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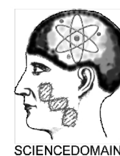

Screening of Locally Isolated *Aspergillus* species for Their Cellulolytic Potential and Their Optimization on *Vigna mungo* in Solid State Fermentation

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Authors' contributions

The study was carried out in collaboration with all authors. Authors UI and FG designed the study, performed the experimentation. Authors SS and ZB managed the literature searches. Author HI carried out statistical analysis. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To evaluate cellulolytic potential of locally isolated *Aspergillus* species and emphasis the importance of screening on qualitative bases. Also, to enhance cellulase production by optimizing the process parameters of the solid state fermentation of *Vigna mungo* with the isolated *Aspergillus* species.
Place and Duration of Study: Institute of Biological Sciences, Department of Botany, University of Gujrat, Gujrat, Pakistan, from February, 2012 to November, 2012.
Methodology: *Aspergillus* species were isolated from different deteriorated plant materials and maintained on potato dextrose agar. The purified isolated species were qualitatively screened on caboxymethylcellulose agar plates. The cellulolytic ability was further tested by culturing *Aspergillus* species on *V. mungo* in solid state fermentation. Various nutritional and cultivation parameters were optimized for the production of cellulases by *Aspergillus* species on *V. mungo.*
Results: Most of the isolated *Aspergillus* species showed potential for cellulase production. However, *A. terreus* gave qualitatively, the highest cellulase activity by fermenting *V. mungo* of 0.566 IU/g while *A. niger* gave quantitatively, the highest cellulase

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activity of 0.435 IU/g. Cellulase production by *A. terreus* reached at its maximum with 0.1% of urea as nitrogen source and 80% of initial moisture level at 35°C after 144 hours of fermentation.
Conclusion: *A. terreus* was identified *as* highest cellulase producer among the isolated *Aspergillus* species. The cellulolytic ability of *Aspergillus* specie greatly depends upon the nutritional and cultivation parameters of fermentation.

Keywords: Aspergillus species; Vigna mungo; cellulase; fermentation; nitrogen source.

1. INTRODUCTION

Cellulases are one of the largest industrial enzymes for lignocellulose conversion [1]. Cellulases are widely used in textile industry for softening and finishing of cotton and denim, added in detergents for cleaning and colour care purposes, in food industry, for maceration to make ketchup and purees, in pulp and paper industries for paper recycling, and pollution treatment [2,3]. Cellulases are glycoside hydrolase, which depolymerize cellulose by hydrolyzing β-1, 4 glycosidic bonds [4]. The complete hydrolysis of cellulose involves synchronized action of three enzymes: exoglucanase (EC. 3.2.1.91), endoglucanase (EC. 3.2.1.4) and β-glucosidase (EC. 3.2.1.21) [5].

Cellulases are synthesized by microorganisms, plants and animals. In higher plants such as *Lantana camara* and *Cuscuta reflexa,* cellulases are synthesized at leaf senescence and fruit ripening [6]. Few animals are reported to produce cellulases such as *Helix pomatia* [7] and *Mytilus edulis* [8]. However, numerous microorganisms including actinomycetes, bacteria, and fungi produce cellulases in large amount as compared with plants and animals [9]. The filamentous fungi belongs to genus *Aspergillus* and *Trichoderma* are thought to be a good producer of cellulases. *Aspergillus* species have excellent potential to secrete wide range of enzymes in large quantities to degrade lignocellulosic mass and this quality make them model organism for industrial applications [10].

Cellulose is chief component of plant material, which may serve as immense reservoir of renewable energy source and can be converted into many valuable products [11,12]. Agriculture, forestry and agro based industries generated agro wastes in huge amounts and dispose of these wastes creates a lot of pollution problems. Utilization of these agro wastes for enzyme production through the microbial action will be a remarkable and sustainable approach to manage agro wastes. Solid state fermentation (SSF) has great potential for enzyme production by utilizing wide range of agro wastes including wheat bran, rice bran, sugarcane bagasse, rice husk, fruit pulps, corn cobs, sawdust, sugar beet pulp, coffee husk and coffee pulp, tea waste etc. The present studies were aimed at screening locally isolated fungi for their cellulolytic potential and optimization of process parameters of solid state fermentation of *V. mungo* by *Aspergillus* species.

2. MATERIALS AND METHODS

2.1 Microorganism

Many deteriorated and spoiled plant tissues such as leaf litter, wood bark, and maize cob scales were collected in sterilized polyethene bags from the vicinity of Gujrat, Pakistan. Isolation was performed by cutting a thin slice (10 x 2mm) of damaged tissue. Then, surface sterilized with 1% NaOCl (v/v) and placed on Potato Dextrose Agar (PDA) in Petri dish for 5

days at 30 ± 1ºC. The fungal cultures were isolated from leaf litter by sprinkling a pinch of crushed litter on PDA plate. The isolated cultures were purified upto single colony from enriched culture by multiple re-culturing.

The purified *Aspergillus* species were identified on the basis of colonial morphology (colour and growth pattern of colony) and microscopic characteristics such as shape of conidial heads, arrangement of conidia, pattern of spores arrangement on conidia, shape and size of conidia with the aid of Universal key of identification [13,14,15].

2.2 Screening of *Aspergillus* Species

2.2.1 Qualitative screening

The identified *Aspergillus* species were screened for their cellulolytic activities on screening media with composition (g/l): CMC, 1; $CaCl_2$, 0.5; $(NH_4)_2SO_4$, 3; Agar, 20 was used. A 2µl of spore suspension (~10^6) of each species was added in the small well created in the center of solidified screening plate and incubated at 30±2ºC for 48h. The inoculated plates were stained with 1% Congo red for 30min, and then treated with 1M NaCl solution for 15min. The zone of clearing appeared on screening media in Petri dish indicated the cellulolytic activity of *Aspergillus* species.

2.2.2 Quantitative screening

Quantitative screening was performed by solid state fermentation (SSF) of *Vigna mungo,* which served as the sole carbon source. *V. mungo* was collected from the surroundings of Gujrat, Pakistan. The substrate was dried, chopped and sieved through 2mm mesh. SSF, which served as control, was carried out in flasks (250ml) with 5g of powdered substrate. The liquid salt medium (LSM) as described by Juhász et al. [16] was used. The substrate was moistened with distilled water and 5ml of LSM to give 80% initial moisture and autoclaved at 121ºC for 30min. Then, the autoclaved substrate was aseptically inoculated with 1ml of spore suspension (with strength of $1x10^6$) of each *Aspergillus* species and incubated for 8 days at 30±1ºC under static conditions.

2.3 Optimization of Process Parameters of SSF

After screening, the substrate was supplemented with different nitrogen sources to find out the best source, which enhanced the cellulase production of selected *Aspergillus* species in SSF. Many organic sources such as Peptone, Urea, Yeast extract, Tryptone and Malt extract were used. Also, different salts such as Ammonium nitrate, Ammonium chloride, Ammonium phosphate, Ammonium sulphate and Sodium nitrate were used as inorganic nitrogen sources. Each nitrogen source was added at the rate of 0.01g per 5g of substrate. The control was maintained without addition of any nitrogen source. The initial moisture content was optimized by moistening the substrate with different volumes of water but the volume of LMS (5ml) was kept constant. The optimum fermentation time was established by determining cellulase activity after 24h upto10 days. SSF was carried out at different temperatures ranging from 25 to 45ºC to select the best temperature for cellulase production by the selected *Aspergillus* species.

2.4 Enzyme Assay

The crude enzyme was extracted by following the method of Acharya et al. [17]. Cellulase activity was determined by using 1% carboxymethylcellulose as substrate. The amount of reducing sugar released was estimated by using 3, 5-dinitrosalicyclic acid [18]. One International Unit (IU) was taken as the amount of glucose (mM) released per minute per milliliter of enzyme solution.

2.5 Analysis of Data

The data was subjected to analysis of variance (ANOVA) by using software package Co-stat version 3.03. Differences among means of obtained data were analyzed by applying Duncan's multiple range test at the significance level $P = 0.05$.

3. RESULTS AND DISCUSSION

From the different lignocellulolytic sources (deteriorated wood, scales of corn cob, leaf litter and unbleached pulp and paper effluent) investigated in this study, 123 fungal isolates were isolated, out of which, 57 were *Aspergillus* species (Table 1). The genus *Aspergillus* was the most frequent in isolated fungi, so, it was selected for further experimentations. The isolated *Aspergillus* species were further purified to single colony on PDA plates and stored at 4ºC.

3.1 Qualitative Screening of *Aspergillus* Species

The purified species were screened qualitatively and quantitatively. The qualitative screening was based on diameter of clear halo made by each species, in plate screening with incubation time of 48h at 30±1ºC. The largest halo zone observed among tested species was made by *A. niger*, which was 4.5±.32cm (Table 1). *A. fumigates* and *A. terreus* had clearing zone of 3.4±0.24cm and 3.1±0.16cm, respectively. *A. ornatus* and *A. deflectus* had a little activity in qualitative screening.

Table 1. Qualitative screening of *Aspergillus* species for their cellulolytic potential

***Aspergillus* species**	**Frequency of each of *Aspergillus* species (%)**	**Diameter of halo zone(cm)**
A. nidulans	7.01	1.2±.35
A. fumigates	15.01	3.4±.24
A. flavus	14.03	2.1±.41
A. ornatus	5.26	0.3±.07
A. deflectus	1.75	0.5±.09
A. candidates	3.50	1.4±.11
A. oryze	5.26	2.4±.21
A. niger	24.56	4.5±.32
A. nives	7.01	2.1±.13
A. terreus	15.78	3.1±.16
Total	99.94	-

3.2 Quantitative Screening of *Aspergillus* Species

In quantitative screening, production of cellulases was significantly higher in *A. terreus* 0.564 IU/g (Fig. 1) as compared with *A. niger* (0.435 IU/g**)** and *A. fumigates* (0.391 IU/g**)**, which

produced larger halo zone in screen plate method. In the present studies, the nutrients were supplied in inorganic form e.g. CMC salt was supplied as carbon source and Ammonium sulphate was used as nitrogen source in plate screening. But in quantitative screening, the nutrients were supplied in complex organic form (*V. mungo*). *A. terreus* may have consumed nutrients of *V. mungo* more efficiently than *A. niger* and showed more cellulase production.

So, *A. terreus* was selected for further experimentation and optimized on *V. mungo* in SSF. It is evident from present work that selection of *Aspergillus* species with plate method is a partial screening. So, screening process becomes more established when the microorganism is provided with selected substrate as a screening media. According to Safique et al. [19], screen plate method provides selection on partial bases only. The ability of *Aspergillus* species to produce cellulases is significantly influenced by the chemical composition of substrate and the physical conditions of culturing parameters such as moisture, pH, temperature and aeration also [20].

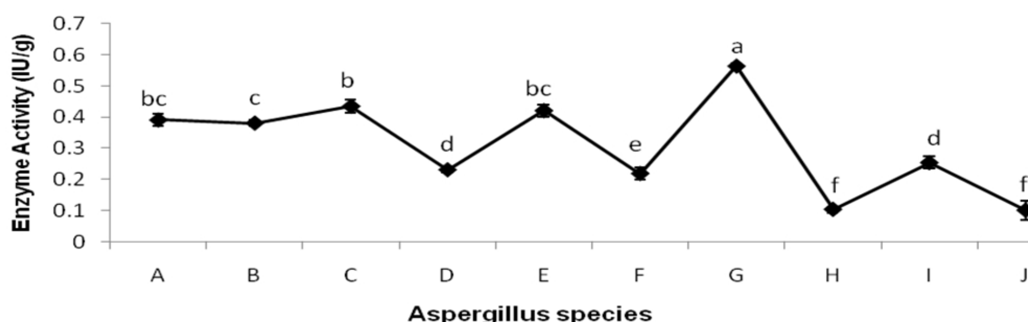

Fig. 1. Cellulase activities of isolated *Aspergillus* species in quantitative screening
***A**, A. fumigates; **B**, A. nives; **C,** A. niger ; **D**, A. nidulans; **E**, A. flavus; **F**, A. ornatus; **G**, A. terreus, **H**; A. candidates; I, A. oryze; **J**, A. deflectus.*
Verticals bars show standard error of means of three replicates. The values with different letters show the significant difference at (P= 0.05) as determined by DMRT.

3.3 Optimization of Nitrogen Source

Enzyme activity of *A. terreus* was significantly high (0.648 IU/g) when Urea was used as nitrogen source (Fig. 2). For inorganic sources, Ammonium sulphate increased the cellulase activity compared with the control. The results were insignificant (P=0.05) with Peptone and Ammonium nitrate. The optimum concentration of urea for cellulase production was found to be 0.1% (w/w) and further increase did not have significant improvement on the amount of cellulase produced (Fig. 3). However, Singh and Narang [21] reported 0.2% Ammonium sulphate as the optimum nitrogen source for cellulase production by *A. niger* grown on forage while Gao et al. [22] reported that 0.8% (w/w) yeast extract was preferred nitrogen source for cellulase production by *A. terreus* on corn stover.

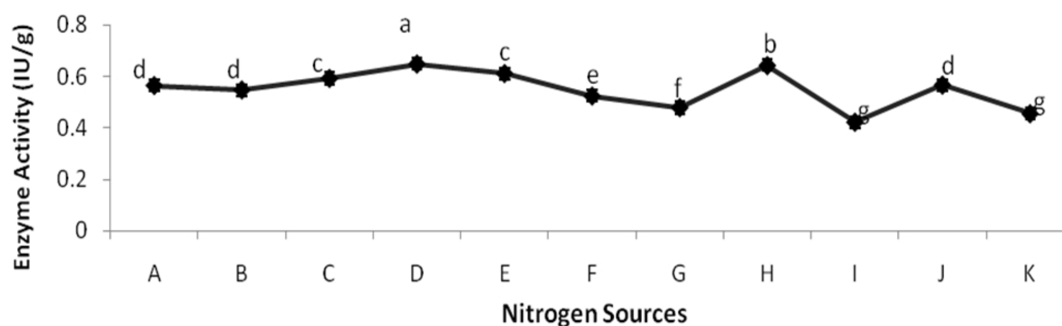

Fig. 2. Effect of various nitrogen sources on cellulase activity of *A. terreus* on *V. mungo* in SSF

***A**, Control; **B**, Peptone; **C**, Tryptone; **D**, Urea; **E**, Yeast extract; **F**, Malt extract; **G**, Ammonium chloride; **H**, Ammonium sulphate; **I**, Ammonium phosphate; **J**, Ammonium nitrate; **K**, Sodium nitrate. Verticals bars show standard error of means of three replicates. The values with different letters show the significant difference at (P = 0.05) as determined by DMRT.*

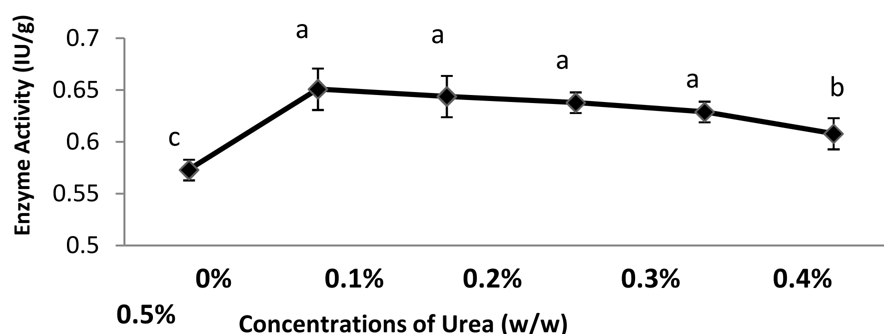

Fig. 3. Effect of various concentrations of Urea on cellulase activity of *A. terreus* on *V. mungo* in SSF

Verticals bars show standard error of means of three replicates. The values with different letters show the significant difference at (P= .05) as determined by DMRT.

3.4 Optimization of Initial Moisture Level

In the present study, the production of cellulase enzyme reached its maximum at 80% initial moisture level (Fig. 4).The results agreed well with the findings of Szendefy et al. [23] *i.e*. the optimum moisture level was 83% in SSF of eucalyptus and 80% in SSF of bagasse pulp for xylanase production by *A. niger*.

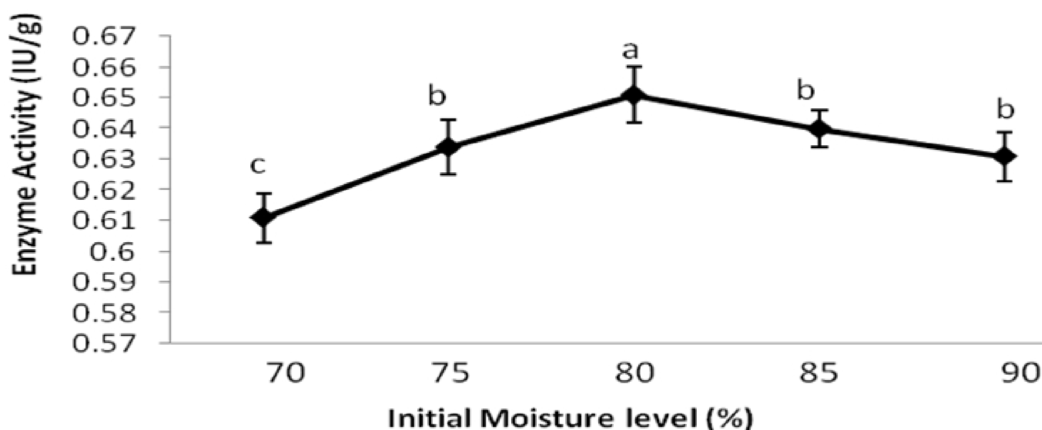

Fig. 4. Effect of initial moisture level on cellulase activity of A. terreus on *V. mungo* in SSF

Verticals bars show standard error of means of three replicates. The values with different letters show the significant difference at (P= .05) as determined by DMRT.

3.5 Optimization of Fermentation Temperature and Fermentation Time

The highest enzyme activity of *A. terreus* was attained at 35ºC after 144 h of fermentation as shown in Figs. 5a and b. Vyas *et al.* [24] optimized *A. terreus* on groundnut shells for cellulase production at 28±1ºC with 8 days of fermentation period. Ali et al*.* [25] reported that cellulase activity of *A. terreus* on water hyacinth was highest after six days of inoculation at 40ºC.

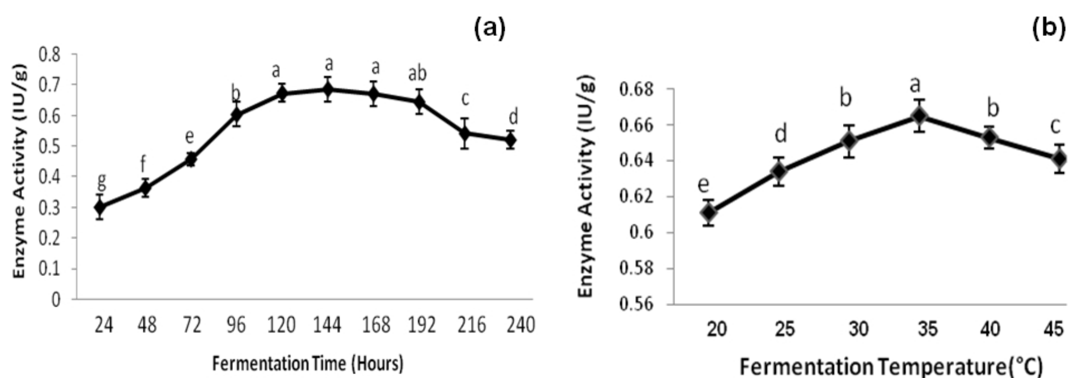

Fig. 5a & b. Effect of fermentation Time and fermentation Temperature on cellulase activity of *A. terreus* on *V. mungo* in SSF at 80% initial moisture level

Verticals bars show standard error of means of three replicates. The values with different letters show the significant difference at (P = 0.05) as determined by DMRT.

4. CONCLUSION

This study showed that screening should be performed on both qualitative and quantitative bases before selecting microorganism for cellulase production. Diameter of halo zone in

plate method only indicated the cellulolytic ability of microorganism. Quantitative screening proved to be a better method of determining cellulolytic potential among different species. So, the selection of screening method is of primary importance especially in SSF. Additional supply of nitrogen to fermenting substrate i.e. *V. mungo* increased the cellulase production to a certain limit. The optimization of cultivation conditions such as initial moisture level, fermentation time and fermentation temperature increased the cellulase production of *A. terreus.*

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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