An Improved Method of Arachidonic Acid Production by *Mortierella alpina*

Nagao TOTANI*, Akira WATANABE, and Kenkichi OBA

Biological Science Laboratory, Lion Corporation, (202 Tajima Odawara-shi 〒256)

Highly unsaturated fatty acids such as γ -linolenic and eicosapentaenoic acids are now synthesized in large quantities by microorganisms but production methods of arachidonic acid are still inadequate due to its low yield for each type of medium used. To obtain lipids containing arachidonic acid at high efficiency, a detailed study was made of the conditions for incubating *Mortierella alpina* on potato paste-dextrose medium, addition of trace metals, thickness of the medium layer and pH. Mycelium and pellicle were found to grow on a medium, 2 mm thick, containing 6% dextrose and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 750 mg/kg medium at 20°C for 20 d. Fatty acid analysis of the lipids extracted from the dried cell indicated the yield of arachidonic acid to be 13.1 g/kg medium.

Introduction

Gamma-linolenic acid is now available in a big amount by the profound studies by Suzuki *et al.*¹⁾ followed by industrial cooperation. The biochemical synthesis of the acid could reduce the price about a half regardless of climate conditions and provide uniform quality of the product. Icosapentaenoic acid is also obtainable by cultivation of *Chlorella* which is a new species fused between ones living in sea and fresh waters^{2),3)}. Toyo Jozo⁴⁾, Japan provides arachidonic acid obtained from lipids of *Euglena gracilis* grown in the dark. However, it should seem that the method is not successful enough to expel the conventional production method because of the low yield.

The authors reported^{5),6)} that *Mortierella alpina* was a potential fungus for the production of arachidonic acid and that the fungus grew substantially at 20°C for 20 d on a solid medium comprising potato paste and dextrose; methyl esters derived from the extracted lipids contained 56.6% of arachidonic acid which yield was 11.8 g/kg medium. This yield was more than 20 times higher than that of Toyo Jozo mentioned above and the content of the acid was desirably so high that purification should be quite easy. In the present paper detailed examination was made on the incubation conditions to get better yield of arachidonic acid per amount of potato.

Experimental^{7)~10)}

Preparation of potato paste-dextrose medium

Washed potatoes were peeled and cut into 1 cm cubes. Six hundred grams of potato cubes were boiled with 400 mL of distilled water for 20 min and passed through 0.5 mm $\times$ 0.5 mm mesh with a spatula to make potato paste where remaining water was mixed with the paste. The paste was added distilled water to make 1,000 g followed by good mixing and autoclaving for 15 min. Before cooled to room temperature the paste was mixed with sterilized dextrose solution diluted in the least amount of water and poured equally into sterilized dishes of 80 mm in diameter, flattened by a vibrator, then allowed to stand at room temperature for an hour.

Incubation

Mortierella alpina IFO 8568 purchased from Institute for Fermentation Osaka, Japan was used throughout the present experiment. Several days after inoculation of the fungus white mycelia and pellicle covered the whole surface of the medium. Incubation was kept at 20°C for 20 d; these condition were settled by the result shown in another paper⁶⁾.

Fatty acid analysis

Mycelium and pellicle grown were harvested, dried in a desiccator, and ground in a mortar with pestle using chloroform/methanol

2:1 vol/vol. Lipids were extracted further with the same solvent mixture and interesterified with sodium methoxide. Colorless clear methyl esters, thus obtained, were analyzed by DEGS 15% column at 190°C contained in a Hitachi 663-50 gaschromatograph fitted with a flame ionization detector.

Concentration of dextrose

Relation between yield of arachidonic acid and dextrose concentration in the potato paste medium was examined using 4~15% of dextrose (Fig.-1).

Effect of Ca^{2+} addition

In order to improve yield of arachidonic acid $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and sodium acetate¹¹⁾ were added respectively to the potato paste medium containing 6% of dextrose (Table-1). Thickness of the medium layer used was 2 mm by the reason mentioned later.

Thickness of the medium layer

Efficiency of the plates was checked in terms of thickness; 2 mm, 4 mm, and 6 mm which corresponded respectively to 10g, 20g, and 30 g of the potato paste medium containing 6% of dextrose in a dish of 80 mm in diameter. Thickness of 2 mm was the thinnest layer possible (Fig.-2).

pH

Conventional potato extract-dextrose broth⁶⁾ made from 100 g of potato and 20 g of dextrose in a litre broth was employed to presume optimum pH for the arachidonic acid production on the potato paste-dextrose medium. After inoculation of the fungus into the 50 mL media at pH between 4.0~8.0 each contained in a 500 mL Sakaguchi-flask incubation was kept at 20°C for 10 d under shaking (Fig.-3).

Result and Discussion

The fungal growth and yield of arachidonic acid showed optimum dextrose concentration at 9%. It seems that more than 10% of dextrose inhibited the growth intensively and at 15% the fungus could hardly grow. Arachidonic acid content in the total fatty acids was constant up to 6% but it decreased gradually because of the inhibition.

Preliminary incubation at 20°C for 20 d using a broth⁶⁾ comprising extract from 600 g potato and 6% dextrose showed that the fungus

Fig.-1 Relation between dextrose concentration in the potato paste medium and production of arachidonic acid.

consumed dextrose at the rate of 4 g/d since two days after inoculation. If the result could be applied to the case of the potato paste-dextrose medium 72 g of dextrose would have been consumed after 20 d. Then the optimum dextrose concentration, 9% was acceptable although content of arachidonic acid in the total fatty acids tends to decrease under too nutritious conditions and *vice versa*.

Table-1 Effect of Ca^{2+} added in the potato paste-dextrose medium on the growth of *Mortierella alpina* IFO 8568.

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ added mg/kg medium	DCW ^a g/kg medium	MA ^b content in the methyl esters (%)	MA content per DCW (%)	MA yield per the medium (g/kg medium)
375	88.2	47.5	13.6	12.0
750	95.6	49.2	13.7	13.1
1500	81.1	47.8	12.2	9.9
0	82.4	46.9	12.5	10.3

^aDCW=dry cell weight, ^bMA=methyl arachidonate

There was a tendency that calcium chloride made content of arachidonic acid in the total fatty acids higher and also the cell body slightly stiff. Sodium acetate which is reported to be one of the substances activating the metabolism in the fatty acid synthesis in the cell¹¹⁾ helped increase the yield of neither arachidonic acid nor total fatty acids.

The thickness of medium layer, 2 mm, was the most efficient on the base of potato used. Arachidonic acid content in the total fatty acids was remarkably high in the fungus grown on a thin layer because of high consumption of nutrition by the fungus. The fungus can probably absorb most nutrition in the medium from down to 4 mm of depth although cost of the medium used would not be a decisive factor for the production of arachidonic acid. Consequently it is important how to let the fungus assimilate as much nutrition in the medium as possible, that is, the bigger area with certain amount of medium produces more arachidonic acid. However, thin plates have to be taken for the incubation under the good control of humidity during the fungal growth.

The optimum pH, at which the highest arachidonic acid content in the total fatty acids was observed, was shown between 5.0~6.0 although dry cell weights and yields of total methyl esters were almost constant irrespective of the initial pH in the range of 4.0~8.0. The final pH after 10 d of incubation was between 6.6~7.0. This result indicated that the fungus was not so sensitive towards pH between 4.0~8.0. Conveniently potato paste-dextrose medium prepared as mentioned above

Fig.-2 Influence of medium layer thickness on the yield of arachidonate.

Fig.-3 Influence of initial pH of the potato dextrose medium on the growth of *Mortierella alpina* IFO 8568.

had pH around 5.6.

Consequently the potato paste-dextrose medium, 2 mm thick containing 6% dextrose (and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 750 mg/kg medium) was employed for the practical use considering possibility of losing water from the medium under dry environment which brings increase of dextrose concentration in the medium.

The lipids and methyl esters obtained from the fungus had slightly dark color when it was cultivated on the medium containing pigments which occurred during autoclaving.

As general, the incubation conditions relatively poor or insufficient for the fungal growth increase the content of arachidonic acid in the total fatty acids.

The neutral glycerides obtained have special character, that is, arachidonic acid is attached not only to the beta position of the glycerol backbone but also the alpha positions. Among these, there are tri-, di-, and mono-arachidonyl glycerol where rests of glycerol backbone were occupied mostly with palmitic, stearic, oleic, linoleic, or γ -linolenic acid.

Conclusion

Mortierella alpina IFO 8568 was inoculated on 2 mm thick layer comprising 1,000 g paste made of 600 g potato boiled in water, 60 g

dextrose, and 750 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Incubation was kept at 20°C for 20 d followed by collection of the mycelium and pellicle. Lipids were extracted from the dried and ground cell and subjected to fatty acid analysis after interesterification. The yield of arachidonic acid accounted for 13.1 g/kg medium which corresponded to 49.2% of the total fatty acids.

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モルティエセラ・アルピナによる アラキドン酸の改良生産法

戸谷永生・渡辺 晃・大場健吉
ライオン株式会社生物科学研究所
(〒256 小田原市田島 202)

γ-リノレン酸やイコサペンタエン酸のような高度不飽和脂肪酸は、現在、微生物を用いて大量に合成されるが、アラキドン酸の生産法は低収率のため、まだ検討の余地があると考えられる。アラキドン酸含有脂質を効率よく得るために、*Mortierella alpina* をポテトペースト・デキストロース培地上で培養する際の詳細な条件を、培地中のデキストロース濃度、微量金属の添加、培地層厚、pH の点から検討した。その結果、菌糸と菌床を 6 % のデキストロースと $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 750 mg/kg 培地を含む厚さ 2 mm の培地上で、20°C 20 d 間培養する条件を選定した。乾燥菌体よりそれらの脂質を抽出し脂肪酸分析したところ、アラキドン酸の収量は 13.1 g/kg 培地となった。

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☆ 新刊図書

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