J Am Oil Chem Soc (2009) 86:791–797
DOI 10.1007/s11746-009-1409-0

ORIGINAL PAPER

Influence of Temperature on Growth and Peak Oil Biosynthesis in a Carbon-Limited Medium by *Pythium irregulare*

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Received: 11 February 2009 / Revised: 4 May 2009 / Accepted: 17 May 2009 / Published online: 12 June 2009

Abstract Kinetic analysis was investigated for a carbon-limited medium (C/N ratio = 5.0) supporting the growth of the 5,8,11,14,17-eicosapentaenoic acid (20:5; ω-3) (EPA)-accumulating fungal organism *Pythium irregulare*. The productivity and yield parameters at three temperatures, 14, 21, and 28°C, demonstrated growth-coupled synthesis for lipid-free biomass growth and lipid accumulation. For this system, the maximum specific growth rate and theoretical maximum biomass yield based on logistic growth kinetics were used to determine an activation energy of the growth process, E_g, of 36.5 kJ mol^{-1}. At 14, 21, and 28°C, peak lipid yield occurred after culturing for 7, 4, and 3 days, respectively, with peak lipid yields of 8.14, 12.8, and 6.69 g lipid 100 g^{-1} glucose. At these peak yields, the maximum lipid-free biomass productivity was achieved at the colder 14°C temperature as well as an increased concentration of EPA—10.9 wt%. Despite these enhancements, the maximum relative lipid production ($P_{R(FA/B)}$) was achieved at 21°C—19.1%.

Keywords PUFA · Lipid production · Eicosapentaenoic acid · Fungal oil · Fermentations · Growth kinetics

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Introduction

Due to an increasing number of potential health benefits associated with them, omega-3 polyunsaturated fatty acids (ω-3 PUFAs) are of prime medical interest. PUFAs, including 5,8,11,14,17-eicosapentaenoic acid (20:5; ω-3) (EPA), have proven beneficial in prevention and treatment of coronary heart disease, blood platelet aggregation, and artherosclerosis. These compounds are capable of alleviating inflammatory conditions such as arthritis as well as retarding growth of tumor cells. PUFAs are precursors to a variety of metabolites, including prostaglandins and leukotrienes, that regulate critical biological functions [1–3]. These same fatty acids have also been linked to visual and mental health as well as neonatal development [4, 5].

Major PUFA commercial production and distribution are achieved through encapsulating fish oil. Unfortunately, the oil can contain objectionable tastes and odors; furthermore, it can require removal of cholesterol and potential toxic pollutants (e.g., mercury) [6, 7]. Another downside to encapsulating fish oil is the variation in the oil quality and quantity of individual PUFAs; the EPA content of fish oil can range from 9 to 27% of the total oil weight [8]. Despite fish oil's dominance on the consumer market, the greatest PUFA diversity is encountered in microorganisms, especially algae and fungi.

Extensive reviews are available describing PUFAs from various algal and fungal sources [5, 6, 9, 10]. Two genera of filamentous fungi can synthesize relatively high amounts of EPA; these are *Pythium* and *Umbelopsi* (formerly *Mortierella*) [1, 7, 11–18]. For these two fungi, submerged culture fermentation conditions such as medium composition, pH, and temperature influence both intracellular lipid accumulation and composition of the PUFA-rich oil

[11, 16, 19, 20]. Some of these fermentation conditions have been optimized in order to maximize EPA production from *P. irregulare*. For example, when testing glucose, lactose, and sweet whey permeate as sole carbon sources, Stinson et al. [16] concluded that fermentations at 12°C using a medium with a 1% glucose concentration and pH between 6.0 and 7.0 were most effective for promoting biomass growth and EPA yield (12–15 wt%).

Many fermentation studies have cultured oleaginous microorganisms to address the kinetics of biomass and lipid production by PUFA-producing fungi [15, 17, 21]. Other studies have modeled the relationship among biomass growth, lipid accumulation, and substrate consumption [22–25]. These kinetic relationships are critical to develop fermentation models essential for design and control of bioprocesses. One model of bioprocess productivity performance describes growth of fungal organisms with a logistic growth kinetic [26–29]. This logistic equation has two adjustable parameters: the maximum specific growth rate, μ_{max}, and the theoretical maximum biomass yield, X'_{max}. The growth rate of a microorganism is influenced by temperature; this temperature influence is similar to that observed for enzyme activity. Consequently, the temperature sensitivity of μ_{max} over a range of temperatures can be expressed by the Arrhenius function [30].

Our objectives were to undertake the following at three fermentation temperatures, 14, 21, and 28 °C: (1) analyze kinetics and temperature effects of lipid-free and lipid accumulation of *P. irregulare*, (2) quantify the activation energy of the growth process, and (3) compare fatty-acid profiles of lipids at peak lipid biosynthesis.

Materials and Methods

Microorganism

Pythium irregulare (ATCC 10951) was maintained on cornmeal agar plates and transferred every 3 weeks to potato dextrose agar (PDA) plates. To transfer the culture, a 1-cm^2 plug containing mycelium was placed on the new PDA petri dish. The culture was grown at 25°C for 2 days, and then stored at 4°C until fermentations were conducted. The liquid medium used for experimental fermentations was a modified yeast-malt extract (YM) medium [31] previously optimized for this organism with respect to glucose and K_2HPO_4 concentrations and initial pH [16]. This YM medium with an approximate C/N molar ratio close to 5.0 consisted of yeast extract, 3.0 g l^{-1}; malt extract, 3.0 g l^{-1}; peptone, 5.0 g l^{-1}; glucose, 10 g l^{-1}; and K_2HPO_4, 0.684 g l^{-1}; pH to 6.0 (1 N HCl).

Inoculum Preparation and Fermentation

Once *P. irregulare* was actively growing on PDA, a 1-cm^2 plug was added to a 250-ml Erlenmeyer flask containing 50-ml of sterile YM medium. This inoculated medium was incubated in an orbital shaker at 150 rpm and 25°C for 2 days. A Kinematica Polytron handheld homogenizer (Newark, NJ, USA) was used to blend this seed culture for 10 s at the highest rpm setting. Experimental flasks containing 45 ml sterile YM media were inoculated with 5-ml portions of blended seed culture and incubated at selected temperatures in the orbital shaker at 150 rpm.

The effect of temperature on biomass yield and lipid production was studied at 14, 21, and 28°C. Triplicate fermentation flasks were sampled daily to determine biomass growth, lipid production, and residual glucose remaining in the liquid YM medium over a period of 7–12 days.

Analytical Methods

The mycelial growth of *P. irregulare* was expressed as dry cell weight per volume of medium in mg/ml. Daily biomass samples were harvested by filtration of the sample through Whatman No. 1 filter paper (Florham Park, NJ, USA) and washed with deionized water. These biomass cells were transferred to preweighed tubes and then freeze-dried at −50°C and 0.15 Mbar vacuum until a constant weight was achieved, typically within 36 h.

Undiluted filtrate was retained and stored at −20°C until the residual glucose concentration of the undiluted filtrate was quantified using a Shimadzu VP Series HPLC system (Columbia, MD, USA) equipped with a Shimadzu ELSD-LT detector with a gain setting of 6 and a temperature of 70°C. Filtered house air was the nebulizer gas. HPLC separation was performed using a Prevail C18 column (150 × 4.6 mm i.d.; Alltech Assocociates; Columbia, MD, USA) packed with 5-μm spherical particles. The mobile phase was distilled water set to a flow rate of 0.50 ml min^{-1}. Injected filtered samples were 25 μl in volume.

The lipids were extracted from the dried biomass by a modified procedure using a 3:2 hexane isopropanol mixture (v/v; HiP) [32]. This method was chosen due to familiarity [11, 33] and its use of low toxicity solvents. Freeze-dried mycelia (~0.25 g) were suspended in 10 ml of HiP, masticated for 1 min at high speed with the Polytron homogenizer, and then centrifuged at 1,300*g* for 10 min. The supernatant was decanted and saved. This extraction procedure was repeated twice for the residue. At the end of this extraction procedure, the three supernatants were combined. The addition of 10 ml of 0.47 M sodium sulfate to the combined supernatants broke the emulsion and

removed any extracted nonlipids. The upper hexane-rich phase containing the lipids was filtered with Whatman No. 1 paper and transferred to a preweighed tube for complete hexane evaporation under nitrogen at roughly 50°C. The dry weight of this residue was considered the total weight of lipids.

For fatty-acid methyl ester (FAME) analysis, dried lipids were redissolved in 2 ml of hexane, and aliquots containing 2 mg of lipid residue were esterified with a rapid micro-scale procedure using CH_3NaO [34]. These FAMEs were extracted with hexane and analyzed by gas chromatography. Gas chromatography was performed with a Shimadzu GC-17A gas chromatograph (Columbia, MD, USA) equipped with a flame ionization detector (FID) and a split injector with a split-to-splitless ratio of 11:1. This apparatus was fitted with a DB-Wax bonded phase capillary column (60 m × 0.25 mm × 0.25 μm; Agilent Technologies; Palo Alto, Ca, USA). Samples (5 μl) were injected at 180°C; after 6 min the oven temperature was raised at 3°C min^{-1} to 204°C; and then 5°C min^{-1} to 240 °C, where it was held for 25 min. Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. The injector and detector temperatures were 250 and 275°C, respectively. A known amount of heptadecanoic acid methyl ester (Sigma Chemical; St. Louis, MO, USA) serving as an internal standard was added to the aliquots after derivatization to FAMEs. All FAMEs were identified by the retention index of the standards in a 37-FAME mix (Sigma Chemical). Additionally, FAME quantification was accomplished using the internal standard method.

The SAS software (version 9.1; SAS Institute; Cary, NC, USA) was used for all statistical analyses among biomass productivity, lipid productivity, and FAME oil compositions.

Parameter Estimation

From the basic measured variables—total biomass accumulation (B), total intracellular lipids (FA), and glucose concentration in residual medium (GLU)—other parameters were determined: lipid-free biomass (X) and consumed glucose (GLUc). The productivity (P) of X and FA (g l^{-1}) was assumed to be the final value of the product; the relative productivity (P_R) (%, w/w) was the accumulation of FA in B; the yield (Y) of X and FA indicated accumulation of X or FA from the total GLUc (%, g 100 g^{-1} GLUc).

Frequently, fungal growth has been described by a logistic equation [27] defined as

$$\frac{dX}{dt} = \mu_{max} \cdot \left(1 - \frac{X}{X'_{max}}\right) \cdot X \quad (1)$$

where μ_{max} is the maximum specific growth rate theoretically obtained for $X(t = 0)$ (h^{-1}), and X'_{max} is the theoretical maximum attainable amount of lipid-free biomass (g l^{-1}). Integration of Eq. 1 yields an equation for the actual amount of total lipid-free biomass (g l^{-1}) or $X(t)$:

$$X(t) = \frac{X'_{max}}{\left(\frac{X'_{max}}{X_0} - 1\right) \cdot e^{-\mu_{max} \cdot t} + 1} \quad (2)$$

The temperature dependence of μ_{max} can be explained by the Arrhenius equation [30]:

$$\mu_{max} = A \cdot \exp\left(-\frac{E_g}{RT}\right) \quad (3)$$

where the Arrhenius constant is A (h^{-1}), E_g is the activation energy of the growth process (J mol^{-1}), R is the universal gas constant in J mol^{-1} K^{-1}, and fermentation temperature, T, is in K.

Results and Discussion

Batch Cultivations

Submerged culture growth of *P. irregulare* and its respective lipid accumulation differed among the experimental temperatures of 14, 21, and 28°C (Fig. 1). It was notable that, despite the carbon-limited conditions (i.e., nitrogen-excess conditions), *P. irregulare* was able to accumulate lipid upwards of 20% w/w. This phenomenon was contrary to most cases of single-cell oil accumulation and production. Lipid accumulation was understood to commonly accumulate in organisms grown in a medium with excess carbon substrate and a limited amount of nitrogen whereby once the organism consumes all available nitrogen, the organism directs the remaining consumed carbon to lipid biosynthesis [35]. Additionally, for these conditions, *P. irregulare* exhibited growth-coupled lipid synthesis. This suggested the organism was capable of synthesizing essential cellular materials while accumulating lipids. The growth-coupled synthesis has been documented previously with *M. alpina* under batch cultivation at 25°C and 5% glucose medium [36].

As part of growth-coupled synthesis, *P. irregulare* consumed significant amounts of glucose for simultaneous production of lipid-free biomass and lipid accumulation. The maximum lipid-free biomass production values ($P_{(X_{max})}$) were found to be statistically different ($\alpha = 0.05$) with a decrease in temperature promoting lipid-free biomass production. The maximum $P_{(X)}$ was achieved at 14°C at 7 days with a value of 5.87 g l^{-1} (Fig. 1). This corresponded to a lipid-free biomass yield, $Y_{(X)}$, of 73.7% (Fig. 2). Lipid-free biomass productivity reached a maximum value of 5.11 and 3.87 g l^{-1} during fermentations at

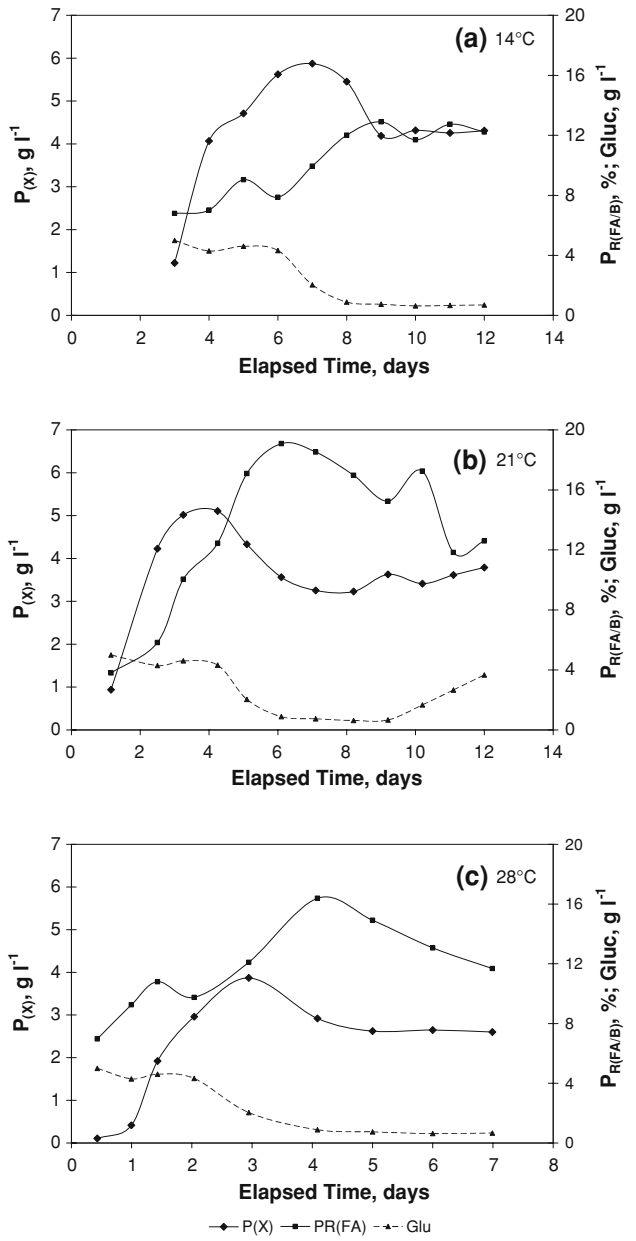

Fig. 1 Time-course effects of lipid-free biomass productivity ($P_{(X)}$), relative lipid productivity ($P_{R(FA/B)}$), and glucose consumed (GLUc) during batch growth of *P. irregulare* (ATCC 10951) in submerged culture using modified yeast-malt extract medium: **a** 14°C, **b** 21°C, **c** 28°C

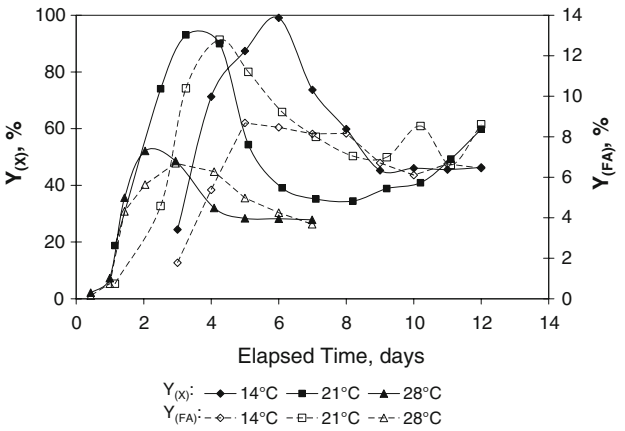

Fig. 2 Time-course effects of yield of lipid-free biomass (*X*) and lipid (*FA*) of *P. irregulare* (ATCC 10951) in submerged culture using modified yeast-malt extract medium

21 and 28°C on days 4 and 3, respectively, with associated yields of 90.0 and 30.5%. The overall trend of $P_{(X)}$ was similar to the trend for $P_{R(FA/B)}$ where after $(P_{(X_{max})})$ was achieved and glucose was completely consumed, there was degradation of both biomass and lipids. This trend has been noted elsewhere for yeast cultivated on stearin [37]. Thus, for the present study, it was inferred that the decrease in lipid and biomass was due to the potential death of cells after no available glucose could support growth and utilization of these components as a carbon-source. Some cellular lipid degradation did occur during stationary growth. Yet there was no evidence of new lipid-free biomass as has been documented elsewhere [23]. This further supported possible autolysis of mycelia to supplement the carbon-limited conditions.

Peak biomass accumulation and yield did not occur at the same time as peak lipid accumulation (Figs. 1, 2). Lipid accumulation was a maximum for this study when fermentations were conducted on day 4 at 21°C with a $P_{R(FA/B)}$ value of 19.1%. This value decreased for fermentation temperatures 14 and 28°C to 12.9 and 17.0%, respectively. Consequently, with an increased $P_{R(FA/B)}$ at 21°C, there was an increase in the lipid yield, $Y_{(FA)}$. This value was 12.8% compared to a low at 28°C of 6.69%.

Modeling of Fungal Growth

A logistic model was applied to describe each temperature-dependent biomass profile of *P. irregulare*. The logistic equation described the growth phase well; however, it could not provide a complete representation of the entire growth curve, which includes the death phase [28]. For parameter estimation, it was assumed that the theoretical maximum biomass production, X'_{max}, corresponded to the experimental biomass production, X_{max}, determined at 7, 4, and 3 days of cultivation for 14, 21, and 28°C fermentations, respectively. Estimates for X'_{max} and the maximum specific growth rate (μ_{max}) were obtained by a least-squares procedure in Excel, applying the experimental data during growth phase to Eq. 2. The logistic model biomass profile obtained described the experimental profile reasonably well with r^2 values in the range of 0.952–1.000 (Table 1; Fig. 3, solid lines).

Table 1 Estimates for kinetic parameters ($n = 3$) obtained by fitting the logistic model to lipid-free biomass productivity

Kinetic parameter	Fermentation temperature (°C)		
	14	21	28
X'_{max} (mg/ml)	5.896	5.153	3.937
μ_{max} (h^{-1})	0.0512	0.0958	0.104
r^2	0.952	1.000	0.990

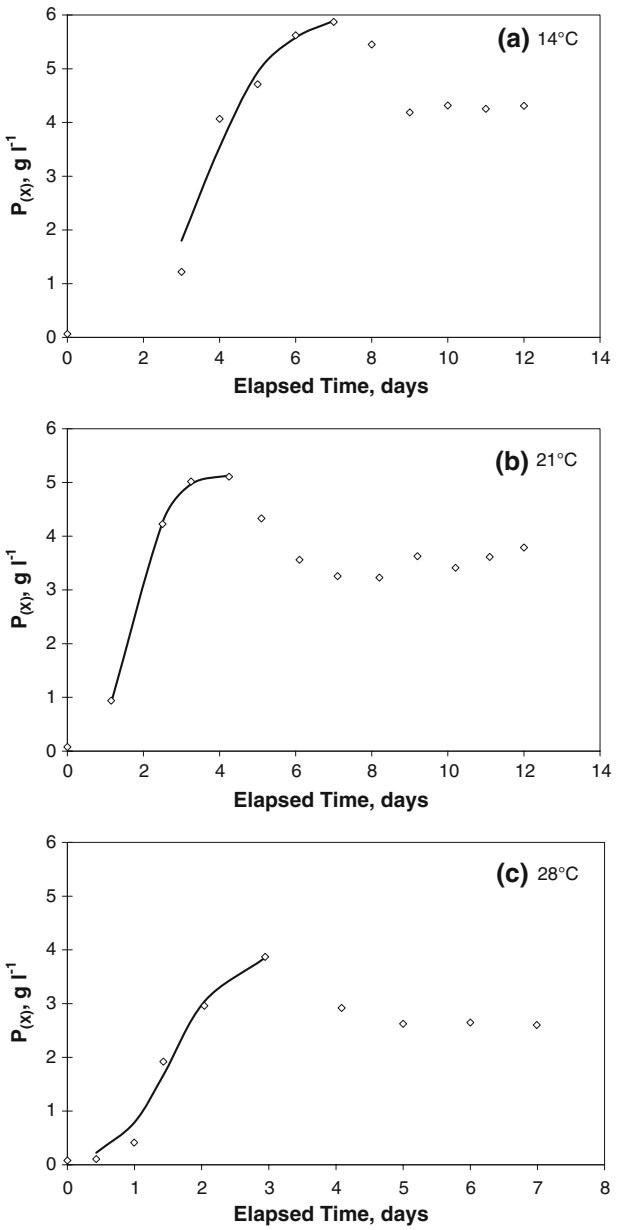

Fig. 3 Experimentally determined and modeled lipid-free biomass productivity ($P_{(X)}$) of *P. irregulare* (ATCC 10951) in submerged culture using modified yeast-malt extract medium: **a** 14°C, **b** 21°C, **c** 28°C. *Solid lines* represent biomass estimated from the logistic equation

Alongside a temperature increase was an increase in μ_{max} values. The temperature dependence of μ_{max} was explained by rearranging the Arrhenius equation (Eq. 3) in order to determine E_g from a linear regression of the three points. The slope of a plot of $\ln(\mu_{max})$ versus $1{,}000/T$ defined E_g as 36.5 kJ mol^{-1}, while the intercept of this plot allowed for the calculation of the Arrhenius constant (A), 2.50×10^5 h^{-1}.

Composition of the Lipid Extract

Composition of the individual and total fatty-acid content of the lipid extract differed slightly at each temperature-dependent $Y_{(FA)}$ (Table 2). The main components of all the oils tested were palmitic (C16:0) and oleic (C18:1) acids. The 28°C oil had the only significant difference in wt% of these compounds; palmitic acid contribution was greatest at 29.8 wt%. Other statistically significant differences were noted with myristic (C14:0), γ-linoleic (C18:3), ARA, and EPA. Fungal production of myristic acid was maximized at 14°C, while growth at 28°C promoted greater γ-linoleic acid.

EPA was the most abundant PUFA, comprising 3.5–10.9 wt% of the total oil; maximum EPA composition was realized for *P. irregulare* growth at 14°C. The EPA content at 14 and 21°C fell within the range (6–12%) reported elsewhere for *P. irregulare* growth on various substrates [16]. However, growth at 28°C yielded a significantly lower EPA and ARA content and has not been documented in the literature previously.

Significant increases in the ratio of ARA to EPA were noted for an increase in temperature (Table 2). This phenomenon was attributed to temperature-sensitive desaturase and elongase enzymes required for ω-3 PUFA production. Oleic (C18:1ω-9), linoleic (C18:2ω-6), and α-linolenic (C18:3ω-3) fatty acids are the basic precursors of longer-chain PUFAs. Omega-3 PUFA production involves two possible routes: (1) conversion of ω-6 fatty acids to a corresponding ω-3 via a desaturase enzyme system and (2) α-linolenic acid conversion through the ω-3 pathway through a system of both elongase and desaturase enzymes. The first route is considered temperature-dependent [1, 20]; therefore, conversion of linoleic to either γ-linolenic or to α-linolenic as well as ARA to EPA is optimized at lower temperatures. The latter route is temperature-independent and operates simultaneously with the first [7, 38]. Greater activation of desaturases at lower temperatures was possible because EPA production was maximized at 14°C and the ARA/EPA ratio decreased with decreasing temperature. This phenomenon of increased EPA production at lower temperatures has been documented in previous research efforts involving *P. irregulare* as well [11, 12, 16].

Table 2 Individual fatty-acid composition (wt%) of *Pythium irregulare* oil obtained from biomass on the day of maximum lipid yield

Fatty acid	$C_{q:r,\ \omega}$	LSD[a] (%)	Weight %		
			14°C (7 days)	21°C (4 days)	28°C (3 days)
Myristic	$C_{14:0}$	1.40	11.1*	9.47	9.17
Palmitic	$C_{16:0}$	2.25	23.8	25.9	29.8*
Palmitoleic	$C_{16:1}$	2.45	6.73	5.75	6.61
Stearic	$C_{18:0}$	0.26	5.11	5.14	4.94
Oleic	$C_{18:1\ \omega9}$	4.32	22.5	21.5	23.7
Linoleic	$C_{18:2\ \omega6}$	0.89	10.7	11.0	11.2
γ-linolenic (GLA)	$C_{18:3\ \omega6}$	0.33	1.01*	0.56	3.62*
Arachidic	$C_{20:0}$	1.08	0.71	1.06	0.96
cis-11-Eicosenoic	$C_{20:1\ \omega9}$	1.40	1.28	2.10	1.28
Arachidonic (ARA)	$C_{20:4\ \omega6}$	0.64	5.32	5.12	3.03*
Eicosapentaenoic (EPA)	$C_{20:5\ \omega3}$	2.02	10.9*	8.84*	3.47*
Total			98.5	96.4	97.8
Unknown			1.5	3.6	2.6
100 × ARA/EPA			48.8	57.9	87.3

$C_{q:r,\ \omega}$: q Number of carbons, r number of double bonds, ω position of the first double bond

*Weight% is statistically different from the other two temperatures

[a] Based on $t = 2.7765$, $\alpha = 0.05$, error degrees of freedom = 4, r = 3

Fermentation of *P. irregulare* in carbon-limited conditions demonstrated this organism's ability to both generate new lipid-free biomass and accumulate lipids in growth-coupled synthesis. As fermentation temperature decreased, greater experimental lipid-free biomass production was realized along with slower growth (i.e., μ_{max} decreased). The Arrhenius relationship between μ_{max} and temperature yielded an activation energy of the growth process, E_g, of 36.5 kJ mol^{-1}. Fermentation temperature was inversely related to the lipid-free biomass yield with increasing temperatures reducing the effective biomass concentrations on the same initial amounts of glucose. Temperature also had an effect on the fatty-acid composition of the oil. The lower the temperature, the greater the weight contribution of EPA to the oil; EPA contributed 10.9 wt% of the fungal oil cultivated at 14°C compared to 3.5 wt% at 28°C. Temperature did not seem to have a discernable relationship to lipid yield or relative lipid production. Lipid yield and production were maximized at 21°C with values of 19.1 and 12.8%, respectively.

Acknowledgements We are grateful to Cheng-Yi Kuan, Yen Hui Chen, and Meidui Dong for their technical assistance. We thank the Southern Regional Canola Research Board for partial funding of this research.

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References

1. Li D, Bode O, Drummond H, Sinclair AJ (2003) Omega-3 (n-3) fatty acids. In: Gunstone FD (ed) Lipids for functional foods and nutraceuticals. Oily Press, Bridgewater, pp 225–262
2. Simopoulos AP, Kifer RR, Martin RE, Barlow SM (1991) Health effects of ω-3 polyunsaturated fatty acids in seafood, 7th ed. Karger, Basel
3. Youdim KA, Martin A, Joseph JA (2000) Essential fatty acids and the brain: possible health implications. Int J Dev Neurosci 18:383–399
4. Uauy R, Hoffman DR, Peirano P, Birch DG, Birch EE (2001) Essential fatty acids in visual and brain development. Lipids 36:885–895
5. Cohen Z, Ratledge C (2005) Single cell oils. American Oil Chemists' Society, Champaign
6. Belarbi EH, Molina E, Chisti Y (2000) A process for high yield and scaleable recovery of high purity eicosapentaenoic acid esters from microalgae and fish oil. Enzyme Microb Technol 26:516–529
7. Certik M, Shimizu S (1999) Biosynthesis and regulation of microbial polyunsaturated fatty acid production. J Biosci Bioeng 87:1–14
8. Robles Medina A, Molina Grima E, Gimenez Gimenez A, Ibanez Gonzalez MJ (1998) Downstream processing of algal polyunsaturated fatty acids. Biotechnol Adv 16:517–580
9. Leman J (1997) Oleaginous microorganisms: an assessment of the potential. Adv Appl Microbiol 43:195–243
10. Wen ZY, Chen F (2003) Heterotrophic production of eicosapentaenoic acid by microalgae. Biotechnol Adv 21:273–294
11. Cheng MH, Walker TH, Hulbert GJ, Raman DR (1999) Fungal production of eicosapentaenoic and arachidonic acids from industrial waste streams and crude soybean oil. Bioresour Technol 67:101–110
12. O'Brien DJ, Kurantz MJ, Kwoczak R (1993) Production of eicosapentaenoic acid by the filamentous fungus *Pythium irregulare*. Appl Microbiol Biotechnol 40:211–214
13. Singh A, Ward OP (1997) Microbial production of docosahexaenoic acid (DHA, C22:6). Adv Appl Microbiol 45:271–312
14. Stredansky M, Conti E, Salaris A (2000) Production of polyunsaturated fatty acids by *Pythium ultimum* in solid-state cultivation. Enzyme Microb Technol 26:304–307
15. Certik M, Shimizu S (2000) Kinetic analysis of oil biosynthesis by an arachidonic acid-producing fungus, *Mortierella alpina* 1S–4. Appl Microbiol Biotechnol 54:224–230
16. Stinson EE, Kwoczak R, Kurantz MJ (1991) Effect of cultural conditions on production of eicosapentaenoic acid by *Pythium irregulare*. J Ind Microbiol 8:171–178

17. Papanikolaou S, Komaitis M, Aggelis G (2004) Single cell oil (SCO) production by *Mortierella isabellina* grown on high-sugar content media. Bioresour Technol 95:287–291
18. Lardizabal K, Effertz R, Levering C, Mai J, Pedroso MC, Jury T, Aasen E, Gruys K, Bennett K (2008) Expression of *Umbelopsis ramanniana* DGAT2A in seed increases oil in soybean. Plant Physiol 148:89–96
19. O'Brien DJ, Senske GE (1994) Recovery of eicosapentaenoic acid from fungal mycelia by solvent extraction. J Am Oil Chem Soc 71:947–950
20. Shimizu S, Kawashima H, Akimoto K, Shinmen Y, Yamada H (1991) Production of odd chain polyunsaturated fatty acids by *Mortierella* fungi. J Am Oil Chem Soc 68:254–258
21. Gema H, Kavadia A, Dimou D, Tsagou V, Komaitis M, Aggelis G (2002) Production of γ-linolenic acid by *Cunninghamella echinulata* cultivated on glucose and orange peel. Appl Microbiol Biotechnol 58:303–307
22. Aggelis G, Sourdis J (1997) Prediction of lipid accumulation–degradation in oleaginous micro-organisms growing on vegetable oils. Anton Leeuw Int J G 72:159–165
23. Papanikolaou S, Aggelis G (2003) Modeling lipid accumulation and degradation in *Yarrowia lipolytica* cultivated on industrial fats. Curr Microbiol 46:398–402
24. Papanikolaou S, Galiotou-Panayotou M, Chevalot I, Komaitis M, Marc I, Aggelis G (2006) Influence of glucose and saturated free-fatty acid mixtures on citric acid and lipid production by *Yarrowia lipolytica*. Curr Microbiol 52:134–142
25. Ykema A, Verbree EC, Kater MM, Smit H (1988) Optimization of lipid production in the oleaginous yeast *Apiotrichum curvatum* in wheypermeate. Appl Microbiol Biotechnol 29:211–218
26. Mitchell DA, Krieger N, Stuart DM, Pandey A (2000) New developments in solid-state fermentation II. Rational approaches to the design, operation and scale-up of bioreactors. Process Biochem 35:1211–1225
27. Sangsurasak P, Nopharatana M, Mitchell DA (1996) Mathematical modeling of the growth of filamentous fungi in solid state fermentation. J Sci Ind Res 55:333–342
28. Lareo C, Sposito AF, Bossio AL, Volpe DC (2006) Characterization of growth and sporulation of *Mucor bacilliformis* in solid state fermentation on an inert support. Enzyme Microb Technol 38:391–399
29. Ooijkaas LP, Buitelaar RM, Tramper J, Rinzema A (2000) Growth and sporulation stoichiometry and kinetics of *Coniothyrium minitans* on agar media. Biotechnol Bioeng 69:292–300
30. Shuler ML, Kargi F (2002) Bioprocess engineering: basic concepts, 2nd ed. Prentice Hall, NJ
31. Wickerham LJ (1951) Taxonomy of yeasts. Tech. Bull. No. 1029. US Department of Agriculture, Washington DC
32. Hara A, Radin NS (1978) Lipid extraction of tissues with a low toxicity solvent. Anal Biochem 90:420–426
33. Walker TH, Cochran HD, Hulbert GJ (1999) Supercritical carbon dioxide extraction of lipids from *Pythium irregulare*. J Am Oil Chem Soc 76:595–602
34. Christie WW (1982) A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. J Lipid Res 23: 1072–1075
35. Ratledge C (2004) Fatty acid biosynthesis in microorganisms being used for single cell oil production. Biochimie 86:807–815
36. Eroshin VK, Satroutdinov AD, Dedyukhina EG, Chistyakova TI (2000) Arachidonic acid production by *Mortierella alpina* with growth-coupled lipid synthesis. Process Biochem 35:1171–1175
37. Papanikolaou S, Chevalot I, Komaitis M, Aggelis G, Marc I (2001) Kinetic profile of the cellular lipid composition in an oleaginous *Yarrowia lipolytica* capable of producing a cocoa-butter substitute from industrial fats. Anton Leeuw Int J G 80:215–224
38. Certik M, Sakuradani E, Shimizu S (1998) Desaturase-defective fungal mutants: useful tools for the regulation and overproduction of polyunsaturated fatty acids. Trends Biotechnol 16:500–505