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Continuous production of the lipopeptide biosurfactant of *Bacillus licheniformis* JF-2

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Abstract *Bacillus licheniformis* JF-2 synthesizes a surfactin-like lipopeptide that is the most effective biosurfactant known. In shake-flask cultures the biosurfactant is produced by actively growing cells (mid-linear phase), but subsequently it becomes rapidly internalized by the cells as soon as the culture enters the stationary phase. This deactivation phenomenon is a major hurdle in the efficient production of the biosurfactant. We have shown that the synthesis of the JF-2 lipopeptide is strongly dependent on O_2 concentration with substantial production observed only in cultures grown under O_2-limiting conditions. In continuous cultures the biosurfactant was produced only within a narrow window of low dilution rates. At a dilution rate of 0.12 h^{-1} and low dissolved O_2, the biosurfactant concentration was maintained at 33 mg/l, which is virtually the same as the maximum concentration obtained in optimized batch fermentations.

Introduction

Biomolecules that exhibit particularly high surface activity are classified as biosurfactants (Georgiou et al. 1992). A wide spectrum of microbial compounds, including lipopeptides, glycolipids and fatty acids, have been found to be surface active. Microbial surfactants offer two important advantages relative to synthetic detergents. First, the structural diversity of biosurfactants offers a potentially wider range of interfacial properties, some of which may be better suited for specific applications. Second, as environmental compatibility is becoming an increasingly important factor for the selection of industrial chemicals, microbially produced surfactants, which are more biodegradable, appear promising for applications such as bioremediation and the dispersion of oil spills (Harvey et al. 1990; Oberbremer et al. 1990; Van Dyke et al. 1991; Müller-Hurtig et al. 1993). At present, however, biosurfactants do not represent a viable economic alternative to synthetic detergents because of relatively high production and recovery costs.

We have been investigating the structure, interfacial properties and production of a very promising biosurfactant produced by *Bacillus licheniformis* JF-2 (Lin et al. 1993a, b). This organism has been shown to produce a very effective biosurfactant under both aerobic and anaerobic conditions (Jenneman et al. 1983; Javaheri et al. 1985; Lin et al. 1990). We have shown that the JF-2 biosurfactant is a lipopeptide having a heterogeneous C_{15} fatty-acid tail linked to a peptide moiety similar to that of surfactin, a lipopeptide antibiotic, produced by *B. subtilis* (Arima et al. 1968; Lin et al. 1993b). The *B. licheniformis* JF-2 lipopeptide is the most effective biosurfactant found so far: at concentrations as low as 25 mg/l, the purified biosurfactant reduces the interfacial tension of saline against decane to about 10^{-3} dyne/cm.

Interestingly, in shake-flask cultures, the JF-2 biosurfactant is produced during active growth (in the mid-linear growth phase) but subsequently disappears from the fermentation broth within 6 h (Lin et al. 1993a). We have shown that the disappearance of the biosurfactant from the fermentation broth is due to its internalization by stationary-phase cells. This process was found to be independent of metabolic energy and was not simply mediated by hydrophobic or electrostatic interactions. Thus, addition of synthetic surfactants or changes in the ionic strength of the fermentation broth failed to release the biosurfactant from the cells or to significantly change the rate of internalization.

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The unusual kinetics of surfactant formation and the short duration of the production phase present a major hurdle in optimizing the fermentation. Since the time between the first appearance of the biosurfactant in the fermentation broth and its complete disappearance is only about 10 h and each biosurfactant assay takes about 1 h to complete, it is practically impossible to determine the optimal time for harvesting the culture. Production is further complicated by the apparent dependence of the fermentation on the age and density of the inoculum (Javaheri et al. 1985; Lin et al. unpublished results).

In this study we show that the synthesis of the JF-2 lipopeptide is markedly affected by low dissolved O_2 (DO) concentrations and is almost completely inhibited by high O_2 concentrations. We also demonstrate that high lipopeptide concentrations can be attained in continuous culture operated under O_2-limiting conditions and at optimal dilution rates.

Materials and methods

Fermentations. B. licheniformis JF-2 ATCC no. 39307 was used in all experiments. Fermentations were conducted in a New Brunswick BioFlo III fermenter (Edison, N.J., USA) equipped with a polarographic O_2 electrode. The working volume for batch fermentations was 2 l and the starting working volume for continuous fermentations was 2.5 l. All fermentations were conducted at 42°C using a mineral salt medium supplemented with 1% glucose and sodium chloride as needed (Lin et al. 1993a). The medium pH was maintained at 7.0 by the addition of 1 M NaOH or 1 M HCl using an external pH controller. For inoculation, an overnight culture grown under identical conditions was added to the fermentor at a concentration of 5% (v/v). The DO level was maintained at either 85% or 30% by varying the agitation rate automatically.

Product analysis. Bacterial concentrations were determined by measuring the optical density of samples at 600 nm (OD_{600}). The bacterial dry cell mass was determined as a function of the OD_{600}, cell dry weight (g/l) = 0.83OD_{600} (Lin et al. 1990). Glucose concentration was determined by an enzymatic glucose analyzer (Yellow Spring Instrument Co., Yellow Springs, Ohio, USA).

To determine the concentration of the biosurfactant, culture samples were withdrawn asceptically and centrifuged at 8,000 *g* for 10 min to pellet the cells. The biosurfactant in the clarified culture supernatant was measured by reverse phase C_{18} HPLC using a Waters HPLC system (Milford, Mass., USA) equipped with a Waters C_{18} μBondapak column (7.8300 mm). The solvent system consisted of mobile phase A: 10 mM KH_2PO_4 buffer at pH 6.0 and mobile phase B: 20% tetrahydrofuran in acetonitrile (HPLC grade, Fisher Scientific, Fair Lawn, N.J., USA). For each assay, 200 μl of cell-free culture was injected and eluted with 53% B isocratically at a flow rate of 1 ml/min. The absorbance of the eluent was monitored at 210 nm. The biosurfactant eluted at 18.4 min and the peak area was calibrated using known amounts of the purified compound (Lin et al. 1993b).

Results and discussion

A series of batch fermentations were performed to determine the optimal conditions for biosurfactant production. The surfactant concentration in the fermentation broth was determined by HPLC (Lin et al. 1993a). In all earlier studies of biosurfactant production, the concentration of the active species had been determined by interfacial tension or surface tension measurements or by thin layer chromatography (Cooper et al. 1981; Jenny et al. 1993). However, the resolution of these techniques is low and the results are not quantitative. In contrast, the HPLC assay used here is specific for the surface-active lipopeptide and is highly sensitive and reproducible.

In shake-flask cultures we noticed that the production of the surfactant was dependent on the culture density at the time of harvest, which in turn was affected by the inoculum density and agitation speed (Lin et al. unpublished data). These observations suggested that production may be affected by the availability of DO. Since the synthesis of the JF-2 lipopeptide is substantially reduced under anaerobic conditions (Goursaud 1989; Lin et al. 1990), we investigated only the effect of O_2 sufficient (85% DO saturation) and O_2-limited (30% DO saturation) conditions. Figure 1A and B

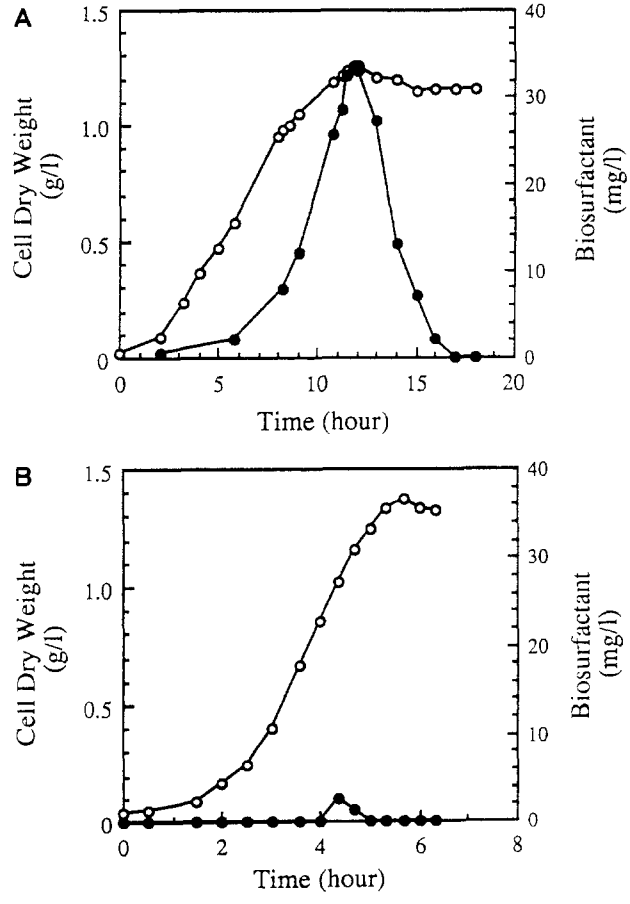

Fig. 1A, B The microbial growth and biosurfactant production profiles of *Bacillus licheniformis* JF-2 in mineral salt medium with 0.5% sodium chloride in batch fermentation under 30% (**A**) and 85% (**B**) saturation of dissolved O_2 (DO): ○, cell dry weight; ●, biosurfactant concentration

Table 1. Doubling times and maximum biosurfactant concentrations of *Bacillus licheniformis* JF-2 grown in the presence of different concentrations of sodium chloride under low and high dissolved O_2 (DO) concentrations

	Concentration of NaCl in medium (%)					
	0.0		0.5		0.2	
DO (% of saturation)	Biosurfactant conc[a] (mg/l)	t_D (h)	Biosurfactant conc (mg/l)	t_D (h)	Biosurfactant conc (mg/l)	t_D (h)
85	0.70	0.76	2.68	0.80	6.08	0.87
30	5.30	1.55	34.00	2.58	34.64	2.70

t_D, Doubling time
[a] Maximum biosurfactant concentration (mg/l) determined by HPLC

illustrate the biomass accumulation and biosurfactant production profiles in *B. licheniformis* JF-2 fermentations with 0.5% NaCl. During active growth in this medium, the cell dry weight of *B. licheniformis* JF-2 increased linearly with time. Although the rate of biomass growth during the linear, active growth phase was lower in the 30% DO fermentation, the final dry cell weight and biomass yield were not significantly affected by the level of DO. Both the duration of the biosurfactant production phase and the maximum concentration were markedly higher in the 30% DO fermentation. On the other hand, when the DO concentration was maintained at 85% of saturation, the production phase was barely detectable and, because of its short duration, could be identified only by frequent sample collection. These results were reproducible in independently run fermentations (data not shown). The effect of O_2 concentrations lower than 30% was not investigated because it has previously been shown that a significantly lower amount of biosurfactant was produced under anaerobic conditions (Goursaud 1989; Lin et al. 1990).

Fermentations were conducted in medium containing 0%, 0.5% or 2% (w/v) NaCl under both O_2 sufficient and O_2 limited conditions. The linear growth rates and biosurfactant production for all the batch fermentations are summarized in Table 1. For all NaCl concentrations tested, the biosurfactant concentration was higher under O_2 limited conditions. An increase in NaCl concentration resulted in a higher peak biosurfactant concentration as well as an increase in the doubling time of the culture. In the high DO fermentations, the maximum biosurfactant concentration increased from 0.70 mg/l to 6.08 mg/l in the absence or presence of 2.0% sodium chloride, respectively. Similarly, in the 30% DO saturation fermentations the maximum biosurfactant concentration increased from 5.30 mg/l to approximately 34 mg/l. No difference in biosurfactant production was observed between fermentations with either 0.5% or 2.0% NaCl. For all conditions examined, the duration of the production phase was proportional to the peak biosurfactant concentration. For example in the presence of 0.5% NaCl and under O_2 saturation conditions, the biosurfactant was detectable in the fermentation broth only for 1 h, whereas in the 30% DO fermentation measurable concentrations of biosurfactant were sustained for 15 h.

It is interesting to note that in shake-flask cultures the addition of NaCl actually resulted in a decrease rather than an increase in biosurfactant production (Lin et al. 1993a). The difference between the results of shake-flask cultures and those of the 2-fermentations reported here is probably due to the variation in DO concentration as a function of time and the different pattern of biomass accumulation in shake flasks.

The production of biosurfactant in continuous culture was investigated as a means of minimizing the deactivation process that was observed in the batch fermentations. The biomass and biosurfactant concentrations as a function of dilution rate under conditions of high and low DO are shown in Fig. 2A and B, respectively. Consistent with the results of the batch experiments, appreciable biosurfactant production was observed only when the DO concentration was low. Furthermore, in continuous culture the concentration of biosurfactant in the effluent was also a strong function of the dilution rate. Substantial production was observed only within a narrow window of dilution rates, namely between 0.1–0.2 h^{-1}. Maximum lipopeptide concentrations of 34.0 mg/l and 2.0 mg/l were detected under low and high DO concentrations, respectively. In the 30% DO continuous fermentation at the optimal dilution rate ($D = 0.12\ h^{-1}$), the biosurfactant concentration was almost the same as the maximum value obtained in batch culture. The biomass decreased with increasing dilution rates and washout was observed at $D = 0.45\ h^{-1}$.

The glucose concentration data in Fig. 2A indicate that complete utilization of the carbon source occurred only at $D < 0.05\ h^{-1}$. In the 85% DO continuous fermentation washout was not observed until $D = 1.3\ h^{-1}$ and glucose was never completely utilized throughout the experiment. As expected, when the DO concentration was maintained near saturation both the

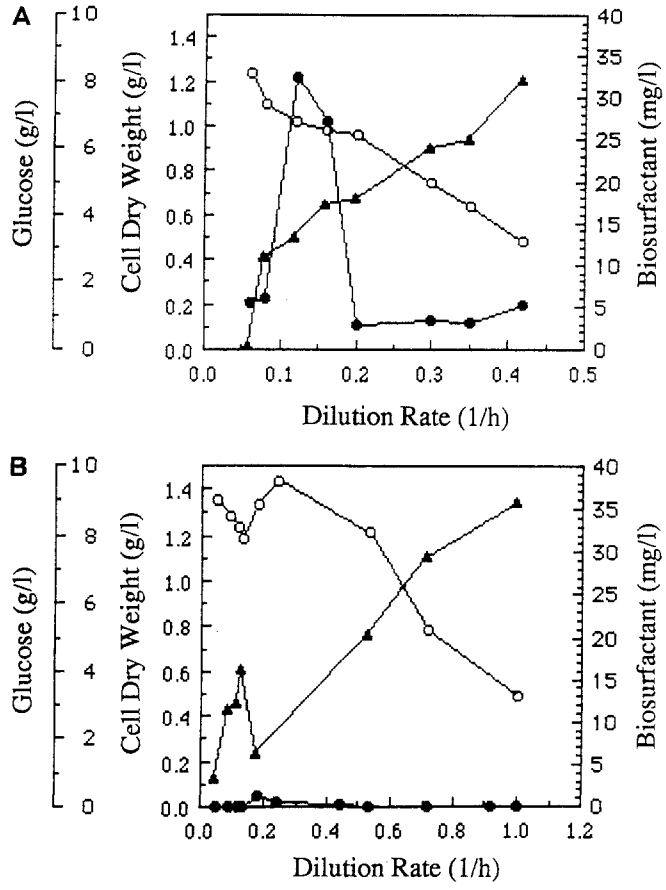

Fig. 2A, B The microbial growth and biosurfactant production profiles of *B. licheniformis* JF-2 in mineral salt medium with 0.5% sodium chloride in continuous fermentation under 30% (**A**) and 85% (**B**) saturation of DO: ○, cell dry weight; ●, biosurfactant concentration; ▲, glucose concentration

biomass concentration and the cell yield on glucose were higher than in the 30% DO experiments. Specifically the cell yield varied from 0.149 (D = 0.05) up to 0.510 (D = 1.00) g cell dry weight/g glucose consumed compared to a cell yield of between 0.125 and 0.233 in 30% DO fermentations. The fact that glucose was never completely exhausted except at very low dilution rates indicates that glucose was not the rate-limiting nutrient. In addition, the higher biomass production and cell yield in the 85% saturation fermentations indicate that growth was strongly affected by the availability of DO.

Figure 3 shows the profile of biomass accumulation and biosurfactant concentration for *B. licheniformis* JF-2 grown under low DO conditions in the absence of NaCl. Again the JF-2 lipopeptide was detectable only when the dilution rate was maintained between 0.1 and 0.2 h^{-1}. In this case the maximum biosurfactant concentration obtained in continuous culture was 11.2 mg/l compared to a peak concentration of only 5.3 mg/l in batch culture.

In this report we have shown that the lipopeptide biosurfactant from *B. licheniformis* JF-2 can be produced

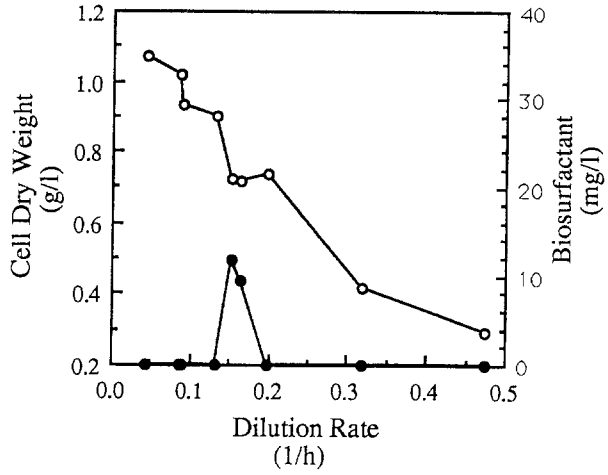

Fig. 3. The microbial growth and biosurfactant production profiles of *B. licheniformis* JF-2 in mineral salt medium without sodium chloride in continuous fermentation under 30% DO saturation: ○, cell dry weight; ●, biosurfactant concentration

continuously at concentrations at least comparable to and even higher than the maximum value that can be obtained in batch cultures. The production of this biomolecule occurs only under low DO conditions and within a narrow window of dilution rates. The strong dependence of biosurfactant production on the dilution rate is reminiscent of other *Bacillus* secondary metabolites. For example, Ataai et al. (1989) showed that the production of the major acidic protease by *B. subtilis* decreased over tenfold as the dilution rate was increased from 0.1 to 0.2 h^{-1}. The concentration of the acidic protease decreased monotonically with increasing dilution rates whereas in *B. licheniformis* JF-2 the production of the biosurfactant exhibited a distinct maximum at dilution rates of between 0.1 and 0.2 h^{-1}.

The concentration of biosurfactant in the fermentation broth is determined by the outcome of two competing processes: lipopeptide synthesis and deactivation via internalization by resting cells (Lin et al. 1993a). The observed optimal biosurfactant production is most likely a result of "decoupling" the synthesis and deactivation processes that occur in *B. licheniformis* JF-2 fermentations. At steady state the biomass presumably consists of three sub-populations: premature cells that have not yet become competent for surfactant production, cells that produce the surfactant, and finally mature slow-growing cells that are responsible for the internalization and removal of the surfactant from the fermentation broth. At the optimal dilution rate the fraction of surfactant-producing cells relative to the other two is maximal. As the dilution rate is increased, premature, non-producing cells become increasingly predominant resulting in a lower surfactant concentration. At the other end (i.e. at sub-optimal dilution rates) the fraction of resting cells is higher and thus also leads to poor biosurfactant production.

We have found that the concentration of the JF-2 biosurfactant is strongly dependent on the DO concentration both in batch and continuous cultures. Interestingly, the production of surfactin, an almost identical compound from *B. subtilis*, is not known to be O_2 dependent (Cooper et al. 1981). Jenny et al. (1993) recently reported that the surface tension of cultures of a *B. licheniformis* strain that produces a lipopeptide with a slightly different peptide chain is lower under O_2-limiting conditions. Assuming that the surface tension is proportional to the concentration of lipopeptide, these results may be indicative of another case of O_2-dependent biosurfactant production. However, in contrast to our results, in the study of Jenny et al. (1993) the surface tension of the fermentation broth was not significantly affected by the dilution rate.

As mentioned earlier, the concentration of biosurfactant in the fermentation broth is determined by the rates of biosurfactant production and deactivation. Thus, at a macroscopic level, there are four possible explanations for the dependence of the biosurfactant production on the DO concentration:

1. The O_2 level directly affects the biosurfactant synthesis rate.

2. The rate of biosurfactant production is independent of the growth conditions but the rate of deactivation (via adsorption onto the cell mass) is inhibited under O_2-limiting conditions.

3. Both the synthesis and deactivation rate are affected by O_2.

4. Biosurfactant production is not directly controlled by the DO level per se but rather by the growth rate, which in turn depends on the availability of O_2 and NaCl concentration.

Although the effect of DO on growth, surfactant deactivation and production cannot be easily delineated from the available data, hypotheses 2 and 3 can be ruled out because the adsorption of the JF-2 biosurfactant is independent of metabolic energy (Lin et al. 1993a). It is, therefore, unlikely that the level of DO significantly affects the rate of JF-2 biosurfactant deactivation.

Hypothesis 4 can best explain the dependence of the biosurfactant production on the availability of DO. It is possible that a lower growth rate extends the production phase while at the same time it delays the onset of stationary phase and the concomitant deactivation of the surfactant. This explanation is supported by the results obtained in batch fermentations. As the level of DO is increased and/or sodium chloride concentration is decreased, the doubling time of the culture increases and thus, the overlap between production and deactivation increases resulting in a lower surfactant concentration, as shown in Table 1.

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References

Arima K, Kakinuma A, Tamura G (1968) Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. Biochem Biophys Res Commun 31:488–494

Ataai MM, Strohm EJ, Jeong JW (1989) *Bacillus subtilis* as a host of recombinant vectors: the protease problem. In: Todd P, Sikder SK (eds) Frontiers in Bioprocessing. CRC press, pp. 167–171

Cooper DG, Macdonald CR, Duff SJB, Kosaric N (1981) Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Appl Environ Microbiol 42:408–412

Georgiou G, Lin SC, Sharma MM (1992) Surface-active compounds from microorganisms. Bio/Technology 10:60–65

Goursaud JC (1989) Production of a biosurfactant by *Bacillus licheniformis* JF-2. MS Thesis, The University of Texas, Austin

Harvey S, Elashvili I, Valdes JJ, Kamely D, Chakrabarty AM (1990) Enhanced removal of Exxon Valdez spilled oil from Alaskan gravel by a microbial surfactant. Bio/Technology 8:228–230

Javaheri M, Jenneman GE, McInerney MJ, Knapp RM (1985) Anaerobic production of a biosurfactant by *Bacillus licheniformis* JF-2. Appl. Environ Microbiol 50:698–70

Jenneman GE, McInerney MJ, Knapp RM, Clark JB, Ferro JM, Revus DE, Menzie DE (1983) A halotolerant, biosurfactant-producing *Bacillus* species potentially useful for enhanced oil recovery. Dev Ind Microbiol 24:485–492

Jenny K, Deltrieu V, Käppeli O (1993) Lipopeptide production by *Bacillus licheniformis*. In: Kosaric N (ed) Biosurfactants. Dekker, New York, p 135

Lin SC, Goursaud JC, Kramer PJ, Georgiou G, Sharma MM (1990) Production of biosurfactant by *Bacillus licheniformis* strain JF-2. In: Donaldson EC (ed) Microbial enhancement of oil recovery—recent advances. Elsevier, Amsterdam, p 219

Lin SC, Sharma MM, Georgiou G (1993a) Production and deactivation of biosurfactant by *Bacillus licheniformis* JF-2. Biotechnol Prog 9:138–145

Lin SC, Sharma MM, Georgiou G (1993b) Purification, structural and immunological characterization of a biosurfactant produced by *Bacillus licheniformis* JF-2. Appl Environ Microbiol 60:31–38

Müller-Hurtig R, Blaszczyk R, Wagner F, Kosaric N (1993) Biosurfactants for environmental control. In: Kosaric N (ed) Biosurfactants. Marcel, New York, p 447

Oberbremer A, Müller-Hurtig R, Wagner F (1990) Effects of the addition of microbial surfactants of hydrocarbon degradation in a soil population in a stirred reactor. Appl Microbiol Biotechnol 32:485–489

Van Dyke MI, Lee H, Trevors JT (1991) Applications of microbial surfactants. Biotechnol Adv 9:241–252