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Improvement of medium composition for heterotrophic cultivation of green microalgae, *Tetraselmis suecica*, using response surface methodology

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ABSTRACT

Optimization of medium composition for the improvement of heterotrophic cultivation of green microalgae, *Tetraselmis suecica*, was performed using response surface methodology (RSM). Heterotrophic cultivation of *T. suecica* was conducted in total darkness using Walne medium formulated with natural sea water. Initially, the effect of two types of carbon source (glucose and sodium acetate) and various types of nitrogen source (peptone, yeast extract, meat extract, malt extract, urea, sodium nitrate and ammonium nitrate) on growth of *T. suecica* was studied. The concentration of medium component that was found to significantly influence the heterotrophic growth of *T. suecica* (glucose, peptone, yeast extract and meat extract) was further optimized using RSM. The medium that consists of 5.78 g/L glucose, 9 g/L peptone, 4.48 g/L yeast extract and 3.01 g/L meat extract was found optimal for heterotrophic cultivation of *T. suecica*. The final cell concentration (28.88 g/L) obtained in heterotrophic cultivation using this optimized medium was about 3 and 2 times higher than obtained in photoautotrophic culture (8.40 g/L) and non-optimized medium for heterotrophic cultivation (13.81 g/L), respectively. In addition, the cell yield based on glucose consumed (9.31 g cell/g glucose) was increased by about 3 times as compared to non-optimized medium (3.61 g cell/g glucose).

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1. Introduction

Algal biomass has been widely used as fertilizer [1], food source for both humans and animals [2], biological cells for secondary waste water treatment [3] and bioremediation [4,5]. Through the advances of processing technology, algal biomass is currently used as the possible source of fuels, fine chemicals, and pharmaceuticals [6]. Several species of microalgae, capable to produce useful chemicals such as amino acids, vitamins, carotenoids, fatty acids, polysaccharides, and antibiotics, have been reported [7,8,9]. Development of processes that utilize microalgal biomass as renewable energy sources has also been reported [10,11].

The heterotrophic cultures may offer several advantages over culture systems requiring light for photosynthesis. Photoautotrophic cultivation is considered the most desirable method of culturing microalgae, in which, sunlight is used as "freely available" photosynthetic energy source. Furthermore, the photosynthetic efficiency of microalgae is substantially higher than plants [12]. However, photoautotrophic cultivation in open ponds or indoors under natural or artificial lighting suffers limitation in the supply of light. The continuously growing algae with increasing density eventually reduce light penetration that limit growth to a final cell concentration rarely exceeds 0.5 g dry cell weight/L [13]. The availability of algal feeds in abundance is recognized as major bottleneck in fishes, crustaceans and molluscs farming. Hatcheries relying upon continuous supply of microalgae feeds demand for large volumes of water to be handled in photoautotrophic cultivation systems, invariably leading to increasing requirement for land, labor forces and energy. Photoautotrophic cultivation also faces a difficulty in process control with fluctuation in quality, frequent predatory grazing, unwanted contaminants and unpredictable algal culture "crashes" (massive die-off) causing severe financial repercussions [14].

Knowledge and experience in bioreactor design with efficient control systems have been established for many industrial processes employing microorganisms, mammalian cells and plant cell culture. Similar bioreactor system can be easily exploited into microalgae cultivation [15]. A number of aquaculture algae species have been successfully cultivated under heterotrophic culture conditions in stirred tank bioreactor. For examples, heterotrophic *Nitzschia laevis* [16], *Crypthecodinium cohnii* [17], *Chlorella protothecoides* [18] and *Galdieria sulphuraria* [19] were cultivated for the production of eicosapentaenoic acid, docosahexaenoic acid, biodiesel and c-phycocyanin, respectively. Cultivation in bioreactor, which has small reactor surface-to-volume ratio, presents a much simpler scale-up possibilities without the need for auxiliary

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Table 1
Effect of two different carbon sources, glucose and sodium acetate, and their concentration on growth of *T. suecica* cultivated in heterotrophic condition.

Carbon source	Concentration (g/L)	Total carbon (g/L)	Cell concentration[a] (g/L)
Glucose	5	1.8	11.7 ± 0.9
	10	3.6	7.9 ± 0.6
	15	5.4	6.6 ± 1.2
Sodium acetate	0.5	0.15	6.0 ± 1.2
	1	0.29	4.1 ± 0.9
	2	0.58	3.3 ± 0.7

Walne medium formulated using natural sea water was used as a basal medium with yeast extract (0.75 g/L) as a nitrogen source.
Values are mean ± standard deviation ($n = 3$).
[a] Cell concentration was measured after 120 h cultivation.

solar receiver installation [20]. Other benefits include the use of axenic culture free from pathogenic bacteria for serial production of pure batches of algal products with consistent biochemical composition. Microalgae cultivated using this method has very high specific growth rate [21] and high final cell concentration, ranging from 50 g dry cell weight/L [13] up to 100 g dry cell weight/L [22]. High cell density culture obtained via heterotrophic cultivation may reduce the downstream processing cost [23]. Thus, heterotrophic culture shall also be considered as the potential method for commercial production of algae cells to broaden the range of economically viable microalgal products [24]. Mass cultivation of *Chlorella* in heterotrophic conditions with a final cell concentration of 14 g/L for subsequent use as health food and animal feed has been reported [25].

Formulation of medium is important in the cultivation of microalgae to obtain high final cell concentration [26]. Moreover, the medium constituents must satisfy the basic requirements for cell build-up and metabolite production, by providing an adequate supply of energy for biosynthesis and cell maintenance [27]. For heterotrophic cultivation of microalgae, glucose [28] and acetate [8] have been successfully used as carbon source. Growth of heterotrophic culture of microalgae was also greatly influenced by the type and concentration of nitrogen sources supplemented to the medium [10,29].

Tetraselmis sp. is widely used in aquaculture facilities such as feed for juvenile bivalve molluscs, penaeid shrimp larvae and rotifers [14]. This marine genus has a large spectrum of antimicrobial activity [30] and its members have probiotic properties [31]. *Tetraselmis* sp. has also been proposed as a source of vitamin E for human and animal consumption [32]. *Tetraselmis* sp. is ideal for mass cultivation because they are euryhaline and eurythermal [33,34]. To our knowledge reports on the formulation of medium for improvement of heterotrophic cultivation of *Tetraselmis* sp. is not available in the literature.

The main objective of this preliminary study on the nutrient requirement by *Tetraselmis suecica* grown under heterotrophic condition was to optimize the medium composition for the enhancement of growth performance in shake flask culture using response surface methodology. Formulation of medium was focused on the use of different types and concentrations of carbon and nitrogen sources.

2. Materials and methods

2.1. Microalgae and inoculum preparation

T. suecica (Kylin) Butcher (Chlorophyta, Prasinophyceae) was obtained from the Institute of Bioscience Culture Collection Unit, Universiti Putra Malaysia. The cells culture was maintained as a stock culture at −80 °C on agar plates containing Walne medium [35] added with 10 g/L glucose and 12 g/L bacteriological agar. Stock culture of heterotrophic *T. suecica* was inoculated into 100 mL Walne medium containing 10 g/L glucose in 250 mL Erlenmeyer flasks. The flasks were incubated at 30 °C in an orbital incubator shaker (TS-560, Germany), agitated at 130 rpm for 100 h in the dark condition, to obtain final cell concentration of around 32×10^6 cell/mL. This culture was used as a standard inoculum for all cultivations in shake flasks.

2.2. Medium formulation

Walne medium [35] formulated using natural sea water was used as a basal medium for heterotrophic cultivation of *T. suecica*. Two different types of carbon source; glucose and sodium acetate were initially tested for heterotrophic growth of *T. suecica*. Different concentrations of glucose (5, 10 and 15 g/L) and sodium acetate (0.5, 1 and 2 g/L) were added to the basal medium. The amount of each carbon source used was based on data available in the literature for heterotrophic culture of *Tetraselmis* spp. using glucose and sodium acetate as carbon source [8]. Subsequently, the effect of different types and concentrations of nitrogen source was tested using 5 g/L glucose as a carbon source. The C:N ratio used was ranged from 2 to 52. The selection of concentration range for each nitrogen source was based on the cultivation of mixotrophic and heterotrophic culture of *Spirulina* sp., *Tetraselmis* sp. and *Chlorella* sp. [14,23]. All microbiological-grade carbon and nitrogen sources used in the experiments were known to be stable when subjected to sterilization at 121 °C, 15 psi for 15 min [36]. However, some of the components were autoclaved separately as to prevent any occurrence of Maillard-type reaction, while the heat labile components such as vitamins B_{12}, B_1 and H solutions used in Walne medium were filter sterilized using 0.2 μm Whatman syringe filter.

2.3. Heterotrophic cultivation

All heterotrophic cultivations of *T. suecica* were performed in 250 mL Erlenmeyer flasks containing 100 mL medium. The sterile flasks were inoculated with 10 mL of a standard inoculum and incubated in orbital incubator shaker (TS-560, Germany) at 30 °C, agitated at 130 rpm without light exposure for 120 h. All cultivations were carried out in triplicates.

2.4. Analytical procedures

The microalgae cell number was determined using Neubauer haemocytometer by fixing the cells with methanol according to the method described by Guillard [37]. On the other hand, microalgae cell concentration, expressed as dry cell weight per culture volume, was determined using filtration and oven drying method [38]. The culture samples of known volume and cell density were washed with 0.5 M ammonium bicarbonate to remove salts and filtered through a pre-weighed dried filter paper (Whatman No. 1). The filtered cells and filter papers were later dried in an oven at 80 °C for at least 24 h until a constant weight was obtained.

Glucose and acetate were determined using High Performance Liquid Chromatography (HPLC) (Hewlett Packard 1050). Culture samples were centrifuged at 5000 × *g* for 10 min and then the supernatant was filtered through a 0.2 μm cellulose acetate membrane prior to injection into HPLC column. For glucose determination, the filtered sample was injected into Aminex HPX-87H column (Bio-Rad, Hercules, CA) eluted with 0.4 (mL/min) of acetonitrile 0.5 (mM) at 30 °C. Detection was performed by a Knauer K-2300 refractive index detector. For acetate determination, the filtered sample was injected into C18 column (Bio-Rad, Hercules, CA) using methanol–acetonitrile–triethylamine buffer solution (0.05 mol/mL, pH was adjusted to 2.5 with H_3PO_4) as

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