Isolation and Identification of Protease Producing Bacteria from Food Processing Industries

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Abstract

The objective of the present study was to isolate and identify protease producing bacteria from soil and wastewater collected from bakery industry especially halwa manufacturing units in Kollam & Karunagappally region (Kerala), India and detection of protease production on gelatine agar plates. Samples were serially diluted and 0.1 ml was spread on nutrient agar plates at 37°C for 48 hours. Total 87 dissimilar colonies were selected and detected protease activity on gelatine agar using 0.8 % mercuric chloride and the activity was observed by the occurrence of clear zone around the isolates. Among the 87 isolates, 27 isolates were showed zone formation around colonies and 8 isolates were selected based on zone diameter and named as, TKMFT8, TKMFT10, TKMFT19 TKMFT22, TKMFT25, TKMFT39, TKMFT53, TKMFT61. The selected were identified using cultural characterization, microscopic observation and biochemical identification using Biomerieux VITEK 2 system as Cedecea davisae, Staphylococcus intermedius, Enterobacter asburiae, Alloiococcus oitis and Proteus mirabilis. These bacterial isolates can be use as microbiological tool for recycling of waste from food processing industries.

Keywords

Soil, Waste water, Bacteria, Protease, Gelatine agar, Biomerieux, VITEK 2 system.

Introduction

Proteases are a group of enzymes catalyze hydrolysis of polypeptide chains into smaller polypeptides or free amino acids. Huge percentages (Ca.59%) of the international market of industrially important enzymes are occupied by proteases (Deng et al., 2010) Proteases represent the important group among the industrial enzymes with a long array of industrial applications. (Ashis and Sudhir, 2011; Singhal et al., 2012; Silva et al., 2012; Roja Rani et al., 2012; Sankeerthana et al., 2013; Adriana et al., 2013; Global Industrial Enzyme Market Research: 2013). Microbial proteases are one of the important groups of industrially and commercially produced enzymes and the initial screening methods for detection of protease production are of greatest importance (Kasana et al. 2011). Protease production is an inherent capacity of all microorganisms. (Padma Singh et al., 2015) Microbial sources play a major role in the production of all the three important
types of proteases – acidic, neutral and alkaline. Proteases have tremendous applications, especially in the detergents, food processing industries, waste treatment in the waste management system, leather tanning, and Chemical industries as well as in metal recovery. (V.N.Jisha et.al.2013)

Bakery industry is the rapidly growing industry in our country and it is considered to be one of the sources of food industry wastes. In many food processing units, separation of useful food constituents from undesired one generates huge fraction of solid waste in the initial stage of processing (Abdalla S.M. Ammar,2014). Halwa is a sweet Indian dish made up of different substrates primarily maida-the finely milled and refined wheat flour. Major Halwa manufacturing units in Kerala are located in Kollam and Karunagappally region. The waste from halwa manufacturing units comprises of gluten which is obtained from maida flour in the initial stage of processing. The two viable alternatives for waste management are application of waste as animal feed and to landfill. (Brown et al.1989).

In the present study, soil was collected from gluten waste discharged soil of halwa manufacturing units in Kollam and Karunagappally region as well as waste water from the processing centres for isolation and identification of protease producing bacteria. Protease producing bacteria can be used for the degradation of gluten waste.

**Materials and Methods**

**Sources of Sample Collection**

Soil (from the close premises of food processing centres) and water (waste water from the processing units) samples were collected and stored in sterile containers at 4 °C with date and time.

**Isolation of Protease Producing Bacteria**

The technique used for isolation of bacteria from soil and water samples was serial dilution agar plate technique described by Sjodahl et al. (2002).1 gram/ 1 ml of soil and water sample was added to 9 ml of sterile distilled water and performed serial dilution up to 10^-6 dilution under aseptic environment of laminar airflow cabinet. From each dilution 0.1 ml was spreaded on nutrient agar medium plates. Inoculated plates were incubated at 37° C for 48 hours. Nutrient agar slants of bacterial isolates were prepared and maintained at 4° C.

**Screening for Best Strain Produced Protease**

87 dissimilar colonies (TKMFT01 to TKMFT 87) from nutrient agar plates were selected and were subjected to primary screening for the production of protease by plate assay using protease specific medium containing (g/l) glucose 1.0, K2HPO4 2.0, Peptone 5.0, gelatin15.0, and agar 15. After 24h incubation at 28° C, the clear zone diameters were measured by flooded the plates with mercuric chloride solution, this method was described as gelatine clear zone method (Abdel Galil, 1992).

**Identification of Protease Producing Bacteria**

**Cultural Characterization**

Bacterial isolates were subjected to microscopic observation to obtain the colony morphology according to size, pigmentation, form, margin, elevation and colour.

**Microscopic Observation**

The selected bacterial isolates were gram stained and observed under a light microscope in high power magnifying lens.
Qualitative Test for Protease

Proteolytic activities of selected bacterial isolates were detected on the basis of formation of clear zones around the bacterial isolates. Gelatine agar plates were used for this purpose.

Identification of Bacteria using Biomerieux VITEK 2 System

The selected organisms were identified using Biomerieux VITEK 2 system and the test method was A O A C OMA 2012.02. and the selected protease producing bacterial isolates were identified as Cedecea davisae (TKMFT 8 ), Proteus mirabilis (TKMFT 19), Staphylococcus intermedium (TKMFT 22 TKMFT 10,TKMFT53 ), Enterobacter asburiae (TKMFT 39, TKMFT25) and Alloiococcus otitis (TKMFT 61)

Results and Discussion

Screening and Isolation of Proteolytic Bacteria

Microbial proteases are preferred to proteases from plant and animal sources, since they comprises of almost all desired characteristics for their applications in biotechnology field(Gouda et al.,2006). The proteolytic ability of 87 bacterial isolates from soil and waste water samples were evaluated using Gelatine agar medium as shown in Fig.2.

It was reported as appearance of clear zone (zone of hydrolysis) around bacterial colonies. Among 87 isolates 27 isolates were found positive based on zone of hydrolysis and out of them 8 isolates (TKMFT 8, TKMFT10, TKMFT19, TKMFT22, TKMFT25, TKMFT39, TKMFT53, and TKMFT61) were chosen for further study based on diameter of zone of hydrolysis.

The results of bacterial isolates showing zone of inhibition (Diameter in mm) are presented in Table.1 Arun Kumar Sharma (2015) reported that gelatine agar medium was best than skim milk agar medium for qualitative test of protease production because zone of hydrolysis were developed with more clarity in gelatine agar plates.

Alnahdi (2012) reported screening and isolation of 6 different bacterial strains on gelatine agar medium. Proteolytic activity was expressed as diameter of clear zone of hydrolysis around bacterial colonies. The results of zone of hydrolysis on Gelatine agar are presented in Table.1. Similar screening method using gelatine agar has been adopted earlier by Abirami et al.,2011;Geethanjali and Subash (2011); Sevine and Demirkan,(2011); Najla O Ayaz (2012), Smitha et al., 2012; Sinha et al, (2013).

Identification of Protease Producing Bacterial Isolates

The selected 8 isolates were identified using cultural characterization, microscopic observation and biochemical identification using Biomerieux VITEK 2 system. Among the identified species,TKMFT8 belonged to Cedecea davisae,TKMFT 10, TKMFT22, TKMFT53 to Staphylococcus intermedium, TKMFT 25, TKMFT 39 to Enterobacter asburiae, TKMFT61 to Alloiococcus otitis and TKMFT19 to Proteus mirabilis. The results are presented in Tables 2,3,4,5.
Table 1: Bacterial Isolates Showing Zone of Inhibition (Diameter in Mm)

| Sl No | Bacterial isolates | Diameter of Zone of hydrolysis (mm) |
|-------|--------------------|-------------------------------------|
| 1     | TKMFT 8            | 7                                   |
| 2     | TKMFT 19           | 8                                   |
| 3     | TKMFT 22           | 15                                  |
| 4     | TKMFT 39           | 11                                  |
| 5     | TKMFT 61           | 10                                  |
| 6     | TKMFT 10           | 8                                   |
| 7     | TKMFT 25           | 9                                   |
| 8     | TKMFT 53           | 9                                   |

Table 2: Results of Gram Staining and Colony Characters of Selected Isolates

| Sl No | Bacterial isolates | Result of Gram staining | Colony characters on Nutrient agar |
|-------|--------------------|--------------------------|-----------------------------------|
|       |                    |                          | Size     | Pigmentation | Form     | Margin   | Elevation | Texture   |
| 1     | TKMFT 8            | Gram negative cocci     | Small    | No           | Circular | Entire   | Flat      | Rough     |
| 2     | TKMFT 19           | Gram negative cocci     | Small    | No           | Circular | Entire   | Undulate  | Smooth, oily |
| 3     | TKMFT22, TKMFT10, TKMFT53 | Gram negative cocci | Small    | Yellow       | Circular | Entire   | Raised    | Smooth     |
| 4     | TKMFT 39 TKMFT 25  | Gram negative cocci     | Moderate | No           | Circular | Entire   | Flat      | Rough, cottony |
| 5     | TKMFT 61           | Gram positive cocci     | Large    | No           | Irregular| Entire   | Flat      | Rough     |

Figure 1: Isolation of Organisms from Soil and Waste Water
Table 3: Biochemical Details of Organisms Identified Using Biomerieux Vitek/Gn Cards

| Well | Test                              | Mnemonic                  | Result |
|------|-----------------------------------|---------------------------|--------|
| 2    | Ala-Phe-Pro-ARYLAMIDASE           | APPA                      | TKMFT8 TKMFT19 TKMFT25,39, |
| 3    | ADONITOL                          | ADO                       |        |
| 4    | L-Pyrrolydonyl-                   | PyrA                      |        |
| 5    | L-ARABITOL                        | IARL                      |        |
| 7    | D-CELOBIOSE                       | dCEL                      |        |
| 9    | BETA-GALACTOSIDASE                | BGAL                      |        |
| 10   | H2S PRODUCTION                    | H2S                       |        |
| 11   | BETA-N-ACETYL-                    | BNAG                      |        |
| 12   | Glutamyl Arylamidase pNA          | AGLTp                     |        |
| 13   | D-GLUCOSE                         | dGLU                      |        |
| 14   | GAMMA-GLUTAMYL-                   | GGT                       |        |
| 15   | FERMENTATION/GLUCOSE              | OFF                       |        |
| 17   | BETA-GLUCOSIDASE                  | BGLU                      |        |
| 18   | D-MALTOSCE                        | dMAL                      |        |
| 19   | D-MANNITOL                        | dMAN                      |        |
| 20   | D-MANNOSE                         | dMNE                      |        |
| 21   | BETA-XYLOSIDASE                   | BXYL                      |        |
| 22   | Beta-Alanine arylamidase pNA      | BAalap                    |        |
| 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-DTALGATOSE                      | 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-                    | NAGA                      |        |
| 44   | ALPHA-GALACTOSIDASE               | AGAL                      |        |
| 45   | PHOSPHATASE                       | PHOS                      |        |
| 46   | Glycine ARYLAMIDASE               | GlyA                      |        |
| 47   | ORNITHINE DECARBOXYLASE           | ODC                       |        |
| 48   | LYSINE DECARBOXYLASE              | LDC                       |        |
| 53   | L-HISTIDINE assimilation          | IHISa                     |        |
| 56   | COUMARATE                         | CMT                       |        |
| 57   | BETA-GLUCONONIDASE                | BGUR                      |        |
| 58   | O/129 RESISTANCE                  | O129R                     |        |
| 59   | Glu-Gly-Arg-ARYLAMIDASE           | GGAA                      |        |
| 61   | L-MALATE assimilation             | IMLTka                    |        |
| 62   | ELMAN                             | ELM                       |        |
| 64   | L-LACTATE assimilation            | ILATa                     |        |
Table 4: Biochemical Details of Organisms Identified Using Biomerieux Vitek/Gp Cards

| Well | Test                              | Mnemonic | Result |
|------|-----------------------------------|----------|--------|
| 2    | D-AMYGDALIN                       | AMY      | -      |
| 4    | PHOSPHATIDYLINOSITOL              | 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        | PpyrA    | +      |
| 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 alkalinization          | ILATk    | +      |
| 42   |LACTOSE                           | LAC      | -      |
| 44   |N-ACETYL-D-GLUCOSAMINE            | NAG      | +      |
| 45   |D-MALTOSE                         | dMAL     | -      |
| 46   |BACTITRACIN 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 5 Results of Microbial Identification Using Biomerieux Vitek 2 System

| Sl No. | Bacterial isolate | Organism              | Test Method          |
|--------|-------------------|-----------------------|----------------------|
| 1      | TKMFT22,10,53     | *Staphylococcus intermedium* | VITEK/GP CARDS      |
| 2      | TKMFT25,39        | *Enterobacter asburiae* | VITEK/GN CARDS      |
| 3      | TKMFT8            | *Cedecea davisae*     | VITEK/GN CARDS      |
| 4      | TKMFT19           | *Proteus mirabilis*   | VITEK/GN CARDS      |
| 5      | TKMFT61           | *Alloiococcus otitis*  | VITEK/GP CARDS      |

Figure 2 Zone of Hydrolysis on Gelatine Agar

In conclusion, soil and waste water collected from food processing industries in Kollam and Karunagappally region shows presence of protease producers. A total number of 87 bacterial isolates were tested for protease production in gelatine agar plates and 27 isolates exhibited zone of hydrolysis and 8 isolates were selected based on zone
diameter. All 8 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 8 isolates, TKMFT22, 10, 53 are representing Staphylococcus intermedius, TKMFT25, 39 are representing Enterobacter asburiae, and TKMFT8, TKMFT 19, and TKMFT61 are representing Cedecea davisae, Proteus mirabilis and Alloiococcus otitis respectively according to the test results. Further these protease producing isolates can be used for degrading gluten waste from bakery industry viz. halwa manufacturing units. Hence the present study can play a significant role in the recycling of food industry wastes.

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