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RESEARCH ARTICLE

DIVERSITY OF PROTEASE PRODUCING *BACILLUS* SP. ISOLATED FROM SOIL SAMPLES OF BUTCHER'S SHOP

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ABSTRACT

The paper presents experimental One of such enzymes that are widely distributed in biological systems proteolytic enzymes that are capable of corrupting proteins and other derivative Proteolytic enzymes like protease are very important in digestion as they breakdown the peptide bonds in the protein foods to liberate the amino acids needed by the body. In the present study diversity of protease producing *Bacillus* sp. isolated from soil samples of butcher's shop were enumerated. Totally 30 Bacillus sp. were isolated during the study period. Above isolates were identified based on the morphological test and biochemical test.

INTRODUCTION

The microbial world is the largest undiscovered field of biology on this period. It is an important topic inviting research and development in biology under vigorous investigations. Microbial diversity is the study of microorganisms, regarding to their biotic and abiotic period. The deep world of microbial activities and their greatness in the atmosphere of earth and human economics, conservation and exploitation. Microbial diversity leads to evolution of life as well as nutrient cycles. It has vast industrial applications. The prospect of new industrial applications from microorganisms is as large as the variety of environments they confront. Industrial enzymes are used in technical products and processes, used in a large scale. Microbial enzymes are more advantageous with less toxic metal ions hence creating more friendly process (Comfort *et al.* 2004). Proteases are the most important industrial enzymes that implement a wide variety of functions and have various important biotechnological applications. They constitute two-thirds of the total enzymes used in various industries and account for at least a quarter of the total global enzyme production (Kumar *et al.,* 2002). Though several microorganisms such as bacteria, fungi, yeast, plant, and mammalian tissues are known to produce alkaline proteases (Ellaiah *et al*., 2002), with rising industrial demand for proteases, it is expected that hyperactive strains will emerge and that the enzymes produced by new exotic microbial strains could be used as biocatalysts in the presently growing biotechnological era. Among the various proteases, microbial proteases play an important role in biotechnological processes. Alkaline proteases produced are of special interest as they could be used in produce of detergents, food, pharmaceuticals and leather (Saeki, *et al.*, 2007; Dias *et al*., 2008).

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MATERIALS AND METHODS

Sample Collection

Soil sample collected from butcher's shop, Coimbatore was used for isolating protease producing organisms.

Isolation and screening of the protease-producing organism

Isolation and screening of the protease-producing organism was done by plating the serially diluted samples on 2% skim milk agar plates.

Skim milk agar

Two of skim milk and 1.5 g of agar were weighed and dissolved in 100 mL of water, sterilized and plated on sterile petri plates.

Serial dilution and plating

Soil sample was weighed and mixed in sterile saline in 1:1 w/v ratio. From that, 100/µL aliquot was transferred to a micro centrifuge tube containing 900µL of saline (10^{-2} dilution). Likewise dilutions upto 18^{-8} were prepared. Aliquots of 100/µL from each dilution were plated on skim milk agar plates by spread plate technique.

Identification of proteolytic bacteria

After 2 days of incubation at 30°C, the plates were observed. Those organisms, which produced protease enzyme, showed zone clearance on the plates. On the basis of zone clearance, single colony was selected and two slants were prepared. One slant was used as working slant and other was maintained as stock.

Gram Staining

- A thin smear was prepared, air-dried and heat fixed.
- The smear was flooded with crystal violet for one minute.
- Washed the slide with water and flooded with Grams Iodine for one minute.
- Washed the slide with water and decolorized with 95% ethanol until no further violet colour could be washed out.
- The slide was washed with water and counter stained with 0.5% safranin for about 1 minute.
- Washed with water, dried and observed under oil immersion microscope.

Catalase test

Used a sterile inoculating loop or wooden applicator stick, collected a small amount of organism from a well isolated 18-24 hour colony. Placed it onto the microscopic slide . Care was taken not to pick agar. Using a dropper or Pasteur pipette placed 1 drop of 3 % Hydrogen-peroxide onto the organism on the microscope slide. The two were not mix and immediately were covered the perti dish with a lid. No bubble formation indicated the absence of catalase enzyme.

Sugar fermentation test

Used a single carbohydrate for each medium was prepared.After autoclaved the broth inoculated the three types of sugar fermentation broth with the bacteria and inserted a Durham tube.Kept one tube as un-inoculated as a control.Incubated at optimum temperature for 24 hours. If the organism has the ability to ferment sugar the medium changes its color from yellow to pink.

Indole test

Following incubation, few drops of Kovac's reagent were added. Kovac's reagent (which contains hydrochloric acid and p-dimethy laminobenzaldehyde in amyl alcohol) the solution turns from yellow to cherry red. Because amyl alcohol is not water soluble, the red coloration will form in an oily layer at the top of the broth. HCl. Ehrlich's reagent (using ethyl alcohol in place of isoamyl alcohol, developed by Paul Ehrlich) is used when performing the test on non fermenters and anaerobes. Formation of a red or pink coloured ring at the top is taken as positive.

Casein hydrolysis test

Prepared milk agar plate, inoculated theplatewith bacterial species and kept the plate in incubator for overnight. The zone of clearing around the colonies confirmed the production of enzyme caseinase.

MR-VP test

Inoculated two tubes containing MR-VP Broth with a pure culture of the microorganisms under investigation.Incubated at 35°C for 4 days. Added about 5 drops of the methyl red indicator solution to the first tube(for MR test), Barrit's reagent was added to other tube (VP test). A positive reaction is indicated, if the colour of the medium changes to red within a few minutes in first test tube, while change of color to cherry red in the second tube indicates positive result for VP test.

Urease test

Inoculated a urea broth tube with the microbe and incubated at optimum temperature for 24 hours. A positive reaction is indicated, if thecolour of the medium changes from yellow to pink.

Starch hydrolysis test

Hydrolysis of Starch was carried out of 10 gm soluble starch in 100 ml distilled water was heated in water bath until dissolved. 20 ml of this solution was mixed with 100 ml of melted nutrient agar and poured in the petridish after sterilization. A loopful of fresh bacterial culture was picked up by the sterile needle and stabbed on to the agar plate; After 24 hrs of incubation at 37° C, the plate was flooded with dilute iodine solution. Hydrolysis of starch was indicated by a clear zone around the growth and unchanged starch gave a blue color.

RESULTS

Sample collection from selected sites

Soil sample were collected from butcher's shop, Coimbatore (plate 1-3).

Isolation of Bacterial Isolates

A total of thirty bacterial isolates were obtained from Soil samples of Butcher shop using skim milk medium.

Plate 1. Sample collection

Plate 2. Sample collection

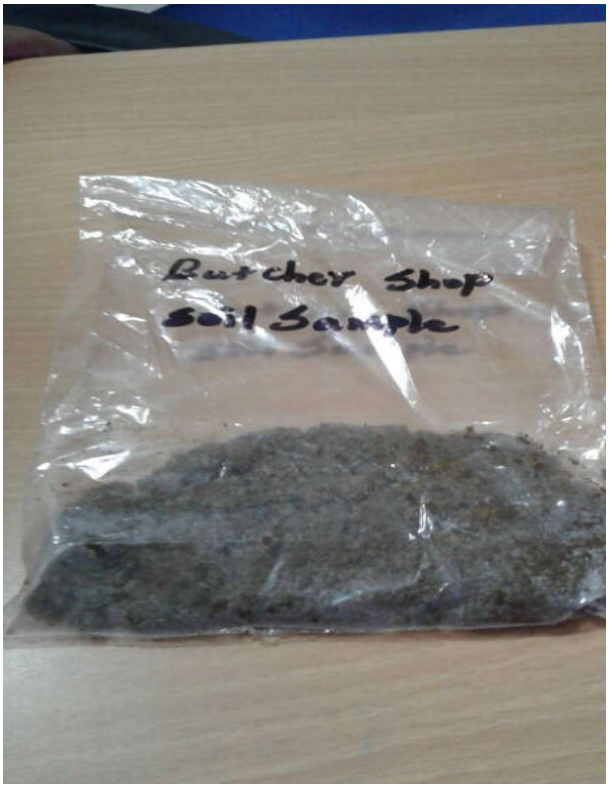

Plate 3. Collected sample
















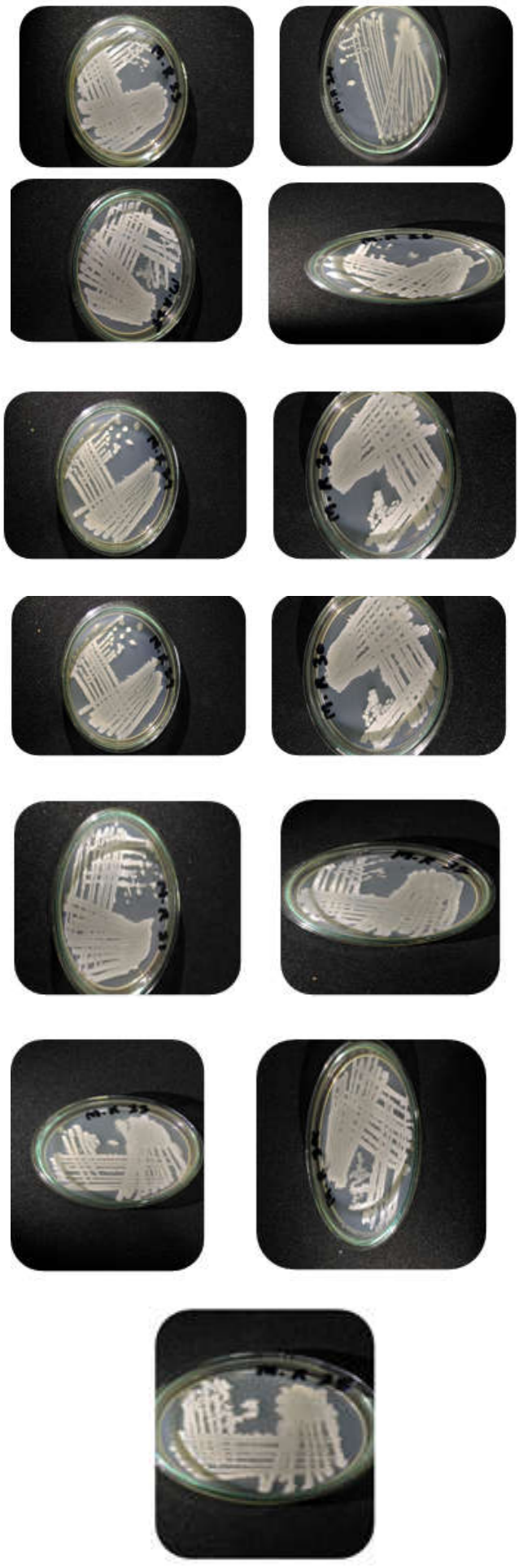

Plate 4. Isolation of Bacterial Isolates

The detectable growth of bacterial population was observed in all the thirty samples and different types of colony morph types differing in their size, color and appearance were obtained (Plate 4).

Characterization of Isolates

Morphological Characterization of Isolates

The thirty isolates obtained were further studied for various morphological characters which were classified into the following categories:

- Colony characterization
- Microscopic characterization

Colony characterization

Colony characteristics of all the thirty isolates showed significant variation for various morphological descriptors of colour, size and texture of the colony along with zone of clearance and zone size (Table- 1).

Colour

On the basis of the colour of the colonies three coloured morphotypes were identified viz., white, creamish and light yellow (Table-1). Maximum of twenty seven bacterial isolates were found to possess white in colour, two were creamish and one is light yellow in colour.

Size

Size of the colonies varied from a minimum of 1.0 mm to a maximum of 12.0 mm. (Table-1)

Form

The form of thirty bacterial isolates was found irregular in form (Table-1).

Texture

The texture of the colonies was observed as smooth or rough. Twenty four bacterial isolates were found to produce smooth texture and six were rough in texture (Table-19).

Margin

The irregular margin was possessed by thirty bacterial isolates (Table-1).

Form

The form of twenty six bacterial isolates was found irregular, whereas four isolates were found circular (Table-1).

Elevation

The elevation of the colonies was observed as either flat or raised (Table- 1).The flat elevation was observed in twenty eight isolates, whereas two bacterial isolates possessed raised elevation.

Zone of clearance

The next significant colony character of zone of clearance formed as a result of digestion of casein by only protease

producing bacterial isolates. Out of thirty, twenty eight bacterial isolates were protease producers and two bacterial isolates did not produce any zone of clearance (Table-19). And thus did not possess the ability to produce protease enzyme (Table-1).

Zone size

Another significant colony character depicting zone size of the twenty eight protease producers highlighted that the zone size varied from minimum of 1.0 mm to maximum of approximately 12.0 mm. Maximum zones of 12.0 mm was again produced by the bacterial isolate MR12 followed by 11.0 mm zone size produced by two bacterial isolates MR21 and MR8. Three bacterial isolates produced 3.0 mm zone size. 1.0 mm to 2.0 mm zone size produced by remaining bacterial isolates (Table-1).

Microscopic characterization

The microscopic characteristics studied were gram reaction, shape and pellicle formation (Brock 1984 & Degryse *et al*. 1978). These characteristics of the isolates are depicted in Table-2.

Gram's reaction

Gram's staining of this thirty seven isolates revealed that twenty two isolates were gram positive in nature (Table-2).

Shape of the cells

The morphological character of shape of the cells was rod-shape (Table-2).

Spore formation

Twenty seven bacterial isolates were found to form spores whereas three bacterial isolates were found negative for spore formation (Table-2).

Biochemical Characters

Catalase test

This test depicted variation in the twenty eight protease producing bacterial isolates. Only two protease producing bacterial isolates were tested negative and rest of the twenty eight were tested positive for catalase (Table-3).

Oxidase test

This test also depicted variation within the thirty protease producing bacterial isolates. Only thirteen protease producing bacterial isolates tested negative and rest of the isolates were tested positive for oxidase enzyme activity (Table-3).

Urea production

Thirty isolates were found to be negative for urease enzyme test (Table-3).

IMViC test

IMViC test done for thirty protease producing bacterial isolates. In case of indole production test, all thirty forty isolates were tested negative for indole production. Methyl red test was found negative for the thirty isolates and whereas VP and citrate test marked for all the thirty bacterial isolates was found to be positive. (Table-3).

Fermentation of sugars (glucose, sucrose, lactose)

Bacterial isolates showed significant variation to ferment different sugars viz., glucose, sucrose, lactose. It was depicted that lactose sugar was fermented by only sixteen isolates whereas fourteen isolates were not able to ferment lactose respectively. Glucose and sucrose were fermented by thirty bacterial isolates (Table-3).

Starch hydrolysis

Thirty isolates were found to be positive for starch hydrolysis enzyme test (Table-3).

Isolation and screening of the protease-producing organism

Totally 30 *Bacillus subtilis* were isolated from butcher's shop, Coimbatore. The formation of clear zones around the colonies confirmed the production of alkaline protease. Twenty eight isolates showed a clear zone in skim milk agar plate. Out of twenty eight isolates, an isolate that produced intense proteolytic zone and hence, it was selected as the best strain MR12 and used for further identification. Normally *Bacillus sp* produces high quantity of protease enzyme. The isolated organism is grown in skimmed milk agar as it contains casein, major source of protein. Casein is cleaved by the hydrolytic action of the enzymes secreted by the organisms. Hence, a zone of clearance is observed around the colonies secreting protease. The growth pattern was studied by constructing a growth curve. The growth curve gives us invaluable information on the onset of the various phases of growth of the organism. In general, production of protease is during the late log phase and early stationary phase.

DISCUSSION

In the present study, skim milk agar medium was used for the isolation of protease producing organisms. 28 isolates gave positive result for protease production. Similarly skim milk medium has been shown to be the best medium for the isolation of thermos table protease producing bacterial isolates using which putative thermos table protease producing bacterial isolates could be distinguished by the formation of a zone of clearance, as casein which on hydrolysis by proteolytic microorganisms leads to the formation zone of clearance. Similar reports of skim milk medium for successful isolation of thermos table protease producing bacteria have been communicated (Habib *et al.* 2012 and Azlina and Norazila 2013). However nutrient with skim milk medium and minimal synthetic medium supplemented with skim milk have also been used for the isolation of thermo stable protease producing thermophilic bacteria (Zilda *et al* 2012).

REFERENCES

Azlina I N and Norazila Y. 2013.Thermostable Alkaline Serine Protease from Thermophilic *Bacillus* Species. *International Research Journal of Biological Sciences* 2(2): 29-33.

Comfort D A, Chhabra S R, Conners S B, Chou C J, Epting K L, Johnson M R, Jones K L, Sehgal A C and Kelly R M. 2004. Strategic biocatalysts with hyperthermophilic enzymes.*Green Chemistry* 6:459-465.

Dias D.R, Vilela DM, Silvestre MPC, Schwan RF (2008). Alkaline Protease from Bacillus sp. Isolated from Coffee bean Grown on Cheese Whey. *World J Microbiol Biotechnol* 24: 2027-2034.

Ellaiah P., B.Srinivasulu and K.Adinarayna ., 2002. A review on microbial alkaline proteas. *J.sci. Industrial Research*, 61: 690-704.

Habib SMA, Fakhruddin ANM , Begum S and Ahmes MM. 2012. Isolation and screening of thermostable extracellular alkaline protease producing bacteria from tannery effluents. *Journal of Scientific Research* 4(2):515-522.

Kumar, C.G. 2002. Purification and characterization of a thermostable alkaline protease from alkalophilic*Bacillus pumilus*. *Letters in Applied Microbiology.* 34:13-17.

Saeki K, Ozaki K, Kobayashi T, Ito S 2007. Detergent Alkaline Proteases: Enzymatic Properties, Genes, and Crystal Structures. *J Biosci Bioeng* 6: 501-508.

Zilda D S, Harmayani E, Widada J, Asmara W, Irianto HE , Patantis G and FawzyaY N. 2012. Screening of thermostable protease producing microorganisms isolated from indonesian hotspring. *Squalen* 7(3): 105- 114.

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