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Production of Pectin Lyase by fungi isolated from agro waste

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ABSTRACT

In the present investigation totally 25 fungal species alone were isolated during the study period from three different samples such as dead organic, fruit and vegetable soil samples. The fungal cultures were identified by standard manual and kept on PDA medium.The present data demonstrate that maximum enzyme activity was exhibitedin *A.niger* (5.2 IU/ml)and *P.citrinum* (4.8 IU/ml)in both plate assay and quantification assay. The minimum enzyme activity was expressed as *Curvularia lunata, Rhizoctonia solani; Aspergillus flavus*, *A.lunchensis* and *A.nidulans.* Pectin lyase production was increased by optimizing various fermentation parameters. The enzyme activity was assayed over a pH range of 5.0 to 9.0. The enzyme activity was assayed at different temperature (15-35°C).This was agreed with present findings i.e., 6.1 IU/ml of pectin lyase activity was recorded after 9 days of incubation by *A.niger* and *P.citrinum* was 6.9 IU/ml maximum amount of protein was also recorded after 9 days of incubation.In the present study, partial purification of Pectin lyase enzyme from*A.niger* and *P.citrinum* was carried out by dialysis followed by Ammonium sulphate precipitation method.

Keywords: Agro waste, *Aspargillus Niger, Penicillium Citrinum,* and Pectin lyase.

INTRODUCTION

Microorganisms have various advantages and can be used for enzymes production at higher level. Pectinolytic enzymes have great biotechnological potential and can be employed in many important industrial processes (TewariR, 2005 & ZhongW.H., 2005). *Aspergillus niger* belongs to Ascomycota group of fungi, genus *Aspergillus*. It is an opportunistically infectious microbe to human being and well adapted to environmental changes. The optimization of production of pectin lyase by *A. niger* and then its characterization was done only after partial purification (SamsonR.A, 2001&Baker S.E., 2006).Pectinolytic enzymes can be produced in large amount by microorganisms, using citrus peel as a substrate because it contains considerable quantity of pectin. It works as inducer for the synthesis of pectinolytic enzymes by microbial systems (DhillonS., 2004). These enzymes have the ability to degrade and chemically modify pectin (Zhang J., 2006).

Pectinases are common lyemployed injuice, textile, paper and pulp industries. These enzymes catalyzed the conversion of complex polysaccharides into simpler molecules like galacturonic acids (GieseE.C, 2008). These have wide industrial applications like oil extraction, tea extraction, juice clarification and waste water treatments (Botella C, 2007 &Mohnen D, 2008).In the present work, the enzyme producing fungi was isolated from waste soil

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samples of Erode district and it's used to produce pectin lyase enzymeby using solid state fermentation process.

MATERIALS AND METHODS

Sample collection

The samples were collected from agro waste soil samples in Erode District. Three different soil samples viz., vegetable waste soil sample (VS) from Gobichettipalayam Vegetable market, fruit degrading soil sample (FS) from Gobichettipalayam fruits market and dead organic matter sample (DOS) from Household material were randomly collected, just 1cm below the soil surface and digested plant litters were aseptically transferred to sterile polythene bags.

Isolation of fungi from soil

Ten gram of the soil sample was taken in a 250 ml conical flask containing 100ml sterile distilled water. The flask was shaken on an electric shaker to get a homogenous suspension and different dilution of the soil sample viz., 10^{-1}, 10^{-2} and 10^{-3} were prepared by transferring serially about 10 ml of the soil suspension to 90 ml of sterile distilled water. One ml of 10^{-3} dilutions was plated in Petri dishes containing potato dextrose agar medium. The pH of the medium was adjusted to 5.6 and streptomycin sulphate (100 mg/L^{-2}) was added to the media to prevent the bacterial growth. The plates were incubated at 25±2°C for five days and the fungal colonies appearing on the potato dextrose agar media were picked and isolated. Purified strains were obtained by streaking repeated in PDA medium and observed under compound microscopy. The cultures were characterized to genus level on the basis of macroscopic (colonial morphology, colour and appearance, shape) and microscopic characters (septation of mycelium, shape, diameter and texture of conidia)(Warkcup J.H, 1958). Population of fungi was calculated by using following standard formula:

$$Percentage\ of\ contribution = \frac{Mean.no.of\ propagules\ Indilution\ plate}{Weight\ of\ the\ dry\ soil} \times Dilution\ factor$$

Identification of fungi

The fungi were identified by using standard manual, such as Manual of Soil Fungi (Gillman J.C., 1957), Dematiaceous Hyphomycetes (Ellis M.B., 1971), More Dematiaceous Hyphomycetes (Ellis M.B., 1976),Hyphomycetes(Subramanian C.V., 1971).The fungion PDA plates were identified based on characteristic features of colony morphology and reproductive structural characteristics like sporangiospore position, columella and sporeshape (Pitt J.I., 1979, Domsch K.A, 1980,Ellis M.B, 1971 and NagamaniA, 2006). Freshly grown mycelia with small amount of medium was stained with LCB and examined under stereobinocular microscope. Identified fungal cultures were maintained on the PDA medium in the laboratory by using conventional methods and sub cultured at regular intervals.

Screening for the Pectin lyase Enzyme Production

Pectinlyase Screening Agar Medium (PSAM) is used as selective medium for the growth of microbes which release pectin. After the preparation and sterilization of the media, block of fungal culture were made using cork borer from the master plate and transferred to Petri dish with PSAM medium. The plates were stored at room temperature in inverted position for proper microbial growth. After incubation, the plates were screened for the identification of zones of hydrolysis which indicating the positive or negative results for pectinlyase production (TewariR, 2005).

Pectin lyase enzyme production by liquid state fermentation

Fungal isolates were placed in a basal medium used for pectinase production, the medium consist of 0.3%$(NH_4)_2HPO_4$;0.2%KH_2PO_4;0.01%K_2HPO_4;0.01% $MgSO_4$; 2.5% Pectin. The type of fermentation used submerged liquid state fermentation. The culture was incubated for 10-12 days at 25°C (ZhongW.H., 2005).

Pectin lyase enzyme assay

Assay of pectin lyase was performed by the method described by Preiss and Ash well. 0.5mLofenzyme was incubated for 1h with 0.5mL of 0.5% pectin and 1 mL of 50 mM Tris HCl buffer (pH 8) and 1 mL of 0.2 mM $CaCl_2$. After 1 h, absorbance was measured at 548 nm against blank. One unit of pectin lyase activity was defined as "the amount of enzyme present in 1 mL of original enzyme solution which released 1 M of galacturonic acid in 1 min (Preiss J and Ashwell G, 1963). Enzyme activity was calculated by using the following formula:

$$\text{Enzyme activity (IU/ml)} = \frac{Absorbance\ of\ enzyme\ solution \times standard\ factor}{Time\ of\ incubation}$$

Whereas,

$$\text{Standard factor} = \frac{\text{Concentration (}\mu\text{ M/ml) of standard}}{\text{---}}$$

Absorbance

Optimization of pectinlyase enzyme production by fungi

Optimization of pectinlyase enzyme production by fungi was performed in Erlenmeyer flask method to determine the effect of pH, temperature, incubation period and nutrient sources on pectinlyase enzyme production was assayed. For optimization studies various parameters that influence the pectinlyase enzyme production, eight days of fungal spores of *Aspergillusniger* and *Penicilliumcitrinum* were inoculated into100ml of selective medium in 250 ml Erlenmeyer flask (Goyal N, 2003).

Effect of pH

Fungi inoculated in culture medium were incubated at different pH (5.0, 6.0, 7.0,8.0 and 9.0).The pH of the solution was altered using 0.1NHCl and 0.1NNaOH solution. After incubation period, pectinlyase enzyme production was determined (Goyal N, 2003).

Effect of temperature

Aspergillus niger and *Penicillium citrinum* were used to determine the effectiveness of different temperatures such as 15, 30 and 45°C. Fungi inoculated culture medium was incubated at temperature of 15,30and45°Cforoneweek.After incubation period, the culture medium was filtered and analysed for pectinlyase enzyme production (Goyal N, 2003).

Effect of incubation period

The optimum incubation period required by *A.niger* and *P.citrinum* was determined for the period of 3,6 and 9 days at room temperature. After each end point of 3, 6, 9 and 12 days, the culture medium was filtered and analyzed to determining the pectin lyase enzyme production (Goyal N, 2003).

Characterization of Pectin lyase enzyme activity

Ammonium sulphate fractionation

Ammonium sulphate was added to the extract with stirring to bring the saturation 30% and after standing it for 4 hours at 4^{o}C, the precipitate and supernatant was determined. Additional ammonium sulphate was added to the supernatant to bring the saturation to 60% and the mixture was left overnight. The supernatant was further subjected to saturation 90% the precipitates were collected, dissolved in distilled water and the solution was dialyzed against water for 48 hours using dialysis bag. The dialyzed enzymatic fractions were subjected to protein and Pectin lyase activity was determined (Whelan H, 1969).

Dialysis

The dialysis bag was sterilized. The bag was cut into pieces of convenient length (10-20cm) and boiled for 10 minutes in a large volume of 2% (w/v) sodium bicarbonate solution which removes salt from pores. On rinsing the bag thoroughly with distilled water and boiled for 10 minutes of followed by boiling in 1mm EDTA pH 8.0 which removes heavy metal ions form pores again boiled at 100^{o}C for 10 minutes. Bag was allowed to cool and used for dialysis. Filled the bag with enzyme and tied tightly with band on both the sides and kept immersed in buffer solution (Tris Hcl buffer pH 7) for 5 hours (OgunbanwoS.T, 2003).

RESULTS AND DISCUSSION

Isolation of fungi from dead organic matter

In dead organic matter sample, totally 33 fungal colonies were recorded, 25 in fruit sample and 20 in vegetable sample. Totally 14 species belonging to 6 genera were observed in dead organic matter sample, 9 species belonging to 5 genera were observed in fruit sample and 9 species belonging to 3 genera were observed in vegetable sample. Maximum number of *A.niger* were observed in DOS and fruit sample.13coloniesof*A.niger*indeadorganicmattersampleand 8coloniesinfruit sample. Maximum number of *P.citrinum* (17 colonies) were recorded in vegetable sample. The minimum number of *A.terreus* in dead organic matter samples; *Chetomium globosum* in fruit samples and *A.niger* in vegetable samples were recorded.

Screening of pectinolytic enzyme from fungal isolates

In the primary screening studies of pectinolytic enzyme, totally 25 fungal isolates were oberseved and selected. Among that 21 fungal isolates had the ability to produce pectinolytic enzymes but 4 fungal isolates gave negative results. Belonging to the 21 fungal isolates *A.niger* and *P.citrinum* were showed excellent activities for pectinlyase enzyme production purpose (Table-1 and Figure-1).

Table 1: Screening of pectin lyase enzyme from fungal isolates

S.No	Name of the fungi	Pectin lyase activity
1	*Aspergillus awamori*	++
2	*A. candidus*	++
3	*A. clavatus*	++
4	*A. flavus*	++
5	*A. fumigates*	++
6	*A. luchuensis*	++
7	*A. nidulans*	+
8	*A. niger*	+++
9	*A. sydowi*	+
10	*A. sulphureus*	+
11	*A. versicolor*	++
12	*A. terreus*	++
13	*A. terricola*	++
14	*A. variecolor*	+
15	*Bipolaris oryzae*	++
16	*Colletotrichum falcatum*	++
17	*Curvularia lunata*	++
18	*Chaetomium globosum*	++
19	*Fusarium chlamydosporum*	++
20	*F.oxysporum*	++
21	*Penicillium citrinum*	+++
22	*P.chrysogenum*	++
23	*Rhizoctonia solani*	++
24	*Trichoderma harzianum*	++
25	*T.viride*	++

(+) low, (++) moderate, (+++) highly recommended

Figure 1: Screening of pectin lyase enzyme producing fungi

Quantification assay of pectinolytic enzymes from fungi

Secondary screening quantification assay was done for 25 fungal isolates. Maximum amount of enzyme activity was recorded in *A.niger* (5.2 IU/ml) and4.8 IU/ml recorded in *P.citrinum*. Minimum enzyme production was observed in *A.sydowi*(0.1IU/ml).Remaining*Aspergillus*sp.producedonly amoderateamountof pectin lyase enzyme.

Effect of pH and temperature optimized for pectinlyase enzymes by potential fungi

pH and temperature were optimized for pectin lyase enzyme production of *A.niger* 1.67 IU/ml of enzyme produced in pH 6, minimum amount (0.2 IU/ml) was

observed in pH 5. In temperature optimization, 30ºC was suitable for maximum enzyme production (1.78 IU/ml). But 20ºC was not suitable for enzyme production, because enzyme production was very low (1.4 IU/ml) in this temperature. *P.citrinum* produced 0.76IU/ml of enzymein pH6, which was the maximum and minimum amount (0.1 IU/ml) was recorded in pH 5. 1.0 IU/ml of enzyme was recorded at 35 ºC, and minimum (0.16 IU/ml) was recorded at 20ºC (Table-2; Figure-2 and 3).

Table2: Effect of pH and temperature optimized for pectinlyase enzyme by potential fungi

S.No	Parameters	Concentrations	*A.niger* (IU/ml)	*P.citrinum* (IU/ml)
1	pH	**5**	0.2	0.1
		6	1.67	0.76
		7	1.61	0.72
		8	1.16	0.13
		9	0.9	0.8
2	Temperature (^{0}C)	**15**	1.1	0.9
		20	1.5	0.16
		25	1.74	0.6
		30	1.78	0.74
		35	1.49	1.0

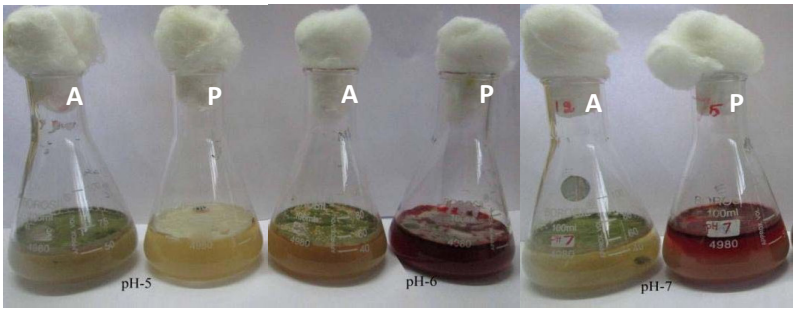
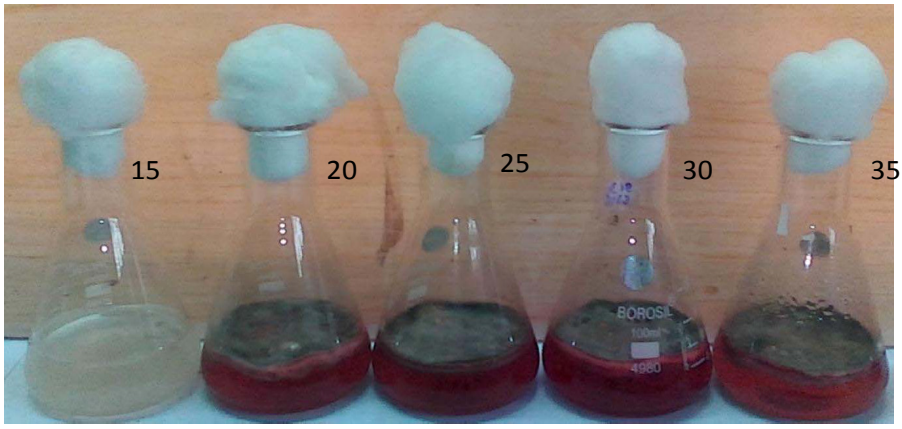
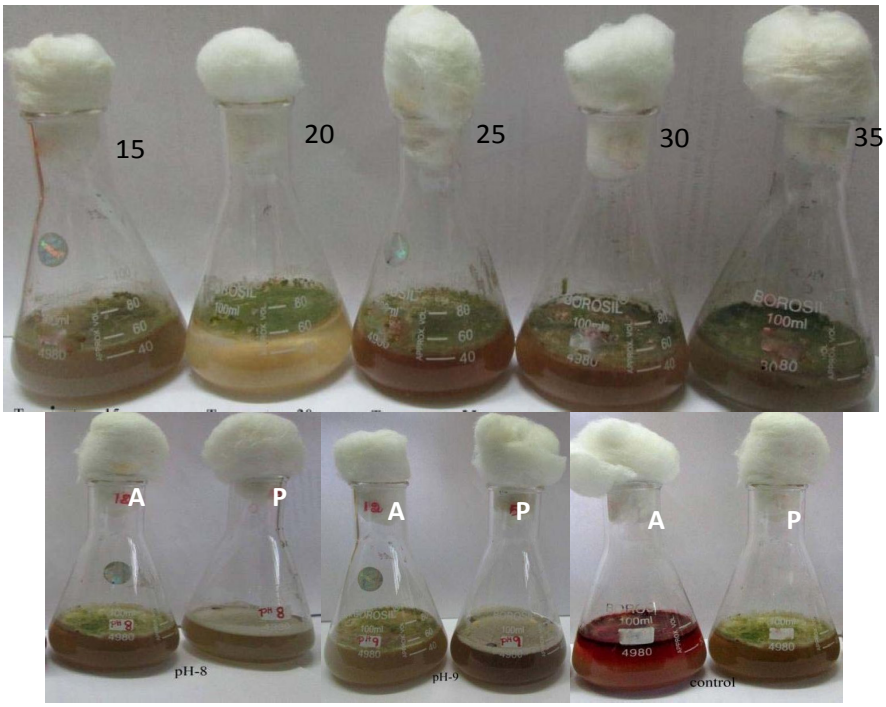


Figure2:EffectofpHonthegrowthof*A.niger*and*P.citrinum*after7daysof incubation



A.niger



P.citrinum

Figure3: Effect of Temperature (°C) on the growth of *A.niger* and *P.citrinum*after 7 days of incubation

Effect of incubation period on production of pectinlyase activity by potential fungi

Various incubation periods were tested for pectinlyase activity by *A.niger* and *P.citrinum* .6.1IU/ml pectinlyase activity was recorded after 9 days of incubation by *A.niger,* whereas 6.9 IU/ml was recorded after 9 days of incubation by *P.citrinum*. Minimum activity was recorded after 3 days of incubation *A.niger* (2.6 IU/ml) and *P.citrinum* (2.7 IU/ml). Maximum amount of protein was recorded after 9 days of incubation by both species. 10.3 IU/ml protein was recorded by *P. citrinum* and 8.5 IU/ml protein by *A.niger* after 9 days of incubation. After 3 days of incubation period, protein production was very low.

Purification of Pectinlyase enzyme from *A.niger*

Partial purification of Pectinlyase enzyme was carried out by dialysis followed by ammonium sulphate precipitation method. The specific activity of the crude sample was found to be 49.0IU/mg of dialyzed sample and ammonium sulphate precipitated sample was found to be 61.09IU/mg and 78.0IU/mg respectively.

Purification of Pectinlyase enzyme from *P.citrinum*

Partial purification of Pectin lyase enzyme was carried out by dialysis followed by ammonium sulphate precipitation method. The specific activity of the crude sample was found to be 56.7IU/mg of dialysed sample and ammonium sulphate precipitated sample was found to be 65.0IU/mg and 42.8IU/mg respectively.

CONCLUSION

The overall investigation concluded that the *A.niger* and *P.citrinum* were found to utilize agricultural waste and byproducts for enzyme production and also *A.niger* potential fungus had the ability to produce high level of pectinlyase and protein with s pecified parameters and compared with other fungus *P.citrinum*. This study revealed that the potential of utilizing agricultural wastes provided cost effective and eco-friendly method for pectinlyase production on large scale.

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