Production of Polyunsaturated Fatty Acids by *Mucor recurvus* sp. with Sugarcane Molasses as the Carbon Source

Nan Li[1], Zhi-Nian Deng[2], Yong-Ling Qin[1], Chun-Lan Chen[1] and Zhi-Qun Liang[1]*

[1]College of Life Science and Technology, Guangxi University, Nanning, CN-530004 Guangxi, PR China

[2]Key Laboratory for Crop Genetic Improvement and Biotechnology, Guangxi Academy of Agricultural Sciences, Nanning, CN-530007 Guangxi, PR China

Summary

Sugarcane molasses is rich in nutriments and vitamins. It may be used as the carbon source for the production of polyunsaturated fatty acids (PUFA) by *Mucor recurvus* sp. Using sugarcane molasses, the effects of fermentation parameters and media components on polyunsaturated fatty acid production through both linear and orthogonal array experiments were investigated. The best fermentation conditions for PUFA production were found as follows: 15 % sugarcane molasses, pH=6.0, 28 °C, 5 days, 160 rpm. It was also found that molasses and urea enhanced PUFA production with the optimal carbon to nitrogen (C/N) ratio of 35. Under the most favourable conditions, the total lipid content at 7.13 g/L and PUFA up to 5.74 g/L including (0.82±0.05) g/L of linolenic acid (LA), (1.35±0.02) g/L of γ-linolenic acid (GLA), (0.17±0.06) g/L of α-linolenic acid (ALA), (0.57±0.06) g/L of arachidonic acid (ARA), (0.46±0.07) g/L of eicosapentaenoic acid (EPA) and (0.34±0.08) g/L of docosahexaenoic acid (DHA) were obtained. Our study suggests that sugarcane molasses is a superior alternative carbon source for industrial PUFA production.

Key words: Mucor recurvus sp., polyunsaturated fatty acids, sugarcane molasses, fermentation, cultivation condition, fatty acid analysis

Introduction

Polyunsaturated fatty acids (PUFA) have unique biological activities (*1,2*) and clinical effects (*3,4*). Nutritional studies have indicated their potential benefits to human health (*5–8*). Marine fish is the traditional source of PUFA. However, marine resources are not sustainable due to limited fishing seasons, geographical locations and declining fish populations (*9*). Moreover, the fish oil concentrates contain cholesterol and have unpleasant odour (*10*). Therefore, there is a need for alternative sources of PUFA. Living organisms, including fungi, marine algae, diatoms, and some bacteria, are some potential sources (*11–13*).

The production of PUFA by microbial fermentation has been shown to be an ideal alternative owing to its amicability for the separation, purification, and industrialization (*14*). For efficient microbial PUFA production, it is critical to obtain a microbial strain with both high biomass and high PUFA content. Recent research has focused mainly on maximizing PUFA yield by genetic manipulation as well as by medium optimization (*15,16*). In traditional cultivation methods, glucose, starch, and/or sucrose were often used as the carbon source for producing PUFA. Here, we demonstrated that sugarcane molasses, a by-product of sugarcane processing, is a good carbon source substitute for PUFA production by *Mucor recurvus* sp. We identified the optimal fermentation conditions as well as medium composition for maximal PUFA production. Our study also points out the feasibility of using sugarcane molasses as a substrate for the general fungal fermentation.

*Corresponding author; Phone: ++86 771 3270 733; Fax: ++86 771 3271 181; E-mail: zqliang@gxu.edu.cn

Materials and Methods

Chemicals

Medium components in the experiments were from Guanghua Chemical Factory Co., Ltd. Guangdong, PR China; solvents were of reagent grade (RG). The standards of fatty methyl esters were from Sigma (USA); sugarcane molasses was from Nanning Sugar Ltd., Guangxi, PR China.

The molasses was diluted with distilled water at the ratio of 1:1.5 and the pH was adjusted to 3.5 with H_2SO_4. After 8 hours, the supernatant was adjusted to pH=7.2 with lime cream and whisked at 60 °C for 30 min. After 12 hours, molasses was percolated, adjusted to pH=6.0 and diluted into proper concentrations with distilled water (17).

Components of sugarcane molasses analysis

Components of sugarcane molasses were analyzed according to Wang's method (18).

Determination of dissolved oxygen

The dissolved oxygen in the media was measured with an oxygen analyzer (Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, PR China).

Fungal strain

Mucor recurvus sp. was obtained from Food Research and Fermentation Institute, Guangxi University, Nanning, PR China.

Media and cultivation conditions

Transferring medium was composed of 10 % molasses with 0.01 g/L of $(NH_4)_2SO_4$ and 0.002 g/L of K_2HPO_4, with the initial pH=6.0. The production medium was composed of 15 % molasses with pH=6.0. The 5 % (by volume) mycelial suspension of isolated culture was inoculated in 500-mL flask containing 200 mL of broth and incubated in a shaker (SKY-211B, Shanghai Sukun Industry and Commerce Co., Ltd., PR China) at 25 °C and 140 rpm for 4 days (19).

Nitrogen source in the basal medium was provided with 1.5 % of NH_4Cl, $(NH_4)_2SO_4$, KNO_3 and urea. When the sugarcane molasses concentration was fixed, the C/N ratio varied from 20:1 to 40:1.

An orthogonal design method was used to study the PUFA production in *Mucor recurvus*. The method was based on L_{64} (4^3) with three factors and four levels (16,20).

Cell dry mass determination and lipid extraction

Mycelia were harvested by filtering the fermented media through Chinese No. 1 filter paper, washed thoroughly with distilled water, and finally dried at –50 °C. Lipid was extracted from the dried mycelia with a mixture of chloroform and methanol (volume ratio=2:1) (21). Residual moisture in the extracted lipid was removed by adding anhydrous sodium sulphate and filtering through filter paper. The dried lipid was then concentrated under vacuum drying (Labconco, USA) (22).

Methyl ester preparation and analysis of fatty acid composition

A rapid transmethylation method was applied in this study (23). Briefly, the dried *Mucor recurvus* was directly transmethylated with 10 % methanolic HCl at 50 °C for 3 h. Then, the fatty acid methyl esters (FAME) were extracted with hexane, concentrated and analysed by gas chromatography.

Gas chromatography settings

The gas chromatograph GC-17A (Shimadzu Co., Japan) was equipped with a fused silica capillary column (30 m×0.25 mm i.d.×0.25 μm film thickness) and a flame ionization detector. The injector and detector temperatures were maintained at 220 and 260 °C, respectively. The oven was programmed as follows: 160 °C for 2 min, increased to 180 °C at 6 °C/min, maintained at 180 °C for 2 min, increased to 220 °C at 4 °C/min and finally maintained at 220 °C for 10 min. The carrier gas, nitrogen, was used at a flow rate of 1.5 mL/min. The injection volume was 1 μL with a split ratio of 60:1. Methyl esters of available PUFA were used as standards for fatty acid identification and quantitation. Total fatty acid production was calculated from the total peak areas of the chromatogram relative to the peak area of an internal standard (24).

Results and Discussion

Effect of carbon sources on PUFA production

We tested the effect of carbon sources on dry biomass (DBM), total lipid, γ-linolenic acid (GLA) and PUFA production. As presented in Table 1, the effects of carbon sources on cell growth, total lipid, GLA and PUFA

Table 1. Effect of carbon source on DBM, total lipid and PUFA production*

Carbon source	γ(DBM) g/L	γ(total lipid) g/L	γ(PUFA) g/L	γ(LA) g/L	γ(GLA) g/L	γ(ALA) g/L	γ(ARA) g/L	γ(EPA) g/L	γ(DHA) g/L
Sucrose	8.97±0.14	4.46±0.08	2.74±0.02	0.39±0.05	0.64±0.03	0.08±0.04	0.35±0.05	0.22±0.03	0.16±0.04
Starch	8.70±0.26	3.58±0.06	2.29±0.03	0.32±0.02	0.54±0.01	0.07±0.03	0.22±0.02	0.18±0.01	0.13±0.02
Glucose	10.85±0.32	6.10±0.05	4.48±0.07	0.64±0.01	1.06±0.01	0.13±0.03	0.44±0.01	0.36±0.04	0.27±0.02
PDA	10.95±0.16	5.62±0.08	4.43±0.04	0.63±0.01	1.04±0.02	0.13±0.05	0.43±0.04	0.36±0.02	0.26±0.01
Molasses	9.58±0.19	4.75±0.07	3.07±0.06	0.44±0.02	0.72±0.03	0.09±0.03	0.30±0.01	0.24±0.02	0.19±0.03

*Cultures were incubated for 5 days at 25 °C, 140 rpm

production are in the order of potato dextrose agar (PDA) =glucose>molasses>sucrose=starch. Compared with sucrose, the sugarcane molasses is rich in nutriments and vitamins (Tables 2 and 3). The cost of molasses is significantly lower than of the PDA and glucose although it is slightly less efficient. Therefore, together with the previous report that molasses is a good carbon source for lipid production (*25*), we believe that molasses could be a good alternative for PDA or glucose.

Table 2. Components of sugarcane molasses

Component		w/%
	Sucrose	52
Total sugar	Reducing sugar	35
	Non-reducing sugar	16
Organic compounds (non-sugar)	Soluble colloids	4
	Organic acids (including citric acid, malate, succinate, *etc.*)	3
	Sugar cane wax, sterol, *etc.*	1
Nitrogenous substance	Protein	3
Components of ash	Sodium	0.4
	Potassium	3.8
	Calcium	0.6
	Phosphorus	0.3

Effect of molasses concentrations

The production of dry biomass (DBM), total lipids, γ-linolenic acid (GLA) and total PUFA were boosted as the molasses concentration increased. The maximum yields were observed with the molasses concentration at 15 % (volume ratio), as the yields decreased when the concentration was 20 % or higher (Table 4). With the molasses at 15 %, each litre of the medium contained 10.18 g of DBM and 2.64 g of PUFA, which was composed of (0.61±0.01) g of linolenic acid (LA), (0.99±0.02) g of γ-linolenic acid (GLA), (0.12±0.03) g of α-linolenic acid (ALA), (0.41±0.01) g of arachidonic acid (ARA), (0.34±0.02) g of eicosapentaenoic acid (EPA) and (0.25±0.01) g of docosahexaenoic acid (DHA). Our data are different from those of *M. alpina* ATCC 32222, where the yield reached a maximal level with the glucose concentration at 10 % (*26,27*). These data are also different from the previous report that fatty acid production increased continuously with the glucose concentration from 2 to 12 % in *M. alpina* CBS754.68, while ARA content and ARA yield were reversed (*28*).

Effect of different nitrogen sources and C/N ratio on PUFA production

Nitrogen source affects the mycelial morphology and ARA production by *M. alpina* CBS754.68 (*29*). Therefore, we investigated the effect of nitrogen supplement on DBM, total lipid, GLA and total PUFA production. As illustrated in Table 5, urea gave the highest DBM, total lipid, GLA and PUFA yields, followed by potassium nitrate, ammonium sulfate and ammonium chloride. Therefore, urea was the best nitrogen source for DBM, lipid, GLA, and PUFA production.

The effect of C/N ratio on DBM, total lipid, GLA and total PUFA production was then investigated. As shown in Table 6, total PUFA and GLA production did

Table 3. Mass ratio of vitamins in sugarcane molasses

Component	Vitamin H	Folic acid	Pantothenate	Pyridoxin	Vitamin B_2	Vitamin B_1	Niacin	Choline
w/(mg/kg)	2.2	0.04	58	3.8	2.5	1.8	400	600

Table 4. Effect of sugarcane molasses concentration on DBM, total lipid and PUFA production*

w(molasses) %	γ(DBM) g/L	γ(total lipid) g/L	γ(PUFA) g/L	γ(LA) g/L	γ(GLA) g/L	γ(ALA) g/L	γ(ARA) g/L	γ(EPA) g/L	γ(DHA) g/L
10	8.82±0.14	3.64±0.04	2.26±0.02	0.46±0.01	0.76±0.03	0.09±0.01	0.32±0.02	0.26±0.04	0.19±0.02
15	10.18±0.23	4.64±0.06	2.64±0.09	0.61±0.01	0.99±0.02	0.12±0.03	0.41±0.01	0.34±0.02	0.25±0.01
20	9.84±0.34	4.15±0.03	2.53±0.08	0.51±0.02	0.83±0.06	0.11±0.03	0.34±0.08	0.28±0.03	0.21±0.03
25	7.86±0.24	3.15±0.09	2.10±0.04	0.44±0.01	0.73±0.05	0.09±0.04	0.30±0.07	0.25±0.02	0.21±0.03

*Cultures were incubated for 4 days at 25 °C, 140 rpm

Table 5. Effect of nitrogen sources on DBM, total lipid and PUFA production*

Nitrogen source	γ(DBM) g/L	γ(total lipid) g/L	γ(PUFA) g/L	γ(LA) g/L	γ(GLA) g/L	γ(ALA) g/L	γ(ARA) g/L	γ(EPA) g/L	γ(DHA) g/L
NH₄Cl	7.46±0.12	2.98±0.09	1.65±0.07	0.23±0.02	0.39±0.01	0.05±0.01	0.16±0.04	0.13±0.04	0.11±0.02
(NH₄)₂SO₄	8.23±0.21	3.30±0.04	2.39±0.06	0.34±0.01	0.56±0.02	0.07±0.01	0.23±0.02	0.19±0.02	0.17±0.05
KNO₃	10.14±0.23	4.02±0.08	2.91±0.04	0.41±0.03	0.68±0.02	0.09±0.03	0.29±0.04	0.23±0.04	0.17±0.03
Urea	10.16±0.32	4.94±0.05	4.31±0.03	0.61±0.02	1.01±0.03	0.13±0.02	0.43±0.01	0.35±0.01	0.26±0.07

*Cultures were incubated for 5 days at 25 °C, 140 rpm

not change significantly when the C/N ratio was between 20 and 30. However, the production of GLA and total PUFA increased significantly to the maximal level at a C/N ratio of 35. Interestingly, GLA content decreased sharply when the C/N ratio was lower than 30 or higher than 40, which is probably due to the fact that molasses contains significant amounts of organic nitrogen. Thus, a C/N ratio of 35 gives the optimal production. However, Šajbidor *et al.* (*30*) reported that *Mortierella* sp. S-17 had high ARA concentration in the cell cultivated at C/N ratio 10, and high ARA production at C/N ratio 20. Koike *et al.* (*28*) also reported that the optimal C/N ratio of the medium was around 15–20 for ARA production in a culture of *M. alpina* CBS 754.68. When the C/N ratio was higher than 20, the mycelium mass and ARA decreased due to nitrogen limitation.

Effect of fermentation time on PUFA production

The accumulation of PUFA and GLA was also affected by the fermentation time. Yamada *et al.* (*31*) and Bajpai *et al.* (*26*) also reported that *M. alpina* IS-4 and ATCC 32222 had their maximal production after 3 and 4 days of incubation, respectively. That is why the effect of fermentation time was examined in our system. As a result, we found that the time duration for maximum yields of DBM, γ-linolenic acid and total PUFA was 5 days (Table 7). PUFAs are the primary metabolites of cells. Their yields depend on cell growth. Consequently, the production of PUFA decreased gradually in prolonged cultivation due to cell lysis (*31,32*). The time needed for maximum yields of PUFA also depends on the kind of fatty acid since the γ-linolenic acid and linoleic acid were synthesized prior to arachidonic acid and eicosapentaenoic acid.

Effect of shaking speed and dissolved oxygen levels

The relationship between shaking speed and yields of DBM, total lipids, GLA and PUFA was investigated. As shown in Table 8, shaking speed of 160 rpm is the best with the highest DBM, total lipid, GLA and PUFA yields. At 140 or 200 rpm, lower PUFA yields were obtained. Lipid and PUFA yields were reduced sharply when the shaker speed was over 180 rpm, although DBM production was not obviously changed between 160 and 200 rpm.

The effects of dissolved oxygen on fatty acid production have not been well studied possibly due to technical problems encountered in growing cells at a rigor-

Table 6. Effect of C/N ratio on DBM, total lipid and PUFA production*

C/N ratio	γ(DBM) g/L	γ(total lipid) g/L	γ(PUFA) g/L	γ(LA) g/L	γ(GLA) g/L	γ(ALA) g/L	γ(ARA) g/L	γ(EPA) g/L	γ(DHA) g/L
20/1	7.52±0.14	4.47±0.06	2.95±0.08	0.42±0.04	0.69±0.07	0.09±0.02	0.29±0.01	0.24±0.04	0.17±0.09
25/1	8.16±0.16	3.59±0.04	3.18±0.06	0.45±0.07	0.74±0.08	0.10±0.02	0.31±0.03	0.26±0.08	0.19±0.03
30/1	9.21±0.23	3.65±0.08	3.38±0.04	0.49±0.03	0.79±0.05	0.10±0.08	0.33±0.04	0.28±0.02	0.21±0.01
35/1	10.51±0.26	4.78±0.05	4.47±0.03	0.64±0.02	1.05±0.02	0.14±0.07	0.44±0.01	0.36±0.03	0.27±0.02
40/1	9.58±0.18	4.91±0.04	4.05±0.07	0.58±0.02	0.95±0.03	0.12±0.03	0.40±0.01	0.33±0.09	0.25±0.05

*Cultures were incubated for 5 days at 25 °C, 140 rpm

Table 7. Effect of culture time on DBM, total lipid and PUFA production*

Time/day	γ(DBM) g/L	γ(total lipid) g/L	γ(PUFA) g/L	γ(LA) g/L	γ(GLA) g/L	γ(ALA) g/L	γ(ARA) g/L	γ(EPA) g/L	γ(DHA) g/L
3	9.55±0.17	4.65±0.04	3.89±0.05	0.56±0.02	0.92±0.03	0.11±0.03	0.38±0.01	0.32±0.04	0.23±0.03
4	10.04±0.13	5.01±0.08	4.46±0.02	0.64±0.01	1.05±0.03	0.13±0.07	0.44±0.02	0.36±0.02	0.27±0.02
5	10.95±0.18	5.48±0.06	5.04±0.06	0.78±0.02	1.83±0.02	0.15±0.02	0.49±0.02	0.41±0.05	0.30±0.03
6	9.56±0.16	4.78±0.03	3.84±0.08	0.55±0.02	0.91±0.01	0.12±0.04	0.37±0.08	0.31±0.06	0.23±0.08

*Cultures were incubated for 5 days at 25 °C, 140 rpm

Table 8. Effect of shaker speed and dissolved oxygen concentration on DBM, total lipid and PUFA production*

Shaker speed rpm	γ(dissolved oxygen) mg/L	γ(DBM) g/L	γ(total lipid) g/L	γ(PUFA) g/L	γ(LA) g/L	γ(GLA) g/L	γ(ALA) g/L	γ(ARA) g/L	γ(EPA) g/L	γ(DHA) g/L
140	18.62±0.02	11.23±0.12	4.87±0.02	3.53±0.03	0.51±0.01	0.83±0.03	0.11±0.01	0.34±0.01	0.28±0.04	0.21±0.07
160	21.21±0.05	11.84±0.14	5.91±0.04	5.44±0.05	0.78±0.07	1.28±0.01	0.16±0.06	0.53±0.02	0.44±0.01	0.33±0.02
180	23.08±0.06	11.61±0.13	5.38±0.03	4.78±0.02	0.68±0.01	1.13±0.05	0.15±0.08	0.47±0.02	0.39±0.03	0.29±0.04
200	26.02±0.03	11.56±0.15	4.64±0.01	3.40±0.06	0.48±0.02	0.81±0.01	0.11±0.02	0.33±0.08	0.28±0.01	0.21±0.01

*Cultures were incubated at 25 °C, for 5 days

ously controlled pressure. Nevertheless, it is reported that *Saccharomyces cerevisiae* (*33,34*) and *Candida utilis* (*35*) produce less unsaturated fatty acids when the dissolved oxygen level is below the optimum level.

The desaturation of long-chain fatty acids by yeast requires molecular oxygen. Cell lipids vary with the content of dissolved oxygen in the culture (*33*). The preferential inhibition of the conversion of saturated fatty acid to unsaturated fatty acid at low level of dissolved oxygen might be caused by the inhibition of the dehydrogenase system with a relatively low affinity for O_2. Lower shaking speed resulted in slower growth and lower PUFA yields because of the lower level of dissolved oxygen. However, lower PUFA yields were also obtained in the experiment with too high shaking speed, because the shear stress was increased with the increased shaking speed.

Effect of temperature and pH

Microbacteria generally grow well between 20 and 28 °C. Cell viability decreases dramatically when the incubation temperature is higher than 28 or lower than 20 °C. The effects of incubation temperature on DBM, total lipid, GLA and PUFA production by *Mucor recurvus* sp. were examined. As shown in Table 9, the production of DBM, total lipids, GLA and PUFA was the highest at 28 °C, moderate at 25 and 20 °C, and the lowest at 30 °C. *Mucor recurvus* stops growing at 35 °C. At 28 °C, the optimum temperature, a maximum production of DBM at 11.54 g/L, total lipids at 5.74 g/L and overall PUFA at 5.02 g/L was obtained.

Low incubation temperature favours the production of the overall PUFA and the unsaturated PUFA in *Mortierella* as well as in *Mucor recurvus* (*36*). Unsaturated fatty acids in cell membranes can maintain the membrane function and the cell growth of *Mucor recurvus* at low temperatures (*37*) and they are preferentially synthesized by the organism at low incubation temperatures.

Low incubation temperature enhances long-chain fatty acid production, such as LA, ARA, GLA, EPA and DHA, but slows the mycelial growth. Shimizu *et al.* (*38*) reported that EPA can be produced by *Mortierella* at 6–16 °C and could not be detected at 20–28 °C.

The effect of initial pH was also investigated. The media had a pH range from 4.0 to 8.0 (pH of the medium was adjusted before autoclaving with 1.0 M HCl/ NaOH). We found that the production of DBM, total lipids, GLA and PUFA were highest at pH=6.0 (Table 10). In *Mucor recurvus*, total lipid and PUFA production increased as the pH increased from 4.0 to 6.0. However, the production decreased sharply as the pH was raised from 7.0 to 8.0. Our data indicated that the optimal pH was 6.0 for maximal PUFA production.

Orthogonal experiments

To examine what the best overall fermentation conditions for PUFA production by *Mucor recurvus* with sugarcane molasses as the carbon source are, we designed the L_{64} (4^3) orthogonal experiments. Temperature, fermentation time and rotating speed were used as inspecting factors at four levels, and the iodine value was used as the inspecting marker (Table 11).

The results showed that, among the three factors, the rotating speed was the most significant factor, giving the widest range (32.13) of PUFA yields. Temperature and fermentation time were of moderate and little importance, giving a range of 11.84 and 4.4, respectively (Table 12). Thus, the optimum conditions for PUFA production by *Mucor recurvus* were as follows: temperature 28 °C, fermentation time 5 days, rotating speed 160 rpm. Un-

Table 9. Effect of temperature on DBM, total lipid and PUFA production*

Temp. °C	γ(DBM) g/L	γ(total lipid) g/L	γ(PUFA) g/L	γ(LA) g/L	γ(GLA) g/L	γ(ALA) g/L	γ(ARA) g/L	γ(EPA) g/L	γ(DHA) g/L
20	10.42±0.18	4.87±0.06	4.13±0.07	0.59±0.01	0.97±0.02	0.12±0.04	0.41±0.03	0.33±0.02	0.25±0.01
25	10.91±0.14	5.31±0.08	4.69±0.04	0.67±0.04	1.09±0.07	0.14±0.02	0.46±0.02	0.38±0.01	0.28±0.02
28	11.54±0.12	5.74±0.07	5.02±0.06	0.72±0.02	1.18±0.02	0.15±0.03	0.49±0.01	0.49±0.01	0.31±0.01
30	8.69±0.15	3.75±0.04	2.97±0.05	0.42±0.05	0.69±0.03	0.09±0.01	0.29±0.03	0.29±0.03	0.18±0.04
35	0	0	0	0	0	0	0	0	0

*Cultures were incubated for 5 days at 140 rpm

Table 10. Effect of media pH on DBM, total lipid and PUFA production*

pH	γ(DBM) g/L	γ(total lipid) g/L	γ(PUFA) g/L	γ(LA) g/L	γ(GLA) g/L	γ(ALA) g/L	γ(ARA) g/L	γ(EPA) g/L	γ(DHA) g/L
4.0	10.08±0.14	3.67±0.04	1.41±0.02	0.20±0.05	0.33±0.01	0.04±0.01	0.13±0.01	0.11±0.04	0.08±0.01
5.0	10.09±0.12	4.53±0.05	3.33±0.05	0.47±0.01	0.78±0.04	0.11±0.02	0.32±0.03	0.27±0.01	0.20±0.03
6.0	10.12±0.10	5.78±0.04	5.20±0.08	0.74±0.03	1.22±0.03	0.15±0.01	0.51±0.04	0.42±0.08	0.31±0.04
7.0	10.06±0.15	5.18±0.06	4.34±0.06	0.62±0.01	1.02±0.02	0.13±0.04	0.42±0.05	0.35±0.05	0.26±0.03
8.0	9.94±0.13	4.51±0.08	3.27±0.03	0.47±0.02	0.77±0.01	0.10±0.06	0.32±0.04	0.26±0.07	0.19±0.02

*Cultures were incubated for 5 days at 25 °C, 140 rpm

Table 11. L$_{64}$ (4^3) orthogonal experiments

Level	Factor		
	Temperature/°C	Rotation speed/rpm	Culture time/day
1	20	140	3
2	25	160	4
5	28	180	5
4	30	200	6

Table 12. Total iodine values from the orthogonal experiments L$_{64}$ (4^3)

Number of experiment	Iodine value			Iodine value
	Temperature/°C	Rotation speed/rpm	Culture time/day	
K$_1$	1100.10	926.20	1177.50	
K$_2$	1289.60	1440.30	1179.40	
K$_3$	1178.40	1188.30	1247.80	
K$_4$	1235.70	1258.80	1199.10	
K$_1$/16	68.76	57.89	73.59	
K$_2$/16	80.60	90.02	73.71	
K$_3$/16	73.65	74.27	77.99	
K$_4$/16	77.23	78.68	74.94	4803.8
Range	11.84	32.13	4.40	

der the optimized conditions, we were able to obtain total lipids at 7.13 g/L and PUFA at 5.74 g/L. To our knowledge, these yields were higher than previously reported.

Fatty acid analysis

The analysis of the fatty acid composition in total lipids was performed by a gas chromatograph (Fig. 1).
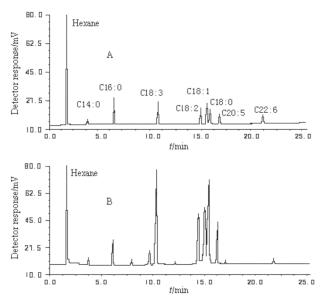

Fig. 1. Gas chromatography analysis of fatty acid methyl esters by flame ionization. A, FAME extracted from standards; B, FAME extracted from *Mucor recurvus* sp. The peaks appearing in both A and B were identified as C14:0, C16:0, C18:3, C18:2, C18:1, C18:0, C20:5, C22:6. The peak in B corresponds to the retention time of PUFA

The relative percentages of fatty acids were calculated by measuring the peak areas. Fatty acids were identified by comparing their retention time with that of the FAME standards. The PUFA, which were produced at 5.74 g/L, included LA ((0.82±0.05) g/L), GLA ((1.35±0.02) g/L), ALA ((0.17±0.06) g/L), ARA ((0.57±0.06) g/L), EPA ((0.46±0.07) g/L) and DHA ((0.34±0.08) g/L). Therefore, the greatest PUFA production was achieved at the optimum culturing conditions.

Conclusions

The data of this research show that *Mucor recurvus* sp. could be an excellent source for PUFA production. The optimal conditions for maximal production of PUFA using sugarcane molasses as the carbon source were also identified. Notably, the maximal yields are higher than previously reported, suggesting that sugarcane molasses is a superb alternative carbon source considering its considerably lower costs than those of PDA and sucrose.

References

1. P.C. Calder, Polyunsaturated fatty acids, inflammation and immunity, *Lipids, 36* (2001) 1007–1024.
2. G. Fontani, F. Corradeschi, A. Felici, F. Alfatti, S. Migliorini, L. Lodi, Cognitive and physiological effects of ω-3 polyunsaturated fatty acid supplementation in healthy subjects, *Eur. J Clin. Invest. 35* (2005) 691–699.
3. R.A. Siddiqui, S.R. Shaikh, L.A. Sech, H.R. Yount, W. Stillwell, G.P. Zaloga, Omega 3-fatty acids: Health benefits and cellular mechanisms of action, *Mini. Rev. Med. Chem. 4* (2004) 859–871.
4. C.H. MacLean, S.J. Newberry, W.A. Mojica, P. Khanna, A.M. Issa, M.J. Suttorp, Y.W. Lim, S.B. Traina, L. Hilton, R. Garlan, S.C. Morton, Effects of omega-3 fatty acids on cancer risk: A systematic review, *JAMA, 295* (2006) 403–415.
5. S.M. Møller, J.C. Hansen, E.B. Thorling, G. Mulvad, H.S. Pedersen, P. Bjerregaard, Effects of dietary seal oil on fat metabolism, *Int. J. Circumpolar Health* (Suppl. 1), *57* (1998) 322–324.
6. L.A. Horrocks, Y.K. Yeo, Health benefits of docosahexaenoic acid (DHA), *Pharmacol. Res. 40* (1999) 211–225.
7. P. Bjerregaard, H.S. Pedersen, G. Mulvad, The associations of a marine diet with plasma lipids, blood glucose, blood pressure and obesity among the Inuit in Greenland, *Eur. J. Clin. Nutr. 54* (2000) 732–737.
8. R. Uauy, D.R. Hoffman, P. Peirano, D.G. Birch, E.E. Birch, Essential fatty acids in visual and brain development, *Lipids, 36* (2001) 885–895.
9. S.G. Armstrong, G.S. Wyllie, D.N. Leach, Effects of season and location of catch on the fatty acid compositions of some Australian fish species, *Food Chem. 51* (1994) 295–305.
10. S.L. Cao, Z.F. Guan, Y.P. Cai, The analysis and research of fatty acids in deep-sea fish oil and seal oil, *J. Chin. Mass Spectrom. Soc. 20* (1999) 70–75 (in Chinese).
11. N.J. Russell, D.S. Nichols, Polyunsaturated fatty acids in marine bacteria – A dogma rewritten, *Microbiology, 145* (1999) 767–779.
12. M. Satomi, H. Oikawa, Y. Yano, *Shewanella marinintestina* sp. nov., *Shewanella chlegeliana* sp. nov. and *Shewanella sairane* sp. nov., novel eicosapentaenoic acid producing marine bacteria isolated from sea animal intestines, *Int. J. Syst. Evol. Microbiol. 53* (2003) 491–499.

13. N. Morita, T. Nishida, M. Tanaka, Y. Yano, H. Okuyama, Enhancement of polyunsaturated fatty acid production by cerulenin treatment in polyunsaturated fatty acid-producing bacteria, *Biotechnol. Lett.* **27** (2005) 389–393.

14. P. Bajpai, P.K. Bajpai, O.P. Ward, Production of docosahexaenoic acid (DHA) by *Thraustochytrium aureum*, *Appl. Microbiol. Biotechnol.* **35** (1991) 706–710.

15. A.M. Lindberg, G. Molin, Effect of temperature and glucose supply on the production of polyunsaturated fatty acids by the fungus *Mortierella alpina* CBS 343.66 in fermentor culture, *Appl. Microbiol. Biotechnol.* **2** (1993) 9450–9455.

16. H.D. Jang, Y.Y. Lin, S.S. Yang, Effect of culture media and conditions on polyunsaturated fatty acids production by *Mortierella alpina*, *Bioresour. Technol.* **96** (2005) 1633–1644.

17. Q.R. Jin, J.M. Zhang, Q. Xu: *Technology of Organic Acid Fermentation*, Chinese Light Industry Press, Beijing, PR China (1997) pp. 113–127 (in Chinese).

18. L.J. Wang: *Sugar Analysis*, Light Industry Press, Beijing, PR China (1982) pp. 114–122 (in Chinese).

19. P.K. Bajpai, P. Bajpai, O.P. Ward, Optimisation of culture condition for production of eicosapentaenoic acid by *Mortierella elongata* NRRL5513, *J. Ind. Microbiol.* **9** (1991) 11–18.

20. R.X. Wang: *Mathematical Statistics*, Xi'an Jiaotong University Press, Xi'an, PR China (2000) pp. 120–158 (in Chinese).

21. Z.F. Li, L. Zhang, X.J. Shen, A comparative study on four method of fungi lipid extraction, *Microbiology*, **28** (2001) 73–76 (in Chinese).

22. J.W. Li, R.Y. Yu, M.X. Yua: *Experimental Principle and Method of Biochemistry*, Beijing University Press, Beijing, PR China (1994) pp. 139–141 (in Chinese).

23. E.J. Sakuradani, M. Kobayashi, T. Ashikari, S. Shimiz, Identification of Δ^{12}-fatty acid desaturase gene from arachidonic acid-producing *Mortierella* fungus by heterologous expression in the yeast *Saccharomyces cerevisiae* and the fungus *Aspergillus oryzae*, *Eur. J. Biochem.* **261** (1999) 812–820.

24. S.G. Bian, X.Y. Zhang, L. Zhang, A fast determination of polyunsaturated fatty acids produced by marine fungus, *Microbiology*, **28** (2001) 73–76 (in Chinese).

25. R.M. Alvarez, B. Rodriguez, J.M. Romano, A.O. Diaz, E. Gomez, D. Miro, L. Navarro, G. Saura, J.L. Garcia, Lipid accumulation in *Rhodotorula glutinis* on sugar cane molasses in single-stage continuous culture, *World J. Microbiol. Biotechnol.* **8** (1992) 214–215.

26. P.K. Bajpai, P. Bajpai, O.P. Ward, Arachidonic acid production by fungi, *Appl. Environ. Microbiol.* **57** (1991) 1255–1258.

27. P.K. Bajpai, P. Bajpai, O.P. Ward, Production of arachidonic acid production by *Mortierella alpina* ATCC 32222, *J. Ind. Microbiol.* **8** (1991) 179–186.

28. Y. Koike, H.J. Cai, K. Higashiyama, S. Fujikawa, E.Y. Park, Effect of consumed carbon to nitrogen ratio on mycelial morphology and arachidonic acid production in cultures of *Mortierella alpina*, *J. Biosci. Bioeng.* **91** (2001) 382–389.

29. E.Y. Park, Y. Koike, K. Higashiyama, S. Fujikawa, M. Okabe, Effect of nitrogen source on mycelial morphology and arachidonic acid production in cultures of *Mortierella alpina*, *J. Biosci. Bioeng.* **88** (1999) 61–67.

30. J. Šajbidor, S. Dobroňová, M. Čertík, Arachidonic acid production by *Mortierella* sp. S-17: Influence of C/N ratio, *Biotechnol. Lett.* **12** (1990) 455–456.

31. H. Yamada, S. Shimizu, Y. Shinmen, Production of arachidonic acid by *Mortierella elongata* IS-5, *Agr. Biol. Chem.* **51** (1987) 785–790.

32. P.K. Bajpai, P. Bajpai, Review: Arachidonic acid production by microorganisms, *Biotechnol. Appl. Biochem.* **15** (1992) 1–10.

33. D. Jollow, G.M. Kellerman, A.W. Linnane, The biogenesis of mitochondria. III. The lipid composition of aerobically and anaerobically grown *Saccharomyces cerevisiae* as related to the membrane systems of the cells, *J. Cell Biol.* **37** (1968) 267–279.

34. P.J. Rogers, P.R. Stewart, Mitochondrial and peroxisomal contributions to the energy metabolism of *Saccharomyces cerevisiae* in continuous culture, *Gen. Microbiol.* **79** (1973) 205–217.

35. C.M. Brown, A.H. Rose, Fatty acid composition of *Candida utilis* as affected by growth temperature and dissolved oxygen tension, *J. Bacteriol.* **99** (1969) 371–378.

36. J. Terrados, J.A. Lopez-Jimenez, Fatty acid composition and chilling resistance in green algae *Caulerpa prolifera* (Forrskal) Lamouroux (Chlorophyta, Caulerpales), *Biochem. Mol. Biol. Int.* **39** (1996) 863–869.

37. L. Toivonen, S. Laakso, H. Rosenguist, The effect of temperature on growth, indole alkaloid accumulation and lipid composition of *Catharanthus roseus* cell suspension cultures, *Plant Cell Rep.* **11** (1992) 390–394.

38. S. Shimizu, H. Kawashima, K. Akimoto, Y. Shinmen, H. Yamada, Production of eicosapentaenoic acid by *Mortierella* fungi, *J. Am. Oil Chem. Soc.* **66** (1988) 342–347.