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Production of exo pectinases by six fruit rot fungi and their application in fruit juice technology

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Abstract

Six fruit rot fungi were isolated and tested for exo pectinase production by using (Pectin or pectic Acid supplemented Asthana and Hawkers broth for invitro and six fruits (invivo). Exo pectinases act on pectin or pectic acid in a terminal cleavage mechanism. Exo pectinases are, Exo PME, Exo PMG, Exo PL, Exo PG and Exo PAL. *Aspergillus flavus* found to be potential in production of Exo PME, after days in supplemented Asthana and Hawkers broth (1.24 mg/ml). The maximum exo PMG was noticed in *Penicillium citrinum* during its 7 days (1070 mg/ml) while exo PG was maximum in *Mucor racemosus* during 21 days (420 mg/ml). The highest exo PL was observed in *Penicillium citrinum* (70.2 U/ml) in 21 days and exo PAL, 27 U/ml after 14 days. *Invivo* studies showed highest exo PME production in grapes (4.50 mg/ml) and in sapota (3.10 mg/ml) after 4 days by *Aspergillus flavus*. The maximum Exo PL showed by *A. niger* (89 U/ml) in grape fruit after 8 days and Exo PMG by *A. niger* (995 U/ml) after four days. The maximum exo-PG (1020 mg/ml) by *M. racemosus* (1020 mg/ml) after 16 days in apple fruit while the maximum exo PAL (52 U/ml) in Tomato after 2 and 4 days of incubation by *A. flavus* and *A. niger* respectively. These four fungal strains i.e., *Aspergillus flavus, Penicillium citrinum, Mucor racemosus* and *Aspergillus niger* were potential microorganisms in the production exo-pectinases and grapes sapota, apple fruits are the substitutents for the pectins substrate and most useful in the fruit juice technology.

Keywords: Pectin, Exo Pectinases, Fruits, Fruit rot fungi and 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 wall of plants (Sharma and Satyanarayana 2006). Pectinases are group of enzymes that attack pectin and de-polymerase it by hydrolysis and trans elimination as well as de-esterification it by reaction which hydrolysis the ester bond between carboxyl and methyl groups of pectin (Saatyanarayana and Pada 2003). Pectinases are classified based on their preferred substrate (Pectin, Pectic acid or Poly Galacturinic Acid) and on

the degradation mechanism (Trans elimination or hydrolysis) and the type of cleavage random-endo or terminal –Exo (Kashyap *et al* 2001). Enzymes action on pectin (i) Exo Pectin Esterase or Pectin Methyl esterase (E.C. 3.1.1.11) (ii) Endo PMG (EC 3.2.1.41) (iii) Exo PMG (E.C.3.2.1.40) (iv) Endo Pectin Lyase (E.C. 4.2.23). (v) Endo Pectin Lyase (E.C.4.2.2.3) (v) Exo PL (E.C.4.2.2.10) and Enzymes acting 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).

The Commercial preparation of pectinases are produced mainly from fungi Microbial pectinases account for 25% of the global food enzyme sale etc. (Jayani *et al*, 2005). With the use of agricultural wastes generates gallons of wastes during preparations of different juices. Its dumping nature causes pollution, this problem can be solved by exploiting these agro wastes for pectinase production by using potential microorganisms (Preeti *et al* 2015). Pectinases are eco friendly in nature in degrading or decomposting the material in the surroundings (Garge *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 clarification of the fruit juices. This scientific approach showed its enhanced advantage in the collection, 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. The selected strains i.e., *Rhizoctonia solani, Penicillium citrinum, Mucor racemosus, Rhizopus stolinifer, Aspergillus flavus* and *A.niger,* were thoroughly investigated and critically monitored are the safe candidates for the application in fruit juice technology.

Materials and methods

A) Collection of infected fruits and fungal isolation method:

The infected fruits of tomato (*Lycopersicum esculentum*), mango (*Mangifera indica*), apples (*Malus pumila*), sapota (*Achras sapota*), orange (*Citrus sinensis*) and grapes (*Vitis vinifera*) were collected carefully in the separate polyethylene 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 on to the agar slants of Asthana & Hawker's Agar medium (A) (Glucose- 5 g, KNO_3-3.5 g, KH_2PO_4–1.75 g, $mgso_4$ – 0.75 g, Agar-Agar 20 g) 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 *Aspergillus niger*.

B) Extraction of pectinases from fruits (*in vivo*):

Healthy fruits were inoculated with six fruit rot fungi viz. *Rhizoctonia solani, Penicillium*

citrinuim, 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 centimeters. 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.

C) 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. Aspetically 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.

(i) Assay of Pectin Methyl Esterase (Exo-PME):

The PME was estimated by the method suggested by Kartez (1937). Ten ml of enzyme preparation from different incubation periods was taken and added with 5 ml of distilled water followed by 25 ml of 1% pectin solution in 250 ml Ehrlen Meyer conical flask. Two drops of methyl red indicator was added to the reaction mixture and adjusted the ph to 6.2 with the help of 0.1N naoh and incubated for 3 hours.

During the digesting period enough 0.1N naoh was added to the reaction mixture to prevent it from turning pink at every 10 minutes and recorded the volume of naoh consumed. The amount of methoxyl groups (in mg) which got split off during the reaction time by 1 ml of enzyme was calculated by the following formula.

$$\text{Number of methoxyl groups split off by one ml of enzyme} = \frac{\text{Total ml of 0.1N NaOH consumed}}{\text{Total volume of enzyme used}} \times 3.1$$

Ii) Assay of Exo-Polymethyl Galacturonase (Exo-PMG):

Exo-PMG was estimated by DNS method (Miller, 1959).The following solutions were prepared and used in the assay of exo-PMG.

(i) Pectin solution (0.5%) (0.5 g / 100ml DW) (ii) Citrate buffer-ph (5.5) (dissolved 35.6 g of $Na_2HPO_42H_2O$ in 1000 ml distilled water to prepare solution A and dissolved 19.21 g of citric acid in 1000 ml of distilled water to prepare solution B and mixed 580 ml of solution A and 420 ml of solution B to get citrate phosphate buffer of 5.5 ph) (iii) DNS reagent (dissolved 1g of DNS, 200 mg crystalline phenol and 50 mg sodium sulphate in 100 ml of 1% naoh solution) (iv) Na-K tartarate solution (20%); 20 g Na.K Tartarage in 100ml DW.

3.5 ml of 0.5% pectin 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^{o}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 using 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 Methyl Galacturonase (PMG) activity was expressed in terms of mg of reducing groups (as mg/ml) liberated in 6 hours.

(iii) Assay of Exo Poly Galacturouase (Exo-PG):

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^{o}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 using spectrophotometer. A blank was prepared with the same procedure by using a heat killed enzyme.

(iv) Assay of Exo-Pectin Lyase (Exo-PL)

The exo-PL was assayed *in vivo* and *in vitro* by the method suggested by Sherwood (1967). The reaction mixture in exo-PL consisted of substrate (pectin –1%), tris-hcl buffer (dissolved 24.2 grams of tris in 100 ml distilled water to prepare solution – A; 16.1 ml of hcl made up to 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 8.1) and enzyme in 4:1:2 ratio. The contents were taken into a test tube and incubated at 30^{o}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 Thio Barbutaric Acid reagent (3.5 ml TBA solution, 1.5 ml 1N hcl, 0.5 ml 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 using spectrophotometer. A blank was prepared with the same procedure, but boiled enzyme was taken in place of active enzyme.

(v) Assay of Exo – Pectic Acid Lyase (Exo-PAL):

The exo-PL was assayed *in vivo* and *in vitro* by the method suggested by Sherwood (1967). The reaction mixture in exo-PL consisted of substrate (pectic acid –0.5%), tris-hcl buffer (dissolved 24.2 grams of tris in 100 ml distilled water to prepare solution – A; 16.1 ml of hcl made up to 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 8.1) and enzyme in 4:1:2 ratio. The contents were taken into a test tube and incubated at 30^{o}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 Thio Barbutaric Acid reagent (3.5 ml TBA solution, 1.5 ml 1N hcl, 0.5 ml 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 using spectrophotometer. A blank was prepared with the same procedure, but boiled enzyme was taken in place of active enzyme.

(vi) Extraction of more juice from fruits:

Ripened and healthy Apple, Sapota, Mango, Grapes, Orange and Tomato fruits were collected from the fruit markets in and around Warangal and Hanamkonda and immediately carried to the laboratory.

The fruits were washed and cleaned with muslin cloth and chapped in to small pieces (5 mm x 5mm x 5mm). The 50 g of fruit pieces / pulp was transferred in to four beakers each and was added with 2ml of commercial pectinase enzyme, culture extract six fungal strains and distilled water. The beakers were stirred thoroughly and incubated for 15-20 minute the room temperature. After incubation and extraction of the total juice from the pulp the resultant clear fruit juice solution was filtered through glass funnel containing 3-4 folds of muslin cloth and the volume was quantified. Based on the obtained quantities with commercial enzyme, culture extract with pectinase enzyme was evaluated and compared. The fruit juice extracted with distilled water served as control. The fruit pulp was also extracted after heat treatment and the volume of fruit juice was compared with the normal treatments.

Results

Exo PME:

From the results it was evident that *Aspergillus flavus* found to be potential in production of PME, after 14 days of incubation period, in pectin supplemented Ashthana and Hawkers broth (1.24 mg/ml). *A. Niger* also showed high PME activity after the same incubation period (0.95 mg/ml). The PME activity was increased by 1.8 mg/ml. In *Penicillium citrinum* in 21 days of incubation; while remaining organisms showed maximum PME activity in 14th day of incubation.

Table - 1 : Exo-Pectin Methyl Esterase (PME) activity of six fruit rot fungi on Ashthana & Hawker's medium supplemented with pectin after 7, 14 and 21 days of incubation

Fungi	No. of methoxyl group split by 1ml of enzyme after incubation period (days)		
	7	14	21
Rhizoctonia solani	0.60	0.80	0.60
Penicillium citrinum	0.60	0.70	1.80
Mucor racemosus	0.60	0.56	0.16
Rhizopus stolanifer	0.30	0.16	0.16
Aspergillus flavus	0.93	1.24	0.62
A. niger	0.87	0.90	0.62

Table - 2 : Exo Pectin Methyl Esterase (Exo PME) activity of six fruits rot fungi on six fruits after 4,8,12,16 and 2,4,6,8 days of incubation

No. of methoxy groups split by 1 ml enzyme

Fungi	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	0.62	0.84	1.08	1.40	0.93	2.02	1.24	0.93	0.62	0.90	1.24	1.24	2.17	2.48	2.01	1.86	1.86	3.10	3.70	2.90	1.18	1.86	1.39	1.11
Penicillium citrinum	0.37	0.56	0.78	0.78	0.78	1.55	1.08	1.08	0.78	0.93	1.08	1.24	1.70	2.63	1.70	1.39	1.86	3.10	2.60	2.00	1.24	1.55	1.55	1.27
Mucor racemosus	0.48	0.62	0.78	0.93	0.62	2.17	1.18	1.08	0.78	0.90	1.00	1.24	1.86	2.17	2.01	1.86	2.17	3.40	2.80	2.00	0.93	1.86	1.86	1.08
Rhizopus stolanifer	0.81	1.18	1.24	1.55	0.93	1.86	2.02	1.40	1.71	1.55	1.08	1.08	2.01	2.17	2.01	1.70	2.48	3.70	2.90	2.20	1.08	1.86	1.86	1.11
Aspergillus flavus	0.62	1.24	1.24	0.87	0.78	1.40	1.71	1.40	1.71	1.33	0.93	0.93	2.01	3.10	1.70	1.70	2.79	4.50	3.40	2.60	0.93	1.86	1.70	1.18
A. niger	0.40	0.78	0.93	1.86	0.68	1.71	2.48	1.53	1.55	1.24	1.08	0.87	1.86	2.63	1.86	1.86	2.94	4.30	3.80	3.10	1.24	1.86	1.55	1.08

The results showed that with the increase in the incubation period in apple extract, the PME activity was increased by *Aspergillus niger*, *Rhizopus stolanifer*, *Rizoctonia solani* and *Mucor racemosus*, The lowest PME activity (0.37 mg/ml) was recorded by *Penicillium citrinum* at 4th day of incubation while the maximum was recorded at 12th day of incubation by *Aspergillus flavus* 1-24 mg/ml) and *Aspergillus niger* (1.86 mg/ml).

In the Mango extract the lowest PME activity was recorded (0.02 mg/ml) in *Mucor racemosus* in 2nd day of incubation, while the maximum was recorded at 6th day of incubation (2.48 mg/ml) by *A. niger.*

In the Sapota fruit extract the PME activity was lowest (1.39 mg/ml) was observed in 8th day of incubation by *Penicillium citrinum*, while the maximum was recorded at 4th day of incubation by *A. flavus* (3.10 mg/ml) and by *A. niger* (2.63 mg/mol.).

In the Grapes extract PME activity was maximum at in *Aspergillus flavus* (4-5 mg/ml) *A. niger* (4.3 mg/ml) and on the tomato extract the maximum PME activity (1.71 mg/ml) was observed in 2nd day of incubation by *Rhizopus StolanIfer* and in *A. flavus* and in *Orange* fruit, extract maximum activity (1.86 mg/ml) showed by four organisms except *Penicillium citrinum* (1.55mg/ml) after four days of incubation.

Exo PL:

The maximum exo-PL was in 21 days of incubation which gradually increased from 7 days of incubation, while *A. niger* showed its maximum exo-PL Activity in 14 days. In general 21 days of incubation was viewed to be ideal for optimum enzyme production. The highest was recorded in *P. citrinum* (70.2 U/ml) in 21 days of incubation, the next was *Rhizoctonia Solani* (62.5 U/ml). Among the six fungi *A. flavus* was responsible for least production (45U/ml). In view of these results it is suggested that for maximum area exo PL production the ideal incubation is 21 days in Asthana and Hawker's medium supplemented with pectin (Table 3).

Table - 3 : Exo pectin lyase (Exo PL) of six fruit rot fungi on Ashthana & Hawker's medium supplemented with pectin after 7, 14 and 21days of incubation

Fungi	Exo PL*		
	7	14	21
Rhizoctonia solani	10.5	19.0	62.5
Penicillium citrinum	22.5	27.5	70.2
Mucor racemosus	17.0	48.5	56.5
Rhizopus stolanifer	42.0	47.0	51.5
Aspergillus flavus	41.0	43.0	45.0
A. niger	55.0	58.5	48.5

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

Table - 4 : Exo-Pectin lyase (Exo-PL) activity of six fruit rot fungi on six fruits after 4,8,12,16 and 2,4,6,8 days of incubation

Fungi	Exo-PL*																							
	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	13.5	92.2	13.5	3.5	0.18	0.48	0.19	0.07	4.8	25.0	7.0	5.7	2.5	13.0	5.5	3.5	1.0	1.3	9.1	12.0	5.0	9.0	17.0	52
Penicillium citrinum	9.5	11.0	7.5	1.5	0.43	0.41	0.21	0.08	13.0	52.0	17.0	9.0	5.6	14.0	8.0	5.0	0.6	0.8	17.1	50.0	11.0	16.0	21.0	42
Mucor racemosus	8.0	5.1	3.5	1.0	0.23	0.45	0.22	0.04	40.6	33.0	28.0	5.5	5.0	26.0	14.0	13.0	--	0.5	37.0	89.0	16.0	22.0	31.0	37
Rhizopus stolanifer	13.5	3.2	2.5	1.1	0.02	0.20	0.06	0.04	45.0	35.0	13.0	2.0	2.0	20.0	6.0	4.0	--	0.4	23.0	56.0	9.0	15.0	33.0	47
Aspergillus flavus	12.5	8.7	8.0	2.9	0.02	0.16	0.15	0.03	44.0	13.0	12.0	10.0	1.8	11.0	3.0	3.0	2.0	6.4	38.0	39.0	9.0	16.0	21.0	31.0
A. niger	24.0	5.4	3.5	2.8	0.03	0.19	0.08	0.04	40.6	20.0	3.0	1.0	3.5	11.0	8.0	6.0	3.0	8.0	38.0	38.0	52.0	71.0	85.0	46

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

The exo-PL in vivo was studied in six fruits and the results are shown in Table 4. The apple fruits, showed the maximum activity during eight days of Incubation (29.2U/ml) by *Rhizoctonia solani.* The lease producer was *M. racemosus* with 8 U/ml after four days of incubation.

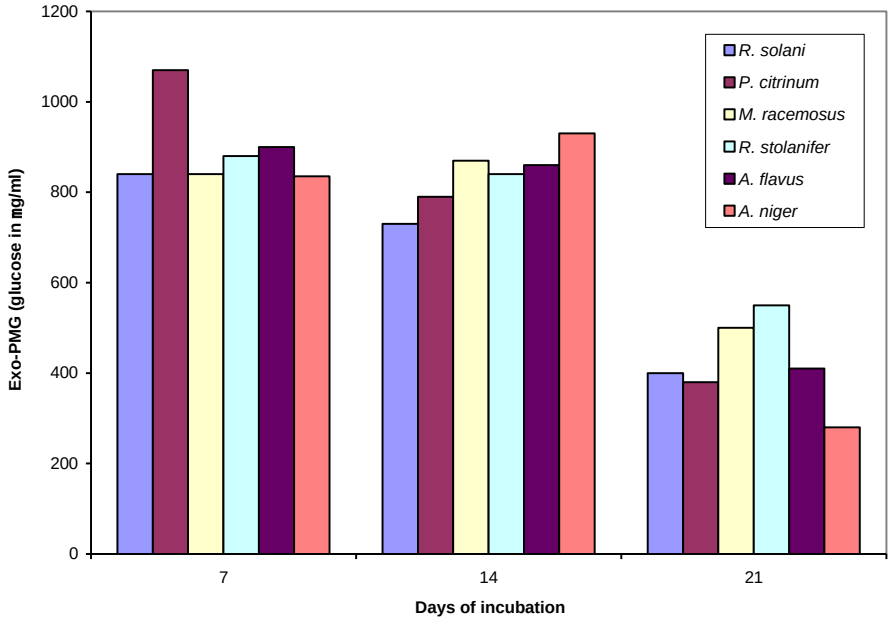
In the *Mango* fruit, the maximum exo PL was in 4 days of incubation (0.48 U/ml) in *R. solani*, the least in *A. flavus* (0.16U/ml) after four days. In tomato fruit the maximum production was obtained by *P. citrinum* (52U/ml) and the least by *R. Solani* (25 U/ml) after four days of incubation. In Sapota fruit, six fungi showed their maximum exo PL activities after four days and growth rate of fungi and enzyme activity decreased upto 8 days of incubation under pathogenesis. The maximum was in *M. raceumsus* (26 U/ml) and next in *R. stolanifer* (20 U/ml), being the least in *A. flavus and A. niger* with production rate of 11U/ml for both. In the Grape fruit, the six fungi showed their maximum exo-Pl activity after 8 days of incubation with increasing trend. The highest was recorded in *M. racemosus* (89 U/ml) and the least in *R. solani* (12 U/ml) after eight days of incubation. In the Orange fruit six fungi showed their maximum exo-PL production after eight days of incubation. With gradual increase in growth rate and enzyme production.

The highest was noticed in *A. niger* (85 U/ml) after six days, while the least in *A. flavus* 31U/ml) after 8 days of incubation. Quick spoilage of the fruit vigorous infection and rapid growth of the fungi was reasons for decrease of exo-PL activity, with increase in incubation time in all the fruits.

Exo PMG

The production of exo-PMG by six fruit not fungi during 21 days of incubation was assayed in 7, 14, and 21 days and presented in Figure 1. from the figure it was evident that the maximum exo PMG was noticed in *Penicillium citrinum* during its 7 days of incubation (1070 mg/ml), but in general the 14 days incubation was viewed to be ideal for optimum production and subsequently 21 days the production rate was decreased. The next best organisms were *A niger* (930 mg/ml) after 14 days and *A. flavus* after 7 days (900 mg/ml). In the 21 days of incubation the growth rate was decreased and subsequently the enzyme production was also declined, which ranged from 280 to 550 mg/ml six fungi. During 7 days of incubation the fungi were initiated their growth and acclimatized for the condition and started production of exo-PMG which ranged between 835 to 1070 mg/ml. *P. citrinum* and *A. flavus* were showed their maximum production in 7 days of incubation (1070 and 900 mg/ml). In view of these results it was noticed that for maximum exo-PMG production the ideal incubation period is between 7-14 days in Asthana and Hawker's medium supplemented with pectin.

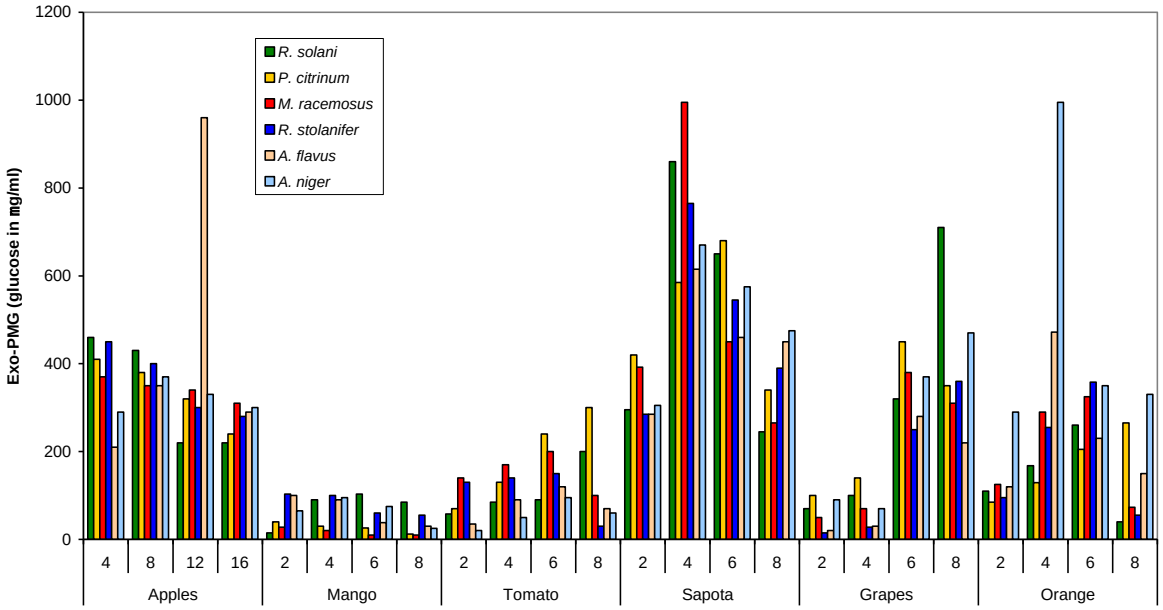
Fig. 1 : Exo Poly Methyl Galaturonase (Exo-PMG) activity of six fruit rot fungi on Ashthana & Hawkers medium supplemented with pectin after 7, 14 and 21 days in incubation



***In vivo*:**

The exo-PMG *In vivo* was studied in six fruits and the obtained results were incorporate in Fig. 2. The figure clearly indicated that the production rates were increased up to 16 days and subsequently the quantities were decreased. The apple fruit was incubated after the inoculation by six fruit not fungi and after 4, 8, 12 and 16 days of incubation, the fruit pulp was assayed for exo PMG Activity The maximum activity was recorded during 12 days of incubation (960 mg/ml) by *A. flavus.*

Fig. 2 : Exo-polymethyl galacturonase (Exo-PMG) activity of six-fruit rot fungi on six fruits after 4, 8, 12, 16 and 2, 4, 6, 8 days of incubation

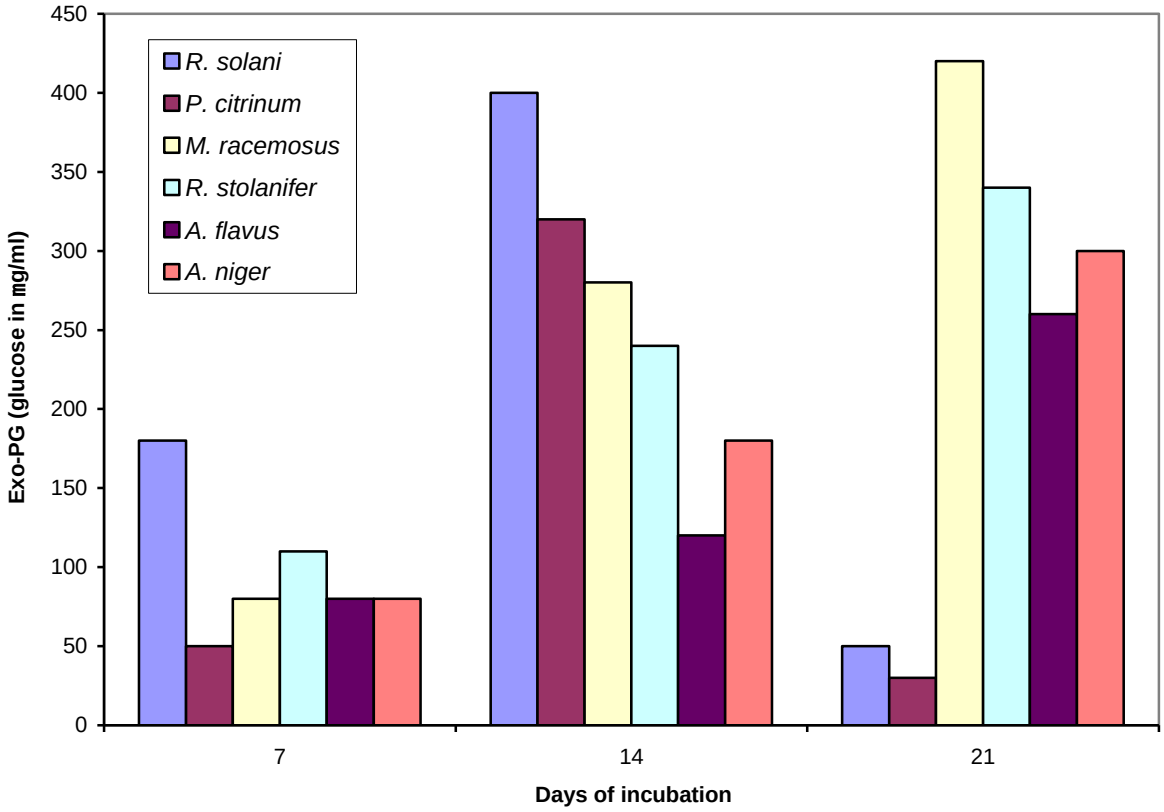


In mango fruit maximum exo PMG was reported in six days of incubation (103 mg/ml) by *R. solani* and *Rhizopus stolanifer*. The moderate activity was noticed in *A. flavus* after 2 days (100 mg/ml) and *A. niger* (95 mg/ml) after four days. In Tomato fruit the highest exo PMG activity was obtained after eight days of incubation period in *R. Solani* and *P. citrinum* while remaining four organisms showed after six days of incubation and enzyme activity was declined up to 8 days. The highest exo-PMG was recorded in *P. citrinum* (300 mg/ml) after 8 days of incubation. In *Sapota* fruit the highest range was recorded in four days of incubation period and highest (995 mg/ml) *Mucor racemosus* there was much decline in the production rates during 8 days of incubation and their range between 245 to 475 mg/ml. In the Grape fruit the maximum exo-PMG was noticed during 8 days of incubation in *R. Solani* (740 mg/ml). Moderate activities was showed by *A. niger* (470 mg/ml) after 8 days and *P. citrinum* (450 mg/ml) after six days of incubation periods. In the orange fruit the maximum exo-PMG activity was (995 mg/ml) was noticed after 4 days of incubation in *A.niger* and activity was much declined up to eight days. *A. flavus* (472 mg/ml) showed moderate activity after four days of incubation

Exo-PG

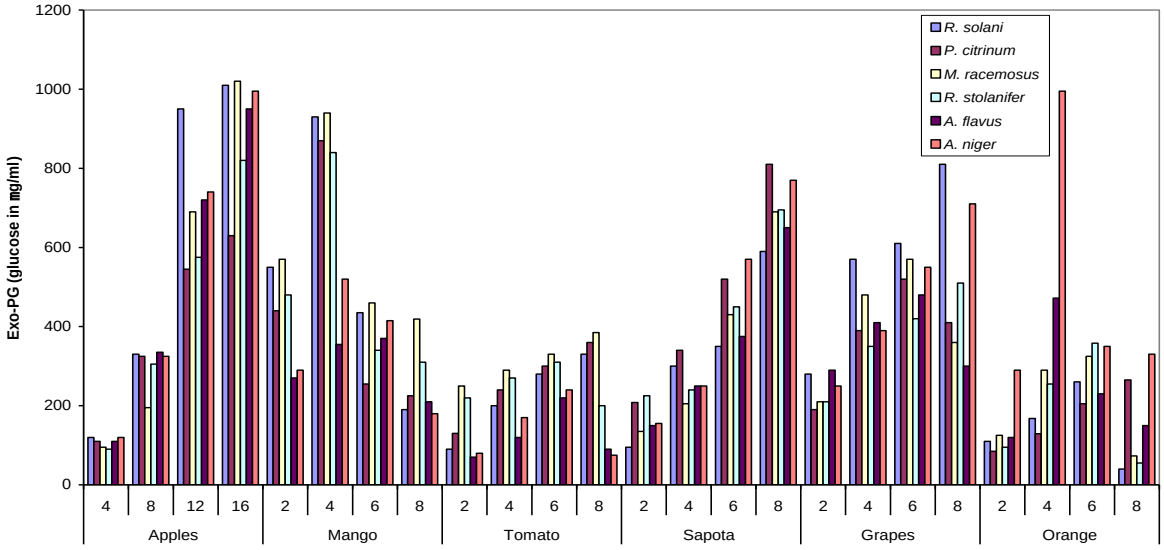
In vitro studies the exo PG was maximum in *Mucor racemosus* during its 21 days of incubation (420 mg/ml). The next best organism was *R. Solani* (400 mg/ml) while *A. flavus* was responsible for least enzyme secretion(260 mg/ml) after its 21 days of incubation (Fig. 3).

Fig. 3 : Exo Poly Galaturonase (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 exoPG production in six fruits was studied and presented in Fig.4. In *Sapota* fruit the highest exo-PG (995 mg/ml) was noticed in *M. racemosus* after its 4 days of incubation and less production rate was recorded in *A niger* (590 mg/ml) after its 6 days of incubation. In the Grape fruit the highest production was noticed in *R. solani* (710mg/ml) after its 8 days of incubation. In Orange fruit the maximum exo-PG (995mg/ml) was noticed in *A. niger* during its 4 days of incubation, while the least production was noticed in *R. solani* (260 mg/ml) after its 6 days of incubation period. In the Mango fruit Exo-PG maximum was reported in four days of incubation in five fungi, while only one organism i.e., *A. flavus* showed its maximum activity after six days. The highest Exo PG was noticed in *M.racemosus* after its 4 days of incubation (930 mg/ml). The next best production was showed by *R.solani* after four days of incubation (910 mg/ml). The least production was showed by *A. flavus* after its 6 days of incubation (370 mg/ml). In *Tomato* fruit, the highest exo PG was recorded in *M. racemosus* (385 mg/ml), after 8 days of incubation. The next best production was noticed in *P.citrinum* (360 mg/ml), after its 8 days of incubation. The least production rate was noticed in *A. flavus* (220 mg/ml), after its 6 days of incubation period.

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



The apple fruits were incubated after the inoculation by six fungi after 4,8,12 and 16 days of incubation and the pulp was assayed for exo-PG activity. The maximum activity was recorded during 16 days of incubation (1020 mg/ml) by *M. racemosus,* while *R.solani* was the next best producer of exo-PG (1010 mg/ml) and the least producer among the six fungi was *P.citrinum* with the production rate of (630 mg/ml)

Exo-PAL:

The production of Exo-PAL by fruit rot fungi during 21 days of incubation was assayed in between 7,14 and 21 days and prescribed in Table: 5. From the table it was evident that the maximum (27 U/ml) exo-PAL was noticed in *P.citrinum* during its 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). 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 pectin) was 14 days.

Table – 5 : Exo pectic acid lyase (Exo PAL) activity of six fruit rot fungi on Ashthana & Hawker's medium supplemented with pectin after 7, 14 and 21 days of incubation

Fungi	Exo-PAL*		
	7	14	21
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)

Table - 6: Exo-Pectic Acid Lyase (Exo-PAL) activity of six fruit rot fungi on six fruits after 4,8,12,16 and 2,4,6,8 days of incubation

Fungi	Exo Pectic Acid Lyase*																							
	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	5.1	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)

The exo PAL production *in vivo* was studied in six fruits and the obtained results were incorporated in table 6. 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 six fungi was *R. stolanifer* with production rate of 24 U/ml after 16 days.

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* (21.5 U/ml) after 4 days and the activity was gradually decreased up to 8 days. In tomato, the highest production rate was 52 U/ml after four days in *R. stolanifer*, *A. niger*, while low range of activity was observed in *P. citrinum* (33U/ml) after 6 days.

In sapota fruit, the highest exo PAL was recorded in four days by all fungi. The maximum (34 U/ml) enzyme was noticed in *P. citrinum* and the least (19.5U/ml) in *A. niger* and *R.solani.* Interestingly the growth rate was decreased along with production rate upto 8 days (8 to 26 U/ml). In the grape fruit 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 PALwas maximum after six days by 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 produced (26.5 U/ml) and lowest activity (13.5 U/ml) was recorded in *P.citrinum, A. flavus* and *A. niger* after six days.

Discussion

Exo-PME

Crotti *et al* (1999) observed the Pectin Esterase activity in a passion fruit peel, lemon or orange peels after 3-6 days of incubation. The present results are in accordance with the above results and Orange fruit extract showed the maximum PME activity during 4-6 days of incubation.

PME, Poly galactoronase, pectate, lyase were present during ripening (Nunan Kylic *et al* 2001), Our studies showed that Pectin Methyl Esterage was present during ripening of different fruits, this supports the pectinase activity.

Vinod Kumar *et al* (2006) Observed the maximum PME activity at 96 hours of incubation period from Apple pomace by *Aspergillus niger* and when apple pomace medium was supplemented with Ammonium

Sulphate. In our results also showed the maximum activity in Asthana and Hawker's medium supplemented with pectin by *A. niger* and in apple fruit extract after 96 hours of *A. flavus* and *A. niger*.

The maximum Pectin Methyl Estate was 447.93 U/g dry substrates (Yellow Passion fruit peels) and pH 3.5 at an optimum temperature of 30°C. The PME was stable at 3.5 to 5.0 at 30 to 60°C (Marilia *et al* 2014). PME can be classified on the basis of presence or absence of the PRO domain into type-I and Type-II, (500-900 amino acids, 52-105 KDa). There are 1-3 PRO domains and two or three introns, whereas PME Type-II (250 to 400 amino acids, 27-45 KDa) contain no PRO domain with five or six introns (Pooja Kohli *et al* 2015).

Within 30 min. the juice methanol concentration increased from 35 to over 400 mg/g, while the pH dropped from 4.45 to 4.20 on average (Gordon and Diane 2012). In creased resistance was related to the trade ability of this fungal pathogen to grow on methyl esterified pectin and to a reduced activity of the fungal PG to hydrolyse methyl esterified pectin (Chiara *et al* 2011).

The activity of PME has been enhanced by NaCl particularly at 0.15M. Km and Vmax values for Alyanak apricot PME using apple as a source was found to be 1.69 mg/ml and 3.41 Units/ml respectively. The enzyme was maximum at 30-45°C / 10 min. whereas it lost nearly all of its activity at 80°C /10 min. (Omitnal & Aysun 2015).

It was concluded that among six fruit rot fungi *A. niger* and *A. flavus* were proved to be ideal in PME production in pectin supplemented Asthana and Hawker medium and can be used in fruit juice technology and grape fruits, Sapota fruits and Mango fruits also used as pectin source for industrial production of PME.

Exo-PL:

Szajar and Szajer (1982) clarified the apple juice by pectin Lyase from *Penicillium paxilii* and stated that enzymatic clarification was stimulated by Ca^{+2} ions in 10^{-2}M concentration, but according to the present studies not only the purified enzyme even crude enzymes also are most useful to clarify the fruit juice.

Valle *et al* (2001) Stated that the capacity of microorganisms to produce extra cellular enzymes was influenced by environmental conditions such as temperature, pH, medium inoculum age and the presence of inducer or repressor as a substrate.

Production of PL by three isolates of *Syncephalastrum racemosum* isolated from fruit rot of lemon, orange and mosombi was studied by Jagadeesh and Reddy (2006) and stated that orange isolate showed ExoPL (25 U/ml) after 8 days of incubation in Asthana and Hawkers medium. In the present studies also maximum exo-PL was noticed (89U/ml) by *Mucor racemoses* after 8 days of incubation but *A niger* showed maximum (exo-PL 85 U/ml) after 6 days of incubation.

Sangeetha *et al* (2008) purified and characterised Alkaline PL from *Aspergillus flavus* and striated that the enzyme was found to be stable for 24 hours in the pH range 4-10. Sara *et al* 2009 hydrolysed the Orange peel by PL of *Aspergillus flavipes*) *A. niveus* and observed that the PL was stable to hydrolyse 56% of orange peel biomass with potential application in the pectin industry.

The optimum Pectin Lyase production was analysed from *Aspergillus niger* after 96 h at 30°C, 8pH, 4ml inoculum and 0.1 % peptone Tween 80 was used as a surfactant and showed negative effect on Pectin Lyase production. It was purified by the addition of 60% of ammonium sulphate and showed maximum activity at 30°C and 8 pH (Batool *et al* 2013). The production of PL was substantially induced up to the level of 875 U/ml, when fermentation medium of Lemon peel waste inoculated with 5ml spore suspension of *Aspergillus oryzae* (Koser *et al* 2014).

A pectin lyase named PL-III was purified from the culture filtrate of *Aspergillus giganteus* grown in submerged culture containing. Orange peel waste as a carbon source, PL-III was able to digest apple pectin and citrus pectins with different degrees methyl esterification. Interestingly the PL-III activity was stimulated in the presence of some divalent cations including pb2+ and was not significantly affected by Hg^{+2} (Pendrolli and Carona, 2014). 2-deoxy-D-glucose can be used for the isolation of catabolite repression resistant mutants in *Penicillium griseoroseum* for the first time, pH over producing mutants in *P. griseoroseum* was reported, mutation leading to 2-deoxy Dglucose resistance in the mutants could be related to the glucose dessication that increase the endogenous C-Amp level resulting in higher PL production (Lima *et al* 2017).

It is concluded that among six fruit rot fungi, *P.citrinum* and *R.solani* were ideal in exo-PL production in Pectin supplemented Asthana and Hawkers medium in 21 days of incubation which can be used in fruit juice technology.

Exo-PG

The maximum production of PG (500U/ml) was reported in *Aspergillus japanicus* when the fungus was grown on liquid medium containing pectin and galactose (Teixeira *et.al.* 2000). In this present study pectin supplement Asthana and Hawker's broth also showed the maximum production of PG. The clarification of grape juice was greatly effected by *Aspergillus niger* and clarified juices *Penicillium citrinum* was found to be the was stable upon storage 4°C and 25°C Mohsen *et al* 2009). In the present study Asthana and Hawker's medium supplemented with pectin acid or fruit juice as source of nutrients increased the Exo-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 (Ivana *et al* 2013). In the present studies also the genous *Aspergillus* with two species, *A. flavus* and *A.niger* produced substantial amounts of exo and endo P.G. *Colletotricum truncatum* strain BAFC 3097, yielded high titles of the PGase (108U/ml) after a short incubation period 7 to 10 days with (Aracelli *et al* 2010).

Penicillium citrinum was found to be the potent source for pectinase, Polygalacturonase and Pectin Lyase production was higher in solid state fermentation by using wheat bran as the solid substrate and submerged fermentation at room temperature and large amount of agro-industrial residues and Orange peel can be used as a carbon source for pectinase production in large scale (Ramachandran Sandhya and Kurup 2013).

A thermophilic fungi *Rhisomuco pusillus, PG age* was optimally active at 55°C and pH 5.0. It was stable upto 50° for 120 minutes of incubation and pH condition between 4 and 5 (Siddiqui *et al* 2013). The average PG production was 36.5U/ml, which was enhanced by 4.1 fold, the production from Mango peel waste with optimized submerged fermentation using *A. foetidus* (Sudheer *et.al.,* 2014). The highest production levels obtained by *A. soze* using sugar beet as a carbon source with yield of 1111 and 449U/g for exo and endo pG respectively (Marco *et al* 2015).

The production of P.G. by *Aspergillus oryzae* RR103 was enhanced by optimization of different nutritional parameters. Highest PG activity was observed at 192 hours at 37°C. Optimum pH for the production of enhanced polyglacturonic acid was at 6.0, the carbon and Nitrogen sources, which gave enhanced enzyme activity, were cellulose and mal extract respectively. Highest enzyme specificity was recorded when the substrate used as Citrus Pectin. *PGase* from

Aspergillus oryzae RR 103 is suitable for industrial scale production as it has high productivity (Ravi and Raghuram 2018). Treatment with NaHCo3 under Alkaline pH delayed the polygalactunase activity for 72 hours (Tullionenditti, *et al* 2018).

The Pectinases (PG) secreted by two 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.

Exo-PMG

Gimgihong *et al* (1991) identified the Pectinolytic enzymes production and activity by *Botryosphaeria dothedia* and stated that exoPG and Exo PMGin apple medium showed maximum activity upto 6.4 to 7.2 units at six days of culture respectively. But in our studies the maximum exo PMG activity was noticed in Asthana and Hawker's medium supplemented with pectin by *Penicillium citrinum* after seven days of incubation period and in apple fruit the exo-PMG activity was maximum (916mg/ml) after 12 days by *A.flavus.* Levin and Forshiasson (1998) noticed by the white rot fungi *Tremetes trogii* and good results were obtained in growth as well as in enzyme production and the addition of Tween 80 promoted the growth and gave the highest yield of PMG and PL 0.32 and 36.2 U/ml). But in our investigation pectin promoted the growth and gave the highest yield of PMG in Asthana and Hawkers broth supplemented with pectin. The exo PMG from *Aerophilophora nainiana* was most active at pH 7.0 and 60°C, while the exo PMG optimal activity from *Trichoderma. harzianum* strain T6 was obtained at pH 4.3 and 40°C (Celestino and Filho 2005). The mixture of two substrates such as sugar beet pulp and wheat bran, having different ratios of carbon, nitrogen and moisture levels were used for the highest PG and PMG production under solid state conditions were determined on wheat bran (Tuskin and Eltem, 2008).

Colletotrium truncatum strain BAFC 3097, yielded high 1.05U/ml PMG. The disparity observed in enzyme productivity among strains cannot be related to fungal growth. Since no major difference in mycelial yield were found, it was not connected with their geographic origin, but might be associated with different in virulence among strains not yet evaluated (Araceli *et al* 2010) With increasing incubations time, the activity of three enzymes i.e., cellulose, PG and PMG has been enhanced in *Fusarium oxysporum* (Shukla and Dwivedi, 2012).

Among pectin degrading enzymes PMG is known to be secreted by pathogenic fungi (Shridha Chourasia *et al* 2015). Maximum inhibition of PME, and exo and endo PMG and pectin transeliminase was showed by culture filtrate of *Trichoderma viridae* + Psueduomonas fluroscens (Rajeshwari and Kapoor, 2017). The maximum exo-PMG was noticed in *Penicillium citrinum* (1070mg/ml) during its 7 days of incubation and in vivo studies. The maximum activity was in Sapotas fruits 995 mg/ml by *Mucor racemosus* and in Orange fruit by *A.niger* (Suryam *et al* 2018).

It was clearly noticed that, the exo-PMG from these four fungal strains i.e., *Aspergillus niger, A flavus, P.citrinum, and M racemosus* strains is very effective and replace the application of costly commercial enzymes in classifications and extraction of fruit juice.

Pectic Acid Lyase (PAL)

PAL exhibited gradual increase in enzyme activity and *A. flavus* showed maximal production (42.69 and 36.19U/ml) at 1M KCl and NaCl respectively (Makkey 2009). But in our studies, we found that the maximum exo PAL (52 U/ml) produced by *A. flavus* after two days in tomato fruit, without KCl as a stresser. PAL plays an

important role in plant pathogensis. 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). The maximum activity of enzymes from solid state fermentation (SSF) was observed at 35°C, but crude enzyme was more thermo tolerant than PL-III, maintaining its maximum activity up to 40°C (Viviani *et al* 2010). PAL is a kind of enzyme that is abundantly used in to the textile industry for cotton souring. Previously the PAL gene to enhance the production of PAL combined strategy was formulated by combining on line methanol control, two stage pH Control strategies and the strategy proved to be very useful for the enhancement of PAL production (Qureshi *et al* 2010). PAL activity was up to 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 PAL (Wang *et al* 2010). The 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 in *A.niger* (Suryam *et al* 2018).

The funal strains *Penicillium citrinum, Rhizopus stolanifer, Aspergillus flavus and A.niger* are optimal producers of ex PAL and most useful in fruit juice extraction and clarification.

Conclusions

It was concluded that among six fruit rot fungi *Aspergillus niger* and *A. flavus* were to be ideal in exo PME, *Exo PG* exo PMG, exo PAL. Production and *Penicillium citrinum Rhizoctonia solani, Rhizopus stolanifer* are the moderate producers in Asthana and Hawker's medium supplemented with pectin and pectic acid most useful in fruit juice extraction and clarification and shall pave the way for commercialization and exploitation of these enzymes in fruit juice industries.

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