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Effective production of *Pseudomonas fluorescens* lipase by semi-batch culture with turbidity-dependent automatic feeding of both olive oil and iron ion

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Summary. An automatic feeding system to supply olive oil in semi-batch culture was established by monitoring cell concentration with a laser turbidimeter combined with a microcomputer and a pulse motor. In this automatic feeding system, specific olive oil supply rate (g olive oil) · (g dry cell)$^{-1}$ · h^{-1}, q_0, was changed in an appropriate range. Attempts were made to produce lipase by a turbidity-dependent automatic fed-batch culture of *Pseudomonas fluorescens*. It was found from the semi-batch cultures with turbidity-dependent feeding of olive oil and with varied initial Fe ion concentrations that excess Fe ion was inhibitory to formation of the lipase. Turbidity-dependent automatic simultaneous feeding of olive oil and Fe ion was performed to obtain semi-deficiencies of both the oily substrate in the culture liquid and Fe content of the cells. Using this semi-batch culture, high lipase activity, 5600 units/ml, was attained at an optimal value of q_0.

Introduction

Lipase catalyses hydrolysis of fats, and mono- and di-glycerides to give free fatty acids and glycerol, and conversely catalyses synthesis of glyceride from glycerol and free fatty acids. Also, it catalyses trans-esterification reactions which cover acidolysis, alcoholysis, ester exchange and aminolysis. Lipase-catalysed trans-esterification permits one to obtain many industrially important products. The main disadvantage of lipase-catalysed reactions is the cost of lipase. Several papers (Morinaga et al. 1986; Omar et al. 1987; Lechner et al. 1988; Sztajer and Maliszewka 1988) have been published recently on the production of microbial lipases. In order to lower the cost of microbial lipase production, we are studying lipase production by fed-batch cultures. In a previous paper (Suzuki et al. 1988), we reported that *Pseudomonas fluorescens* lipase was produced effectively by supplying olive oil, the feed rate of which was controlled automatically by the CO_2 evolution rate in the exhaust gas. At the optimal ratio of the feed rate of olive oil to the CO_2 evolution rate, a high activity of lipase, 2000 units/ml, was achieved.

In relation to the development of off-flavors in microbially-contaminated milk, the influence of iron on extracellular lipase formation by *P. fluorescens* B 52 was recently reported (McKellar et al. 1987). Excess iron reduced the biosynthesis of lipase. Investigations were, however, performed only in shake-flask cultures. We report here the effect of Fe ions on the formation of *P. fluorescens* lipase, and the effective production of lipase by a semi-batch culture technique with turbidity-dependent automatic feeding of both olive oil and Fe ion simultaneously.

Materials and methods

Strain and medium

The strain used in this study was one reported previously (Suzuki et al. 1988). The composition of the basal medium was as follows (per litre distilled water): 20 g olive oil, 6 g $(NH_4)_2SO_4$, 20 g KH_2PO_4, 10 g Na_2HPO_4, 5 g $MgSO_4$, 3 g $CaCl_2 \cdot 2H_2O$, 0.02 g $MnSO_4 \cdot 4 \sim 6H_2O$, 0.005 g $CoCl_2 \cdot 6H_2O$, 0.005 g $CuSO_4 \cdot 5H_2O$, 0.005 g $ZnSO_4 \cdot 7H_2O$, 5 g antifoamer (Adekanol LG = 109, Asahi Denka Industry Co. Tokyo, Japan) and varied amounts of $FeSO_4 \cdot 7H_2O$.

Cultivation

Culture conditions were the same as used previously (Suzuki et al. 1988) except that the composition of the preculture me-

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dium was (per 100 ml distilled water): 1.0 g olive oil, 0.3 g $(NH_4)_2SO_4$, 0.2 g KH_2PO_4, 0.2 g Na_2HPO_4, 0.05 g $MgSO_4$ (pH was adjusted to 7.0 with NaOH) and that the initial volume of the culture was 1.3 l.

Control of olive oil and Fe ion supply

For investigation of the effects of olive oil and Fe ions on both cellular growth and lipase production, three different methods of cultivation were compared.

Batch culture. The initial concentration of Fe ions in the basal medium was 0, 10, 30 or 100 ppm. No olive oil was supplemented after the initial olive oil was consumed.

Semi-batch culture with turbidity-dependent feeding of olive oil. Cultivation was divided into two phases. The initial Fe ion concentrations were set at 0, 10, 20, 30, 40, 70 or 100 ppm, and the early period (ca. 20 h) was the cell growth phase. When the initial olive oil was consumed after about 20 h of batch culture, the dissolved oxygen (DO) rose sharply and the cell concentration reached about 10 $g \cdot l^{-1}$. At this time, feeding of olive oil was started using a turbidity-dependent feeding policy. The later phase was the lipase production phase. In the turbidity-dependent fed-batch culture, the feed rate of olive oil (f in g/h) was automatically varied according to the following equation,

$$f = q_0/(XV)$$

where X, V and q_0 are the cell concentration (g/l), the total volume of the culture broth (l) and specific olive oil feed rate (g oil · (g dry cell)$^{-1}$ · h^{-1}), respectively; XV is total cell mass in the bioreactor. Values of X were estimated by a turbidity sensor-turbidimeter-microcomputer system (see Analytical procedures). Utilizing a relationship predetermined between the turbidity and cell concentration (g dry cell · l^{-1}), the microcomputer calculated X and then f from the input of turbidity transmitted from the turbidimeter. The output of the computer controlled a pulse motor which changed the rotational speed of a tubing pump; V was assumed constant. Since the lipase was most effectively produced when q_0 was controlled below 0.06 (g oil · (g dry cell)$^{-1}$ · h^{-1}) for this type of fed-batch culture (Suzuki et al. 1988), q_0 was set at 0.05 (g oil · (g dry cell)$^{-1}$ · h^{-1}) in these experiments. The total amount of olive oil supplied was 100 g/l in all the experiments.

Fed-batch culture with turbidity-dependent feeding of both olive oil and Fe ion. The culture was divided into two phases. In the early phase the cells grew batchwise in the basal medium containing 43 $g \cdot l^{-1}$ olive oil and 10 $mg \cdot l^{-1}$ Fe. After 40 h batch culture, when DO rose sharply, the turbidity-dependent feeding of both olive oil and Fe ion solution (1.08 g $FeSO_4 \cdot 7H_2O$/l) started. The cell concentration reached about 35 $g \cdot l^{-1}$ at this time. The values of q_0 were set at 0.0065, 0.008, 0.011, 0.023 and 0.033 (g oil · (g dry cell)$^{-1}$ · h^{-1}). It was expected that the cell growth rate could be controlled by changing the value of q_0. The principle of turbidity-dependent automatic fed-batch culture will be published elsewhere. The amount of olive oil and Fe ion fed totally were 43 g/l and 10 mg/l, respectively.

The turbidity-dependent fed-batch culture system is drawn schematically in Fig. 1.

Analytical procedures

Turbidity of the culture was monitored by an on-line laser turbidimeter (model LT-201, Komatsugawa Kakoki Co., Tokyo, Japan). Basal medium exhibited some turbidity value due to the antifoamer as well as insoluble precipitates of mineral ions. Bubbles of oxygen also gave small additional turbidity. These turbidities were cancelled to zero at the beginning of the measurement when the culture was started. It was found that the increase in turbidity by the emulsion of mono-, di- and tri-glycerides was quite small because, in the phase of the fed-batch cultures, their concentrations were very low (see Fig. 4) and the cell concentration was high.

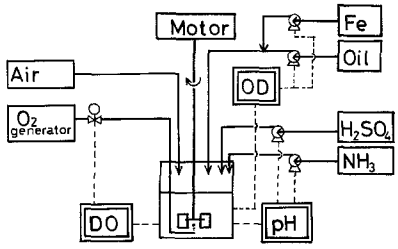

Fig. 1. System for turbidity-dependent fed-batch culture; OD = turbidity; DO = dissolved oxygen

Eleven millilitres of culture broth were taken from the bioreactor for analysis of the concentrations of tri-, di-, monoglycerides, fatty acids, turbidity, intracellular content of Fe, Fe concentration of the culture supernatant, and activity of lipase. From the sample, 10 ml was centrifuged at 15000 g for 15 min at 4° C. The lipase activity of the supernatant was determined by the olive-oil non-emulsion method (Yamane 1987) using an enzymic reaction incubator (Model Ms-50, Matsumoto Manufacturing Co., Osaka, Japan). The cell precipitate was washed twice with distilled water and then suspended in 0.1 M phosphate buffer (pH 7.0).

Cells were disrupted by sonication for 3 min with 20 kHz, 50 W (Model 5202, Ohtake Works, Co. Tokyo, Japan). The disrupted solution was used to determine the lipase activity of cells and the intracellular content of Fe. The intracellular content of Fe was analysed with an atomic absorption spectrophotometer (Model 208, Hitachi Seisakusho Co., Tokyo, Japan). The intracellular lipase activity was also analysed by the olive-oil non-emulsion method (Yamane 1987).

Tri-, di-, mono-glyceride and free fatty acids were extracted from the supernatant twice with the same volume of hexane and their contents were analysed by a TLC/FID analyser (Model TH-10, Iatron Laboratories Co., Tokyo, Japan) (Yamane et al. 1986).

Results and discussion

Effects of Fe ions on lipase activity and on lipase formation in batch culture

Residual lipase activity was determined intermittently after the *P. fluorescens* lipase solutions were incubated with (50 ppm) or without ferric ions. The lipase activity was not lost for 70 h in the absence of ferric ions. But it was gradually lost, obeying first-order decay kinetics, in the presence of ferric ions. At 70 h incubation the residual activity was only 60% of the original; this result suggested that excess ferric ions might deactivate lipase during cultivation. It has also been reported

(Iwai et al. 1970) that *A. niger* lipase is inhibited by Fe ions. Therefore, we performed batch cultures with different initial Fe ion concentrations. Although the cell mass concentration increased slightly with increasing initial Fe ion concentration, the lipase activity of the culture supernatant did not (data not shown). This result suggested that the reduction of initial Fe ion concentration alone was not effective for lipase formation. As reported previously (Suzuki et al. 1988), oil-feeding was suggested to be important.

Semi-batch culture with turbidity-dependent feeding of olive oil

The time courses of these cultures are depicted in Fig. 2. The cell mass concentration increased as the amount of initial Fe ions increased. From the data in Fig. 2 the maximum lipase activities of the culture supernatant are plotted as a function of initial Fe ion concentrations in Fig. 3. There was Fe ion concentration at which the final lipase activity was the highest. It might be suggested that Fe ion concentration affected both the production and secretion of the lipase, and that the extracellular lipase was inactivated by Fe ions more than the intracellular lipase. It was possible to calculate the initial Fe ion concentration necessary for the finally attained cell mass concentration from the Fe content of dry cell mass (0.05%). From the calculations it was found that below 40 ppm initial Fe ion concentration, cells in the later phase of cultivation were in a state of iron deficiency. Figure 3 and the above calculation suggested that

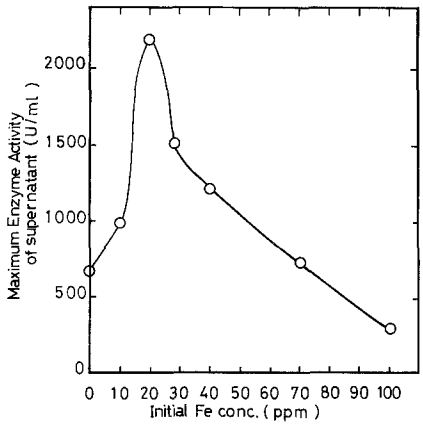

Fig. 3. Effect of initial Fe ion concentration on the maximum lipase activities of the supernatant in semi-batch culture with turbidity-dependent feeding of olive oil

a little Fe ion deficiency in the fed-batch culture was effective for lipase formation.

The fat composition of the culture broth of both batch and fed-batch cultures is shown in Fig. 4. There was abundant fat in the early phase of the batch culture. However, low levels of triglyceride and trace amounts of di- and mono-glyceride and of free fatty acids were maintained during the period of turbidity-dependent fed-batch culture.

Semi-batch culture with turbidity-dependent feeding of both olive oil and iron ion

The time courses of cell concentration, extracellular and intracellular lipase activities, and extracel-

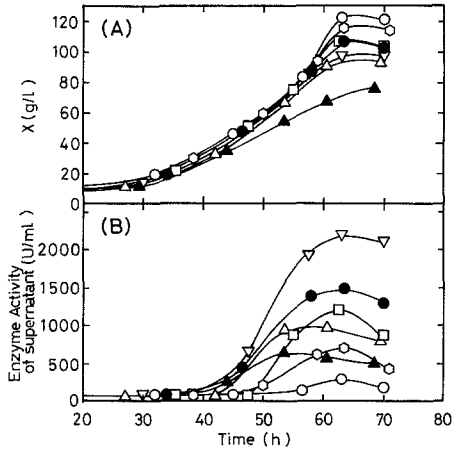

Fig. 2A, B. Semi-batch culture with turbidity-dependent feeding of olive oil. **A.** Growth curves. **B.** Profiles of enzyme activities in the supernatant. The initial Fe ion concentrations: ▲, 0; △, 10; ▽, 20; ●, 30; □, 40; ◇, 70; ○, 100 ppm. Olive oil feeding was started after 20 h batch culture

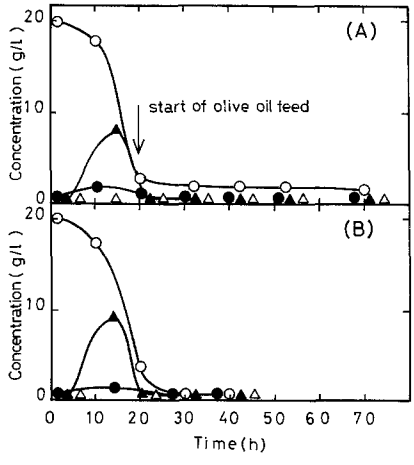

Fig. 4. Time courses of the concentration of glycerides and free fatty acids in semi-batch culture with turbidity-dependent feeding of olive oil **(A)** and in batch culture **(B).** ○, tri-glyceride; ●, di-glyceride; △, mono-glyceride; ▲, free fatty acid

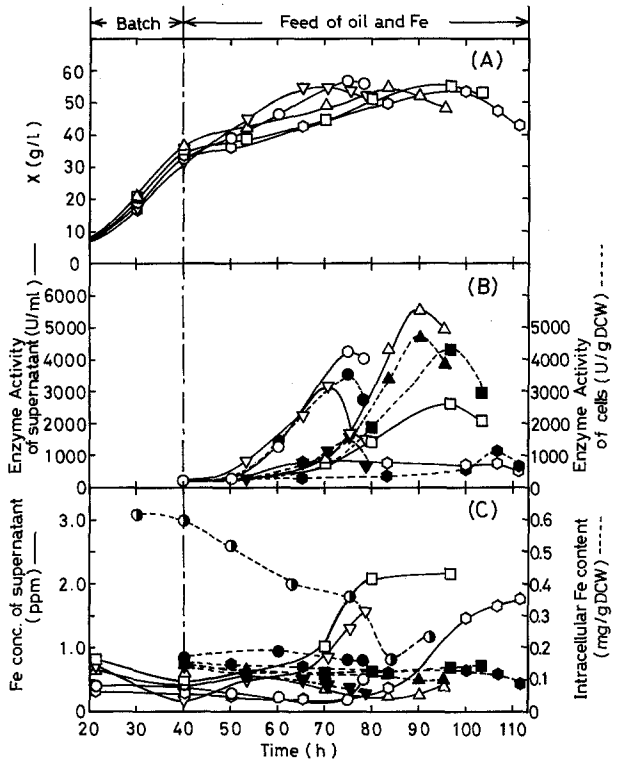

Fig. 5A-C. Semi-batch culture with turbidity-dependent simultaneous feeding of olive oil and Fe ion. Olive oil feeding was started after 40 h batch culture. **A.** Cell concentration. **B.** Lipase activities. **C.** Fe ion concentrations. DCW = dry cell weight. The values of q_0 were set at: —◇—, ---◆---, 0.0065; —□—, ---■---, 0.008; —△—, ---▲---, 0.011; —○—, ---●---, 0.023; and —▽—, ---▼---, 0.033 (g oil) · (g dry cell)$^{-1}$ · h^{-1}. For comparison, the intracellular Fe contents in the semi-batch culture with turbidity-dependent feed of olive oil are shown by the symbol ---◑--- in Fig. 5C (the initial Fe ion concentration was 20 ppm)

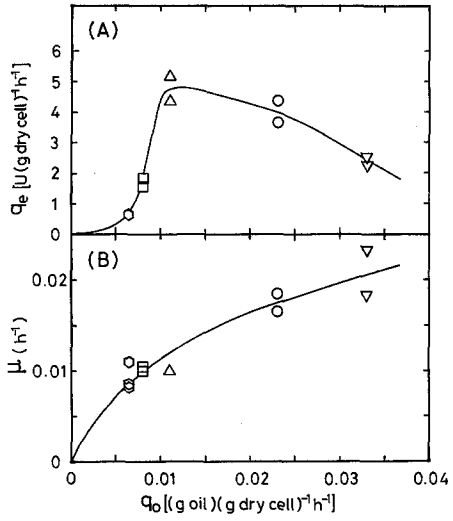

Fig. 6A, B. Relationships between q_e and q_0 and between μ and q_0. **A.** q_e as a function of q_0. **B.** μ as a function of q_0. Data were obtained from Fig. 5. ◇, 0.0065; □, 0.008; △, 0.011; ○, 0.023; ▽, 0.033 (g oil) · (g dry cell)$^{-1}$ · h^{-1}

lular and intracellular Fe ion concentrations are shown in Fig. 5. Although the lipase activity was low at the end of the early batch period, the extracellular lipase activity increased at the later phase of the fed-batch culture. The optimal q_0 for lipase production was 0.011 (g oil · (g dry cell)$^{-1}$ · h^{-1}). The discovery in a previous study (Suzuki et al. 1988) that the excessive addition of olive oil suppressed lipase production was reconfirmed in this study. The maximum lipase activity, 5600 U/ml, was obtained after 90 h of culture by setting q_0 at 0.011 (g oil · (g dry cell)$^{-1}$ · h^{-1}). In Fig. 5c the time course of the Fe ion concentrations in semi-batch culture with turbidity-dependent feeding of olive oil are plotted for comparison. By feeding Fe ion, the intracellular ion content was controlled at a low level.

Figure 6 shows the relationships between the specific lipase production rate, q_e, and q_0, and between the specific growth rate, μ, and q_0, which were calculated from Fig. 5. It is clear that q_e is maximal at $q_0 = 0.011$, and that μ is controlled by q_0. The results shown in Fig. 6 indicate that the turbidity-dependent fed-batch culture technique is a very effective feeding strategy and could be successfully applied to other microbial processes in order to increase the productivity of various microbial metabolites.

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