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Production of Polygalacturonase by Some Fruit Rot Fungi and its use in Fruit Juice Extraction and Clarification

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Abstract

Pectinase breaks the pectin, which is a polysaccharide substrate found in cell wall-middle lamella of fruits and vegetables. About 50 fungi were isolated from infected fruits and tested for their Polygalacturonase (PG) production, among which *Rhizoctonia solani, Mucor racemosus, Penicillium citrinum, Rhizopus stolanifer, Aspergillus flavus* and *A. niger* were proved to be maximum PG Producers. Among these six fungi, *Aspergillus flavus* and *A. niger* were responsible for production of maximum juice and hence selected. Exo PG and endo PG production was assayed *in vitro* and *in vivo* and i*n vitro* studies showed that the maximum endo PG was noticed in *Aspergillus flavus* during its 14 days of incubation (52.6 RVU. In the production of exo PG, maximum was noticed in *Mucor racemosus* during its 21 days of incubation (420 µg/ml). *In vivo* studies showed that in grape fruit, the maximum endo PG was 66.7 RVU in *Aspergillus niger* after its four days of incubation while in the exo PG activity, the maximum (995 µg/ml) was noticed in *A. niger* during four days of incubation in orange while the least production was noticed in *R. solani* (260 µg/ml) after its six days of incubation period. The PG was most useful in fruit juice extraction and clarification.

Keywords: Pectin, Pectinases; Polygalacturonase, Fruit Juice technology.

Introduction

Pectin found in primary cell wall and middle lamella of fruits and vegetables (Favela *et al*., 2006). Pectin contain α1, 4 linked D-galactosyluronic residues. Three pectic polysaccharides, homogalacturonan, rhamno galacturonan-I and substituted galacturonans have been isolated from primary plant cell walls (Ridely *et al*., 2001; Sharma and Satyanarayana 2006). Pectinases are group of enzymes that attack pectin and depolymerize it by hydrolysis and trans elimination as well as by de-esterification reaction, which hydrolysis the ester bond between Carboxyl and methyl group of pectin (Satyanarayana and Panda 2003). Pectinases are classified based on their preferred substrate (pectin or pectic acid) and on the degradation mechanism (Trans elimination or hydrolysis) and the type of cleavage (random-endo or terminal - exo (Kashyap *et al*., 2001). Polygalacturonases belongs to family of glycosyl hydrolases polygalacturonases (PG) divided into three groups. Endo-PG randomly attack the (1-4) α-glycosidic linkages of the polysaccharide chain producing a number of galacturonic acid oligomers, Exo-PG types I hydrolyses D-galacturonic acid from the non-reducing end and Exo-PG-II releases di-galacturonate from the non-reducing end of polygalacturonic acid. Endo and Exo type I were identified in fungi, but only two exo PG type II were isolated until now from the bacteria (Sheychick *et al*., 1997).

The commercial preparations of pectinases are produced mainly from fungi (Palanivelu 2006). Microbial pectinases account for 25% of the global food enzyme sale (Jayani *et al*., 2005). Fruit industries generates gallons of wastes and dumping in nature causes pollution problems, such problems can be solved by exploiting these agro wastes for pectinase production (Preeti *et al*., 2015) Fungal pectinases used in the food industry for the production of fruit juices to increase the fruit juice and in clarification (Henriksson *et al*., 1999). When compared to the commercial products the use of the crude preparations resulted in similar clarification of apple juice (Ivana *et al*., 2011). Poly galucturonase was successfully decolourised, purified by activated charcoal with significantly high purification fold and recovery (Tapati and Rintu 2014). The present study is oriented to isolate the fungal organisms capable of producing high quantities of PG and its application in fruit juice technology.

Materials and Methods

The infected fruits of Sapota (*Achras sapota*), orange (*Citrus sinensis*) and grapes (*Vitis vinifera*) were collected carefully in the separate poly ethylene bags from the fruit markets of Kumarpally, Hanamkonda, Kazipet and Warangal areas and carried to the laboratory.

The infected portions of fruits indicate post-harvest fungal or bacterial diseases. The fruit was surface sterilized with 0.1% mercuric chloride for one minute and washed thoroughly and a small transitional portions of infected and healthy regions was separated and transferred on to the agar slants of Asthana and Hawker's Agar medium (A) (Glucose-5g, KNO_3-3.5g, KH_2PO_4 –1.75G, $MgSo_4$ – 0.75 g, Agar –20g) and incubated at room temperature for 3 days. After incubation period the emerged hyphal tips were picked up and transferred to Asthana and Hawker's Agar (A) slants in aseptic condition and incubated them at room temperature for one week to obtain pure cultures. About 50 fungal species were isolated and identified from different fruits and among these the dominant cultures occurred very frequently were selected for the present study on (*in vivo* and *in vitro*) pectinase production. The important six fungal species used in the present study are viz., *Rhizoctonia solani, Penicillium citrinum, Mucor racemosus, Rizhopus stolanifer, Aspergillus flavus* and *A. niger*.

Extraction of Pectinases from fruits (*in vivo)*:

Healthy fruits were inoculated with six fruit rot fungi viz., *Rhizoctonia solani, Penicillium citrinum, Mucor racemosus, Rizhopus stolanifer, Aspergillus flavus* and *A. niger* by giving a small incision on the surface of fruit and sterilized cotton was wrapped on the infected part and after an adhesive tape was fixed in aseptic condition. The fruits were incubated 2-8 days and after incubation period, 20 grams of infected portion of the fruits was separated in aseptic condition and cut the tissue into small pieces of 1-2 centimeters. The cut pieces were transferred into waring blender and added 100 ml 0.15M NaCl and macerated. This was filtered through two layers of cheese cloth and transferred the filtrate to centrifuge tubes and centrifuged at 2000 rpm. for 30 minutes and supernatant was separated into culture flask and this filtrate was used as an enzyme source. Few drops of toluene were added and enzyme was stored at 4°C in case when enzyme was not immediately used.

Extraction of Pectinases from pathogen (*in vitro*):

Pectic acid or polygalacturonic acid supplemented Asthana and Hawker's medium (Pectic acid-5g, KNO_3-3.5g, KH_2PO_4 –1.75g, $MgSo_4$ – 0.75 g) was prepared and 100 ml of broth was transferred into 250 ml conical flasks. Aseptically the flasks were inoculated with 2 ml of spore suspension or 7 mm mycelial disc from the growing margin of 5 days old culture of respective fungi. The inoculated flasks were incubated at 27°C for 7, 14 and 21 days. After the incubation period the contents were fixed through whatman No.1 filter paper and mycelial mat was separated. The filtrate was centrifuged at 2000 rpm for 30 minutes and supernatant was taken as enzyme source. Few drops of toluene were added to the enzyme, when enzyme assay was delayed.

Assay of Exo-Polyglaacturonase (Exo-PG):

Exo PG was estimated by DiNitrosalisylic acid method proposed by Miller (1959).

3.5 ml of 0.5% pectic acid solution was taken into a test tube and added with one ml of citrate buffer followed by 0.5 ml enzyme (fruit extract/broth culture) and 3-4 drops of toluene and incubated at 30°C for six hours. After incubation period, 0.2 ml of aliquot was withdrawn from the above reaction mixture and added

with three ml of DNS reagent. The contents were thoroughly mixed and kept for 15 minutes in a hot water bath. Two ml of 20% sodium potassium tartarate solution, was added to the test-tube while, it was hot and cooled the tube under running tap water. The developed brown colour was read at 575 nm by spectrophotometer. A blank was prepared with the same procedure by using a heat killed enzyme.

Reducing sugars liberated were calculated with the help of standard curve drawn for glucose. Poly galacturonase (PG) activity was expressed in terms of mg of reducing groups (as µg/ml) liberated in 6 hours.

Assay of Endo Poly Galacturonase (Endo-PG):

Wood's viscometric method (1955) was followed to estimate the exo-PG. Pectic acid (0.5%) was prepared by dissolving 0.5g of Pectic acid in 100 ml citrate (buffer (pH 5.5) and heated at 50°C - 60°C and the contents were blended for 3 minutes and filtered through two layers of cheese cloth. The pH was adjusted to 5.2 by the addition of 1N HCl or 1N NaoH using pH meter.

The reaction mixture for the estimation of endo PG was with Pectic acid (0.5%) substrate citrate buffer (pH 5.5) and enzyme source in 4:1:2 ratio. The contents were mixed in a 100 ml beaker and immediately transferred into an Oswald viscometer. The efflux time of the contents were determined with the help of a stop clock at the initial time. The contents were incubated for three hours in the viscometer at room temperature and reduction in the efflux time of the contents in the viscometer were calculated after every 10 minutes.

The percentage of reduction in viscosity was calculated by applying the following formula.

$$V = \frac{ETo - ETt}{ETo - ETw} x100$$

Where, V = Percent Loss of viscosity

ETo = flow time of water in seconds at zero time.
ETt = flow time of reaction mixture at 't' intervals.
ETw= flow time of distilled water.

The Relative Enzyme Activity (REA) of endo PG was calculated by dividing 1000 with time required for 50% loss of viscosity (t_{50}) and expressed the activity in Relative Viscometric units (RVU).

$$REA = 1000 / t50$$

Results and Discussion

Poly galacturonases (PG) are hydrolytic enzymes with endo and exo activities. In *in vitro* studies the endo PG was maximum in *A. flavus* during its 14 days of incubation (52.6 RVU). Among the six fungi *R. solani* was responsible for least enzyme secretion (33.3 RVU) in 14 days of incubation, while the exo PG was maximum in *Mucor racemosus* during its 21 days incubation (420 µg/ml). The next best organism was *R. solani* (400 µg/ml), while *A. flavus* was responsible for least enzyme secretion (260 µg/ml) after its 21 days of incubation. (Table 1 & Fig – 1).

Table 1 Endopolygalacturonase (Endo PG) of six fruit rot fungi on Asthana & Hawker's medium supplemented with pectic acid after 7, 14 and 21 days of incubation

Fungi	Relative Enzyme Activity (REA) in Relative Visco metric units(RVU)		
	7	14	21
Rhizoctonia solani	22.5	33.3	33.3
Penicillium citrinum	22.5	40.0	33.3
Mucor racemosus	33.3	50.0	16.7
Rhizopus stolanifer	11.1	44.6	33.4
Aspergillus flavus	33.3	52.6	33.4
A. niger.	44.6	40.0	22.2

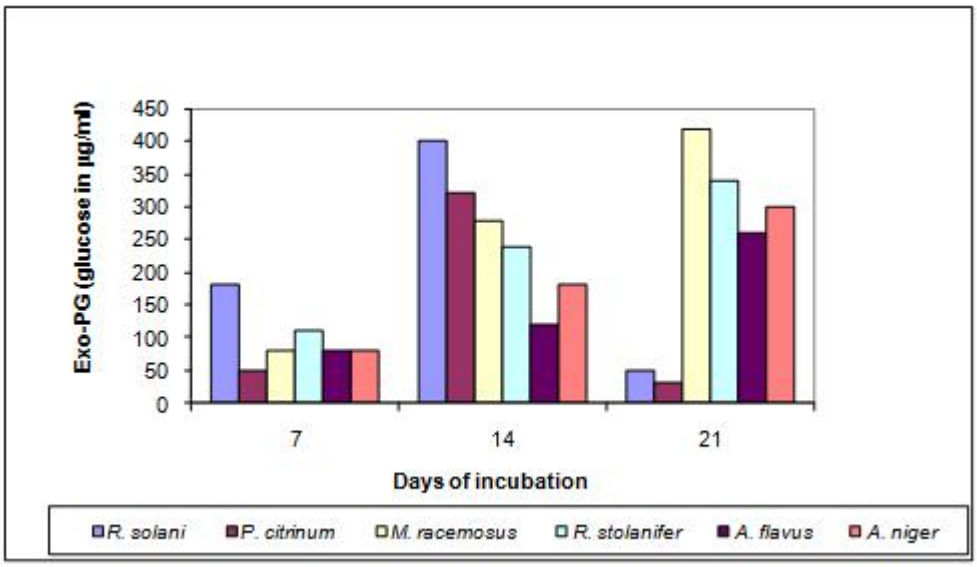

Fig. 1 : Exo Polygalaturonase (Exo-PG) activity of six fruit rot fungi on Ashthana & Hawkers medium supplemented with pectic acid after 7, 14 and 21 days in incubation

The endo PG production *in vivo* was studied in three fruits (Table – 2).

In Sapota fruit highest range of endo PG was recorded again in six days (533 RVU) by *R. stolanifer* and *A. flavus.* In the remaining fungi the endo PG activity was quite moderate and ranged in between 42.7 to 50.0 RVU. In the grape fruit the maximum activity was 66.6 RVU by four fungi i.e., *A. flavus, A. niger, R. stolanifer, M. racemosus.* While *P. citrinum* (50.0 RVU) and *R.solani* (49.5 RVU) were showed their moderate activity after 4 and 6 days respectively. In orange fruit the maximum endo PG was (66.6 RVU) noticed in *M. racemosus, R. stolanifer* and *P.citrinum* after their 4 days of incubation. The remaining three fungi i.e., *R. solani, A. flavus* and *A.niger,* secreted moderate (33.3 RVU) quantities of enzyme.

Table - 2: Endo-polygalacturonase (Endo-PG) activity of six-fruit-rot fungi on three fruits after 2,4,6,8 days of incubation

Fungi	Relative Enzyme Activity *											
	Sapota				Grapes				Orange			
	2	4	6	8	2	4	6	8	2	4	6	8
Rhizoctonia solani	32.4	44.4	42.7	40.0	21.5	44.4	49.5	4.7	11.1	33.3	33.3	33.3
Penicillium citrinum	32.4	38.9	42.7	40.0	28.6	50.0	32.4	3.4	33.3	66.6	33.3	22.2
Mucor racemosus	22.3	42.5	50.0	47.6	31.5	66.6	42.5	3.4	33.3	66.6	66.6	11.1
Rhizopus stolanifer	22.3	41.5	53.3	50.0	31.7	66.6	41.5	4.0	33.3	66.6	66.6	22.2
Aspergillus flavus	19.4	40.5	53.3	40.0	30.8	66.6	40.5	4.0	16.7	33.3	22.2	14.3
A. niger	17.5	38.9	44.4	40.0	31.6	66.6	40.6	5.6	14.3	33.3	22.2	16.7

* Activity expressed in Relative Viscometric Units

The exo PG production in three fruits was studied and presented in Fig. 2.

In sapota fruit the highest exo PG (995 µg/ml) was noticed in *Mucor racemosus* after its 4 days of incubation and the less production rate was recorded in *A.niger* (590 µg/ml) after its 6 days incubation. In the grape fruit the highest production of exo-PG was recorded in *R. solani* (710 µg/ml) after its 8 days of incubation and the production rate was recorded in *A. flavus* (480 µg/ml) after its 8 days of incubation. In the orange fruit the maximum exo-PG (995 µg/ml) was noticed in *A. niger* during four days of incubation, while the least production of exo-PG was noticed in *R. solani* (260 µg/ml) after its six days of incubation period.

The fruit juice in orange was 82 ml and 86 ml by *A. flavus* and *A. niger* respectively while, it was extracted 84 ml with commercial enzyme. The sapota juice with water extraction was 59 ml, while with *A. flavus* and *A. niger* it was 76 and 74 ml respectively. The commercial pectinase enzyme secreted the fruit juice by 75 ml. Grapes were very much prone for fungal pectinase attack and 64 ml of fruit juice was extracted with water, while 71 and 70 ml were extracted with *A. flavus* and *A. niger* enzymes and 74 ml of fruit juice was separated with commercial pectinase enzyme.

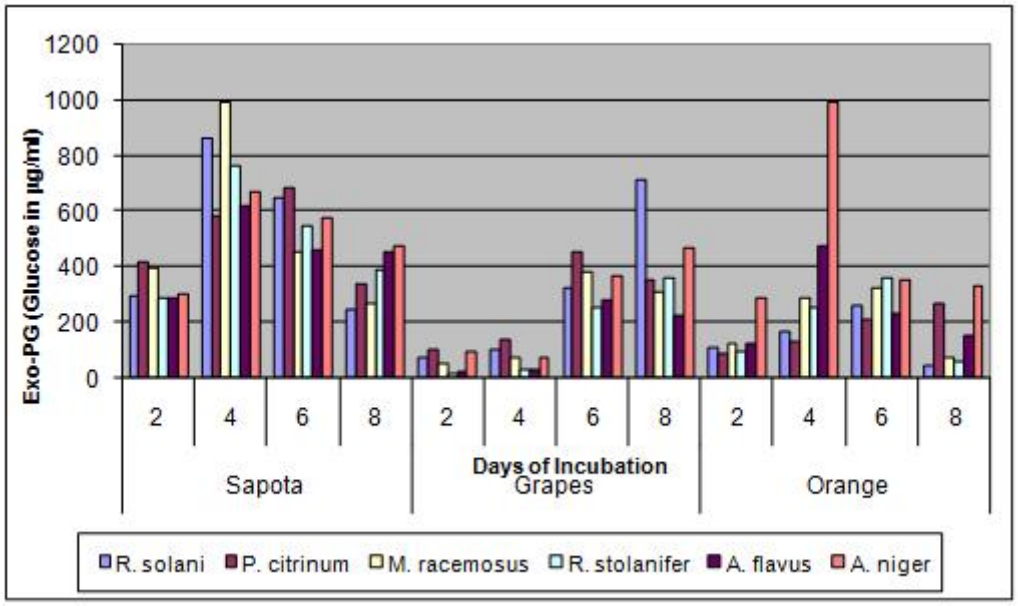

Fig. 2: Exo-Poly Galacturonase (Exo-PG) activity of six-fruit rot fungi on three fruits after 2, 4, 6, 8 days of incubation

It was clearly noted that, the enzyme extraction from these two fungal strains is very effective and replaces the application of costly commercially enzyme in clarification and extraction.

Similar to the present observations, many researchers (Kester and Visser 1990; Solis *et al*., 1993; Rao *et al*., 1996; Kavitha and Umesh 2000; Blandino *et al*., 2002; Niture and Panth 2004) reported the PG activities *in vivo* and *in vitro* under the fungal pathogenesis. Pectin de-polymerization during fruit ripening has been shown to be largely due to pectinolytic enzymes, including PG and these enzymes are not primary determinants of softening although participation in texture changes during the later stages of ripening. Pectin depolymerization differs significantly between various fruit types, notably avocado, tomato, apples and Mango (Muda *et al*., 1995). When orange peel alone was used as carbon source, a better production of PG was observed (Preethi *et al* 2015). Cotoras and Silva (2005) observed the hydrolytic oxidative enzyme activity by the *Botrytis cinerea* isolates in solid state fermentation (SSF) on tomato peels.

PG production was also observed by Favela *et al*. (2006) by the species of *Aspergillus, Penicillium, Rhizopus, Fusarium* grown on a solid medium containing wheat bran and orange pulp resulted in a threefold increase in comparison with production obtained in submerged fermentation. The clarification of grape juice was greatly affected by *Aspergillus niger* PG and clarified juices were stable upon storage at both 4 and 25^{0}C (Mohsen *et al*., 2009). In the present work Asthana & Hawker's medium supplemented with polyglacturonic acid or fruit juice as source of nutrients increased the endo PG/exo PG activity.

Devries *et al*., (2000) studied that the genus *Aspergillus* is widely used for the production of plant cell wall, polysaccharide degrading enzymes. The experimental extract enzyme (EE) produced by *Aspergillus niger* and *A.oryzae* showed results statistically similar or superior to those obtained with the commercial enzyme preparations (Ivana *et al* 2013). In the present studies also the genus *Aspergillus* with two species, *A. flavus* and *A. niger* produced substantial amounts of endo and exo-PG. It is generally agreed that the optimum medium for the enhanced production of extra-cellular pectinase is that containing pectic material as an inducer (Crotti *et al*., 1999).

According to Martin *et al*., (2004) polygalacturonases are produced substantially by *Penicillium* sp. and *Aspergillus* sp. using orange fruit, orange bagasse and sugarcane bagasse which served as a good source for PG Production for many fruit rot fungi. The highest production levels were obtained by *A. sojae* using sugar beet as a carbon source with yields of 1111 and 449 U/g for exo-PG and endo-PG respectively (Marco *et al*., 2015). The average PG production was 36.5 U/ml, which was enhanced by 4.1 fold, the production from mango peel waste with optimized submerged fermentation using *Aspergillus foetidus* (Sudheer *et al*., 2014).

The maximum production of PG (500 U/ml) was reported in *Aspergillus japanicus* when the fungus was grown on liquid medium containing pectin and galactose (Teixeira *et al*., 2000). It has been shown that 0.2% glucose along with 1% pectin is essential for the maximum production of PG from *Fusarium moniliforme*, whereas, higher glucose concentration decreased the production (Niture *et al*., 2006). Satyanarayana and Panda (2003) reported the role of different carbon sources and other fermentation parameters for the production of pectinase including PG. During the pathogenesis of tomato and cauliflower plants, *Fusarium moniliforme* produced more PG In tomato host tissue which is having slightly acidic cell sap (pH 6.8) compared with cauliflower host tissue with cell sap pH 7.7 (Niture 2008). When the fermentation medium supplemented with different carbon sources like glucose, sucrose and galacturonic acid and agricultural by products such as wheat bran (Kavitha and Umesh 2000), rice bran and sugar cane bagasse (Solis *et al*., 1993) with the beet peel, citrus peel (Siessere and Said 1989) and orange peel (Niture and Panth 2004) were used as additional pectin sources and enhanced the production of PG. Favela *et al*. (2006) reviewed the comparative production of PG from different fungi in submerged culture and in solid state fermentation.

Fruit juices contain colloids that are mainly polysaccharides (Pectin, cellulose, lignin and starch), Proteins and metals (Vaillant *et al*., 2001). One of the major problems encountered in the preparation of fruit juice is cloudiness primarily due to the presence of pectin. The cloudiness that pectin cause is difficult to remove except by enzymatic depectinization (Liew *et al*., 2007). Several studies have reported on depectimization using enzymatic treatment such as pectinase which could affectively clarify the fruit juices (Kashyap *et al*., 2001; Vaillant *et al*., 2001; Alvarez *et al*., 1998; Ceci and Lozana 1998) especially for tropical fruit juices (Lee *et al*., 2006; Sin *et al*., 2006; Grebecheva *et al*., 2007). Recently Garg *et al* (2016) reported the eco-friendly nature of the petinases in degrading/decomposing the materials available in the surrounding.

Conclusion

The characterization and purification of the pectinases secreted by these two potential fungal strains *A. niger* and *A. flavus* and their application in fruit juice technology shall pave the way for commercialization and exploitation of these enzymes in fruit juice and related technologies.

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