Optimization of bioprocess conditions to enhance xylanase production from *Bacillus coagulans*

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Abstract

A soil-isolate of *Bacillus coagulans* was studied for high-level production of cellulase-free xylanase. Batch cultivation showed diauxic growth on two substrates viz glucose and xylan with xylanase production taking place only after glucose was depleted from the medium. The cells were grown in chemostat culture at different dilution rates ranging from 0.1 h\(^{-1}\) to 0.4 h\(^{-1}\), to study product formation kinetics. Xylanase production was observed to be primarily growth-associated with repression of product formation at high dilution rates because of the presence of glucose. Optimized media was used to grow the cells in fed-batch culture where concentrated media consisting of a mix of glucose and xylan was fed exponentially in order to maintain a constant specific growth rate. A specific growth rate of 0.2 h\(^{-1}\) gave the best volumetric activities of 1330 IU/mL with oatspelt xylan and 1400 IU/mL with birchwood xylan after 16 h of cultivation. Given the short cultivation time, these translate to very high productivity in the range of 80–90 IU/mL/hr. Xylanases being a low value product and required in bulk amounts by the pulp and paper industry, the media was designed keeping the cost factor in mind. Shake flask studies using alkali treated wheat straw yielded enzyme levels of 150 U/mL indicating that oatspelt xylan can be replaced by alkali treated wheat straw. Yeast extract (small amount of 0.1%) was a critical component of the media to get high activity and peptone could be replaced by soya flour. Scale-up experiments using low cost media components showed xylanases activity of 525 U/mL, which is the highest activity, reported so far in literature using cheap substrates.

Key words: *Bacillus coagulans*, cellulase-free-xylanase, fed-batch, CSTR, wheat straw

Introduction

Xylan is the principal constituent of plant hemicelluloses, which is located in the cell wall of plants. It consists of \(\beta\)-1,4-glycosidically linked D-xylene backbone with branches containing pentoses, hexoses and uronic acids (Gomes et al., 1994). The key enzymes of the xylanolytic system are endoxylanases, which cleave the glycosidic linkages in the xylan backbone, and \(\beta\)-xylosidases, which hydrolyze the soluble xylo-oligosaccharides to xylose (Deshpande et al., 1986). The synthesis of xylanolytic enzymes is regulated by induction and repression systems in many microorganisms. Xylanase production is induced by xylan while readily metabolizable sugars like glucose repress enzyme synthesis (Samain et al., 1997). The regulatory systems for biosynthesis of xylanase studied so far are mainly from mesophilic organisms. Several thermophilic organisms have been reported to produce thermostable xylanase. The application of thermophilic microorganisms in the production of thermostable enzymes has technical and economical advantages (Gomes et al., 1993). The lignocellulosic products can have high temperature stability in process conditions in the range of 55-60 degrees C (with present industrial products at 45-50 degrees C) and clearly can be improved specific activity, essentially decreasing the protein dosage required for an efficient hydrolysis of lignocellulosic substrates (Vikari et al., 1990). Xylanases are of potential importance in the bioconversion of lignocellulosic materials to sugar, alcohol and other useful products. The
other important use of xylanase has been in the pretreatment of pulps for enhancing bleaching (Wong et al., 1988). This enables a significant reduction in the use of chlorine in subsequent bleaching steps, thereby lowering the production of toxic chlorinated compounds from paper and pulp wastes. However the paper and fibre industries require xylanases to be free from cellulase with high activity under high temperature and alkaline conditions (Hoq et al., 1994; Kulkarni and Rao, 1996).

Xylanase production has been extensively studied in both bacterial and fungal systems. Thus Bacillus SSP-34 gave a xylanase activity of 506 IU/mL using optimized media (Subramaniyan and Prema, 2000). The xylanase activity from Bacillus circulans was 400 IU/mL at an optimum pH of 7.0 and 40% of the activity was retained at pH 9.2. However, there was also a low cellulase activity of 1.88 IU/mL (Ratto et al., 1992). Bacillus sp. strain XE and its mutant derivative strain D3 could reach an activity up to 1000 IU/mL using a continuous supply of exogenous amino acids. Bacillus sp. strain NCL 87-6-10 produced cellulase-free xylanase with an activity of 93 IU/mL in a zeolite induced medium (Balakrishnan et al., 2000; Kinegam et al., 2007).

Fungal systems usually give higher activities (Kitpreechavanich et al., 1984; Siedenberg et al., 1997; Kvesitadze et al., 1998). Thus Trichoderma reesei, which gave a xylanase activity of 960 IU/mL along with a cellulase activity of 9.6 IU/mL (Biely, 1985). Schizophillum commune is also one of the high xylanase producers with an activity of 1244 IU/mL along with a cellulase activity of 65.3 IU/mL and FPase activity (Filter paper activity) of 5.0 IU/mL (Steiner et al., 1987). Thermomyces lanuginosus was found to produce cellulase-free xylanase having an activity of 650-780 IU/mL (Gomes et al., 1993). However fungal strains grow slowly and thus their productivity compared to bacterial systems is lower.

Biopulping reduces electrical energy consumption—a major cost in mechanical pulping, improves paper strength and lower effluent toxicity. Analogously to pulping, isolation of hemicellulose from intact wood or pulp also leads to modification in the structure of the substrate. The degree of polymerization may also change during the isolation procedure. Considerable chemical differences may exist between fibre-bound and isolated substrates. The isolated substrates have been used for elucidation of the mechanism of action of xylanolytic enzymes. Under alkaline extraction conditions the side groups are cleaved to varying degree depending on the method used (Gould, 1984; Wang et al., 2004).

Recent interest in xylanases production and application is a welcome development, because xylanases are important in the bioconversion of hemicelluloses into their constituent sugars, for commercial applications, xylanases should ideally be produced quickly and in large quantities from simple and inexpensive substrates. Abundantly available agro-residues are an obvious source of substrate. Agricultural wastes, which are inexpensive and abundant, are more attractive to be used as carbon sources for the production of enzyme. Wheat straw, which contains around 40% hemicellulose, is a useful source of xylan. Many authors (Lowe et al., 1987; Zillox and Debeire, 1998; Wang et al., 2004) have used wheat straw to induce xylanases given its low cost and easy availability. The crude enzyme could hydrolyze xylan in corn hull, sugarcane bagasse and eucalyptus wood directly, without any pretreatment of those agriculture products and it hydrolyzed xylan in pulps especially sugarcane bagasse and eucalyptus pulps (Reese, 1972; Viikari et al., 1994; Pham et al., 1998). Even though the crude enzyme can be used without purification in pre-bleaching of Kraft pulps, the production cost of the enzyme was high because xylan, a sole source of carbon in the production of the enzyme, is expensive.

In this paper, we report a xylanase producer Bacillus coagulans isolated from soil, which under optimized bioprocess conditions resulted in high level production of cellulose-free xylanase in the range of 1400 IU/mL. This paper further describes in three steps. First shake flask experiments were done to optimize the concentrations of costly media. And second one studies the kinetics of cell growth and product formation in order to design a fed-batch strategy to grow the cells to high cell density and hence obtain high volumetric activities.
Finally, costly components were replaced by cheap nutrients without effecting in xylanases production.

Materials and methods

Microorganism

Screening of xylanase producing organisms was done in our laboratory using cong-red and Remazole Brilliant blue as substrates. The best xylanases producer *Bacillus coagulans* was selected for further studies.

Strain and growth media

A microorganism pool was isolated from soils of semi-arid zone of India. The microorganisms were screened and identified using the standard methods of gram staining (Jacob and Gerstein, 1965) congo red (Teather and Wood, 1982) and Remazole brilliant blue (Bailey et al., 1993) assays were used to screen for xylanase producers. The best producer identified by the plate assay, was chosen and the organism was grown in LB media (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) containing 1% xylan. Aliquots of cells were mixed with glycerol (15% final concentration) and stored at −70°C until use.

Cultivation methods

Shake flask cultures were grown in 10 mL and 50 mL volumes in 100-mL and 500-mL Erlenmeyer flasks, respectively, with shaking at 200 rpm at 37°C on an orbital shaker (Labline Inc., USA). Bioreactor studies were conducted in a 2 L bioreactor (Bioflo2000, New Brunswick Scientific Inc., USA) in a working volume of 1 – 1.5 L. The dissolved oxygen tension was maintained at 40% of air saturation by automatic adjustment of the agitation rate through a PID controller, in which the aeration rate was kept constant at 2 vvm. The medium pH was maintained at a constant value of 7.5 by automatically adding 1N NaOH or 1N HCl. Foam level was controlled by adding antifoam (Sigma, Catalogue No. A-5551) through an electronic controller. For fed batch studies concentrated media consisting of 50 g/L xylan, 100 g/L glucose, 50 g/L yeast extract, 50 g/L peptone, 20 g/L NH₄NO₃, 0.1g/L CaCl₂, 1 g/L NaCl, 0.2 g/L MgSO₄.7H₂O, 1 g/L KH₂PO₄, 1g/L KH₂PO₄, and 1x trace elements solution, was fed via peristaltic pump (Masterflex, USA). The flow rate was increased exponentially to get the desired value of specific growth rate.

Growth rate measurements

Agitation rate (RPM) was used to measure the growth rate indirectly as it was difficult to estimate biomass in terms of wet weight or optical density because of the insolubility of oatspelt xylan. Thus the dynamic gassing out technique was used to estimate *K₉a* at different agitation rates keeping the aeration rate constant. Antifoam addition was avoided as far as possible since it tends to effect *K₉a*. A fairly good correlation was obtained with *K₉a* being proportional to (RPM)².

In fed-batch cultivation, specific growth rates (µ) were determined from the agitation rate vs cultivation time profiles using the relation

\[(RPM)^2 \propto \frac{dX}{dt} = \mu X \text{ (where } X = X_0 e^{\mu t}) \]  \hspace{1cm} (1)

Thus proportionality can be assumed if the yield coefficient with respect to oxygen (*Yₓ/s*) is constant. A plot of ln (RPM)² vs t would thus give a slope of µ. These graphically obtained µ values were compared with the exponential feeding profiles, where a match would ensure complete utilization of the feed. In the case of the CSTR also, the change in biomass concentration was obtained by taking (RPM)² \propto Dx and thus plotting (RPM)²/D vs D to obtain the biomass profile. The α and β values corresponding to growth associated and non-growth associated product formation kinetics were determined from the product and biomass profiles using the following relationship

\[D_p = \alpha Dx + \beta x\]  \hspace{1cm} (2)

\[\Rightarrow p/x = \alpha + \beta/D\]  \hspace{1cm} (3)

A plot of p/x vs 1/D was used to determine α and β.

Xylanase assay

The culture was centrifuged at 8000–g in a table-top centrifuge (Heraeus, Germany) for 5 min to pellet down the cells and the supernatant assayed for xylanase activity using the DNSA-method (Leudeking and Piret, 1959). Briefly, the reaction mixture contained 0.5% oatspelt xylan (Sigma, Catalogue No. X-0627) in a total volume of 750 µl at pH 7.0, was incubated for 20 min at 50°C. The reaction was stopped by adding dinitrosalicylic acid
(DNSA) reagent (Miller, 1959) and immersing in boiling water for 5 min. After cooling, the color intensity was measured in terms of optical density at 540 nm using spectrophotometer (Hitachi, model U2000). The reducing sugar thus liberated was quantified by following the same method on xylose (Sigma) as standard. One unit (IU) of xylanase was defined as the amount of enzyme required to release 1 μmol reducing sugar as xylose equivalent in 1 min under the standard incubation conditions. It should be noted that there is a large variation in xylanase activity measurements as reported by many authors (Bailey et al., 1992). Thus increasing the substrate concentration to 1% xylan as has been used by many authors leads to an over reporting in the measured activity primary because the reaction $K_M$ is very large. Similarly reducing the time of incubation leads to an over estimation of the activities obtained either due to product inhibition or enzyme deactivation. We have used the most conservative assay protocol while reporting the activity.

Treatment of wheat straw

For alkali pretreatments, 5 g wheat straw were treated with 100 ml of 1.5 N sodium hydroxide by soaking overnight and grinding it by using mixie. As a control, oatspelt xylan was used at the similar percentage with wheat straw.

Results and discussions

When oatspelt xylan was used as an inducer

Shake flask experiments produced only 6 IU/ml of xylanase activity. We, therefore, designed to optimize the media to give higher production of xylanase. The Plackett-Burman experimental design (Plackett and Burman, 1946) showed that the positive effective values of various nutrients showed the inducing effect of oatspelt xylan in xylanase production. Yeast extract and peptone also helped in enhancing the xylanase production showing the complete consumption of these complex nitrogen sources while negative effect of glucose was seen possibly because of catabolite repression. Being glucose a good carbon source, it is needed to determine the optimum concentration of glucose below which there is no inhibition in xylanase production. The delayed enzyme production was observed with components such as NH$_4$NO$_3$, NH$_4$Cl and trace metal-vitamin solution were used. The more effective values of NH$_4$NO$_3$ was observed both in log and late log phase in comparison to that of another inorganic source NH$_4$Cl. Similarly yeast extract was giving the more effective values comparatively with peptone (Table 1).

The best three identified variables, which would be used for further studies using Box-Wilson media design (Box and Wilson, 1951), were oatspelt xylan, glucose and yeast extract. The optimum relative concentrations of the three selected variables i.e., oatspelt xylan, glucose and yeast extract were determined by this method. The best-fit equation was established using multivariate regression analysis as follows:

$$Y_{xylanase} = 64.0617 - 143.1742x_1 + 7.5486x_2 - 44.6383x_3 + 106.8287x_1^2 - 2.0942x_2^2 + 6.7618x_3^2 - 9.7266x_1x_2 + 46.1178x_1x_3 + 2.2094x_2x_3$$

Where, $x_1$ = glucose, $x_2$ = yeast extract, $x_3$ = xylan

High yeast extract has a positive effect when 0.5% glucose is present. At 1.0% xylan, maximum xylanase activity could be achieved when glucose concentration was increased to 0.5% and yeast extract concentration of 1.5%. Glucose represses xylanase production at high concentrations (Figures 1A). It was therefore decided to use 5 g/l glucose, which was observed to be non-inhibitory for xylanase production in shake flask cultures. An optimized media was designed which contained 5g/L oatspelt xylan, 5g/L Glucose, 2.5g/L Yeast extract, 2.5g/L Peptone, 1g/L NH$_4$NO$_3$, 0.1g/L CaCl$_2$, 1g/L NaCl, 0.2g/L MgSO$_4$7H$_2$O, 1g/L K$_2$HPO$_4$, 1g/L KH$_2$PO$_4$, 1x trace elements (100 x trace elements solution contained 0.4 g/L CoCl$_2$, 0.1 g/L CuSO$_4$, 5H$_2$O, 0.05 g/L H$_3$BO$_3$, 1 g/L MnCl$_2$, 0.2 g/L Na$_2$MoO$_4$, 2H$_2$O, 0.2 g/L ZnSO$_4$, 7H$_2$O and 0.1 g/L FeCl$_3$).

Shake flask cultivation

Using this optimized media, the xylanase producer *B. Coagulans* was initially grown in baffled flask to monitor the enzyme production profile. It observed that sudden increase in activity observed after certain period of cultivation. The maximum xylanase productivity of 60 U/mL was obtained after a total cultivation of 32 h (data not shown) which was ~ 10
times higher than that of the unoptimized media. One of the main factors behind non-use of xylanase by Indian industry has been lack of cost-effectiveness. The different constituents of media that were sought to be replaced were xylan (sigma), glucose, yeast extract and peptone since they form the major components of media cost.

**Fig. 1A.** Surface response curve to optimize the media concentration. **Fig. 1B.** Profiles of product formation and agitation rate.

Bioprocess optimization: Batch cultivation
Various xylanase-producing organisms were isolated from ecological niches from soils and termite-infested mound soils of the semi-arid zones in our laboratory. They were screened by congo red based assay and Remazol Brilliant Blue (RBB) xylan assay. The best producer of xylanase was used for further study. Optimized media was used to grow the soil isolate Bacillus coagulans in a 2-L bioreactor in a working volume of 1 – 1.5L. Agitation rate and product formation was monitored over time. A diauxic growth was observed with the RPM increasing sharply as the glucose was utilized (Figure 1B). The RPM then declined followed by an increase as glucose got consumed and xylan utilized as the carbon source. Maximum product formation which was in the range of ~ 60 IU/mL, took place primarily in this phase, which is similar to earlier reports where the xylo-oligosaccharides formed induce xylanases expression (Biely et al., 1985; Steiner et al., 1987; Gomes et al., 1994).

**Fig. 2A.** Measurement of glucose present in the culture at different dilution rates in CSTR. **Fig. 2B.** Product formation kinetics in CSTR.

Product formation kinetics
The cells were grown in continuous culture using optimized media to study product formation kinetics. The dilution rate was varied in steps from 0.4 h\(^{-1}\) to 0.1 h\(^{-1}\). For achieving steady states at each dilution rate, the continuous culture was run till medium equivalent to at
least three-reactor volumes was fed. Samples were withdrawn at various time-points during the steady-state period to analyze xylanase activity. The steady-state xylanase activity declined from 698 IU/mL at a dilution rate of 0.1 h\(^{-1}\) to 595 IU/mL at a dilution rate of 0.3 h\(^{-1}\) and then fell sharply to 216 IU/mL at a dilution rate of 0.40 h\(^{-1}\) (Figure 2A). This sharp drop in activity could be because of the catabolite repression of glucose, which remained in the media. The biomass concentration as determined from the agitation rate profile was fairly constant with increasing dilution rate till D reached 0.3 h\(^{-1}\) after which it fell sharply thereby implying a rise in the residual substrate concentration (Figure 2B). The relative values of α and β were calculated for D between 0.1 h\(^{-1}\) and 0.3 h\(^{-1}\) and the α value was found to be 10 times higher than β showing that the product formation was primarily growth associated.

**Fed batch cultivation**

Fed-batch cultivation techniques have been used to obtain high-cell densities, in order to get enhanced product concentrations in microbial cultures. The feeding profile of fed-batch cultures can be used to maintain a low residual concentration of medium components in the culture, which otherwise may be inhibitory to product formation at high concentrations. Exponential feeding strategies were employed, which would maintain a constant specific growth rate and hence a constant

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**Fig. 3A.** Xylanase activity and ln (RPM)\(^2\) profile in fed batch cultivation at specific growth rate (μ) 0.1 h\(^{-1}\), **Fig. 3B.** Xylanase activity and ln(RPM)\(^2\) profile in fed-batch cultivation at specific growth rate (μ) of 0.15 h\(^{-1}\), [3C (i)] Xylanase activity and ln(RPM)\(^2\) profile in fed-batch cultivation at specific growth rate (μ) of 0.2 h\(^{-1}\) in presence of Birchwood xylan, **Fig. 3C (ii)** Xylanase activity profile and ln (RPM)\(^2\) in fed-batch cultivation at specific growth rate (μ) of 0.2 h\(^{-1}\) in presence of oatspelt xylan.
residual substrate concentration in the medium. The feed was increased in small increments, in accordance with the desired exponential function and oxygen transfer rate was monitored to check whether it matched the exponential increase in the feed.

Fed-batch cultivation at a specific growth rate of 0.10 h\(^{-1}\)

As observed from the previous experiments, product formation was maximum when the cells grow on xylan. However, it is difficult to feed concentrated xylan in comparison to glucose. The strategy therefore adopted was to use a mix of high concentration of glucose and lower concentration of xylan in the feed. The glucose would be preferentially consumed in increasing biomass yield while xylan would induce the product formation. However, for this strategy to be successful, it was necessary for residual glucose concentration to remain below inhibitory levels. Xylan addition was continuous required since it is both an inducer as well as substrate for xylanases.

An exponentially growing culture was used to inoculate 1.2 L of 1X medium in a bioreactor. Concentrated feed, which contained glucose, yeast extract, ammonium nitrate and peptone (all at 20 × concentration), xylan (10×concentration) and the remaining components of optimized media (1× concentration), was started when the agitation rate started declining and concomitantly xylanases activity was observed in the medium. The feed rate was increased every hour so as to keep the specific growth rate at 0.10 h\(^{-1}\). Xylanase activity continued to increase during the course of cultivation and reached a maxima of ~600 IU/mL after 32 h of cultivation. This activity was ~10 times higher than the activity achieved in batch culture. A specific growth rate (µ) of ~ 0.11 h\(^{-1}\) was obtained from the graph of ln (RPM)\(^2\) vs time of cultivation (t) which was comparable to the feeding profile demonstrating the efficient consumption of substrate (Fig. 3A).

Fed-batch cultivation at a specific growth rate of 0.15 h\(^{-1}\)

In a similar set-up as described previously, the feeding profile of the concentrated feed was changed to get a specific growth rate of 0.15h\(^{-1}\). This led to an increase in the maximum xylanase activity to the level of 1000 IU/mL which is ~17 times more than that of batch culture. Also growing the cells at a higher specific growth rate resulted in lowering the cultivation time from 32 h to ~23 h in which the maximum activity could be obtained. The specific growth rate (µ) obtained experimentally from the ln (RPM)\(^2\) vs t graph was 0.14 h\(^{-1}\) which again was close to the expected value (Figure 3B).

Fed-batch cultivation at a specific growth rate of 0.20 h\(^{-1}\)

It was decided to further increase the specific growth rate since the cells were able to efficiently utilize the substrate and the strategy of feeding glucose and xylan seemed to give the desired results. At a specific growth rate of 0.20 h\(^{-1}\) only 18 h were needed to complete the run. The xylanase activity also increased to 1330 IU/mL (in oatspelt xylan) (Fig. 3C(ii)) while with birchwood xylan an activity of 1400 IU/mL was observed after 16 h of cultivation (Fig. 3C (i)). A slight increase with birchwood xylan was possibly due to its higher solubility which makes it a better inducer for xylanases synthesis. The specific growth rates obtained experimentally were found to be 0.204 h\(^{-1}\) in oatspelt xylan and 0.23 h\(^{-1}\) in birchwood xylan.

### Table 2. Y.E and corn steep liquor

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Y.E</th>
<th>CSL</th>
<th>Activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
<td>1.25</td>
<td>123.56</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>1.38</td>
<td>129.63</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>1.5</td>
<td>134.56</td>
</tr>
<tr>
<td>4</td>
<td>0.15</td>
<td>1.63</td>
<td>137.81</td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>1.75</td>
<td>145.87</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>2</td>
<td>7.05</td>
</tr>
</tbody>
</table>

If oatspelt xylan was replaced by cheap wheat straw

The 1.5 N NaOH was used to pretreat the wheat straw. Experiment using alkali treated 40% whole-wheat straw extract could give maximum xylanase activity of 98 U/mL (Figure 4A). At all concentrations of the alkali treated whole wheat straw, the xylanase productivity gradually increased with time. Maximum activity of ~138 U/mL when 40% of the alkali treated whole wheat
straw was used (Fig. 4B). This xylanase activity is comparable with that of 2% oatspelt xylan.

**Media redesign to replace the yeast extract by corn steep liquor**

Yeast extract concentrations were reduced gradually while the concentrations of corn steep liquor was increased keeping the rest components constant (Table 2). The highest and lowest concentrations were chosen in such a condition that the optima should fall within that range. The results showed that the activity of ~145 U/mL was maintained till the concentration of yeast extract fall to 0.1% even in the presence of excess corn steep liquor. It shows that corn steep liquor is not a good nitrogen source to achieve high xylanase activity. A further reduction in the concentration of yeast extract resulted in a drastic decrease in enzyme activity (~7 U/mL). Though small amounts of yeast extract was a critical component of the media, there is a possibility of reducing the concentration of yeast extract to 0.1% to get high xylanase activity.

**Table 3.** Corn steep liquor and Soya flour concentrations

<table>
<thead>
<tr>
<th>Experiments</th>
<th>CSL (%)</th>
<th>SF (%)</th>
<th>Activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.13</td>
<td>1.13</td>
<td>6.83</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>1.13</td>
<td>6.9</td>
</tr>
<tr>
<td>3</td>
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<td>7.13</td>
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<tr>
<td>4</td>
<td>1.13</td>
<td>2.0</td>
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<tr>
<td>5</td>
<td>1.13</td>
<td>0.25</td>
<td>61.35</td>
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<tr>
<td>6</td>
<td>0.69</td>
<td>0.69</td>
<td>69.15</td>
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<tr>
<td>7</td>
<td>1.56</td>
<td>1.56</td>
<td>12.83</td>
</tr>
<tr>
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</tr>
<tr>
<td>9</td>
<td>1.56</td>
<td>0.69</td>
<td>6.53</td>
</tr>
</tbody>
</table>

**Media redesign to know the relative concentrations of corn steep liquor and soya flour to remove the yeast extract and peptone**

One fractional factorial experiment was redesigned to optimize the relative concentrations of corn steep liquor and soya flour. Since a small amount of yeast extract was necessary for achieving high xylanase activity, it was needed to remove peptone, which is also one of the cost components in the media. Here rests of the components were kept constant varying the concentrations of these two nutrients. Table 3 shows that high productivity of ~69.15 U/mL was obtained with similar concentrations of corn steep liquor and soya flour. Similarly xylanase activity of 61.35 U/mL was achieved with relative concentrations of 1.125% corn steep liquor and 0.25% soya flour (Table 3). These experiments concluded that optimized media (II) should contain as follows:

- Glucose – 0.25%.
- Yeast extract – 0.1%.
- Corn steep liquor – 1.0%.
- Soya flour – 0.3%.
- NH₄NO₃ – 0.1%.
- MgSO₄ – 0.02%.
- KH₂PO₄ – 0.1%.
- K₂HPO₄ – 0.1%.
- Trace elements – 1X.

**Fig. 4A.** Xylanase activity profile at different percentages of alkali treated wheat straw extract, **Fig. 4B.** Comparative Xylanase activity profile between alkali treated wheat straw extract and oatspelt xylan.

**Scale up experiment using wheat straw as an inducer**

Using the optimized concentrations, the Bacillus coagulans was cultivated in 1.0L bioreactor to determine the yield of xylanase. Activity started measuring after 12
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hrs of inoculation in the bioreactor. The maximum xylanase activity was 512 IU/ml after 36 hrs of cultivation (Fig. 5, Table 4).

**Fig. 5.** Xylanase activity profile in fed-batch cultivation at specific growth rate (μ) of 0.2 h⁻¹ in presence of alkali treated wheat straw mixture feeding with wheat straw extract and glucose

Table 4. Summary of xylanase activity obtained using different cultivation strategies (X-Volumetric xylanase activity (IU/mL))

<table>
<thead>
<tr>
<th>Cultivation strategy</th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
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</tr>
<tr>
<td>Continuous culture (μ = 0.10 h⁻¹)</td>
<td>698</td>
</tr>
<tr>
<td>Continuous culture (μ = 0.30 h⁻¹)</td>
<td>595</td>
</tr>
<tr>
<td>Continuous culture (μ = 0.40 h⁻¹)</td>
<td>216</td>
</tr>
<tr>
<td>Fed-batch (μ = 0.10 h⁻¹)</td>
<td>600</td>
</tr>
<tr>
<td>Fed-batch (μ = 0.15 h⁻¹)</td>
<td>1000</td>
</tr>
<tr>
<td>Fed-batch (μ = 0.20 h⁻¹)</td>
<td>1330</td>
</tr>
<tr>
<td>Fed batch using wheat straw</td>
<td>525</td>
</tr>
</tbody>
</table>

Discussion

In the present studies, a soil isolate Bacillus was used for the production of xylanase enzyme. Bioprocess optimization led to enhanced xylanase activity from 60 IU/mL in batch culture to 1330 IU/mL in fed batch culture thus yielding a 22-fold increase (Table 4). Additionally since the cultivation time was short (~18–20 h) the volumetric productivities were close to 80 IU/mL/hr. This is one of the highest levels reported so far in literature. Additionally the xylanases produced by this organism was found to be cellulase free, which makes it a potential candidate for application in pulp and paper industry. The increase in xylanase activity was primarily because we were able to maintain a high μ even at high biomass concentrations while maintaining a low residual glucose concentration in the culture medium. The use of a mix of xylan and glucose in the feed, allowed us to overcome the limitations of feeding concentrated xylan which tends to increase the broth viscosity.

The indirect method of estimating biomass by monitoring oxygen transfer rates does have inherent errors. Firstly, the dynamic gassing out techniques has errors and also the medium rheology changes with time making the correlation between RPM and KLa problematic. However these problems were more than compensated since this technique allowed easy on-line continuous evaluation of growth rate. The fact that the xylanases activity increased almost exponentially in the fed batch cultures matched the results of the continuous cultures where product formation was largely growth associated. While we did attempt to grow the cells at higher μ in fed batch mode of cultivation, the results did not lead to higher activities possibly due to substrate accumulation in the culture media. Further studies were done replacing this media by low cost substrate. Wheat straw containing around 40% hemi-cellulose is a useful source of xylan. It was, therefore, decided to use alkali treated wheat straw as a xylan source in our media redesign experiments. Serious problems arise with respect to mixing when increased amount of wheat straw saw dust is present in the culture and therefore oxygen transfer.

At lower concentrations, there was clearly a limitation in terms of xylan for proper induction availability resulting in lower activity. At concentrations greater than 50%, multiple reasons can be postulated for lower activity such as excess salt or inhibitory compounds leaching into the supernatant during the soaking step. The above results proved that cheap nutrient wheat straw could replace the high cost oatspelt xylan for
xylanase production. The peptone also can be replaced by the combination of corn steep liquor and soya flour. However, to maintain the C:N ratio it was decided to use 1% corn steep liquor and 0.3 % soya flour in the alkali treated whole-wheat straw based media.

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**References**


