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Surface active agent production from olive oil in high salt conditions and its process optimization

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Microbial surfactants or biosurfactants are surface active amphiphilic macromolecules that are produced by a number of microorganisms (bacteria, yeast and fungi). These compounds have extensive application in various industries especially in food, pharmaceutical and oil industry. The aim of this paper is to optimize the culture conditions for the biosurfactant production from olive oil by a novel halophilic isolate microorganism. The Taguchi experimental design methodology based analysis of olive oil as carbon source, yeast extract as nitrogen source and KH_2PO_4 as phosphorus source revealed that the olive oil and yeast extract significantly affect biosurfactant production in high salt conditions. Maximum biosurfactant (E_{24}= 40%) produced in the presence of 4% (v/v) olive oil, 0.2% (w/v) yeast extract, and 0.002% (w/v) KH_2PO_4 . In conclusion, halophilic archaeon *Haloarcula* sp. IRU1 could be a potential microorganism for the production of biosurfactant from olive oil as carbon source in high salt conditions. The optimal parameters obtained during the optimization process were: olive oil 4%, yeast extract 0.4% and KH_2PO_4 0.004%.

Keywords: Surface active agent, Olive oil, optimization, Taguchi experimental design methodology.

INTRODUCTION

Surfactants or surface active agents are widely used in the petroleum, pharmaceutical, cosmetic and food industries. Most of these compounds are chemically synthesized and it is only in the past few decades that surface-active molecules of biological origin have been described[1]. Biosurfactants are surfactants produced by microorganisms, either directly in microbial cell surfaces or by extracellular secretion. As amphiphilic molecules, biosurfactants contain hydrophilic and hydrophobic portions, and their structures are typically composed of one or more classes of compounds, including mycolic acids, glycolipids, polysaccharide-lipid complexes, lipoproteins, lipopeptides, phospholipids, and/or the microbial cell surface itself[2–4].These microbial compounds have extensive application in various industries, mining, leather, agricultural, oil recovery with functional properties as foaming, surface activity, wetting agent, emulsification in agricultural, pharmaceutical, cosmetics and a wide range of petrochemical industries[5]. They can be active at extremes of temperature, pH, and salinity and can be synthesized from renewable feedstocks besides, making an ecofriendly environment[6–10]. The type, effectiveness, and efficiency of biosurfactants are influenced by the nature of the carbon sources and the concentrations of nitrogen, phosphorus, magnesium, iron, and sulfur ions in the medium[6]. The search for biosurfactants in extremophiles seems to be particularly promising since the biosurfactants of these organisms have particular adaptations to increase stability in adverse environments that can potentially increase their stability in the harsh environments in which they are to be applied in biotechnology[11,12].

Halophiles, which have a unique lipid composition (phytanylglycerol), may have an important role to play as surface-active agents. The archae bacterial ether-linked phytanyl membrane lipid of the extremely halophilic bacteria has been shown to have surfactant properties[12]. Yakimov et al. reported the production of biosurfactant by a halotolerant Bacillus species and its potential in enhanced oil recovery. In that study, *Bacillus licheniformis* strain BAS 50 was able to grow and produce a lipopeptide surfactant in different salinities[13]. In the present study, we aimed to optimize for the first time, the culture medium composition for the biosurfactant production from olive oil by a novel halophilic isolate *Haloarcula* sp. IRU1 using Taguchi experimental design methodology.

EXPERIMENTAL

Microorganism, Growth Medium and Culture Conditions

Haloarcula sp. IRU1 isolated from hypersaline Lake Urmia, Iran was cultured in 50mL liquid basal salt medium composed of (g/L): NaCl, 250; $MgCl_2.6\ H_2O$, 34.6; $MgSO_4\ .7H_2O$, 49.4; $CaCl_2.2\ H_2O$, 0.92; NaBr, 0.058; KCl, 0.5; NaH_2CO_3, 0.17. The growth medium supplemented with various nutrients compositions by varying olive oil as carbon source at 0.5–4% (v/v), yeast extract as nitrogen source at 0.05–0.4% (w/v) and KH_2PO_4 as phosphorus source at 0.001–0.008% (w/v) according to the details following the experiment design (Table 1 and 2). The cultivation temperature for all experiments was 42°C.

Emulsification activity assay

After 5 days of incubation in an orbital shaker at 200 rpm and 42°C, the cells were removed by centrifugation

Table 1. Parameters and levels used in this experiment

Factor	Parameter	Level1	Level2	Level3	Level4
Factor A	Olive oil % (V/V)	0.5	1	2	4
Factor B	Yeast extract % (W/V)	0.05	0.1	0.2	0.4
Factor C	KH_2PO_4 % (W/V)	0.001	0.002	0.004	0.008

Table 2. Taguchi experimental design for three variables affecting biosurfactant productionby *Haloarcula* sp. IRU1

Trial	Olive oil % (V/V)	Yeast extract % (W/V)	KH_2PO_4 % (W/V)	E_{24} (%)
1	0.5	0.05	0.001	13.33
2	0.5	0.1	0.002	20.00
3	0.5	0.2	0.004	28.33
4	0.5	0.4	0.008	31.67
5	1	0.05	0.002	21.67
6	1	0.1	0.001	18.33
7	1	0.2	0.008	25.00
8	1	0.4	0.004	33.33
9	2	0.05	0.004	23.33
10	2	0.1	0.001	26.67
11	2	0.2	0.008	30.00
12	2	0.4	0.002	36.67
13	4	0.05	0.008	28.33
14	4	0.1	0.004	35.00
15	4	0.2	0.002	40.00
16	4	0.4	0.001	38.33

at 10000 rpm for 10 min at room temperature. Two milliliters of the cell-free supernatant was mixed with 2 ml kerosene in a test tube. This mixture was shaken for 2 min and then left to stand. The emulsification stability was measured after 24 h and the emulsification index (E_{24}) was calculated by dividing the measured height of the emulsion layer by the total height of the liquid layer and multiplying by 100[11,14].

Experimental design and statistical analyses

Taguchi experimental method was used to describe the number of experimental situations. All experiments were preformed in 250 ml Erlenmeyer flasks containing 50 ml of the basal medium and incubated in a shaker at 42°C and 200 rpm for 5 days. The culture broth was separated and analyzed for biosurfactant. Qualitek-4 software was used for the design and analysis of Taguchi experiments.

RESULTS AND DISCUSSION

The Taguchi experimental design differentiates between control factors and the noise or uncontrollable factors, and treats them separately by means of special design matrices called "Orthogonal Arrays"(OA). Columns and rows of an OA are arranged in a fixed way indicating the combination of factor levels in each experiment to be run, and allowing the simultaneous evaluation of several parameters with the minimum number of trials[15,16]. The Taguchi experimental design is a good positive option for the optimization of biotechnological processes for microbial synthesis[17]. The effect of olive oil as carbon source, KH_2PO_4 as phosphorus source, yeast extract as nitrogen source on the biosurfactant production by *Haloarcula* sp. IRU1 was tested in the Taguchi experimental design that resulted in a total of 16 experiments. Based on the experimental design, the factor combinations resulted in different biosutfactant amounts. The range of the responses was 40% in experiment No. 15 (maximum) and 13.3% in experiment No. 1 (minimum). The results indicated that *Haloarcula* sp. IRU1 gave highest biosutfactant (E_{24}=40%) in the presence of 4% (v/v) olive oil, 0.2% (w/v) yeast extract, and 0.002% (w/v) KH_2PO_4 (Table 2).

Effect of carbon source

Olive oil has been suggested to be one of the best carbon sources for biosurfactant synthesis[18]. The effect of olive oil concentration on biosurfactant production was evaluated at 42°C varying olive oil of 0.5, 1, 2, and 4% (v/v). The kinetic curves obtained at this step are presented in Fig. 1. Using different concentrations of olive oil we can observe that biosurfactant was produced. Using 0.5% of olive oil in a different experiment, lower biosurfactant production was obtained. The highest production in these experimental conditions was obtained only using 4% of olive oil. From Fig. 1, it can be verified that when olive oil concentration is increased from 0.5 to 4%, the biosurfactant increased from 23.3% to 35.4%. Then olive oil concentration has a significant effect on biosurfactant production. A possible explanation for this fact might be related to the fact that microbial ability to synthesize biosurfactant is often coupled with their ability to grow on immiscible carbon sources, such as hydrocarbons. Biosurfactants have been demonstrated to play a role in different stages of the interaction between microorganisms and hydrocarbons by overcoming the low solubility of these substrates, accessing to hydrocarbons before transportation into cells, and regulating the adhesion-deadhesion of microbial cells from and to hydrocarbon surfaces[19,20].

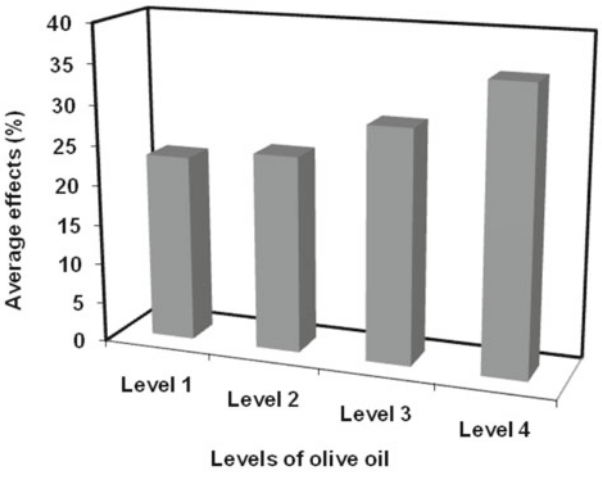

Figure 1. Average effects of olive oil on biosurfactant production obtained by Qualitek-4 (W32b) software

Effect of nitrogen source

The effect of four yeast extract concentrations (0.05, 0.1, 0.2, and 0.4%) on the level of biosurfactant produced by *Haloarcula* sp. IRU1 is presented in Fig.2. The increase in yeast extract concentration caused the increase in the amount of biosurfactant produced by *Haloarcula* sp. IRU1. Studying the different concentrations of yeast extract we found that the highest concentration (0.4%) was the best concentration for the production of Biosurfactant (Fig.2). Nitrogen source plays an important role in the production of biosurfactant by microorganisms[14]. It has been reported that the type of nitrogen influenced

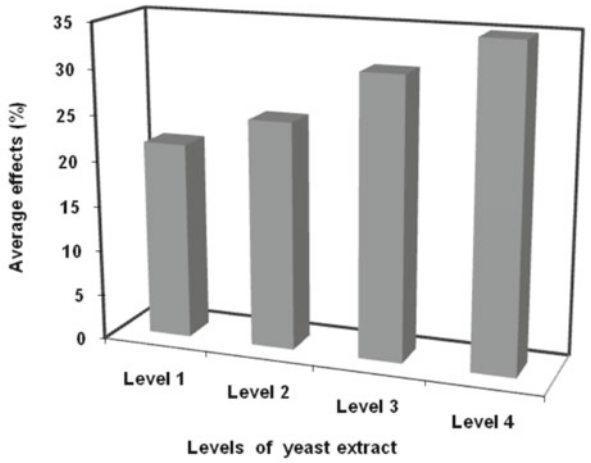

Figure 2. Average effects of yeast extract on biosurfactant production obtained by Qualitek-4 (W32b) software

the biosurfactant produced by microorganisms.[21] Previous studies have reported that the enhancement of nitrogen concentration increased the microbial biosurfactant production[6,21,22].

Effect of phosphorus source

When measure E_{24} for the produced biosurfactant in different concentrations of KH_2PO_4 (0.001, 0.002, 0.004, 0.008%) we found that concentration 0.004% was the best for biosurfactant production (Fig.3). An increase in the concentration of phosphorus source in the medium for the production of biosurfactant was not associated with any remarkable changes in the biosurfactant production. Some studies have reported that the enhancement of phosphorus concentration did not significantly increase the microbial biosurfactant production[6].

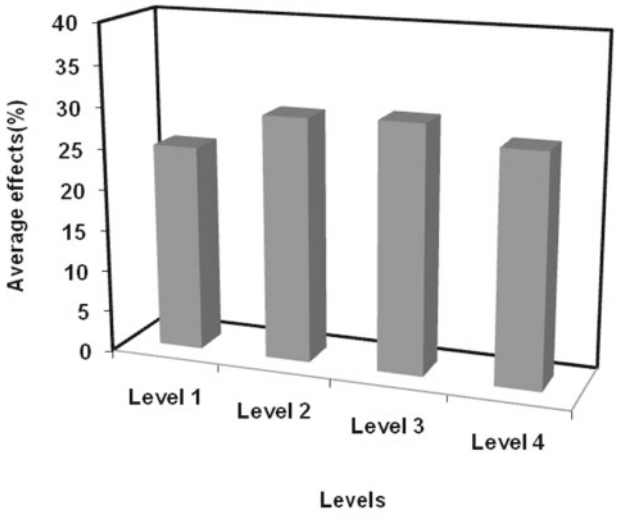

Figure 3. Average effects of KH_2PO_4 on biosurfactant production obtained by Qualitek-4 (W32b) software

Analysis of variance

The purpose of the analysis of variance (ANOVA) for orthogonal array is to determine which process factors significantly affect the yield. There is a tool called an *F*-test to see which process factors have a significant effect on the quality characteristic. In performing the *F*-test, the mean of squared deviation due to each factor needs to be calculated. The *F*-test value for each factor is simply the ratio of the mean of squared Deviation to the mean of squared pooled error. The process factors with a large *F*-test value have a more significant effect on the quality characteristic. In addition, for significant factors their percentage contributions can be determined from the mean of squared pooled error and the pure sum of squared deviation due to each factor[23–25]. Table 3 shows the results of ANOVA for biosurfactant production by *Haloarcula* sp. IRU1. The last column of the table shows the percentage contribution of each factor on the total variation which indicates the degree of influence on the yield of biosurfactant. The percentage contributions, sum of squares and maximum variances of parameters affecting the yield of biosurfactant in a decreasing order are: yeast extract (39.77, 391.02 and 130.43 respectively), olive oil (36.07, 358.99 and 119.66 respectively), KH_2PO_4 (5.94, 61.848 and 5.94 respectively). From ANOVA, it is clear that nitrogen source (yeast extract) is the most significant process factor affecting biosurfactant production, followed by the carbon source (olive oil). The change of phosphorus source (KH_2PO_4) in the range tested (0.001–0.008%) has little effect on biosurfactant.

Optimization of parameters using Taguchi's approach

Table 4 shows a predicted yield of biosurfactant produced by *Haloarcula* sp. IRU1 under optimal conditions for process parameters. By using the optimization function of the Taguchi experimental design the maximum yield of biosurfactant (E_{24} =42.50%) was predicted at a reaction condition of olive oil 4%, yeast extract 0.4%, KH_2PO_4 0.004%. Good agreement is observed between the predicted value and experimental value of E_{24}. It can also be seen that the yield of biosurfactant under optimal conditions for factor levels is higher than any of the experimental yields shown in Table 2.

The contribution of the tested factors in biosurfactant production by Taguchi experimental design showed that olive oil played a more important role than the other

Table 4. Prediction of optimum conditions for biosurfactant production *Haloarcula* sp. IRU1

Factors	Level Description	Level	Contribution
Olive oil	4%	4	7.29
Yeast extract	0.4%	4	5.208
KH_2PO_4	0.004%	3	1.873
Total Contribution From All Factors			14.37
Current Grand average Of Performance			28.124
Expected Results At Optimum Condition			42.495

Table 3. Analysis of Variance (ANOVA) for biosurfactant production *Haloarcula* sp. IRU1

Factors	DOF (f)	Sum of Sqrs (S)	Variance (V)	F-Ratio (F)	Pure Sum (S')	Percent P (%)
Olive oil	3	358.993	119.664	7.705	312.404	36.07
Yeast extract	3	391.023	130.341	8.393	344.435	39.768
KH_2PO_4	3	22.896	7.632	0.491	0	0

factors. The contributions of the parameters affecting the yield of biosurfactant in optimal conditions were: yeast extract (5.21), olive oil (7.29) and KH_2PO_4 (1.87). The total contribution and grand average performance from all factors were 14.37, 28.12 respectively.

CONCLUSIONS

In this work, we used Taguchi orthogonal array design method to optimize biosurfactant synthesis from olive oil by *Haloarcula* sp. IRU1. The following conclusions can be drawn based on the experimental results of this study:

– Taguchi methodology design is suitable to optimize the conditions for biosurfactant production by *Haloarcula* sp. IRU1.

– The yeast extract as nitrogen source and olive oil as carbon source significantly affect the biosurfactant production.

– The optimal levels of nitrogen source, carbon source and phosphorus source were estimated for the best biosurfactan production.

– The optimal conditions for biosurfactan production were: olive oil 4%, yeast extract 0.4%, KH_2PO_4 0.004%.

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