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Production and Optimization of Xylanase from Thermotolerant and Alkali Tolerant *Emericilla nidulans* AS210136.01 isolate from Lignocellulosic Wastes

Ghada A. Youssef[1]*, Sanaa S. Kabeil[2], Samah K. Elkady[1] and Samy A. El-Aassar[1]

[1]Faculty of Science, Botany and Microbiology Department,
Alexandria University, Moharram Bek 21511, Alexandria, Egypt.
[2]Genetic Engineering and Biotechnology Research Institute,
Scientific Research and Technology Applications City, Egypt.

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The objectives of the present study were isolation, identification and characterization of xylanase producing fungi, optimization of medium composition and cultural conditions for xylanase enzyme production using cheaper sources. Among the lignocellulosic substrates tested, wheat bran supported a high xylanase (EC 3.2.1.8) activity. The aim of the work was to search for a new thermostable and alkaline tolerant xylanase producing organisms and characterize the molecular feature, 18S rRNA sequence and phylogenetic analyses of the most potent selective isolates. The thermophilic fungi were screened in Egyptian soil at 40°C. The *Emericilla nidulans* AS210136.01 was a thermotolerant, alkali tolerant strain was found to be the best isolate that produced higher xylanase activity than the other strains isolated in solid-state fermentation (SSF). Xylanase production was approx 1.33-fold higher in SSF than in submerged fermentation (SmF). The maximum xylanase production in SSF culture (25.54U/ml) was observed at optimized conditions, incubation temperature of 40°C at pH 8.0 after 120h of incubation period followed by a decline thereafter. The best organic nitrogen source was 5.0g/l peptone, 3.0g/l yeast extract achieved maximum production of enzyme. Thus the present study proved that the fungal strain *Emericilla nidulans* used is highly potential and useful for xylanase production. As *Emericilla nidulans* produced highest xylanase activities, 18S rRNA sequence was analyzed and identified. Phylogenetic analysis revealed the *Emericilla nidulans* AS210136.01 strain isolated in this study is a genetically new isolate.

Key words: *Emericilla nidulans* AS210136.01, Thermophilic fungi, Wheat bran, Xylanase optimization, Solid-state fermentation (SSF).

Xylan is a major component of the plant cell wall and the most abundant form of renewable hemicellulose. The xylan structure, however, can differ greatly depending on its origin[1]. Basically, a xylan structure consists of D-xylopiranose units, linked by β -1,4 bonds, which can be exhibited either in linear or branched form[2]. Xylanases (EC 3.2.1.8; endo- β-1,4-D-xylanase) are enzymes that can catalyze the hydrolysis of xylan with β-1,4-xylanolytic linkages[3,4]. In recent years, xylanases have attracted considerable research interest because of their potential applications in industrial processes such as bleaching of pulp in the paper industry, to obtain cellular proteins, liquid fuels, and other chemical substances[5], in xylitol and ethanol production[6], bioconversion of biomass

* To whom all correspondence should be addressed.
Tel: (020100)407- 3123; Fax: ?20 203 3911794;
E-mail: aminghada66@gmail.com

wastes to fermentable sugars and clarification of fruit juices and wines[7,8]. They also have potential application in the production of rare xylo-ligosaccharides with degrees of polymerization ranging from 2 to 5 xylose residues[9,6]. Xylanases are extracellular enzymes produced by microorganisms such as bacteria (saprophytic and phytopathogenous), mycorrhizic fungi, and some yeasts. The enzyme is also found in protozoa, insects, crustaceans, snails, seaweed, and also seeds of plants during the germination phase in the soil[10]. Although bacterial xylanase were reported to have higher values which are beneficial from the view point of applying in biobleaching process but xylanase produced from fungi usually has higher activity[11,12]. Fungal strains usually prefer solid state fermentation media which has low water content. These media types are one of the most responsible factors for the higher value of crude enzyme activity obtained from fungal strains.

From an industrial point of view, filamentous fungi are particularly interesting as producers of xylanases because they excrete substantially greater amounts of xylanolytic enzymes into an extracellular culture medium than do bacterial or yeast cultures[13]. Xylanase obtained from thermophilic microorganisms have unique physico-chemical and catalytical properties, such as thermostability and high temperature optima. Many industrial processes must operate at high temperatures, making availability of thermostable enzymes highly attractive[3]. Some of the thermophilic fungi, *Chaetomium thermophile, Humicola insolens* (syn. *Scytalidium thermophilum), Thermomyces lanuginosus* and *Thermoascus aurantiacus* have been reported to produce biotechnologically-important, thermostable xylanases. A thermostable, cellulase-free xylanase from the filamentous fungus *Thermomyces lanuginosus* has been isolated by[14]. The xylanase from this fungus is not only remarkably thermostable, but it is also active over a wide pH range[15]. The use of cheaper lignocellulosic residues viz. wheat bran, wheat straw, corn cobs and sugarcane bagasse can be used as growth substrates in culture to produce xylanase and also to replace the xylan as a inducer for cost reduction in production. Special attention has been paid to the development of an economical culture medium that uses inexpensive and readily available ingredients, to reduce the cost and to best suit industrial applications. A cellulase-free xylanase preparation, produced by *T. lanuginosus* grown in a culture medium based on waste corn cobs, has been successfully used to enhance the bleaching of kraft pulp with no adverse effect on the final paper strength[16]. The optimal design of the culture medium is a very important aspect for successful use of microorganisms in industrial biotechnology, such as the substrate used[17], pH, temperature, and cultivation time[13] as well as spore concentration[18,19]. Culture medium optimization by the traditional 'one-factor at-a-time' techniques requires a considerable amount of effort and time[20]. Statistical experimental design techniques can aid in the optimization of a culture medium, as they can provide statistical models that reveal the interactions among the process parameters at varying levels. Furthermore, calculations of the optimal level of each parameter for a given target can be performed. Therefore, in order to search for a new producer of xylanolytic thermophilic fungi and characterize the molecular and enzyme feature of the selective isolate. The objective of this research was to evaluate the best cultivation conditions for optimizing the production of xylanase by *Emericilla nidulans.*

MATERIALS AND METHODS

Microorganism

The fungal organism used in this study was *Emericilla nidulans* (GenBank accession number, AS210136.01) isolated from Egyptian soil samples during a screening study for thermostable xylanase-producing microorganisms. The isolated fungi were kindly identified in the Mycological Center, Assiut University as *Emericella nidulans*, *Humicola grise*a, *Aspergillus tamarii, Penicillium oxalicum, Aspergillus fumigates, Trichoderma harzianum, Fusarium solani, Rhizopus stolonifer, Mucor racemosus, Phoma betae, Penicillium corylophilum.* The culture was kept in malt extract agar, performing constant replications every four weeks and maintained at 4°C. The isolates screened for xylanase production on xylan-agar medium, containing 1% w/v Birchwood xylan from Sigma Chemical Company, St. Louis, USA, 2% agar agar[21], contained the following ingredients (g/l):, 2.0g K_2HPO_4, 0.3g $MgSO_4.7H_2O$, 0.3g $CaCl_2$, 5.0g

peptone, 3.0g yeast extract. The initial pH of media was adjusted to 6.0. Two agar blocks (2mm) from 5 day- old colony of each strain grown into 50 ml cultured media on 250 ml of Erlenmeyer flasks at different temperatures.

Screening of agro-industrial waste material as submerged fermentation (SmF) and solid state fermentation (SSF) substrate for maximum enzyme production

Various agro-industrial waste materials such as Wheat bran (Wb), Oat split (Os), Corn cobs (Cc), Rice straw (Rs) and Wheat straw (Ws) were selected as substrates and screened for the maximum production of xylanase through SmF and SSF. The substrates were dried in an oven at 80°C for 2 hours, ground with blender, sieved into finer particles and then used[22]. At SmF 1g/50ml of each substrate was dispensed in 250 ml Erlenmeyer flasks then sterilized at 121°C for 20 minutes. After sterilization, each flask was inoculated with two cubes of the 5 days cultured xylan plates; the flasks were then incubated at 40°C for 5 days. In SSF 5g of Wb, Os, Cc, Rs, Ws, were transferred to 250 ml Erlenmeyer flasks and moistened with 10ml of culture medium [23]. At the end of the incubation period the fermentation mass was extracted by the simple method of extraction using distilled water as extracting agent[24]. Forty ml distilled water was added for one hour on a rotary shaker (200 rpm) followed by centrifugation for 10 minutes. The supernatant was used for estimation of xylanase activity and protein content.

Assay for xylanase activity

This was done according to the reaction mixture (1ml) containing 0.05ml culture filtrate + 0.45ml dis.H_2O +0.5ml 0.05M acetate buffer using Birch wood xylan (Sigma, U.S.)[25]1% (w/v) in sodium acetate buffer 50mM (pH 5.0). Solution was incubated in water bath at 50°C for 30 min, 1.5ml dinitrosalycilic acid (DNS) was added to the reaction mixture. This mixture was boiled for 15 minutes and then the absorbance was measured at 550 nm. The released xylose due to xylanase activity was determined by dinitrosalycylic acid (DNS) method[26]. One unite of xylanase activity is defined as the amount of enzyme necessary to produce 1µmol/ml/min of glucose or xylose under the assay conditions.

Quantitative protein determination

The protein concentration was determined by Lowry's method[27], using bovine serum albumin as the standard.

Optimization of culture conditions for enzyme production

Culture conditions for maximum production of xylanase by *Emericilla nidulans* AS210136.01, were optimized with respect to temperature, mode of cultivation medium, pH, incubation period, inoculums size, organic and inorganic nitrogen sources and additives of carbon sources. The effect of cultivation temperature was studied at different temperatures starting from 35°C to 50°C at pH 6.0. Different modes of cultivation, solid-state fermentation and submerged fermentation (SSF and SmF) were tested for higher xylanase activity at 40°C. The effect of initial pH of the medium was observed by adjusting the initial pH to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 with 50mM citrate and phosphate buffer. The fungal organism was cultivated for a period of 14 days at pH 8.0 and 40°C to examine the effect of incubation period. The inoculums size was also reported to be an important factor in microbial fermentation, so it was raised from 1 to 5 cubes/ flask. The effect of the supplementing nitrogen sources was studied by adding different concentrations of peptone and yeast as organic nitrogen source 2.5, 5, 10 g/l of peptone and 1.5, 3, 6g/l of yeast and absence of nitrogen source. The effect of different additives (xylan, fructose, glucose, sucrose, lactose, carboxymethyl cellulose (CMC), and starch) to the Wb as a sole carbon source on the enzyme production was tested. The control assay was also conducted simultaneously without the addition of any additives.

18S rRNA Sequence Determination and Phylogenetic Analyses

PCR amplification of fungal DNA was performed using universal primers targeting a highly conserved region of the fungal 18S small-subunit rRNA multicopy gene to generate a PCR product from fungal DNA of 625 bp. PCR was carried out using a Thermocycler Gene Amp 9700, (*Applied Biosystems* (ABI), USA) in a final reaction volume of 25 µl. The reaction mixture contained approximately 2 µl of DNA from the fungal isolate, 1.5µl of each of the 18S-forward primer 5V TCCGCAGGTTCACCTACGGA -3V and 18S-reverse primer 5V- AACTTAAGGAAATT GACGGA -3V ,0.5 µl DNTP's, 2.5 µl MgCl2, 1U of

Dreem Taq DNA polymerase, and 2.5µl Dreem reaction buffers. The PCR conditions consisted of an initial denaturing step of 1min at 94°C followed by 40 cycles of denaturation at 94°C, for 30s, annealing at 40°C for 30 s and extension at 72°C for 30s. The reaction was completed with a final extension at 72°C for 5 min and then cooled and held at 4°C. A second cycle was composed by a denaturation step of 94°C for1 min followed by 35 cycles of 94°C for 1 min, 40°C for 1 min, 72°C for 1 min and final extension of 72°C for 3 min. PCR products were first analyzed by electrophoresis in 2% (wt/v) agarose gels and ethidium bromide staining. PCR products were purified using a high pure PCR product purification kit (Roche Molecular Systems, INC.) and then sequenced. All DNA sequences were determined by the Genetic Engineering and Biotechnology Research Institute, Scientific Research and Technology Applications City, Egypt.

QIAquick PCR purification kit (Qiagen, Germany) was used to purify the amplified products of the 18S rRNA gene. Five volumes of the buffer PS were mixed with one volume of the PCR product and the mixture was loaded into the PCR purification column placed into 2.0 ml collection tube. The column was centrifuged at 13000 rpm for 1.0 min. The column was then placed into new collection tube and 750.0 µl of PE buffer were pipetted into it and was followed by centrifugation at 13000 rpm for 1.0 min. The centrifugation step was repeated to ensure getting rid of ethanol. The amplified fragment was eluted from the column using 50µl Milli-Q water with centrifugation step at 10,000 rpm for 1.0 min.

Sequence alignment and sequence analysis

DNA sequence analysis of resultant 600 bp as a partial sequence from the tested fungus species using CLUSTALW program was performed. The nucleotide sequences were analyzed with the BLAST database (http//:www.ncbi.nlm.nih.gov.)[28]. BLAST search confirmed that the obtained partial sequences are related to the Ascomycetes and Basidiomycetes which is conquered with the morphological identification of the tested fungi.

Statistical analysis

Data obtained was subjected to analysis of variance and the means were compared using the Least Significant Difference (LSD) at 0.05 level of probability[29]. *In vitro* experiments were conducted in triplicate.

RESULTS

Screening of xylanase producing strains

Eleven fungal strains were isolated from different Egyptian soil samples by enrichment culture technique, where selective culture media and incubation conditions were used to isolate microorganisms directly from nature by growing them on xylan-agar medium, containing 1% w/v Birchwood xylan and 2% agar agar at different temperatures. All the 11 isolated fungal strains were found to be xylanase positive, showing variable

Table 1. The activity of different isolated fungi for xylanase production at different temperatures

Fungal isolates	Xylanase activity (U/ml)			Protein content (mg/ml)			Dry wt (g/50 ml)		
	35°C	40°C	50°C	35°C	40°C	50°C	35°C	40°C	50°C
Aspergillus tamarii	11.92b	-	-	7.42d	-	-	1.02b	-	-
Trichoderma harzianum	11.22c	-	-	8.58c	-	-	0.97c	-	-
Penicillium oxalicum	9.51e	-	-	9.69a	-	-	0.89d	-	-
Fusarium solani	10.86d	-	-	3.49h	-	-	0.94c	-	-
Rhizopus stolonifer	8.68f	-	-	5.61f	-	-	0.81e	-	-
Mucor racemosus	7.86g	-	-	6.32e	-	-	0.79e	-	-
Phoma betae	8.84f	-	-	9.51b	-	-	0.85d	-	-
Humicola grisea	7.32h	-	-	5.91f	-	-	0.64f	-	-
Emericilla nidulans	8.40g	12.89a	6.39a	5.8f	7.62d	5.41a	0.82e	1.32a	0.92a
Aspergillus versicolor	5.80i	6.17g	3.72c	4.8j	6.41e	2.08c	0.82e	0.77e	0.47c
Penicillium corylophilum	7.12h	7.54h	4.07b	5.59f	4.14g	2.92b	0.62f	0.60f	0.53b
$LSD_{0.05}$	0.36	0.32	0.43	0.31	0.31	0.26	0.07	0.07	0.05

Means followed by the same letter(s) are not significantly different at 0.05 level of probability

activities but they differ in ability to grow at 35°C, 40°C and 50°C. *Emericella nidulans* shows the highest xylanase activity at 40°C (12.89U/ml) 2-fold of that at 50°C Table1.While the lowest activity (3.72U/ml) was that produced by *Aspergillus versicolor* at 50°C. Similarly the highest value of the dry weight obtained from *Emericella nidulans* (1.32g/50ml). The highest extracellular protein content was produced by *Penicillium oxalicum* at 35°C (9.69mg/ml). It was also noticed that the growth as a dry weight increased by increasing the xylanase activity. *Emericella nidulans* could therefore be considered as a satisfactory and promising producer of thermostable xylanase. All experiments were analyzed using analysis of variance procedure. Experiments including two factors were analyzed as a factorial experiment in a randomized complete block design with three replications. Comparison of means was made using LSD (Least Significant Difference) at 0.05 level of probability.

The optimum temperature ranging between 40 to 45°C, indicating their thermotolerant nature[30] . *Emericella nidulans* NK-62 was able to grow at 45°C with maximum xylanase activity[31]. In contrast[32] reported that the optimum temperature for *A. nidulans* was 56°C. The best growth temperature for the fungus is not always the best for enzyme activity. However, *Aspergillus niger*, verified that optimal temperatures for enzyme activity and fungal growth were similar[33], which is also in agreement with our results and the results obtained by [34]. According to [35, 36], the large decreases at very low or high temperatures are because the fungal growth is inhibited at these temperatures, leading to a decrease in enzyme synthesis.

Effect of using different agricultural wastes on xylanase production under different modes of cultivation, solid-state fermentation and submerged fermentation (SSF and SmF)

Xylanase activity was higher in solid-state fermentation (SSF) when compared to that

Table 2(a). Influence of different concentrations of agricultural wastes as a sole carbon source on xylanase activity produced by *Emericella nidulans* at Submerged fermentation (SmF)

Substrate	Conc (g/50ml)	Xylanase activity (U/ml)	Protein (mg/ml)	Dry wt. (g/50ml)
Os	0.2	7.27i	6.80bc	0.50e
Wb		8.47f	6.00d	0.72d
Ws		7.09i	5.60d	0.78d
Cc		7.96h	4.70g	0.43e
Rs		8.03g	5.90d	0.65d
	0.5			
Os		8.30fg	4.73g	0.83c
Wb		10.29c	5.29f	0.89c
Ws		7.50h	4.59g	0.92c
Cc		7.80h	5.30e	0.68d
Rs		8.11g	6.30e	0.73d
Os	1	10.46c	4.33g	0.91c
Wb		11.95a	7.31b	1.02c
Ws		9.13e	7.1b	1.15c
Cc		8.65f	4.67g	0.83c
Rs		9.41d	5.78e	0.94c
Os	2	9.62d	4.39g	1.87b
Wb		10.25c	6.50c	1.92b
Ws		11.03b	8.10a	2.31a
Cc		7.76h	4.10h	1.85b
Rs		10.58c	5.10f	2.09a
LSD		0.36	0.27	0.30

Means followed by the same letter(s) are not significantly different at 0.05 level of probability.

under submerged fermentation (SmF) by using different agricultural wastes locally available lignocelluloses namely, Wheat bran (Wb), Oat split (Os), Corn cobs (Cc), Rice straw (Rs) and Wheat straw (Ws) to determine the xylanase activity, and supernatant protein concentrations of the crude enzyme preparations (Tables 2a and 2b). The highest xylanase activity (15.90 U/ml) was obtained when 5g/10ml/flask of Wb at SSF was used as the carbon source comparing to that of SmF (11.95U/ml) at 1g/50ml/flask concentration. Ten g of Cc at SSF gave the lowest activity (10.51 U/ml). On the other hand the highest value of extracellular protein content obtained when use 10g of Os (10.40mg/ml) and the lowest value obtained at media contain 3gCc. On the other hand the dry weights do not give an accurate indication to growth rate. Each treatment was carried out in triplicate and the results obtained throughout the work were the arithmetic mean of at least 3 experiment. It also analyzed as a factorial experiment in a randomized complete block design with three replicas.

Table 2(b). Influence of different concentrations of agricultural wastes as a sole carbon source on xylanase activity produced by *Emericella nidulans* at solid state fermentation (SSF)

Substrate	Conc (g/flask)	Xylanase activity (U/ml)	Protein (mg/ml)	Dry wt. (g/50ml)
Os	3	13.91b	8.63b	3.10h
Wb		14.20a	8.98a	2.93i
Ws		13.90b	6.48c	2.83i
Cc		12.60d	5.63d	2.41j
Rs		13.30c	6.29c	2.90i
Os	5	15.30b	9.04b	4.80e
Wb		15.90a	9.51a	5.80e
Ws		14.50c	6.87d	5.15f
Cc		13.800e	6.45e	5.07f
Rs		14.10d	7.32c	4.50g
Os	7	14.10a	10.21a	6.76d
Wb		14.92a	9.85b	6.54d
Ws		14.03b	8.15c	5.97e
Cc		13.50c	7.20e	7.22c
Rs		13.40c	7.94d	7.09c
Os	10	11.20c	10.40a	9.32a
Wb		12.50a	10.2b	9.55a
Ws		11.30c	9.91c	8.79c
Cc		10.51d	7.86e	10.01a
Rs		11.70b	7.91d	8.94b
LSD		0.21	0.19	0.6

Means followed by the same letter(s) are not significantly different at 0.05 level of probability.

Table 3. The activity of xylanase produced by *Emericilla nidulans* at solid state using different inoculums size

Inoculums size (cube/flask)	Xylanase activity (U/ml)	Protein content (mg/ml)	Dry wt (g/50ml)
1	12.39e	7.33d	4.05e
2	15.92d	9.50c	5.82c
3	16.40c	9.97b	5.90b
4	17.97b	10.03b	6.08a
5	19.83a	10.41a	6.27a
6	15.77d	8.43d	5.03d
LSD	0.26	0.25	0.19

Means followed by the same letter(s) are not significantly different at 0.05 level of probability

In liquid state fermentation (SmF), the fungus is exposed to hydrodynamic forces, while in SSF; growth is restricted to the surface of the

Table 4. Classification and Accession number of the tested fungus

Isolate name	*Emericella nidulans*
Anamorph	*Aspergillus nidulans*
Accession number	AS210136.01
Classification	Eukaryota; Fungi;Dikarya; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Eurotiales; Trichocomaceae; *Emericella.*

solid matrix, with no such negative effects [37]. Also, catabolic repression and protein degradation by proteases that are severe problems in SmF, have often been reported to be reduced or absent in SSF[38]. According to[39], xylanase produced by *Humículala nuginosa* was approximately 23-fold higher in SSF than in SmF. Production of pectinases by *Bacillus* sp. in SSF was higher than that produced in SmF[40]. The use of solid-state fermentation bioreactor is not only good for control of microbial fermentation that affects the growth of some important parameters, but also for substantially increase production[41,42]. These results are similar to those obtained with endoglucanases and β-glucosidades [43], xylanases [44], α-amylases[45], and pectinases produced by *A. niger*[46]. Many authors report the advantages of using wheat bran as a substrate for xylanase

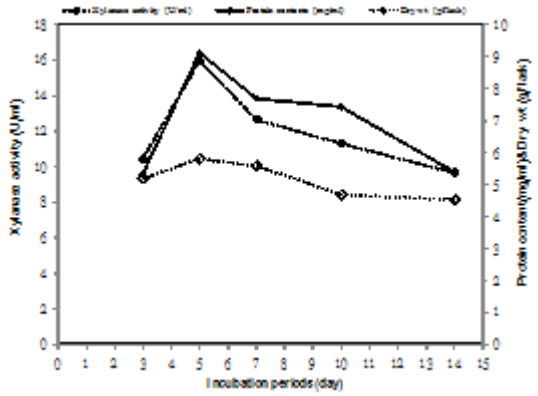

Fig. 1. Xylanase activity of *Emericilla nidulans* at different incubation periods at solid state fermentation

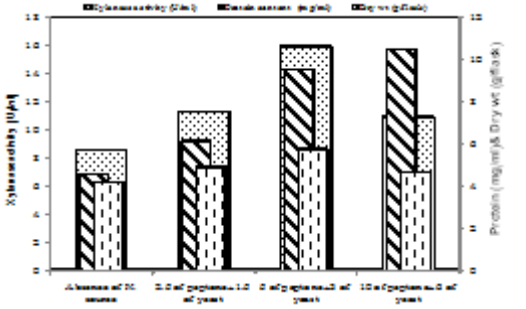

Fig. 2. Effect of different yeast and peptone concentrations on xylanase produced by *Emericilla nidulans* in solid state

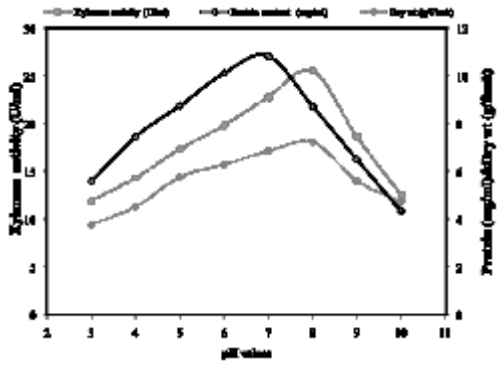

Fig. 3. Effect of different initial pH values on xylanase activity produced by *Emericilla nidulans* at solid state

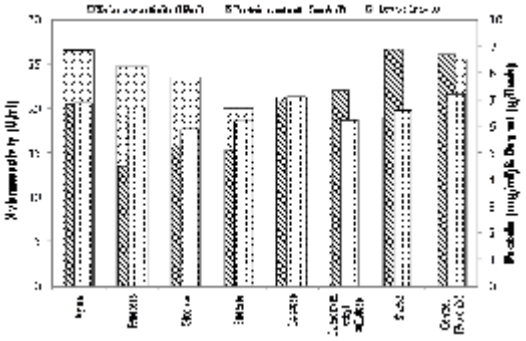

Fig. 4. The effect of additional carbon sources 1% on xylanase activity produced by *Emericilla nidulans* using Wb as a sole carbon source

production in SSF when compared to other solid wastes. Wheat bran holds greatest promise for low cost production of the xylanase enzyme[47]. Cultivation of *Aspergillus tamari* in wheat bran, was obtained a higher enzymatic activity than in sugar cane bagasse and corn cobs[48,49]. *Aspergillus terreus*[50], and *Pleurotus ostreaus*[51], tested the xylanase activity of these fungi in different substrates, and a highest production was obtained with wheat bran, possibly because wheat bran is rich in proteins and hemicelluloses, among which xylan represents about 40% of dry matter[52] which are used by microorganisms as energy and carbon sources, through specific enzymes such as xylanase and α –amylases[53]. According to[48], wheat bran among other substrates used in solid systems, is the most efficient because it does not perform catabolic repression in the enzymatic activity. Some

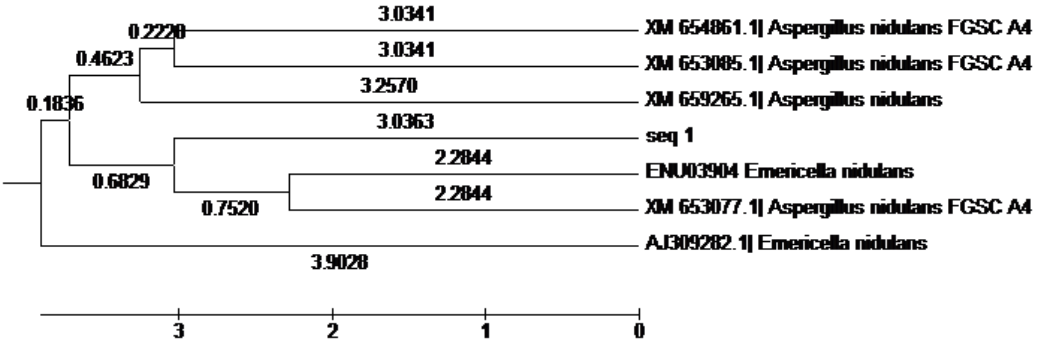

Fig. 5. Phylogenetic tree based on the 18S rRNA sequence data indicating the position of isolate among representatives of the species of the *Emericella nidulans* AS210136.01

ATGATACCAAAAGCTGGTAAAATTAAAATATAAACTTCAGGATGACCAAAAAACCA

AAATAAATGTTGATATAATACTGGATCACCTCCTCCAGATGGATCATAAAAAGAAGT

ATTTAAATTTCTATCAGTTAATAACATAGTAATTGCACCAGCTAATACAGGTAAAGTT

AATAATAAAAGAAAAGCTGTAATTAATACAGACCAAACAAATAAAGGTAATCTATG

AAAACTTAAACCAGGAGCTCGCATATTATAAATTGTTGAAATAAAATTAATAGCACC

TAATAATGATGAAATACCTGTTAAATGTAAACTAAAAATAGCTAAATCTACAGAAGG

TCCTGAGTGTGCTTGTACACTTGATAAAGGTGGATAAACAGTCCAACCAGTACCAGC

ACCTGATTCAACAATAGCTGATGATACTAATAATAATAAAGCAGGTGGTAATAACCA

AAAACTAATATTATTCATACGTGGAAAAGCCATATCAGGTGCTCCTATCATTAAAGG

AACAAACCAATTACCAAAACCACCAATTAAAGCAGGCATAACTAAAAAGAAAACCA

TAATAAAAGCATGTGCTGTAACAATAACATTATATAATTGATGATTCCCCATAAAAA

TTTGATTACCCGGTTGTGCTAATTCCATTCGAATTAAAAGTGATAAAGTTGTACCAAC

AATACCAGCAAAAGCACTAAAAATTAAATATAAAGT

Fig. 6. The sequence of 18s rRNA of the tested fungus. Seq1 [organism=*Emericella nidulans*]

authors suggest that the lack of catabolic repression is because the diffusion processes are slow due to the low water activity in the system[54].

Effect of cultivation time

The xylanase activity and extracellular protein in the culture increased gradually during the first 120h of incubation with the highest activity (15.93U/ml) and maximum extracellular protein production (9.50mg/ml) were obtained at the 5th day of incubation, while on the seventh day there was a large decrease (Fig.1). Several researches on xylanase production report similar data, it was obtained maximum production, using *Aspergillus foetidus,* on the fourth day (80.50 U/ml) and also observed a decrease from the sixth day[55]. *Aspergillus terreus* showed maximum activity (22.03U/ml) on the fourth cultivation day; after this period, a decrease in enzyme production was observed[56]. The maximum production of xylanase 56.31 U/ml for *Fusarium solani* was 6 days of incubation period at 30ºC[57]. Other enzymes have also shown better activities at similar times, such as pectinase and cellulase produced by fungi when cultivated in solid-state fermentation[58].

Effect of different peptone and yeast concentrations as a sole nitrogen source on xylanase production by *Emericilla nidulans* at solid state fermentation

The activity was increased with increasing the peptone and yeast levels, showing its maximum value (15.94U/ml) by using 5g/l of pepton+3g/l of yeast (Fig.2). On the other hands the highest value of extracellular protein content obtained after adding 10g/l of peptone+6g/l of yeast (10.49 mg/ml). Therefore, a concentration of 5g/l of pepton+3g/l of yeast appeared to be the most adequate for xylanase activity as compared to the other concentrations. Organic nitrogen sources like peptone and yeast extract supported better synthesis of the enzyme than any of the organic and inorganic nitrogen sources used alone[59]. Similar result was also reported in xylanase production in solid state fermentation by *Penicillium canescens* where combination of yeast extract and peptone gave the best result[60]. In the absence of any nitrogen sources, indicating nitrogen content in wheat bran was not sufficient to support higher level of enzyme synthesis[61,51]. In contrast among the inorganic nitrogen sources, ammonium sulfate was known to promote xylanase synthesis in certain fungal found unsuitable in the present investigation[62].

Effect of the inoculums concentration

The inoculums concentration was raised from 1 to 5 cubes/flask, the enzyme activity, dry weight and extracellular protein content progressively increased reaching maximum values (19.83 U/ml, 6.27g/flask, 10.41 mg/ml respectively) with 5 cubes inoculums size (Table 3).

The inoculums size was also reported to be an important factor in other microbial fermentation of agro-industrial wastes [63, 6]. In order to verify the enzyme activity, the spore concentration in fungi cultivation must be high enough to colonize the substrate particles[64].

Effect of pH on the culture medium

The xylanase showed a high activity in the pH range of 6.0–9.0, with the optimal pH at 8.0 (Figure 3).The highest xylanase activity (25.54 U/ml), was achieved at initial pH 8.0 with 1.3fold of that at pH 6.0. A maximum protein content (10.83 mg/ml) of culture filtrate was obtained at pH 7.0. Present organism of our study is one among the few alkali tolerant fungal strain founded.

Other alkali tolerant fungi producing xylanase are *Aspergillus flavus*[65], *A. niveus*[59], *A. nidulans*[66], *A. fischeri* [67]and *A. fumigates*[68]. The optimum pH for *A. nidulans* was 6.0[31]. *A. candidus* give maximum activity at pH 8.0[69]. The rise of medium pH may be associated with release of nitrogenous protein metabolites in the media[70].

Effect of the addition of other carbon sources on xylanase synthesis (additives)

Seven sugars were individually tested as additives to the sole carbon source (Wb) to evaluate *Emericilla nidulans* growth and xylanase activity. Following to the culture conditions used here, xylan as the co- carbon source showed the highest xylanase activity (26.60 U/ml), followed by fructose (24.88U/ml) which had almost nearly xylanase activities as on control (Wb only) (25.54 U/ml) respectively (Figure 4). Addition of carboxymethyl cellulose (CMC) to wheat bran did not induce higher levels of enzyme production because CMC could be produced during the fermentation, which hydrolyzes the substrate in cellobiose, leading to a repressive action on xylanase production[71]. The highest activity was exhibited by the pure substrate, such results may be due to the catabolic repression processes when easily assimilated carbon sources (e.g. sorbitol)

were added[34]. Xylanase production by *T. harzanium* was also more advantageous without the addition of other carbon sources[72,73]. However, mechanisms of synthesis control vary considerably among microorganisms[74]. Using *Streptomyces* A-151, a low efficiency of xylanase production after the addition of other carbon sources in cultures using rice bran[75], which also corroborates the results obtained by[76-78].

PCR amplification and sequencing of 18S rRNA

The fungus-specific primers 5‘ AACTTAAGGAAATTGACGGA 3‘ and 5‘TCCGCAGG -TTCACCTACGGA 3‘ were used to amplify a 750-bp fragment within the gene coding for the small ribosomal subunit18S rRNA of fungus. Amplification was performed in a Thermal Cycler Gene Amp PCR System9700, Applied Biosystems, and Norwalk, USA. Thermal amplification cycle was used to amplify the fungal 18S rRNA. The sequence was submitted to the Gene Bank and the sequences Fig. (6) have the accession numbers in Table (4). The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 12.61036518 was shown Fig. (5), the tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distance was computed using the Maximum Composite Likelihood method and is in the units of the number of base substitutions per site. Phylogenetic analysis was conducted in MEGA4.

Six of fungi which have similar nucleotide sequence (according to BLAST) and may be on molecular basics related to the fungus we tested were compared and an analytical phylogenetic tree was drown Fig. (5). The data showed that the tested isolate have a molecular relationship with other *Asperigellus nidullans* from the same cluster of Ascomycota.

CONCLUSION

We optimized the culture conditions for high xylanase production with best activity (25.54U/ml) by the industrially important thermophilic alkali tolerant fungus *Emericella nidulans* AS210136.01 on inexpensive agricultural by-product wheat bran as a carbon source (pH8.0, 45°C, 120h) using an inoculum of 5 cubes/flask. The results obtained indicate a significant increase in xylanase production with the use of nutrients and suitable growth conditions under SSF, as compared to SmF. As demonstrated in other studies, pure wheat bran was the best substrate for enzyme production. The high xylanase activity and the low production cost may further broaden this application to the industrial scale. Commercial applications for xylanase include the chlorine-free bleaching of wood pulp and paper industry. More studies are required to evaluate and explore the potentiality of this fungus. The results indicated a potentiality of xylanase, as functional food additives to poultry.

REFERENCES

1. Huisman, M.M.H., Schols, H.A., Voragem, A.G.J. Glucuronoarabino xylan from maize kernel cell walls are more complex than those from Sorghum kernel cell walls. *Carbohyd. Polym.*, 2000; **43**: 269-279.
2. Bastawde, K.B. Xylan structure, microbial xylanases, and their mode of action. *World J. Microbiol. Biot.*, 1992; **8**: 353- 368.
3. Maheshwari, R. Thermophilic fungi: their physiology and enzymes. *Microbiol. Mol. Biol. Rev.*, 2000; **64**: 461–488.
4. Singh, S. *Thermomyces lanuginosus:* Properties of strains and their hemicellulases. *FEMS Microbiol. Rev.*, 2003; **27**:3–16.
5. Biely, P. Microbial xylanolitic systems. Trends in *Biotechnol.*, 1985; **3**: 286-290.
6. Beg, Q.K., Kapoor, M., Mahajan, L., Hoondal, G.S. Microbial xylanases and their industrial applications. *Appl. Microbiol. Biot.*, 2001; **56**:326-338.
7. Sonia, K.G. Sorghum straw for xylanase hyper-production by *Thermomyces lanuginosus* (D2W3) under solid state fermentation. *Bioresour Technol*., 2005; **96**: 1561–156.
8. Bajpai, B. The impact of xylanases on bleaching of eucalyptus kraft pulp. *Biotechnol.*, 1994; **38**: 1–6.
9. Crittenden, R.G. Production, properties and application of food-grade oligosaccharides. Trend *Food Sci. Technol*., 1996; **7**:353-361.
10. Wong, K.K.Y., Tan, L.U.L., Saddler, J.N. Multiplicity of 3-1,4-xylanase in microorganisms: Functions and applications. *Microbiol. Rev*., 1988; **52**: 305-317.
11. Kulkarni, N.A., Shendye, A., Rao, M. Molecular and biotechnological aspects of xylanases. *FEMS.Microbiol. Rev*., 1999; **23**:411-456.

12. Subramaniyan, S., Prema, P. Biotechnology of microbial xylanases: Enzymology, molecular biology and application. Crit. *Rev. Biotechnol.*, 2002; **22**: 33-46.
13. Haltrich, D.; Nidetzky, B. Kulbe, K.D. Steiner, W. Zupancic, S. Production of fungal xylanases. *Bioresour. Technol*.,1996; **58**: 137-161.
14. Singh, S. Thermal stability of ²-xylanases produced by different *Thermomyces lanuginosus* strains. *Enzyme Microb. Technol*., 2000a; **26**: 502–508.
15. Singh, S. Relatedness of *Thermomyces lanuginosus* strains producing a thermostable xylanase. *J. Biotechnol*., 2000b; 81: 119–128.
16. Sinner, M. VAIBiobleiche. *Das Papier*, 1991; **45:**, 403–410.
17. Lenartovicz, V., Souza, C.G.M., Moreira, F.G., Peralta, R.M. Temperature effects in the production of multiple xylanases by *Aspergillus fumigates*. *J. Basic Microb.*, 2002; **42**: 388-395.
18. Kuhad, R.C., Manchanda, M., Singh, A. Optimization of xylanase production by a hyperxylanolitic mutant strain of *Fusarium oxysporum. Process Biochem*., 1998; **33**: 641-647.
19. Fadel, M. Production physiology of cellulases and glicosidases enzymes of *Aspergillus niger* grown under solid state fermentation conditions. *J. Biol. Sci*., 2000; **1**: 401-411.
20. Cazetta, M.L. Fermentation of molasses by *Zymomonas mobilis*: effects of temperature and sugar concentration on ethanol production. *Bioresour. Technol.*, 2007; **98**: 2824–2828.
21. Addleman, K., Archibald, F.S. Kraft pulp bleaching and delignification by dikaryons and monokaryons of *Trametes versicolor*. *Appl. Environ. Microbiol*., 1993; **59**:266-273.
22. Park, Y.S., Kang, S.W., Lee, J.S., Hong, S.I. Xylanase production in solid state fermentation by *Aspergillus niger* mutant using statistical experimental designs. *Appl. Microbiol. Biotechnol.*, 2002; **58**: 761-766.
23. Singh, S.K., Garg, A.P. Bioconversion of pea crop residues using *Gliocladium virens*. *J. Indian Bot. Soc*., 1996; **75**: 245-250.
24. La-Grange, D.C., Pretorius, S.I., Claeyssens, M., Vanzyl, W.H. Degradation of xylan to D-xylose by recombinant *Saccharomyces cerevisiae* co-expressing the *Aspergillus niger* ²- Xylosidase (XIND) and the *Trichoderma reesei* xylanase ll (xyn2) Genes. *Genome*, 2001; **67**: 5512-5519.
25. Bailey, M.J., Biely, P., Poutanen, K. Inter laboratory testing of methods for assay of xylanase activity. *J. Biotechnol*., 1992; **23**: 257-271.
26. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 1959; **31**: 426-428.
27. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*., 1951; **193**: 265-275.
28. Liebmann, B., Friedl, A., Filipa, J. Lignocellulosic biomass dissolution and Fractioning using ionic liquids as a solvent. *Chemical Engineering*., 2012; **29**:555-558.
29. Sendcor, G.W., Cochran, W.G. Statistical Methods. 7th edn., The Iowa State University Press, Ames, Iowa, USA., 1982; pp 507.
30. Deacon, J.W. Environmental conditions for growth and tolerance of extremes. In: Modern Mycology, 3rd edn., Blackwell Science Oxford, 1997; pp 121-135.
31. Subramaniyan, S., Prema, P. Biotechnology of microbial xylanases: Enzymology, molecular biology and application. *Crit. Rev. Biotechnol.*, 2002; **22**: 33-46.
32. Raj, K.C., Chandra, T.S. Purification and characterization of xylanase from alkali tolerant A*spergillus fischeri* fxn1.*FEMS. Microbiol. lett*., 1996; **1453**: 457-461.
33. Kheng, P.P., Ibrahim, C.O. Xylanase production via solid-state fermentation. *Sci. Technol*., 2005; **27**: 332.
34. Biswas, S.R., Jana, S.C., Mishra, A.K., Nanda, G. Production, purification and characterization of xylanase from a hyperxylanolitic mutant of *Aspergillus achraceus*. *Biotechnol. Bioeng*., 1990; **35**: 244-251.
35. Yaun, Q., Rugyu, M. Study on temperature oscillation in production of xylanase by *Aspergillus niger*. *Beijing Hugagong Daxue Xuebao*., 1999; **26**:11-16.
36. Rahman, A.K., Sugitani, N., Hatsu, M., Takamizawa, K. A role of xylanase, alpha-arabinofuranosidase, and xylosidase in xylan degradation. *Can. J. Microbiol*., 2003; **49**: 58-64.
37. Da Silva, R., Lago, E.S., Merheb, C.W., Macchione, M.M., Park, Y.K. Production of xylanase and CMCase on solid state fermentation in different residues by *Thermoascus aurantiacus*. *Braz. J. Microbiol.*, 2005; **36**: 235-241.
38. Aguilar, C.N., Augur, C., Favela-Torres, E., Viniegra-González, G. Production of tannase by Aspergillus niger Aa-20 in submerged and solid-state fermentation: influence of glucose and tannic acid. *J. Ind. Microbiol. Biotechnol*., 2001; **6**(5): 296-302.

39. Kamra, P., Satyanarayana, T. Xylanase production by the thermophilic mold *Humicolala nuginosa*in solid state fermentation. *Appl. Biochem. Biotechnol*., 2004; **119**: 145-158
40. Soares, M.C.N., Silva, R., Gomes, E. Screening of bacterial strains for pectinolytic activity: Characterization of the polygalacturonase produced by *Bacillus* sp. *Rev. Microbiol.*,1999; **30**: 299-303.
41. Xian, J.D., Ming, Q.L., Hong, X.J., Ming, Y.J. Optimisation of solid-state fermentation of *Aspergillus niger* JL-15 for xylanase production and xylo-oligosaccharides preparation. *Czech J. Food Sci*., 2011; **5**: 557–567.
42. Nan, Jin., Shuhan, Ma., Yan, Liu., Xiuli, Yi., Rong, He., Hui, Xu., Dai, Rong., Yi, Cao**.** Thermophilic xylanase production by *Aspergillus niger* in solid state fermentation using wheat straw and corn cob. *African J. of Microbiol. Research*., 2012; **6**(2): 2387-2394.
43. Grajek, W. Comparative studies on the production of cellulases by thermophilic fungi in submerged and solid-state fermentation. *Appl. Microbiol. Biotechnol.*, 1978; **26**: 126-129.
44. Deschamps, F., Huet, M.C. Xylanase production in solid-state fermentation: A study of its properties. *Appl. Microbiol. Biotechnol*., 1985; **22**: 177-180.
45. Lonsane, B.K., Ramesh, M.V. Production of bacterial thermostable enzymes by solid state fermentation: A potential tool for achieving economy in enzyme production. *Adv. Appl. Microbiol*., 1992; **15**: 1-48.
46. Acunã-Arguelles, M.E., Gutiérrez-Rojas, M., Viniegrea-González, G., Favela-Torres, E. Production and properties of three pectinolytic activities produced by *Aspergillus niger* in submerged and solid-state fermentation. *Appl. Microbiol. Biotechnol.*, 1995; **43**: 808-814.
47. Okafor, U.A., Okochi, V.I., Onyegeme, B.M., Nwodo-Chinedu, S. Xylanase production by *Aspergillus niger* ANL 301 using agro – wastes. *African J. of Biotechnol.*, 2007; **6** (14): 1710-1714.
48. De Souza, D.F., De Souza, C.G.M., Peralta, R.M. Effect of easily metabolizable sugars in the production of xylanase by *Aspergillus tamarii* in solid-state fermentation. *Process Biochem.*, 2001; **36**,: 835-838.
49. Ferreira, G., Boer, C.G., Peralta, R.M. Production of xylanolytic enzymes by *Aspergillus tamari* in solid state fermentation. *Microb.Lett.*, 1999; **173**:335-339.
50. Gawande, P.V., Kamat, M.Y. Production of xylanases by immobilized *Aspergillus* sp.using lignocellulosic waste. *World J. Microb.Biot*., 2000; **16**:111-112.
51. Qinnghe, C., Xiaoyu, Y., Tiangui, N., Cheng, J., Qiugang, M. The screening of culture condition and properties of xylanase by white rot fungus *pleurotus ostreatus. Process Biochem*., 2004; **3**: 1561-1566.
52. Beaugrand, J.,Crônier, D., Debeire, P., Chabbert, B. *Cereal Science*, 2004; **40**: 223–230.
53. Adolph, S., Müller, S.R., Siedenberg, D., Jager, K., Lehmann, H., Schüerl, K., Giuseppin, M. Cultivation of *Aspergillus awamorii*on wheat bran: ultrastructural description of growth and localization of xylanase by immunogold labeling. *Biotechnol.*, 1996; **46**: 221-234.
54. Ramesh, M.V., Lonsane, B.K. Ability of a solid-state fermentation technique to a significantly minimize catabolic repression of amylase production by *Bacillus licheniformis*M27. *Appl. Microbiol. Biotech*., 1991; **35**: 591-593.
55. Shah, A.R., Madamwar, D. Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterization. *Process Biochem*., 2005; **40**: 1763-1771.
56. Ghanem, N.B., Yusef, H.H., Mahrouse, H.K. Production of *Aspergillus terreus* xylanase in solid-state cultures: Application of the Plackett-Burman experimental design to evaluate nutritional requirements. *Bioresource Technol*., 2000; **73**: 113-121.
57. Kumar, K.S., Manimaran, A., Permaul, K., Singh, S. Production of β-xylanase *Thermomyces lanuginosus* MC134 mutant on corn cobs and its application in biobleaching of bagasse pulp. *Biosci. Bioeng*., 2009; **107**: 494-498.
58. Desgranges, C., Durand, A. Effect of pCO2 on growth conidiation and enzyme production in solid-state culture of *Aspergillus niger* and *Trichoderma viride*. *Enzyme Microb*. Tech., 1990; **12**: 546-551.
59. Gang, Z.Z., Zhuol, Y., Xiao, Q.P., Zhong, L.W. Improving the thermostability of *Geobacillus stearothermophilus* xylanase XT6 by directed evolution and site-directed mutagenesis. *Bioresour. Technol*., 2010; **101**: 9272-9278.
60. Bakri, y., Jacques, P., Thonart, P. Xylanase production by *Penicillium canescens* 10-10c solid state fermentation. *Appl. Biochem. Biotechnol*., 2003; **48**:105-108.
61. Sudan. R., Bajaj, B.K. Production and biochemical characterization of xylanase from an alkali tolerant novel species *Aspergillus niveus* RS2. *World J. Microbiol. Biotechnol.*,

2007; **23**: 491-500.
62. Seyis, I., Aksoz, N. Effect of carbon and nitrogen sources on xylanase production by *Trichoderma harzianum* 1073 D3. Int. *Biodeter. Biodeg*., 2005b; **55**: 115-119.
63. Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller, R.C., Warren, R.A. Domains in microbial beta-1,4-glyconases, sequence conservation, function and enzyme families. *Microbiol. Rev*.,1991; **55**: 303-315.
64. Sikyta, B. Development of microbial process. *Method Ind. Microb*., 1983; 250-274.
65. De Souza, C.G.M., Girardo, N.C., Peralta, R.M. Influence of growth conditions on the production of xylanolytic enzymes by *Aspergillus flavus*. *Basic Microbiol*., 1999; **39**: 155-160.
66. Taneja, K., Gupta, S., Kuhad, R.C. Properties and application of a partially purified alkaline xylanase from an alkalophilic fungus *Aspergillus nidulans* KK-99. *Bioresour Technol*., 2002; **85**: 39-42.
67. Senthilkumar, S.R. Optimization of medium composition for alkali-stable xylanase production by *Aspergillus fischeri* Fxn 1 in solid-state fermentation using central composite rotary design.*Bioresour. Technol*., 2005; **96**: 1380-1386.
68. Achouri, I.M., Guerfali, M., Gargouri, A., Belghith, H. Production of xylo-oligosaccharides from agro-industrial residues using immobilized *Talaromyces thermophilus* xylanase. *Molecular Catalysis*., 2009; **59**: 145-152.
69. Kumar, V., Garai, D. Response surface optimization for xylanase with high volumetric productivity by indigenous alkali Tolerant *Aspergillus candidus* under submerged cultivation. *Biotech*., 2012; **10**: 3205-012.
70. Silva, L.A., Camona, E.C. Production and characterization of cellulase-free xylanase from *Trichoderma inhamatum*. *App. Biochem. Biotechnol*., 2008; **150**: 117-125.
71. Ahmed, S., Ain, Q., Aslan, N., Naeem, S., et al. Induction of xylanase and cellulase genes from *Trichoderma harzianum* with different carbon sources. *Pakistan J. Biol. Sci*., 2003; **6**: 1912-1916.
72. Seyis, I., Aksoz, N. Investigation of factors affecting xylanase activity from *Trichoderma harzianum* 1073 D3. *Braz. Arch. Biol. Tech*., 2005a; **48**: 187-193.
73. Seyis, I., Aksoz, N. Effect of carbon and nitrogen sources on xylanase production by *Trichoderma harzianum* 1073 D3. *Int. Biodeter. Biodeg*., 2005b; **55**: 115-119.
74. DeVries, R.P., Kester, H.C.M., Poulsen, C.H., Benen, G.A.E., Visser, J**.** Synergy between enzymes from *Aspergillus* sp. involved in the degradation of plant cell wall polysaccharides. *Carbohyd. Res*., 2000; **327**: 401-410.
75. Wang, S.L., Yen, Y.H., Shih, I.L., Chang, A.C., Chang, W.T., Wu, W.T., Chai, Y.D. Production of xylanases from rice bran by *Streptomyces actuosus* A-151. *Enzyme Microb*. Tech., 2003; **33**: 917-925.
76. Chan, A.Y., Chan, M.M., Lo, H.M., Leung, Y.C., Lim, B.L. A dual protein expression system in *Bacillus subtilis*. *Protein Expres. Purif*., 2002; **26**: 337- 342.
77. Rani, D.S., Nand, K. Purification and characterization of xylanolytic enzymes of a cellulose free thermophilic strain of *Clostridium absonum* CFR-702. *Anaerobe*., 2001; **7**: 45-53.
78. Bakir, U., Yavascaoglu, S., Guvenc, F., Ersayin, A. An endo-1,4- xylanase from *Rhizopus oryzae*: Production, partial purification and biochemical characterization. *Enzyme Microb. Tech.*, 2001; **29**: 328-334.