**Journal of Applied Molecular Biology (JAMB)**
**Molecular Biology, Research & Studies Institute, Assiut University, Egypt.**
ISSN 2974-4008
Vol. 1(1): Sep.(2023)
Journal of Applied Molecular Biology (ekb.eg)

# Screening of Bacterial Isolates for Protease Production with Special Reference to Molecular Identification of Highly Producer Strains

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**ARTICLE INFO**

**Article History:**

***Received:2023-06-15***
***Accepted:2023-08-04***
***online:2023-09-12***

**Keywords:**

***Microbial,proteases;***
***Bacillus sp;***
***Genetic identification.***

**ABSTRACT**

Proteases play a critical role in many industrial applications. The most important advantage of microbial proteases is that they can tolerate harsh industrial conditions. In this study, 17 bacterial strains were isolated from different milk products. The bacterial isolates were screened for their capabilities to produce protease enzyme in normal and alkaline conditions with pH values 7.4 and 9.4 on skimmed milk and casine agar plate respectively at 30°C. The enzyme index (EI) for protease was measured for the highly protease producer strains in normal and alkaline media (1.6±0.02, 1.4±0.1, 0.9±0.05 in normal medium but 2.2±0.04, 2.5±0.06, 1.7±0.04 in alkaline medium for ASU2, ASU1,Sm-26 respectively). Biochemical tests were performed for the highly protease producer isolates. Genetically identification of bacteria was carried out through 16S rRNA gene sequencing. The current results indicated that the highest protease-producing bacterial strains were *Bacillus subtilis*AUS2 , *Bacillus tequilensis* AUS1, and *Serratia marcescens.* This study emphasized the necessity of utilizing microorganisms as promising enzyme producers employed in several industrial applications.

## INTRODUCTION

Proteases are proteolytic enzymes which can hydrolyze the protein peptide links (also known as peptidases and proteinases) [1]. Depending on the position where they work on the peptide chain, proteases roughly classified into two groups: exopeptidases and endopeptidases[2]. Proteases are further divided into four distinct classes demonstrated on the functional group located at the active site: metalloproteases, aspartic proteases, cysteine proteases, and serine proteases[2]. They are widespread and can be found in a wide variety of sources, including plants, animals, and microbes, because of being physiologically important for living organisms[2]. Microbial proteases are successfully used as a substitute for chemicals, due to they are eco-friendly indicator for the environment, in addition to their cost comptition and availability[3]. The world enzyme market is dominated by microbial proteases, which have a wide range of applications in bioindustries including food, pharmaceuticals, textile, photography, leather, and detergents[4].

The most common effective producers of extracellular alkaline proteases in the industry are *Bacillus* species. Proteases are one of the three major groups of industrial enzymes, and their yearly global market growth is very significant. They represent 20% of the total enzymes sold worldwide [5-7]. According to estimates, proteases make about 1-5% of the genomes of pathogenic organisms and 2% of the genomes of humans[8].Researchers proved that proteases control the production, activation, and turnover of proteins to control physiological processes[9]. Because of their critical role in the life cycle and the mimicking and spread of infectious illnesses, proteases are essential for drug discovery[3].In more than 50 human proteases,a single amino acid mutation may cause a genetic disease[10]. The proteases sold on the market are manufactured by microorganisms and are suited for biotechnological usage due to their high yield, little time and space requirements, advanced genetic manipulation, and low cost[11].

Despite the fact that proteases are found throughout nature, microbes are a superior source of these enzymes on account of their speed growth, the small amount of space for cultivation, and the simplicity with which they can be genetically altered to produce new enzymes with different properties that are desirable for their various applications[2]. The most active and dynamic extracellular alkaline protease producer in the industrial sector is *Bacillus sp.*[3]. *Bacillus* genus is superior source of proteases and is able to producemassive yields of neutral and alkaline proteases with exceptional characters, as extreme temperatures, pH, organic solvents, detergentsstability, and oxidising compounds[12].*Bacillus* is essential for the development of the alkaline protease (EC.3.4.21-24.99), which is active at alkaline pH levels between 9 and 11[7, 13, 14].

In the pharmaceutical, food, and other related industries, alkaline proteases are used in a variety of formulations due to their distinctive activity and ability to maintain a stable alkaline pH[15-18].Researchers are increasingly interested in a variety of uses for these alkaline proteases in the hopes of finding new strains with distinct characteristics and high activity[19, 20]. Neutral proteases are those that are active at a pH that is neutral, low acidic pH, or low alkaline pH[3]. The genus Bacillus produces mostly neutral proteases with a pH range from 5 to 8 and a rather low thermotolerance[3]. Because they

hydrolyze food proteins at a medium rate and produce less bitterness as a result, they are useful in the food industry[3].Because Neutrase is resistant to plant natural proteinase inhibitors, it is used in the brewing industry. Neutral proteases are recognised and described based on their strong affinity for hydrophobic amino acids.Because of low thermotolerance, it is a bit helpful to regulate the reactivity of neutral proteases due to low thermotolerance within the formation of hydrolysate. The neutral metalloprotease must be in the presence of a divalent metal ion to be active[21-23].

*Bacillus Subtilis* APO1 is capable of producing alkaline protease enzyme at all pH range 7.0–11.5, however it has the potential to produce alkaline protease for potential commercial usage as a component of detergent formulations[24]. It was shown that *Bacillus tequilensis* (JQ904626) produces an extracellular protease that is solvent- and detergent-tolerant as well as stable in the presence of surfactants, commercial detergents, and bleach-oxidant ($H_2O_2$). It has been shown that this protease has an activity in removing blood stains from clothing, dehairing hide, and removing the gelatin from expired photographic films[25]. Due to the importance of bacteria in the production of proteases which are suitable for industrial uses and can adapt to harsh conditions, in this study we perform screening of 17 bacterial isolates recovered from different plant and animal protein sources for alkaline and neutral proteolytic activities in two media and two pHs. Also, identifiying the highly producers based on their phenotypic and genotypic identification.

## MATERIALS AND METHODS

### Isolation of proteolytic bacteria

Different contaminated protein sources were collected from milk products stores. The samples were put in clean plastic bags, transported to the mycological lab, and stored at 4°C to use for the isolation of proteolytic bacteria.

Skimmed milk medium containing(g/L):$NH_4Cl$, 0.5; $K_2HPO_4$,0.3;skimmed milk,10; $KH_2PO_4$,0.4;bacteriological agar, 15 $MgCl_2$, 0.2;$CaCl_2$, 0.22; and was used for the isolation as discriped in [26]. Bacteria were isolated using dilution and direct plate methods. For the dilution method, one gram of protein contaminated sample was taken and mashed into small pieces with sterile loop, and suspended in 9 mL of double sterile distilled water. Then tubes were vortexed for homogeneity 5 min, one ml transferred to each Petri dish and covered with 20 ml sterile Skimmed milk medium. For direct method, four approximately 0.5 cm sample cuts were transfered into Petri dishes contaning solidified sterile Skimmed milk medium. All plates were incubated in static incubator at 30 °C for 48h then the developed bacterial isolates were taken, purified and preserved. *Bacillus subtilis, Escherichia coli, Serratia marcescens, Micrococcus luteus* and*Staphylococcus aureus* were kindley provided from previous studies of Dr. Ghada Abd-Elmonsef Mahmoud.

## Qualitative screening of the bacterial proteolytic activities

Two media were used for screening the proteolytic activities of the 17 bacterial isolates.Alkaline casin agar media containing (g/L):bacteriological agar, 15.0; peptone, 5.0; casein, 5.0 and yeast extract, 1.0 with pH(9.4) as described in[27]. Skimmed milk agar medium contaning(g/L):bacteriological agar, 15; $K_2HPO_4$,0.3; $CaCl_2$,0.22; $NH_4Cl$, 0.5;$KH_2PO_4$,0.4;$MgCl_2$,0.2 andskimmed milk,10 with pH(7.4) as discriped in[26].The media plates were inoculated with 24 h. single colony bacterial isolates, and incubated in static incubator at 30°C for 48h. Clear zones indicated the proteolytic activities around the bacterial colonies. The proteolytic index for each isolates was calculatedaccording to this equation

$$PI= \frac{\text{(clear zone diameter-colony diameter)}}{\text{Colony diameter}}$$

## Identification of isolates

All isolates were identified according to cell morphology, and biochemical tests [28, 29] .

## Cell morphology

According to the Hucker procedure, the Gram's stain was applied to an 18–24 h. culture [28]. A glass slide was air-dried and heat fixed with a loop of an overnight culture. Crystal violet stain was added and allowed to stand for 1 minute at 0.3% w/v. A moderate spray of water was used to removethe excess stain. After adding Gram's iodine (0.4% w/v), the solution was left to stand for 30 seconds before being washed off. The stain was first rinsed with ethanol (95% v/v), and then it was stained for 1 minute with safranin (0.4% v/v). The film was then washed with water for 5 seconds. Under a microscope, Gram-negative bacteria appeared pink, while Gram-positive bacteria appeared purple. For endospore test; bacterial smear was prepared from cultures after 24 hours of growth. Then it was placed over the steaming water bath and malachite green (primary stain) was applied for 5 minutes and then it was rinsed with water. The slide was flooded with the counter stain safranin for 20 second and rinsed with water then, dried and observed under the light microscope.

## Biochemical identification of the highely protease producers

Utilising the procedure outlined in Bergey's Manual, the bacterial characteristics of the highly protease-producing isolates were assessed using the catalase reaction, oxidation-fermentation reaction, arginine dihydroxylase activity, nitrate reduction, utilisation of carbohydrates, gelatin hydrolysis, starch hydrolysis, and urease test[30]. Each test was carried out in three duplicates, twice, for each isolate.

**a. Ryu's test**

A drop from 3% potassium hydroxide was combined with a drop of concentrated bacterial solution. If you pick up the suspension with a toothpick or a pin, the suspension

becomes sticky and makes threadlike slime if the bacteria are Grame negative. A Gram-positive bacterium is present if a watery suspension forms[31].

**b**. **Catalase test**

The purified suspected colonies were picked up with a sterile loop and transferred to the surface of a clean glass slide. One or two drops of hydrogen peroxide solution (3%) were added then, the cover slide was applied. The rapid appearance of gas bubbles was considered a positive reaction [32].

**c. Utilization of different carbon sources.**

By bacterial inoculation of basal medium containing certain carbohydrates (L-arabinose,maltose, xylose, D-fructose,sucrose, citrate, sorbose, glucose, lactose, maltose, and mannitol), the ability of bacterial isolates to utilize carbon sources were tested. the component of the basal medium composed of (g/l) (0.33 M Na-K phosphate buffer pH 6.8): $NH_4Cl_2$ 1.0;ferric ammonium citrate 0.05; $MgSO_4.7H_2O$ 0.5 and $CaCl_2$ 0.005. The evaluated carbohydrates were autoclaved individually, and then sterilised solution was added aseptically to give a final concentration of 1% v/v. 10.0 ml of the medium was combined and dispensed aseptically into test tubes that had undergone sterilisation. Incubating the culture medium at 30°C for 48 hours. According to Palleroni and Doudoroff [33].The turbidity of the culture media indicates bacterial growth.

**d. Nitrate reduction test**

Test cultures were inoculated into 5 ml of peptone broth containing 0.1 % potassium nitrate. The nitrate broth composed of (g/l): peptone (10.0), $K_2HPO_4$ (5.0), yeast extract (1.0), and $KNO_3$ (1.0).Within 48 hours, the tubes were incubated at 30°C. By adding 0.5 ml of Griess reagent I (0.8% sulphanilic acid solution in 5.0 N acetic acid) and 5–10 drops of Griess reagent II (1.0 g of L-naphtylamine in 200.0 ml 5.0 N acetic acid), A positive test was indicated by development of a red colour[34]. .

**e. Gelatin hydrolysis test**

Using the medium of Frazier's gelatin agar, the hydrolysis of gelatin was tested. This medium contains (g/l):NaCl, 5.0;peptone, 10.0; beef extract, 10.0; agar, 18.0; and gelatin, 4.0. The plates were then covered with mercuric chloride reagent, which consisted of distilled water 100.0 ml, $HgCl_2$ 15.0 gram and HCl 20.0 ml, after 72 hours of incubation at 30°C. A distinct halo region was seen to surround the bacterial growth and showed liquefaction of the medium [35].

**f. Starch hydrolysis**

Nutrient agar (NA), which contains (g/l): soluble starch, 10.0; beef extract, 10.0; peptone, 10.0; NaCl, 5.0; and agar, 15.0 was used to conveniently assess the hydrolysis of starch [36]. The starch plates were streaked, inoculated, and then put for two to three days at 30°±1C. By streaking the starch agar plates inoculated and then incubated for two to three days at 30°C. After growth, Lugol's iodine solution (1.0 gram of iodine, 2.0 gram of potassium iodide, and 100.0 milliliter of distilled $H_2O$) was poured over the plates.Hydrolyzed starch has no colour in the areas where it has been applied [37].

**h. Arginine dihydrolase**

Suspected colonies were inoculated into arginine decarboxylase medium just below the surface. Thornley's medium, which contains (g/l)agar, 3.0; peptone, 1.0; K2HPO4, 0.3;NaCl, 5.0;phenol red, 0.01 dissolved in ethanol and L-arginine HCl, 10.0, was used for the arginine dihydrolase test. To create anaerobic conditions, arginine agar medium was stab-inoculated, and a layer of sterilised mineral oil was pipetted onto the surface to a depth of about 1 cm. This oil had previously been sterilised by autoclaving for 10 min at 121°C, three times. For five days, the tubes were incubated at 30°C. The indicator's colour changed to violet with a positive result[38].

**i. Urease test**

Bacterial isolates were cultured on the slant surface of urea base agar supplemented with urea solution. The medium, which was divided into sections A and B, was used for the urease test. Peptone 1.0, Glucose 1, $KH_2PO_4$2.0, Phenol Red 12.0,NaCl 5.0, Agar 12.0, and 1.0 (g/900) ml were the ingredients in Portion A. Urea 40.0 was present (g/100 ml) in Portion B.With the exception of the portion B of the urease medium, which was sterilised using a membrane filter, the medium was autoclaved at 121°C for 15 minutes.The medium was used to make slants after part A in the tube (5.0 ml/tube) had been sterilised and part B(0.25ml)was added. When combining parts A and B, the temperature of part A shouldn't be higher than 55°C. Tested bacteria were used to inoculate the urease medium, which was then incubated at 30±1°C.Red colour appearance denoted a positive result [39].

**j. Hydrogen sulphide production test**

The agar medium was used for $H_2S$ production, the medium contains (g/L); peptone 30.0, sodium thiosulphate 0.025, beef extract 3.0, ferrous ammonium sulphate 0.2, and agar 3.0 [37].Test tubes were filled with 5.0 ml of media, stab-inoculated with the selected bacterial isolates, and then incubated at 30°C±1. Black colour indicated a positive result for $H_2S$ production [29][40].

**k. Motility test**

Motility test medium was inoculated by stabbing in semisolid agar medium. A colony of fresh culture (18-24 h.) which grown on nutrient agar medium was touched with a straight needle. The tube was stabbed once in the middle at a depth of just 1/3 to 1/2 inch. When removing the needle from the medium, make sure to keep it in the same line that it entered. For up to 4 days, the tube was put at 35 °C and checked every day. Keep an eye out for a widespread growth zone that flares out from theinoculation line. For positive result: The medium is slightly opaque due to the diffuse, hazy growing that have spread over it. A circular growth from the line of stabbing represented a positive test [41].

**l. Growth on MacConkey agar test**

The bacterial isolates which streaked on MacConkey agar plates was incubated for up to 3 days at 35°C. Lactose-fermenting strains was developed as red or pink growth and could have a zone of acid-precipitated bile surrounding them. The red colour was a result of lactose producing acid, neutral red being absorbed and the dye changing colour when the medium's pH decreases below 6.8. Lactose non-fermenting strains were translucent and colourless, and they usually did not change the appearance of the medium[42].

**m. Bile esculin test**

A loopful from the isolated bacteria was inoculated into a test tube containing bile esculin agar slant. The medium contains (g/L), Beef extract 11, ferric ammonium citrate 0.5 , esculin 1 , ox bile 2, agar 15 ,enzymatic digest of gelatin 34.5 and pH 6.6. One to two colonies from an 18- to 24-hour bacterial growth were inculated upon the surface of slant for 48 hours at 35 °C in natural air. An eye was kept out for the medium's growth and blackening. For the positive results: The agar slant was turning black and growth of bacteria.No medium blackening and no bacterial growth was the indication of negative result [43].

**n. Oxidase Test**

Oxidase test was done by streaking of the pure culture onto filter paper moistened with oxidase reagent. A small piece of filter paper was dried after immersing it in 1% Kovács oxidase reagent. A well-isolated colony was picked from a fresh (18–24 hour culture) bacterial plate using a sterile loop, and was scrubbed onto filter paper that had been treated. The colour variations were checked, when the colour quickly turned to dark purple (5–10 seconds) that indicates to positive result.. [44].

**o. Indol production test**

A mineral broth medium supplemented with L-tryptophan was used to estimate indol acetic acid (IAA) production. After sterilization the medium was inoculated with bacteria and incubated for 120 h at 28°C ± 1°C in a shaking incubator at 200 rpm. In the next steps, bacterial cultures were harvested and centrifuged at 6,000 g for 15 min. 1.5 ml of the bacterial supernatant was then combined with 1 ml of Salkowski reagent The formation of a red ring (surface layer) after 10 minutes was considered a positive reaction.

**p. Methyl red test**

Buffered glucose broth (5 ml) tube was inoculated with pure culture. The media and chemicals; MRVP broth (with pH 6.9), media composition per litre of deionized $H_2O$: glucose 5gm , dipotassium phosphate 5gm, buffered peptone; 7.0 gm. Methyl red solution, 0.02%; mix 300 ml of 95% ethyl alcohol with 0.1 g of methyl red. Enough distilled water was included to make 500 ml. The medium was left to balance with room temperature before inoculation. The medium was inculated with organisms from an 18–24 hour pure culture. 24 h. of aerobic incubation at 37° C. Aliquot 1ml of the brothinto a clean tube after the broth has been incubating for 24 hours. The residual broth was re-incubated for a further day.To an aliquot, 2 to 3 drops of methyl red indicator were added. The development of a red colour was considered a positive test[46].

**q. Voges–Proskauer (VP) Test**

Define whether an organism makes acetylmethyl carbinol from glucose fermentation using the Voges-Proskauer (VP) test.Acetylmethyl carbinol, if present, is changed into diacetyl in the existence of α-naphthol, strong alkali (40% KOH), and oxygen from the air. Barritt discovered that the – naphthol acts as a colour intensifier and that it must be

put first even though it was not a part of the original technique. The peptones of the broth is diacetyl- and quanidine-containing molecules which then compress to create a pinkish red polymer. In the Voges-Proskauer Test, the following media and reagents were used: MRVP broth (pH 6.9),dipotassium phosphate (5.0 gram), glucose (5.0 gram) andpeptone (7.0 gram) per litre of deionized water. Barritt's reagent B had Potassium Hydroxide (400 gram) and $H_2O$ (1L), whereas Voges-Proskauer Reagent A contains Alpha-Naphthol, 5% (50 gram) and Absolute Ethanol (1L). Allow the medium to reach room temperature before inoculation. A colony of fresh culture (18-24 h.) pure culture and slightly were inculated the medium. 24 hours of aerobic incubation at 37°C. Aliquot 2mlfrom the broth into a clean tube afterthe 24-hour incubation period was complete. Re-incubate the residual broth for a further day. Mix completely to aerate after adding 6 drops of 5% alpha-naphthol. Add 2 drops from 40% KOH, and mix completely to aerate. During 30 minutes, look for a pink-red colour at the surface and vigorously shake the tube during this time. Positive results: when A pink-red color was observed on the surface, indicates to positive results. The results is negative in an absence of a pink-red color [46].

**Quantitave screening for the bacterial proteolytic activities**

The bacterial isolates which have a clear zone were a protease producing bacteria. To measure the protease activity, liquid media was prepared which composed of (g/L) peptone, 5.0;yeast extract, 1.0; casein, 5.0; the same as solid without agar as dicribed in [27] with modified pH (7.4). 100ml sterile liquid medium inoculated with 0.5 OD bacterial suspenstion, incubated at 30°C for 48h. After that the liquid media was centrifuged under cooling at 4°C 7000 rpm for 15 minutes. The bacterial cells were discard and the supernatant was collected to calculate the activity. The activity of this isolates was measured at wave length 280 using spectrophotometer. Protease activity was estimated by adding 1 mL of the enzyme to 1 mL of 1.0% (w/v) casein dissolved in 0.1 M glycine–NaOH buffer, pH 10.0. The reaction mixtures were then incubated at 45°C for 30 min. The reaction was ended by the addition of 2 mL of 10% (w/v) trichloroacetic acid stop solution. The $A_{280}$ of the supernatant was measured and converted to L-tyrosine equivalents. One unit of protease activity (U) was known as the quantity of protease releasing one µmole of L-tyrosine equivalents per minutes under the criterion conditions of the assay [47].

**Molecular identification of the highest protease producing bacteria**

On nutrient agar medium, the bacterial isolates were grown[48]and transfered to the molecular Biology Research Unit, Assiut University after being put at 28ºC for 48 hours. The Patho-gene-spin DNA/RNA extraction kit from Intron Biotechnology Company, Korea, was utilised to do the DNA extraction. The DNA samples and delivered to SolGent Company in Daejeon, South Korea for polymerase chain reaction (PCR) and gene sequencing. Two universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTT ACGACTT-3'), were used in the PCR process. By electrophoresis on a 1% agarose gel, the purified PCR products (amplicons) were reconfirmed using a size nucleotide marker (100 base pairs). With the addition of dideoxynucleotides (dd NTPs) to the reaction mixture, purified amplicons were sequenced in both sense and antisense orientations using 27F and 1492R primers[49]. Using (BLAST) available on the (NCBI) website.Mega11 software was used to perform a phylogenetic analysis of sequences.

## RESULTS and Discussion

### Qualitative screening and proteolytic index for all bacterial isolates

17 bacterial strains (ASU1, ASU2, ASU3B, ASU4B, ASU5B, ASU6B, ASU7B, ASU8S, ASU9S, ASU10S, ASU11S, ASU12SR, KU55987, Sm-26, Ec-25, Sa-33 and Ml-27) were isolated from different milk products and screened for their proteolytic activity through calculation of their proteolytic index on skimmed milk (neutral) and casein (alkaline) agar plates as shown in **(Figure1)** formation of clear zone around the highest protease producing bacteria ASU1 **(Figure1.a,b)**, ASU2 **(Figure1.c,d)** and Sm-26 **(Figure1.e,f)** on skimmed milk and casein agar plates . Out of 17 bacterial isolates one bacterial isolate (ASU7B) was found to be negative on nutral medium but other 16 isolates were found to be positive on neutral medium.For neutral medium; as shown in **(Figure2.a)** 7 bacterial isolates (ASU3B, ASU4B, ASU8S, ASU9S, ASU10S,ASU11S and ASU12SR) were found to have proteolytic index values≤0.5(weak producers), 6 bacterial isolates (ASU5B, ASU6B, Sm-26, Ec-25, Sa-33 and Ml-27) were found to have proteolytic index ≤1(moderate producers), three bacterial isolates (ASU1, ASU2 and KU55987) were found to have highly proteolytic index >1(strong producers). All bacterial isolates could grow and produce protease enzyme on alkaline medium.For alkaline medium; as shown in **(Figure2.b)** 5 bacterial isolates (ASU5B, ASU6B, ASU7B, ASU9S and ASU11S) had proteilytic index ≤1 (weak producers), 10 bacterial isolates(ASU3B, ASU4B, ASU8S, ASU10S, ASU12SR, KU55987, Sm-26, Ec-25, Sa-33 and Ml-27) had proteolytic index ≤2 (moderate producer), two bacterial isolates (ASU1and ASU2) had higher proteolytic index>2.

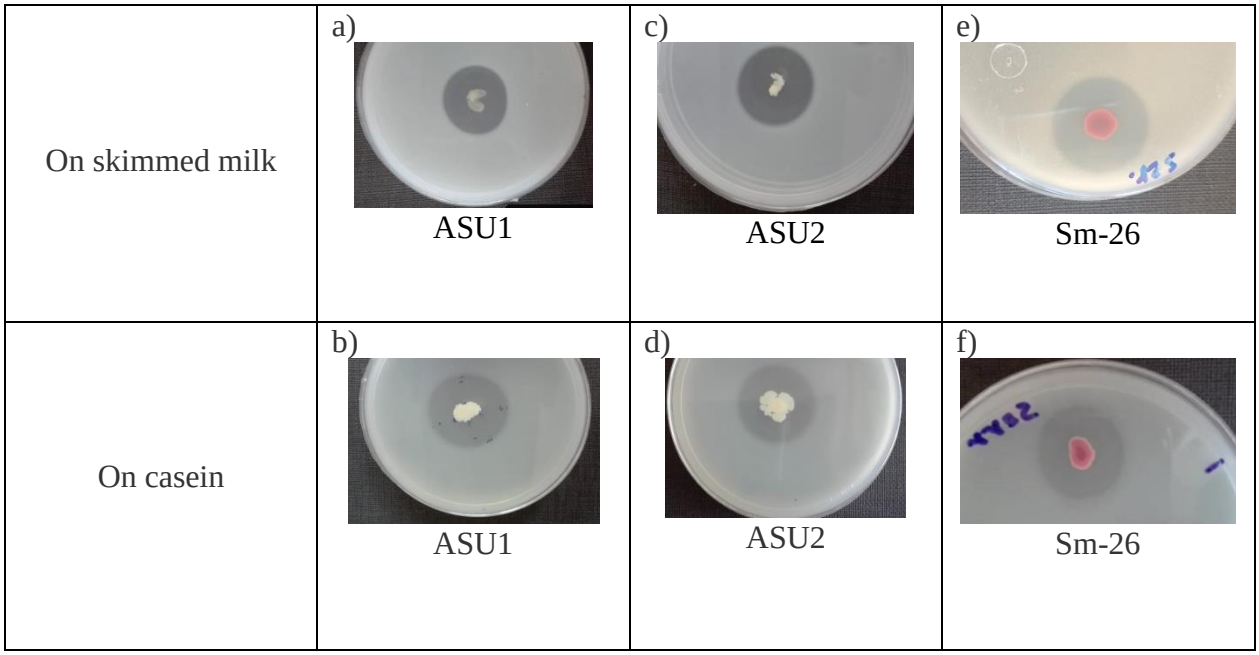
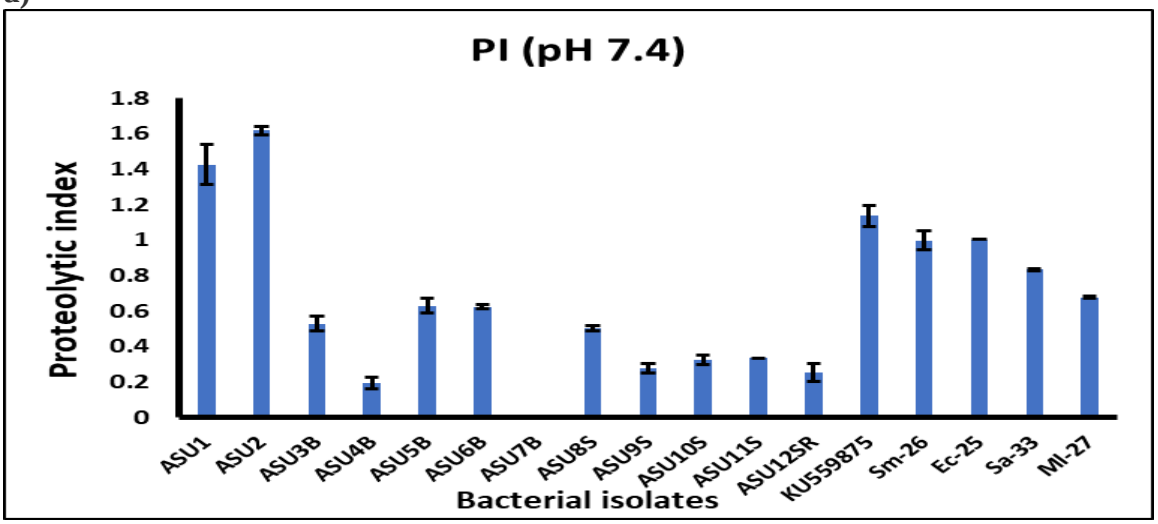



**Figure1**. showed the clear zone of the highest protease producing bacterial isolates onskimmed milk (neutral medium) and casein agar plates(alkaline medium).

a)




b)

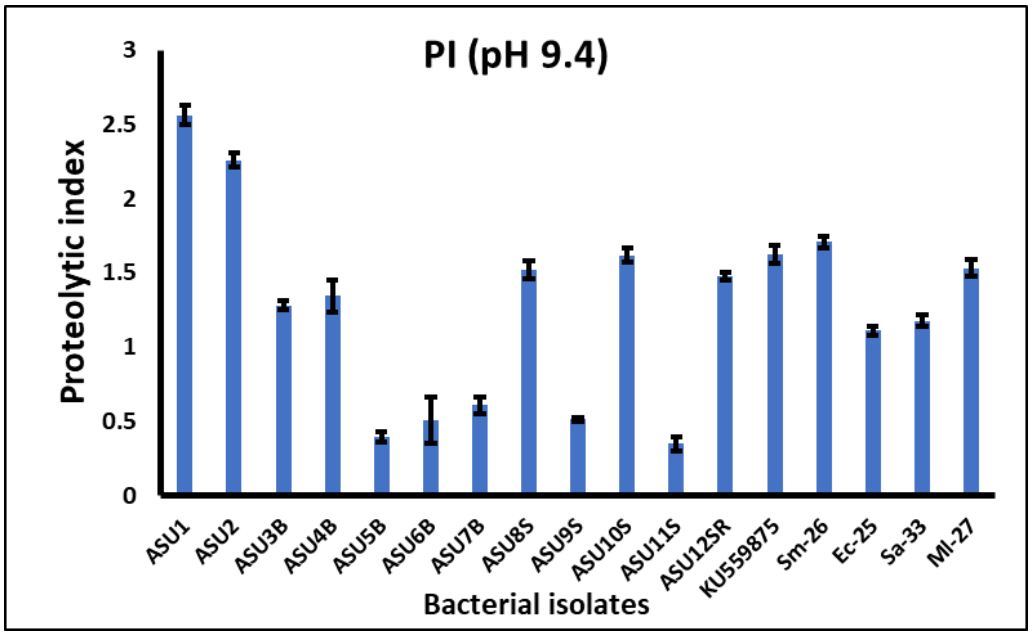



**Figure2**. proteolytic index (PI) values for all bacterial isolates; **(a)** proteolytic index on normal pH(7.4)**. (b)** proteolytic index (PI) on alkaline pH(9.4).

**Morphological identification of bacterial isolates**

Simple staining (crystal violet) and Gram staining were used to identify the morphological characteristics of bacterial isolates microscopically**(Table1)**. The bacterial isolates were varied in their morphological characteristics. The Gram positive bacteria were 14 isolates, out of them 12 bacterial isolates(ASU1, ASU2, ASU3B, ASU4B, ASU5B, ASU6B, ASU7B, ASU8S, ASU9S, ASU10S, ASU11S and KU559875) were bacilliin their shape but Sa-33 and Ml-27 were cocci. The Gram negative bacteria were

three bacterial isolates (ASU12SR, Sm-26 and EC-25) and all were short rodes in their shapes. As shown in **(Table1)** eight bacterial isolates (ASU1, ASU2, ASU3B, ASU4B, ASU5B, ASU6B, ASU7B and KU559875) were spore forming bacteria and were all *Bacillus sp.* but other bacterial isolates (ASU8S, ASU9S, ASU10S, ASU11S, ASU12SR, Sm-26, Ec-25, Sa-33 and Ml-27) were non-spore forming bacteria, four isolates out of them (ASU8S, ASU9S, ASU10S, ASU11S) were *lactobacillus sp.* but other non-spore forming bacteria(ASU12SR, Sm-26, Ec-25, Sa-33 and ML-27) were *Serratia marcescens, Escherichia coli, Staphylococcus aureus, and Micrococcus luteus* respectively.

**Table (1). Morphological identification of the bacterial isolates**

| No. | Isolate no. | Identification | Gram staning | Shape | Spore forming |
|---|---|---|---|---|---|
| 1 | ASU1 | *Bacillus subtilis* ASU1 | G +ve | bacilli | +ve |
| 2 | ASU2 | *Bacillus tequilensis*ASU2 | G +ve | bacilli | +ve |
| 3 | ASU3B | *Bacillus* sp. | G +ve | bacilli | +ve |
| 4 | ASU4B | *Bacillus* sp. | G +ve | bacilli | +ve |
| 5 | ASU5B | *Bacillus* sp. | G +ve | bacilli | +ve |
| 6 | ASU6B | *Bacillus* sp. | G +ve | bacilli | +ve |
| 7 | ASU7B | *Bacillus* sp. | G +ve | bacilli | +ve |
| 8 | ASU8S | *Lactobacillus* sp. | G +ve | bacilli | -ve |
| 9 | ASU9S | *Lactobacillus* sp. | G +ve | bacilli | -ve |
| 10 | ASU10S | *Lactobacillus* sp. | G +ve | bacilli | -ve |
| 11 | ASU11S | *Lactobacillus* sp. | G +ve | bacilli | -ve |
| 12 | ASU12SR | Short rods | G +ve | Short rods | -ve |
| 13 | KU559875 | *Bacillus subtilis* | G +ve | bacilli | +ve |
| 14 | *Sm-26* | *Serratia marcescens* | G +ve | Short rods | -ve |
| 15 | *Ec-25* | *Escherichia coli* | G +ve | Short rods | -ve |
| 16 | Sa-33 | *Staphylococcus aureus* | G +ve | Cocci | -ve |
| 17 | *Ml-27* | *Micrococcus luteus* | G +ve | Cocci | -ve |

**Biochemical tests for the highest producers**

Biochemical tests were performed for the highest protease producing bacteria to identify the bacterial species**(Table2).**The biochemical tests for *Bacillus subtilis* shown that it is bacilli, Gram positive bacteria and was watery in ryu's method, couldn't grow on *MacConkey* agar, spore forming bacteria, could use different carbon sources for their groth such as glucose,lactose, fructose, sucrose,maltose,mannitol and citrate but couldn't utilize arabinose, xylose and sorbose carbon sources. It can make hydrolysis of starch, esculin, casein and gelatin. It could produce hydrogen sulfide and indol. It had the ability to produce catalase enzyme but it couldn't produce arginine dihydrolase, oxidase, urease enzymes. It could make a reduction for nitrate. It shown positive results with motility and methyl red tests but showed negative result with Voges–Proskauer test.The biochemical tests for *Bacillus tequilensis* showed that it is bacilli, Gram positive bacteria which was watery in ryu's test. It couldn't grow on MacConkey agar. It gave positive result in motility test. It could produce enzmes such as arginine dihydrolase, catalase, oxidase but it couldn't produce urease enzyme. It could use some carbon sources for their growth such as citrate, fructose, glucose, sorbose, maltose, mannitol and sucrose but couldn't use xylose, arabinose and lactose for their growth. It had ability to hydrolyze casein, starch, gelatin and esculin. It couldn't make reduction from nitrate to nitrite, while produced hydrogen sufide and indol. It was negative in Voges–Proskauer test and methyl red test. The biochemical tests for *Serratia marcescens* showed that it is short rod, Gram negative bacteria which formed threads in ryu's test. It could grow on MacConkey agar. It was motil and could produce catalase enzyme but couldn't produce arginine dihydrolase, urease, oxidase enzymes. It was capable of hydrolyzing gelatin and casein, but wasn't capable of hydrolyzing starch and esculin. It couldn't produce indol and hydrogen sulfide. It had ability to use different carbon sources for their growth such as citrate, arabinose, xylose, sorbose, glucose, maltose, mannitol and sucrosebut couldn't use fructose and lactose. It showed positive result with Voges–Proskauer test, but it was negative with methyl red test.

**Table (2). Some physiological and biochemical characteristics of *Bacillus subtilis, Bacillus tequilensis,* and *Serratia marcescens***

| **Characteristic of bacterial isolates** | ***Bacillus subtilis*** | ***Bacillus tequilensis*** | ***Serratia marcescens*** |
|---|---|---|---|
| **Shape** | bacilli | bacilli | short rod |
| **Gram stain** | +ve | +ve | -ve |
| **Growth on MacConkey agar** | - | - | + |
| **Spore formation** | + | + | - |
| **Motility test** | + | + | + |
| **Ryu's method** | watery | watery | threads |
| **Arginine dihydrolase** | - | + | - |
| **Hydrolysis of Starch** | + | + | - |
| **Gelatin** | + | + | + |
| **Casein** | + | + | + |
| **Esculin** | + | + | - |

| | | | |
|---|---|---|---|
| **Urease test** | - | - | - |
| **Catalase** | + | + | + |
| **Oxidase** | - | + | - |
| **Indol production** | + | + | - |
| **Methyl red test** | + | - | - |
| **Voges–Proskauer test** | - | - | + |
| **Nitrate reduction** | + | - | + |
| **$H_2S$ production** | + | + | - |
| **Decomposition of citrate** | + | + | + |
| **Carbon sources used for growth:** | | | |
| **Arabinose** | - | - | + |
| **Xylose** | - | - | + |
| **Sorbose** | - | + | + |
| **Fructose** | + | + | - |
| **Glucose** | + | + | + |
| **Lactose** | + | - | - |
| **Maltose** | + | + | + |
| **Mannitol** | + | + | + |
| **Sucrose** | + | + | + |

### Quantitave determination of protease

The proteolytic activity of the highest producer bacterial strains (***Bacillus subtilis ASU1, Bacillus subtilis ASU2*** **and** ***Serratia marcescens*** Sm-26) was measured from tyrosine standard curve at 280 nm **(Figure 3).** The proteolytic activity for the highest producers Sm-26, ASU1, ASU2 were 43.8±2 (U/min), 32.5±5(U/min) and 32.3±7(U/min) respectively.

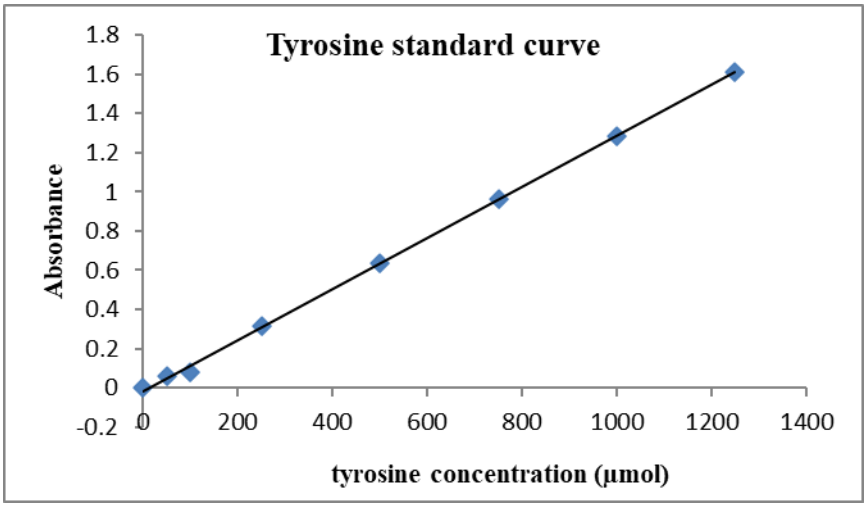
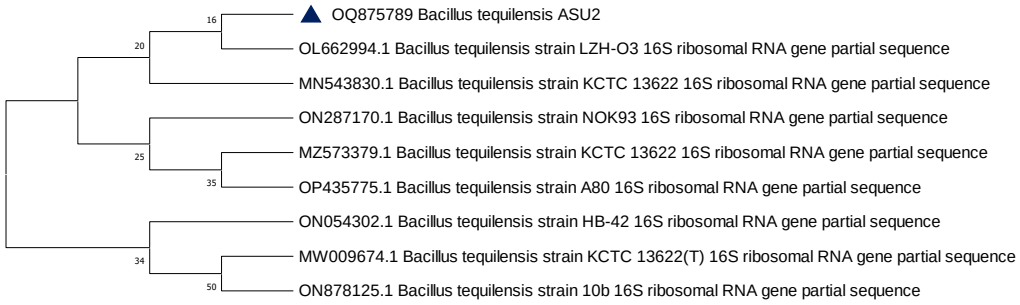



**Figure 3**. Tyrosine standard curve at 280 nm (tyrosine concentration from 50-1250µmol)

## Molecular identification of the highest producers

The highest producers **(*Bacillus subtilis* ASU1, and *Bacillus subtilis* ASU2)** were molecularly identifyed through 16S rRNA gene sequence analysis. When search on NCBI using BLAST the nucleotide sequences results showed that ASU2 bacterial sequence had similarity with ***Bacillus tequilensis*** and ASU1bacterial isolate had asimilarity with ***Bacillus subtilis.*** The sequences of16s rRNA were deposited to GeneBank with the accession numbers OQ875945 and OQ875789 for ***Bacillus subtilis*** and ***Bacillus tequilensis*** respectively. A phylogenetic tree was contructed as shown in **(Figure4)**through the 16s rRNA gene sequences of bacterial isolates and closely related nucleotide sequences using MEGA11 software and neighbor joining method.

**a)**




**b)**

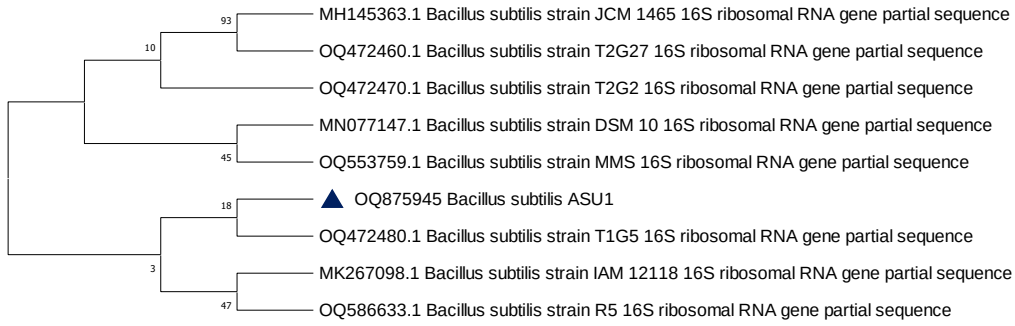



**Figure 4.** phylogenetic tree for the highest producer; **(a).** phylogenetic tree of *Bacillus tequilensis* ASU2 **(b).** pylogenetic tree of *Bacillus subtilis*ASU1.

## DISCUSSION

The important role that proteases play in analytical, physiological, and industrial applications has drawn the attention of researchers all around the world[11]. Microbial proteases are favoured because they can be produced more easily than proteases from animal and plant sources, and have practically the whole qualities needed for their industrial uses[50].Alkaline proteases are mostly produced by microbes, particularly *Bacillus* species, since they have properties that make them suitable for use in a different

industrial applications[51].In this study all 17 bacterial isolates which isolated from proteinacious sources are notable for their ability to produce protease enzyme. In contrast,Mushtaq, Ganai et al.[52] found from screening for protease production ability of 15 bacterial isolates which were isolated from a grassland site in lower part of Dachigam National Park that out of them 8 isolates could produce protease enzyme. Similarly, Ali, Ullah et al.[11] reported that 54 bacterial strains were isolated from salt mines of Karak, Pakistan, screened for their proteolytic activity and found that 23 isolates from them had proteolytic activity. The increased sample size and the greater number of sampling sites are the primary causes of the higher number of positive isolates[53]. In our study all bacterial isolates were positive which prove that different sources of protein are a sutible sources for more number protease positive producers.

The bacterial isolates were screened on two media with different pH (neutral and alkaline) and the proteolytic index was measured for determination of the highest producers. The enzyme index results showed that the highest producers with the highest clearance zone were ASU1, ASU2, KU559875 (1.4cm, 1.6cm, 1cm respectively) on neutral medium but the highest proteolytic index values on the alkaline medium were for ASU1, ASU2, Sm-26 bacterial isolates (2.5cm, 2.2cm, 1.7cm respectively). The enzyme index values for all isolates on neutral medium were lower than the proteolytic index values for the same isolates on alkaline medium which indicates that the bacterial isolates have tendency to produce alkaline protease higher than neutral proteases and the suitable pH for protease production is the alkaline pH. The morphological identification of the highest bacterial isolates (ASU1, ASU2, Sm-26) was performed which showed that ASU1, ASU2 were bacilli, Gram positive bacteria and spore forming bacteria. But Sm-26 was Gram negative bacteria, short rodes and non-spore forming bacteria. The biochemical tests for the highest protease producers was performed for primary identification of the bacterial strains genus. The results of biochemical tests for ASU1and ASU2 bacterial isolates showed that they were bacilli, Gram positive bacteria and motile. They could utilize different carbon sources for their growth such glucose, mannitol, citrate, sucrose, maltose and fructose. ASU1 ferment lactose but not ASU2. ASU1 and ASU2 bacterial isolates could produce catalase enzyme but couldn't produce urease enzyme. ASU1 bacterial isolate could produce oxidase enzyme but not ASU2. ASU1 and ASU isolates hydrolyze starch, casein and gelatin. ASU2 bacterial isolate was positive for methyl red test but not ASU1, this results shown similarity with the biochemical tests in this studies [11, 52, 53] which prove that ASU1 and ASU2 bacterial isolates are *Bacillus* species. For Sm-26 bacterial isolate the biochemical tests showed that it is Gram negative bacteria, short rodes and motile. It showed negative result for indol and methyl red test which indicate that it doesn't belong to enterobacteriaceae as described in this study[54]. It was positive for citrate utilization test and Voges–Proskauer test, according to Anusree, Swapna et al.[54]. These results showed that the bacterial isolate Sm-26 belongs to *Serratia* species.

*Bacillus* may be sutible bacterial source of proteases, *Bacillus* genus produces high yields of neutral and alkaline proteolytic enzymes with remarkable properties, including stability towardsoxidising compounds,high temperatures,organic solvents, pH and detergents [55]. The highest protease *Bacillus sp.* isolates (ASU1 and ASU2) were molecularely identified through 16s rRNAgene sequence analysis and with using

neighbor joining method, the results showed that the isolates were *Bacillus tequilensis*ASU1 and *Bacillus subtilis* ASU2.

## CONCLUSION

This study showed that the proteinatious sources were the most favorable sources for the isolates to produce protease enzyme efficiently. From all isolates, *Bacillus tequilensis* ASU1, *Bacillus subtilis* ASU2 and *Serratia marcescens* Sm-26 were found to have the highest potentiality for production of protease enzyme. The proteolytic index values on normal pH for the highest producers (ASU1, ASU2, Sm-26) were 1.4cm, 1.6cm, 0.99cm respectively but on alkaline pH the values were 2.5cm, 2.2cm, 1.7cm for ASU1, ASU2, Sm-26 respectively which showed that they have higher proteolytic activity on alkaline medium than the normal medium. This findings are interesting for future industrial applications.

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