J. Microbiol. Biotechnol. (2008), **18**(7), 1335–1341

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Mutanase Induction in *Trichoderma harzianum* by Cell Wall of *Laetiporus sulphureus* and its Application for Mutan Removal from Oral Biofilms

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Received: October 4, 2007 / Accepted: March 5, 2008

The cell wall material from fruiting bodies of *Laetiporus sulphureus* has been suggested as a new alternative to mutan for the mutanase induction in *Trichoderma harzianum*. Structural analyses revealed that the cell wall fraction from this polypore fungus contained 56.3% of (1→3)-linked α-glucans. When the strain *T. harzianum* F-340 was grown on a cell wall preparation from *L. sulphureus*, the maximal enzyme productivity obtained after 3 days of cultivation was 0.71 U/ml. This yield was about 1.8-fold higher than that achieved on mutan, known so far as the best, but expensive and inaccessible, inducer of mutanase production. Cell-wall-induced mutanase showed a high hydrolytic potential in reaction with a dextranase-pretreated mutan, where maximal degrees of saccharification and solubilization of this biopolymer (80% and 100%, respectively) were reached in 3 h at 45°C. The mutanase preparation was also effective in degradation of streptococcal mutan and its removal from oral biofilms, especially in a mixture with dextranase.

Keywords: Mutanase, *Trichoderma harzianum*, *Laetiporus sulphureus*, α-(1→3)-glucan

Microbial mutanases are mainly inducible extracellular enzymes capable of hydrolyzing α-(1→3)-glucosidic bonds in streptococcal mutans and of removing dental and denture plaques [5, 18]. Mutan, a mixed-linkage [α-(1→3), α-(1→6)] water-insoluble D-glucan, is a structural and functional component of oral cavity biofilms and is commonly used as a specific and efficient inducer of mutanases [15]. However, this biopolymer is not a suitable stimulant of mutan-degrading enzymes because (i) mutan production is based on a two-stage procedure and requires the use of cariogenic streptococci and medium components, which are expensive and dangerous for humans; (ii) the output of this time-consuming process is still relatively low [20]; and (iii) the obtained mutans possess great structural heterogeneity and differential properties [17]. Given this, bacterial synthesis of mutan has only been realized on a laboratory scale, and this type of exopolysaccharide has not yet been made available as a commercial product.

Fungal α-(1→3)-glucans may also be used as alternative inducers of mutanase synthesis. Basidiocarps of *L. sulphureus* are the richest source of such biopolymers (up to 78% dry weight) [6], but to our best knowledge, there are no reports dealing with their use for mutanase induction. *Laetiporus sulphureus* is a wood-decomposing organism growing on deciduous trees. It produces large and strong fruiting bodies that are edible when young, and whose wet biomass sometimes reaches a few kilograms. This polypore fungus can also be cultivated on a larger scale in the laboratory both as fruiting bodies and as hyphal mycelia [1, 2]. Therefore, a mycelial material of *L. sulphureus*, rich in α-(1→3)-glucans, could represent an inexpensive, easily available, and safe for humans source of inducer for mutanase production.

In these investigations, a cell wall preparation from fruit bodies of *L. sulphureus* was applied as a new inducer of mutanase synthesis in *T. harzianum*, and its alkali-soluble fraction was characterized in detail in a structural study. The aim was also to effectively hydrolyze streptococcal mutan by the cell-wall-induced mutanase or a mixture of mutanase and dextranase and to remove this biopolymer enzymatically from oral biofilms created *in vitro* by pathogenic microorganisms.

Materials and Methods

Microorganisms

Trichoderma harzianum strain F-340 (Czech Collection of Microorganisms, Brno, Czech Republic) was used as a starting culture for mutanase induction by cell wall material from *L. sulphureus*. The cariogenic streptococci used in this study included *Streptococcus mutans* 6067, *S. sobrinus* 6070 (The Collection of

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Animal Pathogenic Microorganisms, Brno, Czech Republic), *S. sobrinus* 20381 (formerly *S. mutans* 20381) (The German Collection of Microorganisms, Braunschweig, Germany) and *S. sobrinus/downei* CCUG 21020 (formerly *S. mutans* OMZ 176) (The Culture Collection, University of Göteborg, Sweden). The pathogenic yeast *Candida albicans* was a gift from Dr. A. Kędzia (Medical University of Gdańsk, Poland). The fruiting bodies of *Laetiporus sulphureus* (Bull.: Fr.) Murrill, representing a combination of young and aged basidiocarps, were collected in summer 2005 from deciduous trees grown in Lublin and the surroundings, Poland.

Trichoderma harzianum Cultivation

Stock cultures of *T. harzianum*, maintained at 4°C on potato dextrose agar slants, were used for inoculations. Liquid medium A (pH 5.3), as described by Mandels *et al.* [8], supported by 0.3% (unless otherwise stated) cell wall material or mutan as mutanase inducers, 0.05% peptone proteose, and 0.1% Tween 80, was used for mutanase production. Shaken cultures were conducted in 500-ml conical flasks containing 100 ml of sterile medium. Unless otherwise stated, the flasks were seeded with conidia to a final concentration of about 2×10^5 conidia/ml and placed on an orbital rotary shaker at 220 rpm and 30°C for 6 days. Samples of the culture media were withdrawn periodically from shaken flasks and analyzed for mutanase activity and protein content.

Partial Purification of Cell-Wall-Induced Mutanase

The cell-free culture supernatant of *T. harzianum*, containing mutanase induced by cell wall preparation from the polypore fungus *L. sulphureus*, was loaded on a Sephadex G-50 column (2.5×54 cm; Pharmacia, Uppsala, Sweden). The column was preequilibrated with 0.2 M sodium acetate buffer (pH 5.5), and proteins were eluted with the same buffer. Fractions showing high mutanase activity were pooled and lyophilized in a freeze-dryer (Model 77535; Labconco Co., Kansas City, MO, U.S.A.). The dry powder with an enzyme activity of 0.077 U/mg and protein content of 0.048 mg protein/mg lyophilizate was used as a partially purified cell-wall-induced mutanase preparation for hydrolysis of mutan and its removal from oral biofilms.

Cell Wall and Alkali-Soluble Glucan Preparation

The extraction and purification of the polysaccharides from *L. sulphureus* (100 g) was initiated by milling the lyophilized fruit bodies (preparation of fresh fruiting bodies) and treating the resulting powder with water at 121°C for 1.5 h (×3). Wall material was removed by centrifugation (10,000 rpm for 30 min) and freeze-dried (cell wall preparation, CWP). To isolate the alkali-soluble fraction, the CWP was suspended in 1 M NaOH under constant stirring. After an overnight incubation at room temperature, the supernatant was neutralized with 1 M HCl. The insoluble fraction was collected by centrifugation, washed with water (×3), and lyophilized to give the white powder [purified α-(1→3)-glucan preparation].

Mutan Preparation and Structure

Native mutan was synthesized from sucrose with the use of a mixture of crude glucosyltransferases of cariogenic *S. sobrinus/downei* 21020 as described previously [20]. It was used in fungal submerged cultures as a mutanase inducer of streptococcal origin. A dextranase-pretreated mutan (DTM) was prepared (50 U/g mutan, pH 6.0, 37°C, 3×24 h) as a material for hydrolysis experiments and a substrate for mutanase activity. Dextranase of *Penicillium* sp. with an enzyme activity of 12.9 U/mg preparation was purchased from Sigma-Aldrich (St. Louis, MO., U.S.A.). The linkage structures of the natural and the dextranase-treated mutans, determined by ^{1}H nuclear magnetic resonance (NMR), showed that they were mixed-linkage α-(1→3) and α-(1→6) biopolymers with a greater proportion of α-(1→3) to α-(1→6) linkages; namely, 59.1 and 40.9 mol% for native mutan and 79.8 and 20.2 mol% for DTM, respectively.

Structural Studies of the Alkali-Soluble Glucan Isolated from Fruiting Bodies of *L. sulphureus*

The ^{1}H NMR spectrum of the alkali-soluble glucan preparation was recorded with an Avance (300 MHz) spectrometer (Bruker BioSpin GmbH, Rheinstetten/Karlsruhe, Germany) at 60°C. ^{1}H chemical shift was obtained using acetone (δ_H–2.225 ppm) as the internal standard. An infrared absorption spectrum (FT-IR) was done using a Perkin Elmer FT-IR spectrophotometer (Model 1725X, Perkin Elmer, Norwalk, CT, U.S.A.) between 400 and 4,000 wave numbers (cm^{-1}). A specimen was prepared by the KBr-disk method. Specific rotation $[\alpha]_D^{25}$ (*c* 1 M sodium hydroxide) was measured at 589 nm in a Perkin Elmer Automatic Polarimeter (Model 341 LC).

Mutanase Assay

The standard mutanase assay mixture contained 0.5 ml of 0.2% (w/v) DTM in 0.2 M sodium acetate buffer (pH 5.5) and 0.5 ml of the suitably diluted enzyme solution. After 1 h incubation at 45°C, the released reducing sugars were quantified by the Somogyi-Nelson method [10, 14]. One unit of mutanase activity (U) was defined as the amount of enzyme hydrolyzing mutan to yield reducing sugars equivalent to 1 μmol of glucose/min, and expressed as units per ml of culture (U/ml). Specific activity was defined as mutanase units per mg of protein (U/mg protein). One U corresponds to 16.67 nkat. Soluble protein was measured by the method of Schacterle and Pollack [13] using crystalline bovine serum albumin as a standard.

Hydrolysis of Dextranase-Treated Mutan

Standard hydrolysis was conducted in plugged Eppendorf tubes in the presence of 0.05% sodium azide as a preservative. The reaction mixtures contained 1 mg of DTM and cell-wall-induced mutanase at concentrations ranging from 0.01 to 1 U/ml in 1 ml of 0.2 M sodium acetate buffer (pH 5.5). The tubes were incubated for 12 h at 45°C in a water bath shaker, agitated at 150 rpm. Samples of mutan hydrolyzates were withdrawn at various intervals of up to 12 h, heated at about 100°C for 5 min to stop the reaction, and analyzed for total reducing sugars. Enzyme and substrate blanks were included. The percentage of mutanolysis was calculated using the equation: saccharification (%)=reducing sugars formed (mg)×0.9×100/mutan (mg). The residual insoluble glucan was determined by turbidimetric analysis at 560 nm and the degree of mutan solubilization (expressed as a percentage) was calculated.

Degradation of Mutan Adhered to Glass Plates

Natural mutans were formed on saliva-coated glass plates immersed in beakers with 24-h-old sucrose-containing post-culture fluid of *S. sobrinus/downei* 21020. The glass plates with the preformed mutans (24-h-old) were placed in buffered solutions (pH 5.5) of cell-wall-induced mutanase (0.25 U/ml) or a mixture of mutanase (0.25 U/ml) and commercial dextranase (1 U/ml). The degradation of mutans was performed for up to 6 h at 40°C. After enzymatic reactions, the remaining glucans were rinsed copiously with water

and dyed with erythrosin. The absorption of the dyed biopolymers was determined at 560 nm.

Degradation of Mixed Biofilms Adhered to Glass Plates

All tests were conducted under conditions that simulate mutan formation on tooth surfaces in the oral cavity. For formation of oral biofilms, saliva-coated glass plates were placed in beakers with autoclaved (30 min, 117°C) sucrose-containing BHI medium (Brain-Heart Infusion Broth; Baltimore Biological Laboratory, Cockeysville, MD, U.S.A.) and inoculated with a mixed culture of cariogenic streptococci (*S. mutans* 6067, *S. sobrinus* 6070 and 20381, *S. sobrinus/downei* 21020) and the pathogenic yeast *C. albicans* isolated from the oral cavity. Batch cultures were incubated at 37°C for 24 h under stationary conditions. The medium was then removed, and the formed biofilms adhering to glass plates were rinsed with water and immersed in flasks with buffered solution (pH 5.5) of a mixture of cell-wall-induced mutanase (0.25 U/ml) and commercial dextranase (1 U/ml). The degradation of biofilms was performed for up to 6 h at 40°C in the presence of 0.05% sodium azide as a preservative. The medium was then removed, and the remaining biofilm adhering to the glass surface (consisting of mutan with embedded cells) was rinsed with water and dyed with erythrosin. After thorough washing, the absorption of the dyed biofilm was measured at 560 nm.

Statistics

Submerged cultures, saccharification and solubilization of mutan, as well as degradation of mutan and mixed biofilms adhered to glass plates were performed in triplicate, and the analyses were carried out at least in duplicate. The values reported here are mean values with standard deviations being less than 5% in all cases. Other methodological details are given in the legends of the tables and figures.

RESULTS AND DISCUSSION

Structural Analysis of the Cell Wall Alkali-Soluble Fraction from *L. sulphureus*

The purified, alkali-soluble, and water-insoluble D-glucan from fruiting bodies of *L. sulphureus* was extracted from this source with a yield of 56.3%, and showed a specific rotation of $[\alpha]_D^{25}$ +238°. In an infrared absorption spectrum of the polysaccharide, two bands at 848.52 and 822.63 cm^{-1} were visible (Fig. 1), which indicates that the isolated material contained α-(1→3)-D-glucans. Similar IR spectra, distinguished by the absorption band at 849 cm^{-1}, were obtained for α-(1→3)-glucans isolated by Zonneveld [22] from cell walls of *Aspergillus nidulans* and by Jelsma and Kreger [6] from cell wall material of *Piptoporus betulinus*, *Schizophyllum commune*, and *Lentinus edodes*.

The determination of α-glucosidic linkages in the tested glucan was based on the ^1H NMR spectrum. The results presented in Fig. 2 showed a single signal resonance at δ 5.33 ppm, which suggests a linear form of α-(1→3)-D-glucan. This ^1H NMR spectrum resembled those from studies concerning to glucans synthesized by some species of yeasts and

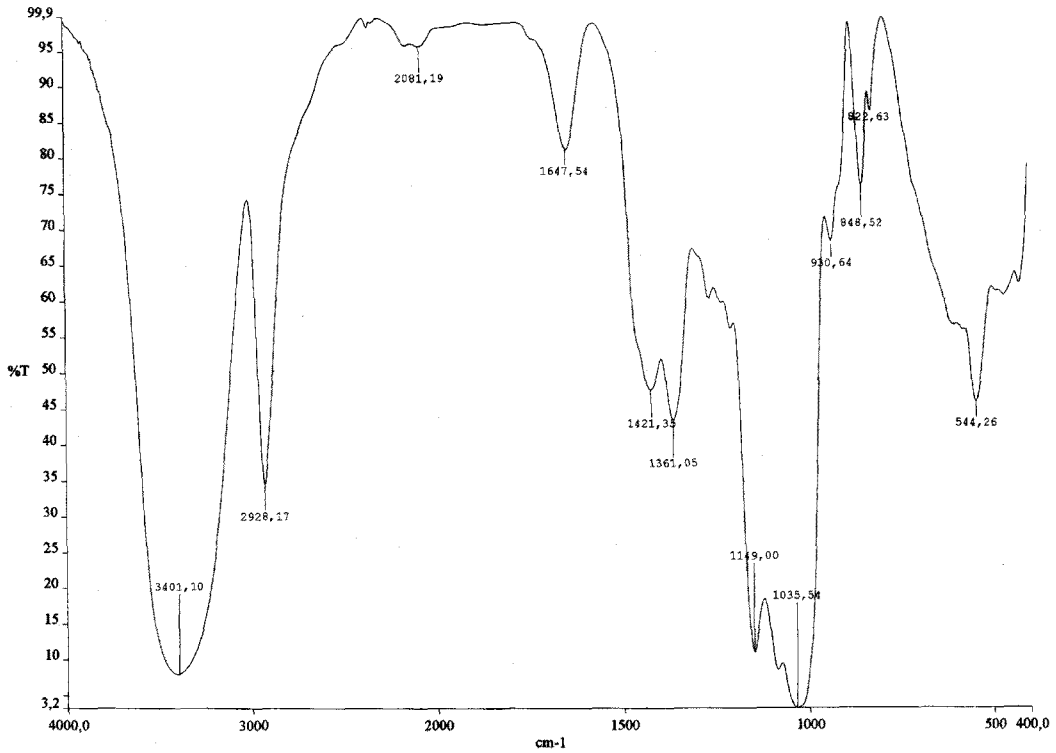


Fig. 1. Infrared absorption spectrum of the alkali-soluble, acid-precipitable, and water-insoluble fraction extracted from powdered fruiting bodies of *L. sulphureus*.

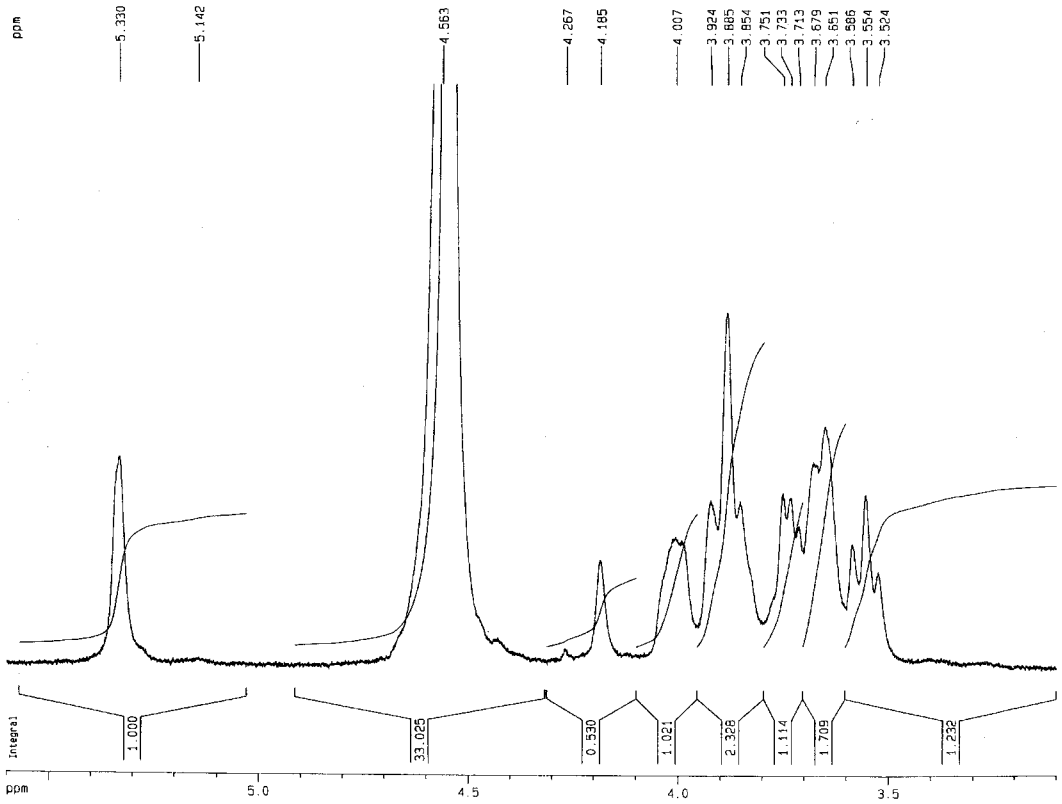


Fig. 2. ^{1}H NMR spectrum of the alkali-soluble, acid-precipitable, and water-insoluble fraction extracted from powdered fruiting bodies of *L. sulphureus*.

filamentous and basidiomycetous fungi [4], and decisively proved the α-(1→3) nature of the analyzed glucan preparation.

Choice of the Best Mutanase Inducer

From among four of the tested preparations, three [lyophilized and milled preparation of fresh fruiting bodies, cell wall preparation (CWP), and purified α-(1→3)-glucan preparation] were derived from *L. sulphureus*, and the fourth one (mutan) was synthesized by cariogenic streptococci and served as a referral trial. These preparations were then used as inducers of mutanase production in shaken flask cultures.

The profiles of mutanase activity stimulated by the respective carbon sources are shown in Fig. 3. The fungal mutanase reached the highest activity (0.61 U/ml) after 3 days of cultivation on CWP, and the enzyme productivity increased up to 0.71 U/ml (1.72 U/mg protein) when the medium was supplemented with 0.4% CWP as a stimulus of mutanase synthesis (Table 1). Increasing of the CWP concentration in the culture medium above 0.5% markedly decreased the mutanolytic activity, and at the CWP dose of 2%, the mutanase yield declined by 81% in comparison with that obtained with 0.4% CWP. In comparison with CWP, streptococcal mutan proved to be a weaker stimulant of mutanase synthesis. After 3 days of cultivation, it induced only 0.4 units of enzyme activity per ml of culture.

The high mutanase productivity (0.71 U/ml) obtained in CWP isolated from fruiting bodies of *L. sulphureus* is at present the best reported in the literature. For example, the

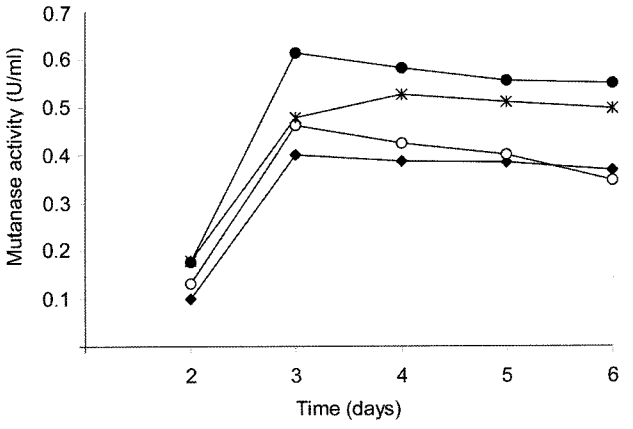
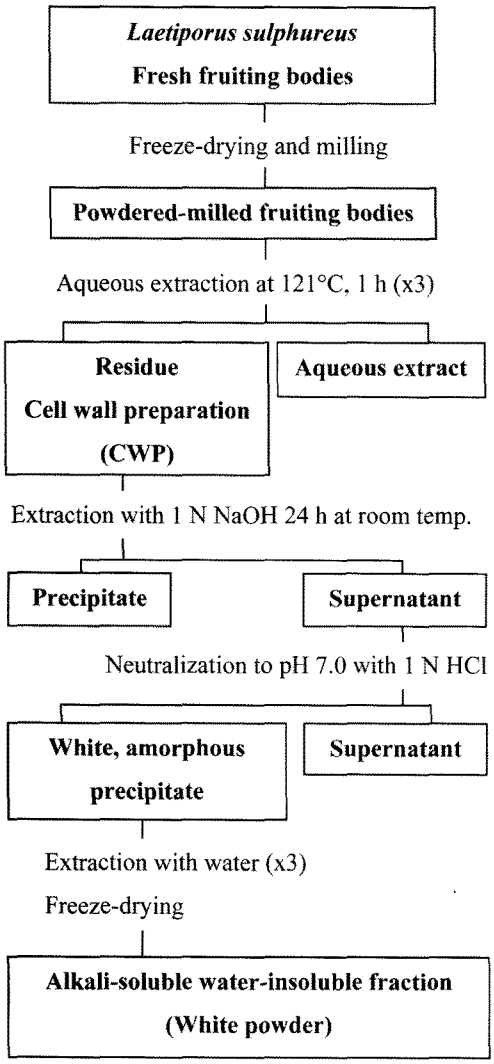


Fig. 3. Inductive effects of different α-(1→3)-glucan preparations on the production of mutanase by *T. harzianum* in 6 days shaken flask cultures.

Mandels medium A (pH 5.3) with 0.3% inducer was used. Native mutan was synthesized by cariogenic streptococci; the other preparations were derived from fruiting bodies of *L. sulphureus* (see Scheme 1). Symbols: mutan preparation (◆), freeze-dried and milled preparation of fresh fruit bodies (○), cell wall preparation (CWP) (●), α-(1→3)-glucan preparation (×).



Scheme 1. Preparation of mycelial cell wall as well as extraction and purification of the alkali-soluble polysacchrides from the fruiting bodies of *L. sulphureus*.

Table 1. Effect of various concentrations of CWP[a] in the culture medium on mutanase production by *T. harzianum* in shaken flasks[b].

CWP concentration (% w/v)	Mutanase activity[c]	
	(U/ml)	(U/mg protein)
0.1	0.326	1.253
0.2	0.497	1.457
0.3	0.645	1.608
0.4	0.711	1.717
0.5	0.695	1.373
1.0	0.278	0.462
2.0	0.135	0.178

[a]CWP, cell wall preparation isolated from fruiting bodies of *L. sulphureus*.
[b]Composition of the Mandel's medium A (pH 5.3) was the same as that of the basal one, but CWP concentrations varied as indicated.
[c]Enzyme activity was determined after 3 days of culture.

strain *T. harzianum* F-470 reached a maximum mutanase yield (0.33 U/ml) after 2–3 days of cultivation on a medium supplemented with 0.3% mutan [19]. Guggenheim and Haller [3], testing the enzyme production of *T. harzianum* OMZ 779 in fermenter runs, obtained an activity of 0.08 U/ml after 155 to 165 h. In shaken flask cultures supplemented with 1% mutan, mutanase activity reached its maximum yield of 0.16 U/ml after 120 h of incubation. Using the same fungal strain, Quivey and Kriger [11] obtained the specific mutanase activity of 0.37 U/mg protein after 4 days in shaken flask cultures.

From these comparative data, it can be concluded that the strain of *T. harzianum* F-340 identified in this laboratory constitutes a culture of high mutanase activity, which gives

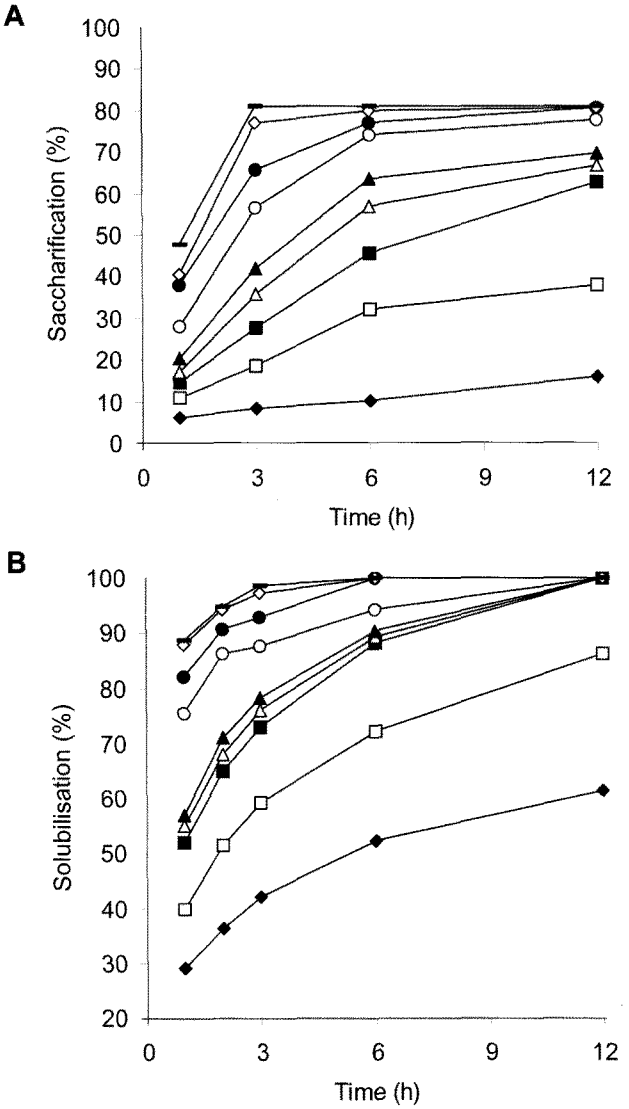


Fig. 4. Enzymatic hydrolysis of dextranase-pretreated mutan (DTM) of streptococcal origin. **A.** Saccharification; **B.** Solubilization. Symbols: Concentration of mutanase (U/ml): 0.01 (◆) 0.025 (□), 0.05 (■), 0.075 (△), 0.1 (▲), 0.25 (○), 0.5 (●), 0.75 (◇), 1.0 (—).

reasonable enzyme yields from cell wall preparation of *L. sulphureus* in a short period of time.

Hydrolysis of Enzymatic-Treated Mutan

The hydrolytic potential of cell-wall-induced mutanase from *T. harzianum* was tested on a dextranase-treated mutanat enzyme concentrations over the range of 0.01 to 1 U/ml. Hydrolysis of glucan was studied as a function of enzyme concentration, and the optimization of the process was carried out with respect to its usefulness in removing native mutan from oral biofilms. Experimental results were obtained by monitoring enzymatic saccharification of DTM and its solubilization (Figs. 4A and 4B). In both cases, hydrolysis reaction rates increased linearly in the initial phase of the process and later became almost constant. As illustrated in Figs. 4A and 4B, the dynamics of hydrolysis yield rose also as the enzyme concentration increased. The maximum degree of mutan saccharification (about 80%) was reached after 3 h of hydrolysis when an enzyme preparation of 0.75–1 U/ml was used. Having considered that dextranase-pretreated mutan, prepared in this laboratory, was a mixed-linkage glucan with 79.8 mol% of α-(1→3)-glucosidic linkages, it is possible to conclude that almost all of these glucosidic bonds were degraded during specific action of mutanase on this biopolymer. It is noteworthy that a high degree of DTM solubilization (about 90%) at those enzyme concentrations was attained just after 1 h of hydrolysis. At the same time, the saccharification of this substrate reached about 40–50%.

During hydrolysis of insoluble mutan, Kopec *et al.* [7] obtained only 15.3% saccharification with mutan-induced mutanase of *T. harzianum* after 4 h of incubation at 30°C. Similar results (hydrolysis yield of up to 20% after 48–64 h at 37°C) were obtained for Dextranase 50 l (a commercial preparation containing several hydrolytic activities) and glucanhydrolase with mixed (dextranolytic and amylolytic) activity from *Lipomyces starkeyi* [9, 12].

Degradation of Native Mutan and Oral Biofilms Adhered to Glass Plates

To estimate the effects of cell-wall-induced mutanase and a mixture of this enzyme with dextranase on breakdown of streptococcal mutan and mixed oral biofilms adhered to the saliva-coated glass plates, optimum amounts of both enzyme preparations were used under conditions that simulated similar processes occurring in the oral cavity. The enzymes were tested in different *in vitro* application studies (*i.e.*, in degradation of preformed mutan or its removal from biofilms), and their hydrolyzing activities were visualized as a decrease in the redness of dyed mutan or mutan-containing oral biofilms.

The data presented in Fig. 5 show the effective action of cell-wall-induced mutanase, used either individually or in a mixture with dextranase, towards the presynthesized

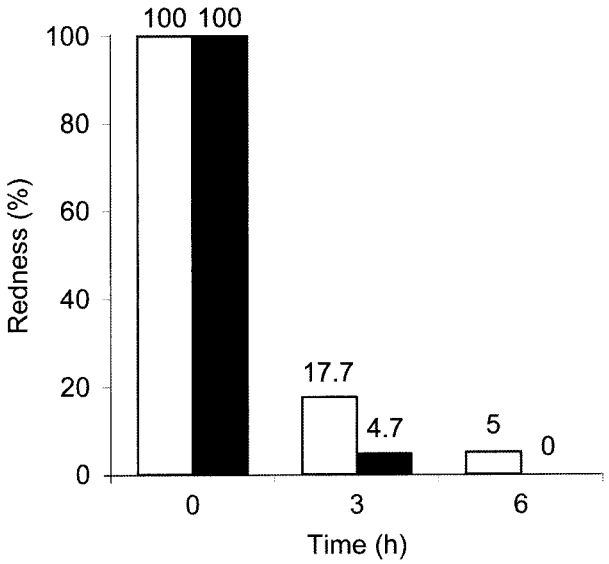


Fig. 5. Action of cell-wall-induced mutanase and its combination with dextranase on pre-formed mutan.
Mutan was formed, hydrolyzed, and reddened with erythrosin as described in Materials and Methods. Hydrolysis of mutan was expressed as a decrease in redness of dyed biofilm (decreasing redness stands for less dyed biofilm). Redness was quantified as optical density at 560 nm, and given as a percentage of the maximum attained in the control test. Symbols: (□) - mutanase, (■) - mutanase with dextranase.

mutan of streptococcal origin. However, the application of both glucanases in combination gave higher levels of native glucan degradation (95.3% and 100% after 3 and 6 h, respectively). As illustrated in Fig. 6, a similar re-inforced effect of the simultaneous action of both glucanohydrolases was observed in removing preformed oral biofilm from a saliva-coated glass plate. The enzymatic mixture reduced the oral biofilm containing pathogenic microorganisms by 86.6% or degraded it completely after 3 and 6 h, respectively. Recently, Wiater *et al.* [21] have reported that a combination of mutan-induced mutanase from *T. harzianum* F-470 and a commercial dextranase hydrolyzes mutan present in the preformed biofilm by about 95% after 24-h incubation. An effective action of mutanase-dextranase mixture in removing mixed biofilm from saliva-coated hydroxyapatite disks was also noted by Tsuchiya *et al.* [16]. Good results (reduction of insoluble glucan formation by 80% and removal of up to 80% of the preformed bacterial films after 24 h at 37°C) were achieved for a novel glucanhydrolase from *Lipomyces starkeyi* during the action of the enzyme on streptococcal glucan [12].

In summary, the present study reveals that the cell wall preparation from *L. sulphureus*, rich in α-(1→3)-glucans, effectively induced mutanase in *T. harzianum* and fully substituted mutan when it was used in the culture medium as the sole carbon source. In contrast to mutan, this new and powerful stimulus for inducible mutanase synthesis is inexpensive, easily available, and safe for humans. Therefore,

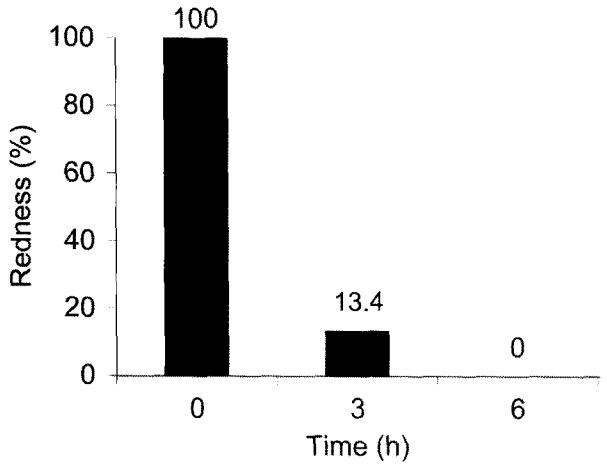


Fig. 6. Action of cell-wall-induced mutanase in a mixture with dextranase on preformed biofilm.
Biofilm was formed, hydrolyzed, and reddened with erythrosin as described in Materials and Methods. Hydrolysis of mutan was expressed as a decrease in redness of dyed biofilm (decreasing redness stands for less dyed biofilm). Redness was quantified as optical density at 560 nm, and given as a percentage of the maximum attained in the control test.

it will be very useful to facilitate the mutanase production on a larger scale and at relatively low costs acceptable for oral applications. The cell-wall-induced mutanase shows a relatively high hydrolytic potential and good compatibility with dextranase in mutan degradation and its removal from mixed oral biofilms. Therefore, the tested enzymes could be applied as active ingredients in chewing gum, mouthwash, dental gel, and toothpaste to prevent the accumulation of glucose biopolymers.

Acknowledgment

This work was financially supported from funds for science in the years 2008–2011 as the development project (No. 325/R/P01/2007/IT1).

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