β -1,3-Glucanase from *Delftia tsuruhatensis* Strain MV01 and Its Potential Application in Vinification^{†‡§}

V. Blättel,¹ M. Larisika,^{1‡} P. Pfeiffer,¹ C. Nowak,^{2‡} A. Eich,³ J. Eckelt,³ and H. König^{1*}

Institute of Microbiology and Wine Research, 55128 Mainz, Germany¹; Max Planck Institute for Polymer Research, 55128 Mainz, Germany²; and WEE-Solve GmbH, 55130 Mainz, Germany³

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During vinification microbial activities can spoil wine quality. As the wine-related lactic acid bacterium *Pediococcus parvulus* is able to produce slimes consisting of a β -1,3-glucan, must and wine filtration can be difficult or impossible. In addition, the metabolic activities of several wild-type yeasts can also negatively affect wine quality. Therefore, there is a need for measures to degrade the exopolysaccharide from *Pediococcus parvulus* and to inhibit the growth of certain yeasts. We examined an extracellular β -1,3-glucanase from *Delftia tsuruhatensis* strain MV01 with regard to its ability to hydrolyze both polymers, the β -1,3-glucan from *Pediococcus* and that from yeast cell walls. The 29-kDa glycolytic enzyme was purified to homogeneity. It exhibited an optimal activity at 50°C and pH 4.0. The sequencing of the N terminus revealed significant similarities to β -1,3-glucanases from different bacteria. In addition, the investigations indicated that this hydrolytic enzyme is still active under wine-relevant parameters such as elevated ethanol, sulfite, and phenol concentrations as well as at low pH values. Therefore, the characterized enzyme seems to be a useful tool to prevent slime production and undesirable yeast growth during vinification.

The conversion of must into wine is a complex microbiological process, in which a great variety of different yeasts and bacteria can possibly be involved. Due to changes of chemical composition during vinification, the microbial diversity varies as well. Generally, about 100 different yeast species, more than 20 lactic acid bacteria, and—under certain conditions—species of three genera of acetic acid bacteria can be found in must and wine (34). Besides desirable microbes such as *Saccharomyces cerevisiae* and *Oenococcus oeni*—which are commercially used as starter cultures—many wild-type yeasts and several lactic acid bacteria are able to produce multiple off-flavors which lead to a decreased wine quality. In this context, spoiling lactic acid bacteria are predominantly responsible for the formation of diacetyl (39), biogenic amines (36, 48), and mousy off-flavor (14) and in particular the production of exopolysaccharide (EPS) slimes (7, 59). Species of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* and even *Oenococcus oeni* have been isolated from ropy wines or ciders (18). The most frequently incriminated species is *Pediococcus parvulus* (17). This bacterium has been isolated from ropy red and white wines, ciders, and beers. It has been identified as causing ropiness by the production of a high-molecular-mass β -1,3-glucan (500 to 2,000 kDa) from the residual sugars in fermented beverages (17). Consequently, the wine gets a

slimy and thick texture (graisse). In this context, a relatively small concentration (20 to 30 mg/liter) of this β -glucan is sufficient to induce visible textural modification (17). Indeed, this kind of wine spoilage has no known impact on human health, but the high viscosity can hamper wine filtration, resulting in lower wine quality and rising costs for the winemaker.

Additionally, several yeasts such as species of the genera *Brettanomyces*/*Dekkera*, *Pichia*, *Metschnikowia*, *Saccharomyces*, and *Zygosaccharomyces* can also influence wine quality negatively by producing different off-flavors and off-odors at several stages of the wine-making process (24). During the alcoholic fermentation, undesirable yeast species or strains are able to produce acetic acid, various esters, volatile phenols, and hydrogen sulfide. During bottling and storage of wines, film formation can be caused by weakly fermentative species of *Pichia*, *Candida*, and *Metschnikowia*. In addition, extensive growth of ethanol-tolerant species of *Zygosaccharomyces*, *Saccharomyces*, *Schizosaccharomyces*, and *Dekkera* could result in cloudiness, gas production, esteriness, and acid off-flavors (40).

The yeast cell wall and the exopolysaccharide of *Pediococcus parvulus* have substantial similarities in their biochemical compositions. Whereas the β -glucan of the exopolysaccharide possesses a β -1,3-linked glucosyl backbone with branches made up of single β -1,2-linked D-glucopyranosyl residues (38), the cell wall of yeasts mainly contains β -1,3 and β -1,6 but also variably linked β -glucans, too (33). Thus, by means of suitable enzymes not only could the slime of *Pediococcus parvulus* be degraded but also the undesirable growth of yeasts could be inhibited. Today, growth of destructive microorganisms is prevented traditionally by extensive sulfuring. However, this treatment also inhibits desirable microorganisms and their metabolism, for example, the malolactic fermentation of *Oenococcus oeni*. Although enzyme preparations are applied for several problems in the wine-making process, these crude preparations contain

* Corresponding author. Mailing address: Institute of Microbiology and Wine Research, Johann-Joachim-Becherweg 15, 55128 Mainz, Germany. Phone: 49-6131-3922662. Fax: 49-6131-3922695. E-mail: hkoenig@uni-mainz.de.

‡ Present address: Austrian Institute of Technology GmbH-AIT, 1220 Vienna, Austria.

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multiple additional activities with unpredictable effects. Therefore, there is a tendency to replace these enzyme preparations with pure enzymes with known activity.

This study presents the characterization and application of a β -1,3-glucanase from *Delftia tsuruhatensis* strain MV01 that is able to degrade the slime of *Pediococcus parvulus* and to inhibit yeast growth.

MATERIALS AND METHODS

Microorganisms and culture conditions. The intestinal tract of various termites is a rich source of glycanolytic microorganisms (54, 62). Several glycanolytic bacteria were isolated from the gut of *Zootermopsis angusticollis*, using medium 1 as described previously (62), and were tested for their activity toward β -1,3-glucans. One bacterial strain (strain MV01) with high β -1,3-glucanase activity was further classified. Therefore, standard physiological analyses such as Gram reaction, oxidase and catalase activity, nitrate reduction, citrate utilization, urease activity, and oxidation-fermentation (O/F) reaction with D-glucose (30) were performed. The utilization of several mono- and disaccharides, amino acids, and organic acids as sole energy and carbon sources (in liquid minimal basal salt medium, pH 7.6 [27], with 5 g/liter of the various compounds) was tested. Degradation of polymers such as glycogen, pullulan (from *Aureobasidium pullulans*), mannan (from *Saccharomyces cerevisiae*), pustulan, and dextran was analyzed by cultivation of the bacterial strain with 5 g/liter of each polymer in medium MM (see below). Then the respective culture supernatant was incubated with 1 g/liter of the corresponding polymer for 24 h, and the cleaving fragments produced were identified by thin-layer chromatography (26). Solid medium MM was supplemented with 1 g/liter of laminarin (from *Laminaria digitata*), xylan from oat spelt, starch, casein, or azure chitin, respectively. The bacterial strain was streaked on these agar plates and incubated for 2 to 3 days. Utilization of the polymers by the growing cells was indicated by clearing zones, when plates were flooded with trichloroacetic acid (casein hydrolysis), with Congo red (cellulose hydrolysis) (3), or with Lugol's solution (starch hydrolysis). Laminarin and xylan degradation could be detected immediately by calcofluor white staining after UV illumination (15). Chitinase activity was detected with azure chitin (Sigma). In addition, the 16S rRNA gene was analyzed by amplification using the universal primer (forward, 5'-AGA GTT TGA TC [AC] TGG CT-3'; reverse, 5'-AGA AAG GAG GTG ATC C-3') and subsequent sequencing (Eurofins MWG Operon, Germany) (accession no of 16S rRNA genes, HM587796). Clustal X 2.0 software (37) was used for the calculation of pairwise nucleotide similarity values based on the Jukes-Cantor algorithm, for construction of the phylogenetic tree by the neighbor-joining method, and for validation of the tree topology by bootstrapping (1,000 resamplings). 16S rRNA gene analysis grouped the bacterial strain in the genus *Delftia*. To define the species precisely, the determination of the fatty acid composition, the G+C content, and the DNA-DNA homology to the other member of the genus was performed at the DSMZ (Braunschweig, Germany). The results showed that strain MV01 belonged to the species *Delftia tsuruhatensis*.

Delftia tsuruhatensis strain MV01 (DSM 23722) was routinely cultivated in LB broth (4). To induce the production of glucanolytic exoenzymes, *Delftia tsuruhatensis* strain MV01 was grown in a minimal medium (medium MM) containing 0.1% NaNO₃, 0.1% K₂HPO₄, 0.05% KCl, 0.05% MgSO₄, and 0.05% yeast extract, supplemented with 0.1% yeast cell walls (Hefacell, Erbslöh, Germany) as a carbon source. Cultivation was carried out at 30°C on a rotary shaker (150 rpm) for 3 days.

Yeast strains, isolated from local wineries in Germany and identified by sequencing of the internal transcribed spacer (ITS) region (63), were grown in YGP medium (1% yeast extract, 2% peptone, 2% glucose) at 30°C on a rotary shaker (150 rpm) for 24 h.

For exopolysaccharide (EPS) production, *Pediococcus parvulus* strain B399, isolated from a Spätburgunder wine and identified by multiplex PCR (51), was cultivated in semidefined broth (SMD1) without yeast extract, beef extract, or peptone, since these ingredients interfered with EPS quantification and purification. SMD1 broth contained the following ingredients (in percent): yeast nitrogen base (Difco), 0.5; glucose, 2; Tween 80, 0.1; K₂HPO₄, 0.2; sodium acetate, 0.5; (NH₄)₂ citrate, 0.2; MgSO₄ · 7H₂O, 0.02; MnSO₄ · H₂O, 0.005. The pH was adjusted to 4.5. Growth occurred at 20°C for 10 days.

To stimulate the EPS production by *Botrytis cinerea* strain ATCC 90769, a medium containing 3% glucose, 1.5% K₂HPO₄, 1.25% KNO₃, 0.5% MgSO₄ · 7H₂O, 1 mg/liter FeSO₄ · 7H₂O, 1 mg/liter ZnSO₄ · 7H₂O, 40 µg/liter CuSO₄ · 5H₂O, and 40 µg/liter MnCl₂ · 4H₂O was used. *Botrytis cinerea* was

cultivated for 14 days at 25°C. The preparation of the EPS was similar to the procedure described for the EPS of *Pediococcus parvulus* (see below).

Isolation, purification, and quantification of the EPS from *Pediococcus parvulus*. Bacterial cells were removed from SMD1 medium by centrifugation (17,700 × g, 50 min, 4°C). The EPS was precipitated from the supernatant by adding 3 volumes of cold ethanol, followed by incubation overnight at 4°C. The precipitate was collected by centrifugation at 3,800 × g for 20 min at 4°C and dissolved in distilled water. The EPS was further purified by precipitation with ethanol three times. Finally, the precipitated EPS was dissolved in distilled water and exhaustively dialyzed against distilled water for 4 days and then lyophilized.

The total carbohydrate content of the EPS was measured by the phenol-sulfuric acid method (31), using glucose as a standard.

The composition and structure of EPS were determined by ¹H and ¹³C nuclear magnetic resonance (¹H NMR and ¹³C NMR, respectively) spectroscopy (200-MHz Bruker Avance and 700-MHz Bruker 700 Ultra Shield, respectively).

The molecular mass of EPS was determined by size exclusion chromatography (SEC), which included a differential refractive index detector (Gynkotek/Dionex, Germany). The apparatus was equipped with a guard column and a Hema Bio 40, a Hema Bio 1000, and a Suprema 3000 column (Polymer Standards Service, Germany) in series. The EPS (0.25%) was dissolved by continuous stirring for at least 3 h and eluted from the columns with a solution containing 0.1 M NaNO₃ and 0.05 M NaHCO₃ at a flow rate of 1 ml/min at room temperature. All samples were filtered with 0.45-µm polytetrafluoroethylene filters (Carl Roth, Germany) prior to the injection. For the calibration curve, dextran standards (180 to 277,000 g/mol) obtained by the Polymer Standards Service Mainz were used. In addition two dextran standards (670,000 g/mol and 1,400,000 g/mol) were obtained from Sigma-Aldrich.

Preparation of yeast cell walls. Cell walls were obtained according to the procedure described by Schleifer and Kandler (55). After disruption of cells with glass beads in a homogenizer (Bühler, Germany), cell wall fragments were separated from other cell debris by extended centrifugation and washing steps. After protein degradation by trypsin (0.5 mg/ml) overnight at 37°C, cell walls were washed thoroughly again and then lyophilized.

Enzyme assay. The β -1,3-glucanase activity was determined by measuring the reducing sugars produced from the degradation of laminarin from *Laminaria digitata* (Sigma-Aldrich), usually used as a substrate for β -1,3-glucanase. One hundred twenty-five microliters of enzyme solution from different stages of protein purification was mixed with an equal volume of distilled water and 450 µl of a laminarin solution (0.25% in 50 mM acetate buffer, pH 6.0). Control assays were done with heat-inactivated enzyme solution. After an incubation of 15 min at 30°C, reducing sugars were measured by using the dinitrosalicylic acid (DNS) method (43) with D-glucose as a calibration standard. One unit of β -1,3-glucanase was defined as the amount of enzyme that liberated 1 µmol of reducing sugars per min under the conditions described above.

To determine the substrate specificity of the β -1,3-glucanase, carboxymethyl pachyman (CM-pachyman; Megazyme, Ireland), carboxymethyl cellulose (CM-cellulose; Sigma-Aldrich), pustulan (Carl Roth, Germany), xylan from oat spelt (Sigma-Aldrich), dextran from *Leuconostoc* sp. (Sigma-Aldrich), and laminarin, as well as the β -1,3-glucan produced by the wine-relevant mold *Botrytis cinerea*, were used.

Effect of parameters on enzyme activity. Enzyme activities were measured at pH values from 3 to 8 under standard assay conditions with laminarin as a substrate. Buffers used were sodium citrate (pH 3 to 4), sodium acetate (pH 4.5 to 6), and Tris-HCl buffer (pH 7 and 8), 50 mM each. Enzyme activities were also assayed at temperatures from 10°C to 80°C at pH 4.0. To test the enzyme stability, enzyme solution was incubated at 20°C and 50°C. After various times up to 48 h, the enzyme activity was measured as described above.

To investigate enzyme activity under wine-relevant parameters, standard enzyme tests were carried out with a laminarin solution, supplemented with different concentrations of ethanol (1 to 17.5% [vol/vol]), sulfite (10 to 100 mg/liter), and phenols (ferulic acid, gallic acid, and catechin in equal concentrations, with final concentrations from 5 to 40 mg/liter). These tests were made at pH 4.0 at 20°C for 60 min.

Purification of β -1,3-glucanase. After cultivation of *Delftia tsuruhatensis* strain MV01 for 3 days, the culture was centrifuged (11,300 × g, 60 min, 4°C) and the supernatant was concentrated and dialyzed against 20 mM Tris-HCl buffer, pH 7.5, by using cross flow-filtration (SartoJet; Sartorius, Germany) with an exclusion size of 5 kDa. Subsequently, the enzyme solution was loaded onto an anion-exchange chromatography column (MonoQ, HR5/5; GE Healthcare, United Kingdom), equilibrated with 20 mM Tris-HCl, pH 7.5. The proteins were eluted with a linear gradient of NaCl (0 to 1 M) with a flow rate of 1 ml/min. Fractions (1 ml) containing the highest β -glucanase activity were pooled and concentrated, whereas fractions with low activity were discarded. Subsequently,

there was a further purification by size exclusion chromatography (Sephadex G75 [HR10/30]; GE Healthcare, United Kingdom); equilibration with 20 mM Tris-HCl buffer, pH 7.5, containing 200 mM NaCl; and an elution with the same buffer with a flow rate of 0.5 ml/min. The eluate was collected in 1-ml fractions. The success of purification was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (35). Thus, the final purified enzyme preparation separated by gel electrophoresis appeared as a single band after staining with Coomassie brilliant blue R-250. Isoelectric focusing and subsequent protein staining were carried out with Servalyt Precotes 3-10 according to the recommendations of the manufacturer (Serva, Germany). Gels were stained with Serva Violet 17 (Serva, Germany).

Activity staining of β -1,3-glucanase (zymogram). SDS-PAGE was carried out as described above. After SDS-PAGE the gel was washed in 25% isopropanol in 10 mM sodium phosphate buffer, pH 6.0, to remove residual SDS for 30 min with gentle shaking. Two washing steps with 10 mM sodium phosphate buffer, pH 6.0, followed for 20 min. The gel was laid onto a gel bed, containing 0.5% laminarin and 0.01% calcofluor white. After incubation at 30°C, enzyme activity was detected as a dark lytic zone under UV illumination.

Determination of protein and N-terminal amino acid sequences. Protein concentration was determined with a bicinchoninic acid assay kit (Uptima, France). The N-terminal amino acid sequence of the enzyme blotted from the SDS polyacrylamide gel to polyvinylidene fluoride membranes was determined by Edman degradation, performed by Toplab (Germany).

Analysis of exopolysaccharide degradation. The ability of the enzyme to degrade EPS produced by *P. parvulus* was demonstrated by various commonly used methods. Reducing sugars were detected after enzymatic hydrolysis of the polymer. For this purpose, the β -1,3-glucan (0.125%, wt/vol; in 50 mM citrate buffer, pH 4.0) was prepared and incubated with the enzyme solution at 50°C over a period of 6 h. Enzyme activity was determined by the dinitrosalicylic acid (DNS) method (43) described above.

To visualize additional effects in polymer degradation, viscometry was applied. Therefore, 8 mg of EPS was dissolved in 1 ml sodium citrate buffer (10 mM, pH 4.0). One hundred microliters of the enzyme solution (3 U/mg) was added to the reaction mixture and incubated for 24 h at 20 and 50°C. Controls were carried out with heat-inactivated enzyme. To prevent microbial growth, sodium azide (0.001%) was added to the mixture. After incubation, aliquots were taken and the reduction of the viscosity was determined by means of an AR 1000 rheometer (TA Instruments), equipped with a cone plate geometry (40-mm diameter, 1° cone angle) at a constant shear rate of 100/s at 25°C. Steady-state flow behavior was studied by measuring 10 points, each for 10 s.

The degradation of the polymer was additionally monitored by size exclusion chromatography (SEC). Therefore, 10 mg EPS was incubated with the enzyme preparation (3 U/mg) over 48 h. After 6, 18, 24, and 48 h, the reaction was stopped by cooling down and shifts in the distribution of the molecular mass of the polymer were analyzed by SEC. By determination of the number- and weight-average molecular weights (M_n and M_w , respectively), the polydispersity D , defined as $D = M_w/M_n$, could be obtained. It follows from this correlation that the greater the parameter D , the wider is the molecular mass distribution of the polymer.

Analysis of yeast cell wall degradation and cell lysis. Enzyme activity against yeast cell walls was tested by measuring the release of reducing sugars from the cell wall by the DNS method (43) with some modifications. Instead of 450 μ l of substrate solution, 550 μ l of an aqueous cell wall suspension (0.25%) was added to the enzyme solution (3 U/mg). After incubation the undigested and water-insoluble fragments were removed by centrifugation (10,000 $\times$ g, 10 min, 4°C) before dinitrosalicylic acid solution was added to the supernatant.

The impact of the enzyme on intact yeast cells was analyzed by turbidity measurements at a wavelength of 578 nm. The test was carried out in microtiter plates according to a modified description by Gacto et al. (26). Cells were suspended in 10 mM sodium citrate buffer, pH 4.0, up to an optical density of approximately 1.0. One hundred twenty-five microliters of this cell suspension was mixed with 100 μ l enzyme solution (3 U/mg). Sodium azide (final concentration, 0.001%) was added to avoid microbiological contamination, as described previously (25). It is known that yeasts with partially damaged cell walls do not reduce the optical density in photometric measurements (41). Therefore, Triton X-100 with a final concentration of 0.1% was added for 15 min to destroy already-affected cells completely. After incubation, the lytic activities were determined as the percent decrease of optical density at 578 nm (OD_{578}) in comparison to a control assay with heat-inactivated enzyme.

The potential damage of the envelope of yeast cells resulting from β -1,3-glucanase treatment in comparison to untreated cells was additionally investigated by using scanning electron microscopy (SEM). For SEM, cells were harvested by centrifugation, applied on coverslips, and air dried. The preparations

TABLE 1. Purification of β -1,3-glucanase from *Delftia tsuruhatensis* strain MV01^a

Step	Vol (ml)	Protein (mg)	Activity (U)	Sp act (U/mg)
Culture broth	2,690	282	94.2	0.35
Ultrafiltration	48	40	81.4	3.19
MonoQ chromatography	24	4.3	13.2	3.04
Size exclusion chromatography	1	0.03	0.34	10

^a In each case only the fraction with the highest enzyme activity was used for further purification steps.

were fixed in a solution of 2.5% glutaraldehyde in sodium phosphate buffer (100 mM, pH 7.4) at room temperature. After 24 h samples were washed and post-fixed in a 1% osmium tetroxide solution for 60 min. Before examinations with a Philips XL30 ESEM electron microscope (Institute of Functional and Clinical Anatomy, University of Mainz) were performed, the preparations were dehydrated in a series of acetone (30 to 100%) and were dried by critical point drying.

RESULTS

Identification of the β -1,3-glucanase-producing strain. Several bacterial strains isolated from the termite gut were screened for the degradation of β -1,3-glucans. Because of its high activity, the isolate strain MV01 was identified before starting further experiments. The isolate MV01 (DSM 23722) was a Gram-negative aerobic motile rod with an optimal growth temperature at 30°C and pH value of 7.0. The isolate was nonfermentative and positive for oxidase, catalase, and urease activities. Nitrate reduction and citrate utilization were observed. An α - and β -glucanase, chitinase, mannanase, and protease were produced. D-Glucose, D-fructose, D-arabinose, D-galactose, D-mannose, D-xylose, D-lactose, glycerol, D-mannitol, maltose, sucrose, maleate, fumarate, acetate, L-tryptophan, L-glutamate, L-proline, L-aspartate, and malate were used as energy and carbon sources. The usage of monomeric sugars and disaccharides has not been described for the genus so far (61). The fatty acids were C_{16:0}, C_{18:1 ω 7c}, C_{16:1 ω 7c}/2-OH iso-C_{15:0}, 3-OH C_{10:0}, C_{12:0}, C_{14:0}, C_{15:0}, C_{18:0}, cyclopropanoic acid (C_{17:0}), and 3-OH C_{8:0}. The G+C content of the DNA was 67.1 mol%. The closest relative is *Delftia tsuruhatensis* with a 16S rRNA gene similarity of 99% (GenBank/EMBL/DBJ accession no. HM587796) and a DNA-DNA homology of 98.8%.

Enzyme purification and characterization. When *Delftia tsuruhatensis* strain MV01 was grown in medium MM containing yeast cell walls as carbon source, optimal glucanolytic activity in the supernatant could be already detected after 3 days. The glucanolytic enzyme was then purified by ultrafiltration, anion-exchange chromatography, and size exclusion chromatography (Table 1). The purified enzyme had a specific activity of 10 U/mg protein with laminarin as substrate. When analyzed by SDS-PAGE, the enzyme preparation appeared as a single protein band corresponding to about 29 kDa. This band of purified protein detected by Coomassie blue staining corresponded fairly well with the clearing zone seen on the agarose gel by zymography (Fig. 1). The pI of the pure enzyme was estimated to be 4.2. N-terminal sequencing of the first 15 amino acid residues of the protein was performed by Edman degradation. The sequence showed a high similarity with members of the glycosyl hydrolase superfamily 16 such as β -1,3-glucanase from

FIG. 1. SDS-PAGE analysis of purified β -1,3-glucanase of *Delftia tsuruhatensis* strain MV01. (A) Protein staining. (B) Zymogram of glucanase activity shown as a lytic zone on a laminarin-containing agarose gel stained with calcofluor white under UV illumination. Lanes 1, molecular mass standard; lanes 2, purified enzyme.

Cellulosimicrobium cellulans (accession no. AAC38290) (23), *Nocardiopsis dassonvillei* subsp. *dassonvillei* (accession no. YP_003679525) (unpublished data), and *Arthrobacter aureus* (accession no. YP_946579) (45) (Table 2).

The activities of the isolated enzyme on various substrates are summarized in Table 3. The highest activity was achieved with CM-pachyman and laminarin as substrates, whereas CM-cellulose, pustulan, xylan, and dextran were not cleaved. This substrate specificity indicated the β -1,3-glucanase activity and confirmed the enzyme classification based on the N-terminal amino acid sequence. The distinct lower activity toward the EPS of *Pediococcus parvulus*, the EPS of *Botrytis cinerea*, and the cell wall of the different yeasts in comparison to the polymer laminarin is not remarkable due to the smaller size of laminarin (23 to 25 glycosyl residues).

The β -1,3-glucanase showed activity over a broad range of tested temperatures and pH values (10 to 80°C; pH 3.0 to pH 8.0) with maximum activity at 50°C and pH 4.0 (see Fig. S1 and

TABLE 2. Comparison of the N-terminal amino acid sequences of various β -1,3-glucanases of the glycosyl hydrolase family 16

Species	Amino acid sequence ^a	Similarity (%)
<i>Delftia tsuruhatensis</i> strain MV01	<u>APG</u> <u>DLLWS</u> <u>DE</u> <u>FDG</u> <u>AA</u>	
<i>Cellulosimicrobium cellulans</i> (accession no. AAC38290)	<u>APG</u> <u>DLLWS</u> <u>DE</u> <u>FDG</u> <u>AA</u>	100
<i>Nocardiopsis dassonvillei</i> subsp. <i>dassonvillei</i> (accession no. YP_003679525)	<u>APG</u> <u>DVVWS</u> <u>DE</u> <u>FDG</u> <u>AA</u>	86
<i>Arthrobacter aureus</i> (accession no. YP_946579)	<u>APG</u> <u>KL</u> <u>VWS</u> <u>DE</u> <u>FN</u> <u>GPA</u>	73

^a Identical amino acids are underlined.

TABLE 3. Substrate specificity of β -1,3-glucanase from *Delftia tsuruhatensis* strain MV01

Substrate ^a	Main linkage type (monomer)	Activity (%) ^b
Laminarin	β -1,3; β -1,6 (Glc)	100
CM-pachyman	β -1,3 (Glc)	135
Xylan	β -1,4 (Xyl)	0
Pustulan	β -1,6 (Glc)	0
CM-cellulose	β -1,4 (Glc)	0
Dextran	α -1,6 (Glc)	0
EPS (<i>Pediococcus parvulus</i>)	β -1,3; β -1,2 (Glc)	23
EPS (<i>Botrytis cinerea</i>)	β -1,3; β -1,6 (Glc)	18
Yeast cell wall		
<i>Pichia fermentans</i>	β -Glucan	67
<i>Zygosaccharomyces bailii</i>	β -Glucan	58
<i>Saccharomyces ludwigii</i>	β -Glucan	47
<i>Dekkera bruxellensis</i>	β -Glucan	58

^a All substrates were used at a final concentration of 1.5 mg/ml, except EPS (0.75 mg/ml).

^b An activity level of 100% corresponds to the activity on laminarin (mean value of three parallel determinations).

S2 in the supplemental material). These optima could also be obtained with *Pediococcus* slime as substrate, when the enzyme was incubated with EPS at 40, 50, and 60°C and pH 3, 4, and 5, respectively (data not shown). Whereas the enzyme is stable at 20°C for a period of 48 h with 75% activity remaining, the enzyme activity decreased to 17% within the first 24 h when the enzyme was incubated at 50°C (Fig. S1).

To analyze the cleavage products of laminarin produced by the β -1,3-glucanase, 100 μ l of a laminarin solution (1 mg/ml in 50 mM sodium acetate buffer, pH 6.0) was hydrolyzed with 25 μ l of the β -1,3-glucanase (3 U/mg) at 37°C overnight. The hydrolysis products were visualized by silica gel thin-layer chromatography according to the work of Gacto et al. (26). The enzyme cleaved laminarin randomly. The lytic products were glucose and laminarioligosaccharides (data not shown).

Effect of wine-relevant parameters on enzyme activity. The influence of typical wine-relevant factors, such as different concentrations of ethanol (up to 17.5% [vol/vol]), sulfite (up to 100 mg/liter), and phenols (up to 40 mg/liter), was tested. Whereas ethanol and phenols had no significant influence on enzyme activity, a loss of up to 20% of relative activity could be detected in the case of all sulfite concentrations tested (data not shown).

Degradation of EPS from *Pediococcus parvulus*. ¹H and ¹³C nuclear magnetic resonance spectroscopy revealed a glucan with a β -1,3 backbone and additional β -1,2-linked glucose at every second glucose molecule of the β -1,3-glucan as described previously (20, 38, 58). ¹H and ¹³C NMR chemical shifts (δ , ppm) and coupling constants (*J*, Hz) for the EPS of *Pediococcus parvulus* strain B399 are summarized in Table 4.

Since slime production by *Pediococcus* could cause problems during wine making, the enzyme activity against the EPS of *Pediococcus parvulus* was tested. Molecular mass determination of the β -1,3-glucan was carried out by SEC using different dextrans as molecular mass standards. The elugram showed the presence of some low-molecular-mass fractions and a major fraction, representing >80% of the material. This peak had a high-molecular-mass fraction of around 5×10^6 g/mol (see

TABLE 4. ^1H NMR chemical shifts (δ , ppm), coupling constants (J , Hz), and ^{13}C NMR chemical shifts (δ , ppm) for the EPS of *Pediococcus parvulus* strain B399

Shift or constant	Deduced linkage type		
	Glucp-(1 $\rightarrow$ $\rightarrow$ 3)-Glc	p-(1 $\rightarrow$ $\rightarrow$ 2,3)-Glc	p-(1 $\rightarrow$ $\rightarrow$ 2,3)-Glc
^1H NMR chemical shift (δ , ppm)			
H-1	ND ^a	ND	ND
H-2	3.19	3.5	3.73
H-3	3.39	3.66	3.87
H-4	3.30	3.45	3.44
H-5	3.35	3.44	3.44
H-6a	3.87	3.82	3.82
H-6b	3.63	3.61	3.61
Coupling constant (J , Hz)			
$J_{1,2}$	ND	ND	ND
$J_{2,3}$	8.7	8.5	8.2
$J_{3,4}$	9.1	9.0	8.5
$J_{4,5}$	9.2	9.8	10.2
$J_{5,6b}$	5.5		5.3
$J_{6a,b}$	-11.6	-11.7	-11.6
^{13}C NMR chemical shift (δ , ppm)			
C-1	103.1	102.4	102.2
C-2	73.5	72.3	80.5
C-3	76.7	86.7	84.9
C-4	70.4	69.0	68.9
C-5	76.6	74.8	76.2
C-6	61.8	61.6	61.5

^a ND, not determined

Fig. S3 in the supplemental material). The degradation of the polymer as a result of the incubation with the β -1,3-glucanase is visualized in Fig. S3. The different average molecular masses and the resulting polydispersities are shown in Table 5. As a result of incubation time, the peak of the high-molecular-mass fraction shifts to lower molecular masses accompanied by a simultaneously increasing polydispersity. From this ascending polydispersity, we were able to infer that the chain length of the polymer became more heterogeneous and that the degradation took place statistically along the polymer chain. In the first 6 h of the incubation, the molecular mass distribution of the polymer changed dramatically, while no further changes could be observed after 24 h.

In addition to the results achieved by SEC, measuring the liberated reducing groups (DNS method [43]) showed a relative activity of 23% of the enzyme on the polysaccharide in comparison to laminarin after the short incubation time of 15 min (Table 3). Forty-seven percent degradation of the polymer was attained by extension of the incubation time. This value was reached as a maximum after 6 h.

Viscosity reduction of approximately 50% was observed at 50°C after 24 h. Moreover, it could be shown that 35% of the initial viscosity could be reduced by the enzyme at 20°C (Fig. 2).

Degradation of yeast cell walls. By means of measuring the released reducing sugars (DNS method [43]) after incubation of the cell walls with the β -1,3-glucanase, relative activities of 47 to 67% compared to laminarin were observed (Table 3).

TABLE 5. Calculated polydispersity (D) of the high-molecular-mass peak during enzymatic degradation^a of the β -glucan of *Pediococcus parvulus*

Degradation time	M_n^b	M_w^c	D
Original (control)	1.8×10^6	4.6×10^6	2.6
6 h	1.1×10^6	3.3×10^6	3.0
18 h	7.5×10^5	2.6×10^6	3.5
24 h	6.3×10^5	2.1×10^6	3.3
48 h	5.5×10^5	1.8×10^6	3.3

^a See Fig. S3 in the supplemental material. For calculation, see Materials and Methods.^b M_n , number-average molecular weight.^c M_w , weight-average molecular weight.

These different values of cell wall hydrolysis could indicate differences in cell wall compositions of varied yeast genera.

In addition, effects of the β -1,3-glucanase on the cell wall of *Schizosaccharomyces pombe* strain 82 were analyzed by scanning electron microscopy (SEM). SEM analysis showed that after β -glucanase treatment, cells had a rough surface and an irregular, uneven, and perforated shape (Fig. 3).

Moreover, lysis of whole cells could be investigated by means of photometric measurements (Table 6). Lysis values reaching from 11% (*Pichia membranifaciens* strain 190.1) to 53% (*Metschnikowia pulcherrima* strain 152) indicate that the enzyme has different lytic efficiencies toward different yeasts. In addition it could be shown that the application of 0.1% Triton X-100 to the reaction mixture only further disintegrated already-damaged cells, resulting in an additional decrease of optical density. By means of CFU counting carried out with *Schizosaccharomyces pombe* strain 82, it could be demonstrated that Triton X-100 has no effect on cell viability without cell wall damage. After the incubation with glucanase, similar CFU could be determined independently of the usage of Triton X-100. Whereas the control showed a cell density of $(5.9 \pm 0.1) \times 10^5$ cells/ml, both the glucanase assay and the glucanase assay with Triton X-100 showed a cell density of $(2 \pm 0.12) \times 10^5$ cells/ml, which corresponded to a cell lysis of 66.1%. It therefore could be shown that cell lysis, as indicated by CFU analysis, was slightly higher than the results achieved by the photometric determination.

DISCUSSION

Extracellular β -1,3-glucanases are widespread in the domain *Bacteria*. They are produced and released from different bacilli (44) and paenibacilli (8), as well as from genera of *Actinomycetales* like *Streptomyces* (29), *Cellulosimicrobium* (57), and *Arthrobacter* (49). Furthermore, many fungi such as *Trichoderma* sp. and *Aspergillus* sp. synthesize these enzymes (1, 10, 21, 60). As far as we know, there is no description of β -1,3-glucanase production within the genus *Delftia*. So far, the degradation of environmental toxins like chloranilines (64) and 2,4-dichlorophenoxyacetic acid (28) is characteristic for these species, although strains of *Delftia lacustris* and *Delftia tsuruhatensis* are able to hydrolyze polymers such as chitin and peptidoglycan (32).

The practical application of β -1,3-glucanases is widespread. Especially in the wine-making process, several enzyme preparations such as hen egg lysozyme for the prevention of undes-

FIG. 2. Viscosity reduction (percent) during β -glucanase treatment at 20°C (A) and at 50°C (B) (solid symbols). Controls were applied as well (open symbols).

ired bacterial growth could be applied (5, 16). Also the usage of crude β -glucanase preparations is established in wine technology (e.g., Trenolin [Erbslöh], Panzym Fino G [Begerow], and Lallzym [Lallemand]). Usually, *Trichoderma* sp. and *Aspergillus* sp. are sources of these β -glucanases, which are primarily utilized to eliminate the β -1,3(1,6)-glucan produced by the mold *Botrytis cinerea*, which grows on damaged grapes. In addition they should enhance the natural autolysis of yeasts after the alcoholic fermentation.

Unfortunately, these crude preparations consist not only of β -1,3-glucanase activity but also of various accompanying activities like pectinases and β -glucosidases. This can be considered a disadvantage as there is less activity in β -glucan degradation as well as no controllable and predictable modifications of must and wine. Consequently, the application of purified β -1,3-glucanases is desirable. The β -1,3-glucanase of *Delftia tsuruhatensis* strain MV01 is highly specific, so that a degrada-

tion of substances other than β -glucans in must and wine could be excluded.

Despite the commercial enzyme preparations which are able to hydrolyze the β -glucan from *Botrytis cinerea*, no investigations in the case of degradation of EPS from pediococci have been carried out so far. In fact, the synthesis of exopolysaccharides by lactic acid bacteria, notably *Pediococcus parvulus*, isolated from various alcoholic beverages had been reported frequently (9, 19, 46, 54), which points out the relevance of β -1,3-glucanases for the degradation of this β -1,3(1,2)-glucan. In order to inhibit the growth and the EPS production of pediococci, winemakers have to take measures like intensive mixing and sulfuring. However, these rough treatments do not ensure the permanent elimination of bacteria and ropiness, especially as ropy bacteria are highly resistant to sulfur dioxide (18). The β -1,3-glucanase from *Delftia tsuruhatensis* is able to degrade the EPS from *Pediococcus parvulus*, which results in a

FIG. 3. Scanning electron micrographs of *Schizosaccharomyces pombe* strain 82 without (A) and with (B) β -1,3-glucanase treatment. The control assay (A) was done with heat-inactivated enzyme. Bars, 5 μ m.

TABLE 6. Lytic activities of the β -1,3-glucanase from *Delftia tsuruhatensis* strain MV01 toward different yeast strains

Species	Strain	Lytic activity (%) ^a
<i>Dekkera bruxellensis</i>	2.6-6	26 ($\pm$ 1.1)
<i>Hanseniaspora uvarum</i>	527	31.8 ($\pm$ 1.1)
<i>Metschnikowia pulcherrima</i>	152	53.8 ($\pm$ 2.1)
	160	32 ($\pm$ 2.7)
<i>Pichia fermentans</i>	167	21.4 ($\pm$ 0.3)
<i>Pichia membranifaciens</i>	190.1	11.5 ($\pm$ 1.0)
<i>Saccharomyces cerevisiae</i>	72	32.3 ($\pm$ 2.4)
<i>Saccharomycodes ludwigii</i>	84	22.5 ($\pm$ 1.4)
<i>Schizosaccharomyces pombe</i>	82	52.1 ($\pm$ 2.7)
<i>Zygosaccharomyces bailii</i>	427	21.2 ($\pm$ 1.3)
<i>Zygosaccharomyces rouxii</i>	68	17.6 ($\pm$ 0)

^a Lytic activities were measured according to descriptions in Materials and Methods and were determined as percent decrease of OD₅₇₈ in comparison to a control assay with heat-inactivated enzyme (mean value of two parallel determinations).

strong reduction of viscosity. Filtration problems arising from EPS production by pediococci could be solved.

Besides the degradation of pediococcal slimes, the enzyme shows activity toward the cell wall of wine yeasts which are responsible for reduced wine quality. For instance, the production of unwanted compounds like volatile ethyl phenols, acetoin, 2,3-butanediol, ethyl acetate, acetate, or sulfur compounds is linked to the metabolism of yeasts of the genera *Brettanomyces* (53), *Hanseniaspora* (47), and *Zygosaccharomyces*, *Schizosaccharomyces*, or *Saccharomycodes* (52). An additional risk is the ability of some ethanol-tolerant yeasts like *Zygosaccharomyces bailii* or *Schizosaccharomyces pombe* to grow in wine with remaining sugar; spoilage, therefore, could occur even in bottled wines (24, 40). Finally, wild-type yeasts are known for their high resistance toward preservatives like sulfite or sorbic acid (2, 42).

Due to the limited application of sulfite dioxide, alternative treatments have been suggested. The usage of commercialized enzyme preparations with β -1,3-glucanolytic activity has proved to be successful in the inhibition of spoiling yeasts such as *Zygosaccharomyces bailii* and *Dekkera bruxellensis*. However, an effect against these species could be observed only if the dosages of the preparation were up to 20 times higher than those recommended by the manufacturer (22). Such a high addition of protein could require a subsequent fining of the wine. Furthermore, the application of killer toxins produced by various yeasts (56) may be possible due to their high specific action against yeasts, though killer toxins inhibit only a minor group of sensitive yeast strains and are not effective against a broad range of different species (11, 12, 13). The β -1,3-glucanase from *Delftia tsuruhatensis* strain MV01 showed activity against all tested yeast species. Moreover, it has to be pointed out that this β -1,3-glucanase additionally affects *Saccharomyces cerevisiae*. Therefore, it is possible that the glucanase from *Delftia tsuruhatensis* strain MV01 has practical applicability not only concerning the inhibition of spoilage yeast growth but also with regard to encouraging the desirable autolysis of *Saccharomyces cerevisiae*, resulting in the liberation of specific flavor compounds (6, 50). Finally, due to high sugar contents in grapes and must, the β -glucanase could be used for ethanol management in order to stop the alcoholic fermenta-

tion, whereby desired ethanol concentrations could be adjusted.

In conclusion, *Delftia tsuruhatensis* strain MV01 produces an endo-type β -1,3-glucanase that is not only active under wine conditions but also applicable for two independent issues in wine making. Beside its ability to hydrolyze the exopolysaccharide from *Pediococcus parvulus*, leading to decreased viscosity, the enzyme affects the cell wall of different yeasts and thus could prevent spoilage at all stages of the wine-making process. Due to its high temperature optimum of about 50°C, the novel β -1,3-glucanase could also be applied for the degradation of glycans in the food industry and other industrial fields.

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