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Optimization of Parameters of Biosynthesis of Surface-Active Rhamnolipids by the Strain *Pseudomonas* sp. PS-17 in the Bioreactor with Injection-Vortex Aeration System

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Abstract The conditions of the cultivation of the strain *Pseudomonas* sp. PS- 17 - the producer of surfactants of rhamnolipid nature in the bioreactor with injection-vortex aeration system were studied. The optimal values of some parameters of rhamnolipid biosynthesis were determined: dissolved oxygen concentration, medium pH, as well as the optimum method of inoculum preparation. The efficiency of the use of two-stage temperature control for biosurfactant synthesis process was shown, which increased the accumulation of the product by 14% (from 12,1 to 13,8 g/dm3) and reduced the total cultivation duration on 22 % (from 90 to 70 h).

Keywords Rhamnolipid surfactants, Biosynthesis, *Pseudomonas*, Bioreactor, Cultivation parameters

1. Introduction

With the development of biotechnology a special attention is drawn to the study of biogenic surfactants (biosurfactants) – products of microbial synthesis. Biogenic surfactants have significant advantages over synthetic ones: they are highly efficient, but at the same time, non-toxic, non-allergenic and biodegradable. The practical significance of microbial surfactants is stipulated by their ability to significantly reduce the surface and interfacial tension of aqueous solutions, their emulsifying, foaming properties and environmental safety[1]. Inexpensive substrates, including waste production can be used for the synthesis of microbial surfactants[2]. Despite the fact that biosurfactants are a relatively new biotechnology product, the expediency of their use in the oil, paper, wood and paper, food, textile, perfume and cosmetics, pharmaceutical industries as well as environmental protection and agriculture was grounded[3].

One of the most promising classes of biosurfactants are rhamnolipids – products of biosynthesis of bacteria of the genus *Pseudomonas*[4]. Currently the technologis of rhamnolipid products are not widely used in industry, primarily due to the low yield of the final product and excessive foaming during fermentation. But given the current world trends of transition to environmentally friendly products, the development of industrial technology of biosynthesis of rhamnolipids is especially topical. Development of an efficient technology of synthesis of surface-active rhamnolipids can be realized primarily as a result of the determination of optimal conditions for culturing the strains-producers and application of new energy-efficient approaches to the process of biosynthesis and the isolation of target products[5].

Production of biosurfactants depends on many factors: the effectiveness of the producer strain, culture medium composition, temperature, pH, aeration, duration of fermentation, bioreactor type and method of cultivation. Previously it was shown that a strain *Pseudomonas* sp. PS-17 is a promising producer of rhamnolipid surfactants[6]. The optimum composition of the nutrient medium for biosynthesis of rhamnolipids was determined[7]. It is shown that to achieve high yield of rhamnolipids the most effective construction of bioreactor is the one with injection-vortex aeration system, which also allows the control of the foaming process in the biosynthesis of surfactants[8]. In this connection, the aim of this work was to develop an effective technology of biosynthesis of rhamnolipids by the strain *Pseudomonas* sp. PS- 17 in a laboratory fermenter - namely,

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the determination of the optimum parameters of aeration, temperature, pH and inoculum.

2. Materials and Methods

The bacterial strain *Pseudomonas* sp. PS-17 – producer of extracellular surfactants – rhamnolipids from the Collection of Cultures of Department of Physico-Chemistry of Combustible Minerals L.M. Lytvynenko In POCC NAS of Ukraine was used for studies. Cultivation of the strain was performed in the nutrient medium with the following composition (g/dm^3): glycerol – 30, sodium citrate – 4,0; $NaNO_3$–3,0; $K_2HPO_4 \times 3H_2O$–2,0; KH_2PO_4–1,2; $MgSO_4 \times 7H_2O$ – 0,5[7].

The fermentation was carried out in the laboratory bioreactor with injection-vortex aeration system and volume of 5 dm^3. Sterilization of the fermenter was performed with nutrient medium for 45 min at 125ºC.

Cultivation was carried out at medium pH 6, 7, 8, 9 or without pH adjustment. Maintainance of the desired pH value was performed via the addition of 1 N solutions of NaOH and HCI. Fermentation temperature – 26, 28, 30, 32, 34, 36ºC – was maintained via external thermostating coil in the shell of the reactor.

The following parameters were controlled in the process of the fermentation: biomass amount, concentration of rhamnolipids, pH of the medium, temperature, dissolved oxygen content in the culture liquid. The values of pH, temperature and dissolved oxygen concentration were determined using an automatic analyzer “Expert-001-4.0.1” (LLC “Ekonyks-Expert”, Russia) in real time conditions.

Aeration of the culture medium was carried out using a compressor (SERA air550R, “Sera”, Germany) at the speed of 1 m^3/m^3·min, which was controlled by a laboratory rotameter. The concentration of dissolved oxygen was controlled by adjusting the velocity of culture fluid flow through the injector using a peristaltic pump.

The amount of bacterial biomass was determined by optical density on a spectrophotometer UVmini - 1240 ("Shimadzu", Japan) at a wavelength of 540 nm in cuvettes 5 mm[9].

Rhamnolipids concentration in the culture liquid was determined with orcinol method using a spectrophotometer UVmini - 1240 ("Shimadzu", Japan)[10].

The biomass productivity Yp/x for rhamnolipids was defined as the ratio of rhamnolipid output (g/dm^3) to a biomass concentration (g ODB/dm^3) and expressed in g/g ODB.

Output of surfactants from the substrate was determined as the ratio of rhamnolipid concentration in culture liquid (g/dm^3) and substrate concentration (g/dm^3) and expressed as g/g of substrate.

3. Results and Discussion

In our previous studies it was shown that the most effective bioreactor for the cultivation of the strain *Pseudomonas* sp. PS-17 is a fermenter with injection-vortex aeration system. It was shown that for the effective synthesis of the target products it is necessary to ensure the supply of oxygen into medium in amount Ks = 1 kg O_2/(m^3·h). Such mass transfer conditions are provided in the fermenter with injection-vortex aeration system, which is a reactor without reflecting walls with bottom-drive turbine stirrer equipped with an external circulation loop with injector for supplying air[8]. At that, only the rate of oxygen saturation of the culture medium was taken into account, and the change in the concentration of dissolved oxygen in the cultivation has not been studied. In fact, the latter parameter is important because it allows the control of aeration efficiency and prevention of the inhibition of the growth of culture and synthesis of the target product. Therefore, our objective was to study the changes in the concentration of dissolved oxygen in the culture medium during fermentation of the strain *Pseudomonas* sp. PS- 17. The dynamics of dissolved oxygen content when culturing the strain in a fermenter with injection-vortex aeration system is shown in Figure 1.

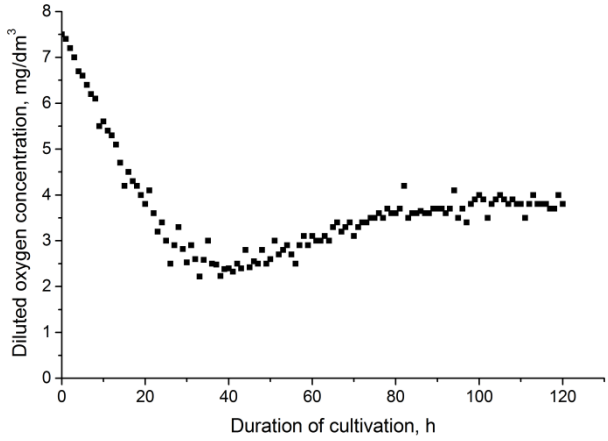

Figure 1. Change in the concentration of dissolved oxygen in culture medium when cultivating the strain *Pseudomonas* sp. PS-17 in injection-vortex bioreactor

The changes in the concentration of dissolved oxygen in the medium can be explained by the fact that it is used for accumulation of biomass and respiration, and the oxygen demand varies in the process of fermentation depending on the phase of growth.

The design of injection-vortex bioreactor allows the maintenance of a constant dissolved oxygen concentration in the medium by changing the velocity of passing of culture liquid through the injector[8]. We investigated the process of cultivation of the strain *Pseudomonas* sp. PS-17 with three constant values of dissolved oxygen in the medium. This indicator during the process of fermentation was maintained at 2, 4 and 6 dm^3, which corresponded to 26, 53 and 79% of saturation for the given conditions. Dependences of biomass accumulation and rhamnolipid synthesis on the concentration of dissolved oxygen are shown in Fig. 2.

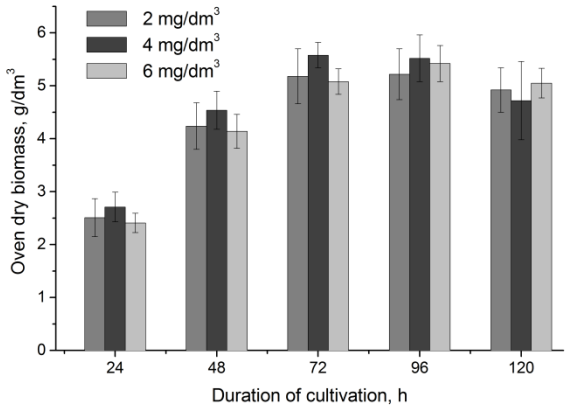

Figure 2. The growth of the strain *Pseudomonas* sp. PS-17 (a) and accumulation of rhamnolipids (b) depending on the concentration of dissolved oxygen in the medium

Table 1. Effect of the duration of cultivation and inoculum amount on growth and synthesis of surfactants by the strain *Pseudomonas* sp. PS-17

Inoculum amount, % v.	Duration of inoculum preparation, h	Maximum oven dry biomass, g/dm^3	Rhamnolipids, g/dm^3	Cultivation duration[1], h
5	12	3,1±0,2	7,4±0,2	100
	24	4,1±0,1	10,1±0,1	100
	36	4,2±0,1	10,6±0,2	90
7	12	3,9±0,1	10,8±0,2	90
	24	4,4±0,1	12,2±0,2	90
	36	4,6±0,1	13,0±0,2	80
10	12	4,3±0,2	11,5±0,2	90
	24	4,6±0,1	12,4±0,3	80
	36	4,8±0,1	13,0±0,2	80
15	12	4,7±0,2	11,8±0,2	90
	24	5,0±0,2	12,6±0,3	80
	36	5,2±0,2	13,1±0,3	80

Thus, the biomass accumulation and synthesis of rhamnolipids by the strain Pseudomonas sp. PS-17 is almost independent of the oxygen concentration in the medium within 2-6 dm^3 (25-80% of saturation).

Duration of cultivation and the amount of inoculum also play an important role in the fermentation process[11]. Therefore, the next task was to determine the optimal values of these parameters. Inoculum was grown for 12, 24 or 36 hours and supplemented in an amount of 5, 7, 10 and 15% in the culture medium for the synthesis of rhamnolipids.

Note. 1 – time of achievement of maximum accumulation of rhamnolipids

The obtained results (Table 1) show that the duration of the lag phase depends strongly on the method of inoculum preparation. The introduction of inoculum from late exponential phase (36h, viable titer - 2×10^8 CFU/cm^3) reduced the duration of the lag phase, increased the accumulation of the final product and reduced the duration of its synthesis. The amount of inoculum also influenced the cultivation parameters of the strain-producer. Maximum surfactant- synthesizing ability of the strain *Pseudomonas* sp. PS-17 (2,83 g/g) was achieved when using inoculum in the amount of 7% (viable titer - 2×10^8 CFU/sm^3).

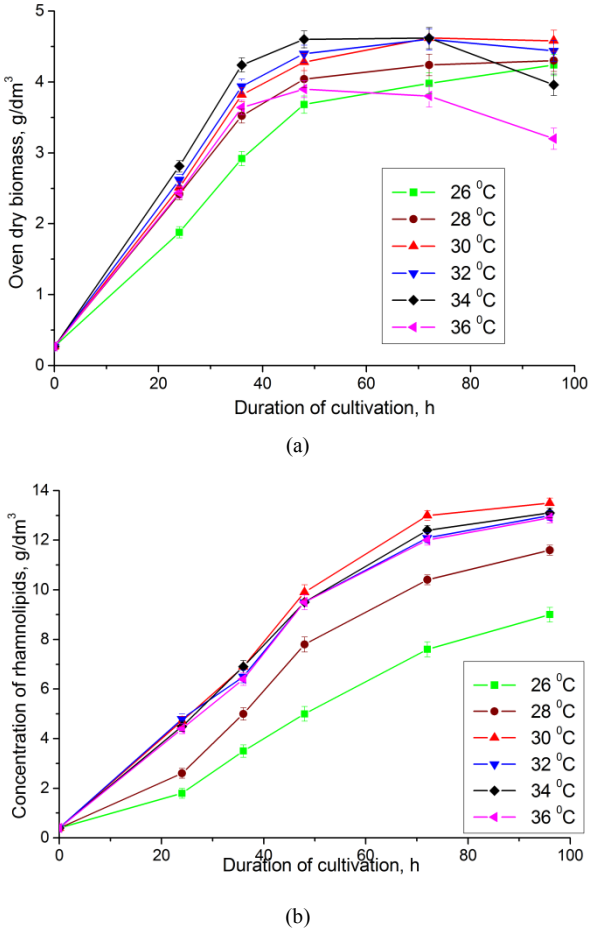

Figure 3. Dynamics of growth (a) and accumulation of rhamnolipids (b) by the strain *Pseudomonas* sp. PS-17 at different temperatures of cultivation

Therefore, the optimal method of inoculum introduction for the biosynthesis of rhamnolipids by the strain *Pseudomonas* sp. PS-17 can be considered the introduction of inoculum (36h of cultivation, viable titer – 2×10^8 CFU/cm^3) in the amount of 7% (v/v). Under these conditions, the maximum accumulation of product made 13,0 g/dm^3, fermentation time – 80 h.

Previously it was determined that the optimum

temperature for the synthesis of biosurfactants by the strain *Pseudomonas* sp. PS-17 is 30°C[6]. Given that the temperature optimum for the accumulation of biomass and synthesis of rhamnolipids may differ the dynamics of the process at different temperatures was investigated (Fig. 3). It was shown that the optimum temperature for biomass accumulation is 34°C, for the synthesis of surfactants – 30°C.

It was determined that in the conditions of two-stage temperature regime the first 36 h of fermentation should be performed at a temperature of 34°C, and then the temperature should be reduced to 30°C (Fig. 4). When the temperature was lowered the culture passed to the stationary phase of growth, which contributed to the intensification of synthesis of rhamnolipids, the maximum accumulation of which was reached on the 70th hour – 13,8 g/dm^3. The surfactant output from substrate and surfactant-synthesizing ability of biomass under such cultivation conditions were $Y_{p/s}$ = 0,484 g/g and $Y_{p/x}$ = 3,000 g/g, respectively.

Thus, the two-stage temperature regime promoted the maximum accumulation of target product by the culture on 6,2 % while reducing the cultivation duration on 12,5% (70 h).

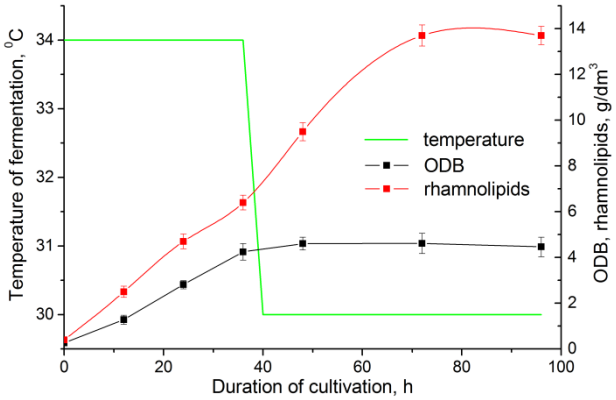

Figure 4. The growth and synthesis of biosurfactants by the strain of *Pseudomonas* sp. PS-17 when applying two-stage temperature regime

The process of the cultivation of the strain *Pseudomonas* sp. PS-17 was studied at different values of medium pH. Greatest biomass accumulation was observed when maintaining the culture medium pH at 7,0, but at the same time the surfactant-synthesizing ability of the culture and the product accumulation decreased while the fermentation duration increased. When reducing the medium pH to 6,0 all values of fermentation decreased if compared to those at pH 7,0. Increasing the pH to 8.0 led to a decrease in biomass growth, but at the same time the surfactant-synthesizing ability of the culture and maximum accumulation of product increased and the duration of the process was reduced. Cultivation of strain at pH 9,0 promoted the reduction of output of the product and increase of the fermentation duration. Interestingly, that the controlled fermentation at all studied pH values of the culture medium has shown the decline in the accumulation of the product and the surfactant-synthesizing ability of the culture if compared with the cultivation without the adjustment of medium pH.

Thus, to obtain the inoculum with maximum biomass concentration the process should be performed at pH 7,0. At the same time, for the maximum accumulation of the product the optimum medium pH was 8,2-8,4, which is achieved without pH adjustment during the fermentation.

4. Conclusions

It was determined, that biomass accumulation and synthesis of rhamnolipids by the strain *Pseudomonas* sp. PS-17 in the injection-vortex fermenter doesn't depend on the dissolved oxygen content in culture liquid if the latter parameter is in the range 2-6 mg/dm^3.

It was shown that for the production of inoculum optimum it is reasonable to cultivate the strain for 36 h at 34°C at pH 7,0. Optimum concentration of inoculum – 7% vol. (viable titer - 2×10^8 CFU/cm^3) in medium.

The efficiency of the application of two-stage temperature regime for the process of biosurfactant synthesis was shown: for the first 36 h temperature was set to 34°C, then (during 4 h) – a gradual decrease in temperature to 30°C. This mode of fermentation allowed the increase of culture productivity and accumulation of the product to 13,8 g/dm^3, as well as reduction of the process duration on 12,5% (to 70 h).

It was determined that the maximum accumulation of rhamnolipids by the strain *Pseudomonas* sp. PS-17 is achieved without pH adjustment (pH 8,2-8,4).

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