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Production of Optically Pure D-Lactic Acid by *Nannochlorum* sp. 26A4

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Abstract

Microalgae were screened from seawater for greenhouse gas CO_2 fixation and D-lactic acid production by self-fermentation and tested for their growth rate, starch content, and conversion rate from starch into D-lactic acid. More than 300 strains were isolated, and some of them were found to have suitable properties for this purpose. One of the best strains, *Nannochlorum* sp. 26A4, which was isolated from Sakito Island, had a starch content of 40% (dry weight), and a conversion rate from consumed starch into D-lactic acid of 70% in the dark under anaerobic conditions. The produced D-lactic acid showed a high optical purity compared with the conventional one. The proposed new D-lactic acid production system using *Nannochlorum* sp. 26A4 should also be an effective technology for greenhouse gas CO_2 fixation and/or conversion into industrial raw materials.

Index Entries: D-lactic acid; optical purity; microalgae; self-fermentation; *Nannochlorum* sp.; Sakito Island.

Introduction

Lactic acid is a chiral molecule that includes some types of optical isomer acids, such as the L type or D type or a mixture of the L and D types. Of these isomers, D-lactic acid has a valuable potential as a raw material for biodegradable plastics, agricultural chemicals, and medical supplies. Up to now, optically high-purity D-lactic acid could be heterotrophically produced from sugar by some bacteria such as *Sporolactobacillus inulinus*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, and *Leconostoc mesenteroides* subsp. *mesenteroides (1–3)*. The optical purity of these D-lactic acids was confirmed

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to be approx 96% *(4,5)*. This optical purity is lower than that of L-lactic acid that was developed a long time ago for increasing the optical purity in the fermentation industry *(6–8)*. Regarding chiral molecule production, because a low optical purity has led to a thalidomide disaster, a high optical purity of the chiral molecule must provide high safety for application to agricultural chemicals and medical supplies. Based on this history, it was postulated that the development of a new method or technology was needed for the production of higher optically pure D-lactic acid, with this technology leading to more applications of D-lactic acid.

It is known that microalgae containing starch have the ability to convert their starch into some organic matters such as lactic acid, ethanol, acetic acid, and formic acid under dark and anaerobic conditions *(9–11)*. There are only a few reports on the productivity and/or optical characteristic of lactic acid from microalgal anaerobiosis. Based on this background, we screened microalgal strains that produce D-lactic acid with high optical purity. In this article, we report on the isolation of this microalga mean isolated alga of *Nannochlorum* sp. 26A4 and its properties of the D-lactic acid production under dark and anaerobic conditions. We also discussed the proposed new system with D-lactic acid production.

Materials and Methods

Cultivation of Microalgae

Each algal strain was isolated from seawater samples from around Japan by micropipet manipulation and colony formation on a gellan gum or an agar gel containing medium A (pH 7.8) *(12)*. The content of medium A was as follows: 0.2 g of $NaNO_3$, 0.01 g of sodium β-glycerophosphate, 0.1 g of ethylenediaminetetraacetic acid iron salt (Fe-EDTA), 0.06 g of Clewat-32 (Teikoku, Osaka, Japan), 0.2 g of vitamin B_{12}, and 0.1 g of Tris (hydroxy methyl) aminomethane in 1 L of seawater. The pH of medium A was adjusted to 7.8, and then the medium was autoclaved. Algal cells from the seawater sample in the stationary growth phase in the main culture using 2-L flat culture bottles sparged with air containing 0.5% CO_2 at 15,000 lux were harvested by centrifugation.

Dark Fermentation

The cells were resuspended in 0.4 *M* potassium phosphate buffer (pH 7.7) for protection against a decreasing pH at a final density of 150–250 mg dry wt/mL and placed in a light-shielded airtight tube (10 mL) for self-fermentation. The starch content was measured by a coupled method with perchloric acid and glucose oxidase *(13)*. The fermentative products were analyzed by gas chromatography, and optical isomers of the lactic acid were detected by both liquid chromatography with a chiral column (SUMICHIRAL OA-5000, Sumitomo, Osaka, Japan) and enzymatic analysis (F-Kit; Boehringer Mannheim).

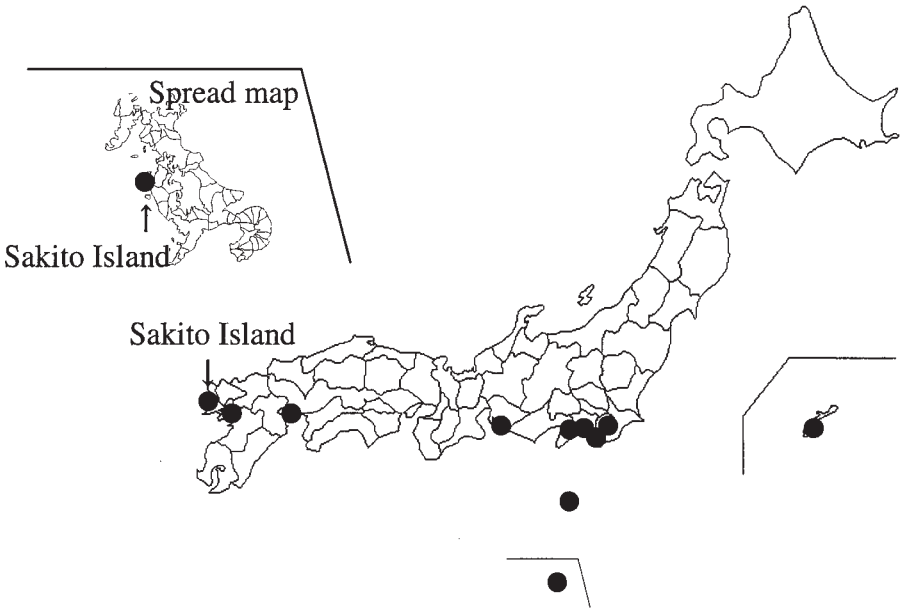

Fig. 1. Location of seawater sampling sites in Japan.

Enzyme Assay

Soluble extracts were made by breaking the microalgal cells in a sonicator followed by centrifugation and dialysis at 4°C. The nicotinamide adenine nucleotide-linked lactate dehydrogenase was measured by monitoring A_{340} *(14)*. One unit of enzyme activity was defined as the amount of enzyme catalyzing the degradation of 1 µmol of NADH/min at 25°C.

Results and Discussion

Isolation of Microalgae From Seawater

The location of the seawater sampling sites around Japan are shown in Fig. 1, and the seawater samples were mainly collected from the southern area of Japan. During the course of screening the microalgae from the seawater, more than 300 strains were isolated, but many of them showed adhesive growth on the preculture flasks and/or flocculated growth. More than 20 strains were tested to examine their algal productivity, starch content, and microalgal lactic acid production. Some strains had a starch content of more than 35% (vs dry weight), and a starch conversion into some organic acids. One of the best strains, *Nannochlorum* sp. 26A4 (see Fig. 2), was isolated from Sakito Island, located in the Nagasaki Prefecture, Japan. Professor I. Inouye and Dr. T. Nakayama of the University of Tsukuba classified and identified this strain.

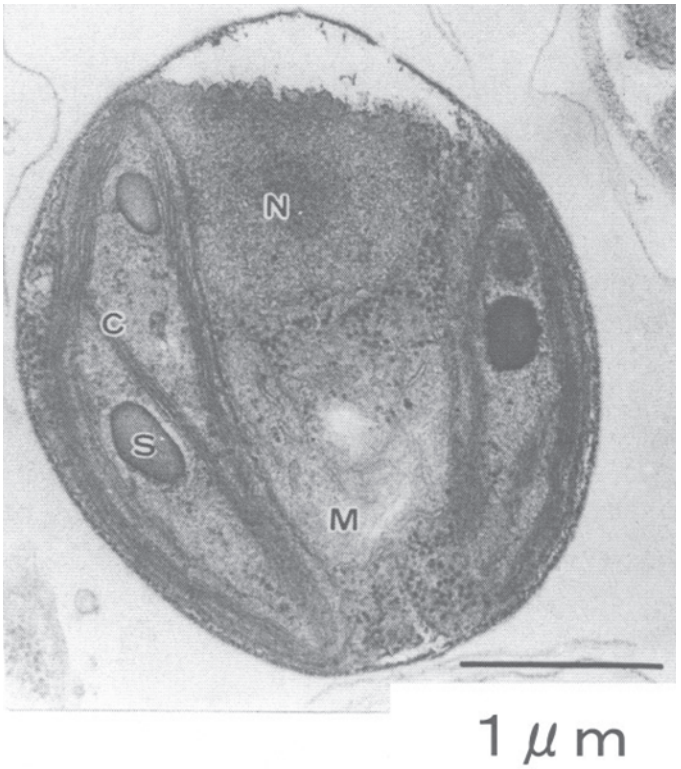

Fig. 2. Electron micrograph of *Nannochlorum* sp. 26A4. The preparation was fixed in 2.5% glutaraldehyde in 0.2 *M* cacodylate buffer (pH 7.2) followed by 2% osmium tetroxide in the same buffer. C, chloroplast; M, mitochondrion; N, nucleus; S, starch.

D-Lactic Acid Production From Isolated Microalga

The resulting cultivation of *Nannochlorum* sp. 26A4 showed a starch content of 40% (dry weight). After the algal cells were harvested and then incubated under anaerobic conditions at room temperature, this strain indicated a conversion rate, which is defined as the theoretical ratio shown by Eq. 1, from consumed microalgal starch to lactic acid of 70%, corresponding to 26,000 mg/L of lactic acid. As minor components, acetic acid, propionic acid, and ethanol were produced, but the main product was lactic acid:

$$C_6H_{10}O_5 + H_2O \rightarrow 2C_3H_6O_3 \quad (1)$$

Because barely no intracellular starch remained after 3 d at 25–35°C, this lactic acid production seemed to finish within 3 d. These results demonstrate that no added energy for temperature control was needed to produce lactic acid at room temperature levels. The resulting supernatant of the algal slurry was filtered and then underwent separation to a chiral isomer by high-performance liquid chromatography. As a result of this chromatography, only D-lactic acid was detected and no L-lactic acid was

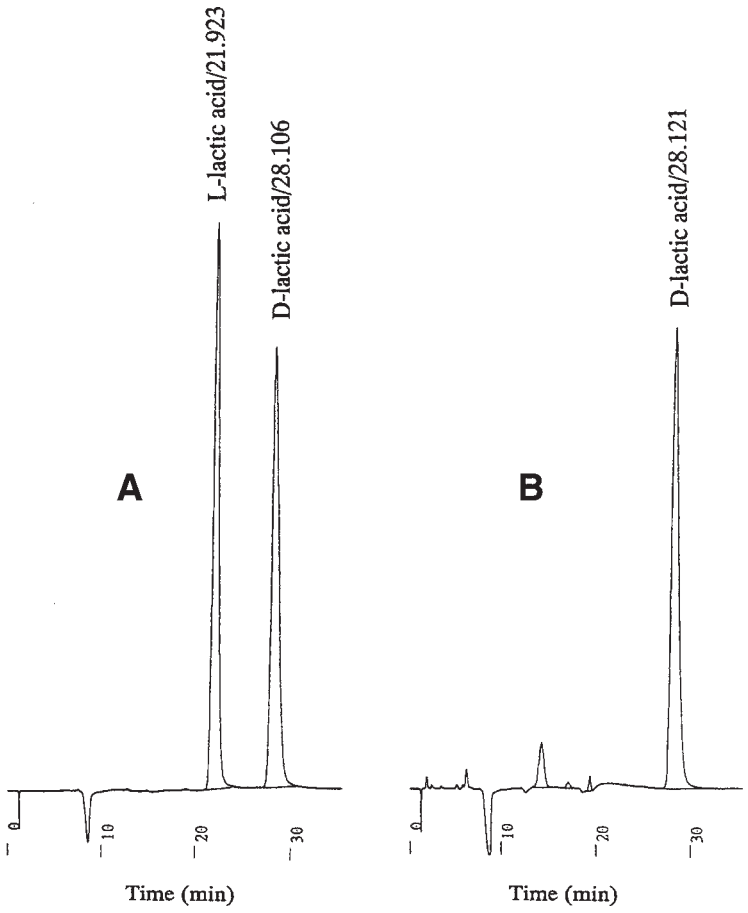

Fig. 3. High-performance liquid chromatograms of lactic acid. **(A)** Standard of D- or L-lactic acid; **(B)** optical purity check of lactic acid in fermentative products of *Nannochlorum* sp. 26A4.

found, as shown in Fig. 3. Because the concentration of the detected D-lactic acid was 5100 mg/L, and the identification limit of the L-lactic acid was <5 mg/L, the optical purity of this D-lactic acid is thought to be at least 99.9%. In addition, because the purity of the D-lactic acid was >99.8% by an enzymatic analysis, the optical purity of D-lactic acid must be at least 99.8%. Phosphate has been used to improve optical purity, but its detailed mechanisms are not clear *(8)*. In the present study, because phosphate was not used for D-lactic acid production under dark and anaerobic conditions, the mechanisms leading to the high optical purity are now being investigated. Such an extremely high optically pure D-lactic acid has never been reported. This optical purity of D-lactic acid seemed to be the highest in the world reported for D-lactic acid production.

Green algae usually produce organic matter, such as lactate and ethanol, only under dark conditions by intracellular starch digestion via the Embden-Meyerhof-Pamas pathway *(9)*. The activity of lactate dehydro-

Table 1
Lactate Dehydrogenase Activities of Cell-Free Extract of *Nannochlorum* sp. 26A4 Cells From Light or Dark Condition[a]

	Activity (nmol/[min·mg] of protein)	
Enzyme	Light condition	Dark condition
Lactate dehydrogenase	0.0 ± 0	3.1 ± 0.1

[a]Values are an average of repeatedly measuring three times.
The extract of light condition was immediately prepared from photoautotrophically grown cells. The extract of dark condition was prepared from the dark fermentation cells for 24 h.

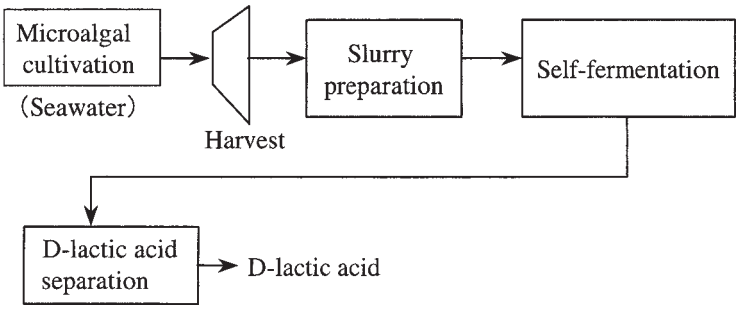

Fig. 4. D-lactic acid production system using microalgal self-fermentation.

genase determined from the cell-free extract of *Nannochlorum* sp. is shown in Table 1. The activities of the lactate dehydrogenase were detected only under dark conditions. These results indicate that D-lactate was produced only under dark conditions by lactate dehydrogenase, not under light conditions.

Designation of D-Lactic Acid Production System

Based on some properties of D-lactic acid production by microalgae, we designed and proposed a new optically specific D-lactic acid production system using this microalgal fermentation under dark and anaerobic conditions. The proposed D-lactic acid production system as a new technology shown in Fig. 4 consists of microalgal cultivation, algal cell harvest, slurry preparation, self-fermentation of the algae, and D-lactic acid extraction processes. The system seems very simple for the production of D-lactic acid with an extremely high optical purity compared with other conventional ones *(4,5)*. In addition, the culture medium in the system does not need careful selection compared with heterotrophic microorganisms such as *Streptococcus faecalis (8)*, thus providing a simple process.

From both the growth rate and conversion rate to the lactic acid, approx 2.3 g of lactate/(m^2·d) is expected as the carbon sequestration

potential of this system. Although the cost of producing lactic acid must depend on its scale and sanitary grade of the main facilities, such as the microalgal cultivation ponds and harvester, the minimum potential costs are expected to be less than the cost ($10–20/dry kg) of microalgal supplement food with sanitary equipment *(15)*.

Because algal cultivation has the advantages of being used in devastated lands and seawater on a large scale, the system also should be an effective technology for greenhouse gas CO_2 fixation and/or conversion into more valuable products.

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