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Isolation, Identification, and Culture Conditions of the Strain Producing Eicosapentaenoic Acid

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The bacterium producing EPA was isolated from the intestines of the marine fishes. The strains were studied for their identification and their culture conditions. The selected strain was gram negative, rod (0.7 × 2.4 μm in size) and motile with a single polar flagellum. This strain was identified as a *Pseudomonas* sp. on the basis of its morphological, cultural and physiological characteristics. The strain showed a maximum productivity of phospholipid at 20°C after 48 hours of culture time with an initial pH of 7.0 in the PYM-glucose medium. Under these culture conditions, the production of phospholipid was about 0.3 mg/ml and 0.06 mg/mg dry cell weight.

Dyerberg et al. (6) reported that Greenland Eskimos had a rare myocardial infarction although they had taken a fairly high amount of lipid. It was elucidated that this was due to the presence of ω-3 group fatty acids such as EPA (eicosapentaenoic acid) in their food (3). Consequently, the ω-3 group fatty acids have been studied extensively. EPA is effective in preventing and curing thrombosis (13), depression in blood pressure (12), and decreasing the blood lipid content (21). However, EPA must be taken as a diet since it cannot be synthesized by mammals *in vivo* (18). It has been known that marine fishes like sardine and mackerel have a high content of EPA (1). Then, the industrial production of EPA has been based on the extraction of EPA from the fish oils. The concentrated products of 20~30% EPA have been supplied as health foods (11). However, there have been a lot of problems in the purification and the supply of the raw materials as well as the unavoidable fishy smell (9). In order to overcome these problems, the microbial production of EPA by fungi (16~19), algae (2) and chlorella (15) have been investigated. In the present work, the EPA-producing bacterium was isolated from the intestines of fishes. The identification and the culture conditions of the strain were studied.

MATERIALS AND METHODS

Isolation of the Strain

After the intestines of marine fishes were diluted with saline, they were spread onto a PYM-glucose agar medium (peptone 1.0%, yeast extract 0.5%, meat extract 0.25%, glucose 2.0%, sea water 50%, pH 7.0) (11). After this strain was cultured in the PYM-glucose liquid medium for 2~3 days, the lipid was extracted from the cells by the Folch method (7). Using a mixture of hexane : ether (18:1, v/v) as the developing solvent for the TLC (Thin layer chromatography), the strain showing the same R_f value as the standard EPA was isolated. The strain exhibiting the highest productivity of EPA was used in this work.

Identification of the EPA-producing Strain

The identification of the isolated strain was carried out by the general method of the bacterial identification as described in Microbes in action (14), Manual of methods for general bacteriology (8), Bergey's manual of determinative bacteriology (5) and Bergey's manual of systematic bacteriology (20).

Determination of Dry Cell Weight

The isolated strain which was inoculated from the slant culture with a loop was cultured in PYM-glucose medium for 48 hours at 25°C. The cells were harvested by the centrifugation at 5000 × g for 20 min. The cell pellets were dried at 105°C and the dry cell weight was determined by measuring the absorbance from the standard curve.

Quantification of Phospholipid

The cell pellets harvested by centrifugation were resuspended into the distilled water. After adding a mixture of chloroform : methanol (1:2, v/v), the layer of chloroform was separated by the centrifugation at 5000 × g for 20 min. This layer containing the phospholipid was

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used as a sample to be measured for the quantification of phospholipid. The concentration of phospholipid was quantified by the method of Fiske-Subbarow method (4). The qualification and quantification of EPA will be discussed in the paper (in preparation).

RESULTS AND DISCUSSION

Isolation and Identification of the EPA-producing Strain

Isolation of the strain: The strain of bacteria was isolated from the intestine of the marine fishes. When the lipids extracted from the isolated strain were spotted on the TLC, the strain exhibiting the same R_f value as the standard EPA and the highest productivity was selected, and then designated as CH-414.

Identification of the Strain

Morphological characteristics: As shown in Table 1, the morphological characteristics of the strain CH-414 were as follows: gram negative, rod and motile with a single polar flagellum. The strain, however, did not have any spore. Fig. 1 shows the electron micrograph of the strain CH-414. The strain was 0.7 × 2.4 μm in size.

Cultural characteristics: The cultural characteristics of the strain CH-414 are presented in Table 2. The colonies on the nutrient agar medium were shown to be in a punctiform. The color was creamy and the surface was mucoid.

Table 1. Morphological characteristics of the isolated strain

Gram stain	negative
Shape	short rod
Cell size	0.7 × 2.4 μm
Motility	motile
Flagella	positive
Spore stain	negative

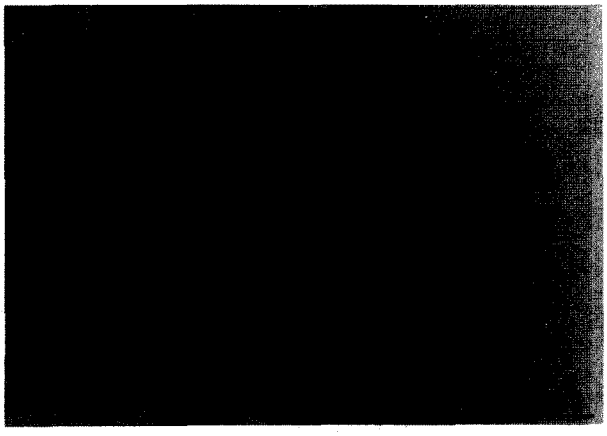

Fig. 1. Electron micrograph of the isolated strain.
TEM : Phosphotungstate negative staining. Bar represents 1μm.

Physiological characteristics: Table 3 represents the physiological characteristics of the strain CH-414. The temperature and pH for the cell growth were within the ranges of 4-42°C and 5-12, respectively. The maximum salt concentration for the cell growth reached 9%. The strain CH-414 was aerobic and positive for catalase and oxidase. It also produced H_2S and lipase, but not urease. It hydrolyzed gelatin and casein, but did not hydrolyze starch or cellulose. The experimental results on the sugar fermentation by the strain CH-414 are shown in Table 4. It was also indicated that while the strain fermented arabinose, dextrin, maltose and soluble starch, it did not ferment other sugars. Gas was not produced from any of the sugars. The sugar utilization by the strain CH-414

Table 2. Cultural characteristics of the isolated strain

Colony on nutrient agar(20°C, 2-3 days)	
Form	punctiform
Surface	mucoid
Elevation	raised
Opacity	translucent
Color	creamy

Table 3. Physiological characteristics of the isolated strain

Temperature range for growth	4°C~42°C
pH range for growth	5~12
NaCl tolerance for growth	≤9%
Catalase	positive
Oxidase	positive
Urease	negative
Lipase(Tween 80)	positive
Arginine dihydrolase	positive
Hydrolysis of:	
Starch	negative
Casein	positive
Cellulose	negative
Indole production	negative
Levan formation from sucrose	positive
NH_3 production from arginine	positive
NH_3 production from peptone	positive
Utilization of citrate	positive
Methyl red test	negative
Voges-Proskauer reaction	negative
Nitrate reduction	positive
Denitrification	positive
Action on milk:	
Coagulation	negative
Peptonization	positive
Hemolysis, human blood	negative
O-F test	oxidation
H_2S production	positive
Phenylalanine deaminase	negative
Gelatin hydrolysis	positive

Table 4. Fermentation of sugars by the isolated strain

Sugar	Acid	Gas
Arabinose	+	−
Cellobiose	−	−
Cellulose	−	−
Dextrin	+	−
Fructose	−	−
Galactose	−	−
Glucose	−	−
Glycerol	−	−
Inositol	−	−
Inulin	−	−
Lactose	−	−
Maltose	+	−
Mannitol	−	−
Mannose	−	−
Raffinose	−	−
Soluble starch	+	−
Sorbitol	−	−
Sucrose	−	−
Xylose	−	−

+ : Positive, − : Negative

Table 5. Utilization of sugars by the isolated strain

Sugar	Utilization
Arabinose	++
Cellobiose	++
Cellulose	−
Dextrin	+
Fructose	+
Galactose	++
Glucose	+
Glycerol	−
Inositol	−
Inulin	+
Lactose	−
Maltose	−
Mannitol	−
Mannose	+
Raffinose	+
Soluble starch	+
Sorbitol	+
Sucrose	+
Xylose	−

++ : Strong utilized, + : Utilized, − : Not utilized

was also investigated. As shown in Table 5, the strain CH-414 utilized arabinose, cellulose and galactose. From the above results, the strain was identified, thus designated as *Pseudomonas* sp. CH-414.

Condition of Phospholipid Production by *Pseudomonas* sp. CH-414

Effect of culture time: The phospholipid production and the growth of *Pseudomonas* sp. CH-414 were studied in PYM-glucose liquid medium (Fig. 2). The cell mass reached the stationary phase at 36 hours of the culture time and then decreased after 72 hours. The production of phospholipid reached the maximum level at 48 hours of cultivation, and then decreased beyond this time. Thus, the following studies for the phospholipid production were accomplished within the culture time of 48 hours.

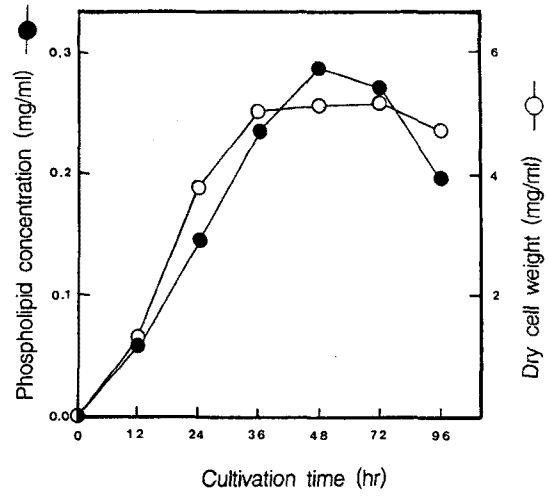

Fig. 2. Effect of culture time on the growth and the phospholipid production by *Pseudomonas* sp. CH-414.

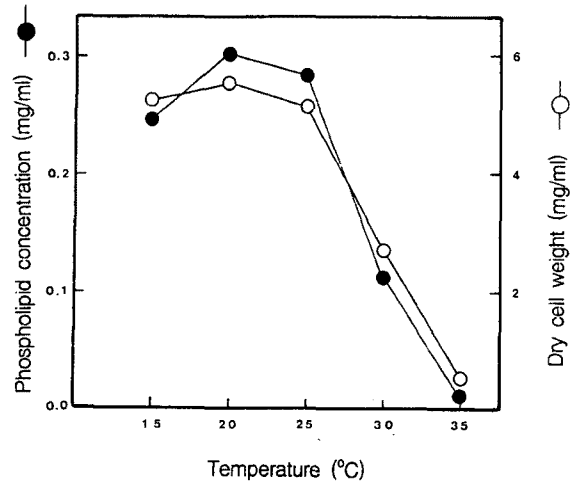

Fig. 3. Effect of temperature on the growth and the phospholipid production by *Pseudomonas* sp. CH-414.

Effect of culture temperature: Fig. 3 shows the effect of the culture temperature on the production of phospholipid. For the highest phospholipid production and cell growth, the optimum temperature was 20°C. Beyond this temperature, the phospholipid production and the cell growth were rapidly decreased. Jareonkitmongkol et

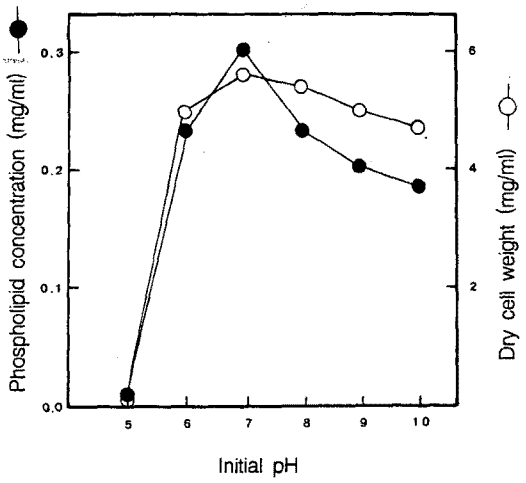

Fig. 4. Effect of the initial pH on the growth and the phospholipid production by *Pseudomonas* sp. CH-414.

al. (10) demonstrated that the production of the fatty acid by *Mortierella alpina* 1S-4 was the highest at 12°C. And Yazawa et al. (22) reported that SCRC-2738 produced a fairly high amount of fatty acid within the temperature range of 8-25°C. The results of the current paper are in accord with those of Yazawa et al. (22).

Effect of the initial pH; By varying the initial pH within the range of 5-10, the effect of the initial pH on the phospholipid production and cell growth was investigated at 20°C (Fig. 4). Although Yazawa et al. (22) reported that the production of the fatty acid using the strain SCRC-2738 was the maximum at pH 8, the phospholipid concentration and the biomass were found to show their maximum level at the initial pH 7.0 in this work.

Under the above culture conditions, the production of phospholipid was about 0.3 mg/ml and 0.06 mg/mg dry cell weight.

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