Hydrobiologia **512:** 27–32, 2004.
P.O. Ang, Jr. (ed.), Asian Pacific Phycology in the 21st Century: Prospects and Challenges.

Microalgal studies for the 21st Century

Norihide Kurano & Shigetoh Miyachi*
Marine Biotechnology Institute, Kamaishi Laboratories, Heita, Kamaishi City, Iwate 026-0001, Japan
**Author for correspondence: E-mail: BXW02571@nifty.ne.jp*

Key words: microalgae, Marine Biotechonology Institute, Japan, CO_2 fixation, culture collection

Abstract

Microalgal photosynthesis is efficient enough to fix CO_2 in both atmosphere and industrially discharged gases, and is a possible future alternative for CO_2 reduction. This paper describes physiological responses of microalgal cells to extremely high CO_2 concentrations, capability of microalgal cells to fix CO_2 at both indoor and outdoor culture experiments, and efforts to establish a culture collection of marine microalgae. Recent researches indicate that microalgae are likely to play a key role in worldwide issues of the coming century.

Introduction

Since Marine Biotechnology Institute (MBI) started its work in April of 1990, one of the research targets at the Kamaishi Laboratories has been marine microalgae. Many microalgae have been collected from various parts of the Pacific Ocean and elsewhere. The features of these microalgae have been characterized and, with some, their capacity for CO_2 fixation has been extensively studied.

Since the continued increase in atmospheric CO_2 in parallel with the increase in fossil fuel consumption was first reported, a possible rise in the Earth's temperature due to greenhouse effect has become a matter of global concern. Photosynthesis seems to be the only feasible way to remove atmospheric CO_2. Reduction of atmospheric CO_2 by means of chemical or physical methods will be too expensive, since its concentration is as low as about 0.04%. The efficiency of photosynthesis is much higher in microalgae than in terrestrial plants including C4s (Miyachi, 1995), so the use of microalgae, especially marine microalgae, for reducing the CO_2 concentration in the atmosphere, attracted us.

Carbonic anhydrase of *Porphyridium*

The enzyme carbonic anhydrase (CA, EC 4.2.1.1), which enhances the equilibrium between CO_2 and bicarbonate in water, is involved in the mechanism underlying the high efficiency of photosynthesis in microalgae (Suzuki et al., 1994). Studies on this enzyme are being carried out at MBI. We will therefore briefly mention first our studies on CA of the red alga, *Porphyridium purpureum.*

This enzyme was purified to nearly a single band on SDS-PAGE, and its nucleotide and deduced amino acid sequences were elucidated (Mitsuhashi & Miyachi, 1996). The primary structure of this enzyme is characterized by two nearly identical domains, each homologous with CAs found in eukaryotes. It was, therefore, assumed that *Porphyridium* CA had evolved through the duplication of an ancestral and hypothetical CA gene with subsequent fusion of the duplicated genes. Mitsuhashi et al. (2000a) have determined the X-ray structure of this enzyme, and revealed a novel catalytic site for CO_2 hydration (Mitsuhashi et al., 2000b)

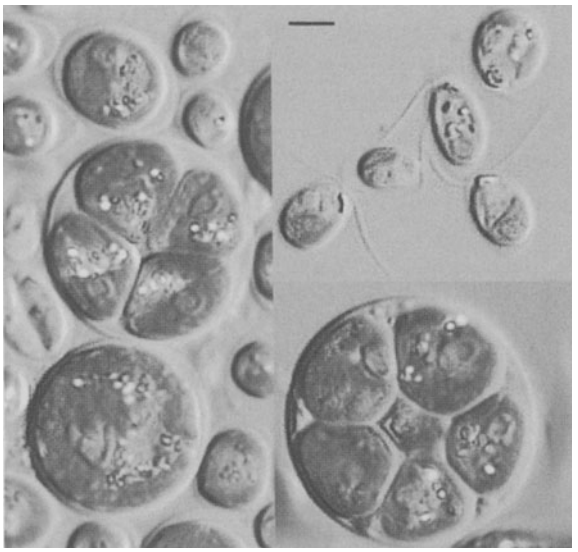

Figure 1. Photomicrographs of *Chlorococcum littorale*. Scale = 2 μm.

High CO_2-tolerant strain

It has been well documented that microalgae can play a very important role in the bioremediation of a low level of CO_2, CA being one of the essential tools. On the other hand, it is considered that microalgae are unsuitable for treating a high partial pressure of CO_2, since studies on some *Chlorella* species have revealed that CO_2 above 5% inhibited algal growth. Although this growth inhibition has been described as 'narcotic', the mechanism for the inhibition remains unclear.

Soon after MBI started its work, one scientist found a new species of a marine green microalga which could grow rapidly in an extremely high CO_2 concentration (Kodama et al., 1993). The new species, *Chlorococcum littorale* (Fig. 1), could grow under bubbled air enriched with 60% CO_2. At 10% CO_2, the growth rate was twice that in *Chlorella regularis* which had shown the highest growth rate in our culture collection. One of the surveys in 1988 indicated that about one third of CO_2 discharged from Japanese industries was from power stations. If we add CO_2 discharged from steelworks, the total would have covered about half of all CO_2 discharged by industry. These industrial flue gases usually contain 10–20% CO_2. The finding that *C. littorale* can grow rapidly under an extremely high CO_2 concentration raised the new possibility of absorbing CO_2 discharged from these industries by means of a biological method. Since the discovery of *C. littorale*, other green and blue-green algae that can grow rapidly under extremely high CO_2 concentrations have been found. After the finding of *C. littorale* had been reported, a report on the red alga *Cyanidium caldarium* (Tilden) Geitler, which could grow under 100% CO_2 (Seckbach et al., 1970), was brought to our attention. Unfortunately, there has been no follow up on this important finding.

Physiology under high CO_2 pressure

In order to understand how *C. littorale* cells can grow under an extremely high CO_2 concentration, we studied the change in photosynthetic characteristics during the course of adaptation of air-grown cells to 40% CO_2.

When air-grown cells were inoculated into a fresh culture medium and aerated with ordinary air supplemented with 40% CO_2, they did not grow for the first 3–4 days (Pesheva et al., 1994). However, logarithmic growth started after that. During the induction period, the specific growth rate was nearly zero and photosynthesis was inhibited. During the initial phase of inhibition of photosynthesis by 40% CO_2, the activity of PS II in intact cells measured by the Hill reaction in the presence of benzoquinone was suppressed. In contrast, the PS I activity in cell homogenates measured by oxygen uptake as a result of the photoreduction of methylviologen was greatly enhanced. These results obtained in batch cultures were confirmed by other measurements from a continuous culture (Iwasaki et al., 1996).

The cell concentration of *C. littorale* was kept constant with constant in- and out-flow of air. When the bubbling gas was changed from air to 40% CO_2, the concentration of algal cells temporarily decreased, then recovered to the original level by the 4th day followed by a further increase. In a green alga, *Stichococcus bacillaris,* which is intolerant to extremely high CO_2, cell concentration was constant in air, progressively decreasing and finally the cells disappeared at 40% CO_2, indicating that the *Stichococcus* cells could not survive at this CO_2 concentration. A quenching analysis of chlorophyll fluorescence carried out by the saturation pulse method supported the suggestion that there was photoinhibition in PS II during the induction period after the cells were transferred to 40% CO_2 from air. The change in excitation energy distribution between PS I and PS II was measured by the ratio of fluorescence at 714 nm and 618 nm (F714/F678) at 77K. The transition from state 1 to

state 2 was enhanced immediately after the *C. littorale* cells had been transferred from air to 40% CO_2 (Iwasaki et al., 1998). No such change in excitation energy distribution was apparent when *S. bacillaris* cells were transferred from air to 40% CO_2. During the transition period, the level of D1 protein in *C. littorale* cells was approximately constant, indicating that PS II (including D1 protein) in these cells was protected from photoinhibition by controlling the state transition. Such a protective mechanism is not likely to have functioned in *S. bacillaris* cells because the level of their D1 protein dropped during the transient period.

The next query was why PS II in *S. bacillaris* was damaged under extremely high CO_2 concentrations. One possibility is acidification of the algal cells due to the formation of protons during the course of CO_2 hydration. ^{31}P-NMR spectroscopy and the DMO method showed a drop in the cytoplasmic pH value when *S. bacillaris* cells were exposed to 40% CO_2, while the cytoplasmic pH value remained constant in *C. littorale* cells exposed to 40% CO_2. This indicated that the inhibition of photosynthesis by an extremely high CO_2 concentration was associated with intracellular acidification (Pronina et al., 1993).

In this connection, an increase in the number and size of vacuoles has been observed in *C. littorale* cells under extremely high CO_2 concentrations, but no such change was apparent in *S. bacillaris* cells (Kurano et al., 1998). With *C. littorale* cells, Sasaki et al. (1999) have further reported that the activity of vacuolar-type H^+-ATPase increased under 40% CO_2. These results suggest that vacuole development associated with enhanced vacuolar H^+-ATPase activity occurred during the acclimatization of *C. littorale* cells to an extremely high CO_2 concentration.

Acidification of the cytoplasm and possibly of the chloroplast would inhibit the Calvin-Benson cycle, decreasing the population of open-state PS II reaction centers. We, therefore, assume that during the early period after the transfer of *C. littorale* cells from air to 40% CO_2, a large increase in their PS I/PS II ratio will support the energy supply for ATP synthesis, which would be enhanced by cyclic electron transfer around PS I. Accordingly, the state 2 transition in photosynthesis will support the pumping activity of H^+-translocating ATPase across the cytoplasmic and chloroplastic membranes to maintain the pH values of the cytoplasm and chloroplast under extremely high CO_2 concentrations. In contrast, *S. bacillaris* cells, which did not show the state 2 transition, cannot maintain the cytoplasmic and chloroplastic pH values constant under extremely high CO_2 conditions.

CO_2 fixation

In parallel to these photosynthetic studies, studies on the growth capability of microalgae are also being carried out at MBI. To evaluate the maximum ability for growth and CO_2 uptake by microalgae, a novel photobioreactor of the flat-plate type has been developed. This reactor is mainly characterized by its short light path, which is less than 2 cm, curved baffles which stimulate liquid mixing, and intensive aeration which enhances gas–liquid mass transfer and the flashing light effect. The flat plate is made of transparent acrylic plastic and is illuminated from both sides by banks of fluorescent lamps. Ordinary air or air enriched with CO_2 is supplied from the bottom of the reactor through a fine perforated tube. The culture temperature is controlled in a water bath made of acrylic plastic. Under continuous illumination by an average light intensity of 2000 μmol m^{-2} s^{-1}, *C. littorale* cells exhibited a very high linear growth rate of 384 $\pm$ 30 mg of dry cells l^{-1} h^{-1} (Hu et al., 1998a) in a small-scale flat-plate photobioreactor with 1 cm light path and 1.5 l working volume. This growth rate corresponds to a CO_2 uptake rate of 16.7 g in 24 h, and is almost 4 times faster than the highest value so far reported for a marine cyanobacterium (Takano et al., 1992). By replacing the culture medium daily with a fresh medium, the maximum cell density reached as high as 84 g of dry cells l^{-1}. Frequent medium replacement has two positive effects: compensation of nutrient limitation and removal of autoinhibitory activity. This high cell concentration holds economic significance, because cell separation from the culture medium requires a large amount of power and a high cell density saves on this cost.

Larger-scale culture experiments have been conducted outdoors to evaluate the CO_2 fixation ability of microalgae under solar irradiation (Zhang et al., 1999). Three to five flat plates, each being 1.5 cm wide, 100 cm long and 80 cm high and giving a 9 l working volume, were stood vertically in parallel at a distance of 25 to 50 cm (Fig. 2). The thermophilic cyanobacterium, *Synechocystis aquatilis* SI-2, which had been isolated from a hot spring for the purpose of CO_2 fixation in a temperature range higher than that at which *C. littorale* could grow, was grown in this reactor system. The optimum temperature for the

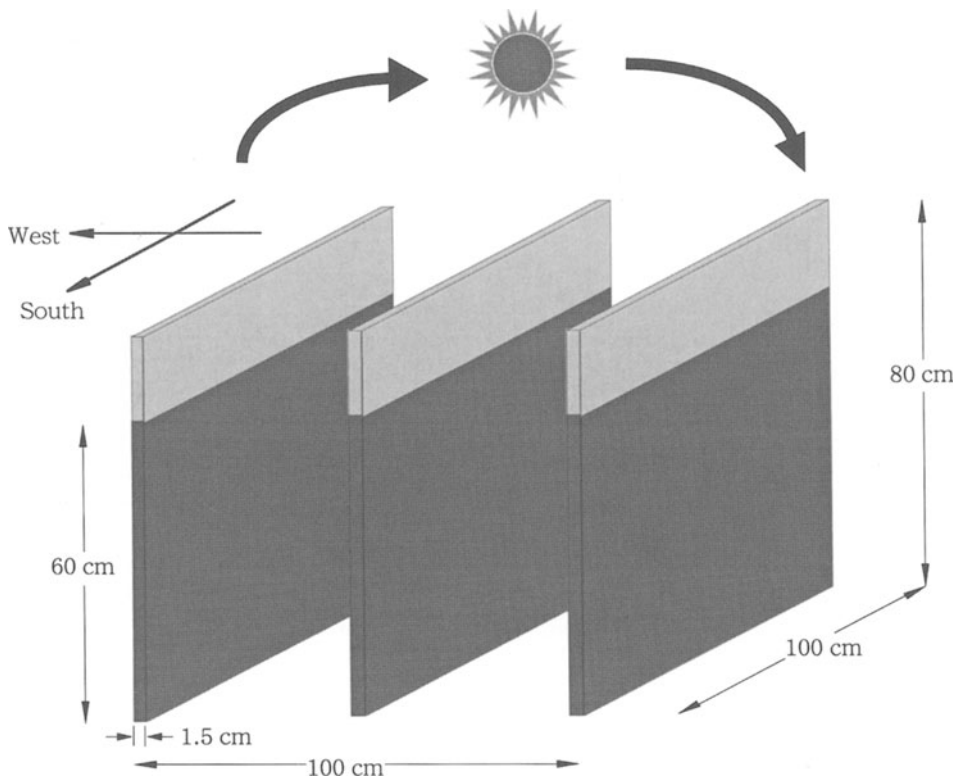

Figure 2. Flat-plate photobioreactor system for outdoor culture.

growth of this alga is 40 °C (being 25 °C for *C. littorale*), and 2 h of doubling time was observed at this temperature. We have chosen *S. aquatilis* SI-2 as a candidate organism for outdoor experiments, because *C. littorale* cannot survive above 30 °C and the temperature of outdoor cultures often exceeds this limit. The effects of the following three factors on cell productivity were studied during the summer of 1998 at Kamaishi (39° N, 142° E): direction of the flat-plate surface, distance between the plates and temperature control. Japan has a shortage of usable land and land is therefore expensive; the smaller the land area needed by the reactor system for CO_2 fixation, the better the economics. In these outdoor experiments, we therefore evaluated the CO_2 fixation rate on the basis of the land area occupied by individual plates. When five plates were placed in an east-west orientation separated by a distance of 25 cm and the culture temperature was regulated in the optimal range (37–43 °C), a CO_2 fixation rate of 53 g of CO_2 m^{-2} of ground area d^{-1} was achieved. This is an average value obtained in the middle three plates during 8 days, the average daily irradiation being 6.3 MJ m^{-2} d^{-1} in this period. This CO_2 fixation ability is about 10 times greater than that of the average forestland in the temperate regions. This high capability implies that the reactor design provides effective utilization of solar irradiation. Unlike conventional open-pond culture facilities, the flat-plate system has a three-dimensional structure which can function to diffuse unnecessarily strong sunlight into a moderate intensity and distribute it uniformly on to the flat surface. The microalgal cells utilize this moderate irradiation for photosynthesis without any unfavorable effect of photoinhibition. The high growth potential of *S. aquatilis* SI-2 also contributes to the performance of this system.

Culture collection

The search for microalgae with high efficiency for CO_2 fixation has resulted in many strains collected and stored at MBI. Based on this culture stock, we are going to establish a specialized and open culture collection of marine microalgae. The purposes of this effort are as follows: (1) to maintain and to supply marine strains for scientific studies as well as for industrial application, (2) to develop a tech-

Table 1. Some characteristic microalgal strains mentioned in this paper.

Species	Unique properties	Reference
Acaryochloris marina Miyashita et al.	Possesses chlorophyll *d* as a major photosynthetic pigment	Miyashita et al. (1996, 1997) Hu et al. (1998b)
Chlorococcum littorale N. Chihara, T. Nakayama et I. Inouye	Grows at high CO_2	Kodama et al. (1993)
Porphyridium purpureum (Bory de Saint-Vincent) K. Drew et Ross	Possesses unique gene structure and novel catalytic site of CA	Mitsuhashi et al. (1996, 2000a, 2000b)
Stichococcus bacillaris Nägeli	Sensitive to high CO_2	Iwasaki et al. (1996, 1998)
Synechocystis aquatilis Sauvageau	Very short doubling time (2 h)	Zhang et al. (1999)

nique for effective preservation and (3) to construct a database containing taxonomic information, strain history, characteristics of each strain, growth conditions, photo- and electron-micrographs, 18S rDNA (16S rDNA for the Cyanophyta) gene sequences, etc.

With strong emphasis on the phylogenetic relationship, the 18S rDNA sequences of nearly all of our green algal strains have been determined. The results indicate that most of these strains form clades consisting of marine microalgae, while a few belong to the fresh-water group. This means some algae of fresh-water origin can be found in seawater, even in pelagic waters. Traditional methods for taxonomy, for example morphological observations by electron microscopy and pigment analysis, are also core activities of this project.

Cryopreservation is an important method to effectively maintain algae; however, many strains cannot survive after being frozen. Periodical subculturing is the only method at this moment, and extending the subculture interval by applying sub-optimum growth conditions for culturing helps to decrease the labor required and the risks accompanying such manipulation. To develop a universal medium for supporting the life of a broad range of marine strains is also one of our targets to achieve effective preservation. One result of this effort, the Daigo IMK medium, is now commercially available and is manufactured by Nihon-Seiyaku of Japan (contact e-mail: life-tc@nihon-pharm.co.jp, or labchem-tect@wako-chem.co.jp). This medium is applicable to Cyanophyta, Rhodophyta, Chlorophyta, Cryptophyta, Chlorarachniophyta, Dinophyta, Heterokontophyta and Haptophyta.

At present, we are privately maintaining more than a thousand strains, with ca. 870 original isolates comprising Cyanophyta, Rhodophyta, Cryptophyta, Dinophyta, Heterokontophyta, Haptophyta, Chlorarachniophyta, Chlorophyta, unidentified strains and protozoa. Some of them produce a large amount of intracellular starch, extracellular polysaccharides (Miyashita et al., 1993), and polyunsaturated fatty acids (Kawachi et al., 1996). It should be noted that the novel marine prokaryote, *Acaryochloris marina*, which has chlorophyll *d* as its major photosynthetic pigment, has been isolated and studied in detail at MBI (Miyashita et al., 1996; 1997; Hu et al., 1998b). Some of these microalgae with outstanding feature are listed in Table 1. New classes, orders, genera and species exist in this collection, showing that the marine environment still embraces a great number of unknowns.

Conclusion

The research activities that have been described indicate that microalgae are likely to play a key role in solving some environmental problems, in studies on photosynthesis, in the production of useful substances, and in understanding the marine ecosystem. Technology and knowledge on these small organisms will contribute to the welfare of humans in the coming century.

Acknowledgement

This work was partly supported by New Energy and Industrial Technology Development Organization (NEDO).

References

Hu, Q., N. Kurano, M. Kawachi, I. Iwasaki & S. Miyachi, 1998a. Ultrahigh-cell-density culture of a marine green alga, *Chlorococcum littorale*, in a flat-plate photobioreactor. Appl. Microbiol. Biotech. 49: 655–662.

Hu, Q., H. Miyashita, I. Iwasaki, N. Kurano, S. Miyachi, M. Iwaki & S. Itoh, 1998b. A photosystem I reaction center driven by chlorophyll *d* in oxygenic photosynthesis. Proc. Natl. Acad. Sci. USA 95: 13319–13323.

Iwasaki, I., N. Kurano & S. Miyachi, 1996. Effects of high-CO_2 stress on photosystem II in a green alga, *Chlorococcum littorale*, which has a tolerance to high CO_2. J. Photochem. Photobiol. B: Biology 36: 327–332.

Iwasaki, I., Q. Hu, N. Kurano & S. Miyachi, 1998. Effect of extremely high-CO_2 stress on energy distribution between PS I and PS II in a 'High-CO_2' tolerant green alga, *Chlorococcum littorale*, and the intolerant green alga, *Stichococcus bacillaris*. J. Photochem. Photobiol. B: Biology 44: 184–190.

Kawachi, M., M. Kato, H. Ikemoto & S. Miyachi, 1996. Fatty acid composition of a new marine picoplankton species of the Chromophyta. J. appl. Phycol. 8: 397–401.

Kodama, M., H. Ikemoto & S. Miyachi, 1993. A new species of highly CO_2-tolerant fast-growing marine microalga for high-density cultivation. J. Mar. Biotech. 1: 21–25.

Kurano, N., T. Sasaki & S. Miyachi, 1998. Carbon dioxide and microalgae. In Inui T. et al. (eds), Advances in Chemical Conversions for Mitigating Carbon Dioxide Studies in Surface Science and Catalysis, vol. 114. Elsevier Science B.V., Amsterdam: 55–63.

Mitsuhashi, S. & S. Miyachi, 1996. Amino acid sequence homology between N- and C-terminal halves of a carbonic anhydrase in *Porphyridium purpureum*, as deduced from the cloned cDNA. J. Biol. Chem. 271: 28703–28709.

Mitsuhashi, S., T. Mizushima, E. Yamashita, S. Miyachi & T. Tsukihara, 2000a. Crystallization and preliminary X-ray diffraction studies of a carbonic anhydrase from the red alga, *Porphyridium purpureum*. Acta Cryst. D56: 210–211.

Mitsuhashi, S., T. Mizushima, E. Yamashita, M. Yamamoto, T. Kumasaka, H. Moriyama, T. Ueki, S. Miyachi & T. Tsukihara, 2000b. X-ray structure of β-carbonic anhydrase from the red alga, *Porphyridium purpureum*, reveals a novel catalytic site for CO_2 hydration. J. Biol. Chem. 275: 5521–5526.

Miyachi, S., 1995. Diversity of microalgae and their possible application. OECD Documents, Environmental Impacts of Aquatic Biotechnology: 28–31.

Miyashita, H., H. Ikemoto, N. Kurano, S. Miyachi & M. Chihara, 1993. *Prasinococcus capsulatus* gen. et sp. nov., a new marine coccoid prasinophyte. J. Gen. appl. Microbiol. 39: 571–582.

Miyashita, H., K. Adachi, N. Kurano, H. Ikemoto, M. Chihara & S. Miyachi, 1997. Pigment composition of a novel oxygenic photosynthetic prokaryote containing chlorophyll *d* as the major chlorophyll. Plant Cell Physiol. 38: 274–281.

Miyashita, H., H. Ikemoto, N. Kurano, K. Adachi, M. Chihara & S. Miyachi, 1996. Chlorophyll *d* as a major pigment. Nature 383: 402.

Pesheva, I., M. Kodama, M. L. Dionisio-Sese & S. Miyachi, 1994. Changes in photosynthetic characteristics induced by transferring air-grown cells of *Chlorococcum littorale* to high-CO_2 conditions. Plant Cell Physiol. 35: 379–387.

Pronina, N. A., M. Kodama & S. Miyachi, 1993. Changes in intracellular pH values in various microalgae induced by raising CO_2 concentrations. XV Int. Botanical Cong., Yokohama, Japan: 419.

Sasaki, T., N. A. Pronina, M. Maeshima, I. Iwasaki, N. Kurano & S. Miyachi, 1999. Development of vacuoles and vacuolar H^+-ATPase activity under extremely high-CO_2 conditions in *Chlorococcum littorale* cells. Plant Biol. 1: 68–75.

Seckbach, J., A. F. Baker & P. M. Shugarman, 1970. Algae thrive under pure CO_2. Nature 227: 744–745.

Suzuki, E., Y. Shiraiwa & S. Miyachi, 1994. The cellular and molecular aspects of carbonic anhydrase in photosynthetic microorganisms. Progress in Phycological Research 10: 1–54.

Takano, H., H. Takeyama, H. Nakamura, H. Sode, J. G. Burges, E. Manabe, E. Hirono & T. Matsunaga, 1992. CO_2 removal by high-density culture of a marine cyanobacterium *Synechococcus* sp. using an improved photobioreactor employed light-diffusing optical fibers. Appl. Biochem. Bioeng. 34/35: 449–458.

Zhang, K., N. Kurano & S. Miyachi, 1999. Outdoor culture of a cyanobacterium with a vertical flat-plate photobioreactor: effects on productivity of the reactor orientation, distance setting between the plates, and culture temperature. Appl. Microbiol. Biotechnol. 52: 781–786.