Vol. 16, No. 3 (2017) 745-756
Revista Mexicana de Ingeniería Química

CULTIVATION OF *Penicillium roqueforti* IN COCOA SHELL TO PRODUCE AND CHARACTERIZE ITS LIPASE EXTRACT

CULTIVO DE *Penicillium roqueforti* EN CÁSCARA DE CACAO PARA PRODUCIR Y CARACTERIZAR SU EXTRACTO DE LIPASA

T.P. Silva[1], L.O. Souza[2], N.S. Reis[4], S. A. Assis[3], M.L.O. Ferreira[4], J.R. Oliveira[4], E. Aguiar-Oliveira[4], M. Franco[4*]

[1]*Post-Graduation Program in Chemistry, Department of Exact Sciences and Technology, State University of Santa Cruz (UESC) 45654-370, Ilhéus, Brazil.*

[2]*Post-Graduation Program in Food Engineering, Department of Basic and Instrumental Studies, State University of Southwest Bahia (UESB), 45700-000, Itapetinga, Brazil.*

[3]*State University of Feira de Santana (UEFS), Department of Health, Postal Code: 44.036-900, Feira de Santana, Bahia, Brazil.*

[4]*State University of Santa Cruz (UESC), Department of Exact and Technological Sciences (DCET), Postal Code: 45.662-900, Ilhéus, Bahia, Brazil*

Received May 20, 2017; Accepted September 4, 2017

Abstract

Lipases are enzymes of great interest and have industrial application. In this work, the production of lipase (*Lip*, U/g) was investigated from *Penicillium roqueforti* using cocoa shell as a substrate for semi-solid fermentation. The optimization of cultivation conditions were *aw* = 0.888 / T = 26.7°C / t = 60 h), which showed a productivity of 0.299 ± 0.031 Ug^{-1} h^{-1}. The enzymatic extract presented an optimal pH and a T of 6.8 and 35.5°C respectively; a thermal stability (50°C) showed a half-life estimated at 50 min. and the addition of the solvents dichloromethane or methyl ether or the addition of the salts $CoCl_2$, $Pb(C_2H_3O_2)_2$ or $MgCl_2$ to the incubation buffer favored the *Lip activity*. The results showed that cocoa shell can be used as a viable substrate for *Penicillium roqueforti* to produce enzyme extracts, which is a promising alternative for the production. It also contained, for example, lipases with characteristics like those described in the literature.

Keywords: food waste, fermentation time, response surface, freezing stability, thermostability.

Resumen

Las lipasas son enzimas de gran interés y aplicación industrial, y en este trabajo se investigó la producción de lipasa (*Lip*, U/g) a partir de *Penicillium roqueforti* utilizando cáscara de cacao como sustrato para la fermentación semisólida. La optimización de las condiciones de cultivo *aw* = 0.888 / T = 26.7 °C / t = 60 h) mostró una productividad de 0.299 ± 0.031 Ug^{-1} h^{-1}. El extracto enzimático presentó pH y T óptimos de 6,8 y 35,5 °C, respectivamente; La estabilidad térmica EM 50 °C, mostró una semivida estimada a 50 min y la adición de disolventes diclorometano o metil éter o de sales $CoCl_2$, $Pb(C_2H_3O_2)_2$ o $MgCl_2$ al tampón de incubación favoreció el *Lip*. Los resultados mostraron que el salvado de cacao puede ser utilizado como un sustrato viable por *Penicillium roqueforti* para producir extractos enzimáticos, siendo una alternativa prometedora para la producción de lipasas y presentado conteniendo, por ejemplo, lipasas con características similares a las descritas en la literatura.

Palabras clave: residuos de alimentos, tiempo de fermentación, superficie de respuesta, estabilidad de congelación, termoestabilidad.

1 Introduction

Lipases (glycerol ester hydrolyses, EC 3.1.1.3) are enzymes applied in many different products and processes. The most significant industrial applications being mainly in food, detergent and pharmaceutical sectors and a minor application in diagnostic tools for medicine (Sharma *et al*. 2001; Borrelli and Trono 2015). For the food industry, as an example, lipases are commonly applied in dairy product preparation, egg yolk treatments, lecithin modification and in oil degumming when refining certain vegetable oils (De Maria *et al*. 2007; Patel *et al*. 2016), which proves its versatility and relevance.

These important enzymes can be obtained from animal, microbial and/or vegetable sources; however, the use of plants or animals as a source of enzymes can be economically unfeasible due not only to

* *Corresponding author. E-mail*: mfranco@uesc.br

the high cost involved in the process, but also to controversies especially if the enzyme has a non-food-related purpose (Shea *et al*. 2012; Okino-Delgado and Fleuri 2014).

The use of agricultural and agroindustrial byproducts to produce microbial enzymes is more and more a reality nowadays (Okino-Delgado and Fleuri 2014; Álvarez-Cervantes *et al*., 2016; Patel *et al*., 2016; Ravindran and Jaiswal 2016; Ramos-Ibarra *et al*., 2017; dos Santos *et al*. 2017). The production of enzymes through the growth of fungi on solid substrates (solid state fermentation) is a technology with several attractive features, such as the fact that the fungi are very versatile and the possibility of using food substrates. Using food substances are attractive because its nutritional composition produces the conditions necessary for the growth of microorganisms (dos Santos *et al*. 2011; Santos *et al*. 2013; Thomaz *et al*. 2013). Among the various byproducts that can be used as substrates for microbial cultivation, a few can be mentioned: orange by-products (Ramos-Ibarra *et al*., 2017) and wheat and rice bran and soybean meal. Among these, the cocoa shell is a byproduct of cocoa bean (*Theobroma cacao* l.) processing for chocolate manufacture. More specifically, it consists of dry and crushed shells of cocoa almonds and represents around 10% of the production of dried cocoa almonds (Afoakwa *et al*. 2013).

Since the genus *Penicillium* stands out as a great producer of extracellular enzymes by SSF (Li and Zong, 2010) and because the accumulation of such cocoa residues on the South of Bahia (Brazil) needs to be better addressed, in this present work, the commonly food-related (therefore, not pathogenic) fungus, *Penicillium roqueforti*, was cultivated in cocoa shells by solid state fermentation (SSF) without the addition of nutrients or additives to obtain an enzymatic extract with good lipase activity using a simpler and more effective bioprocess.

2 Materials and methods

2.1 Cocoa shell (CS)

Cocoa shells (CSs) were provided by the chocolate industries located in the South of Bahia (Brazil), which were dried in an oven at 50°C for 24 h. They were crunched in a mill of knives of the Wiley (ACB LABOR®) type down to a particle size of 2 mm and then stored in plastic container until they were ready to use. The physico-chemical composition was determined through the analysis of dry matter (DM), mineral material (MM), crude protein (CP), crude fiber (CF), neutral detergent fiber (NDF), acid detergent fiber (ADF) and ether extract (EE) according to the methodology of the Association of Official Analytical Chemists (AOAC, 2006); the values obtained were expressed as "g/100 g of dry matter." Microphotographs of the CM particles at different magnitudes (300x, and 1000x, and 3000x) were obtained with an FEI Quanta 250 scanning electron microscope (SEM) located at the Center for Electron Microscopy da State University of Santa Cruz (UESC, Brazil) and using the secondary electron technique (SEI).

2.2 Microorganism and inoculum

The filamentous fungus *Penicillium roqueforti* was provided by *Fundação Oswaldo Cruz* (FIOCRUZ, Rio de Janeiro, RJ, Brazil) and duly deposited at the *Instituto Nacional de Controle de Qualidade em Saúde* (INCQS, Rio de Janeiro, RJ, Brazil) under the registration number 40075 and lot number 079840075. This fungus was preserved in silica and glycerol and maintained in an ultrafreezer at a temperature of -80°C. The spore suspension was prepared with fungus cultivated in Potato-Dextrose-Agar (VETEC®) and Agar Agar (VETEC®), which were placed in 250-mL Erlenmeyer flasks, over a period of 7 days in a bacteriological greenhouse (SL 222, Solab) maintained at 26.5°C; the collection of spores was made by adding Tween 80 (VETEC®) (0.01% v/v) sterile glass beads to the Erlenmeyer flasks. For counting the number of spores in suspension, a doubly mirrored Neubauer Chamber and a binocular microscope (BIOVAL® L1000) were used. The titer of the inoculum used was 10^7 spores/g of dry cocoa shell (dos Santos *et al*., 2016).

2.3 Solid state fermentations

2.3.1 Fermentation profile

10 g of CS were autoclaved (121°C/1 atm/15 min) in 125-mL Erlenmeyer flasks; after cooling, the sterile substrate was inoculated with 10^7 spores/g and moistened with sterile distilled water (Novasina labswift, TECNAL®) until the value of the desired water activity (a_w) was determined. The fermentations

were incubated in bacteriological greenhouses under different temperatures (T, °C).

2.3.2 *Experimental design and statistical analysis*

The optimization of the production of lipase by SSF was evaluated using the experimental methodology design with a Central Composite Rotational Design (CCRD) 2^2 composed of 4 different experiments, 4 axial points and 3 central points. The correspondent matrix is represented in Table 1. The independent variables (factors) analyzed and their ranges were *aw* = 0.923 - 0.854 and T = 15 - 30°C. The dependent variable (response) evaluated was the activity of lipase (*Lip*, U/g), which was measured in the enzymatic extract that was obtained. The fermentations proceeded for 60 h and the obtained data was analyzed with the aid of STATISTICA® v. 10.0 (Statsoft, the USA) software.

2.3.3 *Determination of enzymatic activity*

After fermentation, 50 mL of sodium phosphate buffer (0.1 M/pH 7.0) was added to the fermented substrate and the mixture was stirred (shake incubator, TECNAL) at 35oC and 200 rpm for 20 min. The liquid phase was separated by mechanical pressing was permeation achieved gauze followed by centrifugation (704.34g) for 5 minutes to remove impurities. This solution was tittered from raw enzymatic extract.

2.3.4 *Lipase activity (Lip)*

The lipase activity was established using two different methodologies: titration and spectrophotometry. The titration methodology (Gutarra *et al*. 2009; Carvalho *et al*. 2017) was performed by employing olive oil as the substrate and 0.04M NaOH as the titter solution; one unit of lipase activity (U) was defined as the amount of enzyme capable of releasing 1.0 μmol of oleate methyl per minute. This method was used to optimize lipase production, the determination of the temperature and the pH optimums, the thermostability and the stability of freezing the extract and the fermented biomass. The spectrophotometric methodology employed *p*-nitrophenyl palmitate as the substrate; one unit of lipase activity (U) was defined as the amount of enzyme capable of hydrolyzing 1.0 μmol of *p*-nitrophenyl palmitate per minute. This methodology was used to the evaluation of the effect of the salts and the solvents and to their partial purification.

2.4 *Enzymatic characterization*

The obtained enzymatic extract containing lipase activity was characterized, in triplicate, to obtain the temperature and pH stability, thermostability, freezing stability, effect of the salts and the solvents and the saline fractionation of the raw enzymatic extract.

2.4.1 *Optimal pH and temperature*

The optimal conditions for the pH and temperature (T, °C) in regard to the lipase activity were analyzed by means of a Central Composite Rotational Design (CCRD) 2^2 composed of 4 different experiments, 4 axial and 3 central points (Table 1). The independent variables (factors) were analyzed and their ranges were pH (5.0 - 8.0) and T (20 - 50°C) and the dependent variable (response) was the lipase activity (U/g).

2.4.2 *Thermostability*

Thermostability for the lipase activity was established through the incubation of the obtained enzymatic extract at temperatures between 35 and 80°C with samples collected between 10 and 50 min.

2.4.3 *Freezing stability*

The enzymatic extract, as well as the fermented substrate (fermented biomass), were maintained at -4°C for 90 days to evaluate the stability of lipase activities; samples were collected and analyzed every 10 days.

2.4.4 *Effect of the addition of salts*

One μL of each of the selected salt solutions (1.0 M) - $Pb(C_2H_3O_2)_2$, $MgCl_2$, $CaCO_3$, Na_2CO_3 and $CoCl_2$ - was added to the reactive medium that contained the substrate *p*-nitro phenyl palmitate. The enzymatic activity was determined and expressed as residual activity (%) in relation to the condition without salt, which had a lipase activity of 48.64 U/g.

2.4.5 *Effect of the addition of solvents*

One μL of each of the selected solvents - acetone, methyl ether, methanol and dichloromethane -were added to the reactive medium that contained the substrate *p*-nitro phenyl palmitate. The enzymatic activity was determined and expressed as residual activity in relation to the condition without solvent, which had a lipase activity of 48.64 U/g.

2.5 Saline fractionation of the crude enzymatic extract

The crude enzymatic extract was fractionated by precipitation with ammonium sulphate [$(NH_4)_2SO_4$] at 20%, 40%, 60% and 80% (g/100 mL) (Englard and Seifter 1990). The salt was added slowly to 50 mL of the crude enzymatic extract, under stirring in an ice bath, until the intended saturations were obtained. Afterwards, the samples were kept cool in the refrigerator for 1 hour. Later, the samples were centrifuged at 15.000g and 4°C for 20 min. The supernatant was removed and the precipitate was re-suspended in 1.0 mL of sodium phosphate buffer with a pH of 7.0. Then, the final volume was measured and the lipase activity and the total protein analyses were performed. The efficiency of the precipitation process with ammonium sulphate was evaluated through recovery (R, %) of the enzymatic activity. The total protein content was determined by the Lowry method with an absorbance reading at 660 nm and bovine serum albumin (BSA) as the pattern.

3 Results and discussion

3.1 Characterization of the substrate

The chemical characterization of cocoa shell in natura confirmed the nutritious potential of this material as a substrate for microbial cultivation especially as a source of carbon and nitrogen. The compositions (%, g/100 g of dry matter) obtained were: DM = 89.91 ± 0.03%, MM = 6.87 ± 0.09%, CP = 12.15 ± 0.28%, NDF = 43.1 ± 0.2%, ADF = 36.18 ± 0.24% and EE = 14.32 ± 0.05%. This residue is highly advantageous compared to others because it has an intrinsic lipid content (EE = 14.32% ± 0.05%) that, in turn, stimulates lipase production using the microorganism. Coradi *et al*. (2012) used sugar cane (EE = 3.5%) and Trichoderma harzianum to produce lipase, which infers that supplementation with olive oil was necessary because, in the absence of the oil, the enzymatic activity was not detected. The same phenomenon was also observed in cultures of *A. niger* by Falony *et al*. (2006). This, in turn, corroborated the choice to use cocoa shell in natura as a matrix for SSF and for the cultivation of *P. roqueforti* to obtain the lipase activity of the enzymatic extract.

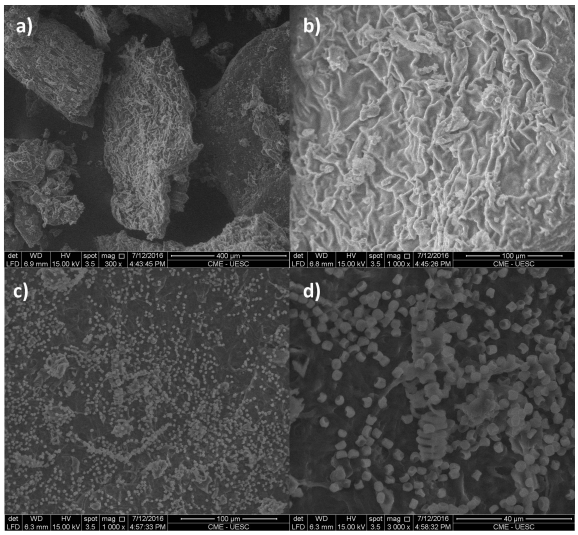

Fig. 1. Microphotographs (MEV) of the cocoa meal particle surface before - a) and b) (magnitudes of 300x and 1000x, respectively) - and after - c) and d) (1000x and 3000x, respectively) - fermentation by *Penicillium roqueforti*.

In addition, Figure 1 presents microphotographs obtained from the surface of a cocoa shell before and after fermentation, which makes it possible to identify the growth of the fungi in a uniform way. Similar microphotographs were obtained by Panwar *et al*. (2014) when evaluating the cultivation of *Bacillus* sp. PKD-9 grown on wheat bran.

3.2 Effect of the fermentation time on lipase production

Extracellular lipases are usually produced intracellularly during the first hours of fermentation and are then released to the exterior during the stationary phase. Its production decreases significantly by the end of fermentation (Sharma *et al*. 2001; Stergiou *et al*. 2013). The lipase from *P. roqueforti* presented a similar profile in which the optimum fermentation time was determined to be around 60 h due to the higher obtained lipase activity (17.33 ± 2.3 U/g) during the total fermentation process (Figure 2). Fermentation times between 20 and 144 h were reported as the best times for producing lipases through SSF with different fungi and substrates (Kumar and Ray 2014). In this present work, the excretion of lipase by *P. roqueforti* was performed without the need to add an inductor (in general, an oil) or other nutrients as is normally mentioned in literature (Gutarra *et al*. 2009; Amin

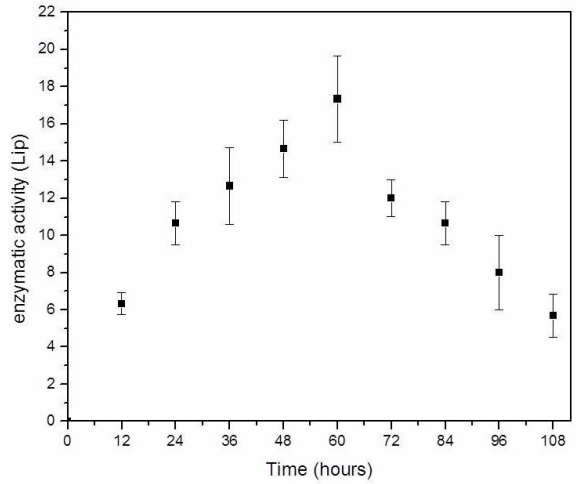

Fig. 2. Production of lipases (U/g) in solid state fermentations by *Penicillium roqueforti* in 10 g of cocoa meal at 26.5ºC and water activity (a_w) of 0.854. Bars represent the deviation of triplicates and the figure was obtained with software OriginPro® v. 8.0.

et al. 2011; Malilas *et al*. 2013; Fleuri *et al*. 2014; Venkatesagowda *et al*. 2015). This can be understood by analyzing the composition of a cocoa shell (presented above) especially in regard to its lipid content (ether extract), which could be interpreted as a natural inductor.

3.3 Optimization of lipase production

The Pareto analysis (Figure 3.a) on the lipase activity (*Lip*, U/g) obtained from the performed composite design (Table 1) allowed for the selection of statistically significant terms at 95% of significance; the non-significant terms ($p > 0.05$) were removed from the mathematical model. The ANOVA performed (Table 2) in sequence approved the model (Equation 1) and the response surface and contour curve were obtained (Figure 3.b and 3.c).

$$Lip(U/g) = 15.10 - 1.49(a_w^2) + 3.32(T) - 2.08(T^2) \quad (1)$$

According to the model (Eq. 3) and the surface analysis (Fig. 3.b and 3.c) obtained, a theoretical maximum lipase activity of 16.43 U/g (equivalent theoretical productivity of 0.274 U/g.h) was obtained under the conditions of aw = 0.888 and T = 26.7°C. To validate the model, new fermentations were carried out in triplicate under the same conditions and a lipase activity of 17.93 ± 1.87 U/g (equivalent productivity of 0.299 ± 0.031 U/g.h) was obtained. This experimental value represents a 5.48% error in relation to the value predicted by the model. In addition, the values obtained for R^2_{adj} (< 0.80) and

Table 1: Matrixes for the Central Composite Rotatable Design (DCCR) 22 for the optimization of lipase production from Penicillium roqueforti cultivated in coca meal and for the optimization of residual lipase activity for the enzymatic extract obtained under optimized conditions. The evaluated factors were: water activity (a_w), temperature (T, °C) and pH in their codified and real (in parenthesis) values. The responses were the enzymatic activity (Lip, U/g) and the residual activity (Lip%, %). All fermentations were conducted at 60 h; under the optimized conditions (26.7°C/a_w = 0.886), the initial lipase activity obtained was 17.33 U/g.

Runs	Fermentations			Enzimatic extract		
	Factors		Response	Factors		Response
	aw	***T* (ºC)**	***Lip* (U/g)**	***pH***	***T* (ºC)**	***Lip%* (%)**
1	-1 (0.864)	-1 (17.18)	7.00 ± 0.1	-1 (5.43)	-1 (24.36)	16.156 ±0.2
2	+1 (0.913)	-1 (17.18)	8.33 ± 0.17	+1 (7.56)	-1 (24.36)	43.277 ± 0.12
3	-1 (0.864)	+1 (27.81)	17.33 ± 0.27	-1 (5.43)	+1 (45.63)	38.084 ± 0.45
4	+1 (0.912)	+1 (27.81)	16.00 ± 0.4	+1 (7.56)	+1 (45.63)	9.578 ± 0.19
5	0 (0.888)	0 (22.5)	15.33 ± 0.52	0 (6.5)	0 (35)	73.860 ± 0.67
6	0 (0.888)	0 (22.5)	15.66 ± 0.35	0 (6.5)	0 (35)	83.669 ± 0.29
7	0 (0.888)	0 (22.5)	14.33 ± 0.43	0 (6.5)	0 (35)	93.479 ± 0.57
8	-1.41 (0.854)	0 (22.5)	10.66 ± 0.54	-1.41 (5.0)	0 (35)	9.578 ± 0.38
9	+ 1.41 (0.923)	0 (22.5)	12.33 ± 0.57	+1.41 (8.0)	0 (35)	71.552 ± 0.25
10	0 (0.888)	-1.41 (15)	7.33 ± 0.77	0 (6.5)	-1.41 (20)	13.444 ± 0.13
11	0 (0.888)	+1.41 (30)	13.33 ± 0.32	0 (6.5)	+ 1.41 (50)	0 ± 0

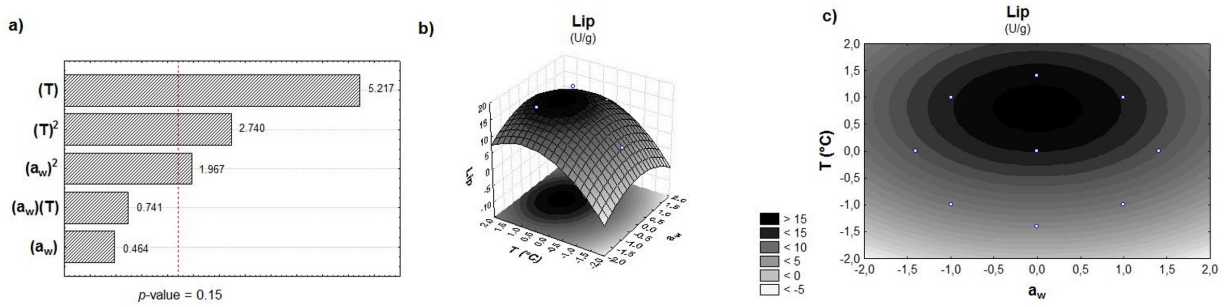

Fig. 3. a) Pareto Diagram b) Response Surface and c) contour curve for the production of lipase (*Lip* U/g) by *Penicillium roqueforti* cultivated in 10 g of cocoa meal for 60 h. The water activity variables (a_w) and temperature (T, °C) are represented in their coded values and the figures were obtained in STATISTICA® v.10.

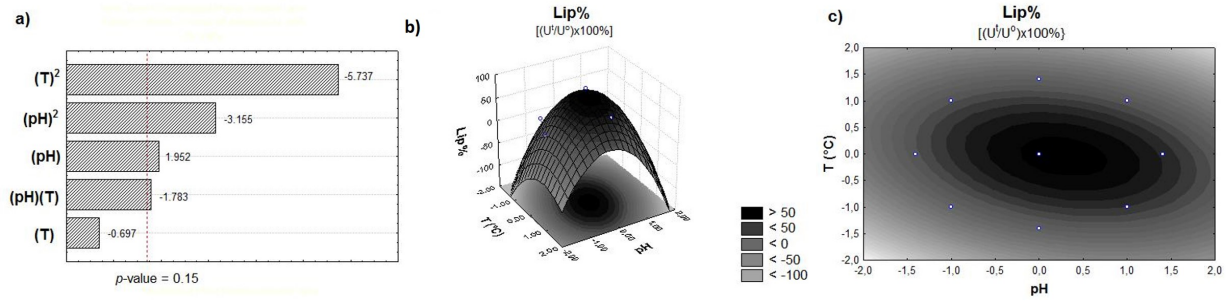

Fig. 4. a) Pareto Diagram, b) Response Surface and c) Contour Curve for the enzymatic activities were expressed as residual activity ($Lip\% = (U_t/U_o)$x(100%)) as a result of the initial activities: (U_o = 17.93 U/g) and final after the incubation under the conditions analyzed (U_t), of the extract produced under optimized conditions of cultivation (26.7 °C/a_w = 0.886/60h) of *Penicillium roqueforti* in 10 g of cocoa meal. The variables (pH and temperature) are represented in their coded values and the figures were obtained in STATISTICA® v.10.

R^2 (> 0.85) can be considered to satisfactory validate the mathematical models of complex systems such as microbiological/enzymatic systems. Compared with the conditions performed prior to the optimization, very similar activity values were obtained since the conditions of *aw* and T were also like those determined by the model. But it must also be considered that the standard deviation obtained before optimizing was equivalent to 13.27% of the average value, while after optimization, we obtained a deviation of 10.42%.

For comparison, Khayati and Kiyani (2012) used rice straw enriched with oil and urea and with the fungus *Rhisopus oryzae* to obtained the higher value of lipase activity of 56.44 U/g; Vaseghi *et al*. (2012) observed lipase productivity (> 2.0 U/g.h) when cultivating the same microorganism but in sugarcane bagasse at 45°C. Ferraz *et al*. (2012) obtained higher productivities (> 1.0 U/g.h) using different substrates (soy, sugarcane bagasse and rice bran) also without supplementation at 30°C and 60% humidity and with a different microorganism. Lipase production employing solid state fermentation of agro-industrial waste has been investigated extensively by Singh *et al*. (2014), who used seeds of *Leucaena leucocephala* to grow *Basidiomycetes Schizophyllum* (146.5 U/g). Colla *et al*. (2014) used wheat or soybean meal and rice bran to produce lipase (42.82 U/g) using Aspergillus niger. However, mineral oils, olives, olive oil and other compounds were used to supplement the used residues, which could lead to higher production costs. In this work, only the cocoa shell was used as a source of carbon, nitrogen and energy (do Santos *et al*. 2015). By observing these results, it was possible to observe that the cocoa shell has potential for microorganism cultivation, because even after drying, it was still possible to produce lipases from it, which was not a characteristic observed in any of the substrates mentioned above.

Table 2. Analysis of the variance for the optimization of the lipases production by *P. roqueforti* through the solid state fermentations and determination of the optimum conditions of pH and temperature for the lipases obtained in the enzymatic extracts after the optimization of the fermentation.

Source of variation	Sum of Squares	Degree of Freedom	Medium Squares	F-test	p-value
Solid state fermentations					
Regression	116.8	3	38.9	14.7	0.002*
Residue	18.6	7	2.6		
Lack of Fit	17.6	5	3.5		
Pure Error	0.96	2	0.48		
Total Sum of Squares	135.4	10			
R^2	0.863				
R^2_{adj}	0.788				
Enzymatic extract					
Regression	10298.2	4	2574.6	11.6	0.006*
Residue	1335.3	6	222.5		
Lack of Fit	1142.9	4	285.7		
Pure Error	192.5	2	96.2		
Total Sum of Squares	11633.6	10			
R^2	0.885				
R^2_{adj}	0.822				

* values statistically significant at less than 95% of confidence.

3.4 Enzymatic characterization

3.4.1 Determination of optimal pH and temperature

The crude enzymatic extract, which was obtained after optimization of the fermentation step, was characterized to determine the optimal conditions of pH and temperature for the residual lipase activity (*Lip%*, %) using a DCCR 2^2, whose matrix is shown in Table 1. The obtained results were analyzed by Pareto analysis (Figure 4a) at 85% of confidence and the non-significant terms ($p > 0.15$) were removed from the model. The ANOVA (Table 2) approved the model (Equation 2) and the response surface and contour curve were obtained (Fig. 4b and 4c, respectively).

$$Lip\%(\%) = 83.66 + 10.78(pH) - 20.80(pH)^2 - 37.82(T)^2 - 13.91(T) \quad (2)$$

The obtained model (Eq. 2) allowed the estimation of the theoretical optimum residual activity (Lip% = 84.75%) when the pH was 6.8 and the temperature was 35.5°C; the experimental value obtained under these same conditions was 81.36%, which represents a 4% error. The values determined for R^2 and R^2_{adj} (Table 3) were greater than 0.8 and just like the model presented for the optimization of lipase production, it was considered satisfactory and validated the model.

Based on the standard conditions applied to the methodology for determining the lipase activity (pH 7.0 and 35°C), the conditions determined as optimal in this study (pH 6.8 and 35.5°C) confirm the maintenance of enzyme characteristics with respect to these standard conditions; the central points (pH 6.5 and 35°C), for example, presented an average of 83.67% ± 9.81%. These results indicate that the use of cocoa shell did not cause drastic changes in the characteristics of the lipases of *P. roqueforti* because the literature reports a slightly acid nature for most lipases from fungi and yeasts (Ferrer *et al*. 2000; Lima *et al*. 2004) at optimal temperatures ranging from 35 to 45°C (Dheeman *et al*. 2011; Supakdamrongkul *et al*. 2010).

3.4.2 Thermostability

The obtained results (Figure 5) indicated a better stability at 50ºC (less inclination over time) and the estimated half-life ($t_{1/2}$), i.e. *Lip%* = 50%, for this condition was 50 min, whereas the half-life at 60-70°C was around 35 min and approximately 20 min. at 80°C. Higher thermostabilities are cited in literature

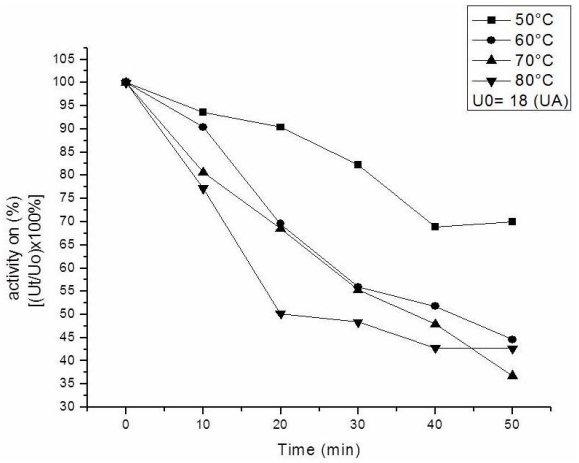

Fig. 5. Thermostability for lipase activity, in an enzymatic extract from *Penicillium roqueforti* cultivated in cocoa meal, expressed as residual activities (*Lip%*) at different temperatures (°C) and incubation time (min). The initial lipase activity (U_o) was 17:33 U/g. The figure was obtained with software OriginPro® v. 8.0.

for purified lipases, e.g. a lipase from *Aspergillus* sp. (Fleuri *et al*. 2014), which presented good stability after 60 min. of incubation at 60°C and a lipase from *Mucor hiemalis f. Corticola* (Ülker and Karaoglu 2012) had a $t_{1/2}$ of 90 min. at 50°C.

3.4.3 Freezing stability

For the fermented biomass (Fig. 6.a), the residual activities (*LiP%*) were greater than 50% for approximately 50 days, whereas for the crude enzymatic extract (Fig. 6.b), a significant reduction in residual enzymatic activity was only observed after 40 days of freezing. It is important to consider that *P. roqueforti* is a mesophilic fungus that tends to produce enzymes that are less stable at low temperatures; however, their cooled storage for more than 1 month (with *Lip%* between 60-70%) without additives is a promising result if it also considers the stock of the fermented biomass. Lazari (2013), while evaluating a crude enzymatic extract from an *S. thermophilum*-containing lipase, observed losses of more than 50% of the residual activity after 1 day at -20°C and 7 days at 5°C.

3.4.4 Effect of the addition of salts

The effect of the addition of salts indicated that the lipase activity presented in the crude extract showed descending residual activities (*Lip%*): $CoCl_2$ = 160.75%, $Pb(C_2H_3O_2)_2$ = 107. 91%, $MgCl_2$ = 103.86%, $CaCO_3$ = 32.26% and Na_2CO_3 = 11.60%. Certain salts may contribute to the enzyme reactivity directly or indirectly; therefore, values of Lip% greater than 100% indicate that there was an increase in the lipase activity due to the presence of the salt. The effect of the salts varies for each enzyme and its production source. It is useful to promote the syntheses in which the enzymes are applied (Liu and Zhang 2011).

3.4.5 Effect of the addition of solvents

Lipases are capable of acting in both emulsions (water:oil) and organic solvents (Venkatesagowda *et al*. 2015); therefore, the effect of certain solvents

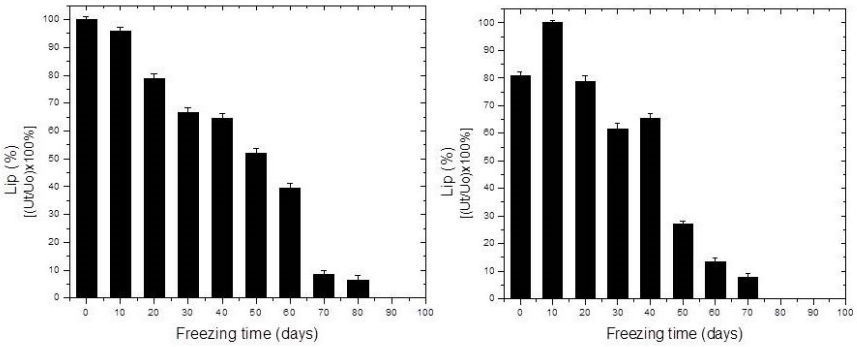

Fig. 6. Residual activity of lipase during frozen stock (-4°C) of a) cocoa meal fermented by *Penicillium roqueforti* and b) enzymatic extract obtained from the process. Initial lipase activity (U_o) was 17:33 U/g, respectively. The figures were obtained with software OriginPro® v. v.8.

Table 3. Evaluation of the partial purification of lipase from *P. roqueforti* by precipitation with ammonium sulphate [$(NH_4)_2SO_4$] in different concentrations (%, g/100g). The specific (Lip_{ptn}, U/μg of protein) activities lipase (Lip, U/g), and recovery (R,%) relative to the initial lipase activity in the crude enzyme extract (48.64 U/g, 0.106 U/μg).

$(NH_4)_2SO_4$ (g/100g)	Lip (U/g)	Lip_{ptn} (U/μg)	R (%)
20%	32.14	0.0105	66.07%
40%	28.44	0.0158	58.47%
60%	34.67	0.0233	71.28%
80%	30.04	0.0216	61.76%
100%	28.12	0.0156	57.81%

on the residual lipase activity (Lip%, %) were, in descending order: dichloromethane = 162.62%, methyl ether = 115. 93%, acetone = 52.71% and methanol = 32.26%. Among the solvents employed, dichloromethane and methyl ether acted as lipase activity activators ($Lip\% > 100\%$). Demir and Tükel (2010) also obtained inhibitory effects with a lipase purified with acetone and methanol.

3.5 *Precipitation of lipase with ammonium sulphate*

The concentration of the lipase activity present in the crude extract was further evaluated by precipitation with ammonium sulphate [$(NH_4)_2SO_4$]. According to the obtained results (Table 3), the highest recovery ($R > 70\%$) was obtained when 60% of the solution was $(NH_4)_2SO_4$. Considering lipases from *Aspergillus* and the same precipitant salt, a higher recovery was reported (R = 90%) with a higher concentration of salt (90%) by Mhetras *et al*. (2009), as well as a lower recovery (R = 44%) with the same concentration of salt (60%). According to Maldonado *et al*. (2015), recoveries above 100% were obtained with the precipitation (same salt, but at 80%) followed by the lyophilization of lipase from *Geotrichum candidum* NRRLY-552. The purity can be increased with the application of different combinations of downstream techniques, particularly the chromatographic ones, but the total cost of the process increases as the purity is increased.

Conclusion

The application of cocoa shells as a substrate for the cultivation of *Penicillium roqueforti* and its subsequent lipase production did not require the addition of additives or minerals. The use of an agro-industrial residue can contribute to the reduction of negative environmental impacts. Therefore, the enzymatic extract obtained presented lipase activities with very similar characteristics to other lipases described in the literature and its precipitation with ammonium sulphate is promising as a pre-purification technique.

Acknowledgements

Authors would like to thank to the Banco do Nordeste do Brasil (BNB, Brazil), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) and the Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB, Brazil) for their financial support.

References

AOAC International Official Methods of analysis of AOAC International. AOAC International.

Afoakwa, E.O., Quao, J., Takrama, J., Budu, A.S. and Saalia, F.K. (2013). Chemical composition and physical quality characteristics of Ghanaian cocoa beans as affected by pulp pre-conditioning and fermentation. *Journal of Food Science and Technology 50*, 1097-1105. doi: 10.1007/s13197-011-0446-5.

Álvarez-Cervantes, J., Sánches, C., Díaz, R. and Díaz-Godínez, G. (2016). Characterization of production of laccases, cellulases and xylanases of *Pleurotus ostreaus* grown on solid-state fermentation using an inert support. *Revista Mexicana de Ingeniería Química 15*, 323-331.

Amin, F., Bhatti, N.H. and Rehman, S. (2011). Optimization of growth parameters for

lipase production by *Ganodermalucidum* using response surface methodology. *African Journal of Biotechnology 10*, 5514-5523. doi: 10.5897/AJB10.1714.

Borrelli, G.M. and Trono, D. (2015). Recombinant lipases and phospholipases and their use as biocatalysts for industrial applications. *International Journal of Molecular Sciences 1*, 20774-20840. doi: 10.3390/ijms160920774.

Carvalho, T., Finotelli, P.V., Bonomo, R.C.F., Franco, M., Amaral, P.F.F. (2017). Evaluating aqueous two-phase systems for *Yarrowia lipolytica* extracellular lipase purification. *Process Biochemistry 53*, 259-266. doi: 10.1016/j.procbio.2016.11.019.

Colla, L.M., Ficanha, A.M.M., Rizzardi, J., Bertolin, T.E., Reinehr, C.O. and Costa, J.A.V. (2014). Production and characterization of lipases by two new isolates of *Aspergillus* through solid-state and submerged fermentation. *BioMed Research International 2015*, 1-9. doi: 10.1155/2015/725959

Coradi, G.V., Visitação, V.L., Lima, E.A., Saito, L.Y.T., Palmieri, D.A., Neto, P.O. and Lima, V.M.G. (2012). Comparing submerged and solid-state fermentation of agro-industrial residues for the production and characterization of lipase by *Trichoderma harzianum*. *Annals of Microbiology 63*, 533-540. doi: 10.1007/s13213-012-0500-1

De Maria, L., Vind, J., Jensen, M.T., Van Haesendonck, I. and Van der Biest, G. (2012). Methods for preparing cakes using phospholipases and cake batter and cake mix compositions comprising phospholipases. U.S. Patent 2012/0156328 A1.

Demir, B.S. and Tukel, S.S. (2010). Purification and characterization of lipase from *Spirulina platensis*. *Journal of Molecular Catalysis B: Enzymatic 64*, 123-128. doi: 10.1016/j.molcatb.2009.09.011.

Dheeman, S.D., Babud, A.S., Frias, J.M. and Henehana, G.T.M. (2011). Purification and characterization of an extracellular lipase from a novel strain *Penicillium* sp. DS-39 (DSM 23773). *Journal of Molecular Catalysis B: Enzymatic 72*, 256-262. doi:10.1016/j.molcatb.2011.06.013.

Do Santos, T.C., Diniz, G.A., DE Brito, A.R., Pires, A.J.V., Franco, M. (2015). Effect of solid state fermentation on nutritional content and evaluation of degradability in cactus pear. *Revista Caatinga 28*, 248-254. doi: 10.1590/1983-21252015v28n328rc.

dos Santos, T.C.D., Cavalcanti, I.S., Bonomo, R.C.F., Santana, N.B. and Franco, M. (2011). Optimization of productions of cellulolytic enzymes by *Aspergillus niger* using residue of mango a substrate. *Ciência Rural 41*, 2210-2216. doi: 10.1590/S0103-84782011005000145.

dos Santos, T.C., Reis, N.S., Silva, T.P., Machado, F.P.P., Bonomo, R.C.F., Franco, M. (2016). Prickly palm cactus husk as a raw material for production of ligninolytic enzymes by *Aspergillus niger. Food Science and Biotechnology 25*, 205-211, 2016. doi: 10.1007/s10068-016-0031-9

dos Santos, T.C., Brito, A.R., Bonomo, R.C.F., Pires, A.J.V., Aguiar-Oliveira, E., Silva, T.P. and Franco, M. (2017). Statistical optimization of culture conditions and characterization for ligninolytic enzymes produced from rhizopus sp. using prickly palm cactus husk. *Chemical Engineering Communications 204*, 55-63. doi: 10.1080/00986445.2016.1230851

Englard, S. and Seifter, S., (1990). Precipitation techniques. *Methods in Enzymology 182*, 287-300. doi: 10.1016/0076-6879(90)82024-V.

Falony, G., Armas, J.C., Julio, C.M.D. and Hernández, J.L.M. (2006). Production of extracellular lipase from *Aspergillus niger* by solid-state fermentation. *Food Technology and Biotechnology 44*, 235-240.

Ferraz, L.R., Oliveira, D.S., Silva, M.F., Rigo, E., Di Luccio, M., Oliveira, J.V., Oliveira, D. and Treichel, H. (2012). Production and partial characterization of multifunctional lipases by *Sporobolomyces ruberrimus* using soybean meal, rice meal and sugarcane bagasse as substrates. *Biocatalysis and Agricultural Biotechnology 1*, 243-252. doi: 10.1016/j.bcab.2012.03.008.

Ferrer, M., Plou, F.J., Nuero, O.M., Reyes, F. and Ballesteros, A. (2000). Purification and properties of a lipase from *Penicillium*

chrysogenum isolated from industrial wastes. *Journal of Chemical Technology and Biotechnology 75*, 569-576. doi: 10.1002/1097-4660(200007)75:7<569::AID-JCTB258>3.0.CO;2-S.

Fleuri, L.F., Novelli, P.K., Delgado, C.H.O., Pivetta, M.R., Pereira, M.S., Arcuri, M.L.C. and Capoville, B.L. (2014). Biochemical characterisation and application of lipases produced by *Aspergillussp*. on solid-state fermentation using three substrates. *International Journal of Food Science & Technology 49*, 2585-2591. doi:10.1111/ijfs.12589.

Gutarra, M.L.E., Godoy, M.G., Maugeri, F., Rodrigues, M.I., Freire, D.M.G. and Castilho, L.R. (2009). Production of an acidic and thermostable lipase of the mesophilic fungus *Penicillium simplicissimum* by solid-state fermentation. *Bioresource Technology 100*, 5249-5254. doi: 10.1016/j.biortech.2008.08.050.

Khayati, G.H. and Kiyani, F. (2012). A statistical approach for optimization of lipase production by using rice straw: analysis of different inducers and nitrogen sources effect. *Minerva Biotechnologica 24*, 83-9.

Kumar, D.S. and Ray, S. (2015). Fungal lipase production by solid state fermentation - An Overview. *Journal of Analytical & Bioanalytical Techniques 6*, 230-10. doi: 10.4172/2155-9872.1000230.

Lazari, S.A., Salgado, J.C.S., Masui, D.C., Peralta, R.M., Jorge, J.A. and Furriel, R.P.M. (2013). Production and secretion of a multifunctional β-glucosidase by humicola grisea var. thermoidea: effects of l-sorbose. *Annals of Microbiology 64*, 1089-1097. doi: 10.1007/s13213-013-0748-0.

Li, N. and Zong, M.H. (2010). Lipases from the genus *Penicillium*: Production, purification, characterization and applications. *Journal of Molecular Catalysis B: Enzymatic 66*, 43-54. doi: 10.1016/j.molcatb.2010.05.004.

Lima, V.M.G., Krieger, N., Mitchell, D.A. and Fontana, J.D. (2004). Activity and stability of a crude lipase from *Penicillium aurantiogriseum* in aqueous media and organic solvents. *Biochemical Engineering Journal 18*, 65-71. doi: 10.1016/S1369-703X(03)00165-7.

Liu, J. and Zhang, Y. (2011). Optimisation of lipase production by a mutant of *Candida antarctica* DSM-3855 using response surface methodology. *International Journal of Food Science & Technology 46*, 695-701. doi: 10.1111/j.1365-2621.2011.02548.x.

Maldonado, R.R., Aguiar-Oliveira, E., Fogaça, F.M., Ramos, G.G., Macedo, G.A. and Rodrigues, M.I. (2015). Evaluation of partial purification and immobilization of lipase from *Geotrichum candidum. Biocatalysis and Agricultural Biotechnology 4*, 321-326. doi: 10.1016/j.bcab.2015.05.005.

Malilas, W., Kang, S.W., Kim, S.B., Yoo, H.Y., Chulalaksananukul, W. and Kim, S.W. (2013). Lipase from Penicilliumcamembertii KCCM 11268: Optimization of solid state fermentation and application to biodiesel production. *Korean Journal of Chemical Engineering 30*, 405-412. doi: 10.1007/s11814-012-0132-y.

Mhetras, N.C., Bastawde, K.B. and Gokhale, D.V. (2009). Purification and characterization of acidic lipase from *Aspergillus niger* NCIM 1207. *Bioresource Technology 100*, 1486-1490. doi: 10.1016/j.biortech.2008.08.016

Okino-Delgado, C.H. and Fleuri, L.F. (2014). Obtaining lipases from byproducts of orange juice processing. *Food Chemistry 163*, 103-107. doi: 10.1016/j.foodchem.2014.04.090.

Patel, A.K., Singhania, R.R. and Pandey, A. (2016). Novel enzymatic processes applied to the food industry. *Current Opinion in Biotechnology 7*, 64-72. doi:10.1016/j.cofs.2015.12.002.

Ramos-Ibarra, J.R., Miramontes, C., Arias, A., Arriola, E., Guatemala, G. and Corona-Gonzáles, R.I. (2017). Production of hidrolytic enzymes by solid-state fermentation with new fungal strains using orange by-products. *Revista Mexicana de Ingeniería Química 16*, 19-31.

Ravindra, R. and Jaiswal, A.K. (2016). A comprehensive review on pre-treatment strategy for lignocellulosic food industry waste: Challenges and opportunities. *Bioresource Technology 199*, 92-102. Doi: 10.1016/j.biortech.2015.07.106.

Santos, T.C., Filho, G.A., Oliveira, A.C., Rocha, T.J.O., Machado, F.P.P., Bonomo, R.C.F., Mota, K.I.A. and Franco, M. (2013). Application of response surface methodology for producing cellulolytic enzymes by solid-state fermentation from the puple mombin (*Spondias purpurea* L.) residue. *Food Science and Biotechnology 22*, 1-7. doi: 10.1007/s10068-013-0001-4

Sharma, R., Chisti, Y. and Banerjee, U.C. (2001). Production, purification, characterization, and applications of lipase. *Biotechnology Advances 19*, 627. doi: 10.1016/S0734-9750(01)00086-6.

Shea, N.O., Arendt, E.K. and Gallagher, E. (2012). Dietary fibre and phytochemical characteristics of fruit and vegetable by-products and their recent applications as novel ingredients in food products. *Innovative Food Science and Emerging Technologies 16*, 1-10. doi: 10.1016/j.ifset.2012.06.002.

Singh, M.K., Singh, J., Kumar, M. and Thakur, I.S. (2014). Novel lipase from basidiomycetes *Schizophyllum commune* ISTL04, produced by solid state fermentation of *Leucaena leucocephala* seeds. *Journal of Molecular Catalysis B: Enzymatic 110*, 92-99. doi: 10.1016/j.molcatb.2014.10.010

Stergiou, P-Y., Foukis, A., Filippou, M., Koukouritaki, M., Parapouli, M., Theodorou, L.G., Hatziloukas, E., Afendra, A., Pandey, A. and Papamichael, E.M. (2013). Advances in lipase-catalyzed esterification reactions. *Biotechnology Advances 31*, 1846-1859. doi: 10.1016/j.biotechadv.2013.08.006.

Supakdamrongkul, P., Bhumiratana, A. and Wiwat, C. (2010). Characterization of an extracellular lipase from the biocontrol fungus, *Nomuraearileyi* MJ, and its toxicity toward *Spodopteralitura. Journal of Invertebrate Pathology 105*, 228-235. doi: 10.1016/j.jip.2010.06.011.

Thomaz, L., Larroche, C. and Pandey, A. (2013). Current developments in solid-state fermentation. *Biochemical Engineering Journal 81*, 146-161. doi: 10.1016/j.bej.2013.10.013.

Ülker, S. and Karaoglu, S.A. (2012). Purification and characterization of an extracellular lipase from *Mucor hiemalis* f. corticola isolated from soil. *Journal of Bioscience and Bioengineering 114*, 385-390. Doi: 10.1016/j.jbiosc.2012.04.023.

Vaseghi, Z., Najafpour, G.D., Mohseni, S. and Mahjoub, S. (2013). Production of active lipase by *Rhizopus oryzae* from sugarcane bagasse: solid state fermentation in a tray bioreactor. *International Journal of Food Science & Technology 48*, 283-289. doi: 10.1111/j.1365-2621.2012.03185.x.

Venkatesagowda, B., Ponugupaty, E., Barbosa, A.M. and Dekker, R.F.H. (2015). Solid-state fermentation of coconut kernel-cake as substrate for the production of lipases by the coconut kernel-associated fungus *Lasiodiplodia theobromae* VBE-1. *Annals of Microbiology 56*, 129-142. doi: 10.1007/s13213-014-0844-9.