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Full Length Article

Production of Antifungal Gelatinase by *Enterococcus durans* S2C and its Potential Role in the Biological Control

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Abstract

Probiotic bacteria are good sources for antimicrobial and are normal microbiota of the gastrointestinal tract of animals and humans. Enterococci are found everywhere in nature and have been utilized as probiotics in the food industry. In the present study, *Enterococcus durans* S2C strain was isolated from raw cow's milk using the culture-dependent method and evaluated for antifungal properties. The strain S2C showed promising extracellular proteolytic activity and the extracellular peptide was an important source of antifungal activity. Besides, a low rate of antibiotic resistance and non-hemolytic activity was detected in *E. durans* S2C. Strong antifungal activity of *E. durans* against two plant pathogenic fungi namely *Fusarium oxysporum* and *Rhizoctonia solani* was also recorded. Antifungal activity evaluation showed that *R. solani* colony was the most inhibited fungus followed by *F. oxysporum*. Gelatinase enzyme having antifungal activity was also purified from E. durans S2C isolate. This study concludes that the environmental-derived *E. durans* S2C strain can be selected for technological application to control pathogenic fungi and to protect the economic value of the crops. © 2021 Friends Science Publishers

Keywords: Antifungal activity; Antibiotic resistance pattern; *Enterococcus durans*; Gelatinase; Raw milk

Introduction

Serious economic losses are incurred annually because of disease-causing microbial agents. Among these microbial agents, fungal plant pathogens are the most important ones (Almeida *et al.* 2019). The genus *Fusarium* alone has been known to infect over 100 host organisms. This has resulted in significant losses of a wide variety of crops including cotton, banana, tomato, onion, and melon (Michielse and Rep 2009; Jamal *et al.* 2015; Akhtar and Javaid 2018). *Fusarium oxysporum* invades host plants through their roots or stems and propagates within their vascular system. This leads to wilting and eventually death of the plant. This becomes a serious issue because of its persistence in the soil, which makes it difficult to eradicate (Sun *et al.* 2017. Similarly, *Rhizoctonia solani* has been reported to affect a wide range of hosts such as grasses, potatoes, and sugar beets. Its infection results in seed and fruit decay, foliage diseases, damping-off, and stem cankers (Xia *et al.* 2017). An issue of using chemicals to control these infections is that the substances tend to bio-accumulate in the plants and then move up the food chain, which can have disastrous consequences (Özkara *et al.* 2016). Along with this issue, the rising in fungal resistance against the most common chemical agents and a push in the market for more fungicide-free fruits and vegetables have begun driving researchers to look for alternative solutions for fungal control. The desire, which has seen substantial growth in the past decades, prompted a search for microbial products to control plant maladies. Use of biocontrol to inhibit the growth of pathogenic microorganisms has long been considered a potential alternative to chemical fungicides (Carmona-Hernandez *et al.* 2019; Ali *et al.* 2020; Sharf *et al.* 2021).

Lactic acid bacteria (LAB) are known to produce lactic acid as a major product by the carbohydrate metabolism of food. Other properties of this group include a Gram-positive nature, catalase-negative, immobility, and lack of spore formation. They have generally been recognized as safe status (Fhoula *et al.* 2013). Thriving in carbohydrate-rich environments, they are commonly found in milk and meat along with plants, animals, and the intestinal mucosa of humans (Bintsis 2018). They are particularly prolific in various fermented foods (Tamang *et al.* 2020). Owing to their ability to prevent the growth of entero-pathogenic bacteria and promote health, they have seen use as probiotics. LABs have found many industrial applications owing to their distinctive properties, such as their ability to produce organic acids, exopolysaccharides, aromatic and antimicrobial compounds. Numerous researchers have reported the ability of certain LAB strains to suppress food-borne pathogens like *Escherichia coli* and *Salmonella typhimurium* (Darsanaki *et al.* 2012), as well as

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phytopathogenic bacteria such as *Erwinia carotovora* and fungi including *Aspergillus flavus*, *F. graminarum*, and *Penicillium expansum* (Daranas *et al.* 2019).

The genus *Enterococcus* is a member of LAB and widespread in nature. They are part of the gastrointestinal flora and are detected in samples from all over the animal kingdom, insects, lower vertebrates, and humans (David *et al.* 2012; David and Onifade 2018). They produce small, extracellular metabolites that have been reported against food-borne pathogens and, more recently, against plant pathogens (Belguesmia *et al.* 2013). In this study, we evaluated the anti-fungal ability of an Enterococcus species. Some additional properties of this species regarding safety aspects were also investigated in this study.

Materials and Methods

Isolation of bacterial and fungal species

Eight raw milk samples were randomly obtained from apparently healthy cows owned by locals from different localities in Islamabad, Pakistan. The milk samples were collected in sterile 50 mL tubes stored in an icebox and transferred to the lab within 24 h. From raw milk samples, the lactic acid bacteria (LAB) were isolated on de Man, Rogosa, and Sharpe (MRS) agar using the spread-plate method. Inoculated plates were incubated at 37°C for 48 h. After incubation, the bacterial colonies were purified to investigate for LAB characteristics (Gram-positive, catalase-negative, and oxidase-negative). Isolates with Gram-positive and catalase-negative characters were considered for further identification.

For the antifungal assay, two plant pathogen isolates *i.e.*, *F. oxysporum* and *R. solani* were collected from Applied Microbiology and Biotechnology lab, COMSATS University Islamabad Pakistan, and maintained on 2% Potato Dextrose Agar (PDA) for further use.

Molecular characterization and selection of bacterial isolates

Genomic DNA was extracted from fifteen LAB isolates using phenol/chloroform method with some modifications (Cheng and Jiang 2006). The 16S rRNA gene was amplified using primers P1 "CGGGATCCAGAGTTTGATCCTGGTCAGAACGAAC GCT" and P6 "CGGGATCCTACGGCTACCTTGTTACGACTTCACC CC" (Tan *et al.* 1997). An aliquot of 25 μL reaction mixture consisting of 1.5 m*M* $MgCl_2$, 1X Taq buffer, 10 m*M* dNTPS, 1–1.5 U Taq DNA polymerase and 10 μM primer was subjected to amplification (Applied Biosystems). The cycling conditions for PCR were as follows: initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and final extension at 72°C for 4 min. Amplified product were sent for sequencing.

Obtained sequences were aligned using chromas software version 2.6.6 with default parameters and BLAST searched to find their pairwise identities on NCBI database. Similar sequences were aligned using Clustal W and phylogenetic tree was constructed using the neighbor-joining method in Mega software. On the basis of molecular identification, only two isolates were identified as *Enterococcus durans*. Due 16S rRNA gene similarity, only *E. durans* S2C was used in further biochemical tests and pathogenicity assay. The fresh culture was maintained in Tryptic Soy Broth (TSB), at 37°C for 24 h and stored at -80°C as frozen stock culture in TSB containing 20% (v/v) glycerol.

Biochemical characteristics of the *enterococci*

Detection of bio-film formation: To assess *Enterococci* virulence, biofilm formation test was also conducted using tube method (Deka 2014). A loopful inoculum of *E. durans* S2C was taken from overnight grown culture in nutrient agar and was inoculated into 10 mL of TSB broth containing 1% glucose. After incubation at 37°C for 24 h, the cultures were decanted, and tubes were washed with Phosphate buffer saline (at pH of 7.3) followed by drying and stained with 0.1% crystal violet. The de-ionized water was used to wash out the excess stain and the tubes were dried in an inverted position to observe bio-film formation. Results were considered positive if a visible film lined produced in the base and wall of the tube. The experiment was performed in triplicate and repeated three times.

Hemolytic and gelatinase hydrolysis test

To understand the virulence potential of dairy enterococci, *E. durans* S2C strain was also tested for hemolytic and gelatinase activities. For hemolytic activity, commercial blood agar plates were inoculated with fresh culture of S2C strain and incubated at 37°C under anaerobic conditions (Valenzuela *et al*. 2009). Hemolytic reaction was recorded after 24–48 h and the test was conducted in triplicate.

The gelatinase activity of the *E. durans* S2C strain was evaluated by tube method. A 12 h pure culture of cells was inoculated into tubes containing 5 mL of growth medium [0.5% Bacto Peptone, 0.25% yeast extract, 0.5% glucose, 0.1% $MgSO_4.7H_2O$, and 0.02 *M* phosphate buffer with 12% gelatin (pH 7)]. Gelatinase activity was discovered as medium liquefaction at room temperature for 1 week of incubation.

Assessment of antibiotic susceptibility of *E. durans*

Disk diffusion method according to Clinical Laboratory Standards Institute guidelines (CLSI, 2017), was used to determine antibiotic resistance in *E. durans* S2C and the

following eight antibiotic discs (Oxoid) were tested: cholorompheniсol (30 μg disc^{-1}), tetracycline (30 μg/disc), vancomycin (30 μg disc^{-1}), streptomycin (10 μg disc^{-1}), piperacillin (100 μg disc^{-1}), levofloxacin (5 μg/disc), minocycline (30 μg/disc) and amoxicillin (10 μg disc^{-1}). Based on the measured inhibition zones, the strains were categorized as susceptible, intermediate, or resistant according to the criteria of the CLSI.

Antifungal assay

A mycelial disc (5 mm) from 3 days old fungal culture was placed in the center of 2% PDA plates. The bacterium inoculum was sown with a sterile stick at a distance of 2.5 cm from the fungal disc and inoculated plates were kept in darkness for 7 days at 25°C. The inhibition of fungal growth was quantified by measuring the colony diameter and calculating percentage inhibition using the following formula:

Percentage inhibition = (C-T) × 100/C

Where, C = colony diameter (mm) of the control and

T = colony diameter (mm) of the test plate.

Partial purification of proteins involved in biofilm formation

Partial purification of proteins was performed by method as described by Chiba *et al.* (2015). To produce biofilms, single colonies grown on TSA plates were picked and inoculated in 3 mL of TSB. They were placed overnight in a shaking incubator at 37°C. 1000-fold dilution was then carried out in 10 mL of TSB medium, followed by static incubation at 37°C for 24 h. After incubation, TSB with bacterial culture was centrifuged at 25°C for 10 min at 8000 rpm. To extract the ECM (Extracellular Matrix) components, the supernatant was discarded, while the pellet was re-suspended. Centrifugation was again carried out this time at 5000 rpm and the supernatant obtained was transferred to another clean tube. These partially purified extracellular proteins were used to assess protease and gelatinase activities and characterization in separate assays.

Protease activity from partially purified extracellular proteins

Protease activity was screened by agar well diffusion assay (Vijayaraghavan and Vincent 2013). Autoclaved, sterile skim milk was added to LB agar medium at 37°C with a pH of 6.5, and then poured into a Petri plate. Thirty μL of the extracted ECM was added to the well of the plate. Formation of a clear zone around the well as a result of skim milk hydrolysis indicated that the sample was protease positive.

Molecular size approximation SDS-PAGE analysis for protease

Molecular weight of protease in ECM was estimated by SDS-PAGE with some modifications (Fitriani and Guven 2018). The 4% stacking gel (pH 6.8) and 15% separating gel (pH 8.8) was utilized. The Running buffer consisted of 0.1% (w/v) SDS (pH 8.35), 192 m*M* glycine and 25 m*M* Tris. 4 μL of (Thermo Scientific Pre-stained Protein Ladder) was used as a standard. Voltage of 150 V was applied for 2 h and staining was carried out in a solution with 50% methanol, 10% acetic acid and 0.25 g 100 mL^{-1} Coomassie Brilliant Blue. De-staining was done in a 50% methanol, 50% distilled water solution.

Purification of gelatinase using ammonium sulphate precipitation and membrane filters

Precipitation of protein using ammonium sulphate was carried out using 10 mL of protein extract. After adding the ammonium sulphate, solution was stirred to reach some degree of saturation and then placed on ice for around 30 min. Centrifugation was then carried out at 4°C for 15 min at 13000 rpm. The obtained pellet was suspended in 1 mL PBS buffer at pH 7. The above procedure was carried out multiple times to obtain 20, 30, 40, 50, 60, 70 and 80% saturation. Further purification was then done by application of molecular weight cut size membrane filters. Aliquots were then again analyzed on SDS-PAGE.

Determination of molecular weight of protein with SDS PAGE

After running gel, the relative migration distance (*RF*) was determined of the standards of protein and the unknown protein. The migration distance can be determined using the following equation:

RF = Migration distance of the protein/Migration distance of the dye front

Gelatinase activity assay

The phenotypic assay of gelatinase activity performed through the gelatin liquefaction method (Cruz and Torres 2012). Purified protein (30 µL) was added in a falcon tube containing Gelatin medium (nutrient gelatin) with the following formulation per litter: peptone 5 g, beef extract 3 g, gelatin 120 g, pH 7. After placing the tube overnight at 4°C, tube was observed for liquefaction. The test was considered gelatinase positive if the gelatin liquefied.

Antifungal activity of purified gelatinase

Purified gelatinase was tested for inhibitory activity against spore germination of *F. oxysporum* and *R. solani*. The antifungal activity of gelatinase was estimated using a

growth inhibition assay described earlier (Zandvakili *et al.* 2017). Purified gelatinase (100 µL) was mixed with the spores of each pathogenic species in the broth separately and was spread on 2% PDA agar plates. The test microorganism's spore's broth without gelatinase served as control group. After 4 days of incubation at 25°C, the fungal colonies were checked for spore germination. The inhibition percentage of gelatinase activity was measured by formula described below:

Inhibition ratio (%) = (C-E) /C × 100%

Where *C* is the average diameter of colonies in the control, *E* is the average diameter of colonies in the experimental group. All experiments were conducted in triplicate.

Results

Molecular identification and biochemical characteristics of *enterococci* isolate

In this work, 35 bacterial isolates were randomly selected from eight collected milk samples. They were all Gram-positive bacteria without catalase activity. Obtained sequences of isolates were identified by pairwise numerical comparison with an extensive existing database (Ez Taxon) comprising multiple well characterized reference strains of all validly described bacterial species. On the basis of 16S sequencing, only two bacterial isolates were identified as *Enterococci durans* without catalase activity (Fig. 1; Fig. 2). The sequence of *E. durans* S2C has been submitted to Gene bank under accession number (MG877665) and its closest homologue is shown in Table 1. The *E. durans* S2C was capable to grow at 45°C, protease positive and also showed positive indication for biofilm formation.

Biochemical tests and antibiotic susceptibility of bacterial isolate

In the present study, *E. durans* S2C presented moderate capability of biofilm formation on abiotic surfaces; while, exhibited multiple antibiotic sensitivity against antibiotics i.e. cholоromphenicol, streptomycin, tetracycline, piperacillin, vancomycin, levofloxacin, minocycline, amoxicillin as shown in Table 2. Hemolytic test was also negative for this bacterial isolate; however, liquefaction of the growth medium inoculated with *E. durans* S2C, indicates gelatinase activity in the medium, one of the virulence factors of Enterococcus.

Antagonist effect of *E. durans* against phytopathogens

In the dual-culture plate test, the bacterial isolate S2C showed antagonistic activity against the mycelia growth of *F. oxysporum* and *R. solai* after four days' incubation (Fig. 3), and the inhibition rates were 50 and 52.6%, respectively as shown in Table 3.

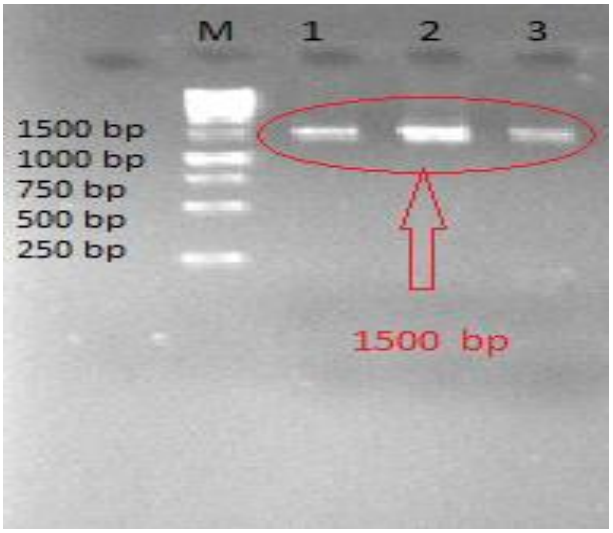

Fig. 1: PCR Amplification of 16S rRNA gene: Lane (1-3) showing replicate of 16S rRNA gene, lane M showing DNA markar of 1kb

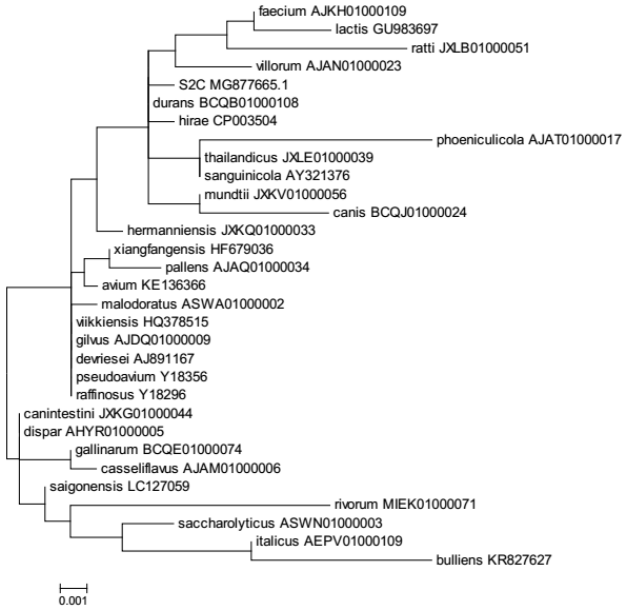

Fig. 2: Phylogenetic tree analysis on the basis of 16S rRNA gene

Purification and characterization of gelatinase

The proteins were partially purified and analyzed by SDS-PAGE. The partially purified proteins were protease positive. The gelatinase enzyme was purified from partially purified proteins by combination of ammonium sulfate precipitation method and molecular cut size membrane filters. The molecular weight of 37.9 kDa gelatinase was estimated (Fig. 4). Gelatin liquefaction test was positive as the tube containing purified gelatinase was liquefied while that of the control has remained solid.

Table 1: Identification of bacterial S2C isolate on the basis of 16S rRNA

Strain Name	Source	Closest strain	Percentage similarity	Accession number	Database
Enterococcus spp. S2C	Milk	*Enterococcus* sp. strain CAU7950	100%	MG877665	NCBI
Enterococcus spp. S2C	Milk	*E. durans* NBRC 100479(T)	99.90%	MG877665	Ez-Taxon

T indicate "type strain"

Table 2: Antibiotics resistance patterns of bacterial S2C isolate

Antibiotic Name	Disk content (μg)	Zone diameter (mm)	Mean (mm)	Resistant/intermediate/sensitive
Cholorophenicol	30	25, 24, 24	24.33	S
Streptomycin	10	19, 19, 18	18.66	S
Tetracycline	30	19, 20, 20	19.66	S
Piperacillin	100	17, 19, 20	18.66	S
Vancomycin	30	22, 20, 21	21	S
Levofloxacin	5	32, 33, 34	33	S
Minocycline	30	36, 37, 35.6	36.2	S
Amoxicillin	10	22, 21, 22	21.66	S

S indicate "sensitive"

Table 3: Antifungal activity of bacterial S2C isolate and purified enzyme against phytopathogens

Fungal Pathogen	Control colony diameter (mm)	Experimental colony diameter (mm)	Percent inhibition	Fungal spores production /100 μL in the presences of purified enzyme
Rhizoctonia solani	19	10	52.6	12
Fusarium oxysporum	20	10	50	9

Fig. 3: Antifungal activity of *Enterococcus* S2C against *Fusarium oxysporum* (**A**) and *Rhizoctonia solani* (**B**)

Antifungal activity of purified gelatinase

Result showed that 100 μL of purified gelatinase significantly inhibited the conidial growth of *R. solonai* and *F. oxysporum* as shown in Table 3. It also indicates that *E. durans* possess remarkable antifungal activity toward tested fungi compared to control.

Discussion

Enterococcus spp. known for their diversity, found everywhere in nature, are present in fermented foods. The research interests are increasing in enterococci as probiotic candidates (Hussein *et al.* 2020). LAB strains were isolated from various sources which inhibited the growth of *F. oxysporum* and they were belonged to the members of various LAB genera like *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Pediococcus* (Varsha *et al.* 2014). In this study, *Enterococcus* species was isolated with the idea of using the bacterial isolate as bio-control agent against phytopathogens. The isolated strain S2C showed the antifungal activity against *F. oxysporum* and *R. solonai*.

The existence of enterococci in food products as a natural flora has led to a lot of controversy over the safety aspects for their use, as scientists have found a few confirmations about the association of these microorganisms with clinical infections (Bondi *et al.* 2020). Enterococci food strains have also been found to contain antibiotic resistance genes normally found in conjugative plasmids, increasing the risk of genetic transmission. Despite these considerations, the enterococci present in many cheeses at high densities and are supposed to play a helpful part in the development of flavors (Hanchi *et al.* 2018; Garcia-Solache and Rice 2019). Verification of virulence factors was

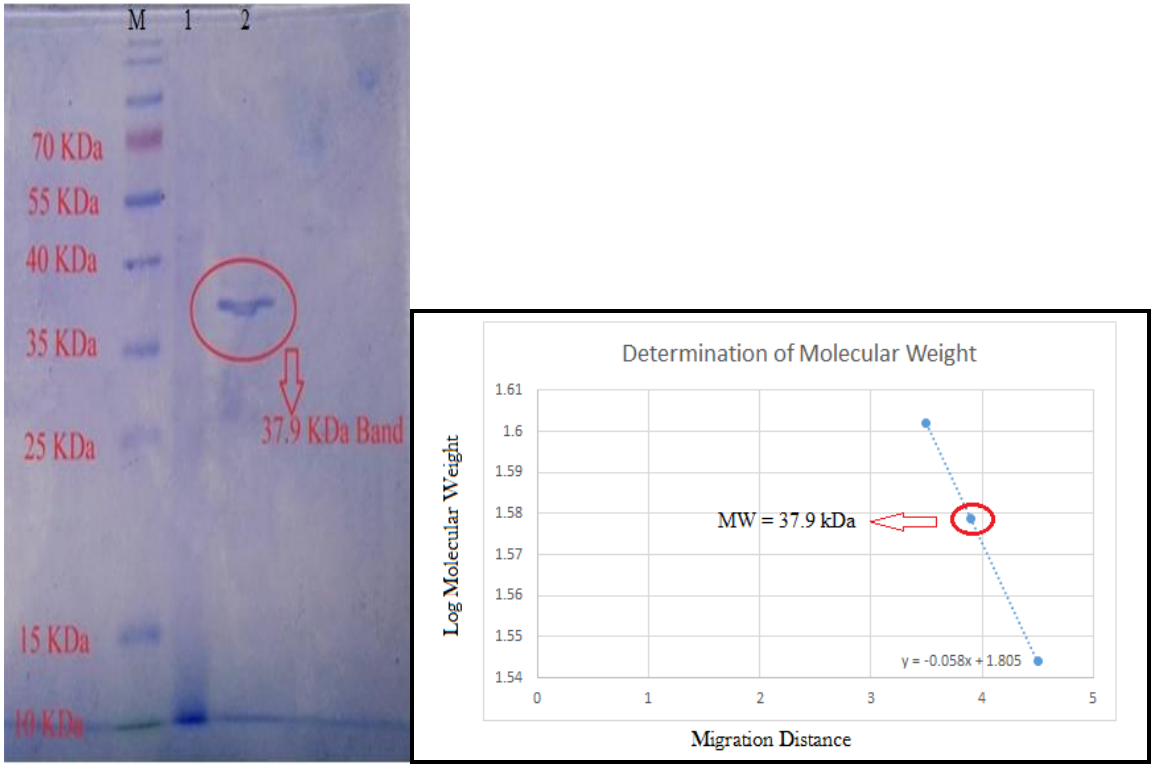

Fig. 4: (**A**) SDS PAGE analysis of purified enzyme: Lane M shows Protein Markar, Lane 2 shows purified enzyme of 37.9 kDa (**B**) Molecular weight estimation of purified enzyme

therefore of utmost importance among enterococci through phenotypic methodologies. Biofilm formation, hemolytic and gelatinase activities by enterococci have been recently recognized as a factor that contributes for pathogenicity. In the present study, stain S2C isolates presented moderate ability to form biofilm on abiotic surfaces and gelatinase activity. A positive correlation among gelatinase production and formation of biofilm was observed in present study. However, Mohamed and Murray (2005) found no association between gelatinase production and biofilm formation in enterococci of clinical isolates. The development of resistance to high levels of glycopeptides like vancomycin is a major concern for enterococci (Zalipour *et al.* 2019). *Enterococcus* species examined in this study, however, was found to be sensitive to vancomycin and other clinically significant antibiotics (carbenicillin, penicillin, chloramphenicol and amoxicillin/clavulanic acid), therefore can be considered as biocontrol agent.

E. faecalis present in filled root canals produced gelatinase as one of the virulence factors that may be linked with their survival. *E. faecalis* in large proportion are isolated from hospitalized patients that can express gelatinases (Guneser and Eldeniz 2016). Under the consideration from an endodontic point, the expression of gelatinase has been reported to be higher in biofilm-positive strains (Wang *et al.* 2011). The genes related to virulence factors are sporadically present in enterococci isolates which are tested in dairy products, so that are not associated with cytotoxic activity, suggesting that adhesion and biofilm formation are more associated with gut colonization (Popović *et al.* 2018). In the present study, gelatinase was also isolated from the *Enterococcus* species S2C from raw milk source and the purified gelatinase showed antifungal activity against phytopathogens.

Conclusion

Raw milk associated *E. durans* S2C is considered to be safe and has good potential to control fungal pathogens in agriculture sector. The results produced in this study encourage us to carry out other tests to evaluate the probiotic potential of this strain and also investigate the deep characterization of the gelatinase enzyme produced.

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Author Contributions

AU gives the idea of presented study and supervised the research work. ZH planned and conducted the research work. FS planned and conducted antifungal assay. IM, KU, AH and FYH helped in preparing manuscript and finalized the manuscript by giving feedback.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

The data will be available upon reasonable requests to the corresponding author.

Ethics approval

Not applicable.

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