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Production of Cellulase-Free Xylanase from *Bacillus megaterium* by Solid State Fermentation for Biobleaching of Pulp

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Abstract. Xylanase production from *B. megaterium* was enhanced using solid state fermentation with respect to the use of solid substrate, moistening solution, moisture content, inoculum, sugars, soyabean meal, amino acids, and extraction with surfactant. An increase of ≈423-fold in xylanase production and complete suppression of CMCase production was achieved over submerged liquid fermentation. Biobleaching using this cellulase-free xylanase, 8 U/g of oven dried pulp of 10% consistency, showed 8.12% and 1.16% increase in brightness and viscosity, 13.67% decrease in kappa number, and 31% decrease in chlorine consumption at the CD stage.

Xylanases have attracted considerable research interest because of their potential application in the biobleaching of pulp and other industrial systems [1]. Viikari et al. [2] first reported that xylanases decrease the use of chlorine needed for bleaching kraft pulp and play an important role when the use of hazardous chemicals is to be decreased in bleaching processes. Many researchers have confirmed and extended this observation, and the technology is now being commercialized [3, 4].

In the paper production process, pulping is a step during which cellulose fibers are broken apart and most of the lignin is removed. The remaining lignin is then removed by a multistep bleaching process [4]. Pulping and bleaching are both performed at high temperatures. Hence, the paper industry needs xylanases that are thermostable and, preferably, active at neutral and alkaline pH [4]. Also, xylanases are mostly contaminated with cellulases, which destroy the structure of cellulose and diminish pulp quality. This means xylanases with a high degree of cellulase-free purity are required. So the application of an alkali- and heat-stable cellulase-free xylanase for large-scale pulp bleaching biotechnology requires efforts that are aimed at process optimization, simplification, and cost reduction. The production of xylanases must, therefore, be improved by finding potent fungal or bacterial strains or by inducing mutant strains to produce and excrete greater amounts of enzymes or by enhancing production by solid state fermentation (SSF). SSF is a well-adapted and cheaper process than submerged liquid fermentation (SLF) and the amounts of products obtained by SSF are many-fold higher. In addition, the products obtained have slightly different properties (e.g., more thermotolerance). Besides, low-moisture content reduces the possibilities of contamination by bacteria and yeast. Higher levels of aeration, simple culture media that provide all the nutrients necessary for growth, simple design reactors with few spatial requirements that can be used due to the concentrated nature of the substrates and low energetic requirements (mechanical agitation and aeration are not necessary), are other advantages [5]. There are several reports on xylanase production by SSF using fungi, but few on alkaline xylanase productions by SSF using bacteria [6, 7]. Bacteria are preferred over fungi as they are a good source of alkaline and thermostable enzymes. SSF by bacteria are primarily confined to *Bacillus* spp., which could be attributed to their ability to adhere to the substrate particles to produce filamentous cells for penetration and to their specific need for water activity [8]. The present work reports improvement in culture conditions and medium composition so as to obtain high

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Table 1. Moistening solutions used in the study

Moistening solutions (MS)	Reference
MS1 (1X M162): L^{-1}: $CaSO_4.2H_2O$, 0.04 g; $MgCl_2.6H_2O$, 0.2 g; Nitrilotriacetic acid, 0.1 g; Micronutrient solution (10X), 0.1 ml; Ferric citrate (0.01 M), 0.5 ml.	[28]
Micronutrient solution (10X): L^{-1}: H_2SO_4 (conc.), 0.5 ml; $MnSO_4.H_2O$, 2.28 g; $ZnSO_4.7H_2O$, 0.5 g; Boric acid, 0.5 g; $CuSO_4.5H_2O$, 25 mg; sodium molybdate, 25 mg; $CoCl_2.6H_2O$, 45 mg	
MS2: MS1, yeast extract (0.2%), tryptone (0.2%)	
MS3: 5X MS1	
MS4: 5X MS1, yeast extract (0.2%), tryptone (0.2%)	
MS5: 10X MS1	
MS6: 10X MS1, yeast extract (0.2%), tryptone (0.2%)	
MS7: *mg/g of solid substrate*: K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.1	
MS8: 10X MS7	[7]
MS9: *mg/L*: Na_2HPO_4, 11; NaH_2PO_4, 0.1; $Na_4\ H_2PO_4$, 1; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.1; $FeSO_4$, 0.1; $MnSO_4$, 0.1.	[6]
MS10: Tap water	

xylanase yields from *B. megaterium* and its application in biobleaching.

Materials and Methods

Microbial Strain. An alkalophilic *Bacillus* strain was isolated from soil/water samples, collected from the vicinity of a paper mill in Mohali (Punjab), on seed M162 agar plates, pH 8, containing 0.2% xylan. The microscopic, morphological, physiological, and biochemical tests were performed according to Bergey's Manual of Systematic Bacteriology [9].

Xylanase Production.

Submerged liquid fermentation (SLF).

Xylanase production was carried out in 25 ml of MS2 medium, inoculated with 1–2% of overnight inoculum, made in the same medium, and incubated for 24 h at 37°C and 200 rpm. Enzyme was obtained after centrifugation at 10,000*g* for 10 min.

Solid state fermentation (SSF)

Five grams of solid substrate, moistened with mineral salt solution, was inoculated and incubated at 37°C in an incubator humidified by keeping a tray of sterile distilled water (relative humidity 60–70%). The contents were shaken intermittently. The enzyme from each flask was extracted with 50 ml of distilled water. The whole content was squeezed through wet muslin cloth. The extract was centrifuged at 10,000*g* for 30 min and the supernatant was used as enzyme source. Enzyme productions were done at least in triplicates and average values and standard errors were calculated.

Conditions for the Optimization of Xylanase Production in SSF.

1. Moistening solutions: Various mineral salt solutions and tap water were used. (Table 1)
2. Moisture level: Different substrate to moistening solution ratios (1:1, 1:1.5, 1:2, 1:2.5) were used.
3. Soya bean meal: 1–5 % was added as a nitrogen source in the medium.
4. Inoculum size and age: An 8–24-h-old culture at 1–20% size was used as inoculum.
5. Incubation period: SSF was carried out for 24–168 h.
6. Sugars: Xylose, glucose, lactose, mannose, arabinose, and maltose were added at 1%.
7. Polysaccharides: Xylan, soluble starch, chitin, and guar gum were added at 1%.
8. Amino acids: Glycine, isoleucine, proline, leucine, alanine, valine, serine, threonine, and cysteine were added at 0.2%.
9. Solid substrates: Wheat bran, rice bran, and oil cakes were used.
10. Extraction: Xylanase was extracted with distilled water and 0.01, 0.02, 0.04, 0.07, and 0.1% concentrations of Tween 80.

Enzyme Assays. Xylanase and CMCase activities were determined by the dinitrosalicylic acid (DNSA) method [10, 11]. One unit of xylanase (CMCase) activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to xylose (glucose) per ml per minute. Xylanase production was expressed as units/g of solid substrate.

Scale Up of Solid State Fermentation. Enamel metallic trays of sizes $35 \times 25 \times 5$ and $40 \times 30 \times 5$ cm^3 containing 250 and 500 g of solid substrate, respectively, were moistened with suitable moistening solutions and used to cultivate the bacterial strains after covering with aluminium foil and autoclaving. The wet solid bacterial cultures were extracted and assayed for xylanase and CMCase.

Biobleaching of Kraft Pulps ($ECDED_1D_2$). Pulp used for biobleaching was hardwood pulp obtained from Ballarpur Industries Limited (BILT), Yamunanagar, Haryana, India, and was a mixture obtained from woods of six different trees, namely poplar, eucalyptus, eucalyptus rulla, small vaneer, bamboo, and debarka bamboo hardwood (DBH). Extensively washed pulp (50 g) of 10% consistency was treated with xylanase (8 U/g pulp) in a plastic bag under optimum conditions. Xylanase-treated pulp was bleached with chemical sequence $CDED_1D_2$ (Table 2). Reduction in chlorine consumption to obtain same amount of brightness in both xylanase-treated and control (non-treated) was seen by conducting an experiment in which all the parameters and treatment conditions were kept the same except for the dose of chlorine treatment given in the CD stage.

Analytical Techniques. Kappa number, a measure of lignin content, was determined by reaction of pulp samples with acidified potassium permanganate (Tappi method T 236). Viscosity, which indicates cellulose chain length, was determined by dissolving delignified pulp

Table 2. Conditions for biobleaching sequence ($ECDED_1D_2$)

Treatment	Pulp consistency	Temperature (°C)	Time (min)	pH
E (enzymatic prebleaching)	10%	50	180	8.0
CD (chlorine-chlorine dioxide bleaching)	3%	Ambient	45	1–2
E (alkali extraction)	10%	65–70	120	11–12
D_1 (chlorine dioxide stage 1)	10%	70–75	180	3.8–4.0
D_2 (chlorine dioxide stage 2)	10%	70–75	180	3.8–4.0

in cupriethylenediamine (CED) and measuring the viscosity of a 0.5% solution with an Ostwald viscometer (Tappi method T 230). Brightness of a paper sheet was measured as % ISO with ISO (TechniBright, USA). % ISO (International Organisation of Standardisation) brightness is the measure of diffused reflectance at 457 nm. Total organic chlorine (TOCl) was determined by the method of Hong et al. [12].

Results and Discussion

Microbial Strain. The isolated strain was a gram-positive, spore (central, ellipsoidal) -forming rod ($2.2–2.5 \times 0.4–0.6$). It grew optimally at 40°C (range 20–50°C), pH 8.0 (range 5–10.5) and in the presence of 0.02% sodium azide and 10% sodium chloride, which were inhibitory for most other *Bacillus* strains. The microscopic, morphological, physiological, and biochemical characters of the isolate revealed that it belonged to genus *Bacillus* and on the basis of similarity coefficients (S_J) measured pair wise with *B. brevis* (0.437), *B. cereus* (0.57), *B. coagulans* (0.53), *B. circulans* (0.53), *B. megaterium* (0.85), and *B. stearothermophilus* (0.43), using 25 characters, the identity of the isolate was found closest to *B. megaterium*. This isolate was used for the optimization of the following parameters of SSF.

Moistening Solutions. Xylanase production was supported maximally by MS9 (96.8 U/g) followed by MS8 (90 U/g) and minimally by MS1 (52 U/g). It appears that high phosphate content (amount or buffering) of MS9 and MS8 may be important.

Moisture Level. Xylanase titers were highest at 1:1.5 (105 U/g) and lowest at 1:2.5 (73 U/g). The importance of moisture level in SSF media and its influence on microbial growth and product biosynthesis may be attributed to the impact of moisture on the physical properties of the solid substrate [13]. A higher than optimum moisture level causes decreased porosity, alteration in wheat bran particle structure, gummy texture, lower oxygen transfer, and enhancement of the formation of aerial mycelia [13, 14]. Likewise, a lower than optimum moisture level leads to reduced solubility of the nutrients of the solid substrate, lower degree of swelling, and higher water tension.

Soya Bean Meal. Five percent concentration increased xylanase production maximally by 1.4-fold (130 U/g).

Inoculum Size and Age. Sixteen-hour-old inoculum at 10% size yieded maximum xylanase production (400 U/g).

Incubation Period. Maximum production (415.5 U/g) was achieved after 96 h and was constant thereafter.

Sugars. The objective was to devise a medium that will enhance the production of xylanase and inhibit the production of CMCase. This happened in the presence of xylose (1%), as it resulted in an increase in xylanase production (460 U/g) and a decrease in CMCase production (from 20 to 4 U/g). Lactose (390 U/g), maltose (450 U/g), mannose (430 U/g), and arabinose (385 U/g) also supported good xylanase production. Maltose was not selected as it also enhanced CMCase production to 40 U/g. CMCase production by *B. megaterium* was completely repressed on the addition of 1% glucose to the medium though there was a slight (5%) decrease in xylanase production also. Bataillon et al. [15] and Beg et al. [16] reported that xylanase production was inducible in nature and a high yield of xylanase was obtained when xylose was supplemented in the medium. Catabolite repression on the addition of xylose to the medium was also reported in many *Bacillus* spp. [15, 17]. There are reports of bacterial xylanase production, resistant to repression by glucose and xylose [18].

Polysaccharides. The presence of xylan, soluble starch, chitin, and guar gum at 1% concentration in the medium decreased xylanase production by 8, 9, 27, and 10%, respectively. A strong inducing effect of xylan on xylanase production has been reported [19]. The induction of xylanase biosynthesis by xylan occurs via the low molecular weight soluble catabolites, which are generated from xylan by the action of constitutively

Table 3. Effects of xylanase (8U/g of oven dried pulp) treatment on kraft pulp obtained at different stages of $CDED_1D_2$ based bleaching of pulp

	Brightness (% ISO)			Kappa number			Viscosity		
Stage	Control	Xylanase treated	% increase	Control	Xylanase treated	% decrease	Control	Xylanase treated	% increase
E	33.33	35.0	4.7	16.5	14.24	13.67	15.1	17.2	12.0
CDE	45.0	50.18	10.3	11.2	10.8	3.45	15.1	17.4	13.0
D_1D_2	78.1	85.0	8.11	—	—	—	17.0	17.2	1.16

Table 4. Reduction of chlorine charge for the treatement of biobleached pulp

Parameters	$CDED_1D_2$	*B. megaterium* Xylanase (Enz-$CDED_1D_2$)
Brightness	33.3 (control)	35.0 (after enzyme treatment)
Kappa number	16.5 (control)	14.2 (after enzyme treatment)
CD stage: % Cl_2 added	3.3	2.3
CD stage: % Cl_2 consumed	3.2	2.2
Extraction stage: 4 M NaOH added (ml)	0.3–1	0.3–1
Brightness	45.0	45.1
Kappa number	11.2	10.8
D-1 stage: % Cl_2 added and consumed	0.8	0.8
D-2 stage: % Cl_2 added and consumed	0.6	0.6
Final brightness	78.1	78.2
Final viscosity	17.0	17.3
Total Cl_2 added (%)	4.7	3.7

produced extracellular or cell surface located xylan degrading enzymes [20].

Amino Acids. Among all the amino acids tried, the maximum stimulatory effect was seen in the medium supplemented with hydrophobic compared to hydrophilic amino acids. Among the hydrophobic group, glycine exhibited the highest (31.14%) increase in yields to 465 U/g followed by isoleucine, proline, and leucine producing 420, 410, and 370 U/g, respectively. Alanine and valine are also hydrophobic but decreased xylanase production slightly by 0.14 and 0.15%, respectively. Among the hydrophilic amino acids, only serine resulted in 0.11% increase while threonine and cysteine decreased xylanase production by 0.12 and 0.24%, respectively. Balakrishnan et al. [21] reported a 2.5-fold enhancement in xylanase production by *Bacillus* sp. NCL 87-6-10 using glycine and casamino acids. The combination of DL-norleucine, L-leucine, and DL-isoleucine stimulated endoxylanase production from *Streptomyces* sp. QG-11-3 by 6.72-fold [22].

Solid Substrates. Wheat bran supported maximum xylanase production (650 U/g) compared to rice bran (94 U/g), wheat + rice bran (120 U/g), and wheat bran + oil cakes (200 U/g). The universal suitability of wheat bran may be because it contains sufficient nutrients and is able to remain loose even in moist conditions, thereby providing a large surface area [23]. However, use of other solid substrates like grain byproducts, cassava, potato, beans, and sugar beet pulp have also been reported [24]. dos Santos et al. [25] used sugarcane bagasse for xylanase production by *Thermoascus aurantiacus*.

Extraction. Maximum extraction was achieved with 0.01% Tween 80 (746 U/g) compared to 540 U/g with distilled water.

Final SSF Conditions. Ten percent of 16-h-old culture was used as inoculum for 5 g wheat bran moistened (1:1.5) with MS 9 containing 1% xylose, 1% glucose, 0.2% glycine, and 5% soyabean meal and incubated at 37°C for 96 h for xylanase production. Enzyme extraction was done with Tween 80 (0.01%). The final yields of xylanase and CMCase were 846 and 0 U/g, respectively (Fig. 1).

Scale Up of SSF. Scale up from 5 g in flasks to 500 g in enamel trays resulted in a slight decrease (12.14%) in xylanase production. Xylanase production in enamel trays was comparable with that in culture flasks and scaling up did not result in a reduction of xylanase titers in another study [6].

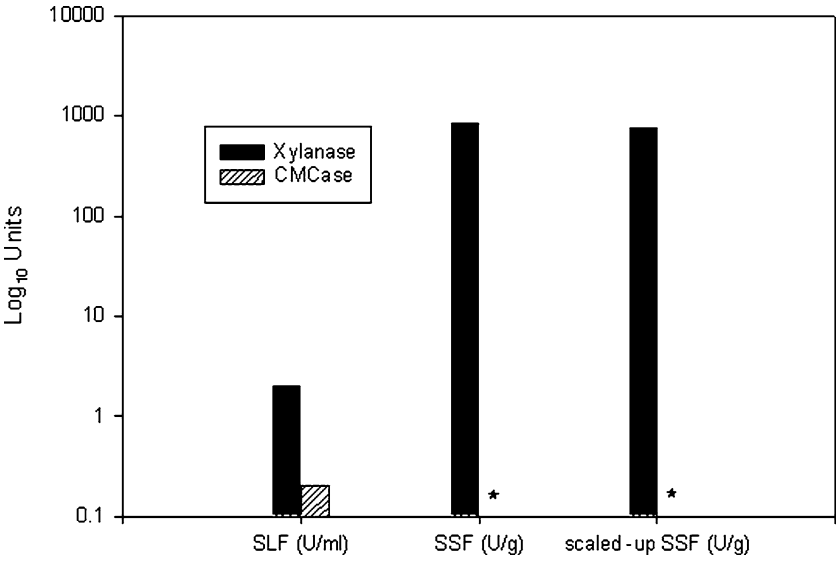

Fig. 1. Production of xylanase under submerged liquid fermentation (SLF) and final optimized solid state fermentation (SSF) conditions. *CMCase production was suppressed.

Biobleaching of Kraft Pulp. The kraft pulp was pretreated with xylanase. Pulp brightness and viscosity increased by 8.12% (7 brightness points) and 1.16%, respectively, and kappa number reduced by 13.67% (2.26 points) (Table 3) as compared to control (not treated with xylanase). Also, prebleaching with xylanase from *B. megaterium* caused 31.25% reduction in chlorine consumption at CD stage (Table 4). Xylanase from *Thermatoga maritima* increased the brightness of oxygen-delignified hardwood kraft by 3.8% ISO [26]. Xylanase P (a commercial xylanase from Sappi forest Products, Southern Africa) improved the brightness of kraft pulp by 5.6 brightness points when used at 7 U/g of moisture-free pulp and caused an approximately 10% reduction in chlorine dioxide consumption [27].

The SSF conditions optimized in this work have resulted in ~423-fold increase in xylanase yield compared to SLF. Also, the CMCase has been completely suppressed under these conditions, thus circumventing the need to find cellulase-negative isolates or mutants. The slight increase in the viscosity of xylanase-treated pulp showed the absence of CMCase. Optimized SSF thus serves as an alternative to the generation of xylanase hyperproducing recombinants. The xylanase preparation has shown potential in bleaching the pulp and reducing the consumption of chlorine dioxide significantly. This would, in turn, result in the reduction of absorbable organic halogens and chloride levels of the bleach wastewaters and alleviate the environmental impact of the industry.

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