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Properties of *Tetragenococcus halophilus* Strains Isolated from Myeolchi (anchovy)-jeotgal

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Halophilic lactic acid bacteria (LAB) were isolated from myeolchi-jeotgal (23% NaCl, w/v) fermented in jangdok (Korean earthenware) located outside a house. They were identified as *Tetragenococcus halophilus* by 16S rRNA and *recA* gene sequencing. Four *T. halophilus* isolates showing high protease activities were selected for further studies. Four strains grew well, reaching OD_{600} values of 0.75–0.92 at 18% NaCl content (w/v) and 0.28–0.44 at 23% salt. They showed rapid growth, attaining OD_{600} values of 1.1–1.2 at 20–30℃, but did not grow at 4℃. At 15℃, the highest OD_{600} values, which exceeded 0.6, were observed at 20 days, and were higher than those of cultures at 37℃ and 42℃ (approximately 0.5). Four isolates grew best in broth where the initial pH was adjusted to 8 and did not grow at pH ≤ 4. *T. halophilus* BS2-36 showed the highest survival ratio of 18.7% after 2 hours of exposure at pH 3. BS2-36 showed the highest survival ratio (1.29%) in presence of 0.3% bile salts. *T. halophilus* BS2-36 seems a promising candidate as a starter for jeotgal and other fermented foods with high salinities.

Keywords: ***Tetragenococcus halophilus*, jeotgal, starter, fermented foods**

Introduction

Myeolchi (anchovy) jeotgal, a Korean traditional salted-fermented food, is made by adding salt (20–30%, w/w) to myeolchi and storing at room temperature for several months. Myeolchi jeotgal is mostly consumed as side dishes, but also used as a seasoning for Kimchi. Myeolchi jeotgal is the most popular jeotgal in Korea, but microbiota of myeolchi jeotgal has not been studied at detail.

Tetragenococcus strains are halophilic lactic acid bacteria (LAB) which have been isolated from various fermented foods with high salinities such as doenjang [1, 2], fish sauce [3, 4], jeotgal [5, 6], and soy sauce [7, 8]. *Tetragenococcus* strains were also isolated from kimchi [9] and fermented sausage [10]. *Tetragenococcus* strains can grow in the presence of NaCl up to around 20% (w/w) and grow fast at 20–30℃. But they grow slowly at 37℃ and above. Currently, 5 species are registered and they are *T. halophilus*, *T. muriaticus*, *T. koreensis*, *T. soliatarius*, and *T. osmophilus* [11].

Because of their high salt tolerance and the status as a member of LAB, *Tetragenococcus* strains have been tested for their potentials as possible starters for jeotgal for accelerating fermentation and improving flavor of products [12]. Recently, *T. halophilus* was tested as a starter for soy sauce fermentation and the organism was believed to contribute to the development of flavor of soy sauce [13]. But so far few studies have been done and the exact roles of *Tetragenococcus* strains and their effects on the quality of foods are largely unknown. More studies are required before *Tetragenococcus* strains are actively used as starters for various fermented foods. In addition to their positive effects, safety issues also

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should be investigated at detail [14]. In this work, properties of 4 *T. halophilus* strains isolated from myeolchi-jeotgal were investigated.

Materials and Methods

Isolation and identification of *Tetragenococcus* strains from myeolchi-jeotgal

Myeolchi-jeotgals were prepared with 3 different types of salts, pure salt (PS), solar salt (SS), and bamboo salt (BS). The final salt concentration was 23% in terms of NaCl content (w/w). Jeotgals were fermented in jangdok, Korean traditional earthenware used for preparation of fermented foods such as ganjang (soy sauce) and doenjang (soy paste). Aliquots of myeolchi-jeotgal were taken out at 4 week and 12 week of fermentation, mixed with peptone water (0.1%, w/v), and homogenized by using a stomacher (stomacher®80, Seward, USA). Homogenates were serially diluted with peptone water, and diluents were spread on deMan-Rogosa-Sharpe agar (MRS, Acumedia, USA) plates (cyclohexamide, 50 μg/ml). The plates were incubated for 7 days at 30℃. Catalase test was done for colonies on MRS plates by pouring hydrogen peroxide solution (3%). Catalase negative colonies were selected and preliminary identification was done such as Gram staining. Colonies were spotted onto MRS plates with skim milk (Acumedia, USA, 1%, w/v) to check their proteolytic activities.

For further identification, molecular biological methods were used. 16S rRNA genes of isolates were amplified by PCR. *Taq* DNA polymerase was used together with primer pairs: 27F (5'-AGAGTT TGATCMTGGCTCAG-3') and 1492R (5'-GGYTACCTTGTTACGACTT-3'). Part of *recA* genes were amplified by using following primer pairs: TrecAF (5'-GATCAACRRATTTCAACTAT-3') and TrecAR (5'-CCWACTTGTGAAATACCTTC-3'). A MJ mini personal thermal cycler (BioRad, USA) was used. Initial denaturation was done at 94℃ for 4 min followed by 30 cycles of 94℃ 1 min, 45℃ 45 s and 51℃ 45 s, and 72℃ 2 min. The final extension was done at 72℃ 4 min. Sequences were determined at Cosmogenetech (Seoul, Korea), and analyzed by BLAST (NCBI, Bethesda, USA).

Salt tolerance of *T. halophilus* isolates

Isolates were inoculated into MRS broth with NaCl (8%, w/v), and incubated for 3 days at 30℃. Isolates showing good growth were selected, and inoculated into fresh MRS broth (3%, v/v) with NaCl (10–25%, w/v). Absorbances at 600 nm (OD_{600}) of cultures were measured after 15 days at 25℃. Isolates showing significant salt tolerance were tested for their proteolytic activities.

Proteolytic activities of *T. halophilus* isolates

T. halophilus isolates were grown in MRS broth containing NaCl (8%, w/v) and skim milk (Acumedia, USA 1%, w/v) for 72 h at 30℃. *B. subtilis* HK176 actively secretes proteases into culture medium, and used as a positive control [15]. *B. subtilis* 168, a lab strain with low protease activity, was used as a negative control [16]. *B. subtilis* HK176 and 168 strains were grown in LB broth containing skim milk (Acumedia, USA 1%, w/v) for 72 h at 37℃ with aeration. Culture supernatant was obtained after centrifugation at 12,000 ×*g* for 10 min. One hundred μl of supernatant was mixed with 1 ml of casein (Junsei, Japan) solution (1%, w/v) and 20 μl of $CaCl_2$ (10 mM). One gram of casein was dissolved in either 100 ml of 0.4 M lactic acid buffer (pH 3.0, for acid protease activity measurement), 0.5 M sodium phosphate buffer (pH 6.0, for neutral protease activity measurement), or 0.2 M boric acid-borate buffer (pH 9.0, for alkaline protease activity measurement). Mixtures were incubated for 20 min at 37℃, then 2 ml of 5% TCA solution was added, and stood for 15 min at room temp (RT). Samples were centrifuged at 7,000 ×*g* for 15 min. One ml of supernatant was mixed with 2 ml of 0.5 M NaOH and 100 μl of Folin-Ciocalteu reagent. The absorbance of each sample was measured at 660 nm after 10 min at RT. A standard curve was prepared using tyrosine at different concentrations. One unit of enzyme activity was expressed as the amount of enzyme which released 1 μmol of tyrosine per min.

Growth of *T. halophilus* isolates at different temperature

T. halophilus isolates were first grown in tryptic soy broth (TSB, BD Difco, USA) with 5% NaCl for 48 h at 30℃, and the cultures were used to inoculate (3%, v/v) fresh MRS broth with 10% NaCl (w/v). The cultures were cultivated at different temperature (4, 15, 20, 30, 37, 42℃) for 60 days. The growth was monitored by measuring the OD_{600} values at 5 days intervals. At 35 days and 60 days, viable cells of cultures grown at 15℃ were

measured by plate counting method using TSB agar with 5% NaCl (w/v).

Growth of *T. halophilus* isolates in MRS broth with different initial pH

T. halophilus strains were grown for 48 h in TSB (5% NaCl) at 30℃, and then cultures were inoculated into fresh TSB broth with 5% NaCl where the initial pH was adjusted to pH 2–10 using 1 N HCl and NaOH. Inoculated cultures were incubated for 120 h at 30℃, and the OD_{600} values were measured at 24 h intervals.

Viability of *T. halophilus* isolates under acidic pH and bile salts challenges

T. halophilus isolates were grown in TSB (5% NaCl, w/v) for 48 h at 30℃. One ml of culture was centrifuged at 13,000 ×*g* for 10 min, and the cell pellet was obtained. After washing with cold, sterile water, cells were resuspended in 1 ml of distilled water whose pH was adjusted to 2, 3, or 6.5 (control) by 1 N HCl or 1 N NaOH. After 2 h at 30℃, viable cells were counted by plate count method using TSB agar with 5% NaCl. Plates were incubated for 96 h at 30℃. Cells were also resuspended in 0.3% bile salts (B8756, Sigma-Aldrich, USA) solution and stood for 2 h at 30℃, and then viable cells were counted. Survival ratios (SR, %) were calculated by dividing the viable cell numbers under stress by the control cell number (pH 6.5).

Results and Discussion

Isolation and identification of tetragenococci

Several isolates showing significant salt tolerance and protease activities were obtained, and 4 strains were finally selected for further studies. They were gram + cocci. For identification, 16S rRNA genes were amplified and sequenced: BS1-37 (1,180 nucleotides), BS2-36 (1,368 nucleotides), PS1-25 (1,376 nucleotides), and SS3-2 (1,372 nucleotides). BLAST analyses showed that the sequences showed 99–100% identities with those from *T. halophilus* strains. In addition, partial *recA* gene sequences (536–587 nucleotides) were determined. BLAST analyses showed that the sequences had 99–100% identities with those from *T. halophilus* strains (results not shown). From these results, 4 isolates were identified as *T. halophilus* strains. Genbank numbers of 16S rRNA genes from BS1-37, BS2-36, PS1-25, and SS3-2 are MG905357, MG905358, MG905359, and MG905360, respectively. Genbank numbers of *recA* genes from BS1-37, BS2-36, PS1-25, and SS3-2 are MG912952, MG912953, MG912954, MG912955, respectively.

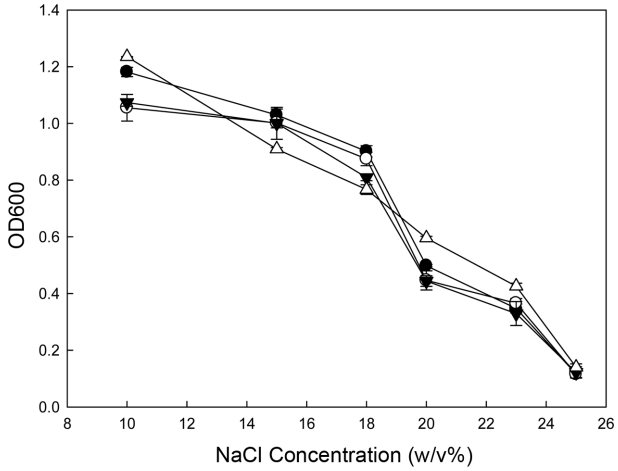

Fig. 1. Growth of *T. halophilus* strains in MRS broth with different NaCl contents. *T. halophilus* isolates were inoculated into MRS broth with NaCl (10, 15, 18, 20, 23, and 25%, w/v) and grown for 15 days at 25℃. -●- *T. halophilus* BS1-37; -○- *T. halophilus* BS2-36; -▼- *T. halophilus* PS1-25; -△- *T. halophilus* SS3-2.

Salt tolerance of *T. halophilus* isolates

Salt tolerance of isolates were examined during growth in MRS broth with NaCl (10–25%, w/v). All isolates grew well up to 18% salt, showing the OD_{600} values of 0.75–0.92 at 18% salt. At 20% salt, SS3-2 grew better than other isolates, and absorbance at 600 nm (OD_{600}) was 0.60 ± 0.01 at 15 days (Fig. 1). BS1-37 was the second, showing OD_{600} of 0.50 ± 0.01. BS2-36 and PS1-25 showed similar values (0.44–0.45). All strains showed some growth activity (0.28–0.44) at 23% NaCl concentration but did not grow at 25% salt. Most LAB can't grow at high salt concentration, and the results confirmed that 4 *T. halophilus* strains are halophilic, and can be used as a starter for jeotgals.

Proteolytic activities of *T. halophilus* isolates

For acid protease activity, SS3-2 showed the highest value, 2.30 ± 0.02 U/ml (Fig. 2). Other strains showed slightly lower values (2.20–2.29 U/ml). but the activities were still higher than that of *Bacillus subtilis* HK176 (1.76 ± 0.07 U/ml). *B. subtilis* 168, a negative control,

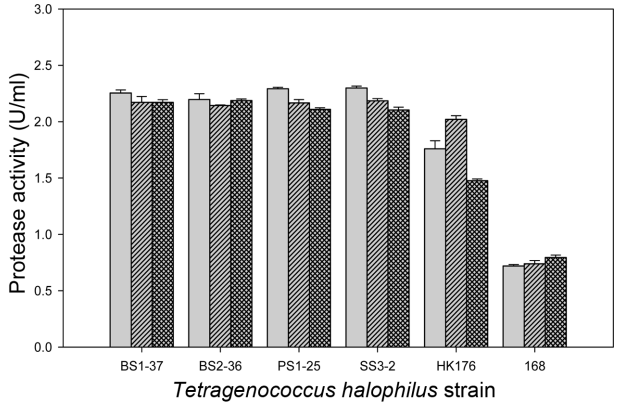

Fig. 2. Proteolytic activities of *T. halophilus* strains. , acid protease activity (pH 3.0); , neutral protease activity (pH 6.0); , alkaline protease activity (pH 9.0). HK176 represents *Bacillus subtilis* HK176 (a positive control) and 168 represents *B. subtilis* 168 (a negative control).

showed 0.72 ± 0.01 U/ml. Similar results were observed for neutral protease activities. SS3-2 showed the highest value (2.19 ± 0.02 U/ml) and other strains showed slightly lower values. All strains showed higher activities than *B. subtilis* HK176. For alkaline protease activity, BS2-36 showed the highest value (2.19 ± 0.01 U/ml), and BS1-37 was the second (2.17 ± 0.02 U/ml). Other 2 strains showed slightly lower values (2.10–2.11 U/ml), but the values were significantly higher than those of *B. subtilis* HK176 (1.48 ± 0.02 U/ml) and *B. subtilis* 168 (0.79 ± 0.02 U/ml).

Growth of *T. halophilus* isolates at different temperature

T. halophilus isolates grew well at 20℃ and 30℃ (Fig. 3). Growth at 30℃ was slightly better than growth at 20℃ within the first 10 days. But no significant differences were observed after 10 days. All isolates grew quickly within the first 5–10 days, and showed the highest OD_{600} values (ca. 1.2). After 10 days, the values decreased gradually, reaching to 1.0–1.1 at 60 days. All isolates did not grow at 4℃. At 37℃ and 42℃, isolates grew quickly within the first 10 days, but the highest OD_{600} values were less than 0.6, and similar values were

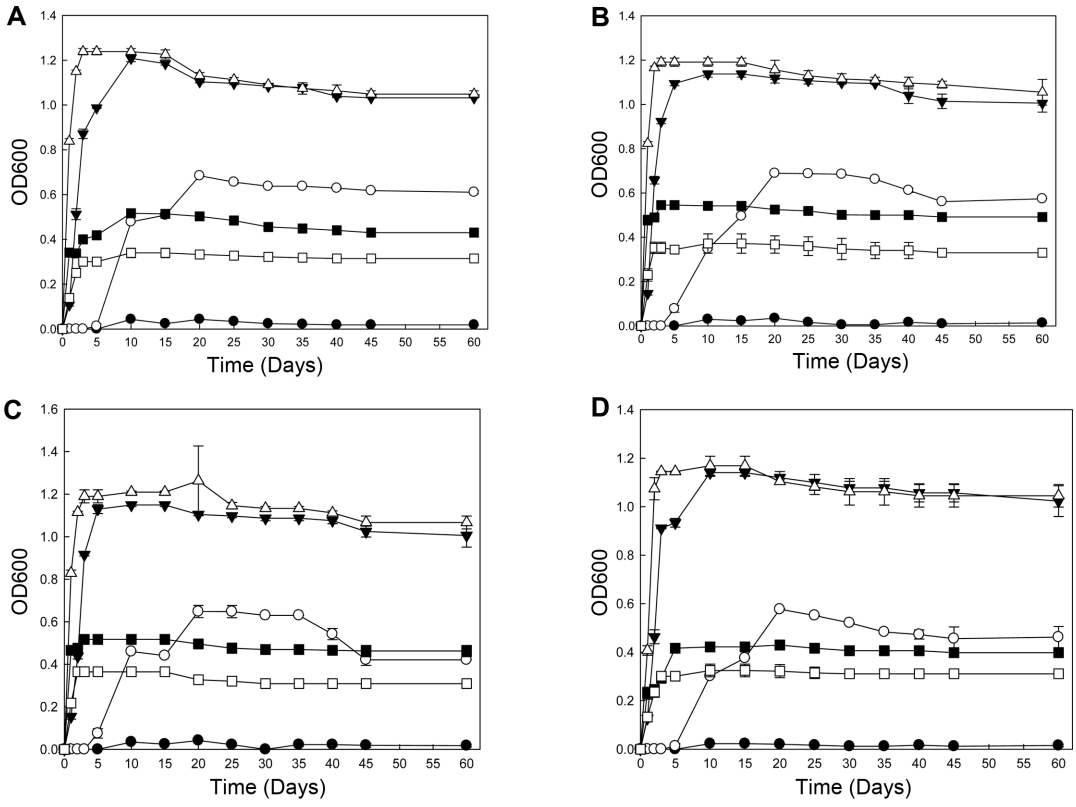

Fig. 3. Growth of *T. halophilus* strains at different temperature. *T. halophilus* isolates were inoculated into MRS broth with NaCl (10%, w/v) and grown for 60 days at different temp. -●- 4℃; -○- 15℃; -▼- 20℃; -△- 30℃; -■- 37℃; -□- 42℃. (A) BS1-37, (B) BS2-36, (C) PS1-25, (D) SS3-2.

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Table 1. Viable cells of *T. halophilus* isolates grown for 35 and 60 days at 15℃.

Strain	35 days	60 days
	CFU/ml	
BS1-37	4.1×10^7	4.0×10^7
BS2-36	6.5×10^7	3.5×10^7
SS3-2	8.4×10^7	8.0×10^7
PS1-25	6.0×10^7	5.7×10^7

Tryptic soy agar plates with 5% NaCl were used for viable cell counting.

maintained until 60 days. At 15℃, the highest values (0.69–0.58) were observed at 20 days, and the values decreased gradually. At 60 days, the values were in the range of 0.42–0.61. From these results, it was concluded that the optimum temperature for *T. halophilus* isolates was 20–30℃. *T. halophilus* strains isolated from sea water and mountain snow did not grow at 4℃ and 40℃ [17]. Whereas some *T. halophilus* strains isolated from fish sauce mashes grew at 45℃ [4]. These results indicate that variations are present among *T. halophilus* strains. Viable cells of cultures grown at 15℃ were counted at 35 and 60 days and the results were similar among isolates (Table 1). 4.1–8.4 × 10^7 CFU/ml were maintained at 35 days and slightly reduced counts, 3.5–8.0 × 10^7, were observed at 60 days. OD_{600} values of cultures were 0.48–0.66 at 35 days and 0.42–0.61 at 60 days.

Growth of *T. halophilus* isolates in MRS broth with different initial pH

All isolates showed a same pattern of pH changes when grown in MRS broth (NaCl, 8%) for 72 h at 30℃, i.e. pH decreased continuously, and reached to 4.6–4.8 at 72 h (results not shown). The OD_{600} values were 1.1–1.2 at 72 h. *T. halophilus* isolates grew best in TSB with the initial pH of 7 and 8, reaching to the highest OD_{600} values of 1.2 at 48 h (Fig. 4). They also grew well at the initial pH of 6 and 9. They grew slowly at pH 10 except SS3-2. SS3-2 did not grow at initial pH of 10. SS3-2 did not grow at initial pH of 5 whereas other strains grew a little bit (0.18–0.2). All strains failed to grow at pH 4 and below.

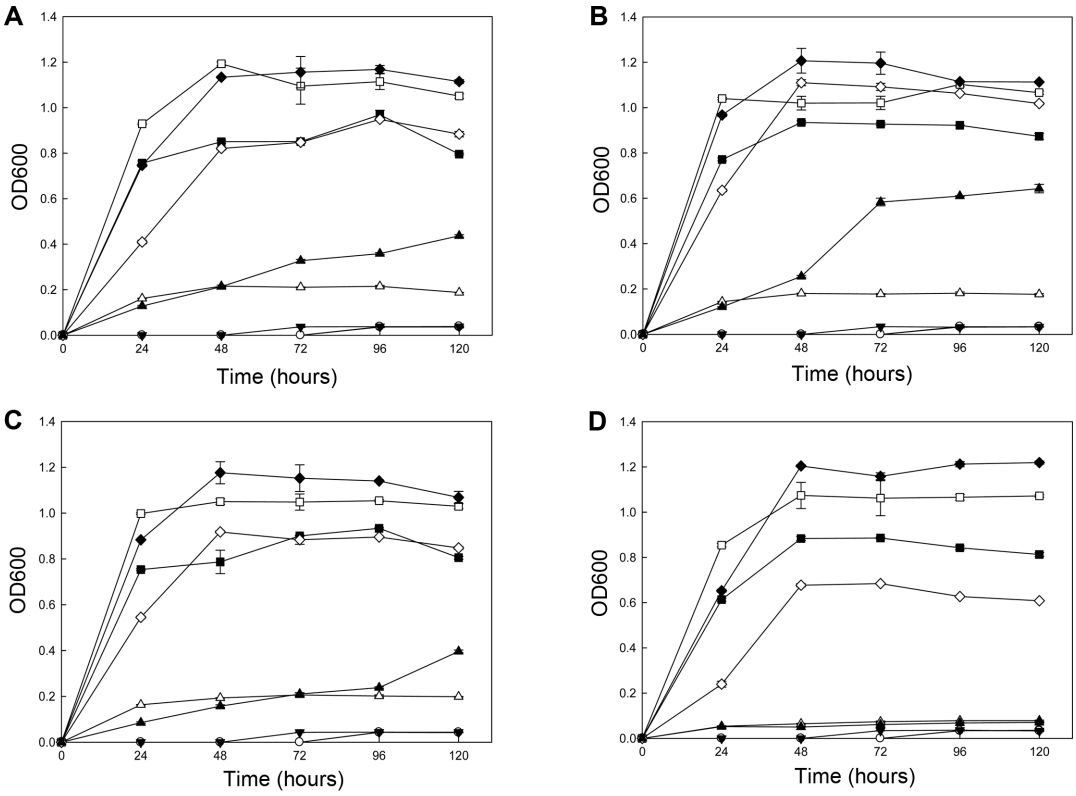

Fig. 4. Growth of *T. halophilus* strains in TSB with different initial pH. -●-, pH 2; -○-, pH 3; -▼-, pH 4; -△-, pH 5; -■- pH 6; -□-, pH 7; -◆-, pH 8; -◇- pH 9; -▲- pH 10. (A) BS1-37, (B) BS2-36, (C) PS1-25, (D) SS3-2.

Table 2. SR of *T. halophilus* isolates after acid, bile salts, and combined challenges.

Strain	Challenge	CFU/ml	Survival ratio (%)
BS1-37	Control (pH 6.5)	1.7×10^8	100
	pH 2.0	0.0	0.0
	pH 3.0	3.6×10^2	0.0
	0.3% bile salts (BS)	1.8×10^6	1.1
	pH 3.0 + 0.3% BS	0.0	0.0
BS2-36	Control (pH 6.5)	1.6×10^8	100
	pH 2.0	1.0×10^2	0.0
	pH 3.0	2.9×10^7	18.7
	0.3% bile salts (BS)	2.0×10^6	1.3
	pH 3.0 + 0.3% BS	7.0×10^1	0.0
SS3-2	Control (pH 6.5)	8.2×10^7	100
	pH 2.0	0.0	0.0
	pH 3.0	1.2×10^6	1.5
	0.3% bile salts (BS)	7.9×10^5	1.0
	pH 3.0 + 0.3% BS	0.0	0.0
PS1-25	Control (pH 6.5)	9.8×10^7	100%
	pH 2.0	0.0	0.0
	pH 3.0	7.0×10^6	7.1
	0.3% bile salts (BS)	9.2×10^5	0.9
	pH 3.0 + 0.3% BS	0.0	0.0

Viability of *T. halophilus* isolates under acidic pH and bile salt challenges

All *T. halophilus* cells were killed after 2 hours exposure at pH 2.0 except BS2-36. Few BS2-36 cells survived but the SR was very close to 0% (Table 2). Most cells were killed by 2 h exposure at pH 3.0, and SRs were 0–18.7%. BS2-36 showed the highest SR, 18.7% whereas BS1-37 was the most sensitive. BS2-36 also showed the highest SR (1.29%) against 0.3% bile salts challenge. After exposure to pH 3 and 0.3% bile salts at the same time, few BS2-36 cells still survived whereas cells from other strains were completely killed. Compared to LAB originated from dairy environments, *T. halophilus* strains isolated from myeolchi-jeotgal showed lower degree of resistance against acidic pH and bile salts challenges [18]. Especially, resistance against 0.3% bile salts was quite lower than LAB isolated from human feces [19]. The results were not surprising considering the natural environments where *Tetragenococcus* strains are often isolated.

Temperature and salt content are the 2 most important factors which determine the types of dominant microorganisms and the final quality of foods during food fermentation [20]. Thus understanding the growth properties of starters is the first step for establishing optimum fermentation conditions for the production of high quality fermented foods. If *T. halophilus* strains in this study are used as starters for myeolchi-jeotgal, fermentation can be carried out at low temperature and high salt conditions. Thus it seems possible to produce high quality myeolchi-jeotgal without deterioration for a long time. Four *T. halophilus* strains might be useful as starters for jeotgal and other fermented foods with high salinities although their safety should be confirmed before use. Among 4 isolates, *T. halophilus* BS2-36 seems to be most suitable as a starter since the strain possesses significant degree of resistance against low pH (pH 3.0) and 0.3% bile salts. Future studies are necessary on the production of fermented foods using *T. halophilus* BS2-36 as a starter.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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