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Isolation and Identification of Protease Producing Bacteria from Food Processing Industries

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A B S T R A C T

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 for48 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 otitis and Proteus mirabilis.* These bacterial isolates can be use as microbiological tool for recycling of waste from food processing industries.

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^{0} C for 48 hours. Nutrient agar slants of bacterial isolates were prepared and maintained at 4^{0} 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 intermedius* (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 intermedius,* 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	TKMFT10	8
7	TKMFT25	9
8	TKMFT53	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 TKMFT25	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

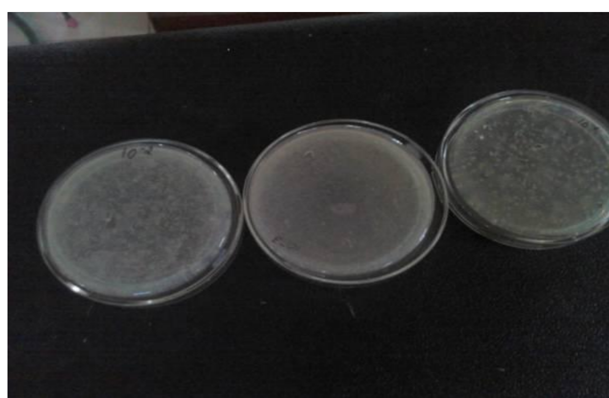


Table.3 Biochemical Details of Organisms Identified Using Biomerieux Vitek/Gn Cards

Well	Test	Mnemonic	Result		
			TKMFT8	TKMFT19	TKMFT25,39,
2	Ala-Phe-Pro-ARYLAMIDASE	APPA	-	-	-
3	ADONITOL	ADO	-	-	-
4	L-Pyrorolydonyl-	PyrA	-	-	-
5	L-ARABITOL	IARL	-	-	-
7	D-CELLOBIOSE	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-MALTOSE	dMAL	-	-	+
19	D-MANNITOL	dMAN	+	-	+
20	D-MANNOSE	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 alkalinisation	ILATk	+	-	+
41	ALPHA-GLUCOSIDASE	AGLU	-	-	-
42	SUCCINATE alkalinisation	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-GLUCORONIDASE	BGUR	-	-	-
58	O/129 RESISTANCE	O129R	+	+	+
59	Glu-Gly-Arg-ARYLAMIDASE	GGAA	-	-	+
61	L-MALATE assimilation	IMLTa	-	-	-
62	ELLMAN	ELLM	-	-	-
64	L-LACTATE assimilation	ILATa	-	-	-

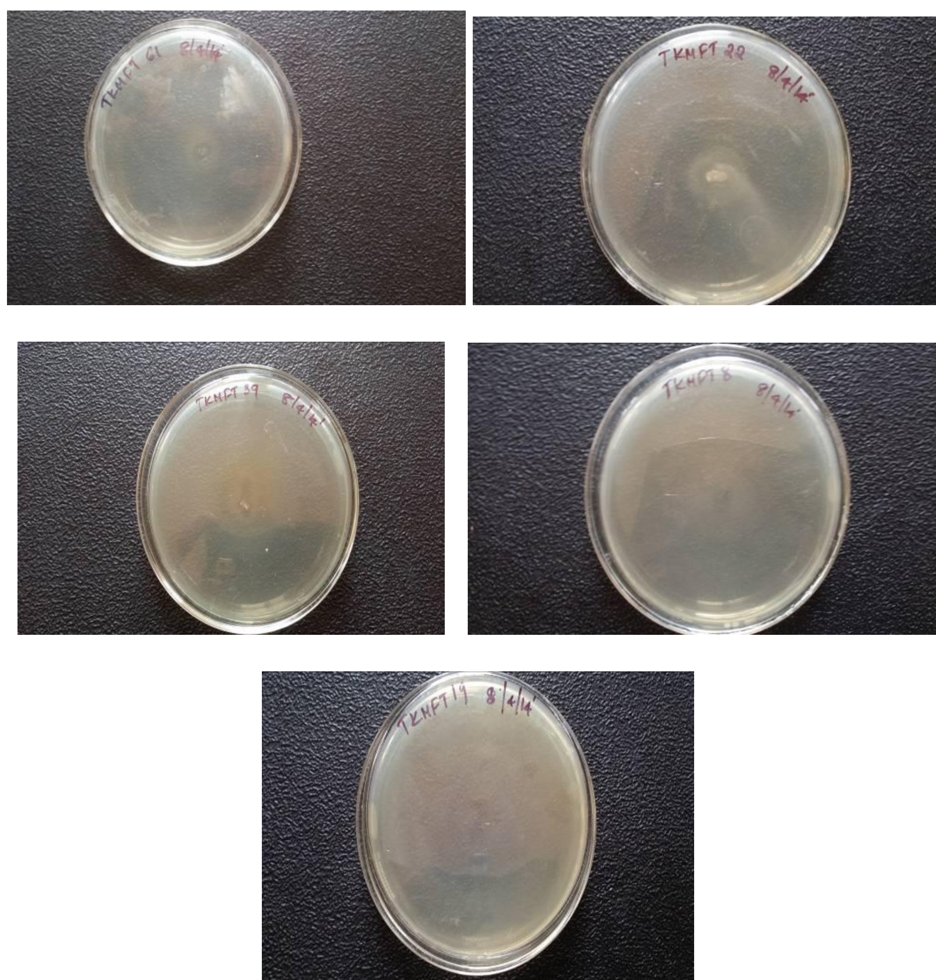
Table.4 Biochemical Details of Organisms Identified Using Biomerieux Vitek/Gp Cards

Well	Test	Mnemonic	Result	
			TKMFT22,10,53	**TKMFT61**
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	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 alkalinization	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.5 Results of Microbial Identification Using Biomerieux Vitek 2 System

Sl No.	Bacterial isolate	Organism	Test Method
1	TKMFT22,10,53	*Staphylococcus intermedius*	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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