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Bioprocessing of Lignocellulosic Biomass for Production of Bioethanol using Thermotolerant *Aspergillus fumigatus* under Solid State Fermentation Conditions

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Abstract: Screening of cellulolytic activity by ten fungal isolates recovered from soil and agricultural wastes indicated that *Aspergillus fumigatus* showed the highest cellulolytic activity, under solid-state fermentation (SSF) using rice straw as substrate. Optimization of fermentation conditions showed that highest cellulolytic enzymes production on 4th day at pH 5.5 and at 40°C. The production of enzymes was reached its maximal value at 5.0 g/flask of rice straw. Avicellase, endoglucanase and CMCase productivity were highly increased by addition of L-asparagine, NH_4Cl and $NaNO_3$, respectively as N-sources to the fermentation medium. Maximum activities of avicellase and endoglucanase were recorded at inoculum size of 2.5 mL, while maximum activity of CMCase was detected at inoculum size of 0.5 mL. The influence of various physico-chemical factors "incubation period, temperature, pH and substrate concentration" on enzyme activity was also investigated. The half life time of avicellase, endoglucanase and CMCase at 60°C were 67.8, 79.1 and 96.5 min, respectively. Rice straw hydrolysate obtained through enzymatic hydrolysis of rice straw was used as a substrate for bioethanol production. Maximum ethanol production by *Saccharomyces cereviseae* using dilute acid pretreated rice straw hydrolysate with initial soluble sugar 4.1055±0.0146% was recorded after 48 h of fermentation (2.26424±0.0206%).

Key words: Cellulases, rice straw, solid-state fermentation, *Aspergillus fumigates*, bioethanol

INTRODUCTION

Currently, the feedstock for bioethanol production is sugar substances, such as sugarcane juice and molasses, as well as starch-based materials such as wheat and corn. The production of ethanol from sugars or starch impacts negatively on the economics of the process, thus making ethanol more expensive compared with fossil fuels. Hence the technology development focus for the production of ethanol has shifted towards the utilization of residual lignocellulosic materials to lower production costs. Production of bio-ethanol from biomass is one way to reduce both the consumption of crude oil and environmental pollution. Lignocellulosic material is regarded as a promising energy source because it is potentially low cost renewable source of mixed sugars for fermentation to fuel ethanol and rice straw is one of the abundant lignocellulosic waste materials in the world (Howard *et al.*, 2003). The main constituents of lignocellulosic biomass (rice straw) are cellulose, hemicellulose and lignin and the minor components include ash and extractives such as waxes (Brown, 2003). Cellulases responsible for the hydrolysis of cellulose are composed of three major enzymes. These are endoglucanses (endo-1, 4-β-glucanase; EC 3.2.1.4), cellobiohydrolase (Exoglucanase; EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Endoglucanases are proposed to initiate attack randomly at multiple internal sites in the amorphous regions of cellulose fiber opening-up sites for subsequent attack by the cellobiohydrolases (Wood, 1991). Exoglucanase remove mono- and dimers from the end of the glucose chain. Subsequently β-glucosidase hydrolyses glucose dimers to glucose. Since the use of cellulose degrading enzymes is related to industrial processing and operating at high temperature, application of thermostable enzymes produced by mesophilic or thermophilic fungi appears to be advantageous. *Aspergillus* is known to be a good producer of cellulases. Cellulase production has been described for many *Aspergillus* species (Wang *et al.*, 2006; Sherief *et al.*, 2010).

Solid-state fermentation (SSF) has emerged as a potential technology for the production of microbial products, it has gained much importance in the

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production of microbial enzymes due to several economic advantages over conventional submerged fermentation include low waste water output, simple and cheap media for fermentation, high product yield, lower risk of contamination, easier scale up of processes, lower capital investment and lower operating expenses (Panagiotou *et al*., 2003). Conversion of lignocelluloses to ethanol employs three major steps including: (1) substrate pretreatment, (2) hydrolysis with a combination of enzymes to reduce the cellulose to glucose and (3) microbial fermentation of glucose to ethanol (Sun and Cheng, 2002).

In the present study, a strain of *A. fumigatus*, proved to be a good producer of cellulase, was isolated and its ability of cellulases production under SSF was investigated. Moreover, some of the critical factors affecting cellulases production and activity by this strain were also optimized. *Saccharomyces cereviseae* was used for bioethanol production from rice straw hydrolysate.

MATERIALS AND METHODS

Substrate: Rice straw was chosen as natural substrate for solid state fermentation because of their abundance in the local area. Rice straw was collected from local farmers at the end of the harvest season (2008), air-dried and milled into 1 cm particles.

Microorganisms and maintenance of cultures: The fungi used in this study were locally isolated from soil samples collected from rice fields in Dakahlia governorate according to the procedures adopted by Johnson *et al*. (1960). The cultures were maintained on potato dextrose agar (PDA) medium at 4°C. Fungal strains were subjected to full identification using the most recent sophisticated facilities; an Imaging analysis system using soft-imaging GbH software (Analysis Pro ver.3.0) at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Egypt.

Survey for cellulolytic enzymes production: The isolated strains were cultured on rice straw for the selection of the most active cellulolytic strain for further investigations according to modified method of Purkarthofer *et al*. (1993).

Dilute acid pretreatment of rice straw: Fifteen grams of rice straw was milled and screened to about 0.1 cm and suspended in 300 mL of 1% H_2SO_4 (v/v). The mixture was then autoclaved at 121°C for 2.0 h in a 500 mL Erlenmeyer flask. The residues were collected and washed extensively with tap water until pH 5, dried at 45°C for 3 days. Grind dried biomass to ensure more uniform particle size distribution.

Alkali pretreatment of rice straw: Twenty grams of milled dried rice straw was suspended in 160 mL 1% (w/v) NaOH and pretreated in an autoclave at 121°C for 1 h. The residues were collected and washed extensively with water until washings turned neutral. The residues were dried at 65°C for two days. Dried biomass is grinded to ensure more uniform particle size distribution.

Solid state fermentation: Dilute acid pretreated rice straw was used as the solid substrate for SSF. Experiments were conducted in 250 mL Erlenmeyer flasks containing 3 g of pretreated rice straw moistened with 3 mL of buffered basal medium (pH 5.0) containing the following (g L^{-1} 0.1 M sodium acetate buffer): $NaNO_3$, 3; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01. The flasks were autoclaved at 121°C for 30 min, inoculated with 3 mL of (3×10^7) spore suspension of *Aspergillus fumigatus* and the contents of the flasks were thoroughly mixed. Flasks were incubated at 30°C, under static conditions for 4 days. After fermentation, the contents of the flasks were harvested for enzyme extraction and assay.

Enzyme extraction and assay: Cellulases were extracted by suspending the fermented rice straw in 10 fold 0.1 M sodium acetate buffer (pH 5.0) and thoroughly mixed on a rotary shaker (200 rpm) for 30 min at 30°C and extract was kept for 6 h in the refrigerator. Following this, the residue of substrate and the fungal biomass were separated by centrifugation (5000 rpm for 20 min) according to Shamala and Screekantiah (1986). The clarified supernatant represented the crude enzyme preparation was used for assaying different enzyme activity. Endoglucanase, avicellase and carboxymethylcellulase (CMCase) activities were assayed by measuring the release of reducing sugars in the reaction mixture of 0.1 mL of crude enzyme and 0.1 mL of 1.0% (w/v) amorphous cellulose (for endoglucanase), carboxymethylcellulose (for CMCase) or avicel PH 101 (for avicellase) dissolved in 0.1 M acetate buffers (pH 5.0) and incubated for 1 h (for endoglucanase and avicellase) and 45 min (for CMCase) at 45°C. The amount of reducing sugars released by active enzyme was determined by Nelson (1944) and Somogyi (1952) method against boiled enzyme as control with D-glucose as standard. One unit (U) of endoglucanase, avicellase and CMCase activity is defined as the amount of enzyme releasing 1 μmol of glucose per minute under assay condition.

Determination of soluble protein: The soluble protein concentration was determined according to Bradford (1976), using crystalline bovine serum albumin as standard.

Optimization of fermentation conditions for cellulolytic enzyme production: Various process parameters influencing the enzyme production during SSF were studied for maximal enzyme production as follows: The effect of incubation period was evaluated through 24 h interval by checking the enzyme activity. The effect of cultivation temperature on the enzyme production was examined at different temperatures (20-60°C). The effect of initial pH on the enzyme production was examined using different pH levels (4-8) of moistening agent (buffered basal medium) which adjusted with 0.1 M sodium acetate and 0.1 M phosphate buffers. The effect of substrate concentration on the enzyme production was done by using different weights of rice straw (1.0 - 7.0 g/flask). The effect of initial total moisture content was carried out using different moisture content (33, 50, 65, 75, 85 and 90%), adjusted using various volumes of buffered basal medium. The effect of supplement of different nitrogen sources was examined on equivalent nitrogen basis. The optimization of inoculum level was carried out by using different inoculum size.

Physicochemical properties of the enzymes: The optimum incubation period of reaction mixture at which the maximum activity of the enzymes was determined at different time intervals. The effect of temperature on the enzyme activity was determined after incubation of the crude extract of enzyme with its specific substrate at different temperatures (30-70°C) for 20 min for CMCase and endoglucanase, 10 min for avicellase. The optimum pH level for enzyme activity was determined by incubating each enzyme with its specific substrate at different pH levels (4-8) under optimal conditions. Effect of different substrate concentration on the enzyme activity was determined using 0.1 mL enzyme incubated with different concentrations of the substrate (0.50-3.0%) under optimal conditions. The thermal and pH stabilities of different enzymes were examined. The calculated half life time ($T_{1/2}$), D value and different deactivation constants were detected through linear regression analysis of obtained data using Graph-Pad Prism 4 software.

Degradation of pretreated rice straw into fermentable sugars: Forty milliliter of crude enzyme produced by *Aspergillus fumigatus* was incubated with 2 g of dilute or alkaline pretreated rice straw for 24 h at 50°C with constant stirring. The free reducing sugars produced were determined by anthrone method (Trevelyan and Harrison, 1952).

Fermentation process: Pooled culture filtrate (initial reducing sugars of rice straw hydrolysate) was further inoculated with Baker's yeast *Saccharomyces cereviseae* strain (identified by Biotech international R and D, Egypt) and allowed for fermentation for 3 days (Harpreet *et al*., 1998). After fermentation it was filtered and ethanol content was determined. In addition the remaining sugar after 3 days fermentation was measured. For calculation of fermentation efficiency, the following equation was applied:

$$\text{Fermentation efficiency} = \frac{\text{Actual ethanol recovery}}{\text{Theoretical recovery}} \times 100$$

Where:

Actual ethanol recovery = Actual ethanol obtained
Theoretical recovery = Amount of initial sugar content in fermentation solution ×0.64

Ethanol estimation: Determination of ethanol content was done by spectrophotomentric method (Caputi *et al*., 1968).

Determination of yeast from fermentation process: The concentration of yeast in the fermentation mash was determined gravimetrically as grams of dry weight in 1000 mL. The Centrifuged and washed cells were dried to a constant weight at 103-105°C for 12 h (APHA, 2005). Duplicate determinations were made on all samples.

RESULTS AND DISCUSSION

Ten fungal species were isolated. These species were belonging to two classes including: Ascomycetes and Deutromycetes. A number of Ascomycetes were the most frequent (i.e., 8 species belonging to the same genus): *Aspergillus terreus*, *A. flavus*, *A. ornatus*, *A. niger*, *A. wentii*, *A. orchraceus*, *A. viridi-nutans* and *A. fumigatus*. Deutromycetes was the second class. This class include two genera were identified as, *Trichoderma viride* and *Penicillium chrysogenum.* Screening of cellulase enzymes production by fungal isolates recovered from agricultural wastes was carried out; all the isolated fungal species were capable of producing cellulolytic enzymes. *Aspergillus fumigatus* and *Trichoderma viride* showed relatively high cellulolytic activity. The remaining isolates were found to be relatively moderate to weak cellulolytic enzymes producers.

Substrate pretreatment: An important goal of pretreatment is to increase the surface area of lignocellulosic material, making the polysaccharides more susceptible to hydrolysis. Along with an increase in

Table 1: Effect of different temperature on the production of cellulolytic enzymes

Temperature (°C)	Enzyme activity (IU g^{-1})		
	Endoglucanase	Avicellase	CMCase
20	1.328	0.428	2.875
30	1.519	0.572	4.921
40	3.217	1.766	6.798
50	2.207	1.277	4.864
60	0.879	0.137	2.290

Table 2: Effect of different moisture levels on the production of cellulolytic enzymes

Moisture levels (%)	Enzyme activity (IU g^{-1})		
	Endoglucanase	Avicellase	CMCase
33	1.05	1.55	3.14
50	2.92	1.87	5.56
67	3.46	1.92	8.91
75	3.10	2.34	7.40
80	2.81	2.31	6.91
83	2.79	2.09	6.39
86	2.80	2.01	6.33

surface area, pretreatment effectiveness and hydrolysis improvement has been correlated with removal of hemicellulose and lignin and the reduction of cellulose cyrstallinity (McMillan, 1994).

Factors influencing the production of cellulolytic enzymes by *Aspergillus fumigates* through SSF: Since *Aspergillus fumigates* was the highest cellulolytic isolate, compared to all isolated fungi, it was selected for further detailed studies. Studies have been subsequently carried out in an attempt to increase the production and activity of the cellulolytic enzymes produced extracellularly by *A. fumigatus* by evaluating the effects of environmental, physiological and nutritional factors on cellulolytic enzymes production including; avicellase, endoglucanase and CMCase under SSF using rice straw as sole carbon source in the production medium.

Effect of incubation temperatures: The data in Table 1 indicate that the temperature is greatly affecting the production of cellulolytic enzymes. Data indicate that maximum activities of CMCase, endoglucanase and avicellase were obtained at 40°C (6.798, 3.217 and 1.766 IU g^{-1} for CMCase, endoglucanase and avicellase respectively). The optimum temperature of the production of avicellase, endoglucanase and CMCase in the present investigation is in good agreement with the reports for other fungal species (Fadel, 2000; EL-Tanash, 2007).

Effect of different moisture levels: The results obtained in Table 2 indicated that the maximum production of CMCase and endoglucanase enzymes was obtained at 67% (8.91 and 3.46 IU g^{-1}, respectively) and at 75% for avicellase (2.34 IU g^{-1}). It is clear from the present results

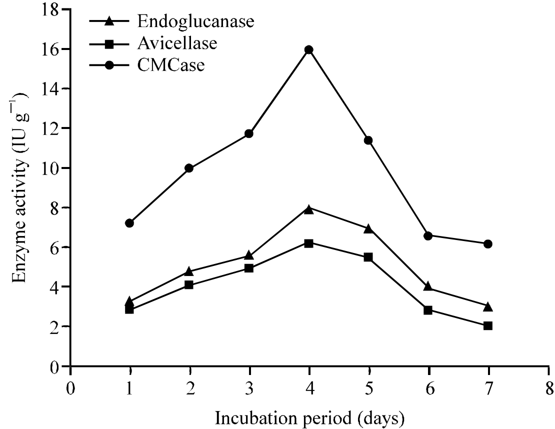

Fig. 1: Effect of different incubation periods on the production of cellulolytic enzymes

that cellulolytic enzyme production are increased at moderate moisture levels (67 and 75%); while, decreased under low levels (33-50%) and higher moisture levels (80-86%). These results are in agreement with those reported by Jecu (2000) and Panagiotou *et al.* (2003) they recorded that an increase in the initial moisture content of the substrate from 60-75% greatly enhanced the cellulase production. However, a further increase to 80% had a negative effect on the production of cellulolytic enzymes. Increase in moisture level is believed to decrease enzyme productivity due to decreasing substrate porosity, alteration in particle structure, lowering oxygen transfer, higher soluble protein and increase bacterial contamination. On the other hand, low moisture content causes reduction in the solubility of nutrients, low degree of substrate swelling as well as reduction in soluble protein (Gervais and Molin, 2003).

Effect of different incubation periods: Data illustrated in Fig. 1 showed that the maximum activities of CMCase, endoglucanase and avicellase were 15.945, 7.972 and 6.245 IU g^{-1}, respectively. The enzyme activity was increased gradually from the 1st day of incubation and reaches its maximum production in the 4th day, then decreased gradually until the 7th day of incubation. This result is more or less similar with that obtained by Jecu (2000), Panagiotou *et al.* (2003) and Narasimha *et al.* (2006) they reported that optimum incubation period of cellulolytic enzymes during SSF of lignocellulosic residues was 3-8 days. On the other hand, the highest cellulase activity was observed on the 10th day in *Rhizopus stolonifer* mediated solid state fermentation using *cassava* tubers (Pothiraj *et al.*, 2006).

Effect of initial pH levels: The results illustrated in Fig. 2 showed that the optimal initial pH for maximum

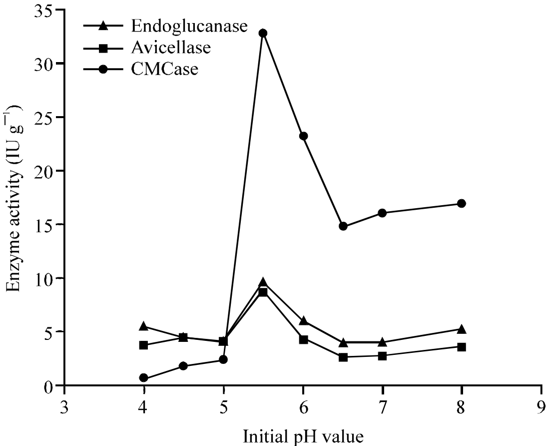

Fig. 2: Effect of different initial pH values on the production of cellulolytic enzymes

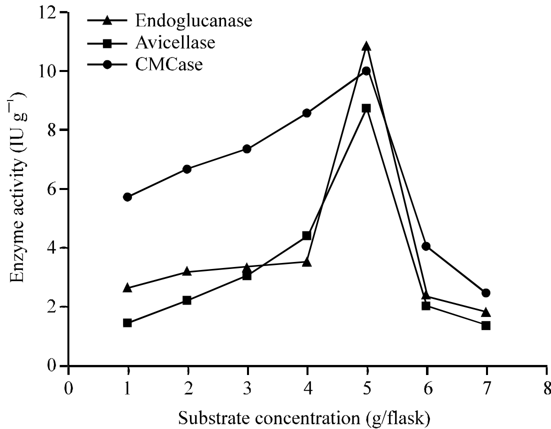

Fig. 3: Effect of different substrate concentrations on the production of cellulolytic enzymes

production of all cellulolytic enzymes was 5.5. Maximum activities of CMCase, endoglucanase and avicellase were 32.872, 9.632 and 8.770 IU g^{-1}, respectively. High or weak acidity was unfavorable for the cellulolytic activity of these enzymes. This result is in accordance with previous findings recorded by several investigators (EL-Tanash, 2007). On the other hand, Fadel (2000) showed that *A. niger* carboxymethylcellulase (CMCase) biosynthesis on radical waste was inhibited at low (under pH 4.0) and high (above pH 5.5); but, maximum level of CMCase biosynthesis was recorded at pH 4.5. In this connection, the effect of culture initial pH on the rate of enzyme productivity was depending on the type of used solid substrate (Lonsane *et al*., 1985). The role of pH factor attributed to affect the permeability of cells as well as stability of produced enzyme (Mase *et al*., 1996). The effect of pH on enzyme stability may be due to denaturation of the enzyme proteins which occur at pH differ or changing from the optimum of such enzyme (Kalra and Sandhu, 1986).

Effect of different substrate concentrations: The data illustrated in Fig. 3 showed that lower (1-4 g/flask) and higher (6-7 g/flask) substrate concentration affected the production of all cellulolytic enzymes. The production of these enzymes were increased gradually and reached their maximum production at 5.0 g of rice straw, where CMCase, endoglucanase and avicellase recorded 10.02, 10.89 and 8.77 IU g^{-1}, respectively. These results are in agreement with previous results which showed that high substrate concentration usually results in lower enzyme yield due to by-product inhibition effect (Ramos *et al*., 1993) and enzyme inactivation (Reese, 1980).

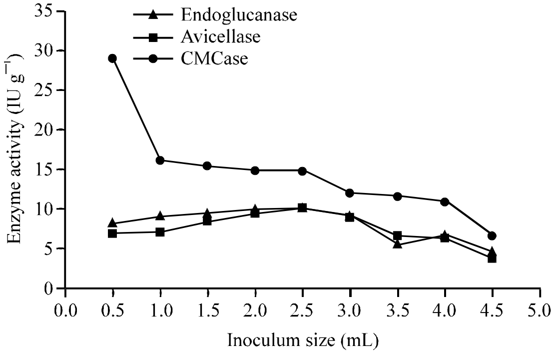

Fig. 4: Effect of different inoculum size on the production of cellulolytic enzymes

Effect of different inoculum size: The data illustrated in Fig. 4 showed that the maximum activities of endoglucanase and avicellase were recorded at inoculum size 2.5 mL (10.164 and 10.175 IU g^{-1}, respectively), while maximum activity of CMCase was detected at inoculum size 0.5 mL (29.048 IU g^{-1}). These results are agreement with Fadel (2000) who proved that CMCase productivity was highly influenced by different inoculum size of *Aspergillus niger* grown on radicle waste. Furthermore, Kumar and Satyanarayana (2004) recorded that effect of inoculum sizes on enzyme production are depending on other culture fermentation conditions such as; incubation period, moisture content, nature of the used microbe and the characteristics of used substrate. In this connection, Domingues *et al.* (2000) showed that fungal sporulation and its metabolic activities are influenced by size of inoculum. Low inoculum may require longer time for fungal multiplication and substrate utilization to produce desired enzyme. On the other hand, high inoculum would

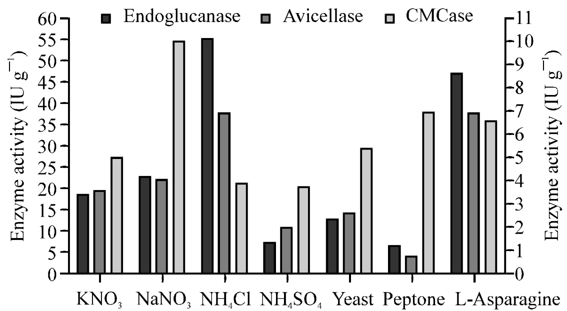

Fig. 5: Effect of different nitrogen sources on the production of cellulolytic enzymes

ensure rapid proliferation of fungal biomass. So, balance between the proliferating biomass and substrate utilization would yield maximum enzyme activity as recorded by Ramachandran *et al.* (2004). Furthermore, further increase in inoculum size resulted in decreasing enzyme yield due to limitation of nutrients as reported by Ghanem *et al.* (2000).

Effect of different nitrogen sources: The results recorded in Fig. 5 showed that avicellase, endoglucanase and CMCase productivity were highly increased by addition of L-asparagine, NH_4Cl and $NaNO_3$ (6.930, 10.098 and 54.760 IU g^{-1}), respectively as N-sources to the fermentation medium. In this connection Desai *et al.* (1982) recorded that production of fungal enzymes is very sensitive to nitrogen source and its level in the medium. On the other hand, many investigators reported that addition of $NaNO_3$ or urea as a nitrogen source stimulates cellulase production by other fungal strains during SSF (Rao *et al.*, 1985; Narasimha *et al.*, 2006).

Physicochemical characterization of cellulolytic enzymes: Cellulase preparations generally contain other enzymatic activities besides cellulase and these may affect the properties of the preparations. The influence of various physico-chemical factors on enzyme activity was investigated.

Effect of different incubation periods on the enzyme activity: Table 3 showed that the optimum incubation period for maximum CMCase and endoglucanase activities was 20 min, while avicellase reached its maximum activity at 30 min. EL-Tanash (2007) found that CMCase reaches its maximum activity after 30 min. On the other hand, El-Azab (2007) reported that the optimum incubation period for maximum avicellase and cellulase activity was 75 min, while, CMCase reached its maximum activity at 30 min.

Table 3: Effect of different incubation periods on the enzyme activity

Incubation periods (min)	Enzyme activity (IU g^{-1})		
	Endoglucanase	Avicellase	CMCase
10	0.98	4.32	9.24
20	4.59	4.87	14.41
30	2.81	7.18	9.77
60	1.03	2.62	5.13
90	0.21	0.92	2.90
120	0.08	0.39	1.87
150	0.04	0.01	0.42

Fig. 6: Effect of different pH values on the enzyme activity

Effect of different pH values on the enzyme activity: The results illustrated in Fig. 6 showed that endoglucanase and avicellase enzymes of *A. fumigatus* exhibited maximal activity at pH 5.5. While in case of CMCase enzyme exhibited its maximal activity at pH 5. This result is close to the data obtained by Shah and Madamwar (2005). They reported that pH 5.0 was the optimum for *Aspergillus niger* CMCase and cellulase activities. On the other hand, Coral *et al.* (2002) recorded that *A. niger* has two major activity peaks at pH 4.5 and 7.5; they attributed the results to the presence of two subunits in enzyme preparation. Furthermore, other CMCase reaches maximal activity at pH 7.0 (Chen *et al.*, 2004).

Effect of different temperatures on the enzyme activity: Figure 7 showed that 40°C was the best for the maximum activity of avicellase, endoglucanase and CMCase produced by *Aspergillus fumigatus*. Sharma *et al.* (1991) reported that 55°C was the optimum for *Trichoderma viride* CMCase. In addition George *et al.* (2001) recorded that 50°C as optimum temperature for *Thermomonospora* sp. CMCase. However El-Azab (2007) reported that the optimum temperature of CMCase activity was ranged from 45 to 55°C.

Effect of different substrate concentration on the enzyme activity: The results illustrated in Fig. 8 showed that the increase in substrate concentration was accompanied by

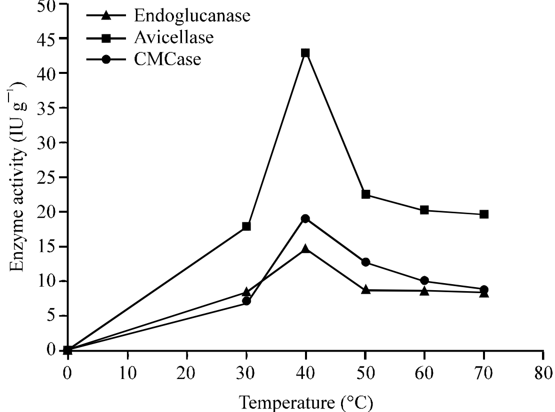

Fig. 7: Effect of temperature on the enzyme activity

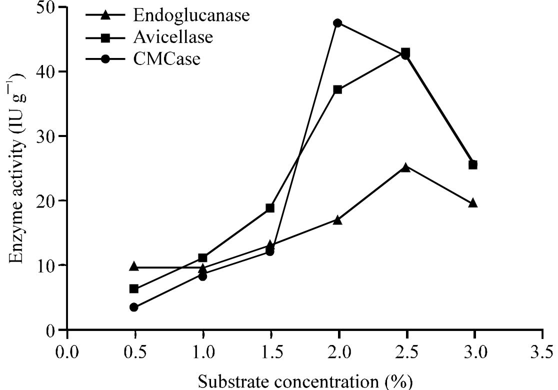

Fig. 8: Effect of substrate concentration on the enzyme activity

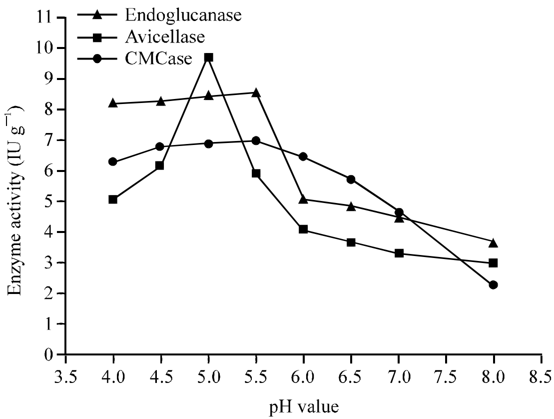

Fig. 9: pH stability of the cellulolytic enzymes

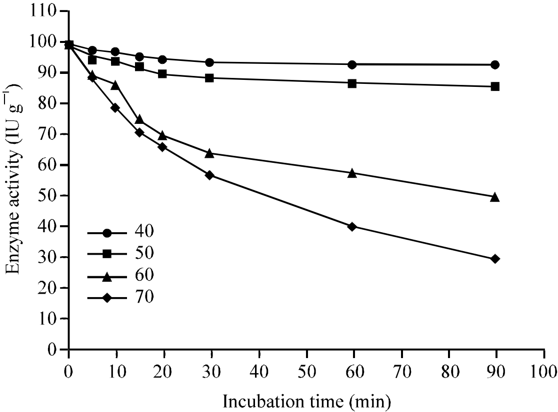

Fig. 10: Thermostability of the avicellase

increase in endoglucanase and avicellase activities till the concentration of 2.5%, at this concentration, enzymes activity reached their highest rate. However CMCase reached its highest rate of activity at the concentration of 2.0%. An increase in substrate concentration made more binding sites available for the enzymes to adhere to and the rate at which product formation would be achieved therefore would be faster (Dixon and Webb, 1971).

pH stability of the cellulolytic enzymes: The results obtained in Fig. 9 showed that CMCase and endoglucanase activities were stable at pH 5.5 and avicellase activity was stable at pH 5.0. These results are agreement with that obtained by Gusakov *et al.* in 2005 who found that *Chrysosporium lucknowense* cellulase was stable at pH 5.0.

Thermostability of the cellulolytic enzymes: Enzyme stability is considered very important tool used in the enzyme selection for industrial uses. Yamagata *et al.* (1994) indicated that thermal inactivation profile of the enzyme was differing according to its half life values. Results of plotting *Aspergillus fumigatus* celluolytic enzymes remaining activity versus time were linear at 40 and 50°C. Similar thermostable properties for other CMCase was obtained by George *et al.* (2001). Figure 10 indicated that 93.2, 88.0, 57.8 and 40.1% of the avicellase activity was retained at 40, 50, 60 and 70°C, respectively at the end of 60 min. The half life time of avicellase at 60 and 70°C was 67.8 and 51.8 min, respectively, these result was in agreement with those obtained by George *et al.* (2001). Results in Fig. 11 showed that about 81, 84, 39.2 and 35.2% of endoglucanase activity was retained after 40, 50, 60 and 70°C, respectively, where the half life time of endoglucanase at 60 and 70°C was 79.1 and 77.1 min, respectively. Results in Fig. 12 indicated that the thermostability of CMCase produced from *Aspergillus fumigatus* was higher than the other tested enzymes during the time of incubation period at the same temperatures. At 70°C after 60 min of incubation it

Table 4: Soluble sugar concentration and yeast productivity

Analysis	Dilute acid pretreatment	Alkaline pretreatment
Total soluble sugar	4.1055±0.0146%	2.5880±0.0195%
Remaining sugar concentration after 72 h fermentation	0.6708±0.0158%	1.7830±0.0024%
Yeast productivity after 72 h fermentation	50.3450±0.9260 g L^{-1}	42.1100±1.1380 g L^{-1}

Table 5: Ethanol yield during fermentation

	Dilute acid pretreatment		Alkaline pretreatment	
Time of fermentation (h)	Alcohol (%)	Fermentation efficiency (%)	Alcohol (%)	Fermentation efficiency (%)
24	1.85727±0.0616	70.69	0.08401±0.0205	5.07
48	2.26424±0.0206	86.17	0.20029±0.0205	12.09
72	2.16977±0.1740	82.58	0.41831±0.0410	25.26

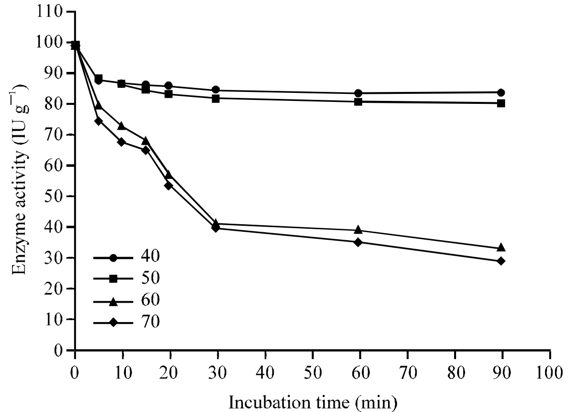

Fig. 11: Thermostability of the endoglucanase

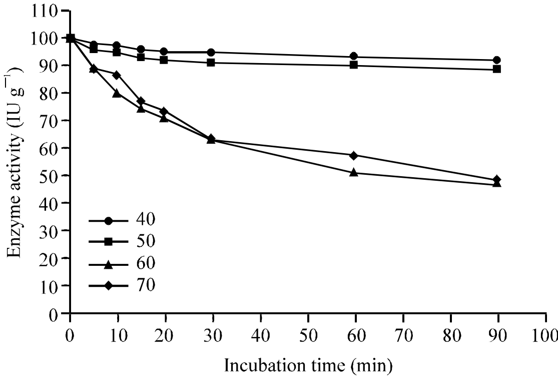

Fig. 12: Thermostability of the CMCase

retained about 57.4% of its relative activity. The half life time of CMCase at 60 and 70°C was 96.5 and 94.0 min respectively.

The production of ethanol by fermentation: As shown in Table 4 and 5, the maximum ethanol production by *Saccharomyces cereviseae* using dilute acid pretreated rice straw hydrolysate with initial soluble sugar 4.1055±0.0146% was recorded after 48 h of fermentation (2.26424±0.0206%) with fermentation efficiency 86.17%. The ethanol production was decreased after 72 h of fermentation, the decrease in ethanol level is probably a result of ethanol's volatility. Gaur (2006) reported that with initial soluble sugar 5% after 48 h of fermentation, ethanol production was 2.56%. The maximum ethanol production by *Saccharomyces cereviseae* using alkaline pretreated rice straw hydrolysate with initial soluble sugar 2.588±0.0195% recorded after 48 h was 0.20029±0.0205% with fermentation efficiency 12.09%. Fermentations of dilute acid pretreated rice straw hydrolysate showed improved sugar consumption and ethanol production when compared to alkaline pretreated rice straw hydrolysate, which showed little sugar consumption and ethanol production. This is more likely to be due to the effectiveness of the chosen pretreatment where, undesirable compounds might originate; thus, besides sugars, compounds such as lignin residues, acids and aldehydes can be released. The concentrations of inhibitors in the hydrolysate have been shown to inhibit not only the yeast, but also the enzymes (Tengborg *et al*., 2001). An inhibitory effect caused by inhibitory compounds formed from the lignin during the alkaline heat pretreatment, makes the alkaline pretreatment less attractive for the ethanol production (Hendriks and Zeeman, 2009). Small pieces of lignin, e.g., monolignols from the degradation of lignocellulose also pose a problem during fermentation. These have an inhibitory effect on the fermenting organism, baker's yeast (*Saccharomyces cereviseae*) - the aromatic rings of the monolignols (i.e., the six membered rings) are actually toxic to yeast (Hansen, 2009).

REFERENCES

APHA, AWWA, WPCF, 2005. Standard Methods for the Examination of Water and Wastewater. 21st ed., American Public Health Association, Washington, DC, USA.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem., 72: 248-254.

Brown, R.C., 2003. Biorenewable Resources: Engineering New Products from Agriculture. Iowa State Press, Ames, IA., ISBN: 978-0813822631.

Caputi, A., M. Veda and T. Brown, 1968. Spectrophotometric determination of ethanol in wine. Am. J. Enol. Vitic., 19: 160-165.

Chen, P.J., T.C. Wei, Y.T. Chang and L.P. Lin, 2004. Purification and characterization of carboxymethyl cellulose from *Sinorhizobium fredii*. Bot. Bull. Acad. Sinica, 45: 111-118.

Coral, G.K., B. Arikan, M.N. Naldi and H.G. Venmez, 2002. Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 Wild-Type Strain. Turk. J. Biol., 26: 209-213.

Desai, J.D., A.J. Deasi and N.P. Patel, 1982. Production of cellulases and glucosidases by shake culture of Scytaidum lignicola. J. Ferment. Technol., 60: 117-124.

Dixon, M. and E.C. Webb, 1971. Enzymes. 2nd Edn., Longman Group Ltd., London, pp: 67-188.

Domingues, F.C., J.A. Queiroz, J.M.S. Cabrad and L.P. Fonseca, 2000. The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* rut C-30. Enzyme Microb. Technol., 26: 394-401.

El-Azab, N.T., 2007. Bioconversion of some agricultural wastes by fungi. M.D. Thesis, Botany Department, Faculty of Science, Mansoura University, Egypt.

El-Tanash, A.B., 2007. Biotechnological studies on some fungal industrial enzymes. Ph.D. Thesis, Botany Department, Faculty of Science, Mansoura University, Egypt.

Fadel, M., 2000. Production physiology of cellulases and β-glucosidase enzymes of *Aspergillus niger* grown under solid state fermentation conditions. Online J. Biol. Sci., 1: 401-411.

Gaur, K., 2006. Process optimization for the production of ethanol via fermentation. M.Sc. Thesis, Thapar Institute of Engineering and Technology, Deemed University, Patiala.

George, S.P., A. Ahmed and M.B. Rao, 2001. Studies on carboxymethyl cellulase produced by an alkalothermophilic actinomycete. Bioresour. Technol., 77: 171-175.

Gervais, P. and P. Molin, 2003. The role of water in solid-state fermentation. Biochem. Eng. J., 13: 85-101.

Ghanem, N.B., H.H. Yusef and H.K. Mahrouse, 2000. Production of *Aspergillus terreus* xylanase in solid-state cultures: Application of the Plackett-Burman experimental design to evaluate nutritional requirements. Bioresour. Technol., 73: 113-121.

Hansen, M., 2009. Bio-fuels and the prospect of converting plant fibers into gasoline using Enzymes. Sci. Creative Quart., Issue 4. http://www.scq.ubc.ca/biofuels-and-the-prospect-of-converting-plant-fibres-into-gasoline-using-enzymes/.

Harpreet, S., K.L. Bajaj and J.S. Arneja, 1998. Biochemical studies on bioconversion of rice straw to ethanol. Ind. J. of Ecol., 25: 62-65.

Hendriks, A.T.W.M. and G. Zeeman, 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol., 100: 10-18.

Howard, R.L., E. Abotsi, J.E.L. van Rensburg and S. Howard, 2003. Lignocellulose biotechnology: Issues of bioconversion and enzyme production: Review. Afr. J. Biotechnol., 2: 602-619.

Jecu, L., 2000. Solid state fermentation of agricultural wastes for endogluconase production industry. Crops Prod., 11: 1-5.

Johnson, I.F., E.A. Curl, J.H. Bond and H.A. Fibourg, 1960. Method for Studying Soil Microflora. Burgess Publishing Co., Minnwapolis, USA.

Kalra, M.K. and D.K. Sandhu, 1986. Optimal production of cellulolytic enzymes and their location in *Trichoderma pseudokoningii*. Acta Biotechnol., 6: 161-166.

Kumar, P. and T. Satyanarayana, 2004. Biotechnological Aspects of Thermophilic Fungal Glucoamylases. In: Emerging Trends in Mycology, Plant Pathology and Bagyanarayana, G., B. Bhadraiah and I.K. Kunwar (Eds.). B.S. Publications, Hyderabad, pp: 539-63.

Lonsane, B.K., N.P. Ghildyal, S. Budiatman and S.V. Ramakrishna, 1985. Engineering aspects of solid state fermentation. Enzyme Microb. Technol., 7: 258-265.

Mase, T., Y. Matsumiya, S. Mori and A. Matsura, 1996. Purification and characterization of a novel glucoamylase *Acremimium* sp. YT.78. J. Ferment. Bioeng., 81: 347-350.

McMillan, J.D., 1994. Pretreatment of Lignocellulosic Biomass. In: Enzymatic Conversion of Biomass for Fuels Production, Himmel, M.E., J.O. Baker and R.P. Overend (Eds.). American Chemical Society, Washington, DC., ISBN13: 9780841229563, pp: 292-324.

Narasimha, G., A. Sridevi, B. Viswanath, M.S. Chandra and R.B. Rajasekhar, 2006. Nutrient effects on production of cellulolytic enzymes by *Aspergillus niger*. Afr. J. Biotechnol., 5: 472-476.

Nelson, N., 1944. A photometric adaptation of somogyi method for determination of glucose. J. Bio. Chem., 153: 375-380.

Panagiotou, G., D. Kekos, B.J. Macris and P. Christakopoulos, 2003. Production of cellulolytic and xylanolytic enzymes by *Fusarium oxysporum* grown on corn stover in solid state fermentation. Ind. Crops. Prod., 18: 37-45.

Pothiraj, C., P. Balaji and M. Eyini, 2006. Enhanced production of cellulases by various fungal cultures in solid state fermentation of cassava waste. Afr. J. Biotechnol., 5: 1882-1885.

Purkarthofer, H., M. Sinner and W. Steiner, 1993. Cellulase-free xylanase from *Thermomyces lanuginosus*: Optimization of production in submerged and solid-state culture. Enzyme Microb. Technol., 15: 677-682.

Ramachandran, S., A.K. Patel, K.M. Nampoothiri, F. Francis, V. Nagy, G. Szakacs and A. Pandey, 2004. Coconut oil cake-a potential raw material for the production of α-amylase. Bioresour. Technol., 93: 169-174.

Ramos, L.P., C. Breuil and J.N. Saddler, 1993. The use of enzyme recycling and the influence of sugar accumulation on cellulose hydrolysis by *Trichoderma cellulases*. Enzyme Microb. Technol., 15: 91-125.

Rao, M., V. Deshpande, R. Seeta, M.C. Srinivasan and C. Mishra, 1985. Hydrolysis of sugar-cane bagasse by mycelial biomass of *Penicillium funiculosum*. Biotechnol. Bioeng., 27: 1070-1072.

Reese, E.T., 1980. Inactivation of cellulase by shaking and its prevention by surfactants. J. Applied Biochem., 2: 36-39.

Shah, A.R. and D. Madamwar, 2005. Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterization. Process Biochem., 40: 1763-1771.

Shamala, T.R. and K.R. Sreekantiah, 1986. Production of cellulases and D-xylanase by some selected fungal isolates. Enzyme Microb. Technol., 8: 178-182.

Sharma, N., T.C. Bhalla and A.K. Bhatt, 1991. Partial purification and characterization of extracellular cellulase from a strain of *Trichoderma viride* isolated from forest soil. Folia Microbiol., 36: 353-356.

Sherief, A.A., A.B. El-Tanash and N. Atia, 2010. Cellulase production by *Aspergillus fumigatus* grown on mixed substrate of rice straw and wheat bran. Res. J. Microbiol., 5: 199-211.

Somogyi, M., 1952. Notes on sugar determination. J. Biol. Chem., 195: 19-23.

Sun, Y. and J. Cheng, 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresour. Technol., 83: 1-11.

Tengborg, C., M. Galbe and G. Zacchi, 2001. Reduced inhibition of enzymatic hydrolysis of steam-pretreated softwood. Enzyme Microb. Technol., 28: 835-844.

Trevelyan, W.E. and J.S. Harrison, 1952. Studies on yeast metabolism I: Fractionation and microdetermination of cell carbohydrates. Biochem. J., 50: 298-309.

Wang, X., J. Bai and Y. Lian, 2006. Optimization of Multienzyme production by two mixed strains in solid-state fermentation. Applied Microbiol. Biotechnol., 73: 533-540.

Wood, T.M., 1991. Fungal Cellulases. In: Biosynthesis and Biodegradation of Cellulose, Haigler, C.H. and P.J. Weimer (Eds.). Macel Dekker Inc., New York, pp: 491-534.

Yamagata, Y., K. Arakawa, M. Yamaguchi, M. Kobayashi and E. Ichishima, 1994. Functional changes of dextran-modified alkaline proteinase from alkalophilic *Bacillus* sp. Enzyme Microb. Technol., 16: 99-103.