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SÍNTESE DE GALACTO-OLIGOSSACARÍDEOS A PARTIR DE CÉLULAS PERMEABILIZADAS DE *Kluyveromyces marxianus*

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(Isaac Newton)

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RESUMO GERAL

Galacto-oligossacarídeos (GOS) são carboidratos não digeríveis por humanos, produzidos a partir da lactose por ação da enzima β-galactosidase. São considerados ingredientes prebióticos e possuem propriedades favoráveis à saúde dos consumidores. Este trabalho teve como objetivo o estudo da produção de GOS a partir de células permeabilizadas de *Kluyveromyces marxianus* CCT 7082. A primeira etapa foi a otimização das condições de cultivo da levedura para a produção da enzima β-galactosidase empregando como substratos os subprodutos agroindustriais, soro de queijo e água de maceração de milho, obtendo 1400 U/g_{cel} em 24 h de fermentação. Em seguida estudou-se a permeabilização das células da levedura. Foram testados sete agentes permeabilizantes: etanol, isopropanol, butanol, acetona, brometo de cetiltrimetilamônio, Tween 80 e Triton X-100, tendo sido selecionado o isopropanol, para a etapa de otimização do processo de permeabilização, onde se avaliou o efeito da relação biomassa/isopropanol e da temperatura. Na caracterização da enzima, a β-galactosidase das células permeabilizadas apresentou pH ótimo de 6,6 e temperatura ótima de 50°C, sendo esta mais estável no pH 7,0 e na temperatura de 30ºC. A energia de desnaturação foi 81,6 Kcal/mol. A cinética enzimática da enzima seguiu o modelo de Michaelis-Menten. O estudo da síntese de GOS, através de delineamentos experimentais, empregando as células permeabilizadas, resultou em 83 g/L de GOS. O emprego de fluidos pressurizados como meio reacional de reações enzimáticas podem favorecer a solubilidade dos compostos, as transferências de massa das reações, e aumentar a atividade e estabilidade de enzimas, assim sendo, estudou-se o comportamento da atividade enzimática da enzima das células permeabilizadas tratadas nessas condições. Foi realizado um delineamento composto central para cada fluido (n-butano, propano, CO_2), sendo observado um aumento na atividade residual, em todos os ensaios dos delineamentos, de 110 a 211% dependendo do fluido. O tratamento com n-butano resultou na maior estabilidade da enzima: após 3 semanas de armazenamento a 10°C a enzima tratada manteve 96% de sua atividade. Estas células tratadas a alta pressão foram aplicadas na síntese de GOS em reator batelada a pressão atmosférica. Obteve-se aproximadamente 75 g/L de GOS para os três fluidos pressurizados e para as células permeabilizadas sem tratamento. Porém, a quantidade necessária de enzima (em gramas de células) para se obter a mesma atividade enzimática, foi bem menor para as enzimas tratadas a alta pressão, tendo em vista o aumento da atividade enzimática após o tratamento. Na etapa seguinte, estudou-se a síntese de GOS em reator batelada empregando como meio reacional fluidos pressurizados (n-butano, propano, CO_2). Realizou-se um delineamento composto central para cada fluido, obtendo-se entre 65 e 83,4 g/L de GOS, dependendo do meio reacional. De acordo com os resultados deste trabalho, usando células de *K. marxianus* CCT 7082, pôde-se definir a metodologia empregando as células permeabilizadas, tratadas a alta pressão com n-butano e síntese em reator batelada a pressão atmosférica como a mais promissora para a produção de GOS

Palavras-chave: galacto-oligossacarídeos; β–galactosidase; permeabilização celular; fluidos pressurizados.

SUMARY

Galactooligosaccharides (GOS) are humans non-digestible carbohydrates, produced from lactose by the action of the enzyme β-galactosidase. They are considered prebiotic ingredients and have beneficial properties to the health of consumers. This work aimed the study of galacto-oligosaccharide production from permeabilized cells of *Kluyveromyces marxianus* CCT 7082. The first step was to optimize the yeast culture conditions in order to produce the β-galactosidase enzyme, employing as substrates by-products from agriculture industries, such as cheese whey and corn steep liquor, it were obtained 1400 U/g in 24 h of fermentation. The next step was to study the yeast cell permeabilization. Seven permeabilizant agents were tested: ethanol, isopropanol, butanol, acetone, cetyl-trimethylammonium bromide, Tween 80 and Triton X-100. Isopropanol was selected for the optimization step of the permeabilization process, where the effect of the biomass/isopropanol ratio and the temperature on cell permeabilization were evaluated. Afterward, the β-galactosidase from permeabilized cells was characterized presenting the optimum pH of 6.6 and the optimum temperature 50°C. The enzyme was more stable at pH 7.0 and 30°C temperature. The denaturation energy was 81.6 Kcal/mol. The enzyme kinetics followed Michaelis-Menten model. The GOS synthesis, studied through experimental designs, employing the permeabilized cells, resulted in 83 g/L of GOS. The use of pressurized fluids as a reactional medium for enzymatic reactions can help the components solubility and the mass transferences of the reactions and to increase the enzymes activity and stability. Therefore, the behavior of the enzymatic activity of the permebialized cell enzymes, treated with pressurized fluids, was studied. A central composite design was performed for each pressurized fluid, and it was observed an increase on residual activity for all pressurized fluids, in all designs essays, from 110 a 211%, depending on the fluid. The enzyme treated with n-butane resulted in the highest enzyme stability. After 3 weeks of storage at 10°C the enzyme kept 96% of activity. These cells treated at pressure were employed at GOS synthesis in batch reactor at atmospheric pressure. Around 75 g/L of GOS were obtained for all three pressurized fluids, as well as for the enzyme without treatment. However, the amount of the enzyme needed (in g of cells) to obtain the same enzymatic activity was lower in the case of the enzymes treated at high pressure, due to the increase of enzymatic activity after the treatment. In the next step of the work, the GOS synthesis was studied in batch mode, using as a reactional medium pressurized fluids (n-butane, propane, CO_2). A central composite design for each pressurized fluid was carried out, obtaining between 65 g/L and 83 g/L of GOS, depending on reactional medium. According to the results of this work, using cells of *K. marxianus* CCT 7082, it can be defined that the methodology of permeabilized cells, treated at high pressure with n-butane and synthesis in atmospheric pressure reactor, is the most promising one for GOS production.

Key words: galactooligosaccharides, β-galactosidase, cell permeabilization, pressurized fluids

CAPÍTULO 1

INTRODUÇÃO GERAL

OBJETIVOS

DESCRIÇÃO DOS CAPÍTULOS

1. INTRODUÇÃO GERAL

O apelo por produtos com características de qualidade que aportem ganhos fisiológicos aos consumidores, além das exigidas vantagens nutricionais, é a nova fronteira de expansão no mercado de alimentos processados. A crescente demanda por alimentos funcionais tem estimulado o mercado desse segmento no Brasil e impulsionado importantes esforços em pesquisa e desenvolvimento desses produtos (Martins & Burkert, 2009).

São vários os fatores que vêm estimulando o desenvolvimento de alimentos funcionais ao longo dos últimos anos. Dentre eles, destacam-se principalmente: o aumento da expectativa de vida em países desenvolvidos (cujas populações necessitam de cuidados hospitalares por maior período de tempo), o elevado custo dos serviços de saúde, os avanços na tecnologia de alimentos e ingredientes, a necessidade que as instituições públicas de pesquisa têm em divulgarem os resultados de suas investigações e a maior cobertura dos diferentes tipos de mídia dada a essas descobertas e às questões de saúde (Arvanitoyannis & Van Houwelingen-Koukaliaroglou, 2005; Komatsu et al., 2008).

Segundo Fooks & Gibson (2002) os oligossacarídeos estão entre os alimentos funcionais que proporcionam efeito positivo na composição da microbiota intestinal, sendo os fruto-oligossacarídeos (FOS) e os galacto-oligossacarídeos (GOS) os oligossacarídeos mais comuns utilizados como ingredientes funcionais.

Os galacto-oligossacarídeos são considerados ingredientes prebióticos por estimular a proliferação de bifidobactérias e bactérias lácticas no intestino. A predominância destes micro-organismos no cólon causa efeitos benéficos para manutenção da saúde, tais como a inibição de certas bactérias potencialmente patogênicas, como o *Clostridium*, redução do nível de colesterol, prevenção do câncer de cólon, aumento da absorção de cálcio e por não serem metabolizados por micro-organismos da cavidade oral, não estão envolvidos na formação de cáries dentárias. A estabilidade dos GOS sob condições ácidas durante o processamento de alimentos torna-os potencialmente aplicáveis como ingredientes para uma ampla variedade de produtos alimentícios (Crittenden & Playne, 1996; Rowland & Tanaka, 1993; Tomomatsu, 1994; Alander et al., 2001; Chonan et al., 2001).

GOS são os produtos da reação de transgalactosilação catalisada pela enzima β-galactosidase, quando se utiliza lactose como substrato (Mahoney, 1998). A hidrólise da lactose em glicose e galactose ocorre predominantemente em baixas concentrações de lactose, enquanto que a produção de GOS aumenta com o incremento na concentração de lactose (Cho et al., 2003).

A β-galactosidase (EC 3.2.1.23) é uma das enzimas mais promissoras empregadas em processos industriais, e tem diversas aplicações nas indústrias de alimentos, de fermentações e em laticínios (Kaur et al., 2009). Entre os micro-organismos, as leveduras têm emergido como uma importante fonte de β-galactosidase, uma vez que essas enzimas têm um pH ótimo adequado para hidrólise de lactose de leites e soro de queijo (Kondo et al., 2000). Porém as aplicações industriais de processos baseados na hidrólise enzimática da lactose são limitadas, pois a β-galactosidase de leveduras é uma enzima intracelular (Panesar, 2008). Como a preparação de extratos isentos de células é trabalhosa além de necessitar da ruptura da parede celular, a permeabilização de células tem sido recomendada como um método alternativo para o estudo das enizmas intracelulares (Alamäe & Järviste, 1995). Os agentes permeabilizantes diminuem o conteúdo de fosfolipídeos da membrana celular permitindo a passagem de solutos incluindo a lactose e seus produtos (Siso et al., 1992; Panesar, 2008).

O emprego de β-galactosidase comercial, livre ou imobilizada para a síntese enzimática de GOS a partir de lactose e soro de queijo, tem sido reportada na literatura empregando a enzima comercial, livre ou imobilizada (Cho et al., 2003; Gaur et al., 2006; Hsu et al., 2007; Park et al., 2008; Martínez-Villaluenga et al., 2008), no entanto, a utilização de células permeabilizadas como fonte de β-galactosidase na síntese de GOS é uma alternativa interessante que tem sido pouco explorada (Park & Oh, 2010).

Os métodos convencionais de síntese de GOS são realizados em reatores a pressão atmosférica, onde a enzima é adicionada ao substrato e a mistura reacional mantida a pressão ambiente, com temperatura constante sob agitação até que ocorra a máxima conversão de substrato em GOS. Outra metodologia ainda não explorada na síntese de GOS é o emprego de fluidos pressurizados como meio reacional.

O tratamento de enzimas e/ou reações a alta pressão pode modificar a estrutura secundária e terciária da proteína, resultando numa conformação diferente da nativa, mas que pode manter ou aumentar a atividade catalítica, sem que ocorra a desnaturação da proteína. Em geral, pressões acima de 3000 bar a temperatura ambiente causam desnaturação protéica irreversível, enquanto pressões menores resultam em mudanças reversíveis na estrutura da proteína (Cheftel, 1995).

A catálise enzimática em fluidos pressurizados apresenta interesse particular para as indústrias farmacêuticas e de alimentos, principalmente devido a facilidade de recuperação dos produtos, livres de solventes ao final do processo (Feirhmann, 2005).

Com base no exposto acima, e considerando a carência de estudos de síntese de GOS empregando células permeabilizadas e a inexistência de dados, na literatura pesquisada, sobre comportamento da enzima β-galactosidase tratada a alta pressão bem como sobre a síntese de GOS em fluidos pressurizados, desenvolveu-se o presente trabalho.

1.2 Objetivos

1.2.1 Objetivo geral

Este trabalho faz parte do projeto "Obtenção de Enzimas de Interesse para a Indústria de Alimentos" aprovado pelo Programa Nacional de Cooperação Acadêmica (PROCAD/CAPES), realizado em conjunto com a Universidade Federal do Rio Grande – FURG e a Universidade Regional Integrada do Alto Uruguai campus de Erechim – URI, e tem como principal finalidade desenvolver metodologias para a síntese de galacto-oligossacarídeos a partir de células permeabilizadas de *Kluyveromyces marxianus* CCT 7082.

1.2.2 Objetivos específicos

- ✓ Estudar as condições de produção da enzima β-galactosidase a partir da levedura *Kluyveromyces marxianus* CCT 7082 empregando subprodutos agro-industriais;
- ✓ Determinar as melhores condições para a permeabilização das células da levedura a fim de se obter a máxima atividade de β-galactosidase;
- ✓ Caracterizar a enzima β-galactosidase das células permeabilizadas quanto ao perfil de temperatura e pH, estabilidade térmica e de pH e determinar os parâmetros cinéticos K_m e $V_{máx}$;
- ✓ Estudar as condições de síntese de galacto-oligossacarídeos a partir das células permeabilizadas empregando três metodologias:
    - Síntese em reator batelada a pressão atmosférica;
    - Tratamento a alta pressão das células permeabilizadas e posterior síntese dos galacto-oligossacarídeos em reator a pressão atmosférica;
    - Síntese em reator batelada usando fluidos pressurizados como meio reacional.

1.3 Descrição dos Capítulos

Para que tais objetivos fossem alcançados, o presente trabalho foi desenvolvido de acordo as etapas descritas a seguir:

Capítulo 1: **Introdução geral**

Breve introdução e objetivos propostos neste trabalho.

Capítulo 2: **Revisão Bibliográfica**

Este capítulo apresenta uma breve revisão da literatura sobre os principais assuntos abordados neste trabalho.

Capítulo 3: Utilização de subprodutos agroindustriais em processo biotecnológico para produção de β-galactosidase de *Kluyveromyces marxianus* CCT 7082

Neste capítulo, estudou-se as condições de produção da enzima β-galactosidase a partir da levedura *Kluyveromyces marxianus* CCT 7082. Foram realizados delineamentos experimentais para avaliar o efeito de subprodutos agroindustriais, sulfato de amônia, e o pH na produção da enzima.

Capítulo 4: Permeabilization of *Kluyveromyces marxianus* cells aiming β-galactosidase activity and characterization

O capítulo 4 apresenta as condições de permeabilização das células da levedura, para obtenção da enzima intracelular, e posterior caracterização da enzima. Testou-se diferentes agentes permeabilizantes a fim de selecionar o que resultasse em maior atividade enzimática, e em seguida através da técnica de delineamento experimental otimizou-se o processo de permeabilização. Posteriormente, comparou-se os resultados de caracterização entre a enzima das células permeabilizadas e a enzima extraída das células. Determinou-se o perfil de temperatura e pH, a estabilidade térmica e de pH e os parâmetros cinéticos K_m e $V_{máx}$.

Capítulo 5: Galacto-oligosaccharide production using permeabilized cells of *Kluyveromyces marxianus*

Este capítulo apresenta a síntese de galacto-oligossacarídeos, em reator batelada a pressão atmosférica, empregando as células permeabilizadas. As variáveis que mais influenciam na produção de GOS - concentração de lactose, concentração de enzima, temperatura e pH - foram estudadas através de delineamentos experimentais.

Capítulo 6: Effect of compressed fluids treatment on the activity, stability and enzymatic reaction performance of β-galactosidase

Neste capítulo, as partir dos dados apresentados no Capítulo 5, realizou-se primeiramente um tratamento a alta pressão nas células permeabilizadas, visando avaliar o efeito da alta pressão na atividade enzimática para posterior emprego destas células na síntese de GOS em reator batelada a pressão atmosférica. As células permeabilizadas foram tratadas a alta pressão com n-butano, propano e CO_2. Realizou-se um delineamento experimental para cada fluido pressurizado avaliando a pressão do sistema, tempo de exposição e a taxa de despressurização, tendo como resposta a atividade enzimática residual. Em seguida realizou-se a síntese de GOS em reator batelada a pressão atmosférica empregando as enzimas tratadas a alta pressão.

Capítulo 7: Enzymatic synthesis of galactooligosacharides using pressurized fluids as reaction media

A partir dos resultados apresentados nos Capítulos 5 e 6, estudou-se a síntese de GOS em reator batelada usando como meio reacional diferentes fluidos pressurizados: butano, propano e CO_2. Para cada fluido pressurizado realizou-se um delineamento experimental avaliando a influência da pressão do sistema, concentração de lactose e concentração de enzima na concentração de GOS.

Capítulo 8: Conclusões

As principais conclusões obtidas com a realização deste trabalho são relatadas neste capítulo.

1.4 Referências Bibliográficas

Alamäe, T.; Järviste, A. Permeabilization of the methylotrophic yeast *Pichia pinus* for intracellular enzyme analysis: a quantitative study. **Journal of Microbiological Methods**, v. 22, p. 193-205, 1995.

Alander, M.; Matto, J.; Kneifel, W.; Johansson, M.; Kogler, B.; Crittenden, R.; Mattila-Sandholm T.; Saarela, M. Effect of galacto-oligosaccharide supplementation on human fecal microflora and on survival an persistence of Bifidobacterium lactis Bb12 in the gastrointestinal tract. **International Dairy Journal,** v. 11, p. 817-825, 2001.

Arvanitoyannis, I.; Van Houwelingen-Koukaliaroglou, M. Functional foods: a survey of health, claims, pros and cons, and current legislation. **Critical Reviews in Food Science and Nutrition,** v. 45, n. 5, p. 385-404, 2005.

Cheftel, J.C. Review: High-pressure, microbial inactivation and food preservation. **Food Science and Technology International**, v. 1, p. 75-90, 1995.

Cho, Y. J.; Shin H. J.; Bucke, C. Purification and biochemical properties of a galactooligosaccharide producing β-galactosidase from *Bullera singularis*. **Biotechnology Letters**, v. 25, p. 2107-2111, 2003.

Chonan, O.; Takahashi, R; Watanuky, M. Role of activity of gastrointestinal microflora in adsorption of calcium and magnesium in rats fed 1-4 linked galactooligosaccharide. **Bioscience, Biotechnology and Biochemistry**, v. 65, n. 8, p.1872-1875, 2001.

Crittenden, R. G.; Playne, M. J. Production, properties and applications of food-grade oligosaccharides. **Trends in Food Science & Technology,** v.7, p. 353-361, 1996.

Feihrmann, A. C. **Avaliação da atividade enzimática de lipases imobilizadas em fluidos pressurizados.** Dissertação de Mestrado, Universidade Regional Integrada – Campus de Erechim, RS, 76p., 2005.

Fooks, L. J.; Gibson, G. R. Prebiotics and modulators of the gut flora. **British Journal of Nutrition,** v. 88, n. 1, p. S39-S49, 2002.

Gaur, R.; Pant, H.; Jain, R.; Kkare, S. K. Galacto-oligosaccharide synthesis by immobilized *Aspergillus oryzae* β-galactosidase. **Food Chemistry**, v. 97, p. 426-430, 2006.

Hsu, C. A.; Yu, R. C.; Chou, C. C. Production of β-galactosidase by bifidobacteria as influenced by various culture conditions. **International Journal of Food Microbiology,** v. 104, p. 197-206, 2007.

Kaur, G.; Panesar, P. S.; Bera, M. B.; Kumar, H. Hydrolysis of whey lactose using CTAB-permeabilized yeast cells. **Bioprocess and Biosystems Engineering,** v. 32, n. 1, p. 63-67, 2009.

Kondo, A.; Liu, Y.; Furuta, M.; Fujita, Y.; Matsumoto, T.; Fukuda, H. Preparation of high activity whole cell biocatalyst by permeabilization of recombinant flocculent yeast with alcohol. **Enzyme and Microbial Technology,** v. 27, p. 806-811, 2000.

Komatsu, T. R.; Buriti, F. C. A.; Saad, S. M. I. Inovação, persistência e criatividade superando barreiras no desenvolvimento de alimentos probióticos. **Revista Brasileira de Ciências Farmacêuticas,** v. 44, n. 3, p. 329-334, 2008.

Mahoney, R. R. Galactosyl-oligosaccharide formation during lactose hydrolysis: a review. **Food Chemistry**, v. 63, n. 2, p.147-154, 1998.

Martínez-Villaluengua, C.; Cardelle-Cobas, A.; Corzo, N.; Olano, A.; Villamiel, M. Optimization of conditions for galactooligosaccharide synthesis during lactose hydrolysis by β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000 L HP G). **Food Chemistry**, v. 107, n. 1, p. 258-264, 2008.

Martins, A. R.; Burkert, C. A. V. Galacto-oligossacarídeos (GOS) e seus efeitos prebióticos e bifidogênicos. **Brazilian Journal of Food Technology**. v. 12, n. 3, p. 230-240, 2009.

Panesar, P. S. Application of response surface methodology for maximal lactose hydrolysis in whole milk using permeabilized yeast cells. **Acta Alimentaria**, v. 37, n. 2, p. 191-203, 2008.

Park, A. R, Oh, D. K. Galacto-oligosaccharide production using microbial β-galactosidase: current state and perspectives. **Applied Microbiology and Biotechnology,** v. 85, p. 1279-1286, 2010.

Park, H. Y.; Kim, H. J.; Lee, J. K.; Kim, D.; Oh, D. K. Galactooligosaccharide production by a thermostable β-galactosidase from *Sulfolobus solfataricus*. **World Journal Microbiol Biotechnol.** v. 24, p. 1553-1558, 2008.

Rowland, I. R.; Tanaka, R. The effects of transgalactosylated oligosaccharides on gut flora metabolism in rats associated with a human faecal microflora. **Journal of Applied Bacteriology**, v. 74, p. 667-674, 1993.

Siso, M. I. G.; Cerdan, E.; Freire, M. A. ; Ramil, E.; Belmonte, E. R.; Torres, A. M. R. Permeabilization of Kluyveromyces lactis cells for Milk whey saccharification: a comprison of different treatments. **Biotechnology and Technology,** v. 6, p. 289-292, 1992.

Tomomatsu, H. Health effects of oligosaccharides. **Food Technology**, v. 48, p. 61-65, 1994.

CAPÍTULO 2

REVISÃO BIBLIOGRÁFICA

2. REVISÃO BIBLIOGRÁFICA

2.1 Prebióticos

Prebiótico é definido como um ingrediente alimentar não digestível que afeta beneficamente o hospedeiro por estimular seletivamente o crescimento e/ou atividade de um número limitado de bactérias no cólon, melhorando assim a saúde do hospedeiro (Gibson & Roberfroid, 1995).

Os critérios utilizados para a classificação de um ingrediente alimentar como prebiótico inclui: resistência à digestão; fermentação seletiva por bactérias benéficas no cólon; alteração da composição microbiana do cólon para uma composição mais saudável; indução de efeitos que são benéficos para a saúde do hospedeiro (Fooks et al., 1999).

Oligossacarídeos nao digestíveis, como os galacto-oligossacarídeos e os fruto-oligossacarideos, são prebióticos devido aos efeitos beneficos causados pela proliferação das bifidobactérias no cólon humano (Roberfroid, 2007).

2.2 Galacto-oligossacarídeos

Oligossacarídeos são geralmente definidos como glicosídeos de diferentes graus de polimerização e podem ser encontrados na natureza ou sintetizados tanto por via enzimática quanto por via química. A síntese química normalmente envolve muitas etapas de proteção e desproteção das hidroxilas dos açúcares, resultando em baixo rendimento dos produtos finais e, muitas vezes, a formação de enantiômeros indesejados (Flowers, 1978). Em contraste com a síntese química, a síntese enzimática de oligossacarídeos geralmente produz poucos subprodutos, evita a necessidade de proteção/desproteção química, e apresenta baixo impacto ambiental.

Os GOS são componentes naturais do leite humano, bem como de alguns vegetais, incluindo cebola, alho, banana, soja e chicória, e podem ser produzidos a partir da lactose pela reação de transgalactosilação da enzima β-galactosidase (Mahoney, 1998).

Embora na síntese enzimática ocorra a formação de tri- a hexa-sacarídeos com 2 a 5 unidades de galactose (Figura 1.2), é muito comum encontrar di-sacarídeos transgalactosilados, consistindo em uma molécula de galactose e uma molécula de glicose

com ligações β-glicosídicas diferentes da lactose ou di-sacarídeos formados somente por galactose. Estes di-sacarídeos podem ser considerados prebióticos desde que tenham características fisiológicas similares as dos GOS (Sako et al., 1999).

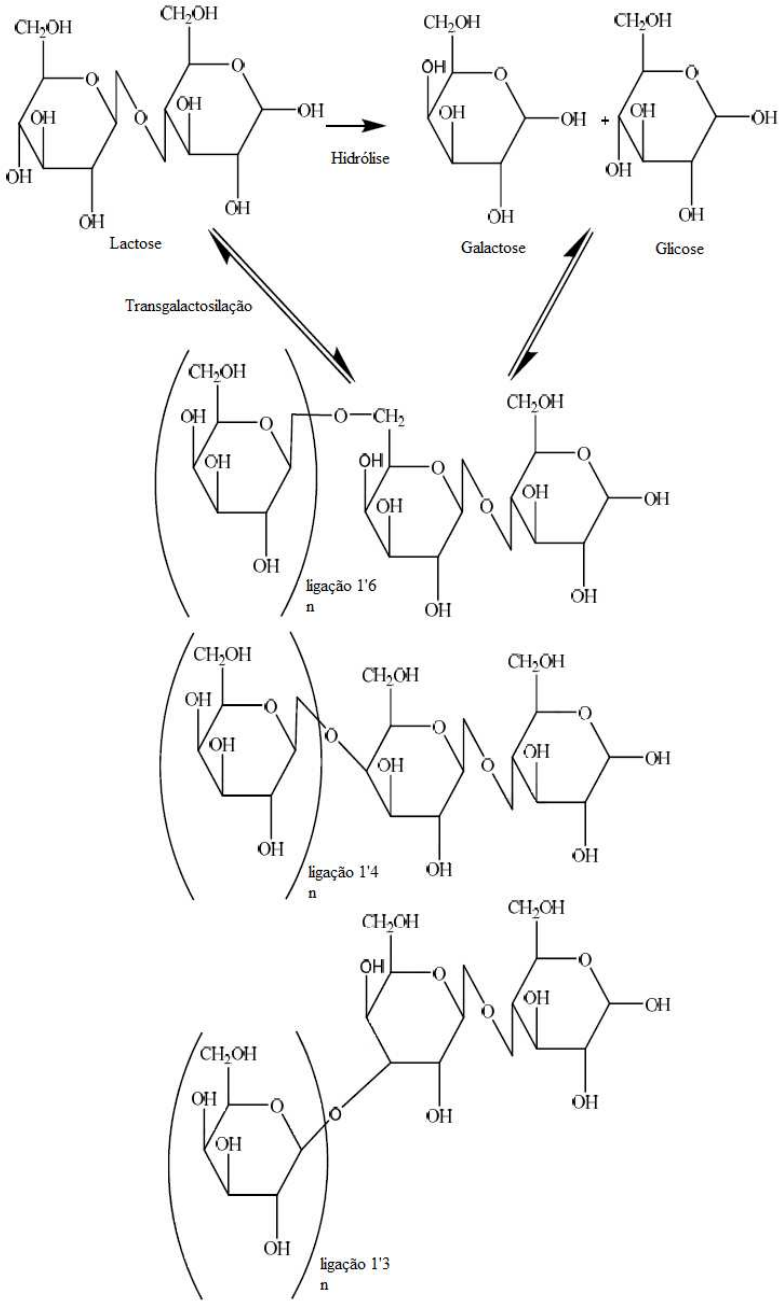

Figura 1.2: Formação de galacto-oligossacarídeos pela reação de transgalactosilação da β-galactosidase (Otieno, 2010).

2.2.1 Efeitos benéficos e aplicações de GOS

A ingestão de GOS aumenta a proliferação de bifidobactérias no trato intestinal e por efeito antagônico, a supressão da atividade de bactérias putrefativas e redução da formação de metabólitos tóxicos. Os efeitos benéficos à saúde dos consumidores, em função da proliferação das bifidobactérias incluem: (a) proteção de infecção intestinal, inibindo bactérias prejudiciais, tais como *Citrobacter* sp. e *Klebsiella* sp.; (b) diminuição do *C. perfringens* na microflora intestinal e diminuição de subprodutos resultantes, como amônia, amina e indol; (c) prevenção da diarréia patogênica; (d) favorecimento da absorção de cálcio, de magnésio e fósforo presente na dieta; (e) redução dos níveis séricos de colesterol; (f) redução da pressão arterial; (g) aumento da imunidade celular ou propriedades anticarcinogênicas; (h) produção de vitaminas do complexo B (Conesa et al., 2007; Rivero-Urgel & Santamaria-Orleans, 2001).

Algumas características dos GOS são interessantes do ponto de vista industrial, já que apresentam solubilidade maior do que a sacarose, não cristalizam, não precipitam, e nem deixam sensação de secura na boca (Bornet, 1994). São muito estáveis em condições adversas de pH e temperatura, resistem a 160°C por 10 min em pH neutro ou a 120°C por 10 min a pH 3,0. Nestas mesmas condições a degradação da sacarose seria superior a 50%. Em condições ácidas na temperatura ambiente, GOS tendem a ser estáveis durante o armazenamento a longo prazo (Sako et al., 1999).

Devido a essas características, os GOS podem ser usados em formulações de sobremesas lácteas, leites fermentados, pães, geléias, bebidas e produtos de confeitaria. Alimentos infantis e alimentos especiais para idosos e hospitalizados são promissores na aplicação de GOS, pois estas pessoas são mais suscetíveis a mudanças na microflora intestinal (Sako et al., 1999; Rivero-Urgel & Santamaria-Orleans, 2001). Além disso apresentam cerca de um terço do poder adoçante da sacarose e não serem calóricos, os GOS podem ser usados de modo seguro por diabéticos.

2.2.2 Produção de galacto-oligossacarídeos

Galacto-oligossacarídeos produzidos pela ação da enzima β-galactosidase sobre a lactose foram identificados pela primeira vez no início da década de 50. Quatro tipos de

GOS foram formados utilizando β-galactosidase de *Kluyveromyces lactis* (Aronson, 1952; Pazur, 1954), e três tipos com β-galactosidase de *E. coli* (Aronson, 1952). Desde então, vários estudos foram realizados sobre a síntese enzimática de GOS e diferentes resultados foram observados.

As principais conclusões obtidas foram as seguintes: (a) dependendo da fonte de β-galactosidase existem grandes diferenças entre a quantidade, o grau de polimerização e o tipo de galacto-oligossacarídeos formados (Boon et al., 2000); (b) o aumento da concentração da enzima não é proporcional ao aumento da concentração de oligossacarídeos (Chockchaisawasdee et al., 2005); (c) a concentração inicial de lactose é o fator mais importante que afeta a formação de GOS. Em concentrações baixas de lactose a reação de hidrólise é predominante, enquanto que a formação de GOS é observada em elevadas concentrações de lactose (Albayrak & Yang, 2002).

Zhou et al., (2001) citam que uma maior quantidade GOS é obtida com maior concentração de lactose, pois numa solução de baixa concentração de lactose, a água é mais competitiva para ser um aceptor para o grupo β-galactosil, do que os açúcares presentes na solução. Os mesmos autores relataram um aumento na produção de GOS de 2% a 32% quando a concentração inicial de lactose aumentou de 14% a 40% (p/v).

No trabalho de Albayrak & Yang (2002) e Hsu et al., (2007), os autores observaram que a formação de GOS é acompanhada por uma rápida diminuição da concentração da lactose no início da reação e com o passar do tempo de reação, a concentração de GOS diminui, enquanto que a concentração de galactose aumenta, indicando que transgalactosilação predomina no início da reação.

De acordo com López-Leiva & Gusman (1995), a fonte e concentração da enzima, o tempo de reação, a temperatura e o pH do processo, a concentração inicial de lactose e a presença de inibidores ou ativadores específicos para a enzima, têm influência sobre a quantidade de GOS formada.

Boon et al. (2000) observaram que as β-galactosidases obtidas de *Bacillus circulans* produzem grande quantidade de GOS com maior grau de polimerização (tetra e pentassacarídeos), enquanto que as β-galactosidases de *A. oryzae* e *Kluyveromyces* sp produzem principalmente trissacarídeos e uma pequena quantidade de tetrassacarídeos.

López-Leiva & Gusman (1995) acompanharam a formação de glicose, galactose e oligossacarídeos durante a hidrólise da lactose de soro de queijo, por β-galactosidase de *Aspergillus oryzae* imobilizada em filme poroso em um reator a 40°C e com 6, 10 ou 20% (p/v) de lactose. O aumento na concentração inicial de lactose, de 6 para 20%, provocou um aumento na concentração de oligossacarídeos, enquanto que com o aumento do tempo de residência, a concentração de oligossacarídeos diminuiu, ocorrendo a formação de mono e di-sacarídeos.

Chen et al. (2003) verificaram que o rendimento total da produção de GOS, utilizando a enzima β-galactosidase de *Aspergillus oryzae*, aumentava gradualmente com o aumento da temperatura de 35 a 47°C, mas diminuía quando a temperatura excedia 47°C, justificado por uma possível desnaturação da enzima.

Martínez-Villaluenga et al. (2008), empregaram a enzima Lactozym 3000 L HP G, obtida de *K. lactis*, para a produção de GOS. As sínteses foram realizadas em diferentes condições: concentração de lactose (150 a 350 g/L), concentração de enzima (3 a 9 U/mL), temperatura (40 a 60°C), e pH (5,5 a 7,5). O tempo total de reação foi de 300 minutos. Os autores observaram que o efeito das condições de síntese, resultaram em diferentes concentrações de di- e tri-sacarídeos. As melhores condições para produção dos di-sacarídeos galactobiose e alolactose foram: 50°C, pH 6,5, 250 g/L de lactose e 3 U/mL de enzima, em 300 minutos de reação, resultando em 5,1% (p/p) de galactobiose e 10,3% (p/p) de alolactose. Já para a produção do tri-sacarídeo 6'galactosil lactose, as melhores condições foram: 40°C, pH 7,5, 250 g/L de lactose, 3 U/mL de enzima, em 120 minutos de reação, nestas condições 17% (p/p) do tri-sacarídeo foi produzido.

Santos et al. (2009), estudaram a produção de GOS a partir da β-galactosidase produzida por uma linhagem de *Scopulariopsis* sp. Foram analisadas as temperaturas 35, 45 e 60°C, os tempos de reação de 12, 24 e 48 h, a concentração de enzima de 0,5 a 10 U/mL e concentração de lactose de 10, 25 e 40% (p/v). As condições ótimas para a síntese de GOS foram concentração de lactose de 40% (p/v), 45°C, 10 U/mL de enzima, em 12 h de reação. Nessas condições, a concentração de GOS foi 80,8 g/L e a enzima converteu 20% de lactose em GOS.

Pocedicová et al. (2010), produziram GOS em reatores em batelada e contínuo empregando como substrato, lactose preparada em tampão (198 g/L); soro de queijo (197 g/L) ou permeado ultrafiltrado (201 g/L). A concentração da enzima Maxilact LX 5000 foi 6 U/mL. As maiores conconetrações de GOS no reator em batelada foram 25,5 g/L para a solução de lactose, 31,1 g/L para o soro de queijo e 32,9 g/L para o permeado ultrafiltrado, obtidas após 30 min de reação. Para o processo contínuo foi obtido 8,8 g/L de GOS para a solução de lactose, 21 g/L para o soro de queijo e 16,7 g/L para o permeado ultrafiltrado.

Outros trabalhos de produção de GOS foram compilados na Tabela 2.1. Observa-se que a β-galactosidase pode ser empregada de diferentes formas, e que existe um grande número de micro-organismos, tanto bactérias, quanto fungos (bolores e leveduras), produtores desta enzima com atividade de transgalactosilação. Observa-se também que a maior concentração de GOS (63%), foi obtida na síntese com β-galactosidase de *Bifidobacterium infantis* (Hung & Lee, 2002), já as maiores produtividades foram obtidas nas sínteses com *Aspergillus candidus* e *A. oryzae*, resultando em 87 e 106 g/L.h, respectivamente, em ambos sistemas operacionais a síntese foi realizada em reator contínuo (Zheng et al. 2006; Albayrak & Yang, 2002).

Em relação à produção de GOS empregando células permeabilizadas, somente um grupo de pesquisa da Ajinomoto do Japão (Onishi et al. 1995; Onishi & Yokozeki 1996; Onishi et al. 1996) investigaram a produção de GOS empregando células de leveduras permeabilizadas com tolueno. Eles empregaram as leveduras *Rhodotorula minuta, Sirobasidium magnum* e *Sterigmatomyces elviae*, cujas condições para a produção de GOS foram similares para as três leveduras (5 mL de suspensão de células permeabilizadas, 360 g/L de lactose, temperatura de 60°C para as leveduras *R. minuta* e *S. elviae* e 50°C para *S. magnum*), obtendo-se uma concentração de GOS de 136 g/L, indicando um rendimento de 38% para todas as leveduras testadas. O tempo de síntese foi de 42 h para as leveduras *S. magnum* e *R. minuta*, e 20 h para *S. elviae*.

Tabela 2.1: Produção de GOS por β-galactosidases microbianas empregando lactose como substrato.

Fonte da enzima	T (°C)	pH	Lactose (g/L)	GOS[a] (%)	P[b] (g/L.h)	Referência
Extrato bruto						
Aspergillus oryzae	40	4,5	380	31	24	Iwasaki et al. 1996
B. longum	45	6,8	400	33	13	Hsu et al. 2007
Lactobacillus reuteri	30	6,5	205	38	3,9	Splechtna et al. 2006
G. stearothermophilus	37	6,5	180	2	0,4	Placier et al. 2009
T. thermophilus	40	6,5	200	50	13	Nakkharat et al. 2006
Enzima purificada						
Kluyveromyces lactis	38	-	219	13	-	Curda et al. 2006
Bullera singularis	50	5,0	180	50	3,9	Cho et al. 2003
L. acidophilus	30	6,5	205	39	7,9	Nguyen et al. 2007
P. simplicissimum	50	6,5	600	31	11	Cruz et al. 1999
S. rectivirgula	60	6,5	600	44	-	Nakao et al. 1994
Enzimas recombinantes						
Bifidobacterim infantis	60	7,5	300	63	13	Hung & Lee 2002
Sulfolobus solfataricus	80	6,0	600	53	5,6	Park et al. 2008
Thermotoga maritima	80	6,0	500	19	18	Ji et al. 2005
Células permeabilizadas						
Rhodotorula minuta	60	6,0	200	38	3,2	Onishi & Yokozeki 1996
Sirobasidium magnum	50	-	360	38	3,2	Onishi et al. 1996
Sterigmatomyces elviae	60	6,0	360	38	6,7	Onishi et al. 1995
Células inteiras						
B. bifidum	39	6,8	500	20	65	Tzortzis et al. 2005
Enzimas imobilizadas						
Aspergillus candidus	40	6,5	400	37	87	Zheng et al. 2006
Aspergillus oryzae	40	4,5	400	27	106	Albayrak & Yang, 2002
Bullera singularis	45	3,7	300	54	4,8	Shin & Yang, 1998
Kluyveromyces lactis	40	7,0	400	25	25	Chockchaisawasdee et al. 2005
Thermus aquaticus	70	6,0	160	34,8	-	Berger et al. 1995
Células imobilizadas						
S. singularis	55	5,0	600	40	8,7	Sakai et al. 2008

[a] porcentagem (p/p) de GOS baseado na concentração inicial de lactose
[b] produtividade

2.3 Enzima β-galactosidase

A enzima β-galactosidase (E.C. 3.2.1.23) ou lactase é classificada como uma hidrolase, com capacidade transferase para grupos galactosil, atuando no resíduo terminal β-galactopiranosil da lactose (Galβ1-4Glc) para formar uma mistura isomolecular de glicose e galactose. No entanto, dependendo das condições, a mistura isomolecular não é alcançada, pois a galactose pode polimerizar ou se unir a outra molécula para formar galacto-oligossacarídeos (Gekas & López-Leivas, 1985; Mahoney, 1998).

Para que ocorra a hidrólise enzimática da ligação glicosídica de lactose, são necessários dois resíduos de aminoácidos, um doador de próton e um nucleófilo. O mecanismo de hidrólise da lactose foi primeiramente descrita por Wallenfels (Wallenfels et al., 1961), usando a enzima de *E. coli*. O mecanismo de reação proposto foi que os resíduos da cistina e histidina atuam como doadores de prótons e nucleófilo, respectivamente, durante o processo de hidrólise enzimática.

No entanto, estudos recentes descrevem que a β-galactosidase de diversas fontes microbianas, possui dois resíduos de ácido glutâmico (Glu482 e Glu551) como doador de prótons e nucleófilo, respectivamente, ao mesmo tempo na reação enzimática. Este mecanismo da reação é apresentado na Figura 2.2. O primeiro passo é a formação do complexo enzima-galactosil e simultânea liberação da glicose. Na segunda etapa o complexo enzima-galactosil é transferido para um aceptor contendo um grupo hidroxila. Se a solução apresentar baixa concentração de lactose, a água ao invés de outros açúcares como a glicose e a lactose pode ser mais competitiva para ser um aceptor, portanto, galactose é formada e o sítio ativo é liberado. Por outro lado, em uma solução de alta concentração de lactose, as moléculas de glicose, galactose e lactose têm mais chances de atuar como aceptor, formando oligossacarídeos (Zhou et al. 2001).

Portanto, o mecanismo de reação da β-galactosidase inclui tanto a hidrólise da lactose como a transgalactosilação (Mahoney, 1998). Dependendo da concentração de lactose, a reação é deslocada para a hidrólise ou para a transgalactosilação. Quando a concentração de água no sistema é alta, ocorre predominantemente a hidrólise da lactose. A reação de transgalactosilação aumenta com a redução da atividade de água (Goulas et al., 2007).

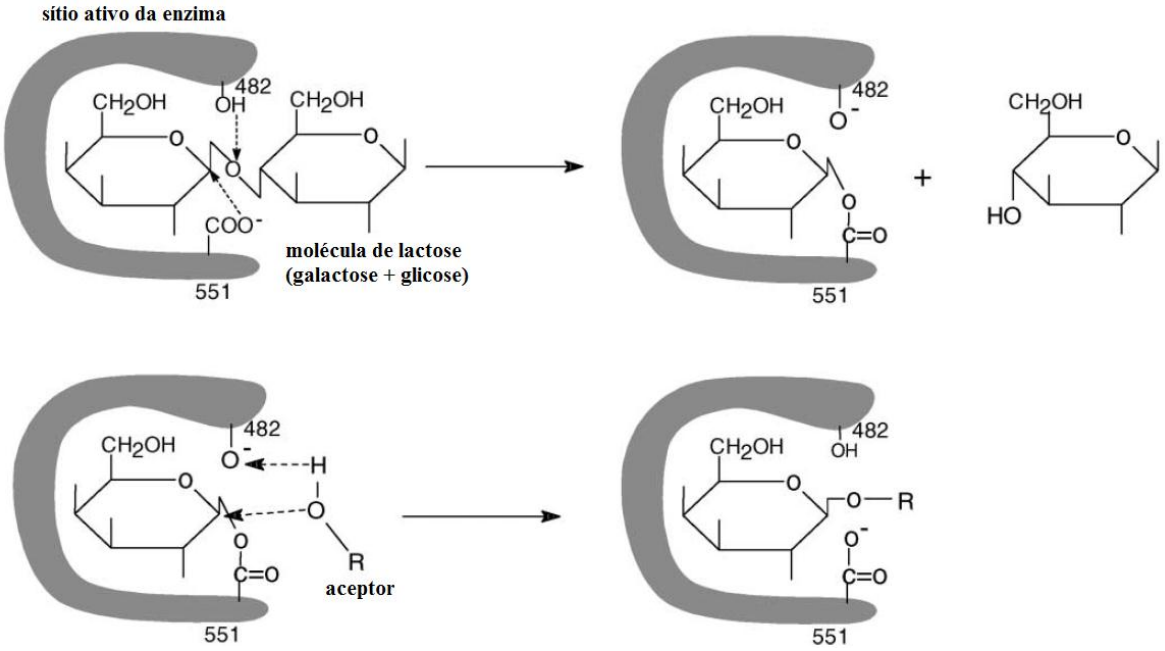

Figura 2.1: Mecanismo de reação de hidrólise e de transgalactosilação da lactose pela β-galactosidase (adaptado de Zhou et al. 2001).

A importância industrial da β-galactosidase está em sua aplicação na indústria de laticínios. Esta enzima hidrolisa a lactose, carboidrato característico do leite, em dois monossacarídeos glicose e galactose, obtendo alimentos com baixos teores de lactose, melhorando a solubilidade e digestibilidade do leite e derivados lácteos, ideais para consumidores intolerantes a lactose (Santiago et al., 2004).

Entre os vários micro-organismos usados para a produção de β-galactosidase (Tabela 2.2), as leveduras são uma importante fonte, uma vez que a enzima de leveduras tem um pH adequado para hidrólise da lactose de leite e soro de leite (Kondo et al., 2000; Husain, 2010). Sendo que a levedura *Kluyveromyces marxianus* apresenta algumas vantagens em relação a outros micro-organismos produtores de β-galactosidase, tais como, bom rendimento de crescimento que tem um impacto econômico importante na indústria de alimentos; aceitabilidade como um microrganismo seguro (GRAS – Generally Recognized as Safe), aspecto técnico importante ao considerar que os produtos fermentados têm aplicações farmacêuticas e alimentícias; e apresentam atividade enzimática maior do que

outras leveduras como a *Candida* quando lactose é usada como substrato (Kaur et al., 2009).

Tabela 2.2: Micro-organismos produtores de β-galactosidase.

Micro-organismos	Referência
Leveduras	
Kluyveromyces marxianus	Martins et al. (2002); Pinheiro et al. (2003); Rajoka et al. (2003); Cortés et al. (2005); Bansal et al. (2008); Manera et al. (2008); Singh et a. (2009); Alves et al. (2010)
Kluyveromyces lactis	Numanoglu & Sungur (2004); Becerra et al. (2004); Kim et al. (2004); Ramírez Matheus & Rivas (2003); Dagbagli & Goksungur (2008); Ornelas et al. (2008)
Saccharomyces cerevisiae	Domingues et al. (2004)
Candida pseudotropicalis	Inchaurrondo et al. (1998)
Guehomyces pullulans	Nakagawa et al. (2006); Song et al. (2010)
Fungos	
Bullera singularis	Shin et al. (1998)
Penicillium simplicissimum	Cruz et al. (1999)
Penicillium chrysogenum	Nagy et al. (2001)
Aspergillus niger	Rodriguez et al. (2006)
Bactérias	
Streptococcus thermophilus	Rao & Dutta (1977)
Bacillus stearothermophilus	Chen et al. (2009)
Lactobacillus bulgaricus	Vasiljevic & Jelen (2001)

Em trabalho desenvolvido anteriormente pelo grupo de pesquisa do Laboratorio de Microbiologia da FURG-RS, foram testadas sete espécies de *Kluyveromyces* na produção da β-galacotosidase, por fermentação submersa empregando lactose como substrato. A levedura *K. marxianus* CCT 7082, apresentou maior atividade enzimática sendo selecionada para etapa de otimização do meio de cutura para produção da enzima (Manera et al., 2008). Esta enzima vem sendo empregada em diversos estudos de purificação e síntese de GOS.

2.3.1 Caracterização enzimática

A literatura apresenta poucos dados sobre as propriedades enzimáticas e parâmetros cinéticos para a enzima β-galactosidase de *K. marxianus*. Samoshina & Samoshin (2005) apresentam uma revisão com 111 artigos publicados entre 1952 e 2004, sobre propriedades enzimáticas e parâmetros cinéticos da β-galactosidase de diversas fontes, destes 67 são sobre enzimas de fungos e 44 sobre enzimas de leveduras e dentre estes apenas 4 são sobre a enzima de *Kluyveromyces marxianus.*

O pH e a temperatura são os principais fatores que influenciam na velocidade de reação da catálise enzimática. Os sítios ativos das enzimas são frequentemente compostos de grupos ionizáveis que devem estar na forma iônica própria para manter a conformação do sítio ativo, do contrário a enzima é desnaturada (Rodriguez-Nogales & Delgadillo, 2005).

O pH ótimo de uma enzima depende de vários parâmetros experimentais, incluindo tempo de reação, temperatura, natureza e concentração de substrato, força iônica do meio e pureza da enzima (Whitaker, 1994). O pH operacional é a característica que define a aplicação de uma dada β-galactosidase. Para o processamento de soro ácido ou produtos lácteos fermentados, as lactases provenientes de fungos filamentosos são mais indicadas, pois possuem pH ótimo de operação entre 3,0 a 5,0. Já as β-galactosidases provenientes de leveduras possuem pH ótimo próximo ao neutro (Gekas & López-Leiva 1985; Holsinger & Kligerman, 1991; Robinson, 1991).

Quanto a temperatura ótima, ao se aumentar a temperatura, espera-se um aumento na atividade da enzima, sendo isto resultado de colisões entre enzima/substrato, este aumento na atividade cessa quando se inicia a desnaturação, ocasionada a elevadas temperaturas, quando as colisões se tornam desfavoráveis à enzima (Rodriguez-Nogales & Delgadillo, 2005). A temperatura ótima de operação da β-galactosidase também depende de sua origem, β-galactosidases provenientes de fungos filamentosos possuem maior atividade e maior estabilidade a temperaturas elevadas que as lactases de leveduras. As primeiras são utilizadas nas hidrólises de lactose entre 50 e 55ºC, enquanto que com as últimas ocorre rapidamente a desnaturação da enzima acima de 50ºC (Rech, 1998).

Outros dados relevantes para a catálise enzimática são os parâmetros cinéticos (K_m e $V_{máx}$). Sabe-se que $V_{máx}$ não é uma característica fundamental da enzima, pois depende da sua pureza e concentração, já a constante de Michaelis-Menten, representa a afinidade da enizma pelo substrato, quanto menor for o valor de K_m maior será esta afinidade. A estimação de K_m pode ser afetada por vários fatores incluindo a origem microbiana da enzima; as condições experimentais, tais como temperatura, pH, concentração de substrato e produto, e o tipo de substrato empregado; e os diferentes métodos para cálculo de K_m, se por linearização ou regressão não linear (Samoshina & Samoshin, 2005). Valores entre 4,5 a 132 mM foram encontrados para K_m de enzimas obtidas de fungos (Wierzbicki & Kosikowski, 1973; Shukla & Chaplin, 1993), já para enzimas de leveduras os valores de K_m foram menores, entre 2,5 até 77 mM (Fontes et al. 2001; Bernal & Pavel, 1985).

Muitos outros fatores podem afetar a atividade e estabilidade enzimática como a força iônica do meio, pressão, o tampão empregado, a pureza dos reagentes e da enzima. Todos estes fatores devem ser experimentalmente determinados ou no mínimo, escolhidos arbitrariamente e mantidos constantes durante estudos (Shuler e Kargi, 2002).

2.3.2 Permeabilização de células

A β-galactosidase produzida por *K. marxianus* é uma enzima intracelular, portanto, as aplicações industriais desta enzima são limitadas (Panesar, 2008). A obtenção de extratos livres de células é trabalhosa, pois necessita da ruptura das mesmas, e pode causar a inativação da enzima. Portanto, a permeabilização celular tem sido recomendada como um método alternativo para o estudo das reações enzimáticas intracelulares (Alamäe & Järviste, 1995).

A permeabilização celular modifica a estrutura da membrana citoplasmática diminuindo o conteúdo de fosfolipídios, permitindo a entrada e saída de solutos (Becker et al., 1996). As células permeabilizadas podem ser consideradas portadoras de enzimas as quais permanecem em um estado naturalmente imobilizado (Somkuti et al., 1998).

Na literatura, diferentes métodos têm sido descritos para a permeabilização de células, empregando solventes, como: etanol (Panesar et al., 2007), isopropanol (Kondo et al., 2000), butanol (Champluvier et al., 1989), acetona (Cánovas et al. 2005) e tolueno

(Zhou et al., 2006); e os detergentes: brometo de cetiltrimetilamônio (Presecki et al 2007), digitonin (Alamäe & Järviste, 1995), Triton (Galabova et al. 1996) eTween (Cánovas et al., 2005).

Decleire et al. (1987) compararam vários solventes para a permeabilização das células de *Kluyveromyces* sp. contendo β-galactosidase. Os agentes permeabilizantes empregados foram: n-butanol, propanol, isopropanol, terc-butanol, etanol e acetona. As concentrações dos agentes variaram de 10 a 70%. As maiores atividades enzimáticas foram obtidas empregando n-butanol 10%, propanol 20%, isopropanol 30%, terc-butanol 30%, etanol 40% e acetona 40%. Os autores observaram uma diminuição de 30% no conteúdo de fosfolipídios das células da levedura.

Bachhawat et al. (1996) estudaram a hidrólise da lactose empregando células de *Kluyveromyces fragilis* permeabilizadas com brometo de cetiltrimetilamônio. Mais de 90% da lactose presente no soro de queijo foi hidrolisada em 2h de reação com 0,5% (p/v) de células permeabilizadas com brometo de cetiltrimetilamônio 0,1% (p/v).

Panesar et al. (2007) empregaram células de *Kluyveromyces marxianus* permeabilizadas com etanol, na hidrólise da lactose. A concentração de etanol na permeabilização variou de 20 a 70%, onde os autores observaram máxima atividade enzimática ao empregar etanol 50%. Concentrações superiores diminuem a atividade enzimática, provavelmente devido a saída da enzima pela parede celular ou lise da célula, em baixas concentrações do agente permeabilizante foram observadas baixas atividades enzimáticas devido a quantidade insuficiente do agente para uma efetiva permeabilização. As células permeabilizadas contendo β-galactosidase hidrolisaram 89% da lactose presente no leite desnatado empregando 120 mg de células, 30ºC, 80 rpm, por 150 min.

2.4 Reações enzimáticas em fluidos pressurizados

O uso de fluidos pressurizados como meio de reação tem sido intensamente pesquisado devido às propriedades favoráveis de transporte destes fluidos, que podem acelerar a transferência de massa das reações, facilitar a separação e a recuperação de produtos e/ou reagentes. Como exemplo de fluidos pressurizados pode-se citar o CO_2 supercrítico, propano e n-butano (Oliveira, 1999).

Em função dessas características favoráveis, os fluidos pressurizados começaram a ser estudados como meio potencial para a catálise enzimática. O uso de solventes não aquosos para reações enzimáticas são atrativos por várias razões. Uma enzima em um solvente não aquoso pode possuir interações solvente/enzima similares àquelas de seu meio nativo e, então, mostrar atividade maior quando comparada a água pura. Os substratos podem, também, ser mais solúveis em um solvente não aquoso, fazendo com que as taxas de reação sejam maiores neste tipo de solvente. Quando alguns destes solventes orgânicos (CO_2, butano, propano) são pressurizados, têm-se outras vantagens no emprego em reações enzimáticas, tais como: a termoestabilidade de biomoléculas em fluidos pressurizados é maior que na água e existe a possibilidade de reciclagem do solvente (Dordick, 1989; Klibanov, 1997; Oliveira, 1999).

Muitas enzimas quando expostas a alta pressão, têm sua estrutura tridimensional alterada de maneira significativa, levando a desnaturação e consequente perda da atividade enzimática, porém a alta pressão também pode induzir ao aumento da estabilidade e atividade de algumas enzimas (Eisenmenger & Reyes-de-Corcuera, 2009).

Vários autores têm proposto o mecanismo detalhado do comportamento das enzimas em solventes pressurizados, mas as conclusões têm sido contraditórias. Alguns declaram que as enzimas perdem sua atividade durante a reação por causa da pressurização, outros alegam que é o processo de despressurização o fator relevante na perda de atividade das enzimas (Oliveira, 1999).

Penniston (1971) observou que em sistemas aquosos, pressões abaixo de 1000 bar não apresentaram efeito significativo na atividade enzimática. Os grupos de Blanch e Chark (Randolph et al., 1985), usando espectroscopia de ressonância magnética a alta pressão, não observaram qualquer mudança significativa na estrutura da colesterol oxidase em misturas de CO_2 – solvente a 35ºC entre 1 e 113 bar. Affleck et al. (1994) mostraram não haver mudança conformacional considerável na subtilisina a 700 bar em n-hexano. Em contraste, estudos usando espectroscopia de fluorescência mostraram que a tripsina sofreu uma mudança conformacional em CO_2 supercrítico durante a despressurização. Contudo, nenhum dado de atividade foi reportado para determinar os efeitos de tais mudanças estruturais (Oliveira, 1999).

A hipótese mais aceita com relação a perda de atividade enzimática em meio supercrítico sugere que a taxa de despressurização do CO_2 supercrítico e o conteúdo de água do solvente têm um efeito direto na taxa de desnaturação e que enzimas que contêm pontes de dissulfeto são menos propensas a desnaturação. Taniguchi et al. (1987) examinaram o efeito do CO_2 supercrítico em 9 enzimas comerciais, divididas entre oxidoredutases, hidrolases, isomerases e transferases, a 35ºC e 203 bar. Após 1 hora de exposição, os autores não observaram perda na atividade das enzimas. Kasche et al. (1988) mostraram que a atividade de algumas enzimas foi adversamente afetada pela despressurização. As enzimas com pontes dissulfeto, α-quimiotripsina e tripsina, foram desnaturadas em menor grau que a enzima penicilina amidase que não contém cisteína.

Steinberger et al. (1999) avaliaram o efeito isolado da despressurização em sistemas contendo CO_2-esterase e CO_2–lipase a 35ºC e 150 bar. Após a realização de ciclos consecutivos de pressurização/despressurização, a atividade residual das duas enzimas foi determinada. No final deste procedimento, a lipase manteve 92,2±9,3% de sua atividade, enquanto a esterase manteve 102,3±3,8%, mostrando que a despressurização, isoladamente, não afetou a atividade das enzimas estudadas.

Oliveira et al. (2006) investigaram a influência da temperatura (35 - 75°C), pressão (10 - 280 bar), tempo de exposição (1 - 6 horas) e a taxa despressurização (2 - 50 bar/min para propano e n-butano e 10 - 200 kg/m^3.min para dióxido de carbono) na atividade das enzimas lipolíticas Novozym 435 e Lipozyme IM. Os resultados mostraram que a Lipozyme IM apresentou perda de atividade para os três solventes, sendo esta perda maior na presença de dióxido de carbono (0,5 - 13%), seguida pelo propano (1,1 - 8,9%), já para o n-butano a enzima teve apenas uma pequena perda de atividade (0,5 - 3,6%). A Novozym 435 apresentou perda da atividade na presença de CO_2 (1,3 - 8,9%), mas teve um ganho de atividade, que variou de 1,7 a 14% e 1,8 a 21,5%, quando submetida ao propano e n-butano, respectivamente.

2.4.1 β-galactosidase em fluidos pressurizados: estabilidade e reação

Na literatura há poucos relatos sobre atividade enzimática e estabilidade da enzima β-galactosidase quando submetida a altas pressões hidrostáticas (Degraeve et al., 1996;

Athès et al., 1997; Degraeve & Lemay 1997; Athès & Combes, 1998;), e há uma falta de informação experimental para esta enzima, tanto de estabilidade quanto de reação enzimática, em dióxido de carbono supercrítico, propano e n-butano pressurizados.

Athès et al. (1997) investigaram o aumento da termoestabilidade sob alta pressão da β-galactosidase de *A. oryzae*, *K. lactis* e *E. coli*. Os autores empregaram água como fluido pressurizado. Após 1 h de incubação a 25°C a atividade enzimática residual não apresentou decréscimo significativo quando a enzima de *A. oryzae* foi tratada a 450 MPa, a enzima de *E. coli* a 300 MPa e a β-galactosidase de *K. lactis* submetida a 200 MPa.

Para as três enzimas testadas, pressões moderadas protegeram a enzima da inativação térmica. A atividade residual da enzima de *E. coli* aumentou de 60% em 0,1 MPa para 80% em 100 MPa a 55°C. A atividade residual da β-galactosidase de *K. lactis* tratada a 100 MPa aumentou 5 vezes em comparação com a enzima tratada a 0,1 MPa a 45°C. Um dado interessante foi observado na atividade residual da enzima de *A. niger*, a qual foi completamente inativada a 0,1 MPa, 55°C e incubada por 1h, no entanto manteve praticamente 100% de sua atividade residual quando tratada a 250 MPa. Em outro trabalho, Degraeve & Lemay (1997), avaliaram a meia vida da β-galactosidase de *E. coli* tratada a alta pressão a 55°C. Os autores observaram que a 250 MPa a meia vida foi de 32 h, já a 0,1 MPa a meia vida foi de 1,5 h.

2.5 Considerações

Tendo como base os aspectos apresentados no decorrer deste capítulo no que se refere ao potencial de aplicação de galacto-oligossacarídeos e a escassez de informações sobre a síntese de GOS empregando células permeabilizadas, além da inexistência de relatos na literatura concernentes ao comportamento da enzima β-galactosidase em fluidos pressurizados, bem como a síntese de GOS sob alta pressão, desenvolveu-se o presente trabalho.

2.6. Referências Bibliográficas

Affleck, R. A.; Clark, D. S.; Kamat, S. V. E Russel, A. J. High pressure EPR studies of protein mobility in reversed micelles. **Biotechnology and Bioengineering**, v. 43, n. 4, p. 342-348, 1994.

Alamäe, T.; Järviste, A. Permeabilization of the methylotrophic yeast *Pichia pinus* for intracellular enzyme analysis: a quantitative study. **Journal of Microbiological Methods**, v. 22, p. 193-205, 1995.

Albayrak, N.; Yang, S. T. Production of galacto-oligosaccharides from lactose by *Aspergillus oryzae* β-galactosidase immobilized on cotton cloth. **Biotechnology and Bioengineering**, v. 77, p. 9-18, 2002.

Alves, F. G.; Maugeri Filho, F.; Burkert, J. F. M.; Kalil, S. J. Maximization of β-galactosidase production: a simultaneous investigation of agitation and aeration effects. **Applied Biochemistry and Biotechnology**, v. 160, n. 5, p. 1528-1539, 2010.

Aronson, M. Transgalactosylation during lactose hydrolysis. **Archives of Biochemistry and Biophysics**, v. 39, p. 370, 1952.

Athès, V.; Combes, D. Influence of additives on high pressure stability of β-galactosidase from *Kluyvermyces lactis* and invertase from *Saccharomyces cerevisiae*. **Enzyme and Microbial Technology**, v. 22, p. 532-537, 1998.

Athès, V.; Degraeve, P.; Cavaillé-Lefebvre, D.; Espeillac, S.;Lemay, P.; Combes, D. Increased thermostability of three mesmophilic β-galactosidases under high pressure. **Biotechnology Letters**, v. 19, n. 3, p. 273-276, 1997.

Bachhawat, N.; Gowda, L. R.; Bhat, S. G. Single step method of preparation of detergent-permeabilized *Kluyveromyces fragilis* for lactose hydrolysis. **Process Biochemistry**, v. 31, n. 1, p.21-25, 1996.

Bansal, S.; Oberoi, Harinder, S.; Dhillon, G. S.; Patil, R. T. Production of β-galactosidase by *Kluyveromyces marxianus* MTCC 1388 using whey and effect of four different methods of enzyme extraction on β-galactosidase activity. **Indian Journal of Microbiology,** v. 48, p. 337-341, 2008.

Becerra, M.; Rodríguez-Belmonte E.; Cerdán, M. E.; Siso, M. I. G. Engineered autolytic yeast strains secreting *Kluyveromyces lactis* β-galactosidase for production of

heterologous proteins in lactase media. **Journal of Biotechnology,** v. 109. p.131-137, 2004.

Becker, J. M.; Caldwell, G. A.; Zachgo, E. A. Exercise 15 - Determination of β-Galactosidase in Permeabilized Yeast Cells. In: Becker, J M.; Caldwell, G. A.; Zachgo, E. A. **Biotechnology: a technology course.** 2. ed. Academic Press, 1996. p. 131-134.

Berger, J. L.; Lee, B. H.; Lacroix, C. Purification, properties and characterization of a high molecular-mass β-galactosidase isoenzyme from *Thermus aquaticus*. **Biotechnology and Applied Biochemistry**, v. 25, p. 29–41, 1995.

Bernal, V. Pavel, J. Lactose hydrolysis by *Kluyveromyces lactis* β-D-galactosidase in skim milk, whey, permeate and model system. **Canadian Institute of Food Science And Technolog,** v. 18, p. 97-99, 1985.

Boon M. A., Janssen A. E. M., and Riet, K. V. Effect of temperature and enzyme origin on the enzymatic synthesis of oligosaccharides. **Enzyme and Microbial Technology**, v. 26, p. 271-281, 2000.

Bornet, F. R. Undigestible sugars in food products. **The American Journal of Clinical Nutrition**, v. 59, n. 3, Suppl., p. 763S-769S, 1994.

Cánovas, M.; Torroglosa, T.; Iborra, J. L. Permeabilization of *Escherichia coli* cells in the biotransformation of trimethylammonium compounds into L-carnitine. **Enzyme and Microbial Technology**, v. 37, p.300-308, 2005.

Champluvier, B.; Marchal, F.; Rouxhet, P. G. Immobilization of lactase in yeast cells retained in a glass wool matrix. **Enzyme and Microbial Technology**, v. 11, p. 422-430, 1989.

Chen, C. W.; Ou-Yang, C. C.; Yeh, C. W. Synthesis of galactooligosaccharidesand transgalactosylation modeling in reverse micelles. **Enzyme and Microbial Technology**. v. 33, p. 497–507, 2003.

Chen, W.; Chen, H.; Xia, Y.; Yang, J.; Zhao, J.; Tian, F.; Zhang, H. P.; Zhang H. Immobilization of recombinant thermostable β-galactosidase from *Bacillus stearothermophilus* for lactose hydrolysis in milk. **Journal of Dairy Science**, v. 92, n. 2, p. 491-498, 2009.

Cho, Y. J.; Shin, H. J.; Bucke, C. Purification and biochemical properties of a galactooligosaccharide producing β-galactosidase from *Bullera singularis*. **Biotechnology Letters,** v. 25, p. 2107–2111, 2003.

Chockchaisawasdee, S.; Athanasopoulos, V. I.; Niranjan, K.; Rastall, R. A. Synthesis of galacto-oligosaccharide from lactose using β-galactosidase from *Kluyveromyces lactis*: Studies on batch and continuous UF membrane-fitted bioreactors. **Biotechnology and Bioengineering**, v. 89, p. 434–443, 2005.

Conesa, D.P., Lopez, G. and Ros, G. Effects of probiotic, prebiotic and symbiotic follow-up infant formulas on large intestine morphology and bone mineralisation in rats. **Journal of the Science of Food and Agriculture**, v. 87, p. 1059-1068, 2007.

Cortés, G.; Trujillo-Roldán, M. A.; Ramírez, O. T.; Galindo, E. Production of β-galactosidase by *Kluyveromyces marxianus* under oscillating dissolved oxygen tension. **Process Biochemistry**, v. 40, p. 773-778, 2005.

Cruz, R.; D'Arcadia Cruz, V.; Belote, J. G.; Khenayfes, M. O.; Dorta, C.; Oliveira, L. H. S; Ardiles, E.; Galli, A. Production of transgalactosylated oligosaccharides (TOS) by galactosyltransferase activity from *Penicillium simplicissimum*. **Bioresource Technology**, v. 70, p. 165–171, 1999.

Curda, L., Rudolfová, J., Stetina, J., and Dryák, B. Dried buttermilk containing galacto-oligosaccharides process layout and its verification. **Journal of Food Engineering**, v. 77, p. 468-471, 2006.

Dagbagli, S.; Goksungur, Y. Optimization of β-galactosidase production using Kluyveromyces lactis NRRL Y-8279 by response surface methodology. **Eletronic Journal of Biotechnology**, v. 11, n. 4, p. 1-12, 2008.

Decleire, M.; Cat, W.; Van Huynh, N. Comparison of various permeabilization treatments on *Kluyveromyces* by determining in situ β-galactosidase activity. **Enzyme and Microbial Technology**, v. 9, p.300-302, 1987.

Degraeve, P.; Delorme, P.; Lemay, P. Pressure-induced inactivation of E. coli β-galactosidase: influence of pH and temperature. **Biochimica et Biophysica Acta**, v. 1292, p. 61-68, 1996.

Degraeve, P.; Lemay, P. High pressure-induced modulation of the activity and stability of E. coli (lac Z) β-galactosidase: potential applications. **Enzyme and Microbial Technology**, v. 20, p. 550-557, 1997.

Domingues, L.; Oliveira, C.; Castro, I., Lima, N.; Teixeira, J. A. Production of *β*-galactosidase from recombinant *Saccharomyces cerevisiae* grown on lactose. **Journal of Chemical Technology and Biotechnology**, v. 79, p. 809-815, 2004.

Dordick, J.S. Enzymatic catalysis in monophasic organic solvents. **Enzyme Microbiology and Technology**, v.11, p.194-211, 1989.

Eisenmenger, M. J.; Reyes-De-Corcuera, J. I. High pressure enhancement of enzymes: a review. **Enzyme and Microbial Technology**, v. 45, 331-347, 2009.

Flowers, H. M. Chemical synthesis of oligosaccharides. **Methods in Enzymology**, v. 50, p. 93-121, 1978.

Fontes, E. A. F.; Passos, F. M. L.; Passos, F. J. V. A mechanistical mathematical model to predict lactose hydrolysis by β-galactosidase in a permeabilized cell mass of *Kluyveromyces lactis*: validity and sensitivity analysis. **Process Biochemistry**, v. 37, p. 267-274, 2001.

Fooks, L. J.; Fuller, R.; Gibson, G. R. Prebiotics, probiotics and human gut microbiology. **International Dairy Journal**, v. 9, p. 53-61, 1999.

Galabova, D.; Tuleva, B.; Spasova, D. Permeabilization of *Yarrowia lipolytica* cells by triton X-100. **Enzyme and Microbial Technology**, v. 18, p.18-22, 1996.

Gekas, V.; López-Leiva, M. Hydrolysis of lactose: a literature review. **Process Biochemistry**, p.2-12, 1985.

Gibson, G. R., Roberfroid M. B. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. **Journal of Nutrition**, v. 125, p.1401-1412, 1995.

Goulas, A.; Tzortzis, G.; Gibson, G. R. Development of a process for the production and purification of α- and β-galactooligosaccharides from *Bifidobacterium bifidum* NCIMB 41171. **International Dairy Journal**, v. 17, p. 648-656, 2007.

Holsinger, V. H.; Kligerman, A. E. Applications of lactase in dairy foods and other foods containing lactose. **Food Technology,** v. 45, p. 92-95, 1991.

Hsu, C. A.; Yu, R. C.; Chou, C. C. Production of β-galactosidase by bifidobacteria as influenced by various culture conditions. **International Journal of Food Microbiology,** v. 104, p. 197-206, 2007.

Hung, M. N.; Lee, B. H. Purification and characterization of a recombinant β-galactosidase with transgalactosylation activity from *Bifidobacterium infantis* HL96. **Applied Microbiology and Biotechnology**, v. 58, p. 439–445, 2002.

Husain, Q. β Galactosidases and their potential applications: a review. **Critical Reviews in Biotechnology**, v. 30, n. 1, p. 41–62, 2010.

Inchaurrondo, V. A.; Flores, M. V.; Voget, C. E. Growth and β-galactosidase synthesis in aerobic chemostat cultures of *Kluyveromyces lactis*. **Journal of Industrial Microbiology & Biotechnology,** v. 20, p. 291-298, 1998.

Iwasaki, K.; Nakajima, M.; Nakao, S. Galacto-oligosaccharide production from lactose by an enzymic batch reaction using β- galactosidase. **Process Biochemistry**, v. 31, p. 69–76, 1996.

Ji, E. S.; Park, N. H.; Oh, D. K. Galacto-oligosaccharide production by a thermostable recombinant β-galactosidase from *Thermotoga maritima*. **World Journal of Microbiology and Biotechnology,** v. 21, p. 759–764, 2005.

Kasche, V.; Scholothaues, R. E.; Brunner, G. Enzyme denaturation in supercritical CO_2: stabilizing effect of S-S bonds during the depressurization step. **Biotechnology Letters**, v.10, n.8, pp.569, 1988.

Kaur, G.; Panesar, P. S.; Bera, M. B.; Kumar, H. Hydrolysis of whey lactose using CTAB-permeabilized yeast cells. **Bioprocess and Biosystems Engineering,** v.32, n. 1, p. 63-67, 2009.

Kim, C. S.; Ji. E. E Oh, D. A new kinetic model of recombinante β-galactosidase from *Kluyveromyces lactis* for both hydrolysis and transgalactsylation reactions. **Biochemical and Biophysical Research Communications**, v. 316, p. 738-743, 2004.

Klibanov, A. M. Why are enzymes less active in organic solvents than in water? **Trends in Biochemical Science**, v.1, p.19-26, 1997.

Kondo, A.; Liu, Y.; Furuta, M.; Fujita, Y.; Matsumoto, T.; Fukuda, H. Preparation of high activity whole cell biocatalyst by permeabilization of recombinant flocculent yeast with alcohol. **Enzyme and Microbial Technology,** v. 27, p. 806-811, 2000.

López-Leiva, M. H.; Guzman, M. Formation of oligosaccharides during enzymic hydrolysis of milk whey permeates. **Process Biochemistry**, v. 30, n. 8, p. 757-762, 1995.

Mahoney, R. R. Galactosyl-oligosaccharide formation during lactose hydrolysis: a review. **Food Chemistry**, v. 63, n. 2, p.147-154, 1998

Manera, A. P.; Ores, J. C.; Ribeiro, V. A.; Burkert, C. A. V.; Kalil, S. J. Optimization of the culture medium for the production of β-galactosidase from *Kluyveromyces marxianus* CCT 7082. **Food Technology and Biotechnology**, v.46, n. 1, p. 66-72, 2008.

Martínez-Villaluengua, C.; Cardelle-Cobas, A.; Corzo, N.; Olano, A.; Villamiel, M. Optimization of conditions for galactooligosaccharide synthesis during lactose hydrolysis by β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000 L HP G). **Food Chemistry**, v. 107, p. 258-264, 2008

Martins, D. B. G.; Souza, C. G.; Simões, D. A.; Morais, M. A. The β-galactosidase activity in *Kluyveromyces marxianus* CBS 6556 decreases by high concentrations of galactose. **Current Microbiology,** v. 44, p. 379-382, 2002.

Nagy, Z.; Kiss, T.; Szentirmai, A.; Biró, S. β-galactosidase of *Penicillium chrysogenum:* production, purification, and characterization of the enzyme. **Protein Expression and Purification,** v. 21, p. 24-29, 2001.

Nakagawa, T.; Ikehata, R.; Uchino, M.; Miyaji, T.; Takano, K.; Tomizuka, N. Cold-active acid β-galactosidase activity of isolated psychrophilic-basidiomycetous yeast *Guehomyces pullulans*. **Microbiological Research,** v. 161, p. 75-79, 2006.

Nakao, M.; Hatada, M.; Kodama, Y.; Nakayama, T.; Shibano, Y.; Amachi, T. Purification and characterization of a thermostable β-galactosidase with high transgalactosylation activity from *Saccharopolyspora rectivirgula*. **Applied Microbiology and Biotechnology**, v. 40, p. 657–663, 1994.

Nakkharat, P.; Kulbe, K. D.; Yamabhai, M.; Haltrich, D. Formation of galacto-oligosaccharides during lactose hydrolysis by a novel β-galactosidase from the moderately thermophilic fungus *Talaromyces thermophilus*. **Biotechnology Journal**, v. 1, p. 633–638, 2006.

Nguyen, T. H.; Splechtna, B.; Krasteva, S.; Kneifel, W.; Kulbe, K. D.; Divne, C.; Haltrich, D. Characterization and molecular cloning of a heterodimeric β-galactosidase from the probiotic strain *Lactobacillus acidophilus* R22. **FEMS Microbiology Letters**, v. 269, p. 136–144, 2007.

Numanoglu, Y. E Sungur, S. β-galactosidase from *Kluyveromyces lactis* cell disruption and enzyme immobilization using a cellulose-gelatin carrier system. **Process Biochemistry,** v. 39, p. 703-709, 2004.

Oliveira, D. **Estudo comparativo da produção enzimática de ésteres a partir de óleos vegetais em solventes orgânicos e CO_2 supercrítico**. Tese de Doutorado. Rio de Janeiro, 1999.

Oliveira, D.; Feihrmann, A. F.; Rubira, A. F.; Kunita, M. H.; Dariva, C.; Oliveira, J. V. Assessment of two immobilized lipases activity treated in compressed fluids. **The Journal of Supercritical Fluids**, v. 38, p. 373-382, 2006.

Onishi, N.; Kira, I.; Yokozeki, K. Galacto-oligosaccharide production from lactose by *Sirobasidium magnum* CBS6803. **Letters in Applied Microbiology**, v. 23, p. 253–256, 1996.

Onishi, N.; Tanaka, T. Purification and properties of a novel thermostable galacto-oligosaccharide-producing β-galactosidase from *Sterigmatomyces elviae* CBS8119. **Applied and Environmental Microbiology**, v. 61, p. 4026–4030, 1995.

Onishi, N.; Yokozeki, K. Gluco-oligosaccharide and galactooligosaccharide production by *Rhodotorula minuta* IFO879. **Journal of Fermentation and Bioengineering**, v. 82, p. 124–127, 1996.

Ornelas, A. P.; Silveira, W. B.; Sampaio, F. C, Passos, F. M. The activity of beta-galactosidase and lactose metabolism in *Kluyveromyces lactis* cultured in cheese whey as a function of growth rate. **Journal Applied Microbiolology**. v. 104, n. 4, p. 1008-13, 2008.

Otieno, D. O. Synthesis of β-galactooligosaccharides from lactose using microbial β-galactosidases. **Comprehensive Reviews in Food Science and Food Safety,** v. 9, n. 5, p. 471–482, 2010.

Panesar, P. S. Application of response surface methodology for maximal lactose hydrolysis in whole milk using permeabilized yeast cells. **Acta Alimentaria**, v. 37, v. 2, p. 191-203, 2008.

Panesar, R.; Panesar, P. S.; Singh, R. S.; Kennedy, J. F.; Bera, M. B. Production of lactose-hydrolyzed milk using ethanol permeabilized yeast cells. **Food Chemistry**, v. 101, p.786-790, 2007.

Park, H. Y.; Kim, H. J.; Lee, J. K.; Kim, D.; Oh, D. K. Galactooligosaccharide production by a thermostable β-galactosidase from *Sulfolobus solfataricus*. **World Journal of Microbiology and Biotechnology**, v. 24, p. 1553–1558, 2008.

Pazur, J. H. The Mechanism of enzymatic synthesis of galactosyl-oligosaccharides. **The Journal of Biological Chemistry**, v. 208, p. 439-444, 1954.

Penniston, J.T. High hydrostatic pressure and enzymic activity: Inhibition of multimeric enzymes by dissociation. **Archives of Biochemistry and Biophysics**, v.142, p.322-332, 1971.

Pinheiro, R.; Belo, I. E Mota, M. Growth and β-galactosidase activity in cultures of *Kluyveromyces marxianus* under increased air pressure. **Letters in Applied Microbiology,** v. 37, p. 438-442, 2003.

Placier, G.; Watzlawick, H.; Rabiller, C.; Mattes, R. Evolved β-galactosidases from Geobacillus stearothermophilus with improved transgalactosylation yield for galacto-oligosaccharides production. **Applied and Environmental Microbiology**, v. 75, n. 19, p. 6312-6321, 2009.

Pocedicová, K.; Curda, L.; Misún, d.; Dryáková, A.; Diblíková, L. Preparation of galacto-oligosaccharides using membrane reactor. **Journal of Food Engineering**, v. 99, n. 4, p. 479-484, 2010.

Presecki, A. V.; Zelic, B.; Vasic-Racki, D. Comparison of the L-malic acid production by isolated fumarase and fumarase in permeabilized baker's yeast cells. **Enzyme and Microbial Technology**, v. 41, p. 605–612, 2007.

Rajoka, M. I.; Khan, S.; Shahid R. Kinetics and regulation studies of the production of β-galactosidase from *Kluyveromyces marxianus* grown on different substrates. **Food Technology & Biotechnology**, v. 41, p. 315-520, 2003.

Ramírez Matheus, A. L.; Rivas, N. Producción y caracterización parcial de β-galactosidase de *Kluyveromyces lactis* propagada en suero de leche desproteinizado. **Archivos Latinoamericanos de Nutrición,** v. 53, n.2, p. , 2003.

Randolph, T.W.; Blanch, H.W.; Prausnitz, J.M.; Wilke, C.R. Enzymatic catalysis in a supercritical fluid. **Biotechnology Letters**, v.7, p. 325-328, 1985.

Rao, M. V. R.; Dutta, S. M. Production of β-galactosidase from *Streptococcus thermophilus* grown in whey. **Applied and Environmental Microbiology**, v. 34, n. 2, p. 185-188, 1977.

Rech, R. **Aproveitamento do soro de queijo para a produção de lactase pro *Kluyveromyces marxianus***. Porto Alegre, 1998. 75 f. Dissertação (Mestrado em Engenharia Química) – Universidade Federal do Rio Grande do Sul.

Rivero-Urgell, R. M.; Santamaria-Orleans, S. Oligosaccharides: Application in infant food. **Early Human Development**. v. 66, s43-s52, 2001.

Roberfroid, M. Prebiotics: the concept revisited. **The Journal of Nutrition**. v. 137, p. 830S-837S, 2007.

Robinson D. S. **Bioquímica y valor nutritivo de los alimentos**. Zaragoza: Editorial Acribia, 1991.

Rodriguez, A. P.; Leiro, R. F.; Trillo, M. C.; Cerdán, M. E.; Siso, M. I. G.; Becerra, M. Secretion and properties of a hybrid *Kluyveromyces lactis-Aspergillus niger* β-galactosidase. **Microbial Cells Factories**. v. 5, n. 41, p. 1-13, 2006.

Rodriguez-Nogales, J. M.; Delgadillo, A. Stability and catalytic kinetics of microencapsulated β-galactosidase in liposomes prepared by the dehydration-rehydration method. **Journal of Molecular Catalysis B: Enzimatic,** v. 33, p. 15-21, 2005.

Sakai. T.; Tsuji, H.; Shibata, S.; Hayakawa, K.; Matsumoto, K. Repeated-batch production of galactooligosaccharides from lactose at high concentration by using alginate-

immobilized cells of *Sporobolomyces singularis* YIT 10047. **The Journal of General and Applied Microbiology**, v. 54, p. 285–293, 2008.

Sako, T., Matsumoto, K., and Tanaka, R. (1999). Recent progress on research and application of non-digestible Galacto-Oligosaccharides. **International Dairy Journal**, v. 9, p.69-80, 1999.

Samoshina, N. M.; Samoshin, V. V. The Michaelis constants ratio for two substrates with a series of fungal (mould and yeast) β-galactosidase. **Enzyme and Microbial Technology.** v. 36, p. 239-251, 2005.

Santiago, P. A.; Marquez, L. D. S.; Cardoso, V. L.; Ribeiro, E. J. Estudo da produção de β-galactosidase por fermentação de soro de queijo com *Kluyveromyces marxianus*. **Ciência e Tecnologia de Alimentos**, v. 24, n. 4, p. 567-572, 2004.

Santos, R.; Simiqueli, A. P. R.; Pastore, G. M. Produção de galacto-oligossacarídeo por *Scopulariopis* sp. **Ciência e Tecnologia de Alimentos**. v. 29, n. 3, p. 682-689, 2009.

Shin, H. J.; Yang, J. W. Enzymatic production of galactooligosaccharide by *Bullera singularis* β-galactosidase. **Journal of Microbiology and Biotechnology**, v. 8, p. 484–489, 1998.

Shukla, H.; Chaplin M. Noncompetitive inhibition of β-galactosidase (*A. oryzae*) by galactose. **Enzyme and Microbial Technology**, v. 15, p. 297-299, 1993.

Shuler, M. L.; Kargi, F. **Bioprocess enginnering**: Basic concepts, 2nd ed., UpperSaddle River: Prentice Hall PTR, 553 p. 2002.

Singh, A. K.; Sinha, S.; Singh, K. Study on β-Galactosidase Isolation, Purification and Optimization of Lactose Hydrolysis in Whey for Production of Instant Energy Drink. **International Journal of Food Engineering**: v. 5, n. 2, Article 5, 2009.

Somkuti, G. A.; Dominiecki, M. E.; Steinberg, D. H. Permeabilization of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus* with ethanol. **Current Microbiology**, v. 36, p.202-206, 1998.

Song, C.; Chi, Z.; Li, C.; Wang, X. β-Galactosidase production by the psychrotolerant yeast *Guehomyces pullulans* 17-1 isolated from sea sediment in Antarctica and lactose hydrolysis. **Bioprocess and Biosystems Engineering**. v. 33, n. 9, p. 1025-1031, 2010.

Splechtna B.; Nguyen, T.-H.; Steinböck, M.; Kulbe, K.D.; Lorenz, W.; Haltrich, D. Production of prebiotic galacto-oligosaccharides from lactose using β-galactosidases from *Lactobacillus reuteri*. **Journal of Agriccultural and Food Chemistry**, v. 54**,** p. 4999–5006, 2006.

Steinberger, D.; Gamse, T. E; Marr, R. Enzyme inactivation and prepurification effects of supercritical carbon dioxide". In: **Proceedings of Fifth Conference on Supercritical Fluids and their Applications,** p. 339-346, 1999.

Taniguchi, M.; Kamihira, M.; Kobayashi, T. Effect of treatment with supercritical carbon dioxide on enzymatic activity. **Agricultural Biological Chemistry,** v.51, n.2, p. 593-596, 1987.

Tzortzis, G.; Goulas, A. K.; Gibson, G. R. Synthesis of prebiotic galactooligosaccharides using whole cells of a novel strain, *Bifidobacterium bifidum* NCIMB 41171. **Applied Microbiology and Biotechnology**, v. 68, p. 412–416, 2005.

Vasiljevic, T. E Jelen, P. Production of β-galactosidase for lactose hydrolysis in milk and dairy products using thermophilic lactic acid bacteria. **Innovative Food Science e Emerging Technologies,** v. 2, p. 75-85, 2001.

Wallenfels, K.; Malhotra, O. P. Galactosidases. **Advances Carbohydrate Chemistry**, v. 16, p.239-298, 1961.

Whitaker, J. R. **Principles of enzymology for the food sciences**. 2. ed. New York: Marcel Dekker Inc., 1994

Wierzbicki, L. E.; Kosikowski, F. V. Kinetis of lactose hydrolysis in acid whey by β-galactosidase from *Aspergillus niger*. **Journal of Dairy Science**, v. 56, p. 1396-1399, 1973.

Zheng, P.; Yu, H.; Sun, Z.; Ni, Y.; Zhang, W.; Fan, Y.; Xu, Y. Production of galacto-oligosaccharides by immobilized recombinant β-galactosidase from *Aspergillus candidus*. **Biotechnololgy Journal**, v. 1, p. 1464–1470, 2006.

Zhou, Q. Z. K.; Chen, X. D. Effects of temperature and pH on the catalytic activity of the immobilized β-Galactosidase from *Kluyveromyces lactis*. **Biochemical Engineering Journal**, v. 9, p. 33-40, 2001.

Zhou, Y.; Yuan, Q.; Gao, H.; Ma, R. Production of trehalose by permeabilized *Micrococcus* QS412 cells. **Journal of Molecular Catalysis B: Enzymatic**, v. 43, p.137-141, 2006.

CAPÍTULO 3

UTILIZAÇÃO DE SUBPRODUTOS AGROINDUSTRIAIS EM PROCESSO BIOTECNOLÓGICO PARA PRODUÇÃO DE β-GALACTOSIDASE DE Kluyveromyces marxianus CCT 7082

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RESUMO

O objetivo deste trabalho foi avaliar a composição do meio de cultura para a produção da enzima β-galactosidase de *Kluyveromyces marxianus* CCT 7082 utilizando a técnica de delineamento experimental. A produção da enzima foi realizada em meio composto por soro de queijo, água de maceração de milho (AMM) e hidrolisado de levedura Prodex-lac® e sais. Foi realizado um planejamento experimental fracionário (2^{5-1}) para determinar as variáveis significativas na produção da enzima. Foram testadas diferentes concentrações de lactose presente no soro de queijo (10 a 70 g/L), AMM (10 a 100 g/L), Prodex-lac (4 a 20 g/L), $(NH_4)_2SO_4$ (0 a 10 g/L) e o pH (5 a 7). As variáveis concentração de lactose, AMM e o pH apresentaram efeito estatisticamente significativo na atividade enzimática dentro das faixas estudadas, sendo estas variáveis empregadas num delineamento composto central rotacional para otimizar a produção da enzima. As faixas testadas foram: concentração de lactose de 40 a 100 g/L, AMM de 10 a 120 g/L e pH de 3,5 a 6,5. As condições que resultaram em maior atividade enzimática (1400 U/g) foram 70 g/L de lactose, 65 g/L de AMM, 4 g/L de Prodex-lac e pH 5, obtendo uma produtividade de 61 U/L.h.

Palavras-chave: delineamento experimental, soro de queijo, água de maceração de milho, Prodex-lac.

3.1 INTRODUÇÃO

O estudo de meios industriais de fermentação para obtenção de produtos biotecnológicos tem recebido grande atenção nos últimos anos. Diversos subprodutos e matérias-primas da indústria de alimentos e da agroindústria têm sido empregados para obtenção de produtos biotecnológicos, pela alta disponibilidade e por representarem fonte alternativa de baixo valor comercial (SILVA et al., 2009; ERNANDES et al., 2010).

Entre esses produtos biotecnológicos destaca-se a produção de enzimas, a qual é frequentemente limitada pelos custos dos substratos utilizados para o cultivo dos micro-organismos. Estima-se que por volta de 30-40% do custo envolvido na produção de enzimas é pelo meio de cultura utilizado para o crescimento dos micro-organismos, portanto sua otimização é de grande importância para a redução de custos (JOO; CHANG, 2005).

A enzima β-galactosidase (EC 3.2.1.23) encontra-se entre as enzimas de grande interesse industrial empregada na hidrólise da lactose de leites e soro de queijo, obtendo, assim, alimentos com baixos teores de lactose, cujo resultado é a melhora da solubilidade e da digestibilidade de leites e derivados lácteos, tornando-os ideais a consumidores intolerantes a este açúcar. Recentemente, têm sido empregada na produção de galacto-oligossacarídeos (GOS), açúcares funcionais que trazem diversos efeitos benéficos à saúde de seus consumidores, tais como redução de metabólitos tóxicos, prevenção do câncer de cólon, aumento da tolerância a lactose e da absorção de cálcio com consequente redução dos riscos de osteoporose (MAHONEY, 1998; CHONAN et al., 2001; MANNING & GIBSON, 2004).

Um dos principais subprodutos agroindustriais empregados na produção da enzima β-galactosidase como fonte de carbono é o soro de queijo (SANTIAGO et al., 2004; PANESAR, 2008; OBEROI, et al., 2008), porém de acordo com Vasiljevic e Jelen (2001), o crescimento do micro-organismo e a produção da enzima em meio, contendo somente soro de queijo, é praticamente desprezível, sendo necessária a sua suplementação com fontes de nitrogênio, sais minerais e vitaminas. Como alternativa para suplementação do soro, e visando o reaproveitamento de outros subprodutos, pode-se empregar a água de

maceração de milho, como fonte de nitrogênio e sais minerais em substituição à peptona; e o Prodex-lac, um produto comercial com composição similar do extrato de levedura, como fonte de nitrogênio e vitaminas.

Considerando que o Brasil gera quantidades expressivas de soro de queijo e água de maceração de milho, desenvolveu-se o presente trabalho para avaliar fontes alternativas de carboidrato, nitrogênio e vitaminas para produção da enzima β-galactosidase por *Kluyveromyces marxianus* CCT 7082 utilizando-se a técnica de delineamento experimental.

3.2 MATERIAL E MÉTODOS

3.2.1 Substratos agroindustriais

Os substratos agroindustriais utilizados foram: soro de queijo em pó cedido pela Cosulati – Pelotas/RS, composto por 45% de lactose, 12% de proteínas e 8% de sais minerais e vitaminas; e a água de maceração de milho (AMM), cedida pela Corn Products – Mogi Guaçu/SP, composta por 53% de matéria seca, 43% de proteínas bruta, 15,5% de sais minerais. O hidrolisado de levedura Prodex-lac® em pó foi adquirido da Indústria Prodesa - Mogi Mirim/SP, com 44% de proteínas e 0,32% de cloreto de sódio em sua composição.

3.2.2 Micro-organismo e condições de cultivo

A levedura *Kluyveromyces marxianus* CCT 7082, adquirida da Coleção de Culturas Tropicais (CCT) da Fundação André Tosello – Pesquisa e Tecnologia – Campinas/SP, previamente selecionada por Manera et al. (2008), foi empregada para produção da enzima β-galactosidase. O micro-organismo foi mantido em tubos com tampa de rosca contendo caldo YM com glicerol 10% (v/v) e congelados em freezer a –18ºC.

A enzima β-galactosidase foi produzida por fermentação submersa, em frascos Erlenmeyers de 500 mL contendo 150 mL de meio de cultura a 30ºC, 180 rpm (LUKONDEH et al. 2005). As fermentações iniciaram com 10% (v/v) de inóculo (NOR et al., 2001). As concentrações de lactose presente no soro de queijo, água de maceração de milho (AMM), Prodex-lac, $(NH_4)_2SO_4$ e o pH inicial do meio variaram de acordo com o

delineamento experimental (Tabela 3.1). As concentrações de KH_2PO_4 e de $MgSO_4.7H_2O$ foram fixadas em 5 g/L e 0,4 g/L, respectivamente, de acordo com Manera et al. (2008).

Antes de ser empregado nas fermentações, o soro de queijo, após dissolução em água destilada para atingir a concentração desejada de lactose, passou por um tratamento termoácido para desproteinização, o qual consistiu em ajustar o pH para 4,6 com acido acético e posterior aquecimento (90ºC por 15 min), e, em seguida, as proteínas precipitadas foram retiradas por filtração a vácuo (MATHEUS; RIVAS, 2003) e a solução de soro de queijo esterilizada por filtração com membrana de 0,2 μm (RAJOKA et al. 2003). A água de maceração de milho, após ser diluída na concentração desejada, foi centrifugada para retirada do material insolúvel de acordo com BURKERT (2003), e esterilizada por filtração com membrana de 0,2 μm. O Prodex-lac e os demais reagentes foram preparados com água destilada e esterilizados em autoclave a 121°C por 15 min.

3.2.3 Delineamento experimental

Os efeitos das variáveis na concentração de lactose presente no soro de queijo, água de maceração de milho, Prodex-lac, $(NH_4)_2SO_4$, e o pH na produção da β-galactosidase foram avaliados usando um delineamento experimental fracionário 2^{5-1} com a adição de três ensaios no ponto central. As faixas estudadas foram selecionadas de acordo com a literatura e estão apresentadas na Tabela 3.1.

Baseados nos resultados obtidos no delineamento fracionário, realizou-se um delineamento composto central rotacional (DCCR) de acordo com Rodrigues e Iemma, (2009). Foram realizados 17 ensaios (8 fatoriais, 6 axiais e 3 no ponto central) As variáveis independentes avaliadas foram concentração de lactose, concentração de AMM e o pH. Os níveis das variáveis estão apresentados na Tabela 3.1. A análise dos dados dos delineamentos experimentais foi realizada utilizando o programa STATISTICA versão 9.1.

Tabela 3.1: Níveis codificados e concentrações das variáveis estudadas nos delineamentos experimentais.

Níveis	Lactose (g/L)	AMM (g/L)	Prodex-lac (g/L)	$(NH_4)_2SO_4$ (g/L)	pH
Delineamento fracionário					
-1	10	10	4	0	5
0	40	55	12	5	6
+1	70	100	20	10	7
DCCR					
-1,68	40	10	-	-	3,5
-1	52,1	32,3	-	-	4,1
0	70	65	-	-	5
+1	87,9	97,7	-	-	5,9
+1,68	100	120	-	-	6,5

3.2.4 Extração da enzima

A enzima β-galactosidase é uma enzima intracelular. Para ruptura da parede celular adicionou-se 1,1 g de pérolas de vidro (R < 0,4 mm) para cada mL de célula em suspensão. A suspensão foi submetida a um tratamento com ondas ultrassônicas, por 10 min a 4°C, o sobrenadante foi coletado por centrifugação a 6.000 × *g* por 10 min a 4ºC e utilizado para determinação da atividade enzimática (MANERA et al., 2008).

Um mililitro de célula em suspensão foi obtido por ressuspensão em tampão KH_2PO_4 50 mM com $MnCl_2.4H_2O$ 0,1 mM pH 6,6 de células equivalente a 2,62 mg de células secas (NUMANOGLU; SUNGUR, 2004).

3.2.5 Determinações analíticas

A atividade enzimática foi determinada usando *o*-nitrofenil-β-D-galactopiranosideo (ONPG) como substrato, segundo metodologia descrita por Inchaurrondo et al. (1994). Em tubo de ensaio contendo 2 mL de ONPG 1,25 mM, preparado em tampão KH_2PO_4 50 mM com $MnCl_2.4H_2O$ 0,1 mM pH 6,6, foram adicionados 50 μL da solução enzimática. A solução foi mantida a 37 ºC por 5 min em banho agitado, a reação foi paralisada adicionando 0,5 mL de carbonato de sódio 1 M, em seguida determinou-se a absorbância a 420 nm, e calculou-se a atividade enzimática. Uma unidade de atividade enzimática (U) é

definida como 1 μmol de *o*-nitrophenol produzido por minuto, sob as condições do ensaio. O coeficiente de extinção molar do *o*-nitrophenol determinado experimentalmente nestas condições foi 4,64 $cm^2/\mu mol$.

A concentração celular foi estimada por leitura da absorbância a 620 nm e convertida para peso seco conforme curva-padrão. As células do caldo foram coletadas por centrifugação a 6.000 × *g*, 10 min, lavadas duas vezes com água destilada e ressuspendidas com água para leitura da absorbância. Para a curva-padrão as células foram secas a 90ºC até peso constante (LONGHI et al., 2004).

3.3 RESULTADOS E DISCUSSÃO

As condições experimentais testadas no delineamento experimental fracionário 2^{5-1} estão apresentadas na Tabela 3.2, bem como as respostas para a atividade enzimática máxima (U/g de massa seca) de cada ensaio e o tempo de fermentação em que ocorreu a máxima atividade. As atividades enzimáticas máximas obtidas neste delineamento variaram de 790,0 U/g até 1.733,7 U/g, e o tempo de fermentação necessário para atingir estes valores de atividade variaram de 24 a 72 h.

As maiores atividades, 1.733,7 e 1.718,5 U/g, foram obtidas nos ensaios 4 e 12, respectivamente, em 48h de fermentação. As condições destes ensaios foram 70 g/L de lactose, 100 g/L de AMM, 4 g/L de Prodex-lac, para ambos os ensaios, a concentração de sulfato de amônio foi 0 g/L e 10g/L, para os ensaios 4 e 12, respectivamente e o pH inicial da fermentação foi 7,0 para o ensaio 4 e 5,0 para o ensaio 12.

O efeito de cada variável na atividade enzimática pode ser observado na Figura 3.1. As variáveis concentração de lactose e de água de maceração de milho e o pH apresentaram efeito estatisticamente significativo a 90% de confiança; a concentração de lactose e de AMM apresentaram um efeito positivo, ou seja, ao passar do nível -1 (menor concentração) para o nível +1 (maior concentração), houve incremento na atividade enzimática, já o pH apresentou efeito negativo, ou seja, ao passar do nível -1 para o nível +1, houve diminuição da atividade. As concentrações de Prodex-lac e $(NH_4)_2SO_4$ não apresentaram efeito estatisticamente significativo dentro das faixas estudadas.

Tabela 3.2: Matriz do delineamento experimental fracionário 2^{5-1}, valores codificados e reais (entre parênteses) e atividade enzimática máxima.

Ensaio	Lactose (g/L)	AMM (g/L)	Prodex-lac (g/L)	$(NH_4)_2SO_4$ (g/L)	pH	Ativ. enzimática máxima (U/g)	Tempo (h)
1	-1 (10)	-1 (10)	-1 (4)	-1 (0)	1 (7)	898,3	48
2	1 (70)	-1 (10)	-1 (4)	-1 (0)	-1 (5)	1317,1	48
3	-1 (10)	1 (100)	-1 (4)	-1 (0)	-1 (5)	1280,2	48
4	1 (70)	1 (100)	-1 (4)	-1 (0)	1 (7)	1733,7	48
5	-1 (10)	-1 (10)	1 (20)	-1 (0)	-1 (5)	939,5	48
6	1 (70)	-1 (10)	1 (20)	-1 (0)	1 (7)	1430,0	48
7	-1 (10)	1 (100)	1 (20)	-1 (0)	1 (7)	1249,8	72
8	1 (70)	1 (100)	1 (20)	-1 (0)	-1 (5)	1528,2	48
9	-1 (10)	-1 (10)	-1 (4)	1 (10)	-1 (5)	1334,5	48
10	1 (70)	-1 (10)	-1 (4)	1 (10)	1 (7)	939,5	48
11	-1 (10)	1 (100)	-1 (4)	1 (10)	1 (7)	792,0	24
12	1 (70)	1 (100)	-1 (4)	1 (10)	-1 (5)	1718,5	48
13	-1 (10)	-1 (10)	1 (20)	1 (10)	1 (7)	913,5	24
14	1 (70)	-1 (10)	1 (20)	1 (10)	-1 (5)	1291,0	24
15	-1 (10)	1 (100)	1 (20)	1 (10)	-1 (5)	1459,4	48
16	1 (70)	1 (100)	1 (20)	1 (10)	1 (7)	1423,4	48
17	0 (40)	0 (55)	0 (12)	0 (5)	0 (6)	1323,6	48
18	0 (40)	0 (55)	0 (12)	0 (5)	0 (6)	1210,7	48
19	0 (40)	0 (55)	0 (12)	0 (5)	0 (6)	1484,1	48

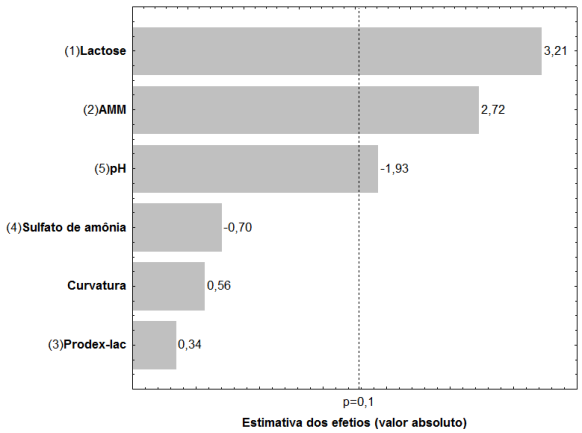


Figura 3.1: Estimativa dos efeitos para as concentrações de lactose presente no soro de queijo, água de maceração de milho, Prodex-lac, $(NH_4)_2SO_4$ e o pH para o delineamento experimental fracionário 2^{5-1}.

Em função dos resultados obtidos neste delineamento, as concentrações de lactose e de água de maceração de milho e o pH foram selecionadas para serem estudadas em um DCCR, em que se aumentaram as concentrações de lactose e de AMM e diminuíram-se os valores de pH. As concentrações de Prodex-lac e $(NH_4)_2SO_4$ foram mantidas no nível -1, ou seja, 4 g/L de hidrolisado de levedura e o sulfato de amônio foi retirado do meio já que tinha sido estudado de 0 a 10 g/L.

O sulfato de amônio foi empregado como fonte inorgânica de nitrogênio, porém pelos resultados obtidos, a água de maceração de milho e o Prodex-lac forneceram a quantidade de nitrogênio necessária para o crescimento do micro-organismo e produção da enzima, não sendo necessária sua adição no meio de cultivo. O mesmo comportamento foi observado nos trabalhos de Rao e Dutta (1977) e Sonawat et al. (1981) em que a produção da β-galactosidase foi pouco influenciada quando o soro de queijo foi suplementado com fontes inorgânicas de nitrogênio, ao passo que quando o soro foi suplementado com fontes orgânicas de nitrogênio a atividade enzimática aumentou consideravelmente.

A partir dos resultados apresentados na Tabela 3.3, observa-se que os ensaios 1 a 4 em que o pH estava no nível -1 (pH = 4,1) foram os ensaios que necessitaram de maior tempo de fermentação para atingir a máxima atividade enzimática, isto provavelmente ocorreu pois o micro-organismo necessitou de um tempo maior para se adaptar ao meio ácido, tendo como consequência uma maior fase lag (fase de adaptação ao meio). No ensaio 13 em que o pH estava no nível -1,68 (pH = 3,5), observa-se a menor produção da enzima, 33 U/g em 96 h de fermentação, indicando que neste pH o micro-organismo não conseguiu se adaptar, logo, a produção da enzima foi muito baixa.

A literatura apresenta alguns estudos sobre a influência da variação do pH na obtenção de β-galactosidases produzidas por leveduras em meio à base de soro de queijo. Bales e Castillo (1979) obtiveram máxima produção enzimática empregando *Candida pseudotropicalis* quando o pH inicial do meio foi ajustado para 3,5. Matheus e Rivas (2003) determinaram como ótimo o pH de 4,68 para a produção da enzima de *Kluyveromyces lactis*, e Panesar (2008), ao fermentar *K. marxianus,* obteve maiores atividades enzimáticas em pH 5,0.

Tabela 3.3: Matriz do DCCR, valores codificados e reais (entre parênteses) e atividade enzimática máxima.

Ensaio	Lactose (g/L)	AMM (g/L)	pH	Ativ. enzimática máxima (U/g)	Tempo (h)
1	-1 (52,1)	-1 (32,3)	-1 (4,1)	844,1	48
2	1 (87,9)	-1 (32,3)	-1 (4,1)	707,4	144
3	-1 (52,1)	1 (97,7)	-1 (4,1)	989,5	72
4	1 (87,9)	1 (97,7)	-1 (4,1)	835,4	168
5	-1 (52,1)	-1 (32,3)	1 (5,9)	679,2	24
6	1 (87,9)	-1 (32,3)	1 (5,9)	982,9	24
7	-1 (52,1)	1 (97,7)	1 (5,9)	874,4	48
8	1 (87,9)	1 (97,7)	1 (5,9)	1403,9	24
9	-1,68 (40)	0 (65)	0 (5)	1071,9	72
10	1,68 (100)	0 (65)	0 (5)	1208,6	24
11	0 (70)	-1,68 (10)	0 (5)	1108,8	48
12	0 (70)	1,68 (120)	0 (5)	1015,5	24
13	0 (70)	0 (65)	-1,68 (3,5)	33,0	96
14	0 (70)	0 (65)	1,68 (6,5)	904,8	48
15	0 (70)	0 (65)	0 (5)	1550,0	24
16	0 (70)	0 (65)	0 (5)	1358,3	24
17	0 (70)	0 (65)	0 (5)	1498,5	24

Sabe-se que leveduras crescem melhor em pH ácido, porém ficou evidente que para a levedura empregada neste trabalho, pH menores de 4,1 prejudicam o crescimento celular e a obtenção da enzima. De acordo com Panesar (2008), o pH de um sistema afeta pelo menos dois aspectos das células microbianas, o funcionamento de suas enzimas e o transporte de nutrientes na célula.

As condições que resultaram nas maiores atividades enzimáticas foram as condições dos pontos centrais, ou seja, 70 g/L de lactose, 65 g/L de água de maceração de milho e pH 5. Por meio da análise estatística dos resultados, obteve-se a equação do modelo codificado (Equação 1) que foi validada pela análise de variância (Tabela 3.4) em que o coeficiente de correlação obtido foi de 0,84 e o F calculado foi 4,88 vezes maior que o valor tabelado.

$$\text{Atividade (U/g)} = 1464{,}6 - 102{,}9(\text{lactose})^2 - 130{,}5(\text{AMM})^2 - 148{,}7(\text{pH}) - 340{,}7(\text{pH})^2 + 140\,(\text{lactose} \times \text{pH}) \quad \text{(Eq. 1)}$$

Tabela 3.4: Análise de variância para o delineamento composto central rotacional.

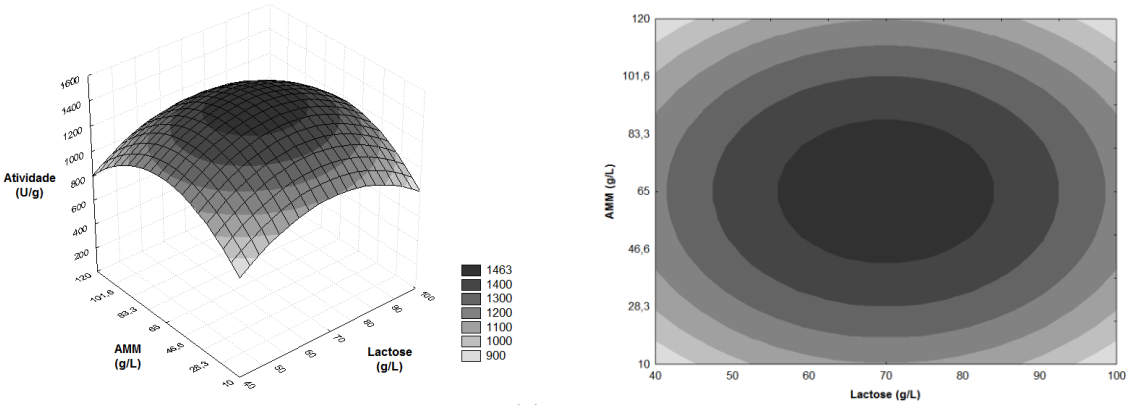
Fonte de variação	Soma dos quadrados	Graus de liberdade	Média quadrática	F calculado
Regressão	1776847,56	5	355369,5	11,98
Resíduo	326428	11	29675	
Total	2103275	16		

R^2=84,5%; $F_{5;11;0,1}$= 2,45

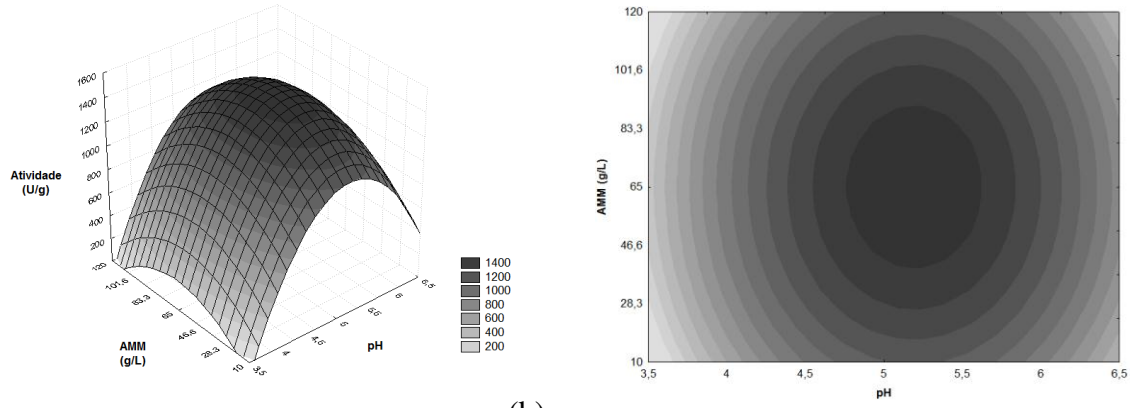
A partir da equação foi possível gerar as superfícies de resposta (Figura 3.2) para analisar as melhores condições de concentração de lactose, de AMM e pH para produção da β-galactosidase que conduzem a um maior valor de atividade enzimática.

Pode-se observar na Figura 3.2 (a) que as concentrações de AMM entre 30 e 100 g/L e lactose entre 50 e 90 g/L conduzem a valores de atividade enzimática superiores a 1.400 U/g. A Figura 3.2 (b) indica que concentrações de AMM entre 30 e 100 g/L e pH na faixa de 4,8 a 5,6 propiciam altos valores de atividade enzimática. Pela Figura 3.2 (c) observa-se que as maiores atividades são obtidas ao empregar concentração de lactose entre 60 e 90 g/L e pH entre 4,8 e 5,7.

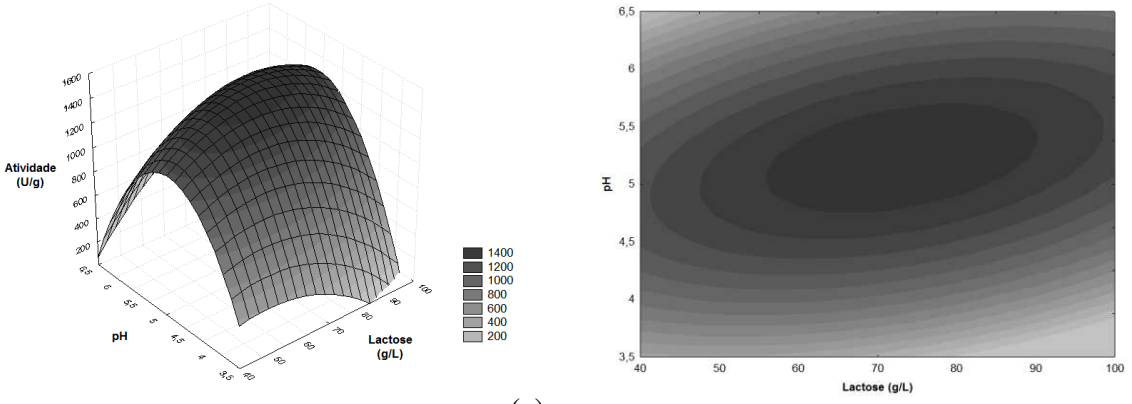
Nas concentrações de AMM acima de 100 g/L para qualquer faixa de concentração de lactose estudada a atividade enzimática diminuiu, de acordo com Pinheiro et al. (2008), esta inibição está associada à composição do substrato, pois tanto a água de maceração de milho quanto o soro de queijo e o Prodex-lac não podem ser considerados como fontes exclusivas de nitrogênio, carbono e vitaminas, pois estes substratos têm uma composição bastante complexa, contendo diversos nutrientes, que quando analisados individualmente podem causar efeito positivo na produção de enzimas, porém a interação e o aumento da concentração podem resultar em excesso de alguns destes nutrientes importantes e consequente inibição indesejada na produção da enzima.



(a)



(b)



(c)

Figura 3.2: Superfícies de respostas para a atividade de β-galactosidase em função: (a) concentração AMM e de lactose; (b) concentração de pH e AMM; (c) pH e concentração de lactose.

Sonawat et al. (1981) observaram que o emprego de água de maceração de milho influenciou positivamente na produção da enzima β-galactosidase, o mesmo

comportamento foi observado no trabalho de Rao e Dutta (1977), em que houve aumento de aproximadamente 28% na atividade enzimática usando soro suplementado com água de maceração de milho. Furlan et al. (2000) estudaram a produção da β-galactosidase empregando *Kluyveromyces marxianus,* a partir de um meio de cultura com melaço de cana- de-açúcar. A máxima produção da enzima foi obtida quando o meio foi suplementado com 100 g/L de água de maceração de milho.

A partir dos resultados, para obtenção de uma atividade enzimática por volta de 1.400 U/g, pode-se empregar as condições dos pontos centrais, ou seja, 70 g/L de lactose, 65 g/L de água de maceração de milho e pH 5, estes ensaios resultaram em uma produtividade média de 61 U/g.h. O maior valor de atividade enzimática obtido no DCCR foi inferior ao máximo obtido no delineamento fracionário, em que se obteve nos ensaios 4 e 12 uma atividade por volta de 1.700 U/g, porém a produtividade nesses ensaios foi aproximadamente 35 U/g.h.

Manera (2006) otimizou a produção da enzima β-galactosidase de *Kluyveromyces marxianus* CCT 7082 empregando um meio sintético composto por lactose, extrato de levedura e sais, a máxima atividade enzimática obtida foi 800 U/g. Comparando esse resultado com os obtidos neste trabalho pode-se verificar o aumento de 75% na produção da enzima β-galactosidase.

3.4 CONCLUSÕES

A produção de β-galactosidase de *Kluyveromyces marxianus* CCT 7082, utilizando um meio de cultura composto por subprodutos industriais, é um processo biotecnológico promissor. O emprego de soro de queijo, um subproduto dos laticínios, água de maceração de milho, um subproduto da indústria de extração do amido, e a substituição do extrato de levedura por Prodex-lac, como substrato para fermentação tem o intuito de reduzir custos e propiciar uma enzima com alta atividade enzimática. Com o emprego de metodologia de delineamento experimental e análise de superfície de resposta foi possível obter uma produção enzimática de aproximadamente 1.400 U/g em 24 h de fermentação.

Agradecimentos

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3.5 REFERÊNCIAS BIBLIOGRÁFICAS

BALES, S. A.; CASTILLO, F. J. Production of lactase by *Candida pseudotropicalis* grown in whey. **Applied and Environmental Microbiology,** v. 37, n. 6, p. 1201-1205, 1979.

BURKET, J.F.M. **Otimização das condições de produção de lipase por *Geotrichum candidum* NRRLY-552**. Campinas, 2003. 172p. Tese (Doutor em Engenharia de Alimentos), Faculdade de Engenharia de Alimentos. Universidade Estadual de Campinas.

CHONAN, O.; TAKAHASHI, R; WATANUKY, M. Role of activity of gastrointestinal microflora in adsorption of calcium and magnesium in rats fed 1-4 linked galactooligosaccharide. **Bioscience, Biotechnology and Biochemistry**, v. 65, n. 8, p.1872-1875, 2001.

ERNANDES, F. M. P. G.; BOSCOLO, M.; CRUZ, C. H. G. Influência da composição do meio para a produção de *Zimomonas mobilis*. **Acta Scientiarum. Technology**, v. 32, n. 1, p 21-26, 2010.

FURLAN, S. A.; SCHNEIDER, A. L. S.; MERKLE, R.; CARVALHO-JONAS, M. F.; JONAS, R. Formulation of a lactose-free, low-cost culture medium for the production of β-D-galactosidase by *Kluyveromyces marxianus*. **Biotechnology Letters,** v. 22, p. 589-593, 2000.

INCHAURRONDO, V. A.; YANTORNO O. M.; VOGET, C. E. (1994), Yeast growth and β-galactosidase production during aerobic batch cultures in lactose-limited synthetic medium. **Process Biochemistry,** v. 29, p. 47-54, 1994.

JOO, H. S.; CHANG, C. S. Production of protease from a new alkalophilic Bacillus sp I-312 grown on soybean meal: optimization and some properties. **Process Biochemistry**, v. 40, n. 3-4, p. 1263-1270, 2005.

LONGHI, L. G. S.; LUVIZETTO, D. J.; FERREIRA, L. S.; RECH, R.; AYUB, M. A. Z.; SECCHI, A. R. A growth kinetic model of *Kluyveromyces marxianus* cultures on cheese whey as substrate. **Journal of Industrial Microbiology and Biotechnology**, v. 31, p.35-40, 2004.

LUKONDEH, T.; ASHBOLT, N. J.; ROGERS, P. L. Fed-batch fermentation for production of *Kluyveromyces marxianus* FII 510700 cultivated on a lactose-based medium. **Journal of Industrial Microbiology and Biotechnology**, v. 32, p. 284-288, 2005.

MAHONEY, R. R. Galactosyl-oligosaccharide formation during lactose hydrolysis: a review. **Food Chemistry**, v. 63, n. 2, p. 147-154, 1998.

MANERA, A. P. **Otimização do meio de cultura para produção da enzima β-Galactosidase de *Kluyveromyces marxianus* CCT 7082 e caracterização parcial da enzima**. 2006. Dissertação (Mestrado em Engenharia de Alimentos) – Engenharia de Alimentos, Fundação Universidade Federal do Rio Grande, Rio Grande.

MANERA, A. P.; ORES, J. C.; RIBEIRO, V. A.; BURKERT, C. A. V.; KALIL, S. J. Optimization of the culture medium for the production of β-galactosidase from *Kluyveromyces marxianus* CCT 7082. **Food Technology and Biotechnology**, v.46, n. 1, p. 66-72, 2008.

MANNING, T. S.; GIBSON, G. R. Prebiotics. **Best Practice & Research Clinical Gastroenterology**, v. 18, n. 2, p.287-298, 2004.

MATHEUS, A. L. R; RIVAS, N. Produción y caracterización parcial de β-galactosidase de *Kluyveromyces lactis* propagada en suero de leche desproteinizado. **Archivos Latinoamericanos de Nutrición**, v. 53, n. 2, p. 194-201, 2003.

NOR, Z. M.; TAMER, M. I.; MEHRVAR, M.; SCHARER, J. M.; MOO-YOUNG, M.; JERVIS, E. J. Improvement of intracellular β-galactosidase production on fed-batch culture of *Kluyveromyces fragilis.* **Biotechnology Letters**, v. 23, p. 845-849, 2001.

NUMANOGLU, Y.; SUNGUR, S. β-galactosidase from *Kluyveromyces lactis* cell disruption and enzyme immobilization using a cellulose-gelatin carrier system. **Process Biochemistry**, v. 39, n. 6, p. 705-711, 2004.

OBEROI, H. S.; BANSAL, S.; DHILLON, G. S. Enhanced β-galactosidase production by supplementing whey with cauliflower waste. **International Journal of Food Science and Technology,** v. 43, p. 1499-1504, 2008.

PANESAR, P. Production of β-D-galactosidase from whey using *Kluyveromyces marxianus*. **Research Journal of Microbiology**, v. 3, n. 1, p. 24-29, 2008.

PINHEIRO, T. L. F.; MENONCIN, S.; DOMINGUES, N. M.; OLIVEIRA, D.; TREICHEL, H.; DI LUCCIO, M.; FREIRE, D. M. G. Production and partial characterization of lipase from *Penicillium verrucosum* obtained by submerged fermentation of conventional and industrial media. **Ciência e Tecnologia de Alimentos,** v. 28, n.2, p. 444- 450, 2008.

RAJOKA, M. I.; KHAN, S.; SHAHID, R. Kinetics and regulation studies of the production of β-galactosidase from *Kluyveromyces marxianus* grown on different substrates. **Food Technology and Biotechnology**, v. 41, n. 4, p. 315-320, 2003.

RAO, M. V. R.; DUTTA, S. M. Production of β-galactosidase from *Streptococcus thermophilus* grown in whey. **Applied and Environmental Microbiology**, v. 34, n. 2, p. 185-188, 1977.

RODRIGUES, M. I.; IEMMA A .F. **Planejamento de experimentos e otimização de processos**. 2ª ed., Campinas: Casa do Pão Editora, 2009.

SANTIAGO, P. A.; MARQUEZ, L. D. S.; CARDOSO, V. L.; RIBEIRO, E. J. Estudo da produção de β-galactosidase por fermentação de soro de queijo com *Kluyveromyces marxianus*. **Ciência e Tecnologia de Alimentos**, v. 24, n. 4, p. 567-572, 2004.

SILVA, G. A. B.; ALMEIDA, W. E. S.; CORTES, M. S.; MARTINS, E. S. Produção e caracterização de protease obtida por *Gliocladium verticilloides* através da fermentação em estado sólido de subprodutos agroindustriais**. Revista Brasileira de Tecnologia Agroindustrial**, v. 3, n. 1, p. 28-41, 2009.

SONAWAT, H. M.; AGRAWAL, A.; DUTTA, S. M. Production of β-galactosidase from *Kluyveromyces fragilis* grown on whey. **Folia Microbiologica,** v. 26, p. 370-376, 1981.

VASILJEVIC, T.; JELEN, P. Production of β-galactosidase for lactose hydrolysis in Milk and dairy products using thermophilic lactic acid bactéria. **Innovative Food Science & Emerging Technology**, v. 2, p. 75-85, 2001.

CAPÍTULO 4

PERMEABILIZATION OF Kluyveromyces marxianus CELLS AIMING β-GALACTOSIDASE ACTIVITY AND CHARACTERIZATION

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PERMEABILIZATION OF *Kluyveromyces marxianus* CELLS AIMING β-GALACTOSIDASE ACTIVITY AND CHARACTERIZATION

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Abstract

Large-scale production of β-galactosidase continues to be hindered by difficulties in recovery intracellular enzyme from microbial cells. To overcome the permeability barrier and prepare whole cell biocatalysts with high activities, permeabilization of *Kluyveromyces marxianus* CCT 7082 regarding β-galactosidase activity was optimized. Permeabilization treatments using detergents (Triton X-100, Tween 80, cetyl-trimethylammonium bromide) and solvents (acetone, ethanol, butanol, isopropanol) were performed and compared by measuring the β-galactosidase activity. The comparison between the different permeabilizing agents has led to the selection of isopropanol for maximal permeabilization of yeast cells. The optimum conditions were a ratio of 0.03 between biomass and isopropanol masses (w/w), 25°C and treatment time of 5 min. Characterization of β-galactosidase from permeabilized cells were performed and compared with the characterization of the free enzyme. There were no big difference on the studied characterization between the two enzymes. The optimum pH and temperature were 6.6 and 50°C, respectively, and were not affected by the intracellular localization. The enzyme of the permeabilized cells showed a small increase in pH and thermal stability when compared with the free enzyme. The enzyme half-lives at pH 7.0 and 45°C were 75 min and 60 min for permeabilized cells and free enzyme, respectively. The stability at 30°C were 144 h and 115 h, for permeabilized cells and free enzyme, respectively. The Michaelis-Menten

constant, K_m, was quite similar for both tipes of the enzyme, been 38.5 mM and 31.9 mM for permeabilized cells and free enzyme, respectively.

4.1 INTRODUCTION

A large proportion of potentially useful microbial products is retained within the cells of their producers. The isolation of intracellular material requires either the cell to be genetically engineered (so that intracellular products can be excreted into the growth medium) or the cells must be disintegrated by physical, chemical or enzymatic means to release their cytoplasmic content (Chisti & Moo-Young, 1986). The permeabilization of cell walls is also possible, making the enzymes in the cytoplasm easily accessible for a substrate (Somkuti et al., 1998).

Permeabilization is known to overcome cell membrane barriers of whole cell biocatalysts. The permeabilizing agent may disrupt the membrane structures by decreasing the phospholipid content to allow the passive passage of low molecular weight solutes in and out of cells, including lactose and its products of hydrolysis (Siso et al., 1992; Panesar, 2008).

The outer wall of a microorganism can be permeabilized by a large variety of chemical compounds, which differ in selectivity and efficiency towards different microbial species. Chemical permeabilization could be accomplished by antibiotics, chelating agents, chaotropes, detergents, solvents, or by hydroxides and hypochlorites (Geciova et al., 2002). Chemical permeabilization with solvents or detergents does not result in the release of intracellular enzymes (Somkuti et al., 1998).

The use of detergents or organic solvents is known to be most effective on the permeabilization because of the low cost and simple procedure. The representative organic solvents that enhance cell membrane permeability are toluene, chloroform, ethanol, isopropanol, butanol, acetone, and the detergents are triton, tween and cetyl-trimethylammonium bromide (Naglak et al., 1990). Moreover, permeabilization with chemical compounds causes the microorganisms to become unviable. Thus,

permeabilization can additionally reduce unwanted side reactions and minimize energy loss for cell growth (Choi t al., 2004).

The β-galactosidase (EC 3.2.1.23) is one of the most promising enzymes, which has several applications in the food, fermentation and dairy industry (Kaur et al, 2009). Amongst various microorganisms, yeast has emerged as an important source of β-galactosidase, since the yeast enzyme has an optimum pH suitable for lactose hydrolysis in milk and sweet whey (Kondo et al., 2000). *Kluyveromyces marxianus* offers several great advantages, such as good growth yield, acceptability as a safe microorganism (GRAS) and higher β-galactosidase activity than other yeast (Kaur et al., 2009).

β-galactosidase produced by *K. marxianus* is an intracellular enzyme, therefore, the industrial applications of processes based on the enzymatic hydrolysis of lactose are limited (Panesar, 2008). The preparation of cell-free extracts is laborious and needs disruption of cells, which destroys the integrity of cells and may cause inactivation of enzymes. Therefore, cell permeabilization has been recommended as an alternative method for the study of intracellular enzymatic reactions (Alamäe and Järviste, 1995).

In this work, *Kluyveromyces marxianus* CCT 7082 was permeabilized with chemical compounds (solvents and detergents) to make the enzyme β-galactosidase accessible. The most efficient solvent for the permeabilization was selected according to a factorial experimental design. Then the enzyme β-galactosidase inside permeabilized cells permeabilized was characterized for optimum pH and temperature, pH and thermal stability, K_m and V_{max} kinetics constants, and compared to data from free enzyme.

4.2 MATERIAL AND METHODS

4.2.1 Enzyme production

Kluyveromyces marxianus CCT 7082, previously selected as the best β-galactosidase producer amongst the tested yeast by Manera et al. (2008), was used in the present study. The strain was maintained at -18ºC on YM broth (yeast extract 3.0 g/L, malt extract 3.0 g/L, peptone 5.0 g/L, glucose 10.0 g/L) with glycerol 10% (v/v). The yeast was cultivated for the enzyme production on a fermentation media composed of 17.0 g yeast

extract, 8.8 g $(NH_4)_2SO_4$, 5.0 g KH_2PO_4, 0.4 g $MgSO_4.7H_2O$ and 28.2 g lactose in 1 L of 0.2 M potassium phosphate buffer, pH 6.0. The cultures were incubated in an orbital shaking incubator for 96 h at 180 rpm and 30ºC. Cultivation was started with a 10% inoculum, prepared with the same culture medium fermentation, incubated at 30ºC, 180 rpm for 24h (Manera et al., 2008).

4.2.2 Screening of the permeabilizing agents

The yeast cell permeabilization procedures were carried out following a modified method by Joshi et al. (1987). The cells of the culture medium, equivalent to 0.16 g dry wt, were centrifuged at 6000 × g, for 5 min, at 4ºC, and washed twice with 10 mL of 0.1 M potassium phosphate buffer pH 7.0. The cells were suspended in 10 mL potassium phosphate buffer containing the permeabilizing agent (solvents: acetone, ethanol, butanol, isopropanol; detergents: Triton X-100, Tween 80, cetyl-trimethylammonium bromide - CTAB) at the desired concentration, incubated for 15 min at 25ºC and then centrifuged to remove the permeabilization solution. The cells were washed twice with potassium phosphate buffer, and suspended in the same buffer and used for determination of β-galactosidase activity.

The effect of different concentrations of permeabilizing agent was studied, 10 – 70% (w/v) to solvents and 0.05 – 1.0% (w/v) to detergents. The data from all the experiments are presented as the mean ± standard deviation of three experiments. The data were treated by an analysis of variance and the differences by a means the Tukey's test.

4.2.3 Optimization of the permeabilization process

The isopropanol performed the highest enzyme activity, so that it was selected for optimization of process parameters. The experiments were carried out according to a central composite design (CCD) (comprising 4 trials factorials and 3 central points) (Rodrigues and Iemma, 2009). The influence of the ratio between biomass and isopropanol masses (w/w) (0.04 – 0.12) and temperature (5 – 25°C) were studied. Before adding the isopropanol to the biomass, it was prepared in buffer at concentration which resulted in a

major enzymatic activity at the previous permeabilizing agent selection phase (30% w/v). The response was the enzyme activity.

The enzymatic activity was measured at 5, 10, 15 and 20 min of incubation for each assay of the CCD. Statistical analyses were performed using the software STATISTICA 9.1 (Statsoft, 2010).

According to the CCD results, 4 additional assays were performed in triplicate in order to optimize the permeabilization of yeast cells, keeping the temperature constant at 25°C and varying the ratio biomass/isopropanol (0.01; 0.02; 0.03 and 0.04 w/w). The data variance and average differences were analysed using the Tukey's test.

4.2.4 Characterization of the β-galactosidase

On the enzyme characterization there were employed two β-galactosidase enzymes forms of *K. marxianus* CCT 7082, the permeabilized cells containing the enzyme and the cells extracted enzyme.

The enzymes were characterized according to the temperature and pH profile, as well as pH and thermal stability and kinetics parameters.

4.2.4.1 Temperature and pH profile

The effect of temperature (33 – 47°C) and pH (5.2 – 8.0) on the activity was studied according to a central composite rotatable design (CCRD) (4 factorial trials, three central points and four axial points) (Rodrigues and Iemma, 2009), for each enzyme.

According to the results of the CCRD additional assays were carried out in order to optimize the temperature and pH of the enzyme activity, where pH was fixed (6.6) and the temperature changed from 47 to 56°C.

4.2.4.2 pH and thermal stability

In order to investigate the pH stability of β-galactosidase, permeabilized cells and free enzyme were incubated in 0.1 M potassium phosphate buffer at different pH (5.0 – 9.0). Samples were taken at regular intervals and cooled in ice bath prior the assays. The residual activity was expressed as percentual of the initial activity. The inactivation rate

constants (K_d) were calculated (Equation 1) from a logarithmic plot of the residual activity versus time, and half-lives were estimated using Equation 2. The half-life ($t_{1/2}$) is defined as the time needed for the residual activity reaches 50%.

$$\ln\left(\frac{U}{U_0}\right) = -K_d.t \quad (1)$$

$$t_{1/2} = \frac{-\ln(0.5)}{K_d} \quad (2)$$

Where:

U/U_0: relative activity; K_d: denaturation constant (min^{-1}); t: time (min); $t_{1/2}$: half-life (min);

The influence of the temperature on enzyme stability was performed as a function of incubation time at different temperature (30, 34, 38, 42, 46, 50°C) in 0.1 M potassium phosphate buffer pH 7.0, for each enzyme. Samples were taken at regular intervals and ice cooled prior assays. The residual activity was expressed as percentual of the initial activity, and K_d were determined for each temperature (Equation 1). The K_d dependence of the temperature was analyzed using the Arrhenius equation (Equation 3). The value of the deactivation energy (E_d) was estimated from the slope.

$$\ln K_d = \ln A_0 - \frac{E_d}{R.T} \quad (3)$$

Where:

E_d: deactivation energy (Kcal/mol); K_d: thermal inactivation constant (min^{-1}); A_0: constant; R: gas constant (1.9872 cal/mol.K); T: absolute temperature (K).

4.2.4.3 Determination K_m e $V_{máx}$

The kinetics of the reaction follows the Michaelis-Menten and the kinetic constants ($V_{máx}$ and K_m) were determined using Lineweaver Burk double reciprocal (1/V versus 1/S) plots, with different lactose concentrations (29 – 1460 mM) as substrate.

4.2.5 Enzyme extraction

Samples of 1 mL of cell suspension containing 2.62 mg of dry cell in phosphate buffer (0.1 M, pH 6.6) were mixed with 1.1 g of glass beads (r<0.4 mm) and ultrasonicated for 10 min at 4°C. The suspension was centrifuged at 5200×*g* for 10 min at 4°C, and the supernatant was assayed for β-galactosidase characterization (Manera et al., 2008). Biomass content in the cell suspension was adjusted according to Numanoglu and Sungur (2004), using a dry cell calibration curve previously established.

4.2.6 β-galactosidase assay

β-galactosidase activity was assayed using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) following the method described by Inchaurrondo et al. (1994). A 50 µL sample of permeabilized cell suspension was mixed with 2 mL of 1.25 mM ONPG in buffer (50 mM of KH_2PO_4 and 0.1 mM $MnCl_2.4H_2O$, pH 6.6) and incubated for 5 min at 37°C. The reaction was stopped by adding 0.5 mL of 1 M sodium carbonate. Liberated *o*-nitrophenol (ONP) was measured spectrophotometrically at 420 nm. The molar extinction coefficient of *o*-nitrophenol under these conditions was 4.64 cm^2/µmol. One enzyme unit is defined as the quantity of enzyme that catalyzes the release of 1 µmol of ONP from ONPG per minute under assay conditions.

The determination of the kinetic constants of β-galactosidase was carried out according the method described by Szczodrak et al. (2000). The activity was determined by mixing 0.5 mL of enzyme with 0.5 mL of lactose in the concentration in 0.05 M potassium phosphate buffer pH 7.0. It was then incubated at 37ºC for 5 min, and measured the glucose released with a glucose-oxidase reagent. One enzyme unit is defined as the amount of enzyme producing 1 µmol glucose per min under the described conditions.

4.2.7 Cell concentration

Cell concentration was estimated by measuring the absorbance at 620 nm and converted into dry weight by using a standard curve. Samples were centrifugated at 6000 × *g* for 5 min, washed twice with distilled water and ressuspended in water, and absorbance read. For the standard curve cells were dried at 90°C to constant weight (Longhi et al., 2004).

4.3 RESULTS AND DISCUSSION

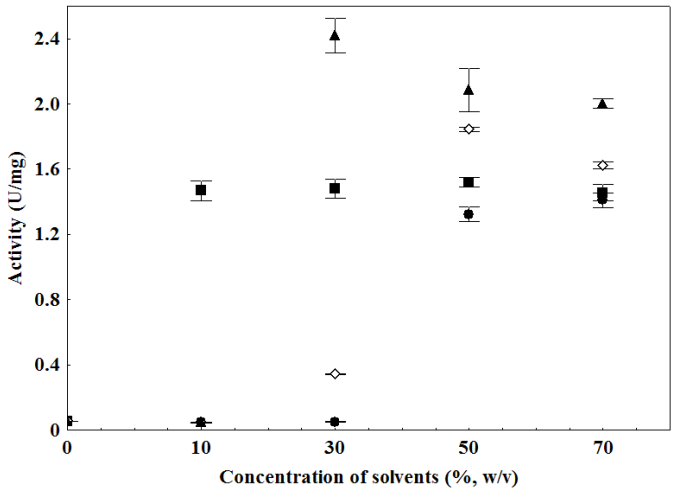
4.3.1 Screening of the permeabilizing agents

The yeast cells were treated with different permeabilizing agents, at different concentrations, in order to select the agent that resulted in higher enzyme activity.

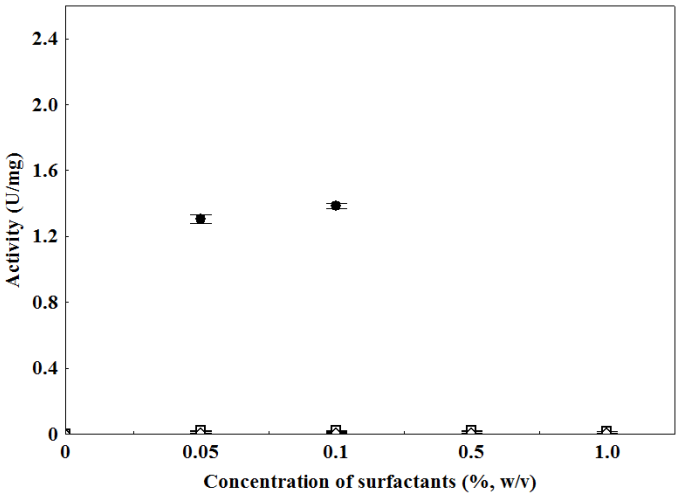
Figure 4.1 (a) shows the enzyme activity of cells permeabilized with solvents, where the highest activities were obtained at a concentration of 70% for acetone (1.4 U/mg), 50% for butanol (1.5 U/mg) and 50% for ethanol (1.8 U/mg) and 30% for isopropanol (2.4 U/mg). Figure 4.1 (b) shows the activities obtained with cells treated with detergents. The maximum activity was obtained with 0.1% (w/v) cetyl-trimethylammonium bromide (CTAB) (1.4 U/mg). Triton X-100 and Tween 80 permeabilization were not efficient in the concentration range studied, since the activities of permeabilized cells were similar to untreated cells.

With the increasing concentration of permeabilizing agent, it can be observed an increase in enzyme activity, up to a maximum value, and then activity decreases. According to Panesar et al. (2007), low permeabilizing agent concentrations can cause low enzyme activities due to insufficient quantity of agent for effective permeabilization. On the other hand high concentrations can cause enzyme denaturation.

The buffer solutions used for cell washing after permeabilization assays were assayed for enzyme activity. In all cases the enzyme activity was nearly zero, indicating that the permeabilization was efficient to allow the substrate access to intracellular enzyme, without enzyme losses through the cell wall.



(a)



(b)

Figure 4.1: Effect of the chemical agents in the permeabilization of *Kluyveromyces marxianus* CCT 7082. a: acetone (●); butanol (■); ethanol (◊); isopropanol (▲). b: CTAB (●); Triton X100 (□); Tween 80 (◊). Bars indicate the standard deviation between triplicates.

The results of this study are consistent with those found in the literature. Panesar et al. (2007) reported that eight permeabilizing agents were tested with cells of *K. marxianus*, including acetone, butanol, ethanol and isopropanol. The maximum activities were around 1.4 U/mg at concentrations of 30, 15, 50 and 35% respectively. The same authors also used

Triton X-100, but it was not an effective permeabilization agent, similar to what was observed in this work. Kaur et al. (2009) reported the permeabilization of cells of *K. marxianus* with CTAB, and found out that the highest enzyme activity, around 1.3 U/mg, was obtained at the detergent concentration of 0.06% (w/v).

For the five most effectives permeabilizing agents, data of maximum activities were analysed according to the data variance and Tukey's test (Figure 4.2).

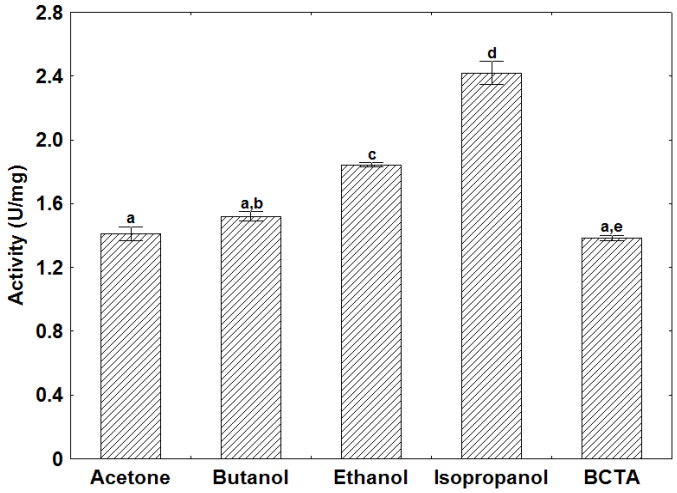


Figure 4.2: Maximum enzymatic activity for each permeabilizing agent. Same letters indicate that there were no statistical differences at 95% of confidence. Bars indicate the standard deviation between the triplicates.

The Tukey's test, based on the difference between averages, led to the conclusion that no significant difference at 95% confidence could be detected between acetone and butanol, and between acetone and CTAB. Isopropanol was the permeabilizing agent that resulted in higher enzyme activity and was statistically different from the other agents at 95% confidence. The latter was then selected for the optimization of the permeabilization process.

4.3.2 Optimization of the cell wall permeabilization

The assays carried out in order to find the most appropriate method for the cell wall permeabilization relied on a CCD where the independent variables were the mass ratio

biomass/isopropanol (w/w) and process temperature. The enzyme activity was measured at the incubation times of 5, 10, 15 and 20 minutes. In all experiments, the highest activities were obtained at the incubation time of 5 min, which results are shown in Table 4.1. It can be observed that the enzyme activity ranged from 1.31 to 1.85 U/mg. The highest activities were obtained in tests 1 and 3, while the lowest ones were obtained in tests 2 and 4.

Table 4.1: Coded and real (in parentheses) values for the variables and resulting activities of the CCD for cell permeabilization.

Essay	Biomass/isopropanol (w/w)	Temperature (ºC)	Activity (U/mg)
1	-1 (0.04)	-1 (5)	1.83
2	1 (0.12)	-1 (5)	1.31
3	-1 (0.04)	1 (25)	1.85
4	1 (0.12)	1 (25)	1.42
5	0 (0.08)	0 (15)	1.54
6	0 (0.08)	0 (15)	1.56
7	0 (0.08)	0 (15)	1.47

The effect estimates for each variable and the interaction between them are shown in Table 4.2. For statistical analysis, only the biomass/isopropanol ratio has shown a statistically significant effect at 95% of confidence. However, this effect was negative, i.e. when the ratio increased from 0.04 to 0.12, there was a decrease in the enzyme activity. The temperature was no statistically significant, within the studied range, so that any value between 5 and 25°C leads to similar results of enzyme activity.

Table 4.2: Effect estimates of the enzyme activity.

Factor	Effect	Standard Error	t(3)	p-valor
Mean*	1.56	0.026	60.67	0.00001*
(1) Relation biomass/isopropanol (w/w)*	-0.46	0.068	-6.84	0.00640*
(2) Temperature	0.06	0.068	0.91	0.42834
1 x 2	0.05	0.068	0.77	0.49878

*significant factors ($p<0.05$)

According to the results from the CCD, four additional trials were carried out, where the biomass/isopropanol ratio changed from 0.01 to 0.04, while the temperature was kept constant at 25ºC (Figure 4.3). The tests were performed in triplicate and the responses were assesses by Tukey's test. Equal letters indicate that the tests did not differ statistically at 95%, and by this mean it can be noticed that the ratios of 0.01, 0.02 and 0.03 were statistically identical. According to the data depicted by Figure 4.3, it is evident that the biomass/isopropanol ratio should be used at 0.03 (w/w), and when this ratio increased there is a decrease in the enzymatic activity.

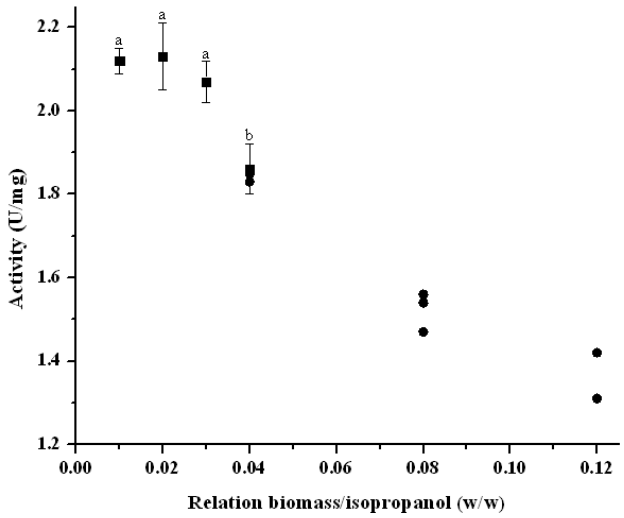


Figure 4.3: β-galactosidase activity after permeabilization as a function of the biomass/isopropanol mass ratio. (●) assay of experimental design; (■) assay of Tukey's test.

4.3.3 Characterization of the β-galactosidase

4.3.3.1 Temperature and pH profile

To determine the effects of temperature and pH over the enzyme activity, two CCRD were performed, one for each enzyme (free enzyme and permeabilized cells), with two independent variables (temperature and pH), and having the enzyme activity as the response variable. Data are presented in Table 4.3.

Results for the activities of the permeabilized cells ranged from 0.30 to 2.3 U/mg, and for the free enzyme, from 0.39 to 1.81 U/mg. These data variability points out that the enzyme was sensitive to the change in temperature and pH, within the range studied. The results for the central points (assays 9 to 12) were similar, what shows reproducibility.

The statistical analysis of the enzyme activity results in Table 4.3 led to the statistical coded models expressed in Equations 6 and 7, for the enzymatic activity of permeabilized cells and free enzyme, respectively, with 95% confidence.

Table 4.3: Coded and real (in parentheses) variables and enzyme activity response according to the CCRD 2^2 plus 4 central points.

Essay	pH	Temperature (ºC)	Activity of the permeabilized cells (U/mg)	Activity of free enzyme (U/mg)
1	-1 (5.6)	-1 (35)	0.90	0.70
2	1 (7.6)	-1 (35)	0.87	0.44
3	-1 (5.6)	1 (45)	0.80	0.81
4	1 (7.6)	1 (45)	1.18	0.72
5	-1.41 (5.2)	0 (40)	0.30	0.39
6	1.41 (8.0)	0 (40)	0.62	0.40
7	0 (6.6)	-1.41 (33)	1.42	1.04
8	0 (6.6)	1.41 (47)	2.30	1.81
9	0 (6.6)	0 (40)	1.88	1.46
10	0 (6.6)	0 (40)	1.85	1.45
11	0 (6.6)	0 (40)	1.90	1.42
12	0 (6.6)	0 (40)	1.89	1.38

$$\text{Activity (permeabilized cells) (U/mg)} = 1.83 - 0.75\text{pH}^2 + 0{,}18\text{temperature} \quad (6)$$

$$\text{Activity (free enzyme) (U/mg)} = 1.38 - 0.57\text{pH}^2 + 0.18\text{temperature} \quad (7)$$

Table 4.4 present the values of the percentage of variance explainded (R^2) and the values of the F_{calc} and F_{tab} for the enzymatic activity of the permeabilized cells and free enzyme.

According to the F test, the model for the activity of the permeabilized cells is predictive, since the calculated F is higher than the listed F, 11.2 times, and the R^2 is good. The model is then considered appropriate to describe the response surface for the enzymatic

activity of permeabilized cells (Figure 4.4 - a). For the free enzyme, the model is also predictive, as calculated F is 8.2 times higher than the listed F, with acceptable R^2, so that the model can be considered appropriate to describe the response surface for the enzymatic activity of the free enzyme (Figure 4.4 - b).

Table 4.4: Values of the percentage of variance explained (R^2) and F for activity of the permeabilized cells and free enzyme.

Enzyme	**R^2 (%)**	**F_{calc}**	**F_{tab}***
Permeabilized cells	91	47.3	4.2
Free enzyme	90	36.8	4.4

*values at 5% significance

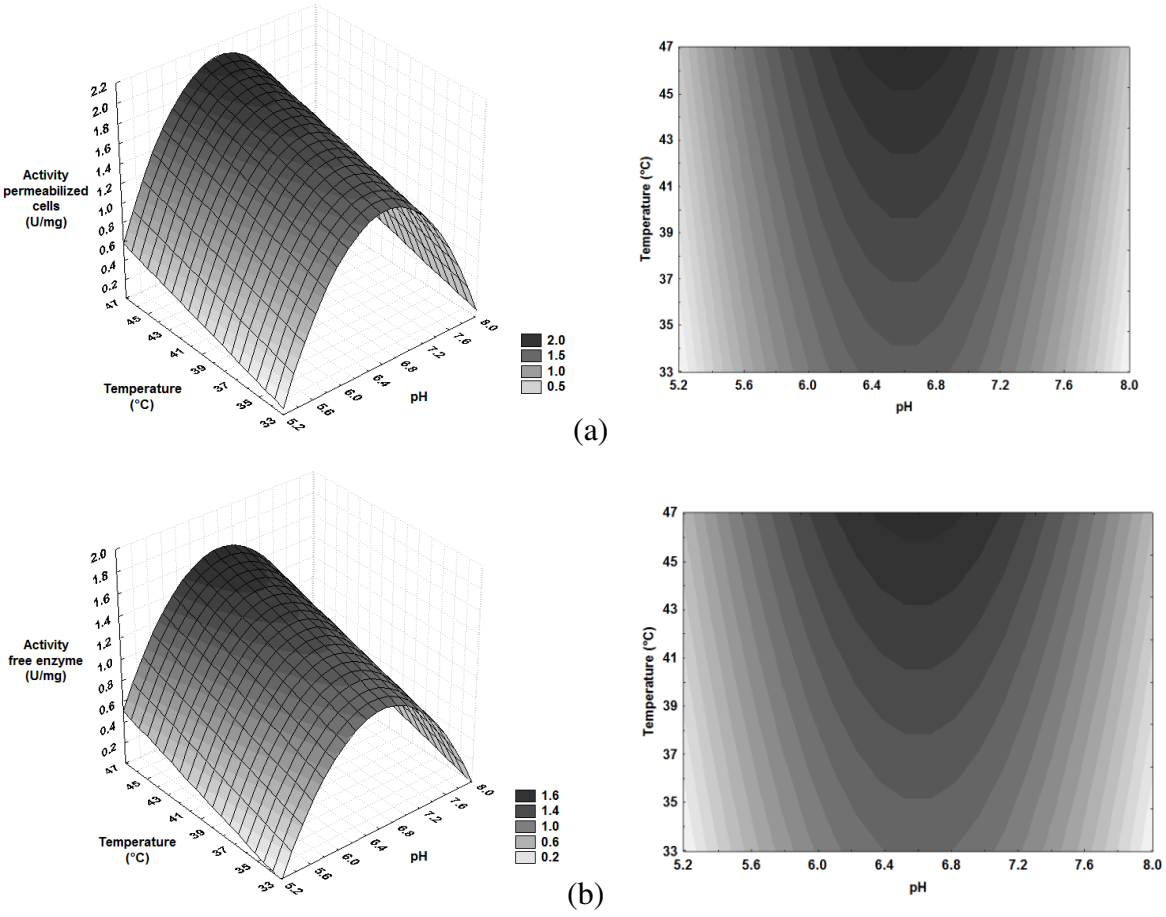


Figure 4.4: Response surface for enzyme activity as a function of pH and temperature. (a) permeabilized cells, (b) free enzyme.

According to the response surfaces depicted by Figure 4.4, the maximum ativity values for both enzymes are found at the pH 6.6 (central point), while the increase in temperature indicates an increase in enzyme activity, without a clear maximum point. For this reason, four additional experiments in triplicate were carried out, with temperature changes from 47-56°C and keeping the pH fixed at 6.6 (Figure 4.5). The Tukey's test was used for checking whether there was a significant difference between the new studied conditions. The test points out that there is no statistically significant difference at 95% confidence between the temperatures 47 and 53ºC, however there is a sligh higher enzyme activity for both enzymes at 50°C.

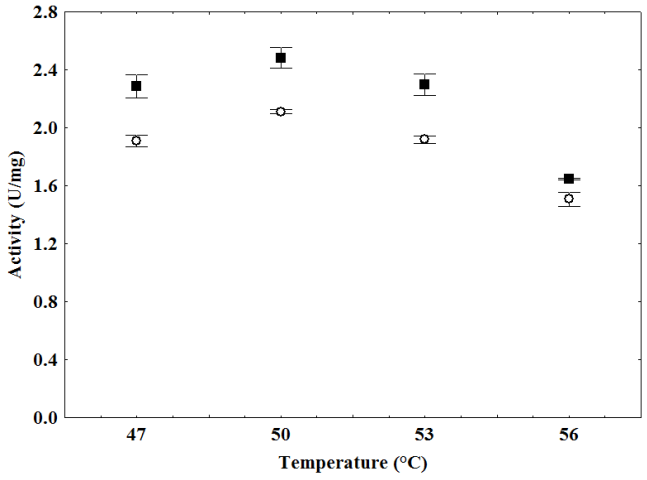


Figure 4.5: Enzymatic activity as a function of the temperature. (○) free enzyme; (■) cells permeabilized. Bars indicate the standard deviation between triplicates.

According to these results it was then defined as the optimum operational conditions pH 6.6 and temperature of 50°C, for both enzymes.

Samoshina and Samoshin (2005) compared published data for optimal pH and temperature of 43 different yeast β-galactosidases, and found out that theoptimum pH ranged between 3.9 and 7.5 and temperature between 30 and 55 ° C. For the enzymes from *Kluyveromyces* species the optimum pH ranged from 6.2 to 7.5 and temperature from 30 to 52°C, what is in agreement with the results obtained in this work with *K. marxianus* CCT 7082. According to Whitaker (1994), the pH and temperature influence the velocity of an

enzyme-catalysed reaction. The active sites on enzyme are frequently composed of ionisable groups that must be in the proper ionic form in order to maintain the conformation of the active site. As the temperature increases, the expected increase in enzyme activity resulting from increased enzyme/substrate collisions is offset by increasing rate of denaturation.

4.3.3.2 pH and thermal stability

According to the results of the enzyme activity during incubation, for each pH, the half-lives ($t_{1/2}$) of both enzymes (Figure 4.6) could be determined. Both enzymes are more stable at pH 7.0 and the permeabilized cell enzyme has a half-life higher than the free enzyme, about 75 min and 60 min, respectively. At pHs lower than 6.5 and higher than 7.0 the losses of enzyme activity are 50% in less than 20 min. The enzyme from the permeabilized cells was more stable than the free enzyme in most of the pH conditions, what suggests that the cells have a protective effect on intracellular enzyme, slowing the adverse effects of less favorable conditions in the conformational structure of the protein.

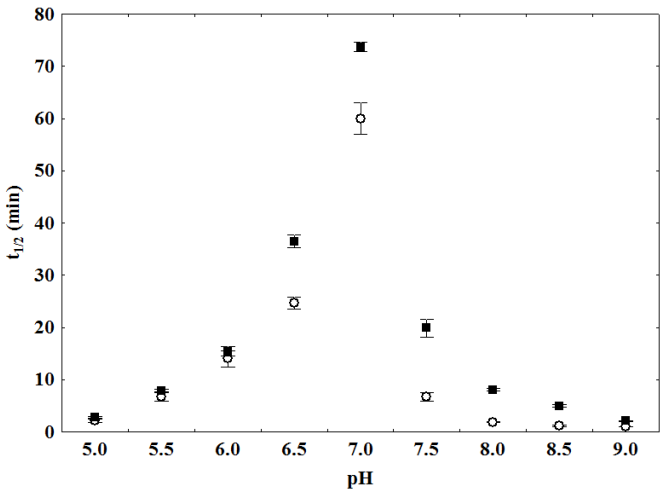


Figure 4.6: Enzyme half-lives ($t_{1/2}$) at different pH values. (○) free enzyme; (■) permeabilized cells.

Ismail et al. (1997), reported that the sharp drop in activity below of neutrality is due to conformational instability of the protein in this pH range. According to Ladero et al.

(2000), at high pH values, the density of active amine groups of the enzyme is much higher than at neutral pH, so that the enzyme could interact further in basic media and lose activity due to a structural change of the enzyme.

Thermal stability of β-galactosidase from permeabilized cells and free enzyme were determined by incubating them in 0.1 M phosphate buffer pH 7.0, at 30 to 50°C. The logarithm of the relative β-galactosidase activity, based on the initial enzyme activity, was plotted against the incubation time. The deactivation step obeys an irreversible first-order kinetic, what suggests that a direct transition occurs from active enzyme to totally inactive protein. As shown in Table 4.5, the deactivation constants (K_d) for the free enzyme were slightly higher than those for the β-galactosidase from permeabilized cells, meaning that the thermal stability of the permeabilized cell enzyme should be higher than that of the free enzyme.

This can be seen in the half-life data of the same table. However, this occurs only for a limited range of the temperature. At temperatures between 30 and 38°C the enzyme of the permeabilized cells is effectively more stable than the free enzyme, whereas at temperatures above 38°C, both enzymes had practically similar half-lives. At 42°C the enzyme retained 50% of initial activity during about 1.8 h, whereas at temperatures of 46 and 50°C the enzymes lose their activities in a few minutes.

Table 4.5: Thermal inactivation constants (K_d) and half-lives ($t_{1/2}$) for β-galactosidase from permeabilized cells and free enzyme.

T (°C)	**K_d (h^{-1})**		**$t_{1/2}$ (h)**	
	Permeabilized cells	**Free enzyme**	**Permeabilized cells**	**Free enzyme**
30	0.005 ± 0.0002	0.006 ± 0,0003	144.41 ± 6,24	115.52 ± 5,55
34	0.015 ± 0.0010	0.020 ± 0,0008	46.49 ± 3,10	34.44 ± 1,28
38	0.090 ± 0.0052	0.135 ± 0,012	7.68 ± 0,46	5.15 ± 0,45
42	0.36 ± 0.011	0.40 ± 0,036	1.89 ± 0,06	1.71 ± 0,15
46	2.98 ± 0.028	2.99 ±0,104	0.23 ±0,002	0.23 ± 0,008
50	19.19 ± 0.40	29.50 ± 0,53	0.036 ±0,0008	0.023 ±0,0004

Deactivation energies (E_d) of β-galactosidase from permeabilized cells and free enzyme were found to be similar, approximately 81 Kcal/mol. These results confirm the above one about stability, since higher the deactivation energy higher the energy needed to deactivate the protein molecule, and therefore higher the stability. Also, these values are in range (50-140 Kcal/mol) estimated for many microbial enzymes (Whitaker, 1994).

4.3.3.3 Determination of kinetic constants K_m e V_{max}

According to Samoshina & Samoshin (2005), $V_{máx}$ is not a fundamental characteristic of enzyme, because it depends on the enzyme purity and concentration, however, the Michaelis-Menten constant, is a more fundamental parameter, it is often used as an affinity parameter, in particular for a comparison of enzyme affinity to substrate, and the smaller the K_m, the larger it is believed to be the affinity.

The kinetics constants of β-galactosidase from permeabilized cells and free enzyme, related to the hydrolysis of lactose, were obtained by the typical double reciprocal Lineweaver Burk plot. K_m and $V_{máx}$ values were 31.9 mM and 2.3 μmol/L.min, for permeabilized cells and 38.5 mM and 1.9 μmol/L.min for free enzyme, respectively. The constants values for both enzymes are quite similar, pointing out that the microorganism cell wall does not hinder the access of substrate to enzyme active sites. The K_m values are in range (2.5 – 76.9 mM) estimated for many β-galactosidase from yeast for the hydrolysis of lactose (Samoshina & Samoshin, 2005).

4.4 CONCLUSIONS

The use of permeabilized cells can be advantageous in industrial processes since the cells can be easily recovered and reused. Isopropanol was the permeabilizing agent selected for the permeabilization of the cells of *K. marxianus* CCT 7082, and the conditions that resulted in highest enzyme activity were biomass/isopropanol mass ratio (w/w) of 0.03, temperature of 25°C and 5 min of incubation. The enzyme of the permeabilized cells and the free enzyme were shown to have the same optimum temperature (50°C) and pH (6.6). The enzyme of the permeabilized cells was more stable than the free enzyme. The half life

at 45°C and pH 7.0 was 75 min for the permeabilized cells and 60 min for the free enzyme. On the other hand, the enzyme half lives at 30ºC were quite similar and about 115 h for both enzymes. The kinetic constants, K_m and V_{max} were 31.9 mM and 2.3 μmol/L.min, respectively, for β-galactosidase of the permeabilized cells and 38.5 mM and 1.9 mmol/min, respectively, for the free enzyme. According to the above it can be concluded that the cell wall causes a mild protective effect on intracellular enzyme, and causes no hindering to the hydrolysis reaction rate.

Acknowledgments

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4.5 REFERENCES

ALAMAE, T.; JARVISTE, A. Permeabilization of the methylotrophic yeast *Pichia pinus* for intracellular enzyme analysis: a quantitative study. **Journal of Microbiological Methods**, v. 22, p. 193-205, 1995.

NAGLAK, T. J., HETTWER, D. J., WANG, H. Y.**Chemical Permeabilization of Cells for Intracellular Product Release In: Separation Process in Biotechnology**. ASENJO, J. A. Marcel Dekker: New York and Basel, p. 177-205, 1990.

CHISTI, Y., & MOO-YOUNG, M. Disruption of microbial cells for intracellular products. **Enzyme Microbiology and Technology**, 8, 194–204, 1986.

CHOI, K. O.; SONG, S. H.; YOO, Y. J. Permeabilization of *Ochrobactrum anthropi* SY 509 cells with organic solvents for whole cell biocatalyst. **Biotechnology and Bioprocess Engineering**, v. 9, p. 147-150, 2004.

GECIOVA, J.; BURY, D.; JELEN, P. Methods for disruption of microbial cells for potential use in the dairy industry - a review. **International Dairy Journal**. v. 12, p. 541–553, 2002.

INCHAURRONDO, V. A.; YANTORNO O. M.; VOGET, C. E. Yeast growth and β-galactosidase production during aerobic batch cultures in lactose-limited synthetic medium. **Process Biochemistry,** v. 29, p. 47-54, 1994.

ISMAIL, S. A.; MABROUK, S. S.; MAHONEY, R. R. Purification and characterization of β-galactosidase from *Mucor pusillus*. **Journal of Food Biochemistry**. v. 21, p. 145-162, 1997.

JOSHI, M. S.; GOWDA, L. R.; KATWA, L. C.; BHAT, S. G. Permeabilization of yeast cells (*Kluyveromyces fragilis*) to lactose by digitonin. **Enzyme and Microbial Technology**, v. 11, p. 439-443, 1989.

KAUR, G.; PANESAR, P. S.; BERA, M. B.; KUMAR, H. Hydrolysis of whey lactose using CTAB-permeabilized yeast cells. **Bioprocess and Biosystems Engineering,** v.32, n. 1, p. 63-67, 2009.

KONDO, A.; LIU, Y.; FURUTA, M.; FUJITA, Y.; MATSUMOTO, T.; FUKUDA, H. Preparation of high activity whole cell biocatalyst by permeabilization of recombinant flocculent yeast with alcohol. **Enzyme and Microbial Technology,** v. 27, p. 806-811, 2000.

LADERO, M.; SANTOS, A.; GARCIA-OCHOA, F. Kinetic modeling of lactose hydrolysis with an immobilized β-galactosidase from *Kluyveromyces fragilis*. **Enzyme and Microbial Technology**. v. 27, p. 583-592, 2000.

LONGHI, L. G. S.; LUVIZETTO, D. J.; FERREIRA, L. S.; RECH, R.; AYUB, M. A. Z.; SECCHI, A. R. A growth kinetic model of *Kluyveromyces marxianus* cultures on cheese whey as substrate. **Journal of Industrial Microbiology and Biotechnology**, v. 31, p.35-40, 2004.

MANERA, A. P.; ORES, J. C.; RIBEIRO, V. A.; BURKERT, C. A. V.; KALIL, S. J. Optimization of the culture medium for the production of β-galactosidase from *Kluyveromyces marxianus* CCT 7082. **Food Technology and Biotechnology**, v.46, n. 1, p. 66-72, 2008.

NUMANOGLU, Y. and SUNGUR, S. β-galactosidase from *Kluyveromyces lactis* cell disruption and enzyme immobilization using a cellulose-gelatin carrier system. **Process Biochemistry**, v. 39, n. 6, p. 705-711, 2004.

PANESAR, P. S. Application of response surface methodology for maximal lactose hydrolysis in whole milk using permeabilized yeast cells. **Acta Alimentaria**, v. 37, v. 2, p. 191- 203, 2008.

PANESAR, R.; PANESAR, P. S.; SINGH, R. S.; KENNEDY, J. F.; BERA, M. B. Production of lactose-hydrolyzed milk using ethanol permeabilized yeast cells. **Food Chemistry**, v. 101, p.786-790, 2007.

RODRIGUES, M. I.; IEMMA A .F. **Planejamento de experimentos e otimização de processos**. 2ª ed. 2009, Casa do Pão Editora, Campinas.

SAMOSHINA, N. M.; SAMOSHIN, V. V. The Michaelis constants ratio for two substrates with a series of fungal (mould and yeast) β-galactosidase. **Enzyme and Microbial Technology.** v. 36, p. 239-251, 2005.

SISO, M. I. G.; CERDAN, E.; FREIRE, M. A. ; RAMIL, E.; BELMONTE, E. R.; TORRES, A. M. R. Permeabilization of Kluyveromyces lactis cells for Milk whey saccharification: a comparison of different treatments. **Biotechnol. Technol.** v. 6, p. 289-292, 1992.

SOMKUTI, G. A.; DOMINIECKI, M. E.; STEINBERG, D. H. Permeabilization of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus* with ethanol. **Current Microbiology**, v. 36, p.202-206, 1998.

SZCZODRAK J. Hydrolysis of lactose in whey permeate by immobilized β-galactosidase from *Kluyveromyces fragilis*. **Journal of Molecular Catalysis B: Enzymatic.** v.10, n. 6, p. 631-637, 2000.

WHITAKER, J. R. **Principles of enzymology for the food sciences**. 2. ed. New York: Marcel Dekker Inc., 1994.

CAPÍTULO 5

GALACTOOLIGOSACCHARIDE PRODUCTION USING PERMEABILIZED CELLS OF Kluyveromyces marxianus

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GALACTOOLIGOSACCHARIDE PRODUCTION USING PERMEABILIZED CELLS OF *Kluyveromyces marxianus*

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Abstract

Galactooligosaccharides (GOS) are non-digestible carbohydrates, and are recognized as important prebiotics for the estimulation of the proliferation of lactic acid bacteria and bifidobacteria in the human intestine. GOS can be produced by a transgalactosylation reaction catalyzed by β-galactosidase enzyme, and microorganisms can be used as a source of β-galactosidase. In this work a process for producing GOS using permeabilized cells of *Kluyveromyces marxianus* CCT 7082 was proposed. The effects of the concentrations of lactose and enzyme, temperature and pH were studied using a fractional design followed by a central composite rotatable design (CCRD). The optimum conditions for Galacto-oligosaccharide production were found to be: lactose concentration 500 g/L, enzyme concentration 10 U/mL, 45ºC and pH 7.0. Under optimized conditions, the GOS concentration, yield and productivity were 83 g/L, 16.5% and 27.6 g/L.h, respectively.

Key words: β–galactosidase, transgalactosylation, experimental design.

5.1 INTRODUCTION

Galactooligosaccharides (GOS) have become the focus of great attention as functional foods, owing to their known health benefits and potential to improve the quality of many foods. Because of these properties, they are currently used as low-calorie

sweetners in fermented milk products, confectioneries breads, and beverages (Park and Oh, 2010). GOS can stimulate the proliferation of lactic acid bacteria and bifidobacteria in the intestine, the predominance of this microorganisms in the colon has been suggested to cause beneficial effects for maintaining human health, such as reduction of the level of cholesterol in serum, decrease the population of pathogenic microorganisms, colon cancer prevention, enhancement of calcium adsorption (Rowland and Tanaka, 1993; Sako et al., 1999; Tomomatsu, 1994; Alander et al., 2001; Chonan et al., 2001; Sinclair et al., 2009).

The stability under acidic conditions of GOS during food processing makes them potentially applicable as ingredients for a wide variety of food products. Their excellent taste quality and relatively low sweetness make GOS interesting functional sweetners. They pass the small intestine without being digested and are therefore of low caloric value. In addition, GOS cannot be metabolized by microorganisms of the oral cavity and are thus not implicated in the formation of dental caries (Crittenden and Playne, 1996; Sako et al., 1999).

GOS are the products of transgalactosylation reaction catalyzed by the enzyme β-galactosidase, when using lactose or other structural related galactosides as the substrate. The hydrolysis of lactose to glucose and galactose, occurs predominantly at low lactose concentrations, while GOS production by the transgalactosylation reaction increases with increasing concentrations of lactose (Mahoney, 1998, Golsing et al., 2010).

β-galactosidase (EC 3.2.1.23) is one of the most promising enzymes, which has several applications in the food, fermentation and dairy industry (Kaur et al, 2009). Amongst various microorganisms, yeast has emerged as an important source of β-galactosidase, since the yeast enzyme has an optimum pH suitable for lactose hydrolysis in milk and sweet whey (Kondo et al., 2000). In this context, *Kluyveromyces marxianus* offers several great advantages, such as good growth yield, acceptability as a safe microorganism (GRAS) and higher β-galactosidase activity than other yeast (Kaur et al., 2009).

β-galactosidase produced by *K. marxianus* is an intracellular enzyme, therefore, the industrial applications of processes based on the enzymatic hydrolysis of lactose are limited (Panesar, 2008). The preparation of cell-free extracts is laborious and needs disruption of cells, which destroys the integrity of cells and may cause inactivation of enzymes.

Therefore, cell permeabilization has been recommended as an alternative method for the study of intracellular enzymatic reactions (Alamäe and Järviste, 1995). The permeabilizing agent may disrupt the membrane structures by decreasing the phospholipid content to allow the passive passage of low molecular weight solutes in and out of cells, including lactose and its products of hydrolysis (Siso et al., 1992; Panesar, 2008).

The enzymatic synthesis of GOS from lactose and cheese whey using various crude, purified and immobilized β-galactosidases has been reported (Cho et al., 2003; Gaur et al., 2006; Hsu et al., 2007; Park et al., 2008; Martínez-Villaluenga et al., 2008) however, the use of yeast permeabilized cells as a source of β-galactosidase on the synthesis of GOS is an interesting alternative, which has been little explored (Park and Oh, 2010).

Several parameters such as the source and concentration of the enzyme, substrate concentration, pH and temperature can influence the equilibrium of the enzyme reaction catalysis in the synthesis of GOS. In this context, the aim of the present research was to optimize the production of GOS from permeabilized cells of *Kluyveromyces marxianus* CCT 7082. The optimization was carried out by experimental design and surface analysis methodology.

5.2 MATERIALS AND METHODS

5.2.1 Enzyme production

Kluyveromyces marxianus CCT 7082, previously selected as the best β-galactosidase producer by Manera et al. (2008), was used in this present study. The strain was maintained at -18ºC on YM broth (yeast extract 3.0 g/L, malt extract 3.0 g/L, peptone 5.0 g/L, glucose 10.0 g/L) with glycerol 10% (v/v).

The fermentation media was composed by 17.0 g of yeast extract, 8.8 g of $(NH_4)_2SO_4$, 5.0 g of KH_2PO_4, 0.4 g of $MgSO_4.7H_2O$ and 28.2 g of lactose in 1 L of 0.2 M potassium phosphate buffer, pH 6.0. The cultures were incubated in an orbital shaking incubator for 96 h at 180 rpm and 30ºC (Manera et al., 2008). Cultivation was started with a 10% inoculum, incubated at 30ºC, 180 rpm for 24 h.

5.2.2 Cells permeabilization

Cell permeabilization was carried out using isopropanol in a ratio biomass/isopropanol (w/w) of 0.03 and held at 25°C for 5 min. Before adding the isopropanol to the biomass it was prepared in 0.1 M potassium phosphate buffer pH 7.0 at concentration 30% Cells were separated from solvent at 6000 × *g* for 5 min and washed twice with cold 0.1 M potassium phosphate buffer, pH 7.0 and lyophilized (Manera, et al., 2010).

5.2.3 Optimization of the galactooligosaccharides synthesis using permeabilized yeast cells

The reaction mixture, containing the enzyme β-galactosidase in permeabilized yeast cells and lactose prepared in 0.1 M potassium phosphate buffer, was incubated in a temperature-controlled water bath for 12 h. At regular intervals, an aliquot of the reaction mixture was removed and heated in boiling water for 5 min to inactivate the enzyme. The samples were stored at -18°C for subsequent analysis of sugars.

Two experimental designs were carried out (Table 5.1); the first one, a fractional factorial design including 2^{4-1} trials with three central points (17 trials) (Rodrigues and Iemma, 2009) was carried out in order to evaluate the effects of lactose concentration, enzyme concentration, temperature and pH (independent variables) on the synthesis of GOS. The responses taken into account were GOS concentration, yield and productivity (dependent variables).

The preliminary fractional factorial design allowed for the selection of the statistically significant variables with respect to GOS concentration, yield and productivity. With these variables, a central composite rotatable design (CCRD) with three replicates at the central point and four axial points (11 trials) (Rodrigues and Iemma, 2009) was performed to obtain a second-order model for the prediction of GOS concentration, yield and productivity as a function of the variables studied. Statistical analyses were performed using the software Statistica 9.1 (Statsoft, 2010). Table 5.1 shows the range of the studied variables and the correspondent coded levels for both experimental designs.

Table 5.1: Values of coded levels and real values in fractional factorial design and CCRD.

Factorial design	Coded variable level	Lactose (g/L)	Enzyme (U/mL)	Temperature (ºC)	pH
Fractional	-1	400	5.0	40	6.0
	0	450	7.5	45	6.5
	+1	500	10.0	50	7.0
CCRD	-1.41	209	5.8	-	-
	-1	250	7.0	-	-
	0	350	10.0	-	-
	+1	450	13.0	-	-
	+1.41	491	14.2	-	-

5.2.4 Chromatographic determination of carbohydrates

The identification and quantification of sugars (lactose, glucose, galactose and GOS) was carried out by ion exchange chromatography with pulsed amperometric detection (HPLC-PAD). A DIONEX (USA) chromatograph, supplied with a Carbopac PA1 (4x250 mm) column, a PA1 (4x50 mm) guard column, with a GP50 gradient pump, ED40 electrochemical detector and PEAKNET software were used for the analysis. Sugars were eluted with 20 mM sodium hydroxide, at a flow rate of 1.0 $mL.min^{-1}$. Before injection, the samples were diluted with water and filtered through 0.22 μm filters.

5.2.5 β-galactosidase assay

β-galactosidase activity was assayed using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) following the method described by Inchaurrondo *et al*.(1994). A 50 μL sample of permeabilized cell suspension was mixed with 2 mL of 1.25 mM ONPG in buffer (50 mM of KH_2PO_4 and 0.1 mM $MnCl_2.4H_2O$, pH 6.6) and incubated for 5 min at 37°C. The reaction was stopped by adding 0.5 mL of 1 M sodium carbonate. Liberated *o*-nitrophenol was measured spectrophotometrically at 420 nm. One unit of β-galactosidase activity is defined as the amount of the enzyme necessary for the hydrolysis of 1 μmol of ONPG per minute under the conditions of the assay.

5.2.6 Cell concentration

Cell concentration was estimated by measuring the absorbance at 620 nm and converted to dry weight by using a standard curve. Samples were centrifugated at 6000 × *g* for 5 min, washed twice with distilled water and ressuspended in water, and absorbance read at 600 nm. For the standard curve cells were dried at 90°C to constant weight (Longhi et al., 2004).

5.3 RESULTS AND DISCUSSION

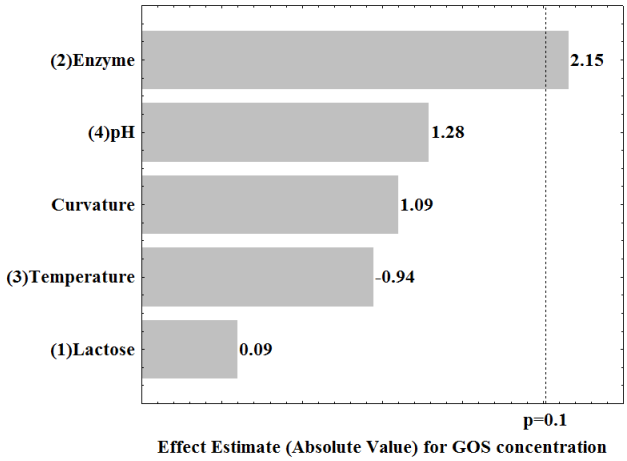
Table 5.2 shows the results of maximum GOS concentration, productivity and yield from the first experimental design. As it can be seen, GOS production changed from 28.3 g/L up to 95.1 g/L, the productivity from 7.1 to 23.8 g/L.h and yield from 5.7 to 19.0%. The maximal concentrations of GOS were obtained between 4 and 6 h of synthesis.

Table 5.2: The 2^{4-1} factorial design for GOS production.

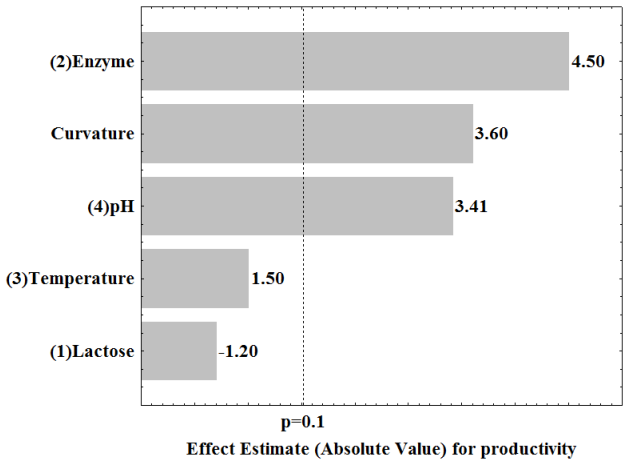
Essay	Lactose (g/L)	Enzyme (U/mL)	T (ºC)	pH	GOS (g/L)	t[a] (h)	P[b] (g/L.h)	R[c] (%)
1	-1 (400)	-1 (5)	-1 (40)	-1 (6.0)	66.8	6	11.1	16.7
2	1 (500)	-1 (5)	-1 (40)	1 (7.0)	75.3	6	12.6	15.1
3	-1 (400)	1 (10)	-1 (40)	1 (7.0)	75.2	4	18.8	18.8
4	1 (500)	1 (10)	-1 (40)	-1 (6.0)	92.1	6	15.4	18.4
5	-1 (400)	-1 (5)	1 (50)	1 (7.0)	71.4	4	17.9	17.9
6	1 (500)	-1 (5)	1 (50)	-1 (6.0)	28.3	4	7.1	5.7
7	-1 (400)	1 (10)	1 (50)	-1 (6.0)	73.6	4	18.4	18.4
8	1 (500)	1 (10)	1 (50)	1 (7.0)	95.1	4	23.8	19.0
9	0 (450)	0 (7.5)	0 (45)	0 (6.5)	81.6	4	20.4	18.1
10	0 (450)	0 (7.5)	0 (45)	0 (6.5)	83.7	4	20.9	18.6
11	0 (450)	0 (7.5)	0 (45)	0 (6.5)	85.7	4	21.4	19.0

a) t (h) = Time course for maximal GOS concentration
b) P (g/L.h) = GOS productivity = [g/L.h]
c) R (%) = GOS yield = [g GOS/g Initial lactose]*100

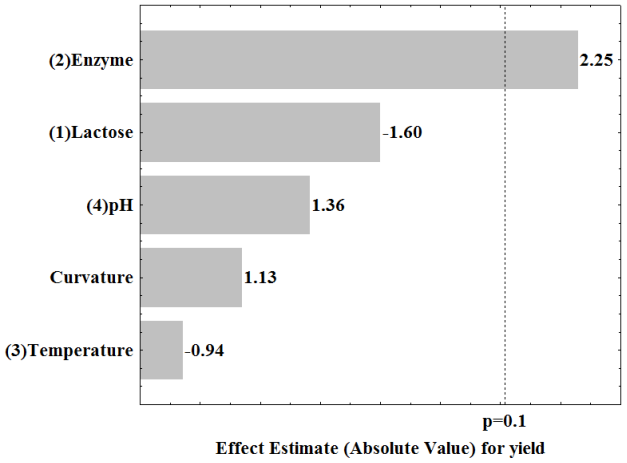
The variable effects were estimated according to the responses, for each variable, which changed from level -1 to +1 (Figure 5.1).



(a)



(b)



(c)

Figure 5.1: Pareto plot for the effect estimates: (a) GOS concentration; (b) productivity and (c) yield.

As shown in Figure 5.1, for the responses GOS concentration and yield, only the enzyme concentration was significant at 90% of confidence, in the range studied, and it was positive, what means that when the enzyme concentration changed from -1 to +1 levels, there was an increase in the GOS concentration. For the response productivity, both enzyme concentration and pH were significant at 90% of confidence, and both positives.

According to the effect estimates concerning the first experimental design, a second one was planned, in which the temperature was fixed at 45°C (central point) and the pH at 7.0 (level +1). This pH value was chosen since it is the pH of maximum enzyme stability (Manera et al., 2010). The new experimental design, a central composite rotatable design, was carried out, whose variables were enzyme and lactose concentrations (Table 5.3). A higher range of enzyme activity was essayed, from 5.8 to 14.2 U/mL. For the lactose concentration, even if this variable was not significant in the factorial design, a lower concentration range was essayed (from 209 g/L to 491 g/L), in order to study the possibilities of using lower concentrations of this substrate, as an attempt for lowering GOS production costs.

Table 5.3: The CCRD for GOS production.

Essay	Lactose (g/L)	Enzyme (U/mL)	GOS (g/L)	t[a] (h)	P[b] (g/L.h)	R[c] (%)
1	-1 (250)	-1 (7)	39.1	2	19.6	15.7
2	1 (450)	-1 (7)	74.8	3	24.9	16.6
3	-1 (250)	1 (13)	39.4	2	19.7	15.8
4	1 (450)	1 (13)	79.6	3	26.6	15.9
5	-1.41 (209)	0 (10)	32.8	2	16.4	15.7
6	1.41 (491)	0 (10)	87.4	3	29.1	17.8
7	0 (350)	-1.41 (5.8)	60.6	3	20.2	17.3
8	0 (350)	1.41 (14.2)	58.4	2	29.2	16.7
9	0 (350)	0 (10)	62.9	3	20.9	18.0
10	0 (350)	0 (10)	65.0	3	21.7	18.6
11	0 (350)	0 (10)	66.5	3	22.2	19.0

a) t (h) = Time course for maximal GOS concentration
b) P (g/L.h) = GOS productivity = [g/L.h]
c) R (%) = GOS yield = [g GOS/g Initial lactose]*100

Equations (1) and (2) were obtained from data in Table 5.3, for GOS concentration and yield, respectively, with 95% of confidence. For the response productivity only the linear correlation of lactose was statistically significant, so that no statistical model was possible.

$$GOS\ (g/L) = 64.80 + 19.17Lactose - 2.74Lactose^2 - 3.02Enzyme^2 \quad (1)$$

$$R\ (\%) = 18.51 + 1.68Lactose - 1.89Lactose^2 - 1.62Enzyme^2 \quad (2)$$

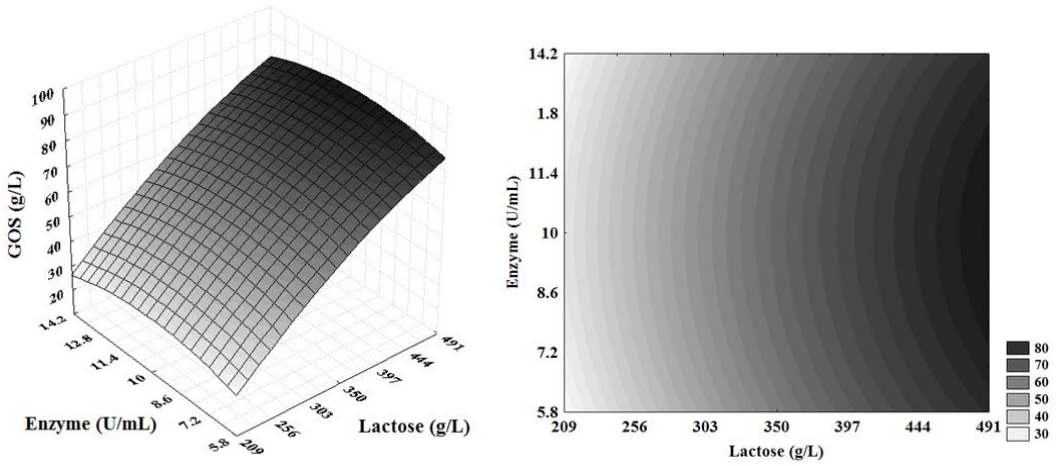
The ANOVA of these equations are shown in Tables 5.4 and 5.5, for GOS concentration and yield, respectively. According to the F test, both models are predictive, since the calculated F is higher than listed one, 61.6 times for GOS concentration and 6.88 times for yield. The regression coefficients were 0.99 and 0.92, for GOS concentration and yield, respectively. Therefore, the models can be used for the surface generations (Figure 5.2).

Table 5.4: The ANOVA for GOS concentration.

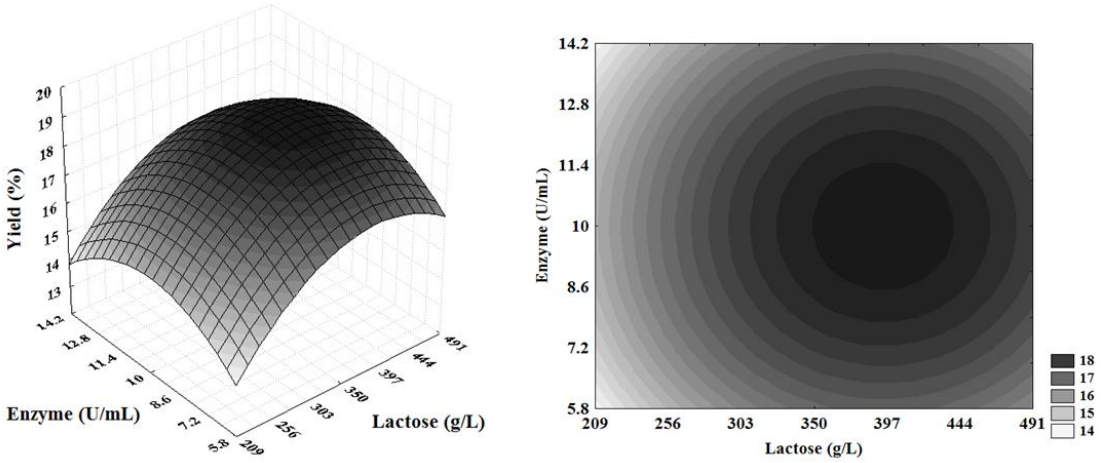
Source of variation	**Sum of squares**	**Degrees of freedom**	**Means squares**	$\mathbf{F_{calc}}$	$\mathbf{F_{tab}}$	$\mathbf{F_{calc}/F_{tab}}$
Regression	3006.7	3	1002.2	265.2	4.3	61.6
Residual	26.4	7	3.7			
Total	3033.1	10				

Table 5.5: The ANOVA for yield.

Source of variation	**Sum of squares**	**Degrees of freedom**	**Means squares**	$\mathbf{F_{calc}}$	$\mathbf{F_{tab}}$	$\mathbf{F_{calc}/F_{tab}}$
Regression	12.4	3	4.1	29.6	4.3	6.88
Residual	0.97	7	0.13			
Total	13.4	10				



(a)



(b)

Figure 5.2: Surface responses for (a) GOS concentration and (b) yield, as a function of enzyme activity and lactose concentration.

According to the surfaces, GOS concentration can achieve more than 80 g/L if lactose concentration is higher than 444 g/L, no matter the enzyme activity, for the range beginning at 5.8 up to 14.2 U/mL (Figure 5.2a). Yields higher than 17% can be achieved with lactose concentration between 303 and 491 g/L and enzyme activity between 7.2 and 12.8 U/mL (Figure 5.2b). Also, it can be seen that the lower lactose concentration the lower GOS production. Considering the essays with low lactose concentration and higher glucose and galactose concentrations, lower GOS concentrations are found. The transgalactosilation

is a reaction where the enzyme β-galactosidase hydrolysis lactose producing glucose and galactose, transferring a galactose molecule to a hydrosylated compound, which can be galactose, lactose or galactooligosaccharides, instead of transferring it to a hydroxyl group of the water molecule. However, at low lactose concentrations, the transgalactosilation is lower than hydrolysis, since the quantity of hydroxyl groups from carbohydrates is low, resulting in a higher glucose and galactose concentrations in the reaction solution (Prenosil et al., 1987). It has also been reported that the lactose initial concentration is a major factor in the GOS synthesis and that higher concentrations lead to higher GOS production (Hsu et al., 2007: Martínez-Villaluenga et al., 2008; Park et al. 2008).

A set of three experiments were carried out, following the indications of the previous experimental designs, in which the enzyme activity was kept at 10 U/mL, lactose concentration at 500 g/L, 45ºC and pH 7.0 (Figure 5.3).

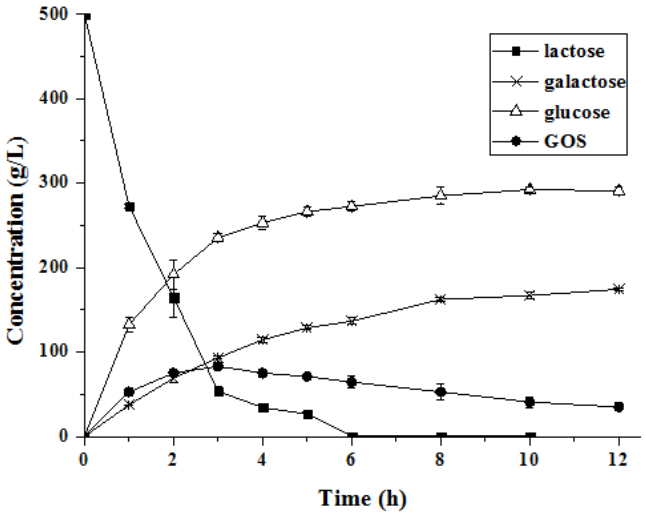


Figure 5.3: Time course of GOS synthesis at the following reaction conditions: 500 g/L lactose, 10 U/mL enzyme activity, 45°C, pH 7.0. The data are from triplicate essays.

The GOS production was monitored and is shown in Figure 5.3, where the experimental data are the average of the three essays. The use of these synthesis conditions led to a maximum GOS production of 83 g/L, after three hours of reaction, with a productivity of 27.6 g/L.h and a yield of 16.5%. The total lactose conversion to glucose,

galactose and GOS was around 90%. After three hours of reaction there was a gradual decrease of galactooligosaccharides as a consequence of the continuous GOS hydrolysis. Similar results about GOS production have been reported using β-galactosidases from different microorganisms (Albayrak & Yang, 2002; Chockchaisawasdee et al., 2005; Hsu et al., 2007). These results show that the transgalactosilation happens predominantly at the beginning of the reaction, resulting in higher GOS concentration, while the β-galactosidase hydrolytic activity increases during the reaction time.

Onishi and collaborators (1995) obtained GOS from permeabilized cells of the yeast *Sterigmatomyces elviae.* In their work, 135 g/L of GOS were produced after 20 hours of synthesis, with a yield of 37.5% and a productivity of 6.75 g/L.h. In a similar work, Onishi *et al.* (1996) studied the synthesis of GOS with permeabilized cells of *Sirobasidium magnum*, in which 136 g/L of GOS were produced after 42 hours of synthesis, with a yield of 37.7% and a productivity of 3.2 g/L.h. Onishi and Yokozeki (1996) working with *Rhodotorula minuta* cells, which had been treated with toluene, obtained a maximum GOS production of 76 g/L after 24 hours of reaction, with a yield of 38% and a productivity of 3.2 g/L.h. When these results of GOS production by permeabilized yeast cells are compared to the one obtained in this work, it can be asserted that in this work the GOS yields were lower and productivity 7 times higher, so that the lower yields can be compensate by higher productivities

5.4 CONCLUSIONS

The use of permeabilized cells for GOS synthesis can be an advantage in industrial processes since the enzyme can easily be recuperated and reused. In this work, the use of permeabilized cells of *Kluyveromyces marxianus* CCT 7082, containing β-galactosidase, to produce galactooligosaccharides was focused. The methodology of experimental factorial design and surface analysis led to the more suitable reaction conditions, which were: 500 g/L lactose concentration, 10 U/mL enzyme activity, 45ºC and pH 7.0. These reaction conditions led to a GOS production of 83 g/L, with a yield of 16.5% and productivity of 27.6 g/L.h. The reaction time for the highest production was 3 hours.

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5.5 REFERENCES

Alamae, T., Jarviste, A., Permeabilization of the methylotrophic yeast *Pichia pinus* for intracellular enzyme analysis: a quantitative study. *J. Microbiol. Methods*, 1995, *22*, 193-205.

Alander, M., Matto, J., Kneifel, W., Johansson, M. *et al*., Effect of galacto-oligosaccharide supplementation on human fecal microflora and on survival an persistence of Bifidobacterium lactis Bb12 in the gastrointestinal tract. *Int. Dairy J***.** 2001, *11*, 817–825.

Albayrak, N. A., Yang, S. T., Production of galacto-oligosaccharides from lactose by *Aspergillus oryzae* β-galactosidase immobilized on cotton cloth. *Biotechnol. Bioeng***.** 2002, *77*, 8-19.

Chockchaisawasdee, S., Athanasopoulos, V. I., Niranjan, K., Rastall, R. A., Synthesis of galacto-oligosaccharide from lactose using β-galactosidase from *Kluyveromyces lactis*: studies on batch and continuous UF membrane-fitted bioreactors. *Biotechnol. Bioeng***.** 2005, *89*, 434-443.

Cho, Y. J.; Shin H. J.; Bucke, C. Purification and biochemical properties of a galactooligosaccharide producing β-galactosidase from *Bullera singularis*. *Biotechnol. Letters*, 2003, *25*, 2107-2111.

Chonan, O., Takahashi, R, Watanuky, M., Role of activity of gastrointestinal microflora in adsorption of calcium and magnesium in rats fed 1-4 linked galactooligosaccharide. *Biosci. Biotechnol. Biochem.* 2001, *65*,1872-1875.

Crittenden, R. G., Playne, M. J., Production, properties and applications of food-grade oligosaccharides. *Trends Food Sci. Technol.* 1996, *7*, 353–361.

Gaur, R.; Pant, H.; Jain, R.; Kkare, S. K. Galacto-oligosaccharide synthesis by immobilized *Aspergillus oryzae* β-galactosidase. *Food Chemistry*, 2006, *97*, 426-430.

Gosling, A., Stevens, G. W., Barber, A. R., Kentish, S. E. *et al*., Recent advances refining galactooligosaccharide production from lactose. *Food Chem*. 2010, *121*, 307-318.

Hsu, C. A., Lee, S. L., Chou, C. C., Enzymatic production of galactooligosaccharides by β-galactosidase from Bifidobacterium longum BCRC 15708. *J. Agric. Food Chem.* 2007, *55*, 2225-2230.

Inchaurrondo, V. A., Yantorno O. M., Voget, C. E., Yeast growth and β-galactosidase production during aerobic batch cultures in lactose-limited synthetic medium. *Process Biochem***.** 1994, *29*, 47-54.

Kaur, G., Panesar, P. S., Bera, M. B., Kumar, H., Hydrolysis of whey lactose using CTAB-permeabilized yeast cells. *Bioprocess Biosyst. Eng*. 2009, *32*, 63-67.

Kondo, A., Liu, Y., Furuta, M., Fujita, Y. *et al*., Preparation of high activity whole cell biocatalyst by permeabilization of recombinant flocculent yeast with alcohol. *Enzyme Microb. Technol*. 2000, *27*, 806-811.

Longhi, L. G. S., Luvizetto, D. J., Ferreira, L. S., Rech, R. *et al*., A growth kinetic model of *Kluyveromyces marxianus* cultures on cheese whey as substrate. *J. Ind. Microbiol. Biotechnol*. 2004, *31*, 35-40.

Mahoney, R. R., Galactosyl-oligosaccharide formation during lactose hydrolysis: a review. *Food Chem***.** 1998, *63*, 147-154.

Manera, A. P., Ores, J. C., Ribeiro, V. A., Burkert, C. A. V. *et al*., Optimization of the culture medium for the production of β-galactosidase from *Kluyveromyces marxianus* CCT 7082. *Food Technol. Biotechnol***.** 2008, *46*, 66-72.

Manera, A. P.; Rodrigues, M. I.; Kalil, S. J.; Maugeri Filho, F. Permeabilization of *Kluyveromyces marxianus* cells aiming β-galactosidase activity and characterization. *J Agric. Food Chem.* 2010, (submitted).

Martínez-Villaluengua, C., Cardelle-Cobas, A., Corzo, N., Olano, A. *et al*., Optimization of conditions for galactooligosaccharide synthesis during lactose hydrolysis by β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000 L HP G). *Food Chem***.** 2008, *107*, 258-264.

Onishi, N., Kira, I., Yokozeki, K., Galacto-oligosaccharide production from lactose by *Sirobasidium magnum* CBS6803. *Lett. Appl. Microbiol*. 1996, *23*, 253-256.

Onishi, N., Yamashiro, A., Yokozeki, K., Production of galactooligosaccharide from lactose by *Sterigmatomyces elvia* CBS8119. *Appl. Environ. Microbiol.* 1995, *61*, 4022-4025.

Onishi, N., Yokozeky, K., Gluco-oligosaccharide and galacto-oligosaccharide production by *Rhodotorula minuta* IFO 879. *J. Ferment. Bioeng.* 1996, *82*, 124-127.

Panesar, P. S., Application of response surface methodology for maximal lactose hydrolysis in whole milk using permeabilized yeast cells. *Acta Aliment.* 2008, *37*, 191- 203.

Park, A. R, Oh, D. K., Galacto-oligosaccharide production using microbial β-galactosidase: current state and perspectives. *Appl. Microbiol. Biotechnol*. 2010, *85*, 1279-1286.

Park, H. Y.; Kim, H. J.; Lee, J. K.; Kim, D.; Oh, D. K. Galactooligosaccharide production by a thermostable β-galactosidase from *Sulfolobus solfataricus*. *World J. Microbiol. Biotechnol.* 2008**,** *24*, 1553-1558.

Prenosil, J. E., Stuker, E., Bourne, J. R., Formation of oligosaccharides during enzymatic lactose: Part I: State of art. *Biotechnol. Bioeng*. 1987, *30*, 1019-1025.

Rodrigues, M. I., Iemma A .F. (2nd Ed.) *Planejamento de experimentos e otimização de processos*, Casa do Pão Editora, 2009.

Rowland, I. R., Tanaka, R. The effects of transgalactosylated oligosaccharides on gut flora metabolism in rats associated with a human faecal microflora. J. *Appl. Bacteriol*. 1993, *74*, 67-674.

Sako, T., Matsumoto, K., Tanaka, R. Recent progress on research and applications of non-digestible galacto-oligosaccharides. *Int. Dairy J.* 1999, *9*, 69-80.

Sinclair, H. R., Slegte, J., Gibson, G. R., Rastall, R. A., Galactooligosaccharides (GOS) inhibit *Vibrio cholerae* toxin binding to its GM1 receptor. J. Agric. *Food Chem*. 2009, *57*, 3113-3119.

Siso, M. I. G., Cerdan, E., Freire, M. A., Ramil, E. *et al*., Permeabilization of *Kluyveromyces lactis* cells for milk whey saccharification: a comprison of different treatments. *Biotechnol. Technol***.** 1992, *6*, 289-292.

Tomomatsu, H. Health effects of oligosaccharides. *Food Technol***.** 1994, *48,* 61-65.

CAPÍTULO 6

EFFECT OF COMPRESSED FLUIDS TREATMENT ON THE ACTIVITY, STABILITY AND ENZYMATIC REACTION PERFORMANCE OF β-GALACTOSIDASE

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EFFECT OF COMPRESSED FLUIDS TREATMENT ON THE ACTIVITY, STABILITY AND ENZYMATIC REACTION PERFORMANCE OF β-GALACTOSIDASE

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Abstract

The observation that galactooligosaccharides can stimulate the growth of bifidobacteria and other health-promoting bacteria has gained growing interest in the transferase reaction of β-galactosidases. Galactooligosaccharides (GOS) are produced from lactose by a glycosyl transfer of one or more galactosyl units onto the galacose moiety of lactose catalyzed by β-galactosidase. The linkage between galactose units and components in the final product depends upon the source of the enzyme and conditions used in the reactions. In this context, the present work investigates as a first step the influence of pressure, exposure time and depressurization rate on the β-galactosidase activity of permeabilized cells of *K. marxianus* CCT 7082 submitted to treatment with compressed carbon dioxide, propane and n-butane. In general, results showed that the enzyme activity changes significantly depending on the experimental conditions investigated, allowing the selection of proper operating conditions for advantageous application of this biocatalyst in hydrolysis reactions. Further, the stability of the enzyme after high-pressure pre-treatment was also experimentally monitored, and the results demonstrated that the activities of this biocatalyst were always higher than the non-treated one. Finally, evaluation of synthesis of GOS using β-galactosidase without treatment and after treatment in compressed fluids was carried out. Results pointed out promising possibilities since the use of n-butane pre-treated enzyme led to very satisfactory reaction conversions and selectivity.

Keywords: β-galactosidase; galactooligosaccharides; permeabilized cells; compressed fluids.

6.1. INTRODUCTION

Galactooligosaccharides (GOS) are non-digestible food ingredients that beneficially affect the host by selectively stimulating the proliferation of bifidobacteria and lactobacilli in the intestine. GOS are considered to be valuable to human health and can be synthesized from lactose when this sugar acts as the acceptor and transgalactosylation is catalyzed by β-galactosidase. However, if this acceptor is a water molecule, galactose is released through a hydrolysis reaction (Neri et al., 2009).

In aqueous systems transgalactosylation competes with hydrolysis, and therefore GOS mixtures always contain considerable amounts of remaining lactose and monosaccharides that decrease the overall yield (Mahoney, 1998). An alternative to overcome such drawback would be the use of organic solvents to conduct the reactions. The solvent can affect an enzymatic reaction both by direct interaction with the enzyme and by influencing the solvation of the substrates and products in the reaction medium. However, the use of organic solvents as reaction medium has some inconvenient such as the solvent interaction with enzymes hence decreasing its stability, the presence of chemical residues affecting the safety of food product and the high costs involved in eliminating the organic solvent from the product (Garrea & Riva, 2008).

Numerous studies have shown that many enzymatic reactions can be conducted in supercritical carbon dioxide and, in some cases, rates and selectivities achieved are greater than those obtained in normal liquid or gas phase reactions (Oliveira & Oliveira, 2000; 2001). However, a serious drawback of those applications may arise from the non-polarity of carbon dioxide, which means non-proper dissolution of both hydrophobic and hydrophilic compounds, affecting negatively the activity of the enzyme. Nevertheless, carbon dioxide is not the only gas whose properties seem to be adequate for biocatalysis (Kao, Ekhorutomwen, & Sawan, 1997). For instance, the comparable dielectric constant of propane and n-butane to carbon dioxide (Habulin & Knez, 2001; Oliveira, Feihrmann,

Rubira, Kunita, Dariva & Oliveira, 2006a), and the fact that much higher pressure phase transition values are generally found in systems formed by carbon dioxide with high molecular weight compounds when compared to the use of propane and n-butane support a firm belief that propane and n-butane may also be suitable as reaction media for enzyme-catalyzed bioconversions (Ndiaye, Lanza, Tavares, Dariva, Oliveira & Oliveira, 2006).

To conduct galactosidase-catalyzed reactions at high pressures, the enzyme behavior in compressed fluids is of primary importance as the loss of enzyme activity may lead to undesirable poor reaction rates and low yields of target products. Enzyme stability and activity may depend on the enzyme species, characteristics of compressed fluid, water content of the enzyme/support/reaction mixture and manipulated process variables. Inspection of the literature cited reveals that, while there are some few reports regarding activity and stability of β-galactosidase submitted to high hydrostatic pressures (Eisenmenger, & Reyes-De-Corcuera, 2009), there is a lack of corresponding experimental information for this enzyme in compressed carbon dioxide, propane and n-butane.

The reaction mechanism of GOS synthesis presented in the review of Mahoney (1998) indicates that enzyme will transfer galactose to nucleophilic acceptors containing a hydroxyl group. Transfer to water produces galactose; transfer to another sugar produces di-, tri- and higher galactosyl-saccharides. These in turn become substrates for the enzyme and are slowly hydrolyzed. Under most conditions, hydrolysis predominates due to high water concentration (necessary for lactose solubilization) and GOS production is low. The yield of GOS can be increased by using higher substrate concentrations and/or by decreasing the water content. Another alternative to improve the process yield is to propose a modification of the enzyme amino groups, as can be the case of using high-pressure treatment of the enzyme before its use in the reaction.

Based on these aspects, the main objective of this study is to investigate the effect of compressed fluids (carbon dioxide, propane and n-butane) treatment on the activity, stability and enzymatic reaction performance of β-galactosidase from permeabilized cells of *K. marxianus* CCT 7082.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals

The chemicals used were all of analytical grade purchased from Synth, Brazil, and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) purchased from SigmaChem., USA.

6.2.2 Enzyme production

Kluyveromyces marxianus CCT 7082 was employed for β-galactosidase production. The microorganism was cultivated in a medium containing 28.8 g/L lactose, 17.0 g/L yeast extract, 5.0 g/L KH_2PO_4, 8.8 g/L $(NH_4)_2SO_4$ and 0.4 g/L $MgSO_4.7H_2O$, at 150 rpm and 30°C for 96 h (Manera, Ores, Ribeiro, Burkert, & Kalil, 2008). Cell concentration was estimated by measuring the absorbance at 620 nm and converted to dry weight following the standard curve. The cells of broth were collected by centrifugation at 6000 × *g* for 5 min, washed twice with distilled water and re-suspended in water to absorbance readings. For the standard curve, cells were dried at 90°C to constant weight (Longhi, Luvizetto, Ferreira, Rech, Ayub, & Sechi, 2004).

6.2.3 Cell permeabilization

Cell permeabilization was carried out using isopropanol in a ratio biomass/isopropanol (w/w) of 0.03 and held at 25°C for 5 min. Before adding the isopropanol to the biomass it was prepared in 0.1 M potassium phosphate buffer pH 7.0 at concentration 30% Cells were separated from solvent at 6000 × *g* for 5 min and washed twice with cold 0.1 M potassium phosphate buffer, pH 7.0 and lyophilized (Manera et al., 2010).

6.2.4 Enzyme activity

β-galactosidase activity was assayed using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) following the method described by Inchaurrondo, Yantorno, and Voget (1994). A 50 µL sample of permeabilized cell suspension without any previous purification was mixed with 2 mL of 1.25 mM ONPG in buffer (50 mM of KH_2PO_4 and 0.1 mM

$MnCl_2.4H_2O$, pH 6.6) and incubated for 5 min at 37°C. The reaction was stopped by adding 0.5 mL of 1 M sodium carbonate. Liberated *o*-nitrophenol was measured spectrophotometrically at 420 nm. One unit of β-galactosidase activity is defined as the amount of the enzyme necessary for the hydrolysis of 1 μmol of ONPG per minute under the conditions of the assay. The enzyme activity were measured in triplicate and all the analyses presented experimental error lower than 2%.

6.2.5 High-pressure treatment of enzyme

The equipment used in all the experiments for evaluation the enzyme activity under compressed fluids consists basically of a solvent reservoir, two thermostatic baths, a syringe pump (ISCO 260D), a stainless steel vessel with an internal volume of 3 mL, an absolute pressure transducer (Smar, LD301) equipped with a portable programmer (Smar, HT201) with a precision of $\pm$ 0.4 bar. The vessel was built to conduct the experiments up to 350 bar and 80°C, as schematically presented in Fig. 6.1 (Fricks et al., 2006; Franken et al., 2008). All lines of the experimental setup consisted of 1/16" OD stainless steel tubing (HIP) and between the pump and solvent reservoir a check (one way) valve (HIP 15-41AF1-T 316SS) was positioned to avoid pressurization solvent back flow to the head of solvent cylinder. Two additional micrometering valves (HIP 15-11AF2 316SS) completed the experimental apparatus, one located after the syringe pump, at the entrance of high-pressure cell, to allow solvent loading and the other just after the cell to perform solvent discharge. The high-pressure cell was submerged into the water bath and was supported by a simple device while the micrometering valves were located outside the bath.

The permeabilized cells of *K. marxianus* containing the β-galactosidase (approximately 0.15 g) were charged into the cell. Afterwards, the system was pressurized and kept at constant temperature and pressure for a pre-established exposure time. Typically, the pressure come up time was less than 0.5 min and accordingly it was not included in the pressure holding time because its relatively small time compared to longer holding times. After the treatment time the system was depressurized and the enzyme activity was measured. The residual activity was defined as the ratio of enzyme activity before (initial) and after (final) treatment with pressurized fluid.

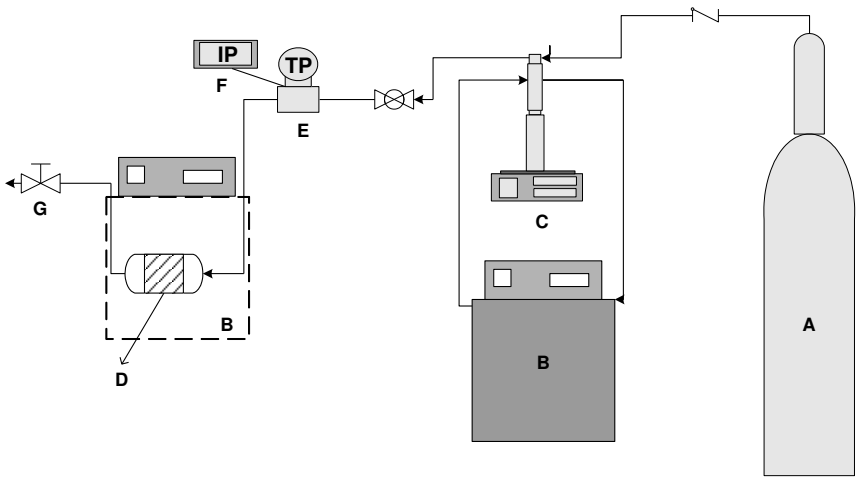

Figure 6.1: Schematic diagram of the apparatus for treatment enzyme with compressed solvents. A-solvent reservoir; B-thermostatic bath; C-syringe pump; D-treatment (cell) vessel; E-pressure transducer; F-pressure indicator; G-micrometric valve.

To evaluate the effects of decompression rate, exposure time and pressure on the activity of β-galactosidase of permeabilized cells of *K. marxianus* submitted to compressed carbon dioxide, propane and n-butane, a central composite design (CCD) was accomplished for each solvent. The experiments were carried out at fixed temperature of 37°C and exposure times (t) from 1 to 6 h for all solvents. For carbon dioxide, the decompression rates (R) covered the range of 10-200 $kg.m^{-3}min^{-1}$ with reduced density (RD) varying from 0.5 to 2.0. For this solvent, system pressure was estimated from Angus, Armstrong, and Reuck (1976) equation. For propane and n-butane, due to their practically incompressible nature within the temperature and pressure range investigated, it was adopted the depressurization (R) range of 2 to 50 $bar.min^{-1}$ and the system pressure (P) varying from 30 to 250 bar for propane and 10 to 250 bar for n-butane.

6.2.6 Synthesis of galactooligosaccharides

The permeabilized cells containing the enzyme (pre-treated and non-treated) (10 U/mL of solution) was added to the lactose solution (500 g/L lactose in 0.1 M sodium phosphate buffer, pH 7.0). The flasks were incubated in a temperature-controlled water

bath at 45ºC under shaking conditions for 12 h. Samples of the reaction mixture were removed at regular time intervals and immediately immersed in a boiling water bath for 5 min to inactivate the enzyme before sugar analysis. The synthesis medium was analyzed for its GOS composition by high performance liquid chromatography with pulsed amperometric detection (HPLC-PAD), using a Dionex (USA) chromatograph and a Carbopac PA-1 column equilibrated with 150 mM NaOH and eluted with 20 mM NaOH at rate of 1 mL/min.

6.3. RESULTS AND DISCUSSION

6.3.1 Effect of compressed propane on the β-galactosidase activity of permeabilized cells of *K. marxianus*

Results obtained from the application of the CCD to evaluate the effects of process variables on the β-galactosidase activity treated with pressurized propane are presented in Table 6.1. For all the experimental runs it was verified that the enzyme activity increased after treatment with compressed propane. The gain of enzyme activity was in the range of 128.0 to 211.1%, an interesting result considering possible applications of enzymes for synthesis of food products, as is the case of galactooligosaccharides or the lactose hydrolysis by β-galactosidase.

To evaluate the main effects of the manipulated variables on the enzyme activity, data of Table 6.1 were treated statistically considering a significance level of 95% ($p<0.05$). Table 6.2 presents the effects of these variables on the final activity of the β-galactosidase submitted to compressed propane, and as one can see the most significant variable was the decompression rate, which presented a positive effect in the experimental range evaluated in this work, thus indicating that higher decompression rates leads to an increase in β-galactosidase activity. Other variables were not significant in the range studied.

In fact, some works presented in the literature show that solvents with low dielectric constant, such as propane, could keep or even enhance enzyme activity and stability (Knez & Habulin, 2002; Oliveira et al., 2006a; Oliveira et al., 2006b; Fricks et al., 2006; Andrade et al., 2008). Since the solvent properties affect the specific interaction with enzymes,

different effects may be obtained depending on the studied enzyme (Kasche, Schlothauer, & Brunner, 1988; Fricks et al., 2006). For the present case, one can observe that the use of pressurized propane as solvent, for all experimental conditions investigated, led to an enhancement in the activity of β-galactosidase. As propane presents relatively low solubility in water, it can be speculated that it might be acting as a piston fluid, enhancing the pressure over the enzyme. Regarding the effect of hydrostatic pressure on enzyme stability, literature indicates that pressure values around those used in this work cause a small effect on enzyme activity (Andrade et al., 2008).

Table 6.1: Matrix of the CCD (real and coded values) with responses in terms of the β-galactosidase activity submitted to compressed propane.

Run	P (bar)	t (h)	R (bar.min^{-1})	Final enzyme activity (U.g^{-1})	Residual activity (%)*
1	30 (-1)	1 (-1)	2 (-1)	450.4	176.0
2	250 (1)	1 (-1)	2 (-1)	327.3	128.0
3	30 (-1)	6 (1)	2 (-1)	356.1	139.2
4	250 (1)	6 (1)	2 (-1)	351.7	137.4
5	30 (-1)	1 (-1)	50 (1)	540.1	211.1
6	250 (1)	1 (-1)	50 (1)	459.3	179.5
7	30 (-1)	6 (1)	50 (1)	519.6	203.1
8	250 (1)	6 (1)	50 (1)	535.4	209.2
9	140 (0)	3.5 (0)	26 (0)	436.6	170.6
10	140 (0)	3.5 (0)	26 (0)	438.8	171.4
11	140 (0)	3.5 (0)	26 (0)	462.5	180.7

*Residual activity defined as the ratio of (Final activity/Initial activity) x 100;
Initial enzyme activity: 255.9 U.g^{-1}.

To our knowledge, there are no reports referring to the β-galactosidase activity treated with compressed propane. However, results obtained in this study were similar to those found by Franken et al. (2008) for immobilized lipase activity in propane, in which the only significant variable was the decompression rate and the activity increased in all experimental conditions. Oliveira et al. (2006a) have also verified that the use of compressed propane while increased the activity of lipase Novozym 435, led to negligible changes for Lipozyme IM.

Table 6.2: Effects of process variables on the β-galactosidase activity submitted to compressed propane, n-butane and CO_2.

	Effect	**Standard Error**	**t-value (7)**	**p-value**
Propane				
Average	443.44	10.66	41.60	<0.001
(1)P(bar)	-48.13	25.00	-1.92	0.10
(2)t(h)	-3.58	25.00	-0.14	0.89
(3)R (bar.min^{-1})	142.23	25.00	5.69	<0.001
n-butane				
Average	383.56	10.58	36.24	<0.001
(1)P(bar)	-25.48	24.82	-1.03	0.34
(2)t(h)	-25.48	24.82	-1.03	0.34
(3)R (bar.min^{-1})	30.93	24.82	1.25	0.25
CO_2				
Average	411.45	21.22	19.39	<0.001
(1)P(bar)	-43.48	49.76	-0.87	0.41
(2)t(h)	-4.77	49.76	-0.10	0.93
(3)R (bar.min^{-1})	-21.88	49.76	-0.44	0.67

6.3.2 Effect of compressed n-butane on the β-galactosidase activity of permeabilized cells of *K. marxianus*

Results obtained from the application of the CCD to assess the effects of process variables on the β-galactosidase activity treated with pressurized n-butane are presented in Table 6.3. As verified for propane, the enzyme activity increased in all the experimental runs after treatment with compressed n-butane. For this solvent, the enzyme activity gain was in the range of 118.5 to 161.8%, a slightly poorer result compared to the use of propane. After the statistical analysis of the experimental data presented in Table 6.3, it is possible to observe that none of the variables were significant ($p<0.05$) in the range evaluated (Table 6.2).

As in the case of propane, no reports were found in the literature referring to the β-galactosidase activity treated in compressed n-butane. For lipases, Oliveira et al. (2006a) verified that treatment of Novozym 435 and Lipozyme IM under compressed n-butane resulted in activity gains for both enzymes in the order of 21 and 4%, respectively.

Table 6.3: Matrix of the CCD (real and coded values) with responses in terms of the β-galactosidase activity submitted to compressed n-butane.

Run	P (bar)	t (h)	R (bar.min^{-1})	Final enzyme activity (U.g^{-1})	Residual activity (%)
1	10 (-1)	1 (-1)	2 (-1)	446.8	161.8
2	250 (1)	1 (-1)	2 (-1)	327.1	118.5
3	10 (-1)	6 (1)	2 (-1)	345.4	125.1
4	250 (1)	6 (1)	2 (-1)	337.2	122.1
5	10 (-1)	1 (-1)	50 (1)	400.4	145.0
6	250 (1)	1 (-1)	50 (1)	395.0	143.0
7	30 (-1)	6 (1)	50 (1)	376.7	136.4
8	250 (1)	6 (1)	50 (1)	408.1	147.8
9	130 (0)	3.5 (0)	26 (0)	414.1	150.0
10	130 (0)	3.5 (0)	26 (0)	371.1	134.4
11	130 (0)	3.5 (0)	26 (0)	397.3	143.9

Initial enzyme activity: 276.1 U.g^{-1}

6.3.3 Effect of compressed CO_2 on the β-galactosidase activity of permeabilized cells of *K. marxianus*

Table 6.4 presents the results of the execution of the CCD to evaluate the effects of process variables on the β-galactosidase activity with supercritical CO_2. It can be seen from this table that the enzyme activity after treatment with supercritical CO_2 increased for all the experimental conditions investigated. The highest increase occurred in experiment 1 (~177%) at the lowest exposure time (1.0 h), slowest depressurization rate (10 kg/m^3.min) and lowest reduced pressure (0.5). Statistical analysis of the experimental data presented in Table 4 revealed that none process variables presented a significant effect ($p<0.05$) on the enzyme activity (Table 6.2).

It may be important to mention that most works available in the literature related to the present subject refer to the use of carbon dioxide as solvent and in the majority of cases the use of this solvent led to enzyme activity losses due mainly to the hydrophilic characteristic of carbon dioxide. According to many authors, supercritical carbon dioxide could remove the essential water responsible for keeping the enzymatic activity (Primo et al., 2007; Oliveira et al., 2006a; Oliveira et al., 2006b; Fricks et al., 2006). Conversely, in

this work it was verified an increase in the enzyme activity after treatment with supercritical carbon dioxide for all experimental conditions. This could be due to the fact that the possible water removed from the permeabilized cells did not affect the water content responsible for keeping the enzyme activity. The improved activity could possibly be due to changes in the tertiary structure of β-galactosidase promoted during exposure to high-pressure carbon dioxide.

Table 6.4: Matrix of the CCD (real and coded values) with responses in terms of the β-galactosidase activity submitted to supercritical CO_2.

Run	RD	t (h)	R ($kg.m^{-3}.min^{-1}$)	Final enzyme activity ($U.g^{-1}$)	Residual activity (%)
1	0.5 (-1)	1 (-1)	10 (-1)	525.0	176.8
2	2.0 (1)	1 (-1)	10 (-1)	328.1	110.5
3	0.5 (-1)	6 (1)	10 (-1)	479.1	161.3
4	2.0 (1)	6 (1)	10 (-1)	345.6	116.4
5	0.5 (-1)	1 (-1)	200 (1)	385.5	129.8
6	2.0 (1)	1 (-1)	200 (1)	405.0	136.4
7	0.5 (-1)	6 (1)	200 (1)	331.4	111.6
8	2.0 (1)	6 (1)	200 (1)	468.4	157.7
9	1.25 (0)	3.5 (0)	105 (0)	413.3	139.2
10	1.25 (0)	3.5 (0)	105 (0)	415.7	140.0
11	1.25 (0)	3.5 (0)	105 (0)	428.9	144.4

Initial enzyme activity: 297.0 $U.g^{-1}$

6.3.4 Evaluation of enzyme stability after high-pressure treatment

After the high-pressure treatment step, the permeabilized cells of *K. marxianus* containing the β-galactosidase were maintained at 4°C and their stabilities were evaluated with time. For propane, the monitored activity was carried out using the permeabilized cells from experiment 8 (activity of 535.4 $U.g^{-1}$ after high-pressure treatment), while for n-butane and carbon dioxide samples were taken from experiment 1 (activities of 446.8 $U.g^{-1}$

and 525.0 $U.g^{-1}$ after treatment, respectively). Results were compared with permeabilized cells without high-pressure pre-treatment.

Fig. 6.2 presents the results obtained, where it is possible to verify that the activity gradually decreases through time for the enzyme treated with CO_2 and propane, while the enzyme treated with n-butane kept 96% of its initial activity after high-pressure treatment until three weeks. For CO_2 and propane it was verified a reduction of around 30% in the initial activity after high-pressure treatment after three weeks, which were similar to that obtained for the enzyme without high pressure treatment. However, it is worth noticing that, in general, the activities of high-pressure treated enzyme were always higher than those of fresh, non-treated one. Such result may be of great technological relevance since it is shown that treatment in compressed fluids, mainly with n-butane, could be employed as a preceding, preparation step, to improve enzyme activity for further synthesis of galactooligosaccharides at ambient pressure, once its activity could be kept nearly unchanged for weeks.

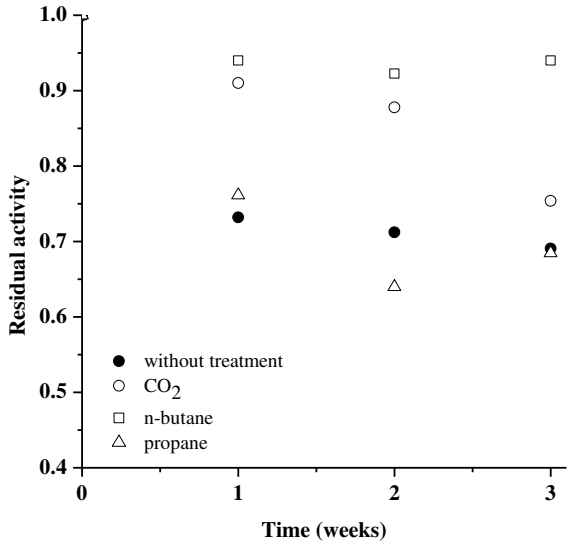

Figure 6.2: Enzyme stability of permeabilized cells after high-pressure treatment (samples maintained under refrigeration).

6.3.5 Synthesis of galactooligosaccharides using the high-pressure treated enzyme

The enhancement in the β-galactosidase activity after high-pressure treatment of permeabilized cells obtained in this work can be considered quite satisfactory. However, this enzyme activity increase does not necessarily lead to an increase in GOS yields, or at least it is not sufficient to change the enzyme conformation to make it more selective for synthesis than for hydrolysis. To evaluate the potential use of the high-pressure treatment as a method to improve the enzyme activity in the synthesis of GOS at ambient pressure, four additional experiments were carried out using permeabilized cells without treatment and permeabilized cells just treated with propane (run 8 of Table 6.1), n-butane (run 1 of Table 6.2) and CO_2 (run 1 of Table 6.3).

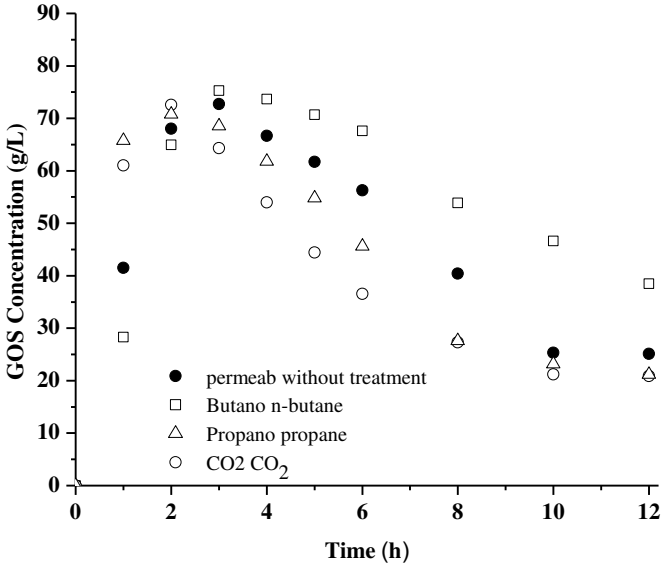

Figure 6.3: Kinetics of GOS synthesis using permeabilized cells without and after treatment with compressed fluids.

Fig. 6.3 presents the results obtained, which reveals that the increase in the enzyme activity obtained after the high-pressure treatment leads to an increase in the maximum yield of GOS, with slightly best results for the enzyme treated with n-butane. This result could be expected, due to the fact that all reactions were carried out with the enzyme

activity units (U) per reaction solution volume, U/mL. From an industrial viewpoint, this result may be of economic interest once a much lower amount of enzyme can be used to provide the same yield obtained using non-treated enzyme.

In addition, it can be seen from the kinetic evaluation of the process, presented in Fig. 6.3 that the competition between synthesis and hydrolysis is less pronounced for the enzyme treated with n-butane compared to the enzymes without treatment and treated with supercritical CO_2 and pressurized propane. This might be an indicative that the GOS yield may be improved by carrying out reactions at high pressure using n-butane as a solvent medium.

6.4. CONCLUSIONS

Results obtained in this work demonstrated that the permeabilized cells of *K. marxianus* containing β-galactosidase enzyme showed higher activities after treatment with compressed fluids, enabling its utilization in the synthesis of GOS in this type of solvent. We concluded that the magnitude of pressure (or reduced density), decompression rate and exposure time affected positively the enzyme activity in a similar degree for all solvents. The enzyme stability after the high-pressure treatment revealed that this procedure could be an attractive pre-treatment step of enzyme before its use in the synthesis of GOS. Regarding the GOS production, it was not verified significant differences in the maximum yield among the treatments, but the enzyme treated with n-butane showed to be more attractive for GOS synthesis. Results obtained here enable the use of β-galactosidase for GOS synthesis under high-pressure conditions and open a promising possibility of improving enzyme activity, hence helping the development of biotransformation processes for a varied of raw materials.

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6.5 REFERENCES

Andrade, J. M., Oestriecher, E. G., Antunes, O. A. C., Oliveira, J. V., Oliveira, D., Soares, C. M., & Dariva, C. (2008). Effect of treatment with compressed CO_2 and propane on D-hydantoinase activity. *The Journal of Supercritical Fluids*, 46, 342-350.

Angus, S., Armstrong, B., & Reuck, K. M. (1976). International thermodynamics tables of the fluid stste: carbon dioxide, Pergamon Press, Oxford, UK, chapter 2, p. 340.

Eisenmenger, M. J., & Reyes-De-Corcuera, J. I. (2009). High pressure enhancement of enzymes: a review. *Enzyme and Microbial Technology*, 45, 331-347.

Franken, L. P. G., Marcon, N. S., Treichel, H., Oliveira, D., Freire, D. M. G., Dariva, C., Destain, J., & Oliveira, J. V. (2008). Effect of treatment with compressed propane on lipases hydrolytic activity. Food Bioprocess and Technology, DOI 10.1007/s11947-008-0087-5.

Fricks, A. T., Souza, D. P. B., Oestricher, E. G., Antunes, O. A. C., Girardi, J. S., Oliveira, D., & Dariva, C. (2006). Evaluation of radish (*Raphanus sativus* L.) peroxidase activity after high-pressure treatment with carbon dioxide. *The Journal of Supercritical Fluids*, 38, 347-353.

Garrea, G., & Riva, S. (2008). Organic synthesis with enzymes in non-aqueous media. Wiley-Verlag, Weinheim, Germany.

Habulin, M., & Knez, M. (2001). Activity and stability of lipases from different sources in supercritical carbon dioxide and near-critical propane. *Journal of Chemical Technology and Biotechnology*, 76, 1260-1266.

Inchaurrondo, V. A., Yantorno O. M., & Voget, C. E. (1994). Yeast growth and β-galactosidase production during aerobic batch cultures in lactose-limited synthetic medium. *Process Biochemistry***,** 29, 47-54.

Kao, F. J., Ekhorutomwen, S. A., & Sawan, S. P. (1997). Residual stability of lipase from *Candida rugosa* in hexane, supercritical CO_2, and supercritical SF6. *Biotechnology Techniques*, 11, 849-852.

Kasche, V., Schlothauer, & Brunner, G. (1988). Enzyme denaturation in supercritical CO_2: stabilizing effect of S-S bonds during depressurization step. Biotechnology Letters, 10, 569-574.

Knez, Z., & Habulin, M. (2002) Compressed gases as alternative enzymatic-reaction solvents: a short review. *The Journal of Supercritical Fluids*, 23, 29-42.

Longhi, L. G. S., Luvizetto, D. J., Ferreira, L. S., Rech, R., Ayub, M. A. Z., & Secchi, A. R. (2004). A growth kinetic model of *Kluyveromyces marxianus* cultures on cheese whey as substrate. *Journal of Industrial Microbiology and Biotechnology*, 31, 35-40.

Mahoney, R. R. (1998) Galactosyl-oligosaccharide formation during lactose hydrolysis: a review. *Food Chemistry*, 63, 147-154.

Manera, A. P., Ores, J. C., Ribeiro, V. A., Burkert, C. A. V., & Kalil, S. J. (2008). Optimization of the culture medium for the production of β-galactosidase from *Kluyveromyces marxianus* CCT 7082. *Food Technology and Biotechnology*, 46, 66-72.

Manera, A. P.; Rodrigues, M. I.; Kalil, S. J.; Maugeri Filho, F. Permeabilization of *Kluyveromyces marxianus* cells aiming β-galactosidase activity and characterization. *J Agric. Food Chem.* (submitted).

Ndiaye, P. M., Lanza, M., Tavares, F. W., Dariva, C., Oliveira, D., & Oliveira, J. V. (2006). Phase behavior of olive oil and soybean oils in compressed propane and n-butane. *Brazilian Journal of Chemical Engineering*, 23, 405-415.

Neri, D. F. M., Balcão, V. M., Costa, R. S., Rocha, I. C. A. P., Ferreira, E. M. F. C., Torres, D. P. M., Rodrigues, L. R. M., Carvalho, L. B., & Teixeira, J. A. (2009). Galacto-oligosaccharides production during lactose hydrolysis by free *Aspergillus oryzae* β-galactosidase and immobilized on magnetic polysiloxane-polyvinyl alcohol. *Food Chemistry*, 115, 92-99.

Oliveira, J. V., & Oliveira, D. (2000). Kinetic of enzymatic alcoholysis of palm kernel oil in SC-CO_2. *Industrial and Engineering Chemistry Research*, 39, 4450-4454.

Oliveira, D., & Oliveira, J. V. (2001). Enzymatic alcoholysis of palm kernel oil in n-hexane and SC-CO_2. *The Journal of Supercritical Fluids*, 19, 141-148.

Oliveira, D., Feihrmann, A. C., Rubira, A. F., Kunita, M. H., Dariva, C., & Oliveira, J. V. (2006a). Assessment of two immobilized lipases activity treated in compressed fluids. *The Journal of Supercritical Fluids*, 38, 373-382.

Oliveira, D., Feihrmann, A. C., Dariva, C., Cunha, A. G., Bevilaqua, J. V., Destain, J., Oliveira, J. V., & Freire, D. M. G. (2006b). Influence of compressed fluids treatment on the activity of *Yarrowia lipolytica* lipase. *Journal of Molecular Catalysis B: Enzymatic*, 39, 117-121.

Primo, M. S., Ceni, G. C., Marcon, N. S., Antunes, O. A. C., Oliveira, D., Oliveira, J. V., & Dariva, C. (2007). Effects of compressed carbon dioxide treatment on the specificity on oxidase enzymatic complexes from mate tea leaves. *The Journal of Supercritical Fluids*, 43, 283-290.

CAPÍTULO 7

ENZYMATIC SYNTHESIS OF GALACTOOLIGOSACCHARIDES USING PRESSURIZED FLUIDS AS REACTION MEDIA

Submetido para:

Enzyme and Microbial Technology

ENZYMATIC SYNTHESIS OF GALACTOOLIGOSACCHARIDES UNDER DIFFERENT PRESSURE CONDITIONS

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Abstract

This work is focused on the evaluation of enzymatic synthesis of GOS catalyzed by β-galactosidase from permeabilized cell of *Kluyveromyces marxianus* in different compressed fluids, namely carbon dioxide, propane and n-butane. To our knowledge, no related study regarding enzymatic synthesis of GOS in pressurized fluids is available in the open literature. To assess the effect of process variables on the GOS synthesis, a factorial experimental design with two levels and three variables was employed for each one of the compressed fluids. The experiments were performed in a batch mode reactor, where the effects of enzyme concentration, lactose concentration and system pressure were evaluated. Results showed significant differences in the maximum GOS concentration among the treatments, with the lactose concentration and enzyme activity were found to be statistically significant. Comparing the GOS maximum production for all the three reaction systems, the values were 83 $g.L^{-1}$, 63.0 and 75.0 $g.L^{-1}$ and yield were 16,7 wt%, 15 and 13 wt% , respectively for the CO_2 ,propane and n-butane .

Keywords: carbon dioxide, propane, n-butane, β-galactosidase, cell permeabilized

7.1 INTRODUCTION

Galactooligosaccharides (GOS) are non-digestible oligosaccharides, comprising 2-5 molecules of galactose and one molecule glucose, and are recognized as important prebiotics for their stimulation of the proliferation of lactic acid bacteria and bifidobacteria in the human intestine. They therefore beneficially affect the human host by selectively stimulating the growth and/or activity of a limited number of gastrointestinal microorganisms that confer health benefits. GOS can be added to foods such as soft drinks, ice cream, cookies, infant formulas, and animal feeds. Naturally, GOS are found in garlic, onion, soybean, and chicory roots. However, natural GOS are not sufficient and convenient to use as food additives to enhance human health. As a result, the development of chemical or enzymatic production of GOS is necessary (Sako et al., 1999).

GOS can be produced by a series of enzymatic reactions catalyzed by β-galactosidase. The β-galactosidase reaction mechanism includes both the hydrolysis of lactose and a transglycosylation reaction (Mahoney, 1998). Depending on lactose concentration, the reaction is shifted towards either hydrolysis or transglycosylation. When water concentration in the system, expressed as water activity (aw), is high, the hydrolysis of lactose occurs predominantly. The transglycosylation reaction increases with a decrease in water activity (Goulas et al., 2007; Neri et al., 2009). Under most conditions, hydrolysis predominates due to high water concentration (necessary for lactose solubilization) and GOS production is low. The yield of GOS can be increased by using higher substrate concentrations and/or by decreasing the water content (Mahoney, 1998). Another alternative to improve the process yield is to propose a modification of the enzyme amino groups, as can be the case of using pressurized fluids as reaction medium.

As for most reactions catalyzed by a biological entity, there are many modes in which GOS producing reactions can be performed. The β-galactosidase can be soluble or immobilized. It can be isolated after intra- or extracellular expression, or provided within whole cells. The mode in which the process is undertaken may change the behavior of the enzyme, the concentrations of product and substrate around the enzyme and will certainly influence the performance and economics of the process. A recent publication reviewed the

immobilization of β-galactosidase for GOS production (Panesar et al., 2006). Gosling et al. (2010) presented a review using two alternative approaches: (1) whole cells as biocatalysts and (2) membrane reactors, emphasizing the differences in the outcomes of the process due to these alternative reactor arrangements. The maximum concentration of GOS typically obtained in a reaction varies widely, depending on reaction conditions, as does the GOS yield (as a percentage of the initial lactose).

Using resting or living whole cells removes the need for the isolation of the β-galactosidase enzyme. Protein isolation can be demanding and costly and requires additional processing steps after the culture of the β-galactosidase producing organism. Using the organism directly can circumvent these problems (Fukuda et al., 2008). GOS yields of 36% (w/v) to 43% (w/w) have been achieved in a whole cell system, demonstrating comparable yields to those achieved with isolated enzymes (Goulas et al., 2007). An advantage of whole cells over isolated enzymes during the development of an optimal biocatalyst is the ability to evolve. Changing an isolated protein typically involves manipulation at the DNA level. Regulation of genetically-modified food ingredients makes this approach inappropriate and removes the most powerful tool for optimizing an isolated enzyme. However, standard breeding and selection techniques offer options for improving food-grade whole cell biocatalysts. It is generally believed that the added complexity of using whole cells for biocatalysis can be a disadvantage compared to isolated enzymes (Woodley, 2006). However, whole cells also provide the opportunity to include additional metabolic functions such as the consumption of the monosaccharide glucose and galactose from a GOS product.

Numerous studies have shown that many enzymatic reactions can be conducted using pressurized fluids as carbon dioxide, propane and n-butane as reaction media and, in some cases, rates and selectivities achieved are greater than those obtained in standard liquid or gas phase reactions (Oliveira & Oliveira, 2000; 2001). To conduct galactosidase-catalyzed reactions at high pressures, the enzyme behavior in compressed fluids is of primary importance as the loss of enzyme activity may lead to undesirable poor reaction rates and low yields of target products. Enzyme stability and activity may depend on the enzyme

species, characteristics of compressed fluid, water content of the enzyme/support/reaction mixture and manipulated process variables.

In a previous work we have evaluated the influence of pressure, exposure time and depressurization rate on the β-galactosidase activity of permeabilized cells of *K. marxianus* CCT 7082 submitted to treatment with compressed carbon dioxide, propane and n-butane. In general, results showed that the enzyme activity changes significantly depending on the experimental conditions investigated, allowing the selection of proper operating conditions for advantageous application of this biocatalyst in hydrolysis reactions. Further, the stability of the enzyme after high-pressure pre-treatment was also experimentally monitored, and the results demonstrated that the activities of this biocatalyst were always higher than the non-treated one. Finally, evaluation of synthesis of galactooligosaccharides (GOS) using β-galactosidase without treatment and after treatment in compressed fluids was carried out (Manera et al., 2010a).

In this context, the main objective of this work was to study the enzymatic synthesis of galactooligosaccharides in batch mode using pressurized carbon dioxide, propane and n-butane as reaction media using permeabilized cells of *K. marxianus* CCT 7082. For this purpose, the effects of lactose concentration, enzyme activity and system pressure on the GOS production were evaluated.

7.2 MATERIAL AND METHODS

7.2.1 Chemicals

Carbon dioxide (99.9%), propane (99.5 mol% purity) and n-butane (99.5 mol% purity) were supplied by White Martins S.A. The chemicals products used were all of analytical grade purchased from Synth, Brazil, and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) purchased from Sigma Chem., USA.

7.2.2 Enzyme production

Kluyveromyces marxianus CCT 7082 was employed for β-galactosidase production. The microorganism was cultivated in a medium containing 28.8 g/L lactose, 17.0 g/L yeast extract, 5.0 g/L KH_2PO_4, 8.8 g/L $(NH_4)_2SO_4$ and 0.4 g/L $MgSO_4.7H_2O$, and incubated at

150 rpm and 30°C for 96 h (Manera et al., 2008). Cell concentration was estimated by measuring the absorbance at 620 nm and converted to dry weight following the standard curve. The cells of broth were collected by centrifugation at 6000 × *g* for 5 min, washed twice with distilled water and re-suspended in water for absorbance measurement. For the standard curve, cells were dried at 90°C to constant weight (Longhi et al., 2004).

7.2.3 Cell permeabilization

Cell permeabilization was carried out using isopropanol in a ratio biomass/isopropanol (w/w) of 0.03 and held at 25°C for 5 min. Before adding the isopropanol to the biomass it was prepared in 0.1 M potassium phosphate buffer pH 7.0 at concentration 30% Cells were separated from solvent at 6000 × *g* for 5 min and washed twice with cold 0.1 M potassium phosphate buffer, pH 7.0 and lyophilized (Manera et al., 2010b).

7.2.4 Enzyme activity

β-galactosidase activity was assayed using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) following the method described by Inchaurrondo et al. (1994). A 50 µL sample of permeabilized cell suspension was mixed with 2 mL of 1.25 mM ONPG in buffer (50 mM of KH_2PO_4 and 0.1 mM $MnCl_2.4H_2O$, pH 6.6) and incubated for 5 min at 37°C. The reaction was stopped by adding 0.5 mL of 1 M sodium carbonate. Liberated *o*-nitrophenol (ONP) was measured spectrophotometrically at 420 nm. The molar extinction coefficient of *o*-nitrophenol under these conditions was 4.64 $cm^2/\mu mol$. One enzyme unit is defined as the quantity of enzyme that catalyzes the release of 1 µmol of ONP from ONPG per minute under assay conditions.

7.2.5 Apparatus and experimental procedure for the synthesis of GOS

The experimental setup used for GOS production from lactose with permeabilized cells of *K. marxianus* CCT 7082 in supercritical carbon dioxide, pressurized propane and n-butane is schematically presented in Fig. 7.1, and consists basically of a jacketed 50mL

reactor (Parr) with mechanical agitation, a gas cylinder and a syringe pump (Isco, model 260D).

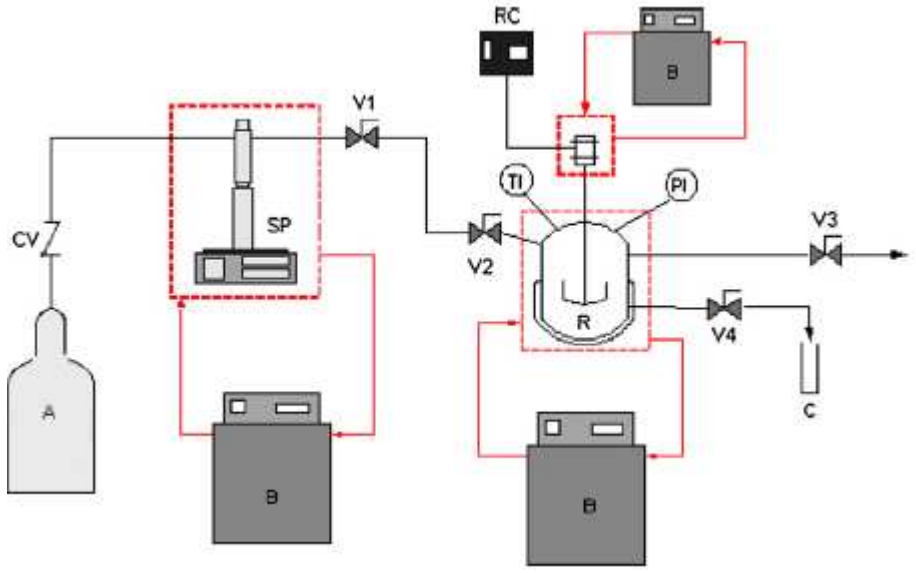

Figure 7.1: Schematic diagram of the experimental apparatus: A, solvent reservoir; B, thermostatic baths; SP, syringe pump; R, reactor vessel; RC, reactor control system; TI, temperature indicator; PI, pressure indicator; C, collector tube; CV, check valve; V1, V2, V3 and V4, ball valves.

The amounts of lactose, permeabilized cells containing the enzyme and the system pressure were defined according to the experimental design. Precise amounts of substrates and permeabilized cells were weighed on a precision scale balance (Ohaus Analytical Standard with 0.0001g accuracy) and loaded into the reaction vessel, which was immediately closed and the temperature control (accuracy of 0.5^{o}C) was turned on. The charge of a known volume amount of gas was performed with the help of the syringe pump (resulting accuracy of ± 0.01g in gas loadings), kept at constant temperature (5^{o}C) and at the system pressure, until the pre-established pressure was achieved. With known values of pressure and temperature in the syringe pump reservoir, solvent density was estimated using the HBT (P-V-T) correlation for compressed liquids (Reid et al., 1987), making possible to estimate the mass of solvent charged into the reaction vessel. Typically, around 3 minutes were sufficient to feed and pressurize the reactor to the desired value, and once

the system pressure had been reached the agitation was turned on and the reaction time was set to zero. The pressurized solvent to aqueous substrate ratio was 35:65 (wt%) for n-butane and propane and 45:55 (wt%) for supercritical carbon dioxide. Throughout this work the agitation was kept constant at 100 rpm. Based on the uncertainty in pressurized fluid loadings, substrates weighing and predictions in solvent feed, pressurized fluid to substrates ratio varied approximately 5% of the value.

To evaluate the effects of enzyme activity, lactose concentration and system pressure on the GOS synthesis, a central composite design (CCD) (Rodrigues and Iemma, 2009) was accomplished for each compressed fluid (Tables 7.1 to 7.3). All the experiments were carried out at fixed temperature of 45°C for 4 h. Samples were taken at regular time intervals and immediately immersed in a boiling water bath for 10 min to inactivate the enzyme before sugar analysis.

7.2.6 Chromatographic determination of carbohydrates

The identification and quantification of sugars (lactose, glucose, galactose and GOS) was carried out by ion exchange chromatography with pulsed amperometric detection (HPLC-PAD). A DIONEX (USA) chromatograph, supplied with a Carbopac PA1 (4x250 mm) column, a PA1 (4x50 mm) guard column, with a GP50 gradient pump, ED40 electrochemical detector and PEAKNET software were used for the analysis. Sugars were eluted with 20 mM sodium hydroxide, at a flow rate of 1.0 $mL.min^{-1}$. Before injection, the samples were diluted with water and filtered through 0.22 μm filters (Manera et al., 2010c)

7.3 RESULTS AND DISCUSSION

7.3.1 Synthesis of GOS using compressed n-butane as reaction media

Table 7.1 presents the results of GOS contents and yields, according to the CCD, using compressed n-butane as reaction medium. The GOS production ranged from 34.0 $g.L^{-1}$ to 75.0 $g.L^{-1}$ in runs 2 and 3, respectively. The highest yielding obtained was 15 wt% at runs 3 and 5. The data of Table 7.1 were used to compute the main effects of the independent

variables on the GOS production, considering a confidence level of 95%. The statistical analysis are presented in Table 7.2.

Table 7.1: Matrix of the CCD (real and coded values) with responses in terms of the GOS concentration and yield, using compressed n-butane as reaction media.

Run	Enzyme ($U.mL^{-1}$)	Lactose ($g.L^{-1}$)	Pressure (bar)	GOS ($g.L^{-1}$)	GOS yield (wt%)	Time (h)*
1	5 (-1)	300(-1)	10(-1)	41.3	13.8	2
2	25 (+1)	300(-1)	10(-1)	34.0	11.3	1
3	5 (-1)	500(+1)	10(-1)	75.0	15.0	4
4	25 (+1)	500(+1)	10(-1)	65.6	13.1	2
5	5 (-1)	300(-1)	250(+1)	45.0	15.0	2
6	25 (+1)	300(-1)	250(+1)	39.5	13.2	1
7	5 (-1)	500(+1)	250(+1)	64.6	12.9	3
8	25 (+1)	500(+1)	250(+1)	58.8	11.8	1
9	15(0)	400(0)	130(0)	50.5	12.6	1
10	15(0)	400(0)	130(0)	49.2	12.3	1
11	15(0)	400(0)	130(0)	50.5	12.6	1

* Time of the synthesis that the maximum GOS concentration was obtained

The statistically significant variables ($p<0.05$) were the enzyme activity and lactose concentration, where the effects were negative and positive, respectively. In this sense, it can be seen from the effect analysis that as the enzyme activity decreases and the lactose concentration increases and higher concentrations of GOS can be obtained. However, the modification of the range of study for enzyme activity and lactose concentration is technically unfeasible, since higher lactose concentration leads to non proper dissolution of the substrate, while decreasing the enzyme activity probably will affect the catalytic power of the enzyme.

The effects of enzyme activity and lactose concentration on GOS production can be observed by the experimental data at runs 1 and 2; when the enzyme activity changes from 5 to 25 $U.mL^{-1}$ and keeping constant others variables, a decreasing GOS production, from

41.3 to 34 $g.L^{-1}$, is noticed. The effect of lactose concentration is visualized in runs 1 and 3, where its increase leads to higher GOS production. The pressure showed little effect on production, as can be seen from runs 3 and 7, where the GOS concentration ranged from 75 to 64.6 $g.L^{-1}$, increasing the pressure from 10 to 250 bar, respectively. Based on these results, the best experimental conditions to produce GOS using compressed n-butane as reaction medium is lactose concentration of 500 $g.L^{-1}$, enzyme activity of 5 $U.mL^{-1}$ and pressure of 10 bar, which is the conditions of run 3. In terms of GOS yields, run 5 presented the same value (15 wt%), but the costs associated to pressurization is higher than that related to lactose concentration, since the experimental conditions of this run is lactose concentration of 300 $g.L^{-1}$, enzyme activity of 5 $U.mL^{-1}$ and pressure of 250 bar.

Table 7.2: Main effects of process variables in the GOS production submitted to compressed n-butane, propane, and CO_2.

	Effect	**Standard Error**	**t-value (7)**	**p-value**
n-butane				
Average	52.19	1.200	43.49	<0.001
Enzyme (U/mL)	-7.00	2.814	-2.49	0.042
Lactose (g/L)	26.05	2.814	9.26	<0.001
Pressure (bar)	-2.00	2.814	-0.71	0.500
Propane				
Average	52.50	0.893	58.77	<0.001
Enzyme (U/mL)	-5.23	2.095	-2.49	0.041
Lactose (g/L)	19.43	2.095	9.27	<0.001
Pressure (bar)	-3.42	2.095	-1.63	0.146
CO_2				
Average	47.51	2.858	16.62	<0.001
Enzyme (U/mL)	36.02	6.703	5.37	0.001
Lactose (g/L)	21.08	6.703	3.14	0.016
Pressure (bar)	-4.62	6.703	-0.69	0.512

Figure 7.2 presents the kinetics of lactose, galactose, glucose and GOS for some selected experimental conditions of Table 7.1. It can be seen that the glucose and galactose increases until reaching the steady-state, while the lactose concentration decreases, since

the first step in the reaction is the hydrolysis. The GOS production presented the highest value in the first hour of reaction for all the experiments, with exception of run 3, where the concentration increased until 4 hours. Due to the presence of water in the reaction media a hydrolytic activity is expected. This phenomenon is verified in the GOS concentration after two hours of reaction, since it decreases for this time on.

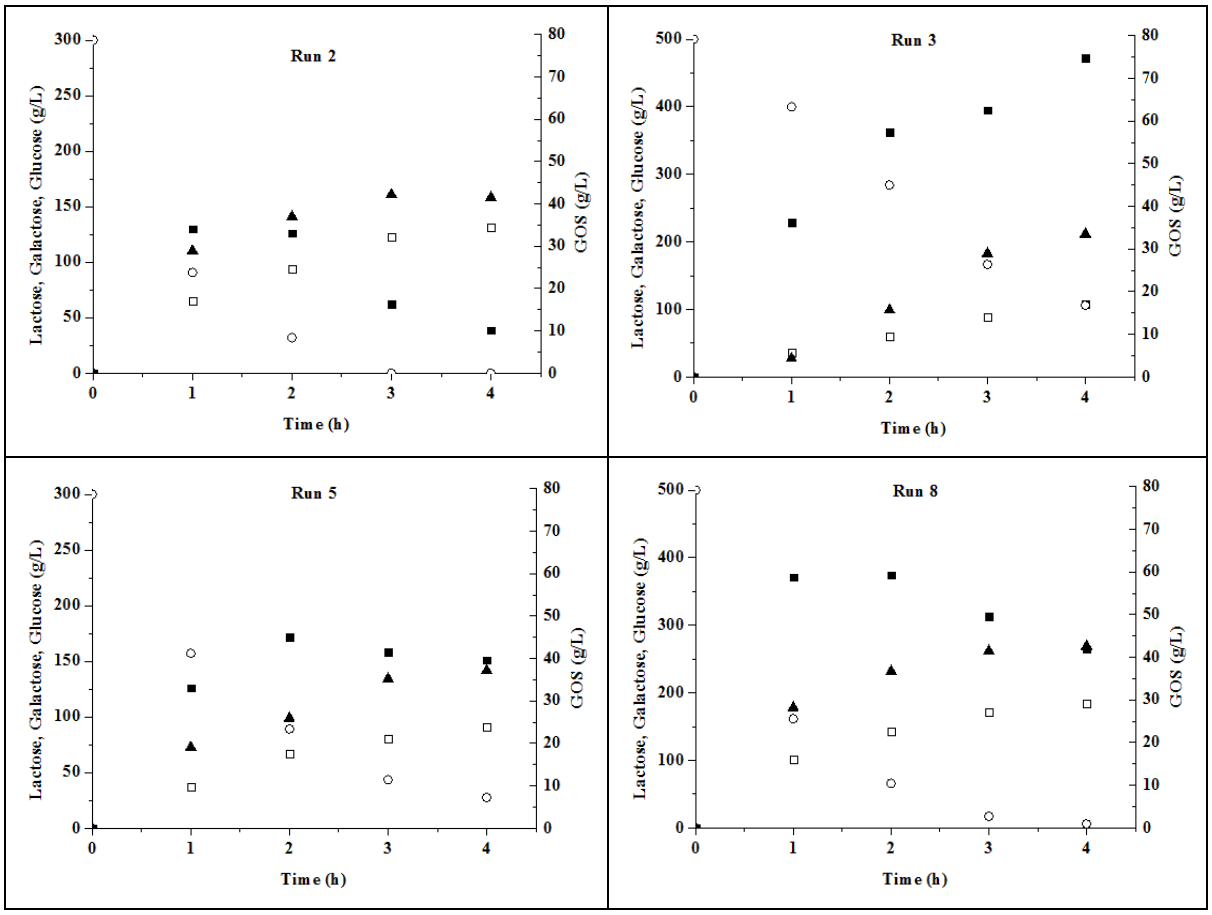

Figure 7.2: Variations in lactose, galactose, glucose and GOS concentrations during reaction time, for some selected experiments carried out with compressed n-butane as reaction media. (○) Lactose; (□) Galactose; (▲) Glucose; (■) GOS.

7.3.2 Synthesis of GOS using compressed propane as reaction media

Table 7.3 presents the results of GOS concentrations and yields according to the CCD, using compressed propane as reaction medium. The GOS production ranged from 35.3g.L^{-1}

to 65.0 $g.L^{-1}$ in runs 6 and 7, respectively. In average, the GOS yields were higher than those obtained using compressed n-butane, where the highest yield was 16.4 wt% in run 1. The data of Table 7.3 were used to compute the main effects of the independent variables in the GOS production, considering a confidence level of 95% and the related statistical analysis are presented in Table 7.2.

Table 7.3: Matrix of the CCD (real and coded values) with responses in terms of the GOS concentration and yield, using compressed propane as reaction media.

Run	Enzyme (U/mL)	Lactose (g/L)	Pressure (bar)	GOS (g/L)	GOS yield (wt %)	Time (h)*
1	5 (-1)	300 (-1)	30 (-1)	49.3	16.4	4
2	25 (+1)	300 (-1)	30 (-1)	42.4	14.1	1
3	5 (-1)	500 (+1)	30 (-1)	62.0	12.4	4
4	25 (+1)	500 (+1)	30 (-1)	63.1	12.6	2
5	5 (-1)	300 (-1)	250 (+1)	44.1	14.7	2
6	25 (+1)	300 (-1)	250 (+1)	35.3	11.8	1
7	5 (-1)	500 (+1)	250 (+1)	65.0	13.0	4
8	25 (+1)	500 (+1)	250 (+1)	58.7	11.7	2
9	15(0)	400 (0)	140 (0)	56.0	14.0	1
10	15(0)	400 (0)	140 (0)	51.1	12.8	1
11	15(0)	400 (0)	140 (0)	50.5	12.6	1

* Time of the synthesis that the maximum GOS concentration was obtained

As previously for n-butane, the statistically significant variables ($p<0.05$) were the enzyme activity and lactose concentration, where the effects were negative and positive, respectively. As it can be seen from the effect analysis, a decrease in enzyme activity and an increase in lactose concentration lead to higher concentrations of GOS. The pressure was not a significant effect for the production, most probably because propane (as is the case of n-butane) behaves like a compressed liquid within the pressure and temperature ranges investigated in this work, which means a low variation of fluid density and accordingly with no appreciable changes in the solvatation power of the fluid.

Figure 7.3 presents the lactose, galactose, glucose and GOS concentrations during reaction time, for some selected experimental conditions of Table 7.3. Although the GOS production was lower than the one verified for n-butane, the kinetic profiles were similar, where the glucose and galactose concentrations increased until reaching an asymptotic value, while the lactose concentration decreases. The lactose concentration decreased to values lower than 50 $g.L^{-1}$ in all the experimental conditions.

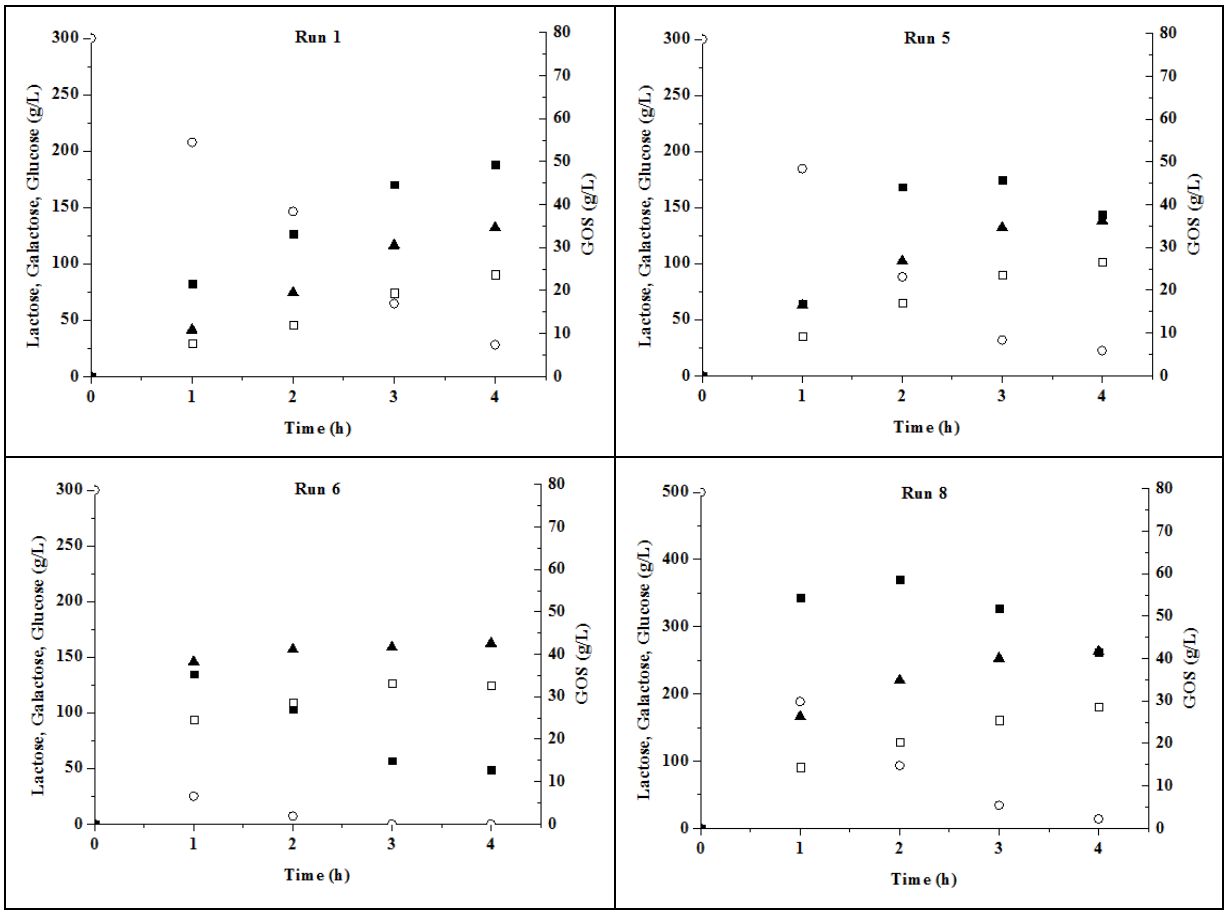

Figure 7.3: Variations in lactose, galactose, glucose and GOS concentrations during reaction time, for some selected experiments carried out with compressed propane as reaction media. (○) Lactose; (□) Galactose; (▲) Glucose; (■) GOS.

7.3.3 Synthesis of GOS using supercritical carbon dioxide as reaction media

The GOS production and yields are shown in Table 7.4, according to a CCD, using supercritical CO_2 as reaction medium. The GOS production was in the range of 20.2 $g.L^{-1}$ to 83.4 $g.L^{-1}$ for runs 5 and 4, respectively. The highest yield was 16.7 wt% in run 4. Again, data of Table 7.4 were used to compute the main effects of the independent variables in the GOS production, considering a confidence level of 95%. The statistical analyses are shown in Table 7.2.

Table 7.4: Matrix of the CCD (real and coded values) with responses in terms of the GOS concentration and yield, using supercritical CO_2 as reaction media.

Run	Enzyme ($U.mL^{-1}$)	Lactose ($g.L^{-1}$)	Pressão (bar)	GOS ($g.L^{-1}$)	GOS yield (wt%)	Time (h)*
1	5 (-1)	300 (-1)	100 (-1)	25.5	8.5	2
2	25 (+1)	300 (-1)	100 (-1)	46.3	15.4	3
3	5 (-1)	500 (+1)	100 (-1)	33.4	6.7	4
4	25 (+1)	500 (+1)	100 (-1)	83.4	16.7	4
5	5 (-1)	300 (-1)	140 (+1)	20.2	6.7	2
6	25 (+1)	300 (-1)	140 (+1)	45.2	15.1	3
7	5 (-1)	500 (+1)	140 (+1)	28.2	5.6	3
8	25 (+1)	500 (+1)	140 (+1)	76.5	15.3	3
9	15 (0)	400 (0)	120 (0)	58.0	14.5	4
10	15 (0)	400 (0)	120 (0)	58.2	14.6	4
11	15 (0)	400 (0)	120 (0)	57.9	14.5	4

* Time of the synthesis that the maximum GOS concentration was obtained

The statistically significant variables ($p<0.05$) were the enzyme activity and lactose concentration, with both effects being positive. As it can be seen from the effect analysis, the increase of the enzyme activity and lactose concentration led to higher concentrations of GOS. The pressure was not a significant variable for the GOS production, although wide variations in the supercritical CO_2 density is verified.

The effect of enzyme activity in the GOS production can be observed by the experimental data of runs 1, 3, 5 and 7 (experiments carried out with enzyme activity of 5

$U.mL^{-1}$) and at runs 2, 4, 6 and 8 (experiments carried out with enzyme activity of 25 $U.mL^{-1}$). Varying the enzyme activity from 5 to 25 $U.mL^{-1}$ there was a considerable increase in GOS production. The effect of lactose concentration is noticeable at runs 2 and 4, where its concentration increases from 300 g/L to 500 g/L, while other variables are kept constant, leading to higher GOS production.

Comparing the maximum GOS production in the three reaction systems, the supercritical CO_2 presented the best result, where the maximum production was 83 $g.L^{-1}$, while for propane and n-butane it was experimentally observed 63.0 and 75.0 $g.L^{-1}$, respectively. The low GOS production verified in the case of propane and n-butane can be explained by the low solubility of these solvents in the aqueous medium used to dissolve the lactose. In addition, propane and n-butane are compressed liquids in the whole range of pressure and temperature studied, which may implicate in the existence of a two liquid-phase system inside the reactor (aqueous and organic phases). As the enzyme is soluble in the aqueous phase, low contact between compressed fluid and enzyme is expected and the positive effect of these fluids on the enzyme activity as previously reported by Manera et al. (2010) is not applicable.

On the other hand, with the pressure increase improves the hydrophilic characteristic of the CO_2 (Nakaya et al., 2001). As a consequence, it is more soluble in aqueous solutions, as the case of this work. In this sense, there is a more efficient contact between the enzyme and CO_2, leading to the highest GOS production compared to the other compressed solvents employed in this study.

Figure 7.4 presents the lactose, galactose, glucose and GOS concentrations changes during time, for some selected experimental conditions of Table 7.3. The profile changes in lactose, glucose and galactose concentrations were similar to those for n-butane and propane, where the concentrations increased until reaching an asymptotic value, while the lactose concentration decreases. However, the GOS production presented distinct behavior, since the decreasing in its concentration after 1 or 2 hours of reaction due to hydrolysis was lower than for n-butane and propane. In addition, the decreasing in the lactose concentration was lower than that verified for the two other fluids.

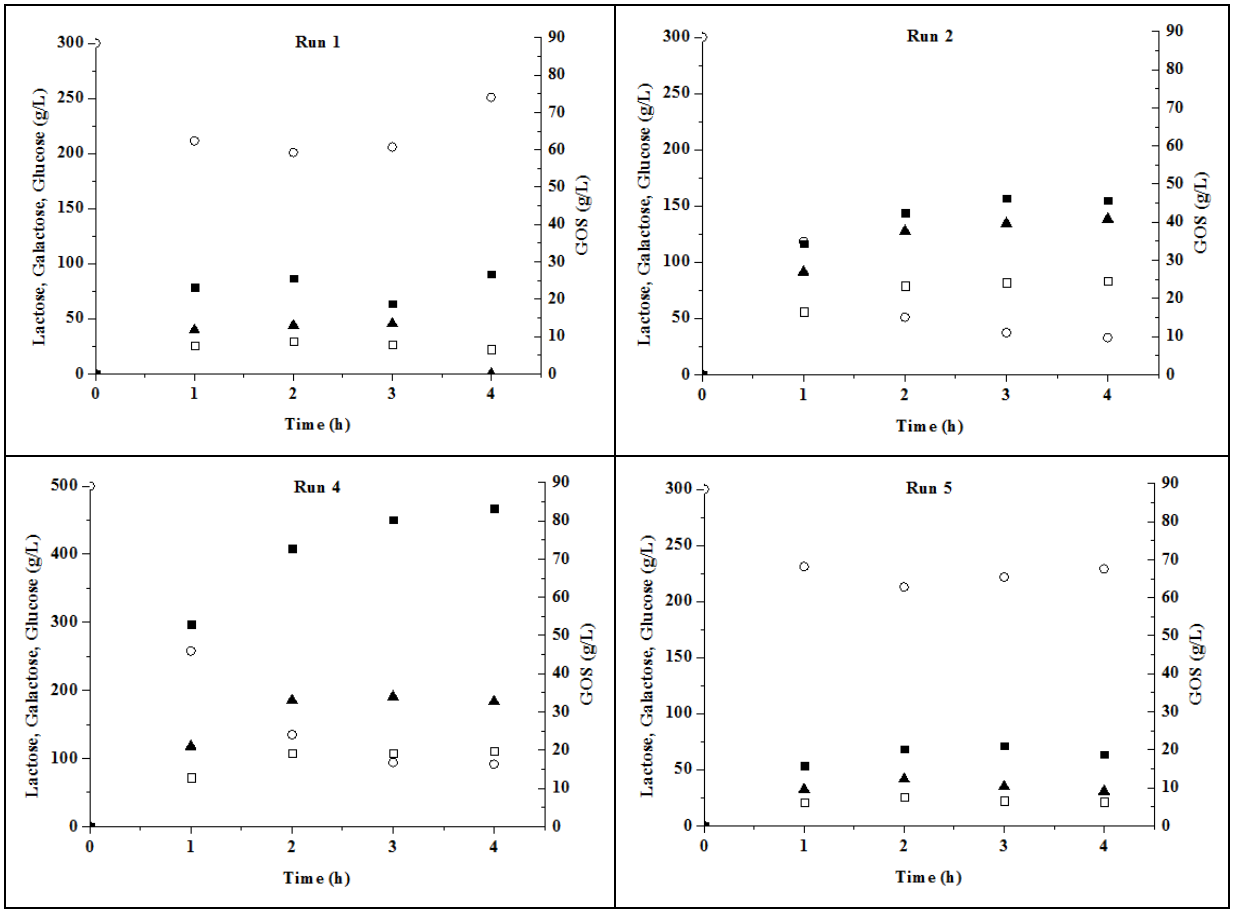

Figure 7.4: Variations in lactose, galactose, glucose and GOS concentrations during reaction time, for some selected experiments carried out using supercritical CO_2 as reaction media. (○) Lactose; (□) Galactose; (▲) Glucose; (■) GOS.

The Table 7.5 shows the results for GOS maximum production for each fluid studied, compared to the results from Manera et al. (2010a), where the permeabilized cells were treated at high pressure and employed in the GOS synthesis, in a batch reactor at atmospheric pressure.

As it can be seen in Table 7.5, the GOS concentration varied between 65 to 83 g/L, in the synthesis with propane and CO_2 at high pressure, as the reactional medium, respectively. For the other assays show in this table, the GOS concentrations are around 70 g/L.

Table 7.5: Data of GOS concentration (g/L), yield (%), productivity, P (g/L.h), and specific productivity, SP (g_{GOS}/g_{cells}.h), for GOS synthesis with permeabilized cells of *K. marxianus* according to the enzymatic activity, EA (U/mL), and cell concentration (g/L).

Enzyme	Synthesis	EA	Cell conc.	GOS	Yield	P	SP
Permeabilized cells *	Atmospheric pressure	10	36.2[a]	73	14.5	24.3	0.7
Permeabilized cells	10 bar with n-butane	5	18.1[a]	75	15	18.8	1.0
Permeabilized cells	250 bar with propane	5	18.1[a]	65	13	16.2	0.9
Permeabilized cells	100 bar with CO_2	25	90.6[a]	83	16,6	21	0.2
Permeabilized cells treated with high pressure n-butane*	Atmospheric pressure	10	22.4[b]	75	15	25	1.1
Permeabilized cells treated with high pressure propane*	Atmospheric pressure	10	18.6[c]	70	14	35	1.9
Permeabilized cells treated with high pressure CO_2*	Atmospheric pressure	10	19,0[d]	72	14,5	36	1,9

* data from Manera et al. (2010)
[a] initial enzymatic activity of permeabilized cells: 276,0 U/g
[b] initial enzymatic activity of permeabilized cells: 446,8 U/g
[c] initial enzymatic activity of permeabilized cells: 535,4 U/g
[d] initial enzymatic activity of permeabilized cells: 525,0 U/g

The yield results were similar for all assays varying between 13 to 16,6%. The productivity varied between 16,2 to 36 g/L.h, where the lower values were obtained at high pressure synthesis applying propane (16,2 g/L.h), and n-butane (18,8 g/L.h). However, the highest productivities were obtained with cells treated with propane and CO_2 at high pressures, and afterward used in the synthesis at atmospheric pressure. The results for these assays were 35 e 36 g/L.h, respectively. These same synthesis resulted also in the highest specific productivity (1,9 g_{GOS}/g_{cells}.h), due to the fact that after the high pressure treatment, the permeabilized cells presented an increased residual enzymatic activity, about 176% and 210%, for CO_2 and propane, respectively.

Evaluating the enzyme stability in the permeabilized cells when stored at refrigeration temperature, the cells treated at high pressure with n-butane have presented practically the same enzymatic activity after three weeks storage. Nonetheless, the cells treated with propane, CO_2 and the also permeabilized cells without treatment have lost about 30% of their activity in the same conditions and storage time. So, it can be considered the GOS synthesis at atmospheric pressure with permeabilized cells, treated at high

pressure with n-butane, as the most suitable, since these cells have presented the highest enzyme stability, enabling them to longer storage time.

7.4 CONCLUSIONS

In this work, the enzymatic synthesis of galactooligosaccharides in batch mode using pressurized carbon dioxide, propane and n-butane as reaction media using permeabilized cells of *K. marxianus* CCT 7082 was studied. The effects of lactose concentration, enzyme activity and system pressure on the GOS production were evaluated. Results pointed out significant differences in the maximum GOS production among treatments, where the lactose and enzyme were the variables statistically significant. Comparing the maximum GOS production in the three reaction systems, the supercritical CO_2 presented the best results, where the maximum production was 83 $g.L^{-1}$, while for propane and n-butane values of 65 and 75 $g.L^{-1}$ were obtained, respectively.

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7.5 REFERENCES

Fukuda, H., Hama, S., Tamalampudi, S., & Noda, H. Whole-cell biocatalysts for biodiesel fuel production. **Trends in Biotechnology**, v. 26, n. 12, 668–673, 2008.

Gosling, A., Stevens, G. W., Barber, A. R., Kentish, S. E. *et al*., Recent advances refining galactooligosaccharide production from lactose. **Food Chemistry**, v. 121, p. 307-318, 2010.

Goulas, A., Tzortzis, G., Gibson, G. R. Development of a process for the production and purification of alpha- and beta-galactooligosaccharides from *Bifidobacterium bifidum* NCIMB 41171. **International Dairy Journal**, v. 17, n. 6, p. 648–656, 2007.

Inchaurrondo, V. A.; Yantorno O. M.; Voget, C. E. Yeast growth and β-galactosidase production during aerobic batch cultures in lactose-limited synthetic medium. **Process Biochemistry,** v. 29, p. 47-54, 1994.

Longhi, L. G. S.; Luvizetto, D. J.; Ferreira, L. S.; Rech, R.; Ayub, M. A. Z.; Secchi, A. R. A growth kinetic model of *Kluyveromyces marxianus* cultures on cheese whey as substrate. **Journal of Industrial Microbiology and Biotechnology**, v. 31, p. 35-40, 2004.

Mahoney, R. R. Galactosyl-oligosaccharide formation during lactose hydrolysis: A review. **Food Chemistry**, v. 63, n. 2, p. 147–154, 1998.

Manera, A. P.; Ores, J. C.; Ribeiro, V. A.; Burkert, C. A. V.; Kalil, S. J. Optimization of the culture medium for the production of β-galactosidase from *Kluyveromyces marxianus* CCT 7082. **Food Technology and Biotechnology**, v.46, n. 1, p. 66-72, 2008.

Manera, A. P.; Kuhn, G.; Polloni, A.; Marangoni, M.; Zabot, G.; Kalil, S. J.; Oliveira, D.; Treichel, H.; Oliveira, J. V.; Mazutti, M. A. Maugeri, F. Effect of compressed fluids treatment on the activity, stability and enzymatic reaction performance of β-galactosidase. **Food Chemistry**, 2010a, Doi: 10.1016/j.foodchem.2010.10.040.

Manera, A. P.; Rodrigues, M. I.; Kalil, S. J.; Maugeri Filho, F. Permeabilization of *Kluyveromyces marxianus* cells aiming β-galactosidase activity and characterization. **Journal of Agricultural and Food Chemistry,** 2010b, (submitted).

Manera, A. P.; Costa, F. A. A.; Rodrigues, M. I.; Kalil, S. J.; Maugeri Filho, F. Galacto-oligosaccharides production using permeabilized cells of *Kluyveromyces marxianus*. **International Journal of Food Engineering,** 2010c (accepted).

Neri, D. F. M., Balcao, V. M., Costa, R. S., Rocha, I., Ferreira, E., Torres, D. P. M., et al. Galacto-oligosaccharides production during lactose hydrolysis by free Aspergillus oryzae beta-galactosidase and immobilized on magnetic polysiloxane-polyvinyl alcohol. **Food Chemistry**, v. 115, n. 1, p. 92–99, 2009.

Oliveira, D., & Oliveira, J. V. Enzymatic alcoholysis of palm kernel oil in n-hexane and SC-CO_2. **The Journal of Supercritical Fluids**, v. 19, p. 141-148, 2001.

Oliveira, J. V., & Oliveira, D. Kinetic of enzymatic alcoholysis of palm kernel oil in SC-CO_2. **Industrial and Engineering Chemistry Research**, v. 39, p. 4450-4454, 2000.

Panesar, P. S., Panesar, R., Singh, R. S., Kennedy, J. F., & Kumar, H. (2006). Microbial production, immobilization and applications of beta-D-galactosidase. **Journal of Chemical Technology and Biotechnology**, v. 81, n. 4, p. 530–543, 2006.

Reid, R. C.; Prausnitz, J. M.; Poling, B. E. **The Properties of gases and liquids**. McGraw-Hill Book Company, Fourth Edition, 1987.

Rodrigues, M. I.; Iemma A .F. **Planejamento de experimentos e otimização de processos**. 2ª ed., Casa do Pão Editora, Campinas, 2009.

Sako, T.; Matsumoto, K.; Tanaka, R. Recent progress on research and applications of non-digestible galacto-oligosaccharides. **International Dairy Journal**, Amsterdam, v. 9, p. 69-80, 1999.

Woodley, J. M. Choice of biocatalyst form for scalable processes. **Biochemical Society Transactions**, v. 34, n. 2, p. 301–303, 2006.

CAPÍTULO 8

CONCLUSÕES GERAIS

8. CONCLUSÕES GERAIS

As principais conclusões deste trabalho são citadas a seguir:

Na etapa de produção da enzima β-galactosidase a partir da levedura *Kluyveromyces marxianus* CCT 7082 utilizou-se subprodutos agroindustriais, sendo possível obter 1400 U/$g_{cél}$ em 24 h de fermentação, empregando 70 g/L de lactose presente no soro de queijo, 65 g/L de água de maceração de milho, 4 g/L de Prodex-lac, pH 5,0.

Na permeabilização das células da levedura, o isopropanol destacou-se como melhor agente permeabilizante entre os sete agentes testados. As condições que resultaram em maior atividade enzimática foram: relação biomassa/isopropanol (p/p) de 0,03, temperatura de 25°C e 5 min de incubação.

Os resultados da caracterização da enzima das células permeabilizadas foram semelhantes aos obtidos para a enzima extraída das células. Para a temperatura e pH ótimos ambas enzimas apresentaram o mesmo resultado, 50°C e 6,6, respectivamente, já na estabilidade térmica e de pH a enzima das células permeabilizadas foi mais estável que a enzima extraída, indicando que a parede celular promoveu um efeito protetor na enzima nas células permeabilizadas. Comparando os valores de K_m, observa-se que a afinidade pelo substrato não foi prejudicada pelo ambiente intracelular, resultando em 38,5 mM para a enzima das células permeabilizadas e 31,9 mM para a enzima extraída.

A síntese de GOS realizada em reator a pressão ambiente resultou em 83 g/L de GOS, nas seguintes condições: 500 g/L de lactose, 10 U/mL de enzima, pH 7,0, 45°C em 3 h de reação, resultando em 16,5% de conversão de lactose em GOS e produtividade de 27,6 g/L.h.

A enzima nas células permeabilizadas tratadas com fluidos pressurizados mostrou um aumento da atividade residual para os três fluidos estudados em todos os ensaios dos delineamentos. Para o propano o aumento da atividade residual variou de 128 a 211,1%, para o n-butano de 118,5 a 161,8% e para o CO_2 variou de 110,5 a 176,8%. Após 3

semanas de armazenamento a 10°C a enzima tratada com n-butano manteve 96% de sua atividade, já as enzimas tratadas com propano e CO_2 e a enzima sem tratamento, perderam cerca de 30% da atividade enzimática.

A síntese de GOS em reator a pressão atmosférica empregando as células permeabilizadas tratadas a alta pressão apresentou praticamente a mesmas concentrações de GOS, aproximadamente 75 g/L, para os três fluidos pressurizados e para a enzima sem tratamento. Vale destacar que a quantidade necessária de enzima (em gramas de célula) para se obter a mesma atividade enzimática, foi bem menor para as enzimas tratadas a alta pressão tendo em vista o aumento da atividade enzimática após o tratamento.

Na etapa da síntese de GOS em reator pressurizado, obteve-se 75 g/L de GOS para o n-butano, nas seguintes condições: 5 U/mL de enzima, 500 g/L de lactose e 10 bar de pressão. Para o propano, as condições experimentais que resultaram em 65 g/L de GOS foram 5 U/mL, 500 g/L de lactose e 30 bar de pressão. Ao trabalhar com CO_2, as melhores condições foram: 25 U/mL de enzima, 500 g/L de lactose e 100 bar de pressão, resultando em 83,4 g/L de GOS.

Avaliando as condições estudadas neste trabalho, observa-se que o emprego de células permeabilizadas tratadas a alta pressão, principalmente com n-butano, para a síntese de GOS, é o método mais atrativo, considerando tanto o aumento da atividade enzimática, quanto a estabilidade da enzima armazenada, podendo ser realizada a síntese de GOS em período posterior ao tratamento das células a alta pressão.