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Disaccharide Synthesis by Enzymatic Condensation of Glucose: Glycoside Linkage Patterns for Different Fungal Species

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Abstract: Four enzyme preparations produced by fungal species belonging to different genera (*Aspergillus niger, Corynascus* sp., *Penicillium verruculosum, Trichoderma reesei*) were used for synthesis of disaccharides by D-glucose (60% w/v) condensation catalyzed by β-glucosidase. Effects of pH and temperature on the disaccharide synthesis were studied, and glycoside linkage patterns for enzymes from different sources were determined. The highest concentration of disaccharides (114 and 118 g/l) was achieved in the case of *A. niger* and *Corynascus* sp. enzymes after 48 h of the condensation reaction carried out at 70 ^{o}C and optimal pH; the *P. verruculosum* sample slightly conceded them in the yield of products (96 g/l), while the *T. reesei* preparation displayed the lowest synthetic activity (35 g/l). Gentiobiose was predominantly formed in the reaction catalyzed by the first three enzyme samples, while in the case of *T. reesei* laminaribiose was the main condensation product.

Keywords: β-Glucosidase, Condensation, Cellobiose, Gentiobiose, Laminaribiose, Sophorose.

INTRODUCTION

Glycoside hydrolases (EC 3.2.1.-) catalyzing the hydrolysis of polysaccharides and/or low-molecular glycosides with overall retention of the anomeric configuration are also able, under certain conditions, to catalyze the formation of glycosidic bonds *via* transglycosylation and condensation (reverse hydrolysis) [1-3]. Transglycosylation is a kinetically controlled reaction, while the reverse hydrolysis is under thermodynamic control. The synthetic activity of glycosidases has been used for production of oligosaccharides [4-12], alkyl-glycosides [12-17], vitamin derivatives [18]. Oligosaccharides are used as therapeutic agents, diagnostic tools, and additives in the food and cosmetic industries [8, 12, 19, 20]. Alkyl-glycosides found applications as non-ionic surfactants in detergents, cosmetics, foods and pharmaceuticals [12, 13, 20]. The enzymatic synthesis of glycosides has certain advantages over chemical synthesis, since it is carried out under mild conditions in one stereoselective step, while being more regioselective [8, 13, 17].

The most widely used enzymes for oligosaccharide and alkyl-glycoside synthesis are β-glucosidases (EC 3.2.1.21), which act on terminal non-reducing β-D-glucosyl residues in cellooligosaccharides and other β-D-glucosides with release of β-D-glucose [20]. Different β-glucosidases from fungi, bacteria and plants have been used in transglycosylation and condensation reactions [4-17, 20-24], which are usually carried at high substrate concentration in order to attain higher yields of products. Trisaccharides and higher oligomers are formed as major transglycosylation products of β-glucosidase-catalyzed reactions with cellobiose as a substrate [5, 6, 8, 10, 12, 20, 22], while the condensation reactions with glucose as a substrate lead to preferential formation of disaccharides [4, 9-11, 20]. Although the typical yields of products in the condensation reaction (10-25%) [10, 11, 20] are usually lower than those obtained with transglycosylation (15-40%) [5, 6, 10, 12, 20, 22], the substrates (monosaccharides) for condensation are cheaper and the reaction can be more easily controlled. With 90% glucose as a substrate, the unusually high yield of disaccharides (40%) was obtained in reverse hydrolysis reaction catalyzed by almond β-glucosidase [4], while the yields of several oligosaccharides up to 65% have been achieved by utilizing the transglycosylation activity of *Agrobacterium* sp. β-glucosidase [24].

The preferential formation of the β-1,6-glucosidic linkage has been observed in both transglycosylation and condensation reactions catalyzed by β-glucosidases of fungal (*Aspergillus* spp., *Trichoderma* spp.) and plant (almond, Thai rose wood) origin [4, 8, 9, 20-23]. Together with gentiobiose (β-1,6-linkage), smaller amounts of sophorose (β-1,2), laminaribiose (β-1,3) and cellobiose (β-1,4) have been detected amongst products of glucose condensation [4,9], while a notable amount of trehalose (α-1,1) has been formed by β-glucosidase from *T. pseudokoningii* in the reaction system containing cellobiose and glucose as initial substrates [23]. Rather unusual biosynthetic activity in the order of sophorose > gentiobiose > cellobiose has been reported for the recombinant *Pichia etchellsii* β-glucosidase II [10].

However, in spite of the numerous publications on β-glucosidase-catalyzed transglycosylation and condensation, the number of organisms studied from this point of view, is rather limited. One may expect that enzymes from different organisms would form oligosaccharides with different yields and various ratios between synthesized products.

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This paper focuses on the reaction of glucose condensation catalyzed by enzyme preparations produced by fungal species belonging to four different genera (*Aspergillus, Corynascus, Penicillium, Trichoderma*). All enzyme samples possessed β-glucosidase activity to catalyze the reverse hydrolysis reaction within reasonable time. Effects of pH and temperature on the synthesis of disaccharides were studied, and glycoside linkage patterns for enzymes from different sources were determined.

MATERIALS AND METHODS

Enzymes

Crude enzyme preparations produced by fungal species belonging to four different genera were used for disaccharide synthesis from glucose, that is, Celloviridin G20x (*Trichoderma reesei*), *Aspergillus niger*, *Corynascus* sp., *Penicillium verruculosum*. The first preparation was a commercial one, produced by "Promferment" company (Russia), while the other enzyme samples were laboratory preparations obtained in the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences. The specific β-glucosidase activity of the preparations determined with *p*-nitrophenyl-β-D-glucoside (Sigma, USA) as a substrate at pH 5.0 and 40 ^{o}C [25] was 0.3, 41.0, 18.5 and 1.3 U/mg protein, respectively.

Condensation Reaction

The condensation reaction was carried out with 60% (w/v) D-glucose (Reakhim, Russia) as a substrate in plastic tubes (1.5 ml volume) placed on the thermostated shaker (50, 60, 65 or 70 ^{o}C) at different pH using 20 mM Na-acetate buffers. The enzyme dosage in a typical experiment was either 4 or 8 mg of protein per 1 ml. Aliquots of the reaction mixture (0.1 ml) were taken at various reaction time, they were diluted with distilled water and analyzed by HPLC.

Analysis of the Reaction Products

The products of glucose condensation were analyzed by HPLC on a Workstation 700 system (Bio-Rad Laboratories, USA) equipped with a refractometric detector, using a Diasorb 130 Amino column (4 x 250 mm, 6 μm) from Bio-ChemMack (Russia) and acetonitrile/water mixture (80:20) as a mobile phase. Sophorose (Serva, Germany), laminaribiose (Megazyme, Australia), cellobiose (Merck, Germany) and gentiobiose (Sigma, USA) were used as standards in the HPLC analysis.

RESULTS AND DISCUSSION

Crude enzyme preparations produced by fungal species belonging to four different genera (*Aspergillus, Corynascus, Penicillium, Trichoderma*) were used for disaccharide synthesis by enzymatic condensation of D-glucose. The condensation reaction is typically carried out at high glucose concentration (30-90%). We used 60% (w/v) glucose as a substrate and carried out the enzymatic process for 5 days at different temperatures (50, 65, 70 and 75 ^{o}C). The formation of the condensation products was monitored by HPLC. A typical chromatogram is shown in Fig. (**1**).

For all enzymes under study, four disaccharides with different glucoside linkages were formed as the condensation products, that is, laminaribiose, cellobiose, sophorose and gentiobiose. In these disaccharides the glucosidic residues are connected by β-1,3-, β-1,4-, β-1,2- and β-1,6-linkage, respectively. The ratio between disaccharides varied depending on the enzyme source (Fig. **1**). Gentiobiose was predominantly formed in the reaction catalyzed by the *A. niger*, *Corynascus* sp. and *P. verruculosum* enzymes, while in the case of the *T. reesei* sample laminaribiose was the main condensation product.

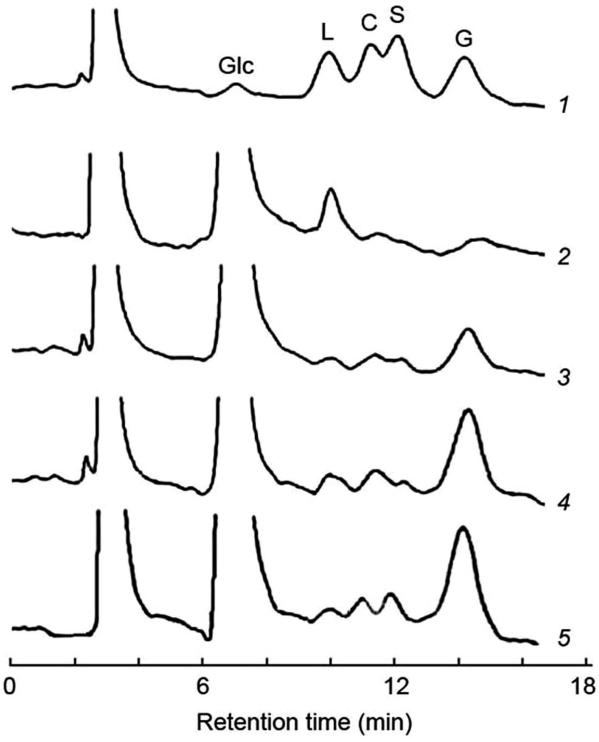

Fig. (1). HPLC analysis of the glucose condensation products using a silica column with bonded amino phase and acetonitrile-water (80:20) as a mobile phase at a flow rate of 1 ml/min. *1*, chromatographic standards: laminaribiose (L), cellobiose (C), sophorose (S), gentiobiose (G); *2*, *T. reesei*; *3*, *P. verruculosum*; *4*, *A. niger*; *5*, *Corynascus* sp.

Since the condensation reaction is known to be controlled by thermodynamics [1-3], one may expect that the increase in temperature would result in shorter time to attain the equilibrium. Indeed, while at 50 ^{o}C the total concentration of disaccharides gradually increased with time and it still did not achieve maximum even after 5 days of the reaction, at higher temperatures (65-75 ^{o}C) the maximum disaccharide concentration was achieved after 2 days of the process. As an example, Fig. (**2**) shows the kinetics of the condensation reaction catalyzed by *P. verruculosum* enzyme preparation at pH 4.5 and protein dosage of 4 mg/ml, while Fig. (**3**) shows the yields of disaccharides after 2 or 5 days for different enzyme samples at various temperatures. For all fungal species the maximum yield of disaccharides was observed at either 65-70 ^{o}C (*A. niger*) or 70 ^{o}C (other enzymes). This result was rather unexpected. Fungal enzymes, including β-glucosidases, are typically unstable at such temperature [25, 26]. So, enzymatic processes lasting a few days, such as enzymatic hydrolysis of cellulose, are usually performed at a lower temperature (~50^{o}C) [27, 28]. On the other hand, substrates, like low-molecular-weight sugars, may cause stabili-

zation effects on enzymes [26]. We carried out the condensation reaction at very high glucose concentration (60%), so the substrate seems to stabilize the enzymes dramatically. All further experiments were carried out at 70 °C for 48 h.

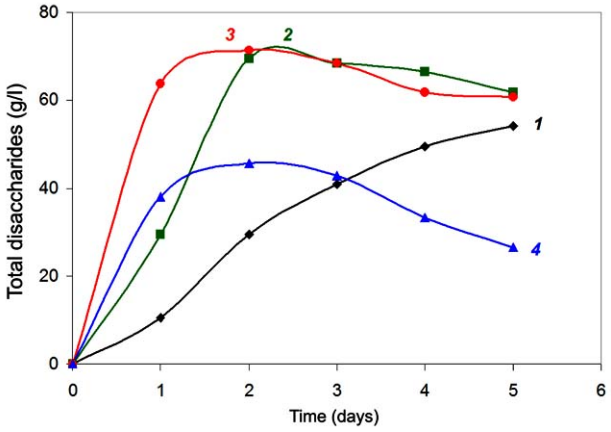

Fig. (2). Kinetics of disaccharide formation in the glucose (60% w/v) condensation reaction catalyzed by *P. verruculosum* enzyme at pH 4.5 and protein dosage of 4 mg/ml. *1*, 50 °C; *2*, 65 °C; *3*, 70 °C; *4*, 75°C.

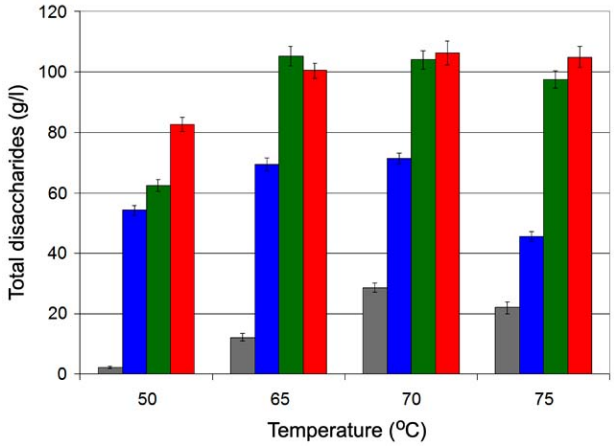

Fig. (3). Disaccharide production from 60% (w/v) glucose by fungal enzymes at pH 4.5 and different temperatures using protein loading of 4 mg/ml: *T. reesei* (grey), *P. verruculosum* (blue), *A. niger* (green), *Corynascus* sp. (red). The reaction time was 120 h (50 °C) or 48 h (65, 70, 75 °C).

The effect of pH on the yields of disaccharides is shown in Fig. (**4**). For *T. reesei* and *P. verruculosum* enzymes, the maximum disaccharide synthesis was observed at pH 4.5, while for the *A. niger* and *Corynascus* sp. preparations the optimum was found at pH 4.0 and 3.5, respectively. It is interesting to note that the purified β-glucosidases isolated in our laboratory from the fungal species under study had pH-optima of hydrolytic activity at pH 5.5 (*P. verruculosum*), 5.0 (*T. reesei*), 4.5 (*A. niger*) [25] and 4.0 (*Corynascus* sp., unpublished data). So, in all cases the enzyme pH-optimum for oligosaccharide synthesis by condensation seems to be shifted to acidic pH region (by 0.5-1.0 unit of pH) compared to the hydrolytic pH-optimum of the same enzyme.

The total concentrations of disaccharides synthesized from 60% (w/v) glucose by different enzyme preparations

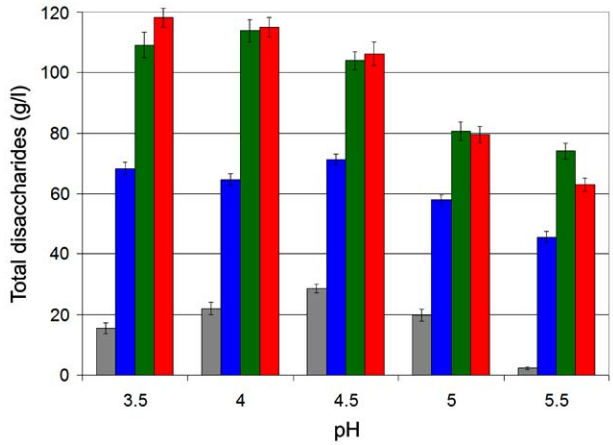

Fig. (4). Disaccharide production from 60% (w/v) glucose at different pH after 48 h of the enzymatic reaction at 70 °C and protein loading of 4 mg/ml: *T. reesei* (grey), *P. verruculosum* (blue), *A. niger* (green), *Corynascus* sp. (red).

after 48 h of the condensation reaction at 70 °C and optimal pH as well as the composition of each product mixture, determined by HPLC, are presented in Table **1**. It should be noted that the dosage of *A. niger* and *Corynascus* sp. enzymes was 4 mg of protein per ml, while in the case of *T. reesei* and *P. verruculosum* the enzyme dosage was 8 mg/ml. These protein dosages correspond to the β-glucosidase activity of 164, 74, 2.4 and 10.4 U/ml, respectively. For the first two samples, the increase in the enzyme concentration did not result in the improvement of the product yield (the yield even slightly decreased), while at the double dosage of *T. reesei* and *P. verruculosum* enzymes the disaccharide yield increased by 25-35% (data are not shown); that was evidently the result of much lower β-glucosidase activity of the last two preparations.

The highest concentration of disaccharides (114-118 g/l) was achieved in the case of *A. niger* and *Corynascus* sp. enzymes; the *P. verruculosum* sample slightly conceded them in the yield of condensation products. The total yields of disaccharides made up 20, 21 and 17% of the theoretical yield, respectively. All the mentioned enzyme preparations formed gentiobiose as a major product (more than 50-60% of the total disaccharides). The *T. reesei* preparation displayed much lower synthetic efficiency (the yield of disaccharides was only 6% of the theoretical), laminaribiose being the main condensation product. Perhaps, the lower yield of products was the result of the lowest β-glucosidase activity of this enzyme sample compared to the other enzymes under study.

Relatively high levels of sophorose in the disaccharide mixtures produced by *Corynascus* and *Penicillium* enzymes (13 and 10%) deserve a special attention. It is believed that sophorose play an important role in the induction of cellulase biosynthesis by fungi [29, 30]. The use of gentiobiose as an inductor of enzyme biosynthesis has also been reported [30]. Cellulases are important commercial enzymes produced on a large scale by many companies in the world, and they are used in different biotechnological applications [31, 32]. The process of renewable lignocellulosic biomass conversion to ethanol and other useful products may become the most im-

Table 1. Disaccharide Synthesis from 60% (w/v) Glucose Using Enzymatic Condensation Reaction (48 h, pH 3.5-4.5, 70°C)

Organism	Total Concentration of Disaccharides (g/l)	Sophorose (%)	Laminaribiose (%)	Cellobiose (%)	Gentiobiose (%)
A. niger	114	8	15	16	61
Corynascus sp.	118	13	9	11	67
P. verruculosum	96	10	16	21	53
T. reesei	35	5	54	18	23

portant cellulase application in the near future. Unlike cellobiose and laminaribiose that can be produced by hydrolysis of widely abundant polysaccharides (cellulose, laminarin, curdlan), sophorose and gentiobiose should be obtained by a different way. The enzymatic synthesis of these disaccharides from glucose by the condensation reaction may become a simple and inexpensive method, if reasonable yields of the products are obtained.

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