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Optimization of Culture Conditions for Production of Cellulase by *Aspergillus tubingensis* KY615746

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TWENTY isolates of cellulase producing fungi were isolated from six samples of rotted rice straw which collected from different localities in El-Gharbia Governorate (Basyion), Kafrelsheikh Governorate (Sakha) and El-Dakahlia Governorate (El-Mansoura). On the basis of hydrolysis zone surrounding the colonies, an efficient fungus isolate (F7) for highest cellulolytic activity were chosen. According to morphological and biochemical characteristics as well as 18S rRNA sequence indicated that the isolate belonged to *Aspergillus tubingensis* KY615746. Effect of culture conditions for production of cellulase enzyme by *A. tubingensis* were investigated under Submerged Fermentation (SMF) and Solid State Fermentation (SSF) techniques. The optimum conditions for cellulase production by *A. tubingensis* were the best inoculum size was found to be 3%, pH 4, temperature 30 °C and incubation period 6th day where the reducing sugars attained (0.675, 0.728, 0.731 and 0.913 mg ml^{-1}) with maximum activity (0.050, 0.055, 0.053 and 0.063 U ml^{-1}), respectively for SMF conditions.On the other hand, the maximum cellulase activity under SSF technique with moisture level 10g : 30ml were (0.16 Ug^{-}), incubation period 9th day, inoculum size 4 ml:10g, pH 5 and temperature 30 °C where the reducing sugars were (1.412, 1.532, 1.551 and 1.521 mg g^{-1}) with maximum activity (0.20, 0.22, 0.23 and 0.23 Ug^{-1}), respectively. Based on the results, it may be concluded that rice straw waste can be a potential substrate for produce cellulase by SSF technique.

Keywords: *Aspergillus tubingensis,* Cellulose, Rice straw waste, Submerged Fermentation, Solid State Fermentation

Introduction

Agricultural residues are a great source of lignocellulosic biomass, which is renewable, chiefly unexploited, and inexpensive (Shaban et al. 2010). These residues include leaves, stems, and stalks from rice straw, corn stover, sugarcane bagasse and woody crops as well as forest residues. In addition, a lot of sources of lignocellulosic waste can produced from industrial and agricultural processes, e.g., municipal solid waste, citrus peel waste, sawdust, paper pulp, and paper mill sludge (Maki et al. 2009).

A large amount of agricultural crop residue was burned directly in the open field especially in grain-producing regions where are high density of population, economic developed areas, and abundant fossil fuel producing (Irfan et al. 2014 and Liska et al. 2014). The direct burning of agriculture crop residue in the open field has been considered as an important source of atmospheric pollution by now with significant impacts on atmospheric chemistry and global climate change and with great threat to human health (Brühl et al. 2015; Pongpiachan et al. 2015 and Udeigwe et al. 2015). Among these series of atmospheric pollutants, CO_2 emission which primarily leads to global warming was mostly focused on fossil fuel combustion by past (Streets et al. 2001).

Therefore, it is very important to reuse of the agriculture wastes in any other environmentally friendly way. To solve this problem a new technology needs to be developed to handle these residues easily and safely, in particular to protect crops against the harmful disease carriers. (Shaban et al. 2010 and García-Torreiro et al. 2016).

A promising method of utilizing microorganism to degrade lignocellulose has become more and more popular because it is

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pollution-free, energy-efficient and economical. Saccharification or bioconversion process to microbial biomass as an improved feed supplement by cellulosic and hemicellulosic components of agricultural residues. Therefore, cellulolytic fungi and bacteria can play an important role in natural biodegradation process by produce cellulolytic enzymes. These enzymes can using in industry by application in production of ethanol, detergents and other chemical. Also, these enzymes have been used in the pulp and paper industry, e.g., in deinking of fiber surfaces and in improving pulp drainage, in the textile industry, animal feed, and even in the food industry, for the processing of paper and cellophane, as well as for biotransformation of wastepaper to fermentable

The microbial flora including bacteria, yeast and fungi can utilize solid substrates which make a perfect supporting and nourishing in the environment. The best microbial flora can used in Solid State Fermentation (SSF) is filamentous fungi because of their hyphal growth. The use of Solid State Fermentation (SSF) for the production of enzymes and other products has many advantages over Submerged Fermentation technique (SMF) (Asgher et al. 2006). Some of the advantages are less volume of liquid required for product recovery, which could reduce the cost of downstream processing and subsequent waste treatment; usability of simple and cheap media for the fermentation; a lower energy demand, and often a high product yield; lower risk of contamination due to the inability of most contaminants to grow in the absence of free-flowing water (Peréz-Guerra et al. 2003 and Florencio et al. 2016).

The aim of this work was conducted for isolation and identification of cellulase producing fungi as well as studied the culture conditions for maximum production of cellulase using Solid State Fermentation (SSF) and compare with Submerged Fermentation (SMF) technique.

Materials and Methods

Sources of microorganism

A total of six samples of rotted rice straw used in the present study were collected from different localities in El-Gharbia Governorate (Basyion), Kafrelsheikh Governorate (Sakha) and El-Dakahlia Governorate (El-Mansoura).

Culture media and growth conditions

Stock cultures were grown on Mandel's Medium (Mandels et al. 1976) composed as follows (g l^{-1}): 0.3, Urea; 1.4, $(NH_4)_2SO_4$; 2.0, KH_2PO_4; 0.3, $CaCl_2$; 0.3, $MgSO_4.7H_2O$; 0.75, Peptone; 0.25, Yeast extract; 10, Carboxymethyl cellulose; 20, agar and adjusted pH 5. All components sterilized at 121 °C for 15 min. One gram of rotted - milled rice straw was weighed and mixed in 9 ml of medium, which was serially diluted from 10^{-1} to 10^{-5} and then 100 µl of each dilution were spread on plates using a glass spreader. Petri plates incubated at 30 °C for 5 days. The fungi maintained on Potato Dextrose Agar (PDA) slants at 4 °C for further analysis (Abo-State 2003).

Potato dextrose agar medium (Tsao, 1970) composed as follows (g l^{-1}): 200 g potatoes, Potato extract; 20, Dextrose; 0.5, Yeast extract; 20, agar and adjusted pH 5.5.

Czapek's agar medium (Fawole and Oso, 1988) composed as follows (g l^{-1}): 30, Sucrose; 3.0, $NaNO_3$; 0.5, KCl; 1.0, K_2HPO_4; 0.5, $MgSO_4.7H_2O$; 0.01, $FeSO_4$ $.7H_2O$ and adjusted pH 6.0.

Plate screening for cellulolytic activity

Agar plates were prepared and spot inoculated with isolates. The plates were incubated at 30 °C for 48 h monitored for appearance of clear zone. The plates were stained with 0.1% (w/v) congo red dye for 15 min followed by destaining with 1 M NaCl solution for 15 to 20 min. Apun et al. (2000); unstained areas (clear zone of hydrolysis) indicate where the CMC was hydrolysed.

The diameter of the clear zone can be measured to provide a quantitative comparison of cellulolytic activity. Cellulolytic isolates were selected on the basis of the hydrolysis zone surrounding the colonies as described by (Bradner et al. 1999; Peciulyte 2007 and Belal 2008). The efficient isolates which showed the most efficient result was selected for further experiments.

Identification of the isolates

Identification of the isolates to species level was carried out through morphological, biochemical tests and 18S rRNA gene sequences. Morphological studies were carried out by microscopic observation and studies on growth characteristics in petri dishes. The isolates were cultured on Czapek's agar at 30 °C. Macroscopical

characters were assessed at 3 and 7 days of age and microscopical characters were assessed after staining with lactophenol cotton blue (Fawole and Oso, 1988).

For 18S rRNA gene sequences was carried out by polymerase chain reaction (PCR) at Sigma Scientific Services Co., Giza, Egypt.

Inoculum preparation

Fungal inoculum was prepared from a 7th days old slant on PDA at 30 °C. By adding 10 ml of sterilized distilled water containing 0.01%, (v/v) Tween 80 (Shaibani et al. 2011).The spores scratched with the help of a sterilized wire loop to make a homogeneous suspension of spores. Spore count measured by direct microscopic counting using haemocytometer. The spore suspension concentration was adjusted at 10^5 to 10^7spores ml^{-1} in the homogenous spore suspension (Juhász et al 2003).

Submerged fermentation conditions

Submerged fermentation for enzyme production was carried out in Erlenmeyer flasks (500 ml) containing 100 ml of the medium, pH 5 was adjusted. The flasks were inoculated with 1ml of inoculums containing of 10^7spores ml^{-1}. The inoculated flasks were incubated at 30°C 5 days on a rotary shaker at 150 rpm. After incubation period, the dry weight of mycelia was measured. The resulting supernatant was used as crude enzyme preparation for estimation of cellulase activity.

Solid State Fermentation Conditions

Production of cellulase enzyme by selected isolates under solid state fermentation using rice straw as the substrate (Lonsane 1994; Pandey et al. 2001). Cellulase production experiments were carried out in 250 ml Erlenmeyer flasks. Ten gram of rice straw was taken in individual flasks and 30 ml of medium was added as moistening agent. The substrate was inoculated with 4 ml of inoculum containing 10^7spores ml-1 of the moistening agent (Belal and El-Mahrouk 2010).

The flasks were incubated at 30°C for 9 days under static conditions. After incubation period the Colony-Forming Units (CFU) counts was determined. Three replicates were maintained for each organism. At the end of fermentation time 0.05 M citrate buffer pH 4.8 was added to give total 50 ml/flask. The flasks were shaking at 200 rpm for 30 min, filtered and then centrifuged to obtain clear filtrate for determining cellulase enzyme activity.

Optimization of cultural conditions for cellulase production

Effect of moisture level on cellulase production

The effect of moisture level on cellulase production was tested by varying the rice straw-to-moisture ratio (w/v) in the range of 10 g to (20, 25, 30, 35 and 40 ml) where medium was added as moistening agent, to compare the enzyme activity for solid state fermentation only. Three replicates were used for each treatment.

Effect of inoculum size on cellulase production

To investigate the effect of inoculum size on cellulase production, different inoculum levels (v/v) for submerged fermentation (1, 2, 3, 4, 5 and 6%), and v/10 g for solid state fermentation, were used. One ml of spore suspension contains $1x10^7$spore ml^{-1} and one ml of bacterial inoculums contains $1x10^s$ CFU ml^{-1}.

Effect of pH on cellulase production

To determine the optimum pH for cellulase production, different pH values (3, 4, 5, 6, 7, 8 and 9) were used. Buffers including citrate buffer (3-6), citrate phosphate buffer (pH 7), Tris buffer (pH 8 and 9) were used to adjust the pH of the medium.

Effect of temperature on cellulase production.

To determine the optimum temperature for cellulase production, fermentation was carried out at various temperatures in the range of (20, 30, 40, 50, 60 and 70 ºC).

Effect of incubation period on cellulase production

The flasks were incubated for 1, 2, 3, 4, 5, 6, 7 and 8 days for submerged fermentation, while solid state fermentation were incubated for 3, 6, 9, 12, 15, 18 and 21 days. Samples withdrawn at the previous intervals and assayed for cellulase activity using the standard DNS method (Miller 1972).

Enzyme assay

The activity of cellulase was assayed by the method of (Miller 1972). The assay mixture contained 1 ml of 1% carboxy methyl cellulose (CMC) suspended in 0.05M citrate buffer (pH 4.8) as substrate and 1 ml of culture filtrates of the strains. The reaction mixture was incubated for 30 min at 50°C.The blanks were made in the same way using distilled water in place of culture filtrate. The reaction was terminated by

adding 3 mL of DNS reagent. The colour was then developed by boiling the mixture for 5 min in water bath. The absorbance was measured at 540 nm and the amount of reducing sugar released was calculated from the standard curve of glucose. One unit (U) of enzyme activity was defined as the amount of enzyme, which liberates 1 µmol of reducing sugars (measured as glucose) per ml per minute. The residue was dried to constant mass at 80°C. The enzyme activity was expressed as Ug^{-1} dried substrate. All values given are means of three determinations.

Results and Discussion

In the present study concentrate on the isolation of cellulase producing fungi from different rice straw samples. The data presented in Table 1, showed that twenty isolates were selected. Screening of all isolates was conducted by using the congo red test (Apun et al. 2000) as a preliminary study to select cellulase producers.

The plates were incubated at 30 °C for 48 h and all isolates were screened for presence or absence of (clear zone) cellulolytic activity. Among the selected twenty isolates, 15 isolates were found to have cellulolytic activities on CMC agar in Fig. 1, therefore the result of the test was strong evidence that cellulase was produced in order to degrade cellulose (Lynd et al. 2002; Wang et al. 2008). In this study, clear zones around the colonies was observed after staining with congo red indicated the hydrolysis of CMC as a result of cellulases production and this phenomenon has been reported by (Abdelnasser and Ahmed 2007).

Selection of the most efficient isolates for cellulase production

Data presented in Table 1, showed that the ratio of clear zone diameter to colony diameter in all fungal isolates were between 1.18 and 4.1 cm, which the highest cellulolytic activity was recorded 4.1 cm for the fungal isolate F7.

Morphological, biochemical identification

The fungal genus was morphologically identified as *Aspergillus* sp. Microscopical characters were assessed after staining with lactophenol cotton blue. In early culture stages (3~5 days), radial villus and white mycelia at the edge of each colony was observed. After seven days, the colony became pitchy at the head of mycelium and white in the reverse side in agar plate. The colony color was observed by naked eye. Colonies consist of a compact white basal felt covered by a dense layer of dark black conidial heads. *Aspergillus* species was described by Mosseray (1934). The most effective isolate F7 was morphologically identified as *Aspergillus tubingensis*.

Genetic identification of the selected isolates by sequencing of the amplified 18S rRNA gene.

Genetic identification of the most effective isolate chosen to confirm identification by gene coding through polymerase chain reaction (PCR) as presented in Fig. 2. For sequence analysis of the selected isolate, data compared with 18S rRNA gene. The data base in Fig. 3, showed that F7 recorded 99% gene sequence similarity with *Aspergillus tubingensis* strain USMI03 using

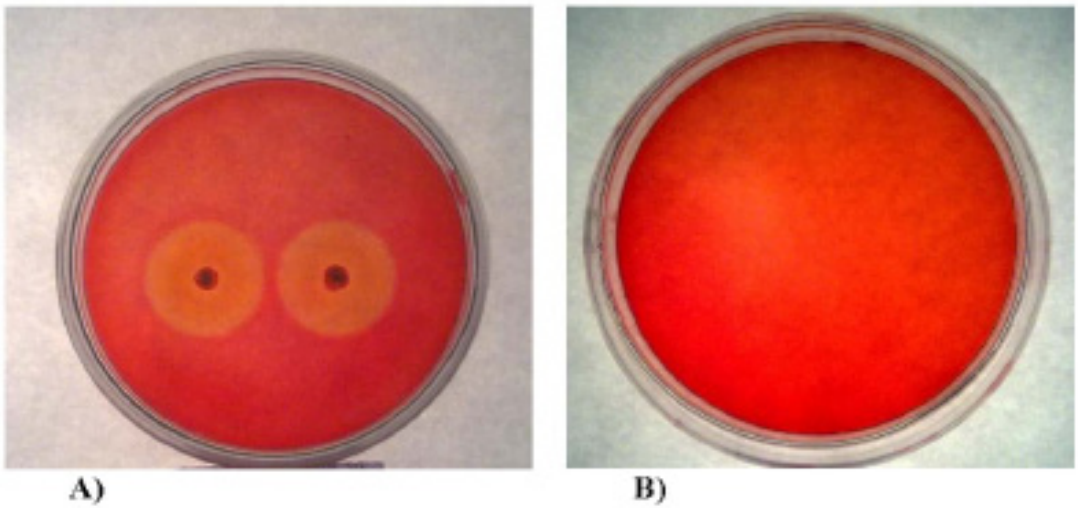

Fig. 1. (A) Clear zone on CMC agar plates after staining with congo red indicates CMC degradation as compared with (B) control without CMC degradation.

TABLE 1. Hydrolysis capacity of carboxymethyl cellulose (CMC) by cellulase producing fungi.

NO. of isolate	Colony diameter (cm)	Clear zone diameter (cm)	HC value
F1	0.91±0.03	2.15±0.07	2.36
F2	0.92±0.01	2.53±0.05	2.75
F3	1.14±0.03	3.27±0.18	2.87
F4	1.31±0.02	2.74±0.06	2.09
F5	0.23±0.01	0	0
F6	1.18±0.05	2.56±0.05	2.17
F7	1.13±0.06	4.6±0.05	4.1
F8	0.29±0.01	0	0
F9	0.21±0.00	0	0
F10	2.00±0.07	2.50±0.06	1.25
F11	1.3±0.04	1.72±0.06	1.32
F12	1.01±0.12	3.84±0.08	3.80
F13	0.45±0.00	0	0
F14	1.25±0.09	2.40±0.03	1.92
F15	1.92±0.12	2.44±0.08	1.27
F16	2.51±0.03	2.96±0.04	1.18
F17	1.5±0.035	2.19±0.07	1.46
F18	0.44±0.014	0	0
F19	1.56±0.12	3.15±0.16	2.01
F20	1.5±0.10	2.59±0.11	1.73

Hydrolysis capacity (HC): diameter of clear zone / diameter of colony.

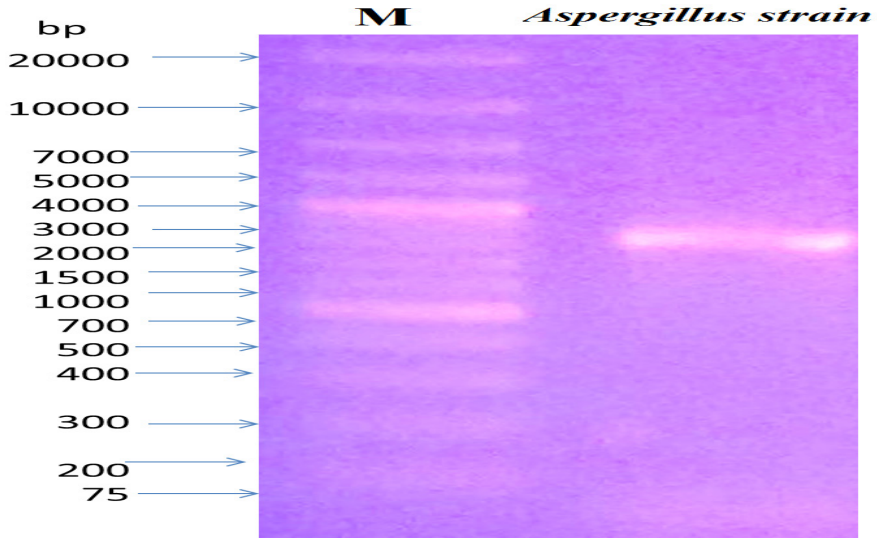

Fig. 2. Agarose gel electrophoresis of purified PCR product for *Aspergillus tubingensis* KY615746 and M: DNA marker.

18S ribosomal RNA gene, partial sequence; 5.8S ribosomal RNA, and genes, complete sequence; and 28S ribosomal RNA gene, partial sequence. Sequence data were submitted to GenBank and it provided a GenBank accession number KY615746.

The previous results showed that F7 strain were exhibited higher cellulase and reducing sugars productivity than the other fungi isolates. These results are in agreement with previous findings reported by Perrone et al. (2008) and Soni et al. (2010) who found that filamentous fungi particularly *Aspergillus* and *Trichoderma* spp. are well known efficient producers of cellulases.

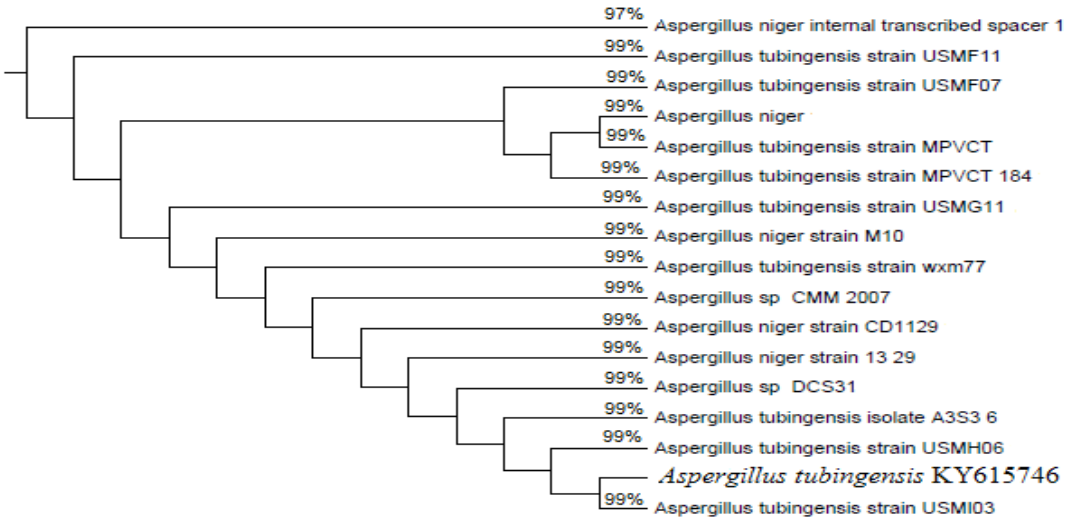

Fig. 3. Phylogenetic tree of 18S rRNA gene sequence of *Aspergillus tubingensis* KY615746.

These results are on line with previous findings reported by (Peciulyte 2007 and Belal 2008).

Optimization of culture conditions for cellulase production by Aspergillus tubingensis USMI03 under submerged fermentation

Aspergillus tubingensis KY615746 were identified as the best cellulase producer. Numerous factors are affecting the culture performance including inoculum size, pH, temperature, and incubation times, similar to any type of fermentation. Optimization of fermentation conditions have been used to enhance cellulase yield and productivity.

Effect of inoculum size on cellulase production

As shown in Fig. (4 - A) the results indicated that the best inoculum size for cellulase production by *A. tubingensis* KY615746 was found to be 3%, where the reducing sugars were (0.675 mg ml^{-1}) with maximum activity (0.050 Uml^{-1}). Subsequently increase in the inoculum size led to decrease in cellulase activity. Variation in inoculums size from this optimal range resulted in decreased in enzyme production. This decrease in glucose production with further increase in inoculum might be due to clumping of cells, which could have reduced sugar and oxygen uptake rate and enzyme release (Haq et al. 2005).

Ray et al. (2007) who found that the cellulolytic enzyme production increased gradually up to 3% inoculum size, then decreased thereafter. The maximum enzyme production was found at 3% of inoculum size subsequently increase in the inoculum size higher than 3% led to decrease in cellulase activity (Haq et al. 2003).

Effect of pH on cellulase production

Growth medium pH strongly influences many enzymatic reactions by affecting the transport of a number of chemical products and enzymes across the cell membrane (Liang et al. 2009 and Kapoor et al. 2008). The results presented in Fig. (4 - B) showed the highest production of reducing sugars were (0.728 mg ml^{-1}) with maximum activity (0.055 Uml^{-1}) at pH 4. Further increase in pH, decreased the cellulase activity. Akiba et al. (1995) it was reported that the optimal pH for a cellulase from *A. niger* was between 6.0 and 7.0, and in another report the optimal pH activity of *A. niger* was found to be between 4.0 and 4.5 (McCleary and Glennie-Holmes 1985). Such different results may appear because of differences within the same genus. Results in this study are accordance with earlier reports that the optimal pH for fungal cellulases varies from species to species, though in most cases the optimum pH ranges from 3.0 to 6.0 (Niranjane et al. 2007).

Effect of temperature on cellulase production

As shown in Fig. (4 - C), the cellulase activity was increased with the increase in temperature from 20 to 30°C then decreased. The maximum cellulase activity was found at 30°C where the highest reducing sugars were (0.731 mg ml^{-1}) with maximum activity (0.053 Uml^{-1}). As the temperature was further increased, there was a gradual reduction in the reducing sugar and enzyme

production. This may be due to the fact that higher temperature denatures the enzymes.

The temperature was found to influence extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane. The production of cellulase enzyme was maximal at 30 °C. These results are in agreement with those of Singh et al. (2009) and Saro et al. (2010) who reported that the maximum enzyme production was observed at 30 °C and Malik et al. (2010) showed that the maximum cellulase production by *T. viride* obtained at 30 °C. In support of these results the highest cellulase activity of *Streptomyces* sp. B-PNG23 was observed at 28 °C (Azzeddine et al. 2013).

Effect of incubation periods on cellulase production

Incubation period is considered one of the most important factors affecting cellulose production. As shown in Fig. (4 - D) the maximum cellulase activity was found after 6th days, where the maximum reducing sugars were (0.913 mg ml^{-1}) with maximum activity (0.063 Uml^{-1}). These results are in agreement with Kang et al. (2004) who reported that *T. harzianum* achieve maximum cellulase activity after 6^{th} days of incubation. The production increased with increasing the fermentation period. Further increase in the incubation period lead to decrease in production of cellulase. Decrease in enzymatic activity with time might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes (Haq et al. 2005).

Optimization of culture conditions for cellulase production by Aspergillus tubingensis KY615746 under solid state fermentation

Optimization of solid state fermentation conditions have been used to enhance cellulase yield and productivity. So, there are numerous factors are affecting the culture performance including different moisture levels, incubation periods, inoculum size, pH and temperature.

Effect of different moisture levels on cellulase production

Data presented in Fig. 5, showed that the maximum cellulase activity was recorded at 10 g: 30ml moisture for *A. tubingensis* KY615746 were (0.16 Ug^{-1}). On the other hand at lower or higher moisture level than 10g: 30ml moisture decrease in the cellulase activity was observed.

These results are in agreement with those results

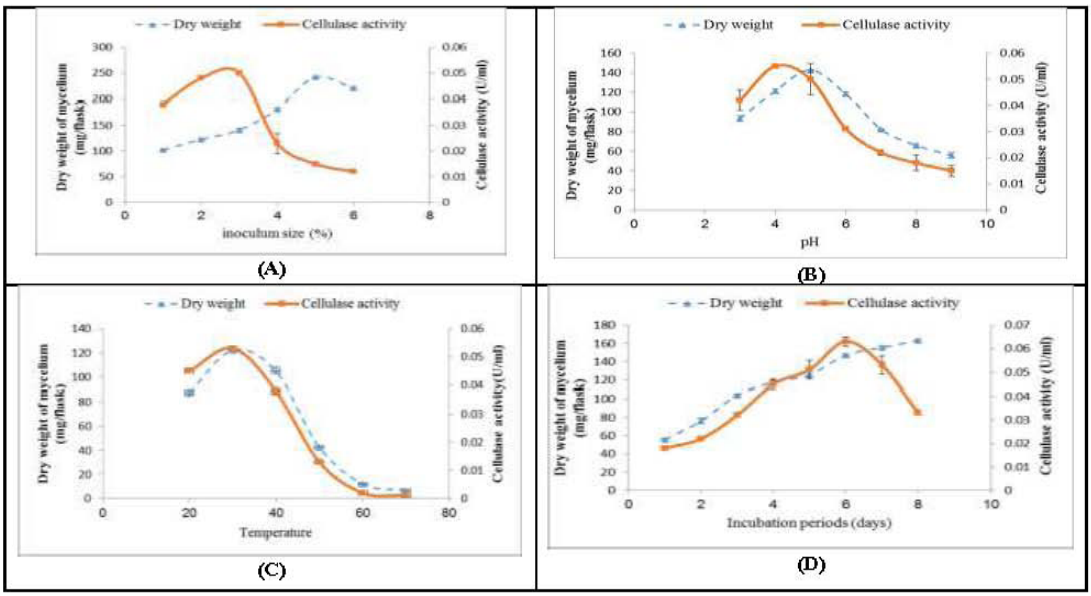

Fig. 4. Effect of different factors on cellulase production by *A. tubingensis* KY615746 under submerged fermentation (A): inoculum size, (B): pH (C): temperature and (D): incubation times.

of Abd El-Zaher and Fadel (2010) who found that the cellulase production was optimum using the lower moisture addition at 30 ml. In support of these results Jadon et al. (2013) showed that the best activity was obtained with initial moisture content was 1:3 and lower moisture content adversely affects the activity of cellulase. These results are on line with Grover et al. (2013) who found that the optimum moisture level was regarded as 1:3 substrates to medium ratio. Moisture is the key element for regulating and optimizing the solid state fermentation process.

Ahmed (2008) and Sodhi et al. (2005) reported that the increase in moisture level lead to decrease in enzyme production due to decreasing substrate porosity, alteration in substrate particle structure, lowering oxygen transfer, reduction of gas volume and decreasing in microbial growth. Low moisture leads to sub-optimal growth, reduces nutrients and protein solubility as well as effect on swelling of used substrate and facilitates good utilization of substrates by the microorganisms. Water activity of the medium significantly affects the productivity and behavior of SSF (Gervais and Molin 2003).

Effect of incubation periods on cellulase production

As shown in Fig. (6 - A) A. tubingensis KY615746 produced low activities of the enzyme but with the passage of time, there was gradual increase in enzyme synthesis up to 9th days. A. tubingensis KY615746 efficiently production enzyme and gave maximum activity (0.20 Ug^{-1}) with highest reducing sugars were (1.412 mg g^{-1}) that reduced to (0.17 Ug^{-}) with reducing sugars were (1.315 mg g^{-1}) at 12th days of incubation.

Melo et al. (2007) reported that the enzyme level decreased with prolonged incubation, due to loss of moisture or denaturation of the enzyme and these result showed from variation in pH during fermentation. Singh et al. (2009) reported that the decrease of enzyme activities might be due to the accumulative effect of cellulobiose. These results are on line with those of Juwaied et al. (2011) who found that the maximum production of enzyme by *A. niger* gave 10.42 Uml^{-1} of cellulase at 10th days. Pothiraj et al. (2006) observed maximum cellulase activity at 10th days of fermentation of cassava waste using *Rhizopus stolonifer.*

Effect of inoculum size on cellulase production

Inoculum size has also significant effect on the production of cellulase. The effect of inoculum size on cellulase production was studied by using various inoculum sizes (1, 2, 3, 4, 5 and 6 ml 10 g-1).

As shown in Fig. (6 - B) the production of enzyme by *A. tubingensis* KY615746 decreased at 2 ml inoculum and reached maximum (0.22 Ug^{-1}) with 4 ml inoculum where the reducing sugars were (1.532 mg g^{-1}). Further, increase in inoculum size resulted in the gradual decrease in production of cellulase. At low inoculum size i.e., 2 ml, conidial cells were not enough to utilize the fermentation medium in a better way hence, resulted in less growth and cellulases biosynthesis. On the other hand, at high concentration of conidial cells, anaerobic condition of fermentation medium, due to the tremendous growth of microorganism may lead to nutritional imbalance in medium, which resulted in gradual reduction of cellulase yield.

In support of these results Haq et al. (2003) showed that the maximum production of cellulase by *Trichoderma viride* GCBT-11 was achieved using 4% inoculum. Lower inoculum size required longer time for the cells to multiply to sufficient number to utilize the substrate and produce enzyme. After a certain limit, enzyme production could decrease because of depletion of nutrients due to the enhanced biomass, which would result in a decrease in metabolic activity (Kashyap et al. 2002).

Effect of pH on cellulase production

Factors such as pH have important effects on the microbial degradation of rice straw and these conditions must be considered when the biodegradability of rice straw is tested (Karpouzas and Walker 2000).

Effect of pH on cellulase production was examined by using different buffers at varying pH ranging from 3.0 to 9.0 in the medium. Data presented in Fig. (6 – C) showed that the optimum pH for *A. tubingensis* KY615746 was found to be pH 5.0, where the highest reducing sugars were (1.551 mg g^{-1}) with maximum activity (0.23 U g^{-1}). These results were confirmed by other previous researches.

Niranjane et al. (2007) found that, the best CMCase produced by *T. viride* was recorded at pH 5. The optimum pH for fungal cellulases and crude protein production varied from species to species. In most of the cases, the optimum pH ranges from 3.0 to 6.0. In support of these results Gomes et al. (2006) found that cellulases produed by *T. reesei* was higher in acidic conditions (4.5-

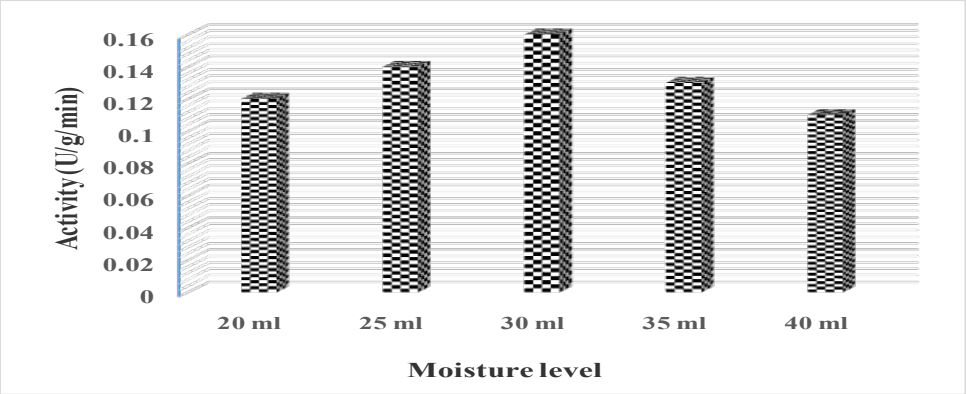

Fig. 5. Effect of different moisture level on cellulase production by *A. tubingensis* KY615746 (Ug^{-1} min^{-1}) under solid state fermentation.

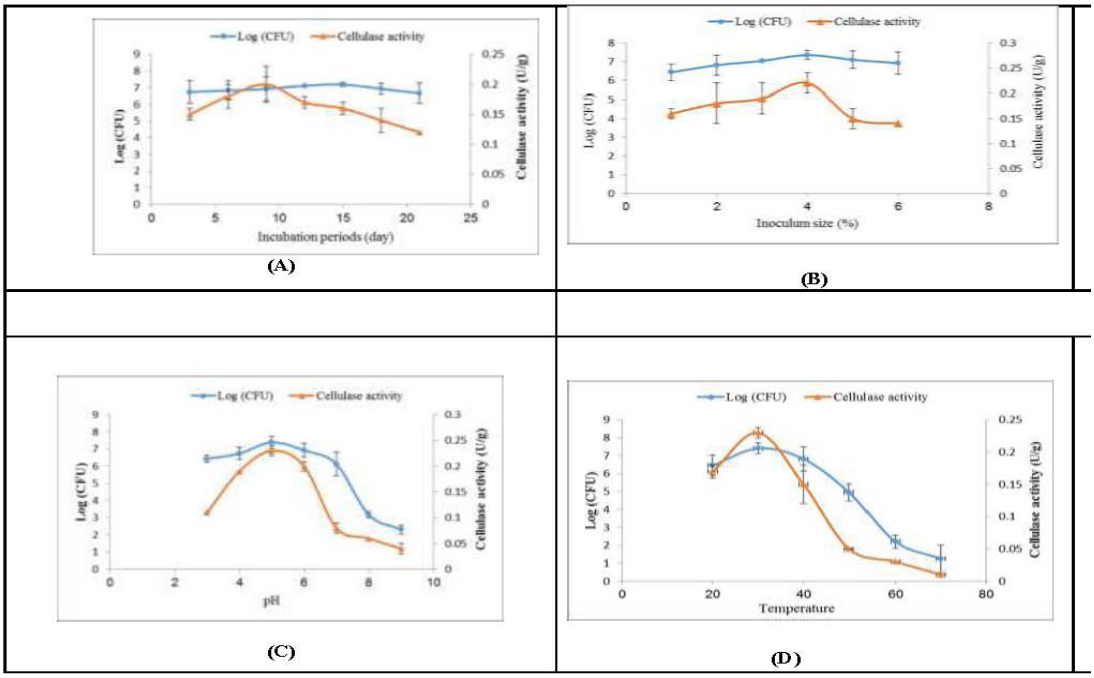

Fig. 6. Effect of different factors on cellulase production by *A. tubingensis* KY615746 under solid state fermentation (A): incubation times (B): inoculum size (C): pH and (D): temperature.

5.0), also Yang et al. (2004) and Ilyas et al. (2010) found that maximum production of cellulase was recorded at pH 4.5.

Effect of temperature on cellulase production

Effect of temperature on the production of cellulase enzyme was determined at temperatures ranging from 20 to 70°C. As shown in Fig. (6 – D) the cellulase activity was increased with the increase in temperature from 20 to 30°C then decreased. The maximum activity was found at 30°C for *A. tubingensis* KY615746. The maximum production of reducing sugars was (1.521 mg g^{-1}) with maximum activity (0.23 U g^{-1}) respectively. The result obtained was in agreement with Alam et al. (2009) who observed that the highest cellulase activity was achieved when carried out SSF was incubated at 32°C using *Trichoderma harzianum* T 2008 grown on empty fruit bunches.

Incubation at lower temperature resulted in longer time to achieve the maximum enzyme activity. On the other hand, incubation at higher temperature affected the fungus harmfully, which reflected on the enzyme synthesis. Since enzyme

is a secondary metabolite produced during exponential growth phase, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield (Sabu et al. 2002). Similarly, Acharya et al. (2008) and Narasimha et al. (2006) reported that the maximum cellulase activity for *Aspergillus niger* was recorded at 28°C infermentation of sawdust. Also, Singhania et al. (2006) showed that *Trichoderma reesei* also revealed maximum cellulase production at 28°C.

Conclusion

This result indicated that suitability of using cheap and abundantly available rice straw waste as solid substrate for maximum production of cellulase under solid state fermentation in comparison with submerged fermentation system in the laboratory. Also, Aspergillus tubingensis KY615746 is a relatively good producer of cellulase under solid state fermentation conditions.

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