CHEMICAL ENGINEERING TRANSACTIONS
VOL. 86, 2021
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Sauro Pierucci, Jiří Jaromír Klemeš

ISBN 978-88-95608-84-6; **ISSN** 2283-9216

The Antarctic yeast *Candida sake*: investigation of fermentation parameters for biosurfactant production

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High-added value inputs and inefficient means of production using the already known microorganisms make it even more difficult the industrial application of biosurfactants. Therefore, this work aimed to optimize the fermentation parameters for biosurfactant production by the Antarctic yeast *Candida sake*, since to the best of our knowledge it was not investigated for this purpose yet. First, the optimized condition for the production of biosurfactant using the yeast *Candida globosa* was adopted, being as follows: residual soybean oil (72 g/L), ammonium nitrate, NH_4NO_3 (1.4 g/L), monobasic potassium phosphate, KH_2PO_4 (0.2 g/L), yeast extract from brewing industry or residual yeast extract (0.4 g/L), and fermentation time (72 h). However, no biosurfactant production was observed. So, the further fermentation assays were performed using the production medium known as Czapek, which was prepared as follows: sodium nitrate, $NaNO_3$ (2 g/L), magnesium sulfate, $MgSO_4$ (0.5 g/L), potassium chloride, KCl (0.5 g/L), iron(II) sulfate, $FeSO_4$ (0.01 g/L), monobasic potassium phosphate, KH_2PO_4 (1 g/L), and residual yeast extract (1.2 g/L). Secondly, the biosurfactant production by the yeast Candida sake was evaluated as regard to the fermentation time (24, 48, 60, 72, and 84 h), residual soybean oil concentration (40, 60, 80, and 90 g/L), KCl concentration (0.3, 0.4, and 0.5 g/L), and how necessary would be the use of $FeSO_4$, $MgSO_4$ and yeast extract. According to the results, the greater biosurfactant yield was obtained for 60 h of fermentation, coupled with 80 g/L of residual soybean oil and 0.4 g/L of KCl. The results also revealed that the process demanded yeast extract, but did not need $FeSO_4$ and $MgSO_4$, providing a biosurfactant yield of 13.8 g/L. Finally, *Candida sake* showed a better performance in relation to *Candida globosa* and *Candida lipolytica* (mostly explored in the literature), proving to be quite effective and promising in producing biosurfactant.

1. Introduction

Biosurfactants are mainly produced by aerobic microorganisms, whether they are bacteria, fungi or yeasts (Secato et al., 2017). Based on their functional properties, biosurfactants can be employed for emulsification, separation, solubilization and reduction of surface tension in agriculture, civil construction, food industry, textiles, paper, metal, pharmaceuticals, cosmetics and in the oil sector (such as for oil leak bioremediation and improved well recovery) (Mulligan et al., 2001). When compared to synthetic surfactants, biosurfactants stand out due to advantages like low toxicity, greater reduction in surface tension, solubility in alkaline waters, high biodegradability, stability in extreme pH and temperature conditions, and the possibility to be obtained from renewable substrates (residues or agro-industrial by-products) (De et al., 2015). Due to recent demand growing for green solutions, mainly in personal and home care industries, the global biosurfactant market is expected to reach $ 5.52 billion in 2022, at an annual growth rate of 5.6% during this period (Marketsandmarkets, 2017).
The biosurfactants can be divided into glycolipids, phospholipids, liposaccharides, lipopeptides, fatty acids and neutral lipids (Mulligan et al., 2001). Glycolipids are divided into trehalose, sophorolipids and raminolipids, being

that in general these biosurfactants are involved in the assimilation of low polar hydrocarbons by microorganisms.
Therefore, the goal of the present work was to evaluate the biosurfactant production by the yeast *Candida sake* as regard to the residual soybean oil concentration (after frying potatoes), the fermentation time and the composition of the fermentation medium in relation to the KCl concentration and about the need to use yeast extract. It is worth mentioning that in the literature, no reference was found to the production of biosurfactant by *Candida sake*.

2. Material and Methods

The microorganisms used in this study were *Candida sake* 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 - Brazil).
The maintenance medium YPD (Yeast Peptone Dextrose) was used with the following composition: 0.3% yeast extract, 0.5% peptone, and 1% glucose diluted in distilled water. The fermentation assays were conducted using shaking table at 150 rpm for 48 hours and at room temperature (28 ± 4 ºC). The inoculum was prepared with YPD liquid medium under the same conditions as the maintenance medium.
Firstly, for the production of the biosurfactant, the medium based on Rufino et al. (2011) and modified by Cardoso et al. (2020) was used, being composed as follows (g/L): residual soy oil (72), NH_4NO_3 (1.4), KH_2PO_4 (0.2), residual yeast extract (0.4) from the brewing industry and $MgSO_4.7H_2O$ (0.2) for 72 hours of fermentation. Subsequently, the modified Czapek liquid medium with the following composition was used as the production medium (g/L): $NaNO_3$ (2.0), $MgSO_4$ (0.5), KCl (0.5), $FeSO_4$ (0.01), K_2HPO_4 (1.0) and yeast extract (1.2). The production medium was autoclaved at 121 ºC for 20 minutes. The yeast extract was added after autoclaving the medium.
The preliminary experiments were divided into tests (Figure 1):
Test 1 - The objective was to evaluate the biosurfactant production using the conditions and means of production of biosurfactant by *Candida globosa,* found in a previous study reported by Cardoso et al. (2020). For this purpose, 500 mL Erlenmeyer flasks containing 200 mL of production medium were used, as well as a shaking table at 150 rpm and at room temperature (28 ± 4 ºC) during a fermentation time of 72 h.
Test 2 - The objective was to evaluate the biosurfactant production using Czapek as the production medium at different fermentation times (24, 48, 60, 72 and 84 h) and residual oil concentrations (40, 60 and 80 g/L). For this second test, 500 mL Erlenmeyer flasks filled with 200 mL of production medium containing an initial cell concentration in the inoculum ranging from 0.8 to 1 g/L, which represented an initial cell concentration in the fermentation flasks varying from 2.8 to 3.1 g/L. Fermentation assays were conducted at room temperature, pH around 6 and using shaking table at 150 rpm.
Test 3 – Similar to the test 2, the objective was to evaluate the biosurfactant production using Czapek as the production medium at different fermentation times (48, 60 and 72 h) and under the same conditions mentioned in test 2, but without the addition of potassium chloride (KCl).
Test 4 - The objective was to evaluate the biosurfactant production using Czapek as production medium in different concentrations of potassium chloride (KCl) (0.3, 0.4 and 0.5 g/L) under the same conditions mentioned in test 2.
Tests 5, 6, 7 and 8 – Aiming to evaluate the need to use all Czapek compounds, these experiments were carried out under the same conditions operational mentioned in test 2.
After the fermentative process and biosurfactant production, samples were prepared for analysis through a process in which 30 mL of the fermentation broth were centrifuged (8000 rpm for 20 minutes) in order to separate the cells from the aqueous solution. The aqueous suspension was stored in penicillin bottles of equivalent volume. In sequence, the bottom body was diluted in distilled water and dried in Petri dishes for 24 h or until constant mass in an oven at 90 ± 2 °C. The result of cells formed (dry mass) was determined by the difference in weight between the Petri dish with the addition of the diluted and dry pellet and the Petri dish before adding the bottom body of the fermentation process.
The samples surface tension of the sample was determined by the duNoüy ring method (1925), with the model K6 tensiometer (KrüssGmbH, Humburgo, Germany), at room temperature (28 ± 4 ºC), using 10 mL of sample of the aqueous solution free of cells.
For the extraction of the biosurfactant, chloroform PA was added in a proportion of 1:1 (v:v) in the fermentation broth and stirred manually in a separating funnel. After stirring, the phases were expected to separate and the organic phase was removed. The biosurfactant phase extracted from the separating funnel was placed in Petri dishes, then taken to an oven at a temperature of 40 ± 1 °C for 48 hours or until the mass was constant. The biosurfactant was removed with a spatula (Silva et al., 2010).

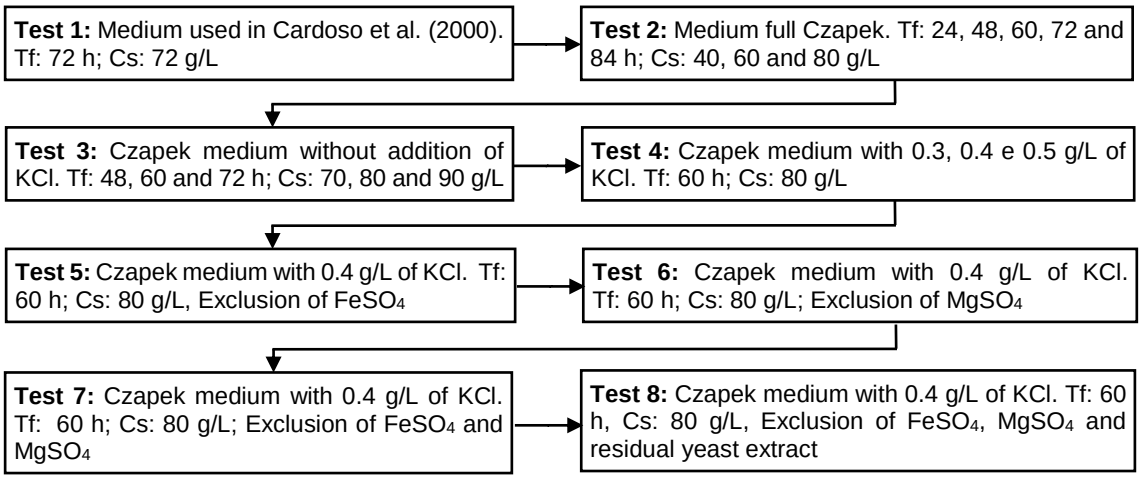

Figure 1: Tests carried out in the preliminary experiments. Where: medium used by Cardoso et al. (2020) (g/L): residual soy oil (72), NH_4NO_3 (1.4), KH_2PO_4 (0.2), residual yeast extract from the brewing industry (0.4) and $MgSO_4.7H_2O$ (0.2); Czapek medium (g/L): $NaNO_3$ (2.0), $MgSO_4$ (0.5), KCl (0.5), $FeSO_4$ (0.01), K_2HPO_4 (1.0) and yeast extract (1.2); Tf: fermentation time; Cs: residual soybean oil concentration.

3. Results and Discussion

Initially, the biosurfactant production was tested by *Candida sake* using the medium modified by Cardoso et al. (2020) during 72 hours of fermentation. However, after several repetitions, no biosurfactant production was observed when the chloroform extraction process was carried out. Thus, the production medium was changed using the modified liquid Czapek medium formulation, which was composed as follows (g/L): $NaNO_3$ (2.0), $MgSO_4$ (0.5), KCl (0.5), $FeSO_4$ (0.01), K_2HPO_4 (1.0), residual yeast extract (1.2) and a concentration of soybean oil from potato fried of 72 g/L. As mainly result, the biosurfactant production was 9.7 ± 0.5 g/L after 72 hours of fermentation. The initial surface tension of the medium, which was 51.2 ± 0.2 mN/m, decreased to 30.8 ± 0.1 mN/m and the final biomass concentration was 7.7 ± 0.4 /L. Therefore, this medium started to be used for the production of the biosurfactant by *Candida sake*. All experiments were carried out at room temperature (28 ± 4 ºC).

The following experiments were performed to evaluate the biosurfactant production in function of fermentation time and the residual soybean oil concentration from the fried potatoes (Table 1).

Table 1: Results of biosurfactant production, surface tension and cell production as a function of fermentation time and residual soybean oil concentration for Candida sake, at the following concentrations (g/L): $NaNO_3$ (2.0), K_2HPO_4 (1.0), KCl (0.5), residual yeast extract (1.2), $MgSO_4$ (0.5) and $FeSO_4$ (0.01).

Time (h)	Oil Concentration (g/L)	Biosurfactant Concentration (g/L)	Surface Tension (mN/m)	Cell Concentration (g/L)
24	40	-	51.2 ± 0.1	3.8 ± 0.3
	60	-	51.1 ±0.1	4.1 ± 0.4
	80	-	51.3 ± 0.2	5.0 ± 0.4
48	40	1.3 ± 0.2	48.2 ± 0.1	4.3 ± 0.3
	60	3.6 ± 0.3	43.3 ± 0.2	5.2 ± 0.4
	80	6.0 ± 0.3	38.0 ± 0.1	6.7 ± 0.4
60	40	3.4 ± 0.3	43.2 ± 0.2	5.0 ± 0.3
	60	7.9 ±0.5	35.9 ± 0.2	6.9 ± 0.4
	80	13.2 ± 0.5	27.5 ± 0.1	8.0 ± 0.2
72	40	3.9 ± 0.2	43.0 ± 0.3	4.9 ± 0.2
	60	7.6 ± 0.4	35.2 ± 0.1	6.7 ± 0.3
	80	13.1 ± 0.5	27.3 ± 0.1	8.1 ± 0.4
84	40	3.2 ± 0.4	43.2 ± 0.2	4.8 ± 0.2
	60	6.7 ± 0.3	38.0 ± 0.3	6.8 ± 0.4
	80	11.8 ± 0.5	28.5 ± 0.1	8.0 ± 0.3

The results revealed that the biosurfactant production increased until 60 h of fermentation, remaining constant after 72 h, but a small reduction was observed after 84 h. As regard the concentration of residual soybean oil, it was found that the highest production of biosurfactant was reached for 80 g/L. At this condition, a biosurfactant yield of 13.2 ± 0.5 g/L was obtained after 60 h of fermentation with Czapek medium, besides a reduction of surface tension from 51.3 ± 0.1 (initial surface tension of the fermentation medium) to 27.5 ± 0.1 mN/m and a final biomass concentration of 8.0 ± 0.2 g/L against an initial cell concentration of 2.7 ± 0.3 g/L.

The obtained results of biosurfactant production were higher than those presented for *Candida globosa* in a shorter fermentation time (60 h), but for a higher concentration of residual soy oil (80 g/L) (Cardoso et al., 2020). Rufino et al. (2008) observed that the biosurfactant produced by the yeast *Candida lipolytica* cultivated in a medium with 0.1% of NH_4NO_3, 0.02% of KH_2PO_4 and 0.02% of $MgSO_4.7H_2O$, supplemented with residues of soy oil, glutamic acid and yeast extract, reduced the surface tension to 26 mN/m in 72 h of cultivation. Luna et al. (2009) used cottonseed oil, glucose and yeast extract for the biosurfactant production by *Candida glabrata* and found a reduction in surface tension to 31 mN/m after 144 hours of cultivation. It is worth mentioning that the reduction in surface tension is used as a primary criterion for the selection of microorganisms that produce biosurfactants, although emulsifiers and dispersants do not necessarily have the ability to reduce surface tension (Yossef et al., 2004).

El-Sheshtawy et al. (2016) obtained biosurfactants yields of 1 and 12 g/L for *Bacillus licheniformis* and *Candida albicans*, respectively, while the surface tension decreased from 72 to 36 mN/m after 72 h using *B. licheniformis* and to 45 mN/m after 4 days of incubation using *Candida albicans*.

As the highest value obtained for the biosurfactant production was in the highest concentration of residual soybean oil investigated (80 g/L) experiments were carried out in the same previous condition using 90 g/L of residual soybean oil (Table 2). In this case, the biosurfactant yield was 13.0 ± 0.4 g/L, while the surface tension of the medium was 27 mN/m and the final cell concentration was 7.9 ± 0.3 g/L (initial cell concentration of 2.8 ± 0.4 g/L). These values are close to those obtained for 80 g/L of residual soybean oil.

Table 2: Evaluation of fermentation time as regard residual soybean oil concentration on biosurfactant production. The following concentrations were employed: $NaNO_3$ (2.0 g/L), K_2HPO_4 (1.0 g/L), residual yeast extract (1.2 g/L), $MgSO_4$ (0.5 g/L), and $FeSO_4$ (0.01 g/L).

Time (h)	Oil Concentration (g/L)	Biosurfactant Concentration (g/L)	Surface Tension (mN/m)	Cell Concentration (g/L)
48	70	5.1 ± 0.5	53.9 ± 0.1	4.0 ± 0.3
	80	6.3 ± 0.6	38.1 ± 0.1	6.6 ± 0.3
	90	6.1 ± 0.5	38.3 ± 0.1	6.3 ± 0.5
60	70	6.9 ± 0.4	37.9 ± 0.2	7.0 ± 0.4
	80	8.5 ± 0.3	32.1 ± 0.2	8.0 ± 0.5
	90	8.5 ± 0.4	32.1 ± 0.1	8.0 ± 0.3
72	70	6.8 ± 0.5	38.5 ± 0.1	7.3 ± 0.4
	80	7.3 ± 0.7	35.1 ± 0.2	8.0 ± 0.5
	90	7.2 ± 0.6	35.4 ± 0.1	8.0 ± 0.3

The evaluation of effect of the concentration of residual soybean oil over fermentation time on the responses presented in Table 2 (biosurfactant concentration, surface tension and cell concentration) was made using 2-way analysis of variance (Two-way ANOVA) with a significance level of 5%. The results of the analyzes indicated significant differences (p-value < 0.05) for all the answers mentioned above, both for the fermentation time factor, as well as for the residual soybean oil concentration factor and for the interaction between these two factors.

The results showed (Table 2) that for residual soy oil concentrations of 80 and 90 g/L of residual soybean oil and fermentation time of 60 h the biosurfactant production was maximum and in similar amounts, with yields of 8.5 ± 0.3 g/L and 8.5 ± 0.4 g/L, respectively. These results were inferior to those obtained with the presence of KCl in the fermentation medium. Confirming once again the fermentation time was 60 h and that there was a need to add this salt in the production medium. Then an experiment was performed with KCl concentrations of 0.3, 0.4 and 0.5 g/L, in the time of 60 h, residual soy oil concentration of 80 g/L and initial cell concentration of 2.9 ± 0.4 g/L. The results are summarized in Table 3.

The results in Table 3 showed that for KCl concentrations of 0.4 and 0.5 g/L the results of biosurfactant production, surface tension and cell production were similar. Thus, for the further experiments, 0.4 g/L of KCl was used.

Table 3: Biosurfactant production in relation to KCl concentration (0.5, 0.4 e 0.3 g/L), for a residual oil concentration of 80 g/L, 60 h of fermentation time, $NaNO_3$ (2.0 g/L), K_2HPO_4 (1.0 g/L), residual yeast extract (1.2 g/L), $MgSO_4$ (0.5 g/L), and $FeSO_4$ (0.01 g/L) for Candida sake.

Time (h)	Oil Concentration (g/L)	Biosurfactant Concentration (g/L)	Surface Tension (mN/m)	Cell Concentration (g/L)
60	0.5	12.3 ± 0.6	27.4 ± 0.1	8.0 ± 0.4
	0.4	13.0 ± 0.5	27.0 ± 0.1	8.0 ± 0.4
	0.3	9.2 ± 0.5	30.5 ± 0.1	7.0 ± 0.5

Sarubbo et al. (2006) observed that the biosurfactant produced by *Candida glabrata* UCP 1002 reduced the surface tension from 68 to 31 mN/m, while the biosurfactant produced by *Candida bombicola* ATTC 22214 reduced the surface tension of water from 72 to 38.9 mN/m (Pekin and Vardar-Sukan, 2006).
Marques et al. (2019) produced 6 g/L of biosurfactant using the filamentous fungus *Mucor circinelloides* and the surface tension of the medium was reduced to 26 mN/m. According to the authors, the biosurfactant showed stability to surface tension under adverse environmental conditions. This biosurfactant was prepared in a pre-optimized medium composed of corn maceration liquor (8.82%) and frying oil (2%), at pH 5 for 96 h, under orbital agitation at 150 rpm and 28 ºC.
Gaur et al. (2019) obtained for *Candida albicans* and *Candida glabata* the biosurfactant production of 1.32 g/L and 1.60 g/L when cultivated using 2% glucose as a carbon source.
The next test consisted of using the fermentation time of 60 h, 0.4 g/L of KCl, 80 g/L of residual soybean oil, while the other variables were fixed. Iron and magnesium sulfates ($FeSO_4$ and $MgSO_4$) were removed and a room temperature (28 ± 4 ºC) was employed. The biosurfactant production was of 13.0 g/L, surface tension was 27.1 mN/m and cell concentration was 8.4 g/L. Thus, two experiments were performed: one removing only $MgSO_4$ and the other one removing $FeSO_4$, with the other variables in the same conditions as before. The results were 12.6 ± 0.4 g/L and 12.9 ± 0.5 g/L of biosurfactant yields, surface tensions of 27.5 and 27.2 mN/m and final cell concentrations of 8.0 ± 0.6 and 8.2 ± 0.5 g/L, respectively. Thus, for the other experiments, these two salts were not used in the fermentation medium.
Andrade et al. (2018) produced biosurfactant from *Cunninghamella echinulata* using sustainable technology for cleaning and degreasing cotton fabrics impregnated with burnt motor oil. The surface tension was 32.4 mN/m in a medium containing residues of instant noodles (2%), maceration liquor (2%) and post-frying oil (0.5%) with a carbon/nitrogen ratio of 30:1, and 6.0 g/L of biosurfactant yield.
Applying a central compound design, Rodrigues at al. (2017) obtained a biosurfactant production of 11.7 g/L of using *Pseudomonas aeruginosa* ATCC 10145, soy molasses as a carbon source and 4 g/L of initial cell concentration in shaken flasks. All experiments were carried out in duplicate on a shaking table at 30.0 ± 1.0 ºC and 120 rpm for 72 h with samples collected every 12 h. The final surface tension was 31.9 mN/m. These results show that *Candida sake* has potential as a biosurfactant producing strain.
Then, the removal of residual yeast extract was evaluated using 0.4 g/L of KCl in the fermentation medium with the absence of the iron and magnesium salts and other variables defined in the best results obtained in the previous experiments. The results were 5.3 ± 0.4 g/L of biosurfactant, 39.5 mN/m of surface tension and 6.0 ± 0.6 g/L of final cell concentration in the absence of $FeSO_4$ and $MgSO_4$ salt, respectively. This result shows the importance of the concentration of residual yeast extract in the production medium. Thus, the yeast extract was kept in the fermentation medium.

4. Conclusions

In this work, it was determined that the optimal fermentative medium to be used for biosurfactant production by the yeast *Candida sake* was 80 g/L of residual soy oil and 0.4 g/L of KCl over 60 h, and there was also a need to use residual yeast extract (extract of yeast after use in the brewing industry) at a concentration of 1.2 g/L. As a source of nitrogen and phosphorus, 2.0 g/L $NaNO_3$ and 1.0 g/L K_2HPO_4 were added to the medium. As results, a biosurfactant production of 13.0 ± 0.5 g/L, a reduction of the medium surface tension from 51.3 ± 0.1 to 27.0 ± 0.1 and an increase in the final cell concentration from 2.9 ± 0.4 to 8.0 ± 0.4 were obtained, indicating that *Candida sake* has shown promise in the production of biosurfactant.

Acknowledgments

This work was financially supported by “Coordenação de aperfeiçoamento de Pessoal de Nível Superior (CAPES)”, “Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)”, “Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)” and “Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG)”.

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