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Pectic Acid Lyase (PAL) Production By Six Fruit Rot Fungi: Role In Fruit Juice Technology

Aruri Suryam*, MD. Raffiyuddin and M.A. Singaracharya
Department of Microbiology, Kakatiya University,
Warangal (Urban), Telangana State, India – 506 009.
*Corresponding author: *arurisuryam69@gmail.com*

Abstract

The exo Pectic Acid Lyase (exo PAL) production in Asthana and Hawker's medium (*in vitro*) and six infected fruits (*in vivo*) by six fruit rot fungi was assayed. The maximum exo PAL (27 U/ml) was observed in *Penicillium citrinum* after 14 days of incubation, while in the infected tomato fruits, the highest enzyme secretion (52 U/ml) was noticed after two days in *Aspergillus flavus* and after four days in *Rhizopus stolanifer* and *A.niger*. These four fungal strains can be successfully used in fruit juice extraction and clarification.

Keywords: Pectin, pectinases, pectic acid lyase, fruit rot fungi, fruit juice technology.

Introduction

Pectin found in primary cell wall and middle lamella of fruits and vegetables (Favela *et al*, 2006). Fruit ripening involves changes in the composition and organization of pectin, hemicelluloses and cellulose poly saccharides of the cell wall, which take place as a co-ordianted series of assembly and disassembly steps (Lous *et al*, 2007). Pectins are rarely simple polymers of poly galacturonic acid (PGA), but normally contains neutral sugars, notably D-galactose, L-arabinose, L-rhamnose, D-xylose and D-apiose (Ridely *et al*, 2001). Pectinases are group of enzymes that attack pectin and de-polymerase it by hydrolysis and trans-elimination as well as by de-esterification reaction, which hydrolyse the ester bonds between carboxyl and methyl groups of pectin (Satyanarayana and Panda, 2003). Pectinases are classified based on their preferred substrate (pectin/pectic acid or poly galacturonic acid) and on the degradation mechanism (trans elimination or hydrolysis) and the type of cleavage (random-endo or terminal-exo (Kashyap *et al*, 2001). Pectic enzymes are classified into two main groups namely de-esterifying enzymes (pectin-esterases) and chain splitting enzymes (de-polymerases). The depolymerisation split the glycosidic bonds of their preferred substrate either by hydrolysis (hydrolases) or by B-elimination (lyases) (Ward and Moo-young, 1989). Pectic acid lyases (PAL) are most active towards pectic acid or pectin with low degree of esterification (20-50%) (Whitaker, 1990). Abundent quantities of PAL are reported in variety of fungi (Satyanarayana and Kumar, 2005). The commercial preparations of pectinases are produced mainly from fungi, especially *Aspergillus niger* (Favela *et al*, 2005). Microbial pectinases account for 25% of global food enzyme sale (Jayani *et al*, 2005). Food Processing enzymes including

pectinases account for 45 percent of enzyme usage (Sangeetha *et al*, 2005). Microbially derived pectinases find more use due to their advantage over plant and animal derived pectinases (Chaudhri and Suneetha, 2012). Fungal pectinases used in the food industry to increase the fruit juice extraction and clarification (Henriksson *et al*, 1999). Mechanical crushing of pectin rich fruit yields a fruit juice with high viscocity, which remains bound to the pulp in the form of a jellified mass. It is difficult to the extract of this juice by pressing or using other mechanical methods, with the addition of pectinases fruit juice is easily obtained and with higher yields (Tapre and Jain, 2014). The production of fruit and vegetable juices is important both from the human health and commercial stand points (Harsh *et al*, 2014). Waste material from agro industrial processing may be used as the substrate for microbial growth in SSF or SMF (Viviani *et al*, 2010).

The present study was aimed to analyze the six fruit rot fungi, for their highest yields of pectinases and given a right direction for selection of fungal pectinases in fruit juice technology. The selected strains, which were thoroughly investigated and critically monitored, are the safe candidates for the application in fruit juice technology.

Materials and Methods

The infected fruits of sapota (*Achras sapota*), orange (*Citrus sinensis*), grapes (*Vitis vinifera*), mango (*Mangifera indica*), apple (*Malus pumila*) and tomato (*Lycopersicon esculentum*) were collected carefully in the separate poly ethylene bags from the fruit markets of Hanamakonda, Kazipet and Warangal areas in Telangana State and carried to the laboratory.

The fruit was surface sterilized with 0.1% mercuric chloride for one minute and washed thoroughly and a small transitional portion 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, $MgSO_4$, $7H_2O$-0.75g, 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 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 which 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 : *Rhizoctonia solani, Penicillium citrinum, Mucor racemosus, Rhizopus stolanifer, Aspergillus flavus and A.niger.*

(a) **Extraction of pectinases from fruits (*in vivo*)**:
Healthy fruits were inoculated with six fruit rot fungi viz., *Rhizoctonia solani, Penicillium citrinum, Mucor racemosus, Rhizopus 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 for 4-16 days in case of apples and 2-8 days for all other fruits. After incubation period, 20 g of infected portion of the fruits was separated in aseptic condition and cut into small pieces of 1-2 cm. The cut pieces were transferred into Waring Blender and added 100 ml 0.15M NaCl and macerated for two minutes. 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 the enzyme source. A few drops of toluene was added and enzyme was stored at 4°C in case when not immediately used.

(b) **Extraction of pectinases from pathogen (*in vitro*)**: Pectic acid supplemented Asthana and Hawker's medium (Pectic acid-5g, KNO_3 – 3.5g, KH_2PO_4-1.75g, $MgSO_4$, $7H_2O$-0.75g) 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 mycelia 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 filtered through Whatman No.1 filter paper and mycelia mat was separated. The filtrate was centrifuged at 2000 rpm for 30 minutes and supernatant was taken as enzyme source. A few drops of toluene was added to the enzyme and stored at 4°C, when enzyme assay was delayed.

(c) **Assay of exo-pectic acid lyase (PAL)**: The exo-PAL was assayed in *vivo* and in *vitro* by the method suggested by Sherwood (1967). The reaction mixture in exo-PAL consisted of substrate (pectic acid or poly galacturonic acid-1%), tris-HCl buffer (dissolved 24.2 g of tris in 100 ml distilled water to prepare solution-A; 16.1 ml of HCl upto 100 ml with distilled water to prepare solution-B and mixed 50 ml of solution A with 23.2 ml of solution-B to get tris-HCl buffer of pH 8.)

and enzyme in 4:1:2 ratio. The contents were taken into a test-tube and incubated at 30°C for 3 hours. After incubation the contents were added with one ml of 0.5 N NaOH and $ZnSO_4$ (9%) to stop the enzyme reaction. Five ml of this solution was taken into a fresh test-tube and added 5 ml of the Thio Barbutaric Acid reagent (3.5 ml T.B.A. solution, 1.5ml HCl, 0.5ml distilled water). The test-tube then placed in boiling water bath for 40 minutes and the developed pink colour was read at 547 nm by spectrophotometer. A blank was prepared with the same procedure, but boiled enzyme was taken in place of active enzyme.

Results and Discussion

The production of exo-PAL by six fungi during 21 days of incubation was assayed in between 7,14 and 21 days and presented in table-1. From the table it was evident that the maximum (27 U/ml) exo PAL was noticed in *P.citrimum* during 14 days of incubation. In general, the 14 days of incubation was viewed to be ideal for optimum enzyme production and subsequently in 21 days the production rate has been decreased. The next best organisms were *A.flavus* (24 U/ml) and *A.niger* (23 U/ml). Among the six fungi, *R.solani* was responsible for lesser enzyme secretion (16 U/ml). The enzyme production also declined which ranged from 9 to 18 U/ml. In view of these results, it was noticed that for maximum exo PAL production, the ideal incubation time in Asthana and Hawker's medium (supplemented with pectic acid) was 14 days.

Table-1: Exo pectic acid lyase* (Exo PAL) activity of six fruit rot fungi on Asthana and Hawker's medium supplemented with pectic acid after 7, 14 and 21 days of incubation.

Fungi	7 days	14 days	21 days
Rhizoctonia solani	0.8	16.0	12.0
Penicillium citrinum	15.0	27.0	18.0
Mucor racemosus	10.0	17.0	10.0
Rhizopus stolanifer	8.0	19.0	16.0
Aspergillus flavus	6.0	24.0	12.0
A.niger	9.0	23.0	9.0

*Expressed in units (0.01 OD change was taken as 1 unit of enzyme activity)

The exo PAL production *in vivo* was studied in six fruits and the obtained results were incorporated in table-2. The table clearly indicated that, the production was increased upto 16 days and subsequently the quantities were decreased. The apple fruits were incubated after 4.8.12 and 16 days, the fruit pulp was assayed for exo PAL activity. The maximum recorded exo PAL was 42.5 U/ml in *A.niger* and the least producer among the six fungi was *R.stolanifer* with production rate of 24 U/ml after 16 days.

Table -2: *In vivo* the production of Exo Pectic Acid Lyase*(PAL) by six fruit rot fungi

Fungi	Apples				Mango				Tomato				Sapota				Grapes				Orange			
	Days of incubation																							
	4	8	12	16	2	4	6	8	2	4	6	8	2	4	6	8	2	4	6	8	2	4	6	8
Rhizoctonia solani	5.1	8.0	13.5	26.5	26.5	34.0	19.5	13.6	25.0	39.5	17.0	13.0	12.1	19.5	9.5	9.0	2.1	3.0	41.0	40.7	12.1	20.0	9.5	9.0
Penicillium citrinum	3.2	4.0	16.7	30.5	24.0	33.0	18.0	13.5	17.0	25.0	33.0	28.0	12.7	34.0	12.5	12.0	2.1	3.0	39.5	39.5	3.0	5.4	13.5	8.6
Mucor racemosus	5.4	7.5	24.0	34.0	26.5	34.0	16.0	14.5	28.0	33.0	40.6	20.0	16.0	31.0	27.0	26.0	0.9	1.8	41.5	39.5	9.5	13.5	24.0	3.5
Rhizopus stolanifer	5.1	6.0	12.3	24.0	17.3	33.5	20.2	5.8	33.0	52.0	41.0	39.5	25.0	25.5	17.0	16.5	1.5	3.4	45.5	42.7	3.2	4.0	26.5	3.2
Aspergillus flavus	3.2	4.0	17.3	35.0	15.5	28.0	18.5	13.5	52.0	45.0	25.0	20.0	19.0	19.8	9.0	8.0	2.5	8.6	38.6	38.4	7.5	12.3	13.5	4.0
A. niger	3.5	9.5	15.5	42.5	12.3	21.5	12.5	6.0	45.5	52.0	28.0	25.0	19.0	19.5	12.5	12.2	3.5	6.5	38.5	38.0	8.0	12.5	13.5	3.5

*Expressed in units (0.01 OD change was taken as 1 unit of enzyme activity)

In the mango fruit the maximum exo PAL reported in 4 days (34 U/ml) in *R.solani* and *M.racemosus*. The least activity was noticed in *A.niger* (21.5U/ml) after 4 days and the activity was gradually decreased upto 8 days. In tomato, the highest production rate was 52 U/ml, after four days in *Rhizopus stolanifer, A.niger*, while low range of activity was observed in *P.citrinum* (33 U/ml) after six days. In sapota fruit the highest exo PAL activity was recorded in four days by all fungi. The maximum (34 U/ml) enzyme was recorded in *P.citrinum* and the least (19.5 U/ml) in *A.niger* and *R.solani*. Interestingly, the growth rate was decreased along with production rate up to 8 days (8 to 26 U/ml), in the grape fruits the maximum exo PAL was noticed in *R.stolanifer* (45.5 U/ml) after six days. Lower production range was recorded in *A.flavus* (38.6 U/ml) and *A.niger* (38.5 U/ml) after six days. In the orange fruit the exo PAL was maximum after six days by five fungi, but only *R.solani* showed after four days. Decreasing growth rate and activity was observed after 8 days and the range was between 3.2 to 9 U/ml. *R.stolanifer* was maximum producer (26.5 U/ml) and lowest activity (13.5 U/ml) was recorded in *P.citrinum, A.flavus* and *A.niger* after six days.

Our *in vitro* and *in vivo* studies supported the statements of perishable horticultural commodities such as fleshy fruits have a relatively short post harvest, shelf life during which the fruit tissues undergo profound changes in texture, colour and flavor as well as becoming more susceptible to pathogenic attack. Fruit softening is associated with cell wall disassembly (Saymour and Gross, 1996). PAL transcripts in many fruits, may indicate that these enzymes have a more important role in ripening. Banana pulp with a substantial fruit tissue, PAL activity has been obtained directly from banana pulp with a substantial increase in activity during ripening (Jamenez *et al*, 2002), but our investigation proved the PAL activity has been obtained by other fruits also, such as apple, mango, sapota, tomato, grapes, and orange.

PAL gene expression has been manipulated in transgenic strawberry fruits and suppression of the m-RNA during ripening resulted in significantly firmer fruits (Marin *et al*, 2002). Pectate lyase (PL) secration was detected when the pH reached 5.8 and the level of secretion increased upto pH 6.5. PL gene (pel) transcript production began at pH 5.0 and increased upto 5.7 (Nir Yakoby *et al*, 2000). The yields of pectic trans eliminases or PAL are less than other pectinases. *Aspergillus* and *Penicillium* species reconstructed in *Pichia pastoris* for the expression and produced Pectin Lyase and bacteria are the major producers of PAL (Satyanarayana and Kumar, 2005). According to our studies not only *Aspergillus* and *Penicillum* species, the other fungi like *Rhizoctonia solani, Mucor racemosus* and *Rhizopus stolanifer* were also able to produce exo PAL, PAL was higher in poly pectate mineral salt medium than in apple (Gimgihong *et al*, 1991). But our *in vitro* and *in vivo* studies showed exo PAL activity was high in apple fruit than in Asthana and Hawker's medium supplemented with pectic acid and fungal growth was maximum after 14 days, while in apple fruit highest production rate was noticed after 16 days. Chemical modification and substrate production studies showed the presence of lysine and tryptophan at or near the active site of the PAL. The substrate affinity studies showed that tryptophan could be essential for substrate binding, where as lysine could be involved in the catalysis (Narasimha Rao *et al*, 1996). PAL secretion was induced by 0.2% glucose and significantly decreased at 2% glucose and glucose may control the expression of PAL at a transcriptional level (Suryakanth *et al*, 2006). Maximum enzyme production was obtained in the medium containing wheat bran as substrate compared to rice bran (Janani *et al*, 2011). Clarification of banana and pine apple juice using the partially purified enzyme resulted in 38 and 41% reduction in viscocity as determined septroscopically (Rashmi *et al*, 2008). PAL exhibited gradual increase in enzyme activity and *A.flavus* showed maximal production (42.69 and 36.19 U/ml) at 1 M KCL and NaCl respectively (Makky, 2009). In this study we found that the maximal Exo PAL (52 U/ml) production by *A.flavus* after two days in tomato fruit without KCl as stresser.

PAL plays an important role in plant pathogenesis. The enzyme is widely distributed in diverse families of microorganisms, biochemical studies such as isozymes, structure, reaction mechanism, purification and properties like molecular mass, optimum pH and temperature, substrate specificity, metal ion requirement, inhibitors and activators, kinetic parameters of the enzymes are reviewed (Anurag *et al*, 2009). Maximum enzymatic activity was observed after 7 days incubation at 40°C temperature (Nazneen Akthar *et al*, 2011). The maximum activity of enzymes from solid state fermentation (SSF) was observed at 35°C, but crued enzyme was more thermo tolerant than PL III, maintaining its maximum activity upto 45°C (Viviani *et al*, 2010). PAL is a kind of enzyme that is abundantly used in the textile industry for cotton souring. Previously the PAL gene

to enhance the production of PAL a combined strategy was formulated by combining online methanol control, two stage pH control strategies and this strategy proved to be very useful for the enhancement of PAL production (Quereshi *et al*, 2010). Poly galacturonate lyase (PGL) or PAL activity was upto 1593 U/ml, which was enhanced 1.85 fold compared to the control (863 U/ml) cultured with sorbitol and an appropriate sorbitol co-feeding strategy not only decreased the cell mortality to 8.8% (the control is about 23.1%) in the end of the fermentation, but also reduced the proteolytic degradation of PGL (Wang *et al*, 2010). The genus *Penicillium* is worldwide known for production of secondary metabolites and extracellular enzymes of commercial value, including pectinases (Rasheedha *et al*, 2010). 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). Commercial pectinase in comparison with the laboratory produced pectinase was also more effective than the commercial produced enzyme (Ajayi *et al*, 2014). Recently Garg et al, (2016) reported the eco-friendly nature of the pectinases in degrading/decomposing the materials in the surroundings.

Conclusion

It was concluded that the fungal strains *P.citrinum*, *R.stolanifer*, *A.flavus* and *A.niger* are optimal producers of exo PAL and most useful in fruit juice extraction and clarification.

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