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Research Article

PRODUCTION OF ENDO PECTOLYTIC ENZYMES BY SIX FRUIT ROT FUNGI AND THEIR USE IN FRUIT JUICE EXTRACTION AND CLARIFICATION

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ABSTRACT

Pectinases are group of enzymes that attack pectin or pectic acid and depolymerize it by hydrolysis and transelimination, endopectinases acts on pectin or pectic acid in a random cleavage mechanism. Endopectinases are Endo PG, Endo PMG, Endo PL and Endo PAL. Six fruit rot fungi isolated and tested for endopectinases production by using pectin/pectic acid as a substrates in Asthana and Hawker's medium (In vitro) and for In vivo studies six fruits were used i.e., Apple, Mango, Tomato, Sapota, Grape and Orange. In vitro studies the maximum endo PG was noticed in Aspergillus flavus during its 14 days of incubation (52.6 RVU) endoPMG was (50.0 RVU) by Rhizoctonia solani during its 14 days of incubation. Endo PL was 22.2 RVU by Penicillium citrinum after 7 days and Endo PAL was 55.5 RVU Penicillium citrinum after 14 days. In In vivo studies the maximum endo PG was recorded in Mango during 6 days (66.7 RVU) by Rhizoctonia solani, R. stolanifer, A flavus and after 4 days in P. citrinum, M.racemosus while endo PMG was noticed in A. niger (62.4RVU) in mango fruit pulp after 6 days. Endo PL was maximum in M.racemosus (51.8 RVU) while the endo PAL was maximum (66.7 RVU) showed by M.racemosus also. While A. flavus and A.niger after 4 days in Mango fruit pulp. Among these 6 fungi P. citrinum, M.racemosus and A. flavus were the potential strains in the endopectinases production and most useful in fruit juice technology.

INTRODUCTION

Pectin found in primary cell wall and middle lamella of fruits and vegetables (Favela *et al*, 2006) pectin contains α,1,4 linked D-galactosyluronic residues. Three pectic polysacchorides. Homogalacturonan, Rhamno galacturonan-1 and substituted galacturonan have been isolated from primary plant cell walls (Sharma and Satyanarayana 2006). Pectinases are group of enzymes that attack pectin and de-polymerase it by de-esterification reaction, which by 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 (transelimination or hydrolysis) and the type of cleavage as random – endo or terminal – Exo (Kashyap *et al* ,2001). Enzymes act on i) Pectin Esterase or Pectin Methyl Esterase (E.C. 3.1.1.1.1) (ii) Endo PMG (EC 3.2.1.41) (iii) Exo PMG (EC 3.2.1.40) (iv) Endo Pectin Lyase (E.C.4.2.2.3) v) Exo Pectin Lyase (EC 4.2.2.10) and enzymes act on Pectic Acid (i) Endo PG (E.C. 3.2.1.15) (ii) Exo P.G. (E.C. 3.2.1.67) (iii) Endo PAL (E.C.4.2.2.2.) and Exo-PAL (E.C.4.2.2.9)

With the use of agricultural wastes generates gallons of wastes during preparation of different juices. Its dumping nature causes pollution, this problem can be solved by exploiding these agro wastes for pectinases production by Using potential microorganisms (Preeti *et al* 2015). Pectinases are eco friendly in nature in degrading or decomposting the material in the surroundings (Garg *et. al.* 2016). Commercial pectinases with comparison with laboratory produced pectinase was also more effective than the commercial produced enzyme (Ajayi *et al* 2014). Fungal pectinases used in the food industry for the production of fruit juices to increase the fruit juice and in the

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clarification of the fruit juices. This scientific approach showed enhanced advantage in the collection of maceration, separation, clarification and liquefaction of variety of fruit juices and this type of biotechnological methods using cost-effective eco friendly, non toxic approaches are of utmost important. (Suryam *et al* 2018). The selected strains i.e., *Rhozoctonis solani, Penicillium citrinum Mucor racemosus, Rhizopus stolonifer, Aspergillus flavus* and *A. niger,* were thoroughly investigated and critically monitored and are the safe candidates for the application in fruit juice technology.

MATERIALS AND METHODS

Collection of Infected Fruits and Fungal Isolation Method

The infected fruits of tomato (*Lycopersicum esculentum*), Mango (*Mangifera indica*), Apple (*Malus pumila*), 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, Warangal areas and carried to the laboratory.

The infected portions of fruits indicate post-harvest fungal / bacterial diseases. 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 onto the agar slants of Asthana & Hawker's Agar medium (A) (Glucose- 5 g, KNO_3-3.5 g, KH_2PO_4–1.75g, $MgSO_4$–0.75g, Agar-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, Rhizopus stolonifer, Aspergillus flavus* and *A. niger*.

Extraction of Pectinases from Pathogen (in vitro)

Pectin or pectic acid supplemented Asthana and Hawker's medium (pectin or pectic acid 5 g, KNO_3 3.5 g, KH_2PO_4 – 1.75 g, $MgSO_4$ – 0.75 g) was prepared and 100 ml of broth was transferred into 250 ml conical flasks. Aseptically inoculated the flasks 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^{o}C for 7, 14 and 21 days. After the incubation period the contents were filtered 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 was added to the enzyme, when enzyme assay was delayed.

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 grams of infected portion of the fruits was separated in aseptic condition and cut the tissue into small pieces of 1-2 centrimeters. The cut pieces were transferred into a waring blender and added 100 ml 0.15 M 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 r.p.m. for 30 minutes and supernatant was separated into culture flask and this filtrate was used as an enzyme source. Few drops of toluene was added and enzyme was stored at 4^{o}C in case when enzyme was not immediately used.

Endo Poly Methyl Galacturonase (Endo-PMG)

Wood (1955) viscometric method was followed to estimate the endo-PMG. Pectin (0.5%) was prepared by dissolving 0.5 g of pectin in 100 ml citrate buffer (pH 5.5) and heated at 50^{o}C – 60^{o}C and the contents were blended for 3 minutes and filtered through two layers 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-PMG was with pectin (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 viscocity was calculated by applying the following formula.

$$V = \frac{ET_o - ET_t}{ET_o - ET_w} \times 100$$

Where, V = percent loss of viscosity.
ET_o = flow time of water in seconds at zero time
ET_t = flow time of reaction mixture at 't' intervals
ET_w = flow time of distilled water.

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

$$REA = \frac{1000}{t_{50}}$$

Endo Poly Galacturouase (Endo PG)

Endo-PG was also assayed similar to the method of Endo-PMG by using Oswald viscometer, but the substrate in Endo-PG was pectic acid instead of pectin, that was used in PMG (endo).

Endo-Pectin Lyase (endo-PL)

The endo-PL was assayed by viscometric method as followed for the estimation of endo-PG and endo-PMG, but the substrate

for endo-PL was pectin (0.5%) and buffer was Tris-HCl buffer (pH 8.1).

Endo-Pectic Acid Lyase (Endo-PAL):

The endo PAL was assayed by viscometric method followed for endo-PL, however, the substrate used was sodium polypectate instead of pectin.

RESULTS

Endo PMG

In vitro: The production of endo-PMG by six fruit rot fungi during 21 days of incubation was assayed in between 7, 14, 21 days and presented in Figure 1. From the Figure it was evident that the maximum endo PMG was noticed in *Rhizotonia solani* during its 14 days of intubation (50 RVU). The next best organisms were *Aspergillus niger* (41.6 RVU) and *A. flavus* (37.0 RVU) after 21 days. Among the six fungi *Rhizopus stolanifer* was responsible for least enzyme secretation (19.0 RVU) and that was stable upto 21 days of incubation. In the 21 days of incubation the growth rate decreased and subsequently the enzyme production also declined during 7 days of incubation the fungi was initiated the production of endo PMG and ranged between 12.7 to 40.0 RVU. *Mucor racemosus* is only one organism showed its maximum in 7 days of incubation (33.3 RVU). In view of these results at was noticed that for maximum endo PMG production the ideal incubation time in Asthana and Hawker medium supplemented with pectin was 14 days.

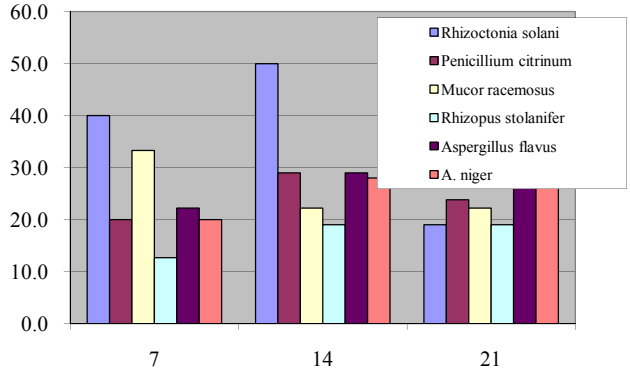


Figure 1 Endo PMG in Asthana Hawkers medium with 7,14 and 24 days of Incubation Period

In vivo

The eno-PMG production *in vivo* was studied in six fruits and the obtained results were incorporated in table 1. The table clearly revealed that the production rates were increased initially upto 4/6 days and subsequently the quantities were decreased. The apple fruits were incubated after the inoculation by six fungi and after 4, 8, 12 and 16 days of incubation, the fruit pulp was assayed for endo-PMG activity. The maximum activity was recorded during 12 days incubation, (23.4 RVU)) by *M. racemosus,* while *P. citrinum* was the next best fungi for endo PMG (22.8 RVU) production, while *A. niger* (21.5 RVU) and *R.solani* (21.3 RVU) were moderate in their endo PMG activity. In mango fruits the maximum endo PMG was reported in 4 days of incubation (49.3 RVU) in *R. solani.* Moderate activity was showed in *M. racemosus* (41.7 RVU) and *R.stolanifer* (41.7 RVU) after 6 days. Where the endo PMG activity and growth rate increased upto six days and declined after 8 days. In *Tomato* fruit during 2, 4, 6, and 8 days of incubation under pathogensis was assayed and recorded. Among the six fungi, only *Aspergillus niger* activity was increased upto 8 days and remaining fungi showed their maximum activity after 6 days. The maximum was recorded in 4 days of incubation (20.0 RVU) in *R. solani.* In *Sapota* fruit the highest range of endo PMG activity was recorded in 8 days of incubation and highest was in *P. citrinum* and *M. racemosus* (47.6 RVU). In the *Grape fruit,* the maximum endo PMG was noticed during six days of incubation (62.4 RVU) by *A. niger.* A moderate activity was showed by *R. solani* (56.0 RVU) and *A. flavus* (55.5 RVU) after 8 days. While *M. racemosus* showed lowest endo PMG activity after six days (32.5 RVU). In the Orange fruits the endo PMG was maximum in 8 days of intubation by *M. racemosus* (11.1 RVU) and gradually endo PMG activity increased upto 8 days by *P.citrinum* and *M.racemosus.* A moderate activity was occurred in *A. niger* (10.3 RVU) and *R. solani* (9.2 RVU) after six days and activity declined in both organisms after 8 days.

Endo PG

In Vitro

The production of endo PG By six fungi during 21 days of incubation was assayed during 7,14 and 21 days and presented in Figure 2. From the figure it was evident that the maximum endo PG was noticed in *Aspergillus flavus* during its 14 days of incubation (52.6 RVU). In general the 14 days incubation was viewed to be ideal for optimum enzyme production and subsequently in 21 days the production rate has been decreased. The next best organism were *Mucor racemosus* (50 RVU) and *Rhizopes stolanifer* (44.6 RVU) for endo PG production. Among the six fungi *R. solani* was responsible for least enzyme secretion (33.3 RVU). In 14 days of incubation and viewed that for maximum endo PG production the ideal intubation time in Asthana and Hawker's medium was 14 days of incubation.

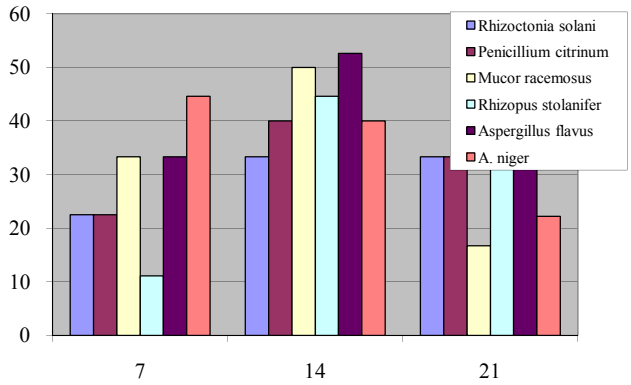


Figure 2 Endo PG in Asthana Hawkers medium with 7,14 and 24 days of Incubation Period

In Vivo

The endo PG production *in vivo* was studied in six fruits and the obtained results were incorporated in table 2. The table clearly revealed that the production rates were increased initially upto 16 days and subsequently the quantities were decreased. The apple fruits were incubated after the inoculation by six fungi and after 4,8, 12 and 16 days of incubation, the

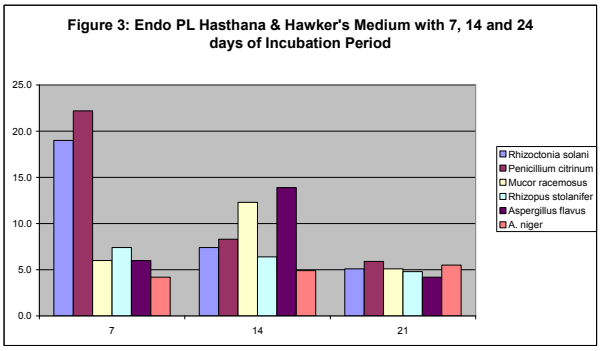
fruit pulp was assayed for endo PG Activity. The maximum was recorded during 16 days of incubation (55.5 RVU) by *R. solani* and *R.stolanifer* was the next best fungi in producing high amounts of endo PG (47.6 RVU), while *P.citrinum* (39.2 RVU). *Mucor racemosus* (39.2 RVU) and *A. niger* (38.0 RVU) were moderate in their activity. The least producer among six fungi was *A flavus* with production rate of 33.3 RVU.

In *Mango* fruit the maximum endo-PG was reported in 6 days of incubation (66.7 RVU) in four fungi i.e., *R. solani, P.citrinum, R. stolanifer* and *A. flavus.* The other two fungi *M.racemosus* (53.5 RVU) and *A.niger* (50.0 RVU) were moderate in their endo PG activity. In *Tomato* fruit during 2,4,6 and 8 days of incubation under pathogensis was assayed and recorded. In all the fungi the maximum endo PG was recorded 6 days of incubation while the maximum endo PG (66.6 RVU) by *R. solani,* while *P.citrinum* and *M.racemosus* showed the moderate endo PG (50.0 RVU) and the subsequent incubation decreased the enzyme production. In *Sapota* fruit the highest was recorded again in 6 days of incubation and highest (53.3 RVU) was in *Rhizopus stolanifer* and *A. flavus.* Interestingly, there was not much decline in the production rates of endo PG during 8 days of incubation and their range was in between 40 to 50 RVU. In *Grape* fruits, the maximum was noticed during four days of incubation and subsequently there was substantial decrease in 6 and 8 days of incubation. The maximum activity (66.6 RVU) was in *A. flavus, A. niger, R stolanifer* and *M.racemosus.* In the remaining fungi enzyme activity was moderate i.e., *P. citrinum* (50 RVU) and *R. solani* (49.5 RVU). In the orange fruit also the rates of endo PG was maximum in four days of inoculation and maintained in six days of incubation and subsequently decreased in 8days. The maximum endo PG (66.6 RVU) was noticed in *M. racemosus* and *R. stolanifer* during 4 and 6 days of incubation under pathogenesis. *P. citrinum* was responsible for maximum (66.6 RVU) activity only in 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 was decreased in the next incubation.

Endo PL Production

In Vitro

The production of endo-PL by six fungi during 21 days of incubation was assayed in between 7, 14 and 21 days and presented in Figure 3. From the Figure it was evident that the maximum endo-PL was noticed in *P. citrinum* during its 7 days of incubation (22.2 RVU) and the next highest endo PL activity was noticed by *R. solani* (19 RVU) after 7 days of incubation. *A. flavus* 13.9 RVU) and *M.racemosus* (12.3 RVU) were showed their moderate activities after their 14 days of incubation. In general, 14 days incubation was viewed to be ideal for optimum enzyme production and subsequently in 21 days the production rate was decreased, but in endo-PL secretion, the ideal incubation for *R. solani* and *P. citrinum* was 7 days in Asthana & Hawker's medium supplemented with pectin. In view of these results it was reported that for the maximum endo PL production, the ideal incubation time was 7 days in Asthana and Hawker's medium supplemented with pectin.



In Vivo

The endo-PL production *in vivo* was studied in six fruits and the obtained results were incorporated in table 3. The table clearly revealed that the production rates increased initially upto 16 days and subsequently the quantities were decreased. The apple fruits were incubated after the inoculation by six fungi and after 4,8,12 and 16 days of incubation. The fruit pulp was assayed for endo-PL activity. The maximum activity was recorded during 16 days of incubation (44.4 RVU) by *R. solani.* The next best production (38 RVU) was recorded in *P. citrinum, R. stolanifer* and *A. niger* after their 16 days of incubation. Moderate activity was recorded in *M.racemosus* (33.3 RVU) after its 16 days of incubation.

In the mango fruit, the maximum endo PL was reported in 8 days of incubation (51.8RVU) with *M. racemosus.* The next best production of endo PL was noticed in *P.citrinum* (43.7 RVU) and *R. solani* (42.9 RVU) after their 4 days of incubation. In tomato fruit, the endo PL was assayed during 2,4,6 and 8 days incubation under pathogenesis. Most of the fungi showed their maximum activity after 4 days, but only one organism i.e., *A. niger* showed maximum activity after 8days of incubation. Quick spoilage was the cause for their maximum production after four days. The maximum endo-PL was in *R. solani* (8.3 RVU) and best activity was showed by *M.racemosus* (8.0 RVU) and *P. citrinum* after their four days of incubation. In sapota fruit the highest range of endo PL activity was recorded in six days of incubation (17.8 RVU) was in *R. solani* and *P.citrinum.* Interestingly, the endo PL activity was so much declined upto 8 days of incubation, in most of fungi and range was between 12 to 1.5 RVU. Quick spoilage, rapid growth and vigorous infection were the reasons for declined in endo PL activity. In the grape fruit the maximum endo-PL ranged after six days (5.5 to 15.6 RVU) and eight days of incubation (7.1 to 11.1 RVU) under pathogenesis. The maximum endo-PL recorded in *R. solani* (15.6 RVU) after six days. The next best organism was *P. citrinum* (15.3 RVU) and the endo PL activity was declined after eight days.

In the orange fruit four fungi showed their maximum activity after 8 days of incubation, while two fungi viz., *A. flavus* and *A. niger* were showed their maximum activity after their six days of incubation. The highest endo-PL activity was recorded in *R.solani* 36.3 RVU) after 8 days and the next best production was noticed in *P. citrinum* (18.5 RVU) after its 8 days of incubation. Delayed spoilage and less infection by the fungi were causes for lowest activity of endo-PL after 8 days.

Endo PAL

In Vitro

The endo PAL activity of six fruit fungi during 21 days of incubation was assayed in between 7, 14 and 21 days and data obtained presented in Figure 4. From the figure it was evident that the maximum endo PAL was noticed in *P. citrinum* during its 14 days of incubation (55.5 RVU). In general, the 14 days incubation was viewed to be ideal for optimum enzyme production and subsequently in 21 days the production rate has been decreased. The next best organism was *Mucor racemosus* (53.5 RVU) for endo PAL production. Among the six fungi *R.solani* and *A. niger* were responsible for least enzyme secretion (44.6 RVU) in 14 days of incubation. *A. flavus* and *R.stolanifer* were moderate in their endo PAL (47.6 RVU) secretion. In the 21 days of incubation the growth rate decreased and subsequently the enzyme production also declined which ranged from 22.2 to 44.4 RVU by six fungi. During 7 days of incubation the fungi were initiated their growth and acclimatized for the condition and started producing endo-PAL, which ranged between 3.7 to 14.5 RVU. *A. niger* is only one organism which showed its maximum endo PAL production in 7 days of incubation (44.6 RVU). In view of these results it was understood that for maximum endo PAL production the ideal incubation time was 14 days of incubation in Asthana and Hawker's medium supplemented with pectic acid.

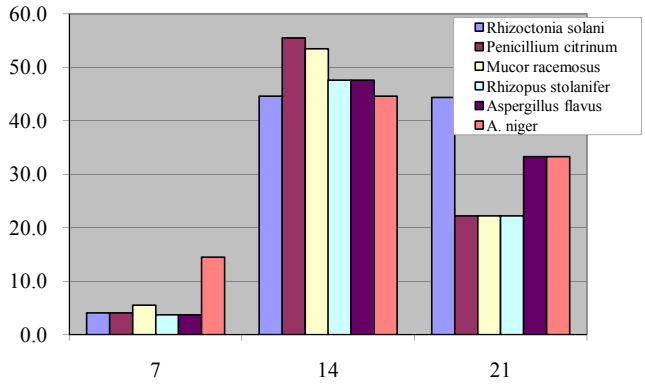


Figure 4 Endo PAL in Asthana Hawkers medium with 7,14 and 24 days of Incubation Period

In Vivo

The endo PAL production *in vivo* was studied in six fruits and the obtained results were incorporated in Table 4.The table clearly revealed that the production rates were increased initially upto 16 days and subsequently the quantities were decreased. The Apple fruits were incubated after the inoculation by six fungi and after 4,8,12 and 16 days of incubation, the fruit pulp was assayed for endo PAL activity. The maximum activity was recorded during 16 days of incubation (45.6 RVU) by *Rhizopus stolanifer and Aspergillus flavus* was the next best fungi in producing high amount of endo PAL (43.7 RVU), while *A. niger* (36.6 RVU) and *Rhizoctonia solani* (33.3 RVU) were moderate in their endo PAL activity. The least producer among the six fungi was *Mucor racemosus* with the production rate of 17.5RVU.

In mango fruit, the maximum endo PAL was reported in four days of incubation (66.7 RVU) in three fungi i.e., *A.niger, A. flavus* and *M.racemous*. Two fungi *P.citrinum* 59.8 RVU) and *Rhizopus stolanifer* (58.5 RVU) were moderate in their endo PAL activity after six and four days of incubation period respectively. In majority of the fungi the subsequent incubation (8 days) decreased the endo-PAL activity, but in few organisms the production rate were stabilized and maintained as in 6 days of incubation. The endo PAL production in Tomato fruits during 2,4,6 and 8 days of incubation under the pathogenesis was assayed and recorded. In four fungi the maximum endo PAL was recorded in four days and remaining two fungi *R.solani* and *P.citrinum* the highest activity was recorded after six days (22.2 RVU). In *M.racemosus, A. flavus* and *A. niger* the endo PAL activity was (22.2 RVU) recorded after four days of incubation. The endo PAL activity was much declined in 8 days of incubation and their range was between 7.9 to 15.6 RVU. In Sapota fruit the highest endo-PAL was recorded again in four days of incubation period and highest (51.8 RVU) was noticed in *P. citrinum* . The endo PAL activity was gradually decreased in three fungi i.e., *R. solani, P. citrinum* and *M.racemosus,* while in remaining three fungi the endo PAL activity was increased upto 8 days of incubation period.

Table 1 Endo-Poly Methyl Galactaronase (Endo PMG) activity of six fruits rot fungi on six fruits after 4,8,12,16 and 2,4,6,8 days of incubation

Fungi	Relative Enzyme Activity																							
	Apples				Mango				Tomato				Sapota				Grapes				Orange			
	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	5.8	16.7	21.3	14.6	2.11	49.3	16.7	13.9	9.3	20.0	16.7	14.8	37.9	46.7	42.7	41.7	12.8	23.4	39.3	5.60	5.6	8.1	9.2	5
Penicillium citrinum	6.8	22.2	22.8	18.9	3.1	36.0	29.0	16.7	7.3	7.4	11.0	6.7	16.7	38.9	42.5	47.6	8.5	16.7	35.8	28.6	2.9	5.6	7.3	8.1
Mucor racemosus	6.4	15.5	23.4	20.8	5.0	39.4	41.7	27.8	8.3	9.9	11.0	3.5	18.9	22.3	38.9	47.6	5.5	11.1	32.5	26.7	2.9	5.6	5.6	11.1
Rhizopus stolanifer	7.7	12.3	18.5	18.2	2.6	29.6	41.7	19.3	2.8	9.5	14.9	11.1	12.7	19.4	33.3	42.4	6.5	9.1	26.8	41.7	4.1	5.0	6.0	2.9
Aspergillus flavus	7.0	16.7	18.2	18.1	2.2	22.2	37.0	19.7	11.1	16.7	8.1	3.7	11.7	19.4	36.0	46.5	11.1	21.2	37.5	55.5	5.6	7.3	8.00	5.2
A. niger	8.0	18.5	21.5	18.2	2.0	20.6	22.7	22.7	5.5	7.4	11.1	13.3	17.4	20.3	34.7	42.4	23.5	57.1	62.4	58.5	8.0	9.2	10.3	6.0

**Activity express in Relative Viscometric Units*

Table 2 Endo-Poly Galacturonase (Endo-PG) activity of six-fruit-rot fungi on six fruits after 4,8,12,16 and 2,4,6,8 days of incubation

Fungi	Relative Enzyme Activity																							
	Apples				Mango				Tomato				Sapota				Grapes				Orange			
	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	22.8	32.4	44.4	55.5	29.0	38.1	66.7	66.7	20.0	44.4	32.4	28.6	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	15.5	22.3	38.9	39.2	39.4	66.7	66.7	50.0	11.0	50.0	50.0	28.6	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	16.7	22.3	32.4	39.2	36.0	66.7	53.5	53.5	28.6	31.5	50.0	32.4	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	18.5	32.4	42.5	47.6	19.3	26.7	66.7	66.7	28.6	44.4	66.6	41.5	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	16.7	19.4	22.3	33.3	16.7	22.7	66.7	44.4	16.7	30.8	44.4	20.0	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	15.5	22.3	32.4	38.0	27.8	55.5	50.0	22.2	11.0	28.6	41.5	21.5	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 express in Relative Viscometric Units*

Table 3 Endo Pectin Lyase (Endo-PL)* activity of six fruit rot fungi on six fruits after 4,8,12,16 and 2,4,6,8 days of incubation

Fungi	Relative Enzyme Activity																						
	Apples				Mango				Tomato				Sapota				Grapes				Orange		
	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	8.6	14.8	17.4	44.4	17.9	42.9	29.8	23.8	5.3	8.3	3.3	2.4	8.0	16.7	17.8	1.3	1.3	4.4	15.6	8.30	2.0	2.5	3.2	33.3
Penicillium citrinum	9.2	10.0	15.9	38.0	19.7	43.7	42.9	31.5	3.7	7.9	2.8	2.0	12.0	17.5	17.8	1.5	2.4	4.9	15.3	11.1	2.0	2.5	4.1	13.3
Mucor racemosus	10.0	13.7	18.1	33.3	11.8	31.2	36.5	51.8	6.0	8.0	5.6	2.4	8.0	8.4	2.9	1.4	2.0	2.6	9.5	7.1	3.0	4.5	5.6	13.3
Rhizopus stolanifer	8.6	12.4	13.3	38.0	3.7	32.8	34.6	19.4	5.9	6.7	6.0	4.4	11.1	16.7	4.7	2.2	1.2	1.8	5.5	8.3	3.5	4.5	5.6	5.5
Aspergillus flavus	8.0	13.2	16.7	19.0	2.4	11.4	22.9	22.7	5.5	5.6	4.4	2.8	8.7	7.2	2.3	1.4	2.3	5.5	13.4	11.1	2.5	3.3	6.50	4.2
A. niger	7.5	12.4	18.5	38.0	2.4	7.8	18.5	40.0	2.5	4.0	4.7	7.8	9.0	16.7	2.2	1.2	1.2	2.3	8.5	9.5	2.5	3.5	5.2	4.2

**Activity express in Relative Viscometric Units*

Table 4 Endo Pectic Acid lyase (Endo PAL)* activity of six fruit rot fungi on six fruits after 4,8,12,16 and 2,4,6,8 days of incubation

Fungi	Relative Enzyme Activity																						
	Apples				Mango				Tomato				Sapota				Grapes				Orange		
	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	12.4	17.3	22.8	33.3	29.8	41.7	51.8	42.9	8.3	15.6	22.2	15.3	16.7	44.4	41	38.6	15.6	22.2	27.8	33.3	4.2	5.5	33.3	33.3
Penicillium citrinum	8.0	12.3	16.7	20.6	31.2	57.1	59.8	25.6	8.3	15.3	22.2	8.3	17.5	51.8	39.2	44.6	15.3	22.2	9.5	--	13.3	22.2	33.3	13.3
Mucor racemosus	7.7	12.3	15.5	17.5	42.9	66.7	48.5	48.5	7.9	22.2	15.1	8.0	12.0	44.4	42.0	20.0	20.5	33.3	4.5	--	22.2	33.3	33.3	13.3
Rhizopus stolanifer	7.0	12.4	33.3	45.6	32.8	58.5	51.8	41.0	8.0	20.5	15.3	7.9	8.4	20.0	38.6	42.0	21.6	33.3	35.6	33.3	5.5	33.3	22.2	5.5
Aspergillus flavus	5.8	7.7	38.0	41.7	43.7	66.7	40.0	40.0	21.6	22.2	20.5	15.6	8.7	20.0	33.3	41.0	15.1	22.2	31.5	41.7	13.3	22.2	44.4	4.2
A. niger	5.4	8.4	23.4	36.6	42.9	66.7	44.4	30.6	20.5	22.2	15.1	8.3	7.2	16.6	38.6	42.0	15.3	22.2	33.5	44.4	13.3	33.3	22.2	4.2

In the grape fruit the maximum endo PAL activity was noticed during eight days of incubation period in *A. niger* (44.4 RVU) another best organism is *A. flavus* (41.7 RVU) after its 8 days of incubation. Interestingly *P. citrinum* and *M.racemosus* ceased their production after 8 days and quick spoilage of the fruit, non availability of substrates may be the reasons for that. In the orange fruit the maximum endo PAL was produced by *A. flavus* (44.4 RVU) after 6 days of incubation. Moderate endo PAL was noticed by remaining fungi, but *A. niger, R.solani* and *Muco racemosus* after 4 days while *P. citrinum* and *R. solani* showed after 6 days. Production upto 8 days showed their decreasing endo PAL activities. Quick spoilage of fruit and vigorous infection were the main reasons for this type of fast decline in the rates of endo PAL activities.

DISCUSSION

Endo PMG

Endo PG and Endo PMG in pectin / polypectate mineral salt medium were showed maximum activity (4.4 and 16.2 units) at six and eight days of culture respectively (Gimgihong *et . al.,* 1991). But in our studies the maximum endo PG/PMG activity was noticed in Asthana and Hawker's medium supplemented with pectic acid/Pectin. Levin and Forchiassin (1998) noticed the pectinolytic enzymes, the white rot fungus *Tramates tragii* on a laboratory scale and observed that high pectinase activities in a media with more alkaline initial p^H values (6.2) and in the range of 23 to 28□C and tween 80 promoted growth and gave the highest yield of PMG. But in our investigation Pectin/fruit pulp promoted the growth and gave the highest yield. Grebechova *et. al.* (2007) observed the pectinolytic enzymes by submerged fermentation from *A. niger* and *A. foetidus* and stated that the channe growth medium is the best for endo PMG filtered by Talto bello medium.

Aspergillus genus especially *A. niger* as frequently responsible for post harvest decay of fresh fruit such as citrus, grapes, tomatoes (Ajayi et. al., 2014). But according to our results other fruits like Apples, Mango, Sapota were also decayed by *Aspergillus genus.* Production of PMG was increased with increasing in temperature upto 30□C it means 30□C was most suitable for the production PMG (Sridha *et. al.* 2015). With the use of agricultural wastes generates gallons of wastes during preparation of different juices, its dumping nature causes pollution. This problem can be solved by using potential microorganisms (Preeti *et. al.* 2015). Maximum inhibition of PME, exo and endo PMG was showed by culture filtrates of *Trichoderma viridae* + *Psuedomonas fluorescents* (Rajeshwari and Kapoor, 2017). Recently Suryam *et. al.* (2018) also reported the endo and exo PMG activities by six fruit rot fungi by using different fruits extraction from four fungal strains. *Aspergillus niger, A. flavus, P.citrinum* and *M.racemosus* were very effective and replace the application of costly commercial enzymes in clarification and extraction of fruit juice.

Endo PG

One of the major problems encountered in the preparation of fruit juices is cloudiness primarily due to the presence of pectin. The cloudness that pectin cause is difficult to remove except by enzymatic depectinization (Liew *et. al.* 2007). The clarification of grape juice was greatly affected by *A. niger* PG and clarified juices were stable upon storage at both 4□ and 25□C (Mohasen *et. al.* 2009). In the present work Asthana and Hawker's medium supplemented with Polygalactoronic acid or fruit juice as source of nutrients increased the endo PG activity. The experimental Extract Enzyme (EE) produced by *A. 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 study also the genus *Aspergillus* with two species *A. flavus* and *A. niger* produced substantial amounts of endo PG. When orange peal alone were used as carbon source, a better production of PG was observed (Preethi *et. al.* 2015), but in our studies fruit pulp was also used as carbon source and better production was observed. The highest production levels were obtained by *A. sozae* using sugar beet as a carbon source with yields of 1111 and 449 U/g exo PG and Endo PG respectively (Marco *et. al.* 2015). *A.*

niger and *A. flavus* were most useful fungal strains for the fruit juice technology (Suryam and Charya, 2018).

Endo PL

Nedjma *et. al.* (2001) detected the PL activity by *A. niger* and *Saccharomyces cervisiae*. PL Production was also studied by Hamdy (2008) by using orange peel as substrate observed in *Rhizopus oryzae* (5.20 U/g). The present *in vivo* studies on orange fruit extract also showed the maximum PL activity by *Rhizopus stolanifer*. Sangeetha and Shastri (2007) observed the PL characteristics, properties and stated that the enzyme produced by *Penicillium oxalicum* had molecular mass of 50 KDa, as determined by SDSPAGE and showed optimum p^H and temperature at 8 and 50□C respectively. Sangeetha et. al. (2008) purified and characterized alkaline PL from *Aspergillus flavus* and stated that the enzyme was found to be stable for 24h in the pH range 4-10 Sara *et. al.* (2009) hydrolysed the orange peel by PL of *Aspergillus flavipes* and *A. niveus* and observed that the increased levels of PL in cultures containing lemon peel as a sole carbon source and stated that the PL was able to hydrolyse 56% of orangle peel biomass, with potential application in the pectin industry.

Pectinolytic fungi were isolated from cheap sources like spoiled fruits and vegetables and *Penicillium citrinum* was found to be the potent source of Pectinase. PG and PL Production was higher in SSF by using wheat bran as the solid substrate than submerged fermentation at room temperature (Sandya and Kurup, 2013).

The optimum PL production was analyzed from PL activity assay. *A. niger* showed maximum production after 96h at 30□C, 8 pH, 4 ml inoculum and 0.1% peptone. Tween 80 was used a surfactant and showed negative effect on PL production. PL was purified by the addition of 60% of Ammonium sulphate and showed maximum activity at 30□C and 8.0 pH (Batool *et. al.* 2013).

The production of PL was substantially induced upto the level of 875 U/ml, when fermentation medium of lemon peel waste inoculated with 5ml of spore suspension of *A.oryzae* (Koser *et. al.* 2014).

PL named PL III was purified to homogenecity from the culture filtrate of *Aspergillus giganteus* grown in submerged culture containing orange peel waste as carbon source. PL-III was able to digest Apple pectin and Citrus pectins with different degrees of methyl asterification, interestingly the PL III activity was stimulated in the presence of some divalent cations including Pb2+ and was not significantly affected by Hg2+ (Pendolli and Carmon, 2014). 2-deoxy-d-glucose can be used for the isolation of carbolyte repression resistant mutants in *P. griseorseum.* For the first time PL over producing mutants in *P.griseorseum* was reported and the mutation leading to 2-DG resistance in the mutants to be related to the glucose dissimilation that increases the endogenous CAmp level resulting in higher PL productivity (Lima *et. al.,* 2017).

Endo-PAL

PAL transcripts in many fruits, may indicate that these enzymes have more important role in ripening. Banana pulp with 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. The yields of pectic trans eliminases or PAL are less than other pectinases *Aspergillus* and *Penicllium* species reconstructed in *Pichia pastaris* for the expression and produced Pectinlyase and Bacteria are the major producers of PAL (Satyanarayana and Kumar, 2005). According to our studies not only *Aspergillus* and *Penicillium* species the other fungi like *Rhizoctonia solani, Mucor racemosus* and *Rhizopus stolanifer* were also able to produce PAL.

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 a substrate compared to rice bran (Janani *et. al.* 2011).. PAL exhibited gradual increase in enzyme activity and *A. flavus* showed maximal production (42.69 and 36.19 U/ml) at 1M KCL and NaCl respectively (Makky 2009). In this study we found that a maximum PAL Production was noticed without KCl as stresser. Two stage pH control strategies proved to be very useful for the enhancement of PAL Production (Quereshi *et. al.* 2010). PAL activity was enhanced 1.85 fold compared to the control cultured with sorbitol (Wang et. al., 2010). Commercial pectinase in comparison with the laboratory produced pectinases was also more effective than the commercial produced enzyme (Ajayi *et. al.* 2014). The fungal strains *P. citrinum, R.stoalnifer, A. flavus* and *A. niger* were the optimal producers of exo PAL and are the most useful in fruit juice industry. These strains also produced maximum Endo PAL (Suryam *et. al.* 2018).

CONCLUSIONS

It was concluded that, three fungal strains i.e., *Aspergillus flavus, Rhizoctonia solani* and *Penicillium citrinum* were the potential strains in the Endopectinases (Endo-PG, PMG, PL and PAL) production, these strains are very effective and replace the application of costly commercial enzymes in fruit juice extraction and clarification. In Asthana and Hawker's medium (supplemented with pectin/ Pectic acid) is most useful medium and ideal incubation period is 14 days, fruit peels and wastage also served as substrate instead of pectin.

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