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Enzymatic redox isomerization of 1,6-disaccharides by pyranose oxidase and NADH-dependent aldose reductase

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Abstract

Pyranose 2-oxidase, a homotetrameric FAD-flavoprotein from the basidiomycete *Trametes multicolor*, catalyzes regioselectively the oxidation of the 1 → 6 disaccharides allolactose [β-D-Gal*p*-(1 → 6)-D-Glc], gentiobiose [β-D-Glc*p*-(1 → 6)-D-Glc], melibiose [α-D-Gal*p*-(1 → 6)-D-Glc], and isomaltose [α-D-Glc*p*-(1 → 6)-D-Glc] at position *C*-2 of their reducing moiety. The resulting glycosyl D-*arabino*-hexos-2-uloses can be reduced specifically at *C*-1 by NAD(P)H-dependent aldose reductase from the yeast *Candida tenuis*. By this novel, two-step redox isomerization process the four disaccharide substrates could be converted to the corresponding keto-disaccharides allolactulose [β-D-Gal*p*-(1 → 6)-D-Fru], gentiobiulose [β-D-Glc*p*-(1 → 6)-D-Fru], melibiulose [α-D-Gal*p*-(1 → 6)-D-Fru], and isomaltulose (palatinose, [α-D-Glc*p*-(1 → 6)-D-Fru]) in high yields. These products could find application in food technology as alternative sweeteners. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Pyranose oxidase; Aldose reductase; Allolactose; Isomaltulose; Aldos-2-ulose

1. Introduction

The enzyme pyranose 2-oxidase (P2O, glucose 2-oxidase, pyranose:oxygen 2-oxidoreductase, EC 1.1.3.10), which catalyzes the oxidation of several aldopyranoses at the position *C*-2 to yield the corresponding 2-ketoaldoses (aldos-2-uloses, 'osones'), is widely distributed among wood-degrading basidiomycetous fungi, in which it is considered a constituent of the ligninolytic system supplying peroxidases with hydrogen peroxide [1]. It has been purified and characterized from several microorganisms including *Phanerochaete chrysosporium* [2,3], *Phlebiopsis (Peniophora) gigantea* [4,5], *Pleurotus ostreatus* [6], *Polyporus obtusus* [7], *Trametes multicolor* [8], *T. (Coriolus) versicolor* [9], and the unidentified basidiomycete no. 52 [10]. The data available at present reveal some general similarities among P2Os from these different fungi. Typically, P2O is a rather large, homotetrameric protein that contains covalently bound flavin adenine dinucleo-

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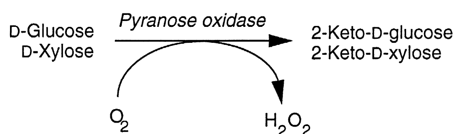

Fig. 1. In vivo reaction catalyzed by the fungal periplasmic enzyme pyranose oxidase.

tide. The in vivo substrates of P2O are most probably D-glucose and D-xylose, which are abundant in lignocellulose and which are oxidized to 2-keto-D-glucose (D-*arabino*-hexos-2-ulose, 'D-glucosone') and 2-keto-D-xylose (D-*threo*-pentos-2-ulose, 'D-xylosone'), respectively (Fig. 1). In addition, P2O also exerts significant activity on a number of other carbohydrates including L-sorbose, D-glucono-1,5-lactone, D-allose or D-galactose [11]. This substrate selectivity, however, varies to some extent among P2Os isolated from different fungal sources. During these oxidation reactions, the electrons are transferred to molecular oxygen, resulting in the formation of hydrogen peroxide.

Pyranose oxidases are gaining increased interest in carbohydrate chemistry as valuable biocatalysts for the synthesis of dicarbonyl sugar derivatives. In contrast to the chemical syntheses of these compounds, which often give low yields and a number of by-products [12–14], the enzymatic oxidations of aldoses and ketoses to dicarbonyl derivatives typically show high regioselectivity on the unprotected sugar substrates, as well as close to complete conversions of these substrates to the corresponding products. Dicarbonyl sugars represent attractive synthons, combining the high number of functional groups and the inherent chiral information of sugars with a higher degree of chemical diversity in their functional groups [11]. They are not produced on an industrial scale at present; however, they could be attractive intermediates for several proposed subsequent reactions. For 2-keto-D-glucose these include the oxidation to 2-keto-D-gluconic acid and further transformation to D-isoascorbic acid, its reduction to D-fructose, D-mannitol or D-sorbitol, as well as its reductive amination yielding 1,2-diaminosorbitol/mannitol [15]. The oxidation of a variety of monosaccharides on laboratory scale using both free and immobilized P2O preparations has been described [11,16,17]. Recently, we reported the use of free P2O from *T. multicolor* for the efficient oxidation of D-glucose and D-galactose to the corresponding aldos-2-uloses, 2-keto-D-glucose ('D-glucosone') and 2-keto-D-galactose (D-*lyxo*-hexos-2-ulose, 'D-galactosone') [18,19]. These could be reduced specifically at *C*-1 by yeast aldose reductase yielding D-fructose and D-tagatose, respectively. Especially the latter is of special interest for food technology as it is a low-calorie noncariogenic sweetener with a sweetness comparable to that of sucrose.

Pyranose oxidase has so far been mainly employed for the oxidation of various monosaccharides, the only exception to this is the report on the activity of the *P. gigantea* enzyme with gentiobiose [11]. Only recently we could show that *T. multicolor* P2O exerts significant activity on several 1,6-disaccharides [20], oxidizing them regioselectively at position *C*-2 of the reducing sugar moiety. Here we report the application of soluble pyranose oxidase from *T. multicolor* for the transformation of four $1 \rightarrow 6$-linked disaccharides, i.e., gentiobiose [β-D-Glc*p*-($1 \rightarrow 6$)-D-Glc], allolactose [β-D-Gal*p*-($1 \rightarrow 6$)-D-Glc], isomaltose [α-D-Glc*p*-($1 \rightarrow 6$)-D-Glc] and melibiose [α-D-Gal*p*-($1 \rightarrow 6$)-D-Glc], into the corresponding glycosyl D-*arabino*-hexos-2-uloses ('disaccharide osones'). In a second step, these products are subject to

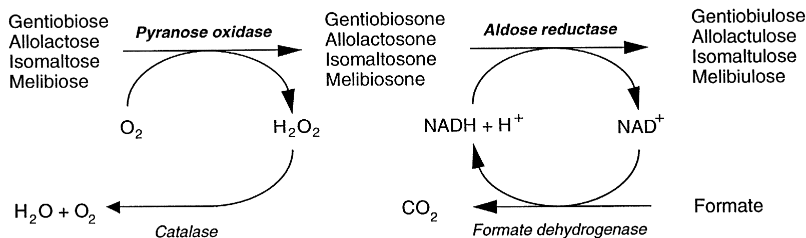

Fig. 2. Isomerization of aldose-disaccharides into the corresponding ketoses by an oxidoreductive route employing pyranose oxidase and aldose reductase.

the enzymatic *C*-1 reduction, yielding the corresponding keto-disaccharides (glycosyl-fructoses), by aldose (xylose) reductase (ALR; alditol:NAD(P)$^+$ 1-oxidoreductase, EC 1.1.1.21). This enzyme is known to perform the initial step in D-xylose assimilation in yeasts and mycelial fungi, i.e., the NAD(P)H-dependent reduction of this pentose to xylitol [21]. By our novel enzymatic redox process, the 1,6-disaccharides gentiobiose, allolactose, isomaltose and melibiose are isomerized to the corresponding keto-disaccharides gentiobiulose, allolactulose, isomaltulose (palatinose) and melibiulose, respectively, in high yields (Fig. 2). These products could find application in food technology as alternative sweeteners. These isomerizations of 1,6-disaccharides are not catalyzed by glucose (xylose) isomerases that are used for the technical-scale conversion of D-glucose into D-fructose (Kieweg and Kulbe, unpublished results).

2. Experimental

2.1. Materials and chemicals

Allolactose was prepared from amygdalin using a new synthetic strategy (Riva and Nonini, unpublished results). All other chemicals were of the highest purity available and obtained from Sigma (St. Louis, MO, USA) unless otherwise stated. Isomaltose was from ICN (Costa Mesa, CA, USA); horseradish peroxidase (EC 1.11.1.7), grade I, was from Roche-Boehringer Mannheim (Mannheim, Germany), and catalase (EC 1.11.1.6) from bovine liver was purchased from Fluka (Buchs, Switzerland). Formate dehydrogenase (FDH, EC 1.2.1.2) from *Candida boidinii* was a kind gift from Dr. Udo Kragl (Forschungszentrum Jülich, Germany). Material for preparative chromatography was obtained from Amersham-Pharmacia (Uppsala, Sweden), and the dye Reactive Red 31 (Procion Red H-8B) was from ICI (Manchester, UK).

2.2. Microbial strains and culture conditions

The wild type strain of the basidomycete fungus *T. multicolor* MB 49 was used as the source of pyranose oxidase. P2O was produced on a basal medium containing whey powder, which was added so that the final lactose concentration was 2.5%, 1.0% peptone from casein and 0.1% KH_2PO_4 as described elsewhere [22]. The average biomass and P2O yields in these laboratory fed-batch cultivations were approximately 15 g dry mycelium and 1600 U P2O activity/l, respectively. For the production of aldose reductase, the yeast *C. tenuis* CBS 4435 (Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) was grown on a medium containing 2% xylose, 1.6% yeast extract, 0.4% peptone from casein, 0.53% NH_4Cl and 0.05% $MgSO_4 \cdot 7H_2O$ as previously described [23]. These fermentations yielded approximately 19 g dry biomass and 3700 U ALR activity/l fermentation broth.

2.3. Enzyme assays

Pyranose oxidase activity was determined spectrophotometrically at 420 nm and 30°C by measuring the formation of H_2O_2 in a peroxidase-coupled assay using ABTS (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); $\varepsilon_{420} = 43{,}200\ M^{-1}\ cm^{-1}$ [24]) as the chromogen [4]. The standard assay mixture (1 ml total) contained 1 μmol ABTS in potassium phosphate buffer (50 mM, pH 6.5), 2 units (U) horseradish peroxidase, 100 μmol D-glucose and a suitable amount of the P2O sample. One unit of P2O activity is defined as the amount of enzyme necessary for the oxidation of 2 μmol of ABTS/min under the given conditions. Aldose reductase activity was assayed spectrophotometrically at 340 nm and 25°C by measuring the consumption of NADH ($\varepsilon_{340} = 6220\ M^{-1}\ cm^{-1}$). The standard assay mixture (1 ml) contained 707 μmol D-xylose and 0.22 μmol NADH in 300 mM phosphate buffer (pH 6.0). One unit of ALR activity refers to the amount of enzyme consuming 1 μmol of NADH/min. Protein concentrations were determined according to the dye-binding method of Bradford [25] using bovine serum albumin (BSA) as standard.

2.4. Enzyme purification

For the partial purification of pyranose oxidase washed mycelia of *T. multicolor* (2 g wet weight) were resuspended in 10 ml phosphate buffer (50

mM, pH 6.5) containing 10 mM EDTA, homogenized and disrupted by using a laboratory homogenizer (24,000 rpm, 4°C, 10 min). Cell debris were removed by ultracentrifugation (30,000 × *g*, 4°C, 20 min) and the clear supernatant loaded onto a Source 30Q column pre-equilibrated with 20 mM BisTris buffer, pH 6.5 (buffer A). Subsequently, P2O was eluted with a linear gradient of 0–100% 1 M KCl in buffer A. Fractions of the eluate were screened for P2O activity and the most active fractions pooled.

Aldose reductase was purified to homogeneity by dye–ligand pseudoaffinity chromatography followed by an anion exchange chromatography step [26]. Washed cells of *C. tenuis* were diluted 1:3 in 30 mM phosphate buffer (pH 7.0) and disintegrated in a continuously operated Dyno-Mill (glass bead diameter 0.25–0.5 mm) at 4°C and an average residence time of 10 min. Cell debris were removed by ultracentrifugation (110,000 × *g*, 40 min) and the clear supernatant was loaded onto a Reactive Red 31-Sepharose CL-4B column equilibrated with 50 mM phosphate buffer, pH 7.0. ALR was then eluted with a linear gradient of 1–2 M NaCl in buffer A, fractions containing ALR activity were pooled and concentrated by 70% ammonium sulfate precipitation. The final purification step was performed on a Mono-Q HR 5/5 column preequilibrated with 20 mM Tris/HCl buffer, pH 7.0, containing 0.1% Tween-20. Elution was accomplished with a linear gradient of 0–100 mM NaCl in the same buffer.

2.5. Carbohydrate conversions

The conversion of the 1 → 6 disaccharides into corresponding glycosyl D-*arabino*-hexos-2-uloses employing P2O was performed batchwise at 30°C in 30-ml conical flasks with a total working volume of 10 ml. The reaction system contained 100 mM of the disaccharide substrates in potassium phosphate buffer (25 mM, pH 6.5), 10 U ml^{-1} partially purified P2O, 10,000 U ml^{-1} catalase and 5 mg ml^{-1} BSA. The reaction system was continuously stirred and oxygenated using pure oxygen (0.5 vol. oxygen/vol. fluid/min), which was bubbled through a porous sintered glass disk. At the times stated, samples were taken to monitor chromatographically the course of the reaction. After completion of the conversion, proteins were separated by ultrafiltration using Millipore Minitan ultrafiltration membranes, 10 kDa cut-off (Bedford, MA, USA). The glycosyl D-*arabino*-hexos-2-ulose solutions thus obtained were not purified by any further step.

For the ALR-catalyzed reduction of the disaccharide osones at 25°C, 10 ml of the crude, ultrafiltered sugar solutions containing 50 mM glycosyl D-*arabino*-hexos-2-uloses in 50 mM phosphate buffer, pH 6.9, was supplemented with 1.4 U ml^{-1} ALR, and 0.5 mM NAD^+. The continuous regeneration of the coenzyme was accomplished using the system FDH/formate [27] at concentrations of 0.7 U ml^{-1} FDH and 100 mM sodium formate, respectively. Reaction products were purified by a cation exchange step (Amberlite CG-120-II, Fluka; H^+ form), a subsequent anion exchange step (Dowex 1 × 8, Fluka; acetate form), and by lyophilization.

2.6. Analytical methods

The identification and quantification of the 1 → 6 disaccharides and the glycosyl D-*arabino*-hexos-2-uloses were done by HPLC using an Aminex HPX-87P column (300 × 7.8 mm; Bio-Rad, Hercules, CA, USA) and refractive index detection. Water was used as a mobile phase at a flow rate of 0.85 ml min^{-1} and a column temperature of 70°C. Alternatively, they were analyzed by employing an Ostion LGKS 0800 Na column (250 × 8 mm; Watrex, Prague, Czech Republic) and refractive index detection. Water was the mobile phase at a flow rate of 0.5 ml min^{-1} and a column temperature of 70°C. The ^{13}C NMR spectra of the keto disaccharide products were recorded in D_2O on a Bruker AC-300 instrument.

3. Results and discussion

For biotransformation purposes, pyranose oxidases have almost exclusively been used for the oxidation of various monosaccharides into the respective dicarbonyl derivatives [11,16–19,28]. The only exception reported so far has been the oxidation of the disaccharide gentiobiose catalyzed by *P. gigantea* P2O, which was investigated in some detail [11]. The *P. gigantea* enzyme, however, did not

oxidize other disaccharides, including the 1,6-disaccharides melibiose and isomaltose. Interestingly, P2O from *T. multicolor* exerts significant activity with all three of these disaccharides, which are regioselectively oxidized at position *C*-2 of the reducing glucosyl moiety as was unequivocally shown by NMR/MS analysis [20]. Up to now, these disaccharide osones have only been synthesized chemically [29–31]. In addition, *T. multicolor* P2O also converts the structurally related disaccharide allolactose [β-D-Gal*p*-(1 → 6)-D-Glc] which has now become available by a new synthetic method. All four of these disaccharides were converted in a batchwise mode to the corresponding glycosyl D-*arabino*-hexos-2-uloses employing a partially purified P2O preparation as a soluble biocatalyst. The time courses of these oxidation reactions are shown in Fig. 3. With the exception of isomaltose, which is a rather poor P2O substrate, conversion to the different glycosyl D-*arabino*-hexos-2-uloses was almost quantitative within the reaction time chosen for these experiments (up to 100 h) and no by-products could be detected by HPLC. Conversion of the two β-1,6-linked disaccharides proceeded considerably faster than that of the α-1,6-linked substrates (Fig. 3) and gentiobiose clearly was the preferred substrate, which is also reflected by some of its kinetic constants (K_m 62.4 mM, v_{max} 22.7% relative to glucose) when compared to melibiose (K_m 120.3 mM, v_{max} 8.6%). In comparison, the Michaelis constant for the natural

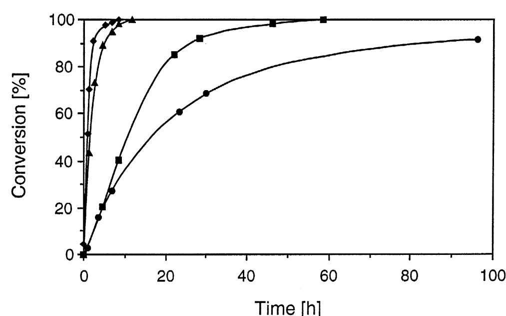

Fig. 3. Discontinuous conversion of 1,6-disaccharides into the corresponding glycosyl D-*arabino*-hexos-2-uloses catalyzed by pyranose oxidase from *T. multicolor*. Conditions: 100 mM substrate; 10 U ml^{-1} P2O; 10,000 U ml^{-1} catalase; 5 mg ml^{-1} BSA; 25 mM phosphate buffer, pH 6.5; 30°C. Symbols: ◆, gentiobiosone; ▲, allolactosone; ■, melibiosone; ●, isomaltosone.

substrate D-glucose was determined to be 0.74 mM. P2O was found to be perfectly stable during these reactions, provided that the two stabilizing agents catalase and BSA were added. The addition of catalase ensuring the fast destruction of hydrogen peroxide is imperative for the operational stability of the biocatalyst, furthermore, BSA was found to increase the stability of P2O during its catalytic action [19]. Under these reaction conditions, more than 95% of the initially added P2O activity could be recovered by ultrafiltration and could be reused for subsequent biotransformations. On the other hand, catalase from bovine liver was unstable under these reaction conditions and could not be recovered in appreciable amounts after one round of batchwise sugar conversion.

The crude glycosyl D-*arabino*-hexos-2-ulose solutions that were obtained by ultrafiltration were directly used in the subsequent, aldose reductase-catalyzed reduction reactions. After adjusting the pH value of these solutions to 6.9, which was necessary since FDH is not active at lower pH values, it was noticed that their color, which initially was clear, turned yellowish, indicating that the dicarbonyl sugars were undergoing some degradation. In addition, at least two so far unidentified by-products were detected by HPLC in these solutions even before the enzyme ALR was added. Most probably, these by-products are formed due to an increase in the pH caused by inhomogeneities when adding dropwise the base Tris for adjusting the pH. These inhomogeneities were certainly more pronounced and hence more deleterious in our small-scale experiments performed in 10 ml reaction volume. It is well known that aldos-2-uloses are not stable in the alkaline region and rapidly decompose under these conditions [12,32].

Whereas disaccharide aldose sugars are not reduced at appreciable rates by *C. tenuis* ALR [26], the enzyme exerts significant activity on the glycosyl D-*arabino*-hexos-2-uloses used in this study. It has been shown for yeast ALRs that the *C*-2 position of the carbohydrate substrate is of great importance for the enzyme–sugar interaction [33]. Introducing a carbonyl group at this position of the reducing glucosyl moiety of the 1,6-linked disaccharides drastically changes the reactivity of the enzyme with these substrates. This is also reflected by the catalytic

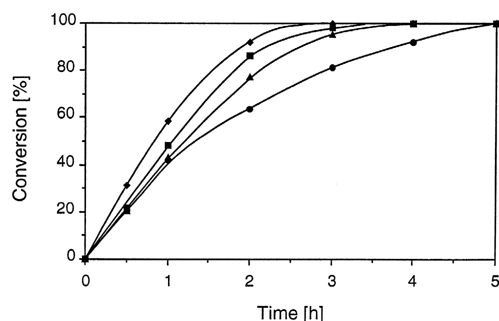

Fig. 4. Discontinuous conversion of the glycosyl D-*arabino*-hexos-2-uloses into the corresponding ketose disaccharides catalyzed by aldose reductase from *C. tenuis*. Conditions: 50 mM substrate; 1.4 U ml^{-1} ALR; 0.5 mM NAD^{+}; 0.7 U ml^{-1} FDH; 100 mM Na formate; 50 mM phosphate buffer, pH 6.9; 25°C. Symbols: ◆, gentiobiulose; ▲, allolactulose; ■, melibiulose; ●, isomaltulose.

efficiencies that were measured for the four disaccharide osones. For isomaltosone, gentiobiosone, melibiosone and allolactosone these were found to be 577, 239, 92, and 73 M^{-1} s^{-1}, respectively. It is interesting to note that these values are in the range of the catalytic efficiency for the in vivo substrate D-xylose which is 211 M^{-1} s^{-1} and significantly higher than that for D-glucose (16 M^{-1} s^{-1}). As is evident from these data, the two glucosyl D-*arabino*-hexos-2-uloses are better substrates than the D-Gal-containing disaccharide osones. However, the value obtained for isomaltosone must be considered with some caution, since the commercially available preparation of isomaltose contained glucose as impurity, which was completely converted to 2-ketoglucose by P2O. This dicarbonyl sugar is an excellent substrate of ALR (catalytic efficiency of 4684 M^{-1} s^{-1}) and certainly interferes with the determination of the kinetic constants.

The time courses of the batchwise conversion of the four glycosyl D-*arabino*-hexos-2-uloses employing NADH-dependent ALR and the system FDH/formate for coenzyme regeneration are shown in Fig. 4. All of the reactions were completed within 5 h. The coenzyme regeneration system employed in this study was well suited for this conversion and a total turnover number of 100 was achieved for the coenzyme. The glycosyl D-*arabino*-hexos-2-uloses that had remained after adjusting the pH value could be almost completely converted into the corresponding aldopyranosyl ketoses. Again, this indicates that the osones were mainly degraded because of unfavorable pH conditions when the base was added to their solution. The overall yield for the conversion of all four disaccharide osones into the corresponding ke-

Table 1
^{13}C chemical shifts of the disaccharides in D_2O (δ in ppm)

Carbon	Chemical shifts (ppm)			
	Isomaltulose	Melibiulose	Gentiobiulose	Allolactulose
C-1, α-Fru	63.8	63.6	63.6	63.5
C-1, β-Fru	63.5	63.4	63.4	63.4
C-2, α-Fru	105.6	105.6	105.5	105.5
C-2, β-Fru	102.6	102.6	102.6	102.6
C-3, α-Fru	82.7	82.6	82.7	82.6
C-3, β-Fru	76.4	76.2	72.9	72.9
C-4, α-Fru	77.1	77.1	77.2	77.2
C-4, β-Fru	75.6	75.5	75.7	75.6
C-5, α-Fru	80.9	80.9	80.8	80.8
C-5, β-Fru	79.9	79.9	80.1	80.1
C-6, α-Fru	67.8	68.0	70.3	70.6
C-6, β-Fru	68.7	68.9	71.8	71.9
C-1′, Glu (Gal)	99.2	99.3	103.5	104.1
C-2′, Glu (Gal)	72.3	70.1	74.0	71.7
C-3′, Glu (Gal)	74.0	70.3	76.5	75.5
C-4′, Glu (Gal)	70.5	69.2	70.5	69.5
C-5′, Glu (Gal)	72.8	71.9	76.8	76.0
C-6′, Glu (Gal)	61.5	62.0	61.6	61.8

toses was approximately 80%. Isomaltulose prepared by this novel two-step enzymatic oxidoreduction process was compared to commercially available, authentic isomaltulose (palatinose, [α-D-Glc p-(1 → 6)-D-Fru]) by HPLC using two different columns and was found to have identical retention times. In addition, the products were isolated by cation and anion exchange followed by lyophilization and their structures were confirmed by ^{13}C NMR. These data are summarized in Table 1. The ^{13}C NMR data obtained for isomaltulose and gentiobiulose were in excellent agreement with data previously published [34–36], thereby confirming the structures of the products obtained in our two-step oxidoreductive isomerization process employing P2O and ALR. Of these products, isomaltulose is produced on an industrial scale (35,000 tons/year) by transglucosylation using immobilized cells of *Protaminobacter rubrum* [37]. Isomaltulose can be used as a noncariogenic, dietetic sweetener with a slow intestinal absorption rate that has a relative sweetness of 40% compared to sucrose. Because of the structural similarities one can expect that the other ketose-disaccharides, that were produced by the combination of P2O and ALR, i.e., gentiobiulose, allolactulose and melibiulose, may have similar favorable properties and could be used in food technology. Furthermore, these keto-disaccharides could be used as starting material for further modifications. Ketoses have only recently been described as attractive organic raw materials for the production of industrially useful chemicals, however, they are far from being fully exploited [34].

Acknowledgements

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