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Isolation and Identification of Protease producing Bacteria through Biodegradation of Protein content of Kitchen Wastes in Gwalior, India

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A B S T R A C T

The current paper investigates skimmed milk (protein) degrading bacteria found in kitchen waste for maximum skimmed milk activity and growth of bacteria at pH and optimum temperature. Sample was collected from household kitchen waste and bacterial strains were isolated using nutrient agar media. Enrichment technique was used for isolating PDB (protein degrading bacteria) strains. The objective of research was to utilize effective bacteria for degrading complex polymer protein into simpler amino acids under optimum conditions. Out of 06 isolated bacterial strains, only two were having effective protein degrading bacteria. These two isolates were screened for qualitative estimation through Skimmed milk medium and quantitatively tested by Protease activity was measured by the standard method. The optimum pH and temperature for most potent isolate was recorded as 7 pH and 37°C correspondingly. These optimum working conditions were recommended for biomass utilization.

Introduction

Biodegradation is the broken down of organic contaminants occurring due to production of extracellular enzymes by microorganisms. These contaminants can be considered as the substrate or microbial food source (Maier *et al.*, 2000). Biodegradable materials have the proven capability to degrade in the most common environment where the material is managed, within a year, through natural biological processes into non-toxic carbonaceous soil, H_2O and CO_2 (Vander Zee, 1997).

Kitchen wastes consist of household kitchen wastes such as vegetables peels, fruit peels, smashed fibre of fruits, spare uneaten food items, food grains. Kitchen Waste is full of nutrients and organic materials and easily biodegradable (Soliva *et al.*, 2010). An Indian city generates about 0.8 to 1 kg solid wastes per capita per day (waste management at military station, 2009). These wastes are collected and dumped into the landfills, causing major pollution (Bouallagui *et al.*, 2003; Bouallagui *et al.*, 2005). Urban solid waste in India mainly made up of the 70% to 80% of domestic waste which are high in organic waste. A standard family produces up to 0.5 to 0.75 kgs of kitchen waste per day (Tweib *et al.*, 2014).

Proteases are a group of enzymes catalyze hydrolysis of bonds in polypeptide chains and split them into smaller polypeptides or else free amino acids. Huge percentages (ca. 59%) of the international market of industrially important enzymes are covered by proteases (Deng *et al*., 2010). Protease production is an inherent capacity of all microorganisms. (Padma Singh *et al*., 2015). Proteolytic enzymes support the natural healing process in local management of skin ulceration by efficient removal of necrotic material (Sjodahl *et al*., 2002). Proteases catalyze or hydrolyze protein (Schallmey *et al*., 2004).

Bacterial Proteases are preferred as they grow rapidly, needless space, can be easily maintained and are accessible for genetic manipulations (Odu and Akujobi, 2012).

Conversion of wastes into useful biomass by microbes and their enzymes is a new trend, and new protease-producing micro-organisms and perfected fermentation technology are needed to meet the ever-growing demand for this enzyme (Rathakrishnan *et al*., 2012). Some examples of potent alkaline protease producing *Bacilli* strains are *B. amyloliquifaciens, B. subtilis* and *B. lichiniformis*. Some other bacterial species which are also known for their protease production potential are *Staphylococcus, Pseudomonas, Serratia, Alcaligenes, Vibrio, Brevibacterium, Flavobacterium* and *Halobacterium* (Gupta *et al*., 2005).

Depending on the nature of the industrial by-products or waste, this would allow obtaining products with higher digestibility and nutritional value, destined to both human and animal food (Nortey *et al*., 2007; Okonko *et al*., 2009). Additionally, it would be possible to use them as renewable carbon sources in large scale fermentation processes. Another alternative could be its utilization as raw-material for the production of high-value products.

In recent years, there has been a marked trend towards the use of novel technologies, mainly focused on biological processes, for recycling and efficient utilization of organic residues (Nigam and Pandey, 2009; Okonko *et al*., 2009).

The major natural organic matters available are polymeric, e.g. polysaccharides (cellulose, hemicellulose, pectins, starch etc.), lignin, and protein, which can be metabolized by different microbes as a source of energy through enzymatic hydrolysis. While there is a wide diversity of microorganism producing hydrolytic enzymes, including bacteria, yeast, and fungi, the use of indigenous microbes (waste native microflora) ensures efficient activity, acting in an environment to which it is adapted (Ponce *et al*., 2008). The aim of this study is to isolate and characterize bacterial strains from kitchen waste by determining their ability to produce protease enzymes and their potential for use as a potential bacteria in the degradation of kitchen waste.

Materials and Methods

Sample collection

The kitchen waste samples were collected from a cafeteria, in Gwalior.

Isolation of Bacteria

The bacteria were isolated through the enrichment technique. The technique consisted of incubating, in 125 mL conical flasks containing 50 mL of Nutrient Agar Medium (composition in g/L): Peptone-5 gm, NaCl-5 gm, Yeast Extract -1.5 gm, Beef

Extract -1.5 gm, Agar-Agar -15.0 gm, pH-7.4.

The technique used for isolation of bacteria from kitchen waste samples was serial dilution agar plate technique. 1 gram/ 1 ml of kitchen wastes 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 of the high protease producing bacteria

Qualitative protease activity assay was evaluated on Modified Basal Medium (MM) that contained (g/L): 1 g glucose, 2.5 g yeast extract and 14 g agar–agar supplemented with 6.2 g/L skim milk protein (5 g/L of casein). The formation of clear zone (12 mm or more) in the medium surrounding the well indicated positive protease activity (Perez *et al*., 2009).

Identification of the isolated bacteria by Biochemical characterization

Gram's staining; morphological studies, physiological and biochemical characteristics of the isolate were investigated according to Bergey's Manuals Bergey *et al*., 1974 (Table-1).

Morphological studies of Bacterial culture characterization

Bacterial isolates were subjected to microscopic observation to obtain the colony morphology according to size, pigmentation, form, margin, elevation and colour (Table-3).

Qualitative Test for Protease

Proteolytic activities of selected bacterial isolates were detected on the basis of formation of clear zones around the bacterial isolates (Fig-3).

Protease enzyme assay

Enzyme assay for Protease enzyme

Protease activity was determined by a modified method of Folin and Ciocalteu (1927). 200 µl of the protease broth was added to the reaction mixture, containing 0.65% (wv-1) casein in 800 µl of 50 mM in phosphate buffer (pH 9). The mixture was incubated at 75^{o}C for 10 min. The reaction was stopped by the addition of 1 ml of 5% (wv-1) Trichloroacetic acid (TCA), followed by centrifugation at 10,000×*g* for 15 min. The supernatant were analyzed by the Folin-Ciocalteu reagent. One unit of protease activity was defined as the amount of enzyme that liberated 1µg tyrosine per min per ml of protease broth.

Results and Discussion

The proteolytic ability of 02 bacterial isolates from kitchen waste samples were evaluated using Skimmed milk agar (Protease) medium as shown in Fig.1. It was reported as appearance of clear zone (zone of hydrolysis) around bacterial colonies.

Table.1 Morphological, biochemical and physiological analysis of isolated bacteria from kitchen wastes

Characteristic	**B1**	**B2**
Gram's staining test	Gram +ve	Gram +ve
Morphology	Rods	Rods
Endospore	Cenral	Central
Oxidase	_	+
Catalase	+	+
Motility	+	+
Indole production		_
H_2S production	_	_
Citrate utilization	_	_
Gelatin hydrolysis	_	_
Lecithinase	_	+
Urease	_	+
Starch hydrolysis	+	+
Methyl red test	_	+
Voges Proskauer test	_	+
Nitrate reduction	_d	+
Acid production from Glucose	+	+
Arabinose	+	+
Xylose	_	_
Mannitol	+	_
Trealose	+	+
Inositol	_	_
Lactose	_	_
Growth at 41 ^{0}C		
Growth at 50 ^{0}C	_	_
Casein hydrolysis	+	+
Phenylalanine	_	_
Growth 7.5% (w/v) NaCl	_	_
Anaerobic growth	Obligate aerobic	Facultative

+, positive reaction; _,negative reactions; d, dubious.

Table.2 Bacterial Isolates Showing Zone of hydrolysis (Diameter in mm)

S.No.	Bacterial Isolates	Diameter of zone of hydrolysis (mm)
1.	B1 Isolates	12
2.	B2 Isolates	14

Table.3 Morphological studies of isolated bacteria

S. No.	Bacterial Isolates	Morphology
01	B1 Isolates	Slightly irregular with undulated margin
02	B2 Isolates	dry, flat and irregular, with lobate margins

Table.1 Protease enzyme activity by bacterial isolates after 2 days of incubation.

S. No.	Bacterial Isolates	O.D. at 660 nm	Concentration of tyrosine released (µM)	Enzyme activity (U/mg)
01.	B1 Isolates	1.21	204.34	36.79
02.	B2 isolates	1.19	201.27	34.89

Fig.1 B1 and B2 - Show zone formation in protease medium

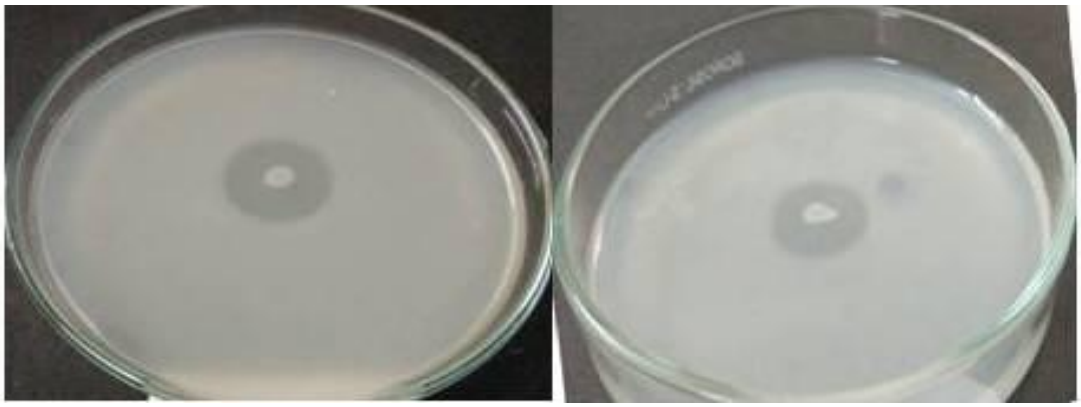


Fig.2 Evaluation of zone formation measurement in mm

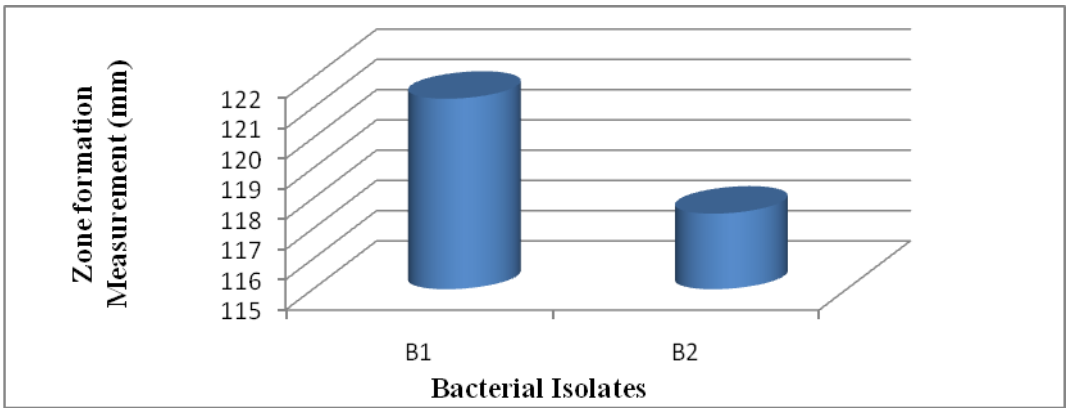
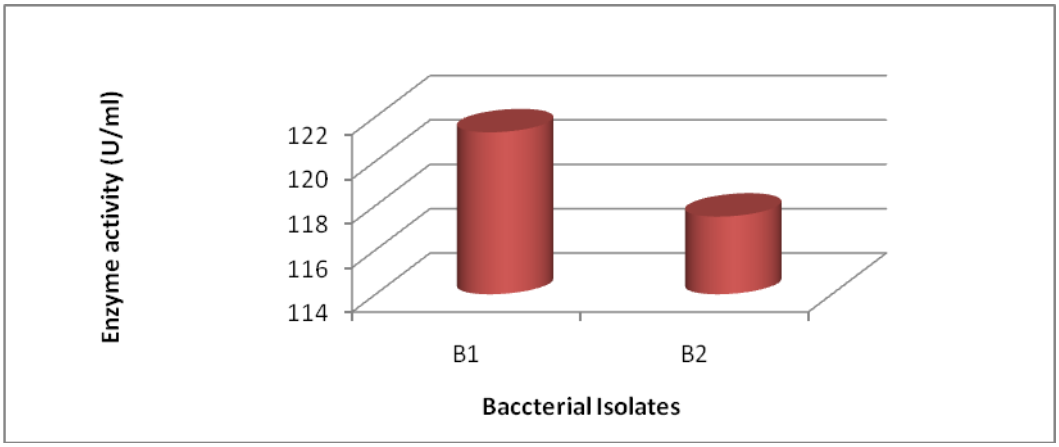


Fig.3 Evaluation of qualitative enzyme activity of isolated Bacteria



Two isolates were found positive based on zone of hydrolysis and out of them 06 isolates. (B1, B2) were selected for further study based on diameter of zone of hydrolysis. And enzyme activity of B1 Bacterial isolates is maximum activity show than the other B2, bacteria. The results of bacterial isolates showing zone of inhibition (Diameter in mm) are presented in Table- 2 and Fig-1 and Fig-2.

Screening of protease producing bacteria

Alnahdi (2012) reported screening of 02 bacterial isolates on skim milk agar plate and gelatin agar plate.

Proteolytic activity was expressed as diameter of clear zone of hydrolysis around bacterial colonies. Two *Bacillus* isolates (B1, B2) were selected for protease production based on appearance of zone of hydrolysis in skim milk agar medium. Similar screening method using skim milk agar medium and gelatin agar medium has been used earlier by Abirami *et al*., 2011; Geethanjali and Subash, 2011; Sevinc and Demirkan, 2011; Smita *et al*., 2012; Sinha *et al*., 2013. A total of 06 bacterial isolates from kitchen wastes were screened on skim milk agar plate and 02 isolates were demonstrated transparent circular zone around colonies indicated protease production (Sinha *et al*., 2013). These halo zones of two bacteria are given below in Table- 2 and Fig-1 and Fig-2.

Graphical studies of screening of protease producing bacteria.

Identification of Protease producing Bacteria

These bacteria are identified B1 as *Bacillus Megaterium* and B2 as *Bacillus Subtilis* bacteria according to Bergey's manual of Determinative Bacteriology 9th (Table-1).

Cultural Characterization

The B1 and B2 Bacteria are identified through morphological Studies of cultures. These morphological studies are given below in table-3.

Qualitative test for protease production

To achieve this objective, a total of 6 isolates (2 bacterial) were tested for extracellular protease production in liquid medium (quantitative test). Fig- 3, showed that maximum protease activity (121.3 U/mL) was obtained by bacterial isolate B1 at 37 ºC after 72 h of incubation. Whereas lowest protease

activity was observed by bacterial isolate B2 with enzyme activity (117.5 U/mL). Therefore we found that proteolytic activity according to Sevinc and Demirkan (2011) replaced glucose in basal medium by different sugars and reported that fructose was found to be the best carbon source for highest protease production (125 U/mL) by *Bacillus* sp. N-40.

Protease enzyme assay

These bacteria are produced enzyme according to protein substrates in particular broth. These numbers of units of activity per mg protein was taken as the specific activity of the enzyme.

In conclusion, basically, *Bacillus spp* are role play in degradation of proteineous content of kitchen wastes changes into different kinds of amino acids as simpler molecules as according to protein composition, due to secreted hydrolytic protease enzymes. These amino acids are used as source of nitrogen and carbon for growth and development of other living organisms. Optimized bacteria can survive in adverse condition for decomposition of protein containing kitchen wastes in environment.

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