Production, purification and molecular level identification of cellulase degrading enzyme from improved strains of *Aspergillus niger*

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Abstract - Cellulase is a degrading-enzyme provides a key opportunity for achieving tremendous benefits of biomass utilization. The objective of this study was production, purification and molecular characterization of cellulase from Aspergillus niger. A total of 7 Aspergillus isolates (WA1-WA7) were isolated from agricultural field soils. WA2 isolate was found to produces highest zone of hydrolysis on cellulase screening media and selected for strain improvement studies. A total of 5 WA2 colonies were isolated after UV radiation for enzyme production. Several agricultural and natural wastes were used for cellulase production by Aspergillus sp. Among the 5 isolates WA2M4 showed higher cellulase production followed by WA2M2 and WA2M3. WA2M4 was found to be the superior in cellulase production with dry leave (5.6U/m), paper (5.2 U/ml) and other substances (2.2-2.8 U/ml). Cellulase was extracted from Aspergillus sp. (WA2M4), used to determine the homogeneity with existed strains and molecular weight of the enzyme.

Keywords: Cellulase, *Aspergillus niger*, mutation, UV radiation, agriwastes, natural wastes, submerged fermentation.

1. INTRODUCTION

Industrial and commercially important enzymes are obtained from three sources namely plants, animals and microorganisms. Due to few difficulties in production and extraction of plant and animal sources for the enzyme production microbial production of enzymes became a very popular from few years. Cellulose is a one of the most abundant organic polymer and dominating waste material from agriculture. Cellulose is commonly degraded by an enzyme cellulase, it is produced by several microorganisms, commonly by bacteria and fungi [1], [2]. Fungi are main cellulase producing organisms, among *Aspergillus* sp. are important which can hydrolyse both soluble and insoluble cellulose [3].

Cellulase is a complex enzyme it is composed of endoglucanases, cellobiohydrolases and glucosidases which synergistically acts to convert carbohydrates present in lignocellulosic biomass into glucose [4]. Cellulases are most important enzymes which are used in several industries like textile, detergent, pulp and paper industry, food industry etc [5]. In fermentation technology both submerged and solid state fermentation processes are used for production of cellulases. Lignocellulosic wastes are the highest wastes with rich carbon sources are present on this earth and causing environmental pollution [6]. In this regard for the past few decades there is an increase in the utilization of agriculture and industrial wastes as substrates for production of cellulase enzyme [7], [8], [9]. The ability of cellulase production from wild organisms is always not effective. The organisms that have been mutanized and genetically modified organisms can able to produce high levels of cellulase [10]. Earlier in few studies physical (UV) and chemical mutants used in strain improvement for the production of cellulase [11], [12], [13]. In view of this the aim of this work is to improve the cellulase production from *Aspergillus* isolates and mutants using different agriculture wastes.

2. MATERIALS AND METHODS

2.1 Isolation and maintenance of culture

Serial dilutions were made with agriculture soil samples, 0.1 ml of inoculum was spreaded on the potato dextrose agar (PDA) plates. Plates were and incubated at 28^{o}C for three days. Fungi were isolated from the mixed isolates and sub cultured on PDA slants. Sub culturing was continued until a pure isolate was obtained and Culture was maintained by repeated sub culturing on appropriate medium.

2.2 Induction of mutations by ultraviolet (UV) irradiation

The isolated subculture strains were exposed to UV radiation at 2600Å for varying time periods of 5, 10, 15, 20 minutes. They were kept in dark (12h duration) for stabilization of thymine-thymine (T-T) dimers after exposure to UV radiation and then were spread onto CMC plates and incubating at 35°C for 48 h, the fungal preparations were used for the percentage survival analysis of organisms after UV radiation [14]. After, plates were strained by Congo red for conformation. All mutants and wild *Aspergillus* sp isolates were raised on PDA slants and plates and are stored for further work. The fungal spore suspension was used as inoculums for enzyme production by submerged fermentation. After four days of fermentation, enzymes were extracted and their activity assayed.

2.3 Cellulase plate assay

Discs of each mutant and wild Aspergillus sp isolates were inoculated on PDA plate containing 0.1% carboxy methyl cellulose (CMC), ampicillin (50 µg/ml) and tetracycline (50 µg/ml) and then incubated at 300C for 3~7 days, and were stained with 0.1% Congo red dye for 30 min followed by de-staining with 1M NaCl for 15 min. Fungal strains forming a large clearing zone surrounding the colonies were picked up and then streaked onto PDA plates, which were subsequently incubated at 30°C for 7 days.

2.4 Enzyme Activity (Cmcase Assay)

Carboxymethyl cellulase (CMCase) activity was determined according to reference [15]. One ml of the crude enzyme supernatant was incubated with 1 ml of 1% CMC in 0.1 M sodium was incubated with 1 ml of 1% CMC in 0.1 M sodium acetate buffer solution pH 5.0 for 30 min at 63°C. The resulted reducing sugars were determined by dinitrosalisylic acid (DNS) method [16]. Carboxymethyl cellulase hydrolyzes to produce free carboxymethyl glucose units which react with DNS reagent to form a colored complex detected spectrophoto metrically at 540 nm.

2.5 Molecular characterization of Aspergillus isolate

Genomic identification of the selected Aspergillus mutant strain was done using 18S rDNA technique. Analyses of 18S rDNA were used to confirm the identity of isolated cellulolytic fungi. DNA was extracted using CTAB method and the 18S rDNA sequences were amplified using primers ITS-4: TGCTCGGCTTATAGATATCC and ITS-5: GCAAGTATTAGTGGTATCTACC. PCR amplification was done in a Mini–cycle and purified the PCR product using PCR purification kit (Genei). The 18s rRNA sequence data was aligned and analyzed for finding the closest homologs for the strain.

2.6 Inoculum preparation

The selected WA2M4 strain of Aspergillus were maintained as stock culture on Potato dextrose agar slants and it was grown at 30° C for 5 days and then stored at 4° C. 50 ml of inoculum was prepared using potato dextrose broth, the inoculum was kept in orbital shaker (300 rpm) at 30° C for 48 hours before it was used for the fermentation process.

2.7 Fermentation process

Raw substrates, groundnut husk, sugar cane baggies, rice brawn, saw dust, dry leaves and paper wastes were sun dried individually, crushed and get powdered form. Individually the substrates were soaked in 1% Sodium Hydroxide solution for 2 hours at room temperature. After soaking, they were washed with distilled water and autoclaved at 121□C for 1 hour.

Prepared culture medium with 3 gms of substrate, 0.03 gm of L-glutamic acid, 0.14 gm of NH_4NO_3, 0.2 gm of KH_2PO_4, 0.03 gm of $CaCl_2$, 0.03 gm of $MgSO_4$, 0.75 gm of peptone, $MnSO_4$-0.16 gm were dissolved in 100 ml distilled water substrates in separate 250 ml Conical flasks with reference flasks. All flasks were incubated at 300° C for 7 days. The cellulase activity was measured at regular intervals. Freshly prepared 5 % inoculums of *Aspergillus* was inoculated into 100 ml of optimized culture medium

3. Results and Discussion

A total of 7 Aspergillus isolates (WA1-WA2) were isolated from agricultural field soils. Identification of fungi was done based on colony characters and microscopic examination. The confirmative Congo red test was positive by forming reddish orange halo-zone of hydrolysis this confirmed *Aspergillus* sp, cellulase producing isolate and was preserved. When discs of 7 isolates (WA1 to WA7) of *Aspegillus* species were inoculated on

cellulase screening media, after incubation the WA2 isolate was found to produces heights zone of hydrolysis (Table 1). This isolate was selected for further strain improvement studies and was used to develop the enhanced cellulose producing mutant.

Table1: The zone of cellulase activities of Aspergillus isolates used in the cellulolytic screening

Isolates	Fungal spp	Diameter of zone (mm)
WA1	*Aspergillus* sp.	16
WA2	*Aspergillus* sp.	20
WA3	*Aspergillus* sp.	12
WA4	*Aspergillus* sp.	9
WA5	*Aspergillus* sp.	11
WA6	*Aspergillus* sp.	7
WA7	*Aspergillus* sp.	14

3.1 Screening of mutants (Cellulase plate assay)

In in-vitro plate screening studies, enzyme production is typically indicated by formation of zones of clearance around the growing colony or by the formation of colored product. The most significant inference from the current study is the surprising degree of variability of enzyme secretion by wild and mutant WA2 isolates. It was observed that enzymatic index (EI) values (Table.2) of in vitro cellulase activity was observed in the range of 0.96-2.32. Among the five isolates WA2M4 was found to be the superior in cellulase production with EI value (Table.2) of 2.32 followed by WA2M2 and WA2M3. However WA2M1was identified to be the least cellulase enzyme producer with EI value of 0.72. The quantitative estimation of cellulase by WA2 mutants, WA2M4 produced high amount of cellulase (3.26 U/ml) than the other mutants of WA2 isolate (Table 3).

Table 2: Enzymatic Index Values of extra-cellular enzymes produced by wild and WA2 mutants Aspergillus sp. isolates.

S.No	Isolate	Cellulase
1.	WA2M1	0.72
2.	WA2M2	1.65
3.	WA2M3	1.72
4.	WA2M4	2.32
5.	WA2M5	1.23
6.	WILD	0.96

Table 3: Quantitative estimation of cellulase enzyme produced by wild and WA2 mutants Aspergillus sp. isolates

S.No.	Isolate	Cellulase (U/ml)
1.	WA2M1	1.72
2.	WA2M2	2.10
3.	WA2M3	2.26
4.	WA2M4	3.26
5.	WA2M5	1.96
6.	WILD	0.81

Molecular identification of *Aspergillus* sp.WA2M4 based on 18 S rRNA selecting Internal Transcribed Spacer Region (ITS) Sequence and its analysis

The isolated genomic DNA from WA2M4 isolate of Aspergillus sp. was checked in agarose gel electrophoresis. The PCR of 18S rRNA gene was carried out by the fungus-specific universal primer pairs ITS4 and ITS5 [17] and both the ITS1 and ITS2 regions were successfully amplified from DNA of WA2M4 isolate of *Aspergillus* sp. The amplified product with 580 bps (Fig. 1) and the results were proved that amplification of 18 S rRNA gene of WA2M4 isolate. The PCR product was purified and used for sequencing. The isolates of Aspergillus spp. (WA2M4) were analyzed by Its sequence analysis targeting from 18S rRNA region of fungus. The sequence data were subjected to MEGA programme to carry out to produce phylogenic tree. The phylogenetic analysis revealed similarity (98-99%) with *Aspergillus niger* (Fig. 8 & 9).

3.2 Fermentation of *A. niger* (WA2M4) using different substrates:

In the present study, different agricultural and natural wastes such as of groundnut husk, rice bran, sugar cane waste, saw dust, dry leaves and paper waste were used for cellulase production by WA2M4Aspergillus sp. and production of cellulase was confirmed by DNS method. The effect of different substrates on cellulase activity determined at concentration ranges (4.2-0.72 U/ml). After 7 days incubation at ambient temperature 28 ± 3°C, dry leaves was used as substrate produced high levels of cellulase (5.6 U/ml) followed by Paper.

Fig 1: PCR-Amplification of ITS1 – 18S rDNA – regions of *Aspergillus* sp. Lane M:

DNA marker, Lane 1 and 2 are isolate of WA2M4.

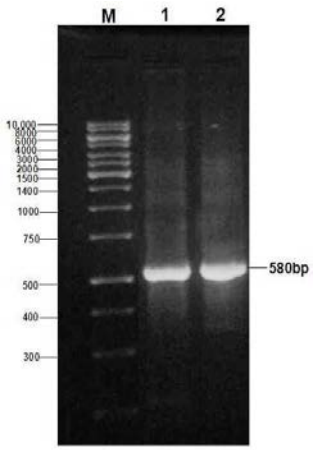


Fig 2: Comparison of ITS region nucleotide sequences of WA2M4 with other *Aspergillus niger* isolates

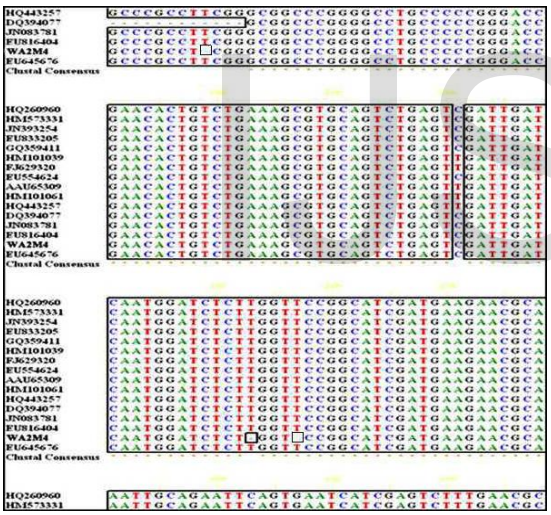


Fig 3: Phylogenetic tree deduced from the sequence of 18s rRNA of WA2M4 with other *Aspergillus sp.*

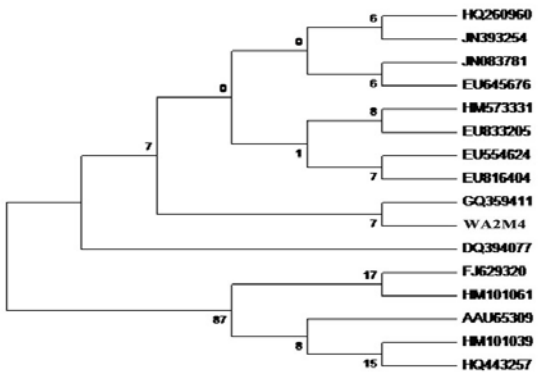
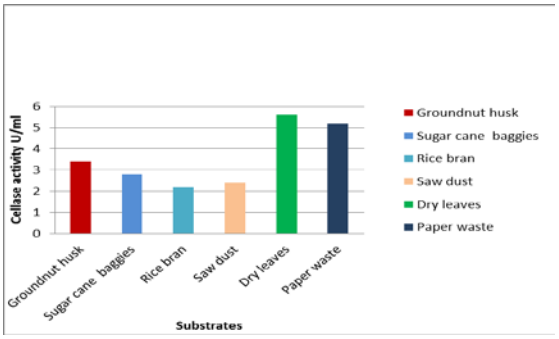


Fig 4: Effect of different substrates on cellulase activity



Mutations with UV rays are important inducers and it is used in strain improvement in fermentation technology. UV light is to modify the structure of pyrimidine (cytosine and thyamine) causing the formation of thyamine dimmer which damage the structure of DNA and block the replication process [18]. In this study mutant (WA2M4) produced higher (3.26U/ml) amount of cellulase activity than wild strains, this results agreement with earlier reports. UV irradiated strains, *Aspergillus* sp. and *Pencillium* sp. produced maximum enzyme production and conformed that UV was a potent mutagen [19]. In strain improvement, UV irradiation was found to be best for strains like *Aspergillus niger* for production high amount of various enzymes [20]. UV treated strains of *Aspergillus niger* UAM-GS1 increases the production of hemicellulolytic and cellulolytic activities [21]. In one study it was reported that UV irradiation showed more amounts of different enzymes than the parental strains of *Aspergillus niger* [22].

The morphological and microscopic characterization are most popular methods to identify fungal sp. but, these are time consuming and are not sufficient to characterize the similar fungal species [23], [24. On other hand molecular characterization is able to distinguish morphologically, similar fungal species and it is rapid process. Therefore, molecular characterization has also been carried out in the present investigation, to identify the WA2M4 isolate of Aspergillus with the help of fungal universal primer ITS4 and ITS5. Use of the PCR technology by internal transcribed spacer (ITS) region, it have already been published [25], [26] which also support the present study results. Using ITS different Aspergillus sp. was classified [27], [28], [29]. Apart this amplification of the internal transcribed spacer 2 (ITS 2) regions confirmed

the *A. flavus* [30].

The production of cellulase is induced by carbon sources, but amount produced is variable and carbon source influenced on the growth of cellulolytic organisms [1]. In the present study dry leaves and paper wastes produced higher (5.6 U/ml and 5.2U/ml) amount of cellulase when compared with other substrates. Earlier it was already reported that agricultural by-product which is a rich nutrient and it can be stimulate fungal growth [31]. In various studies the high amount of cellulase was produced by several natural and agri wastes such as coir waste and saw dust [1], wheat bran [32], [33], pea seed husk [12], wheat straw [31], [33], rice straw and Corn Cob [32], rice bran and orange peel [34] by Aspergillus sp.

4. CONCLUSION

In the present study the production of cellulase on different substrates under submerged fermentation was studied by mutant *Aspergillus niger*. The enzyme activities obtained on dry leaves and paper wastes substrates were maximum than other substrates. The results highlight the potentials of the substrates as possible raw materials for cellulase production using *Aspergilius niger*. It also provides valuable enzymes for cheap and renewable raw materials and environment safety by easy disposal of agriwastes.

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