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Isolation, screening and selection of xylanase producing thermophilic fungi from Raipur

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In the present study, isolation, screening and selection of xylanase producing thermophilic fungi from soil of Raipur was studied. A total of 55 fungal isolates were isolated and screened for xylanase activity by standard plate assay method and activity of xylanase was determined by estimating the amount of released reducing sugar xylose in culture filtrates. Based on screening methods, 18 isolates with higher xylanase activity were selected for studying effect of temperature i.e. 25, 35, 45, 50, 55, 60, 65^{o}C on their mycelia biomass production and for studying percent coagulation of protein at 50^{o}C at different intervals of time. Five isolates namely *Chrysosporium tropicum* NFCCI 2531, *Malbranchea cinnamomea* MTCC 11894, *Aspergillus fumigates* NFCCI 2532, *Aspergillus terrus* NFCCI 2533 and *Emericella nidulans* NFCCI2538 showed maximum biomass production at 45^{o}C (0.19 to 0.32g/100ml/10days) and minimum protein coagulation at 50^{o}C (14% to 25%).

Keywords: Thermophilic fungi, screening, xylanase, temperature biomass, protein coagulation, reducing sugar, xylose.

Thermophilic fungi are those micro-organisms that survive at high temperatures. These are associated with piles of agricultural and forestry products and accumulation of organic matter where warm humid and aerobic environment provides the basic conditions for their development (Maheshwari *et al*., 2000). Among various eukaryotic organisms, only some species of fungi have the ability to thrive at temperatures between 45 and 55°C. These fungi were known as thermophilic and thermotolerant forms and were distinguished on the basis of their minimum and maximum temperature of growth.

Thermophilic fungi are generally found in soil, composts, stored grains, bird's nest, wood chips, municipal waste compost system and plant degrading materials. Thermophiles provide a major advantage for industrial and biotechnological processes because of their efficiency at higher temperature. Enzymes from thermophiles are generally more stable as compared to mesophiles as it can catalyze all the reactions at high temperature. In compost, thermophilic fungi have the ability to produce a variety of cell wall degrading enzymes (Sharma, 1989) like cellulases, hemicellulases and many other important hydrolases. Thermophilic fungi can also degrade polysaccharide constituents of biomass.

The thermophilic fungi now attracting more attention as sources of thermostable xylanases for applications in various fields like making of paper pulps, processing of agricultural food raw materials and for conversion of hemicellulose present in lignocellulosic bio-mass into useful products. Research on those microorganisms that utilizes xylan and on the enzyme systems

involved is becoming more and more significant in ecological and economic terms (Polizeli*et al*., 2005). Xylans are the second most important natural poly-saccharide material after cellulose (Collins *et al*., 2005). These compounds are generally found in the cell wall between lignin and cellulose i.e. at the middle lamella of plant cells.

Xylanase, a hemicellulolytic enzymes is generally required for the hydrolyisis of β 1, 4-xylans present in lignocellulosic biomass (Kheng and Omar, 2005). Xylanases are those microbial enzymes that have created more interest nowadays due to their potential application in many industrial sectors like hydrolysates production from agroindustrial wastes (Kheng and Omar, 2005; Gessesse and Gashe, 1997), lignocellulosic feed stuff improvement (Wallace, 2001), juices and wines clarification (Gable and Zacchi, 2002) and craft pulp biobleaching in paper industry.

In recent years, a lot of effort has been put into the isolation of thermophilic and even extremophilic microorganisms, since they produce very stable enzymes (Lasa and Berenguer, 1993; Maheshwari *et al*., 2000; Bruins *et al*., 2001;Monti*et al*., 2003). Generally, it was found that the level of xylanase in fungal culture is much higher as compared to yeasts or bacteria (Singh *et al*., 2003). The main required properties of xylanases for use in the food industry are high thermostability and optimum activity at an acid pH, in paper industry thermostablility and activity at alkaline pH (Polizeli *et al*., 2005). Due to increase of application of xylanases in various fields in the last few decades demands discovery of new sources which are capable of producing specific xylanase. Thus, keepingthis objective in mind the present work has been planned and designed to investigate the thermophilic fungi with high xylanase activity.

Materials and methods

Collection of samples - The soil samples were collected from the various localities in and around Raipur city. The samples were mainly from Govt. Nagarjun P.G. Science College Botanical Garden, SOS in Life Science garden, dumped soil around canteen of Govt. Nagarjun P.G. Science College, dumped soil around Ravishankar University canteen, garbage soil from (Din Dayal Upadhyay Nagar, Mahoba Bazaar, colonies of Vivekanand Aashram), stable manure (from domestic animal dung farms), compost soil from various municipal wastes of Raipur city.

Isolation of fungi - Isolation of fungi were done from the collected soil samples in Potato dextrose agar medium by using two methods, the dilution plate method of (Waksman, 1922) and direct plate method of (Warcup, 1950).

Identification of fungi - Purified cultures was identified under compound microscope with available literature of Cooney and Emerson (1964). They were further identified and accessed in National Fungal Culture Collection of India, Agharkar Research Institute, Pune. Molecular identification of selected fungal isolate was obtained from Microbial Type Culture Collection of India, Chandigarh.

Screening of fungi for xylanase activity by plate assay and quantitative DNS method- Isolated fungi were primarily screened by plate assay on xylan agar medium containing g/l: K_2HPO_4 2.0, KH_2PO_4 2.0, NH_4NO_3 2.0, $MgSO_4$ 0.6, $CaCl_2$ 0.5, Oat spelt xylan 5, Agar 15 as described by Muthezhilan *et al*.(2007). Secondary screening was done in wheat bran broth medium as described by Kar *et al*. (2006).

Xylanase assay: Xylanase activity was assayed using 1% oat spelt xylan substrate (Sigma chemical). Xylan was dissolved in 50mM phosphate buffer (pH 6.5) using stirrer. The reaction mixture composed of 0.5ml oat spelt xylan and 0.5ml crude enzyme, which was incubated in an incubator for 30 min at 50°C. The enzyme was assayed by calculating the amount of released reducing sugar xylose using 3, 5-

dinitrosalicylic acid method (DNS method) (Millers, 1959). One unit of xylanase activity means 1µmol of reducing sugars (xylose) released in 1 min under the above conditions. Protein content of the crude culture filtrate was estimated by method of Lowry *et al.* (1951) with BSA (bovine serum albumin, Himedia, India) standard protein.

Table 1.Screening of different fungi for xylanase activity

S.No.	Culture code	Fungal isolates	Sources	Zone of clearance	Xylanase Activity µmol xylose/min/ml (Mean±SE)
1.	F1	*Phoma*sp.1	Compost soil	++	0.08±0.003
2.	F2	NI*		+	0.01±0.003
3.	F3	*Chrysosporium tropicum* NFCCI 2531		++	0.20±0.003
4.	F4	*Thelavia* sp.		++	0.08±0.005
5.	F5	*Penicillium*sp.1		++	0.08±0.001
6.	F6	NI*		+	0.02±0.002
7.	F7	*Malbranchea cinnamomea* MTCC 11894		++	0.20±0.009
8.	F8	NI*		+	0.01±0.004
9.	F9	NI*		_	Nil
10.	F10	*Aspergillus fumigates* NFCCI 2532		++	0.19±0.008
11.	F11	NI*	GNPG, botanical garden Soil	_	Nil
12.	F12	NI*		+	0.01±0.005
13.	F13	NI*	Stable Manure	+	0.01±0.001
14.	F14	NI*		+	0.01±0.003
15.	F15	*Aspergillus ustus*		++	0.10±0.007
16.	F16	NI*		+	0.02±0.000
17.	F17	*Aspergillus terrus* NFCCI 2533		++	0.19±0.007
18.	F18	NI*		+	0.01±0.001
19.	F19	NI*		+	0.01±0.003
20.	F20	*Phoma*sp.2		++	0.04±0.004
21.	F21	NI*		+	0.01±0.004
22.	F22	NI*	SOS Garden	+	0.005±0.002
23.	F23	NI*		+	0.01±0.006
24.	F24	*Aspergillus flavus* NFCCI 2534		++	0.03±0.000
25.	F25	NI*		+	0.02±0.003
26.	F26	*Aspergillus terrus* NFCCI 2535	Compost soil	++	0.09±0.003
27.	F27	*Chaetomium* sp.	SOS Garden	++	0.04±0.003
28.	F28	NI*	SOS Garden	+	0.02±0.003
29.	F29	*Penicillium*sp.2		++	0.06±0.003
30.	F30	NI*		+	0.008±0.003
31.	F31	NI*		+	0.004±0.008
32.	F32	NI*	Garbage soil (Mahoba bazaar)	+	0.01±0.001
33.	F33	NI*		+	0.02±0.004
34.	F34	NI*		+	0.003±0.001
35.	F35	NI*		+	0.01±0.003
36.	F36	NI*		+	0.01±0.005
37.	F37	NI*		+	0.01±0.003
38.	F38	*Aspergilliu sterrus* NFCCI 2536	Dumped soil (Canteen of	++	0.09±0.005
39.	F39	NI*		_	Nil

40.	F40	NI*	GNPG)	+	0.007±0.001
41.	F41	NI*		+	0.02±0.004
42.	F42	NI*	Garbage soil (DD nagar)	+	0.01±0.005
43.	F43	NI*		+	0.01±0.006
44.	F44	NI*		+	0.001±0.002
45.	F45	NI*		+	0.01±0.001
46.	F46	NI*		+	0.01±0.003
47.	F47	NI*		+	0.002±0.008
48.	F48	NI*	Dumped soil (Canteen of RSU)	+	0.009±0.004
49.	F49	NI*		_	Nil
50.	F50	*Aspergillus nidulans*		++	0.07±0.011
51.	F51	*Emericella nidulans* NFCCI 2537	Garbage soil (DD nagar)	++	0.11±0.003
52.	F52	*Emericella nidulans* NFCCI2538		++	0.20±0.001
53.	F53	*Penicillium* sp. NFCCI 2539	Garbage soil (Vivekanand Aashram)	_	0.03±0.007
54.	F54	NI*		_	Nil
55.	F55	NI*		+	0.002±0.000

NI*= Unidentified fungal cultures, + = Strain showing xylanase activity, — = Strain not showing xylanase activity (Zone of clearance + = 1-5mm; ++ = ≥ 5mm) ;32 isolates= Lower activity (0.001 to 0.02µmol xylose/min/ml) , 18 isolates= Higher activity (0.03 to 0.20µmol xylose/min/ml)

Effect of temperature on biomass production of different fungal isolates - All the fungal isolates were grown in 100ml of YpSs broth medium in 250ml Erlenmeyer flasks and incubated at different temperatures 25, 35, 45, 50, 55, 60 and 65^{o}C for 10 days. After incubation, the flask content was filtered through Buchner funnel in a filter paper, dried (60^{o}C, 24 h) in an incubator and weighed. Biomass was determined by subtracting the initial weight of filter paper from the weight of the filter paper along with mycelial biomass.

Coagulation of protein at 50^{o}C at different intervals of time- Protein coagulation was done according to the method of Koffler (1957) for assessing the thermostability of protein. 1.0g of fresh mycelial biomass was homogenized in 10.0ml normal saline in ice cold conditions, sonicated in sonicator for 5.0 min with 20 kHz frequency followed by centrifugation (5000rpm, 15min). Supernatant was collected and equal aliquots were incubated at 50^{o}C for protein coagulation for 2, 4, 6, 8 and 10 mins. After incubation, coagulated protein was centrifuged (5000rpm, 15min). The pellet so obtained was washed with ethanol: ether (3:1), dried and dissolved in 1ml of 1N NaOH. 1.0ml of sample was taken and protein content was determined (Lowry *et al*., 1951). The percent coagulation was calculated by calculating the amount of coagulated protein from the total soluble protein.

Results and Discussion

Isolation and screening of xylanolytic fungi– In the present work, a total of 55 fungal isolates were isolated from different soil samples and screened for xylanase activity (Table 1). Xylan agar plate test showing zone of clearance by some fungi was given in Fig 1. In primary screening, 17 isolates showed higher activity with ≥5mm zone whereas other isolates showed 1-5mm zone. In secondary screening,18 isolates showed higher activity (0.03±0.007 to 0.20±0.009 U/ml) whereas other isolates showed lower activity (0.001±0.000 to 0.02±0.002U/ml) (Table 1).

Zone of clearance ranging from 34 to 48mm was recorded in 16 fungal isolates (Gupta *et al*., 2009). Muthezhilan *et al*. (2007) selected *Peniciliu moxalicum* as xylanase producer based on plate assay method with 8-9 mm zone. Kar *et al*. (2006) identified *Trichoderma ressei* based on plate assay method. Sridevi and Charya (2011) recorded 30 fungal species as xylanase producer based on zone formation. Tallapragada *et al*. (2011) confirmed *Aspergillus niger* as xylanase enzyme

producer by Congo red test after growing on oat spelt xylan agar plates. Twenty eight fungal isolates were screened and confirmed as producers of extracellular cellulases based on their ability to grow and form clear zones around fungal colonies in 2% carboxymethyl cellulose (Naveenkumar and Thippeswamy, 2013).

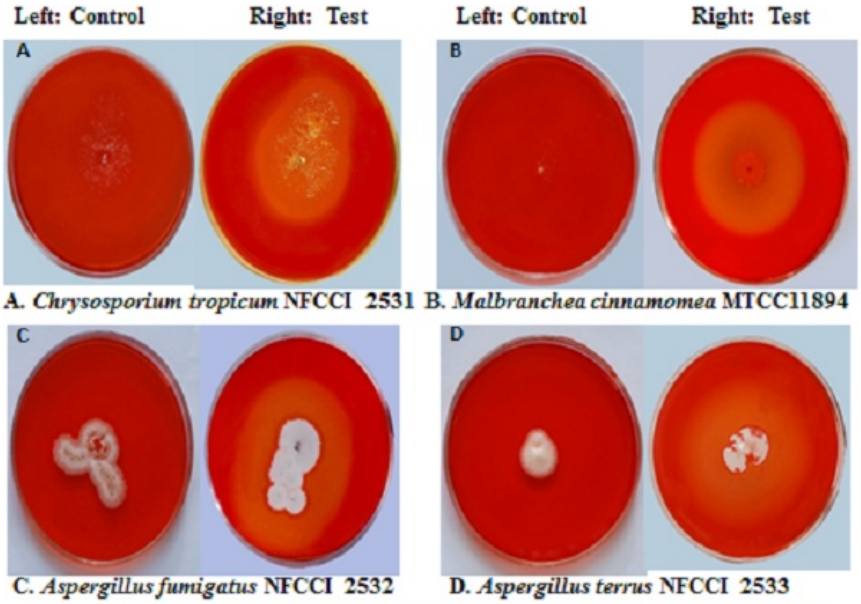

Fig 1 Xylan agar plate test for the presence of extracellularxylanase

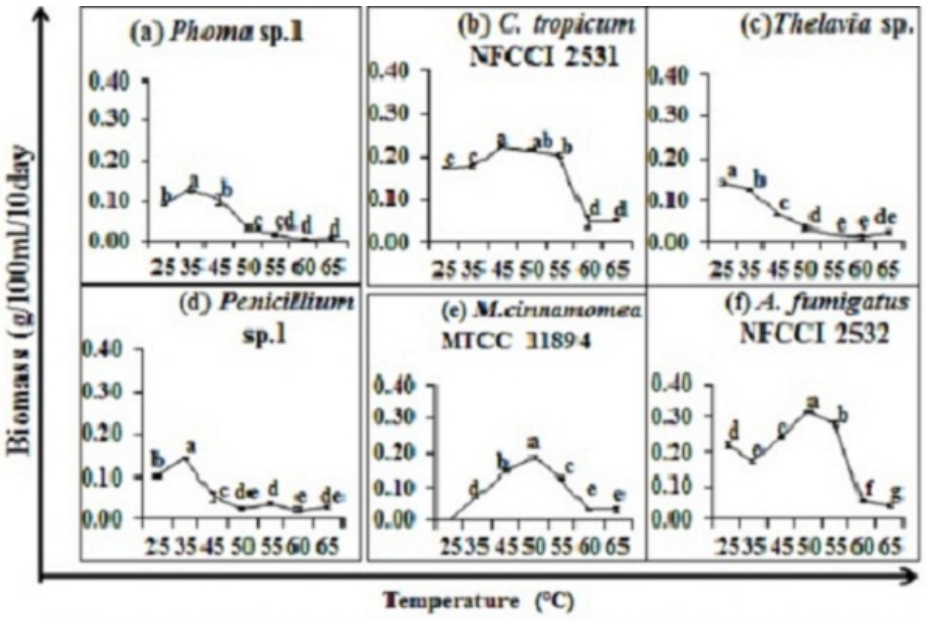

Fig 2(a-f) Effect of different temperatures on biomass production

Means having similar alphabets as superscripts are not statistically significant from each other at $p<0.05$ (Based on Duncan's Multiple Range Test)

In the present study, *Penicillium* sp. NFCCI 2539 (Table 1, Culture code F53) showed positive xylanase activity in quantitative DNS assay which showed negative activity in plate assay method. The present study thus indicate that, there is no relationship between the zone of the clearance and enzyme production. Tseng *et al*. (2000) have found that, some of the strains previously identified as potential-

enzyme producing microbes on solid screening methods, did not produce any enzyme in liquid broth. In contrast, some strains, which were identified as negative, were shown to produce high amounts of enzyme (Teather and Wood, 1982; Tseng *et al*., 2000).

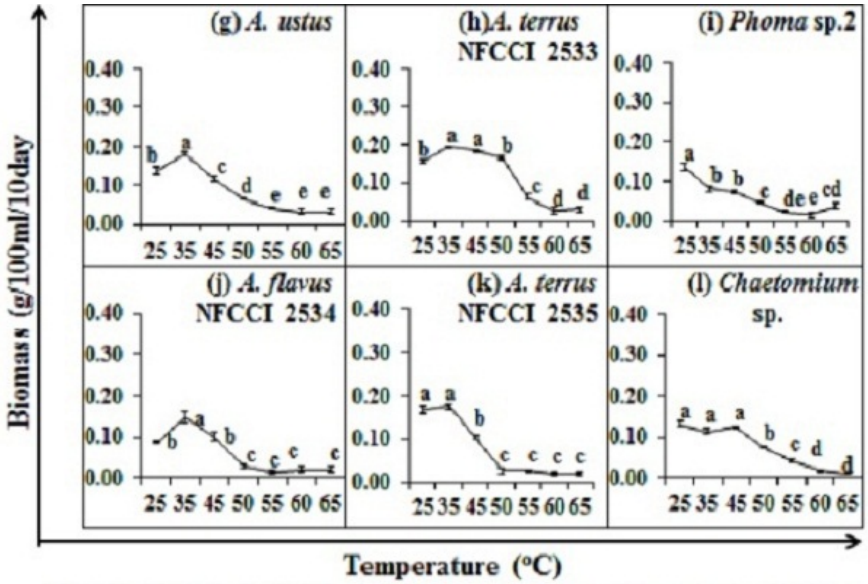

Fig 2(g-l) Effect of different temperatures on biomass production
Means having similar alphabets as superscripts are not statistically significan from each other at $p<0.05$ (Based on Duncan's Multiple Range Test)

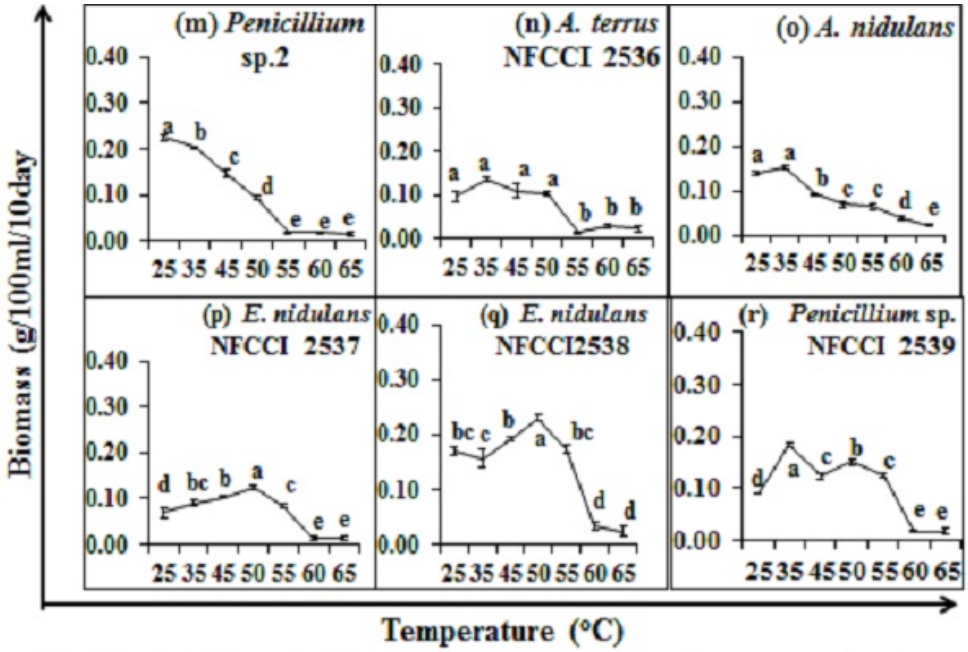

Fig 2(m-r) Effect of different temperatures on biomass production
Means having similar alphabets as superscripts are not statistically significant from each other at $p<0.05$ (Based on Duncan's Multiple Range Test)

Effect of temperature on biomass production of different fungal isolates- Fig 2 (a-f), Fig 2 (g-l), Fig 2 (m-r) show results of effect of temperature on biomass production based on Duncan's multiple range tests. Table 2 represents results of one

way ANOVA. Results indicate that the temperature produced significant effect on biomass production of different fungal isolates (Table 2). Different isolates showed different level of biomass at different temperatures (Fig 2(a-f); (g-l); (m-r)). However, *Malbranchea cinnamomea* MTCC 11894, *Chrysosporium tropicum* NFCCI 2531, *Aspergillus fumigatus* NFCCI 2532, *Aspergillus terrus* NFCCI 2533 and *Emericella nidulans* NFCCI2538 were found to show higher biomass at 45°C and 50°C, respectively. Similar temperature biomass studies were also reported by others in *A. fumigatus, A. nidulans and A. sparsus* at 25, 40, 50 and 60°C (Nazir and Nasim, 2008) and in *Aspergillus japonicas* 40°C (Jaya-prakash and Ebenezer, 2010). Moretti *et al.*(2012) isolated twenty-seven thermophilic and thermotolerant fungal strains from soil, sugarcane piles and decaying organic matter on the basis of their ability to grow at 45°C.

Table 2 One way ANOVA summary for effect of different temperatures on biomass production of different fungal isolates

S.No.	FungalIsolate	df	F	p
1	Phoma	6,14	66.76	p<0.001
2	*Chrysosporium tropicum NFCCI 2531*	6,14	165.55	p<0.001
3	*Thelavia sp.*	6,14	201.04	p<0.001
4	*Penicillium sp.1*	6,14	64.51	p<0.001
5	*Malbranchea cinnamomea*	6,14	166.67	p<0.001
6	*Aspergillus fumigatus NFCCI 2532*	6,14	449.17	p<0.001
7	*Aspergillus ustus*	6,14	82.45	p<0.001
8	*Aspergillus terrus NFCCI 2533*	6,14	157.58	p<0.001
9	*Phoma sp.2*	6,14	51.97	p<0.001
10	*Aspergillus flavus NFCCI 2534*	6,14	50.35	p<0.001
11	*Aspergillus terrus NFCCI 2535*	6,14	155.28	p<0.001
12	*Chaetomium sp.*	6,14	65.96	p<0.001
13	*Penicillium sp.2*	6,14	330.13	p<0.001
14	*Aspergilliu sterrus NFCCI 2536*	6,14	13.38	p<0.001
15	*Aspergillus nidulans*	6,14	92.81	p<0.001
16	*Emericella nidulans NFCCI 2537*	6,14	68.19	p<0.001
17	*Emericella nidulans NFCCI2538*	6,14	75.72	p<0.001
18	*Penicillium sp. NFCCI 2539*	6,14	183.91	p<0.001

Coagulation of protein at 50°C at different intervals of time –A gradual increase in percent coagulation of proteins was recorded in all the isolates with gradual increase of time of incubation at 50°C (Table 3 and Fig 3(a-f); (g-l); (m-r)). Among 18 fungal isolates, six fungal isolates namely *Chrysosporium tropicum* NFCCI 2531, *Malbranchea cinnamomea* MTCC 11894, *Aspergillus fumigatus* NFCCI 2532, *Aspergillus ustus*, *Aspergillus terrus* NFCCI 2533 and *Emericella nidulans* NFCCI2538 exhibited lower percent coagulation of protein (13.89±0.55 to 35.55±0.20) at the end of 10min incubation at 50°C. Remaining twelve fungal isolates showed higher percent coagulation (42.35±0.68 to 58.27±0.39). The essential cell components of thermophiles are found to be comparatively more stable than mesophiles. This may be due to the presence of protective factors or the absence of labilizing materials in the external and internal environment of the cell (Koffler, 1957). Gaughran (1947) found that the lipid content of a mesophile decreases with increase in temperature if it grows above its optimum temperature and that the lipid becomes more saturated. On the other hand, lipid of a thermophile remains comparatively constant in quantity and degree of saturation.

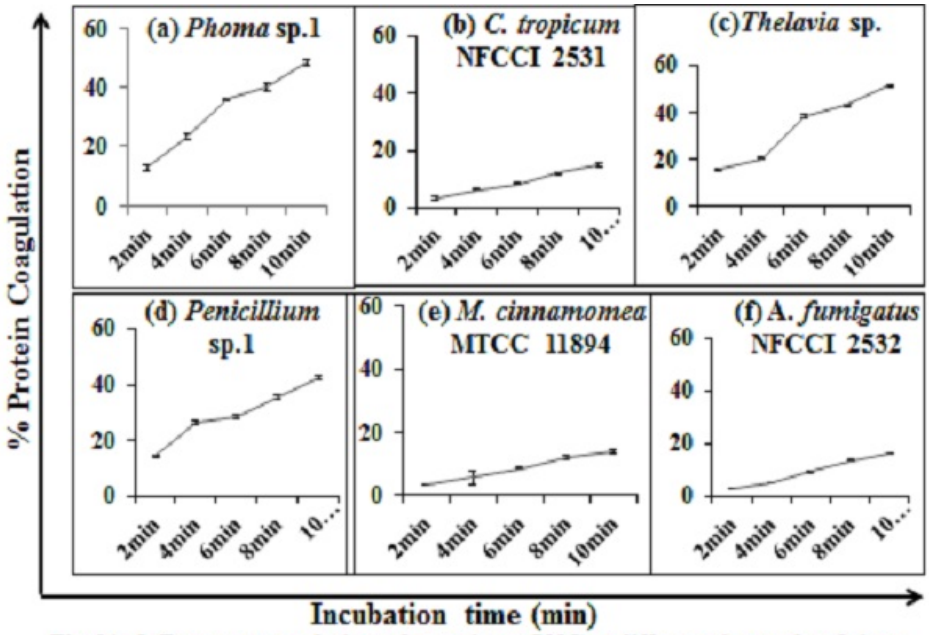

Fig 3(a-f) Percent coagulation of protein at 50°C at different intervals of time

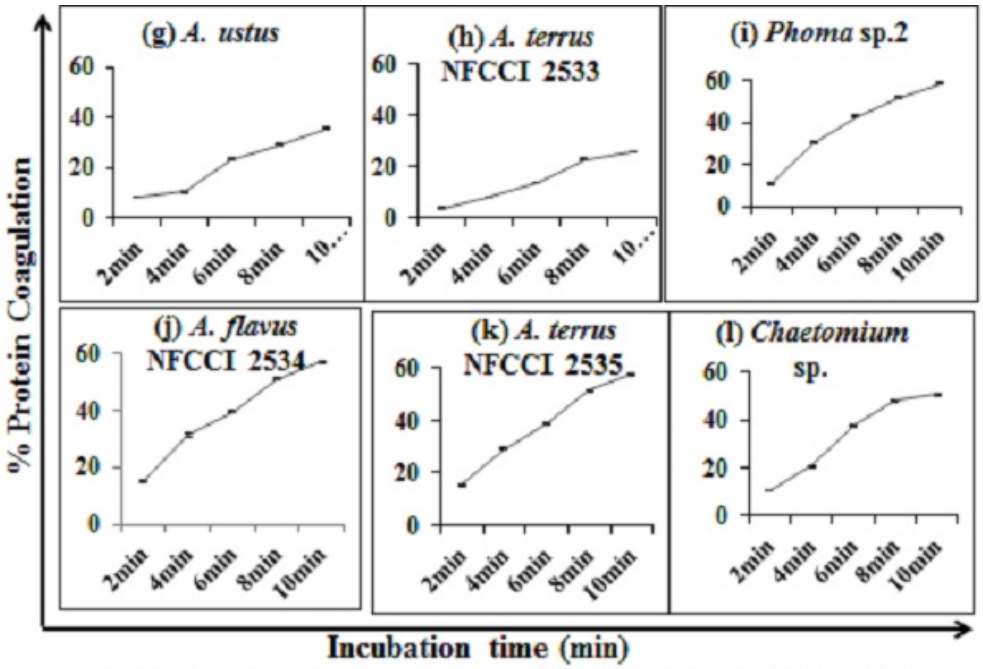

Fig 3 (g-l) Percent coagulation of protein at 50°C at different intervals of time

In microorganisms, it was found that, the enzyme or protein production is always associated with the growth phase (Tlecuitl-Beristain *et al*., 2008). From the Fig 2(a-r) and Fig 3 (a-r), it was cleared that all the fungal isolates which show higher mycelia biomass or growth at 45°C did not produce thermostable protein or enzyme. In the present study, *Aspergillus ustus* did not show higher biomass at 45°C but produced thermostable protein with minimum percent coagulation (Fig 2 (g-l)(g) and Fig 3 (g-l) (g)). Similarly, *Emericella nidulans* NFCCI 2537 showed higher biomass at 45°C but did not produced thermostable protein (Fig 2 (m-r) (p) and Fig 3 (m-r) (p)).

Table 3: Percent coagulation of protein at 50°C at different intervals of time

	Fungal isolates	Total protein (mg)	Percent coagulation of protein at 50°C*				
			2min	4min	6min	8min	10min
1	*Phoma sp.1*	0.481	12.82 ± 0.76	23.04±0.96	35.86±0.30	39.85±1.25	48.33±0.79
2	*Chrysosporium tropicum NFCCI 2531*	0.750	3.00 ± 0.70	6.22±0.44	8.26±0.15	12.00±0.19	14.89±0.48
3	*Thelavia sp.*	0.550	15.45 ± 0.69	20.15±0.55	38.49±0.66	42.91±0.77	51.21±0.66
4	*Penicillium sp.1*	0.425	14.35±0.36	26.27±0.52	28.63±0.52	35.49±0.71	42.35±0.68
5	*Malbranchea cinnamomea*	0.546	3.47±0.11	5.49±2.11	8.42±0.38	11.93±0.48	13.89±0.55
6	*Aspergillus fumigatus NFCCI 2532*	0.490	2.79±0.07	4.96±0.07	9.38±0.41	13.40±0.38	16.15±0.17
7	*Aspergillus ustus*	0.825	8.36±0.07	10.83±0.42	23.33±0.46	28.99±0.44	35.55±0.20
8	*Aspergillus terrus NFCCI 2533*	0.575	3.71±0.29	8.11±0.15	13.67±0.12	23.01±0.21	25.73±0.20
9	*Phoma sp.2*	0.765	11.22±0.48	30.28±0.58	42.81±0.19	51.63±0.38	58.27±0.39
10	*Aspergillus flavus NFCCI 2534*	0.736	15.39±0.30	31.50±0.76	39.63±0.41	50.95±0.39	57.19±0.30
11	*Aspergillus terrus NFCCI 2535*	0.790	15.39±0.56	29.22±0.46	38.81±0.46	51.37±0.46	57.41±0.48
12	*Chaetomium sp.*	0.583	10.72±0.25	20.58±0.49	37.88±0.38	48.03±0.50	50.45±0.62
13	*Penicillium sp.2*	0.568	12.76±0.26	23.04±1.73	39.03±0.39	49.89±0.78	55.75±0.39
14	*Aspergillius terrus NFCCI 2536*	0.555	15.01±0.40	23.57±0.65	37.83±0.78	41.59±0.40	57.05±0.39
15	*Aspergillus nidulans*	0.960	12.08±0.22	15.10±0.30	24.39±0.32	37.93±0.46	44.79±0.30
16	*Emericella nidulans NFCCI 2537*	0.702	10.01±0.53	11.99±0.83	27.66±0.52	40.12±0.52	48.91±0.72
17	*Emericella nidulans NFCCI2538*	0.460	5.43±0.63	9.23±0.31	13.04±0.83	17.57±0.79	24.27±0.96
18	*Penicillium sp. NFCCI 2539*	0.597	14.09±0.37	28.05±0.64	39.22±0.37	45.36±0.37	57.31±0.61

***Average of three replications, Mean** ± SE

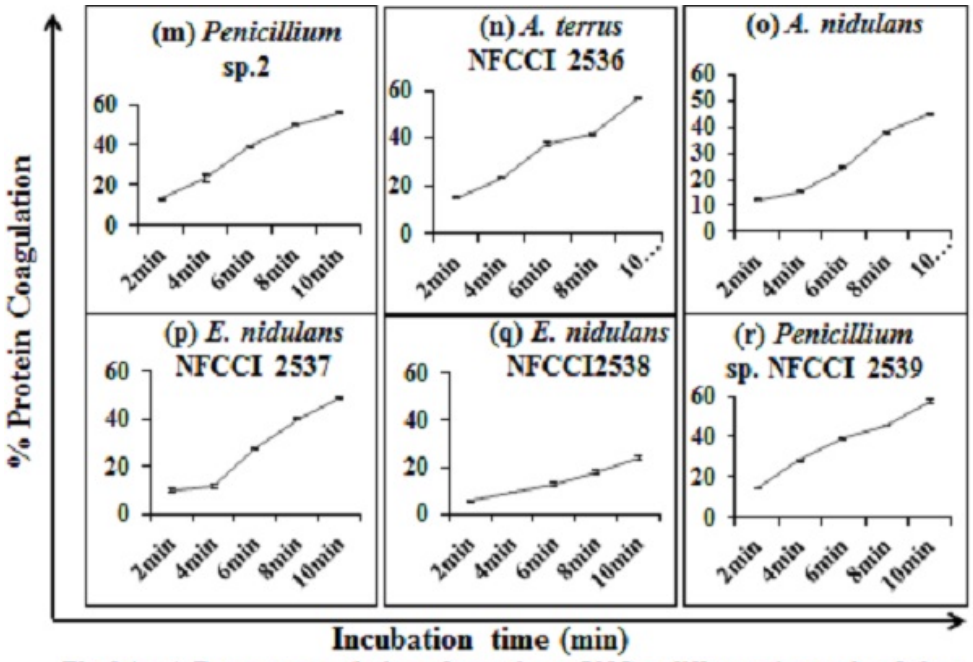

Fig 3 (m-r) Percent coagulation of protein at 50°C at different intervals of time

Conclusions

The result indicates that xylanase screening must be done in both the solid medium as well as the liquid medium. The results also showed that there was no correlation between the dry weight of the mycelium with the production of enzyme and protein.

So, in order to produce thermostable protein or enzyme from thermophilic fungi, thermostability of protein must be checked using percent protein coagulation. In the present study, five fungal isolates may be thermophilic/ thermotolerant as they recorded higher biomass at 45°C and minimum percent protein coagulation at 50°C. Thus, xylanase produced by

Chrysosporium tropicum NFCCI 2531, *Malbranchea cinnamomea* MTCC 11894, *Aspergillus fumigatus* NFCCI 2532, *Aspergillus terrus* NFCCI2533 and *Emericella nidulans* NFCCI2538 may be further assessed for industrial exploitation.

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