

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
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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

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 arteriosclerosis. 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

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[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-cm² 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 K₂HPO₄ 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 K₂HPO₄, 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-cm² 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 Associates; Columbia, MD, USA) packed with 5- μ m spherical particles. The mobile phase was distilled water set to a flow rate of 0.50 ml min⁻¹. Injected filtered samples were 25 μ l 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 1,300g 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 CH₃NaO [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

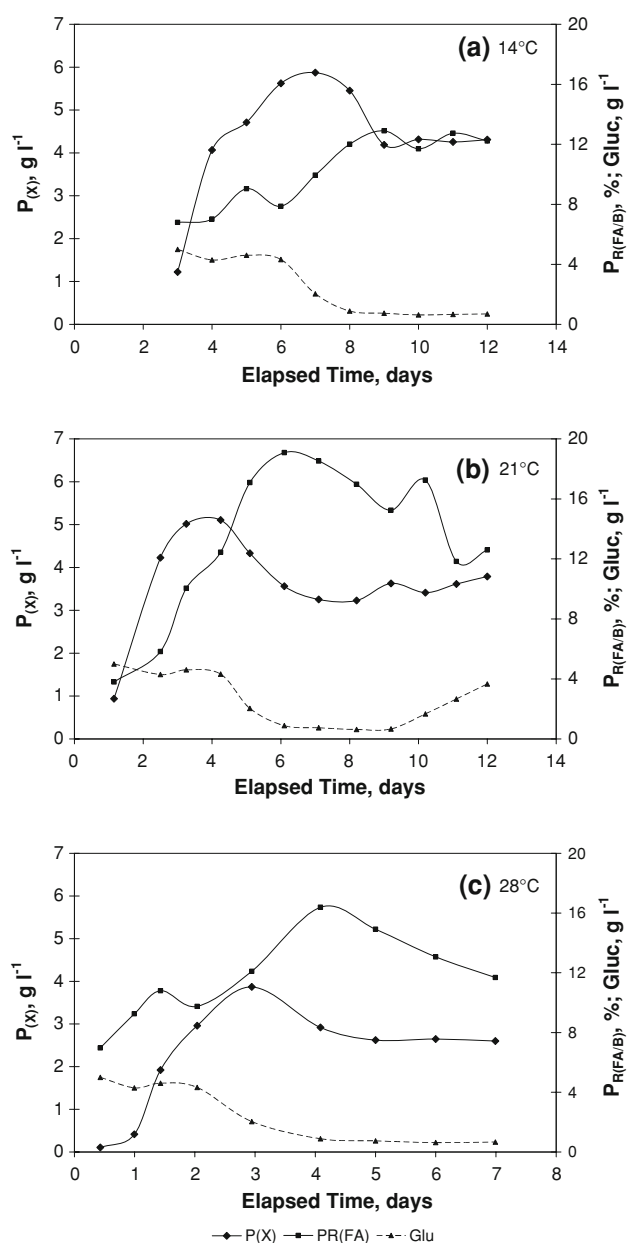


Fig. 1 Time-course effects of lipid-free biomass productivity ($P_{(X)}$), relative lipid productivity ($P_{R(FA/B)}$), and glucose consumed (GLUC) during batch growth of *P. irregularis* (ATCC 10951) in submerged culture using modified yeast-malt extract medium: **a** 14°C, **b** 21°C, **c** 28°C

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

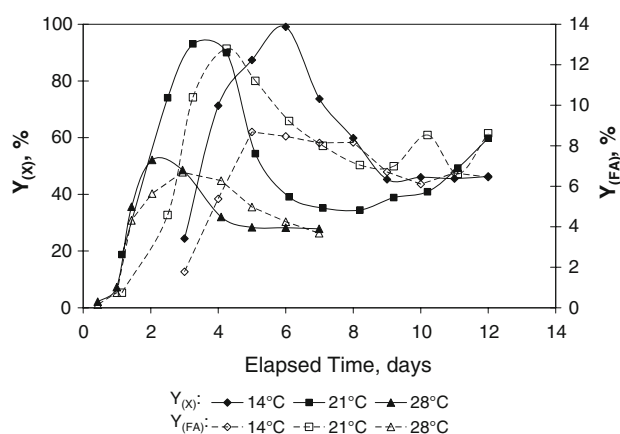


Fig. 2 Time-course effects of yield of lipid-free biomass (X) and lipid (FA) of *P. irregularis* (ATCC 10951) in submerged culture using modified yeast-malt extract medium

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.

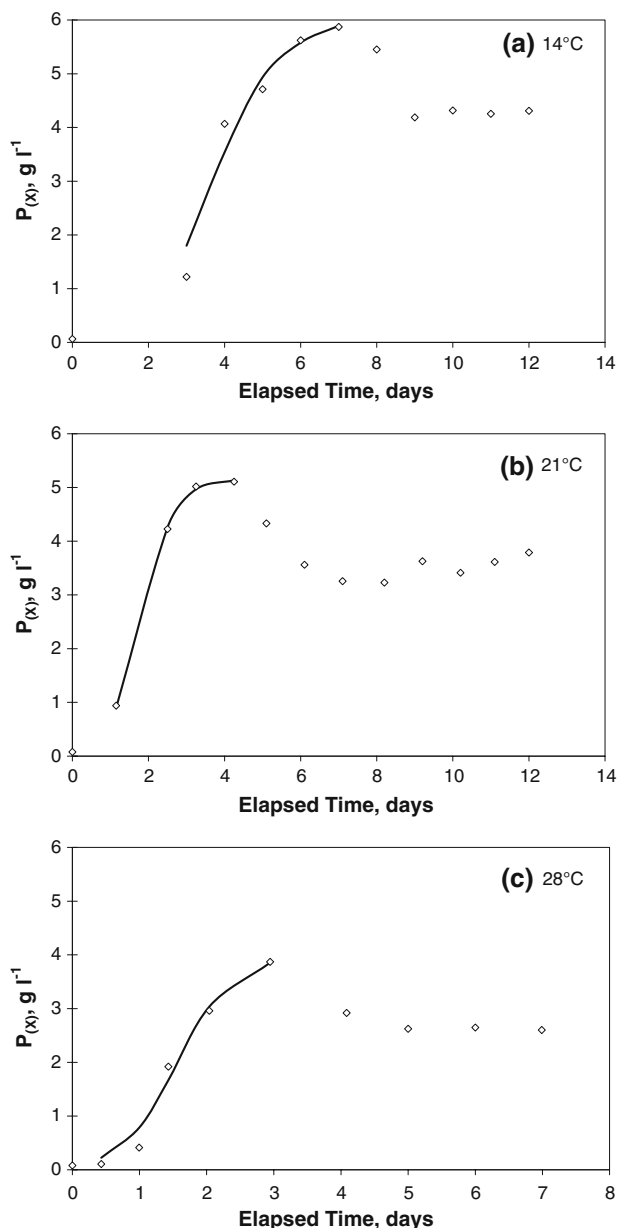
Peak biomass accumulation and yield did not occur at the same time as peak lipid accumulation (Figs. 1, 2). Lipid accumulation was a maximum for this study when fermentations were conducted on day 4 at 21°C with a $P_{R(FA/B)}$ value of 19.1%. This value decreased for fermentation temperatures 14 and 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. irregularis*. 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. irregularis* (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), $2.50 \times 10^5 \text{ h}^{-1}$.

Composition of the Lipid Extract

Composition of the individual and total fatty-acid content of the lipid extract differed slightly at each temperature-dependent $Y_{(\text{FA})}$ (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. irregularis* growth at 14°C. The EPA content at 14 and 21°C fell within the range (6–12%) reported elsewhere for *P. irregularis* 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 (C18:1 ω -9), linoleic (C18:2 ω -6), and α -linolenic (C18:3 ω -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. irregularis* 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} , ω	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} ω 9	4.32	22.5	21.5	23.7
Linoleic	C _{18:2} ω 6	0.89	10.7	11.0	11.2
γ -linolenic (GLA)	C _{18:3} ω 6	0.33	1.01*	0.56	3.62*
Arachidic	C _{20:0}	1.08	0.71	1.06	0.96
<i>cis</i> -11-Eicosenoic	C _{20:1} ω 9	1.40	1.28	2.10	1.28
Arachidonic (ARA)	C _{20:4} ω 6	0.64	5.32	5.12	3.03*
Eicosapentaenoic (EPA)	C _{20:5} ω 3	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}, ω : 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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