

## Clean Production of Xylanase from White Corn Flour by *Aspergillus fumigates* F-993 under Solid State Fermentation

M. Fadel, Abeer A. Keera, Shadia M. Abdel-Aziz and T. Kahil

Department of Microbial Chemistry,  
Genetic Engineering and Biotechnology Division, National Research Center, Dokki, Giza, Egypt

**Abstract:** Ten, non-producing mycotoxin, fungal strains, belonging to *Aspergillus* spp., *Trichoderma* spp. and *Penicillium* spp., were screened for clean production of xylanase using white corn flour (WCF) by solid state fermentation. *A. fumigates* F-993 showed promising xylanase production activity. Culture conditions, i.e., moisture content, initial pH value, temperature, incubation time were optimized to be 60 % (v/w), 3.5, 32°C and 48 hrs, respectively. Solid: solvent ratio of 1:10 was more suitable for enzyme extraction as well as a saline solution of 0.3 M sodium chloride, was more efficient to elute enzyme from fermented substrate. Under the above conditions 720 U/g original WCF was obtained. The enzyme is active at a wide range of pH values, from 4.0-6.5 and a temperature range from 35-70°C. Forty percent (v/v) of cold acetone was more efficient for precipitating the enzyme than ethanol or ammonium sulfate, as 32% of total protein contained 86 % of total xylanase activity was obtained with a specific activity of 38U/mg protein. The precipitated enzyme involved considerable activities of cellulases and glucoamylase. The precipitate was applied for hydrolyzing 15% (w/v) of delignified sugarcane bagasse, wheat straw and rice straw, where reducing sugars reached 46,44,52 g l<sup>-1</sup>, containing 16,15 and 12% (w/w) glucose in the enzymatic hydrolysates of wheat straw, rice straw and sugar cane bagasse, respectively. The remainder fermented substrate, corresponded to 47% (w/w) from the original WCF, was found to be pounded with xylanase, cellulases and glucoamylase and contained 17% crude protein which can be used for fortification at concentrated rations in both poultry and ruminant feed.

**Key words:** *Aspergillus fumigates* F-993 • Optimization • Solid state fermentation • White corn flour  
• Xylanase

### INTRODUCTION

Xylan is a major structural polysaccharide of plant-cell walls being the second most prevalent in nature after cellulose. It is a heterogeneous polymer constituted primarily by a linear B-(1,4)-D-xylose backbone, which is partially acetylated and substituted at different degrees by a variety of side chains, mainly *a*-D-glucuronosyl and *a*-L-arabinosyl units. Due to its structural complexity, several hydrolyses are required for complete xylan degradation. The key enzyme in this process is endo-B-(1, 4)-xylanase (EC 3.2.1.8), which cleaves xylan backbone to xylooligosaccharides [1]. Xylanolytic enzymes have applications in conversion of lignocellulosic materials to chemicals and fuels, animal feed preparations [2], food industries and textile industries, nutritional value improvement of silage and

green feed, food industry and as bleaching agents in the pulp and paper processing. Xylanase production has been reported for bacteria, actinomycetes and fungi. Filamentous fungi which produce xylanases are attracting greater attention than bacteria and yeast because they are particularly interesting from an industrial point of view, due to much higher secretion of xylanolytic enzymes into the medium [3]. Although the industrial uses of xylanase include, petrochemical production [4, 5] and as a supplement to animal feed, new uses of xylanase are expanding to include processing plant fiber sources, such as flax and hemp, clarification of juices along with pectinases and for the preparation of dextrans for use as food thickeners [2].

The majority of the industrial enzymes in the market are produced by submerged liquid fermentation (SmF).

**Corresponding Author:** Shadia M. Abdel-Aziz, Microbial Chemistry Department, Genetic Engineering and Biotechnology Division, National Research Center, Dokki, Giza, Egypt.

However the solid-state fermentation (SSF) technique can improve the yield and the economy of enzymes production [6]. Solid-state fermentation refers to the growth of microorganisms on solid materials without the presence of free liquid. Solid-state fermentation mimics the natural environmental conditions and habitat for some microorganisms [7]. Filamentous fungi are the most commonly used microorganisms in SSF because they are able to grow on solid materials with low water contents [8]. Filamentous fungi are the most widely exploited because of their ability to grow on complex solid substrates and production of a wide range of extracellular enzymes. Filamentous fungi have been widely used to produce hydrolytic enzymes of industrial importance, including xylanases, whose levels in fungi are generally much higher than those in yeast and bacteria. Solid-state fermentation typically uses agricultural residues such as wheat straw, wheat bran, rice straw and cotton stalks. Other advantages of SSF are simpler product recovery and reduction of bacterial contamination due to low moisture levels [8]. The cost of an enzyme is one of the main factors determining the economics of a process. Reducing the costs of enzyme production by optimizing the fermentation medium and the process is the goal of basic research for industrial applications [3]. Recovery of enzyme is also an important aspect of solid-state-fermentation technology. Applications of xylanases can be found in the food and beverage industries, feedstock improvement and the quality improvement of lignocellulosic residues. On an industrial scale, xylanases are produced mainly by *Aspergillus* and *Trichoderma* spp.

The production of high yields of an enzyme using SSF requires optimal environmental conditions to promote maximum growth of the microorganism and increased production of the enzyme. The environmental conditions include incubation temperature, moisture content [9], depth of substrate and the length of time for fermentation [10]. Variety filamentous fungi have been reported to produce xylanases. A large number of mesophilic and thermophilic fungi, e.g. several different species of *Aspergillus* have been reported to produce xylanases, including *A. niger*, *A. ochraceus*, *A. oryzae*, *A. awamori*, *A. tawamarii*, *A. fumigatus* [11-15], *Trichoderma* strains [6, 16, 17], *P. funiculosium* and *Penicillium janczewskii* [7, 18]. Successful application of enzymes depends on optimization of production conditions, which can directly result in cost reduction. A wealth of information is available on medium optimization for xylanase production but studies on xylanase recovery from optimized medium are scarce. Thus, the aim of this study was to investigate the effect of culture conditions on production of xylanase

by *A. fumigatus* F-993 under solid state fermentation and optimal extracts for recovery of the enzyme. The crude extract was examined for hydrolysis of some agro-industrial wastes to be used in animal feed.

## MATERIALS AND METHODS

**Microorganisms and Culture Conditions:** Ten non mycotoxins producing fungal isolates namely *Aspergillus niger* F-119 (F1) *Aspergillus niger* F-93 (F2), *A. oryzae* F-236 (F3), *A. fumigatus* F-993 (F4), *Trichoderma harezinum* F416 (F5), *T. reesei* F-418 (F6), *T. vitide* F-127(F7), *T. viride* F-321(F8), *T. konogii* Fk-77 (F9) and *Penicillium finculosium* NRCE 629 (F10) obtained from Microbial Chemistry Lab. National Research Center, Dokki, Giza, Egypt, were screened for production of xylanase from white corn flour (WCF) by solid state fermentation maintained on potato dextrose agar slants at 30°C for 72 hrs. The spore suspensions of 72 hrs old-cultures were prepared by adding 10 ml of sterilized water to slant cultures and the surface was gently rubbed with a sterilized wire loop. Enzyme production was studied in 250 ml Erlenmeyer flasks containing 5g of WCF (12 % moisture) moistened to 50 % (v/w) with distilled water. One milliliter of spore suspension ( $10^6$  spores) was used as inoculums. The cultures were incubated at 30°C for 3 days. At end of the incubation period fifty ml distilled water were added to each flask, blended and shaking at 150 rpm for 30min, harvested by filtration, the filtrates were saved as sources of crude extracellular enzymes. The selected fungal strain was incubated for 120h at 30°C and culture was taken at interval 24 hrs to detect the optimum incubation period for xylanase production. WCF was moistened to different moisture levels, i.e. 35, 40, 45, 50, 60, 65, 70, 75 and 80% (v/w) under the optimum incubation period to determine the more suitable moisture content for enzyme production. 0.1 M sodium phosphate buffer and 0.1 M glycine buffer were used for adjusting the initial pH of fermentation medium (from 3 to 8) to study the effect of pH on the enzyme secretion. The fungus was incubated under different temperatures (25, 30, 35, 40 and 45°C) to study the effect temperature on the enzyme production.

**Protein Enzyme Precipitation:** Different concentrations of ammonium sulfate, i.e., 20, 40, 60 and 80% (w/v) were used to precipitate the enzyme protein. Each ammonium sulfate treatment was added slowly to a 15 ml centrifuge tube containing 5 ml of the supernatant culture solution (pH 4.0). The tubes were agitated for 15 s in a vortex at room temperature and then refrigerated overnight. The protein precipitate was collected by centrifugation

under cooling (6000 x g for 20 min at 4°C) and then dissolved in 0.05 M acetate buffer (pH 5.5) up to the initial volume (5ml). Similarly different cold ethanol or acetone concentrations were employed for protein enzyme precipitation. The pH of the supernatant was adjusted with 0.1 M acetate buffer to pH 4.0. The cold ethanol or acetone was slowly added to the culture supernatant under agitation (200 rpm) at 4°C. After the addition of ethanol or acetone, the agitation was stopped. After 30 min the mixture was centrifuged (2000 rpm) for 10 min at 4°C. The obtained pellets were re-suspended in 0.05 M acetate buffer pH 5.5 at room temperature (~28°C). The total protein contents were separately determined in the supernatant, in the pellet and the initial samples. Necessary enzymatic activities were then determined.

**Enzymatic Assays:** Xylanase activity was determined by measuring released reducing sugars by the dinitrosalicylic acid method [19] using D-xylose as the standard. The enzyme assay was carried out at 50°C, using 1.0% (w/v) birch wood xylan in 50 mM acetate buffer, pH 5.4, as substrate. An enzyme unit (U) is the amount that releases 1 µmol of reducing sugars per min under standard assay conditions. Filter paper (FPase) and carboxymethylcellulase (CMCase) activities were assayed in acetone recovery fraction dissolved in 0.05 M sodium phosphate buffer pH 5.0 according to the method described by Mandels *et al.* [20]. The enzyme was assayed by the method of Bernfeld [21] as described by Pestana and Castillo [22] using soluble starch as substrate. One unit of enzyme activities was expressed as one mg of glucose liberated in one minute.

**Protein Determination:** The amount of total protein was determined according to the Coomassie blue method described by Ohnisti and Barry [23] using bovine serum albumin as a standard protein.

#### **Determination of Enzyme Properties:**

##### **Effect of pH on Xylanase Activity and Stability:**

The activity of the crude xylanase was measured in pH range of 2.5 to 8.0 at 50°C for 10 min using 0.05 M sodium phosphate buffers. The stability of xylanase was tested by incubating the enzyme solution in appropriate buffer at 50°C for a period of 4 hrs. Aliquots were withdrawn at different times and cooled prior to activity measurements at pH 5.5 and 50°C. For determination of enzymatic properties, the activity corresponding to 470 U g<sup>-1</sup>(365 Uml<sup>-1</sup>) of original white corn flour was set as 100%.

**Effect of Temperature:** The crude xylanase precipitated by acetone (dissolved in 0.05 M sodium phosphate buffer, pH 5.5) was incubated with the substrate at different temperatures ranging from 30 to 80°C for a period of 10 min followed by the estimation of the enzyme activities. For determining the thermostability, the enzyme dissolved in buffer solution pH 5.5 was incubated for 4 hrs at different temperatures and then cooled prior to measure the residual activity under optimum temperature resulted from the above determination (50°C).

**Effect of Substrate Concentrations:** Different concentrations of xylan slurries ranging between 2.5 to 20% (w/v) were used for the determination of effect of substrate concentration on the xylanase activity. The reaction mixtures were incubated at 50°C for 10 min.

##### **Hydrolysis of De-lignified Lingocellulosic Wastes:**

Sugar cane bagasse, rice straw and wheat straw were de-lignified by alkali-treatment according to Sidhu and Sandhu [24]. The de-lignified substrate was air dried and sized to 40 meshes. Hydrolysis was conducted in 250 ml conical flasks containing, 15 % (w/v) slurry of de-lignified substrate in total volume 100 ml 0.05 M sodium phosphate buffer pH 5.5 containing 0.01% toluene. The flasks were incubated at 50°C in shaking water bath. Samples were withdrawn at 4h intervals. Total reducing sugars was determined with DNS [19] and glucose was determined with glucose oxidase-peroxidase kits.

**Analysis of Residual Fermented Corn:** At the end of fermentation period and after the enzyme extraction, the remainder fermentation mass (Fungal biomass with the unutilized part of corn) was air dried till constant weight. Crude protein was measured by micro-Kjeldahl method described by A.O.A.C. [25], total carbohydrates according to Dubois *et al.* [26] and the moisture % was determined after oven drying at 105°C to constant weight.

**Preparation and Enzyme Assays:** The air dried solid fermented substrate of *A. fumigates* F-993 was powdered. Dry powder 0.5 g was added into centrifugation tubes, added 10 ml of 1 % enzyme substrate for each then incubated at 50°C in shaker water bath for 30min., centrifuged at 8,000 x g for 5 min and the liberated reducing sugars were determined for enzyme activities of xylanase, cellulase (FPase and CMCase) and glucoamylase.

## RESULTS AND DISCUSSION

### Screening of Fungal Strains for Xylanase Production:

Selection of microorganism comes on the top when need to produce enzymes with high activity and economic. Fig. 1. illustrates that *A. fumigatus* F-993 have advantages than other tested fungal strains in xylanase activity production, growing on WCF under SSF system as produces 560 U/g original substrate, so was chosen for further studies to optimize the culture conditions and characterization of xylanase enzyme. It has been reported that a large number of mesophilic species of *Aspergillus* have been reported to produce xylanases, including *A. niger*, *A. oryzae*, *A. fumigatus* [11-15, 27], *Penicillium funiculosum* and *Penicillium canescens* [7, 28] and *Trichoderma harzianum* F-418 [29]. For production of high levels of any enzyme, optimizing the growth parameters is of prime importance in industrial enzymology. Of these parameters are the initial pH of the medium, temperature, carbon and nitrogen sources have been reported to strongly influence many enzymatic systems by affecting the transport of a number of chemical products and enzymes across the cell membrane [30].

### Optimization of Culture Conditions:

**Effect of Incubation Period:** Culture conditions were optimized for optimum production of xylanase by *A. fumigatus* F-993. Maximum production of xylanase was recorded after 48h of growth (Fig 2). Further incubation was not correlated with increase in the level of enzyme production probably due to inactivation or hydrolysis by secreted proteases of fungi. This trend was observed by Park *et al.* [31] and Shah and Madamwar [32].

The fact that the organism grows and produces maximum xylanase activity in short incubation time will offer significant advantage in reducing the risk of contamination as well as from economic point of view. Xylanase activity reached to a maximum value (680 U/g) within a period of 48-60 hours and then decreased when *A. niger* was cultivated on wheat bran [33]. The yield of xylanase by *Chaetomium cellulolyticum* [34] and *Penicillium funiculosum* reached a maximum at 6 days [7], whereas, *Melanocarpus albomyces* IIS-68 achieved maximum production after 8 days [4]. Growth and enzyme synthesis was maximum after 3 days fermentation of *A. niger* using starch wastes [35]. High-level xylanase was obtained after 48 hrs when new isolate of *T. harezianum* F-416 was grown on sorghum flour under SSF [6].

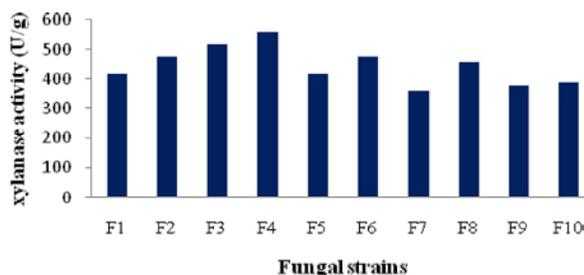


Fig. 1: Xylanase production by different fungal strains cultured on white corn flour, 50% moisture (v/w) under SSF at 32°C.

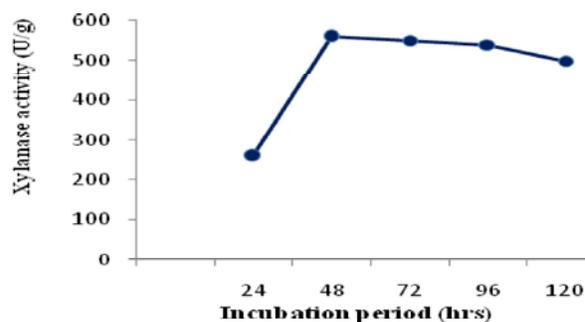


Fig. 2: Effect of incubation time on enzyme production by *A. fumigatus* F-993 cultured on WCF, 50% moisture (v/w) under SS at 32°C.

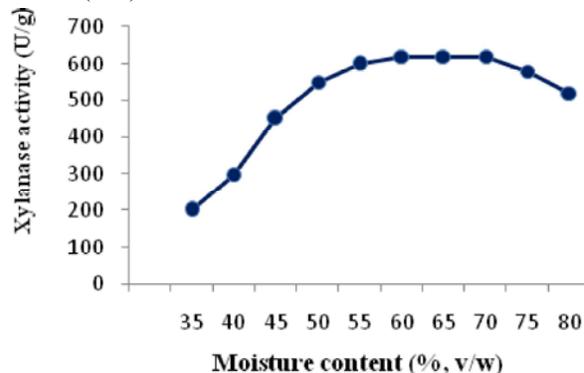


Fig. 3: Effect of moisture content in white corn flour on xylanase production by *A. fumigatus* F-993 after 48 hrs of growth at 32°C by SSF.

**Effect of Moisture Contents:** Initial moisture content is one of the key factors influencing xylanase production. The highest xylanase activity (680 U/g) was achieved when the moisture content was between 60-70 % (v/w) (Fig. 3). It seems that the wet conditions cause swelling of the substrate and facilitates utilization of the medium by the organism. If the substrate is too moistened, the substrate porosity decreases which prevents oxygen penetration, while at very low moisture levels no growth of the organism will occur [6]. Cell growth and oxygen consumption rate increased in conjunction with an

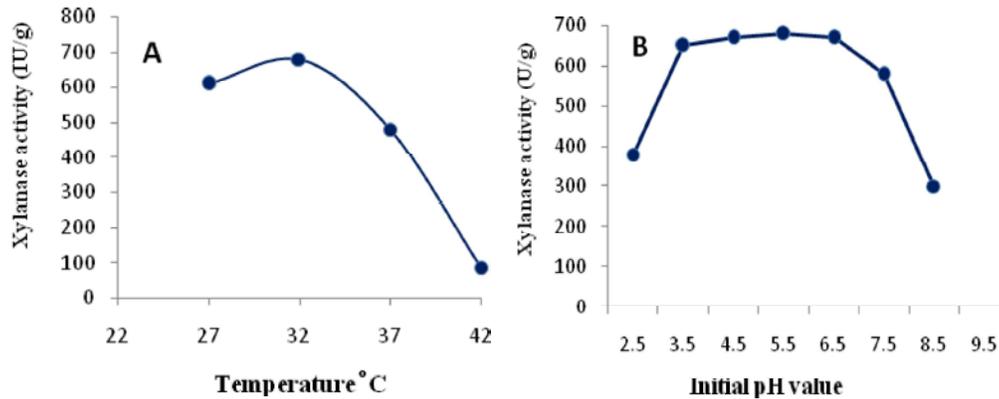


Fig. 4: Effect of temperature (A) and pH (B) on xylanase production by *A. fumigatus* F-993 grown on white corn flour after 48 hrs of growth using SSF.

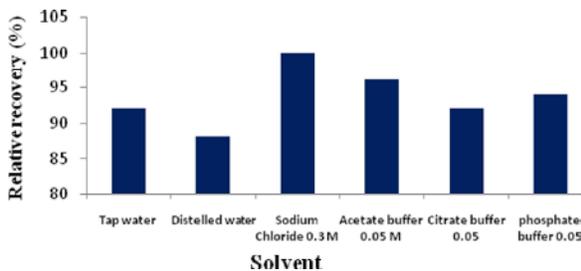


Fig. 5: Effect of different aqueous solutions on xylanase recovery from fermented substrate by *A. fumigatus* F-993. Maximum value was set as 100%.

increase in moisture content [36]. However, the enhancement of enzyme production occurred up to a certain point and then leveled off. Many researchers have reported similar effect. The optimum moisture content (on a wet weight basis) for xylanase production by *Aspergillus ochraceus* and *T. harsezinum* was 55% [6, 37, 38]. Xylanase yield by *Aspergillus niger* was optimal at 60-70% wet basis moisture content on a one to one mixture of straw and wheat bran [39], while, moisture 80 % was the optimum for xylanase production by *A. fumigatus* Sk1 when cultivated on oil palm trunk [15].

**Effect of Initial pH and Temperature on Xylanase Production:** Greater attention is given for optimizing the initial pH value of the moist solid medium [40]. Fig. 4B, illustrates that, the fungus expressed the highest degree of enzyme production (650-690 U/g) at an initial pH range 4.5-6.5 before or after, the enzyme synthesis was reduced. The pH value of the culture medium affected the permeability of cells as well as stability of enzyme [6, 41]. The effect of hydrogen ion on enzyme may be due to the stability of the enzyme at particular pH and denaturations

of the enzyme proteins occur at pH differ, the optimum pH of such enzyme was reported by Ang *et al.* [15]. Earlier reports on xylanase production by several fungi and bacteria also showed marked dependence on the initial pH of the medium. However, there are rare reports on alkali conditions at up to pH 9.0 for xylanase production by terrestrial *Streptomyces* spp. CA24 [30].

Under SSF, temperature is a controlling factor for enzyme formation [42]. Strain *A. fumigatus* F-993 exhibited maximum enzyme production (680 U/g) when incubation was conducted between 27-32°C (Fig. 4A) and decreased sharply above 37°C. The strain *Aspergillus awamori* was not greatly affected by temperature during production of xylanase [43], whereas *Chaetomium cellulolyticum* was highly influenced by temperature with a decrease in production of xylanase to about 50% when the temperature was changed from 37 to 25°C, under SSF [34]. High xylanase activity was obtained when *A. niger* was grown on starch wastes at 28°C [35].

### Xylanase Recovery

**Effect of Different Aqueous Solution for Xylanase Recovery:** NaCl solution 0.3 M was more efficient than other tested solvents in the extraction of xylanase from the fermented WCF (Fig. 5), as an activity of 720 U/g was achieved. About 4 and 6% increase in enzyme activity was attained compared to citrate and phosphate buffers respectively. The efficient solvent to obtain enzyme from fermented substrate may depend mainly on three factors, i.e. type of enzyme, source of enzyme and substrate used as a medium [42]. The yield of recovered enzyme are increased using NaCl as a solvent suggested that the enzyme was bound to the mycelial surface by ionic linkages and could be released by ionic compounds as NaCl into the medium [44].

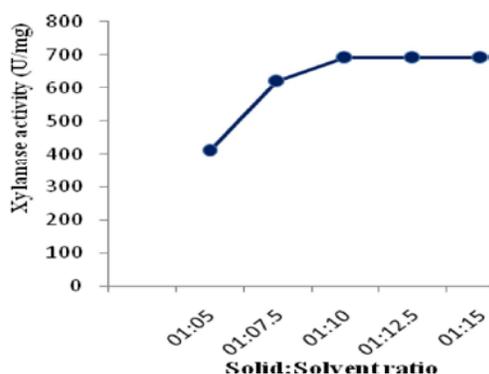


Fig. 6: Effect of solid: solvent ratio on recovery of xylanase produced by *A. fumigatus* F-993 after 48 hrs of grown on white corn flour at 32°C by SSF.

#### Effect of Solid: Solvent Ratio on Xylanase Recovery:

At the end of SSF, the fermented mass consists of non-utilized substrate, microbial cells, spores, the desired product and a number of metabolites formed during the course of fermentation. The optimization of xylanase was performed with the purpose of reducing enzyme losses in order to obtain crude extracts as concentrated as possible.

Several extraction solvents, temperatures, at a time were tested. Fig. 6 shows the level of xylanase obtained by leaching with 0.3 M NaCl at different solid: solvent ratios. Gradual increase in xylanase yield was achieved with the increasing of solvent volume. The highest xylanase production (690U/mg) was observed at solid: solvent ratio of 1:10. This result is in accordance with most commonly ratios used for enzymes extraction from fermented substrates by SSF. Pirota *et al.* [45] reported that a ratio of 1:9 solid: solvent was suitable for optimum recovery of xylanase. Correct selection of the operational parameters is of importance and an indicative for improvement in the recovery of enzymes. The positive effect of the solid to liquid ratio obtained for xylanase recovery means that an increase in the value of this variable should improve the extraction yield. The positive influence can be attributed to the mass transfer process, which is driven by the concentration gradient between the solid (substrate) and liquid (solvent) phases. A higher concentration gradient between the two phases facilitates transfer of the solutes to the liquid medium [45], which favors extraction of the enzyme.

#### Effect of Salt and Solvent on Recovery of Xylanase:

Preliminary experiments were performed in order to determine the extraction time and solvent type that is most favorable for xylanase extraction. An ideal solvent should extract the enzyme selectively and completely at room

temperature, with minimal contact time [45]. Fig. 7 represents applying with different solutions for recovery of xylanase.

#### Precipitation of Xylanase with Ammonium Sulphate:

The higher efficiency of precipitation for xylanase activity was achieved at 40% ammonium sulfate saturation. Total proteins (44.4%) in the crude enzyme solution were salted out by ammonium sulfate, gave a xylanase activity of 64% from the total xylanase activity of enzyme extract (Fig. 7A) and enhanced the specific activity from 14.2U /mg protein to 20.8 U/mg. Previous studies on xylanase precipitation employed high levels of ammonium sulphate to salt out the enzyme from cultures supernatant. Xylanase from *Aspergillus niger* was precipitated when ammonium sulphate increased up to 80% saturation [46], whereas at 90% saturation with ammonium sulphate maximum activity of xylanase from *Penicillium glabrum* was achieved [1].

#### Precipitation of Xylanase with Ethanol:

Total proteins (52.2%) present in crude enzyme solution were precipitated by cold ethanol to give xylanase activity of 68.6% in crude enzyme solution (Fig. 7B) and enhance the specific activity from 14.2U/mg protein present in crude enzyme solution to 18.9 U/mg protein.

#### Precipitation of Xylanase with Acetone:

Precipitation by cold acetone recovered 32.6% of total proteins in crude enzyme solution to give a xylanase activity of 86.2% of total xylanase activity present in the crude enzyme (Fig.7 B) and enhanced the specific activity from 14.2U/mg protein in the crude enzyme solution to 38.1 U/mg protein.

Results in Fig. 7 showed that, recovery with ammonium sulphate occurred at low concentration (40%) in comparison with other studies. Previous studies on xylanase precipitation employed high levels of ammonium sulfate to salt out the enzyme from culture supernatant which reached up to 90% [1]. However, Farinas *et al.* [46] reported that, recovery of xylanase from *Aspergillus niger* was obtained at a concentration of 25% ammonium sulfate. In the present study, ethanol as the precipitant was used at the same concentration of ammonium sulfate (40%, v/v) and great efficiency for precipitation of xylanase was achieved. However, the precipitation of xylanase at 40% concentration of the precipitant agent was more efficient with acetone than with ethanol or ammonium sulfate. The higher concentration may probably lead to protein denaturation [46].

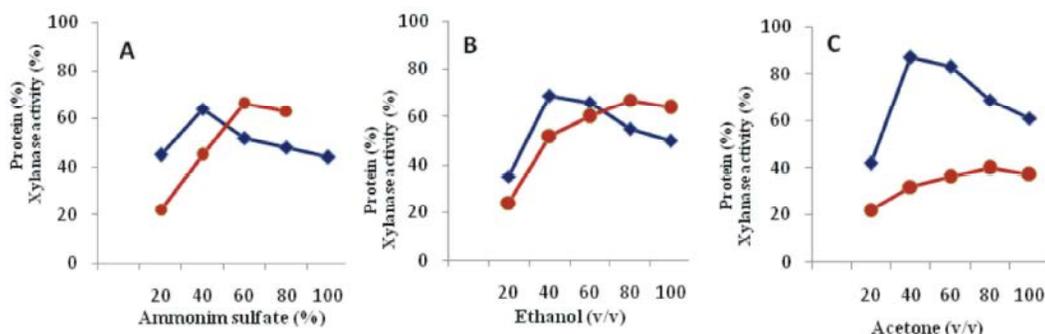


Fig. 7: Percentages of protein (●) and activity of xylanase (◆) % produced by *A. fumigatus*F-993 after recovery with ammonium sulfate (A), ethanol (B), or acetone (C).

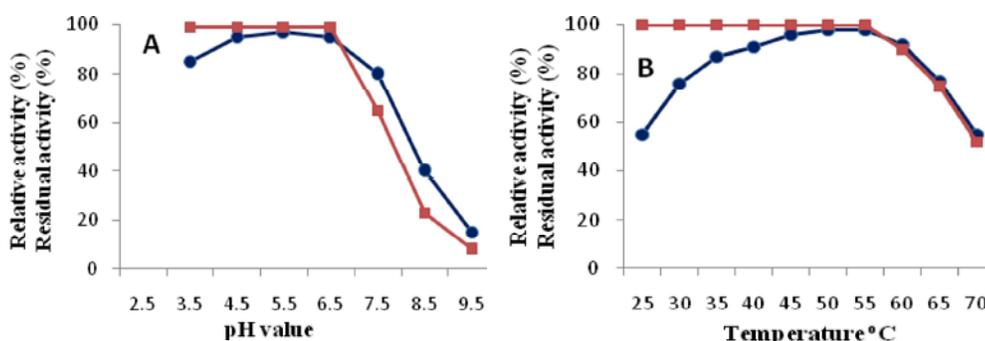


Fig. 8: Effect of pH value (A) and temperature (B) on activity (●) and stability (■) of xylanase.

In *Salting out*, ammonium sulfate reduces the electrostatic repulsion between like-charged groups at the protein surface and disturbs the structure of water molecules around the protein, making the aqueous salt solution a poor solvent for proteins, which precipitate out [46]. *Salting out* is also largely dependent on the hydrophobicity of the protein, since a suitable salt concentration promotes the aggregation of hydrophobic patches on the protein surface [46]. The positive effect of long time on enzyme recovery can be explained by the fact that a longer aging time increases the probability of nuclei formation due to collision among the molecules. Once the nuclei reach a critical size, they continue to grow [46]. Even though the salt concentration range required for precipitation is not an absolute property of the protein because it is dependent on both the properties of the other proteins present and the protein concentration in the starting solution. During precipitation with ammonium sulfate, the solution was kept over-night at 4°C because high temperature affects the solubility of proteins at high salt concentrations in an unusual way because of its effect on the hydrophobic interaction [46] and, in the *salting out* range, the solubility of proteins generally decreases with increasing temperature.

### Some Properties of Xylanase

**Effect of pH and Temperature on Xylanase Activity and Stability:** The enzyme exhibited maximum activity in a pH range of 3.5-6.5 (Fig. 8A). At the same time, the enzyme exhibited remarkable stability at pH values ranged from 3.5- 6.5; it retained its original activity at these pH values (Fig. 8A). Effect of temperature shows an increase in xylanase activity with increasing in temperature (Fig. 8B). Optimum activity reached a maximum between 50-65°C. In an attempt to determine the heat stability, the enzyme solution was pre-incubated in 0.1 M citrate buffer (pH 5.5) for 4 h at 50°C and 75°C to avoid the short incubation period and the protective effect of substrate. It was found that the enzyme expressed nearly its original activity after incubation at 50°C and remained 52% of its original activity after incubation at 75°C. Similar enzyme stability at high temperatures has been reported for a few *Bacillus* strains [47, 48], *Thermotoga* spp. [49] and thermophilic fungi [50]. Similar results are reported previously for xylanase from *A. fumigatus* [51, 52]. However, stability at pH from 2-5 was reported for a xylanase production by *Penicelium glabrum* [1]. Microbial xylanases are usually stable over a wide pH range from 3–10. On other hand, thermal stability is an interesting enzyme property due to the great industrial importance.

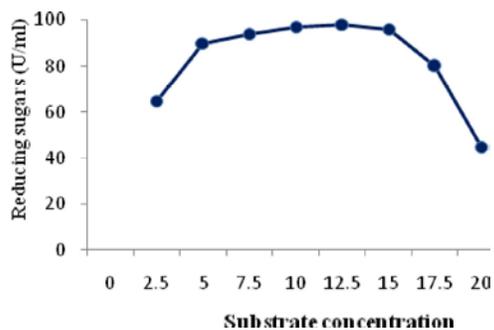


Fig. 9: Effect of substrate concentration on the activity of xylanase produced by *A. fumigatus* F-993.

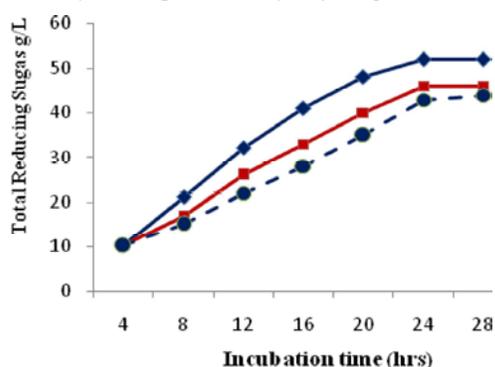


Fig. 10: Hydrolysis of alkali-treated rice straw (•), wheat straw (■) and sugarcane bagasse (◆) with crude xylanase of *A. fumigatus* F-993 grown on WCF at 32°C for 48 hrs.

Table 1: Enzymes activities involved during white corn flour fermentation and recovery

Enzymes	Units
Enzymes bounded to the fermented residual WCF:	
Xylanase	U/g fermented residue
FPase	22.4
CMCase	26.2
Glucoamylase	18.5
Enzymes involved in protein recovery:	
Xylanase	U/mg protein
FPase	11.2
CMCase	14.8
Glucoamylase	08.4

It was found that the enzyme remained 52% of its original activity after incubation at 75°C. Similar results of enzyme stability at high temperatures have been reported in previous studies [1, 53]. In this study, xylanase is more thermostable than many fungal xylanases, such as those from *Penicillium expansum* [54], *Aspergillus niger* B03 [55] and *Penicelium glabrum* [1], however, it is less thermostable than the xylanase from thermophilic *Talaromyces thermophilus* which exhibited its optimum activity at 70°C [56].

**Effect of Substrate Concentration on Enzyme Activity:**

Fig. 9 shows that the enzyme activity was increased with the increasing of xylan concentrations in the reaction mixture up to 15% (w/v). On the other hand the enzyme has the advantage of proceeding while retaining 65% of its activity in high substrate slurry concentrations up to 22.5% (w/v). Considerable activities such as FPase, CMCase as well as glucoamylase at 11.2, 14.8, 8.4 U/mg protein, respectively were involved with xylanase during protein precipitation with 40% (v/v) acetone.

**Enzymes Involved with Xylanase:**

The multi-enzymes produced by different microorganisms was reported by many investigators and utilized in lignocellulosic saccharification [11, 15, 57, 58]. Enzymes bounded to the fermented residual WCF reached 82.6, 22.4, 26.2 and 18.5 U/g for Xlanase, FPase, CMCase and Glucoamylase, respectively (Table 1). Data presented in Table 1 indicated that, considerable activities of cellulases (FPase and CMCase) as well as glucoamylase were involved in protein precipitated with 40% (v/v) acetone. Optimum activity and stability in acidic conditions, thermal stability and high enzyme activity exhibited by xylanase in this study make this enzyme attractive for some industrial applications, such as an animal feed additive and in food industries.

**Hydrolysis of Some Lignocelluloses Wastes:**

Xylanase and multi-enzymes produced by *A. fumigatus* F-993 and recovered by 40% (v/v) acetone, were tested for hydrolysis of some lignocellulose wastes such as wheat straw, rice straw and sugar cane bagasse (at concentrations 15%, w/v), after a pretreatment with an alkali. Total reducing sugars were increased (Fig. 10) despite of a decrease in the hydrolysis rate (the amount of released reducing sugars in time unit) with increase hydrolyzing time. About 46, 44, 52 g l<sup>-1</sup> reducing sugars were obtained after 24hrs (Fig. 10), containing 16, 15 and 12% (w/w) glucose in the enzymatic hydrolysates of wheat straw, rice straw and sugar cane bagasse, respectively.

**Feeding Considerations:**

The nutrient content of co-products classifies feedstuffs as both high-protein and high-energy alternatives. Many reports have been conducted studying the feeding value of these co-products. With respect to energy, grains have an equal to slightly greater feeding value in comparison to corn. The majority of studies suggested that, when used as a protein source, it is equivalent to soybean meal and other

sources of protein for beef cattle diets. In this study, high levels of xylanase using white corn flour as a carbon source were obtained. Enzymes bounded to the fermented residual WCF reached 82.6, 22.4, 26.2 and 18.5 U/g for xylanase, FPase, CMCCase and glucoamylase, respectively (Table 1). In addition, the same enzymes involved during protein recovery. The nutrient profile of distillers grains, a product of the dry corn milling industry is reported as a viable source of supplemental energy to forage-based ruminant diets [59-61]. In the present study, clean production of xylanase was performed with environmental-friendly production consideration.

### REFERENCES

- Knob, A., S. Beitel, D. Fortkamp, C. Terrasan and F. Alex, 2013. Production, purification and characterization of a major *Penicillium glabrum* xylanase using Brewer's spent grain as substrate. *Bio. Med. Res. Int.*, pp: 1-8.
- Wong, L., L. Tan and J. Saddler, 1988. Multiplicity of  $\beta$ -1-xylanase in microorganisms: functions and applications. *Microbiol. Rev.*, 52: 305-317.
- Pal, A. and F. Khanum, 2012. Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation. *Biores. Technol.*, Gela Myan Bueno (presentations online).
- Jain, A., 1995. Production of xylanase by thermophilic *Melanocarpus albomyces* IIS-68 *Process Biochem.*, 30: 705-709.
- Beg, Q., M. Kapoor, L. Mahajan and G. Hoondal, 2001. Microbial xylanases and their industrial applications, *Appl. Microbiol. Biotechnol.*, 56: 326-338.
- Fadel, M., 2001. High level xylanase production from sorghum flour by a newly isolate of *Trichoderma harizianum* cultivated under solid state fermentation. *Ann. Microbiol.*, 51: 61-78.
- Fadel, M. and M.S. Foda, 1993. Physiological studies on xylanase production by *Penicillium funiculosum* on some agricultural wastes. *Zentralbi, MiKrobiol.*, 148: 304-312.
- Pandey, A., 1992. Recent process developments in solid-state fermentation. *Process Biochem.*, 27: 109-117.
- Smits, J., A. Rinzema, J. Tramper, H. Van Sosbeek and W. Knol, 1996. Solid-state fermentation of wheat bran by *Trichoderma reesei* QM 9414: substrate composition changes, C-balance, enzyme production, growth and kinetics. *Appl. Microbiol. Biotechnol.*, 46: 489-496.
- Bailey, M., P. Biely and K. Poutanen, 1992. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.*, 23: 257-270.
- Fadel, M. and T. Kahil, 2004. Production and application of cellulase and hemicellulases degrading enzymes from cotton stalks by fungi in solid state fermentation. *Egypt. J. Biotechnol.*, 3: 416-427.
- Chandra, M., B. Viswanath and B. Reddy, 2007. Cellulolytic enzymes on lignocellulosic substrates in solid state fermentation by *Aspergillus niger*. *Indian J. Microbiol.*, 47: 323-328.
- Diaz, A., I. Ory, I. Caro and A. Blandino, 2010. Enhance hydrolytic enzymes production by *Aspergillus awamori* on supplemented grape pomace. *Food Bioprod. Process*, 10,101.
- Umsza-Guez, M., A. Díaz, I.O. Ory, A. Eleni and I. Caro, 2011. Xylanase production by *Aspergillus awamori* under solid state fermentation conditions on tomato pomace. *Braz. Microbiol.*, 42: 1585-1597.
- Ang, S., E. Shaza, Y. Adibah, A. Suraini and M. Madihah, 2013. Production of cellulases and xylanase by *Aspergillus fumigatus* SK1 using untreated oil palm trunk through solid state fermentation. *Process Biochem.*, 48: 1293-1302.
- Ridder, E., S. Nokes and B. Knutson, 1997. Production of hemicellulolytic enzymes (xylanases) using solid-state fermentation of *Trichoderma longibrachiatum*. An ASAE Meeting Presentation, pp: 1-10.
- Gomes, J., I. Gomes and W. Kreiner, *et al.* 2008. Xylanase production with xylan rich lignocellulosic wastes by a local soil isolate *Trichoderma viride*. *Braz J. Microbiol.*, 39: 535-541.
- Terassan, C., B. Temer, C. Sarto, G. Francides and E. Carmona, 2013. Xylanase and  $\beta$ -xylosidase from *Penicillium janczewskii*: production, physico-chemical properties and application of the crude extract for pulp biobleaching. *Bio. Res. Com.*, 8: 1292-1305.
- Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 31: 426-428.
- Mandels, M., T. Hontz and C. Nystrom, 1974. Enzymatic hydrolysis of waste cellulose. *Biotechnol. Bioeng.*, 16: 1471-1493.
- Bernfeld, P., 1951. Enzyme of Starch Degradation and Synthesis. *Advance in Enzymology* 12: 382-428, F.F. Nord Ed., Interscience Publishers, Inc. New York.
- Pestana, F. and J. Castillo, 1985. Glucoamylase production by *Aspergillus awamori* on rice flour medium and partial characterization of the enzyme. *MIRCEN, J. Microbiol. Biotech.*, 1: 225-237.

23. Ohnisti, S. and J. Barry, 1978. A simplified method of quantitating protein. *Anal. Biochem.*, 86: 193-200.
24. Sidhu, M. and D. Sandhu, 1980. Single-cell protein production by *Trichoderma longibrachiatum* on treated cane bagasse. *Biotechnol. Bioeng.*, 22: 689-692.
25. A.O.A.C. 1980. Official Methods of Analysis of the Association of Official Analytical Chemists. Benjamin Franklin Station Washington, D.C. 220, 2004, U.S.A.
26. Dubois, M., K. Gilles, J. Hamilton, P. Rebers and F. Smith, 1956. Determination of total carbohydrates. *Anal. Chem.*, 28: 350-356.
27. Díaz, A.B., I. Caro, I. Ory and A. Blandino, 2007. Evaluation of the conditions for the extraction of hydrolitic enzymes obtained by solid state fermentation from grape pomace. *Enz. Microb. Technol.*, 41: 302-306.
28. Assamoi, A., J. Destain, F. Delvigne, G. Lognay and P. Thonart, 2008. Solid-state fermentation of xylanase from *Penicillium canescens* 10 in a multi-layer-packed bed reactor. *Appl. Biochem. Biotechnol.*, 145: 87-98.
29. Fadel, M., 2000. Production physiology of cellulose and  $\beta$ -glucosidase enzymes of *Aspergillus niger* grown under solid state fermentation conditions. *Online Biological Sci.*, 1: 64-75.
30. Porsuk, I., S. Ozakin, B. Bali and E. Yilmaz, 2013. A cellulose-free, thermoactive and alkali xylanase production by terrestrial *Streptomyces* sp. CA24. *Turk. J. Biol.*, 37: 370-375.
31. Park, Y., S. Kang, J. Lee, S. Hong and S. Kim, 2002. Xylanase production in solid state fermentation by *Aspergillus niger* mutant using statistical experimental designs. *Appl. Microbiol. Biotechnol.*, 58: 761-766.
32. Shah, A. and D. Madamwar, 2005. Xylanase production by an isolated strain of *Aspergillus foetidus* in India. *World J. Microbiol. Biotechnol.*, 21: 233-243.
33. Shahi, S., I. Alemzadeh, M. Khanahmadi and I. Roostaazad, 2011. Xylanase production under solid state fermentation by *Aspergillus niger* IJE Transactions B: Applications, 24: 197-208.
34. Dubeau, H., D. Chahal and M. Ishaque, 1987. Xylanase of *Chaetomium cellulolyticum* its nature of production and hydrolytic potential. *Biotechnol. Lett.*, 9: 275-280.
35. Norazlina, S. and R. Puvanesvaran, 2011. Production of xylanase enzyme through bioconversion of agricultural waste. Conference: International Symposium & Exhibition in Sustainable Energy & Environment- ISESEE
36. Kim, J., M. Hoe and M. Hosobuchi, *et al.* 1985. Cellulase production by a solid-state culture system. *Biotechnol. Bioeng.*, 27: 1445-1450.
37. Seyis, I. and N. Aksoz, 2003. Determination of some physiological factors affecting xylanase production from *Trichoderma harzianum* 1073 D3, *Microbiologica*, 26: 89-95.
38. Seyis, I. and N. Aksoz. 2005. Xylanase Production from *Trichoderma harzianum*, *Food Technol. Biotechnol.*, 43: 37-40.
39. Wu, M., S. Li, J. Yao, R. Pan and Z. Yu, 2011. Mutant of a Xylanase-producing Strain of *Aspergillus niger* in Solid under solid-state fermentation and its characterization State Fermentation by Low Energy Ion Implantation, 21: 1045-1049.
40. Lonsane, B., N. Ghildyal, S. Budiartman, S. Ramakrishna, 1985. Engineering aspects of solid state fermentation. *Enz. Microb. Technol.*, 7: 258-265.
41. Mase, T., Y. Matsumiya, S. Mori and A. Matura, 1996. Purification and characterization of a novel glucoamylase *Acrominium* sp. YT.78. *J. Ferment. Bioeng.*, 81(4): 347-350.
42. Harris, A. and C. Ramalingam, 2012. Xylanases and its application in food industry: A Review. *J. Exp. Sci.*, 1: 01-11.
43. Smith, D. and T. Wood, 1991. Xylanase production by *Aspergillus awamori*: Development of a medium and optimization of the fermentation parameters for the production of extracellular xylanase and  $\beta$ -xylosidase while maintaining low protease production. *Biotechnol. Bioeng.*, 38: 883-890.
44. Rao, M., R. Seeta and V. Deshp, 1983. Effect at pretreatment of cellulose by *Penicillium funiculosum* and recovery of enzyme. *Biotechnol. Bioeng.*, 25: 1863-1867.
45. Pirota, R., L. Miotto, P. Delapona and C. Farinas, 2013. Improving the extraction conditions of endoglucanase produced by *Aspergillus niger* under solid state fermentation. *Braz. J. Chem. Eng.*, 30: 117-123.
46. Farinas, C., L. Scarpelini, E. Miranda and V. Neto, 2011. Evaluation of operational parameters on the precipitation of endoglucanase and xylanase produced by solid state fermentation of *Aspergillus niger*. *Braz. J. Chem. Eng.*, 28: 17-26.
47. Khasim, A., I. Alchaanati and Y. Shoham, 1993. Purification and characterization of a thermostable xylanase from *Bacillus stearothermophilus*T-6. *Appl. Environ. Microbiol.*, 59: 1725-1730.

48. Nakamura, S., Y. Ishiguro, R. Nakai, R. Aono and K. Horikoshi, 1995. Purification and characterization of thermophilic alkaline xylanase from thermoalkaliphilic *Bacillus* sp. Strain TAR-1. *J. Mol. Catal. Enz.*, 1: 7-15.
49. Sunna, A., J. Puls and G. Antranikan, 1996. Purification and characterization of two thermostable endo 1,4- $\beta$ -D-xylanases from *Thermotagathermarum*. *Biotechnol. Appl. Biochem.*, 24: 177-185.
50. Cesar, T. and V. Mrsa, 1996. Purification and properties of xylanase produced by *Thermomyces lanuginosus*. *Enz. Microb. Technol.*, 19: 289-296.
51. Silva, C., J. Puls, M. Sousa and E. Ferreira-Filho, 1999. Purification and characterization of a low molecular weight xylanase from solid-state cultures of *Aspergillus fumigatus* Fresenius. *Rev. Microbiol.*, 30: 114-119.
52. Polizeli, M., A. Rizzatti, R. Monti, H. Terenzi, J. Jorge and D. Amorim, 2005. Xylanases from fungi: properties and industrial applications. *Appl. Microbiol. Biotechnol.*, 67: 577-591.
53. Tan, L. and J. Saddler, 1987. Purification and characterization of a thermostable xylanase from a thermophilic fungus *Thermoascus aurantiacus*. *Can. J. Microbiol.*, 33: 689-692.
54. Querido, A., J. Coelho, E. Ara'ujo and V. Chaves-Alves, 2006. Partial purification and characterization of xylanase produced by *Penicillium expansum*. *Braz. Arch. Biol. Technol.*, 49: 475-480.
55. Dobrev, G. and B. Zhekova, 2012. Purification and characterization of endoxylanase Xln-2 from *Aspergillus niger* B03. *Turk. J. Biol.*, 36: 7-13.
56. Maalej, I., I. Belhaj, N. Masmoudi and H. Belghith, 2009. Highly thermostable xylanase of the thermophilic fungus *Talaromyces thermophilus*: purification and characterization. *Appl. Biochem. Biotechnol.*, 158: 200-212.
57. Jagtap, S.S., S.S. Dhiman, M. Jeya, Y.C. Kang, J.H. Choi and J.K. Lee 2012. Saccharification of poplar biomass by using lignocellulases from *Pholiota adiposa*. *Biores. Technol.*, 72: 120:264.
58. Jagtap, S., S.T. Dhiman, J. Li, J. Lee and Y. Kang, 2013. Enzymatic hydrolysis of aspen biomass into fermentable sugars by using lignocellulases from *Armillaria gemina*. *Biores. Technol.*, 133: 307-314.
59. Loy, D.D. and K.N. Wright, 2003. Nutritional Properties and Feeding Value of Corn and its By-products. 571-603, in: *Corn Chemistry and Technology*. (P. J. White and L. A. Johnson, Eds.) American Ass: Ociation of Cereal Chemists, Inc. St. Paul, MN.
60. National Research Council (NRC), 1996. Nutrient Requirements of Beef cattle. National Academy Press, Washington, DC.
61. National Research Council (NRC). 2001. Nutrient Requirements of Dairy cattle. 7th Rev. Ed. Nat. Acad. Sci., Washington, DC.