RAPHAEL HERMANO SANTOS DINIZ

OTIMIZAÇÃO DO PROCESSO FERMENTATIVO E ANÁLISE DO SECRETOMA DE *Kluyveromyces marxianus* UFV-3 EM MEIOS CONTENDO LACTOSE EM DIFERENTES CONDIÇÕES DE CULTIVO

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 15 de março de 2013.

_____	_____
Prof. Luciano Gomes Fietto (Coorientador)	Prof. Agenor Valadares Santos
_____	_____
Prof. Cléberson Ribeiro	Prof. Antônio Galvão do Nascimento

_____

Prof. Wendel Batista da Silveira
(Presidente da Banca)

« Se eu ordenasse, costumava dizer, que um general se transformasse em gaivota, e o general não me obedecesse, a culpa não seria do general, seria minha ».

Antoine de Saint-Exupéry

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BIOGRAFIA

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RESUMO

DINIZ, Raphael Hermano Santos, D. Sc., Universidade Federal de Viçosa, março de 2013. **Otimização do processo fermentativo e análise do secretoma de *Kluyveromyces marxianus* UFV-3 em meios contendo lactose em diferentes condições de cultivo**. Orientadora: Flávia Maria Lopes Passos. Coorientadores: Luciano Gomes Fietto e Wendel Batista da Silveira.

Kluyveromyces marxianus UFV-3 é uma levedura que possui metabolismo respiro-fermentativo, ou seja, a fermentação e respiração coexistem, contudo a via fermentativa ou respiratória pode ser favorecida dependendo da concentração de oxigênio e carboidrato no meio de cultivo. Considerando esta característica das leveduras do gênero *Kluyveromyces* o objetivo deste trabalho foi otimizar a produção de etanol a partir de permeado de soro de queijo (PSQ) e caracterizar o secretoma de *Kluyveromyces marxianus* UFV-3, tendo em vista o uso desta levedura como uma hospedeira para produção de proteínas heterólogas. Para otimizar a produção de etanol em PSQ foi utilizada a metodologia de superfície resposta (MSR) com delineamento composto central rotacionado (DCCR) avaliando os efeitos de pH (4,5-6,5), temperatura (30-45 ºC), concentração de lactose (50-250 g L^{-1}) e biomassa celular seca (1-2 g L^{-1}). Foram realizadas 29 fermentações em hipoxia além de 7 fermentações para validar a equação obtida na MSR. Temperatura foi o fator mais significante na

produção de etanol, seguido de pH, biomassa celular e concentração de lactose. As condições para produção de etanol com rendimentos superiores a 90% foram: temperatura entre 33,3-38,3 ºC, pH entre 4,7-5,7, biomassa celular entre 1,26-1,68 g L^{-1} e concentração de lactose entre 50-108 g L^{-1}. A equação gerada no processo de otimização foi validada podendo, deste modo, ser utilizada para futuros processos de escalonamento da produção de etanol utilizando *K. marxianus* UFV-3. Para analisar como as condições de cultivo influenciam a secreção de proteínas de *K. marxianus* UFV-3, esta levedura foi cultivada em batelada, batelada alimentada (taxa de alimentação inicial – D_o - 0.05 e 0.1 h^{-1}) e cultura contínua (diluição - D - 0.1 e 0.3 h^{-1}). Os sobrenadantes dos cultivos foram analisados em SDS-PAGE e em todos os cultivos observou-se baixa diversidade de proteínas secretadas. Entretanto nos cultivos em batelada alimentada (D_o=0.05 h^{-1}) e cultivo contínuo (D=0.1 h^{-1}) detectou-se uma maior concentração e diversidade de proteínas que nos demais cultivos, além de, em ambos os cultivos, obtivemos uma proteína secretada em maior proporção que as demais. Para identificar as proteínas do secretoma *K. marxianus* UFV-3 foi cultivada em cultura contínua (D=0.1 h^{-1}) devido à reprodutibilidade deste método, além de, maior controle e homogeneidade dos cultivos. As amostras dos cultivos contínuos foram fracionadas por tamanho em um gel de digestão seguido de separação reversa dos peptídeos em um sistema de nano-LC e posterior identificação dos peptídeos em MALDI-TOF/TOF. De um total de nove proteínas, oito proteínas foram identificadas como sendo estruturais ou de membrana e a proteína que foi diferencialmente secretada no meio foi identificada como endopoligalacturonase – endoPG – (EC: 3.2.1.15). EndoPG é uma proteína de interesse biotecnológico e foi caracterizada com atividade ótima na temperatura de 59,5 ºC e no pH 5,1. Além disso, verificou-se que esta enzima não é secretada em meio contendo glicose, por outro lado endoPG é secretada em meio contendo lactose, galactose e principalmente em meio contendo glicerol.

ABSTRACT

DINIZ, Raphael Hermano Santos, D. Sc., Universidade Federal de Viçosa, March, 2013. **Optimization of the fermentation and secretome analysis of *Kluyveromyces marxianus* UFV-3 in medium containing lactose in different forms of cultivation**. Adviser: Flávia Maria Lopes Passos. Co-advisers: Luciano Gomes Fietto and Wendel Batista da Silveira.

Kluyveromyces marxianus UFV-3 is yeast that has respiro-fermentative metabolism, i.e., respiration and fermentation coexists, however fermentative or respiratory pathway can be favored depending on the oxygen and carbohydrate concentration in the culture medium. Considering this characteristic of yeasts of the genus *Kluyveromyces* the objective of this study was to optimize the production of ethanol from cheese whey permeate (CWP) and characterize the secretome of *K. marxianus* UFV-3 in view of the use of this yeast as a host for producing heterologous proteins. To optimize the production of ethanol we used the response surface method (RSM) with a central composite rotational design (CCRD) to evaluate the effects of pH (4.5-6.5), temperature (30-45 ºC), lactose concentration (50-250 g L^{-1}), and dry cell mass concentration (1-2 g L^{-1}). We performed 29 fermentations under hypoxia in CWP and 7 fermentations for the validation of the equation obtained via RSM. Temperature was the most significant factor in optimizing ethanol production, followed by pH, cell biomass concentration, and lactose

concentration. The conditions for producing ethanol at yields above 90% were as follows: temperature between 33.3-38.5 ºC, pH between 4.7-5.7, dry cell mass concentration between 1.26-1.68 g L^{-1}, and lactose concentration between 50-108 g L^{-1}. The equation generated from the optimization process was validated and exhibited excellent bias and accuracy values for the future use of this model in scaling up the fermentation process. To analyze how culture conditions influencing protein secretion in *K. marxianus* UFV-3, this yeast was cultivated in five different conditions: batch, fed-batch (initial feed rate of 0.05 and 0.1 h^{-1}) and continuous culture (0.3 and 0.1 h^{-1}) in medium containing lactose. The supernatant of the cultures were analyzed on SDS-PAGE and all the samples had low diversity of proteins. However, in continuous cultures with dilution of 0.1 h^{-1} and in fed batch 0.05 h^{-1} detected a higher concentration and diversity of proteins in other cultures, and in both cultures, we obtained a protein secreted in greater proportion than the others. To identify proteins secretome of *K. marxianus* was cultivated in continuous culture because it has more reproducible results and control of fermentation parameters when compared with fed batch. Samples of the continuous culture supernatant were subjected to protein size fractioning and in-gel digestion, followed by reversed-phase peptide separation in the nano-LC system and subsequent peptide identification by MALDI-TOF/TOF. Eight proteins were identified as structural proteins or membrane proteins, and the protein was secreted differentially was identified as an endopolygalacturonase - endoPG - (EC 3.2.1.15). EndoPG was characterized with optimum activity enzymatic at a temperature of 59.5 ºC and pH 5.1. Furthermore, it was found that apparently is repressed by glucose and shows intense secretion in batch cultures in medium containing glycerol as the carbon source, and cultures on lactose and galactose.

INTRODUÇÃO

Atualmente, o etanol é considerado a mais importante alternativa ao uso de combustíveis fósseis por reduzir a emissão de gases poluentes na atmosfera, diminuir a dependência de petróleo, criar empregos nas áreas rurais e reduzir déficits comerciais entre os países produtores e consumidores de petróleo (PRASAD et al., 2007). A produção de bioetanol a partir da biomassa lignocelulósica de resíduos agroindustriais e florestais é vista como uma tecnologia promissora. No entanto, o alto custo associado à hidrólise da biomassa, que é essencial para disponibilizar os açúcares fermentáveis, ainda inviabiliza o processo industrial em larga escala. Assim, matérias-primas que possuem carboidratos prontamente fermentáveis, como a lactose do soro de queijo, são provavelmente mais viáveis para a produção de etanol (GUIMARÃES et al., 2010). Soro de queijo é o principal subproduto da indústria de queijo e o volume total de soro produzido no mundo ultrapassa 10^8 toneladas de resíduo por ano. O descarte do soro de queijo sem tratamento prévio pode provocar a destruição da flora e fauna aquática devido a alta demanda bioquímica de oxigênio (DBO) do mesmo, entre 50.000 a 60.000 mg de O_2 por litro de soro (GONZÁLEZ-SISO, 1996).

Dentre as leveduras capazes de metabolizar e fermentar lactose, *Kluyveromyces marxianus* tem chamado atenção por sua aplicação biotecnológica, desde a produção de etanol de açucares que não são

metabolizados pela tradicional levedura da fermentação alcoólica, *Saccharomyces cerevisiae,* até a alta capacidade para produção de biomassa e biomoléculas de interesse socioeconômico (LANE e MORRISSEY, 2010). *Kluyveromyces marxianus* UFV-3, isolada de ambientes de laticínios da Zona da Mata mineira, produz etanol com rendimento próximo ao valor teórico, quando cultivada em alta concentração de permeado de soro de queijo e baixo nível de oxigênio (SILVEIRA et al., 2005). De fato, a expressão de genes e atividade de enzimas chave do metabolismo fermentativo de lactose em *K. marxianus* UFV-3 é maior em hipoxia do que em aerobiose (DINIZ et al., 2012).

Embora tenha sido demonstrado o potencial fermentativo de *K. marxianus* UFV-3 a interação de inúmeros fatores que podem influenciar a capacidade fermentativa desta levedura ainda não foram avaliados. Os efeitos da temperatura, pH, concentração de substrato, do produto final (etanol) e de células são relatados como importantes fatores que influenciam na capacidade fermentativa de leveduras. A metodologia de superfície resposta (MSR) pode ser uma importante ferramenta estatística no processo de otimização da produção de etanol por *K. marxianus* UFV-3.

Além de serem utilizadas na produção de etanol, as leveduras têm sido modificadas geneticamente para a síntese de proteínas heterólogas, isto é, nativas de outras células ou organismos na natureza. A disponibilidade da sequencia genomica de leveduras, os avanços da espectrometria de massa (EM) e da elevada capacidade de processamento de dados pela bioinformática tem facilitado o desenvolvimento de abordagens proteômicas nestes micro-organismos (SZOPINSKA e MORSOMME, 2010). Estudos sobre o secretoma de *K. marxianus* são inexistentes, apesar de algumas pesquisas indicarem que esta levedura é de fato uma boa hospedeira para a expressão de proteínas heterólogas. Devido principalmente às suas propriedades fisiológicas como alta velocidade de crescimento em temperaturas superiores a 30 ºC, além da capacidade de secretar e dobrar, de maneira correta, proteínas heterólogas (ROCHA et al., 2010). Além disto, a possível utilização de soro de queijo como meio de cultivo para leveduras que secretam proteínas heterólogas pode ser uma interessante perspectiva (BECERRA et al., 2006). O conhecimento do

secretoma é útil para a compreensão de como o organismo interage com o ambiente e responde aos estímulos externos, podendo assim, desenvolver novas estratégias para otimizar a produção de proteínas heteróloga (MATTANOVICH et al., 2009). Nesse sentido, o açúcar lactose, próprio do nicho ecológico de *Kluyceromyces marxianus*, parece ser capaz de induzir enzimas extracelulares interessantes para assimilar diversas biomassas na natureza.

O objetivo deste trabalho foi otimizar a fermentação de *Kluyveromyces marxianus* UFV-3, em meios contendo lactose, analisando pH, temperatura, concentração celular e de lactose, além de, analisar como as condições de crescimento da levedura influenciam a capacidade de secreção de *K. marxianus* UFV-3 e analisar o secretoma desta levedura em meio contendo lactose.

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ARTIGO 1

TITLE

Optimizing and validating the production of ethanol from cheese whey permeate by *Kluyveromyces marxianus* UFV-3

1. ABSTRACT

The purpose of this study was to optimize the production of ethanol from cheese whey permeate using *Kluyveromyces marxianus* UFV-3. We used the response surface method (RSM) with a central composite rotational design (CCRD) to evaluate the effects of pH (4.5-6.5), temperature (30-45ºC), lactose concentration (50-250 g L^{-1}), and dry cell biomass concentration (1-2 g L^{-1}). We performed 29 fermentations under hypoxia in cheese whey permeate and 7 fermentations for the validation of the equation obtained via RSM. Temperature was the most significant factor in optimizing ethanol production, followed by pH, cell biomass concentration, and lactose concentration. The conditions for producing ethanol at yields above 90% were as follows: temperature between 33.3-38.5ºC, pH between 4.7-5.7, cell biomass concentration between 2.4-3.3, and lactose concentration between

50-108 g L^{-1}. The equation generated from the optimization process was validated and exhibited excellent bias and accuracy values for the future use of this model in scaling up the fermentation process.

Keywords: *Kluyveromyces marxianus*, cheese whey permeate, response surface method, lactose, fermentation.

2. INTRODUCTION

Cheese whey is the main byproduct from the dairy industry and is composed of approximately 93% water, 5% lactose, 0.9% protein, 0.3% fat, 0.2% lactic acid, vitamins, and mineral salts [1]. In the production of 1 kg of cheese, approximately 10 kg of whey is generated, and it is estimated that the total volume of cheese whey produced worldwide surpasses 160 million tons per year, representing approximately eight million tons of lactose [2]. Approximately 50% of all whey produced is discarded prior to any treatment and causes extensive environmental damage, mainly due to its high biochemical oxygen demand (BOD) of between 50,000 and 60,000 mg L^{-1} of O_2 [1]. Several industries recover a portion of the whey proteins via ultrafiltration for use in food supplements or in other milk products. However, cheese whey permeate resulting from this process still contains approximately 85-95% of the whey lactose, the carbohydrate mainly responsible for its high BOD [3]. Therefore, there is strong incentive for the development of a process for cheese whey permeate treatment that can produce a biotechnological product from the lactose. In recent decades, research on ethanol production from permeate has been driven by the growing demand for cleaner, more renewable energy sources [4]. In addition to biofuel, the ethanol produced from permeate is used in the food, beverage, pharmaceutical, and cosmetic industries, due to its potability [5].

Among the few microorganisms able to ferment lactose is the yeast *Kluyveromyces marxianus*. *K. marxianus* stands out for its high metabolic diversity and its substantial degree of intraspecific polymorphism, traits that are reflected by the various environments from which it has been isolated [6]. In addition to lactose fermentation, *K. marxianus* has other desirable attributes for industrial fermentation processes, such as thermotolerance, a high growth rate, and metabolizing capacity, and often ferments a wide variety of carbohydrates, such as pentoses, hexoses, and disaccharides [7]. *Kluyveromyces marxianus* UFV-3, isolated from cheese factories in South eastern Brazil, is able to convert the lactose in cheese whey into ethanol at high yields under conditions of highly concentrated cheese whey permeate and low oxygen levels [8]. This strain's fermentative behavior is mainly due to

its increased expression of key enzymes involved in lactose metabolism [9]. However, other factors that may affect the fermentative capacity of *K. marxianus* UFV-3, such as temperature, pH, substrate concentration, and cell biomass concentration, have not been established for this yeast. Recently, *K. marxianus* has been used for characterizing and optimizing empirical models for biological systems. These models allow us to study the effects of numerous independent variables (e.g., temperature and pH) that may or may not interact with each other or act on a dependent response variable of interest, such as fermentation yield [10]. The response surface method (RSM) is a combination of mathematical and statistical functions for obtaining empirical models for the development, improvement, and optimization of processes using composite experimental designs [11]. Thus, the purpose of this study was to define the optimal conditions for the production of ethanol by *K. marxianus* UFV-3 from cheese whey permeate using the RSM and central composite rotational design (CCRD). The effects of four independent factors (temperature, pH, lactose concentration, and cell biomass concentration) were analyzed with respect to ethanol yield from lactose consumption (response variable).

3. MATERIALS AND METHODS

3.1. Yeast strain and maintenance

The yeast used in this study, *Kluyveromyces marxianus* UFV-3, was isolated from cheese factory in southeastern Brazil and has been stored and maintained in the culture collection at the Laboratory of Microorganism Physiology, BIOAGRO, of the Federal University of Viçosa, Minas Gerais, Brazil. The yeast was kept frozen at -80ºC in medium containing 50% glycerol. The starting inoculum for fermentation was prepared by adding 1% (w/v) of the biomass stored at -80ºC into YNB medium (Sigma®, St. Louis, USA) supplemented with 2% lactose and cultured under agitation (200 rpm) at 37ºC for 18-24 hours. After this period, the active cells were centrifuged (3,000 *g*, 5 minutes), washed three times with distilled water, and then inoculated into the fermentation medium.

3.2. Fermentation medium

Cheese whey permeate (CWP) obtained from a dairy factory in the region (Indústria Maroca & Russo, Cotochés, Minas Gerais, Brazil) was dried and pulverized in a pilot plant of the Department of Food Technology, Federal University of Viçosa, Minas Gerais, Brazil. The permeate powder was reconstituted with distilled water to lactose concentrations ranging from 50-250 g L^{-1}. The permeate was sterilized by filtration (0.22 µm pore size) and added to the culture medium. The YNB (Yeast Nitrogen Base) medium was prepared according to the manufacturer's instructions. Lactose (Sigma®, St. Louis, USA) was separately sterilized when necessary at 121ºC for 20 minutes. The carbon:nitrogen ratio was maintained at 10:1 when the cells were cultured in YNB medium, with ammonium sulfate ($(NH_4)_2SO_4$) used as a nitrogen source. All media were buffered using citrate-phosphate buffer (100 mmol L^{-1} citrate, 200 mmol L^{-1} Na_2HPO_4) at pre-determined pH values ranging from 4.5 to 6.5.

3.3. Fermentation conditions

The fermentations were performed in 50 mL test tubes containing 20 mL of fermentation medium, and the tubes were sealed with silicone plugs to reduce oxygen permeability. The test tubes were kept in a water bath for 144 hours without agitation. Different combinations of lactose concentration, initial cell biomass concentration, temperature, and pH were used in this study (Table 1). All culturing was performed in hypoxic conditions under nitrogen gas (99.9%, v/v) following a 15-minute purge after initial cell biomass inoculation. Samples were taken from all of the fermentations every 24 hours to determine cell growth, lactose consumption, and ethanol production. The pH was measured at the end of each experiment to test the effectiveness of the buffer used.

3.4. Cell growth and the relationship between absorbance at 600 nm (A_{600nm}) and dry cell mass concentration (g L^{-1})

To determine the biomass dry weight, culture samples from the exponential growth phase (10 mL) were centrifuged at 3000 *g* for 5 min at 4 ºC, and the cell pellet was resuspended in 6 mL of demineralid water. A volume of 4 mL was distributed into four aliquots of 1 mL each and dried at 105 ºC for 24 h to determine the dry mass. A linear regression of the plot of the absorbance (A_{600nm}) versus dry mass (g L^{-1}) allowed the determination of the dry mass corresponding to 1 unit of absorbance at 600 nm. One A_{600nm} unit was found to be equivalent to 0.507 g L^{-1} of the dry mass of *K. marxianus* UFV-3.

3.5. Primary metabolite analysis

Samples taken during the various fermentations were centrifuged at 13,200 *g* for 5 minutes, and the supernatants were collected and frozen at -20ºC. To determine the levels of lactose and ethanol, 20 µL of supernatant from the samples was applied to a high performance liquid chromatography (HPLC) system (HP 1050 M Hewlett Packard 1050 series, HP 1047A detector, using a BIO-RAD Aminex HPX-87H column (300 x 7.8 mm)) with

0.005 M H_2SO_4 eluent, a flow rate of 0.7 mL min^{-1}, and a column temperature of 25°C.

3.6. Determining fermentation parameters

The maintenance coefficient of the cell was set to zero, and three fermentative parameters were determined:

$$RF \text{ or } Y_{E/L} = ((Et_f - Et_i)/(L_i - L_f))/4 \quad \text{(Equation 1)}$$

$$Y_{E/X} = (E_f - E_i)/X_m \quad (g\ g^{-1}), \text{ and} \quad \text{(Equation 2)}$$

$$Q_p = (E_f - E_i)/h \quad (g\ L^{-1}\ h^{-1}) \quad \text{(Equation 3)}$$

where Et_i is the initial ethanol concentration (mol L^{-1}), Et_f is the final ethanol concentration (mol l^{-1}), L_i is the initial lactose concentration (mol L^{-1}), L_f is the final lactose concentration (mol L^{-1}), E_i is the initial ethanol concentration (g L^{-1}), E_f is the final ethanol concentration (g L^{-1}), X_m is the average cell biomass concentration in the medium (g L^{-1}), and h is the time (h).

The theoretical ethanol yield is 4 mol per 1 mol of lactose consumed, for this reason the RF value was divided for 4. Thus, the RF value is 1 (or 100% ethanol yield) and minimum value 0 (or 0% ethanol yield).

3.7. Experimental design and validation of methods

The design of this study consisted of two steps: (i) a preliminary analysis of the factors that influence the fermentative behavior of *K. marxianus* UFV-3 in synthetic YNB medium and (ii) the determination of the effects of these factors on the fermentation process in CWP and a subsequent optimization and validation of the process's operating conditions.

To determine the effects of the four factors on ethanol production, we proposed a CCRD (2^K+2K+5, where K is the number of factors) with a total of 29 experimental units and 5 replicates at the central point. The 25 different experimental arrangements are listed in Table 1. The factors that were investigated, pH, temperature, lactose concentration, and cell biomass concentration, were selected due to their known effects on the production of

ethanol by *K. marxianus* UFV-3 [8, 9]. The experiment was initially performed in YNB medium. After confirming the significance of the factors' effects and optimizing their operational ranges, the fermentations were performed in cheese whey permeate. The CCRD was designed using the Minitab®16 software, and the assays were randomized to avoid any experimental or technical bias. The fermentation process was monitored every 24 hours. This experimental design allowed for the fitting of a quadratic model to estimate the response factor (RF) (Equation 1) using the factors pH, temperature, lactose concentration, and cell biomass concentration, as given by:

$$RF = \beta_0 + \Sigma^4_{i=1}\beta_{ii}X_i + \Sigma^4_{i=1}\beta_{ii}X^2_i + \Sigma^3_{i=1}\Sigma^4_{j=i+1}\beta_{ij}X_iX_j \quad \text{(Equation 4)}$$

where β_0 is the constant; x_i are the variables in natural scale (not coded); β_i (i = 1-4) and β_{ii} are the coefficients corresponding to linear and quadratic terms, respectively; and β_{ij} (i and j= 1-4) are the second-order interaction coefficients. The data were analyzed using the F test for regression (analysis of variance), and the polynomial model (Equation 4) was fitted based on the significance ($\alpha = 0.05$) of the coefficients according to the t-test. Statistical analyses were performed using the Minitab®16 software. The quality of the model fit was expressed by the coefficient of determination, R^2 and by the statistical significance of regression and of lack-of-fit from the analysis of variance. In addition, the model for the fermentation of cheese whey permeate was validated through bias and accuracy factors [12], as described in the following equations:

Bias Factor (F_B): $F_B = 10^{(\Sigma|\log(P/O)/n)}$ (Equation 5);

Accuracy Factor (F_A)**:** $F_A = 10^{(\Sigma|\log(P/O)|/n)}$ (Equation 6)

The Bias Factor is related to the reliability of predicting the response, and values lower than 1 indicate reliable models. The Accuracy Factor examines whether the model has adequate accuracy, and acceptable values should be close to 1. This value is appropriate for comparing two or more models [12]. The model was validated under conditions in which it was possible to obtain RF values close to 1, thus favoring maximum ethanol yield.

The following fermentation conditions were used for validating the equation: temperature, 37ºC; pH, 5.4; cell biomass concentration, 1.72 g L^{-1}; and lactose concentration, 94 g L^{-1}.

4. RESULTS AND DISCUSSION

Initially, the factors that affect the fermentative behavior of *K. marxianus* UFV-3 and the functional ranges of their values were investigated in synthetic YNB medium. Table 1 shows the experimental conditions for the fermentations performed and the RF that corresponds to the relationship between ethanol yield and lactose consumed (Equation 1). In addition to the RF, other fermentation parameters were calculated, such as ethanol produced per gram of cell biomass ($Y_{E/X}$) and maximum volumetric productivity (Q_p). In preliminary analyses performed in minimal medium, the RF values ranged from 0.054 to 0.992, indicating that the process can be optimized within the range studied for each fermentation factor. In most of the experiments, the Q_p was higher at 24 and 48 hours of fermentation than at the other periods evaluated. This result indicates that the process is efficient in the early stages of fermentation, which is desirable for industrial fermentation processes. Analysis of the $Y_{E/X}$ values revealed that the factors evaluated influence the fermentative metabolism of *K. marxianus* UFV-3, as the $Y_{E/X}$ values were reduced up to 25-fold under certain conditions. A quadratic model was fitted (*p*-value = 0.043, R^2 = 65.66%) for this preliminary analysis, in which the factors temperature (T), pH, and lactose concentration (L) exhibited significant linear and quadratic coefficients:

$$\mathbf{RF}_{\text{(synthetic medium)}} = -20.111 + 0.538\,(T) + 4.138\,(pH) + 0.011\,(L) - 0.008\,(T^2) - 0.391\,(pH^2) - 0.000\,(L^2) \quad \text{(Equation 7)}$$

All of the coefficients shown in the equation were significant, and non-significant coefficients have been removed. The results of the ANOVA and t-test used in fitting the model (Equation 7) are summarized in Table 2. The high RF values (close to 1) from some conditions used in the preliminary analysis using minimal medium containing lactose suggested that similar conditions may also result in high RF values in cheese whey permeate (CWP). Figure 1 shows the relationship between the RF values predicted by the fitted model (Equation 7) and the values obtained in the fermentations.

Table 1. Central composite rotational design (CCRD) conducted to optimize fermentation by *Kluyveromyces marxianus* UFV-3 in YNB medium containing lactose and cheese whey permeate. The factors evaluated were pH, temperature (T), lactose concentration (L), and dry cell mass concentration initial (C). The following fermentation parameters were analyzed: relationships between ethanol production by lactose consumed (RF), ethanol yield with cell biomass concentration ($Y_{E/X}$), and maximum volumetric productivity (Q_p).

					YNB medium containing lactose			Cheese whey permeate		
Run	pH	Temperature (ºC)	Lactose concentration (g L^{-1})	Dry cell mass concentration (g L^{-1})	RF	$Y_{E/X\ (g/g)}$	$Q_{p\ (g/L)\ (h)}$	RF	$Y_{E/X\ (g/g)}$	$Q_{p\ (g/L)\ (h)}$
01	5.5	37.50	150	1.52	0.944	51.431	1.212 (24)	0.768	24.549	0.899 (48)
02	5.5	37.50	150	1.00	0.980	80.904	1.906 (24)	0.570	17.545	0.787 (24)
03	4.5	37.50	150	1.52	0.810	42.403	0.938 (72)	0.617	18.616	0.489 (48)
04	5.5	37.50	250	1.52	0.427	37.077	0.865 (24)	0.318	14.408	0.462 (24)
05	5.5	45.00	150	1.52	0.429	21.974	1.421 (24)	0.110	3.404	0.309 (24)
06	5.5	37.50	150	1.52	0.937	63.098	1.649 (24)	0.777	26.766	0.818 (48)
07	5.5	37.50	150	1.52	0.895	54.531	1.988 (24)	0.767	27.701	0.760 (48)
08	5.5	37.50	150	1.52	0.960	51.855	1.571 (48)	0.723	27.043	0.724 (72)
09	6.5	37.50	150	1.52	0.682	31.662	0.959 (48)	0.459	12.210	0.506 (24)
10	5.5	37.50	150	2.00	0.911	41.111	1.732 (24)	0.571	17.546	0.959 (24)
11	5.5	30.00	150	1.52	0.992	54.220	1.206 (48)	0.573	17.531	0.821 (24)
12	5.5	37.50	50	1.52	0.902	13.820	1.140 (24)	0.784	7.092	0.601 (24)
13	5.5	37.50	150	1.52	0.908	44.671	1.779 (24)	0.745	27.214	0.703 (48)
14	6.0	41.25	100	1.27	0.303	20.653	0.260 (72)	0.740	13.179	0.565 (24)
15	5.0	33.75	100	1.77	0.925	32.979	0.489 (48)	0.839	22.745	0.793 (48)
16	5.0	41.25	200	1.27	0.213	23.924	0.446 (24)	0.282	13.592	0.453 (48)
17	6.0	33.75	100	1.27	0.599	33.053	0.369 (120)	0.831	17.491	0.495 (24)
18	5.0	41.25	200	1.77	0.197	20.526	0.403 (48)	0.297	14.513	0.522 (24)
19	5.0	33.75	200	1.27	0.270	25.057	0.405 (24)	0.483	23.478	0.504 (72)
20	5.0	41.25	100	1.77	0.591	20.737	0.545 (24)	0.777	18.249	0.792 (24)
21	5.0	33.75	100	1.27	0.862	37.579	0.415 (72)	0.862	22.854	0.586 (48)
22	5.0	33.75	200	1.77	0.338	20.280	0.311 (120)	0.446	21.703	0.486 (24)
23	6.0	41.25	200	1.77	0.054	3.243	0.05 (120)	0.233	9.393	0.364 (24)
24	6.0	33.75	200	1.27	0.087	6.327	0.08 (120)	0.310	12.341	0.356 (24)
25	6.0	33.75	200	1.77	0.096	5.928	0.091 (96)	0.900	15.802	0.480 (24)
26	6.0	33.75	100	1.77	0.712	33.160	0.554 (72)	0.561	12.656	0.423 (48)
27	6.0	41.25	200	1.27	0.077	6.149	0.177 (48)	0.198	7.863	0.325 (24)
28	6.0	41.25	100	1.77	0.290	11.106	0.251 (72)	0.363	16.880	0.638 (48)
29	5.0	41.25	100	1.27	0.572	21.033	0.532 (48)	0.740	14.441	0.562 (24)

Table 2. Analysis of variance – ANOVA – of the model fitted using the Minitab®16 software for the response factor (RF) – relationship between ethanol production and lactose consumed.

	YNB medium containing lactose					Cheese whey permeate				
Source	**DF**	**SS**	**MS**	***F*-value**	***p*-value**	**DF**	**SS**	**MS**	***F*-value**	***p*-value**
Regression	14	2.02	0.38	7.01	0.00	8	1.21	0.15	20.58	0.00
Linear	4	1.31	0.18	3.71	0.03	4	0.91	0.65	8.98	0.00
Square	4	0.72	0.24	4.96	0.01	4	0.29	0.07	10.01	0.00
Error	22	1.06	0.06			18	0.13	0.01		
Lack-of-fit	8	1.04	0.13	107.23	0.00	14	0.13	0.01	19.12	0.01
PureError	14	0.02	0.00			4	0.0	0.0		
Total	28	3.08				26	1.33			

DF: degree of freedom

SS: square sum

MS: mean square

The R value (correlation coefficient) was 0.66 (Figure 1A) for the linear relationship between the predicted and observed values in synthetic medium, similar to that observed in other studies on the optimization of ethanol production from CWP [13-15].

After testing the significance of the factors that affect the fermentation process in minimal medium and confirming their range of values, a new CCRD was conducted to obtain an empirical model for optimizing the fermentation conditions in CWP. Table 1 shows the combinations of the levels of factors tested and the respective responses obtained. As in the synthetic medium, all of the fermentations in CWP were performed under hypoxic conditions similar those under which most industrial fermentations are performed.

The RF values in the CWP reached 0.9, and the lowest yield was approximately 0.2 (Table 1). The $Y_{E/X}$ values were more homogeneous with nine-fold less variation. As observed in the synthetic medium, the Q_p in the permeate was higher within the first 48 hours of fermentation. In some cases, the RF values obtained in the present study with *K. marxianus* UFV-3 were higher than those found with other *K. marxianus* strains [5]. However, the lack of standardization among bioreactors makes identifying the real differences between strains difficult, indicating a need for constructing validated empirical models for comparing fermentative capacities between different yeast strains.

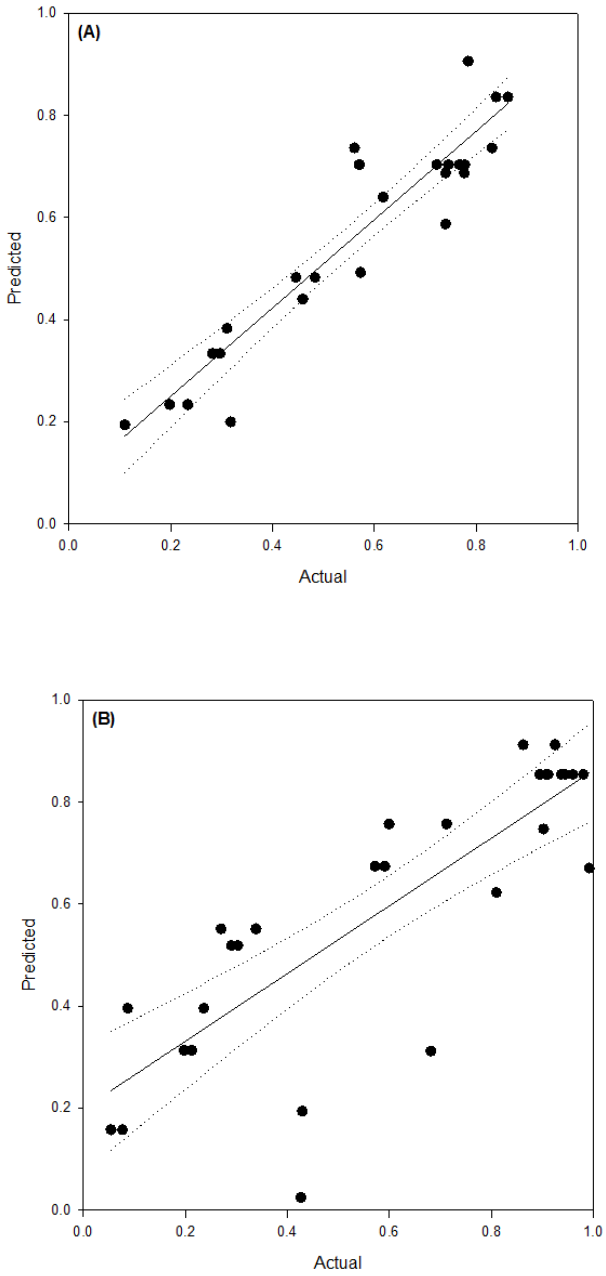


Figure 1. Predicted values and actual values for the RF (relationship between ethanol production and lactose consumed). (A) YNB medium containing lactose (R^2 = 0.66) and (B) cheese whey permeate (R^2 = 0.86). The dotted lines indicate the 95% confidence interval.

The fitted model (Equation 8) for permeate was more complete and significant (*p*-value = 0.000, R^2 = 93%) than that derived in synthetic medium, and the factors temperature (T), pH, lactose concentration (L), and cell

biomass concentration (C) showed significant linear and quadratic coefficients as follows:

$$\mathbf{RF}_{(permeate)} = -15.059 + 0.506\,(T) + 2.060\,(pH) - 0.020\,(L) + 1.924\,(C) - 0.007\,(T^2) - 0.198\,(pH^2) - 0.000\,(L^2) - 0.655\,(C^2) \quad \text{(Equation 8)}$$

The R^2 value observed for this fit was higher than those obtained in different types of cheese whey used in other studies [13-15].

The RF values predicted by the fitted model (Equation 8) showed a high linear correlation (R = 0.86) with the values obtained in the fermentation trials using cheese whey permeate (Figure 1B), which was a considerably high value for experiments on fermentation processes.

Figures 2-4 show the effects of the different factors on ethanol yields from fermenting CWP. Figure 2 reveals that temperature (the factor with greatest effect on the RF) interacts with pH and cell biomass concentration. In both graphs, the 33-42ºC temperature range shows the highest RF values. As the temperature approaches 45ºC, there is a trend of decreasing ethanol yield. The high RF values attained at high temperatures demonstrate the potential for applying *K. marxianus* UFV-3 in the ethanol industry because the temperature in the fermentation vats may increase by up to 10ºC during the period of greatest cell metabolic activity, leading to a loss of cell viability in traditional yeasts [16]. Therefore, using thermotolerant yeasts capable of fermenting at high temperatures, such as *K. marxianus* UFV-3, is appropriate, especially considering the costs associated with the cooling process for fermentation vats. It is estimated that depending on the fermentation process, fermentations at temperatures 5°C above 30°C can save approximately US$ 30,000.00 annually for a medium-sized factory [17]. *K. marxianus* UFV-3 exhibits ethanol yields close to the theoretical values at temperatures up to 5ºC higher than the other *K. marxianus* strains already studied [13-15]. Both pH (Figure 2A) and cell biomass concentration (Figure 2B) showed a wide operational range that allowed for robust RF values close to 0.8, allowing for the moderate variations that are common in fermentation processes without much loss of efficiency.

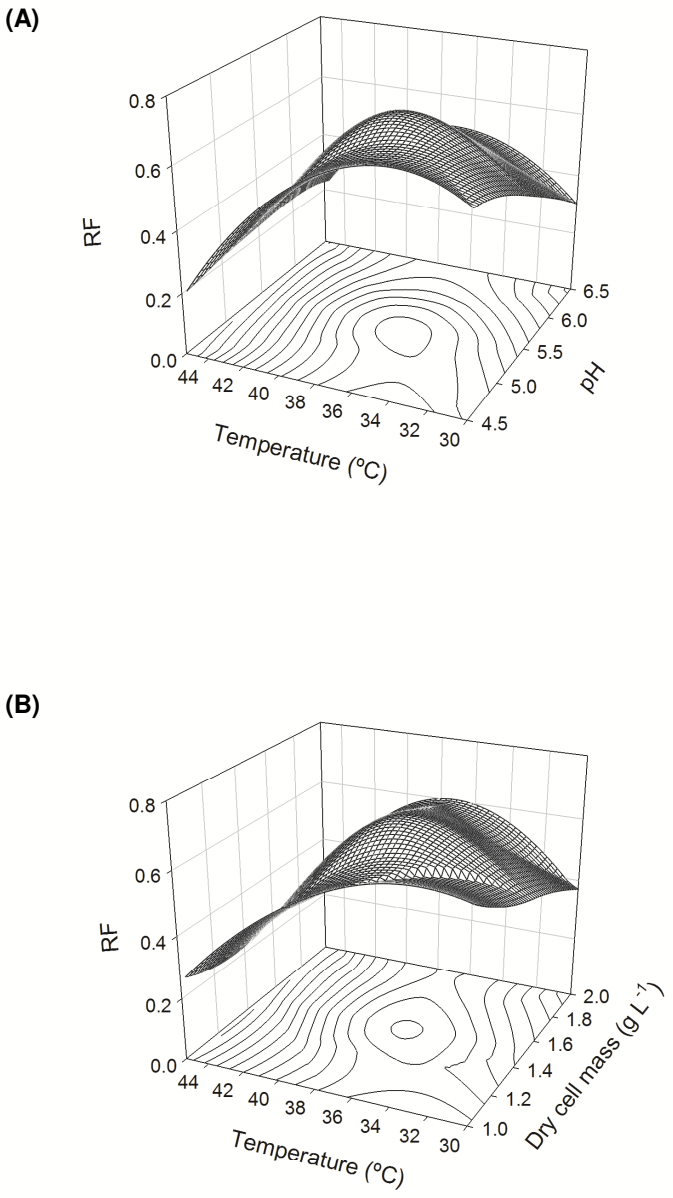


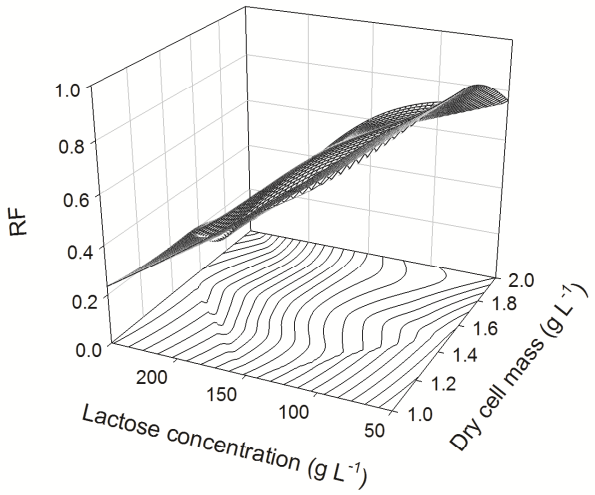
Figure 2. Surface response for the RF as a function of temperature and pH (A) and temperature and dry cell mass (B), in cheese whey permeate.

Although lactose concentration showed the lowest significance value among all of the factors studied, inter-factor analysis of the relationships lactose-cell concentrations (Figure 3A) and lactose concentration-pH (Figure 3B) revealed that variations in lactose concentration resulted in higher variations in the RF. Ethanol yields were high at lactose concentrations near 120 g L^{-1}. At concentrations above 120 g L^{-1}, there was a progressive decrease in the RF, reaching minimal values when the lactose concentration was above 220 g L^{-1}.

Fermentation yields were higher at cell mass concentrations lower than 1.72 g L^{-1} (Figure 3A) and were also high within the 4.5-6 pH range (Figure 3B). Depending on the type of cheese that is produced, sweet or sour, the pH of the permeate/cheese whey will vary [5]. The ability of *K. marxianus* UFV-3 to efficiently convert lactose from the CWP into ethanol in a pH range common to different types of cheese whey can be considered an advantage from an industrial perspective because there is no need for prior correction of substrate pH.

Figure 4A shows that the variation in the factors temperature and lactose concentration significantly alters the RF. Within the ranges of temperature from 33-38ºC and lactose concentration from 50-120 g L^{-1}, ethanol yields are close to the theoretical value. In contrast, at high temperatures and lactose concentrations, minimal yields are attained. In general, the different *K. marxianus* strains analyzed recently, such as MSR Y-8281, CBS 397, and *Kluyveromyces fragilis* (Kf1) [13-15], show higher ethanol yields in lactose concentrations near or below 80 g L^{-1}. Ethanol yields near the theoretical values obtained by *K. marxianus* UFV-3 in CWP with a lactose concentration near 100 g L^{-1} are promising when considering the economic viability of using permeate as the raw material for producing ethanol. Economic feasibility studies show that ethanol production from cheese whey becomes economically viable when it is concentrated two-fold to achieve a lactose concentration near 100 g L^{-1} [18]. It is to be noted that significant variations in the RF were only observed at dry cell mass concentrations near 2 g L^{-1} and pH values near 4.5 or 6.5 (Figure 4B). A weaker response to the cell biomass concentration factor was also observed

(A)



(B)

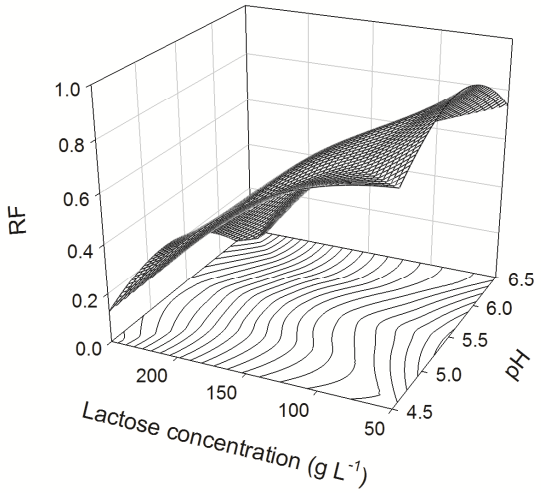
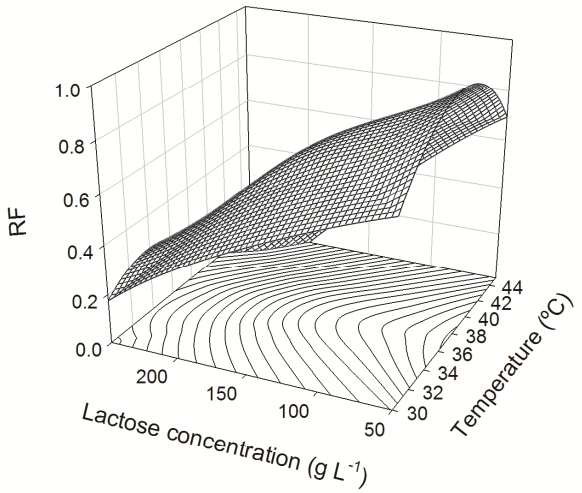


Figure 3. Surface response for the RF as a function of lactose concentration and dry cell mass concentration (A) and lactose concentration and pH (B), in cheese whey permeate.

(A)



(B)

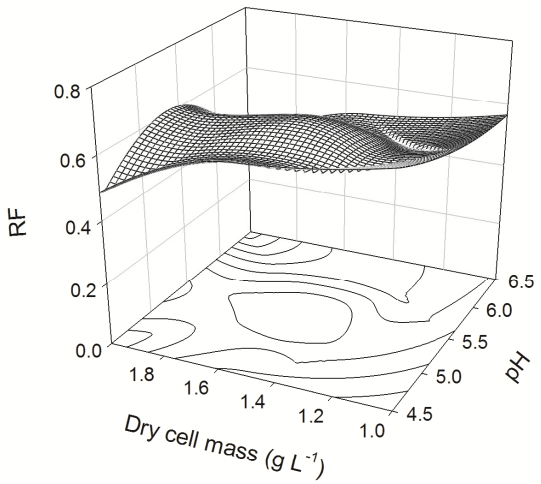


Figure 4. Surface response for the RF as a function of temperature and lactose concentration (A) and pH and dry cell mass concentration (B), in cheese whey permeate.

in ethanol production from cheese whey by *Kluyveromyces fragilis* Kf1 [15]. Apparently, cell biomass concentration only affects the rate at which lactose is converted into ethanol and does not increase the conversion efficiency. In fact, recent studies with the *K. marxianus* DSMZ 7239 strain found that lower cell biomass concentrations led to longer lag phases before initial ethanol production but without significant changes in ethanol yields [19].

Despite the consensus in the scientific community regarding the potential for using *K. marxianus* under industrial conditions, there are few studies on optimizing these processes and validating the resulting optimization models. In the present study, the bias and accuracy factors were evaluated (Table 3) to test the reliability and suitability of the fitted model for predicting RF values in optimizing the fermentation parameters for CWP. Seven fermentations were performed under the following conditions: temperature, 37ºC; pH, 5.4; lactose concentration, 94 g L^{-1}; and cell biomass concentration, 1.72 g L^{-1}. The values obtained for the bias factor (0.944) and the accuracy factor (1.060) indicate that the model is reliable and suitable for estimating the RF values of this process and that modulations of the factors pH, temperature, lactose concentration, and cell concentration all contribute to maximizing the RF in optimizing ethanol production from cheese whey permeate.

Table 3. Validation of the quadratic model (Equation 8) obtained for optimizing ethanol production from cheese whey permeate by *Kluyveromyces marxianus* UFV-3. The culturing conditions were as follows: temperature, 37ºC; pH, 5.4; lactose concentration, 95 g L^{-1}; and dry cell mass concentration, 1.72 g L^{-1}.

Run	Actual FR	Relation Actual/Predicted FR (%)	Bias Factor	Accuracy factor
01	0.954	8.305		
02	0.917	4.097		
03	0.919	4.332		
04	0.969	9.933		
05	0.948	7.649		
06	0.925	4.975		
07	0.904	2.613		
			0.944	1.060

5. CONCLUSIONS

Temperature, pH, lactose concentration, and dry cell mass concentration are factors that significantly affect the fermentation of cheese whey permeate by *K. marxianus* UFV-3. Optimization of the RF values through factors that affect the fermentation process produced ethanol yields above 90%. The quadratic response model was adequately validated and may be used for guiding the scaling of the fermentation process. The ranges in values that obtained ethanol yields above 90% were as follows: temperatures between 33.3-38.5ºC, pH between 4.7-5.7, lactose concentrations between 50-108 g L^{-1}, and dry cell mass concentration between 1.21-1.67 g L^{-1}.

The results indicated that using *K. marxianus* UFV-3 to convert lactose from cheese whey permeate into ethanol is promising because yields close to the theoretical value were achieved over a range of temperatures, pH values, and lactose concentrations, all of which are considered crucial to the economic feasibility of using the permeate as a raw material for ethanol production.

Acknowledgements

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ARTIGO 2

Title

Effect of cultivation methods on *Kluyveromyces marxianus* UFV-3 protein secretion and biotechnological applications

1. ABSTRACT

Kluyveromyces marxianus UFV-3 grows by oxidative or fermentative metabolism depending on both oxygen availability and carbohydrate concentration in the culture medium. The fermentative behavior of *K. marxianus* UFV-3 has been previously studied. However, studies to identify proteins naturally secreted by this yeast or the influence of the cultivation methods in protein secretion has not been previously conducted yeast. We here describe the analysis of protein secretion by *Kluyveromyces marxianus* UFV-3 under five different conditions: batch, fed-batch (initial feeding rate of 0.05 and 0.1 h^{-1}), and continuous culture (dilution rate 0.1 and 0.3 h^{-1}) in medium containing lactose as the sole carbon source. The supernatants of the cultures were analyzed on SDS-PAGE and all the samples had low diversity of proteins. However, in continuous cultures conducted at dilution rate of 0.1 h^{-1} and in fed-batch conducted at feeding rate of 0.05 h^{-1}, the

protein diversity was higher than in other cultures and a protein with apparent Mr of 45 KDa was predominant. Furthermore, one of the bands was clearly stronger than other bands in the gel. The secretome of *K. marxianus* UFV-3 was characterized from growth in continuous culture, because the environmental conditions are highly controlled and therefore the results are more reproducible than during fed-batch cultures. Samples of the continuous culture supernatant were subjected to protein size fractioning and in-gel digestion, followed by a reversed-phase peptide separation in the nano-LC system, and a subsequent peptide identification made by MALDI-TOF/TOF. Eight proteins were identified as structural proteins or membrane proteins, and the protein that was heavily secreted was identified as an endopolygalacturonase - endoPG - (EC 3.2.1.15). EndoPG shows optimum enzymatic activity at 59.5 ºC and pH 5.1. Furthermore, it was found that secretion diminished in glucose while showing intense secretion in batch cultures containing lactose, galactose and mainly glycerol as carbon source.

Keywords: Continuous culture; endopolygalacturonase; fed-batch; *Kluyveromyces marxianus;* MALDI TOF/TOF; extracellular proteome.

2. INTRODUCTION

Yeasts have occupied a prominent position in modern genetics, molecular biology and cell biology. Focus has been set predominantly on *Saccharomyces cerevisiae,* the "conventional" yeast considered a model for eukaryotic cells, *Schizosaccharomyces pombe,* the "fission yeast" model, *Kluyveromyces lactis*, a convenient model for oxidative and lactose metabolism studies, and *Candida albicans* due to its clinical significance. There is an increasing interest in the study and the finding of new biotechnological applications of the so-called ''non-conventional'' yeasts (Lane and Morrisey, 2010). Recently, *Kluyveromyces marxianus* has attracted attention due to its biotechnological potential. This yeast has been isolated from different environments, and the different strains show high metabolic diversity and substantial degree of intra-specific polymorphism. Several biotechnological applications have been investigated in *K. marxianus* such as cellular protein (single-cell protein), aromatic compounds, ethanol and heterologous protein production, bio-ingredients from cheese whey and bioremediation (Lane et al., 2011).

Kluyveromyces marxianus UFV-3, a strain first isolated from a cheese factory in south eastern Brazil, is able to convert lactose into ethanol with yields close to the theoretical maximum, even in media containing high lactose concentrations and low oxygen availability (Silveira et al., 2005). In aerobiosis and with low sugar concentrations, biomass production is very effective with a generation time shorter than 1.5 hour (Diniz et al., 2012). The potential of *K. marxianus* as a convenient host for heterologous protein production has also been proved (Bergkamp et al., 1993; Rocha et al., 2010). In relation with its present and future biotechnological applications, the study of the *K. marxianus* secretome becomes of strategic relevance. The secretome includes all secreted proteins, that is, proteins anchored to the cell surface and proteins involved in the secretory pathway (Tjalsma et al., 2000). Secreted proteins have been implicated in formation and maintenance of the cell wall, cell separation, nutrient-scavenging, sexual reproduction (mating), virulence (in pathogenic yeasts), and defense against competing fungi (Swaim et al., 2008). Bioinformatics might contribute to predict the secretome

composition of a given microorganism once its genome has been sequenced; i.e. computational analyses of *C. albicans* genome predicted approximately 200 potentially secreted proteins (Mattanovich et al., 2009). However, there are obvious limitations in these predictions, such as those attributed to proteins released from the cell wall during growth and budding. Moreover, the actual composition of the secretome is strongly dependent on the growth conditions and on the actual expression of the genes encoding potentially secreted proteins The identification of proteins secreted by *K. marxianu*s UFV-3 will be useful to understand how this yeast responds to external stimuli, providing new insights for improved production of heterologous proteins. (Mattanovich et al., 2009).

We here describe the first secretome analysis of *K. marxianus* UFV-3. The main aim focuses on the analysis of how diverse growth conditions influence protein secretion. This knowledge will allow a better selection of cultivation conditions, which improve protein secretion and with direct applications in heterologous protein production. Besides, the identification in the secretome of high productive secreted proteins and the study of their characteristics will facilitate the construction of expression vectors to produce heterologous secreted proteins in *K. marxianus*.

3. MATERIAL AND METHODS

3.1. Yeast strain and maintenance

Kluyveromyces marxianus UFV-3 used in this study was isolated from a Brazilian regional dairy environment. During the course of this work it was maintained streaking on YPL plates (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} lactose, 15 g L^{-1} agar) at 4 ºC. A pre-culture was prepared by inoculating one isolated colony in YPL agar to synthetic YNB medium (Sigma®, St. Louis, U.S.A.) with lactose (20 g L^{-1} final concentration). The pre-cultures were incubated at 37 ºC in rotary shaker at 150 rpm and grown overnight. After this period, the cells were centrifuged (3,000 *g*, 5 minutes), washed three times with sterile distilled water, and inoculated up to an A_{600nm} 0.1 into the medium.

3.2. Growth conditions

To analyze the diversity caused by growth conditions in the protein secreted by *K. marxianus* UFV-3, this yeast was cultured in three different modes: batch, fed-batch, and continuous culture in a chemostat. All cultures were conducted in a bioreactor 2 L (BIOSTAT®MD, Braun Biotech Technology, Germany) with 300 mL YNB medium supplemented with lactose (20 g L^{-1} final concentration) (Table1). Temperature and stirring were maintained at 37 ± 0.2 ºC and 530 ± 10 rpm, respectively. The dissolved oxygen (DO) was measured with a polarographic electrode. The oxygen electrode was calibrated prior to the inoculation of the cultures. The batch phase of the culture lasted 24 hours (up to late log phase), when lactose in the batch medium was completely consumed. Fed-batches were initiated after 24 hours with initial feeding rate of 0.1 h^{-1} or 0.05 h^{-1}. The feeding medium was two times concentrated YNB with lactose 100 g L^{-1}, the final volume in the bioreactor was 800 mL. The continuous cultures were initiated after 24 hours with dilution rates 0.1 h^{-1} or 0.3 h^{-1} and the feeding medium was identical to the batch culture. The continuous cultures were maintained

for 8-10 generations to reach steady-state when cell mass concentration and the pH of the samples were monitored.

Table 1. Conditions of cultivation of *Kluyveromyces marxianus* UFV-3.

Cultivation	$pH_{(final)}$	A_{600nm}	Final volume (ml)	Cultivation time (h)	Oxygen Soluble
Bath	2.38	3.2	500	24	93%
Feed-bath[*;+] **(D_o=0.1 h^{-1})**	2.71	3.6	1000	34	94%
Feed-bath [*;+] **(D_o=0.05 h^{-1})**	2.65	2.6	1000	44	92%
Continuous culture[*] **(D=0.1 h^{-1})**	2.61	2.9	320	91	94%
Continuous culture [*] **(D=0.3 h^{-1})**	3.02	1.6	330	44	98%

All cultures were performed at 37 ºC ± 0.2 ºC, initial pH 5.4 and agitation 510 rpm ± 10

The culture medium used was YNB 2% lactose

The initial A600nm all cultivation was 0.1

*The feeding were initiated after 24 hours of cultivation

+The culture medium used in the batch feed was fed: YNB (2x) supplemented with lactose10%

After identifying the *K. marxianus* UFV-3 extracellular secretome, experiments were performed to analyze the pattern of secretion of yeast in complex medium (YP) with different carbohydrates. Extracellular protein diversity was analyzed in cultures carried out in YP medium supplemented with glucose, lactose, galactose or glycerol (final concentration 2 g L^{-1} w v^{-1}). The yeast was cultured at 37 ºC at 150 rpm for 24, 48, and 72 hours.

3.3. Protein concentration and SDS-PAGE

The complexity of the extracellular protein collection of K. marxianus UFV-3, in different growth conditions was analyzed by gel electrophoresis. At the end of fermentation, approximately 45 mL, the cultures were centrifuged and the supernatant was concentrated in Amicon filters (MWCO 3,000 Da, Millipore, Billerica, (MA), U.S.A.) to a final volume of approximately 500 µL. The total protein concentration was determined by the Bradford method (1976). Separations were performed in denaturing 12% polyacrylamide (SDS-PAGE), at a constant voltage of 200 V by the method of Laemmli (1970). The gel was stained with colloidal Coomassie Blue R-250 (Neuhoff et al., 1988) or silver stain (Rabilloud, 1992), when necessary.

For the in-gel fractioning, 20 µL of each extracellular concentrated protein sample was loaded in 12% acrylamide in-house gels and separated by SDS-PAGE in a Protean® mini-gel system (Bio-Rad, Hercules, (CA), U.S.A.) at a constant voltage of 100V. The gels were lightly stained with Coomassie Blue for 10 minutes to check protein integrity. Each entire lane was size-fractioned into 4 sections, which were subsequently processed separately. Each section was de-stained with methanol, generating three experimental replicates, diced into small pieces and in-gel digested, following a standard procedure (Shevchenko et al., 2006). Briefly, the samples were desiccated for 16 hours at 37 ºC with acetonitrile, reduced with DTT, alkylated with iodoacetamide and trypsin-digested (6 ng μL^{-1}, PromegaMSgold, Madison, (WI), U.S.A.). Peptides were then extracted, dried in a speed-vac, reconstituted in 0.1% trifluoroacetic acid and de-salted by using self-made frits for nanoscale columns.

In the experiments analyzing the diversity of proteins in YP medium, the supernatant was not concentrated; it was centrifuged and filtered through 0.22 µm membrane (Millipore, Billerica, (MA), U.S.A.).

3.4. NanoLC–MALDI-TOF/TOF

The peptide fractions were separated using reversed phase chromatography in a nanoLC system (Tempo, Eksigent, Dublin, (CA), U.S.A.). Samples were loading through a trapping column into a C18 silica-based column (New Objective, Woburn, MA, U.S.A.) with an internal diameter of 300 Å. Peptides were eluted at a flow rate of 0.35 ìL min^{-1} during 30-minute by a linear gradient from 2% to 40% acetonitrile (mobile phase A: 0.1% trifluoroacetic acid 2% acetonitrile; mobile phase B: 0.1% trifluoroacetic acid 95% acetonitrile), mixed with á-cyano matrix (4 mg mL^{-1} at a flow rate of 1.1 ìL min^{-1}) and deposited onto a MALDI plate using an automatic spotter (SunCollect, Sunchrome, Friedrichsdorf, Germany). Chromatograms, corresponding to each gel section, were composed of 120 spots, each one comprising a 15-second deposition.

The MS runs for each chromatogram were acquired and analyzed in a MALDI-TOF/TOF instrument (4800 ABSciex, Framingham, (MA), U.S.A.)

using a fixed laser intensity of 3800 kV and 1500 shots/spectrum. Automated precursor selection was done using a Job-Wide interpretation method (up to 10 precursors/fraction, signal-to-noise lower threshold 50) with a laser voltage of 4800 and 1500 shots/spectrum at medium CID collision energy range. A second Job-Wide precursor selection was done by excluding those previously fragmented precursors and by using a lower signal-to-noise threshold of 30, to identify peptides coming from low-abundance proteins. Data from both MS/MS acquisitions were used for data processing and subsequent protein identification.

3.5. Database search and protein identification

The complete MS and MS/MS raw data were processed separately using the Protein Pilot® software platform (ABSciex, Framingham, (MA), U.S.A.) and Mascot Search (Pearkins et al., 1999). Peptide identification in Protein Pilot® software was performed against the last uniprot_sprot-release (2012_05 of 20-may-2012, 536029 sequence entries). Search parameters for Mascot and Protein Pilot® included carbamidomethylation of cysteines (fixed), oxidation of methionine (variable, ≤ 4), 1 missed trypsin normal cleavage, precursor ion mass range 800–4000 Da, ± 50-150 ppm tolerance on precursor and ± 0.3 Da on fragmentation of ions. The scoring model was defined by the algorithm, and the peptide acceptance parameters were peptide length ≥ 5; minimum peptide z-score of 5.0; maximum peptide p-value of 1.0 $e^{-7;}$ and an AC score of 8.0. False Discovery Rate (FDR) was estimated by contrasting both uniprot_sprot and decoy uniprot_sprot databases.

3.6. Endopolygalacturonase enzymatic assay

Endopolygalacturonase activity was measured in a spectrophotometer (Beckman DU serial 600) by evaluating its reducing power according to the DNS method (Miller, 1959). Typical reaction mixtures, containing 0.5 mL of sample and 0.5 mL of 0.5% polygalacturonic acid in citrate-phosphate buffer (100 mM citrate, 200 mM Na_2HPO_4 at pH range 2.6-8.0), were incubated for

10 minutes with temperatures ranging from 50 to 70 ºC. One unit of activity was defined as the amount of enzyme that releases 1 µmol of galacturonic acid or the equivalent in reducing power per minute.

3.7. Experimental design of response surface methodology on endopolygalacturonase activity

To determine the effects of the two factors on endopolygalacturonase activity, a CCRD (2^K+2K+5, where K is the number of factors) was proposed, with a total of 13 experimental units and 5 replicates at the central point (K=2). The investigated factors, pH and temperature, were selected after preliminary testing (data not shown). The experiment was performed in YNB medium, supplemented with lactose. The CCRD was designed using the Minitab®16.0 software, and the assays were randomized to avoid any experimental or technical bias. This experimental design allowed for the specification of a quadratic model to estimate the enzymatic activity (EA) (Equation 1) using the factors given by:

$$RF = \beta_0 + \Sigma^4_{i=1}\beta_{ii}X_i + \Sigma^4_{i=1}\beta_{ii}X^2_i + \Sigma^3_{i=1}\Sigma^4_{j=i+1}\beta_{ij}X_iX_j \quad \text{(Equation 1)}$$

Where β_0 is the constant; x_i are the variables in natural scale (not coded); β_i (i = 1-2) and β_{ii} are the coefficients corresponding to linear and quadratic terms, respectively; and β_{ij} (i and j= 1-2) are the second-order interaction coefficients. The data were analyzed using the F test for regression (analysis of variance), and the polynomial model (Equation 1) was fitted based on the significance ($\alpha = 0.05$) of the coefficients according to the t-test. Statistical analyses were performed using the Minitab®16.0 software. The fitness of the model was expressed by the coefficient of determination ($R^{2)}$ and by the statistical significance of regression and of lack-of-fit from the analysis of variance.

4. RESULTS

4.1. Influence of growth conditions upon secreted proteins

In order to determine the influence of the growth control on the profile of secreted proteins by *K. marxianus* UFV-3, this yeast was cultured in lactose as the sole carbon and energy source under five different operation modes: batch, to reach entrance of stationary phase, fed-batch with initial feeding rate of 0.1 or 0.05 h^{-1}, for the later log phase, and continuous culture with the entrance of the stationary phase being attributed to precise low growth rates of 0.3 and 0.1 h^{-1}. In all conditions it was observed a narrow diversity of secreted proteins; however, the pool of secreted proteins changed according to the yeast culture mode (Figure 1).

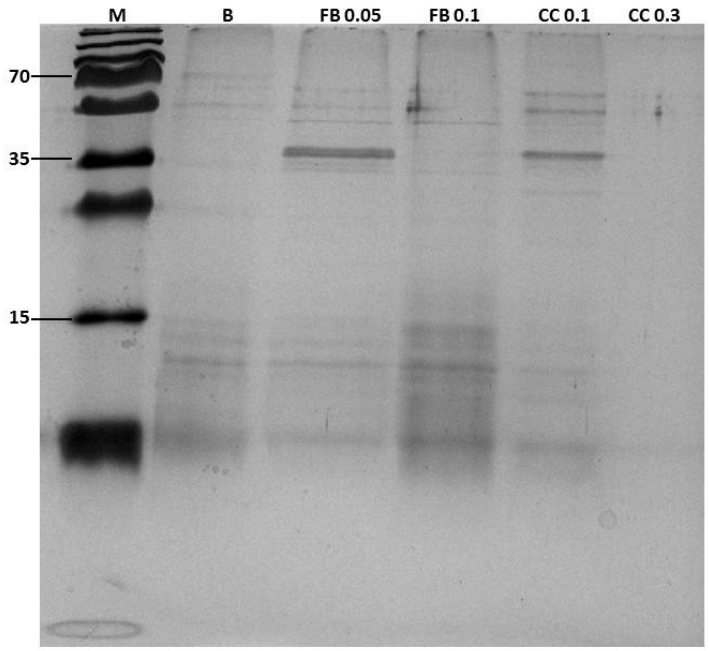


Figure 1. Proteins of the *K. marxianus* UFV-3 secretome subjected to SDS-PAGE at 12% polyacrylamide Tris-Glycine. Samples of 20 µL were loaded and the separated proteins were visualized by colloidal staining (A) and silver staining. M corresponds to protein standards (Molecular masses in KDa; Invitrogen). *K. marxianus* UFV-3 concentrated supernatants with lactose as sugar source were analyzed in: B is batch (24 hours); FB0.05 and FB0.1 is fed-batch (initial feeding rate the 0.1 h^{-1} or 0.05 h^{-1}, respectively); CC0.1 and CC0.3 are culture continuous (dilution rates the 0.3 h^{-1} or 0.1 h^{-1}, respectively).

The diversity of secreted proteins was lower in continuous culture conducted at the growth rate closer to maximum (0.3 h^{-1}) compared to any other culture mode. In both batch and fed-batch conducted at higher initial feeding rate (0.1 h^{-1}), there was a greater number of bands, when compared to continuous culture conducted at higher dilution rate (0.3 h^{-1}). It should be noted that in both culture operation batch and fed-batch conducted at higher feeding rate, the banding pattern was similar. On the other hand, in both fed-batch and continuous culture conducted at lower feeding and dilution rates 0.05 h^{-1} and 0.1 h^{-1}, respectively, the band whose size ranges from 35 to 55 KDa was more strong than the other bands. Furthermore, it was found, in these cultures, a higher number of proteins.

4.2. Secretome characterization

Because the environmental conditions in fed-batch cultures are not as well controlled as in the continuous culture conducted at dilution rate of 0.1 h^{-1}, the latter was chosen to analyze the secretome of *K. marxianus* UFV-3. Nine proteins found in the extracellular extract were identified (Table 2). The most abundant protein in the extracellular medium was excised from the gel for individual identification. It was identified as polyendogalacturonase, endoPG, (EC: 3.2.1.15) with approximately 45 kDa. The other proteins included in Table 2 are related to cell wall or metabolic functions and several are not directly recognized in *Kluyveromyces*, but in other genera probably due to the un-completeness of *K. marxianus* genome database

The identification that at low growth rates for high secretion endoPG establishing culture conditions a cell which must be maintained for a future use of the expression vector using the promoter of this protein.

Table 2. Mass spectrometric analysis of the proteins released in culture continuous (D=0.1 h^{-1}) of *Kluyveromyces marxianus* UFV-3.

n	ORF	Organism	Annoted protein function
1	Q6TA03_KLUMA	*Kluyveromyces marxianus*	Extracellular protein with polygalacturonase activity
2	KLLA0D16214g	*Kluyveromyces lactis*	P-P-bond-hydrolysis-driven protein transmembrane transporter activity
3	KLLA0D18304g	*Kluyveromyces lactis*	Intracellular protein transport
4	KLLA0B07392p	*Kluyveromyces lactis*	Structural constituent of cell wall
5	KLLA0B07370p	*Kluyveromyces lactis*	Structural constituent of cell wall
6	CAGL0M13805g	*Candida glabrata*	Hydrolase activity, hydrolyzing O-glycosyl compounds
7	BRE1_CANAL	*Candida albicans*	E3 ubiquitin-protein ligase
8	C4QVL7_PICPG	*Pichia pastoris*	Cell wall protein with similarity to glucanases
9	DRE2_UNCRE	*Uncinocarpus reesii*	iron-sulfur (Fe/S) protein assembly machinery

4.3. Biochemical characterization of the predominant secreted protein

The activity of the *K. marxianus* UFV-3 endoPG was evaluated as a function of temperatures and pH. It revealed a broad range of activity from 30 to 80 ºC and from pH 2.6-8 (data not shown). Based on this data, the parameters for the response surface were established to maximize the activity of this enzyme with temperatures ranging from 50-70 ºC and pH of 2.6-7.0 (Figure 2). A quadratic model was fitted (R^2 = 90.94%) for this analysis, in which both the factors pH and temperature (T) exhibited significant linear and quadratic coefficients:

$$EA_{(U/ml)}: -1.572 + 0.156(pH) + 0.046(T) - 0.015\,(pH)^2 - 3.899x10^{-4}(T)^2$$

Equation (2)

The relationship between the observed and expected activity was close to 1 (0.91), which shows the reliability of the data. The conditions that had the highest enzymatic activity were: temperature of 59.5 ºC and pH 5.1 with values around enzymatic activity of 0.20 U/ml.

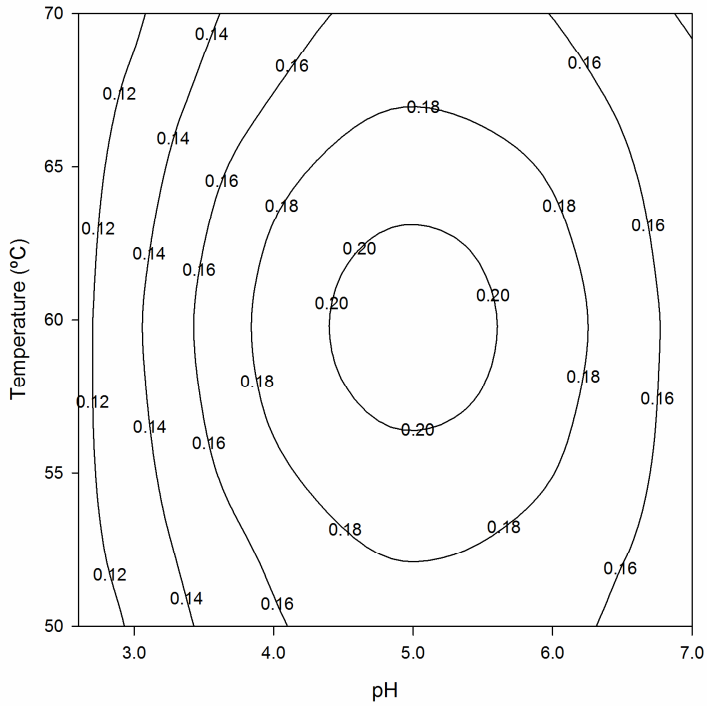


Figure 2. Surface response for polyendogalacturonase activity (EC: 3.2.1.15) as a function of temperature and pH.

4.4. Secretion of endoPG in function of carbon source

Finally, it was evaluated whether endoPG is differentially secreted in function of carbon source availability. *K. marxianus* UFV-3 was grown in YP supplemented with 2% glucose, lactose, galactose or glycerol for 24, 48, and 72 hours (Figure 3). In the medium containing glucose, the endoPG band was not observed in 24, 48, or 72 hours. On the other hand, in the medium containing lactose, the endoPG was strongly expressed at 48 hours. At 72 hours, the concentration of extracellular proteins was superior to the dyeing capacity of the method (Figure 3A). In galactose, there is an apparently constitutive thin band of endoPG at all culture times. In glycerol, the protein concentrations were apparently higher. Moreover, the endoPG concentrations were also higher in glycerol, mainly at 48 and 72 hours (Figure 3B).

(A)

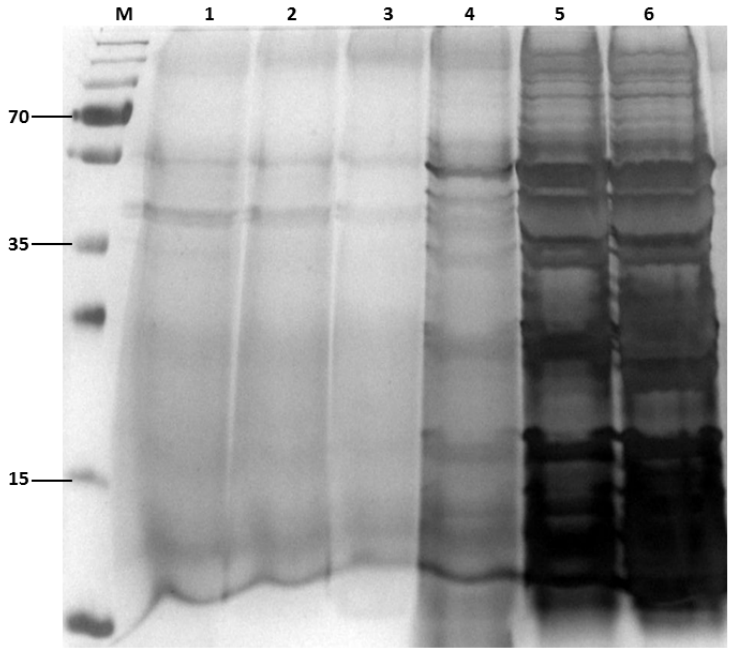
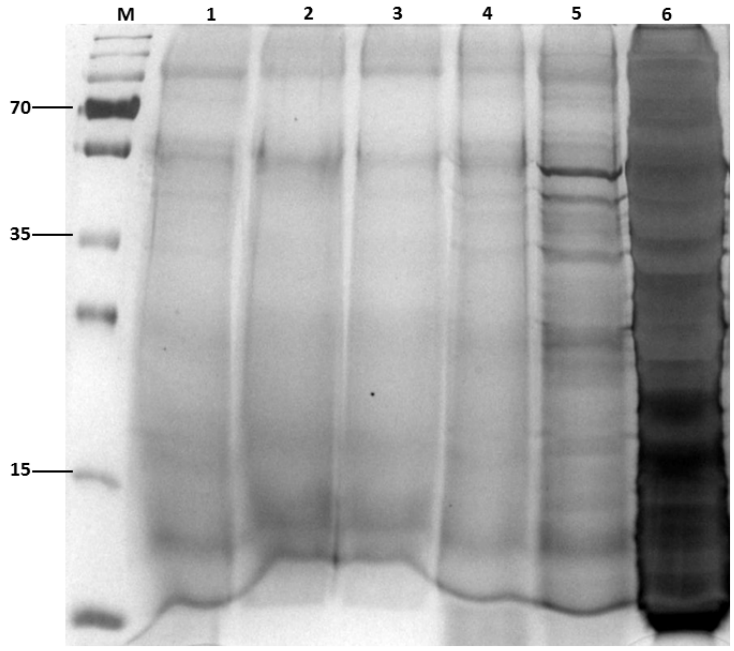


Figure 3. Proteins supernatant of the *K. marxianus* UFV-3 cultured in YP medium containing different carbon sources subjected to SDS-PAGE a 12% polyacrylamide Tris-Glycine. Samples of 20 µL were loaded and the separated proteins were visualized by colloidal staining. M corresponds to protein standards (Molecular masses in KDa; Invitrogen). (A) Lines 1, 2, 3 correspond to the cultures in glucose (24, 48, and 72 hours, respectively) and lines 4, 5, 6 in lactose (24, 48, and 72 hours).

(B)



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5. DISCUSSION

It has been demonstrated that some *Kluyveromyces marxianus* strains are suitable for ethanol production from various carbohydrates (*e.g*. sucrose, lactose, glucose) while other strains have relevant characteristics to be used as host for heterologous proteins production (Rocha et al., 2010; Lane et al., 2011). *K. marxianus* UFV-3 is a strain capable of producing cell mass and ethanol from cheese whey permeate and it reaches high fermentation yields (Silveira et. al., 2005), but its ability for protein secretion has not been evaluated yet.

To serve as a host cell for heterologous protein production, the secretion of low number of proteins is desirable, due to some advantages such as (i) simplified purification processes because of the low number of protein contaminants and (ii) decreased energetic cost in production of proteins which are not of interest (Graf et al., 2009; Idiris et al., 2010). As revealed in this work, K. marxianus secreted a low number of proteins in all evaluated culture operations, thus indicating that this yeast is a potential candidate to host heterologous protein expression systems producing extracellular proteins. The number of secreted proteins in yeast is directly related to a particular physiological state, i.e., growth rate and physical or chemical environment. Changes in growth rate are observed at the transition from log to stationary phase. For example, the yeast Candida utilis has been reported to produce more proteins in stationary phase (74%) than in logarithmic phase (Buerth et al., 2011), but it is difficult to reproduce a broad condition as stationary phase. In C. albicans, the variations in culture medium composition or in growth conditions influence the protein secretion ranging from 25 to over 100 proteins, far below the 225 predicted proteins for secretion by informatics tools (Thomas et al., 2006; Sorgo et al., 2013). The yeast secretomes are generally obtained in cultivations carried out in batch or fed-batch, by using minimal medium containing glucose as the sole carbon source or in continuous cultures using complex media, e.g. YP (Swaim et al., 2008). In secretome studies, the cultivation of micro-organisms in batch and

fed-batch is a routine, because they are simple, quick, and have great diversity of proteins. Furthermore, the proteins secreted are found in high concentrations because cell mass yield is higher. On the other hand, cultivations in continuous culture are more complex and demand more amounts of media, and the proteins secreted are in lower concentrations when compared to batch and fed-batch. This occurs because the culture medium, cells, proteins, and metabolites are continuously discharged from fermentator. Nevertheless, the growth conditions in continuous culture are well defined, providing more reproducible responses and homogeneity of the cell population. Furthermore, due to the constant flow of medium, only secreted proteins are constantly accumulated in the fermentator and, consequently, identified. Thus, the number of proteins identified in continuous culture is lower, because intracellular proteins that remain in the medium due to cell lysis are not identified (Daran-Lapujade et al., 2009).

Most of the proteins identified in the secretome have functions not expected *a priori* in the extracellular medium. KLLA0D18304g, KLLA0D16214g and BRE1_CANAL are proteins related to intracellular transport of other proteins. In *Kluyveromyces lactis,* KLLA0D18304g is mainly related to protein ubiquitination. In *C. albicans*, the BRE1_CANAL is also involved in the protein ubiquitination (Madinger et al., 2009; Swaim et al., 2008). KLLA0D16214g is related to the active transport of proteins and transmembrane protein traffic. KLLA0B07392g and KLLA0B07370g are structural constituents of the yeast cell wall. These proteins are part of the native conformation of the cell wall and can occasionally be released. C4QVL7 is also related to cell wall, and its glucanase function suggests that it is related to the remodeling of the cell wall during budding. DRE2 has an anti-apoptotic protein and participates in the maturation of proteins Fe/S, besides to be present in the control of oxidative stress.

The endopolygalacturonase Q6TA03- endoPG (EC: 3.2.1.15) - was the only protein whose secretion was expected in *K. marxianus*. EndoPG has biotechnological importance and it can be used in the textile, food, pulp and paper industries, among others (Kashyap et al., 2001). This enzyme hydrolyzes α 1-4 linkages in pectin and other galacturonans. Pectin is one of

the major components of the primary cell wall of plants, the main constituent of the middle lamella and is found in dividing cells and also in the areas of contact between cells having a secondary cell wall (Márques et al., 2011). EndoPG is common in filamentous fungi and found in yeasts, in minor amount. *K. marxianus* is one of the few ascomycetes able to secrete this enzyme (Serrat et al., 2011).

The maximum extracellular production of endoPG's in batch culture occurs at early stationary phase because it is reported that involves stress conditions such as decreased rate of oxygen and nutrients (Schwan and Rose, 1994; Serrat et al., 2004). Fed-batch and continuous cultures produce higher concentrations of endoPG when compared to batch cultures (Rojas et al., 2011). In *S. cerevisiae* producing endoPG, the presence of glucose inhibits the expression of this enzyme, whereas cultivations in polygalacturonans or in galactose induce the expression. Therefore, glucose media are used, in general, for obtaining cell biomass (Radoi et al., 2005; Louw et al., 2010; Rojas et al., 2011). Apparently, the same effect occurs in *K. marxianus* UFV-3, because the endoPG was not observed on SDS-PAGE when this yeast was cultured in medium containing glucose as carbon source. Thus, apparently, the endo-PG expression ~~in~~ from *K. marxianus* UFV-3 is repressed by glucose and the enzyme is secreted predominantly in the absence of this carbohydrate, at higher abundance when grown on glycerol.

Generally, the activity of previously isolated endoPGs is maximum in pH ranging from 3.5 to 5.5 and in temperature ranging from 30 to 50 ºC (Jayani et al., 2005). However, few endoPGs have the capacity to hydrolyze pectin in alkaline pH or at high temperatures. Although the endoPG synthesized by *K. marxianus* UFV-3 has maximum activity in pH 5.1 and at 59.5 ºC, the enzyme also has activity in more alkaline pH and at even higher temperatures, thus increasing its biotechnological value. The optimization of the growth conditions for the industrial production of endoPG by *K. marxianus* UFV-3 is an interesting challenge, considering that the enzyme activity liberated to the media (U/ml) has been reported to be lower than in other fungi (Singh et al., 2005). Simultaneous production of ethanol and

endoPG was already investigated using beet sugar as substrate and obtaining higher yields of both products (Serrat et al., 2004). The verification in this work that endoPG of *K. marxianus* UFV-3 is efficiently induced and secreted on lactose enables further studies on the production of ethanol and endoPG from agro-industrial residues containing lactose, as cheese whey.

Pectinase production occupies about 10% of the overall manufacturing of enzyme preparations. For this reason, it is important to investigate the production conditions and physico-chemical characteristics of new enzymes. The identification and characterization of endoPGs, in addition to providing new potential enzymes to participate in this vast international market, also assist the development of new methodologies (Pedrolli et al., 2009). Furthermore, the prominent expression of this protein in specific conditions of growth, as continuous culture at low growth rates in medium containing lactose, is an interesting feature to construct cloning vectors using the endoPG gene promoter. The *K. marxianus* UFV-3 strain secretes abundant protein into extracellular medium, which can be a base for a construction of an expression system. In addition, the limited number of proteins detected in all culture conditions facilitates down-stream purification of the produced recombinant protein. The genome of *K. marxianus* UFV-3 was recently sequenced, obtaining 10.7 Mpb, that will assist in future strategies to construct the cloning vectors since the promoter region of the gene of the endoPG can be identified in the genome. Furthermore, the genome sequence of *K. marxianus* UFV-3 will give support to *in silico* secretome analysis, because they will complement the information obtained in this work.

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CONCLUSÕES GERAIS

No processo de otimização da produção de etanol por *Kluyveromyces marxianus* UFV-3 a partir de permeado de soro de queijo, a temperatura foi o fator mais significativo seguido por pH, concentração de biomassa e de lactose. A faixa de valores para a obtenção de rendimentos de etanol superiores a 90% é: temperatura entre 33,3-38,5 ºC, pH entre 4,7-5,7, concentração de lactose entre 50-108 g L^{-1} e concentração celular seca entre 1,2-1,7 g L^{-1}. O modelo quadrático foi validado podendo assim ser utilizado na comparação com outros micro-organismos com equações também validadas. Os dados obtidos também podem ser utilizados em processos de escalonamento da produção de etanol e viabilidade econômica por *K. marxianus* UFV-3. Com a equação da otimização pode-se analisar rendimentos de etanol utilizando matérias primas com valores diversos de pH e concentração de lactose.

Além da capacidade fermentativa *K. marxianus* UFV-3 possui velocidades de crescimento elevadas (tempo de geração menor que 1,5 hora) e alto rendimento de biomassa, características de interesse para a produção de proteínas heterólogas. Em nosso estudo sobre o secretoma de *K. marxianus* UFV-3, esta levedura apresentou um número reduzido de

proteínas secretadas seja em batelada, batelada alimentada ou cultura contínua em meio contendo lactose. No cultivo em cultura contínua com diluição de 0,1 h^{-1} foram identificadas nove proteínas, sendo que destas oito são estruturais. Apenas uma proteína é considerada como classicamente secretada – endopoligalacturonase (endoPG) – que é utilizada na indústria de celulose e papel, alimentícia, têxtil, dentre outras. A atividade enzimática ótima desta enzima foi: temperatura 59,5 ºC e pH 5,1. Trabalhos para a otimização da produção desta enzima devem ser realizados, pois sua produção foi abaixo do observado em outros fungos. Aparentemente endoPG é reprimida por glicose e é secretada em meios contendo lactose, galactose e principalmente glicerol. Deste modo, existe a possibilidade de caracterizar a produção de endoPG e etanol simultaneamente em permeado de soro de queijo uma vez que a produção otimizada de etanol já foi estabelecida neste trabalho. A identificação do promotor do gene que codifica esta enzima pode propiciar a construção de novos vetores de clonagem ou vetores específicos para esta levedura. As características de secreção de endoPG identificadas neste trabalho podem auxiliar no desenvolvimento de futuros projetos de secreção de proteínas heterólogas em *K. marxianus* UFV-3.