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SHORT COMMUNICATION

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IMPROVEMENT OF LIPASE PRODUCTION FROM *Geotrichum* sp. IN SHAKEN FLASKS

This work is focused on the study of different variables on inoculum build-up aiming to improve the lipase production by Geotrichum sp. by means a sequential strategy of experimental design. The effects of inoculum size, corn steep liquor concentration, volume of inoculum, pH of medium, age of inoculum and soybean oil concentration on lipase activity were assessed by means of two factorial experimental designs. A maximum lipase activity of 35.20±0.8 U/mL was obtained with a inoculum composed of one circular area of 0.78 cm^2 containing spores, 50 mL of inoculum volume medium, 12 hours of inoculum age, 15% w/v of corn steep liquor concentration, 1.0% w/v of soybean oil concentration and initial pH 5.0 at 30 °C and 150 rpm in flasks. This work showed that an enhancement of lipase activity can be obtained using a sequential statistical factorial approach to define the variables for inoculum build-up.

Keywords: lipase; corn steep liquor Geotrichum sp.; soybean oil; factorial design.

Lipases (triacyglycerol acylhydrolases, EC 3.1.1.3) are able to bring about the hydrolysis of triacyglycerols into fatty acids and glycerol at the water-lipid interface and its reverse reaction in non aqueous solvents [1]. The microbial production of lipase has been the object of several investigations due to the important implications of this enzyme in direct and reversed biotransformation reactions [2,3]. Frequently, lipases are used in hydrolysis processes at the oil-water interface, but these enzymes have great versatility and can be used in different processes as oil-chemical, food, fuel, medicine, etc.

Lipases are produced by a wide range of microorganisms, including bacteria [4-6], fungi [7], and yeast [8-10]. Lipases produced by different microorganisms have different applications according to their specificity. Lipases from *Geotrichum* sp. and *Geotrichum candidum* have specificity for unsaturated long-chain fatty acids that allows their utilization in hydrolysis process with various vegetable oils like olive oil and soybean oil [3,11]. Griebeler *et al*. [12] selected potential lipase producer microorganisms from soybean bran and dairy products. The authors found a fungus with good action in the hydrolysis of triacylglycerol different chain length and another with long-chain fatty acids.

The influence of culture medium composition on production of enzymes has been reported in literature, mainly focusing on the use of agroindustrial residues as substrates [13]. In previous reports, different substrates like olive oil, Tween 80, glucose, citric acid, triolein, peptone, soybean meal, corn steep liquor, yeast extract, urea, NH_4NO_3, etc. have been frequently used for lipase production by Geotrichum sp. [9,14-16]. Peptone, yeast hydrolyzate, yeast extract

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and $(NH_4)_2SO_4$ have also been employed for lipase production by Geotrichum candidum [17,18].

In recent years, research on the selection of suitable substrates for fermentative processes has mainly been centered on agro-industrial residues because of their potential advantages. In addition, the utilization of these agro-industrial wastes, on one hand, provides alternative substrates and, on the other, helps in solving pollution problems, which otherwise may cause by their disposal. The nature of the substrate employed is the most important factor affecting fermentative processes, and its selection depends upon several factors mainly related with cost and availability and, thus, may involve the screening of several agro-industrial residues [19]. The inoculum is also an important variable when imperfect fungus like *Geotrichum* is used in fermentation process. A good inoculum is essential to improve the production of the metabolite of interest and to reduce the experimental error found in such fermentations.

Based on this aspect, this work is focused on the study of different factors on inoculum build-up aiming to improve the lipase production by *Geotrichum* sp. by means a sequential strategy of experimental design. Initially, a Plackett-Burman design (PB) was carried out to evaluate the effects of inoculum size (1 to 3 circular disk with 0.78 cm^2 containing spores), volume of inoculum (50 to 150 mL), inoculum age (10 to 20 h), corn steep liquor concentration (8 to 16% w/v), soybean oil concentration (0.2 to 1.0% w/v) and initial pH (5.0 to 7.0). Based on the results of PB design, a 2^{4-1} fractional factorial design was carried out to test the effects of inoculum age (8 to 16 h), corn steep liquor concentration (10 to 20% w/v), soybean oil concentration (0.5 to 1.5% w/v) and initial pH (4.0 to 6.0) maintaining constant the inoculum size and volume of inoculum at one circular area and 50 mL, respectively.

MATERIAL AND METHODS

Strain and cultivation conditions

Geotrichum sp. isolated in Biochemistry Laboratory (FEA/DCA/UNICAMP) in Brazil was maintained on yeast malt agar slants and stored at 4 °C. Before this study, the inoculums' build-up was based on the re-suspension of the stock culture into 20 mL of distillated water. Then, a suspension of 5 mL was transferred to erlenmeyers of 1000 mL containing 200 mL of inoculum. The medium for inoculum containing (wt.%) corn steep liquor 5.0, NH_4NO_3 0.5, and soybean oil 1.0 was incubated at 30 °C and 120 rpm for 24 h. However, many difficulties to maintain the homogeneity of the inoculums were found, increasing experimental error of the results of the fermentations.

Based on this issue, a new procedure was evaluated in previous tests, obtaining good results. In this procedure, the strain was grown in yeast malt agar in Petri dish and incubated at 30 °C for 48 h and the inoculum was prepared by adding colonized agar plugs (0.78 cm^2 of area) to flasks containing the volume of inoculum defined by the experimental design and incubated for different times. The inoculum was composed of peptone (Difco) 5.0%, $NaNO_3$ 0.1%, $MgSO_4$ 0.1% and soybean oil 1%. Aliquots of 10 mL of this inoculum were added to 500 mL Erlenmeyer flasks and grown for 16, 24 or 32 h depending of the experimental design under orbital shaking (150 rpm) at 30 °C. Aliquots of fermentation medium were periodically sampled and subsequently analyzed for lipase activity.

Lipase assay

Lipase activity was determined using a microtitrimetric assay with 0.05 M NaOH, and emulsified olive oil as the substrate [14].

Screening variables of inoculum and medium composition

In this study six variables were selected to start the inoculum build-up through a PB experimental design with 12 trials and 3 central points. The PB design investigated the effects of inoculum size (1 to 3 circular disk with 0.78 cm^2 containing spores), volume of inoculum (50 to 150 mL), inoculum age (10 to 20 h), corn steep liquor concentration (8 to 16% w/v), soybean oil concentration (0.2 to 1.0% w/v) and initial pH (5.0 to 7.0). Lipase activity was measured after 8, 12 and 16 h and results were analyzed by software Statistica 8.0 (StatSoft®) to verify the main effects of these variables on lipase activity.

A 2^{4-1} fractional factorial design was carried out to test the variables selected in the first experimental design. The selected variables here were inoculum age (8 to 16 h), corn steep liquor concentration (10 to 20% w/v), soybean oil concentration (0.5 to 1.5% w/v) and initial pH (4.0 to 6.0). Inoculum size and volume of inoculum were fixed in one circular area and 50 mL, respectively. Lipase activity was measured after 8, 16 and 24 h and results were analyzed in the same way described earlier.

Experimental validation of maximized lipase production

Four different conditions were selected based on the results of 2^{4-1} fractional factorial design to obtain maximized conditions for lipase production. Three trials for each condition were carried out and lipase

activity was measured after 8, 16, 24 and 32 h of fermentation. The results were submitted to Tukey's test considering a significance level of 90% ($p < 0.1$).

RESULTS AND DISCUSSION

Screening variables of inoculum and medium composition

The lipase activities obtained in the PB design after 16 h of fermentation are presented in Table 1. The highest activity was 20.86 U/mL, obtained in trial 8 (1 circular area of inoculum size, 50 mL of inoculum, 20 hours of inoculum age, 16% w/v of corn steep liquor, 1.0% w/v of soybean oil and initial pH of 5).

Data of Table 1 were used to compute the main effects of independent variables considering a significance level of 90% ($p < 0.10$), which are presented in Table 2. As can be seen, inoculum volume (p = = 0.09) and initial pH (p = 0.09) presented negative effect on lipase activity, being both statistically significant. This means that lipase activity was higher when these variables were used at level -1. Based on these results, the inoculum volume was fixed at the lowest level of PB design (50 mL), because it is difficult to reduce the amount of this variable to lower values. For initial pH, lower values are possible and a range from 4.0 to 6.0 was then selected to be evaluated in a second factorial experimental design.

Three variables did not have significant effect on lipase production: inoculums size (p = 0.13), inoculum age (p = 0.86) and corn steep liquor concentration (p = 0.16). Inoculum size was fixed at level -1 because one circular area presented satisfactory results concerning the lipase production. We decided to test the inoculums age at a lower range (from 8 to 16 h) to reduce the total fermentation time. Corn steep liquor concentration also did not have a significant effect on lipase production. Thus a higher range (from 10 to 20% w/v) was selected for the next experimental design to check if this variable would have a significant effect on lipase production at higher concentration range.

Table 2. Effect on lipase activity by Geotrichum sp. after 16 h of fermentation

Variable	Effect U/mL	Standard error U/mL	$t(8)$	p-value
Average activity	13.05	0.42	31.00	<0.01[a]
Inoculum size	-1.56	0.94	-1.66	0.13
Inoculum volume	-1.84	0.94	-1.96	0.09[a]
Inoculum age	0.17	0.94	0.18	0.86
Corn steep liquor	1.47	0.94	1.56	0.16
Soybean oil	4.28	0.94	4.55	< 0.01[a]
Initial pH	-1.84	0.94	-1.95	0.09[a]

[a]Significant effects with $p < 0.10$

Soybean oil concentration presented a positive effect ($p<0.01$) on lipase activity and its concentration was increased from 0.5 to 1.5% w/v. This experimental design was carried out to select the most important variables for the definition of medium, but the maximum value obtained in this experiment (20.86 U/mL after 16 h) was similar to the result obtained in a

Table 1. Plackett Burman experimental design to lipase production from Geotrichum sp. after 16 hours of fermentation (real values in parenthesis)

Trial	Variables												
	Inoculum size (circular disks with 0.78 cm^2)		Inoculum medium volume, mL		Inoculum age h		Corn steep liquor		Soybean oil		Initial pH		Lipase activity, U/mL
1	1	(3)	-1	(50)	1	(20)	-1	(8%)	-1	(0.2%)	-1	(5)	11.55
2	1	(3)	1	(150)	-1	(10)	1	(16%)	-1	(0.2%)	-1	(5)	11.55
3	-1	(1)	1	(150)	1	(20)	-1	(8%)	1	(1.0%)	-1	(5)	15.49
4	1	(3)	-1	(50)	1	(20)	1	(16%)	-1	(0.2%)	1	(7)	11.46
5	1	(3)	1	(150)	-1	(10)	1	(16%)	1	(1.0%)	-1	(5)	14.95
6	1	(3)	1	(150)	1	(20)	-1	(8%)	1	(1.0%)	1	(7)	11.34
7	-1	(1)	1	(150)	1	(20)	1	(16%)	-1	(0.2%)	1	(7)	10.14
8	-1	(1)	-1	(50)	1	(20)	1	(16%)	1	(1.0%)	-1	(5)	20.86
9	-1	(1)	-1	(50)	-1	(10)	1	(16%)	1	(1.0%)	1	(7)	15.76
10	1	(3)	-1	(50)	-1	(10)	-1	(8%)	1	(1.0%)	1	(7)	14.78
11	-1	(1)	1	(150)	-1	(10)	-1	(8%)	-1	(0.2%)	1	(7)	11.32
12	-1	(1)	-1	(50)	-1	(10)	-1	(8%)	-1	(0.2%)	-1	(5)	11.45
13	0	(2)	0	(100)	0	(15)	0	(12%)	0	(0.6%)	0	(6)	11.92
14	0	(2)	0	(100)	0	(15)	0	(12%)	0	(0.6%)	0	(6)	11.45
15	0	(2)	0	(100)	0	(15)	0	(12%)	0	(0.6%)	0	(6)	11.69

previous work, which was 18.0 U/mL after 24 h, with the same microorganism and substrate [9]. So, a second experimental design was defined according to these results obtained in the first experimental design, which is shown in Table 3.

The best results were obtained at central points of the experimental design, with average lipase activity of (25.6±4.6) U/mL. This value is statistically similar to the average lipase activity considering the different treatments (trials 1 to 8), which was 22.4±2.2 U/mL. The main effects of independent variables were calculated from data of Table 3, but all effects were not significant in the evaluated range (data not shown). This result indicates that all conditions used in this experimental design can be used to obtain high level of lipase activity.

Analyzing the results in both experimental designs it is possible to see that lipase activity is more than two times higher than the central points in PB (11.68±0.24) U/mL and the central points in 2^{4-1} factorial design (25.65±4.62 U/mL). These results showed that the changes in levels of variables studied produced an improvement in lipase activity.

Experimental validation of optimized conditions

The four conditions chosen to validate the results obtained are showed in Table 4 and the profile of lipase activity during fermentation time is showed in Figure 1. The analysis of variance (ANOVA) was carried out and the minimum significant difference (MSD) was calculated at 95% of confidence level, which resulted in 2.01 U/mL after 32 h of fermentation.

According to results of the analysis of variance, the condition D was selected as the most appropriated for lipase production by *Geotrichum* sp., because in this condition was obtained the highest lipase activity and a significant difference compared to the other conditions. This condition is the same that one used in central point of the second experimental design, which showed the best result. Then, this test validated the information obtained in previous experimental design. Condition D yielded 35.2 U/mL of li-

Table 3. Fractional factorial design 2^{4-1} to lipase production by Geotrichum sp. after 24 h of fermentation ((real values in parenthesis)

Trial	Variables								
	Inoculum age, h		Corn steep liquor		Soybean oil		Initial pH		Lipase activity, U/mL
1	-1	(8)	-1	(10%)	-1	(0.5%)	-1	(4)	24.61
2	1	(16)	-1	(10%)	-1	(0.5%)	1	(6)	23.26
3	-1	(8)	1	(20%)	-1	(0.5%)	1	(6)	20.08
4	1	(16)	1	(20%)	-1	(0.5%)	-1	(4)	24.09
5	-1	(8)	-1	(10%)	1	(1.5%)	1	(6)	23.36
6	1	(16)	-1	(10%)	1	(1.5%)	-1	(4)	24.05
7	-1	(8)	1	(20%)	1	(1.5%)	-1	(4)	20.49
8	1	(16)	1	(20%)	1	(1.5%)	1	(6)	18.92
9	0	(12)	0	(15%)	0	(1.0%)	0	(5)	27.94
10	0	(12)	0	(15%)	0	(1.0%)	0	(5)	28.34
11	0	(12)	0	(15%)	0	(1.0%)	0	(5)	28.28

Table 4. Lipase activity from Geotrichum sp. after 32 h of fermentation; A and D with 12 h of inoculum age; B and C with 8 h of inoculum age; CSL = corn steep liquor; SO = soybean oil; averages with different letters have significant difference at 95% of confidence interval

Trial	Lipase activity, U/ml	Average, U/mL	Standard error, U/mL	Fermentation conditions
A1	27.73			10%w/v CSL
A2	27.13	27.2[a]	0.40	0.5% SO
A3	26.90			pH 4.0
B1	24.03			15%w/v CSL
B2	28.14	25.7[a]	2.20	1.0% SO
B3	24.84			pH 5.0
C1	27.24			10%w/v CSL
C2	29.85	29.4[b]	2.00	0.5% SO
C3	31.25			pH 4.0
D1	34.63			15%w/v CSL
D2	36.10	35.2[c]	0.80	1.0% SO
D3	34.92			pH 5.0

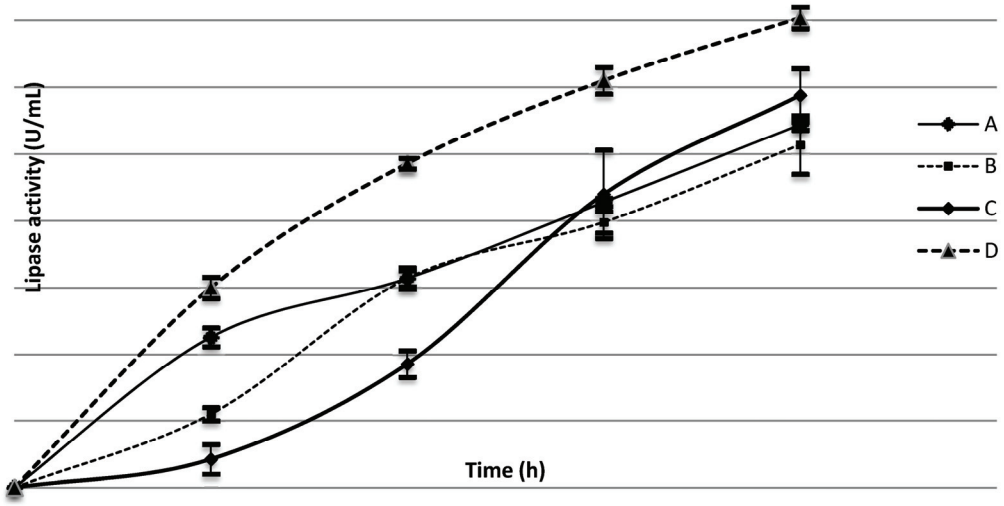

Figure 1. Lipase activity profile during fermentation time. Specific conditions represented by letters A, B, C and D are found in Table 4.

pase activity, a value two times higher than that obtained in a previous work, using the same microorganism [9], reducing the fermentation time from 48 to 32 h.

In addition, the results obtained in this work can be compared with other studies published recently. Gopinath *et al.* [10] evaluated the lipase production from *Geotrichum candidum* using synthetic medium and obtained 89.6 U/mL after 6 days of fermentation at 30 °C. Loo *et al.* [18] obtained a maximum lipase activity of 22.6 U/mL from *Geotrichum candidum* using peptone and yeast extract after 54 h of fermentation. Kamimura *et al.* [20] evaluated the lipase production from *Geotrichum* sp. in a bench-scale bioreactor using corn steep liquor obtaining a maximum lipase activity of 28 U/mL after 10 h of fermentation at 1 vvm, 400 rpm and 30 °C. Yan and Yan [21] evaluated 13 variables in the lipase production using *Geotrichum* sp. in a rotating shaker and obtained a maximum lipase activity of 23.2 U/mL after 24 h of fermentation.

CONCLUSION

This work evaluated six variables during the inoculums build-up to improve the lipase production by *Geotrichum* sp. by means a sequential strategy of the experimental design. A maximum lipase activity of 35.20±0.8 U/mL was obtained with a inoculum build-up composed of one circular area of 0.78 cm^2 containing spores, 50 mL of inoculum volume medium, 12 h of inoculum age, 15% w/v of corn steep liquor concentration, 1.0% w/v of soybean oil concentration and initial pH 5.0 at 30 °C and 150 rpm in flasks. This result is 95 and 52% higher than previous works that studied optimization of lipase production with the same microorganism [9,20] and showed low experimental error, which is very difficult for this kind of microorganism.

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KRATKO SAOPŠTENJE

UNAPREĐENJE PROIZVODNJE LIPAZE IZ *Geotrichum* SP. U ERLENMAJERIMA SA MEŠANJEM

Ovaj rad je usmeren na proučavanje različitih promenjivih pri uvećanju zapremine inokuluma u cilju unapređenja proizvodnje lipaze sa Geotrichum sp. primenom sekvencijalne strategije eksperimentalnog plana. Uticaji veličine inokuluma, koncentracije vode od namakanja kukuruza, zapremine inokuluma, pH medijuma, starosti inokulima i koncentracije sojinog ulja na aktivnost lipaze su procenjeni na osnovu dva faktorijalna eksperimentalna plana. Maksimalna aktivnost lipaze od 35,2±0,8 U/ml dobijena je sa inokulumom sa sporama čija je kružna površina 0,78 cm^2, zapremina 50 ml i starost 12 h, pri koncentraciji vode od namakanja kukuruza od 15 mas.%, koncentraciji sojinog ulja od 1,0 mas.% i početnoj pH od 5.0 na 30 °C pri mešanju od 150 min^{-1} u erlenmajerima. Ovaj rad je pokazao da se povećana aktivnost lipaze može dobiti upotrebom sekvencijalnog statističnog faktorijalnog pristupa kako bi se definisale promenjive u uvećavanju zapremine inokuluma.

Ključne reči: lipaza; voda od namakanja kukuruza; Geotrichum sp.; sojino ulje; faktorijalni dizajn.