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Research Article

FACTORS AFFECTING THE PRODUCTION OF POLY METHYL GALACTURONASE ENZYME BY SCLEROTIUM ROLFSII SACC

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Abstract

The aim of this work was to investigate the effects of different culture conditions on the production of poly methyl galacturonase enzyme by *Sclerotium rolfsii* and their optimization. Activity of poly methyl galacturonase enzyme was assayed by viscometric method. Culture conditions like culture media, incubation period, temperature and pH greatly influenced the production of poly methyl galacturonase enzyme. Out of ten culture media, only six media were proved to be producer of poly methyl galacturonase enzyme. Amongst them, Elliot's medium was found to be the best for the maximum production of poly methyl galacturonase enzyme. Analyzing the different incubation period, the production of poly methyl galacturonase enzyme was found rapid (3 days incubation) in *Sclerotium rolfsii*. Whereas, production of poly methyl galacturonase enzyme, gradually increased with increasing the length of incubation period up to 18 days and further increase in length of incubation up to 10 days, did not show any effect on the production of poly methyl galacturonase. The nine days incubation has found to be the best incubation period for the maximum production of poly methyl galacturonase enzyme. The *Sclerotium rolfsii* was also able to produce the poly methyl galacturonase enzyme at wide range of temperatures (from 15ºC to 35ºC), but 30ºC temperature was found to be the best suitable for the maximum production of poly methyl galacturonase enzyme. Among the wide range of pH tested (pH 3.0 to 9.0), the pH 5.0 was proved to be the best for the maximum production of poly methyl galacturonase enzyme in *Sclerotium rolfsii* when it was cultured in the same medium. On the basis of overall experimental results it could be concluded that *Sclerotium rolfsii* has potential to produce poly methyl galacturonase enzyme constitutively.

Keywords: *Sclerotium rolfsii*; Poly methyl galacturonase; culture media; incubation period; temperature; pH

Introduction

Pectin is an important component of middle lamella and primary cell wall of higher plants. These are high molecular weight acid polysaccharide primary made us of α (1-4) linkage of D-galacturonic acid residues (Singh *et al.*, 2005; Patil and Chaudhari, 2010). It is present in highest concentration in middle lamella, where it acts as a cementing substance between adjacent cells (Hoondal *et al.*, 2002). During pathogenesis cell wall act as the first line of defence that pathogens encounter to colonize the plant tissue and obtain nutritional requirements. A pathogen has to breach the pectin layer for its ramification. A wide range of pectin degrading enzymes are involved in pectin modifications (De Varies and Visser, 2001) which are to be secretes during fungal infection. These enzymes facilitates the entry and expansion of pathogen in the host tissue (Jayani *et al*., 2005). Among pectin degrading enzymes, poly methyl galacturonase enzymes are known to be secreted by pathogenic fungi and bacteria (Mehta *et al.*, 1976; Agarwal *et al.*, 1977; Ramos *et al.*, 2010; Shukla and Dwivedi, 2012; Chaurasia *et al.*, 2013a; Chaurasia *et al.*, 2014a). Poly methyl galacturonase catalyzes the hydrolytic cleavage of α-1, 4 glycosidic bonds in pectin back bone preferentially highly esterified pectin, forming 6-methyl-D-galacturonate (Jayani *et al.*, 2005).

Poly methyl galacturonase has wide application in juice and food industries. It is required for extraction and clarification of fruit juices and wines, extraction of oil, flavors and pigments from plant materials, preparation of cellulose fibers for linen, jute and hemp manufactures etc. (Taragano *et al.*, 1997; Hang and Dornenburg, 2000; Kashyap *et al.*, 2001).

From the available literature it is clear that in any plant pathogens like *Botryodiplodia theobromae*, *Botrytis cinera*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Rhizoctonia solani*, *Phytophthora* species, *Pythium* species, *Rhizopus* species etc. are able to produce pectin degrading enzymes in normal course of their metabolic activities, whether growing on host or on nutrient media (Mehta *et al.*, 1976; Mandavia *et al.*, 1999). In vitro, the production of poly galacturonase (PG) and poly methyl galacturonase largely

depends on the cultural conditions and their production may also be induced by different nutrients, which are incorporated in the medium (Mehta and Mehta, 1985; Fiedurek *et al.*, 1995; Chaurasia, 2000).

In view of above, the present study was carried out to investigate the effect of different culture media, incubation period, temperature and pH on the production of poly methyl galacturonase by brinjal foot-rot pathogen *Sclerotium rolfsii*.

Materials and Methods

Organism

Sclerotium rolfsii Sacc. was obtained from infected foot region of *Solanum melongena* plant. Single Sclerotium culture was obtained and maintained on potato-dextrose agar slants under refrigeration at 4ºC (Chaurasia, 2000; Chaurasia *et al.*, 2014b). The slants were freshly made once a month.

Chemicals and Glass ware

All chemicals were of analytical grade. Agar-agar and pectin were obtained from Merck Germany. All other chemicals were obtained from sigma chemicals Co. Ltd. England.

Corning glass ware and double distilled water were used thoughout the course of present experimentation. Glass were used in the present study was cleaned by immersing in chronic acid (5.0 g sodium dichromate + 500 ml conc. H_2SO_4), followed by repeated washings with distilled water.

Extraction of poly methyl galacturonase enzyme

Active poly methyl galacturonase enzyme preparations of *Sclerotium rolfsii* was obtained by growing the pathogen in different culture conditions.

The pathogen was grown in 150 ml Erlenmeyer flasks containing 25 ml of broth medium. These flasks were sterilized at 15 lb/sq in pressure for 15 minutes. Each flask was seeded with one 8.0 mm diameter mycelial disc, cut from the periphery of 72 hours old culture of the pathogen. The cultures were incubated at 30ºC (except temperature experiment). The fungus filtered after desired incubation period and the filtrates from three flasks were pooled and centrifuged at 10,000 rpm for 20 minutes at 4ºC using ultracentrifuse to obtained clear supernatant liquids, which assayed for poly methyl galacturonase enzyme activity.

Factors affecting poly methyl galacturonase Enzyme production

(a) Effect of different culture media

Table 1 shows ten previously reported broth media (Chaurasia *et al.*, 2013d). These media were used in the present investigation to see the effect of different culture media on poly methyl galacturonase enzyme production.

For poly methyl galacturonase enzyme production, the pathogen was grown in 150 ml Erlenmeyer flask containing 25 ml of the above said broth media. Each flasks was inoculated with an inoculum disc of 8.0 mm diameter of mycelial disc and was incubated at 30ºC for 9 days. After 9 days of incubation, the mycelium was removed, the culture filtrate was centrifuged and the supernatant was assayed for poly methyl galacturonase enzyme activity.

(b) Effect of incubation period

After selection of broth medium, the effect of different incubation periods, i.e., 3, 6, 9, 12, 15 and 18 days were tried to study their influence on the poly methyl galacturonase enzyme production. For this study, the pathogen was grown in 150 ml Erlenmeyer flasks containing 25 ml of Elliot's broth medium. Each flask was inoculated aseptically and then incubated at 30ºC. After 3, 6, 9, 12, 15 and 18 days of incubation, the mycelium was removed, the culture filtrate was centrifuged and the supernatants was assayed for poly methyl galacturonase enzyme activity.

Table 1: Different culture media used in the present investigation

S.N.	Media	Composition (g/L)
1.	Asthana and Hawker's	Glucose 5, KNO3 3.5, KH2PO4 1.75, MgSO4. 7H2O 0.75, Distilled Water to 1 L.
2.	Basal mucor	Dextrose 10, Asparagine 2, KH2PO4 0.5, MgSO4.7H2O 0.25, Thiamine chloride 0.5, Distilled Water to 1 L.
3.	Brown's	MgSO4.7H2O 0.75, KH2PO4 1.25, Asparagine 2, Dextrose 20, Starch 10, Distilled Water to 1 L.
4.	Czapek's	NaNO3 2, KH2PO4 1, MgSO4.7H2O 0.5, KCl 0.5, FeSO4.7H2O 0.01, Sucrose 30, Distilled Water to 1 L.
5.	Dextrose-asparagine phosphate	Dextrose 30, MgSO4.7H2O 0.5, Asparagine 1, KH2PO4 1.5, Distilled Water to 1 L.
6.	Elliot's	Dextrose 5, Asparagine 1, Sodium Carbonate 1.06, MgSO4.7H2O 0.5, KH2PO4 1.36, Distilled Water to 1 L.
7.	Fernando's	MgSO4 5, KH2PO4 6.8, Asparagine 5, Glucose 15, Distilled Water to 1 L.
8.	Glucose-dox	MgSO4.7H2O 0.5, KH2PO4 1, FeSO4.7H2O 0.01, NaNO3 2, KCl 0.5, Glucose 15, Distilled Water to 1 L.
9.	Glucose-nitrate	Glucose 10, NaNO3 1, KH2PO4 1, Distilled Water to 1 L
10.	Potato dextrose	Peeled potato slices 200, Dextrose 20, Distilled Water to 1 L.

(c) Effect of temperature

In order to determine the effect of temperature on poly methyl galacturonase enzyme productin, the pathogen was grown in 150 ml Erlenmeyer flask containing 25 ml of Elliot's broth medium. Each flasks was inculated and then incubated at 15, 20, 25, 30, 35, 40 and 45ºC temperatures for 9 days. After 9 days of incubation, the mycelium was removed, the culture filtrate was centrifuged to obtained supernatant which was later assayed for poly methyl galacturonase enzyme activity.

(d) Effect of pH

The effect of pH on poly methyl galacturonase enzyme production was conducted by adjusting the Elliot's broth medium with 1N NaOH and 0.1 N HCl to pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 before pathogen inoculation. After inoculation the inoculated flasks were incubated at 30ºC for 9 days. After 9 days of incubation period the mycelium was removed, and culture filtrate was centrifuged to obtained supernatant which was later assayed for poly methyl galacturonase enzyme activity.

Assay of poly methyl galacturonase enzyme activity

Enzyme extracts obtained were assayed for the presence and activity of poly methyl galacturonase. The activity of poly methyl galacturonase was measurd by using standard viscometric method (Chaurasia *et al.,* 2013a; Chaurasia *et al.,* 2013b; Chaurasia *et al.,* 2013c; Chaurasia *et al.,* 2014 a).

Oswald viscometers were clamped in stands which were fixed vertically in water bath, with temperature adjusted to 30ºC. For the assaying of poly methyl galacturonase enzyme, the following freshly prepared substrate components were used

1.2% Pectin	3.5 ml
Distilled water	1.5 ml
Citrate phosphate buffer (pH 5.0)	1.5 ml

At the time of determination of poly methyl galacturonase enzyme activity, desired substrate component was taken into the stalk bulb of viscometer. Then, 1.5 ml of freshly prepared enzyme extract was poured into viscometer and soon efflux time of the enzyme reaction mixture was determined at the intervals of 0, 20, 40, 60 and 80 minutes. Efflux time for 8.0 ml of distilled water was also noted in each viscometer.

Determination of per cent loss in viscosity

Per cent loss in viscosity was calculated with the help of the following formula (Chaurasia *et al.,* 2013a; Chaurasia *et al.,* 2013b; Chaurasia *et al.,* 2013c; Chaurasia *et al.,* 2014a).

$$\% \text{ Loss in visvosity} = \frac{ET_0 - ET_t}{ET_0 - ET_w} \times 100$$

where,

ET_0 = Efflux time in seconds at zero time/control.

ET_t = Efflux time in seconds at any specific interval of time.

ET_w = Efflux time in seconds for distilled water.

Determination of relative enzyme activity (REA)

Values for per cent loss in viscosity were determined for 0, 20, 40, 60 and 80 minutes reaction time. These values were plotted against the reaction time, thus a curve was obtained and from this curve the time to bring a 25 per cent loss in viscosity was determined. Relative enzyme activity (REA) was calculated by the following formula:

$$\text{REA} = \frac{1}{\text{Time for 25\% loss in Viscosity}} \times 1000$$

Results and Discussion

Effect of different culture media on the production of poly methyl galacturonase

The effect of ten different broth media, viz., Asthana and Hawker's, Basal mucor, Brown's, Czapeks, Dextrose-asparagine phosphate, Elliot's, Fernando's, Glucose-dox, Glucose-nitrate and Potato-dextrose were tried to study their influence on the production of poly methyl galacturonase enzyme. It is clear from the results (Table 2) that *Sclerotium rolfsii* was not capable to produced poly methyl galacturonase enzyme in Asthana and Hawkers, Czapek's, Glucose-dox and Glucose-nitrate media. Therefore, it can be concluded that these media have not favoured the poly methyl galacturonase enzyme production. Perhaps nitrogen source and other ingradients of the medium may interfered with the poly methyl galacturonase production of pathogen. Rajmane and Koreker (2012) reported that nitrites and nitrates containing media are toxic for enzyme production of post harvest fungi. Several workers like Baijal (1967), Bilgrami (1975) and Chaurasia *et al.* (2013d) also having similar opinion as they have reported that nitrate containing media are very toxic to several members of microorganisms. Amongst all the tested media, Elliot's medium was found to be the most suitable for the production of poly methyl galacturonase enzyme in which 15.0 REA has been calculated. Dextrose-asparagine phosphate and Basal mucor media were proved to be very poor for poly methyl galacturonase production. Brown's, Fernando's and Potato-dextroase media have been found to be satisfactory in which appreciable amount of poly methyl galacturonase enzyme was determined. It has been observed that the type of culture medium greatly influenced the production of the poly methyl galacturonase enzyme. The enzyme constitutive is clearly shown by their production in good amounts in the medium lacking pectin materials.

On the whole, it is concluded that Elliot's medium was found to be the best for the maximum production of poly methyl galacturonase enzyme. Asthana and Hawker's, Czapeks, Glucose-dox and Glucose-nitrate media showed toxic effect for the production of poly methyl galacturonase in which no trace of this enzyme was detected

Table 2: Effect of different culture media on the production of poly methyl galacturonase enzyme.

Media	Enzyme activity (% Loss in viscosity) Reaction time (in mts.)				REA
	20	40	60	80	
Asthana and Hawker's	0.0	0.0	0.0	0.0	0.0
Basal mucor	3.7	6.0	7.5	8.5	0.0
Brown's	6.3	12.5	17.4	21.2	0.0
Czapek's	0.0	0.0	0.0	0.0	0.0
Dextrose-asparagine phosphate	2.8	5.8	7.4	8.2	0.0
Elliot's	8.2	18.0	24.3	30.0	15.0
Fernando's	5.8	11.2	15.3	17.8	0.0
Glucose-dox	0.0	0.0	0.0	0.0	0.0
Glucose-nitrate	0.0	0.0	0.0	0.0	0.0
Potato-dextrose	5.7	9.0	11.2	12.5	0.0

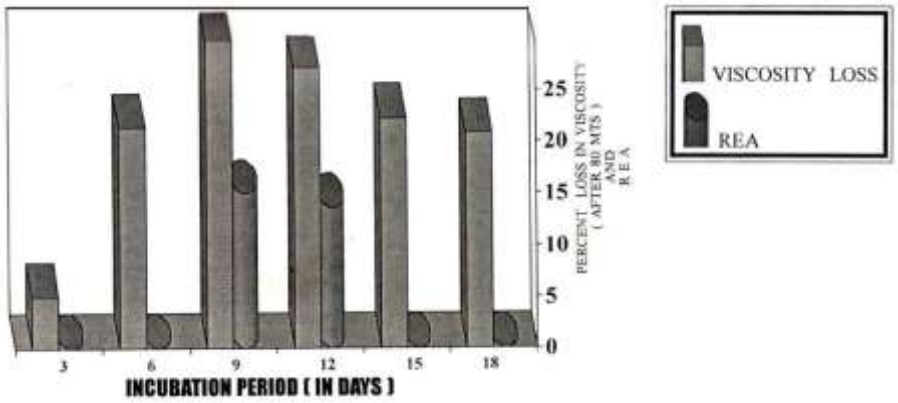

Fig. 1: Effect of different incubation period on the production of poly methyl galacturonase of *Sclerotium rolfsii*.

Effect of incubation period on the production of poly methyl galacturonase

The different incubation periods, viz., 3, 6, 9, 12, 15 and 18 days were tried to study their effect on the production of poly methyl galacturonase enzyme. The results are summarized in Fig. 1. It was observed that *Sclerotium rolfsii* could be able to synthesize poly methyl galacturonase enzyme within a short time i.e., within 3 days. The poly methyl galacturoanse enzyme activity rapidly increased with the length of incubation period upto 9 days. In 9 days of incubation period, the maximum relative enzyme activity (REA 15.0) was recorded. After 9 days, further increase in length of incubation upto 18 days, did not show any effect on the production of poly methyl galacturonase but rather resulted in a gradual fall of poly methyl galacturonase activity. It means that poly methyl galacturonase production is correlated with the incubation period, which was also found from other investigations (Mehta *et al.*, 1974; Agarwal *et al.*, 1977; Agarwal and Gupta, 1978). The result was in more or less consistent with Mehta *et al.* (1974) who reported poly methyl galacturonase production of the *Alternaria solani* and *Alternaria tenuis* isolates to be maximum between 4 to 12 days of incubation. However in another study Chaurasia *et al.* (2014a) found poly methyl galacturonase production to be maximum after 6 days of incubation. The study suggests that the production of poly methyl galacturonase was optimum after a definite period of incubation, however further increase in the incubation time, reduced the enzyme production. The reason for this may be due to the depletion of nutrients in the medium with the lapse in time (Nochur *et al.*, 1993).

On the whole, it is concluded that *Sclerotium rolfsii* has capability of producing poly methyl galacturonase enzyme as this was detected in 3 days of incubation period. 9 days of incubation was found to be the best for the maximum production of poly methyl galacturonase enzyme

Effect of temperature on the production of poly methyl galacturonase

Temperature plays an important role in the metabolic activities of microorganism. In the present study investigation was carried out on the effect of different temperatures, viz., 15, 20, 25, 30, 35, 40 and 45°C on the production of poly methyl galacturonase enzyme. It is evident from the results given in Table 3 that *Sclerotium rolfsii* was able to produce poly methyl galacturonase enzyme between a wide range of temperature, i.e., from 15 to 35ºC. At 15ºC poly methyl galacturonase enzyme production was found to be negligible and only 3.5% loss in viscosity of pectin was recorded. Production of poly methyl galacturonase was increased with increase in temperature up to 30ºC, it means, 30ºC was most suitable for the production of poly methyl galacturonase enzyme. This might be due to better growth of the test pathogen at this temperature (Chaurasia *et al.*, 2013d). Further increase in temperature up to 35ºC resulted in the little decrease in poly methyl galacturonase enzyme production. At 40 and 45ºC, no trace of poly methyl galacturonase enzyme have been detected. The reason might be because a higher temperature (above 30ºC) alters the cell membrane composition and stimulates protein catabolism (Ikram-ul-Haq *et al.*, 2006).

From the results shown in Table 3, it is concluded that 30ºC temperature was found to be the best for the maximum production of poly methyl galacturonase enzyme. Almost similar results have been recorded by Chaurasia *et al.* (2013c) with *Alternaria solani***.**

Effect of pH on the production of poly methyl galacturonase

The hydrogen ion concentration (pH) has a direct effect on the uptake of mineral nutrients which are present in the medium, therefore, the production of enzymes are affected. The effect of different pH viz., 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 were tried on the production of poly methyl galacturonase enzyme and results are summarized in Table 4. It was observed that *Sclerotium rolfsii* could synthesize poly methyl galacturonase enzyme at a wide range of pH, i.e., from pH 3.0 to 9.0. In culture filterate of pH 3.0, only 20.0 per cent loss in viscosity of pectin was recorded at the end of reaction time which was gradually increase up to pH 5.0. At pH 5.0, maximum production of poly methyl galacturonase enzyme recorded which was estimated as 30.0 per cent loss in viscosity of pectin (REA 15.0). Further increase in pH from pH 5.0 have no effect on the production of poly methyl galacturonase enzyme but it rather resulted in gradual fall in production of poly methyl galacturonase up to pH 9.0. This might be due to the fact that pathogen culture require slightly acidic pH i.e., 5.0 for their growth and enzyme biosynthesis (Chaurasia, 2000). Acidic pH was reported to be an important growth parameter in the production of poly galacturonase and poly methyl galacturonase in *Solanum melongena* fruit-rot pathogen *Fusarium moniliformae* var. *subglutinans* (Mehta and Mehta, 1985). Many report on the requirement of pH of culture medium for extra cellular poly methyl galacturonase enzyme production by fungi and bacteria, are there and in most of the cases it lies maximum between pH 4.0 and 5.0 (Mehta, *et al.*,1974; Celestino and Filbo, 2005).

Table 3: Effect of different temperatures on the production of poly methyl galacturonase enzyme.

Temperature (^{0}C)	Enzyme activity (% Loss in viscosity) Reaction time (in mts.)				REA
	20	40	60	80	
15	1.5	2.5	3.0	3.5	0.0
20	3.0	7.2	11.2	15.0	0.0
25	6.3	14.2	20.0	25.0	12.5
30	8.2	17.0	24.3	30.0	15.0
35	5.4	10.6	18.1	23.8	0.0
40	0.0	0.0	0.0	0.0	0.0
45	0.0	0.0	0.0	0.0	0.0

Table 4: Effect of different pH on the production of poly methyl galacturonase enzyme.

pH	Enzyme activity (% Loss in viscosity)				REA
	Reaction time (in mts.)				
	20	40	60	80	
3.0	5.2	12.1	17.2	20.0	0.00
4.0	6.8	14.5	21.3	26.2	13.10
5.0	8.2	17.0	24.3	30.0	15.00
6.0	7.5	16.1	23.0	28.2	14.10
7.0	7.0	14.2	20.0	25.0	12.50
8.0	6.6	10.9	15.2	19.5	0.00
9.0	6.1	10.6	14.0	16.1	0.00

On the basis of aforsaid findings, it is concluded that *Sclerotium rolfsii* possesses capability to synthesize poly methyl galacturonase enzyme at a wide range of pH, i.e., from pH 3.0 to 9.0 and pH 5.0 was found to be most suitable and favourable for the maximum production of poly methyl galacturonase.

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