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β-Glucosidase from the Grape Native Yeast *Debaryomyces vanrijiae*: Purification, Characterization, and Its Effect on Monoterpene Content of a Muscat Grape Juice†

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Six hundred ten yeast colonies isolated from various vineyards in Chile were screened for the presence of a *β*-glucosidase activity as well as the resistance to glucose and ethanol inhibition. Among them, *Debaryomyces vanrijiae* was found to produce high levels of an extracelular *β*-glucosidase which was tolerant to glucose (K_i = 439 mM) and ethanol inhibitions. The enzyme (designated DV−BG) was purified to apparent homogeneity, respectively, by gel filtration, ion-exchange, and chromatofocusing techniques. Its molecular weight was 100 000, and its pI 3.0, optimum pH, and temperature activities were 5.0 and 40 °C, respectively, and had a V_{max} of 47.6 *μ*mol min^{-1} mg^{-1} and a K_m of 1.07 mM. The enzyme was active against different *β*-D-glucosides including glucosidic flavor precursors. The disaccharidic flavor precursors were not substrates for the enzyme. When added to a Muscat grape juice, the concentration of several monoterpenes increased as the consequence of its hydrolytic activity.

KEYWORDS: ***β*-Glucosidase; *Debaryomyces vanrijiae*; glucose inhibition; aroma enhancement**

INTRODUCTION

Beside volatile aroma compounds, grape berries and several fruits contain different aroma precursors. Among them, glycosidic precursors represent an important aroma source (Gunata et al., 1985; Bayonove et al., 1992; Rogerson et al., 1995; Winterhalter and Skouruomounis, 1997). Indeed a main proportion of potent flavorants, monoterpenes, shikimate-derived compounds, and C_{13}-norisoprenoids in grapes is linked to the sugars consisting of *β*-D-glucosides or diglycosides (Williams et al., 1982; Voirin et al. 1992; Gunata et al., 1985; Baumes et al., 1994). Consequently there is a growing interest in the exploitation of this hidden aromatic potential by the use of exogenous enzymes (Cordonnier et al., 1989; Gunata et al., 1993, 1996; Caldini et al., 1994; Janbon et al., 1994; Gueguen et al., 1996; Riou et al., 1998).

Acidic high-temperature conditions can be used for the hydrolysis of the bound aroma fraction, but it may lead to extensive rearrangements of volatiles and loss of the natural aroma profile of the product (Williams et al., 1982; Marais, 1983; Di stefano, 1989; Colagrande et al., 1994). Enzymatic hydrolysis was considered a more natural method that would not modify the natural aroma distribution pattern.

The enzymatic hydrolysis of the diglycosides occurs in two stages, following a sequential mechanism (Gunata et al., 1988, 1993). Primarily, the intersugar linkage is cleaved by a suitable glycosidase, and the corresponding *β*-D-glucosides are released. In the second stage, the aglycon release takes place after the action of a *β*-D-glucosidase. This is the key step, since the efficiency of the hydrolysis is strongly influenced by glucose and gluconolactone, potent inhibitors, and the structure of the aglycon moiety (Colagrande et al., 1994; Gunata et al., 1988; Rosi et al.,1994). Moreover, the inhibition by glucose limits the efficiency of glycosidase enzyme treatment in sweet wines and fruit juices, because of the high concentrations of this sugar. Therefore, the investigations were focused in the search of a fungal *β*-glucosidase tolerant to glucose, gluconolactone, and ethanol as well as possessing a large aglycon specificity and a good activity at low pH (Cordonnier et al., 1989; Gunata et al., 1993; Caldini et al., 1994; Rossi et al., 1994,1995; Riccio et al., 1999; Yanai and Sato 1999). *β*-Glucosidase from yeast strains *Debaryomyces hansenii* (Rosi 1994; Yanai and Sato 1999) and *C. molischiana* (Guegen et al., 1996) was studied for aroma enhancement in winemaking and fruit juices.

We have recently isolated 610 colonies of yeasts originating from different Chilean vineyards and studied them for their

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ability to produce a high-glucose tolerant β-glucosidase. One yeast was chosen, identified as *Debaryomyces vanrijiae*, and this paper describes the properties of the β-glucosidase produced by this yeast and its effect on aroma release from a grape juice.

EXPERIMENTAL PROCEDURE

Yeast Isolation and Screening. The yeast strains originated from Chilean vineyards of four different wine regions and were isolated from grapes (11 varieties) and 14 wines obtained by spontaneous fermentation. For the isolation, the water suspension of grape berries or fermenting juices were diluted and cultured on yeast malt agar and lysine plates. After incubation at 28 °C, the different colony types were counted and representatives of each purified. Thus, 610 colonies were obtained.

The screening of yeast strains for β-glucosidase activity was performed on agar plates as follows: after yeast development on the medium above, the plates were flooded with a 0.5 mM solution of 4-methylumbelliferyl-β-D-glucoside (MUG) in acetate buffer (100 mM, pH 4.0) (van Tilbeurgh and Claeyssens, 1985). β-Glucosidase activity was positive when a blue fluorescent halo appeared around the yeast colony upon transilumination, due to the release of 4-methylumbelliferone. Thereafter, the influence of high glucose content (1 M) and ethanol (10%) were tested on agar plates in the conditions above, but by buffering the medium at pH 3.6 with the strains that showed β-glucosidase activity.

Yeast Strain Identification. The strain numbered 286 was identified as *Debaryomyces vanrijiae* (van der Walt & Tscheuschner) Abadie et al., at the CLIB (Collection of Yeasts of Biotechnological Interest, Grignon, France). It was maintained at 4 °C on YM agar.

Culture Conditions. Aerobic cultures were performed in Erlenmeyer flasks, filled to one-fifth of their volume. Inocula were prepared with YM broth, and culture medium was inoculated with 1×10^6 cells/mL. The composition of the medium was 0.5% cellobiose (Sigma, St. Louis, MO), 1% bacto-peptone (DIFCO, Detroit, MI), and 1% yeast extract (Sigma, St. Louis, MO) buffered with phosphate-tartrate (100 mM, pH 5.0). Cultures were incubated (200 rpm) at 28 °C for 135 h. At different times during culture, cells were counted with a Coulter-counter, and protein concentration from the culture medium was estimated (see protein assay).

Enzyme Assays. *Glycosidase Activities.* β-D-Glucosidase, α-L-rhamnosidase, and α-L-arabinosidase activities were determined using the respective synthetic substrates: *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-L-rhamnopyranoside, or *p*-nitrophenyl-α-L-arabinofuranoside. The activities were assayed by incubating 1 volume of the enzyme solution (diluted if necessary) in 1 volume of the substrate (4 mM in acetate buffer,100 mM, pH 5.0) at 40 °C for 20 min. The reaction was stopped by the addition of 6 volumes of 1 M Na_2CO_3. The release of *p*-nitrophenol (pNP) was determined by absorbance measure at 400 nm ($\epsilon = 18\,300\ M^{-1}\ cm^{-1}$). Enzyme activity was expressed as nanokatal (nkat), i.e., nmol pNP liberated per second under standard conditions.

Protein Assay. The protein content of the samples was determined using the Pierce method, with bicinchoninic acid (BCA) as protein reagent (Pierce, Rockford, IL). The complex BCA−Cu^+−protein was quantified measuring absorbance at 560 nm. A calibration curve was done with bovine serum albumin (Sigma, St. Louis, MO) as standard.

DV−BG Purification. All steps in the purification were carried out at 4 °C. Absorbance at 280 nm was measured for protein estimation.

Gel Filtration Chromatography. Following centrifugation of a culture medium (5 000 g × 10 min), the supernatant (800 mL) was concentrated on an Amicon cell (PM 30 membrane, Amicon, Beverly, MA) to 8 mL and loaded on a Ultrogel AcA 44 (IBF, Villeneuve-La−Garene, France) column (75 × 1.6 cm) equilibrated with 100 mM phosphate-tartrate buffer (pH 7.0). Elution was performed with the same buffer at a flow rate of 12 mL h^{-1}, and 1.5 mL fractions were collected. The fractions containing β-glucosidase activity were pooled, concentrated, and desalted by ultrafiltration (PM 10 membrane).

Ion-Exchange Chromatography. The above β-glucosidase pool was subjected to an anion-exchange chromatography (40 × 1.6 cm) on DEAE Sepharose CL-6B (Pharmacia, Upsala, Sweden) preequilibrated with 25 mM imidazole, pH 6.9. The column was rinsed with 0.1 M NaCl in the same buffer. Elution at 30 mL h^{-1} of adsorbed protein was completed with a linear gradient of NaCl (100−350 mM). The volume of fractions was 3.5 mL. The enzyme containing fractions were pooled, concentrated, and desalted by ultrafiltration (PM 10 membrane).

Chromatofocusing. Ion-exchange chromatography pool was subjected to chromatofocusing technique on a Polybuffer Exchanger 94 (Pharmacia, Upsala, Sweden) column (12 × 1.0 cm) equilibrated with 25 mM histidine-HCl buffer, pH 6.2. The gradient of pH was formed by applying Polybuffer 74 (Pharmacia) (diluted 1:8 with water and adjusted to pH 3.7 with HCl). β-Glucosidase activity was eluted with a solution of glycine−glutamic acid (20 mM, pH 2.6) and concentrated by ultrafiltration (PM 10) to 2.2 mL.

Electrophoresis (PAGE−SDS). The purification steps and molecular weight of protein were checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) with a 12% polyacrylamide according to Laemmli procedure (Laemmli, 1970). The proteins were detected by staining with a commercial silver stain kit (Bio-Rad, CA). To estimate the molecular weight a suitable standard was also run (Bio-Rad, CA) (cf. **Figure 4**). For the detection of protein glycosylation the gel from SDS−PAGE was stained with periodic-acid Schiff's (PAS) reagent (Gerard, 1990).

Properties of β-Glucosidase. *Isoelectric Point.* The isoelectric point of the enzyme was determined by both chromatofocusing technique (see above) and isoelectrofocusing (IEF). For the latter, an IEF agarose gel containing ampholites in the pH range 2.5−5.5 were used. For pI determination, pI standards (pI range 2.5−6.5) were parallely run. Enzyme activity on the gel was revealed with MUG.

Substrate Specificity. Several substrates (1 mM each in acetate buffer, 20 mM, pH 4.6): 6-*O*-β-D-glucopyranosides of nerol, linalool and benzyl alcohol (Voirin et al., 1990), rutinosides (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) of pNP and geraniol (Voirin et al., 1990) and primeveroside (6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside) of eugenyl (Gunata et al., 1998) were incubated (40 °C, 16 h) with *D. vanrijiae* β-glucosidase (1 nKat). The hydrolysis was checked by thin-layer chromatography (TLC) (Gunata et al., 1998).

Cellobiose and pNP- and geranyl-β-D-glucosides were used to determine kinetic parameters (K_m, V_{max}) using Lineweaver−Burk plots. The enzyme solution was incubated at 40 °C for 10 min with different concentrations of the substrate in acetate buffer (pH 5.0, 100 mM), and released glucose was determined using an enzymatic (GOP) method (Boheringer, Mannheim, Germany). Enzyme activity was expressed as IU (international units). One IU correspond to 1 μmol of released glucose per minute under assay conditions.

Effects of pH and Temperature. Optimum pH activity was measured under standard assay conditions in McIlvain buffer of various pHs (2.8 to 7.0). For the pH stability, the enzyme was incubated (22 °C, 24 h) in the phosphate-citrate buffer at various pHs from 3.0 to 7.0, followed by measure of the activity at optimum pH. The optimum temperature activity was checked in the 20−60 °C temperature range in acetate buffer (100 mM, pH 5.0).

Inhibition. Glucose, glucono-δ-lactone, ethanol, various divalent (10 mM) and monovalent (20 mM) cations, EDTA, SDS, and dithiotreitol were added to enzyme assay medium containing pNP-glucoside as substrate to check the effect on β-glucosidase activity. Enzyme inhibition constants (K_i) for glucose and gluconolactone inhibition type were determined using Lineweaver−Burk plot.

Enzymatic Treatment of a Grape Juice and Extraction of Aroma Compounds. A juice (50 mL) from Moscatel rosada was spiked with a DV−BG solution (50 IU) issued from gel chromatography. The experiment was performed in triplicate. The mixture was incubated at 30 °C for 48 h in stoppered glass bottles with continuous agitation (100 rpm). The samples were added with 4-nonanol (36.2 μg) as internal standard and subjected to column chromatography on Amberlite XAD-2 for the isolation of free volatiles and volatiles bound to the sugars (Gunata et al., 1985; Belancic et al., 1997). The free volatiles were eluted with pentane/dichloromethane (2:1 v/v), dried with sodium sulfate, and concentrated to ca. 100 μL at 35 °C by Dufton columns

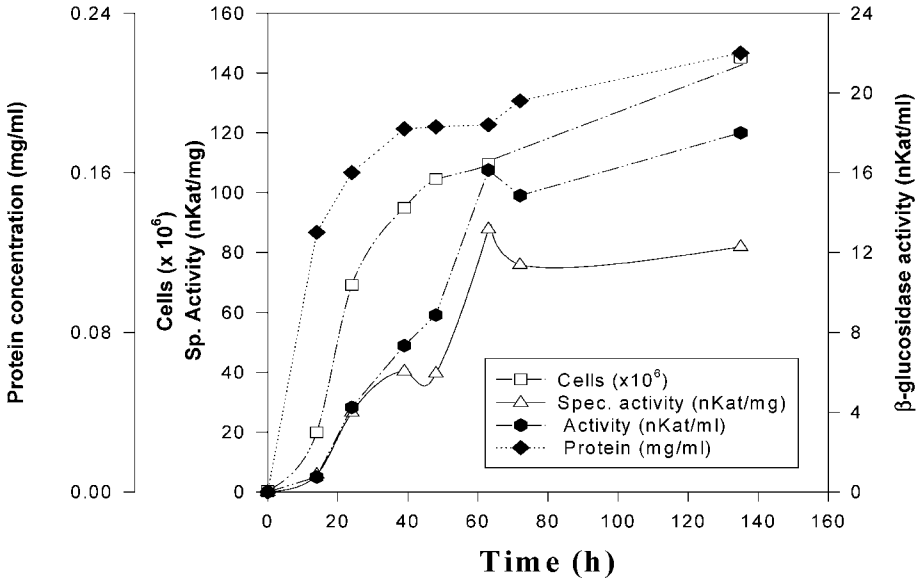

Figure 1. Cell growth, extracellular protein concentration, and exocellular β-glucosidase production by *D. vanrijiae* grown on cellobiose.

and analyzed by GC-MS. The bound fraction was eluted with ethyl acetate, concentrated to dryness, and subjected to enzymatic hydrolysis involving glycosidases from an enzyme preparation, Pektolase 3PA (Belancic et al., 1998). The released aglycones were extracted with pentane/dichloromethane (2:1 v/v), concentrated, and analyzed by GC-MS.

GC and GC-MS Analysis of the Free and Bound Aroma Fractions. The analysis of the free and bound aroma fractions were carried out in a GC-FID (Hewlett-Packard, HP 6890). The identification of the components was performed by GC-MS (HP 5972) on the basis of retention index and the comparison of EI mass spectra with published data or with reference compounds. Both GCs were equipped with a HP−FFAP capillary column (25 m × 320 μm I.D. × 0.5 μm film), and helium was used as carrier gas. The oven was programmed as folllows: 3 min at 60 °C with gradual heating to 230 °C at 2 °C/min. The injection was done in splitless mode (60 °C). The concentration of volatiles was expressed as equivalents of 4-nonanol.

RESULTS AND DISCUSSION

Yeast Selection. Within 610 yeast colonies isolated from grape and fermenting juices, only 22 showed β-glucosidase activity against MUG when developed on plates containing yeast malt agar medium. The positive strains were then cultured on agar plates supplemented with a medium at the pH of grape juice containing high concentrations of glucose (1 M) and ethanol (10%) as well as MUG (**Table 1**). Two were distinguished by a β-glucosidase highly tolerant both to glucose and ethanol (286, 292). The strain 286 was chosen for this work for its higher growth rate in agar plates. It was identified by the Collection of Yeasts of Biotechnological Interest (CLIB, Grignon, France) as *Debaryomyces vanrijiae*.

***β*-Glucosidase Production.** A preliminary investigation showed that little or no extracellular DV−BG was produced when the growth medium contained as carbone source glucose or methyl-β-D-glucoside. β-Glucosidase synthesis by fungi is often inhibited by glucose which is attributed to a catabolic repression (Dekker, 1981). In contrast cellobiose was found to be a good carbon source for β-glucosidase production by *D. vanrijiae*. This sugar has been often used for the production of fungal glucosidases in synthetic media (Dekker, 1981; Vasserot et al., 1989). It induced the production of an intracellular β-glucosidase from *Debarymyces hansenii*, but not that of extracellular enzymes (Riccio et al., 1999; Yanai and Sato, 1999). The exocellular enzymes (two isoforms) are constitutive and non-repressed by glucose and therefore produced and purified in a highly glucose containing media (Yanai and Sato, 1999).

Table 1. Origin and Hydrolytic Activities against a Synthetic β-Glucosidase Substrate (MUG), in the Presence of Ethanol and Glucose, of the Most Promising Native Strains Colonies Selected

strain no.	origin	MUG[a]	MUG + ethanol	MUG + glucose
591	grape	+	++	+–
330	wine	+	++	+
582	wine	+	++	+
413	wine	++	++	+
496	grape	++	++	+–
434	wine	+	+	+
432	wine	++	+	+
412	wine	+	++	+
292	grape	++	++	++
602	wine	++	++	+
426	wine	+	+	+
427	wine	++	++	+
603	wine	++	++	+
286	grape	++	++	++
346	wine	+	++	+
257	grape	++	++	+
433	wine	+	+	+
517	grape	++	++	+–
347	wine	+	+	+
491	wine	+	++	+
492	wine	+	++	+

[a] MUG = methyl umbeliferyl glucoside. +– = low activity; + = medium activity; ++ = high activity.

The time course of formation of extracellular β-glucosidase by *D. vanrijiae* is shown in **Figure 1**. The activity increased gradually during exponential growth phase of the yeast and thereafter slowly. The maximum activity was obtained after 60 h of cultivation (18 nkat/ml or 87 nkat/mg of protein). With respect of the production of β-glucosidase activity, *D. vanrijiae* appears superior to several yeast belonging to *Debaryomyces* et *Candia* sp.; *D. hansenii*, *D. polymorphus* (Rosi et al., 1994), *Candida wickerhamii* (Leclerc et al., 1984), and *C. peltata* (Saha and Pothast, 1996). It is interesting to note that β-glucosidase production by *D. vanrijae* is close to that obtained by filamentous fungi often used for technological applications (Woodward and Wiseman, 1982; Yan and Lin, 1997).

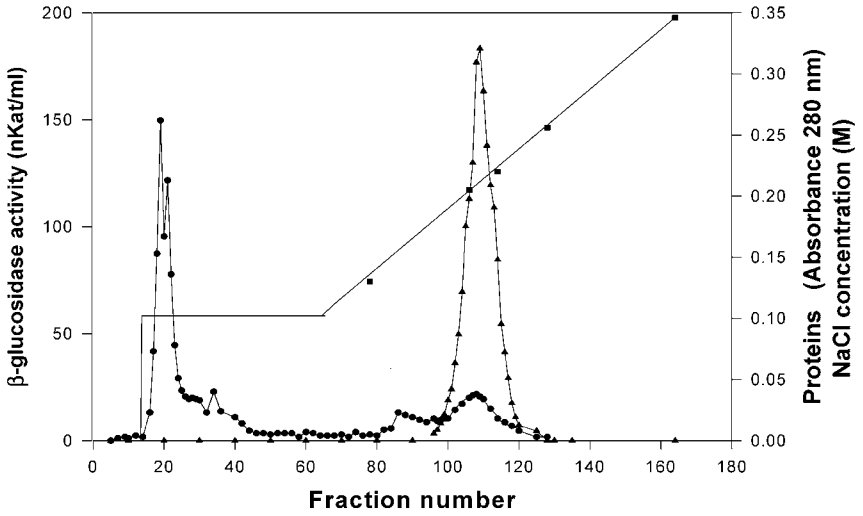

Figure 2. Ion-exchange chromatography of the β-glucosidase on DEAE Sepharose CL-6B. (▲) β-Glucosidase activity, (●) proteins, absorbance 280 nm, (■) NaCl concentration.

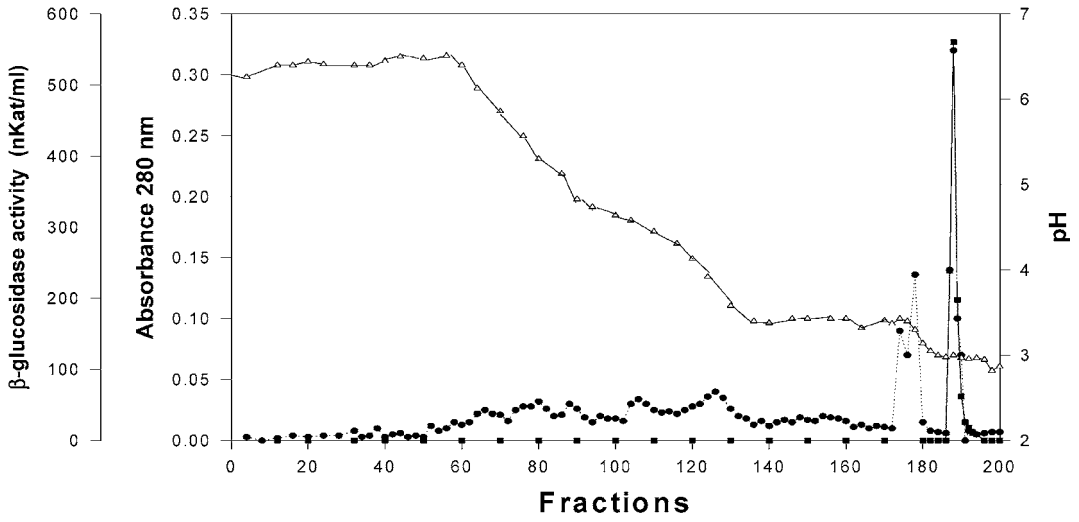

Figure 3. Elution profile of chromatofocusing separation (Polybuffer exchanger PBE 94) of the enzyme. (■) β-Glucosidase activity, (●) proteins, absorbance 280 nm, (△) pH.

Table 2. Purification of β-Glucosidase from *Debaryomyces vanrijiae*

purification step	volume (mL)	total act. (nkat)	total prot. (mg)	spec. act. (nkat/mg)	act. yield (%)	purific factor
initial extract	800	12,704	140.6	90.3	100	1
ultrafiltration (PM30)	8	10,616	19.84	535	83.6	5.9
Ultrogel AcA 44	34	9,465	1.496	6,327	74.5	70.1
DEAE Sepharose CL-6B	74	6,342	0.888	7,142	49.9	79.1
chromatofocusing	2.2	1,597	0.075	21,293	12.6	235.8

Enzyme Purification. The culture supernatant was first subjected to gel filtration after concentration by ultrafiltration. β-Glucosidase active fractions were further purified by anion-exchange chromatography (**Figure 2**) and chromatofocusing technique where the strongly adsorbed protein could be eluted only at pH 3.0, but not during pH gradient from 7.5 to 3.7 (**Figure 3**).

Table 2 summarizes purification yield. The DV−BG enzyme was purified 236-fold with an overall yield of 12.6% and a very high specific activity (21 293 nkat/mg). SDS−PAGE analysis of purification steps is given in **Figure 4**. The purified enzyme showed a single protein band.

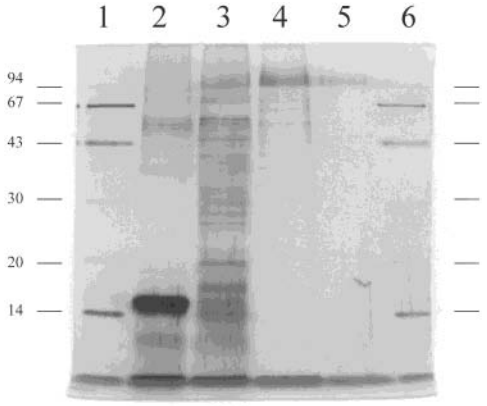

Figure 4. SDS−PAGE electrophoresis of the different steps of the β-glucosidase purification. Lanes 1 and 6: molecular weight markers; lane 2: concentrated supernatant; lane 3: Ultrogel AcA 44 pool; lane 4: DEAE Sepharose CL-6B pool; lane 5: pure enzyme after chromatofocusing.

Characterization. *Molecular Weight and pI*. The molecular weight of DV−BG was estimated by SDS−PAGE to be approximately 100 000 (**Figure 4**). This value is close to those

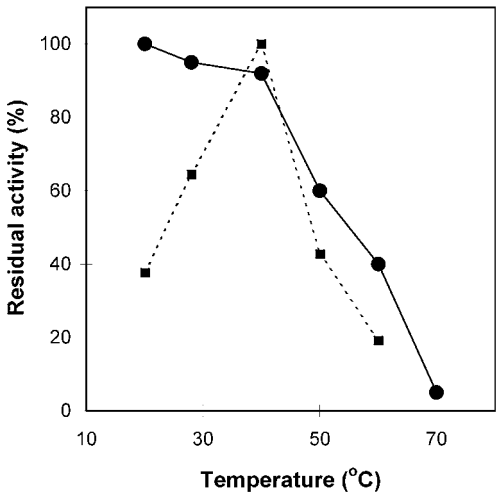

Figure 5. Effect of temperature on activity (■) and stability (●) of pure β-glucosidase from *D. vanrijiae*.

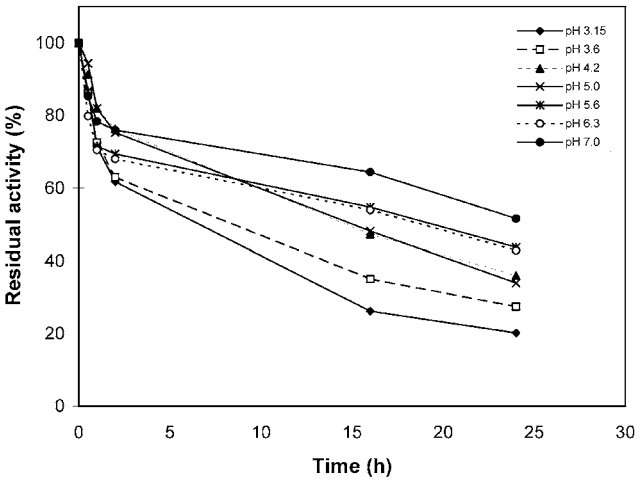

Figure 6. pH Stabilities of the purified β-glucosidase.

Table 3. Effect of Ethanol and Glucose Concentration on Enzymatic Activity of the β-Glucosidase

ethanol (%, v/v)	residual act. (%)	glucose (mM)	residual act. (%)
0	100	0	100
5	98	10	86
10	81	50	90
15	64	100	84
20	40	200	80
30	5	450	78

reported for extracellular β-glucosidase of *D. hansenii* (Riccio et al., 1999) and *C. molischiana* (Vasserot et al., 1991).

By chromatofocusing, the pI obtained for the enzyme was 3.0. The isoelectric point determined by narrow-range IEF was also near 3.0, but in this case the enzyme which gave a unique band in SDS−PAGE analysis appeared as a joined triple band by Coomassie staining. Furthermore the three bands were active against MUG (not shown). This could be attributed to differences in enzyme glycosylation**.** Indeed the PAS method on SDS gels showed that DV−BG was glycosylated (not shown).

To our best knowledge, the pI of DV−BG is the lowest value reported for a β-glucosidase from microbial sources. Only the pI of *A. niger* (3.2) and *A. acuteatus* (3.5) β-glucosidases are close to that of *D. vanrijae* (Yan and Lin, 1997).

Temperature and pH Effects. The enzyme displayed maximal activity at 40 °C (**Figure 5**). At the temperature of fermentation, around 20 °C, most β-glucosidases keep only 10 to 20% of their maximum activity (Gunata et al., 1993). However, DV−BG retains 38% of its maximum activity at this temperature. The enzyme was very stable at temperatures up to 45 °C for 1 h. It was completely inactivated at 70 °C. The optimum pH of activity was 5.0. Activity was maintained in the range 4.0−5.5 with residual activities higher than 89% (**Figure 5**). The optimum conditions for DV−BG enzymatic activity are similar to that reported for a *D. hansenii* enzymatic preparation, pH 4.0−5.0 at 40 °C (Rosi et al., 1994).

DV−BG was found to be sensitive to low pHs: after 24 h incubation (25 °C) at pH 3.15 the enzyme loses 80% of its initial activity (**Figure 6**). At higher pHs, ranged between 5.6 and 7.0, the enzyme was more stable, keeping 42−52% its initial activity. Similar results were reported for *S. cerevisiae* and *C. wickehramii* β-glucosidases (Gunata et al., 1993).

Substrate Specificity and Catalytic Properties. Kinetic parameters were calculated on cellobiose, pNPglucose, and geranyl-β-D-glucoside, with K_m values of 57.9, 0.77, and 1.07 mM and V_{max} values of 84.3, 668, and 47.6 μmol min^{-1} mg^{-1}, respectively. The enzyme had no α-arabinofuranosidase, neither α-rhamnosidase activities.

The hydrolysis of several monoglucosides and diglycosides by the DV−BG was checked by TLC after 16 h of incubation at 40 °C. The enzyme was able to hydrolyze the β-D-glucosides of pNP, nerol, linalool, and benzyl alcohol. Nevertheless, the hydrolysis of linalyl glucoside was incomplete, since a spot of the substrate is still present after 16 h of reaction. This is in accordance with low activity of β-glucosidases toward tertiary alcohols β-D-glucosides (Gunata et al., 1985; Gueguen et al., 1996). Furthermore DV−BG was unable to act toward disaccharidic flavor precursors tested, the rutinosides and primeverosides (not shown). This indicates that it does not possess an endoglycosidase activity capable to cleave heterosidic linkages of disaccharides, in contrast to the enzyme from tea leaves (Ogawa et al., 1997) and grape berry (Gunata et al., 1998).

Potential Inhibitors and Activators. The enzyme was competitively inhibited by glucose and glucono-δ-lactone. The K_i values obtained on the Lineweaver−Burk plots were 439 mM and 5 mM, respectively. This is interesting for technological applications since most microbial enzymes are strongly inhibited by glucose, the inhibition constants (K_i) ranging from 0.6 to 10 mM (Gunata et al., 1993). With 450 mM of glucose the enzyme retains 78% of its activity (**Table 3**). Highly glucose tolerant β-glucosidases from some fungi and yeasts have been recently reported: *Candida peltata* (K_i = 1.4 M, Saha and Bothast, 1996), *Aspergillus niger* (K_i = 543 mM, Yan and Lin, 1997), *Aspergillus oryzae* (K_i = 1.36 M, Riou et al., 1998).

Ethanol had an inhibitory effect on DV−BG (**Table 3**). Under the conditions prevailing in wine, i.e. 10−15% ethanol, the enzyme retains 60−80% of its original activity. High inhibition was observed with ethanol concentrations above 15%, being almost total with 30% of ethanol. Unlike some β-glucosidases that are activated by low ethanol concentrations which is attributed to the implication of glucosyl transferase activity (Shoseyov et al., 1988; Caldini et al., 1994; Rosi et al., 1994; Riou et al., 1998; Yanai and Sato 1999), this phenomenon was not observed in the case of DV−BG.

The effect of several effectors on the β-glucosidase activity is shown in **Table 4**. Copper was the only cation that had a negative effect on enzymatic activity (50% inhibition). β-Glucosidases from microbial origin are usually sensitive to Ag^+, Hg^{2+}, and Cu^{2+} ions (Gunata et al.; 1993).

Table 4. Effect of Different Cations and Reagents on the Activity of the β-Glucosidase

	% of residual activity
cation	
Hg^{2+}	106
Cu^{2+}	52.7
Zn^{2+}	94.4
Co^{2+}	99.8
Ca^{2+}	97.7
Mg^{2+}	97.4
Ce^{2+}	102
Ni^{2+}	88.9
K^{+}	98.3
Na^{+}	98.4
compounds	
EDTA (5 mM)	92
DTT (5 mM)	100
SDS 0.4%	0
ethanol 10%	81

Table 5. Enzymatic Treatment of a Grape Juice from Moscatel Rosada. Concentration of Free and Bound Terpenes[a]

	concentration (μg/L)[g]	
compounds	control	DV–BG[b]
Free Terpene Fraction		
linalool	656.5	816.3
hotrienol	61.5	24.3
α–terpineol	347.2	1289.5
lop	228.9	260.6
citronelol	28.5	34.9
geraniol	169.7	259.5
total	**1492.2**	**2685.1**
Bound Terpene Fraction		
linalool	326.5	324.1
hotrienol	37.2	n.d.[f]
α–terpineol	48.0	41.2
LOF[c]	105.9	111.2
LOP[d]	108.8	23.8
nerol	167.8	156.3
geraniol	388.5	311.7
terpenic diols[e]	6668.4	4945.5
total	**7851.2**	**5930.9**

[a] The samples were incubated at 30 °C during 60 h. [b] DV–BG = *D. vanrijiae* β-glucosidase. [c] LOF = *cis* and *trans* furanic linalool oxide. [d] LOP = *cis* and *trans* pyranic linalool oxide. [e] Terpenic diols = diol 3,6 + diol 3,7 + diol 3,8 (diol 3,8 = *cis* + *trans* 8-hydroxylinalool). [f] n.d. = not detected. [g] CV < 10%.

Dithiothreitol (DTT) had no effect upon enzymic activity, suggesting that in the active site, disulfide bridges were not involved. Sodium dodecyl sulfate (SDS) completely inhibits the enzyme, indicating that the integrity of the three-dimensional structure is critical for the catalytic activity.

Effect of DV−BG on the Monoterpene Content of a Grape Juice. For this purpose, Moscatel rosada was chosen due to its richness in monoterpene compounds (Belancic et al.; 1997). The level of free monoterpenes (2685 μg/L) increased clearly in grape juice treated with DV−BG compared to control juice (1492 μg/L) (**Table 5**). This increase can be explained by the hydrolysis of glycosidically bound compounds. However, the increase in the concentration of each free monoterpene cannot be attributed directly to the enzymic hydrolysis of its corresponding glycoside. Indeed free monoterpenes are prone to acid-catalyzed rearrangements which are accelerated at high temperature (Strauss et al., 1986). The assay conditions used here (30 °C, 48 h) favored certainly such structural modifications. The considerable increase in the concentration of free α-terpineol in DV−BG treated juice is a good indicative of this phenomenon.

CONCLUSION

This study shows the potential of DV−BG in the flavor enhancement of fruit juices and derived beverages and opens several strategies to study with regard to its applications: (i) use of DV−BG as exogenous enzyme supplemented with nowadays available fungal enzyme preparations to improve the liberation of flavor compounds in glucose rich media, such as sweet wines and fruit juices, (ii) construction of *S.cerevisiae* yeast strains expressing DV−BG activity during grape juice fermentation, (iii) coculture of *D. vanrijiae* with *S. cerevisiae* during juice fermentation to supplement the medium in glucosidase activity.

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