ISSN 0975-6299 Vol 3/Issue 2/April – June 2012

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE MICROBIOLOGY

EXPLOITATION OF NATURAL SUBSTRATES AND OIL CAKES FOR PECTINASE PRODUCTION BY *A.tamarii & A.carbonarius*

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ABSTRACT

The present work was carried out with an aim to isolate an Exo-pectinase producing fungal strain, to optimize physical factors of the enzyme activity and to formulate a media for the cost effective production of the enzyme. Screening, isolation and identification of *Aspergillus tamarii* and *Aspergillus carbonarius* were done. Various process variables like pH, temperature and incubation time of the growth medium were optimized. Modified media using natural wastes as C and N sources were used in combination for quantitative estimation of the enzyme activity. Optimization of various physical variables like pH, temperature, incubation period and substrate concentration for enzyme activity were done. Out of the various C and N combinations tested, citrus peel and groundnut oil cake(GOC) were found to be the best C and N source for *A.tamarii*, Groundnut hull and groundnut oil cake were proved to be the best C and N source with respect to *A.carbonarius*. Orange peel and groundnut peel, a major waste by-product of food industries could be effectively used as C-source and GOC, a major waste of oil industry `can be used as N-source thus, making the process cost effective.

KEYWORDS

Exo pectinase, *A.tamarii, A.carbonarius*, oil cakes, fruit wastes

INTRODUCTION

Pectic substances, present in the primary cell wall and middle lamella of higher plants, contribute to the firmness and structure of plant tissues[1]. Pectin was first isolated and described by Henri Braconnot (1825)[2]. The term "pectin" encompasses a group of acidic heteropolysaccharides with distinct structural domains. They are subjected to both biosynthetic and cell wall-based modifications. These are high molecular weight acid polysaccharides, primarily made of a partially methylated poly – α-(1, 4) linked D-galacturonic acid .Sections of α-(1, 2)-L-rhamnosyl, α-(1, 4)-D-galacturonosyl containing branch points with L-arabinose and D-galactose can be incorporated in the main polymeric chain. Pectin may also contain residues of D-glucuronic acid, D-apiose, D-xylose and L-fucose attached to poly-α-(1, 4)-D-galacturonic acid sections.

Different pectinolytic enzymes are involved in the breakdown of pectin and are widely distributed in higher plants and microorganisms. They are important for plants as they help in cell wall extension and fruit softening. They have a role in maintaining ecological balance by causing decomposition of plant materials. According to the reaction mechanisms, pectinases can be classified as esterases, eliminative depolymerises (lyases) and hydrolytic depolymerases (polygalacturonases). Pectin esterase (E.C. 3.1.1.11) catalyses the hydrolysis of methylated carboxylic ester groups in pectin into Pectic acid and methanol. Pectin lyases (E.C. 4.2.2.10) cleave a-(1, 4)-glycosidic linkages by transelimination, which results in galacturonide with a double bond between C-4 and C-5 at the non-reducing end. Polygalacturonases are involved in the hydrolysis of α-(1, 4) - glycosidic linkages in homogalacturonans. Endo-polygalacturonases (E.C. 3.2.1.15) bring about random hydrolysis of the polymer, whereas Exo-polygalacturonases (E.C. 3.2.1.67) act sequentially from the non-reducing end[3].

The commercial preparations of pectinases are produced mainly from fungi, especially *Aspergillus niger*[3]*.* Microbial pectinases account for 25 % of the global food enzyme sale. Applications of pectinases include vegetable oil extraction, coffee and tea leaf fermentation, improvement of the cloud stability of fruit and vegetable juices and nectars, depectinization and clarification in order to produce high density fruit juice concentrates, wine and other alcoholic beverage processing and haze removal[4]. Pectic enzyme preparations are also used in the degumming of natural fibres in the textile industry[4], in the production of low methoxy pectin for diabetic foods, in making commercial softwoods, such as Sitka and Norway spruce, waste water treatment, bleaching of paper, in poultry feed additives and food industries. Purified pectinases have also been developed specifically for use in plant protoplast culture studies. When used with cellulase, purified pectinases have been found to be very useful for generating good yields of viable protoplast in several plant systems, *e.g.* corn, soybean, red beet, sunflower, tomato, citrus etc[6]. Commercial enzymes are generally obtained from fungal sources since the pH optima of these enzymes are in the range found naturally in materials to be processed and the enzymes are secreted into the culture media, making the downstream processing easier[6].

Bio wastes/fruit wastes are highly perishable materials and their disposal often is a problem in processing

industries[6].Traditionally, agro-industrial waste has been used as a feed or as a fertiliser, but currently, the utilisation of bio wastes is on the increase and represents a good alternative that avoids environmental pollution and gives an additional value to plant by-products. Extraction of enzymes from bio wastes using the technology of fermentation, which gained importance recently, is one of the many ways of exploiting them profitably[7]. Oil cakes/oil meals are by-products obtained after oil extraction from the seeds. Oil cakes are of two types, edible and non-edible. Edible oil cakes have a high nutritional value; especially the protein content ranging from 15% to 50% (www.seaoWndia.com). Their composition varies depending on their variety, growing condition and extraction methods. Due to their rich protein content, they are used as animal feed, especially for ruminants and fish. Non edible oil cakes such as castor cake, karanja cake, and neem cake are used as organic nitrogenous fertilizers, due to their N P K content [8].

This project focuses on screening, isolation and identification of pectinase producing fungi using basal media followed by growth and enzyme characterisation of the isolates. Finally, utilisation of bio waste materials as carbon substrates along with the value addition of oil cakes (residue obtained after oil extraction) by their utilisation in production of pectinase enzyme[8] was done. This production has been emphasised by using mango, onion, citrus peels, wheat bran and groundnut hulls as carbon substrates and Sesame oil cake (SOC) , Groundnut oil cake (GOC) and Coconut oil cake (COC) as nitrogen substrates for a considerable improvement in enzyme secretion.

MATERIALS AND METHODS

Sample collection:

The various fungal strains were collected from different soil samples, compost and decomposed matter of vegetable, fruit and decomposing soils of Visakhapatnam, Kakinada cities in Andhra Pradesh (India). The collected samples were first grown on Sabouraud's agar media. All morphologically contrasting colonies were separated and pure cultured individually by repeated streaking.

Subculture and maintenance of microorganism:

The strains were sub cultured on SDA slants and incubated for 72 h at 30°C. The sub cultured strains were maintained in a refrigerator at 4°C in the laboratory by conventional methods and sub cultured at regular intervals.

Screening of pectinolytic strains:

The pectinolytic activity of the isolated strains was assayed qualitatively using modified Pectin Congo red agar plate assay method with composition (gL^{-1}):

Pectin - 10; K_2HPO_4 - 1.0; $MgSO_4 .7H_2O$ - 0.5; $NaNO_3$ - 2.0; $FeSO_4.7H_2O$ - 0.01; KCl - 0.5; Congo red – 0.12% [8] and 2% agar . The initial pH of medium was adjusted to 6 - 6.2.To the above medium ampicillin was added (100mg / ml) to prevent bacterial contamination. The Petri dishes were inoculated and incubated at 28° C for five days. At the end of the incubation period, decolourisation of Congo red and the occurrence of clear zones around the colonies were accepted as an indicator of the pectinolytic activity.

Submerged fermentation experiment:

Pectinolytic Enzyme Fermentation (SbF):

After the preliminary screening tests by plate assay method the enzyme producing efficiency of the isolates was tested quantitatively by submerged fermentation using basal media containing the following composition (gL^{-1}):-

Pectin-10g: Yeast extract-5g

The medium was sterilised and in 250 ml Erlenmeyer flask, 50 ml of the medium was taken and inoculated with positive fungal isolates. This medium was incubated in a rotary shaker (120 rpm) at 30 °C for 5 days, and then assayed for pectinolytic activity. The culture

filtrates used in the enzyme determination were obtained by filtering the fermentation medium through a filter paper (Whatmann No. 4) and subsequently centrifuged at 6000 rpm at 4°C for 10 minutes.

Physiological Characterization of the selected isolates:

The isolates were sent for identification to Xcelris laboratories, Ahmadabad, Gujarat. The physiological and cultural characteristics of the selected isolates were studied according to standard techniques using Sabouraud's dextrose broth media (SDB) (p^H -6) of following composition (g/L):-

Dextrose – 40g: peptone – 10g

Growth time course:

The isolate was inoculated and incubated at room temperature under static conditions for 6 days in SDB media (pH- 6).The growth measurement was done by estimating the dry weight at every 24 hrs. The isolate was filtered using Whatmann filter paper and was dried in hot air oven at 80^0 c till standard weight was obtained.

Optimum pH:

The similar procedure was followed to estimate the optimum pH for the fungal growth. The SDB media was prepared with **pH** ranging from **pH** 3.0-5.0(acetate buffer), **pH** 6.0-8.0 (phosphate buffer) and **pH** 9.0(Tris-HCl buffer).The media were incubated at room temperature for 5 days under static conditions.

Optimum temperature:

The optimum temperature was estimated in the same method by inoculating the isolate in SDB media (**pH**-6) and incubating it for 5days at various temperatures ranging from 10^0 C to 50^0 C.

Enzyme Characterisation:

The basal media crude extract was used to perform the following activities:-

- The pectinase enzyme activity was determined by incubating each reaction mixture at 50°C in different **pH** using acetate **(pH** 3.0-5.0), citrate/phosphate (pH 5.0-7.0), and Tris-HCl (**pH** 7.0-8.0) as buffers.
- The optimum temperature was assayed by incubating each reaction mixture at 20-70°C.
- The reaction speed was determined for the enzyme by varying the substrate concentration from 5-25 mg/ml.
- The effect of incubation period on enzyme activity was determined by incubating the reaction mixtures for 10-60 varying time intervals.

Optimisation of substrates for enzyme production using various agro-industrial wastes:

Carbon source:

The carbon substrates were prepared as follows:

a) Mango, citrus, Onions, and ground nuts were purchased from the local market. The peels and groundnut hulls were removed and sorted out manually based on their fine texture and rigidity. The collected peels and hulls were minced to pieces and were dried in hot air oven at 100°C until constant weight was achieved. The dried peels and hulls were diminuted in a Ball mill and they were clarified in a sieve shaker to obtain a fine powder.
b) Wheat bran: This material was purchased from the local market, dried in hot air oven at 80^oc for 1hr and used untreated.

Nitrogen source:

The nitrogen substrates were prepared as follows:

Sesame oil cake (SOC), Groundnut oil cake (GOC) and Coconut oil cake (COC) were purchased from the local market .The oil cakes were broken into small pieces and were dried in hot air oven at 100°C until constant weight was achieved .Later the pieces were diminuted in a Ball mill and they were clarified in a sieve shaker to obtain a fine powder.

Composition of oil cakes[9]

Oil cakes	Dry matter %	Crude protein %	Crude fibre%	Ash %	Calcium%	Phosphorus%	Reference
Sesame oil cake (SOC)	83.2	35.6	7.6	11.8	2.45	1.11	Kuo(1967)
Groundnut oil cake(GOC)	92.6	49.5	5.3	4.5	0.11	7.4	Kuo(1967)
Coconut oil cake(COC)	88.8	25.2	10.5	6.0	0.08	0.67	Ghol(1970)

Submerged fermentation (SbF) was carried out in a 250 ml Erlenmeyer flask with 50 ml of substrate solution containing carbon, nitrogen substrates and inducer (1% pectin, 0.5% yeast extract solution) in 1.5:1.5:1 ratio as pectinase is an inducible enzyme[10]. These media were compared with the nitrogen substrate deficient media as controls which were also prepared in 3:1 ratio. All the above media were maintained at 6.2 **pH**. The flasks were autoclaved , inoculated and incubated in a rotary shaker (180 rpm) at 30 °C for 5 days, then assayed for pectinolytic activity .The culture filtrates used in the enzyme determination were obtained by filtering the fermentation medium through a filter paper (Whatmann No. 4) and subsequently centrifuged at 6000 rpm at 4°C for 10 minutes.

Enzyme activity measurement:
Exo pectinase activity was assayed by measuring the release of reducing sugars by the method of DNS given by Miller[11] using polygalacturonic acid (Himedia) as substrate. The reaction mixture contains 2ml of 1% pectin substrate in 0.2M acetate buffer at 4.5 **pH** and 1ml of enzyme extract. This reaction mixture was incubated at 50^0 c for 30 min in preheated water bath. After incubation 3ml of DNS was added to stop the reaction and tubes were kept in boiling water bath for 10 min, later 10 ml of distilled water was added to each test tube and cooled to obtain room temperature .The developed colour was read by taking the OD at 575 nm using colorimeter (Systronics). The comparison among the isolate readings determined the highest enzyme producing strain.

Biomass determination: Filter papers containing the biomass from the submerged fermentation were dried at 80^0C until constant weight was obtained and recorded.

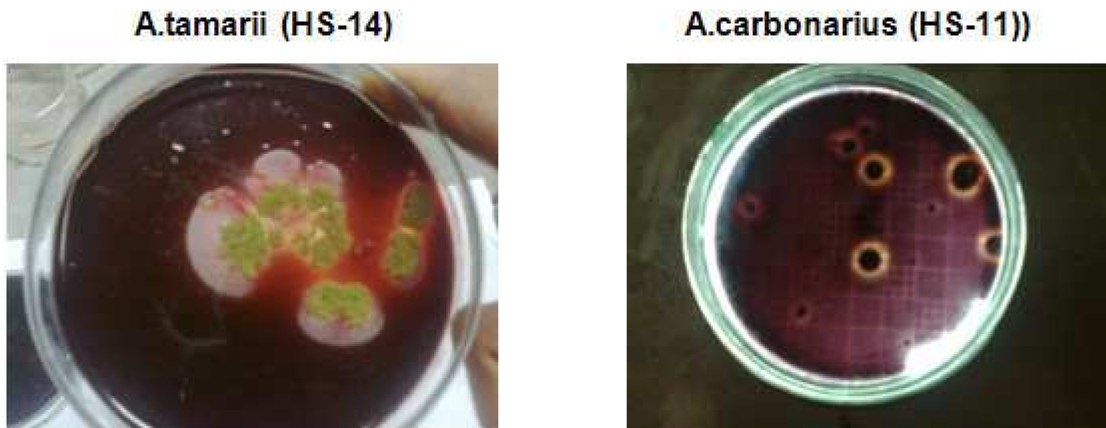
Statistical analysis: The experiments were carried out in triplicate. The means were calculated and graphs are given using Minitab 16 software.

RESULTS AND DISCUSSION

The main aim of this paper is to report the importance of the valueless agro- industrial waste products in the production of industrially most important pectinase by submerged fermentation method.
About 45 isolates were obtained from the decompost and various decaying fruits which were grown on SDA. These fungal strains were further screened by plate assay method in which pectin was the only carbon source. Of the 45 samples only 15 strains were able to grow in the pectin Congo red agar media. The pectinolytic activity was confirmed by observing a concentric transparent ring around the colony against a white background due to decolourisation of Congo red dye.

Figure: 1

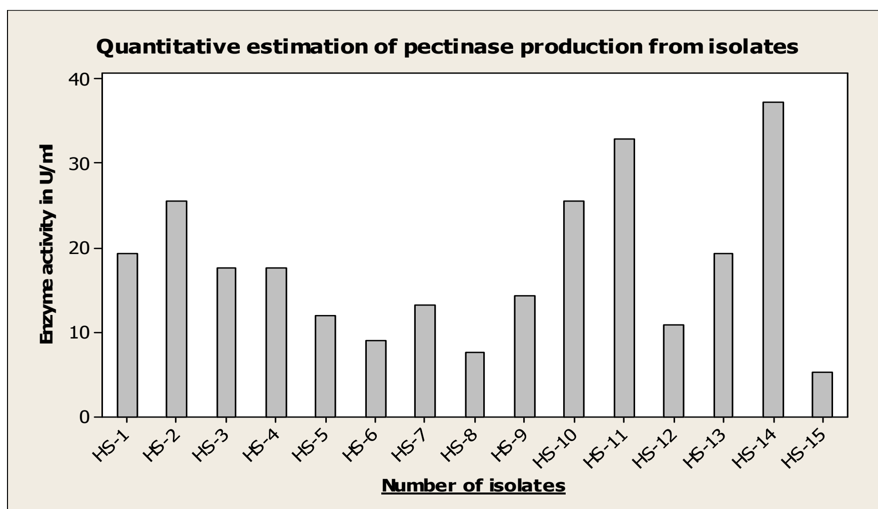
Pictures showing qualitative analysis decolourisation in Congo red- pectin agar plate



All the 15 samples were inoculated in the liquid media for quantitative analysis of enzyme production. The organisms showing maximum growth were isolated, sub cultured and used for further studies.

Figure: 2

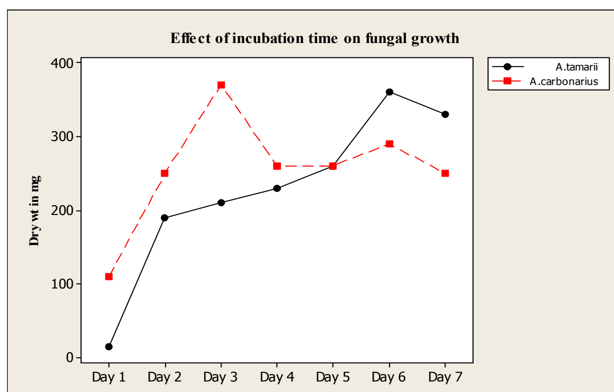
Graph showing preliminary screening results



From the above graph it could be clearly seen that of all the isolates HS-11 and HS- 14 showed maximum pectinolytic activity. Hence high activity isolates were further screened. As per the reports sent by Xcelris Laboratories, Ahmadabad, H -11 was identified as *Aspergillus carbonarius* NRRL -369 (Genbank accession Number: 661204.1) and H - 14 was identified as *Aspergillus tamarii* NRRL-425 (Genbank Accession Number: EF661558.1).

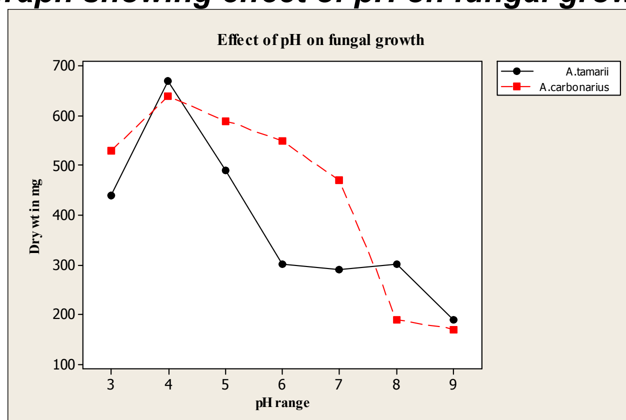
Optimization of growth characteristics:
Aspergillus tamarii showed maximum growth on 6th day of incubation under room temperature in SDB media (**pH**-6) where as *A.carbonarius* showed highest growth on 3rd day of incubation.

Figure: 3
Graph showing optimisation of growth at various time intervals



When the organisms was cultivated in the SDB medium with pH ranging from 3 – 9 at room temperature for 5 days under static conditions, the maximum growth was observed in the medium at pH – 4 for both the organisms .

Figure : 4
Graph showing effect of pH on fungal growth

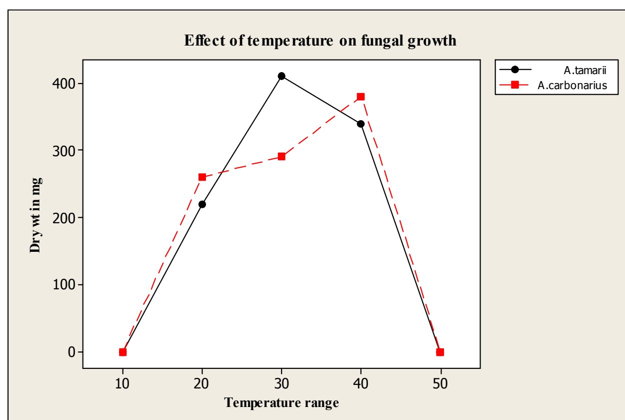


Similarly the maximum growth was observed at 30^0C in *A.tamarii* and at 40^0 C in *A.carbonarius* when the isolates were cultivated in SDB medium for 5days with temperatures ranging from 10 -50^0C under static conditions.

ISSN 0975-6299 Vol 3/Issue 2/April – June 2012

Figure : 5

Graph showing effect of temperature on fungal growth



Optimization of enzyme characteristics:

Further, characterisation was done to check the versatility of the enzyme produced by performing **pH**, temperature, and substrate concentration and incubation time. From optimisation procedures, it was found that both *A. carbonarius* and *A.tamarii* has optimum temperature at 50°c (fig: 6), optimum **pH** at 4.5(fig: 7) and incubation period at 30 min (fig: 9).The optimum substrate concentration for *A.carbonarius* is 1.5% where as for *A.tamarii* the optimum is 1% (fig: 8).

Figure : 6. Graph of effect of temperature on enzyme activity

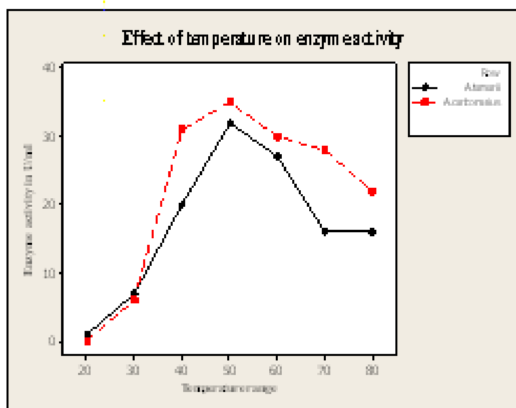


Figure : 7.Graph of effect of pH on enzyme activity

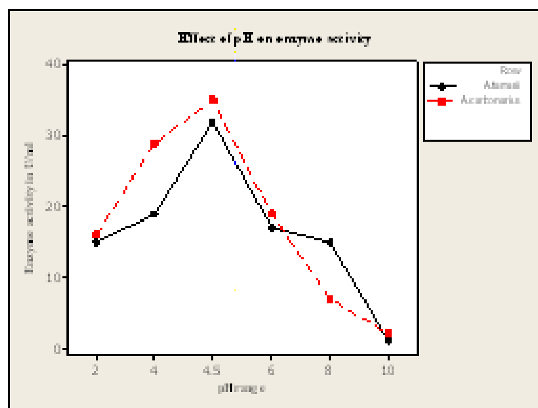


Figure : 8 .Graph of effect of substrate concentration on enzyme activity

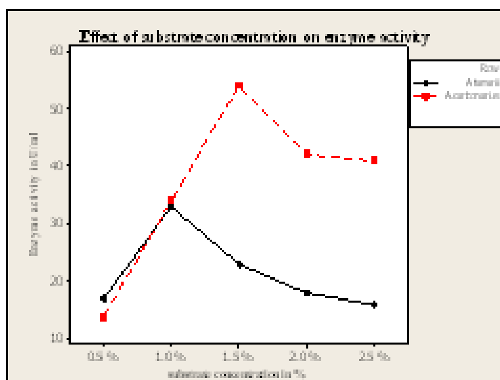
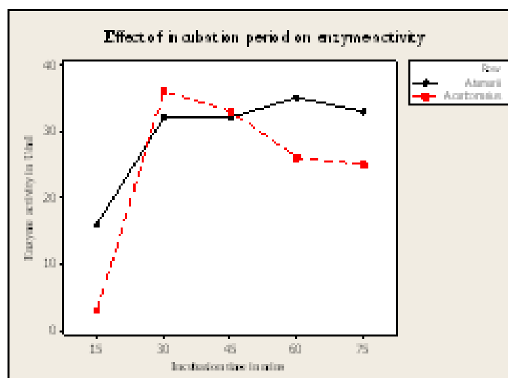


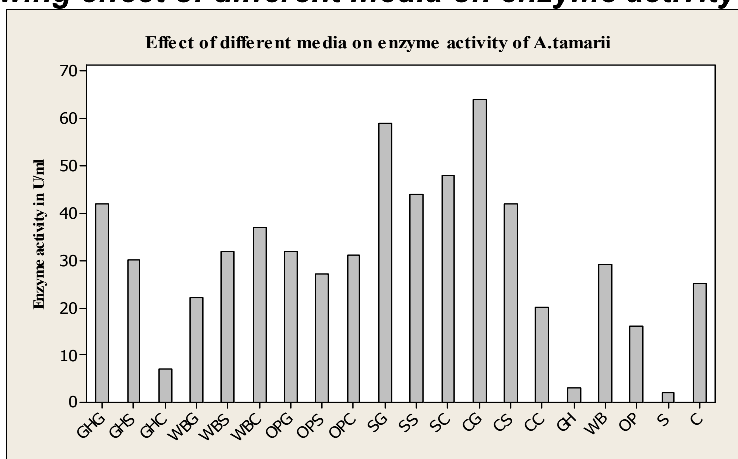
Figure : 9 .Graph of incubation period on enzyme activity



Optimization of production media:
Various carbon and nitrogen sources were used in combinations in the design of production media and it appears to exert a profound effect on the enzyme production behaviour of the fungi.

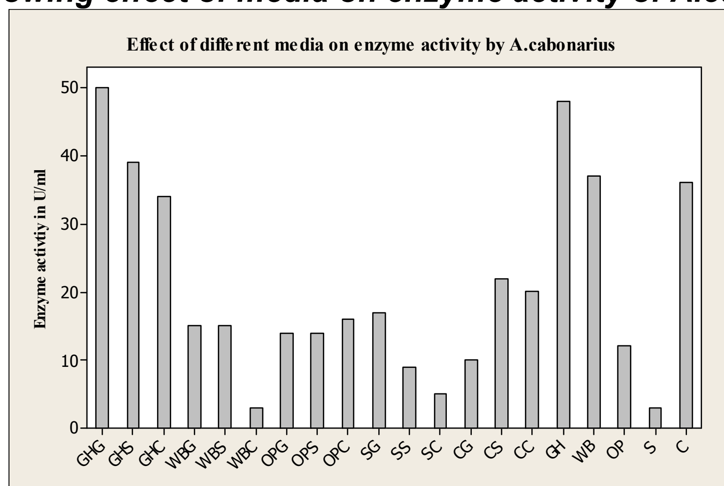
From the given graph it is very clear that *A.tamarii* is having its highest production in citrus peel- groundnut oil cake (GOC). Likewise *A.carbonarius* is having its highest production in Groundnut hull- GOC.

Figure : 10
Graph showing effect of different media on enzyme activity* of *A.tamarii



GH-groundnut hull; WB-wheat bran; OP-onion peel; S- sapota peel; C-citrus peel G-groundnut oil cake (GOC); S-sesame oil cake (SOC); C-coconut oil cake (COC)

Figure : 11
Graph showing effect of media on enzyme activity of A.carbonarius



GH-groundnut hull; WB-wheat bran; OP-onion peel; S- sapota peel; C-citrus peel
G-groundnut oil cake (GOC); S-sesame oil cake (SOC); C-coconut oil cake (COC)

CONCLUSION

The work shows the potential of waste materials in industrial productions of some novel biocatalysts like pectinase. Their proper management in disposal of fruit and other wastes can help keep environmental pollution in control. We have been able to achieve pectinase production using the cheapest agro industrial wastes available. The results also show the potential of increasing production rate of pectinase through strain improvement.

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ACKNOWLEDGEMENT

The authors are very much thankful to the Prof. M.A. Singaracharya sir for their suggestions and support during the tenure of this project.

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