Isolation and Optimization of Lignocellulose Degrading Fungi

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Abstract – The aim of the present study was to determine the type of fungus that can grow and degrade lignocellulose. The research conducted by carrying out isolation, identification and screening of cellulolytic fungi from collected soil samples. Plate screening was done for carboxymethylecellulase activity using Congo red dye and zone of cellulose hydrolysis was apparent as clear area diameter observed. The results showed that among total of 12 isolated fungi maximum CMCase activity was found in Aspergillus sp. R-2, Penicillium sp. R-2 and Trichoderma sp. R-1 recommended further for cellulase production research.

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INTRODUCTION

Today bioethanol has been recognized as the most common non-petroleum based renewable fuel as it provides unique environmental and economic strategic benefits and can be considered as a safe and cleanest liquid fuel alternative to fossil fuels. It has also been reported that total potential bioethanol production from lignocellulosic biomass (LB) is about 16 times higher than current ethanol production from sugars or starch biomass (Kim and Dale, 2004). Hence, LB is considered a future alternative for the agricultural products that are currently used as feedstock for bioethanol production, because it is more abundant and less expensive than food crops, especially when their wastes are used. Furthermore, the use of lignocellulosic biomass is more attractive in terms of energy balances and emissions (Kheshgi *et al*., 2000).

Lignocellulosic biomass consists of three main components, *i.e.*, carbohydrate polymers called cellulose and hemicellulose that can be converted to sugars, and a non-fermentable fraction called lignin (Hayn *et al*., 1993) that can be utilized for the production of electricity and/or heat.

Typical bioethanol production processes using lignocellulosic feedstocks consist of four major steps: pretreatment, hydrolysis, fermentation, and separation (Joanne *et al.*, 2010). The key element in bioconversion process of lignocellulose is the hydrolytic enzymes mainly cellulases. This enzyme is produced by several microorganisms, mainly by bacteria and fungi. Fungi are the main cellulase producing microorganisms, although a few bacteria and actinomycetes have also been reported to yield cellulase activity. Crude enzymes produced by these microorganisms are commercially available for agricultural and industrial uses.

There are growing market for cellulases in the field of detergent industry and saccharification of agriculture wastes for bioethanol technology (Sukumaran *et al*., 2009). The ability of fungi to degrade lignocellulosic materials is due to their ability to secrete highly efficient enzymatic system and many studies have been done on cellulase enzymes complex consists of three major enzymes components that works synergically in cellulose degradation. Filamentous fungi, particularly Aspergillus and Trichoderma species, are well known as efficient producers of these cellulases. Wild type and mutant strains of Trichoderma spp. (T. viride, T. resei, T. longibrachiatum) have long been considered to be the most productive and powerful destroyers of crystalline cellulose (Gusakov *et al.,* 2007). Carboxymethyl cellulase (CMCase), one of the members of cellulase complex, cleaves the internal glycosidic bonds of cellulosic chains and acts synergistically with exoglucanases and β-glucosidases during the solubilization of cellulosic materials. The study was aimed to screen the cellulolytic ability of fungi.

MATERIALS AND METHODS

1. Isolation of fungi

Soil samples were collected from different degrading sites of Rohtak (Haryana). Samples were collected from a depth of 4-6cm and were carried to laboratory in sterilized polythene bags. One gram of soil sample was suspended in 100mL sterilized normal saline and incubated at 30^{o}C at 200rpm for one hour. Hundred microlitre of soil suspension from 10^{-1} to 10^{-7} dilution were spread on the (PDA)

medium plates and incubated at 30^{o}C for 3-5 days. The petriplates were gently rotated clockwise and anticlockwise for uniform spreading of the soil suspensions. The PDA medium was supplemented with 60µg/mL of ampicillin as an antibacterial agent. The petriplates were then incubated at 30ºC in an incubator for 5 days. Isolated fungi were streaked extensively on PDA plates.

Composition of PDA medium (g/L)

Potato peels	-	200g
Dextrose	-	20g
Agar	-	15g
pH	-	7.0

The medium was autoclaved at temperature 121^{o}C and pressure 15psi for 15 minutes. 25mL of the autoclaved PDA medium was taken in sterile petri-dishes under aseptic conditions. The mycelium was picked up from a single colony and streaked on PDA slants, incubated at 30^{o}C for 48 hours and later stored at 4^{o}C. The whole operation was performed under aseptic conditions.

2. Identification of fungal isolates

Each isolate was inoculated onto PDA petriplates. Morphological characteristics of the fungi were observed for one week. Fungi were examined under a compound microscope. The preparations were made in distilled water, then mounted in lactophenol cotton blue and sealed with polyvinyl alcohol. The fungi were identified on the basis of colony morphology and spore characteristics following the standard literature (Barnet and Hunter, 1972).

3. Plate screening for carboxymethylecellulase activity

For plate screening, carboxymethylcellulose-agar Mandles medium was used. This medium consisted of (g/L): $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 2.0; Urea, 0.3; $MgSO_4.7H_2O$, 0.3; $CaCl_2$, 0.3; $FeSO_4$, 5.0(mg/L); $MnSO_4$, 1.6(mg/L); $ZnCl_2$, 1.7(mg/L); Agar, 20 and CMC, 5.0. Fungal discs (dia 4mm) were inoculated on these plates in triplicates and incubated at 30^{0}C for 2-4 days to accelerate the action of extracellular cellulases and thus rapidly develop clear zone around the cellulose-producing colonies. For cellulolytic activity observations, plates were stained with 0.1% (w/v) Congo red dye for 15min followed by destaining with 1M NaCl solution for 15 to 20min. The zone of cellulose hydrolysis was apparent as a clear area in the otherwise congo red stain background and its diameter was measured. The strains that showed the most promising result was selected as potential cellulase producing fungi for further experiments.

4. Optimization of fungal growth

To optimize the growth of fungi, the selected fungi were grown at different temperatures ranging from 20-45^{o}C on PDA medium of varying pH range of 4.0-7.5 to observe the growth. The fungal growth was measured after definite time intervals.

RESULTS AND DISCUSSION

1. Isolation of fungi from soil samples

The soil is always inhabitated by a large number of microorganisms like bacteria, actinomycetes, algae, fungi which form a major biotic component of soil. The PDA medium, meant for the growth of fungi, was utilised for isolating various degrading fungi from 20 soil samples collected from different sites of district Rohtak in Haryana (INDIA) by spread plate method through serial dilutions. Various fungi isolated from different soil samples of Rohtak were identified as follows: *Aspergillus* sp. R-1, *Aspergillus* sp. R-2, *Aspergillus* sp. R-3, *Aspergillus* sp. R -4, *Rhizopus* sp. R-1, *Fusarium sp.* R-1, *Alternaria* sp. R-1, *Penicillium* sp. R-1, *Penicillium* sp. R-2, *Penicillium* sp. R-3, *Mucor* sp. R-1, *Trichoderma* sp. R-1.

2. Plate screening for carboxymethylecellulase activity

The cellulase producing colonies were identified by observing hydrolytic zone/s around colonies against dark background. The zone of hydrolysis was observed and its diameter was measured. *Aspergillus* sp. R-2 and *Trichoderma* sp. R-1 showed the maximum hydrolytic zone than fungus *Penicillium* sp. R-1. The hydrolytic zones of fungus *Aspergillus* sp. R-2 and *Trichoderma* sp. R-1 were also the highest among all the tested fungal isolates. Owing the higher cellulase enzymatic activity of *Aspergillus* sp. R-2, *Trichoderma* sp. R-1 and *Penicillium* sp. R-1 among all the tested fungal isolates, only these fungi were screened for further studies (Table 1).

Table 1: Hydrolytic zone of various fungal isolates for their screening

S.No.	Fungal isolate	Zone of hydrolysis
1.	*Aspergillus* sp. R-1	++++
2.	*Aspergillus* sp. R-2	+++++
3.	*Aspergillus* sp. R-3	+++
4.	*Aspergillus* \sp. R -4	+++
5.	*Rhizopus* sp. R-1	+++
6.	*Fusarium sp.* R-1	++++
7.	*Alternaria* sp. R-1	+++
8.	*Penicillium* sp. R-1	+++++
9.	*Penicillium* sp. R-2	++++
10.	*Penicillium* sp. R-3	++++
11.	*Mucor* sp. R-1	+++
12.	*Trichoderma* sp. R-1	+++++

3. Optimization of fungal growth

For optimizing the growth of all the screened fungi, different temperatures and pH were applied on various fungi and the diameter of hydrolytic zone was measured after a fixed interval of time as also shown in Table 2, 3 and 4.

The Table 2 shows the effect of variable temperature and pH on the growth of fungus *Aspergillus* sp. R-2. The fungus *Aspergillus* sp. R-2 showed least growth rate at temperature 20°C and 45°C in presence of variable pH. Medium growth of the fungus was observed at temperature 25°C and 40°C and variable pH ranging from 4.0-6.5 and 5.0-7.0 respectively for different temperatures. More than 7.0 pH was found to be hindering for the growth of fungus *Aspergillus* sp. R-2 at all the temperatures. It was found that the maximum growth rate of fungi *Aspergillus* sp. R-2 was observed at temperature 30°C and pH 5.5.

Table 2: **Effect of temperature and pH on the growth of fungus *Aspergillus* sp. R-2**

S. No.	Temperature (°C) / Ph	20	25	30	35	40	45
1.	**4.0**	++	+++	++++	++++	++	++
2.	**4.5**	++	+++	++++	++++	++	++
3.	**5.0**	++	+++	++++	++++	+++	++
4.	**5.5**	++	+++	+++++	++++	+++	++
5.	**6.0**	++	+++	++++	++++	+++	++
6.	**6.5**	++	+++	++++	+++	+++	++
7.	**7.0**	++	++	+++	+++	+++	++
8	**7.5**	++	++	+++	++	++	++

Corroborative results have also been observed by Negi and Banerjee (2006) according to whom maximum amylase secretion was obtained at pH 5.5 and protease was found to be maximum at pH 4 and 7. They also found the temperature of 37°C as best for the production of both amylase and protease enzymes which was contradictory to the required temperature of 30°C for the *Aspergillus* sp. R-2 which was corroborative with the results of Ja'afaru and Fagade (2010).

The impact of variable temperature and pH on the growth of fungus *Penicillium* sp. R-1 has been given in Table 3. It was clearly observed that maximum growth rate was observed at temperature 30°C-35°C and pH 4.0-5.5 while minimum growth rate was observed at temperature 45°C and all the pH ranging from 4.0-7.5. It was also noted that if the temperature remains constant at 20-25°C, minimum growth rate could occur at all the pH ranging from 4.0-7.5. Medium growth rate of the fungus could be observed at the temperatures 30-35°C at variable pH ranging from 6.0-7.5.

Vamsi *et al*. (2009) also observed that the best results of *Penicillium* growth could be observed at 30ºC which was found to be corroborative with the present results. They also noted that the optimum pH for production of protease enzyme by *Penicillium* species was 6.6 which was found to be contradictory to the present results as the optimum pH for the growth of fungus *Penicillium* sp. R-1 has been found to be ranging from 4.0-5.5.

Table 3: Effect of temperature and pH on the growth of fungus *Penicillium* sp. R-1

S. No.	Temperature (°C) / pH	20	25	30	35	40	45
1.	**4.0**	++	+++	++++	++++	++++	+
2.	**4.5**	++	++	++++	++++	++	++
3.	**5.0**	++	++	++++	++++	++	++
4.	**5.5**	++	++	++++	++++	++	++
5.	**6.0**	++	++	+++	+++	++	++
6.	**6.5**	++	++	+++	+++	+	+
7.	**7.0**	++	++	+++	+++	+++	+
8	**7.5**	++	++	+++	+++	++	+

The effect of variable temperature and pH on the growth of fungus *Trichoderma* sp. R-1 has been given in Table 4.This Table shows that good growth rate of the fungus occurred at 35°C and in a pH range of pH 4.0-5.0.Temperatures 20-25°C and 45°C were found to show inhibitory effect on the growth of fungus *Trichoderma* sp. R-1 at all the pH ranging from 4.0-7.5 while medium growth rate could be observed at 30°C and 40°C at all the pH, *i.e.*, 4.0-7.5. The effect of temperature 35°C and pH ranging from 5.5-7.5 also showed medium growth rate in fungus *Trichoderma* sp. R-1.

Table 4: Effect of temperature and pH on the growth of *Trichoderma* sp. R-1

S. No.	Temperature (°C) / pH	20	25	30	35	40	45
1.	**4.0**	++	++	+++	++++	+++	++
2.	**4.5**	++	++	+++	++++	+++	+
3.	**5.0**	++	++	+++	++++	+++	++
4.	**5.5**	++	++	+++	+++	+++	++
5.	**6.0**	+	+++	+++	+++	+++	+
6.	**6.5**	++	++	+++	+++	+++	++
7.	**7.0**	++	++	+ ++	+++	+++	++
8	**7.5**	++	++	+++	+++	+++	++

The results of Al-taweil and Osman (2009) indicated that an optimal medium for maximizing the production of biomass in batch cultures of T*richoderma viride* contained temperature 30°C and pH 6.0 which was found to be contrary to the present results as the optimum temperature and pH required for the growth of *Trichoderma* sp. RTK -1 were found to be 35°C and 4.0-5.0 respectively. Contrary to the studies of Al-taweil and Osman (2009), Ahmed *et al.* (2009) reported the optimum fermentation temperature of 28°C for endoglucanase production from

Trichoderma harzianium while they observed the best enzyme production at pH 5.0 which was found to be corroborative with the present results.

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