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The use of raw cheese whey and olive oil mill wastewater for rhamnolipid production by recombinant *Pseudomonas aeruginosa*

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Abstract

This study concerns the potential of whey and olive oil mill wastewater for rhamnolipid production. The production of rhamnolipids in *Pseudomonas aeruginosa* and its recombinant form expressing *Vitreoscilla* hemoglobin gene (*vgb*) was studied. Rhamnolipid production by *P. aeruginosa* and the recombinant was examined using different carbon sources in defined medium containing 30 to 50% olive oil mill wastewater or whey, and cultivated at 30 and 37 °C with agitation speed of 100 to 200 rpm for 96 h. The effect of *vgb* on rhamnolipid production was significant. The highest rhamnolipid level was obtained from whey in cultures grown at 37 °C 100 rpm agitation, and reached 9.6 and 13.3 g L^{-1} for 72 h using *P. aeruginosa* and its recombinant strain, respectively. The study showed that genetic engineering of strains with *vgb* may be an effective method to increase rhamnolipid production.

Key words: olive oil mill wastewater, *Pseudomonas aeruginosa*, rhamnolipid production, *Vitreoscilla* hemoglobin, whey.
Abbreviations: PaJC, *Pseudomonas aeruginosa* expressing *Vitreoscilla* hemoglobin gene; *vgb*, *Vitreoscilla* hemoglobin gene.

Introduction

Olive oil and cheese production are agroindustries that represent a considerable share of the economy in many countries, including Turkey. These industries produce large amount of waste, e.g. olive oil mill wastewater and whey, the disposal of which as a growing problem (Martinez-Garcia et al. 2007). Olive oil mill wastewater and the whey are composed of many complex substances that are not easily degradable and possess a serious environmental risk. Most of the problems associated with olive oil mill wastewater pollution can be attributed to the phenolic fraction (Martinez-Garcia et al. 2007; Assen et al. 2009). Direct discharge of olive oil mill wastewaters is not permissible due to environmental deterioration of natural water bodies as indicated by coloring, appearance of an oily shine, and increased oxygen demand in receiving water bodies (Azbar et al. 2008).

Until recently, the large majority of research for simultaneous remediation and practical use of olive oil mill wastewater and whey have focused on aerobic digestion processes for rhamnolipid production (Aouidi et al. 2009). Among the different classes of biosurfactants, rhamnolipids, members of the glycolipid group, are the most extensively studied and characterized (Aparnaa et al. 2012).

Rhamnolipids are promising surfactants due to several characteristics such as low minimum surface tension, high emulsifying activity, and higher affinity for hydrophobic organic molecules. These properties confer optimal characteristics to rhamnolipids and make them potential carriers of pollutants in soil systems. In order to economize rhamnolipid production using different low-cost substrates, such as molasses, whey, glycerol, orange peelings, vegetable oils and wastes from food industry and coconut oil cake, have been used as carbon source (Costa et al. 2006; Thaniyavarn et al. 2006; Aparnaa et al. 2012).

Pseudomonas aeruginosa is an environmentally versatile Gram-negative bacterium, which can cause disease in particular susceptible individuals and is resistant to antibiotics. The bacterium can use several organic compounds as a food source, which gives it an exceptional ability to colonize ecological niches where nutrients are limited (Sanchez et al. 2007). It is well-known that the genus *Pseudomonas* is able to produce rhamnolipids from hydrophobic renewable sources including hydrocarbons, oils or carbohydrates (Patel, Desai 1997). Although the production of rhamnolipids from pure substrates is relatively well-studied, very few reports on the production of rhamnolipids from inexpensive and renewable sources are available (Patel, Desai 1997). Alternative substrates have been suggested for rhamnolipid production, especially water-miscible waste: molasses, milk whey and distillery waste. Lactic whey from dairy industries has also been reported as a cheap and viable substrate for rhamnolipid

production (Koch et al. 1988). Liquid waste from the dairy industry, known as dairy wastewater, supports good microbial growth and is used as a cheap raw material for rhamnolipid production (Sudhakar-Babu et al. 1996). Several studies have shown that lactic whey waste might be a comparatively better substrate for rhamnolipid production at the commercial scale, compared to synthetic media (Haba et al. 2000; Mukherjee et al. 2006). Recently, rhamnolipids have been found to have important antagonistic effects on economically important zoosporic plant pathogens, thus opening their use as biocontrol agents (Wang et al. 2005). *Pseudomonas aeruginosa* 47T2 was able to grow on olive oil mill wastewater as the sole carbon source, and whey wastes have been used as substrate for rhamnolipid production (Benincasa et al. 2002; Benincasa, Accorsini 2008).

It has been demonstrated that expression of bacterial hemoglobin gene (*vgb*) in heterologous bacterial hosts often results in enhancement of cell density, oxidative metabolism, protein and antibiotic production, and bioremediation, especially, under oxygen-limiting conditions (Dogan et al. 2006). These strains have been shown to be to synthesize rhamnolipids when growing on whey or olive oil mill wastewater.

The aim of this work was to assess rhamnolipid production by *P. aeruginosa* and its recombinant strain as an integrated process, using whey and olive oil mill wastewaters. It was hypothesized that engineering of rhamnolipid producing bacteria with vgb may be a useful way to extend the possible uses of vgb in strain improvement for bioremediation.

Materials and methods

Bacterial strains

Pseudomonas aeruginosa (ATCC 10145) and its recombinant strain containing *vgb* (PaJC; Chung et al. 2001) used in this study were provided by Dr. Benjamin C. Stark (IIT, Chicago, USA).

Shake flask experiments

Rhamnolipid production experiments were carried out in 250 mL Erlenmeyer flasks containing 50 mL medium. The olive oil mill wastewater and whey samples were suspended in distilled H_2O containing 30 and 50% of samples, respectively, and the final pH of the medium was adjusted to 7.0. The medium was autoclaved at 121 °C for 20 min. Cultures were incubated for 96 h in a reciprocal shaker (100 or 200 rpm) at 30 or 37 °C. The culture supernatant was used for rhamnolipid determination. All the experiments were performed in triplicate.

Rhamnolipid purification

The collected culture supernatant was first centrifuged at 9000 × g for 15 min to remove *P. aeruginosa* cells. Then rhamnolipid in the supernatant was precipitated by acidification to pH 2.0 with 1 N HCl. After centrifugation at 9000 × g for 20 min, the precipitate was extracted with ethyl acetate at room temperature. Then the organic phase was transferred to a round-bottom flask and connected to a rotary evaporator, to remove solvent toobtain viscous honey-colored rhamnolipid product (Patel, Desai 1997; Wei et al. 2005; Costa et al. 2006).

Rhamnolipid quantification and analytical methods

Rhamnolipids expressed as rhamnose (g L^{-1}) were measured in cell-free culture medium using the phenol-sulphuric method. One milliliter of the cell-free culture broth was mixed with 0.5 mL of 80% phenol and 2.5 mL concentrated sulphuric acid. After incubation for 20 min at room temperature, the absorbance was measured at 490 nm and the rhamnolipid concentration was calculated using a standard curve prepared using different concentrations of rhamnose (Silva et al. 2010). Rhamnose concentration was measured in the cell free culture broth by the method described by Chandrasekaran and Bemiller (1980). Rhamnolipid concentration was quantified by a colorimetric method as rhamnose content using a rhamnose standard. Rhamnolipid content was calculated by multiplying the rhamnose concentration by a factor of 3. This factor was calculated experimentally using the calibration curve for rhamnose and represents the ratio between rhamnolipid and rhamnose (Haba et al. 2000; Abalos et al. 2002; Benincasa et al. 2002; Costa et al. 2006).

Results

Rhamnolipid production by a wild type and recombinant strain of *P. aeruginosa* was compared using two forms of agroindustrial waste (raw cheese whey and olive oil mill wastewater) as a carbon source, under different agitation speeds and temperature. Rhamnolipid accumulation started after the cells reached stationary phase, and significance differences in production between the strains, depending on cultivation conditions, were evident already within 24 h (Fig. 1 to 4). In general, raw cheese whey was a better carbon source than olive oil mill wastewater in respect to rhamnolipid production. Tthe lowest rhamnolipid content using whey was 50-fold and 18-fold of that obtained using olive oil mill wastewater, at 30 and 37 °C, respectively.

Increase of cultivation temperature from 30 to 37 °C significantly increased rhamnolipid production. For olive oil wastewater, the increase in the basal level of production was more than six-fold (Fig. 1 and 2), compared to a two-fold increase if whey was used as a carbon source (Fig. 3 and 4). In addition, agitation speed significantly affected the rate of rhamnolipid production. In general, increase of speed from 100 to 200 rpm decreased rhamnolipid concentration, depending on temperature, carbon source, and the strain used.

When wild type and recombinant strains of *P.*

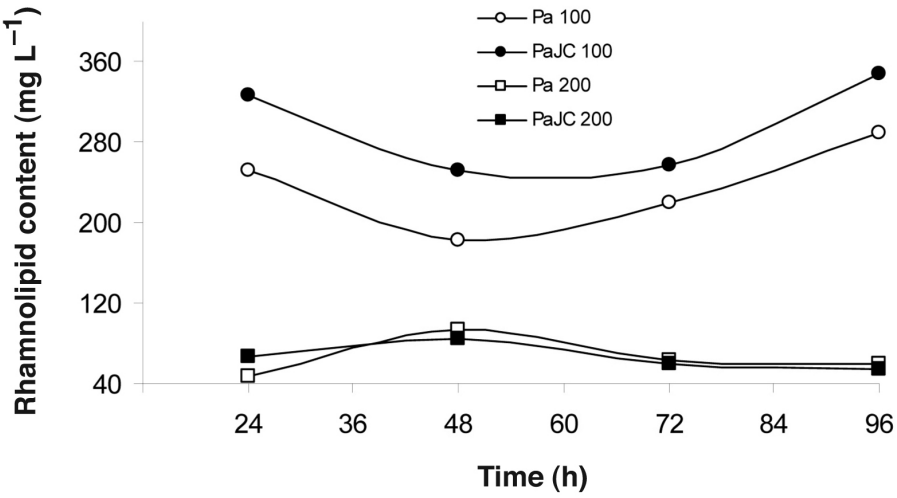

Fig. 1. Rhamnolipid levels of *Pseudomonas aeruginosa* (Pa) and its *vgb* recombinant strain (PaJC) cultivated in olive oil mill wastewater at 30 °C under different agitation speeds. *P. aeruginosa* (❍) and PaJC (●) at 100 rpm and *P. aeruginosa* (❑) and PaJC (■) at 200 rpm. Each value is the average of at least three independent experiments. For clarity, no error bars are given, but they are mostly less than 10 % of the respective data point.

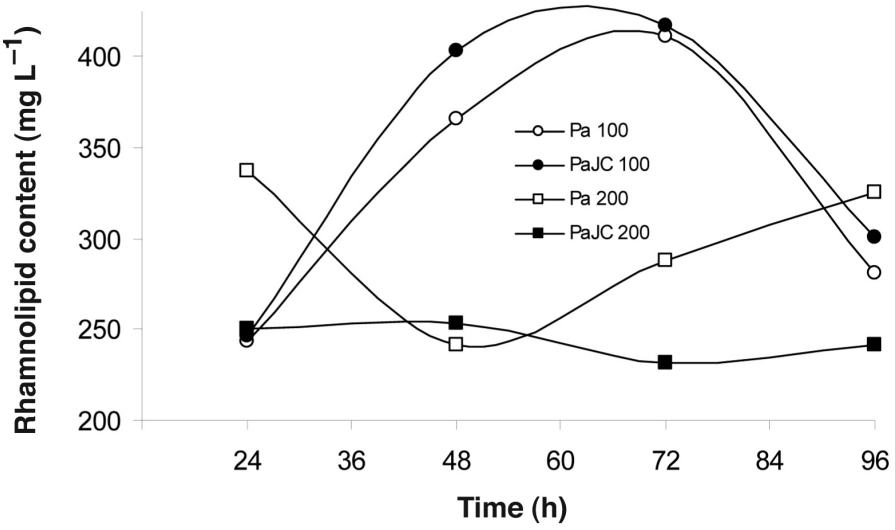

Fig. 2. Rhamnolipid levels of *Pseudomonas aeruginosa* (Pa) and its *vgb* recombinant strain (PaJC) cultivated in olive oil mill wastewater at 37 °C under different agitation speeds. *P. aeruginosa* (❍) and PaJC (●) at 100 rpm and *P. aeruginosa* (❑) and PaJC (■) at 200 rpm. Each value is the average of at least three independent experiments. For clarity, no error bars are given, but they are mostly less than 10 % of the respective data point.

aeruginosa were compared in respect to rhamnolipid production ability, a significant increase of production by the recombinant strain cultivated on olive mill wastewater occurred only at lower agitation speed at 30 °C (Fig. 1). At 37 °C, the effect was significant only after 48 h of cultivation (Fig. 2). When whey was used as a carbon source, the recombinant strain produced significantly higher amounts of rhamnolipids at 100 rpm at both temperatures, but the effect diminished with time (Fig. 3 and 4).

The highest rhamnolipid concentration (more than 13 g L^{-1}) was reached by the recombinant strain of P. aeruginosa 72 h after the start of incubation at 37 °C and 100 rpm agitation speed using whey as a carbon source (Fig. 4). In comparison, the wild type strain produced 9.6 g L^{-1} of rhamnolipids. When olive oil mill wastewtaer was used instead of whey, both strains produced maximum 400 mg L^{-1} of rhamnolipid production under the same conditions (Fig. 2).

Discussion

It is thought that microorganisms produce rhamnolipids as surfactants to emulsify water-insoluble carbon sources for better assimilation (Matsufuji et al. 1997). It has been reported by various researchers that rhamnolipid is "primary metabolite" and that its production coincides

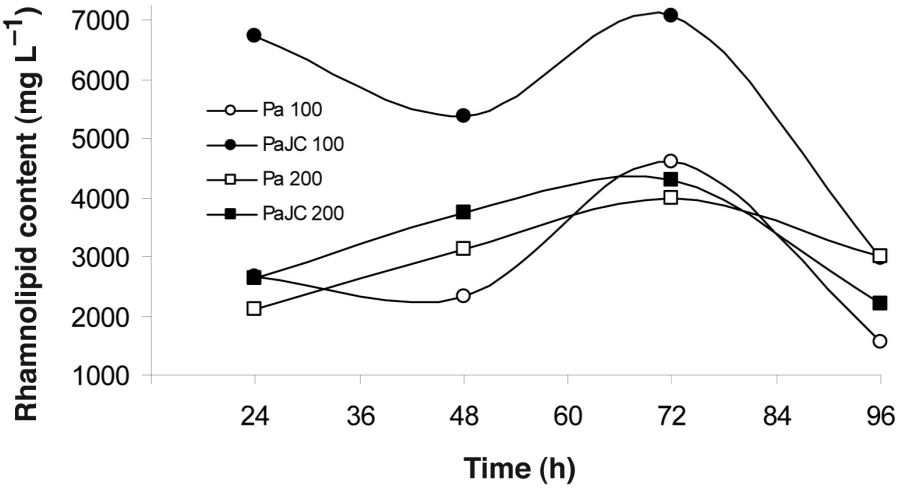

Fig. 3. Rhamnolipid levels of *Pseudomonas aeruginosa* (Pa) and its *vgb* recombinant strain (PaJC) cultivated in raw cheese whey at 30 °C under different agitation speeds. *P. aeruginosa* (❍) and PaJC (●) at 100 rpm and *P. aeruginosa* (❑) and PaJC (■) at 200 rpm. Each value is the average of at least three independent experiments. For clarity, no error bars are given, but they are mostly less than 10 % of the respective data point.

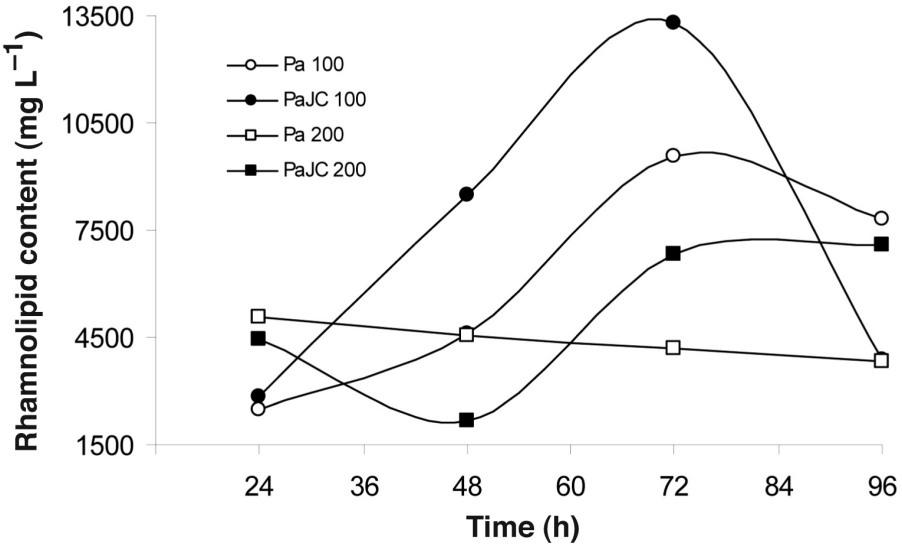

Fig. 4. Rhamnolipid levels of *Pseudomonas aeruginosa* (Pa) and its *vgb* recombinant strain (PaJC) cultivated in raw cheese whey at 37 °C under different agitation speeds. *P. aeruginosa* (❍) and PaJC (●) at 100 rpm and *P. aeruginosa* (❑) and PaJC (■) at 200 rpm. Each value is the average of at least three independent experiments. For clarity, no error bars are given, but they are mostly less than 10 % of the respective data point.

with the exponential growth phase (Aparnaa et al. 2012).

Different rates of rhamnolipid production have been reported for various *P. aeruginosa* strains studied using a number of substrates. Thaniyavarn et al. (2006) showed that rhamnolipid production by *P. aeruginosa* A41 were 6.58 g L^{-1}, when grown on olive oil mill wastewater. In another study, *P. aeruginosa* LBI produced 7.3 g L^{-1} rhamnolipid when grown on a number of water-immiscible substrates in mineral medium: olive oil mill wastewater (Benincasa, Accorsini 2008).

Rhamnolipid production using frying oil, refinery residue, milk whey and molasses as substrate was 2.7, 1.85, 1.78 and 0.24 g L^{-1}, respectively (Silva et al. 2010). In another study, the *Pseudomonas* 47T2 NCIB 400044 strain produced 1.4 g L^{-1} rhamnolipid based on rhamnose content, from olive oil mill wastewater (Haba et al. 2000). Maximum rhamnolipid production (4.97 g L^{-1}) was shown to occurr at 96 h (Aparnaa et al. 2012). In our previous study, *P. aeruginosa* produced rhamnolipids at a rate 326 mg L^{-1} when grown on LB medium (Kahraman, Erenler 2012).

It has been reported that a temperature range of 32 to 34 °C results in high rhamnolipid production by *P. aeruginosa* (Silva et al. 2010). Agitation rate affects the mass transfer

efficiency of both oxygen and medium components and is considered crucial to rhamnolipid formation by the strictly aerobic bacterium *P. aeruginosa* and its recombinant strain, especially when grown in a shake flask (Kahraman, Erenler 2012). Being a product of secondary metabolism, rhamnolipid production in both media started after the stationary phase and generally leveled at 72 h. However, the rhamnolipid yield was affected by the type of carbon substrate used. This suggests that rhamnolipid production can be enhanced to economically viable values using renewable feedstock as a carbon source. This indicates the importance of utilization of industrial by-products and agricultural wastes as cost-effective alternative substrates for microbial growth and rhamnolipid production. Agro-industrial wastes are considered as a promising cost-effective substrate for rhamnolipid production and can reduce many processing industrial waste management problems (Aparnaa et al. 2012).

This work presents an integrated process of waste minimization and disposal of residuals from the whey and olive oil mill wastewater processing industry. Whey can be converted into rhamnolipids in significant amounts (up to 13 g L^{-1}) and with reasonable yields, which can be further improved. In this work, we can conclude that although whey and olive oil mill wastewater are complex substrates, they are suitable for rhamnolipid production. The PaJC cells show favorable properties, including enhanced rhamnolipid productivity, over the wild strain. As a result, genetic engineering of rhamnolipid producing strains with vgb may be an effective method.

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