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Qualitative and Quantitative Screening of Cellulases from Different Local Egyptian Fungal Strains

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ABSTRACT

Eighty four fungal cellulase-producing isolates were isolated from different environmental places in Egypt using cellulose containing medium. Qualitative screening of isolated fungi has been established by cultivating such isolates on selective medium. The promising fungal isolates from the qualitative screening were subjected quantitative screening by cultivating the potent isolates on liquid culture medium for different incubation periods (3, 6 and 9 days) at shack and static conditions. Promising activities for FP-ase, CMC-ase, β- glucosidase, Cottonase and Xylanase were found 0.65IU (isolate 4 at 6th day), 1.49 IU (isolate 4 at 9th day), 3.48IU (isolate 1 at 9th day), 2.19IU (isolate 4 at 9th day), 23.71IU (isolate 4b at 6th day), respectively. The isolates 4, 4b are defined as *Penicillium* sp., while isolate 1 was defined as *Aspergillus* sp. From this study these fungi were selected as potent cellulase–producers for future work.

Key words: cellulases, qualitative, quantitative, screening, fungi.

Introduction

Cellulases are enzymes which catalyze the hydrolytic degradation of cellulosic biomass (Sharada *et al*., 2013). These enzymes can be obtained from microorganisms grown on cheap agro-industrial materials. Cellulases are divided into three major groups: Endoglucanase, which randomly attacks the internal β-1, 4-glycosidic bonds, resulting in glucan chains of different lengths. Exoglucanases or cellebiohydrolases acts on reducing ends of the cellulose chain and releases β-cellobiose as the end product. β-Glucosidase acts specifically on the β-cellobiose disaccharides and produce glucose (Bayer *et al.,* 1994; Singh, 1999). These enzymes work together synergistically to attack cellulose. Enzymatic process to hydrolyze cellulosic materials could be accomplished through a complex reaction of these various enzymes (Lee and Koo, 2001).

Screening for microbial cellulase activity is typically performed on plates containing crystalline cellulose or microcrystalline cellulose such as Avicel in the agar. Clearing zones surrounding microbial growing colonies after incubating them for a suitable period indicating their ability for cellulases production (Kluepfel, 1988).

Soil is inhabited by a large number of microorganisms like bacteria, actinomycetes, algae and fungi. Fungi especially make a unique contribution in these changes which result in the proper maintenance of soil fertility. *Aspergillus niger* produces highly active cellulase when grown in liquid media by both surface and submerged culture methods and recently by solid state fermentation (Ikram-ul-Haq *et al*., 2005). Production of cellulase also reported by using substrates like cellulose, xylose and lactose using *T. reesei* (Muthuvelayudham *et al*., 2005). Saravanan *et al*. (2013) studied the production of cellulose using *Trichoderma reesei* in solid state fermentation. Some species of *Penicillium* i.e. *Penicillium iriensis* and *P. citriviride* produce significant quantities of cellulase, when grown under different conditions. Compost samples of two mushrooms namely Button (*Agaricus bisporus*) and Shiitake (*Lentinula edodes*) were used to isolate cellulytic fungi (Chandel *et al*., 2013). *Chaetomium sp. NIOCC 36* was found better for production of cellulase (Ravindran *et al.,* 2010). Barros *et al*., 2010 study evaluated cellulases, xylanases and beta–glucosidases produced by two fungi, the thermotolerant *Acrophialophora nainiana* and

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Ceratocystis parodoxa using submerged fermentation. Production of cellulases was also reported by Deswal *et al*., 2011 in solid state fermentation using brown rot fungus, *Fomitopsis sp. RCK 2010.*

Materials and Methods

Isolation and Identification of cellulase-producing fungi:

Cellulase-producing fungi were isolated using Shawky and Hickisch, (1984) medium of the following components (g/l): NaCl (3.0), $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (0.5), K_2HPO_4 (0.5), $MgSO_4$. $7H_2O$ (0.1), $CaCl_2$. $2H_2O$ (0.1), yeast extract (0.25), Cellulose (5.0) and agar (20.0). The pH of the medium was adjusted to 5.5. Small pieces of rotten wood, lemon peel, orange peel, infected hay, sugar-cane bagasse undergoing biodegradation, rotten corn stalks, compost, decayed organic residues, soil under decayed rice straw, rotten palm stem, waste water rich in decayed organic residues and aged rice straw were dispersed on the surface of solidified medium and incubated at 30ºC for 7-14 days until growth appeared. The fungal growth appeared were picked out and purified using plate agar streak method. Purified single colonies were maintained on potato dextrose agar (PDA) medium. Soil solution has been done and shakes for 2h and left to settle down for 15min. 200micro-liter were used to inoculate the cellulase isolating medium (Shawky and Hickisch,(1984). The single colonies appeared after incubation at 30ºC were also picked and maintained on PDA medium. The promising fungal isolates were identified according to Barnett (1972).

Qualitative screening for cellulase-producing fungi:

Fungal isolates from different sources were screened qualitatively as described by Wang *et al*. (2003) as follows: on the isolation medium. After 72hr of incubation, the plates were flooded with 1% Congo red solution and the plates were allowed to stand for 20min. at room temperature. Then the plates were thoroughly destained with 1M sodium chloride solution. A clear zone formed around the growing colonies of cellulase positive cultures against dark red backgrounds was taken as the indication of cellulase activity. The contrast was further enhanced by treating the plates with 5% acetic acid for 1 to 2 minutes and then washed off the excess acid with distilled water. The fungus exhibited good clearance beyond the areas of its growth was then selected for further studies (Shankar *et al*., 2011). Detection of cellulases activity was also performed on the culture plate using iodine solution (1% iodine crystals and 2% potassium iodide), which would in 15 min form a bluish-black complex with cellulose or CMC, demarcating a clear zone around the colonies (Sreedevi *et al*., 2013).

Quantitative screening of cellulolytic fungi:

This experiment has been constructed by cultivating cellulose positive fungal isolates that exhibited clear zone in the previous test (with iodine and Congo red) on broth culture medium previously mentioned in the isolation section (Shawky and Hickisch, 1984). The pH of the medium was adjusted to 5.0.This medium were inoculated with 10% of spore suspension (A_{600}=1) and incubated at 30ºC on a rotary shaker (150rpm) for 3, 6 and 9 days. The fungal mycelia were removed from the culture by centrifugation at 5000rpm. The fungal dry weight was also measured. The culture supernatant was used for the determination of cellulase activities of FP-ase, CMC-ase, β- glucosidase, Cotton-ase and xylanase and protein as well.

Assay of cellulolytic enzymes:

Determination of Exo β-1,4- glucanase (FP-ase):

FP-ase activity was measured according to Mandels *et al.* (1976) by mixing 1ml of 0.05M citrate buffer (pH 4.8) and 50 mg of Whatmann filter paper no.1 (strips of 1x6 cm) at 50ºC for 1 hour. Reducing sugars were measured as glucose according to the method of Miller (1959). One unit of enzyme is defined as the amount of enzyme which released 1 micromole of reducing sugars, expressed as glucose, per min. under given condition.

Determination of Endo β-1,4- glucanase (CMC-ase):

Carboxymethyl cellulase (CMC-ase) was measured according to Mandels *et al.,* 1976. Reaction mixture (1ml of enzyme solution, 1ml of 1% CMC dissolved in 0.05M sodium citrate buffer pH 4.8) was

incubated at 50ºC for 1h. The colour of reaction was developed by adding alkaline dinitrosalycilic (DNS) reagent as and the produced reducing sugars (as glucose) were measured according to Miller (1959). The absorbance was measured at 540nm against reagent blank. One unit of enzyme (CMC-ase) is defined as the amount of enzyme which released 1 micromole of reducing sugars, expressed as glucose, per min. under given condition.

β-Glucosidase assay:

β-Glucosidase or Salicin-ase enzyme activity was estimated by incubating 0.5 ml enzyme and 0.5 ml buffer (0.05 M citrate buffer pH 4.8) with 1% salicin at 50º (Mandels *et al.* 1976). Reducing sugars released in assay mixture was measured by dinitrosalicylic acid method (Miller 1959). Enzyme activity was measured in terms of international units defined as the number of micromole of reducing sugar released per min per ml of test solution (measured as glucose).

Xylanase assay:

The enzyme assay has been measured according to Bailey *et al*. (1992) as follows: 1% xylan (beech wood) was dissolved in 0.05M citare buffer (pH 4.8) and used as a substrate. 1ml of the culture supernatant (enzyme source, diluted if necessary) was added to 1ml substrate and the mixture was incubated for 15 min at 50ºC. The released reducing sugars were determined by the method of Miller (1959). Xylan-ase unit is the amount of enzyme that liberates 1µ mole of reducing sugar (measured as xylose) per min.

Cottonase activity:

For enzyme activity on cotton, 50 mg absorbent cotton, 1 ml of 0.02 mol/L sodium citrate buffer (pH 4.8) and 1 ml of the enzyme solution were mixed together into a tube. The samples were incubated for 60min at 50°C according to the method of Vallander and Eriksson (1985) and reducing sugars produced were assayed according to Miller (1959).

Protein assay:

The soluble protein in culture supernatant was measured according to Lowry *et al*. (1951).

Results and Discussion

Screening for cellulases activity:

1- Qualitative screening:

Eighty-four fungal isolates were screened for their ability to produce cellulases. 12 isolates were identified and selected for quantitative screening (Table1). Results present in Figure 1showed both positive and negative cellulase producers for plates flooded with either (a) Iodine solution or (b) Congo red. Clearing zones surrounding microbial growing colonies after incubating for a suitable period indicating their ability for cellulase production. Kasana *et al*. (2008) discovered that Gram's iodine for plate flooding in place of hexadecyltrimethyl ammonium bromide or congo red, gave a more rapid and highly apparent result. Gram iodine was also used for the screening of cellulase producing microorganisms, i.e., fungi (Shahriarinour *et al*., 2011) and bacteria (Sreedevi *et al*., 2013). However, plate-screening methods using dyes are not quantitative method for the poor correlation between enzyme activity and halo size. This problem solved by the development of short cellooligosaccharides possessing modified reducing terminal with chromogenic/fluorogenic groups e.g. fluorescein, resorufin and 4-methylumbelliferone for higher sensitivity and quantification (Fia *et al*., 2005).

2-Quantitative screening:

From the previous experiment only 12 fungal isolates exhibiting cellulase-positive were quantitatively screened. Five enzyme fractions namely: exoglucanase (FP-ase), endoglucanase (CMC-ase),

β-glucosidase (Salicin-ase), cotton-ase and xylanase were assayed. Results in Fig.2 and Fig. 3 showed cellulases activity

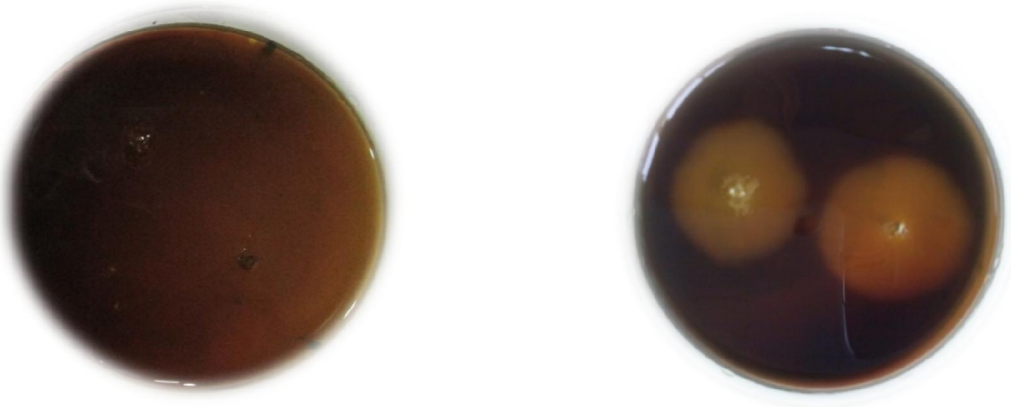


Fig. 1a: Qualitative screening for cellulase activity of isolates grown on agar plates containing cellulose. The plates were flooded with iodine solution.

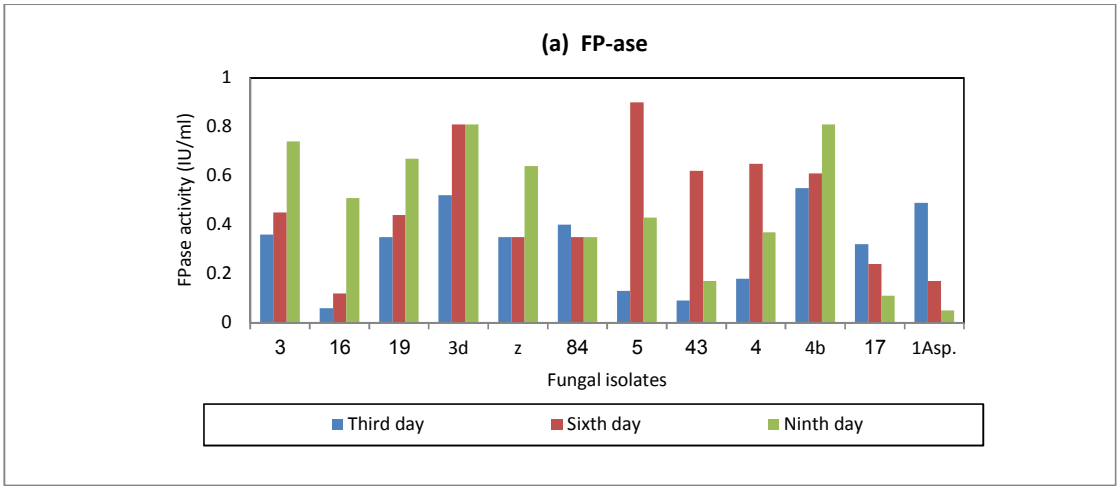
Fig. 1b: Qualitative screening for cellulase activity of isolates grown on agar plates containing cellulose. The plates were flooded with Congo red.

from the12 isolates under static and shacked culture condition. Results in the static are extremely lower than the shake case. Isolates 4, 4b and 1expressed the highest production of different cellulases as compared to the other isolates table 2.The maximum production of FP-ase enzyme was found 0.65 IU,0.61 IU and0.49 IU by isolates 4, 4b and 1 after 6 days incubation respectively, whereas, the potent production of CMC-ase enzyme was 1.49 IU, 1.32 IU and 1.04 IU by isolates 4, 4b after 9 days and isolate 1 after 3 days incubation respectively, moreover, the production of β-glucosidase (Salicinase) enzyme was optimum3.48 IU, 1.79 IU and 1.19IU by isolates 1, 4 after 9 days and isolate 4 after 3 days incubation respectively. Cottonase enzyme has been studied and the highest activity was 2.19 IU by isolate 4b after 9 days, 0.78 IU and 0.65 IU by isolates 4 and 1 after 6 days incubation respectively. Xylanase activity was 23.71 IU, 14.74 IU and 9.53 IU with isolates 4b, 1 and 4 after 6 days incubation respectively. Sri Lakshmi and Narasimha (2012) when studied the production of cellulase from four fungal cultures, three belonged to the genus *Aspergillus* and one related to the genus *Penicillium*, they found that the fungal isolate 3 (*Aspergillus* sp.) exhibited highest cellulase activity (FP-ase, 14.16U/ml; CMC-ase, 64U/ml and b-glucosidase, 0.014U/ml). On the other hand, isolates 1 and 4 (*Aspergillus* sp. and *Penicillium* sp., respectively) produced the lowest cellulase activity (FP-ase, 0.44-0.72U/ml; CMC-ase, 5.55-5,66U/ml and β-glucosidase, 0.006-0.014U/ml). Azzaz *et al*. (2012) studied the production of cellulase from *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium avenacium* and *Cephalosporium acremonium*. *A. niger* was the

potent cellulase producer giving an activity of 0.076U/ml measured as CMC-ase. Moreover, cellulase and xylanase activities were investigated from different strains of *Trichoderma* species (*Trichoderma harzianum* and *Trichderma Harzianum* Th-azad) and *Trichoderma viride* 01PP (Pandey *et al*., 2014). *T. harzianum* Th-azad produced higher cellulase and xylanase than T. *viride* 01PP. T. *harzianum* Th-azad exhibited enzyme activities.Table 2 summarizes the potent cellulase producing fungi that can be used in the next work for hydrolysis and bioethanol production.

Table 1: Identification and characterization of selected fungal isolates

	Isolate number	Source	Characterization	Organism
1	1	Peat	Conidiophores upright, simple, clavate swelling, conidia 1-celled, globose, catenulate.	*Aspergillus sp.*
2	3 d	Soil under decayed rice straw	Conidiophores upright, simple, clavate swelling, conidia 1-celled, globose, catenulate.	*Aspergillus sp.*
3	3	Sugar-cane bagasse undergoing biodegradation	Fored sparse, light, overhead mycelium with numerous olive_green ascocarps, which were distributed evenly on the colony surface..	*Chaetomium sp.*
4	4	Rotten palm stem	Conidiophores a rising the mycelium, conidia hyaline or brightly colored, 1- celled, globose or ovoid.	*Penicillium sp.*
5	4b	Rotten palm stem	Conidiophores a rising the mycelium, conidia hyaline or brightly colored, 1- celled, globose or ovoid.	*Penicillium sp.*
6	5	Rotten corn stalks	Mycelium extensive and cottony in culture, purple or yellow, Conidiophores variable, simple, short and branched.	*Fusarium sp.*
7	16	Soil rich in decayed organic residues	Mycelium extensive and cottony in culture, purple or yellow, Conidiophores variable, simple, short and branched.	*Fusarium sp.*
8	17	Humus	Mycelium extensive and cottony in culture, purple or yellow, Conidiophores variable, simple, short and branched.	*Fusarium sp.*
9	Z	Waste water rich in decayed organic residues	Mycelium extensive and cottony in culture, purple or yellow, Conidiophores variable, simple, short and branched.	*Fusarium sp.*
10	19	Infected hay	Conidiophores upright, hyaline, branched, phialides single and grouped, 1-celled ovoid.	*Trichoderma* sp.
11	43	Decayed organic residues	Conidiophores upright, hyaline, branched, phialides single and grouped, 1-celled ovoid.	*Trichoderma sp.*
12	84	Compost	Syn. = *Aspergillus* sp.	*Emericella sp.*



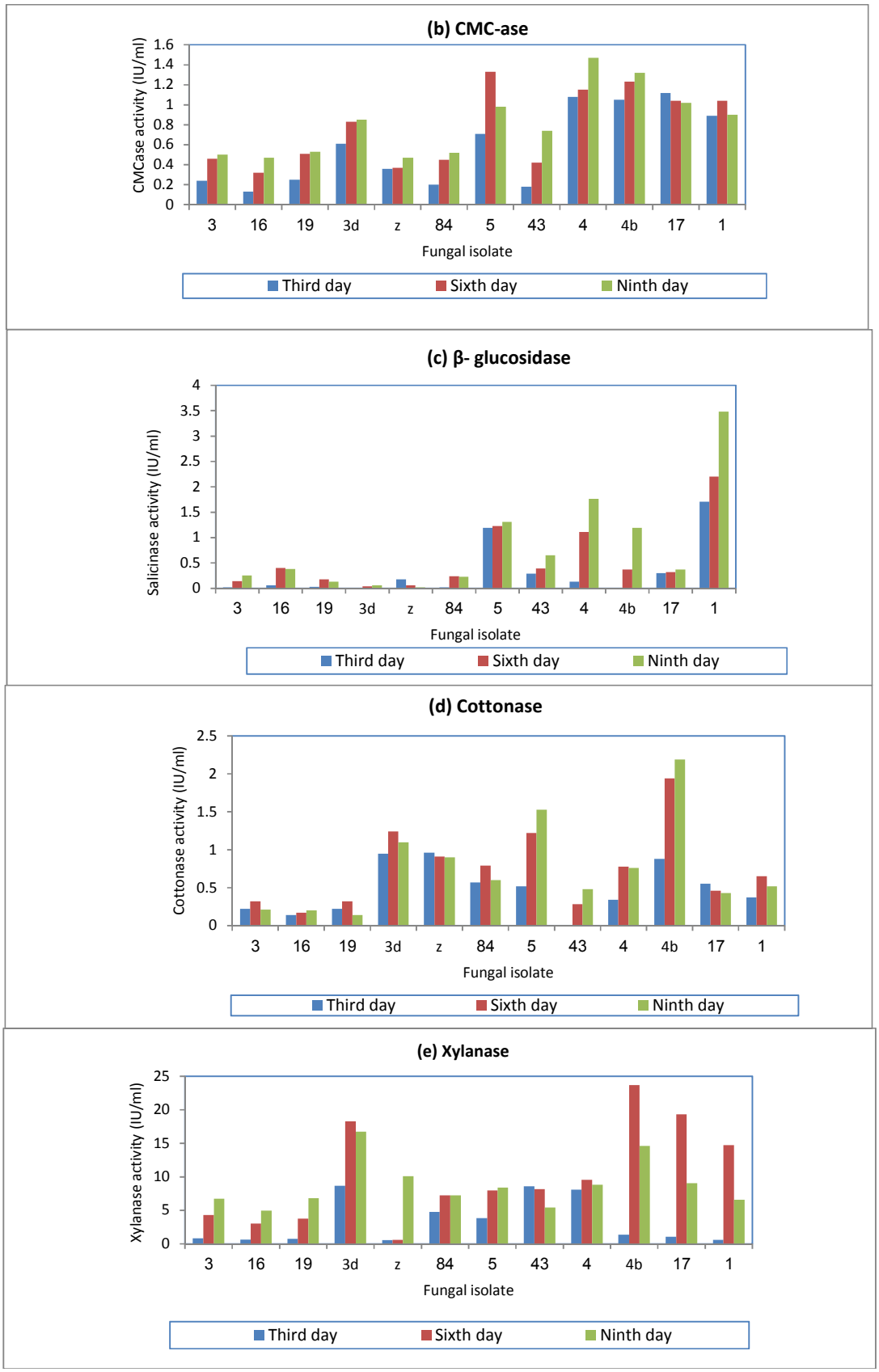
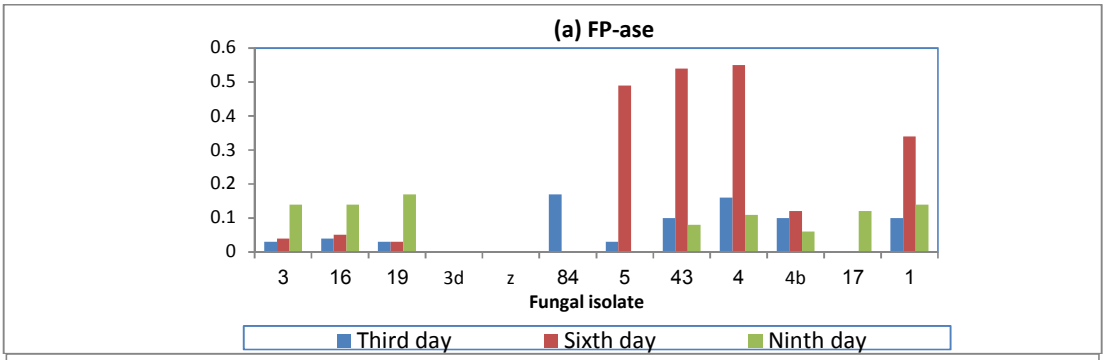
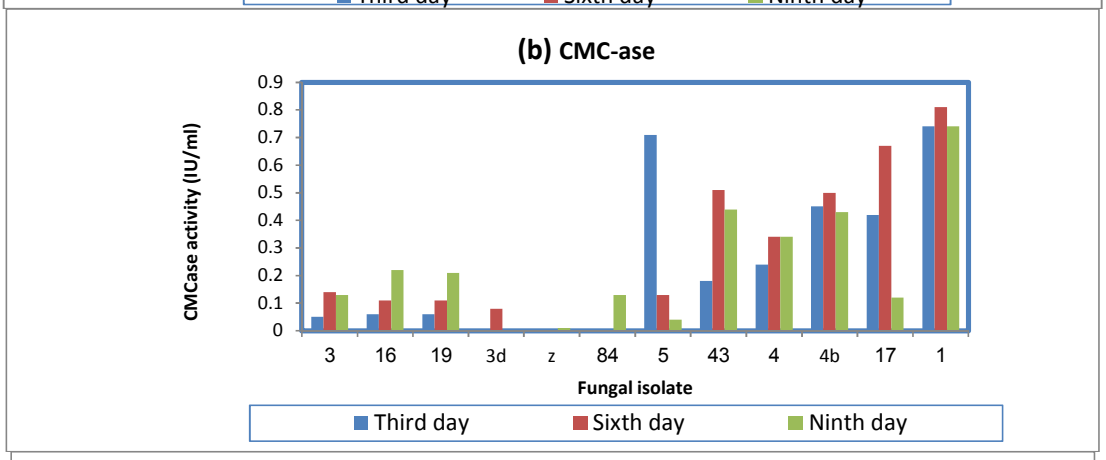
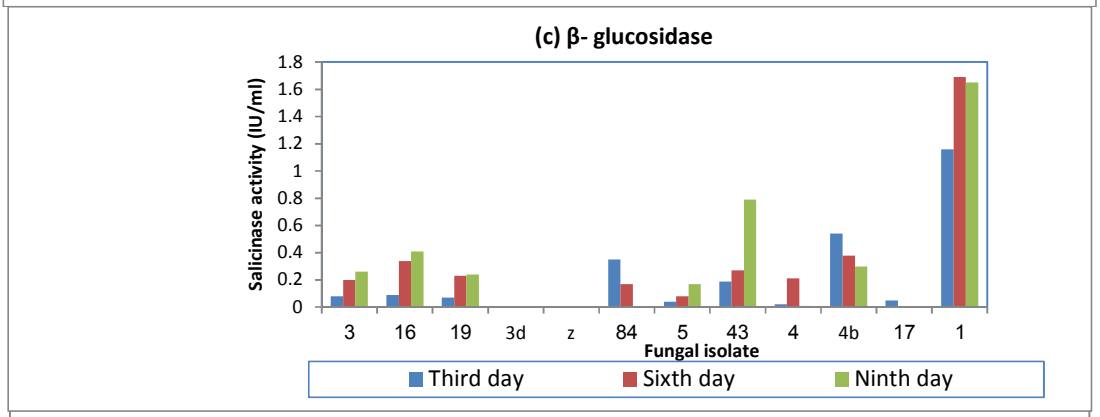
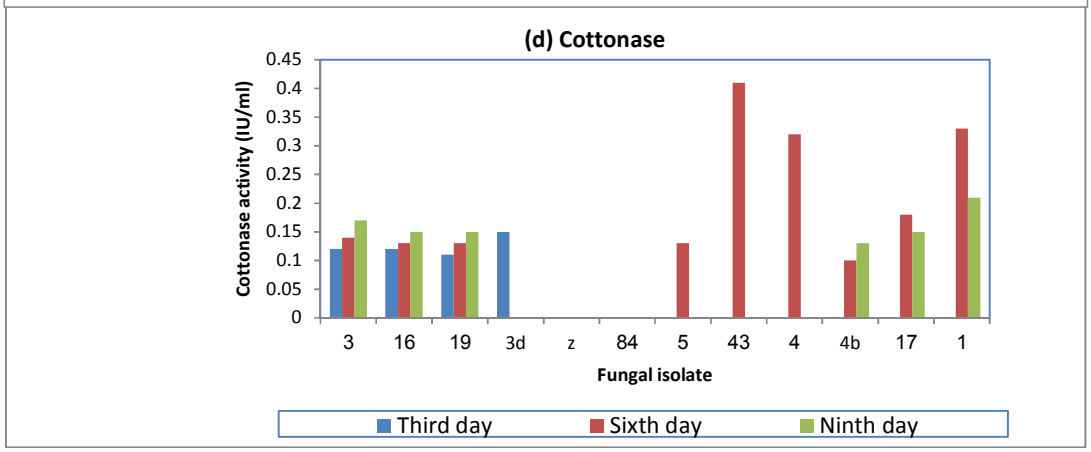


Fig. 2: (a-e): Cellulases and xylanase enzyme production from different fungal isolates under shake culture conditions.


(a) FP-ase
0.6
0.5
0.4
0.3
0.2
0.1
0
3 16 19 3d z 84 5 43 4 4b 17 1
Fungal isolate
Third day
Sixth day
Ninth day


(b) CMC-ase
CMCase activity (IU/ml)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
3 16 19 3d z 84 5 43 4 4b 17 1
Fungal isolate
Third day
Sixth day
Ninth day


(c) β- glucosidase
Salicinase activity (IU/ml)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
3 16 19 3d z 84 5 43 4 4b 17 1
Fungal isolate
Third day
Sixth day
Ninth day


(d) Cottonase
Cottonase activity (IU/ml)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
3 16 19 3d z 84 5 43 4 4b 17 1
Fungal isolate
Third day
Sixth day
Ninth day

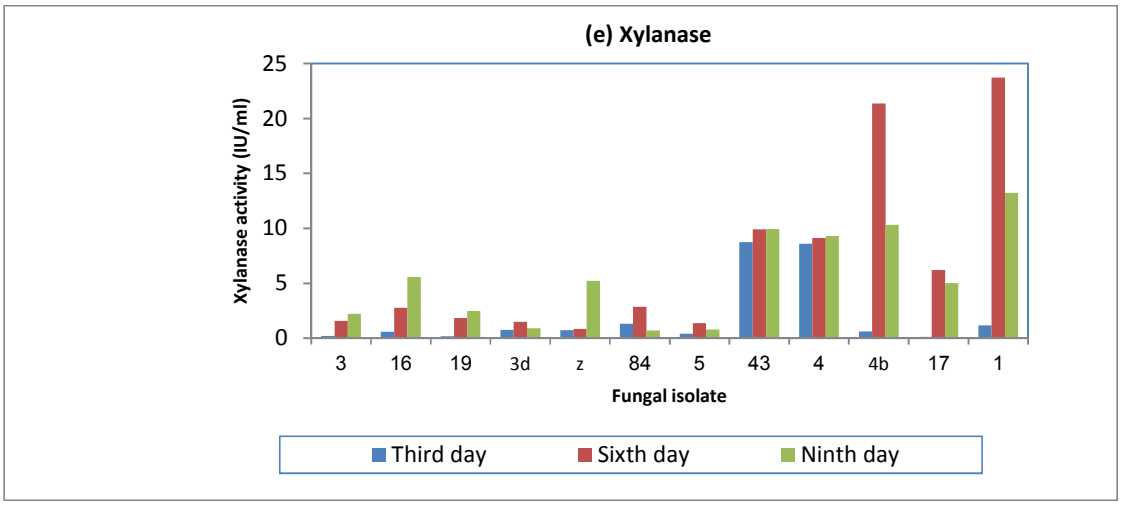


Fig. 3(a-e): Cellulases and xylanase enzyme production from different fungal isolates under static culture conditions.

Table 2: Summarizing of potent cellulase-producing fungi.

Isolate no.	Incubation period	Final pH	Dry weight (g/50ml	Protein (mg/ml)	Enzyme activity (U/ml)				
					FP-ase	CMC-ase	Salicinase	Cottonase	Xylanase
4	3	3.0	0.61	1.05	0.18±0.037	1.08±0.031	0.13±0.024	0.34±0.127	8.10±0.359
	6	3.3	0.85	1.08	0.65±0.044	1.15±0.057	1.11±0.608	0.78±0.049	9.53±0.850
	9	3.1	0.99	1.05	0.37±0.008	1.47±0.040	1.76±0.010	0.76±0.022	8.83±0.140
4b	3	2.8	0.79	1.00	0.55±0.040	1.05±0.068	0.34±0.053	0.88±0.360	1.39±0.056
	6	2.9	0.62	1.05	0.61±0.126	1.23±0.029	0.37±0.017	0.94±0.228	23.71±0.000
	9	3.4	0.50	1.39	0.81±0.124	1.32±0.010	1.19±0.066	2.19±0.139	14.63±0.542
1	3	3.1	0.52	0.99	0.49±0.330	0.89±0.044	1.71±0.038	0.37±0.080	0.63±0.114
	6	3.3	0.96	1.02	0.17±0.029	1.04±0.029	2.20±0.172	0.65±0.086	14.74±0.602
	9	3.9	0.74	1.23	0.05±0.011	0.90±0.037	3.48±0180	0.52±0.088	6.58±0.298

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