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RESEARCH ARTICLE

Chitinases from pitcher fluid of *Nepenthes distillatoria*

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Abstract: Chitinases belong to a diverse family of enzymes among many other hydrolytic enzymes of the pitcher fluid of *Nepenthes* sp**.** There are no existing studies on identification of chitinases in *Nepenthes distillatoria* the only representative of the genus *Nepenthes* in Sri Lanka. In this study an assay procedure was developed and optimized to detect the chitinolytic activity in the pitcher fluid of *N. distillatoria* using colloidal Chitin Azure as the substrate. Crude *Nepenthes* pitcher fluid was subjected to DEAE Cellulose chromatography in an attempt to partially purify and separate chitinases. The optimum pH, temperature and incubation time for the chitinolytic activity were 5.0, 27 °C and 30 min, respectively. DEAE Cellulose Chromatography revealed the presence of more than one type of chitinases within the pitcher juice. Further studies are required to characterize and confirm the origin/s of these chitinases.

Keywords: Carnivorous plant, Chitin, Chitinase assay, Colloid Chitin Azure, Pitcher plant.

INTRODUCTION

The ability to catch and digest prey, mainly insects, allows carnivorous plants growing in very low nutrient habitats to acquire mainly nitrogen and phosphorus and other mineral nutrients. Plants of the genus *Nepenthes* grow in nutrient poor habitats and have evolved modified leaves called pitchers to capture insect preys (Ellison and Adamec, 2011). *Nepenthes* belong to monotypic family Nepenthaceae. According to the classification of angiosperms, at least 583 species of carnivorous plants can be recognized belonging to 20 genera dispersed in 12 families falling under 5 orders. *Nepenthes* which belongs to order Caryophyllales comprises approximately 90 species (Givnish, 2015). *Nepenthes distillatoria* is the only representative of the genus *Nepenthes* in Sri Lanka and it is endemic to the island (Dassanayake and Fosberg, 1980). According to the IUCN red list, *N. distillatoria* can be recognized as a vulnerable plant species (IUCN, 2000). It is mainly distributed in the South West and South-East regions of Sri Lanka (Dassanayake and Fosberg, 1980).

To facilitate the prey digestion and make the nutrients available, carnivorous plants possess a variety of endogenous enzymes including proteases, phosphatases, ribonucleases, peptidases, esterases, and chitinases (Moran and Clarke, 2010). Chitinases are a particularly interesting group of plant enzymes because their substrate chitin is not present in plant tissues **(**Rottloff *et al.*, 2011**).** The induction of chitinases in plants following fungal infection is well documented (Wang *et al*., 2002). Therefore, chitinases are thought to be necessary in plant defense against phytopathogenic fungi, due to their ability to hydrolyze and degrade chitin (Van Loon *et al.,* 2006). Chitinases are widely used in the fields of industry, agriculture and medicine for numerous important functions. This is because the chitinases have the ability of direct digestion of chitin biopolymers into low molecular weight chitooligomers (Yuli *et al*., 2004). Also, elicitor action of chitinases can be used for controlling diseases which are caused by fungi and harmful insects (Mathivanan *et al*., 1998; Mendonsa *et al*., 1996).

Chitin is a linear polysaccharide of β-1, 4-*N*-acetylglucosamine monomers (Shibuya and Minami, 2001; Van Loon *et al*., 2006) and is a major constituent of the outer skeleton of insects, cell wall of fungi as well as in the internal structures of most of the other invertebrates (Theis and Stahl, 2004; Kramer and Muthukrishnan, 2009).

For the conventional chitinase assay procedures, insoluble chitin which is covalently bound to dyes, tritium-labeled chitin or colloidal chitin are used as substrates of chitinases (Gomez-Ramırez *et al*., 2004; Spindler, 1997). In these methods the chitinase activity is determined by measuring the quantity of reducing sugars (e.g., chito-oligosaccharides) (Shen *et al*., 2010). However, the major problem associated with this system was that those results can be affected by other reducing sugars present in the reaction medium (Imoto and Yagishita, 1971; Shen *et al*., 2010; Hackman and Goldberg, 1964; Liu *et al*., 2009). Conversely, a special type of chitin substrate was introduced for the chitinase assay, known as colloidal Chitin Azure. This is a kind of chitin which is covalently coupled with Remazol brilliant violet 5R dye and the resulting products can be measured at 560 nm in a colorimeter. In addition, most significantly the assay is not affected by reducing sugars of the medium (Gomez-Ramırez *et al*., 2001).

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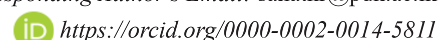
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There are many studies carried out for identification, characterization and purification of proteases present in the pitcher fluid of *Nepenthes* sp. (Thornhill *et al*., 2008; Plachno *et al*., 2006; Fukusaki and Kobayashi, 2002; Athauda *et al*., 2004; Takahashi *et al*., 2005; Stephenson and Hogan, 2006; Hatano and Hamada, 2008), but there are only limited research carried out for identification and purification of chitinases (Eilenberg *et al*., 2006; Ishisaki *et al*., 2012) and there are no studies conducted for identification of chitinases in *N. distillatoria.* The foremost reason for limited research is the unavailability of a soluble and an inexpensive substrate to analyze chitinase activity (Shen *et al*., 2010; Gomez-Ramırez *et al*., 2004).

In this study for the first time, chitinolytic activity of the pitcher fluid of *N. distillatoria was* detected using dispersible, soluble colloid Chitin Azure as the substrate. Following the detection of chitinolytic activity, attempts were made to partially purify and characterize the chitinases of the pitcher fluid. The optimum pH and optimum temperature for chitinase activity of the crude pitcher fluid as well as for the fractions obtained after anion exchange chromatography were determined. Moreover, the thermal stability of the chitinase activity of the different partially purified fractions was investigated. The remarkable stability in the major fraction among the three fractions obtained after anion exchange chromatography over a long period of incubation time seems to indicate that they have evolutionally well adapted to their original habitat.

MATERIALS AND METHODS

Collection of the crude sample

The open (OP) and closed (CP) crude pitcher fluids of *N. distillatoria* were collected from *Hakurugala* forest patch at *Ruwanwella*, Sri Lanka. They were clearly labeled and brought into the laboratory and kept at -20 ^{0}C until use.

Preparation of the colloidal Chitin Azure substrate

The substrate Colloidal Chitin Azure was prepared according to the procedure given in Shen *et al* (2010).

Assay procedure for chitinase activity

The assay was carried out for the crude OP and CP fluid samples separately. Each test and control experiments were conducted in replicates. For the test samples, 1000 µL of 50 mM sodium phosphate buffer solution at pH 5.0, 200 µL of colloidal CA substrate and 100 µL of unpurified open or closed crude pitcher fluid were added to 1.5 ml centrifuge tube. For the controls, similar to test samples, buffer solution and substrate were added except the crude pitcher fluid. The samples were incubated for 3 hrs at 37 °C. The reaction was inhibited by heating both test and control samples at 95 °C for 5 min. After the termination of the reaction 100 µL of heat denatured pitcher fluid was added to each of the controls. Final volumes of the reaction mixtures were maintained at 1.3 ml. All reaction tubes were centrifuged at 13000 rpm for 10 min and the absorbance of the supernatant fraction was measured at 560 nm against the blank (50 mM, sodium phosphate buffer solution). After the detection of chitinase activity, the optimization of the assay procedure was carried out as follows.

For detection of optimum pH, a series of buffer solutions in the range of pH 1.0 to pH 7.0 were used. The optimization of incubation time was done by incubating the reaction mixtures at varying time intervals ranging from 10 min to 60 min while for determination of optimum incubation temperature, four different temperatures (4 °C, 27 °C, 37 °C and 50 °C) were used.

Then the enzyme activity was calculated using the following equation,

$$Enzyme\ activity = \frac{\Delta\ Optical\ density\ (OD) at\ 560\ nm}{Incubation\ time\ (minutes)}$$

Where, $\Delta\ OD = \begin{pmatrix} Average\ absorbance\ of \\ test\ sample\ at\ 560\ nm \end{pmatrix}$

$$- \begin{pmatrix} Average\ absorbance\ of \\ control\ sample\ at\ 560\ nm \end{pmatrix}$$

Comparison of the chitinase activity of the crude open and closed pitcher fluid

After carrying out the optimized chitinase assay procedure mentioned in section 2.3 for both unpurified OP and CP fluids, the activity data were subjected to normality testing followed by GLM and LS means-pdiff mean separation procedure using the statistical package, SAS 9.1.3 (SAS Institute, NC, Cary, USA).

Purification and analysis of chitinase(s) using ion exchange chromatography

All purification procedures were performed at 4 °C. The crude pitcher fluid (100 ml) was dialyzed in about 2000 ml of 0.1 M sodium phosphate buffer at pH 7.0. The proteins in the dialyzed *Nepenthes* fluid were absorbed to DEAE cellulose (pre equilibrated with the same buffer) by batch adsorption and the gel was placed in a 5 ml column. The column was washed with 100 ml of 0.1 M sodium phosphate buffer at pH 7.0 and the proteins were eluted with the same buffer containing 0.5 M NaCl. Five milliliter fractions were collected and the chitinase activity in each fraction was measured.

Then a 2nd DEAE cellulose chromatography was carried out as follows. Five grams of DEAE cellulose powder was suspended in a little volume of phosphate buffer (0.1 M, pH 7.0). Column (10 ml) was evenly packed on to a syringe using a glass rod and a Pasteur pipette. The packed column was equilibrated with 50 ml of phosphate buffer (0.1 M, pH 7.0). Then 400 ml each of crude open pitcher fluid was added to the top of the column and was collected at the bottom of the syringe. The sample collected at the bottom of the syringe was passed through the column for 5 times. The unbound fraction of the sample was collected into a separate container. The column was washed with the 0.1M phosphate buffer (pH 7.0) and washings were collected separately. The chitinases were eluted with a linear gradient of 0.0-1.0 M NaCl in 0.1 M phosphate buffer at pH 7.0. Flow rate was maintained at 0.5 ml/min. Twenty-one eluted fractions (1.5 ml) were collected. The unbound fraction, washing and eluted fractions were subjected to an overnight

dialysis against 500 ml of phosphate buffer (0.1 M, pH 7.0). The dialyzed samples of unbound fraction, washing and eluted fractions were subjected to the chitinase assay (Rao *et al.,* 1983).

Determination of thermal stability of the crude pitcher fluid

The crude OP and CP fluids were incubated at 37 °C and 27 °C for five weeks. Aliquots were removed at one-week time intervals and were subjected to the chitinase assay. The percentages of remaining chitinase activity were calculated using the following equation.

$$Percentage\ of\ remaining\ Chitinase\ activity = \frac{Ai - An}{Ai} \times 100\%$$

Where, Ai = Chitinase activity of 1st week

An = Chitinase activity of nth week

Determination of thermal stability of the purified chitinase(s)

The purified OP fluid subjected to DEAE cellulose column chromatography was pooled and kept at 37 °C for four weeks. Aliquots were removed at one-week time intervals and were subjected to the chitinase assay as mentioned. Then the percentages of remaining chitinase activity were calculated.

RESULTS AND DISCUSSION

Development of an assay procedure for chitinase activity

The determination of chitinase activity was carried at various pH values as shown in the Figure 1. The optimum chitinase activity with Chitin Azure as the substrate is at pH 5 for both OP and CP fluids. The comparison of OP and CP fluids showed that the chitinase activity varies in a similar way for both pitcher fluid samples. Even though OP fluids showed a considerable enzymatic activity for other pH values 2.0, 3.0 and 4.0, the highest activity was obtained at pH 5.0. A sharp decline of activity was observed at pH above 5. The previous studies done by Shen *et al*., (2010) recorded that the Colloid Chitin Azure was optimally activated at pH 5. As well as other studies that have been done for the chitinase secreted by *Nepenthes alata* (Ishisaki *et al*., 2012), *Nepenthes khasiana* (Eilenberg *et al*., 2006) also showed the optimal Chitinase activity approximately at a range of pH 4 to 5.

The incubation time - activity profile is shown in the Figure 2. The optimization of the time for incubation was carried out using 30 min time intervals and according to the results the highest chitinase activity was at 30 min of incubation. Afterward, the activity gradually decreases with the increasing time duration for both samples. The 30 min time period for the chitinase activity was further confirmed by carrying out the assay at 10 min time intervals (Figure 2). Still the highest chitinase activity remains at the 30 min of incubation. Moreover, the values of chitinase activities obtained at the 30 min time intervals and 10 min time intervals were virtually similar. Thus, it confirms the optimum time period for the chitinase assay as 30 min. The studies of chitinase activity where the chitinase is obtained by the *Bacillus thuringiensis* Bt-107 (a chitinolytic bacteria), ASI (a chitinolytic bacterium with low levels of chitinase) and BCTS (an *Escherichia coli* BL21 [DE3] expressing a secretory recombinant chitinase) have given the highest chitinase activity at 30 min time duration (Shen *et al*., 2010).

The incubation-temperature activity profile is shown in Figure 3 the optimum temperature for chitinase activity of both OP and CP fluids is 27 °C and the activity gradually decreases with the increasing temperature. The samples incubated at 37 °C and 50 °C show a gradual decrease of chitinase activity in both OP and CP juice samples compared to the sample incubated at 27 °C. Determination of the chitinase assay was carried out by using the optimized

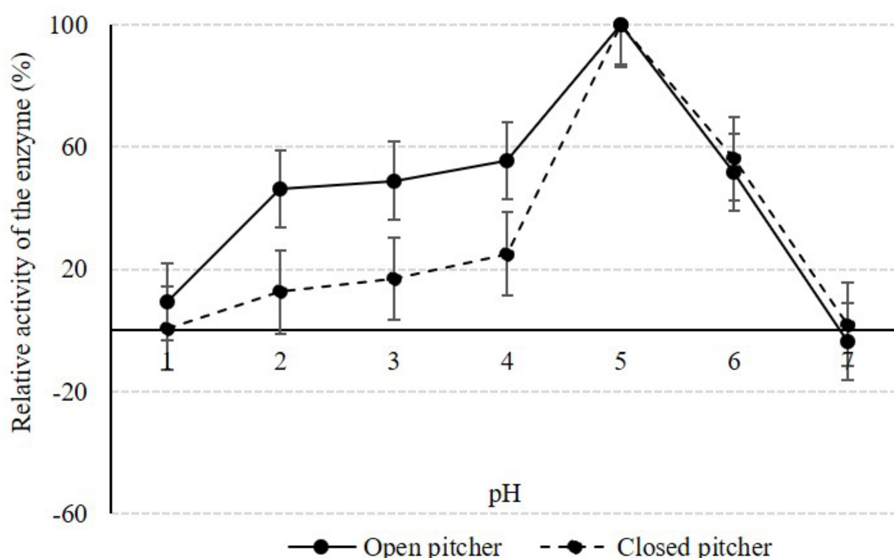

Figure 1: pH dependence of the activity of chitinase toward Chitin Azure. The activity was measured at various pH values (1 pH to 7 pH) of the digestion of Chitin Azure. Y axis indicates the relative activity of the enzyme with compared to the highest activity.

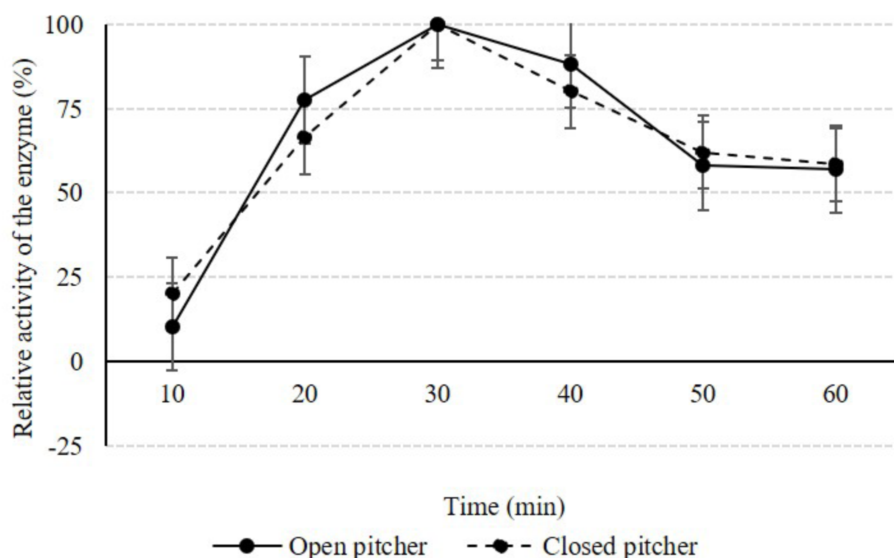

Figure 2: Optimum incubation time of the activity of chitinase in both open and closed pitcher juice toward Chitin Azure. The activity was measured with 10 min intervals up to 60 min. Y axis indicate the relative activity of the enzyme with compared to the highest activity.

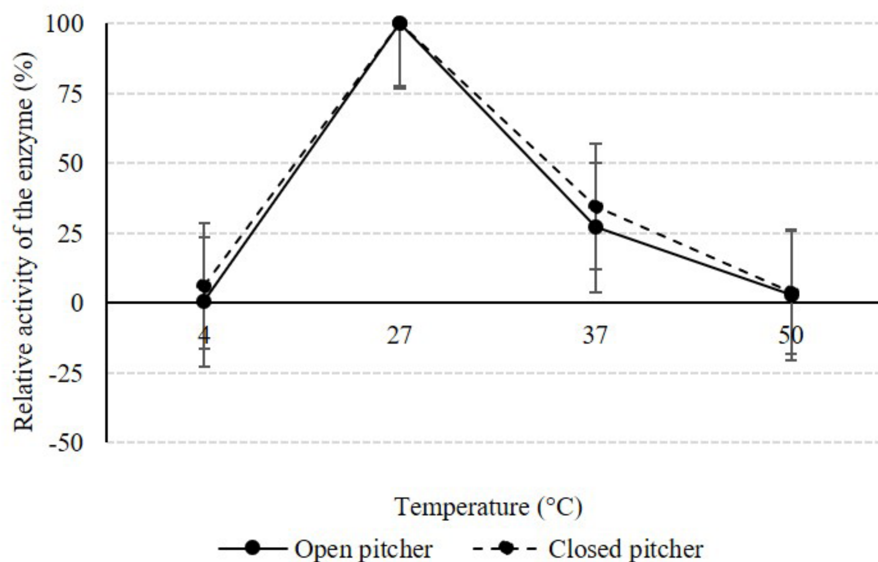

Figure 3: Optimum incubation temperature of the activity of chitinase toward Chitin Azure. The activity was measured at 4 °C, 27 °C, 37 °C and 50 °C toward the digestion of Chitin Azure. Y axis indicates the relative activity of the enzyme with compared to the highest activity.

pH (5 pH) and time duration (30 min). The substrate, CA shows some amorphous nature during incubation at higher temperatures than 27 °C but at 27 °C there are no any notable changes in the substrate. Therefore, it implies that given temperatures are able to change the substrate which in turn gives a low activity in the assay. Moreover, the lesser activity of the samples incubated at 50 °C temperature could be due to the thermal denaturation of the enzymes.

Comparison of the chitinase activity of the both open and closed pitcher fluid

The chitinase activity of both OP and CP crude samples are not significantly different ($P < 0.05$) from each other. The correlation coefficient between the chitinase activity and the pitcher fluid samples is 81.73 % indicating a strong relationship between these two parameters. Most of the results obtained for chitinase assay in both OP and CP juices show approximately similar results. Thus, it infers that chitinases are present in OP and CP fluid samples with an indistinguishable activity against chitin.

Purification and analysis of chitinase(s) from pitcher fluid

DEAE cellulose chromatography using batch adsorption revealed the binding ability of the chitinase(s) in pitcher fluid to the DEAE cellulose column. Since the binding of chitinases to the DEAE cellulose column was successful, separation and purification was carried out using an anion exchange chromatography (Figure 4). The major-eluted peak lies between 650 mM to 800 mM NaCl range (peak iii

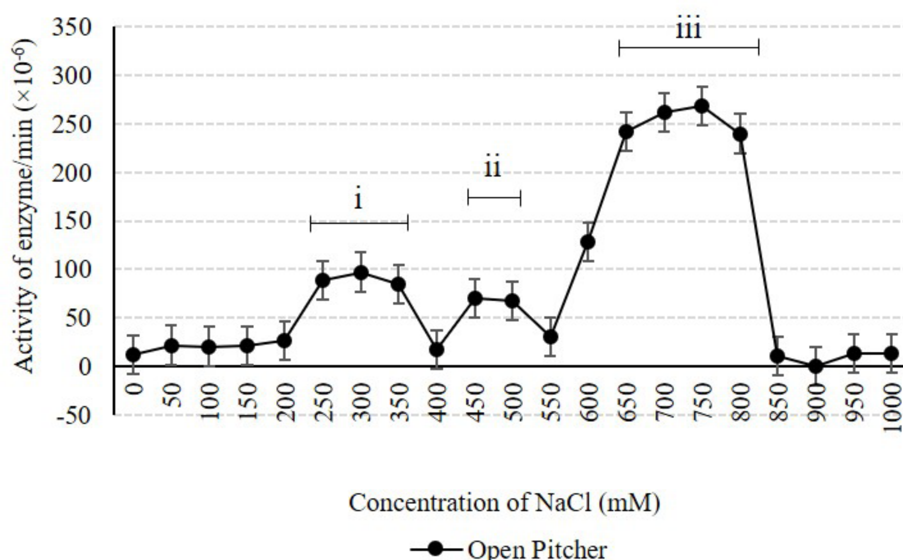

Figure 4: Purification of chitinase(s). DEAE-cellulose chromatography for crude pitcher fluid. Column size, 10 cm. Fraction size, 1.5 ml with 0.1 M pH 7.0 phosphate. The enzymes activities were designated as i, ii and iii, respectively. The enzyme assay was performed with Chitin Azure as a substrate. Y axis indicates the activity of enzyme per minute. In chromatogram B, the fractions under the bar were pooled.

in figure 4) and the minor peak lies between 250 mM to 300 mM NaCl (peak i in figure 4) while a subtle peak lies at 450 mM NaCl (peak ii in figure 4). According to the observed results it is possible to infer that there at least three types of chitinases present in the pitcher fluid. Studies done by Hara *et al*., (1989) and Hamid *et al*., (2013) also confirm the binding ability of chitinase to DEAE cellulose column. As stated by the previous studies, there were several chitinases in the pitcher juice of *Nepenthes khasiana* (Eilenberg *et al*., 2006). Moreover, according to the experimental results cellular extracts of *Metarhizium anisopli* (a fungal species) contained two different chitinases (Kang *et al*., 1999).

Determination of thermal stability of the chitinase activity of the crude pitcher fluid

As indicated in the Table 1**,** neither the chytinolytic activity in OP nor that of CP is thermally stable after 40 °C. At 40 °C, enzyme activity retains 72.22 % (OP) and 80.00 % (CP)compared to the initial activity. They lose the activity gradually after 50 °C. The outcomes of temperature stability tests are indicated in Table 1, in which both OP and CP fluid samples were incubated at different temperatures for Then the remaining chitinase activity was determined and compared with the activity of initial sample. Accordingly, the OP samples possess stability in its chitinase activity up to 40 °C, and started to decrease and cease around 60 °C. Therefore, these experimental data suggest that chitinase is significantly stable at a relatively high temperature. Since Chitinases are widely used in industry, agriculture and medicine (Yuli *et al*., 2004), discovering the thermally stable chitinases from pitcher fluid is significantly important. Similar observations were given by the chitinases secreted by Marine *Streptomyces sp*. (Han *et al*., 2009). In this study, the highest activity was recorded at 60 °C and above that temperature, decreasing of the activity was detected. In addition, another similar approach was done by using *Streptomyces erythraeus* (Hara *et al*., 1989) also revealed the decrease of the chitinase activity after 50 °C.

Determination of thermal stability of the purified Chitinase (s)

The enzyme activity of the sample iii retained 63.24 % of the initial activity after 28 days of incubation at 37^0C. However, chitinase activity of both samples i and ii are unstable at 37 °C under the similar conditions. That was, in the Samples i and ii the enzyme activities retain only up to 2.86 % and 11.32 % of the original activity respectively after 28 days of incubation (Table 2). This indicates the presence of a highly thermally stable enzyme activity within the fraction in peak iii, and the enzymes present within the peaks i and ii were less thermally stable. These types of different thermal stability characteristics indicate the presence of two or more different chitinases within the pitcher fluid. Therefore, it indicates that each of these separated samples contains both relatively unstable chitinases and more thermally stable chitinases. Thus, it was probable for these two-weak peaks to have a bacterial origin since it was well known that pitchers of carnivorous plants are phytotelms that possess specific bacteria that secrete digestive enzymes (Juniper *et al* 1989). Hence, further studies are required to identify the exact origin of those two chitinases. In summary, the present study was the first to report the identification of chitinolytic activity in the pitcher fluid of *N. distillatoria.* This study can be used a platform for effective purification of the chitinases to homogeneity followed by enzymic and structural characterization of the novel chitinases which would probably be unique members within the diverse chitinase family.

CONCLUSIONS

The optimum pH, temperature and incubation time for chitinase activity of both OP and CP crude samples were

Table 1: Percentages of remaining chitinase activity which displays the thermal stability of chitinase(s) in open and closed pitcher juice temperature varied from 4 °C to 90 °C.

Sample	Temperature (°C)							
	4	30	40	50	60	70	80	90
OP	100.00 % - 1.17	100.00 % - 1.19	72.22 % ± 2.18	5.56 % ± 1.23	0.00 % ± 1.85	5.56 % ± 2.11	2.22 % ± 3.51	-16.67 % ± 3.41
CP	100.00 % - 2.41	90.00 % ± 3.2	80.00 % ± 2.15	10.00 % ± 3.20	10.00 % ± 1.51	0.00 % ± 4.11	-15.00 % ± 2.31	-10.00 % ± 3.21

Table 2: Percentages of remaining chitinase activity which displays the thermal stability of the purified chitinase by DEAE cellulose column chromatography method which incubated at 37 °C.

Sample	Time (Days)				
	Day 0	Day 7	Day 14	Day 21	Day 28
i	100 % - 2.15	75.71 % ± 1.21	35.71 % ± 1.18	17.14 % ± 2.17	2.86 % ± 3.24
ii	100 % - 1.17	67.92 %± 1.14	37.74 % ± 1.32	30.19 %± 3.11	11.32 % ± 2.41
iii	100 % - 2.13	95.59 %± 1.15	85.29 % ± 2.21	69.12 %± 1.28	63.24 % ± 1.33

pH 5.0, 27ºC and 30 min respectively. Furthermore, both OP and CP fluids exhibited significantly equal levels of chitinase activities ($P< 0.05$). Chitinases successfully bound to the DEAE ion exchange chromatography column at pH 7.0. There was more than one type of chitinases present in the pitcher juice including thermally stable chitinases. Further characterization approaches are required to detect the exact origins and functions of these different types of chitinases found in pitcher fluids.

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