Isolation, Screening, Characterization and Identification of Alkaline Protease Producing Bacteria from Leather Industry Effluent

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Abstract

Background

A wide variety of Bacterial species produces protease enzyme and the application of same enzyme have been manipulated precisely and used in various biotechnological areas including industrial and environmental sectors. The main aim of this research study was to isolate, screen and identify protease producing bacteria which were sampled from leather industry effluent present in the outer skirts of Addis Ababa, Ethiopia.

Purpose

To isolated alkaline protease producing bacteria from leather industrial effluents and to characterization (Secreening and identification).

Methods

Sample collected from Modji leather industrial effluents and stored in the microbiology lab. After isolated bacteria from effluent using serial dilution and followed by isolate protease producing bacteria using skim milk agar media. After studying Primary and secondary screening using zonal inhibition methods to select potential protease producing bacteria using skim milk agar media. Finally to characterization and identification of potential bacteria using biochemical methods, protein estimation, biomass, protease assay and gene sequencing (16S rRNA) method to finalized best protease producing bacteria.

Results

Twenty-eight different bacterial colonies were isolated initially from the leather industry effluent sample situated at Modjo town of Ethiopia. The isolated bacteria were screened using primary screening method with skim milk agar medium. Three isolates namely MS12, ML5 and ML12 showing highest zone of proteolysis as a result of casein degradation on the agar plates were selected and subjected to secondary screening. Further secondary secreening confirmed that MS12, ML5 and ML12 has efficient proteolytic activity and can be considered as potent protease producer. The three isolates were then subjected to morphological and biochemical tests to identify probably bacterial species and all the three bacterial isolates were found out to be of Bacillus species. Shake flask method was carried out to identify the most potent one having greater biomass production capabilities, protein quantity and protease activity. ML12 isolated from leather effluent waste showed highest Protein(170mg/ml), Protease activity(19U/ml), high biomass production and the same was subjected to molecular identification using 16s sequencing and a Phylogenetic tree was constructed to identify the closest neighbor. The isolate ML12 is 97.87% homologous to Bacillus cereus strain (KY995152.1) and 97.86% homologous to Bacillus cereus strain (MK968813.1).

Conclusions

This study has revealed that the leather industry effluent site has significant feature of housing potent bacterial species producing protease of commercial value. Being one among the most widely used enzyme, comparatively. Protease holds a larger scope for research and commercialization any other type of enzymes. There is a need to develop novel protease enzymes for further necessary applications of these enzymes. Moreover, enzyme produced by bacteria which are present in effluents are a greater boon to establish the significance of converting industrial wastes to a highly valuable enzymes especially like proteases.

Background

Cells of every living organism consist of a chemical substance which possess the ability to catalyze or speed up a biochemical reaction and acts as biocatalysts, which are known as enzymes. Enzymes have better catalytic efficiency, adjustable activity and high specificity when compared to that of catalysts of chemical or synthetic origin. These advantages have broaden the application of enzymes in various industries such as chemical, food, pharmaceutical and etc. (Pires-Cabral et al. 2010; Yücel 2012; Chandran Masi et al. 2017). This has generated a greater demand for enzyme production of high quality by cost-effective and commercial methods. Due to its importance, almost every form of life on earth possess protease enzyme as an important factor of their physiological function. Though the protease enzymes are produced by different forms of life, due to its flexibility towards genetic manipulation, the ones that produced by microbial sources such as bacteria and fungi are more preferred rather than human or plant protease enzyme(Chandran et al. 2014; Tiwari et al. 2015). Proteases are considered as the most useful and a powerful enzymes as they break down complex proteins compounds into amino acids and peptides(Gupta et al. 2002; Verma et al. 2011; Chandran Masi et al. 2015).Around 60% of global enzyme usage is accounted by protease enzymes. Alkaline proteases are the most industrially used or exploited enzymes. Prominent bacterial producers of this enzyme are *Pseudomonas sp.*, *Bacillus sp.*, *Staphylococcus sp.* and *Aeromonas sp.* (Saha et al. 2011; Chandran masi et al. 2017). The main purpose of this study is isolation, screening, Characterization and Identification by morphological and biochemical aspects of potent protease producing bacteria from leather industry effluent.

Methods

1. Materials Used

Chemicals, reagents and culture media necessary for the experiment such as skimmed milk agar, ethyl alcohol, Luria Bertaniagar, casein, nutrient agar, safranin, phenol red, urea, starch, sodium chloride, magnesium sulfate, sodium nitrate, glucose, calcium chloride, hydrogen peroxide, peptone, beef extract, yeast extract, ammonium sulfate, potassium sodium tartrate, bovine serum albumin (BSA), fructose, mannitol, galactose, nutrient broth, di potassium phosphate, Folin’s reagent, tri-chloro acetic acid (TCA) and sucrose of highest purity were used.

2. Description of the Sampling Area

Modjo is a town present in the East Showa Oromia regional state of Ethiopia. The geographical coordinates of the town are 8°35' N and 39°07' E. Its elevation above sea level is seen between 1788 m and 1825 m. The town harbors Modjo tanning industry is located 80 kms south of Addis Ababa. The Modjo Tanning Industry is an average sized leather industry. It has a goat and sheep skin processing unit with a capacity of 844,000 to 1,656,000 skins that can be processed annually. Effluent waste of 3500–5500 cubic meters per day from the Modjo Tanning industry is channeled towards the Modjo River (Amanial 2016).

3. Source of Sample Collection

Soil samples designated as MS and water effluents which were designated as ML are collected from different areas of Modjo leather industry. Soil samples from the mentioned site were collected from the wastes of hides and skin. Effluent samples are collected from the site where the effluents are pumped out from the Modjo Tanning Industry. The soil samples were taken in sterile plastic bags and the water sample were taken in sterile bottle. Both samples were taken to the Microbiology laboratory at Addis Ababa Science and Technology University, Ethiopia. The samples are stored at 4^{o}c for further steps.

4. Isolation of alkaline Protease Producing Bacteria from the leather effluent

Serial dilution technique was carried out to reduce the microbial load. 9 ml of normal saline was prepared in 9 test tubes and 1 ml of the effluent sample was added to the first tube which has a dilution of 10^{-1} and serially diluted up to 10^{-9} dilution. The same procedure was carried out for the soil sample where 1 gram of collected soil was added to the first tube and serially diluted up to 10^{-9} dilution. Serial dilution and spread plate method were the techniques used to isolate the target bacteria. As bacterial sample is the targeted microorganism, the dilution 10^{-5} is used for further tests. Luria Bertani (LB) agar medium was prepared and sterilized by autoclave and poured onto the Petri plates. Spread plating technique is carried out for the growth of bacterial consortium by adding 0.1 ml of the 10^{-5} diluted sample and spread using L-rod. The agar plates were then incubated at 30^{o}c for 24 hours and observed for microbial growth. Consortiums of bacteria showing various colony morphology were observed in the plates. Similar colonies were considered to be same bacteria whereas; different colony morphology is exhibited by different microorganisms. Thus, the distinct colonies are then isolated to obtain pure colonies by streak plate method. The same LB agar medium was prepared and sterilized and then streaked with each distinct colony by quadrant streaking. The plates are incubated at 30 ^{o}c for 24 hours and then observed to isolated pure colonies of bacteria obtained from leather industrial waste site (Sneha et al. 2014).

5. Screening of bacterial isolates for alkaline protease production

5.1. Primary Screening of potential alkaline Protease Producing bacterial Isolates

Primary screening of bacterial isolates was made in order to screen alkaline protease producers using 1% skim milk agar medium by spot inoculation of the isolates using 2 mm toothpick heads and were incubated at room temperature. A clearance zone around the inoculated site as a result of proteolytic activity was observed for 3 days. Isolates seen with clearance zone of more than 10 mm were selected and proceeded for secondary screening (Masi Chandran et al. 2014).

5.2. Secondary screening of potential alkaline Protease Producing bacteria

Selected bacterial isolates from primary screening were grown in Nutrient broth medium. Then, 1% of the grown cultures were again inoculated in a freshly prepared nutrient broth medium and incubated in a rotary shaker for 2 days at room temperature. Post-incubation, centrifugation of the cultures was carried out at 10000 RPM for 15 minutes at 4^{o}c and the supernatant is collected. 1% Skim Milk Agar plates were prepared and wells were punched using a sterile cork borer of 9 mm. Agar plates with 1% skim milk is prepared and sterilized and inoculated with. 100 µl of the collected supernatant of all the selected bacteria and then incubated at 30^{o}c for 2 days. The plates were observed for zone of proteolysis around the inoculated wells. Colonies which show high zone of hydrolysis is selected and cultured in nutrient broth medium and also preserved in agar slants at 4^{o}c for further studies (Sneha et al. 2014, Chandran Masi et al. 2017).

6. Characterization and Identification the best isolates

Colonies which showed efficient proteolytic activity in secondary screening are then subjected to bacterial characterization studies such as morphological and biochemical tests as demonstrated by (Sharmin et al., 2005) which is modified from the Bergey's Manual of determinative Microbiology to find the probable bacteria.

6.1. Morphological characterization of bacterial isolates

Colony characters of all the isolates are observed based on the bacterial growth on nutrient agar plates. Microscopy-based morphological studies including Gram staining, motility test and endospore staining was done (Masi Chandran et al. 2014).

6.1.1. Colony Characters

The selected isolates were inoculated on nutrient agar plates by pour plate method and incubated at 30^{o}c for 24 hours and observed for colony characters such as color, shape, surface and opacity.

6.1.2. Motility Test

The preserved agar slants of the isolates are taken and inoculated in a cover glass using inoculating needle. The cover glass is affixed on a concavity slide and focused under 45x objective to observe the presence or absence of motile rods (Yimer et al. 2016).

6.1.3. Gram Staining

After preparing a 24-hour old culture, it was smeared on the slide following fixing it with heat. it was treated with crystal violet for 2 minutes following rinsing it with running water.it was again flooded with gram iodine solution for 2 minutes following washing it with alcohol. On the last, it was flooded with safranin, washed with water, dried and observed under oil immersion(Harley and P Harley 2002).

6.1.4. Endospore staining

This staining was done by heat fixing the smear on the microscopic slide. Malachite green was used to show the endospore. The slide was kept in hot beaker for 5 minutes. Safranin was added and washed away. it was observed under electronic microscope (Bergey et al. 1994).

6.2. Biochemical Characterization of Isolates

6.2.1. Catalase test

3% H_2O_2 was used to detect Catalase taste. An overnight culture cell was mixed with hydrogen peroxide on the microscopic slide by the help of sterilized loop. The formation of bubble during and after mixing was recorded as positive result (Boominadhan et al. 2009).

6.2.2. Starch hydrolysis

Starch hydrolysis test was done by streaking the culture on starch agar plate using inoculating loop. After labeling the test sample and control, it was incubated for 24hours and observed for zone of hydrolysis of starch(Harley and P Harley 2002).

6.2.3. Gelatin hydrolysis

Preparation and sterilization of gelatin media as agar deep tubes was done using autoclave. Stab inoculation of each isolate on the prepared gelatin media was done using inoculation loop. The inoculated tubes are then subjected to incubation at 30^{o}c for a period of 48 hours and then placed in a refrigerator for a period of 15 minutes at 4^{o}c to observe gelatin liquefaction (Alnahdi 2012).

6.2.4. Urea hydrolysis test

The broth of urea agar medium was prepared and inoculated with isolated bacteria .Both test and control tubes were incubated at 37 °C. The slant was observed for color changes for every 6 hours, and then for every 24 hours for up to 5 days. Phenol red was used as an indicator(Harley and P Harley 2002).

6.2.5. Indole production test

Peptone broth medium was prepared and autoclaved. After cooling the medium, it was inoculated and incubated at 30^{o}c for 48hours by keeping the control. Few drops of Kovac's reagent were added step by step. The tubes were shaken and allowed to stand for 10 minutes, to form the layer. A presence of red colored on the top of the tube is indicative of a positive result (Kiran et al. 2002).

6.2.6. Citrate utilization test

Preparation and sterilization of Simmons citrate agar tube is done and inoculation of all the isolates by the process of stab inoculation and incubated at 37°C for a period of 24 hours. A positive result is indicated by the color change from green to intense Prussian blue color (Pandian et al. 2012).

6.2.7. Carbohydrate fermentation test

Carbohydrate fermentation test using various carbohydrates such as glucose, sucrose, lactose, and mannitol and galactose was carried out for the selected isolates in an anaerobic condition. Nutrient broth which is incorporated with any one of the above-mentioned carbohydrates along with a pH indicator phenol red which is red at pH 7 (neutral) and yellow at a pH of 6.8 (acidic) is used as the medium for carbohydrate fermentation test. This specified carbohydrate containing media is taken and inoculated with the isolates and Durham tubes are placed inside in an inverted position. The media is incubated in an anaerobic condition for 24 hours at 30^{o}c. Fermentation of carbohydrate results in the formation of organic acids which changes the color of the media from pink to yellow, along with liberation of gases which is entrapped in the Durham tubes (Harley and P Harley 2002; Agrahari and Wadhwa 2010)

6.2.8. Methyl red test

Sterile MR-VP broth tubes were prepared and inoculated with the selected isolates and incubated at a temperature of 30^{o}c for 2 days. Post-incubation, the tubes were added with 5 drops of methyl red and were observed for any color change. A positive result is indicated by the color change from yellow to red after the addition of methyl red within 10 to 15 seconds (Mazotto et al. 2010).

6.2.9. Voges-Proskauer Test

Glucose phosphate broth (Voges-Proskauer) broth was autoclaved and cooled to the room temperature. The 24-hour cultures of the selected isolates were inoculated using sterilized loop followed by incubation at 30^{o}c for a period of 48hours.Post-incubation, 1 ml alpha-naphthol was added and shaken then followed by the addition of 0.5 ml of 40%KOH to the broth and shaken. Red color development after the addition of the reagents within one hour was taken as positive result (Minghai Han 2012).

6.2.10. Triple sugar iron test (TSI)

Bacterial gas production can be observed using triple sugar iron (TSI) agar slants. The TSI agar consists of a dye which is pH sensitive known as phenol red along with 1% of three sugars namely lactose, sucrose and glucose and then sodium thio sulfate, ferrous sulfate and finally agar(Harley and P Harley 2002; Sharma 2007). A positive result is indicated by the blackening of the agar slants as a result of H_2S production.

7. Shake flask studies for screening of isolates

The selected isolates from secondary screening is subjected to shake flask study which is done in accordance with the protocol of (Khan et al. 2011) with some modifications. Protease production media (Tryptone 1%, Yeast Extract 0.5%, Skim Milk 1% and NaCl 0.5%) of 25 ml is prepared and sterilized using autoclaved. A loop full of the culture from fresh slants of isolates selected by secondary screening was

taken and inoculated in the sterilized media and incubated in a rotary shaker at 250 RPM for 18 hours at 30 ^{o}c. The biomass production of all the selected isolates was analyzed using UV Visible Spectrophotometer at 660 nm absorbance to determine the efficient protease producing bacteria.

7a. Determination of total protein concentration

The supernatant will be collected from the above method is processed further to determine the total protein concentration present in the medium by Lowry's method. The phenol group of Tyrosine and Tryptophan residues (amino acid) in a protein will produce a blue purple color complex, with maximum absorption in the region of 660 nm wavelength, with Folin- Ciocalteau reagent which consists of Sodium tungstate molybdate and Phosphate.

7b. Determination of protease activity

The universal protease assay with Casein as substrate will be followed to determine the protease activity of the potential protease producing bacterial strains. One ml of potential protease producing bacterial sample will take after 24 hrs of incubation with nutrient broth medium and centrifuged at 10,000 rpm for 10 minutes at 4^{o}C.The tyrosine standard curve will be obtained from the absorbance of the tyrosine concentrations (10 µg, 20 µg, 30 µg, 40 µg, 50 µg, 60 µg) to determine the protease activity.

8. Molecular Identification of the selected isolate

The isolate having highest biomass production in shake flask study was identified using molecular technique. The genomic DNA of the isolate was extracted by using Bacterial Genomic DNA extraction kit according to the manufacturer protocol (QIAGEN, QIAamp DNA Mini Kit) with some modification. The isolated DNA were then amplified using the following PCR mix: 1 µl of Bacterial universal 16S rRNA primers forward E9F (5-GAGTTTGATCCTGGCTCAG-3) (Farrelly et al. 1995)and 1 µl of Reverse primer U1510R (5-GGTTACCTTGTTACGACTT-3) (Reysenbach et al. 1995), 2 µl of Genomic DNA and 6 µl of PCR grade water were added and the PCR amplification was done. Amplified sequence threads were submitted to NCBI database and NCBI BLAST (http:// www.ncbi.nlm.nih.gov/Blast) was carried out to distinguish the nearest neighbors of the isolates and then phylogenetic tree was constructed.

Results

1. Alkaline protease isolation from leather industry effluent bacteria

Bacteria from leather industrial effluent site were subjected to serial dilution and then preceded for pour plating method in LB media to observe bacterial consortium. Based on colony morphology, each distinct morphological character was considered as different bacterial species and was subjected to streak plate method for pure colony isolation and was shown in Figure 1 and Figure 2. A total of 28 different Protease producing bacterial isolates were isolated from the leather industry effluent sample. The isolates were sub-cultured and maintained in LB media for future tests.

2. Primary and Secondary Screening of potential alkaline Protease Producing bacterial Isolates

All the 28 isolates were subjected to primary screening for the process of identification of potential protease producing species. The primary screening of potential protease producing bacteria is indicated in Table 1 and Figure 3. Three distinct isolates namely ML12, MS12 and ML5 having highest zone of inhibition were selected for secondary screening and the results are tabulated in Table 2 and shown in Figure 4.

Table 1. Primary Screening of the Bacterial Isolates

S.No.	Isolate Code	Zone of Lysis	S.No.	Isolate Code	Zone of Lysis
1.	MS1	12 mm	15.	MS15	8 mm
2.	MS2	8 mm	16.	MS16	9 mm
3.	MS3	8 mm	17.	ML1	7 mm
4.	MS4	7 mm	18.	ML2	9 mm
5.	MS5	9 mm	19.	ML3	11 mm
6.	MS6	9 mm	20.	ML4	9 mm
7.	MS7	8 mm	21.	ML5	14 mm
8.	MS8	13 mm	22.	ML6	9 mm
9.	MS9	7 mm	23.	ML7	13 mm
10.	MS10	6 mm	24.	ML8	13 mm
11.	MS11	8 mm	25.	ML9	9 mm
12.	MS12	15 mm	26.	ML10	10 mm
13.	MS13	8 mm	27.	ML11	6 mm
14.	MS14	10 mm	28.	ML12	18 mm

Table 2. Secondary Screening of the Bacterial Isolates

S.No.	Isolate code	Diameter (in mm)
1.	ML5	14±0.75
2.	ML12	19.5±0.66
3.	MS12	15±1.32

3. Characterization and identification of screened bacterial isolates

3.1. Morphological characterization of bacterial isolates

The three selected bacterial isolates namely ML5, ML12 and MS12 were grown on nutrient agar medium to study its morphological characteristics. ML5 and MS12 isolates showed creamy red colonies, whereas ML12 showed white colonies. The colonies of all the three isolates were smooth and opaque. Gram staining results revealed that all the three isolates are motile rods possessing endospores where ML5 and MS12 are gram negative and ML12 is gram positive. Morphological characteristics of the three isolates are tabulated in Table 3 and the gram staining results were shown in Figure 5.

Table 3. Morphological Characteristics of Selected Bacterial Isolates

Morphological Features	ML5	ML12	MS12
Color	Creamy red	White	Creamy red
Shape	Rod	Rod	Long Rod
Surface	Smooth	Smooth	Smooth
Opacity	Opaque	Opaque	Opaque
Gram staining	Positive	Positive	Positive
Motility	Motile	Motile	Motile
Endospore staining	Positive	Positive	Positive

3.2. Biochemical characterization of bacterial isolates

An extensive biochemical characterization of all three isolates was carried out and is tabulated in Table 4. Results of carbohydrate fermentation test revealed that all three isolates ferments sucrose, glucose and galactose. Lactose is fermented only by the isolate ML5 whereas, Fructose is fermented by MS12 and ML12 and finally mannitol is fermented by MS12 and MS5. All the three isolates show positive for hydrolysis of starch and casein and liquefies gelatin. IMViC test shows that ML12 and ML5 were positive for methyl-red test, MS12 and ML5 showed positive results for Voges-Proskauer test and MS12 showed positive for citrate utilization test. The isolate ML5 showed positive for Urease whereas all the three isolates showed positive for Catalase test.

Table 4. Biochemical Characteristics of Selected Bacterial Isolates

Biochemical test	MS12	ML12	ML5
Sucrose	Positive	Positive	Positive
Lactose	Negative	Negative	Positive
Glucose	Positive	Positive	Positive
Fructose	Positive	Positive	Negative
Mannitol	Positive	Negative	Positive
Starch	Positive	Positive	Positive
Galactose	Positive	Positive	Positive
Indole test	Negative	Negative	Negative
Methyl red test	Negative	Positive	Positive
Urease test	Negative	Negative	Positive
Voges-Proskauer test	Positive	Negative	Positive
Casein	Positive	Positive	Positive
Citrate utilization test	Positive	Negative	Negative
Gelatin	Positive	Positive	Positive
Catalase test	Positive	Positive	Positive
Similarity of bacteria	*Bacillus sp.*	*Bacillus sp.*	*Bacillus sp.*

4. Shake flask results for screening of isolates

Three bacterial isolates which were subjected to secondary screening were selected for quantification of its bacterial biomass, protein estimation and protease activity using spectrophotometric method. Protease activity, protein quantification and bacterial biomass of the selected isolates were measured using shake flask method by incubating the isolates on protease production media. The biomass of each isolate, its protein quantity and protease activity were measured by spectrophotometer at 660nm by using casein as a substrate and their results are shown in Figure 6,7 and 8. Out of the three isolates studied, ML12 showed highest intensity of absorbance proving biomass, highest protein (170mg/ml) and protease activity (19U/ml) to be an effective candidate for the commercial production of alkaline protease enzyme.

5. Molecular identification

Genomic DNA of the selected bacterial isolate ML12 was extracted and the same was amplified by polymerase chain reaction (PCR) and visualized using agarose gel electrophoresis (Figure 9). Blast result showed that ML12 was 97.86 % homologous to nucleotide of *Bacillus cereus* strain (MK968813.1) and

97.87% homologous to *Bacillus cereus* strain (KY995152.1). A phylogenic tree was constructed as shown in Figure 10 based on 16S rRNA gene sequences of isolate ML12 (*Bacillus cereus – MN629232.1*) and its related nucleotide sequences was used to construct the phylogenic tree using the mega x software and neighbor-joining method.

Discussions

Our present study reported twenty-eight isolates out of which four bacterial samples (21.4%) are notable for their property of discharging protease enzyme as an extracellular product. On the contrary, the study (Wery et al. 2003;Masi Chandran et al. 2014) conducted with samples collected from Antarctica, revealed successful isolation of seventy-five aerobic heterotrophy with using a packed column bioreactor for the process of production of protease. Similarly, (Ravishankar 2012) reported isolation of six bacterial isolates from fish market waste soil having the ability of producing protease for commercial production. (Hadush et al. 2017) isolated 147 colonies from three sampling sites, out of which 85 isolates were found to be effective for production of protease. The main reasons for getting larger number of positive isolates are due to larger sample size and higher number of sampling site. Results of the study conducted by (Gupta et al. 2005), proved the protease production ability of Streptomycin sp. CD3 isolates. The isolated strains were subjected to primary and secondary screening for the purpose of identification of protease producing ability. In accordance with the results of primary screening of 28 isolates, highest clearance zone was formed by three isolates namely ML 5, ML12 and MS 12 isolates (14, 18 and 15 mm respectively). Similarly, the secondary screening showed a diameter of 14±0.75, 19.5±0.66 and 15±1.32 for ML 5, ML12 and MS 12 revealing the highest hydrolysis zone was recorded by the isolate ML12. Bacterial morphological studies based on microscopic analysis and colony morphology is carried out as stated by (Ahmad and Ansari 2013) of the three isolates revealed that all the three were motile rod-shaped bacteria possessing endospores where ML12 isolate is determined to be Gram positive and the rest two isolates ML5 and MS12 were Gram negative. Colony characteristics of the three isolates when grown on nutrient agar plates revealed that all three isolates are smooth and opaque colonies where ML12 isolate was white in color and the rest MS12 and ML5 isolates are red in color. Biochemical identification of the three isolates were carried out as per the method prescribed by (Harley and P Harley 2002; Boominadhan et al. 2009 ; Pandian et al. 2012;Kiran et al. 2002;Mazotto et al. 2010; Minghai Han 2012) revealed that all the three isolates hydrolyzes starch, casein and gelatin and ferments sucrose, glucose and galactose but not lactose. Fructose is fermented by MS12 and ML12 isolates but not ML5 and mannitol is fermented by MS12 and ML5 but not ML12. The three isolates were. Based on the comparison of the biochemical results with the studies (Bergey et al. 1994), all three isolates were found out to be of *Bacillus species*. Generally bacterial isolates of Bacillus species are proven to be effective for the production of extra cellular proteases and the same fact was proved valid upon comparing the results of the study conducted by (Alnahdi 2012; Varma et al. 2011) stating that out of the six isolates screened for protease production, two were found to be positive. Shake flask test revealed the higher biomass production ratio, protein quantity and protease activity of ML12 and its commercial potential for a larger

product recovery. Hence ML12 isolate was considered for molecular identification and with the aid neighborhood method it was found out to be *Bacillus cereus* (Alnahdi 2012; Kumar et al. 2016).

Conclusions

The present study was focused on isolating, screening, characterization and identification of alkaline protease producing bacteria from leather industry effluents. Twenty-eight different bacterial isolates were identified from the leather industry effluent. Among them, three bacterial isolates showed potent proteolytic activity on skim milk agar plate assay. The proteolytic bacterial isolates were then further screened for protease production using shake flask method and isolate ML12 was observed to have higher protease enzyme producing potential. Based on morphological and biochemical characteristics the strain was identified as *Bacillus* species which was later identified as *Bacillus cereus* using 16S rRNA sequencing techniques.

Declarations

Ethics Approval and Consent to Participate

Not applicable

Consent for Publication

Not applicable

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

All authors are carried out the collection of sample from Modjo leather industry effluent. M. Chandran Masi & Getachew Gemechu carried out isolation of potential bacteria. Mesfin Tafesse & Chandran Masi carried out the identification of bacteria. All authors are contributed by final confirmation of potential

alkaline protease producing bacteria and manuscript revisions. All authors have approved the final version of the manuscript and agree to be held accountable for the content therein.

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Figures

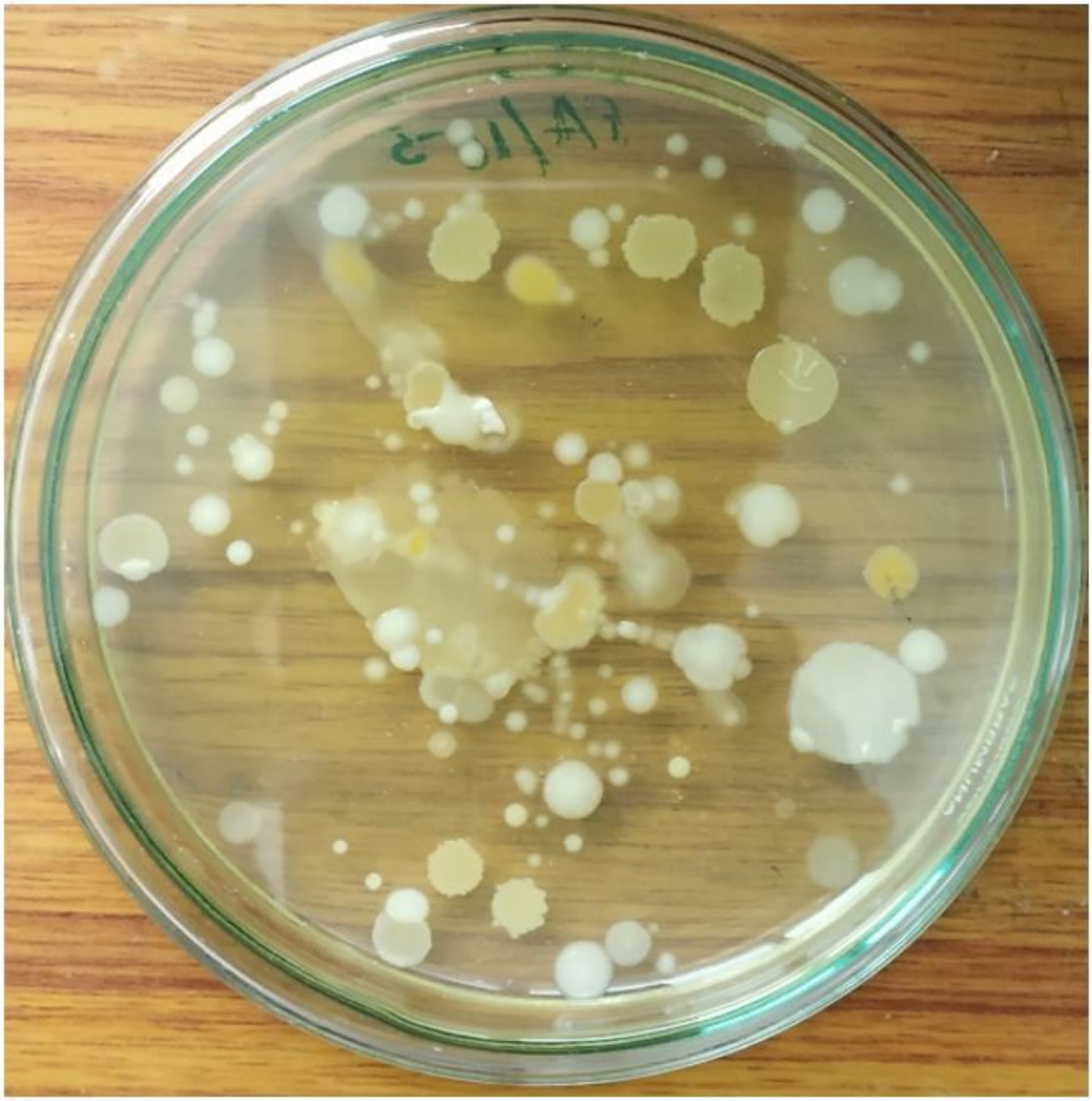

Figure 1

Bacterial Isolation by Pure Plate Technique

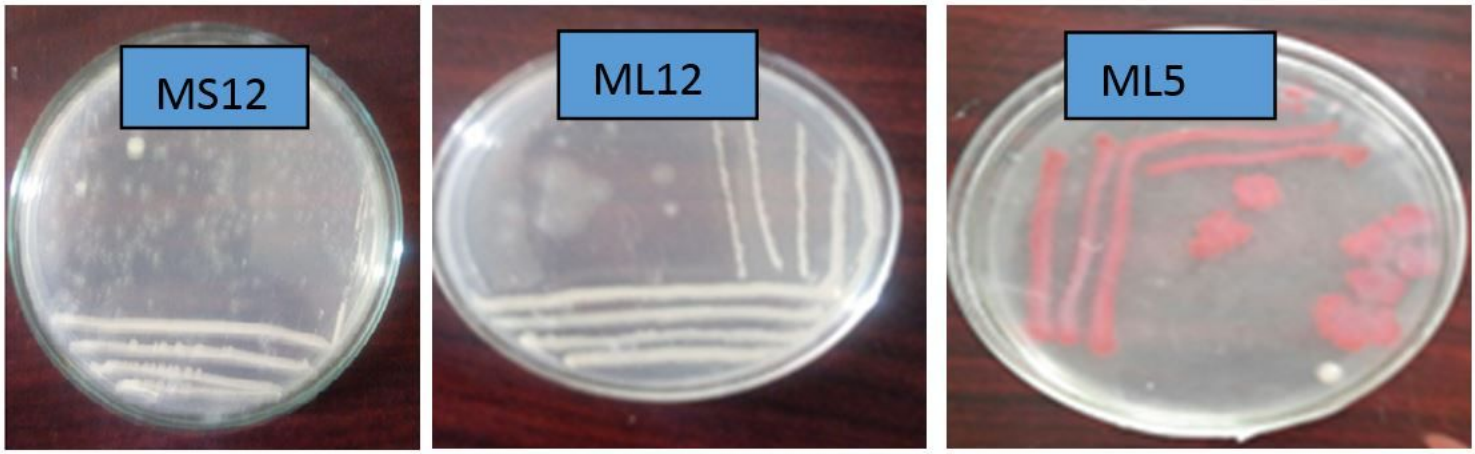

Figure 2

Single Colony Isolation by Streak Plate Technique

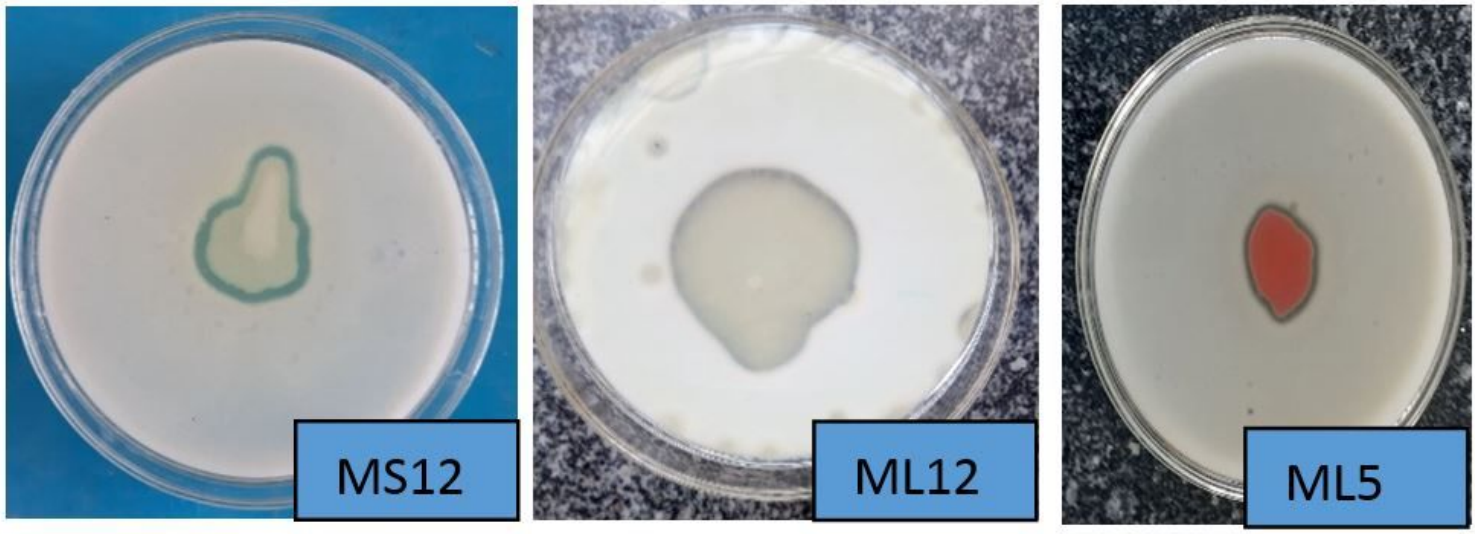

Figure 3

Primary Screening of Bacterial Isolates

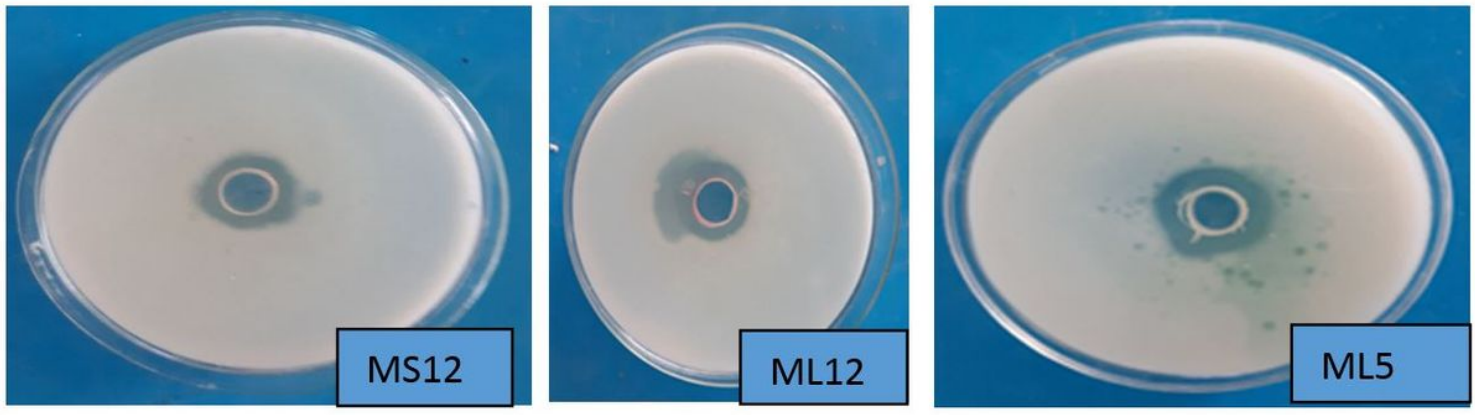

Figure 4

Secondary Screening of Bacterial Isolates

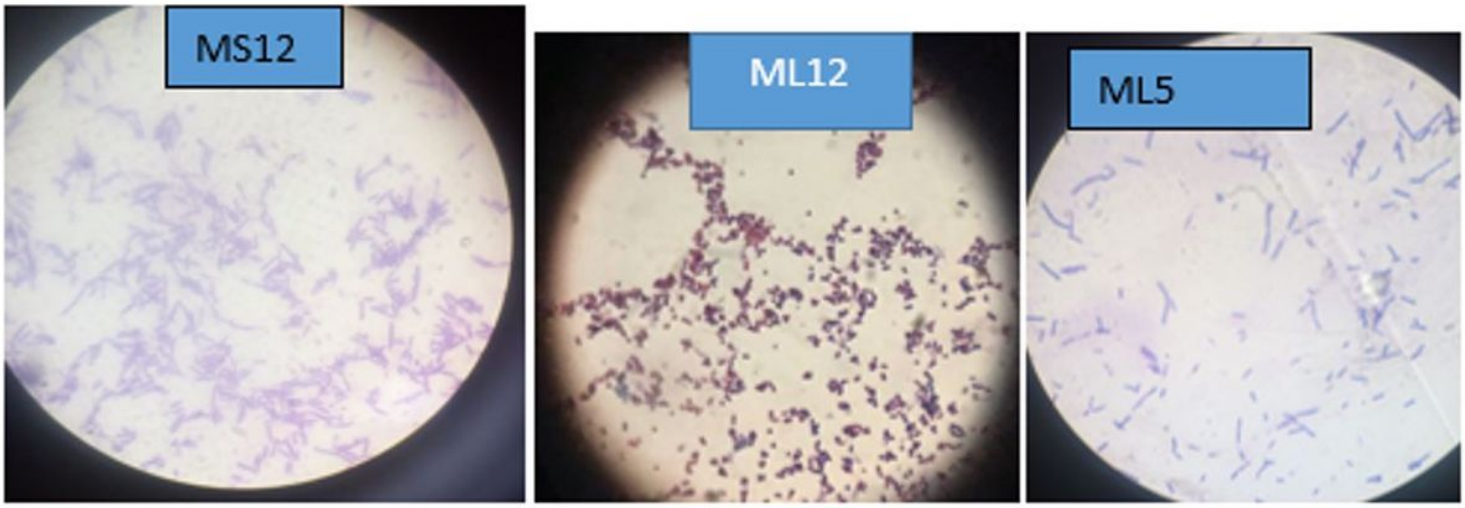

Figure 5

Gram Staining of Selected bacterial isolates

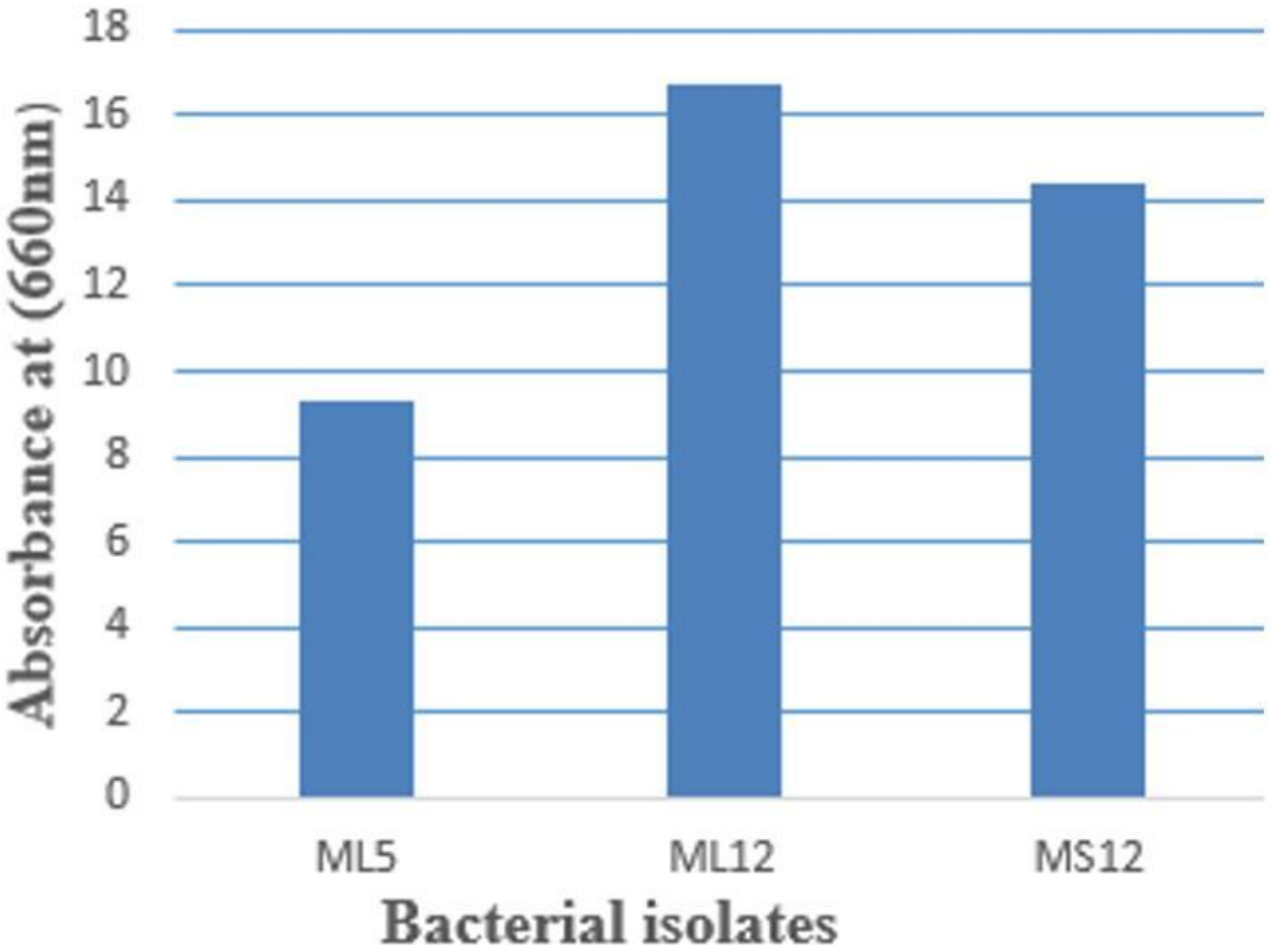

Figure 6

Biomass Production by Selected Bacterial Isolates

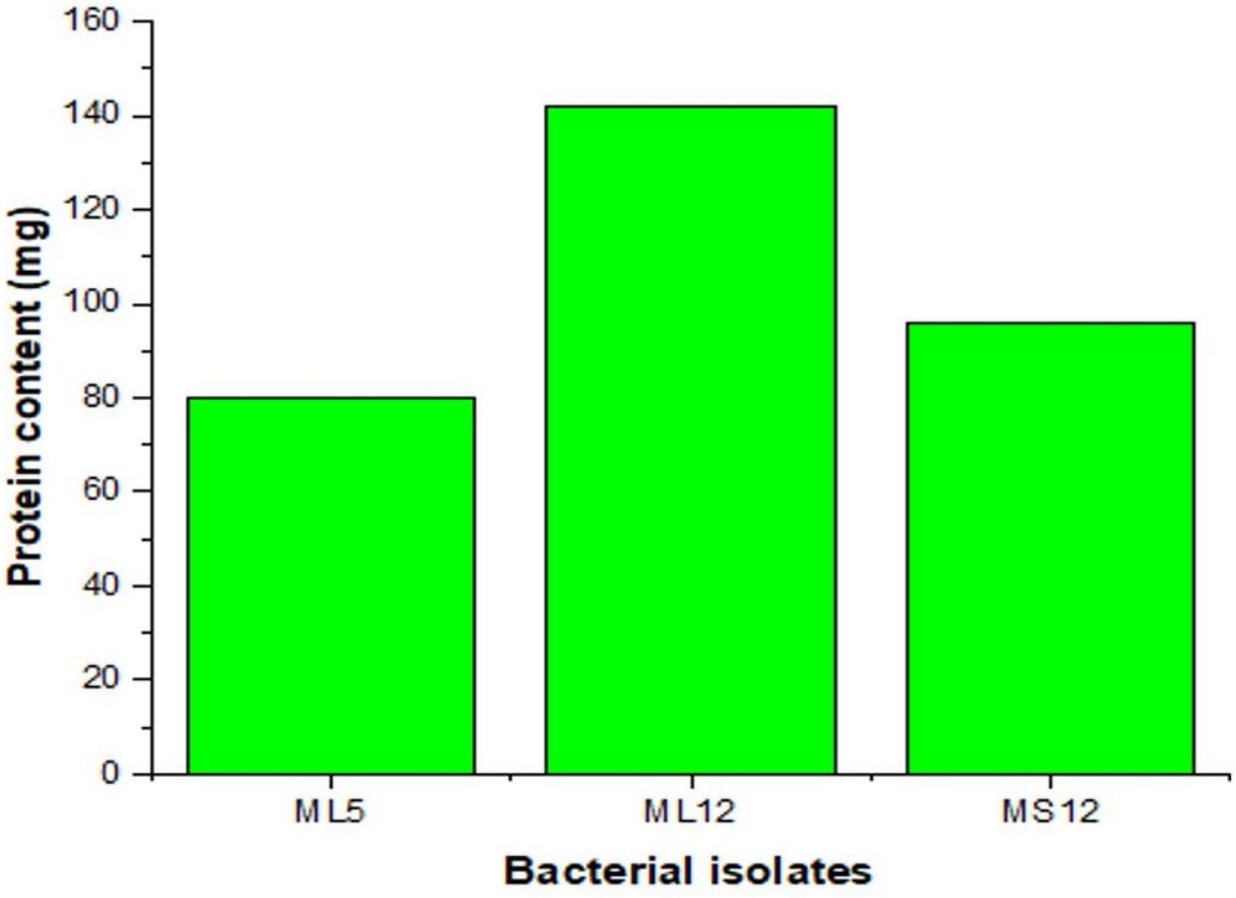

Figure 7

Total Protein estimation of the selected Bacterial isolates

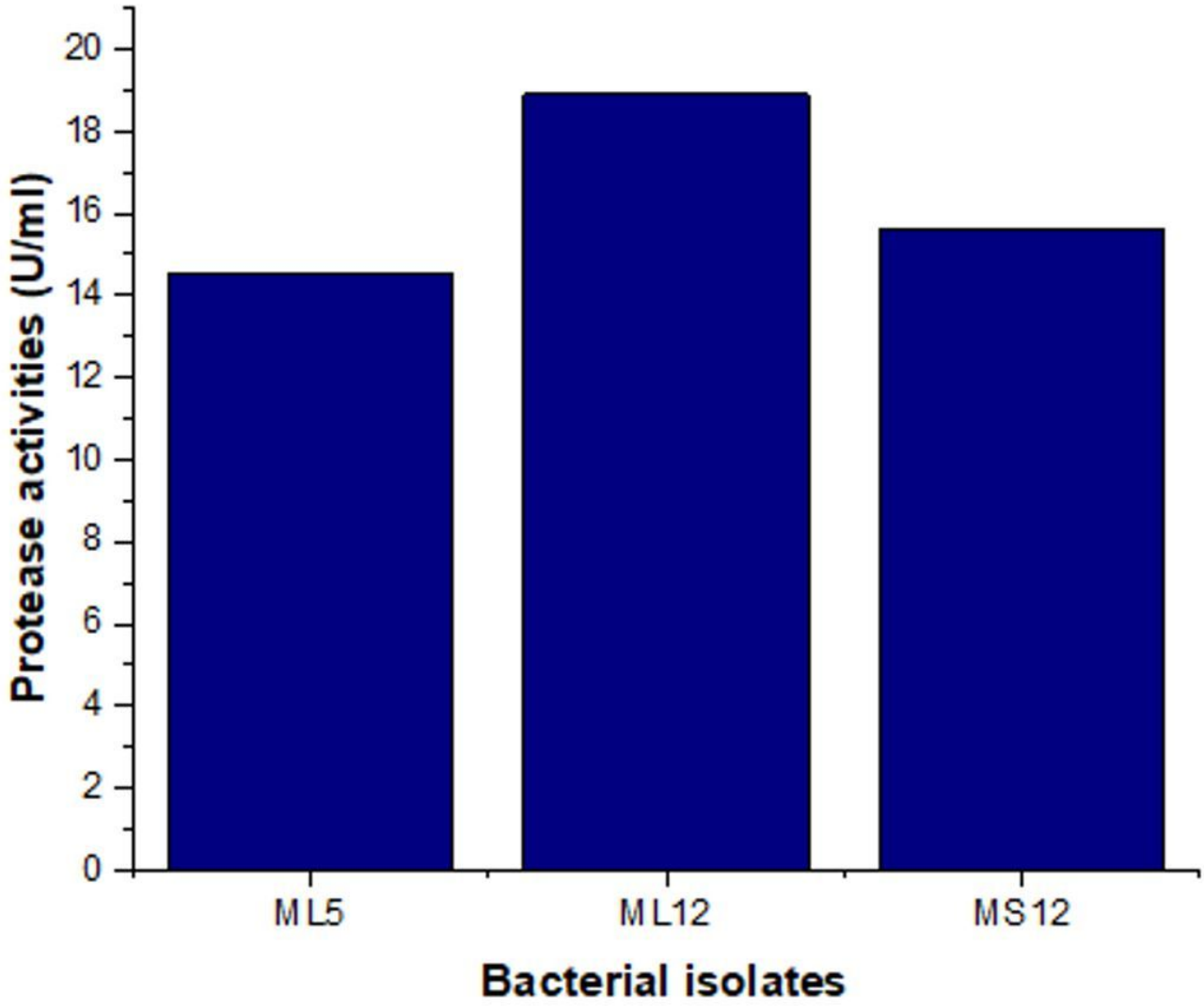

Figure 8

Protease Activities of the selected Bacterial isolates

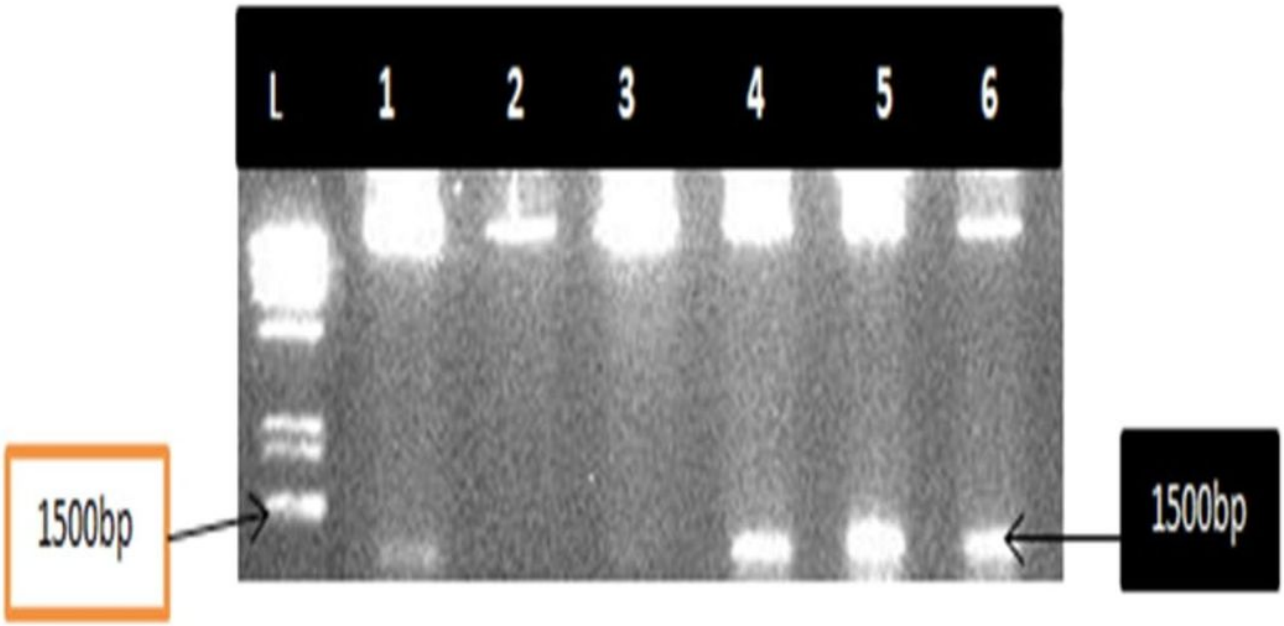

Figure 9

PCR Amplification of bacterial 16S rRNA from Bacillus cereus species. Electrophoresis was performed on a 0.8% agarose gel stained with gelred. Lane L, molecular weight DNA marker (Lamda DNA Ladder, Biobasic, Inc., Canada); lane 1-3, indicates control,4-6 indicates a fragment of ML12 Isolate bacterial 16S rRNA approximately 1500 BP.

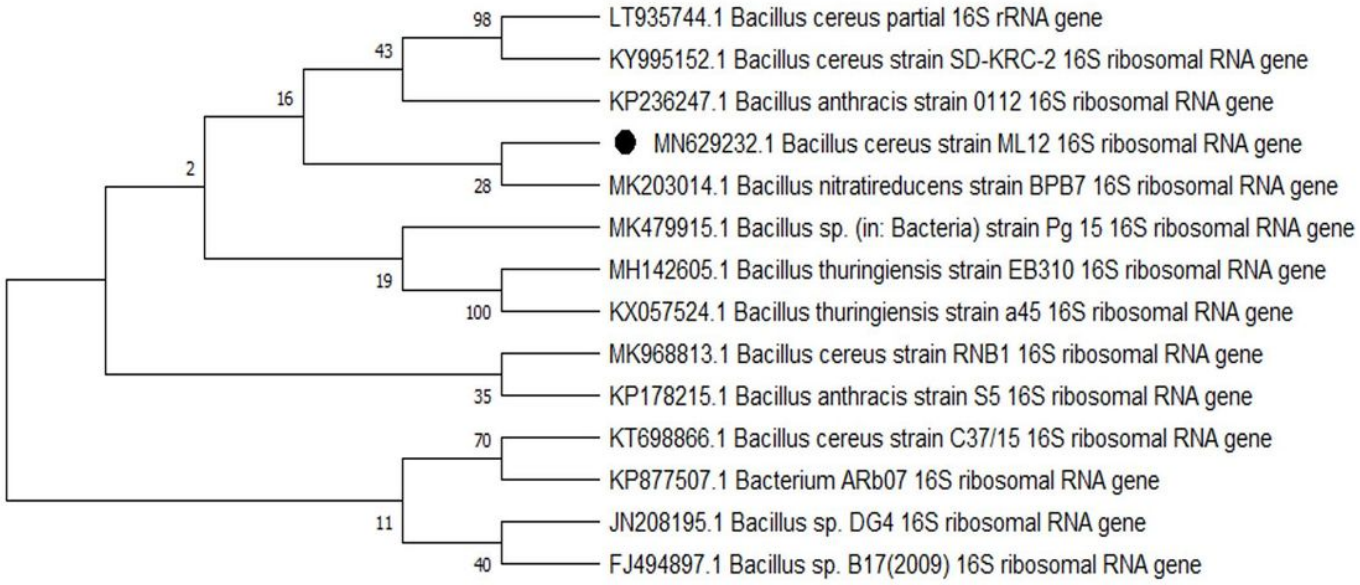

Figure 10

Phylogenetic Tree constructed on the basis of 16S rRNA gene sequences of Bacillus cereus (ML12) with other Bacillus species obtained from Gene Bank database.