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Screening, Production and Characterization of Bacterial Strain for Industrial enzymesa their Potential application in Biodegradation of Plastic and Paper Waste

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ABSTRACT:

Enzymes are widely used in industries like Food, Paper, Textile etc. Recent years focused on recycling and utilising waste efficiently. Numbers of studies based on utilizing agricultural waste, municipal and related waste have been reported. Present study focuses on utilization of vegetable market waste as substrate in order to produce industrially important enzymes. For this, the sample, vegetable market waste was collected from vegetable waste dumping site, Chromepet, Chennai, Tamil Nadu. Bacteria were isolated from it andpure cultures obtained were tested for the production of five different industrially important enzymes namely: protease, amylase, cellulase, lipaseand pectinase. One bacterial strain which was later identified as Bacillus aryabhattai by sequencing 16srDNA regions, that produced most of these enzymes has been selected and used for comparative analysis of enzyme activity of the above mentioned five enzymes produced in both specific media and vegetable market waste media. Results have shown that protease and cellulase produced in vegetable market waste media has 83% and 72.3% higher enzyme activity respectively than their activity in specific media. Enzyme activity of lipase is 50% less in vegetable market waste media whereas enzyme activity of amylase is similar in both the media.

Index terms: Vegetable Market Waste; Protease; Amylase; Lipase; Cellulase; Pectinase; Enzyme Activity.

I. INTRODUCTION

Vegetable market waste has been a major problem of concern as it is generated enormously due to over production for human consumption or unfulfillment of quality standards [1]. India, which is known for its agricultural resources, alone contributes 50 metric tonnes of vegetable waste which is nearly 30% of total vegetables produced by it [2]. According to FAO, approximately 1.30 billion tonnes of food which includes fruits, vegetables, meat, and dairy products are lost from the food supply chain[3]. Conventionally, this solid waste generated, is either incinerated or dumped into open area which may lead to several health and environmental issues [4,5]. Incineration of waste having high content of moisture releases dioxins whichcauses several environmental problems.These released dioxins also hinder recovery of important chemicals and nutrients from the waste incinerated [6]. Besides of all these issues, vegetable waste which is actually packed with high quantities of nutrients

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[7] can be recycled and utilized for the production of necessary products that can benefit many industries [8]. Vegetable market waste is also a rich source of valuable bacterial species.

In recent years, soil microorganisms played a crucial role for their ability to produce high value industrial products [9]. Vegetable market waste which is an organic waste serves as carbon source for bacteria and using it they will produce several metabolites which are industrially important. Bacteria are omnivorous and they are used in many processes like Baking, Brewing, manufacturing of Cheese, Butter and Chemicals such as Ethanol, Organic acids, Acetone, Enzymes etc., They can also produce variety of Antibiotics, Steroids, Vaccines and several other therapeutically useful compounds [10].

Many industrial processes utilize enzymes obtained from microbes and even new ways of applications using microbes are constantly being developed. Enzymes are very important as they modify products by improving their nutritional and functional value [11]. Enzymes are used as alternative for chemical depended processes in industries like Food, Paper, Textile, Brewing, Pharma, Detergents, Fuel etc.

Present study aims to screen, isolate and characterize bacterial strain from regional vegetable market waste, that can produce industrially important enzymes namely: Protease, Amylase, Lipase, Cellulase and Pectinase and compare their hydrolytic profile in both vegetable market waste and specific media and also study their potential degradation of plastic and paper waste.

II. MATERIALS AND METHODS

A. Sample collection:

About 70g of sample (Vegetable Market Waste) was collected from vegetable waste dumping site, Chromepet, Chennai, Tamil Nadu, India, in an aseptically sterilized polythene bags and stored at 4ºC in the refrigerator for further analysis.

B. Serial dilution:

0.5g of sample was aseptically weighed and then serially diluted until 10^{-9} dilutions.

C. Preparation of media:

Nutrient agar media were prepared by adding 5 g of yeast extract, 10 g of NaCl, 10 g of tryptone and 15 g of agar-agar in 1000 ml of distilled water. The pH was set to 7.0 ± 0.5 using pH meter. The media were sterilized at 121˚C for 15 minutes. Approximately 20ml of media was poured into petri plates and let it to dry till it gets solidified.

D. Isolation of bacteria:

0.1ml aliquots of sample from respective dilutions say 10^{-6} to 10^{-9} was taken and inoculated onto solidified nutrient agar media by spread plate method using “L” rod. The cultures were incubated at 30˚C for 24 - 48 hours in the incubator.

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E. Obtaining pure culture and its preservation:

Different colonies were picked based on morphology and purified by streaking onto freshly prepared sterilized agar media and stored at 4ºC in the refrigerator for further use. These cultures were designated with numbers from 1 to 10.

F. Screening for enzymes:

Protease: Pure cultures were screened for protease production for this, Cultures were streaked onto skim milk agar media containing 0.5 g of Peptone, 0.5 g of NaCl, 0.5 g of skim milk powder and 2.0 g of agar in 1000 ml of distilled water and pH then was set to 7.0 ± 0.2 [12]. Formation of clear halo zone around the colony indicates the production of protease by that culture.

Amylase: Cultures were tested for amylase production by streaking them onto starch agar media which contains 0.5 g of Starch, 0.5 g of Peptone, 0.5 g of NaCl, 0.3 g of Yeast extract and 2.0 g of agar-agar in 1000 ml of distilled water with a pH set to 7.0 ± 0.2 using pH meter [13]. Observation was made After 24 hours when the plates were flooded with 1% iodine solution, A clear halo zone around the colony indicates amylase production.

Lipase: production of lipase by pure cultures was screened by streaking them onto Serria's medium which contains 1.0 g of Peptone, 0.5 g of NaCl, 0.001 g of $CaCl_2$, 1.0 % (v/v) of tween 20 and 2.0 g of agar-agar in 1000 ml of distilled water with a pH 7.4 [14]. Observation was made using phenol red solution, when poured, zones around positive cultures turn into red or yellow colour because of decreased pH in the media due to production of fatty acids by lipid hydrolysis.

Cellulase: cellulase production was screened by streaking pure cultures onto screening media containing 0.5 g of carboxy methyl cellulose (CMC) as substrate, 0.1 g of NaNO3, 0.1 g of K_2HPO_4, 0.1 g of KCl, 0.05 g of $MgSO_4$, 0.05 g of yeast extract, 0.1 g of glucose and 2.0 g of agar [15]. In order to visualize the hydrolysis zone, the plates were flooded with an aqueous staining solution of 0.1% Congo red (which was prepared by adding 0.1 g of congo red to 100 ml of distilled water) and then destained using 1M NaCl solution. Clear halo zone can be seen around the positive cultures.

*Pectinase:*pure cultures were screened for the extracellular production of pectinase enzyme using pectinase screening agar medium (PSAM) media [16] which contains 0.5 g of Poly Galacturonic Acid (PGA) as the substrate, 0.17 g of Yeast extract, 0.2 g of Ammonium sulphate, 0.3 g of KH_2PO_4, 0.6 g of Na_2HPO_4, 0.1M of Tris-HCl and 2.0 g of agar. To observe positive cultures, plates were flooded with 3.3% CTAB solution (which was prepared by adding 3.3 g of CTAB in 100 ml of distilled water) and observed for the appearance of clear zones which will be positive cultures.

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G. Identification:

Environmental conditions impactsbacterial morphological and physiological characteristics [21] therefore, it is suggested to perform molecular techniques as they are highly sensitive and species level. DNA was isolated from 24 hours old pure bacterial culture and 16S rDNA was amplified using two primers 16s Forward primer: 5'-AGAGTRTGATCMTYGCTWAC-3' and 16s Reverse primer: 5'-CGYTAMCTTWTTACGRCT-3' in ThermalCylcerABI2720. Amplified product was sequenced using ABI3130GeneticAnalyzer. Sequence similarity search was done using BLAST tool. The unknown sequence was identified from maximum alignment obtained through BLAST search.

H. Production of enzymes:

The culture that produced maximum enzymes was taken and used for producing four industrially important enzymes namely protease, amylase, lipase and cellulase in their respective specific media broth and vegetable market waste media broth.

Preparation of specific media broth: protease, amylase, lipase and cellulase enzymes were produced in specific media broth containing skim milk agar, starch, tween 20 and CMC as substrates respectively with all the minerals and nutrients required. Media broth was then kept on shaker for about 48 hours.

*Preparation of vegetable market waste media broth:*Vegetable market waste (V.M.W) is used as sole source for the production all the four enzymes protease, amylase, lipase and cellulase. About 65 grams of V.M.W was weighed and added to 150 ml of water to make it slurry and then heated at 60ºC for about 25 minutes to kill harmful pathogens. It was then filtered and all the necessary macro and micro minerals were which include 0.5 g of NaCl, 0.001 g of $CaCl_2$, 0.1 g of $NaNO_3$, 0.1 g of K_2HPO_4, 0.1 g of KCl, 0.05 g of $MgSO_4$ were added to it.

I. Enzyme activity assays:

Enzyme assays were performed in order to calculate and compare the enzyme activity of enzymes produced in both specific media broth and vegetable market waste media broth. Before performing the assay, crude extract of each enzyme was prepared by collecting production broth in 15 ml centrifuge tubes and centrifuging at 10,000 rpm for 20 minutes. The supernatant was collected carefully into another tube discarding the pellet and refrigerated at 4ºC until further use. One unit of enzyme activity is the amount of enzyme necessary to release 1 µmol of product produced per minute under the assay conditions used and is given by the formula: µg of product/mol wt × 1000/incubation time.

*Caseinolytic assay:*The reaction mixture which contains 1ml of casein as substrate (which was prepared by adding 2g of casein in Tris buffer having pH 8) was mixed with 0.1ml of enzyme solution and incubated for 2hrs at 30ºC for enzymatic reaction. The reaction is then terminated by adding 2ml of trichloro acetic acid solution (5% v/v) and the precipitate formed was removed by centrifuging

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at10,000 rpm for 20 min at 4 ºC. Supernatant was carefully collected discarding the pellet and reaction mixture is neutralized by adding 4ml of 1N NaOH. To this 500µl of 1N Folin-phenol reagent was the added and allowed for few minutes in order to develop colour. The absorbance was then read at 750 nm using UV- VIS spectrophotometer [17]. The OD values were noted and standard graph of tyrosine was used to calculate enzyme activity.

*Amylase assay:*The reaction mixture containing 2.5ml of 1% starch in sodium phosphate buffer as substrate, 2.5 ml of sodium phosphate buffer, and 1 ml of 1% w/v sodium chloride and 1 ml of enzyme solution was incubated at 37ºC for 15 minutes for enzymatic reaction. In order to terminate the reaction DNSA was then added and heated for 10 minutes in boiling water bath until colour is developed. Absorbency of the solution was checked at 540nm using UV- VIS spectrophotometer [18]. The OD values were noted and standard graph of maltose was used to calculate enzyme activity.

Lipase assay: The reaction mixture containing 2.4ml of p-nitrophenyl palmitate (p-NPP) as substrate (which was prepared by adding two solutions: solution A containing 30mg p-nitrophenyl palmitate in 10ml of isopropanol and solution B containing 207mg sodium deoxycholate and 100mg gum arabica in 90ml of sodium phosphate buffer) and 0.1ml of enzyme was incubated at 37ºC for 15 minutes and the absorbency was checked at 410nm using UV- VIS spectrophotometer [19]. The OD values were noted and standard graph of p-NPP was used to calculate enzyme activity.

Cellulase assay: The reaction mixture containing 1% CMC as substrate and 1ml of enzyme was incubated at 30ºC for enzymatic reaction. The reaction is then terminated by adding 1ml of DNSA and heated at 90ºC for 5-15 minutes until the colour changes to reddish brown. The mixture was cooled and 1ml 1potassium sodium tartrate was added. Absorbency of the mixture was checked at 575nm using UV- VIS spectrophotometer [20]. The OD values were noted and standard graph of glucose was used to calculate enzyme activity.

For all these assays control was prepared same as the test but without adding enzymeand distilled water was used as blank. For each enzyme assay above the original OD value was calculated by subtracting OD value of enzyme with OD value of control.

J. Plastic and paper waste degradation potential of selected bacteria:

Waste degradation was studied using weight loss method. For this, paper and two types of plastic waste say bio degradable and nondegradable was collected aseptically and all these were cut into equal pieces with 6cm long and 6 cm wide weighed and placed in pot filled with soil. To this pot about 40ml of 24 hours old culture was added and kept at 37ºC for 7 and 15 days. Weight loss was determined and so the degradation of bacteria.

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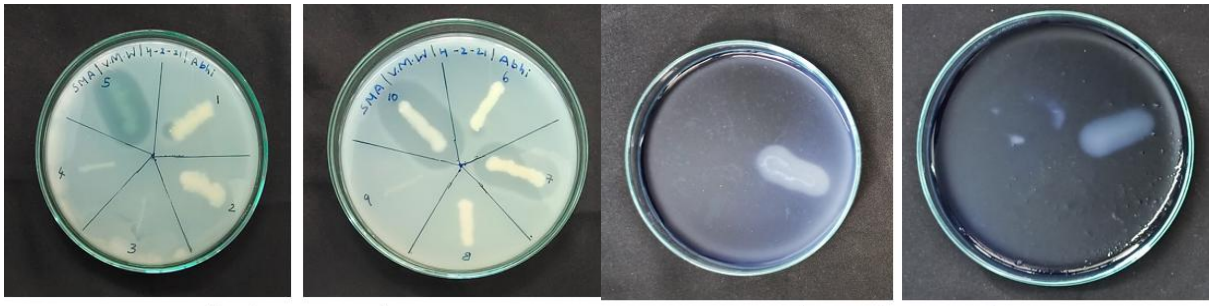
III. RESULTS

Isolation of bacteria from vegetable market waste:

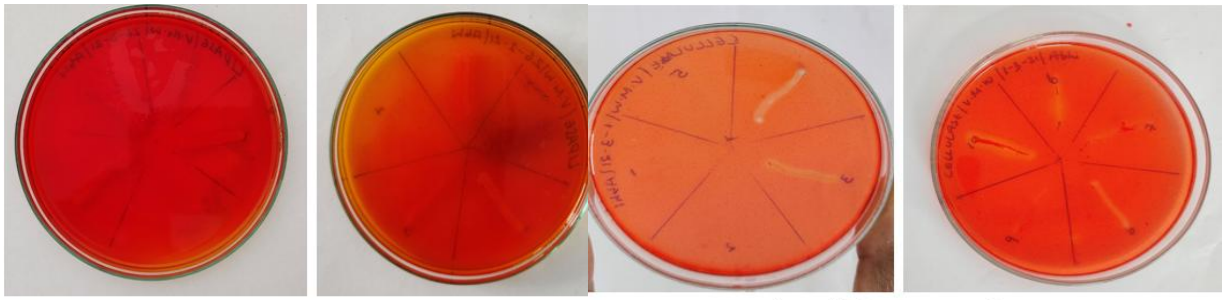
In order to isolate bacteria, collected vegetable market waste sample was serially diluted. Spread plating and streak plating methods were used. In total, ten pure cultures were isolated and they are designated with numbers from 1 to 10.

Screening of bacteria for enzyme production:

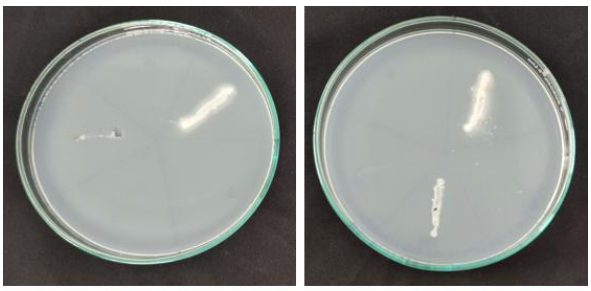
Screening of enzymes is shown in figure 1. In a total of ten pure cultures, isolates numbered 5,7,10 has shown positive for protease. 1,10 have shown positive for amylase and lipase. 3,10 have shown positive for cellulase and lastly 4,8 has shown positive for pectinase. Maximum number of enzymes was produced by the isolate designated '10' which was selected for further study. Clear zone diameter was calculated (Table 1) for all the positive isolates.



a. Protease screening b. Amylase screening



c. Lipase screening d. Cellulase screening



e. Pectinase screening

Figure 1. Screening of enzymes

Table 1. screening bacteria for enzymes production

Isolate number	***Protease***	***Amylase***	***Lipase***	***Cellulase***	***Pectinase***	***Clear zone to colony diameter ratio in (mm)***

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1	-	+	+	-	-	1.85 (Amylase) 1.42 (Lipase)
2	-	-	-	-	-	-
3	-	-	-	+	-	1.67 (Cellulase)
4	-	-	-	-	+	1.50 (Pectinase)
5	+	-	-	-	-	2.2 (Protease)
6	-	-	-	-	-	-
7	+	-	-	-	-	3.6 (Protease)
8	-	-	-	-	+	1.75 (Pectinase)
9	-	-	-	-	-	-
10	+	+	+	+	-	4.0 (Protease) 2.40 (Amylase) 2.0 (Lipase) 2.50 (Cellulase)

Characterisation: Isolated bacteria which has shown to produce maximum number of enzymes was identified at species level using molecular techniques in which genomic DNA was isolated and the purity was checked running agarose gel and was found to be high molecular weight and also intact. 16S rDNA was amplified using 16s Forward primer: 5'-AGAGTRTGATCMTYGCTWAC-3' and 16s Reverse primer: 5'-CGYTAMCTTWTTACGRCT-3' and analysed by performing gel electrophoresis. The PCR product has size is~400bp. It was then sequenced and obtained sequence has shown maximum similarity with target sequence of *Bacillus aryabhattai*. Thus, isolated bacterial numbered '10' was identified to be *Bacillus aryabhattai*.

Production of enzymes: Maximum number of enzymes were produced by isolate designated as 10 which was later identified as *Bacillus aryabhattai*was taken for the production of all the four enzymes that it has produced namely: protease, amylase, lipase and cellulase both in specific media broth and vegetable market waste media broth. Produced enzymes are compared for enzymatic activity.

Enzyme assays: Enzymatic activity of enzymes produced by *Bacillus aryabhattai* in both specific media and vegetable market waste media was compared. Results have shown that protease and cellulase produced in vegetable market waste media has 17% and 72% higher enzyme activity respectively than theiractivity in specific media. Enzyme activity of lipase is 50% less in vegetable market waste media whereas enzyme activity of amylase is similar in both the media.Enzyme activities of all four enzymes are given in the Figure 2.

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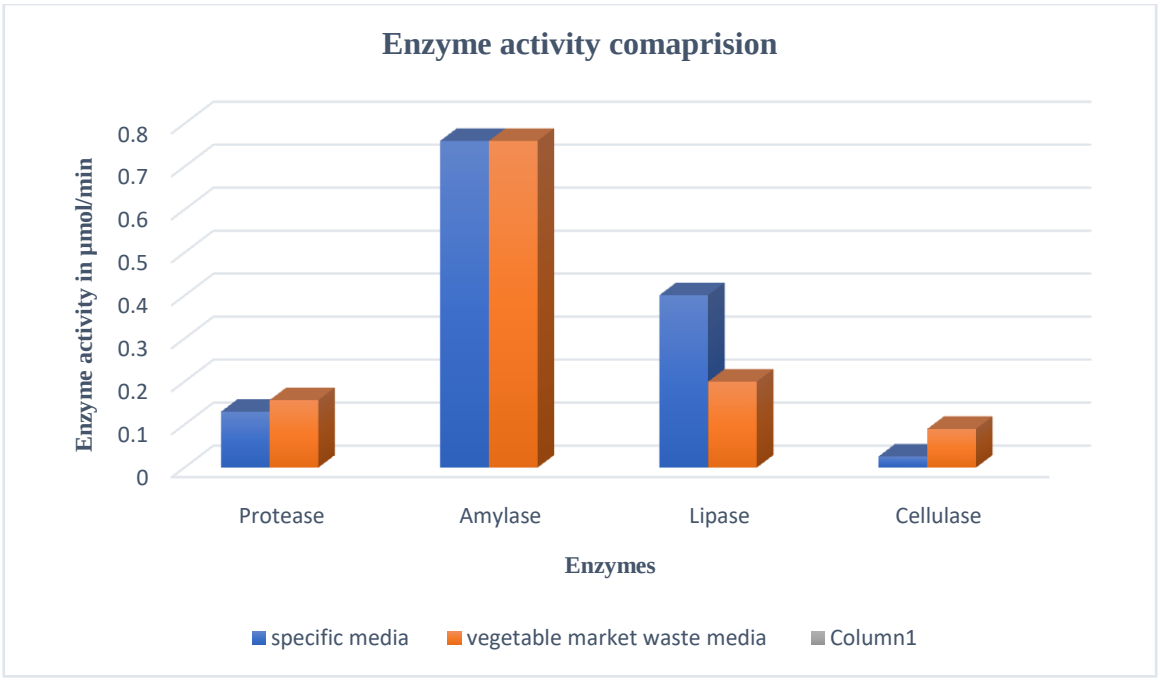

Figure 2. Comparing enzyme activity of enzymes produced by *Bacillus aryabhattai* both in specific media and vegetable market waste media

Plastic and paper waste degradation potential of selected bacteria: *Bacillus aryabhattai*was used in determining the potential degradation of plastic and paper waste.Percentagedegradation was calculated and given in Figure 3.

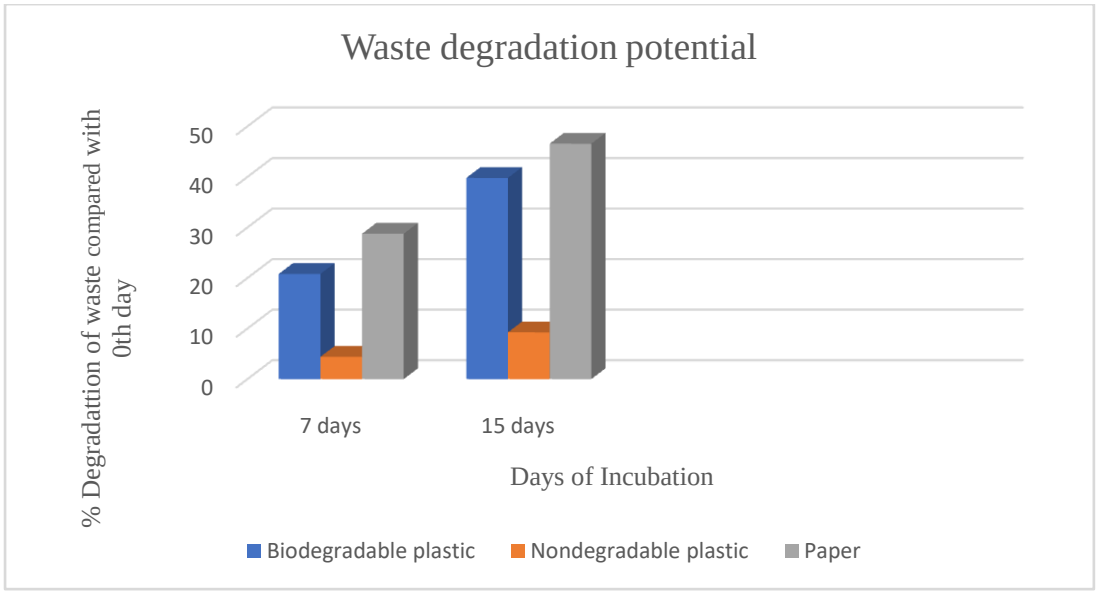

Figure 3. Estimating potential degradation of plastic and paper waste by *Bacillus aryabhattai*

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IV. DISCUSSION

In screening enzymes, most of the pure cultures have shown to produce different combination of enzymes out of five enzymes (protease, amylase, lipase, cellulose and pectinase). This shows group of bacteria isolated from vegetable have the ability to produce wide variety of important enzymes.

In comparative enzyme activity assays, the enzymes protease and cellulase produced in vegetable market waste media has shown 83% and 72.3% greater activity respectively than their activity in specific media. This shows the proteinaceous waste like peas, beans etc present in vegetable market waste media is used as substrate by *Bacillus aryabhattai*for the production of protease enzyme. V.M.W also has plenty of plant material which is rich source of cellulose. This cellulose has been used by *Bacillus aryabhattai* as substrate for the production of cellulase in order to break it down and utilize. We can see that in plastic and paper waste degradation process, paper waste is degraded more rapidly which shows that *Bacillus aryabhattai* produced cellulase in higher quantities.

This study has shown that vegetable market waste can be used as media for producing industrially important enzymes like protease and cellulase very effectively.

V. CONCLUSION

In recent years, focus has shifted towards microorganisms for their potential use in the production of extracellular enzymes used in many industries. Enzymes selected for this study like protease, amylase, lipase, cellulase and pectinase have wide range of applications in industries like food, paper, textile, leather etc.

Vegetable market was can be recycled and utilized as it is a rich source of organic matter and this can not only be used for the production of industrially important enzymes but also to produce many other products like organic acids, antibiotics, vaccines etc for human welfare. Before using bacteria isolated from vegetable market waste or any other waste, it is important to make sure that it is not a pathogen and it is also important to sterile waste media carefully and properly before using it as media so as to confirm killing microbial pathogens.

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