

Research Article

Multiple non-polysaccharide-degrading enzyme production from solid state fermentation of *Aspergillus niger* AK10

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Abstract

Aspergillus niger strain AK10 was isolated from ingredients used for animal feed production. It was found to be capable of producing multi-enzymes required for animal feed supplements, namely phytase, xylanase, cellulase and mannanase. Except for mannanase, solid state fermentation of AK10 for production of the other three enzymes was found to be best done on RB medium which was composed of rice bran mixed with mineral salt solution [0.1% K₂HPO₄, 0.02% MgSO₄, 0.01% CaCl₂ and 0.25% (NH₄)₂SO₄] at a weight by volume ratio of 1:1. The solid medium for high mannanase production was SM medium containing soybean meal and mineral salt solution at 1:1 (w:v) ratio. The optimum temperature for growth of AK10 was at 35°C for 4 days. The mineral salts solution was found to be an important factor for mannanase activity. Each enzyme was characterized for the levels of optimum temperature and pH for the activity and stability. Efficiency of the crude multi-enzyme mixture in digesting grass was tested and found to help increase the metabolizable reducing sugar and protein by 3 and 13.5 folds, respectively. It also helped releasing the free absorbable phosphorus by 10 fold.

Keywords: *Aspergillus niger*, solid state fermentation, phytase, cellulose, xylanase, mannanase, Thailand.

Introduction

In current practice, enzyme supplementation in feed is common for enhancing nutrient consumption of animals. The enzymes used include general digestive enzymes, namely protease, amylase and lipase, etc [1]. However, the major raw materials for production of animal feed are from plant origin which contains high levels of non-starch polysaccharides. Most of these substances are in complex form and cannot be digested by the general digestive

enzymes of animal systems. Furthermore, some of these substances can form complex with protein, starch and essential minerals, and preventing them from being digested and absorbed by the epithelial cells [2]. This causes reduction in animal nutrient consumption. Moreover, the non-digested nutrients will, then be excreted as animal manure and creates unhealthy and unrepresentative environment for the animals [3, 4].

The major composition of plant polymers are cellulose and hemicellulose which constitute for structure and phytate which is a phosphate reservoir for plant use in energy production [2]. The major components in hemicellulose are xylan and mannan. Cellulose and hemicellulose are counted as roughage while phytate is done as anti-nutritional factor. Thus, the additions of cellulolytic enzyme or cellulase, hemicellulolytic enzymes; i.e. xylanase and mannanase, as well as phytate degrading enzyme or phytase, would help improving the nutrient consumption of the animals [5, 6, 7].

In this study, attempts have been made on production of multiple enzyme mixtures containing phytase, cellulase, xylanase and mannanase, for use as animal feed supplement. This was carried out using *Aspergillus niger* AK10 as a producing strain. A medium for solid substrate fermentation of AK10 has been developed. The effects of temperatures and pHs on their activities and stabilities of each enzyme were determined. The ability of the crude multi-enzyme extract in increasing the nutritional value of plant raw materials has been demonstrated.

Materials and Methods

Cultivation

Stock culture was maintained on Potato Dextrose Agar Slant. Solid state fermentation was done in a 100-ml bottle containing 10 gm of dry substrate mixed with 10 ml mineral solution composing of 0.1% K₂HPO₄, 0.02% MgSO₄, 0.01% CaCl₂ and 0.25% (NH₄)₂SO₄. After the media were autoclaved, they were inoculated with 1 ml of spore suspension in 0.05% Tween-80. Incubation was done at room temperature (~28°C) for 72 hours prior to crude enzyme extraction.

Enzyme extraction

The grown culture of solid state fermentation from 10 gm dry substrate was added with 40 ml of distilled water and left agitated at room temperature for 1 hr. The mixture was then pressed through cheese-cloth into a container. This suspension was centrifuged to separate the remained particles from the supernatant, which was collected as the crude enzyme extract.

Phytase Assay

Quantitative assay for phosphate released by phytase was done following Engelen *et al.* [8]. Briefly, the reaction mixture contained 2 ml of enzyme extract and 4 ml of substrate solution containing 0.84 gm sodium phytate in 90 ml acetate buffer, pH 5.5 [0.18 gm acetic acid (100%), 3.0 gm sodium acetate.3H₂O, 0.15 gm calcium chloride.2H₂O, 90 ml distilled water, adjusted to pH 5.5 with acetic acid and diluted to 100 ml]. The reaction was carried out at 37°C for 65 min and was stopped by adding 4 ml freshly prepared color reagent. The colour reagent consisted of 25 ml ammonium molybdate solution [10 gm ammonium molybdate. 4H₂O, 90 ml distilled water, 1 ml NH₃ (25%), adjusted to 100 ml with distilled water] mixed with 25 ml ammonium vanadate solution [0.235 gm ammonium vanadate added to 40 ml distilled water at 60°C followed by slow addition of 2 ml nitric acid and adjustment to 100 ml with distilled water] and with slow stirring, 16.5 ml nitric acid (65%). After cooling to room

temperature, the volume was adjusted to 100 ml with distilled water. The colour developed was measured at 415 nm. One unit of phytase activity was defined as the amount of enzyme that released 1 μ mole of inorganic phosphate in 1 min.

Mannanase Assay

This was done following Hossain et al. [9]. The reaction mixture, containing 0.5% locust bean gum, 50 mM phosphate buffer (pH 7.0) and suitably diluted enzyme solution in a total volume of 1 ml, was incubated at 50°C for 15 min. The reducing sugars formed were determined by the DNS method [10].

One unit of β -mannanase is defined as the amount of enzyme that releases 1 μ mol of reducing sugar equivalent to D-mannose per minute at 50°C.

Xylanase Assay

Xylanase activity was assayed using oat spelt xylan 1% solution as the substrate, as described by Bailey *et al.* [11] and the amount of reducing sugars released was determined by the method of Miller [10] using DNS reagent. One unit of enzyme activity was defined as 1 μ mol of xylose equivalent produced per minute under the assay conditions.

Cellulase Assay

Cellulase activity was assayed by measuring released reducing sugars from carboxymethyl cellulose (Sigma, USA) with 3,5- dinitrosalicylic acid [10]. The reaction mixture containing 0.9 ml of 1% (w/v) carboxymethyl cellulose in 50mM phosphate buffer, pH 6.8 and 0.1 ml of enzyme extract. The enzyme reaction was carried out at 50°C for 30 min, then, 1 ml of DNS was added to stop the reactions. The solution was incubated in a boiling water bath for 5 min for colour development and the absorbance was measured at 540 nm against the enzyme blank. One unit of cellulase was defined as the amount of enzyme required to release 1 μ mol reducing sugar as glucose equivalent per min under the above assay conditions.

Amylase Assay

α -Amylase activity was determined as per Bernfield [12]. The reaction mixture consisted of 1.5 ml of 1% soluble starch, 1.0 ml of 0.1 M phosphate buffer (pH 6.0) and 0.5 ml of crude enzyme extract. After 15 min of incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitrosalicylic acid (DNS) method [10]. The colour developed was read at 540 nm using spectrophotometer. Glucose was used as the standard. The blank contained 1.0 ml of 0.1 M phosphate buffer (pH 6.0), 1.5 ml of 1% starch solution and 0.5 ml of distilled water. One unit of amylase is defined as the amount of enzyme releasing 1 μ mol glucose equivalent per min under the assay conditions.

Protease Assay

Protease activity was determined as per Ason [13] using casein as substrate. In a 6.00 ml reaction mix, the final concentrations are 42 mM potassium phosphate, 0.54% (w/v) casein, 1.7 mM sodium acetate, 0.8 mM calcium acetate, and protease. Colour was developed by Folin & Ciocalteu's reagent and colourimetric measurement was done at OD₆₆₀. One unit will hydrolyze casein to produce colour equivalent to 1.0 mmole (181 mg) of tyrosine per minute at pH 7.5 at 37°C.

Digestion of grass and hay using crude AK10 enzyme extract

The digestion process was done by mixing 1 gm of grass or hay in a reaction mixture containing 18 ml of 0.05 M acetate buffer, pH 5.5, and 2 ml of crude enzyme. The reaction was incubated at 37°C for 1 hour. The increase in the amount of metabolizable reducing sugar and protein and the available phosphate for animal absorption were determined.

DNS Assay for Reducing Sugar concentration

This was done following the method of Miller [10].

Protein Assay

This was done following the method of Bradford [14].

Phosphorus assay

This was done following the method for phytase assay [8] with slight changes in the volume of the assay mixture. To 1.2 ml of sample, mixed in 0.8 ml of colour reagent and then measured for OD₄₁₅.

Results and Discussion***Phytase, cellulase and xylanase production of Aspergillus niger AK10 (AK10) on rice bran, wheat bran and soybean meal based media***

Solid substrate fermentation of AK10 was carried out on the media containing different concentrations of rice bran and/or soybean meal. Rice bran (RB) is a low priced agricultural by-product while soybean meal (SM) is widely used as a protein source in feed production. It was found that AK10 grew well in every media tested but produced the highest phytase, cellulase and xylanase in the medium containing only RB (Table 1). In the case of mannanase, its highest activity was obtained from the culture on medium containing only SM.

Table 1. Phytase, cellulose, xylanase and mannanase production from AK10 on rice bran and soybean meal.

Media *	Phytase U/ml	Cellulase U/ml	Xylanase U/ml	Mannanase U/ml
100% SM	0.2	15.9	45.3	11.3
50% SM + 50% RB	0.2	14.9	42.8	7.3
60% SM + 40% RB	0.2	14.3	37.7	7.4
40% SM + 60% RB	0.3	14.6	47.6	6.4
100% RB	0.3	16.9	52.3	4.1

*The media were mixed with mineral salt solution and incubated at room temperature (~28°C) for 72 hours.

Effect of temperatures and incubation period on the phytase, cellulase and xylanase production from AK10 on RB medium at various temperatures

Cultivation of AK10 in RB medium was carried out at various temperatures for 72 hr prior to determination of the three enzyme activities. It was found that the best temperature for production of the three animal feed enzyme was at 35°C (Table 2). At this temperature, production of each enzyme was determined at every 24-hr intervals and found that maximum phytase and cellulase production was reached at day-3 (Table 3).

Table 2. Phytase, cellulase and xylanase production from AK10 in RB medium at various temperatures.

Temperature(°C)	Phytase (U/ml)	Cellulase (U/ml)	Xylanase (U/ml)
30	0.3	14.6	52.3
35	0.5	18.9	67.7
40	0.4	14.5	68.6
45	0.1	0.8	40.8

Although production of xylanase at day-4 was higher than that at day-3, 3 days of cultivation was considered to be a suitable incubation period since phytase and cellulase production had started to decrease.

Table 3. Phytase, cellulase and xylanase production from AK10 in RB medium at 35°C at various time intervals.

Incubation time (days)	Phytase (U/ml)	Cellulase (U/ml)	Xylanase (U/ml)
0	0.0	0.0	0.1
1	0.0	2.3	6.1
2	0.4	7.4	22.8
3	0.6	18.9	67.7
4	0.5	17.9	78.9

Effect of mineral salt solution on the production of phytase, cellulase, xylanase and mannanase from AK10.

This was done to determine the importance of minerals towards the enzyme productivity of AK10. Cultivation was carried out in RB for phytase, cellulase and xylanase production and done in SM medium for mannanase production. Each medium was prepared in two sets; one with salt solution and one with distilled water. It was found that mineral salts only slightly enhanced the production of phytase, cellulase and xylanase (Table 4). On the contrary, the mineral salts were found to be an important factor for high mannanase activity.

Table 4. Effect of mineral salt solution on the production of phytase, cellulase, xylanase and mannanase from AK10.

Enzymes	Activities at day-4 (U/ml)	
	With salt solution	Without salt solution
Phytase ¹	0.52	0.52
Cellulase ¹	17.9	17.4
Xylanase ¹	92.4	93.4
Mannanase ²	11.3	9.0

¹ Crude enzyme extract from the AK10 culture on RB medium

² Crude enzyme extract from the AK10 culture on SM medium

Effect of pH and temperature on the activities of phytase, cellulase, xylanase and mannanase from AK10.

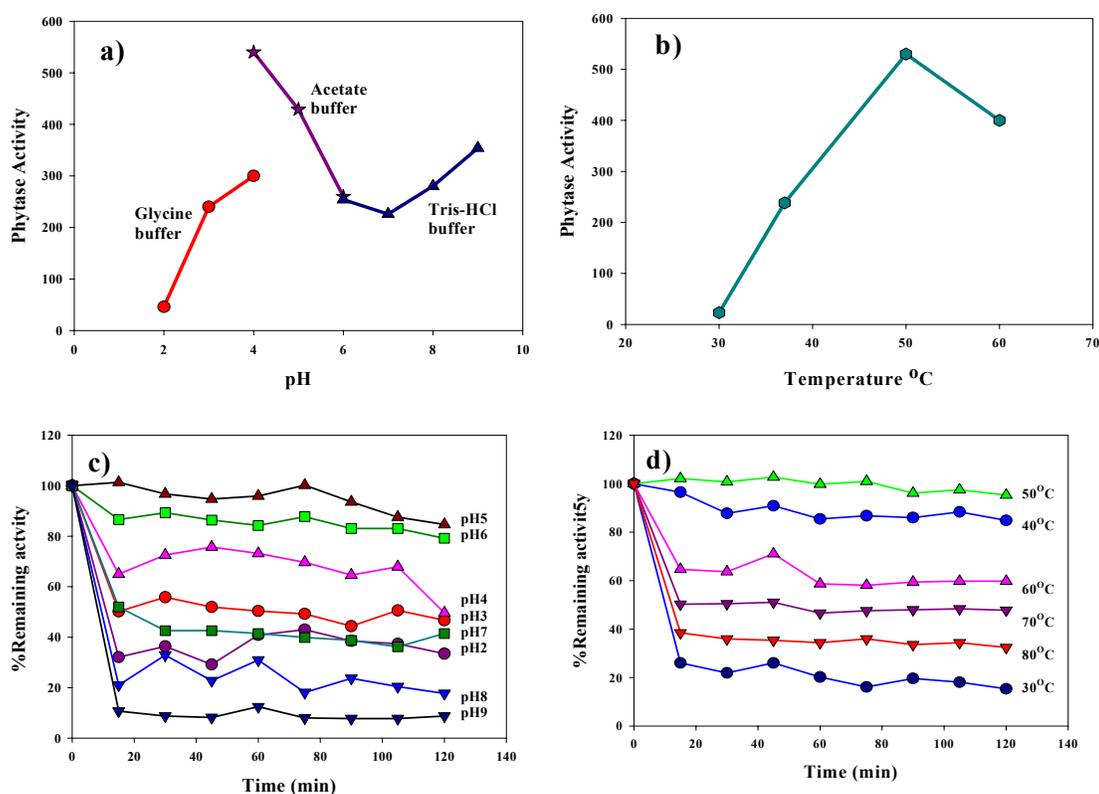
Effect of pH on the activity of enzymes was determined by adjusting the pH in the reaction mixture to various levels with different kinds of 0.1 M buffer; i.e. glycine buffer for pH 2-4, acetate buffer for pH4-6, phosphate buffer for pH6-8 and Tris-HCl buffer for pH8-9. The reactions were carried out at the temperatures as requested in standard assay method for each particular enzyme. In the case of the effect of pH on stability of the enzymes, each enzyme

was stored at different pH adjusted by using different kinds of buffer as above and samples were taken at various periods of time.

For the effect of temperature, the standard assay methods were performed at different temperatures; ranging from 30°C to 60°C. The effects of temperature on stability of enzymes were done by incubating each enzyme at a particular temperature and samples were taken at different time intervals for determination of the remaining activity.

Phytase

The highest phytase activity was obtained when the assay was carried out at pH4.0 and at the temperature of 50°C (Figures 1a,b). The enzyme was highly stable at pH5.0 and 6.0 as its activity remained higher than 80% after 2 hr storage (Figure 1c). At pH 4.0, its activity dropped to around 60% during the first 15 minutes and then retained until 100 min and dropped again to around 50% at 120 min. The activity remained less than 60% when stored at pH 3.0 and 7.0, less than 40% when did at pH 2.0 and 8.0 and less than 10% when did at pH 9.0. In terms of temperatures, AK10 phytase was highly stable at 40°C and 50°C since its activity remained higher than 85% after 2-hr incubation (Figure 1d). Its activity dropped to around 20-70% when stored at other temperatures.



Each line on fig. c) and d) represent the remaining activity during storage at the indicated pH or temperature.

Figure 1. Effects of pH (a) and temperature (b) on the activity and stability of AK10 phytase.

Cellulase

The maximum cellulase activity was obtained when the reaction was carried out at pH 5.0 and at the temperature of 60°C (Figure 2a,b). AK10 cellulase was found to be highly stable at pH6.0 where the activity remained unchanged during the first 90 min and then dropped by around 20% at 120 min of incubation (Figure 2c). When stored at pH 4.0, 5.0 and 7.0, the activity dropped by around 20% during the first 15 min and then did by less than 6.5% during the next 105 min. The activity dropped to 40% after 15 min at pH8.0 and did to less than 20% at pH 9.0, 3.0 and 2.0. In the case of temperature, it was activated during the first 15 min when incubated at 40°C, 50°C and 60°C, and then stayed unchanged until the end of 120 min (Figure 2d). Incubation at 70°C, 80°C and 30°C caused reduction in the cellulase activity down to approximately 80%, 68% and 48%, respectively, during the first 15 min and then stayed relatively unchanged through to the end of 120 min.

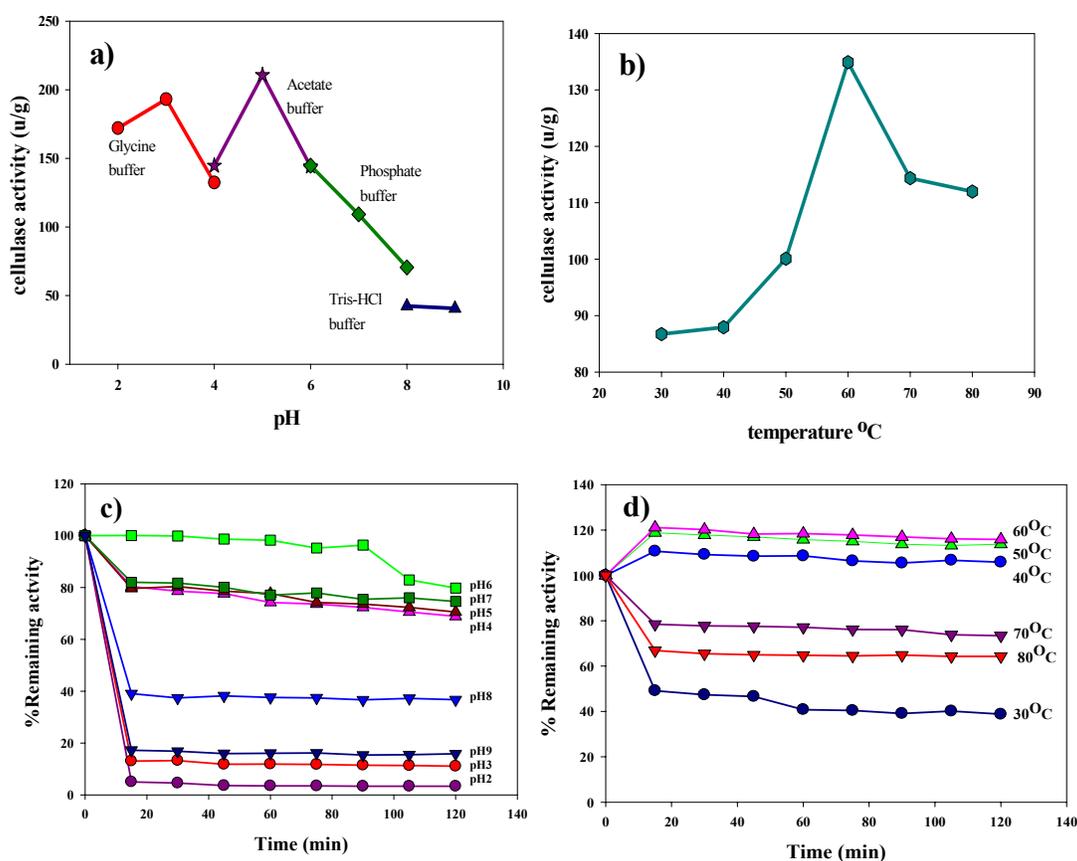


Figure 2. Effect of pH (a) and temperature (b) on the activity and stability of AK10 cellulase.

Each line on fig. c) and d) represent the remaining activity during storage at the indicated pH or temperature.

Xylanase

The xylanase activity was at the highest level when the assay was carried out at pH 6.0 and at the temperature of 45-50°C (Figure 3a,b). AK10 xylanase activity remained higher than 95% at pH 7.0 throughout the 2-hr storage (Figure 3c). At pH 6.0, 5.0 and 8.0, its activity dropped

to around 76%, 48% and 36%, respectively, during the first 15 minutes, and then remained unchanged until the end of 2 hours. At pH 3.0-4.0, the activity remained at less than 10% after

15 minutes of incubation. In terms of temperatures, AK10 xylanase was highly stable at 50°C to 60°C as the activity remained around 95% to 100% during the 2 hr-storage. At 37°C and 70°C the activity after 2 hr storage was at 82% and 78%, respectively (Figure 3d).

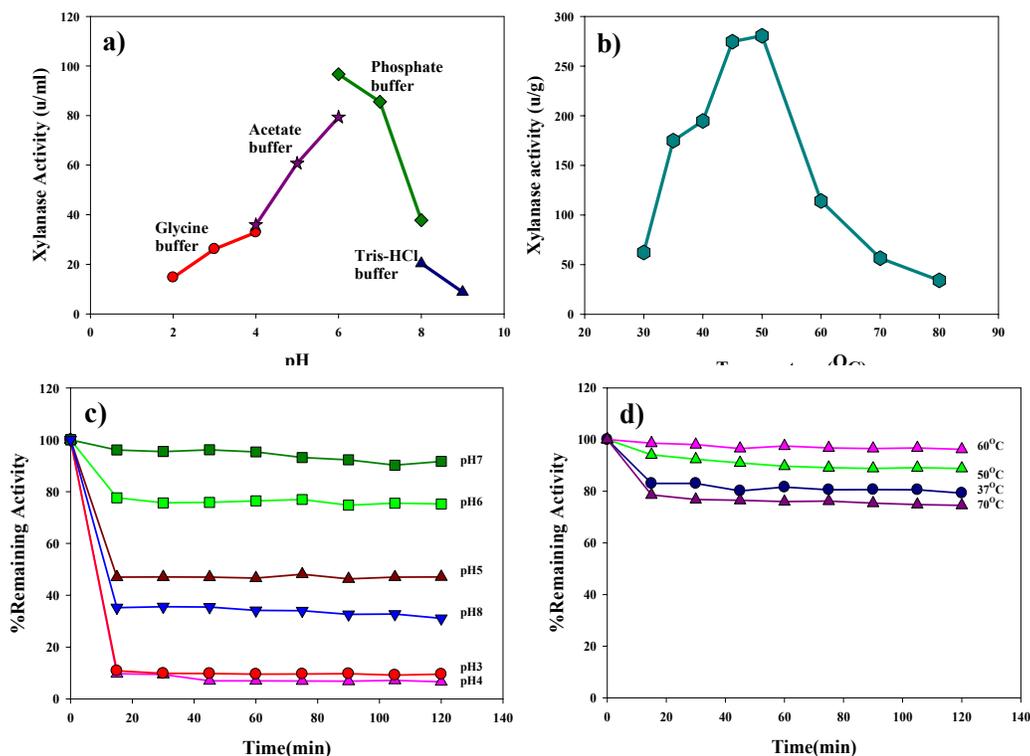


Figure 3. Effect of pH (a) and Temperature (b) on the activity and stability of AK10 xylanase.

Each line on fig. c) and d) represent the remaining activity during storage at the indicated pH or temperature.

Mannanase

AK10 mannanase worked best at pH ranging from pH 5.0 to pH 7.0, and at temperatures of 50-60°C (Figure 4a,b). It was found to be highly stable at pH 5.0, 6.0 and 7.0 (Figure 4c). Throughout 2-hr storage, the activity was activated slightly and remained higher than 100% when stored at pH5.0, remained unchanged when stored at pH 6.0 and maintained at around 90% of the original activity when stored at pH 7.0. At pH 8.0, pH 4.0 and the other testing pH levels, the activity dropped to about 55%, 40% and 20% of the originals when stored, respectively, during the first 15 min of storage and then remained unchanged until the end of 120 min. In terms of temperatures, the activity dropped to around 40% after 15 min incubation at 30°C (Figure 4d). The AK10 mannanase activity remained higher than 80% when stored at 40°C, 50°C and 60°C; and gradually reduced to around 70% when stored at 70°C and 80°C.

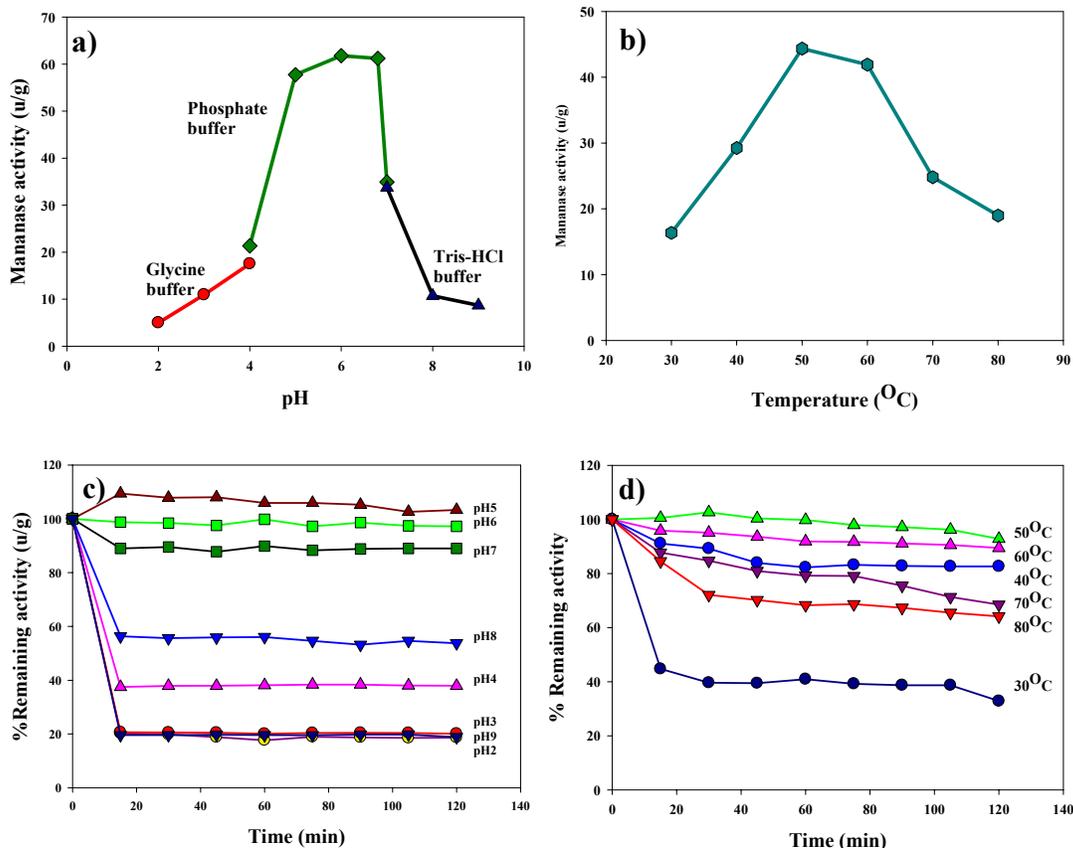


Figure 4. Effect of pH (a) and temperature (b) on the activity and stability of AK10 mannanase.

Each line on fig. c) and d) represent the remaining activity during storage at the indicated pH or temperature.

Efficiency of AK10 crude enzyme extract in digestion of grass and hay

The digestibility of AK10 crude enzyme extract toward plant raw material was determined in order to reveal the potential use as animal feed supplement. The crude enzyme extract was found to contain various enzyme activities, namely, phytase at 0.55 U/ml, xylanase at 67.4 U/ml, cellulase at 17.2 u/ml, mannanase at 4.1 U/ml, protease at 9.2 U/ml and amylase at 0.3 U/ml. From digesting 1 gm of fresh grass, the reducing sugar increased by 3 folds; i.e. from 200 $\mu\text{g/gm}$ to 600 $\mu\text{g/gm}$; the protein content was increased by 13.6 folds; i.e. from 7.7 $\mu\text{g/gm}$ to 104.5 $\mu\text{g/gm}$; and the free absorbable phosphate was increased by 10 folds; i.e. from 0.6 mg/gm to 6.0 mg/gm.

In the digestion of 1 gm of dried hay in 18 ml of acetate buffer, pH 5.5, with 2 ml of crude enzyme, the amount of absorbable reducing sugar increased markedly from 31.5 $\mu\text{g/gm}$ to 712.9 $\mu\text{g/gm}$; the protein content was increased by 8 folds; i.e. from 21.2 $\mu\text{g/gm}$ to 167.4 $\mu\text{g/gm}$; and the free absorbable phosphate was increased from 1.6 mg/gm to 7.4 mg/gm. This revealed that the newly developed enzyme product and production process could be used as feed supplement to increase the nutrient consumption for animals.

Table 5. Summary of the levels of pH and temperature that affected the activity and stability of multienzymes from AK10.

Enzymes	The conditions of pH and temperature ¹							
	Facilitating the maximum activity		Retaining the activity after 2-hr incubation at:					
			≥ 100%		80-99%		60-79%	
	pH	°C	pH	°C	pH	°C	pH	°C
Phytase	4	50	5	50	6	40	4	60
Xylanase	6	45-50	7	60	-	37-50	6	70
Cellulase	5.5	60	-	40-60	7	-	4-5 ²	70-80
Mannanase	6-7	50-60	5-6	50	7	40-60	-	70-80

¹ The levels of pH and temperature which are optimum for the activity and that can maintain the activity, after 2-hr storage, at higher than 60% of the original activity at time 0.

²The activities remained from storage at pH 4 and 5 were 78-79%

Conclusions

A strain of *Aspergillus niger* was isolated from raw materials used for animal feed production. It had been preliminary tested for potential in producing some of the animal feed enzymes. In this study, attempts have been made on developing a suitable process for production of multienzymes that can be readily used as animal feed supplement. One of the advantages of having this strain of fungi as enzyme producer is that this fungus is generally accepted as GRAS (= Generally Regarded As Safe) which is widely used as producing strain for many industrial process. Furthermore, since it is a fungus, it can be cultivated by solid substrate fermentation (SSF) which is a simple process that the technology can be easily transferred to farmers when it is developed.

The first attempt was development of a low cost solid medium suitable for AK10 growth and enzyme production. Agricultural wastes or by-products are generally considered the best substrates for SSF process [15]. The agricultural by-products tested in this work were rice bran and soybean meal which are generally used as ingredients in animal feed formulation. They are plant materials which contain a lot of indigestible substances for animal digestive system, including phytin, cellulose, xylan and mannan. AK10 was found to grow well in these raw materials but only produce each type of enzymes at different levels. Nonetheless, growth of the fungus was not quantitatively measured as it grew mixed through the solid substrate.

In terms of phytase, xylanase and cellulase, AK10 was a good producer when cultivated on RB medium which contained only rice bran with 50% moisture from mineral salt solution. However, mineral salts did not showed much effect on the activities of these three enzymes. On the contrary, the salts solution was found to enhance the mannanase activity since the absence of it caused reduction in the hydrolysis of mannan. Mannanase was also found to produce best in SM medium. This was not unexpected as soybean was a good source of mannan [16].

The levels of xylanase, cellulose and mannanase activities produced by this strain AK10 of *A.niger* were considered higher than some of those produced by other strain of the same species of fungi [17]. Nonetheless, it was difficult to compare the phytase productivity with that from other organisms since the unit definition and the assay methods were varied [18]. The effect of pH and temperature on the activities and stabilities of the four enzymes were summarized in Table 5, which showed that all the tested enzymes produced from AK10 were

found to be stable towards high temperature. This would allow them to retain the activity without much loss during the production process. The activities were also found to be stable at pH 5-7 indicating that they should be stable in the digestive tract of monogastric animals which has the pH condition of around 6.0 -7.0 [19].

Digestion of fresh grass and dried hay at room temperature using the crude enzyme extract from AK10 culture on RB medium was shown to successfully increase the levels of protein, reducing sugar and inorganic phosphate contents, available for animal nutrient absorption. The crude enzyme for this experiment was prepared from RB medium regardless of low mannanase activity obtained in order to simplify the enzyme preparation process. This would allow the technique to be transfer to farmers with no complication. Alternatively, the production of multiple feed enzymes using the newly developed media is also possible. However, experiments on scaling-up process as well as the product formulation would have to be carried out.

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