New Medium for the Production of Exopolysaccharide (OSKC) by *Lactobacillus kefiranofaciens*

Hiroaki MAEDA,^{1*} Xia ZHU¹ and Tomotari MITSUOKA²

¹Research and Development Division, Daiwa Pharmaceutical Co., Ltd., 1–16–19, Sangenjaya, Setagaya-ku, Tokyo 154–0024, Japan

²Professor Emeritus, The University of Tokyo, 2–10–7, Sugano, Ichikawa 272–0824, Japan

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Lactobacillus kefiranofaciens is known to produce an exopolysaccharide (kefiran) that is either excreted in the growth medium or attaches to the cell wall. In order to achieve high exopolysaccharide production, we developed a new rice hydrolyzate (RH) medium using rice starch hydrolyzate as the carbon source and rice protein hydrolyzate as the peptone source. In this study, three fermentation experiments using RH medium, PYG10 medium and PYL10 medium were carried out, respectively. The highest amount of exopolysaccharide production was obtained from the culture grown in RH medium. Among the three various media compositions compared, rice protein hydrolyzate in the RH medium demonstrated the ability to significantly enhance exopolysaccharide production by *L. kefiranofaciens*. In addition, the cultivation of *L. kefiranofaciens* in a 500 l tank was also experimentally conducted, and the maximum yield of exopolysaccharide from the RH culture was 2.5 g/l after a seven-day culture period at pH 5.0 and 33°C. This result indicates that it is possible to produce exopolysaccharide (OSKC) at the level required for large-scale industrial production through the cultivation of *L. kefiranofaciens* in RH medium.

Key words: medium; rice hydrolyzate; *Lactobacillus kefiranofaciens*; exopolysaccharide

INTRODUCTION

Kefir is a popular fermented milk and one type of light alcoholic beverage. Kefir grains are usually used as a starter in the Caucasian regions. It is known that kefir grains consist of a gel matrix in which the microflora of yeasts and lactic acid bacteria are embedded (1, 9). *Lactobacillus kefiranofaciens* isolation from kefir grains has been reported to produce a water-soluble exopolysaccharide known as kefiran (4, 8, 21). The exopolysaccharide produced by *L. kefiranofaciens* has a molecular weight of approximately 1.0×10^6 , and is composed of a branched hexa- or heptasaccharide repeating unit containing approximately equal amounts of D-glucose and D-galactose residues. This structure is believed to be the same as that extracted from kefir grains (6, 11, 12). The gel obtained from the kefiran solution lacked rigidity, but its strength could be improved by adding ethanol or mixing with either κ -carrageenan or xanthan gum. These gel-forming characteristics and rheological properties of kefiran showed that kefiran could serve as a food thickener (13, 17). On the other hand, previous studies on the oral admin-

istration of kefiran to mice have indicated that kefiran is effective for tumor growth and delayed-type hypersensitivity induced by picryl chloride (14, 15, 18). Moreover, kefiran has also been demonstrated to have an enhancing effect on the production of interferon β from animal cells suppressed by stress hormones cortisol and noradrenaline (5).

There are some reports dealing with the isolation of *L. kefiranofaciens* from kefir grains, and the pure culturing conditions required for exopolysaccharide production using *Lactobacillus* sp. (3, 4, 10, 20–22). However, previous studies relating to the cultivation of *L. kefiranofaciens* were carried out on a limited scale in laboratories. In this study, we experimentally developed a new medium, named “rice hydrolyzate (RH) medium,” to produce exopolysaccharide (OSKC) using a pure culture of *L. kefiranofaciens*. In the RH medium, the main components of the carbon and peptone sources were from rice hydrolyzate with a procedure in which the rice was first hydrolyzed by pepsin and then by glucoamylase. The cultivation of *L. kefiranofaciens* in each RH medium, PYG10 medium and PYL10 medium was separately examined. We also investigated the possibility of producing exopolysaccharide in a pure culture of *L. kefiranofaciens* using a 500 l tank.

MATERIALS AND METHODS

Bacterial strain and media. The strain *L. kefirano-*

*Corresponding author. Mailing address: Research and Development Division, Daiwa Pharmaceutical Co., Ltd., 1–16–19, Sangenjaya, Setagaya-ku, Tokyo 154–0024, Japan. Phone: +81–3–5430–4050. Fax: +81–3–3422–5399. E-mail: maeda@daiwa-pharm.com

Table 1. Compositions of the experimental media.

Rice hydrolyzate (RH) medium	PYG10 medium	PYL10 medium
10% Rice starch hydrolyzate	10% Glucose	10% Lactose
0.35% Rice protein hydrolyzate	1.5% Polypepton	1.5% Polypepton
1% Yeast extract	1% Yeast extract	1% Yeast extract
0.1% Tween 80	0.1% Tween 80	0.1% Tween 80
0.2% K ₂ HPO ₄	0.2% K ₂ HPO ₄	0.2% K ₂ HPO ₄
0.5% Sodium acetate	0.5% Sodium acetate	0.5% Sodium acetate
0.2% Triammonium citrate	0.2% Triammonium citrate	0.2% Triammonium citrate
0.02% MgSO ₄ ·7H ₂ O	0.02% MgSO ₄ ·7H ₂ O	0.02% MgSO ₄ ·7H ₂ O
0.005% MnSO ₄ ·5H ₂ O	0.005% MnSO ₄ ·5H ₂ O	0.005% MnSO ₄ ·5H ₂ O

faciens WT-2B^T (JCM 6985 = ATCC 43761) was obtained from Tomohiko Fujisawa, Kanagawa Prefectural Public Health Laboratory, Yokohama, Japan. This strain was separately cultured in RH, PYG10, and PYL10 media. The compositions of the three experimental media are summarized in Table 1. The pH of each medium was adjusted to 6.5 before autoclaving at 115°C for 20 min.

Extraction and preparation of starch and protein fractions from rice. Commercial rice was obtained from a local market, and then powdered. The proteins contained in the rice were extracted twice with five volumes of 0.05 N NaOH by stirring at 25°C for 3 hr. After centrifugation at 10,000 × *g* for 20 min, the precipitate collected was prepared for further experiments as the starch fraction. The supernatant obtained was fractionated with ammonium sulfate, and solid ammonium sulfate was added to the supernatant up to 100% saturation with continuous stirring. After being left for 1 hr, the resulting precipitate was collected by centrifugation at 10,000 × *g* for 20 min, and then dialyzed against distilled water. The dialyzate obtained was used in further experiments as the protein fraction.

Hydrolysis of rice starch by glucoamylase. The above-mentioned starch fraction was homogenized in 6-fold amounts (weight) of distilled water. The pH was then adjusted to 7.0 with 4 N HCl. After centrifugation at 10,000 × *g* for 20 min, lactic acid was added to the resulting precipitate to get a final concentration of 1% lactic acid, and then treated at 121°C for 30 min. The solution obtained was enzymatically hydrolyzed by glucoamylase (EC 3.2.1.3, Sigma Chemical Co., USA, 0.2% of the rice weight) at 50°C and pH 4.5. Samples from the reaction mixture were taken at different indicated times and chromatographed on thin-layer plates of silica gel 70 F₂₅₄ (Wako Pure Chemical Industries, Ltd., Japan) in a solvent system composed of *n*-butanol, pyridine, and distilled water (8:1:1, v/v). The separated

samples were detected by ethanol-sulfuric acid (1:1, v/v).

The reducing sugars contained in each sample from the reaction mixture were measured by the Somogyi-Nelson method (19) using glucose as the standard.

Hydrolysis of rice protein by pepsin and trypsin. The protein fraction was hydrolyzed by pepsin (EC 3.4.23.1, Wako, 0.012% of the rice weight) at 50°C, pH 1.8 and trypsin (EC 3.4.21.4, DIFCO Laboratories, USA, 0.48% of the rice weight) at 50°C, pH 8.0, respectively. Samples from each hydrolysis reaction were taken at different indicated times and examined by SDS-PAGE with a 20% SDS-polyacrylamide gel.

Growth and exopolysaccharide production in a culture broth of *L. kefiranofaciens*. The medium used for both the preparation of inocula and maintenance was MRS broth. *L. kefiranofaciens* was precultured in MRS broth at 33°C for 48 hr, and then inoculated into each experimental medium at an inoculum size of approximately 10⁶ CFU/ml. After the removal of air by introducing carbon dioxide into the broth, *L. kefiranofaciens* was cultivated at 33°C for seven-day with the pH controlled at 5.0 by the addition of 5 N sodium hydroxide solution. The viable cell counts and levels of exopolysaccharide produced were determined.

The sample of cultured broth was suspended, and serially diluted 10-fold in an anaerobic diluent (0.45% KH₂PO₄, 0.6% Na₂HPO₄, 0.05% cysteine hydrochloride, 0.05% Tween 80, 0.1% bacto-agar). One-hundred microlitres of appropriate dilutions were cultured on plates of MRS agar (OXOID Ltd., UK) at 33°C for seven-day under anaerobic conditions established using an ANAERO PACK ANAERO (Mitsubishi Gas Chemical Co., Inc., Japan), and the colonies of *L. kefiranofaciens* were then counted.

To extract the total exopolysaccharide from both culture supernatant and capsulated cells, the culture was heated in a boiling water bath for 30 min and then cen-

trifuged at $10,000 \times g$ for 15 min to remove the cells. The supernatant was collected, and then the exopolysaccharide was precipitated adding an equal volume of cold ethanol, followed by storage overnight at 4°C . The precipitate was recovered by centrifugation at $10,000 \times g$ for 15 min and dissolved in distilled water. The exopolysaccharide was reprecipitated from the solution using the same procedure. The resulting precipitate was redissolved in distilled water, and carbohydrate contents were determined using an anthrone reagent that was prepared by dissolving anthrone (0.2 g/100 ml) in 75% (v/v) sulfuric acid. The sample (0.5 ml) followed anthrone reagent (5 ml) was slowly added to a test tube. The reaction mixture was then incubated in a boiling water bath for 10 min and immediately plunged into a cold-water bath, and finally the carbohydrate content was determined by optical density at 620 nm with lactose as the standard.

Protein assay. Protein concentration was measured using the method of Bradford (2) with bovine serum albumin as the standard.

SDS-PAGE. SDS-PAGE was performed using the method of Laemmli (7) with standard proteins (LMW Electrophoresis Calibration Kit, Amersham Pharmacia Biotech UK Ltd., UK) as the molecular mass markers. Protein bands were stained with silver (16).

RESULTS

Hydrolysis of Starch

In this study, the starch fraction extracted from rice

was enzymatically hydrolyzed by glucoamylase at 50°C and pH 4.5, and the reaction products obtained at the different indicated times were analyzed by TLC as shown in Fig. 1. In addition, the concentrations of reducing sugar for 0.5 hr, 1 hr and 2 hr were 78.0, 92.2 and 97.9%, respectively, based on the value for 5 hr being 100%. Rice starch hydrolyzate for the indicated

Fig. 1. TLC analysis of hydrolytic products from rice starch by glucoamylase.

The rice starch was incubated with glucoamylase at 50°C and pH 4.5, and samples from the reaction mixture at the indicated times were analyzed by TLC.

Lanes: G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; 1, 0 hr; 2, 0.5 hr; 3, 1 hr; 4, 2 hr; 5, 5 hr.

Fig. 2. SDS-PAGE of the enzymatic hydrolyzate of the rice protein.

Samples of the rice protein were hydrolyzed by pepsin (A) and trypsin (B), respectively, as described in Materials and Methods.

Lane M, molecular mass markers; lane 1 to 6 represent 0, 1, 2, 3, 4, 5 hr, respectively, after the addition of pepsin (A) or trypsin (B).

time of 2 hr was used to prepare the RH medium due to the fact that the starch fraction was essentially hydrolyzed into glucose.

Hydrolysis of Protein

The protein fraction was hydrolyzed by pepsin (0.012% of the rice weight) and trypsin (0.47% of the rice weight) at 50°C, pH 1.8 and 50°C, pH 8.0, respectively. The samples obtained at different indicated times up to 5 hr were analyzed and compared with an untreated sample.

The hydrolysis of each individual protein presented in the protein fraction was estimated by 20% SDS-PAGE (Fig. 2). The results showed that bands corresponding to high-molecular-weight proteins gradually disappeared during the incubation period, while those representing several peptidic fragments appeared.

The peptides digested by pepsin for 4 hr were used to prepare the RH medium because the peptides generated after enzymatic cleavage of the proteins by pepsin had more extensive and varied band distributions (Fig. 2A) than those cleaved by trypsin (Fig. 2B). In addition, among the samples digested by pepsin for different times from 1 hr to 5 hr (Fig. 2A), the sample for 4 hr appeared to have the most peptides with low molecular weights between 14.4 kDa and 30.0 kDa.

Preparation of RH Medium

According to the results obtained in the hydrolysis of starch and protein, based on the purpose of making full use of the rice, a simplified procedure to prepare the RH medium without separating the rice into starch and protein fractions was set up. The rice was powdered and suspended in 6-fold amounts (weight) of distilled water, lactic acid was added to a final concentration of 1% lactic acid, and then the solution was treated at 121°C for 30 min. The solution obtained was first hydrolyzed by pepsin at 50°C and pH 1.8 for 4 hr, and then by glucoamylase at 50°C and pH 4.5 for 2 hr. The hydrolyzate, consisting of 17.25% rice starch hydrolyzate and 0.60% rice protein hydrolyzate, was used to prepare the RH medium.

Comparison of Viable Cell Counts and Exopolysaccharide Production in the Experimental Media Cultured under Conditions Using a 5-l Jar Fermentor

L. kefiranofaciens was cultivated in 2.5 l of each experimental medium in 5-l jar fermentors (B. E. Marubishi Co., Ltd., Japan) at pH 5.0 and 33°C. As shown in Fig. 3, the growth of *L. kefiranofaciens* was about the same level for the three experimental media.

Fig. 3. Growth and exopolysaccharide production by *L. kefiranofaciens* in experimental media cultured in a 5-l jar fermentor.

Cultivation was carried out in three different media. Symbols: (●) RH medium, (○) PYG10 medium, (△) PYL10 medium.

The highest exopolysaccharide production was achieved in RH medium, even though the amount of rice protein hydrolyzate in the RH medium was 4.3 times lower than that of the polypepton in both PYG10 medium and PYL10 medium. In this research, the utilization of rice protein hydrolyzate in preparation of the RH medium played an important role for ensuring satisfactory results.

Culture of *L. kefiranofaciens* in a 500-l Tank

L. kefiranofaciens was cultured in 400 l of RH medium in a 500-l tank at pH 5.0 and 33°C. The maximum yield of the exopolysaccharide produced by *L. kefiranofaciens* was about 2.5 g/l after a seven-day culturing period. This result was nearly equivalent to that

obtained using RH medium in the 5-l jar fermentor.

DISCUSSION

Recently, there have been some reports regarding the media used in the cultivation of *L. kefiranofaciens* and optimum culture conditions for the production of exopolysaccharide (3, 10, 20–24). Among the media used in these studies, KPL medium and IKPL medium contained an expensive component of white table wine. The yield of exopolysaccharide was only 20 mg/l in the KPL culture and 80 mg/l in the IKPL culture (20, 21). MRSL medium was used for the production of kefir by *Lactobacillus* sp. KPB-167B isolated from kefir grains. In this medium, trypton, yeast extract, meat extract and triammonium citrate were used as nitrogen sources, and lactose as the carbon source. The highest broth yield obtained was 2.04 g/l (22). Modified MRSL medium also gave a yield of 2 g/l of purified kefiran from the culture supernatant of the LM-17 strain that was a rosy *Lactobacillus* strain isolated from kefir grains (10). However, all of these studies were conducted on a limited scale in laboratories.

In the present study, utilizing traditional food rice as the peptone and carbon sources, we developed an RH medium that does not contain white table wine, trypton or meat extracts (Table 1). Using PYG10 medium as the basal medium, the polypepton and glucose in PYG10 medium were replaced with rice protein hydrolyzate and rice starch hydrolyzate, respectively. Therefore, a new medium was obtained for exopolysaccharide production.

Regarding the peptone source in each medium, polypepton (Nihon Seiyaku Co., Ltd., Japan) digested from casein by trypsin was used for preparing the PYG10 and PYL10 media, while the peptides from rice protein hydrolyzate were used for the RH medium. In comparing the PYG10 and PYL10 media for their abilities to grow *L. kefiranofaciens* and produce exopolysaccharide, the viable cell counts in PYL10 medium were found to be higher than those in PYG10 medium within four-day, and the yield of exopolysaccharide in PYL10 medium was higher than that in PYG10 medium throughout the entire seven-day culturing period. These results reveal that lactose used as the carbon source is more effective than glucose for growing *L. kefiranofaciens* and producing exopolysaccharide. This result is in good agreement with the results of a prior report (22). However, an interesting point was found: when RH medium is used to incubate *L. kefiranofaciens*, the highest level of exopolysaccharide production is achieved, even though the amount of rice protein hy-

drolyzate (0.35%) in the RH medium is 4.3 times less than that of the polypepton (1.5%) in PYG10 and PYL10 media, and glucose from rice starch hydrolyzate (not lactose) was used as the carbon source in the RH medium (Table 1). As shown in Fig. 3B, the yield of exopolysaccharide in RH medium at pH 5.0 for seven-day was 2.16 g/l, while the yields in PYG10 and PYL10 media were 0.54 g/l and 0.90 g/l, respectively. These results suggest that the peptides from rice digested by pepsin are more effective in exopolysaccharide production than those from casein digested by trypsin.

As a continuing study aimed at the large-scale cultivation of *L. kefiranofaciens* for the mass-production of exopolysaccharide, pure culturing of *L. kefiranofaciens* using a 500-l tank was experimentally conducted, even though there were no related reports found. As a result, exopolysaccharide production at a rate of 2.5 g/l was successfully achieved using *L. kefiranofaciens* in RH medium. This result suggests that it is possible to obtain effective industrial-scale production of exopolysaccharide using *L. kefiranofaciens* in RH medium.

In addition, compositional analysis, methylation analysis, specific rotation and ¹H and ¹³C NMR spectroscopy revealed that the structure of exopolysaccharide from RH medium is essentially identical to that of kefiran reported in previous studies (10, 12). The details of these experiments will be reported elsewhere.

Because kefir and rice are both traditional foods, the high safety of exopolysaccharide kefiran produced by the process of using the *L. kefiranofaciens* strain isolated from kefir grains and RH medium that contained rice hydrolyzate could be considered. Physiological effects, such as change in blood pressure and blood serum lipids, resulting from exopolysaccharide (OSKC) produced by *L. kefiranofaciens* in RH medium are now being investigated in our laboratory. It is anticipated that kefiran can be applied more extensively.

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