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Studies on production of pectolytic enzymes by Guignardia citricarpa Kiely

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Abstract

A pure culture of **Guignardia citricarpa Kiely** isolated in this laboratory from diseased mandarin oranges was found to be pathogenic not only to mandarin oranges but to many other fruits. The organism often referred to as black spot pathogen of citrus fruits. The pectolytic enzymes were expected to be produced by **Guignardia citricarpa Kiely**, as the organism mainly affects the citrus fruits which are rich in pectins. Experiments were conducted to study the production of some pectolytic enzymes i.e. polygalacturonase (PG) & polymethylgalacturonase (PMG) by **Guignardia citricarpa Kiely**. The organism elaborated considerable amount of PG & a small amount of PMG. The present study deals with the production of these enzymes on different parameters such as effect of period of incubation, incubation time, pectin concentration & initial pH. The optimum initial pH of the medium for the production of PG & PMG was found to be 6.0, whereas the optimum incubation period required by PG was found to be 20 days & that by PMG was 15 days. Maximum production of these enzymes was observed when the reaction mixtures were incubated for 4 hours.

Key words – **Guignardia citricarpa**, Mandarin orange, PG, PMG.

Introduction

Pectolytic enzymes have been attracting considerable attention of plant biochemists. Such enzymes are a powerful weapon which enable the pathogen to invade the host tissues, leading to successful pathogenesis. Microorganisms are one of the most economical production units of several enzymes, devised by nature. Several workers [5 – 8] have reported that fungi & bacteria which induce rot in fruits have a characteristic property of producing pectolytic enzymes. The present study indicated that the organism **Guignardia citricarpa**, first reported by Kiely [1], is capable of producing pectolytic enzymes, both PG & PMG, extracellularly. PG (EC 3.2.1.15) is an enzyme that hydrolyses α – 1,4 linkages between the galacturonic acid residues in polygalacturan, a significant carbohydrate component of the pectin network that comprises plant cell walls [4]. The action of PMG (EC 3.2.1.67) is same as that of PG, the only difference is that it hydrolyses α – 1,4 linkages between the esterified galacturonic acid residues. Experiments were conducted to establish optimum culture conditions for the extracellular production of PG & PMG by **Guignardia citricarpa Kiely**.

Materials & Methods

After scrutiny of different media, Asparagine-glucose medium consisting of KH_2PO_4 (0.30 %), Asparagine (0.20 %), $MgSO_4.7H_2O$ (0.05 %) with pH (5.0) was found to be most suitable after supplementation with pectin/glucose (0.5 %) as the carbon source [9]. Fifty ml of medium in 250 ml conical flask was inoculated by spore suspension (2 x 10^6 spores/ml) of **Guignardia citricarpa**, prepared in sterile distilled water & was incubated at a temperature of $30^0C \pm 2$ for 10 days.

Harvest of enzyme

Contents of the flask were filtered in cold after incubation through Whatman No. 41 filter paper & centrifuged at $0 – 4^OC$ at 5000 rpm for 10 minutes to get a clear liquid.

Enzyme assay

Culture filtrate obtained after incubation was tested for the presence of PG & PMG. PG activity was assayed by the method of Pressey & Avants [10] containing polygalacturonic acid (M/s Sigma Chemical Co., U.S.A.) as substrate. PMG activity was assayed by the same method using citrus pectin (M/s. Sigma Chemical Co., U.S.A.) as substrate in place of polygalacturonic acid. Reducing groups, in both the cases, liberated were estimated by Nelson's method [2]. Proper boiled enzyme as zero-time controls were run simultaneously. One unit of PG & PMG activity was defined as the amount of enzyme which liberates 1 mg galacturonic acid /hour, under the assay conditions.

Specific activity was expressed as units/mg protein. Proteins were estimated by the method of Lowry et al [3].

Effect of period of incubation on production of PG & PMG

Fifty ml of sterile medium containing 0.5 % glucose & 0.5 % pectin as the carbon source was inoculated in 250 ml conical flask with spore suspension of **Guignardia citricarpa** & activities of both PG & PMG were estimated after 5, 10, 15, 20 & 25 days of incubation.

Effect of incubation time on production of PG & PMG

The reaction mixture was incubated at 37^0C for up to 6 hours & the reducing groups liberated were estimated.

Effect of pectin concentration on production of PG & PMG

Activities of PG & PMG were studied at 0.1 %, 0.2 %, 0.3 %, 0.4 %, 0.5 % & 0.6 %pectin concentration. The reaction mixtures were incubated for 10 days.

Effect of initial pH on production of PG & PMG

Reaction medium was adjusted at pH – 5.0, 5.5, 6.0, 6.5 & 7.0 values before autoclaving. After 10 days of incubation, activities of both PG & PMG were determined.

Results & Observations

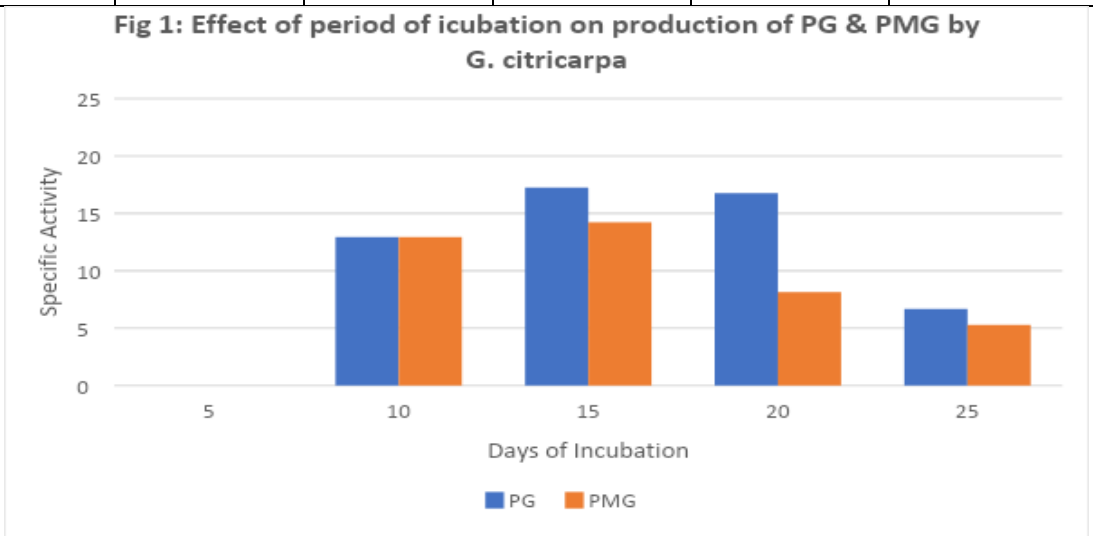
Results obtained are summarized in the given table & figures. In case of the present organism, extracellular activity was maximum between 15 to 20 days & that of PMG was on 15th day of incubation. The reaction was observed to be linear with up to 4 hours of incubation for both PG & PMG, beyond which the rate slowed down & was almost stabilized after 4 hours. Studies on the effect of various pectin concentrations on production of these enzymes shows maximum activity between 0.4 % & 0.5 % concentration. Further increase in pectin concentration was found to be inhibitory. Optimum initial pH for production of PG & PMG by **Guignardia citricarpa** was found to be 6.0. Activities of both these enzymes was greatly affected on both sides in the pH range of 5.5 to 6.5.

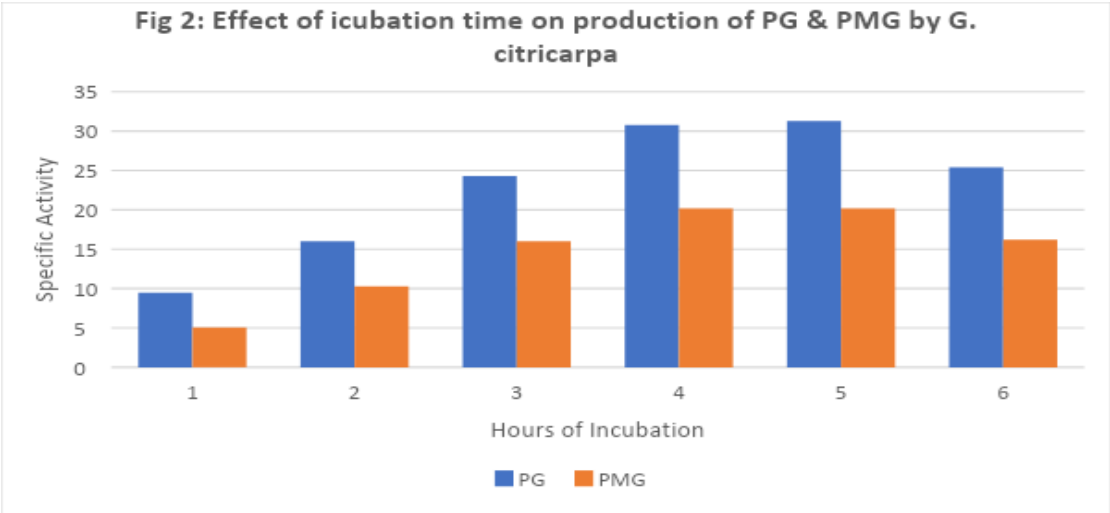
Table: 1

Effect of Incubation period, Incubation time, Pectin concentration & Initial pH on production of PG & PMG by Guignardia citricarpa.

Inc. Period in Days	**5**		**10**		**15**		**20**		**25**		--	
	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**
PG	--	--	4.90	**12.95**	6.40	**17.25**	7.32	**16.77**	4.13	**6.67**	--	--
PMG	--	--	4.90	**12.95**	5.63	**14.21**	3.56	**8.15**	3.28	**5.30**	--	--
Inc. Time in Hours	**1**		**2**		**3**		**4**		**5**		**6**	
	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**
PG	3.28	**9.49**	4.97	**16.01**	9.28	**24.30**	10.30	**30.79**	10.30	**31.25**	10.26	**25.40**
PMG	1.40	**5.05**	2.63	**10.25**	6.10	**15.98**	6.75	**20.18**	6.75	**20.19**	6.90	**16.21**
Pectin Concentration	**0.1**		**0.2**		**0.3**		**0.4**		**0.5**		**0.6**	
	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**
PG	1.64	**3.28**	2.90	**6.45**	2.69	**15.17**	5.14	**25.25**	5.61	**23.74**	4.18	**19.05**

PMG	1.13	**2.26**	1.19	**2.69**	2.65	**8.57**	3.08	**13.03**	3.00	**10.85**	1.65	**7.53**
Initial pH	**5.0**		**5.5**		**6.0**		**6.5**		**7.0**		--	
	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**	U/ml	**S. Ac.**
PG	1.82	**7.69**	5.58	**30.62**	14.09	**69.23**	1.37	**20.02**	0.61	**6.74**	--	--
PMG	6.14	**25.96**	5.46	**30.03**	10.46	**52.29**	2.73	**22.50**	1.60	**8.75**	--	--





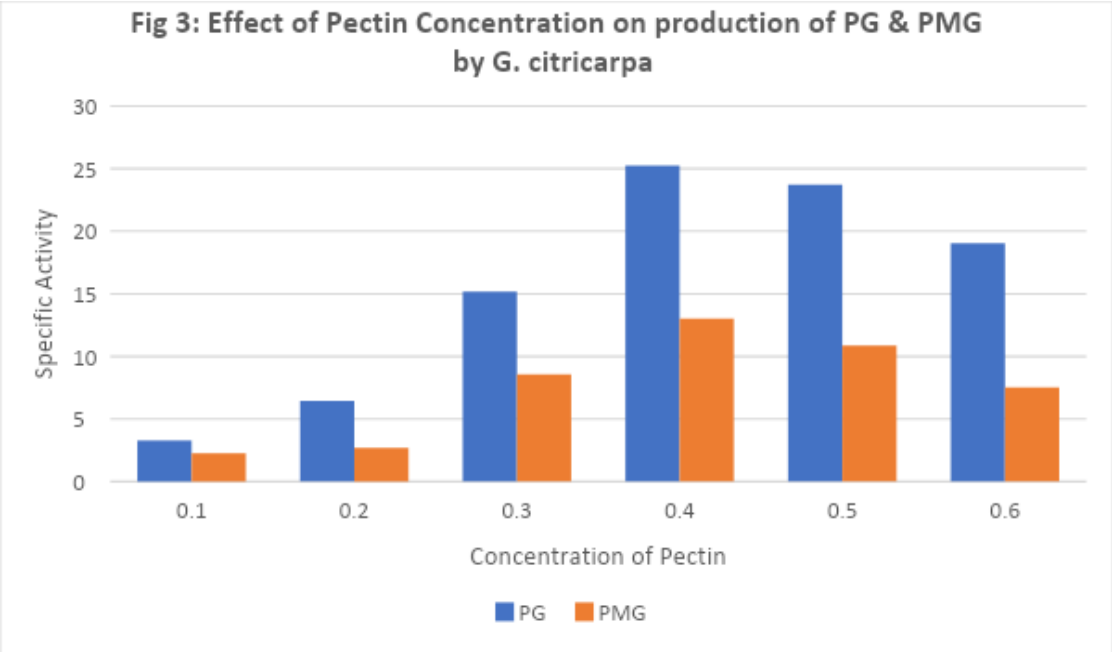


Fig 3: Effect of Pectin Concentration on production of PG & PMG by G. citricarpa
30
25
20
15
10
5
0
Specific Activity
0.1
0.2
0.3
0.4
0.5
0.6
Concentration of Pectin
PG
PMG

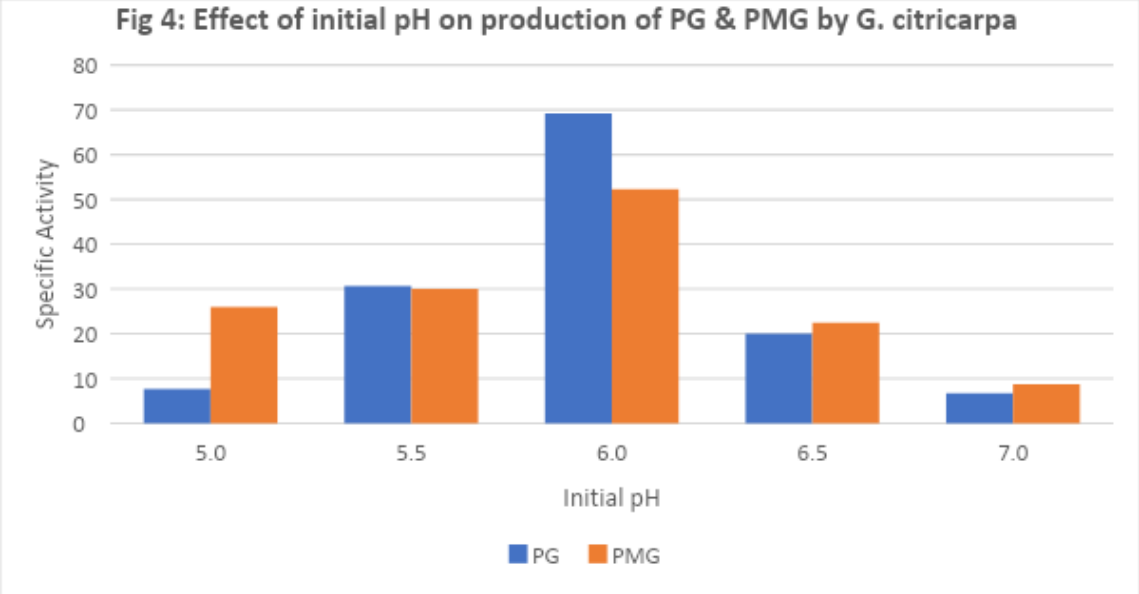


Fig 4: Effect of initial pH on production of PG & PMG by G. citricarpa
80
70
60
50
40
30
20
10
0
Specific Activity
5.0
5.5
6.0
6.5
7.0
Initial pH
PG
PMG

Discussion

Various researchers have studied the production of pectolytic enzymes by microorganisms. Culture conditions, nutrients, initial pH of the medium as well as length of the incubation period have been reported to affect the production [11 – 15].

Several reports indicate that for every enzyme, there is a specific optimum incubation period for each medium. Optimum incubation periods ranging between 4 to 14 days for the production of pectolytic enzymes by different organisms have been reported. Pandey & Gupta [13] & Mehta et al [14] have reported an optimum period of 4 days & 12 days respectively for both PG & PMG by a strain of **Alternaria tenuis**. With a strain of **Alternaria alternata** maximum activity of both PG & PMG was found on 4^{th} day of incubation [16]. Optimum incubation period of 4 & 5 days for PG & PMG respectively have been reported by a strain of **Geotrichum candidum** [17].

In case of pectin concentration, maximum activity between 2 % & 3 % pectin has been reported for **Alternaria alternata** & **Geotrichum candidum** respectively [16, 17]. The results obtained possibly indicates the adaptive nature of both PG & PMG as reported by many workers [3, 18]. The present organism did not grow well below pH – 5.0 & there was complete growth inhibition at pH – 3.0. An optimum pH of 5.0 & 4.5 has been reported for production of PG & PMG respectively by **Geotrichum candidum** [17]. A pH of 4.0 for PG production by **Alternaria alternata** [16] & pH – 4.5 by **Aspergillus awamori** [11] has been found to be optimum.

Conclusion

In the present investigation **Guignardia citricarpa** elaborated considerable amount of PG & a small amount of PMG. Both these enzymes show maximum activity at pectin concentrations between 0.4 % & 0.5 %. The optimum incubation period required by PG was 20 days & that by PMG was 15 days. Maximum production of these enzymes was observed when the reaction mixtures were incubated for 4 hours while the optimum initial pH of the medium was found to be 6.0.

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