Bot. Bull. Acad. Sin. (2000) **41:** 41-48

Polyunsaturated fatty acid production with *Mortierella alpina* by solid substrate fermentation

Hung-Der Jang, Yuh-Yih Lin and Shang-Shyng Yang[1]

Department of Agricultural Chemistry, National Taiwan University, Taipei 10617, Taiwan

(Received April 16, 1999; Accepted May 28, 1999)

Abstract. Polyunsaturated fatty acids (PUFA) were produced with *Mortierella alpina* by solid substrate fermentation. Rice bran was the most effective substrate for PUFA production, followed by peanut meal residue, wheat bran, and sweet potato residue. The optimal conditions for PUFA production were rice bran supplemented with 2.3 to 5% nitrogen at an initial moisture content of 65 to 68% and a pH range of 6 to 7. Each gram of substrate carbon yielded 122.2 mg of total PUFA, including 12.8 mg of eicosapentaenoic acid (EPA), 47.8 mg of linoleic acid (LA), 7.1 mg of α-linolenic acid (ALA), and 54.5 mg of arachidonic acid (ARA) for 8 to 12 days incubation. C/N ratios between 14.5 and 18.5 favored EPA and LA production, while C/N ratios between 19.8 and 21 enhanced ARA and total PUFA production. Total PUFA, EPA and ARA production increased 12, 84.4 and 46.1%, respectively, when the culture temperature was shifted from 20°C to 12°C on the fifth day. Supplement of soybean and linseed oils increased LA by 84.9 and 36%, ARA by 71 and 42.1%, and EPA by 130.6 and 92.1%, respectively.

Keywords: *Mortierella alpina*; Polyunsaturated fatty acid; Rice bran; Solid substrate fermentation.

Introduction

The ω-3 and ω-6 series of polyunsaturated fatty acids have shown tremendous potential for use in food additives and pharmaceuticals for heart and circulatory disorders and cancer as well as inflammatory diseases (Dyerberg, 1986; Reddy and Maruyama, 1986). The submerged culture of *Mortierella* is usually used for PUFA production with glucose or glycerol as a carbon source (Bajpai et al., 1991; Li and Ward, 1994). However, submerged culture needs a higher energy input and produces more wastewater (Canpel and Moo-Young, 1980; Yang, 1988). In addition, culture media and culture conditions affect the quality and the quantity of PUFA production (Granger et al., 1992; Li and Ward, 1994).

Agricultural wastes—such as rice bran, wheat bran, peanut meal residue, and sweet potato residue—are abundant in Taiwan. Both rice bran and wheat bran are good substrates for enzyme and oil production (Deschamps et al., 1985; Yang and Chiu, 1986; Yang and Wang, 1999). Being economically competitive, rice bran was used as the basal substrate in this report to produce PUFA with *Mortierella alpina* by solid substrate fermentation, and the optimal conditions for PUFA production were also investigated.

[1]Corresponding author. Tel: 886-2-23621519; Fax: 886-2-23679827; E-mail: ssyang@ccms.ntu.edu.tw

Materials and Methods

Microorganism

Mortierella alpina ATCC 32222 was purchased from American Type Culture Collection and used for the production of polyunsaturated fatty acids.

Solid Substrate

Rice bran, wheat bran, peanut meal residue, and sweet potato residue were purchased in a local market. The chemical composition of the test solid substrate is listed in Table 1.

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 and Solid State Fermentation

Submerged basal medium contained (mg l^{-1}) soluble starch, 20; Bacto yeast extract, 5; KNO_3, 10; KH_2PO_4, 1 and $MgSO_4 \cdot 7H_2O$, 0.5 at pH 6.5. The broth was inoculated with 5% (v/v) mycelial suspension and shaken at 200 rev min^{-1} and at 20°C for 2 to 10 days. Each ml of mycelial suspension contained 1.0-1.5 $\times$ 10^6 mycelial fragments.

Table 1. The chemical composition of solid substrates.

Substrate	Moisture content (%)	Organic carbon (%)	Total nitrogen (%)	C/N ratio	PUFA (g/Kg)			
					LA	ALA	ARA	Total
Rice bran	12.4±1.4	43.9±4.5	2.1±0.3	16.4-26.9	12.9±0.2	ND	4.2±0.1	17.0±0.4
Wheat bran	20.0±3.4	54.3±4.2	2.6±0.3	17.3-24.9	0.3±0.0	ND	ND	0.3±0.0
Peanut meal residue	8.9±0.8	20.1±2.1	8.1±1.2	1.9-3.2	5.3±0.2	ND	1.8±0.1	6.8±0.4
Sweet potato residue	10.8±0.9	64.3±5.9	1.7±0.2	30.9-46.8	0.3±0.1	ND	ND	0.3±0.1
Linseed oil	-	-	-	-	160.1±3.8	587.4±26.8	20.0±1.1	751.6±16.1
Soybean oil	-	-	-	-	564.6±30.9	69.5±8.3	ND	713.8±25.6

Mean±SD, n=3. PUFA: polyunsaturated fatty acids; LA: linoleic acid; ALA: α-linolenic acid; ARA: arachidonic acid. γ-linolenic acid did not detected. ND: not detectable; -: non available.

Solid substrate fermentation was carried out in a 500 ml flask. Each flask contained (g) solid substrate (rice bran, wheat bran, peanut meal residue, sweet potato residue, or a mixture of sweet potato residue and rice bran), 100; Bacto yeast extract (Difco, Michigan), 2.5; KNO_3, 5; KH_2PO_4, 0.5 and $MgSO_4 \cdot 7H_2O$, 0.25. Rice bran, wheat bran, peanut meal residue, and sweet potato residue were used as the sole basal solid substrates. In addition, the mixture of sweet potato residue and rice bran, wheat bran or peanut meal residue at 1:1 ratios, respectively, or the mixture of sweet potato residue and rice bran in 1:1 to 1:4 ratios were used as the mixed basal solid substrates. The substrate was mixed thoroughly with 5 ml mycelial suspension and incubated statically at 20°C for 2 to 12 days with mixing once daily by rotating the flask. The depth of substrate in each flask was about 3 cm. The pH of the substrate was measured directly by immersing the electrode into it. All the experiments were performed in triplicates.

Effect of Cultural Conditions on PUFA Production with Rice Bran Solid Substrate

Initial moisture content. The initial moisture content of the solid substrate was adjusted from 55 to 80% (w/w) with deionized water at an initial pH of 6.5.

Initial pH. The initial pH of the solid substrate was adjusted with 1 M NaOH or 1 M HCl from pH 5 to 8 at an initial moisture content of 65%.

Incubation temperature. The substrate was cultivated statically at 20°C for 8 to 10 days. In addition, part of the substrate was cultivated statically at 20°C for 5 days, and then shifted to 12°C for an additional 3 to 5 days incubation.

Supplement of nitrogen. The C/N ratio of the solid substrate was adjusted by adding a mixture of KNO_3 and yeast extract (2:1, w/w) at an initial pH of 6.5 and an initial moisture content of 65%.

Supplement of oil. The substrate was supplemented with 1% of soybean oil and linseed oil (w/w), respectively, and adjusted to an initial pH of 6.5 and an initial moisture content of 65%.

Chemical Analysis

The organic carbon was determined by the Nelson and Sommers method (1982) and the total nitrogen was measured by a modified Kjeldahl method (Yang et al., 1991). The protein content was calculated as 6.25 times the total nitrogen content. The moisture content of the culture was measured by drying a sample at -50°C under vacuum for 8 to 12 h to constant mass.

The lipids were extracted with a 5 times volume of chloroform/methanol (2:1, v/v) by an ultrasonicator for 2 h and concentrated by rotary evaporator at 50°C. The residue was dissolved in 1 ml of 0.5 M KOH-methanol solution, and methylated with 1 ml of 20% (w/v) of BF_3-methanol complex. The methylated fatty acids were separated from the water layer by adding saturated NaCl and anhydrous Na_2SO_4, and then dissolving in n-hexane.

PUFA content was determined by gas chromatography (Shimadzu Co., Japan) with a glass column (0.26 mm i.d. × 2 m) packed with 10% diacylglycol succinate (60/80 mesh) and with a flame ionization detector. 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 pentadecanoate was added as an internal standard.

The 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)

Statistical Analysis

Treatments were in triplicates, 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, 1988).

Results

Solid Substrate

The production of PUFA by *M. alpina* using different solid substrate is listed in Table 2. In the case of the sole solid substrates, rice bran was the most effective with the highest yields of linoleic acid (LA), arachidonic acid (ARA), eicosapentaenoic acid (EPA) and total PUFA. The second most effective was peanut meal residue, followed by wheat bran, and sweet potato residue. For the mixed basal substrates, the mixture of rice bran and sweet potato residue was the best. Both total and individual PUFA content increased with the increasing percentage of rice bran in the mixture of sweet potato residue and rice bran. Each gram of substrate carbon supported the production of 103.5 mg of total PUFA when rice bran was used as the sole basal substrate. The yields of PUFA showed significant differences ($p < 0.01$) among these individual basal substrates or the mixed basal substrates.

Time Course of PUFA Secretion

Submerged fermentation. The biomass had a maximal value after 6 days incubation with glucose as the carbon source. The pH of culture broth increased at first, then decreased gradually (Figure 1). PUFA was the primary metabolite, and the production was associated with the growth of the test organism. Each gram of substrate carbon yielded 120 mg of total PUFA, including 90.8 mg of ARA, 12.5 mg of γ-linolenic acid (GLA), and 16.7 mg of LA after 6 days incubation. On a volume basis, each litre of culture broth supported the production of 1021.8 mg of total PUFA, including 826.3 mg of ARA, 96.3 mg of GLA and 99.2 mg of LA.

Solid substrate fermentation. The time course of PUFA secretion in rice bran solid substrate is presented in Figure 2. The moisture content of solid substrate increased from 0.5 to 5.5% for 10 days incubation, while the pH decreased slightly during the first two days, then increased gradually for the remainder of the experiment. The cell protein of substrate increased significantly for the first 6 days, then reached the steady state at the 10th day. Each gram of substrate carbon yielded 122.2 mg of total PUFA, including 47.8 mg of LA, 7.1 mg of ALA, 12.8 mg of EPA, and 54.5 mg of ARA for 10 days incubation. Productivity of each gram of substrate carbon for EPA, LA and ALA were higher in solid substrate fermentation than that in submerged fermentation except ARA and GLA production.

Initial Moisture Content

Moisture content increased during cultivation by the formation of metabolic water of the test organism. However, the pH of solid substrate decreased with increasing initial moisture content from 55 to 75% (Table 3). The optimal initial moisture content of solid substrate for the production of total PUFA, LA, and ARA was about 75% while the highest value for the production of ALA and EPA was an initial moisture content of 65%. Each gram of

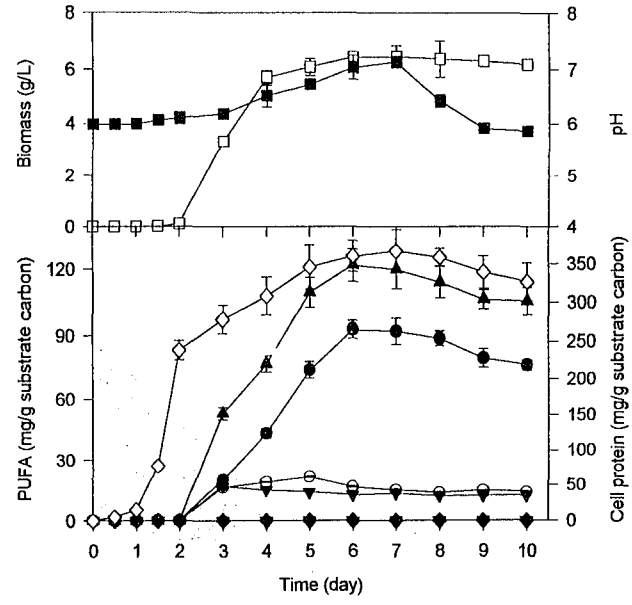

Figure 1. Time course of biomass, pH, cell protein, and PUFA production in submerged fermentation with *Mortierella alpina* ATCC 3222. Culture medium was incubated at 20°C with orbital shaking at 200 rev min^{-1}. □, Biomass; ■, pH; ▲, Total PUFA; ○, Linolenic acid; ▼, γ-Linolenic acid; ▽, α-Linolenic acid; ●, Arachidonic acid; ♦, Eicosapentaenoic acid; ◇, Cell protein.

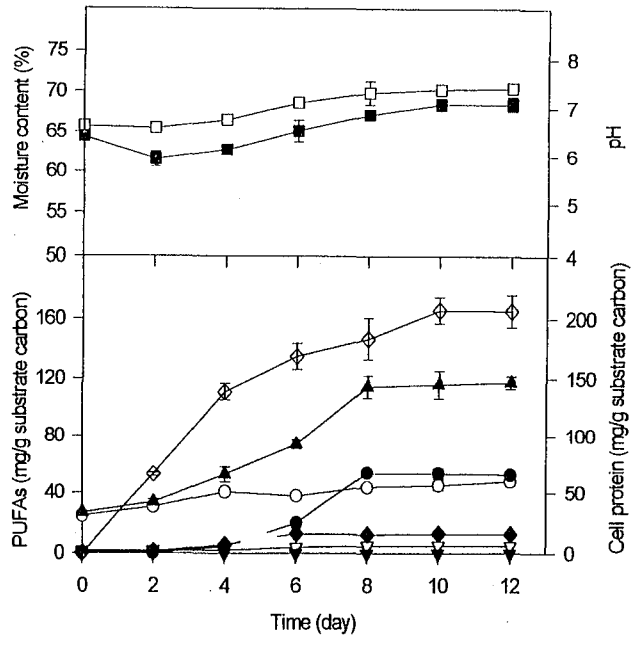

Figure 2. Time course of moisture content, pH, cell protein, and PUFA production in rice bran solid substrate fermentation with *Mortierella alpina* ATCC 3222. Solid substrate with an initial moisture content of 65% and an initial pH of 6.5 were statically incubated at 20°C. □, Final moisture content; ■, pH; ▲, Total PUFA; ○, Linolenic acid; ▼, γ-Linolenic acid; ▽, α-Linolenic acid; ●, Arachidonic acid; ◆, Eicosapentaenoic acid; ◇, Cell protein.

substrate carbon yielded 128 mg of total PUFA, including 59.8 mg of ARA, 54.7 mg of LA, 1.8 mg of ALA, and 11.7 mg of EPA at an initial moisture content of 75% of solid substrate.

Initial pH

The optimum initial pH of solid substrate for PUFA production was between 6 and 7. The yield decreased when the initial pH was higher than 8 or lower than 5 (Table 4). Each gram of substrate carbon supported the production of 123 mg of total PUFA, including 53.5 mg of ARA, 47.6 mg of LA, 18.2 mg of EPA and 3.7 mg of ALA at an initial pH of 6 to 7. The productions were 40.1 mg of total PUFA, 17.8 mg of ARA, 12.4 mg of LA, 1.2 mg of ALA, and 8.7 mg of EPA at an initial pH 9. During the cultivation, the substrate pHs of all tests were between 5.2 and 7.6.

Supplement of Nitrogen Source

Effect of supplementation of the mixture of KNO_3 and yeast extract (2:1, w/w) on PUFA production is presented in Table 5. The kind of individual PUFA produced depended on the amount of nitrogen supplement. Supplementation with 3.75 to 7.5% of a nitrogen source was good for the production of the ω-3 series of PUFA, especially EPA. The concentrations of the carbon and nitrogen sources of the substrate were very important in the quality and quantity of lipid production by *Mortierella*. Substrate with C/N ratios between 14.5 and 18.5 favored EPA and LA production, while C/N ratios between 19.8 and 21 stimulated ARA and total PUFA production.

Oil Supplement

The effect of oil supplement on the yield of PUFA is shown in Table 6. Supplement of 1% of soybean oil and 1% of linseed oil enhanced the yields of total PUFA, LA, ARA and EPA. Each gram of substrate carbon yielded 178.9 mg of total PUFA, including 90.1 mg of LA, 57 mg of ARA, 2.3 mg of ALA, and 29.5 mg of EPA with soybean oil supplement. With linseed oil supplement, each gram of substrate carbon supported the production of 147 mg of total PUFA, including 66.3 mg of LA, 47.4 mg of ARA, 8.8 mg of ALA, and 24.5 mg of EPA. Obviously, the fatty acids in the supplemented oil may act as precursors of PUFA and thus stimulate PUFA production. The supplement of soybean and linseed oils could increase the production of LA by 84.9 and 36%, of ARA by 71 and 42.1%, and of EPA by 130.6 and 92.1%, respectively.

Table 2. Effect of basal substrate on PUFA production.

Basal substrate*	C/N ratio	Final moisture content (%)	Final pH	PUFA (mg/g substrate carbon)**					
				LA	GLA	ALA	ARA	EPA	Total
Peanut meal residue (P)	2.2	65.88±0.02^{d,e,f}	6.37±0.18^{a,b}	29.32±0.21^{c}	ND	4.62±0.47^{b}	8.06±0.48^{c}	5.88±0.78^{b}	42.00±4.19^{c}
Rice bran (R)	14.5	71.31±0.16^{b}	5.93±0.02^{c}	48.72±3.69^{a}	ND	8.70±0.48^{a}	33.35±2.13^{a}	12.77±1.61^{a}	103.54±5.28^{a}
Wheat bran (W)	19.3	66.81±0.62^{d}	6.41±0.13^{a}	1.18±0.02^{g}	ND	ND	0.15±0.03^{e}	0.11±0.03^{e}	1.44±0.14^{f}
Sweet potato residue (S)	24.5	65.70±0.57^{e,f}	6.48±0.22^{a}	1.14±0.28^{f}	ND	ND	0.11±0.06^{e}	0.07±0.02^{e}	1.32±0.07^{f}
S/P (1:1, w/w)	13.4	65.75±0.49^{e,f}	6.37±0.25^{a,b}	15.61±0.31^{e}	ND	ND	3.32±0.25^{d}	2.57±0.32^{c,d}	21.50±1.03^{e}
S/W (1:1, w/w)	21.9	66.05±0.35^{d,e}	6.22±0.40^{a,b,c}	0.42±0.01^{h}	ND	ND	0.06±0.01^{f}	0.04±0.02^{e}	0.52±0.04^{g}
S/R (1:1, w/w)	19.5	68.66±0.43^{c}	6.37±0.11^{a,b}	24.07±1.22^{d}	2.39±0.19^{c}	ND	22.72±1.76^{b}	3.53±0.09^{d}	52.71±2.28^{d}
S/R (1:2, w/w)	17.8	69.04±0.28^{c}	6.36±0.18^{a,b}	28.84±2.94^{b}	3.66±0.22^{b}	ND	26.61±1.19^{b}	4.71±0.21^{c}	63.82±7.72^{c}
S/R (1:3, w/w)	17.0	73.06±0.25^{a}	6.23±0.05^{b}	28.03±2.20^{b}	1.36±0.25^{b}	0.80±0.14^{d}	28.40±1.69^{b}	7.00±0.64^{b}	65.59±5.72c
S/R (1:4, w/w)	16.5	72.02±0.58^{b}	6.16±0.12^{b}	33.82±3.41^{a,b}	4.51±0.39^{a}	1.93±0.22^{c}	35.87±2.24^{a}	8.66±0.48^{c,d}	84.79±8.91^{b}

*Solid substrates with an initial moisture content of 65% and an initial pH of 6.5 were statically incubated at 20°C for 8 day. Mean±SD, n=3. Means in the same column that do not share the same alphabetic superscript show significant difference at 5% level according to Duncan's multiple range test.

**ND: not detectable; PUFA: polyunsaturated fatty acid; LA: linoleic acid; GLA: γ-linolenic acid; ALA: α-linolenic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid.

Table 3. Effect of initial moisture content on PUFA production.

Initial moisture content (%)	Final moisture content (%)	Final pH	PUFA (mg/g substrate carbon)					
			LA	GLA	ALA	ARA	EPA	Total
55	57.94±1.03^{c}	6.43±0.08^{a}	42.29±0.94^{d}	ND	1.09±0.06^{b}	46.27±1.01^{d}	10.10±0.36^{c}	99.75±0.29^{c}
60	61.73±1.12^{d}	6.05±0.39^{a}	43.95±0.34^{d}	ND	1.94±0.11^{a}	46.93±0.55^{c,d}	11.36±0.49^{b,c}	104.18±1.43^{d}
65	65.69±2.23^{c}	5.89±0.52^{a}	47.48±0.46^{c}	ND	2.19±0.23^{a}	47.33±0.10^{c}	13.09±0.10^{a}	110.09±1.96^{c}
70	71.71±2.44^{b}	5.90±0.44^{a}	49.90±1.42^{b}	ND	1.89±0.08^{a}	53.44±0.46^{b}	12.84±0.22^{a,b}	118.07±1.99^{b}
75	76.39±1.01^{a}	5.68±0.65^{a}	54.70±2.66^{a}	ND	1.8±0.18^{a}	59.80±0.26^{a}	11.70±1.06^{a,b,c}	128.00±2.08^{a}

Rice bran was used as the solid substrate. The solid substrate with an initial pH 6.5 was statically incubated at 20°C for 8 days. Mean±SD, n=3. Means in the same column that do not share the same alphabetic superscript are significantly different at 5% level according to Duncan's multiple range test. ND: undetectable. Same abbreviations as described in Table 2.

Table 4. Effect of initial pH on PUFA production.

Initial PH	Final moisture content (%)	Final pH	PUFA (mg/g substrate carbon)					
			LA	GLA	ALA	ARA	EPA	Total
4	67.14±1.31^{c}	5.03±0.07^{a}	11.32±0.74^{c}	ND	1.01±0.05^{b}	15.10±0.81^{c}	7.50±0.26^{c}	34.93±0.12^{c}
5	68.03±0.29^{b}	5.20±0.14^{b}	33.24±0.48^{b}	ND	2.85±0.12^{a}	53.47±0.10^{a}	15.43±0.64^{b}	104.99±0.94^{b}
6	68.21±0.44^{b}	5.68±0.32^{b}	47.08±1.96^{a}	ND	3.50±0.22^{a}	54.07±0.26^{a}	16.29±0.96^{a,b}	120.94±6.08^{a,b}
7	69.21±0.17^{a}	6.71±0.49^{a}	47.60±2.20^{a}	ND	3.70±0.30^{a}	53.50±0.50^{a}	18.20±0.94^{a}	123.00±1.66^{a}
8	67.93±0.33^{b}	7.60±0.59^{a}	42.18±1.32^{a}	ND	2.85±0.36^{a}	45.14±0.98^{b}	16.43±0.64^{a,b}	106.60±1.00^{b}
9	67.51±0.23^{b}	7.60±0.55^{a}	12.40±0.72^{c}	ND	1.20±0.26^{b}	17.80±0.58^{c}	8.70±0.42^{c}	40.10±0.90^{c}

Rice bran was used as the solid substrate. The solid substrate with an initial moisture content of 65% was statically incubated at 20°C for 8 days. Mean±SD, n=3. Means in the same column that do not share the same alphabetic superscript are significantly different at 5% level according to Duncan's multiple range test. ND: undetectable. Same abbreviations as described in Table 2.

Table 5. Effect of nitrogen supplement on PUFA production.

Concentration (%)	C/N ratio	Final pH	Final moisture content (%)	PUFA (mg/g substrate carbon)				
				LA	ALA	ARA	EPA	Total
0	21.0	6.77±0.14^{a}	69.62±0.49^{a}	48.72±3.69^{a}	8.70±0.48^{a}	33.35±2.13^{d}	12.77±1.61^{c}	103.54±5.28^{a,b}
1.0	19.8	6.55±0.23^{a,b}	67.04±0.23^{c}	32.83±1.48^{c,d}	6.33±0.25^{d}	51.62±3.80^{a}	14.16±0.54^{b}	104.94±5.52^{a,b}
2.25	18.5	6.61±0.12^{a}	67.69±0.41^{b,c}	39.01±1.53^{b}	7.60±0.33^{b}	46.79±2.74^{b}	14.81±0.83^{a,b}	108.21±2.43^{a}
2.5	18.3	6.76±0.13^{a}	68.25±0.29^{b}	43.81±2.99^{a,b}	7.81±0.68^{a,b}	45.55±5.38^{a,b}	14.48±1.07^{a,b}	111.65±7.41^{a}
3.75	17.2	6.06±0.44^{b,c}	67.18±0.19^{c}	39.43±2.69^{a,b}	7.27±0.38^{b,c}	39.60±4.94^{c}	15.94±0.87^{a}	102.24±7.26^{a,b}
5.0	16.2	5.92±0.08^{c}	65.79±0.38^{d}	37.92±1.57^{b,c}	7.43±0.29^{b,c}	26.25±3.18^{e}	15.71±1.49^{a}	87.31±10.19^{b,c}
7.5	14.5	6.07±0.32^{b,c}	65.12±0.41^{d}	36.94±1.29^{b,c}	7.01±0.26^{c}	18.28±2.62^{e}	16.88±1.25^{a}	79.11±4.55^{c}
10	13.1	5.88±0.06^{c}	62.12±1.09^{f}	32.90±0.69^{d}	8.43±0.47^{a}	16.14±0.84^{e}	15.25±0.93^{a,b}	72.72±2.89^{d}
15	11.1	5.85±0.05^{c}	63.82±0.46^{e}	28.64±0.65^{e}	7.06±0.28^{c}	11.18±0.66^{f}	12.71±1.22^{c}	60.59±4.03^{e}

Rice bran was used as the basal substrate. The solid substrates with an initial moisture content of 65% and an initial pH of 6.5 were statically incubated at 20°C for 8 days. The mixture of KNO_3 and yeast extract at 2:1 (w/w) ratio was used as the combined nitrogen sources and various amount of nitrogen (w/w) was supplemented to the basal substrate. Mean±SD, n=4. Means in the same column that do not share the same alphabetic superscript show significant difference at 5% level according to Duncan's multiple range test. ND: not detectable. Same abbreviations as described in Table 2.

Table 6. Effect of oil supplement on PUFA production.

Oil	Final moisture content (%)	Final pH	PUFA (mg/g substrate carbon)				
			LA	ALA	ARA	EPA	Total
Control	67.55±0.44^{b}	5.98±0.06^{c}	48.72±3.69^{c}	8.70±0.48^{a}	33.35±2.13^{c}	12.77±1.61^{b}	103.54±5.28^{c}
Linseed oil	68.24±0.12^{a}	6.31±0.22^{a}	66.27±13.12^{b}	8.78±2.09^{a}	47.38±4.39^{b}	24.53±2.04^{a}	146.96±11.77^{b}
Soybean oil	68.82±0.09^{a}	6.25±0.11^{a}	90.08±17.29^{a}	2.31±0.74^{b}	57.02±5.49^{a}	29.45±3.22^{a}	178.86±8.95^{a}

Rice bran was used as the basal substrate. The solid substrates with an initial moisture content of 65% and an initial pH of 6.5 were statically incubated at 20°C for 8 days. Mean±SD, n=4. Means in the same column that do not share the same alphabetic superscript show significant difference at 5% level according to Duncan's multiple range test. ND: not detectable. Same abbreviations as described in Table 2.

Table 7. Effect of incubation temperature on PUFA production.

Culture condition	Final moisture content (%)	Final pH	PUFA (mg/g substrate carbon)					Degree of unsaturation
			LA	ALA	ARA	EPA	Total	
20°C, 8 d	65.22±0.26^{c}	6.62±0.14^{b}	48.72±3.69^{a}	8.70±0.48^{a}	33.35±2.13^{b}	12.77±1.61^{c}	103.54±5.28^{c}	3.28±0.04^{b}
20°C, 10 d	68.30±0.19^{a}	6.97±0.14^{a}	46.01±2.59^{a,b}	2.11±0.13^{d}	49.05±1.18^{a}	17.73±0.39^{b}	114.90±6.61^{b}	3.34±0.04^{b}
20°C 5 d and then 12°C 3 d	66.82±0.27^{b}	6.63±0.22^{b}	40.66±1.57^{b,c}	3.01±0.35^{c}	48.73±2.44^{a}	23.55±0.69^{b}	115.95±8.96^{b}	3.48±0.02^{a,b}
20°C 5 d and then 12°C 5 d	68.49±0.15^{a}	6.89±0.04^{a}	43.37±4.59^{b}	5.72±0.74^{b}	49.78±1.56^{a}	26.93±0.23^{a}	125.80±4.27^{a}	3.48±0.03^{a}

Rice bran was used as the solid substrate. The solid substrate with an initial moisture content of 65% and an initial pH of 6.5 were statically incubated for 8 days. Mean±SD, n=3. Means in the same column that do not share the same alphabetic superscript show significant difference at 5% level according to Duncan's multiple range test. ND: not detectable. Same abbreviations as described in Table 2.

Incubation Temperature

Effect of incubation temperature on PUFA production is listed in Table 7. The incubation temperature, shifting from 20 to 12°C on the fifth day, enhanced the productivity of the total PUFA, EPA and ARA by 11.3, 41.1 and 15.4%, respectively. Prolonging the incubation time to 5 days at low temperature increased the yields of total PUFA, individual PUFA, and the degree of lipid unsaturation.

Discussion

Although the C/N ratios of rice bran and wheat bran were suitable for microbial growth, rice bran, with a high PUFA content, enhanced PUFA production while wheat bran, with a low PUFA content, did not. Overly high or low C/N ratios did not favor PUFA production (Sajbidor et al., 1990). Rice bran was the best solid substrate for PUFA production. Similar phenomena were also observed in eicosapentaenoic acid production with *Mortierella alpina* (Jareokitmongkol et al., 1993), protease production with *Aspergillus niger* (Yang and Chiu, 1986), and oxytetracycline production with *Streptomyces rimosus* (Yang and Swei, 1996).

PUFA production was associated with microbial growth. PUFA production in submerged fermentation decreased gradually in prolonged cultivation due to cell lysis (Yamada et al., 1987; Bajpai et al., 1991). On the other hand, PUFA production in solid substrate fermentation was stable for a 12-day incubation period because of the steady microbial growth and because the product could be stored temporarily without losing significant yield. Similar results were also found in enzyme and antibiotic production in both submerged and solid substrate fermentation (Yang and Huang, 1994; Wang and Yang, 1995; Yang and Swei, 1996; Yang and Wang, 1996, 1999).

Water, especially the initial moisture content of the substrate, was very important in solid substrate fermentation. During cultivation, substrate moisture content increased because of the production of metabolic water by *Mortierella.* High moisture (70 to 75%) favored the production of the ω-6 series of PUFA, while moderate moisture (60 to 65%) stimulated the production of the ω-3 series. Vandamme et al. (1981) reported that the substrate in an aerobic state enhanced the cell growth and gramicidin production of *Bacillus brevis.* Yang and Yuan (1990) and Yang and Swei (1996) also showed that an appropriate initial moisture content (about 65%) stimulated the antibiotic production of *Streptomyces*. Overly high or low moisture content was not good for metabolite production. Individual PUFA production might be adjusted by manipulating the initial moisture content of the solid substrate.

Matsushima et al. (1981) indicated that the pH drop at first might be due to the accumulation of organic acid. Slightly acidic to neutral pH lead to the maximum yield of PUFA with *Mortierella* and algal cultivation (Bajpai et al., 1991; Yongmanitchai and Ward, 1991). Supplementation with 3.75 to 7.5% of a nitrogen source stimulated the ω-3 series of PUFA production, especial EPA. Ben-Amotz et al. (1985) also reported that a high concentration of nitrogen source enhanced EPA production, and similar phenomena were also found in PUFA production with *Scenedesmus* and *Chlorella* (Weete, 1980). Soybean oil and linseed oil were rich in oleic acid, LA and ALA. Supplement of soybean oil and linseed oil stimulated the yields of total PUFA and individual PUFA, which might be due to the rich LA and ALA content of oleic acid and to the use by *Mortierella* of these precursors for PUFA production. Antibiotic production of *Streptomyces rimosus* was also promoted with the supplement of soybean oil and lard oil (Yang and Yuan, 1990; Yang and Swei, 1996). Therefore, individual PUFA production might also be controlled with the C/N ratio of the solid substrate.

Low incubation temperature favored the production of PUFA and increased the degree of unsaturation of PUFA in *Mortiella.* A similar phenomenon was also found in the growth of *Catharanthus roseus* at low temperature (Toivonen et al., 1992). A high degree of unsaturation of fatty acids in cell membranes maintained the membrane function and the cell growth of *Caulerpa prolifera* despite low temperatures (Terrados and Lopez-Jimenez, 1996). Unsaturated fatty acid was synthesized by the organism at low incubation temperature. High temperature was used for mycelial growth, while low temperature was adjusted for PUFA production in solid substrate cultivation.

The productivity of each gram of substrate carbon was higher in solid substrate fermentation than in submerged fermentation. Enzyme and antibiotic productions were 60 to 80% higher in solid substrate fermentation than in submerged fermentation (Yang and Yuan, 1990; Yang and Huang, 1994). In addition, culture in solid substrate fermentation favored production of the ω-3 series of PUFA, and the product could be dried and consumed directly without further extraction treatment. Therefore, production of polyunsaturated fatty acids in solid substrate fermentation as food or feed supplement might be a feasible process in agricultural waste treatment and utilization. Individual PUFA production could be adjusted with an appropriate moisture content and C/N ratio of the solid substrate.

Literature Cited

Bajpai, P.K., P. Bajpai, and O.P. Ward. 1991. Production of arachidonic acid by *Mortierella alpina* ATCC 32222. J. Indust. Microbiol. **8:** 179-186.

Ben-Amotz, A., T.G. Tornabene, and W.H. Thomas. 1985. Chemical profile of selected species of microalgae with emphasis on lipids. J. Phycol. **21:** 72-81.

Canpel, E. and M. Moo-Young. 1980. Solid state fermentation systems. Proc. Biochem. **June/July:** 2-7.

Chen, H.C. and C.C. Chang. 1994. Isolation of microbes for polyunsaturated fatty acid production. J. Chin. Agric. Chem. Soc. **32:** 33-46.

Deschamps, F., C. Giuliano, M. Asther, M.C. Huet, and S. Roussos. 1985. Cellulase production by *Trichoderma harzianum* in static and mixed solid-state fermentation reactors under nonaseptic conditions. Biotech. Bioeng. **27:**

1385-1388.

Dyerberg, J. 1986. Linolenate-derived polyunsaturated fatty acids and prevention of atherosclerosis. Nutr. Rev. **44:** 124-134.

Granger, L.M., P. Perlot, G. Goma, and A. Pareilleux. 1992. Kinetics of growth and fatty acid production of *Rhodotorula glutinis*. Appl. Microbiol. Biotech. **37:** 13-17.

Jareokitmongkol, S., S. Shimizu, and H. Yamada. 1993. Production of an eicosapentaenoic acid-containing oil by a Δ12 desaturase-defective mutant of *Mortierella alpina* 1S-4. J. Amer. Oil Chem. Soc. **70:** 119-123.

Li, Z. and O.P. Ward. 1994. Production of docosahexaenoic acid by *Thaustochytrium roseum*. J. Indust. Microbiol. **13:** 238-241.

Matsushima, K., M. Hayakawa, M. Ito, and K. Stimada. 1981. Features of the proteolytic enzyme system of hyper-acid productive and non-acid-productive fungi. J. Gen. Appl. Microbiol. **27:** 423-426.

Nelson, D.W. and L.E. Sommers. 1982. Total carbon, organic carbon and organic matter. *In* A. L. Page (ed.), Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties. The American Society of Agronomy, Wisconsin, pp. 539-580.

Reddy, B.S. and H. Maruyama. 1986. Effect of diatary fish oil on aroxymethane induces colon carcinogenesis in male F344 rats. Cancer Res. **46:** 3367-3370.

Sajbidor, J., S. Dobronova, and M. Certik. 1990. Arachidonic acid production by *Mortierella* sp. S-17: influence of C/N ratio. Biotech. Lett. **12:** 455-456.

SAS Institute. 1988. SAS/STAT User's Guide, Release 6.03 SAS Institute, Cary, North Carolina.

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

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

Vandamme, E.J., D. Leyman, P. De Visscher, D. De Buyser, and G. Vansteenkiste. 1981. Effect of aeration and pH on gramicidin S production by *Bacillus brevis*. J. Chem. Technol. Biotech. **31:** 247-257.

Wang, J.Y. and S.S. Yang. 1995. Morphogensis, biomass and oxytetracycline production of *Streptomyces rimosus* in submerged cultivation. Chin. J. Microbiol. Immunol. **28:** 21-31.

Weete, J.D. 1980. Lipid Biochemistry of Fungi and Other Organisms. Plenum Press, New York, pp 9-48.

Yamada, H., S. Shimizu, and Y. Shinmen. 1987. Production of arachidonic acid by *Mortierella elongata* 1S-5. Agric. Biol. Chem. **51:** 785-790.

Yang, S.S. 1988. Protein enrichment of sweet potato residue with amylolytic yeasts by solid-state fermentation. Biotech. Bioeng. **32:** 886-890.

Yang, S.S. and C.I. Huang. 1994. Protesae production by amylolytic fungi in solid state fermentation. Chin. J. Agric. Chem. Soc. **32:** 589-601.

Yang, S.S. and J.Y. Wang. 1996. Morphogenesis, ATP content and oxytetracycline production by *Streptomyces rimosus* in solid substrate cultivation. J. Appl. Bacteriol. **80:** 545-550.

Yang, S.S. and J.Y. Wang. 1999. Protease and amylase production of *Streptomyces rimosus* in submerged and solid state cultivations. Bot. Bull. Acad. Sin. **40:** 259-265.

Yang, S.S. and S.S. Yuan. 1990. Oxytetracycline production with sweet potato residue by solid state fermentation. World J. Microbiol. Biotech. **6:** 236-244.

Yang, S.S. and W.F. Chiu. 1986. Protease production with sweet potato residue by solid state fermentation. Chin. J. Microbiol. Immunol. **19:** 276-288.

Yang, S.S. and W.J. Swei. 1996. Oxytetracycline production by *Streptomyces rimosus* in solid-state fermentation of corncob. World J. Microbiol. Biotech. **12:** 43-46.

Yang, S.S., H.L. Chang, C.B. Wei, and H.C. Lin. 1991. Reduce waste production in the Kjeldahl methods. J. Biomass Energy Soc. China **10:** 147-155.

Yongmanitchai, W. and O.P. Ward. 1991. Growth and omega-3 fatty acid production by *Phaeodactylum tricornutum* under different culture conditions. Appl. Environ. Microbiol. **57:** 419-425.

以 *Mortievella alpina* 利用固態發酵生產多元不飽和脂肪酸

詹鴻得　林裕益　楊盛行

國立台灣大學農業化學系

以 *Mortievella alpina* 利用固態發酵可以生產多元不飽和脂肪酸，農業廢棄物中，米糠是最佳基質，而後依次為花生粕、麥麩和甘藷渣。固態發酵生產多元不飽和脂肪酸之最適條件為以米糠為基質，添加 2.3 至 5.0% 氮源在初水分含量 65~68% 和初 pH 6~7。培養 8~12 天，每克基質碳源可得 122.2 mg 總多元不飽和脂肪酸，包括 12.8 mg EPA，47.8 mg LA，7.1 mg ALA 和 54.5 mg ARA。基質 C/N 比介於 14.5 至 18.5 有利 EPA 和 LA 生產，而 C/N 比介於 19.8 至 21 則有利 ARA 和總多元不飽和脂肪酸、EPA 和 ARA 產量分別提高 12.2，84.4 和 46.1%。如於基質中額外添加 1% 大豆油或棉籽油則可分別提高 LA 產量 84.9 和 36，ARA 產量 71 和 42.1% 與 EPA 產量 130.6 和 92.1%。

關鍵詞：多元不飽和脂肪酸；米糠；固態發酵；*Mortierella alpina*。