J. Korean Soc. Food Nutr.
20 (3), 246~252 (1991)

Eicosapentaenoic Acid Production of *Alteromonas putrefaciens* KS-90 Isolated from Fish Intestines

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Abstract

Marine bacteria of fish intestines were screened for high eicosapentaenoic acid (EPA) productivity. An isolated bacteria, isolate, KS-90, identified and designated as *Alteromonus putrefaciens* KS-90. *A. putrefaciens* KS-90 was found to be a rich source of EPA production and it was observed on incubation at 4~12.5°C and pH 7.0. The production of EPA reached 18mg/g of dry cell weight when *A. putrefaciens* KS-90 was grown in the medium containing 1.0 % peptone, 0.5% yeast extract, 0.025% meat extract and 2.0% glucose in 1/2 concentration of an artificial seawater, pH 7.0 for 48 hr at 25°C. This value accounted for 24.7% of the total fatty acid in the extractable lipids.

Key words : eicosapentaenoic acid, *Alteromonas putrefaciens* KS-90

INTRODUCTION

Eicosapentaenoic acid (5, 8, 11, 14, 17-*cis*-eicosapentaenoic acid, EPA) is a rare polyunsaturated fatty acid with five double bonds having unique biological activity. EPA is effective for the prevention and curing of blood circulatory diseases such as of thrombosis, arteriosclerosis[1,2] and antiaggregator substances[3] and reduction of the serum lipid level[4,5]. All these results demonstrated that EPA was essential to maintain homeostasis for mammals. A small amount of EPA was reportedly accumulated in algae[7], fungi[8], moss[9] and fishes[10-13].

For practical purposes, EPA or oil containing EPA was produced conventional source have several fish oils. However, these conventional sources have several disadvantages, such as their low EPA content, the presence of other fatty acids with undesirable properties, and difficulty in purificat-ion of EPA[14-16].

In recent studies, industrial production of EPA from microorganisms is of major interest to obtain more suitable sourses for large-scale preparation of EPA.

Mortierella[9] and *Alteromonas*[17] were previously thought to be the only microorganisms which accumulate significant amounts of EPA intracellular. EPA production by these microorganisms was not sufficient for practical uses.

In order to produce EPA, this paper deals with the screening of high EPA producing strain, the identification of the isolates, and then investigates the conditions for the production of EPA.

MATERIALS AND METHODS

Materials

As a preliminary experiment, marine microor-

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ganisms were isolated from various marine sources such as fish, shellfish, sea water and sediment mainly obtained from the Pusan area.

Media

Media for isolation and growth of EPA-producing bacteria were as follows[17,18] : Nutrient agar, BTB Teepol agar, Sabourand agar, Bacto Marine agar 2216, Malt agar were purchased from Nisui Co. (Japan), and mannitol salt agar, NAC agar and Trypto-soy agar were obtained from Eiken Co. (Japan).

Isolation for microorganisms producing EPA

Each intestine of several fish samples (10~30g) was suspended in 20ml of sterilized 1/2 artificial seawater. After filtration, an aliquot of supernatant was cultured in PYM agar medium (1.0% peptone, 0.5% yeast extract, 0.025% meat extract and 2.0% glucose in 1/2 concentration of an artificial seawater) at 25℃ for 48~72 h. 6,000 colonies of marine microorganisms were isolated from the samples. Each colony was cultured in PYM medium in 1/2 concentration of artificial seawater at 25℃ for 48 h. The cells were harvested by centrifugation at 4,000*g* for 10min[17,18].

Identification of isolates, KS-90

Morphological characteristics and physiological properties of isolates were investigated according to the Bergey's manual of determinative bacteriology [19].

Extraction of total lipid

The wet cells were suspended with organic solvents (chloroform : methanol=2 : 1, v/v) and homogenized for 5min. After phase separation, the bottom chloroform layer was collected and extraction of upper layer with organic solvents was repeated three times. The collected chloroform layer was evaporated to remove organic solvents and the remaining lipid was vacuum-dried to a constant weight.

Analysis of fatty acid composition

Fatty acid was analyzed according to the method of AOCS[21]. The extracted fats were saponified by the usual method and the fatty acid thus obtained were identified for EPA by TLC and then esterified with BF_3-methanol.

The methyl ester of fatty acids were analyzed by GLC equipped with capillary column (0.3mm × 25cm) and flame ionization detector. Solid phase of the column was Chromosorb, W. Column temperature was increased from 175℃ to 240℃ at the rate of 1.5℃/min. Nitrogen carrier gas was flowed at the rate of 0.6ml/min. Fatty acids of samples were identified by comparing the retention times

Table 1. Fatty acid compositions of isolates selected from marine organisms

Isolates	Incubation temperature (℃)	Fatty acid composition(%)						
		14 : 0	14 : 1	16 : 0	16 :1	18 : 1	20 : 6[a]	Others
KS-69	5	4.3	18.5	16.3	26.3	11.7	18.1	4.8
	25	3.7	19.4	18.6	23.7	13.0	16.8	4.8
KS-90	5	3.3	18.4	17.4	22.0	9.6	23.5	5.8
	25	4.0	19.5	16.4	20.4	10.0	24.7	5.0
KS-1027	5	4.6	19.6	20.3	23.5	8.7	20.3	3.0
	25	5.5	20.7	21.5	20.4	8.0	19.9	4.0
KS-3038	5	6.0	18.3	20.4	22.5	10.0	19.0	3.8
	25	4.5	20.0	21.0	20.7	12.0	18.2	3.6

[a] EPA

Each strain was grown under conditions described in methods except for growth temperature (5℃, 25℃) and cultivation periods (2 days).

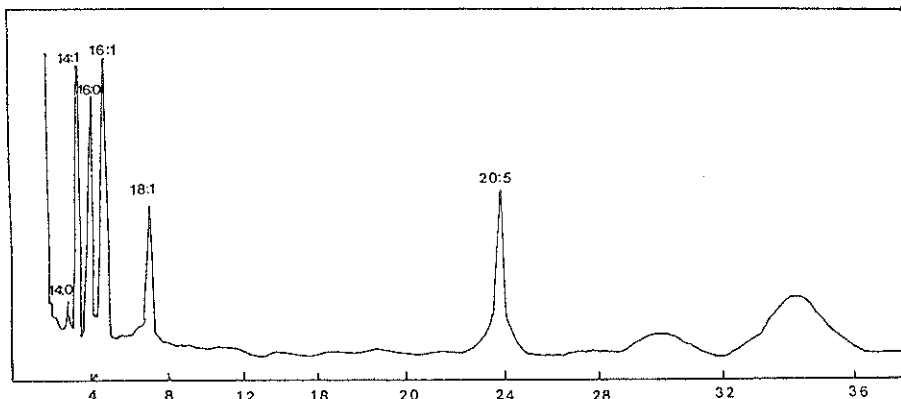

Fig. 1. Gas chromatograms of EPA produced by isolate, KS-90.

with those of authentic fatty acids. EPA was confirmed by the coupled gas chromatography-mass spectrometry (Hewlett Packard, HP 5970, USA).

RESULTS AND DISCUSSION

Screening of microorganisms for high EPA productivity

Microorganisms producing EPA were screened from about 6,000 colonies which were isolated from intestinal tracts of marine fishes such as mackerel, horse mackerel, sardine, saury-pike, salmon and squid. Through the screening, 4 isolates were selected from microorganisms as promising producers of EPA (Table 1).

All strains were grown well on PYM medium. Table 1 also shows the fatty acid compositions of the lipid extracted from cells of these strains. The isolate KS-90 showed the highest EPA in the total extractable fatty acids, when incubated at 25℃ in 500ml flask. This value represents more than 28.9 % of the total polyunsaturated fatty acids and is particularly high when compared with that of any other polyunsaturated fatty acid. Subsequently, strain KS-90 was selected for further study.

Identification of the EPA produced by isolate, KS-90

Gas chromatographic and a mass spectrophotometric analysis were employed. As shown in Fig. 1 on gas chromatographic analysis, the isolated methyl eicosapentaenoic acid showed single peak at 22.5 min of retention time, which corresponded exactly to that of authentic methyl eicosapent-

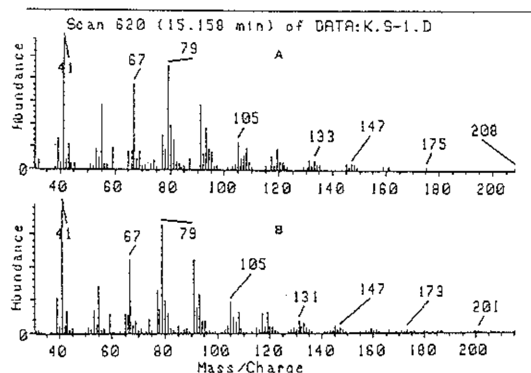

Fig. 2. Mass spectra of authentic EPA(A) and $C_{20:5}$ methyl ester from cellular lipids of the isolate, KS-90 (B).

aenoic acid. The mass spectrum of the isolated sample showed a molecular ion peak at m/c 208 and an intense fragment ion peak at m/c 41, 55, 67, 79, 91 and 105, respectively (Fig. 2). Each peak was well in accordance with the corresponding one in the case of the authentic standard.

Identification of isolate, KS-90

The strain KS-90 grew fast at 25℃ not only on PYM glucose agar medium, but also on nutrient agar and marine agar. As shown in Table 2, strains KS-90 were rod-shaped gram negative bacteria, and motile with a single polar flagellium. Strain KS-90 was 0.8-1.5μm in diameter and 0.8-1.5μm in length. The colony was smooth with entire edge and brownish-yellow. The strain KS-90 required essentially natural seawater or artificial seawater for growth under the aerobic condition. The results indicated that strain KS-90 was similar to *Alte-*

Table 2. Comparison of major morphological characteristics of the strain KS-90 and *Alteromonas putrefaciens* IFO 3909 as a reference

	Strain KS-90	*Alteronomas putrefaciens* IFO 3909
Gram staining	Negative	Negative
Shape of cell	Rod	Rod
Flagellium	Single, polar	Single, polar
Motility	Active	Active
Width of cell(μm)	0.7∼1.5	0.8∼1.5
Length of cell(μm)	1.8∼3.0	1.6∼3.0
Colonies	Smooth, entire edge	Smooth, entire edge
Pigment	Brownish yellow	Brownish yellow

romonas species according to the Bergey's manual of determinative bacteriology,[20] and Hayes, et al[21].

Using *Alteromonas putrefaciens* IFO 3909 as a reference, physiological and biochemical characteristics of the strain, KS-90 were investigated. Table 3 shows the result of ultilization tests. Strain KS-90 ultilized D-xylose, arabinose, glucose and mannitol except for the citrate. Hydrolysis of starch and gelatin were positive and also enzymes such as oxidase, catalase and DNase was positive except for ornithine decarboxylase. The character-istics of the strain, KS-90 do not differ significantly from reference of *Alteromonas putrefaciens* except for in some characteristics. However, we consider that

Table 3. Comparison of physiological and biological characteristics of strain KS-90 to *Alteromonas putrefaciens* IFO 3909 as a reference

	Strain KS-90	*Alteromnas putrefaciens* IFO 3909
Ultilization of xylose	+	+
arabinose	+	+
D-glucose	+	+
mannitol	+	+
citrate	−	+
L-alanine	+	+
L-serine	+	+
Hydrolysis of starch	+	+
gelatin	+	+
Oxidase reaction	+	+
Catalase	+	+
DNase	+	+
Ornithine	+	+
Decarboxylade	−	+
Nitrate reduction	−	−
Growth at 5℃∼25℃	+	−

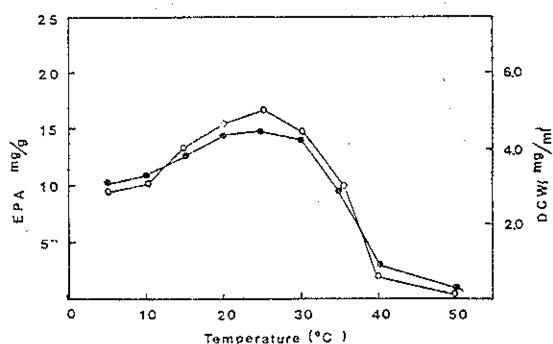

Fig. 3. Effects of temperatures for growth of *A. putrefaciens* KS-90. The strain was cultivated in 300ml flask containing 50ml of PYM medium at the indicated temperatures with reciprocal shaking for 2 days.
○−○ : EPA, ●−● : Dry cell weight (DCW)

these differences are not enough to distinguish the strain KS-90 from *Alteromonas putrefaciens*. Therefore, we identified it as *Alteromonas putrefaciens* KS-90.

Effects of the cultural temperature

The effect of the cultural temperature of the medium on the production of EPA are shown in Fig. 3. *Alteromonas putrefaciens* KS-90 grew well at low temperatures ranging from 5℃ to 18℃, although the growth rate was somewhat lower than that of those at high temperature ranging from 20℃ to 30℃.

The amount of EPA was produced in proportion to cell growth. The production of EPA reached 10.5mg/g at 5℃ and also 16.5mg/g at 25℃. A temperature shift from 5℃ to 25℃ also induced EPA production.

Further elevations of the culture temperature brought about decreases in their EPA contents, although EPA accumulation was still observed up to 25℃. Conversely, other fatty acid increased with elevation of the growth temperature. However, when the temperature was shifted to 25℃ after cultivation at 5℃ for 2 days, no further increase in EPA accumulation was observed.

These experiments seem to suggest that the increase of EPA might be essential for strain KS-90 to maintain cell fludity at low or high temperature[9,17,18]. *A. putrefaciens* KS-90 was found to accumulate monosaturated fatty acid such as pal-

mitic acid and myristic acid. In addition, there was 5% unidentified fatty acid.

However, no n-3 polyunsaturated fatty acid other than EPA was detected.

The EPA content of *A. putrefaciens* cell masses grown at 25℃ was higher than that at 5℃. Yazawa et al.[17] reported that marine bacteria produced the highest level of EPA at 20~25℃. These results were somewhat in accordance with some reports[17, 18].

Effects of optimal pH

To find out optimal pH for EPA production, *A. putrefaciens* KS-90 were grown in PYM medium containing 1/2 concentration of seawater, and the pH of the medium was varied from 4 to 10. As shown in Fig. 4, optimal pH for the EPA production by *A. putrefaciens* KS-90 was found to be 7.0.

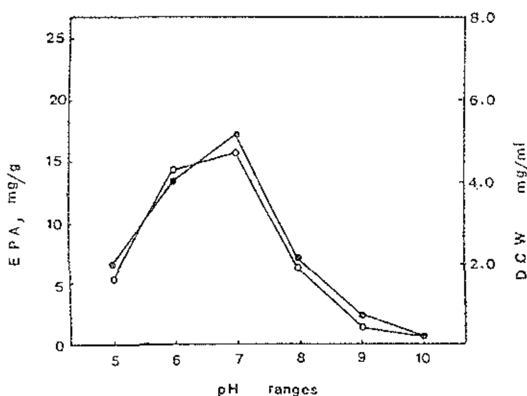

Fig. 4. Effects of the initial pH for growth of *A. putrefaciens* KS-90. *A. putrefaciens* KS-90 was cultivated in 300ml flask containing 50ml of PYM medium at the indicated pH with reciprocal shaking for 2days.
○ – ○ : EPA, ● – ● : Dry cell weight (DCW)

This strain did not grow well in weak acid and alkali regions of pH and thereafter, the EPA production and cell mass was found to be gradually decreased. Yazawa et al.[17] suggested that marine bacteria produced EPA at neutral pH.

Production of EPA under the optimal culture conditions

A. putrefaciens KS-90 was incubated to the PYM medium in the fermentor (Marubishi Co., Japan) under the optimal culture conditions of pH 7.0 and 25℃.

After incubation at 25℃ for 48h, the production of EPA reached 18mg/ml of culture medium as the dry cell weight increased to 6.3mg/ml (Fig. 5). This value accounted for 24.7% of the total extractable intracellular fatty acids (20.4%), myristoleic acid (19.5%), myristic acid (4.0%), palmitic acid (16.4%) and oleic acid (10%).

However, other polyunsaturated fatty acids, such

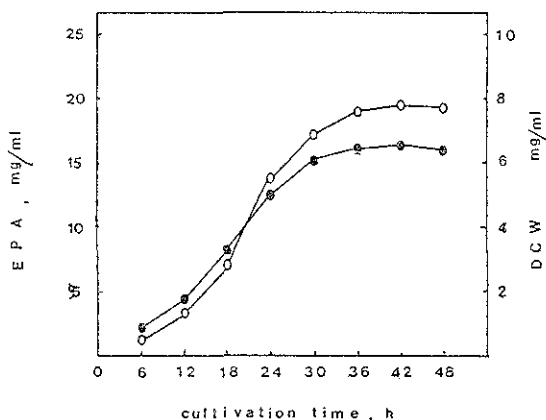

Fig. 5. Time course of EPA production by *A.putrefaciens* KS-90. *A. putrefaciens* KS-90 was cultivated in 5L fermentor containing 1.5L of PYM medium at 25°C for 48h.
○ – ○ :EPA, ● – ● :Dry cell weight (DCW)

as arachidonic acid, linolenic acid and linoleic acid were not detected at all. In addition, there was less than 5% of unidentified fatty acid. EPA productivity by *A. putrefaciens* KS-90 is similar amount of EPA to those reported using *Mortierella*[9], *Alteromonas*[17] and some marine bacteria[18]. These results suggested that it is too easy to separate EPA form fatty acid from *A. Putrefaciens* KS-90, it is because this fatty acid's composition is unique to that of oil containing EPA. In addition, another advantageous characteristic of this strain as an EPA producer is that it contains no polyunsaturated fatty acid other than EPA in high concentrations. These characteristics would make the use of *Alteromonas putrefaciens* KS-90 as a promising source of EPA for its practical preparation even when compared with fish oil.

ACKNOWLEDGEMENT

These works were supported by the grant from the Korean Traders Scholarship Foundation and the Tae-Woo Pharmaceutical Co.

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(Received December 1, 1990)

생선 내장에서 분리한 *Alteromonas putrefaciens* KS-90에 의한 Eicosapentaenoic Acid의 생산

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요 약

EPA를 생산하기 위하여 생선내장에서 분리한 세균에서 EPA를 분비하는 균주 KS-90을 선별하여 분리 동정한 결과 *Alteromonas putrefaciens* KS-90으로 명명하였다. *Alteromonas putrefaciens* KS-90은 우수한 EPA 생산균주였으며, 4℃~25℃와 pH 7.0에서도 EPA의 분비를 확인하였다. *A. putrefaciens* KS-90은 PYM배지 (1.0% peptone, 0.5% yeast extract, 0.025% meat extract, 2.0% glucose 및 1/2 artificial seawater, pH 7.0)로 25℃에서 48시간 배양시켰을 때 건조균체의 g당 18mg의 EPA를 생산할 수 있으며, 이 값은 생산된 전체 지방산의 24.7%를 차지하였다.