Factors Affecting the Production of Succinoyl Trehalose Lipids by *Rhodococcus erythropolis* SD-74 Grown on *n*-Alkanes

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Factors affecting the extracellular production of succinoyl trehalose lipids by *Rhodococcus erythropolis* SD-74 were studied to optimize the culture conditions for the maximum production. Since the optimal pH for the production of the acidic exolipid was about 7, the addition of phosphate buffer (pH 7) of high concentrations (0.3 to 0.4 M) to shake culture media was essential for exolipid production. A high osmotic equivalent of culture broth (about 2% NaCl equivalent value) was favorable for exolipid production. Although K^+ was superior to Na^+ , chlorides at higher concentrations were more inhibitory for exolipid production compared to sulfates or phosphates. Under the optimal conditions in a jar fermentor, the concentration of the exolipid in the culture broth reached about 40 g/l after 10 days.

In the preceding paper,¹⁾ we reported that *Rhodococcus erythropolis* SD-74 abundantly produced acidic exolipids, succinoyl trehalose lipids, from *n*-hexadecane, and that the two main components of the exolipid were 2,3,4,2'-di-*O*-succinoyl-di-*O*-alkanoyl-(C_{16} 87%, C_{14} 13%)-trehalose (STL-1) and 2,3,4-mono-*O*-succinoyl-di-*O*-alkanoyl-(C_{16} 75%, C_{14} 25%)-trehalose (STL-2). Ristau and Wagner²⁾ reported that *R. erythropolis* DSM 43245 produced another exolipid, 2,3,4,2'-mono-*O*-succinoyl-tri-*O*-alkanoyl-($2 \times C_{10}$, $1 \times C_8$)-trehalose, in a small amount. Wagner *et al.*³⁾ reported that the latter exolipid had good surfactant properties in the presence of high salt concentrations and that it was produced in an increased amount (7.9 g/l) with a temperature shift from 30 to 21°C at the late stage and nitrogen limitation. The significance of biosurfactants was recently reviewed by Rosenberg.⁴⁾ This paper describes experiments designed to determine factors leading to maximal production of the exolipid by *R. erythropolis* SD-74.

Materials and Methods

Microorganism. Strain SD-74, used exclusively in this study, was isolated from a soil sample at Sado Island and identified tentatively as *Rhodococcus erythropolis* in the preceding study.¹⁾

Media and culture conditions. The seed culture medium used was the same as described in the preceding paper.¹⁾ The following four basal fermentation media contained 10% (v/v) *n*-hexadecane, 0.3% KNO_3 , 0.01% $MgSO_4 \cdot 7H_2O$, 0.1% yeast extract (except for medium-D), tap water and potassium phosphate buffer (pH 7.0) of different concentrations: medium-A with 0.01 M potassium phosphate buffer; medium-B with 0.1 M buffer; medium-C with 0.3 M buffer; and the optimized medium-D with 0.3 M buffer and an increased amount of yeast extract (0.3%). All inorganic salts used were of guaranteed reagent grade (Wako Pure Chemical Industries, Ltd.). Several ingredients were changed, as indicated in the text and legends.

Seed cultures were prepared as described previously.¹⁾ The seed cultures (2 ml) were transferred to 300-ml Erlenmeyer flasks containing 30 ml of the basal fermentation media. The flasks were shaken at 30°C for 7 days on a rotary shaker (220 rpm). Fermentor experiments were performed in a 5 l Waldhof type jar fermentor (Marubishi model MD-300) with the following culture conditions:

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incubation temperature, 30°C; agitation, 600 rpm; and aeration, 1 v/v/min. The seed culture (200 ml) was inoculated into the jar fermentor containing 3 l of different fermentation media. The pH was automatically maintained at 7.0 with 5 N KOH.

Analytical methods. The total exolipid concentration was estimated by the anthrone method.⁵⁾ The culture broths (whole in flasks or 30 ml) were acidified with 3 N H₂SO₄ to pH 3 and then shaken with ethyl acetate (30 ml) to extract the total exolipid. After standing, the solvent layer was removed and a 20 μ l aliquot of it was analyzed by the anthrone method. The optical density was measured at 625 nm with a Spectrophotometer 100-10 (Hitachi Ltd.). A mixture of succinoyl trehalose lipids-1 and -2, prepared as described previously,¹⁾ was used as the standard.

The ethyl acetate layer (5 ml) was concentrated under reduced pressure and the resultant residue was extracted twice with *n*-hexane. The combined *n*-hexane extracts were used to estimate the concentration of remaining *n*-hexadecane by gas-chromatography. The gas-chromatography was performed with a Shimadzu Gas Chromatograph GC-8A equipped with a flame-ionization detector. Separations were performed on a glass column (4 mm $\times$ 1 m) packed with 15% FFAP on Chromosorb WAW at 150°C. *n*-Heptadecane was used as the internal standard.

Cell growth was measured as dry cell weight, after the remaining water layer in the preceding extraction had been centrifuged, washed with ethyl acetate and 50% methanol, and then dried overnight at 105°C.

Results and Discussion

Effect of the n-hexadecane concentration

Preliminary experiments showed that strain SD-74 was apt to form cell-clumps in shake cultures, which caused the exolipid yield to decrease. The formation of clumps was avoided and reproducible experimental results were obtained with the use of a fairly high initial concentration of *n*-hexadecane (10%, v/v). Concentrations of more than 10%, however, caused a decrease in the yield of the exolipid. The concentration of 10%, accordingly, was used throughout the present study.

The strain was able to grow well on fructose and glycerol, and moderately on glucose, but unable to produce exolipids from these carbohydrates.

Since the exolipid contains trehalose and succinate moieties, the effect of the supplementation of different carbon sources on exolipid

production was examined in medium-B supplemented with fructose, glycerol, glucose, citrate or succinate, at concentrations of 2 to 4%. The supplementation of these different carbon sources failed to give substantial increases in exolipid production.

Exolipid productivity from different *n*-alkanes and other noncarbohydrate carbon sources will be described elsewhere.

Effect of the initial pH

Maintenance of the culture broth at neutral pH was observed to be important for pro-

Table I. EFFECT OF pH ON EXOLIPID PRODUCTION

Initial pH	Final pH	Dry cell weight (mg/ml)	Exolipid (mg/ml)
5.60	5.32	6.2	10.1
6.04	5.68	7.0	14.7
6.28	5.92	7.5	17.8
6.50	6.22	7.8	19.7
6.76	6.53	8.0	21.4
7.00	6.83	8.3	21.8
7.25	7.10	8.5	19.8
7.45	7.26	8.6	18.3
7.95	7.72	7.6	13.0

Fig. 1. Effect of the Concentration of the Phosphate Buffer on Exolipid Production.

Potassium or sodium phosphate buffer, pH 7.0, of different concentrations was added to medium-A containing 0.01 M phosphate buffer. ○—○, exolipid in potassium phosphate buffer; △—△, exolipid in sodium phosphate buffer; ●—●, dry cell weight in potassium phosphate buffer; ▲—▲, dry cell weight in sodium phosphate buffer.

duction of the acidic exolipid. The addition of excess CaCO_3 to medium-A, however, did not maintain a neutral pH or improve the production.

Table I shows the effect of the initial pH on exolipid production in medium-C, which was further supplemented with 0.1 M 1,2,3-propanetricarboxylate and adjusted to different pHs with 4 N H_2SO_4 or 2 N NaOH. In all the culture broths buffered with 0.3 M phosphate and 0.1 M 1,2,3-propanetricarboxylate, the pH dropped considerably after 5 days cultivation, because of accumulation of the acidic exolipid.

The optimal pH for exolipid production was determined to be 6.5 to 7.0; this value was slightly lower than the optimum for cell growth (7.0 to 7.5).¹⁾

Effect of the phosphate buffer concentration

Figure 1 shows the effects of the addition of sodium or potassium phosphate buffer (pH 7.0) of different concentrations to medium-A, which contained phosphate buffer of a low concentration (0.01 M) initially, on exolipid production. The addition of higher than 0.3 M phosphate buffer improved the exolipid production, but this stimulatory effect of the buffers, of high concentrations, was more marked with potassium phosphate, compared with sodium phosphate.

Effects of the addition of different salts

Potassium phosphate buffer was found to be superior to sodium phosphate buffer for exolipid production. Consequently, the effect of the addition of K^+ or Na^+ as the chloride or sulfate at high concentrations on exolipid production was examined in medium-B, which contained phosphate buffer (0.1 M) of a moderate concentration. The addition of these salts gave marked increases in exolipid production (Fig. 2). The optimal concentrations for exolipid production, however, varied when they were expressed in % and M. Therefore, in the figure, the osmotic equivalents of culture broths are conveniently expressed in terms of the total NaCl equivalent values.⁶⁾ The figure also shows the effect of the concentration of

Fig. 2. Effects of the Addition of Different Salts on Exolipid Production.

Different salts were added to medium-B containing 0.1 M phosphate buffer. The salt concentrations are conveniently expressed in terms of total NaCl equivalent values⁶⁾ of culture broths. ●—●, KCl; ▲—▲, NaCl; ●—●, K_2SO_4 ; ▲—▲, Na_2SO_4 ; ○—○, potassium phosphate buffer, see Fig. 1; △—△, sodium phosphate buffer, see Fig. 1.

the phosphate buffer shown in Fig. 1, as dotted lines. The results indicated that an osmotic equivalent corresponding to an NaCl equivalent value of about 2% was favorable for exolipid production, and that K^+ was superior to Na^+ . At higher concentrations, chlorides were more inhibitory for exolipid production, compared with sulfates or phosphates, although cell growth was almost completely unaffected. Media-C and -D had good osmotic equivalents corresponding to about 2% NaCl equivalent values.

In order to increase the osmotic equivalent of the culture broth, the effect of the addition of CaCl_2 or MgCl_2 was examined. Cell-clumps were abundantly observed in these cultures and exolipid production was repressed even with a concentration of 0.02 M.

The omission of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ from the optimized medium-D resulted in a decrease in the exolipid yield (about 10%), but at concentrations ranging from 0.005 to 0.02%, the yield remained unchanged.

Effects of the addition of metal ions

In the cultures shown in Figs. 1 and 2, the respective salts, even though all were of guaranteed reagent grade, were added at extremely high concentrations, thus, the stimulatory or inhibitory effects of these salts on exolipid production might be ascribable in part to metal ions contaminating the salts as impurities. The addition of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ at concentrations of 0.5 to 5 mg/ml to medium-C increased exolipid production to about 5%. The addition of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ or $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ at a low concentration (0.5 mg/ml) decreased the exolipid yield slightly, while $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ decreased it to about one-half.

Effects on nitrogen sources

The effects of different nitrogen sources, instead of KNO_3 in medium-C, on exolipid production were examined at the same concentration, 0.03 M, as nitrogen. Exolipid production and cell growth were almost completely unaffected by $(\text{NH}_4)_2\text{SO}_4$, $\text{NH}_4\text{H}_2\text{PO}_4$,

NH_4NO_3 or urea, all of which were sterilized separately. Polypepton applied at concentrations of 0.4 and 0.6% gave about 55 and 70% exolipid yields, respectively, compared with those obtained with other nitrogen sources. Accordingly, KNO_3 was selected as the nitrogen source in the basal media, because its separate sterilization was not required.

Figure 3 shows the effect of the concentration of KNO_3 in medium-D. A better yield

Table II. EFFECTS OF DIFFERENT SUPPLEMENTARY ORGANIC NUTRIENTS ON EXOLIPID PRODUCTION

Nutrient		Dry cell weight (mg/ml)	Exolipid (mg/ml)
	(%)		
None		5.9	20.2
Casamino acid	0.1	7.9	23.8
(Difco)	0.2	8.4	24.7
Beef extract	0.1	7.4	23.5
(Difco)	0.2	7.9	24.2
Polypepton	0.1	7.3	20.6
	0.2	7.5	20.8
Rice bran	0.1	7.6	23.5
	0.2	8.0	24.4
Corn steep liquor	0.1	8.1	23.3
	0.2	8.8	23.9
Yeast extract	0.05	7.2	24.6
(Oriental)	0.1	8.7	26.4
	0.2	9.9	28.2
	0.3	11.4	29.7
	0.4	12.3	28.4
	0.5	13.5	26.3

Fig. 3. Effect of the Concentration of Potassium Nitrate on Exolipid Production.

The concentration was varied in the optimized medium-D. ○—○, exolipid; ●—●, dry cell weight.

Fig. 4. Effect of Temperature on Exolipid Production.

The strain was cultured in the optimized medium-D. ○—○, exolipid; ●—●, dry cell weight.

Fig. 5. Time Course of Exolipid Production in a 5-l Jar Fermentor.

The strain was cultured in the optimized medium-D. ○—○, exolipid; ●—●, dry cell weight; □—□, residual *n*-hexadecane.

was observed at about 0.3%. Ristau and Wagner,²⁾ and Wagner *et al.*³⁾ reported that *R. erythropolis* DMS 43215 produced the trehalose lipid mainly under nitrogen limited conditions (0.1% (NH₄)₂SO₄).

Effects of the addition of supplementary organic nutrients

As Table II shows, all the tested supplementary nutrients stimulated exolipid production as well as cell growth, yeast extract being the most effective. From these results, the optimized medium-D containing 0.3% yeast extract was selected.

Effects of temperature and aeration

As Fig. 4 shows, 30°C was found to be optimum for exolipid production. On the contrary, Wagner *et al.*³⁾ reported that *R. erythropolis* DMS 43215 produced the trehalose lipid more abundantly at 21 than 30°C.

Different volumes (25~120 ml) of medium-D were put into 300-ml Erlenmeyer flasks to

examine the effect of aeration on exolipid production. Volumes of 30 and 40 ml medium gave higher concentrations of the exolipid, but a larger volume, 120 ml, caused a decrease to about one-half concentration.

Time course of exolipid production in a Waldhof type jar fermentor

Figure 5 shows a typical time course of exolipid production in medium-D when the pH was automatically controlled at 7.0 with 5N KOH. The fermentation was apparently nongrowth associated. After incubation for 10 days, the exolipid concentration reached about 40 mg/ml; this value corresponded to an about 50% yield on the weight basis of *n*-hexadecane consumed.

A similar high yield of the exolipid was obtained in modified medium-D, which contained 0.15 M K₂SO₄ and 0.1 M phosphate buffer, instead of 0.3 M buffer. Medium-A, having a low NaCl equivalent value, gave a low yield of the exolipid (15 mg/ml) after 10 days, even with automatic control of the pH.

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