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Agroindustrial co-products and waste cooking oil in the production of lipases by thermophilic *Bacillus licheniformis* SMIA-3

Aparecida Selsiane Sousa Carvalho[1*], Erica Cruz[1], Vanessa Vicente Vieira Andrade[1], Josilene Lima Serra[2], Marco Antonio Martins[3], Meire Lelis Leal Martins[1] and Luana Pereira de Moraes[1]

[1]Laboratório de Tecnologia de Alimentos, Universidade Estadual do Norte Fluminense, Av. Alberto Lamego, 2000, 28013-602, Campos dos Goytacazes, Rio de Janeiro, Brasil. [2]Laboratório de Tecnologia em Alimentos, Instituto Federal do Maranhão, São Luís, Maranhão, Brasil. [3]Laboratório de Solo, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Rio de Janeiro, Brasil. *Author for correspondence. E-mail: sc.selsiane@hotmail.com

ABSTRACT. The present study investigated the potential lipase production for *Bacillus licheniformis* SMIA-3 using the agro-industrial co-products: orange flour (OF) and grape flour (GF) blend waste cooking oil (WCO). The OF was selected due to its best source for lipase production observed in preliminary tests. Therefore, OF was tested at different fermentation times at 50°C using the statistical design Central Composite Rotatable Design (CCRD) allied to the response surface. An optimal region was found with lipolytic activity of 0.349 U mL^{-1} with OF and WCO filters around (0.50% w v^{-1}) and between (0.55 and 0.75% w v^{-1}), respectively, and the fermentation time at the central point (42h). Data supplied a method to produce lipase using orange flour and frying oil, as a way to reuse these waste as feedstock to obtain employable lipase and lower production costs with biotechnological applications in industrial sector.

Keywords: Orange flour; waste cooking oil; microbial lipase; *Bacillus licheniformis* SMIA-3.

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Introduction

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are enzymes of the hydrolases family that catalyze the hydrolysis of triacylglycerols in diacylglycerides, monoacylglycerides, free fatty acids and glycerol under aqueous conditions (Mendes, Oliveira, & Castro, 2012; Saraswat, Verma, Sistla, & Bhushan, 2017). The hydrolytic catalysis of ester bonds occurs at interface between insoluble substrate phase and aqueous phase. In non-aqueous conditions, the enzyme catalyzes the reverse reaction (esterification, interesterification and transesterification) and produces acylglycerols from glycerol and fatty acids (Saxena, Sheoran, Giri, & Davidson, 2003). Therefore, they are considered to be excellent substances due to their versatility in catalyzing countless reactions, which broadens their application and enables their use in several industrial sectors such as detergents, cosmetics, paper production, food, biodiesel and biopolymers synthesis (Barros, Fleuri, & Macedo, 2010).

Lipases can be produced for animal, vegetable or fermentative process that use microorganisms. The enzymes production by microorganisms is suitable due to the rapid growth of the microorganisms that release the enzymes, and to the high yield enzymatic process bringing speed in the process (Hasan, Shah, & Hameed, 2006). In the lipases of microorganisms, the lipases by thermophilic microorganisms have been applied more and more in the industry for their intrinsic properties as the high stability and activity in high temperatures as well as stability to denaturing agents and pH variations. Therefore, these enzymes are ideal for industrial processes, given that the use of higher temperatures prevent contamination by mesophilic microorganisms, besides reducing viscosity and increasing solubility of the compound (Egorova & Antranikian, 2005; Elleuche, Schröder, Sahm, & Antranikian, 2014; Patel, Matsakas, Rova, & Christakopoulos, 2019). Thermophilic lipase production has been observed in several species of Bacillus (Saun, Mehta, & Gupta, 2014), Neosartorya (Sun et al., 2016), Acinetobacter sp. (Ahmed, Raghavendra, & Madamwar, 2010), Rhizopus (Sun, Xu, & Wang, 2009), and Aspergillus (Mhetras, Bastawde, & Gokhale, 2009).

High cost of lipase production maybe a limiting factor for its development (Anobom et al., 2014). The reduction of cost of the fermentation process can be achieved by replacing some components of the culture medium by agro-industrial residues and co-products from fruit processing (Pereira, Sant'Ana, & Amaral, 2019; Jain & Naik, 2018). Besides that, the use of organic waste to produce value-added bio-products reduces soil and water pollution (Panda, Mishra, Kayitesi, & Ray, 2016). Waste cooking oil is a residue produced from

food frying. Rich in free fatty acids due to the hydrolysis occurred during the frying process its physical and chemical properties do not enable it to be used for human consumption, but it can be applied as a source of carbon in the production of lipase (Xiaoyan et al., 2017). In addition to this residue, orange waste can be used for the production of enzymes because in their constitution there are substances such as pigments, sugars, organic acids and fibers (Ahmed et al., 2016; Marín, Sánchez, & Artola, 2019; Sharma, Oberoi, & Dhillon, 2016). For lipase production the use of orange flour containing peel and orange pomace has not been reported.

In this context, this study aims to investigate the potential of production of lipase for *Bacillus licheniformis* SMIA-3 using agro-industrial co-products and waste cooking oil. In this study, the best co-product for lipase production was selected and studied through the statistical Central Composite Rotational Design (CCRD).

Material and methods

Microorganism

The microorganism used in this study was *Bacillus licheniformis* SMIA-3, kindle provided by the Microbiology Sector of the Food Technology Laboratory of the State University of North Fluminense (UENF).

Enzyme production

Culture medium and inoculum preparation

The culture medium for production of lipase was composed of: KCl (0.3 g L^{-1}); K_2HPO_4 (0.87 g L^{-1}); $MgSO_4$ (0.5 g L^{-1}); NaCl (10.0 g L^{-1}); $CaCl_2$, $2.2x10^{-3}$; ZnO, $2.5x10^{-3}$; $FeCl_3.6H_2O$, $2.7x10^{-2}$; $MnCl_2.4H_2O$, $1.0x10^{-2}$; $CuCl_2.2H_2O$, $8.5x10^{-4}$; $CoCl_2.6H_2O$, $2.4x10^{-3}$, $NiCl_3.6H2O$, $2.5x10^{-4}$, H_3BO^3, $3.0x10^{-4}$; Na_2MoO_4, $1.0x10^{-3}$); commercial OF (0 – 10 g L^{-1}), commercial GF (0 – 10 g L^{-1}), and WCO (0.1 – 10 g L^{-1}) from the university restaurant of the State University of North Fluminense.

B. licheniformis SMIA-3 lipase production was performed in 250 mL Erlenmeyer flasks containing 50 mL of medium. The medium was prepared with distilled water using quantities OF or GF and WCO. The pH was adjusted to 7.5 with 1.0 M NaOH and the flasks containing the medium were autoclaved at 121°C, 1 atm for 15 min. Cells were then inoculated into the medium (initial number of cells 10^4) and incubated at 50°C in an orbital shaker (Thermo Forma, Ohio, USA) operated at 150 rpm for different hours.

Influence of the use of OF, GF and WCO for lipase production

Agro-industrial co-products OF and GF were evaluated at concentrations 0.15, 0.25, 0.50, 0.75 and 1 (% w v^{-1}) with WCO (0.5% w v^{-1}). The medium was prepared in 250 mL Erlenmeyer flasks with final volume of 50 mL of medium. Cultivation of *B. licheniformis* SMIA-3 was performed at 150 rpm, during 36h and 50ºC.

Experimental design

The study made use of a Central Composite Rotational Design (CCRD) 23, with six axial points and three repetitions at the central point, totaling 17 assays. The independent variables studied were orange flour (OF), waste cooking oil (WCO) and fermentation time process. Table 1 demonstrates the levels attributed to each variable determined based on results of preliminaries studies. Surface-response methodology (SRM) was used to obtain a model for lipase activity using the STATISTICA 7.0 software package, and the non-explicit variables were fixed at the central points.

Obtaining the enzymatic extract

After fermentation process, the flasks were removed and the contents were then centrifuged (HERMLEZ 382K, Wehingen, Germany) at 15,500 g for 15 min., at 4ºC, to obtain the cell free supernatant, used to crude enzyme preparation.

Table 1. Levels of the independent variables in the experimental design.

	Levels				
Variable	-1.68	-1	0	1	1.68
OF (% w v^{-1})	0	0.2	0.5	0.8	1
WCO (% w v^{-1})	0.1	0.28	0.55	0.82	1
Time (h)	10	23	42	61	74

Enzyme assay

Lipase activity was determined using the methodology based on the hydrolysis of the substrate p-nitrophenyl butyrate (pNPB) to p-nitrophenol (Virgen-Ortíz et al., 2017). The reactional medium was composed of a mixture containing 50 µL of p-nitrophenylbutyrate solution, 850 µL of Tris-HCl buffer solution, pH 8.5, and 100 µL of the enzyme extract, and was incubated at 70°C for 2 minutes. The reaction was interrupted with 250 µL of sodium carbonate in an ice bath. Stirring was carried out and the absorbance was measured out at 410 nm in a spectrophotometer (SHIMADZU UV - mini 1240). For the control assay the same conditions were performed, however, without the enzymatic extract. One unit (U) of activity was defined as the amount of enzyme that hydrolyzes 1 µmol of p-NPB per minute under the conditions mentioned above.

Results and discussion

Influence of the use of OF, GF and WCO for lipase production

Addition of agro-industrial co-products in medium fermentation was evaluated to verify the potential of the lipase production by thermophilic *Bacillus licheniformis* SMIA-3 (Table 2).

Table 2. Lipase production using agro-industrial co-products in medium fermentation.

Concentration of flour (% w v^{-1})	Lipase production OF (U mL^{-1})	Lipase production GF (U mL^{-1})
0.15	0.0472^{a}	0.0780^{a}
0.25	0.0264^{a}	0.0721^{a}
0.50	0.3480^{bc}	0.0745^{a}
0.75	0.1300^{a}	0.1730^{b}
1.00	0.0131^{a}	0.1310^{ab}
Carbon source control (0.5 % w v^{-1})	0.3050^{c}	0.3050^{c}

Means followed by the same letter are not statistically different by Tukey test at 5% probability.

The composition of the commercial OF was: 46% carbohydrate, 6% protein, 3% total fat, 34% fiber, 0.2% sodium and 10.8% other constituents. The composition of the commercial GF was: 86.6% carbohydrate, 3.6% protein, 5.4% fiber and 4.4% and other constituents. The cooking oil residue presented an acidity of 2.54 mg.KOH.g^{-1}, being above that recommended by the Brazilian legislation for consumption. The higher lipase production was obtained using the OF in a concentration of 0.50% w v^{-1} with values of 0.3480 U mL^{-1} and was statistically similar to control, using the glucose as carbon source. The OF was the only co-product that exhibited a lipid content and high nitrogen value in the composition, and this fact may have contributed to potentialize the lipolytic activity by *Bacillus licheniformis* SMIA-3. The high value of lipolytic activity using the GF as substrate was verified in 0.75% w v^{-1} concentration, with a value (0.173 U mL^{-1}), which was twice as lower in comparison to OF.

The increase of OF concentration in the culture medium (0.75 and 1% w v^{-1}), triggered a decline in the lipolytic activity. It can be explained by the presence of pectin in OF which promotes the formation of the pectin and lipase complex, and does not allow interaction of lipase with the substrate (Kumar & Chauhan, 2010). Best lipase production using waste can be achieved by combining treatments. In the study using orange, pomegranate and pineapple residues, the authors obtained 57.63 U mL^{-1}, when combined the residues and treated with ultrasound for 15 minutes before starting the fermentation process at pH 6, temperature of 33°C, and agitation at 210 rpm for 4 days (Selvakumar & Sivashanmugam, 2017). The treatment of waste before the fermentation process is an alternative to increase the enzymatic activity, as it causes the disintegration of the biological structure of the compounds facilitating the consumption by the microorganisms.

Experimental design

Table 3 shows the responses for lipase production from *Bacillus licheniformis* SMIA-3 and pH in the end of fermentation medium in different conditions. The results of lipolytic activity ranged from 0.119 to 0.444 U mL^{-1} and pH values from 6.89 to 9.17, respectively. The higher concentrations of lipase enzyme (~0.4 U mL^{-1}) was obtained in pH 9, concentration of OF between 0.5 to 0.8, WCO between 0.55 to 0.82 and time from 61 hours or more.

Table 3. Experimental design and responses for lipase production from *Bacillus licheniformis* SMIA-3.

Run	OF ($w\ v^{-1}$)	WCO ($\%w\ v^{-1}$)	Time (hours)	Lipase activity (UmL^{-1})	pH
1	(-1) 0.20	(-1) 0.28	(-1) 23	0.253	7.89
2	(+1) 0.80	(-1) 0.28	(-1) 23	0.146	8.00
3	(-1) 0.20	(+1) 0.82	(-1) 23	0.268	7.79
4	(+1) 0.80	(+1) 0.82	(-1) 23	0.119	8.20
5	(-1) 0.20	(-1) 0.28	(+1) 61	0.243	8.81
6	(+1) 0.80	(-1) 0.28	(+1) 61	0.297	9.04
7	(-1) 0.20	(+1) 0.82	(+1) 61	0.173	8.82
8	(+1) 0.80	(+1) 0.82	(+1) 61	0.444	9.17
9	(-1.68) 0	(0) 0.55	(0) 42	0.184	8.31
10	(+1.68) 1.0	(0) 0.55	(0) 42	0.306	8.33
11	(0) 0.5	(-1.68) 0.10	(0) 42	0.252	8.13
12	(0) 0.5	(+1.68) 1.0	(0) 42	0.261	7.46
13	(0) 0.5	(0) 0.55	(-1.68) 10	0.206	6.89
14	(0) 0.5	(0) 0.55	(+1.68) 74	0.410	9.03
15	(0) 0.5	(0) 0.55	(0) 42	0.349	8.07
16	(0) 0.5	(0) 0.55	(0) 42	0.334	7.98
17	(0) 0.5	(0) 0.55	(0) 42	0.313	8.11

The use of wastes or by-products in the enzyme production is an alternative to lower production costs. Several authors reported the use of orange processing residues in enzyme production (Ahmed et al., 2016; Marín et al., 2019; Sharma et al., 2016), and produced lipase from palm kernel cake (palm kernel) and palm oil refining alkaline sludge (palm oil sludge), both residues from palm oil agroindustry (palm) (Penha et al., 2016).

The study of analysis of variance (ANOVA) test for the quadratic model of the response surface for lipase activity is shown in Table 4. The ANOVA showed that experimental data created coefficient of determinations (R^2) of 0.8878. It was observed that the significant regression coefficients for lipase production with $p < 0.05$ by the F test were the quadratic terms for OF concentration and for WCO, the linear term for the time studied, as well as, for the interaction between OF and time.

Table 4. ANOVA for the quadratic model of the response surface for lipase production from *Bacillus licheniformis* SMIA-3.

Variation source	Sum of squares	Degrees of freedom	Means square	F-value	F-critical	Adj.R^2
Regression	0.119	9	0.013	6.599	3.68	0.8878
Residual	0.014	7	0.002			
Lack of fit	0.013	5	0.002	8.063	19.30	
Pure error	0.000	2	0.000			
Total	0.147	16				

The adjusted model was obtained and is described in Equation (1), where x_A corresponds to OF, x_B is WCO and x_C is Time.

$$\text{Lipase } (U\ mL^{-1}) = 0.334 + 0.020 *x_A - 0.036 *x_A^2 + 0.006 *x_B - 0.032 *x_B^2 + 0.052 *x_C - 0.014*x_C^2 + 0.022*x_A*x_B + 0.073*x_A*x_C + 0.011*x_B*x_C \quad (1)$$

It is important to emphasize that the high concentrations of OF and WCO may be negatively influenced by the lipolytic activity, and the interaction between the OF concentration and fermentation time has a positive influence. These results show the importance of studying the metabolic requirements of the microorganism and the time required for degradation of residues at different concentrations.

The response surfaces were obtained by fixing the middle points of non-explicit variables. Maximum lipolytic activity was obtained when the culture medium containing 0.50 ($\%\ w\ v^{-1}$) of OF, and between 0.55 and 0.75 ($\%\ w\ v^{-1}$) of WCO was used at temperature growth of 50°C during 42 hours of fermentation (Figure 1).

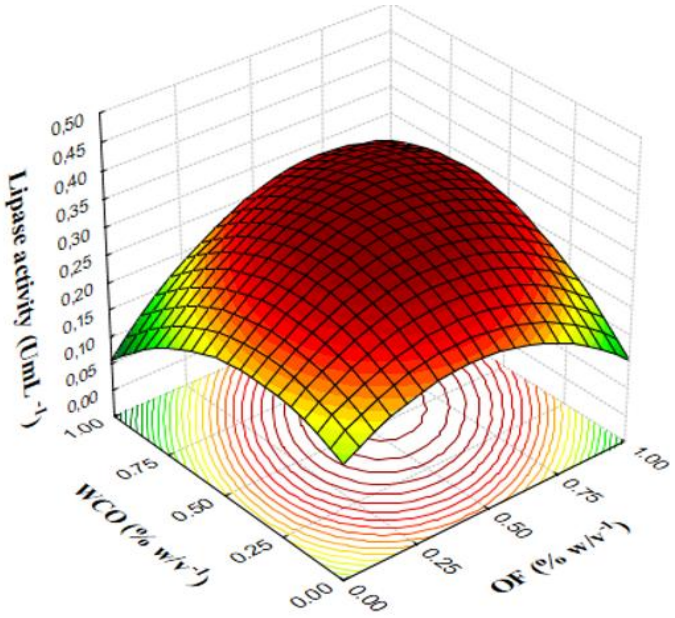

Figure 1. Response surface plot indicating the effects of interactions between WCO and OF on lipase activity.

Simultaneous increase of WCO and OF from 0.75 (% w v^{-1}) in the medium, can decrease the lipolytic activity due to the excess of nutrients in the medium. It can be an inhibitory factor in the production of enzymes. In the present study, the fermentation time for lipase production was higher than the time studied for *Acinetobacter sp* (Gururaj, Ramalingam, Devi, & Gautam, 2016). However, the concentration of inducer required for *Bacillus licheniformis* SMIA-3 to secrete the enzyme was lower when compared to the microorganism used by the authors. The simultaneous increase of OF and fermentation time, caused an increase of values of the lipolytic activity obtained by *Bacillus* (Figure 2) due to the time required for the microorganism to degrade the complex components of the residue and release simple substances that will be used by the microorganism metabolic machine as an energy source, for its growth and subsequent secretion of the enzyme. In the literature, long periods of fermentation have been observed to produce lipases with agro-industrial residues, as found in the present study (Amin, Bhatti, & Rehman, 2011; Selvakumar & Sivashanmugam, 2017; Tacin et al., 2019).

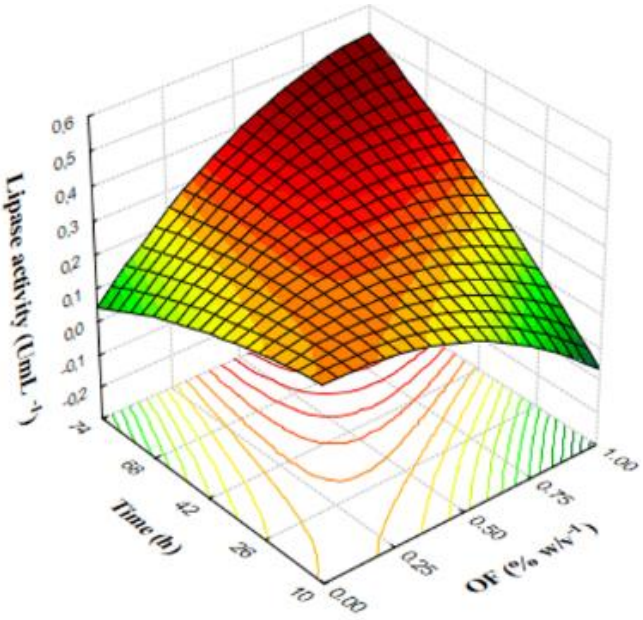

Figure 2. Response surface plot indicating the effects of interactions between time and OF on lipase activity.

Regarding the interaction between fermentation time and oil concentration (Figure 3), it was observed in the present study that with the OF fixed at the central level (0.50% w v^{-1}) it is necessary to increase the fermentation time to achieve higher lipolytic activity, and optimally use of the WCO concentrations between

0.5 to 0.75 (%w v^{-1}). One possible explanation for these results is that lipolytic activity increases with the fermentation time due to the metabolism of the microorganism that initially produces the enzyme and uses it to hydrolyze the oil in the fermented medium, and then accumulates in the medium due to the reduced amount of oil. *P. aeruginosa* KM110, isolated from the wastewater of an oil processing plant and utilizing olive oil as a substrate, produced 0.76 U mL^{-1} of lipase at 24h of time fermentation (Mobarak-Qamsari, Kasra-Kermanshahi, & Moosavi-nejad, 2011). WCO present in the medium age as an inducer for lipase production and as an easier carbon source for the microorganism to assimilate when compared to OF.

Thus, its increased concentration stimulates enzyme secretion, requiring lower OF concentrations. *Yarrowia lipolytica* M53 produced 12.7 U mL^{-1} activity lipase using WCO as an inducing source in the medium and compared to other oily substances such as olive oil, soybean, sunflower, rapeseed and oleic acid. The activity was higher using WCO (Xiaoyan et al., 2017). Lipolytic activity of *Stenotrophomonas maltophilia* was negatively affected by the use olive oil inducer (Beikdashti et al., 2012), but lipase production was optimal by *Bacillus pumilus* RK31 using medium containing 1% olive oil as inducer (Kumar, Mahajan, Kumar, & Singh, 2011). Besides that, was reported that tween 80 is also found to be the best carbon source inducing production of lipase from a *Bacillus* sp. (Sidhu, Sharma, Soni, & Gupta, 1998).

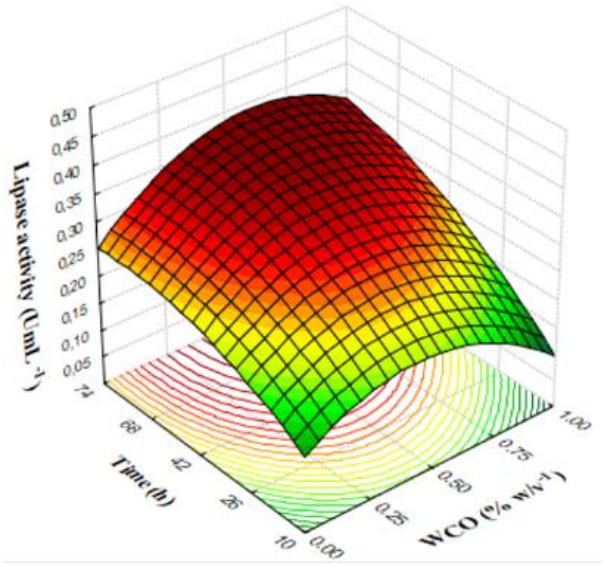

Figure 3. Response surface plot indicating the effects of interactions between time and WCO on lipase activity.

The production and quantification of enzymatic activity in the fermentative medium is related to the growth factors of the microorganism responsible for the process, as the pH (Barbosa, Gentil, Ladeira, & Martins, 2014). This parameter is a factor limiting for cellular growth and multiplication of the microorganism, as well as, for secretion of some product of biotechnological interest. The analysis of variance (ANOVA) for the quadratic model of the response surface for pH is shown in Table 5. The adjusted model was significant by the F test ($p < 0.05$), producing a coefficient of multiple determinations (R^2) of 0.8377.

Table 5. ANOVA for the quadratic model of the response surface for final pH from *Bacillus licheniformis* SMIA-3.

Variation source	Sum of squares	Degrees of freedom	Mean square	F-value	F-statistic	Adj.R^2
Regression	4.824	9	0.536	4.024	3.68	0.8377
Residual	0.932	7	0.133			
Lack of fit	0.923	5	0.185	41.655	19.30	
Pure error	0.009	2	0.004			
Total	6.688	16				

The adjusted model was obtained and is described in Equation (2), where A corresponds to OF, B is WCO and C is Time.

$$pH= 8.025 + 0.083A+ 0.192A^2- 0.065B+ 0.007B^2 + 0.553C+ 0.065C^2 + 0.053A*B+ 0.008A*C+ 0.005B*C \quad (2)$$

The increase of pH of medium at the end of fermentation was observed regardless of the concentration of OF and WCO (Figure 4, 5, 6).

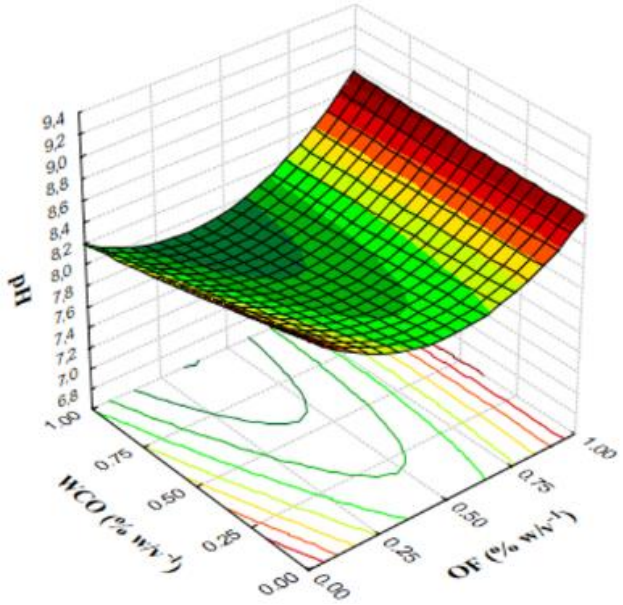

Figure 4. Response surface plot indicating the effects of interactions between WCO and OF on final pH.

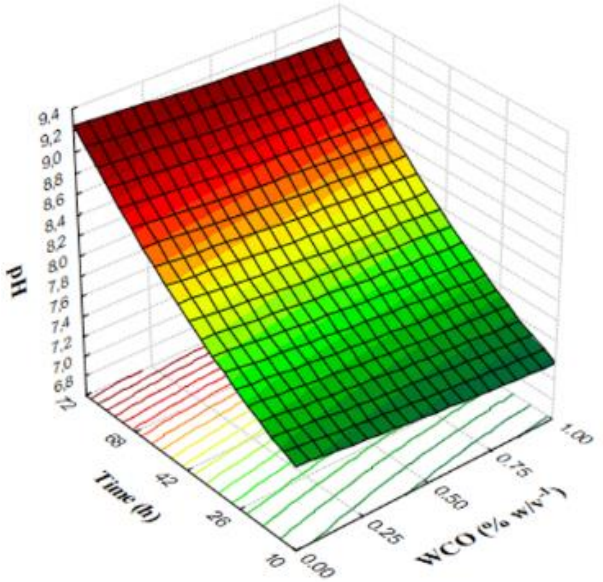

Figure 5. Response surface plot indicating the effects of interactions between time and WCO on final pH.

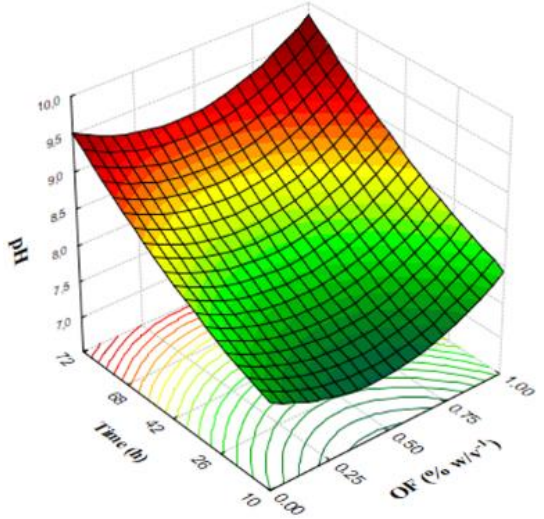

Figure 6. Response surface plot indicating the effects of interactions between time and OF on final pH.

The alkaline pH was due to the consumption of organic nitrogen in the medium by the microorganism (Ladeira, Cruz, Delatorre, Barbosa, & Martins, 2015; Oliveira, Barbosa, Martins, & Martins, 2014). The pH of the medium influences the behavior of the three-dimensional lipase structure. Lipase produced by *Bacillus* sp. PU1 at different pH did not present conformational flexibility when exposed at pH 5 and 7, however, at pH 9 there was necessary double of the enzyme for catalysis (Esakkiraj et al., 2017). Medium pH significantly influences the lipase secretion by *Thermomyces lanuginosus,* and increasing or decreasing pH medium, the lipase activity decreased. The best pH was recorded for authors at 6.0-7.0 (Sreelatha, Rao, Kumar, Girisham, & Reddy, 2017).

Conclusion

Our study established that the feedstocks, OF and WOC were proper sources of lipase. The high lipase production (0.349 U mL^{-1}) by thermophilic *Bacillus licheniformis* SMIA-3 using these alternative sources were obtained with low concentrations of OF and frying oil, ranging from 0.5 to 0.75 (% w v^{-1}). Lipase showed a good stability in alkaline conditions and suggest their use a potential candidate in cleaning products. These dates supplied a method to produce lipase using orange flour and frying oil, as a form of reusing theses wastes as feedstock for obtention of employable lipase and lower production costs with biotechnological applications in industrial sector.

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