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Lipase from *Fusarium solani*: optimization of culture conditions, biochemical properties, and production

Lipase de *Fusarium solani*: otimização das condições de cultivo, propriedades bioquímicas e produção

Lipasa de *Fusarium solani*: optimización de las condiciones de cultivo, propiedades bioquímicas y producción

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Abstract
A Plackett-Burman factorial design with 15 experiments was conducted to evaluate the influence of seven factors on lipases production by *Fusarium solani*. The factors investigated were peptone, tryptone, yeast extract, calcium chloride, potassium phosphate, magnesium sulfate, and copper sulphate. Five fixed variables (cotton oil, pH, temperature, agitation, and time) were maintained and as a response to the enzymatic activity. The concentration of tryptone, calcium chloride, and magnesium sulphate had a significant effect ($p < 0.10$) on lipase production and was studied consecutively through a complete DCCR (central rotational compound design), to optimize lipase production of the fungi *F. solani*. After optimization using DCCR, maximum lipolytic activities of 24.84 U/ml were obtained with the use of 10 $g.L^{-1}$ tryptone, 3.50 $g.L^{-1}$ calcium chloride and 0.50 $g.L^{-1}$ magnesium sulfate, 1 $g.L^{-1}$ potassium phosphate and 1% soybean oil. The statistical model showed a correlation of 85.67% with the experimental data. The biochemical characterization of lipase showed that the enzyme has a better performance at pH 7 at a temperature of 40 °C, where the statistical model had a correlation of 94.15% with the experimental data. In this way, lipases produced by *F. solani* have potential for application and use in biodiesel production.
Keywords: Lipase; Optimization; Hydrolysis, Esterification; *Fusarium solani.*

Resumo
Um planejamento fatorial Plackett-Burman com 15 experimentos foi conduzido para avaliar a influência de sete fatores na produção de lipases por *Fusarium solani*. Os fatores investigados foram peptona, triptona, extrato de levedura, cloreto de cálcio, fosfato de potássio, sulfato de magnésio e sulfato de cobre. Cinco variáveis fixas (óleo de algodão, pH, temperatura, agitação e tempo) foram mantidas e como resposta à atividade enzimática. A concentração de triptona, cloreto de cálcio e sulfato de magnésio teve efeito significativo ($p < 0,10$) na produção de lipase e foi estudada consecutivamente através de um DCCR completo (delineamento de composto rotacional central), para otimizar a produção de lipase do fungo *F. solani*. Após otimização com DCCR, atividades lipolíticas máximas de 24,84 U/ml foram obtidas com o uso de 10 g.L-1 de triptona, 3,50 g.L-1 de cloreto de cálcio e 0,50 g.L-1 de sulfato de magnésio, 1 g.L-1 de fosfato de potássio e 1% de óleo de soja . O modelo estatístico apresentou uma correlação de 85,67% com os dados experimentais. A caracterização bioquímica da lipase mostrou que a enzima tem um melhor

desempenho em pH 7 na temperatura de 40 °C, onde o modelo estatístico teve uma correlação de 94,15% com os dados experimentais. Desta forma, as lipases produzidas por *F. solani* apresentam potencial para aplicação e uso na produção de biodiesel.
Palavras-chave: Lipase; Otimização; Hidrólise; Esterificação; *Fusarium solani*.

Resumen
Se realizó un diseño factorial de Plackett-Burman con 15 experimentos para evaluar la influencia de siete factores en la producción de lipasas por *Fusarium solani*. Los factores investigados fueron peptona, triptona, extracto de levadura, cloruro de calcio, fosfato de potasio, sulfato de magnesio y sulfato de cobre. Se mantuvieron cinco variables fijas (aceite de algodón, pH, temperatura, agitación y tiempo) y como respuesta a la actividad enzimática. La concentración de triptona, cloruro de calcio y sulfato de magnesio tuvo un efecto significativo ($p < 0.10$) sobre la producción de lipasa y fue estudiada consecutivamente a través de un DCCR completo (diseño de compuestos rotacionales centrales), para optimizar la producción de lipasa del hongo *F. solani*. Después de la optimización con DCCR, se obtuvieron actividades lipolíticas máximas de 24,84 U/ml con el uso de 10 g.L-1 de triptona, 3,50 g.L-1 de cloruro de calcio y 0,50 g.L-1 de sulfato de magnesio, 1 g.L-1 de fosfato de potasio y 1% de aceite de soja. El modelo estadístico mostró una correlación del 85,67% con los datos experimentales. La caracterización bioquímica de la lipasa mostró que la enzima tiene un mejor desempeño a pH 7 a una temperatura de 40 °C, donde el modelo estadístico tuvo una correlación de 94.15% con los datos experimentales. De esta forma, las lipasas producidas por *F. solani* tienen potencial de aplicación y uso en la producción de biodiesel.
Palabras clave: Lipasa; Otimização; Hidrólise; Esterificação; *Fusarium solani.*

1. Introduction

Currently, enzymes have several industrial applications (Haack et al., 2006) and among them, lipases are widely used (Colla et al., 2016). Their applications result from the ability to catalyze reactions, especially hydrolysis and inter-and transesterification of lipids, making these enzymes useful in several sectors, including the production of lipids with high levels of unsaturated fatty acids (Reshma et al., 2008) and methyl- esters of fatty acids (biodiesel) (Park et al., 2006).

Industrial enzymes are mainly produced by submerged fermentation in batch and fed-batch cultures (HAACK et al., 2006) using filamentous fungi. Filamentous fungi, especially those belonging to the genera *Aspergillus*, *Fusarium*, *Geotrichum*, *Mucor*, *Penicillium*, *Rhizomucor*, and *Thermomyces* are known as the best microbial agents producing lipases (MESSIAS et al., 2011). Due to the wide versatility of action of this group of filamentous fungi in producing lipases, its industrial and technological use has grown significantly (Maciel et al., 2010). These industrial applications stimulated interest in the isolation of new lipases from new sources (Dheeman et al., 2011). Submerged processes have some advantages over solid-state processes, such as higher homogeneity of the culture medium and easier control of parameters, such as temperature and pH.

Coradi et al. (2013) mentioned that lipases produced by submerged fermentation have a better recovery of extracellular enzymes and the biomass determination is facilitated by simple filtration or centrifugation. Other factors, such as nutrient types and concentrations, pH, agitation, and the presence and concentration of inducers may affect the productivity of these bioprocesses. Researches using microorganisms isolated from new environments and using agro-industrial waste in the composition of medium are necessary to obtain high yields at lower costs.

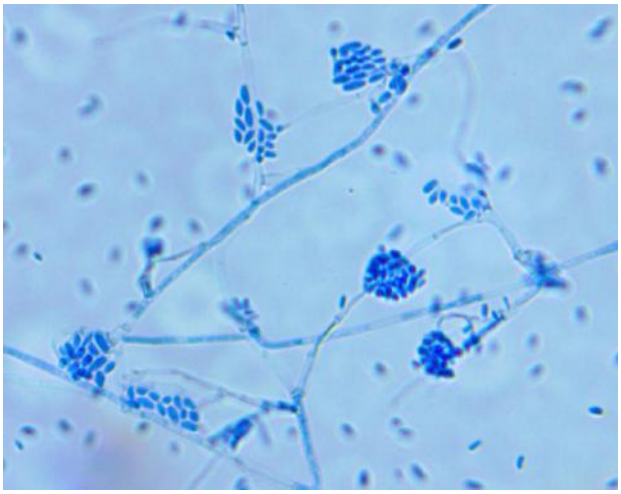
The statistical optimization of the processes has advantages over the classical practice of changing one variable at a time (Box et al., 1978; Kaushik et al., 2006) with a smaller number of experiments and the possibility of evaluating the effects of interaction between the variables. Many researchers have reported the use of these techniques for the production of lipases by microorganisms (Burkert et al., 2004; Wang et al., 2008). An efficient and widely used approach is the application of Plackett-Burman projects that make possible the efficient triage of key variables for additional optimization rationally. The objective of this research was to optimize lipase production by the fungus *F. solani*, evaluate the significant variables for lipase production through submerged fermentation, and optimize these variables through the response surface methodology.

2. Methodology

2.1 Fungal strain used

The fungal Figure 1 strain selected was *F. solani* obtained from a previous work where, after the shaker incubation, the isolate was tested for lipase activity, so this strain was selected because it had the highest enzymatic activity.

Figure 1. Morphology of *Fusarium solani* (zoomed 40 times).



Source: Authors.

2.2 Culture medium

The selected *F. solani* strain was tested for the ability to produce enzymes (lipases) by submerged fermentation in the basal medium containing the following composition: KH_2PO_4 1,00 $g.L^{-1}$; $MgSO_4.H_2O$ 1,123 $g.L^{-1}$; $CuSO_4$ 0,06 $g.L^{-1}$ (PERA et al., 2006), supplemented with 1% cotton oil as carbon source, 2% tryptone, 2% peptone and 2% yeast extract as a nitrogen source.

2.3 Inoculum standardization and the obtaining of the crude enzymatic extract by submerged fermentation

The selected *F. solani* strain was grown in culture medium prepared by diluting 39 $g.L^{-1}$ potato dextrose agar (BDA) in distilled water, plus 0.2% chloramphenicol. The pH was adjusted to 5. The medium was autoclaved at 121 °C for 15 min at 1 atm pressure.

The inoculation was performed punctually in the center of the Petri dishes containing the culture medium. The cultures were kept in a BOD greenhouse at 28 °C for 7 days. The surface of all the plates was added to 5 mL of 0.85% NaCl saline, and was later gently scraped with sterile stiletto to obtain spores. The suspension was filtered and the spore concentration of the filtrate was determined in Neubauer bright-line Optik Labor chamber. The suspension, when necessary, was adjusted so that the spore count was close to the concentration of $1x10^7$ $spores.mL^{-1}$, concentrating it with more inoculum or diluting it with saline.

The crude enzymatic extract was obtained by culturing the lineages in *Shaker Cienlab* CE-725 R for 5 days at 28 °C and 180 rpm. The medium used is described in item 2.2. After the culture, the medium was subjected to filtration of the solids, the filtrate was used as an enzymatic extract for analytical tests, and the crude extracts were stored in glass flasks at -10 °C in the Laboratory of Environmental Chemistry and Biofuels Research (LAPEQ - UFT).

2.4 Experimental design

2.4.1 Plackett and Burman (PB) 7 variables

The experimental design carried out in the first stage of the work aims to evaluate the variables effect for optimizing the composition of the culture medium used for lipase production. This was obtained from a Plackett and Burman type design with fifteen experiments, with three central points, having seven independent variables analyzed (peptone, tryptone, yeast extract, calcium chloride, potassium phosphate, magnesium sulphate and copper sulphate), five fixed variables (cotton oil, pH, temperature, agitation and time) and as a response, the enzymatic activity was obtained.

This experimental design presents an effect table and a Pareto chart, where it is possible to analyze the influence of the main effects, indication of the variables that should be included in the subsequent planning and the new ranges that should be studied for each variable.

2.4.2 Central Rotational Compound Design (DCCR) 3 variables

Through the analysis of the first experimental design, it was possible to carry out a central rotational compound type design with the culture medium used for lipase production. Each parameter was studied in four different levels (-1.68, -1, 0, +1, +1.68). All parameters were taken as a central coded value, considered as an ideal point of production, and a matrix of 17 experiments with three factors was generated by using the program Protimiza (2014). In the DCCR, three central points were used to verify the process reproducibility, whose three independent variables (tryptone, calcium chloride, and magnesium sulfate) and six fixed ones (potassium phosphate, cotton oil, pH, temperature, agitation and time) were analyzed and obtained the enzymatic activity as a response.

This experimental design aimed to study the calculation of the effects, to statistically analyze the experiment and to evaluate the surface response graphs, thus obtaining the optimization of the culture medium for lipase production.

2.5 Validation of the experiment

An experimental design was carried out using the Minitab 18 statistical software, where the results of the central rotational compound design (DCCR) 3 variables were added to validate the lipase production experiment. The optimum condition for lipase production was determined with the program from the strain analyzed and the experiment was conducted to verify the results. Six experiments were carried out with the same conditions to verify the reproducibility of the results, and three independent variables (tryptone, calcium chloride, and magnesium sulphate), six fixed variables (potassium phosphate, cotton oil, pH, temperature, agitation and time) and the enzymatic activity as a response.

2.6 Biochemical characterization

2.6.1 Determination of optimum temperature and pH

A DCCR was performed with eleven experiments, of which three central points, having two independent variables analyzed (pH and temperature) and obtaining as response the enzymatic activity. Each parameter was studied in four different levels (-1.41, -1, 0, +1, +1.41). The objective of this experimental design was to study the effects, statistically analyze the experiment, and evaluate the response surface graphs, thus obtaining the definition of the best temperature and pH for the optimization experiments for lipase production.

2.6.2 Determination of pH stability

The determination of pH stability was performed according to Jinaporn et al. (2016), with modifications.

Optimum pH of lipase activity was determined under lipase assay conditions using different pHs buffers. The Mcilvaine buffer (Na_2HPO_4 and citric acid, pH 3.0 - 8.0) was used. *P*-nitrophenyl palmitate (p-NPP) was used as the substrate at a reaction time of 1 min at 40 ºC. The stability effect on pHs on lipase activity was studied over 0, 24, 48, and 72 hours of incubation.

2.6.3 Determination of thermal stability

The determination of lipase thermal stability was defined by incubating it at optimum pH and different temperatures: 35, 40, 50, and 60 °C throughout the time to its half-life (0, 1, 2, 3, 4, 5, 10, 15, 20, 30, 40, 60 and, 80 minutes). The effect of temperature on lipase activity was studied using the p-NPP test as substrate in Mcilvaine buffer for 1 min reaction at 40 °C.

2.7 Determination of activity and stability in solvents

The effect of various organic solvents on the lipase activity at 10% v / v (50 μL of organic solvent plus 450 μL of crude enzyme) was investigated using several water immiscible organic solvents (toluene, hexane, xylol) and soluble-in-water organic solvents (methanol, ethanol, glycerol, butanol, propanol, 2-propanol, iso-butyl, acetone, acetonitrile and dimethylsulfoxide (DMSO)). The mixture was incubated in tubes and the lipase activity was studied using the p-NPP assay as a substrate in Mcilvaine buffer for 1 min reaction at 40 °C. The activity at incubation was determined at 0, 8, 12, 24, 32, 36, and 48 hours or until its half-life. A sample without addition of organic solvents was taken as control.

2.8 Determination of pH

The determination of pH was performed by reading the samples in a pH meter of the brand *Tecnal*, model TEC – 5.

2.9 Determination of hydrolytic activity

Lipase activity was determined with *p*-nitrophenyl palmitate (*p*-NPP) as substrate, according to Almeida et al. (2013). The *p*-NPP was dissolved in 0.5 mL of dimethylsulfoxide (DMSO) and then diluted to 50 mM with 50 mM sodium phosphate buffer pH 7.0 containing 0.5% (w / v) Triton X-100. The hydrolysis of *p*-NPP was determined discontinuously at 40 °C by measuring the *p*-nitrophenol release. After 5 min of preincubation of 0.9 mL of the substrate and addition of 0.1 mL of properly diluted enzyme sample, the reaction was started. The reaction was then stopped at different intervals by thermal shock at 90 °C for 1 min, followed by the addition of 1 mL of saturated sodium tetraborate solution. The absorbance was measured at 410 nm, which corresponds to the molar extinction coefficient for *p*-NP: $1.8 \times 10^4 M^{-1}$ cm^{-1}. The controls were prepared without enzyme. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-NP per minute.

2.10 Determination of the production of fungal biomass

After cultivation for inoculum standardization, the mycelium was vacuum filtered by using *Büchner*'s funnel and *Wathman* No. 1 filter paper. After the solids filtration, the moist mycelium was oven-dried at 65 °C for 1 day and then weighed with the analytical balance *Shimadzu*, model AX 200, for determining biomass production according to Berovic et al. (2003).

3. Results and Discussion

3.1 Delineamento experimental Plackett e Burman (PB) 7 variáveis

The chosen experimental design of the type of Plackett and Burman was important, because the independent variables were studied to obtain the best formulation for the production of lipase by *F. solani*. The actual decoded values used for this planning are set out in Table 1 with the encoded levels. The experimental design of PB 7 is presented in Table 2.

Table 1. Range of values studied in experimental planning of PB 7 optimization in the production of lipases.

Variable name	**-1**	**0**	**+1**
Peptone*	0	10	20
Yeast extract*	0	10	20
Tryptone*	0	10	20
$CaCl_2.2H_2O$*	0	3,50	7
KH_2PO_4*	0	1	2
$MgSO_4.7H_2O$*	0	0.50	1
$CuSO_4$*	0	0.06	0.12

* Unitis $g.L^{-1}$ Source: Protimiza.

Table 2. First experimental design of PB 7 optimization in the production of lipases.

Experiments	Coded variables							Real variables*							Enzimatic activity (Y1)
	(x1)	(x2)	(x3)	(x4)	(x5)	(x6)	(x7)	Peptone	Yeast extract	Tryptone	$CaCl_2.2H_2O$	KH_2PO_4	$MgSO_4.7H_2O$	$CuSO_4$	
1	1	-1	1	-1	-1	-1	1	20	0	20	0	0	0	0.12	8.04
2	1	1	-1	1	-1	-1	-1	20	20	0	7	0	0	0	0.55
3	-1	1	1	-1	1	-1	-1	0	20	20	0	2	0	0	7.87
4	1	-1	1	1	-1	1	-1	20	0	20	7	0	1	0	0.54
5	1	1	-1	1	1	-1	1	20	20	0	7	2	0	0.12	1.47
6	1	1	1	-1	1	1	-1	20	20	20	0	2	1	0	6.27
7	-1	1	1	1	-1	1	1	0	20	20	7	0	1	0.12	1.09
8	-1	-1	1	1	1	-1	1	0	0	20	7	2	0	0.12	2.04
9	-1	-1	-1	1	1	1	-1	0	0	0	7	2	1	0	0.07
10	1	-1	-1	-1	1	1	1	20	0	0	0	2	1	0.12	0.48
11	-1	1	-1	-1	-1	1	1	0	20	0	0	0	1	0.12	0.77
12	-1	-1	-1	-1	-1	-1	-1	0	0	0	0	0	0	0	0.05
13	0	0	0	0	0	0	0	10	10	10	3.5	1	0.5	0.06	1.64
14	0	0	0	0	0	0	0	10	10	10	3.5	1	0.5	0.06	1.69
15	0	0	0	0	0	0	0	10	10	10	3.5	1	0.5	0.06	1.67

* Unitis $g.L^{-1}$ Source: Protimiza Experimental Desing.

Experiment 1 obtained the highest activity index of 8.04 U/mL after the fifth day of fermentation due to the influence of the interactions between the variables. Experiment 3 also had a result of 7.87 U/mL, close to the maximum found in this step.

Observing the results at the central points (experiments 13, 14, and 15), it is possible to notice that there was no great variation in the values of lipolytic activity, indicating, therefore, a good reproducibility in the data of the process. Burkert et al. (2004), report that central points provide additional degrees of freedom for error estimation, which increases power by testing the significance of effects. The estimate of a main effect is obtained by evaluating the difference in process performance caused by a change from the low level (-1) to the high level (+1) of the corresponding factor.

The lipolytic activity was evaluated at three different pHs: 5, 6, and 7. The major activities were determined at pH 7, which was defined as a parameter for other stages of this work. The analysis of the statistical data obtained from the experimental planning PB 7 allows us to obtain an effect table and a Pareto chart, where it is possible to verify which variables are statistically significant to the process.

Table 3 shows the main effects of the variables in PB 7 planning after the fifth day of fermentation. It was verified that only three of the variables studied had a statistically significant effect on the lipolytic activity when the concentration changed from level -1 to level +1.

Table 3. The main effects of the variables in PB 7 for optimization in the production of lipases.

Name	Effect U/mL	Standard error	Calculated t	p-value
Mean	2.44*	0.45*	5.36*	0.0017*
Curvature	-1.54	2.03	-0.76	0.4773
Peptone (x1)	0.91	0.91	1.00	0.3554
Yeast extract (x2)	1.13	0.91	1.25	0.2589
Tryptone (x3)	3.74*	0.91*	4.12*	0.0062*
$CaCl_2.2H_2O$ (x4)	-2.95*	0.91*	-3.25*	0.0175*
KH_2PO_4 (x5)	1.19	0.91	1.31	0.2372
$MgSO_4.7H_2O$ (x6)	-1.80*	0.91*	-1.98*	0.0950*
CuSO4 (x7)	-0.24	0.91	-0.27	0.7979

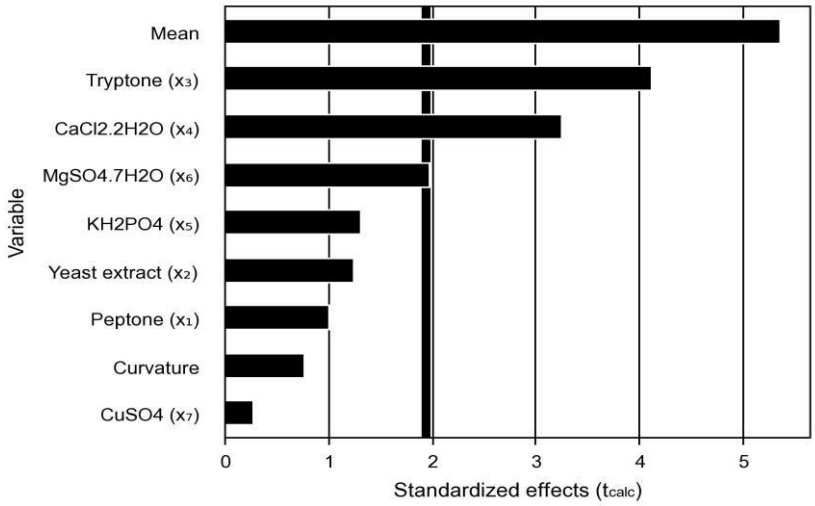
* Statistically significant values at 90% confidence ($p<0.10$). Source: Protimiza Experimental Desing.

The calculated t-value measures the effect amplitude in relation to its standard error. This value was obtained by dividing each effect value by its standard error. The low "p" values were associated with the larger "t" value because they imply that the effects (or coefficients) were much larger than the standard error.

According to the effects presented in Table 3, it was found that tryptone concentration was the only one with a positive effect on lipolytic activity, that is, there was an increase in enzymatic activity when the concentration of this variable went from level -1 to 1 The tryptone concentration had a positive effect of 3.74 U/mL, being statistically significant at 90% confidence. On the other hand, the concentrations of calcium chloride and magnesium sulfate had a negative effect of -2.95 U/mL and -1.80 U/mL respectively, statistically significant at 90% confidence, since there was a decrease in activity when the concentration went from level -1 to +1.

Graph 1 represents the values reported in table 3, where it is possible to observe the three variables that were statistically significant. Considering the results presented and observed in the Pareto graph, potassium phosphate, yeast extract, peptone, and copper sulfate are excluded for the next planning because they are not statistically significant to the process.

Graph 1. The main effects of the significant variables in PB 7.



Source: Protimiza Experimental Desing.

From the results obtained in this experimental design, tryptone, calcium chloride, and magnesium sulfate were selected as variables to be studied in a complete experimental design, with aiming tooptimize the culture medium for the lipases production.

3.2 Central Rotational Compound Design (DCCR) 3 variables

This experimental design of the DCCR type was chosen to optimize the culture medium for the lipases production by *Fusarium solani*. Based on the results obtained from the previous experimental planning PB 7, only 3 variables were analyzed, tryptone, calcium chloride, and magnesium sulfate, standing fixed potassium phosphate, cotton oil, pH, temperature, agitation and time, obtaining as response the enzymatic activity.

In this stage of the work, yeast extract, peptone, and copper sulfate were not included, as these were not statistically significant as seen in Pareto Graph 1 in the previous step. Potassium phosphate, even though having a negative effect and not being statistically significant in the process, such a micronutrient is biologically essential for the development and production of enzymes by the evaluated strain as this is the only source of phosphorus available to the microorganism. As the purpose of this experiment is to optimize the culture medium in order to obtain maximum lipolytic activity, the addition of potassium phosphate was maintained as a necessary micronutrient to the culture medium.

The minimum and maximum ranges of the parameters were investigated and the complete experimental design with their values in real and codified form are listed below in Table 4. The average maximal lipase activity was taken as the dependent variable or response (Y1). A second-order polynomial equation was then fitted to the data by the multiple regression procedure. This resulted in an empirical model that related the response measured in the independent parameters Eq. (1). Only the central point was made in triplicate. For a three-factor system, the equation of the model is as follows:

$$Y1 = 24{,}15 + 1{,}57\,x1 - 6{,}14\,x1^2 - 1{,}54\,x2 - 2{,}23\,x2^2 + 0{,}67\,x3 - 3{,}45\,x3^2 + 2{,}08\,x1\,x2 - 0{,}12\,x1\,x3 + 0{,}51\,x2\,x3$$

Eq.(1)

where Y_1 is the predicted response, X_1, X_2, X_3 are the linear coefficients; X_1^2, X_2^2, X_3^2 are the quadratic coefficients, and X_1 - X_2, X_1 - X_3, X_1 – X_3 are the interaction coefficients.

The actual decoded values used for this planning are arranged in Table 4 with the encoded levels. The experimental design of DCCR 3 is presented in Table 5.

Table 4. Range of values studied in the experimental design of DCCR 3 optimization in the production of lipases.

Variable name *	**-1,68**	**-1**	**0**	**+1**	**+1,68**
Tryptone	0	4.05	10.00	15.95	20
$CaCl_2.2H_2O$	0	1.41	3.50	5.58	7
$MgSO_4.7H_2O$	0	0.20	0.50	0.79	1

* Unitis $g.L^{-1}$ Source: Protimiza Experimental Desing.

Table 5. Experimental planning of DCCR 3 optimization in the production of lipases.

Experiments	**Coded variables**			**Real variables ***			**Enzimatic activity (Y1)**
	(x1)	**(x2)**	**(x3)**	**Tryptone**	**$CaCl_2.2H_2O$**	**$MgSO_4.7H_2O$**	
1	-1.00	-1.00	-1.00	4.05	1.41	0.20	13.29
2	1.00	-1.00	-1.00	15.95	1.41	0.20	12.55
3	-1.00	1.00	-1.00	4.05	5.58	0.20	9.44
4	1.00	1.00	-1.00	15.95	5.58	0.20	9.96
5	-1.00	-1.00	1.00	4.05	1.41	0.79	17.00
6	1.00	-1.00	1.00	15.95	1.41	0.79	8.75
7	-1.00	1.00	1.00	4.05	5.58	0.79	8.16
8	1.00	1.00	1.00	15.95	5.58	0.79	15.27
9	-1.68	0.00	0.00	0.00	3.50	0.50	0.76
10	1.68	0.00	0.00	20.00	3.50	0.50	14.30
11	0.00	-1.68	0.00	10.00	0.00	0.50	22.26
12	0.00	1.68	0.00	10.00	7.01	0.50	14.96
13	0.00	0.00	-1.68	10.00	3.50	0.00	13.58
14	0.00	0.00	1.68	10.00	3.50	1.00	16.68
15	0.00	0.00	0.00	10.00	3.50	0.50	24.30
16	0.00	0.00	0.00	10.00	3.50	0.50	23.06
17	0.00	0.00	0.00	10.00	3.50	0.50	24.84

* Unitis $g.L^{-1}$ Source: Protimiza Experimental Desing.

Analyzing the results shown in Table 5, we can verify that the highest result of lipolytic activity was achieved in experiment 17 with 24.84 U/mL carried out with 10 $g.L^{-1}$ tryptone, 3.50 $g.L^{-1}$ calcium chloride and 0.50 $g.L^{-1}$ magnesium

sulfate, 1 g.L^{-1} potassium phosphate and 1% cotton oil at pH 5. Muralidhar et al. (2001), when optimizing the extracellular production of *Candida cylindracea* lipases, found lower results than this 17.30 U/mL of enzymatic activity, using glucose as carbon source. Salihu et al., 2011, also optimizing lipase production by *C. cylindracea*, found 19.90 U/mL of enzymatic activity, using palm oil as a carbon source.

The results found in experiments 15, 16, and 17, which are the central points, do not present a great difference in characterizing the good reproducibility of the process.

When comparing the maximum lipolytic activities obtained at the higher and lower tryptone concentrations, it seems that increasing the concentration of 10 g.L^{-1} to 20 g.L^{-1} caused a decrease in lipolytic activities. Furthermore, the increase in calcium chloride concentration from 3.5 g.L^{-1} to 7 g.L^{-1} caused a decrease in lipolytic activities. With respect to magnesium sulfate, when an increase in concentration of 0 g.L^{-1} to 0.5 g.L^{-1} caused an increase in lipolytic activities.

According to the results obtained in the DCCR experimental design, the regression coefficients were calculated and a complete statistical analysis was obtained with ANOVA, Pareto graph, and response surface graphs. The results are presented in Tables 6 and 7.

Table 6. Regression coefficients for the second experimental design DCCR 3, optimization in the production of lipases.

Regression				
Name	**Coefficient**	**Standard Error**	**Calculated t**	**p-value**
Mean	24.15	2.12	11.38	0.0000*
$\mathbf{x^1}$	1.57	1.00	1.57	0.1596
$\mathbf{x_1^2}$	-6.14	1.10	-5.60	0.0008*
$\mathbf{x^2}$	-1.54	1.00	-1.55	0.1660
$\mathbf{x_2^2}$	-2.23	1.10	-2.03	0.0821*
$\mathbf{x^3}$	0.67	1.00	0.67	0.5227
$\mathbf{x_3^2}$	-3.45	1.10	-3.15	0.0162*
$\mathbf{x_1 \cdot x_2}$	2.08	1.30	1.60	0.1546
$\mathbf{x_1 \cdot x_3}$	-0.12	1.30	-0.09	0.9321
$\mathbf{x_2 \cdot x_3}$	0.51	1.30	0.40	0.7044

* Statistically significant values at 90% confidence (p<0.10). Source: Protimiza Experimental Desing.

Table 7. ANOVA for the second experimental design DCCR 3, optimization in lipase production.

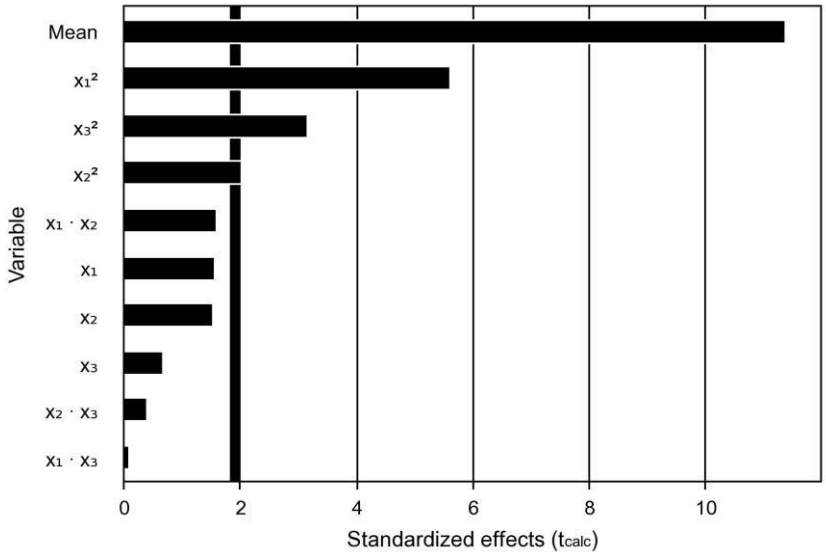
Anova					
Variation source	**Sum of squares**	**Degrees of freedom**	**Mean square**	**f calc**	**p-value**
Regression	567.3	9	63.0	4.65	0.02757
Residuals	94.9	7	13.6		
Lack of fit	93.2	5	18.6	22.4	0.04330
Pure error	1.7	2	0.8		
Total	662.3	16			

% Variation explained (R^2) = 85.67. Source: Protimiza Experimental Desing.

Table 6 shows the regression coefficients for the second DCCR 3 experimental planning. The results found for the variables x_1^2, x_2^2 and x_3^2 are statistically significant at the 90% confidence level. However, they had a negative coefficient of -6.14, -2.23 and -3.45, respectively. It is noteworthy that the R^2 value of this explains 85.67% of the experiment. Kaushik et al. (2006) report that the R^2 value provides a measure of how much the variability in observed response values can be explained by the experimental factors and their interactions. This implies that the sample variation of 85.67% for lipase production was attributed to the independent variables and only 14.33% of the total variation was not explained by the model. Burkert et al. (2004) describe in their work that a model with values above 0.9 is considered good, explain most of the variation in response and the closer the value of the R-squared is to 1.00, the stronger is the model and the better are the predictions of response.

In the same table, it is possible to see that the effect of the variable x_3 and the interaction of the variables x_1 - x_3 and x_2 - x_3 were very small, since they are smaller than the standard error. The value of the interaction effect of variables x_1 - x_3, in addition to being low, had a negative effect. This means that increasing the concentration of magnesium sulfate and the interaction between tryptone X magnesium sulfate, calcium chloride X magnesium sulfate did not influence the value of enzymatic activity at 90% confidence.

Graph 2. The main effects of variables and significant interactions in the second DCCR 3.



Source: Protimiza Experimental Desing.

Graph 2 shows which of the variables and interactions analyzed presented statistically significant results. It is possible to observe that only the quadratic variables were significant for $x1^2$, $x2^2$, and $x3^2$, and the others would not influence the process if an increase of its concentration from the level -1.68 to the level +1.68 occurred.

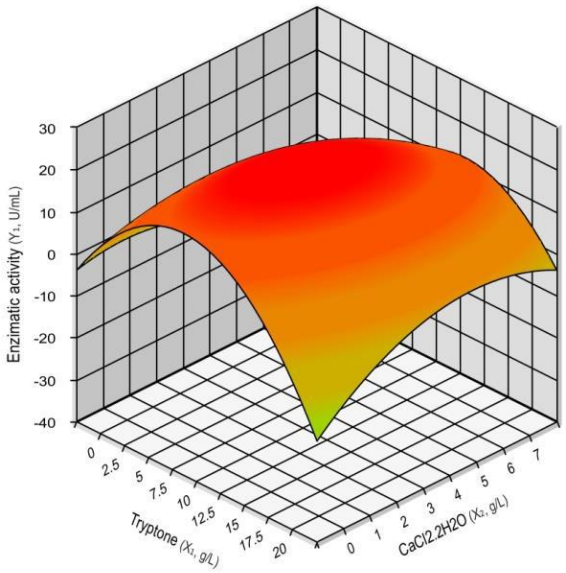
Thus, a readjustment was made in the range of values studied in this experiment with the objective of increasing the enzymatic activity and attending to a higher production.

Figures 2, 3, and 4 below show the response surface found for the optimization of lipase production by the fungus *F. solani*, generated in the prothymatics program. Kaushik et al. (2006) report that the three-dimensional response surface curves plotted by a statistically significant model serve to understand the interaction of the medium components and the optimal concentration of each component required for optimum lipase production. The three-dimensional graphs shown were based on the function of concentrations of two variables, in this way, Muralidhar et al. (2001) report that the significance of the

interactions between the corresponding variables is indicated by an elliptical or saddle characteristic of the surface and contour graphs.

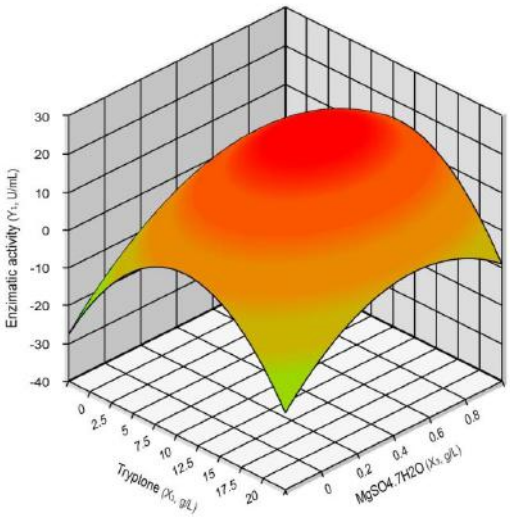
Thus, Figure 2 presents the response surface generated by the enzymatic activity obtained from the interaction of tryptone with calcium chloride, which means that higher levels of both tryptone and calcium chloride did not result in higher enzyme yields. The shape of the response surface curves showed a moderate interaction between these variables tested.

Figure 2. Response surface for enzymatic activity (Tryptone and Calcium Chloride).



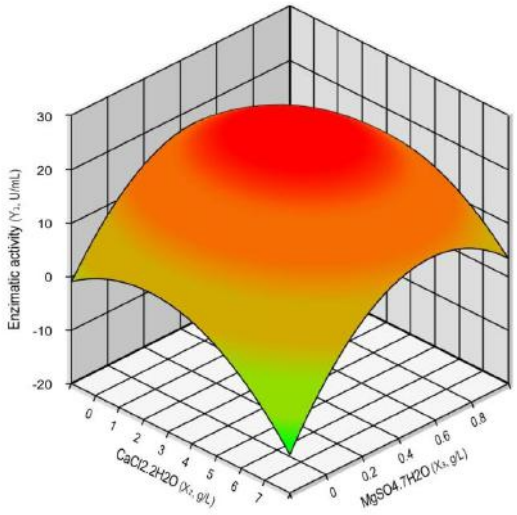
Source: Protimiza Experimental Desing.

Figure 3. Response surface for enzymatic activity (Tryptone and magnesium sulfate).



Source: Protimiza Experimental Desing.

Figure 4. Response surface for enzymatic activity (Calcium chloride and magnesium sulfate).



Source: Protimiza Experimental Desing.

However, Figure 3 shows the response surface generated by the enzymatic activity obtained from the interaction of tryptone with magnesium sulfate, where the best production is in the range of 10 $g.L^{-1}$ to tryptone and 0.5 $g.L^{-1}$ to magnesium sulfate, what means that at lower and higher levels of both tryptone and magnesium sulfate did not result in higher enzyme yields.

Finally, Figure 4 shows the response surface generated by the enzymatic activity obtained from the interaction of calcium chloride with magnesium sulfate, where the best production is in the range of 2.7 $g.L^{-1}$ for calcium chloride and 0.5 $g.L^{-1}$ for magnesium sulfate, that is, lower and higher levels of both calcium chloride and magnesium sulfate would also not result in higher enzyme yields.

3.3 Validation of the experiment

In view of the optimization achieved, a validation of the experiment was proposed to verify the production of lipase by *F. solani*. Based on the results obtained from the previous experimental design DCCR 3, the enzymatic activity and the effectiveness of the compounds (tryptone, calcium chloride, and magnesium sulphate) were analyzed in the validation process. The conditions established in the validation experiment are described in Table 8.

Table 8. Parameters established for validation.

Variable	**Concentration ***
Tryptone	10
$CaCl_2.2H_2O$	1.75
$MgSO_4.7H_2O$	0.5

* concentration in $g.L^{-1}$ Source: Minitab 18.

Table 9 shows the optimization target plotted by the *minitab18* program, which establishes an enzymatic activity target of 13.95 U/mL within the indicated parameters. After the validation process, the maximal enzymatic activity obtained was 13.20 U/mL, which is understood to be promising along the optimization process. To ensure reproducibility, 6 experiments were run under the same conditions.

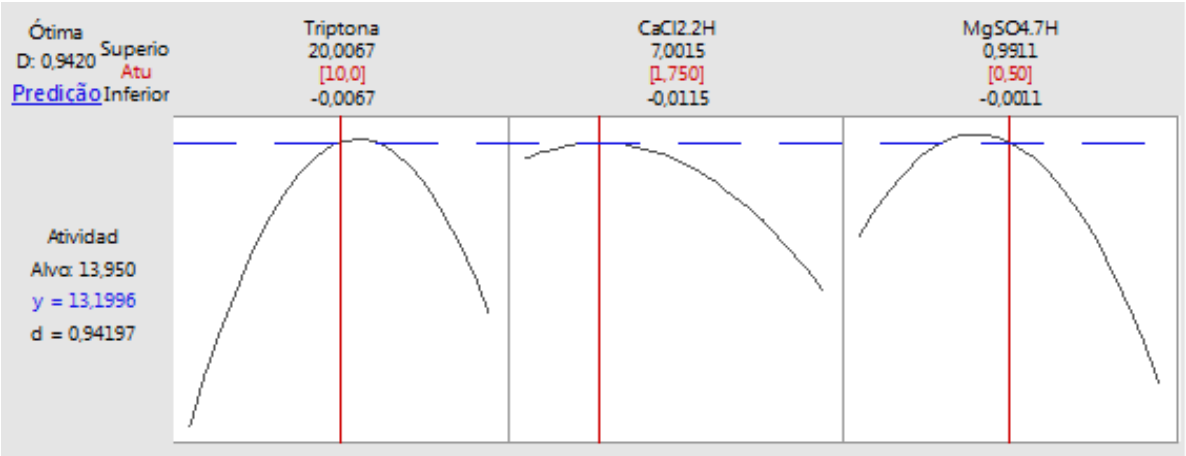
Table 9. Target for optimization of the enzymatic activity response.

Answer	**Goal**	**Bottom**	**Target**	**Higher**
Enzimatic activity	Target	1.02	13.95	15.34

Source: Minitab 18.

Graph 3 shows the behavior profile of the enzymatic activity against the compounds evaluated for the optimization. It can be seen in graph that the target established by Minitab 18 for calcium chloride was reached, but for tryptone and magnesium sulphate, there was a small deviation from the target, making it impossible in the validation process that the enzymatic activity was the maximum defined by it.

Graph 3. Response of experimental validation in lipase production by *Fusarium solani.*



Source: Minitab 18.

3.4 Biochemical characterization

Table 10 shows the ranges of values studied for the DCCR experimental design, temperature and pH. An array of 11 experiments with two factors was generated using the protimiza program. The minimum and maximum ranges of the parameters were investigated and the complete experimental design with their values in real and codified form are listed in Table 11. The average maximal lipase activity was taken as dependent variable or response (Y1). A second-order polynomial equation was then fitted to the data by the multiple regression procedure. This resulted in an empirical model that related the response measured in the independent parameters Eq. (2). All tests were performed where only the central point was performed in triplicate. For a three-factor system, the equation of the model is as follows:

$$Y1 = 13{,}20 + 3{,}28\,x1 - 4{,}11\,x1^2 - 1{,}75\,x2 - 4{,}62\,x2^2 - 1{,}25\,x1\,x2 \quad \text{Eq.(2)}$$

where, Y_1 is the predicted response, X_1 and X_2, are the linear coefficients; X_1^2 and X_2^2 are the quadratic coefficients and X_1 - X_2 are the interaction coefficients.

The experimental design was conducted with 2 variables in 11 experiments under the conditions described in Table 10. The actual decoded values used for this planning are arranged in Table 11, with the coded levels as well as the values of the respective enzymatic activities of each experiment.

Table 10. Range of DCCR experimental planning, temperature, and pH.

Variable name	Units	-1,41	-1	0	+1	+1,41
pH		5.5	6.0	7.0	8.0	8.5
Temperature	° C	25	30	40	50	60

Source: Protimiza Experimental Desing.

Table 11. DCCR experimental design, temperature and pH.

Experiments	Coded variables		Real variables		Enzimatic activity (Y1)
	(x1)	(x2)	pH	Temperature	
1	-1.00	-1.00	6.0	30	0.87
2	1.00	-1.00	8.0	30	8.26
3	-1.00	1.00	6.0	50	1.04
4	1.00	1.00	8.0	50	3.41
5	-1.41	0.00	5.5	40	0.23
6	1.41	0.00	8.5	40	11.88
7	0.00	-1.41	7.0	25	8.34
8	0.00	1.41	7.0	60	1.74
9	0.00	0.00	7.0	40	13.04
10	0.00	0.00	7.0	40	13.35
11	0.00	0.00	7.0	40	13.20

Source: Protimiza Experimental Desing.

When analyzing the results presented in Table 11, we can verify that the highest result of lipolytic activity was reached in experiment 10, with 13.35 U/mL performed at pH 7 and temperature 40 °C. The lowest result was found in experiment 5, with 0.23 U/mL of lipolytic activity performed at pH 5.5 and 40 °C. The results found in the experiments 9, 10, and 11, which are the central points, do not present much difference, characterizing the good reproducibility of the process.

Comparing the minimum and maximum lipolytic activities obtained at the higher and lower pHs, it seems that the increase in lipolytic activity caused an increase in the lipolytic activity, when it increased from the -1.41 level to the 0 level. However, the increase in temperature caused a decrease in lipolytic activity, probably because of the denaturation of the enzyme at such temperatures.

Relating pH and optimum temperature in this work, KEMPKA et al. (2008) have characterized lipase from Penicillium verrucosume and found a pH of 7.0 and temperature of 44 °C. Kamini et al. (1998) characterized the lipase from Aspergillus niger and found a value of pH 7.0. Similar result was obtained by BENJAMIN & PANDEY, 2000, who reported that one of three different forms of lipases produced by Candida rugosa presented optimum activity at pH 7.0 and 40 °C. This temperature value was also found as optimal by Pastore et al. (2003), who studied the characterization of lipase obtained from Rhizopus sp. and observed an optimum pH between 6.0 and 6.5. The same temperature was reported as optimal by SHU et al.

(2006), for lipids of Antrodia cinnamomea, with pH 8.0. Ülker et al. (2010), characterizing the lipase of Trichoderma harzianum, found the optimal activity at pH 8.5 and temperature of 40 °C.

According to the results obtained in the DCCR experimental design, the regression coefficients were calculated and a complete statistical analysis was obtained with ANOVA, Pareto graph, and response surface graph. The results are presented in Tables 12 and 13.

Table 12. Regression coefficients, DCCR experimental design, temperature, and pH.

Regression				
Name	**Coefficient**	**Standard Error**	**Calculated t**	**p-value**
Mean	13.20	1.09	12.16	0.0001*
x_1	3.28	0.66	4.94	0.0043*
x_1^2	-4.11	0.79	-5.20	0.0035*
x_2	-1.75	0.66	-2.64	0.0462*
x_2^2	-4.62	0.79	-5.84	0.0021*
$x_1 \cdot x_2$	-1.25	0.94	-1.34	0.2393

* Statistically significant values at 90% confidence (p<0.10). Source: Protimiza Experimental Desing.

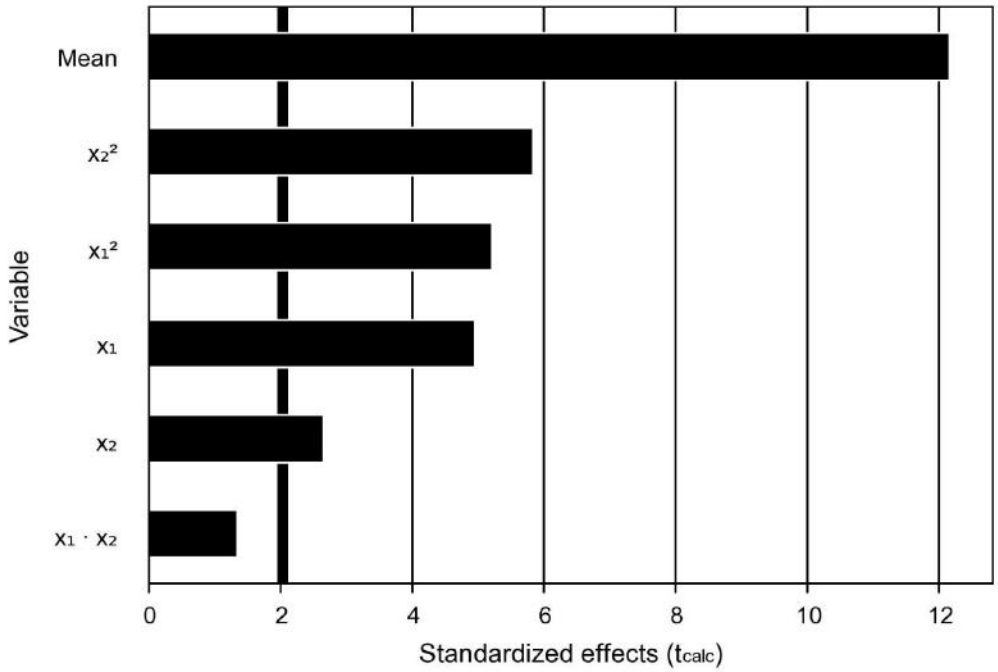
Table 13. ANOVA DCCR experimental design, temperature and pH.

Anova					
Variation source	**Sum of squares**	**Degrees of freedom**	**Mean square**	**f calc**	**p-value**
Regression	284.0	5	56.8	16.08	0.00422
Residuals	17.7	5	3.5		
Lack of fit	17.6	3	5.9	244.26	0.00408
Pure error	0.05	2	0.0		
Total	301.7	10			

% Variation explained (R^2) = 94.15. Source: Protimiza Experimental Desing.

Table 12 shows the regression coefficients for the DCCR experimental design, temperature and pH. The results found for the variables x1, x2, $x1^2$, and $x2^2$ are statistically significant at a 90% confidence level. However, the variables x2, $x1^2$, and $x2^2$ presented a negative coefficient of -1.75, -4.11 and -4.62, respectively. It is worth mentioning that the R^2 value explains 94.15% of the experiment. Kaushik et al., 2006, report that the R^2 value provides a measure of how much the variability in observed response values can be explained by the experimental factors and their interactions. This implies that the sample variation of 94.15% for lipase production was attributed to the independent variables and only 5.85% of the total variation was not explained by the model. In the same Table, it is possible to see that the effect of the interaction of the variables x1 - x2 had the negative coefficient -1.25, which was the lowest one found, not being statistically significant with a *p*-value of 0.2393. This means that the increase in pH or temperature for the interaction did not influence the value of enzymatic activity at 90% confidence.

Graph 4. The main effects of variables and significant interactions on DCCR, temperature, and pH.



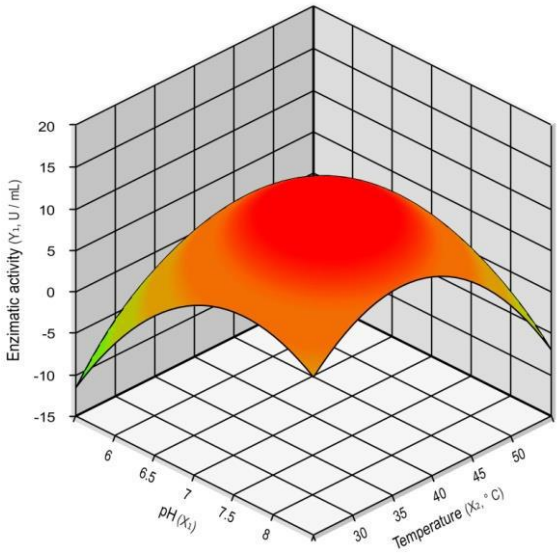
Source: Protimiza Experimental Desing.

Graph 4 shows which of the variables and interactions analyzed presented statistically significant results. It is possible to verify that the linear and quadratic variables, x1, x2, $x1^2$, and $x2^2$, were significant and the interaction of linear variables x1 - x2 would not influence the process if its concentration increased from level -1.41 to level +1.41.

Figure 5 below shows the response surface found for the DCCR, temperature, and pH, which seeks to verify the best range for lipases production by the fungus *F. solani*. Fan et al. (2015) reported that the response surface represents a mathematical and statistical method to optimize an operational condition that is influenced by some independent variables in order to obtain the maximum yield through an economical procedure.

The response surface plot was generated from the six experiments performed, where the best production is in the 40 °C temperature range at pH 7. Through this analysis, it is evident that temperature extremes tend to decrease the lipase production, since low temperatures, lower metabolism, and high temperatures can inactivate lipase.

Figure 5. Response surface for DCCR, temperature and pH.

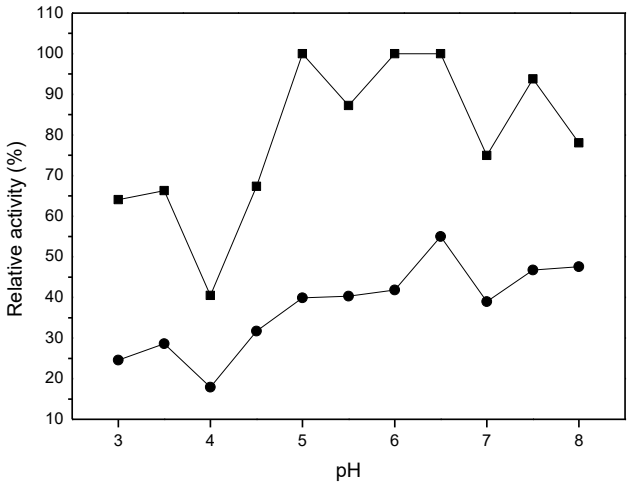


Source: Protimiza Experimental Desing.

3.4.1 Determination of stability in pH

When lipase activity and stability in the crude extract were evaluated in the pH range from 3.0 to 8.0 (Graph 5), both times of 24 and 48 hours presented similar profiles with the highest activity at pH 6.5.

Graph 5. The effect of pH on the stability of *Fusarium solani* lipase in the crude extract. Activity was determined in McIlvaine buffer buffer for pH between 3.0 to 8.0. Lipid activity was determined in McIlvaine buffer pH 7.0 at 40° C (■) crude extract lipase 24 h and C (●) crude extract lipase 48 h.



Source: Authors.

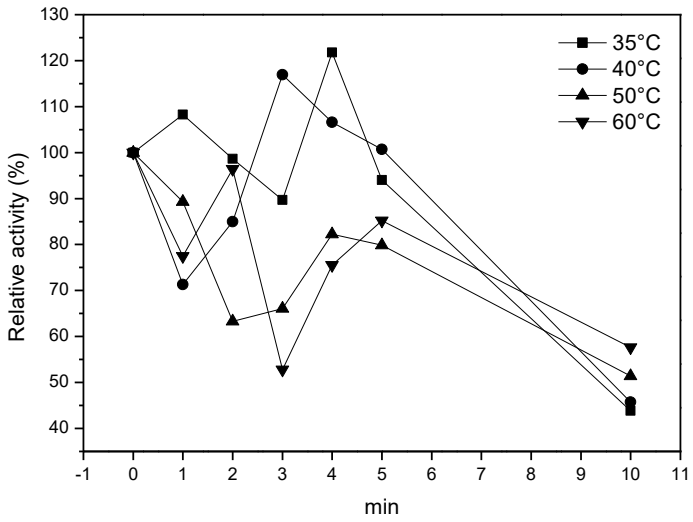
During incubation at different pHs, lipase started to stabilize at 65% activity in the range of pH 3.0 to 3.5, becoming stable over 24 hours in the pH range of 6.0 to 6.5 with 100% activity. At the same time, lipase maintained more than 90% activity in the range of pH 7.5 and with a decrease to 80% in activity at pH 8. Almeida et al. (2018) evaluating the stability at free and immobilized lipase pH of *C. viswanathi*, found for the same pH range, 90% activity for immobilized lipase and between 60-90% activity for free lipase. Üker et al. (2010), when evaluating the lipid pH stability of *Trichoderma harzianum*, found for the same time range a retention of 80% activity at pH 6.5 and around 90% activity at pH 8. Lima et al. (2004), evaluating the activity and stability of a crude lipase of *Penicillium aurantiogriseum*, reported that residual activity remained above 80% between pHs 5 and 9.

At 48 hours, lipase reached its half-life, with its initial activity retained in more than 70% in the range of pH 3.0 to 3.5. The crude lipase of *Fusarium solani* presented maximum stability in 48 hours, between pHs 7.5 and 8.0 with more than 55% of retained activity. It retained 50% of its activity in the range of pH 6.5.

3.4.2 Determination of thermal stability

When the thermal stability of the lipase in the crude extract was analyzed, the enzyme maintained about 120% activity up to 4 minutes at 35 °C and the half-life was in the time of 10 min (Graph 6). ÜLKER et al. (2010), evaluating the thermal stability of lipase of *Trichoderma harzianum*, found a stable profile with a retention of 100% activity at temperatures of 20, 30, and 40 °C.

Graph 6. Thermostability of *F. solani* in the crude extract. Lipase activity was determined in McIlvaine buffer pH 7.0 at 40 ° C. The enzyme was incubated for 10 min.



Source: Authors.

The half-lives of the enzyme at 40, 50, and 60 ºC were all in the time of 10 min. Lipase did not have a desired profile for thermal stability, but maintained 110% activity in the time of 3 min at 40 °C, 90% and 80% activity in the times of 1 min and 4 min respectively at 50 °C and 95% activity in the time of 2 min at 60 °C. Hiol et al. (2000) studied the thermal stability of *R. oryzae* lipase, where it was detected that its activity remained on average with 80% at 40 °C, obtaining intermediate activity of 60% at 45 °C, and in the same thermostability study the enzyme presented half-life with 20% activity at 50 °C.

Lima et al. (2004), evaluating the thermal stability of the crude extract of a *Penicillium aurantiogriseum* lipase, reported that there was only 32% residual activity after incubation at 50 °C, 45% after incubation at 45 °C and 77% after incubation at 37 °C. Ozenl et al. (2004) reported in their study that the decrease in the percentage of residual activity at high temperatures resulted first in some conformational changes in the tertiary structure of enzyme, and then its almost complete inactivation.

3.4.3 Determination of activity and stability in solvents

Stability in organic solvents is an important feature of lipases because it can determine whether the enzyme is ideal for use in synthetic catalysis reactions and to predict which solvent would be best to perform the reaction.

Table 14 shows the effect of organic solvents on the stability of *F. solani* lipase. The solvents are listed according to their hydrophobicity (Log P) varying from -1.67 to 3.90 and according to their polarity (GHAMGUI et al., 2007). The negative hydrophobicity values (Log P) indicate that the solvent is soluble in water, whereas positive values indicate that they are insoluble with the separation of the aqueous phase from the organic phase (SANGSTER, 1989).

Table 14. Effect of organic solvents on free lipase of *F. solani*.

Organic solvents	Log P	Free lipase	
		Relative activity (%)	$t_{1/2}$ (h)
Control		100,00	16
Glycerol	-1.67	84,71	36
DMSO	-1.38	220,04	36
Methanol	-0.76	111,36	48
Acetonitrile	-0.40	55,79	36
Ethanol	-0.24	60,95	48
Acetone	-0.23	85,12	36
2-propanol	0.07	61,16	36
1-propanol	0.25	17,77	36
Tertiary Butanol	0.60	48,14	24
Iso-butyl	0.79	23,97	48
Toluene	2.50	309,92	12
Xylol	3.15	26,86	48
Hexane	3.90	77,69	24

Assay conditions: experiments were conducted in sealed flasks using 10% (v/v) organic solvent in McIlvaine buffer pH 7.0, 40 °C, 180 rpm. Lipase activity was determined using the same buffer at 40 °C. The activities were carried out using 100µL of protein for the free lipase. Relative activity was expressed in relation to the control (absence of organic solvent in the reaction medium). Half-lives were determined by incubating the enzyme in each organic solvent without substrate. Log P logarithm of the partition coefficient of a particular solvent n-octanol and water (SANGSTER, 1989). Source: Authors.

Table 14 shows that the enzyme was very stable in most organic solvents, however, the activity was determined in more than 80% for the enzyme in glycerol, DMSO, methanol, acetone and toluene. As for the enzyme in 1-propanol, iso-butyl and xylol, the smallest activities were detected. This important feature allows several industrial applications such as transesterification reactions, synthesis, and resolution of racemic esters (DANDAVATE et al., 2009).

The lipase half-lives were greater than 40 hours using methanol, ethanol, iso-butyl and xylol. Half-lives greater than 30 hours were observed when using glycerol, DMSO, acetonitrile, acetone, 2-propanol, 1-propanol. Intermediate half-lives were observed with tertiary butanol and hexane, while the shorter half-life was observed with toluene.

The enzyme activity was considerably stimulated after 8 hours of incubation with DMSO, methanol, and toluene (220, 111, and 309%, respectively). Intermediate activities were achieved with acetone, glycerol, hexane, 2-propanol, and ethanol (85, 84, 77, 61, and 60%, respectively). 1-propanol did not significantly affect enzyme activity, with 17%) In addition, the enzyme showed good stability in water-immiscible organic solvents with a residual activity of 113.6% for n-heptane, 92, 3% for hexane, 91.0% for isooctane and 82.3% for toluene.

4. Conclusion

The optimization of lipase production by *F. solani* in submerged fermentation was possible with the use of sequential experimental design, *Plackett-Burman,* and Central Rotational Compound Design, respectively. The optimum conditions were obtained using the fungus *F. solani*, obtaining maximum lipolytic activity of 24.84 U/mL with the use of 10 $g.L^{-1}$ of tryptone, 3.50 $g.L^{-1}$ of calcium chloride and 0.50 $g.L^{-1}$ of magnesium sulfate, 1 g L^{-1} potassium phosphate and 1% cotton oil. With the biochemical characterization of the enzyme, a pH and temperature optimization using a central rotational compound design led to a mathematical model with a correlation coefficient of 94.15%, with the experimental data, showing that the enzyme has better performance at pH 7 at a temperature of 40 °C.

Given the optimization conditions and the results presented, it is expected that in other studies the lipase from *F. solani* can be applied as a catalyst for biofuels.

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