Biochemistry (Moscow), Vol. 70, No. 12, 2005, pp. 1363-1368. Translated from Biokhimiya, Vol. 70, No. 12, 2005, pp. 1656-1663.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-246, November 20, 2005.

Purification and Characterization of an Intracellular β-Glucosidase from the Methylotrophic Yeast *Pichia pastoris*

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Received August 31, 2004
Revision received September 5, 2005

Abstract—*Pichia pastoris* β-glucosidase was purified to apparent homogeneity by salting out with ammonium sulfate, gel filtration, and ion-exchange chromatography with Q-Sepharose and CM-Sepharose. The enzyme is a tetramer (275 kD) made up of four identical subunits (70 kD). The pH optimum is 7.3, and it is fairly stable in the pH range 5.5-9.5. The temperature optimum is 40°C. The purified β-glucosidase is effectively active on *p*-/*o*-nitrophenyl-β-D-glucopyranosides (*p*-/*o*-NPG) and 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG) with K_m values of 0.12, 0.22, and 0.096 mM and V_{max} values of 10.0, 11.7, and 6.2 μmol/min per mg protein, respectively. It also exhibits different levels of activity against *p*-nitrophenyl-1-thio-β-D-glucopyranoside, cellobiose, gentiobiose, amygdalin, prunasin, salicin, and linamarin. The enzyme is competitively inhibited by gluconolactone, *p*-/*o*-nitrophenyl-β-D-fucopyranosides (*p*-/*o*-NPF), and glucose against *p*-NPG as substrate. *o*-NPF is the most effective inhibitor of the enzyme activity with K_i value of 0.41 mM. The enzyme is more tolerant to glucose inhibition with K_i value of 7.2 mM for *p*-NPG. *Pichia pastoris* has been employed as a host for the functional expression of heterologous β-glucosidases and the risk of high background β-glucosidase activity is discussed.

Key words: β-glucosidase, *Pichia pastoris*, yeast enzyme, purification

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolytic cleavage of β-glycosidic linkage between two glycone residues or that between glucose and an alkyl or aryl aglycone. The enzyme constitutes a major group among glycoside hydrolases that have been isolated from members of all three domains (Eucarya, Archaea, and Bacteria) of living organisms. β-Glucosidases play key roles in a variety of fundamental physiological and biotechnological processes depending on the nature and diversity of the glycone or aglycone moiety of their substrates. For instance, plant β-glucosidases have been reported to be involved in phytohormone activation for growth and development [1], chemical defense against pests [2-4], lignification [5], β-glucan synthesis during cell wall development, and cell wall degradation in the endosperm during germination [6, 7], and beverage quality enhancement [8]. Among the mammalian β-glucosidases, the human acid β-glucosidase commonly known as glucocerebrosidase catalyzes the degradation of glucosylceramide in the lysosome. The deficiency of this enzyme leads to the inherited Gaucher's disease [9]. β-Glucosidases in cellulolytic microorganisms have recently been the focus of much research since cellulose is the most abundant substrate on earth and is very likely to be an important renewable energy resource in the future [10-12]. Direct conversion of cellulose to ethanol has been demonstrated in recombinant *Saccharomyces cerevisiae* expressing heterologous genes of three cellulolytic enzymes endo-/exoglucanase and β-glucosidase [13].

The expression system of the methylotrophic yeast *Pichia pastoris* has been extensively employed recently to express several β-glucosidases from different organisms [14-16]. The present paper describes for the first time the purification and characterization of an intracellular β-glucosidase from *P. pastoris*.

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0006-2979/05/7012-1363

MATERIALS AND METHODS

Materials. Prunasin (D-mandelonitrile β-D-glucopyranoside) was obtained from Extrasynthese (France) while other substrates, Sephacryl S-300 HR, Q-Sepharose, and CM-Sepharose were purchased from Sigma-Aldrich (Germany). All other chemicals were of the best available grade.

Yeast strain, medium, and culture conditions. The wild-type yeast *Pichia pastoris* strain X-33 was obtained from Invitrogen (The Netherlands). The cells were cultured to generate biomass with buffered glycerol-complex medium (BMGY) containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 0.34% yeast nitrogen base, 1% ammonium sulfate, $4 \cdot 10^{-5}$% biotin, and 1% glycerol in a rotary shaking incubator at 30°C, 250 rpm, and grown until the culture reached an $A_{600} \sim 6$, approximately 20 h. The cells were harvested by centrifuging at 3000*g* for 5 min at room temperature. The cell pellet was resuspended in BMM (buffered minimal methanol) medium (100 mM potassium phosphate, pH 6.0, 0.34% yeast nitrogen base, 1% ammonium sulfate, $4 \cdot 10^{-5}$% biotin, and 0.5% methanol) using 2× of the original culture volume and returned to the shaking incubator for expression. Methanol (100%) was added to a final concentration of 0.5% every 24 h to maintain induction. The culture was monitored for expression at 0, 12, 24, 36, 48, and 72 h time points by spectrophotometric activity assay. During large scale expression, cells were collected after 48 h by centrifuging at 12,000*g* for 10 min at 4°C, and washed once in breaking buffer (BB) consisting of 50 mM potassium phosphate, pH 7.3, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 5% glycerol.

Preparation of cell lysate. The washed cells were resuspended to an A_{600} of 7.5 in BB. An equal volume of 0.5 mm glass beads estimated by displacement was added, and the mixture was vortexed for 30 sec and then incubated on ice for 30 sec. This step was repeated nine more times. The suspension was centrifuged at 12,000*g* for 30 min at 4°C, and the supernatant was taken as crude extract for further purification.

Purification of β-glucosidase. All steps were performed at 4°C unless otherwise stated. The crude enzymic extract was treated with solid ammonium sulfate to obtain the 55 to 90% fraction after centrifuging at 12,000*g* for 30 min. The precipitate was dissolved in 50 mM potassium phosphate buffer, pH 7.3. The enzyme solution was loaded onto a Sephacryl S-300 HR column (1.5 × 80 cm) pre-equilibrated and eluted with 50 mM potassium phosphate buffer, pH 7.3, at room temperature. The flow rate was 20 ml/h and 1.5-ml fractions were collected. Fractions with β-glucosidase activity were pooled and then applied on a Q-Sepharose ion-exchange column (1 × 14 cm) pre-equilibrated with 50 mM potassium phosphate buffer, pH 7.3. The enzyme was eluted with a linear gradient of 0.0-1.0 M KCl in the same buffer at a flow rate of 30 ml/h and 1-ml fractions were collected. Fractions showing higher activity were pooled and dialyzed overnight against 50 mM potassium phosphate buffer, pH 6.0. The desalted enzyme preparation was applied to a CM-Sepharose ion-exchange column (1 × 14 cm) pre-equilibrated with 50 mM potassium phosphate buffer, pH 6.0, and the effluent was collected in 1-ml fractions. The enzyme was eluted with a linear 0.0 to 1.0 M NaCl gradient in the same buffer at a flow rate of 30 ml/h, and 1-ml fractions were collected. The proteins containing the highest β-glucosidase activity and mostly present in the unbound fractions were combined and concentrated by ultrafiltration (Amicon Ultra-15; Millipore, USA). The concentrated enzyme solution was used as purified β-glucosidase for subsequent studies after confirming homogeneity by gel electrophoresis.

β-Glucosidase assays and protein determinations. During enzyme extraction and purification, β-glucosidase activity was routinely determined using *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) and *o*-nitrophenyl-β-D-glucopyranoside (*o*-NPG) as substrates. Appropriately diluted 70 μl of enzyme solution and 70 μl of substrate were mixed in the wells of a 96-well microtiter plate in quadruplicate. After incubation at 37°C for 30 min, the reaction was stopped by adding 70 μl of 0.5 M Na_2CO_3, and the color that developed as a result of *p*-/*o*-nitrophenol liberation was measured at 410 nm. One unit of β-glucosidase activity was defined as the amount of enzyme that hydrolyzes the substrate to release 1 μmol of glucose per min in the reaction mixture under these assay conditions. When the substrate used did not contain *p*-/*o*-nitrophenol, the enzyme activity was determined by the coupling glucose oxidase-peroxidase assay (Sigma, USA) procedure. Zymogram assays were carried out for detection of β-glucosidase activity after native PAGE under non-denaturing conditions using 6-bromo-2-naphthyl-β-D-glucoside (6-BNG) in Fast Blue BB salt, and alternatively 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG) as a substrate.

Protein concentrations were determined [17] using bovine serum albumin (BSA) as a standard.

Polyacrylamide gel electrophoresis (PAGE). For SDS-PAGE, protein samples were fractionated on 12% SDS-PAGE gels [18] using a Minigel system (Bio-Rad Laboratories, USA). Gels were fixed, stained with Coomassie brilliant blue R-250 (Sigma), and destained using standard methods to detect protein bands. When detection of β-glucosidase activity was required in a non-denaturing electrophoresis process, the enzyme solutions were loaded onto native 6% polyacrylamide gel. After electrophoresis, the gel was equilibrated in two changes of 50 mM potassium phosphate buffer, pH 7.3, for 15 min each. Freshly mixed substrate-coupling dye solution (0.1 g 6-BNG in 1 ml dimethylformamide and 0.15 g Fast Blue BB salt in 200 ml 50 mM potassium phosphate buffer, pH 7.3) was added onto the gel and incubated at

37°C for 3 h to visualize the color development at the site of enzyme activity. When β-glucosidase in native polyacrylamide gel was incubated with 4-MUG for 15 min at 37°C, the released methylumbelliferone was observed and photographed under UV light.

Molecular weight determination. The molecular weight of the native enzyme was determined using a Sephacryl S-300 HR column (1.5 × 80 cm) eluted at a flow rate of 30 ml/h with 50 mM potassium phosphate buffer, pH 7.3, containing 0.15 M NaCl. The column was calibrated with carbonic anhydrase (29 kD), bovine serum albumin (66 kD), β-amylase (200 kD), apoferritin (443 kD), and thyroglobulin (669 kD). The subunit molecular weight was determined by SDS-PAGE.

Determination of the pH optimum and stability. The effect of pH on β-glucosidase activity was determined using citrate-phosphate, phosphate, and glycine-NaOH buffers for the pH ranges of 3.0-6.5, 6.0-8.5, and 8.5-10.5, respectively. For determining the profile of pH stability, samples of enzyme solution were incubated in 25 mM citrate-phosphate buffer (pH 3.0-6.5), 25 mM phosphate buffer (6.0-8.5), and 25 mM glycine-NaOH buffer (8.5-11.0) at 40°C for 2 h. The solutions were diluted 10 times with 100 mM phosphate buffer, pH 7.3 (optimum for the enzyme), and assayed for the residual activity using 5 mM *p*-NPG as substrate in 100 mM phosphate buffer, pH 7.3.

Determination of temperature optimum and thermal stability. For temperature optimum determination, the enzyme and substrate *p*-NPG solution mixtures were incubated in the temperature range 4-80°C for 30 min, and the residual activity was measured. For measuring thermostability, the enzyme was first incubated at different temperatures (4-100°C) in 50 mM potassium phosphate buffer, pH 7.3, in the absence of substrate for 10 min. The activity was subsequently assayed at 37°C as described above.

Kinetic parameters. Various final concentrations of *p*-NPG (0.04-5 mM), *o*-NPG (0.04-5 mM), and 4-MUG (0.015-1 mM) were used to estimate the kinetic parameters K_m, k_{cat}, and V_{max}. Inhibition experiments were performed using *p*-NPG as substrate at concentrations of 1 to 10 K_m with different final concentrations of gluconolactone (0.1-1.5 mM), *p*-nitrophenyl-β-D-fucopyranoside (*p*-NPF) (0.1-1.5 mM), *o*-nitrophenyl-β-D-fucopyranoside (*o*-NPF) (0.1-1.5 mM), and glucose (1-15 mM) as inhibitors. The double reciprocal Lineweaver–Burk plot was used to calculate the parameters.

RESULTS AND DISCUSSION

Since the highest level of specific β-glucosidase activity from *P. pastoris* was obtained in 45-48 h of expression, the cells were harvested after 48 h and disrupted with glass beads. The supernatant fluid of the disrupted cells following centrifugation (at 12,000*g* for 30 min) was taken as crude enzymic extract for further purification. Upon fractionation of the β-glucosidase active fractions with ammonium sulfate, 80% of the activity was obtained in the fraction saturated with 55-90% ammonium sulfate. After gel filtration chromatography on a Sephacryl S-300 HR column, the enzyme was found in fractions 44-74 and pooled. Anion-exchange chromatography of the combined active fraction on a Q-Sepharose column removed most of the contaminants and 92% of the activity from the previous step was retained. When the eluted active fractions from Q-Sepharose column were pooled and applied to a CM-Sepharose column, all of the remaining contaminants bound while β-glucosidase did not. The effluent containing β-glucosidase activity was concentrated by ultrafiltration. The enzyme was purified 99.5-fold to homogeneity with an overall enzyme yield of 25.3% and a specific activity of 10.95 U/mg protein (Table 1). Only one active form of the enzyme was detected during the purification procedures. Multiple forms of β-glucosidase have been found in a variety of yeasts [19, 20].

SDS-PAGE analysis of the purified enzyme showed the presence of a single band when stained with Coomassie brilliant blue (Fig. 1a). The molecular weight of the native β-glucosidase estimated by gel filtration on a Sephacryl S-300 HR column was 275 kD, and by SDS-PAGE analysis it was about 70 kD (Fig. 1a), suggesting the enzyme is a tetramer built of four identical subunits. The subunit molecular mass of *P. pastoris* β-glucosidase is similar to the subunits of polymeric β-glucosidases from various yeast sources [21-24].

In order to confirm the activity data from spectrophotometric assays, native PAGE zymogram assays were performed. The zymogram profiles were developed on gels that yielded a zone of β-glucosidase activity of identical electrophoretic mobility both with the fluorescent substrate 4-MUG (Fig. 1b) and a chromogenic substrate 6-BNG (Fig. 1c).

The pH optimum for β-glucosidase activity was 7.3 (Fig. 2), and the enzyme exhibited 71 and 75% activities at pH 6.0 and 8.0, respectively. This pH optimum is slightly higher than that of other yeast β-glucosidases,

Table 1. Purification of β-glucosidase from *P. pastoris*

Step	Total protein, mg	Specific activity, U/mg	Yield, %
Crude extract	170	0.11	100.0
Ammonium sulfate	43.1	0.37	79.8
Sephacryl S-300 HR	14.2	0.85	60.5
Q-Sepharose	6.2	1.8	56.0
CM-Sepharose	0.46	11.0	25.3

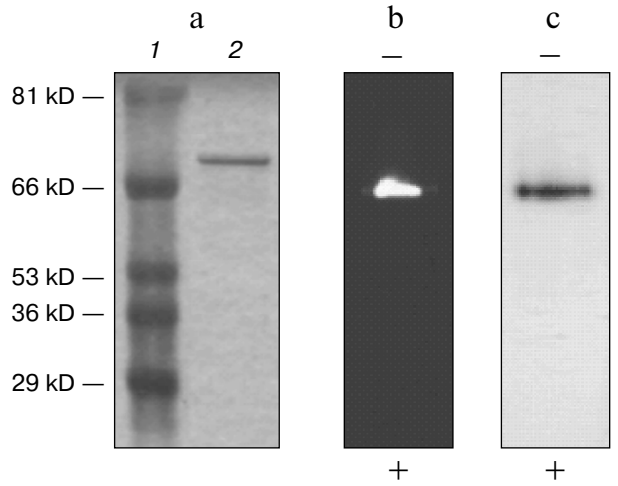

Fig. 1. a) SDS-PAGE of purified β-glucosidase from *P. pastoris*. The enzyme was electrophoresed at pH 8.3 on a 12% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Lanes: *1*) molecular weight standards (creatine phosphokinase, 81 kD; bovine serum albumin, 66 kD; L-glutamate dehydrogenase, 53 kD; glyceraldehyde-3P-dehydrogenase, 36 kD; carbonic anhydrase, 29 kD); *2*) purified β-glucosidase. b, c) Native PAGE (6%) gel zymograms of purified β-glucosidase from *P. pastoris* developed with the fluorogenic substrate 4-MUG (b) and with the chromogenic substrate 6-BNG (c) as described in "Materials and Methods".

since the optimal pH values of β-glucosidases from various yeast sources range between 4.0 and 7.0. However, among the reported β-glucosidases, only the *Pichia etchellsii* enzyme has been reported to have a pH optimum of 7.0 only for cellobiose, and most of the other yeast β-glucosidases have optimal pH values between 5.0 and 6.5 [22-27]. The *P. pastoris* β-glucosidase was fairly stable in the pH range of 5.5-9.5, retaining over 97% activity. The enzyme, however, was shown to be very sensitive to pH below 5.5 since it lost its activity at pH 5.0 after 2 h at 40°C (Fig. 2); conversely, it was found very stable under neutral and alkaline pH since it retained up to 45% of its activity at pH 11.0 under the same conditions (2 h at 40°C). This pH range property of the enzyme for stability is similar to two β-glucosidases of *P. etchellsii* [20] and broader than the pH stability ranges of β-glucosidases from other yeast sources [27-29].

The enzyme displayed maximal activity at 40°C (Fig. 3). Similar temperature optima of β-glucosidases have been reported from several yeasts, such as *Pichia guilliermondii*, *P. nakazawae*, *Candida shehatae*, *C. dendronema*, *Debaryomyces vanrijiae*, and *S. cerevisiae* wine strain [25, 28, 29]. Thermostability of the enzyme at different temperatures was monitored by measuring its activity at 37°C. The enzyme in 50 mM potassium phosphate buffer, pH 7.3, was fairly stable at temperatures up to 53°C for 10 min. It was completely inactivated upon incubation at 60°C for 10 min (Fig. 3).

The substrate specificity of β-glucosidase was determined towards various artificial and natural substrates. The enzyme exhibited different levels of activity against alkyl-glucopyranosides, most aryl-glucopyranosides, and other β-linked disaccharides (Table 2). It was effectively active on *p*-NPG, *o*-NPG, and 4-MUG with relative activities of 1, 1.16, and 0.63, respectively. The aryl-glu-

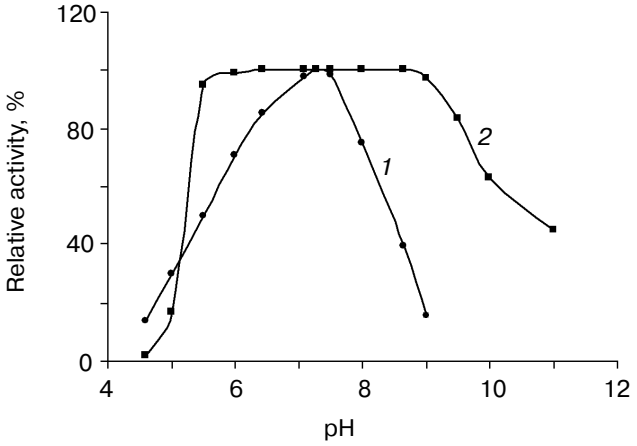

Fig. 2. Effect of pH on activity (*1*) and stability (*2*) of purified β-glucosidase from *P. pastoris*. The influence of varying pH values on the activity was determined using citrate-phosphate, phosphate, and glycine-NaOH buffers for the pH ranges of 3.0-6.5, 6.0-8.5, and 8.5-10.5, respectively. For stability, the enzyme solutions in 25 mM buffers at various pH values were incubated for 2 h at 40°C. After adjustment of pH, the residual activity was assayed by the standard method.

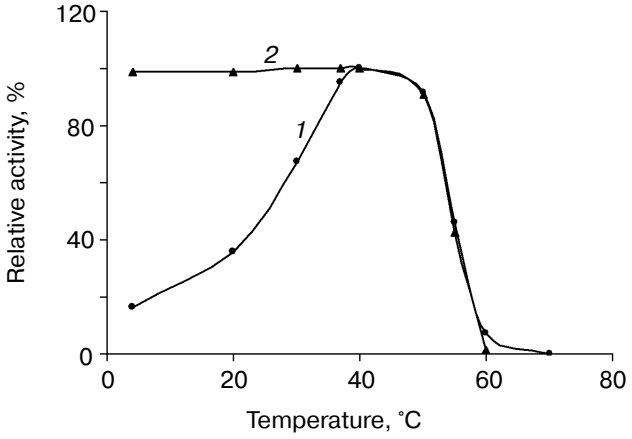

Fig. 3. Effect of temperature on activity (*1*) and stability (*2*) of purified β-glucosidase from *P. pastoris*. For temperature optimum determination, the enzyme and substrate *p*-NPG solution mixtures were incubated in the temperature range 4-80°C for 30 min, and the residual activity was measured. For determining thermostability, the enzyme was first incubated at different temperatures (4-100°C) in 50 mM potassium phosphate buffer, pH 7.3, in the absence of substrate for 10 min. The activity was subsequently assayed at 37°C as described in "Materials and Methods".

copyranoside salicin was hydrolyzed as efficiently as gentiobiose, cellobiose, and an alkyl-glucopyranoside amygdalin. Similar activity rates for these substrates have been reported for the β-glucosidases from the yeast *P. etchellsii* except for salicin, which was hydrolyzed with higher ratio by β-glucosidase-I of *P. etchellsii* [20]. Salicin was also reported to be hydrolyzed effectively by the enzymes of *Candida sake* [23], *C. molischiana* [24], *C. peltata* [27], and *C. cacaoi* [30]. Another naturally occurring β-glucopyranoside, arbutin, a derivative of hydroquinone bound to glucose, was not hydrolyzed by the enzyme of *P. pastoris*. Higher specificity was observed for naturally occurring cyanogenic alkyl-β-glucopyranosides prunasin and linamarin than for amygdalin. However, hydrolysis of these cyanogenic glucopyranosides by *P. pastoris* β-glucosidase was interesting as it is a property usually associated with plant β-glucosidases. *p*-Nitrophenyl-1-thio-β-D-glucopyranoside was also hydrolyzed at 30.9% of that of *p*-NPG, although an S-glucopyranoside is not expected to be hydrolyzed with such efficiency by the activity of a β-O-glucosidase. As some other yeast β-glucosidases have been reported to have little or no activity on *p*-NP-fucopyranoside and *p*-NP-galactopyranoside [22, 23, 27], the *P. pastoris* enzyme also had no activity on aryl-β-glucopyranosides *p*-NP-mannopyranoside, *p*-/*o*-NP-fucopyranosides, and *p*-/*o*-NP-galactopyranosides.

The reaction kinetics of the purified β-glucosidase were determined from Lineweaver–Burk plots with *p*-NPG, *o*-NPG, and 4-MUG as substrates under the explained assay conditions. The enzyme had K_m values of 0.12, 0.22, and 0.096 mM and V_{max} values of 10.0, 11.7, and 6.2 μmol/min per mg protein for the hydrolysis of *p*-NPG, *o*-NPG, and 4-MUG, respectively (Table 3). Affinity of the enzyme for *p*-NPG was considerably higher than those reported for *P. etchellsii* [22], *C. sake* [23], *C. peltata* [27], *Debaryomyces vanrijiae* [28], and *C. cacaoi* [30] β-glucosidases. Catalytic turnovers on *p*-NPG and *o*-NPG were nearly the same, while on 4-MUG it was almost half of that on *o*-NPG.

The inhibition kinetic experiments of the enzyme were performed using *p*-NPG as substrate and gluconolactone, *p*-NPF, *o*-NPF, and glucose as inhibitors. Table 4 shows that the enzyme was competitively inhibited by all the inhibitors investigated. *o*-NPF was the most effective inhibitor of the enzymatic activity with K_i value of 0.41 mM. Inhibition constant values for gluconolactone and *p*-NPF were quite similar. The inhibition kinetics of β-glucosidases from several yeast sources have been extensively studied using glucose as an inhibitor, since glucose inhibition of β-glucosidases undesirable if the enzymatic hydrolysis of cellulose is performed as an industrial process. Highly glucose tolerant β-glucosidases have been reported from yeasts *C. sake*, *P. etchellsii*, *D. vanrijiae*, and *C. peltata* with K_i values of 0.2, 0.3, 0.44, and 1.4 M, respectively [22, 23, 27, 28]. According to Saha and Bothast [25], β-glucosidase activities from

Table 2. Relative activity of *P. pastoris* β-glucosidase on various substrates

Substrate	Relative activity, %
p-Nitrophenyl β-D-glucopyranoside (*p*-NPG)	100
p-Nitrophenyl 1-thio-β-D-glucopyranoside	31
p-Nitrophenyl β-D-fucopyranoside	0
p-Nitrophenyl β-D-mannopyranoside	0
p-Nitrophenyl β-D-galactopyranoside	0
o-Nitrophenyl β-D-glucopyranoside (*o*-NPG)	116
o-Nitrophenyl β-D-galactopyranoside	0
o-Nitrophenyl β-D-fucopyranoside	0
4-Methylumbelliferyl β-D-glucopyranoside (4-MUG)	63
n-Octyl-β-D-glucopyranoside	28
n-Dodecyl-β-D-glucopyranoside	26
D-(+)-Cellobiose	12.9
β-Gentiobiose	6.2
Amygdalin	11.3
Prunasin	27
Arbutin	0
Salicin	10.4
Linamarin	34

Note: Purified β-glucosidase was incubated at its optimum pH (7.3) with potential substrates provided at 10 mM final concentrations. Enzyme activity was determined by measuring the rate of *p*-NPG/*o*-NPG production at 410 nm with subsequent use of respective standard curves. For the substrates that do not contain *p*-/*o*-nitrophenol, the enzyme activity was determined by the coupling glucose oxidase-peroxidase assay procedure. Reaction rates are expressed here as a percentage of that observed with *p*-NPG.

Table 3. Kinetic parameters of *P. pastoris* β-glucosidase

Substrate	K_m, mM	k_{cat}, sec^{-1}
p-NPG	0.12 ± 0.01	11.6 ± 0.4
o-NPG	0.22 ± 0.02	13.7 ± 0.6
4-MUG	0.096 ± 0.01	7.2 ± 0.08

Table 4. Competitive inhibition of *P. pastoris* β-glucosidase

Substrate	Inhibitor, K_i (mM)			
	gluconolactone	*p*-NPF	*o*-NPF	glucose
p-NPG	0.81 ± 0.04	0.72 ± 0.02	0.41 ± 0.02	7.2 ± 0.55

some yeast strains were even stimulated by glucose. However, most microbial β-glucosidases are strongly inhibited by glucose with the inhibition constants ranging from 0.6 to 10 mM [8]. *Pichia pastoris* β-glucosidase activity was also inhibited competitively by glucose with K_i value of 7.2 mM towards *p*-NPG as substrate. This inhibition constant value is similar to those of β-glucosidases from several yeast sources [8, 24, 30].

In conclusion, the present study has revealed the isolation and characterization of an intracellular β-glucosidase from the yeast *P. pastoris* for the first time. *Pichia pastoris* has been extensively used recently as a popular host for functional expression of a broad spectrum of heterologous proteins most of which are eukaryotic, since it utilizes most of the post-translational modification pathways typically associated with eukaryotes and is easy to use [31, 32]. Furthermore, the *P. pastoris* expression system has been successfully employed recently to express several β-glucosidases such as human liver β-glucosidase, cyanogenic β-glucosidases amygdalin hydrolase, and prunasin hydrolase from the plant *Prunus serotina*, and a β-glucosidase from the fungus *Phanerochaete chrysosporium* [14-16]. Moreover, it has been reported that *P. pastoris* is being used as a host for the expression of over 40 putative β-glucosidases of *Arabidopsis thaliana* [33, 34]. Accordingly, these reports also emphasize the importance of the present work describing considerably high background β-glucosidase activity whenever *P. pastoris* is employed as a host for the functional expression of heterologous β-glucosidases.

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