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Pseudomonas aeruginosa Biosurfactant Production in Continuous Culture with Glucose as Carbon Source

LUIS GUERRA-SANTOS, OTHMAR KÄPPELI,* AND ARMIN FIECHTER

Department of Biotechnology, Swiss Federal Institute of Technology, Hönggerberg, 8093 Zurich, Switzerland

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Rsan-ver, a strain of *Pseudomonas aeruginosa* isolated at this department, was used for the development of a continuous process for biosurfactant production. The active compounds were identified as rhamnolipids. A final medium for production was designed in continuous culture by means of medium shifts, since the formation of surface-active compounds was decisively influenced by the composition and concentration of the medium components. In the presence of yeast extract, biosurfactant production was poor. For the nitrogen-source nitrate, which was superior to ammonium, an optimum carbon-to-nitrogen ratio of ca. 18 existed. The iron concentration needed to be minimized to 27.5 μg of $FeSO_4 \cdot 7H_2O$ per g of glucose. A carbon-to-phosphate ratio below 16 yielded the maximum production of rhamnolipids. The final productivity dilution rate diagram indicated that biosurfactant production was correlated to low growth rates (dilution rate below 0.15 h^{-1}). With a medium containing 18.2 g of glucose $liter^{-1}$, a biosurfactant concentration (expressed as rhamnolipids) of up to 1.5 g $liter^{-1}$ was obtained in the cell-free culture liquid.

Interest in microbial surfactants has increased considerably in recent years, especially due to their potential application in enhanced oil recovery. The production of surface-active compounds by microorganisms is well established (1, 5, 13, 19) and has been a matter of discussion at different international meetings (6, 18). Their potential for enhanced oil recovery is based on their application as agents for rock wetting, micellar flooding, emulsification, deemulsification, and viscosity reduction of heavy crude oils (8).

Hydrocarbons are commonly used as the substrate for the production of biosurfactants. It has been postulated that the biological function of surface-active compounds is related to hydrocarbon uptake, and therefore a spontaneous release occurs with these substrates (10, 12, 16, 19). Carbohydrates were rarely used as carbon and energy source for biosurfactant production with the exceptions of *Arthrobacter* sp. (21), *Bacillus subtilis* (3), *Torulopsis bombicola* (4), and *Pseudomonas aeruginosa* (9). Although less qualified for spontaneous formation, the production of biosurfactants from carbohydrate substrates offers some advantages as compared with hydrocarbons. From an engineering point of view, hydrocarbon substrates require more sophisticated equipment and more power input to achieve an adequate dispersion of the insoluble hydrocarbons. In addition, the availability of hydrocarbons is limited if applications of biosurfactants other than in enhanced oil recovery are envisaged.

P. aeruginosa production of surface-active compounds has been reported already (11, 13, 15). In these studies, the minimal surface tension of the cell-free culture broth was about 40 mN m^{-1}. The surface activity was always related to a glycolipid moiety, i.e., a rhamnolipid. Two kinds of rhamnolipids were formed by *P. aeruginosa*. One, called R-1 (7), consisted of two molecules of rhamnose and two molecules of β-hydroxydecanoic acid. Later, a second kind of rhamnolipid, R-2, was identified. It was similar to R-1, but the molecule contained only one rhamnose unit (13). It was demonstrated that both R-1 and R-2 are essential for *P. aeruginosa* growth on hydrocarbons (14). The rhamnolipids were produced when hydrocarbons, glycerol, glucose, or peptone was the substrate (11, 13–15). Best production was obtained with hydrocarbons or glycerol (13).

For the bulk production of biosurfactants, a primary prerequisite is the definition of culture conditions in which high yields of surface-active compounds are obtained by a particular microorganism. This forms the basis for a realistic estimation of the feasibility of biosurfactant production. We report here on such basic studies which aimed at the design of a continuous process for biosurfactant production by a *P. aeruginosa* strain with glucose as the carbon source.

MATERIALS AND METHODS

Microorganism. The Rsan-ver strain used throughout this work was isolated at this institute from soil samples and later identified as *P. aeruginosa* DSM2659. It was maintained on agar slants (1.5% agar in medium 1M) (Table 1) at 4°C and transferred at 3-month intervals.

Media. The compositions of the media used in this study are listed in Table 1.

Cultivation conditions. Growth of microorganisms was in a 5-liter compact loop (color) bioreactor (developed at this department) equipped with a mechanical foam separator. The temperature was 37°C, and the pH was controlled at 6.5 with 1 N KOH. Stirrer and foam separator speeds were set at 1,500 and 2,000 rpm, respectively. The working volume was 1.5 liters, and the aeration was 2.25 liters min^{-1}. The medium optimization was performed at a dilution rate of D = 0.10 h^{-1}. Steady states were considered achieved when the values for dry weight, surface and interfacial tensions, and glucose and biosurfactant concentrations remained constant during at least six volume changes.

Analytical methods. Biomass was determined in triplicate by centrifugation of 10-ml samples of culture liquid at 5,500 × g during 25 min at room temperature in a Heraeus Labofuge 2000 (Heraeus-Christ, Osterode, Federal Republic of Germany). The cell pellet was washed once with distilled water, dried at 105°C for at least 24 h, and then weighed. The supernatant was used for the estimation of glucose, surface and interfacial tensions, and biosurfactant concentration.

Glucose was assayed in a YSI model 23A glucose analyzer (Yellow Springs, Ohio). The surface tension was measured

* Corresponding author.

TABLE 1. Media used for the cultivation of *P. aeruginosa* in the course of medium optimization[a]

Component (amt per g of glucose)	Medium		
	1M	2M	3M
$(NH_4)_2SO_4$ (mg)		220	
$NaNO_3$ (mg)	220	165	137.5
K_2HPO_4 (mg)	110		
KH_2PO_4 (mg)	55		
$MgSO_4 \cdot 7H_2O$ (mg)	220	22	22
KCl (mg)	11	55	55
NaCl (mg)		55	55
$CaCl \cdot 2H_2O$ (mg)	1.1	2.75	2.75
Yeast extract (mg)	0.66		
$FeSO_4 \cdot 7H_2O$ (μg)	1,100	1,100	27.5
$ZnSO_4 \cdot 7H_2O$ (μg)	82.5	82.5	82.5
$MnSO_4 \cdot H_2O$ (μg)	82.5	82.5	82.5
H_3BO_3 (μg)	16.5	16.5	16.5
$CoCl_2 \cdot 6H_2O$ (μg)	8.3	8.3	8.3
$CuSO_4 \cdot 5H_2O$ (μg)	8.3	8.3	8.3
$NaMoO_4 \cdot 2H_2O$ (μg)	5.5	5.5	5.5
H_3PO_4 (ρ = 1.71 g ml^{-1}) (μl)		825	550

[a] Medium 3M is the optimized one for the biosurfactant production (see text).

with a Fisher autotensiomat (Fisher Scientific Co., Pittsburgh, Pa.). The interfacial tension measurements between the cell-free culture liquid and a mixture of aliphatic hydrocarbons ranging from tetradecane to octadecane were performed with the same autotensiomat.

The surface-active rhamnolipids were isolated by liquid chromatography by the method of Itoh et al. (13). Rhamnose was identified by one-dimensional thin-layer chromatography on silica gel plates (Merck, Darmstadt, Federal Republic of Germany) after acidic hydrolysis of the rhamnolipids. Three solvent systems were used: (i) ethyl acetate–*n*-butanol–methanol–water (16:3:3:2 [vol/vol]) and ethyl acetate-acetic acid-methanol-water (6:1.5:1.5:1 [vol/vol]); (ii) *n*-butanol–ethyl acetate–propanol–water (35:10:6:3 [vol/vol]) and acetone-water (9:1 [vol/vol]); (iii) methanol-acetic acid-chloroform (15:5:80 [vol/vol]). Identification was performed by running rhamnose standards simultaneously.

Biosurfactant concentration was estimated by two methods, an indirect measurement and the determination of rhamnose concentration. The indirect estimation was based on the fact that the surface activity is dependent on the concentration of the active compound. When the concentration is below a certain value (critical micelle concentration [cmc]), the surface activity is lost, which is expressed by increasing surface and interfacial tensions. This property was used for the estimation of the concentration of the active compounds. The culture broth was diluted until the interfacial tension increased and the dilution factor (F_{cmc}) was determined. Increasing F_{cmc} values indicate the increasing concentration of active compounds (Fig. 1).

The other method was based on the determination of rhamnose by the following procedure. Cell-free culture broths were diluted to a rhamnose concentration of up to 25 μg ml^{-1} in 0.1 M phosphate buffer (pH 7), and then a 1-ml sample was extracted twice with 3 ml of diethyl ether. After evaporation of the organic phase, 0.5 ml of distilled water was added, and the rhamnose was quantified by the orcinol method (2). The absorbance was measured at 420 nm, with rhamnose as the standard.

Total organic carbon (TOC) content of the supernatant was assayed with a TOC analyzer (Rapid-C; Heraeus, Hanau, Federal Republic of Germany).

RESULTS

Initial biosurfactant formation by *P. aeruginosa*. The isolated strain produced surface-active compounds when cultivated in batch cultures on medium 1M (9). The minimum values for surface and interfacial tensions were 29 and 0.25 mN m^{-1}, respectively, and a maximum concentration expressed as an F_{cmc} of 8 was obtained.

The active compounds were analyzed by thin-layer chromatography as described by Itoh et al. (13). With the same solvent system, the surface-active components exhibited the R_f values established by these authors, i.e., 0.4 and 0.8. Further identification of the sugar moiety as rhamnose indicated that our *P. aeruginosa* strain most probably produced the same rhamnolipids as Itoh et al. (13) reported for their strain. The surface and interfacial tensions of a mixture of enriched rhamnolipids 1 and 2 (50 to 80% pure) were 29 and 0.25 mN m^{-1}, respectively. Therefore, these values can be considered typical for the rhamnolipid biosurfactants.

When the strain was cultivated in continuous culture, the minimum surface and interfacial tensions were 35 and 5.5 mN m^{-1}, respectively (9). It followed that the properties of the spent medium were significantly less favorable with respect to surface activity in continuous culture. Since the composition of the medium influences the amount of the biosurfactant produced (3, 20), a medium optimization was performed in continuous culture.

Influence of nitrogen source and yeast extract on biosurfactant production. Medium 2M with a glucose concentration of 18.2 g $liter^{-1}$ served as the basic medium for the optimization experiments. The influences of the nitrogen source (ammonium or nitrate) and the yeast extract on biosurfactant production were investigated by medium shifts in a continuous culture with a dilution rate of 0.1 h^{-1}. With both nitrogen sources, biosurfactant production was poor when yeast extract was present in the medium (Table 2). Still, slightly better production was obtained with nitrate, as indicated by the lower surface and interfacial tension values of the culture broth as compared for the cultivations in which ammonium served as nitrogen source.

A more pronounced influence was exerted by yeast extract. In its presence, biomass concentration was high and the substrate was completely used by the cells. When yeast extract was omitted, biomass concentration decreased and a moderate accumulation of glucose occurred, indicating a

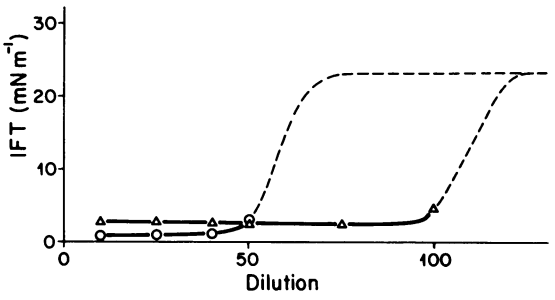

FIG. 1. Indirect measurement of biosurfactant concentration by determination of the F_{cmc}. Samples of the culture liquid were diluted until the interfacial tension (IFT) increased. Sample 1 (○) has an F_{cmc} of ca. 50, and sample 2 (△) has one of 100. It follows that the concentration of the active compound is twice as high in sample 2 as in sample 1.

TABLE 2. Influence of nitrogen source and yeast extract on *P. aeruginosa* growth and biosurfactant production[a]

Addition		Biomass	s	ST	IFT	F_{cmc}
Nitrogen source (g liter^{-1})	Yeast extract (g liter^{-1})	(g liter^{-1})	(g liter^{-1})	(mN m^{-1})	(mN m^{-1})	
$(NH_4)_2SO_4$ (4)	1.5	4.77	0.10	32	3.5	NA
	0	3.11	1.48	30	0.5	5
$NaNO_3$ (3)	1.5	5.0	0.10	30	3.0	NA
	0	3.35	0.48	29	0.25	10

[a] A continuous culture with a dilution rate of 0.10 h^{-1} and medium 2M with a glucose concentration of 18.20 g liter^{-1} were used. Abbreviations: s, residual substrate concentration; ST, surface tension; IFT, interfacial tension; F_{cmc}, dilution factor that yields a concentration of the active compounds below the cmc (Fig. 1). NA, Not applicable, because ST and IFT values were already considerably above the characteristic low values of 29 and 0.25 mN m^{-1}, respectively, and therefore the concentration of the active compounds was already below the cmc.

medium limitation other than carbon. It seems that such conditions favored biosurfactant formation by the cells, since surface and interfacial tensions decreased to 29 and 0.25 mN m^{-1}, respectively, i.e., the typical minimum values observed in the initial batch culture. In addition, better production was noted in the medium containing nitrate as the nitrogen source.

To estimate the dilution rate range for the intended optimization of the nutritional requirements for biosurfactant production, the experiments described above were also done at a dilution rate of 0.28 h^{-1}. With this dilution rate, the surface tension of the culture broth never dropped below 35 mN m^{-1}, indicating that fewer surface-active compounds were released by the cells at the higher dilution rate.

As a consequence of these results, subsequent experiments were carried out at a dilution rate of 0.10 h^{-1} with nitrate as the sole nitrogen source.

Influence of Fe concentration on growth and biosurfactant production. Of the trace elements, Fe had a major influence on *P. aeruginosa* biosurfactant production. At high Fe concentrations (2 mg · g of glucose^{-1} and above), formation of surface-active components did not occur. To establish the correlation between biosurfactant production and Fe concentration, media with different C-to-Fe ratios were used in the continuous culture, and the influence on steady-state biosurfactant concentration was determined. In medium 2M with 18.2 g of glucose liter^{-1} and the concentrations of all other components being constant, the Fe concentration was varied from 0.5 to 10 mg of $FeSO_4 \cdot 7H_2O$ liter^{-1}, yielding C-to-Fe ratios between ca. 3,600 and 72,400 (Fig. 2). The highest biosurfactant concentration (above 500 mg of rhamnose liter^{-1}, corresponding to an F_{cmc} of ca. 70) was reached at a C-to-Fe ratio of 72,400 ($FeSO_4 \cdot 7H_2O$ concentration of 0.5 mg liter^{-1}). With decreasing C-to-Fe ratios, the biosurfactant concentration also decreased. At the lowest C-to-Fe ratio of 3,600, about 150 mg^{-1} of rhamnose liter^{-1} was present (corresponding to an F_{cmc} of 8). Biomass concentration decreased slightly above a C-to-Fe ratio of 18,000, whereas no significant accumulation of glucose took place.

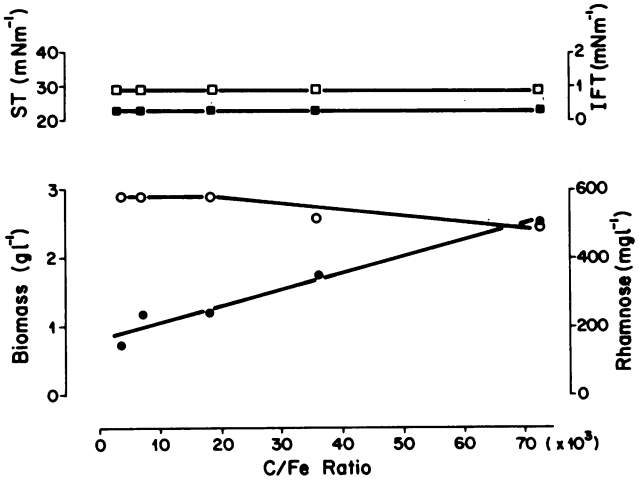

FIG. 2. Influence of the C-to-Fe ratio on biosurfactant concentration in *P. aeruginosa* cell-free culture liquid in a continuous culture with a dilution rate of $D = 0.10$ h^{-1}. Medium 2M with 18.2 g of glucose liter^{-1} and 2.5 g of $NaNO_3$ liter^{-1} was used. Symbols: □, surface tension (ST); ■, interfacial tension (IFT); ●, rhamnose, ○, biomass.

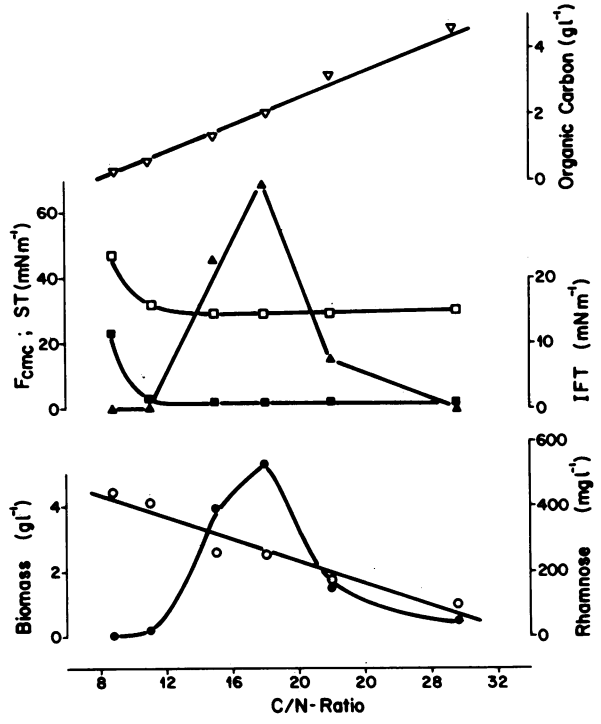

FIG. 3. Influence of the C-to-N ratio on biosurfactant concentration in *P. aeruginosa* cell-free culture liquid in a continuous culture with a dilution rate of $D = 0.10$ h^{-1}. Medium 2M with 18.2 g of glucose liter^{-1} and 0.5 mg of $FeSO_4 \cdot 7H_2O$ liter^{-1} was used. Symbols: ▽, TOC in the cell-free culture liquid; □, surface tension (ST); ■, interfacial tension (IFT); ▲, F_{cmc}; ●, rhamnose; ○, biomass.

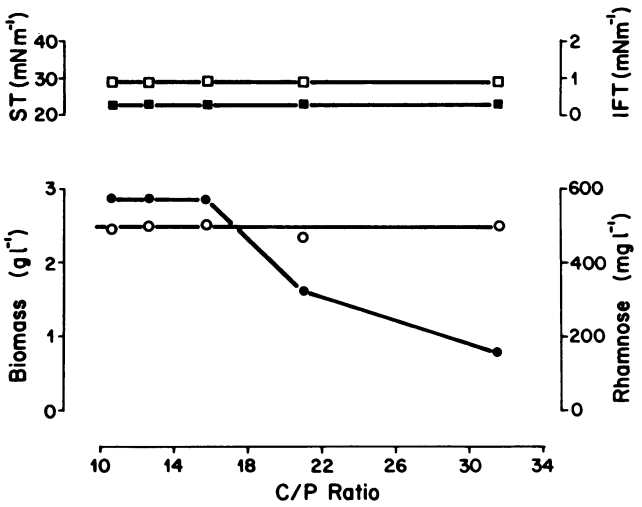

FIG. 4. Influence of the C-to-P ratio on biosurfactant concentration in *P. aeruginosa* cell-free culture liquid in a continuous culture with a dilution rate of $D = 0.10\ h^{-1}$. Medium 2M with 18.2 g of glucose $liter^{-1}$, 2.5 g of $NaNO_3$ $liter^{-1}$, and 0.5 mg of $FeSO_4 \cdot 7H_2O$ $liter^{-1}$ was used. Symbols: □, surface tension (ST); ■, interfacial tension (IFT); ●, rhamnose; ○, biomass.

This indicated that metabolism of the cells can be directed from biomass formation towards biosurfactant production by lowering the Fe concentration of the medium. It is noteworthy that a medium free of Fe could not be used with the equipment employed because no steady state was obtained in this case.

Influence of C-to-N ratio on growth and biosurfactant production. $NaNO_3$ concentration was varied between 1.5 and 5 g $liter^{-1}$ with a concentration of $FeSO_4 \cdot 7H_2O$ of 0.5 mg $liter^{-1}$ (i.e., a C-to-Fe ratio of 72,400), and all other components of medium 2M, with 18.2 g of glucose $liter^{-1}$, being constant. This yielded C-to-N ratios between 8 and 30.

In contrast to the C-to-Fe ratio, there existed an optimum C-to-N ratio of 18 for maximum biosurfactant production of the cells (Fig. 3). A decrease or increase in the concentration of nitrate was expressed in a lower rhamnose concentration and accordingly in a lower F_{cmc} value of the cell-free culture liquid. At C-to-N ratios below 11 (above 4.0 g of $NaNO_3$ $liter^{-1}$), rhamnose was no longer detected. This coincided with a partial loss of the surface activity of the cell-free culture broth as expressed by increasing surface and interfacial tensions.

Biomass concentration decreased above a C-to-N ratio of 9, indicating N-limited growth. This was supported by the absence of nitrate ions at a C-to-N ratio of 14. From the concentrations of biomass and TOC in the cell-free culture liquid, the response of the cells to nitrogen limitation became obvious. At increasing N limitations, there was a shift in the metabolism which was manifested by increasing TOC concentration and decreasing biomass concentration.

One of the released products was the rhamnolipid with a maximum formation around a C-to-N ratio of 18. Since glucose did not accumulate, it follows that the cellular metabolism was directed towards product formation by N limitation.

Influence of phosphorous on growth and biosurfactant production. Phosphorus represents another important element in bacterial metabolism. To evaluate the influence of the phosphate concentration, media with 0.71 to 2.12 g of phosphate $liter^{-1}$ were prepared, covering the C-to-P ratio range from 10 to 32.

Biosurfactant formation of the cells remained at its maximum up to a C-to-P ratio of 16 (Fig. 4). When the media of higher C-to-P ratios were applied, a decrease in biosurfactant concentration occurred. Biomass concentration did not change significantly, indicating that there was no expressed P limitation at all the phosphate concentrations tested. A certain surplus of phosphate was apparently required for *P. aeruginosa* biosurfactant formation.

Growth and biosurfactant production with optimized medium as function of dilution rate. Based on the results of the optimization, medium 3M was formulated (Table 1). With this medium, a final analysis of biosurfactant production and growth as a function of dilution rate was performed. Glucose concentration amounted to 18.2 g $liter^{-1}$.

The biosurfactant production was at its maximum up to a dilution rate of $D = 0.15\ h^{-1}$ (F_{cmc} of 68) (Fig. 5). Higher dilution rates led to a decrease in the biosurfactant and biomass concentrations, and simultaneously, glucose started to accumulate. The surface and interfacial tension values remained at their minimum up to a dilution rate of $0.23\ h^{-1}$ and then increased with increasing dilution rates.

With medium 3M, it was not possible to reach steady states at dilution rates greater than $0.32\ h^{-1}$. Washout of the culture occurred above the mentioned dilution rate.

DISCUSSION

The *P. aeruginosa* strain used in this study produced rhamnolipids when grown in batch cultures with glucose as the carbon and energy source. In view of the possible use of biosurfactants, our goal was to develop a continuous production process. Such a process has several advantages as compared with the production of biosurfactants in batch cultivations: (i) the long-term incubations of several days (3, 4, 19) are avoided, yielding a much improved productivity per unit of reactor volume; (ii) there is a constant mass flow which can be adapted to the capacity of the downstream

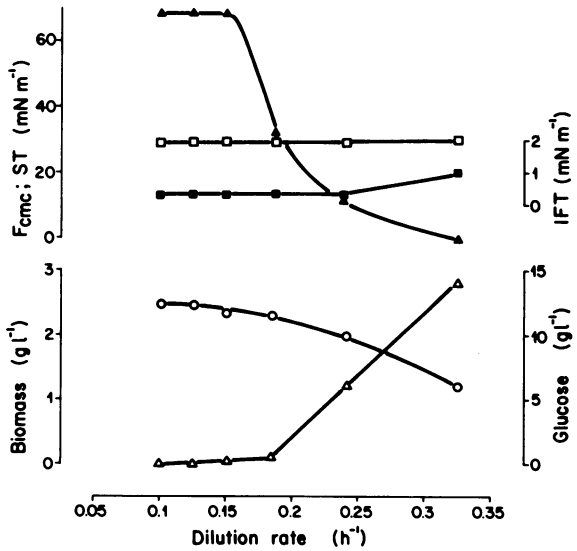

FIG. 5. Biosurfactant concentration expressed as F_{cmc} in *P. aeruginosa* cell-free culture liquid as a function of the dilution rate. Medium 3M with a glucose concentration of 18.2 g $liter^{-1}$ was used. Symbols: □, surface tension (ST); ■, interfacial tension (IFT); ▲, F_{cmc}; ○, biomass, △, glucose.

processing; and (iii) the exact control of the culture conditions which is essential for high biosurfactant formation by the cells is accomplished more easily in a continuous culture.

For the design of the continuous process, it was not possible to take over the batch data directly. When the medium which led to biosurfactant production in batch cultures was used in continuous culture, very poor formation of surface-active compounds resulted, and a medium optimization was necessary.

Several medium components influenced the formation of rhamnolipids by the cells. Additionally, there was no unique pattern of how a particular component affected the performance of the cells. An optimal C-to-N ratio for nitrate concentration (Fig. 3), a minimal iron concentration (Fig. 2), and a surplus of phosphorous (Fig. 4) yielded high biosurfactant production.

From the data of the biomass concentration and that of total organic carbon concentration in the spent medium (Fig. 3), it followed that a certain metabolic state needed to be achieved for a general product formation of the cells. By applying limitations other than carbon (iron and nitrogen here), it was possible to direct cellular metabolism to product formation. This seems to be a general concept, since medium limitations were also leading to polysaccharide production (17, 23). Because of these facts, the use of complex medium additives, such as yeast extract, has to be avoided. This became obvious in the case of biosurfactant production (Table 2).

As far as the growth rate of the cells is concerned, production of the surface-active compounds was bound to low dilution rates. When it was raised above 0.15 h^{-1}, biosurfactant production of the cells dropped (Fig. 5). Again, this observation is in accordance with other reports on the production of extracellular microbial metabolites, which are also related to low dilution rates (17, 22, 23).

Our data indicates that by proper medium design the production of surface-active compounds in continuous culture is possible. The optimization performed here gave rhamnolipid concentrations of up to 1.5 g $liter^{-1}$ with a starting glucose concentration of 18.2 g $liter^{-1}$. As compared with the concentration in the initial batch cultures, an almost 10-fold higher biosurfactant concentration resulted in the continuous process described in this study. This yield can certainly be increased by further process development or strain improvement.

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