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Polygalacturonase production by *Aspergillus nomius* MR103 in solid state fermentation using Agro-industrial wastes

Ravi Ketipally
Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur-522 510 (Andhra Pradesh), India
G. Kranthi Kumar
Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur-522 510 (Andhra Pradesh), India
M. Raghu Ram*
Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur-522 510 (Andhra Pradesh), India

*Corresponding author. E-mail: mraghuram2002@gmail.com

Abstract
The present study was aimed at polygalacturonase production from *Aspergillus nomius* MR103 under solid state fermentation. A total of 57 fungal strains were obtained from mangrove soils collected from Gilakaladindi and Malakayalanka of Krishna District Andhra Pradesh. For the isolation of fungi these Soil samples were serially diluted and plated on pectin agar media plates. Among them, the isolate which showed maximum polygalacturonase activity was selected for this study. This strain was identified as *A. nomius* MR 103 by 18S rRNA sequences analysis. Pectin rich agro-industrial wastes like apple peel, citrus peel, orange peel, wheat bran, rice bran and sugarcane bagasse were used as substrates for polygalacturonase production by *A. nomius* MR 103. This strain was inoculated into the nutrient broth containing agro industrial wastes under solid state fermentation and amount of Polygalacturonase production was estimated. Maximum enzyme production of 4.83 IU/mg was recorded at pH 7.0 and temperature 35^{0}C after 7 days of incubation, when orange peels were used as substrate. Addition of carbon and nitrogen sources to orange peel media improved the Polygalcturonase production. Sucrose as carbon and peptone as nitrogen sources were proved to be the best for maximum production of Polygalcturonase by *A. nomius* MR 103 on orange peel substrate. Utilization of agro-industrial by-products provided the establishment of a cost-efficient and sustainable process for enzyme production.

Keywords: Agro-industrial wastes, Polygalacturonase (PG), Solid state fermentation, *Aspergillus nomius* MR 103

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INTRODUCTION

Production of pectinase from various agro wastes under submerged and solid state fermentations. Solid state fermentation (SSF) condition occurs only the absence of water and is the process of cultivation of microbes grew well and produce maximum enzyme. SSF is cheap and best method for production of industrially important enzymes. Polygalacturonase (PG) production is reported to be significantly higher in solid state fermentation than in submerged fermentation (Castilho *et al*., 2000). Production of pectinase from *Aspergillus* species are very interesting in nowadays. India being an agricultural country produces millions of tons of fruits and vegetables annually. Pectin rich sources are rice bran, wheat bran, sugarcane bagasse, mango, apple, citrus and bananas are the highest yielding fruits in India. Millions of tons of agro wastes were throughout the environment. The enzymes that degrade pectic substances are known as pectic enzymes are wider spread in fungi, bacteria and nematodes (Akhter *et al*., 2011; Batal *et al*., 2013).

The pectic enzymes which are classified into two types i.e. pectin esterases and polygalacturonases. The primary cell wall of the citrus fruits is mainly composed of pectin and these pectins are used in industrially by nutritional and gelling agent due to their properties (Mohnen, 2008). The major components of citrus peels are carbohydrates and proteins and the fats are low components. Citrus peel was used as a substrate under Solid State Fermentation by microorganisms produce pectinases (Aguilar and Huitron, 1990). Different types of microorganisms such as bacteria and fungi pro-

duce different types of enzyme as each of them has different biological process. Fungi usually produce hydrolytic enzymes such as pectinase, cellulose and xylanase. Pectinases are enzymes with a wide range of applications in the food and beverages industries (Castilho *et al*., 1999).

Pectinases have been used in juice and wine processing for the last 70 years. They are extensively used in food industry to increase juice yields, to accelerate juice clarification and to produce concentrates from grapes, berries, pears, green peppers and citrus fruits. These have wide applications in the food industry for clarification of fruit juices, wines, coffee and tea fermentations (Alkorta *et al*., 1998; Whitaker, 1984) and extraction of essential oils (Jayani *et al*., 2005). Pectinases are also used to increase the colour of juices, promoting antioxidants formation, favour of the colour and flavour of the components.

Production of enzymes from agro-industrial wastes contain huge amount of pectinase cellulose, xylanase and hemi cellulose which serve as inducers. Researchers studied the several agro wastes, mainly citrus peel (Jansen, 1954), apple pomace (Hours, 1988) coffee pulp (Boccas *et al*., 1994) and Orange peel (Batal *et al*., 2013) were used for the production of pectinases.

The present investigation was aimed to study the optimization studies for the polygalacturonase production from *A. nomius* MR 103 cultured on different agro waste substrates to produce pectinase under Solid State Fermentation. The substrates that were used in this study are Apple peel, citrus peel, orange peel, wheat bran, rice bran and sugarcane bagasse. We report the nutritional and environmental and optimization conditions requirement in pectinase yield from the substrates which were incubation period, pH and temperature. The effect of carbon and nitrogen sources on the production of pectinases was also studied.

MATERIALS AND METHODS

Chemicals, components and agro-industrial wastes: Analytical grade chemicals and components are used in this study. The agro industrial wastes like i.e., Apple peel, citrus peel, orange peel, wheat bran, rice bran and sugarcane bagasse were collected from a local fruit market and were packed on newspapers for further analysis. All of the collected materials were crushed, several times washed with hot water, oven dried (at 70 ^{0}C), then ground into a mixture by using pistil and mortar. Samples were stored in sterilized containers and used for further experiment.

Mangrove sediments Collected from Gilakaladindi and Malakayalanka of Krishna District were taken up to depth of 20 cm after removing approximately 3cm of the soil surface. Soil samples were obtained from 6-10 cm depth and carry to laboratory by using sterile bags and are dried at room temperature. One gram mangrove soil sample was taken into 100 ml of sterile distilled water and the suspension was kept in rotatory shaker for 30 minutes. One ml of suspension with 9 ml of sterile water was used for the serial dilution process. Serial dilutions were prepared up to 10^{-4} dilutions. 0.1 ml soil suspensions of 10^{-3} and 10^{-4} dilutions were inoculated on Czepak dox Agar plates and spreaded with the help of a spreader. The plates were incubated at 35 ± 2°C for 7 days were observed for fungal colonies. Pure colonies were transferred into slants, further screened for extracellular polygalacturonase using pectin agar media. The fungal strain *Aspergillus nomius* MR 103 was identified by 18 S rRNA sequencing and the sequence was deposited in NCBI Gen bank with accession number of MK192017.

Polygalacturonase production: Polygalacturonase production was carried out in 250 ml of conical flask containing 50 ml of pectin broth medium, which consist of the following constituents (g/L) : $(NH_4)_2$ SO_4 (1g), K_2HPO_4 (1g), KCl (0.5g), NaCl (5g), $MgSO_4$ (0.5g), $FeSO_4$ (0.01g), and Citrus pectin (5 g). The flasks were inoculated with 1 ml of fungal suspension and then incubated at 30° C under shaking condition (2000 rpm) for 7 days. The polygalacturonase activity was determined in the supernatants by performing the standard polygalacturonase assay.

Polygalacturonase assay: Polygalacturonase activity of the fungal growth was assayed by the method of Miller (1959). By using pectin as a substrate the enzyme activity was determined. 1 ml of 1% pectin as a reaction mixture was prepared in sodium acetate buffer (0.1 M with pH 5.5) and suitably diluted to the crude enzyme which were incubated at 40º C in water bath for 20 minutes and the reaction mixture was stopped with 3ml of Di Nitro Salicylic Acid solution (DNS).

Then the mixture was boiled for 5 min, and 1ml of sodium potassium tartarate was added and cooled. The colour intensity was measured at 540 nm using UV-visible spectrophotometer. For the calculation of enzyme activity (IU/mg) as the amount of enzyme required to release 1 micromole (µmol) is equivalent of galactouronic acid/ minute.

The reaction mixture was prepared with 1 ml of 1.2 %w/v pectin in 1 mL of 0.1M citrate – phosphate buffer with pH 5.0 and 1 mL of crude enzyme solution. Control conical flasks were maintained with same amount of substrate added with 1 ml of culture filterate which was boiled for 20 minutes. Both the conical flasks (Experimental and control flasks) were incubated at 35 °C for 3 hours. By using 3, 5-dinitrosalicylic acid (DNSA) reagent the amount of reducing sugar released into the reaction mixture was determined. One unit of PG activity considered as the amount of en-

zyme (1 ml) which liberate reducing sugars equivalent to 1 mg galacturonic acid/minute under standard assay conditions.

Solid state fermentation: Solid state fermentation was carried out in a 500 ml conical flask containing Czepak dox broth along with 5 g each of orange peel, apple peel, wheat bran, rice bran and sugarcane bagasse in separate flasks. Then the test strain *A. nomius* MR103 was inoculated on the flasks. These flasks were then incubated at 35^{0}C temperature for 7 days. The experiments were conducted in triplicate.

Optimization studies for polygalacturonase production: In the present study an attempt was made to investigate the effect of various parameters including incubation period, pH, temperature, carbon (Galactose, glucose, fructose, sucrose, lactose, starch and cellulose) and nitrogen sources (Ammonium sulphate, ammonium nitrate, yeast extract, beef extract, peptone potassium chloride and sodium nitrate) on polygalacturonase production in Solid State Fermentation using orange peel waste (selected from initial screening trial experiment) as substrate for enzyme production by *A. nomius* MR103.

RESULTS AND DISCUSSION

Among the total of 57 fungal strains isolated from mangrove soils of Gilakaladindi and Malakayalanka, Krishna district, Andhra Pradesh which were screened for the pectinolytic activity on pectin agar media, the strain MR 103 showed maximum zone of inhibition on the plates and the strain *A. nomius* was confirmed by 18S rRNA sequencing analysis. The phylogenetic tree constructed based on 18S rRNA gene sequences revealed that it was identified as *A. nomius* MR 103 as the present strain shown 100% relation with it (Fig.1). Much work was published on polygalacturonase production from different *Aspergillus* species. For the first time we are reporting the polygalacturonase production from *A. nomius* MR 103 under solid state fermentation.

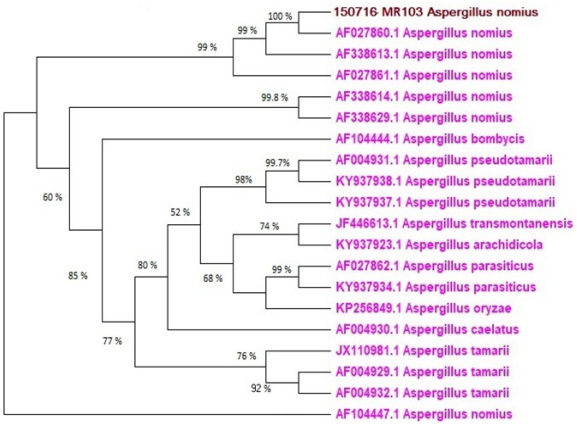

Fig. 1. *Phylogenetic tree based on 18S rRNA sequences of the genus ASPERGILLUS obtained from BLAST search showing the position of isolate (EO3 MR103) and related to Aspergillus nomius MR103.*

Further the enzyme production was observed by different media supplemented into the various agro wastes like Orange peel, Citrus peel, Wheat bran, Rice bran and sugarcane bagasse. Among them orange peel containing the medium supported maximum polygalacturonase production (4.83 IU/mg) by *A. nomius* MR 103 at 35 ^{0}C temperature after 7 days of incubation (Table-1). Phutela *et al*., (2005) reported the highest polygalacturonase production by *A. fumigatus* from decomposing orange peels. Thangaratham and Manimegalai 2014 also reported that the fungal strains *A. oryzae* and *A. flavus* species showed maximum pectinase production (0.79 U/ml) in pineapple substrate under solid state fermentation. For the production of PG by using citrus peels, as reported by Batal *et al*., (2013) the pectin lyase production from *A. niger* was maximum at 96 hours of incubation at 30^{0}C temperature. Sandhya and Kurup *et al*., (2013) also reported that the pectinase from *Aspergillus* and *Penicillium* species had maximum enzyme production under solid state fermentation used as fruit and vegetable wastes.

Effect of incubation period on polygalacturonase production: The present strain exhibited the maximum polygalacturonase production (4.83 IU/mg) after 7 days of incubation. While the increase in incubation time, growth of the fungus also increased with increased biomass upto 7 days (Table-2). However there was a concomitant increase in enzyme production with increase in incubation period upto 8 days. The enzyme production was reduced after 8 days of incubation. The difference in incubation period for en-

Table 1. Screening of Agro wastes for the polygalacturonase production of *Aspergillus nomius* MR103 at 35^{0}C after 7 days of incubation.

Substrates	Polygalacturonase production (IU/mg)
Orange peel	4.83
Apple peel	1.80
Citrus peel	1.94
Wheat bran	1.88
Rice bran	1.56
Sugarcane bagasse	2.01

Table 2. Effect of incubation period on polygalacturonase production *Aspergillus nomius* MR103 at 35^{0}C in orange peel amended media.

Incubation time (Days)	Biomass production (mg/ml)	Polygalacturonase production (IU/mg)
4	90	2.42
5	98	3.88
6	102	4.02
7	100	4.83
8	98	4.39
9	95	3.90

Table 3. Effect of pH on polygalacturonase production by *Aspergillus nomius* MR103 at 35^{0}C in orange peel amended media.

pH	Biomass production (mg/ml)	Polygalacturonase production (IU/mg)
5.0	95	1.30
5.5	110	1.88
6.0	120	2.02
6.5	102	3.47
7.0	98	4.83
7.5	92	4.17
8.0	80	3.90

Table 4. Effect of temperature on polygalacturonase production by *Aspergillus nomius* MR103.

Temperature °C	Biomass production (mg/ml)	Polygalacturonase production (IU/mg)
20	72	1.09
25	80	2.22
28	86	3.49
32	95	3.00
35	106	4.83
37	115	2.16
40	100	1.85

zyme production reported for different fungi may be due to species difference or substrate difference. Thangaratham and Manimegalai (2014) reported that the maximum pectinase production (0.79U/ml) by *A. flavus* at 7 days of incubation.

Effect of pH on polygalacturonase production: Polygalacturonase production was recorded at pH 5.0 with 1.3 IU/mg in *A. nomius* MR103. The enzyme production increased with increase in pH from 5 to 7 and reached maximum enzyme production of 4.83 IU/mg (Table-3). Though the biomass production was increase up to pH the enzyme production enhanced increased enzyme production upto pH 7.0. With further increase in pH the enzyme production decrease along with biomass. For *A. nomius* MR 103 neutral pH was ideal for high production of enzymes. However Anand *et al*., (2016) reported that alkaline pH of 10.0 found to be suitable for polygalcturonase production from *A. fumigatus.*

Effect of temperature on polygalacturonase production: Polygalacturonase production by *Aspergillus* species majorly depends on temperature. Different temperature ranges (20, 25, 28, 32, 35, 37 and 40^{0}C) were maintained for the enzyme production. Maximum enzyme activity of 4.83U/ml was observed at 35^{0}C. At the initial temperature of 20^{0}C *A. nomius* MR103 showed the enzyme production of 1.09 IU/mg and also showed enzyme activity even at 40^{0}C temperature (Table-4). In solid state fermentation using wheat bran and potato starch *A. niger* showed maximum enzyme activity at 40^{0}C temperature (Akhter *et al*., 2011). Similarly by using agro wastes for the PG production was also reported by Pedrolli *et al*., (2008) and Nighoskar *et al*., (2006). The two Thermostable strains of *A. aculeatus* and *Fusarium oxysporum* species could produced enhanced enzyme yield even at higher temperature i.e. 60 to 65 ^{0}C. The difference between temperature ranges may be due to the genetic difference of various species as reported by Ahmed *et al.* (2016).

Table 5. Effect of carbon sources on polygalacturonase production by *Aspergillus nomius* MR103.

Carbon sources(1.0%)	Biomass production (mg/ml)	Polygalacturonase production (IU/mg)
Control	18	0.47
Fructose	103	2.45
Galactose	122	2.38
Glucose	95	3.54
Lactose	88	3.05
Sucrose	111	4.90
Starch	100	1.88
Cellulose	98	1.56

Table 6. Effect of nitrogen sources on polygalacturonase production by *Aspergillus nomius* MR103.

Nitrogen sources (0.5%)	Biomass production (mg/ml)	Polygalacturonase production (IU/mg)
Control	110	0.42
Ammonium sulphate	80	3.86
Ammonium nitrate	98	2.95
Beef extract	120	2.59
Peptone	103	5.94
Potassium chloride	115	2.80
Sodium nitrate	154	4.23
Yeast extract	123	5.60

Effect of carbon and nitrogen sources on polygalacturonase production: Among the different Agro- industrial wastes tested, orange peel supported maximum growth of *A. nomius* MR 103 showed enzyme production. Among the different additional carbon sources glucose, sucrose, maltose, arabinose, fructose, xylose and galactose added to the orange peel medium (Table-5). Sucrose supported the highest enzyme production (4.90 IU/mg) followed by glucose with 3.54 IU/mg. Among the mono, di and polysaccharides tested, the present strain utilized disaccharides followed by mono and polysaccharides. Different nitrogen sources (Sodium nitrate, Ammonium sulphate, potassium chloride, peptone, Yeast extract and beef extract) were screened for PGA activity. Maximum enzyme production (5.94 IU/mg) was recorded when peptone was used as nitrogen source. Peptone contains various amino acids that release nitrogen for the growth of fungi reported by the researchers (Martin *et al*., 2004).

Patil and Dayanand (2006) observed the pectinase production under SSF and SMF, By using the substrates lemon peel, sorghum stem and sunflower head addition of carbon sources sucrose and the nitrogen source ammonium sulphate induced the higher level production of pecti-

nase by *Aspergillus niger* for both solid and submerged fermentation systems. In the present study *Aspergillus nomius* MR 103 also produced maximum amount of Polygalacturonase by using carbon source (sucrose) and nitrogen sources (peptone). This may be due to that suitable carbon and nitrogen sources under fermentation conditions for Polygalacturonase production and their concentrations are also powerful factors that regulate the synthesis of enzymes as reported by Mikiashvili *et al*. (2005) and Iqbal *et al*. (2011).

Conclusion

In conclusion, solid state fermentation is preferred because of simple technique, low capital investment and end- -product inhibition, better product recovery, and maximum quality production. The enhanced enzyme production was obtained only in the presence of cheaper substrate like orange peel. Seven days of incubation period, pH 7.0 and temperature 35^0C were optimum conditions for polygalacturonase production. Addition carbon and nitrogen sources, sucrose and peptone in orange peel medium greatly enhanced the enzyme production.

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