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Isolation and Selection of Thermophilic Fungi for Production of Cellulases Under Submerged and Solid-State Fermentation Conditions.

KEYWORDS: Thermophilic fungi, submerged fermentation, solid–state fermentation, FPase activity

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ABSTRACT *Fungi are the predominant cellulase-producing micro-organisms. In this study, wild-type thermophilic fungal isolates, producing highly effective cellulolytic enzymes were screened and selected using 1% carboxymethyl cellulose and combination of sweet sorghum bagasse and wheat bran under submerged and solid-state fermentations, respectively. Enzyme production among the selected 6 fungal isolates was detected within five days of cultivation under solid state fermentation Among the isolates, DIA-4 strain showed highest cellulolytic activity in 72 hours at 45^{0}C followed by potential strains IA-56 and U3.*

Introduction:
Cellulases play a significant role in saccharifying cellulosic substrates for bioethanol production (Dhillon *et al.*, 2011). However, production cost of these enzymes is high and accounts for 40–60% of the production cost. Hence, current research efforts are focused towards lowering the cost of enzymes. The utilization of abundant renewable lignocellulosic biomass, especially agro-industrial wastes and their by-products as substrates can help to reduce cellulase prices (Rodriguez-Couto and Sanroman, 2005). Also the use of cheaper technologies like solid-state tray fermentation can further improve the production economics (Dhillon *et al.*, 2010).

Cellulases are produced by fungi, bacteria and actinomycetes, but due to higher yields, fungi have been commercially exploited for production of these enzymes. However, majority of commercial enzymes are obtained from mesophilic fungi. Since industrial processes employ high temperatures, thermostable enzymes are in demand (Moretti *et al.*, 2012). Further, thermophilic fungi are known to produce thermostable enzymes with activity at high temperatures, broad tolerance to pH variation and resistance to denaturing agents (Maheshwari *et al.*, 2000; Leite *et al.*, 2008).

Keeping in view the industrial importance of the thermostable cellulases, this study was designed to isolate and select thermophilic filamentous fungi for extracellular cellulase production under submerged and solid state fermentation.

Materials and methods:
Isolation of thermophilic fungal strains

One gram each of samples collected aseptically from field soils of PAU, Ludhiana were serially diluted in distilled water from 10^{-4} to 10^{-7} and spread plated on Potato dextrose agar (PDA) and Rose Bengal Chloramphenicol (RBC) agar medium plates. The plates were incubated at 45°C for 4–6 days and the growing fungal colonies were sub-cultured to obtain pure cultures which were maintained on PDA slants at 4 °C. The isolated strains were identified by morphological characteristics including color of the mycelia and spores and growth pattern studies at different temperatures, as well as their vegetative and reproductive structures observed under the low power light microscope using Lacto phenol cotton blue staining (Murray *et al.*, 1999).

Qualitative screening of cellulase producing thermophilic fungi
Cellulase producing fungi were screened on selective carboxymethyl cellulose (CMC) agar containing 2.0 g $NaNO_3$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 10.0 g carboxymethyl cellulose sodium salt 10.0 g, 0.2 g peptone and 17.0 g agar in 1000 ml distilled water (pH 5.5-6.0). Plates were spot inoculated with spore suspension of pure cultures and incubated at 45°C. After 3 days, plates were flooded with 1% Congo red solution for 15 minutes and then de-stained with 1M NaCl solution for 15 minutes and. The diameter of zone of decolorization around each colony was measured. Cellulolytic index (CI) was determined and expressed by the ratio between the diameter of the degradation halo and the diameter of the colony (Khokhar *et al.*, 2012). Isolates showing CI of 2.0 or more were quantitatively characterized by filter paper (FPase) assay.

Quantitative determination of cellulolytic activity
Submerged fermentation (SF)

The selected fungal cultures were cultivated on PDA plates and incubated at 45 °C for 72-96 h after which the spores were harvested using sterile water containing Tween 80. The spore count of 1×10^8 spores ml^{-1} was used for enzyme production. Submerged fermentation using Mandel Webber medium (KH_2PO_4 2.0g, $MgSO_4.7H_2O$ 0.3g, $CaCl_2.2H_2O$ 0.3g, $(NH_4)_2SO4$ 1.4g, $FeSO_4.7\ H_2O$ 5.0 mg, $MnSO_4.\ H_2O$ 1.6 mg, $ZnSO_4.7\ H_2O$ 1.4 mg, $CoCl_2.6\ H_2O$ 2.0 mg, peptone1.0g, Tween 80 1.0g, pH 5.0) supplemented with 1% CMC was employed for cellulase production in 250-ml Erlenmeyer flasks, with each flask containing 100 ml of fermentation medium. The flasks were inoculated with 1 ml fungal spore suspension and incubated at three different temperatures viz. 45 °C, 50 °C and 55 °C for 5 days. A set of three flasks was removed at 24-h intervals from the incubator centrifuged and analyzed for cellulase production with respect to Filter Paper activity (Wood and Bhatt, 1988). One unit of enzyme activity was defined as the amount of enzyme required to liberate one µmole of the glucose per ml per min under standard assay conditions and expressed as international units per milliliter (IU/ml).

Solid state fermentation (SSF)
The selected fungal cultures were cultivated, harvested and their spores were used for enzyme production as in submerged fermentation. The Solid state fermentation was carried out in 250-ml Erlenmeyer flasks, with each flask con-

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taining 10 g sweet sorghum bagasse and wheat bran in the ratio of 4:1 with an initial moisture content of 75% which was made up with Mandel Weber medium (pH 5.0 adjusted with 5N NaOH before sterilization). The flasks were autoclaved, cooled and inoculated with 1 ml fungal spore suspension. The flasks were incubated at three different temperatures viz. 45 °C, 50 °C and 55 °C for 5 days. Supernatant of 24 hour sample was analyzed for Filter Paper activity as discussed for submerged fermentation.

Statistical analysis
All experiments were carried out in triplicate, and the mean and standard deviation (SD) values were calculated using the MS Excel program.

Result and discussion:
Six fungal isolates which showed characteristic diversity for colony morphology, spore color and microscopic spore characteristics and showed clear zones on the PDA plates containing CMC and Congo red with different CI values were used for enzyme production experiments. DIA-4, IA-56 and KJH showed CI values of 2.42, 2.22 and 2.23, respectively whereas U3 showed least CI value of 2.06 (Table 1). All these values were comparable to the values of standard cultures viz. *Trichoderma harzianum* MTCC 8230 and *Humicola insolens* MTCC 1433, which were 2.10 and 2.88, respectively, showing their potential as efficient cellulase producers. Khokhar *et al* (2012) screened 17 fungal species belonging to three genera i.e. *Trichoderma*, *Aspergillus* and *Penicillium* isolated from different sources and compared these for their ability to degrade cellulose using Index of Relative Enzyme Activity (ICMC).

Table 1: Morphological features and cellulolytic index of cellulolytic thermophilic fungal isolates.

Fungal isolate	Colour of mycelium	Spore colour	Colony diameter (mm)	Zone diameter (mm)	Cellulolytic Index (CI[a])	Vesicle shape
U3	White	Brown	33	68	2.06	Subclavate
IA-56	White	Brown	31	69	2.22	Inflated globose
DIA-4	White	Golden yellow	28	68	2.42	Globose to sub globose
KJH	White	Greenish yellow	26	58	2.23	Inflated globose
L9-39	White	Dull brown	43	45	1.04	Subclavate
L8-38	White	Dull red	44	47	1.06	Inflated globose
Trichoderma harzianum MTCC 8230	Yellow	Light green	40	84	2.10	Subclavate
Humicola insolens MTCC 1433	White	Dark black	26	75	2.88	Globose to sub globose

(CI)[a] cellulolytic index = ratio between the ring diameter (mm) / colony diameter (mm)

All the isolates were evaluated for FPase activity (a relative measure of the overall cellulose-hydrolyzing capacity of microbial cellulase preparations) under SF and SSF conditions. All the isolates showed higher FPase activity under SSF compared to SF. DIA-4 showed the highest FP activity in lesser time among all the strains at 45^{0}C under SSF (Table 2). Filter Paper activity of the selected isolate increased until 72 h, leveling off thereafter. Fungi initially consumed the readily available sugars and produced hydrolytic enzymes; following depletion of the sugar concentration, particularly when the glucose concentration was low, the fungi began to use these hydrolytic enzymes for the production of sugars, resulting in a decrease in enzyme activity. In a previous study, a similar trend was observed in enzyme production using mixed-culture solid-state fermentation (Oberoi *et al*., 2010).

Table 2: Filter paper activity of thermophilic fungal isolates under different fermentation conditions.

Fungal isolate(s)	Temperature (^{0}C)	Fermentation conditions (FPase activity, IU/ml)	
		Submerged fermentation (SF)	Solid state fermentation (SSF)
U3	45	0.231 ± 0.003 (4th day)	1.253 ± 0.020 (4th day)
	50	0.205 ± 0.001 (4th day)	1.051 ± 0.036 (3rd day)
	55	0.106 ± 0.002 (2nd day)	0.531 ± 0.020 (2nd day)
IA-56	45	0.235 ± 0.003 (4th day)	1.131 ± 0.020 (4th day)
	50	0.186 ± 0.002 (4th day)	1.022 ± 0.036 (3rd day)
	55	0.098 ± 0.001 (2nd day)	0.626 ± 0.020 (2nd day)
DIA-4	45	0.215 ± 0.002 (5th day)	1.373 ± 0.015 (3rd day)
	50	0.192 ± 0.002 (4th day)	1.123 ± 0.015 (3rd day)
	55	0.099 ± 0.003 (2nd day)	0.586 ± 0.025 (2nd day)
KJH	45	0.243 ± 0.002 (4th day)	1.183 ± 0.025 (3rd day)
	50	0.196 ± 0.001 (4th day)	0.913 ± 0.020 (3rd day)
	55	0.114 ± 0.002 (2nd day)	0.453 ± 0.025 (2nd day)
L9-39	45	0.194 ± 0.002 (4th day)	0.961 ± 0.026 (4th day)
	50	0.168 ± 0.002 (3rd day)	0.783 ± 0.025 (3rd day)
	55	0.103 ± 0.002 (2nd day)	0.543 ± 0.030 (2nd day)
L8-38	45	0.183 ± 0.002 (4th day)	0.852 ± 0.026 (4th day)
	50	0.134 ± 0.002 (3rd day)	0.646 ± 0.025 (3rd day)
	55	0.094 ± 0.001 (2nd day)	0.386 ± 0.030 (2nd day)
***Trichoderma harzianum* MTCC 8230**	45	0.265 ± 0.004 (5th day)	1.426 ± 0.020 (4th day)
	50	0.128 ± 0.003 (4th day)	1.233 ± 0.020 (3rd day)
	55	0.084 ± 0.002 (2nd day)	0.763 ± 0.015 (2nd day)
***Humicola insolens* MTCC 1433**	45	0.221 ± 0.003 (5th day)	1.206 ± 0.015 (4th day)
	50	0.286 ± 0.002 (4th day)	1.546 ± 0.015 (3rd day)
	55	0.213 ± 0.002 (2nd day)	1.266 ± 0.015 (2nd day)

Values in brackets indicate number of days required for maximum FPase activity.

The values are mean of triplicates.

Conclusion: The present study led to isolation of three potential cellulase producing fungal strains which showed potential under SSF conditions and are being standardized for different physicochemical and fermentation parameters for enhancing their cellulase production.

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