Research Article

MODELING AND OPTIMIZATION OF MAIN FACTORS IN RHAMNOLIPID PRODUCTION PROCESS BY PSEUDOMONAS AERUGINOSA HR

Rashedi H[1]* and M Mazaheri-Assadi[2]

[1]Department of Chemical Engineering, Faculty of Engineering, University of Tehran, P.O.Box: 11365-4563, Tehran, Iran
[2]Biotechnology Center, Iranian Research Organization for Science and Technology, Tehran, Iran

Abstract: Producing bacterium of rhamnolipid in this research is, *P. aeruginosa HR* was isolated from Iranian oil wells. First, the effect of three factors pH (6.5-7.5), Temperature (30-35°C), and Carbon/Nitrogen ratio (C/N) (24-32) on biosurfactant production were investigated using Taguchi model. Lindhard medium which is known as the best culture medium in laboratory scale was used and optimum condition (pH = 7.5, T = 33°C, C/N ratio = 24) was determined. Then, under the obtained optimum condition, in semi-pilot scale (fermenter), the effect of three important factors, C/N ratio (14-24), stirrer rate (100-400 rpm), and aeration rate (0.5-2 Vvm) was evaluated using Taguchi model. The optimum condition was as follows: C/N ratio = 18, stirrer rate = 100 rpm, aeration rate = 1.5 Vvm. Finally, the experimental data for laboratory and fermenter scales were compared with estimated values according to a mathematical model for the production of biosurfactants at this research from *P. aeruginosa*. The appropriate polynomial expansions that coincide with these data were obtained by exponential method.

Keywords: Modeling and Optimization, Biosurfactant, *Pseudomonas aeruginosa*, Taguchi model, Rhamnolipid.

INTRODUCTION

Protect the oil wells to increase their longevity is now one of the major oil-producing countries is difficult. Each year, an extraordinary global budget for research on EOR and MEOR (enhanced oil recovery methods) is allocated. In addition, the owners of these vessels extraordinary efforts to exploit new, lower cost, and highly efficient techniques. A new and promising way to increase the efficiency of oil tanks, the use of biological methods (MEOR). Biosurfactants bioemulsifiers and bioremediation processes are not only used in the oil industry, but also a great way to enhance oil recovery from wells. They may be employed to reduce the viscosity of heavy oil, clean oil storage tanks, increasing the flow through the pipeline, and stabilize water-oil emulsion fuel (Batista *et al.,* 2006; Bognolo, 1998; Makkar and Cameotra, 1997; Ghurye and Vipulanandan , 1994; Yazdian F, 2010).

It is prognosticated that is one of the most efficient methods to increase oil recovery in the future. The use of biotechnology in oil field is growing day by day. Yesterday, biotechnology experts, working with engineers trying to improve oil production from oil wells. The basic idea of the method is the use of certain microbes microbial wells to increase production.

Thought to be the active component of Biosurfactants produced by microorganisms such as bacteria, yeasts and fungi that are either attached to the cell surface or in growth medium (under the 2008 expulsion. Fleming *et al.,* 1994; Kitamoto *et al.,* 1993, grula *et al.,* 1989; Guerra to Santos *et al.,* 1983, Solanas

AM. 2004). They are amphiphilic molecules with both hydrophilic and hydrophobic regions. Hydrophobic molecules of long-chain fatty acids, alpha hydroxy acids, fatty acids and alkyl-β-hydroxy fatty. The hydrophilic portion can be a carbohydrate, amino acid, cyclic peptides, phosphates, carboxylic acid and alcohol. Since the surfactant and hydrophobic moieties like they partition preferentially at the interface between the liquid phase with different degrees of polarity and hydrogen bonding interface as oil / water or air / water (Joshi et al 2008. Koch *et al.,* 1991). The properties of surfactants to reduce surface tension and surface capabilities as well as a small form in which both hydrocarbon emulsions can be dissolved in water or water in the hydrocarbon.

Features that makes it a promising alternative to those commercial microbial surfactants are chemically synthesized lower toxicity, higher biodegradability and thus more environmentally friendly, better foaming properties (useful in mineral processing), and more stable activity at extremes of pH, salinity and temperature. Unlike chemical surfactants, which are mostly derived from petroleum feedstock, these molecules can be produced by microbial fermentation processes using cheaper agro based substrates and waste materials (Das *et al.,* 2009; Joshi *et al.,* 2008; Bodour *et al.,* 2004; Fox and Bala, 2000; Bodour and Miller-Maier, 1998; Lepo *et al.,* 1997; Mulligan and Gibbs, 1990).

*Corresponding Author:

Dr. Hamid Rashedi,
Department of Chemical Engineering,
Faculty of Engineering, University of Tehran ,
P.O.Box: 11365-4563, Tehran, Iran.

Multiplicity of biosurfactants makes them an interesting group of materials applicable in many areas such as agriculture, textiles, petrochemicals, healthcare, food, health care, the use of waste and environmental pollution such as hydrocarbons degradation in soil. Thus, microorganisms that produce biosurfactants great candidate to study on (Desai and Desai, 1993; Mulligan and Gibbs, 1993; Guerra-Santos *et al.,* 1983, Benincasa M. 2007).

The rhamnolipids produced by Pseudomonas strains among a lot of attention because tensioactive and emulsification properties (Mulligan and Gibbs, 1990 Soberon Chavez *et al.,* 2005, Maier R.M. 2004) have received considerable attention. Pseudomonas species is well known for its ability to produce biosurfactants rhamnolipid, with potential surface properties of activated carbon when on different substrates (Mercade and Manresa, 1994, Patel and Desai, 1997, Mukherjee AK, 2007) has been developed. Therefore, they are promising candidates for large-scale production of biosurfactants. Large-scale biosurfactant production costs can be achieved by optimizing the process (Kanha and Leite, 1997 Long, 2002) is reduced. Culture media, carbon source, and growth conditions (pH, temperature, nutrients and trace elements of the restricting) the types and functions of biosurfactants that are generated affect (Lang, 2002, Kuyukina *et al.,* 2001; Makkar and Cameotra, 1998; Fiebig *et al.,* 1997; Ghurye and Vipulanandan, 1994). Add a layer of immiscible medium (for example, hydrocarbons and fatty acids) has been reported to induce the synthesis of biosurfactant (Batista *et al.,* 2006; Makkar and Cameotra, 1998).

Earlier research has biosurfactant produced by the authors (Rashed *et al.,* 2006 A, B, C, 2005, b) investigated in this study, the effects of pH, temperature, T, and C / N ratio using Lindhard models in the Taguchi is known as the best medium was studied at laboratory scale. And then, under favorable conditions, obtained by the other three major causes of agitation rate, aeration rate, and C / N ratio using Taguchi model in pilot-scale (fermenter) was obtained. Finally, the experimental data with estimated values according to a mathematical model for the production of biosurfactants by *Pseudomonas aeruginosa* were evaluated both in laboratory scale fermenter. Polynomial expansion for the same data were obtained using the exponential method. Taguchi model has been studied elsewhere (Joshi et al 2008, Lai *et al.,* 2007; Sen and Swaminathan, 2004).

MATERIAL AND METHODS
Microorganisms:
The microorganism previously was isolated from south of Iran oil wells. This bacterium was designated as *Pseudomonas aeruginosa HR* (Rashedi *et al.,* 2006 b).

The bacterium was maintained at 4°C on nutrient agar slants and sub cultured once every two weeks.

Growth conditions and Media composition
Pseudomonas aeruginosa HR was cultivated at 30°C for 20hr at 200 rpm in a 250ml Erlenmyer flask containing 50 ml of seed medium: Nutrient Broth, pH 7.0.

Seed culture (2% v/v, OD_{610} = 1.0) was added into 1000 ml Erlenmyer flasks, each containing 200 ml of basal fermentation medium consist of different percentages of Lindhard medium as carbon source with composition of (g/l) $NaNO_3$(15), KH_2PO_4 (3.4), K_2HPO_4 (4.4), $MgSO_4.7H_2O$ (0.5), NaCl (1.1), KCl(1.1), Yeast extract (0.5), $FeSO_4.7H_2O$ (0.00028), Corn oil (60), D.W (1000ml), $ZnSO_4.7H_2O$ (0.29), $CaCl_2.2H_2O$ (0.24), $CuSO_4.5H_2O$ (0.25), $MnSO_4.7H_2O$(0.17). Laboratory experiments were carried out in rotary shakers (Kottermann, Germany) under different conditions of pH (6.5, 7, 7.5), T (30, 33, 35°C), and C/N ratio (24, 28, 32) at constant stirrer rate of 200 rpm. Next, to conduct experiments in semi-pilot scale, a 20 lit fermenter of impeller type was used. Each of three impellers has 4 flat blades. Its working volume was 15 lit. The experiments were carried out under different conditions of C/N ratio (14, 18, 24), stirrer rate (100, 300, 400) and aeration rate (0.5, 1.5, 2).

In this study, 96hr and 168 hr duration have been chosen for cultivating in shaker flasks and in fermenter, respectively.

Biomass determination:
Bacterial cells were removed from surfactant containing medium by centrifugation (J2-21M Beckman, U.S.A) at 10000 rpm for 15 min at 4°C. Then, the cells were harvested and dried in oven at 105°C overnight. Finally, they were weighed with a balance (Mettler H-16).

Biosurfactant extraction:
Supernatant obtained more acidic, with pH 2.0 using 6 M hydrochloric acid solution was treated. Acid supernatant overnight at 4° C precipitation is full of biosurfactant (Yakimov *et al.,* 1996) remains. After centrifugation, the precipitate was dissolved in 0. 1 M sodium bicarbonate solution, followed by a solvent extraction process biosurfactant with a 2:1 ratio of chloroform to ethanol at room temperature (Zhang and Miller, 1992). Lower organic phase into a round-bottom flask connected to a rotary evaporator to remove the solvent, the yield of biosurfactant sticky honey-colored moved.

Quantification of rhamnose:
The quantification of rhamnolipid expressed in rhamnose (g/l) was measured in the cell-free culture

medium, using the phenol sulfuric acid method (Chandrasekaran and Bemiller, 1980).

Measurement of emulsification activity:

The method was according to Cooper and Goldenberg (Cooper and Goldenberg, 1987). The emulsion stability was determined after 24 hr. The emulsion index (E24) was calculated by measuring the emulsion layer formed at temperature 25-30°C (all tests were done in triplicate). The results were expressed as percentages of the emulsion layer.

RESULTS AND DISCUSSION

Laboratory scale:

Optimization of rhamnolipid production: At this stage, by changing 3 factors (temperature, pH, and C/N ratio) in various three levels, rate of rhamnose produced in 3-level experiments was determined by the use of phenol-sulfuric acid method in 96 hr.

The obtained results showed that highest value of rhamnose produced (corresponding to 8.8 g/l) was when the temperature, pH, and C/N ratio were 33°C, 7.5, and 24, respectively, while the lowest value (corresponding to 4.2 g/l) was when the temperature, pH, and C/N ratio were 30°C, 6.5, and 24, respectively. The rates of rhamnose produced in other conditions have been represented in Table 1.

Table.1: Rate of rhamnose produced in various 3-level experiments of Taguchi model in laboratory scale

Test No.	Temperature (°C)	pH	C/N ratio	Rate of rhamnose (g/l)	E24 (%)	Dry weight (g/l)
1	30	6.5	24	4.2	58	3.23
2	30	7	28	4.8	62	3.73
3	30	7.5	32	6.2	70	4.89
4	33	6.5	28	5.6	68	4.39
5	33	7	32	6.2	70	4.89
6	33	7.5	24	8.8	88	7.05
7	35	6.5	32	6.2	70	4.89
8	35	7	24	7.2	76	5.72
9	35	7.5	28	7.5	82	5.97

The highest value (88%) and the lowest value (58%) of E24 are related to the test number 6 and 1, respectively. Maximum and minimum values of dried biomass were obtained as 7.05 and 3.23.
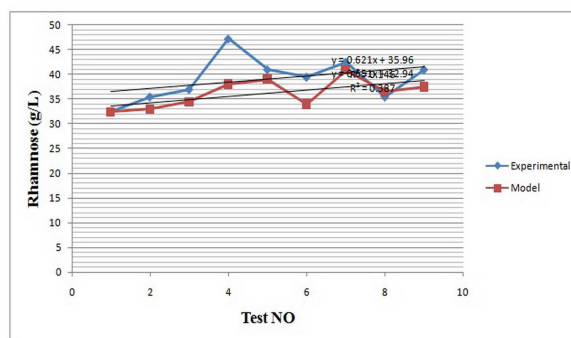

Figure 6. Comparison of the produced rhamnose between the experimental design and predicted values by the model in semi-pilot scale
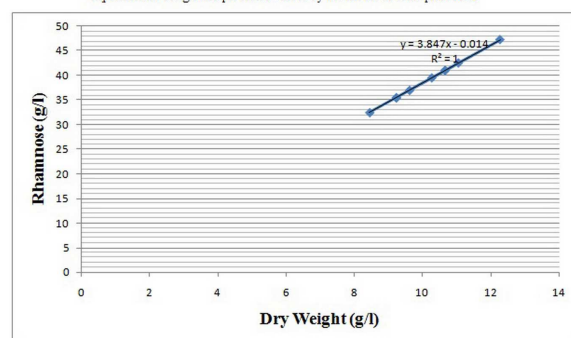

Figure 5. The correlation between the produced rhamnose and dry weight (biomass) in semi-pilot scale
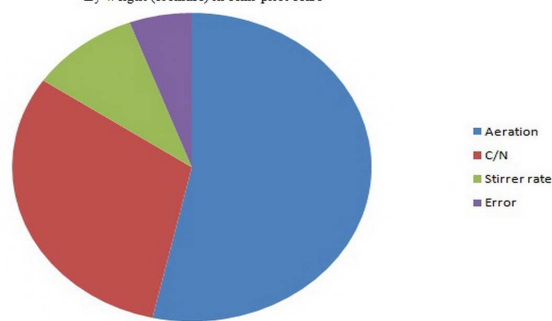

Figure 4. The effect of main factors on biosurfactant production in semi-pilot scale
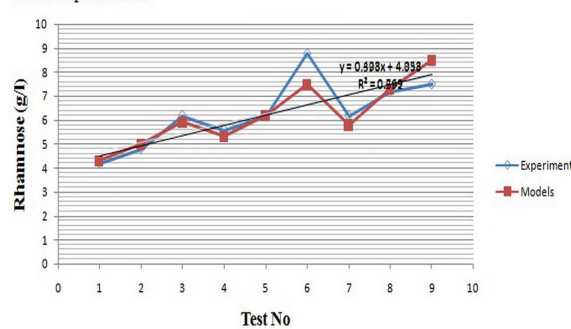

Figure 3. Comparison of the produced rhamnose between the experimental design and predicted values by the model in laboratory scale
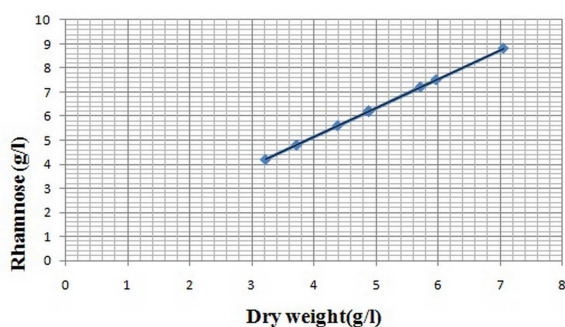
Figure 2. The correlation between the produced rhamnose and dry weight (biomass in laboratory scale
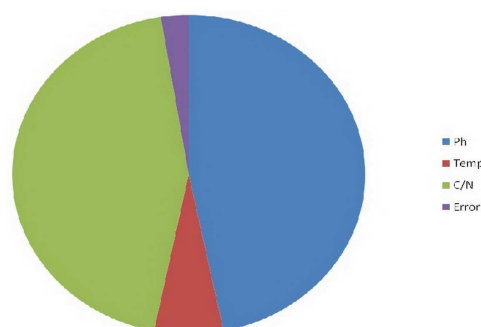

Figure 1. The effect of main factors on biosurfactant production in laboratory scale

The effect of main factors on biosurfactant production and the correlation between the produced rhamnose and dry weight in laboratory scale have been shown in Figures 1 and 2, respectively.

Table.2: Rate of rhamnose produced in various 3-level experiments of Taguchi model in semi-pilot scale

Test No.	Aeration rate (Vvm)	Stirrer rate (rpm)	C/N ratio	Rate of rhamnose (g/l)	E24 (%)	Dry weight (g/l)
1	0.5	100	14	32.5	80.6	8.45
2	0.5	300	18	35.5	87.4	9.23
3	0.5	400	24	37	90.2	9.62
4	1.5	100	18	47.2	98.2	12.27
5	1.5	300	24	41	92.3	10.66
6	1.5	400	14	39.5	87.6	10.27
7	2	100	24	42.5	94.4	11.05
8	2	300	14	35.5	87.4	9.23
9	2	400	18	41	92.3	10.66

By studying the effect of main factors on rhamnose production according to Taguchi model, the following results were obtained:

1. pH has the most effect on the rhamnose production (about 46 ± 2 %).
2. Temperature has the next important role on the rhamnose production (about 43 ± 2 %).
3. C/N ratio has the least important effect on the rhamnose production (about 8 ± 2 %).

Experimental relationship for produced biosurfactant:
To compare experimental data with theoretical values, a model has been proposed in which all parameters are variables, and its precision is determined by an error number. The appropriate model is (Cooper and Goldenberg, 1987):

$$F_1 (\Pi_1, \Pi_2, \ldots, \Pi_{n-m}) = 0 \qquad (1)$$
$$P = a + (pH)^b + (Vvm)^c + (C/N)^d + (T)^e + (speed)^f \quad (2)$$

Where P denotes the rate of production, a, b, c, d, and e are constants of equation, pH is the effect of acidity, Vvm refers to the effect of aeration, C/N is the effect of carbon to nitrogen ratio, T is the effect of temperature, and Speed denotes the effect of stirrer rate.

To formulate the effective parameters in production, the maximum values of experimental data have been used in each case, and the appropriate polynomial expansions that coincide with these data have been obtained by exponential method. The range of validity of each relation has been mentioned together with its maximum error.

At this stage of experiments, by changing three factors pH, T, and C/N ratio at various three levels, the amount of produced rhamnose after 96hr was assessed by the use of phenol-sulfuric method. The obtained results (Table 1) have been evaluated and the parameters were determined.

$$\ln P = \ln a + b \ln pH + c \ln Vvm + d \ln C/N + e \ln T + f \ln speed \qquad (3)$$

By substituting the experimental data in the equation (3), the model has been solved for amount of production. Therefore, we have obtained:

ln a = - 10.5284; b = 2.4524; d = - 0.1438; e = 2.3143; Maximum error = 0.1278

The resulting model read as:

$$\ln p = - 10.5284 + 2.4524 \ln pH - 0.1438 \ln C/N + 2.3143 \ln T \quad (4)$$

Comparison of the produced rhamnose between the experimental design and predicted values by the model is illustrated in Fig. 3.

Semi-pilot scale (Fermenter):

Optimization of rhamnolipid production: After obtaining the results from laboratory experiments and determining the best condition for production of biosurfactant, other parameters were evaluated in the fermenter. At this stage, by changing 3 factors (C/N ratio, aeration rate, and stirrer rate) in various three levels, rate of rhamnose produced in 3-level experiments was determined by the use of phenol-sulfuric acid method in 168 hr.

Results showed that the highest value of rhamnose production (corresponding to 47.2 g/l) obtained when C/N ratio, stirrer rate, and aeration rate were 18, 100, and 1.5 Vvm, respectively, while the lowest value (corresponding to 32.5 g/l) obtained when C/N ratio, stirrer rate, and aeration rate were 14, 100, and 0.5 Vvm, respectively. The rates of rhamnose produced in other conditions have been represented in Table 2.

The highest value (92.3%) and the lowest value (80.6%) of E24 are related to the test number 4 and 1, respectively. The obtained maximum and minimum values of dry weight were 3.4 and 2.45, respectively.

The effect of main factors on biosurfactant production and the correlation between the produced rhamnose and dry weight in semi-pilot scale has been shown in Figures 4 and 5, respectively.

By studying the effect of three factors on rhamnose production according to Taguchi model, the following results were obtained:

1. Aeration rate has main effect on rhamnose production (about 55 ± 6 %).

2. C/N ratio has the next important role on rhamnose production (about 28 ± 6 %).

3. Stirrer rate has the least important effect on rhamnose production (about 11 ± 6 %).

Yield of rhamnose production, Yx/s, Yp/s have been proposed in Table 3.

Experimental relationship for produced biosurfactant:

At this stage of experiments by changing three factors aeration rate, stirrer rate, and C/N ratio at various three levels, the amount of produced rhamnose after 168hr was assessed by the use of phenol-sulfuric method. The obtained results (Table 2)

have been evaluated and the parameters of eq. (3) are determined.

By substituting the experimental data in eq. (3), the model has been solved for the amount of production. Therefore, we have obtained:

ln a = 3.2188; c = 0.1099; d = 0.1693; f = - 0.0206; Maximum error = 0.1454

The resulting model is as:

ln p = 3.2188 + 0.1099 ln pH + 0.1693 ln C/N - 0.0206 ln T (5)

Comparison of the produced rhamnose between the experimental design and predicted values by the model is illustrated in Fig. 6.

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