Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Isolation and identification of keratinolytic probiotic *Bucillus licheniformis* bacteria from the soil below poultry slaughterhouse waste

Isolamento e identificação de bactérias probióticas queratinolíticas *Bacillus licheniformis* do solo sob resíduos de abatedouro de aves

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Abstract

Feathers make up 7% of the total weight of adult chickens and keratin protein makes up 85% of the feathers. Today, the keratinase enzymes of some *Bacillus* strains are used to degrade and process raw keratin waste for animal and poultry feed. According to various studies, the probiotic properties of some spore-shaped *Bacillus* have also been proven. The study aimed to isolation of the keratinolytic *Bacillus* bacteria that they have probiotic properties for using in the livestock and poultry feed industry. We were able to isolate 8 strains of *Bacillus licheniformis* with kreatin degrading properties from the soil of Baharan chicken slaughterhouse (Qom city, Iran) applying heat shock, alcohol- and keratin-rich culture medium, and after microscopic and biochemical analysis, 16S rDNA gene was isolated. The measurement results of keratinase activity showed that the three strains of *Bacillus licheniformis pvkr6*, *pvkr 15*, and *pvkr41* had the highest activity with 124.08, 101.1, and 100.18 U/ml. The results of probiotic properties evaluation also revealed that among all the isolates, only *Bacillus licheniformis pvkr15* and *Bacillus licheniformis PTCC 1595* (positive control) were γ-hemolytic strains. The percentage of surface hydrophobicity of the strains was obtained from 3.27 to 30.57. It was also shown that, on average, all the strains had acceptable susceptibility to the tested antibiotics except penicillin G. *Bacillus licheniformis pvkr15* with highest keratinase activity (101.1U/ml) was considered an optional probiotics due to its abilities such as (biofilm formation, being safe cause of γ-hemolytic activity, high susceptibility to antibiotics such as streptomycin, gentamicin, cefixime, amoxicillin, tetracycline, vancomycin, erythromycin and having a moderate hydrophilic (hydrophobicity: 19.09%), high survivability in pH 2, 2.5 and 3, strong resistance to bile salts and moderate antagonistic activity against pathogenic bacterium like *Proteus mirabilis* and the ability to grow under anaerobic conditions). By using this strain, after hydrolysis of keratin protein in the feather structure, to replace part of the protein of livestock and poultry feed, not only is no need to separate bacteria from the feed, but also the strain play role of an useful and effective additive in animal growth.

Keywords: bacillus, keratinase, probiotic activity, keratinolytic bacteria.

Resumo

As penas representam 7% do peso total das galinhas adultas e a proteína de queratina compõe 85% das penas. Hoje, as enzimas queratinase de algumas cepas de *Bacillus* são usadas para degradar e processar resíduos de queratina brutos para alimentação de animais e aves. De acordo com vários estudos, as propriedades probióticas de alguns *Bacillus* em forma de esporos também foram comprovadas. O estudo teve como objetivo o isolamento das bactérias queratinolíticas *Bacillus* que possuem propriedades probióticas para uso na indústria de ração animal e avícola. Conseguimos isolar 8 cepas de *Bacillus licheniformis* com propriedades degradantes de creatina do solo do abatedouro de frangos de Baharan (cidade de Qom, Irã) aplicando choque térmico, meio de cultura rico em álcool e queratina e, após análise microscópica e bioquímica, o gene 16S rDNA foi isolado. Os resultados da medição da atividade da queratinase mostraram que as três cepas de *Bacillus licheniformis pvkr6, pvkr15* e *pvkr41* tiveram a maior atividade com 124,08, 101,1 e 100,18 U/ml. Os resultados da avaliação das propriedades probióticas também revelaram que dentre todos os isolados apenas *Bacillus licheniformis* pvkr15 e *Bacillus licheniformis PTCC 1595* (controle positivo) eram cepas γ-hemolíticas. A porcentagem de hidrofobicidade superficial das cepas foi obtida de 3,27 a 30,57. Também foi demonstrado que, em média, todas as cepas apresentaram suscetibilidade aceitável aos antibióticos testados, exceto penicilina G. *Bacillus licheniformis pvkr15* com maior atividade de queratinase (101,1U/ml) foi considerado um probiótico opcional devido às suas habilidades como formação de biofilme, sendo causa segura de atividade γ-hemolítica, alta suscetibilidade a antibióticos como estreptomicina, gentamicina, cefixima, amoxicilina, tetraciclina, vancomicina, eritromicina e ter uma hidrofílica moderada (hidrofobicidade: 19,09%), alta

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Received: October 19, 2021 – **Accepted:** March 8, 2022

capacidade de sobrevivência em pH 2, 2,5 e 3, forte resistência aos sais biliares e atividade antagonista moderada contra bactérias patogênicas como *Proteus mirabilis* e a capacidade de crescer em condições anaeróbicas. Ao utilizar esta cepa, após a hidrólise da proteína queratina na estrutura da pena, para substituir parte da proteína da ração de gado e aves, não só não há necessidade de separar as bactérias da ração, mas também a cepa desempenha um papel útil e eficaz aditivo no crescimento animal.

Palavras-chave: bacilo, queratinase, atividade probiótica, bactérias queratinolíticas.

1. Introduction

According to research, millions tons of feathers are produced annually as a by-product of the factory poultry trade worldwide (Tesfaye et al., 2017). Chicken feathers are utilized for a variety of products, including fertilizers, feed, and biofilms. Feather is composed of approximately 90% hard keratin (Ramakrishnan et al., 2018). The use of current added value for feathers is the conversion to feather powder as a digestible dietary protein for animal feed, applying physical and chemical treatments (Tiwary and Gupta, 2012). These methods can eliminate the keratin protein amino acids and reduce the quality and digestibility of the protein (Elkin et al., 1996). Due to this issue, the use of keratin degrading enzymes produced by microorganisms producing amino acids and peptides to compensate for the lack of protein for animal feed has become an important and attractive issue in biotechnological activities (Cai et al., 2008; Zabihi et al., 2011). Alkaline proteases are a group of proteolytic enzymes able to degrade proteins, such as keratin, which are difficult to dissolve more efficiently than other proteases, such as trypsin, pepsin, and papain. Previous studies have indicated that some *Bacillus* species are able to produce proteases for feather digestion (Shamsipur et al., 2012; Sabir et al., 2021; Pustokhina et al., 2021). Keratin is an insoluble protein with a stable structure that forms protective levels in various animals and is a structural component of skin, wool, hair, feathers, and nails (Sharma and Gupta, 2016). Keratinase is able to hydrolyze keratin and degrade feathers. Microorganisms, such as bacteria, fungi, and actinomycetes have been reported to produce keratinase for use as energy and carbon sources. Resistance to proteolytic enzymes has been attributed to the complex structure of beta-keratin filaments. In addition, disulfide cross-links produce a compact three-dimensional network as a result of the intermolecular bonds of disulfide between the rod and the terminal domains of the constituent molecules (Riffel and Brandelli, 2006).

Keratinolytic microorganisms may play an important role in industrial processes and the biotechnology and pharmaceutical industries (Vidmar and Vodovnik, 2018). Some bacteria, such as *Bacillus licheniformis* and *Bacillus subtilis* produce keratinase (Mousavi et al., 2013). The keratinase produced by *B. licheniformis* increases the total digestibility of crude feather amino acids and commercial feather powder. This enzyme can replace up to 7% of dietary protein for growing chickens (Garbeva et al., 2003).

Bacillus species are gram-positive bacteria that can be found widely in soil and plants (Elshaghabee et al., 2017). *Bacillus* species have found a variety of food products, such as probiotics. Probiotics are living microorganisms giving the host health benefits. Probiotics can increase innate immune and resistance to pathogens. The use of probiotics in the feed, poultry, and aquaculture industries is important (Munir et al., 2019). However, removing antibiotics from poultry feed can predispose them to infections caused by certain pathogens, such as *Escherichia coli*, *Clostridium perfringens,* and *Salmonella* species, leading to low yields and serious damage to the poultry industry (Al-Sultan, 2003; Marins et al., 2021; Wekhe et al., 2007). Probiotic products as live microbial foods are beneficial to animal health as a viable alternative to antibiotics to prevent disease and stimulate animals (Jin et al., 1998; Teo and Tan, 2006). The adhesion of probiotics can be related to the characteristics of their cell surface, namely hydrophobicity, which can result in a strong interaction with the mucosa if high enough. However, not only is the hydrophobicity of probiotic cell surfaces responsible for their attachment to epithelial cells, but more specific mechanisms include lipoteichoic acid, extracellular components (exopolysaccharides or proteins), or surface proteins (Grigoryan et al., 2018; Mohanty et al., 2019). The aim of this study was to isolate and investigate the characteristics of *Bacillus* probiotic bacteria producing the enzyme keratinase for use in feather degrading and to provide a protein source for animal feed.

2. Material and Methods

2.1. Sample collection

The soil samples were randomly collected from different areas of the bed soil of Baharan Poultry slaughterhouse (Qom, Iran). A total of 100 samples were collected so that each soil sample was 5 g and was poured sterilely into a separate sterile falcon and delivered to the microbiological laboratory (Islamic Azad University, Ayatollah Amoli Branch), then 1 g of each sample was weighed below the microbial hood (Sarvo Tajhiz Sakoo Company, Iran) and the microbial suspension of the unit was prepared and homogenized by the shaker (MICRO-SPIN – KIAGEN company, Iran).

2.2. Isolation and identification of bacterial strains

Briefly, one gram of the soil sample was diluted 1:10 (w/v) in sterile saline (0.9%) (Samen pharmaceutical company, Iran). Afterwards, the suspensions were allowed to settle in the soil particles for 3 min and 25 ml of the microbial suspension supernatant then reached 50 ml at a ratio of 1:1 with 25 ml of buffered peptone water (QUELAB, Cat No:39-9587, Canada). This solution was divided into two equal parts and treated individually with heat and alcohol to separate *Bacillus* bacteria (Mohamadkhani and Shishegaran, 2020). In this way, for heat treatment,

from the initial microbial suspension, with a volume ratio of 1:10, and with 18 ml of peptone water, it reached to 20 ml and was placed in a 65 ° C water bath for one hour. Then, ten serial dilutions with peptone water were prepared from the suspension treated (from 10^{-1} to 10^{-10}) after homogenization of the last dilution was cultured 100 ml on the LB agar (two replications per dilution) and were incubated for 24 hours at 37 ° C. For alcoholic treatment, the initial microbial suspension was reduced to a volume of 20 ml with a volume ratio of 1:1, and with 10 ml of 96% ethanol, so that the final concentration of alcohol reached 50%. The sample was treated with alcohol for one hour. The next steps were the same as heat treatment (Barbosa et al., 2005). Serial dilution was performed on each treated sample and mass and linear cultures were then prepared from their final concentration; hence, the refined colonies were stored at 70 °C for 24 h (Barbosa et al., 2005). To identify the isolated bacteria, biochemical tests, including catalase, gelatinase, amylase, lecithinase, Simmons citrate agar, urease, caseinase, O/F (obligate or facultative aerobes) and TSI (Triple Sugar Iron) were employed according to the instructions described in Bergey's manual systematic bacteriology (Vos et al., 2011). Finally, a typical colony of each subculture sample was cultured and the bacteria were identified with colony morphology, gram staining compared to standard species (*Bacillus licheniformis PTCC 1595* and *Bacillus licheniformis PTCC 1525* as positive control).

2.3. Feather keratin substrate

The method of preparing keratin powder is described by (Wawrzkiewicz et al., 1987). The feathers obtained from the slaughterhouse were washed with distilled water and detergent (Dishwashing Liquid, Prile Company, Iran) to degrease and remove slaughterhouse contaminants from feathers. Chloroform: ethanol solution (v/v) was then applied to separate the fat from the feathers. Afterwards, 500 ml of DMSO (Dimethyl sulfoxide, Merck Milipore, Germany, Cat: 102950) was added to the lipid-free feathers and they were heated at 100 °C for 120 min. Following the removal of the precipitate, one liter of acetone (-70°C) was added dropwise to the supernatant at 4 °C to precipitate the keratin protein and separate it from the solvent and acetone. Lastly, the keratin precipitate was separated and dried. Keratin powder was employed as a substrate for the keratin culture medium.

2.4. Isolation of keratin degrading bacteria

To identify keratin-degrading *Bacillus* spp, all 42 isolated strains were cultured separately on culture medium containing 1.5% keratin (as the only carbon source) and incubated at 37 °C for 5 days. The degradability of keratin substrates in culture medium was evaluated on the basis of keratin containing NaCl (0.5 g/l) (Merck Company, Germany, Cat No: 106404), K2HPO4 (0.3 g/l) (Sigma-Aldrich Company, USA, Cat No: 1551128), KH2PO4 (0.4 g/l) (Merck Company, Germany, Cat No: 105104), agar (15 g/l) (Merck Company, Germany), and keratin (15 g/l) (keratin is obtained by the method described above (keratin substrate)) (Sangali and Brandelli, 2000). Also, *Bacillus licheniformis PTCC 1595* as positive control and *Bacillus licheniformis PTCC 1525* as negative control were considered as standard strain (National Center of Genetic and Biological Resources, Iran). The plates were incubated at 37 °C for 1 to 5 days and keratinolytic strains were identified and isolated based on the clear keratinolysis area around them.

2.5. Biomolecular identification of bacteria

For biomolecular identification of isolated bacteria, the isolated bacteria were cultured with keratinase activity in LBA (Luria_Bertani Agar, Merck Company, Germany, Cat No: 110283) medium and incubated for 24 hrs at 37 °C. Genomic DNA of the selected isolates was extracted with Peqlab, Cat No: 51304 kit (Dena Zist Company, Iran). The 16S rDNA was utilized to amplify a segment of DNA through the use of the following universal primers of 27F(5'AGAGTTGATCATGGCTCAG-3') and 1492R(5'TACGG ATACCTTGTTACGACTT-3') (Takapouzist Company, Tehran, Iran) (Batisson et al., 2009). PCR was performed on a total volume of 25 µl with 190 ng of template DNA, 10 pmol of each primer, 10 mM dNTP (Thermo Fisher Scientific Company, USA, Cat No: 18427088), 60 mM $MgSO_4$ (Millipore Sigma Company, Germany, Cat No: 105886), 10X Taq buffer (Thermo Fisher Scientific Company, USA, Cat No: B38), and 10 U/µl of Taq polymerase (Roche Company, Swiss). The PCR reaction was as follows: 94 °C for 10 min, 35 cycles 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min, and the final length at 72 °C for 10 min. In addition, *Bacillus licheniformis PTCC 1595* as positive control was used in this process. The amplicon of 16S rDNA was determined applying agarose gel electrophoresis. The obtained sequences were read and processed through three bioinformatics programs Chromas, Edit Seq, and Seq Man. The sequences of genes processed at the NCBI were BLAST (Lin et al., 1995).

2.6. Keratinase enzyme production

The keratinase enzyme was extracted by the use of the modification method of Sangali and Brandelli (Andersen, 2001). The isolated keratinolytic *Bacillus* bacteria were cultured in keratin broth medium (keratin (10 g/l), NaCl (0.5 g/l) (Merck Company, Germany, Cat No: 106404), K2HPO4 (0.3 g/l) (Sigma- Aldrich, USA, Cat No: 1551128), and KH2PO4 (0.4 g/l) (Merck Company, Germany, Cat No: 105104)) at 37 °C/150 rpm and were incubated for 5 days. The supernatant was separated via centrifugation at 12,000 rpm for 30 min at 4 °C and utilized for keratinolytic assay and enzyme analysis.

2.7. Keratinase assay

According to the method described by Senegal and Brandelli with a little contradiction (Sangali & Brandelli, 2000), briefly, 300 µl of medium containing extracted keratin enzyme as explained in the previous step, was prepared. Afterwards, 2700 µl containing 10 g/l keratin in Tris buffer (Sigma-Aldrich, USA, Cat No: T1503) with final concentration 50 mM and pH was added and placed in a water bath at 50 °C for 15 min. After this period, to prevent the activity of keratinase enzymes from continuing, 10% Trichloroacetic acid (Merck Company, Germany, Cat No: 100807) was added to each vial. Subsequently,

to separate the substrate from the enzyme suspension, the vials were centrifuged at 10000 g for 5 min. After separation, the absorbance of the supernatant fluid was measured at 440 nm.

In the next step, to deposit the native protein (enzyme), 76 g of ammonium sulfate (Merck Millipore Company, Germany, Cat No: 101209) was dissolved in 100 ml of distilled water. Through a special decanter (Novin Pyrex Company, Iran), 70 ml of the above solution (at -20 °C) in drops of 30 ml crud enzyme obtained from *pvkr15* strain was added as overnight in cold room at -20 °C. It was then centrifuged at 5000 RPM for 10 min. The sediment was dissolved in 200 µl of Tris buffer with pH 7.5-8. Finally, the dialysis was performed using a microfuge dialysis bag with at 10,000 RPM and using the soup obtained from the final sediment, the enzymatic activity (U/ml) was measured and calculated (Andersen, 2001).

2.8. Safety assessment

2.8.1. Hemolytic activity

The keratinolytic *Bacillus* strains were analyzed for their hemolytic activity on nutrient agar containing 7% (v/v) defibrinated sheep blood (Quelab Company, Tehran, Iran) and incubated at 37°C for 96 hrs. The plates were checked every 12 hrs. Also, *Bacillus licheniformis PTCC 1525* and *Bacillus licheniformis* PTCC 1595 (purchased from National Center of Genetic and Biological Resources, Iran) respectively were positive and negative controls for this experiment (AlGburi et al., 2016).

2.8.2. Resistance to antibiotics

Müller-Hinton Agar (Merck, Germany) were employed to evaluate antibiotic susceptibility of what please clarify via disk diffusion method (Maričević and Žganjar, 2022). Nine antimicrobials were chosen to test for susceptibility to antibiotics so that if there is any antibiotic resistance and the possibility of transmission from probiotic *Bacillus* to livestock and poultry and finally humans, to avoid the use of these *Bacillus* in livestock and poultry feed as a probiotic bacterium; these antibiotics are including streptomycin (10 µg), gentamicin (10 µg), erythromycin (10 µg), cefixime (5 µg), penicillin G (10 µg), amoxicillin (25 µg), tetracycline (30 µg), rifampin (30 µg), and vancomycin (30 µg). All the Müller-Hinton plates were incubated for 24 hrs at 37 °C. In addition, the standard strain were including *Bacillus licheniformis PTCC 1595* and *Bacillus licheniformis PT1525* as the positive and negative control strains, respectively. Due to the size of growth inhibition zone, the results were interpreted as resistant, intermediate susceptible, and susceptible to the Clinical and Laboratory Standards Institute table (CLSI, 2016).

2.8.3. Biofilm formation

Tube adhesion method was applied to detect the ability to form biofilms (Wolfe et al., 2004). The keratinolytic strains were inoculated into the Subwoofer wireless transmitter (SWT) broth and incubated at 37 °C with incubator shaker (GFL Company, 3031 Model, India) for 24 hs. After transferring the contents to glass tubes, the cells were incubated at 37 °C for 24 hrs without shaking (Wolfe et al., 2004). The tubes were stained with 1% crystal violet and washed with distilled water. The biofilm layer, if present, appears purple between the air and the liquid.

2.8.4. Cell surface hydrophobicity

The percentage of surface adhesion to solvent (xylene) was measured and calculated based on the method of Xu et al. (2009). The adhesion to xylene (apolar solvent) indicates surface hydrophobicity. Briefly, *Bacillus* was cultured overnight in LB broth medium, the bacteria were centrifuged at 3000 rpm for 5 min. The bacterial cell deposition were suspended in PBS solution. Afterwards, 1 ml of xylene solvent was added to 3 ml of bacterial suspension and vortexed (Vortex Spin, Micro-Spin Model, Kiagen Company, Iran) for 1 min and kept for 5 min to separate in two phases. We measured the aqueous phases with a spectrophotometer (Beckman Company, DU530 Model, USA) at a wavelength of 600 nm (A_{5min}). The experiment was repeated twice. The values less than 30% (30%>) were considered as (low), between 30-60% (30% ≤, 60%>) as (average), and values greater than 60% (60%≤) were considered as (high). Strains with low solvent adhesion are considered as potential probiotics. The affinities of *Bacillus* to solvents was calculated with the following Formula 1:

$$\text{Adhesion \%} = \left(1 - \frac{A_{5\ min}}{A0\ min}\right) \times 100 \quad (1)$$

2.8.5. Bile salt tolerance

To evaluate bile salt tolerance, as in the previous stage, an 18-hour pre-culture was prepared separately in LB broth in terms of sterile conditions. For the experiment, the falcons containing 10 ml of LB broth (for preparation of control samples) and the falcons containing 10 ml of LB broth with a concentration of 0.3% Oxgall were prepared. The desired colony was removed from the plate and cultured in all media. The falcons were incubated in a shaker incubator at 37 °C and 180 rpm for 6 hrs. The turbidity of the incubated environments under these conditions was then read every 15 min with a spectrophotometer at a wavelength of 600 nm to delay the growth generated with Oxgall for each strain compared to the control sample for the strain until reaching a turbidity of 0.3 was determined (Gilliland et al., 1980).

2.8.6. Resistance to acid

To evaluate resistance to acid, we employed the pre-cultures on LB broth prepared for overnight, after homogenizing them by vortex, 1 ml aliquots of each active culture was poured into falcons containing LB culture medium with HCL acid 37% (Merck Millipore Company, Germany, Cat No: 100317) was reached to pHs 2, 2.5, and 3 using pH Meter device (Beckman Company, Model of 72, USA). Next, the inoculated media were incubated in a shaker incubator at 37 °C for 3-6 hrs. After the first three

hours, the10-µl samples (two samples from the vortex falcons) were cultured as spots in LB agar (two replicates from each medium) and the inoculated media were incubated at 37 °C for 24 hrs. All the experiments were repeated three times (Singhal et al., 2010).

2.8.7. Growth rate of acid resistant strains at acidic pH

For evaluation, as in the previous stage, an 18-hour pre-culture was prepared separately in LB broth under sterile conditions. Then, liquid LB medium with pHs of 2, 2.5 and 3 were prepared and distributed in the sterile falcons, then 10 µl of each sample were inoculated in the falcons. Afterward, the falcons incubated in a shaker incubator at 37 °C and 180 rpm for 4 hours. The turbidity of all the samples was read at 600 nm with a spectrophotometer set.

2.8.8. Antimicrobial assessment

To measure the antibacterial activity of the *Bacillus* isolates, 18-hour pre-cultures on LB broth (Merck Company, Germany) were applied. From all of 8 strains in separate falcons and in a shaker incubator (GFL Company, model 3031, India) was prepared at 37 °C. Then, it was centrifuged at 3000 rpm using refrigerated centrifuge (Kendro Company, D37520 Model, United Kingdom) and 20 µl of supernatant containing possible bacteriocin of all 8 *Bacillus licheniformis* strains was cultured in LB Agar plate wells and purified of any bacterium. Afterwards, a colony was removed from the each plate of purchased pathogenic bacteria (the National Center of Genetic and Biological Resources, Iran) including *Bacillus cereus* (*CCM2010, DSMZ31, ATCC14579, LMG6923, NCBI9373* and *NCTC2599*), *Escherichia coli* (*ATCC11775, DSM30083, CCM 5172*), *Klebsiella pneumonia subsp pneumonia* (*ATTCC700603, CCUG45421* and *LMG20218*), *Salmonella Enteritidis* (*ATCC1307603, DMS 17420, LMG 10395*) and *Proteus vulgaris* (*ATCC25933, CCUG34294*) and cultured through pour plate. At this stage, four wells were installed in each plate and the ends of the wells were blocked with 10 µl of molten agar. The medium with inoculated wells were incubated at 37 °C for 24 hrs. The above experiment was repeated once more. In addition, the pre-cultured strains were centrifuged at 3000 rpm after 18 hrs of incubation and the supernatant was utilized for loading in the wells. Inhibitory zone of the strains were checked after 24 hrs of incubation at 37 °C (Nourouzi et al., 2004).

2.8.9. Phylogenetic analysis of 16S rRNA gene

First, we sent PCR product to Gen Fanavaran Company (Tehran, Iran) for sequencing using Sanger sequencing method. In sequencing, the primers concentration was 10 pmol and the amount of primers was 5 µmol per reading. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. This analysis involved 8 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1392 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021; Stecher et al., 2020; Sabir et al., 2021).

2.8.10. Statistical analysis

SPSS software Ver-20 was used to perform statistical analysis. The data are expressed as a mean ± standard deviation (SD) calculated over three independent experiments performed in triplicate. Analysis of statistical significance was carried out via two-way ANOVA (at a significance level of $P<0.05$) with t-test.

3. Results

3.1. Identification of keratin degrading bacteria

After culturing 42 isolates on culture medium containing 1.5% keratin, 8 strains formed a clear zone around their colonies after 72 hrs (Figure 1), revealing the production of the enzyme keratinase to degrade and use the only carbon source of the culture medium (keratin). There were no clear zones around the other strains; thus, they were not keratinolytic and were excluded from the study at this stage. According to the results, the isolates *pvkr6*, *pvkr8*, *pvkr9,* and *pvkr41* produced the highest amount of clear zone during 72 hrs of incubation at 37 °C. As a result, these strains were classified as strong keratin-degrading bacteria, followed by the *pvkr15* and *pvkr26* strains, which produced a clear zone with a smaller diameter than the mentioned strains and were classified as moderately potent degrading bacteria. Finally, strains *pvkr1* and *pvkr10* produced the lowest levels of the keratinase enzyme and a clear zone with a very small diameter. These strains were classified as weak in terms of keratin degrading activity.

3.2. Biochemical identification of bacteria strains

The bacterial strains were isolated from soil designed as *pvkr1* to *pvkr41*. The results of biochemical experiments of *Bacillus* spp isolates are shown in Table 1. All of 10 isolates (8 isolates and *B. L1525* and *B. L1595* as negative and positive control, respectively) were gram-positive, positive catalase, gelatinase, and α-amylase as positive reaction and were identified as *Bacillus licheniformis*. The microscopic shapes of *Bacillus* spp are depicted in Figure 2. In addition the *B. L1525* and *B. L1595* were used as standard *Bacillus lichenifomis* strain and positive control (purchased from National Center of Genetic and Biological Resources, Iran).

3.3. Biomolecular identification of isolated bacteria

Indicating the *Bacillus* detection, 16S rDNA sequence analysis confirmed the biochemical results and PCR was performed on randomly selected colonies of keratinolytic bacteria (8 isolated strains). The PCR results demonstrated that several colonies were positive for *Bacillus* (Figure 3). All the 8 isolates were different strains

Figure 1. Creating a clear zone with keratin-degrading bacteria. *Bacillus licheniformis* PTCC 1525 and *Bacillus licheniformis* PTCC 1595 were employed as control *Bacillus* strains.

Table 1. Biochemical test of *Bacillus* spp (n=10).

Sample number	Catalase	Lecithinase	caseinase	Urease	Gelatinase	Citratase (Simmons citrate)	α-Amylase	Glucose	H_2S	O/F
Pvkr1	3+	1+	3+	-	1+	-	1+	-	1+	Obligate aerobes
Pvkr6	3+	-	2+	-	1+	1+	1+	-	-	Facultative anaerobes
Pvkr8	1+	-	2+	-	1+	-	1+	-	-	Obligate aerobes
Pvkr9	1+	1+	1+	-	1+	2+	1+	-	1+	Facultative anaerobes
Pvkr10	1+	-	1+	-	1+	1+	1+	-	-	Facultative anaerobes
Pvkr15	3+	1+	3+	-	1+	3+	1+	-	-	Facultative anaerobes
Pvkr26	2+	-	2+	-	1+	1+	1+	-	-	Obligate aerobes
Pvkr41	3+	1+	2+	-	1+	-	1+	-	-	Obligate aerobes
B. L1525	2+	-	3+	-	1+	2+	1+	-	-	Obligate aerobes
B. L1595	3+	1+	1+	-	1+	-	1+	-	-	Obligate aerobes

+: sign of the existence; -: sign of none existence; O: Obligate; F: Facultative.

of *Bacillus licheniformis* and were recorded in ENA as represented in supplemental section.

3.4. Keratinase activity

We examined 10 Bacillus species for keratinase activity before and after sedimentation and concentration of keratin enzyme. All the strains (8 strains and *B. L1595* as positive control) produced keratinase except the negative control (*B. L1525*), isolated and allowed to grow on feathers meal powder as the source of nitrogen and carbon. This result showed that the *pvkr6* and *pvkr15* strains could degrade the feathers completely after 7 days at 37 °C. As shown in Figure 4, the highest level of keratinase activity belonged to the *pvkr6* strain (124.08±0.27 U/ml) and *pvkr15* strain (101.1±0.45 U/ml). The *pvkr1* strain produced the lowest level of keratinase activity (63.74±0.24 U/ml). The *pvkr1*, *pvkr6*, and *pvkr15* strains formed yellow colonies and the *pvkr8*, *pvkr9*, *pvkr10*, *pvkr26*, and *pvkr41* strains formed white colonies on feather meal agar plate. In addition, the effect of enzyme concentration on keratinase activity (Figure 5) implied that maximum keratinase activity belonged the *pvkr*6 strain (specific activity 54.83±1.61 and total activity 372.25±0.81 U/ml) and the *pvkr15* strain (specific activity 50.45±2.6 and total activity 303.29±1.35 U/ml) was recorded at 0.07 M. Minimum keratinase activity also belonged the *pvkr1* strain (specific activity 62.16±2.3 and total activity 191.22±0.72 U/ml) at 0.03 M and the *B. L1595* strain (specific activity 23.28±0.53 and total activity 146.73±1.9 U/ml) at 0.07 M.

Following the enzyme deposition, keratinase activity levels of the isolated *pvkr15* strain and *B. L1595* as positive control were also measured. As shown Figure 6,

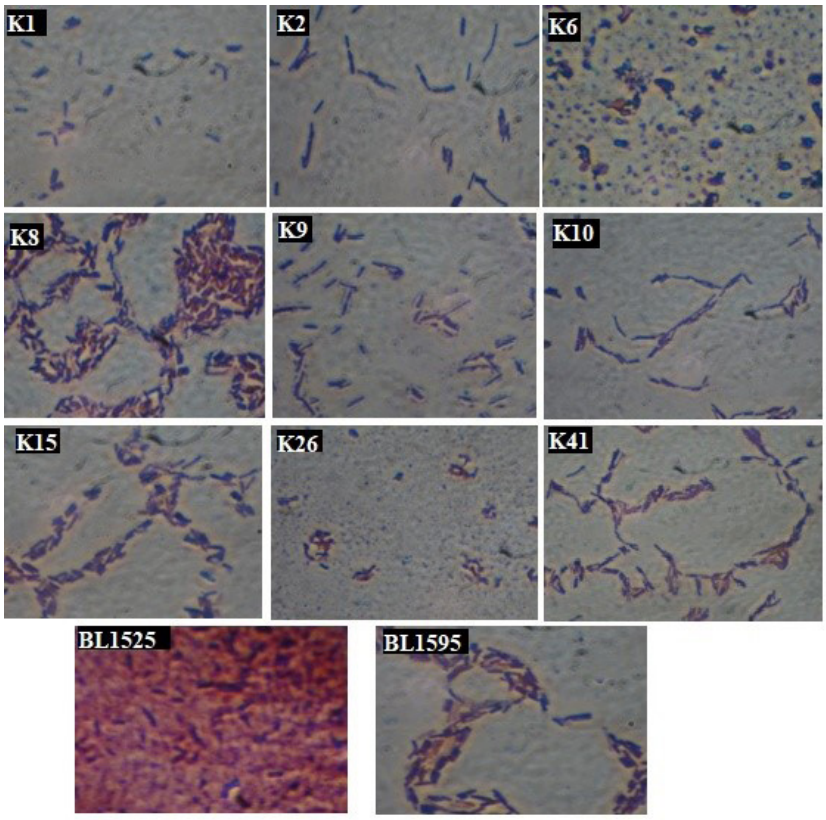

Figure 2. Microscopic shape of *Bacillus* spp. As can be seen in the figure, the *Pvkr10*, *Pvkr41* and *B. L 1595* strains have the longest length in terms of bacilli elongation and the *Pvkr1*, *Pvkr8*, *Pvkr9*, *Pvkr15* and *B. L 1525* have medium length and the *Pvkr6* and *Pvkr26* have very short bacilli. *Bacillus licheniformis PTCC 1525* (negative control), during growth and reproduction, produces a large amount of mucous secretions, which turn red in staining, but rod and gram-positive bacilli (purple) are seen among the red secretions.

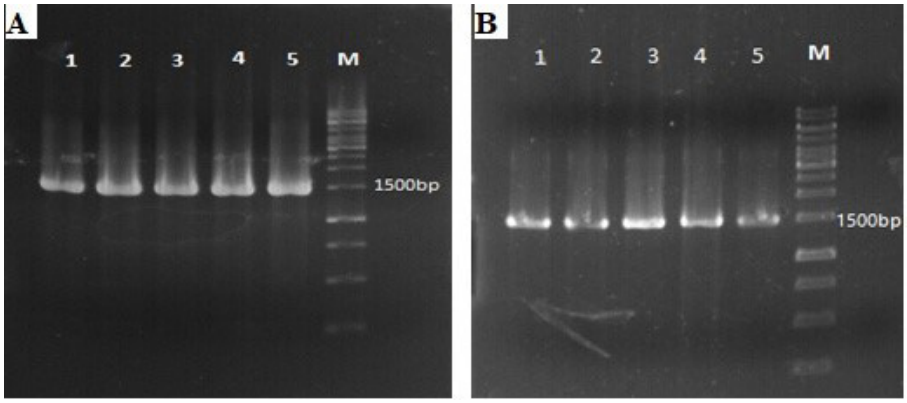

Figure 3. PCR results of 16S rDNA gene (10000 bp). (A) Lane 1: *pvkr1*; Lanes 2-5: *pvkr2*, *pvkr6*, *pvkr8,* and *pvkr9*, respectively; Lane M: ladder marker (10k bp); (B) Lanes 1-4: *pvkr10*, *pvkr15*, *pvkr26,* and *pvkr41*, respectively; Lane 5: *B. L 1595* as positive control; Lane M: ladder marker (10k bp).

both of the strains produced keratinase and keratinase activity of the *pvkr15* strain (160.36±0.85 U/ml) was more than that of *B. L1595* (92.34±0.94 U/ml). Moreover, the effect of enzyme concentration on keratinase activity in the selected strains (Figure 7) showed that keratinase activity of the *pvkr15* strain was recorded at 0.119 M (specific activity 45.73±3.9 and total activity 481.07±2.5 U/ml).

3.5. Hemolytic activity

Hemolytic activity of the *Bacillus* strains was determined using blood agar medium, supplemented with defibrinated blood. After 96 hrs of incubation at 37 °C, the strains of *pvkr1*, *pvkr8,* and *pvkr10* produced α-hemolysis and the *pvkr6*, *pvkr9, pvkr26,* and *pvkr41* strains demonstrated α-hemolysis weakly while the *pvkr15* strain showed no hemolytic activity during 96 hrs (Figure 8); thus, they are γ-hemolytic strains. Therefore, one strain (*pvkr15*) was selected as γ-hemolytic with the probiotic potential.

3.6. Antibiotic susceptibility of the Bacillus licheniformis strains

Selected *Bacillus* strains displayed various levels of antibiotic resistance, ranging from resistance to susceptibility (Table 2). It was found that all the strains were sensitive to streptomycin, gentamycin, amoxicillin, tetracycline, rifampin, vancomycin, erythromycin, and cefixime. The highest resistance belonged to penicillin G and the only strains sensitive to penicillin G were *pvkr8* and *pvkr9*.

3.7. Antagonistic properties of the Bacillus licheniformis strains against pathogenic bacteria

According to the data obtained from this experiment, none of the isolated strains had an inhibitory effect on bacterial pathogens, including *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae* while *pvkr8*, *pvkr10* and both control groups (*Bacillus licheniformis* 1525 and *Bacillus licheniformis* 1595) had an inhibitory effect on the growth of *Salmonella enterica.* Additionally, *pvkr1*, *pvkr6*, *pvkr8*, *pvkr9*, *pvkr10*, *pvkr15,* and *pvkr26* inhibited the growth of *Proteus mirabilis.* In other words, these strains produced bacteriocin against the mentioned pathogenic bacteria and stopped their growth (Figure 9).

3.8. Hydrophobicity

The adhesion of *Bacillus* strains to xylene are represented in supplemental section (Figure 10), showing the most adhesive bacteria to *pvkr8* and *pvkr15* with a 22.44% and 19.58% range of hydrophobicity percentage, respectively. In addition, the relative range of hydrophobicity was 3.27 to 30.57. The highest level of hydrophobicity belonged to *B. licheniformis PTCC 1595* that was 30.57%.

3.9. Biofilm formation with the Bacillus licheniformis strains

Given the importance of the ability of probiotic bacteria to form biofilms to maintain survival, herein, the ability to form biofilms was examined for each of the strains. Based on the results, it can be concluded that all the strains, including isolated strains and control strains, have biofilm formation properties. This result implied that 100% of isolates as biofilm formers were detected (Figure 11).

3.10. The resistance of the isolated Bacillus licheniformis to bile salts and acidic environment

As shown in Table 3, the growth rates of *pvkr9* and *pvkr15* in the medium containing bile salts were between 15 and 40 min relative to their controls and were classified as the group of bacteria resistant to Oxgall 0.3% concentration. However, the growth delay of *pvkr8* was between 40 and 60 min. It can be thus considered that this strain was a bacterium with weak resistance to bile salts while other strains showed a growth delay of more than 60 min classified as bile salt-sensitive bacteria.

Additionally, the results of resistance to low pH (Figure 12) demonstrated the viability of *Bacillus licheniformis* strains.

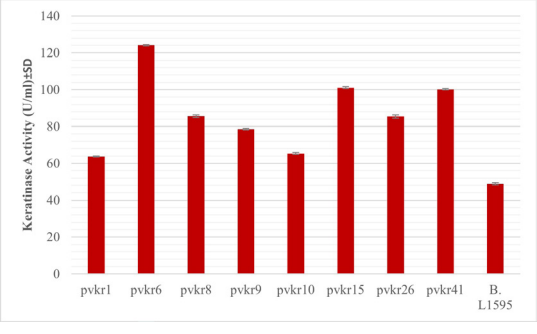

Figure 4. Keratinase activity of *Bacillus* spp before sedimentation of keratin enzyme.

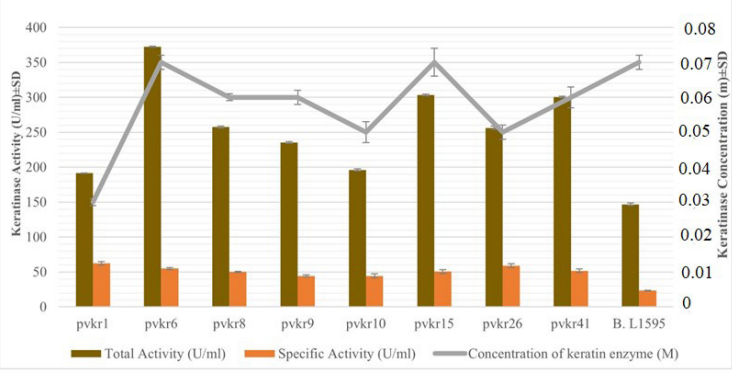

Figure 5. Effect of enzyme concentration on keratinase activity in the isolated strains.

The *pvkr9* strain showed slow growth at pH 2 whereas the growth continued at pH 2.5 and 3 for 3 hours. In the *pvkr26* strain, we observed weak growth at pH 2 and 2.5 for only the first 2 hours, but its growth continued at pH 3 for 3 hours. The growth of *pvkr8* strain also stopped completely at different pH. The other strains had weak and slow growth compared to the control samples (growth of strains in the medium with neutral pH [7.2]). Moreover, the results of pH effect on biofilms showed that all the strains survived the acidic conditions and were able to grow on the LBB agar.

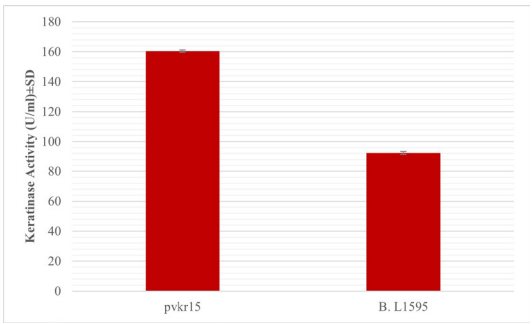

Figure 6. Keratinase activity of the selected strains after sedimentation of keratin enzyme.

3.11. Phylogenetic analysis of 16S rRNA gene

The evolutionary history was inferred through the neighbor-Joining method. The analysis involved eight nucleotide sequences. As can be seen in the Figure 13, pvkr1 and pvkr9 strains have common ancestors and are considered as in-group, which are closer to pvkr6 and pvkr15 strains than other strains. The pvkr26 and pvkr41 also have common ancestors that are considered as outgroup and are closer to the pvkr8 strain (Figure 13).

4. Discussion

To date, numerous studies have been performed on the isolation and identification of *Bacillus* bacteria with keratin degradation properties. The results of these studies showed that several *Bacillus* strains have a very high ability to produce the keratinase enzyme and in some studies, researchers have isolated the strains from soil samples (particularly poultry and slaughterhouse soil) or keratin waste in the nature (Qiu et al., 2020). It has been suggested to employ their keratin degradation potential

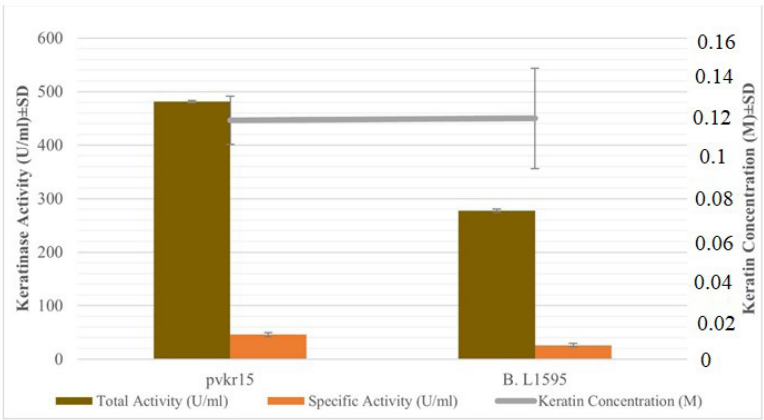

Figure 7. The effect of enzyme concentration on keratinase activity in the selected strains.

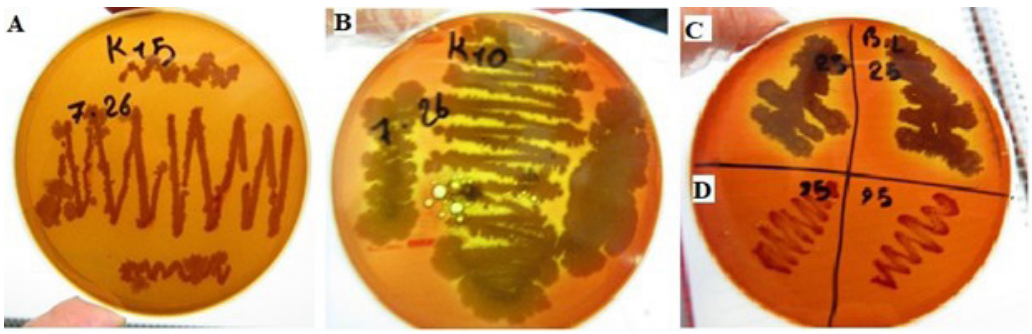

Figure 8. Hemolytic Activity of the strains; (A) *pvkr15*: γ-hemolysis; (B) *pvkr10*: α-hemolysis; (C) *Bacillus licheniformis* PTCC 1525: positive control of α-hemolysis; (D) *Bacillus licheniformis* PTCC 1595: negative control of α-hemolysis.

Table 2. Antibiotic susceptibility.

Strain	Vancomycin	Rifampin	Tetracycline	Amoxicillin	Penicillin	Cefixime	Erythromycin	Gentamycin	Streptomycin
pvkr1	S	S	S	I	R	S	I	S	S
pvkr6	S	S	S	S	R	S	S	S	S
pvkr8	S	S	S	S	S	S	I	S	S
pvkr9	S	S	S	S	S	R	S	S	S
pvkr10	S	I	S	S	R	S	S	S	S
pvkr15	S	I	S	S	R	S	I	S	S
pvkr26	S	I	S	S	R	I	I	S	S
pvkr41	S	I	S	S	R	S	S	S	S
B.l1525	S	R	S	S	R	R	R	I	R
B.l1595	S	R	S	S	R	R	R	S	S

S: Sensitive, I: Intermediate, R: Resistance.

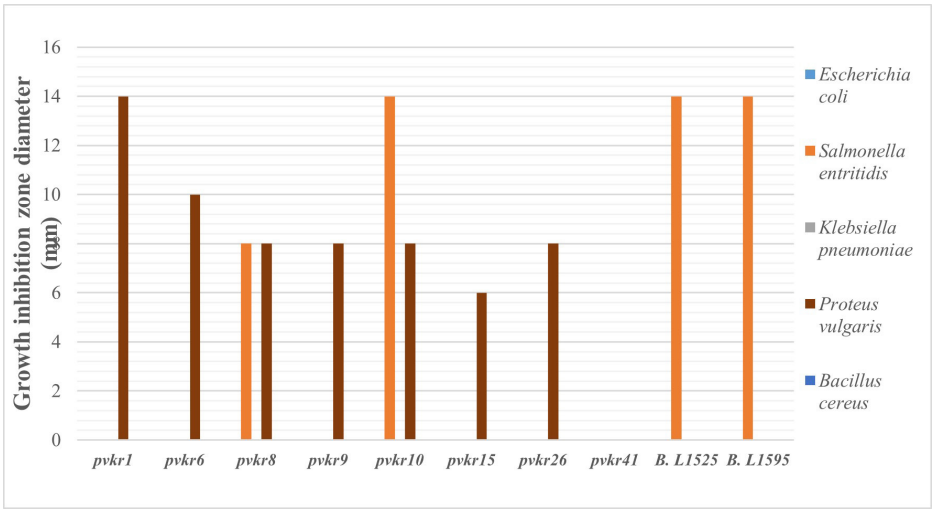

Figure 9. Antagonist properties of the isolated *Bacillus* spp against the pathogenic bacteria.

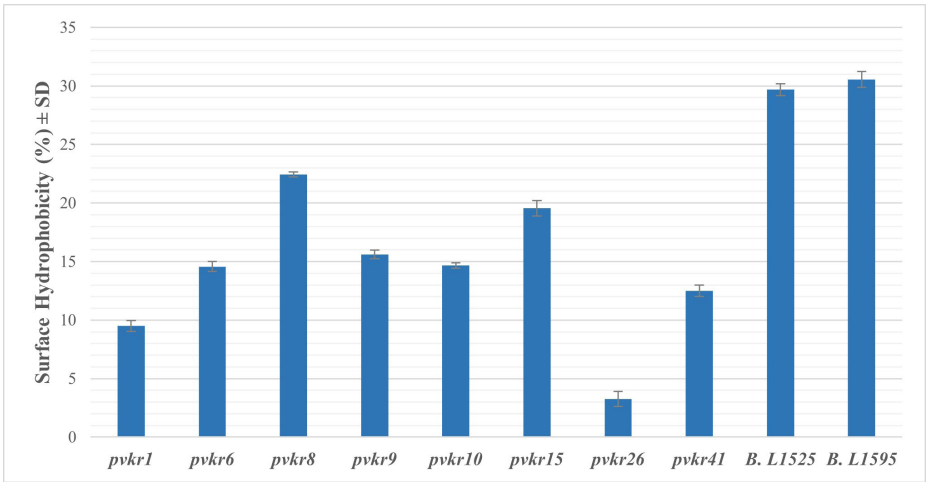

Figure 10. Hydrophobicity percentage of the isolated strains.

Table 3. Resistance of the isolated *Bacillus licheniformis* to bile salts.

Strain	Growth Delay (Min)	Results
pvkr1	D>60 min	Sensitive
pvkr6	D>60 min	Sensitive
pvkr8	40 min <D< 60 min	Weakly tolerant
pvkr9	40 min <D< 15 min	Tolerant
pvkr10	D>60 min	Sensitive
pvkr15	15 min <D< 40 min	Tolerant
pvkr26	D>60 min	Sensitive
pvkr41	D>60 min	Sensitive
B. L1525	D>60 min	Sensitive
B. L1595	D>60 min	Sensitive

Figure 11. Biofilm formation in the isolated strains. The right tube contains one of the isolated strains that has formed biofilm and the left tube is a culture medium

in the livestock and poultry feed industry. There has also been a lot of research on the isolation of spore-shaped *Bacillus* strains with probiotic properties and the results reveal that many *Bacillus* strains have significant probiotic properties and can have very beneficial effects on the host body (Hassan et al., 2013). However, none of the reports examined the simultaneous evaluation of probiotic properties and the ability of these bacteria to degrade keratin. In this experiment, with modified methods and materials, our objective was to isolate *Bacillus* bacteria with probiotic properties and the ability to degrade keratin. Herein, the keratinase-producing bacteria isolated from poultry farm soil identified *Bacillus licheniformis*. In the present work, 42 strains were isolated on suspended soil samples after heat and alcohol treatment. All the isolates were selected and screened to isolate *Bacillus* strains with the selective method. Among all the isolated strains, only eight *Bacillus* strains were able to degrade keratin. The keratinolytic activity of the strains was between 48.91-124.08 U/ml and the highest keratinolytic activity belonged to *Bacillus licheniformis* as *pvkr6*, *pvkr15,* and *pvkr41* strains with 124.08, 101.1, and 100.18 U/ml, respectively. Additionally, the highest keratinase activity was recorded at an enzyme concentration of 0.07 M, which belonged the *pvkr6* with specific activity 54.83 U/ml and total activity 372.25 U/ml and the *pvkr15* with specific activity 50.45 U/ml and total activity 303.29 U/ml at 0.07 M. In our paper, all of the eight *Bacillus* keratinolytic strains produced protease enzyme and the strain isolated from poultry soil revealed that the bacteria producing keratinase enzyme belonged to *Bacillus licheniformis*. Mohamed *et al.* reported that *B. amyloliquefaciens* MA20 and *B. subtilis* have the keratinolytic ability of wool-degrading *bacillus* species and the keratinolytic activity of these strains were in the range of 0.814-0.922 U/ml (Hassan et al., 2013). Mukesh Kumer *et al*. reported that the maximum keratinase and caseinase activity of the *MPTK6* strain were 2.4 and 2.1 U/ml, respectively. Also, the *MPTK6* strain was able to degrade the crude feather to FPH under optimal conditions (30 g/l chicken feather, pH 10.0 and 72 h fermentation) (Kumar et al., 2012). Another study by Deivasigamani and Alagappan (2008) in line with this study indicated that the highest keratinase activity of the isolated *Bacillus* species from slaughterhouse and poultry soil samples applying azo-keratin medium was 0.1225 U/ml).

Previous studies have shown that keratin is digested and degraded in nature with many diverse organisms, including some insects, such as silkworm larvae, carpet beetles, lice (Böckle et al., 1995), and some bacteria, including *Streptomyces pactum* (Seyfi, 2017), *Bacillus licheniformis PWD-1* (Williams et al., 1990), *Chryseobacterium sp.Kr6* and *Streptomyces fradiae* (Riffel et al., 2003), *Bacillus halodurans* (Takami et al., 1992 and 1999), *Fervidobacterium* species (Friedrich and Antranikian, 1996), and some dermatophyte fungi (55), which secrete the enzyme keratinase. In the current research also the strains isolated from poultry soil were keratinase-producing bacteria of the *Bacillus licheniformis* strain.

Kunert (1973) reported that the isolated keratinolytic bacteria from feather with probiotic activities belonged to *Burkholderia*, *Pseudomonas*, *Chryseobacterium,* and *Microbacterium* and were selected and screened on skim milk agar plates for proteolytic activity. Studies have implied that these bacteria could grow on a variety of extract of keratin, such as chicken nail hair and wool, in which all the strains have been reported as keratinolytic protease (Wakil et al., 2011). This study focused on keratinolytic protease production from *Bacillus* strain. The proteolytic activities of all the isolates were detected on the LB agar medium with 1.5% (w/v) skim milk. Among the isolates analyzed, eight isolates showed proteolytic activity. Herein, we investigated the keratinolytic *Bacillus* strains with probiotic potential for antimicrobial susceptibility testing. One of the selection factors of probiotic strains was antibiotic susceptibility of isolated strains (Gueimonde et al., 2013). Therefore, the susceptibility of the strains to antibiotics was measured. The pattern of antibiotic susceptibility has revealed that all the strains were sensitive to the majority of antibiotics except penicillin G. One of the important features of probiotic bacteria is their ability to form biofilm (Salas-Jara et al., 2016). Biofilm formation is one of the

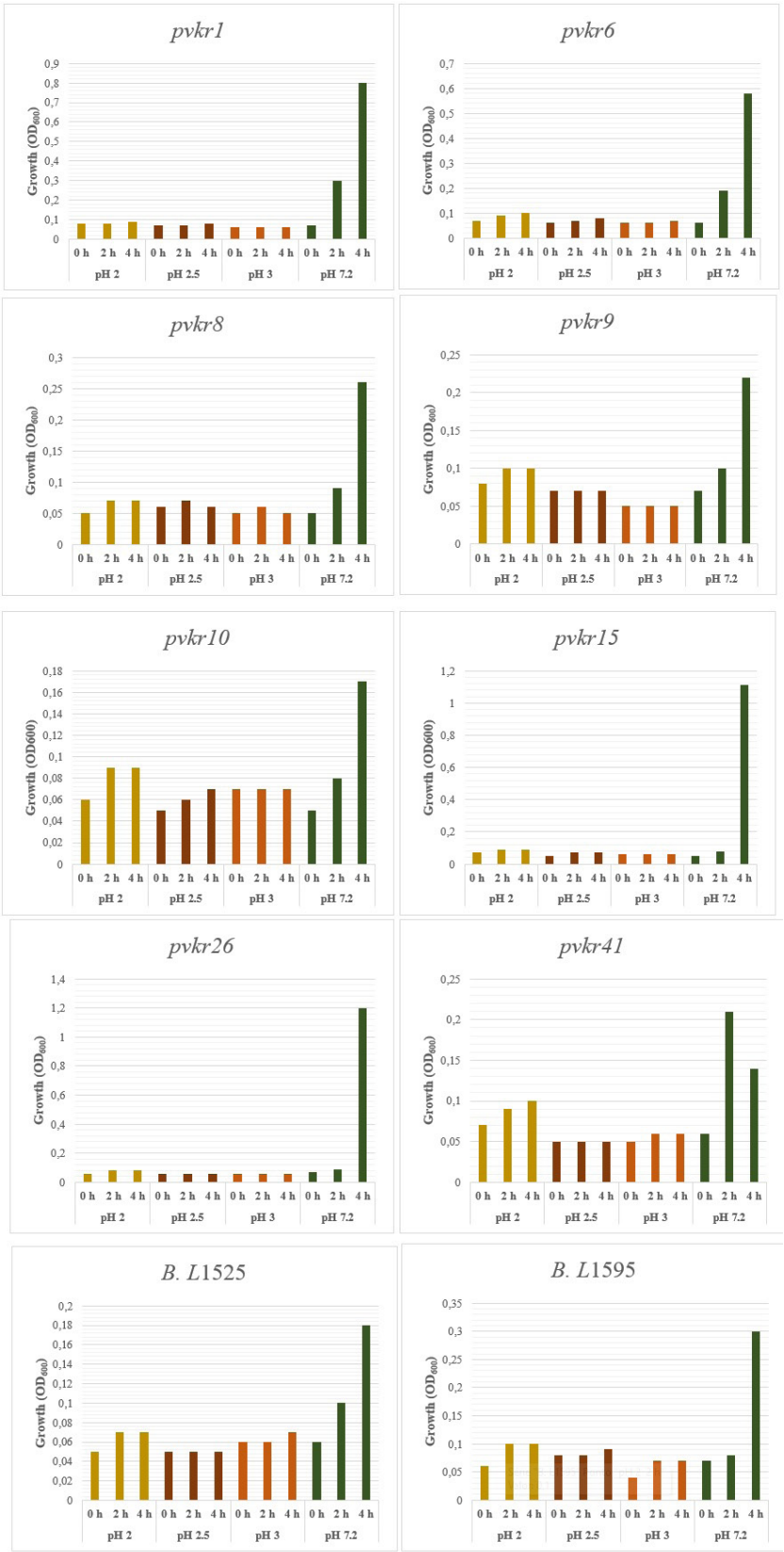

Figure 12. Resistance of the isolated *Bacillus licheniformis* at different pH.

Figure 13. Phylogenic tree illustrates the evolutionary relationships between different 16S rRNA nucleotide sequences of *Bacillus licheniformis*.

central mechanisms in insuring bacterial survival in harsh environments (Zhao et al., 2017). Comparing with free-cells or planktonic cell, the packed bacterial presence in biofilms protects them from environmental factors and anti-microbial treatments. In our work, 100% of isolate were able to form thick biofilms. The range of hydrophobicity was between 3.27-19.65%. To be able to exert their beneficial effects in the gut, probiotic bacteria must have the ability to attach to the cells lining the inner surface of the gut and form a biofilm there. Consequently, the strains with a higher percentage of surface hydrophobicity also had a greater ability to bind to the cells on the inner surface of the intestine. In the present study, the antibacterial activity of the strains was also investigated against some pathogens. *Bacillus licheniformis pvkr15* inhibited the growth of different types of bacteria, such as *Salmonella Enterica,* and *proteus mirabilis*. Acid and bile tolerance in intestinal environment is important for effective functions in the intestines (Kim et al., 2018). This study demonstrated that the high level of survival under acidic conditions belonged to all the strains except *pvkr26*. Bile salts have been shown to inhibit bacterial growth by disordering cell membranes. Some *Bacillus* spps are sensitive or weakly tolerant to bile salt concentrations (Zulkhairi Amin et al., 2019). Although, in our study, the survival rates of *Bacillus pvkr9*, *pvkr15* strains in 0.3% bile salt were higher than of those of other strains. This finding implied that *pvkr9* and *pvkr15* are resistant to bile salts in the intestines and could pass through this area and have probiotic effects. According to a study by Hironimus *et al*., the *Bacillus Laevolacticus, Bacillus racemilacticus*, and *Bacillus coagulans* strains were resistant to pHs 2, 2.5, and 3, and *Bacillus racemilacticus* and *Bacillus coagulans* strains were resistant to bile salts with 0.3% concentration (Hyronimus et al., 2000). As described, the results of these researchers are consistent with the results of the present study. The probiotic bacteria should not lyse red blood cells of the host. Our experiments showed that only the *pvkr15* strain could be classified as probiotic and all the other stains caused the lyses of red blood cells. Hemolytic activity is one of the most common assays to distinguish pathogenic bacteria form probiotics. In this experiment, only γ-hemolytic bacteria were *Bacillus licheniformis pvkr15* and *Bacillus licheniformis PTCC 1595* (control strain). In the isolated *Bacillus licheniformis* strains, only *Bacillus licheniformis pvkr15* was γ-hemolytic and the other strains exhibited α-hemolytic activity, which could not be classifies as probiotic bacteria. The results revealed that the *pvkr8* and *pvkr15* strains respectively had a cell surface hydrophobicity of 22.44% and 19.56%. This adherence is essential for probiotic bacteria affecting the intestines. Thus, *Bacillus licheniformis pvkr15* as a keratinolytic spore forming probiotic *Bacillus* strain is suitable as a nutritional supplement in humans, fish, and animal.

5. Conclusion

The results revealed that only the *Bacillus licheniformis pvkr15* produced acceptable keratinase enzyme and demonstrated potential for surviving in the gut environment with the ability to produce spores. Hence, this strain can be employed as a probiotic in animal and chicken feed industry. Other strains, despite having some probiotic characteristics, showed fundamental disadvantages, disqualifying them from being used for this purpose.

Acknowledgements

The authors acknowledge the Microbiology Department, Ayatollah Amoli Branch, Islamic Azad University And about the name of the last author acknowledge the Biology Department, Ayatollah Amoli Branch, Islamic Azad University.

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