The Bioconversion of Ethanol to Biosurfactants and Dye by a Novel Coproduction Technique

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ABSTRACT: Rhamnolipids, multifunctional glycolipid biosurfactants, and pyocyanine, a phenazine dye, were coproduced by *Pseudomonas* BOP100 from ethanol as the sole carbon source. Bacterial growth was dependent on the ethanol concentration in the medium. Pyocyanine was produced only during the exponential phase, while rhamnolipids production continued during the stationary phase, indicating two different ways of production for each of the products. Maximum coproduction capacity was observed at a concentration of 3% ethanol; yield of rhamnolipids was 3 g/L, and of pyocyanine 0.2 g/L. The products were characterized to confirm their chemical structures. *JAOCS 73,* 851–856 (1996).

KEY WORDS: Biosurfactants, coproduction, ethanol assimilation, glycolipids, phenazine pigments, *Pseudomonas,* pyocyanine, rhamnolipids, surfactant.

Rhamnolipid biosurfactants are expected to have great potential for industrial application (1). Recently, ethanol has become an attractive raw material for biological production of natural coloring materials in some laboratories (2,3). There is no literature dealing with the subject of coproduction, with which we are concerned here. Established techniques and processes for the production of biosurfactants are generally based on the utilization of heterogenous systems, where the bioconversion media are comprised of an aqueous nutrient phase and a water-insoluble carbon source, commonly a hydrocarbon (4–9). Such heterogenous systems have exhibited many drawbacks, including difficulties in handling two-phase media, prolonged cultivation time, inadequate assimilation, poor productivity, and arduous purification. Bearing these matters in mind, a coproduction technique that relies on ethanol, based on a homogenous medium system, was developed as a superior alternative. This approach eliminates the disadvantages of dealing with two-phase media, and it has many other advantages over the conventional methods, such as rapid bacterial growth, generation of less impurities, eco-adaptability of the ethanol process for using many different types of abundant biomass, and possibly lower costs. This paper makes clear the preeminence of ethanol as a carbon source and the novelty of coproduction as a strategy in producing biosurfactants and dyes.

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EXPERIMENTAL PROCEDURES

Chemicals. All chemicals were laboratory-grade reagents. Standard samples of rhamnolipids A and B were provided by Iwata Chemical Co.

Bacteria. Pseudomonas BOP100 was supplied by the Patent Microorganism Depository, National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology.

Coproduction of rhamnolipids and pyocyanine. Batch fermentation was carried out in 500-mL shaking flasks containing 100/200 mL of the following medium: $NaNO_3$ 4 g/L, yeast extract 0.5 g/L, KH_2PO_4 1.5 g/L, Na_2HPO_4 0.6 g/L, $MgSO_4$ 0.5 g/L, $MnSO_4$ 0.001 g/L, Fe_2SO_4 0.01 g/L. Ethanol was used as the sole carbon source and was added in concentrations of 1–8% to test its effect on bacterial growth and on the production of rhamnolipids and pyocyanine. Otherwise, when not stated, 3% ethanol was used in the production of rhamnolipids and pyocyanine. The starting pH in the medium was always adjusted to 7.3, if not otherwise mentioned. Media were sterilized in a top-feed Hirayama Manufacturing Corp. (Mito, Japan) autoclave at 120°C for 20 min. Cultures were incubated in a Takasaki Kagaku Kikai Co., Ltd. (Kawaguchi, Japan) Shaker for 5 d at 32°C and 200 rpm. Inoculum cultures were prepared on a similar medium, containing 1% glucose as a carbon source, and they were incubated for 24 h.

Growth and production monitoring. Bacterial growth and the relation to coproduction were examined in samples drawn periodically by different methods, including foam observation, pigmentation monitoring, turbidimetry, dry-weight estimation, measurement of surface tension, and production of biosurfactants and pyocyanine. Simple observation of foaming indicated the start of biosurfactant production, while pigmentation was an indicator of pyocyanine production.

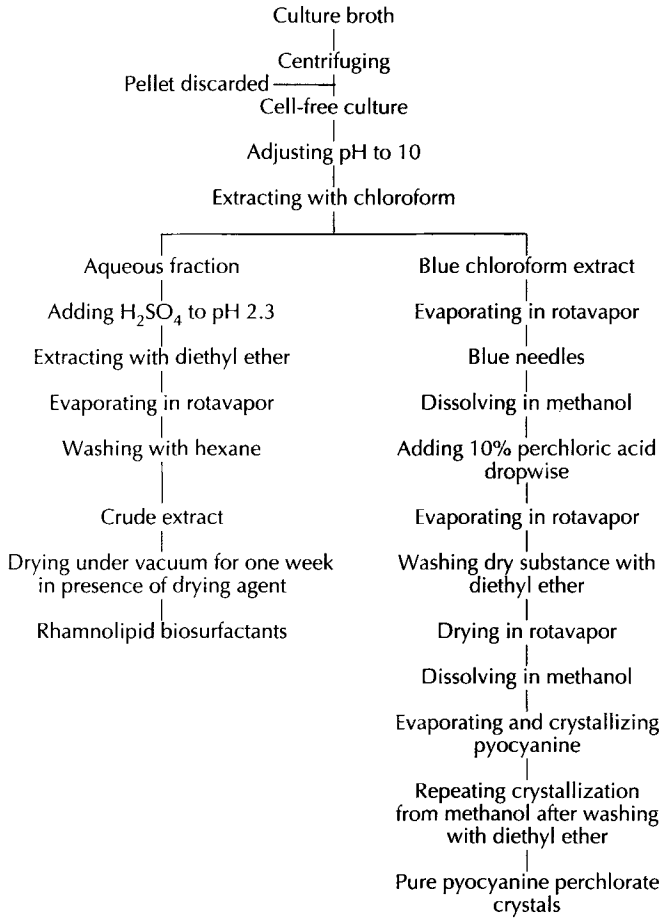
Turbidimetry. Samples of 10 mL culture were collected periodically during the 5-d fermentation period and were centrifuged at 10,000 rpm for 20 min at 10°C in a refrigerated

centrifuge. The supernatant was discarded, and the pellet was suspended in 10 mL distilled water. Then the turbidity of each sample was measured at 660 nm with a double-beam, double-monochromator spectrophotometer. Absorbency was plotted against time as an indicator of bacterial growth.

Dry weight. Samples of 20 mL were collected periodically and centrifuged at 10,000 rpm, as described above. The supernatant was discarded, and the pellet was dried for 24 h at 80°C; then the dry weight of the pellet was estimated and plotted against time as an indicator of growth.

Production monitoring. The production of both products was monitored as follows: (i) Surface tension in cell-free culture was measured with a Wilhelmy-type surface tensiometer Shimadzu ST-1 (Shimadzu Ltd., Kyoto, Japan). (ii) After measuring surface tension, rhamnose sugar in cell-free culture was measured by the anthrone colorimetric method (10). The amount of rhamnose biosurfactants was measured as mg rhamnose/L cell-free culture, and this was plotted against time and compared with growth curves for evaluation of the production-growth relationship. The spectrophotometer was used for colorimetric sugar measurements. (iii) The production of pyocyanine, expressed as absorption, was monitored in the cell-free cultures and plotted against time for comparison of growth and production. The absorption was measured at λ_{max} = 310 nm (11) in the spectrophotometer.

Separation and purification. Separation and purification of rhamnolipids and pyocyanine were performed as described in Scheme 1.



SCHEME 1

Chromatographic analysis. Thin-layer chromatography (TLC) of rhamnolipids A and B was performed on silica-gel plates (Kieselgel 60; Merck & Co., Inc., Tokyo, Japan) with mobile-phase chloroform/methanol/acetic acid/water (79:11:8:2). After drying, TLC plates were developed by spraying with a glycolipid-sensitive reagent (4). Briefly, 2 g diphenylamine were dissolved in 20 mL ethanol, and then 100 mL concentrated hydrochloric acid and 80 mL concentrated acetic acid were added. After spraying, the plates were heated to 150°C for 5 min. Rhamnolipids appeared as a blue-grey zone on a light grey background. Rhamnolipids were also characterized by analytical high-performance liquid chromatography (HPLC) with standard samples of rhamnolipids A and B. HPLC analysis was performed with a μBondasphere 5m C 18-100Å, 3.9 × 15 column with a Waters multifluid system U6K model (Nihon Millipore Ltd., Tokyo, Japan), equipped with ultraviolet (UV) detector (photodiode array detector, Waters 991) and a refractometer (differential refractometer, Waters 410). The mobile phase was a mixture of acetonitrile and ethanol, mixed on-line by the instrument in the ratio of 40 and 60% (vol/vol). A flow rate of 1 mL/min was applied. The running time of analysis ranged from 10–40 min. Purity of obtained peaks was used as indicator of product purity.

Spectroscopy. Fourier transform infrared spectroscopy (FTIR) spectra from KBr disks were obtained by a JASCO FTIR-5000 spectrophotometer (Japan Spectroscopic Co., Ltd., Hachioji, Japan), and data were acquired and processed with JASCO-5000 software, version 2.2. Absorption spectra were recorded with a Shimadzu UV-VIS-NIR recording spectrophotometer UV-3100 (Shimadzu Ltd.).

RESULTS AND DISCUSSION

Ethanol as a substrate for the coproduction process of biosurfactants and pyocyanine. The coproduction strategy was a practical way of producing rhamnolipid biosurfactants and pyocyanine, although coproduction was a demanding process due to its sensitivity to environmental factors, especially under the conditions of batch fermentation. Environmental factors, such as pH, concentration of ethanol, and fermentation time, were critical factors in coproduction and needed strict control. Growth in batch culture was monitored for a period of five days, where the formation of foam indicated the start of surfactant production and pigmentation indicated the start of pyocyanine production. Growth was shown to be dependent on the initial concentration of ethanol. Foaming and pigmentation appeared after 24 h in cultures of 1% ethanol, after 48 h in 2%-ethanol cultures, and after 72 h in 3%-ethanol cultures. No foaming or pigmentation could be observed at concentrations above 3%. Results presented in Figure 1 show that growth reaches a maximum at an ethanol concentration of 3%, and then it drops sharply, as observed by

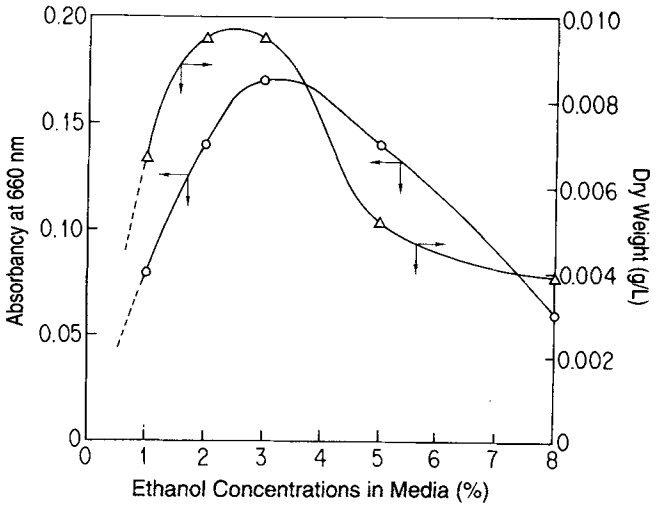


FIG. 1. Effect of ethanol concentration on the growth of *Pseudomonas* BOP100 (Agency of Industrial Science and Technology).

the absorption of culture broths as well as by dry weight of the biomass.

Total production of rhamnolipids was measured at each ethanol concentration and presented in Figure 2A. Maximum biosurfactant production was achieved at a concentration of 3% ethanol. Similar results were obtained by measuring pyocyanine production, shown in Figure 2B.

Therefore the optimum ethanol concentration apparently ranges from 2 to 3% for the coproduction of rhamnolipids and pyocyanine. Ethanol concentration also affected the end pH value of the cultures after five days of incubation. This could mean that there is a correlation between production and ethanol concentration on one hand and the end pH value on the other. Table 1 shows pH values in the cultures at the end of the fermentation for each ethanol concentration.

The higher end pH value in the ethanol concentration range of 1, 2, and 3% corresponds to shorter production times of 24, 48, and 72 h, respectively. Thus it became clear that the rapid growth and production attained at lower concentrations resulted in a higher end pH value. End pH is not affected at concentrations above 3%, which corresponds to the lack of bacterial growth at these high concentrations.

Pseudomonas BOP100 lost its ability to produce pigmentation after repeated fermentations, and fresh inoculum cultures were needed for proper coproduction. This was most probably due to ethanol toxicity, even at low concentration,

TABLE 1
pH Values at Different Ethanol Concentrations After Five Days of Fermentation

Ethanol concentration (%)	Starting pH	Final pH after 5 d of fermentation
1	7.3	8.2
2	7.3	7.7
3	7.3	7.4
5	7.3	7.3
8	7.3	7.1

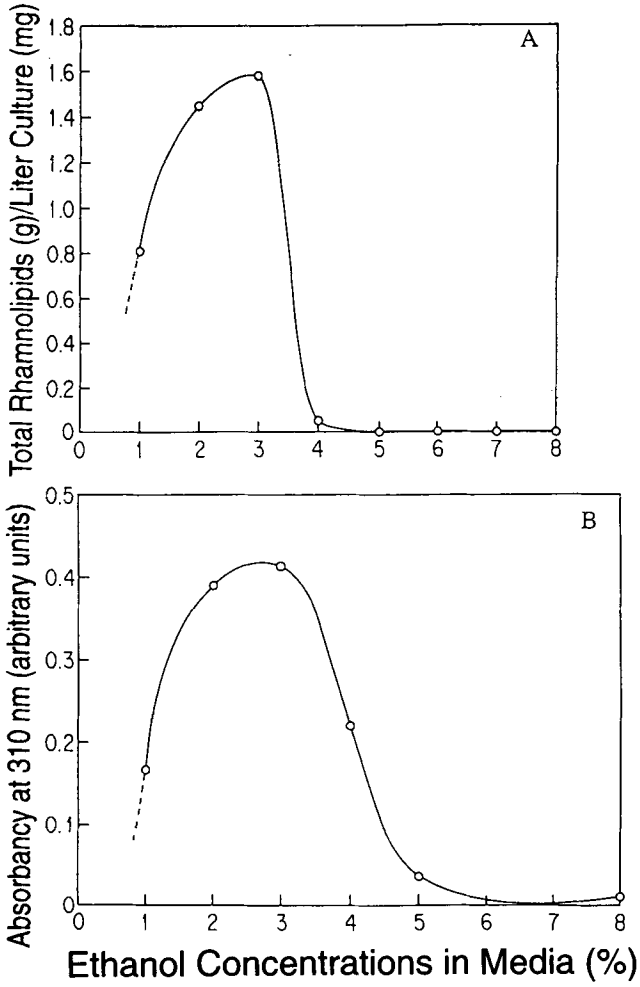


FIG. 2. Effect of ethanol concentration on the production of rhamnolipids A and B as well as on pyocyanine.

and to adaptation problems (12–16). Recently, Heipier and De Bont have reported that adaptation of *Pseudomonas* by growth on hydrocarbons may be necessary before utilizing them in assimilation of alcohols (12).

To investigate other factors that may influence coproduction, we have monitored bacterial growth and maximum production of rhamnolipids and pyocyanine at a concentration of 3% ethanol during a fermentation period of five days. Rhamnolipid production was monitored by the measurement of total rhamnolipids per liter culture and by examining the change in the surface tension. Figure 3A shows that at least 32 h of fermentation are needed before any rhamnolipid production was observed and that the production increased gradually thereafter. Figure 3A also indicates that the production of rhamnolipids between days two and three is relatively stable, and that production is accelerated in the final stage of microbial growth. Measurement of surface tension further confirmed this fact. Samples of 20 mL were collected periodically and centrifuged, and the supernatant was taken to measure surface tension in cell-free culture by the Wilhelmy method. Surface tension was plotted against time to investigate the relationship between growth and biosurfactant production (Fig. 3B). Gradual decrease in surface tension starts

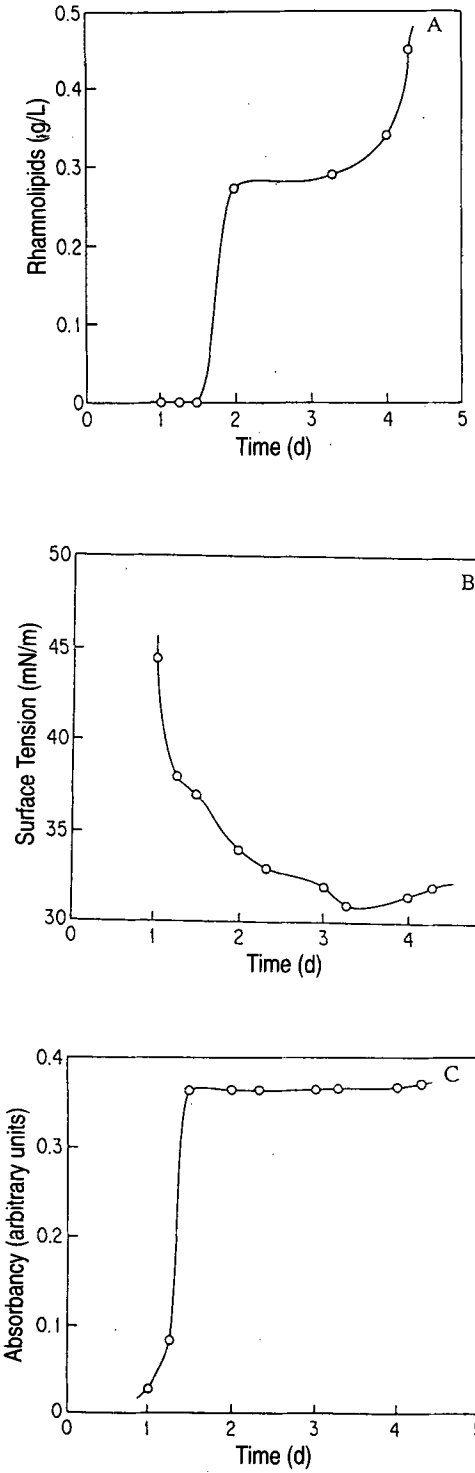


FIG. 3. Production of rhamnolipids and pyocyanine by batch fermentation of BOP100 on 3% ethanol for five days: A, increase of rhamnose concentration; B, decrease of surface tension; C, production of pyocyanine.

after 32 h of fermentation and continues upon further fermentation. This corresponds with an increased concentration of biosurfactant. Conversely, the production pattern of pyocyanine is different from that of the rhamnolipids, as shown in Figure 3C. The growth curve shown in Figure 4 agrees well

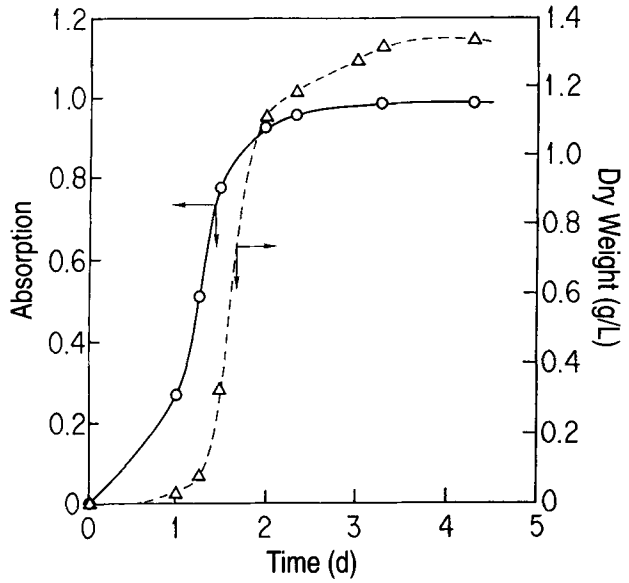


FIG. 4. Growth curves of *Pseudomonas* BOP100 at concentration of 3% ethanol. See Figure 1 for company source.

with the fact that pyocyanine is produced during the exponential phase of the bacterial growth.

Comparison of the growth curves obtained by turbidimetry and by dry-weight measurement (Fig. 4) and production curves (Fig. 3) made it evident that the bacteria start production of biosurfactants at a higher exponential growth phase, and this continues during the whole fermentation period, while the pyocyanine production is stabilized after 48 h. The comparison also shows an enhancement of rhamnolipid production during the stationary phase, which indicates possible increasing rhamnolipids, even after five days of fermentation. Such a production manner of biosurfactants is commonly known. Figure 4 shows the growth curves of *Pseudomonas* BOP100 at an ethanol concentration of 3%.

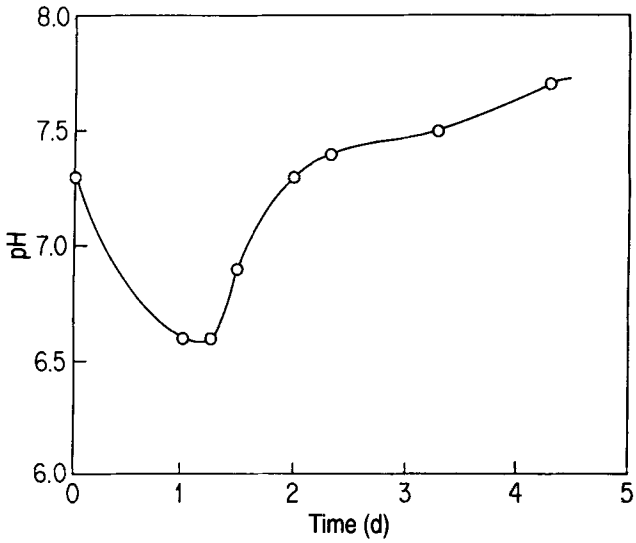


FIG. 5. Change of pH in 3% ethanol culture during fermentation.

Growth of the bacteria affects pH in the culture. During the initial part of the fermentation period, there was a temporary decrease in pH, which stabilized upon further fermentation to near the initial pH. Figure 5 shows the changes in pH during fermentation.

Although an optimum starting pH is needed (8), stabilization of pH, after growth has started, may indicate that it is not necessary to control pH. Stabilization at a wrong pH, however, may affect the production of pyocyanine (11). We have observed that the pH change affects the production of pyocyanine, and at pH values above 7.5, pyocyanine production was halted.

Separation and purification. Although establishing separation and purification procedures was not a simple and straight-forward matter, they have given excellent results when difficulties were solved after a few trials. Rhamnolipids and pyocyanine were purified in fair amounts, and the yield in the culture was 3 g/L for rhamnolipids and 0.2 g/L for pyocyanine. Rhamnolipids were obtained as brownish solid material, while pyocyanine was obtained as perchlorate crystals with a reddish-purple color. The characterization of these products has shown that the coproduction and use of ethanol as a carbon source did no affect or alter their structure or properties. Separation and purification procedures are summarized in Scheme 1.

Chromatographic analyses. TLC analyses of rhamnolipids from BOP100, grown on 3% ethanol, were run against standard samples of rhamnolipids A and B and showed clearly that only rhamnolipids A and B were produced. TLC results were further confirmed by HPLC analysis. HPLC analyses against a standard sample of rhamnolipid A or B have also shown that BOP100, grown on 3% ethanol, produced both types of rhamnolipids. The analyses have also shown that purity was more than 95% because no other peaks appeared on long-time analysis. Figure 6A shows analysis of standard samples of rhamnolipids A and B, while Figure 6B shows results of analyzing samples of rhamnolipids produced by BOP100 on 3% ethanol and prepared according to our methodology. It is also evident from Figure 6B that rhamnolipids A and B from BOP100 cultivation on 3% ethanol are produced in a ratio of 74.3 and 25.7, respectively.

Spectroscopic analyses. FTIR spectra of the alkyl chains of rhamnolipids A and B were compared with the corresponding spectra of the alkyl chain from rhamnolipids produced by growth of BOP100 on 3% ethanol. It showed that the spectra of alkyl chains from standard rhamnolipid samples and the alkyl chain of rhamnolipids obtained by growing *Pseudomonas* on 3% ethanol were identical. This indicated that the rhamnolipids produced by the coproduction process were types A and B rhamnolipids.

Pyocyanine was characterized by examining its absorption spectrum and determining the specific λ_{max} values for each peak (11,17). We obtained results similar to those obtained by other authors (11,17). The absence of peaks attributed to other phenazines indicated purity. The effect of pH on the absorption spectra was examined in a series of phosphate buffers with pH values ranging from 3.4 to 12.4. Figure 7

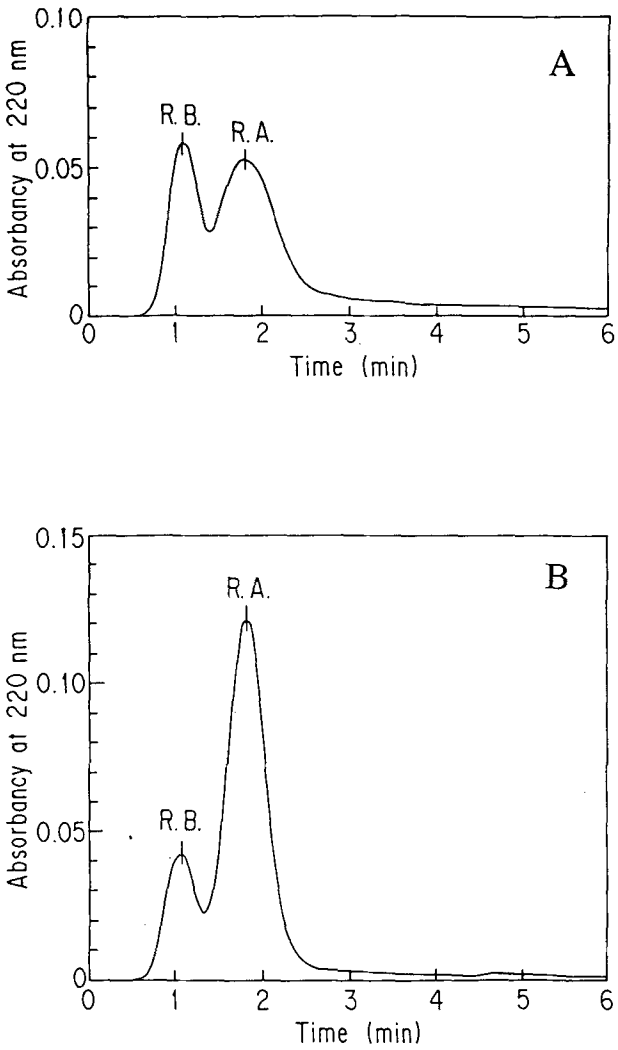


FIG. 6. High-performance liquid chromatographic analysis of rhamnolipids: A, Standard samples; B, samples obtained from BOP100 grown on ethanol. R.A., rhamnolipid A; R.B., rhamnolipid B.

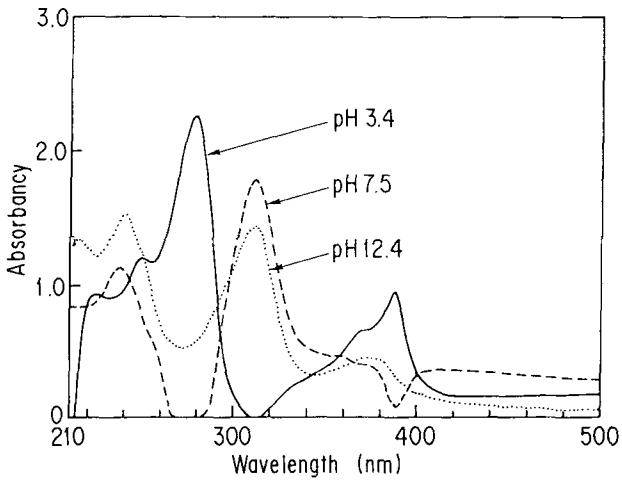


FIG. 7. Absorption spectra of pyocyanine at different pH values.

shows the absorption spectra at three different pH conditions. It shows that λ_{max} has a value of 275 nm at pH 3.4 and of 310 nm at pH values of 12.4 and 7.5. The 380-nm peak, however, is absent at pH 7.5, but is clear at pH values of 3.4 and 12.4. It is evident from this that pyocyanine is highly sensitive to pH.

Pyocyanine has also shown high sensitivity to pH variations as a pH indicator. Namely, the turn point of color from red to blue-purple was at pH 4.8, while a completely blue color was observed at pH values above 5.8. These results correspond to the results reported by other authors (11).

It is evident from the above results that bacteria coproduce rhamnolipids and pyocyanine by ethanol assimilation. It is also clear that a concentration of 3% ethanol is needed to get maximum production, although for industrial purposes, an optimum concentration of approximately 2% could be appropriate. The results also show that pure products can be obtained by the presented separation methodology. It has also become evident that neither the use of ethanol nor the coproduction affected or altered characteristics of the products.

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