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The characterisation of a novel *Pichia anomala* β-glucosidase with potentially aroma-enhancing capabilities in wine

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Abstract - The production and characterisation of β-glucosidase from an isolated yeast strain classified as a *Pichia anomala* MDD24 were studied. The result shows that cellobiose is a good inducer for extracellular β-glucosidase production and optimum concentration is 1.5 percent cellobiose in yeast peptone dextrose medium. The purified β-glucosidase from *Pichia anomala* MDD24 exhibited a specific activity of 614 ± 14 U mg^{-1} of protein and a molecular mass of 42 kDa. This enzyme was slightly inhibited by fructose and sucrose in the range of 4 to 20% (w/v). An ethanol concentration between 4 and 20% (v/v) activated β-glucosidase activity, at presence 16% (v/v) ethanol, β-glucosidases obtained maximum relative activity around 150%. The optimum pH and optimum temperature for β-glucosidase activity were 4.5 and 40 °C, respectively. Although the activity under the pH and temperature of wine production (pH 3.5-4.0 and 15-20 °C) was quite low, the enzyme was stable and the relative activities were higher than commercial enzyme under those conditions. The extracellular β-glucosidase from *Pichia anomala* MDD24 makes it possible to release glucosidically-bound monoterpenes, which are the major contributors to floral and fruity aromas in wines from *Muscat*-type varieties, at final stage of alcoholic fermentation.

Key words: extracellular β-glucosidase; *Pichia anomala*; wine aroma; yeast; monoterpenes.

INTRODUCTION

Glycosidically-bound monoterpenes are important precursors of the varietal aroma of wine, particularly from *Muscat*-type varieties of *Vitis vinifera* and their genetic crosses. These conjugate glycosides are not volatile but soluble in grape must and wine. Several studies have shown that enzymatic hydrolysis occurs in two stages. In the first stage, the intersugar bond is cleaved by an α-L-rhamnosidase, an α-L-arabinofuranosidase, or a β-D-apiofuranosidase cleaving the (1→6) glycosidic linkage, and in a second stage, the aroma compounds are liberated from the resulting monoglucosides by the action of β-glucosidases (Günata *et al*., 1988). Although α-L-rhamnosidase, α-L-arabinofuranosidase and β-D-apiofuranosidase carry out the first step of the enzymatic process involved in the monoterpenes release, most studies have mainly focused on β-glucosidase (Günata *et al*., 1985; Hernandez *et al*., 2002; Arevalo Villena *et al*., 2006). They concluded that by releasing a volatile and thus aromatic aglycon from its monoglucosidic complex, glucosidase activity plays the key role for enhancing the varietal aroma of wine (Rodriguez *et al*., 2007).

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β-Glucosidases constitute a group of well studied hydrolases that have been isolated from plants, bacteria, molds and yeasts (Lecas *et al*., 1991; Riou *et al*., 1998; Ugliano *et al*., 2003; Barbagallo *et al*., 2004; Ducret *et al*., 2006; Rodriguez *et al*., 2007). However, β-glucosidases from grapes and other fruits as well as moulds generally appear to have low stability under winemaking conditions, i.e. they are not active between pH 3.0 to 4.0 and inhibited by sugars (Lecas *et al*., 1991; Riou *et al*., 1998; Barbagallo *et al*., 2004). The β-glucosidases produced from yeasts can be effective volatile aroma liberators. Several authors have reported β-glucosidase activity in wine yeast (*Saccharomyces cerevisiae*) strains that could release wine grape's monoterpenyl glycosides (Fernandez-Gonzalez *et al*., 2003; Palomo *et al*., 2005; Van Rensburg *et al*., 2005). The β-glucosidases from other yeasts such as *Candida molischiana* and *Candida wickerhamii* are less sensitive to glucose and have higher specificity for glycosides (Günata *et al*., 1990). However, the need for a more suitable commercial β-glucosidase for wine processing (low pH, low temperature, high sugar and/or ethanol) has led us to search for novel β-glucosidases.

Exogenous enzyme production is very attractive because it is easy and relatively inexpensive to recover and to purify the enzyme without major side activities. Thus, the discovery of highly active and robust extracellular

β-glucosidases was the focus of this study. Yeasts producing β-glucosidases were isolated in our laboratory from local grapes and native fruits such as pineapple, star gooseberry and tamarind (Swangkeaw *et al*., 2006). Strain *Pichia anomala* MDD24, isolated from table grape (Cardinal), expressed the high β-glucosidase activity was selected for this study. The production, purification and characterisation of extracellular β-glucosidase from *Pichia anomala* MDD24 was investigated with focus on its potential use in commercial wine production.

MATERIALS AND METHODS

Yeast strain. A yeast strain isolated from table grapes grown locally in Thailand, *Pichia anomala* MDD24, which exhibits high β-glucosidase activity was used in this study (Swangkeaw *et al*., 2006). For enzyme activity and stability comparisons, a commercially available β-glucosidase preparation was used (Lallzyme BETA™ by Lallemand, Montreal, Canada).

Effect of cellobiose on cellular growth and β-glucosidase production. *Pichia anomala* MDD24 was grown on yeast malt (YM) agar (malt extract 3 g, yeast extract 3 g, peptone 5 g, glucose 10 g, agar 20 g, in 1 L of distilled water), pH 5.0 and incubated at 30 °C for 24 h. Yeast cells were inoculated into yeast peptone dextrose (YPD) broth (yeast extract 10 g, peptone 20 g and glucose 20 g in 1 L of distilled water), pH 5.0 and incubated at 150 rpm and 30 °C for 15 h. One percent of the inoculum (approximately 10^6 cells. mL^{-1}) was added to the YP media containing different amount of glucose and cellobiose in percent weight by volume which are: YPD (2% yeast extract, 1% peptone, 2% glucose), YPC (2% yeast extract, 1% peptone, 2% cellobiose), YPD + 1.5% C (2% yeast extract, 1% peptone, 0.5% glucose, 1.5% cellobiose), YPD + 1% C (2% yeast extract, 1% peptone, 1% glucose, 1% cellobiose), YPD + 0.5% C (2% yeast extract, 1% peptone, 1.5% glucose, 0.5% cellobiose), YPD + 0.1% C (2% yeast extract, 1% peptone, 2% glucose, 0.1% cellobiose).Yeast cells were grown for 72 h in incubator shaker at 150 rpm, 30 °C. Samples were taken for study cell growth and enzyme activity every 12 h. Microbial cell growth was determined by using counter chamber under microscope. The yeast cells were collected by centrifugation (5000 rpm, 4 ° C, 10 min) and the supernatant (containing extracellular components) was assayed for enzyme activity.

β-Glucosidase assay. β-Glucosidase activity assay was performed by measuring the amount of *p*-nitrophenol (*p*NP) released from an artificial substrate, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG, Sigma) (Rodriguez *et al*., 2004). The 0.1 mL of enzyme solution was mixed with 0.2 mL of 0.002 M *p*NPG solution in a 0.1 M citrate phosphate buffer at pH 5.0. The reaction mixture was incubated at 30 °C for 30 min and the enzymatic reaction was subsequently stopped by adding 2.0 mL of 0.25 M Na_2CO_3 (Merck). The *p*NP released from this mixture was measured spectrophotometrically at 405 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 nmol of *p*NP per min under the experimental conditions.

Purification of β-glucosidase. Supernatant containing extracellular β-glucosidase was subjected to purification in three steps, as follows:

Step 1: Precipitation with acetone. Refrigerated acetone was added to the supernatant to give a final concentration of 50% (v/v). The mixed solution was stored at 4 °C overnight. The precipitate was collected by centrifugation (12000 rpm, 15 min, 4 °C) and resuspended in 0.1 M citrate phosphate buffer (pH 5.0).

Step 2: Anion-exchange chromatography. Partial purified β-glucosidase (from Step 1) was loaded onto an anion-exchanger column (1.8 cm x 9.0 cm), DEAE-cellulose (Sigma), equilibrated with phosphate buffer (0.05 M, pH 7.0) at a flow rate of 0.5 mL. min^{-1}. The unbound proteins were removed from the column by washing with two column volumes of 0.05 M citrate phosphate buffer, pH 7.0. A continuous gradient ranging from 0 to 1.0 M of sodium chloride in 0.05 M citrate phosphate buffer (pH 7.0) with a flow rate of 0.5 mL. min^{-1} was used to elute the bound proteins. Proteins in the column effluents were monitored spectrophotometrically at 280 nm.

Step 3: Ultrafiltration. The fractions from Step 2 exhibiting enzyme activity were pooled and concentrated by Vivaspin 500 ultra-filtration (with a 100 kDa cut-off).

The protein concentration was measured by the Lowry's method using bovine serum albumin (BSA) as a standard protein (Lowry *et al*., 1951).

Gel electrophoresis.
SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was used to estimate enzyme purity and to determine their molecular mass. The 20 µL of sample and 5 µL of molecular mass markers (RPN800, GE Healthcare Amersham) ranging from 10 to 250 kDa were applied to a 12% (w/v) SDS-PAGE at 30 mA. The gels were stained using Coomassie Brilliant Blue R-250 followed by destaining in a methanol-acetic acid-water mix. The distance moved was measured and the corresponding molecular size of the enzymes calculated from a calibration curve of log molecular mass versus distance migrated.

Zymograms analysis. SDS-PAGE was carried out using 12% (w/v) of acrylamide gel. After electrophoresis, the gel was washed by soaking in distilled water for 30 min then rinsed with citrate phosphate buffer (pH 5.0) for 30 min. The β-glucosidase activity was determined by overlaying the gel (0.5% (w/v) agar) with 1 mM 4-methylumbelliferyl-β-D-glucoside (MUG) in citrate phosphate buffer (pH 5.0) for 30 min at 30 °C and visualized under UV light.

Effect of sugars and ethanol on β-glucosidase activity. Effects of glucose, fructose, sucrose and ethanol concentrations on enzyme activities were studied by using sugars and alcohol concentrations over a range of 0 to 20% (w/v).

Effect of pH on β-glucosidase activity and stability. The optimum pH for β-glucosidase activity was examined at pH range 3.0 to 8.0 under standard assay condition using various buffers at 0.1 M concentrations, viz. citrate-phosphate buffer for pH range 3.0 to 6.0 and phosphate

buffer for pH range 6.0 to 8.0. The enzyme activity at each pH was normalized as a percentage of maximum enzyme activity. The effect of pH on the enzyme stability was determined using the buffers described above. The enzyme solution was incubated at each pH value at 30 ° C for 1 h.

Effect of temperature on β-glucosidase activity. The temperature profile for β-glucosidase activity in the purified enzyme preparation was assessed between 20 and 70 °C in 0.1 M citrate-phosphate buffer, pH 5.0. The enzyme activity at each temperature was normalized as a percentage of maximum enzyme activity. The temperature stability was investigated by incubating the purified enzyme in 0.1 M citrate-phosphate buffer, pH 5.0, for 1 h over a temperature range of 20 to 70 °C.

Kinetic parameters and substrate specificity of β-glucosidase enzyme. Kinetic parameters, K_m and V_{max}, were determined from a Lineweaver-Burk plot of β-glucosidase activity using *p*-nitrophenyl-β-D-glucopyranoside as substrate in a range of 0.2 to 2.0 mM (at pH 5.0 and 30 ° C). To assess glucose substrate inhibition, K_i value was calculated at glucose concentrations from 0 to 0.1 M.

The*p*-nitrophenyl-β-D-glucopyranoside,*p*-nitrophenyl-β-D-fucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-β-L-arabinopyranoside (all Sigma) containing glucosidic bonds were employed as substrates instead of *p*NPG in β-glucosidase activity assay. The *p*NP products were spectrophotometrically determined at 405 nm. The cellobiose (Sigma) and arbutin (Sigma) as substrates were determined by assaying the amount of glucose hydrolyzed from the reaction. Glucose concentration was measured by the 3,5-dinitrosalicylic acid method (Miller, 1959).

One unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 nmol of *p*NP or glucose per minute under the conditions of the assay.

RESULTS

Effect of cellobiose on cellular growth and β-glucosidase production

The effect of cellobiose concentrations on cellular growth and β-glucosidase production were studied when yeast cells were cultured in YPD, YPC, YPD + 1.5% C, YPD + 1% C, YPD + 0.5% C and YPD + 0.1% C, pH 5.0 at 30 °C, 150 rpm. The results showed that *Pichia anomala* MDD24 grew well on all experimental medium (Fig. 1A). The maximum enzyme production from *Pichia anomala* MDD24 occurs after 36 h of cultivation and YPD + 1.5% C and YPD + 1% C were suitable medium for extracellular production. The enzyme production was increased around 3.58-3.88 times, compared with cultured yeast cells in YPD (no cellobiose) after 36 h of cultivation (Fig. 1B). The results indicated

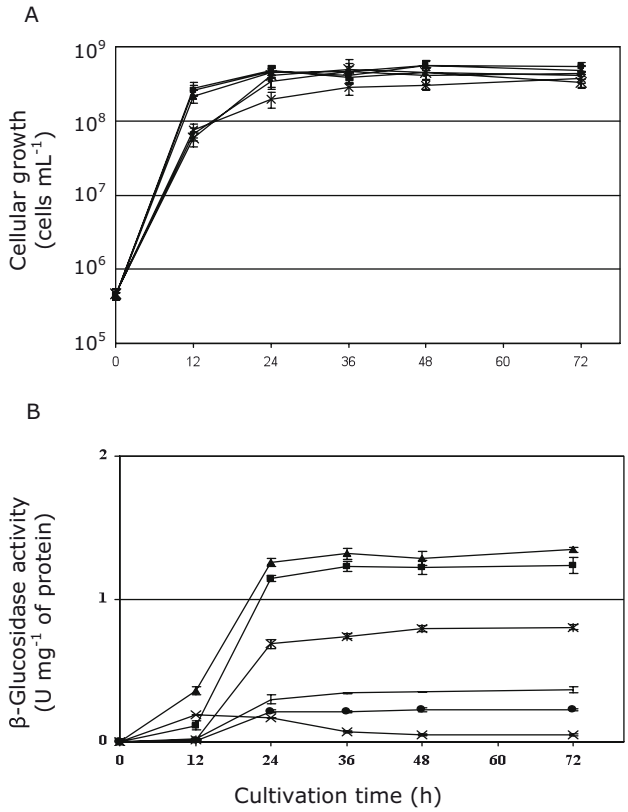

FIG. 1 - Effect of cellobiose on cellular growth (A) and extracellular β-glucosidase production of *Pichia anomala* MDD24 (B) when cultured on YPD (◇), YPC (×), YPD + 1.5% C (▲), YPD + 1% C (■), YPD + 0.5% C (∗) and YPD + 0.1% C (●).

that maximum β-glucosidase production in supernatant was obtained when yeast cells were cultured in YPD plus cellobiose. It is probably yeast cells likely to use glucose as a carbon source for cellular growth and cellobiose acts as a carbon source for inducing β-glucosidase synthesis.

Purification of β-glucosidase

Table 1 summarizes the purification results of the extracellular β-glucosidase from *Pichia anomala* MDD24. The chromatogram of protein, β-glucosidases activity and sodium chloride gradient concentration on a DEAE-cellulose column are shown in Fig. 2. One main peak of unbound proteins was found in fraction 5 to 22. Fractions 23 to 28 exhibited high β-glucosidase activity but low protein concentration. This result implies the discovery of a high efficiency β-glucosidase. The β-glucosidase-active fractions were pooled and concentrated by ultrafiltration with a 100 kDa cut-off. The enzyme was purified to 439-fold with an overall yield of 15% and a high specific activity (614 ± 14 U·mg^{-1} of protein).

TABLE 1 - Purification of extracellular β-glucosidase from *Pichia anomala* MDD24

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} of protein)	Yield (%)	Purification factor
Crude enzyme	6670.0	9193	1.4	100	1
50% Acetone precipitation	128.0	7912	62.0	86	44
DEAE-cellulose	5.5	2031	369.0	22	264
Ultra filtration (100 KDa)	2.2	1351	614.0	15	439

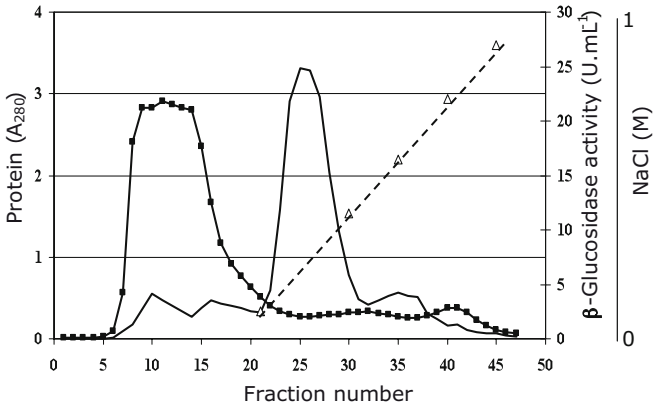

FIG. 2 - Ion-exchange chromatography of extracellular β-glucosidase from *Pichia anomala* MDD24 on DEAE-cellulose column. ■: protein A_{280}, ○: β-glucosidase activity, △: NaCl concentration.

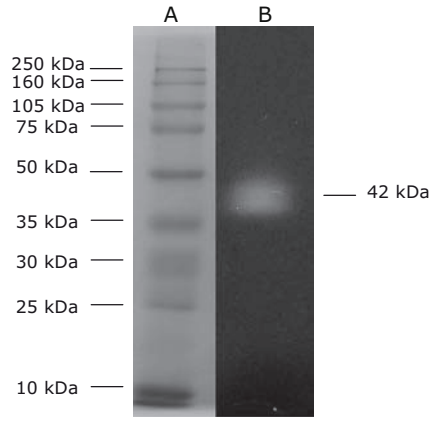

FIG. 3 - SDS-PAGE of molecular mass standard (A) and SDS-PAGE zymogram gel analysis of purified β-glucosidase from *Pichia anomala* MDD24 developed with the fluorogenic substrate MUG (B).

Molecular mass of β-glucosidase from *Pichia anomala* MDD24

A denaturing SDS-PAGE of purified extracellular β-glucosidase from *Pichia anomala* MDD24 was performed. The zymogram was developed on a gel with 4-methylumbelliferyl-β-D-glucoside as substrate and the methylumbelliferone released was detected via fluorescence under UV light. A single active protein band was shown and the molecular mass of this enzyme was 42 kDa when compared to molecular mass markers (Fig. 3).

Effect of sugars on β-glucosidase activity

The effects of sugar concentration (glucose, fructose and sucrose at 0 to 20%, w/v) on both β-glucosidases from *Pichia anomala* MDD24 and a commercial enzyme (Lallzyme BETA™) were also studied. The results are shown in Fig. 4-7. Increasing the glucose concentrations dramatically reduced the β-glucosidase activity from *Pichia anomala* MDD24 and the activity decreased around 80% in the presence of 2% (w/v) glucose. However, the β-glucosidase activity of the commercial enzyme was even more sensitive to glucose inhibition and the activity decreased 95% in the presence of 2% (w/v) glucose (Fig. 4). No activity of enzyme was detected when 20% (w/v) glucose was added.

Subsequently, the kinetics of the inhibition of *p*NPG hydrolysis by glucose was studied. A competitive inhibition at the intersection of the inhibition lines on the X-axis of the Lineweaver-Burk plot is shown in the Fig. 5. The calculated K_i value of glucose inhibition was 2.7 $\pm$ 0.1 mM.

The effect of fructose on β-glucosidase from *Pichia anomala* MDD24 and commercial enzyme is shown in Fig. 6. The β-glucosidase activity from *Pichia anomala* MDD24 was slightly decreased by fructose in the range of 0 to 20% (w/v) and the relative activity reduced around 15% in presence of 20% (w/v) fructose. In case of the commercial enzyme, the enzyme activity was dramatically inhibited when fructose concentrations were increased and the relative activity decreased around 75% in presence of 20% (w/v) fructose.

Figure 7 shows the effect of 0 to 20% sucrose on β-glucosidase activity from *Pichia anomala* MDD24 and commercial enzyme. Both β-glucosidases were inhibited by sucrose. However, the β-glucosidase from *Pichia anomala* MDD24 exhibited more sensitivity (55% activity remaining) to sucrose than the commercial enzyme (80% activity remaining) in presence of 20% (w/v) sucrose.

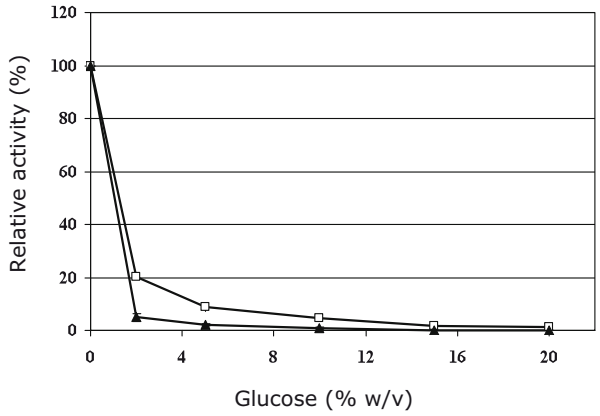

FIG. 4 - Effect of glucose on β-glucosidase activity from purified extracellular β-glucosidase from *Pichia anomala* MDD24 (□) and commercial enzyme (▲).

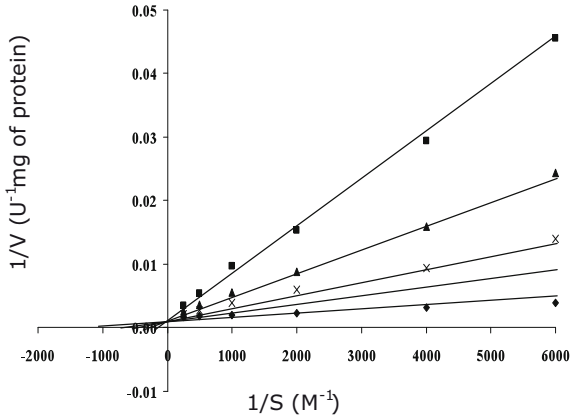

FIG. 5 - Lineweaver-Burk plot of inhibitory effect of glucose on *p*NPG hydrolysis by purified extracellular β-glucosidase from *Pichia anomala* MDD24. Glucose concentrations used were 0.0000 M (◆), 0.0125 M (○), 0.0250 M (×), 0.0500 M (▲) and 0.1000 M (■).

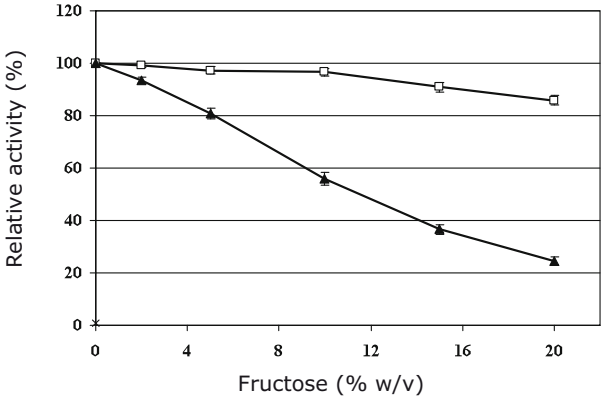

FIG. 6 - Effect of fructose on β-glucosidase activity of purified extracellular β-glucosidase from *Pichia anomala* MDD24 (□) and commercial enzyme (▲).

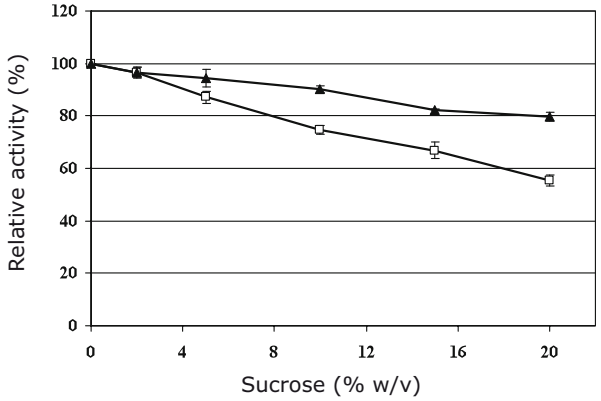

FIG. 7 - Effect of sucrose on β-glucosidase activity of purified extracellular β-glucosidase from *Pichia anomala* MDD24 (□) and commercial enzyme (▲).

Effect of ethanol on β-glucosidase activity

The effect of ethanol (concentration range 0 to 20%, v/v) on β-glucosidase activity is shown in Fig. 8. The *Pichia anomala* MDD24 β-glucosidase activity was enhanced by ethanol concentrations at 4 to 20% (v/v) which is significant for its application in winemaking. In the presence of 16% ethanol, β-glucosidase from *Pichia anomala* MDD24 exhibited maximum relative activity (around 150% of relative activity). In the presence of 20% ethanol, the relative activity was still 140%. In contrast, the commercial enzyme was inhibited by ethanol and the relative activity was around 55% in the presence of 20% ethanol. The alcohol tolerance of the *Pichia anomala* MDD24 enzyme is especially advantageous if it is added during the final stages of alcoholic fermentation or into dry, finished wine (ethanol < 16%, v/v).

Effect of pH on β-glucosidase activity

The results of pH and pH stability on β-glucosidase activity from *Pichia anomala* MDD24 and commercial enzyme were shown in Fig. 9. Normal bell-shaped pH-activity profiles were seen for both enzymes. The optimum pH of the *Pichia anomala* MDD24 β-glucosidase was 4.5 and the pH with 50% relative activity on the acidic side and basic side were 3.7 and 6.0, respectively. The optimum pH of the commercial enzyme was 4.5 as well. The pH with 50% relative activity on the acidic side and basic side were 3.7 and 5.5, respectively. The β-glucosidase from *Pichia anomala* MDD24 was stable in the entire experimental pH range with relative activities greater than 85%. Although the activity of β-glucosidase from *Pichia anomala* MDD24 in wine pH was relatively low, the enzyme was stable within this pH range. The β-glucosidase from *Pichia anomala* MDD24 appears to be rugged enough for use under commercial winemaking conditions.

Effect of temperature on β-glucosidase activity

The results of temperature on the enzyme activity and enzyme stability on the β-glucosidase from *Pichia*

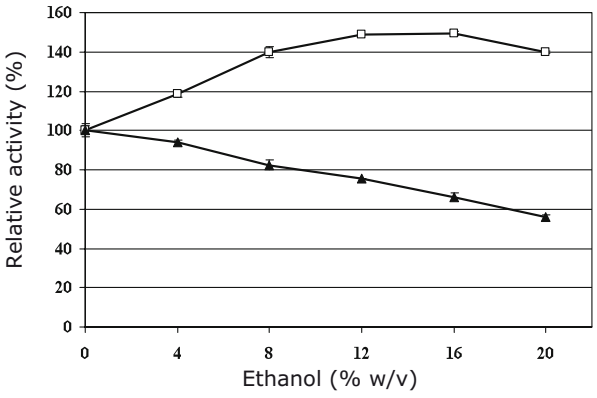

FIG. 8 - Effect of ethanol on β-glucosidase activity of purified extracellular β-glucosidase from *Pichia anomala* MDD24 (□) and commercial enzyme (▲).

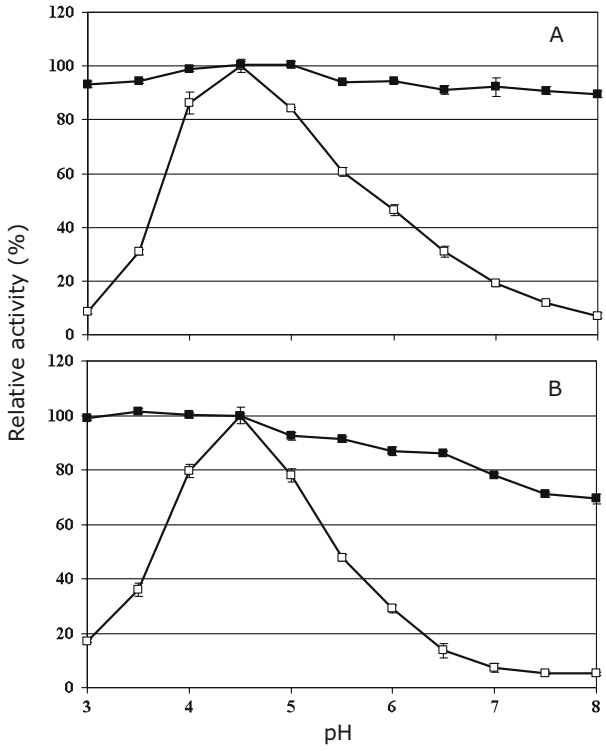

FIG. 9 - Effect of pH on purified extracellular β-glucosidase activity (□) and pH stability (■) from *Pichia anomala* MDD24 (A) and commercial enzyme (B).

anomala MDD24 and commercial enzyme were shown in Fig. 10. The maximum β-glucosidase activity from *Pichia anomala* MDD24 was obtained at temperatures between 40 and 45 °C whereas the commercial enzyme exhibited maximum β-glucosidase activity at 60 to 70 °C. After 1 h of incubation at various temperatures, 100% relative activity of β-glucosidase from *Pichia anomala* MDD24 and the commercial enzyme were obtained at 20 to 35 °C and 20 to 55 °C, respectively. At the winemaking temperature (20 °C), the relative activity of β-glucosidase activity from *Pichia anomala* MDD24 was around 40% whereas the commercial enzyme was only around 10%.

Kinetic parameters and substrate specificity of β-glucosidase enzyme from *Pichia anomala* MDD24

Kinetic parameters (K_m and V_{max}) were determined from Lineweaver-Burk plots. V_{max} and K_m of β-glucosidase from *Pichia anomala* MDD24 were 614 ± 14 U· mg^{-1} of protein and 0.157 ± 0.002 M, respectively.

The substrate specificity of β-glucosidase from *Pichia anomala* MDD24 was determined for various artificial and natural substrates. The results showed that this enzyme exhibited different levels of activity against aryl-glycopyranosides and other β-linked disaccharides (Table 2). Highest specificity (100% relative activity) was observed for aryl-glucopyranoside (glucose contained glycosides) which is *p*-nitrophenyl-β-D-glucopyranoside. However very low activities (5 to 6%) were observed when using aryl-glycopyranosides (fructose, galactose or arabinose contained glycosides) as substrates such as *p*-nitrophenyl-β-D-fucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, and *p*-nitrophenyl-β-L-arabinopyranoside. These results indicate that β-glucosidase from *Pichia anomala* MDD24 is highly specific to glucose in aryl-glucopyranoside. Moreover, natural substrate such as arbutin, a derivative of hydroquinone bound to glucose, was hydrolyzed as efficiently as a β-linked disaccharide, cellobiose (13 to 14% relative activity). These results demonstrate a specificity of this enzyme to phenyl-glucopyranoside containing aromatic rings (aglycon part) and glucose.

TABLE 2 - Relative activity of β-glucosidase from *Pichia anomala* MDD24 on various substrates

Substrate (0.002 M)	Relative activity (%)
p-nitrophenyl-β-D-glucopyranoside	100.0 ± 1.0
p-nitrophenyl-β-D-fucopyranoside	5.8 ± 1.5
p-nitrophenyl-β-D-galactopyranoside	5.1 ± 1.0
p-nitrophenyl-β-L-arabinopyranoside	5.5 ± 0.7
Cellobiose	14.0 ± 2.0
Arbutin	13.0 ± 2.0

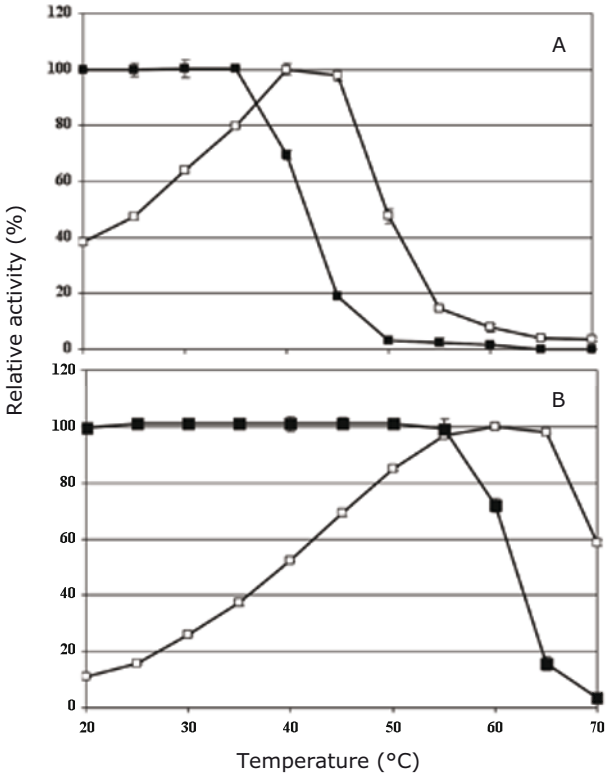

FIG. 10 - Effect of temperature on purified extracellular β-glucosidase activity (□) and temperature stability (■) from *Pichia anomala* MDD24 (A) and commercial enzyme (B).

DISCUSSION

Volatile compounds derived from grape glycosidic complexes make an important contribution to the varietal aroma of certain wine styles. The role of β-glucosidase activity in wine yeasts has been extensively researched. Ferreira *et al.* (2001) reported intracellular β-glucosidase-producing species such as *Metschnikowia pulcherrima*, *Pichia anomala* and *Saccharomyces cerevisiae*. In other studies, *Candida entomophila* (Gueguen *et al*., 1994), *Pichia guillermondii* (McMahond *et al*., 1999), *Saccharomyces cerevisiae* AL41 (Restuccia *et al*., 2002; Quatrini *et al*., 2008), *Candida pulcherrima* V6 (Rodriguez *et al*., 2004) and *Hanseniaspora osmophila* (Arevalo Villena *et al*., 2005) did not show extracellular β-glucosidase activity in the culture medium. Our results show that *Pichia anomala* MDD24 can produce high extracellular β-glucosidase when it is cultured in a medium containing cellobiose as an inducer. Arevalo Villena *et al*. (2006) previously reported that cellobiose can induce the extracellular β-glucosidase production from *Debaryomyces pseudopolymorphus* which supports our findings.

A purified extracellular β-glucosidase from *Pichia anomala* MDD24 had a specific activity of 614 ± 14 U· mg^{-1} of protein and the molecular mass of this enzyme was 42 kDa. The similar size of β-glucosidases from *Candida entomophila* (43 kDa) and *Aspergillus oryzae* (40 kDa) has been reported by Saha and Bothast (1996) and Riou *et al*. (1998), respectively. In comparison, the molecular mass of β-glucosidases from other yeast species and others plants sources varied from 40 to 350 kDa (Gueguen *et al*., 1994, 1995; Saha and Bothast, 1996; Riou *et al*., 1998; Belancic *et al*., 2003; Wallecha and Mishra, 2003; Arevalo Villena *et al*., 2006). Several genes are responsible for producing β-glucosidases in different types of organisms (Quatrini *et al*., 2008) and multiple subunits of a particular β-glucosidase may cause the broad range molecular mass observed.

The β-glucosidase from *Pichia anomala* MDD24 was slightly inhibited by fructose and sucrose. However, the β-glucosidase from *Pichia anomala* MDD24 was a competitive inhibited by glucose. The tolerance of β-glucosidase activity from *Pichia anomala* MDD24 towards fructose may provide a major advantage over existing commercial enzyme preparations.

β-Glucosidase from the grape itself and commercial β-glucosidase prepared from *Aspergillus niger* are inhibited by ethanol (Mateo and Di Stefano, 1997; Aryan *et al*., 1987; Barbagallo *et al*., 2004). Ethanol also inhibits β-glucosidase activities produced from other yeasts species such as *Hanseniaspora uvarum* (Barbagallo *et al*., 2004), *Zygosaccharomyces bailii* (Gueguen *et al*., 1995), *Debaryomyces vanrijiae* (Belancic *et al*., 2003), *Brettanomyces* spp., *Candida oleophila*, *Debaryomyces polymorphus* and *Debaryomyces pseudopolymorphus* (Cordero Otero *et al*., 2003), *Candida guilliermondii*, *Candida pulcherrima* and *Kloeckera apiculata* (Rodriguez *et al*., 2007). Conversely, it has been reported that alcohol stimulates β-glucosidase activity in some yeast strains such as *Saccharomyces cerevisiae* (Spagna *et al*., 2002a) and *Pichia anomala* (Spagna *et al*., 2002b). This phenomenon is similar to our observation that β-glucosidase from *Pichia anomala* MDD24 was actually stimulated by ethanol. According to our result, this may be due to the glycosyl transferase activity of the enzyme (Pemberton *et al*., 1980). The authors showed that ethanol acts as an acceptor for an intermediary "glycosyl" cation and it is more efficient than water because of stronger nucleophillic properties. This result suggests that the *Pichia anomala* MDD24 β-glucosidase would be an advantage when it is added in the final states of alcoholic fermentation (at the highest concentration of ethanol).

The optimum pH of β-glucosidase activity from *Pichia anomala* MDD24 as well as the commercial enzyme was 4.5 which was similar to enzymes from *Aspergillus niger*, *Hanseniaspora uvarum* Y8 (Barbagallo *et al*., 2004) and *Saccharomyces cerevisiae* AL41 (Spagna *et al*., 2002a). At pH 3.5, the average pH found in grape juices and wines, most β-glucosidases from *Zygosaccharomyces bailii* (Gueguen *et al*., 1995), *Candida peltata* Y-6888 (Saha and Bothast, 1996), *Pichia anomala* AL112 (Spagna *et al*., 2002b), *Hanseniaspora uvarum* Y8 and CBS314 (Barbagallo *et al*., 2004), *Candida guilliermondii* V2 and V5 (Rodriguez *et al*., 2007), *Aspergillus oryzae* CBS12559 (Riou *et al*., 1998) exhibited less than 30% of their activities. Alternatively, at this pH, the β-glucosidase activity from *Pichia anomala* MDD24 was still at 35% of maximum activity and the enzyme was physiologically stable at this pH.

The optimum temperature of β-glucosidase activity produced from other microorganisms has been reported between 20 and 40 °C (Rosi *et al*., 1994; Belancic *et al*., 2003). At optimal temperature of winemaking (15 to 20 ° C), the β-glucosidase from *Pichia anomala* MDD24 was observed at up to 40% of maximum activity and was stable at this temperature. In comparison, the commercial enzyme had maximal activity at 60 ° C and kept less than 10% of maximum activity at typical fermentation temperature.

β-Glucosidases have been divided into three groups on the basis of substrate-specificity: aryl-β-glucosidases (which hydrolyze aryl-β-glucosides exclusively), cellobiases (oligosaccharides only) and broad-specificity β-glucosidases (showing activity towards both substrate types), the most commonly observed group in cellulolytic microorganisms (Gueguen *et al*., 1994, 1997). Many β-glucosidases from fungal sources, e.g. *Aspergillus oryzae* (Riou *et al*., 1998), *Melanocarpus* sp. (Kaur *et al*., 2007) and yeast sources, e.g. *Candida entomophila* (Gueguen *et al*., 1994), *Pichia etchellsii* (Wallecha and Mishra, 2003) have been categorized in the broad-specificity glucosidase group. However, the β-glucosidase from *Pichia anomala* MDD24 revealed a specificity on phenyl-glucopyranoside that is aryl-β-glucoside group.

In conclusion, we report the discovery and characterization of an extracellular β-glucosidase from *Pichia anomala* MDD24. Its application in commercial wine production could lead to a more efficient and specific release of aromatic monoterpenes in *Muscat*-type grape juice*s* and wines. While the inhibitory effect of glucose precludes its use in fruit juices, its unique tolerances towards fructose, the main sugar left at the end of the fermentation, as well as ethanol, may make this β-glucosidase superior to currently available technical enzymes.

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