



EXPLOITATION OF FRUIT WASTES FOR PECTINASE PRODUCTION USING *ASPERGILLUS ORYZAE*

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ABSTRACT

Ever increasing population and industrialization has resulted in heavy pollution and waste generation. The main aim of this project lies in waste management and synthesis of pectinase enzyme which plays a crucial role in sewage treatment. Twenty moulds were isolated from decaying fruits and municipal waste soil sample on SDA and were further screened for pectinolytic enzyme production. Screening, isolation and identification of *Aspergillus oryzae* was done based on larger clearance zones in pectin - Congo red agar media and pectinase enzyme production was carried out in YEP media by submerged fermentation. Further production media was formulated using various fruit wastes (peels) in different compositions. Growth characteristics of the isolate and kinetics of the Exopectinase enzyme were optimized. Sapota peel in combination with groundnut oil cake (GOC) had been proved to be the best media for production of high potential Exopectinase. *A. oryzae* has its highest enzyme activity at 50^o C temperature at 4.5 pH concentration level. It takes 60 mins of time duration to give best enzyme activity in 1.5% substrate concentration.

Keywords: *A. oryzae*, YEP media, Sapota peel, ground oil cake, Exopectinase, submerged fermentation



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INTRODUCTION

Proteins are the high complex structures associated with metals, carbohydrates or lipids. In a similar way all enzymes are proteins, but all proteins are not enzymes¹. These are biocatalysts which are synthesized by all living cells. The enzymes are also defined as the catalysts that increase a velocity or rate of a chemical reaction² without interfering in the kinetics of the biochemical reactions. At present, out of 2000 well known enzymes, nearly 30 enzymes are actively used in production. Hydrolases, especially pectinases, which involve in many physiological processes occurring in plants: mainly in fruit ripening, are of great interest in the world of enzymes³.

Pectin, one of the most important component of plant cell wall along with other polysaccharides like cellulose, hemicellulose⁴, was first isolated and described by Henri Braconnot (1825)². It is a jelly like matrix, structural polysaccharide found in primary cell wall and middle lamella of fruits and vegetables and other plant cells². The predominant structure of pectin is homopolymeric, made of a partially methylated poly- α -(1, 4)-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 sections⁵. Pectic substances are negatively charged acidic glycosidic macromolecules having high molecular weight. These pectic substances exist in the form of calcium pectate and magnesium pectate⁶. Pectinolytic enzymes are classified according to their way of attack on the dumped⁶. Disposal of these highly perishable biowastes is a huge problem to the processing industries⁹. Traditionally, industrial waste has been used as a feed or as a fertiliser¹⁰. These residues obtained from the processing industries of wheat, orange, sugarcane and coffee are suitable feed stocks for bioconversion into chemicals, including enzymes by fermentation

galacturonan part of the pectin molecule. They can be distinguished between pectin methylesterases (EC 3.1.11.1) that deesterify pectins to low methoxyl pectins or Pectic acid, and pectin depolymerases, which split the glycosidic linkages between galacturonosyl (methyl ester) residues. Polygalacturonases split glycosidic linkage next to free carboxyl groups by hydrolysis while pectate lyase split glycosidic linkages next to free carboxyl groups by β -elimination. Both endo types of PGs and PALs (EC 3.2.1.15 and EC 4.2.2.2, respectively) are known by splitting randomly the pectin chain. Exo-PGs (EC 3.2.1.67) release monomers or dimmers from the non-reducing end of the chain, whereas Exo-PALs (EC 4.2.2.9) release unsaturated dimmers from the reducing end. Highly methylated pectins are degraded by endo-pectin lyases (PL; EC 4.2.2.10) and also by a combination of PE with PG or PAL⁷.

Among depolymerising microbial enzymes, pectinolytic enzymes are of great commercial value. Various industrial applications like food processing, textile processing, degumming of plant fibres, extraction of pigments from plant materials, preparation of cellulose fibres for linen, jute and hemp manufacture⁸, agro waste treatment and fruit juice treatment, coffee & tea fermentation, oil extraction, paper pulp treatment, waste water treatment, bleaching of paper, adding poultry feed and in the alcoholic beverages and food industries⁶ are done using these enzymes.

Enormous quantities of industrial waste residues are generated throughout the world from processing raw agricultural materials for foods. This generation imposes a high BOD burden on the environment when

processes⁶. Utilisation of such kind of agro-industrial wastes in the production of industrial enzymes can be considered as one of the solid waste disposal process.

Due to the potential and wide applications of pectinases, it is necessary to study several aspects related to pectinase production. The idea of using cheaper agro industrial wastes for pectinase production is

an important parameter useful in technological development. The aim of this experimental paper is to present an overview of the pectinase activity values obtained by fungal strains for pectinase production by utilising cheaply available agro-industrial wastes followed by their optimisation studies.

MATERIALS AND METHODS

Microorganism source

The various fungal strains were collected from different municipal waste soil samples, compost and decomposed matter of vegetable, fruit and decomposing soils of Vishakhapatnam areas in Andhra Pradesh (India). The collected samples were first grown on Sabouraud's agar media (SDA) and maintained in the laboratory by conventional methods.

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.

Qualitative analysis

The obtained fungal strains were tested qualitatively by cultivating in Pectin-Congo red agar¹¹ media (pH-6.2) with following composition in gL⁻¹: Pectin - 10g; yeast extract - 5g; Agar - 15; Congo red - 0.12%¹¹. At the end of the incubation period, the positive plates were identified by observing clear zones around the colonies which was accepted as an indication of pectinolytic activity.

Fermentation experiments

Submerged 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 YEP media¹² (pH-6.2) with following composition in (gL⁻¹) : Pectin - 10g; Yeast extract - 5g. In 250 ml flasks, 50 ml of the medium was taken, inoculated with isolate

spores and incubated in a rotary shaker (120 rpm) at 30°C for 5 days.

Sample extraction followed by fermentation

Upon completion of SbF, the cell free extract was assayed for enzyme activity after removal of residual fungal growth by filtration using pre determined Whatmann (no-4) filter papers¹³ followed by centrifugation at 6000rpm for 10 min. The resulting supernatant broth was assayed for pectinase activity.

Analytical determination

Enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by dinitrosalicylic acid reagent (DNS) method; this method was given by Miller¹⁴. To 1ml of 1% pectin substrate buffer (pH 4.5), 0.5 ml of enzyme extract was added. The reaction mixture was incubated at 50°C for 30 min. After 30min, 1.5 ml of DNS reagent was added to the reaction mixture and were shaken to mix the contents. The test-tubes were heated to 90°C on the boiling water bath for 10–15 min, cooled and 5 ml of distilled water was added to the contents of each tube. The absorbance was measured at 575 nm using digital colorimeter (Systronics). The controls were maintained parallel to tests. The enzyme activity was measured as 1 unit of enzyme activity is equal to 0.01 moles of mono galacturonic acid released.

Physiological Characterization of the selected isolates

The selected isolate was sent for identification to Xcelris laboratories, Ahmadabad, Gujarat. Further characterisation was done by standard methods using Sabouraud's dextrose broth media (SDB) of following composition (gL⁻¹):- Dextrose - 40g; peptone - 10g All the physiological characterisations were done by carrying out all the experiments under static conditions and determining their dry weight.

Growth time course

The isolate was inoculated and incubated at room temperature for 6 days in SDB media (pH 6). Estimation of growth was done by

determining the dry weight in every 24 hrs. Determination of dry weight was done by drying the Whatmann filter paper at 80°C until constant weight was obtained.

Optimum pH

Optimisation of pH was done in likewise manner for the selected isolate. The SDB media was prepared with pH ranging from 3.0-5.0 (acetate buffer), 6.0-8.0 (phosphate buffer) and 9.0 (Tris-HCl buffer). The media were incubated at room temperature for 5 days and growth was estimated dry weight determination.

Optimum temperature

The similar procedure was followed to estimate the optimum temperature for the fungal growth. The SDB media (pH -6) was incubated for 5 days at various temperatures ranging from 10°C to 50°C.

Enzymatic characterisation of selected isolates

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.

Effect of various natural substrates on enzyme production

Carbon source

The carbon substrates were prepared as follows:

A) Sapota, citrus, Onions, and ground nuts were purchased from the local market. The peels of fruits and groundnut hulls were removed, sorted out manually based on their fine texture and rigidity, minced to pieces and were dried in hot air oven at 100°C until constant weight was achieved. The dried peels and hulls were diminished in a Ball mill and they were clarified in a sieve shaker to obtain a fine powder¹⁵.

B) Wheat bran: This material was gathered from the local market, dried in hot air oven at 80°C 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 diminished in a Ball mill and they were clarified in a sieve shaker to obtain a fine powder¹⁵.

Composition of oil cakes¹⁶:-

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)

Formulation of fermentation medium and culture conditions

Presence of pectin in fermentation media affects the enzyme production rate as pectinase is an inducible enzyme¹⁷. 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. These media were compared with the nitrogen substrate deficient media as controls

which were also prepared in 1.5:1 ratio. All the above media were maintained at pH 6.2. The flasks were autoclaved, inoculated and incubated in a rotary shaker (120 rpm) at 30 °C for 5 days, then assayed for pectinolytic activity.

Biomass determination

Filter papers containing the biomass from the submerged fermentation were dried at 70°C until constant weight and measured.

Statistical analysis

All the experiments were carried out in triplicate and means were calculated. The reports are given by plotting graphs using Minitab 16 software.

RESULTS AND DISCUSSION

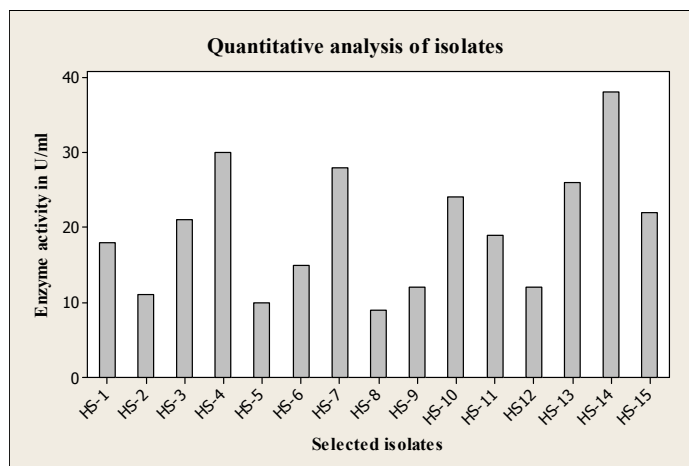
The main objective of this research work is to develop importance of worthless agro – industrial wastes in the field of Industries for making fermentations economic and cost effective. About 20 isolates were obtained from decompost and grown on SDA .Of the 20 samples, 15 strains were able to grow in Pectin - Congo red agar medium. The decolourisations around the colonies observed against a white background were accepted as pectinolytic activity. Of all the 13 samples, only 1 strain was selected based on maximum concentric ring surrounding the colonies.

Figure 1
Figure showing qualitative analysis by decolourisation in Congo red agar plate *A.oryzae* (HS – 14)



These strains were further inoculated in the liquid media for enzyme production for quantitative analysis.

Figure 2
Graph showing quantitative analysis of pectinase producing strains

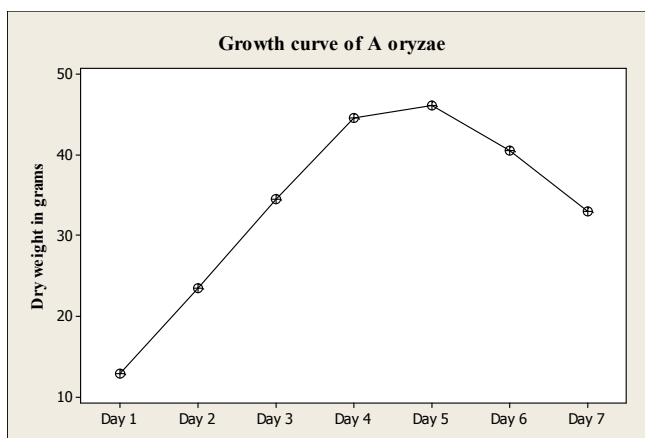


The high pectinase producing strain HS-14 can be easily identified by the above graph showing 42 U/ml of enzyme activity. This organism was subcultured, preserved and utilised in further studies. As per the reports given by Xcelris Laboratories, Ahmadabad, HS – 14 was identified as *Aspergillus oryzae* isolate Y1-SAN (GenBank Accession Number: HQ122941.1).

Optimisation of growth characteristics

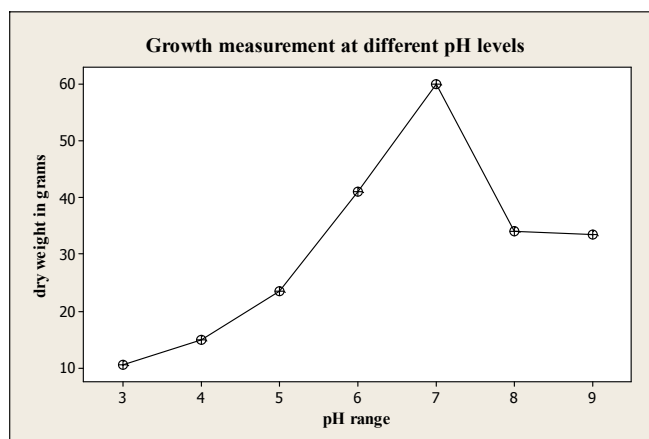
Aspergillus oryzae is a fast growing fungus having light green colour conidia formation. The maximum growth of this isolate is observed on 5th day of incubation in SDB media (pH-6) under static conditions at 30⁰ C.

Figure 3
Graph showing optimisation of growth at various time intervals



Similarly when the isolate was cultivated for 5 days at different pH concentrations in SDB media at 30⁰ C, the maximum growth was observed at 7 pH concentration.

Figure 4
Graph showing effect of pH on fungal growth



In same manner cultivation was done for 5 days in SDB media (pH-6) under various temperatures. Maximum growth was observed at 30⁰ C temperature.

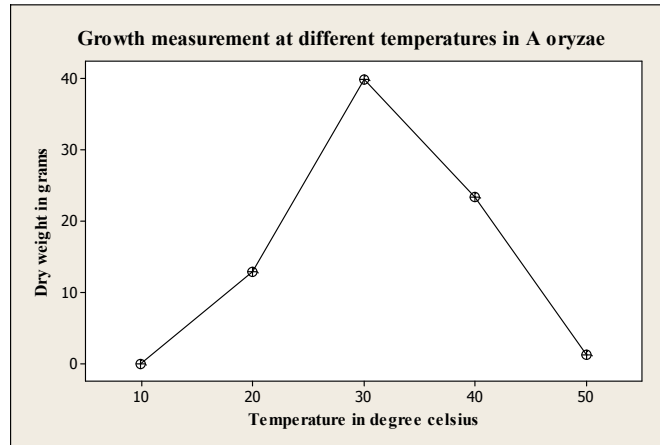


Figure 5
Graph showing effect of temperature on fungal growth

Optimization of enzyme characteristics

Characterisation was done to check the flexibility and compatibility of the enzyme in various process variables. Enzyme assay was performed at various levels of pH, temperature, substrate concentration and incubation time. From optimisation procedures, it was found that Exopectinase produced by *A oryzae* has its maximum activity at 50⁰ C temperature (Fig: 6) and optimum pH concentration at 4.5 (Fig: 7). The optimum incubation period for maximum enzyme activity is 60 min (Fig: 9) and optimum substrate concentration is 1.5% (Fig: 8).

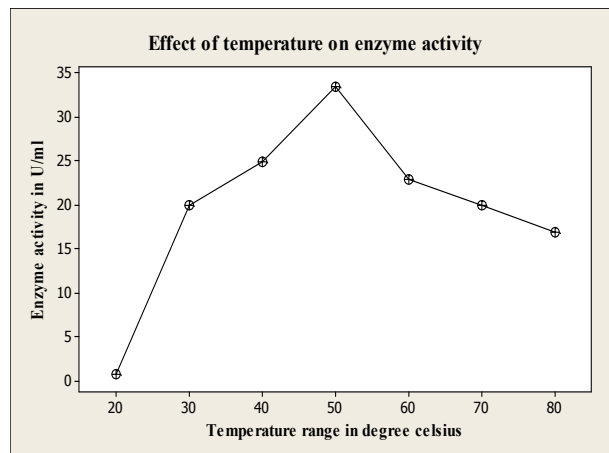


Figure 6
Graph of effect of temperature on enzyme activity

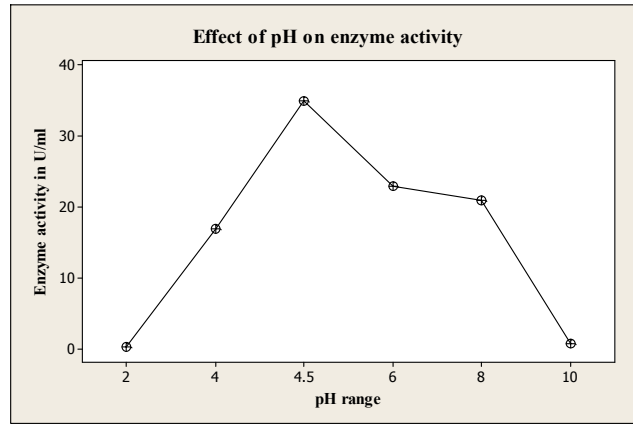


Figure 7
Graph of effect of pH on enzyme activity

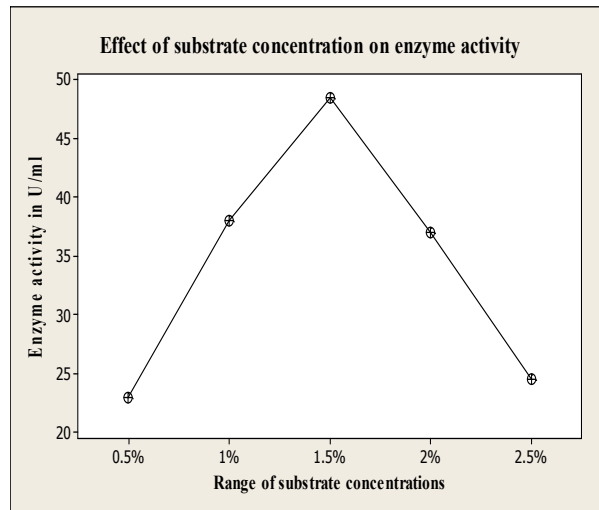


Figure 8
Graph of effect of substrate concentration on enzyme activity

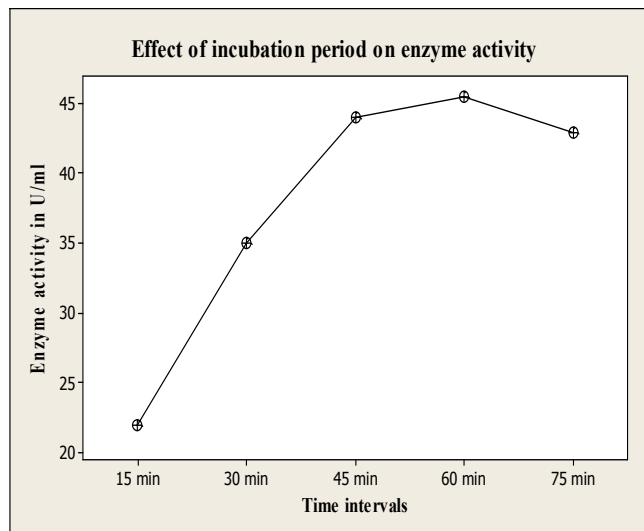
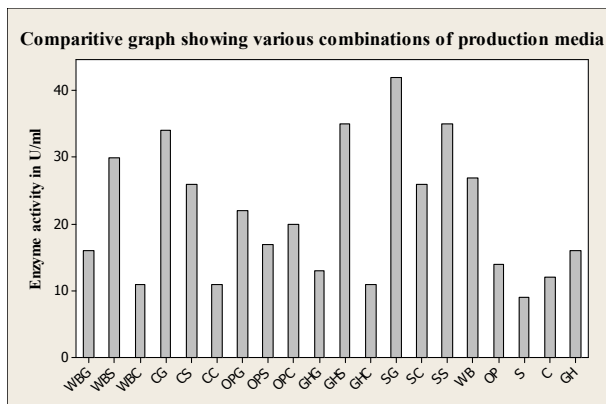


Figure 9
Graph of incubation period on enzyme activity

Formulation of fermentation medium and culture conditions

Several combinations of fruit wastes and oil cakes were used in the formulation of cost effective fermentation media. Oil cake deficient media were kept as controls to reveal the importance of oil cakes in the fermentation media. This economically cheap media designed by using valueless waste materials, plays an important role in bringing out the utmost behaviour of the fungus.



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 10

Graph showing effect of different media on enzyme activity of *A oryzae*

From the above graph, it is clearly understood that Sapota peel – groundnut oil cake (SG) media is the best media for fermentation using *A oryzae* isolate.

CONCLUSION

Utilisation of worthless waste materials to generate a commercial valuable product, Exopectinase is the main objective of this project. This was successfully achieved by increasing the enzyme secretion levels to 42U/ml using Sapota peel – GOC media compared to synthetic purified pectin containing YEP media. Strain improvement methods can be applied to increase the potential enzyme secretion in further research studies.

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