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Carbon-to-nitrogen ratio affects the biomass composition and the fatty acid profile of heterotrophically grown *Chlorella* sp. TISTR 8990 for biodiesel production

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ABSTRACT

Chlorella sp. TISTR 8990 was cultivated heterotrophically in media with various initial carbon-to-nitrogen ratios (C/N ratio) and at different agitation speeds. The production of the biomass, its total fatty acid content and the composition of the fatty acids were affected by the C/N ratio, but not by agitation speed in the range examined. The biomass production was maximized at a C/N mass ratio of 29:1. At this C/N ratio, the biomass productivity was 0.68 g L^{-1} d^{-1}, or nearly 1.6-fold the best attainable productivity in photoautotrophic growth. The biomass yield coefficient on glucose was 0.62 g g^{-1} during exponential growth. The total fatty acids (TFAs) in the freeze-dried biomass were maximum (459 mg g^{-1}) at a C/N ratio of 95:1. Lower values of the C/N ratio reduced the fatty acid content of the biomass. The maximum productivity of TFAs (186 mg L^{-1} d^{-1}) occurred at C/N ratios of 63:1 and higher. At these conditions, the fatty acids were mostly of the polyunsaturated type. Allowing the alga to remain in the stationary phase for a prolonged period after N-depletion, reduced the level of monounsaturated fatty acids and the level of polyunsaturated fatty acids increased. Biotin supplementation of the culture medium reduced the biomass productivity relative to biotin-free control, but had no effect on the total fatty acid content of the biomass.

1. Introduction

Microalgae have attracted much attention as a potential source of triglyceride oils for making biodiesel (Chisti, 2007; Hu et al., 2008; Huang et al., 2010). Microalgae can be grown photoautotrophically, heterotrophically and mixotrophically (Miao and Wu, 2006; Xu et al., 2006; Heredia-Arroyo et al., 2010). The photoautotrophic process requires sunlight, carbon dioxide, water and inorganic nutrients (Chisti, 2013), but the biomass and oil productivities of photoautotrophic cultures are low. This is because of a limited penetration of light in a dense culture of algal cells. Until the limitations of photoautotrophic growth are overcome, the production of oil using heterotrophic growth of algae on organic carbon in the dark appears to offer better commercialization prospects.

Heterotrophic culture is often far more productive than photoautotrophic growth and is easily scaled up in a conventional stirred tank bioreactor. A high concentration of biomass with a high content of triglyceride oil can be obtained in heterotrophic culture (Miao and Wu, 2006; Xu et al., 2006; Heredia-Arroyo et al., 2010; Liu et al., 2011). For example, the lipid content in heterotrophically grown *Chlorella protothecoides* was reported to be 4-fold greater than in the photoautotrophically grown biomass of the same alga (Miao and Wu, 2006). Similarly, heterotrophically grown *Chlorella zofingiensis* was found to have a much higher level of triglycerides compared to the photoautotrophically grown alga (Liu et al., 2011). Heterotrophic culture is especially feasible if a cheap organic carbon source is readily available. This may be sugars from waste molasses or glycerol generated in the ubiquitous biodiesel manufacturing operations. In general, *Chlorella* sp. is capable of rapid heterotrophic growth on glucose and certain other carbon sources (Shi et al., 1999; Xu et al., 2006; Xiong et al., 2008; Heredia-Arroyo et al., 2010; Liu et al., 2010; Cho et al., 2011). For *C. zofingiensis*

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grown in the dark on various carbon sources (lactose, galactose, sucrose, fructose, mannose, glucose), biomass with the highest levels of total fatty acids (TFAs) of 40–45% of dry weight was obtained using sucrose, fructose, mannose and glucose (Liu et al., 2010). Oleic acid (C18:1) constituted 32–36% of the TFAs (Liu et al., 2010).

The suitability of a triglyceride oil for making biodiesel depends on its fatty acid profile, that is the relative proportions of the saturated fatty acids (SFAs), the monounsaturated fatty acids (MUFAs) and the polyunsaturated fatty acids (PUFAs) in TFAs of the oil (Knothe, 2008; Knothe, 2009). The MUFAs are needed for improving the oxidative stability and ignition performance in terms of the cetane number of biodiesel (Knothe, 2008; Knothe, 2009). The fuel properties of a biodiesel can be reliably predicted from its composition measured in terms of the fatty acid methyl esters (Talebi et al., 2014). The suitability of a microalgal oil for making biodiesel may be improved by modifying the growth conditions of the biomass as these conditions influence the fatty acid composition of the oil (Chen, 1996; Xu et al., 2006; Xiong et al., 2008; Cho et al., 2011; Sahu et al., 2013; Talebi et al., 2013). Also, the total content of the triglycerides in an algal biomass is often increased by nitrogen starvation (Illman et al., 2000; Chisti, 2007; Converti et al., 2009) and other possible stress factors. This effect of nitrogen starvation occurs both in photoautotrophic and heterotrophic growth. Nitrogen starvation subsequent to biomass growth under nitrogen sufficiency provides a straightforward method for enhancing the oil content of the biomass. Although several different nitrogen sources are potentially suitable for growing algae such as *Chlorella* sp. (Shen et al., 2010), nitrate is generally preferred on account of being cheap.

The synthesis of fatty acids requires several enzymes. The rate of synthesis is regulated by acetyl coenzyme A carboxylase (ACCase). ACCase plays a key role in elongation of fatty acids by catalyzing the biotin-dependent carboxylation of acetyl coenzyme A to malonyl coenzyme A (Hu et al., 2008). Consequently, biotin is a cofactor in the synthesis of fatty acids (Hu et al., 2008).

Here we report on heterotrophic growth of the microalga *Chlorella* sp. TISTR 8990 on glucose. The effects of the carbon-to-nitrogen ratio, the agitation speed, and supplementation with biotin are reported on biomass production, its fatty acid content and the composition of the fatty acids in the biomass. The duration of the postgrowth nitrogen starvation period is shown to influence the fatty acid composition of the biomass.

2. Materials and methods

2.1. Microalga and medium

Chlorella sp. TISTR 8990 isolated from an outdoor pond at Walailak University, Nakhon Si Thammarat, Thailand, in February 2007, was used. A voucher culture was deposited at Thailand Institute of Science and Technology Research, Thailand, under the accession number TISTR 8990. The stock culture was maintained at 25 °C on agar slants. The agar medium had the following (Horikoshi et al., 1981) components (per liter of distilled water): 2 g KNO_3, 1 g KH_2PO_4, 1 g $MgSO_4{\cdot}7H_2O$, 2 mg $FeSO_4{\cdot}7H_2O$, 2.86 mg H_3BO_3, 1.81 mg $MnCl_2{\cdot}4H_2O$, 0.22 mg $ZnSO_4{\cdot}7H_2O$, 0.08 mg $CuSO_4{\cdot}5H_2O$, 0.021 mg Na_2MoO_4 and 15 g agar. The slants were held under continuous light (3 klux) supplied from 18-W daylight fluorescent lamps.

2.2. Preparation of algal seed

A loopful of the alga was transferred aseptically to a 250-mL Erlenmeyer flask containing 100-mL of the above specified sterile basal medium without agar and with 2 g L^{-1} of glucose. The flasks were incubated (30 °C, 150 rpm) in an illuminated shaker incubator (SANYO Gallenkamp orbital incubator, illuminated model, Japan). The illumination was at 5 klux with a 16 h/8 h light/dark cycle. The incubation period was 3 days, or until the culture attained exponential growth. A mixotrophic preparation of seed was used because with this method the alga rapidly attained exponential growth compared to both phototrophic and heterotrophic methods. To prepare an inoculum for heterotrophic growth, the algal seed was adjusted to a cell concentration of 1.2×10^8 cells mL^{-1} with a sterile aqueous solution of sodium chloride (0.85% w/w NaCl). This standardized seed was used to inoculate the fresh medium such that the inoculum made up 10% of the final volume.

Table 1
Combinations of the C/N ratio and the agitation speed used in the various runs.

Run no.	Conditions	
	C/N ratio (g C g N^{-1})[a]	Agitation speed (rpm)
1	29:1	100
2		150
3		200
4	63:1	100
5		150
6		200
7	95:1	100
8		150
9		200

[a] The C/N ratio was varied at a fixed C-concentration (2 g C L^{-1}, or 5 g L^{-1} glucose).

2.3. Experimental design

2.3.1. C/N ratio and agitation speed

A full factorial design with two factors of C/N ratio (g of carbon in glucose per g of nitrogen in KNO_3) and agitation speed, was used for investigating the effect on the peak biomass production and the lipid content of the biomass grown heterotrophically. Each factor consisted of three levels. These were: a C/N ratio of 29:1, 63:1 and 95:1 and an agitation speed of 100, 150 and 200 rpm. The combination of factors and levels resulted in 9 separate experimental runs (Table 1) that were carried out in duplicate.

The biomass was grown in 500 mL Erlenmeyer flasks. Each flask contained 300 mL of the basal medium supplemented with 5 g L^{-1} glucose (2 g of C L^{-1}) and various concentrations of potassium nitrate to obtain the target C/N ratio. The medium was adjusted to a pH of 6.0 before autoclaving (121 °C, 15 min). The antibiotic amoxicillin (10 mg L^{-1}) was added to the medium before inoculation. The standardized seed culture was added such that the inoculum constituted 10% of the final volume. All cultures were held in the dark at 30 °C on an orbital shaker. The biomass was harvested on day 4. A glucose concentration of 5 g L^{-1} was used as preliminary work had shown it to be fully consumed within 4-days so that the cultures could attain a stationary phase of growth by harvest. Accumulation of storage oils generally occurs in the stationary phase and not during rapid growth. Similarly, an initial pH of 6 was used as it had been found to be optimal in preliminary studies.

2.3.2. Biotin

The effect of biotin at various concentrations (0–1.0 mg L^{-1}) on biomass and lipid production was investigated in heterotrophic growth in 500 mL Erlenmeyer flasks. Each flask contained 300 mL of the above specified basal medium with a specified C/N ratio. The agitation speed was fixed at 150 rpm. The biomass was harvested on day 4. All cultures were grown in duplicate.