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MINI-REVIEW

Microbiological production of tocopherols: current state and prospects

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Abstract Tocopherols are antioxidants that prevent various diseases caused by oxidative stress. Tocochromanols comprise four isoforms of tocopherols and four isoforms of tocotrienols but α-tocopherol is the most abundant and active isoform in human and animal tissues. Tocopherols are used as dietary supplements for human, as food preservatives, in manufacture of cosmetics, and for fortification of animal feed. Only photosynthetic cells are known to accumulate detectable concentrations of tocopherols. Tocopherols can be extracted and purified or concentrated from vegetable oils and other higher plant materials. However, the concentrations in these higher plant materials are very low and there are high proportions of the less-active homologues of tocopherols. Among the many strains of photosynthetic microorganisms known to accumulate tocopherols, *Euglena gracilis* is promising for commercial production of α-tocopherol. The growth rate and α-tocopherol contents are relatively high and α-tocopherol comprise more than 97% of all the tocopherols accumulated by *Euglena gracilis*. Although a lot of work has been done to increase the contents and composition of tocopherols in higher plants through genetic and metabolic engineering, work on genetic modification of microorganisms for increased tocopherol accumulation is scarce. Many cultivation systems have been investigated for efficient production of tocopherol by *Euglena gracilis.* However, those that involve heterotrophic metabolism are more promising. Bubble columns and flat-plate photobioreactors are more suitable for commercial production of tocopherols, than the tubular, internally illuminated, and open-air photobioreactors.

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Introduction

Tocopherols are fat-soluble antioxidants synthesized by photosynthetic cells but not by animals. Reactive oxygen species/free radicals are usually produced in the body, especially under stress. The reactive oxygen species damage DNA, proteins, carbohydrates, and lipids, leading to various disorders such as tissue injury, artheriosclerosis, cardiovascular diseases, and cancer (Rock et al. 1996). Tocopherols act as antioxidants and free-radical scavengers and together with other antioxidants in the body neutralize these free radicals (McCay and King 1980; Fryer 1992). Tocopherols also protect thylakoid components from oxidative damage, play roles in electron transport reactions, cell membrane permeability and fluidity, and thus act as membrane stabilizers (Powls and Redfearn 1967; Lucy 1972; Giasuddin and Diplock 1981). They also have hypocholestemic health benefits (Qureshi et al. 1995). Patients with moderately severe impairment from Alzheimer's disease who received α-tocopherol had a significant improvement in their condition (Sano et al. 1997).

They are involved in the prevention of light-induced pathologies of skin and eyes (Chiu and Kimball 2003) and in photophosphorylation (Vincenzini et al. 1980). Experiments in aquacultures have also demonstrated that tocopherols improve the growth, resistance to stress and diseases, and increase survival of fish and shrimps (Vismara et al. 2003). Vitamin E deficiency causes immature gonads in fish, lower hatching rates, and survival of offspring (Izquierdo et al. 2001). Most of these physiological roles

are due to their anti-oxidative properties but some functions such as inhibition of platelet aggregation and monocyte adhesion, anti-proliferative and neuroprotective effects, and regulation of cell signaling (Rimbach et al. 2002) are not directly linked to their antioxidant activities.

In view of the various physiological roles of tocopherols, they have been extensively used as dietary supplements for humans, food preservatives, and in manufacture of cosmetics and sunscreens. They are also used for fortification of animal feeds, especially in aquaculture. Natural sources of tocopherols are used mainly for human applications because of the relatively high costs (US$20/kg) while chemically synthesized α-tocopherol acetate produced from isophyol and trimethylhydroquinone, is used to fortify animal feed because it is relatively cheap (US$11/kg) (Valentin and Qi 2005).

Vitamin E (tocochromanols) consists of α-, β-, δ-, and γ-tocopherols and α-, β-, δ-, and γ-tocotrienols. However, α-tocopherol is the most predominant and active form of vitamin E in most human and animal tissues. On the whole, the tocopherols are more active than their corresponding tocotrienols and the reactivities of the tocotrienols with peroxyl radical in homogenous solution were in the following order: α>β, γ>δ (Yoshida et al. 2007). Tocotrienols differ from their corresponding tocopherols by the presence of three isolated double bonds in their prenyl side chains. The unsaturated side-chain in tocotrienols makes them penetrate tissues with saturated fatty layers such as brain and liver more efficiently. They are very effective oxygen radical scavengers in lipophilic environments such as oils and the lipid layer of biological membranes. All the four homologues (α, β, δ, and γ) occur in plant tissues and photosynthetic microbial cells but their concentrations and relative proportions depend on the plant species, the tissues, microalgae species, and the culture conditions. Since α-tocopherol is the most active form of the four isoforms, strains, and culture conditions that favor accumulation of α-tocopherol are selected for fermentation production of tocopherols.

Natural sources of tocopherols

1. Higher plants as sources of tocopherols

It has been reported that tocopherols occur ubiquitously in plant tissues, especially leaves and seeds of most dicotyledonous plants (Kamal-Eldin and Appelqrist 1996), *Vitis vinifera* seeds (Bravi et al. 2007), soybean oil, rice bran, and wheat germ (King et al. 1996). They are usually found concentrated in the seed, the germ, and other oil-bearing fractions. Traditionally, various organic solvents such as hexane (Molero-Gomez et al. 1996) are used for extraction of the tocopherols from plant tissues. However, in recent times, a lot of work is being done on the use of super-critical fluids such as super-critical carbon dioxide for extraction of tocopherols from plant tissues (Molero-Gomez et al. 1996; Bravi et al. 2007; King et al. 1996). Various vegetable oils are used for commercial production of tocopherols. They are produced either as tocopherol-enriched vegetable oils, or tocopherol concentrates with different degrees of purity. An Argentinean company (Advanced Organic Material) recently announced that it has started commercial production of tocopherols from sunflower oils. Separation of tocopherols from vegetable oils entails conversion of the volatile alcohols in vegetable oils to less-volatile fatty acid esters through esterification. The tocopherols are then separated from other components with different boiling points by distillation at different temperatures.

The problem with higher plants as sources of tocopherols is that their contents of tocopherol are generally low. Furthermore, the proportion of α-tocopherol, which is the most active form, is very low.

2. Microorganisms as sources of tocopherols

Microorganisms are known to be good sources of tocopherols (Green et al. 1959; Hughes and Tove 1982). However, only photosynthetic microorganisms are known to accumulate detectable amounts of tocopherols (Tani and Tsumura 1989; Taketomi et al. 1983). Microalgae species reported to accumulate tocopherols include *Spirulina platensis* (Powls and Redfearn 1967; Vincenzini et al. 1980), *Dunaliella tertiolecta* (Abalde et al. 1991; Carballo-Cardenas et al. 2003), *Synechocystis* sp and *Nannochloropsis oculata* (Brown et al. 1999; Durmaz 2007), *Tetraselmis suecica* (Carballo-Cardenas et al. 2003; Vismara et al. 2003), species of *Chlorella*, *Clamydomonas*, and *Ochromonas* (Taketomi et al. 1983), *Euglena gracilis* (Hughes and Tove 1982; Ogbonna et al. 1998, 1999b; Vismara et al. 2003), *Dunaliella salina* (Vismara et al. 2003), *Isochrysis galbana* (Fabregas and Herrero 1990), and *Diacronema vlkianum* (Donato et al. 2003). Different intracellular tocopherol concentrations and productivities have been reported for these strains but it is difficult to compare them because of the differences in the culture conditions. There can be as high as seven times difference in tocopherol contents reported for the same strain (Brown et al. 1999; Durmaz 2007).

The desired characteristics of microorganisms for tocopherol production include high intracellular concentration of total tocopherols, high proportion of α-tocopherol, high cell growth rate, high final cell concentrations, simple cultivation conditions, cheap cultivation media, and low susceptibility to contamination. Based on the available literature, *Euglena* spp. seem to be the most extensively

studied strains for tocopherol production. Relatively high intracellular tocopherol concentrations ranging from 1.12 mg/g to 7.35 mg/g cell have been reported. *E. gracilis* was the highest tocopherol producer among 285 strains in 56 genera of microorganisms tested (Tani and Tsumura 1989). Furthermore, high cell density can be achieved (Ogbonna et al. 1998), it grows under photoautotrophic, photoheterotrophic, and heterotrophic conditions (Ogbonna et al. 2002a), it simultaneously accumulates other vitamins such as β-carotene and vitamin C (Takeyama et al. 1997), more than 97% of the tocopherols produced by *Euglena gracilis* is the α-isoform (Tani and Tsumura 1989; Shigeoka et al. 1986), it has no cellulose cell wall so extraction of tocopherol is relatively easy, and if used as whole biomass, it is easily digested and assimilated. However, a major limitation in the use of *Euglena* species for tocopherol is that the culture is easily contaminated by fast-growing microorganisms. In other words, it requires more aseptic culture conditions than other species such as *Dunaliella*, and *Spirulina*. In view of the relative advantages of *Euglena* spp. over other strains of microorganisms used for tocopherol production, most of the discussions that follow are based on this strain.

Biosynthesis of tocopherols

Process development for efficient production of tocopherol requires good knowledge of the organelles responsible for their syntheses. There are conflicting reports on distribution of tocopherols inside the cells. Threlfall and Goodwin (1967) reported that in light-grown *Euglena* cells, more than 95% of α-tocopherols are located inside the chloroplasts while only very small amounts were found in the mitochondria and microsomes. On the other hand, Shigeoka et al. (1986) reported that in the same *Euglena* cells grown under light, greater percentage of α-tocopherol was synthesized inside the mitochondria and only low concentrations were found in the chloroplasts and microsomes. They later reported that γ-tocopherol methyltransferase that converts γ-tocopherol to α-tocopherol was found in chloroplast (Shigeoka et al. 1992). The report of Kusmic et al. (1999) showed that mutants of *Euglena* cells without chloroplasts produced even higher concentration of tocopherols per unit cells than the photosynthetic strain. Again, there was no correlation between chlorophyll *a* and α-tocopherol contents of *Euglena* cells (Carballo-Cardenas et al. 2003) while simultaneous increase in chlorophyll and α-tocopherol was reported for the same strain (Takeyama et al. 1997). In higher plants, α-tocopherols are found both in photosynthetic organs such as leaves, as well as in non-photosynthetic tissues such as germ, and seedlings (Green 1958; Kruk and Strzalka 1995). The question is whether the tocopherols are synthesized in the non-photosynthetic organs or are transported from the photosynthetic tissues to the non-photosynthetic tissues.

All these reports, coupled with the fact that although tocopherols are produced even in dark, the concentration increases with increase in light intensity, imply that both chloroplasts and mitochondria are involved in tocopherol synthesis. The relative contribution of these organelles depends on the culture condition (Ogbonna et al. 1998, 2002a, b; Fujita et al. 2008b).

Improvement of strains for overproduction of tocopherols

Reports on development of strains for efficient tocopherol production are very scarce. Tani and Osuka (1989) constructed analog resistant strains using beta-2-thienylalanine. Many beta-2-thienylalanine-resistant strains were obtained on UV-radiation with or without streptomycin treatment and one of the strains accumulated four times more tocopherols than the mother strain. However, there is apparently no further report on the use of the developed analog strain. Genetic engineering of plants and microorganisms for overproduction of tocopherols require good knowledge of the regulation and rate-limiting reactions in the synthesis of tocopherols. The two approaches used are to convert most of the produced tocochromanols to the most active α-tocopherol or to increase the total tocochromanols produced. However, recent studies have shown that other isoforms of tocochromanols, although having less vitamin E activities, play other important functions that are not played by α-tocopherol. Furthermore, under normal culture conditions, over 90% of the total tocopherols accumulated by most microorganisms are the most active α-tocopherol. Thus, genetic engineering of microorganisms for tocopherol production should be focused on increasing the total tocopherol contents rather than increasing the proportion of the α-tocopherol.

The aromatic precursors of vitamin E are the homogentisate and *p*-hydroxyphenylpyruvate, and it has been shown that the synthesis of *p*-hydroxyphenylpyruvate is the rate-limiting step for accumulation of vitamin E in plants (Rippert et al. 2004; Qi et al. 2005). The synthesis of *p*-hydroxyphenylpyruvate and homogentisate directly at the level of prephenate was achieved by expression of *Saccharomyces cerevisiae* prephenate dehydrogenase gene in *Nicotiana tabacum* plants that already over-express the *Arabidopsis p*-hydroxyphenylpyruvate dioxygenase coding sequence. This led to high accumulation of tocotrienols in the leaves (Rippert et al. 2004). Furthermore, expression of barley homogentisic acid geranylgeranyl transferase (HGGT) in *Arabidopsis thaliana* leaves resulted in a ten-

to 25-fold increase in total tocopherol in the leaves. Again, over-expression of barley HGGT in corn seeds resulted in sixfold increase in tocopherol and tocotrienol (Cahoon et al. 2003). Various approaches aimed at either increasing the proportion of α-tocopherol in plants or increasing the total tocopherol content of plants have been reviewed (Valentin and Qi 2005).

Although a lot of work has been done on genetic/metabolic engineering of higher plants for high accumulation of tocopherols, work on microorganisms is very scarce. A cyanobacterium, *Synechocystis* sp. strain PCC 6803 is a well-characterized model system for genetic engineering for over production of tocopherols and it has been demonstrated that nirA promoter is very useful for engineering the tocopherol metabolic pathway in *Synechocystis* sp (Qi et al. 2005). *p*-Hydroxyphenylpyruvate dioxygenase catalyzes the formation of homogentisic acid from *p*-hydroxyphenylpyruvate, which is a rate-limiting reaction in tocopherol synthesis. When this gene was expressed under inducing conditions, the total tocopherol level increased five times and tocotrienols comprised 20% of the total tocopherols (Qi et al. 2005). It is rather unfortunate that there are no reports on genetic modification of good tocopherols producers such as *Euglea gracilis*. It is hoped that with the knowledge gained with higher plants and *Synechocystis*, it will be possible to develop *Euglena* species that accumulate very high concentrations of tocopherols.

Optimization of culture conditions for α-tocopherol production by photosynthetic microorganisms

It has been reported that addition of organic carbon source resulted in increased mitochondria activity, reactive oxygen species concentration, and α-tocopherol productivity in chloroplast-deficient mutant *E. gracilis* W14ZUL (Fujita et al. 2008b). They also reported that in photoheterotrophic culture of wild strain of *Euglena gracilis* Z, there was a positive correlation between the generation of reactive oxygen species and α-tocopherol productivity. Thus, optimization of culture conditions for production of tocopherol would entail manipulation of those factors that will lead to generation of reactive oxygen species. It is, however, necessary to note that the conditions that are favorable for cell growth may not be the best for accumulation of intracellular tocopherol (Ogbonna et al. 2002a, b). For example, conditions that inhibit photosynthesis but not photolysis of water in photoautotrophic culture would reduce cell growth but increase generation of reactive oxygen species and thus increase α-tocopherol production (Fujita et al. 2008b).

Low-temperature stress can increase generation of reactive oxygen species. Ruggeri et al. (1985) reported that cultivation of *Euglena gracilis* under low temperature resulted in a six- to seven-times increase in α-tocopherol accumulation. Light seem to be the most important factor that affects tocopherol production and a lot of reports have shown positive correlation between light intensity and tocopherol accumulation (Ogbonna et al. 1999b, 2002a, b). Continuous illumination resulted in higher tocopherol accumulation than 12:12 h dark:light cycles (Brown et al. 1999). Although, Carballo-Cardenas et al. (2003) concluded that in *D. tertiolecta*, decreased light availability does not limit tocopherol production, their experimental design did not allow for separation of the effects of light from the effects of culture age. Such conclusion can only be justified by performing the experiments under different illumination intensities. Many workers have suggested that light stimulates tocopherol synthesis through its effects on photosynthesis (Tani and Tsumura 1989; Threlfall and Goodwin 1967). However, Kusmic et al. (1999) reported that light stimulated tocopherol synthesis even in *Euglena* strains that lacked chloroplasts. Media composition also affects tocopherol synthesis. In the case of *N. oculata*, for example, reducing the nitrate concentration from 882 to 441 mol/L resulted in significant increase in tocopherol accumulation per unit cell (Durmaz 2007) but this led to low biomass yield. On the other hand, increase in nitrate concentration led to higher cell concentration but lower intracellular tocopherol contents of the cells. Furthermore, addition of tocopherol precursors such as homogentisate resulted in significant increase in the tocopherol content of the cells. Even in sequential heterotrophic/photoautotrophic cultivation of *Euglena gracilis*, nutrient composition of the photoautotrophic phase affected tocopherol accumulation. Oxygen stress was reported to increase the level of α-tocopherylquinone and substantially decrease the levels of tocopherol and its homologues in *Euglena* culture (Ruggeri et al. 1985). However, there was no direct measurement of the extent of oxygen stress.

Culture systems

Photoautotrophic cultures

Photoautotrophic cultures are the most widely used method of cultivating photosynthetic microorganisms. The intracellular α-tocopherol accumulation is high while the risk of contamination is low in photoautotrophic cultures. However, during the late growth phase, when cells start dying and lysing, contamination by heterotrophs can become a problem. Light supply (in terms of quantity, quality, and distribution) is a major problem limiting cell growth and α-tocopherol accumulation in photoautotrophic cultures (Ogbonna and Tanaka 2000a; Ogbonna 2003). When solar

light energy is used as the sole light source, cell growth and tocopherol accumulation depend to a great extent on weather condition. Depending on the location and season, day length can be very short so that the cultures are light-limited for most period, leading to low accumulation of tocopherols. In order to overcome this problem, an integrated solar and artificial light system for continuous illumination of cultures has been developed (Ogbonna et al. 1999a). The system changes automatically from solar to artificial light whenever solar light intensity decreases below a set value.

Pure photoautotrophic cultures can be used for small household α-tocopherol production. Such systems need to be as simple as possible with very low investment and running costs. However, pure photoautotrophic cultures are not suitable for large-scale commercial production of α-tocopherol because of the low productivity and low biomass concentrations. Comparatively large-volume photobioreactors are required while the cost of harvesting algae from such low concentrations can be very high.

Heterotrophic cultures

Euglena spp. and many other photosynthetic microorganisms such as *Chlorella*, *Chlamydomonas*, *Tetraselmis*, and *Dunaliella* can grow heterotrophically in absence of light, using organic carbon sources (Chen and Johns 1996; Ogbonna et al. 1997; Carballo-Cardenas et al. 2003). Although tocopherol contents of heterotrophically grown *Euglena* cells are, on average, lower than those grown photoautotrophically, heterotrophic bioreactors can be used for production of tocopherols. By optimizing culture conditions in terms of choice of organic carbon source and aeration, very high concentration of cells (39.5 g/L) with relatively high tocopherol contents (1,200 μg/g cell) can be achieved in a fed-batch culture (Ogbonna et al. 1998). During heterotrophic cultivation, *Euglena gracilis* cells lose their chlorophyll. This may have some implications in extraction and purification of tocopherols. Because of the high cost of light energy and the technical problems associated with light distribution inside large-scale photobioreactors, the potential of heterotrophic cultures for commercial production of tocopherols is high.

Photoheterotrophic cultures

Heterotrophic cultures of *Euglena gracilis* (assimilation of organic carbon in dark) leads to production of high concentration of cells with relatively low intracellular tocopherol concentrations while photoautotrophic cultures (photosynthetic growth in absence of organic carbon) leads to production of low concentrations of cells with high intracellular tocopherol concentrations. Photoheterotrophic (mixotrophic) cultures (where both organic carbon and light are supplied simultaneously) can therefore be used to achieve high concentration of cells with high intracellular tocopherol concentrations. Interactions between photoautotrophic and heterotrophic metabolic activities in photoheterotrophic cultures have been studied. Using glucose as the organic carbon source, it was found that under low light intensities, the photoautotrophic and heterotrophic metabolic activities in *E. gracilis* proceeded simultaneously and independently so that the growth rate and final cell concentrations in the photoheterotrophic culture were the sums of the values obtained in heterotrophic and photoautotrophic cultures (Ogbonna et al. 2002a). However, under high light intensities, both photosynthesis and glucose assimilation were inhibited, leading to relatively low cell concentrations but higher intracellular tocopherol concentrations. The results also showed that the heterotrophic metabolism (glucose assimilation) was more sensitive to photoinhibition than photoautotrophic metabolism (photosynthesis). Again, cell growth, glucose assimilation, and tocopherol content were higher with air than with a 5% CO_2 in air (Ogbonna et al. 2002a). On the whole, the conditions that favor *Euglena* cell growth in photoheterotrophic cultures are different from those that favor α-tocopherol accumulation. It appears that cell growth is mainly controlled by organic carbon assimilation while α-tocopherol accumulation is controlled by photoautotrophic metabolism. It is therefore necessary to control the ratios of these metabolic activities in order to obtain high cell concentration with high intracellular α-tocopherol concentrations. Methods of regulating these two metabolic activities in *Euglena* cultures have been studied (Ogbonna et al. 2002b). The relative contribution of photoautotrophic metabolism to cell growth increased with increase in light intensity, carbon dioxide concentration in the aeration gas, and decrease in the feed rate of organic carbon (glucose or ethanol). However, limiting the amount of organic carbon was the most effective method of shifting the metabolic balance to photoautotrophic side. In the presence of excess organic carbon source, the α-tocopherol contents of the cells were low even under high light intensities. However, by limiting the organic carbon supply, the tocopherol contents of the cells were similar to those obtained in pure photoautotrophic cultures (Ogbonna et al. 2002b). Furthermore, although many organic carbon sources can be used (Kusmic et al. 1999; Fujita et al. 2008a), ethanol, lactic acid, and galactose were very effective in α-tocopherol accumulation while glucose and fructose were more effective in cell growth (Fujita et al. 2008a). At the same total substrate concentration (10 g/L), using a mixture of glucose and ethanol was more effective than either of the substrates and the optima ratio of glucose to ethanol for α-tocopherol productivity was 3:2. Using a fed-batch culture,

19.7 g/L cell with intracellular α-tocopherol concentration of 1.19 mg/g cell was obtained in 6 days.

These results demonstrate clearly that photoheterotrophic culture has high potentials for α-tocopherol production by *Euglena gracilis*. This culture system has also been used for α-tocopherol production by *D. tertiolecta* and *T. suecica* (Carballo-Cardenas et al 2003). Furthermore, although α-tocopherol accumulation was not measured, it has been shown that photoheterotrophic culture can be used to achieve high cell concentrations in some strains such as *Chlorella*, *Spirulina,* and *Haematococcus* (Endo et al. 1977; Marquez et al. 1993; Kobayashi et al. 1992). It is necessary to optimize photoheterotrophic cultures of other tocopherol producers in terms of light intensity, type, and concentration of carbon sources. This culture system is suitable even for small-scale production. Relatively high productivity can be obtained by occasional manual mixing because the oxygen evolved by photosynthesis is used for organic carbon assimilation while the carbon dioxide released from organic carbon assimilation is used for photosynthesis.

Sequential heterotrophic–photoautotrophic cultures

Another culture system with high promise for α-tocopherol production is the sequential heterotrophic–photoautotrophic culture. In photoheterotrophic culture, conditions that favor organic carbon assimilation are different from those that favor tocopherol accumulation. In optimizing the culture system, compromise must be made between cell growth and tocopherol accumulation in order to get the highest volumetric tocopherol productivity. This problem can be avoided by separating the organic carbon assimilation from photoautotrophic metabolic activities so that each can be optimized independently. The cells are cultivated heterotrophically to obtain high cell concentrations, harvested from heterotrophic culture, suspended in fresh media and illuminated for photoautotrophic culture (Takeyama et al. 1997) or can simply be illuminated at the end of the heterotrophic phase (Ogbonna et al. 1999b). Again, it can be done in batches or continuously (Ogbonna et al. 1999b). Even in this system, using ethanol is still better than using glucose for the heterotrophic phase. The desired cell concentration at the end of the heterotrophic phase depends on the light supply capacity of the photobioreactor for the photoautotrophic phase. The heterotrophic phase has to be optimized in terms of the type and concentration of organic carbon source, the aeration, other media components and hydrodynamic stress. On the other hand, the photoautotrophic phase has to be optimized in terms of initial cell concentration, light intensity and distribution, carbon dioxide supply and media components, especially nitrogen sources. By using the conventional heterotrophic bioreactor, monoculture can be maintained during the heterotrophic phase, and even if open ponds are used for photoautotrophic phase, contamination would not be a serious problem because of the high cell concentration, the short culture duration and the absence of organic carbon source.

Cyclic photoautotrophic–heterotrophic cultures

Energy cost for photobioreactor illumination is very high and it is always desirable to use solar light energy. However, depending on location and season, less than 6 h per day has enough light to support photosynthetic cell growth. During the dark, the cells do not grow; rather, they metabolize the intracellular organic carbon, leading to decrease in cell concentration (Ogbonna and Tanaka 1996). As a solution to this, cyclic photoautotrophic–heterotrophic culture system where controlled amount of organic carbon is added at night has been investigated (Ogbonna and Tanaka 1998, 2000b). With this method, continuous cell growth under light/dark cycles has been achieved. For maximum productivity, it is necessary to select appropriate organic carbon, and to add only the quantity that can be completely assimilated at night so that residual organic carbon is not carried over to the light period. When glucose was used, the intracellular concentration of α-tocopherol decreased during the heterotrophic phase at night but recovered during the subsequent photoautotrophic phase. However, with ethanol, the intracellular concentration of α-tocopherol remained stable during the two phases (Ogbonna and Tanaka 1998, 2000a). Again, if only solar light energy is used for the photoautotrophic phase, fluctuation in light intensity affected α-tocopherol productivity. However, by using an integrated system of solar light, artificial light, and organic carbon supply, the light energy source changes automatically to solar light during the bright period of the day, and to artificial light during the cloudy period of the day. This ensured stable light supply during the photoautotrophic phase and the α-tocopherol productivity was five times higher than the value obtained in pure photoautotrophic culture under the same culture conditions (Ogbonna et al. 2001).

Photobioreactors for production of tocopherols

Photobioreactors for cultivation of photosynthetic cells have been extensively reviewed (Borowitzka 1999; Ogbonna 2003). These include open-air culture systems such as natural bodies of waters, and various types of artificial ponds; various types of tubular photobioreactors (Torzillo et al. 1993; Ogbonna et al. 1997; Ugwu et al. 2002), flat-plate photobioreactors (Tredici and Materassi 1992; Zhang et al. 1999), vertical column photobioreactors

(Sanchez Miron et al. 2000), and internally illuminated photobioreactors (Mori 1985; Ogbonna et al. 1996, 1999a). Detailed description, characteristics, and evaluation of the various types of photobioreactors are available (Ogbonna 2003).

Open-air culture systems are the simplest and cheapest but are not recommended for production of α-tocopherol by *Euglena gracilis* because of the high risk of contamination. Open-air culture systems can only be used for cultivation of strains with selective growth conditions such as high pH (*Spirulina*) or high salinity (*Dunaliella*). On the other hand, the internally illuminated photobioreactors are complex and too expensive for production of α-tocopherols at competitive prices. Tubular photobioreactors are also not suitable because of low mass transfer and the difficulty in maintaining sterile conditions required for culture systems involving heterotrophic metabolism.

The most economical and efficient photobioreactors for tocopherol production would be carefully designed flat plates or vertical column photobioreactors. Depending on the location, season, and time of the day, the full solar light intensity is too strong for the growth of *Euglena* cells, though such high light intensity would stimulate α-tocopherol accumulation. Relatively long light-paths (>50 cm) or partial shading will be required to achieve a good balance between the cell growth and α-tocopherol accumulation. If the option of long light-path is chosen, then higher degree of mixing will be required to circulate the cells between the surface and bottom of the culture (Ogbonna 2003). The required level of sterility depends on the culture system. If photoautotrophic culture is used, careful washing of the photobioreactor, using relatively high inoculum concentration (initial cell concentration should be more than 10^6cells/mL), and proper covering of the culture may be sufficient to minimize contamination to an acceptable level. However, for any culture system involving addition of organic carbon source, the system must be well-sterilized and high inoculum volume of the pre-culture must be used.

The future

It is unfortunate that in spite of the potentials of production of tocopherols by photosynthetic microorganisms, there is apparently no commercial production by this method. Commercial production of tocopherols depends on the cost effectiveness. The costs of media, energy input for aeration, mixing and illumination, photobioreactors, harvesting, labor, extraction, and purification of tocopherols must be considered.

Because of the vulnerability of *Euglena gracilis* to contamination coupled with the fact that for most parts of the day and seasons, full solar light intensity is too strong for the growth of *Euglena*, commercial scale cultivation of *Euglena* in completely open culture systems is not feasible. Furthermore, the relatively low cell densities obtained in photoautotrophic cultures may not justify the use of internally illuminated photobioreactors for commercial photoautotrophic cultivation of *Euglena*. Commercial production of tocopherols by *Euglena* requires exploitation of its heterotrophic metabolic activities either in photoheterotrophic, sequential heterotrophic–photoautotrophic or cyclic heterotrophic–photoautotrophic culture system. Complete or at least certain degree of sterility must be maintained to avoid contamination by the fast-growing heterotrophic microorganisms. Also, appropriate organic carbon source such as ethanol that favors accumulation of tocopherol and minimizes contamination must be used. A good balance between the heterotrophic and photoautotrophic metabolic activities must be maintained to obtain high concentration of cells with high intracellular tocopherol concentrations. This can be achieved by controlling the amount of organic and inorganic carbon sources as well as the light intensity. Data from small-scale culture systems indicate that commercial scale production of tocopherol by *Euglena gracilis* is feasible. What is required now is scale-up studies and economic analyses. Genetic improvement of photosynthetic microorganisms for efficient accumulation of α-tocopherol is an approach that has not been sufficiently addressed. With the knowledge gained with higher plants, it is feasible to develop microbial strains for efficient production of tocopherols.

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