



## EVALUATION OF SOME INDUSTRIALLY IMPORTANT ENZYMES IN FILAMENTOUS CYANOBACTERIA

Padmapriya V. and Anand N.

Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai, India

E-Mail: [priyajcl@gmail.com](mailto:priyajcl@gmail.com)

### ABSTRACT

Preliminary studies involving nine filamentous cyanobacterial isolates for the screening for amylase, protease, beta lactamase and phosphatase enzymes were carried out. Best growth was observed in *C. membranacea*. *O. proboscidea* showed a complete absence of amylase activity in the external medium, extracellular (EC) activity, and *P. boryanum* showed the highest amount of EC enzyme activity on the 8<sup>th</sup> day amongst non-heterocystous forms. In *P. boryanum*, the amylase intracellular (IC) activity was present consistently throughout the period of study with a gradual increase from zero day (day of inoculation) up to the 20<sup>th</sup> day. Amongst the heterocystous forms, *A. variabilis* showed the highest amylase EC and IC activities. All the non-heterocystous forms showed highest protease EC activity on the 16<sup>th</sup> day except *O. proboscidea*, which registered detectable protease EC activity only on the 16<sup>th</sup> and 20<sup>th</sup> day. The heterocystous forms, *N. paludosum* and *W. iyengarii* showed a consistent presence of the protease EC activity throughout the study period. *N. paludosum* and *W. iyengarii* had a sharp increase of protease IC activity on the 4<sup>th</sup> day, which incidentally was the highest activity. Beta-lactamase EC activity observed in *L. spiralis* on day 4 was the highest amongst the non-heterocystous forms. In *A. variabilis* and *C. membranacea*, the beta-lactamase EC activity was the highest on day 4 compared with other heterocystous forms. The EC activity of acid phosphatase enzyme in the non-heterocystous forms, *P. minnesotensis* and *P. boryanum*, showed a decrease as the growth increased, reaching negligible levels on the 20<sup>th</sup> day. In the heterocystous forms, both the acid phosphatase EC and IC activities were absent up to a period of 8 days. In the case of alkaline phosphatase IC activities, a gradual decrease in the enzyme content from the first interval is observed in almost all the forms. *W. iyengarii* showed higher EC activity compared with other heterocystous forms but the maximum IC activity was observed in *A. variabilis*.

**Keywords:** cyanobacteria, enzymes, protease, amylase, alkaline phosphatase, acid phosphatase, beta-lactamase.

### INTRODUCTION

Cyanobacteria are well known sources of protein rich food, feed supplements, fine chemicals and biofertilizers (Padmapriya and Anand, 2005; Thajuddin and Subramanian, 2005). They are also good sources of a broad spectrum of enzymes like hydrogenase, endonuclease, desaturase, peptide synthetase, lycopene cyclase, ribonuclease P, phosphoglycerate kinase and amino acid oxidases. Reports on the presence of commercially important enzymes like amylase, protease, phosphatase, lactamase and antioxidants have been put forth but their exploitation in industrial scale have not been stupendously successful except in some instances like in the case of *Spirulina*. In cyanobacteria, proteases play a major role in the differentiation and development of heterocysts, spore germination, utilization of phycobilin pigments under nutrient depleted conditions etc. (Dong *et al.*, 2000; Maldener *et al.*, 1991; Reddy and Sarada, 1985; Anantani and Vaidya, 1982). Proteases are always in demand in various industries like the leather industries, meat tenderizing industries, detergent industries, wool industries, dairy, brewing, baking and food processing industries and as food and feed enzymes (Rao and Deshpande, 1998; Rao *et al.*, 1998). Amylases rank among the first three important industrial enzymes and very few reports on this enzyme from cyanobacteria are available (Sarma *et al.*, 1977; Reddy and Lakshminarayana, 1987; Reddy *et al.*, 1987). Alpha-amylases are predominantly used for starch hydrolysis in the alcohol industries and

actively involved in the textile, animal health, detergent, food ingredients, baking, beverage, and sugar and starch industries. Pharmaceutical and dairy industries are currently worried about the increased resistance to the antibiotics. Cyanobacteria are reported to grow in antibiotic enriched medium and also transform the lactam antibiotics, a boon in the dairy processing units (Kumar, 1964; Kushner and Breuil, 1977; Reddy, 1977a; 1977b; Bharati and Bongale, 1976; Prabakaran *et al.*, 1994). Phosphates play a major nutrient limiting factor in the ecosystem and cyanobacteria are known to produce phosphatases to overcome adverse conditions (Reddy and Lakshminarayana, 1988; Strojsova *et al.*, 2003; Tiwari *et al.*, 2002). Phosphatases are also employed in various industries. Acid phosphatases are primarily used as diagnostic chemicals. Alkaline phosphatases are used as fine chemicals in the study of molecular biology as DNA/RNA modifying enzymes. They are primarily used in vector dephosphorylation, clean up of PCR products and as immunolabels in research. This preliminary screening study evaluated the presence of amylase, protease, beta-lactamase and phosphatases in nine filamentous cyanobacterial strains available in the University culture collection.

### MATERIALS AND METHODS

The cyanobacterial strains used in this study were *Lyngbya spiralis* Geitler, *Oscillatoria proboscidea* Gom. *Oscillatoria tenuis* Ag., *Phormidium minnesotensis*



(Tilden) *Dronet*, *Plectonema boryanum*, *Anabaena variabilis* Kütz., *Nostoc paludosum* Kütz., *Calothrix membranacea* Schmidle and *Westiellopsis iyengarii*. Cultures were grown and maintained in BG<sub>11</sub> medium (Rippka *et al.*, 1979). The heterocystous forms were maintained in nitrate free medium. The cultures were axenized according to a modified method given by Droop (1967). Cultures were maintained at a temperature of 27°C±1°C, under fluorescent illumination of 30 to 40 μEm<sup>-2</sup>s<sup>-1</sup> provided by fluorescent tubes (Philips Trulite, Col 82). The cultures were exposed to a 12h light/12h dark cycle photoperiod. Growth was determined every fourth day for a total period of 20 days by measuring the chlorophyll a content as given by McKinney (1941). Protein was estimated by the method of Bradford (1976) and bovine serum albumin was used for standard graph. The results are expressed in mcg/mL. Cells were harvested by centrifugation at 3024 x g for 10 minutes. The pellets were washed twice in Tris-Cl buffer (0.05M; pH 7.6) and suspended in minimum volume of the same. The cells were disrupted using a Labsonic 2000 Sonicator at 50W power with a one-minute burst and a one-minute rest cycle for a total of six minutes. The homogenate was then centrifuged at 12096 x g for 30 minutes at 4°C in a Beckman J2-21 refrigerated centrifuge. The supernatant fraction containing the enzyme was taken for the assay as crude enzyme extract (cell free) for the determination of intracellular enzyme (IC) activity. For extra cellular enzyme (EC) activity determinations, the culture media devoid of cells were taken as crude enzyme samples. Enzyme activities were detected every fourth day up to 20 days. Alpha-amylase (EC 3.2.1.1) was assayed using the method of Bernfield (1955). One gram of 3, 5, dinitro salicylic acid was dissolved in 20 mL of 2N NaOH and then in 50 mL of distilled water. To this, 30 g of Rochelle salt was added and the total concentration was made up to 100 mL with distilled water. The solution was protected from sunlight and carbon dioxide. An enzyme sample of 1mL was incubated for 30 mins with 1 mL of the substrate solution at 37°C. The enzyme reaction was interrupted by adding DNSA reagent 2 mL followed by heating in boiling water for 5 mins. The solution was then cooled under running tap water. After the addition of 20 mL of distilled water, the optical density of the solution containing the brown reduction product was determined spectrophotometrically at 530 nm in a Beckman DU-40 Spectrophotometer. A blank solution was prepared minus the enzyme. A calibration curve with maltose was used to determine the maltose released. Activity of the enzyme is expressed in units where 1U corresponds to 1mg maltose liberated in 30 minutes at 37°C by 1mL of the enzyme solution. Specific activity is expressed as U mg protein<sup>-1</sup>. Alkaline Protease (EC 3.4.21) was assayed as given by Whooley *et al.*, 1983. To 1mL of the crude enzyme, 1 mL of freshly prepared casein substrate solution was added and incubated at 35°C for 30 minutes. 3 mL of ice-cold trichloro-acetic acid solution was added to terminate the reaction and the tubes were left undisturbed for 30 minutes. The precipitate was removed by centrifugation at

1088 x g for 10minutes and the supernatant was collected. To 1 mL of the supernatant, 3 mL of freshly prepared carbonate solution was added. Fifteen minutes later, 1 mL of the Folin-phenol reagent was added. Thirty minutes later, the absorbance of the solution was detected at 660nm in a Beckman DU-40 spectrophotometer against a substrate blank. Enzyme activity was calculated from a standard graph using tyrosine and 1unit (U) corresponded to 1μg tyrosine released from casein/mL/h at 32°C. The specific activity was given as U mg protein<sup>-1</sup>. Beta-lactamase (EC 3.5.2.6) was detected using the micro iodometric method given by Ross and O'Callaghan (1975). For cyanobacterial cultures the pH of buffer ranging from 7.4-7.9 was found to be suitable. To 1mL of the starch-iodine solution, 0.1mL of the crude enzyme, 0.9 mL of the phosphate buffer and 1mL of the substrate solution was added. The absorbance was monitored at 620nm at regular intervals up to a total time period of 3 minutes. The results are expressed in units where 1U corresponds to 1μM of the substrate destroyed per minute per mL of the enzyme. This was calculated using the following formula- (ΔOD/min/initial OD) x 0.015 x 10 = μM destroyed / min/ mL of enzyme. The amount of iodine used in this assay corresponds to 15nM of penicillin conforming to 8 equivalents of iodine utilized by the substrate as given by Alicino (1961). The specific activity of the enzyme is expressed as U mg protein<sup>-1</sup>. Acid phosphatase (EC 3.1.3.2) was determined according to Lee (2000) by assessing the rate of hydrolysis of p-Nitro Phenol Phosphate (p-NPP) to p-Nitro Phenol (p-NP). The reaction mixture contained 100mM sodium acetate buffer, 5mM p-NPP and 1mL of the enzyme sample in a total volume of 3mL. The reaction was started by the addition of 0.5 mL of p-NPP reagent to the enzyme sample and incubating it at 32°C for 30-minutes. 1mL of NaOH solution was added to terminate the reaction. The p-NP released was calculated using a calibration curve drawn using known concentrations of p-NP. One unit (U) of the phosphatase activity is the amount of enzyme producing 1μM p-NP per minute. The specific activity of the enzyme is expressed in terms of U mg protein<sup>-1</sup>. For alkaline phosphatase (EC 3.1.3.1) assay, the reaction mixture contained 0.1M Tris-HCl buffer, 5mM p-NPP and 1mL of the enzyme sample in a total volume of 3 mL. The reaction was initiated by the addition of p-NPP and the samples were incubated at 32°C for 30 minutes. 1 mL of NaOH solution was added to terminate the reaction. The p-NP released was calculated using a calibration curve drawn using known concentrations of p-NP. One unit (U) of the phosphatase activity is the amount of enzyme producing 1uM p-NP per minute. The specific activity of the enzyme is expressed in terms of U mg protein<sup>-1</sup>.

## RESULTS

The non-heterocystous forms comprising *L. spiralis*, *O. proboscidea*, *O. tenuis*, *P. minnesotensis* and *P. boryanum* were grown in BG11 medium amended with sodium nitrate. The heterocystous forms: *N. paludosum*, *A. variabilis*, *C. membranacea* and *W. iyengarii* were grown



in BG11 medium devoid of nitrate. *L. spiralis* showed a peak in growth on the 12<sup>th</sup> day (1.14 µg/mL) while the remaining non-heterocystous forms reached their growth peak on the 16<sup>th</sup> day. In *O. proboscidea* the growth rate was observed to be the highest and doubled within 4 days, followed by *P. boryanum*, which took 9 days. The lowest growth rates were observed in *L. spiralis*. In the heterocystous forms: *A. variabilis* and *C. membranacea* showed a peak in chlorophyll a content on the 12<sup>th</sup> day while in *N. paludosum*, it was on the 16<sup>th</sup> day. In *W. iyengarii*, a slow growing, branched, heterotrichous form, the chlorophyll a content was almost constant from day 1 to day 20 with a standard deviation of  $3 \pm 0.36$  µg/mL. Best growth was observed in *C. membranacea* in 6 days whereas; it took 10 days in the case of *A. variabilis*. *O. proboscidea* showed a complete absence of amylase activity in the external medium (Figure-1) and *P. boryanum* showed the highest amount of EC enzyme activity on the 8<sup>th</sup> day ( $5.8 \pm 0.3$  U / µg protein) amongst non-heterocystous forms. In *L. spiralis*, *O. tenuis*, and *P. minnesotensis*, the amylase was detected in the cells only during the later days of growth (12<sup>th</sup>, 16<sup>th</sup> and 20<sup>th</sup> days, respectively). In *L. spiralis*, both the amylase EC and IC activity seemed to be present in tandem (Figure-1). In *P. boryanum*, the amylase IC activity was present consistently throughout the period of study with a gradual increase from the day of inoculation and an activity of  $2.1 \pm 0.4$  U/µg protein, the highest amongst non-heterocystous forms, was observed on the 20<sup>th</sup> day. In *A. variabilis* and *C. membranacea*, both the amylase EC and IC activities followed a similar pattern with highest activities being observed on the 8<sup>th</sup> day. Amongst the heterocystous forms, *A. variabilis* showed the highest amylase EC and IC activities ( $5.97$  U / µg protein and  $2.23$  U / µg protein, respectively). In the non-heterocystous forms, it is seen that protease EC activities are sporadic rather than following a defined set or pattern (Figure-2). A gradual increase in the protease activity is observed with a complete absence or a sharp increase in the succeeding interval. All the non-heterocystous forms showed highest protease EC activity on the 16<sup>th</sup> day except *O. proboscidea*, which registered detectable protease EC activity only on the 16<sup>th</sup> and 20<sup>th</sup> day ( $6.46$  U/mg protein and  $15.6 \pm 1.8$  U/mg protein, respectively). Both *O. tenuis* and *P. minnesotensis* showed highest protease IC activity on the 4<sup>th</sup> day ( $21.7$  U/mg protein and  $24.4$  U/mg protein, respectively). The activity gradually decreased corresponding to the exponential growth phase of the organism and as the organism entered into the late exponential phase, the protease IC activity resumed again (Figure-2). The heterocystous forms- *N. paludosum* and *W. iyengarii* showed a consistent presence of the protease EC activity throughout the study period ( $16.5 \pm 4$  U/mg protein and  $22 \pm 8$  U/mg protein, respectively). *N. paludosum* and *W. iyengarii* had a sharp increase of protease IC activity on the 4<sup>th</sup> day, which incidentally was the highest activity. *A. variabilis* and *C. membranacea* showed a sharp decrease in the protease IC activity. Highest protease IC activities were observed in *A.*

*variabilis* on the 16<sup>th</sup> day ( $37.2 \pm 2.0$  U/mg protein). Both the species of *Oscillatoria* showed a similar trend in EC and IC activities of beta-lactamase enzyme (Figure-3).  $17 \pm 5$  U/mg protein of the beta-lactamase EC activity was seen in *L. spiralis* (4<sup>th</sup> day), which was highest when compared to all the other non-heterocystous forms. Similarly,  $0.14 \pm 0.02$  U/mg protein of beta-lactamase IC activity in *L. spiralis* on the 12<sup>th</sup> day was the highest activity recorded in comparison to all other non-heterocystous forms in this study. Amongst the heterocystous forms, both *N. paludosum* and *W. iyengarii* showed a decrease in beta-lactamase EC activity from the day of inoculation, which dropped to undetectable levels from the 8<sup>th</sup> day and remained so until the 20<sup>th</sup> day. In *A. variabilis* and *C. membranacea*, a sharp increase in beta-lactamase EC activity on the 4<sup>th</sup> day was seen, corresponding to highest activities ( $17 \pm 0.8$  U/mg protein and  $6 \pm 0.4$  U/mg protein, respectively) during the entire observation period. Beta-lactamase EC activity decreased in all the heterocystous isolates reaching negligible levels on the 20<sup>th</sup> day. In *A. variabilis*,  $12 \pm 0.2$  U/mg protein of beta-lactamase IC enzyme activity was observed on the 12<sup>th</sup> day, which was the highest observed amongst heterocystous forms. The EC activity of acid phosphatase enzyme in the non-heterocystous forms, *P. minnesotensis* and *P. boryanum*, showed a decrease as the growth increased, reaching negligible levels on the 20<sup>th</sup> day (Figure-4). In *L. spiralis* and *O. tenuis*, acid phosphatase EC activity was maximum on the 12<sup>th</sup> day ( $17.8 \pm 4$  U/µg protein and  $7.8 \pm 2$  U/µg protein respectively), which coincided with a peak in their growth rate. Excluding *P. boryanum*, all the other non-heterocystous forms showed a decrease in the acid phosphatase IC activity when compared with zero day (day of inoculation). The highest rate of  $0.6 \pm 0.02$  U/µg protein of acid phosphatase IC activity was recorded on the 16<sup>th</sup> day for *P. boryanum*. In the heterocystous forms, both the acid phosphatase EC and IC activities were absent up to a period of 8 days. High enzyme activities of  $2.8 \pm 0.5$  U/µg protein for EC and  $0.58 \pm 0.05$  U/µg protein for IC were observed in *A. variabilis* on the 12<sup>th</sup> day which subsequently decreased to zero levels on the 20<sup>th</sup> day. In *W. iyengarii* the acid phosphatase IC activity, which appeared only on the 12<sup>th</sup> day, continued to increase till the 20<sup>th</sup> day ( $0.18 \pm 0.02$  U/µg protein). Alkaline phosphatase EC activities were  $6.4 \pm 0.05$  U/µg protein and  $13.6 \pm 1.8$  U/µg protein for *P. minnesotensis* and *P. boryanum*, respectively, on day 4;  $12.1 \pm 2.3$  U/µg protein and  $7.8 \pm 0.9$  U/µg protein, on the 12<sup>th</sup> day for *L. spiralis* and *O. proboscidea*, respectively and  $8.0 \pm 1.2$  U/µg protein in *O. tenuis* on the 16<sup>th</sup> day (Figure-5). In *O. proboscidea* and *P. minnesotensis*, the enzyme activity increased with a decrease in growth. In the case of alkaline phosphatase IC activities, a gradual decrease in the enzyme content from the first interval is observed in all the forms, excepting *O. tenuis* where the highest values of  $0.8$  U/µg protein is observed on the 4<sup>th</sup> day. Both EC and IC alkaline phosphatase activities in *N. paludosum*, *A. variabilis* and *C. membranacea* were observed to be present in tandem, and the highest activities



were observed on the 12<sup>th</sup> day. In *W. iyengarii*, alkaline phosphatase EC activity of  $8.5 \pm 1.4$  U/ $\mu$ g protein on the 4<sup>th</sup> day and IC activity of 0.28 U/ $\mu$ g protein on the 12<sup>th</sup> day was observed. *W. iyengarii* showed higher EC activity compared to other heterocystous forms, however, maximum IC activity was observed in *A. variabilis* ( $0.98 \pm 0.3$  U/ $\mu$ g protein).

## CONCLUSIONS

Applied engineering and intensive research may help in the optimal utilization of promising strains of cyanobacteria for commercially important enzymes. Therefore, further research is warranted to harness these potential microorganisms for various products.

## ACKNOWLEDGEMENTS

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### Legends:

**Figure-1.** Alpha-amylase activity in selected cyanobacteria. Values are mean  $\pm$  standard deviation of triplicate experiments.

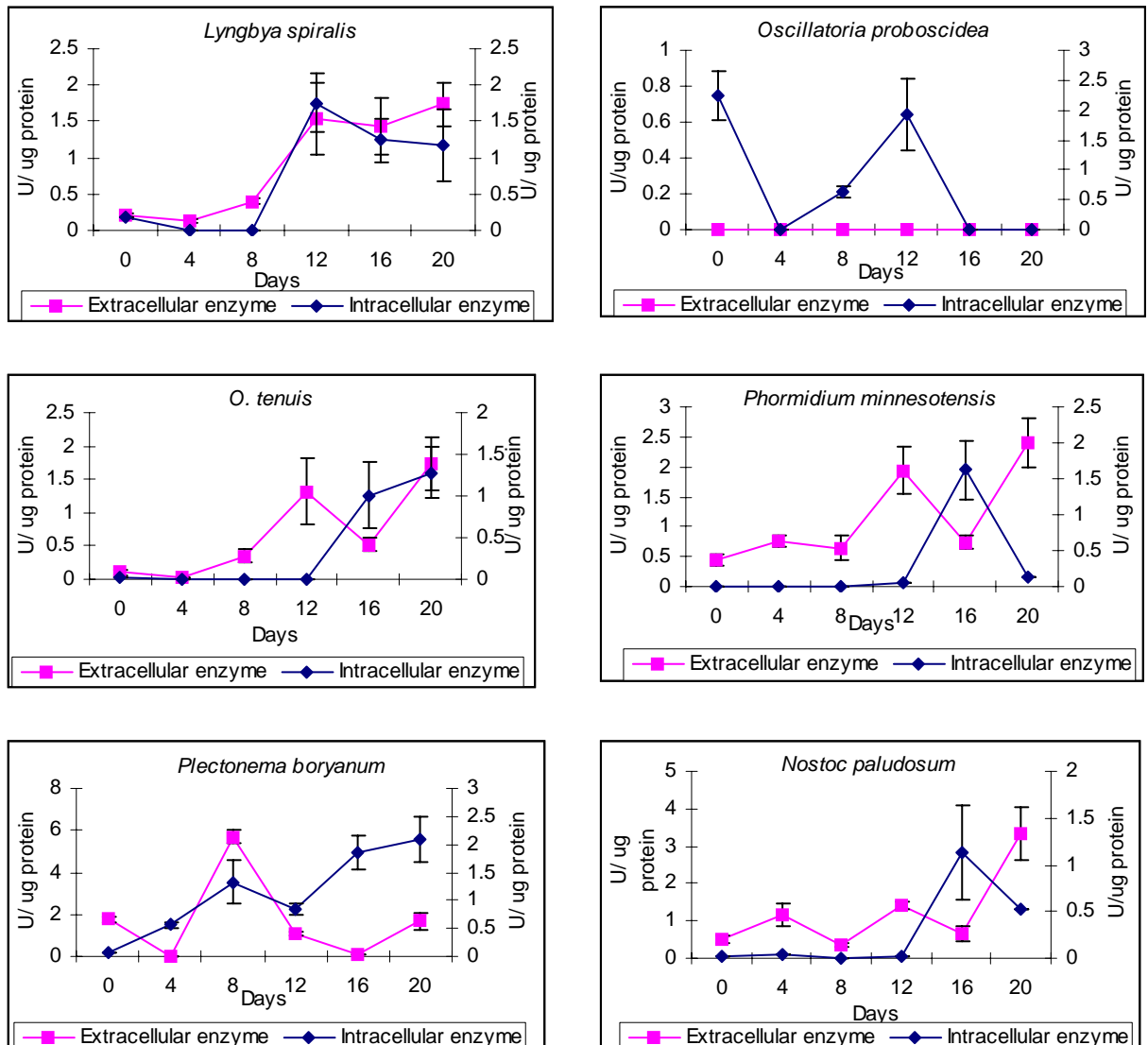
**Figure-2.** Alkaline protease activity in selected cyanobacteria. Values are mean  $\pm$  standard deviation of triplicate experiments.

**Figure-3.**  $\beta$  lactamase activity in selected cyanobacteria. Values are mean  $\pm$  standard deviation of triplicate experiments.

**Figure-4.** Acid phosphatase activity in selected cyanobacteria. Values are mean  $\pm$  standard deviation of triplicate experiments.

**Figure-5.** Alkaline phosphatase activity in selected cyanobacteria. Values are mean  $\pm$  standard deviation of triplicate experiments.

### Figure-1.



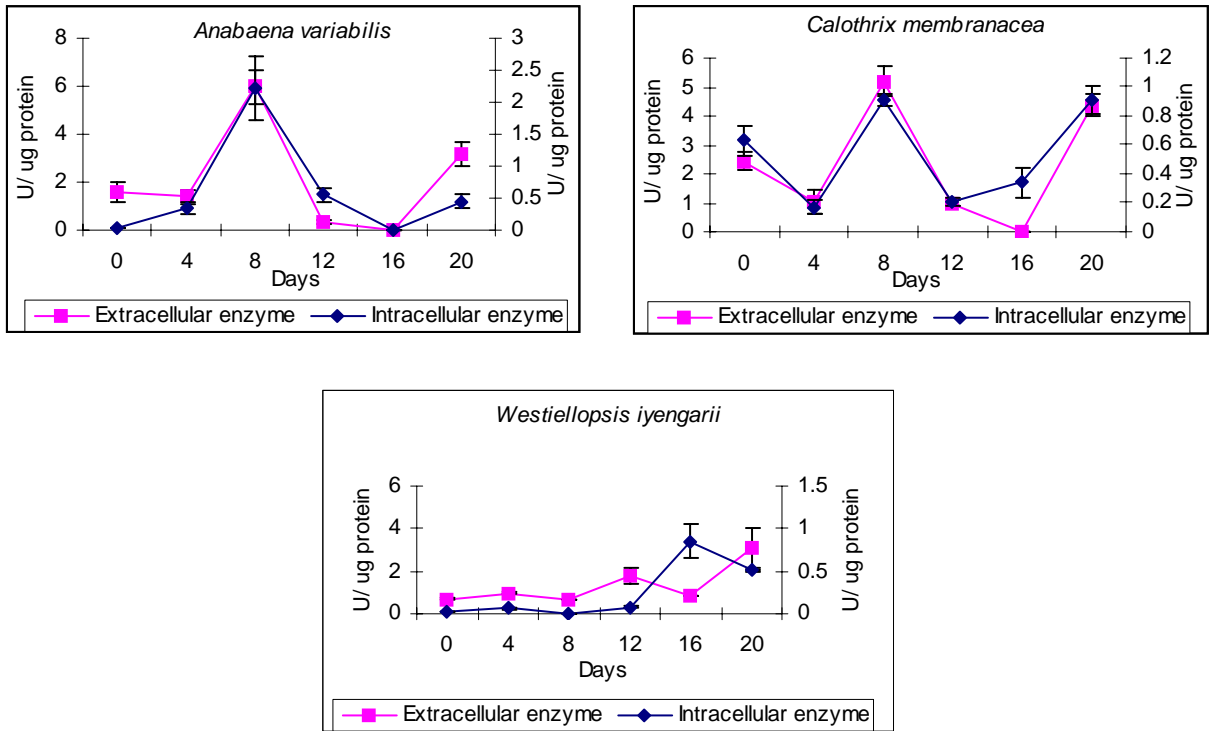
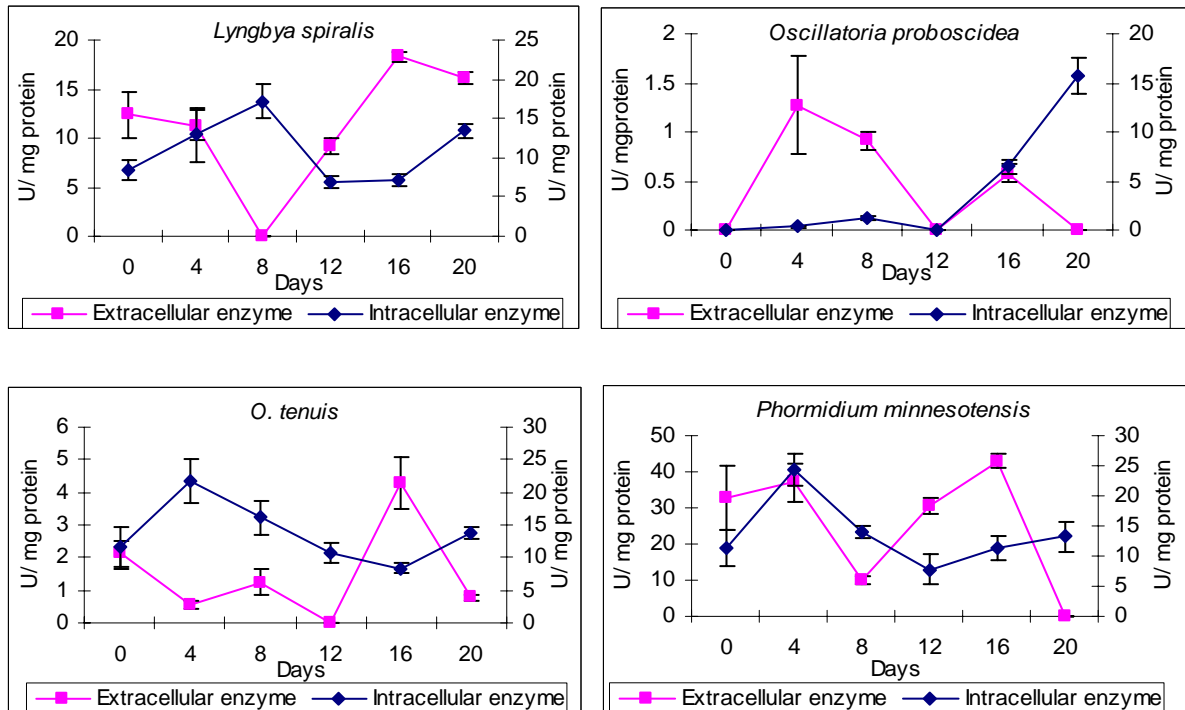


Figure-2.



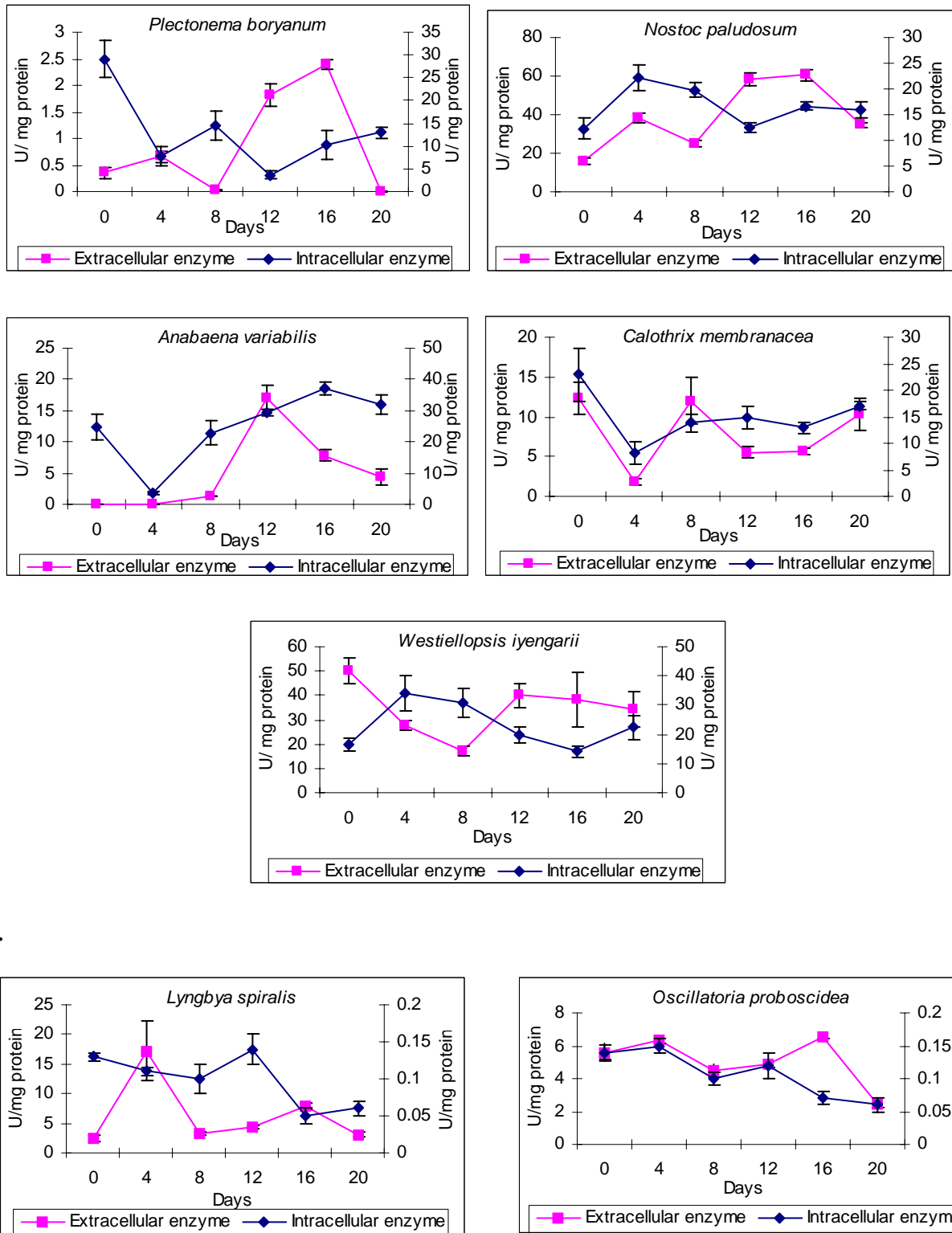


Figure-3.





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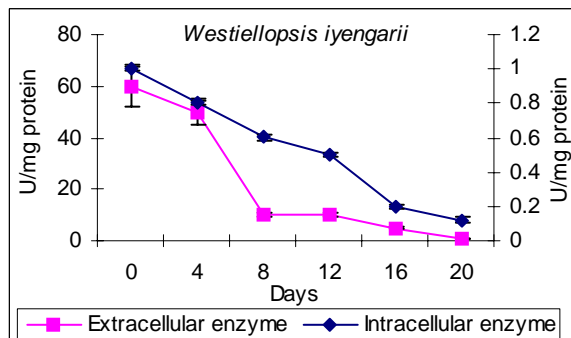
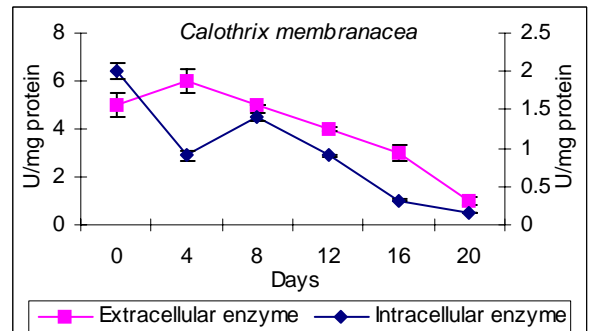
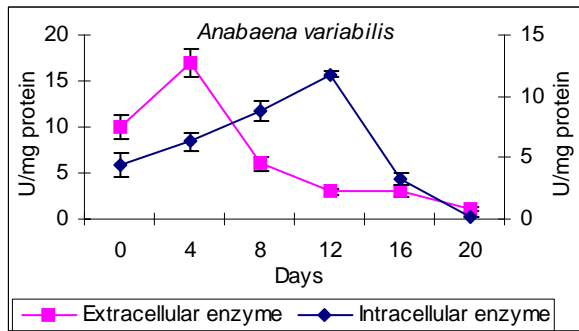
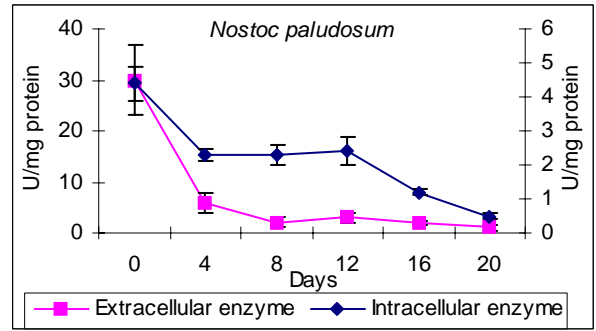
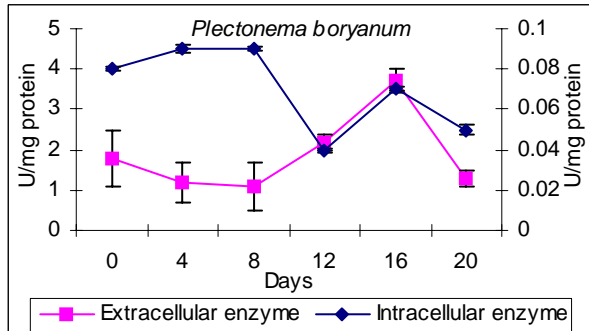
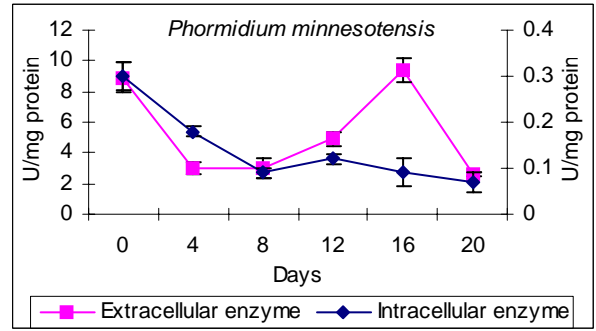
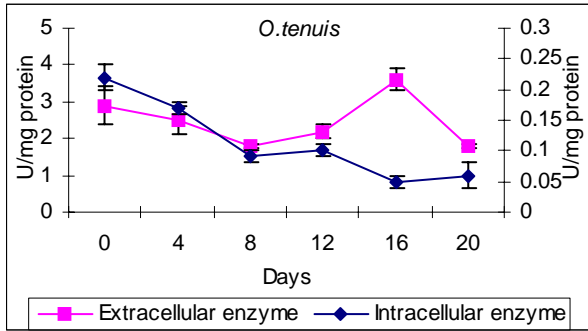
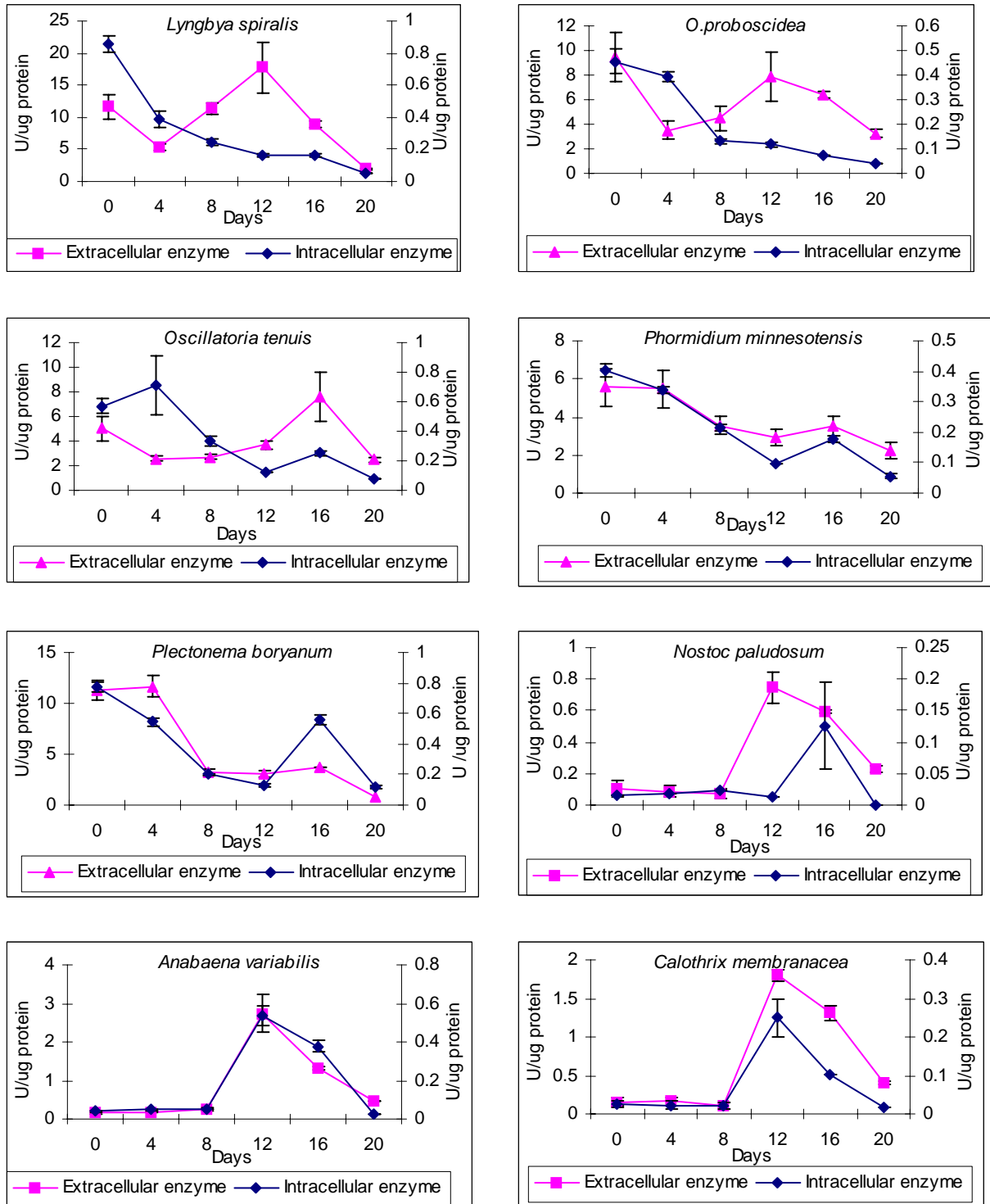




Figure-4.





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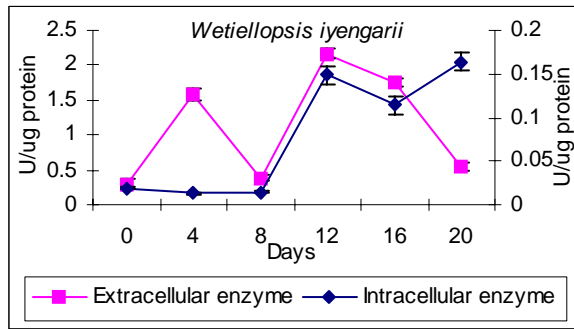
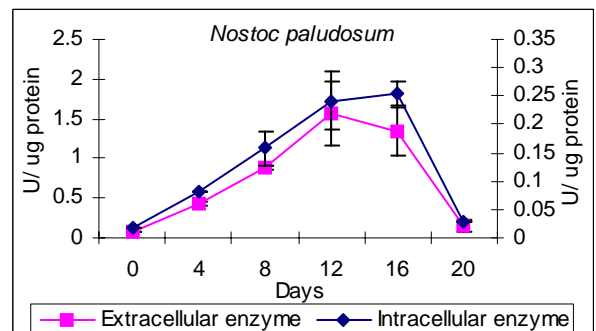
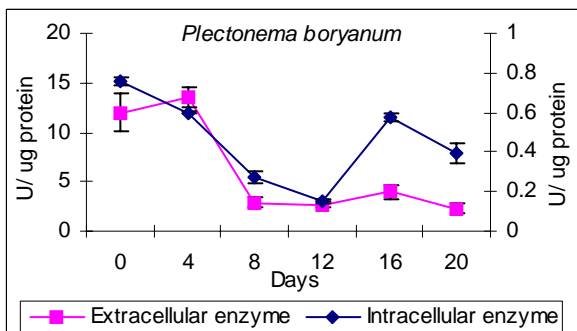
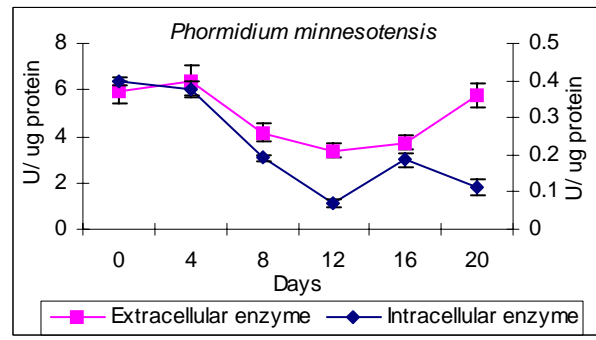
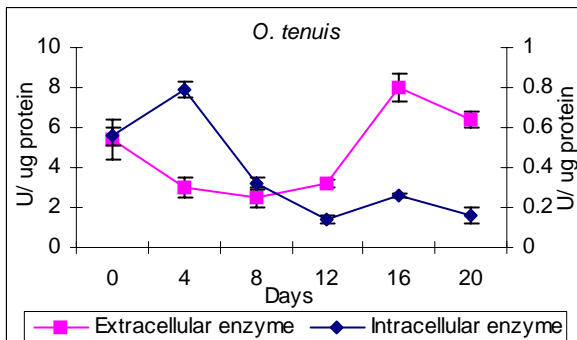
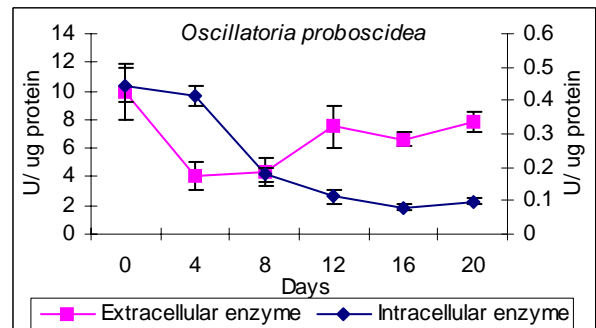
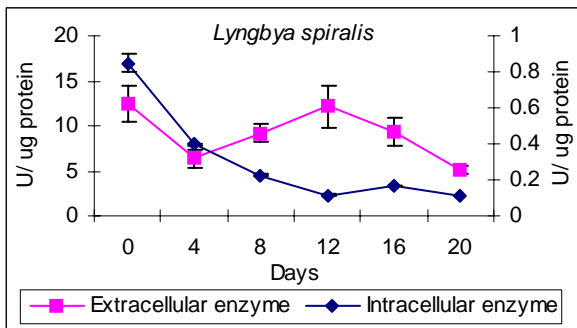


Figure-5.





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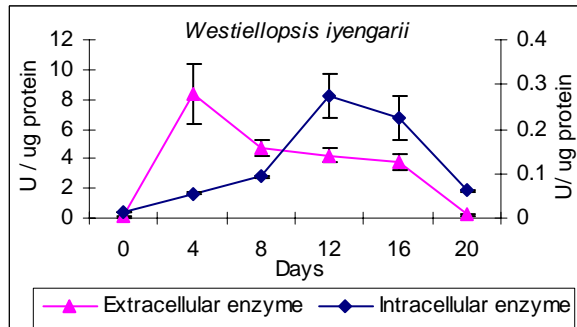
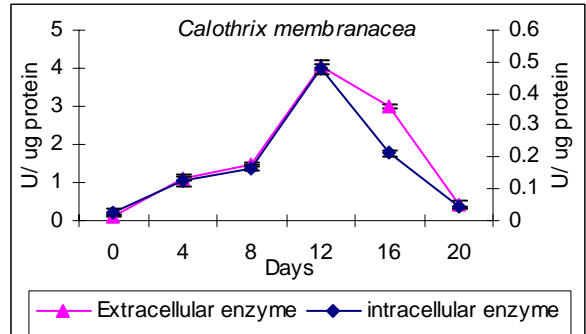
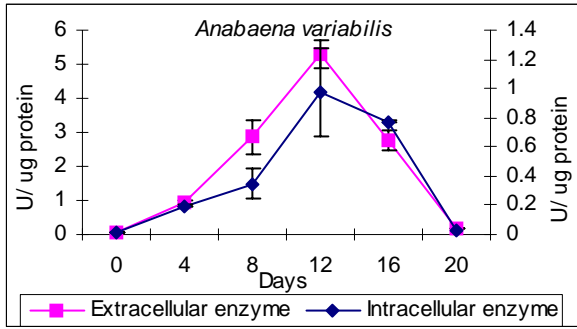


Plate 1: Photomicrographs of the organisms taken for study

