Biochemical Identification of Protease Producing Bacterial Isolates from Food Industries by Vitek 2 Compact System

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The objective of the present study was to identify protease producing bacteria isolated from food processing industries. Isolation of the organism was performed by serial dilution agar plate technique and initial screening of protease production was done using gelatine agar plates by flooded the plates with mercuric chloride solution (HgCl$_2$-15g and 20 ml of 6.0 N HCl made up to 100 ml with distilled water). A total of 5 isolates were selected based on zone diameter in gelatine clear zone method and named as TKMFT8, TKMFT22, TKMFT25, TKMFT39 and TKMFT53. Initial identification of the selected isolates was carried out using cultural characterization, microscopic observation and Biomerieux VITEK 2 system identification based on biochemical reactions and identified as Staphylococcus sciuri (TKMFT8, TKMFT22, TKMFT25, and TKMFT39) and Achromobacter xylosoxidans (TKMFT53). Protease producing bacterial isolates can be used for the degradation proteinaceous waste material from food manufacturing units leading to recycling of food industry waste.

Keywords: Protease, Biomerieux VITEK 2 system, Staphylococcus sciuri, Achromobacter xylosoxidans.
attack the peptide carboxyl group. One way to make a nucleophile is by a catalytic triad, where a histidine residue is used to activate serine, cysteine, or threonine as a nucleophile. Proteases are involved cleavage of long protein chains into shorter fragments by cleaving the peptide bonds that link amino acid residues. Some detach the terminal amino acids from the protein chain (exopeptidases, such as carboxypeptidase A, aminopeptidases), others attack internal peptide bonds of a protein (endopeptidases, such as pepsin, trypsin, papain, elastase).

Proteases represent the class of enzymes which occupy a pivotal position with respect to their physiological roles as well as their commercial applications. Proteases perform both synthetic as well as degradative functions. Since proteases are physiologically necessary for living organisms, they occur ubiquitously in a wide diversity of sources such as microorganisms, plants and animals. Microorganisms are an attractive source of proteases due to the limited space required for their cultivation and their susceptibility to genetic manipulation. Proteases are categorised into exo- and endopeptidases based on their action at or away from the termini, respectively. Depending on the nature of the functional group at the active site, proteases are also classified as serine proteases, aspartic proteases, cysteine proteases, and metalloproteases. Proteases play a significant role in many physiological and pathophysiological processes. Based on their classification, four different types of catalytic mechanisms are operative. Proteases find tremendous applications in the food and dairy industries. Alkaline proteases hold a great potential for application in the detergent and leather industries because of the increasing trend to develop environmentally friendly technologies. There is a renaissance of interest in the application of proteolytic enzymes as targets for therapeutic agents development. Cloning and sequencing of protease genes from several bacteria, fungi, and viruses have been performed with the prime aims of (i) overproduction of the protease enzyme by gene amplification, (ii) delineation of the role of the enzyme in pathogenicity, and (iii) alteration in the properties of enzyme to suit its commercial application. Protein engineering techniques have been exploited to get proteases which show unique specificity and/or enhanced stability at high pH or temperature or in the presence of detergents and to understand the structure-function relationships of protease enzyme. Protein sequences of acidic, alkaline, and neutral proteases from various origins have been studied with the aim of studying their evolutionary relationships. Despite the extensive research on several aspects of proteases, there is a paucity of knowledge about the roles that govern the diverse specificity of these enzymes (Rao et al., 1998).

The major bacterial genera which contribute to proteases include Aeromonas, Alcaligenes, Arthrobacter, Bacillus, Halomonas, Pseudomonas and Serratia (Shafee et al., 2005; Rao et al., 1998), Brevibacterium linens (Rattray, 1995), Alteromonas sp. (Yeo et al., 1995), Hyphomonas jannaschiana VP 3 (Shi et al., 1997), Microbacterium sp. (Gessesse and Gashe, 1997) Pimelobacter sp. z-483 (Oyama et al., 1997) Salinivibrio sp. Strain AF-2004 (Heidari et al., 2007) Streptomyces isolate EGS-5 (Ahmad, 2011) Streptomyces microflavus (Rifaat et al., 2006) Streptomyces rimosus (Yang and Wang, 1999) Thermoactinomyces sp. (Lee et al., 1996) Thermoactinomyces mycelialphilus THM1 (Anderson et al., 1997), Lactobacillus helveticus (Valasaki et al., 2008).

VITEK 2 is an automated microbial identification system that provides highly reproducible and accurate results as shown in
multiple independent studies. With its colorimetric reagent cards and associated hardware and software advances, the VITEK 2 offers a state-of-the-art technology platform for phenotypic identification methods. The GN identification card is based on established biochemical methods and newly developed substrates measuring carbon source utilization, enzymatic activities, and (Chang et al., 2002, Coenye et al., 2001, De Baere et al., 2001, Smith et al., 1991, Vandamme et al., 1999). The GP identification card is based on established biochemical techniques and newly developed substrates (Atlas, 1993, Barros et al., 2001, Collins and Lawson 2000, Collins et al., 2001, Poyart et al., 2002, Schlegel et al., 2000, Whiley et al., 1999).

In the present study, isolation of protease producing bacteria was carried out from soil and water samples collected from food industry surroundings. The protease producing ability was determined using gelatine clear zone method and biochemical identification was carried out by using Biomerieux VITEK 2 system.

**Materials and Methods**

**Isolation of protease producing bacterial isolates**

Soil and water samples were collected and stored in sterilized containers at 4°C until analysis. Soil samples were collected from different areas in Halwa manufacturing units such as close premises of such units, directly from the gluten landfill body by removing the surface soil and subsurface soil dug to a depth of about 1 meter and from the edge of the landfill body. Water samples collected comprised of waste water from the food processing units. The collected samples were used to isolate protease producing microorganisms by serial dilution agar plate technique described by Sjodahl et al., (2002). An aliquot of 1 gram/ 1 ml of soil and water sample was taken and it was added to 9 ml of sterile distilled water and serially diluted up to $10^{-6}$ dilution. From each dilution 0.1 ml was spread on nutrient agar plates and plates were incubated at 37°C for 48 hours.

**Screening of protease producing strains**

Screening of protease producing organism generally composed of growth of organism on the medium that composed of protein as a selective substrate and in the present study Gelatin was used as the substrate. A total of 87 dissimilar colonies from nutrient agar plates were selected and each isolate was given a reference number (TKMFT 01 to TKMFT 87) and each isolate was subjected to primary screening for the production of protease by plate assay using protease specific medium containing (g/l) glucose 1.0, K$_2$HPO$_4$ 2.0, Peptone 5.0, gelatin 15.0, and agar 15. After 24h incubation at 28°C, the clear zone diameters were measured by flooded the plates with mercuric chloride solution (HgCl$_2$- 15g and 20 ml of 6.0 N HCl made up to 100 ml with distilled water), this method was described as gelatine clear zone method (Galil, 1992). Based on the zone diameter, 5 isolates (TKMFT 8, TKMFT22, TKMFT25, TKMFT39 & TKMFT53) were selected for further experimental studies.

**Qualitative test for protease**

Bacterial colonies appeared on agar plates were screened for evaluating their proteolytic potential by inoculating them in gelatin agar medium. Out of 87 isolates, 5 isolates were used in present study for thorough investigation as it exhibited most prominent zones of proteolysis around the colony. Protein hydrolysis was expressed as diameter of clear zone in millimetre (mm). Based on the results obtained in the biochemical identification, 5 isolates were selected for quantitative test for protease activity.
Identification of protease producing bacteria

Colony morphology

The colony morphology of selected bacterial isolates was examined on nutrient agar plates. After the incubation, characterization of individual colonies was performed based on their shape, colour, appearance, size, transparency, pigmentation, form, margin and elevation (Aneja, 2003).

Microscopic observation-Gram’s staining

The selected bacterial isolates were Gram stained in accordance with the standard procedure for Gram’s staining described by Todar et al., (2005).

Biochemical identification of Bacteria using Biomerieux Vitek 2 System

The selected organisms were identified using VITEK 2 compact-Biomerieux, France automatic system in Cashew Export promotion Council of India (CEPCI), Kollam and the test method was A O A C OMA 2012.02. VITEK-2 system imparts an automated, computer based technique of species identifications, relies on advanced colorimetry technology, the measurement of light attenuation associated with each biochemical reactions in VITEK cards (Gram-negative fermenting and non-fermenting bacilli (GN), Gram-positive cocci and non-spore-forming bacilli (GP), yeasts and yeast-like organisms (YST), Gram-positive spore-forming bacilli (BCL)). The reagent cards have 64 wells and each well contain an individual test substrate. Substrates assess various metabolic activities such as alkalisation, acidification, enzyme hydrolysis, and growth in the presence of inhibitory compounds. The VITEK-2 compact system combines several advantages like rapid identification, a simple methodology, a high level of automation and taxonomically updated databases.

Results and Discussion

Isolation and screening of Protease producing bacteria

Protease producing organisms were isolated from soil and waste water collected from the close premises of food processing industries using serial dilution agar plate technique (Rupali, 2015, Sjodahl et al., 2002, Tennalli et al., 2012, Sinha et al., 2013). The proteolysis ability of 87 bacterial isolates from soil and waste water samples were evaluated using Gelatine agar medium. A wide range of methods are available using Gelatin as substrate for detecting proteases (Grubb, 1994). Following inoculation and incubation of the Gelatin agar plates, organisms secreting protease enzyme exhibited a zone of proteolysis which was shown by a clear area around bacterial colonies. Among 87 isolated bacteria, 27 isolates were protease producer based on zone of hydrolysis and out of them 5 isolates (TKMFT 8, TKMFT22, TKMFT25, TKMFT39 and TKMFT53) were chosen for further studies based on the diameter of zone of hydrolysis as shown in Plate.2.

Sharma et al., (2015) reported that gelatine agar medium was best than skim milk agar medium for qualitative test for detecting protease production because zone of hydrolysis were obtained with more clarity in gelatine agar plates. These isolates were streaked on Nutrient agar plates as shown in Plate 4.3 and slants of these isolates were prepared on nutrient agar medium in screw capped tubes and maintained at 4°C for further experimental studies. Clear zone formation around bacterial colonies was considered as the evidence of production of protease. The results of bacterial isolates showing zone of inhibition (Diameter in mm) are presented in table 1.
Screening of protease producing bacteria -

Primary screening on Gelatin agar medium

According to the results presented in Table.1 and Fig.1 the highest zone diameter on Gelatin Agar medium was obtained for TKMFT 8 (26mm) followed by TKMFT22 (25mm) TKMFT39 (23mm), TKMFT25 (20mm) and TKMFT53 (15mm). Five different bacterial isolates showed clear zone indicating enzyme production on gelatine agar plates were selected for secondary screening.

Identification of Protease producing bacterial isolates

Potent protease producers were biochemically identified using Biomerieux VITEK 2 system.

Table.1 Bacterial isolates showing zone of inhibition (Diameter in mm)

| Sl No | Bacterial isolates | Diameter of Zone of hydrolysis(mm) |
|-------|--------------------|-----------------------------------|
| 1     | TKMFT 8            | 26                                |
| 2     | TKMFT 22           | 25                                |
| 3     | TKMFT25            | 20                                |
| 4     | TKMFT 39           | 23                                |
| 5     | TKMFT 53           | 15                                |

Table.2 Colony morphology and microscopic observation of TKMFT8, TKMFT22, TKMFT25, TKMFT39 & TKMFT53

| Sl No | Bacterial isolates | Result of Gram staining | Colony characters on Nutrient agar |
|-------|--------------------|-------------------------|-----------------------------------|
|       |                    |                         | A: Size; B: Pigmentation; C: Form; D: Margin; E: Elevation; F: Texture |
| 1     | TKMFT 8, 22, 25, 39| Gram positive cocci     | Medium | Dark yellow | Circular | Entire | Flat | Rough |
| 5     | TKMFT53            | Gram negative bacilli   | Small  | No           | Circular | Entire | Flat | Smooth |
### Table 3: Biochemical details of organisms identified using BIOMERIEUX VITEK/GP Cards

| Well | Test                               | Mnemonic     | Result |
|------|------------------------------------|--------------|--------|
| 2    | D-AMYGDALIN                        | AMY          | +      |
| 4    | PHOSPHATIDYLINOSITOL               |              |        |
|      | PHOSPHOLIPASE C                    | PIPLC        | -      |
| 5    | D-XYLOSE                           | dXYL         | -      |
| 8    | ARGinine DIHYDROLASE 1             | ADH1         | +      |
| 9    | BETA-GALACTOSIDASE                 | BGAL         | -      |
| 11   | ALPHA-GLUCOSIDASE                  | AGLU         | +      |
| 13   | Ala-Phe-Pro ARYLAMIDASE            | APPA         | -      |
| 14   | CYCLODEXTRIN                       | CDEX         | -      |
| 15   | L-Aspartate ARYLAMIDASE            | AspA         | -      |
| 16   | BETA GALACTOPYRANOSIDASE           | BGAR         | -      |
| 17   | ALPHA-MANNOSIDASE                  | AMAN         | -      |
| 19   | PHOSPHATASE                        | PHOS         | +      |
| 20   | Leucine ARYLAMIDASE                | LeuA         | -      |
| 23   | L-Proline ARYLAMIDASE              | ProA         | -      |
| 24   | BETA GLUCURONIDASE                 | BGURr        | -      |
| 25   | ALPHA-GALACTOSIDASE                | AGAL         | -      |
| 26   | L-Pyrrolidonyl-ARYLAMIDASE         | PyrA         | -      |
| 27   | BETA-GLUCURONIDASE                 | BGUR         | +      |
| 28   | Alanine ARYLAMIDASE                | AlaA         | -      |
| 29   | Tyrosine ARYLAMIDASE               | TyrA         | -      |
| 30   | D-SORBITOL                         | dSOR         | -      |
| 31   | UREASE                             | URE          | -      |
| 32   | POLYMIXIN B RESISTANCE             | POLYB        | -      |
| 37   | D-GALACTOSE                        | dGAL         | +      |
| 38   | D-RIBOSE                           | dRIB         | +      |
| 39   | L-LACTATE alkalisation             | ILATk        | +      |
| 42   | LACTOSE                            | LAC          | -      |
| 44   | N-ACETYL-D-GLUCOSAMINE             | NAG          | +      |
| 45   | D-MALTOSE                          | dMAL         | +      |
| 46   | BACITRACIN RESISTANCE              | BACl         | +      |
| 47   | NOVOBIOCIN RESISTANCE              | NOVO         | +      |
| 50   | GROWTH IN 6.5% NaCl                | NC6.5        | +      |
| 52   | D-MANNITOL                         | dMAN         | +      |
| 53   | D-MANNOSE                          | dMNE         | +      |
| 54   | METHYL-B-D-GLUCOPYRANOSIDE         | MBdG         | +      |
| 56   | PULLULAN                           | PUL          | -      |
| 57   | D-FAFFINOSE                        | dRAF         | -      |
| 58   | O/129 RESISTANCE (comp. vibrio.)   | O129R        | +      |
| 59   | SALICIN                            | SAL          | +      |
| 60   | SACCHAROSE/SUCROSE                 | SAC          | +      |
| 62   | D-TREHALOSE                        | dTRE         | +      |
| 63   | ARGinine DIHYDROLASE 2             | ADH2s        | -      |
| 64   | OPTOCHIN RESISTANCE                | OPTO         | +      |
Table.4 Biochemical details of organisms identified using BIOMERIEUX VITEK/GNCards

| Well | Test                                      | Mnemonic | Result |
|------|-------------------------------------------|----------|--------|
| 2    | Ala-Phe-Pro-ARYLAMIDASE                   | APPA     | -      |
| 3    | ADONITOL                                  | ADO      | -      |
| 4    | ARYLAMIDASE                               | PyrA     | -      |
| 5    | L-ARABITOL                                | IARL     | -      |
| 7    | D-CELLOBIOSE                              | dCEL     | -      |
| 9    | BETA-GALACTOSIDASE                        | BGAL     | -      |
| 10   | H2S PRODUCTION                            | H2S      | -      |
| 11   | BETA-N-ACETYL-GLUCOSAMINIDASE             | BNAG     | +      |
| 12   | Glutamyl Aarylamidase Pna                 | AGLTp    | -      |
| 13   | D-GLUCOSE                                 | dGLU     | +      |
| 14   | GAMMA-GLUTAMYL-TRANSFERASE                | GGT      | +      |
| 15   | FERMENTATION/GLUCOSE                      | OFF      | -      |
| 17   | BETA-GLUCOSIDASE                          | BGLU     | +      |
| 18   | D-MALTASE                                 | dMAL     | -      |
| 19   | D-MANNITOL                                | dMAN     | +      |
| 20   | D-MANNNOSE                                | dMNE     | -      |
| 21   | BETA-XYLOSIDASE                           | BXYL     | +      |
| 22   | BETA-Alanine arylamidase pNA              | BAlap    | -      |
| 23   | L-Proline ARYLAMIDASE                    | ProA     | -      |
| 26   | LIPASE                                    | LIP      | +      |
| 27   | PALATINOSE                                | PLE      | -      |
| 29   | Tyrosine ARYLAMIDASE                     | TyrA     | +      |
| 31   | UREASE                                    | URE      | -      |
| 32   | D-SORBITOL                                | dSOR     | -      |
| 33   | SACCHAROSE/SUCROSE                        | SAC      | +      |
| 34   | D-TAGATOSE                                | dTAG     | -      |
| 35   | D-TREHALOSE                               | dTRE     | +      |
| 36   | CITRATE/(SODIUM)                          | CIT      | +      |
| 37   | MALONATE                                  | MNT      | -      |
| 39   | 5-KETO-D-GLUCONATE                        | 5KG      | -      |
| 40   | L-LACTATE alkalisation                    | ILATk    | +      |
| 41   | ALPHA-GLUCOSIDASE                         | AGLU     | -      |
| 42   | SUCCINATE alkalisation                    | SUCT     | +      |
| 43   | Beta-N-ACETYL-GALACTOSAMINIDASE           | NAGA     | +      |
| 44   | ALPHA-GALACTOSIDASE                       | AGAL     | -      |
| 45   | PHOSPHATASE                               | PHOS     | +      |
| 46   | Glycine ARYLAMIDASE                       | GlyA     | +      |
| 47   | ORNITHINE DECARBOXYLASE                   | ODC      | +      |
| 48   | LYSINE DECARBOXYLASE                      | LDC      | -      |
| 53   | L-HISTIDINE assimilation                  | IHI5a    | -      |
| 56   | COUMARATE                                 | CMT      | +      |
| 57   | BETA-GLUCORONIDASE                        | BGUR     | -      |
| 58   | O/129 RESISTANCE (comp. vibrio.)          | O129R    | +      |
| 59   | Glu-Gly-Arg-ARYLAMIDASE                   | GGAA     | -      |
| 61   | L-MALATE assimilation                     | IMLT a   | -      |
| 62   | ELLMAN                                    | ELLM     | -      |
| 64   | L-LACTATE assimilation                    | ILAT a   | -      |
Table 5 Results of microbial identification using Biomerieux VITEK 2 system

| SL NO. | STRAIN REF. NO.                  | SPECIES IDENTIFIED            | TEST METHOD        |
|--------|---------------------------------|--------------------------------|--------------------|
| 1      | TKMFT8,TKMFT22, TKMFT25,TKMFT39 | *Staphylococcus sciuri*       | VITEK/GP CARDS     |
| 2      | TKMFT 53                         | *Achromobacter xylosoxidans*  | VITEK/GN CARDS     |

Fig. 1 Zone of Diameter of protease producing bacterial isolates

Photo 1 Isolated organisms on Nutrient Agar plates

Photo 2 (a-e). Zone of hydrolysis on Gelatine agar
Biochemical identification - Biomerieux VITEK 2 system

The selected 5 isolates were identified using cultural characterization, microscopic observation and biochemical identification using Biomerieux VITEK 2 system. The results of cultural characterization and Microscopic observation were summarized in Table.2 and biochemical identification results using Biomerieux VITEK 2 system were presented in Table.3 & Table.4. Among the 5 isolates, TKMFT 8, 22, 25 and 39 are representing *Staphylococcus sciuri* and TKMFT 53 is representing *Achromobacter xylosoxidans* according to the test results. The results are presented in Table.5. Wallet *et al.*, (2005) reported the performances of VITEK 2 Colorimetric Cards for Identification of Gram-Positive and Gram-Negative Bacteria. Earlier findings (Funke G *et al.*, 1998) have proved the efficiency of VITEK -2 systems with 85.5% probability of accurate identification of strains. A similar study conducted by Simgamsetty *et al.*, (2016) found to achieve 90-95% probability of identification. In the present study, it was found to achieve 99% probability of identification for *Staphylococcus sciuri* (TKMFT8, TKMFT22, TKMFT25 & TKMFT39) and 91% probability obtained for *Achromobacter xylosoxidans* (TKMFT53).

In conclusion, samples collected from food processing industries shows presence of potent protease producers. A total number of 5 isolates were selected based on zone diameter. All 5 isolates obtained by initial screening of protease production were identified based on cultural characteristics, microscopic observation and biochemical identification using Biomerieux VITEK 2 system, an automated microbiology system for identification of microorganisms. Among the 5 isolates, TKMFT 8, 22, 25 & 39 are representing *Staphylococcus sciuri* and TKMFT53 is representing *Achromobacter xylosoxidans* according to the test results. From the results it is inferred that the bacterial strain TKMFT8 produces maximal protease followed by TKMF22, TKMFT25, TKMFT39 and TKMFT53.
Further these potent protease producers can be used for the degradation of proteinaceous waste from food processing industries. Hence the present study can play a significant role in the recycling of food industry wastes.

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