

# Polygalacturonase by *Aspergillus Niger* Using Seaweed Waste Under Submerged Fermentation: Production, Purification and Characterization

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## ABSTRACT

Polygalacturonases are important enzymes used in industrial applications that treated plant material like Juice extraction, Textile, Clarification etc. Fungi from genus *Aspergillus* are one of the most important sources of this enzyme. We production Polygalacturonase by submerged fermentation using seaweed waste. Five PG P1-P5 were differentiated on DEAE-Sephacryl column. The homogeneity of PGP2a was identified on Sephacryl S-200 chromatography. PGP2a had a molecular weight of 20 kDa by Sephacryl S-200 and SDS-PAGE. The enzyme had an optimal pH of 6 also the temperature optimal of PGP2a was 40 °C. The thermal stability of PGP2a was detected up to 50 °C and the enzyme was highly stable till 60 °C after 30min incubation. The  $V_{max}$  and  $K_m$  values of PGP2a were 4.27 mg/ml and 1.16 $\mu$ mol min<sup>-1</sup>mg<sup>-1</sup>, respectively. The metal ions except only Co<sup>2+</sup> and Hg<sup>2+</sup> was found to enhance the PGP2a activity.

**Keywords:** Polygalacturonase; Purification; *Aspergillus Niger*; Seaweed Waste

## Introduction

Polygalacturonases (PGs) are natural enzymes that are produced by several organisms, such as plants [1-2], bacteria [3-4] and fungi [5-7]. These proteins belong to a large group of pectinases, which synergistically mediate the complete decomposition of pectin substances that are abundantly present in plant tissues, primarily in fruit. Polygalacturonase are pectin-degrading proteins that are classified as exo- or endo-types based on how pectin-degrading proteins are formed. Exo-PGs [E.C. 3.2.1.67] are produced by many fungi [8-13]. Exo-PG is an enzyme that eventually hydrolyses

glycosidic bonds in pectate or other galacturons, yielding the corresponding 1,4-  $\alpha$ -D-galacturonide and galacturonic acid. on the other hand, Endo-PGs [ EC 3.2.1.15] are developed in cultures of many micro-organisms and plants [14]. Their enzymatic reaction involves random in the middle hydrolysis of O-glycosyl bonds in 1,4- $\alpha$ -D-galactosyluronic bonds in homogalacturonans. Microbial polygalacturonase have proven to be instrumental in reducing viscosity and clarifying the juice [15-18]. In general, both submerged state fermentation (SmF) was effectively used in producing

polygalacturonase from specific microbial strains [19-21], Using different agro-industrial by-products such as cotton, sugar beet and coffee, apple pulps, lemon peels, oranges and tomatoes, apple and citrus fruits, sugarcane bagasse, wheat bran, etc., [22-24]. The present study is the first report on using seaweed waste as media for production enzyme from *Aspergillus niger*. In this manuscript we describe the production, purification and characterization of polygalacturonase by *Aspergillus niger* using seaweed waste and its application in fruit juice clarification.

## Materials and Methods

### Chemicals

All the chemicals used for the analytical and laboratory grades were procured from Sigma-Aldrich.

### Microorganism and Culture Conditions

The obtained *Aspergillus niger* (NRC, Cairo, Egypt), kept on potato dextrose agar and slants were grown at 28 °C for seven days and kept at 4 °C.

### Inoculum Medium

The inoculum medium used for of *A. niger* preparation contained (g/l): MgSO<sub>4</sub>·7H<sub>2</sub>O, 4.2; Urea, 4.2; KH<sub>2</sub>PO<sub>4</sub>, 28; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.07; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 19.6; CoCl<sub>2</sub>, 4.2; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.019; CaCl<sub>2</sub>, 0.028; MnSO<sub>4</sub>·7H<sub>2</sub>O, 0.021; glucose, 15; and yeast extract, 7; pH 5.0. The media was sterilized 15 min at 121 °C pressure by autoclaving. Incubation and shake at 30 °C for two days in shaking incubator with speed rotation at 150 rpm prior to the production medium [25].

### Submerged Fermentation (SmF)

SmF was conducted to assess the impact of numerous physical and chemical parameters needed for optimum enzyme saccharification content and production by *A. Niger*. Sterilization of agricultural waste and then incubated at 121 °C for 20 min and 1.2 atm. 5 g of sterilized agricultural waste, 5 × 10<sup>5</sup> spores/g and adequate water (50%) were added to 100 ml of Erlenmeyer flask. Every test is in 3 sets. Crude enzyme was extracted for 10 min at 12000 rpm and the supernatant was designated as a crude extract.

### Purification of Polygalacturonase

Extracts from *Aspergillus niger* are mounted on a DEAE-Sephacryl column balanced with buffer (20 mM Tris-HCl pH 7.2). The enzyme was eluted in the same buffer with a stepwise gradient of 0.0 to 0.3 M NaCl. Fractions in 3 ml of volume at a flow rate of 30 ml / h were collected. The eluted fractions were checked for protein at 280 nm and enzyme activity tested. There were pooled protein fractions with enzyme activity. The most active polygalacturonase (P2) has been concentrated by solid sucrose dialysis and loaded on Sephacryl S-200 column, previously balanced by 20 mM tris-HCl buffer, pH 7.2 and obtained at a rate of flow 30 ml / h and fractions of 3 ml.

### Polygalacturonase Assay

PG activity was assayed using Polygalacturonic Acid (PGA) as substrate as described [26]. The activity for PGA has been established through the formation of reduction groups [27]. The reaction mixture (0.5 ml) included 2% PGA, a pH 5.5 buffer of 0.05 M sod. acetate and an appropriate amount of enzyme. At 37 °C for 1 h, analysis was done. Then a reagent of 0.5 ml of DNS was applied, heated for 10 minutes in water bath. The absorbance was estimated at 560 nm after cooling to room temperature. A unit of enzyme activity was specified as the amount of the enzyme that released 1 μmol per minute of galacturonic acid under standard test conditions.

### Protein Determination

Method of Bradford (1976) was used to determine the protein concentration [28], using BSA as a standard.

### Molecular Weight Determination

The method of determination molecular weight using gel filtration technique and the subunit molecular weight of the pure enzyme was determined according to the method mentioned by Laemmli [29].

### Characterization of Polygalacturonase

**Optimum pH:** Polygalacturonase activity was assessed at different pHs, with various buffers, sodium acetate (pH 4.0-6.0) and Tris-HCl (6.5-9) at 50 mM levels. The maximum activity was 100% and relative activity was compared with different pH values.

**Kinetic Parameters:** K<sub>m</sub> and V<sub>max</sub> values were determined from Lineweaver-Burk plots using 3-7 mg /ml polygalacturonic acid.

**Optimum Temperature:** The activity of polygalacturonase was determined at a 30-80 °C temperature range. Maximum activity was taken as 100% and relative activity plotted against different temperatures.

**Thermal Stability:** The enzyme was incubated at 30-80 °C for 30 min before addition to the substrate.

**Effect of Metal Ions:** The enzyme was implanted for 30 minutes 2mM Pb<sup>2+</sup>, Hg<sup>2+</sup>, Co<sup>2+</sup>, Zn<sup>2+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup> and Ca<sup>2+</sup> prior to substrate addition. The enzyme activity without metal ions was taken as 100% and in the presence of metal ions, relative activity (%) was determined.

**Clarification of Orange Juice:** Fruit juice clarity was studied using method of [30]. 100 μL of polygalacturonase was applied to 2 mL of apple juice, incubated at 37 °C in a water bath for 30, 60 and 120 min. Holding the reaction mixture in water bath for 5 min prevented the reaction. Centrifuged at 3000 rpm for 5 min. The transmittance (%) was calculated at 660 nm for controls containing the same enzyme volume applied just before holding the reaction mixture in water bath.

## Result and Dissection

Polygalacturonase produced by *Aspergillus Niger* using seaweed waste during submerged fermentation was purified in two steps, involving ion exchange chromatography and gel filtration. Table 1 summarizes the purification process. Five peaks of PG activity (Figure 1), negatively adsorbed fraction and eluted portions with 0.05, 0.1, 0.2 and 0.3 M sodium chloride are observed in the elution profile of PG on DEAE-Sepharose and designated as PGP1, P2, P3 P4 and P5. The PGP2 with highest activity was separated on Sephacryl S-200 column to obtain PGP2a (Figure 2) with the highest specific activity 1400 units/mg protein with 10.9 fold. In this study, the purified PGP2a gave a molecular weight of 20 kDa using Sephacryl S-200 column with a single band of protein on SDS-PAGE (Figure 3). The molecular masses of PG ranged from 24 to 34 kDa were detected as *penicillium viridicatum* [31], banana fruit [32], *Aspergillus awamori* [33], *penicillium expansum* [34]. The purified PGP2a showed maximum activity at pH 6 (Figure 4), similar to the result obtained by Esawy [35]. Acidic pH optima ranged from 4.5 to 6 were reported for PGase from *Aspergillus awamori* (pH 4.5) [33], *Aspergillus niger* CFR 305 (pH 4.5) [36], Rice Weevil (pH 5.5) [37]. The optimal temperature of PGP2a activity produced by *Aspergillus niger* during submerged fermentation in seaweed waste was between 40°C (Figure 5). Similarly, optimal activity at 60°C was recorded for exo-PG obtained from *P. viridicatum* RFC3 cultivated

on wheat bran and orange bagasse [31], 43°C for PG obtained from *Penicillium chrysogenum* [38], 40°C for PG obtained from banana fruit [39]. The purified PGP2 enzyme exhibit thermal stability up to 40°C. The purified enzyme preserved 50% of its original activity for 30 min at 60°C, while at 80°C the activity declined to 9% (Figure 6). Similar maximum stability has been reported for PG from *Aspergillus niger* MTCC 478 [40]. Figure 7 demonstrates that the  $K_m$  and  $V_{max}$  values for hydrolyzing PGA by the purified PGP2a were 4.27 mg/ml and 1.16  $\mu\text{mol min}^{-1}\text{mg}^{-1}$ , while  $K_m/V_{max}$  was 3.68. Low  $K_m$  value suggests better affinity to substrate. A PG from *A. tubingensis* has  $K_m$  value of 3.2 mg/mL [41] Many microbial PGs have  $k_m$  of value between 0.1-5.0 [42-47]. The influence of different metal ions was assessed using 2mM concentration of each metal ion in the reaction mixture (Table 2). Amongst all metal ions only  $\text{Co}^{2+}$  and  $\text{Hg}^{2+}$  was found to enhance the PG activity.  $\text{Pb}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Ca}^{2+}$  ions stimulated the enzyme activity to 131%, 180%, 141%, 119% and 123%, respectively. Activity loss can be caused by destabilization of the enzyme as a result of loss of surface charge-charge interaction. Such results are consistent with the enzyme activity actions of other PGs. For example, PG from *Aspergillus niger* MTCC 478 lost 20% and 4% in activity in the presence of  $\text{Co}^{+2}$  and  $\text{Hg}^{+2}$ [39], exopolygalacturonase from a strain of *Bacillus* gained 60%, 36%, 30%, 18% and 16% when  $\text{Mg}^{+2}$ ,  $\text{Mn}^{+2}$ ,  $\text{Pb}^{2+}$ ,  $\text{Ca}^{+2}$  and  $\text{Fe}^{+3}$  were added to the reaction [48].

**Table 1:** Purification scheme for PG.

Step	Total protein	Total activity (units)*	S.A*	Fold purification	Recovery
	(mg)		(units/mg protein)		%
Crude extract Chromatography	20	2580	129	1	100
on DEAE- Sepharose	2	191	95.5		
0.0 M NaCl (P1)	2.2	412	187	0.74	7.4
0.05 M NaCl (P2)	1.7	113	66	1.45	16
0.1 M NaCl (P3)	1.6	89	55.6	0.5	4
0.2M NaCl (P4)	1.9	89	47	0.4	3
0.3M NaCl (P5)				0.36	3
Gel filtration					
on Sephacryl S-200					
PGP2a	0.08	112	1400	10.9	4.3

Note: \*One unit of peroxidase activity is defined as the amount of enzyme which increases the O.D. 1.0 per min under standard assay conditions.

**Table 2:** Effect of metal ions on the activity of purified PGP2a.

Metals (2mM)	% Relative activity
Control	100
Pb <sup>2+</sup>	131
Hg <sup>2+</sup>	66
Co <sup>2+</sup>	75
Zn <sup>2+</sup>	180
Ni <sup>2+</sup>	141
Cu <sup>2+</sup>	119
Ca <sup>2+</sup>	123

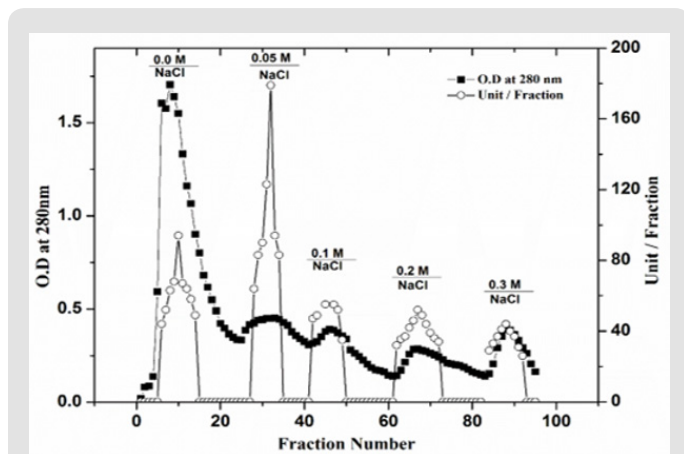


Figure 1: A typical elution profile for the chromatography of PG on DEAE-Sepharose column (10 × 1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 7.2 at a flow rate of 60 ml/h and 3 ml fractions.

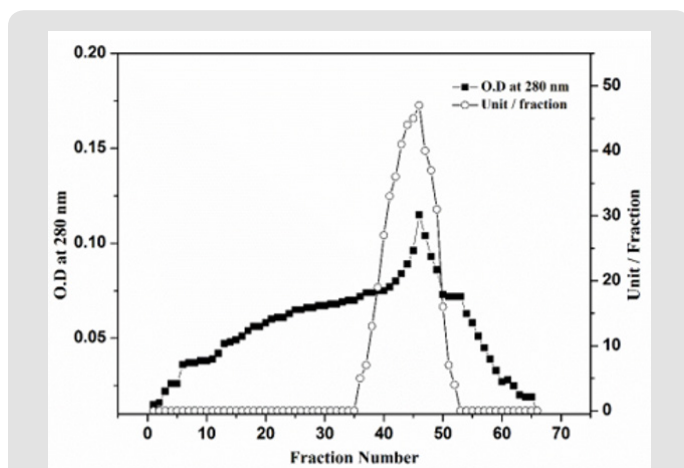


Figure 2: Gel filtration of PGaP2a DEAE-Sepharose fractions on Sephacryl S-200 column (90 × 1.6 cm i.d.). The column was equilibrated with 20 mM Tris-HCl buffer, pH 7.2 at a flow rate of 30 ml/h and 3 ml fractions.

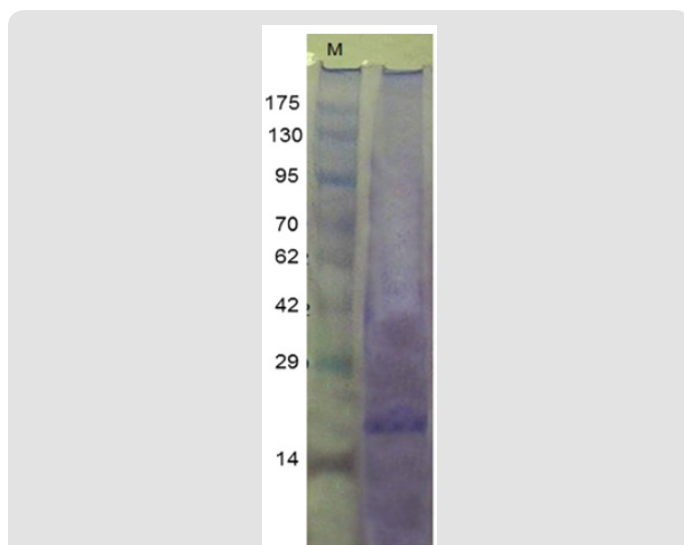


Figure 3: SDS-PAGE for homogeneity and molecular weight determination of PGP2a.

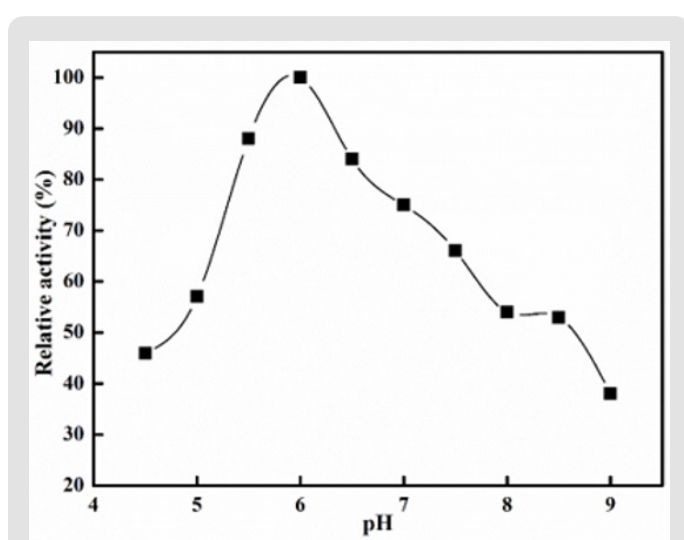


Figure 4: Optimum pH of PGP2a.

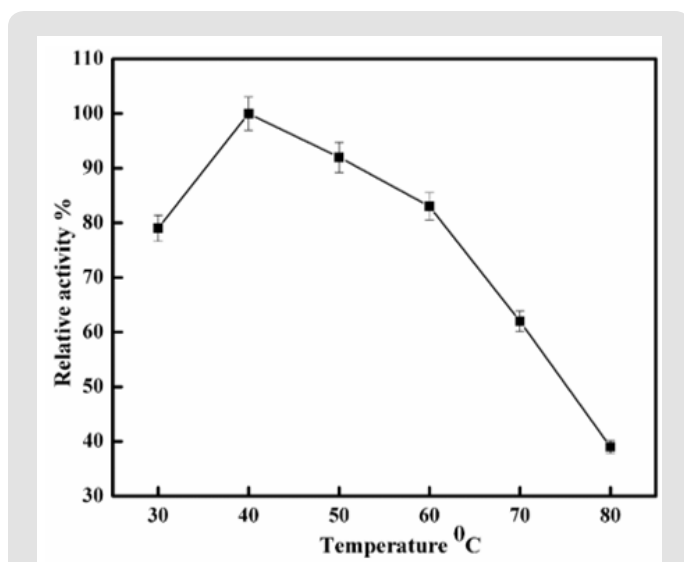


Figure 5: Temperature optimum of PGP2a.

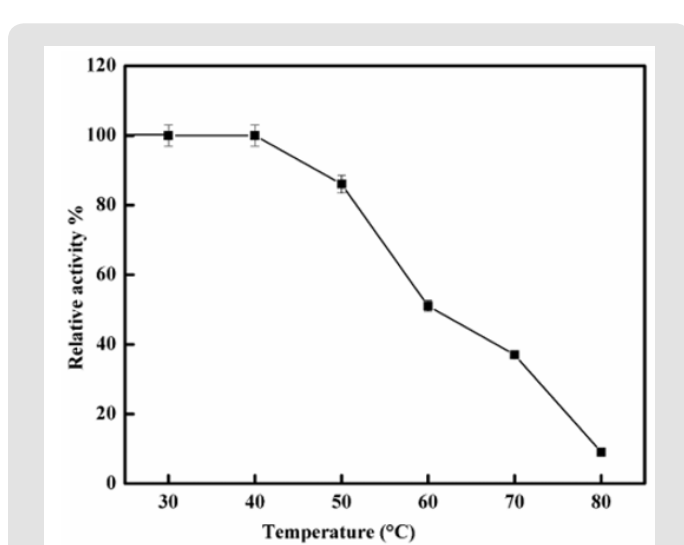


Figure 6: The thermal stability of PGP2a.

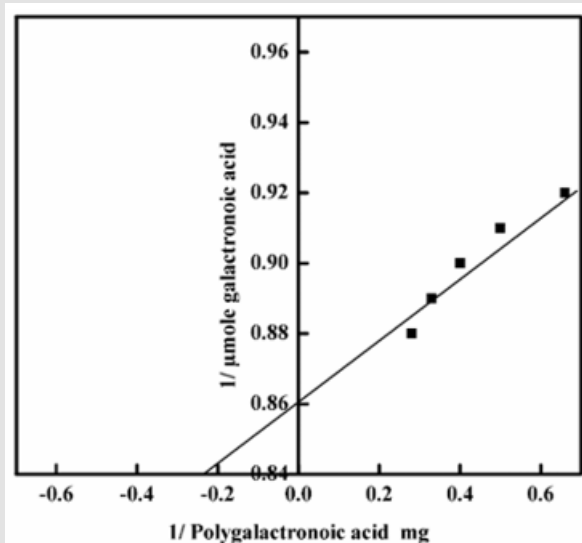


Figure 7: Kinetics parameters ( $K_m$ ) of purified PGP2a.

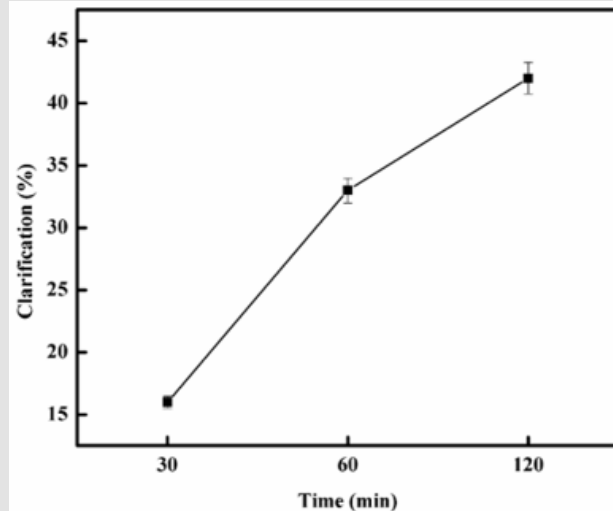


Figure 8: Clarification of orange juices, using 10 units (U) of purified enzyme (PGP2a) activity per mL of juice.

### Application of Purified PG in Clarification of Fruit Juice

Microbial polygalacturonases are generally a significant group of potentially applicable enzymes across different industries such as textile, wine, paper, and food industries [49]. The application of purified PGP2a on orange juice obtained from the local market was studied in clarifying the fruit juice. The clarification of fruit juice was examined using the method [30]. The results of these tests are shown in Figure 8 in which can be seen that the transmittance increased by 16%, 33% and 42% When incubated for 30, 60 and 120 min respectively, with regard to monitoring that the same enzyme amount was applied Just before a mixture of reactions was placed in water bath. The transmission of the juice treated improved by the elimination in particular of pectin of colloidal and suspended particles. Since pectin is present, colloid formation in the fruit juice industry is a major challenge, which reduces the market value of juices. In fruit juices, pectinase degrades pectin in fruit juices, reducing viscosity and cluster formation. The juice is therefore simpler and more intense in taste and colour [50-51]. Fruit juice clarity can be due to the enzyme's biochemical composition. Polygalacturonase had optimum pH at 6.0 and pH stability of 4.5 to 8.5. Therefore, acidic polygalacturonase can be used as a potential candidate for clarifying fruit juice. Several *Aspergillus carbonarius* and *Achaetomium sp Xz8* polygalacturonases has been shown to be able to improve the yield and clarity of the juice [52] and reducing the papaya juice viscosity [53]. Polygalacturonase made by *A. niger* with banana peel as a substratum was used to clarify banana juice [54]. The use of *Neosartorya fisheri* polygalacturonases in clarification of apple and strawberry juice was also reported [55]. An acidic *A. Niger* PG was also used for guava juice clarification [56]. *Penicillium oxalicum* endopolygalacturonase improved the light transmission of papaya pulp by 29.5% [57].

### Conclusion

Polygalacturonase from *Aspergillus niger* was produced by submerged fermentation using seaweed waste was purified simply by DEAE-Sephacryl and Sephacryl S-200 columns chromatography. A relatively molecular weight PGP2a of 20 kDa with pH and temperature optimum of 6 and 40 °C was observed. The  $K_m$  and  $V_{max}$  value of purified PG was found to be 4.27 mg/ml and  $1.16\mu\text{mol min}^{-1}\text{mg}^{-1}$ , respectively, several metal ions under studies found to enhance the PG activity. The potential of purified PGP2a in clarification of orange juice was illustrated owing to its acidic nature.

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