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Production of Xylanase of *Bacillus coagulans* and its bleaching potential

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Summary

The production of xylanase from *Bacillus coagulans* has been studied with respect to the environmental parameters, the carbon source and the concentration of carbon source at the shake flask level. Among the various carbon sources used, wheat straw powder favoured higher enzyme production. Xylan isolated from wheat straw gave higher enzyme production as compared to the birchwood xylan. Maximum enzyme activity of 165 IU/ml was obtained with 2% wheat straw xylan in a shake flask study. Improvement of xylanase production was achieved by increasing the wheat straw powder concentration up to 3%. Enzyme has optimum activity at a temperature of 55 °C and pH of 7. The concentrated crude enzyme was found to reduce the kappa number of enzyme-treated eucalyptus pulp by 5.45% with a marginal increase in the CED viscosity of the enzyme treated pulp as compared to the non-enzymatically treated pulp.

Introduction

In the recent years, the potential of xylanase (EC 3.2.18) as a pre-bleaching agent in the pulp and paper industry was demonstrated on the commercial scale (Subramaniyan & Prema 2002). Most of the published literature on xylanase have reported on the bleach-boosting effect of the enzyme on hardwood and softwood Kraft pulps (Subramaniyan & Prema 2002). The beneficial effect of xylanase has also been reported on non-woody pulps (Chauhan *et al*. 2005). In all these biobleaching applications, either the conventional chlorine bleaching or the non-conventional total chlorine-free bleaching sequences (TCF) was used for the bleaching of pulp (Subramaniyan & Prema 2002). The bleach-boosting effect of the enzyme was explained in terms of either hydrolysis of re-deposited xylan or hydrolysis of the hemicellulose–lignin bond (Subramaniyan & Prema 2002). The suitability of xylanase for biobleaching applications is generally decided with respect to the enzyme stability at high optimum pH and temperature (Subramaniyan & Prema 2002). In the initial stage of development of xylanase as a pre-bleaching agent, fungal sources were explored, but due to cellulase contamination, low thermo-stability and low optimum pH, this source of enzyme finds limited acceptability (Subramaniyan & Prema 2002). In the last two decades, xylanases from extremophilic organisms had got most attention, due to better stability at higher pH and temperature (Subramaniyan & Prema 2002).

Most of the reported literature on xylanase have concentrated on the characterization of the enzyme and its application as a pre-bleaching agent. Relatively less attention was paid on the optimization of xylanase production. In the recent years, solid state fermentation (SSF) has got most attention for the xylanase production using fungal culture (Heck *et al*. 2005; Senthilkumar *et al*. 2005). Many of these SSF studies have concentrated on the parameter optimization using the statistical experiment design method. Another aspect of xylanase production, which was reported in the literature, is aqueous two-phase fermentation. Use of an aqueous two-phase system has resulted in 160 IU/ml of xylanase production and 97% of it partitioned in the upper phase (Oliveria *et al*. 2004). The expression of xylanase from thermophilic microorganisms in fungal and yeast hosts has also received major attention. *Pichia pastoris, S. cerevisiae* and *K. lactis* were used as hosts for the heterologous production of xylanase (Damaso *et al*. 2003; Gorgenes *et al*. 2005). Parameters responsible for the improved production of heterologous protein in yeast were studied and it was reported that amino acid supplementation in defined media favours higher production in *S. cerevisiae (*Gorgenes *et al*. 2005).

In the present study, an attempt was made to describe the optimization studies related to xylanase production from *B. coagulans* culture. Optimization of enzyme production was carried out with respect to pH, temperature, carbon source and concentration of

carbon source. Enzyme production profile in the fermentor under optimized conditions was studied. To evaluate the efficacy of the xylanase on eucalyptus kraft pulp, enzymatic treatment was carried out at optimum temperature and pH. Bleaching potential of the crude enzyme was assessed in terms of kappa number reduction with respect to the non-enzymatically treated pulp (control).

Materials and methods

In this work, xylanase was produced from *Bacillus coagulans*. The culture was kindly provided by Dr K.J. Mukherjee of the Centre for Biotechnology, Jawaharlal Nehru University, New Delhi, India. For *Bacillus coagulans* culture, medium with the following composition was used for the subculturing and the production of the enzyme. Birchwood xylan or other suitable carbon source: 10 g/l, yeast extract: 5 g/l, peptone: 5 g/l, K_2HPO_4: 1 g/l, $MgSO_4$: 0.2 g/l, pH 7. Initially, xylanase production using *Bacillus coagulans* was carried out at shake flask level for 48 h at 45 °C and 200 rev/min and the cell-free broth was used for the enzyme activity determination. Inoculation of shake flask was carried out from freshly prepared slants. The effects of various carbon sources on the enzyme production were tested by using sucrose, xylose, solka floc, lactose, carboxymethylcellulose, cellulose powder, wheat straw powder and rice straw powder. Wheat and rice straw were washed with water and dried and finally ground to powder form. Eucalyptus Kraft pulp was obtained from Star Paper Mills Ltd, Saharanpur, India.

Enzyme assay

The activity of xylanase was determined with 1% birchwood xylan in 50 mM phosphate buffer of pH 7 at 55 °C using the method described by Bailey *et al*. (1992). The enzymatic reaction was carried out for 15 min and the reducing sugar was determined using the DNS method (Miller 1959). 'Filter paperase' activity of the crude enzyme was measured by using the method recommended by IUPAC using filter paper as a substrate (IUPAC 1987). The reaction was carried out at 50 °C for 60 min.

Wheat straw xylan isolation

Dried powder of wheat straw was used for isolation of wheat straw xylan. The method followed was similar to the method described by Biely *et al*. (1988). Lyophilized wheat straw xylan powder was used in the shake flask and fermentor studies.

Fermentation experiments

All the fermentation was carried out in an INFORS 7.5-l fermentor with a working volume of 4 l. The autoclavable fermentor was sterilized at 121 °C for 20 min and 10% (v/v) inoculum (12 h grown culture in the xylose media) was used for inoculation. Temperature and pH were maintained automatically. The pH was maintained at the desired value by the addition of 2 M HCl and 2 M NaOH. Samples were collected at intervals and analysed for enzyme activity. After the fermentation was over, the cell broth was centrifuged at $15{,}000\times g$ for 20 min and the cell-free broth was used for further processing. After harvesting the broth, all operations were carried out at low temperature (<10 °C).

Enzymatic pre-bleaching

The pulps were subjected to the enzyme treatment in polythene bags. For this, two lots of pulp were taken. To one lot enzyme was added and the other lot as a control was subjected to similar conditions without enzyme. An enzyme dose of 7 IU/g OD pulp was used for all the studies and incubated for 1.5 h at 50 °C. After 1.5 h, pulps were washed with hot water. The Kappa number of the treated pulp was evaluated to assess the effect of enzyme treatment on lignin using TAPPI test methods, T236cm-85.

Results and discussion

Recent interest in xylanase as a pre-bleaching agent has resulted in a large number of publications related to the characterization of purified xylanase and its application in the biobleaching process (Subramaniyan & Prema 2002). Production aspects of xylanase have received relatively less attention as compared to the characterization and application studies (Techapun *et al*. 2003). In the case of xylanase production, the carbon source can play an important role as an inducer. In most of these studies, a hemicellulose source was used for higher enzyme productivity. Various hemicelluloses sources used are wheat straw, wheat bran, rice straw, corncob, oat spelt xylan, birchwood xylan, sugar cane bagasse, palm oil meal (Techapun *et al*. 2003).

In the present study, xylanase production was optimized with *Bacillus coagulans* culture at the shake flask level and the production profile was studied in the fermentor under optimized conditions. As, in our study, no standard soluble carbon sources (excepting xylose, lactose, sucrose and carboxymethylcellulose) were used as a substrate so no effort was made to determine the biomass and residual substrate concentration during the optimization studies. To avoid batch-to-batch variation in enzyme activity, a single stock of agri-residues (wheat straw and rice straw) was utilized. Besides the optimization of environmental parameters like pH and temperature, an effort was also made to search for the most suitable carbon source in the xylanase production using B*acillus coagulans* culture.

The optimization of pH and temperature for xylanase production was carried out with 1% wheat straw

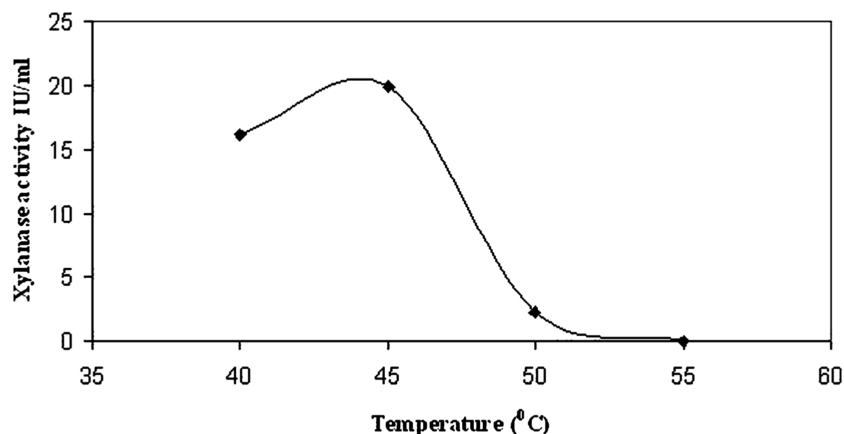

Figure 1. Effect of cultivation temperature on the xylanase production from *Bacillus coagulans* with 1% wheat straw powder at an initial pH of 7 in the shake flask. Standard deviations were within the range of 4–6%.

powder in the shake flask. To find the most suitable temperature, a study was carried out for 48 h within the temperature range of 40–55 °C at an initial pH of 7. Samples were collected at various time intervals and analysed for enzyme activity. Maximum enzyme production was obtained at around 45 °C within 24 h of incubation. From the result (Figure 1) it can be seen that beyond a temperature of 45 °C, enzyme production level decreased significantly. Similarly the production of enzyme was studied in the initial pH range of 6–8 with 1% wheat straw powder at a temperature of 45 °C. Although in the shake flask study, the pH was not controlled, yet there was a distinct optimum pH. From the result (Table 1) it can be concluded that the maximum enzyme production occurred at an initial pH of 7–7.5. However, in all these cases, the final pH increased to 8.7–8.9. The reported optimum pH and temperature for xylanase production from *Bacillus* cultures are in the range 6–10 and 28–50 °C respectively (Techapun *et al.* 2003). In all further enzyme production studies either in the shake flask or fermentor, the initial pH and the temperature were maintained at 7 and 45 °C respectively.

After optimizing the environmental parameters for enzyme production, the effects of various carbon sources on enzyme production were studied to determine the most suitable substrate. The carbon sources used for enzyme production were sucrose, lactose, carboxymethylcellulose, cellulose powder, Solka floc, xylose, wheat straw and rice straw. All of these carbon sources

Table 1. pH optimization for the enzyme production from *Bacillus coagulans* culture in shake flask using 1% wheat straw powder at a temperature of 45 °C.

Initial pH	Time (h)	Xylanase activity (IU/ml)	Final pH
6	24	3.2 ± 0.56	8.7
6.5	24	11.6 ± 1.5	8.7
7	24	16.3 ± 1.87	8.8
7.5	24	13.4 ± 1.08	8.8
8	24	4.5 ± 3.14	8.9

were used at a concentration of 1% in the shake flask under optimum initial pH and temperature conditions. From the results (Figure 2) it can be seen that most of the soluble carbohydrate (sucrose, lactose, xylose and carboxymethylcellulose) did not favour higher enzyme production. Among these soluble substrates, xylose seemed to be a better source for xylanase production. A similar phenomenon was reported for B*acillus circulans* D1 and the maximum enzyme production was observed with sugarcane bagasse, rice straw and wheat straw (Dhillon *et al.* 2000) rather than on simple sugars. In the case of B*acillus subtilis*, enzyme production was inhibited by soluble sugars and disaccharides (Sá-Pereira *et al.* 2002). Damino *et al.* (2003) also observed lower production of enzyme with soluble sugars from *Bacillus licheniformis* 77–2. In the present study, with B*acillus coagulans*, maximum enzyme production of 17.9 IU/ml was obtained with 1% wheat straw powder within 24 h of cultivation. Interestingly, Solka floc was found to be a better carbon source for enzyme production as compared to rice straw powder. Considering the availability of wheat straw in India, further studies were carried out with wheat straw.

Gomes *et al.* (2000) used xylan, alkali-treated bagasse and steamed wheat straw for enzyme production from *Cryptococcus adeliae* and they observed that the maximum production was obtained with xylan. Similarly, Ding *et al.* (2004) obtained maximum enzyme production with corncob xylan using *Streptomyces olivaceoviridis* E-86. So to improve xylanase productivity with *Bacillus coagulans* culture, an effort was made to use alkali-treated wheat straw and xylan isolated from wheat straw as carbon source. To compare the enzyme productivity, birchwood xylan (Sigma) was also used for enzyme production from *Bacillus coagulans*. These entire carbon sources were used at a concentration of 1% in the shake flask study. Results (Table 2) shows that alkali-treated wheat straw improved the enzyme production level by 47% as compared to the wheat straw powder. However, maximum xylanase production occurred with 1% wheat straw xylan and it was almost

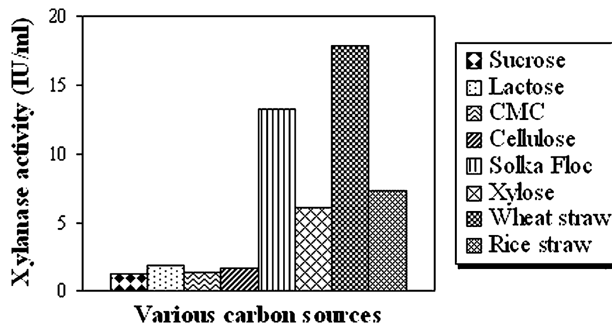

Figure 2. Effect of various carbon sources at a concentration of 1% on the xylanase production from *Bacillus coagulans* in the shake flask study at an initial pH and temperature of 7 and 45 °C. Standard deviations were within the range of 6–9%.

five times higher as compared to the wheat straw powder. Birchwood xylan at the same concentration did not favour higher enzyme production and the activity of the enzyme produced was almost four times lower as compared to wheat straw xylan. Thus it indicates that wheat straw xylan has a higher inducing capability as compared to birchwood xylan. It was observed that around 15% xylan was obtained from wheat straw powder by the method described by Biely *et al.* (1988). If we assume that the xylan content of wheat straw was 15%, than it can be concluded that in 1% wheat straw powder, lower enzyme production was observed due to the low xylan concentration (~0.15%) in the fermentation medium. A similar phenomenon was reported by Pandey & Pandey (2002) for xylanase production by *Aspergillus niger* PPI, and they observed that oatmeal had maximum hemicellulose content among the various carbon sources studied, resulting in highest enzyme production. Gupta *et al.* (2001) observed that when agro-residues such as wheat bran, sugarcane bagasse, corncobs and poplar wood were used as sole carbon source, the xylanase yield was improved by five fold as compared to xylose and xylan for *Staphylococcus* sp. SG-13. However, in case of *Bacillus licheniformis* 77–2, enzyme production levels with birchwood xylan and corn straw was almost similar (Damino *et al.* 2003). Similarly, Bocchini *et al.* (2005) have reported that enzyme production levels from *Bacillus circulans* D1 grown on bagasse and grass hydrolysates were comparable with xylan. Thus it is difficult to generalize on the effect of carbon source on xylanase production using various organisms.

Table 2. Xylanase production from *Bacillus coagulans* culture with different carbon sources in shake flask at an initial pH and temperature of 7 and 45 °C respectively.

Substrate	Concentration (%)	Enzyme activity (IU/ml)
Wheat straw pulp	1	25.0 ± 2.86
Wheat straw xylan	1	98.7 ± 8.97
Wheat straw	1	17.8 ± 1.34
Sigma Birchwood xylan	1	24.2 ± 3.1

Looking into the substrate cost, it is desirable to use cheaper raw material for the enzyme production. Considering the cost of wheat straw xylan (involving solvent precipitation) it was planned to study enzyme production at higher wheat straw powder concentration (to provide higher xylan concentration for the enzyme production). So an optimization study was carried out to determine the wheat straw powder concentration at which maximum enzyme production occurs. It was observed that with the increase in wheat straw powder concentration, enzyme production increased from 15 IU/ml at 1% wheat straw powder to 45.2 IU/ml at 3% wheat straw powder concentration. Thereafter enzyme production decreased with the increase in wheat straw powder concentration (4% (w/v), enzyme produced: 34.2 IU/ml). Interestingly, the increase in xylanase production was almost proportional to the wheat straw powder concentration up to 3% (wheat straw powder concentration:enzyme produced, 1% (w/v):15.6 IU/ml, 2% (w/v):34 IU/ml, 3% (w/v):45.2 IU/ml). The decrease in enzyme production beyond 3% wheat straw powder concentration is either due to substrate inhibition or inhibition by some other component of wheat straw powder. Most reported studies on xylanase production have not considered the substrate concentration optimization but Avcioglu *et al.* (2005) obtained maximum enzyme production with 4% corncob. In the case of *Bacillus* sp., the maximum substrate concentration used for optimum xylanase production was 5% wheat bran (Techapun *et al.* 2003). To understand the inhibition phenomenon, an experiment was designed with 1 and 2% wheat straw xylan in the shake flask. In this case it was observed that enzyme production increased from 110 IU/ml at 1% wheat straw xylan to 165 IU/ml at 2% wheat straw xylan concentration within 40 h of cultivation. Thus it can be concluded that enzyme production has not been inhibited even at the higher wheat straw xylan concentration of 2%. It was observed that at higher wheat straw xylan concentration of 2%, the production of enzyme continued even after 38 h, although at 1% wheat straw xylan concentration, the activity of the enzyme produced decreased after 26 h. Probably the substrate limitation in the fermentation broth induced protease production, which resulted in the decrease in enzyme activity for 1% wheat straw xylan.

The production of xylanase was studied in a 7.5-l Infors fermentor (working volume 4 l) under pH- and temperature-controlled conditions. 1% wheat straw powder was used as a substrate for this study and dissolved oxygen was controlled above 40% saturation level by automatic increase in agitation. Samples were withdrawn at regular intervals of time for analysis of enzyme activity. The profile of enzyme production (Figure 3) showed that maximum enzyme production occured at around 24 h and thereafter it decreased sharply. A similar trend was observed with 1% wheat straw xylan in the fermentor under optimum conditions (Figure 3). The maximum enzyme production of 108 IU/ml was obtained at around 24 h and thereafter it

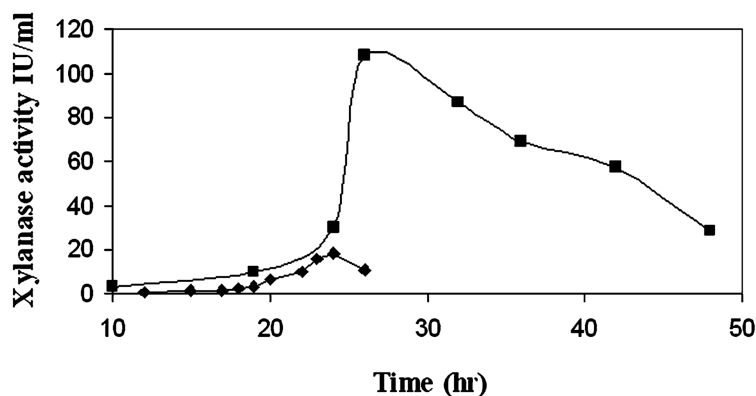

Figure 3. Xylanase production profiles in the fermentor at pH (=7) and temperature (=45 °C) under controlled conditions with 1% wheat straw powder (♦) and 1% wheat straw xylan (■). Standard deviations were within the range of 5–9%.

decreased sharply. As far as the enzyme production level is concerned there was no major improvement in the fermentor (as compared to the shake flask study) under pH-, temperature- and dissolved oxygen-controlled conditions. Thus this indicates that the influence of pH and dissolved oxygen control on enzyme production is insignificant. Decrease in enzyme level after 24 h of cultivation indicated the probable production of protease in the late phase of growth and this can probably be reduced by optimizing the nitrogen source concentration, as was observed with *Bacillus* sp. culture (Subramaniyan *et al.* 2001).

Fermentation broth obtained with 1% wheat straw powder was further processed by centrifugation at 15,000×*g* and 4 °C. The cell-free fermentation broth was further concentrated in a Sartorius tangential filtration system containing a 10 KD polysulphone ultrafiltration membrane. The whole operation was carried out at low temperature (<10 °C) to avoid enzyme deactivation. Cellulase-free xylanase is a prerequisite for successful biobleaching applications (Subramaniyan & Prema 2002; Techapun *et al.* 2003), so in the present study, cellulase activity of the concentrated enzyme was evaluated and expressed as 'filter paperase' activity. It was observed that the concentrated enzyme has xylanase activity of 170 IU/ml with a corresponding filter paperase activity of 0.087 IU/ml. The concentrated enzyme was used for the pre-treatment of eucalyptus Kraft pulp. The potential of the enzyme as a prebleaching agent was assessed with respect to the reduction in Kappa number due to the enzyme treatment. Treatment of enzyme was carried out at optimum pH and temperature of enzyme, which was reported to be 7 and 55 °C respectively (Chauhan *et al.* 2005). Thus the treatment of eucalyptus Kraft pulp was carried out at an initial pH of 7–7.2 and temperature of 50–55 °C. Pulp consistency was 10% and treatment was carried out at an enzyme dosage of 7 IU/(gm OD pulp) for 1.5 h. The efficiency of enzyme was assessed by comparing the kappa number and CED viscosity of enzyme-treated pulp as compared to the non-enzymatically treated pulp (control pulp). Although further bleaching has not been carried out, the potential of this enzyme was evaluated by determining the reduction in kappa number. From the result (Table 3) it can be seen that the enzyme has reduced the kappa number of pulp by 5.45% as compared to the control pulp. Thus it indicates that the enzyme has reduced the lignin content of the pulp. There was a marginal increase in the CED viscosity of pulp due to the enzyme treatment (compared to the control pulp), which is desirable for biobleaching process (Table 3). So the bleaching potential of xylanase of *Bacillus coagulans* on eucalyptus Kraft pulp was assessed and from the initial study it is found to be effective. However, further detail study on the biobleaching process needs to be carried out.

Table 3. Enzymatic treatment of eucalyptus pulp.

Parameters	Control pulp	Enzyme-treated pulp
Consistency	10%	10%
Initial pH	7–7.2	7–7.2
Temperature (°C)	50	50
Enzyme dosage (IU/OD g pulp)	7	7
Time of treatment (h)	1.5	1.5
Kappa number	20.2 ± 0.26	19.1 ± 0.31
CED viscosity (cm^3/g)	645	648

Enzyme activity: 170 IU/ml, Filter paper activity: 0.087 IU/ml.

Acknowledgements

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