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

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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 methodology (RSM) with a central composite rotational design (CCRD) to evaluate the effects of pH (4.5–6.5), temperature (30–45 °C), lactose concentration (50–250 g l^{-1}), and cell biomass concentration (A_{600} 2–4). We performed 29 fermentations under hypoxia in cheese whey permeate and seven 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 and 38.5 °C, pH between 4.7 and 5.7, cell biomass concentration between A_{600} 2.4 and 3.3, and lactose concentration between 50 and 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.

1. 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 (González-Siso, 1996). 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 (OECD-FAO, 2008). 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 (González-Siso, 1996). 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 (Vienne and Stockar, 1985). 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 (González-Siso, 1996). In recent decades, research on ethanol production (e.g. from permeate) has been driven by the growing demand for cleaner, more renewable energy sources (Rana et al., 2013). In addition to biofuel, the ethanol produced from permeate is used in the food, beverage, pharmaceutical, and cosmetic industries, due to its potability (Guimarães et al., 2010).

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 (Lane et al., 2011). 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 (Lane and Morrissey 2010). *K. marxianus* UFV-3, isolated from cheese factories in Southeastern 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 (Silveira et al., 2005). This strain's fermentative behavior is mainly due to its increased expression of key enzymes involved in lactose metabolism (Diniz et al., 2012). 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

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of interest, such as fermentation yield (Uncu and Cekmecelioglu, 2011). The response surface methodology (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 (Myers and Montgomery, 1995). 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).

2. Materials and methods

2.1. Yeast strain and maintenance

The yeast used in this study, *K. marxianus* UFV-3, was isolated from cheese factories 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. *K. marxianus* UFV-3 was kept frozen at −80 °C in medium containing 50% glycerol. The starting inoculum for fermentation was prepared by adding 1% (w/v) of the biomass stored at −80 °C into YNB (Yeast Nitrogen Base) medium (Sigma®, St. Louis, USA) supplemented with 2% lactose and cultured under agitation (200 rpm) at 37 °C for 18–24 h. After this period, the active cells were centrifuged (3000*g* for 5 min), washed three times with distilled water, and then inoculated into the fermentation medium.

2.2. Fermentation media

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 to 250 g l^{-1}. Permeate was sterilized by filtration (0.22 μm pore size) and added to the culture medium. The YNB medium was prepared according to the manufacturer's instructions. Lactose (Sigma®, St. Louis, USA) was separately sterilized when necessary at 121 °C for 20 min. The *C:N* 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 predetermined pH values ranging from 4.5 to 6.5.

2.3. 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 h 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^{-1}) following a 15 min purge after initial cell biomass inoculation. Samples were taken from all of the fermentations every 24 h 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.

2.4. Cell growth and the relationship between absorbance at 600 nm (A_{600}) and dry cell biomass concentration (g l^{-1})

To analyze cell growth, a BECKMAN DU 600 spectrophotometer was used at 600 nm wavelength. One unit of A_{600} was found to be equivalent to 0.507 g l^{-1} of dry cell biomass of *K. marxianus* UFV-3 (Diniz et al., 2012).

2.5. Primary metabolite analysis

Samples taken during the various fermentations were centrifuged at 13,200*g* for 5 min, and the supernatants were collected and frozen at −20 °C. To determine the levels of lactose, ethanol, and glycerol, 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 1047 A detector, using a BIO-RAD Aminex HPX-87 H column (300 × 7.8 mm^2)) with 5 mmol l^{-1} H_2SO_4 eluent, a flow rate of 0.7 ml min^{-1}, and a column temperature of 25 °C.

2.6. Determining fermentation parameters

Since the maintenance coefficient and maintenance yield were fixed at zero, the ethanol production by lactose consumed, designated as response factor (RF) and the fermentative parameters ethanol yield with cell mass concentration ($Y_{E/X}$) and volumetric productivity (Q_p) were determined:

$$RF = [(E_f - E_i)/(L_i - L_f)]/4 \quad (1)$$

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

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

where E_i is the initial ethanol concentration (g l^{-1}), E_f is the final ethanol concentration (g l^{-1}), L_i is the initial lactose concentration (g l^{-1}), L_f is the final lactose 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 0.538 g per 1 g of lactose consumed.

2.7. 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 five 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 (Silveira et al., 2005; Diniz et al., 2012). 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®16.0 software, and the assays were randomized to avoid any experimental or technical bias. The fermentation process was monitored every 24 h. This experimental design allowed for the fitting of a quadratic model to estimate the response factor (RF), Eq. (1), using the factors pH, temperature, lactose concentration,