Production, Purification, Characterization and Comparison of Polygalacturonase from various strains of Aspergillus

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Abstract:- Polygalacturonase is an important enzyme used in food and chemical industries that processed plant material like Juice extraction, Clarification of wine, Textile etc. Fungi from genus *Aspergillus* are one of the most important sources of this enzyme. We compare three strains of *Aspergillus* (*Aspergillus niger, Aspergillus* flavus and *Aspergillus oryzae)* for production Polygalacturonase by submerged fermentation. We also characterized all the Polygalacturonase (isolated from different strains) at different parameters such as pH, Temperature, Incubation time, Concentration Substrate in medium, Concentration of enzyme etc and on the basis of these parameters it is observed that *Aspergillus niger* found to better strain then *Aspergillus flavus and Aspergillus oryzae.*

Keywords:- Polygalacturonase (PG), Submerged fermentation, *Aspergillus niger, Aspergillus* flavus, *Aspergillus oryzae*, Ammonium sulfate

1 INTRODUCTION

Polygalacturonase are hydrolytic depolymerases with endo and exo activities. Endo-PGases (E.C. 3.2.1.15) are important enzymes involved in fruit ripening and in fungal/bacterial attack on plants, and are commonly used in the treatment of certain vegetables like tubers, apples, *etc*. Their enzymatic reaction involves random hydrolysis of *O*-glycosyl bonds in 1,4-a-D-galactosyluronic linkages in homogalacturonans. On the other hand, galacturan 1,4-a-galacturonidases (E.C. 3.2.1.67), or exo-PGases, are enzymes that degrade polygalacturonan by hydrolysis of the glycosidic bonds from the nonreducing ends yielding the corresponding 1,4-a-D-galacturonide and galacturonic acid. Depending on the type of substrate (*i.e.* pectin, polygalacturonate or polymethylgalacturonate) and the mode of action (endo or exo activity), PGase activity can be quantified, and therefore expressed in different units, whether by the reduction of viscosity in the reaction mixture or by the release of reducing groups during the enzymatic reaction under established conditions. Polygalacturonase are naturally present in plants and produced by several microorganisms. Nearly 75 % of the estimated sale value of industrial enzymes in 1995 has been contributed by pectinases (Gummadi and Panda, 2003). Almost all the commercial preparations of pectinases are produced from fungal sources and *Aspergillus* Species is the most commonly used fungal species for the industrial production of pectinases (Gummadi and Panda, 2003). Polygalacturonase can be produced by both submerged and solid state fermentation (SSF).

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Submerged fermentation is cultivation of microorganisms on liquid broth. It requires high volumes of water, continuous agitation and generates lot of effluents. SSF incorporates microbial growth and product formation on or within particles of a solid substrate **(Mudgett, 1986)** under aerobic conditions, in the absence or near absence of free water, and does not generally require aseptic conditions for enzyme production. Many filamentous fungi like *Aspergillus niger, Aspergillus flavus Aspergillus oryzae, Aspergillus. awamori, Penicillium restrictum, Trichoderma viride, Mucor piriformis and Yarrowia lipolytica.*etc are used in both submerged as well as solid state fermentation for production of various industrially important products such as citric acid, ethanol etc. Fungi like *Aspergillus niger*, *Aspergillus oryzae*, *Penicillium expansum*, which are generally regarded as safe (GRAS) by United States Food and Drugs Administration (USFDA) are employed in food industry (**Pariza and Foster**, **1983).** Some bacteria *(Bacillus licheniformis, Aeromonas cavi, Lactobacillus* etc), yeasts like *Saccharomyces, Candida* and *Actinomycetes* like *Streptomycetes* are also used. Amongst these, the filamentous fungi are most commonly employed **(Pandey *et al*, 1999).** In view of the above-mentioned points, the present investigation was undertaken and attempts were made to compare various strains of *Aspergillus* for Production and Characterization of Polygalacturonase.

MATERIAL AND METHODS

Organism and culture conditions

A fungal strain of *Aspergillus niger, Aspergillus flavus and, Aspergillus oryzae* was isolated from soil sample and maintained on Potato dextrose agar (PDA) slant and plates with repeated subculture. For production of PG Aspargine medium of following ingredients are used: 0.3g KH_2PO_4, 0.05g MgSO4.7.H_2O, 0.4g L- Aspargine The initial pH was adjusted to 4.5 and the medium was sterilized at 121 ^oC for 15 min . After Sterilization flask were cooled and inoculated with spore suspension of 3-4 day culture of each strain. In order to induce polygalacturonase activity, Polygalacturonic acid was added to the fermentation broth. Culture flask after inoculation and incubation was filter in cold condition through Whatmann No. 41 filter paper. The filtrated served as crude enzyme.

Enzyme and Protein assay

Polygalacturonase activity was determined or assayed by DNS method (Miller, 1959) by measuring the reducing sugars liberated from Polygalacturonic acid using dinitrosalicylic acid. The solution, containing 0.25 ml of 0.2 M sodium acetate buffer (pH 4.5) and enzyme solution in 0.5 ml, was pre incubated for 5 minutes at 37^{o} C. The reaction was initiated by the addition of 0.25 ml of the above mentioned substrate solution. After addition of substrate the reaction mixture was incubated at 37^{o} C for 1 hour and the reaction was terminated by adding 0.5 ml of DNS reagent. The reaction mixture was then kept in boiling water bath for 10 minutes, cooled and diluted to 4 ml with distilled water. After dilution read this reaction at 540 nm. A calibration curve establish with D-galacturonic acid was used to calculate the Polygalacturonase activity. Proteins were determined by Lowery et al (1951) with bovine serum albumin as standard.

CHARACTERIZATION OF POLYGALACTURONASE ENZYME

1) **Effect Of Incubation Time On Pg Production**

   Asparagine medium used for production of Polygalacturonase various flasks containing this medium were inoculated with fungal spores and kept for Incubation. A flask is removed after 24 hours and the activity of extracellular Polygalacturonase was determined. Results are reported in table 5.1

2) **Effect of Polygalacturonic acid Concentration in the Medium On PG Production**

   The effect of Polygalacturonic acid on PG production was determined by measuring the PGase activity at various concentration of Polygalacturonic acid (0.5, 1, 1.5, 2, 2.5) Results are presented in table 5.2

3) **Effect OF pH On Enzyme Activity**

   The effect of pH on PG production was determined by measuring the PGase activity at various pH (3.5, 4.5, 5.5, 6.5, and 7.5) using 0.2 M acetate buffer which are represented in table 5.3 Results are presented in table 5.3

4) **Effect Of Temperature On Enzyme Activity**

   The effect of Temperature on PG production was determined by measuring the PGase activity at various Temperature (25^{0} C, 30^{0} C, 35^{0} C, 40^{0} C, 45^{0} C and 50^{0}) Results are presented in table 5.4

5) **Effect Of Enzyme Concentration**

   The effect of the enzyme concentration on enzyme activity was determined by measuring PGase activity at different enzyme concentration i.e. 0.3 ml, 0.4 ml, 0.5 ml, 0.6 ml, and 0.7 ml, Results are represented in table 5.5

6) **Effect Substrate Concentration On Enzyme Activity**

   The effect of the Substrate on enzyme activity was determined by measuring PGase activity at different substrate concentration i.e. 0.2, 0.25, 0.30, 0.35, and 0.40 ml Results are represented in table 5.6

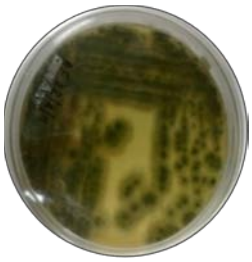
ENZYME PURIFICATION

The purification of Polygalacturonase was carried out by ammonium sulfate precipitation method. PG in culture filter was precipitated using 90% ammonium sulfate. The solution was then centrifuged at 5000x g for 20 minutes. The pellet was dissolved in minimum amount of buffer (0.2 M acetate buffer pH 4.5).The enzyme solution was dialyzed against 0.002 acetate buffer pH of 4.5 for 24 hours. After 24 hours, the dialyzate obtained was then used as the partially purified fungal Polygalacturonase.

OBSERVATIONS AND RESULTS

Three strains of Aspergillus species was isolated from soil i.e. *Aspergillus niger, Aspergillus flavus and Aspergillus oryzae* and cultured on PDA slants. These strains are used for production and partial purification of Polygalacturonase.

Colony Observation



Aspergillus flavus
(Light Green)

Aspergillus oryzae
(Dark Green)

Aspergillus niger
(Black)

Figure 1: Colony characteristics of *Aspergillus* strains

CHARACTERIZATION OF POLYGALACTURONASE ENZYME

1) Effect of incubation time on pg production

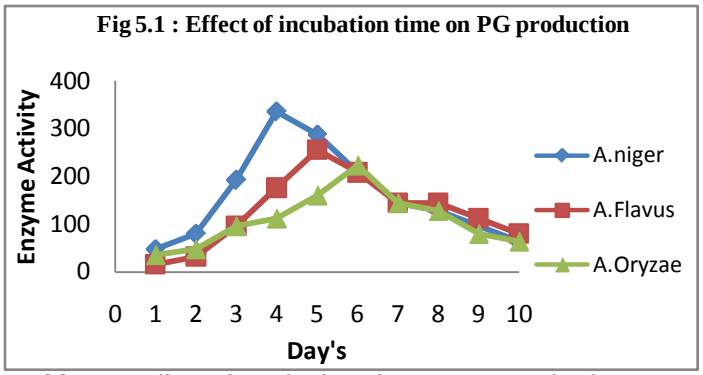
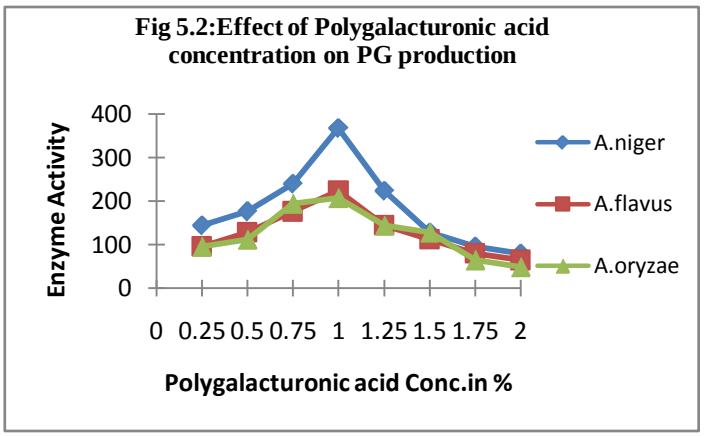


Table 5.1: Effect of Incubation Time on PG Production.

Incubation period in days	Polygalacturonase activity in U/ml		
	A.niger	*A.flavus*	*A.oryzae*
1	48	16	36
2	80	32	48
3	192	96	96
4	336	176	112
5	288	256	160
6	208	208	224
7	144	144	144
8	128	112	128
9	96	80	80
10	64	32	64

The above table and figure shows that highest Polygalacturonase activity was recorded on 4th day for *Aspergillus niger*, 5th day for *Aspergillus flavus* and 6th day for *Aspergillus oryzae.*



2) Effect of Polygalacturonic acid concentration in the medium on pg production

Table 5.2: Effect of *Polygalacturonic acid* concentration in the medium on PG production

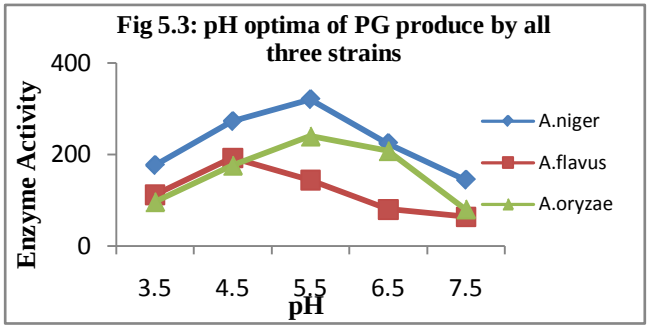
Polygalacturonic acid in %	Polygalacturonase activity in U/ml		
	A.niger	*A.flavus*	*A.oryzae*
0.25	144	96	96
0.50	176	128	112
0.75	240	176	194
1	368	224	208
1.25	224	144	144
1.50	128	112	128
1.75	96	80	64
2	80	64	48

The above table and figure shows 1% of *Polygalacturonic acid* gave highest Polygalacturonase production for all isolate.

3) Effect of pH on Enzyme Activity

Table 5.3: pH optima of Polygalacturonase

pH	Polygalacturonase activity in U/ml		
	A.niger	*A.flavus*	*A.oryzae*
3.5	176	112	96
4.5	272	192	176
5.5	320	144	240
6.5	224	80	208
7.5	144	64	80

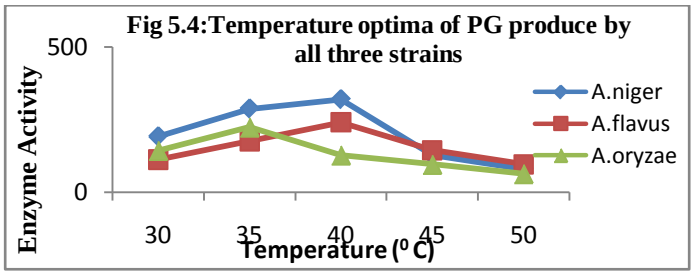


The above table and figure shows that highest Polygalacturonase activity is at optimum pH 5.5 for *Aspergillus niger, Aspergillus flavus* and pH 4.5 for *Aspergillus oryzae.*

4) Effect of temperature on enzyme activity

Table 5.4: Temperature optima of Polygalacturonase

Temperature (^{0}C)	Polygalacturonase activity in U/ml		
	A.niger	*A.flavus*	*A.oryzae*
30	192	112	144
35	288	176	224
40	320	240	128
45	128	144	96
50	80	96	64

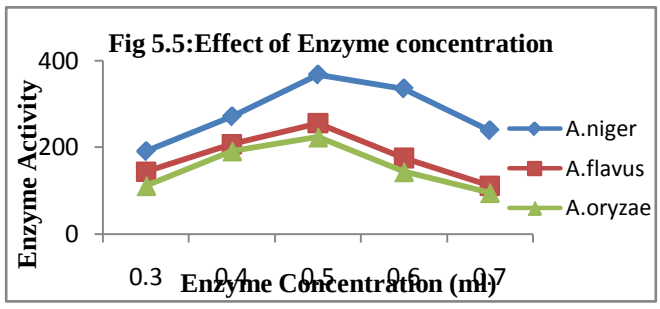


The above table and figure shows that highest Polygalacturonase activity is at optimum incubation temperature of 40^0 C for *Aspergillus niger, Aspergillus flavus* and 35^0 C for *Aspergillus oryzae.*

5) Effect of enzyme concentration Enzymatic activity.

Table 5.5: Effect of Enzyme Concentration, on enzymatic activity

Enzyme concentration (ml)	Enzyme activity in U/ml		
	A.niger	*A.flavus*	*A.oryzae*
0.3	192	144	112
0.4	272	208	192
0.5	368	256	224
0.6	336	176	144
0.7	240	112	96



The above table and figure shows that highest Polygalacturonase activity is given by 0.5 ml of crude enzyme fraction for all isolate.

6) Effect Substrate Concentration On Enzyme Activity

Enzyme concentration(ml)	Enzyme activity in U/ml		
	A.niger	*A.flavus*	*A.oryzae*
0.3	192	144	112
0.4	272	208	192
0.5	368	256	224
0.6	336	176	144
0.7	240	112	96

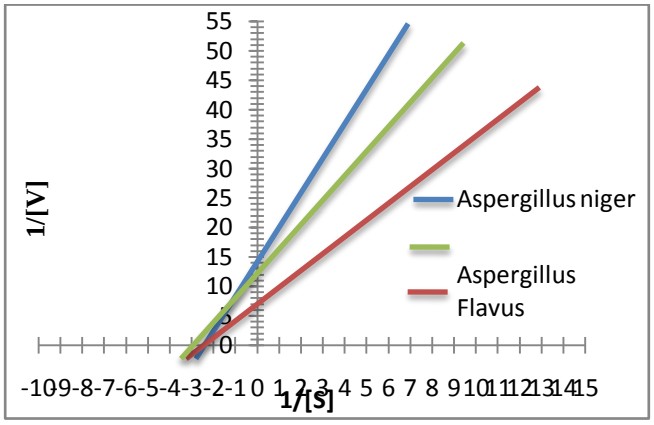


Fig 5.6: Double reciprocal plot

- For Aspergillus niger Km and Vmax was found to be 2.4 and 3336 units respectively.
- For Aspergillus flavus Km and Vmax was found to be 3.1and 3125 units respectively.
- For Aspergillus oryzae Km and Vmax was found to be 2.8 and 2000 units respectively.

PARTIAL PURIFICATION OF POLYGALACTURONAS BY AMMONIUM SULFATE PRECIPTATION

Table 6: Partial purification of polygalacturonase by ammonium sulfate precipitation

For *Aspergillus niger*

Source of Precipitation	Enzyme activity in U/ml	Protein Concentration(µg/ml)	Specific activity In U/ml	Fold of purification
Crude Enzyme	96	400	240	3. 50
Enzyme Pellet	128	270	474	
Ammonium salt fraction	336	240	1400	

For *Aspergillus flavus*

Source of Precipitation	Enzyme activity in U/ml	Protein Concentration(µg/ml)	Specific activity In U/ml	Fold of purification
Crude Enzyme	80	320	250	4.80
Enzyme Pellet	96	270	400	
Ammonium salt fraction	288	2400	1200	

For *Aspergillus oryzae*

Source of Precipitation	Enzyme activity in U/ml	Protein Concentration(µg/ml)	Specific activity In U/ml	Fold of purification
Crude Enzyme	96	400	240	4. 20
Enzyme Pellet	112	320	350	
Ammonium salt fraction	272	270	1007.4	

Conclusion

Thus it may be concluded that *Aspergillus niger* is better strain than *Aspergillus flavus* and *Aspergillus oryzae* for production of Polygalacturonase by Submerge fermentation. Consequently extracellular fungal Polygalacturonase could be easily produced and extracted with the help of Polygalacturonic acid which act as an inducer for Polygalacturonase production. However Further studies required to reduced the cost of PG production by using various agro-waste and by using solid state fermentation for PG production. In addition to strain improvement programs to obtain polygalacturonase hyper-producing mutants, studies on medium composition (Optimization of polygalacturonase production) and culture conditions by using experimental designs have demonstrated that polygalacturonase production might be considerably improved. The selected isolates in the present study would have to be thoroughly characterized before these could be utilized for experiment. Thus once they are deemed as generally regarded as Safe (GRAS) these could be then used in further course of study including scale up trials.

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