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Water availability affects extracellular hydrolytic enzyme production by *Aspergillus flavus* and *Aspergillus parasiticus*

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Abstract

The objectives of this study were to examine the effect of different water activities (a_w; 0.99, 0.96 and 0.94) and time (up to 120 hrs) on quantitative and specific enzyme production during germination and initial growth of *A. flavus* and *A. parasiticus* strains at 25^{o}C. This is an important early indicator of potential for aflatoxin production under conducive conditions. Qualitative API ZYM generic enzyme strips were used to identify key hydrolytic enzymes produced. Subsequently, the temporal effects of a_w on the total/specific activity of the key 4-5 hydrolytic enzymes were determined using 4-nitrophenyl substrates in a 96-well microtitre plate assay. The main enzymes produced by germinating conidia of *A. flavus* were esterase, lipase, acid phosphatase, β-glucosidase and N-acetyl-β-D-glucosaminidase, while for *A. parasiticus* these were alkaline phosphatase, lipase, acid phosphatase and β-fucosidase for both total (μmol 4-nitrophenol min^{-1} g^{-1}) and specific activity (nmol 4-nitrophenol min^{-1} μg^{-1} protein). There were significant increases in the specific activity of all these enzymes of germinating spores of *A. flavus* (0-120 hrs) except

for β-glucosidase which was maximum at 72 hrs. The total/specific activities of the enzymes produced by *A. flavus* were maximum at 0.99 a_w, with the exception of acid phosphatase and N-acetyl-β-D-glucosaminidase at 0.94 a_w. For *A. parasiticus*, maximum total activity occurred at 0.99 a_w for fucosidase activity, while specific activity was found to be higher at lower a_w levels. These enzymes are important in early colonisation of food matrices by these species and single factors (a_w, time) and two-way interactions were all statistically significant for the enzymes assayed for both species. These enzymes could be used as an early and rapid indicator of the activity of *Aspergillus* section *flavi* species and suggests that rapid infection may occur over a wide range of a_w conditions.

1. Introduction

Aspergillus flavus and *A. parasiticus* are the most important fungi colonizing cereal grains, nuts and animal feed during storage and responsible for aflatoxin contamination (Magan and Olsen, 2004; Magan and Aldred, 2007). These fungi germinate, grow and produce aflatoxins over a wider range of environmental conditions. Optimum condition for growth and aflatoxin production differ with the former being optimum at about 30^{o}C and 0.95 a_w, while toxin production being optimum at about 25-30^{o}C and 0.99 a_w (Sanchis and Magan, 2004). Recent studies suggest that aflatoxin can be produced within 2-3 days as indicated by expression of the key genes in the cluster and phenotypic quantification using analytical methods (Heydt-Schmidt *et al.*, 2008). Indeed, kinetic studies with *Penicillium verrucosum* have shown a close correlation between hydrolytic enzyme production and ochratoxin production (Magan and Aldred, 2007a). Pitt and Miscamble (1995) showed that the impact of environmental factors on growth of *A. flavus, A. parasiticus* and *A. oryzae* was similar with minima of 0.82 a_w at 25 ºC, 0.81 a_w at 30 and 37ºC. However, much less information is available on how rapidly colonisation occurs under conducive conditions and little attempt has been made to quantify the key hydrolytic enzymes produced during the early stages of infection (Mellon *et al.*, 2007). This could be an important indicator of the ability for colonisation and infection and may contribute to the competitiveness of this important aflatoxigenic group.

Infection of maize grain by *A. flavus* has received more interest and pectinases, amylases and cutinases have been implicated in this process (Cotty *et al.*, 1990; Woloshuk *et al.*, 1996; Guo *et al.*, 1996; Mellon *et al.*, 2007). The pericarp is the outermost layer of maize kernel and provides effective protection from fungal invasion. It consists of several

layers of cells which differ in their degree of degradation and cell wall thickness (Kent and Evers, 1994). Thus the production of a range of enzymes may be advantageous for infection of maize kernels by *Aspergillus* spp. pre- or post-harvest. It has been suggested that the production of specific hydrolytic enzymes by spoilage fungi on temperate cereals can be a good early indicator of the initiation of moulding and mycotoxin production in grain post-harvest (Magan, 1993a). A range of enzymes is present in healthy cereals grains (Reed and Thorne, 1978) and others may be produced by colonizing spoilage fungi (Stevens and Relton, 1981; Jiminiz *et al.,* 1986; Petruccioli *et al.,* 1988; Jain and Lacey, 1991). These previous studies showed that *Aspergillus* spp. can produce amylase, protease, β-glucosidase, phosphatase, pectinase, lipase and amidase. Amylases and proteases are usually produced in the largest amounts by these species. However, much less information is available in relation to environmental factors such as water availability (Marin *et al.,* 1998; Keshri and Magan, 2000). Steven and Relton (1981) compared hydrolytic enzymes activity in barley seeds alone or when colonized with *Aspergillus* spp. and found more alkaline phosphatase, aryl sulphatase and α-amylase activities in the inoculated grain. Jain and Lacey (1991) using chromogenic 4-nitrophenyl substrates found that the largest quantities of enzymes produced by *Aspergillus, Penicillium* and *Eurotium* spp. were N-acetyl- β-D-glucosaminidase and α-D-galactosidase during colonization of barley/wheat grain. However, specific activities were not determined. Marin *et al.* (1998) showed that *Fusarium verticillioides* and *F. proliferatum* produced large quantities of α-D-galactosidase, β-D- glucosidase and N-acetyl-β-D-glucosaminidase during early colonization of maize kernels. Other semi-quantitative and agar based enzymatic tests have suggested that *Aspergillus* spp. produce large amounts of hydrolytic enzymes such as N-acetyl- β-D-glucosaminidase, β-D-glucosidase, phosphatases, fucosidases and sulphatases although effects of environmental factors were not investigated. However, production of some of these enzymes may also be indicative of early mycotoxin production as part of the ecological strategy for effective competition in ripening cereals pre-harvest (Magan and Aldred, 2007b).

The objectives of this study were thus to (a) evaluate the key hydrolytic enzymes produced by *A. flavus* and *A. parasiticus* qualitatively by using a range of enzyme substrates, and (b) determine the effect of a_w and time (120 hrs) on total and specific activity of up to 5 important extracellular enzymes produced during the initial phases of growth of these species in vitro at 25^{o}C.

2. Materials and methods

2.1 Fungal isolates

One representative strain each of *Aspergillus flavus* (A-2092) and *A. parasiticus* (PRR-2747) was used in all experiments. Initial studies with three strains of each suggested that these were appropriate representatives. The isolates were maintained on malt extract agar (MEA, 20 g glucose, 1 g peptone, 20 g agar, 1000ml distilled water, pH 5.5). The strains were isolated from maize and produced aflatoxins. They are held in the Applied Mycology Group Culture Collection, Cranfield University, Bedford, UK.

2.2 Initial studies using API ZYM strips for determination of extracellular enzyme production

Species were grown in Czapek Yeast Extract (CYE) broth ($NaN0_3$, 3g; K_2HPO_4, 1g; KCl, 0.5g; $MgSO_4.7H_2O$, 0.5g; $FeSO_4.7H_2O$, 0.01g; Yeast extract, 5g; Sucrose, 30 gm and distilled water, 1000ml, pH, 6.25) modified with glycerol to 0.99, 0.96 and 0.94 a_w (Magan and Lacey, 1984). Initial studies involved culturing in broth medium in flasks (conidia, 10^5 spores ml^{-1}) and incubation on an orbital shaker at 25ºC for 6 days before the mycelium was removed by filtration through Whatman No.1 filter paper.

After centrifugation for 5 min the extracellular enzyme activity were tested using API ZYM galleries (API system S. A., Montalieu Vercieu, France). This is a semi-quantitative micro-method that allows a rapid and simultaneous evaluation of 19 enzymatic activities using very small amounts of sample (Table 1). The substrates are contained in support strips, which allow contact between the enzymes and substrates. Each cupule of the API strip was inoculated with 65µL of culture filtrate and incubated at 37ºC for 4 hrs. After incubation, one drop (35 µL) of ZYM A reagent and an equal amount of ZYM B reagent were added simultaneously. The colours became visible within 30 mins and the results were semi-quantitatively graded using the colour chart supplied with the API ZYM kit.

2.3 Effect of water activity and time on quantitative production of enzymes

Both strains of *A. flavus* and *A. parasiticus* were grown on Czapek Yeast Extract Agar (CYA) media modified with glycerol (BDH, U.K) to 0.99, 0.96 and 0.94 a_w. A 0.2 ml volume of a spore suspension (10^5 spores ml^{-1}) were spread plate onto the surface of the different a_w treatments. A total of 12 replicates were used for each treatment a_w and incubated at 25^{o}C. Thus three replicates could be destructively sampled after 48, 72, 96 and

120 hrs. Treatments of the same a_w were kept in sealed polyethylene bags. Three agar discs (5 mm diameter) were removed from each replicate using a surface sterilised cork borer. The agar discs were placed in 4 ml potassium phosphate extraction buffer (10mM; pH 7.2; BDH, U.K.). The bottles were shaken on a wrist action shaker (KS250 basic, IKA labortechnik) for 1hr at 4ºC. The washings were decanted into 1ml plastic Eppendorf tube and centrifuged for 10 min at 1150 x ***g*** in a bench microfuge (MSE Cenetaur 2). The supernatant was removed and specific activity determined as described previously (Marin *et al.,* 1998).

2.4 Total enzyme activity determination

The 4-nitrophenyl substrates and buffers used are listed in Table 2. Enzyme activity was measured by the increase in optical density at 405 nm caused by the liberation of 4-nitrophenol after enzymatic hydrolysis of the substrate, three minutes after stopping the reaction with 1M Na_2CO_3.

For analysis the reaction mixture consisted of 40 µL of substrate solution, 40 µL of enzyme extract and 20 µL of the appropriate buffer. These were placed in the wells of the microtitre plate, together with appropriate controls and incubated at 37ºC for 1hr. The reaction was stopped by the addition of 5 µL of 1M Na_2CO_3 solution. The enzyme activity was measured using a MRX multiscan plate reader (Dynex Technologies Ltd, Billinghurst, West Sussex, U.K.) by the increase in optical density at 405 nm caused by the liberation of *p*-nitrophenol by enzymatic hydrolysis of the substrate. Total enzyme activity was calculated from the calibration curve of absorbance at 405 nm against *p*-nitrophenol concentration and expressed as µmol *p*-nitrophenol released min^{-1}. The calibration curves were made using concentrations in the range 0-3.5 µmol ml^{-1} of *p*-nitrophenol at relevant pH values.

The total enzyme activity provides information on the overall amounts of enzyme produced. It is also important to have information on the specific activity which is related to the amount of protein present provides important complementary information and better reflects the dynamics and behaviour during infection of the substrate (Marin et al., 1998).

2.5 Specific Enzyme Activity determination

Total protein in the extracellular extract was measured using the Bicinchoninic acid protein assay (BCA) procedure. A BCA protein assay reagent kit (Thermo Fisher Scientific, Cramlington, Northumberland, U.K.) consisted of the BCA reagent A (containing sodium

carbonate, sodium bicarbonate, Bicinchoninic acid and sodium tartrate in 0.1N sodium hydroxide), BCA reagent B (solution containing 4% cupric sulphate) and Albumin standard (1mg ml^{-1} in a solution of 0.15M NaCl and 0.05% sodium azide) was used. The concentration range for protein was 0-12 μg to obtain the standard curve (R^2=0.998). Protein reduces alkaline Cu (II) to Cu (I), which forms a purple complex with bicinchoninic acid (a highly specific chromogenic reagent). The resultant absorbance at 550 nm is directly proportional to the protein concentration.

BCA reagent A, 50 parts, was mixed with 1 part of reagent B to obtain the working reagent, which is stable for at least 1 day when stored in a closed container at room temperature. 10μL volumes of each standard or unknown sample were pipetted into the appropriate microtitre plate wells, while 10 μL of the diluents were used for blank wells. A 200 μL volume of the working reagent were added to each well, and after shaking, the plates were incubated at 37ºC for 30 minutes. After cooling the plates to 25^{o}C, the absorbance at 550 nm was measured in a plate reader ((Dynex Technologies Ltd, Billinghurst, West Sussex, U.K.). The protein concentrations in the enzyme extracts were obtained from the calibration curve at 550 nm against BSA concentration. These values were used to calculate the specific activity of the enzymes in nmol p-nitrophenol released min^{-1} μg^{-1} protein.

2.6 Statistical analyses of the results

Variance of both total and specific activity results were analysed and effects of the different factors involved in the experiment and their interactions are shown at 95% level ($P \leq 0.05$) significance. LSD tests were also made (P=0.05) for the significant factors found. All statistical analyses were made using SAS.

3. Results

3.1 Semi-quantitative determination of enzymes by API ZYM system

Figure 1 shows an example of the enzyme profile of *A. flavus* and *A. parasiticus* after 6 days growth in Czapek yeast extract broth at 25°C. Esterase, lipase, acid phosphatase, β-glucosidase and N-acetyl- β-D-glucosaminidase were found in higher quantities with respective means of 4-5 on the colour scale intensity in samples of *A. flavus.* A similar trend was found for *A. parasiticus* with production of high quantities of alkaline phosphatase, β-fucosidase, lipase and acid phosphatase with means of between 4.0-4.3 colour intensity.

Studies were subsequently focussed on the temporal production of total and specific activities of these enzymes in more detail under different a_w regimes.

3.2 Effect of water activity and time on the predominant hydrolytic extracellular enzymes produced by *A. flavus*

Figure 2 compares the effect of a_w and time on the total enzyme activity of the five main enzymes produced by the *A. flavus* strain. In general, the activity of esterase, lipase, acid phosphatase, β-glucosidase and N-acetyl-β-D-glucosaminidase all increased with increasing a_w and time. The lipase, acid phosphatase activities were not significantly affected while esterase, N-acetyl-β-D-glucosaminidase and, in part, the β-Glucosidase activities were higher at 0.99 a_w, but did not show much difference at 0.96 and 0.94 a_w. All the enzymes showed maximum total activity after 120 hrs.

Figure 3 compares the temporal changes in specific activity of all the five major enzymes produced by *A. flavus.* It shows that significantly more lipase and β-glucosidase were produced at 0.99 a_w while the specific activity of acid phosphatase, and N-acetyl-β-D-glucosaminidase was maximum at 0.94 a_w. Acid phosphatase was maximum at 120 hrs at 0.96 a_w. In general, specific activity of all enzymes significantly increased over time. However, specific activity of lipase and N-acetyl-β-D-glucosaminidase were optimum earlier (48-72 hrs) and then varied while that of β-glucosidase activity progressively decreased after 72 hrs.

3.3 Effect of water activity and time on the predominant enzyme production by *A. parasiticus*

A comparison of the total enzyme activity of alkaline phosphatase, lipase, acid phosphatase and β-fucosidase in extracts from *A. parasiticus* grown on CYA is shown in Figure 4. Optimum total activity was at 0.96 a_w for both acid and alkaline phosphatases and 0.94 a_w for lipase. However, differences were non-significant at $P<0.05$. The exception was β-fucosidase where total activity was maximum at 0.99 a_w, and significantly lower and similar at both 0.96 and 0.94 a_w. Furthermore, the total activity of all the four enzymes increased significantly over time.

For specific activity, a comparison of the four enzymes is shown in the Figure 5. Specific activity of alkaline and acid phosphatase were optimum at 0.96 a_w while that of lipase and β-fucosidase was higher at 0.94 a_w. However specific activity of alkaline and acid phosphatase increased up to 72 h but then decreased.

3.4 Interactions between assayed factors

Table 3a shows the analysis of variance of results and the effect of single and two way factors on the total and specific activity of the five enzymes produced by *A. flavus.* For esterase, lipase, acid phosphatase, β-Glucosidase and N-acetyl-β-D-glucosaminidase from *A. flavus*, single factors (a_w, time) and two-way interactions were significant **(**$P < 0.01$) for both total and specific activity. A similar trend was found for alkaline phosphatase, lipase, acid phosphatase and β-fucosidase from *A. parasiticus* (Table 3b). However a_w x time for total activity of alkaline phosphatase was only significant at $P<0.05$.

4. Discussion

This study investigated the very early phases of germination and mycelial colonisation by *A. flavus* and *A. parasiticus* by quantifying the capacity for hydrolytic enzyme production under different water availability conditions. This showed that a range of hydrolytic enzymes were produced over a range of a_w levels for both species. Although initial studies were done in liquid culture to identify key groups of enzymes, we used solid agar based medium for subsequent studies as colonisation would normally on solid grain matrices. Thus the quantitative assays were more relevant to colonisation rates and perhaps indicative of potential for initial mycotoxin production.

The major enzymes produced by *A. flavus* were esterase, lipase, acid phosphatase, β-glucosidase and N-acetyl-β-D-glucosaminidase while for *A. parasiticus* these were alkaline phosphates, lipase, acid phosphatase and β-fucosidase in terms of both total and specific enzyme activity. Previous work has examined enzymes as an indicator of early colonisation of spoilage fungi prior to visible growth in stored grain (Magan, 1992). Some of these enzymes and lipases were found to be variously affected by incubation period, a_w and temperature. Studies with fumonisin producing *F. verticillioides* and *F. proliferatum* strains found that the total and specific activity of α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase, were important during early germination and growth with maximum being produced at 0.98 a_w, with significantly less at 0.95 and 0.93 a_w (Marin *et al*., 1998).

Fungi have evolved different strategies to invade nutritional matrices. Hydrolytic enzyme production is one of the best early indicators of fungal spoilage prior to visible signs of growth and mycotoxin production (Jain and Lacey, 1991; Magan, 1993a; 1993b). A range of enzymes are present in healthy cereal grains (Reed and Thorne, 1978) and others

may be produced by colonizing fungi (Jiminez *et al*., 1986; Petruccioli *et al*., 1988). These enzymes include cutinases, cellulases, pectinases and proteases (Knogge, 1996). There are three major types of enzymes required for the hydrolysis of crystalline cellulose to glucose. These are endoglucanases, exoglucanases and β-glucosidases. Penetration of plant matter may be achieved quite simply by entry through the plant's natural openings or by utilizing a route through non-cellulosic areas. It has been shown that *A. flavus* can produce cell wall degrading pectinases, and may secrete extracellular cutinases to enable effective infection through the pericarp of maize kernels (Guo *et al.*, 1996; Mellon *et al.*, 2007).

The present study suggests that lipase, β-glucosidase, N-acetyl-β-D-glucosaminidase and esterase may be suitable indicators of colonization of *A. flavus* and lipase, acid phosphatase and β-fucosidase for *A. parasiticus*. In general, total activity may be a more useful and rapid method than measurement of specific activity on a protein basis because the former method is more sensitive in detecting fungal production of enzymes than the latter. This study has also shown that the above mentioned enzymes are produced over a wide range of water availabilities, enabling the fungi to rapidly colonize the substrate. This together with the capability for rapid germination, growth and also mycotoxin production as was shown for *Fusarium* section *Liseola* species (Marin *et al.*, 1996; Keshri and Magan, 2000) could partially explain their competitiveness in the substrate niche and their ability for excluding other fungi once becoming established (Magan and Aldred, 2007b).

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5. References

Cotty P.J., Clevland T.E., Brown R.L., Mellon J.E., 1990., Variation in polygalcturonase production among *Aspergillus flavus* isolates. Applied and Environmental Microbiology 56: 3885-3887.

Guo B.Z., Russin J.S., Cleveland T.E., Brown R.L., Bamann K.E., 1996., Evidence for cutinase production in *Aspergillus flavus* and its possible role in infection of corn kernels. Phytopathology 86: 824-829.

Schmidt-Heydt, M., Magan, N., Geisen, R., 2008., Stress induction of mycotoxin biosynthesis genes in relation to abiotic factors. FEMS Microbiology Letters 284: 142-149.

Jain P.C., Lacey J., 1991., Use of API ZYM strips and 4-nitrophenyl substrates to detect and quantify hydrolytic enzymes in media and grain colonized with *Aspergillus*, *Eurotium* and *Penicillium* species. Mycological Research 95: 834-842.

Jiminez M., Sanchis V., Mateo R., Hernandez E., 1986., *Penicillium* in preharvest corn in Valencia (Spain). II. Study of the enzymatic and toxigenic capacities of the species. Mycopathologia 96: 13-18

Kent, N.L. and Evers, A.D. 1994., Technology of Cereals: An Introduction for Students of Food Science and Agriculture. Woodhead Publishing. 334 pp.

Keshri G., Magan N., 2000., Detection and differentiation between mycotoxigenic and non-mycotoxigenic strains of *Fusarium* spp. using volatile production profiles and hydrolytic enzymes. Journal of Applied Microbiology 89**:** 825-833.

Knogge W, 1996., Fungal infection of plants. The Plant cell 8: 1711-1722.

Magan N., 1992., Relationship between rapeseed and contaminant fungi: effects on respiration and enzymatic deterioration. In Extended Abstracts, International Symposium on Stored-Grain Ecosystems, ed. D.S. Jayas, N.D.D. White, W. E. Muir & R. N. Sinha. University of Manitoba, pp. 16-17

Magan N., 1993a., Early detection of fungi in stored grain. Interantional Biodeterioration and Biodegradation 32: 145-160.

Magan N., 1993b., Early detection of mould growth in stored grain. Aspects of Applied Biology: Cereal Quality III 36: 417-426

Magan, N., Aldred, D., 2007a., Why to fungi produce mycotoxins? In: Food Mycology: a multifaceted approach to fungi and food (Dijksterhuis, J., and Samson, R. A., eds), Taylor & Francis, Boca Raton,Florida, USA, pp: 121-133.

Magan, N., Aldred, D., 2007b., Environmental fluxes and fungal interactions: maintaining a competitive edge. Chapter 2 in Stress in yeasts and filamentous fungi (van West, P., Avery, S., Stratford, M.), Elsevier Ltd., Amsterdam, Holland. Pp. 19-35.

Magan N., Lacey J., 1984., Effect of water activity, temperature and substrate on interaction between field and storage fungi. Transaction of the British Mycological Society 82:83-93.

Magan, N., Olsen, M., Eds., 2004., Mycotoxins in food: detection and control**.** Woodhead Publishing Ltd., Cambridge, U.K. pp. 471.

Marin S., Sanchis V., Magan N., 1995., Water activity, temperature and pH effect on growth of *Fusarium moniliforme* and *Fusarium prolifiratum* isolates from maize. Canadian Journal of Microbiology 41: 1063-1070

Marin S., Sanchis V., Teixido A., Saenz R., Ramos A.J., Vinas I., Magan N., 1996., Water and temperature relations and microconidial germination of *Fusarium moniliform* and *Fusarium proliferatum* from maize. Canadian Journal of Microbiology 42: 1045-1050

Marin S., Sanchis V., Magan N., 1998., Effect of water activity on hydrolytic enzyme production by *Fusarium moniliforme* and *Fusarium proliferatum* during colonozation of maize. International Journal of Food Microbiology 42: 185-194.

Mellon, J.E., Cotty, P.J., Dowd, M.K., 2007., *Aspergillus flavus* hydrolases: their roles in pathogenesis and substrate utilization. Applied Microbiology and Biotechnology 77: 497-504.

Petruccioli M., Federici F., Miller M.W., 1988., Extracellular enzymes production in species of the genus *Penicillium*. Mycologia 80: 726-728

Pitt J.I., Miscamble B.F., 1995., Water relations of *Aspergillus flavus* and closely related species. Journal of Food Protection 58: 86-90

Reed G., Thorn J.A., 1978., Enzymes. In Wheat Chemistry and Technology (ed. Y. Pomeranz), pp 453-491. American Association of Cereal Chemists: St Paul, Minn., U.S.A.

Sanchis V., Magan N., 2004., Environmental conditions affecting mycotoxins. In Mycotoxins in Food, ed. N. Magan and M. Olsen, pp 177. CRC Press LLC., U.S.A.

Stevens L., Relton J.M., 1981., Production of hydrolytic enzymes by xerotolerant species of *Aspergillus*. Transactions of the British Mycological Society 77: 637-676.

Woloshuk C.P., Cavaletto J.R., Cleveland T.E., 1996., Inducers of aflatoxin biosynthesis from colonized maize kernels are generated by an amylase activity from *Aspergillus flavus.* Phytopathology 87: 164-169

Table 1. Enzymes detected through API ZYM System

Enzyme	**Substrate**	**Cupule number**
Phosphate alkaline	2-nephtyl phosphate	E2
Esterase (C4)	2-naphthyl butyrate	E3
Esterase lipase	2-naphthyl caprylate	E4
Lipase (C14)	2-naphthyl myristate	E5
Lucine arylamidase	L-leucyl-2-napthylamide	E6
Valine arylamidase	L-valyl-2-napthylamide	E7
Cystine arylamidase	L-cystyl-2-naphtylamide	E8
Trypsine	N-Benzoyl-L-arginine-2-napthylamide	E9
Chymotrypsine	N-glutaryl-phenylalanine-2-naphtylamide	E10
Acid phosphatase	2-naphthyl phosphate	E11
Napthol-AS-BI-Phosphohydrolase	Napthol-AS-BI-phosphate	E12
A-galactosidase	6-Br-2-naphthyl- α-D-galactopyranoside	E13
B-galactosidase	2-naphtyl- β-D-galactopyranoside	E14
B-glucoronidase	Napthol-AS-BI- β-D-glucopyranoside	E15
A-glucosidase	2-naphtyl- α-D-glucopyranoside	E16
B-glucosidase	6-Br-2-naphthyl- β -D-glucopyranoside	E17
N-acetyl-β-glucosaminidase	1-naphtyl-N-acetyl- β –D-glucosaminide	E18
A-mannosidase	6-Br-2-naphthyl- α-D-manopyranoside	E19
A-fucosidase	2-naphtyl- α-L-fucopyranoside	E20

Table 2. Details of the hydrolytic enzymes assayed in this study, their substrates, concentrations, buffer and pH used.

Enzyme	**Substrate**	Conc.(mM)	**Buffer**	**pH**
Esterase(C4)	p-nitrophenyl butyrate	10	0.2M MOPS	7.5
Lipase (C14)	p-nitrophenyl myristate	10	50mM Tris	8.5
B-glucosidase	4-nitrophenyl-β-D-glucopyranoside	1	25nm acetate	5.0
N-acetyl- β-D glucosaminidase	4-nitrophenyl-N-acetyl-α-D-Glucosaminide	2	0.1 Citrate	4.2
B-D-fucosidase	4-nitrophenyl-β-D-fucopyranoside	2	25mMacetate	5.0
Acid phosphatase	4-nitrophenyl-phosphate disodium	1.9	0.6M acetate	4.8
Alkaline phosphatase	4-nitrophenyl-phosphate disodium	2	25mM acetate	8.5

Table 3. Analysis of variance of esterase, lipase, acid phosphatase, β-glucosidase and N-acetyl- β-D-glucosaminidase total (μmol 4-nitrophenol min^{-1}) and specific (nmol 4-nitrophenol mn^{-1} μg^{-1} protein) activity at different levels of water activity (a_w) and incubation time (*t*) for *A. flavus and (b) for A. parasiticus.*

(a)

Enzyme			Total activity		Specific activity	
	Factor	DF	MS	F	MS	F
Esterase	A*w*	2	0.097	7319.26**	6.760	954.34**
	T	3	0.103	7828.77**	4.049	571.57**
	a*w* x *t*	6	0.085	6445.73**	1.762	248.71**
Lipase	A*w*	2	0.001	103.96 **	3.206	482.94**
	T	3	0.001	57.69**	2.968	447.11**
	a*w* x *t*	6	0.001	12.73**	0.121	18.17**
Acid Phosphatase	A*w*	2	0.001	63.59**	7.644	442.22**
	T	3	0.001	146.86**	2.240	129.61**
	a*w* x *t*	6	0.001	32.46**	1.478	85.49**
β-glucosidase	A*w*	2	0.028	1829.67**	10.27	313.64**
	T	3	0.012	770.96**	41.68	1272.24**
	a*w* x *t*	6	0.007	471.01 **	0.951	29.04**
N-acetyl- β-D-glucosaminidase	A*w*	2	0.002	98.36**	0.813	31.56**
	T	3	0.005	295.54**	37.956	1473.92**
	a*w* x *t*	6	0.001	58.27**	3.671	142.54**

**Significant P<0.01

(b)

Enzyme			TA		SA	
	Factor	DF	MS	F	MS	F
Alkaline phosphatase	A*w*	2	0.00005	166.51**	2.08	1287.11**
	T	3	0.0003	135.59**	0.19	120.23**
	a*w* x *t*	6	0.00002	3.35 *	0.12	72.71**
Lipase	A*w*	2	0.001	1031.61**	5.91	2469.64**
	T	3	0.0012	6144.59**	2.58	1078.22**
	a*w* x *t*	6	0.001	1921.44**	1.63	680.75**
Acid Phosphatase	A*w*	2	0.0012	74.39**	10.89	5482.85**
	T	3	0.001	151.76**	101.05	50877.15**
	a*w* x *t*	6	0.0001	11.93**	4.92	2476.33**
β-fucosidase	A*w*	2	0.002	157.87**	2.51	3787.46**
	T	3	0.001	56.64**	1.03	1561.28**
	a*w* x *t*	6	0.0001	9.03**	0.56	857.74**

**Significant P<0.01

* Significant p<0.05

Figure legends

Figure 1. A characteristic enzyme profile of *Aspergillus flavus* and *A.parasiticus*. Results are mean values of API ZYM results. E1 represents the control and E2 to E20 represents 19 enzymes in the API ZYM strip. Colour intensity scale: 0, no activity; 1 to 4 intermediate activity; and 5, maximum activity (≥40nM of chromophore released). Bars represent standard errors of the mean.

Figure 2. Effect of time and water activity, 0.99 a_w (─○─) 0.96a_w (─■─) 0.94a_w (─▲─) on total (a) Esterase, (b) Lipase, (c) Acid phosphatase, (d) β-Glucosidase, (e) N-acetyl-β-D-glucosaminidase activity by *A. flavus.* The blanks have been subtracted from the results given.

Figure 3. Effect of time and water activity, 0.99 a_w (─○─) 0.96a_w (─■─) 0.94a_w (─▲─) on specific (a) Esterase (b) Lipase (c) Acid phosphatase (d) β-Glucosidase (e) N-acetyl-β-D-glucosaminidase activity by *A. flavus.* Blanks have been subtracted from the results given.

Figure 4. Effect of time and water activity, 0.99 a_w (─○─), 0.96a_w (─■─), 0.94a_w (─▲─) on total (a) alkaline phosphatase, (b) Lipase, (c) Acid phosphatise, (d) β-fucosidase activity by *A. parasiticus.* Blanks have been subtracted from the results given.

Figure 5. Effect of time and water activity, 0.99 a_w (─○─), 0.96a_w (─■─), 0.94a_w (─▲─) on specific (a) alkaline phosphatase, (b) Lipase, (c) Acid phosphatise, (d) β-fucosidase activity by *A. parasiticus.* Blanks have been subtracted from the results given.

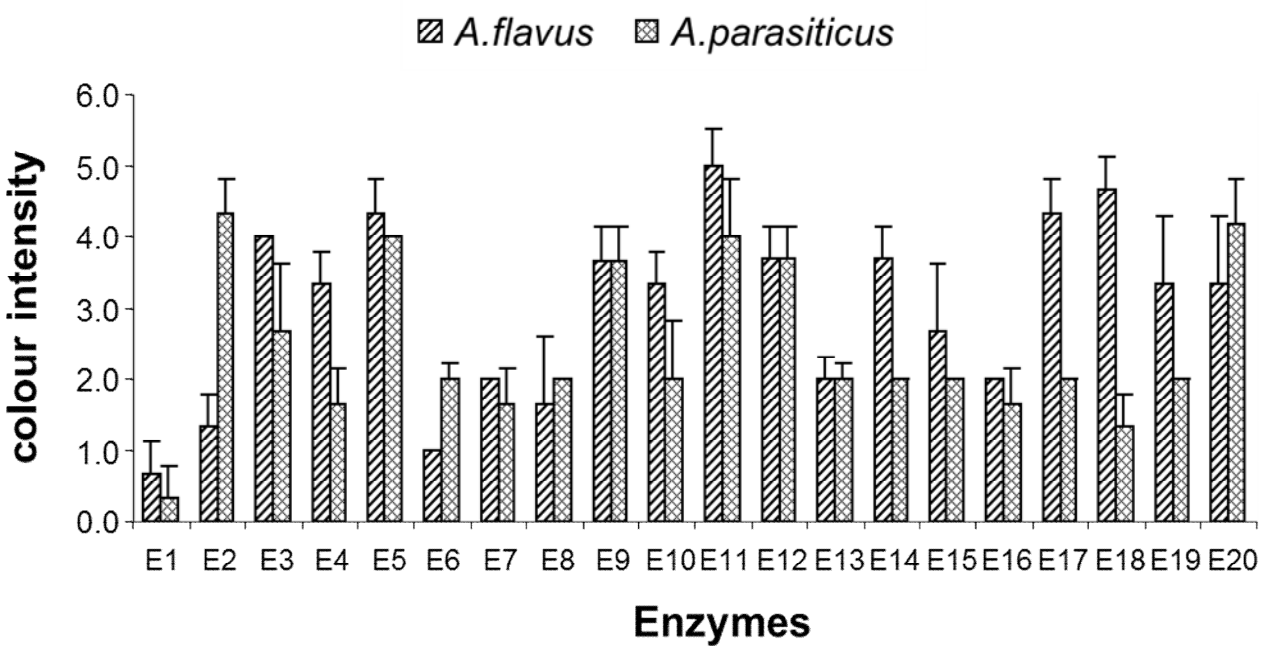

Figure 1. Alam et al.

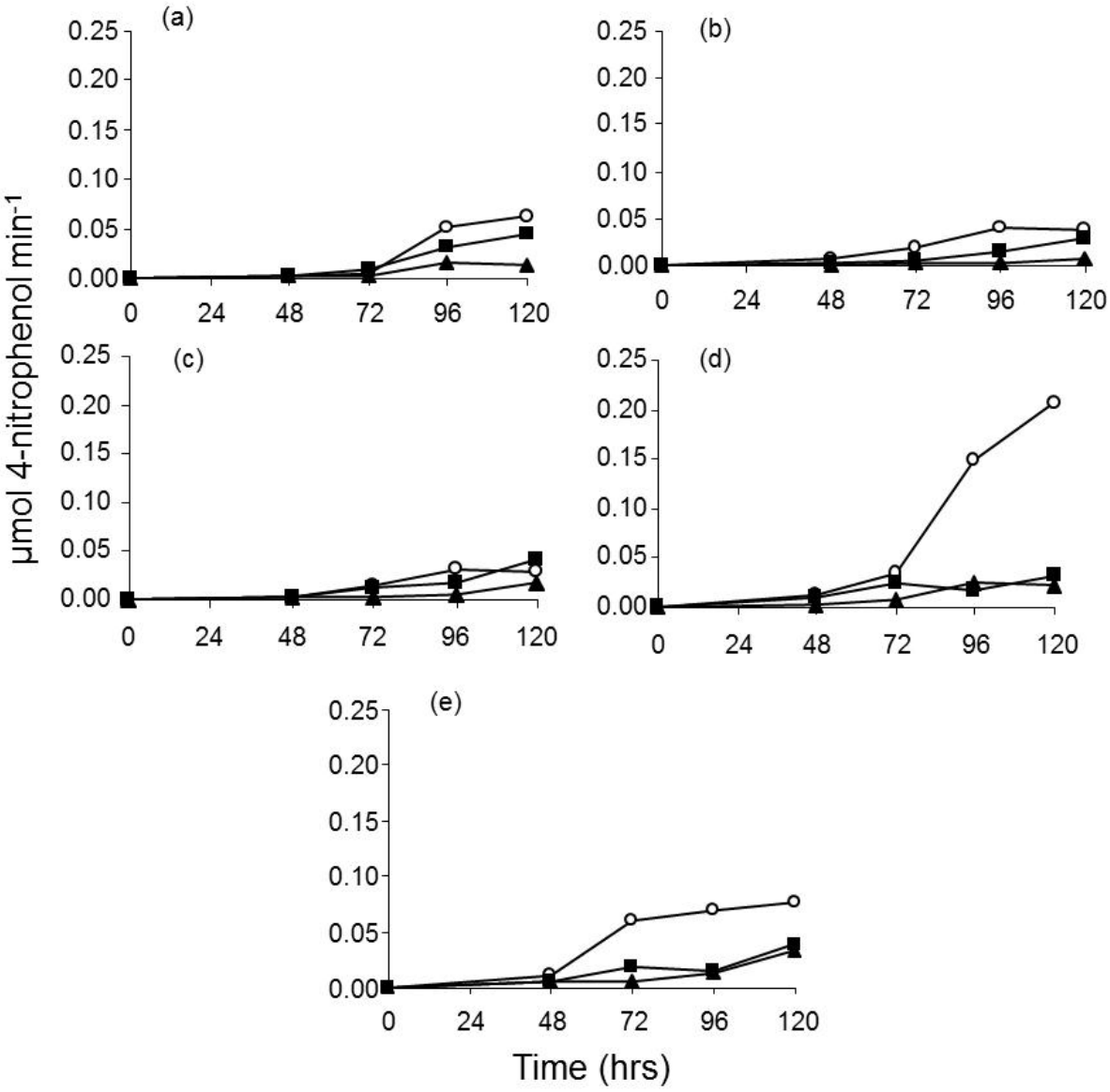

Figure 2. Alam et al.

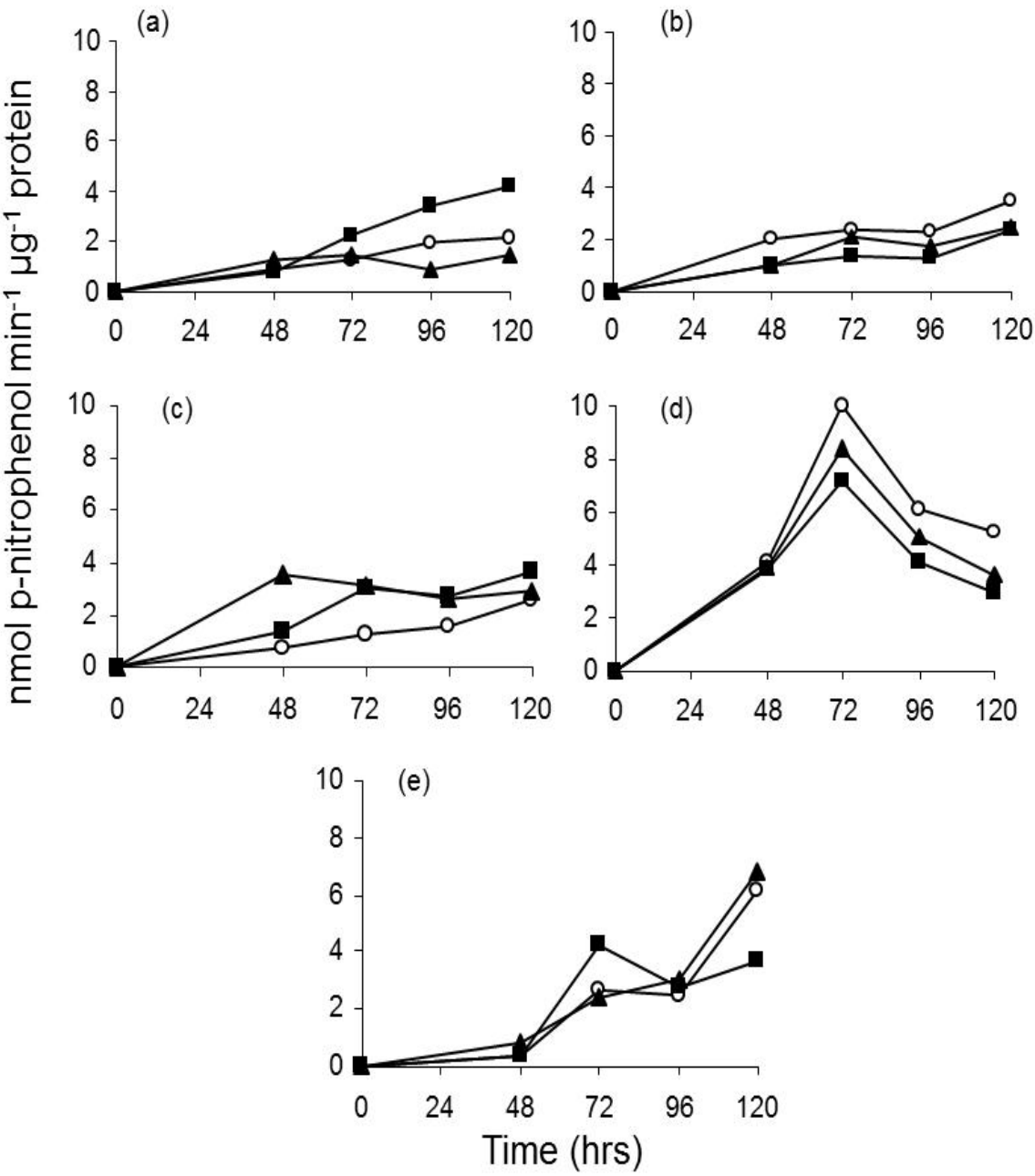

Figure 3. Alam et al.

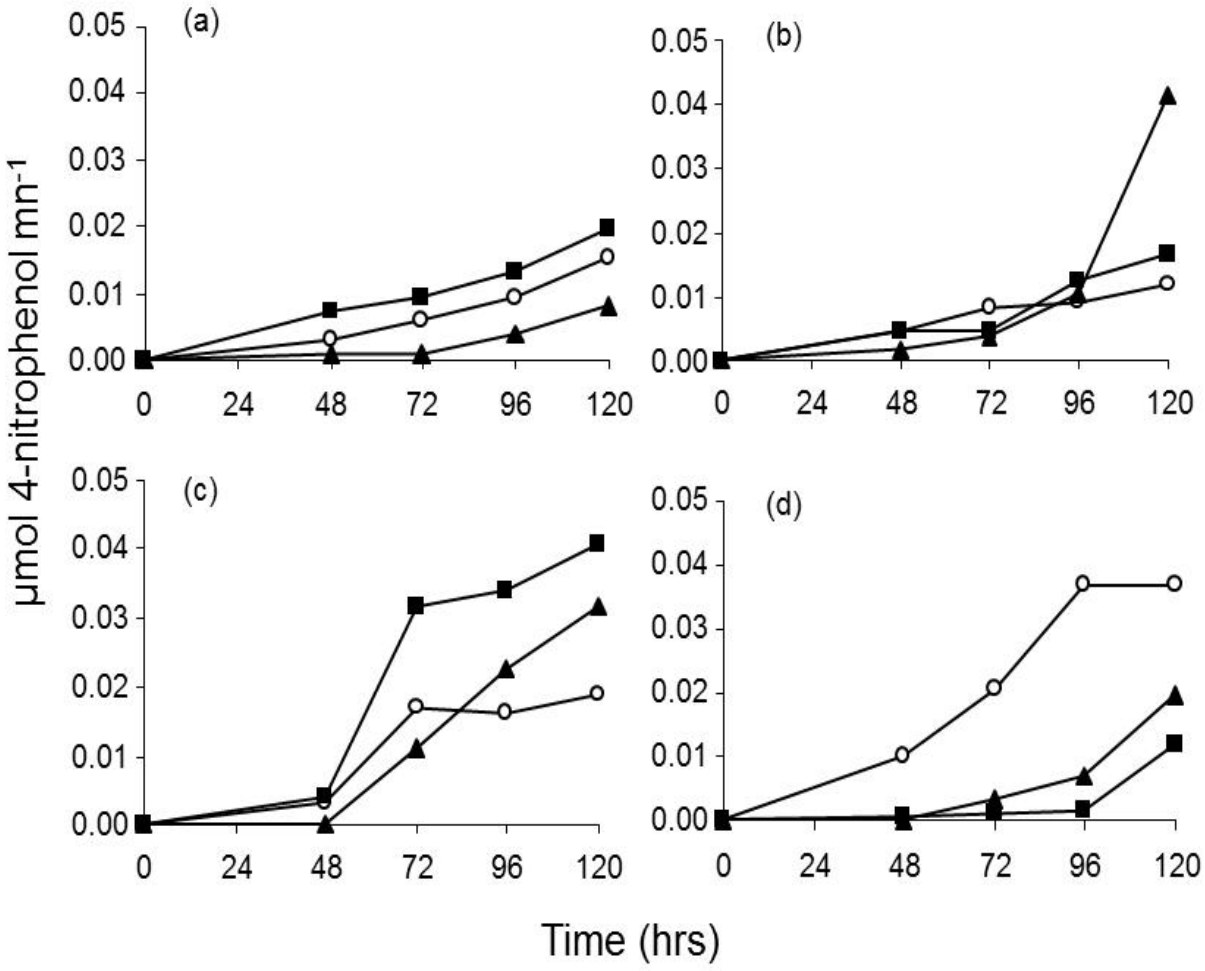

Figure 4. Alam et al.

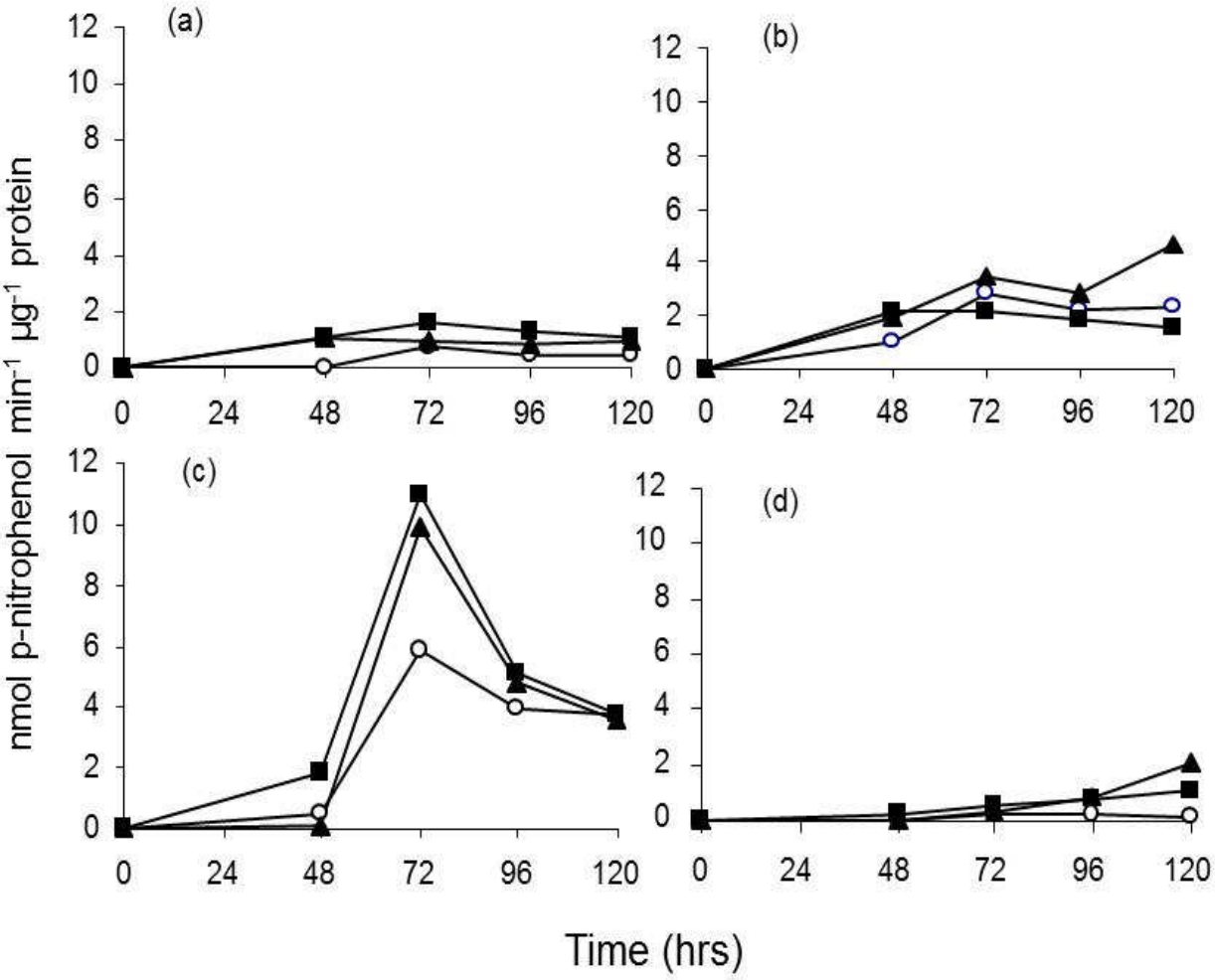

Figure 5. Alam et al.