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Production of Arachidonic Acid by *Mortierella alpina* I_{49}-N_{18}

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Summary

Arachidonic acid (AA), an essential fatty acid in human body, fermented by *Mortierella alpina* I_{49}-N_{18} was investigated in a shake-flask, and a 50-ton fermentor. In order to optimize the culture conditions, the effects of temperature, initial pH, culture time, carbon and nitrogen sources were studied. Furthermore, the way of adding sugar during fermentation was evaluated in a 50-ton fermentor. Under the optimum culture conditions, arachidonic acid produced in shake-flask and 50-ton fermentor was 4.55 and 5.11 g/L media, respectively. It was shown that the highest percentage of AA in lipids in shake-flask and 50-ton fermentor reached 70.20 and 53.01 %, respectively. Gas chromatography/mass spectrometry tests showed that the oil contained 80 % of polyunsaturated fatty acids such as arachidonic acid, γ-linolenic acid, and linoleic acid.

Key words: arachidonic acid, fermentation, gas chromatography/mass spectrometry, industrial scale, *Mortierella alpina*

Introduction

Arachidonic acid (5,8,11,14-eicosatetraenoic acid, AA) is an essential dietary component for human beings and a precursor of many important eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes (*1*). AA has various physiological functions and plays an important role in infant nutrition (*2*). AA, widespread in the animal kingdom, can be isolated from lipids extracted from pig adrenal gland or pig liver and sardines as well; however, the yield of AA is only 0.2 %(ζ(A, B)) or lower (*3*), which is very difficult to industrialize. The production of polyunsaturated fatty acids (PUFAs) such as AA, dihomo-γ-linolenic acid, γ-linolenic acid, and eicosa-5,8,11-trienoic acid by fermentation with fungi has been reported (*4–7*). In addition to the production aspects, there have been some reports on the safety evaluation of *Mortierella* fungi and their products (*8–10*).

The 1995's Report on Fats, Grease and Human Nutrition of FAO/WHO (Food and Agriculture Organization/World Health Organization) indicates that the infant formula shall contain: 40 mg DHA/day/kg of infant weight and 60 mg AA /day/ kg of infant weight (*11*). Therefore, there has been focus on the study of AA produced by microbe in the whole world, especially in America, Japan and Canada (*12–14*). However, these studies were limited to laboratory level or middle-scale level (less than 1 ton). In order to increase the yield of

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AA and shorten the production cycle, since 1992 our laboratory has focused on the study of AA produced by *Mortierella alpina*. *Mortierella alpina* I_{49}-N_{18}, a high-yield arachidonic acid producing strain, was screened by ion implantation (*15*). In this paper, the optimal medium and culture conditions were studied, which were proven to have great effects on the yield of AA. Moreover, the production of AA in a 50-ton fermentor was performed as well.

Materials and Methods

Microorganism

Mortierella alpina I_{49}-N_{18}, screened by ion implantation, was used in the present study. Its content of arachidonic acid was about 2–3 folds higher than that of wild strain.

Medium and culture conditions

Stock culture

Fungal strains were cultured in PDA medium containing the following m/v content: 2.0 % glucose, 2.0 % agar and 20 % potato extract, pH=6.0. Media was sterilized at 0.2 Mpa for 15–20 min. All cultures grew 5–6 days at 28±1 °C.

Shaking batch culture

The fungus was inoculated into a 500-mL shaking flask containing 100 mL liquid medium with 6.0 % glucose, 1.2 % peptone, 0.8 % yeast extract, 1.0 % peanut cake, pH=8.5, and shaken at 200 rpm on an orbital-shaker for 10–13 days.

Industrial-scale batch culture

The flask culture (5–10 %, volume fraction φ(A, B)) was inoculated into 1-ton fermentor for 2 days, then culture (5–10 %, φ(A, B)) was inoculated into 6-ton fermentor for 2 days, at last, the culture (5–10 %, φ(A, B)) was inoculated into 50-ton fermentor for 7 days. The production medium contained 8.0 % glucose, 1.2 % peptone, 0.8 % yeast powder, and 1.0 % peanut cake, pH=8.5.

Lipid extraction and analysis

Fungal mycelia were harvested by suction filtration, washed with 50 mL water and dried at 105 °C for 2 h. Lipids were extracted from the dried fungal cells by Soxhlet method, and methylated (*16*). The fatty acids were analyzed by gas chromatogram-mass spectrometry (GC/MS).

The GC/MS was carried out in a Perkin-Elmer Auto System XL gas chromatography, connected to a flame ionization detector and a silex capillary column of SE-54 (30 m × 0.25 mm × 0.25 μm) made by SULPECOS. Injection temperature was 250 °C. Helium was under the pressure of 100 kPa. N-hexane solution (1.0 μL) was injected into the column, whose temperature was increased from 80 to 240 °C (hold 10 min) at the rate of 4 °C /min. The split was 30:1. The energy of the EI ion source of the Perkin-Elmer Tuto Mass Spectrometer was 70 eV.

Other methods

The growth of fungus was measured by determining the mycelial mass dried at 105 °C for 2 h. The concentrations of glucose in the media were measured by Fehling's solution. Glucose concentration was determined by titration with a standardized solution of sodium thiosulfate (0.1 mol/mL) and compared with the standard curve.

Results and Discussion

Effects of temperature

In Fig. 1 it is shown that the strain grew well when the temperature was 30 °C. However, the highest percentage of AA in lipids was observed at 25 °C, which indicated that the lower temperature was suitable for the accumulation of AA.

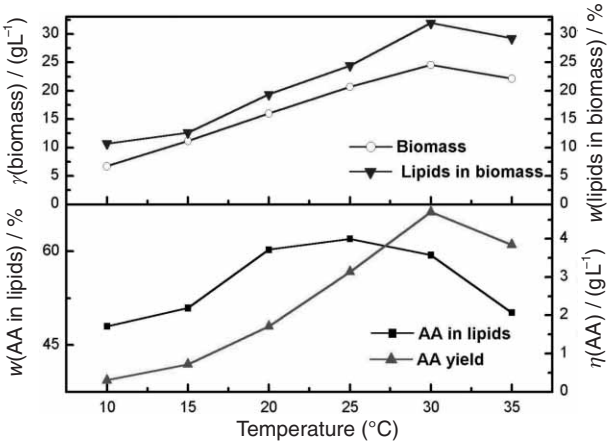

Fig. 1 Effects of fermentation temperature on growth of *Mortierella alpina* I_{49}-N_{18} and its lipid and AA production

Effects of initial pH

Fig. 2 shows the effects of initial pH ranging from 4.0 to 10 on the growth of the strain. The content of biomass and lipids in biomass were the highest at pH=8.0.

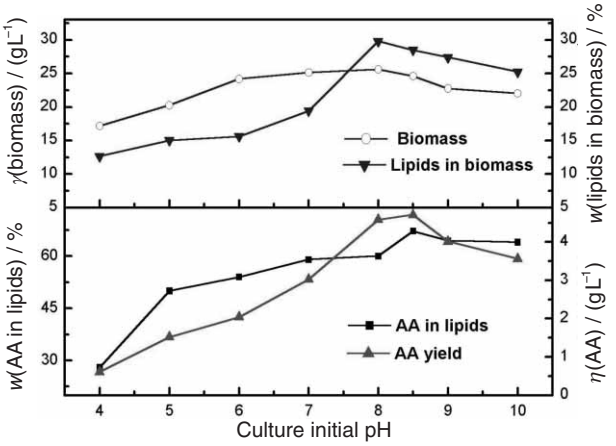

Fig. 2 Effects of culture initial pH on growth of *Mortierella alpina* I_{49}-N_{18} and its lipid and AA production

However, AA in lipids and in media reached the peak when the initial pH was 8.5.

Effects of culture time

It is shown in Fig. 3 that the biomass continuously increased during the first six days and after that slightly decreased. The highest content of the lipids in biomass was observed at the 6th day, but then it decreased intensely. Comparing the accumulation of biomass and lipids, the content of AA in lipids was increased in a time-dependent manner, reaching a peak at the 11th day. However, the highest arachidonic acid content appeared at the 6th day.

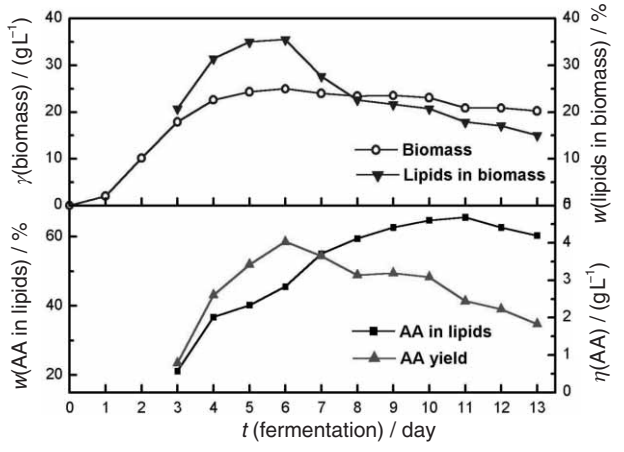

Fig. 3 Effects of fermentation time on growth of *Mortierella alpina* I_{49}-N_{18} and its lipid and AA production

Effects of carbon source

The effects of carbon source on the fermentation are shown in Fig. 4. Different carbon sources at mass concentration of 6.0 % were tested in the medium, containing 1.0 % peanut cake, 2.0 % peptone, and 1.0 % yeast extract. The strain grew very slowly in the medium with sucrose and glycerol as carbon sources. The growth of the strain is moderate with starch and maltose, and very good with glucose.

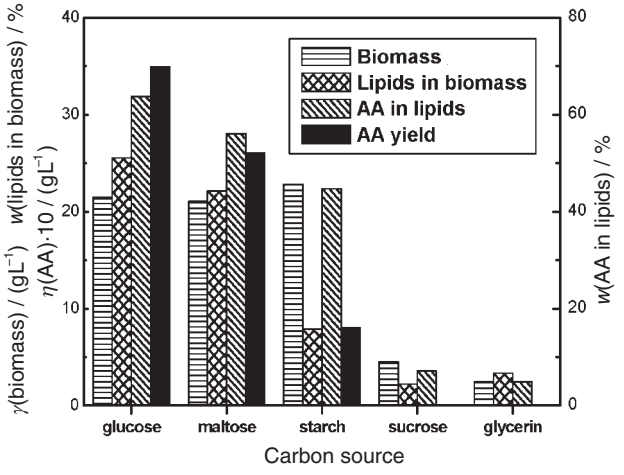

Fig. 4 Effects of carbon source on growth of *Mortierella alpina* I_{49}-N_{18} and its lipid and AA production

Effects of nitrogen source

Various nitrogen sources, at mass concentration of 3.0 %, were added separately to the medium containing 6.0 % glucose. The results are shown in Fig. 5. Yeast extract was the most efficient nitrogen source, which resulted in the highest AA content. The others were followed by yeast powder, peanut cake, and peptone, respectively. The highest percentages of AA in the lipid fraction were observed with yeast extract and peanut cake used as nitrogen source.

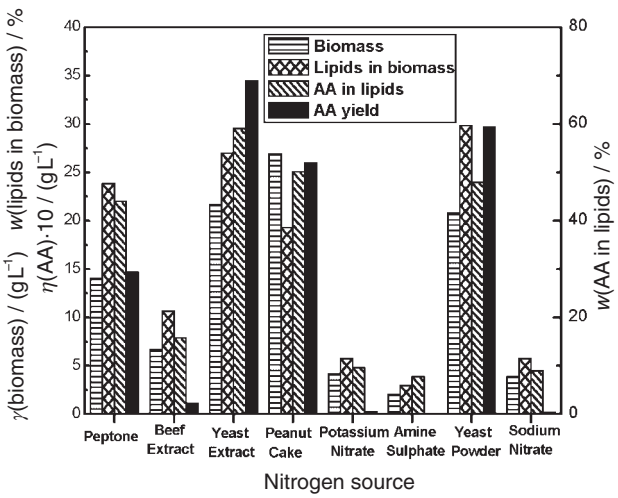

Fig. 5 Effects of nitrogen source on growth of *Mortierella alpina* I_{49}-N_{18} and its lipid and AA production

Analysis of the strain growth curve in shake-flask

Based on the present results, the medium with 6.0 % glucose, 1.0 % peanut cake, 1.2 % peptone, 0.8 % yeast extract, pH=8.5 was shown to be the best one. Fig. 6 shows the growth of AA high-yield strain *M. alpina* I_{49}-N_{18} in 500-mL shake-flask containing 100 mL of medium and shaken at 200 rpm for 11 days at 30 °C.

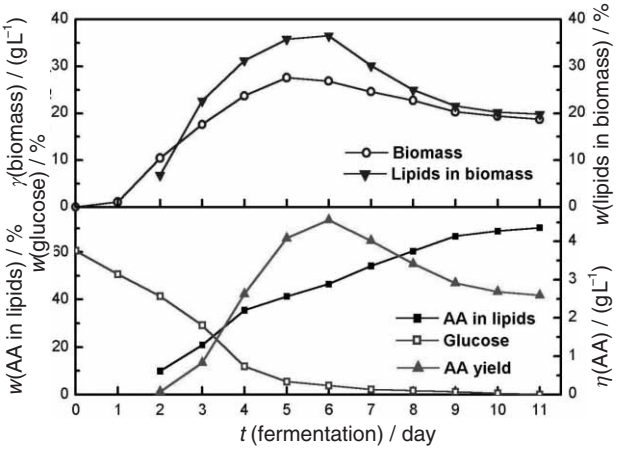

Fig. 6 Growth curve of AA high yield strain *Mortierella alpina* I_{49}-N_{18}

On the first day, the culture got into the lag phase. There was little consumption of sugar and accumulation of biomass. The fungus might require some time to acclimatize to the culture conditions. The content of sugar in the medium was decreased gradually in the exponential phase of growth. In contrast, both biomass and lipids were increased and reached a peak at the 5-6th day. The maximum content of biomass and lipid were 27.58 g/L and 36.52 %(ζ (A, B)), respectively. In the retardation and sedentary phases, although the biomass was decreased slightly, the content of lipids was decreased intensely and at the 11th day they were only 20 %(ζ (A, B)). It should be noticed that the content of AA in lipids increased steadily during the whole period, reaching 70.2 %(ζ (A, B)) at the 11th day, but arachidonic acid content was the highest on the 6th day (4.55 g/L).

Fatty acids analysis

The fatty acid methyl esters of *Mortierella alpina* I_{49}-N_{18} were determined by GC/MS. The concentrations of major fatty acids are listed in Table 1.

Table 1. Fatty acids component and content of the strain

Fatty acid	RT (retention time) min	Content ζ (A, B)/%	Fatty acid	RT (retention time) min	Content ζ (A, B)/%
Myristic acid (C14:0)	21.67	0.221	γ-Linolenic acid (C18: 3)	33.77	1.576
Pentadecanoic acid (C15:0)	24.27	0.174	Arachidic acid (C20: 0)	36.08	0.049
Palmitic acid (C16:0)	26.94	6.315	Arachidonic acid (C20: 4)	38.73	70.201
Stearic acid (C18:0)	31.67	2.236	Bebenic acid (C22: 0)	40.14	2.657
Oleic acid (C18: 1)	32.12	3.676	Lignoceric acid (C24: 0)	45.04	5.302
Linoleic acid (C18: 2)	33.14	3.985	Others		3.806

As it can be seen in Table 1 the content of unsaturated fatty acids in fungus lipids was close to mass ratio ζ (A,B) 80 %; while the content of arachidonic acid (RT= 38.73 min) in total lipid reached 70.2 %.

Industrial-scale culture

After growing in PDA medium for 5–6 days at 28 °C, the culture φ(A,B)= 5 % was inoculated into 500 mL shake-flask with 100 mL production medium, shaken at 200 rpm for 48 h at 30 °C. Then, the culture underwent the three-level fermentation. At first, the culture was inoculated into a 1-ton fermentor (with 400 L production medium) and cultured at 28–30 °C. After 2 days, the fermented media were transferred into 6-ton fermentor (with 2500 L production medium) for 2 days at 28 °C, the inoculating volume ratio was 10 % φ(A,B). At last, the culture 10 % φ(A,B) was inoculated into a 50-ton fermentor (with 30 000 L production medium) and continually fermented for 5–6 days at 25–28 °C. The production medium contained 8.0 % glucose, 1.2 % peptone, 0.8 % yeast powder, and 1.0 % peanut cake, pH=8.5.

In order to increase the final content of AA, different ways of adding glucose were studied in the third fermentation phase: feeding glucose one-off (8 %) or glucose at a concentration of 5 % added at the beginning and the rest (3 %) added during the growth. The results showed the latter was more efficient in accumulating lipids and arachidonic acid in a short period.

In addition, Fig. 1 showed that the strain grew well when the temperature was 30 °C, but the lower temperature was convenient for the accumulation of AA. Therefore, after the mycelium grew steadily (for about 48 h) during the main fermentation, temperature began to decrease 1 °C per 24 h up to 25 °C.

The average content of arachidonic acid in the culture medium was 5.11 g/L. It was found that the content of lipids in biomass in a 50-ton fermentor was higher than that in the shaking batch culture (4.55 g/L). This may be partially because the former contained more carbon source (8 % glucose) than the latter, which contained 6 % glucose.

Based on the above studies, arachidonic acid was produced in large-scale by *Mortierella alpina* I_{49}-N_{18}. The culture was enlarged by the three-phase fermentation, inoculated into a 50-ton fermentor and fermented continually for 7–8 days. Adding glucose in batches, controlling pH, and lowering temperature step by step, the average results in the 50-ton fermentor showed that the biomass, lipids in biomass, AA in lipids, and AA contents in media were 26.4 g/L, 40.5 % (volume ratio A/B), 47.98 % and 5.11 g/L, respectively (see Table 2), which indicated that the content of AA produced in industrial-scale was close to or even better than the level of AA in the shaking batch culture.

Table 2. The AA yields of 10 batches performed in a 50-ton fermentor

Batch (No.)	γ (Biomass) $g\,L^{-1}$	ζ (Lipids in biomass)/%	ζ (AA in lipids)/%	γ (AA) gL^{-1}
1	24.0	34.5	50.3	4.16
2	23.3	40.5	49.9	4.70
3	28.4	46.6	49.6	6.56
4	22.8	39.1	51.8	4.62
5	21.7	45.1	53.0	5.19
6	35.8	40.1	46.1	6.62
7	34.3	39.4	44.4	6.00
8	22.9	40.0	43.0	3.94
9	24.0	41.5	44.3	4.41
10	27.0	38.4	47.4	4.91
Avg.	26.4±5.0	40.5±3.4	48.0±3.4	5.11±0.97

Conclusions

In the present study, it was shown that *Mortierella alpina* I_{49}-N_{18} was a high-yield arachidonic acid strain. The optimal medium for the main fermentation contained 8.0 % glucose, 1.0 % peanut cake, 1.2 % peptone, 0.8 % yeast extract and pH was 8.5. Glucose was added into fermentation medium in batches. Moreover, we observed that higher temperature was favorable for the increase of biomass and lipids, but lower temperature was more suitable for accumulation of AA. Therefore, higher temperature was used in the primary stage of the fermentation, while lower temperature was applied in the late stage of the fermentation. After the fermentation was completed, the average contents of biomass, lipids in biomass, arachidonic acid in lipids and AA contents in media were 26.4 g/L, 40.5 %, 47.98 % and 5.11 g/L, respectively. The production of *Mortierella alpina* I_{49}-N_{18} in industrial scale is a rich source of arachidonic acid.

Because of their higher AA contents, their higher growth rates in simple media, and the simplicity of their manipulation, fungi are thought to be much more advantageous to produce fatty acids than the algal, moss and protozoal sources previously reported *(17–19)*. The content of arachidonic acid in *Mortierella alpina* I_{49}-N_{18} is the highest reported in fungal shake-flask and large-scale cultures, the production scale (50-ton fermentor) is also the largest one *(12–14,20–21)*. Such a low cost and large-scale production of arachidonic acid would have profound and great effects on the areas of production involving AA.

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Proizvodnja arahidonske kiseline iz *Mortierella alpina* I_{49} i N_{18}

Sažetak

Istraživana je fermentacija *Mortierella alpina* I_{49} i N_{18} u tikvicama na tresilici i u 50-tonskom fermentoru za dobivanje arahidonske kiseline, esencijalne masne kiseline u čovjeka. Da bi se postigli najpovoljniji uvjeti uzgoja, ispitan je utjecaj temperature, početnog pH, trajanje uzgoja, izvori ugljika i dušika. Nadalje, ispitan je način dodavanja šećera tijekom uzgoja u 50-tonskom fermentoru. Pod optimalnim uvjetima uzgoja na tresilici i u 50-tonskom fermentoru dobiveno je 4,55 odnosno 5,11 g arahidonske kiseline/L podloge. Najveći je postotak arahidonske kiseline u lipidima na tresilici i u 50-tonskom fermentoru iznosio 70,20 odnosno 53,01 %. Plinskom kromatografijom/masenom spektrometrijom utvrđeno je da ulje sadrži 80 % polinezasićenih kiselina kao što su arahidonska, γ-linolenska i linoleinska kiselina.