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Production of β-xylanase by a *Thermomyces lanuginosus* MC 134 mutant on corn cobs and its application in biobleaching of bagasse pulp

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The production of hemicellulases by *Thermomyces lanuginosus* SK using oatspelts xylan was examined during submerged cultivation. A high level of extracellular xylanase (346 ± 10 U ml^{-1}) production was observed on the fifth day; however, accessory enzyme levels were low. *T. lanuginosus* SK was further subjected to UV and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine mutagenesis. The *T. lanuginosus* MC 134 mutant showed a 1.5 fold increase in xylanase production on oatspelts xylan, compared to the wild type strain. Xylanase production was further enhanced to 3299 ± 95 U ml^{-1} by using corn cobs under optimized growth conditions. A reduction in xylanase production was observed in a 5 L fermenter. Also, the biobleaching efficiency of crude xylanase was evaluated on bagasse pulp, and a brightness of 46.07 ± 0.05% was observed with the use of 50 U of crude xylanase per gram of pulp. This brightness was 3.6 points higher than that of the untreated samples. Reducing sugars (25.78 ± 0.14 mg g^{-1}) and UV-absorbing lignin-derived compound values were considerably higher in xylanase-treated samples. *T. lanuginosus* MC 134 has a potential application in the pulp and paper industries.

[**Key words:** *Thermomyces lanuginosus*; Mutation; Bagasse pulp; Corn cob; Biobleaching; Kappa number; Brightness]

Xylan, the most important hemicellulosic heteropolymer, is composed of a backbone of 1,4-linked β-D-xylopyranose residues and branches of L-arabinofuranose, D-glucuronic acid, or 4-*O*-methyl-D-glucuronic acid (1–3). Hydrolysis of xylan by microbial enzymes is highly specific and an environmentally friendly option when compared to chemical hydrolysis. The unpredictable structure and organization of hemicellulose requires the concerted actions of many enzymes for its complete hydrolysis, including the key enzymes, endo-xylanase (EC 3.2.1.8), and β-xylosidase (EC 3.2.1.37) (4). Xylanases and the associated debranching enzymes are produced by a variety of microbes, including bacteria, yeast, and filamentous fungi (2, 5). Among them, *Thermomyces lanuginosus* (thermophilic fungus) has been reported as one of the superior cellulase-free xylanase producers (6).

To reduce the bioprocessing cost and enhance xylanase production level in microbes, different strategies have been developed. Quantitative enhancement is the foremost approach, and involves strain improvement and optimization of growth parameters to achieve maximum productivity. Examples of spectacular success in strain improvement in industry are mostly attributed to the extensive application of mutation and selection. UV light (7) and chemical mutagenesis (8) using *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) have been reported as successful methods for mutation.

Large quantities of agro-industrial by-products accumulate in the environment, and can be processed for food, fuel, and a variety of chemicals. These negative value agro-wastes can be utilized for cost-effective enzyme production at the industrial level. Several inexpensive substrates have been used as a carbon source for microbial xylanase production (9, 10).

In the paper production process, residual lignin is removed by multistep chemical bleaching processes, resulting in the release of hazardous adsorbable organic halogens into the environment (11). Microbial enzymes are an alternative option that involves the use of hemicellulolytic enzymes to increase the brightness of pulp samples. The main goals of enzyme-aided bleaching are to increase the brightness of the final pulp and to reduce or replace the harmful chlorine compounds used in the bleaching processes (12). Most studies on biobleaching have been confined to kraft and sulphite pulps. Reports on the biobleaching of non-woody plants are limited. Bagasse, one of the most important non-woody materials, is used for paper production in many developing countries. However, reports on the enzymatic prebleaching of bagasse pulp are scant (13, 14). In the present study, xylanase production in *T. lanuginosus* was enhanced via UV/NTG mutagenesis. Fungal growth parameters were optimized for maximum xylanase production using corn cobs as a cheap carbon source in shake flasks and in a lab-scale fermenter. The biobleaching efficiency of crude xylanase was also evaluated on bagasse pulp.

MATERIALS AND METHODS

Microorganism and growth conditions *T. lanuginosus* SK was obtained from the culture collection of the Department of Microbiology, University of KwaZulu-Natal, Durban, South Africa. The strain was periodically subcultured on potato dextrose agar (Oxoid, UK) and stored at room temperature. Fifty milliliters of growth medium (pH 6.5) containing (g l^{-1}) oatspelts xylan (15.0), yeast extract (15.0), and KH_2PO_4 (5.0) were prepared in each 250 ml Erlenmeyer flask. An agar disc (9 mm diameter) of an

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actively growing 5 day old culture was used as an inoculum. The flasks were kept at 50 °C in a shaker incubator (150 rpm). After 5 days, the culture broth was centrifuged (10,000 ×*g* for 10 min), and the supernatant was used to determine the extracellular hemicellulase activities. For intracellular enzyme extraction, 1 g of washed mycelium (wet weight) suspended in 50 ml of sodium citrate buffer (0.1 M, pH 6.5) was kept in an ice bath and sonicated using a Virsonic 100 (Virtis, USA) ultrasonic processor at 5 kilocycles s^{-1} for 5 min. After sonication, the biomass was removed by filtration followed by centrifugation (10,000 ×*g* for 10 min), and the clear supernatant was used as a crude intracellular enzyme source.

Strain improvement through mutagenesis *T. lanuginosus* SK culture grown on a PDA plate was scraped into sodium phosphate buffer (0.1 M, pH 6.5). The spores were uniformly suspended using Tween 80 (0.025%, v/v) and separated from the mycelium by filtering through a sterile muslin cloth. Four ml of spore suspension (10^7 spores ml^{-1}) was transferred aseptically onto a Petri dish and exposed to UV light (254 nm) using a Philips germicidal lamp (30 W) at a distance of 26.5 cm for 180 min (7). The UV exposed spores were stored in dark conditions overnight to avoid photoreactivation and plated on PDA. The colonies were checked for xylanase production in submerged culture using oatspelts xylan. The best xylanase-producing mutant obtained through UV mutation was further subjected to NTG (350 μg ml^{-1}) mutagenesis (8). The treated spores were washed three times with sterile distilled water and plated on PDA. Colonies were again tested for xylanase production using oatspelts xylan as a carbon source.

Optimization of growth parameters for xylanase production by *T. lanuginosus* MC 134 during shake flask cultivation

Effect of various carbon and nitrogen sources The carbon sources (1% w/v) used were glucose, fructose, lactose, maltose, sucrose, xylose, trehalose, mannitol, sorbitol, soluble starch, cane sugar, beechwood xylan, birchwood xylan, oatspelts xylan, coarse corn cob (2–3 mm), fine corn cob (<1 mm), and wheat bran (0.5 mm). All commercial xylans were purchased from Sigma, USA. Corn cobs (coarse and fine) were prepared by stripping the kernels from maize; they were then dried, ground using a mill grinder, and sieved. Corn cobs were autoclaved at 121 °C for 15 min and stored at 4 °C.

The nitrogen sources (1% w/v) used were beef extract, malt extract, meat extract, yeast extract, casein, bacteriological peptone, fish peptone, proteose peptone, vegetable peptone, tryptone, ammonium phosphate, ammonium sulphate, potassium nitrate, sodium nitrate, and urea. For the time course study, 50 ml of the optimized medium containing corn cob and yeast extract was dispensed into 250 ml Erlenmeyer flasks, inoculated with 5 day old fungal cultures, and kept at 150 rpm (50 °C) for 9 days. Samples were withdrawn every 24 h to determine the xylanase activity.

Effect of inoculum size, initial pH, temperature, and agitation rates on xylanase production The tested inoculum sizes of 1, 2, 3, and 4 discs (9 mm, 5 day old from PDA plates) were transferred into 250 ml flasks containing 50 ml of media and grown for 6 days at 50 °C (150 rpm). The optimal initial pH for xylanase production was determined by adjusting the pH of the culture medium from 3 to 8 using 1 N HCl or 1 N NaOH. In the temperature studies, cultures were grown at temperatures between 30 and 70 °C for 6 days at pH 6.5. The effect of agitation on xylanase production was examined by growing cultures at different agitation rates (0–250 rpm). Soluble protein in the culture supernatant was estimated according to the previously described method (15).

Laboratory-scale xylanase production Xylanase production was carried out in a 5 L vertical glass fermenter (Minifors, Infors HT, Switzerland) with 3 L (working volume) of the optimized xylanase production medium (pH 6.5) at 50 °C. A 10% (v/v) culture of the *T. lanuginosus* mutant MC 134 grown in potato dextrose broth was used as an inoculum. The effect of agitation on xylanase production was examined by growing cultures at different agitation rates (200–500 rpm) over a period of 7 days. Aeration was set at 1 vvm (maintained using the cascade mode). Foaming was controlled by the addition of silicon antifoam (Fluka). Samples (20 ml) were withdrawn through the sampling port every 24 h and centrifuged (10,000 ×*g* for 10 min), and the clear supernatant was used to determine the xylanase activity and amount of soluble protein.

Thin layer chromatography (TLC) For enzymatic hydrolysis of xylan, 50 ml (1%) of birchwood xylan (0.5 M citrate buffer, pH 6.5) was incubated with 5 U of crude xylanase obtained from the fermenter after 24 h. Samples were withdrawn at different time intervals (2, 6, 12, and 24 h) and 2 μl aliquots were spotted on silica gel F 60 (Merck, Germany) plates. The chromatogram was developed in ethyl acetate:acetic acid:2-propanol:formic acid:water (25:10:5:1:15, v/v). Sugars were detected by pouring the detecting reagent (1% orcinol in 10% H_2SO_4 in ethanol) on the plates, which were then heated at 100 °C for 5 min. Xylose (Sigma, USA), xylobiose (Sigma, USA), and oligosaccharides (Megazyme, Ireland) were used as standards.

Materials used for biobleaching Unbleached sugar cane bagasse pulp (SAPPI Fine Paper, Stanger, South Africa) was washed with tap water until a neutral pH was observed in the wash water. The pulp was dried in an oven (40 °C) and stored at 4 °C until further use. For biobleaching, pulp (10 g) was treated with 500 U of crude xylanase at 50 °C (pH 6.5) for 3 h at 10% pulp consistency. The reaction was terminated by heating to 100 °C for 20 min. To determine the presence of reducing sugars, 10 ml of test and control samples were centrifuged at 10,000 ×*g* for 15 min and the supernatant was used (15). Lignin-derived compounds in the supernatant were also monitored by measuring the absorption spectrum from 200 to 465 nm using UV–Vis Spectrophotometer (Varian, Cary 100, USA); a control supernatant from sample treated with denatured enzyme was also monitored. The pulp was subjected to standard handsheet-making processes and the final paper product was analyzed (16).

Analytical methods

Enzyme assays According to the previously described method (17), β-Xylanase and cellulase assays were carried out using 1% birchwood xylan (Roth 7500, Karlsruhe, Germany) and carboxymethylcellulose and α-cellulose (Sigma, USA), respectively, as substrates. The activities of accessory enzymes such as xylosidases, arabinofuranosidases, glucosidases, and mannosidase were determined by measuring the release of *p*-nitrophenol from *p*-nitrophenol-β-D-xylopyranoside (3 mg ml^{-1}), *p*-nitrophenol-α-L-arabinofuranoside (3 mg ml^{-1}), *p*-nitrophenol-β-D-glucopyranoside (4 mg ml^{-1}), and *p*-nitrophenol-β-D-mannopyranoside (2 mg ml^{-1}) (Sigma, USA), respectively. Mannanase activity was determined at 50 °C (pH 6.5) using locust bean gum (Sigma, USA) as a substrate. Reducing sugars were quantified using the DNSA reagent method (18). One unit of enzyme (U) was defined as the amount of enzyme that released 1 μmol xylose, mannose, or *p*-nitrophenol min^{-1}.

Standard paper sheets were made from enzymatically pretreated and untreated pulps using the handsheet former (PTI Lab equipment, Austria), and air dried according to TAPPI methods (16). The brightness and opacity of the final paper product was measured using a reflectance meter (Technidyne, USA). The ceramic reference TAPPI corresponding to a brightness of 47.5% was used as a standard. The tensile index, burst factor, and tear index of the paper samples were also tested. For the scanning electron microscopy study, xylanase-pretreated and control samples were mounted on stubs, sputter coated with gold palladium, and examined under a scanning electron microscope (Philips, USA) at 10 kV.

Optimization of biobleaching process Optimization of the enzyme dose (5, 10, 50, or 100 U g^{-1} pulp) and reaction time (1, 2, 3, or 5 h) was carried out by treating the bagasse pulp with the crude xylanase of *T. lanuginosus* MC 134 at 50 °C (pH 6.5). The biobleaching efficiency of crude xylanase (50 U g^{-1} pulp) on bagasse pulp was also examined at various temperatures (30–70 °C) and pH (5–9) conditions for 3 h.

RESULTS AND DISCUSSION

Hemicellulase production by the *T. lanuginosus* SK wild type strain *T. lanuginosus* SK produced high levels of cellulase-free xylanase (346 ± 10 U ml^{-1}) on oatspelts xylan medium after 5 days of cultivation. Accessory enzyme levels were found to be minimal, with the highest being an extracellular β-mannanase (0.91 ± 0.04 U ml^{-1}). The activity of accessory enzymes such as xylosidases, arabinofuranosidases, glucosidases, and mannosidases varied between 0.05 ± 0.02 and 0.23 ± 0.02 U ml^{-1}. A high level of extracellular xylanase production was observed in the culture filtrate, whereas intracellular xylanase levels were very low (34 ± 0.70 U ml^{-1}). Accessory enzyme levels in the intracellular fraction were negligible in *T. lanuginosus* SK and similar observations were made in *T. lanuginosus* SSBP (19).

Strain improvement Mutagenesis, a well-recognized strain improvement approach, was employed to enhance xylanase production in *T. lanuginosus* (20). One hundred and eighteen colonies obtained through a UV mutagenesis study were tested for xylanase production using a medium containing oatspelts xylan. Maximum xylanase production was observed in mutant M 13, which showed 17% increase in xylanase (422 ± 11 U ml^{-1}) production after 5 days, compared to the wild-type fungus. The level of xylanase production in the mutant M 13 was also stable even after being subjected to repeated transfers and further NTG mutagenesis. The resulting mutant, MC 134, showed a 1.5 fold increase in xylanase (516 ± 8 U ml^{-1}) production when compared to the wild type *T. lanuginosus* SK strain.

Effect of carbon and nitrogen sources on xylanase production Maximum xylanase production was observed when *T. lanuginosus* MC 134 cultures were grown on coarse corn cob (1202 ± 42 U ml^{-1}) at 1% (w/v) concentration, whereas, fine corn cob showed a production of 626 ± 21 U ml^{-1}. Polymeric pure substrates such as birchwood (519 ± 16 U ml^{-1}), beechwood (497 ± 13 U ml^{-1}), and oatspelts xylan (473 ± 18 U ml^{-1}) demonstrated appreciable xylanase production. Wheat bran also supported moderate xylanase production (209 ± 6 U ml^{-1}). Although, mono- and di-saccharides favored good fungal growth, the xylanase production level was minimum (data not shown). Most of the nitrogen sources favored xylanase production when at a concentration of 1% (w/v), and yeast extract (1499 ± 53 U ml^{-1}) was found to be the best nitrogen source. Comparatively xylanase production was lower when inorganic salts were used as nitrogen sources (data not shown).