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Biosynthetic Activity of Recombinant *Escherichia coli*–Expressed *Pichia etchellsii* β-Glucosidase II

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Abstract

The hydrolysis and biosynthetic reactions of partially purified *Pichia etchellsii* β-glucosidase II from recombinant *Escherichia coli* pBG22:JM109 are described. With 167 mmol/L of initial glucose, the products of synthetic reactions, glucobiose and glucotriose, accumulated to 18 and 6 mmol/L, respectively. In transglycosylation reactions with 79 mmol/L of initial cellobiose, glucotriose and glucopentaose were obtained at 4.5 and 2 mmol/L, respectively. The effects of incubation time and substrate concentration were studied on the yield of synthesized oligosaccharides. In a reaction time of 24 h with 468 mmol/L of initial cellobiose, glucotriose and glucopentaose levels of 21.6 and 6.6 mmol/L, respectively, were obtained. The addition of dimethyl sulfoxide (DMSO) further increased the yields of the products by 10%. Detailed kinetic analysis indicated a significant (about twofold) increase in V_{max}/K_M of synthetic reactions in the presence of DMSO. A study of other disaccharides in transglycosylation reactions indicated biosynthetic activity in the order of sophorose > gentiobiose > cellobiose.

Index Entries: β-Glucosidase; *Pichia etchellsii*; oligosaccharide synthesis.

Introduction

Oligosaccharides and their derivatives are molecules of immense biologic interest and play an important role as regulatory molecules, receptors in cellular recognition mechanisms *(1)*, structural components, and energy source. This variety of functions is directly related to the complex structure of carbohydrate units and the ways in which monomeric units can be linked. Short-chain cellooligosaccharides, e.g., are involved in the induction of cellulase enzyme in cellulolytic microorganisms, thus facilitating the hydrolysis of cellulose *(2,3)*. Oligosaccharides such as fructo-, isomalto-,

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and galactooligosaccharides are some of the most popular food ingredients because of their excellent functionalities *(4,5)*. Cellotriose has been used as an additive in sugar syrups as a low-calorie sweetener *(6)*. Cellooligosaccharides are also used extensively to describe reaction mechanisms or subsite mapping of cellulase enzymes *(7)*. Sophorolipids such as lactonic sophoro- and dodecyl-sophoroside have applications as important biosurfactants *(8)*.

The large-scale availability of oligosaccharides of defined chemical complexity is important to functionally understand their biochemical roles. Synthesis by chemical routes is difficult because several successive protection and deprotection steps are required *(9)*. The present method of preparation of cellooligosaccharides is by controlled acid hydrolysis of commercially available cellulose (Avicel cellulose) followed by separation using chromatography methods. However, acid hydrolysis may result in complete degradation to soluble sugars. Similarly, oligomers with a desired degree of polymerization or linkage specificity are also difficult to prepare by this method. The enzymatic route of biosynthesis of such compounds is promising and economical. The ability of almond β-glucosidase to catalyze the synthesis of glucooligosaccharides has been demonstrated using a high concentration (>90%) of glucose at high temperature (55°C). The main product of reverse-hydrolytic activity was a disaccharide *(10)*. The synthesis of oligosaccharides from cellobiose using β-glucosidase from various microbial systems has also been reported; these enzymes are from *Aspergillus foetidus (11)*, *Fusarium oxysporum (6)*, sesame *(12)*, and *Aspergillus niger (13)*.

In one of our previous studies, we described the biosynthetic property of a purified yeast (*Pichia etchellsii*) β-glucosidase enzyme; β-glucosidase I or *Bgl*I (encoded by *bglu*1) isolated from recombinant *Escherichia coli* pBG55:XL1-Blue *(14)*. A limited number of cellooligosaccharides were synthesized using this enzyme. Another β-glucosidase gene, *bglu*2, encoding β-glucosidase II (*Bgl*II) was cloned from the same yeast in *E. coli (15)*. The properties of the purified enzyme were studied, and its potential to synthesize various categories of glycoconjugates (cellooligosaccharides, alkyl- and terpene–glucosides) was demonstrated *(16)*. In the present study, the biosynthetic properties of *Bgl*II were investigated in detail for synthesis of cellooligosaccharides.

Materials and Methods

Chemicals

Cellobiose, cellotriose, cellotetraose, cellopentaose, gentiobiose, sophorose, laminaribiose, and *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) were purchased from Sigma (St. Louis, MO). *Bgl*II was isolated from recombinant *E. coli* cells as described in the next section. Precoated silica gel 60 F_{254} thin-layer chromatography (TLC) plates were obtained from Merck. Ampicillin (sodium salt) was from Amresco. All other chemicals were of analytical grade.

Production and Partial Purification of BglII

The recombinant *E. coli* pBG22:JM109 expressing *Bgl*II was constructed in our laboratory *(15)*. This clone was found to give stable enzyme expression. It was maintained in the form of glycerol stocks, stored at –70°C. Large-scale fed-batch cultivation of cells was carried out in a 14-L bioreactor (Chemap) at constant temperature and pH of 37°C and 6.8, respectively, in complex medium. In batch mode, the medium contained 10 g/L of yeast extract, 5 g/L of peptone, 5 g/L of glucose, salts (Na, K, Mg), trace elements (Fe, Cu, Mn, Co, B, Mo, Zn), thiamine, and 100 mg/L of ampicillin. In the fed-batch mode, 100 g/L of yeast extract, 50 g/L of peptone, 300 g/L of glucose, salt (Mg), trace element (Fe), and ampicillin were added as determined from the exponential feeding equation *(15)*. At the end of fermentation, the cells were collected, subjected to sonication, and the clear supernatant was obtained as described previously *(15)*. Protease inhibitor phenylmethylsulfonyl fluoride was added to a final concentration of 1 m*M*. *Bgl*II was concentrated from the supernatant using 40–80% fractional ammonium sulfate precipitation. The protein pellet was suspended in a minimum volume of 50 m*M* sodium phosphate buffer, pH 7.0, and dialyzed overnight (at 4°C) with three buffer changes to remove the salt. The dialyzed enzyme preparation was assayed for enzyme activity and used for subsequent experiments. The specific activity of the enzyme was 291 U/g of protein with a purification factor of 7.0.

Enzyme Assay and Protein Estimation

The β-glucosidase activity was routinely assayed using *p*NPG as the substrate *(17)*. One unit (IU) of enzyme activity defined the release of 1 µmol of *p*-nitrophenol/min. The protein was estimated according to Lowry *(18)*.

Solvent Stability of BglII

The enzyme was incubated in the presence of varying concentrations of different organic solvents—acetone, acetonitrile, dimethyl sulfoxide (DMSO), ethanol, methanol, and ter-butanol—at 45°C with shaking at 210 rpm. These were incubated for 24 h, and residual enzyme activity was measured on *p*NPG as described earlier.

Effect of Time of Incubation and Substrate Concentration on Oligosaccharide Yield

A time course study of synthesis of di- and higher oligosaccharides was carried out using glucose or cellobiose as the substrates. Partially purified *Bgl*II (2.7 IU/mL) was incubated with either 167 mmol/L of glucose or cellobiose (79 mmol/L) in 50 m*M* sodium phosphate buffer, pH 7.0, at 45°C with shaking at 210 rpm in a reaction volume of 1.5 mL. Aliquots (300 µL) were removed at regular intervals (as shown in Fig. 1), and the reaction was terminated by boiling the vial for 2 min. Substrate blank and

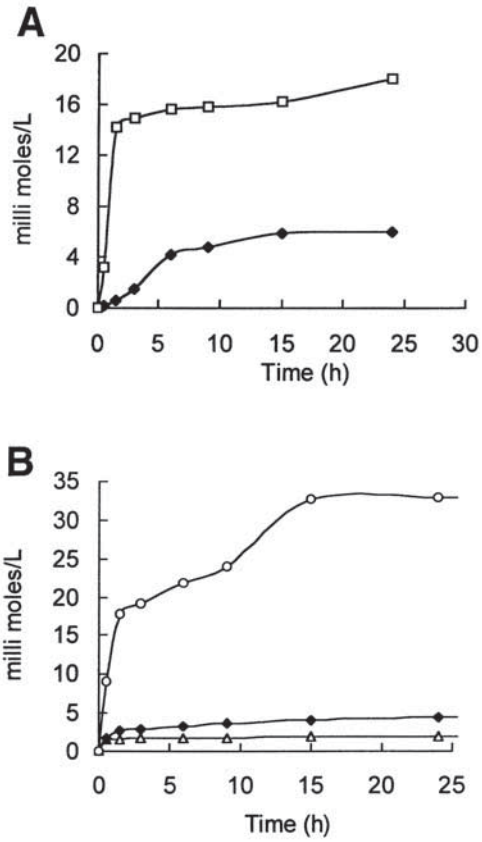

Fig. 1. Time course profile of hydrolysis and biosynthetic reactions with **(A)** glucose and **(B)** cellobiose as initial substrates. (○) Glucose (G1); (□) glucobiose (G2); (◆) glucotriose (G3); and (△) glucopentaose (G5).

heat-killed enzyme served as controls. All samples were passed through 0.45-µm filters and analyzed by high-performance liquid chromatography (HPLC) to quantify the reaction products. The optimized time at which maximum accumulation of oligosaccharides was detected was used to study the effect of substrate concentration (glucose and cellobiose) on oligosaccharide yield. Different concentrations of glucose (0.16–3.33 mol/L) or cellobiose (60–468 mmol/L) were incubated with 2.7 IU/mL of enzyme in 50 m*M* sodium phosphate buffer, pH 7.0, in a total volume of 1.5 mL. The reaction mixtures were incubated at 45°C with shaking at 210 rpm for 24 h. The samples were processed as already described and oligosaccharides quantified. Reactions were also carried out at 50 and 55°C using different concentrations of cellobiose. In experiments involving the addition of solvent, DMSO was added at a concentration of 20% (v/v). The oligosaccharide yields were also determined with gentiobiose and sophorose as the donors of the glucosyl groups in aqueous or DMSO-added reaction environment. The reactions were set in a total volume of 1.5 mL as already described except for substitution of cellobiose with either gentiobiose

(468 mmol/L) or sophorose (468 mmol/L). The product yields were determined after 24 h. All reactions were carried out in triplicate along with suitable controls in which heat-denatured enzyme was added.

Kinetic Constants for Hydrolysis and Biosynthetic Reactions

The dependence of the initial rate of hydrolysis (or oligosaccharide biosynthesis) on cellobiose concentration was studied in a reaction volume of 1.5 mL containing 2.7 IU/mL of enzyme and different cellobiose concentrations. The reaction mixtures were incubated at 45°C for 1 h with shaking at 210 rpm during which samples were removed at regular intervals and the reactions terminated. The samples were prepared for oligosaccharide analysis as described in the previous section. A plot of initial reaction rate vs cellobiose concentration was used to determine kinetic constants for hydrolysis and biosynthetic reactions. The rates were also determined in the presence of 20% (v/v) DMSO, and the data were used to calculate the kinetic parameters. All experiments were performed twice in sets of duplicate reaction vials.

Analysis of Oligosaccharides

The products of hydrolytic and biosynthetic activity of *Bgl*II were monitored on HPLC using a Waters system composed of a 501 pump, a 401 refractive index detector, and an Aminex HPX-87H column (Bio-Rad, Hercules, CA). The mobile phase was 5 m*M* H_2SO_4 in Milli-Q water and detection was carried out at 50°C with a flow rate of 0.5 mL/min. The substrates and products were quantified based on peak areas using standard sugars: glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, sophorose, and gentiobiose. An aliquot (20 µL) of the reaction mixture was also spotted on precoated silica gel 60 F_{254} plates (Merck) along with suitable standards. Ascending TLC was carried out using a solvent system of ethyl acetate:2-propanol:water (40:30:10). Glucose, disaccharides, and higher oligomers were visualized by spraying the dried plates with naptho-resorcinol reagent, followed by heating at 110°C for 10 min. The R_f values of sugars obtained in the reaction mixture were compared with those of standard sugars.

Results

High-Cell-Density Cultivation of Recombinant E. coli

High-cell-density cultivation of recombinant *E. coli* pBG22:JM109 was carried out by fed-batch method. The batch phase lasted for 16 h. The fed-batch phase was initiated by exponential feeding of the medium and lasted for another 24 h. The samples were removed periodically and analyzed for cell OD_{600nm}, β-glucosidase activity, and dry cell weight. A final cell OD_{600nm} of 36, corresponding to 24 g dry cell wt/L, was obtained at the end of the fermentation with an enzyme activity of 180 IU/L.

Stability in Organic Solvents

Study of the stability of *Bgl*II in organic solvents indicated that the enzyme retained 100 and 93% of its initial activity in 10 and 20% (v/v) DMSO, respectively. Similarly, 100% residual activity was displayed by the enzyme in 5% (v/v) acetone. The enzyme was fairly stable in the presence of up to 3% (v/v) ethanol. However, the enzyme activity declined sharply in the presence of ter-butanol and acetonitrile. A residual activity of 39% was obtained with 3% (v/v) acetonitrile after 3 h of incubation, while complete loss of enzyme activity was seen beyond 6 h (data not shown). Thus, 20% DMSO was used in various biosynthetic experiments involving the use of solvent.

Optimization of Reaction Time for Biosynthesis Reactions Catalyzed by Bgl*II*

The effect of incubation time was studied on hydrolytic and biosynthetic activity of *Bgl*II using glucose and cellobiose as initial substrates. As shown in Fig. 1A, a slow but steady increase in levels of glucotriose (G3) was observed up to 15 h of incubation, after which there was no further increase. The disaccharide product, however, continued to accumulate until 24 h after an initial rapid rate during the first 2 h. Maximum levels of 18 mmol/L of disaccharide and 6 mmol/L of trisaccharide were obtained at the end of 24 h. With cellobiose, there was a steady increase in the amount of glucose until the end of 15 h. While the level of glucopentaose (G5) did not increase significantly beyond the first 3 h, there was a low but steady increase in the level of G3 (Fig. 1B). At the end of 24 h, maximum synthesis had been achieved with an accumulation of triose at 4.5 mmol/L and pentaose at 2 mmol/L levels. Analysis of an aliquot of 24-h-incubated sample indicated the presence of G3, glucotetraose (G4), and G5 (data not shown). It appears that G4 and G5 coeluted in these samples, although the two were resolved separately in the G4 and G5 mixture; hereafter, G5 refers to both G4 and G5.

Effect of Increasing Substrate Concentration on Synthesis of Oligosaccharides

The effect of increasing glucose concentration on oligosaccharide yield was studied, and the results are shown in Fig. 2. With an increase in initial glucose concentration from 0.16 to 3.33 mol/L, the levels of G3 and glucobiose (G2) increased by 1.6- and 2.3-fold, respectively, indicating a larger increase for disaccharides. The effect of increasing cellobiose concentration on the release of glucose and oligosaccharide synthesis is shown in Fig. 3A. The release of glucose was maximum at 240 mmol/L of cellobiose, indicating saturating levels (V_{max}). The triose concentration steadily increased up to 468 mmol/L of cellobiose concentration. The extent of the increase was less in the case of G5. An increase in cellobiose concentration to 702 mmol/L did not lead to any enhancement in the concentration of prod-

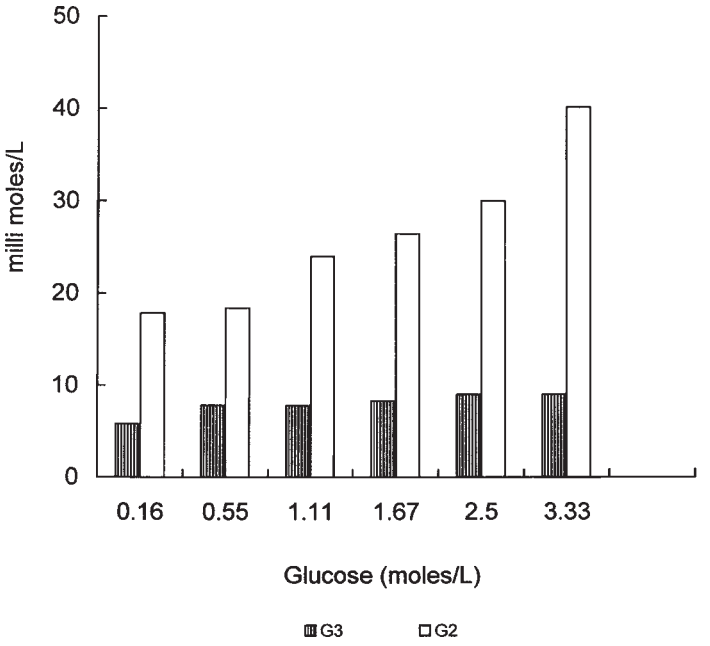

Fig. 2. Effect of initial glucose concentration on hydrolysis and biosynthesis reactions catalyzed by *Bgl*II.

ucts (data not shown). Higher concentrations of cellobiose were also used by increasing its solubility by heating, but no effect on oligosaccharide yield was observed (data not shown). An increase in the reaction temperature to 50 or 55°C also did not have any effect on the oligosaccharide yield compared to the data shown for 45°C (Fig. 3A). When an identical set of reactions was carried out in the presence of 20% (v/v) DMSO, there was a sharp decline in the amount of glucose released (Fig. 3B). This difference was more pronounced at lower cellobiose concentrations as glucose levels were reduced by 62% at 60 mmol/L of initial cellobiose concentration. However, higher yields (about 10%) of G3 were obtained, particularly up to an initial cellobiose concentration of 240 mmol/L.

Determination of Kinetic Constants

The initial rate of hydrolytic and biosynthetic reactions was studied on cellobiose under both reaction conditions (in the absence and presence of organic solvent DMSO). The dependence of initial reaction rate on initial substrate concentration followed the classic Michaelis-Menten form of kinetics, as shown in Fig. 4A,B, in the absence and presence of DMSO, respectively, and the data are summarized in Table 1. The kinetic efficiency of hydrolysis, estimated from the parameter V_{max}/K_M, was reduced from 0.17 to 0.12 h^{-1} in the presence of DMSO. The efficiency for the biosynthetic reaction was, however, increased from 0.04 to 0.07 h^{-1}, about twofold, suggesting that these reactions were favored in the presence of DMSO.

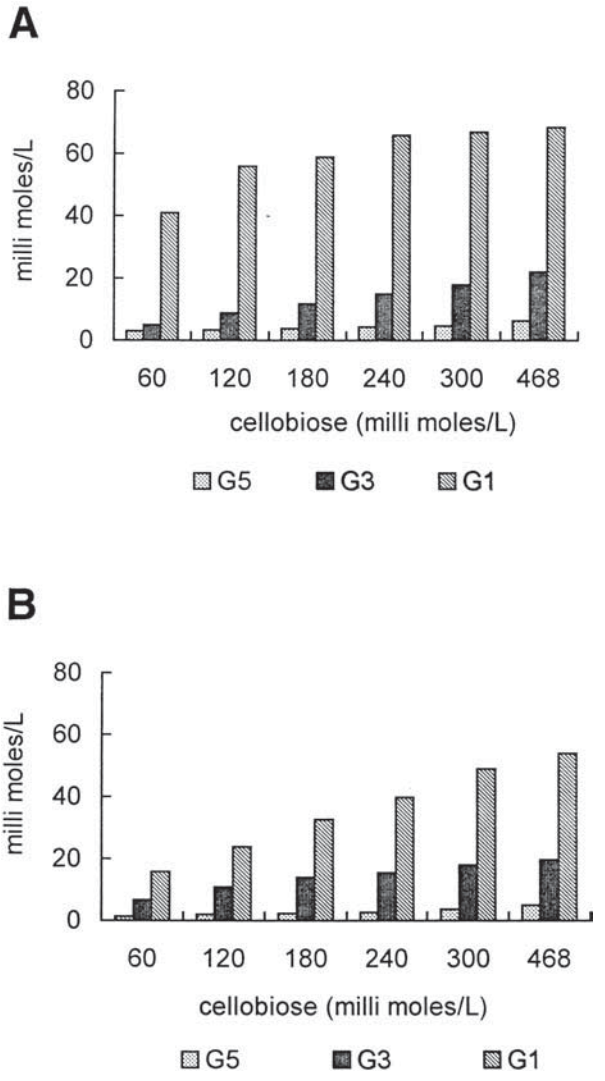

Fig. 3. Effect of initial glucose concentration on hydrolysis and biosynthesis reactions catalyzed by *Bgl*II under **(A)** aqueous and **(B)** DMSO-supplemented reactions.

Table 1
Kinetic Constants for Hydrolytic and Biosynthetic Reactions Catalyzed by *Bgl*II

	Kinetic constants					
	Without DMSO			With DMSO		
Reaction	K_M (mmol/L)	V_{max} (mmol/[L·h])	V_{max}/K_M (h^{-1})	K_M (mmol/L)	V_{max} (mmol/[L·h])	V_{max}/K_M (h^{-1})
Hydrolysis	234	40.2	0.17	184	22.3	0.12
Biosynthesis	634	28.2	0.04	158	11.4	0.07

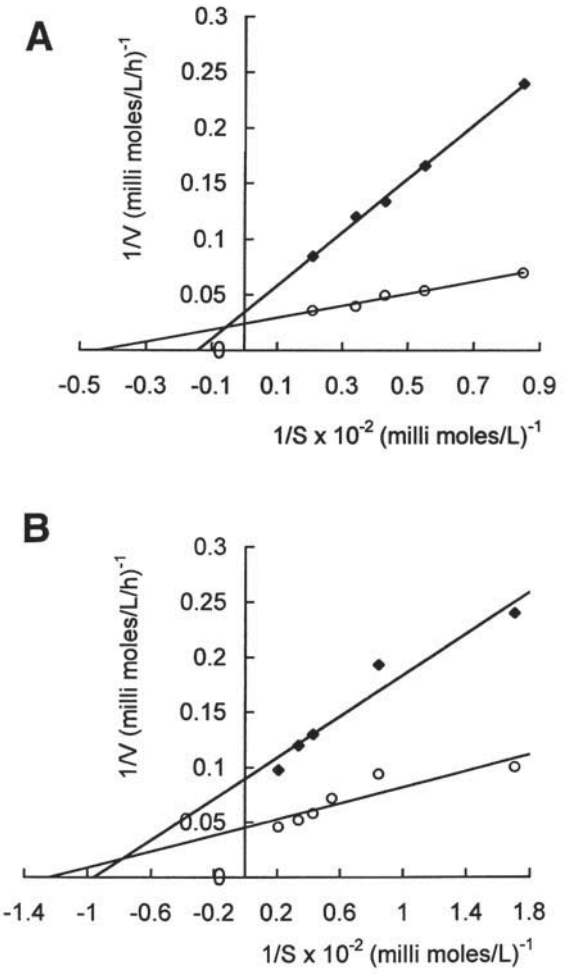

Fig. 4. Kinetic constants for transglycolytic and hydrolytic activities of *Bgl*II in **(A)** absence and **(B)** presence of DMSO: (○) G1; (◆) G3.

Reaction with Other β*-Linked Disaccharides*

The disaccharides sophorose and gentiobiose, containing β(1→2)- and β(1→6)-linked glucose monomers, respectively, were used as substrates to study the effect of β-linkage on biosynthetic activity of the enzyme. Two sets of reaction mixtures were prepared, one with 468 mmol/L of sophorose and the other with 468 mmol/L of gentiobiose, in both the absence and presence of DMSO. Analysis of reaction mixtures revealed the formation of glucose, G3, and G5 (Fig. 5A,B) with both disaccharides. The highest yields of G3 and G5 were obtained with sophorose, irrespective of the solvent conditions. After 24 h of incubation, up to 78% of initial sophorose was utilized by the enzyme yielding 72 mmol/L of G3 and 42 mmol/L of G5 under aqueous reaction conditions, corresponding to conversion efficien-

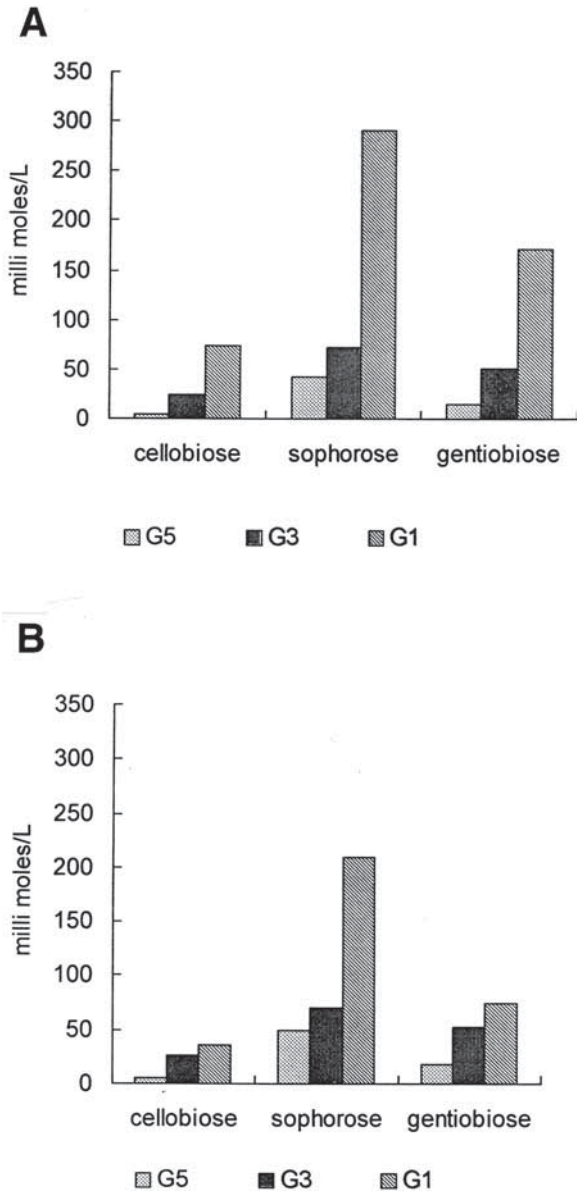

Fig. 5. Biosynthetic and hydrolytic activity of *Bgl*II on different disaccharides under **(A)** aqueous reaction and **(B)** DMSO-supplemented reaction conditions.

cies of 15.4 and 9.0%, respectively. The yield of G5 was further increased by 18% in the presence of DMSO. With gentiobiose, the synthesis of these oligomers was lower compared with that obtained with sophorose, but higher against the yields obtained with cellobiose. The amount of glucose released was less in the presence of solvent, with both disaccharides, as was the case with cellobiose.

Discussion

We have described the biosynthetic activity of recombinant *Bgl*II from *E. coli*. The enzyme exhibited a different substrate specificity profile in comparison to *P. etchellsii Bgl*I purified earlier *(14)*, with the highest hydrolytic activity on sophorose > gentiobiose > *p*NPG > cellobiose. In view of the lack of hydrolytic activity on these substrates by control *E. coli* (containing only pUC18 vector), partially purified β-glucosidase from pBG22:JM109 recombinant was used for detailed studies. Since several experimental conditions such as initial substrate concentration, temperature, and type of substrate influence oligosaccharide synthesis, these parameters were investigated in detail for biosynthesis of cellooligosaccharides.

The biosynthetic reactions catalyzed by β-glucosidases are proposed to proceed by (1) reverse hydrolysis or (2) by transglycosylation reaction. In the first method, in high concentrations of glucose, the equilibrium is shifted toward biosynthetic reactions. In the second method, activated donors such as cellobiose (or other disaccharides, sophorose, or gentiobiose) or *p*NPG can be used, and the formation of the oligosaccharide product is determined by competition between water and another acceptor and the reaction is under kinetic control *(19)*. The enzyme used in the present study indicated use of both methods for biosynthetic purposes. In time course experiments with glucose or cellobiose, synthesis of oligosaccharides was obtained during initial hours of incubation, but higher (25%) yields were obtained with cellobiose.

Substrate concentration is reported to influence enzyme activity, and the underlining basis for the same is a reduction in active water concentration. We also observed an increase in G2 and G3 levels by 2.3- and 1.6- fold, and G3 and G5 by 5- and 3.3-fold, with an increase in glucose and cellobiose concentration, respectively. Such an increase in the yield is expected and has been reported with other systems as well *(6,10)*. A further increase in oligosaccharide yield was observed in the presence of DMSO, particularly up to an initial cellobiose concentration of 240 mmol/L. The application of organic solvents is widely emphasized and is based on several factors such as lowering of water activity in the reaction medium to bring a shift of equilibrium in the reverse direction, enhanced enzymatic thermostability, and even reusability of the enzyme. Ter-butanol and acetonitrile media have previously been used in the synthesis of alkyl-glucosides by almond β-glucosidase *(19,20)*. Mixed buffer/organic solvent system has also been used in cellulase-catalyzed synthesis of oligosaccharides *(21)*.

An attempt was made to analyze hydrolysis and biosynthetic reactions through determination of the kinetic constants K_M and V_{max} for these reactions. As shown in Table 1, an apparent K_M of 634 mmol/L and V_{max} of 28.2 mmol/(L·h) was obtained for biosynthetic reactions in the absence of DMSO. This value of K_M was lower than the value of 1.77 mol/L of cellobiose and 2.3 mol/L of gentiobiose as reported for synthetic reactions carried out with *F. oxysporum* β-glucosidase *(6)*, indicating enhanced affinity

of *Bgl*II for biosynthesis. We also found a further decrease in the value of K_M, to 158 mmol/L, in the presence of DMSO. Although a slight change in V_{max} was also noticed, calculation of catalytic efficiency (V_{max}/K_M) indicated that biosynthetic activity was favored while hydrolysis was suppressed in the presence of DMSO.

Our studies with other β-linked disaccharides (sophorose and gentiobiose) indicated highest oligosaccharide yields with sophorose, the substrate on which maximum hydrolytic activity has been reported with this enzyme *(16)*. Highest conversion efficiency of 25% from disaccharide sophorose was also observed, which is among the best values reported in the literature. A wide variety of disaccharides such as gentiobiose, laminaribiose, and cellobiose were synthesized from sophorose, indicating that the addition of a glucosyl group from the enzyme-glucosyl complex (proposed as an intermediate in the reaction mechanism of β-glucosidase) *(22)* to acceptors occurred in β(1→2), β(1→3), β(1→4), β(1→6) fashion. The same relaxed specificity may hold true for biosynthesis of higher oligomers of glucose.

Based on our work it is therefore indicated that condensation reactions catalyzed by *Bgl*II retained the anomeric specificity of the enzyme but that there was little regiospecificity of synthesis. Equilibrium reaction mixtures contained several products (both di- and oligosaccharides). Synthesis of all β(1→2)-, β(1→3)-, β(1→4)-, β(1→6)-linked glucobioses were confirmed by HPLC and TLC analysis. Hence, a wide range of oligosaccharides, many of which may not occur in nature, can be synthesized using this enzyme. While the synthetic procedure is straightforward and should be scalable, analysis of the products and isolation of individual compounds still remain challenging. Recently, we (Bacchawat and Mishra, unpublished data) have used a combination of gel filtration and charcoal adsorption chromatography to purify components of degree of polymerization 2–6 to homogeneity.

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