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Evaluation of the Medium Fermentative Parameters for the Production of Biosurfactant Using the *Candida Gloebosa*

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The environmental pollution issue related to the widespread use of synthetic surfactants in detergents since 1960s has worried both the scientific community and regulatory agencies, in view of the foaming in rivers and lakes that makes the aquatic environment visually polluted. In this context, biological surfactants have stood out over synthetics due to their biodegradability and biological origin, since they are naturally produced by the microorganisms' metabolism. However, the production usually requires expensive fermentative media and still demands more efficient methodologies to make the process economically advantageous. To the best of our knowledge, there are no studies in the literature reporting the use of Candida gloebosa, which was collected in Antartica, for producing of biological surfactants. Faced with this, the present study aimed to evaluate some fermentative medium parameters in order to obtain ideal conditions for biosurfactant production using Candida gloebosa. For this purpose, the fermentation time (24, 48, 72 and 96 h), the concentration of residual potato frying oil (40, 60 and 90 g.L-1), the concentration of hydrated magnesium sulfate (MgSO4.7H2O) (0.1, 0.15, 0.2 and 0.3 g.L-1), and cells concentration (1 ± 0.2, 2 ± 0.3, 3 ± 0.2 e 4 ± 0.3 g.L-1) were evaluated. According to the results, all the four parameters investigated revealed similar production yields at their best condition with a biosurfactant production of about 4 g/L when were employed 72 h of fermentation time, 0.2 g.L-1 of MgSO4, 60 g.L-1 of residual potato frying oil and a cell concentration of 3 g.L-1. Finally, the obtained results were comparable to those already reported in the literature for the yeast of the same genus known as Candida lipolytica, which is used for biosurfactant production. These findings can be used as basis for future studies involving the modeling through neural networks, for instance, in order to obtain an optimized condition or even for biosurfactant production in larger scales.

1. Introduction

The environmental issue has become a worldwide concern, considering that, as the world population grows, more and more raw materials, nonrenewable resources, and methods to improve the life quality has been demanded, such as agricultural chemical use, crude oil exploration, and fossil fuel extraction (Jimoh and Lin, 2019). The main problem with these activities is that in addition to being harmful to human health, they also can cause the contamination of the environment (Wilton et al., 2018).

Faced with this, some bioremediation techniques are being studied for remediation and helping against these environmental problems. Among them, the use of biosurfactants has been widely employed, since in the last years, they have become good remediation agents whether in aquatic or soil environments (Chebbi et al., 2017).

Biosurfactants can be produced by specific microorganisms and, thus, they have lower toxicity, are biodegradable, present ecological acceptability (Chrzanowski et al., 2012), are efficient in extreme environments (wide pH range, temperature and salt concentrations) (Anjum et al., 2016) and can be stored for over long periods of time (Bezza and Chirwa, 2015). *Bacillus subtilis* (Parthipan et al., 2017) and *Enterococcus faecium* (Sharma et al., 2015) are same examples.

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However, even with several environmental advantages, the cost of biosurfactant production is still very high, due to a low yield and difficult to recover after use (Araujo et al., 2019). Therefore, a thorough study of the fermentation medium for the production of biosurfactant is required, in order to find cheaper and more efficient fermentative processes (Jimoh and Lin, 2019).
Thus, this study aims to investigate the fermentation medium composition and to determine the optimal conditions for biosurfactant production by *Candida gloebosa* yeast. For this purpose, cells concentration, residual potato frying oil, magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) and the fermentation time were explored. It is also worth mentioning that the authors did not find in the literature similar studies related to this yeast species.

2. Material and Methods

2.1 Microorganism and Production medium

The microorganisms used in this study were *Candida gloebosa* yeast, which were collected in Antarctica and were supplied by the Microbial Resources Division of the Pluridisciplinary Center for Chemical, Biological and Agricultural Research of the State University of Campinas (CPQBA/UNICAMP). The maintenance medium had the following composition (%): 2 agar, 0.3 yeast extract, 1 glucose, and 0.5 peptone; pH 6.2 ± 0.2. For biosurfactant production, the concentrations of NH_4NO_3 and KH_2PO_4 were fixed at, respectively, 1 and 0.2 $g.L^{-1}$, while the amounts of ($MgSO_4.7H_2O$), cell concentration and residual potato frying oil were tested as summarized in Table 1:

Table 1: Concentration of $MgSO_4.7H_2O$, cell Concentration and residual potato frying oil tested in the production medium.

$MgSO_4.7H_2O$ ($g.L^{-1}$)	Cell Concentration ($g.L^{-1}$)	Residual potato Frying Oil ($g.L^{-1}$)
0.1	1 ± 0.1	40
0.15	2 ± 0.2	60
0.2	3 ± 0.1	90
0.3	4 ± 0.2	-

2.2 Methods

Yeasts were grown on solid maintenance medium for 24 h at 28 ± 2 °C. Cells obtained after this growth time were inoculated directly into 200 mL of production medium, using a 500 mL Erlenmeyer. Firstly, the biosurfactant production was evaluated varying the residual oil concentration according with summarized in Table 1, at different fermentation times (24, 48, 72 and 96 h), using a rotary shaker (150 rpm). For this purpose, the amounts of $MgSO_4.7H_2O$ and cell concentration were kept constant at, respectively, 0.2 and 3 ± 0.1 $g.L^{-1}$ (Rufino et al., 2011). As aforementioned, the amounts of NH_4NO_3 and KH_2PO_4 were fixed at, respectively, 1 and 0.2 $g.L^{-1}$. After selecting the best conditions of residual potato frying oil concentration and fermentation time, the required amount of $MgSO_4.7H_2O$ and cell concentration were also evaluated according with specified in Table 1. Samples were collected and centrifuged (Beckman J-25) at 8000 rpm (or 12.096 g) for subsequent biosurfactant extraction.
The cell-free broth obtained after centrifuging was extracted twice with chloroform (1:1 v/v) in a separator funnel at room temperature. The extracted biosurfactant was dried for 48 h in an oven at 50 ºC and then weighed to quantify the production yield.
Finally, after discovering the best production conditions, the kind of residual soy frying oil was varied in order to investigate the influence impurities on production yield: raw soy oil (RSO), meat oil (MO), potato oil (PO) and breaded oil (BO). In addition, a mixture containing equal amounts of MO, PO and BO oils was prepared, composed of combined oil (CO). All experiments were conducted in triplicate.

3. Results and Discussion

As previously mentioned in section 2.2, the amounts of $MgSO_4.7H_2O$ and cell concentration were respectively set at 0.2 and 3 ± 0.2 $g.L^{-1}$ (Rufino et al., 2011), while the residual oil concentration and the fermentation time were varied. Thus, the biosurfactant production by *Candida gloebosa* yeast is shown in Figure 1.

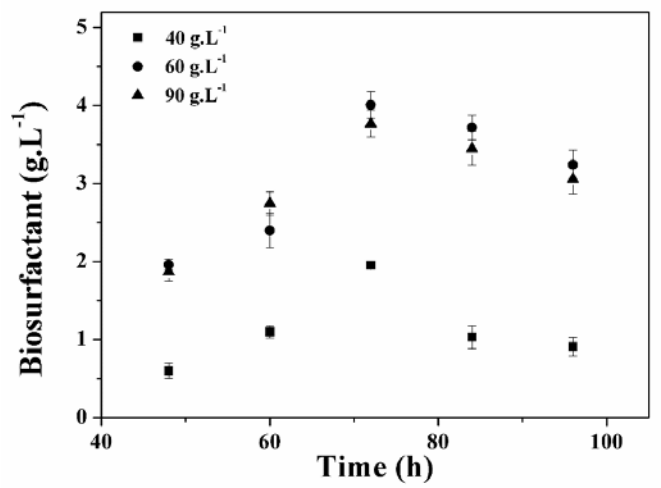

Figure 1: Biosurfactant production yield according with fermentation time and residual oil concentration

In Figure 1, it can be observed that the beginning of biosurfactant production occurred after 36 h of fermentation and reached the maximum production in 72 h (4.01 ± 0.21 $g.L^{-1}$) for 60 $g.L^{-1}$ of residual oil. For Candida lipolytica, Santos et al (2013) reported a maximum biosurfactant production of 2.0 $g.L^{-1}$ after 144 h, also using Erlenmeyers for fermentation. Subsequently, the authors investigated the biosurfactant production by the same microorganism in a 2 L reactor and reported a greater yield of 10 $g.L^{-1}$ in a shorter process time of 96 h (Santos et al., 2017). This finding supports the importance of optimizing the fermentation medium to improve the production of this biomolecule. As already mentioned in the literature, a drop in production yield after 72 h of fermentation was observed (Goswami and Deka, 2019). Ferreira et al. (2019) evaluated the production of Rhamnolipid biosurfactant with glycerol and observed a drop in production after 48 h of fermentation. This behavior suggests the occurrence of biosurfactant degradation and may be related to the action of enzymes and/or to other metabolites produced by the microorganism.

Once defined the fermentation time (72 h) and residual potato frying oil concentration (60 $g.L^{-1}$), the concentration of $MgSO_4.7H_2O$ was varied in order to verify whether this nutrient has relevance in the production of biosurfactant or not. Results were obtained with the cell concentration fixed at 3 ± 0.1 $g.L^{-1}$. The results obtained are shown in Table 2.

Table 2: Biosurfactant yield according with the $MgSO_4.7H_2O$ concentration in fermentation medium.

$MgSO_4.7H_2O$ ($g.L^{-1}$)	Biosurfactant yield ($g.L^{-1}$)
0.1	2.89 ± 0.31
0.15	3.09 ± 0.38
0.2	4.08 ± 0.29
0.3	3.63 ± 0.27

Table 2 depicts a greater biosurfactant production at 0.2 $g.L^{-1}$ of $MgSO_4.7H_2O$. This result is in agreement with the reported by Rufino et al (2011) for *Candida lipolytica*. However, a decrease in biosurfactant yield was observed for 0.3 $g.L^{-1}$. According with Santos et al. (2017), the biosurfactant molecules are considered secondary metabolic products for most microorganisms, including *Candida lipolytica*, which justifies the dressing. Similar behavior was reported by Shivaji et al. (2006) for production of pigments and antibiotics. Thus, when there is a greater availability of nutrients in the fermentative medium, the microorganisms tend to preferentially produce primary metabolites, disrupting the production of biosurfactant.

For evaluation cell concentration, fermentation time (72 h), residual oil concentration (60 $g.L^{-1}$) and $MgSO_4.7H_2O$ concentration (0.2 $g.L^{-1}$) were maintained constant. The results are summarized in Table 3.

As observed in Table 3, the results reveal that the biosurfactant production was improved up to the intermediary cell concentration of 3 ± 0.1 $g.L^{-1}$. However, there was no significant change in production for the higher initial cell concentration.

In this study, an addition of cell directly from the maintenance medium to the production medium was performed and, thus, inoculum preparation was not prepared as already mentioned in most studies (Rufino et al., 2011; Goswami and Deka, 2019; Santos et at., 2017). It is noteworthy that the inoculum growth is commonly carried out in 24 h and, thus, the direct cells loading in fermentative medium allows saving process time and reduces the costs with nutrients consumption that compose the inoculum. Therefore, this procedure may be advantageous even if the initial biomass load is higher (Lima et al., 2009; Borges et al., 2015). In addition Veena-Kumara-Adi and Savithri (2019) evaluated the influence of the initial cell volume in biosurfactant production using software for design of experiments and the best results were using a 7 mL volume of maintenance medium directly on the production medium.

Table 3: Biosurfactant yield according with cell concentration in fermentation medium.

Cell Concentration ($g.L^{-1}$)	Biosurfactant ($g.L^{-1}$)
1 ± 0.2	0.78 ± 0.31
2 ± 0.3	2.89 ± 0.38
3 ± 0.2	3.96 ± 0.29
4 ± 0.3	3.78 ± 0.27

3.1 Comparison of biosurfactant production using different residual soy frying oils

Although there are still no details about the economy of biosurfactant production in the literature, the limiting factor for commercialization and establishment of using these fermented products consists in the reduction of large-scale production costs (Muthusamy et al., 2008). For achieving this purpose, some approaches can be established, such as selection of low cost substrates and increased biosurfactant yields. Besides those, improving steps of preparation of materials, fermentation, extraction and purification can contribute for reducing the process time.

Many researchers have been investigating the preparation of fermentative medium for improving biosurfactant production. In this sense, different kinds of frying oils were investigated in this study in order to reduce the production costs of the fermentation medium. The results are summarized in Table 4.

Table 4: Comparison of biosurfactant production by Candida gloebosa using different carbon sources.

Carbon source	Biosurfactant ($g.L^{-1}$)
Raw soy oil (RSO)	4.04 ± 0.17
Meat oil (MO)	2.97 ± 0.25
Potato oil (PO)	4.08 ± 0.14
Breaded oil (BO)	2.61 ± 0.09
Combined oil (CO)	1.92 ± 0.18

The results presented in Table 4 demonstrate that there were no significant differences on biosurfactant production provided by either RSO or PO, indicating that potato oil can be advantageous and economically attractive. On the other hand, the combined oil provided the worse yield, followed by meat oil and breaded oil. Using reactors for fermentation, Santos et al. (2017) also observed a poor biosurfactant yield when employed animal fats as carbon source for their process.

As already mentioned, biosurfactants are considered secondary metabolites for *Candida* species and, thus, greater yields trend to be obtained when the microorganisms are in a stress condition. Therefore, when a combination of nutrients from all residual soy frying oils is loaded into the fermentation medium, it is acceptable to assume that microorganisms prefer to produce primary metabolites than secondary, producing enzymes able to degrade the biosurfactant.

Based on these findings, residual potato frying oil was used for all fermentation assays carried out in this study considering that the triplicate demonstrated a similar biomolecule production when compared to the RSO. Therefore, the costs related to the feedstock purchase and the process can be reduced.

Sarubbo et al. (2007) investigated the biosurfactant production by *Candida lipolytica* yeast using canola oil as carbon source and observed a yield of 8 $g.L^{-1}$. This result may appear significantly greater than that obtained in this study, but the fermentation time required to reach observed yield was 144 h, which is 50% longer than

the current study (72 h). Faced with the necessity of successfully operating in large-scales, the shorter fermentation time required by *Candida gloebosa* (72 h) suggests that this process has potential to be scaled up for future studies involving this microorganism.

4. Conclusion

It was concluded that fermentation medium optimization is quite important for improving biosurfactant production. In this study, *Candida gloebosa* yeast provided a maximum yield of about 4 $g.L^{-1}$ for the following optimized conditions: fermentation time (72 h), residual potato frying oil concentration (60 $g.L^{-1}$), magnesium sulfate concentration, $MgSO_4.7H_2O$ (0.2 $g.L^{-1}$), and cells concentration (3 ± 0.2 $g.L^{-1}$). When investigating which would be the best source of residual carbon, it was concluded that the production yield was similar either for potato oil or for raw crude oil. Thus, the experiments were performed using potato residual frying oil in order to reduce the process costs.

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