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PRODUCTION OF BIOEMULSIFIERS BY *Yarrowia lipolytica* IN SEA WATER USING DIESEL OIL AS THE CARBON SOURCE

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Abstract - The objective of this work was to investigate, on a flask scale, the production of bioemulsifiers by *Yarrowia lipolytica* in the presence of sea water, supplemented with nitrogen and phosphate sources, using diesel oil as substrate. A full 2^4 factorial design was conducted to investigate the effects and interactions of the nutrient concentrations (diesel oil, urea, ammonium sulfate and monobasic potassium phosphate) on the response variables: emulsification activity and surface tension of the cell-free cultures. High emulsification activities (> 5,4 UEA) were determined after 168 h in all the experiments. The interactions among diesel oil, urea and monobasic potassium phosphate favored the emulsification with statistical significance. A correlation between the increase of emulsification activity and the reduction of surface tension was not identified.
Keywords: Biosurfactant; Bioemulsifier; Diesel oil; Sea water; *Yarrowia lipolytica*; Factorial design.

INTRODUCTION

Accidents during the transport of oil and its derivatives have caused serious damage to the environment. Pollution in marine water, especially oil spills, is a great danger to living organisms. All over the world, research and development of technologies for bioremediation and prevention of oil spills are stimulated.

Conventional techniques for cleaning areas contaminated with petroleum and its derivatives can be complemented in the remediation process by the presence of chemical dispersants or surfactants. Compared with synthetic surfactants, biosurfactants have greatest tolerance to variations of pH, temperature and salinity. These compounds are more selective, less toxic and biodegradable. In addition, they can be produced *in situ* by microorganisms in the presence of organic contaminants as substrates (Desai and Banat, 1997).

The microbial degradation is influenced by the diesel oil hydrophobicity, which limits the mass transfer to microorganisms. This limitation may be overcome either by the addition of emulsifiers/ surfactants or by microorganism growth that produces emulsifiers/surfactant. These compounds are widely used to increase diesel oil bioavailability (Herman *et al*., 1997). When a major oil spill occurs, in either a marine environment or fresh water, the supply of carbon increases and the availability of nitrogen and phosphorus becomes the limiting factor for oil degradation (Atlas, 1984). On the other hand, excessive concentrations of nutrients can inhibit the biodegradation process (Challain *et al*., 2006).

According to Ochoa and Vazquez-Juaréz (2004), sea water used in the formulation of culture medium for the development of marine yeast provides minerals and nutrients, promotes growth selectively, and reduces costs and risks from contamination.

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The objective of this study was to investigate the production of biosurfactants/bioemulsificants by *Yarrowia lipolytica* in sea water, supplemented with sources of nitrogen and phosphorus, using diesel oil as the carbon source.

MATERIALS AND METHODS

Microorganism

A strain of *Y. lipolytica* was isolated from mangrove sediments in the city of Rio Formoso, in Pernambuco state and belongs to the culture bank of the Núcleo de Pesquisas em Ciências Ambientais (NPCIAMB) at the Universidade Católica de Pernambuco. This strain is named UCP 0988. The yeast was maintained at 5ºC in Yeast Mold Agar (YMA) with the following composition: glucose 10 g, tryptone 5 g, yeast extract 3 g, malt extract 3 g, agar 15 g, distilled water 1000 mL, pH 5.

Diesel Oil

Diesel oil used in the experiments was bought at a Petrobras gas station with the specifications: metropolitan B4 type (4% biodiesel), maximum of 0.05% sulfur; with additives; contains paraffinic, naphthenic and aromatic hydrocarbons (10 to 40%). This product is stable under the usual conditions of handling and storage.

Physical-Chemical Characterization of the Sea Water

The sea water was collected in the Port of Suape, Pernambuco. Its composition was Ca^{++} 1.00%; Mg^{++} 0.97%; Na^{+} 11.13%, K^{+} 0.88%, Cl^{-} 20.50%, SO_4^{--} 3.41%; the salinity, specific gravity, pH and surface tension were equal to 37‰; 1026 kg/m^{-3}, 8.22 and 53.43 mN/m, respectively.

Experimental Design

A full 2^4 factorial design with four replicates at the central point, corresponding to a set of twenty experimental runs, was carried out to investigate the main effects and interactions of the independent variables: diesel oil, urea [$(NH_2)_2CO$], ammonium sulfate [$(NH_4)_2SO_4$] and monobasic potassium phosphate (KH_2PO_4) concentrations, on the response variables emulsification activity and surface tension of the cell free cultures of *Y. lipolytica* in sea water after 168 hours (in order to allow for the production of a high amount of emulsifier). The independent variables and their minimum and maximum levels (Table 1) were chosen according Albuquerque *et al*. (2006). The inoculum was obtained from a pure culture of *Y. lipolytica*, maintained at a temperature of 28ºC for 48h; the yeast cells were inoculated in sea water supplemented with urea (0.4%), ammonium sulfate (1.1%) and potassium monobasic phosphate (6.12%). All the medium components were dissolved in sea water and the pH was adjusted to 5.3 with NaOH or HCl; they were sterilized by autoclaving at 120ºC for 20 minutes. The diesel oil (5% v/v) was used as substrate and was sterilized by flowing steam. The inoculum was incubated at 28ºC and 150 rpm for 48 h. The final concentration of inoculum was around 10^6 cells/mL that were determined by counting in a Neubauer chamber. The components of the production media were weighed with the specifications as shown in Table 1. The diesel oil was used as substrate at 1, 3 or 5% (v/v), sterilized by flowing steam and distributed as specified by the full 2^4 factorial design (Table 1). Then, the inoculum (5% v/v) was transferred directly to 500 mL Erlenmeyer flasks, with a working volume of 300 mL which were kept under agitation (150 rpm) for 168 hours at 28°C. The statistical analysis of the results was carried out with the program Statistic version 8.0 (Statsoft - USA).

Table 1: Levels and values of independent variables of the full 2^4 factorial design

Independent variables	Levels		
	-1	0	+1
Diesel oil (%v/v)	1.0	3.0	5.0
(NH2)2CO (%p/v)	0.10	0.25	0.40
(NH4)2SO4 (%p/v)	0.10	0.60	1.10
KH2PO4 (%p/v)	0.68	1.36	2.04

Analytical Determinations

The analysis of the emulsification activity was performed according to the method described by Cirigliano and Carman (1984). Samples collected during the microorganism growth were filtered through Millipore® membranes (pore diameter of 0.22 μm). Cell-free aliquots (2 mL) were transferred to test tubes and diluted with 2 mL of 0.1 M sodium acetate buffer (pH 3.0). Then, 1 mL of corn oil was added; the mixture was vortexed for 2 minutes at 25°C in shaker tubes. After 10 min (at rest), the absorbance was measured. The culture medium was used as blank. One unit of emulsification activity (UEA) was defined as the amount of emulsifier required to produce an emulsion with absorbance equal to one at 540 nm.

The surface tension was determined by the ring method of du Noüy (ASTM D971, 1999), while the salinity and the density were determined by refractometry and the pH by potentiometry.

RESULTS AND DISCUSSIONS

The yeast *Y. lipolytica* was able to use diesel oil as substrate and to grow in natural sea water, supplemented with urea, ammonium sulfate and monobasic potassium phosphate, producing emulsification activities for corn oil after 168 hours. The main effects and interactions of independent variables on the response variable emulsification activity in the factorial design are illustrated in the Pareto Diagram (Figure 1). The decoded factorial design matrix and the results of each experimental condition of the 2^4 factorial design are shown in Table 2.

Effects of Nutrient Concentrations on Emulsification Activity and Surface Tension

In the Pareto Diagram, shown in Figure 1, monobasic potassium phosphate did not present statistically significant effects on the emulsification activity. The interaction of this phosphate with diesel and with urea, showed highly significant effects from the statistical point of view, favoring an increase in emulsification activity. A curvature test was performed and revealed a lack of adjustment in the linear approximation. Figure 1 shows that the curvature crosses the confidence level of 95%, indicating the proximity to the optimum point and the requirement for a second-order design and a quadratic model that incorporates the effect of the curvature. The Pareto Diagram of the dependent variable surface tension is not shown because the effects of the independent variables and their interactions were not statistically significant.

Culture Medium Investigation for Bioemulsifier/ Biosurfactant Production

In each of the twenty runs performed (Table 2), after 168 h the cell-free broth showed high emulsification activities, ranging between 5.45 and 6.00 UEA, compared to the values obtained by Albuquerque *et al*. (2006), who determined a maximum emulsification activity of 4.5415 UEA. Correlation was identified between high levels of emulsification activity and low surface tension. Despite the pH values between 4.92 and 6.00 (Table 2) and the literature information about yeasts preference for acid pH, this parameter needs to be investigated for the production of biosurfactant by *Y. lipolytica*, since sea water had pH 8.22. The salinity of the cell-free broth was between 42 and 65‰ (twice the amount of the sea water salinity). Few microorganisms are tolerant to seawater salinity. The genus *Yarrowia* is reported in the literature as presenting a high halophilic capacity (Ochoa and Vazquez-Juaréz, 2004).

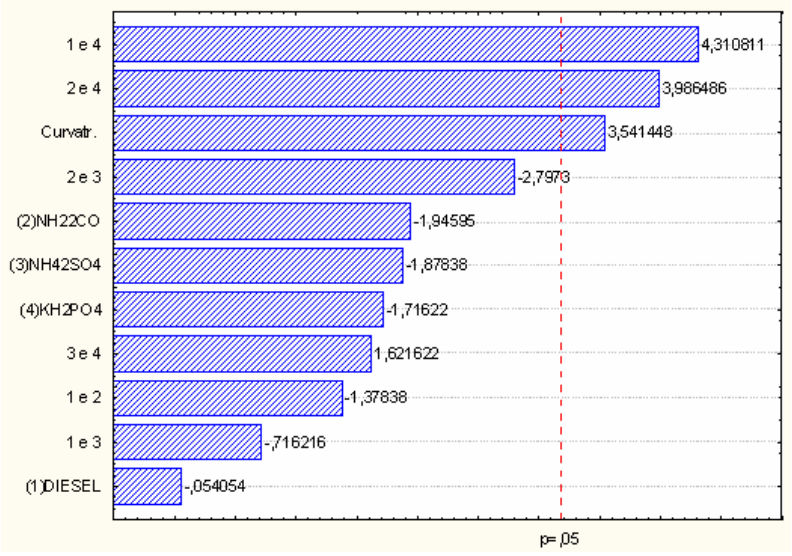

Figure 1: Pareto Diagram for 2^4 factorial design; response variable: emulsification activity. The point, in which the estimated effects were statistically significant (p = 0.05), is indicated by a vertical dashed line.

Table 2: Matrix for 2^4 factorial design and decoded results of emulsifying activity (EA) for corn oil-in-water emulsion, surface tension (ST), pH and salinity of the cell-free cultures after 168 hours of cultivation in the presence of diesel oil as substrate.

Run	Diesel oil (%v/v)	$(NH_2)_2CO$ (%p/v)	$(NH_4)_2SO_4$ (%p/v)	KH_2PO_4 (%p/v)	EA *(UEA)	ST (Mn/m)	pH	Salinity (‰)
1	1.0	0.10	0.10	0.68	6.000	49.22	5.14	46
2	5.0	0.10	0.10	0.68	5.996	47.33	5.19	45
3	1.0	0.40	0.10	0.68	5.872	49.14	6.08	42
4	5.0	0.40	0.10	0.68	5.784	43.48	5.65	50
5	1.0	0.10	1.10	0.68	6.000	52.55	5.44	53
6	5.0	0.10	1.10	0.68	5.836	49.15	5.11	56
7	1.0	0.40	1.10	0.68	5.844	46.28	5.87	46
8	5.0	0.40	1.10	0.68	5.454	52.43	5.68	57
9	1.0	0.10	0.10	2.04	5.654	50.84	4.97	53
10	5.0	0.10	0.10	2.04	5.692	53.61	4.92	53
11	1.0	0.40	0.10	2.04	5.830	49.21	6.02	55
12	5.0	0.40	0.10	2.04	5.982	50.13	5.93	53
13	1.0	0.10	1.10	2.04	5.658	49.13	5.07	61
14	5.0	0.10	1.10	2.04	5.984	48.16	5.07	65
15	1.0	0.40	1.10	2.04	5.682	47.16	5.85	61
16	5.0	0.40	1.10	2.04	5.796	51.94	5.72	60
17	3.0	0.25	0.60	1.36	6.000	51.44	5.39	49
18	3.0	0.25	0.60	1.36	6.000	46.68	5.35	45
19	3.0	0.25	0.60	1.36	5.852	49.56	5.41	51
20	3.0	0.25	0.60	1.36	6.000	48.91	5.69	48

* Unit of Emulsification Activity (UEA)

The highest emulsification activity (6.00 UEA) was determined in run 1 and in run 5, in the presence of 0.10% and 1.10%ammonium sulfate, respectively. Both of these experiments used diesel oil at 1% as substrate in the presence of 0.10% urea and 0.68% monobasic potassium phosphate. The maximum value was also determined at the central point of the factorial design (runs 17, 18 and 20); these conditions used diesel oil at 3% in the presence of 0.25% urea, 0.60% ammonium sulfate and 1.36% monobasic potassium phosphate. The emulsification activity was independent of ammonium sulfate concentration (Table 2).

The nitrogen source is very important in the physiology of microorganisms in order to produce protein during cell growth. This nitrogen source (ammonium sulfate) is ammoniacal, commonly metabolized by microorganisms. On the other hand, urea is a nitrogen source of lower cost, sold as fertilizer.

The interaction between urea and monobasic potassium phosphate favored the increase of the emulsification activity. The interaction between the nitrogen sources was not statistically significant under the experimental conditions. These results are shown in the Pareto Diagram (Figure 1).

However, runs 1 and 5 presented surface tensions equal to 49.22 and 52.55 mN/m, respectively, and showed a pH close to the initial value of the medium (pH 5.3) and a salinity higher than 46‰.

Although runs 1 and 5 showed higher emulsification activity (6.00 UAE), using diesel oil at lower concentration (1% v/v), run 4 had a lower surface tension (43.48 mN/m) in the presence of diesel fuel at the higher concentration (5% v/v), urea at 0.40%, ammonium sulfate at 0.10% and monobasic potassium phosphate at 0.68%. The initial surface tension of run 4, after autoclaving, was equal to 60.25 mN/m, making the reduction to 43.48 mN/m significant. Considering the great influence exerted by salinity (50‰) and pH (5.65) on the surface tension of the medium, run 4 demonstrated that its cell-free broth is a promising emulsifier.

There are several biosurfactants that lower the surface tension and stabilize emulsions (Singer *et al*., 1985; Rapp *et al*., 1979). One example is the sophorolipids of *Torulopsis bombicola* that have shown the ability to reduce surface tension and also proved to be good emulsifiers (Cooper and Paddock, 1984). On the other hand, the extracellular bioemulsifier Liposan from *C. lipolytica* is not able to reduce the surface tension of water (72.8 mN/m), but it effectively emulsifies and stabilizes water-in-oil emulsions (Cirigliano and Carman, 1985).

Candida tropicalis in the presence of distilled water as a solvent of a mineral medium and n-hexadecane as sole carbon source produced an extracellular emulsifier under nitrogen limitation in fed-batch culture. This emulsifier showed high emulsification activities for various hydrocarbons

where the maximum value was related to the aromatic compounds. In these experimental conditions, although the surface tensions ranged from 49 to 52 mN/m, the culture medium was equal to 68 mN/m. This implies that the material excreted by the cell was a better emulsifier than a biosurfactant (Singh and Desai, 1989, Singh *et al*., 1990).

Cladosporium resinae was isolated from an aircraft tank. This microorganism grew in a mineral medium in the presence of distilled water and jet fuel as a sole carbon source. It produced an extracellular biosurfactant called Cladosan after twenty five days that reduced the surface tension of the aqueous phase from 72 mN/m to 50 mN/m and, after being partially purified, reduced the tension to 40 mN/m (Muriel *et al*., 1996).

Plaza *et al*. (2006) investigated the biosurfacatant/ bioemulsifier productions to sixteen species of bacteria, isolated from contaminated soil with petroleum hydrocarbons. The experiments were carried out in liquid cultures containing crude oil under thermophilic conditions. The results showed that although the reduction of surface tension was a good measure of biosurfactant production, it was not correlated with the emulsification activity.

In the experiments carried out with *Y. lipolytica*, high values of emulsification activities were not correlated with low surface tension. These results agree with those obtained by Singh *et al*. (1990); the authors produced a biopolymer with high emulsification activity, but no significant results for surface tension reduction during the *Candida tropicalis* growth. On the other hand, Albuquerque *et al*. (2006) obtained an emulsification activity for a water-in-n-hexadecane emulsion equal to 4.415 UAE and a surface tension of 32.750 mN/m in optimal production medium, containing 0.544% of urea, 2.131% of ammonium sulfate, 2.628% of potassium phosphate, 5% v/v of corn oil, 50% v/v of distilled water and 50% v/v of sea water. According to the literature, this can be explained by the fact that the microorganism produces in some conditions only biossurfactants with emulsifier properties and, in others, biosurfactant with surface tension reducing activity (Cirigliano and Carman, 1984).

A consortium of bacteria isolated from a soil sample from Long Beach, contaminated with diesel oil, reduced the surface tension to 41.4 mN/m and increased the rate of emulsification to 64% after 14 hours of growth (Bento *et al*., 2005). Therefore, the surface tension reductions achieved by *Y. lipolytica* may be considered promising under the conditions of runs 2, 4, 7 and 15 of this work. The importance of monobasic phosphate potassium in bioemulsifier production in sea water is corroborated by studies that reported the production of emulsifiers by *C. lipolytica* in sea water diluted to 50%, supplemented with sources of nitrogen and phosphorus, using corn oil (Albuquerque *et al*., 2006) and babassu oil (Harrop-Vance *et al*., 2003) as carbon sources.

Mariano *et al*. (2008) evaluated the production of a biosurfactant by *Staphylococcus hominis*, *Kocuria palustris* and *Pseudonomas aeruginosa* LBI using a mineral medium in the presence of diesel oil (weathered and commercial) at 1, 5, 10, 20 and 30% v/v as a low cost substrate. The production of biosurfactant was monitored by the surface tension. The experiments were carried out in Erlenmeyer flasks with a 50 mL working volume and 1 mL of a bacteria suspension (inoculum). The flasks were incubated at 27±2ºC and 240 rpm for 144 hours. The initial surface tensions of the culture media were in the range between 50 and 61 mN/m, while the minimum surface tension determined was 45 mN/m after cultivation. The surface tension of the *P. aeruginosa* LBI grown in the presence of 10% v/v of weathered diesel decreased to approximately 45 mN/m (the maximum reduction determined). These authors adopted the criteria used by Habba *et al*. (2000): a good biosurfactant decreases the surface tension to a value equal to or less than 40mN/m. In other studies, *P. aeruginosa* LBI was able to produce rhamnolipid biosurfactant in the presence of soapstock (Moraes *et al*., 2002), diesel oil (Mariano *et al*., 2008) and n-hexadecane, glycerol, babassu oil and paraffin oil (Santa-Anna *et al*., 2002).

According to the criteria used by Habba *et al*. (2000), mentioned previously in this work, the yeast *Y. lipolytica* was not a good microorganism producer of biosurfactants, but it was a good producer of emulsifiers in the presence of diesel oil and sea water, supplemented with sources of nitrogen and phosphorus in all experimental conditions (Table 2).

Pornsunthorntawee *et al*. (2009) produced a biosurfactant by *P. aeruginosa* SP4 in a mineral medium supplemented with palm oil at different rates. The inoculum was grown in Erlenmeyer flask at 37°C and 200 rpm for 22 h. The experiments were carried out in a bioreactor with a capacity of 3000 mL and working volume of 1500 mL at 37±1ºC. The authors determined a surface tension reduction of 59% when palm oil was added at 2 kg/m^3.day with a cycle time of 2 days/cycle.

Rufino *et al*. (2011) produced a biosurfactant with antimicrobial properties from *Candida lipolytica* UCP 0988 in the presence of 0.1% NH_4NO_3, 0.02% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, supplemented with soybean oil refinery residue and glutamic acid in 72 hours fermentation at 28°C in an

orbital shaker at 150 rpm. The biosurfactant produced was able to reduce the surface tension from 50 mN/m to 25 mN/m.

According to Sarubbo *et al*. (2007), *Candida lipolytica* synthesized a surfactant in a cultivation medium supplemented with canola oil and glucose as carbon sources after 48 h of fermentation. The cell-free broth was particularly influenced by the addition of salt, the pH and temperature depending on the emulsified substrate (hexadecane or a vegetable oil).

The emulsifier from *Y. lipolytica*, named Yansan, was different from previous emulsifiers. It presented high emulsification activity and stability in the pH range of 3.0 - 9.0 and was capable of stabilizing oil-in-water emulsions with several aliphatic and aromatic hydrocarbons (Amaral *et al*., 2006).

In the literature, bioemulsifier/biosurfactant production was not found associated with the utilization of diesel oil by *Y. lipolytica* in natural sea water, supplemented by sources of nitrogen and phosphorus.

In this study, the application of Y. lipolytica in the bioremediation of oil in seawater was investigated for the first time. The physiology of this yeast was investigated under saline conditions of sea water in the presence of diesel oil. In a second step, experiments under natural conditions are fundamental for investigation of the nutritional interactions of the microbial consortium between *Y. lipolytica* and the indigenous microorganisms of seawater.

Because *Y. lipolytica* is strictly aerobic, the investigation of oxygen transfer in the medium is very important to increase the metabolite production. Thus, an increase of stirring in the bioreactor should promote, in a second step, greater aeration and enhance the production of metabolites.

CONCLUSIONS

The production of emulsifiers by *Y. lipolytica* in saline conditions in the presence of diesel oil enables the application of this yeast in sea bioremediation of petroleum and oil spills, due to its ability to produce a bioemulsifier in the presence of sea water.

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