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Kinetic studies on surfactant production by *Pseudomonas aeruginosa* 44T1

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SUMMARY

Biosurfactant accumulation occurred in the exponential and stationary phases. Production started when the nitrogen level was very low. Surfactant was produced with a diauxic pattern. Rhamnolipid concentration increased as nitrogen levels increased. Maximum product yield ($Y_{p/x}$) 2.9 was detected when C/N ratio was 6.6 and specific rate of product formation (p_q) was calculated. The examination of these kinetics parameters such as product yield and specific rate of product formation should be taken into account to develop a high efficient production process.

INTRODUCTION

Bacillus subtilis [2], *Corynebacterium lepus* [5], *Acinetobacter calcoaceticus* [7] and *Pseudomonas* [19] are reported as biosurfactant producers [12, 21]. Much attention has been focused on the potential of biological surfactants to replace conventional surfactants in textiles, pharmaceutical, cosmetics and food industries [11].

It is of industrial interest to develop a large scale production process to be economical competitive with synthetic surfactants. Therefore in order to produce biosurfactants in a large scale, studies concerning the kinetics and cultural conditions which affect production are needed. Cooper et al. [3] reported the influence of metal ions on surfacting production; Guerra-Santos et al. [20] published the environmental effect on rhamnolipid production by *Pseudomonas aeruginosa* in continuous culture. However, little information has been published concerning the effect of the carbon and nitrogen supply.

In the present work, we examined the kinetics of rhamnolipid production by *Pseudomonas aeruginosa* 44T1 [17] in relation to the C/N ratio of a medium, containing vegetable oil. This study represents a basis to develop a process for a large scale rhamnolipid production with *Pseudomonas aeruginosa* 44T1.

MATERIALS AND METHODS

Organism and growth conditions

Pseudomonas aeruginosa 44T1 was originally isolated in our laboratory [1]. It was maintained on nutrient agar slants and subcultured every two weeks. A 1% v/v cell suspension of a 24 h culture was used as inoculum. Cells were grown in a liquid medium containing (g/l): KCl, 0.1; KH_2PO_4, 0.5; K_2HPO_4, 1; $CaCl_2$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; yeast extract, 0.1; and 0.05 ml/l of the following solution of trace elements: B (0.026%), Cu (0.05%), Mn (0.05%), Mo (0.006%) and Zn (0.07%). Olive oil used (20 g/l) as carbon source was supplied by Cooperativa San Gregorio, Spain. $NaNO_3$ was the nitrogen source, added at 7, 5, 3, 1 g/l. pH was adjusted at 6.8.

In order to study the kinetics of biosurfactant accumulation fermentations were carried out in 2000 ml baffled Erlenmeyer flasks with 200 ml of medium and incubated at 30 °C with an agitation speed of 200 rpm.

Analytical methods

Nitrate content of the medium was followed by nitrate test (Merck, 8032). Rhamnose content was measured as rhamnose by the method described by Chandrasekaran and Bemiller [4]. Rhamnolipid values were determined by multiplying rhamnose values by a standard coefficient of 3. This coefficient represent the ratio rhamnolipid/rham-

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nose (w/w) content of the biosurfactant. The linear correlation was demonstrated between the quantity of rhamnolipid and optical density determined by the method reported by Itoh [10].

Biomass content

Cell growth measurements were carried out by the method of Lowry [13] after cell lysis with equal volume of 2 N NaOH for 3 min at 100 °C. Standard bovine serum albumin (Sigma) was treated in an identical manner.

RESULTS AND DISCUSSION

Production of surface-active rhamnolipids by different strains of *Pseudomonas* has been previously reported [8,10,12]. Studies have been published on the effect of culture medium components either on batch [18] or continuous culture fermentation [20].

In our earlier studies on the environmental control of the production of rhamnolipid with surface active properties by *Pseudomonas aeruginosa* 44T1, different responses to medium components were found [6]. Sodium nitrate appeared to be the best nitrogen source for rhamnolipid production. Olive oil, among other hydrophobic and hydrophilic carbon sources tested, was found to be the best carbon supply for rhamnolipid accumulation [17].

In the aerobic fermentation processes the biosurfactant was produced during the exponential and non-exponential growth phases (Figs. 1–4). Production started when nitrogen concentration reached low values (below 0.5 g/l). Finally it was recovered by liquid-liquid extraction from the supernatant of the culture.

Between 40–60 h of incubation depending on the C/N ratio (Figs. 1–4), an increase on the slope of the surfactant production curve could be detected. At this stage of fermentation growth has ceased due to the exhaustion of the nitrogen source. Only 40% of the total surfactant produced was observed. Thus rhamnolipid accumulation has

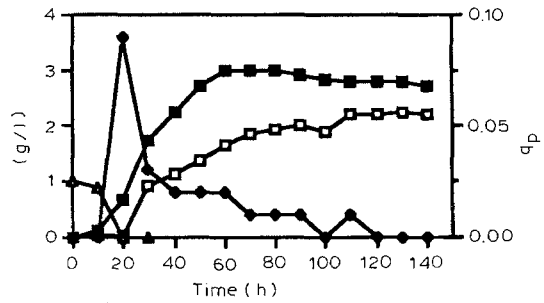

Fig. 1. Time course of rhamnolipid accumulation by *Pseudomonas aeruginosa* 44T1 on olive oil mineral medium at C/N ratio of 20. (■) Cell growth (g/l); (○) rhamnolipid (g/l); (◆) q_p (g product/g cell × h); (△) residual nitrogen (g/l).

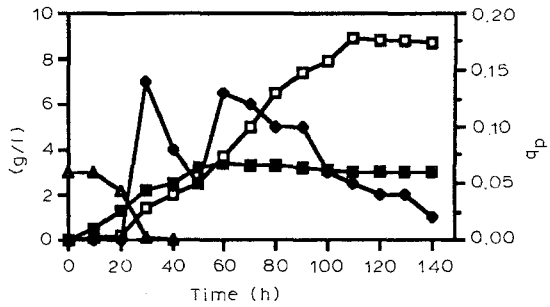

Fig. 2. Time course of rhamnolipid accumulation by *Pseudomonas aeruginosa* 44T1 on olive oil mineral medium at C/N ratio of 6.6. (■) Cell growth (g/l); (○) rhamnolipid (g/l); (◆) q_p (g product/g cell × h); (△) residual nitrogen (g/l).

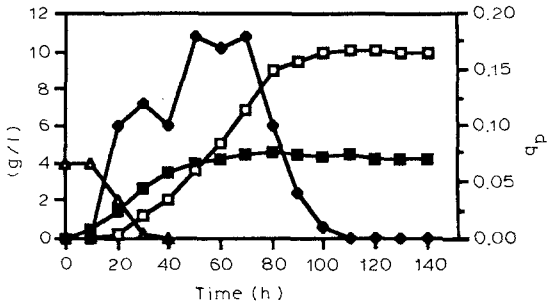

Fig. 3. Time course of rhamnolipid accumulation by *Pseudomonas aeruginosa* 44T1 on olive oil mineral medium at C/N ratio of 5. (■) Cell growth (g/l); (○) rhamnolipid (g/l); (◆) q_p (g product/g cell × h); (△) residual nitrogen (g/l).

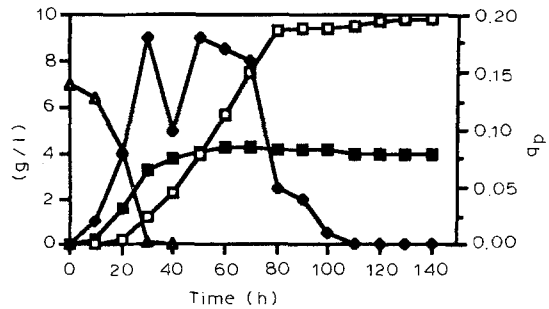

Fig. 4. Time course of rhamnolipid accumulation by *Pseudomonas aeruginosa* 44T1 on olive oil mineral medium at C/N ratio of 2.8. (■) Cell growth (g/l); (○) rhamnolipid (g/l); (◆) q_p (g product/g cell × h); (△) residual nitrogen (g/l).

a diauxic profile, results which are in accordance with those reported by Wagner et al. [19].

Other carbon sources tested either hydrophobic, *n*-alkanes mixture or hydrophilic, glucose, surfactant accumulation followed a diauxic pattern as reported elsewhere [16]. The diauxic profile of rhamnolipid accumula-

tion seemed to be independent of the carbon source supplied. This phenomenon may be due to a change in the permeability of the cell when the nitrogen source was exhausted and bacterial growth ceased. However the carbon excess continued to be taken up by *Pseudomonas aeruginosa* 44T1 and it was converted into rhamnolipids.

The profile of rhamnolipid formation by *Pseudomonas aeruginosa* 44T1 could be clearly observed when the specific rate of product formation (q_p) was calculated [15]. Under our conditions examined, the effect of different C/N ratios can also be clearly observed when this parameter versus incubation time is plotted (Figs. 1–4).

As shown in Fig. 1 nitrogen limiting conditions did not favor biosurfactant accumulation. Under these conditions, product yield ($Y_{p/x}$) was very low (0.8; Table 1). The specific rate of product formation was also very low (0.09 h^{-1}) at the end of the exponential phase (Fig. 1).

When the initial nitrogen content of the medium (Fig. 2) was increased, the rate of product formation was similar in both the exponential and the stationary phases. Under these conditions, the C/N ratio is 6.6 and the highest product yield was achieved (Table 1).

High values of specific rate of product formation at C/N ratios of 4 (Fig. 3) and 2.8 (Fig. 4) were observed. About 44–50% of the utilized carbon was used for synthesis of rhamnolipid. Production yield after 100 h of incubation was only 2.14 g/l and 2.8 g/l. The total amount of rhamnolipid accumulated was 9.1 and 10 g/l, respectively.

Growth and surfactant production were found to be affected by the concentration of nitrogen. When the initial nitrogen concentration was 5 or 7 g/l, the total biomass did not increase as it was expected (Table 1). This observation suggests that rhamnolipid production from olive oil is a fairly complex phenomenon. The nitrogen source was obviously channeled to other metabolic ways. *Pseudomonas aeruginosa* 44T1 probably produces extracellular lipases to break down triglycerides, in order to catabolize the fatty acids released. The exact mechanism by which the nitrate ion affects cell growth and rhamnolipids biosynthesis is not known. One possible explanation is that *Pseudomonas aeruginosa* 44T1 may reduce nitrate by anaerobic respiration even in presence of oxygen, although the significance of this regulation in presence of different amounts of nitrogen is not known [9].

TABLE 1

Effect of C/N ratio on product yield formation

C/N	Biomass (g/l)	Product (g/l)	$Y_{p/x}$ (g/l)
2.8	4.24	9.1	2.14
4	4.2	10	2.38
6.6	3	8.8	2.9
20	2.6	2.2	0.84

Although rhamnolipid production from vegetable oils may have technical difficulties in fermentation and downstream processing, due to the interface of the hydrophobic-hydrophilic mixture, they do have different advantages: (1) they contain a high percentage of assimilable substrate and (2) they are suitable substrates to induce high biosurfactant production.

Our observations give technical support in order to design and develop future processes for large scale rhamnolipid production in a continuous or resting cells culture by *Pseudomonas aeruginosa* 44T1 grown on the water insoluble triglyceride mixture of olive oil. The examination of the kinetic parameters such as product yield formation should be taken into account to develop a high efficient production process.

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