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Delignification of wood pulp by a thermostable xylanase from *Bacillus stearothermophilus* strain T-6

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Abstract

During the bleaching of wood pulp for the paper industry, large amounts of chlorinated aromatic compounds are produced and released into the environment. These compounds are extremely toxic and are a major source of pollution. The paper and pulp industry is seeking for alternative methods for bleaching pulp. One such method involves the use of hemicellulases to release the colored lignohemicellulose. We have isolated and characterized several thermophilic bacteria which produce xylanases. One such strain, T-6, produced high levels of extracellular xylanase, free of cellulase and proteinase activities. Strain T-6 was classified as a strain of *Bacillus stearothermophilus* and was able to grow on defined medium containing xylose, methionine and asparagine at 65 °C. Xylanase activity was induced by either xylose or xylan; no activity was detected with other carbon sources, such as glycerol, acetate, lactose, glucose, maltose, fructose, mannose, galactose or sucrose. Xylanase constitutive mutants were obtained following mutagenesis and detection on p-nitrophenol β-D-xylopyranoside containing agar plates. Xylanase T-6 was produced on large scale, and was purified and concentrated by a single adsorption-desorption step from a cation exchanger. The overall purification yield of a 1000 liter fermentation was 45%, resulting in a 98% pure enzyme. Xylanase T-6 was shown to partially remove lignin from unbleached pulp at 65 °C and pH 9.0, without loss in pulp viscosity. The enzyme-treated pulp was used to make handsheets that had higher brightness than untreated pulp.

Introduction

One of the major sources of environmental pollution today is the pulp and paper industry. During the process of making white paper pulp, large amounts of toxic effluents are released into the environment. Although the major raw material of this industry, wood, is a biodegradable organic material, it is only in the last decade or so that the power of biotechnology has been applied to solve problems in the pulp and paper industry (Eriksson & Kirk 1985; Hakulinen 1988; Jurasek & Paice 1986, 1988; Kirkpatrick 1991). To understand the possible applications of biotechnology in the pulp industry concerning pollution, we would like first to briefly summarize some of the principle technologies involved in making pulp and paper.

Wood is composed of three major components: cellulose, hemicellulose and lignin. These compounds, respectively, comprise about 35–50%, 20–39% and 20–30% of the dry weight of woody plants (Browning 1963). Cellulose is a linear macromolecule consisting of anhydro-β-glucopyranose (glucose) units joined together through β-1,4 glycosidic linkages. Cellulose molecules have a fully extended flat conformation and they are usually present in tightly packed microfibrils that form insoluble crystalline material. Hemicellulose is composed of line-

ar and branched polysaccharides, that can be either hetero- or homopolymers made of various five or six carbon sugars. These sugars include, for example, xylose, arabinose, mannose and galactose; the homopolymer saccharides for these sugars would be xylan, arabinan, mannan and galactan, respectively. Xylan is more flexible than cellulose, and the xylan backbone can be substituted with arabinose, glucuronic acid and methylglucuronic acid. Hemicelluloses are bound through hydrogen bonds to cellulose and by covalent bonds to lignin. Lignin, the third component of wood, is an amorphous, three-dimensional, aromatic polymer composed of oxyphenylpropane units. The three main precursors of lignin are coumatyl alcohol, coniferyl alcohol and sinaphyl alcohol which are polymerized randomly forming a variety of carbon-carbon and ether linkages. The biological role of lignin in living plants is to form, together with celluloses and hemicelluloses, a tissue with enhanced strength and durability against physical and biological degradation. It is the complex, random structure of lignin that allows it to hold and protect the cellulose and hemicellulose fibers.

Wood is converted to pulp for the manufacture of paper and paperboard. Paper, in essence, is a two-dimensional cross-linked cellulolytic fibers that form random hydrogen bonds. In wood, the cellulose fibers are attached to lignin and hemicellulose. Therefore, the cellulose must first be released to give separate fibers that can form intermolecular hydrogen bonds. The process of fiber separation, called pulping, results in paper pulp which is a slurry of wood fibers. Pulping procedures can be divided into four main classes: mechanical pulping, chemical pulping, semichemical pulping and chemimechanical pulping, where the last two procedures are, in essence, combinations of the first two (Sanyer & Chidester 1963). In mechanical pulping, fiberilization is accomplished by grinding bolts of wood with a grindstone, or, by disk milling of chipped wood. The separation of the fibers takes place entirely by mechanical means producing high yield pulp. In this procedure, lignin modification and removal is negligible and the yield of the pulp is about 95%. In chemical pulping the wood is treated with harsh chemicals at high temperature and pH values which degrade and solubilize most of the lignin (delignification). The most common processes are sulfate (Kraft), soda and sulfite pulping. In the Kraft process, wood chips are cooked in a solution of Na_2S/NaOH at about 170 °C for two hours resulting in degradation and solubilization of lignin (Sanyer & Chidester 1963). The lignin in the chemical pulping process is extensively modified, and 60% to 90% of it is removed from the wood. The pulp yield varies from 45 to 60% of the wood weight.

In all of the pulping processes, the resulting pulp has a characteristic brown color which is primarily due to the residual lignin and lignin derivatives. The intensity of the pulp color is a function of the amount and chemical state of the remaining lignin. To obtain white and bright pulp suitable for the manufacture of printing and tissue grade papers, it is necessary to bleach the pulp and to remove most of the constituents of the unbleached pulp, such as lignin and its degradation products, resins and metal ions (Singh 1979). The methods of bleaching pulp with high lignin content (mechanical pulp), differ fundamentally from those of bleaching chemical pulps. Bleaching mechanical pulps is usually conducted in such a way as to avoid loss of lignin. Bleaching agents such as hydrogen peroxide and sodium hydrosulfite are used. The bleaching of mechanical pulp, which improves the eye-appeal of the pulp, does not yield a high brightness paper with stability against brightness reversion on aging. To obtain pulp of a very high brightness and brightness stability, all of the lignin must be removed from the pulp. For this reason, chemical pulping (where most of the lignin is removed during the cooking process), is the method of choice for producing high quality white paper. The residual lignin in chemical pulp is very dark in color because it has been extensively oxidized and modified in the cooking process. This residual lignin is difficult to remove due to the fact that it is covalently bound to the hemicellulose and perhaps to cellulose fibers. Covalent lignin-carbohydrate linkages in wood consist mainly of ester and ether linkages through sugar hydroxyl groups to the a-carbonyl of phenylpropane subunits in lignin (Eriksson et al. 1980; Jeffries 1990). In addition, unnatural covalent

bonds between lignin and carbohydrates are thought to form during the chemical pulping process (Iversen & Waennstroem 1986). The process of bleaching chemical pulp is the main source of pollution in the paper industry (Trubacek & Wiley 1979). The bleaching can be regarded as a purification process involving the destruction, alteration or solubilization of the lignin, colored organic matters, and other undesirable residues on the fibers (Singh 1979).

The traditional bleaching of chemical pulp is performed in a number of stages, known as a bleaching sequence. It has become common practice to use a single letter as a symbol for a particular stage. Some of the symbols and the stages which they represent are, for example: C – chlorination; E – alkaline extraction; H – hypochlorite bleaching; D – chlorine dioxide bleaching; P – peroxide bleaching; O – oxygen bleaching; A – acid treatment; Z – ozone bleaching. The exact choice of the bleaching sequence depends upon the type of pulp to be bleached and the level of brightness to be achieved (Singh 1979; Sanyer & Chidester 1963). The most relevant step regarding pollution is chlorination. Chlorination of pulp, as it is usually carried out, does not show any decoloring effect, and in fact, the color of the pulp may increase with chlorination (Loras 1980). The dominant role of chlorine in bleaching is to convert the residual lignin in the pulp to water or alkali soluble products (bleaching of chemical pulps to a higher brightness without complete removal of lignin has not been successful so far). At low pH the main reaction of chlorine is chlorination rather than oxidation. That is, chlorine selectively chlorinates and degrades lignin compounds rather than the carbohydrates moieties in the unbleached pulp. It is for this reason that chlorine is so effective in the bleaching process. Following the chlorination step, the chlorinated lignins can be efficiently removed from the pulp by alkaline extraction. Under basic conditions, the phenolic compounds are more soluble and, in addition, at high pH, further degradation of chlorinated lignins takes place. Other treatments that usually follow the chlorination and alkaline extraction stages include bleaching with hypochlorite, chlorine dioxide, peroxide and oxygen. These agents are oxidative in nature and perform true bleaching of the pulp; however, they are not as selective as chlorine and can react with the polysaccharides. Today, modern Kraft pulp bleaching is carried out in five or six stages. Common bleaching sequences are, for example, CEDED, CEHDED and CEDEDP.

The effluents that are produced during the bleaching process, especially those following the chlorination and the first extraction stages, are the major contributors to waste water pollution from the pulp industry (Trubacek & Wiley 1979; Eriksson & Kirk 1985). These effluents, which contain large amounts of chlorinated phenols and Kraft lignin, are usually treated biologically by processes such as activated sludge or aerated lagoons. However, some of these harmful chemicals pass the treatment plant and find their way into the recipient water way. A study presented by Larsson et al. (1988) showed that bleached Kraft mill effluents, disposed into the sea, exert biological effects on fish, caught even 10 km from the pulp plant. Typical symptoms were: reduced gonad growth, enlarged liver, strong induction of enzymes in the hepatic mixed function oxidase system, disturbed ion balance and marked effects on the red and white blood cell pattern.

Another aspect of bleaching pulp with chlorine involves the paper itself. Following the bleaching process, some of the chlorinated compounds are likely to be associated with the paper. The most worrying chemical is the animal carcinogen dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin, which often finds its way into finished paper products. There is some concern, therefore, as to how safe it is to use chlorine-bleached chemical pulp paper for purposes such as baby diapers and food packaging.

The pulp and paper manufactures are testing different approaches to reduce and, if possible, to eliminate the use of chlorine in the bleaching process. While paper mills in the United States have preferred to focus on waste water treatment, European companies, particularly in Sweden, Finland and Germany, have addressed process changes instead (Samdani 1990).

For over a decade, it is recognized that white-rot fungi can remove color out of effluents coming

from Kraft pulp mills (Fukuzumi et al. 1977) and degrade ^{14}C-labelled chlorolignins (Lundquist et al. 1977). Based on these findings, several methods have been developed for the treatment of pulp mill effluents. Patented procedures include the MYCOR and the MYCOPOR processes which utilize the white-rot fungus *Phanerochaete chrysosporium*. (Chang et al. 1987; Messner et al. 1989; Eriksson & Kirk 1985; Pellinen et al. 1988a,1988b). Other white-rot fungi that were used for bleaching and purifying bleach plant effluents include: *Coriolus versicolor* (Livernoche 1981); *Phlebia radiata* and *Merlius tremellosus* (Lankinen et al. 1991). For more details on biotechnology in waste water treatments see: Jurasek & Paice 1986; Lankinen et al. 1991; Heinzle et al. 1992; Eriksson & Kirk 1985; Hakulinen 1988.

A different biotechnological approach for reducing or even eliminating the use of chlorine in the bleaching process involves the use of enzymes or fungi for treating the pulp. Two general enzyme categories were considered and tested; ligninases and hemicellulases. Ligninases are produced mainly by white-rot fungi, which are the natural lignin degraders. The complicated biology and chemistry of ligninases had been elucidated in the last years (Kirk & Farrell 1987). Ligninases are peroxidases that catalyze the formation of oxygen radicals from H_2O_2. These oxygen radicals attack and oxidize the aromatic nuclei of lignin and form unstable cation radicals, which decompose through several different reactions (Kersten et al. 1985). Because the mechanism of lignin degradation involves the formation of oxygen radicals, ligninases are nonspecific in the substrate they attack. For example, Kraft lignin is readily metabolized by white-rot fungi (Lundquist et al. 1977; Eriksson & Kirk 1985).

Kraft pulp can be bleached directly with white rot fungi by incubating the fungi with the pulp for several days (Kirk & Yang 1979; Tran & Chambers 1987; Paice et al. 1989; Fujita et al. 1991). Kirk & Yang (1979) achieved with *Phanerochaete chrysosporium* a reduction of 50–75% in Kappa number (a measure of lignin content) during 6–8 days of incubation. Paice et al. (1989) reported that hardwood Kraft pulp can be bleached by the fungus *Coriolus versicolor*. Following a five-day treatment, the pulp brightness increased by 15 points (ISO) and the Kappa number decreased from 11.6 to 7.9. The bleaching effect of *C. versicolor* appeared to be limited to hardwood since experiments with spruce (softwood) pulp failed to show a bleaching effect. However, Reid et al. (1990) recently reported the use of this fungus for bleaching softwood Kraft pulp. Biobleaching directly with fungi is probably not a viable approach in industry for several reasons. Among them are the time required for treatment and the need for nutrients for the growth of the fungi.

The use of purified or crude preparation of ligninases was also considered and, in fact, a US patent has been published, claiming that this enzyme was able to bleach Kraft pulp when used with a subsequent alkaline extraction procedure (Farrell 1987). Trials in other laboratories failed to reproduce biobleaching using enzyme(s) from *P. chrysosporium* (Kirkpatrick 1991; Viikari et al. 1987), and the effectiveness of the ligninase treatment is not clear. One possible explanation could be, that following the ligninase treatment the lignin components polymerize back onto the pulp. A recent patent application by International Paper Company claims, that lignolytic enzymes can be used to bleach pulp in a process which maintains low steady-state concentration of hydrogen peroxide (0.001 to 0.1 mM). The hydrogen peroxide concentration may be maintained by *in situ* enzymatic generation, for example, through the action of glucose oxidase on glucose (Enzymatic delignification of lignocellulosic material, European patent application EP 0 406 617 A2; 1991).

Hemicellulases are another type of enzymes that were tested for biobleaching. Hemicellulases comprise a group of enzymes that can hydrolyze the hemicellulose polymer chains at various positions or act on side groups attached to the backbone polymer. These enzymes are produced by various microorganisms, among them are fungal yeast and bacterial species and include, for example: endoxylanases, β-xylosidase, arabinfuranosidase and acetyl-xylanesterase. (Dekker & Richards 1976; Reily

1979; Dekker 1985). Viikari et al. (1986, 1987) were first to demonstrate that hemicellulases can be used to enhance delignification and bleaching. Fungi hemicellulases were used to treat unbleached pine sulfate pulp. Following the enzymatic treatment the pulps were bleached with hydrogen peroxide. In these experiments, although the enzymatic treatment resulted in a reduction in chlorine consumption, there was also an unacceptable loss of yield and viscosity. The yield loss was mainly a result of hemicellulose solubilization, where the viscosity drop was probably a result of contaminating cellulases. Further experiments with purified and cloned hemicellulases indicated that enhanced bleaching can be achieved without yield and viscosity loss (Paice et al. 1988; Farrell 1991; Senior et al. 1988; Clark et al. 1990). Comparison between different hemicellulases indicated that endo-β-xylanases have the major impact on delignification, even in softwood pulp, where mannan is the major component. It was suggested by Kantelinen et al. (1991) that two types of phenomena are involved in the enzymatic pretreatment. The major effect is due to hydrolysis of reprecipitated and readsorbed xylan or xylan-lignin complexes, that are separated during the cooking process. As a result of the enzymatic treatment, the pulp becomes more accessible to the bleaching chemicals. A minor effect is due to the hydrolysis of the residual, non-dissolved hemicellulose. In this latter phenomenon other enzymes beside endoxylanases can be used.

From the number of patents and patent applications filed in the last years, it is evident that hemicellulases, and xylanases in particular, are considered a viable option to facilitate pulp bleaching. Indeed, full scale mill trials of enzyme pre-bleaching are already under way (Grant 1991; Koponen 1991; Viikari et al. 1991a, 1991b). The results from these trials indicate that the enzymatic treatment can reduce chlorine requirement by 30% and allow a brightness of 89% ISO to be reached without chlorine.

Most of the hemicellulases studied so far are active at neutral or acidic pH and their optimum temperature is below 45 °C. Hemicellulases that are active at higher temperatures and basic pH are of great potential since they can be introduced more freely in the different stages of the bleaching line without the need for cooling and pH changes. We have recently isolated several thermophiles that produce thermostable xylanases (Shoham et al. 1993). In this report we describe the characterization of these isolates and a large scale production and bleaching ability of one of these enzymes, xylanase T-6.

Materials and methods

Organisms

Bacillus stearothermophilus T-6 was isolated following an enrichment procedure for bacteria capable of producing extracellular thermostable xylanases (Shoham et al. 1993). Strain T-6 was identified as *B. stearothermophilus* by the NCIMB (National Collection of Industrial and Marine Bacteria, England), and was designated as NCIMB 40221. Fatty acid composition of strain T-6 was determined by Microbial ID Inc., Newark, DE, USA. Strain M-7 is a xylanase constitutive mutant of T-6 isolated in this work.

Growth conditions

Growth media were composed of a basic salt medium (BSM) supplemented with various carbon sources. BSM contained (g/l): K_2PO_4 $7H_2O$, 0.75; KH_2PO_4, 0.15; $MgSO_4$ $7H_2O$, 0.1; $(NH_4)_2SO_4$, 2; MOPS buffer (3-N-morpholino propane sulfonic acid), 10.4; and 1 ml of trace elements (trace elements solution contained (g/l): $CaCl_2$ $2H_2O$, 0.37; $CuSO_4$ $5H_2O$, 0.62; $FeSO_4$ $7H_2O$, 0.60; $MnSO_4$ $4H_2O$, 0.59; $ZnSO_4$ $7H_2O$, 0.42; $CoCl_2$ $6H_2O$, 0.79; Na_2 MoO_4, 0.696; the pH of the solution was adjusted to 2.0 with sulfuric acid). The pH of the medium was adjusted to 7.0 and the carbon sources were added after sterilization. ND medium was BSM with 0.5% xylose and 0.3% vitamin free casamino acids. Growth was carried out in 125 ml flasks

containing 25 ml medium and reciprocally shaking (180 strokes/min) in a water bath (Tuttnauer, Jerusalem Israel) at 60 °C.

Large scale purification

A large scale fermentation (2 × 500 l) was carried out with strain M-7 in the fermentation facilities of the Hebrew University, Hadasa, Jerusalem. The growth medium was BSM containing 0.1% urea instead of $(NH_4)_2SO_4$, 0.5% glucose and without MOPS buffer. The pH was maintained between 6.5–7.0 with NaOH and H_2SO_4 and the growth temperature was 60 °C. At the end of the fermentation the cell free supernatant fluid contained about 4.5 xylanase units/ml. Fifteen kilograms of sulphoxyethyl cellulose (SE-52, Whatman, Maidstone, England) were added to the cell free broth (500 l) with gentle mixing for one hour. After allowing the adsorbent to settle, it was collected (about 40 l), washed with 20 mM phosphate buffer, pH 7.0, and resuspended in 60 l of 1.0 M KCl. The adsorbent was removed by filtering through a Whatman No. 3 filter paper, and the enzyme was concentrated by ultrafiltration using a Pellicon Cassette Filter Acrylic Holder (Millipore, Bedford, MA) containing an 8K 5 sq. ft. cassette (Filtron, Clinton MA) (flow rate of 300 ml/min with retentate:filtrate ratio of 1:4).

Enzymatic assays

Xylanase was assayed by mixing an aliquot of appropriately diluted enzyme with 0.25 ml of 2% oat spelt xylan (Sigma, St Louis, Mo), 0.5 ml of 0.1 M phosphate buffer, pH 7, and water to a final volume of 1 ml (xylan was prepared by sonicating a 2% xylan solution for 3 min at output 7 on an Ultrasonic W375 sonicator, Heat System Ultrasonics, Plainview, NY). Aliquots of 0.1 ml were taken out of this mixture and placed in four 16 ml glass tubes. Two tubes served as time zero control and were kept at room temperature or ice, the two other tubes were placed in a water bath at 65 °C for 10 or 15 min. The reaction was terminated by placing the tubes in a water bath at room temperature. The reducing sugars content in the tubes was determined by the DNSA method (Miller 1959), with D-xylose as a standard (0.004% xylose was added to the DNSA reagent just before the color reaction). One unit of xylanase activity was defined as that amount of enzyme which produces 1 micro mol of xylose equivalent per min. Cellulytic activity was measured essentially as for xylanase with carboxymethyl cellulose (CMC) as a substrate and glucose as standard. Proteolytic activity was assayed by incubating 0.1 ml of an enzyme sample together with 4.5 ml 0.2% casein in 100 mM phosphate buffer, pH 7.4, at 65 °C. The reaction was terminated by addition of 0.22 ml cold 50% TCA. After centrifugation, the absorbance at 280 nm was de-

Table 1. Characterization of extracellular xylanase activity from several thermophilic isolates.

Strain	Xylanase				CMCase	Protease
	U/ml	Half life[a] (h)	pH 9 (%)[b]	pH 10 (%)	(U/ml)	(U/ml)
T-1	0.63	0.90	67	58	0.026	nd[c]
T-2	0.89	0.71	59	53	0.014	15.7
T-3	0.51	2.04	58	51	0.031	nd
T-4	0.90	0.80	59	45	0.015	7.3
T-5	1.50	0.60	54	27	0.017	nd
T-6	2.33	4.62	66	54	0.021	nd

[a] Half life of xylanase activity at 70° C.

[b] Percentage activity at pH 9 and 10 compared to pH 7.

[c] nd – not detected.

termined. One unit of activity is defined as 0.001 A_{280}/min.

Protein

Protein content was determined by the method of Bradford (1976) using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) with bovine albumin fraction V (Sigma) as standard.

Thermostability

Eppendorf tubes (1.5 ml) containing 0.2 ml of purified enzyme solution (40 units/ml in 10 mM phosphate buffer, pH 7) were incubated at 70 °C. At various times the tubes were removed and placed at −20 °C for 10 to 24 h. The residual enzymatic activity in each tube was determined with the standard assay.

Isolation of constitutive mutants

Mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine (NTG) was performed at 60 °C according to Adelberg et al. (1965). Mutagenized cells were plated on LB agar plates (Difco or Oxid Bacto Agar) incubated for 12 h at 60 °C and then overlaid with 5 ml of soft agar (0.7% agar) containing 2 mg of p-nitrophenyl β-D-xylopyranoside (PNPX). Colonies of constitutive mutants produced yellow color after 10 min at room temperature.

Enzymatic delignification

Enzymatic delignification of softwood pulp was done as described by Shoham et al. (1993).

Table 2. Fatty acids profile of strain T-6.

Fatty acid	%
9:0	0.64
12:0	0.46
14:0	1.92
15:0 iso	46.20
15:0 anteiso	2.35
16:0 iso	4.15
16:1	5.53
16:0	6.14
17:1 iso H	2.60
17:0 iso	22.58
17:0 anteiso	5.82
18:0	1.09

Results and discussion

Initial characterization of extracellular xylanase activity from several thermophilic isolates

Following enrichment procedures, several different thermophilic strains capable of producing extracellular xylanase were isolated (Shoham et al. 1993). To identify the best strain for the production of xylanase, several criteria were tested including: level of extracellular xylanase activity at high pH, thermostability and lack of proteolytic and cellulolytic activities in the supernatant fluid. Table 1 summarizes the results obtained with 6 different isolates. Strain T-6 gave the highest xylanase activity (2.33 units/ml); its enzyme was the most thermostable and had the highest activities at pH 9 and 10. In addition, the extracellular fluid of strain T-6 had no detectable level of proteolytic activity. All of the strains showed very low cellulolytic activity. Based on these results, strain T-6 and its xylanase were further characterized.

Table 3. The effect of phosphate on the growth of strain T-6.

Phosphate (mM)	Growth rate[a] (1/h)	Extent of growth (Turbidity units KU)
2.7	0.94	256
4.1	0.96	260
5.5	0.84	160
8.1	0.26	87
10.8	No growth	

[a] Growth was carried out on ND medium in 125 ml shake flasks at 60° C.

Classification of strain T-6

Strain T-6 was a Gram positive, aerobic, spore-forming rod, capable of growing at 65 °C. The spore shape was either elliptical or cylindrical and spore position terminal or subterminal. The strain failed to grow at 25 °C or 37 °C or in the presence of 5–10% NaCl, was catalase and oxidase positive and was capable of decomposing both casein and gelatin. Based on these results and its fatty acid profile (Table 2), strain T-6 was assigned to the heterogeneous species *Bacillus stearothermophilus*. Strain T-6 was also identified as a *Bacillus stearothermophilus* strain by the National Collection of Industrial & Marine Bacteria (NCIMB), England, and was designated as NCIMB 40221.

Growth characteristics

Strain T-6 can readily grow on rich or semi defined media. On the semi defined media, ND, the optimum temperature and pH for growth are 65 °C and 6.5, respectively, with a doubling time of about 40 min. Strain T-6 is inhibited by high phosphate concentrations (Table 3). At concentrations above 4 mM, there was some inhibition of growth; at concentrations above 10 mM, there was no growth at all. A defined medium was also constructed for strain T-6. Although the strain can grow to some extent on basic salt medium, BSM, in the presence of either methionine or asparagine, addition of the two amino acids together strongly stimulated growth (Table 4). Addition of trace elements to either the defined or semi defined media improved growth, and the most important metals for growth were Zn, Fe and Mn. The growth yield of strain T-6 on glucose and xylose were 0.27 and 0.17 g dry cell weight per g sugar, respectively.

Xylanase production

Strain T-6 produces extracellular xylanase activity when grown in the presence of either xylan or xylose. When grown on ND medium, strain T-6 reached a turbidity of 600 KU and xylanase activity was about 2 units/ml. No activity was detected with other carbon sources, such as glycerol, acetate, lactose, glucose, maltose, fructose, mannose, galactose or sucrose. The rate of xylanase production was usually highest towards the stationary phase; enzyme synthesis continued during the stationary phase if enough xylose was present. Combinations of xylose, together with other readily metabolized carbon sources such as glucose or glycerol, resulted in lower levels of xylanase production. These results suggest that xylanase production is inducible, can be repressed by other carbon sources and probably can be controlled by stationary phase genes such as degQ and degU (Fisher & Sonenshein 1991; Sonenshein 1989). In *B. subtilis*, enzymes responsible for the degradation of xylose and xylan are induced when grown in xylose-containing medium and repressed when rapidly metabolized carbon sources are available (Gartner et al. 1988).

Isolation of xylanase constitutive mutants

We were interested in obtaining xylanase constitutive mutants for the following reasons: a) xylose is a relatively expensive carbon source; economical large scale production of the enzyme would require a less expensive source; b) in many cases, constitutive mutants are over-produces of the desired protein; and c) constitutive mutants can facilitate some understanding on the regulation of the xylanolytic system in strain T-6. Screening for constitutive mutants on xylan containing agar plates (looking

Table 4. Growth of strain T-6 on basic salt medium (BSM) with amino acids.

BSM + amino acid[a]	Growth rate[b] (1/h)	Extent of growth (Turbidity units KU)
Met	0.32	61
Asn	0.35	55
Asn, Met	0.66	253
Asn, Met, Asp, Lys, Thr, Ile	0.71	260

[a] Amino acid concentration was 50 mg/l.
[b] Growth was carried out in 125 ml shake flasks at 60 °C.

for clear zones) was not possible since xylan is an inducer. We, therefore, took advantage of the fact that in many cases, xylanase and xylosidase are under the same regulatory control (repressor). Xylosidase producing strains can be easily detected on agar plates containing the chromagenic substrate p-nitrophenyl β-D-xylopyranoside (PNPX). Only constitutive mutants will produce yellow color on agar plates in the absence of xylose or xylan. To obtain xylosidase constitutive mutants, cells were mutagenized with NTG and then plated on LB agar plates. Plates containing 12 to 24 h old colonies were then overlaid with 5 ml of soft agar containing 2 mg PNPX. Colonies of constitutive mutants were identified after 10 min at room temperature by the yellow color they produced. Out of about 30,000 colonies screened, 30 colonies that produced yellow color were isolated. All of the isolates produced both xylosidase and xylanase activities in LB liquid media. From the relatively high frequency of mutants obtained and the mode of action of NTG (NTG induces multiple mutations in localized regions), it is likely that the mutations are in the repressor (inactivation of a gene) rather than mutations in the promoter/operator region. One of the constitutive mutants, strain M-7, was used for further studies. Strains M-7 and T-6 showed identical growth patterns on LB or ND media. However, on DM containing glucose instead of xylose, strain M-7 produced about 2 units/ml of xylanase, whereas strain T-6 gave no detectable levels of xylanase activity.

Large scale purification of xylanase T-6

Preliminary results during the purification of xylanase T-6, indicated that the enzyme can be highly purified and concentrated after a single adsorption step to a cation exchanger (CM-11 or CM-52 Whatman). Batch adsorption has the advantage of speed and ease of scaling the process. However, unless the partition coefficient P_c is well above 0.98, some losses are inevitable (Scopes 1987) (the coefficient P_c is defined as $P_c = Q/(C + Q)$ where Q is the concentration of the adsorbed protein and C is the concentration of the free protein). To assess the possibility of using batch adsorption for purifying xylanase T-6, it was necessary to determine the partition coefficient (P_c) and the maximum adsorbent capacity (Qmax) of CM-52. According to the mathematical model for an ion exchange process formulated by Cowan et al. (1986), the solid phase concentration of the adsorbed material will vary with the concentration of the adsorbate in a manner described by the equation $Q^* = C^* \times Qmax/(Kd + C^*)$, where Qmax is the maximum adsorbent capacity, C^* and Q^* are the equilibrium protein concentrations in liquid and solid phase, respectively, and Kd is the dissociation constant of the equilibrium reaction. The maximum adsorbent capacity and dissociation constant of an adsorbent can be estimated using the linear forms of this equation.To determine Qmax and Kd for xylanase T-6 and CM-52, different amounts of purified enzyme were mixed together with a constant amount of CM-52 adsorbent. Flasks containing 0.02 to 0.1 mg/ml of xylanase T-6 in 10 ml of phosphate buffer (20 mM, pH 6.5) and 0.1 g CM-52 were shaken for 12 h to reach the equilibrium. The adsorbent was filtered and the protein content in the solutions was determined using the Bio-Rad assay. The amount of the enzyme bound to the adsorbent (Q^*) was calculated from the difference between the amount

Table 5. Purification of xylanase T-6 from a 1000 liter fermentation of strain M-7.

Steps	Volume (l)	Protein (mg/ml)	Act. (U/ml)	Sp. act. (U/mg)	Yield (%)
Supernatant	1150	0.23	0.23	17.4	100
Residual broth[a]	1000	0.22	0.22	4.03	20
KCl elution	63	–	37.8	–	45.5
Ultra-filtration	7	0.78	3410	437	46.2

[a] Residual broth after adsorption.

of the enzyme present in the beginning of the experiment and the amount still left in the soluble phase. From the linear forms of adsorption isotherms, Qmax and Kd were calculated to be 25 mg/g and 0.017 mg/ml, respectively. The partition coefficient was calculated as $P_c = Q^*/(C^* + Q^*)$ (1.05 g CM-52 ~ 1 ml of packed bed) for the four experimental conditions, and the average value was 0.999. This high partition coefficient implies that batch adsorption of xylanase T-6 by CM-52 Cellulose is a viable approach. Another commercially available cation exchanger is SE-52 (the negative groups are sulfoxyethyl attached to cellulose). SE-52 is known to have a larger adsorption capacity then CM-52 and since the purification process is also a concentrating step, it is advantageous to use as little as possible of the adsorbent. We found that 2–3% of SE-52 will result in a yield of 48% of the purified enzyme. Based on these results, a simple scaleable process was developed for purifying xylanase T-6 directly from the fermentation broth. The process was based on the use of a single adsorption step for purifying and concentrating the enzyme. The results from a 1000 l purification process are summarized in Table 5. The overall yield of the process was 45%, and the enzyme obtained was over 98% pure as estimated by SDS-PAGE and FPLC gel filtration. The purified xylanase had a molecular weight of 43,000 and a pI of 9.0. At pH 7 and 65 °C, the enzyme was stable for over 10 h; at pH 9 and 65 °C, the half life of the enzyme was about 6 h.

Table 6. Xylanase T-6 treatment of softwood Kraft pulp[a].

Treatment[b]	% Lignin released	
	Total	Net
Buffer, 2 h	4	–
Buffer, 4 h	5	–
Xylanase, 2 U/ml, 2 h	10	6
Xylanase, 5 U/ml, 2 h	17	13
Xylanase, 20 U/ml, 4 h	15	10
Xylanase, 50 U/ml, 4 h	23	18

[a] Partially oxygen bleached softwood Kraft pulp (Kappa No. = 17.5) was used at a final concentration of 5% dry weight pulp.
[b] pH 9.0, 65° C, 20 mM $(NH_4)_2SO_4$.

Delignification

The delignification activity of pure xylanase T-6 was tested in laboratory using partially oxygen bleached soft wood pulp obtained from Korsnas paper and pulp company. The net release of lignin was 18% and 10% for 5 units/ml and 2 units/ml, respectively, for 4 h at pH 9.0 and 65 °C (Table 6). Larger quantities (10 g) of the pulp were then treated with 5 units/ml xylanase T-6 at pH 9.0 and 65 °C for 2 h and sent to Korsnas for analysis (Table 7). Compared to the control (no enzyme), the enzyme reduced the lignin (Kappa no.) and pentosan content without loss in the viscosity of the cellulose. The slight but significant increase in viscosity was probably due to the loss of lignohemicellulose, which does not contribute to the viscosity. Handsheets prepared from the pulps demonstrated that the enzyme-treated pulp yielded higher brightness with no significant loss of fiber strength. More significantly, the ClO_2, Cl_2 required to completely bleach the enzyme-treated pulp was reduced by 35%.

The xylanase T-6 treatment is now being optimized and scaled up. We anticipate that xylanase, as well as possibly other hemicellulases, will soon be used in the pulp industry to reduce or eliminate the use of chlorine-containing compounds for preparation of bleached pulp.

Table 7. Analysis of xylanase T-6 treated Kraft pulp.

Parameter	Control	Enzyme treated[a]
Kappa no.	17.1	15.4
Viscosity (dm^3/kg)	1061	1072
Pentosan (%)	7.3	7.3
Handsheets		
Brightness (% ISO)	32.6	34.4
Tensile index (Nm/g)	21.2	20.7
Zero-span (Nm/g)	100	103

[a] pH 9.0 and 65° C for 2 h with 50 U/ml xylanase T-6.

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