Pilot Plant Production of Rhamnolipid Biosurfactant by *Pseudomonas aeruginosa*

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Rhamnolipid biosurfactants were continuously produced with *Pseudomonas aeruginosa* on the pilot plant scale. Production and downstream processing elaborated on the laboratory scale were adapted to the larger scale. Differences in performance resulting from the scale-up are discussed. A biosurfactant concentration of approximately 2.25 g liter^{-1} was achieved. The biosurfactant yield on glucose was 77 mg g^{-1} h^{-1}, and the productivity was 147 mg liter^{-1} h^{-1}, corresponding to a daily production of 80 g of biosurfactant. The first enrichment step consisted of an adsorption chromatography which was followed by an anion-exchange chromatography. The resulting product was 90% pure, and the overall recovery of active material was above 60% with the downstream processing used.

Pseudomonas aeruginosa produces rhamnolipids that exert an important role when the cells grow at the expense of hydrocarbons (5, 7). The active fraction contained two compounds, R-1 and R-2 (1, 6). They differed in rhamnose content, with R-1 having two rhamnose units and R-2 having one. The lipid part was β-hydroxydecanoic acid in both cases. Recently, Wagner and co-workers (11) reported that compounds with a variable length of the carbohydrate moiety are produced under appropriate conditions (resting cells).

An increased interest for potential application of microbial surface active compounds is based on their broad range of functional properties which mainly comprise emulsification, phase separation, wetting, foaming, surface activity, and viscosity reduction of heavy crude oils (3, 8). Potential applications can be envisaged in several industries such as agriculture, food, textiles, cosmetics, petrochemical, and petroleum production.

In view of wide-spread utilization, process development for large-scale production of biosurfactants is an obvious necessity. In a previous study we have shown the dependence of biosurfactant production on the medium composition (4). It became obvious that environmental parameters considerably affect biosurfactant release. Here, we report on the production of biosurfactant on the pilot plant level and on the downstream processing adapted to the scale.

MATERIALS AND METHODS

Microorganism. A strain of *Pseudomonas aeruginosa* (DSM 2659) was used throughout this work which was originally isolated from soil samples in the vicinity of an oil refinery. The cells were maintained as frozen glycerol cultures (1:1 mixture of freshly grown cells and 30% [wt/vol] glycerol solution) at −70°C.

Medium. The optimized medium 3M which was used for *P. aeruginosa* growth and biosurfactant production was the same as described previously (4), except that iron sulfate was omitted and phosphoric acid (84%) was added to a final concentration of 55 μl g^{-1} of glucose supplied. The main

characteristics of medium 3M are: carbon and phosphorus excess as well as nitrogen and iron limitation.

Glucose supply normally amounted to 30 g liter^{-1}, but concentrations up to 80 g liter^{-1} were also tested. The medium for the continuous culture was prepared in 2,000-liter batches in an in situ sterilizable 3,000-liter stirred-tank bioreactor. Inocula were grown in a 2% (wt/vol) glucose medium 3M including iron sulfate but 4.1 g of Na$_2$HPO$_4$ · 2H$_2$O liter^{-1} plus 5.9 g of KH$_2$PO$_4$ liter^{-1} were used as buffering components in place of phosphoric acid. Unless unavailable, all chemicals used were of technical grade. Because of the severe influence of trace elements on growth and production, deionized water was used throughout.

Cultivation conditions. Medium optimization with respect to biosurfactant production in continuous culture was performed with the used organism on lab scale in a 5-liter compact loop reactor (COLOR; Braun, Melsungen, Federal Republic of Germany) (4). On the pilot plant scale continuous production was carried out in a 50-liter COLOR bioreactor which was described in detail elsewhere (2).

The working volume was 23 liters, growth temperature was 33°C, aeration was at 0.5 volume of air per volume of liquid per min (vvm), stirrer speed was 1,000 rpm, and the pH was maintained at 6.2 with 6 N H$_3$PO$_4$ plus 6 N KOH. The bioreactor was equipped and operated with a centrifuge-type mechanical foam separator operated at 1,000 rpm. In addition, a foam recycling system was connected to the air outlet for security reasons. The outlet air was fed into a separation funnel from which the air escaped on top, and the liquid accumulating at the bottom was recycled to the bioreactor. The system was operated at a dilution rate of 0.065 h^{-1} during the production process. A constant liquid volume was maintained in the bioreactor by a weight control. Inocula were grown in several baffled 1-liter shake flasks with 200 ml of medium on a rotary shaker at 33°C. Late-exponential-phase cells (1 liter) served as the inoculum for the start-up batch cultivation in the bioreactor. When the glucose was exhausted, the culture was switched from batch to continuous operation.

Analytical methods. Determination of biomass, residual glucose concentration, surface and interfacial tension, and F_{cmc} (an indirect measure for biosurfactant concentration) values has been described elsewhere (4). Nitrate and nitrite

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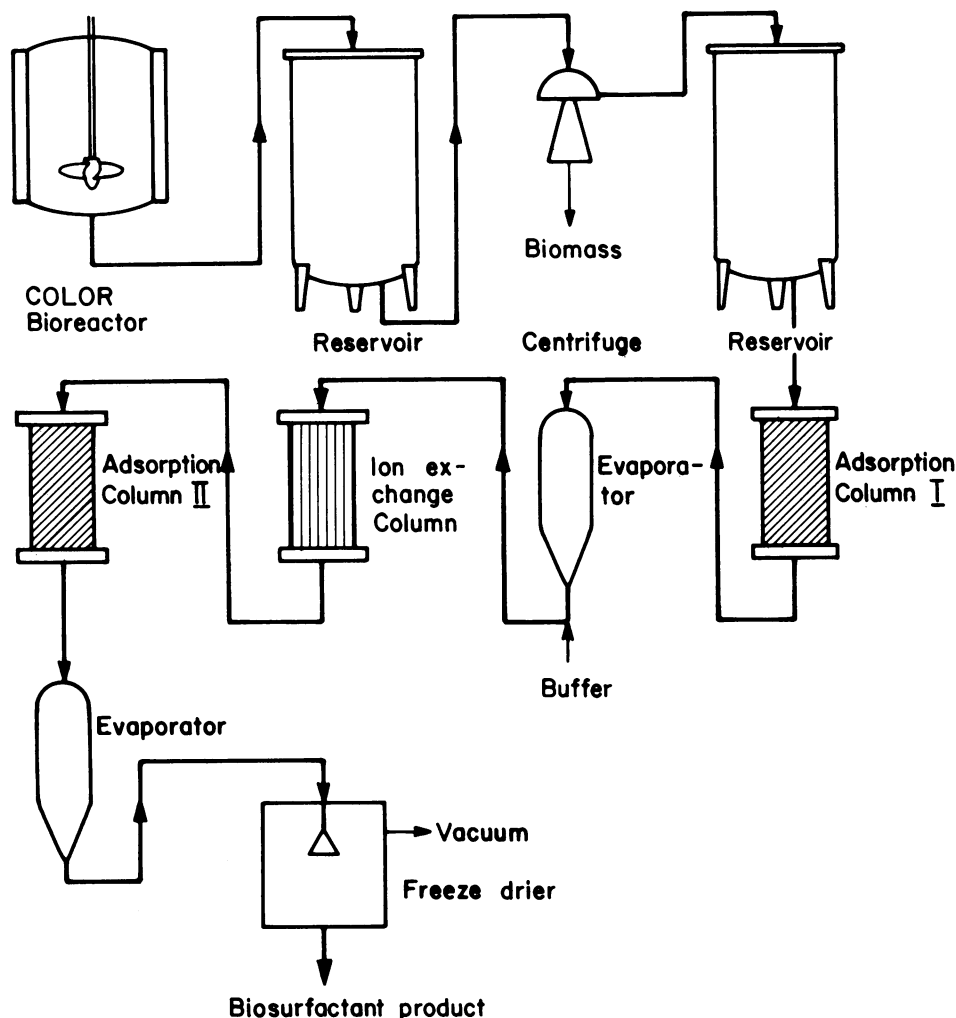‡ Present address: Transgene S.A., F-67000 Strasbourg, France.

FIG. 1. Flow sheet for the pilot plant production and downstream processing of rhamnolipid biosurfactants from *P. aeruginosa*.

were measured semiquantitatively with Merckoquant test sticks (E. Merck AG, Darmstadt, Federal Republic of Germany). Oxygen partial pressure and the composition of the exit air were recorded continuously as reported earlier (4, 9).

Downstream processing. The general procedure for downstream processing of the produced biosurfactant was developed for laboratory-scale investigations but was principally transferable to pilot plant scale. A scheme of the whole downstream process is given in Fig. 1. The outlet of the continuous production in the bioreactor was collected in a cooled reservoir tank (300 liters). Periodically the cells were separated by centrifugation (Westfalia, type SA14-47-476), and the cell-free culture liquid was stored in a second reservoir where 0.2% (vol/vol) formaldehyde was added to prevent microbial growth. All chromatographic steps were installed in duplicates. In this way, loading and regeneration of the columns could be done in parallel.

Loading of the columns. A supply line was installed to the columns which ended in a special device consisting of five stainless steel tubings arranged spiderlike to feed the liquid evenly to the surface of the resin. A sieve (mesh size, 0.2 mm) was placed on top of the resin to protect it from solids still in the liquid and from being whirled up. Flow to the

columns was induced by peristaltic pumps (type 5025; Watson-Marlow, Falmouth, United Kingdom).

The liquid level in the column was monitored by a capacitive probe (NTK 20; Visolux, Baden-Baden, Federal Republic of Germany) attached to the outer surface of the column. The supply pump controlled by this probe, maintained a constant liquid level in the column, which allowed a continuous unattended loading of the columns.

Adsorption chromatography. The primary enrichment of the rhamnolipids was achieved by adsorption chromatography on a polystyrene resin (Amberlite XAD-2; Rohm and Haas, Philadelphia, Pa.). The resin (7 kg) was placed in a glass column (650 by 200 mm), yielding a bed height of approximately 330 mm and corresponding to a bed volume of about 10.4 liters. The column was equilibrated with 0.1 M potassium phosphate buffer (pH 6.1), and then the cell-free culture liquid was applied at a flow rate of approximately 20 liters h^{-1} at the beginning and 10 liters h^{-1} towards the end of the loading procedure. The adsorption of the active compounds was assayed by measuring the surface tension at the column outlet. Adsorption chromatography was terminated when the surface tension dropped below 35 mN m^{-1}. The column was then washed with 2 to 3 bed volumes of distilled water. The elution of the biosurfactant was subse-

TABLE 1. Comparison of continuous growth and biosurfactant production of *P. aeruginosa* on laboratory and pilot plant scales

Bioreactor size, dilution rate (h^{-1})	Initial glucose concn (g liter^{-1})	Residual glucose concn (g liter^{-1})	Biomass concn (g liter^{-1})	Biosurfactant concn (g liter^{-1})[a]	$y_{X/S}$ (mg g^{-1})	$y_{P/S}$ (mg g^{-1})	r_P (mg liter^{-1} h^{-1})
5 liters							
0.05	18.2	0.2	2.5	1.7	140	97	88
0.12	36.5	0.6	3.8	2.6	140	72	309
50 liters (0.065)	30.0	0.7	2.5	2.25	85	77	147

[a] Calculated from an average rhamnose content on the laboratory scale (4) and from F_{cmc} on the pilot plant scale.

quently effected with methanol. Fractions of 1 to 2 liters were collected and analyzed for biosurfactant content by measuring interfacial tension and F_{cmc} values. Fractions with F_{cmc} values above 10 were pooled, and the solvent was evaporated (Rotavapor R 151; Büchi, Flawil, Switzerland).

The adsorbent was regenerated by eluting the column with 2 to 3 bed volumes of 1 N NaOH in methanol. An additional flushing of the column with 20% acetone in 1 N NaOH was performed after five adsorption-desorption cycles and then the column was rinsed with distilled water until the pH of the outlet was neutral. Subsequently 2 to 3 bed volumes of 1 N H$_2$SO$_4$ were applied, followed by a distilled water rinse. Finally, the column was equilibrated with potassium phosphate buffer (pH 6.1).

Ion-exchange chromatography. Further purification of surface-active compounds was achieved by ion-exchange chromatography on DEAE-Sepharose CL 6B (Pharmacia, Uppsala, Sweden). DEAE-Sepharose (6.5 liters) was packed into a column (400 by 200 mm), yielding a bed volume of approximately 6.2 liters and a bed height of 200 mm.

The column was equilibrated with 10 mM Tris hydrochloride buffer (pH 8) containing 10% (vol/vol) ethanol. The concentrated residue from the adsorption column was diluted 10-fold with the same buffer containing 20% (vol/vol) ethanol and applied to the column after filtration through a cotton filter. The flow rate was 6 liters h^{-1}. The effluent was again assayed for surface-active compounds to survey the charging of the column.

The column was washed with 2 to 3 bed volumes of 0.1 M NaCl in 10 mM Tris hydrochloride buffer containing 10% (vol/vol) ethanol. The biosurfactants were then released by 0.8 M NaCl in the same buffer. Fractions (1 to 2 liters) were collected. The fractions with low surface tension (below 35 mN m^{-1}) were pooled and subjected to a second adsorption chromatography on XAD-2 resin. The methanol eluate was evaporated in vacuo, and the residue was lyophilized.

Regeneration of the affinity column was carried out with 2 M NaCl in Tris hydrochloride buffer (pH 8) containing 20% ethanol. After 5 cycles, the column was additionally treated with 0.2 M NaOH containing 20% methanol. This removed a reddish pigment that also accumulated in the ion-exchange column.

RESULTS AND DISCUSSION

Biosurfactant production. The best conditions for biosurfactant production in the pilot plant were obtained with the slightly modified medium 3M of Guerra-Santos et al. (4). With a dilution rate of 0.065 h^{-1} and a 3% glucose concentration, steady states were maintained for several weeks. Residual glucose concentration was approximately 0.7 g liter^{-1}, and oxygen partial pressure was always above 50% of air saturation.

Biosurfactant concentration stood at an F_{cmc} of about 150. When an average critical micelle concentration of 15 mg

liter^{-1} (10) was used, the product concentration p amounted to 2.25 g liter^{-1} (Table 1), corresponding to a product yield on glucose ($Y_{P/S}$) of 77 mg of biosurfactant per g of glucose. The productivity of the system r_p thus amounted to 0.146 g liter^{-1} h^{-1}. It follows that a daily production of 80.6 g of biosurfactant resulted.

The biomass concentration in the bioreactor (X) was 2.5 g liter^{-1} (Table 1), giving a yield of biomass on glucose ($Y_{X/S}$) of 85 mg of biomass per g of glucose. This is a low yield but indicates that the process is not designed for biomass production. Growth is limited by iron and nitrogen (4).

The surface tension of the culture liquid was below 30 mN m^{-1}, and the interfacial tension measured against a mixture of aliphatic hydrocarbons (chain length, C$_{14}$ to C$_{18}$) was below 0.5 mN m^{-1}.

A comparison of the data from the pilot plant with that of the laboratory scale is given in Table 1. It becomes apparent that the scale-up of the production process did not excessively change the performance of the system. On the laboratory scale, higher steady-state dilution rates were possible at which the organism still produced biosurfactants. On the pilot plant scale, steady states for biosurfactant and biomass production could be established up to a dilution rate of 0.08 h^{-1}. At higher dilution rates, the system was unstable, and washout of the culture occurred. This may be a consequence of the lower biomass yields observed in the pilot plant. Considering the differences between the cultivations on laboratory and pilot plant scales, iron content of the medium seems to be the most likely reason for this. On the laboratory scale, iron needed to be added to obtain steady-state conditions (4). Due to the fact that in the pilot plant all containments were made of stainless steel (glass was used in the laboratory), iron could be omitted completely from the medium. Actually, iron introduced from the equipment and with the technical-grade chemicals seemed to be inhibitory for the cells in relation to the other medium components. With additional iron added, operation of the system at the dilution rate used here (0.065 h^{-1}) was not possible. Washout of the culture occurred.

Our data confirm that biosurfactant production is related to slowly growing cells (4). Wagner et al. (11) used resting or immobilized cells of *P. aeruginosa* for rhamnolipid production. This is in accordance with the view that the rhamnolipids are derived from the cell surface and have to be considered as secondary metabolites. Wagner et al. (11) reported somewhat higher product yields ($Y_{P/S}$) when glycerol was the carbon source (approximately 100 mg g^{-1} of glycerol) and considerably higher product yields when *n*-alkanes served as the substrate in experiments with resting cells (230 mg g^{-1} alkanes). However, the productivity of the system was substantially lower when the long incubation periods (several days) are taken into account. The production of biosurfactants in continuous culture represents a most practicable system to control environmental aspects of
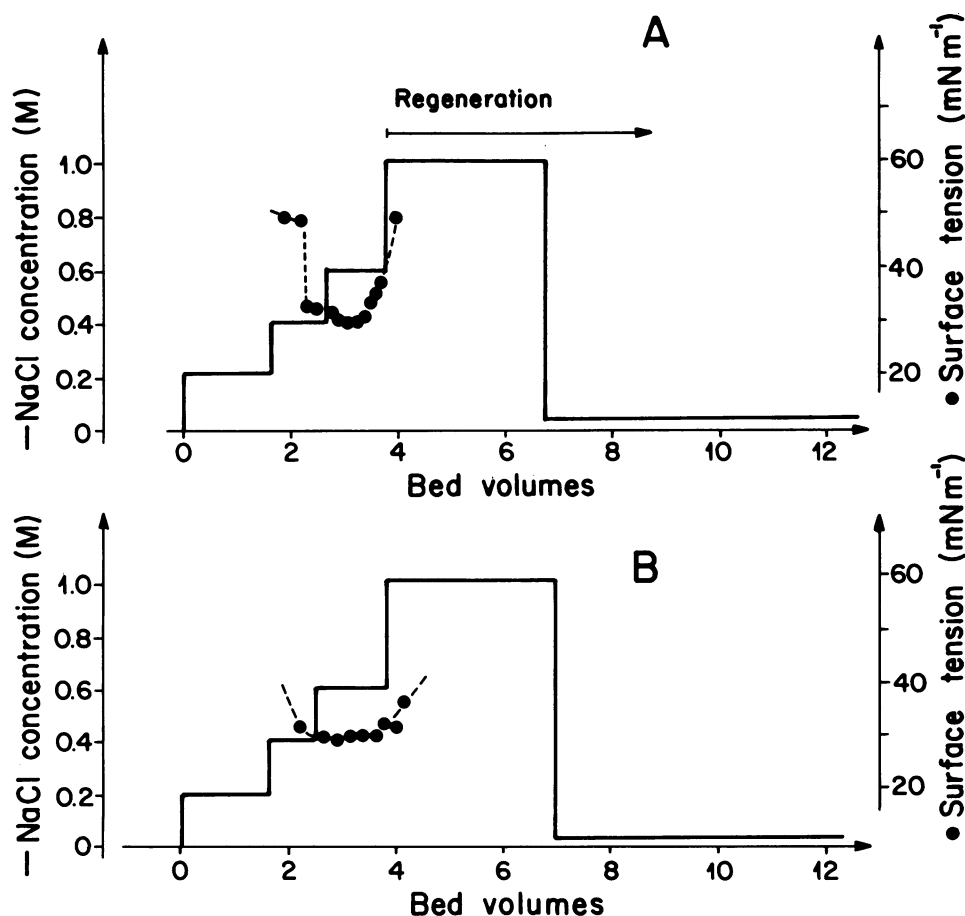
FIG. 2. Release of surface-active material from DEAE-Sepharose CL 6B after the first loading of the column (A) and after several regenerations (B).

biosurfactant production and obviously allows efficient exploitation of the potential of the cells.

A problem of the continuous production process is the excessive foaming of the culture. The amount of foam is dependent on the initial glucose concentration and on the pH of the culture. It increases with both increasing glucose concentrations and increasing pH values. In the pilot plant, it was not possible to apply glucose concentrations higher than 30 g liter^{-1}, whereas, on the lab scale, glucose concentrations of up to 70 g liter^{-1} were used. Generally, the culture in the 50-liter bioreactor was more labile than the one in the 5-liter bioreactor, most probably because the chemicals were of different purity and the materials for the containments were different from those used in the pilot plant. As a consequence, disturbances from excess foaming resulted in a decrease of biosurfactant production. Only with the conditions described and properly functioning process control could steady states be maintained for extended periods of time.

Foam formation was decreased at pH values below 6, whereas the optimum for biosurfactant production was found to be at pH 6.25. Again, reliable pH control was necessary for avoiding disturbances of the steady-state production.

Downstream processing. The adsorption on Amberlite XAD-2 proved to be the best method for the initial enrich-

ment of the rhamnolipid biosurfactants on both laboratory and pilot plant scales. The flow rate to the column had to be adjusted according to the biosurfactant concentration and the degree of loading of the column. It varied between 10 and 20 liters h^{-1}. Clogging of the column during the loading procedure probably resulted from the fact that the cell-free culture liquid contained polysaccharide material which was also formed by the cells.

The capacity of the resin for biosurfactant was calculated to be 60 g of surface-active material kg^{-1} of XAD-2. The methanol eluate from this enrichment step contained on the average 60% pure active material. The elution volume of the active fractions amounted to 1 bed volume which corresponded to approximately 10 liters. Recovery of surface-active compounds on the pilot plant level from the adsorption column was above 75%. The adsorption column was regenerated 15 to 20 times without losing efficiency or capacity.

Anion-exchange chromatography on DEAE-Sepharose CL 6B was chosen as a further purification step. A specific enrichment is possible by this type of exchanger because rhamnolipids contain a carboxylic acid group. The elution of the surface-active compounds was elaborated on the laboratory scale (Fig. 2). The material was released from the matrix with 0.6 M NaCl in a volume corresponding to approximately 2 bed volumes. Regeneration of the column did not

change the characteristics of the exchanger. On the pilot plant scale it was found that, with 0.6 M NaCl, too many bed volumes were required to release the active compounds. With the 0.8 M NaCl solution, the active material was released in 8 to 10 bed volumes. This increase in elution volume on the pilot plant scale was probably due to changing flow characteristics from the small to the larger scale. Furthermore, the capacity of the exchanger was more exhausted before elution in the pilot plant than in the laboratory.

Before elution, the column was flushed with 0.1 M NaCl to remove weakly bound products. Ion-exchange chromatography yielded an about 90% enriched product which was passed once more over an adsorption column (Amberlite XAD-2), and the biosurfactant was released with methanol. The solvent was evaporated, and the highly viscous residue was lyophilized. A slightly brownish powder resulted which was stored in a dessicator because the biosurfactants were highly hygroscopic.

The anion-exchange chromatography proved to be the best method for further enrichment of the biosurfactants. It was, for example, superior to acid precipitation of the biosurfactant, which gave rise to higher losses. The recovery of the products from ion-exchange chromatography was over 90%. The binding capacity of the DEAE-Sepharose CL 6B was approximately 50 g of biosurfactant per liter of gel.

The disadvantages of ion-exchange chromatography concern the cost of the exchanger and the relatively low flow rates of about 6 liters h^{-1} applicable. Further improvement should be possible by changing the design and packing of the column. On the laboratory scale, we also tried other ion-exchange materials (e.g., DEAE-Sephadex), but none of them was as well suited as DEAE-Sepharose with respect to elution and regeneration.

The overall recovery of the surface-active compound by the methods described (centrifugation and three chromatography steps) was approximately 60%. This value also contains inaccuracies originating from the exact amount of the two rhamnolipids present in culture liquid. The calculations reported were based on rhamnose content and the relative amount of both rhamnolipids, which was determined in the early stages of the work to be of R-1 (one rhamnose unit) and of R-2 (two rhamnose units). The exact composition may have changed slightly, depending on the cultivation conditions and therefore, makes the significance of the calculated values only relative. Nevertheless, the methods elaborated proved to be applicable on the pilot plant level, and it was possible to demonstrate that large-scale extraction can be avoided in a production process for biosurfactants.

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