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# Actinobacteria hydrolase producer in solid-state fermentation using licuri

## Actinobactéria produtora de hidrolase em fermentação em estado sólido com licuri

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## ABSTRACT

In recent times, green technologies have gained space and interest in many industrial sectors. Examples of this are enzymes, which can be obtained by solid-state fermentation using microorganisms and agro-industrial residues as substrates. In this context, this study aimed to verify the production of hydrolases leading to *Arthrobacter polychromogenes* and *Streptomyces violaceoruber* strains as producers and the influence of moisture and residues of licuri [*Syagrus coronata* (Martius) Beccari] and wheat as substrates. The solid-state fermentation was performed at 28 °C/12 days, with different ratios of wheat bran/degreased almond licuri cake: 0/70%, 10/60%, 35/35%, 60/10% and 70/0%; and licuri peel, set at 30%. The medium moisture ranged from 29 to 67%. Licuri peel was added to facilitate aeration of the medium; wheat bran is commonly used to produce lipases and pectinases; and degreased almond licuri cake is the substrate of interest in this work, since it was inspired by the extractive production of licuri in the semiarid region of Brazil. The wheat bran/degreased almond licuri cake ratio did not affect enzyme production by *A. polychromogenes*; however, with *S. violaceoruber*, the ratio value was significant for lipase production but not for pectinases. The medium prepared with 67% moisture and equal ratios of wheat bran and degreased almond licuri cake (35/35%) presented the highest enzymatic activities for both enzymes, 840.46 U/gds for lipases and 15.53 U/gds for pectinases, during Composite Central Design assays. With validation experiments, it was verified that licuri residues can be used as substrates for the production of hydrolases by solid-state fermentation and that actinobacteria evaluated are suitable microorganisms.

**Index terms:** Lipase; pectinase; oil extraction waste; central composite design; response surface methodology.

## RESUMO

Nos últimos tempos, as tecnologias verdes ganharam espaço e interesse em muitos setores industriais. Exemplo disso são as enzimas, que podem ser obtidas por fermentação em estado sólido usando microrganismos e resíduos agroindustriais como substratos. Nesse contexto, este trabalho teve como objetivo verificar a produção de hidrolases, utilizando as cepas de *Arthrobacter polychromogenes* e *Streptomyces violaceoruber* como produtoras, e a influência, da umidade e resíduos de licuri [*Syagrus coronata* (Martius) Beccari] e trigo como substratos. A fermentação em estado sólido foi realizada a 28 ºC/12 dias, com diferentes proporções de farelo de trigo/ torta de amêndoa de licuri desengordurada: 0/70%, 10/60%, 35/35%, 60/10% e 70/0%; e casca de licuri, fixada em 30%. A umidade média variou de 29 a 67%. Casca de licuri foi adicionada para facilitar a aeração do meio; o farelo de trigo é comumente utilizado para produção de lipases e pectinases; e a torta de amêndoa de licuri desengordurada é o substrato de interesse deste trabalho, uma vez que ele foi inspirado pela produção extrativa de licuri na região do semiárido do Brasil. A relação farelo de trigo/torta de amêndoa de licuri desengordurada não afetou a produção de enzimas por *A. polychromogenes*; no entanto, com *S. violaceoruber*, o valor da razão foi significativo para a produção de lipase, mas não para pectinases. O meio preparado com umidade de 67% e razões iguais de farelo de trigo e torta de amêndoa de licuri desengordurada (35/35%) apresentou as maiores atividades enzimáticas para ambas as enzimas, 840,46 U/gds para lipases e 15,53 U/gds para pectinases, durante os ensaios de Delineamento Central Composto. Com os experimentos de validação, verificou-se que os resíduos de licuri podem ser utilizados como substrato para a produção de hidrolases por fermentação em estado sólido, e que as actinobactérias avaliadas são adequadas para esse fim.

**Termos para indexação:** Lipase; pectinase; extração de óleo de resíduo; delineamento central composto; metodologia de superfície de resposta.

## INTRODUCTION

Currently, the search for green technologies has been triggered/driven by the use of biocatalysts (enzymes) and less polluting substances, which do not need other reaction aids and have high specificity, motivating the enlargement of their applications and the interest of researchers in various parts of the world who focus on reducing production costs (Sánchez; Tonetto; Ferreira, 2017). Solid-state fermentation (SSF), whose use for the production of these biocatalysts has increased in the last two decades (Pandey, 2003; Amin; Bhatti; Bilal, 2018; Šelo et al., 2021), presents the advantages of proper productivity and the use of agro-industrial waste as a substrate (circular economy); however, there are still some technological "bottlenecks", such as interference of the type of substrate employed (medium heterogeneity), the microorganism used, process control (temperature, moisture), and the presence of inhibitors or activators (Abrunhosa et al., 2011; Oumer, 2018; Ruiz et al., 2012; Sethi; Kumari; Santilata, 2016; Ashok et al., 2016). The most widely used agro-industrial waste is from fruit and vegetable processing (Šelo et al., 2021; Kaur; Gupta, 2017; Oliveira et al., 2016; Umsza-Guez et al., 2011; Bastos; Carvalho; Alegre, 2014). An emerging substrate for SSF, not widely studied, is the almond licuri [*Syagrus coronata* (Martius) Beccari] cake, a residue obtained after the extraction of its oil by cold press. This residue is composed of 41% non nitrogenous substances, 19% protein, 16% cellulose and 11% to 12% oil (Bondar, 1939).

Among the microorganisms used in SSF, the most frequent are fungi (Šelo et al., 2021; Rodrigues et al., 2020) and bacteria (Kaur; Gupta, 2017; Soccol et al., 2017). However, some recent studies have pointed out actinobacteria as emerging biocatalyst producers of important enzymes of industrial and environmental interest and of high resistance to extreme conditions (Orozco et al., 2008). In this group, *Streptomyces* gender has been used for enzyme production (Cadirci; Yasa; Kocyigit, 2016; Sutto-Ortiz et al., 2017); however, only two studies conducted with *Streptomyces violaceoruber* have been found (Giarrizzo; Budis; Taddei, 2007; Matoba; Sugiyama, 2003) for *Arthrobacter* gender, no work aiming at enzyme production has been published to date.

Concerning the enzymes of industrial interest, in addition to carbohydrases and proteases, lipases (triacylglycerol hydrolases, EC 3.1.1.3) and pectinases have a wide variety of industrial uses (Rodrigues et al., 2020). Lipases are hydrolases that act by hydrolyzing, esterifying, trans esterifying or interesterifying oils and fats and releasing free fatty acids, diacylglycerols, monoacylglycerols and glycerol. Among hydrolases, lipases have a wide range of applications and have been used in the production of cosmeceuticals, biosensors, emulsifiers, building blocks, biodiesel, beyond the leather, paper and cellulose industries; animal feed; fabrics; and wastewater treatment and bioremediation (Šelo et al., 2021).

Pectinases (in their different isoforms) are able to use polysaccharides constituting the middle lamella and the primary wall of plant cells as substrates and are therefore widely used in the food industry mainly for the processing of fruits and vegetables with the aim of improving clarification yield, improving the colour and taste intensity of the products and assisting in the extraction of compounds of interest (e.g., phenols). The enzyme is also used in other industrial sectors, such as paper, textiles, biorefineries and animal feed (Garg et al., 2016; Kashyap et al., 2001).

Currently, for the production of enzymes of industrial interest, scientists and industries are looking for ways to reduce the time, the amount of laboratory material used and the number of experiments to optimize the results. A widely used alternative has been the use of response surface methodology (RSM), which allows the evaluation of multiple variables at a time, as well as their interaction and cumulative effect, allowing process optimization with a small number of experiments. Its use in SSF to obtain the most varied products has been expanding due to the good results obtained by this methodology (Said; Hamid, 2018; Handa; Sharma; Pathania, 2016; Wong et al., 2017).

Thus, this study aimed to verify the influence of moisture and substrate proportion on SSF for the production of lipases and pectinases using two strains of actinobacteria not yet used for this purpose and RSM for process analysis.

## MATERIAL AND METHODS

### Substrate preparation

Licuri cake and peel were purchased from the Cooperativa de Produção da Região do Piemonte da Diamantina (COOPES) (11°22′51″S, 40°0′46″W), and wheat bran (WB) was acquired in a local market. The approximate composition of the substrate is wheat bran (*Triticum* spp.) - Dietary fibre (33.4–63.0%), moisture (8.1–12.7%), ash (3.9–8.10%), protein (9.60–18.6%) (Curti et al., 2013) and Licuri Cake - moisture (77.4 ± 0.16%), ash (1.4 ± 0.06%), protein (3.2%), lipids (4.5 ± 0.3%), carbohydrates (13.2%) (Crepaldi et al., 2001). The difference in the hundredth composition of the licuri pie depends on the extraction method used.

Due to the high lipid content (49.27% left after cold extraction) of licuri cake, it was necessary to degrease it to allow medium humidification for subsequent fermentation. The oil extraction was carried out with hexane solvent at a 1:6 oil/solvent ratio under constant stirring at 180 RPM and 25 °C in a shaker (Model TE-424, Tecnal). The final lipid content in the degreased licuri cake (DLC) and WB was quantified by the method of Bligh and Dyer (1959).

Actinobacteria prospects and identification for enzyme production Actinobacteria, provided by the Laboratory of Microbiology located at the State University of Feira de Santana (LAPEM/UEFS), isolated from Chapada Diamantina (12°52′49″S, 41°22′20″W) and Caatinga Bioma (10°57'14"S, 40°34'33"W), located in the State of Bahia, northeastern Brazil, were used. An amount of 42 strains were tested. They were stored at -20 °C in cryotubes containing 20% glycerol solution until use. The isolates were identified as *Arthrobacter polychromogenes* (PI30) and *Streptomyces violaceoruber* (PA32) by Biotyper Matrix-Assisted Laser Desorption Ionization-Time of Flight mass spectrometry (MALDI-TOF MS).

## Inoculum preparation

Actinobacteria were activated in petri dishes containing yeast malt medium and incubated at 28 °C in an incubator (Model Q316B25, Chimis) for 12 days. Six plugs approximately 5 mm in diameter containing the microorganisms were removed from the plates (with sterile tips) and inoculated into 50 g of parboiled rice (previously hydrated and sterilized at 120 °C/55 min) in 250 mL flasks, which were incubated at 28 °C in an incubator for 12 days. At the end of this period, 75 mL of 0.85% sterile NaCl solution was added to the fermented parboiled rice in the flasks, and then it was kept under light agitation (100 RPM) at room temperature for 30 min (for microorganism suspension), followed by filtration on sterile gauze. This suspension was used as inoculum for the licuri, wheat or licuri/wheat medium.

After growth, actinobacteria were incubated in solid-state fermentation medium containing 25 g DLC, 20 g WB, 15 g LP and 2% (v\w) nutrient solution, adapted from Mandels and Sternberg (1976). As an alternative medium, fermentations were carried out only with WB and LP in equal proportions (25 g each supplemented with nutrient solution). Each isolate was individually tested for lipase and pectinase production. Actinobacteria were inoculated in induction medium and incubated in a shaker at 28 °C for 10 days.

## Fermentative design and analysis procedure

Fermentation was carried out in 250 mL conical flasks containing 20 g of substrate, whose compositions of WB and DLC were varied from 0 to 70% of the medium, as described in the abstract. To improve the porosity of the fermentation substrate, LP was used at 30% w/w. Moisture levels were adjusted to the values described in Table 1 with water, discounting the 2% (v\w) nutrient solution and 10% (v/w) inoculum. A moisture content of 67% was adopted as the maximum because, in a preliminary saturation test, it was noticed that, above this value, it was possible to observe free water in the environment, which would mischaracterize the SSF. Moisture analyses were performed in triplicate using an infrared moisture balance (Engineering, Quimiserv) using a temperature of 90 °C and 3 g of sample.

After the nutrient solution was added to the substrates in the conical flask, they were autoclaved at 121 °C for 15 min. A 2 mL (10% inoculum volume/medium weight ratio) aliquot of the bacterial suspension (resuspended from parboiled rice, as per item Inoculum preparation) was added to each flask containing sterilized substrates and incubated at 28 °C for 12 days, adapted from Santos et al. (2016).

Assays were conducted according to a $2^2$ Composite Central Design (CCD), including four (04) axial points and three (03) centre point repetitions combined with the Response Surface Methodology (RSM), totalling 11 trials.

## Enzyme extraction

The fermented solid was homogenized, and 10 g of sample was removed, to which 100 mL distilled water was added and stirred at 100 rpm and 26 °C for 1 h. Then, vacuum filtration was performed using sterile gauze, and the filtrate was centrifuged at 5500 rpm for 15 min at 5 °C. The supernatant (enzyme extract) was stored in an amber flask and frozen at -20 °C, according to Umsza-Guez et al. (2011), adapted.

## Enzyme activity measurements

To determine lipase activity, the methodology described by Ferrarezi et al. (2014) was used. Calculations for lipase activities were made using the *p*-nitrophenol calibration curve ($R^2 = 0.9957$).

Pectinase activity was determined according to the methodology described by Martin et al. (2010). The released D-galacturonic acid was measured by the DNS method (3.5-dinitrosalicylic acid) proposed by Miller (1959), and the activity was calculated through its calibration curve and expressed in U/gds ($R^2 = 0.9937$). All analyses were done in triplicate.

### Statistics evaluation

Statistical analysis of the data was performed using Statistica Software. CCD was rated at 5% significance. An analysis of variance (ANOVA) was performed in addition to the analyses by RSM.

### Experimental validations at the axial points

Validation of lipase production was conducted for the two strains of actinobacteria evaluated were carried out with a humidity of 67% and a WB/DLC ratio of 70/0 (%) and 0/70 (%). The percentage of LP was kept constant and equal to 30%. Validation at the axial points was chosen because of the interest in obtaining enzymes only with licuri as the substrate, thus improving its use by the population of the region where it is extracted. In addition, lipase production was validated because of the smaller values of pectinase activities.

## RESULTS AND DISCUSSION

In the prospection assays, among the 42 actinobacteria evaluated, it was determined that two actinobacteria were suitable for lipase and pectinase production. It was obtained activities of 4.09 U/gds for pectinase production by PI30 (*Arthrobacter polychromogenes*), using WB and LP as substrates (50% each), and 154.01 U/gds for lipase production by PA32 (*Streptomyces violaceoruber*), using WB, DLC and LP as substrates, in proportions of 20%, 50% and 30%, respectively.

Therefore, these two actinobacteria had their lipase and pectinase production optimized. The results obtained are described in Table 1. Both actinobacterial strains grew in all prepared mediums and showed activity for the evaluated enzymes. The E6 assay (moisture 67% and WB/DLC ratio 35/35 (%)) showed the highest activity values for both enzymes and both actinobacteria strains.

Concerning the licuri composition, it is possible to estimate the influence of the licuri oil presence as a lipase inductor, since it has approximately 11% oil content, according to Bondar (1939). Pectinase, however, may have been produced as a constitutive enzyme (Dusœková; Marounek, 2001).

### Lipase production

The activity of lipases obtained with *A. polychromogenes* ranged from 139.09 U/gds (E3) to 840.46 U/gds (E6) with a difference of 83.45% under the different fermentation conditions. For *S. violaceoruber*, the activity ranged from 197.42 U/gds (E3) to 457.63 U/gds (E6), representing 56.86% of the difference (Table 1). The lipolytic activity of the E3 assays (moisture 34.5% and WB/DLC ratio 60/10(%)) presented the lowest activities for both strains. ANOVA showed (Table 2) that the enzymatic activity varied significantly ($p < 0.05$) with moisture for the *S. violaceoruber* strain and that the model was not significant for *A. polychromogenes*, which possibly occurred because of the high variance at the central points and the elevated activity at assay E6, leading to a significant lack of fit ($p < 0.05$).

**Table 1:** Lipase and pectinase activities of *A. polychromogenes* and *S. violaceoruber.*

| Assay | Enzyme activity | | | | | |
|---|---|---|---|---|---|---|
| | Levels | | *A. polychromogenes* | | *S. violaceoruber* | |
| | Moisture (%) | WB/DLC Ratio (%) | Lipases (U/gds) | Pectinases (U/gds) | Lipases (U/gds) | Pectinases (U/gds) |
| E1 | -1 (34.5) | -1 (10/60) | 143.07 | 7.25 | 203.38 | 6.79 |
| E2 | +1 (61.5) | -1 (10/60) | 246.79 | 12.38 | 273.85 | 11.03 |
| E3 | -1 (34.5) | +1 (60/10) | 139.09 | 6.99 | 197.42 | 6.59 |
| E4 | +1 (61.5) | +1 (60/10) | 349.40 | 11.83 | 418.19 | 11.91 |
| E5 | -1.41 (29) | 0 (35/35) | 287.30 | 6.22 | 146.05 | 6.09 |
| E6 | +1.41 (67) | 0 (35/35) | 840.46 | 14.62 | 457.63 | 15.53 |
| E7 | 0 (48) | -1.41 (0/70) | 309.62 | 8.70 | 251.18 | 8.61 |
| E8 | 0 (48) | +1.41 (70/0) | 279.56 | 9.41 | 328.82 | 8.99 |
| E9 | 0 (48) | 0 (35/35) | 225.30 | 8.95 | 302.94 | 7.90 |
| E10 | 0 (48) | 0 (35/35) | 247.00 | 8.67 | 311.29 | 7.98 |
| E11 | 0 (48) | 0 (35/35) | 287.91 | 8.49 | 343.01 | 7.61 |

**Table 2:** ANOVA for lipase activity.

| ANOVA for lipases activity | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Factor | *A. polychromogenes* | | | | | *S. violaceoruber* | | | | |
| | Sum of squares | df | Mean Square | F value | p | Sum of squares | df | Mean Square | F value | p |
| Model | 236447.8 | 5 | 47289.6 | 1.96 | Not significant | 83020.7 | 5 | 16604.14 | 17.16 | Significant |
| Moisture (%)(L) | 150046.5 | 1 | 150046.5 | 148.4 | 0.007 | 66913.01 | 1 | 66913.01 | 149.68 | 0.007 |
| Moisture (%)(Q) | 59150.7 | 1 | 59150.7 | 58.51 | 0.017 | 1160.65 | 1 | 1160.65 | 2.60 | 0.248 |
| WB/DLC (%)(L) | 396.8 | 1 | 396.8 | 0.39 | 0.595 | 7701.68 | 1 | 7701.68 | 17.23 | 0.053 |
| WB/DLC (%)(Q) | 5986.8 | 1 | 5986.8 | 5.92 | 0.135 | 2319.61 | 1 | 2319.61 | 5.19 | 0.150 |
| 1L x 2L | 2840.5 | 1 | 2840.5 | 2.81 | 0.236 | 5647.75 | 1 | 5647.75 | 12.63 | 0.071 |
| Residual | 120367.1 | 5 | 24073.4 | | | 4837.27 | 5 | 967.45 | | |
| Lack of fit | 118345.3 | 3 | 39448.4 | 39.02 | 0.025 | 3943.20 | 3 | 1314.40 | 2.94 | 0.264 |
| Pure error | 2021.9 | 2 | 1010.9 | | | 894.06 | 2 | 447.03 | | |
| Total SS | 356814.9 | 10 | | | | 87857.97 | 10 | | | |

*A. polychromogenes*: $R^2$ = 66.26%; $R^2$adj = 32.53%.
*S. violaceoruber*: $R^2$ = 94.49%; $R^2$adj = 88.99%.

The WB/DLC ratio did not significantly affect, at a 5% level of significance, lipase production by either strain. Since the model for lipase activity produced by *A. polychromogenes* was not significant, data not shown. Below the model (Equation 1) with the independent variables for lipase production, it is possible to observe the positive influence of medium moisture on lipase activity produced by *S. violaceoruber*:

Equation 1 (*S. violaceoruber*):

$$U/gds = 319.17 + 91.59 \times M(\%) - 14.39 \times M^2(\%) + \\ +31.07 \times WB/DLC(\%) - 20.35 \times WB/DLC^2(\%) + \\ +37.58 \times (M \times WB/DLC)(\%) \quad (1)$$

where $M$ is the moisture content and $WB/DLC$ is the wheat bran/degreased almond licuri cake ratio, both in percentage expression.

In the literature, lipase activities obtained by SSF ranging from 18 U/gds, using sugarcane bagasse, with soybean oil as the inductor, and *Thermomucor indicae-seudaticae* N31 as the producing microorganism (Ferrarezi et al., 2014), up to a value of 932 U/gds, with degreased *Jatropha curcas* seed cake and *Pseudomonas aeruginosa* as the producing microorganism (Joshi; Mathur; Khare, 2011), covering all the results obtained by this work. When these results are compared with those obtained with *Streptomyces* specifically, the results obtained for lipase production are much higher than those obtained by Cadirci et al. (2016) (1.74 ± 0.0005 U/gds) with olive oil as an inducer in a wheat bran substrate. For both actinobacteria, the results were also superior to those obtained with *Aspergillus candidus* using almond bran licuri by Farias et al. (2015), who obtained a maximum of 397.15 U/gds of lipase activity.

Concerning lipase production by *A. polychromogenes*, even though the model was not significant, the activities in all the conditions evaluated were high, with values up to 840.46 U/gds, as shown before, with a positive influence of the moisture content. It can also be emphasized that no work about enzyme production by this strain was found in the literature, evidencing the novelty of this work and the importance of the results obtained.

## Pectinase production

Pectinolytic activity for both strains (*A. polychromogenes* and *S. violaceoruber*) in assays 5 and 6 represented the lowest and highest activities, showing variations of 57.45 and 60.78%, respectively, as shown in Table 1. These results were analysed, and by ANOVA (Table 3), it was found that the enzymatic activity varied significantly ($p<0.05$) only with moisture for both strains. Thus, pectinase activity is independent of the WB/DLC ratio.

**Table 3:** ANOVA for pectinases activity.

| ANOVA for pectinases activity | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Factor | *A. polychromogenes* | | | | | *S. violaceoruber* | | | | |
| | Sum of squares | df | Mean Square | F value | p | Sum of squares | df | Mean Square | F value | p |
| Model | 63.663 | 5 | 12.73 | 63.65 | Significant | 75.73 | 5 | 15.15 | 25.68 | Significant |
| Moisture (%)(L) | 59.727 | 1 | 59.73 | 1105.92 | 0.0009 | 65.551 | 1 | 65.55 | 1778.29 | 0.0006 |
| Moisture (%)(Q) | 3.852 | 1 | 3.85 | 71.33 | 0.014 | 9.661 | 1 | 9.66 | 262.08 | 0.004 |
| WB/DLC (%)(L) | 0.005 | 1 | 0.005 | 0.09 | 0.792 | 0.187 | 1 | 0.19 | 5.09 | 0.153 |
| WB/DLC (%)(Q) | 0.116 | 1 | 0.12 | 2.15 | 0.280 | 0.510 | 1 | 0.51 | 13.83 | 0.065 |
| 1L x 2L | 0.020 | 1 | 0.02 | 0.38 | 0.602 | 0.290 | 1 | 0.29 | 7.88 | 0.107 |
| Residual | 1.019 | 5 | 0.20 | | | 2.971 | 5 | 0.59 | | |
| Lack of fit | 0.911 | 3 | 0.30 | 5.63 | 0.155 | 2.897 | 3 | 0.97 | 26.20 | 0.037 |
| Pure error | 0.108 | 2 | 0.05 | | | 0.074 | 2 | 0.04 | | |
| Total SS | 64.682 | 10 | | | | 78.701 | 10 | | | |

*A. polychromogenes*: $R^2$ = 98.42%; $R^2$adj = 96.85%.

*S. violaceoruber:* $R^2$ = 96.22%; $R^2$adj = 92.45%.

The pectinase activity values obtained are within the minimum and maximum values consulted in the literature, which were 5.6 U/gds, using *Penicillium viridicatum* in dry orange peel waste and wheat bran (Silva et al., 2005), up to 265 U/gds, using *Aspergillus niger* in citrus peel (Rodríguez-Fernández et al., 2011).

The models (Equations 2 and 3), with the independent variables for pectinase production, present the positive linear and quadratic influences of medium moisture on activities for both strains of actinobacteria.

Equation 2 (*A. polychromogenes*):

$$U/gds = 8.70 + 2.74 \times M(\%) + 0.83 \times M^2(\%) + \\ + 0.02 \times WB/DLC(\%) + 0.14 \times WB/DLC^2(\%) - \\ - 0.07 \times (M \times WB/DLC)(\%) \quad (2)$$

Equation 3 (*S. violaceoruber*):

$$U/gds = 7.83 + 2.87 \times M(\%) + 1.31 \times M^2(\%) + \\ + 0.15 \times WB/DLC(\%) + 0.30 \times WB/DLC^2(\%) + \\ + 0.27 \times (M \times WB/DLC)(\%) \quad (3)$$

## Response surface analysis

Analysing the results of the ANOVA tables and considering graphs (a), (b), (c) and (d) of Figure 1 for the evaluated enzymes with both strains of actinobacteria, the highest medium moisture ranges analysed (61.5% to 67%) positively influenced fermentation, increasing enzyme production, except for lipase by *A. polychromogenes*. The maximum moisture value (67%) tested corresponds to the moisture value frequently used in SSF for optimal growth of filamentous fungi (65-70%) for lipase and pectinase production, as previously mentioned. Orozco et al. (2008) applied a similar moisture range in SSF with coffee grounds (dregs) and actinobacteria.

In SSF, moisture is directly related to mass, heat, and gas transfer, especially in larger-scale systems such as fermentation columns. Low moisture content may hinder nutrient availability and increase heat build-up and high moisture content may decrease medium porosity and impair oxygen transfer (Abrunhosa et al., 2011). According to the results obtained it can be assumed that the medium compositions, especially those corresponding to the E6, on a bench scale, were sufficient to provide the conditions for actinobacteria to produce lipases and pectinases.

Analysing the ANOVA and Figure 1, it is observed that the variations in the proportions of WB and DLC were not significant for the enzymatic productions by both strains. According to the literature, different sources of nitrogen (including wheat bran) and different microorganisms may improve lipase production. Oliveira et al. (2016) observed in SSF using

olive residue and wheat bran a positive effect on lipase production with *Aspergillus ibericus* but not with *A. niger* and *A. tubingensis*. Sun and Xu (2008) reported that monoammonium phosphate had a positive effect on lipase production by *Rhizopus chinensis*, which was not observed with other nitrogen sources. This work does not show this relation between both the substrate and microorganisms evaluated here, which could be explained by the protein and oil content of the licuri cake used.

The licuri cake initially with 49.27% lipids after the extraction process of the remaining oil presented a lipid content of 2.54% (DLC). DLC with higher lipid content was not tested. The WB used contained 6.29% lipids. DLC and WB, in addition to having lipids, which are substrates for the production of lipases, also naturally have proteins in their composition, 19% for licuri cake and 13.8% for WB (Silveira; Furlong, 2007), which produces lipase.

The results suggest that DLC could be used as an alternative fermentation medium to produce pectinases and lipases, and WB has been used as a medium in SSF, emerging as an interesting alternative for industrial scale production in Brazil, especially for the northeastern region of the country, as this is a licuri-producing region that does not produce wheat due to the climate. In addition, it offers an alternative with higher economic value for this waste, which is currently used as animal feed by small licuri producers.

## Experiment validation

Aiming at the validation of the models obtained through the statistical analysis conducted, the two strains of actinobacteria evaluated were used for production of lipases in the highest moisture content of the medium used (67%), only with wheat bran and licuri peel, in one of the conditions and licuri cake and peel, in another. The results obtained with these experiments are shown in Table 4.

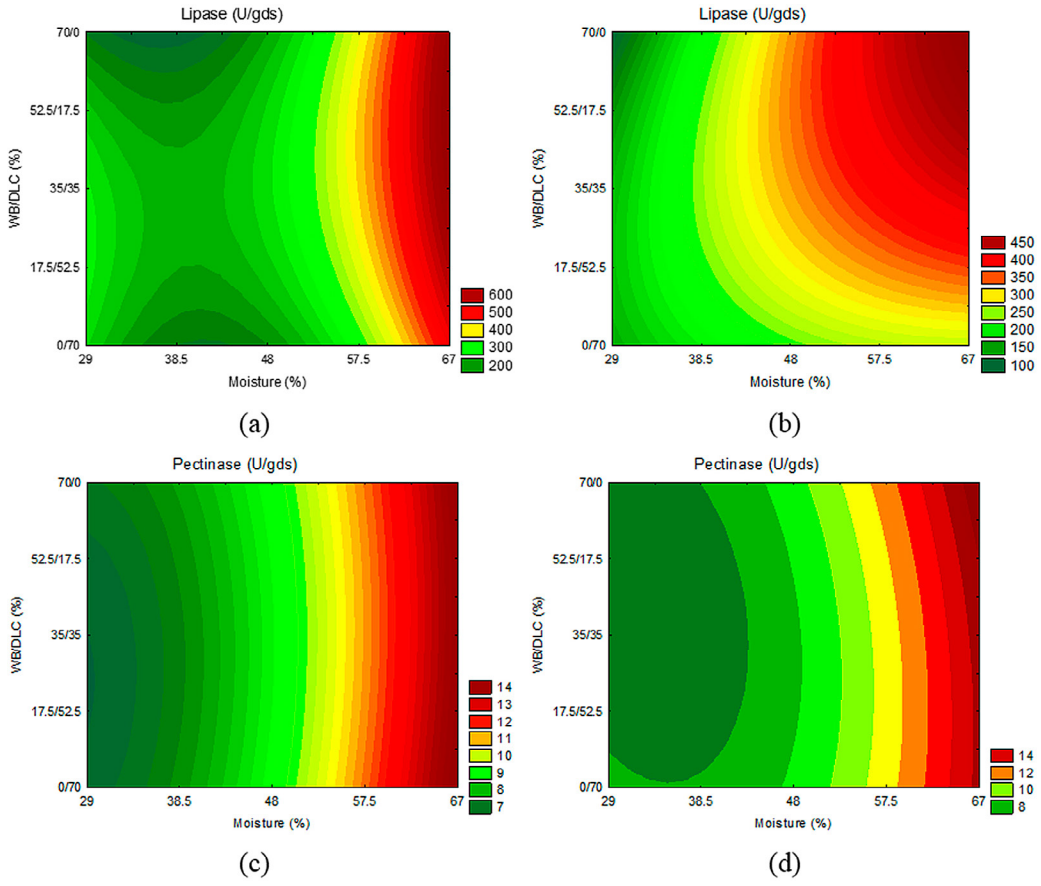


**Figure 1:** Lipase production by *A. polychromogenes* (a) and *S. violaceoruber* (b), and pectinase production by *A. polychromogenes* (c) and *S. violaceoruber* (d) as a function of moisture and WB/DLC ratio during SSF.

**Table 4:** Enzymatic activity of lipases and pectinases and their respective deviations and Tukey's test for media applied to experimental data.

| | DLC/WB (%) | Lipase experimental value (U/gds) | Lipase predicted value (U/gds) |
|---|---|---|---|
| *Arthrobacter polychromogenes* | 70/0 | 466.61 ± 15.73[a] | Model not significant |
| | 0/70 | 149.79 ± 53.93[b] | |
| *Streptomyces violaceoruber* | 70/0 | 399.21 ± 11.23[c] | 260.72 |
| | 0/70 | 850.85 ± 0.00[d] | 497.76 |

From the data in Table 4, it can be seen that the two strains used showed good capacity for the production of lipase. It is also observed that lipase activity produced by *A. polychromogenes* varied 67.89%, depending on the substrate, being higher when the licuri cake was used. Comparing the enzymatic activity obtained in the CCD experiments, which predicted values above 600 U/gds, as shown in Figure 1(a), a reduction in lipolytic activity of approximately 22% was observed. However, it was still possible to observe that there was high enzyme production (466.61 U/gds) and that the production was higher when only licuri cake and peel were used as substrates compared to the use of wheat bran and licuri peel only, as seen in E7 (309.62 U/gds) and E8 (279.56 U/gds) runs of the CCD experiments.

As the model for lipase production by *A. polychromogenes* was not significant ($p<0,05$), it was not possible to compare the results obtained from the model with those obtained experimentally, although it was possible to confirm that only licuri residues can be used as substrates for lipase production.

For *S. violaceoruber*, in turn, the results presented for the activity varied by 95.4%, indicating a difference in the production of lipase between the two substrates evaluated. From the CCD experiments, the model predicted activities above 497.76 U/gds when using only wheat bran and licuri peel as substrates and 260.72 U/gds, using only licuri residues. In the model validation experiments, in turn, an increase in the enzymatic activity of the lipase was observed for both evaluated substrate compositions, with activities of 850.85 U/gds and 399.21 U/gds, respectively, which confirms the use of licuri residues as a promising substrate for lipase production.

When comparing the results obtained by the model, with 67% moisture and substrate proportions of 0/70 (%) and 70/0 (%), with those obtained in the CCD experiments for the axial points, in which the moisture was 48% (E7 and E8 - Table 1), it can be seen that they did not show much difference between them. Thus, it can be inferred that, after some time (and some repetitions), the actinobacteria had their metabolism adapted to the substrate used, improving the production of lipases.

Evaluating the results obtained in the CCD statistical analysis, which includes the axial points, the model provides a prediction of the behaviour of the SSF process in the evaluated range; however, looking at the surface curve obtained, it is possible to see that, with a moisture content of 67%, specifically for *S. violaceoruber*, there was a large variation in lipase activity. This antagonistic effect may have occurred due to the composition of the substrate in the experimental data and that for the data in the validation step, they were performed separately.

Comparing both strains for lipase and pectinase production, using a 50% DLC/WB ratio as substrate and 48% moisture (CCD central points – Table 5), and of lipase in validation experiments (Table 4), by both actinobacteria, it was possible to notice that there was a significant ($p<0.05$) difference between the enzyme activities obtained, suggesting that the *A. polychromogrenes* are better for pectinase production, at central points; and *S. violaceoruber* for lipase production, at central points and using WB as inductor; however, the inverse was obtained for lipase production using DLC as inductor at validation experiments, showing that it is higher for *A. polychromogenes*.

**Table 5.** Tukey tests for enzymatic activity of lipases and pectinases at central points.

| | Pectinase (U/gds) | Lipase (U/gds) |
|---|---|---|
| *Arthrobacter polychromogenes* | 8.704 ± 0.232[a] | 253.404 ± 31.795[a] |
| *Streptomyces violaceoruber* | 7.829 ± 0.192[b] | 319.081 ± 21.143[b] |

## CONCLUSIONS

The licuri residues (cake and peel), generated in the extraction of extra virgin licuri oil, are viable for use in SSF with actinobacteria after a remnant oil extraction step. Licuri residues can contribute as a promising raw material for the production of enzymes such as lipases and pectinases, in addition to adding value to these products and promoting greater use of natural resources from this source. The

results obtained indicated that two strains of actinobacteria, *A. polychromogenes* and *S. violaceoruber*, are good producers of pectinases and lipases among the 42 strains evaluated. RSM indicated that higher moisture positively influences enzyme production and that the use of DLC has similar results to WB during CCD assays. Additionally, it was possible to replicate the positive results obtained, as shown in the validation assays, with a better response for lipase activities, with DLC as an inducer produced by *A. polychromogenes*, which was the goal of this work.

## AUTHOR CONTRIBUTION

Conceptual Idea: Carvalho, A.L., Umsza-Guez, M.A.; Methodology design: Rodrigues, H.C.S.R, Carvalho, A.L., Umsza-Guez, M.A.; Data collection: Rodrigues, H.C.S.R, Carvalho, A.L., Santos, L.M., da Silva, A.B, Data analysis and interpretation: Rodrigues, H.C.S.R, Carvalho, A.L., Umsza-Guez, M.A., and Writing and editing: Carvalho, A.L., Umsza-Guez, M.A.

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