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Statistical experimental design applied to extracellular lipase production by the marine Antarctic yeast *Leucosporidium scottii* CRM 728

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ABSTRACT

The search for microbial enzymes from extreme environments may result in the discovery of biomolecules with different properties. In this sense, the aim of this study was to evaluate and optimize the conditions for lipase production by the marine-derived Antarctic yeast *Leucosporidium scottii* CRM 728 using an experimental design with different sources of carbon and nitrogen. The applied strategy was composed of three steps, including Plackett-Burman, Central Composite Design (CCD), and validation assay. Among nine variables applied, corn steep liquor, olive oil, and soybean oil showed a positive effect in the lipase production, while urea showed a negative effect. The production of lipase increased 9-fold in comparison to the basal activity when olive oil and corn steep liquor were used as nutrient sources. On the other hand, there was an increase of 4.8-fold when soybean oil and corn steep liquor were used as nutrient sources. The highest amounts of lipase were achieved in non-saline conditions and at 20.0 °C. In this study, the lipase production by the marine-derived psychrotolerant yeast *L. scottii* was for the first time reported, providing new knowledgement in the field of enzymatic production by extremophilic and marine microbial resource.

1. Introduction

Lipases (EC 3.1.1.3) are hydrolases that act on the carboxylic ester bonds and catalyze the reaction of hydrolysis, esterification, and interesterification of fats with extreme simplicity of the process, higher quality of the final product, and excellent performance (Maiangwa et al., 2015). The search for microorganisms that produce lipases is especially interesting due to the different possibilities of applications for this enzyme (Sarmah et al., 2018).

Although the expression of recombinant lipases by host organisms through cloning and genetic engineering has been widely reported in recent years (Contesini et al., 2020), the study of new sources of native microorganisms from the extremophile environment is justified by the search for enzymes with better performances in adverse conditions such temperature, pH, and other extreme conditions. In addition, since lipases have different applications due to characteristics such as regioselectivity and stereoselectivity, the discovery of new lipase-producing organisms can mean an expansion in the possibilities of using this enzyme (Andualema and Gessesse, 2012; De Luca and Mandrich, 2020).

Enzymes produced by psychrophilic and psychrotrophic microorganisms exhibit high catalytic activity in processes that occurred at low and moderate temperatures (Duarte et al., 2018). Among the mechanisms of cold tolerance developed by microorganisms is the basal level production of esterase and lipase to the maintenance of the cell membrane fluidity (Maiangwa et al., 2015). This would explain the high number of yeast strains with lipase activity isolated from Antarctic environment (Duarte et al., 2013, 2018; Maharana and Singh, 2018a; Martorell et al., 2019).

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Fungal lipases are preferred because they are generally secreted in the extracellular medium, which facilitates its extraction from fermentation medium, and have higher stability to temperature and pH, in addition to their capacity of being active in organic solvents (Sing and Mukhopadhyay, 2012). Moreover, the microbial lipase production can be influenced by carbon and nitrogen sources, presence of inducers and inhibitors, presence of agents that affect the oil/water interface, pH, and incubation temperature (Melani et al., 2020). The optimum conditions for lipase production shall be accessed by studying the influence of these factors in the biosynthesis of the enzyme (Lima et al., 2019).

Cold active/adapted lipase can be applied to a variety of biotechnological process, including food and beverage industries (Kavitha, 2016), decomposition of milk fat (Tsuji et al., 2015), wastewater treatment in temperate regions (Tsuji et al., 2013), detergent formulation (Sahay and Chouhan, 2018), and ethanol fermentation (Tsuji et al., 2014). Among the Antarctic yeast reported as lipase producers are: *Candida antarctica* (Guo et al., 2020; Yasuda and Yamamoto, 2020), *Candida zeylanoides*, *Vishniacozyma victoriae* (formerly *Cryptococcus victoriae*), *Leucosporidium creatinivorum* (Vaz et al., 2011), *Mrakia blollopsis* (Tsuji et al., 2013), *Leucosporidium scottii* (Duarte et al., 2015), *Phenoliferia glacialis*, *Mrakia frigida*, *Pichia caribbica*, *Leucosporidium muscorum* (Martorell et al., 2017), and *Rhodotorula* sp. Y-23 (Maharana and Singh, 2018a). Moreover, Antarctic filamentous fungi have been also reported as lipase producers, including *Geomyces* sp. P7 (Florczaka et al., 2013) and *Penicillium expansum* SM3 (Mohammed et al., 2013).

Considering the aforementioned, the aim of this study was to optimize the conditions for lipase production by the marine-derived Antarctic yeast *Leucosporidium scottii* CRM 728 using different carbon and nitrogen sources. Individual tests and experimental design were applied to select the independent variables (factors) and each level. This approach may increase the knowledge about the physiological profile of *L. scottii* CRM 728 lipase production, which could be important in biotechnological processes.

2. Material and methods

2.1. Microorganism

The Antarctic yeast *Leucosporidium scottii* CRM 728 (=L117, original code) used in this study was recovered from marine sediment collected from the Admiralty Bay (King George Island, Maritime Antarctica), by the Brazilian Antarctic Program team during an expedition in the southern polar summer of 2010 (OPERANTAR XXVIII). The taxonomic identification (based on 26S rDNA sequencing) and the screening for lipase production in solid and liquid culture media was reported previously by Duarte et al. (2013). This strain is deposited at the UNESP Central of Microbial Resources - CRM-UNESP (UNESP, SP, Brazil) under the acronym CRM 728.

2.2. Culture conditions for lipase production

The initial inoculum (10^7 cells) was cultured in medium containing (g L^{-1}: peptone 5, yeast extract 3, ammonium sulfate 1, sodium nitrate 1), and olive oil 20 mL L^{-1} at 20.0 °C. The freshly grown cells were transferred to a 150 mL Erlenmeyer flask containing 50 mL of the same medium and incubated at 20.0 °C and 180 rpm. Every 24 h (24–144 h) the yeast cells were centrifugated at 10,000 g for 10 min to remove the cells and the supernatant phase was used to determine the extracellular lipase activity.

2.3. Statistical experimental design

The strategy used in the experimental design was composed of one Plackett-Burman design (P&B), one central composite design, and the validation assay.

Table 1
Central composite design (CCD 2^3) with 17 assay and six axials points and three central points to increase culture conditions for lipase by *L. scottii* CRM 728 (120 h at 20.0 °C and 180 rpm).

Assay	Corn steep liquor (mL L^{-1})	Olive oil (mL L^{-1})	Soybean oil (mL L^{-1})	Lipase activity (U mL^{-1})
1	−1 (2.6)	−1 (16)	−1 (16)	0.188
2	1(9.4)	−1 (16)	−1 (16)	0.932
3	−1 (2.6)	1 (64)	−1 (16)	0.655
4	1 (9.4)	1 (64)	−1 (16)	0.957
5	−1 (2.6)	−1 (16)	1 (64)	0.382
6	1 (9.4)	−1 (16)	1 (64)	0.969
7	−1 (2.6)	1 (64)	1 (64)	0.802
8	1 (9.4)	1 (64)	1 (64)	0.957
9	−1.68 (0)	0 (40)	0 (40)	0.064
10	1.68 (12)	0 (40)	0 (40)	0.638
11	0 (6)	−1.68 (0)	0 (40)	0.709
12	0 (6)	1.68 (80)	0 (40)	1.627
13	0 (6)	0 (40)	−1.68 (0)	1.120
14	0 (6)	0 (40)	1.68 (80)	0.772
15	0 (6)	0 (40)	0 (40)	1.310
16	0 (6)	0 (40)	0 (40)	1.250
17	0 (6)	0 (40)	0 (40)	1.310

2.3.1. Plackett-Burman design (P&B)

The integrated statistical design was initialized by the selection of significant factors using P&B model. The P&B 16 was used to assess the effect of the nine independent factors on the extracellular lipase activity of *L. scottii* CRM 728 (Supplementary Table 1): corn steep liquor (provided by Corn Products Mogi Guaçú, São Paulo, Brazil) in different concentrations, and pH variations. Data from literature were employed to the initial values of all factors: peptone (Wolski et al., 2009), pH, glucose and oils (Papagora et al., 2013), yeast extract, and urea (Ali et al., 2010) and corn steep liquor (Edwinoliver et al., 2009).

The variables were screened by Plackett-Burman design and matrix of 16 assays was employed with two-level factorial design (+1 or −1). Three assays on center point were added to the matrix in order to determine the standard error. The design matrix was created by the statistical STATISTICA 7 from Statsoft Inc. (2325 East 14th Street, Tulsa, OK, 74104, USA). The standardized effect was based on the following first-order polynomial model:

$$y = \beta 0 + \sum \beta i x i \quad (1)$$

Where y was the predicted response, β0 was the model intercept, βi was the linear coefficient and xi was the independent variable level.

2.3.2. Central composite design (CCD)

Following the strategy based on the results of P&B the experiment was further expanded to a central composite design CCD (Table 1) with three variables: olive oil, soybean oil, and corn steep liquor. All other independent variables evaluated in P&B were excluded in this new test (peptone, yeast extract, urea, sunflower oil, and glucose) and the pH 8.0 was fixed.

A 2^3 randomized factorial central composite design (CCD) with six axial points $\alpha = (2^3)$ ¼ and four replicates at the center points leading to a total 17 experiments being six axial and three central points. After analyzing the results of CCD 2^3 by statistical analysis new ranges of some independent factors (variables) were again evaluated. This round was attended by two experimental design that were performed using CCD 2^2 with two variables each: i) olive oil and corn steep liquor; and ii) soybean oil and corn steep liquor, both with four axial and three central points, making a total of 11 experiments for each experimental design. These experiments were performed in pH 8.0 at 20.0 °C for 120 h at 180 rpm. For the inoculation and enzymatic broth obtention were used the strategy described in item 2.2.

These experiments were performed to obtain a second-order model to predict the enzymatic activity on functions of different variables. The quadratic model for predicting the optimal point was expressed as

follow:

$$y = \beta 0 + \sum \beta ixi + \sum \beta iix2\ i + \sum \beta ijxixj \quad (2)$$

Where y was the predicted response, β0 was the model intercept, xi and xj were the independent variable levels, βi, βii, and βij were the linear quadratic and interaction coefficients, respectively. The quadratic model and data analysis were performed using STATISTICA software version 7.0 (STAT SOFT, INC. 1995). The quality of fit of the model equation was expressed by the coefficient of determination R^2 and its statistical significance was determined by F test (analysis of variance—ANOVA). The level of significance used for P&B was $p < 0.1$ and for the CCD 2^3 and CCD 2^2 the level was $p < 0.05$.

The response surface plot was generated to analyze the effect of independent variables on the lipase activity. To confirm the model equation adequacy, confirmatory experiments (validation assay) under the optimized condition were carried out. All the confirmatory experiments were conducted in triplicate and the values predicted by the optimization model were set as controls.

2.4. Independent evaluation of other variables for the optimization of lipase production

Lipase production was also evaluated with different carbon sources, such as tributyrin (C4), tricaprilin (C8), and oleic acid (C18) in concentration ranging from 1 to 4%. Corn steep liquor was used as nitrogen source. In addition, the influence of concentration of NaCl in culture medium (1–5%) and temperature of incubation (15.0, 20.0, and 25.0 °C) were also evaluated.

2.5. Precipitation of lipase to partial characterization

After the enzyme production optimization, cold ethanol 95% and acetone were used to define the best ratio of enzyme extraction following the proportion: enzyme extract: ethanol or acetone (1:1, 1:2, 1:3, 1:4, and 1:5). Protein sample volume was adjusted and cold ethanol or acetone was slowly added, mixing lightly with a glass stick. The mixture was allowed to stand in refrigerated environment for different times (2, 4, 6, and 12 h). After standing, the samples were centrifuged at 10,000 rpm, 2.0 °C for 20 min. Precipitation using solution of ammonium sulfate in a range of 25–90% saturation was also performed. The material was centrifuged at 10,000 rpm for 20 min at 4.0 °C. Precipitated and supernatant phases were evaluated.

The precipitate was suspended in 50 mM sodium phosphate buffer (1 mL buffer to 2.5 mL initial extract volume) and the final volumes were recorded. Enzymatic activity and total protein were evaluated for the precipitate suspended in sodium phosphate buffer (sodium phosphate pH 7.0, 50 mM). This precipitate was used in the SDS-page, which was carried out according to Oakley et al. (1980), using silver nitrate for enzyme visualizing.

2.6. Lipase activity

In all assays lipase activity from *L. scottii* CRM 728 was performed using *p*-nitrophenyl palmitate (*p*-NPP) Sigma-Aldrich® (St. Louis, MO, USA) as substrate and measured by reading at 405 nm as described by Duarte et al. (2015). One unit of enzyme was defined as the amount of lipase capable of releasing 1 μmol of *p*-NPP/mL/min of reaction. All assays were performed in triplicate.

3. Results and discussion

3.1. Optimization of lipase production

In the previous study reported by Duarte et al. (2013), the marine-derived yeast *L. scottii* CRM 728 showed the highest enzymatic activity (0.230 U mL^{-1}) among 97 Antarctic yeasts, after 120 h of incubation in basal medium (composed of in g L^{-1}: peptone 5, yeast extract 3, and olive oil 20).

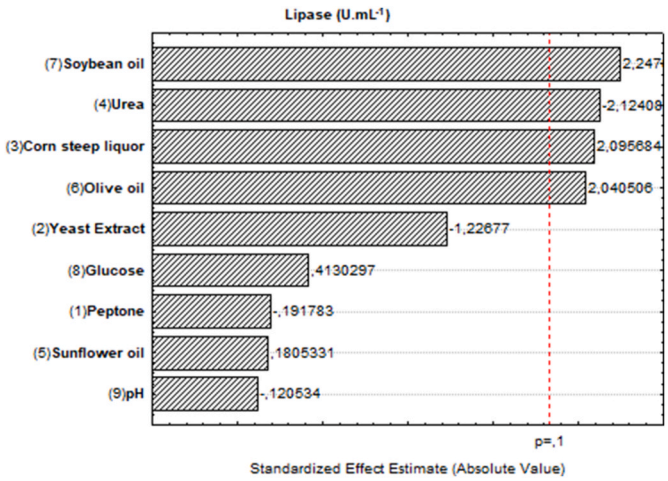

Fig. 1. Pareto plot show the effect of nine variables on lipase activity by *L. scottii* CRM 728.

In order to understand the effect of the carbon and nitrogen sources in the lipase activity of *L. scottii* CRM 728 the experimental design strategy was performed. In the first experimental design (P&B) four independent variables showed significant effects ($p < 0.1$): olive oil, soybean oil, and corn steep liquor (positive effect) and urea (negative effect) (Fig. 1.). The highest lipase activity was 0.409 U mL^{-1} (Supplementary Table 1, assay 14).

Based on these results a new experimental design (CCD 2^3) was carried out considering the variables corn steep liquor, olive oil, and soybean oil in new concentration rates. The variable urea was eliminated due to the negative effect, as well as other variables not significant (peptone, yeast extract, sunflower oil, and glucose). Additionally, the variables not present in the assay with higher lipase activity (assay 14 from P&B) were not considered in the CCD 2^3. The pH of the culture medium was fixed at 8.

In the CCD experiments glucose showed negative effect on lipase production by *L. scottii* CRM 728. In fact, different carbon sources have been used for lipase production by yeast (Balocha et al., 2019; Maharana and Singh, 2018a,b). Maharana and Singh (2018a) assessed the effect of carbon source such as maltose, fructose, lactose, galactose, sucrose, and xylose and noted that *Rhodotorula* sp. Y-23 (isolated from Antarctic sediment) showed negative effect in cold-active lipase production with glucose, maltose, and sucrose at 15.0 °C. On the other hand, Balocha et al. (2019) evaluated the effects of glucose on extracellular and cell-bound lipase production by the yeast *Dipodascus capitatus* A4C and only the cell-bound lipases were inhibited. These authors also observed that double carbon sources (palm oil and glucose) cause an elongation to the mid and late exponential phases of the fermentation process and an increase in the production of extracellular lipase, with no prejudice to cell-bound lipases production. *Candida rugosa* showed the highest yields of lipase using lipids or fatty acids as carbon sources, and combinations of two types of substrates (carbohydrates and fatty acids) did not improve the lipase production, instead, the glucose showed a depressing effect on the enzyme production (Dalmau et al., 2000).

In general, higher production of lipase has been reported with organic sources such as peptone in comparison to inorganic sources such as NH_4NO_3 and $NaNO_3$ (Maharana and Singh, 2018a; Pereira et al., 2019). According to Pereira et al. (2019) among 10 sources of nitrogen, the yeast extract was the best for extracellular lipase production by *Yarrowia lipolytica*. In our study, the best source in the P&B experiment was corn steep liquor, which was even better than peptone and yeast extract. Statistical analyses revealed that all independent variables

Table 2
Regression coefficient obtained from CCD $2^3$for lipase production by *L. scottii* CRM 728 (120 h at 20.0 °C and 180 rpm). The factors with significance (p-value <0.1).

Factors	Regression coefficient	p-value (p < 0.1)
Media	1.294130	0.000238
Corn steep liquor (L)	0.201712	0.002154*
Corn steep liquor (Q)	−0.346517	0.000888*
Olive oil (L)	0.178987	0.002734*
Olive oil (Q)	−0.057047	0.031268*
Soybean oil (Q)	−0.135704	0.005746*
Corn steep liquor by olive oil	−0.109250	0.012335*
Corn steep liquor by soybean oil	−0.038000	0.090065*

(L) - linear effect; (Q) - quadratic effect.

showed significant effect ($p < 0.1$). Corn steep liquor and olive oil showed positive effect ($p < 0.1$). The highest lipase activity (1.627 U mL^{-1}) was achieved in assay 12 (Table 1). This result was 4-fold higher than that one obtained in the P&B (0.409 U mL^{-1}) and 7-fold higher than that one obtained in the initial screening (0.230 U mL^{-1}).

Lipase production showed statistically significant results ($p < 0.1$) in the regression coefficient for the model that predict corn steep liquor and olive oil (linear and quadratic terms), quadratic soybean oil and the interactions: corn steep liquor with olive oil, and corn steep liquor with soybean oil interactions (Table 2). The multiple regressions from the results obtained led to the proposition of a mathematical model according to the significant variables, corn steep liquor and olive oil. The equation of the reparametrised model is: $y = 1.294130 + 0.201712$ (corn steep liquor) – 0.346517 (corn steep liquor)^2 + 0.178987 (olive oil) – 0.057047 (olive oil)^2–0.135704 (soybean oil)^2–0.109250 (corn steep liquor x olive oil) – 0.038 (corn steep liquor x soybean oil). In which y is the predicted response (lipase activity).

The statistical significance (mathematical model) was checked by F test (ANOVA). The model could be considered to be predictive since the value of the F test (226.7) for the regression was highly significant [higher than the tabulated F (2.45)] and the percentage of variation (R^2 = 92%, Supplementary Table 2) explained by the model was high. So, it was used to generate a contour plot and response surface (Fig. 2.). The results derived from contour and surface curves showed that the test was close to the optimization point, mainly considering the corn steep liquor concentration. On the other hand, adjustments are necessary for the concentrations of olive oil and soybean oil (Fig. 2.).

According to Fig. 2 the best condition of corn steep liquor was close to the central point (6 mL L^{-1}), independent of the carbon source. In addition, it is possible to note that the oils used as carbon sources showed different effects on the lipase production. To understand this effect, two new experimental designs CCD 2^2 were performed separately, one using steep corn liquor (nitrogen source) and olive oil (Table 3) and the second with steep corn liquor and soybean oil (Table 4). The highest lipase production using olive oil was 2.07 U mL^{-1} (assay 8, Table 3) and the highest enzymatic activity in the presence of soybean oil was 1.12 U mL^{-1} (assay 4, Table 4). It is important to note that the best production using olive oil was exactly at the point indicated on the CCD 2^3 model (Fig. 2B), while for soybean oil the greatest activity occurred in the highest concentration of both variables, but in the range previously predicted by the model.

In the CCD 2^2 using olive oil only corn steep liquor (quadratic) was statistically significant ($p < 0.1$). However, since the regression coefficient was low ($R^2 = 0.38$) it was not possible to predict the statistical model.

In the results of CCD 2^2 with soybean oil all parameters were

Table 3
Central composite design (CCD 2^2) with 12 assays (corn steep liquor and olive oil) included four axials points and four central points to increase culture conditions for lipase by *L. scottii* CRM 728 (120 h at 20 °C and 180 rpm).

Assay	Corn steep liquor (mL L^{-1})	Olive oil (mL L^{-1})	Lipase Activity (U mL^{-1})
1	−1 (1.76)	−1 (11.6)	0.783
2	1 (10.24)	−1 (11.6)	1.615
3	−1 (1.76)	1 (68.4)	0.996
4	1 (10.24)	1 (68.4)	1.192
5	−1.41 (0)	0 (40)	0.218
6	1.41 (12)	0 (40)	0.713
7	0 (6)	−1.41 (0)	0.225
8	0 (6)	1.41 (80)	2.077
9	0 (6)	0 (40)	1.770
10	0 (6)	0 (40)	1.724
11	0 (6)	0 (40)	1.728
12	0 (6)	0 (40)	1.725

Table 4
Central composite design (CCD 2^2) with 12 assays (corn steep liquor and soybean oil) included four axials points and four central points to increase culture conditions for lipase by *L. scottii* CRM 728 (120 h at 20.0 °C and 180 rpm).

Assay	Corn steep liquor (mL L^{-1})	Soybean oil (mL L^{-1})	Lipase activity (U mL^{-1})
1	−1 (1.76)	−1 (11.6)	0.692
2	1 (10.24)	−1 (11.6)	0.664
3	−1 (1.76)	1 (68.4)	0.409
4	1 (10.24)	1 (68.4)	1.128
5	−1.41 (0)	0 (40)	0.121
6	1.41 (12)	0 (40)	0.926
7	0 (6)	−1.41 (0)	0.375
8	0 (6)	1.41 (80)	0.743
9	0 (6)	0 (40)	1.025
10	0 (6)	0 (40)	1.011
11	0 (6)	0 (40)	0.980
12	0 (6)	0 (40)	0.983

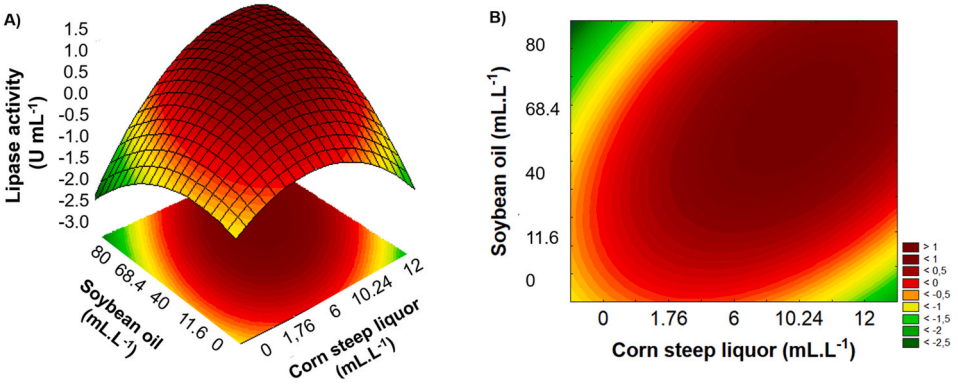

Fig. 2. Surface and contour response plots for lipase production by *L. scottii* CRM 728 (matrix of the experimental design 2^3 CCD). **A, B)** Corn steep liquor and olive oil; **C, D)** Corn steep liquor and soybean oil.

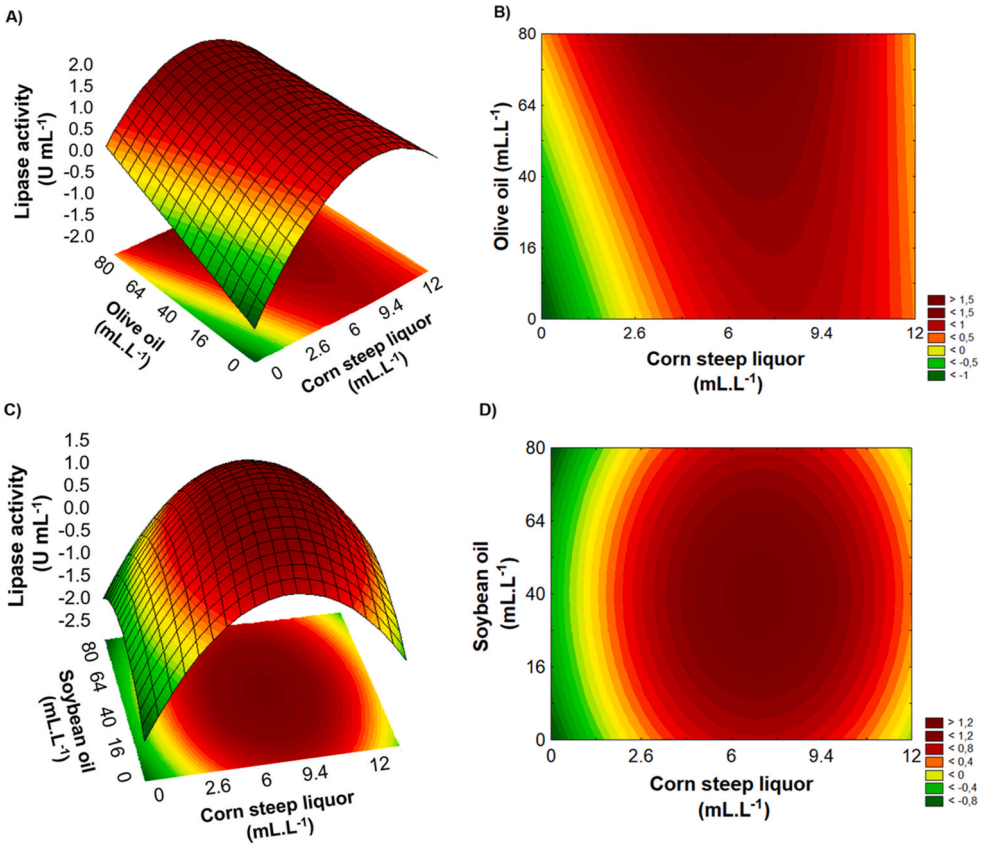

Fig. 3. Surface and contour response plots for lipase production by *L. scottii* CRM 728 (matrix of the experimental design 2^2 CCD*) for corn steep liquor and soybean oil* ($p < 0.05$).

statistically significant ($p < 0.05$), except linear soybean oil, a behavior similarly found in CCD 2^3 (Supplementary Table 3). The statistical significance of the analyzes was confirmed by the F test (ANOVA), as the value of the F test (9.4) for the regression was significant [higher than the tabulated F (4.12)] and the percentage of variation explained by the model was adequate (R^2 = 82.7%, Supplementary Table 4). So, the model was considered predictable and used to generate the response and contour surfaces (Fig. 3). The equation of the reparametrised model is: y = 0.999200 + 0.228937 (corn steep liquor) - 0.192813 (corn steep liquor)^2–0.174957 (soybean oil)^2 + 0.186750 (corn steep liquor x soybean oil).

Comparing the models for soybean oil (Fig. 2C and D, and 3) it is possible to identify that the lipase production by *L. scottii* CRM 728 was better evaluated using the CCD 2^3 and this design was selected as the model of culture medium for the lipase production using soybean oil as carbon source and corn steep liquor as nitrogen source. However, better results of enzymatic activities were achieved in the presence of olive oil as carbon source. In the validation assay, carried out under the optimum conditions in central point with olive oil (40 mL L^{-1}) and corn steep liquor (6 mL L^{-1}), lipase activity (1.7 U mL^{-1}) was closer to the predicted.

Soybean and olive oil have a similar chemical composition, meanwhile the proportion of C18:1 unsaturated fatty acid is different, with 75% and 29% of C18:1 in olive and soybean oil, respectively (Alves et al., 2019). Possibly, lipase induction in *L. scottii* CRM 728 was influenced by C18:1 unsaturated fatty acid rate.

Regardless of the carbon sources used, this study showed a great impact of the corn steep liquor used as nitrogen source, which promotes an increase in the production of lipase by *L. scottii* CRM 728 when used as the only source of nitrogen. This is a great achievement, since the costs related to the enzyme production could be reduced. Corn steep liquor is a product of the corn processing industry and is obtained from maize grain maceration water, containing in its composition the nutrients necessary (amino acids, soluble carbohydrates, mineral salts, and vitamins) for the development and metabolism of fungi (Mironescu et al., 2016; Nurfarahin et al., 2018). Cipolatti et al. (2019) reported the use of corn steep liquor as an alternative substrate for nitrogen source in the culture media. Other studies have been reporting the use of this industrial waste for the production of biomolecules, including lipase (Maldonado et al., 2016; Pereira et al., 2019), surfactant (Santos et al., 2017), carotenoid (Cipolatti et al., 2019), and n-butanol (Cao et al., 2020).

3.2. Independent evaluation of other variables for the optimization of lipase production

Considering the best results of lipase activity using olive oil as carbon source, lipase production was evaluated using others different inducers: tributyrin (C4), tricapriline glycerol (C8), and oleic acid (C18:1) in different concentrations (1, 2 and 4%) together with corn steep liquor (0,6%) at pH 8.0.

Results showed that oleic acid was the best inducer since in the presence of this compound 101.2% of relative activity was observed in comparison to the use of olive oil as carbon source (Fig. 4A). Although there were positive results of lipase activities when tributyrin and tricapriline were used as carbon sources, the relative activities were low. True lipases have greater activity in long-chain triglycerides. These results confirm the preference of *L. scottii* CRM 728 lipase for long-chain triglyceride. According to Maharana and Singh (2018), the Antarctic yeast *Cryptococcus* sp. Y-32 showed better lipase production with olive oil (which contains high amounts of oleic acid) than with tributyrin.

Lipase production by *L. scottii* CRM 728 was reduced in the presence

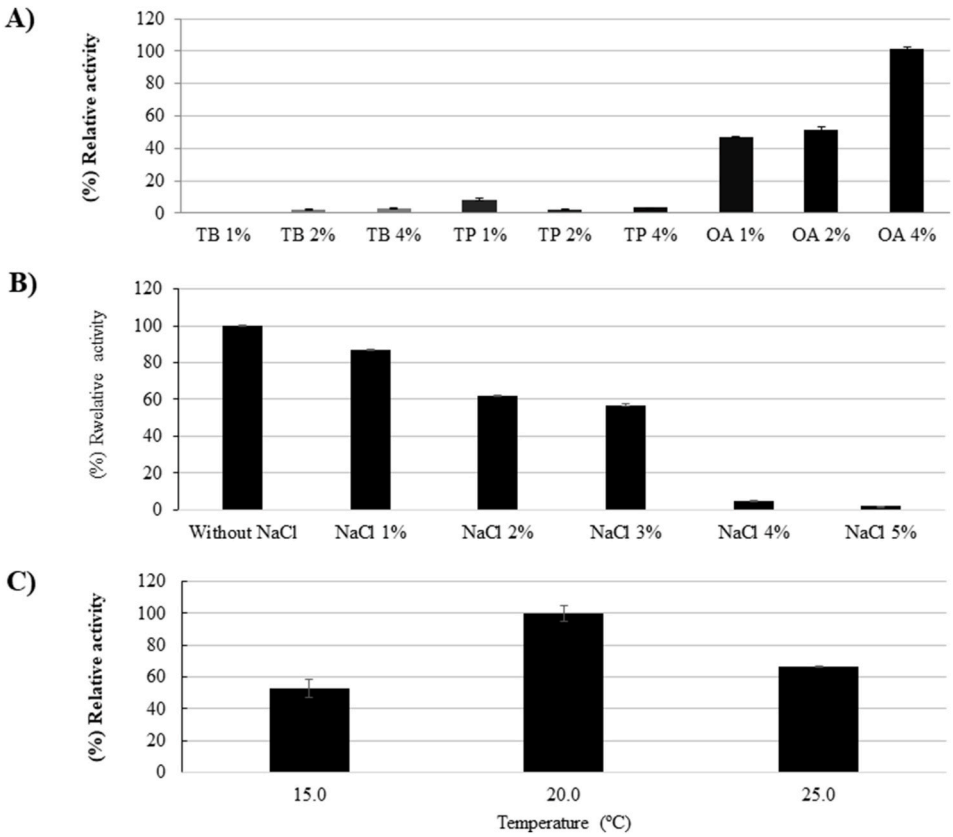

Fig. 4. Relative lipase activity produced by *L. scottii* CRM 728 in different conditions of incubation in comparison to the use of olive oil as carbon source. Different concentration (1–4%) of carbon source. TB – Tributyrin, TP – Tricaprylin, OA – Oleic Acid (A); NaCl concentration (%) (B); temperature of incubation (C).

of NaCl (Fig. 4B). Results revealed a sharp reduction of lipase production from 3 to 4 and 5% of NaCl. This result can be associated with the average salt concentration (34 PSU = 3.4%) in the upper mixed layer of seawater (0.15 and 30 m depth) in the region and period of the sampling collection (Admiralty Bay, OPERANTAR XXVIII, polar summer 2010) (Cascaes et al., 2011). Different result was reported by Smaniotto et al. (2012), where the maximum production of lipase (26.9 U mL^{-1}) by *Sporidiobolus pararoseus* (no origin information) was obtained with 7% of NaCl. On the other hand, *Cryptococcus* sp. Y-32 (isolates from the core sample of Nella Lake, Larsemann Hills region, East Antarctica) showed the ability to grow and produce lipase at 10% w v^{-1} of NaCl (Maharana and Singh, 2018b). It is important to highlight that possibly the increase in the concentration of NaCl had a negative effect on the activity because this salt isolated does not represent the marine environment and may have unbalanced the physiological process of the yeast.

The best temperature for lipase production by *L. scottii* CRM 728 was 20.0 °C (Fig. 4C), with lower enzymatic production at 15.0 and 25.0 °C. This result highlights the origin of *L. scottii* CRM 728 and its cold-adaptation. According to Duarte et al. (2015) the optimum temperature for *L. scottii* CRM 728 lipase activity is 40.0 °C. Similar result was observed for lipase produced by *Meyerozyma guilliermondii* cultivated at 28.0 °C, which showed optimal enzymatic activity at 35.0–40.0 °C (Knob et al., 2020).

3.3. Precipitation of the enzymatic broth

For an initial and exploratory analysis of the enzyme produced by *L. scottii* CRM 728 a precipitation test was applied to eliminate some contaminants from the crude broth. Precipitation of proteins with 95% of cold ethanol was carried out in five conditions (1:1 to 1:5 of crude enzymatic broth: ethyl alcohol, v v^{-1}). The best result for the precipitated fraction (1: 2 – crude enzymatic broth: alcohol ethyl, v v^{-1})

Table 5
Precipitation of lipase from *L. scotti* CRM 728 with cold acetone.

Ratio (enzymatic extract: cold acetone)	Lipase activity U mL^{-1}
1.1	1.54
1.2	2.49
1.3	3.38
1.4	2.81
1.5	2.37

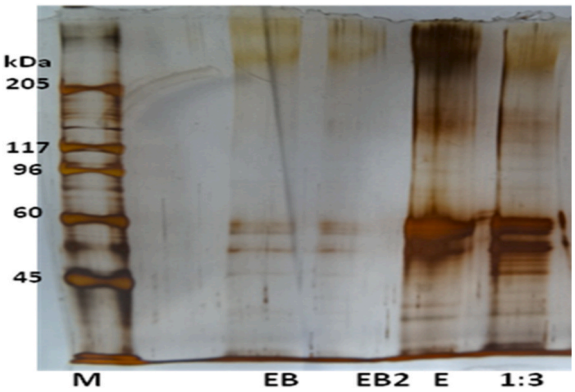

Fig. 5. Polyacrylamide gel of lipase produced by *L. scottii* CRM 728. Protein bands were stained with silver nitrate. Lane M: molecular weight markers. Lanes EB and EB2: Crude broth.; Line E – Fraction precipitate in cold ethanol (1:2); Line 1:3 - Fraction precipitate in cold acetone (1:3). Zymogram (B): Line 1: Fraction precipitate in cold acetone (1:3); Line 2: Fraction precipitate in cold ethanol (1:2).

showed 78.52% of enzymatic recovery and 1.5-fold purification factor in comparison to crude broth. However, the precipitated fraction showed intense viscosity, possibly due to the precipitation of triglyceride residues from the culture medium. So, a new precipitation was carried out with cold acetone and resulted in a higher activity in the ratio 1:3 crude enzymatic broth: cold acetone (Table 5, Fig. 5.), yield of 90.5% and a purification factor of 5.08.

According to SDS-PAGE (Fig. 5.) the crude broth did not have a load of significant contaminating proteins, highlighting only a few low molecular weight (<45 kDa) compounds in small amounts. After precipitation the two bands between 45 and 60 KDa stood out. As reported by Vanklu and Kour (2006), the majority of lipases produced by yeast are extracellular monomeric glycoproteins with molecular weight ranging between ~33 and ~65 kD and more than 50% of the lipases produced by yeast are isozymes, as showed by *L. scottii* CRM 728.

4. Conclusions

The experimental design approach was successfully applied, allowing the evaluation of the effect and the interaction of different carbon and nitrogen sources on the lipase production by the marine-derived Antarctic yeast L. *scottii* CRM 728. The yeast was able to use corn steep liquor as sole nitrogen source for the production of lipase, which can result in a reduction of the costs associated with the enzyme production, and also adding value to this agro-industrial waste, minimizing the environmental problems related to its disposal/treatment.

As far as our knowledge goes, this is the first study to report the optimization of culture conditions for the production of lipase by the psychrotolerant yeast *L. scottii*. The best conditions were achieved in a non-saline medium and at 20.0 °C. These results highlight the potential use of lipases and/or yeast *L. scottii* CRM 728 in several industrial sectors that require low or mild temperatures, as well as in the environmental sector, for the treatment of effluents and environments contaminated with fatty/oil compounds at mild temperatures.

Declaration of competing interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2021.101954.

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