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Isolation and Screening of Cellulolytic Fungi by Baiting Method from Soils of Jalandhar.

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ABSTRACT

The aim of the study was isolation of cellulose producing fungi by baiting method followed by screening and identification upto genus level. Soil samples from different sites of Jalandhar which included garden soil, agricultural field and saw mills were collected for the isolation of cellulolytic fungi. Cellulose bait in form of filter paper, jamun seeds, rotting bark and match stick were introduced in above soil samples and kept for a month. A total of 24 species belonging to 6 genera were isolated and identified based on morphological characteristics. The fungi so isolated belong to 6 species from *genus Penicllium, Helminthosporium, Alternaria, Curvularia, Aspergillus* and *Botrytis*. The relative enzyme activity of isolates were determined on Czapek Dox media supplemented with 1% CMC and subsequent flooding with 0.1% Congo red after incubation period. Among the isolates, the *Aspergillus* spp SP01 showed highest enzyme activity 15.44U/ml and least by Penicillium sp. JM03 (8.31U/ml) amongst the isolate.
Keywords: Cellulase, Baiting, cellulolytic fungi, relative enzyme activity.

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INTRODUCTION

Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials. These are hydrolytic enzymes capable of hydrolysing cellulose to smaller sugar components like glucose units. Cellulases degrade cellulose by the synergistic action of three enzyme activities; endoglucanase also called as carboxymethyl cellulase (CMCase) (endo-1,4-β-Dglucanase, EG, EC 3.2.1.4), exoglucanse (also called as cellobiohydrolase) (exo-1,4- β -D-glucanase, CBH, EC 3.2.1.91) and β-glucosidase (1,4- β -Dglucosidase, BG, EC 3.2.1.21) [1-2].

These cellulolytic microorganisms play an important role in the biosphere by recycling cellulose, the most abundant and renewable biopolymer on earth and forms a key link in the carbon cycle. The abundant availability of cellulose makes it an attractive raw material for producing celluloses. The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest [3-4]. These enzymes have enormous potential in industrial applications. Glucose produce from cellulosic substrate could be further used as substrate for subsequent fermentation of other processes which could yield valuable end products such as ethanol, butanol, methane, amino acid, single-cell protein etc.[5].

Consequently, the present work is focused on the isolation of cellulolytic fungi from natural habitats further leading to selection of the best strain showing maximum cellulase activity for enzyme production.

MATERIALS AND METHODS

Collection of samples for baiting and isolation of fungi

A total of five soil samples were collected from different sites of Jalandhar which can be placed into three categories *viz*. field soil, garden soil, saw mill soil. The surface debris was cleared and 200-250g of soil from a depth of 10cm was collected into plastic bags. Each sample of soil was mixed well in the bag and smaller duplicate samples, approximately 50 g each, were placed into plastic glasses and baited with cotton, match stick and small pieces of filterpaper. Plastic glass was moistened with 5-10 ml of water. All baited samples were incubated in the dark and moistened at intervals of 3-4 days to prevent the soil from completely drying.

Isolation of cellulolytic fungi and identification

Czapek Dox agar medium supplemented with 1% CMC as carbon source was prepared. The molten media was cooled and poured into sterile petriplates and allowed to solidify. The fungal baits were retrieved from soil samples and subjected to serial dilution. Spread plate method of isolation was carried out on Czapek Dox agar medium and plates were incubated at 28±2°C for three days. Fungi isolated from the plates were subcultured on fresh plates of Czapek's dox agar [6]. The petriplates were observed for macroscopic characteristics by observing colony colour, texture, elevation, margin and colour on reverse side of colonies [7-9]. Microscopic study was done using lactophenol cotton blue staining to study morphological characteristics of mycelia, conidiophores and conidia [10].

Measurement of relative enzyme activity of fungal isolates

Fungal colonies were screened for cellulolytic activity and relative enzyme activity was determined using method given by Gopinath *et al*, 2005, Javed *et al*, 2011, Aneja, 2003[11-13]. After incubation of 48 hours, 10ml of 0.1% Congo Red solution was added to the plates and kept for 30 minutes. The Congo Red solution was then discarded and plates were washed with 10 ml of 1 N NaCl and zones of clearance was observed around the colonies. The value of zones of clearance was calculated by using the formula: Relative Zones of hydrolysis (cm) = Diameter of zone of clearance (cm)/Diameter of zone of colony (cm).

Solid State Fermentation of selected fungal isolates for enzyme production

A combination of rice husk and rice bran in 1:1 ratio was used for production of cellulase enzymes. The substrate was collected and sun dried for 15 days. Substrate was ground and sealed in air tight container. Solid-State fermentation with the combination of rice husk and rice bran was done by moistening with

mineral salt medium having composition, NaCl 0.5%, KH_2PO_4 0.5%, $MgSO_4.7H_2O$ 0.1%, $CaCl_2$ 0.05%, NH_4NO_3, 0.5%, Peptone 0.1%, $MnSO_4.7H_2O$ 0 .001%, $ZnSO_4.7H_2O$ 0.001%, $FeSO_4.7H_2O$ 0.005%, $CoCl_2.6H_2O$ 0.0002%. The pH of the media was adjusted to 6.0. The spores maintained in slants a week were wetted by using sterile water and tween 80 in the ratio of 9:1. The spores were scratched with a sterilized inoculating needle and the suspension was used as inoculum for solid state fermentation [14]. The substrate was taken in 250 ml flasks and was moistened mineral salt solution. The flasks were autoclaved at 121^0C for 15 min. The cooled substrate was then inoculated with 2 ml spore suspension and incubated at 28 ºC for 5 days [15].

Enzyme Extraction and estimation

After incubation of 5 days, the enzyme was extracted by mixing the contents of flasks with 15 ml of sodium acetate buffer 0.2M (pH 5). The flasks were placed in shaker at 200 rpm for 60 minute and the content was filtered through muslin cloth. Filtrate collected was centrifuged at 3000 rpm for 20 minutes at room temperature. Supernatant was carefully collected and stored in refrigerator. The obtained crude enzyme extract was used for protein estimations and CMCase activity. Protein content was determined by using method given by Bradford, 1976. The crude enzyme extract was assayed for CMCase activity and reducing sugar released was measured method [16-17]. Enzyme activity was expressed as U/mL.

RESULTS AND DISCUSSION

A total of 24 cellulolytic fungal species were isolated from different soil samples using baiting method. The isolated strains were studied for cultural and microscopic characteristics. Nineteen out of the total number of isolates (Fig. 1, Table 1, Table 2) were identified as *Aspergillus* sps.(12), *Alternaria* sp(1). *Botrytis* sp.(1),*Curvularia* sp.(3), *Helminthosporium* sp.(1), *Penicillium* sp(1). Identification of fungal isolates based on morphological characterstics was done using standard references as mentioned earlier. Due to undistinguishable features during microscopy, some isolates are reported as unidentified (Table 2). The screening of isolated fungi for cellulose degrading activity was carried out on carboxy methyl cellulose (CMC) medium. The appearance of light yellow zone around the colony after addition of congo red solution was evidence for production of cellulase. It was observed that degradation of cellulose by isolates differs from isolate to isolate (Table 2).

Out of 24 fungal isolates, 8 isolates (Table 3) exhibited higher relative enzyme activity *viz. Penicillium sp.* (JM03), *Aspergillus sp.* (SP01), *Aspergillus sp*.(WSeIIA2IA), *Aspergillus sp.*(JM01), *Aspergillus sp.*(TE1(b)), *Aspergillus sp.*(FP), *Curvularia* sp. TE1(a) and *Aspergillus sp* (SP02). The zone of hydrolysis obtained were 1.85±0.32 cm, 1.75±0.074 cm, 1.66±0.025 cm, 1.54±0.038 cm, 1.50±0.065, 1.4±0.42 cm, 1.39±0.038 cm and 1.36±0.003 cm respectively. Judging from the ratio between the clearing zone diameter and colony diameter, these 8 isolates were chosen for enzyme production studies. Crude cellulose enzyme produced on rice husk and rice bran by these 8 isolates were preliminarily tested for cellulose enzyme activity by agar diffusion method using CMC agar (Fig 2). In this test, it was observed that all crude extracts gave cellulose degradation to varying degrees and were further quantified.

Out of the total number of isolates, *Aspergillus* sp. was found to be having highest frequency of occurrence followed by *Curvularia* sp. This may be due to the reason that growth rate of *Aspergillus* sp. is fast as compared to other isolates, thus predominating the population in natural habitat also. Laxshmi and Narasimha, 2012, also reported *Aspergillus* as most frequently occurring cellulolytic fungi in their study involving forest litter. According to Haung and Monk [18], cellulolytic fungi are likely to be detected in habitat rich in cellulolytic substrates, hence the baiting method proved successful in isolating cellulose producing fungi. These isolates have been reported as cellulase producers but with varying degrees. Ezekeil *et al* [19] isolated 22 different cellulolytic fungi from different sites. Duncan *et al* [20] isolated 72 fungi and screened for cellulose activity by using CMC and congo red detection method.

The study was undertaken in order to search for new strains capable of producing commercially important cellulose enzyme from Jalandhar area. Many researchers all over the country have done similar studies with aim of isolating novel cellulose producers [21]. From the isolates obtained in the study, it was observed that *Aspergillus* sp. had highest frequency of occurrence. It shows the ubiquitous presence of *Aspergillus* sp. and confirms its potential as major cellulase producer as compared to other fungus species. The

isolate *Aspergillus* sp. SP01 which showed highest enzyme activity of 15.44U/ml can further be identified up to species level and characterized for cellulose production.

Table 1: Macroscopic cultural characterstics of fungal isolates obtained

Characteristics / Isolates	Colour	Texture	Elevation	Margin	Reverse side Colour
MS1(a)	White	Cottony	Elevated	Regular	White
MS1	Whitish pink	Velvety	Elevated	Regular	Pink
MS1(b)	Green	Powdery	Flat	Regular	Green
MS1(c)	Black	Velvety	Flat	Regular	Black
MS1(d)	Black	Cottony	Raised	Irregular	Black
WSe1B2	Black	Velvety	Flat	Regular	Black
WSe1IA2a	Green	Powdery	Flat	Regular	Green
WSeIIA2IA	Black	Powdery	High raised	Regular	Light yellow
WSe1	Whitish pink	Velvety	Elevated	Regular	Pink
WSe1IB2a	Moss green	Powdery	Flat	Regular	Moss green
GN	White	Velvety	Flat	Regular	White
GN01C1AI	Green	Powdery	Flat	Regular	Green
JM01	Black	Powdery	High raised	Regular	Light yellow
JM02 (MG)	Moss Green	Powdery	Flat	Regular	Moss green
JM03	Green	Powdery	Flat	Regular	Green
TE1(a)	Black	Velvety	Flat	Regular	Black
TE1(b)	Green	Powdery	Flat	Regular	Green
LF01	Black	Velvety	Flat	Irregular	Black
LF02	Whitish orange	Cottony	Elevated	Regular	Orange
BE	Brown	Powdery	High raised	Irregular	Brown
FP	Moss Green	Powdery	Flat	Regular	Moss green
SP01	Black	Powdery	High raised	Regular	Light yellow
SP02	Brown	Powdery	High raised	Irregular	Light brown
SP03	Green	Powdery	Flat	Regular	Green

Table 2: Relative enzyme activity of isolates.

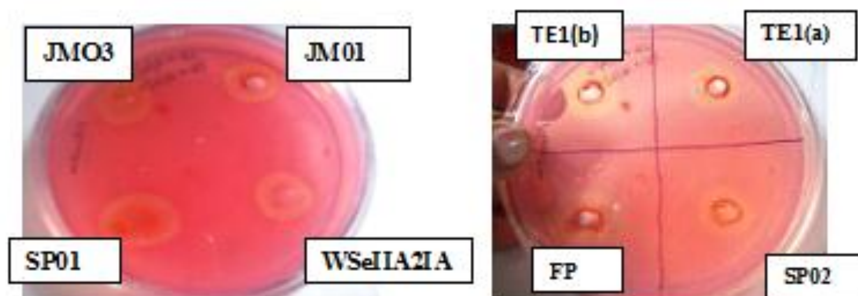
Isolates	Genus	Relative enzyme activity (mm)
MS1(a)	*Botrytis sp.*	1.15±0.069
MS1	Hyphae (Not identified)	1.14±0.070
MS1(b)	*Aspergillus sp.*	1.21±0.043
MS1(c)	*Curvularia sp.*	1.16±0.018
MS1(d)	*Helminthosporium sp.*	1.087±0.009
WSe1B2	*Curvularia sp.*	1.11±0.015
WSe1IA2a	*Aspergillus sp.*	1.32±0.013
WSeIIA2IA	*Aspergillus sp.*	1.662±0.025
WSe1	Hyphae (Not identified)	1.14±0.044
WSe1IB2a	*Aspergillus sp.*	1.06±0.21
GN	Hyphae (Not identified)	1.30±0.029
GN01C1AI	*Aspergillus sp.*	1.14±0.059
JM01	*Aspergillus sp.*	1.54±0.038
JM02 (MG)	*Aspergillus sp.*	1.20±0.012
JM03	*Penicillium sp.*	1.85±0.32
TE1(a)	*Curvularia sp.*	1.39±0.038
TE1(b)	*Aspergillus sp.*	1.50±0.065
LF01	*Alternaria sp.*	1.23±0.021
LF02	Not identified	1.1±0.027
BE	*Aspergillus sp.*	1.26±0.066
FP	*Aspergillus sp.*	1.4±0.42
SP01	*Aspergillus sp.*	1.75±0.074
SP02	*Aspergillus sp.*	1.36±0.003
SP03	*Aspergillus sp.*	1.26±0.177

Table 3: Enzyme Activity of selected isolates.

Isolates	Enzyme activity (U/ml)
WseIIA2IA	8.63
JM01	11.55
JM03	8.31
TE1(a)	8.49
TE1(b)	8.68
FP	13.03
SP01	15.44
SP02	12.25

Figure 1: Microscopic images of isolates.

Figure 2: Preliminary determination of CMCase Activity produced by selected isolates by agar diffusion method.



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