Purification and properties of polygalacturonase associated with the infection process of *Colletotrichum truncatum* CP2 in chilli

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Abstract*—In this study, polygalacturonase enzyme produced by Colletotrichum truncatum CP2 was partially purified by aqueous two-phase system and the properties of this enzyme was characterized. The highest yield (57.4%) and purification fold (5.1) was obtained using 22% PEG 6,000/15% sodium citrate comprising crude load of 16% (w/w) at pH 7.0 with addition of 1.0% (w/w) sodium chloride. The partially purified PG remained active over a wide range of pH (2.5-6.0) and the optimum activity was obtained at pH 5.0. Incubation of the partially purified PG at 40 and 50 °C for 30 min caused the activity of PG to decrease up to 20% and 40%, respectively. However, no significant changes in the activity when the enzymes were incubated up to 4 h at 40 and 50 °C. The results from this study suggested that ATPS comprising of PEG and sodium citrate could be potentially used as an alternative method for purification of PG.*

Keywords*— *Chilli, Colletotrichum truncatum, polygalacturonase, aqueous two-phase system.

I. INTRODUCTION

The plant cell wall is a major barrier to the establishment of fungal infection on a host. Most plant-pathogenic fungi produce a number of cell wall-degrading enzymes when grown in liquid culture containing pectin. One of these enzymes, PG has been implicated routinely in facilitating the invasion and colonization of host tissue during pathogenesis of fungal pathogens (Choi *et al.*, 2013). Highly purified PG from many fungal pathogens has been proved by several researchers to have the ability to cause cell maceration and kill tissues in a similar way to that seen in soft-rot diseases (Protsenko *et al.*, 2010; Herbert *et al*., 2004; Oeser *et al*., 2002).

Many organisms produces polygalacturonases, for example, bacteria, parasites and yeast (Jurick *et al.*, 2009; Latif & Sohail, 2012). Microbial PG from different microbial sources shows wide variety in their physicochemical and biological properties. Most of the PG was found optimum at pH range of 3.5 to 5.5 and temperature between 30 to 50 °C. Molecular mass of the PG also varies from 25 kDa to 85 kDa.

Microbial PG has to be purified for the complete understanding of its properties. Numerous purification strategies have been reported for PG all with varying degree of success. The purification methods commonly employed include ammonium sulphate precipitation, ion-exchange chromatography, Sephadex G-25 gel filtration, ultrafiltration, gel permeation chromatography and ethanol precipitation (Deshmukh *et al*., 2012; Jurick *et al*., 2009; Thakur *et al.*, 2010). Each purification method has its own drawback associated with low yield and purity, cost and the requirement for a skilled operator (Shaligram & Singhal, 2010).

The ATPS has been proposed as an ideal and versatile strategy for the extraction and purification of biomolecules because of its high productivity, environmental-friendly, simplicity, short processing time, cost effectiveness and ease of scaling-up (Naganagouda & Mulimani, 2008; Raja *et al*., 2012). ATPS which consist of PEG/ salt system has been generally employed for the bioseparation of proteins due to its availability at low cost and wide range of hydrophobic differences between the two phase systems which allow enhancement of the partition selectivity of the target protein (Mehrnoush *et al*., 2011). ATPS has been applied in the extraction and purification of various compounds such as enzymes, biopharmaceuticals ad natural products (Srinivas & Raghavarao, 2000).

Selection of ATPS as a purification method is usually dependent on the types of biomolecules and economic considerations. Since the polymer/polymer system is very costly, the aqueous two phase polymer/salt systems are often used compared to the polymer/polymer systems (Abbasiliasi *et al.*, 2014). Moreover, polymer/salt systems have significant differences in density,

greater selectivity, lower viscosity, lower cost and the larger relative size of the drops (Nadar *et al*., 2017). Phosphates and sulfates are commonly used salts in polymer/salt ATPS. But the use of these salts has contributes to environmental problems, especially high concentrations of phosphate and sulfates in the effluent streams. Currently, the use of citrate salts as one of the ATPS components with PEG is preferred since citrate salts are biodegradable and non-toxic (Glyk *et al.*, 2015).

In view of the fact that ATPS is an ideal purification technique for biomolecules such as enzymes, this study evaluated the partitioning efficiency of a PG produced by *C. truncatum* CP2 using ATPS which comprised of PEG and sodium citrate. Since the partitioning mechanism in ATPS is still unknown, the effects of various parameters on the partitioning of the PG, such as the molecular weight, salt concentration, pH, crude load and addition of sodium chloride (NaCl) were investigated. The physico-chemical properties of partially purified PG were also characterized.

II. MATERIAL AND METHOD

2.1 Chemicals

Different molecular weight (MW) of PEG, ranging from 4,000 (g/mol) to 10,000 (g/mol), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium citrate was supplied from SAFC (St. Louis, MO, USA). The protein assay kit and albumin standard were obtained by Bio-Rad, USA and Thermo Scientific Pierce, respectively. All chemicals and reagents used were analytical grade.

2.2 Microorganism and polygalacturonase production

The polygalacturonase producing strain used in this study was *C. truncatum* CP2 which was isolated from lesions of chilli anthracnose**.** Polygalacturonase enzyme was produced in a shaking flask in the basal medium containing pectin from citrus as a carbon source. The fermentation broth was filtered through Whatman filter paper and centrifuged prior to purification by ATPS. The filtrate was used an enzyme source.

2.3 Construction of ATPS

The aqueous two phase system was prepared from stock solution of 50% (w/w) PEG with different MW (PEG 4,000, 6,000, 8,000 and 10,000 g/mol) and 40% (w/w) of sodium citrate stock solutions. The ATPS system was prepared in 15 mL centrifuge tubes. The amount of PEG solution, citrate solution and distilled water were weighed appropriately and mixed with crude enzymes 16% (w/w) to form a 10 g system. The solution was stirred thoroughly using a vortex mixer and then centrifuged at 2,860 × g for 10 min to achieve the phase separations. With the use of pipette, the upper phase was removed and the lower phase was then collected. The volumes of both phases were measured and the PG activity and total protein concentration of each phase were then analysed.

2.4 Polygalacturonase activity assay

Polygalacturonase (PG) activity, the method of Nelson and Somogyi (Nelson, 1944; Somogyi, 1952) was used to measure the release of reducing groups from polygalacturonic acid. The reaction mixture contained 1.8 mL of 1% PGA in 50 mM sodium acetate buffer (pH 4.8) and 0.2 mL of crude enzyme. For the control, the reaction mixture contained the same components except the enzyme was boiled for 5 min. The reaction mixture was then incubated at 40 °C for 30 min followed by addition of 3 mL of 3,5-dinitrosalicyclic (DNS) reagent. The reaction mixture was then heated at 100 °C for 15 min. Then, it was allowed to cool down at room temperature before addition of 1 mL Rochelle salt. The absorbance was measured at 545 nm using a spectrophotometer. The formation of reducing sugars was calculated using D-galacturonic acid as a standard. The amount of enzyme releasing 1 µmol of galacturonic acid per min at pH 4.8 and 40 °C was considered as one enzyme unit.

2.5 Protein concentration determination

The amount of protein concentration was determined using a Bicinchonic Acid (BCA) assay kit with the used of Bovine serum albumin (BSA) as a standard. A total of 50 µL of the sample was mixed with 1 mL of working reagent and then incubated at room temperature for 2 h. The absorbance was measured at 562 nm using a UV spectrophotometer (GENESYS 20, Thermo Scientific, UK).

2.6 Optimization of ATPS parameters

Standard experimental parameters in the aqueous two-phase extraction including MW of PEG, pH, concentration of PEG and sodium citrate in each phase, the addition of sodium chloride and the amount of loaded crude enzyme (C_L) were optimized

for PG recovery. Optimization was done (in triplicates) based on the experimental default conditions set at: total mass of aqueous phase = 10 g, crude load, 16% (w/w), PEG concentration = 50% (w/w) and pH = 7.0.

2.7 Determination of partition coefficient (K), yield of PG (%) and purification factor (P_F)

The partition coefficient of PG was determined by dividing the PG activity in the top phase with the PG activity in the bottom phase as shown below:

$$K = \frac{AT}{AB} \quad [1]$$

Where:

A_T and A_B is the PG activity in the upper and bottom phases, respectively.

In order to evaluate the purification process, the PG specific activity **(SA)**, the purification factor **(P_F)** and the enzyme yield recovered in the upper **(Y_T)** phases were also calculated, according to the given equations:

$$SA\left(\frac{U}{mg}\right) = \frac{PG\ activity\ (U/mL)}{[protein\ ](mg/mL)} \quad [2]$$

$$PF = \frac{SA\ of\ phase\ sample}{SA\ of\ crude\ feedstock} \quad [3]$$

$$YT = \frac{100}{1+[\frac{1}{VR}\times KE]} \quad [4]$$

$$VT = \frac{VT}{VB} \quad [5]$$

Where:

VT is the volume ratio, and V_T and V_B are the volumes of upper and bottom phases, respectively.

2.8 Characterization of partially purified polygalacturonase

The partially purified enzyme from *C. truncatum* CP2 was characterized and its different properties were examined. The characteristic studies include: substrate specificity and effect of temperature and pH on enzyme stability. The experimental procedures are performed as below.

2.8.1 Substrate specificity

Substrate specificity of the partially purified enzyme was studied by using different substrates in the reaction mixture for enzyme assay. The enzyme assays were performed under standard conditions with a fixed substrate concentration of 0.5% (w/v). The various substrates used were citrus pectin, apple pectin, xylan and CM-cellulose (Kant *et al*., 2013).

2.8.2 Effect of pH on enzyme activity and enzyme stability

The optimal pH for enzyme activity was determined by incubating the reaction mixture at different pH values using different buffers (50 mM sodium acetate for pH 3-4.5, 50 mM sodium citrate for pH 5-5.5 and 50 mM sodium phosphate buffer for pH 6-9). The PG activity was measured under standard assay conditions. For pH stability determination, the enzyme was preincubated at different pH values ranged from pH 2.5-7.5 at 4 °C for 2 h. The residual activity for PG enzyme was assayed with PGA as a substrate soon after incubation.

2.8.3 Effect of temperature on enzyme activity and stability

To determine the effect of temperature on the enzyme activity, the reaction mixture was incubated at different temperatures (30 to 90 °C) for 30 min and the activity was measured. For determination of thermal stability, the enzyme was incubated for variable durations (30 min to 4 hours) at fixed temperatures (40 to 50 °C). The residual activity was assayed soon after the incubation period.

III. RESULTS AND DISCUSSION

3.1 Effect of PEG molecular weight on partitioning of PG

The properties of polymer including concentration and its molecular weight are probably the most vital factors affecting the separation of biomolecules in different phases (Yang *et al*., 2013). Therefore, the effect of different molecular weight of PEG

(4,000-10,000 g/mol) on partitioning of PG was studied. The results show that the partition coefficient decreased as the PEG molecular weight increased (Table 1). This was due to a reduction in the volume to accommodate the target enzyme in the upper phase (PEG-rich phase) as PEG molecular weight increased. Volume exclusion usually occurs when high molecular weight of PEG is used due to the lack of molecular space for the enzyme (Benavides *et al.*, 2011; Grilo *et al.*, 2016). This will ultimately partitioned the PG enzyme in the bottom phase and consequently decreased the partition coefficient of the system.The highest purification factor was obtained with PEG 6,000 (2.10) indicating that there was an appropriate volume available to accommodate PG in the upper phase. PG yield was decreased when the PEG molecular weight increased which was most likely due to increase partitioning of PG to the lower phase. In addition, decreasing of purification factor of PG at high PEG molecular weight is due to the addition of PG to lower phase compared with other proteins (Mokhtarani *et al.*, 2008). Based on the results obtained, PEG 6,000 was selected as the most suitable molecular weight for further studies.

TABLE 1
INFLUENCE OF PEG MOLECULAR WEIGHT AND CONCENTRATIONS ON THE PARTITIONING BEHAVIOR OF PG

MW of PEG[a] (g/mol)	Partition coefficient (K)	Yield (%)	Purification factor
4,000	0.55^{A}	69.39^{A}	0.96^{A}
6,000	1.12^{B}	42.02^{B}	2.10^{B}
8,000	0.63^{C}	36.36^{C}	0.80^{C}
10,000	0.43^{C}	31.81^{C}	0.72^{C}

Note: [a] 22% (w/w) PEG + 16% (w/w) sodium citrate. Values within a column followed by different letters are significantly different at ($p < 0.05$).

3.2 Influence of salt concentration on the partitioning behavior of PG

The effect of different concentration of sodium citrate (12-16%, w/w) on the partition behaviour of PG from *C. truncatum* CP2 was investigated. Based on the results obtained, the purification factor and yield of PG was increased when the concentration of sodium citrate was increased from 12 to 15 % (w/w) (Fig. 1). The increased in purification factor and yield is due to the salting out effect of sodium citrate which caused increased PG partition in upper phase of the system. However, further increase in sodium citrate concentration up to 16% (w/w) has led to a decrease in the yield and purification factor. This could be due to the contaminating proteins being moved to the top phase which reduced the purity of the enzyme. These results are in agreement with the finding of Mehrnoush *et al*. (2011) who reported that increased of potassium phosphate concentration up to 20% (w/w) would decrease the purification factor and yield of pectinases from mango. Thus, the subsequent experiment was performed with 22% PEG 6,000/ 15% sodium citrate combination.

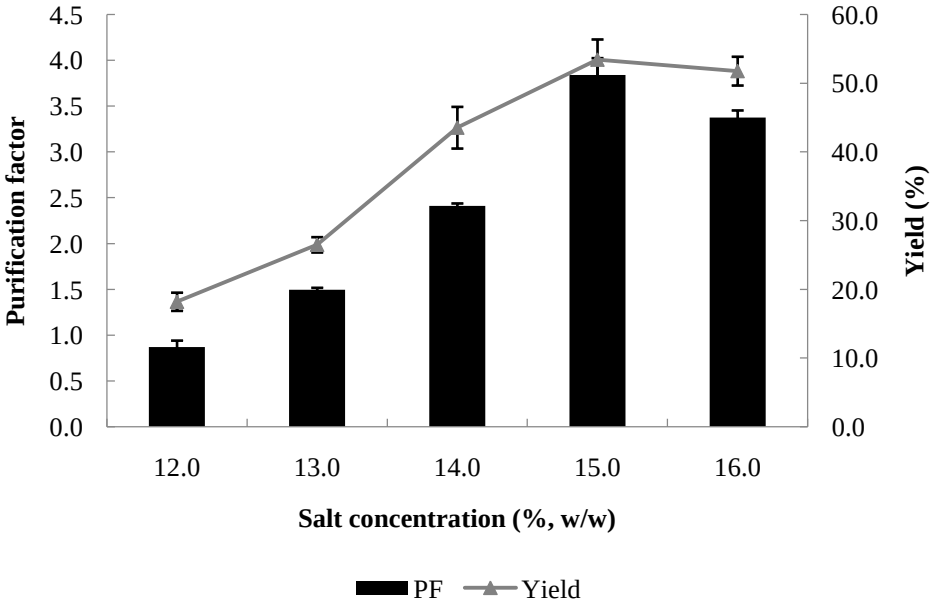

FIG. 1: INFLUENCE OF SALT CONCENTRATION ON THE PARTITIONING BEHAVIOUR OF PG. VALUES ARE MEANS OF 3 REPLICATES WITH ± SD.

3.3 Influence of pH on the partitioning behavior of PG

The effect of pH on partitioning of PG is important as the surface charge of protein is highly influenced by the pH of the system (Ooi *et al*., 2009). To achieve higher performance in partitioning of PG in ATPS, a suitable pH should be selected (Rahimpour & Baharvand, 2009). The partitioning behaviour of PG in ATPSs with different pHs are shown in Table 2. As shown in the table, the purification factor, partition yield and selectivity were increased with an increase of pH from 6.0 to 7.0. The purification factor (3.6), partition coefficient (0.91), yield (55%) and selectivity (1.84) were the highest when pH 7 was used. At this pH, most of the PG was partitioned to the upper phase. This was due to the effect of protein charge of PG (Benavides *et al*., 2011). The isoelectric point of PG is 6.1. At the isoelectric point of the protein, the sum of all the charges on the protein is zero. When the solution pH changes from acidic value to basic value, the protein become less positively or more negatively charge (Ratanapongleka, 2010). The charge of PG tends to be negative at pH 7 while PEG tends to be positive. This caused the attraction of PG to the positively charge PEG. However, a sudden reduction in the purification factor, partition coefficient, yield and selectivity were observed at pHs above 7. Higher pH was not favourable as the partition coefficient, yield and purification factor were decreased. This was due to the change of electrostatic interaction of contaminating proteins (Md Sidek *et al.*, 2016). The negatively charge contaminating proteins tends to be partitioned into the top phase at higher pH. Thus, pH 7 was selected as the optimum and used for the subsequent experiments.

TABLE 2
EFFECT OF PH ON THE PARTITIONING BEHAVIOUR OF PG. THE PH WAS VARIED FROM 6.0 TP 9.0. THE PURIFICATION FACTOR (PFT), PARTITION COEFFICIENT (K), YIELD (%) AND SELECTIVITY (S) OF PG WERE CALCULATED AND PLOTTED AT DIFFERENT PHS.

pH value[a]	Purification factor (P_{FT})	Partition coefficient (K)	Yield (%)	Selectivity (S)
6.0	1.0^{A}	0.03^{A}	10.9^{A}	0.01^{A}
6.5	2.1^{B}	0.34^{B}	45.2^{B}	1.50^{B}
7.0	3.6^{C}	0.91^{C}	55.0^{C}	1.84^{C}
7.5	1.4^{C}	0.41^{D}	50.1^{C}	0.20^{D}
8.0	1.0^{C}	0.39^{D}	44.5^{D}	0.16^{D}
8.5	1.1^{C}	0.34^{D}	42.5^{D}	0.14^{D}
9.0	0.9^{C}	0.33^{D}	43.3^{D}	0.11^{D}

Note: [a]22% (w/w) PEG + 15% (w/w) sodium citrate. Values within a column followed by different letters are significantly different at ($p < 0.05$).

3.4 Influence of crude load on the partitioning behavior of PG

The crude load has its own significance effect on the partitioning behaviour of PG. Fig. 2 shows the influence of crude load ranging from 10% (w/w) to 22% (w/w) on the partitioning behaviour of PG. The purification factor and yield were increased linearly with an increase of crude load from 10% (w/w) to 16% (w/w). However, the yield and purification factor start to decrease when crude stock loading higher than 16% (w/w) were used. Higher amount of crude extract may alter the properties of ATPS, which in turn affected the biomolecules partitioning (Asenjo & Andrews, 2012; Raja *et al*., 2012). The higher accumulation of PG at the Peg and salt interfaces was not favourable as it may cause the loss of PG activity. Hence, 16% (w/w) crude stock loading was chosen for further study.

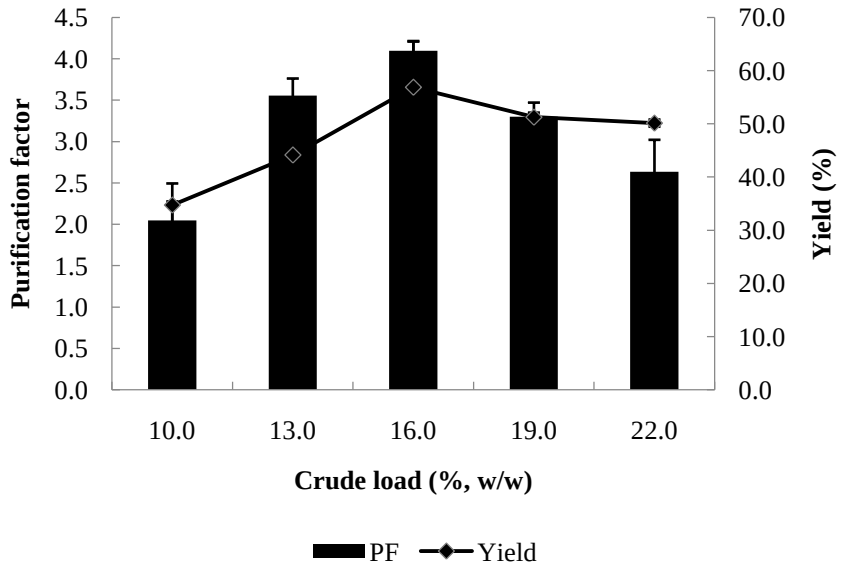

FIG. 2: INFLUENCE OF THE CRUDE LOAD ON THE PARTITIONING BEHAVIOUR OF PG. VALUES ARE MEANS OF 3 REPLICATES WITH ± SD.

3.5 Influence of NaCl on the partitioning behavior of PG

The effect of adding of NaCl, ranging from 0 to 3.0% (w/w) was studied for 22% PEG 6,000/15% sodium citrate with crude load 16% at pH 7.0 and the results is illustrated in Fig. 3. The addition of NaCl to the ATPS causes a hydrophobic difference in both phases increases thereby enhances the partitioning of hydrophobic proteins into the top phase. The addition of NaCl to the ATPS also improves the interaction between hydrophobic chain of PEG and the hydrophobic surface of PG. The optimum partitioning condition for PG was achieved with 1.0% (w/w) addition of NaCl. At this optimum point, PG exhibited the highest purification factor of 5.1, as well as the yield (57.4%). However, the yield and purification factor were decreased with an increase in NaCl concentration more than 1.0% (w/w). Ratanapongleka (2010) suggests that the addition of salt at high concentrations may lead to salt aggregation followed by protein precipitation because most of the water molecules were strongly attached to the salts. The interactions between proteins become stronger than between protein and water. Hence, the purification of PG in the subsequent experiments was carried out with addition 1.0% (w/w) of NaCl.

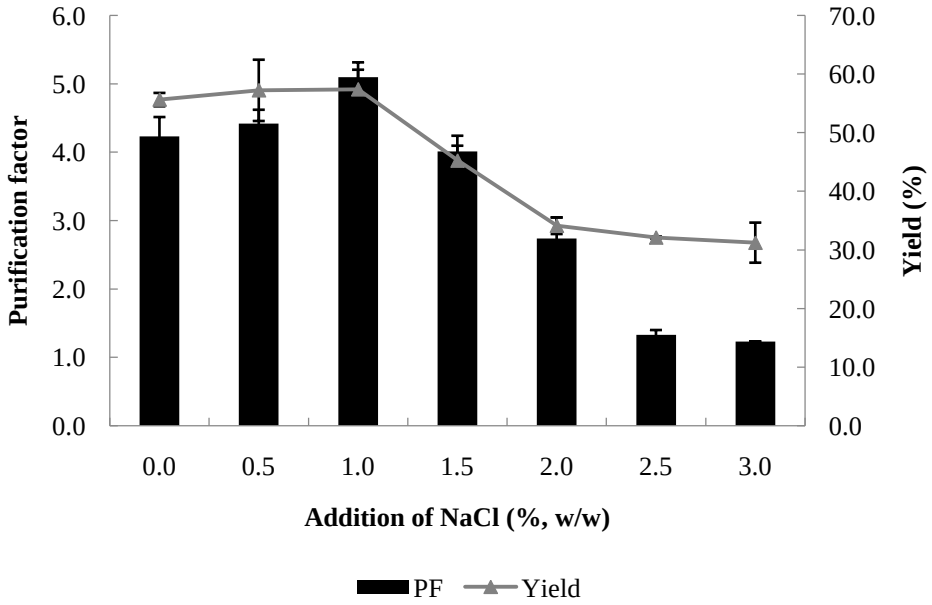

FIG. 3: INFLUENCE OF NACL ADDITION ON THE PARTITIONING BEHAVIOUR OF PG. VALUES ARE MEANS OF 3 REPLICATES WITH ± SD

3.6 Properties of partially purified PG

3.6.1 Substrate specificity

The specificity of the partially purified enzyme was evaluated with various substrates. The maximum PG specificity was observed when PGA was used as a substrate (Table 3). The enzyme also had a remarkable specificity with citrus pectin (81.2%) and apple pectin (64.9). Similar results were obtained by Martins *et al.* (2007) for PG from *Thermoascus aurantiacus*. Very low activity of enzyme was observed toward non-pectic polysaccharides (xylan and CMC). These results revealed that PG produced by *C. truncatum* CP2 had very high affinity and specificity toward PGA and pectins.

TABLE 3
SUBSTRATE SPECIFICITY OF PG FROM *C. TRUNCATUM* CP2 TOWARD DIFFERENT SUBSTRATES

Substrate	Relative activity (%)
PGA	100
Citrus pectin	71.2
Apple pectin	64.9
Xylan	10.1
CMC	8.4

3.6.2 Effect of pH on the activity and stability of PG

The influence of pH on the activity and stability of PG is shown in Fig. 4. Partially purified PG from *C. truncatum* CP2 was found to have optimum at pH 5.0. PG retained above 50% of its maximum activity in a broad pH range of 2.5-6.0. The pH optimum for activity of PG from *C. truncatum* CP2 is in good agreement with the optimum pH reported for PGs from *Aspergillus flavus*, *Trichoderma harzianum* and *Cylindrocarpon destructans* (Anand *et al*., 2017; Sathiyaraj *et al*., 2011). It is also very close to the pH optimum of *Rhizomucor pusilis* PG found by Siddiqui *et al*. (2012). Either increase or decrease in pH beyond the optimum value showed decline in enzyme activities. This PG was inactive at pH 8.0. Previous reports on biochemical properties shows that majority of fungal PGs are optimum at pH in acidic range (El-Batal *et al.*, 2013; Pathak *et al.*, 2000; Quiroga *et al.*, 2009). In the present study, it was observed that the maximum stability of PG enzyme was between pH 4.5 and 5.5. The results showed that the enzyme was very stable at pH 5.0 and retained 84.5% and 79.2% of its activity at pH 4.5 and 5.5, respectively. The enzyme lost about 80-90% of its activity at pHs 2.5 to 3.0 and pHs 7.0 to 9.0. This observation agreed with the results reported for PGs from *Trichoderma harzianum.* PG from *Mucor flavus* was found to be optimum at pH 4.0 to 6.0 but at pH 7.0 the stability decreased up to 80% (Saad *et al.*, 2007). Similar observation was reported by Gadre *et al*. (2003) in which the stability of PG from *Mucor flavus* decreased to 60% at pH 7.0.

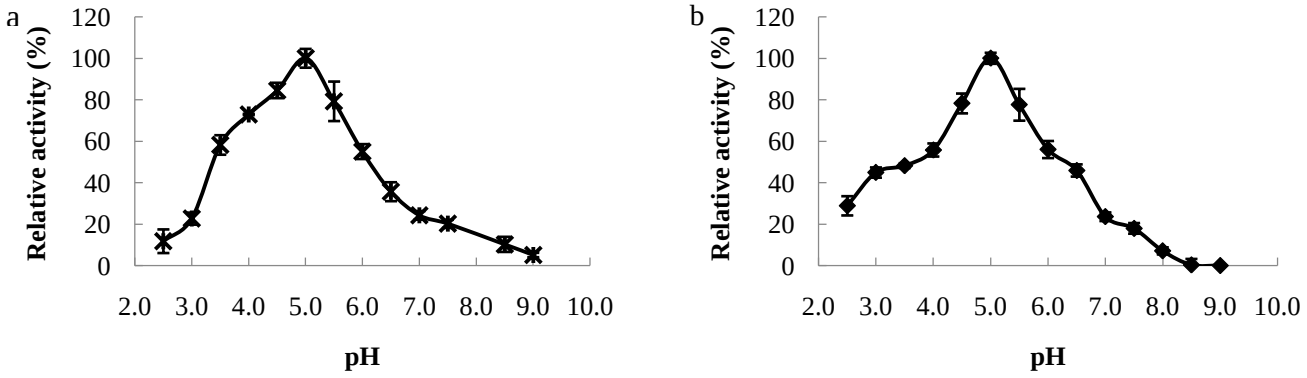

FIG. 4: EFFECT OF PH ON THE ACTIVITY AND STABILITY OF PARTIALLY PURIFIED PG (A) PH OPTIMA (B) PH STABILITY. VALUES ARE MEANS OF 3 REPLICATES WITH ± SD.

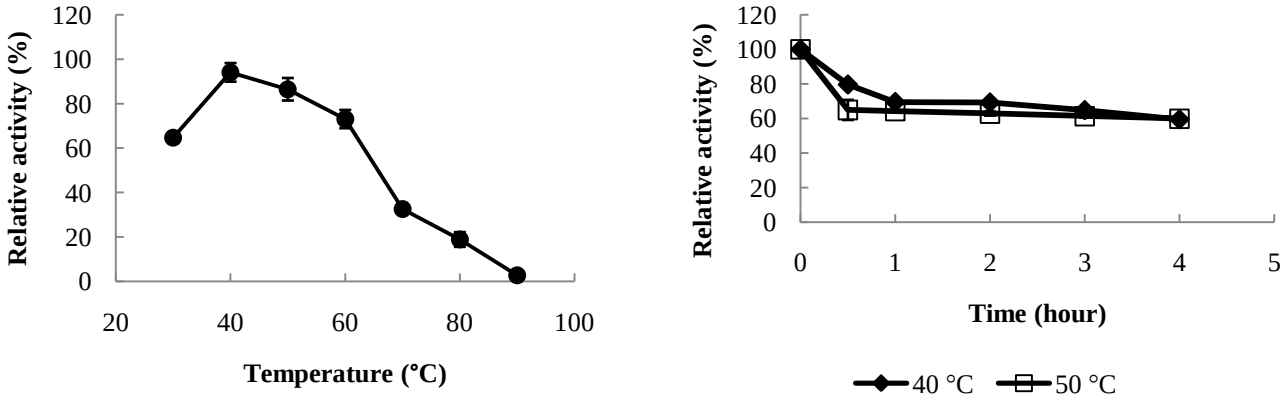

FIG. 5 EFFECT OF TEMPERATURE ON THE ACTIVITY AND STABILITY OF PARTIALLY PURIFIED PG (A) TEMPERATURE OPTIMA (B) TEMPERATURE STABILITY. VALUES ARE MEANS OF 3 REPLICATES WITH ± SD.

IV. CONCLUSION

In brief, ATPS comprising PEG and sodium citrate could potentially be used as an alternative method for purification of PG from the fermentation broth of *C. truncatum* CP2. The optimum condition for purification of PG was achieved using PEG/sodium citrate comprising crude load of 16% (w/w) at pH 7.0 with addition of 1.0% (w/w) sodium chloride. Partial purification of PG with ATPS led to 57.4% recovery of enzyme with 5.1-fold purification.

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