Characterization of lactic acid bacterium *Pediococcus pentosaceus* 4I1 from fresh water fish *Zacco koreanus* and its antibacterial mode of action

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The present study aimed to characterize a lactic acid bacterium *Pediococcus pentosaceus* 4I1, isolated from the intestine of fresh water fish sample *Zacco koreanus*. Biochemical analysis using the API 50 CHL kit and molecular characterization of 4I1 revealed its identity as a lactic acid bacterium *Pediococcus pentosaceus* 4I1. Further, cell free supernatant (CFS) of *P. pentosaceus* 4I1 exhibited significant ($p<0.05$) antibacterial effect as diameters of inhibition zones (16.5–20.4 mm) against the tested foodborne pathogenic bacteria with minimum inhibitory (MIC) and minimum bactericidal (MBC) concentration values found in the range of 250-500 and 500-1,000 µg/mL, respectively. Further, to confirm the efficacy of 4I1 on membrane permeability against foodborne pathogens, antibacterial mode of action of CFS of *P. pentosaceus* 4I1 against two selected bacteria *Staphylococcus aureus* KCTC-1621 (gram-positive) and *Escherichia coli* O157:H7 (gram-negative) was determined by measuring cell viable count, release of 260-nm absorbing materials, leakage of potassium ions and measurement of relative electrical conductivity of the bacterial cells treated at MIC concentration. The CFS of *P. pentosaceus* 4I1 revealed its mode of action on membrane integrity as confirmed by reduction in viable cell count, increased release of potassium ions (900 and 800 mM/L), loss of 260-nm absorbing materials (3.99 and 3.77 OD), and increase in relative electrical conductivity (9.9 and 9.7%) against the tested bacteria *S. aureus* KCTC-1621 and *E. coli* O157:H7, respectively. The above findings hypothesize that *P. pentosaceus* 4I1 compromised its mode of action on membrane integrity, suggesting its enormous potential in food and pharma industries.

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ABSTRACT

The present study aimed to characterize a lactic acid bacterium *Pediococcus pentosaceus* 4I1, isolated from the intestine of fresh water fish sample *Zacco koreanus*. Biochemical analysis using the API 50 CHL kit and molecular characterization of 4I1 revealed its identity as a lactic acid bacterium *Pediococcus pentosaceus* 4I1. Further, cell free supernatant (CFS) of *P. pentosaceus* 4I1 exhibited significant ($p<0.05$) antibacterial effect as diameters of inhibition zones (16.5–20.4 mm) against the tested foodborne pathogenic bacteria with minimum inhibitory (MIC) and minimum bactericidal (MBC) concentration values found in the range of 250-500 and 500-1,000 µg/mL, respectively. Further, to confirm the efficacy of 4I1 on membrane permeability against foodborne pathogens, antibacterial mode of action of CFS of *P. pentosaceus* 4I1 against two selected bacteria *Staphylococcus aureus* KCTC-1621 (gram-positive) and *Escherichia coli* O157:H7 (gram-negative) was determined by measuring cell viable count, release of 260-nm absorbing materials, leakage of potassium ions and measurement of relative electrical conductivity of the bacterial cells treated at MIC concentration. The CFS of *P. pentosaceus* 4I1 revealed its mode of action on membrane integrity as confirmed by reduction in viable cell count, increased release of potassium ions (900 and 800 mM/L), loss of 260-nm absorbing materials (3.99 and 3.77 OD), and increase in relative electrical conductivity (9.9 and 9.7%) against the tested bacteria *S. aureus* KCTC-1621 and *E. coli* O157:H7, respectively. The above findings hypothesize that *P. pentosaceus* 4I1 compromised its mode of action on membrane integrity, suggesting its enormous potential in food and pharma industries.

Keywords *Pediococcus pentosaceus* 4I1; *Zacco koreanus*; Foodborne pathogens; Antimicrobial mode of action; Cell free supernatant

INTRODUCTION

Lactic acid bacteria (LAB) have long been used in fermentations to preserve the nutritive qualities of various foods. The primary antimicrobial effect of LAB is associated with reduced pH values and production of lactic acid (Ammor *et al*., 2006). Also low molecular weight compounds such as hydrogen peroxide, carbon dioxide, diacetyl (2,3-butanedione) and low molecular weight compounds called bacteriocins significantly contribute to the therapeutic potential of LAB (Ammor *et al*., 2006). All of which can antagonize the growth of some spoilage and pathogenic bacteria in foods. Biopreservation systems in food are becoming increasingly interesting for the industry and consumers. LAB and/or their bioactive molecules, including bacteriocins are considered safe additives (GRAS), useful to control the frequent development of pathogens and spoiling microorganisms in food and feed (Parada *et al*., 2007; Djadouni *et al*., 2012).

Recently, severe concerns have been emerged with increasing incidence of foodborne diseases, which have become relevant public health issues (Oussalah *et al*., 2007; da Silveira *et al*., 2012). In spite of the advances in the sanitation techniques and inspection services, the contamination of foods with undesirable microorganisms is a potential risk during food processing, re-processing, storage and distribution both in developing and developed countries (Runyoro *et al*., 2010). Also development of pathogen resistance to commercially available antimicrobials has urged the urgent need to find more effective and natural classes of antimicrobials (Millitello *et al*., 2011).

During the last few years, utilization of natural food preservatives has been widely accepted by the consumers, who increasingly seek natural and healthy products, containing less synthetic additives (da Silveira *et al*., 2012). Consumers are used to the presence of spices in food products that are mainly used to enhance taste and flavor, and therefore, essential oils derived from

spices applied as natural food preservatives should not cause any rejection (Millitello *et al*., 2011; da Silveira *et al*., 2012)

Since decades, synthetic chemicals have been used to control microbial growth of hazardous pathogens and showed potential efficacy to reduce the incidences of foodborne diseases, however, they might be detrimental to human health (Zurenko *et al*., 1996). Hence, application of lactic acid bacteria (LAB) as safe and effective alternatives to synthetic preservatives in controlling pathogenic bacteria may able to reduce the risk of foodborne outbreaks and may assure consumers with safe and microbially-contaminated free food products (Callewaert *et al*., 2000; Mataragas *et al*., 2003). Numerous studies have suggested that LAB have remarkable ability to control the growth of various foodborne pathogenic microorganisms in food matrix (Callewaert *et al*., 2000; Mataragas *et al*., 2003; Ammor *et al*., 2006).

Hence, present study was designed to isolate a potentially effective LAB strain *Pediococcus pentosaceus* 4I1 from the intestine of fresh water fish sample *Zacco koreanus*, and to evaluate its antimicrobial mode of action against foodborne pathogenic bacteria along with its biochemical and molecular characterization.

MATERIALS AND METHODS

Media, reagents and test sample preparation

The Bromocresol purple (BCP) agar medium was used for isolation and identification of lactic acid bacteria, whereas nutrient broth (NB) medium was used for cultivation of test pathogenic bacteria. Both the media were purchased from Sigma-Aldrich (Sigma, MO, USA). The de Man, Rogosa and Sharpe (MRS) agar medium used for culture of lactic acid bacteria was purchased

from Difco (USA). Other chemicals and reagents used were of high purity. Spectrophotometric measurements were made using an enzyme-linked immunosorbent assay (ELISA) instrument. An 18~24 hours grown culture of *Pediococcus pentosaceus* 4I1 was freeze-dried and desired test concentrations were prepared in double-distilled sterilized water. In brief, to prepare the CFS of 4I1, a 24 h grown culture of 4I1 was centrifuged (8,000 x g; 10 min) and the supernatant was collected. The collected supernatant was freeze-dried to make a powdered form which was served as a CFS of 4I1.

Test foodborne pathogens

The test foodborne pathogens included *Staphylococcus aureus* KCTC-1621, *Bacillus subtilis* KCTC-3569, *Listeria monocytogenes* KCTC-3569, *Salmonella enterica* ATCC-4731 and *Escherichia coli* O157:H7. All the bacterial strains were grown in the nutrient broth (NB) at 37°C. The bacterial strains were maintained on nutrient agar slants at 4°C.

Sample collection and isolation of *P. pentosaceus* 4I1

Fresh water fish samples, belonging to *Zacco koreanus*, were collected from a local river in Daegu city, Korea, supplied by Daejeon National Science Museum, Daejeon. All sampling procedures were reviewed following catch per unit effort (CPUE) methods, and the collected samples were transported in ice-packed boxes to the Microbiome laboratory, Yeungnam University and stored at -20^{o}C for further analysis. Taxonomic identification of the fish species was conducted by the fish expert at the National Science Museum of Korea, according to the methods of species identification (Kim and Park, 2002). National ethical approval was obtained for fish samples on "Animal Care and Use" by the ethical committee of Daejeon National Science Museum (Approval

NSMD-MSIFP-KOR208), Daejeon, Korea.

For the isolation of LAB from fresh water fish samples, a previously developed standard serial dilution method was adopted (Cho *et al*., 2013). In brief, scarification of the experimental fish was done in a sterilized clean bench using sterilized knife and forces using 70% ethanol. To isolate LAB, dissected fish tissues of intestine were used since these are known as major reservoirs of microbial community in fish, and homogenized followed by serial dilution in phosphate buffer saline (PBS) using BCP agar medium. Samples were put in 1 mL of PBS and vortexed vigorously in order to make a uniform inoculum size followed by its serial dilution to the maximum serial dilution factor from 10^{-1} to 10^{-9} cells/mL. Finally an inoculum of 100 µL was spread on BCP agar plates, and plates were sealed using paraffin and incubated at 37°C for 24 h. Selection of the diluent was based on the density of the inoculum. Identification of the isolates was based on the clear zone around the colony on BCP agar plates. Each set was prepared in triplicate and positive results were confirmed. Representative colonies were picked from plates and well-isolated colonies were inoculated into fresh MRS broth for stock preparation. For long term storage, stock cultures were maintained at -20°C in MRS broth.

Morphological and biochemical identification of LAB isolate

Morphological identification of 4I1 was conducted by observing the colony shape on BCP agar medium, gram-staining, and cell morphology using a microscope (Holt *et al.,* 1994). Further, the isolate was identified biochemically using API 50 CH strips with API 50 CHL medium at species level based on the instructions of the manufacturer (API 50 CHL, BioMerieux, France). In brief, freshly-grown bacterial colony of the selected LAB isolate was picked-up and inoculated in MRS medium at 36°C for 24 h, and then the bacterial culture was serially diluted to prepare desired

concentration of 10^8 CFU/ml. From this, aliquot (2 mL) was inoculated into APL 50 CHL medium (10 mL), and mixed by gentle inversion. Then, a bacterial suspension (120 µL) was inoculated into API 50 CH strips that were pre-overlaid with mineral oil followed by further incubation for 48 h before measuring the color change abilities. Finally, the strips were processed for analyzing the API profiles using computer APILAB Plus Version.

Molecular Characterization of LAB Isolate

In this study, LAB isolate 4I1 was characterized by partial 16S rRNA gene sequencing analysis. To determine the identity of 4I1, genomic DNA from 4I1 was isolated using TaKaRa MiniBEST universal genomic kit (Seoul, Korea) Universal primers F44 (5'RGTTYGATYMTGGCTCAG-3') and R1543 (5'- GNNTACCTTKTTACG ACTT-3') were used for the amplification of the 16S rRNA gene by PCR. PCR reactions were carried out using a Biometra thermal cycler (M Biotech Inc., Canada) with the following cycle parameters: an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and elongation at 72°C for 1 min. The PCR products of 16S rDNA were sequenced and analyzed. The gene sequences were compared in the National Center for Biotechnology Information (NCBI) for homology using BLAST and multiple-aligned with 16S rRNA gene sequences of different strains for similarity using the ClustalW program coupled with MEGA 5. A neighbor-joining method was employed to construct the phylogenic tree using the MEGA 5 software.

Determination of antibacterial activity

Standard agar well diffusion method was used for the determination of antibacterial efficacy of cell free supernatant (CFS) of *P. pentosaceus* 4I1 (Murray *et al.*, 1995). To get CFS of 4I1,

supernatant from a 24 h grown culture of 4I1 was collected by centrifugation and freeze-dried. Petri-plates were prepared by pouring 20 mL of nutrient agar medium and allowed to solidify. Plates were dried and 1 mL of standardized inoculum containing 10^7 CFU/mL of bacterial suspension was poured and uniformly spread, and the inoculum was allowed to dry for 5 min. The wells were made by using sterilized borer where 100 µL CFS of *P. pentosaceus* 4I1 was poured into each well against each of the tested pathogen. Negative controls were prepared using the same solvent (sterilized distilled water or MRS medium) employed to dissolve the samples. Antibacterial activity was evaluated by measuring the diameter of the zones of inhibition (including diameter of well: 6 mm) against the tested bacteria. Each assay in this experiment was replicated three times.

Determination of minimum inhibitory (MIC) and bactericidal (MBC) concentrations

The MIC of CFS of *P. pentosaceus* 4I1 was tested by the two-fold serial dilution method (Bajpai *et al*., 2013). The freeze-dried CFS of *P. pentosaceus* 4I1 (4 mg) was first dissolved in 1 mL distilled water as a stock, and incorporated into nutrient broth (NB) medium for bacterial pathogens to obtain an initial concentration of 2,000 µg/mL, and serially diluted to achieve 1,000, 500, 250, 125, 62.5, 31.25, 15.62 and 7.81 µg/mL concentrations of cell free supernatant of 4I1. A 10 µL standardized suspension of each tested organism (approximately 10^7 CFU/mL) was transferred to each tube. The treatment and control tubes which contained only bacterial suspensions were incubated at 37°C for 24 h. The lowest concentration of CFS, which did not show any visible growth of test organisms after macroscopic evaluation, was determined as MIC, which was expressed in µg/mL. Further, the concentrations showing complete inhibition of visual growth of bacterial pathogens were identified, and 50 µL of each culture broth was transferred onto the agar

plates and incubated at 37°C for 24 h. The complete absence of growth of bacterial colonies on the agar surface is the lowest concentration of the sample and was defined as MBC. Each assay in this experiment was replicated three times.

Determination of effect of CFS on viable counts of bacterial pathogens

In this assay, freshly-grown bacterial colonies of the selected pathogenic bacteria were picked-up and inoculated in nutrient broth medium at 36°C for 24 h, and then the bacterial cultures were serially diluted to prepare desired concentration of 10^7 CFU/mL (Shin *et al*., 2007). To determine the effect of CFS of *P. pentosaceus* 4I1 on cell viability, two selected foodborne pathogenic bacteria *S. aureus* KCTC-1621 (gram-positive) and *E. coli* O157:H7 (gram-negative) were used for further studies. Briefly, for viable count assay, each of the tubes containing bacterial suspension (10 µL; approximately 10^7 CFU/mL) of *S. aureus* KCTC-1621 and *E. coli* O157:H7 was inoculated with 100 µL of CFS at MIC concentration in 890 µL NB broth, and kept at 37°C. Samples for viable cell counts were taken out at 0, 40, 80, 120, 160 and 200 min time intervals. The viable plate counts were monitored on NB agar following our previously described methods (Bajpai *et al*., 2013). Colonies were counted after 24 h of incubation at 37°C. The controls were inoculated without CFS for each bacterial strain with the same experimental condition as mentioned above. Each assay in this experiment was performed in triplicates.

Determination of effect of CFS on potassium ion efflux

Effect of CFS of *P. pentosaceus* 4I1 on the efflux of potassium ions from the treated cells was determined using our previously reported method (Bajpai *et al*., 2013). The concentration of free potassium ions in bacterial suspensions of *S. aureus* KCTC-1621 and *E. coli* O157:H7 was

measured after the exposure of bacterial cells to CFS of *P. pentosaceus* 4I1 at MIC concentration in sterile peptone water (8.5 g NaCl + 1 g peptone in 1 L sterilized distilled water) for 0, 30, 60, 90 and 120 min. At each pre-established interval, the extracellular potassium concentration was measured by a photometric procedure using the Calcium/Potassium kit (Quantofix, GmbH, Wiesbaden, Germany). Similarly control was also tested without adding CFS. Results were expressed as the amount of extracellular free potassium (mMol/L) in the growth media in each interval of incubation.

Determination of effect of CFS on release of 260-nm absorbing materials

The measurement of the release of 260-nm-absorbing materials from *S. aureus* KCTC-1621 and *E. coli* O157:H7 cells was carried out in aliquots of 2 mL of the bacterial inocula in sterile peptone water (0.1 g/100 mL). The reaction solution containing MIC of CFS of *P. pentosaceus* 4I1 was incubated at 37°C. At 0, 30 and 60 min time interval of treatment, cells were centrifuged at 3,500×g, and the absorbance of the obtained supernatant was measured at 260 nm using a 96-well plate ELISA reader (Bajpai *et al*., 2013). Similarly control was also tested without adding CFS of *P. pentosaceus* 4I1. Results were expressed in terms of optical density (OD) of 260-nm absorbing materials in each interval with respect to the ultimate time.

Determination of effect of CFS on cell membrane permeability

Effect of CFS of *P. pentosaceus* 4I1 on cell membrane permeability of selected pathogens *S. aureus* KCTC-1621 and *E. coli* O157:H7 was determined as described previously (Patra *et al*., 2015), and expressed in terms of relative electrical conductivity. Prior to the assay, cultures of test pathogens were incubated at 37°C for 10 h, followed by centrifugation (5,000×g) for 10 min, and

washed with 5% glucose solution (w/v) until their electrical conductivities reached close to 5% glucose solution to induce an isotonic condition. MICs of CFS of *P. pentosaceus* 4I1 acquired for both the test pathogens were added to 5% glucose (isotonic solution), incubated at 37°C for 8 h, and the electrical conductivities (L_a) of the reaction mixtures were determined. Further, electrical conductivities of the bacterial solutions were measured at 2 h of intervals for a total duration of 8 h (L_b). The electrical conductivity of each test pathogen in isotonic solution killed by boiling water for 5 min served as a control (L_c). The relative electrical conductivity was measured using an electrical conductivity meter. The permeability of bacterial membrane was calculated according to the following formula:

$$\text{Relative conductivity (\%)} = L_a - L_b / L_c \times 100.$$

Statistical analysis

All data are expressed as the mean ± SD by measuring three independent replicates. Analysis of variance using one-way ANOVA followed by Duncan's multiple test.

RESULTS

Morphological, biochemical and molecular characterization of 4I1

Small yellow colonies of similar sizes that appeared on BCP agar using pour-plating method confirmed the presence of the LAB isolate *P. pentosaceus* 4I1 which was further confirmed as rod-shaped under microscopic evaluation. Biochemical analysis of 4I1 was done using the API 50 CHL strip kit and selected strain was identified as a gram-positive and rod-shaped isolate (Table 1). The API web software confirmed that strain 4I1 showed utilization of carbohydrates that

included L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannitol, D-sorbitol, N-acetylglucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-raffinose, gentiobiose, and D-turanose (Table 1). Color change from violet to yellow in the strip capsule indicated complete fermentation of sugar by 4I1. Further, on the basis of molecular analysis using 16S rDNA gene sequencing, the selected strain showed 99.9% similarity with different *Pediococcus* spp. (Figure 1), thus, the strain was finally confirmed as *Pediococcus pentosaceus* 4I1. The sequence was submitted to GenBank with nucleotide accession number KT372700.

Antibacterial activity

The antibacterial activity of CFS of *P. pentosaceus* 4I1 against the tested foodborne pathogenic bacteria was determined by the presence or absence of inhibition zones. As presented in Figure 2, the CFS of *P. pentosaceus* 4I1 exhibited potent inhibitory effect against the tested foodborne pathogenic bacteria. In this assay, it was found that of *P. pentosaceus* 4I1 exerted consistent antibacterial effect against both gram-positive and gram-negative bacteria, and their zones of inhibitions were found in the range of 16.5 to 20.4 mm (Figure 2). Sterilized distilled water and/or MRS medium used as a negative control had no inhibitory effect.

Minimum inhibitory and minimum bactericidal concentrations

This assay revealed different susceptibilities of test pathogens to the CFS of *P. pentosaceus* 4I1, and exhibited potent inhibitory effect as the MIC and MBC values. In this assay, the MIC and MBC values of 4I1 CFS against the tested foodborne pathogens were found in the range of 250 to 1,000 µg/mL (Figure 3). The CFS of 4I1 exhibited potential antibacterial effect as the MIC and

MBC values against all the test pathogens (Figure 3). Interestingly, one of the pathogens *S. enterica* ATCC-4731 was found to be the highest susceptible pathogen to the CFS of *P. pentosaceus* 4I1 in this assay. It was noted that both gram-positive and gram-negative bacteria were inhibited by the CFS of *P. pentosaceus* 4I1.

Effect on cell viability

In this assay, the effect of CFS of *P. pentosaceus* 4I1 on the growth of tested bacterial pathogens *S. aureus* KCTC-1621 and *E. coli* O157:H7 demonstrated reduced viability at the used concentration (Figure 4). Exposure of 0 to 80 min of CFS of *P. pentosaceus* 4I1 did not exhibit severe inhibitory effect on viable cell counts of test pathogens, however, remarkable decline in CFU counts of *S. aureus* KCTC-1621 and *E. coli* O157:H7 was observed when exposed to the CFS of *P. pentosaceus* 4I1. Interestingly, the exposure of CFS of *P. pentosaceus* 4I1 for 200 min exhibited complete inhibition of CFU of both the test pathogens *S. aureus* KCTC-1621 (Figure 4A) and *E. coli* O157:H7 (Figure 4B).

Effect on potassium ion leakage

This test assay confirmed the antibacterial effect of the CFS of *P. pentosaceus* 4I1 as revealed by the release of potassium ions from the treated cells of *S. aureus* KCTC-1621 and *E. coli* O157:H7 when compared with control (Figure 5). The MIC of CFS of *P. pentosaceus* 4I1 when added to bacterial cell suspension resulted in the marked release of potassium ions from the bacterial cells following a sturdy loss along the specified time period (Figure 5A & 5B). However, no leakage of potassium ion was observed in control cells of *S. aureus* KCTC-1621 and *E. coli* O157:H7 during the study (Figure 5).

301

302 **Effect on release of 260 nm materials**

303 Release of 260-nm absorbing materials (DNA and RNA) from the cells treated with specific
304 antimicrobials could be an indication of bacterial cell death. Hence, this study evaluated the effect
305 of the CFS of *P. pentosaceus* 4I1 on the release of 260-nm absorbing materials from the bacterial
306 cells of *S. aureus* KCTC-1621 and *E. coli* O157:H7 treated at MIC. Interestingly, exposure of the
307 CFS of *P. pentosaceus* 4I1 to *S. aureus* KCTC-1621 and *E. coli* O157:H7 caused rapid loss of 260-
308 nm absorbing materials from the bacterial cells. The optical density of the culture filtrates of *S.*
309 *aureus* KCTC-1621 and *E. coli* O157:H7 cells exposed to the CFS of *P. pentosaceus* 4I1, and
310 measured at 260 nm, revealed a significant increase in the release of 260-nm-absorbing materials
311 with respect to exposure time (Figure 6). However, no changes in the optical density of control
312 cells of test pathogens were observed during the study. As a result, 60 minute exposure of the CFS
313 of *P. pentosaceus* 4I1 caused about two-fold increase in the optical density of treated bacterial cell
314 culture filtrates when compared with their respective controls (Figure 6A & 6B).

315

316 **Effect on cell membrane permeability**

317 This assay visualized the effect on membrane permeability of tested bacteria in terms of their
318 relative electrical conductivities when exposed to the CFS of *P. pentosaceus* 4I1 at MIC. As a
319 result, the CFS of *P. pentosaceus* 4I1 exhibited time-dependent inhibitory effect on membrane
320 permeability of the tested pathogens, and the relative electrical conductivity of each test pathogen
321 was increased timely. However, the CFS of *P. pentosaceus* 4I1 exhibited higher inhibitory effect
322 on cell membrane of *S. aureus* KCTC-1621 (Figure 7A) than *E. coli* O157:H7 (Figure 7B) as
323 confirmed by their relative electrical conductivity values (Figure 7). No relative electrical

conductivity was observed in untreated samples as a control. In this assay, the CFS of *P. pentosaceus* 4I1 showed an ability to disrupt the plasma membrane as confirmed by the changes observed in the relative electrical conductivity of both the tested bacteria.

DISCUSSION

In this study, morphological identification of LAB isolate 4I1 was carried out as per the schemes outlined in the Bergey's manual of Systematic Bacteriology (Holt *et al.,* 1994). Small yellow colonies of similar sizes that appeared on BCP agar using pour-plating method confirmed the presence of *Lactobacillus* strain 4I1 as also reported previously (Bajpai *et al*., 2016). The efficiency of detection of other Bifidobacterium such as *B. infantis* on BCP was very low, while *B. bifidum* did not grow on BCP even under anaerobic conditions (Ashraf and Shah, 2011). Although plate count agar with Bromocresol purple is a recommended medium for enumeration of LAB from a variety of samples, it does not support the differentiation of each LAB in a mixed culture. It is known that BCP agar also prevents formation of colonies by concomitant bacteria and hence widely considered specific medium for the selective enumeration of LAB as also reported by others (Bielecka et al., 2000). Kunene *et al.* (2000) identified several *Lactobacillus* strains at morphological and biochemical levels, which were characterized based on the following features such as rod-shaped cell morphology, production of DL-lactate, fermentation of ribose, and an inability to utilize arginine. Similar findings were also observed in our study, thus confirming the biochemical identification of 4I1. Recently Casaburi *et al.* (2016) phenotypically and biochemically identified *Lactobacillus* species with the use of the API 50 CHL kit isolated from fermented sausage. Jini *et al.* (2011) also isolated two potential isolates of LAB such as *E. faecalis*

347 and *P. acidilactici* from fresh water fish microbiota having anti-pathogenic effect against human
348 pathogens. Zapata (2013) studied the intestinal microflora of *Oreochromis niloticus* fish to isolate
349 and identify LAB as new probiotics. Molecular methods are important for bacterial identification,
350 and possibly more accurate for LAB than conventional phenotypic methods. Hence, 4I1 was
351 further identified as *P. pentosaceus* at the molecular level using 16S rDNA gene sequencing as
352 also reported previously (Bajpai *et al*., 2016).
353
354 Further, LAB isolate 4I1 exhibited antibacterial effect against various pathogenic bacteria
355 as confirmed by the inhibitory zones in agar plates. Similarly, LAB isolated from Ethiopian
356 traditional fermented milk "Ergo" were found to exhibit antimicrobial activity against a panel of
357 pathogenic bacteria (Amenu 2013). In addition, LAB isolated from fresh vegetables have shown a
358 profound antimicrobial effect against foodborne pathogenic bacteria (Darsanaki *et al*. 2012;
359 Tadesse *et al*., 2005). To support these findings, thermophilic LAB isolated from hot spring water
360 in Indonesia were found to exhibit a remarkable antibacterial effect against foodborne pathogenic
361 bacteria with a considerable amount of MIC and MBC values (Carina Audisio *et al*., 2011; Yah *et*
362 *al*. 2014). In further assays, randomly selected two pathogens *S. aureus* KCTC-1621 (gram-
363 positive) and *E. coli* O157:H7 (gram-negative) were used to evaluate the possible antibacterial
364 mode of action of CFS of *P. pentosaceus* 4I1 in various *in vitro* assays.
365 This study also revealed the inhibitory effect of the CFS of 4I1 against *S. aureus* KCTC-
366 1621 and *E. coli* O157:H7 and reduced viable counts when exposed to CFS of 4I1. Previous reports
367 have confirmed inhibitory effects of various LAB strains on the cell viability of foodborne
368 pathogenic bacteria (de Barros *et al*., 2012). Similar to our findings, a lactic acid bacterium *L.*
369 *johnsonii* CRL1647 significantly reduced the cell count numbers of *L. monocytogenes in vitro*

(Carina Audisio *et al*., 2011).

In addition, the plasma membrane of bacteria provides a permeability barrier to the passage of small ions including potassium ions which are necessary electrolytes, facilitating cell membrane functions and maintaining proper enzyme activity. This impermeability to small ions is regulated by the structural and chemical composition of the membrane itself. Increases in the leakage of potassium ions could be an indication of disruption of this permeability barrier. Maintaining ion homeostasis is integral to the maintenance of the energy status of the cell, including solute transportation, regulation of metabolism, control of turgor pressure and motility (Cox *et al*., 2001). Therefore, even relatively slight changes to the structural integrity of cell membranes can severely affect cell metabolism and lead to cell death, and potassium ion efflux (Cox *et al*., 2001). We previously confirmed the inhibitory effect in terms of release of potassium ions from the bacterial cells when treated with specific antimicrobial substances such as *Cudrania tricuspidata* fruit derived essential oil (Bajpai *et al*., 2013).

This study also exhibited the remarkable effect of the CFS of *P. pentosaceus* 4I1 on the release of 260 nm materials (DNA and RNA) from the cells of tested bacteria when exposed to MIC concentration, which confirmed its role as a potent antibacterial agent. The observation that the amount of loss of 260-nm-absorbing materials was as extensive as the leakage of potassium ions might indicate that the membrane structural damage sustained by the bacterial cells resulted in release of macromolecular cytosolic constituents (Farag *et al*., 1989). This suggested that monitoring of release of 260-nm absorbing materials from *S. aureus* KCTC-1621 and *S. enterica* ATCC-4731 might be more sensitive indicators of membrane damage and loss of membrane integrity. Similarly, Alakomi *et al* (2000) reported the effect of lactic acid produced by lactic acid bacteria on the release of nucleic acid (DNA and RNA) and other cell electrolytes and confirmed

its inhibitory effect on membrane permeabilization against various foodborne pathogens.

Maintaining membrane permeable integrity is essential for the overall metabolism of a bacterial cell, hence, changes in the relative electrical conductivity on membrane integrity may severely hamper the cell metabolism, which may eventually lead to cell death (Cox *et al*., 2001). Based on the findings of this study, we hypothesized that CFS of *P. pentosaceus* 4I1 severely hampered the plasma membrane of tested foodborne pathogens which resulted in the instant loss of the cell integrity and became increasingly more permeable to ions and other essential metabolites that might be the reason for the establishment of the antibacterial activity of the CFS of *P. pentosaceus* 4I1. These findings were in strong agreement of Roth and Keenan (1971) who reported that lactic acid bacteria were able to cause sub-lethal injury to a foodborne pathogen *E. coli*, and similar properties have also been assigned to acetic acid (Przybylski and Witter, 1979) indirect evidence inferred that such injury involved disruption of the lipopolysaccharide layer. Similarly, antimicrobials of other microbial origins have shown remarkable effect on relative electrical conductivity parameters of foodborne pathogens (Patra *et al*., 2015).

Moreover, the antimicrobial potential of *Lactobacilli* could be attributed to several factors, including production of acids, hydrogen peroxide and bacteriocins (Anas et al., 2008). A number of reports have confirmed that strains of *Lactobacilli* were not able to regenerate hydrogen peroxide in anaerobic condition and no effect of acidity has been observed on the antimicrobial activity of *Lactobacilli* strains (Julliard *et al*., 1987)). However, as reported previously, most of the *Lactobacilli* strains have been found to produce bacteriocins or bacteriocin-like substances which have a large antimicrobial spectrum (Klaenhammer, 1988; Piard *et al*., 1991; Nettles and Barefoot, 1993). Also, in our preliminary screening, we treated the CFS of 4I1 by the proteolytic enzyme trypsin, and it was found that CFS of 4I1 after treatment lost its activity which confirmed

that the antimicrobial activity of 4I1 could be due to the production of proteinous substances such as bacteriocin-like substances (data not shown). Other reports have also confirmed that when treated the supernatants derived from *Lactobacilli* strains by proteolytic enzymes (trypsin and α–chymotrypsin) lost their activity indicating that the active components secreted extracellularly could be proteinaceous in nature, thus confirming that inhibitory effect of *Lactobacilli* strains could be bacteriocin-mediated (Ghrairi *et al*., 2008). Based on the above literature survey, we hypothesized that the antimicrobial activity of 4I1 observed in this study could be mediated through the production of bacteriocin-like substances by 4I1. However, the synergistic effect of acids, hydrogen peroxide and bacteriocin-like substances cannot be ruled out (Anas *et al*., 2008).

CONCLUSION

This study confirmed that newly identified lactic acid bacterium *P. pentosaceus* 4I1, and first time isolated from intestinal microbiota of fresh water fish sample *Zacco koreanus* exhibited significant inhibitory effect against foodborne pathogenic bacteria in various *in vitro* models affecting different membrane permeability parameters. The CFU of *P. pentosaceus* 4I1 caused a significant reduction in the number of viable bacteria, loss of 260-nm absorbing materials, and leakage of potassium ions with dramatical increase in relative electrical conductivity of test pathogens being indicative of loss of membrane integrity. These findings reinforce the suggestions that *P. pentosaceus* 4I1 exhibiting significant antibacterial activity, can be used as an effective antimicrobial agent in the food industry to control and/or to retard the growth of foodborne pathogens.

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554 **Table 1** Biochemical characterization of *P. pentosaceus* (4I1) based on carbohydrate interpretation

555 using API 50 CHL kit.

Active ingredient	Result	Active ingredient	Result
Glycerol	-	Salicin	+
Erythritol	-	D-cellobiose	+
D-arabinose	-	D-maltose	+
L-arabinose	+	D-lactose (bovine origin)	+
D-ribose	+	D-melibiose	+
D-xylose	+	D-saccharose	+
L-xylose	-	D-trehalose	+
D-adonitol	-	Inulin	-
Methyl-β-D-xylopyranoside	-	D-melezitose	-
D-galactose	+	D-raffinose	+
D-glucose	+	Amidon (starch)	-
D-fructose	+	Glycogen	-
D-mannose	-	Xylitol	-
L-sorbose	-	Gentiobiose	+
L-rhamnose	-	D-turanose	+
Dulcitol	-	D-lyxose	-
Inositol	-	D-tagatose	-
D-mannitol	+	D-fucose	-
D-sorbitol	-	L-fucose	-
Methyl-α-D-glucopyranoside	-	D-arabitol	-
N-acetylglucosamine	+	Potassium gluconate	-
Amygdalin	+	Potassium 2-ketogluconate	-
Arbutin	+	Potassium 5-ketogluconate	-
Esculin	-		

556 (-): The bacterium does not use this carbohydrate; (+): The bacterium uses this carbohydrate.

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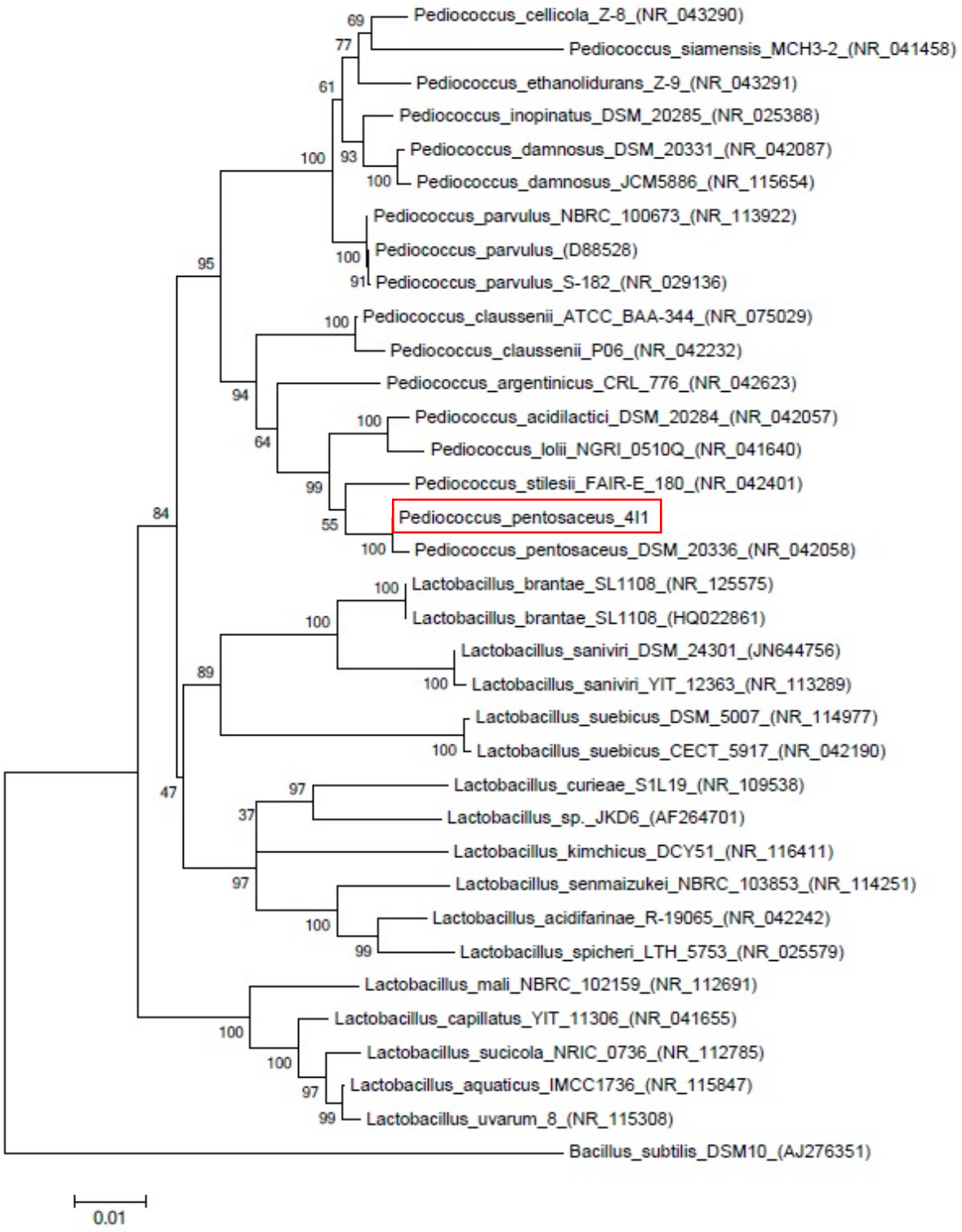

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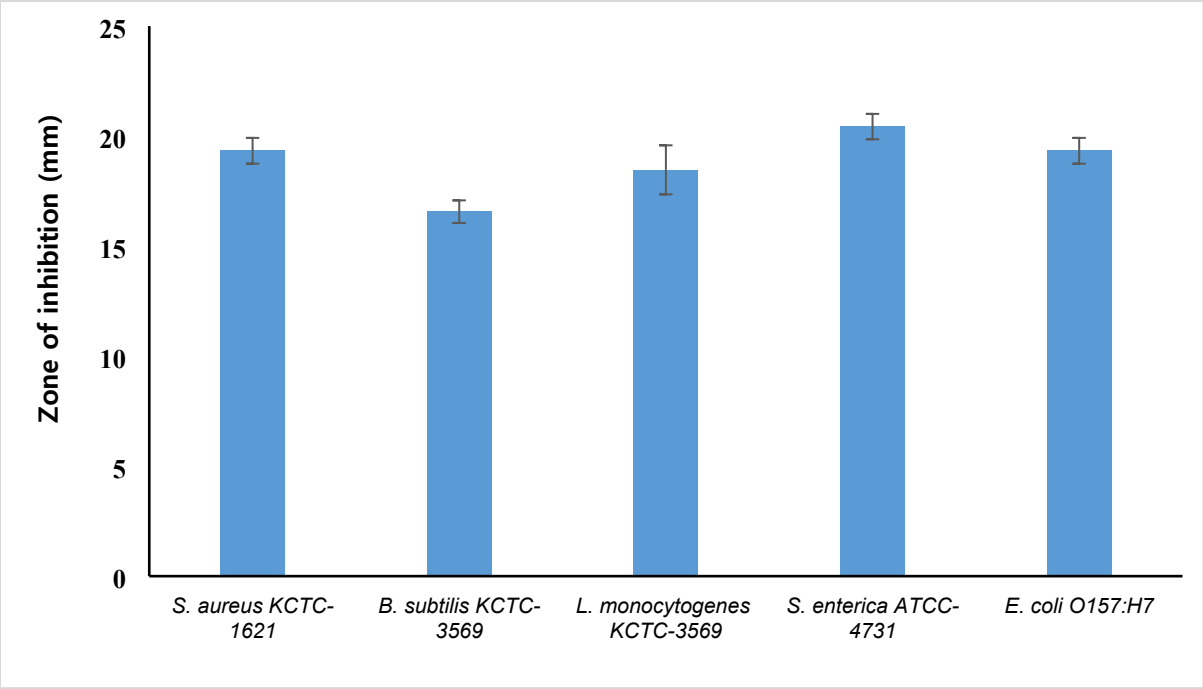
560 **Figure 1** Neighbor-joining phylogenetic tree showing the position of strain *P. pentosaceus* 4I1

561 among the different *Pediococcus* strains based on 16s rDNA sequences.



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563 **Figure 2** Antibacterial activity of cell free supernatant of *P. pentosaceus* 4I1 against foodborne
564 pathogenic bacterial in agar well diffusion assay. Data are expressed as mean ± SD (n = 3).

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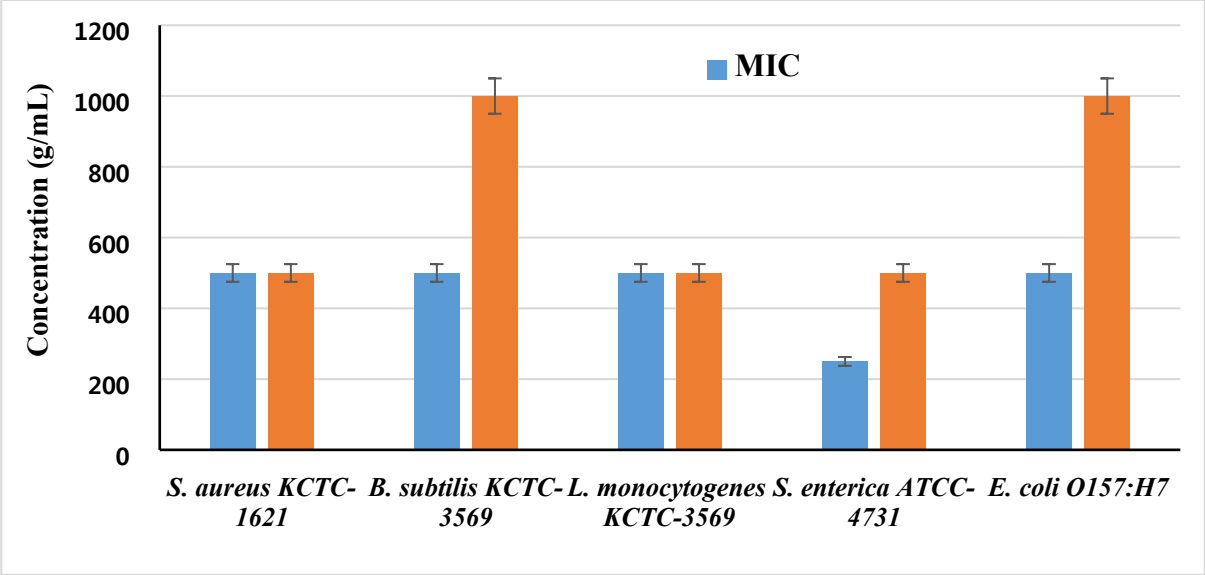

576

577 **Figure 3** Determination of minimum inhibitory (MIC) and minimum bactericidal concentration
578 (MBC) concentrations of cell free supernatant (CFS) of *P. pentosaceus* 4I1 against foodborne
579 pathogenic bacteria. Data are expressed as mean ± SD (n = 3).

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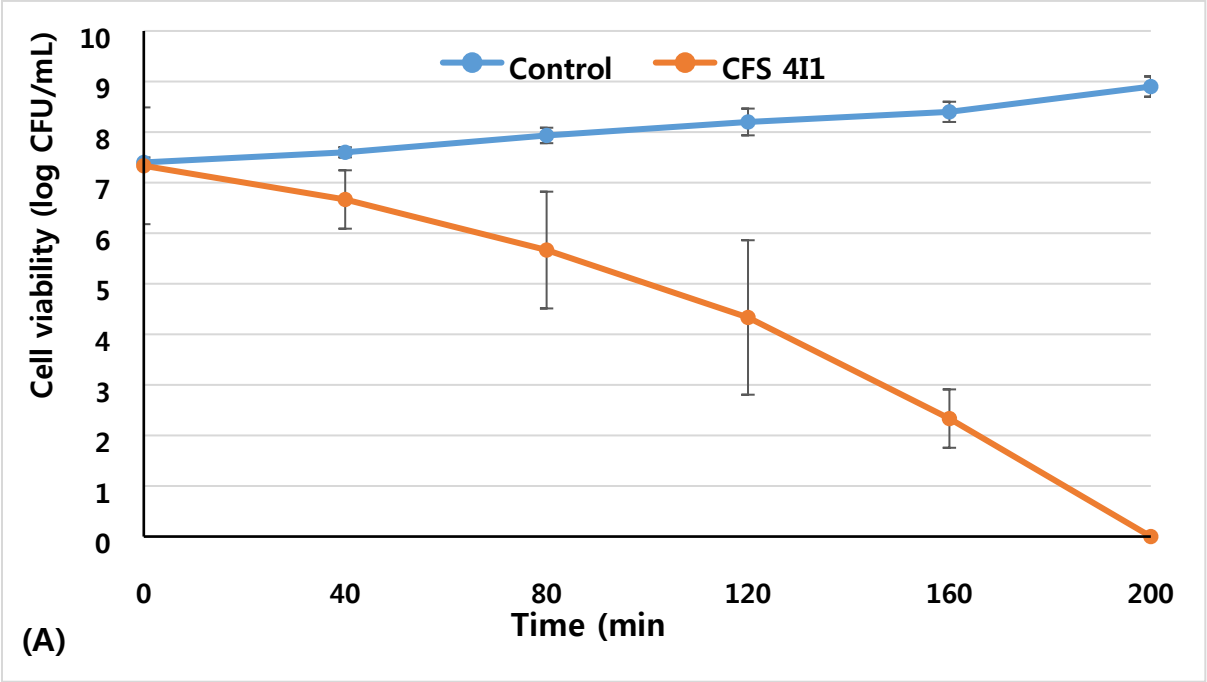

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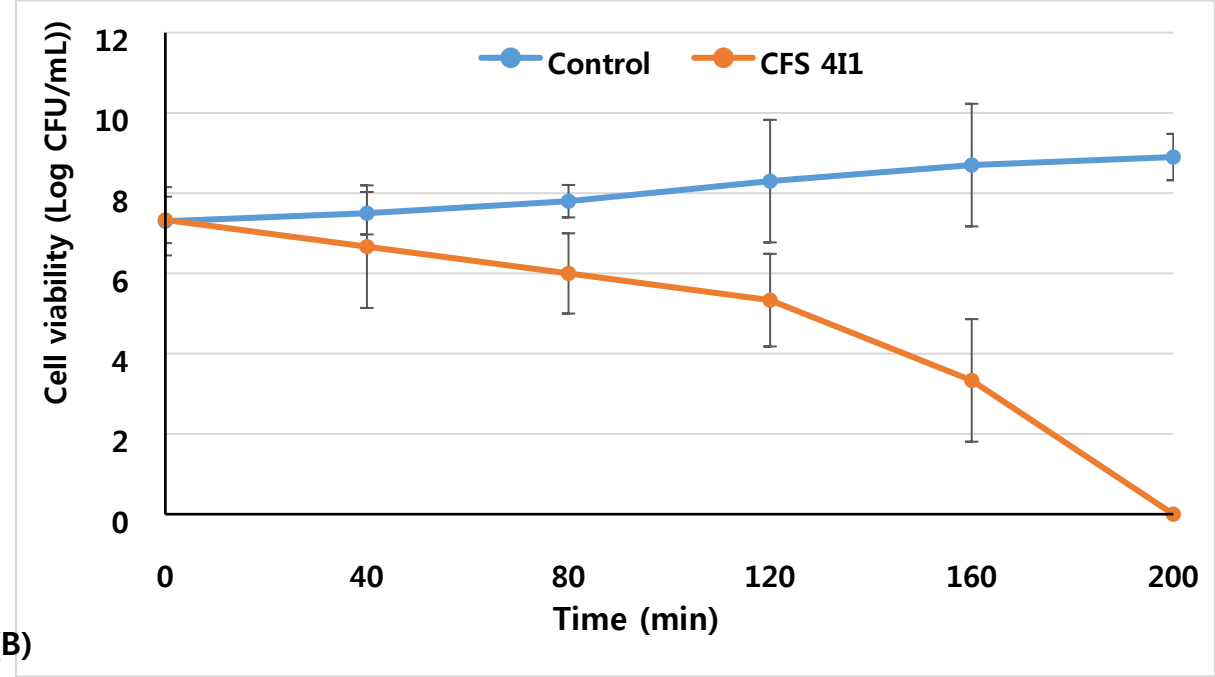
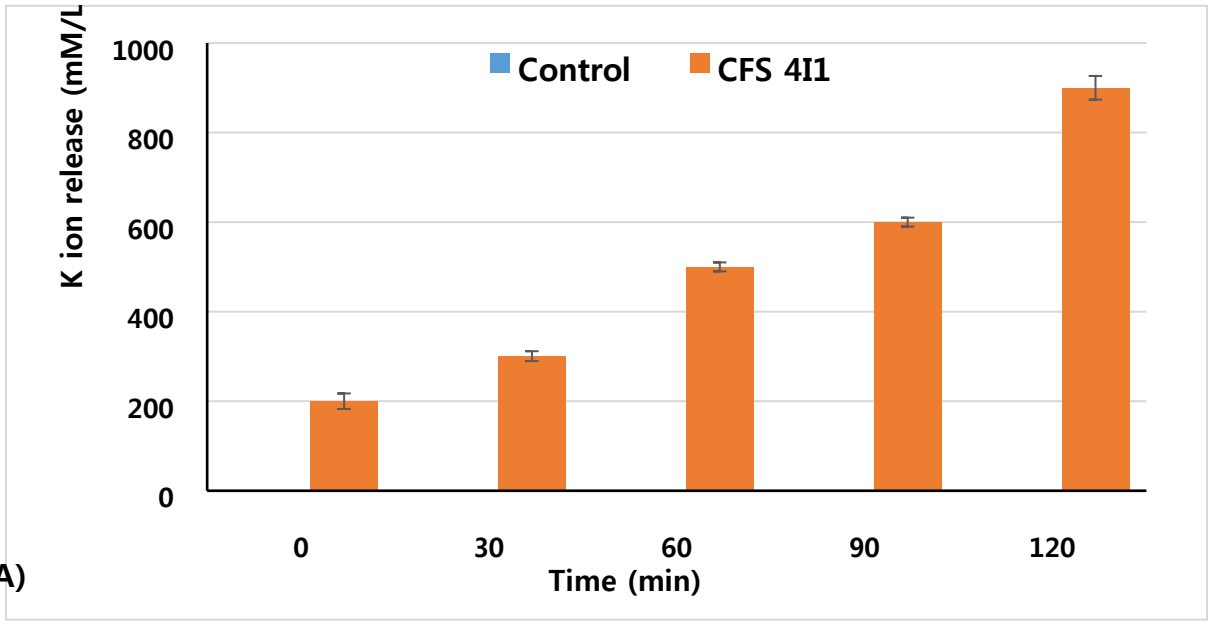


591 **Figure 4** Effect of cell free supernatant (CFS) of *P. pentosaceus* 4I1 on the viability of

592 the tested pathogenic bacteria of *S. aureus* KCTC-1621 (A) and *E. coli* O157:H7 (B). Control

593 without treatment. Data are expressed as mean ± SD (n = 3).



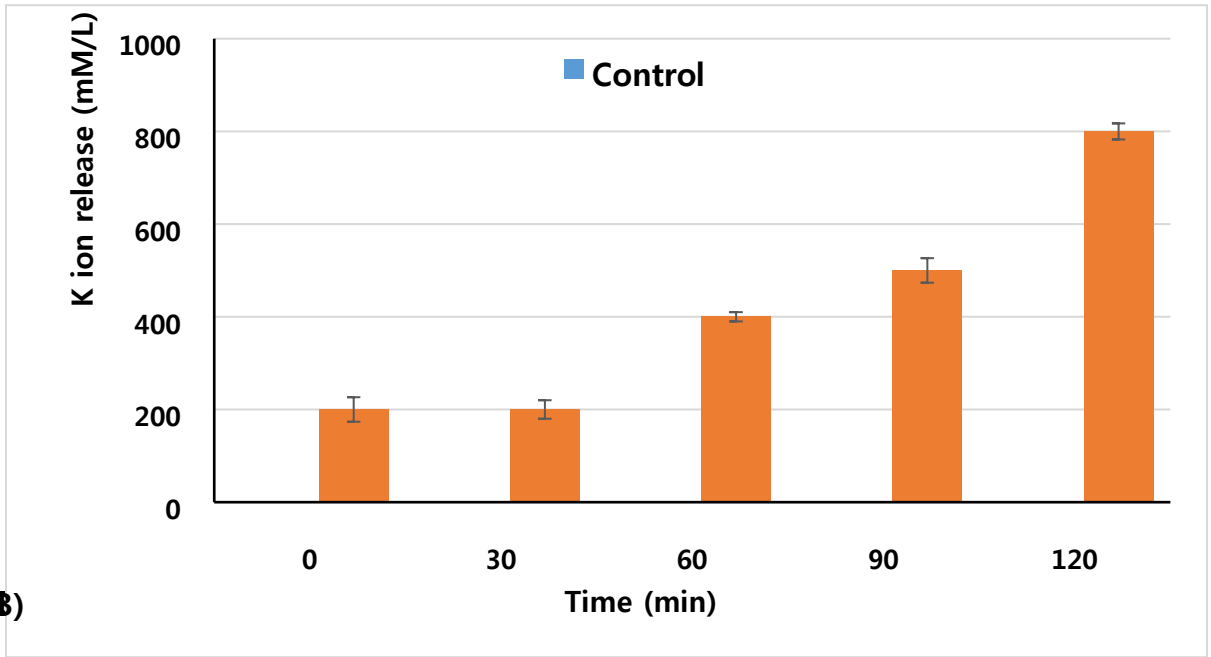


Figure 5 Effect of cell free supernatant (CFS) of *P. pentosaceus* 4I1 on leakage of potassium ions from the tested pathogenic bacteria of *S. aureus* KCTC-1621 (A) and *E. coli* O157:H7 (B). Control without treatment. Data are expressed as mean ± SD (n = 3).

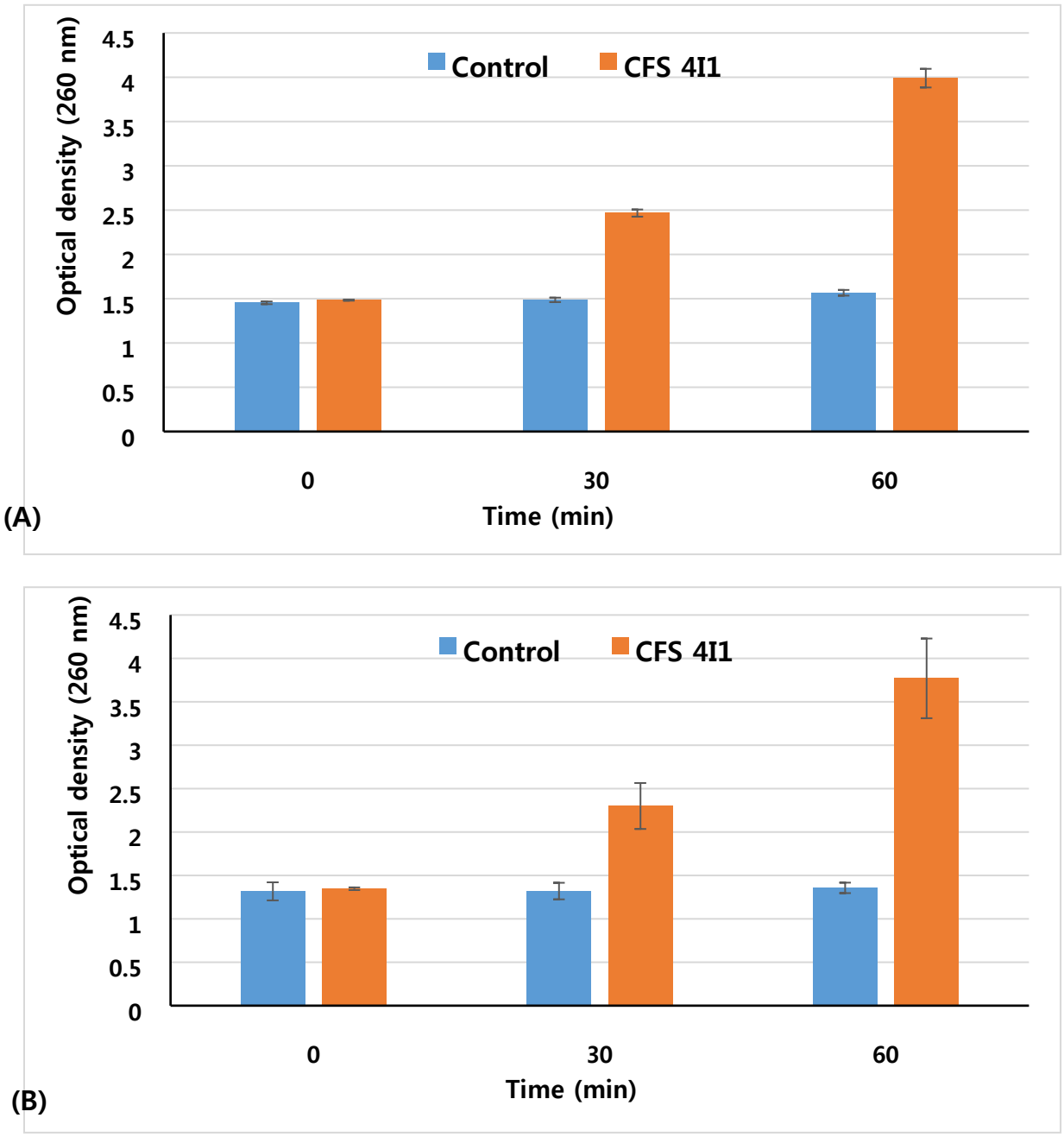
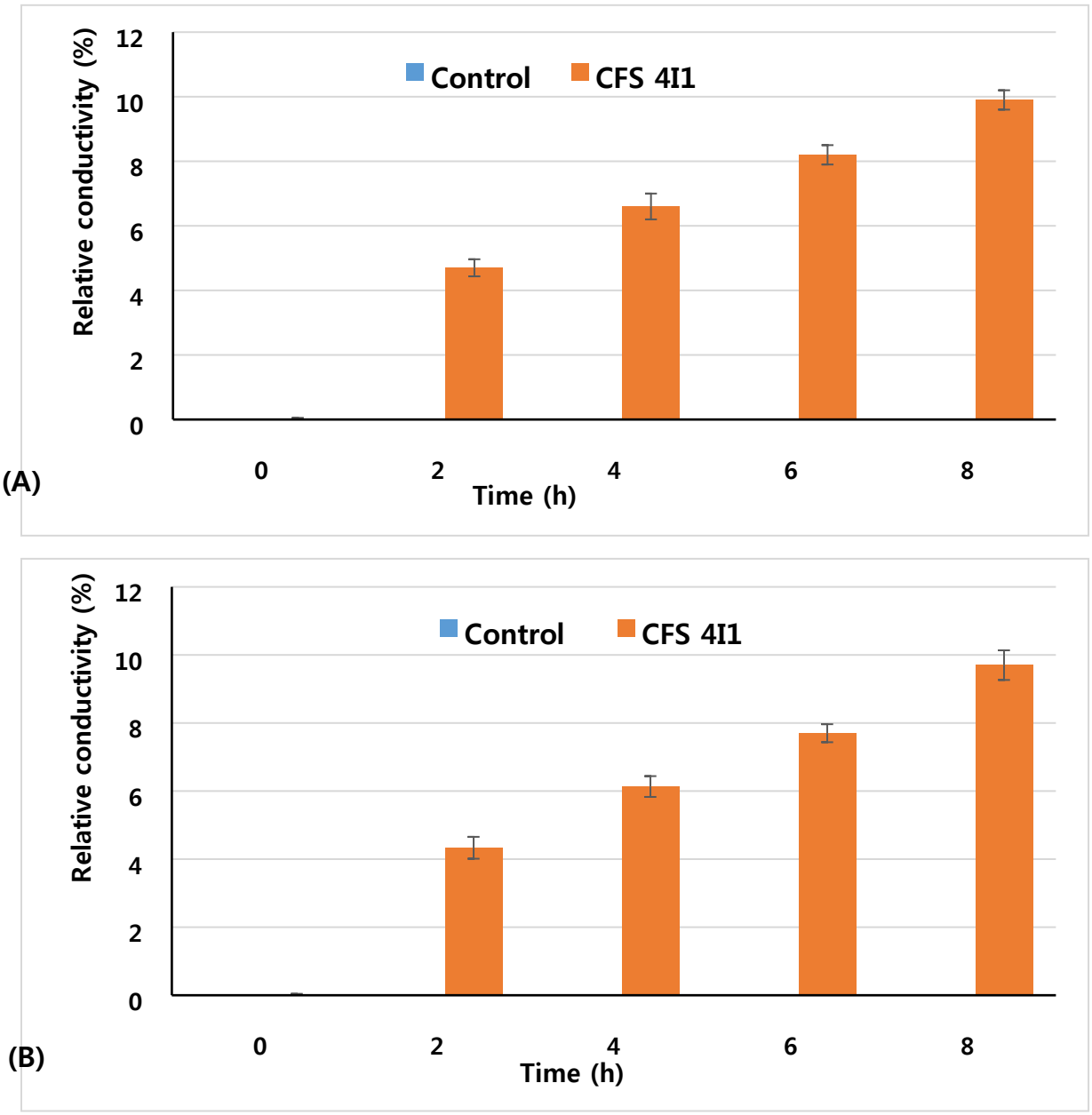


Figure 6 Effect of cell free supernatant (CFS) of *P. pentosaceus* 4I1 on the release rate of 260-nm absorbing material from *S. aureus* KCTC-1621 (A) and *E. coli* O157:H7 (B). Data are expressed as mean ± SD (n = 3).



604

605 **Figure 7** Effect of cell free supernatant (CFS) of *P. pentosaceus* 4I1 on membrane permeability

606 of *S. aureus* KCTC-1621 (A) and *E. coli* O157:H7 (B). Data are expressed as mean ± SD (n = 3).

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