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Utilization of dairy effluent as alternative fermentation medium for microbial lipase production

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Abstract

Annually, dairy industries produce a lot of crude materials as waste that are difficult to treat and valorize. Using dairy effluent as fermentation medium, this study aiming to optimize the lipase production by Trichoderma atroviride 676. The experimental mixture design (EMD) was adopted to optimize the enzyme production by the supplementation of effluent with tween 80, olive oil and Vogel's medium. According to the results, the optimum medium containing 5 ml/l of olive oil, 5.62 ml/l of Tween 80, 15 ml/l of Vogel's medium led to a maximum lipase production of 1327.28 U/ml, which was 123-fold higher that the dairy effluent without supplementation. These data clearly demonstrates the possibility of using dairy effluent as a potential fermentation medium for production of lipases. The search for alternatives substrates for lipase production can contribute in getting lipases with a reduction of the production cost in industrial scale, and help to reduce the massive environmental impact caused by dairy industries.

Keywords: dairy effluent, lipase, *Trichoderma atroviride 676*, experimental mixture design, optimization.

Introduction

The dairy industry is generally considered the biggest source of food processing wastewater and producers of large amount of effluent (0.2 to 10 liters of effluent per liter of processed milk) [1]. In general, this residue contains high concentrations of organic compounds (lipids, proteins and carbohydrates), suspended solids, oil-grease and elevated levels of BOD (biochemical oxygen demand) and COD (chemical oxygen demand), requiring specialized treatments to prevent or minimize environmental problems [2]. Searching for the rational use of industrial wastes, several studies have been focused on their utilization for synthesis of high value metabolites, such as organic acids, biosurfactants and lipases [3-5].

Lipases (EC 3.1.1.3) constitute one of the most important groups of industrial enzymes that have been extensively used in different biotechnology process such as in wastewater

treatments [6], ester synthesis [7], detergents [8], synthesis of food additives [9], and in biodiesel production [10]. Particularly, lipases from microorganisms, mainly bacterial and fungal, are more appropriate for industrial applications due to their stability, selectivity, and broad substrate specificity [11].

Trichoderma sp. is a well-known enzyme producing fungi. Several authors have been reported this genus as a producer of multiple enzymes, including cellulase, xylanase, and β-glucosidase [12-15]. However, the lipase production by this genus has been little explored. In this study, we investigated the lipase production by *Trichoderma atroviride 676* using a fermentation medium based on dairy effluent. The optimization of lipase production was carried out by statistical approach using the experimental mixture design (EMD). This tool has been used by various researchers for optimization of culture conditions [16-19] and present great advantages compared to conventional methods that fail to consider the interaction of different factors involved on the enzyme production. Our results showed that the dairy effluent constitute a useful medium for lipase production by *T. atroviride 676*, contributing to reduce the fermentation cost and helping to minimize environmental problems caused by dairy industry. To our knowledge, this is the first report on the optimization of lipase production from *T. atroviride 676* using dairy effluent based medium.

Materials and methods

Characterization of dairy effluent

A dairy effluent was collected from homogenization tank of a dairy industry of Londrina, Paraná, Brazil, and immediately stored at 4ºC. Total lipids, proteins and carbohydrates were quantified according to previously described by Frings and Dunn (1970) [20], Hartree (1972) [21], and Dubois et al. (1956) [22], respectively. The COD was analyzed as defined in Standard Methods [23].

Microorganism and inoculum preparation

T. atroviride 676 (IOC 4503) was originally isolated from Amazon forest soil and are available at Coleção de Culturas de Fungos Filamentosos, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. The strain was grown in a potato dextrose agar medium (PDA), in Erlenmeyer flasks (125 ml), at 28 ºC for 4 days, using an inoculum of 10^6 spores/ml. The strain was subculture every 15 days, and preserved at 4 ºC.

Fermentation medium preparation

Lipase production medium was prepared using dairy effluent as a basal medium, containing different concentrations of glycerol, olive oil, tween 80 and Vogel's medium, according to statistical design of experiments. The pH was adjusted to 6.0 and then sterilized at 121°C, for 15 minutes. One percent (1 % v/v) of the prepared inoculum was added to each 25 ml of medium in Erlenmeyer flasks (125 ml), according to experimental design. The flasks were incubated for 5 days at 28 °C, under orbital shaker at 105 rpm. The cell-free supernatant was used as a source of extracellular lipase.

Determination of lipase activity

The lipase activity was assayed using *p*-nitrophenyl-palmitate (*p*NPP) as substrate, according to Winkler e Stuckmann (1979) [24]. The cell-free supernatant was incubated with *p*NPP (8.0 mM dissolved in isopropanol), 50mM Tris-HCl buffer (pH 8.5) containing Triton X-100 (0.4 % w/v), at 37 °C, for 10 minutes. The release of *p*NPP was measured at 410nm.

One unit of enzyme activity (U/ml) was defined as the amount of enzyme required to release 1µmol/min de *p*-NPP.

Optimization of lipase production by statistical approach

To optimize the conditions for the lipase production in the dairy effluent based medium, a simplex-centroid EMD consisting of 17 runs with 4 components, olive oil (X_1), Tween 80 (X_2), glycerol (X_3) and Vogel's medium (X_4), was employed. The lipase activity was taken as the response of the design (U/ml) (Table 1). In the EMD the proportions can not be negative and they are expressed as fractions of the mixtures. In addition, their sum must be equal to unity [25]. The volumes of the 4 components were added as shown in Table 1 and the final volume was completed to 25 ml with the effluent.

In the simplex-centroid EMD response data are collected and the polynomial model is composed of the same number of parameters to be estimated, as demonstrated by the canonical equation [25]:

$$Y = \sum_{i=1}^{q} \beta_i x_i + \sum_{i}^{q} \sum_{<1}^{q} \beta_{ij} x_i x_j \sum_{i}^{q} \sum_{<1}^{q} \sum_{k}^{q} \beta_{ijk} x_i x_j x_k + ... + \beta_{12...q} x_1 x_2 ... x_q \text{ (Eq.1)}$$

where Y is the response variable (lipase activity), β_i is the coefficient for the linear effect, β_{ij} is the coefficient for the binary interaction effect, β_{ijk} is the coefficient for the ternary interaction effect, and x is the encoded level of the variable. Results were assessed by an analysis of variance (ANOVA) and multiple regression analysis at the 5 % significance level using Statistica 9.0 software.

Results and discussion

Nowadays, the use of statistical models to optimize culture medium conditions has increased due its propensity and relevance. In this study, the EMD was employed to evaluate the lipase production by *T. atroviride 676,* using the dairy effluent as fermentation medium. Initially, the physicochemical characteristics of the dairy effluent were studied. The pH and the COD of collected samples are found to be 9.0 and 1,376.00 mg/l, respectively. Among the organic compounds quantified, total proteins (189.49 mg/l) was found to be higher than total lipids (70.0 mg/l) and carbohydrates (55.83 mg/l). These results are in agreement with the literature that reports a dairy effluent as a source of proteins, carbohydrates and lipids, containing a high concentration of COD [2].

The next step was to evaluate the lipase production by *T. atroviride 676* using the dairy effluent without any supplements. The results showed the maximum catalytic activity of 10.74 U/ml, showing that this residue could be used as a substrate for lipase production. However, the supplementation of the dairy effluent may favor the lipase production. The EMD design was then adopted to optimize the lipase production using dairy effluent enriched with the different concentrations of glycerol, tween 80, olive oil and Vogel's medium. The results showed that lipase activity ranged from 16.64 U/ml (run 1: 20 ml/l olive oil) to 1313.58 U/ml (run 12: 6.6 ml/l olive oil, 4.99 ml/l Tween 80 and 13.2 ml/l Vogel's medium). The large variation in enzymatic activity indicates that the combinations of the tested components strongly affected the enzyme production (Table 01).

Table 1- Experimental mixture design of three variables for evaluation of the lipase production in the dairy effluent by *T. atroviride* 676

Runs	Mixtures	Components[*]				
		Olive oil (ml/l) X_1	Tween 80 (ml/l) X_2	Glycerol (ml/l) X_3	Vogel's medium (ml/l) X_4	Lipase activity (U/ml/l) Y_1
1	(1;0;0;0)	20	0	0	0	16.64
2	(0;1;0;0)	0	15	0	0	50.12
3	(0;0;1;0)	0	0	10	0	17.20
4	(0;0;0;1)	0	0	0	40	140.74
5	(0.5;0.5;0;0)	10	7.5	0	0	164.44
6	(0.5;0;0.5;0)	10	0	5	0	33.09
7	(0.5;0;0;0.5)	10	0	0	20	124.61
8	(0;0.5;0.5;0)	0	7.5	5	0	10.65
9	(0;0.5;0;0.5)	0	7,5	0	20	770.04
10	(0;0;0.5;0.5)	0	0	5	20	50.62
11	(0.33;0.33;0.33;0)	6.6	4.99	3.3	0	274.20
12	(0.33;0.33;0;0.33)	6.6	4.99	0	13.2	1,313.58
13	(0;0;0.33;0.33)	6.6	0	3.3	13.2	90.25
14	(0;0.33;0.33;0.33)	0	4.99	3.3	13.2	658.11
15	(0.25;0.25;0.25;0.25)	5	3.75	2.5	10	848.07
16	(0.25;0.25;0.25;0.25)	5	3.75	2.5	10	851.36
17	(0.25;0.25;0.25;0.25)	5	3.75	2.5	10	835.56

*The components were selected based on the basis of literature reports of lipase production

The regression analysis was carried out to fit the response function (lipase production) with the experimental data (Table 2). The response revealed that tween 80 ($p = 0.005$) and Vogel's medium ($p = 0.0002$) had positive effect on lipase production. On the contrary, olive oil ($p = 0.092$) and glycerol ($p = 0.086$) were not. This agrees with findings of Sifour and co-workers (2010) [26], where olive oil did not considerably influence the enzyme production by *Geobacillus stearothermophilus*. However, other authors observed that olive oil significantly stimulated the lipase production by *Rhizopus arrhizus* [27] and *Stenotrophomonas maltophilia* [28]. Contrarily to describe on the literature [29-30], glycerol also did not increase the lipase production by *T. atroviride 676*.

Table 02- Regression analysis of the effects of variables on lipase production by *T. atroviride* 676

Components	Coefficient	Error	t-value	p-value
Olive oil (X_1)	16.64	6.81	2.44	0.092
Tween 80 (X_2)	50.12	6.81	7.36	0.005
Glycerol (X_3)	17.20	6.81	2.53	0.086
Vogel's medium (X_4)	140.74	6.81	20.68	0.0002
X_1X_2	524.19	33.15	15.81	0.0006
X_1X_3	64.63	33.15	1.95	0.146
X_1X_4	183.63	33.15	5.54	0.012
X_2X_3	− 92.09	33.15	-2.78	0.069
X_2X_4	2,698.39	33.15	81.39	0.000004
X_3X_4	− 113.45	33.15	-3.42	0.042
$X_1X_2X_3$	5,158.64	211.18	24.43	0.0001
$X_1X_2X_4$	23,381.60	211.18	110.72	0.000002
$X_1X_3X_4$	462.17	211.18	2.19	0.116
$X_2X_3X_4$	8,418.95	211.18	39.87	0.00003

R^2=0.99; lack of fit: p= 0.99

It is well documented that some compounds, such as surfactants, can increase cell permeability, facilitating the export of several molecules across the cell membrane, increasing protein secretion or facilitating contacts between the enzyme and the substrate. In this study, Tween 80 (15 ml/l) showed a positive effect on the lipase production by *T. atroviride 676*, leading to a 4.76 fold increase in the production of lipase. Studying the effect of different surfactants on the lipase production by *Metarhizium anisopliae*, Silva and co-workers (2005) [31] showed that higher catalytic activities were obtained in cultures containing sodium dodecyl sulfate (4.54 U/ml) and Tween 80 (4.15 U/ml). The positive effect caused by Tween 80 was also related to the lipase production by *Botryosphaeria ribis* [32] and *Bacillus pumilus* ([33].

In this study, Vogel's medium, a basal culture medium used primarily for fungal growth, also showed a positive impact on the lipase production by *T. atroviride 676*. The supplementation of dairy effluent with Vogel's medium strongly enhanced the lipase production by *T. atroviride 676*, presenting catalytic activity of 1313.58 U/ml (run 12), 845.00 U/ml (central point average), 770.04 U/ml (run 9) and 658.11 U/ml (run 14).

The experimental data were used to construct a canonical equation that describes the lipase production, using a special cubic model. The ANOVA indicated that the linear (p = 0.60) and quadratic (p = 0.12) models were not significant, whereas the special cubic model was significant (p = 0.00005). Based on the experimental results, a canonical equation was developed (Eq.2):

Y_1= 16.64 x_1 + 50.12 x_2 + 17.20 x_3 + 140.74 x_4 + 524.19 x_1x_2 + 64.63 x_1x_3 +183.63 x_1x_4 − 92.09 x_2x_3 + 2698.39 x_2x_4 − 113.45 x_3x_4 + 5158.64 $x_1x_2x_3$ + 23381.60 $x_1x_2x_4$ + 462.17 $x_1x_3x_4$ + 8418.95 x_2x_3x (Eq.2)

where Y_1 is the response (lipase activity), x_1, x_2, x_3 and x_4 are the codified variables: olive oil, Tween 80, glycerol and Vogel's medium, respectively.

For this model, the lack of fit was not considerable (p = 0.99) and the determination coefficient (R^2) was 0.99, implying that 99% of the variance of the experimental data can be explained. Thus, the obtained equation can be used for predictive purposes. The ternary

interactions, especially between the variables X_1, X_2 and X_4 (p = 0.000002) demonstrated a strong effect which is confirmed by the region on the response surface plot (Figure 1).
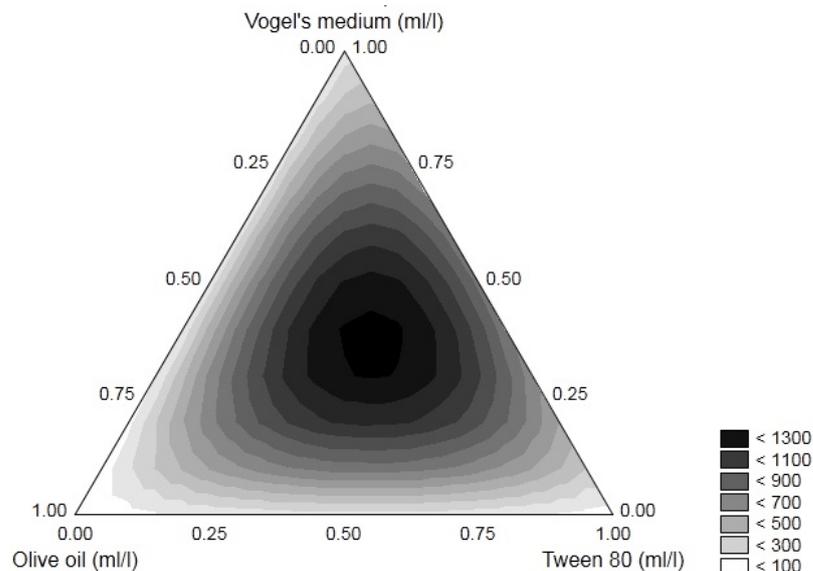

Figure 1- The response surface plot showing the effect of olive oil, Tween 80 and Vogel´s medium on the lipase production by *T. atroviride 676*.

The predictive model specified that the theoretical maximum lipase activity of 1343.60 U/ml can be achieved under the following conditions: 5 ml/l olive oil; 5.62 ml/l Tween 80; 15 ml/l Vogel's medium and no glycerol (Figure 2).
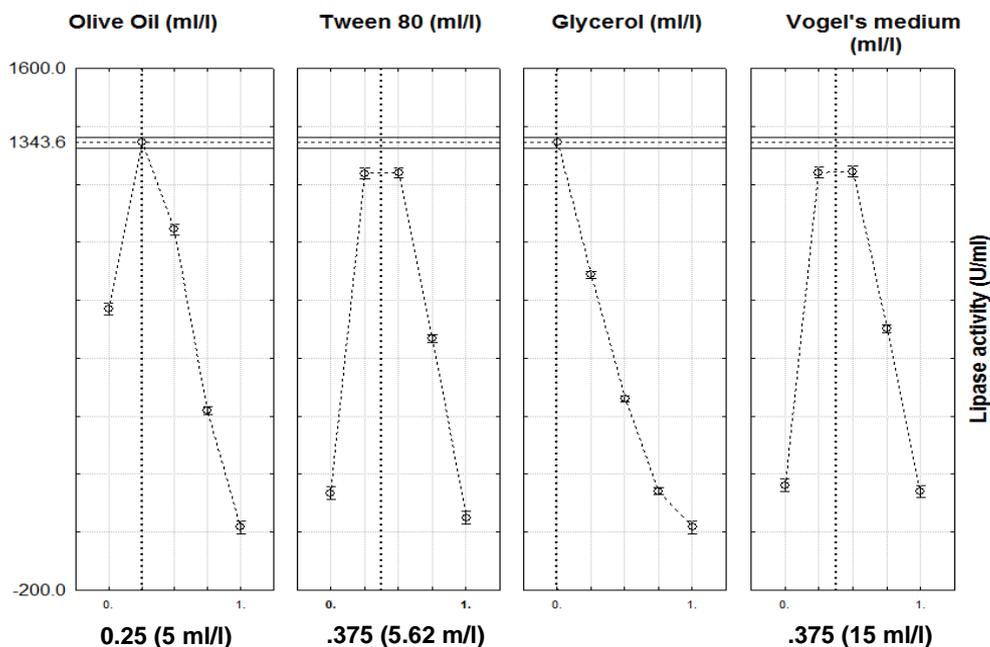

Figure 2- Optimum conditions for lipase production by *T. atroviride 676*.

Although the olive oil did not considerably influence the enzyme production by *T. atroviride 676* the interaction of Tween 80, Vogel's medium and olive oil significantly increased the lipase production. The model validation was performed under these optimal conditions and test t single sample (5 % significance level) was applied. The average lipase activity obtained (1327.28 U/ml) was close to the predicted optimal value.

Conclusions

In summary, the data showed here clearly demonstrates the possibility of using dairy effluent as a fermentation medium for production of lipases. The optimization of lipase production by *T. atroviride 676* results in an increase of 123 times when compared to effluent without supplementation. Therefore, the utilization of low-cost and accessible substrates like dairy residue as an alternative to commonly used and expensive medium would result in a considerable reduction of production costs. In addition, the rational use of industrial residues could help to reduce the massive environmental impact caused by dairy industries.

Acknowledgments

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