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Biochemical Characteristics of Psychrotrophs Isolated from Kimchi

Oh-Sik Kwon*, Min Son, Seung-A Hwangbo

Department of Microbiology, Keimyung University, Daegu 704-701, Korea
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ABSTRACT

Isolation of psychrotrophic lactic acid bacteria (LAB) from Chonggak kimchi was attempted in order to study characteristics of LAB growing at the low temperature. Among isolated strains, CL-1 showed exactly the same fermentation characteristics of *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 in tested carbohydrates except melezitose and the CL-1 showed very wide range temperature profiles (16˚C-28˚C) for growth comparing to other isolates. Its lowest temperature for growth was 16˚C which was much lower than other isolates. However all isolates couldn't grow at 30˚C. In order to identify sudden inhibition of cell growth occurred at the transition temperature (16˚C → 14˚C), the CL-1 was tested for fermentation ability of hexoses and disaccharides at the lowered temperature. Among 5 tested hexoses, significant change was resulted from mannose fermentation. The CL-1 fermented mannose very well at 24˚C that gave a final pH 4.7 after 48 hr incubation but at 16˚C its final pH was only 5.7. This means that the CL-1 could not ferment mannose for its proper cell growth at 16˚C and inhibition of the cell growth was to begin. Similar tendency was found in fermentation of disaccharides at the lowered temperature. Among 6 tested disaccharides, inhibition of fermentation occurred only in melibiose. The CL-1 showed decreasing fermentation ability with the melibiose from pH 4.8 at 24˚C to pH 5.2 at 16˚C. Fermentation of maltose, sucrose and trehalose was not affected by the lowered temperature at all. Thus, such growth inhibition of the psychrotrophs by lowered temperatures is related to occurrence of inability of fermenting the sugars and it leads to sudden growth inhibition of the psychrotrophic CL-1. From the test for bile salt tolerance, the CL-1 showed strong tolerance for glycocholate as much as *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 but both strains were strongly inhibited by 0.5% taurocholate. In case of taurocholate, the CL-1 could overcome the bile salt effect on cell growth with 0.2% taurocholate as incubation time goes by from 12 hr to 24 hr. The taurocholate tolerance of CL-1 was much better than that of *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722.

Key words : Bile tolerance, Fermentation, LAB, *Leuconostoc mesenteroides*, Psychrotroph

Introduction

Lactic acid bacteria (LAB) are anaerobic Gram positive bacteria which produce lactic acid as the major product during fermentation of carbohydrates [1]. The group of LAB consists of non-spore forming cocci or rods in morphological characteristics [2] and most of LAB are mesophilic bacteria that can grow in the temperature ranges of 20˚C-37˚C [3]. The LAB are specially associated with bacteria involved in food and food fermentation, including related bacteria that normally reside in the mucosal surfaces of animals and human [4]. And the organisms are of interest in the food industries because of their typical roles in inhibiting the growth of food spoilage bacteria [5] and in preservation [6,7]. Among the organisms, the genus *Leuconostoc* contains many commercially useful species for food industries [8,9]. Typically it consists of species of *mesenteroides*, *dextranicum*, *citreum*, *cremoris*, *lactis* and *parame-*

* Correspondence should be addressed to Dr. Oh-Sik Kwon, Department of Microbiology, College of Natural Sciences, Keimyung University, Daegu 704-701, Korea. Tel: +82-53-580-5539, Fax: +82-53-585-6949, E-mail: biv@kmu.ac.kr

senteroides isolated from kimchi that were determined by their physiological and biochemical characteristics [10]. Among them, *Leuc. mensenteroides* subsp. *mensenteroides* is usually found in various kimchi known as fermented products [11-13]. Presence of the bacteria gave us many benefits such as improving food preservation, flavors, nutrition and human health [14,15]. Potentiality of the LAB was noticed by many investigators worldwide [16], for examples, anti-cancer effects [17-19] and improvement of immune system of our bodies [20,21]. Since *Leuconostoc* is a very useful bacterial genus for human health and is understood as probiotic lactic acid bacteria [22-24], in this study, we attempted to isolate psychrotrophic *Leuconostoc* species from kimchi as Korean favorite diet which can grow less than 20˚C. Growth profiles as a function of temperature were determined to characterize the isolated LAB. We examined essential biochemical tests and a bile tolerance test that are involved in human diet concerns as desirable characteristics of the probiotics. And it is expected that this kind of effort will make the psychrotrophic *Leuc. mensenteroides* promising for preservation industry of chilled fermented food products such as kimchi.

Materials and Methods

1. Bacterial strains

Strains of lactic acid bacteria (LAB) used in this study are listed in Table 1. Among them, *Leuconostoc mesenteroides* subsp. *mesenteroides* KCTC 3722 was purchased from the Korean Collection for Type Cultures (KCTC), Genetic Resources Center, Daegeon, Korea. The bacterial strains were cultured in a lactobacilli MRS (Difco, USA) and stored in the solution containing 20% glycerol (Sigma) in a −80˚C deep freezer until reactivation.

2. Isolation of LAB strains

Three lactic acid bacterial strains were isolated from kimchi broth fermented in a refrigerator and tentatively named as CL-1, CS-1 and CS-5. For isolating the strains, equal volume of the refrigerated kimchi broth was mixed with 6% saline solution to make the final concentrations of 3% saline samples. After serial dilution (10^{-3}-10^{-6}), the kimchi broth was spreaded onto MRS plates containing 2% Bactoagar (Difco) for detecting numerable colonies. After successive pure culture, colonies were cultured in an incubator (Sanyo, MIR-153) at 25˚C for 72 hours. In order to determine growth kinetics, isolated strains were grown in the same incubator mentioned above and collected after 3 days cultivation. The culture media were measured by a pH meter (Mettler, Model 225) after 20 minutes centrifugation at 3,000 rpm, 4˚C.

Table 1. Lactic acid bacterial strains used in this study

Species & Strains	Sources
Leuconostoc mesenteroides subsp. *mesenteroides* KCTC 3722	Silage
Laboratory isolate LAB CL-1	Chonggak kimchi
Laboratory isolate LAB CS-1	Chonggak kimchi
Laboratory isolate LAB CS-5	Chonggak kimchi

3. CFU test

Samples were subjected to a colony forming unit (CFU) test for determining precise and accurate numbers of bacterial cells alive. In brief, the cultured bacterial solutions were diluted from 10^{-6} to 10^{-12} by successive 10 fold dilution. Each 100 μL of the diluted solution were spreaded onto the 2% MRS agar plates for counting number of viable bacterial cells.

4. Determining psychrotrophic nature of the isolates

In order to investigate psychrotrophic nature of the isolates, 10 mL MRS media (Difco) were prepared in 15 mL plastic tubes (Corning, USA) and were sterilized by an autoclave. Each 10^9 bacterial cells of CL-1, CS-1 and CS-5 were innoculated and were placed into an incubator (Sanyo, MIR-153). The samples were incubated at different temperatures ranged from 14˚C to 38˚C. After 48 hr incubation in the incubator set from 14˚C to 38˚C, the culture tubes were centrifuged for 20 minutes at 3,000 rpm, 4˚C. The supernatant solution of the cultured tubes were measured by a pH meter (Mettler, Model 225) in order to check acidity resulted from cultured organisms.

5. Determining carbohydrate fermentation characteristics

For investigating fermentation characteristics of the isolates, MRS media without sugars was prepared. Each of 10% carbohydrate stock solution [15] was mixed with the media at a ratio of 1 : 10. 20 μL of isolated bacterial solution was inoculated into the 5 mL of carbohydrate containing media in a 15 mL plastic tube (Corning, USA), After 48 hr incubation in an incubator at designated temperatures, the acidity of the cultured

media were measured by a pH meter (Mettler, Model 225) after centrifugation at 3,000 rpm, 4˚C for 20 minutes.

6. Bile salt tolerance test

In order to identify bile salt tolerance of the isolated psychrotrophic CL-1, two kinds of bile salts such as sodium taurocholate and sodium glycocholate (Sigma Chemicals) were used. MRS broth was titrated with 1 N NaOH solution to making the MRS to pH 7.0 and autoclaved in the pressurized sterilizing machine (Sanyo MLS 3020). 5% and 10% stock solution of the sodium taurocholate and sodium glycocholate were added to the MRS broth (pH 7.0) to make final concentration of the bile salts to 0.2% and 0.5% according to Usman and Hosono [25]. Activated LAB were inoculated to the bile salt added MRS broth and incubated in an incubator at 25˚C for 12-24 hours. After the incubated samples were centrifuged at 3,000 rpm for 20 min, acidity of the supernatant was measured by a pH meter (Mettler, Model 225) in order to measure cell growth inhibition.

Results and Discussion

1. Determination of LAB strain CL-1 as *Leuc. mesenteroides*

Fermentation characteristics of the isolate CL-1 showed difference only in one carbohydrate with a standard strain *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 (Table 2). The *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 could not ferment melezitose (a trisaccharide built from two glucose and one fructose) but the isolate CL-1 could ferment it as a sole source of carbohydrate for growth. Other isolated strains such as CS-1 and CS-5 revealed very different fermentation characteristics comparing to the isolate CL-1, that is, they were different in many ways of carbohydrates fermentation. Each of them showed differential growth in four kinds of carbohydrates among cellobiose, gluconic acid, lactose, maltose, mannose, melezitose and salicin. Therefore the CL-1 can be easily identified as another subspecies of *Leuc. mesenteroides.*

2. Salt tolerance of the isolated LAB

Salt tolerance is one of the most unique characteristics that can characterize new strains of LAB because most bacteria residing in animals and plants can not sustain their natural

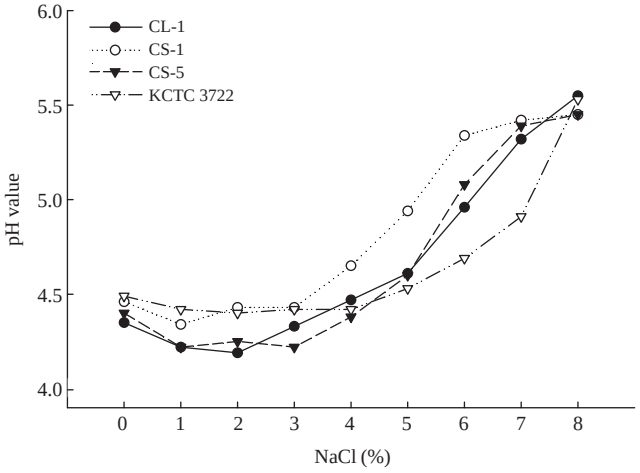

Fig. 1. Comparison of salt tolerance among the isolated LAB from Chonggak kimchi. Tentatively the strains were named as CL-1, CS-1 and CS-5. *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 was used as a standard strain. They were grown at different NaCl concentrations (0-8%). In this case, the increasing pH values indicate growth inhibition occurred by higher NaCl concentration. Symbol descriptions: CL-1 (●), CS-1 (○), CS-5 (▼) and *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 (▽).

Table 2. Fermentation characteristics of the tested lactic acid bacteria that can determine species of *Leuconostoc*

	3722	CL-1	CS-1	CS-5		3722	CL-1	CS-1	CS-5
Amygdalin	−	−	−	−	Mannose	+	+	+	−
Arabinose-D	(+)	−	(+)	−	Melezitose	−	+	+	+
Arabinose-L	+	+	+	+	Melibiose	+	+	+	+
Cellobiose	−	−	+	+	Raffinose	+	+	+	+
Esculin	−	−	−	−	Rhamnose	−	−	−	−
Fructose	+	+	+	+	Ribose	+	+	+	+
Galactose	(+)	−	−	−	Salicin	(+)	−	+	−
Glucose	+	+	+	+	Sorbitol	−	−	−	−
Gluconic acid	+	+	+	−	Sucrose	+	+	+	+
Lactose	(+)	(+)	+	−	Trehalose	+	+	+	+
Maltose	+	+	(+)	+	Xylitol	−	−	−	−
Mannitol	−	−	−	−	Xylose	+	+	+	+

+: strong positive; (+): almost negative; −: negative

growth in higher saline conditions except halophiles and lactic acid bacteria. From the results of salt tolerance test as showed in Fig. 1, it was found that the tested LAB strains could grow in MRS media containing 5% NaCl (w/v). However, as increasing salt concentration in MRS media, each strains showed different salt tolerance that indicated inhibition of cell growth. The growth inhibition of the CS-1 (○) was increased sharply as much as salt concentration in media containing more than 4% of NaCl and growth was stopped at 6% of NaCl concentration. Other isolated strains such as CL-1 (●) and CS-5 (▼) could grow in 6% of NaCl and growth was stopped in 7% of NaCl. Interestingly, *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 (▽) could grow in 7% of NaCl but its growth was suddenly inhibited at 8% of NaCl concentration.

3. Determining psychrotrophic nature of the isolates

All the isolates turned out to be psychrotrophic lactic acid bacteria when they were compared to a standard strain *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 (Fig. 2). Among the isolated psychrotrophs, the CL-1 (●) revealed the most psychrotrophlic nature than others. The CL-1 was able to grow very well, that is, its final pH values were less than 5.0 throughout very wide range temperature profiles (18°C-28°C). At the low temperature (16°C), the CS-5 (▼) could not grow at all. Optimum growth temperature of the CL-1 was 20°C-26°C. The tendency was also observed in the CS-1 (○) that did not give same fermentation characteristics as showed in Table 2. Among them the CL-1 was enabled to outmost growth at the temperature profiles (final pH 4.4±0.1). The CS-5 (▼) showed exceptionally very narrow growth profiles (22°C-28°C) and resulted in poor growth nature except 24°C.

Interestingly enough, growth of the all the isolates was suddenly inhibited at 30°C. At that temperature the standard strain *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 (▽) revealed almost maximal growth (pH 4.6) and its optimal growth temperature was turned out to be 32°C (pH 4.5). This discrepancy was reasoned out that the *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 is a mesophilic species that can grow very well at temperature ranges more than 30°C like most lactic acid bacteria. Growth of *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 was also suddenly inhibited at 36°C as showed in Fig. 2. Thus, the isolated strains of LAB from kimchi broth can be characterized as psychrotrophic bacteria although the CL-1 was characterized as a same species like *Leuc. mesenteroides*.

4. Fermentation characteristics of the CL-1 at low temperature

From the study of psychrotrophic nature of the isolates, it was noticed that sudden inhibition of cell growth occurred dramatically at temperatures ranged from 16°C to 14°C (see Fig. 2, CL-1 and CS-1). And we tried to identify the reason of sudden inhibition of cell growth occurred at the transition temperature (16°C) just prior to the low dead end temperature (L-DET) which could not sustain cell growth at all. The CL-1 was cultured with sugars at 16°C because the growth of CL-1 was totally inhibited at 14°C named as L-DET. A test with hexoses

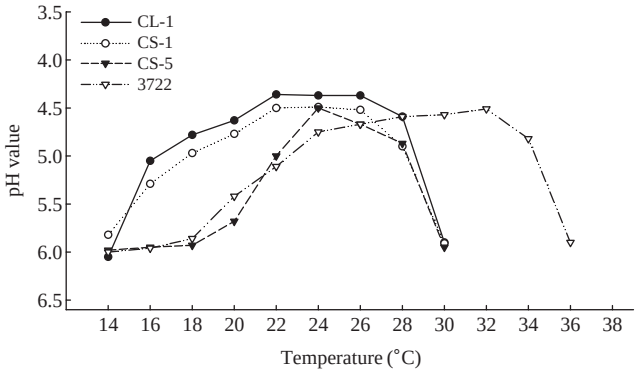

Fig. 2. Growth characteristics of the isolated LAB as a function of temperature. Isolated strains were incubated at variable temperatures ranged from 14°C to 36°C. Symbol descriptions: CL-1 (●), CS-1 (○), CS-5 (▼) and *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 (▽).

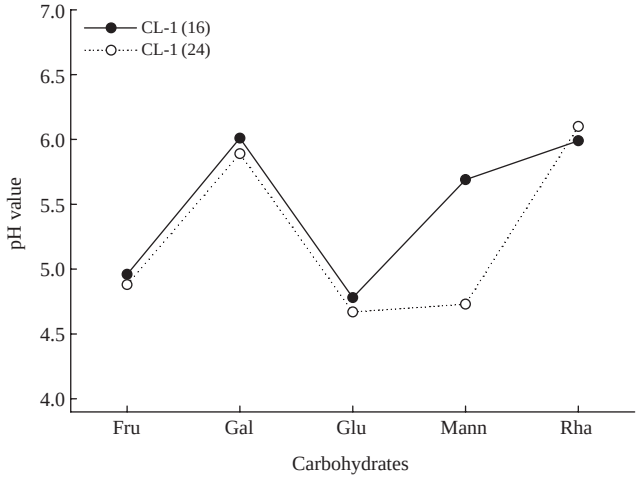

Fig. 3. Comparison of hexose fermentation characteristics of the psychrotrophic LAB CL-1 at different incubation temperatures such as 16°C (●) and 24°C (○). Abbreviations of the carbohydrates: Fru (fructose), Gal (galactose), Glu (glucose), Mann (mannose) and Rha (rhamnose).

revealed very characteristic fermentation results showed in Fig. 3. Among 5 tested sugars (fructose, galactose, glucose, mannose and rhaffinose), significant change was resulted from mannose fermentation. The CL-1 fermented mannose very well at 24˚C (○) that gave a final pH 4.7 after 48 hour incubation but at 16˚C its final pH was only 5.7. This means that at the lowered temperature the CL-1 could not ferment mannose for its proper cell growth and inhibition of the cell growth was to begin. However, at the temperature (16˚C), there was no difference in fermentation of other hexoses such as fructose, galactose, glucose and rhamnose. Because the mannose is an epimer of glucose ($C_6H_{12}O_6$), this result is very valuable to study further for hexose fermentation mechanism in psychrotrophic *Leuconostoc mesenteroides* species. Furthermore, since mannose ($C_6H_{12}O_6$) is a simple sugar for cell growth and energy, it is assumed that the inhibition in fermentation of mannose was resulted from adverse effects on an energy production mechanism. From results (Fig. 3), we can understand the reasons of inhibition of cell growth at the low temperature in an aspect of carbohydrate fermentation especially mannose as a hexose.

Similar tendency was found in fermentation of disaccharides containing hexose (Fig. 4). The tested disaccharides were cellobiose, lactose, maltose, melibiose, sucrose and trehalose. Among 6 tested disaccharides, inhibition of fermentation was occurred only in melibiose that is composed of galactose and glucose. The CL-1 showed decreasing fermentation ability with the melibiose from pH 4.8 at 24˚C (○) to pH 5.2 at 16˚C (●). Fermentation of maltose, sucrose and trehalose was not effected by the low temperature at all. The final pH of the sugars after fermentation were 4.7-4.8 which indicated normal cell growth occurred at the temperatures of either 16˚C or 24˚C. Since the CL-1 and *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 were the same species of *Leuc. mesenteroides*, they could not ferment cellobiose and lactose at all at the both temperatures. From the successive results (Figs. 3 and Fig. 4), we can understand the reasons of sudden inhibition of cell growth occurred at the transition temperature (16˚C) just prior to L-DET. Problems may be occurred during utilization of the sugars that caused irreversible malfunction in fermentation process although both mannose and melibiose contained hexoses being fermented. And it is suggested that more precise mechanisms will be studied further in future. Conclusively, it is found that such growth inhibition of the psychrotrophs by lowered temperatures is related to occurrence of inability of fermenting the sugars and it leads to sudden declining growth of the psychrotrophic lactic acid bacteria CL-1.

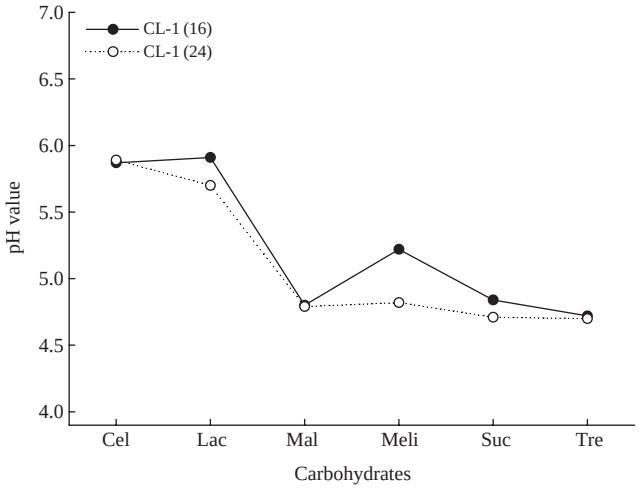

Fig. 4. Comparison of disaccharide fermentation characteristics of the psychrotrophic LAB CL-1 at different incubation temperatures such as 16˚C (●) and 24˚C (○). Abbreviation of the carbohydrates: Cel (cellobiose), Lac (lactose), Mal (maltose), Meli (melibiose), Suc (sucrose) and Tre (trehalose).

5. Bile salt tolerance test

Bile salt tolerance is very important biochemical characteristics for probiotic LAB because the LAB must contact with bile salt in human gastrointestinal tract of a healthy human and animals. Thus we identified bile salt tolerance against the isolated CL-1 with two kinds of bile salts such as sodium taurocholate and sodium glycocholate. As a result, growth of the CL-1 and a standard strain *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 was strongly inhibited in MRS media containing 0.5% taurocholate (Table 3) and it is understand that the 0.5% taurocholate was fairly enough to inhibit LAB cell growth tested. However with treatment of 0.2% taurocholate, the CL-1 showed better growth (final pH 5.2) than *Leuc. mesenteroides*

Table 3. Effect of bile salts on the psychrotrophic CL-1 measured by pH

Strains	Bile salts	Incubation			
		12 hr		24 hr	
		Conc.			
		0.2%	0.5%	0.2%	0.5%
CL-1	Taurocholate	6.0	6.8	5.2	6.7
	Glycocholate	4.9	5.1	4.6	4.9
	Control	4.8	4.8	4.5	4.5
3722	Taurocholate	6.3	6.6	6.1	6.6
	Glycocholate	4.7	4.9	4.5	4.9
	Control	4.7	4.7	4.6	4.6

subsp. *mesenteroides* KCTC 3722 (final pH 6.1) after 24 hr culture. This implies that the CL-1 is more tolerant to bile salt (taurocholate) than *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722. When the strains were inoculated with glycocholate containing MRS, growth of the strains were not effected by the bile salt at all either 0.2% or 0.5% treatments. Although they showed better growth in media containing 0.2% glycocholate than 0.5% glycocholate, the CL-1 could overcome bile salt effect as incubation time goes by from 12 hr to 24 hr.

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