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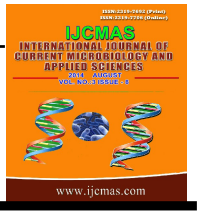

Original Research Article

Optimization and Production of Pectinase Using Agro Waste by Solid State and Submerged Fermentation

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A B S T R A C T

The present study investigated pectinase production and optimization by fungal species isolated and identified from agro waste dumped soil. The selected fungal isolates namely *A.oryzae, A.flavus* and *R.oryzae* were screened for pectinase production by using Solid State Fermentation and Submerged Fermentation. Of the three selected fungal species *A. flavus* produced high amount of pectinase. The maximum pectinase production was observed in Pine apple (0.79 IU/ml) using Solid State Fermentation (15.55 U/ml). High pectinase production was observed at 72 hrs of incubation at 35^{o}C with the initial pH of 5.5. This study has potential of utilizing agricultural waste provides cost effective and ecofriendly method for pectinase production on large scale.

Keywords

Pectinase, Solid state fermentation, Submerged fermntation, Agrowaste

Introduction

Pectin is a polymeric material having carbohydrate group esterifies with methanol. It is an important component of plant cell wall. It is present in highest concentration in the middle lamella, where it acts as a cementing substance between adjacent cells. Plant pathogens attack target cells by producing number of cell degrading enzyme which facilitate the entry and expansion of pathogen in the host tissue.[1]

Pectinolytic enzymes are of significant importance in the current biotechnological era with their all embracing applications in fruit extraction and its clarification. In addition, are involved in degumming of plant fibers, vegetable oil extraction, tea and coffee processing and alcoholic beverages etc. They have a share of 25% in the global sales of food enzymes . Carbon sources especially of agrarian source are more suitable because they are cost effective, renewable and available in large quantities.The agro waste such as Sugarcane, Rice straw, Wheat bran, coconut coir pith, Rice husk and bagasse, Maize bran can be used the best substrate for bioconversion.[2]

These enzymes not only provide an economically viable alternative, but are also environmental friend.[3]The microbial pectinase accounts approximately for 25% of the total worldwide enzyme sale.[4] Although a number of pectinases have been studied there are few reports about the production of pectinase by thermophilic fungi. Pectinase production from *A. niger*was performed in submerged fermentation (SmF) and solid state fermentation (SSF). The increasing energy demand has been focused worldwide attention on the utilization of renewable agricultural and industrial wastes as their disposal pose environmental problems.[5]The pectinolytic enzyme at on pectin, a complex polysaccharide which occurs mainly middle lamella of higher plants.[6]Pectinase is extensively used in food processing industry, souring of cotton, degumming of plant fibers, waste water treatment vegetables oil extractions, tea and coffee fermentation, bleaching of paper, in the alcoholic beverage.

In view of the above, the present study was focused on pectinase production by newly isolated strain of *A. flavus, A. oryzae, R.oryzae* under solid and submerged fermentation using agrowaste.

Materials and Methods

Sample collection

Soil samples were collected from the site where the fruit wastes were dumped and placed in sterile polythene bag. Then, the soil samples were transferred to the laboratory.

Isolation and identification of pectinolytic micro fungi

The filamentous fungi were isolated from decaying agro waste matter using a basal medium containing commercial pectin as the sole carbon source. The solid medium contained (gL-1): 10.0 pectin, 3.0 peptone, 2.0 yeast extract, 0.5 KCL, 0.5 $MgSo_4.7H_2O$, 0.01 $MnSo_4.5H2o$, and 2.0 $(NH_4)_2SO_4$ and 20.0 agar. A supplement of 0.1% ampicillin and 1.0 mlL-1 of trace mineral solution (composed of 0.04 g $CuSO_4.5H_20$, 0.08 g $FeSO_4$, 0.08 g Na_2MoO_4, 0.8 g $ZnSO_4$, 0.04 G $Na_2B_4O_7$, and 0.008 g $MnSO_4$ in 100 ml of distilled water) was added to the medium. P^H value was adjusted to 5.5 before autoclaving at 121^0C for 15 min. Inoculated plates were incubated at 30^0C for 5 to 7 days. Pure cultures were obtained by repeated sub-culturing on PDA plates and maintained at 40^oC on PDA slants. The isolate fungal colonies were identified based on their colonial and morphological characters.

Screening of fungal isolates for pectinolytic activity

The isolates were cultivated on modified Czapek-Dox agar, with commercial citrus pectin as the sole carbon source, and screened for pectinolytic activity by a modified plate method [7]. The clearance zone formed around the colonies was determined using Potassium iodide – iodine solution (5.0 g potassium iodide and 1.0 g iodine in 330 ml of distilled water).

Pectinase production with different agro-wastes

Three of the fungal isolates, *Aspergillus oryzae, Aspergillus flavus* and *Rhizopus oryzae* were studied for pectinase production using the different agro wastes, including, pineapple peel, lemon peel, saw dust, cassava waste and wheat bran, as the sole carbon sources. The modified Czapek-Dox media contained 10 g L-1 of the different

agro wastes as sole carbon a source. One hundred milliliter (100ml) of the sterile medium (pH 5.5) was inoculated with 2.0 ml of spore suspension (106 spores ml-1) of the organism and incubated at 30^0C with agitations (100 Osci min-1) using shaker.Culture was harvested at 24-h intervals by centrifugation at 4000 g for 10 min. The culture supernatants were used as the crude enzyme sources.

Fermentation Process

Solid state fermentation Vs submerged fermentation

The comparative study of the solid state fermentation (SSF) and a submerged fermentation (SmF) was carried out using Rice bran as the sole carbon source. The medium for SmF contained per liter of distilled water: Rice bran 10.0 g, $(NH_4)_2SO_4$ 6.0 g, K_2HPO_4 6.0g, KH_2PO_4 6.0 g and $MgSO_4.7H_2O$ 0.1 g. pH value was adjusted to 5.5 before inoculating with 2.0 ml of spore suspension containing 106 cells ml-1 of each fungus. The medium for SSF contained 5 g of Rice bran and 10 ml of the mineral salt solution: 6.0 g $(NH_4)_2SO_4$, 6.0 g K_2HPO_4, 6 g KH_2PO_4 and 0.1 g $MgSO_4.7H_2O$. Cultures were incubated at 30^0C with agitations at 100 Osci/min using shaker and harvested at 48 h by centrifugation at 4000 g for 10 min. The SSF culture was harvested after addition of 100 ml of sodium acetate buffer (0.05 M, pH 5.5) to the content of the flask. The culture supernatant were used as the crude enzyme source [8].

Determination of proteins pectinase activity

The protein content of the crude enzyme was determined by the Folinciocalteau method of Lowry *et al*., (1951) using Bovine Serum Albumin(BSA) as standard

Enzyme assay

3 ml of diluted culture filtrate was taken as enzyme source into the test tube and 1 ml of DNS acid reagent was added. The mixture was kept in boiling water bath for 5 minutes until the yellow colour developed. Then the tubes were cooled and 5 ml of distilled water was added to the mixture. Absorbance change (OD) was measured Spectrophotometrically at 540 nm. Enzyme activity was determined using citrus pectin as substrate. The reaction mixture, containing equal amounts of 1% pectin prepared in sodium acetate buffer (0.05 M; Ph 5.5) and suitably diluted crude enzyme, was incubated at 50^0C in water bath for 30 min. The reaction was stopped with 1.0 ml dinitrosalicyclic acid solution[8] after which the mixture was boiled for 10 min and cooled. The colour was read at 540 nm using a spectrophotometer. The amount of reducing sugar released was quantified using galacturonic acid as standard.The specific enzymatic activity (IU mg-1 protein) was calculated as the amount of enzyme required release one micromole (1µmol) equivalent of galacturonic acid per minute mg protein under the assay condition. The results are expressed as Mean ± standard error of mean.[10]

Effect of incubation time, temperature, pH

The fungal isolate was subjected to different culture conditions to drive the optimum conditions for pectinase production. Growth and pectinase production were estimated at regular intervals(12 hrs, 24 hrs, 36 hrs, 48 hrs, 72 hrs, 96 hrs 120 hrs& 144 hrs) and selected temperature (25^oC, 35^oC, 40^oC, 45^oC, 50^oC, & 55^oC) pH(3.5, 4.5, 5.5, 6.5). All the experiments were carried out in 500

ml Erlenmayer flask containing 100 ml of basal medium [11].

Results and Discussion

In the present study, fungal species were isolated from the agro waste dumped soil. This identified fungal species *A.flavus* was used for the production of pectinase by solid and submerged fermentation using various substrate such as Pine apple, Lemon peel, Rice bran, Saw dust, Cassava waste.

Isolation and identification of fungal species

From the 10 isolates, three fungal colonies were identified using routine morphological tests. The observed characteristics were compared with manual of soil fungi. The identified fungal species namely *A. oryzae, A. flavus* and *R. oryzae* (Table.1).

Screening of fungal isolates for pectinase production

The selected fungal isolates were further screened for pectinolytic activity by CzapekDox agar plate method.*A. flavus, A. oryzae* cultures had a zone of clearance above 3 mm, *R. oryzae* cultures had a zone of clearance between 1 and 2 mm. The three isolates had a zone of clearance around the colonies.

Formulation of fermentation medium and culture conditions

The selected fungal isolates were tested for its pectinase activity by using different substrate namely Pine apple, Lemon peel, Rice bran, Saw dust and Cassava waste. The result indicated that enzyme activity was higher in Pine apple and Lemon peel.

Several conditions of agro waste were used in the formulation of cost effective fermentation media. This economically cheap media designed by using valueless waste materials, plays important role in bringing out the almost behaviour of the fungus (Table-2).

Comparison of Solid State Fermentation with Submerged Fermentation

Duplicate flasks containing the optimum growth medium for pectin lyase production were used. It was observed that Solid State Fermentation is better then Submerged Fermentation. Solid State Fermentation showed more enzyme production as compared to Submerged Fermentation (Table-3).

Of the three selected isolates, *A. flavus* high amount of pectinase produced than the other two fungi. So it was subjected to optimization of culture conditions.

Optimization of Pectinase production

p^H Vs pectinase production

The pectinase production was optimized by supplementation using different pH range medium from 35 to 6.5. In solid state fermentation maximum pectinase production was *A.flavus* noticed pH 5.5 in Pine apple (0.60±0.080IU/ml) respectively. (Table-4: Figure-1)

Temperature Vs Pectinase Prodution

The pectinase production was optimized for Pine apple using different temperature range of medium from 25^0C- 55^0C. In solid state fermentation maximum pectinase production was noticed*A.flavus* at 35^0C (0.65±0.070 IU/ml) respectively (Table-5;Figure-2).

Table.1 Characteristics of fungal isolates

Fungal species	Characteristics
A.oryzae	Green to olive brown to black with slight yellowish mycelium was observed.
A.flavus	Lime yellow colourmycelial growth. The conidia give various green yellow to green shades to the spore heads and dark sclerotia.
R.oryzae	Brownish grey to blackish grey depending on the amount of sporulation.

Table.2 Comparison of SSF and SmF under optimum conditions

Types of Fermentation	Pectin lyase activity (IU/ml)
	Mean
SSF	15.55
SmF	10.57

Table.3 Effect of substrate on pectinase production by Mixed culture

Substrate	Enzyme activity (IU/ml)
Pine apple	0.79
Lemon	0.66
Cassava	0.51
Rice bran	0.55
Saw dust	0.48

Table.4 Effect of Incubation period on Pectinase activity by *A. flavus*

Substrate	Enzyme activity (IU/ml)		
	4^{th} day	6^{th} day	7^{th} day
Pine apple	0.65±0.030	0.68±0.010	0.79±0.070
Lemon	0.66±0.050	0.65±0.060	0.70±0.019
Cassava	0.54±0.008	0.50±0.004	0.65±0.019
Rice bran	0.54±0.05	0.50±0.061	0.56±0.090
Saw dust	0.60±0.080	0.58±0.020	0.62±0.071

Table.5 Effect of pH on pectinase activity by *A. flavus*

Substrate	Enzyme activity (IU/ml)		
	pH 4.5	pH 5	pH 5.5
Pine apple	0.50±0.030	0.59±0.010	0.60±0.080
Lemon	0.52±0.050	0.50±0.060	0.55±0.019
Cassava	0.48±0.008	0.46±0.004	0.57±0.019
Rice bran	0.55±0.050	0.45±0.061	0.46±0.090
Saw dust	0.49±0.080	0.52±0.020	0.58±0.071

Table.6 Effect of temperature on Pectinase activity by *A. flavus*

Substrate	Enzyme activity (IU/ml)		
	$15^{O}C$	$25^{O}C$	$35^{O}C$
Pine apple	0.55±0.030	0.60±0.010	0.65±0.070
Lemon	0.54±0.050	0.55±0.060	0.50±0.019
Cassava	0.45±0.008	0.48±0.004	0.50±0.019
Rice bran	0.59±0.05	0.45±0.061	0.49±0.090
Saw dust	0.45±0.080	0.55±0.020	0.62±0.071

Figure.1 Effect of pH on pectinace activity by *A.flavus*

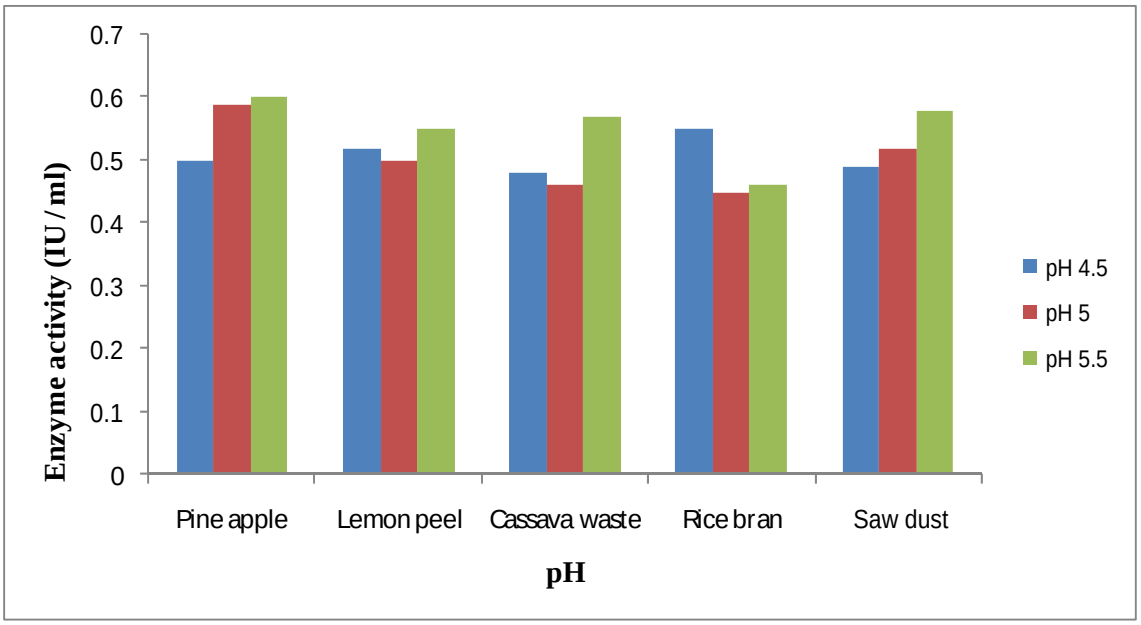


Figure.2 Effect of Temperature on Pectinase activity by *A.flavus*

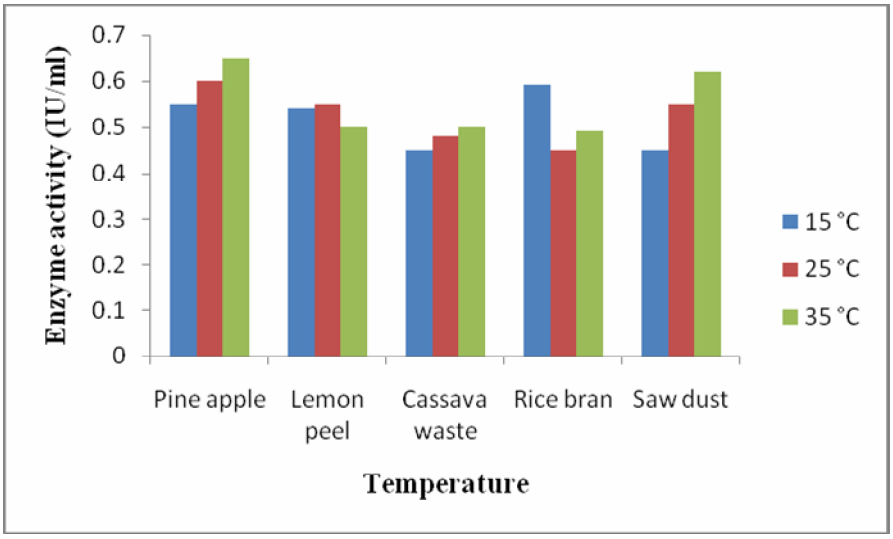
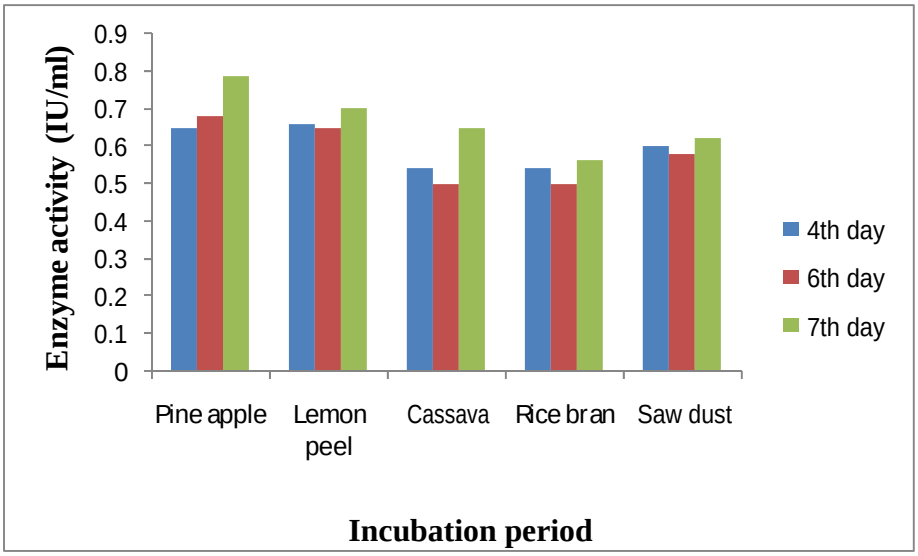


Figure.3 Effect of Incubation period on pectinase activity by*A.flavus*



Incubation Time Vs Pectinase Production

The pectinase production was optimized for Pine apple using different periods range of medium from 12 hrs to 144 hrs. In solid state fermentation maximum Pectinase Production was noticed *A.flavus* maximum pectinase production at 7th day 0.79±0.070 IU/ml (Table-6; Figure-3).

In the present study, pectinase activity were estimate in the isolated fungal strains namely *A. flavus*, *A. oryzae*, *R. oryzae*. Three species namely *A. flavus*, *A. oryzae*, *R. oryzae*isolated and identified from agrowaste. The identified fungal species were subjected to screening for pectinase production. In the present study, among five substrates screened, Pine apple was found to be the most significant for

pectinase production by all the three organism *A. flavus, A. oryzae,* and *R. oryzae.* Similar results was also reported by for pectinase and polygalacturonase production by *Penicilliumsp.*,[7]

Maximum pectinase production by *A. flavus* was observed at an incubation temperature of 35^{o}C. At higher temperature, the yield was less. This might be due to the denaturation of the enzyme. In other work, it was reported as 25^{o}C for effective leaching of the α-amylase from the fermented bran[12]. The incubation temperature has a profound effect on the enzyme yield and the duration of enzyme synthesis phase.[13] Most of the fungi investigated for pectinase production showed optimum growth in the range of 15 to 35^{o}C [14].

The pH regulates the growth and the synthesis of extracellular enzyme by several microorganisms particularly fungal strains[15]. The ideal pH for pectinase production by *A. flavus* has been found to be 5.5. These results were comparable with the findings of[16] for the pectinase prodution by *A. oryzae*. The optimum pH of 6 for *A. niger* was reported using citrus peel and sugarcane bagasse, respectively for the production of pectinase in SSF.[17] The period of fermentation depends up on the nature of medium, fermenting organisms, concentration of nutrients and the process physiological conditions.[12]

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