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Effects of mixed substrates on growth and vitamin production by *Euglena gracilis*

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The effects of mixed carbon sources on growth and production of vitamins E and A by *Euglena gracilis* cells were investigated in batch culture. The cells were grown mixotrophically in glucose (G), ethanol (E) and a mixture of glucose and ethanol (EG). Cell growth was measured by counting the cell number with microscope, while vitamin concentrations were measured spectrophotometrically. Growth of the cells was found to be much better in the mixed carbon culture (EG) than in either glucose (G) or ethanol (E) alone. Significance difference was detected ($P < 0.05$) on cell growth and ranked as [EG] > [G] > [E]. The average cell density in the mixed carbon culture reached $2.34 \pm 0.109 \times 10^7$ cells/ml after 7 days. It was about $1.63 \pm 0.089 \times 10^7$ cells/ml in glucose and $0.70 \pm 0.084 \times 10^7$ cells/ml in ethanol. The result also showed that ethanol alone was the best carbon source for production of the vitamins. The results indicated that the mixed carbon culture enhanced growth of the cells, but cellular contents of the antioxidant vitamins were low. Thus, the mixed substrate system has a high potential for large-scale production of *Euglena* biomass.

Key words: mixed substrate culture, *Euglena gracilis,* cell growth, vitamin production.

INTRODUCTION

The role of *Euglena gracilis* as a good source of nutritionally and medically important substances has called for more research on how to maximize its production. The main importance of *E. gracilis* is that it contains high amounts of proteins, vitamins and polyunsaturated fatty acids (PUFAs). Consequently, it is used as health food and animal feed. Antioxidant vitamins such as α-tocopherol, L-ascorbic acid and β-carotene, which function to prevent diseases caused by oxidative damages among other functions, are accumulated in significant amounts by *E. gracilis* (Takeyama et al., 1997). Although many higher plants are known to produce these vitamins, their production in photosynthetic microorganisms is much higher owing to higher growth rates and absence of seasonal influences, which make it possible for the cells to be cultivated all year round.

Polyunsaturated fatty acids (PUFAs), especially linoleic and α-linolenic acids, which have a number of physiolo-

gical functions, have also been reported to be produced in significant amounts by the microorganism (Hayashi et al., 1993). PUFAs are important in the body for synthesis of membrane lipids, reduction of blood cholesterol levels, reduction of risk of heart attack, normal development of brain and retina and for synthesis of other physiologically important substances like prostaglandins (Taylor et al., 1998a). It is known that fishes, the major sources of PUFAs, obtain these fatty acids from photosynthetic cells in a food chain.

E. gracilis is among the many photosynthetic microorganisms employed for production of single cell protein (SCP), a new food source, used as an alternative source of proteins in developed countries like Europe, America and Asia. Protein remains the most costly food component in countries like Nigeria, where SCP technology has either not been adopted or developed to supplement the conventional sources, such as meat, eggs, fishes and legumes. SCP from photosynthetic cells (algae) is considered better than those from bacteria or fungi. This is because, algae including *E. gracilis*, accumulate less amounts of nucleic acids than bacteria and fungi. High le-

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vels of nucleic acids increase the risk of occurrence of a disease condition known as gout.

Furthermore, photosynthetic cells, have been recognized as a potential tool for environmental bioremediation because of their ability to metabolize toxic substances such as nitrogen and sulphur oxides (Negoro et al., 1991; Yoshihara et al., 1996; Ogbonna et al., 2002), and for production of biological fuels (Zarborsky et al., 1998; Kishimoto et al., 1994; Yamaberi et al., 1998; Taylor et al., 1998b).

E. gracilis is capable of growing as a strict photoautotroph, a photoheterotroph or a strict heterotroph lacking differential chloroplasts (Osafune et al., 1990). Although antioxidant content in cells grown under photoautotrophic conditions is higher, biomass production under photoheterotrophic or heterotrophic conditions is higher (Shigeoka et al., 1980; Hosotani and Kitaoka, 1984).

A mixed substrate culture is one that contains a multiple sources of nutrients that serve a similar physiological function. For example, a culture containing both glucose and ethanol as carbon sources is a mixed substrate culture. It has been recognized as a technique for yield improvement (Bull, 1985). Glucose has been identified as the best carbon source for biomass production in E. gracilis while ethanol was the best for α-tocopherol production (Ogbonna et al., 1998). It was against this background that this study was initiated with the aim of developing a new culture system for efficient production of E. gracilis biomass. We expected that the high growth of the cells observed in glucose and high α-tocopherol content in ethanol would be integrated in cells grown in a mixture of the two carbon sources.

MATERIALS AND METHODS

Microorganism and media composition

E. gracilis z was obtained from the culture collection centre of the Institute of Applied Microbiology, University of Tokyo, Japan and subcultured at OGB Biotechnology Research and Development Centre Enugu, Nigeria. Three types of media, labeled G, E and EG on the basis of carbon sources, were used. G contained glucose (10 gl^{-1}), E contained ethanol (10 gl^{-1}), while EG contained a mixture of glucose and ethanol (10 gl^{-1} of each) as carbon sources. Also added was a source of mineral elements, liquid fertilizer (2.5 ml l^{-1}). The fertilizer (Boost xtra foliar fertilizer complex from Candel) was composed of nitrogen (20%), potassium (20%), phosphorus (20%), magnesium (1%), manganese EDTA (0.075%), copper EDTA (0.075%), cobalt EDTA (0.0012%), zinc EDTA (0.075%), iron (0.15%), boron (0.0315%) and molybdenum (0.0012%). The media were sterilized by autoclaving at 121°C for 15 min.

Effect of mixed carbon sources on cell growth

Erlenmeyer flask (500 ml) containing 100 ml of the medium was used for the cultures. It was equipped with a high quality foam plug and a narrow heat resistant plastic tube passed into the medium through the plug and extends to the outside for aseptic collection of samples for analysis. The exterior opening of the narrow tube was sealed with masking tape.

Approximately 0.5 ml of a pure culture of E. gracilis was aseptically transferred into the medium in a clean box and then incubated at room temperature (25°C) indoors, between two parallel daylight fluorescent tubes (T9 18 watt, 220 – 240 V). The distance of flask from the light source was 5 cm. Cell growth was measured daily for a period of one week by counting the cell number with microscope and Neubaur counting chamber. Growth of the cells was determined in the three media to investigate the effect of the combined carbon sources by comparing the plots of their time courses of growth.

Effect of mixed carbon sources on production of vitamins E and A

Erlenmeyer flask (500 ml) was also used for the cultures as described above. The intracellular concentrations of the vitamins were determined spectrophotometrically using the method described by David (1978). A known volume of the culture was centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the pellet washed with distilled water.

Acetone (5 ml) was added and allowed to stand for 10 min to extract the cell contents. This was necessary because most of the vitamins are contained inside the cells. Distilled water (2 ml) and 5 ml of petroleum ether were then added. There was a separation of oily layer on top of the aqueous liquid. The top layer was gently collected with pipette and used to determine the concentration of the fat soluble vitamins E and A by reading their absorbance at 450 nm and 493 nm respectively, with spectrophotometer model 722S. The dry weight of the cells used in the process was predetermined by counting the cell number in the broth and estimating the corresponding dry weight from a calibration curve. The cellular concentration of the vitamins were then calculated from the equation,

Conc. (mg/g) = (A x DF) / (Ext. Coeff. x light path x wgt)

A = absorbance, DF = dilution factor, Ext. Coeff = absorption, Coefficient (1026 for vitamin E and 2592 for vitamin A in $mg^{-1}cm^{-1}ml$), light path = thickness of cuvette (usually 1 cm) and wgt = dry weight of cells used (gml^{-1}).

Determination of glucose and ethanol concentrations

Glucose kit (Quimica clinica Aplicada S.A., Amposta, Spain) was used to measure glucose concentration daily throughout the period of cultivation. Ethanol concentration was measured using the boiling/iodometric method (Gwarr, 1987).

RESULTS

Effect of mixed carbon sources on cell growth and vitamin production

The effect of a mixture of glucose and ethanol on growth of E. gracilis cells is shown in Figure 1. The growth of the cells was better in the mixed substrate medium (EG) than in either of the single substrate media, G or E. The figure also showed that the cells grew better in glucose than in ethanol. Statistical analysis of the result using the LSD
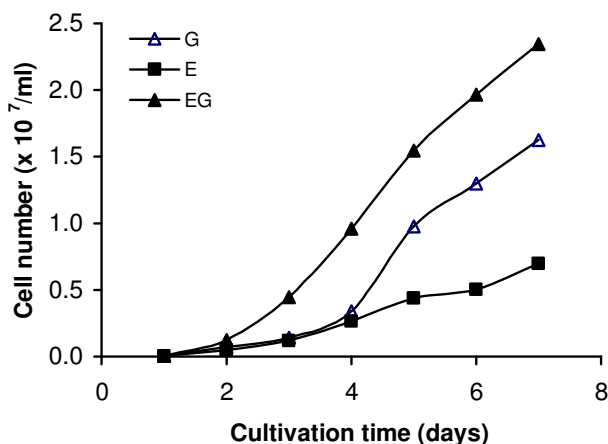
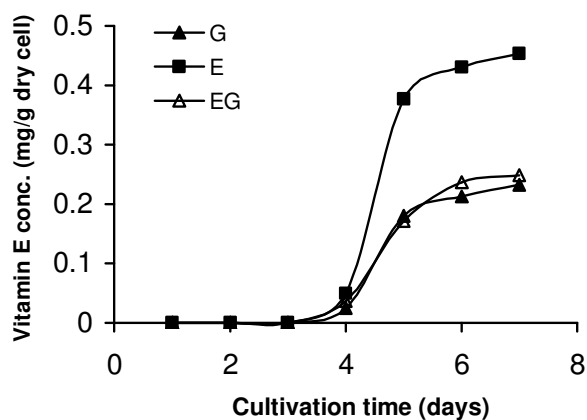**Figure 1.** Effect of mixed substrates on growth of *Euglena gracilis.*

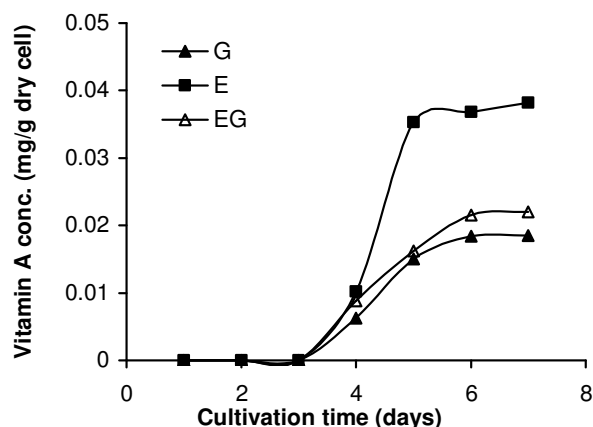**Figure 3.** Effect of mixed substrates on vitamin A production by *Euglena gracilis.*

Figure 2. Effect of mixed substrates on vitamin E production by *Euglena gracilis.*
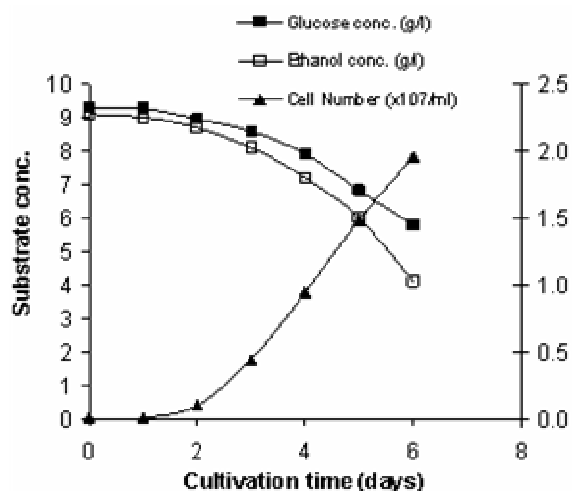

Figure 4. Pattern of utilization of glucose and ethanol by *Euglena gracilis* in a mixed carbon culture.

value at 95% confidence level showed a ranking order of (EG) > (G) > (E).

As shown in Figures 2 and 3, the combination of glucose and ethanol had no advantage on production of the vitamins under the culture conditions used. Ethanol alone was better for intracellular content of the vitamins than either glucose alone or the combination.

Utilization of glucose and ethanol in the mixed carbon culture

This study was carried out to help us understand the cause of the rapid cell growth obtained in the mixed carbon culture. The result (Figure 4) showed that the concentration of the two substrates declined simultaneously with time in the mixed carbon culture. This indicates that the cells utilized both substrates simultaneously.

DISCUSSION

Mixed substrate culture has been recognized as a technique for yield improvement in microbial cultivations. Consequently, culture media for industrial fermentations are frequently formulated so that they contain complex mixture of nutrients, and mixed carbon sources in particular have been exploited for improvement of antibiotic yields (Bull, 1985). In this work, the growth of *E. gracilis* was found to be enhanced by the mixed substrate system relative to either of the single substrate cultures. The actual cause of the rapid cell growth observed in the combined substrate culture is not precisely known. However, one of the possible reasons may be due to simultaneous utilization of the two carbon sources. It has been reported that some microorganisms utilize some

substrate mixtures simultaneously. For example, *Kluveromyces fragilis* has been reported to utilize glucose and fructose simultaneously (Bull, 1985). The result of the study on pattern of consumption of the two substrates in the mixed carbon culture (Figure 4) showed that the glucose and ethanol were simultaneously utilized by *E. gracilis*. However, the rate of consumption of ethanol was higher than that of glucose. About 6.0 gl⁻¹ of ethanol was consumed in 7 days, but only about 4.9 gl⁻¹ of glucose was consumed during the same period. This agrees with a report that when ethanol and glucose were added simultaneously to a culture of *E. gracilis* at a concentration of 1.0% respectively, ethanol was consumed more rapidly than glucose (Tani and Tsuruma, 1989). Simultaneous utilization of substrates may occur when the substrates are catabolized via different metabolic pathways. Glucose is catabolized by most microorganisms through two major routes; glycolytic and pentosphosphate pathways (Okafor, 1987). Ethanol, on the other hand, is also catabolized through two routes, the alcohol dehydrogenase and the

glyoxylate pathways (Ono et al., 1995). Simultaneous utilization of substrates may also occur when one of the component substrates in the mixture is required by the cells for production of certain metabolites (Bull, 1985). Reports exist that *E. gracilis* requires ethanol as a precursor for α-tocopherol production (Ogonna et al., 1998).

The results of vitamin production (Figures 2 and 3) showed that there was no advantage of the mixed substrate system on production of the vitamins tested under the culture conditions used. We suspect that one of the possible reasons may be that the resulting high cell density in the mixed carbon culture limited light distribution among the cells in the culture. This situation is known to affect the accumulation of light dependent metabolites such as α-tocopherol (Ogbonna et al., 1997; Ogbonna and Tanaka, 2000; Ogawa and Aiba, 1981). It is therefore important to investigate the effect of light intensity on mixed substrate cultivation of *E. gracilis*.

Cultivation of micro algae just for production of vitamins or proteins is very expensive and not economically feasible. However, the fact that the microorganism simultaneously produces a number of useful metabolites such as protein, vitamins, PUFAs and carbohydrates, which could be isolated separately, makes the process potentially economical. Besides, unlike higher plants and animals (conventional sources of the useful metabolites) microorganisms generally require much less space and could be grown all year round since they are not affected by seasonal and environmental changes. Its development could therefore guarantee steady supply of the useful products.

REFERENCES

Bull AT (1985). Mixed culture and mixed substrate system, compre-
hensive Biotechnology 1: 281-299, Pergamon press, New York.

David P (1978). Chemical analysis of food, Churchill Livingstone. pp 130-140.

Gwarr SG (1987). Measurement of ethanol concentration. *In*: Ezeogu LI, Okolo BN, Ogbonna JC (2005). Tryptic digests of sorghum: an assessment of their usefulness as organic nitrogen sources for the yeast saccharomyces cerevisiae. J. Am. Soc. Brew. Chem. 63(2): 50-56.

Hayashi M, Toda K, Kitaota S (1993). Enriching *Euglena* with unsaturated fatty acids, Biosci. Biotech. Biochem. 57: 352-353.

Hosotani K, Kitaoka S (1984). Determination of provitamin A in *Euglena gracilis z* by high performance liquid chromatography and changes of the contents under various culture conditions. J. Japan. Soc. Food Sci. 37: 519-254.

Kishimoto M, Okakura T, Nagashima H, Minowa T, Yokoyama S, Yamaberi K (1994). CO₂ fixation and Oil production using microalgae. *J*. Ferment. Bioeng. 78: 479-482.

Negoro M, Shioji N, Miyamoto K, Miura Y (1991). Growth of microalgae in high CO₂ gas and effects of SOₓ and NOₓ. Appl. Biochem. Biotechnol. 28: 877-886.

Ogawa T, Aiba S (1981). Bioenergetic analysis of mixotrophic growth in *Chlorella vulgaris* and *Scenedesmus acutus,* Biotechol. Bioeng. 23: 1121-1132.

Ogbonna JC, Masui H, Tanaka H (1997). Sequential heterotrophic /autotrophic cultivation: an efficient method of producing chlorella biomass for health food and animal feed, J. Appl. Phycol. 9: 359-366.

Ogbonna JC, Ichige E, Tanaka H (2002). Interaction between photoautotrophic and heterotrophic metabolisms in photoheterotrophic cultures of *Euglena gracilis*. Appl. Microbiol. Biotechnol. 58: 352-538.

Ogbonna JC, Tanaka H (2000). Light requirement and photosynthetic cell cultivation: development of processes for efficient light utilization in photobioreactors, J. Appl. Phycol. 10: 555-559.

Ogbonna JC, Tomiyama S, Tanaka H (1998). Heterotrophic cultivation of *Euglena gracilis z* for efficient production of α-tocopherol. J. Appl. Phycol. 10: 67-74.

Okafor N (1987). Catabolism of carbohydrates, industrial microbiology, pp 43-48, University of Ife Press Ltd, Ile-Ife.

Ono K, Kawanaka Y, Inui H, Miyatake S, Kitaoka S, Nakano Y (1995). Mitochondrial alcoholdehydrogenase from ethanol grown *Euglena gracilis,* J. Biochem. 117(6): 1117-1182.

Osafune T, Sumida S, Ehara T, Ucno N, Hase E, Schiff JA (1990). Lipid (wax) and paramylum as sources of carbon and energy for the early development of proplastids in dark-grown *Euglena gracilis* cell transferred to an inorganic

Shigeoka S, Nakano Y, Kitaoka S (1980). Occurrence of L-ascorbic acid in *Euglena gracilis z*. Bull. Univ. Osaka Ser. B 32: 43-48.

Takeyama H, Kanamaru A, Yoshino Y, Kakuta H, Kawamura Y, Matsunaga T (1997). Production of Anti-oxidant vitamins, β-carotene, vitamin C and vitamin E, by two-step culture of *Euglena gracilis z*, Biotech. Bioeng. 53: 185-190.

Tani Y, Tsuruma H (1989). Screening for tocopherol producing microorganisms and α-tocopherol production by *Euglena gracilis z,* Agric. Biol. Chem. 53: 305-312.

Taylor DJ, Green NPO, Stout GW (1998a). Functions of polyunsaturated fatty acids and the main vitamins required in the human diet, Biological Science 3ʳᵈ ed. pp 252-255, Cambridge University Press, New York.

Taylor OJ, Green NPO, Stout GW (1998b). Fuel from biomass, Biological Science 3rd edn, Pp 406-407, Cambridge University Press, New York. medium. J. Electron Microsc. 39: 372-381.

Yamaberi K, Takagi M, Yoshida T (1998). Nitrogen depletion for intracellular triglyceride accumulation to enhance liquefaction yield of marine microalgal cells into a fuel oil. J. Mar. Biotechnol. 6: 44-48.

Yoshihara K, Nagase H, Eguchi K, Hirata K, Miyamoto K (1996). Biological elimination of nitric oxide from fuel gas by marine microalga NOA-113 cultivated in long tubular photobioreactor. J. Ferment. Bioeng 82: 351-354.

Zarborsky O, Benemaun JR, Miyake J, Matsunaga T, Sun Pietro A (1998). Biohydrogen, 2 edn, pp. 542-552, Plenum Press, New York.