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Isolation of Fungi and Optimization of pH and Temperature for Cellulase Production

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ABSTRACT

The crystalline structure of cellulose makes it difficult to degrade and so most of the cellulosic waste in nature is disposed of by biomass burning. Cellulase enzyme system is potent enough to convert cellulose into glucose. Fungi are known to produce an array of hydrolytic enzymes. This study involves isolation of high potential cellulolytic fungal strains from the soil and optimizing pH and temperature conditions for enhanced cellulase production. The fungal strains were isolated from soil using serial dilution and pour plate techniques and screened using Congo red test and FPase method. Based on hydrolytic zones formation and cellulase enzyme production, *Aspergillus fumigatus, Aspergillus terreus* and *Aspergillus flavus* were found to show the highest potency for hydrolytic enzyme production at pH 5.8-6.0 and temperature range of 40°C-50°C.

INTRODUCTION

Cellulose is a linear polysaccharide of glucose residues with β-1,4-glycosidic linkages. Copious availability of cellulose renders it a desirable raw material to produce imperative products for industrial use. Unfortunately, most of the cellulosic waste is often disposed of by biomass burning, which is not restricted to developing countries alone but is considered a global phenomenon. Cellulase enzyme system can convert cellulose to glucose economically and favourably. Cellulase enzyme system harbours three types of enzymes that are produced extracellularly: 1, 4-β-endoglucanase, 1, 4-β-exoglucanase, and β-glucosidase. Endoglucanase aids in the conversion of the crystalline cellulosic structure into a soluble polymer. While exoglucanase is responsible for cleavage of these polymers to produce tri and di-saccharides, β-glucosidase breaks these tri and di-saccharides to finally produce glucose (Shewale 1982, Woodward & Wiseman 1983). The synergistic effect of these three enzymes brings about this complex process effectively to produce glucose molecules (Ryu & Mandels 1980, Wood 1989, Samdhu & Bawa 1992). To make this possible, it is desirable to involve certain cellulase producing microorganisms (Béguin & Aubert 1994, Singh & Hayashi 1995, Lynd et al. 2002).

In-plant cell walls, cellulose is bound by lignin which makes it difficult to degrade the crystalline and complex structure of cellulose. Hence, there is a need to harness cellulase enzyme through economically feasible and effective techniques. Fungi are the most influential and dominant groups present in soils. They produce an array of hydrolytic enzymes and are more effective than bacteria in acidic soils and decomposing cellulose rooted in lignin. Fungi are filamentous and produce prolific spores. Therefore, they invade the substrate easily and are quite effective in composting of lignocellulosic waste. Also, they can stand a wide range of pH as compared to bacteria and hence are more preferred than bacteria for the decomposition of complex organic wastes. With regard to this, filamentous fungi are more appealing owing to their ease of cultivation and substantial yield of extracellular enzymes with significant prospective for industrial application (Mishra & Dadhich 2010). Also, these extracellularly produced enzymes are easily retrievable using the culture media (Abe et al. 2015). Other advantages associated with cellulase production through fungi include rapid enzyme production and the cost-benefit aspect.

Plants are capable of generating 4×10^9 tons of cellulose annually. Cellulases are mainly employed in the textile industry for bio-polishing of fabrics. They are also used to improve the nutritional value and digestibility of animal feeds and in de-inking of paper (Tolan & Foody 1999). The cellulases that are used in these cases are harnessed from fungal sources. The importance of cellulose as a replenishable energy source generates a dire need for keen industrial interest and research in the hydrolysis of cellulose (Bhat et al. 2000). Profita-

ble bioconversion of cellulosic resources is subject to the characteristics of cellulose, sources of cellulolytic enzyme and favourable circumstances for the catalytic activity and enzyme yield. The crystalline structure and insoluble nature of cellulose make it less susceptible to degradation. For the biotechnological implication of cellulases, the present study has been formulated to screen and isolate high potential fungal cultures for cellulases production.

MATERIALS AND METHODS

Isolation of cellulolytic fungi from soil: For isolation of cellulose-degrading fungi, three random soil samples were collected from rice field of Kanheli village, Rohtak, Haryana by digging at a depth of 10 cm and stored in a sterile polythene bag at 4°C. Pure isolates of fungal strains were obtained by Pour Plate technique and streaking on PDA (Potato Dextrose Agar) medium supplemented with (CMC) Carboxymethylcellulose (Viji et al. 2015). Serial dilutions of the experimental soil samples using sterile distilled water were made up to 10^{-5} dilution. PDA media was prepared and autoclaved. 0.3% sodium azide was added to the media as an antibacterial solution to restrict the growth of bacterial colonies on agar plates. Around 50 µL of diluted soil samples were spread on the solidified agar plates. The Petri plates were then sealed and incubated at 28°C for 48 hours. Fungal colonies were found to appear on the agar plates. These plates were labelled as Fungal Master plates (Fig.1). The isolates were further inoculated on sterile PDA plates by point inoculation and incubated at 28°C for 48 hours to obtain pure fungal plates (Fig. 2). Streaking was done to purify the fungal colonies further (Fig. 3). Following media was used for plating:

$NaNO_3$ – 2g/L, K_2HPO_4 – 1g/L, KCl – 0.5g/L, $MgSO_4$.7H2O – 0.5g/L, Peptone – 2g/L, Agar – 15g/L, CMC – 0.5% = 5g/L, PDA – 39g/L (Neethu et al. 2012)

Primary screening - Congo red test: The pure fungal plates were flooded with Congo red solution (1%), and then the solution was discarded after 5 minutes. The plates were then counterstained with 1N NaCl solution and allowed to rest for 15 to 20 minutes. Clear hydrolytic zones could be seen around the fungal colonies indicating cellulose hydrolysis by the enzymes (Fig. 4). High potential cellulolytic fungi were selected depending on their ability to form clear and wider hydrolytic zones around the fungal colony.

Secondary screening - Determination of exoglucanase (total cellulase): Standard Filter Paper Assay (FPase) method described by Eveleigh et al. (2009) was used to determine the total cellulase activity of the isolated fungal strains. Cellulase activity is defined as 1 µmols of the substrate converted to

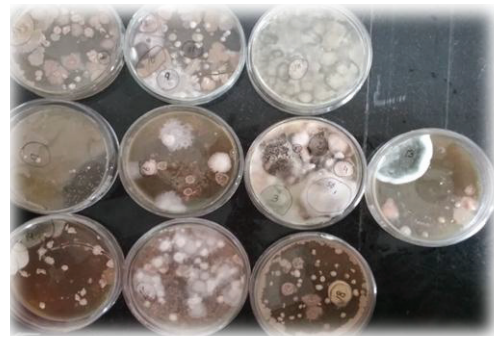

Fig. 1: Fungi Master plates

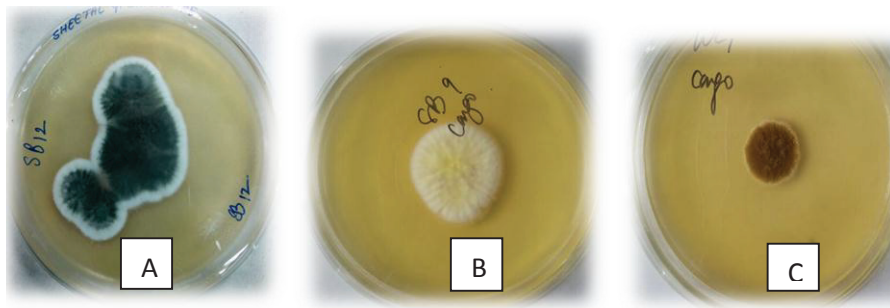

Fig. 2 (A-C): Purified fungal plates

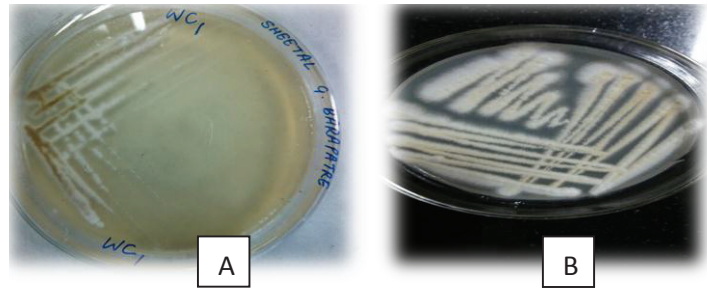

Fig. 3 (A-B): Streaked fungal plates

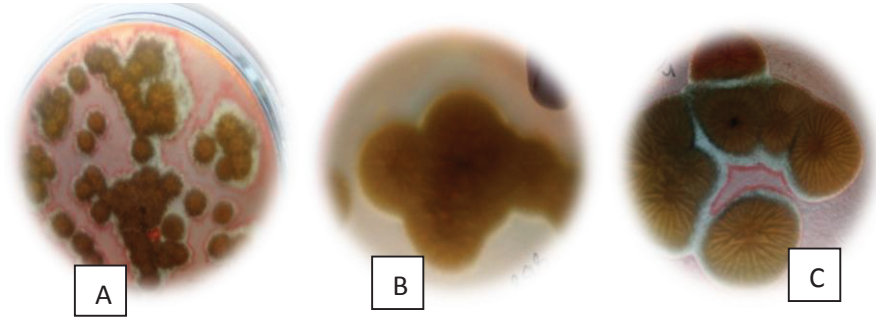

Fig. 4 (A-C): Plates showing Congo Red Test

glucose (Gilna & Khaleel 2011). 0.5 mL culture supernatant from each culture was added to 1 mL of sodium citrate buffer (pH 5.8). A 50 mg strip of Whatman No.1 filter paper (1.0 × 6.0 cm) was added to each tube and incubated at 50°C for 1 hour. Tubes were vortexed till filter paper settled at the bottom of the tube. Glucose standards, Enzyme blank and Substrate blank were prepared and incubated at 50°C for 1 hour. 3 mL of dinitrosalicylic acid (DNS) was added to each tube and mixed well (Fig. 5). The sample mixtures, glucose standards, enzyme blank and substrate blank were boiled together for 5 min in a water bath and then transferred to a cold water bath. Twenty mL of distilled water was added to all tubes and mixed properly. The absorbance values of these sample tubes were measured at 540 nm. Reagent blank was used to set the spectrometer at zero. Cellulase activity was expressed in Filter Paper Unit (FPU) per mL of undiluted culture filtrate (Mandels et al. 1976).

Calculation:

$$\text{FPU/mL} = \frac{0.37}{\text{Enzyme releasing 2 mg of glucose}}$$

Determination of optimum pH for cellulase production: Following buffers were used to maintain different pH values:

- 50 mM acetate buffer (for pH 3 and 4),
- 50 mM Sodium Citrate buffer (for pH 5.8)
- 50 mM Tris-base buffer (for pH 6)
- 50 mM sodium phosphate buffer (for pH 8-10).

0.5 mL culture supernatant pre-incubated at 50°C for 10

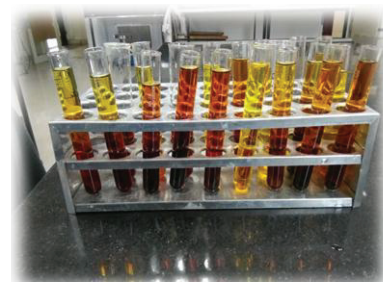

Fig. 5: Sample tubes for Filter Paper Assay

minutes was added to 0.5 mL buffer and incubated at 50°C for 30 minutes. 0.5 mL of DNS was added to terminate the reaction. The test tubes were then placed in a hot water bath for 5 minutes and then allowed to cool down. 2.5 mL of distilled water was added to all test tubes and the amount of reducing sugar liberated was determined by reading the absorbance at 540 nm (Mendel et al. 1969)

Determination of optimum temperature for cellulase production: To assess the effect of temperature on cellulase enzymatic activity, 0.5 mL sodium citrate buffer (pH 5.8) pre-incubated at 50°C for 10 minutes was combined with 0.5 mL of culture supernatant and incubated at 30°, 40°, 50° and 60° Celsius for 30 minutes. 0.5 mL DNS was added to stop the reaction and all mixtures were subjected to the hot water bath for 5 min. All test tubes were then allowed to cool down to room temperature and 2.5 mL of distilled

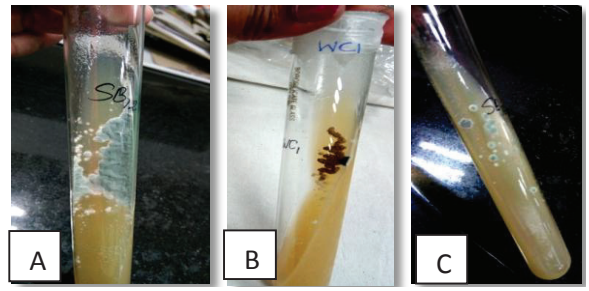

Fig. 6 (A-C): Fungal growth on media slants.

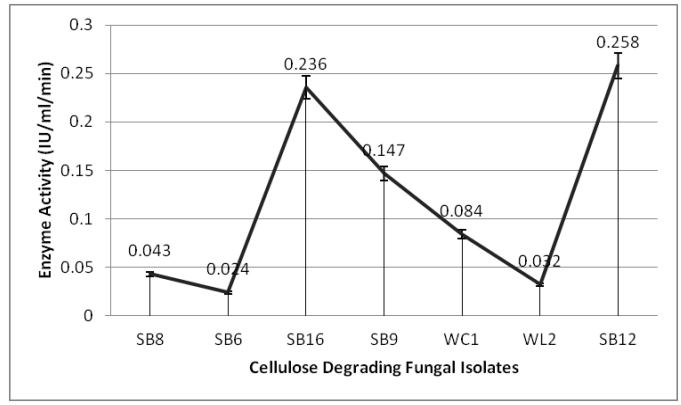

Fig. 7: Total cellulase activity of isolated fungal strains.

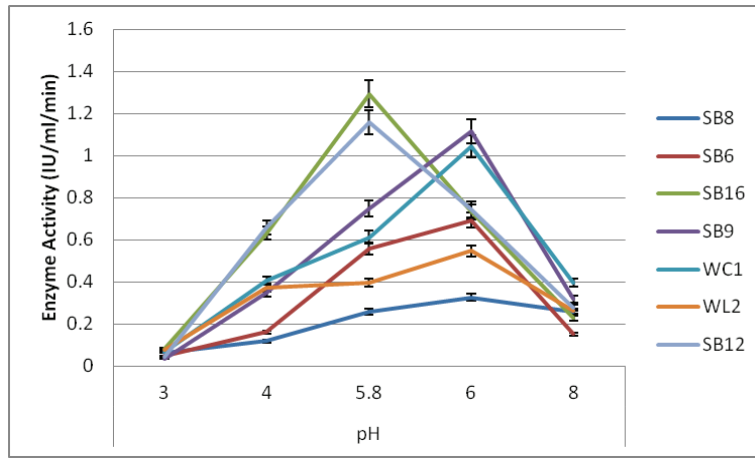

Fig. 8: Effect of pH on cellulase activity of isolated fungal strains.

Table 1: Hydrolytic zone diameter produced by isolated cellulolytic fungal strains.

S. No.	Fungal Isolates	Fungal Colony Diameter (mm)	Fungal Colony + Zone Diameter (mm)	Hydrolysis Zone Diameter (mm)
1	WC1	13	16.3	3.3
2	WL2	10.2	11.5	1.3
3	SB6	14	16.5	2.5
4	SB8	11.1	13.5	2.4
5	SB9	15	18.6	3.6
6	SB16	11	14.1	3.1
7	SB12	6.2	9.4	3.2

Table 2: Effect of pH on cellulase activity of isolated fungal strains.

Fungal Strains	Enzyme Activity (IU/mL/min)				
	pH 3	pH 4	pH 5.8	pH 6	pH 8
SB8	0.065±0.06	0.118±0.03	0.257±0.16	0.326±0.21	0.256±0.27
SB6	0.048±0.06	0.161±0.21	0.559±0.19	0.693±0.14	0.151±0.02
SB16	0.082±0.19	0.631±0.11	1.293±0.29	0.733±0.03	0.227±0.11
SB9	0.036±0.27	0.348±0.21	0.748±0.17	1.115±0.16	0.318±0.24
WC1	0.068±0.13	0.406±0.09	0.611±0.21	1.042±0.06	0.397±0.07
WL2	0.077±0.24	0.372±0.11	0.397±0.26	0.546±12	0.261±0.02
SB12	0.042±0.18	0.658±0.13	1.157±0.03	0.745±0.22	0.276±0.18

(n=3; Mean ± SD)

Table 3: Effect of temperature on cellulase activity of isolated fungal strains.

Fungal Isolates	Enzyme Activity (IU/mL/min)			
	30°C	40°C	50°C	60°C
SB8	0.048±0.09	0.151±0.26	0.382±0.18	0.202±0.18
SB6	0.051±0.12	0.183±0.04	0.341±0.23	0.107±0.26
SB16	0.076±0.03	0.398±0.12	0.986±0.14	0.359±0.17
SB9	0.047±0.11	0.912±0.03	0.415±0.27	0.361±0.19
WC1	0.213±0.06	0.419±0.01	1.013±0.04	0.241±0.07
WL2	0.061±0.17	0.554±0.15	0.361±0.16	0.253±0.08
SB12	0.167±0.22	0.358±0.05	0.901±0.21	0.269±0.21

(n=3; Mean ± SD)

water was added to them. Absorbance was read at 540 nm (Balamurugan et al. 2011).

RESULTS AND DISCUSSION

A total of 11 fungal strains (B1, WC1, WC3, WL1, WL2, SB6, SB9, SB12, SB16, SB8 and I12), were purified by Pour Plate technique and streaking on PDA media supplemented with CMC. CMC degradation was verified by staining the fungal plates with 1 mg/mL Congo red dye and counter-stained using 1N NaCl. Seven fungal strains (WC1, WL2, SB6, SB8, SB9, SB12, SB16) gave positive results in primary screening by forming visible hydrolytic zones around the fungal colonies. Table 1 shows the hydrolytic zone diameters formed by isolated fungal strains.

These 7 fungal strains were then subjected to secondary screening using enzymatic assay FPase for production of exoglucanase. FPase results revealed that the exoglucanase activity was observed to be highest for the isolated fungal

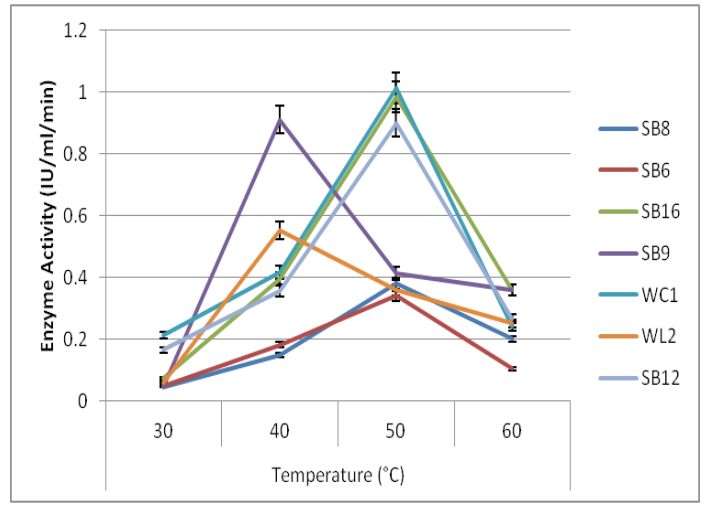

Fig. 9: Effect of temperature on cellulase activity of isolated fungal strains.

strains SB12 (0.258 IU/mL) followed by SB16 (0.236 IU/mL), SB9 (0.147 IU/mL) and WC1 (0.084 IU/mL) as depicted in Fig. 7.

The effect of pH on the activity of the cellulase enzyme was studied by varying pH in the range of 3 to 8 (Table 2). The maximum cellulase activities were noticed at pH 5.8-6.0 with SB16 (1.293 IU/mL), SB12 (1.157 IU/mL), SB9 (1.115 IU/mL) and WC1 (1.042 IU/mL) showing highest enzyme activity. On further increase in pH, a reduction in the cellulase activity was obtained (Fig. 8). The reason for decreasing production at higher pH was probably due to proteolytic inactivation of the cellulase. Hence, it is suggested that slightly acidic pH values favoured cellulase production, which is in agreement with earlier results of other researchers (Ander & Eriksson 1976).

The effect of different temperatures on cellulase production by fungal strains was evaluated by assessing the number of glucose molecules released over temperatures ranging from 30 to 60°C using DNS method (Table 3). Maximum cellulase activities were observed between 40-50°C with WC1 (1.013 IU/mL), SB16 (0.986 IU/mL), SB9 (0.912 IU/mL) and SB12 (0.901 IU/mL) showing highest enzyme activities (Fig. 9).

Based on hydrolytic zones formation and cellulase enzyme production, three fungal strains - SB12, SB9 and WC1 were selected as high potential cellulolytic strains. Then they were grown on media slants (Fig. 6) and sent for identification to Pathology Department, IARI, Pusa. The strains were identified as *Aspergillus fumigatus* (SB12), *Aspergillus terreus* (SB9) *and Aspergillus flavus* (WC1).

CONCLUSION

This study suggests that *Aspergillus fumigatus, Aspergillus terreus and Aspergillus flavus* are potent cellulase producers and can contribute to cellulase production under optimized conditions of pH and temperature. The effect of pH and temperature on the synthesis of cellulase enzyme can be studied by optimizing these parameters. Fungi need optimum pH and temperature to achieve substantial growth and show cellulase enzyme production activity which can be rendered beneficial to be utilized for several industrial applications. In accordance with the results, considering the influencing factors, the optimal cultural process for *Aspergillus* species was considered as PDA media supplemented with CMC at pH 5.8-6.0 within a temperature range 40-50°C.

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