Hyper-Productivity, Characterization, and Exploitation of a Cellulase Complex from a Novel Isolate of *Aspergillus tubingenesis* S2 using Lignocellulose-based Material

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The hyper-production potential of a cellulase complex from a local strain of *Aspergillus tubingensis* S2, indigenously isolated from rotten tomato, was investigated. A total of nine fungal species of *Aspergillus* and *Trichoderma* were isolated and confirmed through triple-phase screening *via* 18S ribosomal DNA sequencing and construction of a phylogenetic tree. Congo red testing and the zone of clearance method were used to confirm the cellulase production from *A. tubingenesis* S2 isolate. *A. tubingenesis* S2 revealed maximum cellulase production (78 µg/mL/min) and was selected for further study. The optimum fermentative conditions, including the incubation period, pH, and temperature values, were determined to be 96 h, pH 4.8, and 40 °C, respectively, for obtaining the cellulase activity of 86.4±2.1 µg/mL/min. The cellulase was 5.14-fold purified by ammonium sulfate fractionation and gel permeation chromatography. Characterization revealed that maximum activity (130.5 µg/mL/min and 133.5 µg/mL/min) was achieved at 4.5 pH and 40 °C, respectively. A monomeric protein with an apparent molecular weight of 76 kDa was evident after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cellulase revealed maximal activity with 40-mesh size corn stover as compared with 20-mesh size corn stover and 80-mesh size corn stover after 36 h of incubation at 40 °C.

Keywords: *Cellulase; Aspergillus tubingenesis S2; Congo red; Purification; SDS-PAGE*

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INTRODUCTION

In recent years, both scientific researchers and industrialists have been concerned about sustainability, renewability, recyclability, and cost-efficacy issues in the production of cellulose-degrading enzymes. In this context, the utilization of numerous lignocellulose-based natural materials and their waste streams has presented a great potential for the production of industrially relevant fine chemicals, including enzymes. So far, various lignocellulose-based waste materials, including various straws (wheat straw, rice straw, *etc.*), rice husk, fruit peels (mango peels, banana peels, citrus peels, *etc.*), sugar cane bagasse, corn cobs, and corn stovers, have been exploited and reported in the literature (Asgher and Iqbal 2011; Iqbal *et al.* 2011a,b; Irshad *et al.* 2011). Sadly, a major portion of the above-mentioned potential raw materials is left behind following agricultural and

industrial practices, thus posing serious ecological pollution issues, particularly in developing regions. Approximately 4.4 billion tons of solid wastes are produced annually in Asia alone (Gautam *et al*. 2012). Many biotransformation-based efforts have already been made to convert lignocellulose-based solid wastes into high-value-added products with industrial interest, including ligninolytic and cellulolytic enzymes, many of which have been successful (Asgher and Iqbal 2011; Asgher *et al.* 2012).

Solid lignocellulosic materials comprise cellulose (45% to 50%), lignin (10% to 15%), hemicellulose (9% to 12%), and some other macromolecules. Naturally occurring cellulose consists of thousands of glucose monomers linked by glycosidic linkages. Hemicellulose comprises xylans and other pentoses (xylose, arabinose), hexoses (glucose, mannose, galactose), and many sugar acids (Saha 2003). There are two ways to utilize these agricultural wastes: either by a chemical method or by converting these wastes into useful products through enzymes. Microorganisms including fungi and bacteria convert these waste products into useful products (biofuel) by producing various enzymes (Anwar *et al*. 2012; Imran *et al*. 2016). Filamentous fungi produce a variety of cellulases by consuming cheaper carbon sources such as cellulosic waste biomass or lignocellulosic biomass, which will minimize the cost of the industrial fermentation process (Alriksson *et al*. 2009). Fungal species such as *Aspergillus*, *Penicillium*, *Humicola*, *Trichoderma*, and other microbial strains have been reported with enzyme secretion potentialities including cellulases, hemicellulases, and many other cell wall-degrading enzymes. Hemicellulose is degraded by xylanase, while cellulose is digested by cellulase (Chinedu *et al*. 2011). Cellulase production is regulated transcriptionally and is carbon source-dependent (Foreman *et al*. 2003; Stricker *et al*. 2008).

Cellulase is a complex of several enzymes, *i.e.*, endoglucanase (endo-1, 4-β-D-glucanase, EG, EC 3.2.1.4); cellobiohydrolase or exoglucanase (exo-1, 4-β-D-glucanase, CBH, EC 3.2.1.91); and β-glucosidase (1, 4-β- D-glucosidase, BG, EC 3.2.1.21) (Gao *et al.* 2008). Agricultural cellulosic waste materials are considered to be an alternative source of energy because of their abundant and renewable nature. Such agricultural byproducts do not have a valuable role in daily life and ultimately offer environmental, tactical, and economic advantages (Perez *et al*. 2002).

Enzymes can be produced from these agricultural wastes in the course of hydrolyzing polysaccharides into simple monomeric sugars, which have a variety of uses (Olsson *et al*. 2003). Cellulolytic gene expression enhances the growth of fungi using cellulosic materials with a variety of enzyme production (Sehnem *et al*. 2006) capabilities. More than 15,000 wild and genetically modified strains of fungi and bacteria are aggressive against cellulosic biomass and other insoluble components of fiber cell walls (Gautam *et al*. 2012). The main hurdle in enzyme production is that the yield with cellobiose is very low as compared with cellulose as a substrate. Some inactivating or inhibitory compounds formed may affect the yield of enzymes. Reducing sugars such as glucose and xylose cause enzyme inhibition most frequently, but the process is poorly understood.

Among the various potent cellulose-degrading microbial cultural strains, both *Aspergillus spp.* and *Trichoderma spp.* can produce abundant extracellular cellulases that are suitable for a wide range of biotechnological applications. Therefore, in this article, a novel fungal strain was isolated from a local source and investigated for a hyper-cellulase production. The active cellulase fraction was purified to homogeneity, characterized, and

exploited for glucose release potential. To sum up, information is also given on the sugar-release practicability of the newly isolated cellulolytic enzymes from agricultural wastes, which might be beneficial in bioethanol production.

EXPERIMENTAL

Chemicals and Agro-Industrial Substrate

Dinitrosalicylic acid (DNSA), Nessler's or Biuret reagent, Congo red dye, zinc chloride ($ZnCl_2$), copper(II) sulfate ($CuSO_4$), iron(II) sulfate ($FeSO_4$), cobalt(II) chloride ($CoCl_2$), manganese(II) sulfate ($MnSO_4$), and other chemicals used in fermentation studies were from Sigma-Aldrich (USA). All other chemicals or reagents used in this study were of analytical laboratory grade and used as received unless otherwise stated. Agro-industrial waste, *i.e.* corn stover was collected from the Pattoki main bazaar and nearby fields of Pattoki, Pakistan, for the screening of hyper cellulase complex-producing fungal species. The collected substrates were oven dried at 60 °C for 24 h, ground to fine particles of 80-mm mesh size, and stored in airtight plastic jars.

Isolation of Fungal Species

All collected samples in 10^{-3} dilution were used in the spread plate method on basal salt agar that was additionally supplemented with cellulose. All agar plates were then incubated at various temperatures with an aim to investigate a wide spectrum of growth characteristics for each fungal species (Rao *et al*. 2008).

Fungal Species – Primary Screening

Qualitative analysis was carried out using Congo red dye for cellulase complex-producing fungi. Fungal species were collected from various vegetable and fruit wastes and then grown on carboxymethylcellulose supplemented with the basal salt medium at pH 5 and 30 ± 5 °C for 3 to 5 days. Agar plates were flooded with 0.1% Congo red after the specified incubation period and then washed with 1 M sodium chloride (NaCl) solution for 10 to 12 min. A clear zone formation around fungal cultures such as *Aspergillus niger* (S1), *Aspergillus tubingensis* (S2), *Aspergillus awamori* (S3), *Aspergillus nidulans* (S4), *Aspergillus japonicas* (S5), *Trichoderma reesai* (S6), *Trichoderma viride* (S7), *Trichoderma harjianum* (S8), and *Trichoderma branchianum* (S9) indicated the production of a cellulase complex (Ariffin *et al*. 2006).

Fungal Species – Secondary Screening

Minimal basal medium along with 1% cellulose at pH 5 was utilized for cellulase complex production. The trace elements $ZnCl_2$, $CuSO_4$, $FeSO_4.7H_2O$, $CoCl_2.6H_2O$, $CuSO_4.5H_2O$, and $MnSO_4.H_2O$, were supplemented in the media. After autoclaving, 6 mL of freshly prepared fungal inoculum was poured in flasks at 35±5 °C for 1 to 5 days. The pH, reducing sugars, and cellulosic contents were analyzed. The cellulase complex activity with DNSA after regular intervals (Gautam *et al*. 2012) was also determined.

Fungal Species – Tertiary Screening

A conserve sequence of 18S ribosomal DNA for *Aspergillus* species was checked, which showed hypercellulase activity. The DNA of *Aspergillus* species was isolated using the cetyl N-N-N-trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980). Smaller subunits of rRNA were amplified using reverse and forward primers (Alwakeel 2013). Gene sequencing was carried out using sequence tools and aligned clustal X (Windows version, Clustal X 2.0), after which the alignments were manually corrected. Then, sequences were compared with already existing sequences aligned by GenBank for possible alignment (Makarenkov *et al*. 2010).

Cellulolytic Production and Extraction Protocols

The moisture-free finely powdered substrates, *e.g.*, corn stover (10 g), were pretreated with 2% HCl solution in an Erlenmeyer flask (250 mL) at room temperature (30 °C) for approximately 2 h. The HCl-assisted pretreated substrate was then autoclaved at 121 °C and 103 kPa for 15 min. After this, the substrate slurry was filtered through three layers of muslin cloth; both the filtrates and the residues were saved and consecutively washed four to five times with deionized water. The collected residues were used for the production of cellulase enzymes and further analysis. A basal fermentation media in an Erlenmeyer flask (250 mL) was used to moisten the pretreated substrates (10 g) for cellulase production, as described earlier by Iqbal *et al*. (2010). Major ingredients of the media were as follows: $(NH_4)_2SO_4$, 10 $g \cdot L^{-1}$; KH_2PO_4, 4 $g \cdot L^{-1}$; $MgSO_4 \cdot 7H_2O$, 0.5 $g \cdot L^{-1}$; and $CaCl_2$, 0.5 $g \cdot L^{-1}$. The initial pH value of the medium was adjusted to 4.5 before sterilization, then inoculated with 5 mL of freshly prepared fungal spore suspension, and incubated at 30±1 °C in a temperature-controlled incubator for a stipulated fermentation period. Various pH buffers (of pH values 3 to 6) and temperatures (20 to 70 °C) were utilized to optimize the growth conditions of *Aspergillus tubingenesis* S2. At the end of each 24 h of fermentation, the fermented substrates were harvested by adding 0.05 M citrate buffer at 4.8 pH in a 1:10 w/v ratio. The buffered solutions in Erlenmeyer flasks were shaken at 120 rpm for 30 min (Iqbal *et al*. 2010). The contents were filtered and washed twice with the 0.05 M citrate buffer described earlier. Finally, the centrifugation was performed for 10 min at 10,000 *g* and 4 °C. The collected supernatants were used as a crude enzyme extract to determine the activity and then used for purification purposes.

Cellulase Complex Activity

Cellulase activity was recorded using a filter paper assay. One FPU is defined as the amount of enzyme that releases 1 μmol of glucose equivalents from Whatman No. 1 filter paper per min. Briefly, the assay conditions were as: in a test tube, 0.5 mL of enzyme solution, 1 mL citrate buffer (pH 4.5), 1 mL of distilled water (only for dilution purposes if required), and 50 mg of filter paper were incubated for 30 min at 30 °C. The reaction was stopped by adding 1 mL of DNS reagent, then boiled for 10 min and cooled. The absorbance was read at 540 nm against a blank solution (without enzyme) with 1 mL of DNS and 1.5 mL of distilled water. The absorbance was measured using a UV-Vis spectrophotometer (4 nm bandwidth, AMV09, TLead, Qingdao Tlead International Co. Ltd. Qingdao, Shandong, China) (Douglas *et al*. 2009).

Purification and Protein Profile

Before purification, to maximize clarity, the *Aspergillus tubingenesis* S2 extract was centrifuged for 15 min at 10,000 *g* and 4 °C. The cell debris-free supernatants were then subjected to ammonium sulfate fractionation as described by Iqbal *et al*. (2011b). The total proteins and the activity of the partially purified enzyme were determined before and after the dialysis of the ammonium sulfate precipitation. The dialyzed active fractions were subjected to gel filtration chromatography using a Sephadex-G-100 (Sigma-Aldrich, USA) with 120×2 cm column specifications (Iqbal *et al*. 2011b). Up to 20 fractions were recorded using a 0.5-mL/min flow rate. The enzyme activity and protein content profiles were determined with the help of Nessler's or Biuret reagent (Sigma-Aldrich, USA) and the Lowry method at 660 nm for each separate fraction.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a 5% stacking and a 12% resolving gel to determine the molecular weight of cellulase, according to the method of Laemmli (1970). Molecular markers of different proteins were used to compare the molecular weight of cellulase complex.

Applications of Cellulase Complex

Different mesh sizes (20, 40, and 80) of corn stover were collected and analyzed for cellulase activity using the crude cellulase complex extracted after dialysis. Crude cellulase complex (3 mL) was inoculated on 5 g of corn stover of various sizes and diluted with 100 mL of citrate buffer (pH 4.5). The cellulase complex activity was determined at various incubation levels (12, 24, 36, 48, 60, and 72 h).

Data Analysis

All reported data were trialed using three replicates under the same working environment. The collected data were subjected to analysis of variance (ANOVA), and treatment means were compared by Duncan's New Multiple Range Test (DMRT) at $P < 0.05$ using a computer-based statistical software package (IBM SPSS Statistics 21).

RESULTS AND DISCUSSION

Isolation of Fungal Species

A total of five *Aspergillus* isolates and four *Trichoderma* isolates were grown on basal salt media supplemented with cellulose powder for cellulase complex production. Fungal strains were identified through colony characterization, biochemical tests, and microscopic methods. The fungal species identified by microscopic method and colony characterization were *Aspergillus niger* (S1), *Aspergillus tubingensis* (S2), *Aspergillus awamori* (S3), *Aspergillus nidulans* (S4), *Aspergillus japonicas* (S5), *Trichoderma reesai* (S6), *Trichoderma viride* (S7), *Trichoderma harjianum* (S8), and *Trichoderma branchianum* (S9). Of these species, six fungal isolates were cellulase complex producers in nine samples, and three were genetically determined (Amouri and Gargouri 2006) samples.

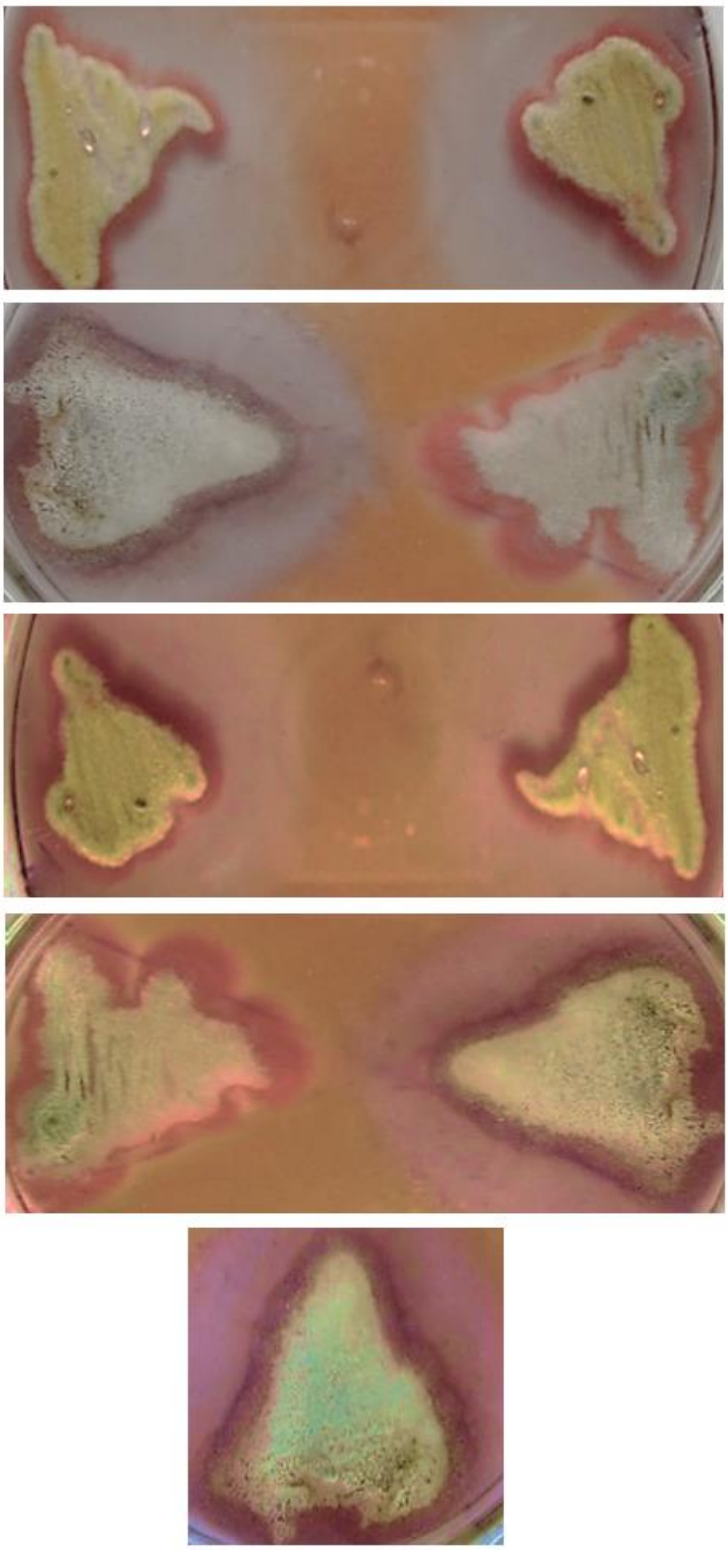

Fig. 1. Primary screening of cellulase producing organisms *via* Congo red dye test – clear zone agar plate because of cellulase complex activity

Fungal Species – Primary, Secondary, and Tertiary Screening

Primary screening was performed as a qualitative technique with Congo red dye. Results revealed that the cellulolytic fungi showed a much greater zone of clearance as compared with less-cellulase-complex-producing fungi. Among the nine fungal species, only three species of *Aspergillus* achieved the maximum clearance zone. The zone of clearance (45 mm) was observed with *Aspergillus tubingensis* (S2), *Aspergillus awamori* (S3), and *Aspergillus niger* (S1). The *Trichoderma* species revealed good cellulase complex activity, but they were not further analyzed because of their already reported cellulase activity (Table 1 and Fig. 1). Secondary screening was carried out using DNS reagent as a dye, and the maximum value was observed with *Aspergillus tubingensis* S2, *Aspergillus awamori* S3, *Aspergillus niger* S1, and *Trichoderma* species (Fig. 2).

Table 1. CMC Congo Red Microbial Clear Zone Diameter (mm) and Well Diffusion Method (mm)

Fungi	Culture Name	S1	S2	S3	S4	S5	S6	S7	S8	S9
	CMC supplemented agar	27	45	35	07	16	43	33	20	23
	Clear zone diameter (mm) by well diffusion method									
	CMC supplemented agar	28	44	39	08	16	41	31	20	21

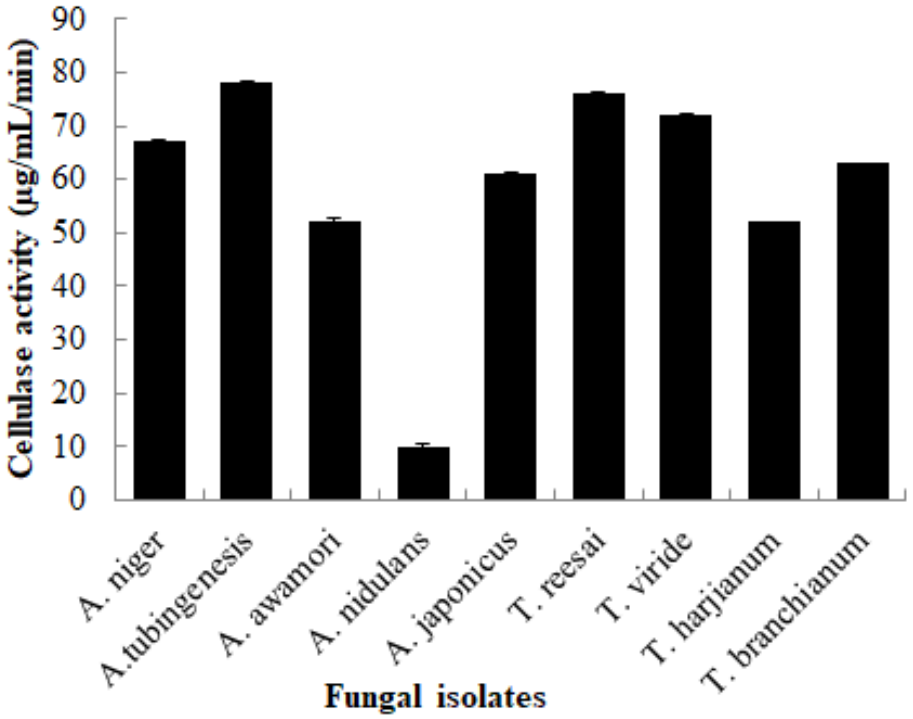

Fig. 2. Secondary screening of fungal isolates for cellulase complex activity

Out of nine isolates, five isolates of fungal species showed maximum cellulolytic activity. For tertiary screening, DNA was extracted from the fungal species and was sequenced (18S ribosomal DNA). Three cellulase complex-producing *Aspergillus* species were reconfirmed by gel electrophoresis (Fig. 3), and the phylogenetic tree of *Aspergillus tubingenesis S2* was constructed (Fig. 4).

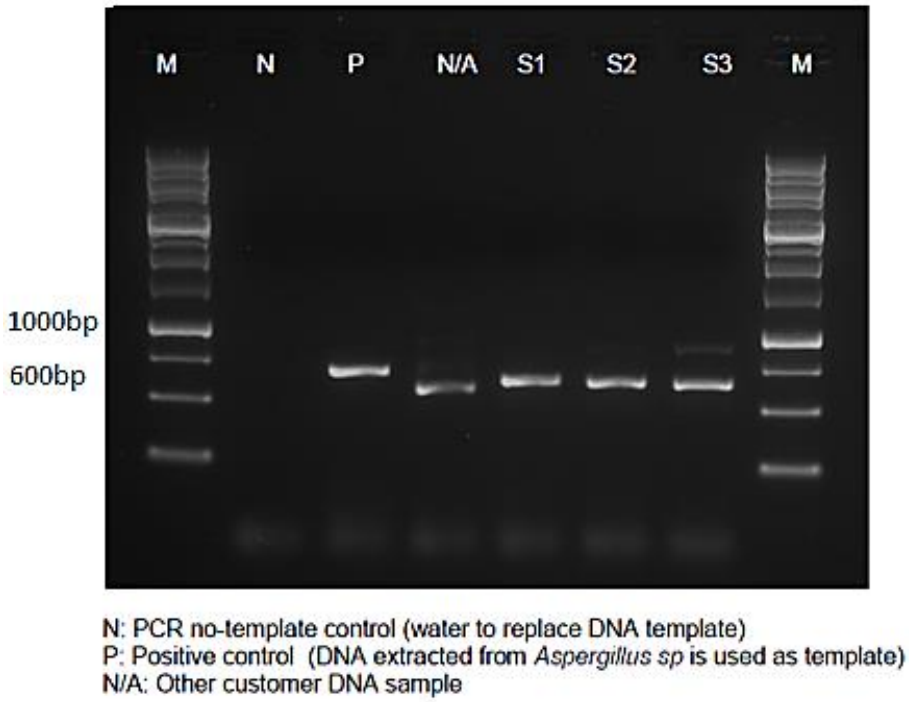

Fig. 3. Native gel electrophoresis *Aspergillus* strains isolates (S1, S2, and S3)

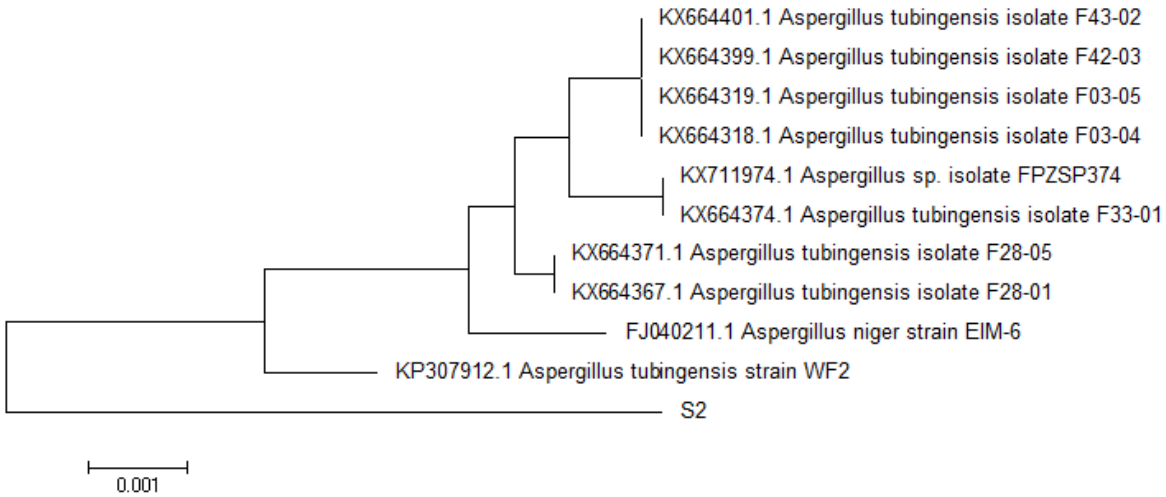

Fig. 4. Phylogenetic Tree for *Aspergillus tubingenesis* S2

Cellulase Activity Profile

The cellulase complex produced by *Aspergillus tubingenesis* S2 was allowed to react with CMC, filter paper, and cellulose powder to access its activity. *Aspergillus tubingenesis* S2 was identified in corn stover medium, and it exhibited a range of carbon source consumption (cellulose) for 1 to 5 days after the inoculum addition. *Aspergillus tubingenesis* S2 growth was quite high and increased continuously up to 4 days (83.7 µg/mL/min), after which it decreased slightly (Table 2). The optimum pH and temperature for *Aspergillus tubingenesis* S2 growth and enzyme production were pH 4.8 (85.4 µg/mL/min) and 40 °C (83.9 µg/mL/min) (Table 2). *Aspergillus tubingenesis* S2 also exhibited maximum production of the cellulase complex in all optimized conditions (86.4±2.1 µg/mL/min). The cellulase complex activity was checked with 1% filter paper, cellulose, and CMC, and quantitative results were achieved with the production of reducing sugars. The amounts of reducing sugar glucose produced during the assay were 2.4, 2.8, and 1.4 mg/mL of glucose from filter paper, cellulose, and CMC, respectively.

Table 2. Influence of Incubation Time, pH, and Temperature on Cellulase Activity Produced from *Aspergillus tubingenesis* S2

pH	Cellulase activity (µg/mL/min)	Incubation period (days)	Cellulase activity (µg/mL/min)	Temperature (°C)	Cellulase activity (µg/mL/min)
2	17.0±0.5^{e}	1	19.0±1.2^{f}	20	26.6±1.6^{de}
3	39.5±0.5^{d}	2	34.5±0.4^{e}	30	56.7±0.3^{c}
4	66.7±1.5^{c}	3	56.7±0.2^{d}	40	83.9±1.4^{a}
4.5	85.4±2.1^{a}	4	83.7±1.6^{a}	50	82.3±1.1^{ab}
5	82.6±0.1^{ab}	5	73.6±2.5^{b}	60	43.6±0.8^{c}
6	66.7±2.3^{c}	6	70.5±0.7^{bc}	70	30.2±1.7^{d}

The collected data were subjected to analysis of variance (ANOVA), and treatment means were compared by Duncan's New Multiple Range Test (DMRT) at $P < 0.05$ using a computer-based statistical software package (IBM SPSS Statistics 21).

Biuret Assay and Purification Profile

Biuret reagent was used to determine the quantity (mg/mL) of protein in the stepwise purification process (Table 3). The crude cellulase enzyme contained a maximum concentration of protein but less active cellulase complex (86.4 µg/mL/min), as shown in Table 3. The total protein content decreased during 80% ammonium sulfate purification, but the amount of active cellulase complex increased (114 µg/mL/min) (Table 3). After the Sephadex-100 gel filtration, a maximal of 1.54-fold purification was achieved. The gel filtration results revealed that the 12th elution led to the maximum value (131 µg/mL/min) for the cellulase complex (Fig. 5). This gel filtration was performed after 80% salting out of cellulase complex (114 µg/mL/min) using ammonium sulfate salt (Table 3).

Table 3. Purification Summary Cellulase Complex from *Aspergillus tubingenesis* S2

Sr. No.	Purification steps	Enzyme activity (µg/mL/min)	Protein content (mg/mL)	Specific activity (µg/mg)	Yield (%)	Purification fold
1	Crude enzyme	86.40	7.26	11.90	100.0	1.00
2	$(NH_4)_2SO_4$ dialysis	114.0	4.53	25.17	132.0	2.12
3	Sephadex-G-100	131.0	2.14	61.21	151.6	5.14

The following formulas were used to calculate the final percent yield and purification fold after Sephadex-G-100.

$$\text{Yield (\%)} = 100\ \text{X}\ \frac{\text{Enzyme activity after Sephadex G100}}{\text{Enzyme activity of crude enzyme}} \quad (1)$$

$$\text{Purification fold} = \frac{\text{Specific activity after Sephadex G100}}{\text{Specific activity of crude enzyme}} \quad (2)$$

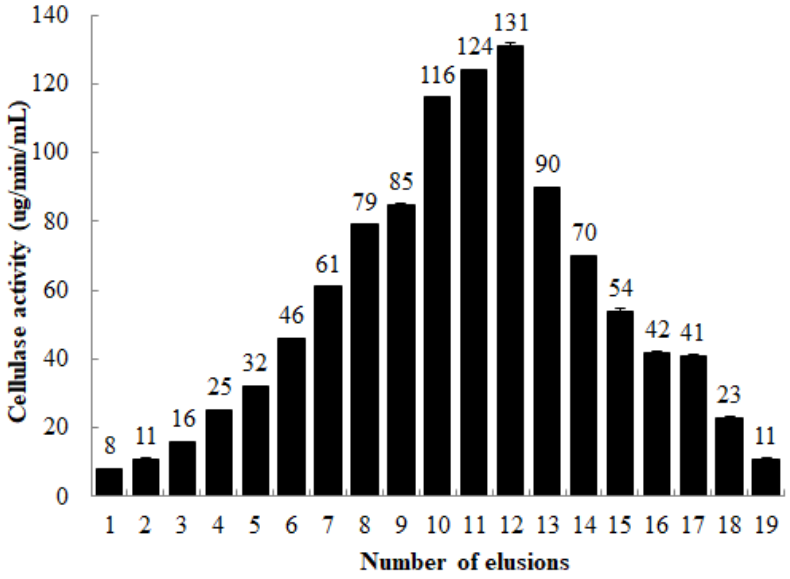

Fig. 5. Gel filtration chromatography for cellulase complex

Characterization of Cellulase Enzyme

For cellulase complex production, pH and temperature optimization for cellulase are highly recommended. The maximum yield of cellulase complex was obtained at optimum pH 4.5, and the cellulase (130.25 µg/mL/min) maximally utilized the substrate (Fig. 6). The cellulolytic enzymes were mostly active at acidic pH but displayed a wide range of pH adaptability of 3.5 to 6.0 (Fig. 6), retaining up to 40.0 µg/mL/min of original activity. The cellulose enzyme- complex assay was also carried out to check the optimum temperature for enzymatic reaction. The optimum temperature (Fig. 6) for cellulase complex was 40 °C (133.5 µg/mL/min). Lane 2 of SDS-PAGE analysis revealed that the molecular weight of the cellulase complex was 76 kDa (Fig. 7).

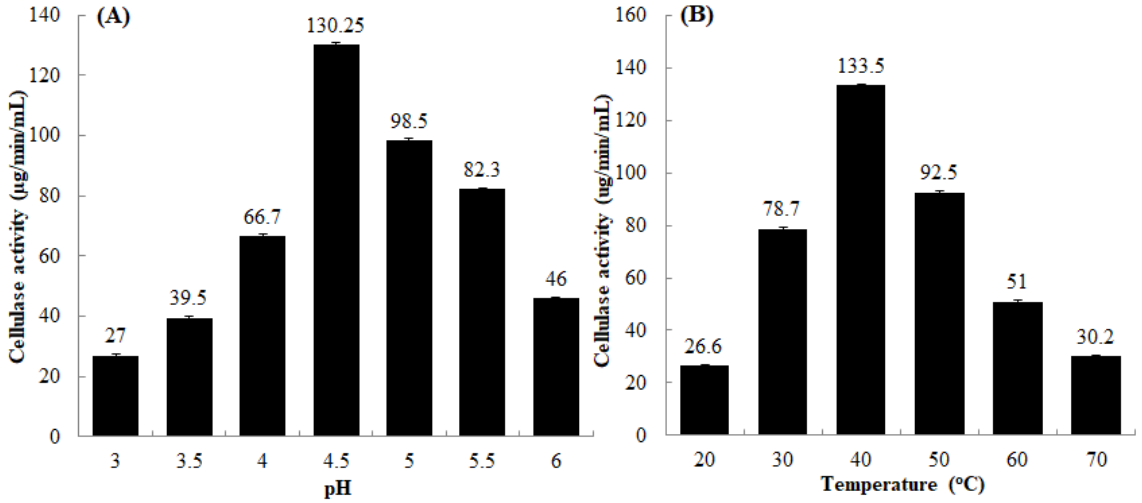

Fig. 6. Characterization of cellulase complex activity with respect to pH (A) and temperature (B)

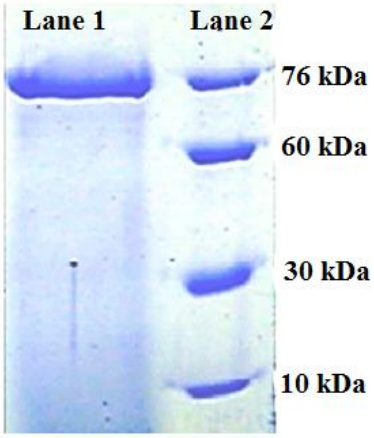

Fig. 7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (8%) for cellulase complex: Lane 1 = Cellulase (76 kDa); Lane 2 = Protein markers)

Applications of Cellulase Enzyme

Crude cellulase was used for the conversion of corn stover with various mesh sizes into glucose. The collected mesh sizes, *i.e.* 20, 40, and 80, were incubated up to 72 h with 12 h intervals. The representative glucose release profile is shown in Fig. 8. After the stipulated incubation period (72 h), the maximal glucose release was recorded with 40-mesh size, *i.e.* 170 mg/L, as compared with the 20-mesh size and 80-mesh size corn stover. During initial hours, a significant difference was observed in the glucose release behavior subject to the respective mesh size use. Towards the end, the almost same level of glucose yield was recorded with all three tested mesh sizes. The glucose production remained almost constant for different mesh sizes after maximal incubation in the presence of crude cellulase enzyme (Fig. 8). In solid-state fermentation, *Aspergillus* cellulase-producing species hydrolyzed cellulose into glucose and *Aspergillus* species used glucose as its carbon source (Vega *et al.* 2012).

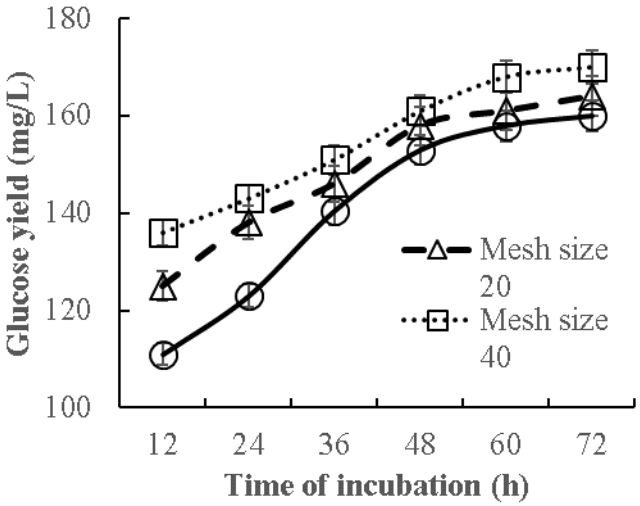

Fig. 8. Enzymatic saccharification of 3 mL of crude cellulase complex on various mesh sizes of corn stover at 40 °C

CONCLUSIONS

1. The current work presented a novel approach for waste management of agro-based waste materials. A total of nine fungal species of *Aspergillus* and *Trichoderma* were isolated and confirmed through triple-phase screening *via* 18S ribosomal DNA sequencing and construction of a phylogenetic tree.
2. The results of this study indicated the remarkable enzyme production potential of locally produced fungal isolates, particularly *Aspergillus tubingenesis* S2, by using the lignocellulose-based material, *e.g.*, corn stover, as an inexpensive fermentative substrate.
3. A maximal of 5.14-fold purification was achieved via ASF and gel permeation chromatography techniques. The homogenously purified cellulose was found monomeric in nature with an apparent molecular weight of 76 kDa.

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REFERENCES CITED

Alriksson, B., Rose, S. H., van Zyl, W. H., Sjöde, A., Nilvebrant, N. O., and Jönsson, L. J. (2009). "Cellulase production from spent lignocellulose hydrolysates by recombinant *Aspergillus niger*," *Appl. Environ. Microbiol.* 75(8), 2366-2374. DOI: 10.1128/AEM.02479-08

Alwakeel, S. S. (2013). "Molecular identification of isolated fungi from stored apples in Riyadh, Saudi Arabia," *Saudi J. Biol. Sci.* 20(4), 311-317. DOI: 10.1016/j.sjbs.2013.05.002

Amouri, B., and Gargouri, A. (2006). "Characterization of a novel β-glucosidase from a *Stachybotrys* strain," *Biochem. Eng. J.* 32(3), 191-197. DOI: 10.1016/j.bej.2006.09.022

Anwar, Z., Gulfraz, M., Imran, M., Asad, M. J., Shafi, A. I., Anwar, P., and Qureshi, R. (2012). "Optimization of dilute acid pretreatment using response surface methodology for bioethanol production from cellulosic biomass of rice polish," *Pak. J. Bot*. 44(1), 169-176.

Ariffin, H., Abdullah, N., Umi Kalsom, M. S., Shirai, Y., and Hassan, M. A. (2006). "Production and characterization of cellulase by *Bacillus pumilus* EB3," *Int. J. Eng. Technol.* 3(1), 47-53.

Asgher, M., and Iqbal, H. M. N. (2011). "Characterization of a novel manganese peroxidase purified from solid state culture of *Trametes versicolor* IBL-04," *BioResources* 6(4), 4317-4330. DOI: 10.15376/biores.6.4.4317-4330

Asgher, M., Iqbal, H. M. N., and Asad, M. J. (2012). "Kinetic characterization of purified laccase produced from *Trametes versicolor* IBL-04 in solid state bio-processing of corncobs," *BioResources* 7(1), 1171-1188. DOI: 10.15376/biores.7.1.1171-1188

Chinedu, S. N., Okochi, V. I. and Omidiji, O. (2011). "Cellulase production by wild strains of *Aspergillus niger, Penicillium chrysogenum* and *Trichoderma harzianum* grown on waste cellulosic materials*," Ife J. Sci.* 13(1), 57-62.

Douglas, E., Eveleigh, D. E., Mandels, M., Andreotti, R. and Roche, C. (2009). "Measurement of saccharifying cellulose," *Biotechnol. Biofuels*. 2(21), 1-8. DOI: 10.1186/1754-6834-2-21

Foreman, P. K., Brown, D., Dankmeyer, L., Dean, R., Diener, S., Dunn-Coleman, N. S., and Meerman, H. J. (2003). "Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*," *J. Biol. Chem.* 278(34), 31988-31997. DOI: 10.1074/jbc.M304750200

Gao, J., Weng, H., Zhu, D., Yuan, M., Guan, F., and Xi, Y. (2008). "Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover," *Bioresour. Technol*. 99(16), 7623-7629. DOI: 10.1016/j.biortech.2008.02.005

Gautam, S. P., Bundela, P. S., Pandey, A. K., Awasthi, M. K., and Sarsaiya, S. (2012). "Diversity of cellulolytic microbes and the biodegradation of municipal solid waste by a potential strain," *Int. J. Microbiol*. 2012, 325907. DOI: 10.1155/2012/325907.

Imran, M., Anwar, Z., Irshad, M., Asad, M. J., and Ashfaq, H. (2016). "Cellulase production from species of fungi and bacteria from agricultural wastes and its utilization in industry: A review," *Adv. Enzy. Res.* 4(2), 44-55. DOI: 10.4236/aer.2016.42005

Iqbal, H. M. N., Asgher, M., Ahmed, I., and Hussain, S. (2010). "Media optimization for hyper-production of carboxymethyl cellulase using proximally analyzed agro-industrial residue with *Trichoderma harzianum* under SSF," *IJAVMS* 4(2), 47-55.

Iqbal, H. M. N., Asgher, M., and Bhatti, H. N. (2011a). "Optimization of physical and nutritional factors for synthesis of lignin degrading enzymes by a novel strain of *Trametes versicolor*," *BioResources* 6(2), 1273-1287. DOI: 10.15376/biores.6.2.1273-1287

Iqbal, H. M. N., Ahmed, I., Zia, M. A., and Irfan, M. (2011b). "Purification and characterization of the kinetic parameters of cellulase produced from wheat straw by *Trichoderma viride* under SSF and its detergent compatibility," *Adv. Biosci. Biotechnol.* 2(3), 149-56. DOI: 10.4236/abb.2011.23024

Irshad, M., Asgher, M., Sheikh, M. A., and Nawaz, H. (2011). "Purification and characterization of laccase produced by *Schyzophylum commune* IBL-06 in solid state culture of banana stalks," *BioResources* 6(3), 2861-2873. DOI: 10.15376/biores.6.3.2861-2873

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature* 227(5259), 680-685. DOI: 10.1038/227680a0

Makarenkov, V., Boc, A., Xie, J., Peres-Neto, P., Lapointe, F. J., and Legendre, P. (2010). "Weighted bootstrapping: a correction method for assessing the robustness of phylogenetic trees," *BMC Evolution. Biol.* 10(1), 250. DOI: 10.1186/1471-2148-10-250

Murray, M. G., and Thompson, W. F. (1980). "Rapid isolation of high molecular weight plant DNA," *Nucleic Acids Res.* 8(19), 4321-4326. DOI: 10.1093/nar/8.19.4321

Olsson, L., Christensen, T. M., Hansen, K. P., and Palmqvist, E. A. (2003). "Influence of the carbon source on production of cellulases, hemicellulases and pectinases by *Trichoderma reesei* Rut C-30," *Enzyme Microb. Technol.* 33(5), 612-619. DOI: 10.1016/S0141-0229(03)00181-9

Pérez, J., Munoz-Dorado, J., de la Rubia, T. D. L. R., and Martinez, J. (2002). "Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview," *Int. Microbiol.* 5(2), 53-63. DOI: 10.1007/s10123-002-0062-3

Rao, R. S., Bhadra, B., and Shivaji, S. (2008). "Isolation and characterization of ethanol-producing yeasts from fruits and tree barks," *Lett. Appl. Microbiol.* 47(1), 19-24. DOI: 10.1111/j.1472-765X.2008.02380.x

Saha, B. C. (2003). "Hemicellulose bioconversion," *J. Ind. Microbiol. Biotechnol.* 30(5), 279-291. DOI: 10.1007/s10295-003-0049-x

Sehnem, N. T., de Bittencourt, L. R., Camassola, M., and Dillon, A. J. (2006). "Cellulase production by *Penicillium echinulatum* on lactose," *Appl. Microbiol. Biotechnol.* 72(1), 163-167. DOI: 10.1007/s00253-005-0251-z

Stricker, A. R., Mach, R. L., and De Graaff, L. H. (2008). "Regulation of transcription of cellulases-and hemicellulases-encoding genes in *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*)," *Appl. Microbiol. Biotechnol.* 78(2), 211-220. DOI: 10.1007/s00253-007-1322-0

Vega, K., Villena, G. K., Sarmiento, V. H., Ludena, Y., Vera, N., and Correa, M. G. (2012). "Production of alkaline cellulase by fungi isolated from an undisturbed rain forest of Peru," *Biotechnol. Res. Int.* 2012, 934325. DOI: 10.1155/2012/934325

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