Rhamnolipid and lipase production by *Pseudomonas aeruginosa* san-ai: The process comparison analysis by statistical approach

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Abstract

Pseudomonas aeruginosa has been repeatedly reported as a powerful producer of rhamnolipid biosurfactants as well as hydrolytic enzymes. In this study, the effects of four fermentation factors were evaluated using response surface methodology and experiments were performed in accordance with a four-factor and five-level central composite experimental design. The investigated factors were: fermentation temperature, time of fermentation, concentration of sunflower oil and concentration of Tween® 80. The most important finding was that regression coefficients of the highest values were those that describe interactions between factors and that they differ for lipase and rhamnolipid production, which were both investigated in this study. Production of both metabolites was optimized and response equations were obtained, making it possible to predict rhamnolipid concentration or lipase activity from known values of the four factors. The highest achieved rhamnolipid concentration and lipase activity were 138 mg dm^{-3} (sunflower oil concentration: 0.8%, Tween® 80 concentration: 0.05%, temperature: 30 °C and fermentation time: 72 h) and 11111 IU dm^{-3} (sunflower concentration: of 0.4%, Tween® 80 concentration: 0.05%, temperature: 30 °C and fermentation time: 120 h), respectively.

Keywords: *Pseudomonas aeruginosa*, rhamnolipid, lipase, response surface methodology.

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Pseudomonas aeruginosa is a gram-negative opportunistic pathogen, known for its ability to survive in a wide range of habitats such are water, plants, oil, etc. This ubiquitous environmental bacterium produces and secrets numerous virulence factors which conduce to its high environmental adaptability [1,2]. Humans, animals, plants, nematodes, amoebae are all prone to infections with *P. aeruginosa*, and all virulence factors take place in processes of infection initiation or establishment [3–5].

Pseudomonas aeruginosa san-ai strain was isolated from water-soluble, rancid mineral cutting oil utilized as an aid for cooling and lubrication in metalworking processes [6–9]. This water-soluble cutting oil is mixture of surfactants and mineral oils with high alkaline pH (pH 10), which makes it unaccommodating for bacterial growth [8]. Nevertheless, *P. aeruginosa* san-ai strain has the ability to survive in these extreme conditions owing to its potential to product enzymes with very distinct properties [8]. Extracellular hydrolytic enzymes produced by this strain have been proven to have exceptional properties suitable for several biotechnological applications. These enzymes are especially interesting for application in detergent formulations and, therefore, have been tested for stability in presence of several oxidizing agents and commercial surfactants [6,7]. These enzymes have already been characterized and it has been known that protease exhibits optimal behavior in alkaline environment, pH 9, and at 60 °C, while lipase shows optimal behavior at pH 11 and 70 °C [8,9].

In addition, other than highly applicable hydrolytic enzymes, *Pseudomonas aeruginosa* strains also produce surface-active compounds known as rhamnolipids. This was firstly reported by Jarvis *et al.* more than sixty years ago, but the chemical nature of these biosurfactants was not elucidated [10]. Nowadays, it is known that *P. aeruginosa* strains produce primarily two forms of rhamnose containing glycolipids: mono-rhamnolipid (rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate) and di-rhamnolipid (rhamnosyl-rhamnosyl-β--hydroxydecanoyl-β-hydroxydecanoate) [11–13].

During the last twenty years, numerous efforts have been made with purpose of increasing yield of rhamnolipid production by *Pseudomonas* species, since various areas of their application emerged. Predominantly, rhamnolipids and modified rhamnolipids are applied instead of chemical surfactants due to biodegradability and non-toxicity of rhamnolipids. Additionally, the pos-

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sibility of their application in bioremediation, food industry, and in the production of fine chemicals has been reported [11,14,15]. Recently, rhamnolipids were proved to have antimicrobial, anti-adhesive and immunomodulating properties making them worth for further research in biomedical area [15].

The main obstacles for substitution of synthetic surfactants by biosurfactants are high production costs of biosurfactants, related with complex process control due to foam formation during fermentation and expensive downstream processing [16]. Therefore, the main goal of majority of studies focused on rhamnolipid production is selection of appropriate carbon and nitrogen source, which allows high yields and reduction of a number of downstream processing steps. Literature data reveal the most diverse list of carbon sources tested in rhamnolipid production by *P. aeruginosa* strains including different vegetable oils (soybean, olive, sunflower and corn) as well as petrochemicals, such as diesel and kerosene [17–21]. In order to decrease production costs, several authors have proposed an alternative strategy, featuring the development of technologies based on cheap waste carbon sources, which are usually a significant environmental problem. A waste fraction from soybean oil refining process, frying oil waste, molasses from sugarcane refining, soap stock from oil refining, whey from dairy industry, spent wash as distillery waste, crude glycerol from vegetable oil industries were all successfully employed in rhamnolipid production as carbon sources [17,22-28]. Rhamnolipid production is also very dependent on carbon/nitrogen ratio in growth medium, and it could be significantly improved by optimization of these factors [22].

The aim of this study was the optimization of process factors for production of two principal metabolites, namely rhamnolipid and lipase, using multifactorial experimental design and response surface methodology (RSM). The effects of several fermentation factors on rhamnolipid and lipase production were investigated including temperature, fermentation time, concentration of sunflower oil and concentration of Tween® 80. Application of experimental design facilitates optimization process and on the other hand, it provides information regarding effects of each experimental factor as well as their interactions. Another aim of this research was to understand the interaction of rhamnolipid production with lipase production and to find a correlation between these outputs.

EXPERIMENTAL

Chemicals and bacterial strain

Growth medium components were purchased from Torlak, Institute of Immunology and Virology, Serbia. Surfactant Tween® 80, *p*-nitrophenyl palmitate (*p*-NPP), and orcinol were obtained from Sigma, St. Luis, CA. *P. aeruginosa* san-ai strain was isolated from mineral oil used in metalworking processes. This strain was originally isolated in San-ai Oil (Tokyo) and it was provided to us by the courtesy of Dr. N. Fujiwara (Technology Research Institute of Osaka Prefecture, Osaka, Japan). Agar slant used for microorganism maintenance consisted of peptone I (10 g dm^{-3}), yeast extract (5 g dm^{-3}), NaCl (5 g dm^{-3}) and agar (15 g dm^{-3}) at it was kept at 4 °C.

Fermentation

P. aeruginosa san-ai was cultivated at 30 °C for 24 h with shaking (250 rpm) in tryptic soy broth, which was prepared in accordance with the instructions provided by the manufacturer.

Growth medium used for *P.aeruginosa* san-ai cultivation was LB medium comprising peptone (10 g dm^{-3}), NaCl (5 g dm^{-3}), and yeast extract (5 g dm^{-3}). Fermentations were carried out in Erlenmeyer flasks on a horizontal shaker (Kühner, Switzerland), set at 250 rpm and temperature predetermined by the experimental plan. During the fermentation, samples were taken, in sterile conditions, from the liquid culture to monitor rhamnolipid concentration, and lipase activity.

Lipase activity assay

Determination of lipase activity was conducted using *p*-nitrophenyl palmitate method (*p*-NPP), which is based on spectrophotometric measurement of *p*-nitrophenol released in enzymatic hydrolysis of *p*-NPP. The substrate was prepared by dissolving 30 mg of *p*-NPP in 10 cm^3 of isopropanol and 90 cm^3 of 50 mmol dm^{-3} phosphate buffer (pH 8). Prior to spectrophotometric measurement both substrate and enzyme were incubated at 37 °C. Lipase solution, 0.1 and 0.9 cm^3 of substrate were mixed directly in spectrophotometric cuvette and absorbance was measured at 410 nm during the first 3 min of reaction. One unit of lipase activity (IU) was defined as the amount of lipase that released 1 μmol of *p*-nitrophenol per minute (ε = 1500 $dm^3\ mol^{-1}\ cm^{-1}$) under the conditions defined in the assay [6,9].

Determination of rhamnolipid concentration

The concentration of rhamnolipids, glycolipids secreted by *P. aeruginosa* san-ai, in the bacterial culture supernatant was evaluated by measuring the concentration of hydrolysis-released rhamnose by the orcinol method, which has been previously described [5,29].

Experimental design

The effects of four fermentation parameters on lipase activity and rhamnolipid production by *P. aeruginosa* san-ai were investigated using a five-level-four-factor central composite rotatable experimental design.

The experimental design included 30 experimental points, which consisted of 16 factorial points, 8 axial points and 6 central points [30]. Based on the preliminary study and literature survey, four experimental factors were analyzed in given ranges: sunflower oil concentration (0.2–1% (w/v)); Tween® 80 concentration (0–0.2% (w/v)); temperature (20–60 °C), and incubation time (48–144 h). The relation between actual values and coded values are given in Table 1.

Experimentally obtained data were fitted using the following equation:

$$Y = \beta_{k0} + \sum_{i=1}^{4} \beta_{ki} X_i + \sum_{i=1}^{4} \beta_{kii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{kij} X_i X_j \quad (1)$$

where Y is the response variable, in our case rhamnolipid concentration (mg dm^{-3}) or lipase activity (IU dm^{-3}), β_{k0}, β_{ki}, β_{kii} and β_{kij} are the regression coefficients variables, for the intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are independent variables. The least square method was used both for the response function coefficients calculation and evaluation of their statistical significance. Fisher test was used to evaluate adequacy of model, and Student distribution was used to evaluate the significance of the coefficients.

RESULTS AND DISSCUSSION

Pseudomonas aeruginosa san-ai strain has already been established as an efficient producer of extracellular hydrolytic enzymes [6–9]. Nevertheless, *Pseudomonas aeruginosa* strains have been known as producers of rhamnolipids, microbial surfactants that deserve at least equal attention due to increasing areas for their utilization.

RSM Analysis: Influence of fermentation factors on rhamnolipid production

The main goal of this study was to ameliorate the lipase production as well as rhamnolipid production by *P. aeruginosa* san-ai and to establish the most influential fermentation factors and their correlations. The data showing the lipase activity and rhamnolipid concentration for the 30 experiments conducted according to the experimental design are presented in Table 2.

Among the various treatments, the highest rhamnolipid concentration (138 mg dm^{-3}) was achieved in experiment no. 2 (sunflower oil concentration 0.8%, Tween® 80 concentration 0.05%, temperature 30 °C, and fermentation time 72 h), while the highest lipase activity (11111 IU dm^{-3}) was achieved in run no. 12 (sunflower concentration of 0.4%, Tween® 80 concentration of 0.05%, temperature of 30 °C, and fermentation time of 120 h). The experimental results were fitted with a second order regression model which included interaction of factors. The regression coefficients were determined using least square method and following regression model was obtained:

$$\begin{aligned} Y = {} & 11.42 + 4.66X_1 - 8.86X_2 - 2.30X_3 + 2.24X_4 - \\ & - 8.07X_1X_2 - 18.2X_1X_3 - 22X_1X_4 + 13.5X_2X_3 + \\ & + 1.86X_2X_4 + 10.9X_3X_4 + 6.66X_1^2 + 5.58X_2^2 + \\ & + 5.82X_3^2 + 5.54X_4^2 \end{aligned} \quad (2)$$

Equation (2) represents quantitative effects of process variables and their interactions on the response, which is in this case rhamnolipid concentration (mg dm^{-3}).

After statistical analysis of experimental results and results obtained by the regression model, the Fischer test of 3.0779 was obtained. Since this value is lower than table values for adequate degree of freedom, obtained regression model can be used for describing obtained experimental results. The analysis of obtained regression coefficients implies that significant interaction between effects of experimental factors occurred. The coefficient of highest value (coefficient –18.24) is the one that describes negative interaction between sunflower oil concentration and temperature. The influence of these factors on rhamnolipid concentration, under fixed values of other factors, is presented in Figure 1.

It can be easily observed that temperature exhibits mild positive effect on rhamnolipid production at lowest oil concentration. On the other hand, at the highest examined oil concentration (1%), a steep decrease of the yield of rhamnolipids occurred with the temperature rise. Hence, maximum rhamnolipid concentration was obtained at maximum sunflower oil concentration and minimum temperature. This is correlated with a higher lipase activity produced at high sunflower concentration and low temperature (Table 2, experiments No. 10 and 12). These results cannot be simply

Table 1. Experimental and coded values of fermentation factors

Experimental factor	Coded values				
	–2	–1	0	1	2
X_1, Sunflower oil conc., % (w/v)	0.2	0.4	0.6	0.8	1
X_2, Tween® 80 conc., % (v/v)	0	0.05	0.1	0.15	0.2
X_3, Temperature, °C	20	30	40	50	60
X_4, Time, h	48	72	96	120	144

Table 2. Experimental design

Run No.	Sunflower oil	Tween® 80	*t* / °C	Time, h	Lipase activity, IU dm^{-3}	Rhamnolipid concentration, mg dm^{-3}
1	–1	–1	–1	–1	533.34	47.17
2	1	–1	–1	–1	155.56	138.06
3	–1	1	–1	–1	544.44	5.46
4	1	1	–1	–1	166.67	40.78
5	–1	–1	1	–1	0	25.39
6	1	–1	1	–1	0	18.00
7	–1	1	1	–1	0	40.72
8	1	1	1	–1	0	17.46
9	–1	–1	–1	1	7377.8	30.65
10	1	–1	–1	1	10844	87.42
11	–1	1	–1	1	7422.2	0
12	1	1	–1	1	11111	40.11
13	–1	–1	1	1	0	44.91
14	1	–1	1	1	0	55.70
15	–1	1	1	1	0	65.70
16	1	1	1	1	0	27.19
17	–2	0	0	0	33.33	20.79
18	2	0	0	0	22.22	0
19	0	–2	0	0	0	12.26
20	0	2	0	0	0	0.53
21	0	0	–2	0	2288.9	1.33
22	0	0	2	0	0	24.25
23	0	0	0	–2	0	0
24	0	0	0	2	111.11	21.99
25	0	0	0	0	19.82	23.59
26	0	0	0	0	22.22	0
27	0	0	0	0	26.73	0
28	0	–1	–1	–1	21.54	0
29	0	–1	–1	–1	28.21	4.66
30	0	1	–1	–1	22.22	37.31

explained since the control of both rhamnolipids and lipase production is complex, influencing by numerous factors at both genetic control and environmental/nutritional levels. It is well known that production of the biosurfactant is subjected to cell density-dependent regulation and limitation of specific nutrients. The rhamnolipid synthesis is positively controlled in a cell-density manner by a cell-to-cell communication system called quorum sensing (QS) [31,32]. It might be plausible that under suboptimal conditions for the cell growth (higher temperature and high oil concentration), the suppressed cell growth had negative effects on QS regulation.

Significant positive interaction between Tween® 80 concentration and temperature (coefficient 13.5) was also observed. The influence of these factors on rhamnolipid concentration, under fixed values of other factors, is presented in Figure 2. At high Tween® 80 concentration the effect of temperature seemed to be negligible. Nevertheless, at low Tween® 80 concentration positive interaction triggered a steep increase of rhamnolipid concentration with decrease of fermentation temperature.

Concentrations of sunflower oil and Tween® 80 have shown a negative interaction (coefficient -8.068) and the influence of these factors is illustrated in Figure 3. At low oil concentrations, as well as at high Tween® 80 concentrations the influence of other factor is very mild. Nevertheless, at highest oil concentrations the effect of surfactant is more intensive, leading to steep increase of rhamnolipid production with the decrease of Tween® 80 concentration.

Model proposed that maximum rhamnolipid concentration of 270 mg dm^{-3}, is achieved when the highest sunflower oil concentration was used. Reports on rhamnolipid production indicate that rhamnolipid concentrations obtained in large scale batch bioreactors (30 dm^3) exceed up to 100 folds those obtained in the

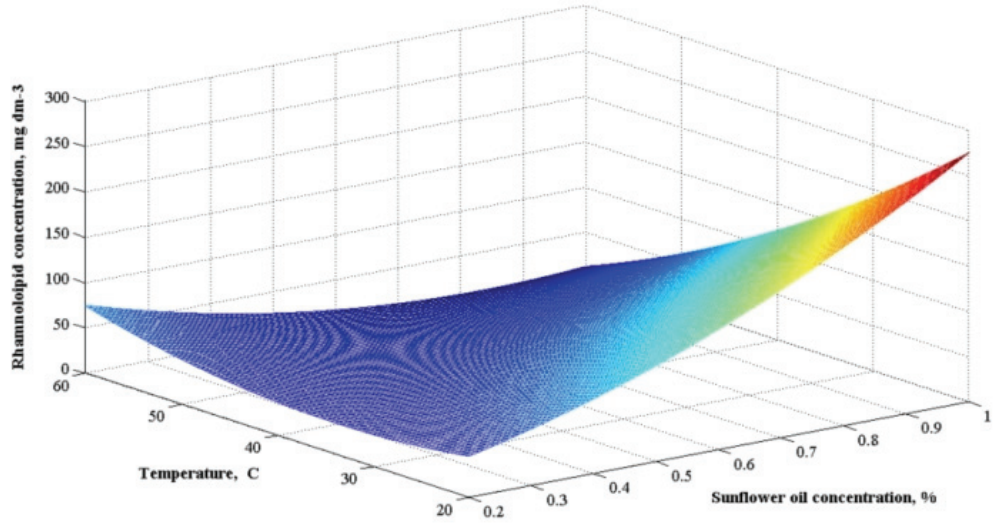

Figure 1. The effect of sunflower oil concentration and fermentation temperature on rhamnolipid production.

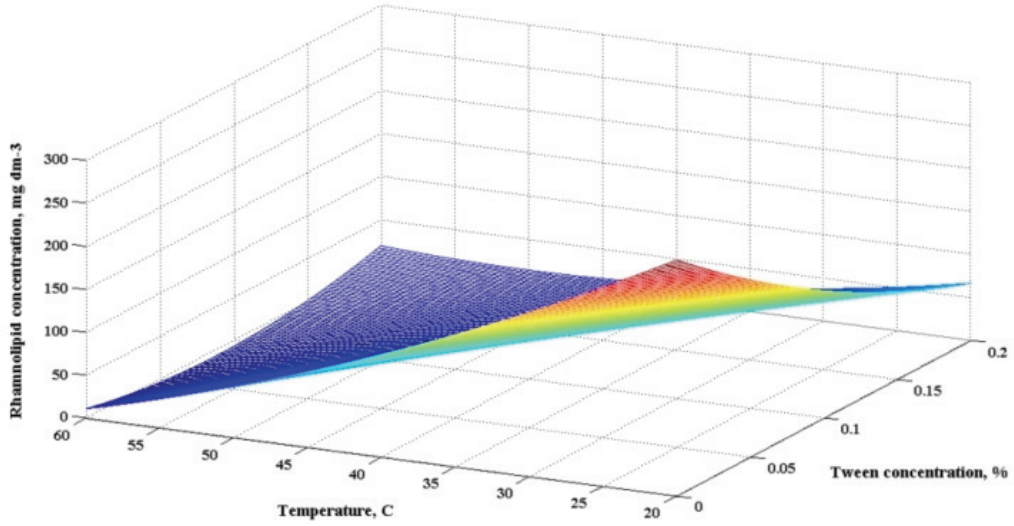

Figure 2. The effect of Tween® 80 concentration and fermentation temperature on rhamnolipid production.

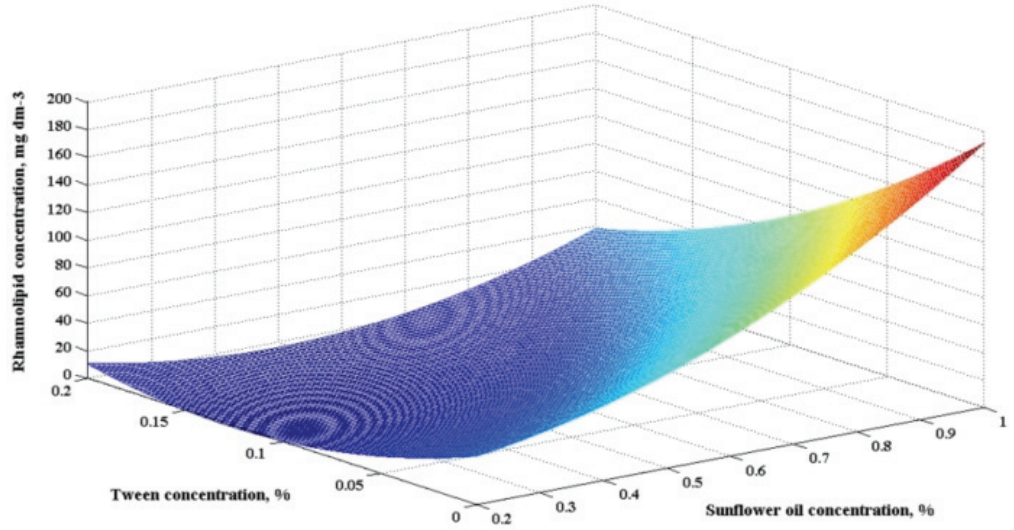

Figure 3. The effect of Tween® 80 concentration and oil concentration on rhamnolipid production.

shake flasks with the same strains [20]. This could be an explanation for relatively low rhamnolipid concentrations obtained in this experiment comparing to literature data. Müller *et al.* [20] reported rhamnolipid concentration of 39 g dm^{-3} after 90 h in bioreactor, while the highest concentration of rhamnolipid obtained with the same strain in shake flask was 2.2 g dm^{-3}. Martínez--Toledo *et al*. [33] recently reported usage of RSM for improving of biosurfactant production by *Pseudomonas putida*. Reported rhamnolipid concentrations were almost 5-fold lower than those obtained in this study.

RSM Analysis: Influence of fermentation factors on lipase activity

Unlike rhamnolipid production, the most relevant variables for the lipase production appear to be temperature and incubation time. The effect of temperature incubation time interaction was the most significant ($p < 0.05$). While incubation time had a positive effect (coefficient 1484), temperature and incubation time-temperature interaction had a significant negative influence on lipase production (coefficients of –1788 and –2220, respectively). The final response equation obtained after eliminating the insignificant terms was as follows:

$$Y = -1782X_3 + 650X_3^2 - 2220X_3X_4 + 1484X_4 + 378X_4^2 \quad (3)$$

where Y presents predicted response (IU dm^{-3}) and other variables have been previously defined.

Proposed model excludes sunflower oil and Tween® 80 concentrations as statistically insignificant although the preliminary study showed their favorable effect on lipase production (unpublished results). Some considerations should be made in the terms of these predictions. This model incorporates a greater number of lipase production curves over an appreciably wide temperature range, including some temperature values highly inimical to the growth of *Pseudomonas* spp. [34]. The inclusion in our model of a considerable amount of data from environmental conditions that adversely affect growth may have the diminishing effect on the importance of medium constituents, sunflower oil and Tween® 80.

Results obtained mathematically confirmed the experimental results regarding an inverse relationship between the influence of temperature and fermentation time on lypolytic activity. The shape of the three-dimensional surface-representing lipase activity *versus* temperature and incubation time is shown in Figure 4.

In addition, contour plot was also generated which delineates predicted response over a range in the design surface (Figure 5). It appears that the surface is smooth, showing increase/decrease in one axis and decrease/increase in the other axis, which reflect that the temperature may affect lipase production in opposite ways. In particular, the lipase activity increased as the temperature increased at initial period. For example, as the temperature increased from 20 to 60 °C, the lipase activity increased from minor to 6460 IU dm^{-3} at 48 h. At intermediate and high levels of incubation period, however, different behavior was observed as the surface decreased when the reaction temperature increased. Such influence could be explained due to the heat sensitivity of lipase-synthesizing reactions or lipase inactivation by the simultaneously produced proteases [6].

CONCLUSION

Pseudomonas aeruginosa san-ai has been proven to be a producer of hydrolytic enzymes with properties

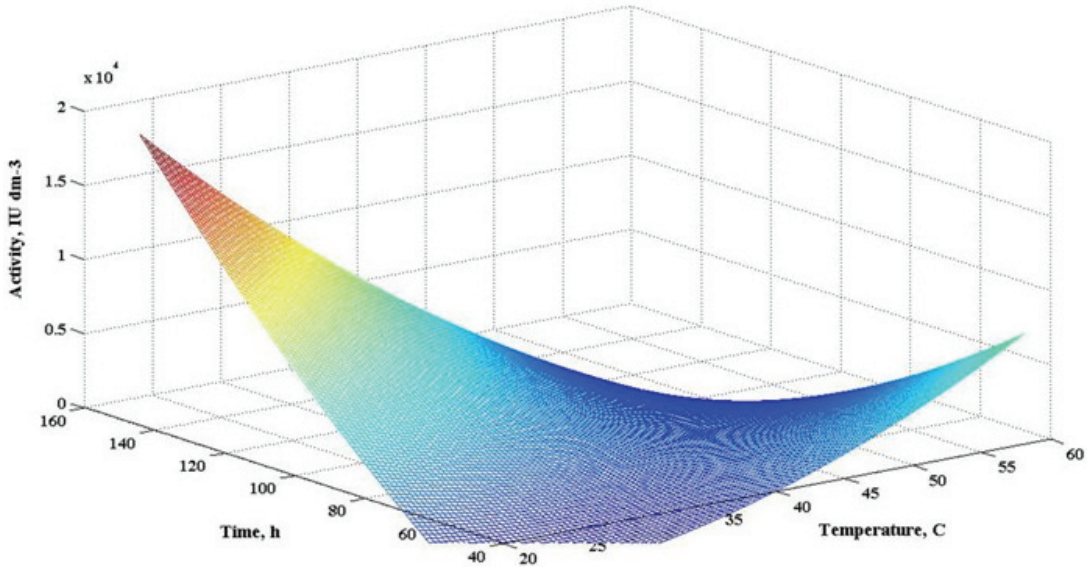

Figure 4. The effect of temperature and fermentation time on lipase activity.

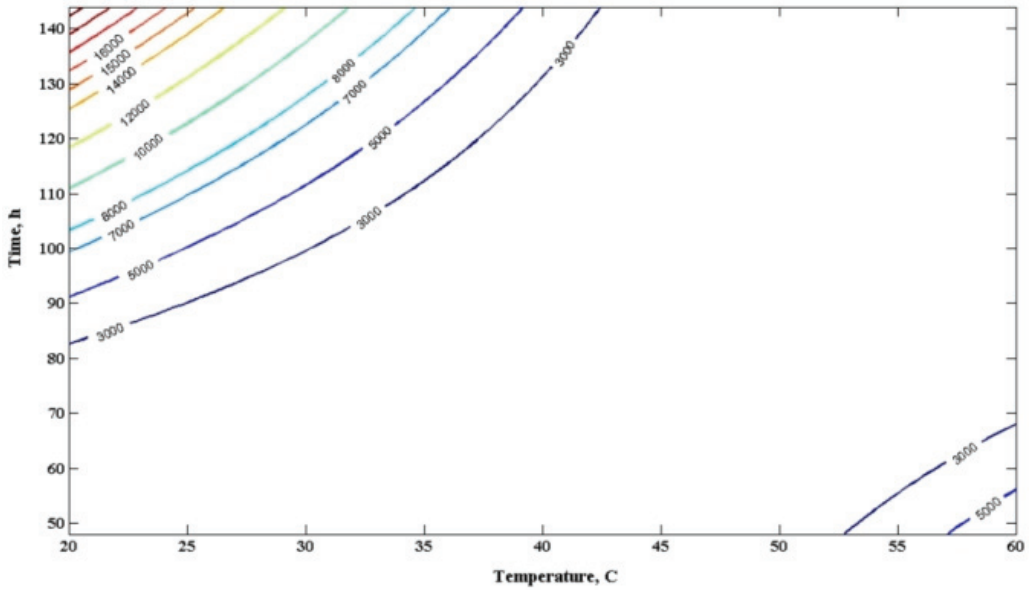

Figure 5. Isoresponse contour plot for lipase activity.

interesting for several biotechnological applications. Although an efficient producer of proteolytic and lypolytic enzymes, this strain was previously poorly understood as a producer of rhamnolipids, microbial surfactants characteristic for *Pseudomonas* spp. [35].

The most important finding of this research related to rhamnolipid production was that regression coefficients of highest values were those that describe interactions between factors.

Negative interaction between sunflower oil concentration and temperature is the most noticeable at high sunflower oil concentrations, when rhamnolipid concentration has steep fall with temperature increasing. On the other hand, temperature and Tween® 80 showed positive interaction, which was most evident in the fermentations with low Tween® 80 concentrations, when the decrease of temperature led to a sudden increase in rhamnolipid concentration. Negative interaction between sunflower oil and Tween® 80 concentrations was obvious at fermentations with high sunflower oil concentrations where the decrease of Tween® 80 concentration caused an increase in rhamnolipid production. Summarizing these findings, rhamnolipid production appeared to be stimulated with high sunflower oil concentrations, and diminished when surfactants were present in the growing medium and at high temperatures.

On the other hand, for lipase production, only temperature and incubation time were shown as significant among four tested fermentation factors.

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IZVOD

PROIZVODNJA RAMNOLIPIDA I LIPAZE IZ *Pseudomonas aeruginosa* san-ai: OPTIMIZACIJA PROCESA PRIMENOM METODE ODZIVNIH POVRŠINA

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Pseudomonas aeruginosa san-ai, izolovan je iz izrazito alkalne emulzije koja je korišćena kao mazivo u industriji pri obradi metala. Sposobnost da preživi u visoko alkalnoj sredini (pH 10) učinila je ovaj mikroorganizam veoma interesantnim za istraživanje, budući da je za preživljavanje u tako ekstremnim uslovima neophodno da mikroorganizam proizvodi enzime specifičnih karakteristika. Prethodna istraživanja su pokazala da ovaj ekstremofilni mikroorganizam ekstracelularno produkuje hidrolitičke enzime, koji zbog izuzetno atraktivnih karakteristika imaju potencijal za primenu u nizu biotehnoloških postupaka. Ipak, iako je pokazano da ovaj atraktivni soj produkuje industrijski veoma interesantne biomolekule (proteaze i lipaze), produkcija ramnolipida, jedinjenja čija oblast primene svakodnevno raste, pomoću ovog soja je malo ispitana. Ramnolipidi su amfifilna jedinjenja, koja se sastoje iz hidrofilne šećerne komponente i hidrofobne komponente koju čine β-hidroksi masne kiseline. Spadaju u grupu mikrobioloških surfaktanata ili biosurfaktanata, koji bi trebalo u budućnosti da se koriste kao zamena za sintetičke surfaktante koji nisu biodegradabilni i kao takvi predstavljaju opasnost za životnu sredinu. Sve veće interesovanje za industrijsku primenu ramnolipida, dovelo je do potrebe za optimizacijom njihove proizvodnje. Cilj ovog rada bila je optimizacija produkcije ramnolipida kao i lipaze pomoću *Pseudomonas aeruginosa* san-ai. Ispitan je uticaj četri fermentaciona faktora: koncentracije suncokretovog ulja u intervalu: 0,2-1,0 % (w/v), Tween® 80 u intervalu: 0–0,2 % (v/v), temperature: 20––60 °C i vremena trajanja fermentacije: 48–-144 h. Uticaj fermentacionih faktora na prinos navedenih metabolita ispitan je pomoću centralnog kompozitnog rotatabilnog eksperimentalnog plana, na pet nivoa vrednosti ispitivanih faktora. Analizom dobijenih regresionih koeficijenata ustanovljeno je da su vrlo izražena interaktivna dejstva nekoliko parova faktora. Kod produkcije ramnolipida, najveća je vrednost koeficijenta koji opisuje negativnu interakciju između koncentracije suncokretovog ulja i temperature, a kao bitne pokazale su se i pozitivna interakcija između koncentracije Tween® 80 i temperature, kao i negativna interakcija između koncentracija suncokretovog ulja i Tween® 80. Interesantno je da su se kod produkcije lipaze kao značajni faktori pokazali samo temperatura i vreme fermentacije. Najveći prinos ramnolipida, 138 mg dm^{-3}, postignut je pri niskoj koncentraciji Tween® 80 (0,05 %) i visokoj koncentraciji ulja (0,8 %) na 30 °C posle 72 h, dok je najveća lipolitička aktivnost, 11111 IU dm^{-3}, ostvarena pri istoj koncentraciji Tween® 80 (0,05 %) i istoj temperaturi od 30 °C, nešto nižoj koncentraciji suncokretovog ulja (0,4 %) i dužem vremenu fermentacije od 120 h.

Ključne reči: *Pseudomonas aeruginosa* • Ramnolipid • Lipaza • Metoda odzivnih površina