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Nitrate concentration-shift cultivation to enhance protein content of heterotrophic microalga *Chlorella vulgaris*: Over-compensation strategy

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HIGHLIGHTS

- Over-compensation of nitrogen can improve protein content of heterotrophic *Chlorella*.
- Convenient nitrate-added culture was adopted to implement concentration shift.
- Operating parameters were optimized to maximize protein content by one factor RSM.
- *Chlorella* was a potential single-cell protein source according to amino acid profile.

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ABSTRACT

Protein production from microalgae requires both high cell density during cultivation and high protein content in cells. Heterotrophic microalgae can achieve high cell density, and yet are confronted with the problem of low protein content. Based on over-compensation strategy, a new concentration-shift method was proposed to cultivate heterotrophic *Chlorella vulgaris*, aiming to increase protein content. With a prior starvation period, microalgae utilized more nitrate and accumulated more proteins compared to one-stage cultivation. Considering the convenience of operation, nitrate-added culture was adopted for producing heterotrophic microalgae, rather than sterile centrifugal culture. Operating parameters including nitrate concentration in N-deficient medium, N-starved time and nitrate concentration in N-rich medium were optimized, which were 0.18 g l^{-1}, 38 h and 2.45 g l^{-1}, respectively. Under the optimized conditions, protein content in heterotrophic *Chlorella* reached 44.3%. Furthermore, the heterotrophic microalga was suggested to be a potential single-cell protein source according to the amino acid composition.

1. Introduction

The investigation by WHO/FAO/UNU (2007) showed that the requirement for proteins has been sharply increased. Single-cell proteins (SCP), referring to the dried cells of microorganisms, are alternative protein sources to conventional proteins in human food or animal feed (Zepka et al., 2010). One of the most promising candidates for SCP is microalgae. Microalgae have been utilized in human nutrition for centuries in oriental countries, owing to the proteins and other beneficial nutrients (Prabhasankar et al., 2009). Besides, microalgae are increasingly utilized as feed additives both in aquaculture and in animal husbandry, since they have the ability to support animal growth and positively affect the physiology (Pulz and Gross, 2004). The traditional technique for obtaining microalgae is photoautotrophic cultivation. However, its commercialization faces some technical and economic challenges, largely due to low biomass productivity (Ogbonna et al., 1997; Xie et al., 2012). There is thus a need for the development of efficient systems for microalgae cultivation.

Heterotrophic cultivation of microalgae has the potential to overcome or minimize the problems associated with autotrophic cultivation (see Appendix A). First, heterotrophic culture systems get rid of the reliance of light, and scale-up is easy. Second, contamination from other microorganisms can be effectively prevented under closed systems, and culture conditions can be properly controlled so as to optimize cell yield. Third, organic carbon can greatly increase cell density in a short time, consequently achieving high biomass productivity. Fourth, high-cell-density culture can reduce the cost of down-stream processing, and help to

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reduce production cost (Bumbak et al., 2011; Miao and Wu, 2006; Shen et al., 2009). In this context, significant attention has been drawn to microalgae heterotrophic cultivation. Nevertheless, protein content of heterotrophic cells (10.3–25.8%) was much lower than that of autotrophic cells (up to 52.6%) (Miao and Wu, 2006; Ogbonna et al., 1997). As SCP, high protein content is important for animal health, especially in the rapidly growing juvenile life stage and in the active gametogenesis period, because proteins are the fundamental building blocks for tissue biosynthesis and enzyme production (Gatenby et al., 2003). For instance, *Mytilus trossulus* (mussel) grew significantly slow when dietary protein content dropped below 40% (Kreeger and Langdon, 1993). Hence, the search for the approach to enhance protein accumulation is well expected for more efficient microalgae application.

Excellent environmental adaptability provides opportunities to control microalgal protein formation, on account of the flexibility of cell composition affected by culture conditions (Bumbak et al., 2011). Several methods were explored for high protein yield, including sequential heterotrophic/autotrophic cultivation, the utilization of plant growth substances, *etc.* (Bajguz and Piotrowska-Niczyporuk, 2014; Ogbonna et al., 1997). Notably, microalgae can be triggered to uptake more substrates than are necessary for survival. In this way, nitrogen is absorbed in excess and stored in the form of proteins. Two mechanisms are involved in the excess storage of a particular substrate. When microalgae are starved of an element and then re-exposed to it, the consequent storage is referred to as "over-compensation". The other mechanism is defined as "luxury uptake", which means the continued uptake of a resource beyond what is required for immediate growth without a prior starvation stage (Brown and Shilton, 2014). The researches of algal luxury uptake focused on phosphorous, and nitrogen was found to be difficult to implement sustained assimilation (Wu et al., 2012). Contrastively, Cho and Komor (1984) observed an unusually high rate of nitrogen assimilation when nitrogen became available to N-starved cells. The phenomenon might be related to an improved level of carbon skeletons. Carbon skeletons in the form of keto-acids (2-oxaloglutarate and oxaloacetate) are required in the primary assimilation of inorganic nitrogen (ammonium) to synthesize amino acids (Perez-Garcia et al., 2011); and Millbank (1957) found that the level of keto-acids was considerably high in *Chlorella* under N-starved conditions. Yet, over-compensation of nitrogen has not caused enough attention.

Over-compensation strategy can be implemented by nitrogen concentration-shift cultivation. The method is different from usual two-stage culture techniques. In general, the first stage of two-stage culture is considered as a cell growth period when cell number increases rapidly; the second stage is considered as a product accumulation period when cell number increases little but non-growth-linked product content is increased (Aflalo et al., 2007). In contrast, the N-deficient stage of over-compensation culture provides a starvation stimulation to cells, and cell number increases little; substantial accumulations of both biomass and protein product occur in the N-rich stage, simultaneously.

This paper aims to develop an over-compensation method to enhance protein accumulation of heterotrophic microalgae. The work investigated the effect of nitrogen concentration on cell growth and protein accumulation. Two concentration-shift procedures were proposed and compared, *i.e.* sterile centrifugal transfer culture and nitrate-added culture. Operating parameters were optimized to maximize protein content. In addition, the nutritive value of heterotrophic microalgae was evaluated by the analysis of amino acid pattern.

2. Materials and methods

2.1. Microorganism and cultivation

The microalga *Chlorella vulgaris* FACHB-8 was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The basal culture medium was Brostol's solution (also known as soil extract, SE) (Cheng et al., 2013).

In heterotrophic cultivations, SE medium was supplemented with 10 g l^{-1} glucose, namely SE^+ medium. *Chlorella* was precultured with sterilized SE^+ medium for 5 days at 25 °C and 150 rpm without light exposure. Microalgal samples were diluted with sterile water to a concentration of 5.0×10^7 cells ml^{-1}, and prepared for inoculation. All cultivations were performed in 250-ml Erlenmeyer flasks containing 100 ml liquid media. The sterile flasks were inoculated with 10 ml of microalgal suspension and incubated in the thermostatic incubator shaker at 25 °C and 150 rpm without light exposure. Autotrophic cultivations were conducted as control, which used SE medium. *Chlorella* was cultured autotrophically for 12 days at 25 °C under continuous illumination of 4000 lux, and the flasks were hand shaken six times daily. Each experiment was carried out in triplicate and average values with standard deviations were reported.

Nitrogen concentration of SE^+ medium was adjusted in order to examine its effect on microalgal growth, nitrogen consumption and protein accumulation. Including the original concentration, six different sodium nitrate concentrations (0.25, 0.75, 1.00, 1.25, 1.50 and 2.00 g l^{-1}) were tested. Other conditions were the same as described above. Samples were regularly taken for the detection of biomass and residual nitrogen concentration. Protein content was measured on the 7th day.

2.2. Concentration-shift procedures

2.2.1. Sterile centrifugal transfer culture

Microalgal cells were first cultivated in N-deficient medium (using SE^+ medium). At 48 h, cells were collected by centrifugation (3556*g*, 3 min) under sterile conditions, and resuspended in fresh N-rich medium (*i.e.* modified SE^+ medium with increased nitrate concentration) to continue the culture for a total of 7 days. Three levels of sodium nitrate concentration in N-rich medium (1.00, 1.25 and 1.50 g l^{-1}) were tested. Biomass, protein content and residual nitrogen concentration were measured at the end of culture. One-stage cultivation in the corresponding N-rich medium was performed as control, which had similar nitrogen supply neglecting nitrogen in N-deficient medium. Differences were evaluated by *t*-test, and were considered significant when $p < 0.05$.

2.2.2. Nitrate-added culture

Microalgal cells were first cultivated in N-deficient medium (using SE^+ medium). At 48 h, 1.00, 1.25 and 1.50 g l^{-1} of sodium nitrates were added into the media respectively, and then the culture continued for a total of 7 days.

2.3. Method optimization

Response surface methodology (RSM) is a collection of mathematical and statistical techniques based on the fit of empirical models to the experimental data, and describes the behavior of a data set with the objective of making statistical previsions (Bezerra et al., 2008). One factor RSM was carried out to study and model the impacts of method parameters on protein accumulation using Design-Expert software (trial version 8, Stat-Ease, MN, USA). The change of protein content with varying each parameter