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NaCl Effect on Dextran Producing *Leuconostoc mesenteroides* CS-5 that Isolated from Chonggak Kimchi

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ABSTRACT

Characteristics of *Leuconostoc mesenteroides* strains isolated from chonggak kimchi fermented in a refrigerator were studied in order to identify salinity nature. Among the isolates, a strain named CS-5 showed an optimal growth temperature ranged from 24°C to 28°C. Interestingly the CS-5 was turned out to be a dextran producing strain. It could grow in the media containing 6% NaCl. At 5% NaCl the CS-5 revealed much better growth in media containing glucose or fructose as a monosaccharide than a strain of CL-1 that could not grow at all. For disaccharides (maltose, sucrose), the CS-5 showed strong growth nature at 6% salinity in which the CL-1 could not grow at all. This kind of salt tolerance of CS-5 also appeared in the media containing trehalose, melibiose or cellobiose that were known to be hard to ferment. Based on a test for dextran production, the CS-5 showed higher values of viscosity (0.153 $Pa.S^n$) in media containing 30% sucrose (w/v) comparing to the controls (0.01 $Pa.S^n$). Optimum fermentation time was determined between 48 hours and 60 hours for highest dextran production by *Leuc. mesenteroides* CS-5.

Key words : Chonggak kimchi, Dextran, *Leuconostoc mesenteroides*, Viscosity

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 *paramesenteroides* isolated from kimchi that were determined by their physiological and biochemical characteristics [10]. Among them, *Leuc. mesenteroides* subsp. *mesenteroides* is 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], such as anti-cancer effects [17-19] and improvement of immune system [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 salt tolerant *Leuconostoc* species from chonggak kimchi as Korean favorite diet. Growth kinetics as a function of fermentable carbohydrates was determined to characterize salt tolerance of the isolates that is to be important in preservation industries. We also investigated its ability to produce dextran that is involved in human health

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Table 1. Lactic acid bacterial strains used in this study

Species & Strains	Sources
Leuconostoc mesenteroides subsp. *mesenteroides* KCTC 3722	Silage
Leuconostoc mesenteroides subsp. *dextranicum* KCTC 3530	ATCC 19255
Laboratory isolate CL-1	Chonggak kimchi
Laboratory isolate CS-5	Chonggak kimchi

concerns as desirable characteristics of the probiotics. The production of dextran in an edible liquid culture was investigated by evaluating its consistency and optimum conditions were determined for enhancing dextran production.

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

Lactic acid bacterial strains were isolated from chonggak kimchi broth fermented in a refrigerator and tentatively named as CS-5 and CL-1. 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, USA) 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.

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. Determination of psychrotrophic nature

In order to investigate psychrotrophic nature of the isolates, 10 mL MRS media (Difco, USA) were prepared in 15 mL plastic tubes (Corning, USA) and were sterilized by an autoclave. Each 10^9 bacterial cells of tested strains were innoculated and were placed into a refrigerate incubator (Sanyo, MIR-153). The samples were incubated at different temperatures ranged from 14°C to 36°C. After 48 hr incubation, 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. Determination of 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. For measuring bacterial cell mass, values of spectrophotometry was determined at 600 nm by a BioPhotometer (Eppendorf, Germany).

6. Measurement of dextran production

The defined media for dextran production consist of 0.5% K_2HPO_4, 0.02% $MgSO_4$, 0.1% $CaCO_3$ and 0.2% yeast extract composed by weight per volume percent. In addition, sucrose was fortified to the media in the 10-30% (w/v) level. The con-

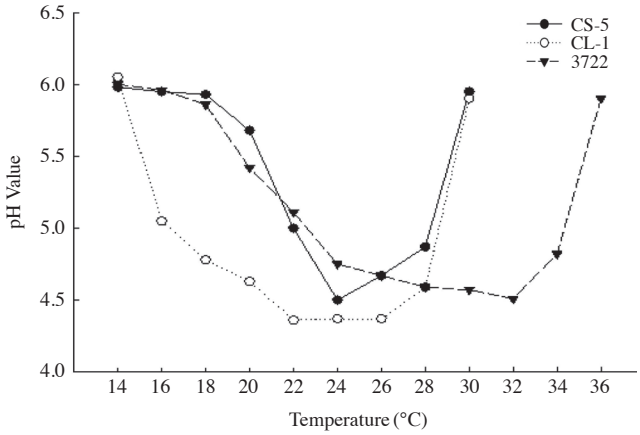

Fig. 1. Growth characteristics of the isolated *Leuc. mesenteroides* as a function of temperature. Isolated strains were incubated at variable temperatures ranged from 14°C to 36°C for 48 hours. Symbol descriptions: CS-5 (●), CL-1 (○) and *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 (▼) as a standard strain.

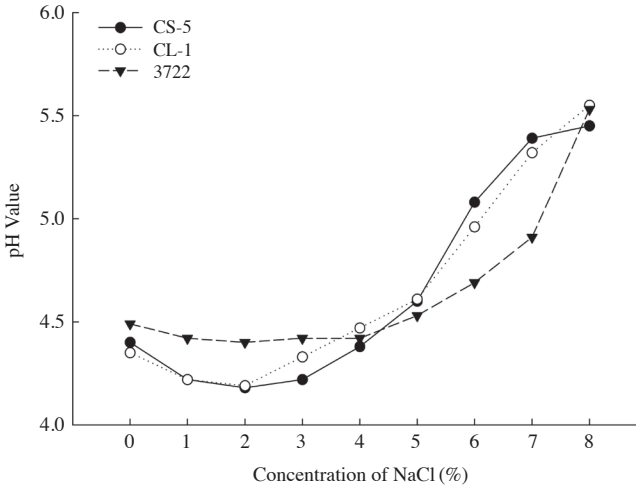

Fig. 2. Comparison of salt tolerance among the isolated *Leuc. mesenteroides* from Chonggak kimchi. They were incubated at different NaCl concentrations (0%-8%) for 48 hours. In this case, the increasing pH values indicate growth inhibition occurred at higher NaCl concentrations. Symbol descriptions: CS-5 (●), CL-1 (○) and *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 (▼) as a standard strain.

centration of dextran in fermentation media were determined by its viscosity. The rheological properties of the viscous culture broth were determined by a Rheometer system (HAAKE RheoStress 1, Germany) equipped with a cone plate device (Platte PP35 Ti, 3.5 cm diameter). Values of produced dextran were described as consistency index ($Pa.S^n$). The viscosity values were measured three times to provide measure of reliability and average with standard deviation was obtained by dividing the sum of replicate measurements by the number of results in the tested set.

Results and Discussion

1. Determining psychrotrophic nature of the isolates

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. 1). Among them, the CL-1 revealed the most psychrotrophic 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 (16°C-28°C). At a low temperature (below 18°C), the CS-5 could not grow at all. Optimum growth temperature of the CS-5 was ranged from 22°C to 28°C but that of the CL-1 was 18°C-28°C. Interestingly enough, growth of the both 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 28°C-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. 1. Thus, the isolated strains from chonggak kimchi can be characterized as psychrotrophic lactic acid bacteria.

2. Salt tolerance of the isolates

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 growth in higher saline conditions except halophilic lactic acid bacteria. From the results of salt tolerance test as showed in Fig. 2, 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-5 was increased sharply as much as salt concentration in media containing more than 6% of NaCl and growth was stopped at 8% of NaCl concentration. In case of the CL-1, it showed same patterns of growth inhibi-

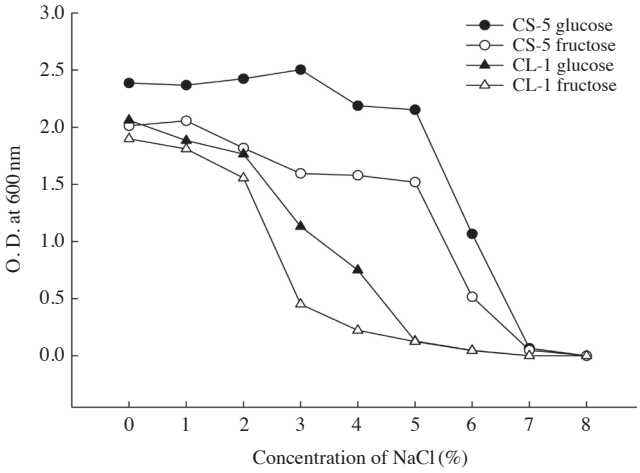

Fig. 3. Effect of NaCl among the isolated *Leuc. mesenteroides* growing in hexoses as a sole source of carbohydrates. They were incubated at different NaCl concentrations (0%-8%) at 25°C for 72 hours. In this case, the decreasing O. D. values indicate growth inhibition of the cells occurred at higher NaCl concentrations. Tested hexoses were glucose (● for CS-5, ▲ for CL-1) and fructose (○ for CS-5, △ for CL-1).

tion like CS-5. Interestingly, a standard strain of *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 could grow in media containing 7% of NaCl but its growth was also suddenly inhibited at 8% of NaCl concentration like isolated strains. Thus it was found that isolated strains such as CS-5 and CL-1 are very salt tolerant *Leuc. mesenteroides*. This fact would be explained by the original source of the LAB isolation, i. e., a salty nature of chonggak kimchi.

3. Effect of monosaccharides in cell growth of the CS-5 under the high salinity conditions

A test with hexoses revealed very characteristic fermentation results showed in Fig. 3. In case of glucose fermentation with 1% glucose (w/v), *Leuconostoc mesenteroides* CS-5 showed strong cell growth until 5% NaCl concentration (value of O. D., 2.3) but its cell growth was declined sharply in the media containig 6% NaCl (value of O. D., 1.2). Under the condition of 7% NaCl the cell growth of CS-5 was stopped (value of O. D., 0.1). In case of fructose fermentation with 1% fructose (w/v), *Leuconostoc mesenteroides* CS-5 showed same patterns of cell growth under the saline conditions but gave a little retarded cell growth. Outmost cell growth of the CS-5 was appeared in the media with 3% NaCl (value of O. D., 2.5). However, in case of the CL-1 that showed same patterns of salt tolerance in Fig. 2, its cell growth was stopped at 5% NaCl condition. Typically its cell growth was heavily inhibited in the media containing 3% NaCl during fermentation of fructose. Thus salt tolerance of isolates was not same and could be changed by kinds of carbohydrates added in the media during fermentation. With the results, it is understood that salt tolerance of the CS-5 was better than that of the CL-1 although they were isolated from a same source of chonggak kimchi. Furthermore, this result gave a clue for usage of preservation additives for making kimchi.

4. Effect of disaccharides in cell growth of the CS-5 under the high salinity conditions

Similar patterns of salinity effects was occurred during fermentation of disaccharides containing two hexoses. The tested disaccharides were sucrose, maltose and lactose (Fig. 4) and trehalose, melibiose and cellobiose which disaccharides are difficult to ferment by species of *Leuconostoc* (Fig. 5). Concentration of sugars in the media was 1% (w/v). Unexpectedly both strains of *Leuconostoc* revealed much better cell growth in the media containing sucrose that gave the highest O. D. values, i. e. 3.0 under the condition of 0%-3% NaCl concentrations (A, CS-5; B, CL-1 in Fig. 4). This implies that salinity can be overcome by adding sucrose in the media for preservation purpose in kimchi production. However response on such saline conditions was very different between the CS-5 and the CL-1. The cell growth of the CS-5 can be sustained in the media containing 6% NaCl with sucrose but its cell growth was stopped in the media containing 7% NaCl with sucrose (Fig. 4A). In case of the CL-1, it can be sustained only in the media containing 4% NaCl with sucrose but its cell growth was stopped in the media containing 5% NaCl with sucrose (Fig. 4B). Similar pattern of fermentation was found in the media containing maltose. However, they grew very poorly in the media containing lactose. This indicates that lactose was not an appropriate disaccharide for fermentation by the strains.

From the successive results with disaccharides such as trehalose, melibiose and cellobiose, it was found that cell growth of both strains were more severely effected by NaCl added in the media. In case of the CS-5 that was more salt tolerable than the CL-1, its cell growth was stopped at 6% NaCl that was 2% below previous results showed on Fig. 3 (glucose, fructose) and Fig. 4 (sucrose, maltose and lactose). Interestingly, the CS-5 showed strong cell growth in the media containing trehalose as same as sucrose (Fig. 5A). Same was true in the CL-1 that showed growth inhibition occurred at 4% NaCl condition (Fig. 5B). Therefore we can understand that trehalose will be a good substitute for sucrose in case of pres-

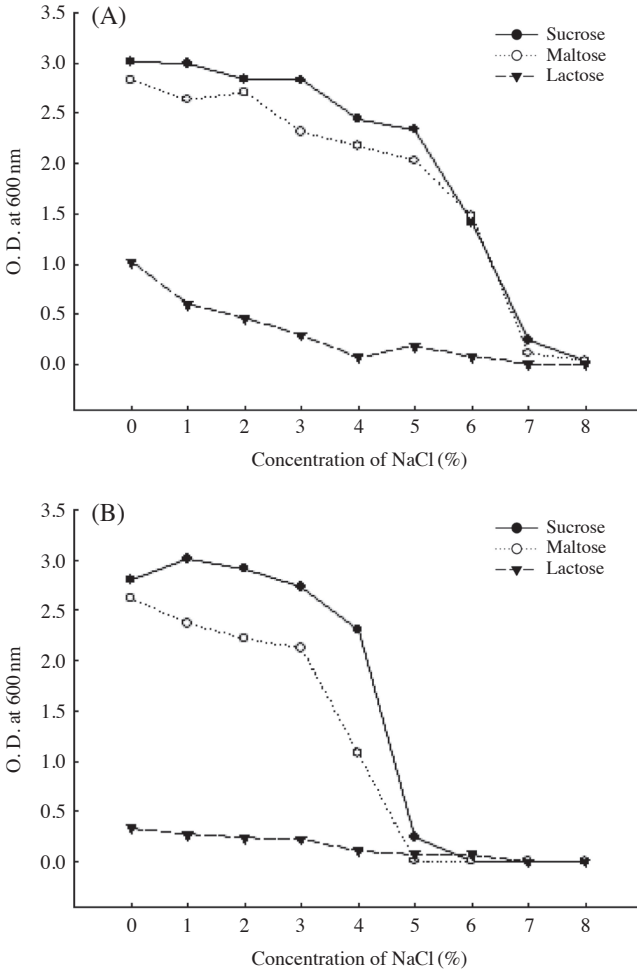

Fig. 4. Effect of NaCl on the *Leuc. mesenteroides* CS-5 (A) and CL-1 (B) during disaccharide fermentation. They were incubated at different NaCl concentrations (0%-8%) at 25°C for 72 hours. In this case, the decreasing O. D. values indicate growth inhibition of the cells occurred at higher NaCl concentrations. Tested disaccharides were sucose (●), maltose (○) and lactose (▼).

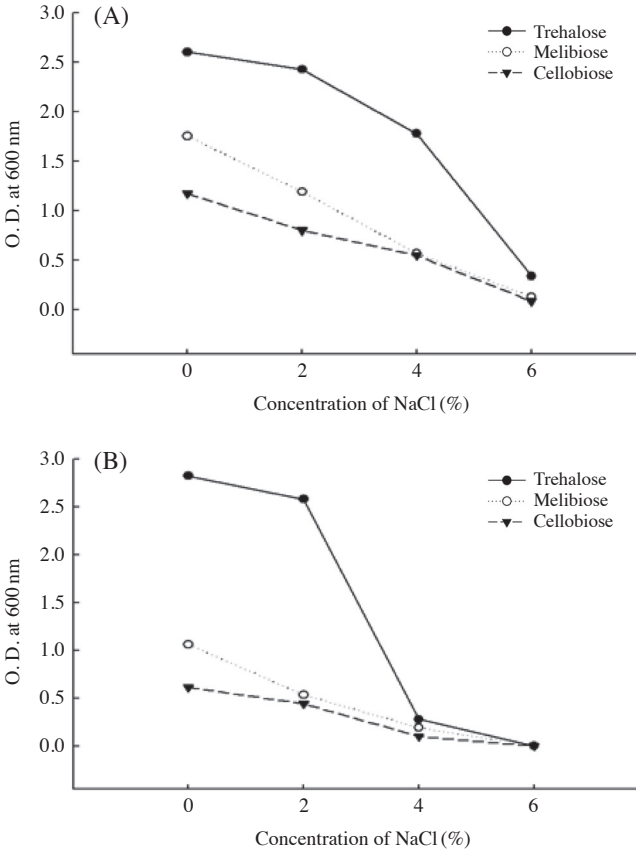

Fig. 5. Comparison of disaccharide fermentation characteristics of the *Leuc. mesenteroides* CS-5 (A) and CL-1 (B). They were incubated at different NaCl concentrations (0%-8%) at 25°C for 72 hours. In this case, the decreasing O. D. values indicate growth inhibition of the cells occurred at higher NaCl concentrations. Tested disaccharides were trehalose (●), melibiose (○) and cellobiose (▼).

ervation of chonggak kimchi under such a salt concentration. Conclusively, it is noticed that such a cell growth inhibition under typical saline condition would be avoided by a choice of sugars (monosaccharides and disaccharides) added to the media or kimchi broth that contained *Leuconostoc mesenteroides* CS-5, a psychrotrophic lactic acid bacteria. As additives for preservation of kimchi, sucrose or maltose will be a good choice for the purposes under the saline conditions tested.

5. Measurement for dextran production in the CS-5

Since the CS-5 was turned out to be a dextran producer, further experiments were carried out to find dextran productivity that depended on composition of media being fermented by the organisms. Addition of sucrose in the media for fermentation will induce cellular activities for degrading sucrose and increasing dextran production in the media [25,26]. Fermentation was done with different concentration of sucrose (10%-30%, w/v) in the MRS broth. As a result, among the tested *Leuconostoc mesenteroides* strains, only the CS-5 as a dextran producer showed higher viscosity index by increasing concentration of sucrose (Fig. 6). Typically all the tested organisms showed no difference in viscosity in the media containing 10% sucrose (0.01 $Pa.S^n$) but viscosity owing to dextran production was differed from 15% sucrose concentration. In the media adding 25% sucrose, viscosity of the CS-5 resulted in 0.13 $Pa.S^n$. Optimum concentration of sucrose for the CS-5 producing dextran was determined as 30% of sucrose (0.153 $Pa.S^n$) because the slope of increase was getting to be flat. This result implied that 15 fold increase of dextran production was achieved by the CS-5 in the media of 30% sucrose added all at

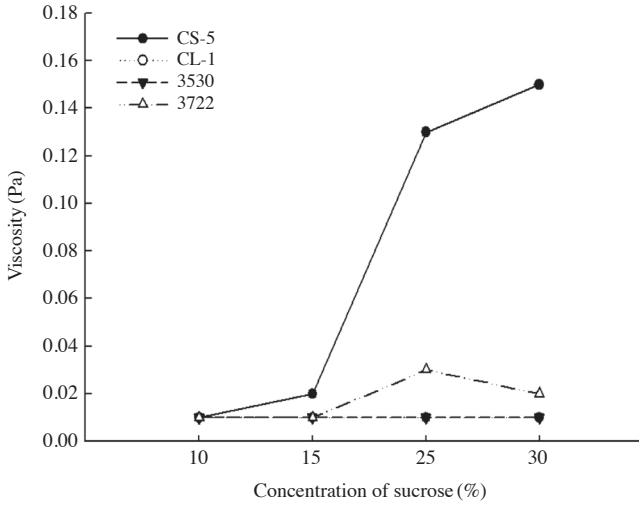

Fig. 6. Effect of sucrose concentration on the consistency of viscous culture broth fermented by *Leuc. mesenteroides* CS-5 (●). Other tested strains were *Leuc. mesenteroides* CL-1 (○), *Leuc. mesenteroides* subsp. *dextranicum* KCTC 3530 (▼) and *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3722 (△). They were incubated at different sucrose concentrations (10%-30%) at 25°C for 72 hours.

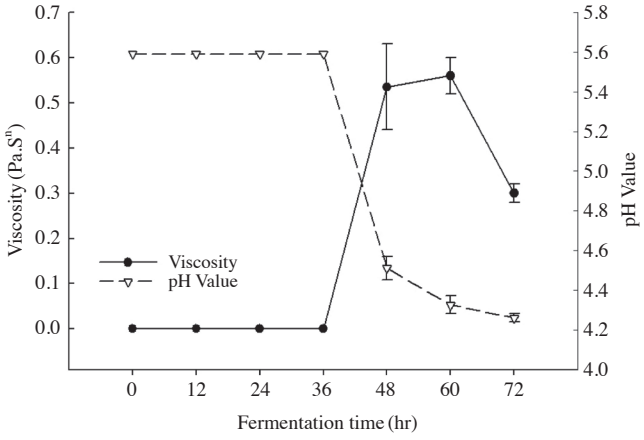

Fig. 7. Determination of optimum fermentation time for dextran production in viscous broth by *Leuc. mesenteroides* CS-5 related to final pH (▽) and viscosity (●). Culture media contained 30% sucrose and 1.5% skim milk that enhanced viscosity of final fermented broth. Data were expressed as mean ± standard error values (n = 3).

once while other strains of tested *Leuconostoc mesenteroides* including standard strains was to be flat.

6. Determination of optimum fermentation time for dextran production by *Leuc. mesenteroides* CS-5

In addition, it was tested in order to enhance dextran production by the CS-5 in relation to time of fermentation at 12 hours period of incubation. As a result, it was found that the CS-5 did not produce dextran until 36 hours of fermentation and a value of viscosity was 0.01 Pa.S^n (Fig. 7). The viscosity resulting from dextran production was sharply increased after 48 hours of incubation up to 0.52 ± 0.10 Pa.S^n. Production of dextran was increased until 60 hours of incubation period (0.55 ± 0.03 Pa.S^n). However production of dextran by CS-5 decreased to a value of 0.30 ± 0.02 Pa.S^n at a time of 72 hours of incubation period and acidity of the fermentation media reached at pH 4.26 ± 0.02. Thus lowered pH of the fermented media by *Leuc. mesenteroides* CS-5 would cause adverse effect on dextran production during a prolonged fermentation period. Conclusively, it was found that optimum fermentation time was 48-60 hours for highest dextran production in *Leuc. mesenteroides* CS-5.

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