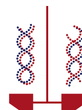

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Extracellular protease derived from lactic acid bacteria stimulates the fermentative lactic acid production from the by-products of rice as a biomass refinery function

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A lactic acid producing bacterium, *Lactobacillus rhamnosus* M-23, newly isolated from a rice washing drainage storage tank was found to produce L-(+)-lactic acid from a non-sterilized mixture of rice washing drainage and rice bran without any additions of nutrients under the simultaneous saccharification and fermentation (SSF) process. This strain has the ability to utilize the non-sterilized rice washing drainage and rice bran as a source of carbohydrate, saccharifying enzymes and nutrients for lactic acid production. Observation of extracellular protease activity in SSF culture broth showed that a higher protease activity was present in strain M-23 than in other isolated lactic acid producing bacteria (LABs). To investigate the structural changes of solid particles of rice washing drainage throughout LAB cultivation, scanning electron microscopic (SEM) observation and Fourier transform infrared-spectroscopy (FT-IR) analysis were performed. The results of the SEM observation showed that the surface material could be removed from solid particles of rice washing drainage treated by culture broth (supernatant) of strain M-23, thus exposing the crystal structure of the starch particle surface. The results of the FT-IR analysis revealed that the specific transmittance decrease of the C–C and C–O stretching and OH– group of the solid particles of the rice washing drainage were highly correlated with the produced lactic acid concentration and extracellular protease activity, respectively. These results demonstrate the high lactic acid producing ability of strain M-23 from a non-sterilized mixture of rice washing drainage and rice bran under the SSF condition due to the removal of proteinaceous material and exposure of the starch particle surface by extracellular protease.

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[**Key words:** Rice bran; Rice washing drainage; Simultaneous saccharification and fermentation; Lactic acid; Protease]

Lactic acid is used in food technology as a preservative or taste enhancing additive and is the source of poly lactic acid, a polymer used as a biodegradable plastic (1). In addition, lactic acid can be polymerized to form the biodegradable and recyclable polyester poly (lactic acid), which may help with solving the world-wide environmental problem (2). Therefore, various companies have done extensive work on the development of lactic acid-based products such as polylactide-based resins (Nature-Works PLA), which are used for plastics, packaging applications and 3D printing and Ingeo polydiactide-based fibers, which are used in specialty textiles and fiber applications (3).

Highly purified preferably 100% optically pure L(+)-lactic acid anhydrous monomer is required for the production of bio-based polymer, a poly lactic acid that is an environmentally friendly replacement of plastics derived from petrochemical materials (4). Optical pure lactic acid can be produced by the homo lactic acid bacteria. However, it is known that lactic acid producing bacteria (LAB) such as *Lactobacilli* need a large number of nutrients (e.g., amino acids, vitamins) for their growth and lactic acid production (1–4). Since, the cost of culture media for LABs is mainly attributed to nutrient sources, new and inexpensive nutrients for the industrial process are desired. Consequently, various low-cost nutrient sources have been put under screening for lactic acid production (3,5,6).

Recently, the demand for polished rice, so called rinse-free rice, has increased at restaurants and among families in Japan. At rice manufacturing sites, a large amount of rice washing drainage has been discharged and treated using expensive procedures involving equipment such as filter presses or spray driers. Therefore, the cost of waste-water treatment has increased (7,8). There are currently a few methods for use with rice washing drainage that contains a relatively high amount of solid particles composed of starch, some proteins (mainly glutelin), and vitamins (7,8).

Rice bran, another major by-product of rice polishing, contains germ and several histologically identifiable soft layers including pericarp, seed coat, nucellus, and aleurone layers (9,10). The weight ratio of bran is approximately 10% of an entire rice grain (paddy

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rice), so rice bran has attracted interest throughout the world as a potential resource for nutrients and elements.

In a previous study, in order to reduce and/or promote the utilization of both these by-products of rice, we investigated whether rice washing drainage derived from a rinse-free rice manufacturing machine and rice bran could be used as cheap material for lactic acid and bioethanol production by simultaneous saccharification and fermentation (SSF) as a source of carbohydrate and saccharifying enzymes (3,7). A newly isolated lactic acid producing bacterium *Lactobacillus rhamnosus* M-23, was found to produce L-(+)-lactic acid from a non-sterilized mixture of rice washing drainage and rice bran without any additions of nutrients under the SSF process. The maximum lactate yield of strain M-23 attained was 59 g/l with a productivity of 1.23 g/l/h and a product optical purity of 95% corresponding to a conversion of 0.85 g of lactic acid per gram of sugar equivalent (3).

However, until now, the mechanism behind the high lactic acid production ability of strain M-23 from a non-sterilized mixture of rice washing drainage and rice bran without any additions under the SSF process has not been clarified.

In the present study, we evaluated the relationship of lactic acid production ability, digestive enzyme activity (saccharifying enzyme and protease) in SSF culture broth, and structural changes of the solid particles of rice washing drainage throughout LAB cultivation under the SSF process.

MATERIALS AND METHODS

Materials Raw rice bran was prepared from brown rice (*Oryza sativa*, cv. Koshihikari, harvested in Niigata prefecture, Japan) using a rice polishing machine (NCP100B; Satake Co., Ltd., Higashi-Hiroshima, Japan) (3). The rice-polishing ratio of the brown rice was controlled to between 88% and 90%. Rice washing drainage was prepared using a rinse-free rice manufacturing machine (SJR-2A; Satake Co., Ltd.). Milled rice was washed for each condition to prepare rice washing drainage using a rinse-free rice manufacturing machine with a drive capacity of 2000 kg of milled rice/h, water consumption of 300 l/h, and a loading time of 8 h. Dry raw material in rice washing drainage was assumed to consist of rice polish (the by-product of polishing rice, consisting of inner bran layers of kernels with part of the germ and a small portion of the starchy interior).

Strains and growth condition For this study, we selected from the rice washing drainage storage tank five isolated LABs (*L. rhamnosus* M-23, *Lactobacillus paracasei* S-27, K-25, M-32 and S-4) having high lactic acid producing ability (more than 35 g lactic acid/l) produced from rice washing drainage and rice bran mixture without pH control or any additives such as nutrients (3).

Experimental procedures A pre-culture LAB was prepared by inoculating LAB from an agar slant into a 200-ml Erlenmeyer flask containing 100 ml PYG medium and the pre-culture was then grown at 37°C for 3 days without shaking. The harvested LAB cells were recovered by centrifugation at 5000 ×*g* for 10 min at 4°C and re-suspended with the same volume of sterile water (100 ml) in order to eliminate the effect of PYG medium constituents in the pre-culture LAB broth for later LAB cultivation and lactic acid production. All fermentation processes were terminated after 3 days. Fermentation experiments were performed in a 1-L bioreactor (MJ-1, Able & Biott, Tokyo, Japan) with a 0.6-L working volume that contained rice washing drainage (500 ml) and either rice bran (50 g) at 37°C. The pre-culture LAB was added (1% v/v) to the bioreactor. The initial pH of the medium was controlled at 6.5 ± 0.1 with 6N NaOH. Suspended culture and anaerobic conditions were maintained by continuous agitation (300 rpm) and intermittent nitrogen gas sparging of the culture media, respectively (3).

Assays for α-amylase, α-glucosidase, and protease activity The α-amylase activity in SSF culture broth was assayed using a commercial kit (cat. 60213, Kikkoman, Noda, Japan) and procedures were performed in accordance with the manufacturer's instructions. The α-glucosidase activity was assayed as follows: 0.1 ml of SSF culture broth was added into 1 ml of 100 mM acetate buffer (pH 4.5) containing 6 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) and the reaction was then performed at 37°C for 10 min. The reaction was terminated by the addition of 0.5 ml of 200 mM Na_2CO_3 solution (11). Protease activity was measured by azocasein and azocollagen hydrolytic activity (12). The enzyme solution was diluted to the appropriate concentration by an enzyme buffer [50 mM Tris-hydrochloride (pH 7.5), 5 mM $CaCl_2$]. Then, the enzyme solution and substrate solution (600 μl of 5% of azocasein and azocollagen, Sigma Chemical Co., dissolved in enzyme buffer) were incubated separately at 37°C. The enzyme reaction was started by mixing the two solutions and the mixture was kept at 37°C for 20 min. The reaction was stopped by the addition of 480 μl of 15%v/v trichloroacetic acid. After centrifugation (15,000 ×*g*, 10 min), 600 μl of the supernatant was neutralized with the 30 μl of 10N NaOH and the optical density was measured at the wavelength of 440 nm. One unit of protease activity was defined as the quantity required to increase the optical density by 0.1 at 440 nm.

Scanning electron microscopic and Fourier transform infrared-spectroscopy analysis Scanning electron microscopic (SEM) observations were

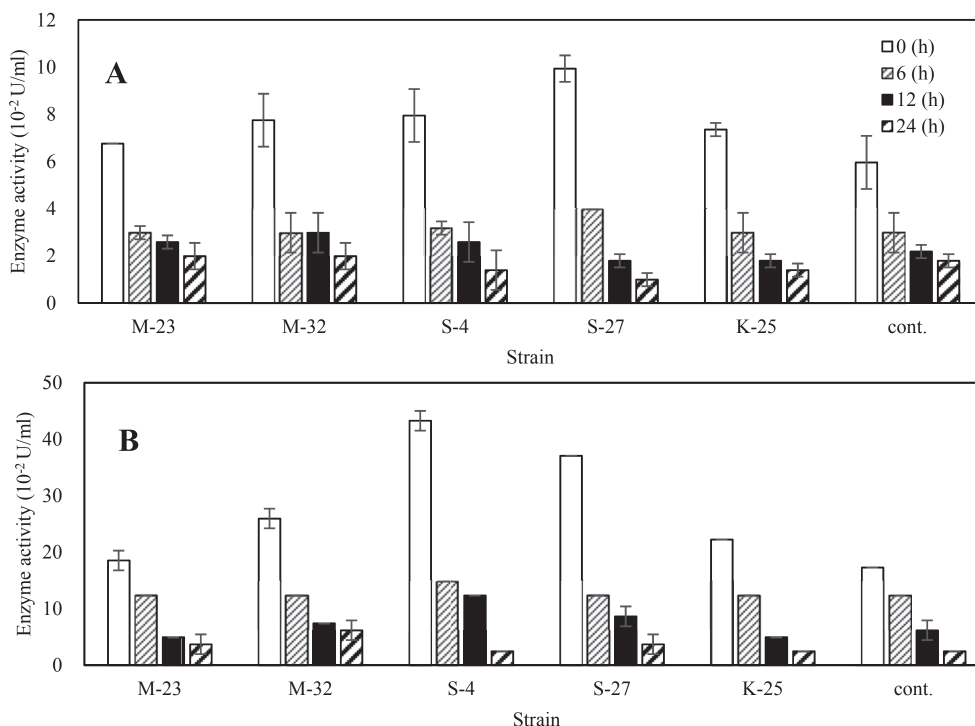

FIG. 1. Saccharifying enzyme activity of pH-controlled SSF culture broth of isolated LABs: (A) α-amylase, (B) α-glucosidase. Bars indicate standard deviations ($n = 3$).

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