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Camerini, Felipe Vieira; Chanin, Carlo do Amaral; Borba, Carina Molins; Costa, Jorge Alberto Vieira; Burkert, Carlos André Veiga; Burkert, Janaína Fernandes de Medeiros

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Biosurfactant production by *Phialemonium* sp. using agroindustrial wastes: influence of culture conditions

Felipe Vieira Camerini[1], Carlo do Amaral Chanin[1], Carina Molins Borba[2], Jorge Alberto Vieira Costa[1], Carlos André Veiga Burkert[2] and Janaína Fernandes de Medeiros Burkert[2*]

[1]Laboratório de Engenharia Bioquímica, Escola de Química e Alimentos, Rio Grande, Rio Grande do Sul, Brazil. [2]Laboratório de Engenharia de Bioprocessos, Escola de Química e Alimentos, Av. Itália, km 8, 96203-900, Rio Grande, Rio Grande do Sul, Brazil. *Author for correspondence. E-mail: jfmb@vetorial.net

ABSTRACT. Biosurfactant are surface active compounds with emulsifying capacity and are produced by microorganisms, and they may be affected by factors related to microbial cultivation, such as pH, salinity, incubation time, carbon and nitrogen sources. The aim of this work was to study the influence of the culture conditions on the production of biosurfactants by *Phialemonium* sp using agroindustrial wastes. The processing parameters of temperature, humidity and pH produced the most significant effects on the production of biosurfactant and emulsifying activity. The maximum concentration of biosurfactant obtained in this study was equivalent to a surface tension reduction of 8.5 mg L^{-1} surfactin commercial solution using wheat bran, pH of 4.5, and 0.5% of soybean oil added at 30°C. Under these conditions, 83.4 EU g^{-1} of emulsifying activity, 16.4 g L^{-1} of emulsifier index and 18.3 U g^{-1} lipolytic capacity were obtained.

Keywords: fermentation; fungi; surfactin; rice; wheat; surfactant; lipase.

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Introduction

Biosurfactants can be defined as compounds with high levels of surface activity and emulsifier capacity, and they are produced by microorganisms such as the bacterium *Lactobacillus*, *Bacillus*, *Pseudomonas* and fungus as *Aspergillus* and *Candida*, and others. Biosurfactants are characterized chemically as glycolipids, lipopeptides, fatty acids, phospholipids, neutral lipids or lipopolysaccharides (Colla, Rizzardi, Pinto, Reinehr, Bertolin, & Costa, 2010; Damasceno, Cammarota, & Freire, 2012; Vaz, Gudinã, Alameda, Teixeira, & Rodrigues, 2012; Rufino, Luna, Marinho, Farias, Ferreira, & Sarubbo, 2013; Vecino, Devesa-Rey, Cruz, & Moldes, 2013; Elshafie, Joshi, Al-Wahaibi, Al-Bemani, Al-Bahry, Al-Maqbali, & Banat, 2015; Ishaq et al., 2015).

These biotechnological substances have several principal advantages of environmental compatibility and high activity under extreme conditions of temperature, pH, and salinity. In addition, they can be used together with other surfactants and solvents, with the ability to reduce the surface and interfacial tension of the medium. They exhibit strong emulsifying power to form stable emulsions and are used in bioremediation processes, tank cleaning, and recovery oil reservoirs and as emulsifiers and solubilizers in the food, cosmetics, and pharmaceutical industries (Zheng, Wang, Wang, & Huang, 2012; Zilio, Furlong, Oliveira, Radmann, Santos, Treichel, & Costa, 2016; Yan et al., 2012; Nitschke & Silva, 2018).

From an environmental and economic point of view, biosurfactants can still be produced using low-cost substrates such as lignocellulosic waste resulting from the pruning of plants, oil sludge, glycerol, soybean oil, wheat bran, soya beans, and rice husks which are produced abundantly in southern Brazil. There is low commercial value to processing the by-products of these types of grains. Wheat processing, for example, on average gives rise to 25% of wheat bran (Colla et al., 2010; Zheng et al., 2012; Vecino et al., 2013; Inoh, Furuno, Hirashima, & Kitamoto, 2013; Pereira, Pacheco,. Tavares, Neves, Kronemberger, Reis, & Freire, 2013).

To enable the production of biosurfactants with low cost and high productivity, several factors must be considered, particularly those related to microbial cultivation, such as growth time, pH, salinity, and carbon and nitrogen sources (Colla et al., 2010; Nalin & Parthasarathi, 2013; Kaskatepe, Yildiz, Gumustas, &

Ozkan, 2015). Therefore the aim of this work was to study the influence of the culture conditions on the production of biosurfactants by *Phialemonium* sp. using agroindustrial wastes.

Material and methods

Reagents

Potato-dextrose agar, Dichloran Rose Bengal Chloramphenicol, yeast extract, and peptone were purchased from Himedia (West Chester, USA); Tween 80, $MgSO_4$, $NaNO_3$, and KH_2PO_4 were purchased from Synth (Diadema, Brazil); NaOH was purchased from Dinâmica (Diadema, Brazil) and gum Arabic was purchased from Sigma Aldrich (Darmstadt, Germany). All the other reagents were of analytical grade.

Microorganism

The filamentous fungi *Phialemonium* sp. were kindly supplied by the Food Microbiology Laboratory of the Food Engineering Faculty, State University of Campinas (FEA/UNICAMP), Brazil. The fungi were maintained at 4°C on potato-dextrose agar–PDA (Martins, Kalil, Bertolin, & Costa, 2006). In 5 mL of 0.2% (v v^{-1}) Tween 80 the spores were scraped, and 0.5 mL of the suspension was transferred to flasks containing PDA and incubated at 30°C for 7 d to allow complete covering of the surface and sporulation of the fungi. Spores were suspended in 0.2% (v v^{-1}) Tween 80, plated on to DRBC agar (Dichloran Rose Bengal Chloramphenicol), with posterior maintenance at 30°C for 3 d. The experimental assays started with 4 x 10^6 CFU g^{-1} (Gabiatti Jr, Vendruscolo, Piaia, Rodrigues, Durrant, & Costa, 2006).

Solid State Process

The production of the biosurfactant was performed for 144 h in 1 L Erlenmeyer flasks using two fermentation mediums. The first consisted of rice husk (15% w w^{-1}) and defatted rice bran (85%), and the second medium contained wheat bran (100%). Both fermentation mediums were milled in a knife mill and sieved (0.420-0.500 mm). In the medium, 40 mL of nutrient solution composed of (g L^{-1}) 0.5 $MgSO_4$, 3 $NaNO_3$, 1 KH_2PO_4, 1 yeast extract and 0.3 peptone.

In the experimental fractional factorial design 2^{5-1} (Table 1), the different combinations of experiments were used to evaluate the effects of the substrate (rice or wheat bran), initial moisture humidity (40, 50 and 60%), processing temperature (25; 30; 35°C), initial pH of the non-sterile medium (4.0; 4.5; 5.0) and use of soybean oil (purchased in local market) as an additional carbon source (0; 0.5; 1.0%) in the biosurfactant production.

This strategy reduces the number of tests that would be needed in a Central Composite Rotational Design (2^5 - DCCR) by half, and is a very interesting option because it allows for evaluating the effects of key variables with the same safety in relation to a complete design with 32 assays. It is a design that provides us with information on the importance of the effects on the response (s) and if the selected range of study was the most appropiate one, as well as how the next design should be modeled. For this design, referred to as 1/2 fraction, the 4 columns are formed as a 2^4 design and the 5^{th} column is the result of multiplying the 4 previous columns (5 = 1234) (Rodrigues & Iemma, 2014). It is a resolution V design since the identity I = 12345 have 5 factors. Three replicates (n = 3) were added at the central points with wheat bran (assay 17 to 19) and three replicates (n = 3) at the central points with rice bran (assay 20 to 22).

Extraction of Biosurfactant

Biosurfactant was extracted in a shaker (BRAUN CERTOMAT BS-1, Melsungen, Germany) at 160 rpm and 50°C for 30 min., adding 10 parts (w v^{-1}) of water in the solid phase followed by vacuum filtration (Gabiatti Jr. et al., 2004).

Analytical Methods

To quantify the biosurfactant concentration, a volume of 5 µL of biosurfactant extract was added with the aid of a micro syringe on a hydrophobic surface (Parafilm®) for subsequent measurement of droplet diameter. A standard commercial surfactin curve was used to relate the concentration of the biosurfactant produced with diameters of the droplets measured using the software ImageTool version 3.0.

Soy oil (2 mL) and extract (3.5 mL) were used for the emulsifying activity, on vortex the mixture it was stirred (1 min.), then allowed to stand (1h) and the absorbance of the emulsion determined at 610

nm using a spectrophotometer (Biospectro, Curitiba, Brazil) and calculating the EA value with the Equation 1.

$$EA = \frac{(A * DL)}{M(1-W)} \quad (1)$$

Where:
EA is the water-in-oil emulsifying activity (EU g^{-1}),
A is the absorbance,
DL is the dilution of the sample in water,
M is the wet mass (g),
W is the water content of the fermented medium.

One unit of emulsifying activity was defined as the quantity of biosurfactant necessary to increase the absorbance at 610 nm by one unit above that of the control and was expressed in emulsifying units per gram (EU g^{-1}) (Johnson, Sigh, Saini, Dilip, Sista, & Yadav, 1992).

To determine the emulsifier content the Equation 2 was used.

$$EI24H = \frac{(TH * DL)}{M(1-W)} \quad (2)$$

Where:
EI24H is the emulsifier index (EU g^{-1}),
TH is the percentage ratio between the height of the emulsion and total height,
DL is the dilution,
M is the wet mass (g), and
W is the water content of the fermented medium.

For this, 3.5 mL of the filtrate obtained from the extraction of biosurfactant and 2 mL of soybean oil was added to test tubes, stirred for 1 min and left to stand for 24h. After this period, the emulsion height and halo were read with ImageTool version 3.0. The emulsifier index was estimated after 24h by calculating the ratio between the total height of the oil and the height of the emulsified oil and expressed as EU g^{-1} (Broderick & Cooney, 1992).

The lipase produced was extracted for subsequent determination of lipase activity using the method based on titration with NaOH and the fatty acids resulting from the lipase activity on olive oil triacylglycerides emulsified with gum Arabic (Gilles, Frank, Tatsadjieu, Nicolas, & Mbofung, 2015).

Statistical Analysis

The samples were collected in triplicate for determination of lipase activity, emulsifying activity, emulsifying index and drop-collapse. Over time in each experiment the maximum of each response was considered (Table 1). Three replicates (n = 3) were added at the central points with wheat bran (assay 17 to 19) and three replicates (n = 3) at the central points with rice bran (assay 20 to 22).

The data were tested for normality by Shapiro Wilk (SW), Kolmogorov Smirnov (KS) and chi-square (X^2) at 95% confidence level.

The use of experimental design enables the study on influence on the levels of one variable on the response variable. Thus, the statistical analysis was made by the main effects (Figure 1) of such a design may be simply calculated as the difference between the average of measurements made on the upper level (+1) and the average of the measurements made on the lower level (-1) (Rodrigues & Iemma, 2014). Results were statistically analyzed using Statistica Software V 5.0 (Statsoft) and the graphics were made with Origin 8 (OriginLab Corporation). All analyses considered 90% confidence level ($p < 0.1$) according to Box, Hunter and Hunter (1978) and Rodrigues and Iemma, (2014) for fractional factorial design 2^{5-1}.

Results and discussion

The responses biossurfactant concentration (SW p = 0.19630, KS p = no significance and X^2 p = 0.61571), emulsifier activity (SW p = 0.53079, KS p = no significance and X^2 p = 0.50987), lipolitic activity (SW p =

0.07378, KS p = no significance and X^2 p = 0.00218) and emulsifying index (SW p = 0.38276, KS p = n.s and X^2 p = 0.29888) were tested for normality. All responses adhered to all normality tests except lipolitic activity that did not adhere to chi-square.

The fractional factorial design 2^{5-1} (Table 1) performed for different initial humidity combinations of the fermentation medium, types of substrate, temperatures, initial pH before the sterilization process, and concentrations of soybean oil resulted in variations in the biosurfactant concentration from 0 (assays 2, 5 and 9) to 8.5 mg L^{-1} (assay 17), emulsifying activity from 0 (assay 1 and 9) to 112.7 EU g^{-1} (assay 16), an emulsifier index from 0 (assay 6) to 19.2 EU g^{-1} (assay 18) and lipolytic activity from 18.3 (assay 17) to 125.5 U g^{-1} (assay 4).

Table 1. The 2^{5-1} fractional factorial design with real and coded values (in parentheses).

Assay	H(%)	T(ºC)	pH	SO(%)	S	BC	EA	LA	EI24H
1	40(-1)	25 (-1)	4.0(-1)	0 (-1)	RB(+1)	2.5	0	64.0	9.3
2	60(+1)	25 (-1)	4.0 (-1)	0 (-1)	WB(-1)	0	31.7	100.3	7.2
3	40(-1)	35(+1)	4.0 (-1)	0 (-1)	W (-1)	2.8	32.7	96.1	1.7
4	60(+1)	35(+1)	4.0 (-1)	0 (-1)	RB(+1)	4.87	43.6	125.5	8.3
5	40(-1)	25(-1)	5.0(+1)	0 (-1)	WB(-1)	0	5.3	64.7	3.8
6	60(+1)	25(-1)	5.0(+1)	0 (-1)	RB(+1)	0.5	35.2	86.0	0
7	40(-1)	35(+1)	5.0(+1)	0 (-1)	RB(+1)	7.3	69.5	73.4	3.5
8	60(+1)	35(+1)	5.0(+1)	0 (-1)	WB(-1)	5.7	98.5	91.8	5.7
9	40 (-1)	25(-1)	4.0(-1)	1 (+1)	WB(-1)	0	0	64.9	2.3
10	60(+1)	25(-1)	4.0(-1)	1 (+1)	RB(+1)	1.9	12.6	125.1	15.6
11	40(-1)	35(+1)	4.0(-1)	1 (+1)	RB(+1)	1.6	19.9	72.9	3.3
12	60(+1)	35(+1)	4.0(-1)	1 (+1)	WB(-1)	2.9	56.3	105.5	4.0
13	40(-1)	25(-1)	5.0(+1)	1 (+1)	RB(+1)	3.2	7.1	69.5	8.4
14	60(+1)	25(-1)	5.0(+1)	1 (+1)	WB(-1)	4.6	46.9	107.6	10.6
15	40 (-1)	35(+1)	5.0(+1)	1 (+1)	RB(+1)	2.6	47.6	89.5	1.7
16	60(+1)	35(+1)	5.0(+1)	1 (+1)	WB(-1)	4.5	112.7	67.6	9.4
17	50 (0)	30 (0)	4.5(0)	0.5 (0)	WB(0)	8.5	83.4	18.3	16.4
18	50 (0)	30 (0)	4.5(0)	0.5 (0)	WB (0)	6.9	81.9	23.8	19.2
19	50 (0)	30 (0)	4.5(0)	0.5 (0)	WB (0)	7.1	86.7	29.3	15.3
20	50 (0)	30 (0)	4.5 (0)	0.5 (0)	RB (0)	6.8	75.4	22.3	13.0
21	50 (0)	30 (0)	4.5 (0)	0.5 (0)	RB (0)	7.0	55.6	21.5	9.3
22	50 (0)	30 (0)	4.5 (0)	0.5 (0)	RB (0)	4.8	51.6	20.1	12.4

H (humidity), T (temperature), SO (soy oil), S (substrate), RB (rice bran), WB (wheat bran), BC (biosurfactant concentration – mg L^{-1}), EA (emulsifier activity – UE g^{-1}), LA (lipolytic activity – U g^{-1}), EI (emulsifying index - UE g^{-1}).

Therefore, in some experimental conditions, the absence of biosurfactant production was observed even with the presence of emulsifying activity or a positive emulsifier index because of the concomitant production of the lipase enzyme, which is involved in the biosurfactant synthesis process through hydrolysis of triacylglycerol inducing constituents. This hydrolysis causes the release of free fatty acids and mono- and diglycerides, which are compounds that have structures with polar and nonpolar groups on the same molecule and consequently can reduce surface tension and act as emulsifiers (Colla et al. 2010).

Figure 1 shows the main effects of the variables on the responses evaluated, emulsifying activity (Figure 1a) and lipase activity (Figure 1a), concentration of biosurfactant (the main variable of this study) (Figure 1b) and emulsifying index (Figure 1b). The temperature of the process was the parameter that had the most significant effect on the production of the biosurfactant. Increasing temperature from 25 to 35°C caused significant ($p < 0.1$) increases in the concentration of the biosurfactant of 2.4 mg L^{-1}. The change in the humidity of the production medium, initial pH of the production medium, the presence of up to 1% soybean oil and the change from wheat bran to rice bran change did not produce significant effects ($p > 0.1$) on the concentration of the biosurfactant produced.

The emulsifying activity of the biosurfactant produced (Figure 1a) was the response variable more affected by changes in process conditions. In addition to temperature and pH, humidity significantly influenced ($p < 0.1$). The most important effect on the emulsifying activity occurred with an increase of 10°C in the fermentation process temperature, causing an increase in activity of 42.7 UE g^{-1}. Increasing the humidity of the medium from 40 to 60% and pH from 4.0 to 5.0 caused average increased emulsifying activity of 31.9 UE g^{-1} and 28.2 UE g^{-1}, respectively.

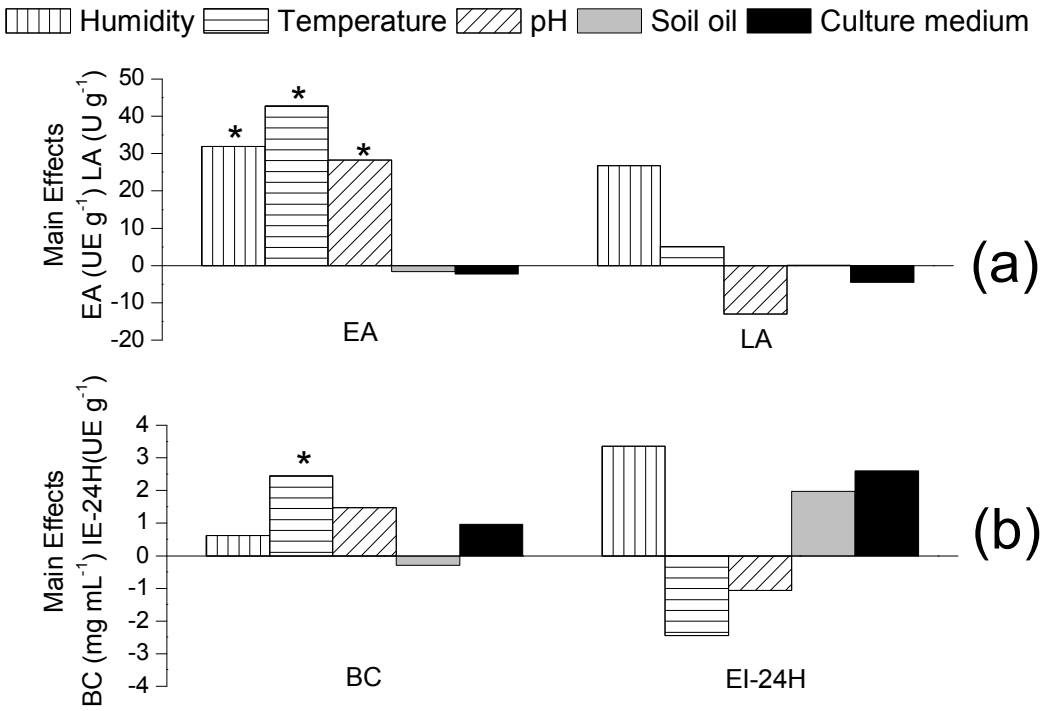

Figure 1. Effect of independent variables humidity, temperature, pH, soy oil, and culture medium in the on the EA (emulsifier activity – UE g^{-1}) and LA (lipolytic activity – U g^{-1}) (a) and BC (biosurfactant concentration – mg L^{-1}), EI-24H (emulsifying index - UE g^{-1}) (b). *$p < 0.1$.

However, the increase of the same variables in the range of variation studied did not significantly influenced ($p > 0.1$) the other two responses, lipolitic activity (Figure 1a) measured concurrently with biosurfactant production, and emulsifier index (Figure 1b). Most likely, the additional carbon source did not influence the production of lipase, probably because both wheat bran and rice bran oil contain sufficient residues to induce the production of the enzyme.

The maximum lipase production by *Phialemonium* sp. under the conditions studied in this work occurred during the tests of the maximum concentration of biosurfactant, in the assay 4 (humidity 60%, 35°C, pH 4.0 and rice bran) reached the maximum of 125.5 U g^{-1} lipase activity.

This production of lipase was superior to a comparative study of the production of biosurfactants by the *Aspergillus* fungus under submerged fermentation (6 days, 30°C with agitation of 120 min.$^{-1}$) and solid state fermentation (12 days at 30°C) that reached lipolytic activity of 4.5 U g^{-1} and 25.0 U g^{-1} after 96h, respectively. This difference demonstrates that the solid state fermentation characteristics generate more concentrated products, making the process more productive (Colla et al. 2010).

In the production of biosurfactants by submerged fermentation at different pH values (5, 6, 7 and 8), fermentation times (48, 72, and 96h) and carbon sources (glucose, sucrose, mannitol and sugar cane juice, fructose and glucose added to fructose) at different concentrations (1, 2, 3, 4 and 5%) using *Bacillus pumilus*, it was found that the highest emulsification rates, 48.8 and 44.4%, respectively, were obtained at pH 5.0 and 7.0 with 72h of fermentation. Under the same conditions, they also resulted in the highest reduction of surface tension, 56.0 and 57.8 cm^{-1} Din. Higher emulsification rates have been obtained (15 to 80%) when testing carbon sources with low concentrations of sucrose sufficient to stimulate biosurfactant productivity (Bueno, Silva, & Garcia-Cruz, 2010), which can be produced using cheap and regional raw materials such as wheat bran, rice bran and rice hulls as proposed in this work.

Emulsifying activity reached 112.7 EU g^{-1} in assay 16 (60% humidity, 35°C, pH 5, 1% soybean oil and wheat bran), and EI24H reached 19.2 UE g^{-1} in run 18 (50% humidity, 30°C, pH 4.5, 0.5% soybean oil and wheat bran), which were superior to a similar study (Martins et al., 2006) using the same microorganism. They achieved EA and EI24H of 7.3 and 12.2 UE g^{-1}, respectively, with diesel oil as the carbon source and an airing at 60 mL g^{-1} h^{-1}. The control of the fermentation conditions, as was done in the present study, can contribute to an increase in the emulsifying properties of the resulting byproduct, improving efficiency in future applications. Because the compound produced by *Phialemonium* sp. reduced the surface tension

following solid state fermentation, this compound can be characterized not only as an emulsifier but also as having characteristics of a biosurfactant.

The drop-collapse analysis demonstrated that the maximum concentration of biosurfactant obtained was equivalent to reducing the surface tension to 8.5 mg L^{-1} surfactin commercial solution obtained under the experimental conditions of run 17 (50% humidity, 30°C, pH 4.5, 0.5% soybean oil and wheat bran). Under these conditions, an emulsifying activity of 83.4 UE g^{-1}, an IE24H of 16.4 UE g^{-1}, and a lipolytic capacity of 18.3 UE g^{-1} were obtained.

All inferences referring to this work are based on the study range of each evaluated variable. Therefore, outside these ranges, initial moisture humidity (40 to 60%), processing temperature (25 to 35°C), initial pH of the non-sterile medium (4.0 to 5.0), soybean oil (0 to 1.0%) and rice or wheat bran statistical analyses are not valid.

The results obtained in this work of biosurfactant production by *Phialemonium* were used for new bioproduction works in fixed-bed reactors (Martins et al., 2006). The biosurfactant produced through this microorganism is mentioned for application in the bioremediation of soils contaminated by vegetable oils and hydrocarbons (Martins, Kalil, & Costa, 2008). However, up to the present time this application in bioremediation occurred only with *Aspergillus fumigatus* (Martins, Kalil, & Costa, 2009).

Conclusion

The humity, temperature and the initial pH of the production medium were the parameters that had the most significant effects on the production of biosurfactant. The maximum concentration of biosurfactant obtained in this study was equivalent to reducing the surface tension to 8.5 mg L^{-1} surfactin commercial solution using wheat bran with 50% moisture, a pH of 4.5, and 0.5% of soybean oil added at 30°C. Under these conditions, an emulsifying activity of 83.4 UE g^{-1}, an emulsifier index of 16.4 UE g^{-1}, and a lipolytic capacity of 18.3 U g^{-1} were obtained.

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