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ISOLATION AND SCREENING OF CELLULASE PRODUCING FUNGAL ISOLATES FROM SEA SANDS

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Abstract

Cellulases are the enzymes which hydrolyse cellulosic biomass and are being produced by the microorganisms grown over cellulosic substrates. The cellulase producing fungal isolates were obtained from sea sands and cellulosic waste materials. Four fungal species were isolated and screened by using Carboxy Methyl Cellulose (CMC) agar medium as a selective medium. Production of clear zones by the fungal isolates on CMC agar medium supplemented with 1% CMC was considered as indicative of extracellular cellulase activity. The size of transparent zone diameter was considered as proportional to the level of cellulase production. These fungal isolates were identified as *Aspergillus sp., Penicillium sp. and Fusarium sp.* by studying their microscopic and macroscopic characters. A basal medium containing CMC, KH_2PO_4, K_2HPO_4, $MgSO_4$, $(NH_4)_2SO_4$, $CaCl_2$ and $FeSO_4$ at pH 7.0 was used for cellulase production. The assay of cellulase in term of CMCase was performed by measuring the release of reducing sugar. The crude cellulase produced by these fungal isolates was partially characterized. Optimum temperature for maximum cellulytic activity was 35°C and was active at 35°-40°C. The cellulase activity was significantly active over a broad pH range from 5.0 to 8.0 having maximum activity at pH 6.0.

Keywords: Cellulase, Carboxy Methyl Cellulose (CMC), Marine fungi, CMCase activity.

Introduction

The cellulose constitutes the major form of stocking glucose obtained through photosynthesis and in the same time the major component of solar energy conversion to the biomass. The cellulose is also major constituent of all the plant materials and that is why it is the most abundant organic material in nature, which is renewed every year. Because of its highly ordered structure, the cellulose is very hard to be degraded and that is why it is unusable and stocked in nature as waste. The capacity to degrade the natural cellulose implies the synthesis of the entire cellulolytic system. Cellulose has been used by man for centuries, however, its enormous potential as a renewable source of energy was recognized only after cellulose degrading enzymes or 'cellulases' had been identified (Bhat and Bhat, 1997).

A cellulosic enzyme system consists of three major components: endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Knowles *et al*., 1987). These components act synergistically in the conversion of cellulose to glucose (Eveleigh, 1987). The cellulase complex of *Aspergillus niger* has been most thoroughly studied. It can convert native cellulose as well as derived celluloses to glucose (King and Nessal, 1969). (Ahmad *et al*., 2003) worked on *Trichoderma harzianum* for cellulase enzyme production by using different carbon sources and reported that Carboxy methyl cellulose is the best for substantial amount of enzyme production (Shaziashafique *et al*., 2009). Growth of fungi as well as the enzyme production depends on the composition of the growth media, pH, and temperature. The effect of environmental factors on the growth of fungi is generally less specific and restricted than the effect on secondary metabolite production. For example, the ranges of water activity, growth medium and pH within which formation of certain secondary metabolites occur, was narrower, than the range of conidial growth (Northolt and Bullerman, 1982). Several studies were carried out to produce cellulolytic enzymes from biowaste degradation process by many microorganisms including fungi such as *Trichoderma, Penicillium, Aspergillus* spp. etc. (Mandels and Reese, 1985; Hoffman and Wood, 1985).

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Similarly celluloytic property of bacterial species like *Pseudomonas*, *Cellulomonas*, *Bacillus*, *Micrococcus*, *Cellovibrio* and *Sporosphytophaga* spp. were reported (Nakamura and Kappamura, 1982; Immanuel *et al*., 2006). Cellulases are relatively costly enzymes, and a significant reduction in cost will be important for their commercial use in biorefineries. Hence, the need for natural and cheaper sources of cellulases is the driving force of this investigation. The present study was aimed to isolate and screen potential cellulase producing native fungal species from sea sands. A novel methodology of isolation and primary screening of cellulolytic fungi was adopted by exposing carboxymethyl cellulose agar, a selective media for cellulolytic microorganisms and enzyme activity was determined.

Materials and Methods

Isolation and identification of marine fungi: Different marine samples were collected from Cox's bazar sea coast in Bangladesh. Isolation was done on sand dilution (serial dilution) plate method. Serial dilution was done till 10^{-4}. One ml of desired dilution (10^{-3} and 10^{-4}) was transferred aseptically into a potato dextrose agar (PDA) prepared from marine water adding one drop of 20% lactic acid to suppress fungal growth. Plates were incubated at 30°C temperature for 3 days. After incubation, small portion mycelium from each fungal colony was transferred into CMC media. The sporulated fungi were identified based on the colony morphology and microscopical features using standard taxonomic keys and monographs (Carmichael *et al.,* 1980).

Production of crude cellulase: The culture was grown under optimal condition for cellulase production. The strain cultured in basal medium containing 1% CMC and incubated for 3-10 days on a rotary shaker (110 rpm min^{-1}) at 30°C. Each of the cultures was centrifuged at 8,000 rpm min^{-1} for 15 min. The clear supernatants were collected aseptically as extracellular cellulase preparations.

Enzyme assay: Cellulase activity was determined by CMCase enzyme assay. CMCase activity was assayed using a method described by Mandels and Weber (1969). The reducing sugar released from carboxymethyl cellulose per ml per min at 575nm was determined by dinitrosalicylic acid (DNS) method. One unit of total cellulase activity was defined as the amount of enzyme releasing 1µmole of reducing sugar per minute.

Effects of temperature and pH on cellulase activity and stability: To investigate the effects of temperature and pH on cellulase activity, 500 µL of the crude enzyme was added to 500 µL of 1% CMC in 50 mM citrate buffer (pH 4.8). The reaction mixture was incubated for 30 min at various temperatures 30, 35, 40, 45, 50, 55 and 60°C. Cellulase activity was then measured as described above. To study the effects of pH on cellulase activity, different buffers such as 50 mM of sodium citrate (pH 4.0 and 5.0), potassium phosphate (pH 6.0 - 7.0) and Tris- HCl (pH 8.0 - 9.0) were used to assay the CMCase activity. To 0.5 ml of 1% CMC prepared in a suitable buffer of a particular pH (pH 4 - 9), 0.5 ml of crude enzyme was added. To investigate the temperature stability, 1 ml of crude enzyme was treated for 1 h at various temperatures 30, 35, 40, 45, 50, 55 and 60°C. The residual CMCase activity of crude cellulase was measured according to the standard assay procedure. To study the pH on cellulase stability, the crude cellulase enzyme was treated for 1 h at room temperature with buffers of different pH 4 - 9 as described above. Standard assay procedure was followed to measure the residual activity of crude cellulase.

Results and Discussion

Isolation of cellulolytic fungi: Cellulolytic fungi were isolated from the sea sands using selective media which contained CMC as carbon source. Isolated fungal colonies with higher cellulase activity were further selected from mix cellulolytic fungal colonies according to clear zone formation capability, where clear zones were visualized by using Grams iodine stain as shown in Fig. 1.

Cellulase strength due to zone and colony diameter ratio: Halo zone is created on the CMC agar media due to the cellulolytic degradation of the fungal isolates (Samira *et al*., 2011). So, the ratio between zone diameters to colony diameters expresses the degrees of cellulase production capacity. Isolate 1 zone diameter $colony^{-1}$ ratio was 6.5, isolate 4 ratio was 5.4, isolate 8 ratio was 7.5 and isolate 9 ratio was 6.25 whereas we considered ratio 5.0 was the significant level in this study (Table 1). Data indicated that isolate 8 had the highest and 4 had the lowest ratio among the four isolates.

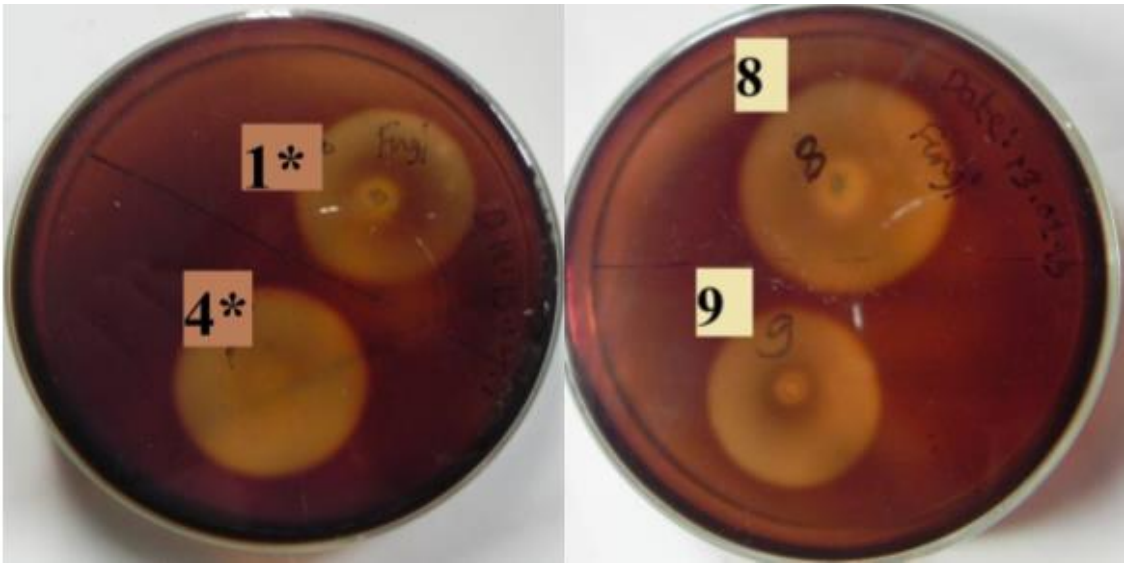

Fig. 1. Visualization of cellulase activity with Grams iodine stain

Table 1. Zone and colony diameter ratio of fungal isolates

Isolates	Zone Diameter (cm)	Colony Diameter (cm)	Ratio
Isolate 1	2.6	0.4	6.5
Isolate 4	2.7	0.5	5.4
Isolate 8	4.5	0.6	7.5
Isolate 9	2.5	0.4	6.25

Morphological identification of fungal genus: The identification of cellulase producing fungi was performed by studying their microscopic and macroscopic characters. Congo red staining was performed so that better microscopic observations could be made (Samira *et al*., 2011). Isolates 1 and 8 were identified as Aspergillus sp. The species generated colonies, compiled of white or yellow felt that was covered by dark asexually produced fungal spores. Conidiophores (asexually produced fungal spores) of *Aspergillus* sp. contain globose (globular) vesicles. Each globose vesicle was utterly covered with phialides which were blistering from entire surface. The conidia were one celled, colored in mass and arranged in basipetal chains. Isolate 9 was also identified as *Fusarium* sp. They were nurtured as white mold in culture. Mycelial or thread like hyphae were alienated by a transparent septum. They produced distinctive conidiophores which were tiny and straight forward. The conidia were one celled, ovoid in shape and smooth. Isolate 4 were also belonging to *Penicillium* sp. These isolates produced colonies which were cottony white in colour with green tinge. The conidiophores cropped up from mycelium and were pronged. The conidia were smooth and ovoid (Figs. 2-5).

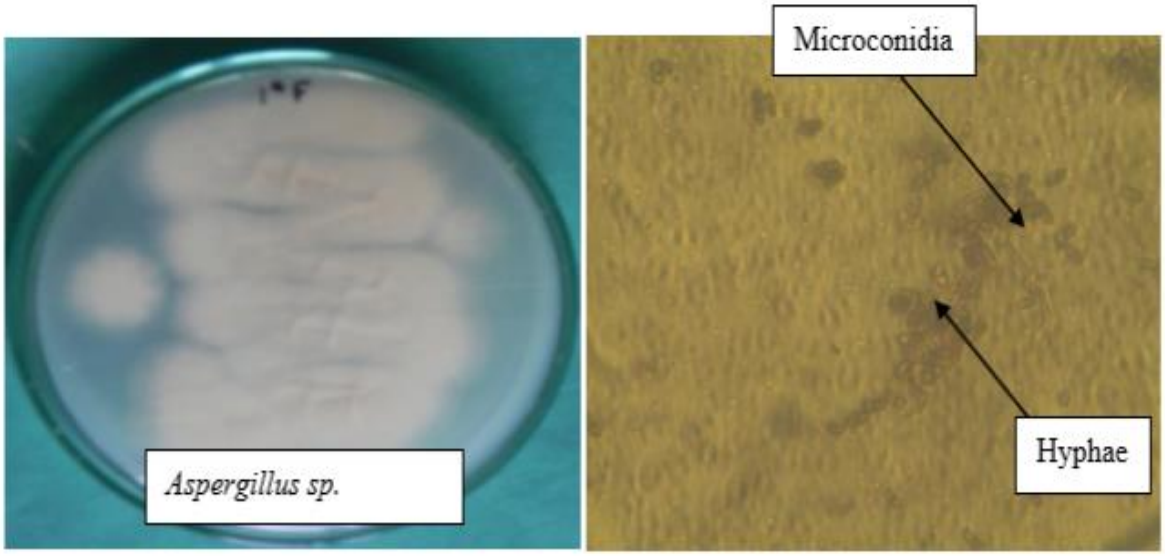

Fig. 2. Colony morphology and microscopy of fungal isolates 1*

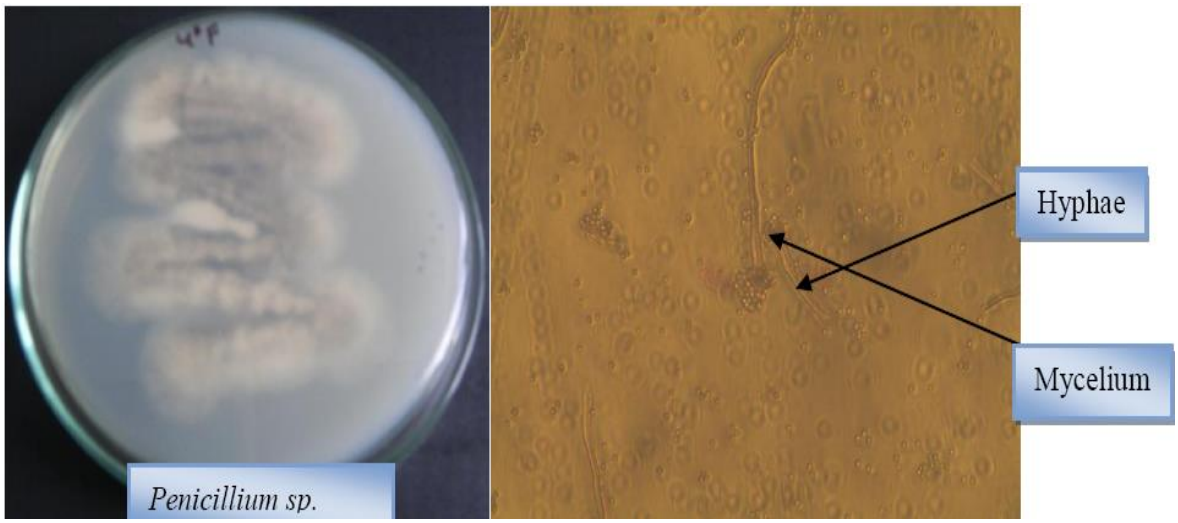

Fig. 3. Colony morphology and microscopy of fungal isolates 4*

Production of CMCase in submerged state fermentation: For the production of cellulase in submerged state fermentation, the selected fungi were inoculated separately in 100 ml fermentation media in 250 ml capacity flask. The fungi utilize the media for its growth and secret various secondary metabolites including cellulases into the culture flask. The enzyme quantity expected to increase with increase in fungal growth and period of incubation. The cultivation time allows maximum growth of microorganism and product formation to a certain degree in a fermentation system. The enzyme activity of crude extract was determined by using spectrophotometer. The results revealed increasing trend of enzyme activity (Uml-1) for both tested fungal isolates up to 9 days of incubation and then decline. This may be due to increase in concentration of certain toxic wastes and depletion of nutrients in fermentation media which leads to decreased fungal growth and enzymes production. Another reason may be the high viscosity of the medium, which decreases the oxygen supply to the microorganism. The enzyme production was recorded higher after 9 days of incubation time.

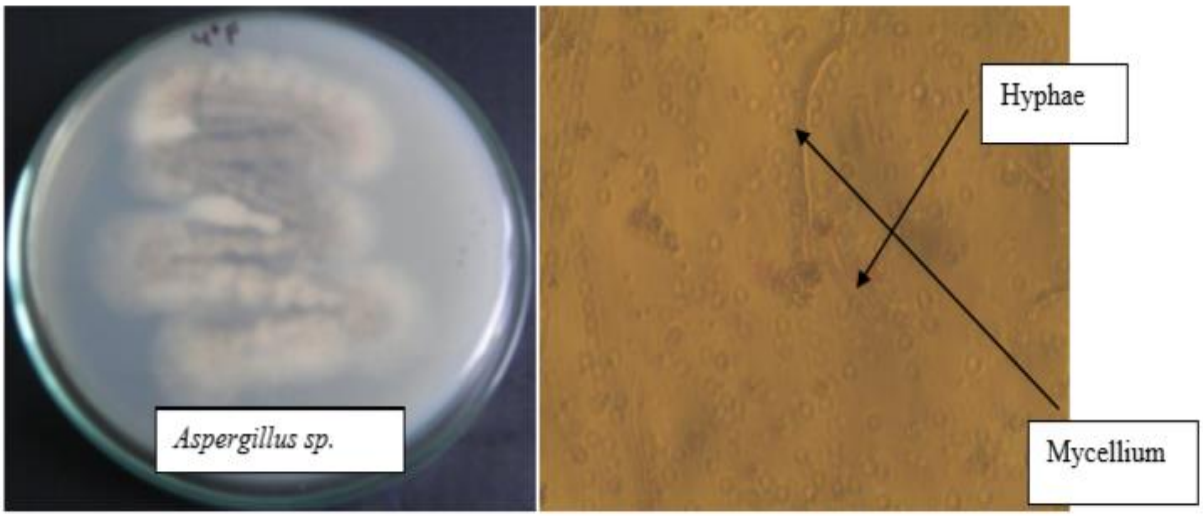

Fig. 4. Colony morphology and microscopy of fungal isolates 8*

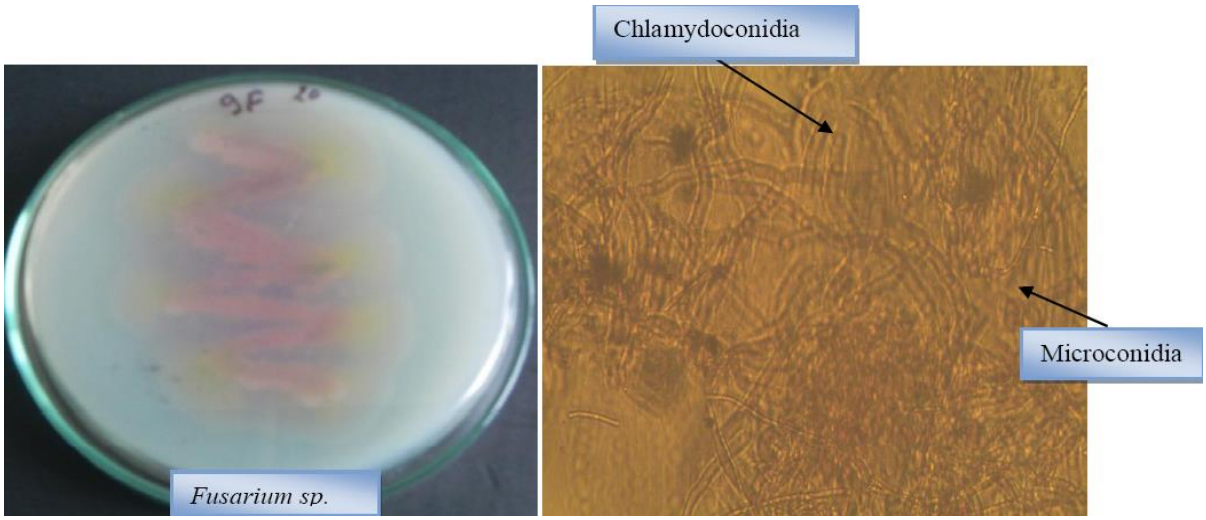

Fig. 5. Colony morphology and microscopy of fungal isolates 9*

Effects of temperature and pH on cellulase activity and stability: The optimum temperature of cellulase activity by four fungal isolates was assayed. The optimum temperature for cellulase activity was found 35°C for isolate 1 (*Aspergillus* sp.), isolate 4 (*Penicillium* sp.) and 40°C for isolate 8 (*Aspergillus* sp.) (Fig. 6). The effects of pH on cellulase activity were investigated using CMC as substrate in 50 mM buffer solutions ranging from pH 4.0 to 10.0. The cellulase produced by the isolates 1 and 8 (*Aspergillus* sp.) was significantly active over a broad pH range from 5.0 to 8.0 having the maximum activity at pH 6.0 while isolate 4 (*Penicillium* Sp.) showed the highest activity at pH 7.0 (Fig. 7). The temperature stability of crude celluase by fungal isolates was assayed. Crude isolate was almost stable from 25 – 45°C for isolates 1 and 8 (*Aspergillus* sp.) while isolate 4 (*Penicillium* sp.) was stable up to 50°C (Fig. 8). The effects of pH on cellulase stability were investigated by treating crude cellulase preparation at the range of pH 4.0 to 10.0. Crude cellulase from the isolates was almost stable at pH 4.0 - 7.0 (Fig. 9). However, the activity was severely lost when the enzyme solution from four fungal isolate was treated at pH 8.0 - 10.0.

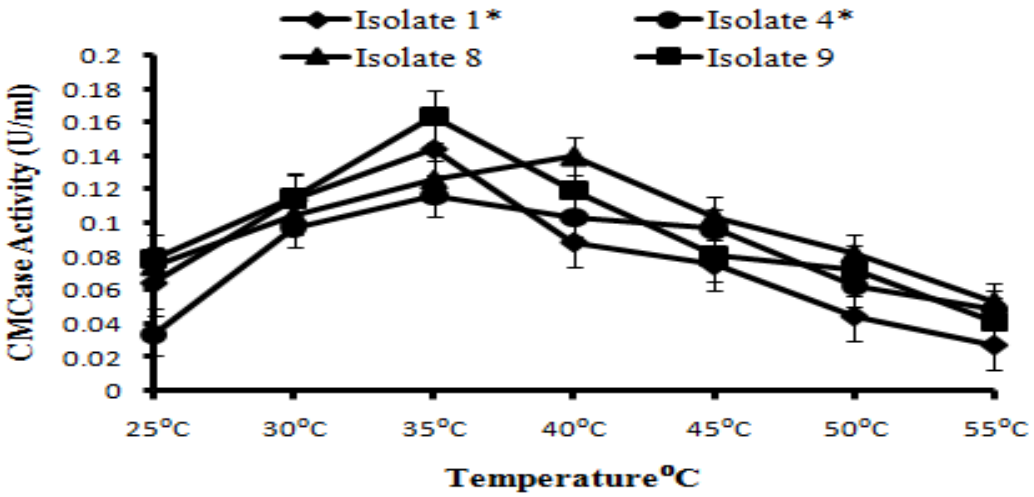

Fig. 6. Effects of temperature on cellulase activity

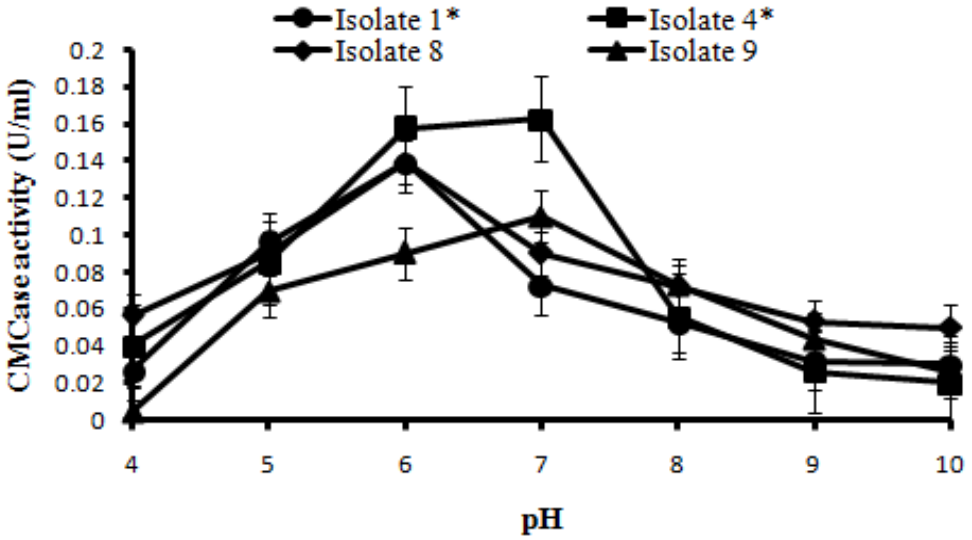

Fig. 7. Effects of pH on cellulase activity

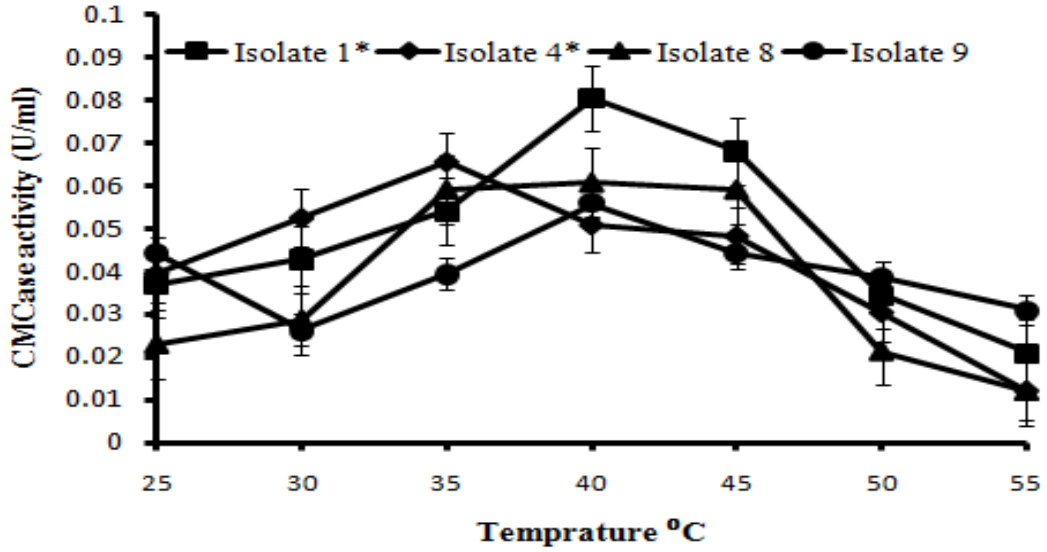

Fig. 8. Effects of temperature on cellulase stability

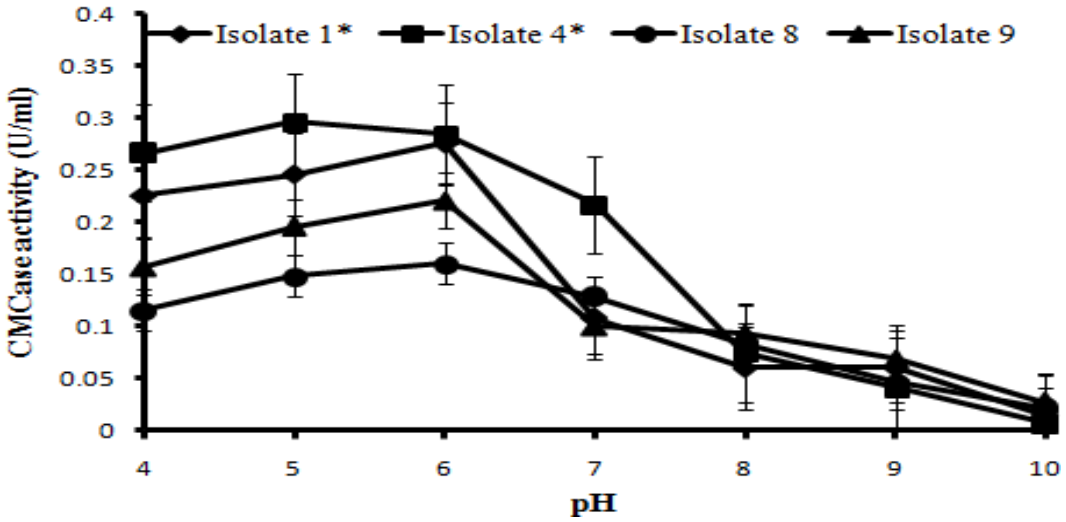

Fig. 9. Effects of pH on cellulase stability

Cellulose is abundant in nature and awaited to be converted into more valuable products used for mankind. Several microorganisms capable of converting cellulose into simple carbohydrates had been discovered for decades. CMC was used as a carbon source in selective media to isolate cellulolytic microorganisms as described by Hankin and Anagnsostakis (1975). CMC is not only used for isolation of cellulolytic fungi but also for production of cellulase. Among the various cellulases, fungal cellulases are the most significant, compared with bacterial cellulase. And among fungi, *Aspergillus*, *Fusarium* and *Penicillium* species are significantly high producers of extracellular cellulase. These cellulases have wide applications in laundry, food and waste processing industries (Uhlig *et al.*, 1998, Galante *et al.*, 1998, Freiermuth *et al.*, 1994). Screening for cellulase-producing microorganisms is routinely done on CMC plates. CMC plates were flooding with Gram's iodine, as described by (Ramesh *et al.*, 2008). Gram's iodine formed a bluish-black complex with cellulose but not with hydrolyzed cellulose, giving a sharp and distinct zone around the cellulase-producing microbial colonies within 3 to 5 minutes. This method is rapid and efficient;

therefore, it can be easily performed for screening large numbers of microbial cultures of both bacteria and fungi. It was the first report on the use of Gram's iodine for the detection of cellulase production by microorganisms using plate assay (Ramesh *et al.,* 2008). Clear zone ratio of isolated cellulolytic fungi was measured. For measurement of clear zone, CMC plates were flooding with Gram's iodine which formed a bluish-black complex with cellulose but not with hydrolyze cellulose. The highest zone forming strain indicated highest cellulase producing activity. Isolated cellulolytic fungal isolate 1* zone diameter/colony diameter ratio was 6.5; isolate 4 ratio was 5.4; isolate 8 ratio was 7.5 and isolate 9 ratio is 6.25 and we considered the significant level of this study. From this zone forming capacity it can be said that isolated four isolates had significantly high cellulase producing activity respectively.

Morphological chacterization and cultural features was done for the identification of unknown genus as described by (Krull *et al.,* 2012). Isolates 1 and 8 were identified as Aspergillus species. The species produced colonies, composed of white or yellow felt was covered by dark asexually produced fungal spores. Conidiophores (asexually produced fungal spores) of Aspergillus sp. contain globose (globular) vesicles. Each globose vesicle was completely covered with phialides which were radiating from entire surface. The conidia were one celled, coloured in mass and arranged in basipetal chains. Isolates 9 were identified as Fusarium species. They grew as white mold in culture. Mycelial or threadlike hyphae were divided by a transparent septum. They produce distinctive conidiophores which were short and simple. The conidia were one celled, ovoid in shape and smooth. Isolate 4 belongs to *Penicillium* species. The *Penicillium* sp. produces colonies which were cottony white in colour with green tinge. The conidiophores arised from mycelium and were branched. The conidia were smooth and ovoid.

The growth and production of enzyme by microorganisms were markedly influenced by the different pH. So the effect of pH on the production of cellulase by four isolates was investigated (pH 4-7) and it was found that these isolates formed the highest level of cellulase at pH 6.5. For the enzyme production these identified fungal species was then inoculated into 10 ml production media at pH 6.5, 30 and 110 rpm for 9 days. Then the crude enzyme (cell free supernatant) was collected by centrifugation and subjected to cellulase assay. The measurement was done by spectrophotometer. CMC was most effective as a carbon source for cellulase enzyme production by fungal isolates results in increase in enzyme activity. Four isolates showed significantly high activity in CMCase assay respectively. This study was accomplished by isolation and morphological characterization of the cellulase producing fungal isolates and the ability of the isolated crude cellulases to degrade cellulose rich substance such as paper. From this study, we found four fungal isolates namely *Aspergillus sp.* (1* and 8.), *Fusarium sp.* (9) and *Penicillium sp.* (4*), respectively with cellulolytic activity and they are capable to biodegradation of cellulosic materials.

Conclusion

Fungi are key agents for degradation of cellulosic materials. The present study has isolated four cellulolytic fungi that have been identified as *Aspergillus sp*., *Penicillium sp.* and Fusarium *sp.* based on morphological, cultural and biochemical characteristics. The crude cellulase of these identified isolates was characterized in terms of effects of temperature and pH on the CMCase activity and stability. The collection of more fungal isolates from cellulosic solid wastes and genetic engineering approach would provide more pacesto degrade the organic wastes as well as xenobiotic compounds. It is now a very important concern to develop ecologically sound and health promoting ways to manage cellulosic waste material for the sustainable development of the society.

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