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Secretion of type II extracellular cell wall degrading enzymes from *Pantoea agglomerans* phytopathogen

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ABSTRACT

In the present study the bacteria were isolated from infected Cajanus cajan seed and identified by 16S rDNA sequencing as Pantoea agglomerans. The virulence of the plant pathogen is dependent on the production and secretion of a large variety of plant cell wall–degrading enzymes. Different cell wall degrading enzymatic activities such as cellulase, polygalacturonase, xylanase, invertase and glycosidase were assayed from the P. agglomerans growing in four nutrient media with different compositions (A) N-broth with 5% sucrose (B) N-broth (C) Minimal medium consisted of sucrose - 5g, peptone - 0.8g, Na_2HPO_4 - 1g, KH_2PO_4 - 0.6g, $MgSO_4.7H_2O$ - 1g per 200ml and (D) Minimal medium with small Gossypium hirsutum leaves (500mg/100ml media). Secretion of enzymes has been shown to play an important role in pathogenicity and to understand the interaction of P. agglomerans and Gossypium hirsutum, a biochemical analysis of secreted proteins in the presence of G. hirsutum leaves was performed. Amongst the studied enzymes cellulase, polygalacturonase and xylanase activity observed higher in media C and glycosidase activity in media D. The probable role of these enzymes in the mechanism of pathogenicity is discussed.

Keywords: Plant–pathogen interaction, *Pantoea agglomerans*, 16S rDNA, Cell-wall degrading enzymes

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INTRODUCTION

The plant cell wall is a natural physical barrier against pathogens and is at the forefront of the interaction between plants and pathogens. To gain access to the contents of plant cells many phytopathogenic bacteria secreted T2SS virulence factors: cell-wall degrading enzymes (CWDE) such as cellulase, pectinase, xylanase and glycosidase [1, 2]. CWDE function to break down the components of host cell walls and may play a crucial role in virulence and bacterial nutrition [3, 4].

Another enzymatic activity which is not related to cell wall degradation but can be an important to pathogen is the invertase, a sucrose hydrolyzing enzyme. Sucrose is the major transportable product of photosynthesis connecting source to sink organs via the phloem. Sucrose is present in the intercellular spaces of source organs and pathogens may use sucrose as source of carbon as well as energy [5].

Bacteria from the genus *Pantoea* have become increasingly important plant pathogens around the world. Many plant pathogenic *Pantoea* species are seed borne and seed transmitted such as *P. agglomerans* in cotton [6]. It also cause leaf blight and bulb rot in onion [7, 8]. Despite the increasing frequency of plant disease caused by *P. agglomerans* it is necessary to study the mechanisms of pathogenicity. Howerever, till date the secretion of cell wall degrading enzymes by *P. agglomerans* is not reported. Considering these point, in the present work bacteria were isolated from infected plant material and identified by 16S rDNA sequencing. The present study is aimed to evaluate the extracellular cell wall degrading enzymes (CWDE) *in vitro* produced from *Pantoea agglomerans* with time course using different media with or without sucrose, as it is a well known fact that sucrose is the common carbon source translocated in the plant system.

MATERIALS AND METHODS

Test organisms

Pantoea agglomerans (SU01) was isolated from *Cajanus cajan*. It was cultured on N-agar media and identified on basis of biochemical tests and 16S rDNA sequencing.

Identification of Bacteria

DNA extraction

Bacterial cells were pelleted by centrifugation, resuspended in 500 µl of 10 mM Tris-EDTA buffer, and treated with 30 µl SDS (10% W/V), 2 µl Proteinase K (10mg/ml). It was mixed well and incubated for 1 h at 37ºC of. Then after 20 µl of Cetyltrimethylammonium bromide (10%, W/V) and 100 µl of NaCl (5 M) were added, and incubated for 10 min at 65°C. DNA was purified by two 1:1 extractions in which we used (i) Chloroform: Isoamyl alcohol (24:1) (ii) Phenol: Chloroform: Isoamyl alcohol (25:24:1) and then was precipitated with isopropanol, washed with ethanol (70%), and dissolved in Tris-EDTA buffer. The quality and concentration of the DNA was confirmed by measuring optical density 260/280 nm ratio.

16S rDNA gene amplification

The 16S rDNA gene was amplified using universal primer pair 8F (5-AGA GTTTGATCCTGGCTCAG-3') and 1525R (5'-ACGGCTACCTTGTTACGA CTT-3'), 946F (5'-CCCGCACAAGCGGTGGA-3') and 1389R (5'-ACGGGCGGT-GTGTACAAG 3'). DNA was amplified in a total volume of 25 µl. The reaction mixture contained 2.5 µl 10X buffer (10mM Tris-HCl pH 9.0, 50mM KCl, 0.1% Trion X100), 1.5mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate, 10 µM primer and 1U of *Taq* DNA polymerase, 200 ng bacterial DNA. Profile of PCR was: initial denaturation 95ºC – 5min: followed by 35 cycles: denaturation 95ºC - 30 s , annealing 52ºC- 45 s, extension 72ºC - 2 min and final extension 72ºC-12 min. Amplified DNA fragments were separated by electrophoresis through 1.5% low melting agarose gel. DNA fragments were eluted from low melting temperature agarose gels. The band of interest is excised with a sterile razor blade, placed in a microcentrifuge tube, frozen at -20ºC, and then melted. TE-saturated phenol was added to the melted gel slice and the mixture was again frozen and then thawed. After this second thawing, the tube centrifuged and the aqueous layer removed to a new tube. The DNA was concentrated by ethanol precipitation.

Sequencing of 16S rDNA gene

The eluted PCR products were sequenced using a Big Dye Terminator V 3.1 Cycle Sequencing Kit using ABI 3130 genetic analyzer. The sequencing reaction required 1 µl of Premix, 10 pmol of sequencing primer and 200 ng of the PCR product template in a total volume of 10 µl. 16S rDNA partial sequence was determined using 8F and 1525R sequencing primers. All sequencing reactions were performed using the Veriti ™ Thermal Cycler with 45 cycles of denaturation (95°C, 30 s), annealing (52ºC, 20 s) and extension (60°C, 4 min).

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been submitted to the NCBI GenBank database (Accession number SU01 strain- JF501472).

Estimation of cell wall degrading enzymes

Media preparation and culture conditions

Four different media were used for assay (A) N-broth with 5% sucrose (B) N-broth (C) Minimal medium consisted of sucrose - 5g, peptone - 0.8g, Na_2HPo_4 - 1g, KH_2PO_4 - 0.6g, $MgSo_4.7H_2O$ - 1g per 200ml and (D) Minimal medium with small *Gossypium hirsutum* leaves (500mg/100ml media). All media were autoclaved for 15 min and allowed to cool to room temperature. The inoculums (0.1ml) was added to each flask and maintained at 37ºC. At regular intervals of 24 h, 30ml aliquot from each medium was obtained and the supernatant was separated from bacterial cells by centrifugation at 10,000 g for further assay. The supernatant of each medium was directly used as a source of enzymes.

Cellulase assay

Cellulase activity was determined according to the method of Lowe et al. [9]. In this assay, Whatman filter paper no. 1 strips (6 x 1 cm; ca 50mg) were used as a substrate to which 1ml 0.2M Sodium-phosphate buffer (pH 7.2) and 1ml culture supernatant were added. This mixture was incubated at 45ºC for 4 h. The reaction was stopped by addition of 1ml of Miller's reagent (Dinitrosalicylic acid 5g, 2M NaOH, Na-K tarterate 40g). The reducing sugar produced in the reaction was quantified with Miller's reagent and absorbance of the reaction mixture was measured at 550 nm.

Polygalacturonase and Xylanase assay

Polygalacturonase and Xylanase activity was determined according to the method of Patel and Thaker [10]**.** The assay medium consisted of equal volume of buffered substrate [2mg/ml polygalacturonic acid or xylan in 100mM sodium acetate buffer (pH 5)] and enzyme solution. After 60 min incubation at RT (30 ± 2ºC), the reaction was terminated by the addition of Miller's reagent and reducing sugars were estimated by measuring the absorbance of reaction mixture at 520 nm.

Invertase assay

The reaction mixture contained 0.15M sucrose and 1ml enzyme solution. After 60min incubation at RT (30 ± 2ºC), the reducing sugars were estimated by Miller's reagent. Control was prepared by addition of Miller's reagent prior to the substrate. Absorbance was measured at 520nm.

The quantity of sugar released was calculated from the standard curve prepared using glucose (100-1000mg/ml). The cellulase, polygalacturonase, xylanase and invertase activities were expressed as mg sugar released/mg protein. The assay was performed in triplicates for each enzyme extract and mean values with ± standard deviation were calculated.

Glycosidase assay

This activity was determined as described by Thaker et al. [11]. The assay medium consisted of equal volume of enzyme extract and buffered substrate (mg/ml p-nitrophenyl α/β-D galactopyranoside or p-nitrophenyl β-D-glucopyronoside in 100mM sodium acetate buffer pH 5). After 60 min incubation at RT (30 $\pm$ 2 ºC), the reaction was terminated by the addition of 1M Na_2CO_3 solution in one and a half times the volume of the reaction mixture.
In control reaction, Na_2CO_3 was added prior to the addition of enzyme. The absorbance of the yellow pNP released was measured at 405nm. The quantity of pNP released was calculated from the calibration curve (100 to 1000µg pNP/ml) prepared in same buffer. The activity was expressed as µg pNP/mg protein for all the four media. The assay was performed in triplicates for each enzyme extract and mean values with ± standard deviation were calculated.

RESULTS AND DISCUSSION

In the present study, bacteria were identified by biochemical tests and 16S rDNA sequencing. Sequence of 16S rDNA from strain SU01 was shown to have a 93 % similarity with *Pantoea agglomerans* by BLAST analysis. The Accession number derived for this sequence was JF501472.

Plant-pathogenic bacteria grow in the intercellular spaces of plant tissues and rely on nutrients available there. Most phytopathogenic microorganisms produce different enzymes that can degrade plant cell wall polymers [12]. Considering this, the presented work was aimed to study *in vitro* release of cell wall degrading enzymes (CWDE) by *P. agglomerans* using four media with different composition. Very few reports are available about studies on the CWDEs in plant pathogenic bacterial system. Sucrose is the major transport form for photoassimilates in higher plants and hence known concentration of sucrose was added to study their influence on enzyme production at different time intervals. Moreover it is the principle carbohydrate substrate for the synthesis of cytoplasmic and cell wall constituents [13] and hence was used in this study as a sole source of carbon. In the present study, extracellular bacterial cellulase, polygalacturonase, xylanase and invertase activities were expressed as mg sugar released/mg protein. Glycosidases enzyme activities were expressed µg pNP released/mg protein.

Evaluation of extracellular protein of *P. agglomerans* was more in D at 168 h followed by B, A and C, respectively (Figure 1a). The protein content of *P. agglomerans* was gradually increased in all media with the day of bacterial growth.

Cellulase is very important enzyme in phytopathogenesis and it has been produced by many microorganisms [14]. In this study, the highest cellulase activity of *P. agglomerans* was observed in media A and C with 5% sucrose, at initial and end phase of experiment, (Figure 1b). The cellulase activity was high at initial and the later hours of growth suggesting that it may have the role in the initial degradation of host cell wall and their penetration in host tissue but later it may be utilized to obtain nutrients for their active growth. Many plant-pathogenic bacteria secrete extracellular depolymerising enzymes, such as cellulases or pectinases [15], which play an important role in the pathogenesis [16].

The highest polygalacturonases (PG) activity of *P. agglomerans* was found in media C with sucrose at initial and end time growth hours analyzed at 24 h, 96 h, 120 h, 144 h and 168 h compared to media A,B,D (Figure 1c). It might be possible that sucrose and leaves led the bacteria to produce more PG so that it could strive in such nutrient

conditions. Phytopathogenic microorganisms utilize PG as a component of their offensive arsenal to penetrate and colonize the plant tissues [17]. Previous studies showed that the PG of phytopathogens belonged to the CWDE, which were secreted to the milieu via the T2SS [18, 4]. However PGs are also important virulence factors in other plant-pathogenic bacteria, such as *Ralstonia solanacearum*, *Agrobacterium tumefaciens*, and *Erwinia carotovora* [19, 20, 21].

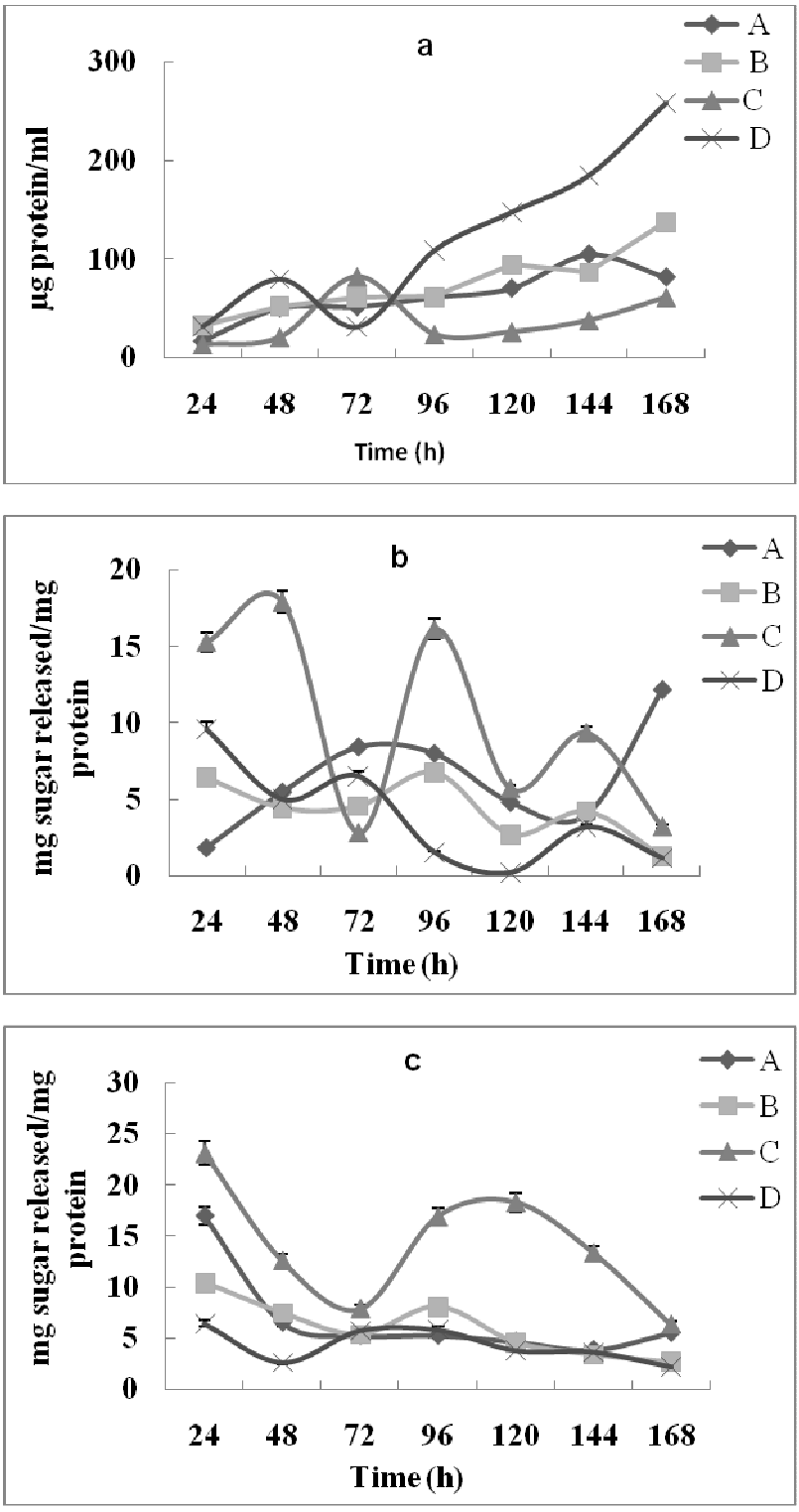

Figure 1 : Extracellular enzyme activity of *Pantoea agglomerans* (a) Protein content (b) Cellulase (c) Polygalacturonase

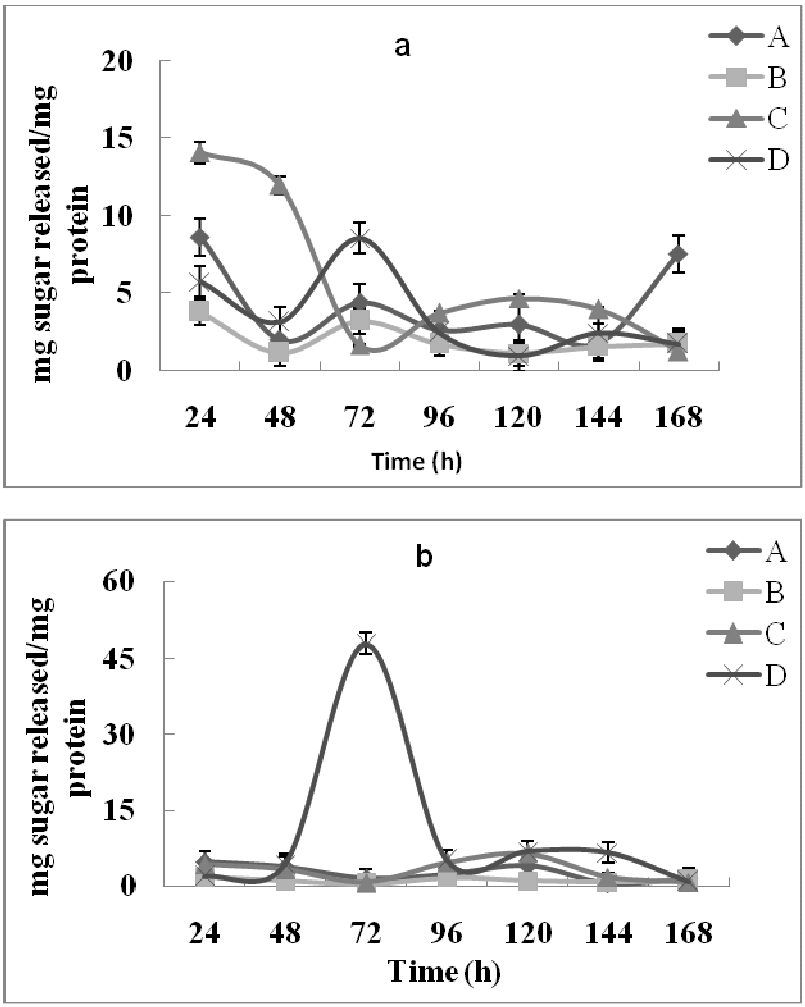

Figure 2 : Extracellular enzyme activity of *Pantoea agglomerans* (a) Xylanase (b) Invertase

The maximum xylanase activity of *P. agglomerans* was observed in media C at 24h compared to D, A, and B respectively. No clear trend was observed in all tested media (Figure 2a). In medium D maximum activity was observed at 72h. These results suggested that presence of sucrose or a leaf in a media induced the xylanase activity which played a role in bacterial growth. Xylanases have been isolated from a wide range of microorganisms [22] and [23] shown in *Bacillus* and *Streptomyces.* The rice pathogen *M. grisea* has been reported to produce five different xylanases [24]. This indicates that xylan degrading enzymes are important for the colonization of the host tissue.

The changes of α, β - galactosidase and β - glucosidase were investigated throughout the growth of the P. *agglomerans*. The maximum α - galactosidase activity of *P. agglomerans* was recorded in D with two peaks at 72 h and 144 h respectively. In media C activity was started from 96 h and remained up to 168 h. The activity in B and D showed similar trend and remained low throughout entire growth phase (Figure 3a). β - D - galactosidase activity of the *P. agglomerans* was observed very high in D at 72 h, while remained low in media A, B and C (Figure 3b). The β - glucosidase activity was not detected in any medium at 24 h. The maximum activity was observed in D, followed by C, A and B (Figure3d). Gradual declined in activity was observed in media C and D (Figure 3c). β - galactosidase and β-glucosidase activity was moderate in the initial hours but gradually increased in the middle hours suggesting that it may have an important role initially in degradation of cell wall but more active in the middle phase of growth. kian et al. [25] reported that pathogen secrete glucosidase to degrade the plant cell wall and release potential cell wall elicitors.

Considering our results it was possible that the bacterial glycosidase such as α - galactosidase, β - galactosidase and β - glucosidase might release sugar moieties that could be used as nutritional source for bacteria during its growth through plant tissue. β - glucosidase of *Botrytis cinerea* is the key enzyme in the enzymatic hydrolysis of cell wall of fruits and vegetables [26]. β - galactosidase and β - glucosidase are considered as the main enzymes associated with the mobilization of xyloglucans [27,28] while α - galactosidase possess sucrose-hydrolyzing activity [29]. Earlier

Bateman et al. [30] reported that α - galactosidase, β - galactosidase and β - glucosidase played an important role during pathogenesis of *Rhizoctonia solani* in *Phaseolus vulgaris* L.

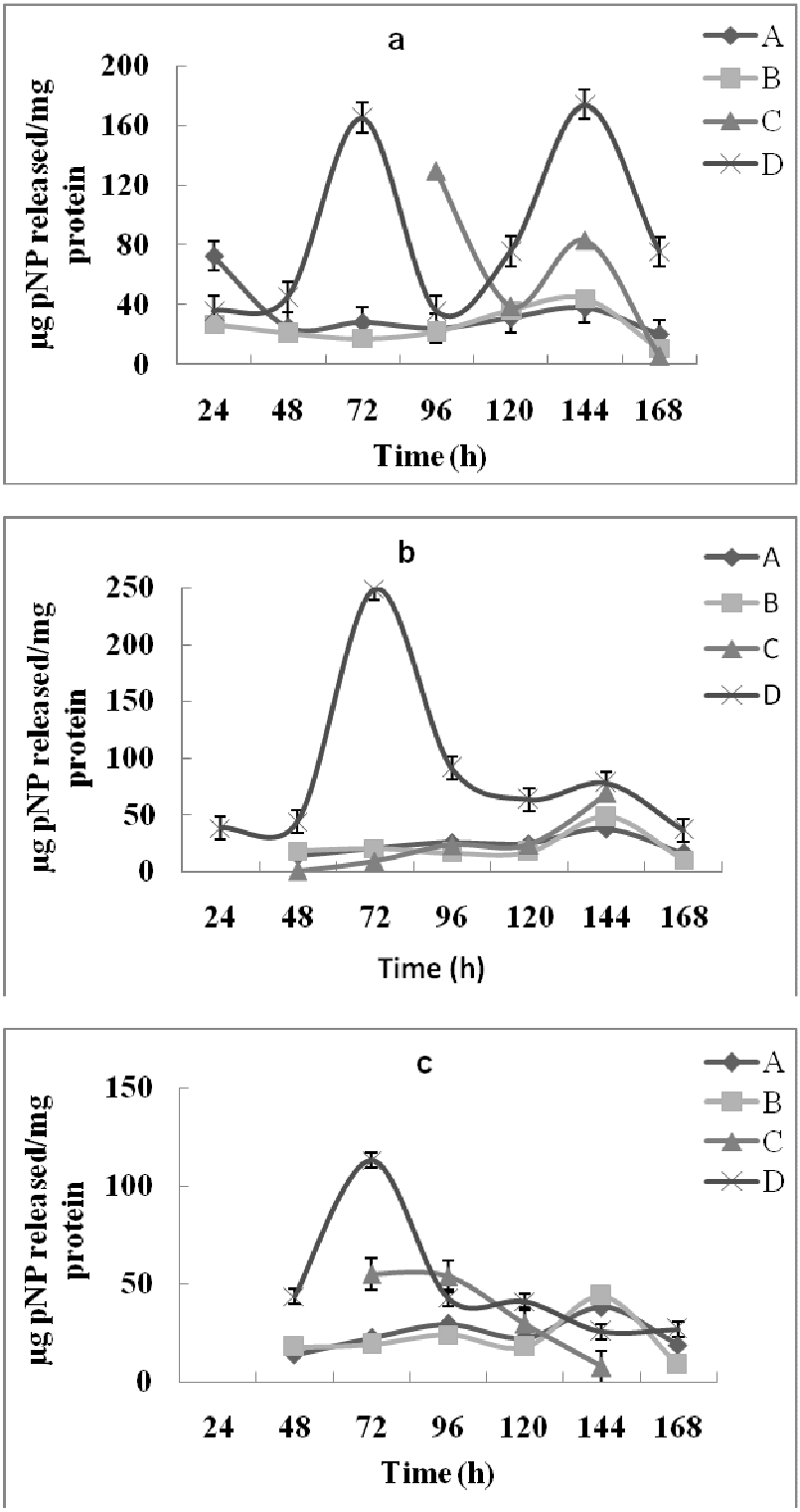

Figure 3: Extracellular enzyme activity of *Pantoea agglomerans* (a) α - D- galactosidase (b) β - D- galactosidase (c) β - D - glucosidase

Invertase activity responsible for sucrose hydrolysis was investigated despite not being related to cell wall degradation. In the present study, the maximum invertase activity of *P. agglomerans* showed in media D at middle growth phase (Figure 2b). The upregulation of invertase has been found in several plant–pathogen interactions [31], such as high invertase activity in clubroots was a cause of increased starch synthesis after the uptake of glucose and

fructose in infected cells. Thus the results obtained in this study showed that presence of sucrose as a carbohydrate has induced invertase activity in *P. agglomerans*.

CONCLUSION

From the results obtained in this study it is concluded that presence of sucrose or leaf tissue in a nutrient medium was responsible for induction of more amount of different cell wall degrading enzymes in the studied pathogen. The results showed that though genome sequence of *P. agglomerans* is available in databank, only biochemical study i.e., enzymatic study provides view about expression of specific enzyme(s) during pathogen infection in host plant. It is possible that these enzymes play a role in virulence during plant microbe interaction.

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