Production of Eicosapentaenoic Acid by a Bacterium Isolated from Mackerel Intestines

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Optimization of culture conditions for the growth rate, 5,8,11,14,17- *cis*-Eicosapentaenoic acid (EPA) content and EPA productivity of a bacterium isolated from Pacific mackerel intestines was investigated by use of a culture medium containing 1.00 wt% peptone and 0.50 wt% yeast extract in an artificial sea water (ASW). Cultivation temperature affected the growth rate and cellular EPA content of the bacterium. The cellular EPA content at 8°C was as great as 16.8 mg/g of dry cells, which was more than two times greater than that at 25°C (7.3 mg/g of dry cells), although the growth rate showed a maximum at 25°C. Both the yield of bacterial cells and the cellular EPA content at 25°C reached maximum values when the pH of the culture medium was nearly 7.0 and when the concentration of ASW was 100% (v/v). Under optimum culture conditions [25°C pH 7.0 and 100% (v/v) ASW], the amount of EPA accumulated in the cellular lipids reached 45.6 mg/L of culture broth after 8 hr.

KEY WORDS: Alteromonas putrefaciens, eicosapentaenoic acid, mackerel, marine bacterium.

5,8,11,14,17-*cis*-Eicosapentaenoic acid (EPA) is a C_{20} polyunsaturated fatty acid of potential pharmaceutical value. This acid has been shown to be effective for prevention and cure of thrombosis and arteriosclerosis (1). The high nutritional value of oils containing EPA for some marine fishes also has been reported (2,3). Recently, several marine fish oil products have become available as sources of EPA. However, these conventional sources of EPA are not satisfactory for practical purpose due to their low EPA contents and the intermixing of other fatty acids having less desirable properties. Thus, the production of EPA by a marine alga, *Chlorella minutissima* (4), a freshwater alga, *Monodus subterraneus* (5), and *Mortierella* fungi (6–8) have been examined extensively.

Yazawa and co-workers have attempted to find EPA-producing marine microorganisms (9,10). Out of some 7,000 marine bacterial strains screened, they found that a bacterial strain isolated from the intestinal contents of Pacific mackerel *Pneumatophorus japonicus* contained a remarkably high level of EPA in its cellular lipids (10). This bacterial strain, tentatively named SCRC-2738, was Gram-negative, obligate aerobic, rod-shaped and motile by means of peritrichous flagella, and was identified as a new species close to *Alteromonas putrefaciens* on the basis of these biological and biochemical characteristics. This bacterial strain required natural or artificial sea water for growth (10).

In the present work we examined the effect of culture conditions on the growth rate, cellular EPA content and EPA productivity of SCRC-2738 and determined the EPA productivity under the optimum culture conditions.

MATERIALS AND METHODS

The bacterial strain SCRC-2738 was from the stock in Sagami Chemical Research Center and its cultivation was carried out aerobically. In the optimization of culture conditions, a preculture (1 mL) in the medium P-Y (peptone-yeast extract) containing 1.00 wt% peptone and 0.50 wt% yeast extract in 50% (v/v) ASW (artificial sea water prepared by mixing Jamarin S with an equal volume of distilled water) was transferred to 100 mL of culture medium in a 500 mL shaking flask and was incubated at 8 ~ 30°C with reciprocal shaking of 120 strokes/min. The EPA productivity under the optimum culture conditions was determined by use of a 3-L jar fermenter equipped with four baffles containing 1 L of culture medium and agitated at 450 rpm. Growth was followed turbidimetrically at 610 nm and was stopped when the OD_{610} leveled off. Peptone, yeast extract, and artificial sea water ("Jamarin S") were purchased from Kyokuto Pharmaceutical Industries Co. Ltd. (Tokyo, Japan), E. Merck (Darmstadt, Germany) and from the Jamarin Laboratory (Osaka, Japan), respectively. The cells were harvested by centrifugation at 6,000 rpm for 25 min and were dried at room temperature under vacuum after being washed twice with 50% (v/v) ASW.

The content of EPA and the fatty acid composition in dry cells were determined by means of methanolysis and gas-liquid chromatography (GLC). Dry cells (ca. 100 mg) were well mixed with 2 mL of chloroform and 3 mL of 10 wt% methanolic HC1 in a 10 mL, screw-capped, glass tube and were then heated at 80°C for 1 hr. Fatty acid methyl esters were extracted four times with 5 mL each of n-hexane, and were then analyzed by GLC. Two meters of glass spiral column containing 5 wt% Advance-DS on 80 ~ 100 mesh Chromosorb W with helium as the carrier gas were used at 150°C (C_{14} ~ C_{19} esters) and 165°C (C_{19} ~ C_{21} esters), respectively. Methyl heneicosanoate was employed as an internal standard for GLC. Fatty acid methyl esters used as the standards for GLC were purchased from Sigma Chemical Co. (St. Louis, MO). The turbidity or optical density of the culture broth at 610 nm (OD_{610}) was determined by a Hitachi 124 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Both the yields of bacterial cells and EPA are expressed as the mass obtained per 1 L of culture broth.

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TABLE 1

Effect of Nutrient Concentrations on the Production of EPA[a]

Nutrient concentrations (wt%)		Cell yield		Total fatty acid content[b]	EPA content[b]	EPA yield	
Peptone	Yeast extract	(g/L)[c]	(g/100 g Nu)[d]	(mg/g)	(mg/g)	(mg/L)[e]	(mg/100 g Nu)[f]
0.50	0.25	2.82	37.6	35.1	7.4	20.7	276
1.00	0.50	5.28	35.2	37.7	7.3	38.5	257
1.50	0.75	6.32	28.1	43.1	6.8	42.7	190
2.00	1.00	6.98	23.3	36.8	6.0	42.2	141

[a]Medium P-Y: ASW 50% (v/v) initial pH 7.0. Cultivation temperature, 25°C. Cultivation time, 8 ~ 11 hr. Final OD_{610}, 6.32 ~ 12.76.
[b]Content of total fatty acids or EPA in one gram of dry cells.
[c]Cell yield per one liter of culture broth.
[d]Cell yield per 100 grams of nutrients. Nutrients = peptone + yeast extract.
[e]EPA yield per one liter of culture broth.
[f]EPA yield per 100 grams of nutrients. Nutrients = peptone + yeast extract.

RESULTS

Factors affecting EPA production.

Nutrient concentrations. The marine bacterium SCRC-2738 contains stearic acid $C_{18:0}$, oleic acid $C_{18:1}$, *cis*-vaccenic acid $C_{18:1}$, linoleic acid $C_{18:2}$ and EPA $C_{20:5}$, in addition to C_{13} to C_{17} saturated and unsaturated fatty acids. Table 1 summarizes the effect of initial nutrient concentrations in 50% (v/v) ASW on the yield of bacterial cells and the cellular EPA content at 25°C. When the initial concentrations of peptone and yeast extract were 0.50 and 0.25 wt%, respectively, the yield of bacterial cells was 2.82 g/L of culture broth. The yield of bacterial cells increased when the initial concentration of these nutrients was increased and reached 6.98 g/L of culture broth at 2.00 wt% peptone and 1.00 wt% yeast extract. Although the cellular EPA content decreased from 7.4 to 6.0 mg/g of dry cells with such a rise in the initial concentration of nutrients, the yield of EPA increased from 20.7 to 42.2 mg/L of culture broth (Table 1). When the mass of nutrients used was taken into account, however, the yield of bacterial cells decreased from 37.6 to 23.3 g/100 g of nutrients with this rise in the concentration of nutrients. The yield of EPA decreased from 276 to 141 mg/100 g of nutrients. Both of these two yields decreased markedly when the concentrations of peptone and yeast extract exceeded 1.00 and 0.50 wt%, respectively (Table 1). Hence, we used the nutrient concentrations, 1.00 wt% peptone and 0.50 wt% yeast extract, in the following experiments.

Cultivation temperature. Table 2 summarizes the results of cultivation of SCRC-2738 at different temperatures. Although the cultivation at 8°C was performed for as long as 50 hr, the yield of bacterial cells was as low as 0.85 g/L of culture broth. Raising the cultivation temperature to 15 and 25°C increased the yield to 3.70 and 3.61 g/L, respectively, but it then decreased to as low as 0.78 g/L at 30°C. Both the total fatty acid and EPA contents in the dried bacterial cells decreased with rising cultivation temperature. For example, the cellular EPA content was as great as 16.8 mg/g of dry cells at 8°C, but it decreased to 7.2 and 7.3 mg/g of dry cells, respectively, at 15 and 25°C, and it became negligible at 30°C. The composition of EPA in the total fatty acids was thus as high as 23.8 wt% at 8°C, whereas it was 12.8 wt% at 15 and 25°C. The relatively higher contents of $C_{15:0}$, $C_{16:0}$ and $C_{16:1}$ fatty acids (total 72.9 wt%) were characteristic of the bacterial strain SCRC-2738 grown at 30°C (Table 2). The yield of EPA was greatest (26.6 and 26.4 mg/L of culture broth, respectively) at 15 and 25°C. Taking the cultivation time into account, we determined that 25°C is the optimum temperature for this bacterial production of EPA.

H^+ concentration. Figure 1 shows the effect of initial pH on the production of EPA. Different initial pH values were used and the cultivation was performed at 25°C for 7 hr. The yield of bacterial cells was negligible at pH 4.9, but it increased gradually with increasing initial pH of the culture medium and reached a maximum (3.94 g/L) at pH 7.2. The yield of bacterial cells then decreased with further rise in the initial pH. The cellular EPA content was the greatest (8.7 ~ 8.9 mg/g of dry cells) at pH 7.2 ~ 7.6, and the yield of EPA showed a maximum value (34.3 mg/L) at pH 7.2 (Fig. 1). This result clearly shows that the optimum pH of medium P-Y is about 7.0 for the bacterial production of EPA.

ASW concentration. The effect of ASW concentration at pH 7.0 on the yields of bacterial cells and EPA was similarly investigated. As shown in Figure 2, the yield of bacterial cells showed a maximum value of 2.48 g/L at an ASW concentration of 100% (v/v), although the yield did not change markedly at 50 ~ 100% (v/v). Similarly, the cellular total fatty acid and EPA contents did not change much at 50 ~ 100% (v/v) — the cellular EPA content was 8.5 ~ 8.7 mg/g of dry cells at these ASW concentrations. The yield of EPA reached a maximum value 21.1 mg/L at 100% (v/v) (Fig. 2).

Aeration rate. The effect of aeration rate on the growth rate and cellular fatty acid composition at 25°C was examined at pH 7.0. Cultures (1 L) were aerated at 0.50, 1.00 or 1.50 L/min and were agitated at 450 rpm. From the initial stage of the cultivation to the late logarithmic growth stage at which foaming began to take place, the OD_{610} of the culture broth was determined at every 1 hr interval. Variation of OD_{610} with elapsed time at the logarithmic growth stage was kinetically analyzed by using the following equations:

$$\mu = 2.303 \log([OD_{610}]_2/[OD_{610}]_1)/(t_2 - t_1) \quad [1]$$

$$t_g = 2.303 \log 2/\mu \quad [2]$$

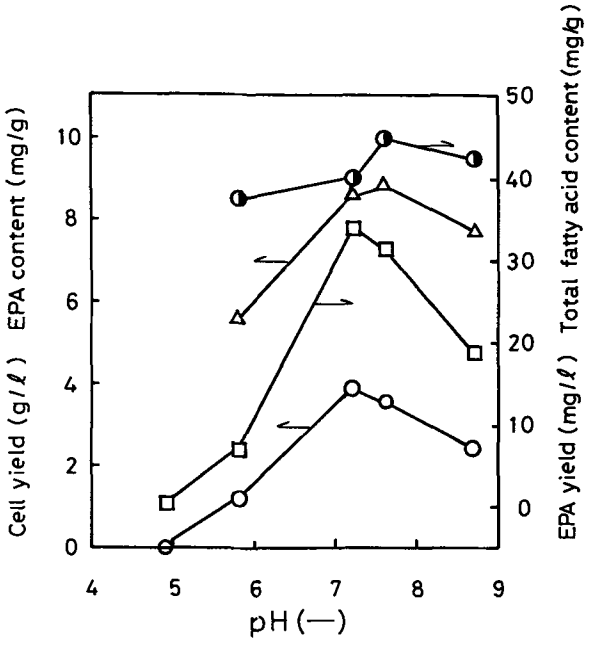

FIG. 1. Effect of the initial pH of medium P-Y on the production of EPA. Medium P-Y: peptone, 1.00 wt%, yeast extract 0.50 wt% and ASW, 50% (v/v). Cultivation, 25°C, 7 hr. ○, cell yield; △, EPA content; □, EPA yield; and ◑, total fatty acid content.

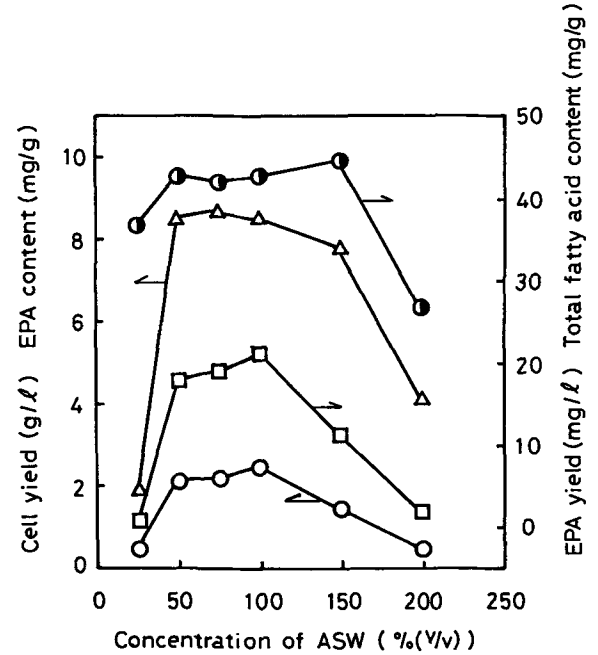

FIG. 2. Effect of the concentration of ASW in medium P-Y on the production of EPA. Medium P-Y: peptone, 1.00 wt%, yeast extract, 0.50 wt%, initial, pH 7.0. Cultivation, 25°C, 6 hr. Symbols used are the same as those in Figure 1.

TABLE 2

EPA Yields and Cellular Fatty Acid Compositions of SCRC-2738 Grown at Different Cultivation Temperatures[a]

Cultivation		Cell yield[b]	Total fatty acid content[b]	EPA content[b]	EPA yield[b]	Cellular fatty acid composition (wt%)							
Temperature (°C)	Time (hr)	(g/L)	(mg/g)	(mg/g)	(mg/L)	13:0	14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	EPA	Others
8	50	0.85	70.8	16.8	14.3	13.6	3.7	19.2	5.4	17.5	7.9	3.9	0.2	4.0	23.8	0.8
15	50	3.70	56.3	7.2	26.6	5.1	2.0	16.5	9.4	21.5	7.8	16.9	1.3	5.8	12.8	0.9
25	10	3.61	57.1	7.3	26.4	3.8	1.5	16.8	11.0	15.9	7.8	18.0	1.7	9.1	12.8	1.6
30	50	0.78	10.4	0	0	10.2	1.4	34.0	12.4	26.5	0	1.4	2.8	11.1	0	0.2

[a]Medium P-Y: peptone 1.00 wt%, yeast extract 0.50 wt%, ASW 50% (v/v), initial pH 7.2.
[b]See Table 1.

Here, μ is the growth rate constant $[h^{-1}]$. $[OD_{610}]_1$ and $[OD_{610}]_2$ are the values of OD_{610} at the cultivation time, t_1 and t_2 ($t_2 > t_1$), respectively; t_g is the generation time (hr).

When the aeration rate was 0.50 L/min, values of OD_{610} observed at cultivation times of 2 and 4 hr were 0.174 and 1.323, respectively. Assuming the absence of inactivated bacterial cells during the middle logarithmic growth stage, the growth rate constant and the generation time were calculated to be 1.00 h^{-1} and 0.693 hr, respectively. The same values of the growth rate constant and the generation time were obtained at both 1.00 and 1.50 L/min aeration. The cellular contents of total fatty acids and EPA were constant, 39.7 ~ 40.4 and 8.4 ~ 8.6 mg/g of dry cells, respectively, at these aeration rates. Thus, variation of aeration rate between 0.50 and 1.50 L/min did not cause any effect on the growth rate and cellular fatty acid content.

Determination of EPA productivity. The EPA productivity was determined with Nissan disfoam FDS-2224 as the defoaming agent. A preliminary experiment with a shaking culture showed that this defoaming agent had no inhibitory effect on the growth rate and cellular EPA content of SCRC-2738.

Figure 3 shows the variation of OD_{610}, the yield of bacterial cells, the cellular EPA content, and the yield of EPA with elapsed time. Although the yield of bacterial cells increased from 4.44 to 5.32 g/L of culture broth from 7 hr to 10 hr, the cellular EPA content decreased from 9.6 to 8.0 mg/g of dry cells. Thus, the yield of EPA reached its maximum value, 45.6 mg/L of culture broth, at 8 hr and then decreased rapidly with further cultivation.

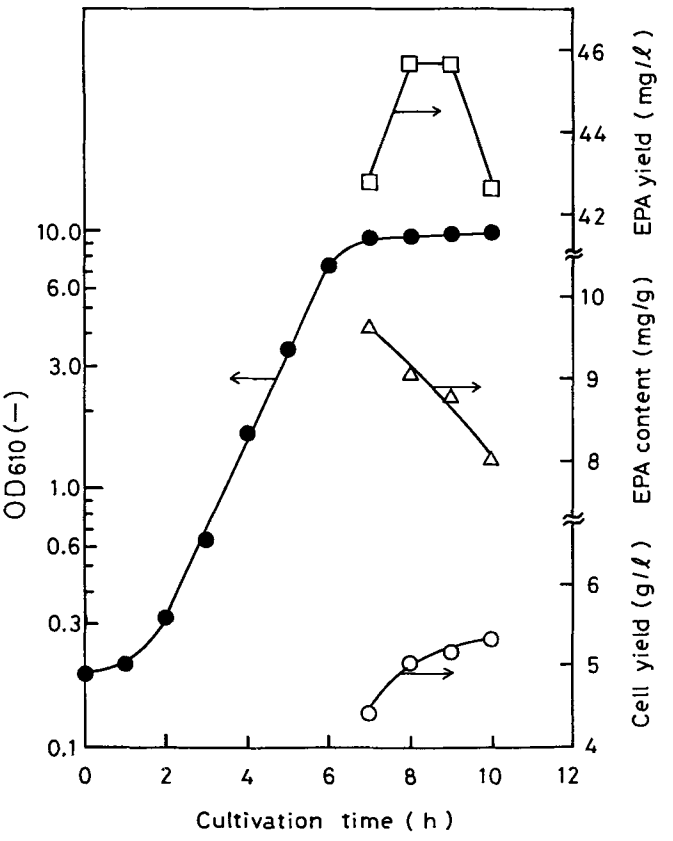

FIG. 3. Variation of the production of EPA with cultivation time in the pH-controlled jar fermenter. Medium P-Y (1 L); peptone, 1.00 wt%; yeast extract, 0.50 wt%; defoaming agent, 0.01 wt%; ASW, 100% (v/v), pH 7.0. Cultivation temperature, 25°C; air, 1.0 L/min; agitation, 450 rpm. ●, OD_{610}. Other symbols used are the same as those in Figure 1.

DISCUSSION

The growth of bacterial strain SCRC-2738 in the medium containing no glucose in ASW (Table 1) suggests that this bacterium requires only proteins and/or amino acids as nutrients. In addition, observation of the greater yield of bacterial cells at pH 8.7 (Fig. 1) and the maximum yield of the cells in 100% (v/v) ASW (Fig. 2) suggests that this bacterium is fairly alkalophilic and, at the same time, halo-philic. This bacterium also showed its maximum growth rate at 25°C (Table 2). All of these characteristics for SCRC-2738 could be attributed to the nutritional and physiological conditions in the Pacific mackerel intestines in which it once lived.

The observed increase in cellular EPA content with decreasing cultivation temperature (Table 2) is consistent with the view that EPA occurs as a component of cellular membrane lipids (10). The increase in cellular EPA content at low temperatures could be important for the bacterium to maintain membrane fluidity (11).

The bacterial strain SCRC-2738 contains EPA as the sole C_{20} polyunsaturated fatty acid (Table 2). This is very advantageous from the viewpoint of separation and purification. Another advantage of this bacterial EPA production is that it can be carried out under normal growth temperature conditions (25°C). Under such conditions, the growth is rapid (generation time = 0.693 hr), and the energy costs for temperature control could be minimized.

The production of EPA by *Mortierella* fungi has extensively been studied. For *Mortierella alpina* 20-17, the mycelial EPA content was as great as 41.5 mg/g of dry mycelia at 28°C and the EPA productivity reached 1.35 g/L of culture broth, although cultivation time was as long as 13 days (7). For *Mortierella alpina* 1S-4, the mycelial EPA content and the EPA productivity at 12°C reached 66.6 mg/g of dry mycelia and 1.88 g/L of culture broth after 16 days (8). The cellular EPA content and EPA productivity of the bacterial strain SCRC-2738 are relatively low—8.0 ~ 9.6 mg/g of dry cells and 45.6 mg/L of culture broth, respectively, at 25°C. However, this value of EPA productivity at 25°C is obtained after 8 hr (Fig. 3).

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