

Screening of Amazonian *Bacillus* strains for lipase production using glycerol as substrate

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Abstract: The industrial application of lipolytic enzymes has been studied mainly due to the ability of these enzymes in catalyze reactions of synthesis and their stability in various organic solvents. One possibility is the use of lipase the organic synthesis, taking advantage as the generation of waste and difficult recovery of sub bioproducts. In this work, we carried out a selection of eighty-four isolates of *Bacillus* amazonian for lipase production, of which 30 strains showed lipolytic activity. The study of the culture conditions was performed through a Plackett-Burman experimental design using the strain that presented the highest lipolytic activity in a culture medium using glycerol as substrate. The studied conditions were: concentration of soybean oil, olive oil, triton X-100, gum arabic, glycerol, and $(\text{NH}_4)_2\text{SO}_4$, pH, temperature and concentration of inoculums. The best result obtained were 27 U/L in 48 h of cultivation by *Bacillus circulans* BL53. This work shows that the search and selection of microorganism with lipolytic activities can facilitate the discovery of new lipases, with potential use as by-product surplus.

Keywords: Screening; Glycerol; Lipase.

INTRODUCTION

Lipases belong to the enzyme class of hydrolases (E.C.3) and they are an important group of biocatalysts with a swiftly growing biotechnology that is based mainly on their remarkable ability to carry out novel reactions in aqueous and non-aqueous media (SINGH & MUKHOPADHYAY, 2012). Thus with the advent of rapid methods for discovering novel enzymes or altering the properties of enzymes, there is paramount interest in the development of screening tools that can be used to search for the best performance with respect to specific property. As each industrial application may require specific properties of the biocatalysts, there are still an interest in finding new lipases that could create novel applications (BASSEGODA *et al.*, 2013). Therefore, the screening of microorganisms with lipolytic activities is essential research aiming to the discovery of novel lipases.

Numerous carbon sources, such as Tween 80, triolein, yeast extract, olive oil, soybean oil, silk oil, sesame oil, coconut oil, and corn oil, have been used as substrates to produce lipases in cultures of bacteria (LI *et al.*, 2005; BURKERT *et al.*, 2004). However, few investigations have been carried out using glycerol as substrate (LIN *et al.*, 2006; VOLPATO *et al.*, 2008; RECH *et al.*, 2012). Glycerol can be utilized as a carbon source in microbial bioconversions for the production of a range of chemicals that might be utilized either as end-products or as precursors for the production of other chemicals, since this biofuel will be produced on a large scale in the coming years, large amounts of glycerol will be discarded and can be used in bioprocesses.

The determination of culture conditions for overproduction of the enzyme is an important step for its commercial usage and involves a number of physico-chemical parameters such as the composition of production medium, the carbon and nitrogen sources, pH and temperature (GUPTA *et al.*, 2004). The use of statistical approach involving Plackett–Burman (PB) statistical designing have been widely used for media screening and for the understanding of interactions among various parameters using a minimum number of experiments (RAJENDRAN *et al.*, 2008; ABU *et al.*, 2017). Recent studies have shown the use of statistical approaches to culture conditions optimization to lipases production by different microorganisms such as

Pichia guilliermondii (ABU *et al.*, 2017), *Bacillus cereus* (VASIEE *et al.*, 2016), and *Yarrowia lipolytica* (IMANDI *et al.*, 2014).

This work deals of screening of Amazon *Bacillus* strains to produce lipase using glycerol as substrate and to study the effects of culture conditions on lipase production using Plackett–Burman statistical designing.

MATERIAL AND METHODS

Screening of Amazon isolates

The eighty-four bacterial used in this study were isolated from samples of soil and water of the Amazon region and stocks of these bacteria are kept at Microbiology Culture Collection of BiotecLab (UFRGS, Brazil). Strains were maintained on LB agar medium containing 1% of peptone, 0.5% of yeast extract, and 1% of NaCl. Two assay methods were used to screen the lipolytic activity: a solid system (agar plates), for bacteria preselecting, and a liquid system (microplates).

The solid system was based on the spread plate technique content tributyrin agar. Cultures were grown at 37 °C for 72 h and positive colonies were identified by the presence of a translucent halo around the colonies; commercial lipase was used as a positive control, and water as negative control (SOUZA *et al.*, 2003).

In the liquid system, sterilized medium (LB supplemented with 10 % of olive oil and 1 % of gum arabic) was then homogenized in a blender, test tubes were inoculated with a single colony from an agar plate culture and incubated for 48 h at 37°C under agitation (180 rpm), the tube cultures were centrifuged at 15,000 g and 4 °C for 10 min, and an aliquot of supernatant was removed to check extracellular activity, using SpectraMax Microplate Reader, with 96-well polystyrene microplates, according to methodology proposed by Winkler and Stuckmam (1979), with modifications.

Media composition

Through the tests described above were selected the strains with higher lipolytic activity. We tested six different media of cultivation, varying the source of carbon (glucose and glycerol), nitrogen source (organic and inorganic) and source of minerals (raw yeast extract and medium Bushnell-Haas), showed Table 1. Inoculum for all experiments were prepared in 250 mL Erlenmeyer flasks containing 40 mL of

the culture medium. Flasks were inoculated with 5 % of pre inoculum (medium LB, 18 h of culture, and agitation of 250 rpm), the initial pH was adjusted to 7.0, incubated for 48 h at 37 °C, and 250 rpm. Samples were taken along the 48 h of cultivation to determine lipolytic activity, pH and optical density.

Table 1 - Culture media composition.

Culture Medium	Carbon source (g/L)		Nitrogen source (g/L)		Mineral source (g/L)	
	Glycerol	Glucose	Peptone	(NH ₄) ₂ SO ₄	Raw yeast extract	Bushnell-Haas extract
1	10	0	10	0	5	0
2	0	10	10	0	5	0
3	10	0	0	10	5	0
4	0	10	0	10	5	0
5	10	0	0	10	0	5
6	0	10	0	10	0	5

Plackett–Burman statistical design

To determine which nutrients and conditions had a significant effect on lipase production, P-B design was used (PLACKETT & BURMAN, 1947), with the strain that presented the highest lipolytic activity in previous experiments. In this paper, nine variables (concentration of soybean oil and olive oil; concentration of Triton X-100; concentration of gum arabic; concentration of glycerol; concentration of (NH₄)₂SO₄; initial pH; temperature, and inoculum optical density) and two dummies variables were screened in 12 trials, in duplicate. The minimum and maximum ranges selected for the nine parameters are given in Table 2, in which each column represents an independent variable. Variables with confidence levels > 95% were considered to have significant influence on lipase production.

Table 2 - Plackett-Burman experimental design matrix with lipolytic activity in 48 h of cultivation by *Bacillus circulans* BL53.

Trial no.	Variables ^a /levels ^b									Lipolytic Activity (U/L)
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	X ₉	
1	+	-	+	-	-	-	+	+	+	7.05
2	+	+	-	+	-	-	-	+	+	1.46
3	-	+	+	-	+	-	-	-	+	4.15
4	+	-	+	+	-	+	-	-	-	4.41
5	+	+	-	+	+	-	+	-	-	4.23
6	+	+	+	-	+	+	-	+	-	8.64
7	-	+	+	+	-	+	+	-	+	0.48
8	-	-	+	+	+	-	+	+	-	1.89
9	-	-	-	+	+	+	-	+	+	26.73
10	+	-	-	-	+	+	+	-	+	11.41
11	-	+	-	-	-	+	+	+	-	19.32
12	-	-	-	-	-	-	-	-	-	16.30
Effects	-4.4*	-4.9*	-8.2*	-4.7*	1.2*	5.1*	-3.1*	3.1*	0.5	

^a X₁ soybean oil: (-) = 5 g/L, (+) = 20 g/L; X₂ olive oil: (-) = 5 g/L, (+) = 20 g/L; X₃ Triton X-100: (-) = 0 g/L, (+) = 5 g/L; X₄ gum arabic: (-) = 0 g/L, (+) = 10 g/L; X₅ glycerol: (-) = 10 g/L, (+) = 30 g/L; X₆ (NH₄)₂SO₄: (-) = 0 g/L, (+) = 5 g/L; X₇ initial pH: (-) = 5, (+) = 8; X₈ temperature: (-) = 25 °C, (+) = 37 °C; X₉ inoculum optical density (-) = 5 % (v/v), (+) = 10 % (v/v). ^b (-) lower level, (+) higher level. * p-values < 0.05.

Analytical methods

The cell-free supernatant of the culture broth, referred to as the crude extract, was used for the estimation of enzymatic activities and for pH determinations using a standard lab pHmeter.

Lipolytic activity was determined using a colorimetric method described for Winkler and Stuckmam (1979), using p-nitro-phenyl palmitate (pNPP) as the substrate. A volume of 0.15 mL 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. Control containing heat-inactivated enzyme was also incubated with each assay. The reaction was measured as absorbance at 410 nm. One unit of enzyme was defined as the amount of enzyme that liberated 1 μmol p-nitrophenol min⁻¹ at 37°C.

RESULTS AND DISCUSSION

Thirty strains from the eighty-four studied, were selected respect with their lipolytic activities. Table 3 shows the results to of halos formed in agar tributyrin by strains pre-selected in this test, and lipolytic activity of these isolates. It was verified that the major lipolytic activities were obtained by the strains that presented the largest halos. The similar behavior was found by Bhatnagar *et al.* (2005) in the selection of lipase-producing halobacteria, these data indicate that although this method is qualitative, it can be considered very reliable. These authors have maximum lipolytic activity of 10.8 U/L, using ρ -NPP as a substrate. Among the pre-selected strains in this work, the isolated BL13 presented the higher lipolytic activity (22.5 U/L).

We performed the statistical analysis of the results, through ANOVA and Tukey test, to verify the differences between the lipolytic activities obtained for each isolated. When the activities were considered statically equal, with confidence level of 95 %, they were grouped and identified by a superscript letter. The strains that showed higher lipolytic activity (groups A and B) were selected for the study of culture media composition. Thus, eight strains were selected, BL13, BL49, BL53, BL58, BL60, BL61, BL64 and BL74, as is evident in Table 3.

After select microorganisms with lipolytic activity, different source of carbon (glucose and glycerol), nitrogen (peptone and $(\text{NH}_4)_2\text{SO}_4$), and mineral (Bushnell-Haas and yeast extract) were tested for the lipase production of such strains. Table 4 presents the results of lipolytic activity for the previously selected strains, in the different cultures using glycerol (media 1, 3 and 5) or glucose (media 2, 4 and 6).

The higher lipolytic activity (26.9 U/L) was obtained by isolated BL53, previously identified as *Bacillus circulans*, in the culture medium containing glycerol as substrate (medium 1), suggesting that glycerol is a better source for microorganism growth and lipase production than glucose, for the studied strains. Volpato *et al.* (2008) and Lin *et al.* (2006), working with *Staphylococcus caseolyticus* and *Antrodia cinnamomea*, respectively, also described the induction of this enzyme by glycerol. Moreover, the lipolytic activities were higher in the media 1 and 2, indicating that the use of peptone as a nitrogen source and raw yeast extract, a cheap substrate, are more interesting for lipase production by these strains, than the use of $(\text{NH}_4)_2\text{SO}_4$ and mineral medium Bushnell-Haas, respectively. From these

results, we selected the isolated BL53 because it presented higher lipolytic activity in cultures containing glycerol as carbon source.

Table 3 - Amazonian isolates that showed lipolytic activity.

Strain	Tributyryn Agar Plates	Lipolytic Activity (U/L)
BL7	+	7.4 ± 0.2 ^{FG}
BL9	+	9.9 ± 0.2 ^{DEF}
BL10	+	10.3 ± 0.0 ^{DEF}
BL12	+	10.3 ± 0.4 ^{DEF}
BL13	++	22.5 ± 0.2 ^A
BL14	++	10.9 ± 0.3 ^{DE}
BL20	+	10.6 ± 0.0 ^{DEF}
BL21	+	10.3 ± 0.0 ^{DEF}
BL22	+	7.9 ± 0.5 ^{FG}
BL23	+	8.2 ± 0.1 ^{FG}
BL32	+	8.9 ± 0.4 ^{EF}
BL37	+	6.7 ± 0.0 ^F
BL38	++	11.3 ± 0.1 ^{DE}
BL48	++	10.9 ± 0.5 ^{DE}
BL49	++	14.0 ± 0.0 ^{BCD}
BL53	++	14.1 ± 0.2 ^{BCD}
BL58	++	14.1 ± 0.5 ^{BCD}
BL60	++	15.4 ± 0.3 ^{BC}
BL61	++	14.9 ± 0.0 ^{BC}
BL62	+	10.5 ± 0.2 ^{DEF}
BL63	++	12.7 ± 0.5 ^{CDE}
BL64	++	16.1 ± 0.0 ^B
BL65	++	12.9 ± 0.0 ^{CD}
BL66	+	10.8 ± 0.9 ^{DE}
BL70	++	11.7 ± 0.5 ^{DE}
BL71	+	10.8 ± 0.2 ^{DE}
BL74	++	15.5 ± 0.0 ^{BC}
BL77	++	11.5 ± 1.6 ^{DE}
BL84	++	11.9 ± 0.4 ^{DE}
BL86	++	12.4 ± 0.4 ^{CDE}

++: very clear halo; +: clear halo. Results are the mean ± standard deviation of three experiments. Means followed by different letters are statistically different ($p < 0.05$).

Table 4 - Lipolytic activity of pre-selected strains in different media studied.

Strain	Lipolytic activity (U/L)					
	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5	Medium 6
BL13	8.8 ± 0.9 ^E	8.1 ± 0.3 ^E	5.6 ± 0.2 ^{EF}	9.3 ± 1.0 ^{DE}	7.2 ± 0.7 ^{EF}	1.4 ± 0.1 ^G
BL49	6.9 ± 0.7 ^{EF}	4.1 ± 0.1 ^{EF}	2.5 ± 0.8 ^{FG}	2.1 ± 0.7 ^{FG}	3.3 ± 1.5 ^{EF}	1.7 ± 0.4 ^G
BL53	24.9 ± 1.1 ^A	19.5 ± 1.4 ^B	8.3 ± 1.6 ^E	11.1 ± 1.5 ^{DE}	12.8 ± 0.4 ^{DE}	2.8 ± 0.5 ^{FG}
BL58	8.3 ± 0.5 ^E	6.9 ± 0.1 ^{EF}	4.3 ± 0.7 ^{EF}	6.8 ± 0.4 ^{EF}	5.9 ± 0.8 ^{EF}	1.8 ± 0.1 ^G
BL60	8.1 ± 0.4 ^E	6.9 ± 0.3 ^{EF}	4.6 ± 0.8 ^{EF}	5.6 ± 0.3 ^{EF}	5.3 ± 0.5 ^{EF}	2.5 ± 0.8 ^{FG}
BL61	8.6 ± 0.7 ^E	10.4 ± 0.7 ^{DE}	3.9 ± 1.0 ^{EF}	5.9 ± 0.4 ^{EF}	6.4 ± 0.1 ^{EF}	1.0 ± 0.1 ^G
BL64	5.4 ± 0.4 ^{EF}	3.9 ± 0.6 ^{EF}	2.1 ± 0.7 ^{FG}	5.0 ± 0.2 ^{EF}	5.5 ± 0.5 ^{EF}	2.2 ± 0.3 ^{FG}
BL74	24.3 ± 2.3 ^A	18.0 ± 2.3 ^{BC}	6.6 ± 1.6 ^{EF}	10.8 ± 1.0 ^{DE}	14.3 ± 0.4 ^C	3.3 ± 0.6 ^{FG}

Means followed by different letters are statistically different ($p < 0.05$).

Table 2 shows the PB experimental design for 12 trials with two levels for each variable and its corresponding lipolytic activity, which varied between 0.5 and 26.7 U/L. This variation reflects the importance of optimization to obtain high enzyme production. Although the kinetics of growth has been followed up to 60 hours of cultivation, the largest enzyme activities were observed with 48 hours of cultivation.

The maximum lipolytic activity (26.73 U/L) was obtained in the following conditions: soybean oil and olive oil, 5 g/L; $(\text{NH}_4)_2\text{SO}_4$, 5 g/L; glycerol, 30 g/L; initial pH, 5.0; 37 °C; inoculum optical density, 5 %; absence of Triton X-100 and gum arabic. Lin *et al.* (2006), working with *Antrodia cinnamomea*, obtained lipolytic activity, 27 U/L using glycerol (50 g/L) as a carbon source for producing lipase. Bhatnagar *et al.* (2005), found lipolytic activity maximum of 45 U/L, working with pH 7.5 and temperature in the range of 35 to 40 °C in a culture of halobacteria *Natronococcus* sp.

Among the studied variables, only the inoculum concentration presented no significant effect on confidence level of 95 % in lipase production. Some authors reported that lipases generally are heavily influenced by presence of oils in the culture medium (VOLPATO *et al.*, 2008; BURKERT *et al.*, 2004). In this study, in contrast, the oils (olive and soybean) presented negative effect in the PB experimental design, i.e., when raised from the lower level (5 g/L) to the higher level (20 g/L) the lipolytic activity decreased.

Iqbal and Rehman (2015) utilized different concentrations of oil and when the concentration has exceeded 2% microbial growth decreased. The microorganisms have the ability to use oil as carbon source, however the high concentration of this

substrate may be toxic to the cell. According to Magdouli *et al.* (2017) the depletion of the carbon source (glucose or glycerol) may lead the microorganism to consume the sources of oil, and the increased concentration can lead to decreased activity.

The source of nitrogen is also an important factor for lipase production. In our study, the addition of an inorganic nitrogen source, $(\text{NH}_4)_2\text{SO}_4$, showed significant effect on lipolytic activity and consequently decreased the proteolytic activity, according to previous experiments. This result is in line to that achieved by Chen *et al.* (1999), which studied the production of lipase by *Acinetobacter radioresistens* in a medium containing 1 g/L of NH_4Cl to inhibit proteolytic activity. The emulsifiers, Triton X-100 (X_3) and gum arabic (X_4), which was added in order to facilitate and maintain emulsified the oils in the cultivation medium had negative effects on lipolytic activity. This result agrees with obtained by our group when study the lipase production by a *Staphylococcus* strain (VOLPATO *et al.*, 2008).

Thus, from this result, it was possible to select the variables that most influence in the lipase production by *Bacillus circulans* BL53, and their levels. The variables that showed significant positive effect, that is the increase in their value leads to an increase in lipase production were: glycerol and $(\text{NH}_4)_2\text{SO}_4$ concentration, and temperature. The variables that showed significant negative effect, that is the increase in their value leads to an increase in lipase production were: soybean oil, olive oil, Triton X-100, gum Arabic, initial pH. The experimental PB experimental design is exploratory, that is, it only indicates the variables and suggests the values of the levels to be studied to obtain the optimal conditions for cultivation.

CONCLUSIONS

In this study, we screened and selected Amazon *Bacillus* strains with lipolytic activity. The results demonstrated that the use of the qualitative solid agar tributyrin technique was an efficient way for the pre-selection of lipase-producing microorganisms, confirmed by the quantitative assay. The Plackett-Burman experimental design was successful used to select the variables that most influence the lipase production by *Bacillus circulans* BL53. Moreover, it was possible to select a microorganism with high lipolytic activity in a medium containing glycerol as a source of carbon. Furthermore, this work showed that the search and selection of microorganisms with lipolytic activities can facilitate the discovery of new lipases, with

potential use as by-product surplus, and this result is very important in the economic point of view, with respect to biotechnological processes.

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