Int.J.Curr.Microbiol.App.Sci (2016) *5*(3): 268-276

EXCELLENT PUBLISHERS

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 3(2016) pp. 268-276**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.503.033

Extraction and Bio-chemical Characterization of Protease Enzyme from a Proteolytic bacteria Isolated from Dry Mixed Kitchen Waste

Diptendu Sarkar[1*] and Goutam Paul[2]

[1]New Horizon College of Engineering, Dept. of Biotechnology, Ring Rd, Kadubisanahalli, Bellandur Post, Near Marathalli, Bangalore-560103, India
[2]Dept. of Physiology, Environmental Physiology Division, Microbiology Unit, University of Kalyani, Nadia, West Bengal-741235, India
**Corresponding author*

Keywords

Submerged Fermentation, Proteolytic Activity, Protease.

Article Info

Accepted:
15 February 2016
Available Online:
10, March 2016

A B S T R A C T

The present research finding have shown that isolation and biochemical characterization of protease enzyme from proteolytic bacteria isolated from mixed vegetable kitchen waste. Three bacterium were isolated from kitchen waste and were screened for proteolytic enzyme production when grown on protein containing solid media, and designated them as DSP1, DSP2 and DSP3. DSP2 (found as *Bacillus sp*) was selected based on clearance zones and protease enzyme production was carried out in submerged fermentation. Enzyme production by DSP2 was higher at pH 6.0 and a temperature of 30°C using sucrose and ammonium sulphate as carbon source and nitrogen source, respectively. The maximal activity of DSP2 protease was at 30°C, pH 5.0 and was thermostable up to 60°C. Manganese sulphate had high effect on protease enzyme activity.

Introduction

Protease is a group of enzymes that break down the complex protein into amino acids. Proteases of commercial importance are produced from microbial, animal and plant sources (Gupta *et al.,* 2005). Microbial proteases are produced from high yielding strains including species of *Bacillus sp., Alcaligenes faecalis, Pseudomonas fluorescens and Aeromonas hydrophylia* grown under submerged culture conditions. Among these, *Bacillus sp.* is the most important group of bacteria that are involved in the enzyme industry and this bacterium is also known to produce proteolytic enzymes quite effectively. Moreover, these enzymes are essential in the decay of dead plant material by nonpathogenic microorganisms and thus assist in recycling carbon compounds in the biosphere (Moon *et al.,* 1991; Micales, J.A., 1992). Several literature indicates that enzymes such as protease can be easily extracted from vegetable waste like beet, carrot, cabbage, tomatoes, by simple extraction processes. New enzymes for use in commercial applications with desirable biochemical and physico-chemical characteristics and a low cost production have been the focus of much research.

Enzyme breakdown of the biomolecules depends upon the type of enzyme, application, temperature, incubation time, agitation, concentration, pH and use of different enzyme preparations (Patel *et al.,* 1985).

In this context, the objective of the present study was to utilize the kitchen waste and bacterial isolates were extracted, that produce protease enzyme at a cheaper rate. These protease enzymes have importance in industries like fruit industry, textile industry, in waste water treatment and therefore have shown future utility.

Materials and Methods

Culture Isolation

Approximately 1.5kg of kitchen mixed vegetable waste collected from boy's hostel kitchen of Acharya College of Science, Bangalore and 500 g of it used to make suspension in 50ml D/W. From this suspension, 1ml was dispensed in 100ml Nutrient Broth (NB) & Potato Dextrose Broth (PDB) respectively. The flasks were incubated at shaker conditions of 400 rpm for 48 hr at 30°C. After incubation, one loopful of suspended broth was inoculated onto Nutrient Agar (NA) and Potato Dextrose Agar (PDA) and incubated at 30°C for 48hrs (Kaur *et al.,* 1998).

Screening of Isolates

Colonies that were grown on NA and PDA media were picked up and spotted on plates containing Hankins's agar medium (Hi-Media). These plates were incubated at 30°C for 48 hrs. After incubation 1 % BSA was overlaid on these plates and kept for half an hour. Clear zones were observed around the colonies indicating the presence of pectinase producing organisms. Cells from these colonies were then picked up and sub-cultured on NA slants and maintained for further studies. Cultures showing larger zones of clearance were chosen for identification based on morphology and biochemical characteristics as per mentioned in Bergy's Manual (Gupta *et al.,* 2005; Fermor *et al.,* 1981).

Preparation of Inoculum

The Hankins broth medium was prepared in 500ml Erlenmeyer flasks and the flask was inoculated with loopful of cells grown for 48hr on NA slants. The flasks were kept on shaker conditions of 600 rpm for 48hr at 30°C with media pH of 7.0 (Gupta *et al.,* 2005; Patel *et al.,* 1985).

Production of Pectinase using Dry Mixed Kitchen Waste

In 500ml Erlenmeyer flasks, 200g of crushed mixed dry kitchen waste was added and was dispensed with 100ml of D/W. To this flask, 10ml of bacterial suspension of O.D. 0.05 from inoculum flask was added. Similar set up was followed for studying the effect of temperature (30°C to 60°C), pH (6 to 8) and concentration of vegetable waste (50g to 300 g) on enzyme production. The flask was incubated on a shaker at 400rpm for 48hr. The supernatant was removed from the cultural broth by centrifuging it at 8000 rpm for 20 min and the clear filtrate was used for determining protease activity. C_x ratio was calculated according to the following formula.(Gupta *et al,* 2005 and Patel *et al.,* 1985).

C_x Ratio= [(Diameter of zone of clearance) / (Diameter of Colony)].

Protease Activity Measurement

The enzyme was assayed in the reaction mixture containing 2.0 ml of 0.5 % casein solution in 0.1 M CO3-HCO3 buffer (pH

9.5) and 1ml enzyme solution in a total volume of 3.0 ml. After inoculation at 30^0C for five minutes, the reaction was stopped by adding of 3.0 ml of 10 % ice cold TCA and centrifuged at 10,000 rpm for five minutes. Protein in the supernatant was estimated by the method of Lowry *et al*. (Patel *et al.,* 1985; Sabota *et al.,* 1995).

Results and Discussion

Eight isolates showing protease activity were obtained based on zones of clearance seen around them. The three isolates showing good C_x ratio on agar plate were selected from 8 isolates, of which 3 isolates showing maximum C_x ratio were than selected and were named DSP 1, DSP 2 and DSP 3 (Table 1 and Fig 1, 2).

Based on their enzyme activity study, out of three isolates, only one (DSP2) was finalized for further studies. Morphological and Biochemical studies of the culture DSP2 was also done. This culture was found to be Gram negative in nature. Biochemically the culture of DSP2 was found to be belonging to the *Bacillus* group of sp.16S rRNA gene was amplified and sequencing of partial sequence of 980 bp was carried out. Using BLAST analysis, the organism was found to be *Bacillus* sp. which was incorporated in to the collaborative nucleotide database of GenBank, EMBL and DDBJ and assigned an accession number (Table1).

The temperature and pH of the cultivation medium are important factors in the protease production, which may influence the sort and content of those enzymes produced by filamentous fungus and bacteria (Sabota *et al.,*1995; Suseela R, 1998; Ogrydziak *et al.,* 1998).The strong effect of pH on the production of protease was clearly observed in flask cultures, where pH value 6-8, and temperature 30^0C-50^0C were tested for crude enzyme production (Fig 3,4,5). The maximum pectinase activity was found with an initial pH of 6 at temperature 30^0C, activity reaching 0.69µg/ml/min on fifth day of incubation. When the temperature was maintained at 40^0C and 50^0C with pH 6, the enzyme activity was found to be 0.51 µg/ml/min and 0.46µg/ml/min respectively on fifth day of incubation. By keeping the pH constant at pH 7, the enzyme activity was measured as 0.57µg/ml/min at 30^0C, 0.504 at 40^0C and 0.39µg/ml/min at 50^0C whereas by keeping pH 8 as constant, it was seen that the enzyme activity was found to be 0.61µg/ml/min at 30^0C, 0.44µg/ml/min at 40^0C and 0.33 µg/ml/min at 50^0C. So optimum pH for enzyme production was found to be 6 and optimum temperature was found to be 30^0C. Either increase or decrease beyond the optimum value show decline in enzyme production. However, the mechanism by which the pH and temperature both acts on the same time for the production of protease enzyme is not clearly known.

The temperature optima of 30^0C, was obtained from a purified culture fluid of *P.frequentans* by Patel *et al.,* 1985. This can give the support to our obtained result. From the observation it was clear that 30^0C was the optimum temperature for the growth of microorganisms, variation was done in different pH and it was seen that pH 6 was optimum (Fig 4, 5, 6).

Supplementation of different carbohydrate sources (dextrose, mannitol, sucrose and starch) to the production medium increase the protease activity of *DSP2*(Fig 7, 8). When pH 6 and temperature 30^0C (both optimum for production) were maintained constant in production medium, the different carbohydrate sources showed different effect on protease production respectively.

Table.1 Cx Ratio and Enzyme Activity of the Final Three Pectinase Producing Isolates

Isolate Name	GenBankAccn No	C_x ratio	Enzyme activityµg/ml/min
DSP1	-----	3.8	2.78
DSP2	JN836944	4.2	4.89
DSP3	------	3.7	3.76

Table.2 Morphological and Biochemical Characteristics of Isolated *Bacillus sp.*(dsp2)

Morphological, Cultural, Physiological and Biochemical characteristics	Results
Morphology	
Cell shape	Rod
Size	5.25 µm
Gram's reaction	Gram negative
Capsule	Present
Spore	spore forming
Cultural condition	
Growth	Aerobic
Motility	Motile
Growth in NaCl (20%)	+
Growth temperature (°C)	35-37
Biochemical activities	
Catalase	+
Oxidase	-
Glucose	Acid
Sucrose	Acid/Gas
Lactose	Acid
Fructose	Acid
Arabinose	Acid/Gas
Galactose	Acid/Gas
Mannitol	Acid
Raffinose	Acid/Gas
Xylose	Acid/Gas
Methyl red	-
VP-test	+
Indole	-
Citrate	-
Nitrate	+
Urease	+
Casein Hydrolysis	+
Starch	+
Gelatin	+
Identification (DSP2)	Above characteristics indicates that the isolate belongs to the genus *Bacillus gr*

Fig.1 Comparison of Cx Ratio among Final Three Pectinase Producing Isolates

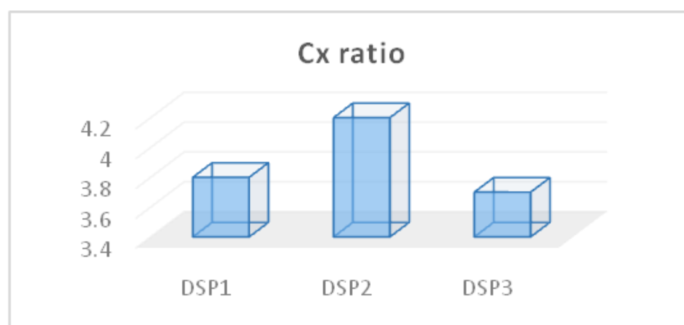


Fig.2 Comparison of Enzyme Activity among Final Three Pectinase Producing Isolates

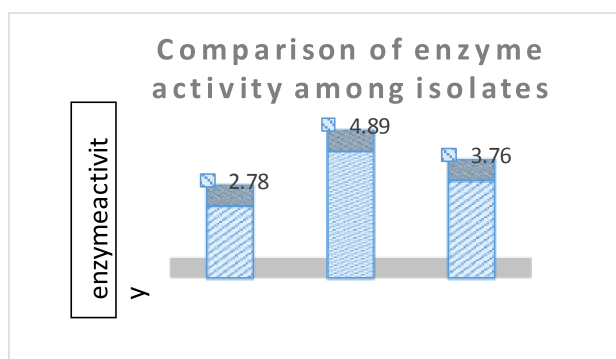


Fig.3 Gram Negative *Bacillus* Dsp2 (100x)

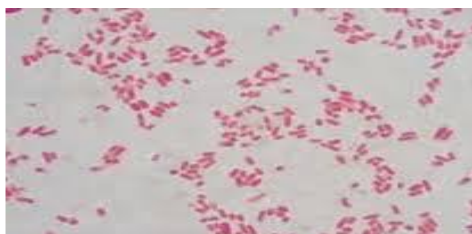


Fig.4 Enzyme Activity Measured at Different Temperatures Keeping pH 6

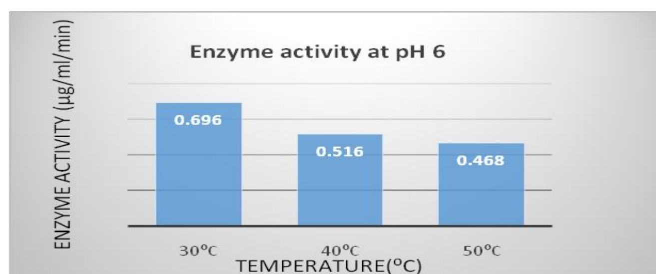


Fig.5 Enzyme Activity Measured at Different Temperatures Keeping pH 7

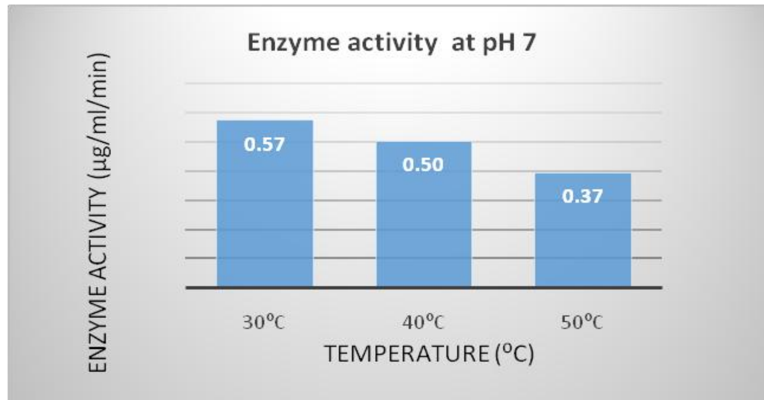


Fig.6 Enzyme Activity Measured at Different Temperatures Keeping pH 8

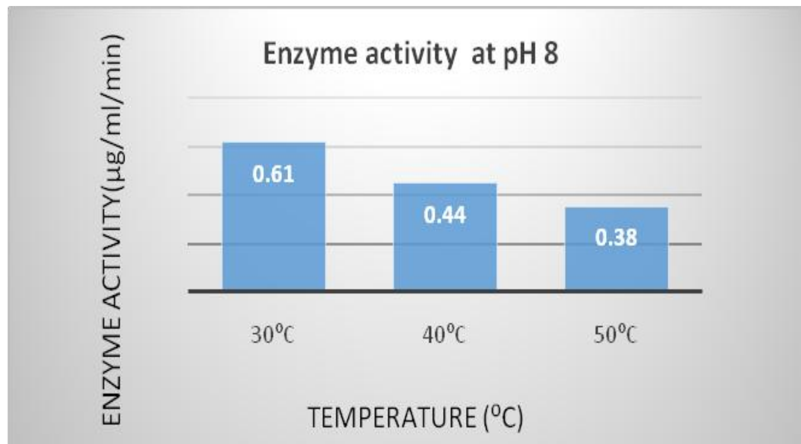


Fig.7 Graph to Show the Enzyme Activity with Different Carbohydrates

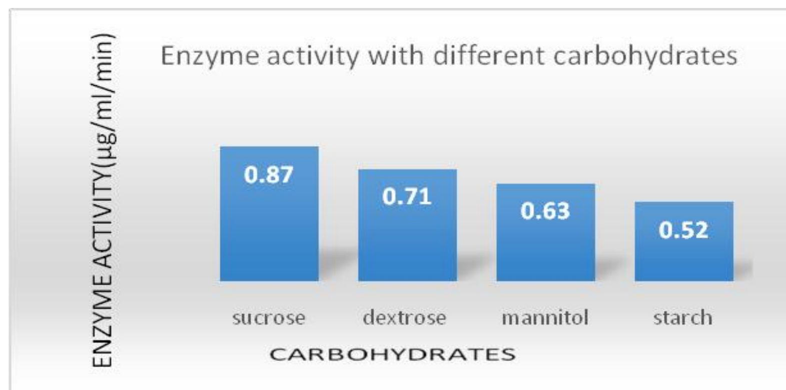


Fig.8 Graph to Show the Protein Concentration with Different Carbohydrates

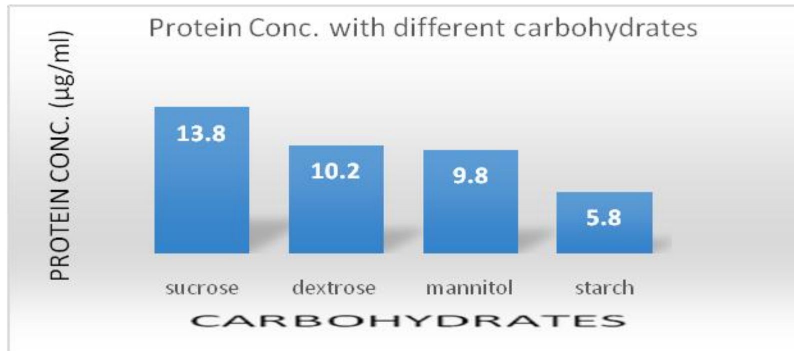


Fig.9 Graph Showing Enzyme Activity with Different Nitrogen Sources

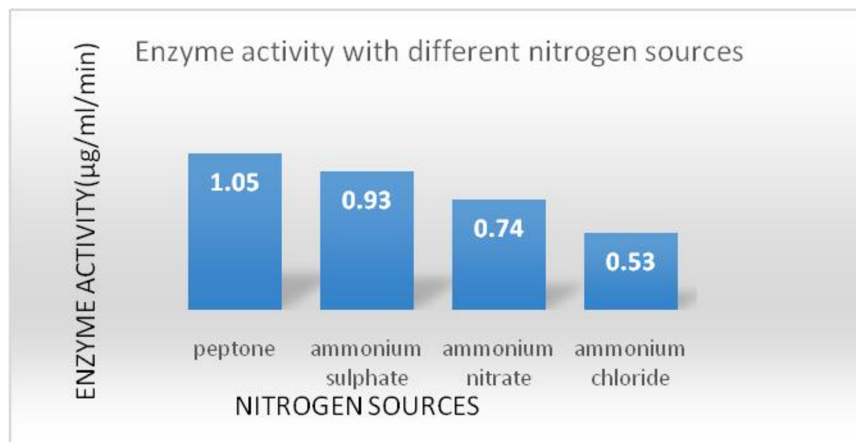


Fig.10 Graph Showing Protein Concentration with Different Nitrogen Sources

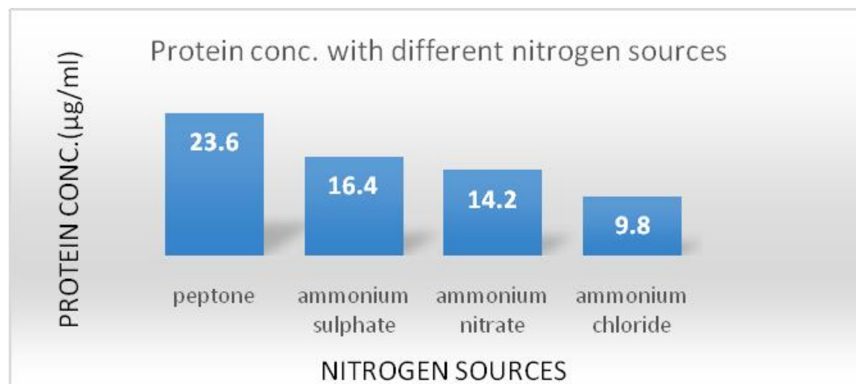
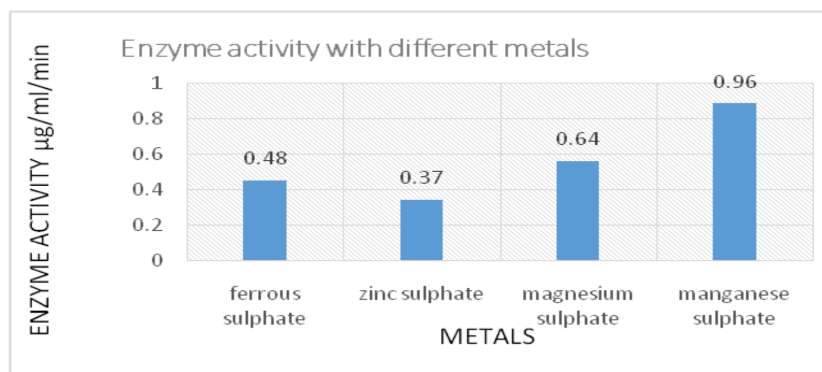


Fig.11 Enzyme activity after addition of various metals



In presence of sucrose the crude production was maximum (13.7 µg/ml) and enzyme activity was found 0.876 µg/ml/min. In case of dextrose, mannitol and starch, the production was found 10.2µg/ml, 9.7µg/ml and 5.8µg/ml respectively and enzyme activity was found 0.73µg/ml/min, 0.63µg/ml/min and 0.51µg/ml/min respectively. So from our data it can be concluded that the protease production rate was highly repressed in presence of starch and highly induced for enzyme production in presence of sucrose. Kaur *et al.,* 1998; Marzan *et al.,* 2002 obtained significant protease production by growing *P.griseorosum* in medium containing 60-74 µM sucrose.

Of the different nitrogenous sources (ammonium sulphate, ammonium nitrate, ammonium chloride and peptone) used, peptone has enhanced the production of *Bacillus sp* protease when medium optimum pH and temperature were 6 and 30^{0}C respectively (Fig 9, 10). Sabota *et al.,* 1995 and Matsushima *et al.,* 1981, reported that peptone followed by ammonium sulphate stimulated protease production more, as in their absence bacteria displayed a slight proteolytic activity and did not produce extracellular protease.

Different metals (zinc sulphate, magnesium sulphate, manganese sulphate and ferrous sulphate) influenced on enzyme activity was also studied (Fig 11). Optimal activity was observed at 1mg/ml of manganese sulphate (0.88µg/mL/min) and least activity was found in presence of 1mg/ml of zinc sulphate (0.34µg/mL/min). It may be suggested that the active sight of protease was influenced by magnesium sulphate to catalyse the hydrolytic reactions and negative cooperativity was shown by zinc sulphate (Marzan *et al.,* 1999 and 2002).

In conclusion, the present investigation reports a new, effective and easy method for obtaining protease producing organisms from vegetable kitchen wastes. Thus in future, similar cheaper sources such as carrot waste can be employed for the extraction of enzyme protease which holds numerous industrial applications.

References

Fermor, T.R., Wood, D.A. 1981. Degradation of bacteria by *Agaricus bisporus* and other fungi. *Gen. Microbial,* 126: 377–387.

Gupta, A., Roy, I., Patel, R.K., Singh, S.P., Khare, S.K., Gupta, M.N. 2005. One step purification and characterization of an alkaline protease from

haloalkaliphilic *Bacillus sp*. *J. Chromatogr.,* 1075: 103–108.
Kalisz, H.M., Moore, D., Wood, D.A. 1986. Protein utilization by basidiomycete fungi. *Trans. Brit. Mycol. Soc.,* 86: 519–525.
Kaur, M., Dhillon, S., Chaudhary, K., Singh, R. 1998. Production, purification and characterization of a thermostable alkaline protease from *Bacillus polymyxa*. *Ind. J. Microbiol.,* 38: 63–67.
Marzan, L.W., Abdul Manchur, M.D., Towhid Hossain, M.D., Anwar, M.N. 1999. Isolation of protease producing fungi and their protease activity. *The hittagong Univ. J. Sci.,* 23: 101–106.
Marzan, L.W., Abdul Manchur, M.D., Towhid Hossain, M.D., Anwar, M.N. 2002. Protease production by a strain Aspergillus funiculosus. *Bangladesh J. Life Sci.*
Matsushima, K., Hayakawa, M., Ito, M., Shimada, K. 1981. Features of the proteolytic enzyme system of hyper-acid-productive and non-acid-productive fungi. *J. Gen. Appl. Microbial.,* 27: 423–426.
Micales, J.A., 1992. Proteinases of the brown rot fungus *Postia placenta*. *Mycologia,* 84: 815–822.
Moon, S.H., Paruleker, S.J.A. 1991. parametric study of protease production in batch and fed-batch cultures of *Bacillus firmus*. *Biotechnol. Bioengg.,* 37: 467–483.
Ogrydziak, D.M., Yamada, T. 1983. Extracellular acid proteases produced by Saccharomycopsis lipolytica. *J. Bacteriol.,* 154: 23–31.
Patel, P.R. 1985. Enzyme isolation and purification. In: Biotechnology: Applications and Research. Technomic Publishing Co. Inc., USA. 534–564.
Sabota, R.R., Rahman, M.M., Choudhury, N. 1995. Extra cellularproteolytic activity of *Saccharomyces cerevisiae* strain DSM 1848. *Bangladesh J. Microbiol.,* 12: 45–49.
Sabota, R.R., Rahman, M.M., Choudhury, N. 1995. Extracellular proteolytic activity of *Saccharomyces cerevisiae* strain DSM 1848. *Bangladesh J. Microbiol.,* 12: 45–49.
Suseela, R. 1998. Hyolysis of chrome-tanned leather waste and production of alkaline protease BBY A Penicillium species. International Symposium on Ecology of Fungi, Goa University, Goa-403 206, India.

How to cite this article:

Diptendu Sarkar and Goutam Paul. Extraction and Bio-chemical Characterization of Protease Enzyme from a Proteolytic bacteria Isolated from Dry Mixed Kitchen Waste. *Int.J.Curr.Microbiol.App.Sci.* 5(3): 268-276. doi: http://dx.doi.org/10.20546/ijcmas.2016.503.033