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PRODUCTION OF PECTINOLYTIC ENZYMES BY FUNGI CAUSING BIODEGRADATION OF SUGARCANE BAGASSE

Botany

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ABSTRACT

Background: Sugarcane Bagasse is the fibrous remains of sugarcane stalk left after extracting the juice. It is a lingo-cellulosic waste used for the production of enzymes, amino acids, drugs, ethanol and single cell protein. Bagasses are subjected to microbial attack that causes degradation by way of decomposition. They are composed of cellulose, pectin, hemicelluloses and lignin. **Aim:** The aim of the present study is to evaluate the production of extracellular Pectinase enzymes by some filamentous fungi growing on sugarcane Bagasse. **Methods:** The pectinase enzymes viz. Pectin lyase (PNL), and Exo-polygalacturonase (PG) were assayed in crude enzyme extract produced by some filamentous fungi growing on sugarcane Bagasse. **RESULTS:** Eleven Pectinolytic fungi viz. Penicillium wortmanni, P. islandicum, P. citrinum, Aspergillus niger, A. flavus, A. fumigatus, A. ustus, Alternaria alternata, Fusarium oxysporum, Verticillium sp. and Curvularia sp. were isolated from sugarcane Bagasse These showed different diameters of pectin degradation in solid medium, and tested for pectin hydrolysis by plate assay at pH 6.0. The isolated fungal species were classified into three categories viz. very good producers (+++) of pectin depolymerizing enzyme when presented clear halos around colonies of at least 1.5 cm, good producers (++) when halos were at least 1.0 cm, weak producers (+) when halos were at least 0.5 cm and poor producers when no clear halo zones were observed after 24 and 48 hours of growth. 4 % pectin caused enhanced production of Exo-polygalacturonase in comparison to 2 % pectin. Media supplemented with 2 % pectin + 2 % galactose and 2 % pectin + 2 % polygalacturonic acid also caused enhanced Exo-polygalacturonase activity. 4 % pectin in media in submerged fermentation (SmF) was found to be most suitable carbon source for Exo-polygalacturonase production by present fungi. The PNL activity increased with increased concentration of pectin in the media. The maximum PNL activity was observed when media were supplemented with 5% pectin. **CONCLUSIONS:** The Bagasse fungi showed highest PNL and PG activities in submerged fermentation when the media were supplemented with pectin as substrate.

KEYWORDS

Sugarcane Bagasse, Pectin lyase, Polygalacturonase, Filamentous fungi, Submerged Fermentation.

INTRODUCTION

Sugarcane Bagasse is the fibrous remains of cane stalk left over after crushing and extracting juice from sugarcane (Saccharum officinarum). Sugarcane is mostly cultivated in tropical countries (FAOSTAT, 2019)[1]. It is used in sugar mills and alcohol mills. Sugarcane cannot be consumed by these mills entirely. About 30% of pulpy fibrous residue is left after being utilized in these mills (Michel et al., 2013)[2]. These residues are called sugarcane Bagasse (Pandey et al., 2000)[3]. Sugarcane Bagasse is used in various industries viz., paper and pulp, feedstock, biofuel, etc (Hern andez-Salas et al., 2009; Loh et al., 2013)[4,5]. Sugarcane Bagasse is a lingo-cellulosic matter (W. Sn, 2008)[6] and is generally considered as waste (Michel et al., 2013)[2]. Bagasse is used for the production of enzymes, amino acids, drugs, ethanol, and single cell protein as animal feed after cultivating it with a large number of bacteria, fungi and yeasts (Eriksson, 1990)[7].

Bagasses are subjected to microbial attack that causes deterioration by way of decomposition. Cellulose, hemicelluloses, pectin and lignin are the basic constituents of sugarcane bagasses. Under aerobic conditions a wide range of saprophytic fungi such as Chaetomium, Stachybotrys, Trichoderma, Penicillium, Cladosporium, Memnoniella, Alternaria, Aspergillus, Thermoascus etc. colonize the sugarcane bagasses. Growth of cellulolytic, hemicellulolytic, pectinolytic and lignolytic microorganisms help in the decomposition of bagasses causing weakening of fibres, perforation and even complete destruction of bagasses into its constituent residues.

Pectinolytic enzymes are used in food processing industries to yield yields, improve liquefaction and clarification (Alkorta et al., 1997; Kashyap et al., 2001; Pilnik and Voragen, 1993)[7-10]. Hydrolysis of pectin is brought about by pectinases which are classified into three main groups:

- Pectinesterases: catalyzing de-esterification of the methoxyl group of pectin.
- Depolymerising hydrolytic enzymes (including polymethyl-galacturonases and polygalacturonases) catalyzing the hydrolytic cleavage of 1, 4-glycosidic linkages.
- Lyases catalyzing the cleavage of glycosidic bonds by transelimination.

In the present investigation, the conditions necessary for the growth and production of extracellular pectinases i.e. Exo-polygalacturonase (PG) and Pectin lyases (PNL) by some Sugarcane Bagasse filamentous fungi have been evaluated.

MATERIALS AND METHODS

About 25 gm of sugarcane Bagasse fibers infested with fungi was transferred to a sterile flask containing 100 mL of distilled water. The flasks were then shaken vigorously. The sample was the diluted to 10^{-6} dilution with sterile distilled water and was used as inoculum. 1 mL of this dilution was pipette out into the medium, and plated into Petri dish of diameter 10 cm containing a medium which consisted of pectin (20 g/L), ammonium sulphate (2 g/L), magnesium sulphate (2 g/L), potassium phosphate (0.2 g/L) and agar (2.0 g/L).

Fungi exhibiting Pectinolytic activity were selected on colonies for 24 and 48 hours at 30^0C employing ruthenium dye (0.5 %) on their colonies as suggested by McKay (1988)[11]. The Pectinolytic activity of fungi was determined by measuring the diameter of clear zone and the size of the colonies in millimeter.

The Pectinolytic fungi were grown separately on medium containing KH_2PO_4 (2 g/L), $(NH_4)_2SO_4$ (1.4 g/L), $MgSO_4$ (0.3 g/L), $CaCl_2$ (0.01 %). This medium was supplemented with 1 mL of a solution containing $MnSO_4$ (1.6 mg) and $ZnSO_4$ (1.4 mg). The medium contained 2 % w/v citrus pectin as the sole source of carbon. The culture was grown at 30^0C in an incubator for seven days. The pectinase activity was assayed on submerged fermentation after seven days of incubation.

The production of pectin lyase (PNL) by Pectinolytic fungi was performed in 250 mL Erlenmeyer flasks holding 50 mL of the medium. The initial spore density was 1 X 10^7 units/mL. The flasks were incubated at 30^0C for seven days without shaking. The different concentrations of pectin ranging from 1 – 5 % w/v were added to the medium for the ability to produce PNL production by fungi. Similarly, Exo-polygalacturonase (PG) production was assayed in flasks holding 50 mL of the medium. The initial spore density was 1 X 10^8 units/mL. the flasks were incubated at 30^0C for 48 hours in an orbital shaker (120 rpm). The Exo-polygalacturonase activity (PG) was assayed in media

containing two different concentrations of pectin (2 % and 4 % w/v).The total protein was estimated in the culture filtrate of Pectinolytic fungi by the method of Bradford (1976)[12], with bovine serum albumin (BSA) as the standard.

The pectinase activity was assayed in supernatant culture fluids (crude enzyme) after 24 and 48 hours of incubation by the method suggested by Okafor et al., (2010)[13]. One unit of pectinase is defined as the amount of enzyme which liberates 1 µmole of reducing sugar per mL per minute under assay conditions. Similarly, PNL activity was measured by method suggested by Gattas et al., (1999)[14]. The reaction mixture consisted of 1.75 mL of 0.1 M citrate buffer at pH 5.0, 1.75 mL of 1 % w/v) citrus pectin and 0.7 mL of culture filtrate. The culture was incubated at 30^{0}C for 10 minutes and started by adding the culture filtrate. One unit of PNL was defined as the amount of enzyme catalyzes the liberation of 1 µ mole of 4, 5- unsaturated oligogalacturonides per unit volume of the culture filtrate per minute under assay conditions. The absorbance was measured at 235 nm in spectrophotometer and the enzyme activity was measured by following formula:

$$\text{Enzyme activity (µmole/mL.min)} = \frac{ABS.V1.10^3}{OC.V2.\epsilon\, time}$$

Where, ABS was calculated as the difference between sample absorbance and white values; V1 = 3.5 mL (solution volume added to the sample volume); V2 = 0.7 mL (volume of enzyme used); Optical path is 1 cm; ϵ is the molar extinction coefficient 5500 $M^{-1}cm^{-1}$ (Yadav et al., 2009)[15]; time is 10 minutes.

Exo-polygalacturonase (PG) was assayed by determining the reducing sugars released during assay condition by the method suggested by Miller (1959)[16] using the reagent dinitro salicylic acid. The reaction mixture contained 2.50 mL of 0.2 M citrate buffer at pH 5.0, 2.50 mL of 1 % galacturonic acid (w/v) and 0.05 mL of culture filtrate. The reaction was carried out at temperature 30^{0}C for 15 minutes and started by adding the culture filtrate. One unit of PG activity was expressed as the amount of enzyme that catalyzes the release of 1 µmole of galacturonic acid per unit volume of culture filtrate per minute under assay conditions.

All chemicals and reagents used were of AR grade. Experiments were conducted in replicates of three and data were analyzed by measuring standard error of mean at 5 % level of significance.

RESULTS AND DISCUSSION

Eleven Pectinolytic fungi were isolated viz. Penicillium wortmanni, P. islandicum, P. citrinum, Aspergillus niger, A. flavus, A. fumigatus, A. ustus, Alternaria alternate, Fusarium oxysporum, Verticillium sp. and Curvularia sp. These showed different diameters of pectin degradation in solid medium, and tested for pectin hydrolysis by plate assay at pH 6.0. The isolated fungal species were classified into three categories viz. very good producers (+++) of pectin depolymerizing enzyme when presented clear halos around colonies of at least 1.5 cm, good producers (++) when halos were at least 1.0 cm, weak producers (+) when halos were at least 0.5 cm and poor producers when no clear halo zones were observed after 24 and 48 hours of growth. The Pectinolytic activity of the present fungal isolates is illustrated in Table-1.

Table-1: Pectinolytic Activity Of The Present Fungal Isolates Showing Different Rate Of Pectinolytic Activity As Indicated By Halo Diameter/Colony Diameter On Pectin Agar Medium Using Red Ruthenium Dye As Test Indicator.

Fungal isolates	Color zone/colony size in mm		Pectinolytic activity*(%)
	After 24 hours	After 48 hours	
Penicillium wortmanni	++	++	75.5 ±0.65
Penicillium islandicum	++	++	74.6 ±0.61
Penicillium citrinum	+++	+++	95.5 ±0.70
Aspergillus niger	+++	+++	99.7 ±0.72
Aspergillus flavus	+++	+++	96.8 ±0.67
Aspergillus fumigatus	+++	+++	95.5 ±0.65
Aspergillus ustus	++	++	74.6 ±0.65
Alternaria alternata	+	++	70.5 ±0.70
Fusarium oxysporum	++	+++	90.6 ±0.67
Verticillium sp	+	++	65.0 ±0.45
Curvularia sp.	+	++	64.5 ±0.51

*Medium contained 2 % pectin after 7 days of growth

The different fungal isolates showed different diameters of pectin degradation in on solid media, and were tested for pectin hydrolysis by plate assay at pH 6.0. The fungal isolates were classified into three categories viz. very good producers of pectin depolymerizing enzymes (+++), which produced clear halos around colonies of at least 1.5 cm, good producers (++) which produces halos of diameter of about 1.0 cm and weak producers (+) which produced halos of diameter 0.5 cm after 48 hours of incubation. Among eleven Pectinolytic fungi, Penicillium citrinum, Aspergillus niger, A. flavus, A. fumigatus showed strong pectin hydrolytic activity (+++) whose pectinolytic activities were 95.6 ±0.70, 99.7 ±0.72, 96.8 ±0.67 and 95.5 ±0.65 percent respectively (Table-1). The Pectinolytic activity of Fusarium oxysporum was also high after 48 hours of growth and exhibited 90.6 ±0.76 % of Pectinolytic activity. Penicillium wortmanni, P. islandicum, Aspergillus ustus, Alternaria alternata, Verticillium sp. and Curvularia sp. showed moderate pectinolytic activity. The present findings gain support from the work of Reddy and Screeramulu (2012)[17] who observed strong pectinase activity of Aspergillus flavus by measuring the clear zones formed around the colonies stained with ruthenium red. Sandhya and Kurup (2013)[18] also observed more or less similar results under solid state and submerged fermentation condition.

The effect of different carbon sources viz. pectin, galactose and polygalacturonic acid on the production of Exo-polygalacturonase by present fungi was studied in submerged fermentation system at 30^{0}C for 48 hours at pH 4.5. The results have been presented in Table-2. The results revealed that 4 % pectin caused enhanced production of Exo-polygalacturonase in comparison to 2 % pectin. Media supplemented with 2 % pectin + 2 % galactose and 2 % pectin + 2 % polygalacturonic acid also caused enhanced Exo-polygalacturonase activity but lower than in media with 4 % pectin. 4 % pectin in media in submerged fermentation (SmF) was found to be most suitable carbon source for Exo-polygalacturonase production by present fungi (Table-2). Penicillium citrinum, Aspergillus niger, A. flavus, A. fumigatus, A. ustus, Alternaria alternata, Fusarium oxysporum, Verticillium sp. and Curvularia sp. showed 100 % polygalacturonase activity in at 30^{0}C and pH 4.5 after 48 hours of fermentation when media were supplemented with 4 % pectin. The present findings gain support from the work of Solis-Pereira et al., (1993)[19] who also found that the exo- and endo-pectinase activities of Aspergillus niger increased with increasing the concentration of pectin in submerged and solid fermentation. More or less similar results for Exo-polygalacturonase have also observed by Gattas et al., (2003)[20], Pashova et al., (1999)[21] and Aquilar and Huitron (1987)[22].

Table-2: Effect Of Carbon Source On Exo-polygalacturonase Activity (%) By Fungi

Fungi	Percent Exo-polygalacturonase activity in different concentration of carbon sources			
	2 % pectin	4% pectin	2% pectin + 2% galactose	2% pectin + 2% polygalacturonic acid
Penicillium wortmanni	48.7	84.7	69.5	72.5
P. islandicum	50.8	86.7	74.5	77.5
P. citrinum	55.6	100	86.5	92.6
Aspergillus niger	60.5	100	87.6	91.8
A. flavus	60.7	100	88.5	93.5
A. fumigatus	60.4	100	85.6	90.2
A. ustus	50.5	83.5	71.5	76.7
Alternaria alternata	49.5	82.6	70.6	77.3
Fusarium oxysporum	52.7	100	85.7	91.5
Verticillium sp.	48.6	80.5	71.6	76.5
Curvularia sp.	45.5	80.6	70.5	73.4

The extracellular crude enzyme of each of the eleven fungal isolates was assayed for pectin lyase (PNL) activity which was expressed in µmole/mL.min after seven days of incubation. The PNL activity has been presented in Table-3. The results revealed that the PNL activity was greatly influenced by the concentration of pectin in the culture media. The PNL activity increased with increased concentration of pectin in the media. The maximum PNL activity was observed when media were supplemented with 5% pectin. At this concentration Penicillium wortmanni, P. islandicum, P. citrinum, Aspergillus niger, A. flavus, A. fumigatus, A. ustus, Alternaria alternata, Fusarium oxysporum, Verticillium and Curvularia showed maximum PNL activity which was 52.64, 55.65, 53.45, 61.35, 63.42, 60.25, 50.25, 46.65, 64.45, 48.27, and 46.28 µmole/mL.min respectively. Above 5%

concentration of pectin all the present fungal isolates showed decline in the level of PNL activity. A 5% concentration of pectin was found to be optimum for enhanced activity of PNL. Alana et al., (1990)[23] have studied the production of PNL by Penicillium italicum CECT 2294 and found the same results. Maisa et al., (2019)[24] have also found that the PNL activity in the culture media increased from the medium containing 1% pectin to reach a maximum at 5% concentration by Aspergillus sp. 391 strains and Aspergillus niger.

Pectic substance and their degradable products are inducers for the synthesis of Pectinolytic enzymes (Tahara et al., 1972; McMillan et al., 1992)[25,26]. Camargo et al., (2005)[27] found that the pectin lyase activity of Aspergillus sp. 0492 increased on increased concentration of inducers (pectic substance). They found pectin lyase activity of 11.3 U/mL after 96 hours of fermentation when media were supplemented with 4% (w/v) citrus waste with mineral salts.

Table-3: PNL Activity Of Pectinolytic Fungi In µmole/ml.min In Various Concentrations Of Pectin

Fungi	Concentration of Pectin (Percent)						
	1	2	3	4	5	6	7
Penicillium wortmanni	28.45	33.15	42.27	45.55	52.65	47.26	43.25
P. islandicum	29.25	34.35	45.05	48.75	55.65	50.15	47.31
P. citrinum	28.75	33.36	42.15	46.25	53.45	49.65	46.35
Aspergillus niger	30.45	37.65	54.45	57.47	61.35	52.25	50.15
A. flavus	31.75	40.45	55.65	59.27	63.42	50.15	49.75
A. fumigatus	30.15	36.25	52.55	53.45	60.25	51.35	48.65
A. ustus	28.35	35.40	43.15	45.25	50.25	43.25	40.25
Alternaria alternata	26.25	29.35	37.15	41.35	46.65	40.15	37.25
Fusarium oxysporum	29.65	38.25	55.75	60.25	64.45	53.15	29.25
Verticillium	27.25	31.35	40.15	44.35	48.27	43.25	38.35
Curvularia	26.35	29.17	38.25	42.15	46.28	40.15	36.25

CONCLUSIONS

The highest PNL activity was obtained in assay with 5% (w/v) pectin by Aspergillus niger, A. flavus, A. fumigatus and Fusarium oxysporum after seven days of fermentation, while the highest Exo-polygalacturonase (PG) activities were observed by the same fungi (100%) with 4 % pectin. The optimization of Pectinolytic enzymes and their kinetics will require further investigations at the scientific level.

Conflict Of Interest: Authors declare no conflict of interest directly or indirectly.

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