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SHORT COMMUNICATION

Biobleaching of banana fibre pulp using *Bacillus subtilis* C O1 xylanase produced from wheat bran under solid-state cultivation

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Abstract A cellulase-free xylanase produced by *Bacillus subtilis* C 01 from wheat bran under solid-state cultivation was tested for its efficacy in biobleaching of raw banana fibre and banana pulp obtained through a mechanical pulping process. Banana pulp samples treated with crude xylanase (450 nkat g^{-1} pulp) resulted in a 19.6% increase in the brightness as compared to untreated pulp. The presence of chromophores, hydrophobic compounds and an increased reducing sugar (10.79 mg g^{-1} pulp) quantity in the bleached solution after enzymatic treatment indicated the removal of materials that were absorbed at 237 nm from the banana pulp.

Keywords *Bacillus subtilis* · Solid-state cultivation · Banana pulp · Biobleaching · Brightness

Introduction

For decades, the consumption of paper and paper products has been increasing rapidly all over the world. To supply the consumers with sufficient amount of paper, the pulp and paper industries not only need to look for alternate feedstocks but also have to introduce environmentally sound methods in paper making processes. Apart from paper recycling, the use of secondary fibres for paper production has increased significantly. This will undoubtedly diminish the dependence on wood materials. Bananas are grown more than any other fruit crop in 132 countries worldwide. Banana is one of the major fruit crops grown in India and its fibre, a very good source of cellulose, has been found to be a promising alternative raw material for paper production. Banana stem fibres (after harvesting the fruit) are waste materials that pose a problem of disposal, and since there is a shortage of raw materials for paper making, the banana stem fibres can be used economically.

"Biopulping and biobleaching" are promising cost effective alternatives involving the use of microbes or their enzymes to reduce and/or replace the harmful chemical extraction of hemicelluloses and lignin without affecting the cellulose and fibre strength of paper products [3, 14, 25]. Among the microbial hemicellulases, xylanases are effective in biopulping and bleaching processes and widely distributed in both prokaryotes and eukaryotes [2, 4, 7]. The search for microbes with high level of xylanase and other desirable characteristics is being actively pursued for commercially viable industrial applications [13, 21].

The utilization of inexpensive lignocellulosic biomass for xylanase production would ultimately reduce the overall enzyme production cost. Various inexpensive substrates have been used for xylanase production [10, 19]. Biobleaching processes so far have been developed for both hard wood and soft wood pulps. The effective bleaching process depends on developing a specific xylanase system according to the chemical nature of raw materials; however, the exact mechanism of xylanase aided pulping and bleaching processes are still not completely understood [2, 4].

Cellulase-free xylanases have recently attracted considerable attention due to their potential application in biopulping and bleaching processes. A search for bacterial strains producing high levels of xylanase at alkaline pH condition

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resulted in the isolation of several *Bacillus* sp [22]. Thus, our present study was aimed at utilizing inexpensive substrates viz., wheat bran for cellulase-free xylanase production by *Bacillus subtilis* C 01 and checking the efficacy of enzymes in biobleaching banana fibre and its pulp in the existing biomechanical, ecofriendly paper production unit.

Materials and methods

Organism and growth conditions

Bacillus subtilis C 01, one of our own isolates, was used in this study. This strain was periodically subcultured in nutrient agar medium and maintained at 4°C.

Solid-state cultivation (SSC) and enzyme extraction

In a 5 l Erlenmeyer flask, 200 g of wheat bran substrate was taken and 180 ml of stock nutrient solution (pH 8.0) containing 0.3% KH_2PO_4, 0.6% K_2HPO4, 0.12% $MgSO_4.5H_2O$, 0.5% tryptone and 0.3% yeast extract was added to the substrate. A 10% (v/w) of 16 h old *B. subtilis* C 01 culture grown on tryptic soy broth was used as inoculum, thereby wheat bran to moisture ratio was maintained as 1:1 (all the liquid content added to the flask was taken into consideration for moisture ratio calculation) and incubated at 37°C for 72 h. The enzyme was extracted twice with 2 l of distilled water and each time the whole content was squeezed through a wet muslin cloth. The extract was then centrifuged at 10,000 g for 15 min. and the clear supernatant used as a crude xylanase source.

Enzyme assay

The xylanase activity was determined according to the method of Baily et al. [1]. The substrate solution contained 1% birchwood xylan (Sigma) dissolved in 0.05 M sodium phosphate buffer (pH 8.0). The reaction mixture consisted of 1.8 ml substrate solution and 0.2 ml of appropriately diluted enzyme. After 5 min incubation at 50°C, the liberated reducing sugars (xylose equivalent) were estimated by the DNS reagent method. Cellulase activity was determined according to the method of Ghose [11]. The enzyme activity was expressed in nkat. One nkat of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 nmol of xylose per second.

Materials used for biobleaching treatment

Banana (*Musa acuminata*, Musaceae) fibre from the plant stem was collected from local farms and washed thrice in hot water to remove the debris and water-soluble particles. Two types of materials were used: moisture-free raw banana fibre (500 g) after heating at 100°C for 15 min and banana fibre pulp (10% pulp consistency) prepared using a Hollander beater (Sheeba, India). These materials were treated with 450 nkat of crude xylanase g^{-1} of test samples at 50°C (pH 8) for 48 h, then subjected to a hand-made paper making process [23] and the final paper product was analyzed.

Optimization of xylanase dose and reaction time for biobleaching

The enzyme dosage and reaction time were optimized by treating the banana pulp with varying amount of crude xylanase ranged from 113 to 563 nkat g^{-1} moisture-free pulp for variable time intervals up to 12 h and the pulp properties were studied at every 1 h intervals. The release of reducing sugars was quantified by the method of Ghose [11] and the bleached solution analysed for its absorption maxima (λ_{max}) at 237 and 465 nm for chromophores and hydrophobic compounds, respectively, using a UV vis spectrophotometer (Chemito, India) against the control (pulp without enzyme) as a blank.

Brightness and opacity measurement

The brightness and opacity of the final paper product was measured using a reflectance meter [Pap Tech (Photo volt), India]. The ceramic reference TAPPI (Brightness: 47.5%) was used as a standard.

Results and discussion

B. subtilis C 01 produced high levels of xylanase (2,264 nkat g^{-1}) when grown on wheat bran as a solid support, but no cellulase production was observed. Banana fibre and its pulp treated with xylanase derived from *B. subtilis* C 01 grown on wheat bran showed a variation in the percentage of brightness (BR) and opacity (OP), as shown in Table 1. The fibres in raw banana fibre materials are highly integrated, whereas in the pulp made as a result of mechanical process, the fibres are separated. Hence, the pulp sample was more accessible to enzymatic attack than raw fibre. This could be one of the reasons for effective bleaching in pulp fibres. This conforms with the results of the treatment of fibres of various lengths with commercial xylanases, indicating that fibre composition played a vital role in the effectiveness of xylanase treatment [17].

Raw banana fibre and its pulp treated with xylanases

A raw banana fibre sample treated with crude xylanase obtained from *B. subtilis* C 01 showed 35.2% (Table 1)

Table 1 Effect of xylanase pretreatment on the brightness and opacity of paper sheets made from raw banana fibre and its pulp

Samples	Brightness (%)		Opacity (%)	
	24 h	48 h	24 h	48 h
Raw banana fibre (untreated)	27.2 ± 0.4	27.1 ± 0.21	38.6 ± 0.14	38.4 ± 0.21
Raw banana fibre + xylanase	34.8 ± 0.3	35.2 ± 0.14	44.2 ± 0.28	44.1 ± 0.35
Banana pulp (untreated)	29.5 ± 0.21	29.8 ± 0.24	39.3 ± 0.28	39.2 ± 0.21
Banana pulp + xylanase	49.4 ± 0.07	49.2 ± 0.35	58.3 ± 0.21	58.3 ± 0.14

For enzymatic treatment, 450 nkat of crude xylanase g^{-1} of test samples was kept at 50°C (pH 8) for 48 h. The data presented are average values of three replicate experiments and the corresponding standard deviations

brightness (BR) and 44.1% opacity (OP) when compared with control (27.2% BR; 38.6% OP). Similar results were obtained in xylanase pretreated banana fibre pulp. The paper produced from the pulp treated with xylanase showed 49.4% BR (Table 1) and 58.3% OP when compared with control (29.5% BR; 39.3% OP). In all the treatments, prolonged incubation (48 h) did not result in much difference in the BR and OP.

Optimization of xylanase dose for biobleaching of banana fibre pulp

Of the various dosages of enzyme tested, a maximum brightness of 49.1% was obtained with 450 nkat of crude xylanase g^{-1} of pulp, which was 19.6% higher than in untreated samples (Table 2). However, the increased dosage of enzyme (563 nkat g^{-1} of pulp) resulted only in a 0.1% increase in the brightness. Reducing sugar (10.82 mg g^{-1}), chromophores (1.01; A_{237} nm) and hydrophobic compounds (0.13; Ab_{465}) were observed as maximum in 450 nkat of crude xylanase g^{-1} of pulp treated samples (Fig. 1). Similarly, a 14% increase in the brightness was also obtained when the pulp treated with culture filtrate of *B. subtilis* contained both mannanase and xylanase [15].

The biobleaching efficiency of xylanase from our isolate *B. subtilis* C 01 was better as compared to biobleaching of bagasse pulp with xylanase of alkalophilic *Bacillus* sp., which showed 2.5% increase in the brightness [16]. Application of crude xylanase from *Bacillus licheniformis* for bleaching of kraft pulp resulted in 5% increase in brightness [8]. Interestingly, the xylanase of *B. subtilis* C 01 treated banana pulp samples resulted in 19.6% increase in the brightness as compared to untreated samples. Xylanase was found to be effective in biobleaching of banana fibre pulp due its chemical composition of 61.7% cellulose, 9.7% lignin and 14.9% of pentosans [20]. Since the fibre has more pentosans (14.9%), these are easily accessible to xylanase attack. The low molecular weight nature of the enzyme (results not shown) helps in promoting the entry of xylanases of *B. subtilis* C 01 into the interior part of the fibre very easily, which in turn results in the successful removal of material, with absorption at 237 nm from the banana pulp.

Table 2 Optimization of crude xylanase dose for biobleaching of banana fibre pulp at 50°C (pH 8) for 12 h

Xylanase dose (nkat)	Brightness (%)	Reducing sugar released (mg g^{-1}pulp)
Control	29.5 ± 0.27	0.86 ± 0.02
112.5	34.6 ± 0.14	2.86 ± 0.01
225	39.4 ± 0.25	5.69 ± 0.02
337.5	43.6 ± 0.28	8.57 ± 0.01
450	49.1 ± 0.24	10.82 ± 0.01
562.5	49.2 ± 0.15	10.83 ± 0.01

The data presented are average values of three replicate experiments and the corresponding standard deviations

Optimization of reaction time for biobleaching of banana fibre pulp

The results of varying reaction time for bleaching of banana pulp with crude xylanase from *B. subtilis* C 01 showed that the maximum brightness (49.3%) and release of chromophores (1.01 at Ab_{237} nm), hydrophobic compounds (0.14 at Ab_{465}) and reducing sugar (10.79 mg g^{-1} pulp) were noticed at 8 h (Table 3; Fig. 2). The extended incubation of crude xylanase with pulp did not contribute much to the brightness of the pulp. The results of chromophores, hydrophobic compounds and reducing sugar released and concomitant increase in the brightness indicate that the removal of material absorbing at 237 nm from the pulp fibres occurred during the treatment. There was no considerable weight loss in the pulp samples after enzymatic treatment. This would be an added advantage of *B. subtilis* C 01, compared with enzymatic bleaching of fungal culture filtrate, which resulted in a weight loss of 16% [12].

Several studies have been reported on kraft pulp bleaching with xylanase from different microbial sources, mostly for reducing the amount of chlorine requirement in the bleaching process, kappa number reduction and brightness enhancement [5, 9, 18, 24]. Literature on biobleaching of alternate cellulosic fibres are scanty. Studies on biobleach-

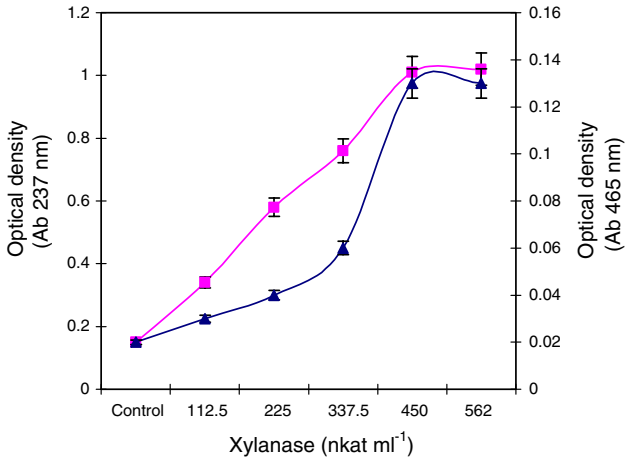

Fig. 1 Spectrum of chromophores (*filled square*) at 237 nm and hydrophobic compounds (*filled triangle*) at 465 nm released during treatment with different doses of crude xylanase g^{-1} of banana pulp at 50°C (pH 8) for 12 h. The data presented are average values of three replicate experiments and the corresponding standard deviations

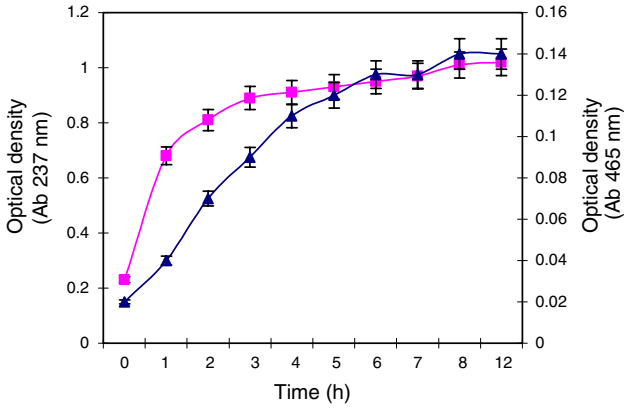

Fig. 2 Spectrum of chromophores (*filled square*) at 237 nm and hydrophobic compounds (*filled triangle*) at 465 nm released during crude xylanase treatment (450 nkat g^{-1} of banana pulp) at different time intervals at 50°C (pH 8). The data presented are average values of three replicate experiments and the corresponding standard deviations

Table 3 Optimization of reaction time for biobleaching of banana fibre pulp with crude xylanase (450 nkat g^{-1} of pulp) at 50°C (pH 8)

Incubation time (h)	Brightness (%)	Reducing sugar released (mg g^{-1} pulp)
0	29.5 ± 0.14	0.98 ± 0.01
1	31.9 ± 0.15	4.65 ± 0.01
2	34.4 ± 0.14	7.21 ± 0.02
3	36.6 ± 0.28	9.31 ± 0.01
4	39.3 ± 0.25	10.21 ± 0.01
5	41.9 ± 0.16	10.43 ± 0.02
6	44.2 ± 0.14	10.65 ± 0.09
7	46.8 ± 0.12	10.73 ± 0.01
8	49.1 ± 0.15	10.79 ± 0.03
12	49.3 ± 0.21	10.80 ± 0.03

The data presented are average values of three replicate experiments and the corresponding standard deviations

ing of bagasse pulp with xylanase of alkalophilic *Bacillus* sp. showed 2.5% increase in the brightness [16]. The application of crude xylanase from *Bacillus amyloliquefaciens* for bleaching of kraft pulp resulted in 5% increase in brightness [8]. Interestingly, the xylanase of *B. subtilis* C 01 treated banana pulp samples resulted in 19.6% increase in the brightness as compared to untreated samples.

In the enzyme aided bleaching of soft and hard wood kraft pulps with family 10 and family 11 xylanases and family 26 mannanases, it was found that xylanases belonging to family 11 were most effective in improving the final brightness [6]. The xylanase of *B. subtilis* C 01 also fit in to family 11 (results not shown) and was found efficient in biobleaching. The capability of pulp bleaching by crude xylanase preparation is an attractive option for pulp bleaching, because the organism can be easily grown on inexpensive substrates like wheat bran by SSC. Hence, the overall cost of xylanase production would be minimized.

For economic reasons, the paper industry remains almost exclusively dependent on the vegetable kingdom for its raw material. The majority of the pulp (50.5%) produced in India is from agro-based fibres [26]. These experimental results showed that xylanase from *B. subtilis* C 01 has the potential application in biopulping and bleaching of secondary fibres. Further studies on the combination of this xylanase with other non-chlorinated processes will help to develop a cost-effective, ecofriendly bleaching process.

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