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RESEARCH ARTICLE

Isolation of protease producing bacteria from a biofertilizer generated from a municipal solid waste

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SUMMARY : Micro-organisms, one of the most potent of organisms on earth, show pronounced capacities to produce different enzymes depending on their source of isolation. Proteases are one among the most important industrial enzymes that execute a wide variety of functions and have various important biotechnological applications. There are various microorganisms which are capable of producing the enzyme protease. In the present study, a biofertilizer generated from Municipal Solid Waste was prospected for proteolytic micro-organisms. For this, the total microbes in the product were isolated and then each of the isolate was separately screened for proteolytic activity by plate assay using casein as substrate. A total of 18 colonies were selected from the total isolated bacteria based on colony morphology. Of these, two colonies (C5 and C16) showed halo zone in casein agar medium. These two colonies were later subjected to enzyme activity testing to compare the level of activity among them. Isolate C5 showed to be potent. Gram staining reactions and standard biochemical tests showed that the two cultures with protease producing capacity belonged to *Pseudomonas* sp. and *Klebsiella* sp., respectively.

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Key Words :
Protease, Casein agar, Municipal solid waste, Biofertilizer, Protease assay

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Micro-organisms are one among the first formed life on earth. As the earth was hostile to life during that period, the microbes had developed the capacity to live in such harsh situation. Depending on the source from where they are isolated, they show different capacities to produce different enzymes. Bacterial cells consume wastes of different types by producing enzymes specific to digest the waste.

Proteases are the most important industrial enzymes which accounts for about 60% and that execute a wide variety of functions and have various important biotechnological applications (Mohen *et al.,* 2005). Two thirds of the total enzymes used in various industries are protease and it account for at least a quarter of the total global enzyme production (Kumar and Hiroshi., 2002). They are reported to be used widely for leather processing, in detergent industry, food industries, for bioremediation process, in pharmaceutical industries, textile industry, waste processing companies, and in the film industry (Rao *et al.,* 1998).Proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Anwar and Saleemudeen, 1997). Proteases isolated from plants and animals were used earlier to meet demands of industries. Regardless of the importance of bacterial proteases, there are not many studies on these (Udandi *et al.,* 2009). Most of the proteases also seem not sufficiently stable under various conditions (Kuberan *et al.,* 2010). Thus there is a need for the isolation of novel micro-organisms that produce protease which satisfies the demands of the industry.

There is a build-up of Municipal Solid Waste (MSW) in almost all places around the world. But there are some methods by which this waste can be converted to a resource. One such method is the generation of energy from it which is called biogas energy. Another technique by which these wastes can be utilized is by digesting it completely to produce non-toxic by-products. The by-products generated are identified as excellent biofertilizers. The digestion of the biowaste is done by microorganisms that are seen indigenously in the waste itself. Viswanatha *et al.*, 2010 proposed that protein containing wastes are excellent source for the isolation of protease producing microorganisms. Effective Micro-organism (EM) Technology, is the one in which organisms from natural sources are utilized to convert waste into a reusable byproduct. It has desirable effect of increase in organic carbon (OC), organic matter (OM), nitrogen (N), phosphate (PO) and potassium (K) content of waste (Kale and Anthappan, 2012). Biowastes are usually rich in protienaceous materials. Hence the microbial diversity in this will be having proteolytic microbes also which are able to digest the protein matter. The industrial demand of highly active preparations of proteolytic enzymes with appropriate specificity and stability to pH, temperature, metal ions, surfactants and organic solvents continues to stimulate the search for new enzyme sources. Proteases with high activity and stability in high alkaline range and high temperatures are required for bioengineering and biotechnological applications.

Isolation of microbes with the capacity to produce desired activity forms the first step of such studies. Optimization of enzyme activity can be carried out in the later stages of studies. If a high activity can be obtained from isolated microbes then they can be recommended as potent sources of the enzyme which can be characterized and stored. The objectives of the present study are to isolate microorganisms from a biofertilizer generated form of bio-waste and to screen these microorganisms for proteolytic activity and also to identify the microorganism which shows the best activity.

EXPERIMENTAL METHODOLOGY

Dried Biofertilizer generated from Municipal Solid Waste were collected from Brahmapuram Waste Treatment Plant. Brahmapuram Waste treatment plant is located in the outskirts of Ernakulam District. The complex organic matter in the waste is degraded by the microorganisms which are native to the waste dumping yard. Samples of biofertilizer generated from this waste was collected in sterile plastic containers (disposable) and were carried to the laboratory in ice box and stored in refrigerator till isolation.

The micro-organisms present in the fertilizer were enriched by pour plate method using Nutrient agar after serial dilution. The culturable bacteria present in the sample were isolated by pour plate method. Pure cultures of the isolated bacteria were prepared by doing streaking. The isolated bacterial colonies were analyzed morphologically using a standard chart. Eighteen different colonies were selected and used for screening for protease activity. The pure cultures of the morphologically selected bacterial colonies isolated were subjected to gram staining technique to categorize into gram

Table 1 : Morphological diversity of bacterial isolates

Culture	Form	Size	Surface	Texture	Color	Elevation	Margin
C1	Circular	Medium	Rough	Dry	Opaque	Convex	Entire
C2	Circular	Medium	Dull	Moist	Cloudy	Raised	Entire
C3	Rhizoid	Large	Veined	Dry	Transparent	Flat	Curled
C4	Irregular	Medium	Wrinkled	Moist	Opaque	Umbonate	Lobate
C5	Circular	Large	Dull	Viscous	Translucent	Raised	Entire
C6	Oval	Medium	Dull	Moist	Cloudy	Pulvinate	Entire
C7	Circular	Medium	Dull	Moist	Transparent	Raised	Entire
C8	Rhizoid	Medium	Wrinkled	Dry	Cloudy	Raised	Filiform
C9	Oval	Punctiform	Rough	Dry	Opaque	Pulvinate	Entire
C10	Irregular	Medium	Dull	Buttery	Cloudy	Pulvinate	Undulate
C11	Circular	Medium	Rough	Dry	Opaque	Convex	Entire
C12	Circular	Punctifom	Glistening	Viscous	Translucent	Umbonate	Entire
C13	Circular	Large	Rough	Moist	Cloudy	Pulvinate	Entire
C14	Irregular	Medium	Dull	Dry	Opaque	Umbonate	Undulate
C15	Irregular	Punctiform	Wrinkled	Mucoid	Translucent	Crateriform	Lobate
C16	Irregular	Large	Glistening	Dry	Cloudy	Umbonate	Entire
C17	Irregular	Large	Rough	Dry	Cloudy	Raised	Undulate
C18	Ovoidal	Medium	Rough	Dry	Cloudy	Pulvinate	Entire

positive and gram negative following the procedures of Hucker and Conn, 1923.

Screening of the isolated microbes for protease production was conducted using casein agar plates. Formation of halo zone around the colonies were identified which results from casein hydrolysis and was taken as evidence of proteolytic activity. The protease activity was measured according to standard method (Anson, 1938). 1mL of 2% casein in phosphate buffer (pH 7.4) and 0.5 mL of enzyme was used as the reaction mixture. After 10 minutes incubation at 30°C, the reaction was stopped by adding 2.5 mL of 5% TCA. After separation of the unreacted casein precipitated by filtration, the protein in the supernatant was estimated by the method of Lowry *et al.,* (1988). The concentration of the protein in the sample was calculated from a BSA standard graph. After the experiment, the potent organisms were identified and stored for further biochemical identification and characterization.

The cultures which showed protease activity was subjected to biochemical identification procedures such as catalase, indole ring test, glucose fermentation, lactose fermentation, starch fermentation, nitrate reduction and motility according to Bergey's Manuel, 1985 and was identified up to genus level.

EXPERIMENTAL FINDINGS AND DISCUSSION

In this study, a biofertilizer produced from Municipal Solid Waste was subjected to microbial isolation procedures. The isolated bacteria were pure cultured and morphologically identified. Morphologically different cultures were selected for further studies. The serially diluted samples were subjected to pour plate procedure for isolating individual colonies. The sample was calculated to contain 213 x 10-4 CFU/g. The results of morphological analysis and the Gram staining results of isolated colonies are presented as Table 1 and Table 2, respectively.

Screening for protease activity :

Formation of halo zone around the colonies, resulting from casein hydrolysis, was taken as evidence of proteolytic activity. Of the 18 colonies tested for proteolytic activity, only two; C5 and C16 showed halo zone in Casein agar medium (Fig. 1).

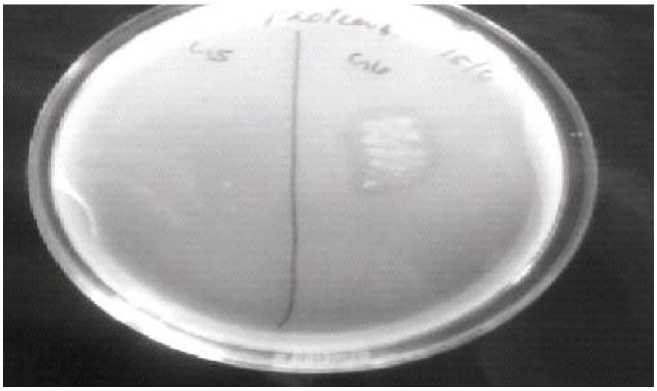

Fig. 1 : Lysis zone produced by proteolytic bacteria

Table 2 : Results of gram staining

Sr. No.	Culture	Gram (+/-)	Shape	Forms
1.	C1	'-'ve	Minute cocci	Clusters
2.	C2	'-'ve	Minute cocci	Chain
3.	C3	'+'ve	Small cocci	Chain
4.	C4	'-'ve	Cocci	Clusters
5.	C5	'-'ve	Rod shaped	Clusters
6.	C6	'-'ve	Small cocci	Clusters
7.	C7	'-'ve	Small cocci	Clusters
8.	C8	'-'ve	Rod shaped	Clusters
9.	C9	'+'ve	Small cocci	Chain
10.	C10	'-'ve	Minute cocci	Clusters
11.	C11	'+'ve	Small cocci	Cluster
12.	C12	'-'ve	Cocci	Chain
13.	C13	'+'ve	Cocci	Cluster
14.	C14	'-'ve	Cocci	Chain
15.	C15	'+'ve	Cocci	Ring
16.	C16	'-'ve	Rod shaped	Clusters
17.	C17	'+'ve	Cocci	Chain
18.	C18	'-' ve	Cocci	Cluster

Protease assay :

From the standard Bovine Serum Albumin (BSA) graph, the concentration of protein after incubation with the supernatant of the broth culture of the bacterial colony C5 was calculated to be 0.1 g/L and that of C16 was calculated and was found to be 0.9g/L. The values for the protein content showed that of the two cultures which showed protease activity in the preliminary plate assay, one culture (C 5) showed more competence (Fig. 2). Nihan and Elif, 2011 screened 31 different Turquish soil using similar methods and isolated one *Bacillus* strain which showed highest activity. In the present study, the standard biochemical identification tests showed that the two isolated species belong to *Pseudomonas* and *Klebsiella*, respectively (Table 4). These cultures have to be further characterized up to molecular level and optimized. Khan *et al.*, 2011 did a similar work where they isolated proteolytic bacteria from soil. Biochemical identification showed the activity was shown by bacterium belonging to *Bacillus* sp. María *et al.*, 2013 reviewed the advantages of using halophilic bacterial isolates in the production of halo-tolerant protease enzymes.

The results of the analysis showed that the biofertilizer has so many microorganisms in it and a portion of the culturable microbial mass is capable of producing protease enzyme and hence are proteolytic in nature (Fig. 3).

Conclusion :

The microbial cultures used for the study was isolated from a biofertilizer which is generate from the microbial biodegradation of Municipal Solid Waste. Since the waste

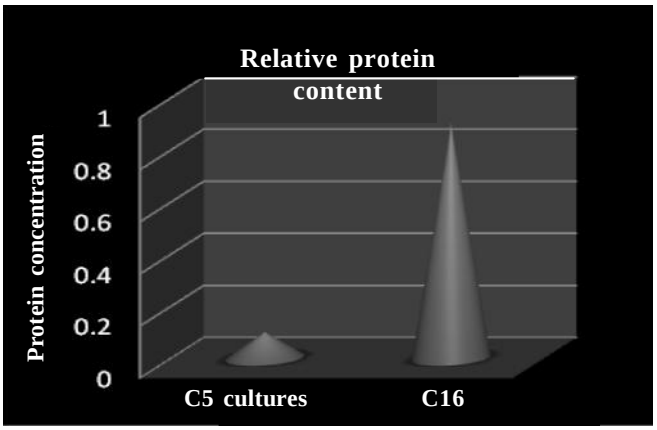

Fig. 2 : Relative protein content in the medium after incubation time

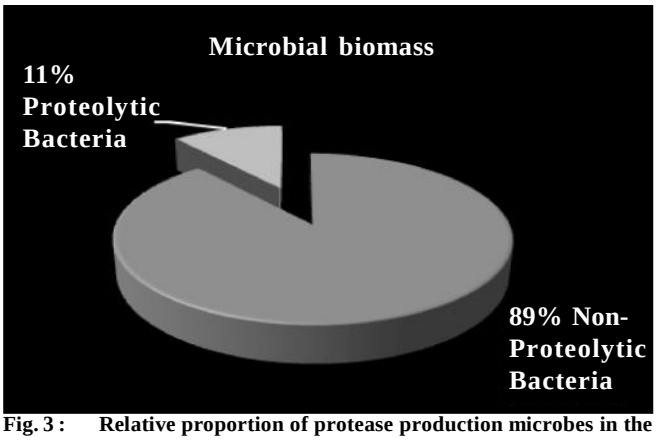

Fig. 3 : Relative proportion of protease production microbes in the biofertilizer

Table 3 : The result of the activity checking is given as

Trial	Vol. of substrate	Vol. of enzyme	After 10 minutes inhibition at 30 ^{0}C	Vol. of TCA	After separation un reacted casein precipitated by filtration	Vol. of filtrae	Vol. of Na_2CO_3	Vol. of folins reagent	After 30 minutes at room temperature	OD at 750 mm	Mean OD
C5R1	1 ML	0.5		2.5 ML		0.5	2 ML	0.5		0.079	0.013
C5R2	1 ML	0.5				0.5				0.128	
C16R1	1 ML	0.5				0.5				0.168	0.264
C16R2	1 ML	0.5				0.5				0.361	

Table 4 : Biochemical Identification of the bacterial isolates

Name of Test	C5	C16
Gram staining	Gram negative rods	Gram negative rods
Morphology	Circular, Large, Viscous, Translucent, Raised and Entire	Irregular, Large, Glistening, Dry, Cloudy, Umbonate and Entire
Catalase	Positive	Positive
Indole ring test	Negative	Negative
Glucose fermentation	Negative	Positive
Lactose fermentation	Negative	Positive
Starch fermentation	Negative	Negative
Nitrate reduction	Positive	Positive
Motility	Motile	Immotile
Result	*Pseudomonas sp.*	*Klebsiella sp.*

materials were MSW, they would have been rich in proteinaceous waste. These protein containing wastes will be degraded by microbes which are capable of producing protease enzymes. Hence it was assumed that the biofertilizer also would be harnessing microbes producing protease enzyme. Though it comes to a small percentage, they show considerable activity.

There are so many research works going on in this regard. But till now a protease enzyme will have all the desired characteristics as required by industries is now available. The result generated from this study has to be further backed up with optimization using various substrates, pH as well as temperature. On getting acceptable results, the novelty of the microbe and the gene has to be looked for before the organisms are proposed as a candidate for industrial applications.

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