

Use of Soybean Oil Fry Waste for Economical Biosurfactant Production by Isolated *Pseudomonas aeruginosa* Using Response Surface Methodology

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Abstract

The present study sought biotensioactive production from soybean oil fry waste using *Pseudomonas aeruginosa* ATCC 10145 and *Pseudomonas aeruginosa* isolated from the soil of a petroleum station having undergone gasoline and diesel oil spills. The results of the experiments were analyzed using a complete factorial experimental design, investigating the concentration of soybean oil waste, ammonia sulfate and residual brewery yeast. Assays were performed in 250-mL Erlenmeyer beakers containing 50 mL of production medium, maintained on a rotary shaker at 200 rpm and a temperature of 30 ± 1 °C for a 48-hour fermentation period. Biosurfactant production was monitored through the determination of rhamnose, surface tension and emulsification activity. The *Pseudomonas aeruginosa* ATCC 10145 strain and isolated *Pseudomonas aeruginosa* were able to reduce the surface tension of the initial medium from 61 mN/m to 32.5 mN/m and 30.0 mN/m as well as produce rhamnose at concentrations of 1.96 and 2.89 g/L with emulsification indices of 96% and 100%, respectively.

Keywords: *Pseudomonas aeruginosa*, Biosurfactant, Rhamnose, Surface-active, Emulsification index, Soybean oil.

Introduction

Surfactants are an important class of chemical compounds widely used in different industries, acting as dispersants and/or solubilizers of organic compounds. The vast majority of commercially employed surfactants are synthesized from petroleum derivatives (1). In the past few decades, however, the interest in surfactants of a microbial origin has increased significantly, above all, due to their biodegradability (2,3,4).

Compounds of a microbial origin that exhibit surfactant properties (reduction of surface tension and/or high emulsifying capacity) are denominated biosurfactants and are metabolic byproducts of bacteria and fungi (5). Glycolipids are the best known microbial surfactants. These compounds are made up of carbohydrates associated to a long chain of aliphatic or hydroxy-aliphatic acids. Rhamnolipids are among the most-studied glycolipids and are compounds that have one or two rhamnose molecules linked to one or two α -hydroxydecanoic acid molecules (6).

Surfactants produced microbiologically offer a number of advantages over their chemical equivalents, such as low toxicity, tolerance to temperature, pH and ionic strength as well as the possibility of being produced from renewable substrates (7,8,9). Biosurfactants can be applied in fields such as agriculture for the formulation of

pesticides and herbicides; the food industry as additives in condiments; and in pharmaceutical, textile, cosmetic and petroleum industries, where there are employed for the secondary recovery of petroleum, such as in the removal and mobilization of oil residuals and bioremediation (10).

Despite their advantages, biosurfactants are not widely used by industries due to the high production costs associated to low productivity and the use of expensive substrates. One possible strategy for reducing production costs is the use of alternative substrates, such as agricultural or food industry wastes, which generally contain the high levels of carbohydrates and lipids necessary for the biosynthesis of biosurfactants (11). Moreover, the use of wastes contributes toward a reduction in environmental pollution and the economic valuation of such products. Alternative substrates, such as oil dregs, used oils, molasses and wastes from the processing of cheese, potatoes and cassava, are examples of byproducts with potential for the production of biosurfactants (12,13,14,15).

The aim of the present study was to determine suitable replacements for chemical surfactants byproducts with either low or no toxicity using wastes as raw materials to reduce the cost of these byproducts. The main objectives of the study were to determine the potentiality of an isolated strain of *Pseudomonas aeruginosa* in producing biosurfactant from soybean oil waste used in the frying of different foods, ammonia nitrate and residue from an autolyzed brewery biomass; and compare its performance to that of *Pseudomonas aeruginosa* ATCC 10145, using a complete factorial experimental design.

Materials and Methods

Microrganism

P. aeruginosa ATCC 10145 was kindly donated by Dr. Ivano de Fillipis from the Instituto Nacional de Controle de Qualidade em Saúde

(INCQS/FIOCRUZ) – Rio de Janeiro, Brazil. *P. aeruginosa* was isolated from the soil of a petroleum station having undergone gasoline and diesel oil spills located in the city of Uberlândia, Minas Gerais, Brazil. The bacterial strain was identified as *P. aeruginosa* called strain UFU. The cultures were maintained at 4°C in a bacto nutrient broth (BD, cod. 234000) supplied by the Becton Dickinson and Company, USA.

Culture Isolation

The medium proposed by Vecchioli (16), added with 0.5% (v/v) of soybean oil fry waste as the sole carbon source, was used for the bacterial cultures using the pour-plate technique. Among the isolated microorganisms, the one that demonstrated the best surface tension reduction of the culture medium after fermentation was selected and identified. The isolated microorganism was identified at the Enterobacteria Laboratory of the Oswaldo Cruz Institute (Rio de Janeiro, Brazil), following traditional procedures based on bacterial cytomorphology, biochemistry and physiology.

Growth Medium and Conditions

Growth of the bacterial culture was performed on the medium proposed by Santos (17), consisting of (g/L) NH_4NO_3 (1.7), Na_2HPO_4 (7.0), KH_2PO_4 (3.0), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2), yeast extract (5.0) and glucose (10.0). Biosurfactant production assays were conducted on the same mineral medium used for microbial growth, with the addition of soybean oil fry waste (g/L between 5 and 15), residual brewery yeast (g/L between 0 and 10), NH_4SO_4 (g/L between 1 and 13). The residual brewery yeast, consisting of 100% inactivated, dried cells of *Saccharomyces cerevisiae* was provided by a local brewery. The product composition was 8.0% moisture, 40.0% protein, 3.0% fibrous matter, 8.0% mineral matter and aflatoxin (50 ppb). All media were autoclaved at 121°C for 15 min after adjusting the pH to 7.0 with 0.1 N HCl.

The inoculum was prepared by adding three loopfuls of cells from the stock culture to a 500 mL Erlenmeyer beaker containing 100 mL of the growth medium. The inoculated medium was incubated at $30 \pm 1^\circ\text{C}$ for 24 hours on a rotary shaker (New Brunswick, USA) at 170 rpm. Afterwards, optical density (600 nm) of bacterial suspension was adjusted to 0.4 and an aliquot of 1 mL of inoculum (2%) was transferred to a 250-mL Erlenmeyer beaker containing 50 mL of medium and incubated at 30°C for 48 hours on a rotary shaker at 170 rpm. Samples were collected at defined time intervals and submitted to analysis.

Complete Factorial Experimental Design

The literature indicates that carbon and nitrogen sources play a critical role in the performance of rhamnase production by *P. aeruginosa* strains (17). To investigate the effects of soybean oil fry waste (WFSO), ammonium sulfate (AS) and residual brewery yeast (RBY) on the selected dependent variables (rhamnase synthesis, emulsification index and surface tension), a complete factorial experimental design (CFED) was used on two levels (18). Statistical calculations were performed using the Statistic 5.1 software program (State Ease Inc.,

Table 1. Real values of variables used in complete factorial experimental design

Independents Variables	Range and levels		
		-1	+1
WFSO (g/L)	X_1	5	15
SA (g/L)	X_2	1	13
RBY (g/L)	X_3	0	10
Microorganism isolated	X_4	<i>P. aeruginosa</i> ATCC 10145	<i>P. aeruginosa</i>

Minneapolis, MN, USA). Using the CFED method, a total of 16 experiments were conducted with combinations of FSO, AS, RBY and the two *Pseudomonas aeruginosa* strains. Table 1 displays the range and levels of the variables investigated.

Analytical Methods

Cell growth was determined by measuring the optical density of samples, using a UV-160A visible spectrophotometer (Shimadzu, Co., Tokyo, Japan) at 540 nm. Cell concentration was determined by dry weight filtering through a $0.45 \mu\text{m}$ previously weighed Millipore membrane (19).

Surface tension (ST) was measured at 25°C using a Tensiometer (Fisher Scientific, USA), which was previously calibrated with surveyor weights. A decrease in surface tension was used as a qualitative measurement of surfactant concentration and a quantitative indicator of efficiency.

The biosurfactant emulsification index (EI) was determined according to Cooper and Goldenberg (20). Cell-free culture samples and kerosene (at a ratio of 4:6) were vigorously mixed for 2 min using a vortex (Phoenix, Brazil, model AP-56) and left undisturbed for 24 h at room temperature. EI 24 is the percentage of the height

of the emulsified layer (cm) relative to the total height of the liquid column, determined at the 24-h time point.

The rhamnase concentration was determined according to the methodology described by Rahman et al. (21).

Results and Discussion

Effects of carbon and nitrogen concentrations on rhamnase production

Table 2 displays the results of the complete factorial designs from the FSO, AS and RBY concentrations.

Table 2. Results of rhamnase production (RM), EI (E24) and ST obtained in the Experiments with *P. aeruginosa* ATCC 10145 and isolated *P. aeruginosa* UFU

Experiments	WFSO (g/L)	SA (g/L)	RBY (g/L)	Microorganism	RM (g/L)	E24 ^a (%)	ST ^b (mN/m)
1	5	1	0	ATCC 10145	0.56±0.02	60±0.0	38.5±0.5
2	5	1	0	Isolated	0.79±0.02	71±0.0	36.5±0.5
3	5	1	10	ATCC 10145	0.73±0.01	71±2.0	36.5±0.5
4	5	1	10	Isolated	0.98±0.03	75±3.0	34.0±1.0
5	5	13	0	ATCC 10145	0.25±0.02	40±2.0	41.0±0.0
6	5	13	0	Isolated	0.36±0.01	42±1.0	40.5±1.0
7	5	13	10	ATCC 10145	0.41±0.04	41±2.0	40.5±1.0
8	5	13	10	Isolated	0.59±0.01	60±1.0	39.5±0.0
9	15	1	0	ATCC 10145	1.43±0.01	96±1.0	32.5±0.0
10	15	1	0	Isolated	1.89±0.03	100±0.0	30.5±1.0
11	15	1	10	ATCC 10145	1.96±0.04	96±0.0	32.5±0.0
12	15	1	10	Isolated	2.81±0.03	100±0.0	30.0±0.0
13	15	13	0	ATCC 10145	0.82±0.02	67±1.0	35.5±0.5
14	15	13	0	Isolated	0.91±0.03	67±2.0	35.5±0.5
15	15	13	10	ATCC 10145	1.07±0.03	81±2.0	33.5±1.0
16	15	13	10	Isolated	1.15±0.05	85±1.0	33.0±1.0

EI^a 24 emulsification index measured after 24-h incubation in kerosene

Inicial ST^b do meio de produção was 61mN/m

Table 2 displays the statistical delineation used in the production of rhamnase by *P. aeruginosa* ATCC 10145 and isolated *P. aeruginosa* UFU under the different conditions. Both strains were able to use the residue tested (FSO) and synthesize the biosurfactant. Experiments 11 and 12 employed extreme FSO, RBY values and a minimal concentration of AS, obtaining the highest production of rhamnase and

emulsification index as well as the lowest surface tension value. According to Lang and Wullbrant (22), high concentrations of carbon and nitrogen in the fermented medium are needed for the obtainment of high concentrations of rhamnase. In the present study, the highest amount of rhamnase (2.81 g/L), highest emulsification index (100%) and lowest surface tension (30.5 mN/m) were obtained from the isolated *P. aeruginosa*

UFU, whereas the least adequate condition occurred for *P. aeruginosa* ATCC 10145, with the lowest production of rhamnase (0.25 g/L), lowest emulsification index (40%) and highest surface tension (41.1 mN/m).

Haba et al. (14) selected the *P. aeruginosa* 47T2 NCIB 40044 strain from 36 screened strains due to its capacity to produce 2.7 g/l of rhamnase from FSO. Previous studies found that this strain produced only 6.4 g/l of rhamnase through cultivation in olive oil waste (23). De Lima et al. (24) obtained a final concentration of 2.3 g/L of rhamnase when *P. aeruginosa* PACL was cultivated in WFSO.

As Table 2 shows, both strains are able to reduce surface tension to below 35 mN/m. According to Cooper (25), an organism is considered a promising biosurfactant producer when it produces tensoactive compounds with a surface tension below 40 mN/m. In order for a biosurfactant to be considered efficient, however, this value must be below 35 mN/m. Studies on rhamnolipid homologues extracted and purified from the fermentation broth by *Pseudomonas aeruginosa* 47T2 cultivated in oil dregs have described a value of 32.8 mN/M for surface tension (26). In a study carried out with vegetable oils (olive, soybean and sunflower) at a concentration of 20 g/L, Andrés et al. (27) found that the broth fermented by *P. aeruginosa* 42 A2 achieved surface tension values of 32.0, 34.0 and 335.5 mN/m, respectively.

For all experiments, the biosurfactant produced had intense emulsification properties. The complete kerosene in water emulsions proved stable for 24 hours. This analysis is a practical measurement of biosurfactant use, as it gives the compound the ability to emulsify non-miscible liquids with the formation of a stable emulsion. The results obtained in the present experiment suggest that isolated *Pseudomonas aeruginosa* UFU has both a better degradation capability

regarding soybean oil waste in fermented media as well as a greater potential for producing biosurfactant.

Statistical analysis of the data

From the CFED, the operational conditions of the WFSO (X_1), AS (X_2), RBY (X_3) and *P. aeruginosa* strains (X_4) were determined. As X_4 is a qualitative variable and the equation of the empirical model must be in function of the quantitative variables, it was necessary to perform a correction of the adjusted equation, replacing the X_4 variation with either -1 or 1 in order to maximize the response. Thus, the adjusted empirical model for rhamnase synthesis containing only significant parameters ($p=0.05$; Student's t test) is represented by Equation 1. Table 3 displays the parameters and significance levels of the model variables.

$$RM = 1.185 + 0.48X_1 - 0.33X_2 + 0.187X_3 - 0.187X_1X_2 \quad (1)$$

The results show that X_1 and X_3 are highly significant among the independent variables, as they have positive coefficients (Eq. 1), according to which an increase in their concentration increases the production yield. The X_2 variable and the X_1X_2 interaction also have a significant effect, as their negative signs initiate when their concentrations are lower in the system, thereby promoting an increase in the response (RM).

Table 3. Results of the regression for rhamnase synthesis

Codified Factor	Parameter	T Student	Probability
Constant	1.0250	22.2485	0.0000
X_1	0.4800	10.4188	0.0004
X_2	-0.3300	-7.1629	0.0020
X_3	0.1875	4.0698	0.0152
X_4	0.1600	3.4729	0.0255
X_1X_2	-0.1875	-4.0698	0.0152

$$R^2=0.9832; \text{Adjusted } R^2=0.9414; R=0.9916$$

The goodness-of-fit of the model was checked by determining the coefficient (R^2) and the multiple correlation coefficient (R). The R^2 value (0.9832) for the complete equation (data not shown) indicates that the sample variation of 98.32% for rhamnose was attributed to the independent variables and only 1.68% of the total variation cannot be explained by the model. The value of the adjusted determination coefficient (adjusted $R^2 = 0.9414$) for Equation (1) is also high, which demonstrates the high significance of the model. The high R value (0.9916) demonstrates high agreement between the experimental observations and predicted values. This correlation is also evident on the plot of predicted versus experimental rhamnose values in Figure 1, as all points cluster around the diagonal line, meaning that no significant violations of the model were found.

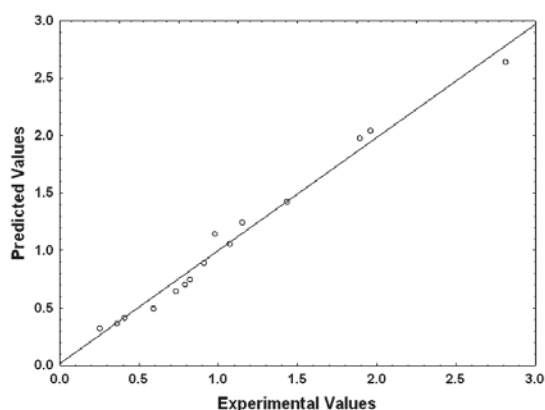


Fig 1. Predicted vs. experimental values plotted for rhamnose

The 3D response surface (Fig. 2) represents the empirically adjusted equation (Eq. 1) and is plotted to visualize the interactions of the independent variables (WFSO, AS) as well as locate the optimum level of each variable for maximum response (rhamnose synthesis).

The response surface in Figure 2 reveals that an increase in FSO concentration and a decrease in AS concentration cause an increase in rhamnose synthesis.

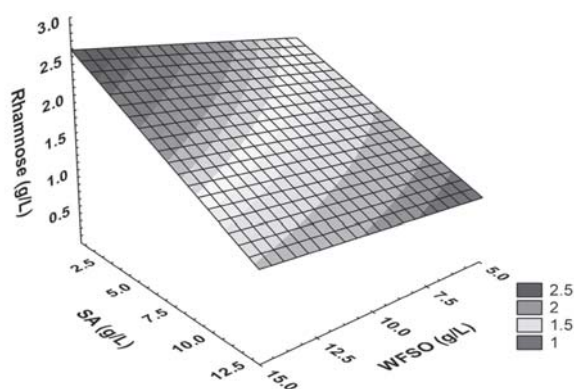


Fig 2. Response surface for rhamnose in relation to WFSO and AS.

Kinetics of rhamnose production by isolated *P. aeruginosa* UFU and *P. aeruginosa* ATCC 10145

Figure 3 illustrates the kinetics of rhamnose production from the best results obtained by *P. aeruginosa* ATCC 10145 (Experiment 11) and isolated *P. aeruginosa* UFU (Experiment 12)

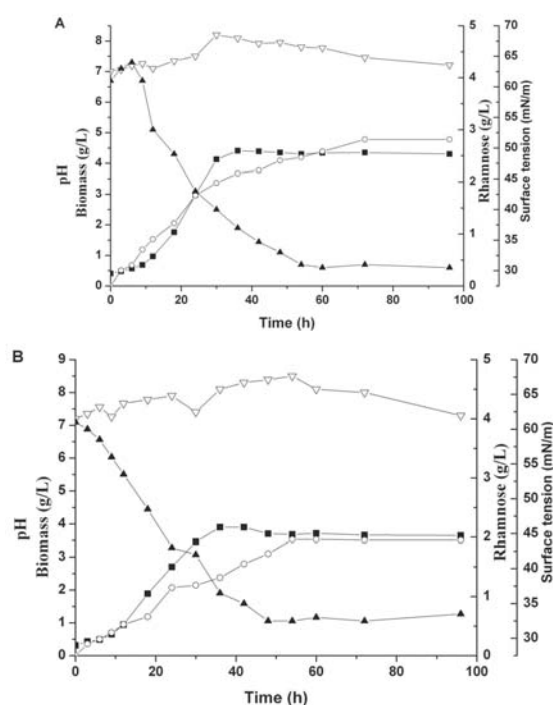


Fig. 3. Kinetics of growth (|), rhamnose (O), pH (▲) and surface tension (?): (A) Isolated *P. aeruginosa* UFU and (B) *P. aeruginosa* ATCC 10145 cultivation from soybean oil fry waste.

using soybean oil waste from fried food preparation as the carbon source.

There was intense cell growth up to 36 h of fermentation, when the stationary phase was established, corresponding to biosurfactant production (Y_p/x) of 0.54 for the isolated strain (Fig.3A) and 0.36 g rhamnolipids/g cells for the ATCC 10145 strain (Fig.3B). At 72 h, the isolated strain achieved rhamnolipid synthesis level of 2.81 g/L, while the ATCC 10145 reached 1.96 g/L of rhamnolipid at 54 h. In previously published studies, Guerra-Santos et al. (28), Haba et al. (14) and Dubey et al. (29) achieved 0.97-2.7 g/L of biosurfactant production with different strains of *P. aeruginosa* using glucose and fry oil waste as carbon sources.

As Figure 3 shows, biosurfactant production initially follows an exponential growth phase, but when microbial growth ceases and a stationary phase is achieved, rhamnolipid synthesis continues, which suggests biotensioactive production partially associated with microbial growth. These observations were also described by Mayer et al. (30), Benincasa et al. (31) and Yu-Hong et al. (32). Perhaps the production of biosurfactant can be classified as a secondary metabolite. Biosurfactant production accompanies bacterial growth in fry oil waste, which may help in the adherence of cells to the substrate molecules and their metabolism (33,34).

Due to the biotensioactive accumulation in the medium, there was also a drop in surface tension (Fig.3). Regarding pH, there was a variation ranging from 7.01 to 8.5 and a tendency toward final values greater than 7.2.

The type of carbon source affects the properties (surface tension and emulsification activity) and final concentration of the rhamnolipid produced. These differences may be associated to the composition of triglycerides in the substrates used (35) as well as the activity of the microbial lipase on these substrates (36).

Differences were found between the two microorganisms tested in the present study with regard to rhamnolipid production when the same substrate was used. Differences in rhamnolipid number in the composition of the rhamnolipids may also result in differences in biotensioactive properties (15).

Conclusion

With the complete factorial experimental design, it was possible to determine the behavior of the independent variables on rhamnolipid production, the emulsification index and surface tension. The present study demonstrated the biosurfactant-producing potential from the re-use of a fried soybean oil substrate by *P. aeruginosa* ATCC10145 and a new isolated strain, which obtained the best results regarding rhamnolipid production (2.89 g/L), surface tension (30 mN/m) and emulsification index (100%).

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