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High and Rapid L-lactic Acid Production by Alkaliphilic *Enterococcus* sp. by Adding Wheat Bran Hydrolysate

Kazuaki Yoshimune[1], **Mika Yamamoto**[2], **Tomo Aoyagi**[3], **and Isao Yumoto**[2*]

[1]*College of Industrial Technology, Nihon University, Izumi-cho 1-2-1, Narashino, Chiba 275-8575, Japan*

[2]*Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Sapporo 062-8517, Hokkaido, Japan*

[3]*Environmental Management Research Institute, National Institute of Advanced Industrial Science and Technology, Onogawa, Tsukuba, Ibaraki 305-8569, Japan*

[*]**Corresponding author**: Isao Yumoto, Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Sapporo 062-8517, Japan, Tel: +81-11-857-8925; E-mail: i.yumoto@aist.go.jp

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Abstract

Two strains of alkaliphilic lactic acid bacteria, L-120 and AY103, which can produce L-lactic acid extensively, were isolated during trials of L-lactic acid production under unsterilized conditions at pH 9. Strains L-120 and AY103 are similar to *Enterococcus casseliflavus* and *Enterococcus faecalis*, respectively, as determined by 16S rRNA gene sequencing. It was found that wheat bran hydrolysate (1.5 or 3.0% dry w/v) strongly stimulates the production of lactic acid in both strains. Strain L-120 produced 149 g L^{-1} L-lactic acid at 35°C under pH 9 from 180 g L^{-1} glucose with the production rate of 3.9 g L^{-1} h^{-1}. Strain AY103 produced 153 g L^{-1} L-lactic acid from 180 g L^{-1} glucose with the production rate of 4.2 g L^{-1} h^{-1}. Both strains produced high-optical-purity (100%) L-lactic acid. In addition, strain L-120 produced 12% (w/v) L-lactic acid from degraded inedible materials, i.e., rice straw and soy bean curd refuse, within 72 h under unsterilized conditions. The obtained results indicate that these two strains are very useful for L-lactic acid production with the advantages of high productivity and rapid production with reduced costs of product purification, raw materials and fermenter sterilization.

Keywords: L-Lactic acid; High concentration of lactic acid; *Enterococcus*; Alkaline fermentation

Introduction

Lactic acid is an industrially important chemical that is utilized as a food additive of beverages or fermented foods, as a solvent for paints and in pesticides, pharmaceuticals and cosmetics [1]. In addition, it is a raw material for the production of polylactic acid, which is an environment-friendly biodegradable plastic [2]. Lactic acid can be produced as a fermentation product in the form of L-isomer, D-isomer or a racemic mixture from lactic acid bacteria. Chemical synthesis can only produce the racemic mixture, which is based on the hydrolysis of lactonitrile derived from acetaldehyde and hydrogen cyanide. For the synthesis of polylactic acid, high-optical-purity L-isomer or D-isomer is required. Therefore, fermentation is required for the synthesis of polylactic acid.

L-lactic acid production by microorganisms has been investigated using lactic acid bacteria (LABs) such as *Lactobacillus* spp., *Enterococcus* spp. or *Streptococcus* spp. [3-6]. The major problems associated with using these LABs are their high nutritional requirements, high risk of contamination and inhibition of lactic acid production when the lactic acid concentration becomes higher than 3% [3]. In addition, the optical purity is sometimes not always high when using LABs and the purification cost becomes high depending on the lactic acid concentration of the spent medium. Although a *Rhizopus* sp. has also been used for L-lactic acid production, its high oxygen requirement is the main limitation [7]. Thermophilic *Bacillus* spp. have also been used for L-lactic acid production [8,9]. Although this type of production has the merits of low risk of contamination and nonrequirement for cooling water, the lactic acid concentrations produced are lower than when using other LABs.

To reduce the production cost of L-lactic acid, lignocellulosic (e.g., straw) and starchy (e.g., potatoes and corn) materials are used as the major feasible raw material candidates for industrial production [10,11]. Starchy materials have the merit of being easily hydrolyzed into fermentable sugars such as glucose or oligosaccharides by relatively inexpensive enzymes [11]. Furthermore, several LABs and lactic acid-producing *Bacillus* spp. produce amylases [3,9]. However, starchy materials are also used as food. Therefore, nonfood plants including inedible parts of rice and oats, such as straw and bran, are preferable materials for lactic acid production. Hydrolysis using sulfuric acid could be employed for the glycosylation of lignocellulosic materials. However, this method is costly because of the neutralization required after the treatment and the use of sulfuric acid burdens the environment.

As described above, lactic acid production by microorganisms without any shortcomings is difficult. To overcome several hindrances in the production of lactic acid by LABS, the application of alkaliphiles will be a good approach, especially to avoid the risk of contamination and growth inhibition by produced lactic acid because alkaliphilic lactic acid bacteria exhibit high salt tolerance. Alkaliphilic lactic acid bacteria such as *Alkalibacterium* spp., *Halolactibacillus* spp., *Paraliobacillus* spp. and *Marinilactibacillus* spp. have been reported [12-15]. On the other hand, the production of high concentrations of lactic acid is important not only for the productivity but also the product purification cost. Actually, the separation and concentration of lactic acid in the fermentation product are difficult because lactic acid has a high boiling point (approximately 120°C).

In this study, two strains of alkaliphilic lactic acid bacteria, L-120 and AY103, which can produce L-lactic acid extensively, were isolated during the trials of L-lactic acid production under unsterilized conditions at a high pH. Growth conditions and the additive (wheat bran hydrolysate) for appropriate L-lactic acid production using strains L-120 and AY103 were adjusted.

Materials and Methods

Isolation of microorganisms

A water stain at a drain outlet was added to nonsterilized fermentation using processed rice straw (delignified and hydrolyzed) and hydrolyzed soy bean curd refuse (the procedure for the medium preparation is described below). As pronounced production of lactic acid was ascertained after 72 h of incubation using the fermentation procedure described below (the pH was maintained at 9 by adding 10 N NaOH under anaerobic condition by the purging N_2 gas), isolation was performed using yeast extract-glucose-alkaline (YGA) medium (pH 10.2) consisting of 1% yeast extract (Kyokuto, Tokyo, Japan), 10% glucose, and 1% $NaCO_3$. Single-colony isolation was performed several times on YGA agar plates (containing 1.5% agar), and the isolate obtained was named strain L-120.

An alkaliphilic lactic acid-producing strain isolated from a fermentation fluid was inoculated into yeast extract-glucose-fish meat extract-alkaline (YGFA) medium (pH 9.0) consisting of 0.8% yeast extract (Kyokuto, Tokyo, Japan), 10% glucose and 0.3% fish meat extract (Bacterion [Maruha-Nichiro, Tokyo, Japan]). The fermenter and medium without glucose were sterilized by autoclaving, whereas the glucose and wheat bran hydrolysate were not sterilized. An unsterilized degraded product of wheat bran (30 g), which was hydrolyzed with 0.5 g of cellulase (cellulase A [Amano Enzyme]), was added before starting the cultivation. As pronounced production of lactic acid was ascertained after 72 h of incubation using the fermentation procedure described below (the pH was maintained at 9 by adding 10 N NaOH under anaerobic condition by the purging of N_2 gas), isolation was performed using peptone-yeast extract-glucose-alkaline (PYGA) medium (pH 10.2) consisting of 0.3% peptone (Kyokuto, Tokyo, Japan), 2% glucose, 0.8% yeast extract (Kyokuto, Tokyo, Japan) and 1% $NaCO_3$. Single-colony isolation was performed several times on the PYGA agar plate (containing 1.5% agar), the isolate obtained was named strain AY103.

16S rRNA gene sequence analysis

To determine the 16S rRNA gene sequence of the isolates (strains L-120 and AY103), DNA was extracted from the isolates, and PCR was performed using the universal primer sets 9F (5'-GAGTTTGATCGTGGATCAG-3') and 1514R (5'-AAGGAGGTGATCCAGCC-3'). The PCR solution (100 µl) consisted of 10 µl of 10 × sequence buffer, 8 µl of 2.5 mM dNTP mix, 100 ng of isolated DNA, 5U of Ex Taq DNA polymerase (Takara) and 20 pmol of each primer. The reaction mixture was subjected to PCR under the following conditions: 94°C for 1 min followed by 30 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1.5 min. The PCR product was purified with a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instruction. The DNA sequence was determined by the dideoxy chain termination method with a BigDye terminator cycle sequence kit (Applied Biosystems, Foster City, CA) and an automated DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems). Sequences were assigned by searching for matching sequences using BLAST [16]. Phylogenetic analysis was performed using the determined 16S rRNA gene sequence of *Enterococcus* spp. The sequences were aligned and the consensus sequence was determined using CLUSTAL W [17]. A phylogenetic tree was constructed by the maximum-likelihood method [18] in MEGA 6 [19]. The distance between sequences (K_{unc} value) was calculated using Kimura's two-parameter model [20,21]. The confidence values for the branches of the phylogenetic tree were determined using bootstrap analysis [22] based on 1000 resamplings.

Glycosylation of rice straw

To remove lignin in the rice straw, 1 L of NaOH (1N) was added to 100 g of rice straw (dry state), which was cut to 10 cm in length or less, followed by heating at 120°C for 20 min. After cooling, the liquid fraction was passed through a gauze and the residue that remained on the gauze was washed several times to remove lignin-containing liquid. To monitor the amount of extracted lignin in the alkaline solution, OD_{329} was monitored. As a result, 100 g of wet cellulose was obtained from 50 g of rice straw (dry state). Two hundred milliliters of tap water was added to 50 g of wet cellulose (delignified rice straw) and the pH was adjusted to 4.5. Cellulase (Cellic CTec [Novozyme], 1.5 ml) and xylanase (Cellic HTec [Novozyme], 1.5 ml) were added, followed by incubation at 37°C for 12 h. After the reaction, the same amounts of cellulose and enzymes were added, followed by incubation at 37°C for 24 h. The addition and incubation were continued 4 times to obtain a higher concentration of glucose (500 ml of 150 g L^{-1} glucose solution). The amount of glucose produced during the reaction was measured by the DNS (3,5-dinitro salicylic acid) method [23].

Fermentation by adding degraded wheat bran

Twenty milliliters of preculture in PYGA medium (cultured for 24 h) was inoculated in 1 L of medium containing 18% glucose, 0.8% yeast extract, 0.3% fish meat extract and degraded wheat bran (15 or 30 g of wheat bran plus 0.5 or 1.0 g of cellulase A [Amano Enzyme]) in a 2-L fermenter. The temperature was maintained at 35°C. Using a pH controller, we maintained the pH at 9.0 by adding 10 N NaOH during cultivation. Nitrogen gas was introduced to maintain an anaerobic condition. The fermenter and medium without glucose were sterilized by autoclaving, whereas the glucose and wheat bran hydrolysate were not sterilized.

Fermentation using treated rice straw

Twenty milliliters of preculture in the PYGA medium was inoculated in 0.5 L of medium containing 1.5% glucose (obtained by glycosylation). Thirty grams of soy bean curd refuse (wet) treated with cellulase (Cellic CTec [Novozyme], 0.45 ml) at 37°C and pH 4.5 for 24 h was added to 500 ml of the glucose solution obtained from rice straw. The fermenter (2-L size) and the medium (approximately 530 ml) were not sterilized. The temperature was maintained at 30°C. Using a pH controller, we maintained the culture pH at pH 9 in the first 24h and then at pH 7 in the following 48 h by adding 10 N NaOH during cultivation. Nitrogen gas was introduced to maintain an anaerobic condition.

Analysis

Lactate concentration was analyzed using an HPLC system equipped with a GL-C610H column using bromothymol blue as the postcolumn reagent (Hitachi High-Technologies Corporation, Tokyo,

Japan). The optical purity of lactate was analyzed using an HPLC system equipped with SUMICHIRAL OA-5000 (Sumika Chemical Analysis Service, Ltd., Osaka Japan) by monitoring OD245. The optical purity of lactate was determined as ([L] - [D]) ×100/ ([L]+[D]). Sugar concentrations were analyzed using the HPLC system equipped with a TSK gel (tandemly connected G6000PWXL, G3000PWXL and G2500PWXL [7.8 mm × 30 cm × 3 column], Tosoh) by monitoring using a refractive index (RI) detector.

Results and Discussion

Identification of strains

In our attempt to isolate lactic acid-producing bacteria from a water-stain-inoculated culture using medium constituents derived from biomass materials, i.e., rice straw and soy bean curd refuse, strain L-120 was isolated. Strain L-120 was identified by 16S rRNA gene sequencing which showed that its sequence (1245 bp) exhibited 99.9% similarity to that of *Enterococcus casseliflavus* NCIMB 11449T (Y18161). The phylogenetic position of strain L-120 based on the determined sequence is shown in Figure 1. From the sequence similarity and phylogenetic position of strain L-120 within the genus *Enterococcus*, strain L-120 was determined to be closely related to *E. casseliflavus*.

On the other hand, in another attempt to isolate lactic acid-producing bacteria from using conventional medium at pH 9 containing unsterilized wheat bran hydrolysate, strain AY103 was isolated. The 16S rRNA gene sequence of strain AY103 was determined. The determined sequence (1435 bp) exhibited 99.4% similarity to that of *Enterococcus faecalis* ATCC 19433T (DQ411814). The phylogenetic position of strain AY103 based on the determined sequence is shown in Figure 1. From the sequence similarity and phylogenetic position of strain AY103 within the genus *Enterococcus*, strain AY103 was determined to be closely related to *E. faecalis*.

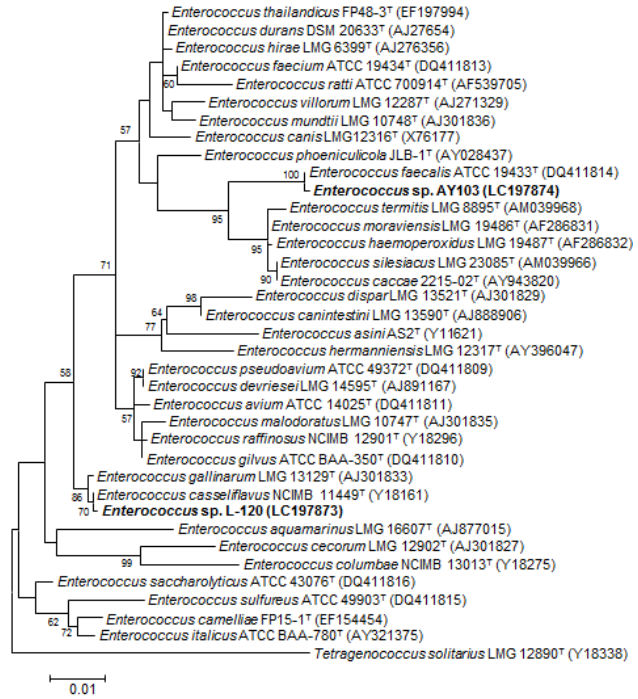

Figure 1: Maximum-likelihood phylogenetic tree showing the positions of 2 isolates obtained from two different lactic acid production trials within the genus *Enterococcus*. The isolate from fermentation using inedible biomass is denoted as strain L-120. The isolate from fermentation using ordinary medium is denoted as strain AY103. Bootstrap percentages (based on 1000 replicates) > 50% are shown at branch points. Scale bars=0.01 substitution per nucleotide position. *Tetragenococcus solitaries* was used as an out group.

Lactic acid production of *Enterococcus* spp. using general medium constituents at pH 9

The lactic acid production of strain L-120 was estimated in batch culture using conventional medium constituents at pH 9 under controlled conditions (Figure 2). The lactic acid concentration without the addition of digested wheat bran at 27°C in 116 h of incubation was 9.8 g L^{-1}. The yield and the production rate were 63% and 0.98 g L^{-1} h^{-1}, respectively. When 20 g of wheat bran digested with 0.5 g of cellulase was added to the medium and the incubation temperature was changed to 35°C, the lactic acid concentration became 14.8 g L^{-1} in 92 h of culture. The yield and production rate were 91% and 1.9 g L^{-1} h^{-1}, respectively. However, after 65 h of incubation period, the production rate was 2.5 g L^{-1} h^{-1}. This indicated that the lactic acid production was quite rapid within the 65 h under this culture condition. When 15 g of wheat bran digested with 0.5 g of cellulase was added to the medium, followed by incubation at 35°C, the lactic acid concentration became 16.0 g L^{-1} in 92 h of culture. The yield and production rate were 106% and 2.1 g L^{-1} h^{-1}, respectively. However, within the 45 h of incubation, the lactic acid concentration was 14.9 g L^{-1}. The yield percentage and production rate were 97% and 3.9 g L^{-1} h^{-1}, respectively. This indicated that the conversion from glucose to lactic acid is almost complete within 45 h.

The lactic acid production of strain AY103 was estimated in batch culture using general medium constituents at pH 9 under controlled conditions (Figure 3). The lactic acid concentration without the addition of digested wheat bran at 32°C in 99 h of culture was 10.2 g L^{-1}. The yield from glucose and the production rate were 64% and 1.16 g L^{-1} h^{-1}, respectively. These values are comparable to those of strain L-120 in 116 h of culture at 27°C (data not shown). The difference in the incubation period may be attributed to the difference in the incubation temperature. When 15 g of wheat bran digested with 0.5 g of cellulase was added to the medium and the incubation temperature was changed to 35°C, the lactic acid concentration became 14.0 g L^{-1} in 92 h of culture. The yield percentage and production rate were 91% and 1.8 L^{-1} h^{-1}, respectively. These values were comparable to those obtained when 20 g of wheat bran digested with 0.5 g of cellulase was added to the medium for strain L-120 (data not shown). When 30 g of wheat bran digested with 1.0 g of cellulase was added to the medium, followed by incubation at 35°C, the lactic acid concentration became 15.3 g L^{-1} in 42 h of culture. The yield percentage and production rate were 99% and 4.2 g L^{-1} h^{-1}, respectively. Under the culture conditions described above, both strains produced high-optical-purity (100%) L-lactic acid.

As described above, the stimulatory effect of wheat bran hydrolysate was demonstrated. Presently, the mechanism of the effect is unknown.

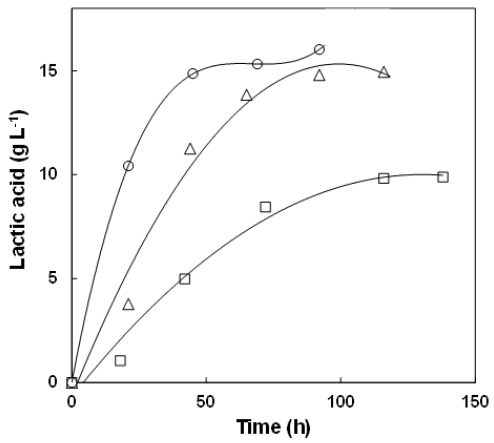

Figure 2: Effect of wheat bran hydrolysate on the production of L-lactic acid using batch fermentation in Enterococcus sp. strain L-120. The fermentation medium contains 18.0% glucose, 0.8% yeast extract, and 0.3% fish meat extract. The culture was incubated at 27°C (square) or 35°C (triangle and circle). Square: no wheat bran hydrolysate; 0.5g of cellulase plus 15 g (circle) or 20 g (triangle) of wheat bran hydrolysate in 1 L of medium in a 2-L fermenter. These values are based on the analyses of obtained samples. Real amounts of lactic acid production were higher due to changing of medium volume by adding alkaline solution during culture.

It can be surmised that the oligosaccharide produced as the intermediate reaction product stimulates the production of L-lactic acid, or debris produced from enzymatic reactions provide the microorganism with the appropriate environment to resist the high concentration of lactic acid. Although the application of wheat bran hydrolysate to the medium for lactic acid production using *Lactobacillus rhamnosus* has been reported [24,25], there has been no report on studies demonstrating of high concentrations of lactic acid (>10%).

Treatments of inedible biomass materials

Although soy bean curd refuse has potential as a low-cost material for the production of lactic acid, it is easily decomposed and it exhibits high viscosity in the medium owing to its high water retention. In this study, soy bean curd refuse was treated with cellulase at 37°C under pH 4.5 to reduce its high viscosity in the medium. Results showed that the treatment reduced its water retention and prevented its easy decomposition by microorganisms owing to the low pH. Therefore, 30 g of bean curd refuse (wet) decomposed with cellulase was added to the medium containing decomposed rice straw.

Rice straw is one of the promising inedible materials for the production of glucose, and it is produced at 9 million tons per year in Japan. Firstly, the delignin reaction condition was optimized by alkaline treatment. It is considered that the procedure will not inhibit microbial growth in later steps of the fermentation process if the lignin residue is appropriately washed away with alkaline solution.

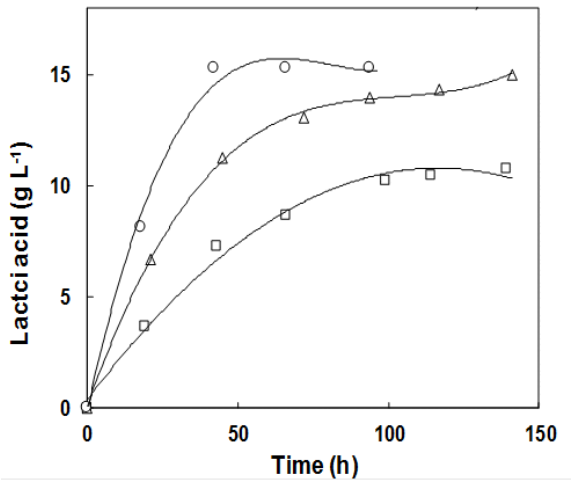

Figure 3: Effect of wheat bran hydrolysate on the production of L-lactic acid in batch fermentation using Enterococcus sp. strain AY103. The fermentation medium contains 18.0% glucose, 0.8% yeast extract, and 0.3% fish meat extract. The culture was incubated at 32°C (square) or 35°C (triangle and circle). Square: no wheat bran hydrolysate; triangle: 0.5 g of cellulase plus 15 g of wheat bran hydrolysate; circle: 1.0 g of cellulase plus 30 g of whe at bran hydrolysate in 1-L of medium in a 2-L fermenter. These values are based on the analyses of obtained samples. Real amounts of production of lactic acid were higher due to changing of medium volume by adding alkaline solution during culture.

When the NaOH concentration was lower than 0.2 N, the relationship between the NaOH concentration and the amount of extracted lignin was linear (data not shown). When the NaOH concentration was higher than 0.2 N, the amount of extracted lignin hardly increased (data not shown). Therefore, 0.2 N NaOH was used for the delignification reaction (120°C for 20 min) to reduce the amount of NaOH used in this step.

The purpose of this study is to produce spent medium that contains a high concentration of lactic acid to reduce the purification cost. For that purpose, a medium with a high glucose concentration should be prepared. It was found that to obtain a medium with 16% glucose, 4 successive and simultaneous treatments of delignated cellulose with cellulase and xylanase during incubation at 37°C and pH 4.5 for 12 h are necessary.

Lactic acid production of *Enterococcus* sp. strain L-120 using medium derived from biomass materials

We inoculated *Enterococcus* sp. strain L-120 into 500 ml of 150 g L^{-1} glucose solution degraded from rice straw plus 30 g of cellulase-degraded soy bean curd refuse. The amounts of lactic acid produced were 8.9, 11.0 and 12.0 % (w/v) after 24, 48 and 72 h of incubation, respectively. Under the culture conditions described above, both strains produced high-optical-purity (100%) L-lactic acid. L-Lactic acid production by *Enterococcus faecalis* from wood hydrolysate has been carried out in a previous study [26]. In that study, the

productivity was very high (3.2 g L^{-1} h^{-1}) when the glucose concentration derived from the wood hydrolysate was 50 g L^{-1}.

Stain	Substrate	C^a (g/L)	Y^b (g/g)	P^c (g/L/g)	Isomer and Optical Purity (%)	Reference
Enterococcus sp. L-120	Glucose	149.4	0.97	3.9	L, 100	This study
Enterococcus sp. AY103	Glucose	153	0.99	4.2	L, 100	This study
Enterococcus casseliflavaus 79w3	Glucose	103	0.8	2.2	L, 99.5	(13)
Bacillus sp. MC-07	Starch	16.6	0.98	0.7	L, 100	(9)
Lb. paracasei subsp. paracasei CHB2121[d]	Glucose	192	0.96	3.99	L, 96.6	(30)
Bacillus sp. Na-2	Glucose	106	0.94	3.53	L, 99.5	(31)
Rizopus oryzae GY18	Glucose	115	0.81	1.6	L, 98.5	(32)
H. halophilus JCM 21694[e]	Sucrose	65.8	0.83	1.1	L, 98.8	(33)
[a]*C*, concentration; [b]*Y*, yield; [c]*P*, productivity; [d]*Lb.*, *Lactobacillus*; [e]*H.*, *Halolactibacillus*						

Table 1: Lactic acid production in batch fermentation with different methods.

However, the productivity decreased to 1.7 g L^{-1} h^{-1} when the glucose concentration was 100 g L^{-1}. This productivity is comparable to the present result when the glucose concentration was 150 g L^{-1}. In the production step from wood to glucose, the pulverization of wood is very costly. On the other hand, such a process is unnecessary when using rice straw. The presented results indicated that, with the use of inedible materials such as rice straw and soy bean curd refuse, high-optical-purity L-lactic acid can be produced at a relatively high concentration using an unsterilized fermentation facility and medium. Therefore, it can be said that low-cost lactic acid production without using food resources is possible.

Enterococcus spp. have been used in L-lactic acid production from xylose or inedible biomass materials such as wood hydrolysate [26-29]. The high concentration of L-lactic acid produced from rice bran and soy bean curd may be attributed to the taxon's high adaptability to harsh environmental conditions such as high pH and high concentration of lactic acid.

To date, several LABs including *Bacillus* species or fugues, such as *Entrerococcus* species, *Lactobacillus* species, *Halolactobacillus* species or *Rizopus oryzae*, have been reported to produce L-lactic acid from glucose or sucrose in batch fermentation (Table 1). To the best of our knowledge, the production of L-lactic acid by strain AY103 is most rapid in the ordinary batch fermentation. In addition, to the best of our knowledge, this is the first achievement all together in production of high concentration (>120 g/L) of high optical purity L-lactic acid (100%) with high yield (>95%) in the ordinary batch fermentation. In the future, there is possibility of further increasing of the productivity by introduction of additional fermentation procedure such as fed-batch, cell immobilization or cell recycle.

Conclusions

Strains L-120 and AY103 were found to be closely related to *Enterococcus casseiflavus* and *Enterococcus fecalis*, respectively, on the basis of their 16S rRNA gene sequences. These strains produced high concentrations of L-lactic acid (14.9 – 15.3%) and the productivity was also high (3.9-4.2 g L^{-1} h^{-1}) in the medium containing hydrolyzed wheat bran. In addition, strain L-120 produced 12% (w/v) L-lactic acid from the degraded inedible materials, rice straw and bean curd refuse, in 72 h under unsterilized conditions. The above-mentioned results suggest that alkaliphilic *Enterococcus* spp. have a very high potential for lactic acid production, not only for the rapid production of lactic acid but also the cost reduction in fermenter sterilization, raw materials and product purification. In addition, by using *Enterococcus* spp., the wheat bran hydrolysate will be applicable to wide range of bioprocess.

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