Kinetic Characterization of a β-Glucosidase from a Yeast, Candida wickerhamii*

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The extracytoplasmic, cell-bound β-1,4-glucosidase of Candida wickerhamii was characterized kinetically. The enzyme was found to produce glucose from cellobiose and cellodextrins (degree of polymerization from three to six) by catalyzing the removal of the terminal glucose moiety from the nonreducing end of these β-glucans. The $K_m$ values for the series, cellobiose through cellohexaose, were 210.7, 106.6, 106.3, 105.9, and 79.8 mM, respectively, whereas the $k_{cat}$ values were 14.79, 13.24, 13.78, 15.13, and 7.66 μmol of glucose·min$^{-1}$·mg$^{-1}$ of protein, respectively. A computer program was developed to estimate the integrated rate equation. When the above kinetic constants were used in the computer model, the predicted rates of glucose formation agreed well with the experimental data.

Saccharomyces cerevisiae, which is unable to ferment cellobiose or cellodextrins, ferments glucose about twice as fast as C. wickerhamii. If S. cerevisiae is cultured on cellobiose or cellodextrins and the purified C. wickerhamii β-glucosidase is added to the S. cerevisiae culture at levels that mimic the production of β-glucosidase by a C. wickerhamii culture with time, the two cultures produce ethanol at equivalent rates. This suggests that the rate-limiting step in the fermentation of cellobiose/cellodextrins by C. wickerhamii is the production of β-glucosidase.

Cellulose, a homopolymer of D-glucose, is the most abundant carbohydrate available from plant biomass. It composes about 40% of the dry weight of most plants and is estimated that about 40 × 10$^{10}$ tons/year are synthesized via photosynthesis (1). As such, it is an attractive substrate to try to utilize as a renewable source of fuels and chemicals.

The enzymatic saccharification of cellulosic materials to D-glucose is known to require the synergistic action of three classes of enzymes: 1,4-β-D-glucan 4-glucanohydrolase (endo-1,4-β-cellulase; EC 3.2.1.4), 1,4-β-D-glucan glucohydrolase and 1,4-β-D-glucan cellobiohydrolase (exo-1,4-β-glucosidase and exo-1,4-β-d-cellobiohydrolase; EC 3.2.1.74 and EC 3.2.1.91, respectively), and 1,4-β-glucosidase (β-glucosidase; EC 3.2.1.21). Endo-1,4-β-cellulases act at random on cellulose, whereas exo-1,4-β-glucosidases release either glucose or cellobiose from the nonreducing ends of β-glucan substrates. The third class of enzymes, β-glucosidases, act to liberate D-glucose units from cellobiose, cellodextrins, and other glucosides (1–3). When Trichoderma reesei Simmons crude culture filtrates are used to saccharify cellulose, the rate of saccharification decreases rapidly because of glucose inhibition of β-glucosidase (4) and subsequent accumulation of cellobiose, resulting in the inhibition of exoglucanase. Cellobiose has been shown to be a more potent inhibitor of cellulase than is glucose (5). When T. reesei cellulase preparations are supplemented with fungal culture filtrates that are rich in β-glucosidase, the amount of glucose produced is increased significantly (4, 6). Thus, β-glucosidase not only generates glucose from cellobiose, it also reduces cellobiose inhibition, allowing the cellulases to function more efficiently.

Candida wickerhamii (syn. Torulopsis wickerhamii) was the first yeast shown to ferment soluble cellodextrins to ethanol (7). To date, only one other yeast, Candida molischiana (syn. Torulopsis molischiana) has been reported to ferment cellodextrins to ethanol (8, 9). Candida guilliermondii has been shown to metabolize aerobically, but not ferment, soluble cellodextrins (9). In all of these yeasts, the ability to metabolize soluble cellodextrins appears to be correlated with the biosynthesis of an extracytoplasmic β-glucosidase (8–11).

C. wickerhamii appears to produce at least two, if not three, distinct β-glucosidases: a cytoplasmic, a secreted, and an extracytoplasmic, cell-bound enzyme. Only the last two enzymes can utilize cellodextrins as substrates. Each of the enzymes has been purified and partially characterized (12–14); however, only the secreted enzyme has been kinetically characterized using soluble cellodextrins as substrates (13).

Since it was shown previously that under certain growth conditions more than 85% of the β-glucosidase activity detected is the cell-associated, extracytoplasmic enzyme (14), this purified enzyme was further characterized kinetically using soluble cellodextrins as substrates. The results indicated that this enzyme hydrolyzes glucose units from the nonreducing end of cellodextrins with a degree of polymerization (DP) $\leq 6$. No significant differences were found in the relative binding affinities ($K_m/K_a$) of the enzyme with the various cellodextrins, suggesting that it is an enzyme that is different from the secreted one described by Himmel et al. (13). A computer program was developed which accurately described the reaction kinetics of the C. wickerhamii β-glucosidase when individual cellodextrins or a mixture of cellodextrins were used as substrates. Finally, results are presented which indicate that the rate-limiting factor in the fermentation of cellodextrins of DP $\geq 2$ by C. wickerhamii is the amount of β-glucosidase produced.

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1The abbreviations used are: DP, degree of polymerization; pNPG, p-nitrophenyl β-D-glucopyranoside; HPLC, high performance liquid chromatography.
C. wickerhamii Cellodextrin Kinetics

Materials and Methods

Source of Yeasts and Fermentation Conditions—The basal growth medium (YM) consisted of 0.5% peptone, 0.3% yeast extract, and 0.5% malt extract. The basal medium plus carbohydrate source was adjusted to pH 4.5 with HCl prior to autoclaving. Inocula preparation and fermentation conditions were identical to those described previously (9, 10, 19).

In the experiment in which the C. wickerhamii β-glucosidase was added to the *Sarcosporomas cerevisiae* fermentations (results presented in Fig. 1), the amount of β-glucosidase produced in a C. wickerhamii fermentation was quantified at 8-h intervals utilizing pnitrophenyl β-D-glucopyranoside (pNPG) as substrate. The *S. cerevisiae* fermentation was initiated 8 h after the *C. wickerhamii* fermentation, and purified β-glucosidase was added to the *S. cerevisiae* culture such that it contained the average amount of β-glucosidase produced in the *C. wickerhamii* culture per each 8-h time interval.

*C. wickerhamii* (Capriotti) Meyer et Yarrow NRRL Y-2565 and *S. cerevisiae* Hansen NRRL Y-2034 were obtained from the Agriculture Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL. *Clostridium thermocellum* ATCC 27405 was obtained from the American Type Culture Collection, Rockville, MD.

Cellodextrins and Chemicals—A mixture of cellodextrins was prepared as described previously (7, 18). Individual cellodextrins were purified from the cellodextrin mixture by charcoal-Celite chromatography as described previously (16) and crystallized from ethanol. Cellodextrins specifically radiolabeled in the nonreducing glucose moiety were prepared by incubating α-β-[14C]glucose 1-phosphate with the G₃ cellodextrin and a crude enzyme preparation from *C. thermocellum* that was enriched for cellodextrin phosphorylase (17, 18). This resulted in the formation of the specifically radiolabeled G₃ cellodextrin. The radiolabeled cellodextrins were purified by charcoal-Celite chromatography as above (16). From high performance liquid chromatography (HPLC) analysis, the purified cellodextrins were all greater than 95% pure, and the radiolabeled cellodextrins were greater than 99% pure. Glucose, yeast extract, malt extract, and peptone were purchased from Difco Laboratories. Cellulose (Sigma, type 100), β-D-(+)-cellobiose, Tris, pNPG, and the glucose detection kit, which was based upon the glucose oxidase-peroxidase reaction, were purchased from Sigma. α-β-[14C(U)]Glucose 1-phosphate (>70 kCi/mmol) was purchased from Du Pont-New England Nuclear. All other chemicals were purchased from Fisher Scientific Co.

Enzyme Kinetics—The C. wickerhamii β-glucosidase was purified to homogeneity, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described previously (14). Soluble cellodextrins (cellotetraose through cellohexaose) were analyzed kinetically as substrates for the β-glucosidase. Prior to initiation of the reactions, the enzyme was incubated at 0 °C for 30 min in the presence of 1.0 mM dithiothreitol. All substrates were boiled briefly and cooled to 28 °C with stirring to ensure solubility. The reactions (0.1 ml in 50 mM sodium acetate, pH 4.75) were initiated by the addition of enzyme. After 30 min, the reactions were quenched at 0 °C, and the amount of glucose produced was determined by the addition of 4.0 ml of the glucose oxidase-peroxidase reagent. The kinetic constants, *Km* and *Vmax*, were determined from linear regression analysis of Lineweaver-Burk plots. The values presented in Table I represent the average of at least three independent determinations. Preliminary experiments were performed to ensure that the reactions were linear with respect to time and enzyme concentration.

Carbohydrate and Ethanol Analysis and β-Glucosidase Assay—Carbohydrates were analyzed by HPLC on a Waters chromatograph fitted with a reverse-phase amino column (Regis, Morton Grove, IL). Acetonitrile/water (70:30) was used as the mobile phase. Carbohydrates were detected with a Waters R401 refractive index monitor (9). When radiolabeled cellodextrins were chromatographed, 0.5-ml fractions were collected, mixed with ACS II scintillation fluid (American Corp.), and counted in a Beckman LS 7800 liquid scintillation counter. Ethanol was quantified by gas-liquid chromatography with a Packard model 402 GLC using a Porapak Q column at 150 °C. Methods described previously (10, 20) were used to quantitate β-glucosidase activity using pNPG as substrate.

Computer Program—Once the gross mechanism of the C. wickerhamii β-glucosidase was determined experimentally (see "Results"), an integrated rate equation was derived, and a computer program was written to estimate the equation. Since it was not possible to integrate the differential equations, the computer program incorporated the Hewlett-Packard Math Pac 83/85 program designed to find the solution of *f(x) = 0* on an interval. This program is based upon the Newton-Raphson algorithm. The program was run on a HP-86b computer.

In the model proposed, multiple substrates of the β-glucosidase behave as competitive inhibitors of each other. For example, if cellollose and cellotriose are present in the same reaction, cellotriose behaves kinetically as a competitive inhibitor when one estimates the amount of product (glucose) formed from cellotriose, and cellotriose behaves kinetically as a competitive inhibitor when one estimates the amount of product (glucose) formed from cellotriose. In the most complex example in this paper, the reactions contain five substrates (G₂ through G₅) and one product (glucose) which vary with time and do not allow expression of each other in terms of a common variable to allow integration.

The gross enzymatic mechanism was found to be Gₙ → Gₙ₋₁ + G₁ (see "Results") for every cellodextrin. The total amount of glucose produced in any given interval of time will be the summation of the amount of glucose produced from each cellodextrin. Also, the individual substrate (cellodextrin) concentration can be estimated with time since each cellodextrin will decrease in concentration by the amount of glucose produced when it is used as substrate and increase in concentration by the amount of glucose produced from the Gₙ₋₁ substrate. Therefore, if the initial enzyme level and substrate concentration(s) are known, the amount of product (glucose) formed in any time interval can be estimated since all variables in the integrated rate equation are known, except for the product variable. After the initial time period, new cellodextrin concentrations can be calculated by estimating the amount of glucose produced when each cellodextrin serves as substrate. If the time interval between estimates is small, the error introduced into the estimates by assuming that the concentration of the cellodextrins remains constant for a given time interval becomes insignificant. This was empirically found to be 10 min for the substrate and enzyme concentrations used herein. Shorter times (1 min) between estimates did not significantly change the computer estimates of glucose formation (data not shown).

Results

Prior to determining the kinetic constants of the C. wickerhamii β-glucosidase, it was important first to characterize the gross mechanism of the enzyme reaction. This arises because the hydrolysis of glucose units from cellodextrins of DP ≥ 3 presents a rather unique enzymological situation in that one of the products of the reaction can also serve as a substrate for the enzyme.

\[ E + Gₙ \rightleftharpoons E.Gₙ \rightleftharpoons E + Gₙ₋₁ + G₁ \]  

**Reaction 1**

\[ E + Gₙ₋₁ \rightleftharpoons E.Gₙ₋₁ \rightarrow E + Gₙ₋₂ + G₁ \]  

**Reaction 2**

Therefore, it is necessary to determine how far the reaction can proceed before the Gₙ₋₁ products accumulate to a level at which they also become substrates for the enzyme. To determine this level, a small amount of the enzyme was incubated with the individual cellodextrins, and the products of the reaction were analyzed by HPLC. Fig. 1 shows the results of the experiment in which 2 µg of β-glucosidase was incubated with 25 mM cellotetraose. The initial products of the reaction (15 min) were equimolar amounts of glucose and cellotetrose. After about 7% of the initial substrate had reacted (30 min), minute amounts of cellotriose were observed. When approximately 20% of the cellotetraose had reacted (45 min), about 2% of the total carbohydrate present in the reaction mixture was cellotriose. Similar results were obtained for the other individual cellodextrins (data not shown).

Since the Gₙ₋₁ products of the reaction are also substrates, other possible enzymatic mechanism could conceivably be either

\[ E + Gₙ \rightleftharpoons E.Gₙ \rightarrow G₁ + E.Gₙ₋₁ \rightarrow G₁ + E.Gₙ₋₂ \rightarrow \text{etc.} \]  

**Reaction 3**
in which the enzyme-product complex does not completely dissociate, or

\[ E + G_n \rightleftharpoons E \cdot G_n \rightarrow E + G_{n-1} + G_1 \]  
**REACTION 4**

\[ E + G_n + G_{n-1} \rightleftharpoons E \cdot G_{n-1} \rightarrow E + G_{n-2} + G_1 \]  
**REACTION 5**

in which the enzyme preferentially binds to the \( G_{n-1} \) product/substrate because of the enzyme-\( G_{n-1} \) proximity. Both models would predict similar reaction products, i.e. the disappearance of the original substrate without the appearance of significant amounts of the smaller cellodextrins. To test these models, cellopentaose was incubated with relative high concentrations of \( \beta \)-glucosidase, and the products of the reaction were analyzed by HPLC. The results (Fig. 2) showed that there was an orderly appearance and disappearance of the cellodextrins of \( DP < 5 \). This confirmed that the gross reaction mechanism depicted by Reactions 1 and 2 are correct.

To determine if the \( \beta \)-glucosidase hydrolyzed glucose units from either the nonreducing or reducing ends of the cellodextrins, cellodextrins specifically radiolabeled in the terminal nonreducing glucose moiety were synthesized and employed as substrates. The results (Fig. 3) showed that when celiotriose was used as substrate, the only radiolabeled product detected, even very early in the time course of the reaction, was glucose. Similar results were obtained when celletetraose and cellopentaose were used as substrates (data not shown). Thus, the enzyme hydrolyzes glucose moieties from the nonreducing ends of the cellodextrins.

Many \( \beta \)-glucosidases possess glucosyltransferase activity, which could potentially interfere with the determination of the kinetic constants by removing glucose from the reaction. To test for this, the *C. wickerhamii* \( \beta \)-glucosidase was incubated with (a) 50 mM glucose, (b) 50 mM cellobiose, or (c) 50 mM glucose and 50 mM cellobiose for 24 h. No significant transferase activity was observed under any of these conditions.

The kinetic characterization of the different cellodextrins is presented in Table I. Because of the relative high \( K_m \) values and the limited solubilities of some of the cellodextrins, the double-reciprocal plots yielded intercepts that passed near the origin (Fig. 4). However, it was possible to derive consistent values for the kinetic constants, \( K_m \) and \( k_{cat} \), from the intercepts. The results (Table I) showed that the \( K_m \) and \( k_{cat} \) values...
for the individual cellodextrins varied by only a factor of 2.5. The values of \( k_{cat}/K_m \) are a reflection of the relative specificities of the enzyme for the different substrates. However, this increase appears nominal when compared with the almost 1,500-fold increase in this factor when \( \mu \text{NPG} \) was used as substrate.

To determine if the kinetic constants obtained experimentally were accurate, computer-generated estimates of the integrated rate equation were compared with the actual reaction kinetics using the individual cellodextrins and a mixture of cellodextrins as substrates. Since multiple substrates of a single enzyme behave kinetically as if they are competitive inhibitors of each other (15), the following integrated rate equation were compared with the actual reaction rate course of the reactions (Fig. 5). In all cases, during the initial stages of the reactions, the computer model predicted the production of slightly less glucose than was experimentally determined. Once the reactions reached 75-80% of completion, the opposite was observed. Late in the time course of the reactions, the experimental reactions produced slightly less glucose than predicted by the computer model. However, considering the experimental error associated with the glucose determinations as well as the determination of the various \( K_m \) and \( V_{max} \) values, the computer-simulated and experimental data agree well. Similar results were obtained when cellobiose and cellobiose were used as substrates (data not shown).

To test the computer model further, \textit{C. wickerhamii} fermentations were performed using either cellobiose or a mixture of cellodextrins as substrate. The \( \beta \)-glucosidase activity and the ethanol concentration were quantified with time. The amount of \( \beta \)-glucosidase activity produced with time was then used in the computer model to try to predict the amount of glucose produced with time. From previous experiments, \textit{C. wickerhamii} produced about 70% of the theoretical amount of ethanol from 50 g/liter cellobiose (data not shown); therefore, the predicted amount of glucose produced was multiplied by 0.511, the theoretical amount of ethanol formed from glucose on a w/w basis, and 0.70 to estimate the theoretical amount of ethanol produced. The results (Fig. 6) indicated that by knowing the initial substrate(s) concentration, the efficiency of fermentation, and the amount of \( \beta \)-glucosidase present with time, one could reasonably estimate the amount of ethanol produced with time. The simulated and experimental data sets agreed well when cellobiose was the substrate (Fig. 6A). However, the computer-simulated data and the experimental data did not agree as closely (Fig. 6B) as when the computer program was used to estimate only the amount of glucose produced from a mixture of cellobiose (Fig. 5). With both substrates tested, the fermentations produced ethanol slightly more rapidly than the computer program predicted (Fig. 6).

The kinetic modeling above suggests that the rate-limiting factor in the fermentation of cellobiose and cellodextrins by \textit{C. wickerhamii} is the production of \( \beta \)-glucosidase. From the data presented in Fig. 6, the actual fermentations proceeded faster than the kinetic model predicted. If the \( \beta \)-glucosidase were present in excess and another step in the fermentation pathway was rate-limiting, one would expect the fermentations to proceed more slowly than the computer projections. Since \textit{C. wickerhamii} ferments glucose about twice as fast as cellobiose (10), this also suggests, but does not prove, that the synthesis of \( \beta \)-glucosidase is rate-limiting.

\textit{S. cerevisiae} ferments glucose about twice as fast as \textit{C. wickerhamii}, but it is unable to metabolize either cellobiose or cellodextrins. If the \textit{C. wickerhamii} \( \beta \)-glucosidase were added to a \textit{S. cerevisiae} cellobiose/cellobiose fermentation at the same levels as found in the \textit{C. wickerhamii} fermentation.
C. wickerhamii Cellohexose Kinetics

DISCUSSION

C. wickerhamii appears to produce at least three distinct \( \beta \)-glucosidases: a cytoplasmic, a secreted, and an extracytoplasmic cell-associated enzyme. Each of these enzymes has been purified and partially characterized. The secreted \( \beta \)-glucosidase is a glycoprotein (4-12% carbohydrate) with a native molecular mass of 130-143 kDa, a subunit molecular mass of 83.5-98 kDa, a pH optimum of 4.5 (12, 13), and an isoelectric point of 3.2 (13). Cellohexotransfers of DP \( \leq 7 \) served as substrates for this enzyme, which catalyzed the removal of single glucose moieties from the nonreducing ends of the cellohexotrans. Himmel et al. (13) were unable to determine \( K_m \) or \( k_{cat} \) values for this enzyme; however, they suggested that because of the 5-fold increase in the kinetic parameter \( k_{cat}/K_m \) as the substrates increased in size from cellohexotrans to cellohexotetraose, this enzyme is not a \( \beta \)-glucosidase but rather a 1,4-\( \beta \)-D-glucan glucohydrolase. The cytoplasminic \( \beta \)-glucosidase is a nonglycosylated enzyme with a native molecular mass of 130 kDa, a subunit molecular mass of 48 kDa, and a pH optimum of 6.0-6.3. Both cellohexotrans and pNPG served as substrates for the enzyme; however, the enzyme showed little reactivity toward cellohexotriose and no reactivity toward cellohexotrans of DP > 3 (12). The extracytoplasmic, cell-associated \( \beta \)-glucosidase used in this study is a glycoprotein (30.5% carbohydrate) with a native molecular mass of 198 kDa, a subunit molecular mass of 94 kDa, a pH optimum of 4.5 (13), and an isoelectric point of 3.89 (14). Cellohexotrans of DP \( \leq 6 \) served as substrates for this enzyme, and, like the secreted enzyme, the extracytoplasmic enzyme catalyzed the removal of single glucose moieties from the nonreducing ends of the soluble \( \beta \)-glucans (Fig. 5). Kinetic constants (\( K_m \) and \( k_{cat} \)) were obtained for this enzyme (Table I). The data indicated that the enzyme did not show a strong "preference" for the larger cellohexotrans as the kinetic constants were approximately equal for all of the cellohexotrans. Although the values determined for \( k_{cat}/K_m \) increased slightly as the size of the substrates increased from cellohexotrans to cellohexopentaose, the increase was only about 2-fold. Since no methionine was detected in the extracytoplasmic enzyme (14) and the secreted exoglucanase contained 5 m mol of methionine/mol of enzyme,

C. wickerhamii Cellodextrin Kinetics

FIG. 5. Comparison of the experimental reaction rates and the computer estimation of the integrated rate equation using cellodextrins, cellotriose, cellopentaose, and a mixture of cellodextrins as substrates. The solid line represents the computer estimation of the integrated rate equation, and the solid dots represent the amount of glucose present in the reaction mixtures at various times, as determined by the glucose oxidase-peroxidase method. The initial enzyme concentration was 0.209 mg of protein/ml, in all cases.

FIG. 6. Comparison of ethanol formation in C. wickerhamii fermentations (●) and the computer estimations (solid line) of ethanol production from the integrated rate equation using either 50 g of cellodextrins/liter (panel A) or 60 g of cellodextrins/liter (panel B) as substrate.

FIG. 7. Comparison of ethanol formation in a C. wickerhamii fermentation (○) and a S. cerevisiae fermentation (□) in which \( \beta \)-glucosidase was added at levels that mimicked the production of \( \beta \)-glucosidase in the C. wickerhamii fermentation. The substrate for both fermentations was 60.9 g of cellodextrins/liter. The control S. cerevisiae fermentation in which no C. wickerhamii \( \beta \)-glucosidase was added is also shown (×).

ulation, the rate of ethanol production in the two cultures should be equal if the \( \beta \)-glucosidase is the rate-limiting enzyme. The results of such an experiment utilizing a mixture of cellodextrins as substrate are presented in Fig. 7. Both the C. wickerhamii- and the \( \beta \)-glucosidase-"spiked" S. cerevisiae fermentations produced ethanol at approximately the same rate, whereas the "unaltered" S. cerevisiae fermentation produced virtually no ethanol. It should be noted that if corrections are made for the slightly better fermentation efficiency of S. cerevisiae on glucose, the rates of the two fermentations become almost identical. Similar results were also obtained using 50 g/liter cellodextrins as substrate (data not shown).
The cell wall-associated \( \beta \)-glucosidase appears to be the rate-limiting enzyme in the fermentation of cellulosic/cellodextrins by \textit{C. wickerhamii}. \textit{S. cerevisiae} ferments glucose at about twice the rate as \textit{C. wickerhamii}. For example, \textit{S. cerevisiae} fermented 50 g of glucose/liter in 16 h, whereas \textit{C. wickerhamii} cultures required 32-40 h (data not shown). \textit{S. cerevisiae} cultures that were supplemented with \textit{C. wickerhamii} \( \beta \)-glucosidase produced ethanol at the same rate as the \textit{C. wickerhamii} fermentations (Fig. 7). If the \( \beta \)-glucosidase were present in excess, the \textit{S. cerevisiae} culture should have fermented the cellbiose/cellodextrin substrates more rapidly than the \textit{C. wickerhamii} culture.

The \textit{C. wickerhamii} extracytoplasmic \( \beta \)-glucosidase possesses at least three unique features that might make it useful in the conversion ofcellulosic materials to glucose. First, besides being the rate-limiting factor in cellbiose/cellodextrin fermentations by \textit{C. wickerhamii}, this enzyme is one of a very few yeast \( \beta \)-glucosidases which utilizes cellodextrins as substrates (9). Most yeast \( \beta \)-glucosidases are unable to act upon cellodextrins of DP > 3. Second, this enzyme is unusual in that it is not readily inhibited by glucose. No inhibition of \( pNPG \) hydrolysis was observed even when the \( pNPG \) concentration was 1.68 \( K_a \), and glucose was present at a concentration of 50 mM (14). In contrast, the \( K_a \) values (for glucose) of some different yeast \( \beta \)-glucosidases (on \( pNPG \)) are 8.5 mm for \( Rhodotorula minuta \) (20), 3.44 mm for \textit{Kluyveromyces marxianus} (21), 8.47 mm for \textit{Kluyveromyces dothamanski} (21), 2.0-2.6 for \textit{Kluyveromyces lactis} (22), and 3.5 mm for \textit{Bekkera intermedia} (23). Third, the physiological factors that regulate the synthesis of the \textit{C. wickerhamii} \( \beta \)-glucosidase are uncommon. The enzyme is produced constitutively; however, when grown aerobically, high concentrations of glucose repress its expression. Surprisingly, anaerobiosis appears to overcome the glucose (catabolite) repression (24). Thus, this enzyme might be of use not only for the conversion ofcellulosics to glucose, but also the regulatory portion of its gene might be useful in biotechnology. Experiments are currently in progress to isolate the gene encoding this enzyme.

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