Int. J. Chem. Sci.: 8(5), 2010, S84-S91

MILKING OF *SPIRULINA PLATENSIS* FOR THE PRODUCTION OF CAROTENOIDS BY AQUEOUS TWO PHASE BIOREACTOR SYSTEMS

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ABSTRACT

The low productivity of algal cultures in the production of high-value compounds is the most significant bottleneck for commercialization of this technology. Cultures in which cell mass is reused for continuous production are proposed as a solution to overcome this problem. Recently, a method was developed in which carotenoids were as harvested from the microalga *Sirulina platensis* grown in a two-phase bioreactor. Specific growth rate was high only during day time. It was 60% at 25 hours and reduced to 30% at 95th hour. 90% of the cells were viable in the presence of propanol. After 24 hours 21% of the cells were viable. Furthermore, microscopic observations showed both dead and live cells after 24 hours and only dead and destroyed cells after 48 hours in aqueous two phase bioreactor systems. Total carotenoids were 135 microgram/L and chlorophylls were 5 microgram/L. So the present investigation reveals that aqueous two phase bioreactor system is an alternative algal technology for milking of *Spirulina platensis*.

Key words: *Spirulina platensis,* Aqueous two phase bioreactor systems, Growth kinetics, Carotenoids.

INTRODUCTION

Natural pigments have long been used in foods, medicines and cosmetics. They are widely distributed in living organisms with a large number of structures reported. Most biological pigments are classified into six kinds of structures : tetrapyrroles, isoprenoids, quinines, benzopyrans, N-heterocyclic compounds, and metalloproteins[1]. Carotenoids are a class of hydrocarbons, collectively called carotenes, and their oxygenated derivatives are called xanthophylls. Carotenoids are distributed widely in bacteria, fungi, algae, plant and in animals. They have diverse functions in photosynthetic organisms. They serve two major recognized functions during photosynthesis, one that they act as accessory pigments for light-harvesting and the other as triplet quenchers to protect from photo oxidation[2,3]. Carotenoids are widely used in the agro-food industry due to their colorant properties, or in the cosmetics and pharmaceuticals industries due to their antioxidant properties and photo-protective qualities.

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Carotenoids are strong antioxidants, scavenging potentially harmful oxy radicals, which are commonly associated with the induction of certain cancers[4]. Therefore, carotenoids, mainly β-carotene, are widely used by the food, pharmaceutical, and cosmetic industries. Increasing demand for β-carotene, mostly natural β-carotene, has resulted in growing interest in extracting β-carotene from different natural sources such as vegetable and fruit wastes[5].

Micro algae are photosynthetic microorganisms which utilize light energy and CO_2 for the production of high-value compounds. Although microalgae are a unique source for high-value compounds, their commercial application is still limited[6]. A major bottleneck for the application of most microalgae is the low productivity of the processes used up till now. One fundamental reason for this is the relatively low growth rate, mainly because of inefficient use of light (usually from solar illumination). Therefore, it takes a long time to just produce the biomass. The blue-green algae, *Spirulina platensis*, has been used for hundreds of years as a food source for humans and animals due to the excellent nutritional profile and high carotenoids content. Spirulina is relatively high in protein with values ranging from 55-65% and includes all of the essential amino acids[7].

Carotenoids can be prepared by spray-drying of algal biomass and sold in the form of β-carotene-rich biomass tablets or capsules. It can also be separated from the algal cells by extraction with organic solvents or edible oils[2]. β-Carotene and chlorophyll are extracted by contacting the cells with organic solvents or edible oils. To increase the extraction yield, the cells are destroyed before adding organic solvents or during the extraction process by strong solvents with high polarity. After separation of organic phase, purification of β-carotene can be done by using several methods. The conventional techniques used for product recovery, for example precipitation and column chromatography, are not only expensive but also result in lower yields. Therefore, there is an ongoing need for new, fast, cost-effective, ecofriendly simple separation techniques.

Thus, in light of the above, the present project is aimed at Milking of Micro algae: Production and selective extraction of carotenoids in two-phase bioreactors from *Spirulina platensis* as an alternative algal technology to traditional organic-water solvent extraction systems.

EXPERIMENTAL

Materials and methods

Algal culture

Liquid culrure of *Spirulina platensis* was obtained from Spirulina Farms, Auroville, Pondicherry. It was grown and maintained in Zarrouks medium for 8 days.

Cell density

The measurement based on turbidity of cell culture was performed in a spectrophotometer. Optical density (OD) was measured at 750 nm and was linear over a range of

2×10^5 to 1×10^7 cells per mL. OD obtained at this wavelength would not be interfered by chlorophyll absorbance[8] .

Cell counting

Cell culture was sampled and transferred to a neubauer counting chamber, and counting was done under a light microscope. For motile cell culture, 0.1% glutaraldehyde was added to immobilize the cells. The suspension was allowed to flow by capillary action under a cover slip. For cell densities up to about 1×10^6 cells per mL, the centre 25 larger squares were counted. For greater densities, five of these squares (either diagonal) were counted and multiplied by five to determine the density of the original culture. An equation of cell density for a 25-square counting is presented here[9].

$$\text{Cell density (cells mL}^{-1}) = (\text{No. of cells}) \times (1 \times 10^4) \times (D) \quad \ldots(1)$$

Growth kinetics of spirulina platensis

Growth kinetics of *Spirulina Platensis* was calculated using the following equation.

$$\text{Specific growth rate (\%)} \frac{\ln (W_2) - \ln (W_1)}{T_2 - T_1} \times 100 \quad \ldots(2)$$

Where W_2 = Dry weight (g) at T_2, W_1 = Dry weight (g) at T_1 and T = Time (days)

$$\text{Doubling time (hours)} = \frac{\ln (2)}{\log (W_2)/(W_1)} \times (T_2 - T_1) \quad \ldots(3)$$

Where, W_2 = Dry weight (g) at T_2, W_1 = Dry weight (g) at T_1 and T = Time (hours)

Determination of the viability and the physiological activity of the cells

Viability of the cells was checked by microscopic observations. Living cells move. Cells that are destroyed or that do not move are considered dead. Because of the influence of solvent on the OD, the cell number after addition of the solvents was determined by direct cell counting. Absorbance measurements were done in duplicate and counting of the cells was carried out 4 times for each sample.

Pigment analysis

Carotene and chlorophyll contents were estimated in aqueous and solvent phase by extracting 10 mL cell culture with propanol. Absorbance was measured at 663.2, 646.8 and 449.0 nm. Carotenoids and chlorophyll were calculated using Beer-Lambert's law with their absorption coefficients.

RESULTS AND DISCUSSION

Growth kinetics of *Spirulina platensis* is represented in Fig. 1. Growth of spirulina clearly indicates its light dependence. Specific growth rate was high only during day time. It was 60% at 25 hours and reduced to 30% at 95th hour. After 195 hours the culture attained stationary phase. These specific growth rates are in agreement with those of[10], who reported generation times for members of the *D. salina*. The same result was supported by the variations in doubling time at the restective hours from Fig. 2. This result indicates that temperature and light intensity are not independent variables. Presumably, growth at a fast rate at high temperature can only be maintained when sufficient energy is provided by photosynthesis.

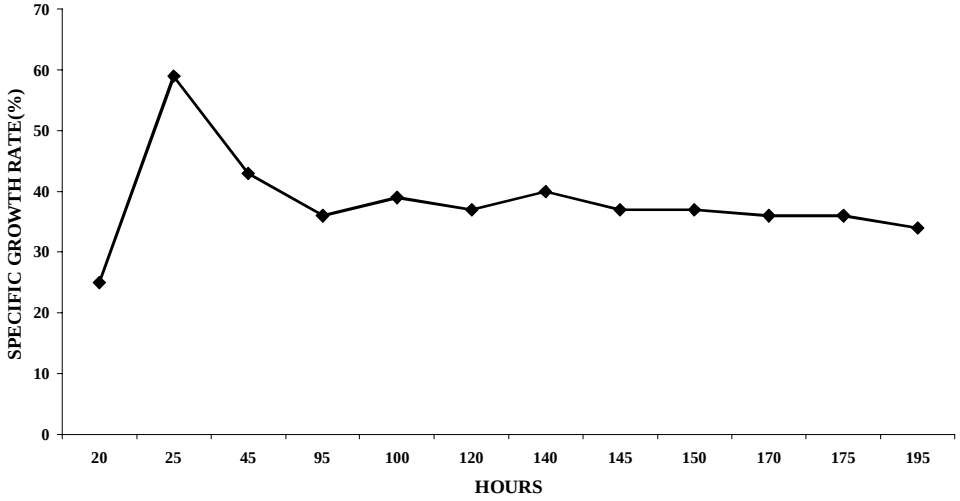

Fig. 1: Growth kinetics of spirulina

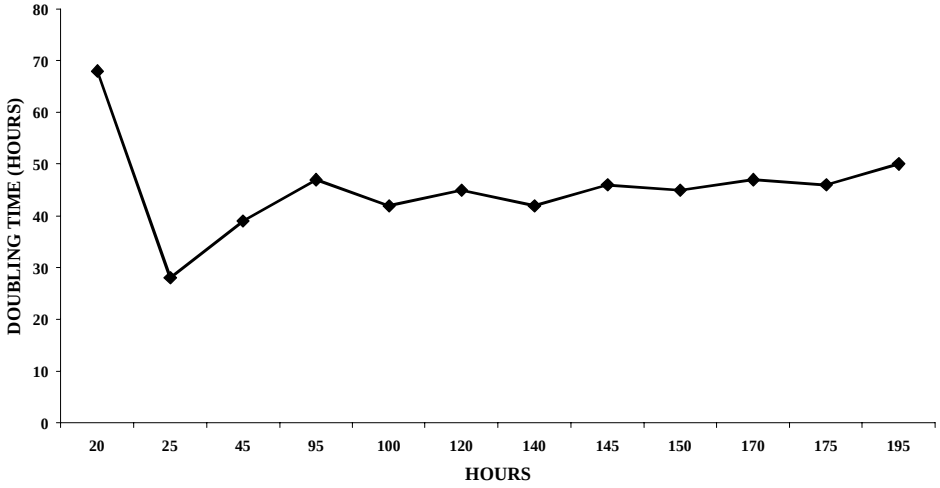

Fig. 2: Doubling time of spirulina in control

Viability of cells in the presence of different organic solvents

The viability of the cells in the presence of different organic solvents was judged by the appearance of the culture media and microscopic observations. The color of the culture media with propanol turned from green to white after 48 h. Furthermore, microscopic observations showed both dead and live cells after 24 hours and only dead and destroyed cells after 48 hours. The living cells appeared to be not as active and mobile as the normal cells after 24 hours. This is due to the impact of propanol on *Spirulina platensis*.

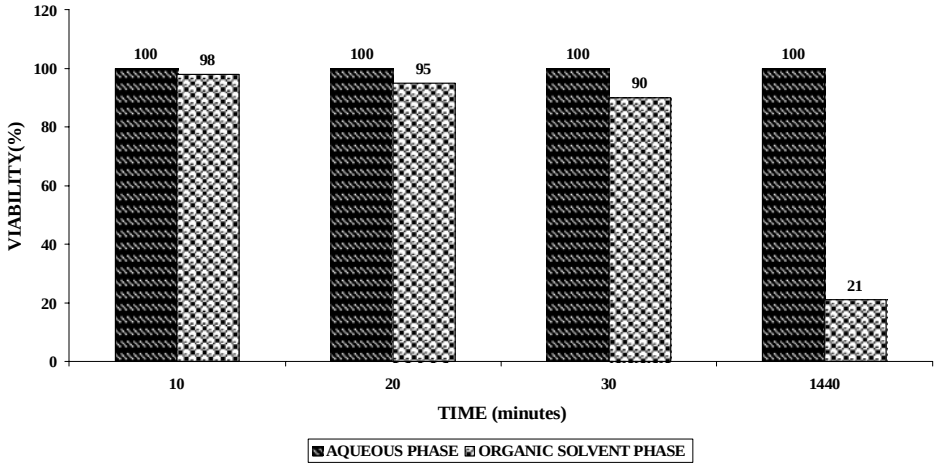

Fig. 3: Visbility of cells in aqueous two phase bioreactor systems

The concentration of total chlorophyll and total carotenoids is presented in Fig. 4. Two-phase bioreactors consist of an aqueous phases and an organic propanol phase. Our previous studies showed that the selective extraction of carotenoids was well with propanol. So propanol was used as an organic phase in the aqueous two phase bioreactor system. It indicates that the cells contain both carotenoids and chlorophyll a. However, the concentration of chlorophyll was very less 5 microgram/L and the concentration of total carotenoids was 132 microgram/L. It indicates that there is a preference for the extraction of carotenoids over chlorophyll by the biocompatible solvents.

According to the literature chlorophyll of plant cells is heterogenically bound to other compounds in the chloroplast and most of these bonds are strong hydrophilic bonds. Highly polar solvents are needed for breaking down these strong chemical bonds[11,12] Complete chlorophyll extraction is only possible by using polar solvents. Meanwhile, carotenoids can be easily extracted by the solvents with lower polarity.

It is obvious that the effect of the solvents on the cell membrane has an important role on the extraction of intracellular compounds by the solvents. Solvents with lower hydrophobicity reach critical concentrations more easily, necessary for inactivation and breaking down of the cell membrane[13]. These solvents can break down the cell membrane and release more intracellular

compounds such as pigments. By increasing the hydrophobicity the effect of solvents on the cell membrane decreases and the extraction ability for both chlorophyll and β-carotene decreases, as well. However, this decrease is stronger for chlorophyll. The results are in agreement with the results of previous researchers. One of the important advantages of the in situ extraction of β-carotene is selectivity of its extraction over chlorophyll[14]. It seems that selective extraction of β-carotene might be because of the strong bonds between chlorophyll and other cell components. Whole-cell biocatalysis in a two-phase system is used for the production of metabolites with a greater affinity to another phase, immiscible with the aqueous cell phase. In such a system the extraction rate is often less than in conventional ones but growth of microorganisms as well as production and separation of metabolites occur simultaneously and continuously[15]. "Milking" products from bacteria has been reported[16,17]. Therefore, overall productivity in nonconventional systems can be higher and downstream processing is often easier.

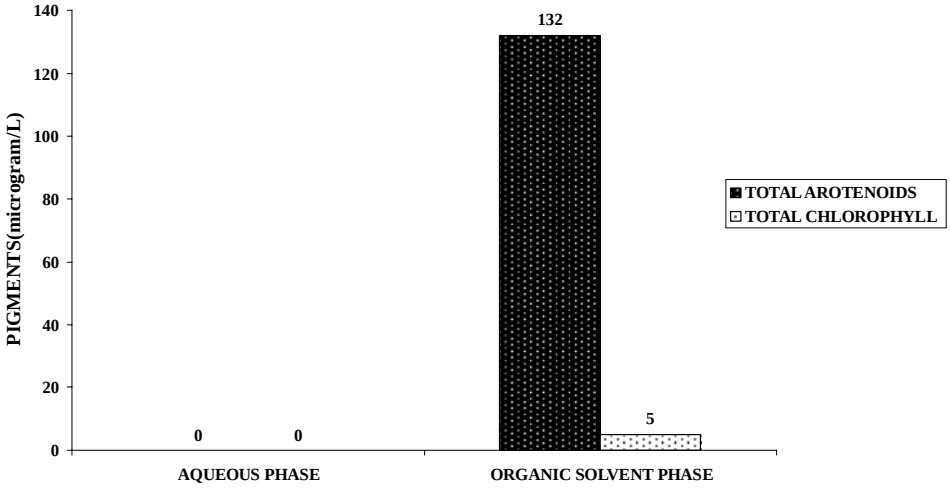

Fig. 4: Pigment profile in aqueous and organic solvent phase

Hypothesis for the extraction process

Propanol dissolves in the cell membrane and causes some alterations there. Subsequently, it is taken up by the cells into the space between cell membrane and chloroplast membrane by diffusion. This leads to more activity in the membranes and to more active endo- and exo-cytosis. As a result the globules are moved from the chloroplast to the space between the cell and chloroplast membranes and afterwards released from there to the medium. Another hypothesis suggests that molecules of β-carotene are released from the globules as a result of alterations in the membranes of the globules. The molecules diffuse from the chloroplast to the space between the chloroplast and cell membranes. From there the molecules either directly diffuse to the medium or first are accumulated inside the vesicles and then released from the cells by exo-cytosis. The extraction rate was affected at least by two parameters: contact area between the cells and the organic solvent and β-carotene content of the cells[18]. During this process the medium in which the algae grow is taken up in small vesicles. After a few seconds the vesicles are released again.

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