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Purification and Properties of Extracellular β-Glucosidase from the Antarctic Yeast Strain *Cryptococcus Albidus* AL_3

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Abstract

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Extracellular β-glucosidase from the Antarctic yeast strain *Cryptococcus albidus* AL_3 was isolated and purified by ultrafiltration, ion exchange chromatography and gel filtration. The purity of the enzyme preparations obtained was controlled by SDS-PAGE. The enzyme activity after gel filtration was 109 U/mg protein, the pH optimum was 5.0 and the temperature optimum was 45 ^{0}C. This enzyme retained 18 - 20 % activity at 10 ^{0}C. It was inhibited by glucose and activated by ethanol. Some metal ions sach as Cu^{+2}, Mg^{+2} and Mn^{+2} stimulated β-glucosidase activity, but it was inhibited by Hg^{+2} and Pb^{+2}. The enzyme was active against different β-glucosides. The kinetic parameters for p-Nitrophenil-β-D-glucopyranoside were K_m 0.64 mMol and V_{max} - 140 U/mg.

Key words: Antarctic yeast, β-glucosidase, isolation, purification

Introduction

Antarctic yeast are an interesting object of investigation both in relation to the scientific research done on the new ecosystem and its variety of species, and because of their possible application as producers of bioactive substances in biotechnology. In view of the severe living conditions in the Antarctic, the enzymes isolated from that yeast species could be expected to possess different properties of practical value.

The enzyme β-glucosidase (b-glucoside glucohydrolase EC 3.2.1.21) catalyzes the hydrolysis of b-glucoside bonds in different natural compounds and that offers opportunities for its extensive use. This is due to the fact that a great part of plant aromatic substances, flavonoids and other use-

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ful components are in the form of inactive β-glucosides.

Therefore, β-glucosidase is of great significance to the formation of aromatic and flavour properties of fruit and fruit drinks as well as to the essential oils industry (Gueguen et al., 1996; Fernandez et al., 2000; Lomolino et al., 2006).

In nature, the β-glucoside hydrolysis and aroma formation are due to the catalytic action of endogeneous β-glucosidases. These enzymes are characterized by low activity, hence they cannot liberate the whole quantity of linked aromatic compounds (Gueguen et al., 1996). In the last years, this has led to intensive investigations of the application of exogeneous β-glucosidases to essential oil and wine manufacture (Cordonnier et al., 1989). For the purpose, highly active strain producers of β-glucosidase need to be isolated and selected.

There have been reports in scientific literature of research on the biosynthesis, isolation and properties of endo- and exogeneous β-glucosidases produced by different yeast genera. The enzyme is most frequently purified by a combination of ion exchange and gel chromatography. The activity of yeast β-glucosidases varies within the wide range of 8 to 266 U/mg protein. The majority of the enzymes obtained are thermosensitive. Their temperature optimum is usually within the 50 - 55 °C range and the pH optimum varies between 4.0 - 4.5 (Drider et al., 1993a,b; Gueguen et al., 1994; Gueguen et al., 1996; Saha and Bothast, 1996). Extracellular β-glucosidases are more stable than the intracellular ones.

Antarctic yeast strains *Cryptococcus* which produce extracellular β-glucosidase have been isolated and identified by Pavlova et al. (2002). The authors have not come across any data on the biosynthesis of the enzyme by that yeast genus and no Antarctic yeast strains have been reported as its producers. Therefore the investigations of the production and properties of the β-glucosidase by the isolated Antarctic strains of the *Cryptococcus* genus are novel, hence they are of interest from a scientific and practical point of view.

This paper presents the results of the isolation and purification of b-glucosidase produced by *Cryptococcus albidus* AL_3 as well as some of the enzyme properties.

Materials and Methods

Strain, culture and cultivation conditions

The strain *Cryptococcus albidus* AL_3 was isolated from a sample taken from Livingston Island by the Bulgarian Antarctic Expedition. The strain was identified using the yeast classification criteria proposed by Kurteman and Fell (1998) and deposited at the National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria.The culture media and cultivation conditions were described earlier by Pavlova et al. (2002).

Enzyme Assay

The β-glucosidase activity against 4-nitrophenyl-β-glucopyranoside (pNPG) was determined by Blondin et al. (1983). One β-glucosidase activity unit (U) was defined as the quantity

of enzyme required for the hydrolysis of one micromole substrate per min under the above experimental conditions.

Protein assay. Proteins were determined by the method of Lowry et al. (1951).

Enzyme purification

After centrifugation at 4000 g for 10 min the culture medium supernatant was concentrated in an Amicon cell (PM 10 filter).This concentrated supernatant was then eluted on a DEAE-Sephadex A-50 column (26x 400 mm). The elution was performed with a linear gradient of phosphate buffer from 0.25 to 0.5M (pH 5.6). 450 cm^3 phosphate buffer was eluted at 13.2 $cm^3.h^{-1}$ and collected in 10 cm^3 fractions. Fractions No 13 - 16 were combined and concentrated in the Amicon cell (PM 10 filter). The concentrated eluate was then analyzed by gel filtration on a Sephadex G-200 column. The elution rate was 10 $cm^3.h^{-1}$ and the eluate was collected in 5 cm^3 fractions. Fractions No 8-15 were combined and analyzed.

Sodium dodecylsulphate polyacrylamide gel elecrophoresis (SDS-PAGE)

SDS-PAGE was conducted by Laemmli's method (Laemmli, 1970) with a 15% (w/v) acrylamide and 0.4% (w/v) bis-acrylamide in Tris-Glycine buffer (pH 8.4). The enzyme and standards were denatured in the presence of 4% (v/v) β-mercaptoethanol and 4% (w/v) SDS at 100°C for 5 min. Electrophoresis was conducted at room temperature and a 30 mA constant current on vertical slabs. The protein bands were detected with Coomassie Brilliant Blue R-250.

Results and Discussion

Purification of β-glucosidase from C. albidus AL_3

The results for the β-glucosidase activity of the preparations obtained at different stages of purification have been shown in Table 1. As seen from the data in Table 1, the culture medium contained β-glucosidase with activity 2.32 U/mg protein. After ultrafiltration, the enzyme activity increased slightly. After ion exchange and gel chromatography, it reached 12.72 and 109.3 U/mg protein respectively. The results obtained were commensurable with the data reported by other authors and excelled some of them (Drider et al., 1993 a,b; Gueguen et al., 1994; Gueguen et al., 1996).

SDS-PAGE (Figure 1) proved that ion exchange chromatography was not very efficient for the elimination of ballast protein (c). However, after gel chromatography only one color zone was seen. It corresponded to the protein with molecular mass of 100 000±10 000 D (d).

Properties of purified β-glucosidase from C. albidus AL_3.

The β-glucosidase obtained after gel chromatography and concentration in Amicon cell (PM 10 filter) was used for the determination of some of the enzyme properties. For comparison, some characteristics of an unpurified enzyme obtained after concentration of *C. albidus* AL_3 culture medium have been shown.

Table 1

Enzyme activity versus purification steps

Purification step	Total protein, mg	Specific activity, $U.mg^{-1}$	Purification factor	% Total activity
Cultutal supernatant	55	2.32		100
Ultraconcentrate	43.4	2.91	1.25	98.98
DEAE-Sephadex A-50	5	12.72	5.48	49.84
Sephadex G-200	0.18	109.3	45.1	15.41

The Effect of Temperature on Enzyme Activity

As seen from Figure 2, β-glucosidase reached its maximum activity at 45 °C. The enzyme activity quickly declined at 40 °C and 50 °C. Unlike the purified enzyme, the β-glucosidase in the ultra-concentrate was characterized by a higher temperature optimum (50 °C). It retained considerable activity (more than 70 % of the maximum activity) in a wider temperature range: from 40 °C to 55 °C. It is worth noting that at lower temperatures of 10 °C and 20 °C, the enzyme activity remained about 20 % regardless of the purity of the enzyme obtained. This property makes the β-glucosidase from *C. albidus* AL_3 different from the yeast β-glucosidases studied by other authors (Drider et al., 1993 a,b; Gueguen et al., 1994) and is probably due to the Antarctic origin of the strain investigated in the present study.

The Effect of pH on β-glucosidase Activity

The pH effect was determined in citric - phosphate buffers within a 3 to 8 pH range (Figure 3). The pH optimum was at pH 5.0 but at pH 5.5 and 6.0 the β-glucosidase activity reached values close to the maximum activity.

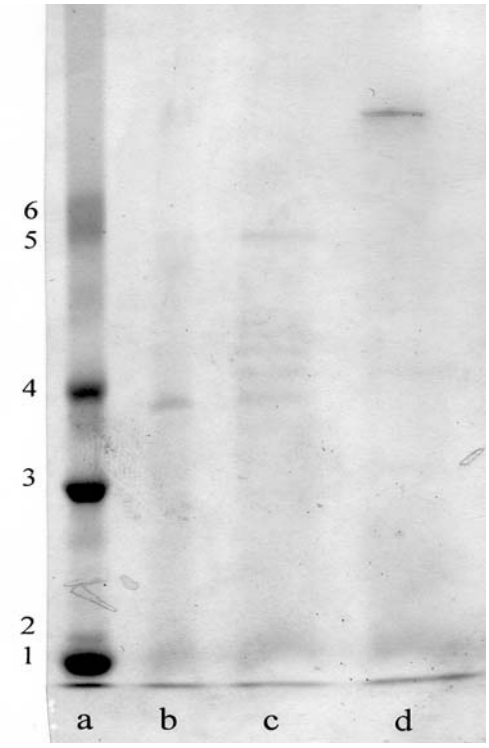


Fig. 1. SDS-PAGE of β - glucosidase from *Cryptococcus albidus* AL_3: a-standards: 1-12.3kDa (Cytochrom C); 2 – 17.3 kDa (Myoglobin); 3 – 30 kDa (Carboanhydrase); 4 – 45 kDa (Ovalbumin); 5 – 66.2 kDa (Albumin); 6 – 78 kDa (Ovotransferin); b - Ultraconcentrate; c - β - glucosidase after DEAE-Sephadex A-50; d - β - glucosidase after Sephadex G-200

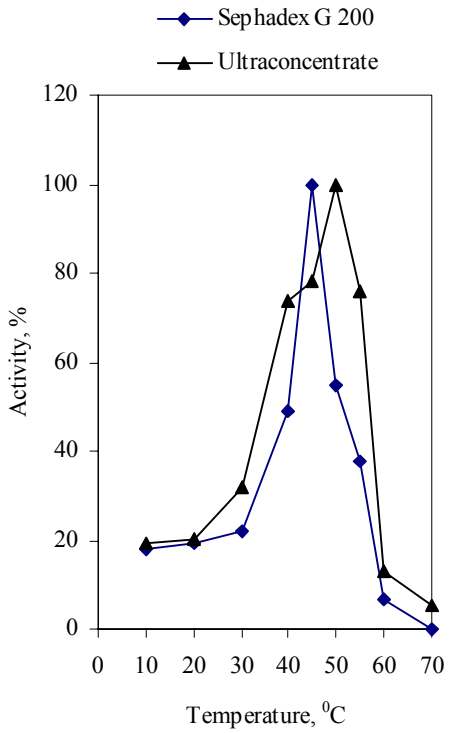


Fig. 2. Effect of temperature on the β - glucosidase activity

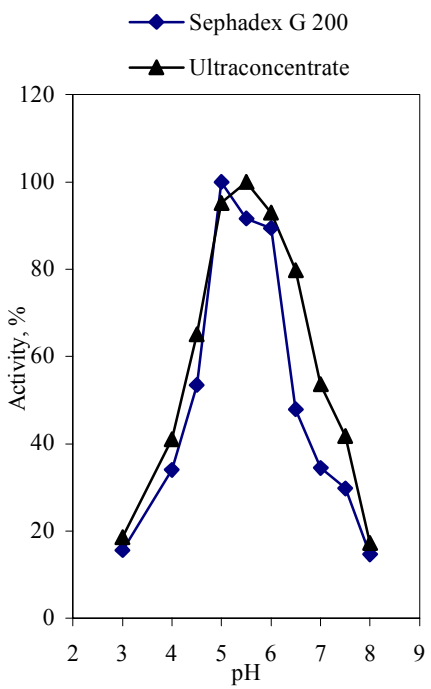


Fig. 3. The Effect of pH on β-glucosidase Activity

Outside that pH range (at 6.0<pH <5.0) the enzyme quickly reduced its activity: it was about 50 % of the maximum value at pH 4.5 and 30 % at pH 4.0. Compared to the purified preparation, the unpurified β-glucosidase was characterized by a pH optimum at pH 5.5 and high activity in the 4.0 - 7.5 pH ranges.

Thermal Denaturation

The thermal denaturation was monitored by incubating the enzyme solution for 10, 20 and 30 minutes at temperatures from 30 °C to 60 °C. The enzyme activity was determined at 30 °C by the method described earlier. As seen from Figure 4, the β-glucosidase produced by *C. albidus* AL_3 retained more than 85 % of its activity after incubation for 30 minutes at 30 ÷ 40 °C. It quickly decreased after thermal treatment at 60 °C. After 30 minutes at 60 °C, the β-glucosidase activity was only 10 % of the maximum value. The enzyme showed relatively good thermal stability at 45 °C and 50 °C: after 30 minutes it retained 55 % of its activity. The results obtained proved that the enzyme investigated by our research group was characterized by higher thermal stability compared to the extracellular β-glucosidases obtained by Drider et al. (1993); Gueguent (1996); Saha and Bothas (1996) and other authors.

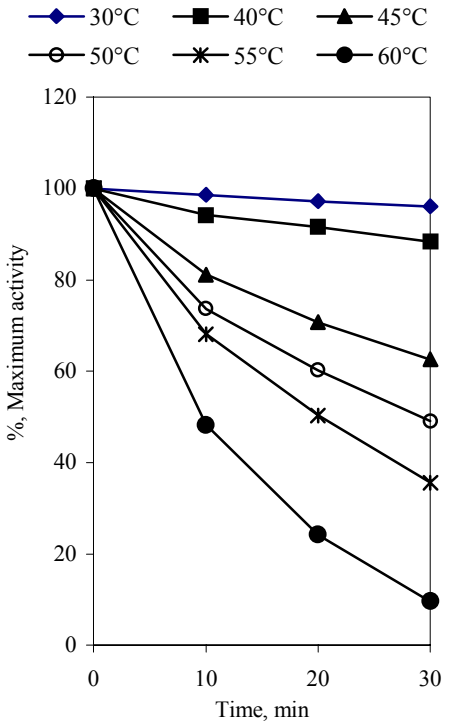


Fig. 4. Thermal denaturation of the β-glucosidase

The Effect of Glucose on the Activity

The β-glucosidases produced by yeast are known to be less sensitive to glucose than plant and fungal β-glucosidases. From a practical point of view it is very important that some of them are glucose-tolerant. The results obtained for the glucose effect on the β-glucosidase activity have been shown in Figure 5. It can be seen that the β-glucosidase produced by *C. albidus* AL_3 was highly sensitive to glucose. When the glucose concentration was increased in the reactivity mixture, the enzyme activity decreased continuously and only 19 % of its maximum value was retained at 5 % glucose concentration.

The Effect of Ethanol on Enzyme Activity

The effect of ethanol on the β-glucosidase activity is of great practical value, especially with regard to its possible application in the winemaking industry. The β-glucosidases described in literature react to ethanol in different ways. Some of them retain their activity, others increase it, and still others are inhibited by ethanol (Dreider et al., 1993b; Gueguen et al., 1994).

As seen from the results represented in Figure 6, the 0.5 to 3.5 M ethanol concentration activated the

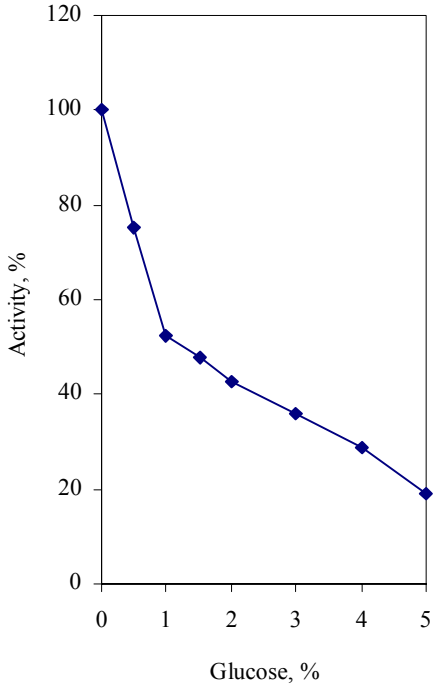


Fig. 5. Effect of glucose on the β- glucosidase activity

enzyme obtained from *C. albidus* AL_3 whereas a higher concentration inhibited it. The β-glucosidase reached its maximum activity when 1 M ethanol was added to the mixture.

The Effect of cations

The effect of various cations at 10^{-2}M was studied. Ca^{2+}, Zn^{2+}, Fe^{2+}, Ag^{+}, Na^{+} have little positive effect on the enzyme. The enzyme was activated by Cu^{2+}, Mg^{2+}, Mn^{2+} (10%, 12%, 29% respectively) and inhibited by 10^{-3} M Hg^{2+} and Pb^{2+} (50% and 23% respectively). The absence of inhibition by the EDTA chelating agent indicated that the enzyme does not have metal co-factors.

Enzyme specificity

The specificity of the β-glucosidase from *C. albidus* AL_3 was studied for six substrates (Table 2). The enzyme exhibited high substrate selectivity against β-glucosides. It did not hydrolyze β-glucosides and o-nitrophenyl -β-D-galactopyranoside.

The K_m and V_{max} values for p-nitrophenil- β-D-glucopyranoside calculated from Lineweaver-Burk plot were 0.64 mMol and 140 U/mg. It was in the same order as most Km and Vmax reported for other β-glucosidases (Blondin et al.,1983; Dreider et al.,1993b).

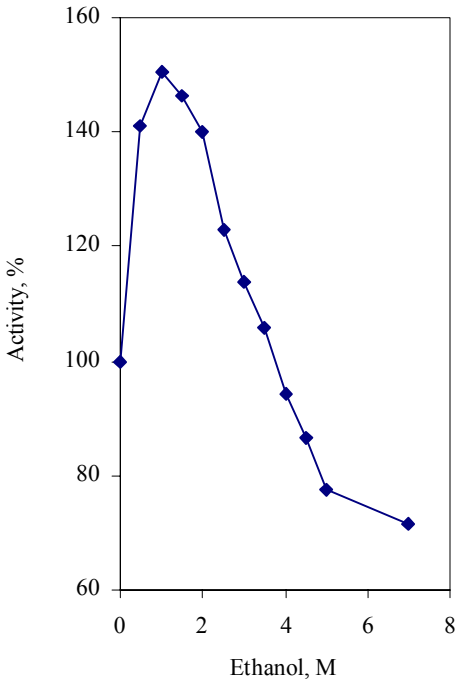


Fig. 6. Effect of ethanol on the β-glucosidase activity

Table 2

Substrate specificity of β-glucosidase from *C. albidus* AL_3

Substrate	Activity, % of max
p-Nitrophenil- β-D-glucopyranoside	100
Methyl- β-D-glucopyranoside	59.11
p-Nitrophenil- α-D-glucopyranoside	0
o-Nitrophenil- β-D-galactopiranoside	0
Cellobiose	6.96
Trehalose	0

Conclusion

In conclusion, the Antarctic strains *C. albidus* AL_3 produced β-glucosidase with a comparatively high activity-2.32 U/mg protein in the culture medium. The temperature optimum of the purified enzyme (45 °C) was lower in comparison with most yeast β-glucosidases isolated by other authors - 55 °C (Drider et al., 1993a; Gueguen et al., 1996) and 60 °C (Gueguen et al., 1994). The relatively high thermal stability of the enzyme, its satisfactory activity at 10 °C and 20 °C and the considerable increase in its activity by 1 M ethanol are of significant practical value. The activation of the enzyme by ethanol remained of presence of glucosiltrans-ferase activity.

References

Blondin, B., R. Ratomahenina, A. Arnaud and P. Galzy, 1983. Purification and properties of the β- glucosidase of yeast capable of fermenting cellobiose to ethanol: Dekkera intermedia. *European J. Appl. Microbiology*, **17**: 1-6.

Cordonnier, R., Y. Gunata, R. Baumes and C. Bayonove, 1989. Recherche d'un materiel enzymatique adapte a l'hydrolyse de precurseurs d'arome de nature glucosidique du raisin. *Connaissances de la vigne et du Vin*, **23**: 7-23.

Drider, D., P. Chemardin, A. Arnaud and P. Galzy, 1993a. Isolation and Characterization of the Exocellular b-glucosidase of Candida cacaoi: Possible Use in Carbohydrates Degradation. *Lebensm. Wiss. u. Technol.*, **26**: 198-204.

Drider, D., P. Pommares, P. Chemardin , A. Arnaud and P. Galzy, 1993b. Purification and Properties of the endocellular b-glucosidase of Candida cacaoi Buckey and Van Uden CBS 2020. *J. Appl. Bacteriology*, **74**: 473-479.

Fernandez, M., J. F. Ubeda and A. I. Briones, 2000. Typing of non-Saccharomyces cerevisiae yeasts with enzymatic activities of interest in wine-making. *Int.J.Food Microbiology*, **59**: 29-36.

Gueguen, Y., P. Chemardin, A. Arnaud and P. Galzy, 1994. Purification and Characterization of the Exocellular b-glucosidase of a new strain of Candida entomophila isolated from fermenting agave (Agave sp.) juice. *Biotechnol. Appl. Biochem.*, **20**: 185-198.

Gueguen, Y., P. Chemardin, G. Janbon, A. Arnaud and P. Galzy, 1996. A Very Efficient β-glucosidase Catalyst for the Hydrolysis of Flavor Precursors of Wines and Fruit Juices. *J. Agric. Food Chem.*, **44**: 2336-2340.

Kurteman, C. and J. Fell, 1998. The Yeasts: Taxonomic Study, 4th ed. *Elsevier Scientific Publishers*, Amsterdam (Netherlands).

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685.

Lomolino, G., F. Zocca, P. Spettoli and A. Lante, 2006. Detection of β- glucosidase and Esterase Activities in Wild Yeast in a Distillery Environment. *J. Inst. Brew.*, **112** (2): 97-100.

Lowry, O., N. Rosebrough, A. Farr and R. Randal, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**: 265-275.

Pavlova, K., G. Angelova, I. Savova, D. Grigorova and L.Kupenov, 2002. Studies of Antarctic yeast for β-glucosidase production. *World J. Microbiol. and Biotechn.*, **18**: 569-573.

Saha, B. and R. Bothast, 1996. Glucose tolerant and thermophilic β-glucosidase from yeast. *Biotechnology Letters*, **18**: 155-158.

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