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Docosapentaenoic acid (C22:5, ω-3) production by *Pythium acanthicum*

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The physiological roles of omega-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid have been investigated in detail and microbial strains producing these polyunsaturated fatty acids have been characterised. It has recently been suggested that docosapentaenoic acid may have an important role, especially in infant nutrition, and that its positive health effects have been overlooked. Docosapentaenoic acid (C22:5, ω-3) production by a strain of *Pythium acanthicum* ATCC 18660 was thus investigated. Optimum conditions for growth of *P. acanthicum* ATCC 18660 and docosapentaenoic acid production were: pH 6.0, temperature 20°C and incubation time, 10 days. Among different saccharides and complex nitrogen sources tested, glucose and sodium glutamate were preferred carbon and nitrogen sources, respectively. Maximum biomass content (10.4 g L^{-1}) and docosapentaenoic acid yield (49.9 mg L^{-1}) were obtained in 10 days. An increase in docosapentaenoic acid volumetric yields to 108–110 mg L^{-1} was obtained when linseed oil was used to supplement glucose or soy flour-containing medium. Batch feeding of additional glucose or linseed oil further enhanced the docosapentaenoic acid volumetric yield to 132 mg L^{-1} and 125 mg L^{-1}, respectively, in 14 days. The specific production of docosapentaenoic acid in preliminary experiments ranged from 1.0–5.0 mg g^{-1} biomass. As conditions were optimised, docosapentaenoic acid specific production titers were generally in the range of 4.0–5.5 mg g^{-1} and increases in docosapentaenoic acid volumetric production could be attributed to increased biomass production. The limited improvement obtained by modifying culture conditions indicates that increasing volumetric yields of docosapentaenoic acid by modifying culture conditions appears to represent a significant barrier to commercialisation of such a process and suggests a more fundamental manipulation of metabolism and physiology is required.

Keywords: *Pythium acanthicum*; lipids; polyunsaturated fatty acids; docosapentaenoic acid

Introduction

Polyunsaturated fatty acids (PUFAs) of dietary origin (ω-6 and ω-3) cannot be synthesized by mammals and, therefore, are essential because they are required for the optimal biologic function of specialized cells and tissues. Nutritional studies have shown the potential benefits of including long-chain PUFAs in the diet [5,9,10]. PUFAs of the omega-3 family, ie. eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), have been shown to have positive effects in the prevention of atherosclerosis, treatment of different cardiovascular disorders, and reduction of incidence of thrombosis and rheumatoid arthritis [10,11]. Results of several studies have suggested an inverse association between DPA and coronary heart diseases such as myocardial infarction and coronary artery disease [14,17].

Docosapentaenoic acid (DPA, C22:5), a precursor of docosahexaenoic acid (DHA, C22:6), is synthesized from eicosapentaenoic acid (EPA, C20:5) by a desaturation and elongation mechanism and shares a common biosynthetic pathway with omega-3 fatty acid precursors of ecosanoid compounds [13,28]. The retroconversion of DPA and DHA to EPA has also been demonstrated [21]. The commonly accepted pathway for DPA and DHA synthesis is: 18:3α (ω-3)→18:4 (ω-3)→20:4 (ω-3)→20:5 (ω-3)→22:5 (ω-3)→22:6 (ω-3). The metabolism of 18:3α requires the sequential use of 6-, 5- and 4-desaturases, and the last step in the biosynthetic pathway is catalyzed by an acyl-CoA-dependent 4-desaturase. However, it has recently been demonstrated that 22:5 is the precursor of 22:6 but via a pathway that is independent of a 4-desaturase [28]. This pathway involves the chain elongation of 22:5 to 24:5, followed by its desaturation to 24:6 and then, via β-oxidation, to 22:6.

At the recent International Congress on Essential Fatty Acids and Eicosanoids, Edinburgh, Scotland, Ackman and Gibson emphasised that docosapentaenoic acid had been overlooked as a potentially beneficial omega-3 fatty acid [1]. The positive health effects of eicosapentaenoic and docosahexanoic acids have been investigated in detail. However, DPA is present in significant amounts in seal oil, a major component of the Eskimo diet. The high omega-3 content of the Eskimo diet is thought to contribute to their low rates of heart disease. In addition, DPA is present in human breast milk, like DHA, and, like DHA, it is incorporated into brain tissue. DPA may have an important role in infant nutrition [1].

Although the current principal commercial source of ω-3 fatty acids is fish oil, alternative sources are being sought among phytoplankton, algae, fungi and bacteria [6,16,31,32]. Several fungal species within the class Phycomycetes, a class of lower fungi, seem to be promising sources of long-chain PUFAs [27,31]. We have previously described cultural conditions for the microbial production

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of high yields of long chain PUFAs such as DHA [2,18,24,25], EPA [4,32,33] and AA [3,19]. Production of PUFAs by lower fungi and some heterotrophic microalgae is typically in the form of triglycerides within the cell [19,20]. A considerable amount of work has been done on the production of AA, EPA and DHA. However, no systematic study has been carried out so far with regard to the microbial production of DPA. In this paper we have screened a range of ω-3 fatty acid-producing lower fungi for DPA production and investigated factors affecting DPA production by *Pythium acanthicum*.

Methods

Chemicals

Methyl esters of standard fatty acids were purchased from Sigma (St Louis, MO, USA). Sugars and media components were obtained from Difco (Detroit, MI, USA) or BDH (Toronto, ON, Canada). Cereal flours and vegetable oils were purchased from Sigma. Solvents, obtained from Fisher Scientific (Fair Lawn, NJ, USA) or EM Science (Gibbstown, NJ, USA), were either analytical or HPLC grade.

Microorganisms

Pythium acanthicum ATCC 18660 and the other strains from the genera *Pythium*, *Thraustochytrium*, *Schizochytrium*, *Mortierella*, *Entomophthora*, *Conidiobolus* and *Saprolegnia* were obtained from the American Type Culture Collection (Rockville, IL, USA). Cultures were maintained on 3% agar slants containing medium components and subcultured every 2 months.

Culture conditions

For all the cultures except marine fungi and species of *Mortierella*, basal medium contained (per liter): glucose, 20 g; sodium glutamate, 2.0 g; $(NH_4)_2SO_4$, 0.2 g; KH_2PO_4, 0.2 g; $MgSO_4$, 1.0 g; $CaCl_2$, 0.1 g; and yeast extract, 2.0 g. The pH of the medium was adjusted to 6.0 before sterilization. For marine fungi belonging to genera *Thraustochytrium* and *Schizochytrium*, the above medium contained additional NaCl (25 g L^{-1}) and KCl (1 g L^{-1}) and $MgSO_4$ (5 g L^{-1}). The basal medium for the species of *Mortierella* contained (per liter): glucose, 20 g; yeast extract, 5 g; $NaNO_3$, 3 g; KH_2PO_4, 1 g; $MgSO_4$, 0.5 g; KCl, 0.5 g; and $FeCl_3$, 1.45 mg. The pH of the medium was adjusted to 6.0 before sterilization. The glucose solution was autoclaved separately. The effect of other nutrients on growth and lipid production of *P. acanthicum* ATCC 18660 was studied by growing the culture in the basal medium in which glucose and sodium glutamate were replaced by other carbon or nitrogen sources, respectively.

An inoculum was prepared in a 250-ml Erlenmeyer flask containing 50 ml medium. Cultures were grown at 20°C for 48 h with orbital shaking at 200 rpm. Erlenmeyer flasks (250 ml) containing 50 ml of production medium (pH 6.0) were inoculated with this 48-h inoculum at a rate of 5% v/v and incubated on an orbital shaker (New Brunswick Scientific, NJ, USA) at 200 rpm.

The values given for each experiment are the means of two to three treatment replications and the variation between the replicates was less than 5%. Selected experiments were repeated to confirm the reproducibility of results.

Analytical methods

The dry weight of biomass was determined by centrifugation of fungal cell suspension, washing with distilled water and drying it at 100°C for 12–16 h. When the medium contained vegetable oil as carbon source, cells were washed with 50 ml ether (acidified with 0.5 ml of 2 M HCl) and then with 50 ml distilled water following centrifugation [23].

Dried cells (20–30 mg) were weighed in 10 ml teflon-lined screw cap tubes and the lipids were extracted following the method of Bligh and Dyer [7]. Extracted lipids were dried at 36°C under nitrogen and methylated using the method of Holub and Skeaff [15]. The methyl esters were dissolved in hexane and analyzed by gas chromatograph, GC-14A (Shimadzu). The GC was fitted with a DB-17 (30 m $\times$ 0.25 mm) fused silica capillary column (Chromatographic Specialties) and flame ionization detector, and connected with a CR601 Chromatopac data integrator. Helium was used as the carrier gas. The fatty acid methyl ester peaks were identified by comparison of their retention times to relative methyl pentadecanoate with those of authentic standard fatty acids obtained from Sigma and standard DPA obtained from Dr B Holub, University of Guelph, Guelph, Ontario. They were quantified by the internal standard method using the same standard.

Results and discussion

Several species of lower fungi were screened for their lipid and DPA-producing capabilities. Growth, lipid production and fatty acid composition of these strains, cultured in the basal medium (described in Methods section) at 20°C for 7 days, were compared (Table 1). The lipid of marine fungi, ie species of *Thraustochytrium* and *Schizochytrium*, accumulated EPA and a high level of DHA but no DPA was detected. *Pythium ultimum* ATCC 13647, *Entomophthora thaxteriana* ATCC 42118, *Saprolegnia australis* ATCC 38487 and *Saprolegnia diclina* ATCC 36147 were found to accumulate a significant amount of EPA in their lipid. Among all the cultures tested only *Pythium acanthicum* ATCC 18660 produced DPA, and was, therefore, used for studies of factors affecting the production of DPA. Under none of the conditions tried was the formation of DHA by *P. acanthicum* observed.

Although the capability of pythiaceous fungi to produce long-chain PUFAs such as AA and EPA has been suggested by Shaw [22], these fatty acids were not detected in *P. ultimum* [8]. More recently, the presence of both AA and EPA in some species of *Pythium* such as *P. ultimum*, *P. irregulare*, and *P. aphanidermatum* was reported [12,26,29]. However, DPA or DHA were not detected in these species.

The effect of initial culture pH on DPA production was tested in the pH range 4.0–9.0. An initial pH of 6.0 was optimum for DPA production (Figure 1). Although no significant differences were observed in biomass and lipid content between pH 5.0 and 8.0, DPA yield drastically reduced when the initial culture pH level was above 7.0.

Table 1 Biomass production and lipid composition of cultures from lower fungi screened for DPA production[a]

Organism/ATCC No.[b]	Biomass (g L^{-1})	Lipid (%, w/w)	Fatty acid (% w/w)									
			14:0	16:0	18:0	18:1	18:2	18:3	20:4	20:5	22:5	22:6
P. acanthicum 18660	10.2	17.9	6.6	18.5	2.0	36.3	13.6	0.1	7.2	2.1	2.2[c]	—
P. ultimum 13647	3.4	7.7	7.2	21.0	1.2	12.8	14.4	0.7	7.7	12.5	—	—
T. aureum 28211	4.6	17.3	0.6	17.4	2.9	7.9	3.0	—	4.7	5.1	—	39.0
T. visurgence 29208	1.7	15.6	1.5	20.2	3/4	15.7	0.2	—	3.8	2.7	—	16.1
T. roseum 28210	7.1	19.6	3.2	7.8	4.2	16.0	2.2	1.5	3.0	—	—	42.3
Thraustochytrium sp 20891	2.1	6.9	2.4	23.0	5.2	14.6	31.7	3.5	4.7	5.1	—	3.9
Sch. aggregatum 29209	1.9	8.8	3.2	33.4	0.8	2.7	3.0	—	10.9	3.9	—	22.9
M. alpina 42430	9.6	7.1	1.7	18.9	4.1	37.7	5.4	7.4	19.5	0.9	—	—
M. elongata 32325	9.3	17.6	1.3	21.5	7.5	34.3	6.4	6.7	12.7	1.1	—	—
E. nouryi 14269	10.2	19.6	10.2	27.4	6.1	25.4	0.4	1.0	5.2	—	—	—
E. thaxteriana 42118	0.6	14.6	3.5	26.3	1.1	1.6	0.6	0.2	2.2	4.3	—	—
C. coronatus 28565	11.2	34.4	26.7	12.2	—	33.1	8.3	1.5	3.3	—	—	—
C. lampraugus 14651	10.2	15.2	2.5	12.9	7.0	13.6	1.4	0.5	23.8	0.3	—	—
S. australis 38487	0.8	12.0	10.6	20.4	2.8	9.8	7.0	—	7.0	5.7	—	—
S. parasitica 22284	8.9	4.4	7.8	26.7	3.9	8.6	6.5	—	9.7	—	—	—
S. diclina 36147	9.9	7.5	5.9	23.1	4.6	19.7	9.1	0.8	9.7	8.6	—	—

[a]Cultures were incubated for 7 days at 20°C, 200 rpm.
[b]Genus: *P., Pythium; T., Thraustochytrium; M., Mortierella; Sch., Schizochytrium; E., Entomophthora; C., Conidiobolus; S., Saprolegnia*.
[c]Equivalent to 40 mg L^{-1} culture.

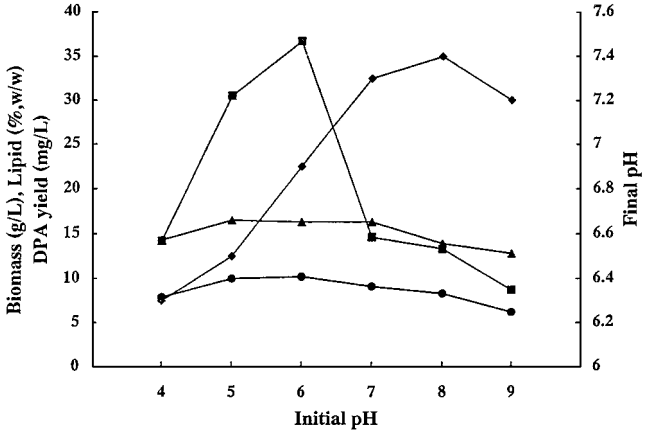

Figure 1 Effect of medium pH on biomass and DPA production by *Pythium acanthicum* ATCC 18660. Cultures were incubated in glucose (20 g L^{-1}) containing medium for 7 days at 20°C on a rotary shaker (200 rpm). (—●—) Biomass; (—▲—) lipid; (—■—) DPA yield; (—◆—) final pH.

The effect of incubation temperature on growth and DPA production by *P. acanthicum* ATCC 18660 was studied in shake flask cultures in a temperature range of 15–25°C for 7 days and the results are shown in Table 2. Maximum biomass (10.2 g L^{-1}) and DPA yield (40.8 mg L^{-1}) were observed at 20°C. The optimum temperature for growth and PUFA production by lower fungi is generally reported to be between 25 and 28°C [2,18,25,30]. The proportion of DPA in total fatty acids of *P. acanthicum* declined with an increase in temperature. Maximum DPA (2.8% w/w of total fatty acids) was observed at 15°C.

The time course of growth of *P. acanthicum* ATCC 18660 and DPA production is shown in Figure 2. Biomass content increased with cultivation time and reached the maximum value (10.9 g L^{-1}) on the eighth day. About 90% of the added glucose was utilized by *P. acanthicum* within

Table 2 Effect of temperature on DPA production by *Pythium acanthicum* ATCC 18660[a]

Parameters	Temperature (°C)		
	15	20	25
Biomass (g L^{-1})	6.5	10.2	9.6
Lipid in biomass (%, w/w)	18.4	17.9	15.4
DPA			
in biomass (mg L^{-1})	5.1	4.0	2.6
yield (mg L^{-1})	33.2	40.8	30.7
in lipid (% w/w)	2.8	1.18	1.11

[a]Culture conditions: glucose (20 g L^{-1}) containing medium (pH 6.0); incubation time, 7 days; shaking speed, 200 rpm.

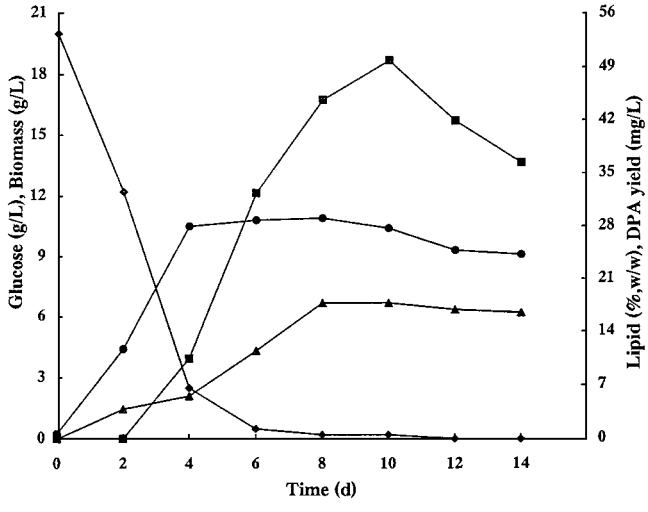

Figure 2 Time course of growth, lipid and DPA yields of *Pythium acanthicum* ATCC 18660. Cultures were incubated in glucose (20 g L^{-1}) containing medium (pH 6.0) at 20°C on a rotary shaker (200 rpm). (—●—) Biomass; (—◆—) glucose; (—▲—) lipid; (—■—) DPA yield.

Table 3 Effect of nitrogen source on DPA production by *Pythium acanthicum* ATCC 18660[a]

Nitrogen source	Biomass ($g\ L^{-1}$)	DPA yield ($mg\ L^{-1}$)	DPA in biomass ($mg\ g^{-1}$)
Glutamate	9.8	43.1	4.40
Peptone	9.7	3.9	0.40
Tryptone	9.9	5.9	0.60
Casamino acid	10.8	35.6	3.30
Malt extract	6.4	8.9	1.39

[a]Culture conditions: glucose ($20\ g\ L^{-1}$) containing medium (pH 6.0); nitrogen source, $2.0\ g\ L^{-1}$; incubation time, 7 days; temperature, 20°C; shaking speed, 200 rpm.

4 days of incubation. Biomass and DPA content were maximum after 4 and 10 days, respectively. A maximum DPA yield of $49.9\ mg\ L^{-1}$ was observed.

Among different complex nitrogen sources tested, the volumetric yield of DPA was highest with sodium glutamate ($43.1\ mg\ L^{-1}$) and lowest with peptone ($3.9\ mg\ L^{-1}$) (Table 3). Specific production of DPA ranged from 0.4 with peptone to $4.4\ mg\ g^{-1}$ biomass for glutamate.

The effect of carbon source on DPA production by *P. acanthicum* ATCC 18660 was investigated using different mono-, di- and polysaccharides as well as vegetable oils and cereal flours in shake flask cultures incubated at 20°C for 10 days. The results are presented in Table 4. Growth on different carbon sources ranged from 2.9–16.9 $g\ L^{-1}$ and values for specific production of DPA ranged from 1.0 to $5.0\ mg\ g^{-1}$ biomass. Vegetable oils and cereal flours appeared to be promising carbon sources for growth and lipid production by *P. acanthicum*. Maximum DPA volumetric yields ($69.5\ mg\ L^{-1}$) and specific production ($5.0\ mg\ g^{-1}$ biomass) were observed with soy flour.

DPA volumetric yield was further increased when soy flour or glucose-containing media were supplemented with

Table 4 Effect of carbon source on DPA production by *Pythium acanthicum* ATCC 18660[a]

Carbon source	Biomass ($g\ L^{-1}$)	DPA yield ($mg\ L^{-1}$)	DPA in biomass ($mg\ g^{-1}$)
Carbohydrates			
Glucose	10.0	42.0	4.2
Xylose	2.9	2.9	1.0
Glycerol	12.0	42.3	3.5
Maltose	9.5	33.3	3.5
Sucrose	5.8	5.8	1.0
Starch	9.2	34.0	3.7
Cereal flours			
Soy	13.9	69.5	5.0
Rye	12.3	50.4	4.1
Cottonseed	11.4	34.2	3.0
Corn gluten	13.2	29.0	2.2
Vegetable oils			
Canola	16.9	59.2	3.5
Corn	14.3	57.2	4.0
Linseed	16.0	64.0	4.0

[a]Culture conditions: basal medium, pH 6.0; -carbon source, $20\ g\ L^{-1}$; incubation time, 10 days; temperature, 20°C; shaking speed, 200 rpm.

Table 5 Effect of vegetable oil supplementation to glucose or soy flour containing media on DPA production by *Pythium acanthicum* ATCC 18660[a]

Substrate[b]	Biomass ($g\ L^{-1}$)	DPA yield ($mg\ L^{-1}$)	DPA in biomass ($mg\ g^{-1}$)
Glucose + corn oil	19.1	87.9	4.6
Glucose + canola oil	17.8	85.4	4.8
Glucose + linseed oil	20.9	110.8	5.3
Soy flour + corn oil	20.2	82.8	4.1
Soy flour + canola oil	18.1	68.8	3.8
Soy flour + linseed oil	21.6	108.0	5.0

[a]Culture conditions: basal medium, pH 6.0; incubation time, 10 days; temperature, 20°C; shaking speed, 200 rpm.
[b]Vegetable oil ($10\ g\ L^{-1}$) was supplemented to glucose or soy flour ($20\ g\ L^{-1}$) containing medium.

linseed oil (Table 5). However, specific production (5.0–5.3 $mg\ g^{-1}$) was not significantly enhanced and, consequently, the DPA increase reflected the increase in biomass.

By extending the fermentation time with batch feeding of additional linseed oil or glucose to the basal media containing glucose and sodium glutamate, DPA volumetric yield was increased to $125\ mg\ L^{-1}$ and $132\ mg\ L^{-1}$ respectively (Figure 3).

Despite the fact that Greenland Eskimoes live to an average age of more than 60 years, they have a much lower incidence of death from ischemic heart disease than is observed in populations with diets rich in 'Western food' [5]. Analysis of fatty acids in the Eskimo diet revealed 9–20 fold higher contents of EPA and DHA as compared with Danish food and such studies have led to intensive investigations on the nutritional and pharmacological values of these omega-3 fatty acids and a search for alternative

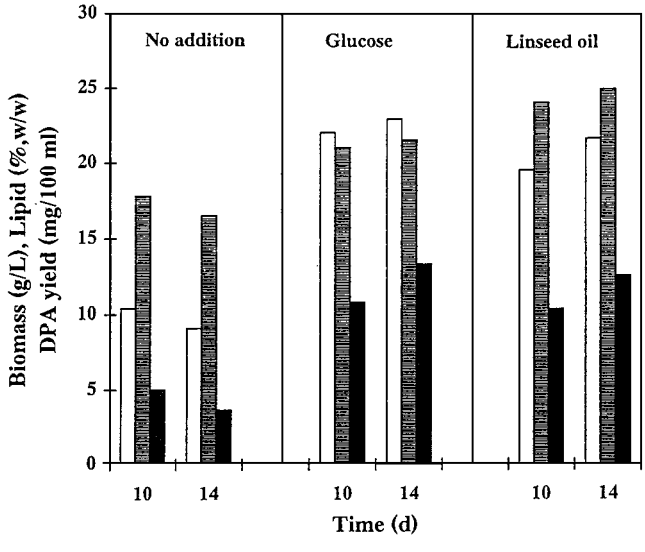

Figure 3 Effect of batch supplementation of glucose or linseed oil to glucose-containing medium on DPA production by *Pythium acanthicum* ATCC 18660. Culture was inoculated to glucose ($20\ g\ L^{-1}$) containing medium and incubated at 20°C on a rotary shaker (200 rpm). Additional glucose ($20\ g\ L^{-1}$) or linseed oil ($10\ g\ L^{-1}$) was supplied after 3, 6 and 9 days and the cells then harvested after 10 and 14 days. (□) Biomass; (▤) lipid; (■) DPA yield.

sources of these compounds. Eskimo diets also contain substantial amounts of DPA which was completely absent in Danish diets. The data suggest that the healthy benefits of DPA should be examined in detail, together with methods for production of this intermediate. Our results show that DPA is not produced by most strains which produce AA, EPA or DHA and that increasing DPA content, where it is produced, appears to represent a significant challenge. Production of high amounts of DPA may require manipulation of the activities of enzymes in the synthesizing pathway by induction/repression, activation/inhibition and/or mutation.

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