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Review

Industrial carbohydrate biotransformations

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Abstract—Nearly all major industrial processes which involve carbohydrates, include biotechnological transformations. This is due to the complex nature of carbohydrates where stereo- and regioselectivity are highly complex and difficult to control. Enzymes and microorganisms work highly selectively and efficiently in water solution, and provide high yield in general. The article focuses on different types of reactions, including large-scale processes. Topics are hydrolytic reactions, including starch processing, oxidation and reduction transformations including organic acids, such as gluconic and ketogluconic acids and vitamin C synthesis, and isomerization and transfer reactions, which are established on a very large scale to produce glucose/fructose syrups and sucrose isomers. The article will further discuss some mechanistic aspects which are relevant for technology and present selected details of industrial-scale processing. Finally an outlook outlines perspectives of future processes.

Keywords: Enzyme catalysis; Industrial biocatalysis; Large scale processes; Enzyme engineering; Substrate engineering; Metabolic engineering

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1. Introduction

The majority of industrial processes involving carbohydrates include biotechnological transformations due to the requirements of specificity and stereoselectivity. Table 1 provides an overview of some selected products out of a large number of industrially established processes. Selected examples are discussed in order to illustrate the potential of biotransformations, including hydrolytic, synthetic transfer, isomerization reactions, oxidation/reduction processes, and complex transformations.

As an example for a hydrolytic reaction, both basic considerations, including genetic engineering of the biocatalyst—relevant for technical application—and process data are discussed in the context of starch processing, in order to highlight the intimate interrelation of these. The subsequent step of glucose isomerization also shows the principle of the enzymatic mechanism as well as enzyme immobilization, including a typical reactor, to make obvious the importance and convenient use of immobilized biocatalysts. The following example of sucrose isomerization (a synthetic transfer reaction, as is cyclodextrin formation) presents similar aspects as well as the combination of chemoenzymatic processing, including a catalytic hydrogenation step, to yield the final product. Several examples of oxidation processes are mentioned, all selective, that should highlight the potential of this type of microbial transformations. Among more complex transformations, vitamin C production has been a process at the large scale including chemical and microbial steps, a traditional chemical process that was substituted in recent years by more straight microbial transformations. The outlook finally provides more examples of potential new tailored reactions to achieve improved processing, as in the case of vitamin C, or modified biocatalysts, and new products.

Certainly, there are already major classical and highly efficient chemical processes, including sorbitol, mannitol and isomalt production, as well as the manufacture of cellulose and starch derivatives, such as esters (acetates, succinates, etc.) and ethers (carboxymethyl, etc.).[1,2]

Biocatalysts in general exhibit excellent substrate specificity, regioselectivity, stereospecificity, thus satisfying the increased demand for optically pure compounds. Biotransformations proceed at mild reaction conditions. They may save additional reaction steps compared to organic synthesis, reducing production costs. Biocatalysis often results in sustainable technology, using renewable raw materials. Many enzymes have become commercially available and cheap.

A considerable number of processes using immobilized biocatalysts for converting sugars and polysaccharides into new products, the synthesis of oligosaccharides and derivatives, mostly sweeteners and functional food, are applied industrially, and several are under development. They comprise hydrolytic as well as synthetic reactions by hydrolases and glycosyltransferases.[6]

Enzymatic processes fully exhibit their potential in the carbohydrate field, because they control regio- and stereospecificity, which makes them superior to chemical

Table 1. Selected products and value of products from carbohydrates by biotechnology[3–5] F = fermentation; E = enzyme technology

	Product	World production 10^3 (Tons/year)	Value 10^9 (€/year)	Production method
Food and feed	Beer/wine	170×10^3	200	F, E
	Glucose (incl. sub-prod.)[b]	ca. 30×10^3		E
	Glucose–fructose mix	12×10^3	5	E
	Isomaltulose	100		E
	Oligosaccharides	>6		E
Chemicals	Vitamin C	>100	5	F, E[a]
	Citric acid	800	1	F
	Gluconic acid	100	0.1	F
	Cyclodextrins	5		E
	Polysaccharides			F, E
Basic chemicals	Ethanol	46×10^3 m^3/a	15	E, F

[a] Several chemical steps are involved in the classical Reichstein process, see below.
[b] incl. sub-prod.: including subsequent products made from glucose, such as ethanol, glucose–fructose syrup, organic and amino acids.

and classical catalytic reactions. The potential to build up different linkages via α- and β-bonds to positions 1 through 6 in hexose sugars makes the synthesis of oligosaccharides dramatically complex. In general, enzymes easily form a single bond or one main product in high yield (mostly >80%) with low byproduct formation of isomers. Synthesis of *gluco*- and *fructo*-oligosaccharides based on sucrose by glycosyltransferases (GTF) furthermore utilizes the advantage of the high glycosidic bond energy which nearly equals that of nucleotide activated sugars (−23 kJ/mol).[7] In water as solvent, this drives the synthesis to high yields which is a prediction for food grade and economic processing. Thus a major range of oligo- and polysaccharides of the glucan as well as fructan type are produced.[8,9]

2. Hydrolytic/transfer reactions

2.1. Starch processing

Starch is the most important carbon and energy source among plant carbohydrates, and it is the second following cellulose, with 10^{10} t/a, in total biosynthesis. Its industrial production is about 45 Mio t/a (EU ca. 7.5×10^6 t/a). The most important starch sources are corn (maize, 3.6 Mio t/a starch in the EU), wheat and potato.[3] Starch therefore represents the source of a wide range of products with major economic importance. In particular starch (amylose, amylopectin) hydrolysis yields: (i) dextrins of various glucan chain length, with α-amylase (endo-acting); (ii) maltose, with β-amylase (exo-acting); (iii) glucose, with α-amylase and glucoamylase (exo-acting), depending on the enzymes' active centre, with an array of consecutive subsites that extends throughout the active-site cleft (Fig. 1).

2.1.1. Enzymes and products. Enzymes for starch processing, including α-amylase, glucoamylase, β-amylase, pullulanase, isoamylase and glucose isomerase, comprise about 30% of the world's industrial enzyme production.[3]

Amylolytic enzymes bind substrate glucosyl residues at an array of consecutive subsites that extends throughout the active-site cleft. In typical endo-acting enzymes such as α-amylases the substrate-binding region comprises from five to eleven subsites.[10] The structure of the binding region decides on substrate and product selectivity (Fig. 1).

2.1.2. Hydrolytic enzymes: selectivity and mechanistic aspects

2.1.2.1. α-Amylases. Enzymes have been extensively used in industrial starch hydrolysis.[11,12] Commercial products from α-amylase hydrolysis comprise dextrins of various glucan chain length, for example, soluble starch (DE, dextrose equivalent of 10–15), a major product with a DE typically near DE 42, with about 19% glucose, 14% maltose, 12% maltotriose and 55% higher oligosaccharides. The production of crystalline glucose or of glucose–fructose syrup requires a product with a DE of 95–96%.[13] A number of starch-converting enzymes, the α-amylases, belong to family 13 glycosyl hydrolases (GH 13). The structure of a range of α-amylases has been elucidated.[14] This group of enzymes shares a number of common characteristics such as a $(\beta/\alpha)_8$ barrel structures, the hydrolysis or formation of α-glycosidic bonds, and a number of conserved amino acid residues in the active site. Three-dimensional structures, mechanistic principles deduced from structure–function relationships, and properties such as kinetics, selectivity and stability have been investigated, reported and summarized in several reviews.[15–17] The established catalytic mechanism of the α-amylase family is

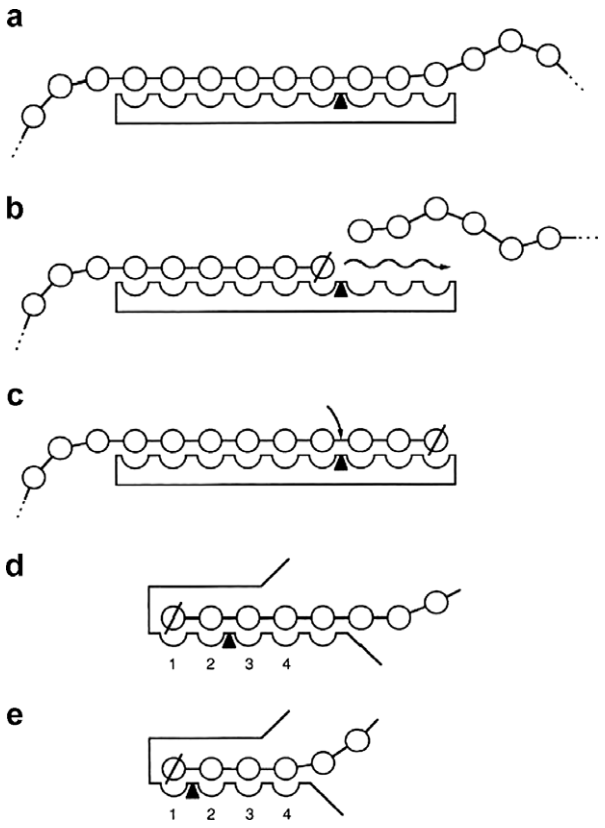
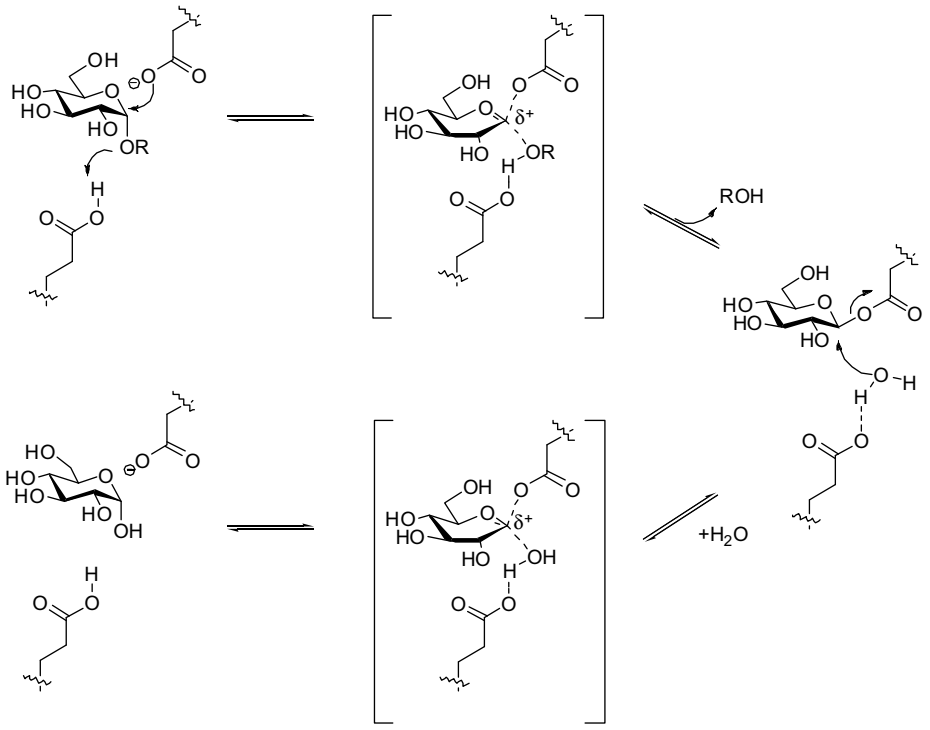


Figure 1. Scheme of subsites in α-amylase, β-amylase and glucoamylase. (a–c) Reaction steps with α-amylase, (a) accommodation/fitting and binding of an amylose chain to the sites near active centre; (b) one product leaving the active centre after the hydrolytic step; (c) reaccommodation (one out of different possibilities) of the remaining part of amylose, ready for the next hydrolytic step (these steps illustrate the range of products typically formed by α-amylase); (d) short amylose or dextrin chain, respectively, fitting into β-amylase, where maltose is released after the hydrolytic step (the active centre accommodates the product only this way), with two binding sites at left; (e) fitting of a dextrin chain to the active centre of glucoamylase, only one glucose moiety can fit left of the active centre, releasing glucose subsequent to the hydrolytic step.

α-retaining, thus including two S_N2 type reactions (Scheme 1).[16] The (general acid) glutamate (Glu 230 in *Aspergillus niger* numbering) donates a proton to the glycosidic bond between two substrate glucosyl units bound in the active site, at subsites −1 and +1 followed by the nucleophilic attack of Asp206 to C-1 of the glucosyl unit at subsite −1. A β-glucopyranosyl-enzyme intermediate is formed via an (assumed) oxocarbonium-like transition state. Then the protonated glucosyl unit at subsite +1 leaves the active site while a water molecule moves in. While the glutamate accepts a hydrogen atom from water, the oxygen atom of which attacks the covalent bond between the glucosyl unit at subsite −1 and the aspartate, going in turn via an oxycarbonium-like transition state and forming a new hydroxyl group at the C-1 position of the glycosyl unit at subsite −1. When another glucose molecule, instead of water, enters the active site, a new glycosidic bond [preferentially with an α-(1→6) bond] is formed. This occurs more frequently with increasing glucose concentrations, leading to products such as isomaltose (α-D-glucopyranosyl-(1→6)-D-glucose). A third conserved residue, a second aspartate (Asp297), binds to the OH-2 and OH-3 groups of the substrate through hydrogen bonds and plays an important role in substrate distortion.[16]

2.1.2.2. Glucoamylases. The step following dextrin production proceeds to glucose formation using glucoamylase (and additional enzymes).[18] Glucoamylase (GA, known industrially as amyloglucosidase, EC 3.2.1.3, GH 15) is an exo-acting inverting glycoside hydrolase [α-(1→4)-D-glucan glucohydrolase] that catalyses the release of β-D-glucose. It is exo-acting since it hydrolyses bonds only from the nonreducing end of starch (Fig. 1). Glucoamylase is produced by filamentous fungi, mainly *Aspergillus* sp., in higher tonnage than almost any other industrial enzyme and thus it is available at low price.[13] Some structural details will be discussed subsequently in order to illustrate the relevance of basic knowledge for improving the technical process. The catalytic domain of GA consists of a $(\alpha/\alpha)_6$ barrel with six interior α-helices surrounded by six exterior α-helices. The helices form a bed supporting a network of loops, in the centre of which is found the active site. This is a well 10 Å deep and 15 Å wide at its mouth (Fig. 2). The substrate must deeply penetrate the well and undergo cleavage, followed by the remaining chain. Then the liberated glucose must leave the well before the next reaction can occur. This explains the exo-acting nature of GA.[19] Glu179 acts as the catalytic acid by protonating the glycosidic oxygen, while Glu 400 as the catalytic base abstracts a proton from the water. The nucleophilic hydroxyl group then attacks the glycosidic carbon from the bottom side and results in the inversion of the configuration at the anomeric centre to form a β-linked hydroxyl.

2.1.3. Enzyme engineering. Two major problems were the motives for extensive research into the genetic



Scheme 1. Simplified scheme of the mechanism with nucleophilic attack of the glycosidic bond by the nucleophile, Glu or Asp, respectively, followed by the formation of an intermediate with a covalently bound glycosyl residue and its release by nucleophilic displacement by a water molecule.[16]

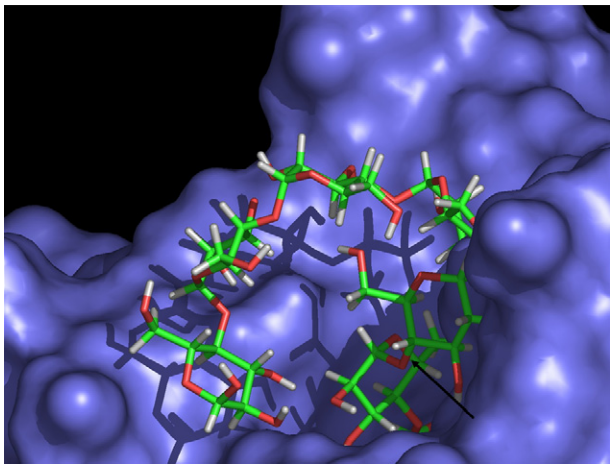


Figure 2. Front view of the *Aspergillus awamori* GA catalytic domain.[20] The figure displays a computationally docked maltoheptaose molecule bound in the active site of *Aspergillus niger* glucoamylase. The reducing-end glucosyl residue is deep in the active-site well, with only a small portion of it being visible. The α-glycosidic bond about to be cleaved to produce β-glucose and maltohexaose appears at the lower right of the illustration. The second through the fourth glucosyl residues are also bound in the well, while the fifth through the seventh residues are on the enzyme surface. Green: carbon; red: oxygen; silver: hydrogen. [Illustration prepared by Anthony Hill, Department of Chemical Engineering, Iowa State University, Ames, Iowa, USA. Docking with AutoDock (Scripps Research Institute, La Jolla, CA, USA) and visualization with PyMOL (DeLano Scientific, South San Francisco, CA, USA).]

modification of glucoamylase. First, at high dissolved solid concentrations it condenses some of the glucose formed to di-, tri- and tetrasaccharides, the most important being isomaltose. Combining favourable mutations was successful in decreasing the kinetics of the enzyme towards the formation of isomaltose, increasing its glucose yield from 96% to 97.5% (equivalent to 200,000 extra tons at actual production scale).[13] Second, it is not as stable as α-amylase and glucose isomerase, being used at 60 °C in starch processing. Stiffening α-helices by Gly→Ala and Ser→Pro mutations as well as creating disulfide bonds across two loops contributed to a fourfold increase of thermostability of *A. niger* glucoamylase.[13,21] A condition for the successful design of mutations of single amino acids in glucoamylase is the advanced knowledge of its structure and function.

Major efforts have also been devoted to researching thermostable α-amylases with much success, revealing structural determinants responsible for the high thermostability of *Bacillus* enzymes.[22]

Genetic engineering and recombinant technologies thus had significant impact on enzyme technology by: (i) a tremendous increase in the productivity of enzyme fermentation with recombinant organisms (as mentioned before); (ii) a significant improvement in thermostability (α-amylases, glucoamylases, glucose isomerase), by the screening of thermophilic organisms, directed evolution and rational design via site-directed mutagenesis; (iii) process optimization by improved selectivity (fewer side products, glucoamylases) and reduced or no Ca^{2+} dependence (α-amylase).

2.1.4. Production technology. Some details of starch processing are subsequently given in order to illustrate the technology.[3,11] In an initial step gelatinization of suspended ground starch is performed by thermal treatment (105–110 °C) in a so-called jet cooker [tubular reactor(s)] in order to make the polysaccharide particles accessible for α-amylase. A part of the thermostable α-amylase (bacterial hyperthermophilic amylase) is added in the gelatinization step (at 105 °C) by making use of the synergism of thermal gelatinization and partial hydrolysis. The second part of the α-amylase is added in the second step (partial hydrolysis) at 85–95 °C (Fig. 3).[11] The degree of hydrolysis (for obtaining dextrins of a specific DE-value) is determined by rapid inactivation of the enzymes (acidification and/or thermal treatment).

The hydrolysis to produce glucose syrup (DE 96–97) is subsequently performed at lower temperature (55–60 °C) and pH 4.5, requiring heat exchange and mixing for adjusting the pH. The conversion takes place in large stirred tanks at high residence time (24–72 h). Yields for glucose syrups are in the range of 96–97%, byproducts are 2–3% disaccharides (maltose and isomaltose) and 1–2% higher oligosaccharides.

2.2. Transfer reactions

Glycosyltransferases are applied as immobilized biocatalysts in a range of processes for manufacturing oligo- and polysaccharides such as isomaltulose, malto- and isomaltooligosaccharides, galacto- and fructooligosaccharides, cyclodextrins and dextran, mostly in the range of 3000–7000 t/a.[8]

Cyclodextrins are torus shaped molecules of cyclic α-(1→4)-linked glucose, with 6, 7 or 8 glucose residues

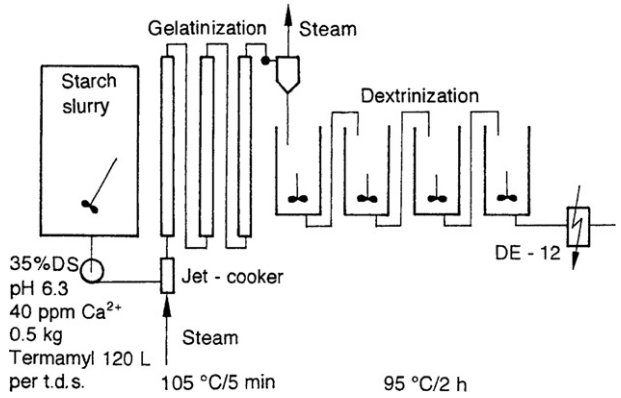


Figure 3. Reactor configuration for the starch liquefaction process (Termamyl is a bacterial thermophilic α-amylase).[11]

in the ring (α-, or β-, or γ-cyclodextrins). They are products of major importance in the fields of food (aroma complexation and slow release, stabilization of flavours), pharmaceuticals (drug protection, slow release) and commodities, for example, used in textile drying (for odour removal and as a perfume carrier). The enzymes used for their manufacture from starch or dextrins are cyclodextrin glycosyltransferases, several of which have been crystallized, their structures elucidated and characterized in detail, including the reaction mechanism.[23]

The Wacker company has optimized, via genetic tools, the enzyme production including improved selectivity to increase the yield of specific cyclodextrins, α-, or β-, or γ-, corresponding best to the special application.[24]

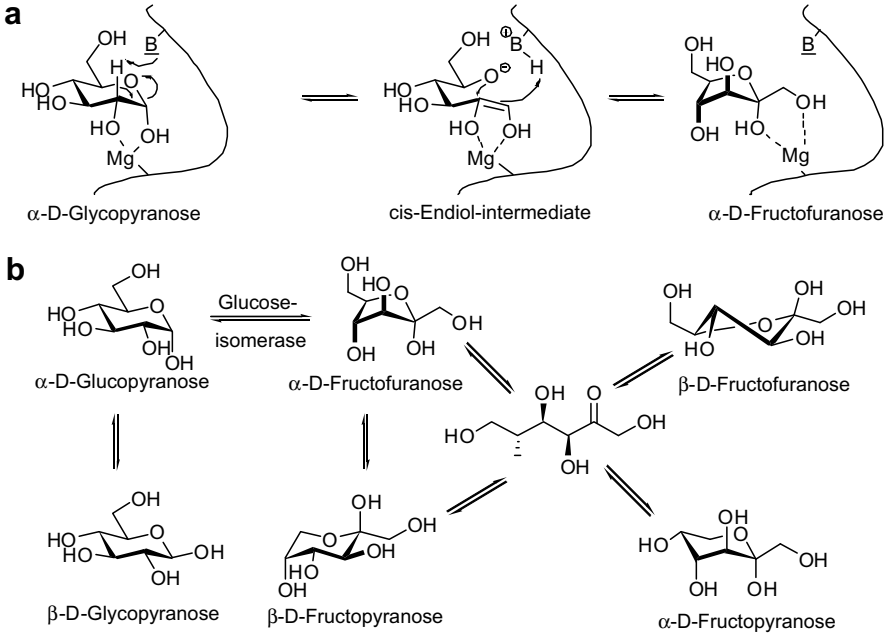
3. Isomerization reactions

3.1. Glucose isomerization

Fructose exhibits a significantly (about twofold) higher sweetening power as compared to glucose, which is produced in large amounts from corn (maize) starch. Therefore glucose is isomerized in part to fructose. This is the largest process performed with about 1500 t of immobilized enzymes producing a glucose–fructose syrup (HFCS, high fructose corn syrup) with more than 10 million t of product (dry matter) per year.[6,25–27]

3.1.1. Mechanistic aspects. The isomerization of glucose to fructose is an equilibrium controlled reaction, with fructose equilibrium concentrations of 48% at 45 °C. The mechanism of the isomerization by glucose isomerase (GI) consists of ring opening, isomerization through a hydride shift from C-2 of the open form glucose to C1 of the product fructose mediated by two Mg^{2+} ions and then the ring closure to give the α-ketol (Scheme 2).[18,28] Many glucose isomerases (which basically are D-xylose ketol-isomerases EC 5.3.1.5) have been characterized and their tertiary structures are now known. Monomers have $(\beta/\alpha)_8$ barrels, with eight β-strands being surrounded by eight α-helices.[18,28] Glucose isomerase has successfully been genetically engineered with respect to thermal stability and tight binding of Mg^{2+}. Furthermore GI has been modified with reference to substrate binding and catalytic activity on glucose and xylose, exhibiting a higher V_{max} and a lower K_M on glucose, which provides advantages for glucose isomerization. Rational genetic engineering succeeded in increased flexibility of the active site to accommodate the larger substrate.[29] This provides a significant advantage insofar as two steps up- and downstream, providing Mg^{2+} ions, and their removal from the product syrup by ion exchange is not necessary any more.

3.1.2. Enzyme immobilization. For immobilization several different procedures have been developed, and different commercial biocatalysts are utilized at the technical scale. One established method of immobilization is the crosslinking of the cells of *Streptomyces murinus* with glutaraldehyde while maintaining nearly all of the GI activity. In another procedure the enzyme is isolated and purified by chromatography and



Scheme 2. (a) Mechanism of glucose isomerization;[28] (b) chemical equilibria in solution.

crystallization, and finally adsorbed to an ion exchange matrix (Fig. 4).[27,30]

The productivity of GI is in the range of 12–20 t (dry substance, d.s.) per kg of biocatalyst with a half-life of 80–150 days.[24,26] Reactor dimensions typically are 1.5 m diameter and 5 m height of fixed bed bioreactors, with operation at 58–60 °C (Fig. 5).[3]

3.2. Sucrose isomerization

Isomaltulose (Palatinose) manufacture is another large process using a glucosyltransfer are activity of a sucrose mutase of *Protominobacter rubrum*. Sucrose is the substrate, in which the α-(1→2′)-β-glycosidic bond is rearranged to an α-(1→6′)-β glycosidic bond (Scheme 3). This is obviously an intramolecular rearrangement without further subsequent reactions of the primary product isomaltulose (side reactions include leucrose and trehalulose formation) with a yield of 80–85%. The catalyst consists of immobilized cells of *P. rubrum* (not living, only the sucrose mutase remaining active) in alginate. At high initial sucrose concentration (550 g/L) the biocatalysts exhibit a high stability (half-life over 5000 h), when used in large fixed bed reactors. Figure 6 presents the scheme of the technical process.[5,31]

Isomaltulose is produced with about 100,000 t/a. In a following reaction it is hydrogenated by classical Raney nickel catalysts to yield isomalt. Isomalt is a mixture of two isomer sugar alcohols, glucosyl-sorbitol and glucosyl-mannitol, that is used in the food sector as an alternative sweetener, with noncariogenic properties, and suited for diabetics as main characteristics. It is largely applied in caramels, chewing gum, tablets, etc.[31]

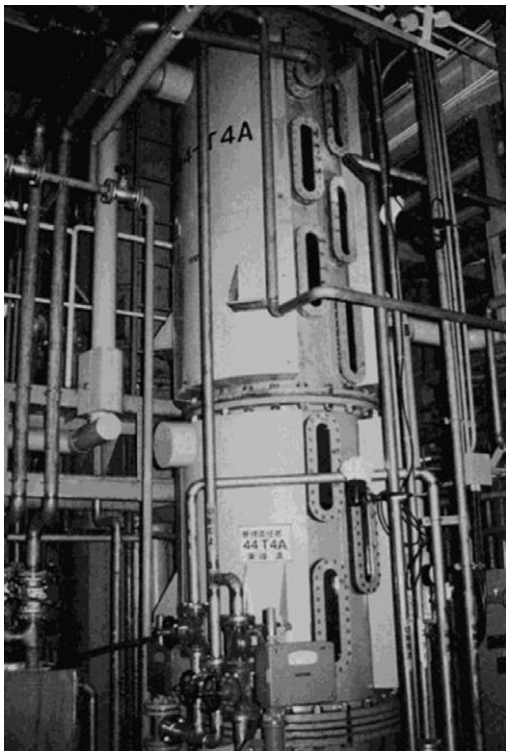


Figure 5. Typical fixed bed reactor.

4. Oxidation and reduction processes

Enzymatic oxidation and reduction reactions are performed in several technical processes. Examples are citric and gluconic acid production as the largest processes (Table 1), but also other acids and keto-acids, such as

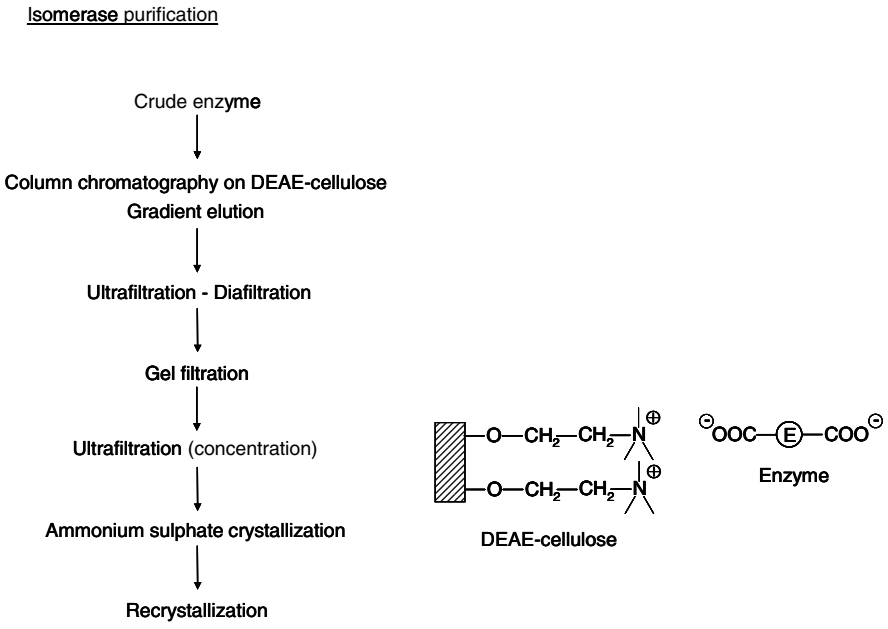
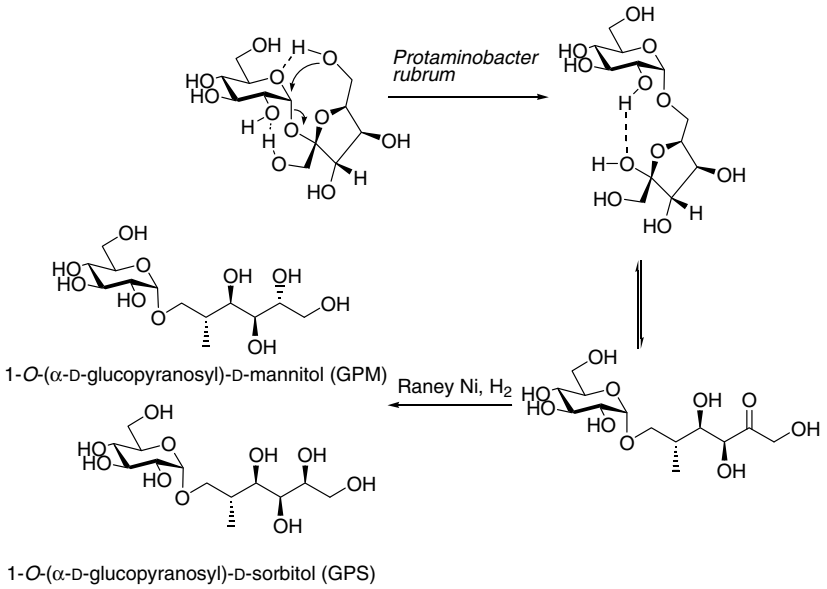


Figure 4. Immobilization of GI. Left: enzyme purification scheme; right: immobilization by adsorption on ion exchanger.



Scheme 3. Rearrangement of sucrose to give isomaltulose which is reduced to GPM and GPS.[31]

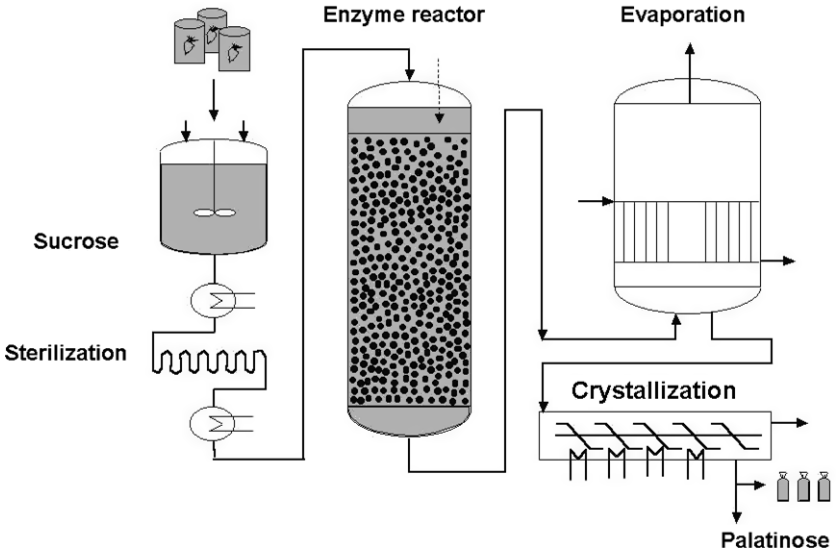


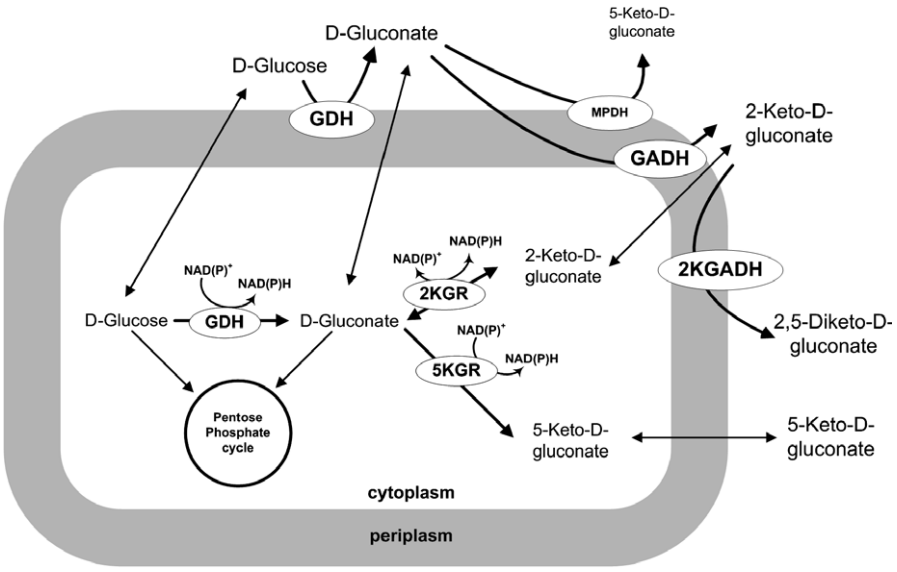
Figure 6. Scheme for technical isomaltulose production.[31]

2,5-diketogluconic acid and 5-ketogluconic acid are produced in the industrial scale. The major organisms used are *Aspergillus* sp. (using three industrial strains, *A. niger*, *Aspergillus oryzae* and *Aspergillus terreus*) and *Gluconobacter oxydans*.[32,33] The knowledge of the metabolic pathways and recently of the genome makes optimization and potentially new products more feasible (Scheme 4). Citric acid is produced from glucose by *A. niger* with high efficiency, (>200 g/L, over 95% of the theoretical yield—1 mol of glucose giving 1 mol of citric acid). More recently products of pharmaceutical interest like 1-deoxynojirimycin, a potent α-glucosidase inhibitor, are produced via the regioselective oxidation of aminosorbitol with *G. oxydans*.[33,34]

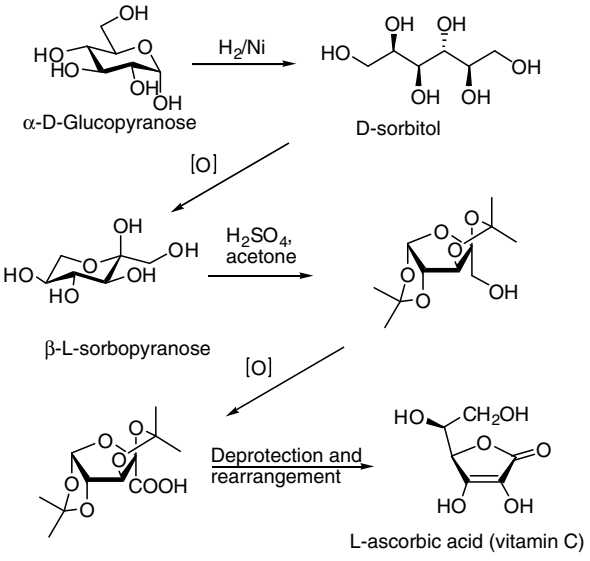
4.1. Vitamin C

4.1.1. Reichstein process. Vitamin C has been a major product (80.000 t/a) over many decades dominated by the Reichstein process with an overall yield of 60%. It includes one regioselective biocatalytic step, the oxidation of D-sorbitol to L-sorbose (Scheme 5). It is still used by Roche and Takeda on a large scale by making use of economies of scale.[35]

The Reichstein synthesis is a sequence of easy, high-yielding steps without the cleavage or formation of a carbon–carbon bond. The synthetic pathway includes the reduction of C-1 of D-glucose and oxidation at positions 5 and 6. The chirality at C-2 and C-3 is preserved



Scheme 4. Pathways of glucose oxidation in *G. oxydans*; GDH glucose dehydrogenase; GADH gluconate dehydrogenase; 2KGADH 2-keto-D-gluconate dehydrogenase; 2KGR 2-keto-D-gluconate reductase; 5KGR 5-keto-D-gluconate reductase; MPDH major polyol dehydrogenase.[33]



Scheme 5. Scheme of the Reichstein process.

to yield the L-threo-configuration at C-4 and C-5 of L-ascorbic acid.

The classical Reichstein–Grüssner synthesis starts with the reduction of D-glucose to D-sorbitol by hydrogenation with a nickel catalyst. The microbiological oxidation of D-sorbitol to L-sorbose is carried out with *Acetobacter xylinum*, followed by isopropylidenation with acetone at low temperature in the presence of sulfuric acid, which affords 2,3:4,6-di-*O*-isopropylidene-α-L-sorbofuranose. The di-*O*-isopropylidenyl protection of the hydroxy-groups at C-2, C-3 and C-4, C-6 allows selective oxidation to di-*O*-isopropylidene-2-ketogulonic acid. The oxidation is carried out with potassium permanganate in alkaline solution and the treatment with hot water affords 2-keto-L-gulonic acid, which is converted to L-ascorbic acid by heating in water at 100 °C (20% yield) or by esterification and treatment with sodium methoxide in methanol followed by acidification with hydrogen chloride, yielding ca. 70% of vitamin C.

The development of Reichstein's classical procedure into an industrial process is marked by great efforts to improve each reaction step. As a result of many technical and chemical modifications each step gives over 90% yield. The overall yield of ascorbic acid from D-glucose is now ca. 60%.

Much less expensive oxidation methods are applied in modern continuous processes: sodium hypochlorite, electrochemical oxidation, or catalytic air oxidation. That is, the oxidation with hypochlorite in the presence of catalytic amounts of nickel chloride or sulfate at 60 °C gives yields >93%. The active oxidant is presumably nickel peroxide.[36]

4.1.2. Whole-cell biocatalysis. It is only recently that a new two-stage fermentation process has replaced the chemical steps of the Reichstein process. Several approaches are devoted in order to replace the Reichstein process by a one-step conversion, including modified microorganisms (see Section 5).

A new process for vitamin C production has been established recently on the industrial level by BASF and Cerestar. It comprises the conversion of sorbitol to sorbose and subsequently to 2-keto-L-gulonic acid by a mixed culture of *G. oxydans* and *Bacillus thuringiensis*, with a yield of 85%.[37,38] The process was developed in China and is used by all Chinese producers, with an estimated production of 43.000 t/a. It has also

been licensed to some Western producers including Roche, ADM and a joint venture involving BASF, Cerestar and Merck.[35] In a first step of the process D-sorbitol as the substrate is oxidized to L-sorbose, the second step yields keto-gulonic acid (KGA) that is transformed into ascorbic acid in two straight chemical and purification steps. It has lower fixed and capital cost and furthermore makes less use of toxic solvents and reagents, thus less processing of waste, resulting in the overall production cost savings of about one third as compared to the Reichstein process.[35]

5. Outlook

5.1. New and modified enzyme activities

Extending the range of enzymes for application as well as the search for new solutions in synthesis, notably of chiral compounds, has been a continuing challenge.[39,40] More recently, new strategies have been developed to include the plethora of 'nonculturable' biodiversity in biocatalysis: (i) the metagenome approach and (ii) sequence-based discovery.

Basically, in the metagenome approach, the entire genomic DNA from uncultivated microbial consortia (i.e., soil samples) is directly extracted, cloned and expressed. Next, distinct enzymatic activities are identified by suitable assay methods.[41–43] The major advantage of this approach is that not only huge numbers of new biocatalysts can be found. Phylogenetic analyses revealed that new subclasses of enzymes can be identified, which show very broad evolutionary diversity and thus the chance to identify biocatalysts with unique properties is substantially increased. In addition, the enzymes identified are already expressed by recombinant methods and thus in principle available at large scale. Sequence-based discovery is increasingly attractive with the tremendously growing knowledge base (for lipases, epoxide hydrolases and dehalogenases, see, for example, http://www.led.uni-stuttgart.de) built from sequencing singular genes and whole genomes.

Sequencing of the *A. niger* genome offers new opportunities for the production of speciality chemicals, and enzymes—incorporating the latest view of citrate synthesis, including the involvement of multiple malate dehydrogenase-like sequences, and further gene multiplicity. Similar findings also play a role in gluconic acid production, where, for example, 11 catalases were detected.[44]

The industrial potential of *Gluconobacter* sp. is highlighted by De Muynck et al. including future potential obvious from the knowledge of the genome. Thus different organic acids, for example, gluconic acid and keto-acids are made or can be made by *Gluconobacter* sp.[33]

The potential for extended and new synthetic pathways is obvious from recent success and the perspectives of enzyme modification by genetic engineering tools. A highly efficient rational strategy was developed by Kelly et al. for altering the synthetic (cyclization) activity of a cyclodextrin transferase (CGTase) into a hydrolytic activity.[45]

Another example is the engineering of a hydrolase (α-amylase) to function as a CGTase, thus a synthetic enzyme. Based on structural homology with the cyclodextrin glucosyltransferases, a loop insertion consisting of five residues, likely to be a steric hindrance for cyclization, was deleted. A correct insertion of an aromatic residue in the active site essential for cyclization was required furthermore. The variant was able to produce β-cyclodextrin and exhibited a significantly reduced hydrolytic activity (Fig. 7).[46]

5.2. Designed cells

Biotransformation by designed recombinant whole-cell systems represents a field of high current interest, and a range of examples have been developed successfully for several multi step reactions. They are mostly used as suspended cells. The potential of using such systems continuously as immobilized biocatalysts is still an open question. A limiting factor seems to be their stability notably with respect to coenzyme regeneration and resynthesis of the enzymes involved in the reaction sequence. Metabolic engineering is a key tool in this context in order to identify limitations (e.g., in redox equivalents) and to improve yields by optimising metabolic fluxes.[48,49] Thus a whole-cell biotransformation system for the conversion of D-fructose to D-mannitol was developed in *Escherichia coli* by constructing a recombinant oxidation/reduction cycle.[50]

Much effort has been devoted to new routes for L-ascorbate (vitamin C) synthesis, in particular by the construction of designed recombinant microorganisms, which are able to synthesize this compound.[37,51,52] Thus, an efficient recombinant strain of *Erwinia* sp. was developed which oxidizes D-glucose to 2,5-diketo-D-gluconic acid. Furthermore this strain encodes a recombinant reductase from *Corynebacterium* sp. producing 2-keto-L-gulonic acid, which can easily be rearranged by cyclization to vitamin C (Scheme 6). A culture of the *Erwinia* strain, fed with glucose to a total of 40 g/L, converts glucose to 2-keto-L-gulonate with a yield of 49.4% during a 72-h-bioconversion.[53]

Still the production of sugar nucleotides is challenging. Some progress has been achieved with selected examples like uridine diphosphate galactose (UDP-Gal) by combining metabolically engineered recombinant *E. coli* with a nucleoside 5′-triphosphate producing microorganism for the manufacture of oligosaccharides.[54,55]

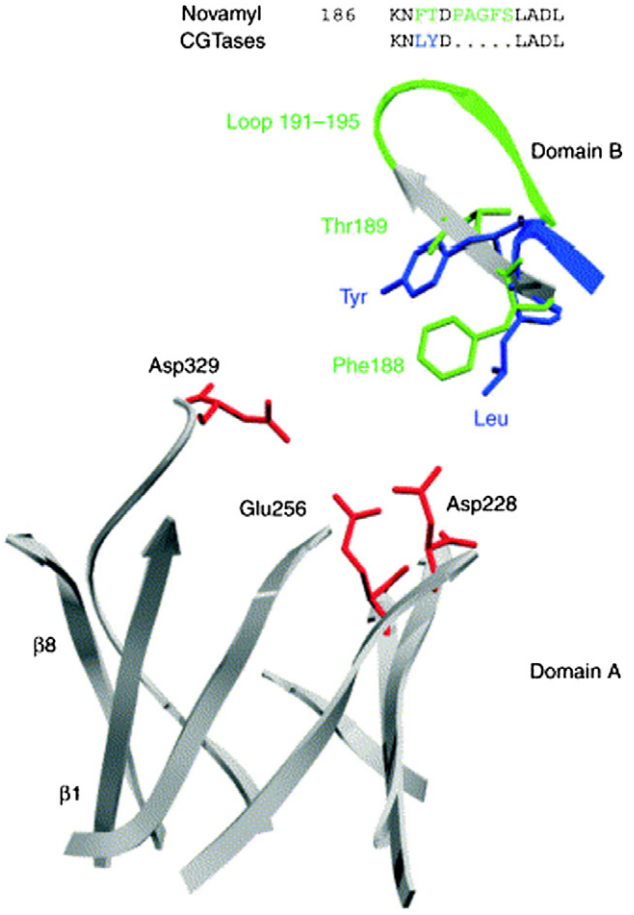
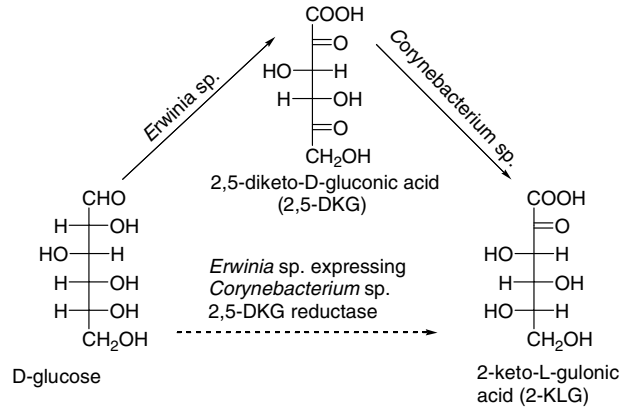


Figure 7. Conversion of a maltogenic α-amylase into a CGTase.[46] The product specificity of the glycoside hydrolase Novamyl was extended by rational design to convert Novamyl into a cyclodextrin-producing enzyme. Therefore a loop insertion (green) was deleted and the two amino acid changes Phe188Leu (blue) and Thr189Tyr (blue) were introduced. The illustration is taken from Hocker et al.[47]



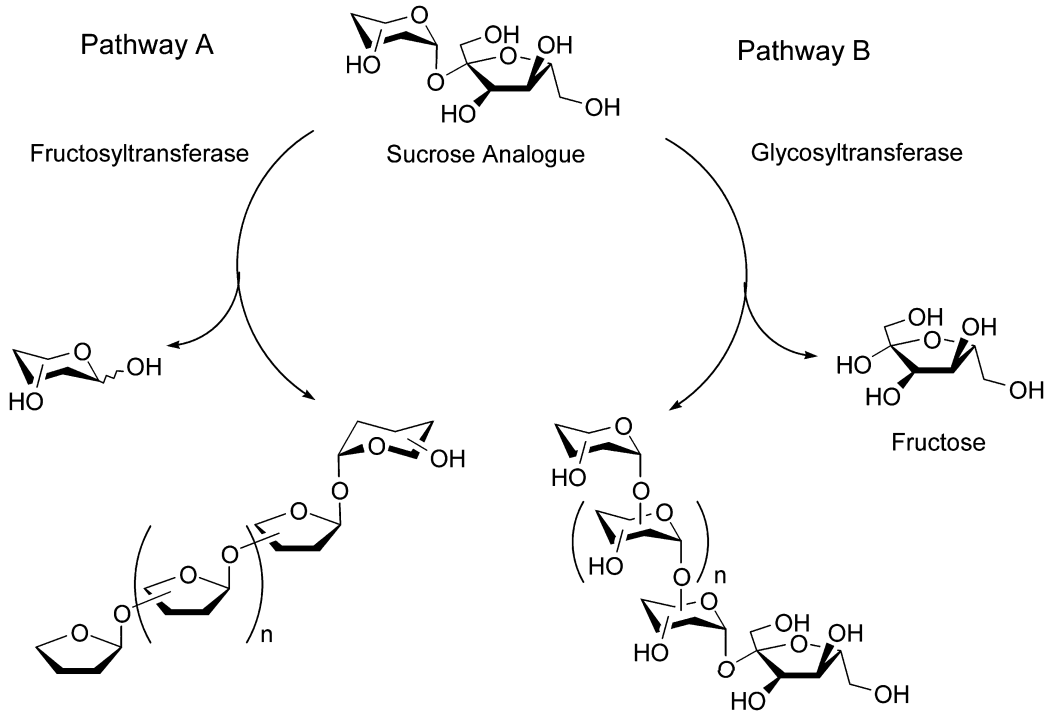
Scheme 6. Whole-cell biotransformation of D-glucose to 2,5-diketo-D-gluconic acid and 2-keto-L-gulonic acid by a recombinant strain of *Erwinia* sp. (in solution, glucose and sugar acids are present in equilibrium as cyclic pyranose and lactones).[53]

5.3. Substrate engineering—sucrose analogs

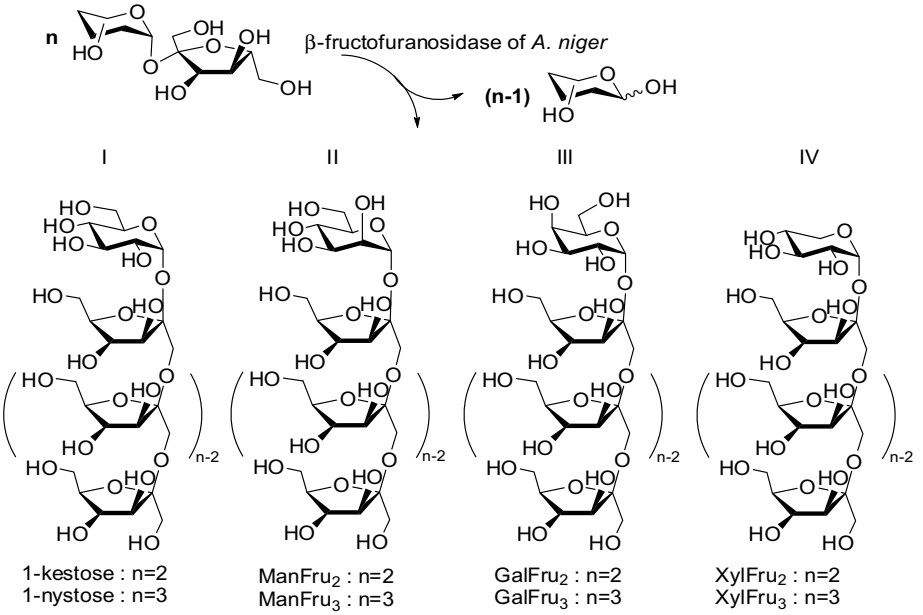
The generation of enzymes with new catalytic activities remains difficult, but 'substrate engineering' has been reported to be an alternative approach for expanding the substrate specificity of a given enzyme.[56] With this concept, it has been demonstrated that acceptor substrates can direct the regioselectivity of an glucansucrase for acceptor reactions without mutating the enzyme. This novel approach was expanded to donor substrates—in this case sucrose analogs. Sucrose itself is a highly activated substrate for glucosyltransferases ($\Delta G^0_A = -26.5$ kJ mol^{-1})[7,57] compared to other disaccharides, that is, isomaltose, lactose and maltose ($\Delta GA^0 = -7,\ -8.8,\ -15.5$ kJ mol^{-1}).[58] This difference in the Gibbs energy change is available for the synthesis of oligo- and polysaccharides by sucrase type enzymes using sucrose as a substrate. However, the convenient synthetic routes are limited to the transfer of glucose and fructose with sucrose as substrate. Thus, in searching for broader applications we expanded our engineering approach on donor substrates and synthesized various sucrose analogs (β-D-fructofuranosyl-α-D-glycopyranosides). The synthesis of the sucrose analogs has been recently established in our laboratories as an inexpensive enzymatic process by the exo-fructosyltransferase (EC 2.4.1.162) from *Bacillus subtilis* in high yields and high concentrations making it feasible for industrial use.[59–61] The new substrates may be used for OS and PS synthesis (a) using fructansucrases in pathway A for the synthesis of new glycopyranosyloligofructosides or (b) alternatively in pathway B using modified glucansucrases for the transfer of the glycopyranoside (Scheme 7).[9,62] The transfer of either fructose or the glycopyranoside to other sugars or different natural products as acceptors is a challenging perspective. Advantages of this system apart from the currently employed enzymes such as glycosynthases and glycosyltransferases[63,64] are that industrially established glucansucrases and fructansucrases may be usable for extended substrate and product spectra.

Very recently, we demonstrated that sucrose analogs indeed provide a new powerful tool for the highly efficient and inexpensive preparative synthesis of tailor-made saccharides (Scheme 8). In the first studies the novel substrates have been converted by levansucrase enzymes to unique oligo- and polyfructans.[9,62] Moreover in further studies a genetically optimized strain of *A. niger* with an overexpressed β-fructofuranosidase transformed sucrose analogs efficiently and with high yield to 1-kestose and 1-nystose analogs headed with different monosaccharides of potential interest (Scheme 8).[65]

As a conclusion, different ways, including efficient search for new enzyme activities, rational enzyme and substrate engineering, as well as tailoring whole-cell systems, offer opportunities for improved or new oligosaccharide synthesis.



Scheme 7. A concept for oligosaccharide synthesis using sucrose analogs as substrate and fructan- and glucansucrase enzymes in pathways A and B. In pathway A novel fructans, fructo-oligosaccharide are synthesized with a terminal glucose analog. In pathway B novel glycan poly- and oligosaccharides may be synthesized, different from the normal glucan and gluco-oligosaccharide, such as mannan and xylan.[9]



Scheme 8. Enzymatic synthesis of 1-kestose, 1-nystose and their analogs by β-fructofuranosidase of *A. niger*. Structures of FOS: (I) commercial products, (II) mannose- (III) galactose- and (IV) xylose-headed analogs.

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References

1. Lichtenthaler, F. W. In *Ullmann's Encyclopedia of Industrial Chemistry*; Wiley-VCH: Weinheim, 2003.

2. Schiweck, H.; Bär, A.; Vogel, R.; Schwarz, E.; Kunz, M. In *Ullmann's Encyclopedia of Industrial Chemistry*; Wiley-VCH: Weinheim, 2000.
3. Buchholz, K.; Kasche, V.; Bornscheuer, U. *Biocatalysts and Enzyme Technology*; Wiley-VCH: Weinheim, 2005.
4. United-Nations *Industrial Commodities Statistics Yearbook 2001*; United Nations: New York, 2003.
5. Buchholz, K.; Ekelhof, B. In *Winnacker-Küchler. Chemische Technik: Prozesse und Produkte*; Dittmeyer, R., Keim, W., Kreysa, G., Oberholz, A., Eds.; Wiley-VCH: Weinheim, 2005; Vol. 8, pp 315–354.
6. Swaisgood, H. J. In *Handbook of Food Enzymology*; Whitaker, J. R., Voragen, A. G. J., Wong, D. W. S., Eds.; Marcel Dekker: New York, 2003; pp 359–366.
7. Goldberg, R.; Tewari, J.; Ahluwalia, J. *J. Biol. Chem.* **1989**, *264*, 9901–9904.
8. Fuji, S.; Komoto, M. *Zuckerindustrie* **1991**, *116*, 197–200.
9. Seibel, J.; Buchholz, K.; Jördening, H. J. *Biocatal. Biotransform.* **2006**, *24*, 311–342.
10. Svensson, B. et al. In *Recent Advances in Carbohydrate Bioengineering*; Gilbert, H. J., Ed.; The Royal Society of Chemistry: Cambridge, 1999; pp 272–281.
11. Olsen, H. S.. In *Biotechnology*; Reed, G., Nagodawithana, T. W., Eds.; Wiley-VCH: Weinheim, 1999; Vol. 9, pp 663–736.
12. Bentley, I. S.; Williams, E. C. In *Industrial Enzymology*; Godfrey, T., West, S. I., Eds.; Macmillan Press: London, 1996; pp 339–357.
13. Reilly, P. In *Handbook of Food Enzymology*; Whitaker, J. R., Voragen, A. G. J., Wong, D. W. S., Eds.; Marcel Dekker: New York, 2003; pp 727–738.
14. (a) CAZY (Carbohydrate Active Enzymes database; http://www.cazy.org/); (b) Coutinho, P. M.; Henrissat, B. Carbohydrate-active enzymes: an integrated database approach. In *Recent Advances in Carbohydrate Bioengineering*; Gilbert, H. J., Davies, G., Henrissat, B., Svensson, B., Eds.; The Royal Society of Chemistry: Cambridge, 1999; pp 3–12.
15. Gottschalk, T. E.; Fierobe, H. P.; Mirgorodskaya, E.; Clarke, A. J.; Tull, D.; Sigurskjold, B. W.; Christensen, T.; Payre, N.; Frandsen, T. P.; Juge, N.; McGuire, K. A.; Cottaz, S.; Roepstorff, P.; Driguez, H.; Williamson, G.; Svensson, B. *Biochem. Soc. Trans.* **1998**, *26*, 198–204.
16. van der Maarel, M. J.; van der Veen, B.; Uitdehaag, J. C.; Leemhuis, H.; Dijkhuizen, L. *J. Biotechnol.* **2002**, *94*, 137–155.
17. Horvathova, V.; Janecek, S.; Sturdik, E. *Gen. Physiol. Biophys.* **2001**, *20*, 7–32.
18. Reilly, P. J.; Antrim, R. L. In *Ullmann's Encyclopedia of Industrial Chemistry*, 7th ed.; Wiley-VCH: Weinheim, 2003.
19. Coutinho, P. M.; Dowd, M. K.; Reilly, P. J. *Proteins* **1997**, *28*, 162–173.
20. Coutinho, P. M.; Reilly, P. J. *Proteins* **1997**, *29*, 334–347.
21. Suvd, D.; Fujimoto, Z.; Takase, K.; Matsumura, M.; Mizuno, H. *J. Biochem. (Tokyo)* **2001**, *129*, 461–468.
22. Declerck, N.; Machius, M.; Wiegand, G.; Huber, R.; Gaillardin, C. *J. Mol. Biol.* **2000**, *301*, 1041–1057.
23. Dijkhuizen, L.; van der Veen, B. In *Handbook of Food Enzymology*; Whitaker, J. R., Voragen, A. G. J., Wong, D. W. S., Eds.; Marcel Dekker: New York, 2003; pp 615–627.
24. Schulz, G. E.; Candussio, A. Patent application US, 5, 474, 917, 1995.
25. Antrim, R. L.; Lloyd, N. E.; Auterinen, A. L. *Starch/Stärke* **2006**, *41*, 155–159.
26. Misset, O. In *Handbook of Food Enzymology*; Whitaker, J. R., Voragen, A. G. J., Wong, D. W. S., Eds.; Marcel Dekker: New York, 2003; pp 1057–1077.
27. Pedersen, S.; Christensen, M. W. In *Applied Biocatalysis*; Straathof, A. J. J., Adlercreutz, P., Eds.; Harwood Academic: Amsterdam, 2000.
28. Lavie, A.; Allen, K. N.; Petsko, G. A.; Ringe, D. *Biochemistry* **1994**, *33*, 5469–5480.
29. Genencor International, EP1264883A2, 1990.
30. Antrim, R. L.; Auterinen, A. L. *Starch/Stärke* **1986**, *38*, 132–137.
31. Rose, T.; Kunz, M. In *Landbauforschung*; Bundesanstalt für Landwirtschaft: Braunschweig, 2002; Vol. 241, pp 75–80.
32. Kubicek, C. P.; Schreferl-Kunar, G.; Wohrer, W.; Rohr, M. *Appl. Environ. Microbiol.* **1988**, *54*, 633–637.
33. De Muynck, C.; Pereira, C. S.; Naessens, M.; Parmentier, S.; Soetaert, W.; Vandamme, E. J. *Crit. Rev. Biotechnol.* **2007**, *27*, 147–171.
34. Schedel, M. In *Biotechnology*; Wiley-VCH, 2000; Vol. 8a, pp 295–307.
35. BASF AG; Takeda Chemical Industries Ltd. A report on the acquisition by BASF AG of certain assets of Takeda Chemical Industries Ltd, 2001.
36. Eggersdorfer, M. In *Ullmann's Encyclopedia of Industrial Chemistry*, 7th ed.; Wiley-VCH: Weinheim, 2002.
37. Rueckel, M.; Hoffmann-Laroche AG, EP 0972843, Schweiz, 2000.
38. J.-C. Troostemberg; I. A. Debonne; W. R. Obyn; C. Peuzet; Cerestar Holding BV, Ed. Netherlands, 2003.
39. Koeller, K. M.; Wong, C. H. *Nature* **2001**, *409*, 232–240.
40. Bevan, M. W.; Franssen, M. C. *Nat. Biotechnol.* **2006**, *24*, 765–767.
41. Lorenz, P.; Liebeton, K.; Niehaus, F.; Eck, J. *Curr. Opin. Biotechnol.* **2002**, *13*, 572–577.
42. Lorenz, P.; Eck, J. *Nat. Rev. Microbiol.* **2005**, *3*, 510–516.
43. Handelsman, J. *Nat. Biotechnol.* **2005**, *23*, 38–39.
44. Cullen, D. *Nat. Biotechnol.* **2007**, *25*, 189–190.
45. Kelly, R. M.; Leemhuis, H.; Dijkhuizen, L. *Biochemistry* **2007**, *46*, 11216–11222.
46. Beier, L.; Svendsen, A.; Andersen, C.; Frandsen, T. P.; Borchert, T. V.; Cherry, J. R. *Protein Eng.* **2000**, *13*, 509–513.
47. Hocker, B.; Jurgens, C.; Wilmanns, M.; Sterner, R. *Curr. Opin. Biotechnol.* **2001**, *12*, 376–381.
48. Edwards, J. S.; Palsson, B. O. *J. Biol. Chem.* **1999**, *274*, 17410–17416.
49. Segre, D.; Vitkup, D.; Church, G. M. *Proc Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15112–15117.
50. Kaup, B.; Bringer-Meyer, S.; Sahm, H. *Appl. Microbiol. Biotechnol.* **2004**, *64*, 333–339.
51. Troostemberg, J.-C.; Debonne, I. A.; Obyn, W. R.; Peuzet, C. WO 03016508, Cerestar Holding BV, Netherlands, 2003.
52. Smirnoff, N. *Nat. Biotechnol.* **2003**, *21*, 134–136.
53. Grindley, J. F.; Payton, M. A.; van de Pol, H.; Hardy, K. G. *Appl. Environ. Microbiol.* **1988**, *54*, 1770–1775.
54. Koizumi, S.; Endo, T.; Tabata, K.; Ozaki, A. *Nat. Biotechnol.* **1998**, *16*, 847–850.
55. Drouillard, S.; Driguez, H.; Samain, E. *Angew. Chem., Int. Ed. Engl.* **2006**, *45*, 1778–1780.
56. Hellmuth, H.; Hillringhaus, L.; Hobbel, S.; Kralj, S.; Dijkhuizen, L.; Seibel, J. *ChemBioChem* **2007**, *8*, 273–276.
57. Goldberg, R.; Tewari, J. *J. Phys. Chem. Ref. Data* **1989**, *18*, 809–822.

58. Tewari, J.; Goldberg, R. *Biophys. Chem.* **1991**, *40*, 59–67.
59. Seibel, J.; Moraru, R.; Götze, S. *Tetrahedron* **2005**, *61*, 7081–7086.
60. Seibel, J.; Moraru, R.; Götze, S.; Buchholz, K.; Na'amnieh, S.; Pawlowski, A.; Hecht, H. J. *Carbohydr. Res.* **2006**, *341*, 2335–2349.
61. Baciu, I. E.; Jördening, H. J.; Seibel, J.; Buchholz, K. *J. Biotechnol.* **2005**, *116*, 347–357.
62. Seibel, J.; Beine, R.; Moraru, R.; Buchholz, K. *Biocatal. Biotransform.* **2006**, *24*, 157–165.
63. Hancock, S. M.; Vaughan, M. D.; Withers, S. G. *Curr. Opin. Chem. Biol.* **2006**, *10*, 509–519.
64. Trincone, A.; Giordano, A. *Curr. Org. Chem.* **2006**, *10*, 1163.
65. Zucarra, A.; Götze, S.; Kneip, S.; Dersch, P.; Seibel, J. *ChemBioChem* **2008**, *9*, 143–149.