Production of EPA by *Shewanella putrefaciens* MAC1 in Selected Culture Media

Houshang Nikoopour[1*] and Mansel Griffiths[2]

1. Associate Professor, Department of Food Science and Technology, Shaheed Beheshti University of M.C.
2. Professor, Department of Food Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada

The EPA production of a marine bacterium, *Shewanella putrefaciens* MAC1 in four selected culture media and at incubation temperatures of 4°C for 72h and 10°C for 24h was studied. Eicosapentaenoic acid was found to be the sole polyunsaturated fatty acid produced by this bacterium. The major saturated fatty acids produced were palmitic and myristic acids. The highest yield of EPA production was 34.7 mg/g dry cell weight which was obtained in a basic culture medium containing 1% peptone and 0.5% yeast extract in ½ strength artificial sea water (ASW) at 10°C. The least EPA was produced when 2% glucose was added to the basic ingredients, indicating that EPA production is enhanced by culture media having limited source of carbon. Addition of 2 mg/l desaturase cofactors NADPH (Nicotinamide Adenine Dinucleotide Phosphate) and FAD (Flavin Adenine Dinucleotide) to the basic ingredients had a significant effect on the EPA production (21mg/g dry cell weight) at 4°C.

Keywords: Eicosapentaenoic Acid, *Shewanella putrefaciens*, Bacteria

1. Introduction

Humans & most animals have no systems to synethesis Eicosapentaenoic Acid ,EPA (a n -3 type fatty acid with 20carbon chain and 5 double bonds) effectively ;therefore, it is necessary to obtain this fattyacid from the diet.EPA is an important component of the cell membranes,also has many pharma cological properties such as anticoagulation of platelets(thrombosis),lowering of plasma triglyceride level,lowering of VLDL &LDL cholesterol in blood(anti-atherosclerosis), lowering of blood viscosity and pressure, anti- inflammation & antitumorigenesis[1]. In order intestine of blueblack fish,are able to produce EPA to overcome the problems of fishy odor & purification difficulties of EPA from fish oil,the microbial production of EPA by culturing marine algae and fungi was attempted,but the industrial production of EPA from these sources was not large enough to replace EPA from fish oil. Some researcher looked for EPA-producing marine bacteria [2]. Yazawa , et al. [2] screened about 5000 trains of marine micro organism for EPA producing ability, which was detected in 88 of them . All of these were found to be Gram-negative , aerobic , short rod–shaped bacteria. They could isolate a strain capable of produc ing the highest amount of EPA when cultured aerobically in a P-Y-M glucose medium using a ½ concentration, Artificial Sea Water at PH 7 & 20-25°C.Optimozation of culture conditions for growth rate, EPA content and productivity of a bacterium isolated from Pacific mackerel intestine was investigated by Akimoto , et al.[3] who found under optimum culture conditions (25°C,pH 7.0 and 1.0 wt % peptone and0.5% wt yeast extract in 100% v/v ASW) the EPA-production reached 45.6 mg/l of culture broth after 8hours. A marine bacterium, judged as a new species close to *Shewanella putrefaciens* was isolated by Yazawa [4] from the intestine contents of the Pacific mackerel. The isolated strain SCRC-2378 produced EPA as the sole

* Corresponding author E-mail address: h_nikoopour@yahoo.com

Polyunsaturated fatty acid,which amounts to 24-40% of the total fatty acids in the cell, corresponding to 2% dry cell weight. Under the optimum growth conditions (pH 7.0, 20 C and grown aerobically for 12-18 h) the yield of SCRC 2378 reached 15g of dry cells per liter.The effect of environmental conditions on production of PUFA (polyunsaturated fatty acid) by bacteria was studied by Nichols, et al. [5]. Bacterial fatty acid composition maybe manipulated by alteration of nutrient levels, osmotic forces, carbon source and biosynthetic cofactors. *Shewanella putrefaciens* strain ACAM 342 produced the maximum EPA during exponential phase, but EPA production decreased in stationary phase. The production of EPA was higher at 15°C as compared to 25°C. An increase in the salinity of the medium from 3.5 to 7.0% greatly reduced cell yield and the overall level of fatty acid unsaturation. Addition of desaturase cofactors such as NADPH (nicotinamide adenine dinucleotide phosphate) and FAD (flavin adenine dinucleotide) significantly increased the percentage of total PUFA, specially the EPA in total fatty acids. In a study by Amiri-Jami, et al. [6] transposon Tn 5 mutagenesis was used to generate random mutations in Shewanella bacteria MAC 1. Three mutants produced 3-5 times more EPA compared to the wild type at 10°C. One of the mutants produced 0.3 mg EPA when grown at high temperature (30°C). Six marine bacterial strains that produce polyunsaturated fatty acids were isolated by Ivanova, et al. [7] from sea water samples collected from sea of Japan and characterized to clarify their taxonomic position. It was found that *Shewanella japonica* was the closest relative (99% similarity). The novel organisms grew between 4 and 33° C. The EPA formed at 28°C was present at up to 5.3% of total fatty acids.The production of EPA by a bacterium isolated from mackerel entrails and identified as *Shewanella putrefaciens* MAC1, using unsalted sweet whey as growth substrate was studied by Cadieux [8,9]. It was shown that this bacterium is capable of synthesizing EPA at a concentration of 7% of the selected fatty acids at 4°C. EPA level reached a maximum after 96 hours (late lag phase), but decreased after 108 hours (stationary phase). In the present study, it was attempted to compare EPA production and the growth of the bacterium *Shewanella putrefaciens* MAC1 in selected culture media and two incubation temperatures. The effects of desaturase cofactors such as NADPH and FAD, also addition of glucose on the growth and EPA production were studied.

2. Materials and Methods

All chemicals and vitamins were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) and the ingredients for microbiological culture media were supplied by Difco Laboratories (Detroit, MI, USA).

2.1 Preparation of pre-culture

The bacterium used in this study was previously isolated from mackerel entrails in the Department of Food Science, University of Guelph, Canada and was identified by MIDI (Microbial Identification Incorporated) clinical library at the Ontario Ministry of Agriculture and Food and Rural Affairs (OMAFRA, Guelph,Ontario) as being *Shewanella putrefaciens*. It was a Gram-negative, aerobic, rod-shaped bacterium having a single flagellum and was named *Shewanella putrefaciens* MAC1. In order to prepare an active pre-culture, a loop of the bacterial culture grown on selected culture broth (SCB) plus agar plates at 4°C was transferred into 100 ml of Luria-Bertani broth (LB) in a 250 ml Erlenmyer flask and left on a shaker at 30°C for 24 hours. One ml of culture was transferred into 100 ml fresh sterilized LB broth and left on shaker at 30°C for 24 hours. The composition of SCB and LB media are given in Table 1, [9].

Table 1 Comparison of selected culture broth (SCB) and Luria-Bertani (LB) media

Ingedients	SCB (g/l)	LB-Difco (g/l)
Nutrient Broth	8.00	0
Tryptone	0	10
Yeast Extract	5.00	5
NaCl	17.56	10
KCl	0.76	0
MgSO4	6.02	0

Table 2 Composition of bulk salt solution

Ingredients	Amount (g/l)
NaCl	24.0
Na2SO4	4.0
KCl	0.678
KBr	0.098
H3BO3	0.026
NaF	0.002
MgCl2.6H2O	10.835
CaCl2.2H2O	1.517

2.2 Preparation of artificial sea water (ASW)

The artificial sea water used for preparing the selected media was made basically as described by Schneider and Marshall[10]. The procedure for preparing ASW was as follows:

1. A bulk salt solution was prepared as given in Table 2. Deionized water was used as solvent.
2. The bulk salt solution was autoclaved at 121°C for 15 min. then cooled.
3. A 1M NaOH (40g/l) solution was prepared and filter sterilized.
4. A mineral salt stock Solution was prepared as given in Table 3**.**
5. 6.5 ml of 25% HCl was added to one liter of mineral salt stock solution.
6. 6.5 ml of 25% HCl was added to one liter of mineral salt stock solution.
7. The mineral salt stock solution was filter sterilized using a 0.2 micron filter.
8. One liter of vitamin stock solution was prepared as given in Table 4.
9. Vitamin stock solution was filter sterilized through a Nalgen 0.2 micron syringe filter, then 0.5 ml was added to one liter of bulk salt solution. This bulk salt solution, so called artificial sea water was used for preparation of selected culture media in this study.

2.3 Preparation of selected culture media

Preparation of four selected culture media used in this study was done according to the following procedure:

Four one- liter flasks (M1, M2, M3 and M4) containing 500 ml of prepared ASW were taken. Ten grams of peptone and 5 grams of yeast extract were added to each flask. Twenty grams of glucose was also added to the flasks M1 and M2. All flasks were made up to one liter volume with deionized water and autoclaved at 121°C for 15 min. After cooling, 0.5 ml of the filter-sterilized vitamin stock solution was also added to each flask. Ten mg of NADPH and 10 mg of FAD were dissolved in 5ml deionized water and filter-sterilized. Then one ml of each was added only to the flasks M1 and M3. Therefore, these two selected media each contained two mg/l of desaturase cofactors NADPH and FAD. Finally, the pH of all four selected media was adjusted to 7.2 with filter-sterilize 1M NaOH solution. The composition of four selected media used in this study is given in table 5.

Table 3 Composition of mineral salt stock solution

Ingredients	Amount (g/l)
FeCl3.6H2O	8000
ZnCl2	70
MnCl2.4H2O	100
CoCl2.6H2O	120
NiCl2.6H2O	25
CuSO4.5H2O	22
Na2MoO4.2H2O	25

Table 4 Composition of vitamin stock solution

Ingredients	Amount (g/l)
p-Amino-Benzoic Acid	50
Pyridoxine-HCl	100
Thiamine-HCl	50
Riboflavin	50
Nicotinic Acid	50
D-Ca-Panthothenate	50
Lipoic Acid	50
Nicotinamide	50
Vitamin B12	50
Biotin	20
Folic Acid	20

2.4 Experimental procedure

In this study, 4 selected media x 2 temperature conditions x 3 replicates, a total of 24 samples were studied. Six 120 ml portions of each selected media were poured aseptically into 250 ml sterile flasks. Each flask was inoculated with 0.6 ml of active Pre-culture of *Shewanella putrefaciens* MAC1. All inoculations were performed under a laminar flow

microbiological hood. Three replicates of each inoculated Selected Media were placed on automatic shakers in 4°C and 10°C incubators and the speed of shakers was set at 60 rpm.

The samples at 4°C were removed after 72 hours of incubation and the O.D. of samples were measured at 600 nm by a Spectrophotometer Model Ultrospec 1000 E (Pharmacia Biotech Co.). The samples at 10°C were also removed after 24 hours and the O.D. measurements were done as mentioned above. The 100 ml portions of all samples were centrifuged at 8000 rpm and 4°C. The bacterial cells were washed twice with distilled water, then stored at –20°C. The frozen samples were freeze-dried for 48 hours, using a freeze-drying unit made by Storks Co., USA. The weights of all dried samples were determined by an analytical balance.

2.5 Esterification of samples

The esterification of samples was carried out according to the following procedure:

The freeze-dried samples were ground to a fine powder form then transferred to screw capped test tubes. The esterification was performed by adding 4 ml of 12% boron trifluoride in methanol to each tube and heating them in a boiling bath for 15 min. The samples were cooled to room temperature, then 4 ml of distilled water was added to each sample. The extraction of fatty acids was carried out by addition of 1 ml hexane and mixing over a test tube vibrator. The hexane layer was transferred to a clean screw caped test tube and extraction with hexane was repeated once more. The hexane was evaporated completely by blowing nitrogen gas into the tubes, then 1 ml of iso-octane was added to dissolve fatty acids. Finally, 10 mg Na2SO4 was added to each sample to remove the moisture. All samples were stored at –20°C until they were analyzed by Gas Chromatography.

2.6 GC Analysis

The identification of fatty acids was carried out by using a Shimadzu Gas Chromatograph Model GC-14A (Mandel Scientific Co. Inc., Guelph, ON, Canada), equipped with a split mode injection system, Flame-Ionization Detector, and a 60m x 0.22mm i.d., 0.25 micron film thickness BPX70 (70 % cyanopropyl polysilphenylene) fused silica capillary column (SGE, Inc., USA). The GC conditions were as given below:

Column initial temperature	110°C
Initial time	2 min
Heating rate	6°C/min
Column final temperature	230°C
Final holding time	10 min
Injection port temperature	260°C
Detector port temperature	280°C
Hydrogen gas pressure	0.5 Kg/Cm2
Air pressure	0.7 Kg/Cm2
Hydrogen gas carrier (p)	1.5 Kg/Cm2
Injection sample volum	0.1 micro-liter

The data were integrated by a Shimadzu Model C-R4A Chromatopac Integrator (Mandel Scientific Co.). The fatty acids were identified by comparing the retention times to a pure standard supplied by Nu-Chek-Prep Inc. (Elysian, Minnesota, USA).

Table 5. Comparison of the prepared selected media

Ingredients	Amount	M1	M2	M3	M4
Peptone	% w/v	1	1	1	1
Yeast Extract	% w/v	0.5	0.5	0.5	0.5
Glucose	% w/v	2	2	0	0
NADPH	mg/l	2	0	2	0
FAD	mg/l	2	0	2	0
Artificial Sea Water	% v/v	50	50	50	50

2.7 Statistical analysis

All data obtained from this study were analysed using a Least Significant Difference Test (LSD-Test) and a Dunnet T-test.

3. Results and Discussion

A summary of data obtained from the growth of *Shewanella putrefaciens* MAC1in selected culture media at 4°C for 72 hours is shown in Table 6. The highest production of EPA (21 mg/g dry cell weight) and lowest cell weight (286 mg/100 ml) were found in medium M3,

where 2mg/l of both desaturase cofactors NADPH and FAD were added to the basic ingredients. Similar results were previously reported by Nichols et al. The EPA production was low in both media M1 and M2, containing extra 2% of glucose, suggesting that EPA production was suppressed by addition of glucose as a carbon source. It was found that Eicosapentaenoic Acid was the sole polyunsaturated fatty acid produced by this bacterium. The major saturated fatty acids found to be palmitic acid, C16:0 (32.79%) and myristic acid, C14:0 (11.4%).The results for the growth and EPA production of *Shewanella putrefaciens* MAC1at 10°C for 24 hours are summarized in Table 7.

Table 6 Production of EPA and growth of *Shewanella putrefaciens* MAC1 at 4°C for 72 hours

M4	M3	M2	M1	Culture Media
1.989	1.283	1.836	2.906	Av. O.D.(600 nm)
448	286	374	430	Av. Cell Wt. (mg/100ml)
5.473	4.746	3.955	4.168	Av. EPA/ Total F.A.(percent)
15.57	21.02	12.38	11.27	Av. EPA yield (mg/g dry cell wt)

The maximum EPA production (34.7 mg/g cell weight) and the lowest cell weight (168 mg/100 ml) were obtained in medium M4 containing the basic ingredients of 1.0% peptone and 0.5% yeast extract in 50% artificial sea water (ASW). Again EPA production was the lowest (17.93 mg/g cell weight) in medium M2 containing 2% extra glucose. The maximum growth (219 mg/100 ml) was obtained in medium M1. Similarly, EPA was found to be the sole polyunsaturated fatty acid produced by this bacterium. The major saturated fatty acids were also palmitic acid, C16:0 (33.8%) and myristic acid, C14:0 (11.4%).

Table 7 Production of EPA and growth of *Shewanella putrefaciens* MAC1 at 10°C for 24 hours

Culture Media	Av.O.D. (600nm)	Av.CellWt. (mg/100ml)	Av.EPA/ Total F.A.%	Av. EPA yield (mg/g)
M1	2.268	219	3.997	22.62
M2	1.645	183	2.659	17.93
M3	1.375	190	3.237	20.99
M4	1.884	168	4.454	34.71

4- Conclusions

Shewanella putrefaciens MAC1was capable of producing Eicosapentaenoic Acid, as the sole polyunsaturated fatty acid in all four selected culture media used in this study.The highest EPA production (34.71 mg/g dry cell wt) was obtained in medium M4, containing the basic ingredients of 1% peptone, 0.5% yeast extract in 50% Artificial Sea Water at the incubation temperature of 10 C. Addition of glucose to the basic ingredients had an adverse effect on EPA production at both incubation temperatures. Addition of desaturase cofactors NADPH and FAD to the basic ingredients, medium M3, resulted in maximum yield (21mg/g dry cell wt) at 4°C. The main saturated fatty acids produced by *Shewanella putrefaciens* MAC1were palmitic acid and myristic acid, respectively

5- References

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