OPTIMIZATION OF SUBMERGED FERMENTATION CONDITIONS FOR TWO XYLANASE PRODUCERS *COPRINELLUS DISSEMINATUS* MLK-01NTCC-1180 AND MLK-07NTCC-1181 AND THEIR BIOCHEMICAL CHARACTERIZATION

MOHAN LAL, DHARM DUTT, AMIT KUMAR and ARCHANA GAUTAM

Department of Paper Technology, Indian Institute of Technology Roorkee, Saharanpur campus, Saharanpur 247 001, India

✉ *Corresponding author: Dharm Dutt, dharm_dutt@rediffmail.com*

*Received*October 6, 2013

Out of 15 isolates, two white-rot basidiomycetes, namely MLK-01 and MLK-07, showed good growth in the pH range of 5.0 to 10.0. Both isolates produced maximal fungal growth and xylanase activity with minimal cellulase contamination at incubation periods of 8 and 9 days, respectively, and pH 10.0 with wheat bran as substrate under submerged fermentation conditions. The maximum xylanase, CMCase and lignin peroxidase activitiesof isolate MLK-01 were 30.32, 0.32 and 0.25 IU/mL, respectively, with protein concentration of 1.76 mg/mL at pH 7.5 and temperature of 75 °C, while those of isolate MLK-07 were 36.37, 0.25 and 0.15 IU/mL with protein concentration of 1.69 at pH 8.0 and temperature of 65°C. Hg^{2+} and Cu^{2+} inhibited the xylanase activity for isolate MLK-01 to 17.6 and 20.20%, and for MLK-07 to 82.2 and 70.5%, respectively. All the metallic ions like Ca^{2+}, Mn^{2+}, Fe^{2+}, Pb^{2+}, Mg^{2+}, Ni^{2+}, K^{+}, Na^{+} and Zn^{2+} improved the enzyme activity, but the enzyme activity doubled with $Zn^{2+}$for isolate MLK-01and with Fe^{2+} for isolate MLK-07.

Keywords: *Coprinellus disseminatus*, alkali-thermo-tolerant enzymes, biochemical characterization, metal ions

INTRODUCTION

The growing public awareness over the environmental impactof pollutants discharged from the pulp industry promoted a technological revolution aiming to remove chlorine partially or completely from the bleaching sequences for mitigating the emissions of chlorinated phenolics, chlorinated dihydroabietic acids and polychlorinated biphenyls in bleach plant effluents.[1]Various old and novel technologies are available for mitigating kappa factor before pulp bleaching,such as oxygen delignification,[2] the use of hydrogen peroxide, ozone, peracetic acid and dioxirane, the substitution of chlorine with chlorine dioxide, and xylanase treatment. Xylanase along with a concoction of cellulase, xylanase, amylase and lipase causes the hydrolysis of ink particles to form bonding on the surface of cellulose, hemicelluloses, starch and fatty acids, which facilitates ink detachment from the fibre surface – this process is known as "bio-deinking".[3] Mesophilic enzymes become inactive in pulp obtained after brown stock washing due to high temperature (about 70°C) and alkaline pH (about 8.5).[4] Likewise, waste paper pulp when subjected to the bio-deinking process has alkaline pH anda temperature of 65°C.[3] Most of the first-generation commercial enzymes do not satisfy the pulp treatment test conditions. The use of xylanase for pulp prebleaching or waste paper bio-deinking in paper industry has been slowed down due tothe loss of enzyme activity at pH above 8.0 and temperature in the vicinityof 65°C, because these were the prevalent conditions for many pre-bleaching and bio-deinking processes. The readjustment of the pH and cooling of the pulp increased the cost of enzymatic pulp prebleaching or bio-deinking of recycled paper.

In the last decade, the production of xylanase attracted the attention of many researchers since this enzyme is essential for the degradation of plant biomass. Xylanase has various applications, including biodegradation of lignocelluloses in

animal feed, foods, and textiles, bio-deinking of waste paper, kraft pulp bleaching and bio-pulping in the pulp and paper industry.[5] The most promising application of xylanaseis in pulp prebleaching. The application of xylanase in pulp could also improve pulp fibrillation and water retention, reduce the beating times in virgin pulps, restore bonding, increase the freeness of recycled fibres and remove xylan from dissolving grade pulps.[6] Xylanase is of great importance to pulp and paper industries since the hydrolysis of xylan facilitates the release of lignin from pulp, thereby reducing the use of chlorine as a bleaching agent.[7]Pulps obtained during kraft pulping and waste paper deinking processes at high temperature and alkaline pH need novel microbial isolates with the ability to produce thermo-tolerant and alkalo-philic xylanases.[8,9] Some of the thermo-philic fungi,such as *Chaetomium thermophile*, *Humicola insolens*, *Thermomyces lanuginosus* and *Thermoascus aurantiacus*, were reported to produce biotechnologically important, thermo-stable xylanases.[10] The optimum temperature for the activity of endoxylanase from bacterial and fungal sources varied from 40 to 100°C[11] and they commonly had a broad optimal pH range of 3.6-10.0. Xylanase from two strains of *T. aurantiacus* and *T. Lanuginosus* were optimally active at 70 and 80°C, respectively.[12] The pH stability of the xylanase produced from *Thermomyces lanuginosus* strains DSM 5826 and ATCC 46882 was in the pH range of 5.0 to 9.0.[13] *Coprinopsis cinerea* HK-1 NFCCI-2032 retained 50% of its activity at pH 8.0 when incubated at 55°C for 15 min and, likewise, it retained 78, 43, and 23% of its activities at temperatures of 65, 75 and 85°C, respectively, proving its alkali-thermo-tolerant nature for biobleaching.[14]

With these considerations, the present study aimed at investigating twopotential xylanase producersisolated from wild strains of *Coprinellusdisseminatus*, i.e. MLK-01NTCC-1180 and MLK-07NTCC-1181, keeping their alkali-thermo-tolerant nature in view. The study also covered the complete optimisation of SmF conditions for high xylanase production and biochemical characterization using cheap and readily available lignocelluloses as substrates.

EXPERIMENTAL

Isolation of fungi

A total of 30 decaying wood samples were collected from different sites in the vicinity of Saharanpur, located at the foothills of Shivalikof western Uttar Pradesh (India). Out of 30 wood samples, 15 white-rot fungalstrains from decaying wood were isolated by the enrichment technique, in which the decaying wood samples were kept in 9 mm diameter glass Petri plates enriched with moist wheat bran. These decaying wood samples were incubated at 40°C in a BOD incubator and the growth was observed every day. The moisture of plates was maintained with sterile tap water. Growing fungal cultures were isolated on wheat bran-agar medium (1:1) and examined under light microscope. When the growth of white-rot basidiomycetes was observed, they were purified and stabilized on same medium by frequent sub-culturing. The purified cultures were maintained at 4°C on potato dextrose agar (PDA) slants in screw capped culture tubes. These were frequently sub-cultured every 2-3 months. The spores were preserved in 15% sterile glycerol and stored at –20°C in deep freeze for further studies.

Effect of pH on fungal growth and xylanase activity

The screening of alkali-tolerant/alkalo-philic fungi was carried out as per the method described by Nagai *et al.*[13] The isolated fungi were cultivated underSmF conditions by the shake flask culture method, using 2% wheat bran (w/v) as substrate, 40 mL of nutrient salt solution (NSS) having 1.5 g/L KH_2PO_4, 4 g/L $NaNO_3$, 0.5 g/L $MgSO_4$, 0.5 g/L KCl and 1 g/L yeast extract in distilled water with 0.04 mL/L trace elements solution (200 µg/L $FeSO_4.7H_2O$, 180 µg/L $ZnSO_4.7H_2O$, 20µg/L $MnSO_4.7H_2O$). The pH of the flasks was adjusted to 5.0 to 11.0 with 1.0 N NaOH by mixing 90% of NSS and 10% of appropriate buffer solution according to pH. These were autoclaved at 15 psi for 15 min and the pH of the flasks was readjusted with 0.1 N NaOH. The flasks were incubated at 40°C for 10 days in an incubator shaker at 100 rpm. After 10 days of incubation, the fermentation broth was filtered through a cheese cloth followed by centrifugation at 15000 rpm for 1 h at 4 °C. The supernatants obtained were analyzed for xylanase activity at different pH levels and two fungal isolates, namely MLK-01 and MLK-07, were selected for further study.

Effect of different agar media on fungal growth

The effect of six distinct agar media, including wheat bran-agar (WBA), malt extract-agar (MEA), PDA, fungal-agar (FA), lactose-yeast extract-agar (LYEA) and malt-glucose-yeast-peptone agar (MGYP),wasstudied on fungal growth. The pH of the media was maintained at 10.0 with 1.0 N NaOH after autoclaving. A disc of 6 mm diameter from a 4-day old culture of test fungi was aseptically inoculated in the center of each Petri plate. Then, the plates were incubated at 40°C in a BOD incubator and growth was measured after 36 and 72 h of incubation, respectively, in terms of colony diameter (cm). The appearance of the fungi was observed with the naked eye every day.

Screening for extracellular enzymes

Both fungal isolates were screened for amylases, cellulase, xylanase, mannanase, laccase, ligninase and protease activities. The screening of the fungal cultures was carried out based on their extracellular enzyme activities on agar medium using 1% of each xylan for xylanase, CMC for cellulase, mannan for mannanase, soluble starch for amylase, gelatine for protease and 0.02% tannic acid for ligninase, 10 mMguaiacol for laccase and supplementing the media with 1% yeast extract. A 6 mm diameter disc from a 4-day old culture of each test fungi was inoculated at the centre of each Petri plate and incubated at 40°C for a period of 3 days. The Petri plates were flooded with 1% iodine and 0.2% KI solution to enhance the visibility of the hydrolysed area. The rest of the Petri plates were flooded with an aqueous solution of 0.1% Congo red and after 30 minthe plates were frequently washed with 1.0 N NaCl solution to enhance the visibility of the hydrolysed area. The activities of laccase, ligninase and protease were observed visually without staining.

Preparation of extracellular enzymes under Smf conditions

Both fungal strains were cultivated separately under Smf conditions in 250 mL Erlenmeyer conical flasks using 0.8 g wheat bran powder, 40 mL of NSS having the aforesaid composition. The desired pH of NSS was adjusted with $NaOH/H_2SO_4$ with the help of a microprocessor controlled pH meter. Each flask was plugged with cotton and sterilized at 15 psi for 15 min. The pH of each flask was checked on cooling at room temperature and readjusted again aseptically. Two discs of 6 mm diameter of a 4-day old culture of both fungal strains were aseptically inoculated in separate flasks. These flasks were incubated at 40°C with shaking at 100 rpm. The shaking was stopped before 24 h of harvesting to preserve the lignin peroxidase activity.

Then, the flasks were taken out from the incubator and the contents were filtered through a cheese cloth followed by vacuum filtration with a microfilter of 0.45 µm pore size at room temperature and the lignin peroxidase activity was determined immediately. The rest of the filtrate was centrifuged at 15000-x g for 1 h at 4 °C. The dark brown coloured supernatant was stored at –20°C until use and the pallets were analyzed for mycelial growth.

Estimation of fungal growth and enzyme assays

The growth of fungus was determined as per the method described by Ball andMcCarthy.[14]The pellets retained on the cheese cloth were washed four times with distilled water to remove the attached wheat bran powder. The washed pellets were then boiled in 20 mL of 1.0 M NaOH for 10 min. After cooling at room temperature, the required solution of 1.0 M NaOH was added to make up a final volume of 20 mL. The protein concentration of the filtrate was determined by Lowry's method,[15] using bovine serum albumin as the standard, and fungal growth was expressed in terms of mycelial protein concentration (mg/mL).

The xylanase activity was determined by measuring the release of reducing sugars using birch wood xylan as the substrate by the 3,5 dinitrosalicylic acid reagent (DNS) method.[16] 1.6 mL of the enzyme preparation was added in a sterile tube, which contained 0.4 mL of substrate suspension (10 mg/mL birch wood xylan in 0.1 M potassium phosphate buffer). Proper controls, in which substrate and/or enzyme preparation had been omitted, were included. The assay mixture was incubated at 55°C for 15 min with constant shaking at 100 rpm. Then, the assay mixture was cooled and centrifuged at 10,000 x g. 1.0 mL of supernatant was poured into a fresh tube, which contained 3.0 mL of the DNS reagent and was kept for five min on boiling water bath. Optical density was measured at 540 nm by a double beam UV-visible spectrophotometer. The enzyme activity is expressed as µ moles of D-xylose equivalents released per minat 55°C (IU).

The cellulase activity in terms of CMCase was determined by the DNS method.[16]Two mL of crude enzyme preparation diluted to 10 times with distilled water was taken in screw capped universal sterile tubes containing 2.0 mL of 2% (w/v) carboxyl methyl cellulose (CMC) as the substrate maintained at pH 4.8 with 0.05 M citrate buffer. The reaction mixture was incubated at constant temperature of 50°C for 30 min. The reaction mixture was centrifuged at 5000 x g for two min after cooling in ice bath. One mL of supernatant and 3.0 mL of DNS reagents was mixed thoroughly and kept on boiling water bath for five min. The contents were cooled rapidly and optical density was measured at 575 nm in a double beam spectrophotometer. The procedure was repeated as a control using distilled water instead ofcrude enzyme preparation. Reducing sugars were measured by comparing the standard curve prepared for D-glucose. The enzyme activity is expressed as µ moles of D-glucose equivalents released per min at 50°C (IU).

Lignin peroxidase activity was measured spectrophotometerically as described by Mercer,[17] using 2,4-di-chlorophenol as the substrate. One mL of reaction mixture contained 200 µL of 50 mM 2,4-4-di-chlorophenol; 200 µL of 1 mM 4-minoantipyrine and 200 µL crude enzyme preparation maintained at pH 6.5 with 200 µL of 100 mM phosphate buffer was taken in a cuvette of 2.0 mL capacity for determining peroxidase activity. Then, 200 µL of 50 mM H_2O_2 was added to the reaction mixture and the absorbance at 510 nm was measured immediately every 30 s until constant with a double beam spectrophotometer.The same procedure was repeated as a control using distilled water instead of crude enzyme preparation. The enzyme activity was expressed as the amount of enzyme produced with an increase of 1.0 absorbance unit per 30 s.

Optimization of incubation period, pH, different sugars and different substrates

Two sets of fifteen Erlenmeyer flasks of 250 mL capacity were prepared for each fungal strain as described earlier for the optimization of the incubation period. 2% wheat bran and 40 mL of NSS were added to each flask and maintained at pH 10.0 with $NaOH/H_2SO_4$. After autoclaving the flasks at 15 psi for 15 min,two discs of 6 mm diameter of 4-day old cultures of both fungi were aseptically inoculated separately in each set. These were incubated at 40°C and harvested periodically every day fromthe 3rd to the 20th day.

In the 2nd series of experiments, two sets of eight Erlenmeyer flasks (250 mL) were prepared for the optimization of pH. Two percent wheat bran and 40 mL of NSS were added to each flask and the pH was varied from 5.0 to 12 with a gap of one pH value using different buffer solutions, as described by Nagai.[13] The pH of each flask was checked and readjusted aseptically in a laminar air flow chamber at room temperature.

In the 3rd series of experiments, two sets of five Erlenmeyer flasks (250 mL) were prepared for each fungal strains and the effects of birch wood xylan and different sugars, i.e. glucose, xylose, galactose, on fungal growth and enzyme induction were studied. A fixed dose of 2% wheat bran and 40 mL NSS were added and further, the concentration of xylan, glucose, xylose and galactose was varied from 1.0 to 5.0 g/L with an interval of 1.0 g/L in each flask separately.

In the 4th series of experiments, two sets of five Erlenmeyer flasks (250 mL) were prepared for the optimization of different lignocelluloses wastes like wheat bran (WB), sugarcane bagasse (BG), wheat straw(WS), saw dust (SD) of wood and ground nut shell (GNS). These lignocelluloses wastes were milled separately into powder in a laboratory Wiley mill and the fraction retained on +100 mesh size was used as substrate. 0.8 g of the above mentioned lignocelluloses wastes were added separately to each flask containing 40 mL of NSS.

In the 3rd and 4th series of experiments, these flasks were autoclaved at 15 psi for 15 min and the pH in each set was adjusted to 10.0 with $NaOH/H_2SO_4$. In all the experiments except the Ist series, two discs of 6 mm diameter of 4-day old culture of both fungi were aseptically inoculated separately. These flasks were incubated at 40°C and harvested after the 8th day of incubation.In all the experiments, all the sets were estimated for fungal growth[14] and their extracellular xylanase,[16]cellulases[16] and lignin peroxidase[17] activities.

Effect of reaction pH on xylanase activity

The xylanase activity of both isolates was measured at various pH levels ranging from 4.0 to 10.0 with an interval of 0.5, while keeping other variables constant, such as incubation period: 15 min, birch wood xylan concentration: 10 mg/mL and temperature: 65°C for fungal strain MLK-01, and temperature: 75°C for fungal strain MLK-07.The pH levels varied from 4 to 6 were maintained with sodium silicate buffer solution, pH levels 6.5 to 8.0 with phosphate buffer solution and pH levels 8.5 to 10.0 with NaOH and glycine solution. The xylanase activity was measured by the DNS method.[16]The maximum value obtained was taken as 100% activity and xylanase activity measured at different reaction pHswas expressed as (%) relative activity.

Effect of temperature on xylanase activity

The xylanase activity of both fungal strains were measured at different temperatures ranging between 45-90°C with an interval of 5°C for one h, while keeping other variables constant,such asincubation period: 15 min, birch wood xylan concentration: 10 mg/mL and pH 7.5 for fungal strain MLK-01, and pH 8.0 for fungal strain MLK-07. The maximum value obtained was taken as 100% activity and xylanase activity measured at different temperatures was expressed as relative (%) activity.

Effect of different metal ions on xylanase activity

The effect of different metals ions, i.e. $HgCl_2$,$ZnSO_4.7H_2O$, NaCl, KCl, NiO, $MgCl_2.6H_2O$, $CuSO_4.5H_2O$, Pb $(NO_3)_2$, $FeSO_4.7H_2O$, $MnSO_4.7H_2O$ and $CaCl_2$, on the xylanase activity was observed while keeping other variables constant, such as incubation period: 15 min,birch wood xylan concentration: 10 mg/mL,pH 7.5 at 65°C for fungal strain MLK-01, and pH 8.0 at 75°C for fungal strain MLK-07. These metal ions were added separately at a concentration of 1.0 mM to each assay mixture. A control was also repeated without metal ions. The xylanase activity of the control was taken as 100% and xylanase activity measured for different metal ions was expressed as (%) relative activity.

RESULTS AND DISCUSSION

Screening of alkali-tolerant white-rot fungi and extracellular enzymes

Out of 15, two white-rot fungal isolates, namely MLK-01 and MLK-07, show good growth in the pH range of 5.0 to 10.0. Conversely, the rest of the isolates grow well in the pH range of 5.0 to 7.0 (Table1). The crude xylanases isolated from isolates MLK-01 and MLK-07 is active inthe pH rangeof 5.0 to 9.5. Meanwhile, crude xylanase isolated from the rest of the fungal isolates are also active in the pH range of 5.0 to 8.0 (Table1). Therefore, fungal isolates MLK-01 and MLK-07 are more alkali-tolerant compared to the rest of the isolates and have been selected for further investigation. Both theseisolates grow well up to a pH level of 10.0, but their xylanases are

active only up to a pH level of 9.5. The reason is that the plasma membrane of microorganisms is unstable above pH 9.5 and the cell wall of alkalophiles, which contains acidic polymers functioning as negatively charged matrix, may reduce the pH at the cell surface. Horikoshi reported that α-galactosidase, isolated from *Micrococcus* sp. strain 31-2 – an alkalophile, had its optimal activity at pH 7.5, indicating that the internal pH of the bacterium is almost neutral.[18]The fungal isolate MLK-01 shows good xylanase and lignin peroxidase activities, as well as average amylase and laccase activities. Contrary to this, isolate MLK-07 shows good xylanase, amylase and laccase and average lignin peroxidase activities (Table2).

Table 1
Effect of initial pH and birch wood xylanon mycelial growth and extracellular enzyme production by *Coprinellusdisseminatus* isolates MLK-01 and MLK-07 under SmF conditions

Variable parameters	Xylanase activity, IU/mL	CMCase activity, IU/mL	Lignin peroxidase activity, IU/mL	Fungal growth as mycelia protein, mg/mL
C. disseminates isolate MLK-01				
[1]Initial pH (After autoclave)				
5.0 (6.28) *	16.22±1.2	0.85±0.02	0.55±0.01	0.31±0.02
6.0 (7.05) *	19.88±1.6	0.82±0.03	0.52±0.00	0.35±0.01
7.0 (7.14) *	21.76±1.1	0.76±0.01	0.49±0.02	0.36±0.02
8.0 (7.50) *	22.08±1.2	0.74±0.04	0.44±0.03	0.38±0.03
9.0 (7.85) *	24.02±2.8	0.62±0.02	0.36±0.01	0.41±0.05
10.0(8.60) *	26.32±1.2	0.58±0.03	0.35±0.01	0.42±0.04
11.0(8.93) *	25.66±2.8	0.25±0.02	0.21±0.03	0.42±0.02
[2]Birch wood xylan dose, g/L				
Control	29.20±1.8	0.50±0.03	0.36±0.03	0.32±0.03
1.0	14.35±2.1	0.42±0.03	1.02±0.02	0.41±0.02
2.0	11.37±2.0	0.39±0.01	1.21±0.01	0.53±0.04
3.0	10.60±1.8	0.38±0.02	0.94±0.02	0.54±0.03
4.0	10.17±1.2	0.32±0.02	0.45±0.02	0.59±0.05
5.0	08.54±0.6	0.28±0.01	0.26±0.03	0.46±0.04
C. disseminatus isolate MLK-07				
[1]Initial pH (After autoclave)				
5.0 (6.30) *	20.43±2.2	0.62±0.05	0.35±0.01	0.17±0.04
6.0 (7.20) *	21.33±1.4	0.63±0.01	0.22±0.00	0.19±0.03
7.0 (7.25) *	22.76±2.1	0.46±0.03	0.24±0.02	0.22±0.06
8.0 (7.60) *	25.08±1.0	0.33±0.02	0.19±0.03	0.28±0.04
9.0 (7.90) *	28.12±1.2	0.24±0.04	0.16±0.01	0.36±0.02
10.0 (8.64) *	31.45±2.6	0.25±0.01	0.15±0.01	0.38±0.03
11.0 (8.90) *	25.43±2.1	0.18±0.02	0.13±0.02	0.40±0.01
[2]Birch wood xylan dose, g/L				
Control	32.86±1.8	0.25±0.02	0.36±0.03	0.33±0.04
1.0	16.43±2.3	0.19±0.03	0.42±0.02	0.44±0.02
2.0	13.37±1.8	0.16±0.01	0.48±0.01	0.48±0.03
3.0	12.60±2.2	0.13±0.04	0.44±0.02	0.54±0.04
4.0	10.27±1.4	0.10±0.02	0.35±0.02	0.41±0.01
5.0	8.98±1.5	0.08±0.03	0.32±0.03	0.36±0.03

Conditions for fermentation:[1]Incubation period = 8 days for isolate MLK01 and 9 days for isolate MLK07, temperature = 40 °C, substrate = 2% wheat bran (w/v); [2]Initial pH = 10.0, pH after fermentation = 8.60 ± 0.5, incubation period = 8 d for isolate MLK01 and 9 d for isolate MLK07, temperature = 40 °C, substrate = 2% wheat bran (w/v);
Conditions for xylanase assay: pH = 7.5 for isolate MLK-01 and 8.0 for isolate MLK-07, temperature = 55 °C, incubation time = 15 min, substrate = 10 mg/mL birch wood xylan;
Conditions for cellulase assay: pH = 6.0, temperature = 50 °C, incubation time = 30 min, substrate = 2% CMC;
Conditions for lignin peroxidase assay: Buffer pH = 6.5, temperature = 25 °C, incubation time = 20 min, substrate = 50 mM 2, 4-DCP);
* values in parenthesis show pH after fermentation

Table2
Effect of reducing sugars at different concentrations on the growth and production of extracellular enzymes by *Coprinellusdisseminatus* MLK-01 and MLK-07 under SmF conditions

Variable parameters	Dose, g/L	Xylanase activity, IU/mL	CMCase activity, IU/mL	Lignin peroxidase activity, IU/mL	Fungal growth as mycelia protein, mg/mL
C. disseminatus isolate MLK-01					
D-glucose	Control	27.10±1.2	0.41±0.03	0.46±0.05	0.42±0.04
	1.0	15.37±4.1	0.26±0.04	1.02±0.04	0.45±0.05
	2.0	12.87±2.2	0.16±0.02	1.21±0.03	0.48±0.02
	3.0	12.40±1.6	0.10±0.03	0.94±0.03	0.44±0.01
	4.0	10.12±2.6	0.09±0.02	0.85±0.02	0.41±0.03
	5.0	09.23±1.1	nd	0.77±0.03	0.36±0.02
D-xylose	1.0	11.86±1.3	0.28±0.05	0.64±0.05	0.55±0.04
	2.0	10.65±1.7	0.19±0.04	0.87±0.02	0.59±0.06
	3.0	08.09±0.8	0.17±0.03	0.95±0.03	0.62±0.04
	4.0	04.76±1.0	0.12±0.02	0.94±0.04	0.63±0.03
	5.0	03.98±0.3	0.10±0.02	0.94±0.02	0.63±0.02
D-galactose	1.0	15.07±0.6	0.17±0.05	0.60±0.03	0.40±0.05
	2.0	14.98±0.8	0.15±0.04	0.42±0.03	0.36±0.02
	3.0	10.00±1.1	0.12±0.03	0.31±0.04	0.32±0.03
	4.0	05.45±0.5	0.11±0.04	0.25±0.01	0.21±0.02
	5.0	01.89±0.3	nd	0.20±0.03	0.10±0.05
C. disseminates isolate MLK-07					
D-glucose	Control	32.87±2.0	0.27±0.03	0.36±0.04	0.32±0.03
	1.0	18.36±2.1	0.12±0.01	0.42±0.03	0.34±0.04
	2.0	16.76±2.8	0.09±0.03	0.51±0.03	0.42±0.03
	3.0	14.60±1.7	0.08±0.02	0.54±0.03	0.51±0.02
	4.0	13.14±2.2	0.05±0.02	0.65±0.02	0.53±0.02
	5.0	11.26±2.3	nd	0.47±0.03	0.36±0.04
D-xylose	1.0	18.86±1.2	0.18±0.02	0.54±0.04	0.38±0.03
	2.0	12.65±1.8	0.15±0.01	0.65±0.02	0.41±0.05
	3.0	10.09±1.4	0.10±0.03	0.87±0.04	0.46±0.03
	4.0	08.76±1.2	0.08±0.02	0.73±0.04	0.57±0.02
	5.0	05.98±0.5	0.06±0.02	0.62±0.02	0.62±0.01
D-galatose	1.0	08.97±1.1	0.08±0.02	0.32±0.03	0.24±0.04
	2.0	06.98±1.3	0.05±0.01	0.27±0.03	0.21±0.03
	3.0	05.07±1.2	0.04±0.01	0.21±0.04	0.18±0.02
	4.0	03.85±1.0	0.02±0.01	0.12±0.01	0.13±0.01
	5.0	03.02±0.6	nd	0.10±0.01	0.10±0.03

Conditions for fermentation: Initial pH = 10.0, pH after fermentation = 8.60 ± 1.0, incubation period = 8 d for isolate MLK-01 and 9 d for isolate MLK-07, temperature = 40°C, substrate = 2% wheat bran (w/v);
Conditions for xylanase assay: pH = 7.5 for isolate MLK-01 and 8.0 for isolate MLK-07, temperature = 55°C, incubation time = 15 min, substrate = 10 mg/mL birch wood xylan;
Conditions for cellulase assay: pH = 6.0, temperature = 50°C, incubation time = 30 min, substrate = 2% CMC);
Conditions for lignin peroxidase assay: pH = 6.5, temperature = 25 °C, incubation time = 20 min, substrate = 50 mM 2, 4-DCP,nd = not detected

Pathology Division, Forest Research Institute, Dehradun (India) confirms the fungal isolates (MLK-01 and MLK-07) as different strains of *Coprinellus disseminatus* on the basis of fruiting bodies, spore print and microscopic examination. The two isolates were deposited at National Type Culture Collection (NTCC), Forest Pathology Division, Forest Research Institute, Dehradun (India) and were allotted NTCC Culture Nos. 1180 and 1181, respectively.

Effect of different media on the growth and appearance of *C. disseminatus*

The decreasing growth pattern for isolate MLK-01 is MEA>FA>WBA> MGYP> PDA> LYEA on different media at pH 10.0 after 36h

and FA=MEA=WBA=MGYP>PDA>LYEAafter 72 h (Table3). Alike, the decreasing growth pattern for isolate MLK-07 is MEA>FA>MGYP> WBA>PDA>LYEA after 36h and FA=MEA= WBA=MGYP>PDA>LYEA after 72h on the afore said media (Table3). The growth diameter of both isolates MLK-01 and MLK-07 on wheat bran-agar medium is 6.0 and 6.2 cm respectively after 36h and 9.0 cm after 72 h. Isolate MLK-07 starts mating after 72 h of growth and dark brown coloured basidiospores appear on the onset of fruiting bodies. Vigorous growth after 72 h indicates the fast growing nature of fungi. Meanwhile, the poorest growth of both fungal isolates is observed on lactose-yeast extract-agar medium because both isolates are unable to utilize lactose as carbon source. However, the growth of bothisolates is good on malt extract-agar medium after 36 h and on wheat bran-agar medium attain the same growth as on malt extract-agar medium after 72 h. Wheat bran is a cheap and easily available substrate compared to other commercial media and it contains 71% fibre carbohydrate, a number of amino acids and trace elements necessary for enzyme induction.[19]

Effect of incubation period on growth and extracellular enzyme production

Fungal isolate MLK-01 produces maximum xylanase activity of 28.04 IU/mL on the 8th day of incubation and maximum cellulase activity of 0.64 IU/mL on the 7th day of incubation (Figure1). The maximum fungal growth in terms of protein concentration is 0.48 mg/mL on the 9th day of incubation. In a similar way, the fungal strain MLK-07 produces maximum xylanase (30.76 IU/mL) and cellulase (0.68 IU/mL) activities on the 9th and 11th day of incubation, respectively (Figure2). Meanwhile, the maximum protein concentration is 0.38 mg/mL on the 7th day of incubation in order to reflect the growth of fungi. It shows that the extracellular xylanase and cellulase are produced simultaneously along with fungal growth when wheat bran was used as core substrate. Steiner[20] also reported that fungal xylanase was generally associated with cellulases. It means that the xylanase and cellulase productions depend on the growth of fungi up to some extent because cellulase and xylanase are the part of primary metabolites. Therefore, cellulase and xylanase, which are produced during the exponential phase of growth, begin to decrease at the beginning of the death phase of growth. Besides this, the metabolic enzymes such as proteases and transglycosidases secreted by xylanase producing microorganisms may hydrolyze xylanase and cellulose, which can affect the enzyme yield adversely.[21] The maximum peroxidase activity is 0.73 and 0.33 IU/mL on the 12th and 13th days of incubation for isolates MLK-01 and MLK-07, respectively. It means that the production of lignin degrading enzymes is a secondary metabolic event and is produced in the stationary phase of growth. DelRio found 14 days as the optimum period for fungal growth on *Eucalyptus globules* wood chips.[22]

Table3
Effect of metal ions on xylanase activity at 1.0 mM concentration

Sl. No	Metal ion concentrations, 1.0 mM	Xylanase activity, IU/mL	
		Isolate MLK-01	Isolate MLK-07
1	Control	30.32±1.2	36.87±2.0
2	$HgCl_2$	6.06±0.5	7.37±0.4
3	$ZnSO_4$. $7H_2O$	90.96±2.5	105.08±0.8
4	NaCl	78.47±1.6	92.18±0.8
5	KCl	73.11±1.4	95.90±1.3
6	NiO	80.23±1.3	105.15±1.3
7	$MgCl_2$. $6H_2O$	64.52±1.4	85.72±1.5
8	$CuSO_4$. $5H_2O$	24.92±1.0	25.99±1.6
9	Pb $(NO_3)_2$	71.34±1.2	84.21±1.5
10	$FeSO_4$. $7H_2O$	84.71±1.2	110.68±1.2
11	$MnSO_4$. $7H_2O$	65.98±1.3	85.61±2.0
12	$CaCl_2$	68.40±1.3	78.28±1.6

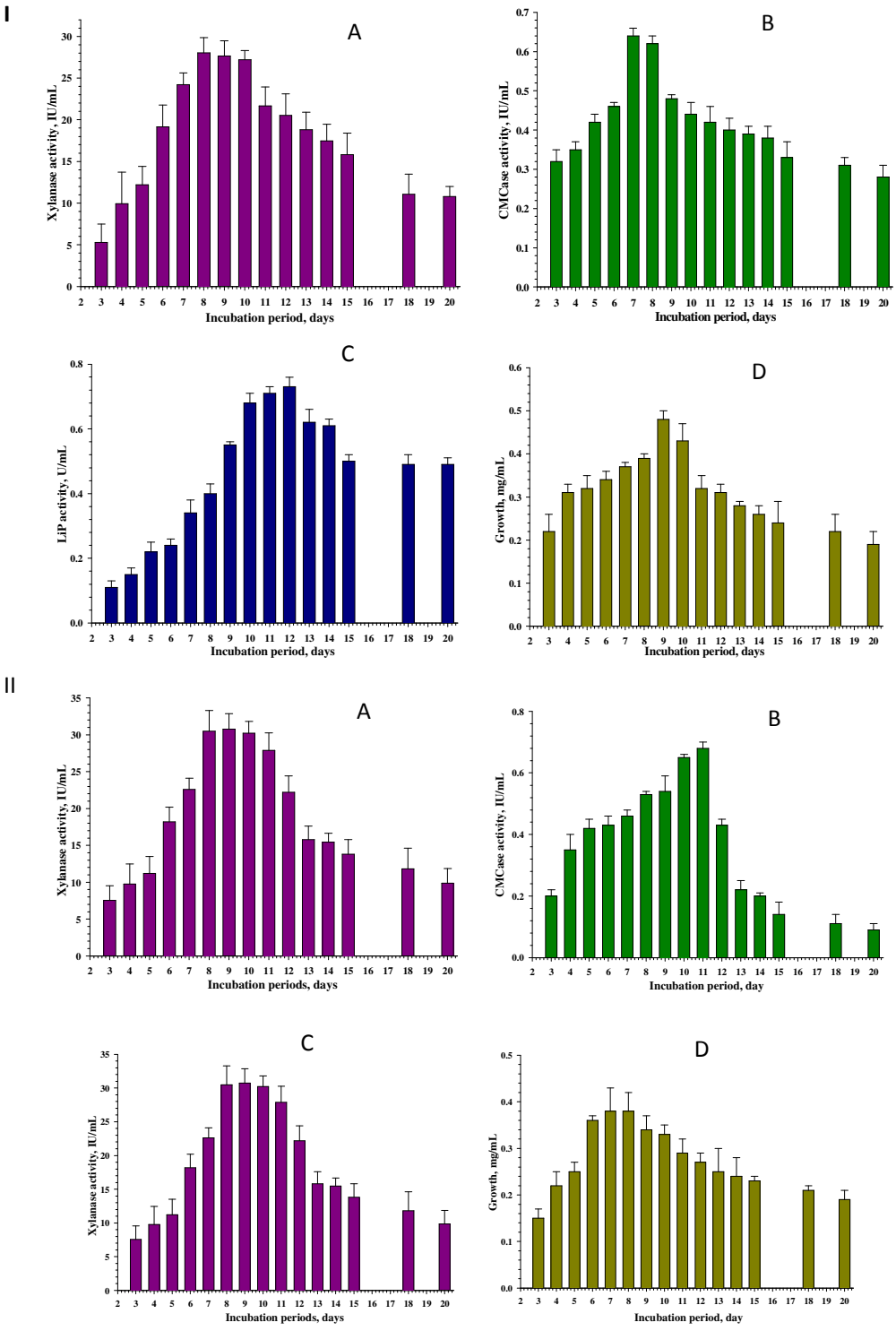

Figure1: Effect of incubation period on the production of (A) xylanase, (B) cellulase, (C) lignin peroxidase and (D) growth of strain MLK-01 (I) and MLK-07 (II) at initial pH 10.0 with 2% wheat bran as substrate at 40°C

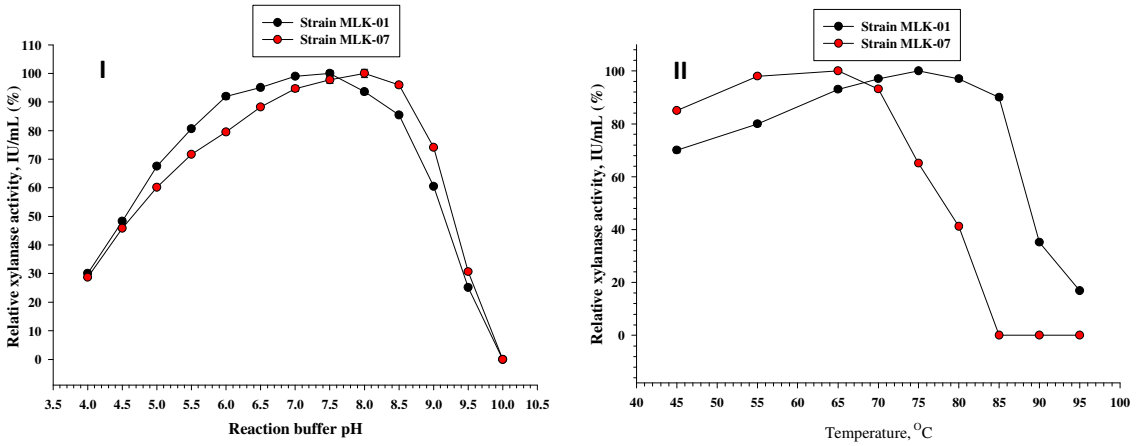

Figure2: Effect of reaction buffers of different pH (I) and reaction temperature (II) on xylanase activity

Effect of initial pH on the growth and extracellular enzyme production

Both fungal isolates exhibit a broad pH range, i.e. pH 5.0 to 11.0,for the growth and production of extracellular enzymes (Table1).The higher fungal biomass achieved at higher pH might have been due to the effect of pH on nutrients availability.[23] The xylanase activity of both isolates increases with increasing pH of the medium and no fungal growth is observed at pH 12.0. Both isolates MLK-01 and MLK-07 show maximum xylanase activity of 26.32 and 31.45 IU/mL, respectively, at pH 10.0. The cellulase and lignin peroxidase activities follow the reverse pattern and gradually decrease with increasing the pH of the medium, showing maximum cellulase and lignin peroxidase activities at pH 5.0. This pH range was significantly higher than that reported for other basidiomycetes.[24] Kirk *et al*. found the optimum pH between 4.0-4.5 for lignin degradation, but probably broader for the growth of *P.chrysosporium.*[25] Thus, both fungal isolates may be considered as alkali-tolerant fungi according to the definition given by Nagai *et al*.[13]

Effect of birch wood xylan on the growth and enzyme production

The suitable inducer and optimum medium composition are key factors for an efficient production of xylanolytic enzymes.[9] The xylanase activity of both isolates decreases with increasing birch wood xylan concentration and remain almost constant between concentrationsof 2-4 g/L for both fungal isolates (Table1). The minimum xylanase activities, i.e. 8.54 and 8.98 IU/mL, are observed at 5.0 g/L for birch wood xylan in the case of isolates MLK-01 and MLK-07, respectively. Cellulase activity decreases gradually with increasing the dose of xylan,but the lignin peroxidase activity of both fungal isolates increases with increasing birch wood xylan concentration up to 2.0 g/L and then declines gradually.Alternatively, fungal growth increases up to 4.0 and 3.0 g/L birch wood xylan concentration for fungal isolates MLK-01 and MLK-07, respectively, and then declines. In fact, xylan being a high molecular mass polymer cannot penetrate the cell wall of fungi. The low molecular mass fragments of xylan play a key role in the regulation of xylanase biosynthesis. These fragments include xylose, xylobiose, xylo-oligosaccharides, hetero-disaccharides of xylose and glucose and their positional isomers, which might act as a catabolic repressor, hence repressed the extracellular xylanase production.[9]

Effect of reducing sugars on fungal growth and enzyme production

D-xylose is found to be the major catabolic repressor for both of the fungal isolates with minimum xylanase activity, i.e. 3.98 and 5.98 IU/mL, respectively, at a concentration of 5.0 g/L (Table2).The lignin peroxidase activity and fungal growth is found maximum at a glucose concentration of 2 g/L for fungal isolate MLK-01 and 4 g/L for isolate MLK-07. The lignin peroxidase activity and fungal growth increase with increasing D-xylose concentration up to 3.0 and 4.0 g/L respectively for fungal isolate MLK-01 and beyond that there are no significant changes in lignin peroxidase activity as well as in fungal growth. The lignin peroxidase activity for fungal isolate MLK-07 increases up to a D-xylose concentration of 3.0 g/L and then declines. Whereas the growth of fungal isolate MLK-01 increases up to D-xylose concentration of 5.0 g/L

and then remains constant. The addition of D-galactose to the medium represses the fungal growth and extracellular enzyme activities of both fungal isolates. It means that D-galactose is a poor medium for fungal growth, as well as enzyme production.

Generally, the reducing sugars act as a catabolic repressor for xylanase as well as cellulase production. Catabolic repression by glucose and xylose was also reported by other authors.[26] De Souza *et al*. found a resistance of *Aspergillus tamari* to catabolic repression with glucose when wheat bran was used as the sole substrate in solid-state fermentation condition.[27] Some other researchers reported the induction of cellulase and xylanase by homo- and hetero-disaccharides composed of glucose and xylose.[21] For both fungal isolates, the lignin peroxidase activity was found to increase with the addition of D-xylose and D-glucose compared to the control. From the above results it has been concluded that the lower concentration of reducing sugars consumed during primary metabolic events increased the fungal mycelium growth. On the other hand, lignin peroxidase was produced as a secondary metabolite when the culture medium was deficient of carbon, sulphur or nitrogen; while the lignin peroxidase activity increased due to the increase in mycelial growth.[28]

Effect of different lignocelluloses as substrate on fungal growth and enzyme production

The decrease xylanase and cellulase activity pattern for fungal isolate MLK-01 on different lignocelluloses is as follows: wheat bran>ground nut shell>bagasse>wheat straw>wood dust, and for isolate MLK-07 as: wheat bran> bagasse> ground nut shell>wheat straw>wood dust (Figs.3-4). The descending order for lignin peroxidase and fungal growth of isolate MLK-01 on different lignocelluloses is as follows: wheat bran> bagasse> wood dust>ground nut shell>wheat straw, and for isolate MLK-07 as: wheat bran>bagasse>wheat straw>wood dust>ground nut shell. The maximum xylanase and cellulose activities on wheat bran for both fungal isolatesMLK-01 and MLK-07 are 26.24 and 0.32 IU/mL and 30.43 and 0.36, respectively. The few key factors that affect enzyme activity are: openness and accessibility of the substrate, the rate and amount of xylo-oligosaccharides release and their chemical nature, and the amount of xylose released, which acts as carbon source and as inhibitor of xylanase synthesis in most cases. Usually, the slow release of inducer molecules and the possibility of the culture filtrate to convert the inducer to its non-metabolizable derivative are believed to boost up the level of xylanase activity.[29]

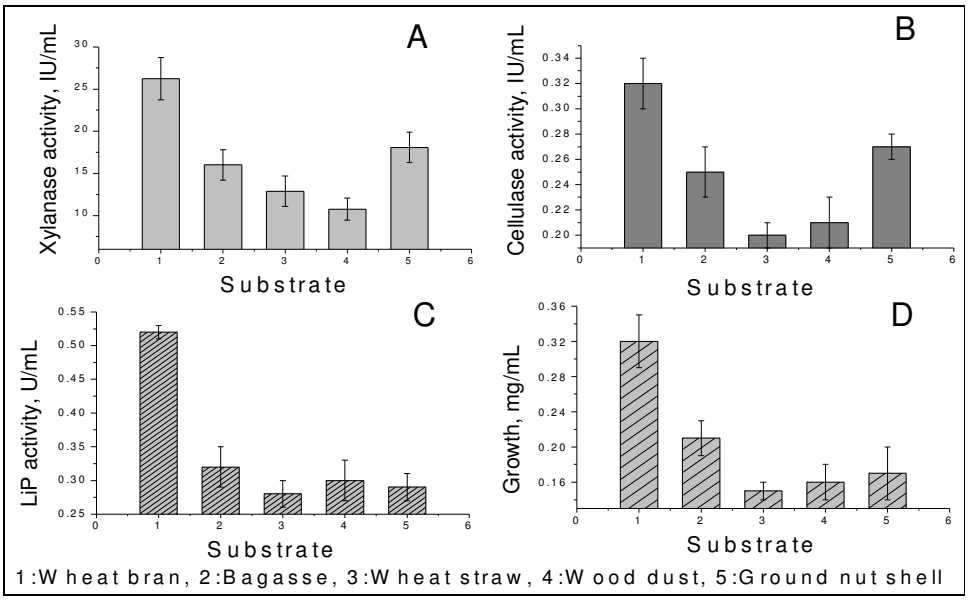

Figure3: Effect of different lignocellulosic substrates on the production of (A) xylanase, (B) cellulase, (C) lignin peroxidase and (D) growth of strain MLK01 at initial pH 10.0 after 8 days of incubation at 40°C

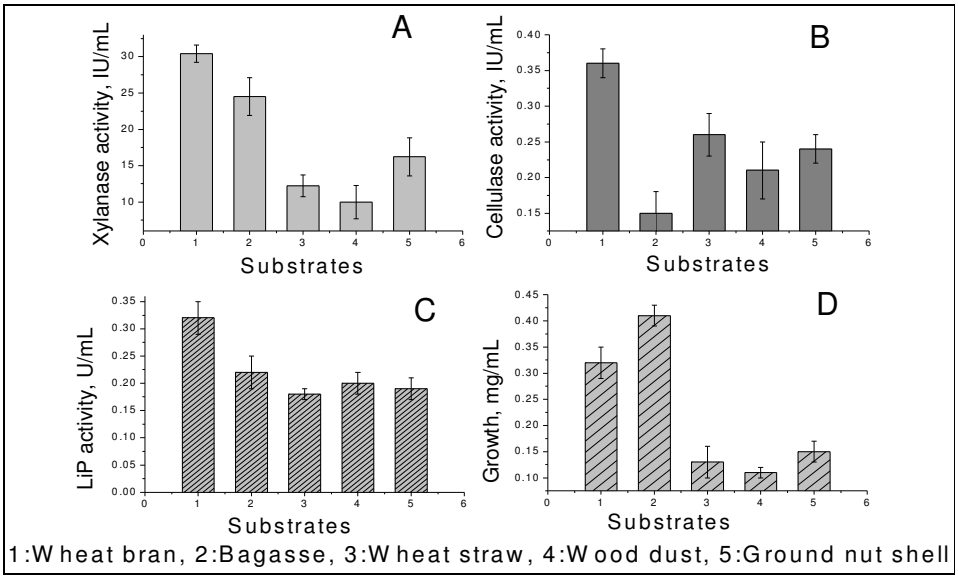

Figure4: Effect of different lignocellulosic substrates on the production of (A) xylanase, (B) cellulase, (C) lignin peroxidase and (D) growth of strain MLK07 at initial pH 10.0 with 2% substrate after 9 days of incubation at 40 °C

Effect of pH on xylanase activity

The crude xylanase obtained from both fungal isolates are active over a wide pH range of 4.0 to 9.0 with the maximal activity of isolate MLK-01 found at pH 7.5 and for isolate MLK-07 at pH 8.0. Xylanase from both fungal isolates MLK-01 and MLK-07 retain 67.56 and 60.16% of their relative activities at pH 5.0. On the other hand, at pH 9.0, xylanases obtained from both fungal isolates retain 60.49 and 74.09% of their relative activities (Figure 2). Both xylanases are found to lose about 70-75% of their activities at pH 9.5, while maintaining about 30% of their activities at pH 4.0. The above results indicate that xylanase produced from both isolates are alkaline in nature. However, under harsh conditions, such as change in pH, high temperature or presence of a high concentration of metal ions,denaturation of protein structure and subsequently, loss of active sites may occur, which may result in the loss of enzyme activity. Since enzymes are proteins, the ionic character of the amino and carboxylic acid groups on the protein surface are likely to be affected by the pH change and the catalytic property of the enzyme is markedly influenced. The pH activity profiles of the enzyme are highly dependent on pKa value of the catalytic residues, which are themselves dependent on the local environment and hence, on the nature of the amino-acids in the vicinity of the catalytic residues. The lower the pKa value, the higher the pH stability.[30] Gupta *et al*. reported an alkali-stable xylanase from a haloalkalophilic *Staphylococcus* sp., which exhibited dual pH optima at 7.5 and 9.5.[31] Broad pH optima of 7.5-9.0, 7.0-9.5 and 6.0-10.0 have been reported in xylanase from *Micrococcus* sp. AR-135.[32]

Effect of temperature on xylanase activity

The maximum xylanase activity of isolates MLK-01 and MLK-07 are found at 75 and 65°C respectively. Xylanase from isolate MLK-01 shows 70.04% of its relative activity at 45°C. On the other hand, xylanase from isolate MLK-07 exhibits 84.95% of its relative activity at 45°C. Xylanase from fungal isolate MLK-01 retains 94.11% of its relative activity at 85°C. In the same way, xylanase from isolate MLK-07 retains 65.07% of its relative activity at 75°C. It means that xylanase obtained from fungal isolate MLK-01 is more thermo-tolerant compared to MLK-07 and xylanasefrom both fungal isolates maybe considered as thermozyme. The thermo-stability of enzymes seems to be a property acquired by a protein through a combination of many small structural modifications that are achieved with the exchange of some amino acids. The variation of the canonical forces, *e.g*. hydrogen bonds, ion-pair interactions, hydrophobic interactions, found in thermozymes provided resistance to them at high temperature.[33] Because of their stability at higher temperature, thermozyme reactions were less susceptible to microbial contamination and

often exhibited higher reaction rates than mesozymecatalyzed reactions.[33]

Effect of metal ions on xylanase activity

In pulp and paper industry, pulp itself and process water contain a number of metal ions, which affects the enzyme accessibility on pulp during biobleaching. Aiming this, the effect of some common metal ions on xylanase activity was examined. Metal ions like Zn^{2+} and $Fe^{3+}$are strong stimulators; they enhance the relative xylanase activity from 179.4 to 200.2% for both fungal strains (Table 3). The decreasing order of metal ions, which stimulates xylanase activity is: Zn^{2+}, Na^{+} > Fe^{+2} > Ni^{2+} > Na^{+}>K^{+}> Pb^{2+}>Ca^{+2}> Mn^{+2}> Mg^{2+} for isolate MLK-01, and Fe^{+2} > Zn^{2+} Ni^{2+} >K^{+}> Na > Mg^{+2}> Mn^{+2}> Pb^{+2}> Ca^{+2} for isolate MLK-07. These ions may bind to the enzyme, causing conformational changes that result in increased enzyme activity. Khandeparkar and Bhosle also reported similar results for *Enterobacter* sp. MTCC 5112.[33] On the other hand, Hg^{2+} inhibits about 80% relative enzyme activity in the case of both isolates. Cu^{2+} is found to inhibit the relative enzyme activity by 17.8% and 29.5% for isolates MLK-01and MLK-07, respectively. The activation of enzyme also depends on maintaining the integrity of sulphydryl (-SH) groups in the enzyme protein since these groups constitute the active centre of the enzyme. When sulphydryl (-SH) groups are oxidized to the disulphide (-S-S-), the enzyme becomes inactive. The inhibition of the enzyme activity by metal ions, which reacted with sulphydryl groups of enzyme such as Hg^{2+} ions, suggested that there was an important cysteine residue in or close to the active sites of the enzyme. Similar results have also been reported by other researchers.[31,32]

CONCLUSION

Both fungal strains MLK01 and MLK07 are xylanase, cellulase, mannanase, laccase, lignin peroxidase and amylase producers. Both isolates produced maximal fungal growth and xylanase activity with minimal cellulase contamination at incubation periods of 8 and 9 days, respectively, and pH 10.0 with wheat bran as substrate under submerged fermentation conditions, while cellulase and lignin peroxidase activities were found to be maximum between pH 4-5. All the sugars (xylan, glucose, xylose, and galactose) were found to repress the xylanase as well as cellulase activities. The lignin peroxidase activity increased by 4 times in the presence of xylose, while lignin peroxidase activity in the presence of glucose and xylan separately increased by 2 and 3 times respectively. Wheat bran was found to be the best substrate for mycelial growth and xylanase production and the maximum xylanase activity was observed to be 30.32 IU/mL for strain MLK01 and 36.87 IU/mL for strain MLK07. Metal ions like Zn^{2+} and $Fe^{3+}$are strong stimulators; they enhanced the relative xylanase activity from 179.4 to 200.2% for both fungal strains

ABBREVIATIONS

BOD:	Biochemical oxygen demand
CMCase:	Caboxylmethylcellulase
IU:	International unit
SmF:	Submerged fermentation

REFERENCES

[1] S. Singh, D. Dutt and C.H. Tyagi, *BioResources*, **6**, 3876 (2011).

[2] M. Nasman, S. Backa and M. Ragnar, *Nordic Pulp Pap. Res. J.*, **22**, 42 (2007).

[3] D. Dutt, C.H. Tyagi, R.P. Singh and A. Kumar, *Cellulose Chem. Technol.*,**46**, 611 (2012).

[4] M. Lal, D. Dutt and C.H. Tyagi, *World J. Microbiol. Biotechnol.*, **28**, 1375 (2012).

[5] S. Subramaniyam and P. Prema, *Crit. Rev. Biotechnol.*, **22**, 33 (2002).

[6] S. Singh, B. Pillay and B.A. Prior, *Enzyme Microbial. Technol.*, **26**, 502 (2003).

[7] C. Techapun, T. Charoenrat, M. Watanabe, K. Sasaki and N. Poosaran, *Biochem. Eng. J.*, **12**, 99 (2002).

[8] S.K. Ghatora, B.S. Chadha, A.K. Badhan, H.S. Saini and M.K. Bhat, *Bioresour. Technol.*, **1**, 18 (2006).

[9] N. Kulkarniand M. Rao, *J.Biotechnol.*,**51**, 167 (1996).

[10] R. Maheshwari, J. Bharadwajand M.K. Bhat, *Mol. Biol. Rev.*, **64**, 461 (2000).

[11] N.A. Bennett, J. Ryan, P. Biely, M. Vrsanska, L. Kremnicky*et al.*, *Carbohyd. Res.*, **306**, 445 (1998).

[12] H. Kaur, D. Dutt and C. H. Tyagi, *BioResources*, **6**, 1376 (2011).

[13] K. Nagai, T. Sakai, R. M. Rantiatmodjo, K. Suzuki, W. Gams *et al.*, *Mycoscience*, **36**, 247 (1995).

[14] A. S. Ball and A. J. McCarthy, *J. Gen. Microbiol.*,**134**, 139 (1998).

[15] O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265(1951).

[16] G.L. Miller, *Anal. Chem.*, **31**, 238 (1959).

[17] D.K. Mercer, M. Iqbal, P.G.G. Miller, and A.J. McCarthy, *Appl. Environ. Microbiol.*, **62**, 2186 (1996).

[18] K. Horikoshi, *Microbiol. Mol. Biol. Rev.*,**63**, 735 (1999).

[19] United States Department of Agriculture (USDA) Food Survey Research Group (FSRG), 2006. The USDA food and nutrient database for dietary studies, 2.0documentation and user guide. http://www.ars.usda.gov/ba/bhnrc/fsrg (retrieved 2007-02-05).
[20] W. Steiner, R. M. Lajerty, I. Gomes, and H. Esterbauer, *Biotechnol. Bioeng*., **30**, 169 (1987).
[21] M. Hrmova, P. Biely, M. Vranskfi, and E. Petrfikovfi, *Arch. Microbiol*., **144**, 307 (1986).
[22] J. C. del Rio, A. Gutiérrez, M. J. Martinezand A. T. Martínez, *J. Anal. Appl. Pyrol*., **58**, 441 (2001).
[23] M.C.N. Saparrat, A.M. Arambarri and P.A. Balatti, *Biol. Fertil. Soils*, **44**, 383 (2007).
[24] V.T. Antonopoulos, M. Hernandez, M.E. Arias, E. Mavrakos and A.S. Ball, *Microbiol. Biotechnol.* ,**57**, 92 (2001).
[25] T.K. Kirk and H.H. Yang, *Biotechnol. Lett*., **1**, 347 (1987).
[26] M.K. Kadowaki, C.G.M. Souza, R.C. Simao and R. M. Peralta, *Appl. Biochem. Biotechnol.* ,**66**, 97 (1997).
[27] D. F. de Souza, C. G. de Souza and M.R.M. Peralta, *Process Biochem*., **36**, 835 (2001).
[28] T.W. Jeffries, S. Choi and T.K. Kirk, *Appl. Environ.Microbiol*., **42**, 290 (1981).
[29] N. Kulkarni, A. Shendye and M. Rao, *FEMS Microbiol. Rev*., **23**, 411 (1999).
[30] T. Collins, C. Gerday and G. Feller, *FEMS Microbiol. Rev*., **29**, 3 (2005).
[31] S. Gupta, B. Bhushan and G.S. Hoondal, *J. Appl. Microbiol*., **88**, 325 (2000).
[32] A. Gessesse, and G. Mamo, *J. Ind. Microbiol. Biotechnol*., **20**, 210 (1998).
[33] R. Scandurra, V. Consalvi, R. Chiaraluce, L. Politi and P.C. Engel, *Biochimie*, **80**, 933 (1998).
[34] R. Khandeparkar and N.B. Bhosle, *Res. Microbiol*., **157**, 315 (2006).