JASEM ISSN 1119-8362

Full-text Available Online at www.ajol.info and www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage. *Sept.,* 2012
Vol. 16 (3) 223 - 226

Isolation and Screening of Highly Cellulolytic Filamentous Fungi

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ABSTRACT: A large number of microorganisms are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of enzymes capable of completely hydrolysing cellulose. Fungi are the main cellulase-producing microorganisms. In this purposed study, seventeen fungal species belonging to three genera i.e. *Trichoderm*, *Aspergillus* and *Penicillium* were isolated from different sources, screened and compared for their ability to degrade cellulose. The plate screening assay recommended by International Union of Pure and Applied Chemistry (IUPAC) were used in the investigation. Cellulolytic fungi were evaluated after 7 days for the production of cellulolytic enzymes by staining with 1% Congo red. The diameter of clear zone on fungal plates, gave an approximate indication of cellulase activities. Fungal species were grouped as high and low celluloytic isolates on the basis of cellulase activity using Index of Relative Enzyme Activity (ICMC). Fungal species i.e. *T. harzianum*, *T. viride*, *T. koningii*, *A. japonicus*, *A. nidulans* ver. *dentatus P. lanosum*, *P. expansum* and *P. oxalicum* gave the highest cellulase activity. Whereas, *A. flavus*, *A. raperi*, *A. acculeatus*, *A. tamarri*, *A. niger*, *A. terreus*, *A. nidulans*, *P. citrinum* and *P. simplicissimum* showed least or no enzyme activity. @JASEM

Keywords: Cellulose; cellulolytic fungi; *Trichoderma*, fungal isolates, Congo red

Cellulases are a group of hydrolytic enzymes capable of hydrolyzing the most abundant organic polymer i.e. cellulose to smaller sugar components. Cellulolytic enzymes also play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi, bacteria, actinomycetes and protozoa (Lynd et al. 2002; Dale Peciulyte 2007). However, fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular (Lynd *et al*., 2002). Cellulases have enormous potential in industries and are used in food, beverages, textile, laundry, paper and pulp industries etc (Jahangeer et al. 2005**;** Miettinen-Oinonen et al. 2004; Cavaco-Paulo and Gübitz2003; Walsh 2002). As lytic enzymes, they are of also major importance is the protoplast production (Davis, 1985; Mandels 1974; Bhat 2000) for tissue culture and plant metabolites production. The demand for more thermostable, highly active and specific cellulases is on the increase; therefore, cellulase systems of local fungi should be investigated keeping in view the importance and application of the cellulases, this study was designed to screen the native fungal isolates as hyper-producers of cellulose.

MATERIALS AND METHODS

Isolation and screening of fungi: Fungi were isolated from different sources from soil, industrial effluent, seeds fruit, vegetable, bread and wood during 2005-2010 (Table. 1). Fungi were isolated as mono-cultures on three media: malt extract agar (MEA), potato dextrose agar (PDA) and Czapek's agar (CA). Fungi were identified to the species on the basis of morphological characteristics (Domsch et al. 1980; Samson et al. 2000; Pitt 1979).

*Plate screening***:** Cellulose activities of the fungal isolates were determined by using plate screening medium (PSM) contained Mendel's mineral salt grams per litter (g/L) solution that is: Urea -0.3, $(NH_4)_2SO_4$ -1.4, KH_2PO_4- 2.0, $CaCl_2$- 0.3, $MgSO_4$ - 0.3, yeast extract- 0.25 and proteose peptone -0.75 with 10 g L-1 of carboxymethyl cellulose(CMC) and 17.5 g L-1 agar (Mandels, 1974). Agar blocks (8 mm in diameter) from one-week old fungal colony grown on MEA plates were cut and inoculated in the centre of the basal media plates. The plates were incubated at 25$\pm$ 2 ºC for seven days. Cellulolytic fungal species were selected on the basis of the diameter of the hydrolysis zone surrounding the colonies.

For observations, plates were stained with 1% Congo red dye (30 min), followed by destaining with 1 M NaCl solution for 20 min. clear zones could be observed only around colonies of the active fungal strains. Cellulose activity on carboxymethyl agar was recorded as the Index of Relative Enzyme Activity (ICMC) was recorded as clear zone ratios = clear zone diameter / colony diameter (Teather and Wood, 1982; Bradner et al. 1999; Dale Peciulyte, 2007). Growth of fungal isolates on MEA was taken as control. Growth simulation/inhibition index was computed as the colony diameter on pectin agar/colony diameter on control agar ratio.

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Table 1 Fungal isolates from different sources

Acc#	Fungal isolates	Sources
1121	*Aspergillus aculeatus* Iizuka	Guava rhizospheric soil
0231	*Aspergillus flavus* Link	*Litchi chinensis,* rhizospheric soil
0990	*Aspergillus japonicus* Saito	Shisham seed
1122	*Aspergillus nidulans* (Eidam) G. Winter	Citus rhizospheric soil
1124	*Aspergillus nidulans* var. *dentatus* D.K. Sandhu & R.S. Sandhu	Citus rhizospheric soil
0002	*Aspergillus niger* Tiegh.	Garden soil
1007	*Aspergillus raperi* Stolk & J.A. Mey.	Rhizosphere of sponge luffa
1027	*Aspergillus tamarii* Kita	Effluents of paper mill
0059	*Aspergillus terreus* Thom	Field soil
1100	*Penicillium citrinum* Sopp	Bread
0622	*Penicillium expansum* Link	*Citrus limonia* fruit
1091	*Penicillium lanosum* Westling	Onion peel
1010	*Penicillium oxalicum* Currie & Thom	Rhizosphere of lady finger
1029	*Penicillium simplicissimum* (Oudem.) Thom	Rhizosphere of guava
0946	*Trichoderma koningii* Oudem.	Citrus fruit
0944	*Trichoderma viride* Pers.	Textile effluent
0755	*Trichoderma harzianum* Rifai	Wood

RESULTS AND DISCUSSION

*Screening of fungal isolates for cellulytic activity***:** Seventeen fungal species belonging to three genera i.e. *Trichoderm*, *Aspergillus* and *Penicillium* were isolated from different sources, screened and compared for their ability to degrade cellulose. Screening of fungal isolates was performed by plate method. Among 17 fungal isolates, 8 fungal isolates were identified as cellulose producer. Most of the cellulase producers belonged to *Trichoderma* (3) and *Penicillium* (3) followed by *Aspergillus* (2). However, some isolates of *Aspergillus* (7) and *Penicillium* (2) did not show any cellulytic activity (Table 2).

Table 2. Growth and enzyme activity of Fungal isolates

Acc#	Fungal isolates	Hydrolysis zone diameter [cm]	Colony diameter on CMC agar [cm]	Hydrolysis activity index (ICMC)	Colony diameter on control agar [cm]	Growth stimulation/ inhibition index
1121	*Aspergillus aculeatus*	0.0 ± 0.0	9.0 ± 0.0	0.0 ± 0.0	6.2± 0.06	1.5± 0.0
0231	*Aspergillus flavus*	0.0± 0.0	9.0 ± 0.0	0.0 ± 0.0	7.2 ± 0.07	1.3± 0.0
0990	*Aspergillus japonicus*	8.3 ± 0.09	8.3 ± 0.09	0.99± 0.01	7.5± 0.09	1.1± 0.0
1122	*Aspergillus nidulans*	0.0 ± 0.0	9.0 ± 0.0	0.0 ± 0.0	6.5± 0.09	1.4± 0.0
1124	*Aspergillus nidulans* var.	9.0 ± 0.0	9.0 ± 0.0	1.0± 0.0	8.3± 0.09	1.1± 0.0
0002	*Aspergillus niger*	0.0 ± 0.0	9.0 ± 0.0	0.0 ± 0.0	7.6 ± 0.09	1.2± 0.0
1007	*Aspergillus raperi*	4.2 ± 0.06	4.2 ± 0.06	0.99 ±0.02	3.7± 0.09	1.1± 0.03
1027	*Aspergillus tamarii*	0.0 ± 0.0	9.0 ± 0.0	0.0 ± 0.0	6.8± 0.18	1.3± 0.0
0059	*Aspergillus terreus*	0.0 ± 0.0	9.0 ± 0.0	0.0 ± 0.0	7.3 ± 0.12	1.2± 0.0
1100	*Penicillium citrinum*	0.0 ± 0.0	3.0± 0.06	0.0 ± 0.0	1.6 ± 0.09	1.9± 0.03
0622	*Penicillium expansum*	2.7 ± 0.17	2.7 ± 0.17	1.01± 0.05	4.1 ± 0.09	0.7± 0.03
1091	*Penicillium lanosum*	2.7 ± 0.16	2.7 ± 0.16	0.99 ±0.06	2.1 ± 0.1	1.3± 0.06
1010	*Penicillium oxalicum*	9.0 ± 0.0	9.0± 0.0	1.0 ± 0.0	5.8 ± 0.05	1.5± 0.0
1029	*Penicillium simplicissimum*	0.0± 0.0	6.2± 0.09	0.0 ± 0.0	5.1 ± 0.06	1.2± 0.03
0946	*Trichoderma koningii*	9.0± 0.0	9.0± 0.0	1.0± 0.0	5.8± 0.06	1.6± 0.0
0944	*Trichoderma viride*	9.0± 0.0	9.0± 0.0	1.0± 0.0	6.8± 0.06	1.3± 0.0
0755	*Trichoderma harzianum*	9.0± 0.0	9.0± 0.0	1.0± 0.0	7.6± 0.07	1.2± 0.0

Growth simulation/inhibition index was computed as the colony diameter on carboxymethyl agar/colony diameter on control agar ratio. The index value <1, represented substrate inhibited fungal growth, while the index value >1, exhibited substrate rendered growth stimulation. In case of *Aspergillus aculeatus* and *Penicillium citrinum*, CMC medium enhanced the growth and was least effective in rest of isolates. The hydrolysis zone diameter correlated well with the colony diameter on carboxymethyl agar medium. However, hydrolysis zone diameters were not greater than colony diameter in case some isolates. Therefore, hydrolysis activity indices were found to be only slightly >1 in case of *P. expansum* but equal to 1 in case of *A. nidulans* var. *dentatus*, *P. oxalicum* and *Trichoderma* species. Subsequently, among the

isolates tested, *A. nidulans*, *A. flavus*, *A. aculeatus, A. niger*, *A. tamari, A. terreus, P. citrinum*, *P. simplicissimum* exhibited the lowest hydrolytic activity on pectin. Furthermore, the colony growth of all tested fungi on CMC agar was found to be the slightly greater as compare to control. None of the isolates were found to be resistant for CMC agar as the colony growth was greater to control (Table 2). Difference between isolates was statistically significant at $p \leq 0.05$.

Fungi are well known agents of decomposition of organic matter in common and of cellulosic substrate in particular (Lynd et al. 2002). Cellulose is world's most abundant organic substance (Ruttloff 1987) and comprises a major storage form of organic compound and major component of biomass energy (Scott et al. 1987). Because a large proportion of vegetation cellulose added to soil, decomposition of cellulose has a special significance in the biological cycle of carbon (Lederberg 1992). In industry, these enzymes have found novel application in production and processing of chemicals, food and manufactured goods such as paper, rayon etc., and extraction of valuable components from plants and improvement of nutritional values of animal feed (Wiseman 1995). Pervious studies were concentrated mainly on soil fungi (Lynd et al. 2002) for the celulitic activity. All raw materials are contaminated by microorganisms (bacteria, fungi and actinomycetes) whose viability and activity depend on the conditions to which substrates are exposed. During our study, fungal isolation was done from different sources and also from soil for the variation in fungal isolates, able to grow in the medium with the sole carbon source – cellulose. Among 17 fungal isolates only 8 fungal isolates were identified as cellulose producer. Most of the cellulase producers identified belonged to *Trichoderma* and *Penicillium* followed by *Aspergillus.* However, some isolates of *Aspergillus* and *Penicillium* did not show any cellulytic activity. Cellulose hydrolysis activity index were found to be only slightly >1 in case of *P. expansum* but equal to 1 in case of *A. nidulans* var. *dentatus*, *P. oxalicum* and *Trichoderma* species.

P. expansum is one of the important microorganisms taking part in such biodegradation processes by virtue of its ability to produce cellulolytic enzymes (Graber et al. 1965; Marsh et al. 1949; Memon et al. 1985). On the other hand, *Aspergillus* produces extensive range of enzymes capable of degrading plant cell wall polysaccharide. Different species of genus *Aspergillus,* have been identified to possess all component of cellulase enzyme system (Vries & Visser 2001) which is in agreement with the present study. The most common and most effective cellulase producers are *Trichoderma ressei, T. koningii, Fusarium* sp., *Aspergillus* and *Penicillium* sp., (Yalpani 1987). Results obtained during this study indicated that cellulase activity of tested *Aspergillus Trichoderma,* and *Penicillium* species were found relatively higher and comparable with some results of other investigators (Updegraff 2004; Kluczek and Turpeinen et al. 2005). The research covered some distribution and biochemical characteristics of the fungi adapted to cellulose containing materials.

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