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Xylanases of marine fungi of potential use for biobleaching of paper pulp

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Abstract Microbial xylanases that are thermostable, active at alkaline pH and cellulase-free are generally preferred for biobleaching of paper pulp. We screened obligate and facultative marine fungi for xylanase activity with these desirable traits. Several fungal isolates obtained from marine habitats showed alkaline xylanase activity. The crude enzyme from NIOCC isolate 3 (*Aspergillus niger*), with high xylanase activity, cellulase-free and unique properties containing 580 U l^{-1} xylanase, could bring about bleaching of sugarcane bagasse pulp by a 60 min treatment at 55°C, resulting in a decrease of ten kappa numbers and a 30% reduction in consumption of chlorine during bleaching. The culture filtrate showed peaks of xylanase activity at pH 3.5 and pH 8.5. When assayed at pH 3.5, optimum activity was detected at 50°C, with a second peak of activity at 90°C. When assayed at pH 8.5, optimum activity was seen at 80°C. The crude enzyme was thermostable at 55°C for at least 4 h and retained about 60% activity. Gel filtration of the 50–80% ammonium sulphate-precipitated fraction of the crude culture filtrate separated into two peaks of xylanase with specific activities of 393 and 2,457 U (mg protein)$^{-1}$. The two peaks showing xylanase activity had molecular masses of 13 and 18 kDa. Zymogram analysis of xylanase of crude culture filtrate as well as the 50–80% ammonium sulphate-precipitated fraction showed two distinct xylanase activity bands on native PAGE. The crude culture filtrate also showed moderate activities of β-xylosidase and α-L-arabinofuranosidase, which could act synergistically with xylanase in attacking xylan. This is the first report showing the potential application of crude culture filtrate of a marine fungal isolate possessing thermostable, cellulase-free alkaline xylanase activity in biobleaching of paper pulp.

Keywords Marine fungi · Thermostable · Cellulase-free xylanase · Biobleaching · *Aspergillus niger*

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Introduction

The paper and pulp industry is a potential source of major pollution, generating large volumes of intensely coloured effluent for each metric ton of paper produced. In the production of paper, residual lignin from wood pulp is chemically liberated by using chlorine bleaching. Elemental chlorine reacts with lignin and other organic matter in the pulp, forming chlorinated compounds that are extracted with alkali. Only about 40–45% of the original weight of the wood goes into paper production and therefore the effluent produced is rich in organic matter [1]. The effluents have high biological oxygen demand (BOD) and chemical oxygen demand (COD), chlorinated compounds measured as adsorbable organic halides, chlorinated lignin derivatives such as chlorolignols, dioxins, and sulfur compounds. Some of these compounds are carcinogenic, toxic and recalcitrant to degradation and are known as persistent organic pollutants.

In response to stricter government rules regarding pollution control and to public awareness, paper mills are substituting elemental chlorine bleaching with ozone and oxygen delignification, and hydrogen peroxide bleaching. Biobleaching is an important alternative to reduce the use of chlorine and chlorine compounds in the bleaching process. Biological bleaching is carried out using white-rot fungi to degrade residual lignin in the pulp by using ligninolytic enzymes such as manganese peroxidase and laccase, or by using hemicellulolytic enzymes such as xylanases [5].

Xylan is the predominant hemicellulose in both hardwoods and softwoods that are used for paper

making. The cellulose fibres are protected by lignin and hemicellulose. The bond between lignin and hemicellulose is primarily between lignin and xylan, which can be removed by xylanase. For this reason, xylanase is the most important hemicellulase. Once this layer of hemicellulose is removed, the lignin layer is easily available for the degradative action of ligninolytic enzymes [9]. In addition, during chlorine bleaching, the exposed lignin layer thus requires less chlorine to achieve the required brightness. As a result, reduced amounts of chlorinated compounds of lignin are discharged as effluent, causing less environmental pollution and damage.

For pulp treatment, xylanase must be free of cellulase. Xylanase treatment is carried out after the initial alkali extraction of the pulp at high temperatures and therefore xylanase that is active at alkaline pH and stable at high temperature, as well as being active in the presence of residual sulfated lignin compounds, is highly desirable [28]. Effluent discharged after alkaline extraction of wood pulp containing residual sulfated lignin is called black liquor. The pulp going on to further enzymatic treatment contains traces of this black liquor that might be inhibitory to the enzyme. Therefore, xylanase active in the presence of trace amounts of black liquor is also desirable [28].

A few terrestrial fungi having cellulase-free alkaline xylanase activity are reported [3, and references therein]. The use of xylanases derived from fungi occurring in the alkaline pH environment of marine habitats has received little attention. Our objective was therefore to explore the potential of these fungi as a source of alkaline xylanases suitable for use in the biobleaching of paper pulp.

Materials and methods

Materials

All chemicals used were either from Sigma (St Louis, Mo.) or of analytical grade from other reputable companies.

Isolation and initial screening of fungi

Fungi were obtained from detritus of decaying mangrove leaves and other plant parts from the mangrove swamps of Chorao Island, Goa, India, and from calcareous material from 850 m depth in the Bay of Bengal. Isolation of fungi was carried out as described earlier by surface sterilisation of detritus using 0.5% sodium hypochlorite solution for 2 min, followed by two washings with sterile sea water [22]. The detritus was plated on corn meal agar medium (Himedia, Mumbai, India) prepared with half-strength sea water and supplemented with 0.05 g streptomycin sulfate and 10,000 units (U) sodium benzyl penicillin in 100 ml medium to inhibit bacterial growth. Fungi thus obtained were initially screened for xylan-degrading activity by growing them on basal medium containing 1% oat spelt xylan (Sigma, St. Louis, Mo.) at 28–30°C. The basal medium contained the following ingredients (l^{-1}): 2 g KH_2PO_4, 1.45 g $MgSO_4{\cdot}7H_2O$, 0.132 g $CaCl_2{\cdot}2H_2O$, 1 mg thiamine-HCl, 28 ml 1.2 mM ammonium tartrate, 18 g agar. The medium was prepared with distilled water and adjusted to pH 4.5 with 20 mM sodium acetate buffer or to pH 8.5 with 20 mM glycine-NaOH buffer as required. As these isolates were obtained from marine habitats, production of xylan-degrading enzymes under marine conditions was tested, wherein the same media were prepared in natural half-strength sea water at a salinity equivalent to 15 parts per thousand (which is the average salinity in mangrove swamps) or 30 parts per thousand for growing the deep sea isolate. Clearance zones produced around the fungal colonies in these media identified the isolates as xylanase positive [14]. The fungi were identified using conventional taxonomic identification keys [7, 17].

Production of xylan-degrading enzyme

The xylan-clearing cultures were grown at 30°C for up to 7 days in basal medium (pH 4.5 or 8.5) containing 1% oat spelt xylan suspended in natural half-strength or full-strength sea water or distilled water. Production of xylanase in different xylan sources such as breakfast oats (commercial preparation), birchwood xylan (Sigma) and sugarcane bagasse powder (supplied by Pudumjee Paper Mills, Pune, India) suspended in distilled water or half-strength sea water was also carried out with some of the isolates. The cultures were filtered through Whatman GF/F filters, the filtrate centrifuged at 9,000 *g* for 15 min at 5°C and the clear supernatants used for enzyme assay. All the assays were carried out in triplicate and repeated at least twice for confirmation. Xylanase from one of the isolates with highest activity was partially purified and characterised. For this purpose, the culture was grown in basal medium prepared with half-strength sea water containing oat spelt xylan.

Enzyme assays

Xylanase activity was assayed using 3,5-dinitrosalicylic acid (DNSA) to measure the amount of xylose-equivalent reducing sugars liberated from oat spelt xylan (Sigma). The enzyme preparation, containing up to 10 μg crude enzyme protein, was used for measuring the activity at pH 3.5 (50 mM sodium acetate buffer) or at pH 8.5 (20 mM glycine-NaOH buffer) at 50°C for 30 min. One unit of activity (U) is defined as the amount of enzyme capable of releasing reducing sugars equivalent to 1 μmol xylose per minute under the conditions described [21]. All results are averages of triplicate measurements. Protein concentrations were measured by

the method of Lowry et al. [19] using bovine serum albumin as the standard.

β-D-Xylosidase and α-L-arabinofuranosidase activities in the crude culture filtrate of one of the isolates were assayed at 50°C at pH 4 in acetate buffer using *p*-nitrophenol-*β*-D-xylopyranoside and *p*-nitrophenol-α-L-arabinofuranoside, respectively. The reaction was terminated by adding glycine-NaOH buffer adjusted to pH 10.8 and the absorbance of *p*-nitrophenol released was read at 430 nm [27]. One unit of activity (U) is defined as the amount of enzyme capable of releasing 1 μmol nitrophenol per minute under the conditions described. Cellulase activity was determined according to the standards of the International Union of Pure and Applied Chemistry [13].

Effect of black liquor on xylanase activity

Black liquor (obtained from Pudumjee Paper Mills, Pune, India) at final concentrations of 0.1 and 0.2% was added to an enzyme reaction mixture containing buffer and oat spelt xylan. Xylanase activity was measured after incubation for 30 min as described above.

Enzyme characterisation

To study the properties of xylanase, the culture filtrates from cultures grown in oat spelt xylan medium prepared in half-strength sea water were used. The effect of pH on the reaction was studied in the presence of different buffers. The buffers used were 50 mM citrate (or acetate) buffer (pH 2.5–5.5), 100 mM phosphate buffer (pH 6.5–7.5) and 25 mM borate buffer (pH 8.5–10.5). The reaction of DNSA with known amount of xylose at different pH was also carried out to check the sensitivity of the DNSA assay to pH. The effect of temperature on the reaction was assessed by incubating the reaction mixtures (pH 3.5) at different temperatures in the range of 30 to 100°C. Thermostability was monitored by incubating the enzyme at pH 3.5 for the specified period at different temperatures followed by rapid cooling in ice and carrying out the routine assay at 50°C and pH 3.5. Enzyme blanks and substrate blanks were similarly treated at the corresponding pH values and temperatures for the same lengths of time and their absorbances subtracted from those of reaction mixtures to arrive at actual enzyme activities.

Enzyme purification

The crude culture filtrate was brought to 50–80% saturation with ammonium sulphate. The pellet obtained after centrifugation was suspended in a minimum volume of 50 mM acetate buffer (pH 4.5) and loaded on a 130 ml bed volume Sephadex G-100 column at a flow rate of 20 ml h^{-1}. The protein was eluted with acetate buffer and 3 ml fractions were collected. The fractions were monitored at 280 nm for protein and assayed as above for xylanase activity. The active fractions were pooled and concentrated by saturating to 80% with ammonium sulphate. All the above operations were carried out at 4°C in a cold room.

Gel electrophoresis

Polyacrylamide gel electrophoresis on native gels (10% separating gels) was carried out in Tris-glycine buffer as described by Sambrook et al. [26], and the gels subjected to silver staining to visualise the protein bands.

For in situ detection of the enzyme, electrophoresis was carried out at 8°C on native composite gels of 10% polyacrylamide containing 1% xylan. The gels were then incubated in 50 mM acetate buffer (pH 4.5) for 30 min at 50°C. After a further incubation for 30 min (at room temperature) in a 0.1% Congo red solution, the gels were destained in 1 M NaCl until clearance bands of xylanase activity were obtained [20]. We found that the bands were more prominent when the gels were finally transferred into 50 mM acetate buffer (pH 4.5) for a few minutes.

Estimation of molecular weight

Gel filtration of the protein standards alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and cytochrome *C* (12.4 kDa) was carried out on a Sephadex G-100 column as described above. The void volume was calculated using blue dextran as marker and a calibration curve prepared.

Pulp treatment and determination of kappa number

The pulp from sugarcane after extraction of sugar is called sugarcane bagasse and is used alone or mixed with wood chips for making paper. The screened, unbleached pulp from sugarcane bagasse (10% consistency) was incubated with crude culture filtrate at a ratio of 10 g pulp to 100 ml enzyme (containing 58 U xylanase, 0.32 U α-L-arabinofuranosidase and 26 U *β*-D-xylosidase activity) at 55°C for 1 h. After incubation, the pulp suspension was filtered through a Whatman No.1 filter paper and air-dried. The delignification was measured as change in kappa number, which is indicative of the extent of delignification and bleachability of the pulp. The kappa number is the volume of 0.1 *N* potassium permanganate solution consumed by 1 g moisture-free pulp under the conditions described in the standard procedure of Technical Association of Paper and Pulp Industries (TAPPI) test method T236-cm-85 [2]. The kappa number ×0.15 gives the percentage of residual lignin.

Results

Five marine fungi out of six screened showed substantial alkaline xylanase activity (Table 1). Of these, isolates 3, 9 and 289 showed xylanase activity when grown in a variety of xylan-containing substrates (Table 2). The crude enzyme preparation from the high xylanase-producing isolate 3 showed optimum activity at pH 3.5 with a second peak of activity at pH 8.5, where about 40% of the activity was detected (Fig. 1). There was no effect of pH on estimation of reducing sugars by the DNSA assay. The optimum temperature for enzyme activity was 50°C and a second peak of activity was seen at 90°C when measured at pH 3.5; when assayed at pH 8.5, maximum activity was observed at 80°C (Fig. 2). The activation energy up to 50°C was about 16 kJ mol^{-1} when the assay was carried out at pH 3.5. Thermostability studies in the absence of substrate showed that the enzyme retained 60–65% of its activity after 1–4 h incubation at 55°C (Fig. 3). At 60°C it retained only 28% of the activity at the end of 2 h and incubation at 70°C inactivated the enzyme within 1 h.

Xylanase activity of isolate 9 was optimum at pH 4.5 with another peak at pH 8.5 (Fig. 1). The optimum temperature for the activity was at 50°C (Fig. 4). Thermostability of the enzyme showed that only <40% of the maximum activity was retained between 1 and 3 h incubation at 55°C (Fig. 5).

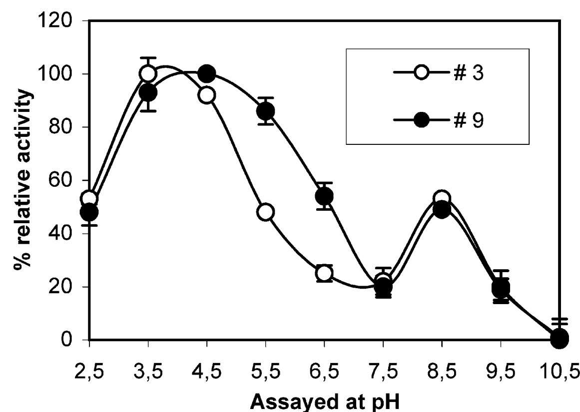


Fig. 1 Effect of assay pH on xylanase activity of culture filtrates from isolates 3 and 9. The enzyme was assayed at 50°C in a range of pH 2.5 to 10.5 as described in Materials and methods. *Bars* One standard deviation (1 SD)

As isolate 3, obtained from mangrove detritus, showed the best thermostability, it was studied further. Xylanase production was maximum at 15 ppt salinity, which is the normal salinity in its natural environment (Fig. 6). About 95–98% of conidia of this fungus ger-

Table 1 Xylanase production by marine fungi (U l^{-1})[a]

Isolate number	Identity	Source	Growth at pH 4.5		Growth at pH 8.5	
			Assayed at pH 3.5	Assayed at pH 8.5	Assayed at pH 3.5	Assayed at pH 8.5
3	*Aspergillus niger*	Mangrove detritus	737 ± 13	857 ± 27	745 ± 15	864 ± 10
289	Unidentified ascomycete	Mangrove detritus	613 ± 4	751 ± 19	594 ± 18	654 ± 21
312	*Flavodon flavus*	Decaying seagrass from a coral lagoon	177 ± 18	144 ± 10	207 ± 15	144 ± 17
313	*Gongronella* sp.	Mangrove sediment	282 ± 10	237 ± 16	351 ± 20	237 ± 30
321	*Halosarpheia ratnagiriensis*	Mangrove wood	57 ± 10	60 ± 5	45 ± 7	69 ± 9
9	*Aspergillus ustus*	Calcareous sediment from 860 m depth in the Arabian Sea	2,924 ± 40	1,533 ± 27	ND[b]	ND

[a]Unit of enzyme activity (U) expressed as μmol xylose equivalents released per minute
[b]No data

Table 2 Effect of substrate and salinity on the production of xylanase (U l^{-1}) by selected isolates

Substrate	Isolate number	Basal medium prepared with distilled water	Basal medium prepared with half-strength sea water[a]
Sugarcane bagasse	3	1,356 ± 178	1,245 ± 377
	9	1,295 ± 101	1,131 ± 97
	289	1,345 ± 77	1,395 ± 177
Commercial breakfast oats	3	958 ± 75	625 ± 65
	9	789 ± 79	534 ± 50
	289	702 ± 23	709 ± 37
Oat spelt xylan	3	1,624 ± 7	1,100 ± 41
	9	876 ± 45	1,297 ± 25
	289	699 ± 32	729 ± 41
Birchwood xylan	3	1,645 ± 58	1,369 ± 138
	9	790 ± 101	502 ± 87
	289	753 ± 85	637 ± 49

[a]Equivalent to 15 ppt salinity, which is the normal salinity in the mangrove ecosystem

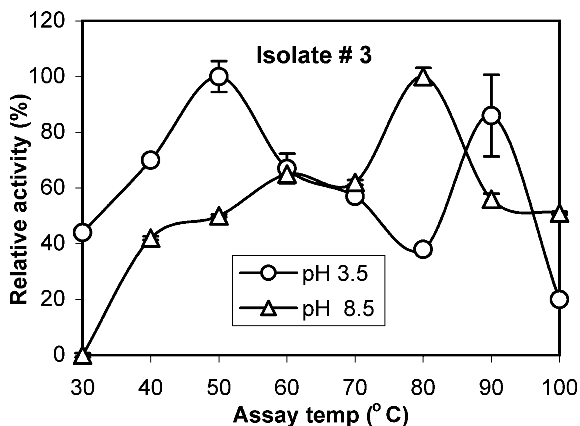


Fig. 2 Effect of assay temperature on xylanase activity in culture filtrate of isolate 3. The enzyme activity was assayed at pH 3.5 and 8.5 at temperatures ranging from 30 to 100°C for 30 min. The enzyme blank and substrate blanks were also incubated at these temperatures and the reducing sugars released were estimated using 3,5-dinitrosalicylic acid (DNSA) reagent. The values for the blanks were subtracted from the reaction mixture to calculate enzyme activity. *Bars* 1 SD

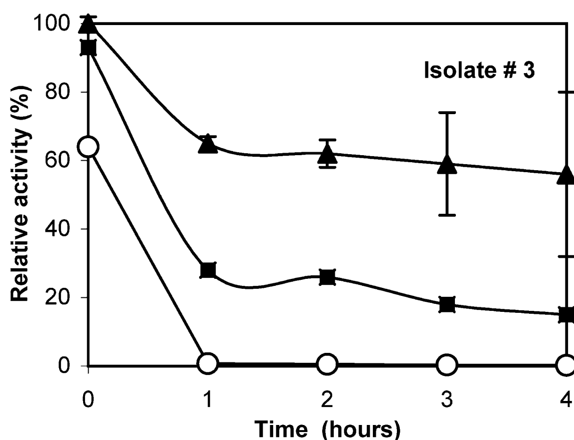


Fig. 3 Effect of temperature on xylananse stability in culture filtrate of isolate 3. The filtrate was incubated at different temperatures for various time periods followed by rapid cooling and measurement of residual activity by incubating with the substrate. ▲; 55°C, ■ 60°C, ○ 70°C. *Bars* 1 SD

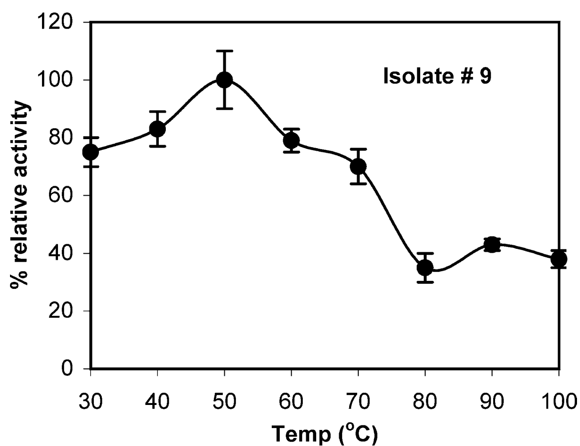


Fig. 4 Effect of assay temperature on xylanase activity in culture filtrate of isolate 9. *Bars* 1 SD

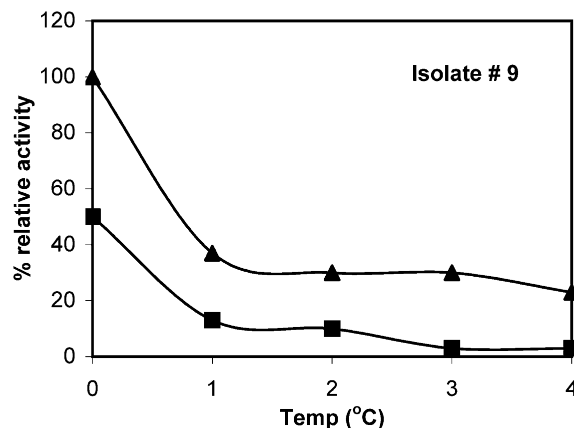


Fig. 5 Effect of temperature on xylanase stability in culture filtrate of isolate 9. The filtrate was incubated at different temperatures for various time periods followed by rapid cooling and measurement of residual activity by incubating with the substrate and buffer at 50°C for 30 min. ▲ 55°C, ■ 60°C

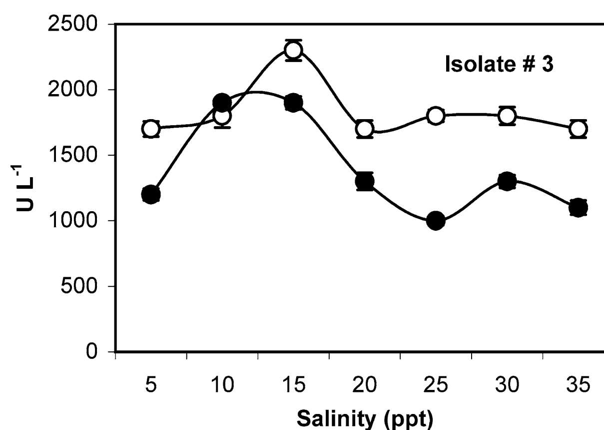


Fig. 6 Effect of salinity of the growth medium on xylanase production by isolate 3 when measured at pH 3.5 at 50°C (○) and 80°C (●). *Bars* 1 SD

Table 3 Ammonium sulphate fractionation of culture filtrate and further purification of xylanase from the isolate 3

Fraction	Total activity (U)	Specific activity (U mg protein^{-1})
Crude culture filtrate	48.8	3.8
0–35%	15.3	1.9
35–50%	2.6	5.8
50–80%	20.1	18.9
80–90%	2.0	5.8
Sephadex G-100 fraction (Xyl I)	6.1	393.3
Sephadex G-100 fraction (Xyl I)	2.4	2,457.0

minated within 6 h in 1% glucose solution prepared with half-strength, as well as full strength, sea water and also in distilled water. The fungal biomass in basal medium prepared with distilled water, half-strength and

Fig. 7 In situ detection of xylanase derived from culture filtrate of isolate 3 after non-denaturing electrophoresis on a polyacrylamide gel containing oat spelt xylan. Lanes: *1*, *2* Crude extract containing 78 and 156 μg protein, respectively; Lane *3* 0–35% ammonium sulphate fraction, 15 μg protein; Lane *4*, *5* 50–80% ammonium sulphate fractions containing 20 and 40 μg protein, respectively. The two xylanase activity bands (*Xyl I* and *Xyl II*) are prominent

full-strength sea water was 99, 87 and 157 mg respectively (dry weight in 20 ml medium on day 7). It also showed good growth on plain powdered sugarcane bagasse suspended in distilled water or half-strength sea water.

The crude culture filtrate of isolate 3 was tested for its biobleaching potential. The culture filtrate of this isolate grown on oat spelt xylan or sugarcane bagasse containing 580 U l^{-1} enzyme could bleach sugarcane bagasse pulp in a 60 min treatment at 55°C, resulting in a 30% reduction in chlorine consumption and a ten point reduction in kappa number (trials conducted independently by Pudumjee Paper Mills, Pune and Seshasayee Paper Mills, Chennai, India). The unbleached bagasse showed a kappa number of 48 and the enzyme-treated pulp showed a kappa number of 38. The drop in kappa number after biobleaching indicates reduction in chlorine consumption during further bleaching of the pulp.

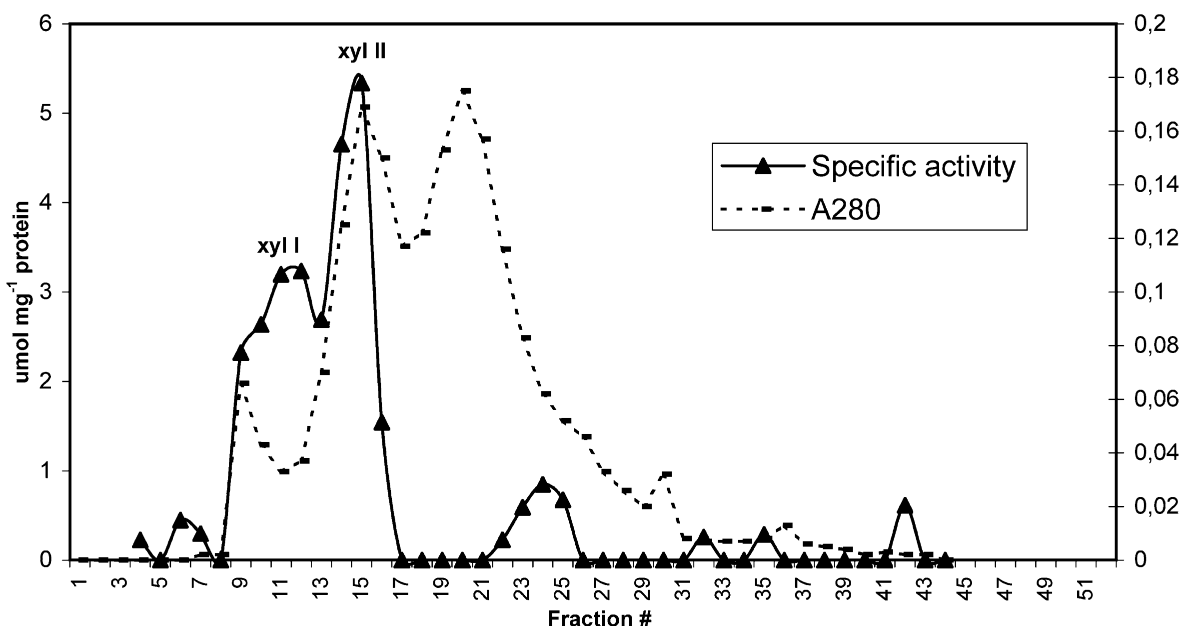
No cellulase activity on either CMC-cellulose or filter paper was detected in culture filtrate from isolate 3. β-Xylosidase activity in the filtrate was optimum at pH 4 and 70°C with about 260 U l^{-1} and α-arabinofuranosidase activity was optimum at pH 4 and 60°C with about 50 U l^{-1} produced in the filtrate of a 4-day-old culture. About 79 and 76% of the xylanase activity could be detected when the enzyme was assayed in the presence of 0.1 and 0.2% black liquor, respectively.

Partial purification of xylanase from isolate 3

Ammonium sulphate fractionation of the culture filtrate yielded maximum xylanase activity at 50–80% saturation (Table 3). Two bands of xylanase activity in the crude (lanes 1 and 2) and 50–80% ammonium sulphate fraction (lanes 4 and 5) and only one band of activity (lane 3) in the 0–35% fraction (Fig.7) were seen on a polyacrylamide gel containing 1% xylan.

The elution profile of the 50–80% ammonium sulphate fraction subjected to column chromatography on

Fig. 8 Gel filtration profile of the 50–80% ammonium sulphate fraction derived from culture filtrate of isolate 3 on a Sephadex-G100 column. Fractions (3 ml) were assayed for xylanase activity (π) and protein (A_{280})



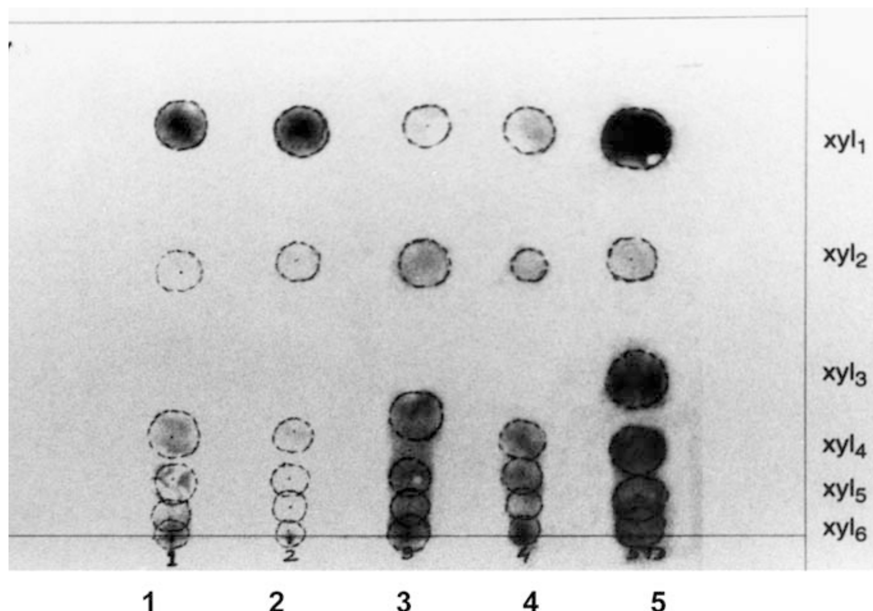


Fig. 9 Paper chromatography of the hydrolysis products of oat spelt xylan after incubation for 30 min with the 50–80% ammonium sulphate fraction derived from culture filtrate of isolate 3. Lanes: *1* Culture grown at pH 4.5 and assayed at pH 4.5/50°C; *2* culture grown at pH 4.5 and assayed at pH 4.5/80°C; *3* culture grown at pH 8.5 and assayed at pH 8.5/50°C; *4* culture grown at pH 8.5 and assayed at pH 8.5/80°C; *5* standard, containing a mixture of xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetrose (X_4), xylopentose (X_5) and xylohexose (X_6)

a Sephadex G-100 matrix showed three distinct protein peaks, two of which had xylanase activity (Fig. 8). The active fractions in each peak were pooled, concentrated by saturating them to 80% with ammonium sulphate and designated Xyl I and Xyl II. The Xyl I and Xyl II fractions showed specific activities of 393 and 2,457 U mg^{-1} protein, respectively (Table 3).

The xylanase-active peaks on Sephadex G-25 matrix corresponded to molecular weights of 18 and 13 kDa, respectively. These results indicate successful separation of two distinct xylanases. Purification by almost 104-fold for Xyl I and 647-fold for Xyl II activity with respect to the crude extract was achieved.

Degradation products of xylanase activity

The degradation products of oat spelt xylan incubated with xylanase from 50 to 80% ammonium sulphate fractions for 30 min at 50°C and 80°C at pH 3.5 and 8.5 were identified by paper chromatography as a mixture of xylose, xylobiose, xylotriose, xylotetrose and xylopentose (Fig. 9), confirming the xylanase to be an endoxylanase type. The presence of xylose also indicated β-xylosidase activity.

Discussion

The best xylanase-producing isolates were not obligate marine, but rather facultative marine fungi (*Aspergillus niger* and *Aspergillus ustus*). This was not surprising as many terrestrial species of fungi appear to have adapted themselves to marine conditions, where they play an important role. Such "facultative marine fungi" [17] are associated with coastal marine plant litter ecosystems [6, 10, 25]. Novel secondary metabolites have been isolated from marine isolates of terrestrial species of fungi [15]. Our present studies indicate that facultative marine fungi may produce enzymes with unique properties. The facultative marine nature of the strain of *A. niger*, NIOCC isolate 3, obtained from mangrove leaf detritus after rigorous surface sterilization, is further proved by the fact that the conidia showed germination in sea water and produced higher fungal biomass in sea water medium. Xylanase production was also seen in media prepared with natural sea water.

A number of species of *Aspergillus* isolated from terrestrial habitats produce xylanase in acidic media and also show activity under acidic assay conditions [4, 27]. Our marine isolate of *A. niger* produced xylanase in acidic as well as in alkaline media and the enzymes thus produced showed good activity at both acidic and alkaline pH. An extremely high specific activity (2,457 U mg^{-1} protein for Xyl II) appears to be a novel feature of the enzyme from this isolate [24]. High specific activity has also been reported for a xylanase from *Aureobasidium pullulans* (de Bary) Arnaud, which produces low molecular weight extracellular xylanase with a specific activity of 2,000 U mg^{-1} protein [18].

A. niger isolated from a terrestrial source was reported to produce endoxylanases I and II with low molecular weights of ca. 13 kDa [11]. Our strain of *A. niger* produced two endoxylanases with molecular masses of 13 and 18 kDa. The two endoxylanases of the terrestrial isolate of *A. niger* showed only one temperature optimum of 45°C and pH optima of 6.0 and 5.5 [11]. Xylanase activity in the crude culture filtrate of our isolate showed a pH optimum around 3.5 with a second peak of activity at pH 8.5. When assayed at pH 3.5, it showed a temperature optimum at 50°C and a second peak of activity at 90°C. At pH 8.5, the enzyme had optimum activity at 80°C. Up to 40°C the energy of activation for Xyl I and Xyl II was 30.4 ± 12.0 kJ

mol^{-1} and 38.8 ± 8.6 kJ mol^{-1}, respectively, for the terrestrial isolate of *A. niger* [11], while for our isolate, the activation energy up to 50°C was only about 16 kJ mol^{-1}. The terrestrial isolate did not produce xylose upon hydrolysis of xylan. On the contrary, the 50–80% ammonium sulphate fraction yielded xylose, as well as xylobiose, xylotriose, xylotetrose and xylopentose, upon hydrolysis of oat spelt xylan, confirming the presence of endoxylanase [20]. Xylose production might also be due to the activity of β-xylosidase in our marine isolate.

The stability of the enzyme for nearly 4 h at 55°C (Fig. 3) suggests that a crude enzyme solution could be used directly for bleaching of cooked paper pulp without requiring any substantial decrease in the temperature of the pulp. Crude culture filtrate of another strain of *A. niger* was reported to bleach Kraft pulp by virtue of its xylanolytic enzymes [8]. *Thermomyces lanuginosus*, which produces cellulase-free exo-and endo-xylanase, could bring down the kappa number of hardwood pulp from 20 to 17 and 13, respectively, when 100 and 300 U xylanase (g pulp)$^{-1}$ were used [29]. About 50 U xylanase from *Thermomonospora fusca* reduced the kappa number of softwood Kraft pulp from 18.5 to 13.4 within 2 h at 70°C [5]. Upon incubation with 10 g sugarcane bagasse pulp, about 100 ml of the crude culture filtrate of our strain, with 58 U endoxylanase, 26 U β-xylosidase and 0.32 U α-L-arabinofuranosidase activities, reduced the kappa number from 48 to 38 within 1 h of incubation at 55°C. A crude culture filtrate having a xylan-degrading enzyme system (devoid of cellulase activity) that could be used in the biobleaching process would eliminate steps involved in purification of the enzyme(s), bringing down the costs involved in their production, thus increasing economic viability.

The presence of multiple xylanases has been reported in a number of fungal and bacterial isolates from terrestrial sources [30]. A species of *Cephalosporium* produced two xylanases varying in their pIs, K_m constants and molecular weights [16]. Similarly, a *Bacillus* species also produced two xylanases differing in their temperature and pH optima [12]. The presence of two pH and temperature optima, and the differing specific activity of the two peaks of xylanase activity separated on gel filtration, suggest the presence of multiple xylanases also in our isolate NIOCC 3. The total yield of Xyl I and Xyl II separated by column chromatography was not very high, largely because the focus was on elimination of other minor xylanase activities in the ammonium sulphate fractionation step. Such situations are commonly reported in the literature [11, 20], wherein a multiplicity of xylanase activities has been encountered.

We have previously reported fungi from marine habitats capable of producing lignin-modifying enzymes [22, 23]. The co-production of endoxylanase, β-D-xylosidase and α-L- arabinofuranosidase in a cellulase-free enzyme system by isolate 3 from marine habitats, with potential in biobleaching, indicates the importance of marine fungi in biotechnological applications. Although xylanase production is not very high in this isolate, its novel properties make it worthwhile to overproduce the enzyme through various approaches.

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