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ACID-TOLERANT LACTIC ACID BACTERIUM ISOLATED FROM RICE VINEGAR

KENJI TANAKA[1], SHINTARO TAJIRI[2], RYO SAWADA[3], YOSHIHIRO KAWAMOTO[4], TERUAKI MATSUBARA[5], MUNEHIRO HOSIHINO[6] & HIROMI MATSUSAKI[7]

[1, 2, 3] Department, of Biological and Environmental Chemistry, Faculty of Humanity-Oriented Science and Engineering, Kinki University, Kayanomori, Iizuka, Japan

[4, 5, 6] Maruboshi Vinegar Co., Ltd., Tabara, Kawasaki, Tagawa, Japan

[7]Department, of Food and Health Sciences, Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, Tsukide, Kumamoto, Japan

ABSTRACT

A lactic acid bacterium which was grown in fermented rice vinegar (pH 2.9, acetic acid 6%) was isolated. The bacterium was identified as *Lactobacillus acetotolerans* according to the homology of 16S rDNA and named *Lb.acetotolerans* HT. The cell concentration of the strain HT was higher than any other species of lactic acid tested when they were grown in MRS medium. The strain HT grew vigorously in the acidic medium added with 2 v/w/% acetic acid of which the growth of other lactic acid bacteria was seriously inhibited. In the batch culture, this acid tolerant strain HT produced 59.5 g/L of DL-lactic acid without maintaining pH and 197.5 g/L of DL-lactic with maintaining pH at 5.5. However, the growth rate of the strain HT was very slow then the fermentation time was long. It is speculated that there exists some unknown growth factor for the strain HT which will shorten the fermentation time

KEYWORD: Lactic Acid Bacterium, *Lactobacillus acetotolerans*, Lactate Fermentation

INTRODUCTION

Lactic acid bacteria are widely used in food industry and the production of lactic acid and bacteriocins. It is also known that lactic acid bacteria contribute as probiotics to human health with the intestine regulating function, protecting action on infectious disease, immunopotentiative action and carcinogen suppression action [1]. Lactic acid is used for manufacturing various kinds of industrial goods, particularly the demand as raw material of biodegradable plastic, poly-L-lactic acid is increasing [2]. In the manufacture of poly-L-lactic acid, the recovery and purification of lactic acid from the fermented broth was the most expensive process. However, lactic acid is inhibitory to the growth of microorganisms including lactic acid bacteria. The inhibitory effect of lactic acid is much stronger than in the undissociated form than the dissociated form. The neutralized salts like sodium lactate also inhibit the growth and acid production of lactic acid bacteria. Then, the concentration of lactic acid produced in culture broth of lactic acid bacteria is usually from 50 g/L to 100 g/L. It is difficult to increase the lactic acid concentration in lactate fermentation. On the other hand, probiotic lactic acid bacteria are also suffered by highly acidic condition (pH2.0) in the stomach after ingestion then most of them are perished before they reach the intestinal tract. Therefore, acid tolerant is important property for lactic acid bacteria as the probiotics and the producer of high concentration of lactic acid

We isolated highly acid tolerant lactic acid bacterium grown in rice vinegar (acetic acid 6%, pH2.9) and herewith report the characteristics and the lactic acid production of the isolate.

MATERIALS AND METHODS

Isolation of Lactic Acid Bacteria

The lactic acid bacterium used in this study was isolated from rice vinegar produced in the Maruboshi Vinegar Co., Ltd., Japan. The isolation of the bacterium was achieved by the double-layer agar plate method. The agar plates were made with MRS medium with the following composition per 1.0 L of distilled water: proteose peptone, 10 g; beef extract, 10 g; yeast extract, 5 g; D-glucose, 20 g; polysorbate 80, 1.0 g; ammonium citrate 2.0 g; sodium acetate, 5.0 g; magnesium sulfate, 0.1 g; dipotassium phosphate 2.0 g; manganese sulfate 0.05 g. The pH was adjusted to 6.5. Two types of MRS agar media (agar 0.5 w/v% and 1.5 w/v%) were autoclaved at 121°C for 15 min. The rice vinegar including the cells was diluted with the soft MRS agar (0.5 w/v% agar) which was kept at 45°C then it was poured into Petri dish and left to solidify. One of the colonies formed in the agar was transferred into the MRS liquid medium in 30-mL test tube with a screw cap. That was cultivated for 3 days then used as the stock culture. The subculture was repeated every two weeks.

All other lactic acid bacteria tested in this study was purchased from Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Tsukuba 305-0074, Japan.

PCR of 16S rDNA

Genomic DNA of the isolated bacterium was extracted by freezing–thawing method then purified with a DNA purification kit, Mag Extractor-Genome (Toyobo Co., Ltd, Japan). 16S rRNA gene fragment was amplified with the following set primers: upstream primer (8UA), 5-AGAGTTT GATCCTGGCTTA-3’; downstream primer (1492r), 5’-GGTTACCTTGTTACGACTT-3’. The reaction was performed using the Gene Amp PCR System 2700 (Applied Biosystems, USA). The thermal program consisted of one cycle at 94°C for 5 min, 35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min, one cycle of 2°C for 15 min, and stored at 4°C. The PCR product was purified with the High Pure PCR Product Purification Kit (Roche Diagnostic Corporation, USA). Sequence analysis of the purified PCR product was conducted by Hokkaido System Science Co.,Ltd., Japan. Database search of the 16S rRNA gene sequence determined was conducted by a BLAST program using the GenBank database.

Fermentation Test

All fermentation tests and subculture of the isolated bacterium were carried out using MRS medium. In the culture test for taxonomical characteristics, D-glucose was replaced with other sugars. In the fermentation test for lactic acid production, glucose concentration in MRS medium was changed. Lactic acid production of the isolated bacterium was tested using 30-mL test tube with a screw cap and 300-mL Erlenmeyer. A glass jar fermenter (total volume 1L) was used for pH-stat batch culture. The working volume was 600 mL. Agitation speed of a magnetic stirrer bar was kept at 300 rpm. The pH was maintained by automatic addition of 6 M NaOH with a pH controller, PHC-2201 (Biott Co., Ltd., Japan). Neither air nor nitrogen gas was fed to the culture medium in the fermenter.

RESULT AND DISCUSSIONS

Identification and Growth Characteristics of the Isolate

Physiological and taxonomical characteristics of the isolated lactic acid bacterium are summarized in Table 1. The bacterium produces racemic DL-lactic acid from glucose according to the result of analysis of the culture supernatant using L-lactate dehydrogenase and D-lactate dehydrogenase. Other product was not detected by HPLC analysis. The sequence of the 16S rRNA gene of this isolate showed 98.2% homology to *Lactobacillus acetotolerans*. From these results, the isolated bacterium was named as *Lactobacillus acetotolerans* HT.

Lactic acid bacteria are known as obligate or facultative anaerobic bacteria. Tolerance to aerobic condition of the strain HT was investigated in 300-mL flask. Five milliliter of the MRS medium was added in the flask and the head space was replaced with the gas mixture prepared to various ratio of N_2/O_2 using the gas mixture exchanging system [3]. A rubber stopper was plugged into the flask after inoculation then it was incubated at 30°C. Cell growth was monitored measuring the absorbance at 600 nm of the culture broth after 3 days. The strain HT could not grow above the oxygen concentration 10 v/v% and the cell growth was completely inhibited in aerobic condition using air (the data is not shown). However, the cell growth was not inhibited in atmosphere when over half volume of the flask was filled with the medium.

Table 1: Physiological and Taxonomical Characteristics, and Assimilation for Sugar and Organic Acid of the Isolated Lactic Acid Bacterium

Physiological and Taxonomical Characteristics		Assimilation of Sugar and Organic Acid	
Cell morphology	Rod	D-Glucose	+
Gram staining	Positive	D-Fructose	+
Spore	−	D-Galactose	+
Motility	−	D-Mannose	+
Anaerobic growth	Grown less than	D-Mannitol	−
	10% O_2	D-Xylose	−
Catalase	−	L-Arabinose	−
Oxidase	−	D-ribose	+
Growth temperature	20°C − 45°C	D-Rhamnose	−
Optimum temperature	30°C	D-trehalose	+
H_2S formation	−	D-Sorbose	−
Growth pH	3.2−6.8	D-Sorbitol	−
Optimum pH	5.5	Sucrose	−
Nitrate reduction	−	Maltose	+
Sulfate reduction	−	Cellobiose	−
Hydrolysis of gelatin	−	Starch	−
		Acetate	−
		Lactate	−
		Citrate	−
		D-Gluconate	−

Cell growth of the strain HT in test-tube culture was compared with other 10 species of lactic acid bacteria (Figure.1). It was found that the cell concentration of the strain HT was very higher than that of the other lactic acid bacteria

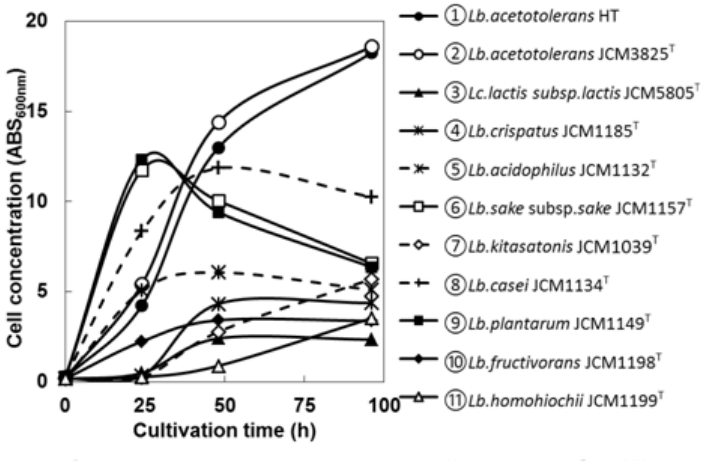

Figure 1: Comparison of Cell Growth between L.B acctotorelans HT and Other Lactic Acid Bacteria in Test Tube Culture

Tolerance to Acidic Condition

Tolerance to acetic acid was compared between the strain HT and other lactic acid bacteria. Test tube culture was carried out using the MRS medium added with 1.0 and 2.0 w/v% of acetic acid and the cell concentration (ABS600nm) were measured (Figure.2). The strain HT showed the highest cell concentration among these lactic acid bacteria. *Lb.acetotolerans* JCM 3825^{T} also showed very high growth in the acidic medium. Especially, in case of these two *Lb.acetotolerans* strains, relatively high cell concentration was obtained even at addition of 2 w/v% acetic acid while the growth of the other bacteria was seriously inhibited. Figure.3 shows the cell growth of the strain HT in the MRS medium added with different amount of acetic acid. The growth rate decreased as the acetic acid concentration increased however even at addition of 4.0 w/v% acetic acid, the cells concentration increased to 4.10 of ABS600nm.

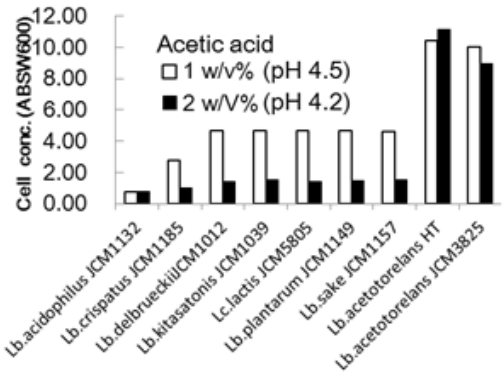

Figure 2: Cell Growth in MRS Medium Added With 1 W/V% and 2 W/V% Acetic Acid

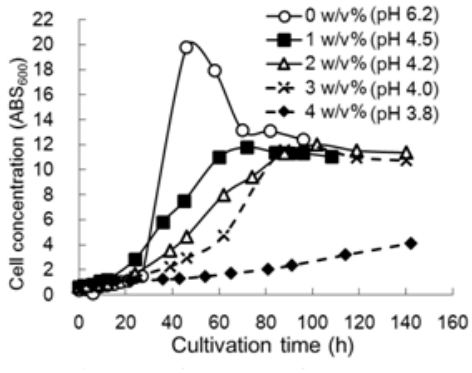

Figure 3: Cell Growth of the Strain HT at Different Concentration of Acetic Acid

Lactic acid Production

To compare lactic acid production of the strain HT and other lactic acid bacteria, batch culture experiment was carried out using a glass jar fermenter and 100 g/L glucose medium. The pH of the medium in the fermenter was not maintained at a constant level during cultivation. Three species of *Lactobacillus* of which the cell concentration was high in Figure.1 and *Lactococcus lactis* that are often used in the manufacture of L-lactic acid were also tested. The results are summarized in Table 2.

Table 2: Cell Growth and Lactic Acid Production in Batch Culture without Maintaining Ph Using the MRS Medium Containing 100 G/L Glucose

Strains	Cell Growth (ABS_{600nm})	Ph At End of Cultivation	Residual Glucose (G/L)	Lactate Production (G/L)
Lb.acetotolerans HT	18.1	3.0	41.1	58.7
Lb.plantarum JCM1149^T	15.9	3.5	63.1	36.8
Lb.sake JCM1157^T	15.4	3.5	59.5	38.2
Lb.casei JCM1134^T	14.1	3.5	61.9	38.2
Lactococcus lactis JCM5805^T	2.1	4.3	89.9	11.0

The concentration of lactic acid was the highest (58.7 g/L) in case of the strain HT among these 5 strains. The strain HT produced 46.6 g/L of lactic acid even in 300-g/L glucose medium which is very inhibitory to the growth of microorganisms due to the high osmotic pressure (the data is not shown).

To enhance the lactic acid production of the strain HT, pH-stat batch culture was carried out using a pH-controller for automatic feeding of 6M NaOH. The pH in the fermenter was maintained at 5.5 during cultivation and the result is shown in Figure.4A. The pH-stat batch culture of *Lc.lactis* JCM5805^T was also carried out with maintaining the culture pH at 6.0 (Figure.4B). The initial concentration of glucose was prepared to 220 g/L in the both cultivations.

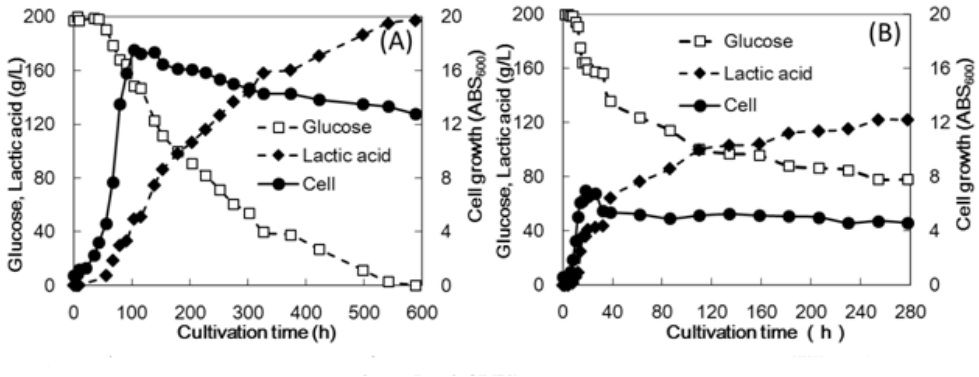

Figure 4: Time Course of pH-Stat Batch Culture of Lactobacillus Acetolerance HT at Ph 5.5 (A) and Lactococcus Lactis JCM 5805 at Ph 6.0 (B) in 200-G/L Glucose Medium

In the pH-stat culture of the strain HT, lactic acid concentration increased to 197.5 g/L while that of *Lc.lactis* ceased at about 120.5 g/L. However, the growth rate of the strain HT was very slow (specific growth rate, □;□0.121 h^{-1} in 100 g/L glucose medium and 0.091 h^{-1} in 220 g/L glucose medium) while that of *Lc.lactis* was fast (□; 1.20 h^{-1} in 100 g/L glucose medium and 0.35 h^{-1} in 220 g/L glucose medium). Therefore, the fermentation time for the strain HT was longer than that for *Lc.lactis.*

CONCLUSIONS

Our *Lb.acetotolerans* HT isolated from rice vinegar is highly tolerant to culture condition acidified by addition of acetic acid. The strain HT grows up to very high cell concentration, which may be the highest among in all lactic acid bacteria. Nevertheless, the growth rate was very slower other lactic acid bacteria. We speculated that the strain HT may require some unknown growth factor then searching the substances to faster the growth rate. *Lb.acetotolerans* was first isolated from rice vinegar by Entani et al. in 1986 [4] however the research report for this species have been very few, especially for the growth and fermentation characteristics.

Many researchers have suggested the mechanism for acid tolerance of microorganisms but that is not completely understood yet. We are now focusing on the relations between the permeability in cell membrane, the fatty acid composition, and the acid tolerance of the strain HT.

The strain HT produces lactic acid to 59.5 g/L without maintaining the culture pH and produces it to 197.5 g/L by maintaining the pH at 5.5. However, the strain HT produces racemic DL-lactic acid which is not available for manufacture of poly-L-lactate. Ding and Tan (2006) reported that 180 g/L of L-lactic acid (210 g/L of lactic acid) was produced by fed batch culture of *Lactobacillus casei* with exponential feeding glucose solution (850 g/L) [5].

We are now breeding the recombinant of the strain HT of which the D-lactate dehydrogenase gene is knocked out. Our goal is the production of 300 g/L of L-lactic acid with high optical purity using the recombinant of the strain HT and the growth promoter.

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