RESEARCH ARTICLE

Production of Fungal Cellulase under Solid State Fermentation Conditions

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ABSTRACT

Filamentous fungi are important due to their high enzyme production potential. Many enzymes produced by fungi have been related to biotechnological applications in several industrial sectors. The purpose of this study was to isolate filamentous fungi from different sources and to screen for cellulase production potential. This study was focused on the evaluation of cellulolytic fungi isolated from different soil samples collected from different Textile Sizing sites of Bhiwandi city. Amongst the twenty isolated fungi, two cellulase producing isolates identified as *P. citrinum* and *A. flavus* were subjected for Solid State Fermentation. SSF was carried out using two substrates namely sugarcane bagasse and sweet lime bagasse. Both the fungi showed maximum cellulase activities at 72 hours of incubation. *P. citrinum* was found to be producing cellulase higher than *A. flavus* using both the substrates. Isolation of cellulase producing fungi from Textile Sizing Site will help in the bioremediation of sizing processing wastes' environment.

Key words: Cellulase, Solid State Fermentation, *P.citrinum, A.flavus*

INTRODUCTION

Enzymes are globular proteins and are important biocatalyst for various industrial and biotechnological purposes and produced by microorganisms, animals and plants. Each single strain of organism produces a large number of enzymes, hydrolyzing, oxidizing or reducing, and metabolic in nature (Sri Lakshmi and Narasimha, 2012). Nowadays, the use of enzymes in industrial sector is increasing due to increase in the number of industries especially food, beverages, textile, leather and paper industries (Ogbonna et al., 2014). Enzymes can work in many adverse conditions compared to chemical catalyst. Microorganisms are preferred as a source of enzyme because of their short life span, high productivity rate, cost effective, and also free of harmful chemicals that are found in enzyme from plant and animal source (Khatri et al., 2015). Fifty percent of available enzymes are originated from fungi and yeast; 35 % from bacteria, while the remaining 15 % are either of plant or animal origin (Khatri et al., 2015). Fungi constitute a group of micro-

organisms that are widely distributed in environment especially in soil (P. Lakshmi Narasimha, 2014). Filamentous fungi are important organisms for production of useful enzymes and biological active secondary metabolites due to extracellular release of various enzymes like amylases, cellulases, xylanases, etc. These fungi produce high levels of polysaccharide degrading enzymes and are frequently used for the production of industrial enzymes. Therefore, there is a growing interest in assessing soil biodiversity and its biological functioning (Barrios E, 2007).

Cellulases

Cellulases are enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze hydrolysis of cellulose. Degradation of the cellulosic materials is achieved chemically, enzymatically or by the combination of both chemical and enzymatic methods (P. Lakshmi Narasimha, 2014). Cellulases bring about the hydrolysis of cellulose, a homopolymer of -1,4 linked glucose units that comprises amorphous and crystalline regions, by synergistic action of its constituent enzymes. Cellulases are group of extracellular enzymes commonly employed in many industries for the hydrolysis of cellulolytic material. Bioconversion of cellulosic biomass to fermentable sugars through biocatalyst cellulases derived from cellulolytic organisms has been suggested as a feasible process and offers potential to reduce the use of fossil fuels and reduce environmental pollution (Lynd et al., 1999). Cellulases have been used for several years in food processing, feed preparation, waste-water treatment, detergent formulation, textile production and other areas. Additional potential applications include the production of wine, beer and fruit juice. Adverse spectrum of extensively studied mesophilic fungi degrades organic material aerobically (Falcon MA et al., 1995). Nearly, all the fungi that have been reported for the production of cellulases are mesophilic fungi and the best known cellulase producers include *Trichoderma sp., Aspergillus sp., Acremonium sp., Penicillium sp., Rhizopus sp., F. solani and Chaetomium sp.,* among other mesophiles (Kuzmanova S et al., 1991; Teeri T and Koivula A, 1995; Bhat M and Bhat S, 1997; Schulein M, 1997 and P. Lakshmi Narasimha et al., 2014). Cellulase enzymes have a wide range of applications, economic importance in agriculture, biotechnology, and bioenergy (Nathan VK et al., 2014; Phitsuwan P et al., 2012).

Solid State Fermentation

Solid-state fermentation is traditionally defined as those processes in which microbial growth and products formation occur on the surfaces of solid substrates in the mere absence of free water. Due to this low amount of water available in solid-state bioprocessing, the class of microorganisms that are most commonly used is fungi (Martin et al., 2004). SSF is suitable for fungi growth because of its low moisture content and permitting of the penetration of fungi mycelium through the solid substrates. This technology usually uses agro-industrial waste as support and carbon source for production of various value added products, such as single cell protein, industrial enzymes, secondary metabolites and fine chemicals (Grover et al., 2013). Several agro-industrial waste and by-products such as orange bagasse, sugar cane bagasse, wheat bran (Martin et al., 2004) and other food processing waste are effective substrates for depolymerizing enzyme production by solid-state fermentation. Solid-state fermentations are simple, low cost, and provide high yields of appropriate enzymes. Solid-state fermentation has gained renewed interest and fresh attention from researchers owing to its importance in recent developments in biomass energy conservation, in solid waste treatment and in its application to produce secondary metabolites (Elliaiah P et al., 2002; Grover et al., 2013).

Taking all the above facts into consideration the present research work was aimed to study various fungal isolates isolated from textile sizing site for the production of cellulase using cheaper substrates under the solid state fermentation conditions.

MATERIALS AND METHODS

Selection of sample sites

Five different textile sizing sites of Bhiwandi city were selected for the sample collection i.e Ram Raheem Sizing, Moksh Sizing, Master Sizing, Kulsum Sizing, Indra Sizing.

Collection of soil samples

The samples were collected from 6 different spots in each site in zip lock polythene bags. Almost 5-6 soil samples were taken from each sizing industries. The soil sample was mixed well and processed next day.

Isolation Of fungi

Fungal colonies were isolated from soil samples by serial dilution method where in SDA (Sabouraud dextrose agar) media was prepared, autoclaved and poured in sterile petriplates. 50µl of soil samples diluted upto 10-5 dilutions were spread on respective solidified SDA plates with the help of sterile spreader. The inoculated petriplates were incubated at 28°C for 48 h. 20 different fungal isolates differentiated on the basis of physical characteristics obtained after incubation were selected for the further processes. The isolates were further inoculated on SDA plates by point inoculation and incubated at 28°C for 48 h in order to obtain pure fungal cultures (Khan and Yadav, 2011).

Primary Screening of Fungal Isolates for Cellulase Production

The isolated fungal cultures were screened for their ability to produce cellulase. Czapek-Dox medium used in this method contained (g/l): sucrose – 30, $NaNO_3$ – 2, K_2HPO_4 - 1, $MgSO_4$ – 0.05, KCl – 0.5, $FeSO_4$ – 0.01, carboxyl- methyl cellulose – 1%, Agar agar – 20 g. pH of the medium was adjusted to 5. After autoclaving at 121°C and 15 lbs pressure the medium was poured into petri plates and allowed to solidify. The plates were incubated at room temperature (28 ± 2 °C) for three days to allow fungal growth, then again incubated for 18 h at room temperature for cellulase activity. After incubation, 10 ml of 1% Congo-red staining solution was added to the plates. The Congo - red staining solution was then discarded and then de-stained with 10 ml of 1 N NaOH solution for 15 minutes. Finally 1 N NaOH was also discarded and the staining of the plates was analyzed by noticing the formation of yellow zones around the fungal growth **(**Sri Lakshmi A. and G. Narasimha., 2012). The diameter of zone of decolorization around each colony was measured. The fungal colony showing largest zone of decolorization was selected for cellulase production.

Identification of isolates

The isolated strains were carefully identified by morphological characteristics include color of the colony and growth pattern studies, as well as their vegetative and reproductive structures observed under the microscope (M. Charitha Devi and M. Sunil Kumar., 2012). Among the characteristics used were colonial characteristics such as surface appearance, texture and colour of the colonies. In addition, microscopy revealed vegetative mycelium including presence or absence of cross-walls, diameter of hyphae. Mycological identification keys and taxonomic descriptions (Ogbonna Cet al., 2014). Appropriate references were made using monograph and morphological characters (Pitt John, 1988; Raper and Thom, 1949).

Production of cellulases by Solid State Fermentation using Cheap Substrates

Cheap substrates used were Sugarcane bagasse and Sweet lime bagasse. Both the substrates were collected from the local market of Bhiwandi and were washed thoroughly with water twice and thrice to remove excess dirt and fungal spores. After that these substrates were sundried for 4 days. Dried substrates were cut in small pieces and finely grinded into powder.

Sugarcane Bagasse Media:(hence forth referred to as **S1**)

Six gram of sugarcane bagasses were transferred into 250 ml cotton plugged conical flasks (13) and moistened with 40ml of defined Media (Murashige and Skoog, 1962), mixed and sterilized for 15 minute in an autoclave and cooled thereafter at room temperature.

Sweet lime Bagasse Media:(hence forth referred to as **S2**)

Six gram of sugarcane bagasses were transferred into 250 ml cotton plugged conical flasks (13) and moistened with 20ml of defined Media (Murashige and Skoog,1962), mixed and sterilized for 15 minute in an autoclave and cooled thereafter at room temperature.

Solid state fermentation

The highly productive isolates in primary screening were selected. Spore suspension was prepared by dipping 4mm disc of fully grown fungal isolates in 10ml of sterile distilled water.1ml of spore suspension of each isolates was added in both the media under the sterile conditions. The cultures were incubated at 28ºC and were harvested at the gap of 24 h for 8 consecutive days.

Extraction of Crude Enzyme

Crude enzyme was extracted from fermented media by adding 50ml of 100mM Tris buffer pH 6.2, agitating the flask in shaker at 180rpm for 1h, the mixture was filtered and centrifuged at 8000 rpm at 4ºC for 10 min. The supernatant was collected and treated as crude enzyme (Khan and Yadav, 2011).

RESULTS AND DISCUSSION

Isolation of Fungi

The soil samples were collected from sizing industry sites, following spread plate technique. Twenty different fungal colonies were obtained and pure culture of these isolates was obtained for the further processes.

Primary Screening of Isolates for Cellulase Production:

All the 20 pure cultures were screened for their Cellulase producing potential. The isolates were subjected to screening procedure on Carboxy Methyl Cellulose (CMC) plates and incubated for 3 days. After completion of incubation period, varied degree of cellulose utilization was seen after flooding with Congo red solution and observed for zone of hydrolysis. The two isolates which gave maximum zone were selected for fermentation (Table. 1).

Identification of the Isolate Showing Maximum Hydrolysis

Based on morphological and microscopical studies, the isolate no. 9 was identified as *Penicillium citrinum* and isolate no. 1 was identified as *Aspergillus flavus.*

Solid State Fermentation Using Cheap Substrates

The cultures showing maximum zone during screening and identified as *A.flavus* and *P. citrinum* were inoculated in SSF flasks containing different substrates.

CELLULASES

The local isolate of *A. flavus* and *P. citrinum* in the present study were able to efficiently utilize sugarcane bagasse and sweet lime bagasse for production of cellulase enzyme by solid state fermentation method.

In *P. citrinum* the amount of extracellular cellulase in sugarcane bagasse (S1) gradually increased since 48 h. Maximum secretion of extracellular cellulose enzyme was found to be 42.89 mg/ml equivalent of glucose at 72 h followed by 144 h and expressed as 36.12 mg/ml equivalent of glucose. Least secretion of extracellular cellulase was recorded at 24 h of incubation (Fig.1). When S2 was used as growth medium, cellulase activity was maximum on the second and third day of incubation period and expressed as 23.93mg/ml equivalent of glucose and 16.84mg/ml equivalent of glucose respectively followed by a rapid decline. Least activity was observed at first day of incubation period and expressed as 0.25mg/ml equivalent of glucose (Fig.2).

For *A. flavus* the cellulase activity in S1 gradually increased at 48h of incubation. The content of extracellular cellulase on sugarcane bagasse was maximum with 37.47mg/ml equivalent of glucose at 72 h followed by 144 h and expressed as 31.83mg/ml equivalent of glucose. Minimum activity was found at 24 h of incubation period (Fig.3). Highest amount of cellulase activity for S2 was recorded with 15.46 mg/ml equivalent of glucose at 120 h of incubation period followed by 72 h of incubation and expressed as 14.21mg/ml equivalent of glucose. Least activity was observed at first day of incubation (Fig.4).

Both the fungi showed maximum cellulase activity at 72 h after which decline was observed in both the substrates. But again rise was observed in the later period of incubation i.e at 144 h. The production was highest done by *P.citrinum* when S1 is used as a substrate at 72 h. *P.citrinum* is found to be producing cellulase higher than *A. flavus* using S1 and S2 both. It is reported earlier that cellulase activity is exhibited maximum at 3rd day of incubation (Lakshmi, 2014). Also in many of the earlier reports *Aspergillus sp*. are found to be the potential cellulase producers. However, it would be concluded that sugarcane bagasse served the best substrate followed by sweet lime bagasse in supporting maximum production. And among these two fungi *P. citrinum* was found to be the best for the cellulase production than that of *A. flavus* for both S1and S2 substrates. Fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular. Even though there are many reports on fungi producing cellulases (Shin et al. 2000), only a few have proved high activities for commercial success (Sri Lakshmi A. and G. Narasimh., 2012). A wide range of *Aspergillus sp.* have been identified to possess all components of cellulases complex (Vries and Visser, 2001).

Large demand of cellulases has increased their prices to a large extent and the major reason is the cost of substrate, and fermentation procedure. It is the need of the time to search for cheaper substrates and reduced fermentation cost so that the production cost can be reduced to a large extent. The present investigation also focused on the same and hence search for substrates with zero cost was attempted.

Table 1: Primary Screening for Cellulase Production

Isolate no.	Result	Isolate no.	Result
1	++++	11	-
2	-	12	++
3	-	13	-
4	++	14	+++
5	-	15	++
6	+++	16	++
7	++	17	-
8	-	18	-
9	++++	19	-
10	++	20	-

*Maximum zone of hydrolysis: ++++, each + sign shows level of rise in hydrolysis

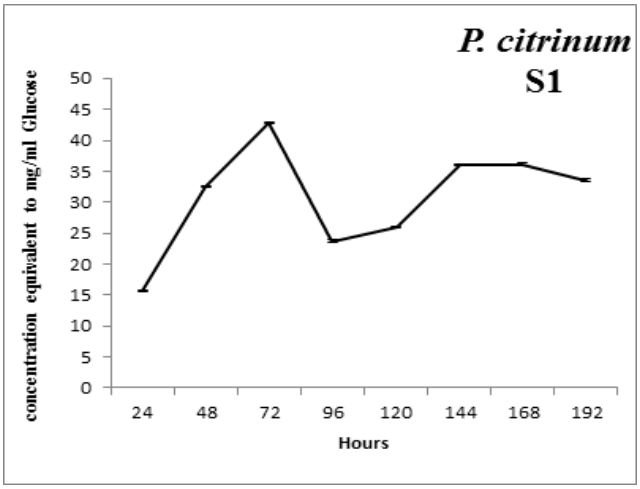

Fig.1: Cellulase activity of *Penicillium citrinum* in Subtrate1

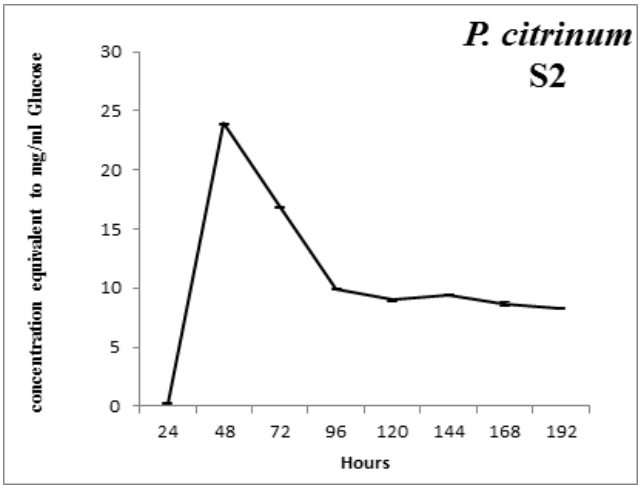

Fig.2: Cellulase activity of *Penicillium citrinum* in Subtrate2

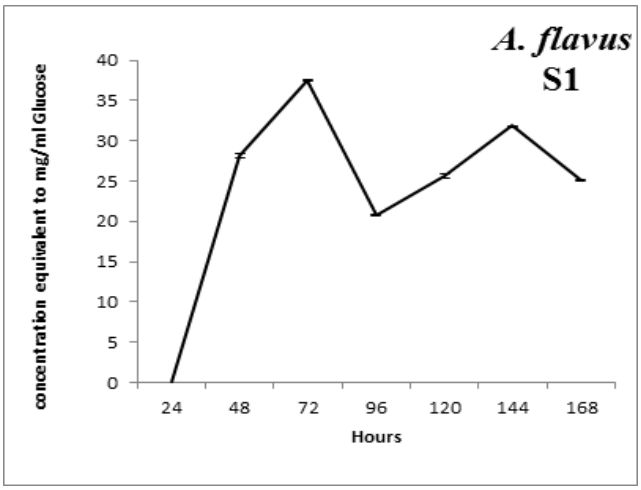

Fig.3: Cellulase activity of *Aspergillus flavus* in Subtrate1

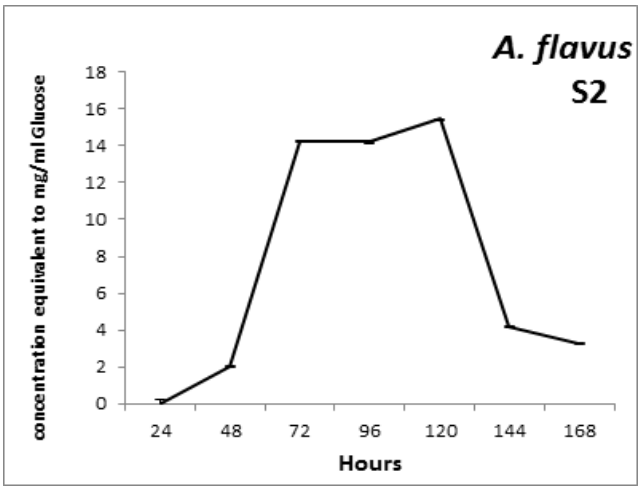

Fig. 4: Cellulase activity of *Aspergillus flavus* in Subtrate2

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