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RHAMNOLIPID PRODUCTION BY *Pseudomonas aeruginosa* ENGINEERED WITH THE *Vitreoscilla* HEMOGLOBIN GENE

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The potential of *Pseudomonas aeruginosa* expressing the *Vitreoscilla* hemoglobin gene (*vgb*) for rhamnolipid production was studied. *P. aeruginosa* (NRRL B-771) and its transposon mediated *vgb* transferred recombinant strain, PaJC, were used in the research. The optimization of rhamnolipid production was carried out in the different conditions of cultivation (agitation rate, the composition of culture medium and temperature) in a time-course manner. The nutrient source, especially the carbon type, had a dramatic effect on rhamnolipid production. The PaJC strain and the wild type cells of *P. aeruginosa* started producing biosurfactant at the stationary phase and its concentration reached maximum at 24 h (838 mg/l^{-1}) and at 72 h (751 mg l^{-1}) of the incubation respectively. Rhamnolipid production was optimal in batch cultures when the temperature and agitation rate were controlled at 30°C and 100 rpm. It reached 8373 mg l^{-1} when the PaJC cells were grown in 1.0% glucose supplemented minimal media. Genetic engineering of biosurfactant producing strains with *vgb* may be an effective method to increase its production.

Microbial surfactants have many advantages over the chemical analogs. Lower toxicity for organisms and environment, biodegradability, high selectivity and specific activity at extreme conditions are just a few to mention [1–2]. Further, the ability of microorganisms to synthesize these compounds from renewable feedstock makes biosurfactants economically comparable to chemical synthesis. Biosurfactants are of increasing industrial interest because of their broad range of potential applications, including emulsification, wetting, phase separation, and viscosity reduction. They belong to a group of secondary metabolites with surface active properties and are synthesized by a great variety of microorganisms. These metabolites are complex amphiphilic molecules whose hydrophobic and polar domains depend on the carbon substrate and the type of microorganism used [3]. A large variety of microbial surface active compounds are produced by bacteria, yeasts, and fungi [4, 5]. Rhamnolipids are the most effective among them today [6]. Many potential applications of these compounds have been described. They have been shown to exhibit antimicrobial activity against competing microorganisms, to be ejective in biological control of zoosporic phytopathogens, and to facilitate the removal of heavy metals from soil [7]. The type and proportion of the rhamnolipids produced depends on the bacterial strain, the carbon source used and the culture conditions. The genus *Pseudomonas* is capable of using different substrates, such as glycerol, mannitol, fructose and glucose, to produce rhamnolipid type biosurfactants [8–10]. *Pseudomonas aeruginosa* is an environmental bacterium that can be isolated from many different habitats, including water, soil, and plants, where it survives due to its extraordinary metabolic abilities. This bacterium was shown by Jarvis and Johnson [11] to produce the biosurfactant rhamnolipids, which are amphiphilic molecules composed of a hydrophobic fatty acid moiety and a hydrophilic portion composed of one or two rhamnose molecules [12, 13]. Rhamnolipids from *P. aeruginosa* have been studied extensively. They produce two types of rhamnolipids containing two rhamnoses attached to β-hydroxydecanoic acids or one rhamnose connected to the identical fatty acid from glucose and hydrocarbon substrates. Mono- and di-rhamnolipids have quite different physicochemical properties [3, 7, 14]. Rhamnolipids from *P. aeruginosa* are glycolipid biosurfactants produced during growth phase especially in stationary phase of growth on hydrocarbons or carbohydrates as the sole carbon source [15, 16]. It was shown that their production is under quorum sensing control [17] and depends on several environmental and nutritional factors, including nitrogen and iron limitation, pH, and temperature [18]. The stimulating effect of rhamnolipid was attributed to enhanced transport of substrate to the bacteria and its inhibitory effect induced flocculation of the cells. It is known that the stimulation of many *P. aeruginosa* strains is more pronounced for rhamnolipid than for other surfactants [19].

Vitreoscilla hemoglobin (VHb) is the first well characterized prokaryotic hemoglobin. The role of VHb in bacteria is to raise the effective dissolved oxygen tension within the cells and to scavenge and release oxygen to terminal oxidases during oxygen-limited growth conditions. Bacteria engineered with the *vgb* gene had 2.0- to 10-fold higher oxygen uptake rates than the *vgb*$^-$ counterparts [20]. It has been demon-

strated that expression of bacterial hemoglobin VHb in heterologous bacterial hosts engineered by *vgb* gene often results in enhancement of cell density, oxidative metabolism, protein and antibiotic production, and bioremediation, especially under oxygen limiting conditions [21].

The aim of the study was to optimize the production of rhamnolipid by *P. aeruginosa* and its *vgb* transferred recombinant strain, PaJC in different conditions of cultivation including various carbon sources, agitation rate and temperature.

MATERIALS AND METHODS

Chemicals. L-(+)-rhamnose monohydrate was purchased from MP Chemicals (USA). Phenol, H_2SO_4 and $NaHCO_3$ were obtained from Carlo Erba Chemicals (Italy). Ethyl acetate was purchased from Riedelde Haen Chemicals (Germany). All other chemicals used were of analytical grade.

Bacterial strains. *P. aeruginosa* (NRRL B-771) and its transposon mediated *vgb* transferred recombinant strain, PaJC [22] were used in the study (PaJC strain was obtained from Illinois Institute of Technology (USA) Benjamin C. Stark, Ph.D. Professor of Biology).

Cultivation of bacteria for rhamnolipid production. For rhamnolipid production the bacteria were cultivated in LB medium (g l^{-1}): peptone – 10.0; NaCl – 10.0 and yeast extract – 5.0; or in mineral salts medium (MM) (g l^{-1}): KH_2PO_4 – 0.7; Na_2HPO_4 – 0.9; $CaCl_2$ – 0.1; $FeSO_4$ – 0.001; $NaNO_3$ – 2.0; and $MgSO_4 \cdot 7H_2O$ – 0.4 [7], both at pH 7.0. MM was supplied with selected carbon sources (glucose, sucrose and glycerol). Autoclaved separately, carbon sources were added to MM at a final concentration of 1.0%. The effect of carbon sources on rhamnolipid production was also determined in LB with 1.0% glucose (LBG). 250 μl of overnight cultures grown in 20 ml LB or carbon supplemented MM in 125 ml Erlenmeyer flasks was inoculated into 50 ml of the medium in 150 ml volume flasks. Shake-flasks were incubated at 30 or 37°C in a 100 or 200 rpm in a gyratory water-bath, drawing the samples at certain intervals 12, 24, 48, and 72 h.

Rhamnolipid extraction and assay. At selected intervals, 3 ml culture was withdrawn and centrifuged at 10000 *g* for 10 min at 4°C. The supernatant was pipetted into a new set of 10 ml plastic tubes. The pH of supernatant was adjusted to 2.0 with 1 M HCl. After adding an equal volume of ethyl acetate, the mixture was briefly vortexed and left at room temperature for 20 min. The aqueous phase (i.e., the lower phase) was discarded by dipping a pipette through the organic phase (i.e., the upper phase). The solvent organic phase was evaporated in about 4–5 h at 55°C. The dry material was dissolved in 1 ml 0.1 M $NaHCO_3$, pH 8.6 [23].

The rhamnolipid level of cell-free cultures was determined using the phenol sulfuric acid method [24]. 250 μl of mixture containing $NaHCO_3$ was pipetted into 5 ml glass tubes and after adding 250 μl of 5.0% phenol and a brief vortexing, 1.25 ml of concentrated H_2SO_4 was added, gently vortexed and left at room temperature for 20 min for color development. The color intensity of each sample was read at 480 nm against a blank (i.e., the sample missing the rhamnolipid material) and the rhamnolipid level in each sample was determined from a standard curve utilizing rhamnose.

Each value is the average of at least 3 independent experiments. For clarity, no error bars are given on the figures, but they are mostly less than 10% of the respective data point.

Rhamnolipid content was calculated by multiplying the rhamnose concentration by a factor of 3. This factor was calculated experimentally using rhamnose calibration curve, representing rhamnolipid/rhamnose correlation [25].

RESULTS AND DISCUSSION

Rhamnolipid production in various media with glucose as a carbon source – effect of temperature and agitation. As the agitation rate and temperature are two leading factors and crucial for the cell growth and rhamnolipid formation, *P. aeruginosa* and its *vgb* bearing strain (PaJC) were cultivated in shake flasks under different agitation rates (100 and 200 rpm) and temperature (30 and 37°C). The cell density in MM was significantly lower than in complex LB medium (data not shown). For the cultures grown in LB at 37°C, the agitation rates had no significant effect on rhamnolipid production (Fig. 1b), while at 30° C there was nearly 2-fold rhamnolipid increase at higher agitation rate (200 rpm) (Fig. 1a). Rhamnolipid levels (drawn as the average from the values at all incubation time points, i.e., 12, 24, 48, and 72 h) of *P. aeruginosa* and PaJC in both media, however, showed a similar trend (Figs. 1c and 2a). At 37°C and 100 rpm agitation, the average level of rhamnolipid in LBG (LB + 1% glucose) cultures were 973 (±27) and 982 (±16) mg l^{-1} for *P. aeruginosa* and PaJC, respectively. These values were 1061 (±20) and 1065 (±51) mg l^{-1} in that respect at 200 rpm agitation (Fig. 1d). The average level of rhamnolipid in MM plus 1% glucose cultures grown at 37°C was 1012 (±40) and 1060 (±34) mg l^{-1} at 100 rpm, 980 (±14) and 969 (±32) mg l^{-1} at 200 rpm agitation for *P. aeruginosa* and PaJC, respectively (Fig. 2b). The temperature effect on rhamnolipid accumulation was even more pronounced in LBG. Both strains had a substantial increase in rhamnolipid at 30°C. Compared with cultures at 37°C (Fig. 1d), there was up to 7-fold rhamnolipid increase at 30°C (Fig. 1c). Being a product of secondary metabolism, rhamnolipid production in both media started after stationary phase and generally leveled up at 48 h. In LBG cultures at 30°C under 100 rpm agitation, the average rhamnolip-

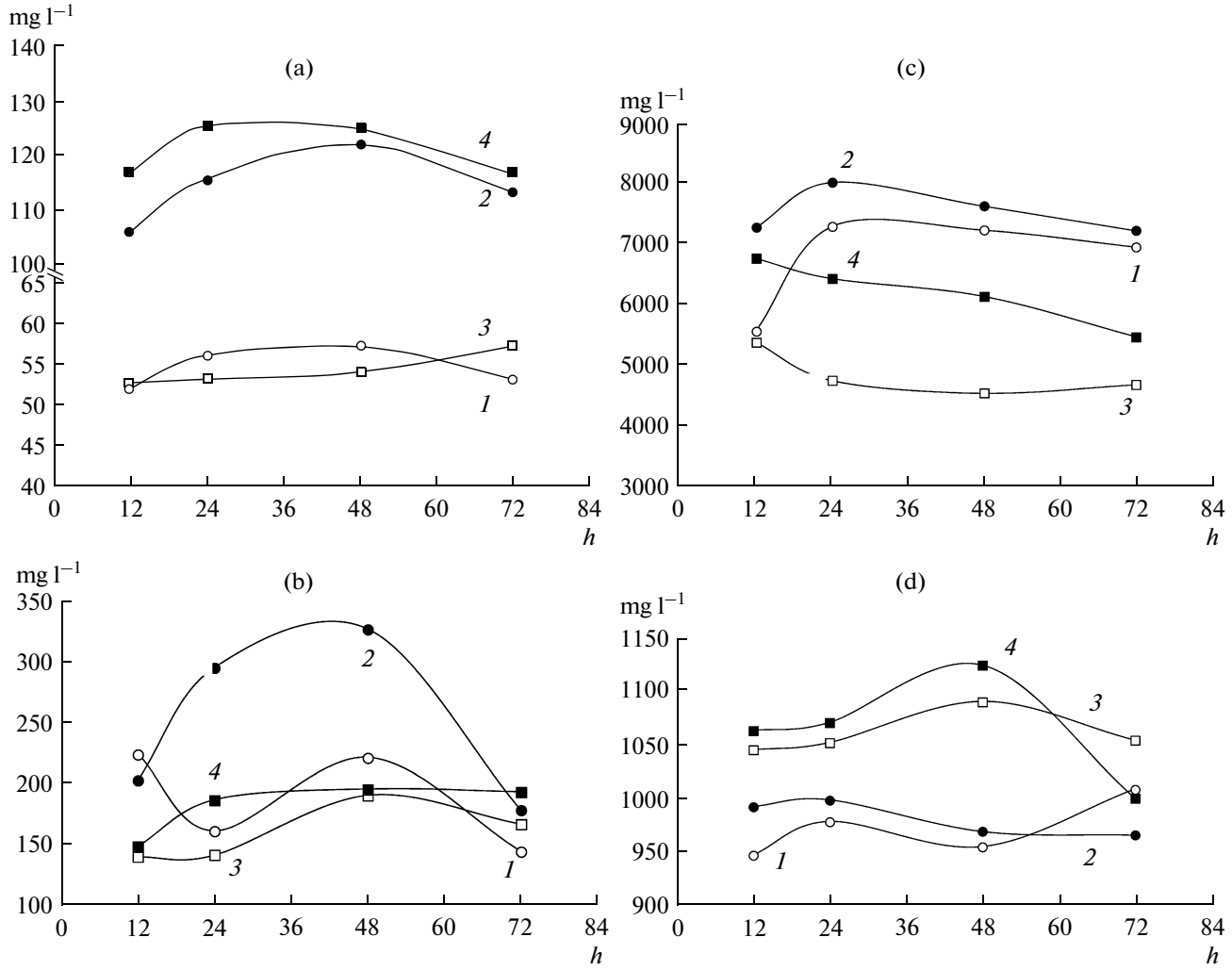

Fig. 1. Rhamnolipid levels of *P. aeruginosa* and its *vgb* recombinant strain, PaJC, under different agitation conditions at 30 (a, c) and 37°C (b, d), grown in LB (a, b) and LB supplemented 1% glucose (c, d) culture medium. *1* – *P. aeruginosa,* 100 rpm; *2* – PaJC, 100 rpm; *3* – *P. aeruginosa*, 200 rpm, *4* – PaJC, 200 rpm.

id values were 6765 (±811) and 7543 (±367) mg l^{-1} for *P. aeruginosa* and PaJC, respectively. These values were 4847 (±370) and 6207 (±551) mg l^{-1} during cultivation at 200 rpm. In MM plus 1% glucose medium at 30°C (Fig. 2a), *P. aeruginosa* showed a rhamnolipid level of 6918 (±643) and PaJC 7593 (±857) mg l^{-1} under 100 rpm, while these values were 3884 (±714) and 5827 (±781) mg l^{-1} for *P. aeruginosa* and PaJC, respectively, under 200 rpm.

Effect of other carbon sources on rhamnolipid production. Two carbon sources other than glucose (glycerol and sucrose) were also investigated for their effectiveness on rhamnolipid production. *P. aeruginosa* and PaJC in glycerol supplemented (1%) MM medium (Fig. 2c) showed an average 9.2 and 9.3-fold lower rhamnoipid values, respectively, than their counterparts in glucose supplemented medium (Fig. 2a) under similar physical conditions (i.e., 30°C and 100 rpm). At the same temperature but 200 rpm agitation, they were 3.8 and 4.0 fold in that respect. Under both agitation rates (100 and 200 rpm) the glycerol cultures grown at 37°C (Fig. 2d), however, showed similar level of rhamnolipid to the glucose cultures (Fig. 2b) under the same agitations.

Sucrose was a much better substrate for rhamnolipid production compared to glycerol. As it is apparent from Fig. 3a, the level of rhamnolipid in *P. aeruginosa* and PaJC cultures in sucrose supplemented MM medium (30°C and 100 rpm) was 5.3 and 3.5-fold higher than for the respective cultures in glycerol (Fig. 2c). At the same temperature but 200 rpm agitation, these values were 3.5 and 4.8-fold in favor of sucrose. These differences between glycerol and sucrose, however, were not observed at 37°C 100 rpm cultures, while *P. aeruginosa* and the PaJC cells showed 3.5 and 2.9-fold higher rhamnolipid levels than the corresponding cultures in glycerol at 200 rpm agitation (Figs. 3b and 2d).

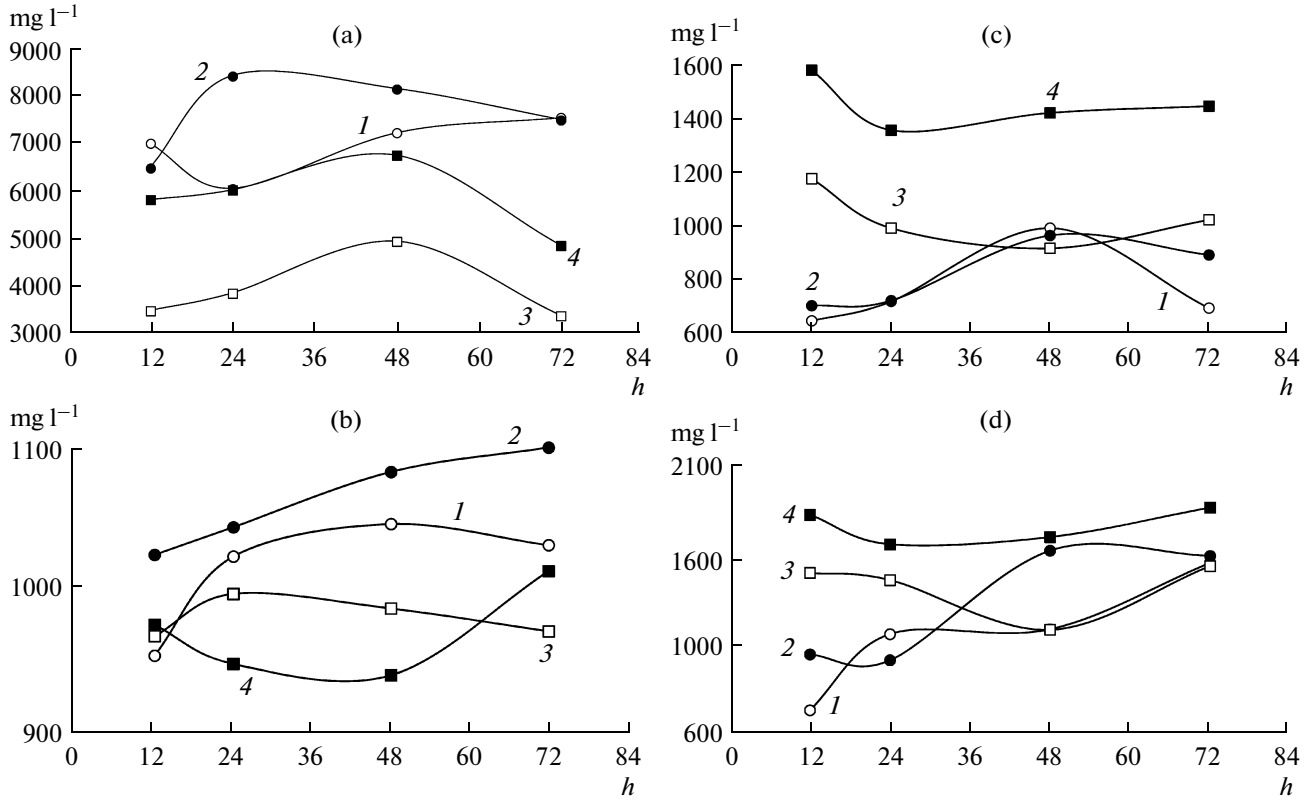

Fig. 2. Rhamnolipid levels of *P. aeruginosa* and its *vgb* recombinant strain, PaJC, under different agitation conditions at (a, c) 30°C and (b, d) 37°C, grown in MM supplemented 1% glucose (a, b) and 1% glycerol (c, d). *1* – *P. aeruginosa,* 100 rpm, *2* – PaJC, 100 rpm, *3* – *P. aeruginosa*, 200 rpm, *4* – PaJC, 200 rpm.

LB medium used since it is one of the most commonly adopted culture media for *P. aeruginosa* strains [23]. As it shown in Fig. 1c at 30°C and 100 rpm, PaJC started producing rhamnolipid at the stationary phase and the concentration of rhamnolipid reached its maximum 8373 mg l^{-1}, at the 24 h of the incubation. *P. aeruginosa*, started producing rhamnolipid at the stationary phase and the concentration of rhamnolipid reached maximum (7507 mg l^{-1}) at the 72 h of the incubation (Fig. 1c). Agitation rate affects the mass transfer efficiency of both oxygen and medium components and is considered crucial to the rhamnolipid formation of the strictly aerobic bacterium *P. aeruginosa* and its recombinant strain, especially when it was grown in a shake flask. *P. aeruginosa* has been used carbon sources such as fructose, lactic acid, glucose, mannitol, mannose and glycerol [9]. Under 37°C, the recombinant strain improved the maximum rhamnolipid level in the MM +1% sucrose, MM +1% glycerol, LB +1% glucose, respectively. At 30°C, the same advantages of the recombinant strain were rhamnolipid production in MM +1% glucose, MM +1% sucrose, and MM +1% glycerol, respectively. Therefore, it would be more economical to use 30°C in practical applications. Rhamnolipid production was optimal in batch cultures when the temperature and agitation rate were controlled at 30° C and 200 rpm, respectively [23]. Increase in agitation rate from 100 to 200 rpm indicates that elevation of dissolved oxygen level seemed to have a positive effect on rhamnolipid production. Normal aeration, however, supported the higher surfactant production of both bacterial strains than limited aeration. This is consistent with the fact that rhamnolipid production was inefficient under oxygen-limiting conditions [6]. This further supports that MM gave the higher rhamnolipid production and the difference between *vgb*-bearing and untransformed strains was greater at normal than at limited aeration. In the case of PaJC, the positive effects (rhamnolipid production) are similar or greater at normal versus limited aeration. Most rhamnolipids were found to accumulate at the stationary stage of cell growth. Their accumulation in the supernatant started at the end of the logarithmic phase because rhamnolipids are secondary metabolites.

Rhamnolipid production by bacteria grown in the medium with glucose or sucrose is much higher than that with other substrates including glycerol. It differs from the results of Monteiro et al. [26] obtained 3.9 g l^{-1} of a rhamnolipid type biosurfactant or Rashedi et al. [9] and Santa Anna et al. [8] shown the production 690 mg l^{-1} and 2650 mg l^{-1} rhamnolipid by *P. aeruginosa* grown in the medium containing 1.0% and 3.0%

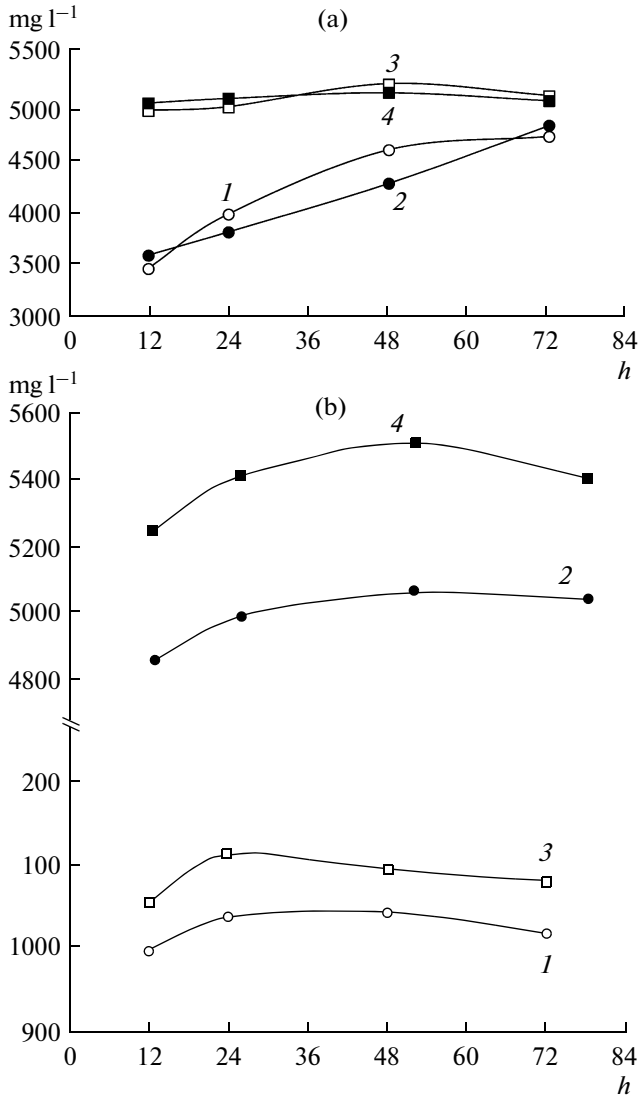

Fig. 3. Rhamnolipid levels of *P. aeruginosa* and its *vgb* recombinant strain, PaJC, under different agitation conditions at (a) 30°C and (b) 37°, grown in MM supplemented 1% sucrose. *1* – *P. aeruginosa,* 100 rpm, *2* – PaJC, 100 rpm, *3* – *P. aeruginosa*, 200 rpm, *4* – PaJC, 200 rpm.

glycerol as a carbon and energy source respectively. Besides that, Wei et al. worked with the strain *P. aeruginosa*, which produced ramnolipid (733 mg l^{-1}) when grown in LB media [23]. According to our results, *P. aeruginosa* produced rhamnolipid (326 mg l^{-1}) when grown in LB media. The poor performance of the rich media (LB) on rhamnolipid production may be attributed to their abundance in nitrogen sources, which are known to limit rhamnolipid production [27]. Thus, at 72 h of incubation, where the PaJC strain showed slightly better rhamnolipid production than *P. aeruginosa*. The results show that PaJC strain was able to produce rhamnolipid efficiently with in 1.0% glucose supplemented MM. When LB media was used the final pH reached values 9.0 (data not shown). Our results are in agreement with those obtained by Gautam and Tyagi who shown that rhamnolipid production in *Pseudomonas* sp. was maximal at a pH range from 6 to 6.5 and decreased sharply above pH 7 [28].

The PaJC cells exhibit favorable properties including enhanced rhamnolipid productivity over the wild strain. In this work glucose and sucrose were the most effective carbon sources for rhamnolipid production. As a result, genetic engineering of rhamnolipid producing strains with *vgb* may be an effective method to.

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