Figure 16b).

The use of HCR as substrate for single cell protein production in fed-batch culture demonstrated to be more appropriated in comparison to SB and single batch culture of HCR. During batch culture with HCR *W. onychis* LABI2 reached 1.4×10^9 cells/mL, 2.2 times lower than fed-batch culture with this residue (3.2×10^9 cells/mL) (Figure 16b). Maximum specific growth rate was higher for batch culture mainly because log phase finished at 72 h while in fed-batch culture stationary phase was not reached.

Regarding *S. cerevisiae* M26, only fed-batch culture was tested with HCR and a maximum of 3.2×10^8 cells/mL ($\mu_{\max} = 0.04 \text{ h}^{-1}$) was reached, 10 times lesser cell concentration than *W. onychis* LABI2 culture at the same conditions. Yeast cells viability in all cultures ended above 80% showing the conditions for these cultures were healthy for the yeasts.

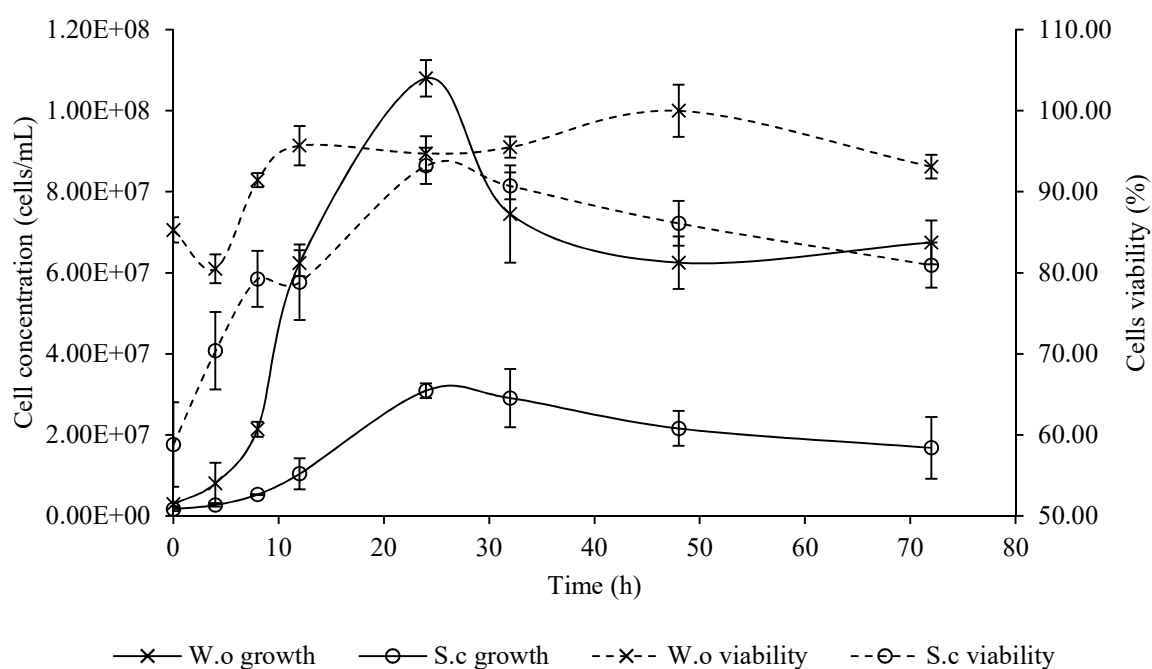
The different performance of these yeasts cultures in residues with lignocellulose and starch can be due to some enzymes metabolism. Absence or low production of certain enzymes, like xylanases and amylases by *S. cerevisiae* M26 may explain the different performance than *W. onychis* LABI2 (Figure 17). While in batch culture of *W. onychis* LABI2 with SB xylanases concentrations started from 0.25 U/mL and reached up to 0.34 U/mL, *S. cerevisiae* M26 culture started with 0.14 U/mL, decreasing during the culture. In addition, xylanases found in *S. cerevisiae* M26 culture may be due to contaminants found along cultivation (max 1×10^3 cells/mL) or the own SB since this yeast do not produce xylanases by itself (La Grange et al., 2001). Xylanases are responsible to degrade xylan component of hemicellulose and release fermentable sugars that can be used for cell maintenance and growth (Alponti et al., 2016). An increase of reducing sugars released after xylanase activity can be observed demonstrating the enzymatic action. Since lignocellulosic residues requires a pool of enzymes to hydrolyze

(Sandgreen et al., 2005), other enzymes may be responsible to the break and release of reducing sugars used for cell growth although they were not quantified in this study.

The production of amylases by both yeasts was not detected in any cultures. Probably, *W. onychis* LABI2 produces amylases for its common isolation in “lafun”, a fermented cassava food consumed in Africa (Hahn et al., 1992). Since the production of enzymes are required to break and release of substrates fermentable sugars, previous hydrolysis performed for CR with *R. oligosporus* amylases lead to an unnecessary production amylases by the yeast justifying its absence.

Quantification of xylanases by *W. onychis* was not found for comparison. *Cryptococcus albidus* is a producer of xylanases, which was demonstrated to produce this enzyme in xylan formulated medium, being detected activities of 0.3 U/mL for crude extract (Morosoli et al., 1986) a similar value of xylanases produced by *W. onychis* in the present work. Other yeasts producers of xylanases are: *Candida boidinii*, *Cryptococcus laurentii*, *Galactomyces geotrichum* (Morais et al., 2013).

a.



b.

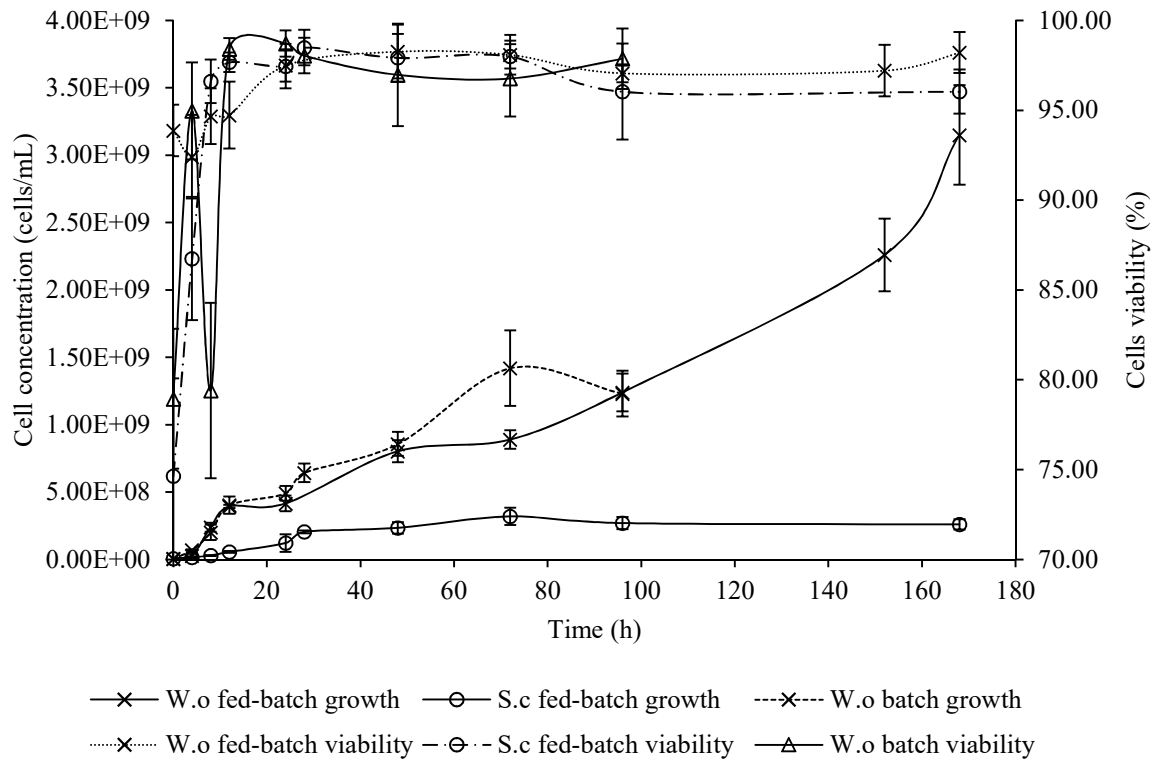


Figure 16 – Kinetic parameters of *Wickerhamomyces onychis* LABI2 (W.o) and *Saccharomyces cerevisiae* M26 (S.c) in bioreactor using respectively, a) sugarcane bagasse in batch culture (28 °C, pH 5.5, 1 vvm, 200 rpm) and b) hydrolyzed cassava residue in batch and fed-batch culture (28 °C, pH 5.5, 1 vvm).

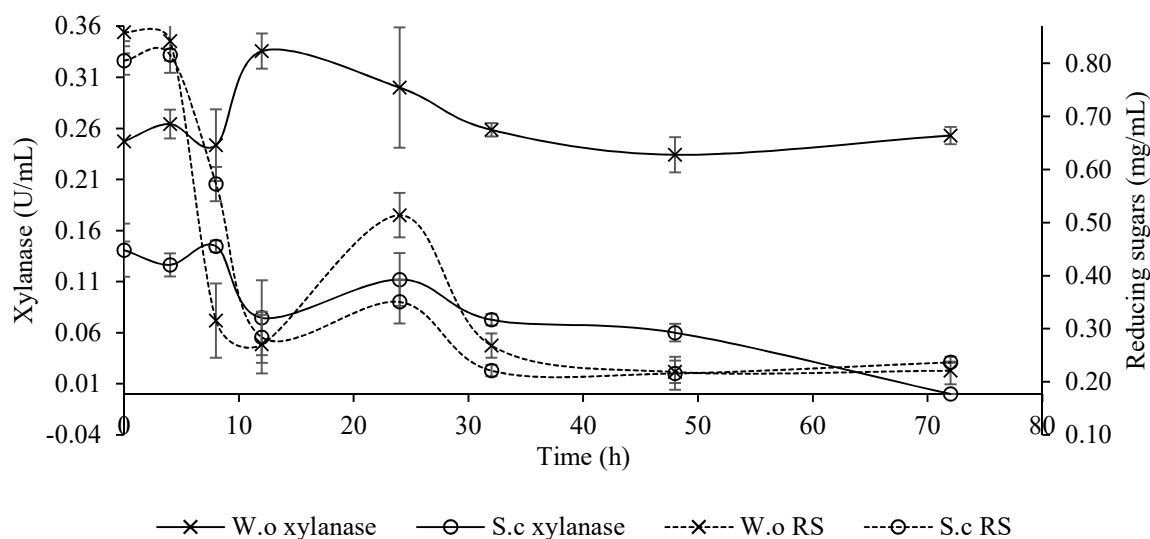


Figure 17 – Xylanase and reducing sugars produced during batch culture of *Wickerhamomyces onychis* LABI2 (W.o.) and *Saccharomyces cerevisiae* M26 (S.c.) in bioreactor using sugarcane bagasse at 28 °C, pH 5.5, 200 rpm, 1 vvm.

The specific rate of oxygen consumption or demand for dissolved oxygen (DO) for both yeasts in batch culture using SB demonstrated an increase in the beginning of log phase (Figure 18). However, there was an abrupt decrease or this rate achieving almost zero at the end of the exponential phase. This behavior is according to the intense metabolism during the log phase of cell growth. Such decrease can be related to availability of nutrients in the culture medium, oxygen solubility, oxygen transfer rate (K_{La}) and how fast it is consumed (Gonçalves et al., 2001). The greater growth of *W. onychis* LABI2 in comparison to *S. cerevisiae* M26 demonstrated that *S. cerevisiae* M26 needed a lesser DO than *W. onychis* LABI2 for the same air flow (1.7 L/min). In addition, a faster consume of oxygen of *W. onychis* LABI2 than *S. cerevisiae* M26 was verified in first hours of the culture. Oxygen consumption rate is a trustful biological activity indicator and provides a rapid response method to substrate consumption (Campos et al., 2006).

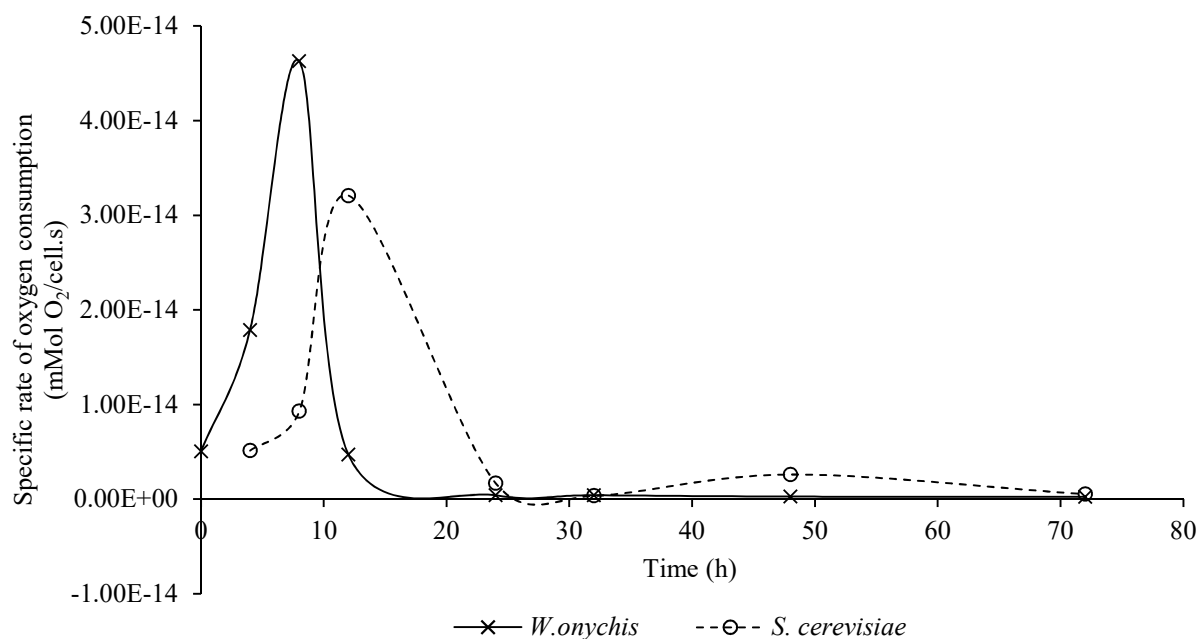


Figure 18 – Specific rate of oxygen consumption of *Wickerhamomyces onychis* LABI2 and *Saccharomyces cerevisiae* M26 in batch culture in bioreactor using sugarcane bagasse (28 °C, pH 5.5, 200 rpm, 1 vvm).

W. onychis LABI2 biomass in the culture using HCR was 10.3 (batch culture) and 19.8 times (fed-batch culture) higher than SB, reaching up to 10.9 g/L in fed-batch process (Tables 23 and 24). Certainly, if SB was submitted to enzymatic hydrolysis, biomass production would be increased. In addition, the cost to hydrolyze lignocellulosic residues is more expensive and complex than amylases hydrolysis.

Table 23 – Kinect and nutritional parameters of *Wickerhamomyces onychis* LABI2 (W.o) and *Saccharomyces cerevisiae* M26 (S.c) in batch culture using sugarcane bagasse (SB) in bioreactor (28 °C, pH 5.5, 200 rpm, 1 vvm).

Parameters	Supplied SB	After submerged culture			
	before culture	Final residue		Yeast biomass	
		<i>W.o</i>	<i>S.c</i>	<i>W.o</i>	<i>S.c</i>
Residue (g)	34.0	31.8	27.6	0.9	0.7
Crude protein (%)	1.6±0.2 ^a	4.0±0.5 ^b	3.7±0.3 ^b	19.1±0.7 ^c	28.2±0.4 ^d
Yx/s (g/g)	-	-	-	0.41	0.10
Yeast biomass (g/L)	-	-	-	0.6	0.8
Biomass productivity (g/L.h)	-	-	-	0.01	0.01
μ_{\max} (h ⁻¹)	-	-	-	0.26	0.09

^{a, b, c, d} = Different letters correspond to statistically different averages (P<0.05)

Table 24 – Kinect and nutritional parameters of *Wickerhamomyces onychis* LABI2 (W.o) and *Saccharomyces cerevisiae* M26 (S.c) in batch or fed-batch cultures using hydrolyzed cassava (HCR) in bioreactor (28 °C, pH 5.5, 1 vvm).

Parameters	Supplied residue before culture			Final residue			Yeast biomass		
	Batch	Fed-batch		Batch	Fed-batch		Batch	Fed-batch	
	<i>W.o</i>	<i>W.o</i>	<i>S.c</i>	<i>W.o</i>	<i>W.o</i>	<i>S.c</i>	<i>W.o</i>	<i>W.o</i>	<i>S.c</i>
Residue (g)	50.0	150.0	130.0	19.3	70.1	51.2	5.7	13.6	3.6
Yx/s (g/g)	-	-	-	-	-	-	0.18 ^a	0.17 ^a	0.05 ^b
Yeast biomass (g/L)	-	-	-	-	-	-	5.7 ^a	10.9 ^b	3.1 ^c
Biomass productivity (g/L.h)	-	-	-	-	-	-	0.06	0.07	0.02
μ_{\max} (h ⁻¹)	-	-	-	-	-	-	0.03	0.02	0.04
Non-nitrogenous extractive (%)		70.9±3.7 ^a		46.8±0.8 ^b	50.1±1.2 ^b	51.2±0.8 ^b	51.4±0.6 ^e	68.7±2.2 ^f	55.8±1.5 ^g
Ethereal extract (%)		1.0±0.6 ^a		0.9±0.5 ^a	1.0±0.4 ^a	0.8±0.1 ^a	0.8±0.1 ^e	0.8±0.7 ^e	1.1±0.7 ^e
Crude fiber (%)		22.8±2.2 ^a		39.6±1.2 ^b	35.6±1.2 ^c	35.9±1.3 ^c	0.8±0.1 ^e	2.9±0.5 ^f	1.9±0.4 ^f
Dry matter (%)		88.4±0.5 ^a		90.6±0.6 ^a	90.8±1.7 ^a	93.0±0.7 ^a	99.2±0.6 ^e	96.9±0.1 ^f	96.6±0.6 ^f
Ashes (%)		3.4±0.8 ^a		5.2±0.2 ^b	7.6±0.8 ^c	5.6±0.2 ^b	6.4±0.5 ^e	7.2±1.7 ^e	7.4±0.4 ^e
Total digestible nutrients (%)		56.9±2.5 ^a		50.7±1.5 ^b	49.8±2.6 ^b	51.4±1.0 ^b	85.7±0.2 ^e	81.0±1.9 ^e	83.6±0.7 ^e
Crude protein (%)		1.9±0.2 ^a		7.5±0.3 ^b	5.6±0.1 ^c	6.6±0.3 ^d	40.7±0.3 ^e	20.3±0.5 ^f	33.8±0.8 ^g
Moisture (%)		11.9±0.5 ^a		9.4±0.6 ^a	9.2±1.7 ^a	7.0±0.7 ^a	0.8±0.6 ^e	3.1±0.1 ^f	3.4±0.1 ^f

a, b, c, d, e, f = Different letters between the same parameter correspond to statistically different averages (P<0.05)

The parameter $Y_{x/s}$ was higher for SB culture than HCR respectively, 0.41 g/g and 0.17-0.18 g/g for *W. onychis* LABI2, 0.10 g/g and 0.05 g/g for *S. cerevisiae* M26 (Tables 23 and 24). This difference may be due to the formulation of SB culture medium with yeast extract. Such nutrient was not taken into account for $Y_{x/s}$ calculation. Furthermore, the use of 0.2% (w/v) yeast extract is economically unfeasible due to its high cost (US\$ 18/kg – Biorigin, Quatá/SP, Brazil). Productivity of *W. onychis* LABI2 in fed-batch culture with HCR was 7 times higher than sugarcane batch culture (0.01 g/L.h versus 0.07 g/L.h). Besides higher biomass concentration, culture medium with HCR has another advantage over culture medium with SB, since nitrogen source in the first was corn steep liquor instead of yeast extract, which may reduce culture medium cost.

Other aspect is the importance of fed-batch for obtaining higher yeast biomass concentration. While single batch culture of *W. onychis* LABI2 a total of 50 g of HCR was supplied, for fed-batch this value was three times greater (150 g) which lead to a consumption of 61.4% and 53.3% of the residue respectively. Therefore, fed-batch culture of *W. onychis* LABI2 produced almost 2 times more yeast biomass (10.9 g/L) than single batch culture. Moreover, yeast biomass produced in fed-batch by this yeast was 3.5 times higher than *S. cerevisiae* M26 (3.1 g/L) fermentations (Table 24).

Biomass productivity using SB in batch culture was low for both yeasts (0.01 g/L.h) (Table 23). Cultures with HCR obtained 0.02 g/L.h of *S. cerevisiae* M26 biomass against 0.06-0.07 g/L.h for *W. onychis* LABI2 (Table 24). These results demonstrate higher affinity to sugars released by HCR for the last yeast, and/or less influence for any kind of inhibitor, indicating *W. onychis* is more robust and convenient strain for biomass production in this kind of substrate.

Although protein content of *S. cerevisiae* M26 cells was higher than *W. onychis* LABI2, respectively 33.8% and 20.3%, in fed-batch with HCR, highest productivity makes *W. onychis* LABI2 more interesting to single cell protein (SCP) production from HCR than *S. cerevisiae* M26. In addition, fed-batch culture leads to bigger biomass concentration than batch culture. However, if protein and biomass combination were considered, *W. onychis* LABI2 batch culture would be more interesting for SCP than fed-batch since 5.7 g/L of yeast biomass with 2 times more protein content (40.7%) was produced (Table 24).

Díaz et al. (2005) evaluated the determination of optimal conditions for *W. onychis* biomass production through statistical design. Using different carbon and nitrogen sources (molasses, hydrolyzed soy, hydrolyzed yeast and hydrolyzed corn) the authors achieved 6.3 g/L of yeast biomass, corresponding to 8.95×10^9 cells/mL with the use of 43.42 g/L of carbon source and 0.26 g/L of organic nitrogen source at 110 rpm, pH 6.0 under submerged culture (SmF)

using flasks. In contrast, the present work using hydrolyzed cassava residue (without yeast extract) in a batch culture obtained lower biomass (5.7 g/L) and almost 2 times more in fed-batch culture in bioreactor (10.9 g/L). Moreover, the determination of optimal culture conditions was not performed and probably a higher biomass could be achieved.

SmF with cellulolytic fungus *Penicillium janthinellum*, with cassava peel and sugarcane bagasse pretreated with acid yielded, respectively, 1.30 g/L and 0.50 g/L and maximum specific growth rates of 0.013 h⁻¹ and 0.030 h⁻¹ (Oliveira et al., 2006). These values are far lesser than results obtained with *W. onychis* LABI2 in the present work (up to 10.9 g/L and μ_{\max} of 0.02 h⁻¹ using hydrolyzed cassava and 0.26 h⁻¹ with sugarcane).

Candida utilis and *Saccharomyces cerevisiae* culture in hydrolysate rice straw in formulated medium was responsible for obtaining biomass concentrations of 6.56 and 6.48 g/L after 48 h of SmF in pretreated residue with cellulases (Araújo and D'Souza, 1986). *Eucalyptus* hydrolysate is also used for microorganisms productions, as *Paecilomyces variotii*, with biomass production of 6.35 g/L, 0.44 g/g yield, 0.10 h⁻¹ and a productivity of 0.26 g/L.h (Silva et al., 1995). Based on these results, HCR in culture with *W. onychis* LABI2 is probably more interesting for biomass production (single cell protein) since a higher biomass concentration was obtained without the need to use a more expensive pretreatment with cellulases.

On the other hand, hydrolyzed sugarcane bagasse was used to formulate a hemicellulose hydrolysate medium as substrate for obtainment of single cell protein from *Candida langeronii* in SmF (Nigam, 2000). This culture produced 26.67 g/L of biomass from an initial concentration of 50.0 g/L of sugars, yielding 0.40 g/g ($\mu_{\max} = 0.43$ h⁻¹), composed by 48.2% protein and 23.4% carbohydrate. The culture of *C. utilis* and *Geotrichum candidum* obtained 15.0 g/L (0.27 g/g) and 19.5 g/L (0.33 g/g) of biomass respectively, with same medium (Holder et al., 1989), and *Candida tropicalis* yielded 0.31 g/g, at 0.196 g/L.h and μ_{\max} 0.137 h⁻¹ (Pessoa Jr et al., 1996). Although these results of biomass production are higher than those found by the present study, cost with physical-chemical pretreatment and cellulases hydrolysis of lignocellulosic residues are higher than a simple amylase hydrolysis. Probably if these treatments were used in the present work, a greater conversion of biomass would be obtained with a high cost of the process.

7.4.4 Nutritional parameters of yeast biomass and residues after submerged culture in bioreactor

The culture of *W. onychis* LABI2 using SB improved crude protein from 1.6% (before batch in bioreactor) to 4.0% an increase of 157% (Table 22). For HCR the increase was from 1.9% (before culture in bioreactor) to 7.5% or 295% more (after batch culture) and to 5.6% or 195% more (after fed-batch culture) (Table 23). *S. cerevisiae* M26 culture using SB improved crude protein in this residue from 1.6% to 3.7%, or an improvement of 137%. For HCR improvement of protein was from 1.9% to 6.6% or 246% more. In addition, these protein values of residues do not include yeast biomass protein, since the final residue was separated of yeast biomass by simple filtration. Combination of yeast biomass and residue would reach protein content up to 15% and 8% for *W. onychis* LABI2 culture in batch and fed-batch with HCR and to 8.3% for *S. cerevisiae* M26 culture with the same residue.

Protein of final residue after SmF for both yeast was higher for HCR than SB (Tables 23 and 24), showing advantage in the use of HCR for single cell protein from this residue. Other nutritional parameters for HCR residue were assessed and non-nitrogenous extractive decreased about 30% in batch or fed-batch cultures (Table 24). Probably, this decrease was due to glucose consumption from starch hydrolysis, which increased percentage of fibers and ashes and decrease the total digestible nutrients since the yeast do not consume lignin.

Nutritional composition of these residues is well established in literature. Quantities of 1.6% SB crude protein determined by this work is similar to the 1.8% proposed by Neumann et al. (2016). Nutrients concentration present in cassava residue are within the range found in literature, being: 0.53% to 1.06% of ethereal extracts, 14.88% to 50.55% of crude fiber, 0.32% to 1.61% of crude protein, 0.66% to 1.55% of ashes, 5.02% to 11.20% of moisture and 40.50% to 63.84% of carbohydrates (Pandey et al., 2000b). Although crude protein and ashes are slightly higher from the range found for non-hydrolyzed cassava residue, the level of nutrients determined in the present study was for cassava residue treated with amylase (Table 24).

SmF of fungus *Pleurotus ostreatus* with SB without treatment after 14 days increased protein content to 22.6% (El-Sayed et al., 1994). In the present work, the crude protein in final residue of SB increased from 1.6% to 4.0% or an increase of 158% after only 4 days of *W. onychis* LABI2 culture.

Other organism studied to protein improvement of SB are: the algae *Spirulina platensis* in solid-state fermentation (SSF), with an increase to 7.2% (Pelizer et al., 2007). Submerged mixed culture of *Trichoderma reesei* and *Rhizopus* sp. obtained 7.7% crude protein (Monteiro et al., 1991) and SSF of *Chaetomium cellulolyticum* raised to 8.65% SB protein (Bravo et al.,

1994). These values are higher than SmF of *W. onychis* LABI2 with SB although yeast cells from this study were not dried together with the residue, which would enhance this concentration.

Using cassava residue (CR) as substrate, Vandenberghe et al. (2000) improved protein concentration from 13.1% to 23.1% after SSF with *Aspergillus niger*. *Rhizopus oryzae* improved protein content of CR during SSF to 12.0% in laboratorial scale, and to 13.5% scale-up (Soccol et al., 1995a; Soccol et al., 1995b). These results are for SSF process and culture started with 13.1% of residue protein, while in the present work for SmF and the initial protein content of 1.9%. Moreover, combination of *W. onychis* LABI2 biomass and residual HCR would reach protein concentration up to 15%.

Another residue from sugar factory is sugarcane blunting. After 60 days with inoculation with probiotic bacteria, the resultant silo obtained values of protein, ashes and lipids of 1.4%, 3.6% and 0.47% respectively (Apás et al., 2008). In comparison, submerged fed-batch culture of *W. onychis* LABI2 using HCR obtained higher parameters than the authors did, especially protein (5.6%) after 7 days of culture. According to these results, pretreated cassava could be used for animal feed after SmF with this yeast taken into account its nutritional parameters. In addition, although agro-industrial residues are currently used in animal feed (Lima Jr et al., 2010; Yang et al., 2001) complementation with some compounds are necessary to improve protein, minerals and amino acids content (Pandey et al., 2000a; Silva et al., 2007). Cultivating microorganisms as a biological pretreatment would enhance these parameters because nutritional values of residue are improved and microorganisms are a valuable source of protein, vitamins, acids and minerals (Apás et al., 2008; Lima Jr et al., 2010). In contrast to chemical and physical pretreatments, biological cultures can also provide probiotic effect into the animal gastrointestinal tract and enhance animal performance and productivity (Lima Jr et al., 2010).

7.4.5 Chemical composition of yeasts biomass

Cellular composition of *W. onychis* LABI2 cells demonstrated higher concentration of magnesium, copper, manganese and zinc compared to *S. cerevisiae* cells (Table 25). Therefore, considering only cell composition, *W. onychis* LABI2 could be used for the same purpose of *S. cerevisiae* as food and feed ingredient. High protein content turns this yeast into a valuable SCP source and high affinity to residues makes this bioprocess more valuable once protein content of the waste is improved. Organisms cannot synthesize minerals and yeast cells are a valuable source of such compounds. Minerals are involved with nucleic acids metabolism as well as cofactors, vitamins, amino acids and pigments synthesis (Alterthum, 2001; Cooper and

Hausman, 2004). The cultivation of agro-industrial residues with microorganisms solves low protein content problem and provide other essential minerals to the host metabolism.

Table 25 – Chemical composition of *Wickerhamomyces onychis* LABI2 and *Saccharomyces cerevisiae* biomass after submerged fermentation at 28 °C.

Element	<i>W. onychis</i> (g/kg)	<i>S. cerevisiae</i> (g/kg)*
Carbon	393.0±6.33	460.0±10.0
Nitrogen	81.3±2.80	82.5±7.50
Phosphorus	15.1±0.31	15.2±0.20
Potassium	16.7±0.04	20.4±0.005
Calcium	0.1±0.01	1.5±0.014
Magnesium	1.8±0.003	1.4±0.003
Sulfur	3.7±0.002	4.0±1.00
Copper	0.07±0.001	0.05±0.0003
Manganese	0.04±0.002	0.01±0.0001
Zinc	0.17±0.01	0.13±0.001

*Belluco (2001)

7.4.6 Amino acids and vitamins single-omission test

W. onychis LABI2 showed to be very sensitive with absence of most amino acids and all vitamins used for its grow in tubes cultures of amino acids and vitamins single-omission tests (Table 26). Absence of histidine (His) and arginine (Arg) caused the total absence of cell growth indicating the importance of these amino acids for this strain. Moreover, asparagine (Asn) demonstrated to be another amino acid necessary to the normal metabolism of *W. onychis* LABI2 with a slightly higher growth than control tubes.

Some other amino acids showed to act as a growth promoters. Yeast cell concentration almost reached positive control growth when proline (Pro), valine (Val), tyrosine (Tyr), alanine (Ala), serine (Ser), isoleucine (Ile), cysteine (Cys), lysine (Lys), aspartic acid (Asp), phenylalanine (Phe), glutamic acid (Glu), methionine (Met) and glycine (Gly) were absent. Perhaps growth metabolism was only retarded by the absence of such amino acids and more incubation days would be necessary to *W. onychis* LABI2 achieve equal cell concentration of positive control tubes.

Some amino acids demonstrated to be non-essential to the regular growth of this yeast: tryptophan (Trp), leucine (Leu), threonine (Thr) and glutamine (Gln), since their absence did not affected the cell growth. Therefore, *W. onychis* LABI2 probably have the enzymes necessary to produce these amino acids.

Table 26 – Tubes culture with *Wickerhamomyces onychis* LABI2 amino acids and vitamins single-omission tests after 72 h at 28 °C.

Amino acid		Vitamin	
Nutrient	Growth	Nutrient	Growth
Trp	+++	Thiamin	+
Leu	+++	Riboflavin	+
Pro	++	Folic Acid	+
Val	++	Ascorbic Acid	+
Tyr	++	Pyridoxine	+
Ala	++	Panthenic Acid	-
Ser	++	Nicotinic Acid	+
Ile	++	Aminobenzoic Acid	++
Cys	++		
Gln	++		
Thr	+++		
Lys	++		
Asp	++		
Phe	++		
Glu	+++		
His	-		
Arg	-		
Met	++		
Gly	++		
Asn	+		

P<0.05

- = Absence of growth; + = Little growth; ++ = Partial growth; +++ = Full growth

Regarding vitamins single-omission test, absence of the majority affected growth cell (Table 26). Pantothenic acid was the unique essential vitamin since its absence in the formulated synthetic medium led to the absence of yeast growth. Thiamin, riboflavin, folic acid, ascorbic acid, pyridoxine and nicotinic acid absence resulted in a growth close to the negative control, noting that their presence are necessary to the normal function of *W. onychis* LABI2 metabolism. In addition, aminobenzoic acid showed to be unnecessary to the yeast growth.

One strain of *W. onychis* isolated from a fermented vegetable in Korea demonstrated to produce an enzyme responsible to vitamin C (ascorbic acid) production, known as L-galactono- γ -lactone oxidase, when galacturonic acid was used as substrate (Oh and Han, 2003),

demonstrating presence of this vitamins synthesis apparatus on its own. Although in the present research the strain tested did not grow very well in the absence of vitamin C, medium was also absent of galacturonic acid for its production. Absence of thiamin (vitamin B1) also affect others microorganisms growth (Sierra et al., 1999; Strzelczyk et al., 1991).

7.4.7 Different carbohydrates assimilation test

Carbohydrates assimilation tests and μ_{\max} of *W. onychis* LABI2 demonstrated high affinity to maltose, xylose, glucose, sucrose and fructose (Table 27). Greater cell concentrations of this yeast were verified when carbohydrates were maltose (3.9×10^8 cells/mL), xylose (3.8×10^8 cells/mL) and glucose (3.7×10^8 cells/mL) respectively. An intermediary level of cell concentration was obtained for sucrose and fructose, while arabinose, lactose and galactose demonstrated to be unviable to use as carbon source for this specific yeast having a slightly growth compared to control absent of carbohydrate.

In addition, yeast biomass obtained from cultures using sucrose, xylose, fructose, maltose and glucose were, respectively, 1.86 g/L, 1.67 g/L, 1.65 g/L, 1.62 g/L and 1.58 g/L. The range of biomass yield of these carbohydrates is from 0.81 g/g to 0.92 g/g with same biomass productivity for all (0.02 g/L.h). Interestingly, despite arabinose and galactose biomass reached 0.48 g/L and 0.36 g/L, values close to the control (0.37 g/L), a decrease of total reducing sugars (TRS) from the beginning of culture was verified. For this reason, there were biomass yields of 1.67 g/g (arabinose) and 0.96 g/g (galactose), values higher than other carbohydrates tested (Table 27).

Table 27 – Kinect parameters of *Wickerhamomyces onychis* LABI2 using different carbohydrates cultivated in orbital shaker incubator for 96 h at 28 °C and 180 rpm.

Carbohydrate	Maximum cell concentration (cell/mL)	μ_{\max} (h ⁻¹)	Biomass concentration (g/L)	Biomass yield (g/g)	Biomass productivity (g/L.h)
Arabinose	8.7×10^7	0.12	0.48 ^{a*}	1.67*	0
Fructose	2.6×10^8	0.17	1.65 ^{b,d*}	0.92*	0.02**
Galactose	6.1×10^7	0.15	0.36 ^{c*}	0.96*	0
Glucose	3.7×10^8	0.19	1.58 ^{b*}	0.84*	0.02**
Lactose	7.4×10^7	0.14	0.40 ^{c*}	0	0
Maltose	3.9×10^8	0.09	1.62 ^{b*}	0.87*	0.02**
Sucrose	3.1×10^8	0.21	1.76 ^{d*}	0.85*	0.02**
Xylose	3.8×10^8	0.20	1.67 ^{b,d*}	0.81*	0.02**
Control	5.0×10^7	0.10	0.37 ^{c*}	-	-

^{a, b, c, d} = Different letters correspond to statistically different averages (P<0.05)

Standard deviation: * <0.07 and ** <0.01

The growth curves of carbohydrates assimilation could be separated in two groups. One with maximum cell concentration higher than 1.0×10^8 cells/mL, which include glucose, sucrose, xylose, maltose and fructose, and a second group with cell concentration below 1.0×10^8 cells/mL (Figure 19). In general, log phase ended within 8 – 28 h of cultivation. Although maltose was the best sugar consumed by the yeast obtaining the highest cell growth, its consumption was slow leading to the lower maximum specific growth rate of all (0.09 h^{-1}). The high affinity for xylose and maltose can explain yeast ability to grow in SmF with the fermentable sugars released by the hydrolysis of SB and HCR (Figure 16a and 16b1). In addition, low growth observed for *S. cerevisiae* M26 (Figure 16a and 16b) in submerged culture with those residues can be explained by the same reason, which is the yeast low affinity to those carbohydrates (Vaughan-Martini and Martini, 2011).

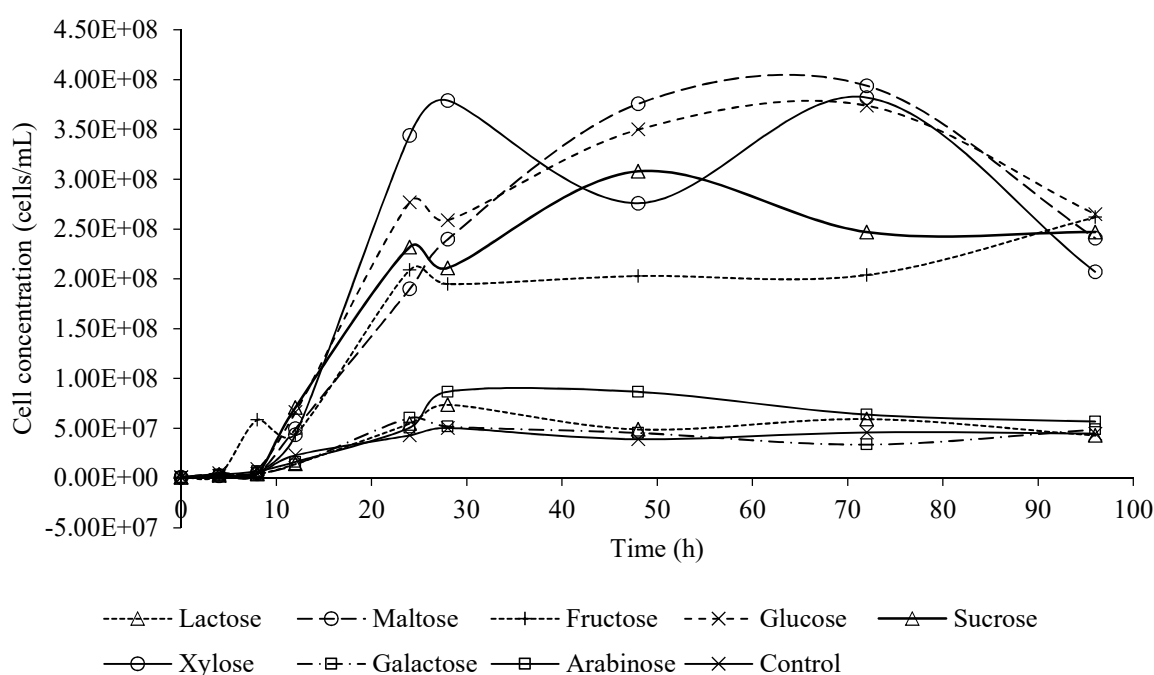


Figure 19 – Growth curve of *Wickerhamomyces onychis* LABI2 in submerged culture in orbital shaker incubator in aerobiosis with different carbohydrates after 96 h at 28 °C and 180 rpm.

Kurtzman (2011) affirms that *W. onychis* is capable to grow in submerged cultures using glucose, sucrose, maltose and xylose, and arabinose assimilation being variable according to the strain. Such affirmative is in accordance to those found in this study. The yeast is also capable of using methyl- α -D-glucoside, celobiose, trealose, raffinose, melezitose, ethanol, glycerol, manitol, lactate, citrate, and incapable to assimilate lactose and galactose. Although literature affirms the inability to assimilate galactose (Kurtzman, 2011), the use of TRS from

galactose by this strain was observed (Table 27). Perhaps, strain variation could explain the ability to use galactose.

The use of yeasts as food and feed is more suitable due to its higher content of nitrogen and ash than fungi, algae and bacteria. The ability to assimilate arabinose is another advantageous on choosing new strains for biomass production, as well as capacity to grow in absence of vitamins and temperatures up to 40 °C which lower cost and turns the bioprocess more attractive (Nigam, 2000). Among these characteristics, *W. onychis* is capable to assimilate arabinose as was confirmed by this research. Kurtzman (2011) and Zapata et al. (2014) attest tolerance to high temperatures and although the strain did not growth in absence of vitamins, cultures using corn steep liquor demonstrate one viable alternative to use as vitamins and amino acids source for its growth

Besides the potential to be used as SCP source and improve protein content of the residues, *W. onychis* may induce probiotic effects into gastro-intestinal tract of the host. Among the characteristics to consider development of new probiotics is the ability to grow under anaerobic conditions, tolerate a wide range of pH and different temperatures. The production of ethanol from grape using *W. onychis* (Ibarra, 2015) and contamination by the species in brewery (Campbell, 2003) demonstrated facultative characteristic by the yeast also attested by the present work. Besides the anaerobic tolerance, according to Zapata et al. (2014), this specie owns a high tolerance to pHs from 3.0 to 9.0 and temperatures from 5 to 37 °C. Kurtzman (2011) also states the *W. onychis* capacity to grow in 37 °C and Cevallos (2014) affirms the pH tolerance range from 3.5 to 7.5.

Another interesting probiotic feature is the production of antimicrobials, which control harmful organisms inside digestive tract (Martins et al., 2005). *W. onychis* is studied for its bio control capacity demonstrating to produce some antagonist compound against *Botrytis cinerea* in roses petals (Cotes et al., 2011) and onions (García et al., 2002) and *Rhizopus stolonifer* in tomatoes (García et al., 2002). Moreover, the use of microorganisms, either as a bio control agent or as SCP requires that they are not harmful to the environment and humans (Kurtzman, 2014). In this sense, *W. onychis* presence in fermented human foods (Hahn, 1988; Oh and Han, 2003) and absence of works relating this species pathogenicity may demonstrate the potential to use this yeast as SCP.

Production of xylanolytic enzymes and possibly amylases by *W. onychis* is another advantage when considering probiotic effects. Such enzymes degrade the residues components helping on digestion and digestibility in the rumen, which may increase animal performance (Newbold et al., 1996). Among other interesting characteristics, the ability to grow in hard

residues, high versatility in assimilating sugar and nutritious biomass, this yeast emerges as an alternative to the production of SCP from residues and maybe a potential probiotic yeast.

7.5 Conclusion

Wickerhamomyces onychis LABI2 demonstrated to be a yeast capable of growing using harsh residues such as sugarcane bagasse and hydrolyzed cassava residue in submerged culture. This species produced xylanases when cultivated with sugarcane bagasse and was able to grow in anaerobic condition in Petri dishes. Submerged culture improved nutritional parameters of both residues, especially crude protein content, which could be studied for feed aiming to improve animal productivity. The use of agro-industrial waste for single cell protein production from this species was more favorable than *Saccharomyces cerevisiae* M26 due to its interesting characteristics of biomass production, high affinity to maltose and xylose, xylanases production and nutritional advantages. However, more studies are required to confirm other probiotic effect of this special yeast and the nutrients quality for animal nutrition.

8 CAPÍTULO 5

Production of single cell protein from agro-industrial residues and some physiological aspects of *Rhodotorula mucilaginosa* LABI1

8.1 Abstract

The objective of this research was to evaluate the yeast biomass production from *Rhodotorula mucilaginosa* LABI1 with potential use as single cell protein from agro-industrial residues as well as to characterize some aspects of yeast physiology. The submerged culture using formulated medium with solid residues was performed and yeast biomass production and final residue was nutritionally evaluated. Yeast ability to assimilate different carbohydrates and to grow in absence of specific amino acids and vitamins were tested. *R. mucilaginosa* LABI1 demonstrated capacity to grow in fed-batch culture using sugarcane bagasse or hydrolyzed cassava residue in aerobic conditions reaching respectively values of 2.4×10^8 cells/mL (4.0 g/L with 18.0% of protein) and 1.5×10^9 cells/mL (8.5 g/L with 25.1% of protein), 7 and 5 times bigger than *Saccharomyces cerevisiae* M26 at the same conditions. An increase from 1.9% to almost 7% crude protein of pretreated cassava after culture of *R. mucilaginosa* LABI1 was achieved. Production of xylanases (0.31 U/mL), cellulases (0.43 U/mL) and amylases (0.33 U/mL) may confirm the ability of this special yeast to use harsh residues. Besides, the capacity to grow in anaerobic condition, media without addition of amino acids and the high versatility in the use of different carbohydrates for its growth are additional indicators the importance of *R. mucilaginosa* LABI1 applied in biotechnological process. The use of this yeast as microbial feed additive emerges as an alternative to reduce the high amount of waste discarded into the environment. In addition, derived products with high-added values at relative low cost can also be obtained. The improvement of the nutritional parameters of some residues by culture with this yeast reveal the necessity to study these final residues in animal feed including its potential probiotic properties.

Key words: Single cell protein, nutritional requirements, biomass production, waste enrichment.

8.2 Introduction

The increase of world population consequently increases the demand for nutrients like animal protein. In order to increase animal productivity, genetic improvement brought as consequence the need of more nutritional feed (Villas-Bôas et al., 2002). Studies on the use of non-conventional substrates have been performed aiming the reduction of costs. Investment in processing co-products or wastes from food industries has shown to be a strategic option to solve the demand for productivity and animal performance like ruminants and monogastric (Villas-Bôas et al., 2002; Apás et al., 2008). The use of some residues, such as sugarcane bagasse and cassava residue, for animal feed is restricted and associated with physical (pressure and steam) or chemical (caustic soda and ammonia) treatments, which are responsible for cost increase and limitation of its use as well as the production of some toxic compounds (Lima Jr et al., 2010). Another problem of such residues is the low concentration of vitamins, proteins, and low digestibility. The conversion of waste by microorganisms is a potential solution for these problems in view of their high protein content, with essential amino acids and favorable vitamins and minerals levels (Ugalde and Castrillo, 2005; França and Rigo, 2011; Tripathi and Karim, 2011; Durmic et al., 2013).

Fermentation process by microorganisms increases food durability and its digestibility. Such microorganisms can serve as starters in the inoculation in silages and residues contributing to texture, flavor and aroma of foods (França and Rigo, 2011). These fermented products with microorganisms have positive effects on animal performance, food assimilation and weight gain, although its causes are not exactly known (Apás et al., 2008; Gaggia et al., 2010; Marrero et al., 2014). Microorganisms responsible for enhancing animal performances are classified as probiotics, defined as "preparation of microbial cells or components of cells that have a beneficial effect on health and well-being of the host" (Salminen et al., 1999; Guarner et al., 2005; Gaggia et al., 2010). Microbial additives used to improve animal performance are also defined by the Foods and Drugs Administration (FDA) as Direct-Fed Microbials (DFM), or "natural source of viable microorganisms", replacing the term probiotic which was used before by producer companies of these supplements (França and Rigo, 2011). Studies on microbial additives have grown taking into account especially the nature of the feed used for animals, which mostly alter the gastro-intestinal environment causing health problems and decrease of their performance (França and Rigo, 2011).

Probiotics currently used are mostly bacteria and yeasts species (Martins et al., 2005) and the use of the species *Saccharomyces cerevisiae* in feed often requires the addition of other compounds like enzymes, antibiotics and vitamins (Villas-Bôas et al., 2002). Considering that

these compounds addition increases the feed cost and the limited probiotic strains and microorganisms able to grow in lignocellulosic materials for biomass production, the study of new microorganisms for this purposes is relevant (Martins et al., 2005).

The use of microbial cells can also be a valuable source of protein, being known as single cell protein (SCP) (Anupama and Ravindra, 2000). Algae, fungi and bacteria can be used as SCP sources, being rich on protein, amino acids, carbohydrates, vitamins, fats and essential minerals that can serve as dietary supplement either for humans or animals (Anupama and Ravindra, 2000; Ghorai et al., 2011).

The yeast *Rhodotorula mucilaginosa* belong to the phylum Basidiomycota, order Sporidiobolales and has elliptic ovoid cells, isolated or in pairs with polar budding. This species is found worldwide both in terrestrial and aquatic environments and for a variety of substrates including extreme conditions (Sampaio, 2011a). Because of its pink color this species has been studied for pigment production, mainly β - carotene, tolurene and torularhodin (Libkind et al., 2004; Aksu and Eren, 2005; Hainal et al., 2012; Maldonade et al., 2012; Cheng and Yang, 2016; Yoo et al., 2016). Some researchers also indicate the production of enzymes such as cellulases (Arcuri et al., 2014; Li et al., 2014), lipases (Arcuri et al., 2014; Li et al., 2014; Yang et al., 2013), esterase (Lee et al., 1987), proteases (Yang et al., 2013; Lario et al., 2015) and pectinases (Luh and Phaff, 1951; Arcuri et al., 2014).

The aim of this work was to define preliminary bioprocess using sugarcane bagasse or hydrolyzed cassava residue inoculated with *Rhodotorula mucilaginosa* LABI1 in aerobic submerged culture, including some physiological aspects of this special yeast focusing on a low cost single cell protein production and nutritional enrichment of the final waste.

8.3 Materials and Methods

8.3.1 Strain, culture medium and anaerobiosis test

The yeast *Rhodotorula mucilaginosa* LABI1 used in this study was isolated from rice bran and deposited in the Laboratory of Industrial Biotechnology, Universidade Estadual Paulista (UNESP/Assis). The strain of *Saccharomyces cerevisiae* M26 isolated from sugar industry (Oliva Neto et al., 2004) was used as comparison for growth and enzyme production in bioreactor using the same agro-industrial residues as *R. mucilaginosa* LABI1 culture.

Basal culture medium formulated for the tests was composed by (w/v): 0.13% $(\text{NH}_4)_3\text{PO}_4$, 0.10% $(\text{NH}_4)_2\text{SO}_4$, 0.031% K_2SO_4 , 0.0028% ZnSO_4 , 0.0012% MnSO_4 and 0.024% MgSO_4 , modified from Oliva Neto et al. (2004). All media were sterilized in autoclave (SP215-

26, Phoenix, Araraquara/SP, Brazil) at 121 °C for 20 minutes or through filtration using 0.22 µm membrane (Millipore, Merck, Darmstadt, Germany).

The ability of *R. mucilaginosa* LABI1 to grow in absence of oxygen was assessed through its cultivation in Petri dishes incubated under anaerobic jar (Anaerocult, Merck, Darmstadt, Germany) with an anaerobic generator (Anaerobac, Probac do Brasil, São Paulo, Brazil) at 33 °C for 72 h. Capacity to grow was visually evaluated in comparison to the control Petri dish grown in presence of oxygen.

8.3.2 Submerged fermentation using agro-industrial residues

The concentration of solid cassava residue (CR) as well as pretreatment need with amylase were performed after inoculation of *R. mucilaginosa* LABI1 in flasks with 3.0% or 5.0% (w/v) of CR or hydrolyzed cassava residue (HCR) mixed with 0.3% or 0.5% of corn steep liquor (w/v) and basal culture medium without (NH₄)₂SO₄ and (NH₄)₃PO₄. Culture incubation occurred in orbital shaker incubator for 96 h at 180 rpm and 28 °C in aerobiosis (TE421, Tecnal, Piracicaba/SP, Brazil).

Pretreatment of CR occurred by hydrolysis at 10% (w/v) of concentration in sodium acetate buffer (0.05 M, pH 5.5) for 24 h at 50 °C using 1.2 U/mL of amylase from *Rhizopus oligosporus* (Gonçalves, 2016). The final residue was dried in oven for 72 h, 50 °C (TE394/2, Tecnal, Piracicaba/SP, Brazil) after hydrolysis.

Sugarcane bagasse (SB) and HCR were evaluated as carbon sources in aerobic submerged fermentation by the culture in bioreactor (Bioflo 115, New Brunswick, New Jersey, USA). Inoculums were prepared through yeasts culture with 50 mL of the same medium formulated for bioreactor culture with 1.0% glucose without residue, with incubation in orbital shaker incubator (TE421, Tecnal, Piracicaba/SP, Brazil) for 24 h, 28 °C and 180 rpm. Conditions of bioreactor cultures were 28 °C, 1 vvm of air supply and pH 5.5. Dissolved oxygen was measured with a polarographic oxygen electrode (Mettler Toledo AG, Greifensee, Switzerland) and pH was measured with a glass electrode (Mettler Toledo AG, Greifensee, Switzerland) controlled with addition of 1.0% (v/v) H₂SO₄ or 1.0 M NaOH.

The culture of *R. mucilaginosa* LABI1 and *S. cerevisiae* M26 using SB occurred in batch and fed-batch processes. Medium composition for cultivation in single batch was composed by basal culture medium with 2.0% of sugarcane bagasse, 0.05% glucose and 0.2% yeast extract and fermentation occurred for 72 h and 200 rpm with 1.5 L for *R. mucilaginosa* LABI1 and 1.7 L for *S. cerevisiae* M26. Medium formulated for fed-batch (initial 500 mL) was composed by 5.0% SB, basal culture medium without (NH₄)₃PO₄ and (NH₄)₂SO₄ and with

addition of 0.5% corn steep liquor. Three feds of 300 mL each occurred at 12, 36 and 60 h and supply of SB was performed according to maintaining the same level of concentration of solids at the beginning of culture (5.0%) measured with humidity balance (BTS110, Scientific Industries, Poland). Mechanical agitation was increased from 200 rpm to 300, 400 and 500 rpm respectively after each fed. Fed-batch culture ended after 120 h of fermentation.

Submerged culture using HCR occurred in fed-batch process for 96 h for *R. mucilaginosa* LABI1 and 168 h for *S. cerevisiae* M26 in bioreactor. Medium contained 0.5% of corn steep liquor, initial 5.0% of HCR (w/v) and basal culture medium without $(\text{NH}_4)_2\text{SO}_4$ and $(\text{NH}_4)_3\text{PO}_4$ with initial 500 mL. During fed-batch, three feds of 300 mL were added at 12, 36 and 60 h for *R. mucilaginosa* LABI1 and at 12, 36 and 72 h for *S. cerevisiae* M26. The supply of the culture medium was performed according to maintaining the same level of initial soluble solids (Brix) concentration (approximately 3.0%) at the beginning of culture measured using a refractometer (Portable Refractometer, Biosystems). Due to the increase of viscosity, mechanical stirring was increased from 200 to 300, 400 and 500 rpm after each for *R. mucilaginosa* LABI1 and from 350 rpm to 450, 550 and 650 rpm respectively after feds for *S. cerevisiae* M26.

The separation of liquid medium and residual SB or HCR after submerged culture occurred through manual filtration using polymer fabric. The liquid was centrifuged at 5000 x g for 30 minutes, 4 °C (Megafuge 16R, Heraeus, Thermo Fisher, Massachusetts, USA) for cell separation. Both yeast and residue were dried in oven (TE394/2, Tecnal, Piracicaba/SP, Brazil) for 72 h at 50 °C before nutritional tests.

8.3.3 Carbohydrates culture test

The ability of *R. mucilaginosa* LABI1 to assimilate different carbohydrates: carboxymethylcellulose (CMC), arabinose, galactose, xylose, fructose, glucose, lactose, maltose, sucrose, starch and xylan was performed in flasks with medium containing basal culture medium, 0.2% (w/v) of yeast extract and 0.2% (w/v) of the respective carbohydrate. Flasks were incubated at 180 rpm at 28 °C for 96 h in orbital shaker incubator (TE421, Tecnal, Piracicaba/SP, Brazil). The culture without any sugar concentration was conducted as control.

8.3.4 Amino acids and vitamins single-omission tests

Each pure amino acid was evaluated in yeast tube cultures by synthetic medium with 19 from 20 of amino acids (listed on Table 34) at 0.03% (w/v), basal culture medium, 2.0% of glucose (w/v) and the following 8 vitamins: aminobenzoic acid (0.2 µg/mL), ascorbic acid (2

$\mu\text{g/mL}$), folic acid (0.02 $\mu\text{g/mL}$), nicotinic acid (2 $\mu\text{g/mL}$), pantothenic acid (1 $\mu\text{g/mL}$), pyridoxine (2 $\mu\text{g/mL}$), riboflavin (2 $\mu\text{g/mL}$) and thiamin (1 $\mu\text{g/mL}$). Evaluation of vitamins was conducted in yeast tubes cultures using 7 from 8 vitamins in the basal culture medium, 2.0% of glucose and 20 pure amino acids.

Results were expressed as absence, partial or full growth. The ability of this yeast to grow in absence of one specific amino acid or vitamins was evaluated. Tubes containing all the essential amino acids and vitamins (positive control) and without amino acids or vitamins (negative control) were performed. Cell growth was assessed after 120 h of incubation at 28 °C by measuring the culture turbidity at 600 nm using spectrophotometer (UV-M51, Bel Engineering, Monza, Italy) (Oliva Neto and Yokoya, 1997).

8.3.5 Analytical procedures

Growth curve was performed through direct cell concentration using Neubauer counting chamber (7301-1B, New Optics) and yeast cells viability was assessed after using methylene blue (Lee et al., 1981). Dried biomass was performed according to Chen et al. (2012) through drying at constant weight at 105 °C. Reducing sugars (RS), xylanase and amylase were quantified according to Miller (1959), Carvalho et al. (2015) and Gonçalves (2016) respectively. One enzymatic unit means the releasing of 1 μmol of RS per minute per mL of enzymatic extract. When necessary, total reducing sugars (TRS) quantification occurred after acid hydrolysis with H_2SO_4 (2.0 M), at the proportion 1:1, for 10 minutes at 100 °C followed by neutralization with NaOH (2.0 M) (1:1:1) and quantification through the Miller method (Miller, 1959).

The kinetic parameters maximum specific growth rate (μ_{max}), yield coefficient of cell growth ($Y_{x/s}$) and biomass productivity were determined according to Shuler and Kargi (2002) and specific rate of O_2 consumption in batch culture using sugarcane bagasse was calculated according to Núñez et al. (2014) considering saturated O_2 concentration at 28 °C (0.244 mMol/L).

Nutritional analysis and chemical composition of biomass were quantified according to the Association of Official Analytical Chemists (AOAC) methods (Helrich, 1990). Ashes quantification occurred after burning in muffle oven (SP2707-20, Sppencer, São Paulo/SP, Brazil) for 6 h at 700 °C. Dry matter was measured after drying at constant weight at 105 °C (BTS110, Scientific Industries, Poland). Ethereal extraction occurred using the solvent petroleum ether for 6 h with a Soxhleht equipment. Crude protein was quantified through the Kjeldahl method and crude fiber was quantified after acid (H_2SO_4) and basic (NaOH) – 1.25%

– hydrolysis at 400 °C for 40 min and drying at 105 °C for 24 h. Non-nitrogenous extracts was determined subtracting the percentage of ashes, fat content, crude fiber and crude protein. Total Digestible Nutrients was calculated according to Kearn (1982). Yeast's centesimal composition of carbon was determined according to Walkley (1947), sulfur and phosphorus through “visible light spectrum”, calcium, potassium, magnesium and zinc with digestion and atomic absorption and nitrogen through Kjeldahl method.

Tests were performed in triplicates, and statistical analysis occurred through ANOVA and Tukey ($P < 0.05$) comparison between replicates using the software GraphPad Prism 5.01.

8.4 Results and Discussion

8.4.1 Anaerobiosis test

The yeast culture in anaerobic jar demonstrated *R. mucilaginosa* LAB11 was capable to grow under anaerobic environment proving to be a facultative microorganism as stated by Brad et al. (2008), Ollivier et al. (2011) and Villegas et al. (2009).

8.4.2 Submerged fermentation in orbital shaker incubator with cassava residue hydrolyzed or not

The hydrolysis of cassava residue with *Rhizopus oligosporus* amylases produced 35.4 g/L of reducing sugars (RS) after 24 h. This content of RS are smaller than found by Gonçalves (2016) and Shi et al. (2014) which obtained respectively 39.9 g/L and 43.3 g/L.

The cultivation of this specie in different concentrations of non-treated solid cassava residue (CR) or hydrolyzed cassava residue (HCR) combined with corn steep liquor (0.3% - 0.5%) revealed a preference by the yeast for the HCR (Table 28).

Flasks cultures of *R. mucilaginosa* LAB11 with HCR achieved higher biomass concentrations than flasks with CR. While the culture with media formulated with HCR obtained from 7.7 g/L to 11.6 g/L of yeast biomass (dry weight), the culture with CR achieved only up to 3.6 g/L (at least 2 times lesser). The maximum cell concentration for cultures with CR was up to 2.4×10^8 cells/mL, while the cultures with HCR was up to 1.1×10^9 cells/mL, confirming the necessity of CR enzymatic pretreatment. In addition, concentration of 5.0% of this residue treated or not, demonstrated to be preferable by the yeast, which achieved higher biomass than cultures with 3.0% of the residue. In the same way, the higher the concentration of corn steep liquor (CSL), the higher yeast biomass concentration (Table 28).

Table 28 – Kinect and proteic composition of *Rhodotorula mucilaginosa* LABI1 batch culture in medium formulated with solid cassava residue hydrolyzed (HCR) or not (CR) and corn steep liquor (CSL) in aerobic condition in orbital shaker incubator for 96 h, 180 rpm at 28 °C.

Conditions of medium composition			After submerged culture								
CR (g)	HCR (g)*	CSL (%)	Log phase	μ_{\max} (h ⁻¹)	Max cell concentration (cell/mL)	Biomass concentration (g/L)	Yeast protein (%)	Biomass productivity (g/L.h)	Residual bagasse (g)	Residual bagasse protein (%)**	Y _{x/s} (g/g)***
3.0	-	0.3	12-28 h	0.10	9.7E+07	2.5±0.4 ^a	28.8±1.0 ^{a,c,d}	0.03	1.47	4.2±0.2 ^{a,b,c}	0.12
3.0	-	0.5	12-72 h	0.05	1.6E+08	2.8±0.1 ^a	26.1±1.1 ^{a,c,d}	0.03	1.57	4.3±0.4 ^{a,b,c}	0.14
5.0	-	0.3	12-96 h	0.02	2.4E+08	3.6±0.4 ^a	15.8±3.0 ^b	0.04	3.08	3.7±0.5 ^b	0.13
5.0	-	0.5	12-48 h	0.05	1.4E+08	3.6±1.1 ^a	26.1±0.7 ^{a,c,d}	0.04	2.81	4.3±0.3 ^{a,b,c}	0.12
-	3.0	0.3	12-72 h	0.06	7.2E+08	7.7±0.5 ^b	32.3±0.6 ^a	0.08	1.36	4.4±0.5 ^{a,b,c}	0.38
-	3.0	0.5	12-96 h	0.03	1.1E+09	8.5±0.3 ^b	34.1±0.8 ^c	0.09	1.37	4.5±0.4 ^{a,b,c}	0.42
-	5.0	0.3	12-96 h	0.04	8.0E+08	11.4±0.7 ^c	22.8±0.7 ^d	0.12	2.12	4.0±0.4 ^{a,b,c}	0.28
-	5.0	0.5	12-72 h	0.06	7.1E+08	11.6±0.2 ^c	24.7±1.4 ^a	0.12	2.09	4.9±0.4 ^c	0.27

*Enzymatic hydrolysis of cassava; **Protein in CR and HCR were 1.8% and 1.9% respectively; ***Calculated according to the amount of bagasse

^{a, b, c, d} = Different letters between the same parameter correspond to statistically different averages (P<0.05)

Log phases of *R. mucilaginosa* LABI1 culture started at 12 h in all cultures, ended before 48 h for two combinations, not reaching stationary phase for another three combinations. Perhaps for these flasks if culture continued until the end of log phase, biomass concentrations would be higher. Extended log phase decreased the value of μ_{\max} , which ranged from 0.02 h⁻¹ to 0.10 h⁻¹.

Biomass yields as well as productivities were higher for medium formulated with HCR. While cultures with HCR biomass yields and productivities reached 0.42 g/g and 0.12 g/L.h respectively, CR reached 0.14 g/g and 0.04 g/L.h, also confirming *R. mucilaginosa* LABI1 preference for hydrolyzed cassava (Table 28).

Protein concentration of the *R. mucilaginosa* LABI1 biomass ranged from 15.8% to 34.1% while protein in the cassava residue increases from 1.9% (before culture) up to 4.9%, after culture with 5.0% HCR and 0.5% CSL. In these conditions, a higher biomass concentration (11.6 g/L) and productivity (0.12 g/L.h) were reached. This combination was chosen for the formulation medium in fed-batch culture in bioreactor.

8.4.3 Kinect of *R. mucilaginosa* LABI1 and *S. cerevisiae* M26 in bioreactor with sugarcane bagasse (batch and fed-batch) or hydrolyzed cassava residue (fed-batch)

Bioreactor cultures demonstrated *R. mucilaginosa* LABI1 and *S. cerevisiae* M26 were able to grow in media containing sugarcane bagasse (SB) and hydrolyzed cassava residue (HCR) as carbon source, and achieved higher cell concentrations for HCR cultures (Figure 20). Submerged culture (SmF) of *R. mucilaginosa* LABI1 had higher growth compared to *S. cerevisiae* M26 in all conditions demonstrating that the first yeast is more suitable for single cell protein (SCP) production in this low-cost culture medium.

SmF with SB in single-batch achieved maximum cell concentration of 1.1x10⁸ cells/mL (μ_{\max} of 0.106 h⁻¹) for *R. mucilaginosa* LABI1 2 times lower than fed-batch process (2.4x10⁸ cells/mL, 0.26 h⁻¹). Moreover, the culture with this yeast reached 3.4 and 6.5 times more cell growth and higher μ_{\max} in both processes when compared to *S. cerevisiae* (3.1x10⁷ cells/mL and 3.7x10⁷ cells/mL).

The use of HCR in fed-batch process in bioreactor was more suitable for biomass production for both yeasts. Cell concentration for *R. mucilaginosa* LABI1 with this residue was 6.4 times higher (1.5x10⁹ cells/mL, 0.09 h⁻¹) than SB fed-batch culture and 14.4 times more than SB batch culture. For *S. cerevisiae* M26, hydrolyzed cassava achieved up to 3.2x10⁸ cells/mL (0.04 h⁻¹), 10.4 and 8.8 times more than SB SmF in batch and fed-batch respectively. Results showed that *R. mucilaginosa* LABI1 highest cell concentration was 4.8 times higher

than the highest achieved by *S. cerevisiae* M26 in culture with HCR confirming its highest affinity to the residue.

Log phase ended between 24 - 28 h for both yeasts during batch and fed-batch cultures with SB. Fed-batch culture using HCR demonstrated a log phase initiating at 4 h without reaching a stationary phase for *R. mucilaginosa* LABI1, while *S. cerevisiae* M26 stationary phase started around 72 h. The extended log phase was responsible for higher yeast cell concentration and the decrease of *R. mucilaginosa* LABI1 μ_{\max} . Perhaps, a prolonged time for *R. mucilaginosa* LABI1 in culture with HCR would increase biomass concentration.

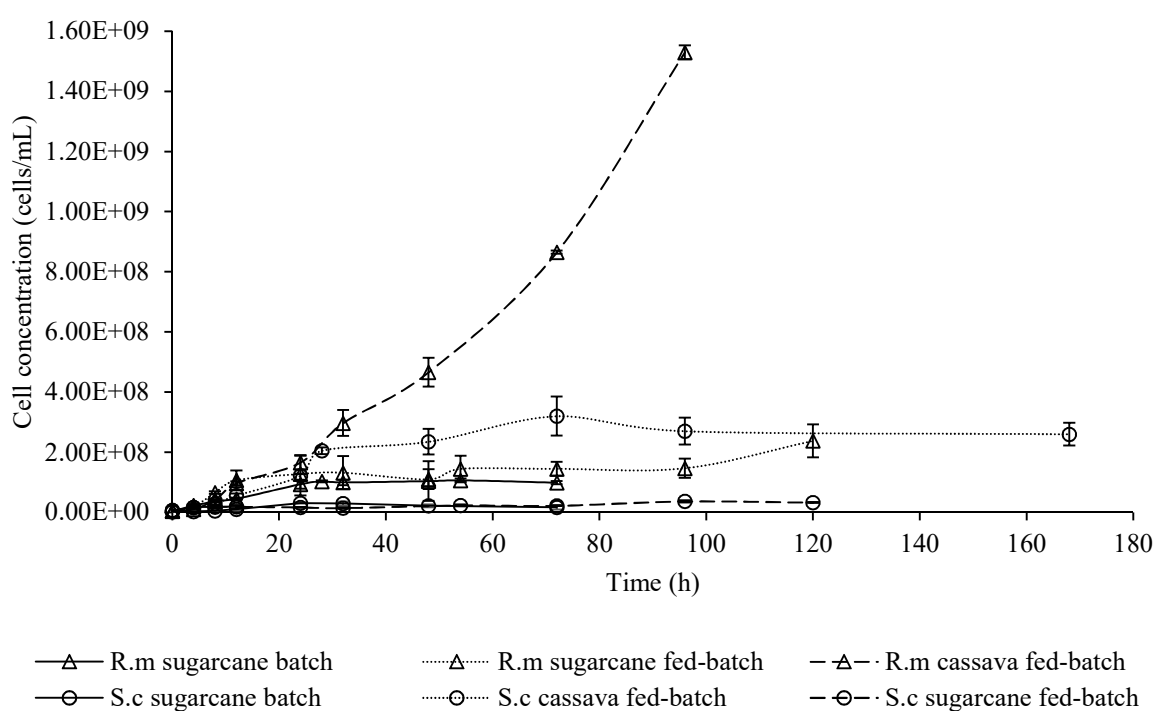


Figure 20 – Growth curves of *Rhodotorula mucilaginosa* LABI1 (R.m) and *Saccharomyces cerevisiae* M26 (S.c) bioreactor culture using sugarcane bagasse in batch or fed-batch culture and with hydrolyzed cassava residue in fed-batch (28 °C, pH 5.5, 1 vvm).

Growth curves for both yeasts demonstrate higher affinity of *R. mucilaginosa* LABI1 with the agro-industrial residues, pretreated or not, compared to *S. cerevisiae* M26. Kinetic parameters of yeast cultures also confirm HCR residue and the yeast *R. mucilaginosa* LABI1 as better source and microorganism for SCP production (Tables 29 and 30).

R. mucilaginosa LABI1 produced 1.9 g/L of biomass in SmF culture with SB while *S. cerevisiae* M26 culture at the same conditions achieved only 0.4 g/L. The fed-batch process with SB increased the yeast biomass in 2.2 (4.0 g/L) and 5.2 times (2.2 g/L) respectively for *R.*

mucilaginosa LABI1 and *S. cerevisiae* M26 cultures. However, the highest biomass was obtained in fed-batch culture using HCR for both yeasts with 2.1 more for *R. mucilaginosa* LABI1 (8.5 g/L) and 1.4 more for *S. cerevisiae* M26 (3.1 g/L). This last residue proved to be more efficient than SB yeast biomass production in submerged culture. In addition, *R. mucilaginosa* LABI1 fed-batch culture was more suitable for biomass production in medium formulated with HCR since there was a 2.75 times more biomass than *S. cerevisiae* M26 culture at the same conditions.

Table 29 – Kinect and nutritional parameters of *Rhodotorula mucilaginosa* LABI1 (R.m) and *Saccharomyces cerevisiae* M26 (S.c) in batch culture using sugarcane bagasse (SB) in bioreactor at 28 °C, 200 rpm, 1 vvm, pH 5.5 for 72 h.

Parameters	Supplied SB		After submerged culture			
	before culture		Final residue		Yeast biomass	
	R.m.	S.c.	R.m	S.c	R.m	S.c
Residue (g)	30.0	34.0	24.8	27.6	2.8	0.7
Crude protein (%)	1.6±0.2 ^a		3.1±0.9 ^b	3.7±0.3 ^b	20.56±0.7 ^c	28.2±0.4 ^d
Yeast biomass (g/L)	-	-	-	-	1.9 ^a	0.4 ^b
Max cell concentration (cell/mL)	-	-	-	-	1.1x10 ⁸	3.1x10 ⁷
Biomass productivity (g/L.h)	-	-	-	-	0.03	0.01
Y _{x/s} (g/g)*	-	-	-	-	0.51 ^a	0.10 ^b
μ _{max} (h ⁻¹)	-	-	-	-	0.11	0.09
Yeast cells viability at the end of culture (%)	-	-	-	-	87.1	81.0

*Calculated according to the amount of residue used, yeast extract not considered for this calculation

^{a, b, c, d} = Different letters between the same parameter correspond to statistically different averages (P<0.05)

Table 30 – Kinect and nutritional parameters of *Rhodotorula mucilaginosa* LABI1 (R.m) and *Saccharomyces cerevisiae* M26 (S.c) in fed-batch cultures using sugarcane bagasse (SB) or hydrolyzed cassava residue (HCR) in bioreactor at 28 °C, 1 vvm, pH 5.5.

Parameters	Supplied residue before culture				Final residue				Yeast biomass			
	SB		HCR		SB		HCR		SB culture		HCR culture	
	<i>R.m</i>	<i>S.c</i>	<i>R.m</i>	<i>S.c</i>	<i>R.m</i>	<i>S.c</i>	<i>R.m</i>	<i>S.c</i>	<i>R.m</i>	<i>S.c</i>	<i>R.m</i>	<i>S.c</i>
Residue (g)	115.0	86.0	89.5	130.0	91.9	61.9	35.5	51.2	3.2	1.8	9.4	3.6
Yeast biomass (g/L)	-	-	-	-	-	-	-	-	4.0 ^a	2.2 ^b	8.5 ^c	3.1 ^d
Max cell concentration (cell/mL)	-	-	-	-	-	-	-	-	2.4x10 ⁸	3.7x10 ⁷	1.5x10 ⁹	3.2x10 ⁸
Biomass productivity (g/L.h)	-	-	-	-	-	-	-	-	0.03	0.02	0.09	0.03
Y _{x/s} (g/g)	-	-	-	-	-	-	-	-	0.14	0.08	0.17	0.05
μ _{max} (h ⁻¹)	-	-	-	-	-	-	-	-	0.26	0.01	0.09	0.04
Yeast cells viability at the end of culture (%)	-	-	-	-	-	-	-	-	91.3	77.5	97.6	96.0
Moisture (%)	8.7±0.8 ^a		11.6±0.5 ^b		8.0±1.7 ^a	6.1±0.4 ^c	7.0±0.6 ^b	7.0±0.6 ^b	1.5±0.2 ^f	1.3±0.1 ^f	2.4±1.1 ^f	3.4±0.1 ^f
Dry matter (%)	91.3±0.8 ^a		88.4±0.5 ^b		92.0±1.7 ^a	93.9±0.5 ^a	93.0±0.6 ^b	93.0±0.6 ^b	98.5±0.2 ^f	98.7±0.1 ^f	97.6±1.1 ^f	96.6±0.1 ^f
Ashes (%)	5.5±1.3 ^a		3.4±0.8 ^b		3.9±0.4 ^a	3.5±0.3 ^c	5.1±0.2 ^d	5.6±0.2 ^d	5.7±0.9 ^f	7.1±1.9 ^g	6.7±0.2 ^f	7.4±0.4 ^g
Ethereal extract (%)	0.7±0.4 ^a		1.0±0.6 ^b		1.5±0.9 ^a	1.9±0.3 ^c	1.3±0.5 ^b	0.8±1.3 ^b	0.7±0.4 ^f	2.1±1.7 ^g	0.5±0.2 ^f	1.1±0.7 ^{f,g}
Crude fiber (%)	38.3±1.2 ^a		22.8±2.2 ^b		41.3±0.3 ^c	42.2±1.2 ^c	48.4±0.4 ^d	35.9±1.2 ^c	5.8±1.3 ^f	5.2±2.2 ^f	1.4±0.5 ^g	1.9±0.4 ^g
Crude protein (%)	1.6±0.2 ^a		1.9±0.2 ^b		3.2±0.3 ^c	3.5±0.2 ^c	6.7±0.4 ^d	6.6±0.3 ^d	18.3±0.2 ^f	28.2±1.5 ^g	25.1±3.1 ^g	33.8±0.8 ^h
Non-nitrogenous extract (%)	53.9±1.8 ^a		70.9±3.7 ^b		50.2±0.8 ^c	48.8±1.3 ^c	38.6±0.2 ^d	51.2±1.0 ^c	69.4±0.8 ^f	57.4±2.9 ^g	66.4±2.8 ^h	55.8±1.5 ^g
Total digestible nutrients (%)	48.6±0.9 ^a		56.9±2.5 ^b		50.7±1.8 ^a	51.6±0.7 ^a	47.9±0.9 ^d	51.4±0.8 ^c	78.0±0.2 ^f	80.4±4.0 ^{f,g}	83.1±0.9 ^g	83.6±0.7 ^g

a, b, c, d, e, f, g, h = Different letters between the same parameter correspond to statistically different averages (P<0.05)

Batch culture with SB showed higher biomass yield for *R. mucilaginosa* LABI1 (0.51 g/g) and *S. cerevisiae* M26 (0.10 g/g) than SB fed-batch culture (0.14 g/g for *R. mucilaginosa* LABI1 and 0.08 g/g for *S. cerevisiae* M26) (Tables 29 and 30). On the other hand, formulated medium used for batch cultures contained 0.2% yeast extract while fed-batch medium corn steep liquor substitute this compound. Probably, the yeast extract was responsible for the higher biomass yield since some nutrients present in this product additionally stimulate the yeast growth. Moreover, yeast extract is economically unfeasible due to its high cost (US\$ 18/kg, Biorigin, Quatá/SP, Brazil) and the use of corn steep liquor can reduce culture medium costs.

The biomass yield for *R. mucilaginosa* LABI1 (0.17 g/g) in fed-batch culture with HCR was higher than *S. cerevisiae* M26 (0.05 g/g) at the same conditions. Biomass productivities was equal for fed-batch and batch process with SB with 0.03 g/L.h for *R. mucilaginosa* LABI1. *S. cerevisiae* M26 biomass productivities were lower than *R. mucilaginosa* LABI1. The highest productivity was achieved for fed-batch with HCR for *R. mucilaginosa* LABI1 (0.09 g/L.h) a value 2.8 times higher than *S. cerevisiae* M26 at the same conditions (Table 30)

At the end of cultures, a total of 17% and 20% of SB were consumed by *R. mucilaginosa* LABI1 in batch and fed-batch process respectively. This yeast consumed a higher amount of HCR, with 60% of the total residue added during the culture, confirming the higher affinity of this yeast to HCR than SB. *S. cerevisiae* M26 consumed 9% and 18% of SB supplied in batch and fed-batch respectively, and 61% of HCR.

Results of biomass concentration, biomass productivity and yield demonstrated higher performance *R. mucilaginosa* LABI1 culture than *S. cerevisiae* M26, indicating that the first yeast is more robust and convenient strain for SCP using HCR. High yeast cells viability at the end of the process (up to 81%) demonstrates that conditions were favorable to yeasts healthy. The high affinity of *R. mucilaginosa* LABI1 to xylose and maltose (Table 33) helps to elucidate higher biomass concentration than *S. cerevisiae* M26 since *S. cerevisiae* cannot assimilate xylose (Arora et al., 2015; Vaughan-Martini and Martini, 2011).

The difference observed between the yeasts ability to grow in SB or HCR can also be explained by the xylanases, cellulases and amylases produced by *R. mucilaginosa* LABI1 (Table 31). While this target yeast produced up to 0.31 U/mL, 0.43 U/mL and 0.33 U/mL of xylanases, cellulases and amylases respectively, *S. cerevisiae* M26 culture almost did not express enzymatic activities (maximum of 0.14 U/mL of xylanases and 0.18 U/mL of endoglucanases) without production of amylases. Moreover, *S. cerevisiae* does not have the metabolism to produce xylanases and cellulases, so the activities detected in the beginning of

culture may be due to the own SB or contaminants along the fermentation (max 1×10^3 cells/mL) (La Grange et al., 2001).

Microorganism needed to break the hemicellulose and cellulose from SB to release fermentable sugars, so the activities of xylanases and cellulases during the entire culture time are needed. The highest enzymes produced by *R. mucilaginosa* LABI1 in batch and fed--batch culture were found in the range of 12 to 28 h and 24 to 48 h respectively. These range times are in accordance with log phase of the yeast growth, so there is a greater demand for carbon source. In addition, *R. mucilaginosa* LABI1 showed to assimilate xylose (Table 33) while *S. cerevisiae* is not able to consume this sugar (Arora et al., 2015; Vaughan-Martini and Martini, 2011). *R. mucilaginosa* LABI1 higher cell concentration than *S. cerevisiae* M26 (Figure 20) may be due to higher concentration of xylanases and amylases secreted by the first yeast, which are partially responsible for the hydrolysis of the residues, releasing fermentable sugars that can be used for cell growth and maintenance (Alponti et al., 2016).

Table 31 – Enzymes and reducing sugars (RS) released during batch culture with sugarcane (SB) and fed-batch cultures with sugarcane (SB) or hydrolyzed cassava residue (HCR) of *Rhodotorula mucilaginosa* LAB11 or *Saccharomyces cerevisiae* M26 in bioreactor at 28 °C, pH 5.5, 1 vvm.

Time (h)	<i>R. mucilaginosa</i> LAB11							<i>S. cerevisiae</i> M26					
	SB batch		SB fed-batch			HCR fed-batch		SB batch		SB fed-batch			HCR fed-batch
	Xylanase (U/mL)	RS (g/L)	Xylanase (U/mL)	CMCase (U/mL)	RS (g/L)	Amylase (U/mL)	RS (g/L)	Xylanase (U/mL)	RS (g/L)	Xylanase (U/mL)	CMCase (U/mL)	RS (g/L)	RS (g/L)
0	0.29 ^{a*}	1.15 ^{a*}	0.19 ^{b*}	0	1.96 ^{e*}	0	9.36±0.12 ^a	0.14 ^{d*}	0.81 ^{b*}	0.07 ^{e*}	0	0.59 ^{d*}	5.13±0.17 ^c
4	0.25 ^{b*}	0.81 ^{b*}	0.19 ^{b*}	0.16±0.05 ^a	1.40 ^{f*}	0.18±0.03 ^a	8.95±0.18 ^b	0.13 ^{d*}	0.82 ^{b*}	0.09 ^{e*}	0	0.63 ^{d*}	2.30±0.19 ^f
12	0.27 ^{a*}	0.94 ^{c*}	0.24 ^{b*}	0.16±0.09 ^a	0.76 ^{b*}	0	7.57±0.13 ^c	0.07 ^{e*}	0.28 ^{e*}	0.09 ^{e*}	0	0.70 ^{d*}	2.62±0.14 ^f
24	0.29 ^{a*}	0.85 ^{b*}	0.31 ^{a*}	0.19±0.04 ^a	0.71 ^{d*}	0.06±0.01 ^b	9.53±0.02 ^a	0.11 ^{d*}	0.35 ^{f*}	0.11 ^{d*}	0	0.69 ^{d*}	3.95±0.03 ^g
32	0.26 ^{b*}	0.87 ^{b*}	0.24 ^{b*}	0.43±0.10 ^b	0.73 ^{d*}	0	6.28±0.17 ^c	0.07 ^{e*}	0.22 ^{e*}	0.08 ^{e*}	0	0.80 ^{b*}	-
48	0.26 ^{b*}	0.70 ^{d*}	0.22 ^{c*}	0.18±0.08 ^a	0.72 ^{d*}	0	8.18±0.09 ^d	0.13 ^{d*}	0.22 ^{e*}	-	-	-	5.40±0.20 ^e
54	0.21 ^{b*}	0.18 ^{e*}	0.25 ^{b*}	0.13±0.07 ^a	0.77 ^{b*}	0.33±0.08 ^c	5.56±0.17 ^c	-	-	0.14 ^{d*}	0.18 ^a	0.81 ^{b*}	-
72	0.24 ^{b*}	0.68 ^{d*}	0.20 ^{c*}	0	0.78 ^{b*}	0.18±0.06 ^a	6.55±0.04 ^c	0	0.24 ^{e*}	0	0	1.04 ^{g*}	5.38±0.13 ^e
96	-	-	0.24 ^{b*}	0.17±0.02 ^a	0.69 ^{d*}	0.11±0.00 ^b	2.68±0.04 ^f	-	-	0.10 ^{e*}	0.00	1.18 ^{g*}	5.66±0.27 ^c
120	-	-	0.29 ^{a*}	0.16±0.02 ^a	0.83 ^{b*}	-	-	-	-	0	0.05 ^c	0.99 ^{g*}	-
168	-	-	-	-	-	-	-	-	-	-	-	-	4.63±0.09 ^h

*Standard deviation <0.04

a, b, c, d, e, f, g, h = Different letters between the same parameter correspond to statistically different averages (P<0.05)

The decrease of reducing sugars (RS) observed are explained by the use by the yeasts for cell maintenance and growth (Table 31). Batch culture with *R. mucilaginosa* LABI1 and *S. cerevisiae* M26 decreased RS concentration from 1.15 g/L to 0.68 g/L and from 0.81 g/L to 0.24 g/L respectively. After enzymes production, a higher concentration of RS was observed. During fed-batch culture an increment of RS happened after each fed. Since *S. cerevisiae* does not produce amylases (Van Zyl et al., 2012) any residual starch was not degraded during HCR culture. In addition, high amounts of reducing sugars at the end of *S. cerevisiae* M26 culture with cassava residue demonstrated a difficulty to assimilate RS by the yeast. Maybe some amount of RS could be released at the beginning of SB culture due to autoclaving process, breaking some bonds and releasing sugars into culture medium.

There are some studies on the production of xylanases and amylases by yeasts even by *R. mucilaginosa*. This species has been reported to produce cellulolytic enzymes (Arcuri et al., 2014; Li et al., 2014), such as the present study. Lipases (Arcuri et al., 2014; Li et al., 2014; Yang et al., 2013), esterase (Lee et al., 1987), proteases (Lario et al., 2015; Yang et al., 2013) and pectinases (Arcuri et al., 2014; Luh and Phaff, 1951) also confirming the ability of this yeast to use agro-industrial substrates to grow.

Batch culture with SB demonstrated a decrease in the specific rate of cell respiration (Figure 21), as expected. With the increase of cells concentration, specific rate of respiration decreases once air supply was at the same rate during the entire culture (1 vvm). In addition, a higher metabolism for *R. mucilaginosa* LABI1 than *S. cerevisiae* M26 cells was observed. Specific rate of O₂ consumption by *S. cerevisiae* M26 increased concomitantly with the first stage of log phase, showing a lower activity of the metabolism in this stage, and decreased at the end of this phase reaching almost zero. However, this parameter only decreased for *R. mucilaginosa* LABI1 during all log phase, showing the intense metabolism of this yeast or a higher requirement for dissolved oxygen by *R. mucilaginosa*. The oxygen consumption rate is considered a trustworthy indicator of biologic activity and provides a quickly answer to substrate consumption (Campos et al., 2006).

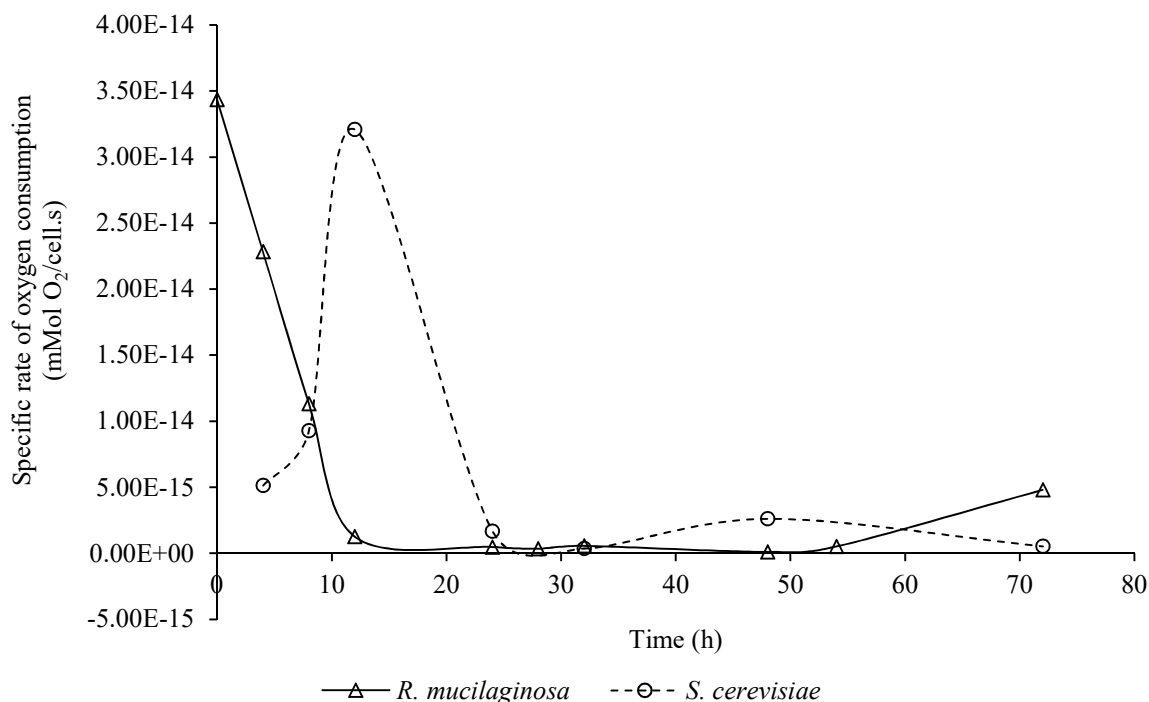


Figure 21 - Specific rate of oxygen consumption of *Rhodotorula mucilaginosa* LABI1 and *Saccharomyces cerevisiae* M26 batch culture in bioreactor with sugarcane bagasse at 28 °C, pH 5.5, 200 rpm and 1 vvm.

The use of agro-industrial residues as feed often requires some pretreatments, and the addition of mineral, vitamins, amino acids and other compounds turns the process more expensive and less viable (Lima Jr et al., 2010; Pandey et al., 2000a; Silva et al., 2007; Yang et al., 2001). Biological treatment helps to enhance nutritional parameters of the waste and are a valuable source of protein, vitamins, acids and minerals, raising digestibility and palatability and often providing probiotic effects on host tract (Apás et al., 2008; Lima Jr et al., 2010). The use of agro-industrial residues as feed would also help to solve the problem of its accumulation into the environment (Pinto et al., 2005).

The culture with *R. mucilaginosa* LABI1 obtained higher biomass concentration in comparison to other studies with different fungus and residues. The fungus *Penicillium janthinellum* obtained 1.30 g/L with SmF with cassava peel and 0.5 g/L with pretreated sugarcane bagasse (Oliveira et al., 2006). The yeast *Sporidiobolus pararoseus* achieved 5.1 g/L using glycerol, corn steep liquor and parboiled rice water (Valduga et al., 2014) and 8.27 g/L using sugarcane molasses and corn steep liquor (Machado and Burkert, 2014). Hydrolyzed rice straw produced 6.56 g/L of *Candida utilis* and 6.48 g/L of *S. cerevisiae* (Araújo and D'Souza, 1986) and eucalyptus hydrolisate achieved 6.35 g/L of *Paecilomyces variotii* (0.44 g/g yield,

0.10 h⁻¹ and 0.26 g/L.h productivity) (Silva et al., 1995). All studies achieved lower values compared to *R. mucilaginosa* LAB11 culture with hydrolyzed cassava residue, which demonstrated to be a more interesting substrate for SCP production with this species. In addition, costs with physical-chemical pretreatment and cellulases to the hydrolysis of lignocellulosic residues are higher than a simple amylase hydrolysis.

Silva et al. (2011) obtained biomass from *S. cerevisiae* and *Candida parapsilosis* with 0.70 g/L and 15 g/L respectively and productivities from 0.04 to 0.10 g/L.h when cultivated in vinasse and different concentrations of glucose, yeast extract and peptone. Results from the cultivation of *R. mucilaginosa* LAB11 are within this range but the use of yeast extract, malt extract, peptone and glucose increase the cost with culture medium due to the high cost of these compounds, turning more interesting the use of medium formulated without those compounds. The determination of the optimal conditions for *R. mucilaginosa* LAB11 production would lead to higher biomass concentrations (SCP). Increasing aeration rate is one possible way to increase biomass concentration (Nigam, 2000; Pessoa Jr et al., 1996; Pfeifer et al., 1996).

8.4.4 Nutritional parameters of yeasts biomass and residues after submerged culture in bioreactor

R. mucilaginosa LAB11 biomass obtained by the fed-batch culture using HCR (Table 30), presented less ashes (6.7%), ethereal extract (0.5%), crude fiber (1.4%) and protein (25.1%) than *S. cerevisiae* M26 biomass, with similar total digestible nutrients concentration (83%). In SB fed-batch culture *R. mucilaginosa* LAB11 was composed by more crude fiber (5.8%) and non-nitrogenous extract (69.4%). On the other hand, protein (28.1%) and ashes (7.1%) were higher for *S. cerevisiae* M26 culture.

In general, the same nutritional parameters of the both final residues (SB and HCR) improved after submerged fed-batch culture for both yeasts (Table 30) except SB ashes which decreased from 5.5% to 3.7% (average) and HCR non-nitrogenous extracts from 56.9% to 49-51%. The decrease of this last parameter was expected since carbohydrates from cassava residue were hydrolyzed and consumed by the yeasts.

Ethereal extract was similar for both residues after cultures (1.2%-1.7%) and crude fiber was similar (41-42%) in the final residue for both yeasts after SB culture, but superior for *R. mucilaginosa* culture in HCR (48.4%). This parameter decreased when compared to HCR before culture since starch was consumed and consequently the percentage of fiber increased. (Table 30).

Crude protein was two times higher in HCR (average of 6.6%) than SB (average of 3.3%) for both strains. Considering the protein in HCR (1.9%) and SB (1.6%) before cultures, an increase of 3.5 times and 2.14 times was verified. These relevant protein enrichments in the final residues after cultures were not taken account the protein of yeast biomass, since in the present process the culture is filtered. Residual HCR with all yeast biomass would reach up to 10.5% crude protein.

Sugarcane bagasse contains 1.81% ethereal extract and crude protein, 5.21% ashes, 6.3% moisture and 46.66% carbohydrates (Neumann et al., 2016). Those values are close to percentages found by the present work (0.7% ethereal extract, 5.5% of ashes, 8.7% moisture and 48.6% carbohydrates). Cassava residue composition range from 0.53% - 1.06% ethereal extract, 14.88% - 50.55% crude fiber, 0.32% - 1.61% crude protein, 0.66% - 1.5% ashes, 5.02% - 11.2% moisture and 40.50% - 63.84% carbohydrates (Pandey et al., 2000b). The present results obtained similar compositions for hydrolyzed cassava residue.

Sugarcane blunting, a sugar factory residue, was used as culture medium for inoculation of probiotic bacteria of ruminants flora mixed with lactic acid bacteria. The authors state this waste could be used as ruminant feed supplement after silo inoculation with bacteria, obtaining good values of dry matter (32.1%), carbohydrates (14.22%) and fiber (11.57%) and the growth of probiotic bacteria in the rumen. The percentage of crude protein of the residue increased from 1% to 1.4% and ash and lipids were 3.6% and 0.47%, respectively, after 60 days of fermentation (Apás et al., 2008). In the present work, after only 96 h of *R. mucilaginosa* LAB11 culture using HCR was obtained 5.1% of ashes, 1.3% of ethereal extract, 48.4% crude fiber and 6.7% of crude protein, values higher than results obtained by Apás et al. (2008). According to these results, hydrolyzed cassava residue could be assessed for animal feed after submerged cultivation with this yeast taken into account its nutritional parameters. Therefore, the yeast biomass would provide protein, carotenoids, amino acids, enzymes and other supplements in animal nutrition. In addition, if this strain would have probiotic activity, this yeast could contribute to promote a beneficial effect on animal health (Villas-Bôas et al., 2002).

The minimum percentage of crude protein required to use a waste in feed is 7% (Bravo et al., 1994; Lima Jr et al., 2010). Residual cassava with some yeasts cells reached 6.7% demonstrating an improvement of almost 354% of crude protein comparing to initial bagasse. This value may increase up to 10.5% protein by drying bagasse and yeast together in view of the high value of crude protein of the yeast biomass obtained with this culture in pretreated cassava residue (25.1%) (Table 30).

There are some studies demonstrating the improvement of nutritional parameters of substrates after inoculation with microorganisms. Submerged fermentation with *Pleurotus ostreatus* increased protein content of non-treated sugarcane bagasse to 22.6% after 14 days of incubation (El-Sayed et al., 1994). In the present work, crude protein of the final residue of sugarcane improved from 1.6% to 3.2% after only 4 days of *R. mucilaginosa* LABI1 culture.

Submerged mixed culture of *Trichoderma reesei* and *Rhizopus* sp. achieved 7.7% of crude protein of sugarcane (Monteiro et al., 1991). Those protein concentrations are higher than achieved by *R. mucilaginosa* LABI1 with sugarcane bagasse, but similar to hydrolyzed cassava protein, so the last residue is more suitable to serve as substrate. Vandenberghe et al. (2000), Soccol et al. (1995a) and Soccol et al. (1995b) improved protein concentration of cassava residue from 13.3% to 23.1% using *Aspergillus niger* and to 12.0% and 13.5% using *Rhizopus oryzae*, in SSF. These results are for solid state fermentation process and start the culture from 13.1% of protein in the residue, while in the present work the culture is submerged and the protein content on the beginning of the culture is 1.89%. Different conditions could explain the difference of results within the literature. In addition, *R. mucilaginosa* LABI1 increased sugarcane and hydrolyzed cassava protein content to 3.2% and 6.7% respectively, and these values would be enhanced if all yeast biomass were dried with the residues.

8.4.5 Chemical composition of yeasts biomass

According to *R. mucilaginosa* LABI1 and *S. cerevisiae* chemical composition the most important components in yeast biomass are carbon, nitrogen, phosphorus and potassium (Table 32). Although the concentration of carbon, potassium and calcium from *R. mucilaginosa* LABI1 cells are smaller compared to *S. cerevisiae* cells, these are not substantial differences. As *S. cerevisiae* is widely used in feed and food, *R. mucilaginosa* LABI1 demonstrates the possibility of its use for the same purpose when considering the cellular composition of both.

Table 32 - Chemical composition of *Rhodotorula mucilaginosa* LABI1 and *Saccharomyces cerevisiae* biomass after submerged culture in orbital shaker incubator.

Parameter	<i>R. mucilaginosa</i> (g/kg)	<i>S. cerevisiae</i> (g/kg)*
Carbon	441.0±4.7	460.0±10.0
Nitrogen	78.1±2.3	82.5±7.5
Phosphorus	14.7±1.0	15.2±0.2
Potassium	15.6±0.6	20.4±0.005
Calcium	0.1±0.01	1.5±0.01
Magnesium	1.6±0.003	1.4±0.003
Sulfur	4.0±0.04	4.0±1.0
Copper	0.05±0.002	0.05±0.0003
Manganese	0.06±0.001	0.01±0.0001
Zinc	0.15±0.001	0.13±0.001

*Belluco (2001)

The presence of potassium, magnesium, phosphorus and sulfur in higher amount in *R. mucilaginosa* LABI1 biomass is important since unlike other nutrients, minerals cannot be synthesized by living organisms and are involved in many aspects of cellular metabolism, so they are closely related to cellular functions (Cooper and Hausman, 2003). Macronutrients are represented by phosphorus (P) in the form of phosphates important in energy metabolism and synthesis of nucleic acids. Sulphur (S) is needed to be part of amino acids such as cystine and cysteine and in the synthesis of vitamins such as biotin and thiamin. Potassium (K) acting as cofactor, an enzyme activator and as regulator of osmotic pressure. Magnesium (Mg), also a cofactor, activating extracellular enzyme. Iron (Fe) required for the synthesis of cytochromes and certain pigments (Alterthum, 2001). Another advantage on the use of *R. mucilaginosa* LABI1 is the versatility to use agro-industrial residues as carbon source and achieve high biomass concentration.

8.4.6 Different carbohydrates assimilation

High assimilation of glucose, followed by fructose, sucrose, galactose, xylose, maltose and arabinose by *R. mucilaginosa* LABI1 was observed (Table 33). The highest μ_{\max} was obtained with xylose (0.40 h^{-1}), but the μ_{\max} for xylan (0.18 h^{-1}) was higher than some monosaccharides (sucrose and galactose). Biomass yielded from 1.6 g/L to 2.1 g/L with medium formulated with glucose, fructose, sucrose, galactose, xylose and arabinose. These carbohydrates also achieved the highest cell concentrations, above 1.5×10^8 cells/mL.

Table 33 – Kinect parameters of *Rhodotorula mucilaginosa* LAB11 using different carbohydrates cultivated in orbital shaker incubator (28 °C at 180 rpm for 96 h).

Carbohydrate	Maximum concentration (cell/mL)	μ_{\max} (h ⁻¹)	Biomass concentration (g/L)	Biomass yield (g/g)	Biomass productivity (g/L.h)
Arabinose	1.5E+08	0.21	1.7 ^{a*}	0.89*	0.02**
CMC	4.7E+07	0.16	0.9 ^{b*}	0	0
Fructose	2.1E+08	0.26	2.0 ^{a*}	1.10*	0.02**
Galactose	1.9E+08	0.10	1.8 ^{a*}	0.95*	0.02**
Glucose	2.9E+08	0.25	1.9 ^{a*}	1.03*	0.02**
Lactose	9.3E+07	0.03	0.8 ^{b*}	0	0
Maltose	1.5E+08	0.12	1.9 ^{a*}	0.99*	0.02**
Starch	7.7E+07	0.09	0.9 ^{b*}	2.25*	0.01**
Sucrose	2.0E+08	0.14	2.1 ^{a*}	1.02*	0.02**
Xylan	5.4E+07	0.18	0.9 ^{b*}	2.56*	0.01**
Xylose	1.7E+08	0.40	1.6 ^{a*}	0.78*	0.02**
Control	3.0E+07	0.16	0.8 ^{b*}	-	-

*Standard deviation <0.06; ** = <0.003

a, b, c, d = Different letters between the same parameter correspond to statistically different averages (P<0.05)

Although lactose maximum cell concentration was three times higher than control flasks, total reducing sugars (TRS) concentration was not different from the beginning of the culture, which indicates that the yeast do not have the apparatus to assimilate such carbohydrate. The carboxymethylcellulose (CMC) yield coefficient was also zero with a poor cell growth with no assimilation by the yeast. Although starch and xylan formulated medium achieved a biomass yield slightly higher than control flasks, TRS diminish at the end of cultivation. Therefore, the specie was capable to use a small amount of these carbohydrates to cell maintenance and growth. Almost all carbohydrates achieved productivities of 0.02 g/L.h except starch and xylan, with 0.01 g/L.h. Log phases started around 4 to 12 h, and ended between 28 to 72 h (Figure 22).

These results showed to be in accordance to Sampaio (2011a) who states this yeast is capable to grow in culture medium containing glucose, sucrose, raffinose, galactose, trehalose, maltose, melezitose, xylose, arabinose or ribose, and absence of growth with lactose and starch. The last carbohydrates was an exception since a moderate growth was observed in the present work. These differences are due to the fact that different strains of the same species may have different behavior even under the same conditions (Hainal et al., 2012). Libkind et al. (2004) also found the grown capacity using xylose, arabinose, glucose, sucrose and maltose. *S.*

cerevisiae, differently from *R. mucilaginosa*, does not assimilate xylose and arabinose (Vaughan-Martini and Martini, 2011; Arora et al., 2015).

The high affinity for xylose confirms the yeast's ability to grow using sugarcane bagasse as substrate (Tables 29 and 30), xylanases production (Table 31) and the use of xylan fraction of bagasse.

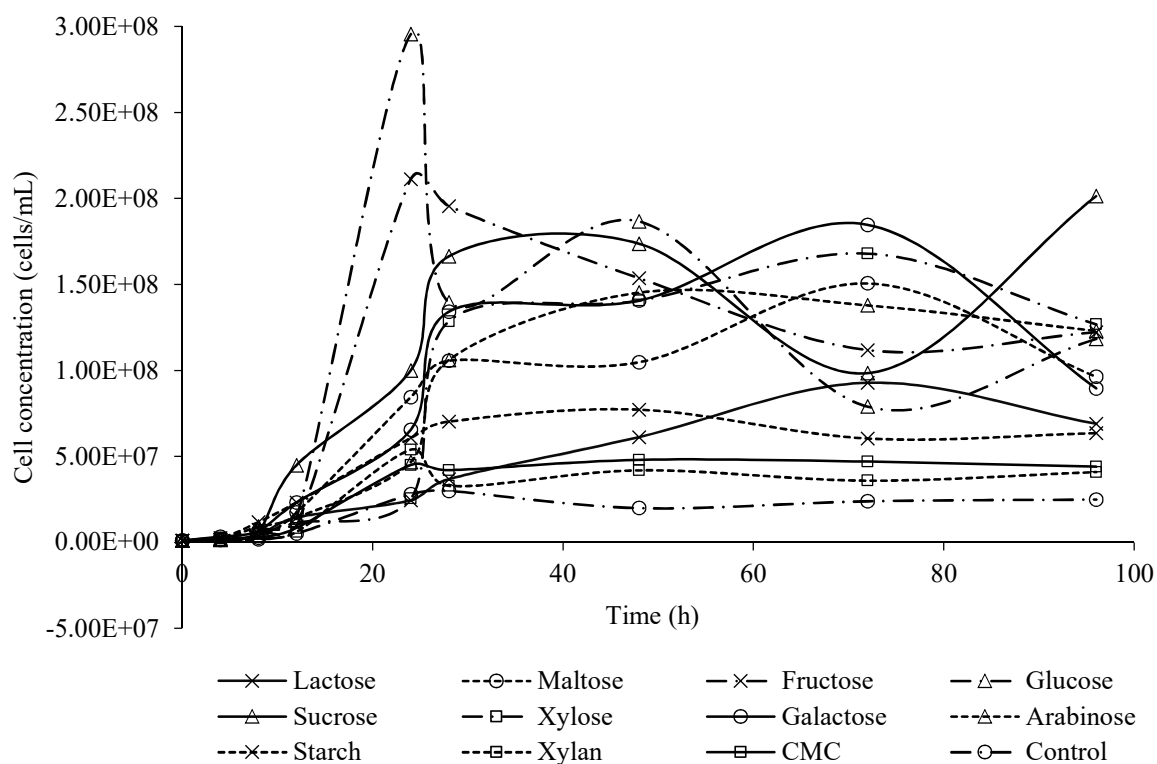


Figure 22 – Growth curves of *Rhodotorula mucilaginosa* LABI1 with different carbohydrates cultivated in orbital shaker incubator at 28 °C and 180 rpm.

8.4.7 Amino acids and vitamins single-omission test

The amino acids and vitamins single-omission tests showed that *R. mucilaginosa* LABI1 does not depend on external amino acids sources and is able to produce them by its own (negative control without any amino acids had equal growth as positive control) (Table 34). Thus, the yeast does not require a complex medium for growth, cheapening the formulation of a medium for production of biomass using agro-industrial residues.

Thiamin seems to be the only vitamin that affected the yeast growth. A minor growth in the tubes without thiamin could be observed, which demonstrates that *R. mucilaginosa* LABI1 is dependent on the addition of such vitamin for regular growth or this vitamin is a growth promoter for this yeast. Under anaerobic conditions, the yeast also showed absence of growth

in medium lacking thiamin (Tabak and Cooke, 1968). Thiamin, also known as vitamin B₁, is produced in small quantities by certain species of microorganisms. The production of this vitamin can be affected depending on the used carbon source (Sierra et al., 1999) and pH (Strzelczyk et al., 1991). Certain microorganisms such as *Rhizobium* are dependent on or stimulated by the presence of certain vitamins, particularly thiamin, biotin and pantothenic acid (Elkan and Bunn, 1992; Sierra et al., 1999).

Studying essential nutrients for growth are valuable due to the simple addition of the essential elements and omission of the non-essentials lead to the microorganism to grow more efficiently. In addition, such studies lower cost of the culture medium by removing elements that are not essential.

Table 34 - Amino acids and vitamins single-omission tests of *Rhodotorula mucilaginosa* LABI1 in tubes culture after 120 h of incubation at 28 °C and aerobiosis.

Amino acid		Vitamin	
Nutrient	Growth	Nutrient	Growth
Trp	+++	Thiamin	+
Leu	+++	Riboflavin	+++
Pro	+++	Folic Acid	+++
Val	+++	Ascorbic Acid	+++
Tyr	+++	Pyridoxine	+++
Ala	+++	Panthotenic Acid	+++
Ser	+++	Nicotinic Acid	+++
Ile	+++	Aminobenzoic Acid	+++
Cys	+++		
Gln	+++		
Thr	+++		
Lys	+++		
Asp	+++		
Phe	+++		
Glu	+++		
His	+++		
Arg	+++		
Met	+++		
Gly	+++		
Asn	+++		

+ = Absence; ++ = Partial; +++ = Full
P<0.05

The feed offered to ruminants must have at least 7% crude protein or 1% of total nitrogen due to the minimum total protein supply to the small intestine. In addition, the low level of sulfur in the crude bagasse is incompatible with the production of amino acids by rumen

microorganisms (Lima Jr et al., 2010). The use of this yeast as a nutritional enrichment treatment of the wastes like sugarcane bagasse and cassava residue shows an alternative for both barriers, in view of the high protein content of the yeast cells and the sulfur provided by the yeast biomass. The mineral uptake by the yeasts is important for the production of essential amino acids, which therefore serve to the ruminant. According to this research, *R. mucilaginosa* LABI1 is able to synthesize its amino acids without relying on the addition of them in the culture medium.

Nitrogen and ashes high content turns yeasts more suitable than fungi, algae and bacteria for the use as food and feed (Nigam, 2000). Capacity to grow in temperatures up to 40 °C and assimilation of arabinose makes the bioprocess cheaper for biomass production (Nigam, 2000). Although only some strains *R. mucilaginosa* is capable to grow in temperature above 37 °C (Sampaio, 2011a), this species is capable to assimilate arabinose being an advantage stated by Nigam (2000).

Among the positive effects of probiotics, there is stimulation of microflora in the rumen, increase of the growth of cellulolytic bacteria, which also increases the rate of degradation and digestibility of fiber, and the higher total protein contained in the yeast cell and control of ruminal pH (Newbold et al., 1996). The production of fibrolytic enzymes is important to probiotic microorganisms since they would increase food digestibility (Laconi and Pompei, 2007). As results of this research, the production of xylan-degrading enzymes by *R. mucilaginosa* LABI1 brings a beneficial effect in the animal's rumen, helping to break down hemicellulose and increasing substrates digestibility. In addition, cellulolytic activity tests (CMCase) performed by *R. mucilaginosa* LABI1 showed the presence of endoglucanases which are responsible for breaking cellulose fraction of sugarcane bagasse contributing to a possible use of this species as DFM. Arcuri et al. (2014) and Li et al. (2014) also detected the production of cellulases by this species and the benefits to the degradation of waste fibers.

Carotenoids production by this species is well known in the literature (Aksu and Eren, 2005; Cheng and Yang, 2016; Hainal et al., 2012; Libkind et al., 2004; Maldonade et al., 2012; Yoo et al., 2016). The production of such compounds increases the added value of the waste. β -carotene, tolurene and torularhodin are the main pigments produced by *R. mucilaginosa* having antioxidant and pro-vitamin effects. They are known to prevent certain cancers, and enhance immune system (Silva et al., 2011; Yoo et al., 2016) being desired effects to use as additives in animal nutrition. The high cost of carotenoid production is an industrial limiting factor and the use of waste and process optimization decreases this value as well as the use of

residual yeast biomass as protein source in animal feed with possible probiotic effects (Maldonado et al., 2012).

Due to the anaerobic condition of gastric environment of animals, another desirable characteristic for a microorganism to be used as DFM is the ability to grow in an environment in absence of oxygen. Results obtained by this study through cultivation in anaerobic jar demonstrated that *R. mucilaginosa* LAB11 was capable to grow aerobic, in low oxygen concentration and anaerobic environment proving to be a facultative microorganism (Alterthum, 2001; Brad et al., 2008; Ollivier et al., 2011; Villegas et al., 2009).

The main yeast species used as probiotics are *Saccharomyces boulardii* and *Saccharomyces cerevisiae*. There are cases of fungemia recorded by the use of *S. boulardii* and cases of bacteremia by the use of probiotic bacteria, but cases are related to immunologically impaired individuals (Martins et al., 2005). Therefore, the opportunism observed in immunosuppressed individuals by *R. mucilaginosa* (Sampaio, 2011a) would not be a limiting factor for its use as DFM nor in the enrichment of waste for use as animal feed since the ruminant animal metabolism is different from human.

Inhibition of other harmful microorganisms to the gastrointestinal tract is also an interesting factor when considering microorganisms with probiotic properties (Martins et al., 2005). Libkind et al. (2004) demonstrated the killer effect of the strain CRUB 0138 in *Rhodospiridium kratochvilovae* CRUB 0121 cells and *R. mucilaginosa* CRUB 0137 cells. This effect is due to the production of a glycoprotein or protein compound by some yeast species that inhibits the growth of sensitive cells from other microorganisms (Golubev, 2011) and shows evidence of an antagonism against other microbial cells.

In addition, pigments produced by *R. mucilaginosa* were able to inhibit some species of Antibiotics Resistant Bacterium (ARB) (Yoo et al., 2016). The use of Antibiotics as Growth Promoters (AGPs) in animal feed in order to control pathogenic bacteria that can be passed along the food chain is an abolished practice since January 2006 in the European Union in view of the emergence of resistant microorganisms (Gaggia et al., 2010; Mathur and Singh, 2005). In this sense, the antagonist activity of *R. mucilaginosa* (Libkind et al., 2004; Yoo et al., 2016) could be an alternative to control pathogenic microorganisms and increase efficiency and output of animals.

The present work showed the potential of *R. mucilaginosa* LAB11 as an economic source of SCP production from agro-industrial residues due to its more favorable physiological characteristics compared to *S. cerevisiae*. Other important probiotic characteristics that this strain showed are the advantage in xylanases, cellulases and amylases production, ability to

grow in anaerobic conditions and assimilation of xylose. However, future studies with this fermented residue in the animal nutrition and probiotic ability are necessary to prove the nutritional and healthy advantages of this biomass.

8.5 Conclusion

The culture of *Rhodotorula mucilaginosa* LAB11 with an economic material like sugarcane bagasse and cassava residue enriched this biomass content of lipids, protein and total digestible nutrients in aerobic submerged bioprocess in a short time. Single cell protein production from agro-industrial residues was possible due to favorable physiological characteristics of this yeast comparing with *Saccharomyces cerevisiae* M26. Xylanases, cellulases and amylases production, ability to grow in anaerobic conditions, assimilation of xylose and several other carbohydrates and the growth in a culture medium with agro-industrial residues as carbon source partially indicate the potential of this species as Direct-Fed Microbial (DFM). However, additional study with this fermented residue in the animal nutrition and DFM ability are necessary to prove the health advantages of this biomass for animal production.

9 CONSIDERAÇÕES GERAIS

O crescimento das leveduras em biorreator utilizando bagaço de cana em batelada simples e condições aeróbicas produziu 1,9 g/L (*R. mucilaginosa* LABI1), 1,6 g/L (*S. pararoseus* Sia 33.1), 1,4 g/L (*S. japonicus* Sia 70a), 0,9 g/L (*W. onychis* LABI2) e 0,4 g/L (*S. cerevisiae* M26) de levedura, com 20,5%, 39,2%, 26,2%, 19,1% e 28,1% de proteína, respectivamente (Tabela 35). O alto teor proteico, em especial de *S. pararoseus* Sia 33.1, demonstra a possibilidade do uso dessas linhagens pouco exploradas como fonte de proteína unicelular produzidas com bagaço de cana, em alternativa à espécie *S. cerevisiae*, amplamente utilizada com esta finalidade (ANUPAMA e RAVINDRA, 2000). Outro fator importante é o aumento do teor proteico do bagaço, de 1,6% para 4,7%, 4,6%, 4,0%, 3,1% após fermentação submersa com *S. japonicus* Sia 70a, *S. pararoseus* Sia 33.1, *W. onychis* LABI2 e *R. mucilaginosa* LABI1, respectivamente. Vale ressaltar que ao considerarmos a proteína total final, correspondente à proteína unicelular e à proteína do bagaço residual, a biomassa final alcançaria valores de 7,3% (*S. pararoseus* Sia 33.1), 6,2% (*S. japonicus* Sia 70a) e 4,7% (*R. mucilaginosa* LABI1 e *W. onychis* LABI2) de proteína.

Ao utilizar o modo de fermentação em batelada alimentada com bagaço de cana e *R. mucilaginosa* LABI1, a biomassa de levedura obtida foi de 4,0 g/L. Entretanto, ao considerarmos uma biomassa final residual composta de levedura e bagaço residual, o teor proteico desta seria de 3,7%, 1,0% menos que a fermentação em batelada. Tal fato deve-se principalmente a uma maior quantidade de bagaço utilizado para o cultivo alimentado com menor teor de proteína.

Tabela 35 – Comparação entre as leveduras em biorreator de bancada em fermentação submersa (28 °C, pH 5,5) e condições aeróbias (1vvm) quanto a biomassa de levedura obtida e teor proteico de levedura e resíduo remanescente.

	<i>R. mucilaginosa</i>			<i>W. onychis</i>			<i>S. pararoseus</i>		<i>S. japonicus</i>		<i>S. cerevisiae</i>			
	LABI1			LABI2			Sia 33.1		Sia 70a		M26			
	BC1	BC2	RMH2	BC1	RMH1	RMH2	BC1	RMH2	BC1	RM1	BC1	BC2	RM1	RMH2
Tempo de cultivo (h)	72	120	96	72	96	168	72	96	72	96	72	120	96	168
Xilanase máx (U/mL)	0,29 ^a	0,31 ^a	-	0,34 ^a	-	-	0,25 ^b	-	0,52 ^c	-	0,14 ^d	0,10 ^d	-	-
CMCase máx (U/mL)	-	0,43 ^a	-	-	-	-	-	-	-	-	-	0,18 ^b	-	-
Amilase máx (U/mL)	-	-	0,33 ^a	-	0	0	-	0,26 ^b	-	0,05 ^c	-	-	0	0
Biomassa de levedura (g/L)	1,9 ^a	4,0 ^b	8,5 ^c	0,9 ^d	5,7 ^c	10,9 ^f	1,6 ^a	5,2 ^c	1,4 ^a	8,1 ^c	0,4 ^g	2,2 ^h	4,6 ⁱ	3,1 ^b
Proteína da levedura (%)	20,5 ^a	18,3 ^a	25,1 ^b	19,1 ^a	40,7 ^c	20,3 ^a	39,2 ^c	43,3 ^c	26,2 ^b	35,6 ^c	28,2 ^b	28,2 ^b	41,9 ^c	33,8 ^c
Proteína do resíduo remanescente (%)	3,1 ^a	3,2 ^a	6,7 ^b	4,0 ^a	7,5 ^b	5,6 ^c	5,6 ^c	8,3 ^d	4,7 ^c	6,7 ^b	3,7 ^a	3,5 ^a	7,0 ^b	6,6 ^c
Proteína total (levedura + resíduo) (%)	4,7	3,7	10,5	4,6	15,0	8,0	7,3	11,6	6,2	11,8	4,3	4,2	11,3	8,3

a, b, c, d, e, f, g, h, i: Letras diferentes para o mesmo parâmetro representam médias estatisticamente diferentes (P<0,05)

BC1 = Bagaço de cana e batelada simples

BC2 = Bagaço de cana e batelada alimentada

RM1 = Resíduo de mandioca sem hidrolisar e batelada alimentada

RMH1 = Resíduo de mandioca hidrolisado e batelada simples

RMH2 = Resíduo de mandioca hidrolisado e batelada alimentada

Enquanto a fermentação em batelada possui uma concentração limitada de nutrientes, a batelada alimentada caracteriza-se por alimentações com nutrientes ao longo da fermentação, tornando o volume de meio variável durante o cultivo. A batelada alimentada possibilita um crescimento linear máximo das células ao longo da fermentação, levando a uma densidade celular maior uma vez que os nutrientes não serão um fator limitante do crescimento (CARVALHO e SATO, 2001; SHULER e KARGI, 2002), fato que pôde ser observado neste estudo.

Da mesma maneira que ocorreu com o bagaço de cana, pode ser observada uma maior quantidade de biomassa de *W. onychis* LABI2 obtida após o modo de fermentação de batelada simples para batelada alimentada com resíduo de mandioca hidrolisado. Enquanto o cultivo em batelada única produziu-se 5,7 g/L, a batelada alimentada levou ao dobro de biomassa (10,9 g/L). Vale ressaltar que apesar de uma maior quantidade biomassa de levedura produzida, o teor proteico da mesma foi de 40,7% e 20,3% para as fermentações em batelada e batelada alimentada, respectivamente. De modo similar, a batelada simples alcançou um maior incremento do teor proteico do resíduo final, sendo de 7,5% contra 5,6% para o bioprocessamento em batelada alimentada. Considerando a biomassa residual composta de levedura e resíduo, o teor proteico seria de aproximadamente 15,0% e 8,0% para os processos em batelada e batelada alimentada, respectivamente.

A fermentação em batelada alimentada utilizando o bagaço de mandioca hidrolisado levou à produção de 8,5 g/L de *R. mucilaginosa* LABI1, com 25,1% de proteína, e 5,2 g/L de *S. pararoseus* Sia 33.1 que possui 43,3% de teor proteico. Assim como o bagaço de cana, o resíduo de mandioca hidrolisado levou a uma maior produção das espécies estudadas que da levedura *S. cerevisiae* M26 (3,1 g/L com 33,3% de teor proteico) demonstrando uma maior afinidade com os resíduos agroindustriais pelas leveduras alternativas considerando a produção de proteína unicelular. O valor proteico do resíduo foi incrementado para 6,7% e 8,3%, e seria de aproximadamente 10,5% e 11,6% considerando o teor proteico da biomassa de levedura de *R. mucilaginosa* LABI1 e *S. pararoseus* Sia 33.1 e do resíduo remanescente, respectivamente.

Ao utilizar o bagaço de mandioca sem tratamento enzimático e em batelada alimentada, *S. japonicus* Sia 70a produziu 8,1 g/L de levedura com 35,6% de proteína e um resíduo remanescente com 6,7% de proteína. Ao considerarmos o teor proteico de biomassa de levedura e de resíduo, a proteína da biomassa residual seria de aproximadamente 11,8%. Comparando com *S. cerevisiae* M26, a qual produziu 4,6 g/L de levedura com 41,9% de proteína (em torno de 11,3% de proteína considerando toda biomassa residual), *S. japonicus* Sia 70a também

demonstrou ser mais apta para produção de proteína unicelular a partir de resíduos agroindustriais.

Alguns estudos demonstram a possibilidade do uso de diversos resíduos e microorganismos para atingir essa meta, como os hidrolisados de palha de arroz (6,56 g/L de *Candida utilis* e 6,58 g/L de *S. cerevisiae*) (ARAÚJO e D'SOUZA, 1986) e de eucalipto (6,35 g/L de *Paecilomyces variotii*) (SILVA et al., 1995). Valduga et al. (2014) obtiveram 5,1 g/L de *Sporidiobolus pararoseus* e Machado e Burkert (2014) alcançaram 7,18 g/L da mesma levedura, em combinações de glicerol, água de maceração de milho, água de parboilização de arroz e melão de cana. Os valores da biomassa unicelular obtida pelos bioprocessos utilizando bagaço de mandioca hidrolisada (10,9 g/L de *W. onychis* LABI2 e 8,5 g/L de *R. mucilaginosa* LABI1) ou não (8,1 g/L de *S. japonicus* Sia 70a) foram superiores comparados a esta literatura.

Segundo Lima Jr et al. (2010), rações ofertadas aos ruminantes devem possuir pelo menos 7% de proteína bruta ou 1% de nitrogênio total. Isso pois uma concentração baixa desestimula o crescimento microbiano, o que diminui o aporte de proteínas para o intestino delgado. Um enriquecimento com proteína microbiana através da fermentação pode torná-lo mais nutritivo com uma maior porcentagem proteica, e sem a necessidade da adição desses compostos (BRAVO et al., 1994; BEZERRA e RAGAUSKAS, 2016), fato que pode ser obtido após o cultivo submerso com *W. onychis* LABI2 (8,0-15,0%), *R. mucilaginosa* LABI1 (10,5%), *S. pararoseus* Sia 33.1 (11,6%) e *S. japonicus* Sia 70a (11,8%) utilizando bagaço de mandioca hidrolisado ou não, considerando toda biomassa residual (levedura + bagaço) obtida. Ao utilizar o bagaço de cana, somente o cultivo com *S. pararoseus* Sia 33.1 alcançaria tal teor (7,3%), considerando toda biomassa residual.

Além de serem uma atraente fonte de proteína, as leveduras fornecem vitaminas, aminoácidos e minerais, melhorando mais qualidade nutricional dos resíduos agroindustriais que podem ser utilizados como ração após uma fermentação submersa, cortando gastos com adição desses compostos na ração (PELCZAR Jr et al., 1996; KUHAD et al., 1997b; BRUM et al., 1999; LIMA JR et al., 2010; BEZERRA e RAGAUSKAS, 2016). As células de *Sporidiobolus pararoseus* Sia 33.1, *Sporobolomyces japonicus* Sia 70a, *Wickerhamomyces onychis* LABI2 e *Rhodotorula mucilaginosa* LABI1 produzem seus próprios aminoácidos (exceto Arginina e Histidina por *W. onychis* LABI2), e possuem composição mineral pouco diferente de *S. cerevisiae*, a principal espécie utilizada como aditivo na alimentação animal (FRANÇA e RIGO, 2011), com altas concentrações de carbono, nitrogênio, potássio e fósforo. É interessante observar que *Sporidiobolus pararoseus* já foi considerada do gênero *Sporobolomyces* (COBBAN et al., 2016), e o teste de crescimento em omissão pontual de

vitaminas puras demonstrou grande similaridade entre *Sporidiobolus pararoseus* Sia 33.1 e *Sporobolomyces japonicus* Sia 70a: ambas não cresceram em ausência das vitaminas ácido ascórbico, piridoxina, ácido fólico e ácido pantotênico, e cresceram em ausência de tiamina e ácido aminobenzoico, diferenciando somente no crescimento em riboflavina (ausente para *S. pararoseus*) e ácido nicotínico (ausente para *S. japonicus*). Ambas leveduras também cresceram em ausência total de vitaminas, embora na presença de determinadas vitaminas não. Desse modo, estudos acerca das rotas metabólicas dessas espécies devem ser realizados a fim de se determinar se elas desviam o metabolismo conforme presença ou ausência de certas vitaminas.

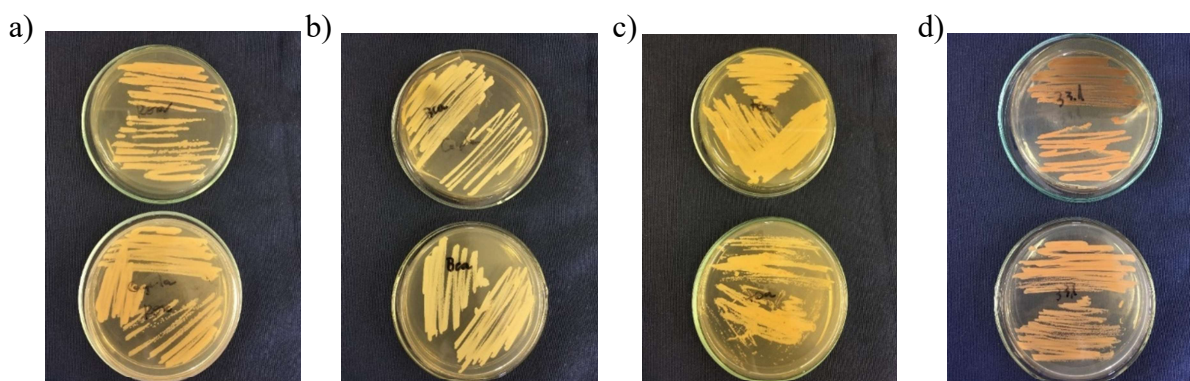
De acordo com as assimilações de alguns carboidratos, as quatro leveduras teste assimilaram frutose, galactose, glicose, maltose, sacarose e xilose. Somente *S. pararoseus* Sia 33.1 não assimilou arabinose, e nenhuma assimilou lactose. A alta afinidade com maltose e xilose pode ser uma das razões pelas maiores concentrações celulares atingidas ao utilizar bagaço de mandioca ou bagaço de cana como principal fonte de carbono. Segundo Nigam (2000) a assimilação de arabinose é uma vantagem ao considerar o crescimento microbiano em hidrolisados de materiais lignocelulósicos como o bagaço de cana. A fração hemicelulósica desses resíduos é constituída principalmente por glicose (17,5% a 50,5%), galactose (11,5% a 39,9%), xilose (15,5% a 28,9%) e arabinose (5,35% a 14,31%) (PENG et al., 2010), sendo estes açúcares disponíveis para crescimento e manutenção celular fornecidos por essa fração. Assim, a vantagem de algumas espécies está na capacidade de assimilar outros açúcares além da glicose e galactose que ficam disponíveis após hidrólise da hemicelulose.

As espécies estudadas destacaram-se nos bioprocessos para produção de proteína unicelular e enriquecimento proteico de resíduos: *W. onychis* LABI2 e *R. mucilaginoso* LABI1 pela biomassa de levedura produzida em batelada alimentada com bagaço de mandioca hidrolisado (10,9 g/L e 8,5 g/L respectivamente); *S. pararoseus* Sia 33.1 pelo alto teor proteico da levedura (43,3%) e do resíduo remanescente (8,31%), incrementado após fermentação submersa com o bagaço de mandioca hidrolisado em batelada alimentada; *S. japonicus* Sia 70a pela biomassa proteica de levedura obtida sem a necessidade de um pré-tratamento enzimático do bagaço de mandioca (8,1 g/L com 35,6% de proteína), sendo todos superiores à *S. cerevisiae* M26, principal espécie utilizada como proteína unicelular e probiótico animal (ANUPAMA e RAVINDRA, 2000; FRANÇA e RIGO, 2011). A presença de *W. onychis* como parte da comunidade biológica de alimentos amiláceos de humanos e animais (HAHN, 1988; OH e HAN, 2003), torna a espécie mais interessante, *a priori*, considerando questões que concernem a biosegurança da utilização de novas espécies como alimentos.

Vale ressaltar que a produção de proteína unicelular a partir de resíduos é uma alternativa para o aumento da demanda por alimentos sem a necessidade de grandes áreas para sua produção, e uma solução para o aproveitamento dos resíduos agroindustriais que são alocados no meio ambiente, muitas vezes causando sérios problemas ambientais pelo seu acúmulo e produção de compostos tóxicos (ANUPAMA e RAVINDRA, 2000), em especial o bagaço de mandioca.

Dentre as características fisiológicas avaliadas, as espécies *Sporidiobolus pararoseus* Sia 33.1, *Sporobolomyces japonicus* Sia 70a, *Wickerhamomyces onychis* LABI2 e *Rhodotorula mucilaginosa* LABI1 demonstraram capacidade em crescer em anaerobiose (Figura 23). Esta é uma das características ao considerar novas espécies potencialmente probióticas pelo ambiente ausente de oxigênio que é o trato gastrointestinal. Dessa maneira, outra alternativa ao uso dessas espécies além de proteína unicelular e enriquecimento do teor proteico dos resíduos, que poderiam ser utilizados como rações, as espécies listadas podem se tornar organismos probióticos ao se comprovar o efeito benéfico no hospedeiro que as ingerir.

Figura 23 – Placas inoculadas com: a) *Rhodotorula mucilaginosa* LABI1; b) *Wickerhamomyces onychis* LABI2; c) *Sporobolomyces japonicus* Sia 70a; e d) *Sporidiobolus pararoseus* Sia 33.1, em aerobiose (placas superiores) – controle –, e em anaerobiose (placas inferiores) a 33 °C por 72 h.



Uma dessas características probióticas é a produção de enzimas hidrolíticas que ajudam na quebra e absorção dos nutrientes e que muitas vezes são adicionadas na preparação de alimentos (VILLAS-BÔAS et al., 2002). As leveduras *Sporidiobolus pararoseus* Sia 33.1, *Sporobolomyces japonicus* Sia 70a, *Wickerhamomyces onychis* LABI2 e *Rhodotorula mucilaginosa* LABI1 produziram xilanases (0,52 U/mL *S. japonicus* Sia 70a) e celulases (0,89 U/mL *S. pararoseus* Sia 33.1), e, com exceção da *W. onychis* LABI2, amilases (0,33 U/mL *R. mucilaginosa* LABI1), enzimas hidrolíticas consideradas benéficas para o trato gastrointestinal

dos animais, sendo uma vantagem na escolha de novos micro-organismos com potencial probiótico. Outros estudos na literatura apontam a produção de outras enzimas por essas espécies (LUH e PHAFF, 1951; LEE et al., 1987; BAFFI et al., 2010; BUSSAMARA et al., 2010; KHANH et al., 2012; SMANIOTTO et al., 2012; TRINDADE et al., 2012; MENDONÇA et al., 2013; QIAO et al., 2013; YANG et al., 2013; ARCURI et al., 2014; LI et al., 2014; LARIO et al., 2015).

10 PERSPECTIVAS FUTURAS

Os testes químico-bromatológicos como proteína bruta, extrato etéreo, cinzas, fibra bruta, são o primeiro passo para determinar o valor nutritivos de rações (SALMAN et al., 2010). Outros testes *in vitro* e *in vivo* devem ser realizados a fim de determinar os possíveis efeitos do uso de novas espécies e biomassa como fonte de proteína unicelular, probióticos ou rações. Após um tratamento adequado para remover quaisquer compostos tóxicos como metais pesados, toxinas e altas concentrações de ácidos nucleicos que podem ter sido produzidos através da fermentação submersa, até a definição de uma possível purificação da biomassa residual, um dos pontos a ser avaliado é o potencial mutagênico e genotóxico da biomassa (ANUPAMA e RAVINDRA, 2000). Além disso, a biomassa, principalmente proteína unicelular, deve ser caracterizada em termos de porcentagem de aminoácidos, ácidos nucleicos, lipídios, gorduras, toxinas e vitaminas (ANUPAMA e RAVINDRA, 2000).

Além da qualidade do produto e segurança para uso, um dos objetivos dos aditivos é contribuir com a produtividade animal com redução do risco de contaminação do consumidor e reduzir excreção de poluentes, como o metano (FRANÇA e RIGO, 2011). Dessa maneira, ensaios *in vitro*, *in vivo* e/ou *in situ* quantificando a produção de gases e digestibilidade geralmente são realizados a fim de avaliar rações animais (SALMAN et al., 2010).

A capacidade probiótica é avaliada de acordo com fatores como: competição de micro-organismos maléficos ao trato gastrointestinal, fornecimento de compostos essenciais ao hospedeiro, capacidade de tolerar o ambiente gástrico, adesão na mucosa intestinal e estímulo ao crescimento de espécies benéficas ao trato. Tais testes compreendem a capacidade de sobreviver no ambiente hostil que é o trato gastrointestinal dos animais, como altas temperaturas e presença de enzimas e suco gástrico, além da manutenção da flora benéfica do animal (NEWBOLD et al., 1996; VILLAS-BÔAS et al., 2002; KUMURA et al., 2004; MARTINS et al., 2005; APÁS et al., 2008). Dessa maneira, uma fermentação submersa anaeróbia simulando o ambiente do trato deve ser realizada a fim de se confirmar o potencial probiótico das espécies.

11 CONCLUSÕES

O *screening* por novas espécies de leveduras para produção de proteína unicelular permitiu a seleção das espécies *Sporidiobolus pararoseus* Sia 33.1, *Sporobolomyces japonicus* Sia 70a, *Wickerhamomyces onychis* LABI2 e *Rhodotorula mucilaginosa* LABI1 com base no crescimento utilizando bagaço de cana em cultivo submerso em batelada em aerobiose. O pré-tratamento do bagaço de cana com CaO, H₂SO₄ e NaOH não foi benéfico para o crescimento celular.

Embora as quatro leveduras tenham atingido concentrações maiores que *Saccharomyces cerevisiae* M26 em cultivo em batelada em fermentador, as melhores concentrações de biomassa foram obtidas para o processo em batelada alimentada em fermentador ao utilizar bagaço de mandioca hidrolisado (para *S. pararoseus* Sia 33.1, *W. onychis* LABI2 e *R. mucilaginosa* LABI1) ou sem tratamento (para *S. japonicus* Sia 70a), também superando as concentrações obtidas de *S. cerevisiae* M26.

As quatro espécies produziram xilanases e celulases, e, com exceção da *W. onychis* LABI2, amilases, durante os cultivos aeróbicos utilizando os resíduos bagaço de cana ou de mandioca. Os parâmetros nutricionais de ambos resíduos foram incrementados após cultivo em fermentador, com destaque para a concentração de proteína, que aumentou consideravelmente após cultivo submerso com as leveduras.

A produção de proteína unicelular pelas espécies selecionadas foi possível por meio de cultivo submerso aeróbio em fermentador em batelada alimentada utilizando bagaço de mandioca e água de maceração de milho. Além da possibilidade de uso do resíduo final como ração animal sem necessidade da adição de outras proteínas, aminoácidos, minerais, vitaminas e enzimas, que encarecem o produto, a biomassa microbiana obtida possui concentrações aptas para uso como proteína unicelular quando comparadas à levedura modelo *S. cerevisiae* M26.

Algumas características desejáveis à micro-organismos probióticos tornam as quatro leveduras especiais, e o bioprocesso de sua produção utilizando resíduos da agroindústria mostra-se uma saída para os problemas ambientais de despejo de resíduos e aumento da performance e produtividade animal. Para tornar esses bioprocessos uma realidade industrial, estudos posteriores devem ser realizados a fim de confirmar os efeitos da biomassa residual (substrato e células) na alimentação animal.

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Anexos

Anexo 1 (Capítulo 1): Curvas de crescimento de *Rhodotorula mucilaginosa* LABI1 em meios com bagaço de cana ou de mandioca com diferentes combinações de extrato de levedura, $(\text{NH}_4)_3\text{PO}_4$ e água de maceração de milho

