Effect of culture media and conditions on polyunsaturated fatty acids production by *Mortierella alpina*

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Received 12 January 2004; received in revised form 15 December 2004; accepted 31 December 2004

Available online 8 March 2005

Abstract

To improve the polyunsaturated fatty acid (PUFA) production by *Mortierella*, culture media and conditions were investigated. *M. alpina* ATCC 32222 had the highest yield of arachidonic acid, γ -linolenic acid and linoleic acid among 11 test microbes. Soluble starch at 10% and the mixture of KNO₃ and yeast extract at 2:1 (w/w) was the best carbon and nitrogen sources for arachidonic acid and total PUFAs production, respectively. The optimal C/N ratio ranged from 5.1 to 9.0. Each gram of carbon produced 17.4 mg of linoleic acid, 17.0 mg of γ -linolenic acid, 103.0 mg of arachidonic acid and 194.2 mg of total PUFAs at 20 °C, while it yielded 21.4 mg of linoleic acid, 25.6 mg of γ -linolenic acid, 2.6 mg of γ -linolenic acid, 110.3 mg of arachidonic acid, 4.3 mg of eicosapentaenoic acid and 218.4 mg of total PUFAs at 12 °C. A high degree of unsaturation was found at low temperature incubation. Linseed oil supplementation (1%, w/v) increased the PUFAs production and each gram of carbon produced 403.4 mg of α -linolenic acid, 123.1 mg of arachidonic acid, 33.6 mg of eicosapentaenoic acid, 1.68 mg of docosahexaenoic acid and 943.2 mg of total PUFAs. From the optimization of culture media and conditions, PUFAs production increased from 30% to 5 times that was optimal for practical use.

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Keywords: *Mortierella alpina*; Polyunsaturated fatty acid; Soluble starch; Linseed oil; C/N ratio; Degree of unsaturation

1. Introduction

The ω -3 and ω -6 series of polyunsaturated fatty acids are identified as potential food additives or pharmaceuticals for their biological activities. The PUFAs could be used in treatment of heart and circulatory disorders, cancer or participation in the inflammatory reaction (Braden and Carroll, 1986; Dyerberg, 1986; Willett, 1993; Demaison and Moreau, 2002; Gil, 2002). Many

species of the genus *Mortierella* have been found to yield exceptionally high amount of PUFAs depending upon the fermentation media and culture conditions (Yamada et al., 1987; Hansson and Dostalek, 1988; Shimizu et al., 1989; Sajbodor et al., 1990; Bajpai et al., 1991a,b,c; Kendrick and Ratledge, 1992; Jareokitmongkol et al., 1993; Lindberg and Molin, 1993; Jang and Yang, 1998; Jang et al., 2000; Yu et al., 2003).

The extensive research and production of PUFA by the fungus *Mortierella* in submerged culture was carried out over the past several years. However, emphasis is put on screening for more efficient strains, using suitable substrates and cultivating the strains at the optimum conditions to achieve the maximal PUFAs yield (Bajpai et al., 1991a,b,c; Li and Ward, 1994; Certik and

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Shimizu, 1999; Koike et al., 2001). Being economically competitive, the producing strains, culture media and conditions of *Mortierella* for PUFAs production were examined in this study.

2. Methods

2.1. Test organism

Mortierella alpina ATCC 32222 was purchased from the American Type Culture Collection, while *M. alpina* CCRC 32365, *M. alpina* CCRC 32738, *M. elongata* CCRC 32709, *M. humicola* CCRC 31720, *M. isabellina* CCRC 31721, *M. isabellina* CCRC 31847, *M. nana* CCRC 31722, *M. ramanniana* CCRC 31391, *M. ramanniana* CCRC 31723 and *M. ramanniana* CCRC 31809 were purchased from the Bioresource Collection and Research Center of Food Industry Research and Development Institute, Hsinchu, Taiwan. All microorganisms were used for the production of PUFAs.

2.2. Culture media and conditions

Mortierella was grown at 20 °C in a membrane culture containing (mg l⁻¹): glucose, 10; yeast extract, 5 and agar, 20 at pH 6.5. Mycelia were harvested from membrane culture and blended with a micro-Waring blender for mycelial suspension. Submerged basal medium contained (mg l⁻¹) soluble starch, 20; Bacto yeast extract, 5; KNO₃, 10; KH₂PO₄, 1 and MgSO₄·7H₂O, 0.25 at pH 6.5. The broth was inoculated with 5% (v/v) mycelial suspension, shaken at 200 rev min⁻¹ and incubated at 20 °C for 2–10 days. Each ml of mycelial suspension contained 1.0–1.5 × 10⁶ mycelial fragments.

2.3. Cell dry weight determination

Mycelia were harvested from culture broth by suction filtration through Whatman No. 4 filter paper. Cell dry weight was determined by washing the mycelia with distilled water twice and then freeze-dried at -50 °C for 24 h to get a constant weight. The dried mycelia were grounded into a fine powder for following extraction by organic solvents. The lipid content of grounded powder was extracted and determined the homogeneity.

2.4. Lipid extraction

Extraction of lipids from mycelia was performed according to the modified procedure of Bligh and Dyer (1959). Lipids were extracted with a 5 times volume of chloroform/methanol (2:1, v/v) by an ultrasonicator for 2 h, filtered through Whatman No. 1 filter paper and concentrated by rotatory evaporator (Buchi, Swiss) at 50 °C.

2.5. Fatty acid analysis

The residue of extracted lipid was dissolved in 1 ml of 0.5 M KOH–MeOH solution, and methylated by 1 ml of 14% (w/v) BF₃ in MeOH. The methylated fatty acids were separated from the aqueous layer by adding saturated NaCl and anhydrous Na₂SO₄, and then dissolving in n-hexane. PUFA content was determined by using gas chromatography GC-8A (Shimadzu Co., Japan) equipped with a 5% diethylene glycol succinate (60/80 mesh) and 1% H₃PO₄ packed glass column (0.26 mm i.d. × 2 m) and a flame ionization detector. Nitrogen was used as the carrier gas. The column temperature was programmed from 190 to 200 °C with an increasing rate of 4 °C min⁻¹. Injection port and detector temperatures were maintained at 230 °C. Methyl esters of palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, α-linolenic acid, γ-linolenic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid were used as standard for fatty acid identification and quantitation. Methyl pentadecanoate was added as an internal standard (Jang et al., 2000).

2.6. Effect of culture conditions on PUFAs production

2.6.1. Incubation temperature, carbon source and oil supplement

The basal medium was incubated with orbital shaking at 12–28 °C for 7 days for temperature effect. Soluble starch in the basal medium was replaced by galactose, glucose, maltose and glycerol and incubated at 20 °C for 7 days for carbon source effect. The effect of carbon source concentration was used 0.0–12.0% of soluble starch or 0.0–10.0% of glucose. The basal medium supplemented with 1% corn oil, lard oil, linseed oil, peanut oil, soybean oil and sunflower oil was used for oil amendment test. The effect of soybean oil concentration was studied from 0.0% to 1.5%. The effect of carbon source and soybean oil was studied using 2.0–8.0% of glucose or soluble starch as carbon source with 1.0% soybean oil supplementation.

2.6.2. Nitrogen source and C/N ratio

Nitrogen source in the basal medium was replaced by 1.5% of NH₄NO₃, (NH₄)₂SO₄, KNO₃, NaNO₃, urea, yeast extract and the different ratios of a mixture of KNO₃ and yeast extract (w/w) from 1:3 to 3:1. The effect of C/N ratio was studied with 2% of soluble starch as carbon source adjusting the ratio of different nitrogen sources and the mixture of KNO₃ and yeast extract at 2:1 (w/w) from 2.5 to 48.3.

2.7. Chemical analysis

pH was measured directly with pH meter. Total nitrogen was determined by a modified Kjeldahl method

(Yang et al., 1991). The protein content was calculated as 6.25 times the total nitrogen content. Degree of unsaturation of fatty acids was calculated as the sum of the product of concentration (% w/w) and the number of unsaturated double bonds of each fatty acid as follows (Chen and Chang, 1994): Degree of unsaturation = 1 (% of monoene) + 2 (% of diene) + 3 (% of triene) + 4 (% of tetraene) + 5 (% of pentaene) + 6 (% of hexaene).

2.8. Statistical analysis

Treatments were done in triplicate, and experimental data was subjected to analysis of variance and Duncan's multiple range test ($p = 0.05$) using the Statistical Analysis System (SAS Institute, 2002).

3. Results and discussion

3.1. Qualitative and quantitative determination of fatty acid

In gas chromatograph, the retention times of test fatty acids increase with increasing number of carbon atoms and double bonds. Palmitic acid and palmitoleic acid had shorter retention time, whereas docosahexaenoic acid had a longer one. The concentration of fatty acids (X) and the sum of absorbance (Y) at a specific retention time had a linear correlation. In the concentrations between 0 and 2000 mg l⁻¹, the equation and correlation coefficient (r) were $Y = 95.66X - 8102$ and 0.9993, $Y = 90.92X + 10674$ and 0.9994, and $Y = 86.71X - 9134$ and 0.9991 for arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid, respectively.

3.2. Effect of test organism on PUFAs production

Polyunsaturated fatty acid production with different species of *Mortierella* is shown in Table 1. *M. alpina*

ATCC 32222, *M. alpina* CCRC 32738 and *M. alpina* CCRC 32365 had high arachidonic acid production, *M. elongata* CCRC 32709 and *M. isabellina* CCRC 31721 had low yield, and the other test strains had undetectable levels. In addition, *M. alpina* ATCC 32222 had the highest arachidonic acid and total PUFAs production. There were significant differences among the test species in linoleic acid, γ -linolenic acid, α -linolenic acid and arachidonic acid production. Therefore *M. alpina* ATCC 32222 was chosen for further study.

3.3. Effect of culture temperature and cultivation period on PUFAs production

Microbial growth had a high rate between 15 and 25 °C, and the value decreased dramatically when the incubation temperature was higher than 25 °C or lower than 15 °C. The effect of incubation temperature on biomass and PUFAs production is presented in Table 2. Biomass production was high at 20 °C, followed by 15 and 12 °C, and 25 °C was the least. The growth of *M. alpina* at 20 °C had a lag phase of 34 h, log phase between 72 and 120 h, stationary phase between 120 and 168 h and decline phase after 192 h. The pH of culture broth increased from 6.0 to 7.1 for 168 h incubation, and then decreased gradually to 5.9 after 240 h. The time for maximum yield was 72 h for γ -linolenic acid, 120 h for linoleic acid and 144 h for total PUFAs and arachidonic acid. PUFAs are primary metabolites and the yield was associated with the cell growth. The production of PUFAs decreased gradually in prolonged cultivation due to cell lysis (Yamada et al., 1987; Bajpai and Bajpai, 1992). Time for maximum yield of PUFAs depends on the kind of fatty acid. γ -Linolenic acid and linoleic acid were synthesized prior to arachidonic acid and eicosapentaenoic acid. Yamada et al. (1987) and Bajpai et al. (1991a) also reported that *M. alpina* IS-4 and ATCC 32222 had their maximal production at 4- and 3-days incubation, respectively. ω -3 and ω -6 series

Table 1
PUFAs production of test organisms

Test strain	LA (mg l ⁻¹)	GLA (mg l ⁻¹)	ALA (mg l ⁻¹)	AA (mg l ⁻¹)	Total PUFAs (mg l ⁻¹)
<i>M. alpina</i> ATCC 32222	50.0 ± 2.6 ^a	17.7 ± 2.4 ^a	4.9 ± 2.6 ^{b,c}	356.8 ± 10.4 ^a	429.4 ± 9.0 ^a
<i>M. alpina</i> CCRC 32738	10.7 ± 0.6 ^b	5.7 ± 0.6 ^b	7.7 ± 1.4 ^{a,b}	56.3 ± 5.0 ^b	80.4 ± 5.1 ^b
<i>M. alpina</i> CCRC 32365	7.0 ± 1.4 ^c	5.3 ± 0.2 ^{b,c}	8.0 ± 1.5 ^{a,b}	25.4 ± 2.1 ^c	45.5 ± 4.3 ^c
<i>M. elongata</i> CCRC 32709	7.0 ± 0.3 ^c	1.8 ± 0.3 ^{d,e}	1.0 ± 0.4 ^d	8.5 ± 1.1 ^d	18.2 ± 1.7 ^d
<i>M. isabellina</i> CCRC 31721	ND	ND	ND	1.3 ± 0.1 ^e	1.3 ± 0.1 ^e
<i>M. humicola</i> CCRC 31720	13.0 ± 0.2 ^b	6.0 ± 0.0 ^b	0.7 ± 1.2 ^d	ND	29.6 ± 1.5 ^d
<i>M. ramanniana</i> CCRC 31391	2.2 ± 0.1 ^d	2.1 ± 0.2 ^{d,e}	3.4 ± 1.3 ^{c,d}	ND	27.0 ± 1.6 ^d
<i>M. isabellina</i> CCRC 31847	9.9 ± 0.6 ^{b,c}	4.3 ± 0.4 ^{b,c,d}	3.9 ± 1.5 ^{c,d}	ND	18.0 ± 1.6 ^d
<i>M. ramanniana</i> CCRC 31809	3.2 ± 0.2 ^d	2.8 ± 0.2 ^{d,e}	3.1 ± 0.4 ^{c,d}	ND	9.1 ± 0.8 ^e
<i>M. ramanniana</i> CCRC 31723	2.3 ± 0.1 ^d	1.3 ± 0.1 ^e	1.3 ± 1.1 ^d	ND	4.8 ± 1.0 ^e
<i>M. nana</i> CCRC 31722	ND	ND	ND	ND	ND

The concentrations of fatty acid are expressed as mean ± standard deviation of triplicate experiments. Values in the same column that do not share the same alphabetic superscript are significantly different at 5% levels according to Duncan's multiple range tests. The basal medium was 2.0% of soluble starch as carbon source at initial pH 6.0 and incubated at 20 °C for 7 days. ND: under detection, PUFAs: polyunsaturated fatty acids, LA: linoleic acid, GLA: γ -linolenic acid, ALA: α -linolenic acid, AA: arachidonic acid.

Table 2
Effect of incubation temperature on biomass and PUFAs production of *M. alpina* ATCC 32222

Temperature (°C)	Cell dry weight (g l ⁻¹)	LA (mg l ⁻¹)	GLA (mg l ⁻¹)	ALA (mg l ⁻¹)	AA (mg l ⁻¹)	EPA (mg l ⁻¹)	Others (mg l ⁻¹)	Total PUFAs (mg l ⁻¹)	<i>Δ</i>
12	6.2 ± 0.2 ^b	131.4 ± 3.4 ^a (8.01 ± 0.19%)	164.9 ± 5.2 ^a (10.06 ± 0.24%)	20.8 ± 1.3 ^a (1.27 ± 0.07%)	882.4 ± 47.5 ^a (53.81 ± 0.25%)	34.4 ± 6.4 ^a (2.10 ± 0.12%)	406.1 ± 10.3 ^a (24.76 ± 0.34%)	1627.0 ± 15.4 ^a (100%)	3.89 ± 0.01 ^a
15	6.3 ± 0.3 ^b	129.9 ± 8.3 ^a (8.01 ± 0.11%)	161.0 ± 2.0 ^a (9.93 ± 0.14%)	17.4 ± 1.0 ^b (1.07 ± 0.17%)	871.9 ± 10.5 ^a (53.76 ± 0.49%)	30.6 ± 5.1 ^a (1.89 ± 0.07%)	411.2 ± 11.2 ^a (25.35 ± 1.04%)	1621.9 ± 15.6 ^a (100%)	3.88 ± 0.01 ^a
20	7.2 ± 0.3 ^a	99.2 ± 0.8 ^b (6.83 ± 0.06%)	96.3 ± 6.3 ^b (6.63 ± 0.03%)	ND	826.3 ± 4.2 ^b (56.91 ± 0.16%)	8.2 ± 2.8 ^b (0.57 ± 0.18%)	422.0 ± 10.6 ^a (29.06 ± 0.06%)	1466.4 ± 6.2 ^b (100%)	3.84 ± 0.01 ^b
25	6.0 ± 0.3 ^b	77.0 ± 5.1 ^c (6.25 ± 0.41%)	62.2 ± 3.5 ^c (5.05 ± 0.12%)	ND	630.6 ± 21.1 ^c (51.22 ± 1.08%)	ND	461.4 ± 15.4 ^a (37.48 ± 0.72%)	1231.2 ± 8.5 ^c (100%)	3.76 ± 0.01 ^c

The concentrations of fatty acid are expressed as mean ± standard deviation (% of total fatty acids) of triplicate experiments. Values in the same column that do not share the same alphabetic superscript are significantly different at 5% levels according to Duncan's multiple range tests. The basal medium was 2.0% of soluble starch as carbon source at initial pH 6.0 and incubated at 20 °C for 7 days. EPA: eicosapentaenoic acid. Others: the difference between total PUFAs and state fatty acid in the table. *Δ*: degree of unsaturation. Same abbreviations as described in Table 1.

Table 3
Effect of carbon source on biomass and PUFAs production of *M. alpina* ATCC 32222

Carbon source	Cell dry weight (g l ⁻¹)	LA (mg l ⁻¹)	GLA (mg l ⁻¹)	ALA (mg l ⁻¹)	AA (mg l ⁻¹)	Others (mg l ⁻¹)	Total PUFAs (mg l ⁻¹)	<i>Δ</i>
Soluble starch	7.2 ± 0.4 ^a	99.2 ± 0.4 ^a (6.89 ± 0.04%)	96.3 ± 6.5 ^a (6.68 ± 0.64%)	ND	826.3 ± 4.3 ^a (57.37 ± 0.42%)	418.6 ± 9.1 ^a (29.06 ± 0.06%)	1440.5 ± 20.8 ^a (100%)	3.87 ± 0.01 ^a
Glucose	7.5 ± 1.1 ^a	58.8 ± 0.1 ^c (4.58 ± 0.01%)	69.9 ± 3.5 ^b (5.45 ± 0.43%)	ND	673.1 ± 14.2 ^b (52.45 ± 1.77%)	481.3 ± 7.4 ^a (37.51 ± 6.31%)	1283.2 ± 17.2 ^b (100%)	3.80 ± 0.05 ^a
Glycerol	6.5 ± 0.5 ^b	42.4 ± 2.1 ^d (5.56 ± 0.31%)	ND	22.6 ± 2.4 (2.96 ± 0.37%)	595.4 ± 10.8 ^c (78.00 ± 1.63%)	102.8 ± 4.9 ^c (13.47 ± 1.79%)	763.3 ± 16.5 ^c (100%)	3.44 ± 0.03 ^b
Galactose	5.1 ± 0.4 ^c	91.8 ± 1.4 ^a (19.04 ± 0.37%)	112.7 ± 3.3 ^a (23.36 ± 0.87%)	ND	171.8 ± 2.0 ^d (35.62 ± 0.53%)	105.0 ± 3.7 ^c (21.76 ± 3.31%)	482.3 ± 11.8 ^d (100%)	3.03 ± 0.06 ^c
Maltose	4.3 ± 0.2 ^d	65.5 ± 0.5 ^b (18.78 ± 0.19%)	99.5 ± 5.1 ^a (28.53 ± 1.91%)	ND	102.3 ± 1.0 ^c (29.33 ± 0.36%)	81.5 ± 4.1 ^c (23.36 ± 2.84%)	348.7 ± 12.9 ^c (100%)	3.23 ± 0.05 ^b

The concentrations of fatty acid are expressed as mean ± standard deviation (% of total fatty acids) of triplicate experiments. Values in the same column that do not share the same alphabetic superscript are significantly different at 5% levels according to Duncan's multiple range tests. The basal medium was 2.0% of carbon source at initial pH 6.0 and incubated at 20 °C for 7 days. ND: under detection. Same abbreviations as described in Table 1.

of PUFAs production also depended on the microbes, culture media and conditions (Shimizu et al., 1989).

Low temperature stimulated the production of high-unsaturated fatty acids, such as arachidonic acid, eicosapentaenoic acid, linoleic acid and γ -linolenic acid. Total PUFAs had high yield at 12 and 15 °C, and it decreased when incubation temperature was higher than 20 °C. Each gram of carbon produced 17.4 mg of linoleic acid, 17.0 mg of γ -linolenic acid, 103.0 mg of arachidonic acid and 194.2 mg of total PUFAs at 20 °C, while it yielded 21.4 mg of linoleic acid, 25.6 mg of γ -linolenic acid, 2.6 mg of α -linolenic acid, 110.3 mg of arachidonic acid, 4.3 mg of eicosapentaenoic acid and 218.4 mg of total PUFAs at 12 °C. Although, low temperature incubation favored the production of PUFAs, but it slowed down the growth rate of test organism.

M. alpina had high cell growth at 15–25 °C, and it decreased at temperature higher than 28 °C or lower than 8 °C. Bajpai et al. (1991b) and Bajpai and Bajpai (1992) indicated that biomass and arachidonic acid production of *M. alpina* ATCC 32222 decreased when the incubation temperature was shifted from 25 °C to 28 °C, and eicosapentaenoic acid could detect only at 10–15 °C incubation. Ahern (1983) reported that in red alga *Porphyridium cruentum*, the arachidonic acid yield per cell was the highest at low temperature and low cell concentration. Low incubation temperature enhanced long chain fatty acid production, such as arachidonic acid and eicosapentaenoic acid, but it retarded the mycelial growth. α -Linolenic acid, arachidonic acid and eicosapentaenoic acid productions were high at 12 and 15 °C. Shimizu et al. (1988) also reported that eicosapentaenoic acid produced by *Mortierella* at 6–16 °C, and it could not detect at 20–28 °C.

3.4. Effect of carbon source on PUFAs production

The effect of carbon source on PUFAs production is presented in Table 3. Cell dry weight had a high value when glucose and soluble starch were used as carbon source, followed by glycerol and galactose, and maltose was the least. The final pH of the culture broth had a similar tendency, soluble starch and glucose gave high pH values (between pH 6.42 and 7.14). Soluble starch was the best carbon source for arachidonic acid and total PUFAs production, glucose and glycerol were the next, and galactose and maltose were the poorest. Degree of unsaturation in the products was the highest when soluble starch was used as carbon source, while galactose was the lowest one. There were significant differences among the test carbon sources in PUFAs production, degree of unsaturation, cell dry weight and final pH value.

M. alpina can be used as arachidonic acid-producing microbes, arachidonic acid content in the total PUFAs

depends on the culture conditions. Arachidonic acid was 57.3–64.8% in the total PUFAs with 2% of soluble starch as carbon source. The value increased to 78.0% with glycerol as carbon source, and it was 52.5%, 35.6% and 29.3% with glucose, galactose and maltose as carbon source, respectively. The arachidonic acid content in the total PUFAs was of same range as that in *M. alpina* (78%) (Totani and Oba, 1987) and in *M. alpina* ATCC 32222 (56%) (Bajpai et al., 1991b), and higher than that in *Mortierella* sp. S-17 at C/N 10 (46%) (Sajbidor et al., 1990), in *M. alpina* ATCC 16266 (43%), in *M. alpina* ATCC 42430 (41%) (Bajpai et al., 1991a), in *M. alpina* IS-4 (35%) (Shinmen et al., 1989), in *M. alpina* CBS 343.66 (35%) and the value increased to 57% after the consumption of glucose in the medium (Lindberg and Molin, 1993), in *M. alpina* IS-4 AKU 39998 (31%) and in *M. alpina* CBS 210.32 (6.9%) (Shinmen et al., 1989). Soluble starch and glucose as carbon source are good for cell growth, arachidonic acid and total PUFAs production. This phenomenon was consistent with Chen and Chang (1994) in *M. alpina* CBS 210.32, and Shinmen et al. (1989) in *M. alpina* IS-4.

Arachidonic acid, total PUFAs production and cell dry weight increased with increasing soluble starch concentration and they had the maximum yield at 10.0%, and then decreased gradually when the concentration was higher than 12.0% (Table 4). At 10.0% of soluble starch, each gram of carbon could support 171.8 mg of total PUFAs, including 5.6 mg of linoleic acid, 5.3 mg of γ -linolenic acid and 114.1 mg of arachidonic acid. In the case of glucose as the sole carbon source, it had the highest cell dry weight, arachidonic acid and total PUFAs production at 6.0% of glucose, and then decreased sharply when the concentration was higher than 8%. Each gram of carbon supported 167.2 mg of total PUFAs production, including 6.6 mg of linoleic acid, 8.3 mg of γ -linolenic acid, and 101.1 mg of arachidonic acid. This effect was different from *M. alpina* ATCC 16266 and *M. alpina* ATCC 32222 with high values at 10% of glucose as carbon source (Bajpai et al., 1991a,b). Koike et al. (2001) reported that fatty acids content increased with the concentration of glucose from 2.0% to 12.0% in *M. alpina* CBS 754.68, while arachidonic acid content and arachidonic acid yield were reversed.

3.5. Effect of nitrogen source and C/N ratio on PUFAs production

The effect of nitrogen supplement on PUFA production is illustrated in Table 5. KNO₃ gave the highest cell biomass and total PUFAs yield. In the mixture of inorganic and organic nitrogen source, yeast extract gave the maximal cell biomass, while the mixture of KNO₃ and yeast extract (2:1, w/w) had the highest total PUFAs,

Table 4
Effect of carbon source concentration on biomass and PUFAs production of *M. alpina* ATCC 32222

Carbon source	Concentration (g l ⁻¹)	Cell dry weight (g l ⁻¹)	LA (mg l ⁻¹)	GLA (mg l ⁻¹)	AA (mg l ⁻¹)	Others (mg l ⁻¹)	Total PUFAs (mg l ⁻¹)	Δ
Glucose	0	1.6 ± 0.1 ^e	1.2 ± 0.1 ^f (8.15 ± 1.30%)	1.9 ± 0.3 ^g (12.96 ± 3.19%)	6.9 ± 1.2 ^g (45.89 ± 12.26%)	4.9 ± 0.2 ^d (33.00 ± 2.25%)	15.0 ± 1.6 ^h (100%)	2.89 ± 0.05 ^c
	20	7.5 ± 2.6 ^c	58.8 ± 0.1 ^d (4.67 ± 0.01%)	69.2 ± 3.5 ^d (5.50 ± 0.43%)	671.1 ± 14.2 ^c (53.28 ± 1.77%)	460.4 ± 10.6 ^c (36.55 ± 0.06%)	1259.5 ± 4.5 ^d (100%)	3.81 ± 0.05 ^{a,b}
	40	11.9 ± 2.6 ^b	142.0 ± 7.7 ^a (4.17 ± 0.35%)	155.9 ± 10.9 ^b (4.58 ± 0.50%)	1887.1 ± 147.0 ^b (55.42 ± 6.73%)	1220.0 ± 73.6 ^a (35.83 ± 0.13%)	3405.0 ± 93.3 ^b (100%)	3.83 ± 0.01 ^a
	60	14.9 ± 3.7 ^b	158.4 ± 5.0 ^a (3.85 ± 0.18%)	200.2 ± 5.4 ^a (4.86 ± 0.19%)	2424.7 ± 68.1 ^a (58.86 ± 2.45%)	1227.9 ± 32.2 ^a (32.43 ± 0.07%)	4011.2 ± 65.3 ^a (100%)	3.80 ± 0.04 ^{a,b}
	80	11.8 ± 1.5 ^b	125.9 ± 1.4 ^a (3.96 ± 0.07%)	110.4 ± 3.2 ^b (3.47 ± 0.15%)	1858.2 ± 2.9 ^b (58.49 ± 0.14%)	1082.3 ± 11.3 ^b (34.06 ± 0.16%)	3176.8 ± 21.7 ^b (100%)	3.84 ± 0.01 ^a
	100	5.3 ± 1.3 ^d	42.9 ± 2.3 ^d (4.91 ± 0.47%)	43.6 ± 2.2 ^d (4.99 ± 0.46%)	393.1 ± 5.7 ^d (44.97 ± 1.18%)	401.0 ± 7.4 ^c (45.87 ± 0.47%)	874.2 ± 9.6 ^c (100%)	3.76 ± 0.01 ^b
Soluble starch	0	1.6 ± 0.0 ^e	1.2 ± 0.1 ^f (8.15 ± 1.30%)	1.9 ± 0.3 ^g (12.96 ± 3.19%)	6.9 ± 1.2 ^g (45.89 ± 12.26%)	4.9 ± 0.2 ^d (33.00 ± 2.25%)	15.0 ± 1.6 ^h (100%)	2.89 ± 0.05 ^c
	10	4.2 ± 0.2 ^d	17.1 ± 0.4 ^e (12.59 ± 0.37%)	8.8 ± 0.1 ^f (6.48 ± 0.15%)	70.5 ± 4.3 ^f (51.87 ± 4.48%)	39.5 ± 2.3 ^d (29.06 ± 0.85%)	136.0 ± 4.0 ^g (100%)	3.85 ± 0.02 ^a
	20	7.2 ± 0.4 ^c	99.2 ± 0.4 ^e (7.01 ± 0.04%)	95.3 ± 6.5 ^b (6.73 ± 0.64%)	826.3 ± 4.3 ^c (58.33 ± 4.24%)	395.8 ± 2.7 ^c (27.94 ± 0.04%)	1416.7 ± 15.2 ^d (100%)	3.87 ± 0.01 ^a
	40	9.6 ± 0.9 ^c	81.0 ± 7.1 ^e (4.08 ± 0.49%)	73.5 ± 3.1 ^d (3.70 ± 0.21%)	1293.6 ± 57.7 ^b (65.09 ± 3.99%)	539.1 ± 10.0 ^c (27.13 ± 0.66%)	1978.1 ± 57.2 ^d (100%)	3.87 ± 0.01 ^a
	60	14.0 ± 2.0 ^b	81.0 ± 5.0 ^e (3.13 ± 0.26%)	74.0 ± 1.0 ^d (2.86 ± 0.05%)	1746.3 ± 74.1 ^b (67.52 ± 3.90%)	685.1 ± 16.5 ^c (26.49 ± 0.04%)	2586.3 ± 45.2 ^c (100%)	3.88 ± 10.01 ^a
	80	17.8 ± 0.5 ^b	91.1 ± 2.4 ^e (3.07 ± 0.11%)	84.5 ± 2.6 ^c (2.85 ± 0.12%)	2007.7 ± 34.6 ^b (67.72 ± 1.59%)	781.5 ± 17.5 ^c (26.36 ± 0.04%)	2964.7 ± 19.1 ^b (100%)	3.94 ± 0.05 ^a
	100	18.4 ± 3.0 ^a	130.0 ± 1.4 ^a (3.28 ± 0.05%)	122.6 ± 4.3 ^b (3.09 ± 0.15%)	2636.9 ± 369.5 ^a (66.42 ± 1.28%)	1080.7 ± 16.2 ^b (27.221 ± 0.31%)	3970.2 ± 53.5 ^a (100%)	3.88 ± 0.10 ^a
	120	17.9 ± 3.9 ^b	104.6 ± 2.9 ^b (17.06 ± 0.67%)	27.4 ± 2.9 ^c (4.48 ± 0.67%)	297.1 ± 11.3 ^c (48.46 ± 2.63%)	184.0 ± 11.2 ^d (30.01 ± 1.00%)	613.1 ± 6.1 ^f (100%)	3.83 ± 0.12 ^a

The concentrations of fatty acid are expressed as mean ± standard deviation (% of total fatty acids) of triplicate experiments. Values in the same column that do not share the same alphabetic superscript are significantly different at 5% levels according to Duncan's multiple range tests. The basal medium was initial pH 6.0 and incubated at 20 °C for 7 days. ND: under detection. Same abbreviations as described in Table 1.

Table 5
Effect of nitrogen source on pH, biomass and PUFAs production of *M. alpina* ATCC 32222

Nitrogen source	C/N ratio	Final pH	Cell dry weight (g l ⁻¹)	LA (mg l ⁻¹)	GLA (mg l ⁻¹)	AA (mg l ⁻¹)	Others (mg l ⁻¹)	Total PUFAs (mg l ⁻¹)	Δ
KNO ₃	6.2	7.5 ± 0.3 ^b	6.5 ± 0.5 ^b	47.0 ± 0.3 ^b (4.51 ± 0.04%)	53.9 ± 1.0 ^b (5.18 ± 0.14%)	622.4 ± 17.1 ^b (59.73 ± 2.36%)	318.7 ± 8.4 ^{b,c} (30.58 ± 0.61%)	1042.1 ± 33.4 ^a (100%)	3.82 ± 0.01 ^b
NaNO ₃	5.4	6.9 ± 0.2 ^b	6.9 ± 0.3 ^b	29.8 ± 0.7 ^c (3.38 ± 0.11%)	37.2 ± 0.4 ^c (4.22 ± 0.06%)	569.2 ± 18.9 ^c (64.57 ± 2.96%)	245.3 ± 7.6 ^c (27.83 ± 2.29%)	881.5 ± 9.2 ^b (100%)	3.73 ± 0.01 ^b
NH ₄ NO ₃	2.5	3.8 ± 0.1 ^c	5.7 ± 0.1 ^b	43.9 ± 2.6 ^b (5.59 ± 0.42%)	46.7 ± 1.6 ^b (5.94 ± 0.25%)	528.1 ± 20.5 ^c (67.21 ± 3.31%)	167.1 ± 7.3 ^d (21.26 ± 0.23%)	785.8 ± 14.6 ^b (100%)	3.59 ± 0.01 ^c
(NH ₄) ₂ SO ₄	4.2	4.7 ± 0.1 ^d	2.6 ± 0.1 ^c	26.9 ± 2.0 ^c (29.42 ± 2.23%)	15.7 ± 0.8 ^d (17.20 ± 0.89%)	48.8 ± 5.5 ^e (53.38 ± 6.06%)	59.3 ± 4.1 ^e (39.32 ± 5.31%)	150.7 ± 4.1 ^c (100%)	3.20 ± 0.01 ^c
Urea	1.9	8.2 ± 0.1 ^a	0.6 ± 0.0 ^d	3.9 ± 0.6 ^d (13.79 ± 3.53%)	2.4 ± 0.2 ^e (8.42 ± 1.31%)	11.4 ± 1.5 ^e (40.70 ± 8.77%)	10.4 ± 1.2 ^e (37.08 ± 1.21%)	27.9 ± 0.9 ^d (100%)	3.47 ± 0.01 ^c
Yeast extract	9.0	8.5 ± 0.3 ^a	9.9 ± 0.1 ^a	54.5 ± 1.5 ^b (3.91 ± 0.25%)	81.5 ± 8.1 ^a (5.85 ± 1.34%)	468.7 ± 8.9 ^d (33.62 ± 1.48%)	789.6 ± 11.3 ^a (56.63 ± 8.05%)	1394.3 ± 18.5 ^a (100%)	3.72 ± 0.02 ^b
KNO ₃ : Yeast extract (1:3, w/w)	7.6	7.8 ± 0.4 ^b	9.3 ± 0.3 ^a	56.9 ± 3.1 ^b (5.83 ± 0.60%)	50.3 ± 4.7 ^b (5.16 ± 0.82%)	473.0 ± 20.2 ^d (48.49 ± 3.48%)	395.2 ± 11.8 ^b (40.52 ± 0.75%)	975.4 ± 19.2 ^a (100%)	3.77 ± 0.01 ^b
KNO ₃ : Yeast extract (1:2, w/w)	7.1	7.6 ± 0.2 ^b	7.2 ± 0.1 ^b	31.4 ± 1.5 ^c (4.63 ± 0.30%)	32.0 ± 0.4 ^c (4.73 ± 0.09%)	435.2 ± 31.3 ^d (64.22 ± 6.28%)	246.9 ± 12.2 ^c (36.43 ± 3.43%)	677.7 ± 20.8 ^b (100%)	3.78 ± 0.02 ^b
KNO ₃ : Yeast extract (1:1, w/w)	6.2	7.6 ± 0.1 ^b	8.5 ± 0.1 ^b	27.2 ± 2.7 ^c (2.88 ± 0.46%)	25.0 ± 1.3 ^c (2.65 ± 0.22%)	538.9 ± 4.1 ^c (57.13 ± 0.69%)	352.2 ± 7.4 ^{b,c} (37.34 ± 0.83%)	943.3 ± 8.1 ^a (100%)	3.79 ± 0.06 ^b
KNO ₃ : Yeast extract (2:1, w/w)	5.2	5.9 ± 0.0 ^c	7.2 ± 0.1 ^b	99.2 ± 0.4 ^a (6.89 ± 0.04%)	96.3 ± 6.5 ^a (6.68 ± 0.64%)	826.3 ± 4.2 ^a (57.37 ± 0.41%)	418.6 ± 7.6 ^b (29.06 ± 0.06%)	1440.5 ± 11.6 ^a (100%)	3.87 ± 0.01 ^a
KNO ₃ : Yeast extract (3:1, w/w)	5.1	5.2 ± 0.2 ^c	6.9 ± 0.4 ^b	52.3 ± 1.9 ^b (4.13 ± 0.23%)	56.4 ± 2.2 ^b (4.45 ± 0.26%)	726.4 ± 13.5 ^b (57.40 ± 1.62%)	430.6 ± 6.5 ^b (34.02 ± 0.07%)	1265.6 ± 13.8 ^a (100%)	3.81 ± 0.01 ^b

The concentrations of fatty acid are expressed as mean ± standard deviation (% of total fatty acids) of triplicate experiments. Values in the same column that do not share the same alphabetic superscript are significantly different at 5% levels according to Duncan's multiple range tests. The basal medium was 2.0% of soluble starch as carbon source at initial pH 6.0 and incubated at 20 °C for 7 days. Same abbreviations as described in Table 1.

linoleic acid, γ -linolenic acid and arachidonic acid production.

Nitrate as nitrogen source showed a high cell growth, total PUFAs, and arachidonic acid production, whereas ammonium and urea had low value. This might be due to the neutral pH in the nitrate salt amendment, while an acidic pH was observed in ammonium salt supplementation. The mixture of KNO_3 and yeast extract as nitrogen source enhanced the cell growth, total PUFAs and arachidonic acid production. Total PUFAs and arachidonic acid production had the maximal value at the ratio of KNO_3 to yeast extract 2:1. Yeast extract as the sole nitrogen source stimulated the cell growth, but not for arachidonic acid and total PUFAs production. Chang and Chen (1994) indicated that KNO_3 was the best nitrogen source for arachidonic acid and total PUFAs production, while Bajpai and Bajpai (1992) reported that 1% of yeast extract was the optimum concentration for arachidonic acid production and high amount of yeast extract supplementation promoted the cell growth. Park et al. (1999) also indicated that nitrogen source affected the mycelial morphology and arachidonic acid production of *M. alpina* CBS 754.68. Feather-like morphology with soybean meal as nitrogen source suitable for arachidonic acid production.

The C/N ratio was 5.2 with 2.0% of soluble starch as carbon source and 1.5% of the mixture of KNO_3 and yeast extract (2:1, w/w) as nitrogen source. The effect of C/N ratio on PUFA production is shown in Table 6. A C/N ratio of 9.0 gave a high cell yield, whereas a C/N ratio of 5.2 gave the maximal arachidonic acid and total PUFAs production. Arachidonic acid content decreased sharply when the C/N ratio was lower than 2.5 or higher than 48.3. There were significant differences among C/N ratios, final pH, cell dry weight and PUFA production.

From the elemental analysis, *M. alpina* ATCC 32222 contained 30.79% of carbon and 6.30% of nitrogen, and C/N ratio was 4.9. The optimal C/N ratio for total PUFAs and arachidonic acid production was 5.2, and the production decreased gradually with increasing of C/N ratio. Cell growth, total PUFAs and arachidonic acid production reduced when the C/N ratio was 2.5 or 48.3, but the percentage of arachidonic acid in total PUFAs increased from C/N ratio 2.5 to 9.0, and then decreased gradually as C/N ratio higher than 12.8. However, Sajbidor et al. (1990) reported that *Mortierella* sp. S-17 had high arachidonic acid concentration in the cell cultivated at C/N ratio 10, and had high arachidonic acid production at C/N ratio 20. Koike et al. (2001) also reported that the optimal C/N ratio of the medium was around 15–20 for arachidonic acid production in a culture of *M. alpina* CBS 754.68. When the C/N ratio was higher than 20 the mycelia weight and arachidonic acid decreased due to nitrogen limitation.

Table 6
Effect C/N ratio on biomass and PUFAs production of *M. alpina* ATCC 32222

C/N ratio	Cell dry weight (g l ⁻¹)	LA (mg l ⁻¹)	GLA (mg l ⁻¹)	AA (mg l ⁻¹)	Others (mg l ⁻¹)	Total PUFAs (mg l ⁻¹)	Δ
2.5	2.6 ± 0.5 ^c	50.5 ± 1.5 ^c (7.65 ± 0.41%)	69.9 ± 3.5 ^c (10.61 ± 0.72%)	109.5 ± 4.2 ^c (16.61 ± 1.13%)	429.3 ± 12.5 ^a (65.13 ± 0.04%)	659.1 ± 14.1 ^c (100%)	3.36 ± 0.01 ^d
5.2	7.2 ± 1.1 ^a	99.2 ± 0.8 ^a (7.04 ± 0.06%)	96.3 ± 6.3 ^a (6.83 ± 0.03%)	826.3 ± 4.2 ^a (58.62 ± 0.18%)	387.8 ± 3.7 ^b (27.51 ± 0.11%)	1409.7 ± 13.8 ^a (100%)	3.79 ± 0.02 ^a
9.0	7.3 ± 0.2 ^a	88.8 ± 1.2 ^b (8.18 ± 0.56%)	86.8 ± 3.2 ^b (7.99 ± 0.12%)	698.1 ± 13.9 ^b (64.26 ± 0.24%)	212.6 ± 7.2 ^c (20.24 ± 0.64%)	1086.3 ± 2.9 ^b (100%)	3.66 ± 0.02 ^b
12.8	7.2 ± 0.9 ^a	82.7 ± 1.1 ^c (7.86 ± 0.01%)	84.3 ± 0.9 ^b (8.02 ± 0.04%)	671.5 ± 5.6 ^{b,c} (63.88 ± 0.35%)	212.8 ± 9.5 ^c (20.24 ± 0.14%)	1051.2 ± 2.4 ^b (100%)	3.66 ± 0.02 ^b
24.6	6.8 ± 0.3 ^b	60.9 ± 3.5 ^d (5.58 ± 0.01%)	60.8 ± 1.3 ^c (5.57 ± 0.14%)	657.2 ± 23.4 ^{b,c} (60.25 ± 0.25%)	311.8 ± 10.1 ^b (28.59 ± 0.14%)	1090.7 ± 3.1 ^b (100%)	3.61 ± 0.01 ^c
48.3	2.8 ± 0.5 ^c	29.9 ± 2.0 ^f (7.30 ± 0.06%)	14.7 ± 1.0 ^d (3.59 ± 0.04%)	214.0 ± 16.9 ^d (52.30 ± 0.08%)	150.6 ± 7.2 ^c (36.81 ± 0.28%)	409.0 ± 2.7 ^c (100%)	3.59 ± 0.04 ^c

The concentration of fatty acids are expressed as mean ± standard deviation (% of total fatty acids) of triplicate experiments. Values in the same column that do not share the same alphabetic superscript are significantly different at 5% levels according to Duncan's multiple range tests. The basal medium was 2.0% of soluble starch as carbon source at initial pH 6.0 and incubated at 20 °C for 7 days. Same abbreviations as described in Table 1.

3.6. Effect of oil supplement on PUFAs production

The effect of oil supplement on the yield of PUFA is illustrated in Table 7. Supplementation with 1% of oil increased the biomass production, and linseed oil gave the highest biomass, and then followed by sunflower oil and soybean oil. In case of PUFAs production, supplementation of linseed oil gave a high arachidonic acid production, followed by soybean oil and sunflower oil. Eicosapentaenoic acid production was high with a linseed oil supplement, followed by lard oil and soybean oil. Docosahexaenoic acid production had high value with the supplement of sunflower oil, and then followed by linseed oil. In the ratios of fatty acid to total PUFAs content, docosahexaenoic acid ratio was high with a peanut oil supplement, eicosapentaenoic acid ratio was high with a linseed oil supplement, and arachidonic acid ratio was high with corn oil and soybean oil amendment. Shinmen et al. (1989) also indicated that olive oil and soybean oil addition increased the accumulation of arachidonic acid.

The effect of soybean oil concentration on PUFAs production is presented in Table 8. Biomass increased with the concentration of soybean oil and it had a plateau at 1.0%. The yields of total PUFAs, linoleic acid, arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid also increased with the increasing concentration of soybean oil supplement, and they had the maximal values at 1.0% of soybean oil. However, soybean oil supplement inhibited the production of γ -linolenic acid. The percentage of arachidonic acid and the degree of unsaturation decreased with the concentration of soybean oil supplement, while the percentages of linoleic acid and other than the stated PUFAs were reversed. Docosahexaenoic acid and eicosapentaenoic acid content had high values at 1.0% of soybean oil supplement.

Linoleic acid is the major fatty acid in soybean oil, corn oil, peanut oil and sunflower oil (contained 56–85%), and α -linolenic acid was the major fatty acid in linseed oil (58%). Saturated fatty acid (23%) and linoleic acid (15%) were the major fatty acids in lard oil. Supplementation of oil stimulated the cell growth, eicosapentaenoic acid, docosahexaenoic acid, arachidonic acid and total PUFAs production in *M. alpina*. Linseed oil was the most effective, followed by soybean oil and sunflower oil. Arachidonic acid production was more obvious in linseed oil supplementation. Jareokitmongkol et al. (1993) suggested that 3% of linseed oil amendment produced 1 g l^{-1} of eicosapentaenoic acid in *M. alpina* (20% of total fatty acid). In case of soybean oil, it contained linoleic acid (56%), oleic acid (16%), palmitic acid (15%), and α -linolenic acid (7%). These fatty acids were the precursors of long chain fatty acids. However, arachidonic acid production decreased with the supplementation of soybean oil, while eicosapentaenoic acid and

Table 7
Effect of oil supplement on biomass and PUFAs production of *M. alpina* ATCC 32222

Kind of oil	Dry cell weight (g l ⁻¹)	LA (mg l ⁻¹)	ALA (mg l ⁻¹)	AA (mg l ⁻¹)	EPA (mg l ⁻¹)	DHA (mg l ⁻¹)	Others (mg l ⁻¹)	Total PUFAs (mg l ⁻¹)	Δ
Linseed oil	17.5 ± 0.9 ^a	2295.4 ± 79.5 ^d (15.58 ± 1.17%)	6302.8 ± 81.4 ^a (42.78 ± 2.00%)	1923.3 ± 69.9 ^a (13.05 ± 0.63%)	524.6 ± 25.5 ^a (3.56 ± 0.23%)	26.2 ± 0.1 ^{ab} (0.18 ± 0.00%)	3661.3 ± 72.5 ^c (24.85 ± 0.39%)	14733.8 ± 96.1 ^a (100%)	3.13 ± 0.01 ^a
Linseed oil*	17.0 ± 0.2 ^{ab}	2224.0 ± 23.0 ^d	5540.9 ± 66.4 ^b	1797.4 ± 11.5 ^a	288.7 ± 8.9 ^b	30.3 ± 3.3 ^{ab}	3352.0 ± 85.6 ^c	13233.2 ± 77.5 ^a	3.08 ± 0.01 ^b
Sunflower oil	17.3 ± 0.4 ^{ab}	7996.9 ± 97.2 ^a (52.05 ± 1.17%)	4187 ± 6.7% (211.9 ± 5.6 ^{cd})	13.58 ± 0.12% (1689.1 ± 31.8 ^b)	2.18 ± 0.09% (53.2 ± 4.5 ^c)	0.23 ± 0.03% (36.3 ± 1.4 ^a)	25.33 ± 0.45% (5374.2 ± 24.5 ^b)	15363.7 ± 72.9 ^a (100%)	2.46 ± 0.02 ^f
Soybean oil	17.1 ± 0.2 ^{ab}	3545.5 ± 23.6 ^c (37.95 ± 0.43%)	26.3 ± 2.2 ^d (0.28 ± 0.04%)	1786.1 ± 19.1 ^a (19.11 ± 0.35%)	70.9 ± 1.3 ^c (0.76 ± 0.02%)	15.7 ± 0.4 ^b (0.17 ± 0.01%)	3899.0 ± 82.5 ^c (41.73 ± 0.03%)	9343.5 ± 43.9 ^b (100%)	2.82 ± 0.02 ^d
Lard oil	13.7 ± 0.5 ^c	4103.4 ± 97.7 ^b (18.13 ± 1.62%)	293.3 ± 21.6 ^c (1.30 ± 0.22%)	688.1 ± 24.1 ^c (3.04 ± 0.27%)	81.6 ± 5.2 ^c (0.36 ± 0.05%)	21.6 ± 2.4 ^{ab} (0.10 ± 0.05%)	17447.2 ± 84.5 ^a (77.08 ± 0.74%)	22635.2 ± 81.9 ^a (100%)	2.59 ± 0.02 ^e
Peanut oil	13.5 ± 0.3 ^c	2368.2 ± 99.5 ^d (46.10 ± 3.25%)	298.1 ± 51.4 ^c (5.80 ± 1.70%)	263.1 ± 23.7 ^d (5.12 ± 0.79%)	68.9 ± 9.2 ^c (1.34 ± 0.73%)	22.2 ± 4.5 ^{ab} (0.43 ± 0.15%)	2116.9 ± 97.5 ^d (41.21 ± 1.82%)	5136.9 ± 191.7 ^c (100%)	2.47 ± 0.01 ^f
Corn oil	15.5 ± 0.2 ^{bc}	806.0 ± 55.3 ^c (29.99 ± 3.84%)	77.8 ± 8.7 ^{cd} (2.90 ± 0.60%)	555.9 ± 45.6 ^c (20.68 ± 3.17%)	ND	ND	1247.8 ± 34.6 ^c (46.43 ± 0.42%)	2687.5 ± 158.7 ^d (100%)	2.90 ± 0.03 ^c

The concentrations of fatty acids are expressed as mean ± standard deviation (% of total fatty acids) of triplicate experiments. Values in the same column that do not share the same alphabetic superscript are significantly different at 5% levels according to Duncan's multiple range tests. The basal medium was 2.0% of soluble starch and 1.0% of oil as carbon source at initial pH 6.0 and incubated at 20 °C for 7 days. DHA: docosahexaenoic acid. Same abbreviations as described as Table 1.

* 2.0% of soluble starch was replaced by glucose.

Table 8
Effect of soybean oil concentration on PUFAs production of *M. alpina* ATCC 32222

Soybean oil (%)	Cell dry weight (g l ⁻¹)	LA (mg l ⁻¹)	GLA (mg l ⁻¹)	ALA (mg l ⁻¹)	AA (mg l ⁻¹)	EPA (mg l ⁻¹)	DHA (mg l ⁻¹)	Others (mg l ⁻¹)	Total PUFAs (mg l ⁻¹)	Δ
0.0	7.2 ± 0.4 ^c	99.2 ± 0.4 ^c (6.85 ± 0.10%)	96.3 ± 6.5 (6.64 ± 0.03%)	ND	826.3 ± 4.3 ^d (57.02 ± 0.08%)	ND	ND	421.1 ± 2.2 ^d (29.06 ± 0.06%)	1449.2 ± 6.4 ^c (100%)	3.87 ± 0.01 ^a
0.2	10.3 ± 0.8 ^d	402.5 ± 36.0 ^d (21.99 ± 0.07%)	ND	ND	798.1 ± 74.8 ^d (43.60 ± 0.03%)	20.6 ± 1.6 ^c (1.13 ± 0.02%)	ND	609.4 ± 52.1 ^c (33.29 ± 0.06%)	1830.7 ± 92.5 ^d (100%)	3.44 ± 0.01 ^b
0.5	12.2 ± 1.2 ^c	864.5 ± 83.8 ^c (29.81 ± 0.41%)	ND	ND	1027.6 ± 65.6 ^c (35.43 ± 0.68%)	37.7 ± 3.0 ^b (1.30 ± 0.01%)	ND	970.4 ± 44.3 ^b (33.46 ± 0.27%)	2900.1 ± 86.4 ^c (100%)	3.23 ± 0.02 ^c
0.8	15.1 ± 0.3 ^b	874.8 ± 85.3 ^c (28.58 ± 0.33%)	ND	ND	1185.3 ± 75.6 ^c (38.77 ± 0.06%)	43.4 ± 3.8 ^b (1.42 ± 0.13%)	1.1 ± 0.0 ^c (0.03 ± 0.01%)	953.5 ± 55.1 ^b (31.19 ± 0.62%)	3057.1 ± 78.1 ^c (100%)	3.07 ± 0.04 ^d
1.0	17.1 ± 0.6 ^a	3545.5 ± 85.5 ^a (37.96 ± 0.52%)	ND	26.3 ± 2.2 ^b (0.28 ± 0.01%)	1786.1 ± 11.5 ^a (19.12 ± 0.51%)	70.9 ± 13.1 ^a (0.76 ± 0.09%)	15.7 ± 0.4 ^a (0.17 ± 0.01%)	3896.1 ± 22.4 ^a (41.71 ± 0.03%)	9340.9 ± 99.4 ^a (100%)	2.82 ± 0.02 ^e
1.5	18.0 ± 2.1 ^a	2191.9 ± 99.9 ^b (32.11 ± 0.46%)	ND	35.2 ± 1.0 ^a (0.52 ± 0.05%)	1511.4 ± 59.1 ^b (22.14 ± 0.15%)	60.1 ± 0.7 ^a (0.88 ± 0.07%)	10.3 ± 1.9 ^b (0.15 ± 0.01%)	3017.1 ± 67.5 ^a (44.20 ± 0.19%)	6826.0 ± 90.2 ^b (100%)	2.77 ± 0.02 ^f

The concentrations of fatty acids are expressed as mean ± standard deviation (% of total fatty acids) of triplicate experiments. Values in the same column that do not share the same alphabetic superscript are significantly different at 5% levels according to Duncan's multiple range tests. The basal medium was 2.0% of soluble starch as carbon source at initial pH 6.0 and incubated at 20 °C for 7 days. Same abbreviations as described as Table 1.

Table 9
Effect of carbon source with 1.0% soybean oil supplement on the PUFAs production of *M. alpina* ATCC 32222

Carbon source (g l ⁻¹)		Cell dry weight (g l ⁻¹)	LA (mg l ⁻¹)	ALA (mg l ⁻¹)	AA (mg l ⁻¹)	EPA (mg l ⁻¹)	Others (mg l ⁻¹)	Total PUFAs (mg l ⁻¹)	Δ
Soluble starch	20	10.2 ± 0.8 ^d	402.5 ± 57.5 ^d (21.72 ± 1.08%)	19.9 ± 1.2 ^c (1.07 ± 0.01%)	798.1 ± 15.4 ^f (43.07 ± 0.36%)	20.6 ± 1.3 ^d (1.11 ± 0.03%)	611.8 ± 8.6 ^c (33.02 ± 0.87%)	1852.9 ± 102.0 ^f (100%)	3.44 ± 0.01 ^d
	40	16.4 ± 0.2 ^c	1676.0 ± 92.4 ^{b,c} (35.54 ± 0.21%)	24.7 ± 1.4 ^d (0.52 ± 0.02%)	1812.71 ± 66.7 ^d (38.44 ± 1.21%)	21.0 ± 1.0 ^d (0.44 ± 0.07%)	1181.4 ± 42.2 ^c (25.05 ± 0.69%)	4716.1 ± 169.8 ^d (100%)	3.56 ± 0.02 ^b
	60	23.0 ± 0.4 ^b	1644.2 ± 88.0 ^{b,c} (24.11 ± 0.61%)	31.0 ± 2.2 ^a (0.45 ± 0.02%)	3750.9 ± 56.2 ^a (55.01 ± 1.27%)	25.8 ± 0.7 ^c (0.38 ± 0.23%)	1367.2 ± 35.4 ^b (20.05 ± 0.13%)	6819.1 ± 156.2 ^a (100%)	3.63 ± 0.01 ^a
	80	23.2 ± 1.0 ^b	1786.7 ± 97.3 ^b (27.69 ± 0.39%)	30.1 ± 1.0 ^a (0.47 ± 0.02%)	3686.5 ± 21.2 ^a (57.14 ± 0.47%)	36.8 ± 1.3 ^a (0.57 ± 0.41%)	911.6 ± 30.0 ^d (14.13 ± 0.52%)	6451.7 ± 40.7 ^b (100%)	3.66 ± 0.01 ^a
Glucose	20	10.7 ± 0.3 ^d	471.7 ± 45.3 ^d (19.14 ± 1.04%)	15.4 ± 2.0 ^f (0.63 ± 0.06%)	1379.1 ± 79.7 ^e (55.97 ± 0.83%)	19.7 ± 0.8 ^d (0.80 ± 0.33%)	578.1 ± 7.2 ^c (23.46 ± 1.06%)	2464.1 ± 57.2 ^c (100%)	3.47 ± 0.02 ^{c,d}
	40	17.3 ± 0.8 ^c	1571.1 ± 35.0 ^c (25.90 ± 1.46%)	26.0 ± 1.4 ^c (0.43 ± 0.02%)	2713.8 ± 99.4 ^c (44.74 ± 0.37%)	21.0 ± 1.2 ^d (0.35 ± 0.12%)	1733.5 ± 62.4 ^a (28.58 ± 1.52%)	6065.3 ± 114.1 ^c (100%)	3.51 ± 0.02 ^c
	60	23.6 ± 0.4 ^{a,b}	1989.2 ± 93.2 ^a (29.81 ± 2.44%)	28.7 ± 0.9 ^b (0.43 ± 0.01%)	3274.4 ± 176.4 ^b (49.07 ± 2.06%)	23.8 ± 0.3 ^c (0.36 ± 0.42%)	1356.6 ± 25.9 ^b (20.33 ± 1.07%)	6672.7 ± 178.9 ^b (100%)	3.40 ± 0.01 ^d
	80	24.9 ± 0.1 ^a	1764.1 ± 28.5 ^b (29.86 ± 0.69%)	30.9 ± 2.7 ^a (0.52 ± 0.01%)	2895.9 ± 91.5 ^c (49.01 ± 0.95%)	33.6 ± 2.2 ^b (0.57 ± 0.14%)	1184.1 ± 41.6 ^c (20.04 ± 0.83%)	5908.6 ± 193.1 ^c (100%)	3.39 ± 0.01 ^d

The concentrations of fatty acids are expressed as mean ± standard deviation (% of total fatty acids) of triplicate experiments. Values in the same column that do not share the same alphabetic superscript are significantly different at 5% levels according to Duncan's multiple range tests. The basal medium was 2.0% of soluble starch as carbon source at initial pH 6.0 and incubated at 20 °C for 7 days. Same abbreviations as described as Table 1.

docosahexaenoic acid production increased slightly with the amendment of soybean oil. In the case of 2% of soybean oil, arachidonic acid production and cell dry weight had high values at 8% of soluble starch, but they had the maximal values at 6% of glucose. A high concentration of glucose increased the osmotic pressure and decreased the efficiency of carbon source and arachidonic acid production.

The effect of glucose and soluble starch concentration with 1.0% of soybean oil supplement on biomass and PUFAs production is shown in Table 9. Biomass, α -linolenic acid and eicosapentaenoic acid production increased with the concentration of carbon source. The yield of total PUFAs and arachidonic acid had a high value at 6% of carbon source. However, γ -linolenic acid and docosahexaenoic acid could not be detected during the cultivation. When compared the results with Table 4, it was observed that 1.0% of soybean oil amendment increased the biomass, α -linolenic acid, eicosapentaenoic acid and total PUFAs production, inhibited γ -linolenic acid production and reduced the degree of unsaturation. Therefore, carbon source concentration and oil supplement had stimulation effect on the PUFAs production. Appropriate concentration of oil supplement could enhance the PUFAs production in *M. alpina* ATCC 32222.

In conclusion, the optimum conditions for polyunsaturated fatty acids production with *M. alpina* ATCC 32222 were 10% of soluble starch as carbon source, the mixture of KNO₃ and yeast extract at 2:1 (w/w) as nitrogen source at C/N ratio 5.2–9.0, supplement with 1% of linseed oil, and incubation at 20 °C for 3 days then shifted to 12 °C for 5 days. Each gram of carbon produced 403.4 mg of α -linolenic acid, 123.1 mg of arachidonic acid, 33.6 mg of eicosapentaenoic acid, 1.68 mg of docosahexaenoic acid and 943.2 mg of total PUFAs.

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