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OPTIMIZATION OF CULTURE CONDITIONS OF SOME THERMOPHILIC FUNGI FOR SELECTION OF A POTENTIAL THERMOPHILIC XYLANOLYTIC STRAIN FROM RAIPUR

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ABSTRACT

The objectives of the present study were optimization of culture conditions for xylanase production from some thermophilic fungal isolates. A total of 55 fungal isolates were isolated and screened for xylanase activity. Five isolates with higher xylanase activity, higher mycelial biomass at 45°C and lowest percent protein coagulation at 50°C were selected as thermophlic fungi. Xylanase production in five fungal isolates namely *Chrysosporium tropicum* NFCCI2531, *Malbranchea cinnamomea* MTCC11894, *Aspergillus fumigatus* NFCCI2532, *Aspergillus terrus* NFCCI2533 and *Emericella nidulans* NFCCI2538 were compared and optimized using different cultural parameters including effect of cheaper raw substrates, different carbon and nitrogen sources, different pH and temperatures. All optimization studies indicated extraordinary capabilities of *M. cinnamomea* MTCC11894 for production of extracellular xylanase in presence of wheat bran as raw substrate, fructose and yeast extract as carbon and nitrogen sources, at pH 6.5 and temperature 45°C to 50°C. Thus, present study originates a thermophlic fungi having capability of growing up to 50°C and so can be used for producing thermotolerant xylanase for industrial applications.

KEYWORDS: Cultural parameters, thermotolerant xylanase, thermophlic fungi, wheat bran, carbon and nitrogen sources, *M. cinnamomea* MTCC11894.

INTRODUCTION

Xylanase are enzymes of glycosidic hydrolase family and catalyses hydrolysis of xylan.[1] Xylan is the hemicellulosic component of plant cell wall. Research on xylan utilizing microorganisms and on the enzyme systems involved is increasing enormously.[2] A number of microorganisms have been noted as xylanase producer. But the level of xylanase in fungal culture is found as much higher than those from yeasts or from bacteria.[3] Among various fungal species, thermophilic fungi are generally more stable at high temperature as compared to their mesophilic counterpart and are known for secreting a variety of cell wall degrading enzymes like xylanase, cellulase, pectinases etc.[4] Different xylanases were noted from thermophilic and mesophilic fungi. However, enzymes from thermophilic fungi has a number of advantages over mesophilic source viz. acceleration of rates of reactions; an increase of reaction temperature by each 10°C, increase of operational stability thus prolongation of half-lives of biocatalysts, high stability towards other denaturing conditions including extremes pH values, organic solvents, preservations from microbial contaminations.[5,6] These are basic properties for utilizing enzymes at high temperature.

Thermostable xylanases has attracted more attention for their potential applications in the preparation of paper pulps, for the processing of raw materials and for enzymatic conversion of hemicellulose in bio-mass into useful chemicals. These processes are useful in ecological and economic point of view. Reduction of chlorine consumption in paper industry and production of biofuels from waste raw materials facilitates bioremediation and minimizes environmental pollution.

Due to the increasing application of themostable enzymes, several efforts have been done to isolate thermophilic and even extremophilic microorganisms. But only few thermophiles are noted to secrete thermostable xylanase.[7,8] Therefore, it is the demand to have more studies on thermophilic microorganisms in order to produce stable thermostable enzyme.

The production of metabolites by microorganisms is highly dependent on the presence of type and concentration of nutrients in the medium. Different

nutrients parameters like carbon and nitrogen source, pH and temperature have been found to play an important role for increasing xylanase production. But from economic point of view, there is the need to reduce the cost of xylanase production by adapting a suitable optimization method.

Thus, keeping these objectives in mind the present paper planned to optimize medium composition for xylanase production under different conditions from different thermophilic fungi. On the basis of comparative optimization study we are targeting to select thermophilic fungi with highest thermotolerant xylanase activity.

MATERIALS AND METHODS

Sampling and isolation of fungi - The soil samples were collected from the various localities in and around Raipur city of Chhattisgarh. The samples were mainly from Garden, dumped soil, garbage soil, stable manure (from domestic animal dung farms) and compost soil from various municipal wastes of Raipur city. Isolation of fungi was done by serial dilution[9] and direct plate method.[10]

Identification of fungi – Identification was based on morphological and microscopic characteristics of different fungal isolates as per available literature.[11] Selected cultures were identified from NFCCI, Agharkar Research Institute, Pune. Molecular identification was obtained from IMTECH Chandigarh.

Screening of fungi for xylanase activity- Isolated fungi were screened for xylanolytic activities on xylan agar medium.[12] Positive xylanolytic fungi were further tested in wheat bran broth.[13]

Xylanase assay: Xylanase activity was assayed using 1% oat spelt xylan substrate (Sigma chemical) as explained by Parihar and Rai[14,15] using Millers[16] method. Protein was estimated by Lowry protocol.[17]

Xylanase production medium*:* Effect of different factors on xylanase production was studied in culture medium of composition as described by Parihar and Rai.[14] Here, Raw substrate is 0.5N NaOH treated-0.8mm size maize straw for *Chrysosporium tropicum*, *Aspergillus fumigatus* and *Aspergillus terrus* whereas 0.5N NaOH treated-0.8mm size wheat bran for *Malbranchea cinnamomea* and *Emericella nidulans*.

Culture amendments for optimum production of extracellular xylanase

The medium composition and culture conditions were optimized by using different parameters like carbon, nitrogen, pH and temperature for xylanase production.

Effect of different carbon and nitrogen sources on xylanase production by fungi- The tested carbon and nitrogen sources were shown in Fig 2(a) and 3 (a). Soluble starch (control for each carbon sources) and yeast extract (control for each nitrogen source) were replaced in the production medium by equal amounts of other carbon and nitrogen sources to be tested, while all the other ingredients remaining the same.

The carbon and nitrogen sources exhibiting higher xylanase activity were selected and their different concentrations were also tried for extracellular xylanase production as shown in Fig 2(b) and 3 (b).

Effect of different pH and Temperature on xylanase production by fungi- The pH of the production medium was adjusted prior to sterilization for testing effect of pH (Fig 4). The fungal isolate was grown at different temperatures shown in Fig (5) to study the influence of temperatures on the enzyme production.

RESULTS AND DISCUSSION

Isolation and screening of thermophilic xylanolytic fungi – Our previous findings, showed that, eighteen out of 55 isolates had higher xylanase activity.[15] However, five fungi i.e. *Chrysosporium tropicum* NFCCI2531, *Malbranchea cinnamomea* MTCC11894, *Aspergillus fumigatus* NFCCI2532, *Aspergillus terrus* NFCCI2533 and *Emericella nidulans* NFCCI2538 were reported as thermophilic fungi.[15] Further study reported maximum xylanase production by these fungi in presence of 0.8mm size of 0.5N NaOH pretreated raw substrates.[14] However, Maize straw and wheat bran were recorded as best raw substrate for maximum xylanase production and thus are selected for further culture optimization study (Fig 1).

Xylanase production by all five fungi was again compared by using different culture amendment studies.

Culture amendments for optimum production of extracellular xylanase

Effect of carbon sources and their concentration on xylanase production by fungi- Fig 2a and Fig 2b represents extracellular xylanase activity (M±SE) of five fungal isolates in presence of different carbon sources and their concentrations. Table 1 (a) and 1 (b) represent results of t-test difference between various carbon sources and their concentrations on extracellular xylanase activity of different fungal isolates. Results indicate that xylanase activity in presence of different carbon sources in all five fungal isolates differ significantly from the control (Starch).

A significant higher xylanase activity was recorded with fructose in *C. tropicum* at 5g/l and *M. cinnamomea* at 1g/l; with xylose in *A. terrus* at 1g/l and *E. nidulans* at 0.5g/l and with lactose in *A. fumigatus* at 0.5g/l, respectively.

In literature, xylose was reported as best carbon sources for higher xylanase production from *Fusarium solani*;[18] from *A.Pullulans*;[19] from *T. lanuginosus*[20] and from *M.*

albomyces.[21] Fructose was recorded as best carbon for xylanase production from *Penicillium oxalicum*[12] and from *Bacillus pumilus*.[22] Lactose was found to increase xylanase level in *Penicillium canescens* 10-10c[23] and in *Trichoderma longibrachiatum*.[24] Lactose, fructose, and xylose were reported as best carbon source for maximum level of xylanase production from *Paenibacillus* sp. KIJ1.[25]

Effect of nitrogen sources and their concentration on xylanase production by fungi - Fig 3a and Fig 3b represents extracellular xylanase activity (M±SE) of different fungal isolates in presence of different nitrogen sources and their concentrations. Table 2(a) and 2(b) represent results of t-test difference between various nitrogen sources and their concentrations on extracellular xylanase activity of different fungal isolates. Results indicate that xylanase activity in presence of different nitrogen sources differ significantly from the control (yeast extract) in three fungal isolates. The three isolates are *Aspergillus fumigatus*, *Aspergillus terrus* and *Emericella nidulans* where urea was recorded for significant increase in xylanase production at 2.0, 1.0 and 1.0 g/l concentrations, respectively. However, no significant increase in xylanase production was noted with different nitrogen sources from *Malbranchea cinnamomea* and *Chrysosporium tropicum* as compared to control carbon. In these two isolates yeast extract at 2.0g/l and 2.5g/l, respectively was recorded to support higher xylanase production.

Yeast extract was considered as best nitrogen source for xylanase production in *T. lanuginosus*;[26] in *Panibacillus sp.* KIJ1 at 0.5%;[25] in *P. oxalicum* at 0.05g/l concentration[12] and in *P. Thermophila* J18.[27] In literature, it has been reported that addition of 2% urea in medium was found to support maximum xylanase production in *A. niger* ATCC 6275.[28] An increased in xylanase production was observed from *Melanocarpus albomyces* in presence of 1.5g/l urea and yeast extract.[29]

Effect of pH and temperature on xylanase production by fungi- Fig 4 represents extracellular xylanase activity (M±SE) of different fungal isolates at different pH of culture medium. Table 3 represent results of t-test difference between various pH of culture medium on extracellular xylanase activity of different fungal isolates. It was observed that three isolates i.e. *C. tropicum*, *A. fumigatus* and *E. nidulans* gave significant higher xylanase production at pH 5.5, 7.5 and 9.5 as compared to control pH (6.5) of culture medium. However, different pH of culture medium did not show any significant effect on xylanase production from *M. cinnamomea* and *A. terrus*. These isolates exhibited highest xylanase activity at control pH 6.5.

Fig 5 represents extracellular xylanase activity (M±SE) of different fungal isolates at different temperature of culture medium. Table 4 represent results of t-test difference between various temperatures of culture medium on extracellular xylanase activity of different fungal isolates. Results indicate that xylanase activity at different temperature (35, 45, 50, 55, 60 and 65°C) of culture medium differ significantly from the control temperature (45°C) in *M. cinnamomea* where maximum production was recorded at 50°C whereas in other four isolates higher xylanase production was recorded at control temperature i.e. 45°C.

In literature temperature and pH parameters were also recorded for enhancing xylanase production from various microorganisms. *H. lanuginosus* were recorded to give maximum xylanase production at pH 5.5 to 6.0 and temperature 45°C[30] and *Coprinellus disseminatus* SW-1 NTCC at pH 6.4 and 37°C.[31] *P. oxalicum* gave maximum xylanase production at pH 8.0 and temperature 45°C, *Thermoactinomyces thalophilus* at pH 8.5 and 50°C[32] and *P. Thermophila* J18 at pH 5.0-8.0 and 50°C temperature.[27]

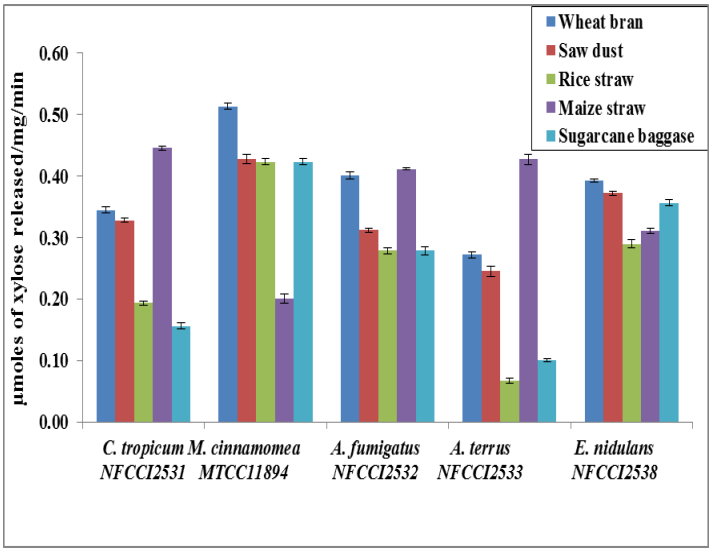

Fig 1 Effect of different raw substrates (0.8mm size/0.5N NaOH treated) on xylanase production from different fungal isolates

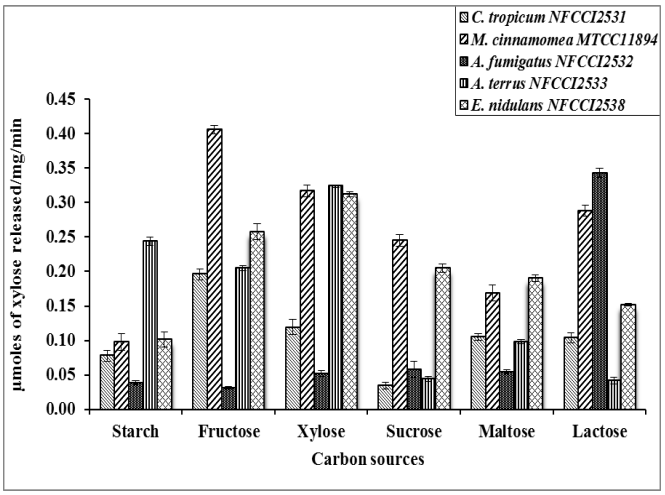

Fig 2(a) Effect of different carbon sources on xylanase production from different fungal isolates

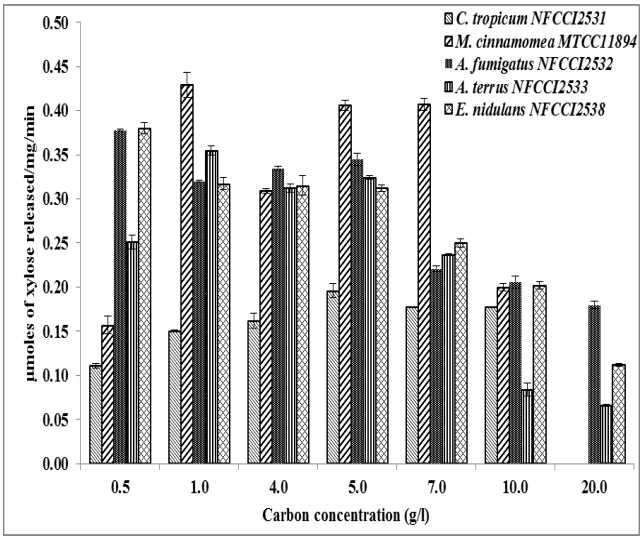

Fig 2(b) Effect of different concentration of carbon sources on xylanase production from different fungal isolates

***fructose- *C.tropicum* & *M.cinnamomea*;**
Lactose-*A.fumigatus
Xylose-*A.terrus & E.nidulans

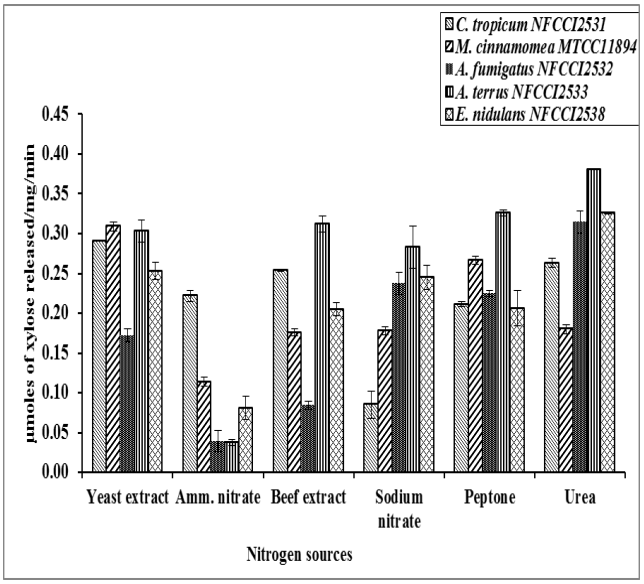

Fig 3(a) Effect of different nitrogen sources on xylanase production from different fungal isolates

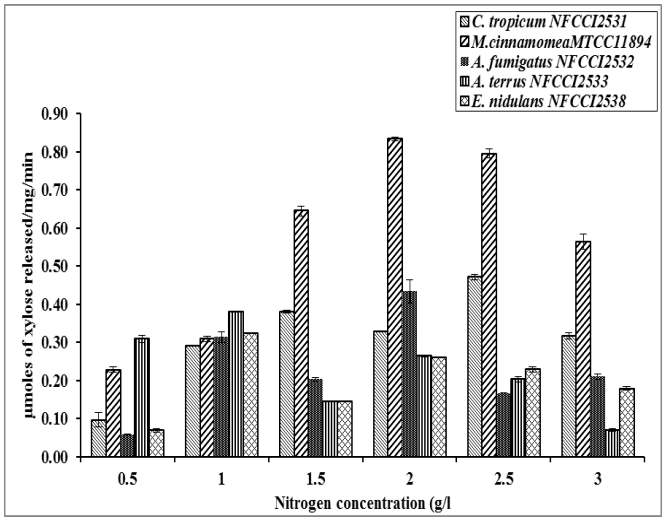

Fig 3(b) Effect of different concentrations of nitrogen sources on xylanase production from different fungal isolates

***Yeast extract- *C.tropicum* & *M.cinnamomea*;**
Urea-*A.fumigatus, A. terrus & E.nidulans

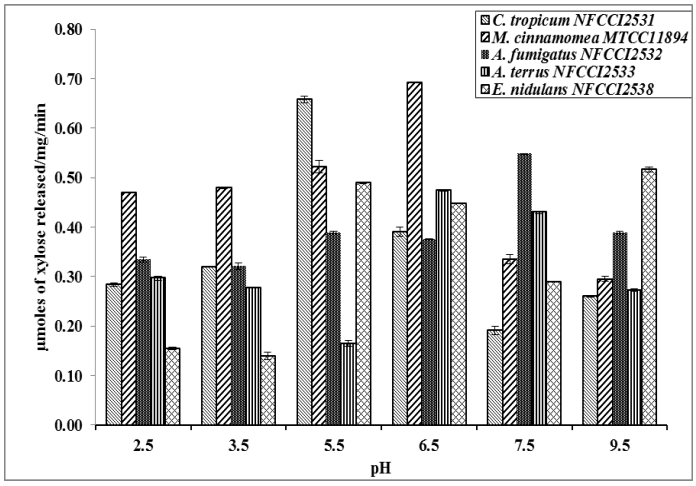

Fig 4 Effect of different pH of YpSs culture medium on xylanase production from different fungal isolates

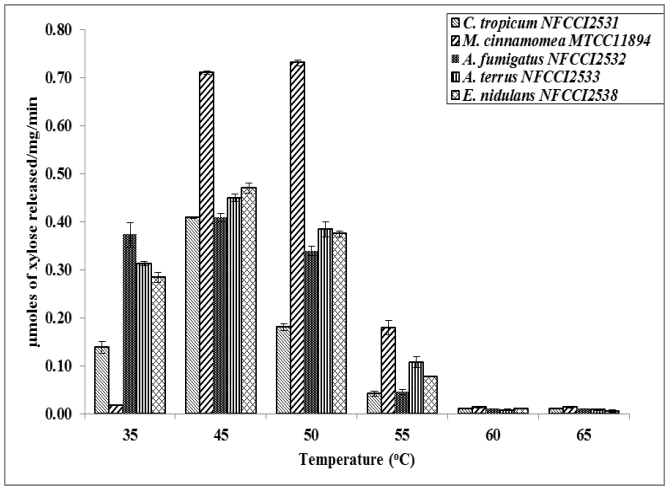

Fig 5 Effect of different temperature of YpSs culture medium on xylanase production from different fungal isolates

Table 1(a): t-test: Difference between various carbon sources on extracellular xylanase activity of different fungal isolates (control carbon- Starch).

Fungal Isolate	Xylanase activity				
	Starch vs. Fructose	Starch vs. Xylose	Starch vs. Sucrose	Starch vs. Maltose	Starch vs. Lactose
	t_{5*} , p	t_{5*} , p	t_{5*} , p	t_{5*} , p	t_{5*} , p
C.tropicum	23.68, <0.001	8.76,<0.001	6.19,<0.01	7.22,<0.001	1.89,0.12
M.cinnamomea	28.68,<0.001	35.26,<0.001	14.55,<0.001	7.42,<0.001	22.24,<0.001
A.fumigatus	1.76,0.14	3.97,<0.01	1.96,0.11	3.26,<0.05	45.89,<0.001
A.terrus	4.95,<0.01	14.80,<0.001	23.86,<0.001	17.79,<0.001	22.93,<0.001
E.nidulans	7.89,<0.001	15.43,<0.001	6.66,<0.001	6.49,<0.001	4.44,<0.01

Table 1(b): t-test: Difference between various concentrations of carbon sources on extracellular xylanase activity of different fungal isolates (Control Starch conc- 5g).

Fungal Isolate	Xylanase activity				
	5g vs. 0.5g	5g vs. 1.0g	5g vs. 4.0g	5g vs. 7.0g	5g vs. 10.0g
	t_{5*} , p	t_{5*} , p	t_{5*} , p	t_{5*} , p	t_{5*} , p
C.tropicum[1]	8.36, <0.001	5.18,<0.01	5.52,<0.01	2.18,0.08	2.22,0.08
M.cinnamomea[1]	33.36, <0.001	2.22,0.08	11.12,<0.001	1.69,0.15	22.03,<0.001
A.fumigatus[2]	4.08,<0.01	2.87,<0.05	1.94, 0.110	11.89, <0.001	1208.37,<0.001
A.terrus[3]	8.85,<0.001	7.26,<0.001	2.50,<0.05	38.59,<0.001	34.26,<0.001
E.nidulans[3]	7.15, <0.001	0.48, 0.65	0.30,0.77	7.64,< 0.001	41.43,<0.001

[1]fructose; [2]Lactose; [3]Xylose

Table 2(a): t-test: Difference between various nitrogen sources on extracellular xylanase activity of different fungal isolates (control- Yeast extract or YE).

Fungal Isolate	Xylanase activity				
	YE vs. Amm.nitrate	YE vs. Beef extract	YEvs. Sodium nitrate	YE vs. Peptone	YE vs. Urea
	t_{5*} , p	t_{5*} , p	t_{5*} , p	t_{5*} , p	t_{5*} , p
C.tropicum	8.18,<0.001	27.34,<0.001	11.33,<0.001	21.78,<0.001	5.33,<0.01
M.cinnamomea	832.31,<0.001	75.91,<0.001	11.63,<0.001	39.096,<0.001	11.73,<0.001
A.fumigatus	27.08<0.001	32.01<0.001	3.02,0.03	4.708,<0.01	6.63,<0.001
A.terrus	27.10.<0.001	2.42,0.06	0.48,0.65	2.387,0.06	6.29,<0.001
E.nidulans	6.92,<0.001	14.30,<0.001	2.17,0.08	1.409,0.22	5.95,<0.01

Table 2(b): t-test: Difference between various concentration of nitrogen sources on extracellular xylanase activity of different fungal isolates (control yeast extract conc. - 1.0g).

Fungal Isolate	Xylanase activity				
	1.0/0.5 (g/l)	1.0/1.5 (g/l)	1.0/2.0 (g/l)	1.0/2.5 (g/l)	1.0/3.0 (g/l)
	t_{5*} , p	t_{5*} , p	t_{5*} , p	t_{5*} , p	t_{5*} , p
C.tropicum[1]	11.78,<0.001	20.57,<0.001	26.62,<0.001	36.51,<0.001	2.92,<0.05
M.cinnamomea[1]	68.61,<0.001	17.72,<0.001	321.81,<0.001	85.15,<0.001	15.20,<0.001
A.fumigatus[2]	17.21,<0.001	11.05,<0.001	2.77,<0.05	11.004,<0.01	15.57,<0.001
A.terrus[2]	9.65,<0.001	215.47,<0.001	43.80,<0.001	34.74,<0.001	116.37,<0.001
E.nidulans[2]	51.60,<0.001	118.53,<0.001	30.82,<0.001	17.15,<0.001	40.75,<0.001

[1]Yeast extract; [2]Urea

Table 3: t-test: Difference between various pH of culture medium extracellular xylanase activity of different fungal isolates (control pH – 6.5).

Fungal Isolate	Xylanase activity				
	6.5/2.5	6.5/3.5	6.5/5.5	6.5/7.5	6.5/9.5
	t_{5*} , p	t_{5*} , p	t_{5*} , p	t_{5*} , p	t_{5*} , p
C.tropicum	16.11,<0.001	8.82,<0.001	37.91,<0.001	134.15,<0.001	13.49,<0.001
M.cinnamomea	121.02,<0.001	139.65,<0.001	12.45,<0.001	35.07,<0.001	70.55,<0.001
A.fumigatus	10.96,<0.001	9.37,<0.001	7.01,<0.001	68.05,<0.001	7.01,<0.001
A.terrus	45.29,<0.001	122.92,<0.001	49.23,<0.001	11.60,<0.001	73.11,<0.001
E.nidulans	63.63,<0.001	34.70,<0.001	20.27,<0.001	131.21,<0.001	10.15,<0.001

Table 4: t-test: Difference between various temperatures of culture medium extracellular xylanase activity of different fungal isolates (control Temperature – : 45°C).

Fungal Isolate	Xylanase activity				
	35°C/45°C	50°C/45°C	55°C/45°C	60°C/45°C	65°C/45°C
	t_{5*} , p	t_{5*} , p	t_{5*} , p	t_{5*} , p	t_{5*} , p
C.tropicum	23.68,<0.001	38.59,<0.001	52.58,<0.001	419.72,<0.001	404.33,,0.001
M.cinnamomea	185.47,<0.001	5.31,<0.01	49.95,<0.001	127.37,<0.001	127.71,<0.001
A.fumigatus	1.05,0.34	52.96,<0.001	97.38,<0.001	45.62,<0.001	44.92,<0.001
A.terrus	40.15,<0.001	9.07,<0.001	108.02,<0.001	55.30,<0.001	63.37,<0.001
E.nidulans	158.18,<0.001	18.72,<0.001	34.13,<0.001	40.84,<0.001	37.96,<0.001

5= degree of freedom*

*(*p<0.05, ** p<0.01, *** p<0.001, p value is calculated with respect to control in all above tables.)*

CONCLUSIONS

The given study revealed that the production of xylanase by thermophilic fungi is highly dependent on the presence of type and concentration of nutrients supplemented in the medium. After examining the different fungal isolates for xylanase production under different culture conditions, found extraordinary capabilities of producing xylanase from *M. cinnamomea* MTCC11894. In fact this fungus found exhibiting higher specific activities as compared to other xylanases. The given study created an optimized medium containing wheat bran, fructose, yeast extract, pH 6.5 and temperature 45°C for better and maximum xylanase production from *M. cinnamomea* MTCC11894 in a cost effective way. The extracellular xylanase so produced may be exploited for industrial purpose like paper industry and biofuel production.

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