ISOLATION AND CHARACTERIZATION OF PROTEASE PRODUCING BACTERIA FROM WASTE SITE OF REWA MUNICIPALITY, MADHYA PRADESH (INDIA)

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ABSTRACT

This study has been undertaken to investigate the proteases producing bacteria in the municipality waste of Rewa. We have sampled about 200 samples during different weather conditions and isolated the protease producing bacterial isolates. We have performed various morphological and biochemical test with some molecular biology techniques to identify the bacterial strain which have the potential to produce maximum enzyme in different environmental and laboratory conditions. We have found the two bacterial isolates R-4 and R-12 as the potential target for further investigation.

IndexTerms – Waste, Bacteria, Environment, Pollution, Protease, Municipality.

1.INTRODUCTION

Wastes are nothing but they are valuable resources about them we are unknown to how we can harvest their value. The rapid growth of urbanization and industrialization are the major factor for growth of municipality related waste generation (Banerjee G & Ray AK 2017). Although the growth of municipality related waste generation rates vary widely within and between countries. The generation rates depend on income levels, social and culture pattern with climatic factors. There is a strong positive inter relation, with the waste generation rates in high income countries being about six fold more than in low income countries (Sonam Singh et al, 2020). Many factors are known; who involved in the degradation of organic compounds, either they are of natural or manmade (Kalwasi nska A et al, 2018). They all are exposed to physical as well as chemical factors as by as biological catalysts which are capable of causing chemical transformation in biological ecosystem (Hidayat, MY et al, 2017). Photo chemical and other non biological catalyzed reactions undoubtedly cause some chemical transformation in many substrates formed or introduced into the nature (Hetemaki, L & Nilsson, S, 2005).

Proteases are the enzymes which occupy a significant position in order to their wide applications in many commercial fields including agriculture (Prakasham RS et al, 2006; Marathe SK et al, 2017). These enzymes catalyze the hydrolysis of proteins, the peptide bonds present in the polypeptide of amino acids are hydrolyzed by these enzymes. With 60% of total enzyme sales, bacterial proteases occupy important place as industrial enzymes (Nonso E Nnolim et al, 2020).

Proteases are enzymes which are secreted by plants, animals, and microbes but microbial proteases are more famous than those produced by plant and animal because they can produced in big scale in a very short time scale by already established fermentation procedures, they produce abundant, regular supply of product categories (Ward OP,1985; Kalisz HM, 1988). Also have a long shelf life and stored under normal conditions for long time without any significant loss of its activity (Paula Monteiro de Souza et al, 2010). The proteases produced by microbes are extracellular. Due to this downstream processing of them become easier than those from plant and animals.

In the industrial sector, *bacillus* species are widely used to produce the extracellular proteases, *Bacillus subtilis* is most frequently used bacillus species for this purpose, it is rod shaped bacterial species; it can survive in extreme environmental condition (Shanthakumari AR & Boominathan M, 2017; Sahay H et al, 2017). The bacterial proteases possess higher thermo-stability with high reaction rate in comparison to fungal proteases, except protease produced by the *Malbranchea pulchella* (Sun L et al, 2019).

In detergent industry protease used to digests food, other body secretions and stain (Yildirim V et al, 2017). For cleaning of contact lenses and dentures, they are preferably used. Advantages of using these enzymes are eco-friendly nature with shorter period of socking and agitation (Allison D Suleiman et al, 2020).

When compared to chemical procedures of peptide synthesis where there are many disadvantages such as, low yield, racemisation issue, health and environmental concern (Dissanayaka D et al, 2019). Protease mediated peptide synthesis offers several advantages such as selectivity, racemisation free, environmental friendly etc. Microbial proteases have been used successfully for synthesis of peptide bonds and hydrolysis of the same (Fanny Guzman et al, 2007).

Small Peptides synthesized through protease are used for nutrition and pharmaceuticals. Leather processing by enzymatic method are more successful and user friendly when compared to chemical methods (Kanagaraj J. Et al, 2006; Lang MF et al, 1999). Proteases are used to modify non-collagenous components of skin and removal of non- febrile protein (Gupta, Munishwar N And Roy, Ipsita, 2004; Taylor MM et al, 1998; Ozgunay H et al, 2007). Application of alkaline proteases during waste disposal helps to shrink waste and save time and energy. Proteases used extensively in food industry. These enzymes modify food protein to enhance nutritional value, solubility, flavors, digestibility value and minimizing allergic compounds.

Proteases can be used to alter various properties of dietary protein such as coagulation, emulsification, foaming, gel strength etc. Proteases hydrolyze connective tissue and muscle fiber thus helps in tenderization of meat. In baking industry these enzymes maintains dough uniformity and gluten strength in bread as well as enhance flavors and texture. Proteases degrade turbidity complex resulting from protein in fruit juices and alcohol liquors. Due to wide diversity and specificity proteases can be used for therapeutic and diagnostic purpose such as anti cancer, clot dissolving, anti microbial, anti inflammatory etc. Serratiopeptidase, a protease has potential effect as anti inflammatory (Kaminishi H et al, 1994). Asparginase from e-coli used in lymphocytic leukemia, Nattokinase from *bacillus subtilis*

is used as cardio-protective. They used in degradation of keratinized skin also and preparation of vaccine for dermatophytosis therapy. From ancient time proteases used to produce sauces and other useful product from soy. Proteases degrade high protein content which improves functional properties during soy processing.

2. MATERIAL AND METHODS

Scientifically proven materials and methods were used to obtain desired aim and objectives.

2.1. Study Site and Collection of Samples

The study site was the waste dumping area of Rewa Municipality, Madhya Pradesh (India) which is also known as the land of White Tiger. Total 05 garbage collection sites were selected for sampling. Total 200 samples were composed of from 05 different garbage collection site of Rewa municipality. From every sites sample were collected four times in a year (January, April, August and December) and every time total 10 samples were taken from the sites. Soil mixed with waste were taken into the sterilized plastic tube in aseptical condition, stored at 4^0C and marked according their source.

2.2. Chemical characterization of waste

Chemical contents of sample were calculated for the following factors: Organic matter (%) Total Nitrogen (In %) Total Phosphorus (In %), Potassium (In %) and organic carbon content was estimated by the titration method (Bremner, 1960; Jackson, 1973).

2.3. Isolation of bacterial isolates and assay of extracellular proteases potential

The serial dilution method was performed to obtain the pure form of bacterial isolates. One milliliter of the original sample solution (stock solution) was mixed with the 10^{-1} dilution zero by the help of a sterile and clean pipette. Again 01 milliliter from the 10^{-1} dilution referred to the 10^{-2} tube in every proceeding step after that the 10^{-2} - 10^{-3}, then to the 10^{-3} mixed to 10^{-4}. With every tube 0.1 ml of diluted fluid were transferred on to NAM media.

For the assay of extracellular proteases enzyme production, agar medium was formulated by addition of Gelatine, casein, skimmed milk 1% (w/v). The crude bacterium suspension was poured into the holes. Plates were incubated for 12 Hrs at 37^0C. These plates was flooded with $C_{21}H_{14}Br_4O_5S$ reagent and incubated for 1/2 hours at 25-30°C. A zone of proteolysis was detected on the casein agar plates.

2.4. Quantitative analysis of protease

After the incubation in 100 ml MRS broth for 10 days at 37^0 C in a rotary shaker each inoculums was taken and subjected to centrifugation at 10,000 Rounds per minute for ten minutes at 4^0 C. The cell free crude enzyme present in supernatant was collected and kept at 4^o C till further use. The protease assay was performed for every bacterial isolates from days one to ten to find out the production potential of each bacterial isolate.

About 200 µl of crude enzyme extract was mixed with 50 mili Molar phosphate buffer and 1 percent casin (w/v). The final amount of this mixture was adjusted to 500 micro liters, the pH was kept at 7, and then this mixture was kept into the waterbath for twenty minutes at temperature 40°C. After that, the enzymatic reaction was blocked by mixing the 1 ml of 10% (w/v) Cl_3CCOOH into the mixture and then it was kept at 27^0C for 15 minutes. After that, the reaction solution was subjected to

centrifugation and after the centrifugation the supernatant was taken, in this supernatant the 2.5 ml of 0.44M Na2CO3, 1 ml of 3-fold diluted Folin-Denis reagent was added. Then this mixture of the reagent was kept for 30 minutes at room temperature in the dark. The blue colour developed which absorbance was calculated at 660 nm beside a blank solution using a tyrosine standard.

One unit of protein (protease) is quantified as the enzyme that liberated one microgram of tyrosine per millilitre/per minute into the supernatant solution under standard conditions.

2.5. Characterization of purified proteases

The collected purified enzyme was now subjected for its optimization of enzymatic activities at different pH, different temperature and substrate specificity determination.

2.6. Molecular weight determination of proteases

Total (100%) precipitated crude Proteases enzyme was collected as the precipitate and stored at 4^0C till further use. Vertical gel apparatus was used to cast the 12% SDS-PAGE. The crude sample of enzyme collected after centrifugation were mixed in the sample buffer and boiled for 12minutes for the proper denaturation of the sample protein and loaded on the gel with six Protein markers containing Phosphorylase b, 97 kDa; Albumin, 66 kDa; Ovalbumin, 45 kDa; Carbonic anhydrase, 30 kDa; Trypsin inhibitor, 20.1 kDa; Alpha lactalbumin, 14.4 kDa were loaded parallel to the sample into the gel to determine the desired enzyme band. Electrophoresis was performed by applying 60 Volts DC electric per gel. After the process was over, the gel was taken out from the apparatus and was dipped in a fixative solution containing 50% (v/v) methanol and 12% (v/v) acetic acid for sixteen hours and then stained by $C_{45}H_{44}N_3NaO_7S_2$ (Sodium salt) solution. After the staining procedure gel was de-stained in a destaining solution. After the destaining procedure gel was brought to gel documentation system and band appeared were analysed and photographed.

2.7. Statistical procedures

During all experiments, the measurements were carried out with duplicated parallel cultures. The values calculated by the standard statistical tools by the Microsoft excel.

3. RESULTS

Results recorded was analysed by standard statistical method.

3.1. Study site

The area of the study for our research was the whole Rewa municipality in which we have selected 05 sampling points for the detailed analysis. Total 200 samples were collected from the study sites in the various weather conditions to fulfil the aim of our research.

3.2. Chemical characterization of waste

The analysis of the chemical composition of all the samples from each site was performed to determine the quantity of organic matter, Nitrogen content, phosphorus content, potassium content and organic carbon content. The result obtained was given in Table No.3.1.

Table No. 3.1. Chemical characterization of waste from all sites

Sample site	Organic matter (%)	Nitrogen (%)	phosphorus (%)	Potassium (%)	Organic carbon (%)
RM-1	18.6	0.7	0.08	0.0	58.2
RM-2	24.5	0.43	0.06	0.0	55.4
RM-3	27.6	0.34	0.0	0.0	53.3
RM-4	20.83	0.39	0.03	0.0	87.13
RM-5	22.3	0.5	0.02	0.0	70.2

3.3. Isolation of bacterial isolates

The serial dilution procedure was used for the isolation of the pure bacterial colonies from the samples. The nutrient agar media was used for the growth of the bacteria. After the pure colonies obtained their morphological characterization also performed. More than 25 different bacterial colonies were isolated and studied during the experiment. After keen observation total 12 colonies were included for the further experimental consideration. Other colonies were excluded due to some important fact such as culture cost, high pathogenesis level and requirement of very high sophisticated operating conditions. All selected isolated bacterial colonies given the code name according their sampling site and sampling number, from R-1 to R-12. (Table No. 3.2).

Table No. 3.2. Isolate code and their respective source samples

Serial No.	Source Sample	Isolate Code
1	RS1J-9	R-1
2	RS1Ap-6	R-2
3	RS2Ap-9	R-3
4	RS2Au-1	R-4
5	RS3J-5	R-5
6	RS3Au-1	R-6
7	RS3D-6	R-7
8	RS4Au-7	R-8
9	RS4D-3	R-9
10	RS5J-6	R-10
11	RS5Ap-1	R-11
12	RS5Au-7	R-12

3.4. Morphological and Biochemical characterization of protease producing bacterial isolates

Various morphological and biochemical test were performed with each isolate. It was recorded that isolate R-4 was a gram positive and spore forming bacteria which is also shown positive results for Catalase test, Voges-proskauer test, triple sugar iron test, simmon's citrate test, motility test, gelatine

hydrolysis test, mannitol salt agar plate test, casein hydrolysis test, haemolysis of blood test, cellulose degradation, have the ability to grow on anaerobic condition and urease activity was positive. Although the oxidase test, methyl red test, nitrates reduction test and indole test given negative results. Isolate R-12 was a gram positive non spore producing bacteria with positive result for catalase test, voges-proskauer test, triple sugar iron test, simmon's citrate test, motality test, gelatine hydrolysis test, nitrate reduction test, mannitol salt agar plate test, casein hydrolysis test, haemolysis of blood, cellulose degradation test, showed growth on anaerobic condition and indole test. This isolate exhibited negative results for oxidase test, methyl red test and urease test.

3.5. Assay of extra cellular protease enzyme production

For protease enzyme production potential all selected bacterial isolates were grown on the Gelatine, casein, skimmed milk 1% (w/v) media and clear zone produced by each isolate measured. It was observed that isolate R-4 and R-12 produced the significant clear zone, and other was found to be non significant. R-4 isolate appeared maximum clear zone area of 2.6 mm and mean value of clear zone calculated was 2.25mm. R-12 maximum clear zone area was 2.9 and mean value was 2.47mm calculated (Fig No. 3.1).

Fig. No. 3.1. Clear zone produced by R-4 and R-12 bacterial Isolates

3.6. Standardization of growth conditions of isolates

Optimisation of growth condition in different media, pH, time and temperature for all selected bacterial isolates were performed. We have observed that in the NAM isolate R-4 showed the maximum growth at pH-7, time 72 hours and at the temperature 37^0C. The good growth condition was recorded at pH-7 after 48 hours at 37^0C. At pH-8 the good growth observed after 72 hours at 37^0C. The optimum growth conditions for R-12 were recorded at pH seven after 72 hours at 37^0C. It is also observed that after 48 hours, at pH-8 and at 37^0C the very good growth condition were recorded (Table No. 3.3).

Table No. 3.3. Standardization of growth conditions in the NAM media

Medi	Isola	pH	Incubation period (In hours)						Temperature (In ^{0}C)				
			6	12	24	36	48	72	25	29	33	37	41

a	te												
NAM	R-4	5	-	-	-	-	-	-	-	-	-	-	-
		6	-	-	-	-	-	+	-	-	-	-	-
		7	-	+	++	++	+++	++++	-	-	++	++++	-
		8	-	-	-	++	++	+++	-	-	+	++	-
		10	-	-	-	-	-	+	-	-	-	-	-
NAM	R-12	5	-	-	-	-	-	-	-	-	-	-	-
		6	-	-	-	-	-	-	-	-	-	-	-
		7	-	+	++	+++	+++	+++	-	-	++	++++	-
		8	-	-	+	+	++	+++	-	-	++	+++	-
		10	-	-	-	-	-	-	-	-	-	-	-

In the BCDA media we have observed that isolate R-4 showed the very good growth condition at pH-7.5 after 48 hours of incubation at **37⁰** C. After 72 hours at pH-7.5 and at 37^{0}C maximum growth was observed. For R-12 isolate the optimum pH was 7.5 after 48 hours and temperature between 33-37^{0}C.At the same pH maximum growth was recorded after 72 hours and temperature 37^{0}C (Table No. 3.4).

Table No. 3.4. Standardization of growth conditions in the BCDA media

Media	Isolate	pH	Incubation period (In hours)						Temperature (In ^{0}C)				
			6	12	24	36	48	72	25	29	33	37	41
BCDA	R-4	7.5	-	+	++	+++	+++	+++	-	-	++	+++	-
		8.5	-	+	++	++	++	+++	-	-	+	++	-

		9.5	-	-	-	-	-	+	-	-	-	+	-
		10.5	-	-	-	-	-	-	-	-	-	-	-
		12	-	-	-	-	-	-	-	-	-	-	-
BCDA	**R-12**	7.5	-	++	++	++	+++	+++	-	-	+++	+++	-
		8.5	-	-	+	++	++	+++	-	-	+	++	-
		9.5	-	-	-	-	-	-	-	-	-	+	-
		10.5	-	-	-	-	-	-	-	-	-	-	-
		12	-	-	-	-	-	-	-	-	-	-	-

The growth condition was optimized in ACDA media for all isolate bacteria. It is observed that for isolate R-4 the optimum pH was 6.9 with temperature 37^0C and duration 48 hours. The massive growth recorded at ph 6.9 after 72 hours at 37^0C temperature. For R-12 the very good growth recorded at pH-6.9 after 48 hours and massive growth was observed at pH 6.9 after 72 Hours and temperature 37^0C (Table No. 3.5).

Table No. 3.5. Standardization of growth conditions in the ACDA media

Media	**Isolate**	**pH**	**Incubation period (In hours)**						**Temperature (In ^{0}C)**				
			6	**12**	**24**	**36**	**48**	**72**	**25**	**29**	**33**	**37**	**41**
ACDA	**R-4**	2.5	-	-	-	-	-	-	-	-	-	-	-
		3.5	-	-	-	-	-	+	-	-	-	-	-
		4.5	-	-	-	-	-	-	-	-	-	-	-
		5	-	-	-	-	-	-	-	-	-	-	-

		.5											
		6.9	-	+	++	++	+++	++++	-	-	++	+++	-
ACDA	**R-12**	2.5	-	-	-	-	-	-	-	-	-	-	-
		3.5	-	-	-	-	-	-	-	-	-	-	-
		4.5	-	-	-	-	-	-	-	-	-	-	-
		5.5	-	-	-	-	-	-	-	-	-	-	-
		6.9	-	+	++	++	+++	+++	-	-	+++	+++	-

3.7. Quantitative analysis of protease

The effect of incubation period (1-10 Days) was tested for R-4 and R-12. It is observed that with the increase in duration the Protease activity (U/ml) also increases till the 6 days and protein content (mg/ml) decreases. The maximum Protease activity (U/ml) for R-4 was recorded 114.43 U/ml at 6th day. The maximum Protease activity (U/ml) for isolate R-12 was recorded 125.32 U/ml at 6th day. After the six days, Protease activity decreased (Table No.3.6).

Table No. 3.6. Effect of Incubation Time on Protease Activity

Incubation period (Days)	Protease activity(U/ml) & protein content (mg/ml)		
	R-4		R-12
1	PA	6.42	44.63
	PC	2.53	1.95
2	PA	17.04	56.46
	PC	2.32	1.93
3	PA	45.23	71.3
	PC	2.12	1.73
4	PA	61.34	80.23
	PC	1.95	1.33
5	PA	91.11	88.94
	PC	1.73	1.21
6	PA	135.34	125.32
	PC	1.6	0.93

7	PA	114.43	117.67
	PC	1.5	0.91
8	PA	92.32	107.56
	PC	1.43	0.89
9	PA	83.43	89.34
	PC	1.39	0.94
10	PA	76.46	82.34
	PC	1.25	0.96

3.8. Characterization of purified Protease enzyme

Optimization of enzymatic activities at different pH, different temperature and substrate specificity was also performed.

3.8.1. Characterization of purified Protease enzyme at different pH

Optimisation of pH was carried out for isolate R-4 and R-12, regarding that the Protease activity was measured at different pH. We have observed that the optimum pH for maximum protease activity was pH-9 for both isolates (R-4 and R-12). As the maximum protease activity for isolate R-4 was 0.089 and for isolate R-12 was 0.087 at pH-9. The pH 7-9 was found suitable for both bacterial isolate (Table No.3.7.1).

Table No. 3.7.1. Effect of pH on Protease Activity

pH	OD at 660 nm	
	R-4	R-12
3	0.046	0.049
4	0.055	0.056
5	0.061	0.063
6	0.070	0.071
7	0.074	0.075
8	0.083	0.081
9	0.089	0.087
10	0.063	0.065
11	0.052	0.055

3.8.2. Characterization of purified Protease enzyme at different Temperature

Experiment were carried out for standerisation of optimum temprature for maximum protease activity. Results showed that temprature between 30^0C to 37^0C has been suitable for enzyme activity. Temprature more than 37^0C was not suited for enzyme activity. It could be due to denaturation of

enzyme at higher temprature. It has been observed that isolate R-4 and R-12 showed the maximum enzyme activity at 37^0C. The maximum activity for R-4 and R-12 was 0.115 and 0.116 respectively (Table No.3.7.2).

Table No.3.7.2. Effect of Temperature on Enzyme Activity

Temprature (In ^{0}C)	OD at 660 nm	
	R-4	R-12
0	0.012	0.010
20	0.071	0.072
30	0.095	0.094
37	0.115	0.116
57	0.079	0.078
80	0.040	0.042
100	0.013	0.012

3.8.3. Characterisation of protease activity with different concentration of substrate

Optimisation of protease activity was measured at different substrate concentration level. Experiment result showed that with the increase in the substrate concentration enzyme activity also increases. This trend was recorded for both bacterial isolates (R-4 & R-12), as maximum enzyme activity recorded at the 22 mg substrate concentration; for R-4 activity was 0.53 IU/ml and for R-12 it was 0.55 IU/ml (Table No.3.7.3).

Table No.3.7.3. Effect of Substrate Concentration on Protease Activity

Substrate concentration (mg)	Protease activity (IU/ml)	
	R-4	R-12
2	0.16	0.16
3	0.20	0.21
5	0.24	0.23
7	0.28	0.26
9	0.31	0.30
10	0.33	0.32
12	0.38	0.36
13	0.44	0.45
15	0.46	0.47
17	0.48	0.49
19	0.51	0.50

20	0.52	0.53
22	0.53	0.55

3.9. Molecular weight determination of protease by SDS-PAGE

Molecular weight of pure protease enzyme were analysed by denaturing PAGE. Result showed the single band of about 19.8 kDa for R-4 and R-12 bacterial protease samples. Single band demonstrated homogeneous preparation of protease enzyme for both sample (Fig. No.3.2).

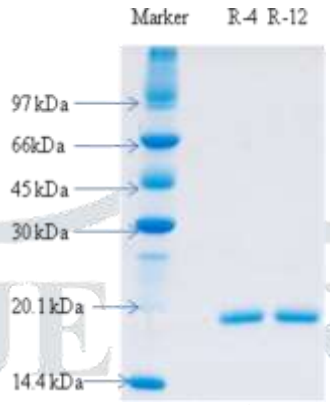

Fig. No. 3.2. SDS-PAGE of Protease enzyme

4. CONCLUSION

Wastes are nothing but they are valuable resources about them we are unknown to how we can harvest their value. The potential of microorganisms in maintaining the ecological and environmental balance is evident from the early knowledge of science and environment. It is evident by the existence of particular biological waste in fewer amounts in the nature. In the industrial sector, *bacillus* species are widely used to produce the extracellular proteases, *Bacillus subtilis* is most frequently used bacillus species for this purpose, it is rod shaped bacterial species; it can survive in extreme environmental condition. The bacterial proteases possess higher thermo-stability with high reaction rate in comparison to fungal proteases, except protease produced by the *Malbranchea pulchella*.

The six bacterial isolate were found to be suitable for the desired enzyme production on the basis of clear zone produced by them. For proteases production two bacterial isolates R-4 and R-12 were selected. Bacterial isolate R-4 was identified as *bacillus megatherium* on the basis of morphological and biochemical examination. On the basis of morphological and biochemical analysis R-12 was identified as *bacillus subtilis* but for proper identification sequencing studied are recommended for both bacterial isolates.

Acknowledgements

We are very thankful for Centre for Biotechnology/Microbiology studies, Awadhesh Pratap Singh University, Rewa (M.P.) for providing all the facilities required for this research work.

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