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ORIGINAL PAPER

Optimization of lipase production by *Staphylococcus warneri* EX17 using the polydimethylsiloxanes artificial oxygen carriers

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Abstract In this research, the combined effects of polydimethylsiloxane (PDMS) and different conditions of oxygen volumetric mass transfer coefficient (k_La) on lipase production by *Staphylococcus warneri* EX17 were studied and optimized in bioreactor cultures. Raw glycerol from biodiesel synthesis was used as the sole carbon source. Full-factorial central composite design and the response surface methodology were employed for the experimental design and analysis of the results. The optimal polydimethylsiloxane concentration and mass coefficient transfer (k_La) were found to be 13.5% (v/v) and 181 h^{-1}, respectively. Under these conditions, the maximal cell production obtained was 10.0 g/l, and the volumetric lipase activities of approximately 490 U/l, after 6 h of cultivation. These results are in close agreement with the model predictions. Results obtained in this work reveal the positive effects of PDMS on oxygen volumetric mass transfer coefficient (k_La) in the *Staphylococcus warneri* EX17 cultivation and lipase production.

Keywords Microbial lipases · *Staphylococcus warneri* EX17 · Oxygen mass transfer coefficient · Response surface methodology · Bioprocess optimization

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Introduction

The major share of the industrial enzyme market is occupied by hydrolytic enzymes such as lipases, amylases, amidases, esterases, and proteases [10]. Lipases (EC 3.1.1.3) represent an important group of biotechnologically valuable enzymes. They are widely distributed in nature and can be obtained from animals, plants, and microorganisms. Microbial lipases are the most interesting due to their potential applications in foods, dairy products, pharmaceuticals, detergents, textiles, in the synthesis of biodiesel, and for new polymeric materials [11]. They not only catalyze the hydrolysis of fats and oils but also mediate the synthesis of long-chain acylglycerols. Microbial lipases show high substrate specificity, enantioselectivity, do not require cofactors, and are stable in organic solvents [12].

The production of microbial lipases is strongly affected by several aspects of the culture, especially the pH, temperature, medium composition, aeration, and agitation [2]. In particular, the mass transfer coefficient, k_La, has been shown to be one of the most important parameters affecting the production of this enzyme [6]. In aerobic submerged cultivations, the supply of oxygen from the gas phase must be carefully controlled in order to keep the microorganism aerobic metabolism for growth and product formation. However, the mass transfer rate of oxygen from the gas phase to a liquid medium is often a rate-limiting factor in the oxidative processes [7]. Some of the new methods formulated to improve oxygen supply in aerobic microbial culture include the manipulation of microbial metabolism through genetic engineering and the use of oxygen carriers such as hydrocarbons, hemoglobin, and perfluorocarbons (PFCs) [5]. PFCs have been shown to efficiently improve oxygen supply in several biological systems and, in microbial cultures, they have been used to improve the

production of some enzymes, including lipases [6]. However, the extremely high prices, and because the PFCs are difficult to use, have hindered their use in industrial-scale bioprocesses [4].

Polydimethylsiloxanes (PDMS) are copolymers of ethylene or propylene oxide that were designed by Leonhard et al. [14] for their versatility and broad range of use. PDMS are cheap, chemically stable, biologically safe, easy to handle, and extensively applied to supply oxygen into enzymatic reactions [21]. It is also used as a detergent in lipase kinetics studies [4, 22]. Notwithstanding these remarkable properties and the fact that oxygen solubility in PDMS copolymers is about 45–50 fold higher than in water, these chemicals have barely been tried for microbial cultivations.

Statistical approaches involving response surface methodology (RSM) have been widely used for the optimization of microbial culture conditions and to aid in the understanding of the interactions among various parameters while using a minimal number of experiments [13]. Factorial design of a limited set of variables is advantageous in relation to the conventional method where a single parameter is individually tested, which frequently fails to define optimal conditions for the process since the effects of interactions among factors are lost [9].

In a previous work, we have reported the isolation of a new strain of *Staphylococcus warneri* (EX 17) for lipase production and presented some data on the production of this enzyme concerning medium composition, optimal pH, and temperature [19]. In the present work, we investigated the effects of the oxygen supply in cultures of *S. warneri*, especially concerning the maximization of lipase production, using the CCD approach. First, we defined the influence of agitation speed, aeration, and PDMS concentrations on oxygen volumetric mass transfer coefficients (k_La) in bioreactors. Then, the combined effects of varying k_La and PDMS concentration on cell growth and enzyme production were investigated. Raw glycerol from biodiesel synthesis was used as the sole carbon source.

Materials and methods

Microorganism, cultures maintenance, and inoculum preparation

A strain of *Staphylococcus* isolated by our group from an abattoir fat-rich wastewater was used in this research. This strain was identified by 16S rDNA gene sequencing and identified as *Staphylococcus warneri* strain EX17 [19]. Stocks of this bacterium are kept at Microbiology Culture Collection of BiotecLab (UFRGS, Brazil). Working stocks of cultures were maintained frozen at −18°C in 20% glycerol suspensions, while for immediate use, cells were kept at 4°C on tributyrin agar plate containing (in g/l): peptone, 5; yeast extract, 3; tributyrin, 10. Pre-inocula for cultivations were carried out in 500-ml Erlenmeyer flasks filled with 200 ml of LB medium inoculated with a loopful from the plate cultures and incubated at 37°C, 180 rpm, until the cultures reached an optical density (OD, Abs at 600 nm) of 1.0 and were then transferred to the bioreactors.

Polydimethylsiloxane

For the purposes of testing improved oxygen transfer mechanisms in the cultures, the chemical polydimethylsiloxane (PDMS; 200 Fluid, Food Grade, 350 cSt., Dow Corning, USA) was chosen. This is a pure silicone fluid that was designed for use in food processing or in other applications where a food-grade product is desired. PDMS is non-polar and chemically inert with high solubility for gases such as oxygen or carbon dioxide.

Bioreactor operation and conditions

Batch cultivations were carried in a 2-l working volume stirred bioreactor (Biostat B model, B. Braun Biotech International, Germany) filled with 2 l of medium. The bioreactor was equipped with temperature, agitation, aeration, and pH controllers and two Rushton turbines with six flat-blades. The culture medium used was optimized in a previous study [20], and contained (in g/l): peptone, 10; yeast extract, 5; raw glycerol from biodiesel synthesis, 30; olive oil, 6; soybean oil, 5. The raw glycerol was used as the sole carbon source for cell growth and its composition was (in a weight basis): 82.8% glycerol, 5.5% ashes, 5.9% NaCl, 11.2% moisture, 0.5% monoacylglycerols, and pH 4.84. The concentration of polydimethylsiloxane was optimized and varied according to the experimental design (Table 2). The medium was sterilized by autoclaving at 121°C for 20 min. The optimized temperature and pH were 37°C and 7.0, respectively [20]. The tested values of the oxygen volumetric mass transfer coefficient, k_La, were determined by the dynamic gassing-out method [15]. The agitation and aeration rates were set at different values, characterizing five different oxygen transfer conditions. Samples were taken along the cultivation in order to quantify lipolytic and proteolytic activities, biomass, and raw glycerol concentration.

Experimental design and optimization of oxygen volumetric mass transfer coefficient

A 2^3 full-factorial composite design with five coded levels leading to 18 sets of experiments was carried in order to optimize the k_La as a function of aeration, agitation, and

PDMS concentration. For statistical calculation, the variables were coded according to Eq. (1):

$$x_i = (X_i - X_0)/\Delta X_i \quad (1)$$

where x_i is the independent variable coded value; X_i is the independent variable real value; X_0 is the independent variable real value on the central point; and ΔX_i is the step change value.

The range and the levels of the variables investigated in this research are given in Table 1. The k_La was the dependent variable or response of the design experiments.

The quadratic model for predicting the optimal point was expressed according to Eq. (2):

$$Y_c = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ij} x_i x_j + \Sigma\beta_{ii} x_i^2 \quad (2)$$

where Y_c is the response variable, β_0 the constant, β_i the coefficient for the linear effect, β_{ii} the coefficient for the quadratic effect, β_{ij} the coefficient for the interaction effect, x_i and x_j are the coded level of variable X_i and X_j. The agitation and aeration rates were set at different values, characterizing nine different oxygen transfer conditions used in planning experiments for the optimization of lipase.

Experimental design and optimization of lipase production

A 2^2 full-factorial composite design with five coded levels leading to 12 sets of experiments was carried in order to optimize the lipase production as a function of k_La and PDMS concentration. For statistical calculation, the variables were coded according to Eq. (1). The range and the levels of the variables investigated in this research are given in Table 2. The lipase production was the dependent variable or response of the design experiments.

The quadratic model for predicting the optimal point was expressed according to Eq. (2).

For both experimental designs, Statistica 7.0 (StatSoft, Tulsa, USA) was used for regression and graphical analysis of the data. The significance of the regression coefficients was determined by Student's t test, and the second-order model equation was determined by Fisher's exact test. The variance explained by the model is given by the multiple coefficient of determination, R^2.

Table 1 Process variables and their levels used in the CCD for the k_La study

Trial no.	Coded variables			Uncoded variables			k_La (h^{-1})
	X_1	X_2	X_3	X_1	X_2	X_3	
1	−1	−1	−1	3	1.9	480	57
2	−1	−1	+1	3	1.9	720	115
3	−1	+1	−1	3	4.1	480	58
4	−1	+1	+1	3	4.1	720	120
5	+1	−1	−1	12	1.9	480	58
6	+1	−1	+1	12	1.9	720	130
7	+1	+1	−1	12	4.1	480	69
8	+1	+1	+1	12	4.1	720	133
9	−1.68	0	0	0	3	600	47
10	+1.68	0	0	15	3	600	120
11	0	−1.68	0	7.5	1	600	109
12	0	+1.68	0	7.5	5	600	112
13	0	0	−1.68	7.5	3	400	57
14	0	0	+1.68	7.5	3	800	180
15	0	0	0	7.5	3	600	85
15	0	0	0	7.5	3	600	86
15	0	0	0	7.5	3	600	87

X_1 PDMS concentration (%), X_2 aeration (vvm), X_3 agitation speed (rpm)

Analytical methods

The cell-free supernatant of the culture medium, referred to as the crude enzyme extract, was used for the estimation of enzyme activities and for raw glycerol concentration determinations.

Lipolytic activity was determined using *p*-nitrophenyl palmitate (pNPP) as the substrate as described by Volpato et al. [19]. A volume of 0.15 ml of crude enzyme extract was mixed with 1.35 ml of the prepared substrate solution and incubated at 37°C for 15 min in a water bath, followed by immersion in an ice bath in order to stop the reaction. A control containing heat-inactivated enzyme was also incubated for each assay. The mixture was centrifuged (14,000 × *g*, 10 min, 4°C) and the reaction was measured as the absorbance at 410 nm. One unit of enzyme was defined as the amount of enzyme that liberated 1 µmol *p*-nitrophenol per min at 37°C. Under these conditions, the

Table 2 Process variables and their levels used in the CCD for the lipase activity study

Trial no.	Coded variables		Uncoded variables		Lipase activity (U/l)
	X_1	X_2	X_1	X_2	
1	−1	−1	2.2	102	232
2	−1	+1	2.2	170	300
3	+1	−1	12.8	102	332
4	+1	+1	12.8	170	536
5	−1.41	0	0	136	108
6	+1.41	0	15	136	428
7	0	−1.41	7.5	89	280
8	0	+1.41	7.5	184	342
9	0	0	7.5	136	260
9	0	0	7.5	136	289
9	0	0	7.5	136	290

X_1 PDMS concentration (%), X_2 k_La (h^{-1})

extinction coefficient of *p*-nitrophenol is $13.23 \times 10^3\ M^{-1}$. Proteolytic activity was measured by the azocasein method according to Sarath et al. [18]. One unit of enzymatic activity was defined as the amount of enzyme needed to produce a change in one unit of absorbance under the conditions of the method.

Biomass was quantified gravimetrically as cell dry weight. Samples were centrifuged, twice washed with cold hydroalcoholic solution (20%), and dried at 80°C to a constant weight. Raw glycerol concentration was determined by HPLC with a refractive index (RI) detector (PerkinElmer Series 200, USA) and a Phenomenex RHM monosaccharide column (300 × 7.8 mm), at 80°C, using ultrapure water as the eluent, a flow of 0.6 ml/min, and a sample volume of 20 µl.

Results and discussion

Optimization of k_La

The use of k_La is important since it allows for comparisons among different bioreactor systems, being the best way to correlate agitation/aeration of different equipments and conditions. It is also the method of choice for scaling-up aerated bioprocesses. Therefore, experiments were performed to analyze the combined influences of agitation speed, aeration rate, and polydimethylsiloxane (PDMS) on k_La of Rusthon turbine-equipped bioreactors. Table 1 shows both coded and real values of the variables at different levels.

The high significances of linear effects of agitation speed and concentration of PDMS indicate that these variables can act as limiting factors and even small variations in their values will alter the k_La. The results also showed that the variations of the aeration rate were not statistically significant for k_La. The second-order polynomial model for k_La can be reduced as expressed in Eq. (3):

$$Y_1 = 86.82 + 23.83X_1 + 67.78X_3 \quad (3)$$

where the variables take their coded values, representing k_La (Y_1) as a function of concentration of PDMS (X_1) and agitation speed (X_3).

ANOVA showed that the model was highly significant ($p < 0.0001$), with $R^2 = 0.9261$, meaning that 92% of the total variation is explained by the model. This suggests a satisfactory representation of the process model and a good correlation between the experimental and predicted values.

The entire relationship between reaction variables and response can be better understood by the contour shapes depicted in Fig. 1, plotted on the basis of the model equation and showing the interaction among the concentration of PDMS and agitation speed for k_La values.

The use of oxygen carriers for improving k_La in microbial cultures has been attempted before for PFCs but not for PDMS. In our study, the use of PDMS produced a 3.8-fold increase of the k_La coefficient. In comparison, Rols et al. [17] demonstrated that the use of the hydrocarbon *n*-dodecane and the perfluorocarbon Forane F66E in a culture of *Aerobacter aerogenes* resulted in a 3.5-fold increase of k_La. Cho and Wang [3] showed a two to five-fold increase in k_La when a spraying of air-saturated perfluorocarbon was added to a bioreactor designed for cultivation of hybridoma cells. Rols and Gona [16] demonstrated that the use of 19% of soybean oil enabled a 1.48-fold increase of the k_La coefficient.

Optimization of lipase production as a function of k_La and PDMS concentration

To determine the operational variables to define k_La, a second CCD was carried out in order to study the effects of the combined effects of k_La and PDMS concentration over lipase production by *S. warneri* EX17. The range of values for k_La and PDMS concentration for this second CCD were chosen to match values normally found in the literature for PFCs. The results are shown in Table 2. The high significance of the linear effects of k_La and PDMS concentration indicates that these variables can act as a limiting factor and even small variations in their values will alter the lipase production. The regression is statistically significant ($p < 0.001$) at 95% of confidence level, and the model has a high determination coefficient ($R^2 = 0.88$) explaining 88% of the variability in the response.

The model expressed by Eq. (4), in its reduced form where the variables take their coded values, represents the lipase activity (Y_2) as a function of PDMS concentration (X_1) and k_La (X_2):

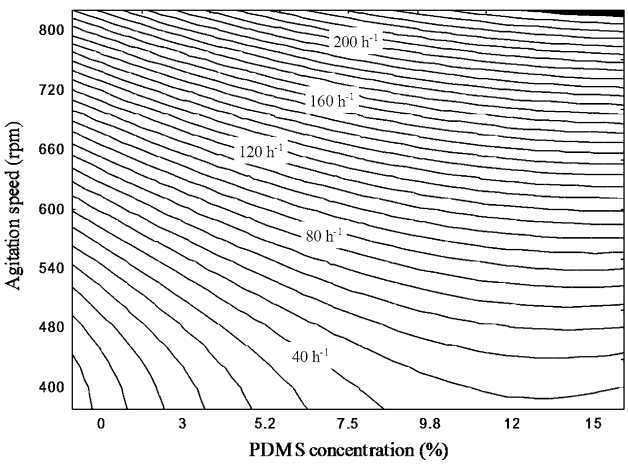

Fig. 1 Contour plots of the k_La determination. The *numbers* inside the contour lines indicate the values of k_La (in h^{-1})

$$Y_2 = 279.66 + 197X_1 + 89.62X_2 \quad (4)$$

The contour plot described by the model equation (Y_2) is represented in Fig. 2. The maximal enzymatic activity (536 U/l) was obtained for the condition 4 (12.8% PDMS and k_La of 170 h^{-1}), while the optimized model shows 13.5% PDMS and k_La of 181 h^{-1} as the optima values for these variables. The lowest enzymatic activity (108 U/l) was obtained for the condition five (0% PDMS and k_La of 136 h^{-1}). Therefore, our results clearly show that the use of PDMS increases lipase activity by a factor of five times.

Validation of the model was performed using the optimized values for k_La and PDMS concentration and results for the cultivation kinetics are shown in Fig. 3. A maximal cell production of 10.0 g/l, and lipase activity of approximately 490 U/l were obtained after 6 h of cultivation. These results are in close agreement with the model predictions. In order to check for proteolysis of lipases, the batches were evaluated for the proteolytic activity in the medium, which was neglectable (results not shown).

Surprisingly, the culture time for the highest lipase activity was reduced by half in the presence of PDMS. In a previous work, we found that the lipase activity of *S. warneri* EX17 always peaked after 12 h of cultivation under optimized conditions, similar to those used in this work, but without PDMS [20]. This is an extremely important result since anticipating the coupled time production of the enzyme reduces costs in the bioprocesses. This result may be related to the aerobic metabolism of the microorganism. Elibol and Mavituna [8] attributed the increase in the antibiotic production of *S. coelicolor* cultures to the enhancement of oxygen transfer due the presence of 50% of PFC in the fermentation medium.

Although a few works have reported on the effects of PDMS addition to microbial cultures for other reactions,

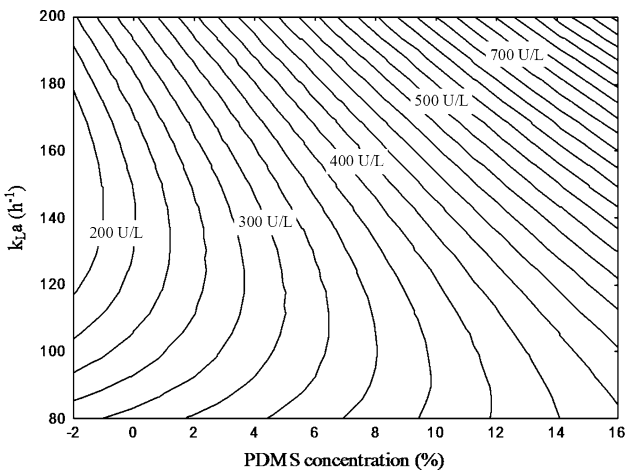

Fig. 2 Contour plot described by the model equation (Y_2) representing the lipolytic activity (U/l) as a function of PDMS concentration and k_La

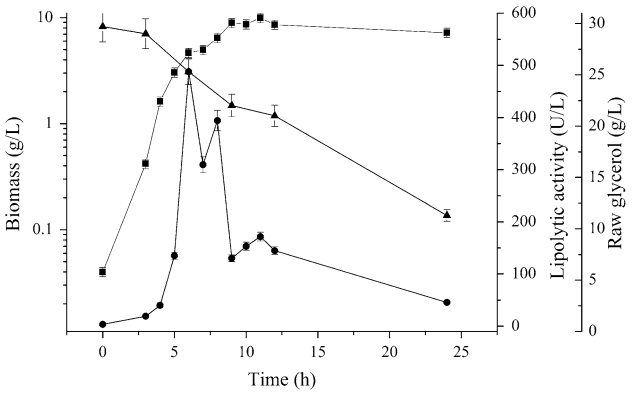

Fig. 3 The time course of lipase production by *S. caseolyticus* EX17 cultivated under optimized culture conditions. Biomass (*filled square*), lipolytic activity (*filled circle*), raw glycerol concentration (*filled triangle*). Experiments were run in duplicate

this research is the first to report on the use of PDMS in an experimental design to enhance lipase activity. Leonhardt et al. [14], PDMS to provide oxygen during α-keto-acid production by immobilized bacteria and found an increase of L-amino acid oxidase activity by a factor of four in the presence of silicon emulsions. Zimoeck et al. [22] studied the biological bleaching of hardwood kraft pulp with *T. versicolor* and observed a three-fold increase in the oxygen uptake rate in cultures of this fungus when PDMS was added to the medium.

Since the synthesis of this enzyme is clearly influenced by the oxygen available in cultures, some authors attempted to demonstrate the positive effects of adding chemical oxygen carriers for this purpose. Elibol and Ozer [6] investigated the addition of 10% (v/v) of the perfluorocarbon perfluorodecalin to culture medium for the production of lipase by the fungus *Rhizopus arrhizus*. The authors showed an increase in lipase production by a factor of 8.5 times. Amaral et al. [1] investigated the effect of PFC on the lipase production by *Yarrowia lipolytica*, reporting an increase of up to 23 times in the enzyme activity when 20% (v/v) of PFC was used in the culture medium compared to the control without PFC. In all of these works, PFCs have been shown to increase the enzyme activities, without reducing the production time.

The low costs of PMDS associated with the reduced time for production of the enzyme makes these compounds attractive for applications in fermentation processes.

In this study, all experiments were carried out using raw glycerol from biodiesel synthesis as the sole carbon source. Although this was not the central objective of the work presented here, this fact is important because our results show that this cheap and abundant substrate can be successfully used for lipase production. In preliminary experiments in shaker, production of lipase in raw glycerol did not differ significantly from cultivation with pure

glycerol (results not shown). In a previous work, we used raw glycerol to produce lipase by *S. caseolyticus* EX17 and found that there was no difference in enzyme production and cell growth compared to pure glycerol [20].

Conclusions

Oxygen supply to microbial cultures is a critical operation to ensure productivity in aerobic cultures. In the present study, we demonstrated that PDMS is an effective oxygen carrier, increasing fivefold the oxygen volumetric mass transfer coefficients in submerged bioreactors, under the conditions of this work. This is the first report on the literature on the effects of PMDS as an oxygen carrier capable of influencing k_La. PMDS is non-toxic and cheap, in contrast with perfluorocarbons, so far the most studied liquid oxygen carriers. The application of statistical tools of CCD in order to study the combined effects of PMDS and k_La on cultures of *S. warneri* EX17 was successful to increase lipase activity and predicting optimal amounts of PMDS and k_La values that could be used in scaling-up this bioprocess. Furthermore, the utilization of low-cost substrates such as residual glycerol from biodiesel synthesis can reduce the overall costs for lipase production. As shown in this research, with the combined increase of productivity and cost reduction, the industrial production of lipase by *Staphylococcus warneri* EX 17 can hold the prospect of being economically attractive.

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