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PRODUCTION AND USE OF MUTANASE FROM *TRICHODERMA HARZIANUM* FOR EFFECTIVE DEGRADATION OF STREPTOCOCCAL MUTANS

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ABSTRACT

Basic cultural parameters affecting mutanase production by *Trichoderma harzianum* F-340 in shaken flasks and aerated fermenter cultures have been standardized. The best medium for enzyme production was Mandels medium A with initial pH 5.3, supplemented with 0.3% mutan and 0.05% peptone and inoculated with 20% of the 72-h mycelium as inoculum. It was shown that mycelial mass, used in the culture medium as a sole carbon source, induced mutanase synthesis and could be utilized as an inexpensive and easily available substitute for bacterial mutan. Application of optimized medium and cultural conditions enabled us to obtain a high mutanase yield (0.6-0.7 U/mL, 2.0-2.5 U/mg protein) in a short period of time (3-5 days), which was much higher than the best reported in literature. The enzyme in crude state was stable in the pH range of 4.5-6.0, and at temperatures of up to 40ºC; its maximum activity was recorded at 45ºC and at pH 5.5. The mutanase preparation obtained from the *T. harzianum* fungus was relatively stable under storage conditions, and showed a high hydrolytic potential in reaction with a mixed-linkage (α-1,3, α-1,6) water-insoluble mutan of streptococcal origin (hydrolysis yield reached a value of 69% in 24 h). Steady-state measurement of the enzymic reaction products during the hydrolysis revealed that mutanase exhibited an exo type of action on mutan. Thin-layer chromatographic analysis showed that glucose was the primary final product of mutan hydrolysis with mutanase. The potential application of mutanase in dentistry is discussed.

Key words*: Trichoderma harzianum*, mutanase, mutan

INTRODUCTION

Mutans, synthesized from dietary sucrose by glucosyltransferases of cariogenic streptococci, are a major virulence factor for dental caries induction in humans and experimental animals (14). These water-insoluble and adherent glucans possess a highly branched structure with a majority of α-1,3 and a minority of α-1,6 glycosidic bonds. The abundance of α-1,3 linkages is associated with water insolubility while the presence of α-1,6-linked side chains contributes to the adhesive properties of these biopolymers (32). Mutans act as a framework for dental plaque, where they mediate attachment of bacteria to the tooth surface and to other bacteria, thus stabilizing the plaque biofilm (18). Accordingly, strategies to reduce the disease potential of dental plaque have included the possibility of using mutan-degrading enzymes to disrupt the molecular architecture of plaque. Some of these enzymes, termed mutanases (α-1,3-glucan 3-glucanohydrolases), have shown high potential as caries preventive agents because they degrade water-insoluble glucans present in dental plaque (11,31,35). However, the use of mutanases in caries prophylaxis has had little commercial success due to the unavailability of enzymic preparations acceptable for oral applications.

Mutanases from various bacteria and filamentous fungi have been investigated; yet, in most cases, the enzyme activity is relatively low, and cultural conditions for mutanase production have not been thoroughly established (9,10,19). Also, search for additional mutanase producers among wild-type fungi is

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necessary to find new potential sources with different characteristics. Moreover, bacterial synthesis of mutan for the specific induction and extracellular secretion of mutanase in microbial cultures has not been realized on a larger scale, and this type of water-insoluble exopolysaccharide is not yet available as a commercial product. Therefore, identification of alternative and inexpensive carbon sources as mutanase inducers would facilitate the enzyme production on a larger scale, and at relatively low costs.

As for the enzymatic hydrolysis of mutans, several papers deal with action patterns of some exo- and endoglucanases (1,9). Mutanases from different sources exhibit diverse action patterns, but fundamentally they degrade mutan to glucose. In the enzymic conversion of mutan, it is essential to obtain a high sugar yield per enzyme unit. Thus, optimized conditions of mutan hydrolysis play a significant role in the economy of the degradation process.

The objective of these investigations was to find the best operating conditions for efficient production of fungal mutanase in shaken flasks and fermenter cultures and to search for alternative inducers of enzyme synthesis as well as to determine some of mutanase properties. The aim was also to effectively hydrolyze mutan by fungal mutanase, to examine the mode of enzyme action towards mutan, and to identify the final degradation products.

MATERIALS AND METHODS

Fungal strain, media and growth conditions

Stock cultures of *Trichoderma harzianum* F-340 (Czech Collection of Microorganisms, Brno, Czech Republic), maintained at 4ºC (± 2ºC) on potato dextrose agar slants, were used for inoculations. The liquid mineral medium A (pH 5.3), as described by Mandels *et al.* (16), supported by 0.5% mutan, 0.1% peptone proteose, and 0.1% Tween 80 was used for mutanase production. This medium was optimized during experiments with respect to carbon source, peptone concentration, initial pH, and medium volume. The media listed in Table 1 were also studied. Shake 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 x 10^5 conidia/mL and placed on an orbital rotary shaker at 220 rpm and 30ºC for 4 days.

Table 1. Effect of different mineral media on mutanase production by *T. harzianum* in shaken flask cultures.

Medium[a]	Days[b]	Mutanase activity	
		(U/mL)	U/mg protein
Mandels *et al.* (16) – medium C	7	0.301	1.316
Mandels *et al.* (16) – medium A[c]	4	0.651	2.170
Saunders *et al.* (25)	2	0.070	0.262
Szczodrak *et al.* (30)	2	0.208	0.629

[a]All media (pH 5.3) were supported by 0.5% mutan; [b]Incubation period for maximum activity; [c]The same as C but with the addition of peptone proteose (0.1%).

For enzyme production on a larger scale, the strain was grown in an optimized Mandels medium in 1.8 L batches in a 2.5 L glass fermenter (Biostat B, B. Braun Biotech International GmbH, Germany). The medium was autoclaved for 20 min at 121ºC and inoculated with 20% (v/v) of the 72 h mycelium of *T. harzianum* grown in shaken flasks in the same medium but with 0.5% glucose as the sole carbon source. The fermenter culture was run at 30ºC for 7 days at the aeration rate of 1 L air/1 L medium/min and with stirrer speed of 300 rpm. The pH was not regulated. Antifoam B emulsion (Sigma-Aldrich, St. Louis, MO, USA) was used for breaking the foam. The mycelium from flasks and fermenter cultures was separated by centrifugation (20 min at 6,000 x g) and the clarified supernatant was used as an enzyme solution for various tests, or it was lyophilized in a freeze-dryer (Labconco, USA). Dry powder with a mutanase activity of 0.026 U/mg, protein content of 0.033 mg/mg lyophilizate, and trace activity of protease was used as an enzyme preparation for determination of mutanase properties and mutan hydrolysis.

Assays

The standard mutanase assay mixture contained 0.5 mL of 0.4% mutan 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 40°C, the released reducing sugars were quantified by Somogyi-Nelson's method (20,28). Appropriate substrate and enzyme blanks were included to correct for any free reducing group not emanating from the mutan. One unit of mutanase activity (U) was defined as the amount of enzyme hydrolyzing mutan to yield reducing sugars equivalent to 1µmol 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), 1 U corresponds to 16.67 nkat. To isolate intracellular mutanase, the washed mycelium was disrupted using a mortar and pestle for 10 min at 4ºC with equal weight of corundum A-320 and four volumes of acetate buffer (pH 5.5). The homogenate was centrifuged at 17,500 x g for 20 min, and the supernatant fluid served as a cell-free extract for estimation of enzymic activity.

The activities of all the accompanying hydrolytic enzymes present in mutanase preparation, such as dextranase, laminarinase, pullulanase, amylase, invertase, chitinase and protease were quantified under the optimized reaction conditions by measuring the reducing sugars, N-acetyl-glucosamine or diazotized aromatic amino acids released by these enzymes from the respective carbohydrates or azocasein (2,7,23,30). One microgram of reducing sugars or N-acetyl-

glucosamine formed per min was taken as the unit of enzymic activity. The reducing power was analyzed by the Somogyi-Nelson's method. In the case of protease, an absorption change of 0.1 in the reaction mixture under the reaction conditions was taken as an activity unit.

Glucose was measured enzymically using glucose oxidase-peroxidase reagent (15). Protein concentration was measured by the method of Schacterle and Pollack (26) using crystalline bovine serum albumin as a standard.

The influence of pH on mutanase activity was examined in a standard assay mixture, except that 0.2 M phosphate-citrate (McIlvaine) buffers (pH 3.0-8.0) were used instead of acetate buffer. The pH stability was determined in a similar way, apart from the fact that after preincubation of the enzyme solution at various pH values and at 40ºC for 24 h, aliquots were removed, and the remaining activities were assayed by the standard method at the optimum pH of 5.5. The effect of temperature on the enzyme activity was estimated in a standard assay mixture containing acetate buffer, but the reaction temperature was changed gradually from 20 to 60ºC and the activity was measured at the optimum pH value. Thermal stability was measured in the same way as temperature optimization. The enzyme solution was incubated without mutan at different temperatures for 1 h under the optimum pH conditions. Then it was cooled, and the residual activity was assayed by the standard method at the optimum pH of 5.5 and the temperature of 45ºC. The relative activity at each pH and temperature was expressed as a percentage of the maximum activity.

Submerged cultures were performed in triplicate, and analyses were carried out at least in duplicate. The values given here are means of all the independent measurements. Mean standard error of the mutanase estimate was $\pm 8.2 \times 10^{-3}$ U and ranged from $\pm 7.5 \times 10^{-5}$ to $\pm 1.9 \times 10^{-2}$. Mean standard error in protein determination was $\pm 4.3 \times 10^{-3}$ mg and ranged from $\pm 5.2 \times 10^{-5}$ to $\pm 8.6 \times 10^{-3}$. Other methodological details are given in tables and figures.

Preparation of mutan

Mutan was synthesized from sucrose with the use of a mixture of crude glucosyltransferases of cariogenic *Streptococcus sobrinus* 21020 (OMZ 176, provided by the Culture Collection, University of Göteborg, Sweden), as reported previously (34). The linkage structure of natural mutan, determined by ^{1}H nuclear magnetic resonance (NMR), turned out to be a mixed-linkage (α-1,3), (α-1,6) polymer with a greater proportion of α-1,3 to α-1,6 linkages (79.8 and 20.2%, respectively).

The ^{1}H NMR spectra of water-insoluble mutan preparation were recorded with the Avance (300 MHz) spectrometer (Bruker BioSpin GmbH, Rheinstetten/Karlsruhe, Germany) at 60ºC. ^{1}H chemical shift values were estimated using acetone (δ_H – 2.225 ppm) as the internal standard. Glucan (20 mg) was dissolved in 0.6 mL of 30% NaOD in D_2O. The percentage of α-1,3- and α-1,6-glucosidic linkages was calculated from the surface areas of anomeric signals.

Hydrolysis of mutan by mutanase and identification of degradation products

Digestion of mutan was performed in plugged conical flasks (100 mL) with 0.05% sodium azide as a preservative. The reaction mixture (50 mL) contained 50 mg of mutan and 0.195 U/mg substrate of mutanase preparation in 0.2 M sodium acetate buffer (pH 5.5). The flasks were incubated for 48 h at 45ºC in a water bath shaker agitated at 150 rpm. Samples were withdrawn at various intervals of up to 48 h, heated at about 100ºC for 5 min to stop the reaction and analyzed for total reducing sugars, glucose and final digestion products. Enzyme and substrate blanks were included. The detailed experimental conditions are described in the subsequent figures and tables. The percentage of mutanolysis was calculated using the following equation: saccharification (%) = reducing sugars formed (mg) x 0.9 x 100/ mutan (mg).

Saccharifications were performed in three replicate experiments and analyses carried out in duplicate. The data given are means of all the measurements. The mean standard error of mutan hydrolysis was ±0.52%, and ranged from ±0.02 to 0.83.

The mutan hydrolytic end products were identified by thin-layer chromatography (TLC). The digests (4 µL) were spotted periodically on TLC-plates (aluminium sheets with Silica Gel 60, Merck, Darmstadt, Germany). TLC chromatography was performed according to the method described by Côté and Biely (5) using glucose (Merck, Germany), nigerose and nigerotetraose (Sigma-Aldrich, USA) as sugar standards. *N*-(1-Naphthyl)ethylenediamine dihydrochloride reagent was used as a sugar locator (3).

RESULTS AND DISCUSSION

Optimization of mutanase production

The starting point of these studies was the choice of an optimal medium for an efficient synthesis of mutanase by the strain of *Trichoderma harzianum* F-340. Among the four tested culture media supported by 0.5% mutan, the mineral medium A of Mandels *et al.* (16) was the most effective with respect to mutanase productivity (Table 1). The enrichment of this medium in peptone proteose (0.1%) shortened the cultivation time by three days and increased (over two times) mutanase activity compared to the medium without the addition of peptone (medium C). Other media gave much smaller effects during a shorter period of cultivation (2 days). Also, a simple medium reported by Szczodrak *et al.* (30) for effective dextranase production was found useless for mutanase synthesis. Hence, culture medium A was used in our further studies.

During initial experiments, the concentration of mutan in the culture medium was 0.5%. To ensure suitable conditions for mutanase production, we studied its activity at mutan concentrations between 0.1% and 1.0%. As manifested by the data in Table 2, an over three-fold increase in the mutan amount (from 0.3% to 1.0%) elevated the enzyme activity by only 37%. Consequently, using such a high mutan dose is not economically justified, the more so as this biopolymer is not yet available as a commercial product. Based on Table 2, it was concluded that the 0.3% mutan concentration giving a relatively high enzyme activity would be an adequate substrate concentration for mutanase production. Also, peptone concentration of 0.05%, an initial medium pH of 5.3, and medium volume of 150 mL were adopted as best conditions, yielding, in shaken flask cultures, about 0.59-0.72 units of mutanase per mL of culture broth, after 4 days of cultivation (Table 2).

Table 2. Culturing factors affecting mutanase production by *T. harzianum* in Mandels medium A[a]: effect of initial pH, medium quantity, and some medium constituents.

Serial No.	Factor varied	Concentration %	Mutanase activity[b] (U/mL)	(U/mg protein)
1.	Mutan	0.1	0.265	0.911
		0.3	0.587	2.010
		0.5	0.690	2.240
		1.0	0.804	2.481
2.	Peptone[c]	0.025	0.568	2.186
		0.050	0.680	2.345
		0.100	0.543	1.989
		0.200	0.135	0.298
3.	Initial pH of the medium[d]			
	4.0		0.502	1.813
	5.0		0.660	2.183
	5.3		0.700	2.450
	5.5		0.628	2.326
	6.0		0.298	1.257
4.	Volume of optimized medium taken (mL)[e]			
	50		0.621	1.966
	100		0.704	2.167
	150		0.724	2.471
	200		0.560	1.958

[a]Composition of the medium was the same as that of the original one, except for the factor or its concentration that varied as indicated; [b]Enzyme activity in culture supernatants was measured after 4 days of submerged cultivation in shaken flask cultures; [c]Concentration of mutan was 0.3%; [d]Concentration of mutan was 0.3%; dose of peptone was 0.05%; [e]Concentration of mutan was 0.3%; dose of peptone was 0.05%; pH was 5.3.

The use of conidia as inoculum extends the cultivation time by a stage indispensable to form a physiologically active mycelium. Therefore, an attempt was made to replace conidia of *T. harzianum* by a suspension of its vegetative mycelium to shorten the cultivation time on the selected medium. The use of 20% of 72 h mycelium as inoculum shortened the incubation time by one day, as compared to the use of conidial suspension, maintaining mutanase activity on a slightly higher level (also in the case of specific activity) than that obtained in control runs (Table 3). Similar results with respect to cellulases were reported by Szczodrak (29) using a 48 h suspension of *Trichoderma reesei* mycelium. Also, Shukla *et al.* (27) observed the highest formation of dextranase by *Penicillium aculeatum*, *P. purpurogenum*, and *P. funiculosum* with the use of an inoculum containing 4% of 48 h mycelium.

Also, a comparison was made between production of mutanase by *T. harzianum* when the organism was grown in optimized medium containing various carbohydrates and when mycelial mass was the sole carbon source and inducer of mutanase synthesis. From 16 sugars tested, mutan was the most specific and effective inducer of enzyme synthesis (Table 4). The maximum mutanase activities (0.7 U/mL, 2.5 U/mg protein) were measured on the third day of cultivation. A slight mutanolytic activity (0.01 to 0.03 U/mL) was also found when pullulan, laminarin, alternan, amylose, amylopectin, raffinose, and maltose were carbon sources. Other carbohydrates had no effect on enzyme induction. Raffinose induced mutanase production was also reported by Quivey and Kriger (22) in shaken flask cultures of *T. harzianum* OMZ 779 strain.

It should be noted here that glucans present in the mycelial cell wall are relatively good stimulants of mutanase synthesis by *T. harzianum*. As shown in Table 4, the enzyme activity reached a significant value of about 0.07 U/mL (0.24 U/mg protein) with mycelial mass as the substrate in a short period of time (2 days). Thus, this raw material could be considered as a useful, inexpensive and easily available substitute for bacterial mutan. The identification of alternative and inexpensive carbon sources (such as vegetative mycelium) capable of inducing mutanase synthesis is justified, as it would facilitate the enzyme production on a larger scale and at relatively low costs. Therefore, further experiments on intensification of fungal mutanase produced on accessible carbon sources (e.g. waste mycelium coming from biotechnological factories) are now in progress in this laboratory.

Based on the results presented in Tables 1-4, Mandels medium A with initial pH 5.3 and a volume of

150 mL, supported by 0.3% mutan, 0.05% peptone and 0.1% Tween 80, inoculated with 20% of 72 h mycelium of *T. harzianum* and incubated for 3 days at 30ºC, was proven to be the best for mutanase production in shaken flask cultures. Under these conditions, the mutanase yield ranged from 0.59 to 0.72 U/mL of culture supernate.

In order to scale up the mutanase production, *T. harzianum* strain was grown on the optimized medium in 1.8 L batches in 2.5 L fermenter under established conditions. Fig. 1 shows a typical culture time course of *T. harzianum* when the organism was grown in fermenter for 7 days. Mutanase activity was gradually increasing for 5 days, and then slowly declined. The maximum mutanase yield (0.56 U/mL, 2.0 U/mg protein) and extracellular protein accumulation (0.32 mg/mL) were monitored on the fifth and fourth days of cultivation, respectively. The pH of the medium rose (from 5.7 to 6.5) over the whole period of incubation. Consequently, a 5 day period was assumed as the optimum time for the synthesis of mutanase by *T. harzianum* in fermenter cultures.

Table 3. Effect of the kind and amount of inoculum from *T. harzianum* on mutanase activity in shaken flask cultures[a].

Inoculum quantity, v/v%	Mutanase activity	
	(U/mL)	U/mg protein
1[b]	0.698	2.350
1[c]	0.177	0.926
2[c]	0.276	1.359
5[c]	0.500	2.145
10[c]	0.583	2.233
20[c]	0.716	2.557

[a]Mandels optimized medium A with 0.3% mutan was used; [b]Control inoculated with aqueous suspension of conidia of *T. harzianum* (2 x 10^5/mL); enzyme activity for the control test was determined after 4 days of culture; [c]Experimental tests inoculated with a suspension of vegetative *T. harzianum* mycelium (72 h) precultured on Mandels medium with glucose (0.5%); enzyme activity was determined after 3 days of culture.

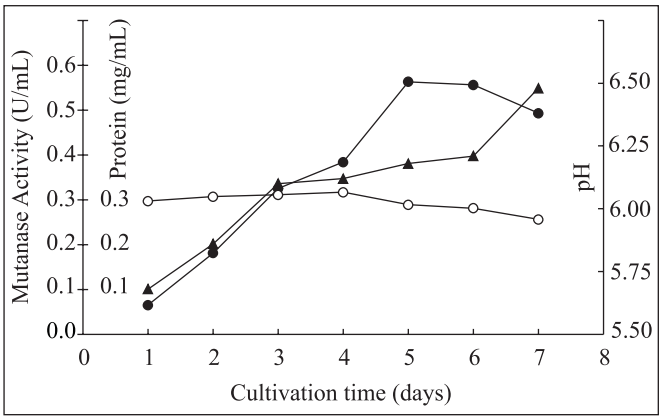

Figure 1. Rates of mutanase (●) and protein production (○), and changes in pH (▲) during fermenter culture of *T. harzianum* on optimized medium containing mutan as the sole carbon source. Initial medium pH was 5.3.

Table 4. Effect of carbon source on mutanase production by *T. harzianum* in shaken flask cultures[a].

Carbon source	Main linkage(s) or Constituent	Days[b]	Mutanase activity	
			(U/mL)	(U/mg protein)
Mutan[c]	α-1,3; α-1,6	3	0.709	2.514
Pullulan	α-1,6; α-1,4	4	0.029	0.139
Laminarin	β-1,3	4	0.026	0.078
Alternan	α-1,3; α-1,6	3	0.016	0.056
Amylose	α-1,4	3	0.020	0.044
Amylopectin	α-1,4; α-1,6	3	0.013	0.035
Raffinose	α-1,6; α,β-1,2	4	0.014	0.086
Maltose	α-1,4	4	0.017	0.053
Wet mycelium[d] (homogenized)	Glucans in mycelial cell wall: β-1,3; β-1,6; α-1,3	2	0.063	0.237

[a]Mandels optimized medium A with 0.3% mutan or 1% other carbon sources was used; [b]Time of maximum mutanase production; [c]Other sugars (dextran, α-cyclodextrin, trehalose, melibiose, sucrose, fructose, glucose) supported only the growth of the organism, but none or trace activity of mutanase was detected in this set of carbohydrates; [d]A 72 h mycelial mass of *Aspergillus wentii*, precultured on growth medium with glucose (1%), was used as the sole carbon source.

Application of optimized medium and cultural conditions in shaken flasks and aerated fermenter cultures enabled us to obtain a high mutanase yield (0.6-0.7 U/mL, 2.0-2.5 U/mg protein) in a short period of time (3-5 days). This productivity is at present the best reported in literature. For example, Guggenheim and Haller (9), 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 would reach its maximum yield of 0.16 U/mL after 120 h of incubation. Meanwhile, Quivey and Kriger (22), using the same fungal strain, reached the specific mutanase activity of 0.37 U/mg protein after 4 days in shaken flask cultures. Also, basing on *Streptomyces chartreusis*,

Inoue *et al*. (10), obtained, after 3 days in shaken flask cultures, the maximum mutanase activity of 0.005 U/mL. In the case of bacterial mutanases, Matsuda *et al*. (19) achieved enzyme activities of 0.17 U/mL and 0.039 U/mg protein in a flask culture of *Bacillus circulans*, grown for 36 h on mutan composed mainly of α-1,3 glucan, whereas Ebisu *et al*. (6) after 24 h in a 10 L fermenter culture of *Flavobacterium* obtained an enzyme yield of 0.003 U/mL.

Characteristics of crude mutanase preparation

Post culture supernatant or cell-free extract of *T. harzianum* obtained after 5 days of fermenter culture on mutan was used as non-purified mutanase preparation in studies of its enzymic characteristics. As seen in Table 5, the preparation showed a high extracellular mutanase activity and trace activity (0.1%) of intracellular enzyme. Besides high mutanolytic activity, the crude enzyme also contained small amounts of accompanying hydrolytic enzymes, such as pullulanase, invertase and chitinase. On the other hand, the mutanase preparation contained practically no dextranase and protease activities. The absence of protease in the culture supernate is very important for successful creation of enzymic preparations for safe oral and caries prophylactic applications. Some of mutanase preparations tested in the past contained large amounts of impurities, which caused certain local side effects in the oral cavity (12).

Enzyme properties

The crude mutanase preparation (post culture supernate) was stable for one day at room temperature and at 4ºC, and for four days at -20ºC. The enzyme in crude state was also found highly stable after one treatment freezing and thawing. The lyophilized enzyme preparation was stable under storage conditions for one month at 4ºC, but it lost 36% of the initial activity after 6 months.

Table 5. Characteristics of the non purified mutanase preparation from *T. harzianum*.

Enzyme	Activity	
	(U/mL)	(U/mg protein)
Extracellular mutanase[a]	0.5700	2.467
Intracellular mutanase[b]	0.0006	0.002
Dextranase[a]	0.0000	0.000
Pullulanase[a]	0.0240	0.104
Invertase[a]	0.0065	0.028
Chitinase[a]	0.1690	0.732
Protease[a]	trace	trace

[a]Post culture supernatant or cell-free extract[b] obtained after 5 days fermenter culture of fungus on optimized medium with mutan.

The effects of pH and temperature on the activity and stability of *T. harzianum* mutanase are illustrated in Fig. 2. The optimum pH for the enzyme activity was pH 5.5, and the enzyme was relatively stable in the pH range of 4.5-6.0. The effect of temperature on mutanase activity was investigated in the range of 20-60ºC. Mutan hydrolysis by the enzyme reached the highest rate at 45ºC. Thermostability studies showed that after 1 h incubation the enzyme was stable at temperatures of up to 40ºC. At 50ºC the enzyme lost about 20% of its maximum mutanolytic activity, which rapidly decreased at temperatures above 50ºC resulting in a 91% loss at 60ºC.

The pH optimum observed is not exactly in agreement with the one reported earlier at pH 6.0 for the *T. harzianum* OMZ 779 mutanase (9), yet comparable to that obtained for the *Microbispora rosea* mutanase (4). Correspondingly, bacterial mutanases from *B. circulans* (19), *Flavobacterium* (6) and *Microbispora rosea* (4) have pH optima similar (5.5) or higher (6.3-6.9) than fungal mutanases, and similar temperature optima

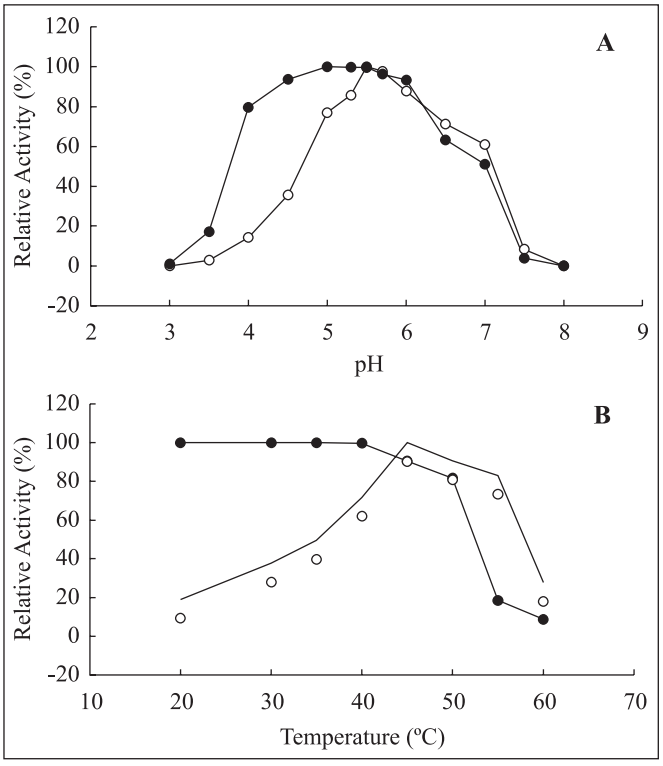

Figure 2. Effects of pH (A) and temperature (B) on activity (○) and stability (●) of *T. harzianum* mutanase. (A) O.2 M McIlvaine buffer of various pH values was used instead of acetate buffer in the standard assay. (B) The activity was assayed by the standard method except for the reaction temperature that changed as indicated. Other experimental details as described in Materials and Methods.

(40-50ºC). Although pH in the oral cavity is around pH 6-7, the slightly acidic pH profile of the fungal mutanase may be of importance in its application for plaque removal, as low pH values have been observed locally in the plaque (21).

Hydrolysis of mutan by *T. harzianum* mutanase

Hydrolysis of a mixed-linkage streptococcal mutan by lyophilized mutanase preparation was performed under standardized conditions in 50 mL batches in 100 mL flasks. The dynamics of mutanolysis and glucose content in hydrolyzates (measured enzymically) during 48 h are depicted in Fig. 3. After 24 h, the degree of mutan saccharification was 69.2% and glucose content 88.5%. It is important to notice that after 12 h of mutanolysis, a relatively high conversion of mutan to soluble digestion products (about 37%) was attained. However, extension of the hydrolysis time to 48 h produced only a negligible increase in hydrolysis and glucose yields (2.4% and 0.7%, respectively). Accordingly, a 24 h period was finally assumed as the optimum time for mutan hydrolysis.

For example, during hydrolysis of insoluble mutan, which was synthesized by glucosyltransferase B derived from the construct of *S. milleri*, Kopec *et al*. (13), reached only 15.3% saccharification of this biopolymer with purified *T. harzianum* mutanase after 4 h of incubation at 30ºC. Similar results (hydrolysis yield of up to 20% after 48-64 h at 37ºC) were obtained for Dextranase 50L (a commercial preparation containing several hydrolytic activities) and glucanhydrolase with mixed (dextranolytic and amylolytic) activity from *Lipomyces starkeyi* (17,24). Hydrolysis of *S. sobrinus* mutan, composed mainly of α-1,3-glucan (99.4%), by mutanase from *Bacillus circulans* resulted in 31% saccharification of this substrate after 10 h at 35ºC (19). After hydrolyzing water-insoluble polyglucans of cariogenic streptococci with *Streptomyces* mutanase for 60 min at 55ºC, Inoue *et al*. (10) reported a degradation in the range from 2.0 to 4.9%, respectively. Also Ebisu *et al.* (6) assayed the susceptibilities of insoluble polysaccharides produced by oral streptococci to bacterial mutanase. In the case of the *Flavobacterium* enzyme, the above mentioned authors obtained about 10.1% saccharification after 24 h of mutan hydrolysis at 37ºC, while at the same time about half of glucan was solubilized. The degradation of insoluble plaque polysaccharides by *T. harzianum* OMZ 779 mutanase attained about 50% saccharification after an overnight incubation at 40ºC (9). A high degree of mutan degradation (31.5%) after 30 min of enzymatic reaction at 37ºC was also reported for highly purified recombinant mutanase (31).

The action pattern of *T. harzianum* mutanase towards mutan was examined by comparing the rate of glucose production to that of reducing sugar production at different times. This method is often used to confirm the exo or endo mechanism of the enzyme action (33). Incubations of mutan with the enzyme resulted in an almost 1:1 correlation between the production of reducing sugars and glucose (Fig. 4). Therefore, glucose accounts for almost all of the reducing sugar and is the major hydrolysis product of the enzyme. A relatively constant (reducing sugars/glucose) ratio obtained over the whole hydrolysis period indicates unequivocally that the tested mutanase exhibits an exo type of action, with glucose as the main early and final hydrolytic product. Also Ait-Lahsen *et al.* (1) observed an identical mode of action for an antifungal exo-α-1,3-glucanase isolated from the strain of biocontrol fungus *T. harzianum* CECT 2413.

Some mutanases split α-1,3 linkages in insoluble glucans of streptococcal origin by endo-type degradation. For example,

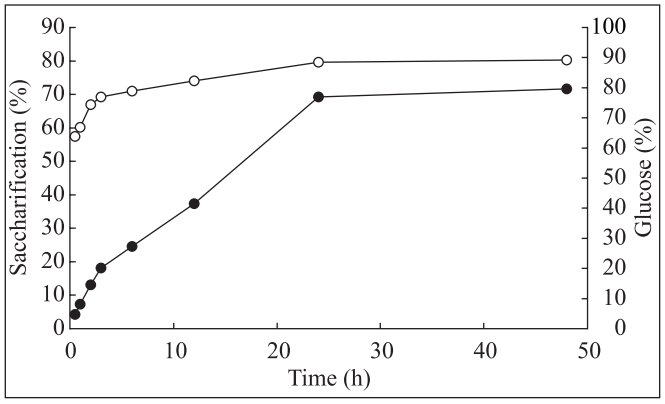

Figure 3. Time course of mutan hydrolysis with *T. harzianum* mutanase (●) and the glucose content in mutan hydrolysates (○). Hydrolysis was conducted in standardized conditions, as described in Materials and Methods. Enzyme concentration: 0.195 U/mg substrate. Glucose was measured enzymically.

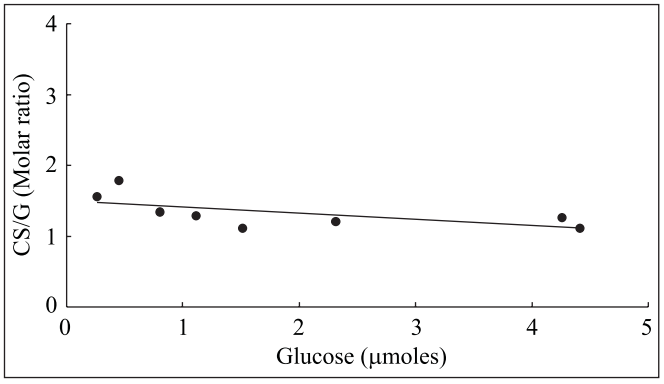

Figure 4. Mechanism of action of *T. harzianum* mutanase. Hydrolysis conditions described in Materials and Methods. CS, total carbohydrate solubilized (reducing sugars); G, glucose (analyzed enzymically).

Guggenheim and Haller (9) have found purified mutanases from the strain of *T. harzianum* OMZ 779 to operate by a completely random attack, that is, by an endo mechanism. Similar results were reported for *Flavobacterium* α-1,3-glucanase hydrolyzing insoluble, sticky glucan of *S. mutans* (6). The action pattern of mutanase separated from a commercial preparation of Dextranase S revealed the enzyme to be composed of an exo- and endo-α-(1→3) glucanase (8).

The soluble end products of mutan hydrolysis were identified by TLC chromatography. Samples were withdrawn for various periods of up to 48 h. As shown by TLC analysis (Fig. 5), glucose was present in hydrolyzates over the whole hydrolysis period as the main initial and final hydrolytic product. Its content increased gradually over the whole digestion cycle as a result of degradation of intermediate higher molecular weight oligomers (compare the size and intensity of glucose spots visible on TLC chromatogram). The heat inactivated enzyme preparation contained end products free of any reducing sugar. After 48 h of mutan hydrolysis, the amount of glucose constituted 89% of the total pool of hydrolytic products (see Fig. 3). These results confirm the issue discussed earlier that the tested mutan-degrading mutanase operates in an exo fashion.

Other researchers (1,9,22) also detected glucose as the major product of mutan hydrolysis with mutanase derived from various strains of *T. harzianum*. Glucose and isomaltose or glucose and fructose were the main degradation products from mutan after incubation with *Penicillium* and *T. harzianum* mutanases, respectively (8,13). Other results were obtained by Ebisu *et al*. (6), and Matsuda *et al*. (19). On hydrolyzing mutan with bacterial mutanases, hydrolyzates contained dimers, trimers and tetramers of glucose but no glucose as the primary soluble products of glucan digestion.

Summarizing, the data presented here imply that the tested *T. harzianum* strain potentially represents a new and highly effective source of extracellular mutanase. The enzymic productivity of this fungus, obtained for mutan in shaken flasks and fermenter cultures under standardized conditions, was much higher than the best reported so far. Moreover, the vegetative mycelium may be used as a substitute for mutan to considerably reduce the costs of mutanase production. The mutanase preparation obtained from this strain was relatively stable during storage, free of dextranase and protease activities, and showed a high hydrolytic potential with an exolytic mode of action on insoluble mutan of streptococcal origin. Therefore, the enzyme could be applied as an active ingredient in dental gel, chewing gum, toothpaste, or mouthwash to prevent the accumulation of mutan in dental plaque and as a useful supplement to mechanical cleaning of teeth and dentures. Thus, further experiments are currently in progress to estimate the operational stabilities and some kinetic properties of mutanase administered in dentifrice preparations.

Figure 5. TLC chromatogram of hydrolysis products of mutan obtained with mutanase preparation from *T. harzianum*. Hydrolysis conditions as in Materials and Methods. The samples applied were: M – marker, G_1 – glucose, G_2 – nigerose, G_4 – nigerotetraose; E – sample from enzymic digestion of mutan at different periods of incubation: 1 (30 min), 2 (1 h), 3 (2 h), 4 (3 h), 5 (6 h), 6 (12 h), 7 (24 h), 8 (48 h); C – heat-inactivated enzyme. Four microliters of each sample were spotted on the TLC plate. The locator was naphthylethylenediamine dihydrochloride reagent. See the text for the other experimental details.

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RESUMO

Produção e utilização da mutanase de *Trichoderma harzianum* para a degradação efetiva dos mutans estreptocócicos de estreptococos mutans

Os parâmetros básicos que afetam a produção da mutanase por *Trichoderma harzianum* F-340 em frascos agitados e em fermentadores foram estabelecidos. O melhor meio para produção da enzima foi o meio Mandels com pH inicial de 5,3, suplementado com 0,3% de mutana e 0,05% de peptona e inoculado com 20% de um micélio de 72h. Demonstrou-se que a massa micelial, usada como única fonte de carbono no meio de cultura, induziu a síntese de mutanase, podendo ser empregada como um substituto barato e de fácil obtenção de mutana bacteriana. Empregando-se condições otimizadas de cultura, foi possível obter um alto rendimento de produção de mutanase (0,6-0,7 U/mL, 2,0-2,5 U/mg de proteína) em pouco tempo (3-5 dias), ultrapassando os melhores resultados relatados na literatura. A enzima bruta foi estável em pH de 4,5 até 6,0 e em temperatura até 40ºC. A atividade máxima foi obtida a 45ºC e pH 5,5. A mutanase obtida deste fungo foi bastante estável durante a seu armazenamento e apresentou um levado potencial hidrolítico sobre as ligações mistas (α-1,3, α-1,6) da mutana estreptocócica insolúvel em água (a taxa de hidrólise em 24 horas foi 69%). Os produtos resultantes da reação enzimática durante a hidrólise indicaram que a mutanase possuía uma ação tipo exo sobre a mutana. A análise cromatográfica em camada delgada mostrou que o principal produto resultante da ação da mutanase sobre a mutana era glicose. O potencial de aplicação de mutanase na estomatologia é discutido.

Palavras-chave: *Trichoderma harzianum*, mutanase, mutana

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