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Enzymatic Synthesis of Oligosaccharides

ROBERT A. RASTALL AND CHRISTOPHER BUCKE

Division of Biotechnology, School of Biological and Health Sciences, University of Westminster, London, UK

Introduction

Oligosaccharides are a class of biomolecules possessing great structural diversity. It is not surprising, therefore, that they have a wide range of roles within living cells, often acting as recognition molecules (Rademacher, Parekh and Dwek, 1988). Oligosaccharides, usually as glycoconjugates, i.e. glycolipids and glycoproteins, have been implicated in cell–cell, cell–virus, and cell–bacterial interactions (Rademacher, Parekh and Dwek, 1988; Schnaar 1991). Many antigens are carbohydrate in nature, for instance the lipopolysaccharide *O*-antigens found on Gram-negative bacterial cells, the blood group determinants and several tumour-associated antigens (Kennedy and White, 1983; Feizi and Childs, 1985). The tumour antigens are of particular interest and have received intensive study due to the potential diagnostic value of changes in antigenicity as a result of changes in oligosaccharides structure. Oligosaccharides are also of great importance in plants, where they have been found to regulate cellular differentiation and to act as inducers of disease-resistance responses following invasion by fungal pathogens (McNeil *et al.*, 1984). Bacterial cell-surface oligosaccharides have also been found to be responsible for host recognition and binding following infection of root hairs with nitrogen-fixing bacteria (Lerouge *et al.*, 1990). The nature of the structure–function relationships in plant-derived oligosaccharides, such as sucrose and its relatives and the fructans, is of great interest to the food industry.

Despite this growing interest in oligosaccharides, very little is understood about the molecular mechanisms underlying the biological roles of these molecules. This is due, in part, to the lack of availability of oligosaccharides

Abbreviations: Ara, arabinose; CMP, cytidine monophosphate; DMF, *N*, *N'*-dimethylformamide; Eryol, erythritol; Fru, fructose; Fuc, fucose; Gal, galactose; GalNAc; *N*-acetylgalactosamine; GDP, guanidine diphosphate; Glc, glucose; GlcNAc, *N*-acetyl glucosamine; Glcol, glucitol; Lyx, lyxose; Man, mannose; Manol, mannitol; Neu5Ac, *N*-acetylneuraminic acid; *p*-NP, *p*-nitrophenol; *p*-NPα-Gal, *p*-nitrophenyl α-galactoside, etc.; Rib, ribose; Ribol, ribitol; UDP, uridine diphosphate; UDP-Man, UDP-mannose, etc.; UV, ultraviolet, Xyl, xylose.

Biotechnology and Genetic Engineering Reviews — Vol. 10, December 1992
0264–8725/92/10/253–281 $20.00 + $0.00 © Intercept Ltd, P.O. Box 716, Andover, Hampshire SP10 1YG, UK

on a suitable scale for experimental work. Many oligosaccharides are commercially available, but they are extremely expensive. This is because most of the molecules of interest occur in very small amounts in very complex biological matrices. Oligosaccharides can, of course, be synthesized chemically, and synthetic strategies have been well developed (Garegg, 1990). Almost any oligosaccharide can be synthesized using such techniques, but the protocols are very complicated and labour intensive. Overall yields are rather low, scale-up is difficult, and the products are necessarily expensive. For these reasons there is a great deal of interest in developing synthetic strategies based on the use of enzymes (Toone *et al.*, 1989; Bucke and Rastall, 1990; Ichikawa, Look and Wong, 1992). Several types of enzyme can be considered as potential synthetic catalysts, including the biosynthetic enzymes themselves, the sugar nucleotide-dependent glycosyltransferases. Glycosyltransferases such as the sucrases, and cyclodestrin-glucosyltransferase, also show useful potential, and recently the use of glycosidase enzymes acting synthetically have received much attention. The use of these enzymes in oligosaccharide synthesis, the advantages and disadvantages of each type, and the potential for the future will now be discussed. Much of this discussion will focus on the use of glycosidases as synthetic catalysts. This is because in recent years there have been many significant developments in this area, and yet this field has received little attention in review articles. The use of sugar-nucleotide-dependent glycosyl transferases in oligosaccharide synthesis has recently been covered in excellent reviews by Toone *et al.* (1989) and Ichikawa, Look and Wong (1992).

Synthesis of oligosaccharides using sugar-nucleotide-dependent glycosyltransferases

INTRODUCTION

Oligosaccharides are synthesized *in vivo* in several stages catalysed by the action of various enzymes (Schachter, 1986; *see Figure 1*). First, monosaccharide residues are activated by conversion to their 1-*O*-nucleotides; second, the sugar residues are transferred from the nucleotide to an acceptor molecule, such as a glycolipid or glycoprotein, to build up complex oligosaccharide chains. Once these oligosaccharide chains are synthesized and have been transferred to the relevant protein or lipid, they are modified by the action of glycosidase enzymes.

The first stage in this sequence of events consists of the formation of sugar-1-phosphates by a kinase enzyme followed by transfer of the sugar residue to a nucleoside triphosphate, catalysed by a nucleoside transferase. Only a few sugar nucleotides are synthesized directly in this way; enzyme-catalysed reactions then convert these into the other sugar nucleotides needed for oligosaccharide synthesis (Toone *et al.*, 1989).

The second stage in the biosynthesis of oligosaccharides is the transfer of sugar units from the sugar nucleotides to sugar acceptors by the glycosyltransferases. These enzymes have a very high degree of specificity, many of them

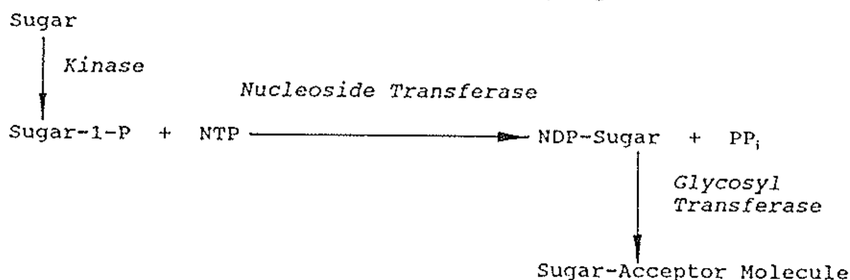


Figure 1. Simplified biosynthesis of oligosaccharides.

catalysing the formation of only one, unique, linkage. This high specificity has led to their application to the synthesis of oligosaccharides *in vitro*.

USE OF GLYCOSYLTRANSFERASES IN *IN VITRO* SYNTHESIS

Many glycosyl transferases have been purified and applied to the synthesis of quantities of oligosaccharides *in vitro* (for reviews see Toone *et al.*, 1989; Ichikawa, Look and Wong, 1992). Galactosyl transferase has been used in the synthesis of a wide range of oligosaccharides ranging in complexity from lactose to branched hexasaccharides (Nunez and Barker, 1980; Zehavi, Sadeh and Herchman, 1983; Augé *et al.*, 1984; Zehavi and Herchman, 1984a, b; David and Augé, 1987; Palcic, Srivastava and Hindsgaul, 1987; for example *see Figure 2*). This enzyme is probably the best characterized of all the glycosyltransferases that have so far been described.

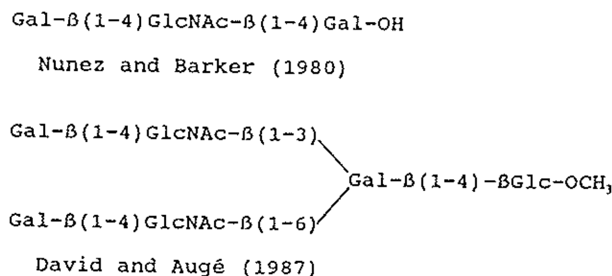


Figure 2. Examples of oligosaccharides synthesized by galactosyltransferase

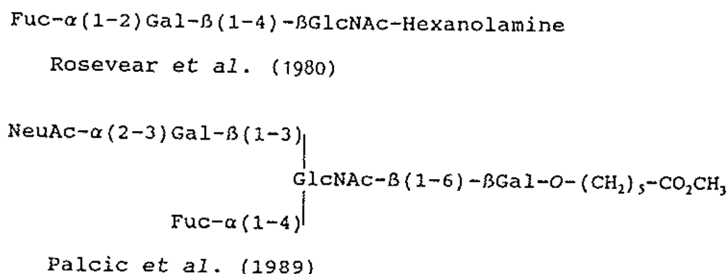
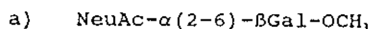
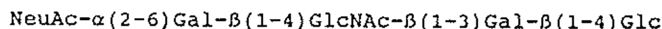


Figure 3. Examples of oligosaccharides synthesized with the aid of fucosyltransferase.

Other glycosyltransferases used in synthesis include: fucosyltransferase,

a)

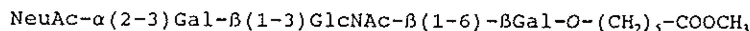




Sabesan and Paulsen (1986)

b)

Sabesan and Paulsen (1986)



Palcic *et al.* (1989)

Figure 4. Examples of oligosaccharides synthesized with the aid of (A) 2–6, and (B) 2–3 sialyltransferases.

which has been used in the synthesis of simple disaccharide-containing glycolipids and more complex molecules (Rosevear, Nunez and Barker, 1980; Palcic *et al.*, 1989; for example *see Figure 3*); glucosyltransferase (Weizemann *et al.*, 1988); *N*-acetylglucosaminyltransferase (Tahir and Hindsgaul, 1986; Hindsgaul *et al.*, 1988); and sialyltransferases (Sabesan and Paulsen, 1986; Thiem and Treder, 1986; Simon, Bednarski and Whitesides, 1988; Augé *et al.*, 1989; Palcic *et al.*, 1989). Two sialyltransferases, 2, 3- and 2,6-specific, have been used widely in the synthesis of complex oligosaccharides (for examples *see Figure 4*).

One of the biggest problems with the use of glycosyltransferases as synthetic reagents is that these enzymes are generally very unstable in solution (Wong, Haynie and Whitesides, 1982). For this reason some workers have immobilized glycosyltransferases for oligosaccharide synthesis (Thiem and Treder, 1986; Augé, Fernandez-Fernandez and Gautheron, 1990). Galactosyltransferase immobilized on Sepharose CL-4B was found to be resistant to mechanical stirring and to have enhanced stability to both high and low temperatures (Demers and Wong, 1985). Thiem and Treder (1986) provide an imaginative example of the use of immobilized glycosyltransferases in the synthesis of the trisaccharide: Neu5Acα(2–6)galβ(1–4)glcNAc. These workers immobilized sialyltransferase, galactosyltransferase, cytidine-5′-monophosphatase, and the enzymes necessary to convert glucose-6-phosphate to UDP-galactose on silica supports. They were then able to synthesize the trisaccharide in useful quantities.

An interesting development is the application of solid-phase synthesis to glycosyltransferases (Zehavi, Sadeh and Herchman, 1983; Zehavi and Herchman, 1984a, b) in which polymeric supports carrying carbohydrate moieties are able to act as acceptors. Product oligosaccharides are then liberated by cleavage with chymotrypsin (Zehavi and Herchman, 1984a), or by exposure to light (Zehavi, Sadeh and Herchman, 1983; Zehavi and Herchman, 1984b). A polymer support consisting of amino-substituted polyvinyl alcohol cross-

linked to 4-carboxy-2-nitrobenzyl-β-glucoside was found to give good accessibility and yields.

ADVANTAGES AND DISADVANTAGES OF GLYCOSYLTRANSFERASES: FUTURE PROSPECTS

The primary advantage of nucleotide-dependent glycosyltransferases is their great specificity, allowing the precise, sequential construction of desired oligosaccharides. This requires, however, that a very wide range of enzymes is available for *in vitro* use. At the present time, the availability of purified enzymes is a major limiting factor, although it is hoped that the application of genetic engineering techniques will improve this situation in the future (Creeger and Rothfield, 1982; Ernst *et al.*, 1989).

Apart from availability, the main disadvantage of these enzymes is their instability (Wong, Haynie and Whitesides, 1982). Although this problem has been partially overcome in some cases by the use of immobilization techniques, this is an area where protein engineering techniques are likely to make an impact. The development of stable, recombinant glycosyltransferases for *in vitro* use would be highly desirable (Ichikawa, Look and Wong, 1992).

Synthesis of oligosaccharides by glycosidases in reverse

INTRODUCTION

The use of glycosidases as synthetic reagents can be traced back to 1898 (Croft-Hill, 1898) and has received sporadic attention ever since. Recently interest has been revived in this field, bringing a remarkable growth in our understanding of glycosidases. Glycosidase reversal can be achieved by two approaches which have been described as the kinetic and the equilibrium approaches (Bucke and Rastall, 1990).

The kinetic approach takes advantage of the fact that the hydrolysis of glycosidic bonds proceeds via a two-stage process involving a covalently linked sugar–enzyme intermediate (Viratelle *et al.*, 1969; Stokes and Wilson, 1972; *see Figure 5*).

In this reaction scheme the covalent bond between the sugar and the enzyme is broken by a nucleophilic displacement reaction involving water as an acceptor molecule. It has been known for some time, however, that many other molecules can act as acceptor, including alcohols (Viratelle *et al.*, 1969; Shifrin and Hunn, 1969) and other carbohydrates (Nilsson, 1987). In order to bring about the synthesis of an oligosaccharide a reaction mixture is set up to contain a reactive donor substrate such as a *p*-nitrophenyl (*p*-NP) glycoside, to promote rapid glycosylation of the enzyme, and a sugar or glycoside acceptor (*Figure 6*).

The equilibrium approach takes advantage of the fact that all enzyme reactions are, in principle, reversible. In order to achieve the reversal of the normal glycoside hydrolysis reaction, it is necessary to increase the concentration of the products of the forward reaction and to reduce the concentration of the reactants. This is done by incubating the enzyme in a highly concen-

trated solution of monosaccharide. Sugar concentrations of the order of 70–80% (w/w) are needed to reduce the water activity to the point at which the reverse reaction becomes dominant (Johannsson *et al.*, 1989; Rastall *et al.*, 1992b). Because reaction rates are rather slow, a temperature of 50–60°C is needed to reach equilibrium on a reasonable time-scale, but the enzyme is protected from denaturation by the stabilizing effects of high sugar and low water concentrations (Johansson *et al.*, 1989). The equilibrium approach is summarized in *Figure 7.*

Many glycosidases are relatively non-specific for acceptor sugars so that hetero-oligosaccharides can be synthesized by the co-condensation of two monosaccharides or a monosaccharide and an oligosaccharide (Rastall, Adlard and Bucke, 1991; Rastall *et al.*, 1992a, b; see below).

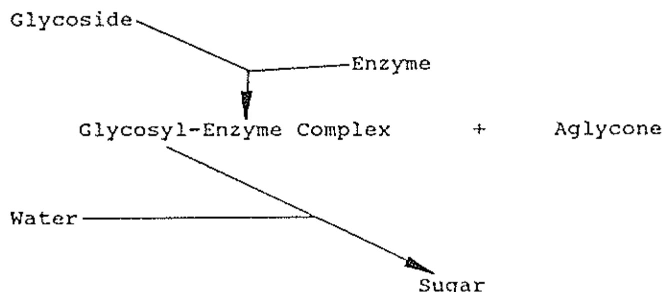


Figure 5. General reaction scheme for glycoside hydrolysis by glycosidases.

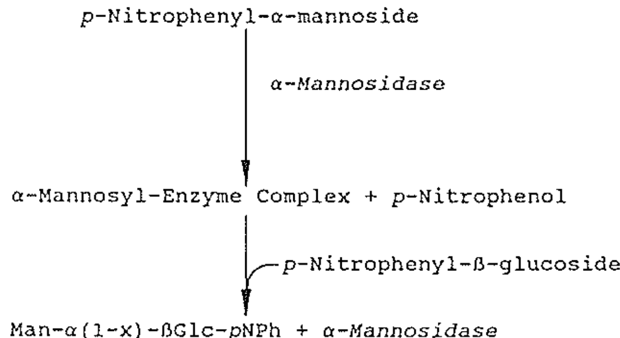


Figure 6. Synthesis of oligosaccharides by the kinetic approach.

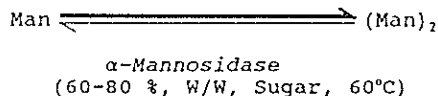


Figure 7. Synthesis of oligosaccharides by the equilibrium approach.

HISTORICAL PERSPECTIVE

α-Glucosidase

Much of the pioneering work on glycosidase reversal was carried out on α-glucosidases dating back to 1898 when Croft-Hill used a yeast extract in glucose solutions to synthesize disaccharides (Croft-Hill, 1898). Subsequently, most workers concentrated on synthesis by the kinetic approach

using the *trans*-glycosylation reactions with maltose as a substrate. α-Glucosidases from several sources were used in these early experiments including *Penicillium chrysogenum* (Saröja, Venkataraman and Giri, 1955), *Cladophora rupestris* (Duncan and Manners, 1958), *Aspergillus oryzae* (Pazur and French, 1952) and *Tetrahymena pyriformis* (Archibald and Manners, 1959; Manners and Stark, 1966; Manners, Pennie and Stark, 1968). The above enzymes were all found to catalyse transfer reactions from maltose with glucose and maltose acting as acceptor molecules to give isomaltose, panose, isomaltotriose, maltotriose, and maltotetraose. The *P. chrysogenum* enzyme also catalysed the formation of higher oligosaccharides such as 4-α-isomaltotriosyl glucose, 4-α-isomaltotetraosyl glucose, and 4-α-isomaltopentaosyl glucose. The ability of the enzymes from *C. rupestris* and *T. pyriformis* to use other carbohydrate acceptors was also examined, and it was found that the *C. rupestris* enzyme could not transfer to D-L-xylose, L-sorbose, *N*-acetyl D-glucosamine, D-fructose, D-galactose or panose (Duncan and Manners, 1958); whilst the *T. pyriformis* enzyme could transfer to D-xylose, L-arabinose and D-galactose, but not to D-fructose. Manners and co-workers extended this work to the use of the more reactive substrate phenyl α-glucoside and found that α-glucosidase from *T. pyriformis* could transfer from this substrate to D-xylose, L-xylose, D-ribose, D-arabinose, D-lyxose, D-mannose, D-galactose, D-glucitol, D-mannitol, ribitol and erythritol (Manners and Stark, 1966; Manners, Pennie and Stark, 1968). The products of these reactions are summarized in Table 1.

Table 1. Products of glucosyl transfer by *T. pyriformis* α-glucosidase

Acceptor carbohydrate	Products
D-Xylose	D-Glc α1–4 D-xyl
D-Xylose	D-Glc α1–4 L-Xyl
D-Ribose	D-Glc α1–4 D-Rib
D-Arabinose	D-Glc α1–2 D-Ara
D-Glc α1–3 D-Ara	
D-Glc α1–4 D-Ara	
D-Glc α1–5 D-Ara	
D-Lyxose	D-Glc α1–2 D-Lyx
D-Glc α1–3 D-Lyx	
D-Glc α1–4 D-Lyx	
D-Mannose	D-Glc α1–6 D-Man
D-Galactose	D-Glc α1–6 D-Gal
D-Glucitol	D-Glc α1–1 D-Glcol
D-Glc α1–6 D-Glcol	
D-Mannitol	D-Glc α1–1 D-Manol
Ribitol	D-Glc α1–1 Ribol
Erythritol	D-Glc α1–1 Eryol

The α-glucosidases of brewer's yeast, *Schizosaccharomyces pombe*, and buckwheat have been intensively studied by Chiba and co-workers. The brewer's yeast enzyme was found to synthesize nigerose, isomaltose, kojibiose and maltotriose from maltose by glycosyl transfer (Chiba *et al.*, 1962), and to transfer to D-glucose, D-xylose (Chiba, Takahashi and Shimomura, 1969), D-mannose (Chiba, Takahashi and Shimomura, 1969), D-fructose (Chiba and

Shimomura, 1971a), D-sorbose (Chiba and Shimomura, 1971b) and sucrose (Chiba *et al.*, 1979). The buckwheat enzyme was found to act on maltose, soluble starch, maltotriose and maltotetraose to produce nigerose, maltose, kojibiose, isomaltose, 2-α-isomaltosylglucose, 2-α-nigerosylglucose, nigerotriose, 3-α-maltosylglucose and maltotriose (Takahashi, Shimomura and Chiba, 1969). The buckwheat and *S. pombe* enzymes were also found to use 2-deoxy glucose as an acceptor to form 3-*O*-α-D-glucosyl-2-deoxyglucose and 4-*O*-α-D-glucosyl-2-deoxyglucose (buckwheat; Chiba *et al.*, 1975), and 6-*O*-α-D-glucosyl-2-deoxy-D-glucose (*S. pombe*; Chiba and Yamana, 1980).

β-*Glucosidase*

Although not as intensively studied as the α-glucosidases, early work also described several potentially useful β-glucosidase enzymes. The enzyme from *Aspergillus niger* was found (Crook and Stone, 1954) to catalyse glucosyl transfer from cellobiose to form gentiobiose, glucose and laminaribiose. Higher oligosaccharides were formed in the first two hours of incubation, then hydrolysed. The enzyme was capable of transfer to D-galactose to form D-Glcβ(1–6)D-Gal, and to D-xylose to form a glucosylxylose of unknown structure. Barker *et al.* (1955) found that the enzyme could also transfer to L-xylose, L-sorbose and *N*-acetyl-D-glucosamine. The Alfalfa-seed enzyme was characterized (Hutson, 1964) as producing trisaccharides from cellobiose, laminaribiose and gentiobiose. Anderson and Manners (1959) characterized the transfer reactions of the β-glucosidase from barley and found that it produced gentiobiose, laminaribiose, gentiotriose, cellotriose and 6-β-glucosylcellobiose. The enzyme could transfer to xylose, lyxose and arabinose.

β-*Galactosidase*

The transfer reactions of β-galactosidase are well known due to the problems they cause in the enzymic removal of lactose from milk (Prenosil, Stuker and Bourne, 1987a, b). Enzymes from a wide range of species have been examined, but until recently (see below), little attention was given to their potential as synthetic reagents for the formation of useful hetero-oligosaccharides. The primary transfer product formed from the action of β-galactosidase on lactose is allolactose (D-Galβ(1–6)D-Glc), the inducer of β-galactosidase in *Escherichia coli* (Huber *et al.*, 1976). Galactobiose (D-Galβ(1–6)D-Gal) is also formed, together with a range of higher oligosaccharides including galactotriose, 4-β-galactobiosylglucose, 6-β-galactobiosylglucose and 6-β-galactotriosylglucose (Asp *et al.*, 1980). The synthesis of oligosaccharides during lactose hydrolysis has been reviewed by Prenosil and co-workers (*loc. cit.*).

Glucoamylase

Glucoamylases are used extensively in the conversion of starch into glucose (Bucke, 1982). However problems have been encountered with obtaining complete conversion of starch into glucose due to the occurrence of reverse

reactions in the presence of high concentrations of monosaccharide (Bucke, 1982). Several species of glucoamylase enzymes have been studied (Bucke, 1982), but the most well-known enzyme is that from *Aspergillus niger*. There have been many studies of the reversion products of glucoamylase (for examples see Pazur and Okada, 1967; Watanabe *et al.*, 1969a, b; Pazur *et al.*, 1977). Recently Nikolov and co-workers published a very thorough investigation of the *A. niger* enzyme in which the enzyme was characterized as producing maltose, isomaltose, nigerose, kojibiose, α, β-trehalose, panose, isomaltotriose and isomaltotetraose (Nikolov, Meagher and Reilly, 1989).

Until recently (see below), no attention had been given to the potential of using glucoamylases to synthesize hetero-oligosaccharides.

RECENT DEVELOPMENTS

Use of the kinetic approach

In recent years there has been a resurgence of interest in the use of glycosidases as synthetic reagents (Bucke and Rastall, 1990). Nilsson has developed the use of glycosidases to synthesize carbohydrates by the kinetic approach (Nilsson, 1987, 1990). In this work Nilsson used a range of glycosidases acting on reactive *p*-nitrophenyl glycoside substrates and obtained glycosyl transfer to a range of *p*-nitrophenyl and alkyl glycoside acceptors. It was found that by careful selection of the aglycone of the acceptor molecule it was possible to manipulate to some extent the nature of the glycosidic bonds formed (*Table 2*).

Nilsson has further developed the technique to include the *in situ* synthesis of acceptor glycosides (Nilsson, 1988). Enzyme reactions were set up to contain lactose, *E. coli* β-galactosidase, and allyl, benzyl or trimethylsilylethyl alcohol; an alternative system contained raffinose or *p*-nitrophenyl galactoside with coffee bean α-galactosidase and allyl alcohol. Galactosyl transfer occurs to the alcohol to form an acceptor glycoside; galactosyl transfer then occurs to form a disaccharide glycoside (*Table 3*).

Use of the equilibrium approach

Recent work has also explored the potential of using glycosidases to synthesize oligosaccharides by the equilibrium approach. Johannsson *et al.* (1989) used the α-mannosidase from jack bean in solutions of 83% (w/w) mannose at 60°C to reverse the normal equilibrium and bring about synthesis. It was found that the equilibrium mixture contained D-Manα(1–1)α-D-Man, D-Manα(1–2)-D-Man, D-Manα(1–3)-D-Man and D-Manα(1–6)-D-Man, trisaccharides and tetrasaccharides in decreasing yields. Rastall *et al.* (1992b) utilized the reversal of jack bean α-mannosidase to bring about the synthesis of a range of novel oligosaccharides. This was achieved by incubation of the enzyme in a mixture of D-mannose and an acceptor sugar at high (45–72%; w/w) concentration at 60°C. The yields obtained with a range of acceptors are given *Table 4*.

These workers classified the acceptor sugars according to their behaviour:

Table 2. Synthesis of oligosaccharides by the kinetic approach

Glycosyl donor	Glycosyl acceptor	Main glycoside products	Yield (%)
α-Galactosidase			
p-NPα-Gal	α-Gal-OMe	α-Gal(1–3)α-Gal-OMe	27
		α-Gal(1–6)α-Gal-OMe	<2
p-NPα-Gal	β-Gal-Ome	α-Gal(1–3)α-Gal-OMe	9
		α-Gal(1–6)α-Gal-OMe	18
p-NPα-Gal	*p*-NPα-Gal	α-Gal(1–2)α-Gal-NP-*p*	2
		α-Gal(1–3)α-Gal-NP-*p*	16
o-NPα-Gal	*o*-NPα-Gal	α-Gal(1–2)α-Gal-NP-*o*	6
		α-Gal(1–3)α-Gal-NP-*o*	<1
β-Galactosidase			
o-NPβ-Gal	α-Gal-OMe	β-Gal(1–6)α-Gal-OMe	14
		+ other isomers	<1
o-NPβ-Gal	β-Gal-OMe	β-Gal(1–6)β-Gal-OMe	3
		β-Gal(1–3)β-Gal-OMe	22
α-Mannosidase			
p-NPα-Man	α-Man-OMe	α-Man(1–2)α-Man-OMe	18
		α-Man(1–6)α-Man-OMe	4
p-NPα-Man	*p*-NPα-Man	α-Man(1–2)α-Man-NP-*p*	8
		α-Man(1–6)α-Man-NP-*p*	<0·1
		+ other isomers	0·4

Data from Nilsson (1987).

Table 3. Synthesis of oligosaccharide glycosides by the kinetic approach: *in situ* generation of acceptor glycosides

Donor	Acceptor	Major glycoside products
α-D-Galactosidase		
Raffinose	Allyl alcohol	α-Gal-OCH$_2$CH=CH$_2$
α-Gal-OPhNO$_2$-*p*	α-Gal-OCH$_2$CH=CH$_2$	α-Gal(1–3)α-Gal-OCH$_2$CH=CH$_2$
β-D-Galactosidase		
Lactose	Allyl alcohol	β-Gal-OCH$_2$CH=CH$_2$
β-Gal(1–3)β-Gal-OCH$_2$CH=CH$_2$		
Lactose	Benzyl alcohol	β-Gal-OCH$_2$Ph
		β-Gal(1–3)β-Gal-OCH$_2$Ph
		β-Gal(1–6)β-Gal-OCH$_2$Ph
Lactose	Trimethylsilylethanol	β-Gal-O(CH$_2$)$_2$Si(Me)$_3$
		β-Gal(1–3)β-Gal-O(CH$_2$)$_2$Si(Me)$_3$

Data from Nilsson (1988).

class 1 sugars act as acceptors at both 45% and 72% (w/w); class 2 sugars appear to inhibit the enzyme when used at 72% (w/w); class 3 sugars do not act as acceptors but do not inhibit mannobiose synthesis by the enzyme. The behaviour of glucose and fucose as acceptors was examined in more detail, and it was found that the absolute concentration of the sugar mixture was critical, and depended on the nature of the acceptor (*Figure 8*). It was also found that the production of hetero-oligosaccharides could be promoted over the production of homo-oligosaccharides by increasing the percentage of acceptor sugar in the mixture, although this led to a decrease in the total yield of oligosaccharide products (*Figure 9*).

Table 4. Acceptor molecules identified with jack bean α-mannosidase

	Percentage yield	
Acceptor sugar	45% sugar	72% sugar
D-Galactose	4·00	4·20
D-Glucose	11·00	11·70
D-Xylose	5·34	4·70
D-Psicose	9·30	8·20
D-Allose	8·05 (m)	2·40 (m)
D-Talose	2·67	0·00
D-Tagatose	7·50	5·50
L-Rhamnose	4·23	1·60
L-Fucose	6·73	0·00
D-Maltose	7·50	5·90
D-Trehalose	13·60	9·20
D-Melibiose	5·60	0·00
D-Maltotriose	7·50	3·20
N-Acetyl-D-mannosamine	9·50 (m)	6·40 (m)
D-Altrose	3·75	ND
D-Fructose	ND	13·10
D-Quinovose	3·05	ND
D-Arabinose	38·00	ND
N-Acetyl-D-glucosamine	3·65 (m)	ND
D-Digitoxose	2·45 (m)	ND
Sucrose	ND	14·40
Raffinose	ND	4·20
D-		

ND, no data available; m, mannobiose as sole product.

Rastall, Adlard and Bucke (1991) have also used *Aspergillus niger* glucoamylase to bring about the synthesis of α-glucose-containing hetero-oligosaccharides in the same manner. Once again the production of hetero-oligosaccharides could be favoured by the use of a high acceptor:donor ratio.

β-Glucosidase enzymes from various sources have received attention in recent years, but have not yet shown promise for the production of hetero-oligosaccharides. Ajisaka, Nishida and Fujimoto (1987a) examined the reversal of the β-glucosidase from almond in glucose solutions of up to 90% (w/w), and at 55°C. These workers found that the enzyme gave good yields of gentiobiose, sophorose, cellobiose and laminaribiose. No attempts were made by Ajisaka and colleagues to synthesize hetero-oligosaccharides, and in our hands such attempts were unsuccessful (B. Patel and R.A. Rastall, unpublished research). In order to develop a system in which β-glucosyl bonds could be synthesized, Rastall *et al.* (1992a) investigated a crude preparation of β-glucanase (laminarinase) from *Penicillium emersonii*. It was found that this enzyme gave rise to low yields (0·58–3·97%) of hetero-oligosaccharides when incubated with mannose, fucose, or fructose as acceptor sugars.

THE USE OF ORGANIC SOLVENTS

In order to induce enzyme reactions to reverse, it is necessary to reduce the concentrations of the reactants of the normal, forward, reaction (see above). The most significant reactant is water, due to its inevitably high concentration. This problem has led many workers to experiment with the use of organic solvents to reduce the water activity of their reaction systems. Much is now known about enzymes in non-aqueous media (Butler, 1979; Waks, 1986), and the use of organic co-solvents has been found to facilitate the synthesis of peptides using proteases as synthetic reagents (Morihara, 1987; Cassells and Halling, 1988). The effects of organic solvents on glycosidase enzymes were investigated some years ago. Shifrin and Hunn (1969) found that β-galactosidase was activated by the presence of lower alcohols in concentrations of up to 20–30% (v/v). This was found to be the glycosylation step because with a reactive substrate such as *p*-nitrophenyl β-galactoside is very rapid (see above), and the subsequent deglycosylation step is rate limiting. The alcohols are better acceptors of the sugar residue and the presence of the alcohol increases the speed of the rate-limiting deglycosylation, resulting in the formation of the appropriate alkyl glycoside. Higher concentrations of alcohol are inhibitory to the enzyme. These observations have been extended recently by A. Patikis and co-workers (unpublished research) to include both α- and β-glucosidases and galactosidases and α-mannosidase. The synthesis of alkyl glycosides using this approach has proved very successful. Shinoyama, Kamiyama and Yasui (1988) used the β-xylosidase from *A. niger* acting on xylobiose in the presence of alcohols to bring about the synthesis of methyl, ethyl, 1-propyl, 1-butyl, iso-butyl 1-pentyl, 1-hexyl, 1-heptyl, 1-octyl, benzyl, β-phenylethyl, 2-propyl, 2-butyl, 2-hexyl, cyclohexyl and *tert*-butyl β-xylosides. Yields varied from 16 to 114%. More recently, Vulfson *et al.* (1990) have used immobilized almond β-glucosidase to synthesize pentyl, hexyl, heptyl and octyl β-glucosides in aqueous–organic two-phase systems. This was achieved both via the kinetic approach, using either cellobiose or methyl β-glucoside as donors, and by the equilibrium approach, employing direct condensation of the sugar and the alcohol. These workers have also used polyethylene glycol-modified β-galactosidase in chlorinated hydrocarbons such as trichloroethane to achieve galactosyl transfer from methyl β-galactoside to hexanol (Beecher, Andrews and Vulfson, 1990). These reactions were carried out in systems containing just 2–6% water (v/v).

Nilsson (1986) investigated the effects of organic co-solvent on the synthesis of oligosaccharide glycosides by α-galactosidase-catalysed glycosyl transfer. Nilsson found that although organic co-solvents such as *N,N*-dimethylformamide increased yields of peptides synthesized by chymotrypsin, they reduced the yield of oligosaccharides formed by α-galactosidase. This is thought to be due to a reduced interaction between CH and CH_2 groups on the substrate and amino acids in the enzyme active site (Nilsson, 1986). More recent reports, however, have described the use of alcohols as aids to oligosaccharide synthesis. Working with a maltohexaoside-

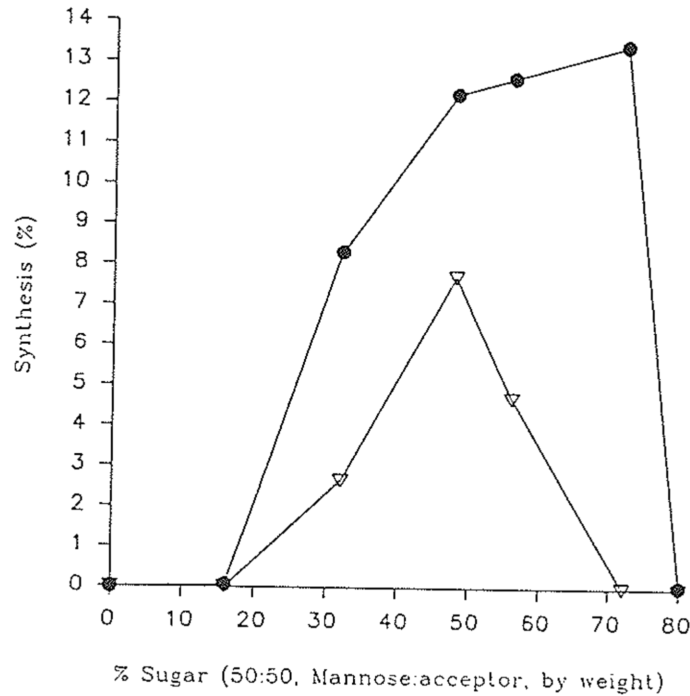


Figure 8. Effect of total sugar concentration on the synthesis of oligosaccharides by α-mannosidase. Key: ● – ●, glucose as acceptor; ▽ – ▽, fucose as acceptor.

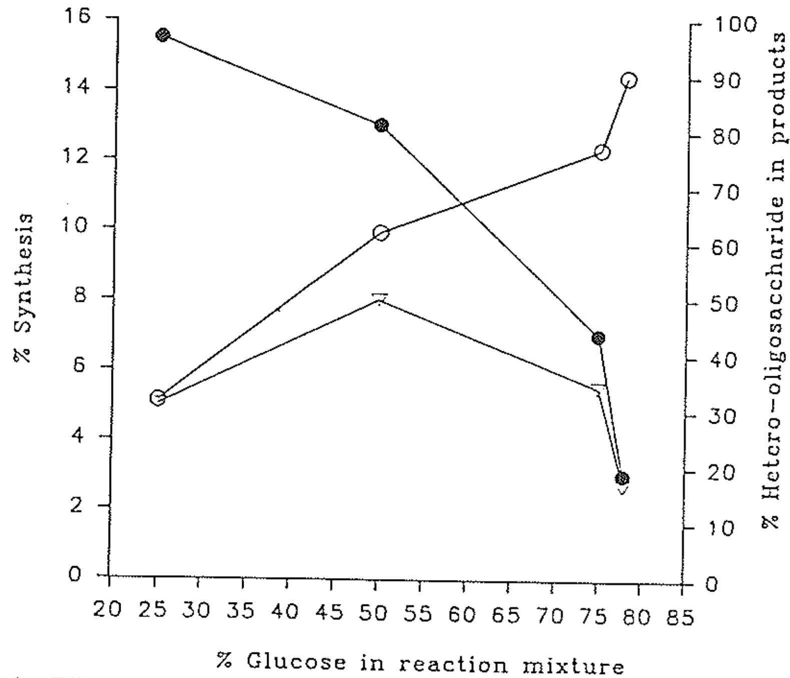


Figure 9. Effect of donor:acceptor ratio on the synthesis of hetero-oligosaccharides by α-mannosidase. Key: ● – ●, total synthesis; ▽ – ▽, total hetero-oligosaccharide synthesis; ○ – ○, % hetero-oligosaccharide in the products.

forming amylase from a UV-mutant of *Klebsiella pneumoniae*, Ogawa *et al.* (1990) found that methanol concentrations of up to 30% (v/v) increased the yield of *p*-nitrophenyl α-maltoheptaoside formed from *p*-nitrophenyl α-glucoside and maltohexaoside. Solvents were found to be effective in the following order: methanol > ethanol > 2-propanol > dimethylsulphoxide >> isopropanol. Propanol was found to be inhibitory. A similar situation was found to exist with the synthesis of *p*-nitrophenyl α-maltopentaoside from *p*-nitrophenyl α-glucoside and maltopentaose by *Pseudomonas stutzeri* (Usui and Murata, 1988). Maximum yield was obtained with 50% methanol, and alcohols were effective in the series: methanol > ethanol > *tert*-butanol > propanol. Increasing alcohol concentrations above 50% decreased yield and increased the time taken to reach peak production.

Asano, Tanaka and Matsui (1991) have found that in one system alcohols can influence the product spectrum of the reaction. These workers used rice α-glucosidase to synthesize *p*-nitrophenyl glucobiosides from maltose and *p*-nitrophenyl α-glucoside, and found that in water the enzyme produced *p*-nitrophenyl α-isomaltoside as the major product. Addition of methanol up to 40% resulted in the synthesis of *p*-nitrophenyl α-nigeroside and maltoside as well as the isomaltoside. The proportions of nigeroside and maltoside were increased further when acetonitrile was used as co-solvent. The enzyme was also immobilized on porous chitosan beads to improve thermostability. When the immobilized enzyme was used in water, *p*-nitrophenyl α-isomaltoside was produced along with nigeroside and maltoside. Addition of 40% methanol resulted in a decrease in isomaltoside production relative to the other glycosides, while the addition of 40% acetonitrile resulted in a marked decrease in isomaltoside with a selective increase in nigeroside synthesis.

THE USE OF AQUEOUS TWO-PHASE SYSTEMS IN OLIGOSACCHARIDE SYNTHESIS

Aqueous two-phase systems and their various applications in biochemistry and biotechnology have recently aroused much interest (Walter, Brooks and Fisher, 1985). Most of this interest has so far concentrated on applications to extractive bioconversions and to protein purification. Aqueous two-phase systems are formed when certain water-soluble polymers are mixed in aqueous solution. Their incompatibility results in the formation of two aqueous phases.

Bartlett *et al.* (1992) have used the novel approach of carrying out oligosaccharide synthesis reactions using the kinetic approach in a polyethylene glycol (PEG)/dextran two-phase system (*see Figure 10*). These workers found that the enzyme jack bean α-mannosidase partitioned very strongly into the dextran phase, whilst *p*-nitrophenyl α-mannoside donor and *p*-nitrophenyl β-galactoside acceptor partitioned evenly. It proved possible to manipulate the phase: volume ratio so that an excess of the PEG phase was present. With this system a 10-fold increase in the amount of 6-*O*-α-mannosyl-*p*-nitrophenyl-β-galactoside formed per unit of enzyme was achieved, compared with normal aqueous systems. The authors also point out

that the two-phase system may have process advantages for large-scale synthetic reactions.

SYNTHESIS OF GLYCOSYL–AMINO ACID CONJUGATES

The carbohydrate chains associated with glycoproteins are anchored to the protein via covalent bonds between the reducing end of the oligosaccharide chain and an asparagine (*N*-linked) or a serine or threonine (*O*-linked) amino acid residue (Rademacher, Parekh and Dwek, 1988). This has prompted attempts to synthesize enzymatically glycosyl amino acids. Johansson, Hedbys and Larsson (1991) have used the equilibrium approach with jack bean α-mannosidase and bovine liver α-*N*-acetylgalactosaminidase. Both of these enzymes catalysed the formation of *O*-glycosylated serine or threonine residues when incubated at 50°C in the presence of 2·9 M L- or D-amino acid and 2·7 M D-mannose or D-*N*-acetylgalactosamine. Both of these enzymes also formed a mixture of disaccharide products together with the glycosyl amino acids, and non-enzymic Maillard reactions also occurred. These workers also examined *E. coli* β-galactosidase, *A. niger* and coffee bean α-galactosidase, and *A. niger* β-xylosidase for the ability to synthesize glycosyl amino acids by both kinetic and equilibrium means; only the α-mannosidase and α-*N*-acetylgalactosaminidase gave enzymically synthesized products.

Cantacuzene and Attal have successfully synthesized galactosyl-L-serine using galactosidases (Cantacuzene and Attal, 1991). These workers used the kinetic approach with lactose or raffinose as glycosyl donor and protected serine derivatives as acceptors. They found that the amino-blocking agent is very important: best yields with the *E. coli* β-galactosidase were obtained using *N-tert*-butoxycarbonylserine methyl ester (15%), with lower yields obtained using the *N*-benzyloxycarbonyl derivative (9%). With the coffee bean α-galactosidase, only 2–3% yields were obtained. The β-galactosidase reaction was not facilitated by the use of organic solvents such as *N,N*-dimethylformamide, acetonitrile, or diethylene glycol diethyl ether.

THE USE OF IMMOBILIZED ENZYMES IN OLIGOSACCHARIDE SYNTHESIS

Often, the most expensive component of an enzymically catalysed oligosaccharide synthesis reaction is the enzyme itself. The mono- and disaccharide reactants can be recycled after separation from the products, but this is not feasible with the enzyme; in addition, immobilization may be expected to stabilize the enzyme when used at high temperatures or in the presence of organic solvents (Larsson *et al.*, 1987). For this reason, it is of great interest to investigate the use of immobilized enzymes in these reactions. It may also prove possible to influence the product spectrum by careful choice of the immobilization conditions.

Using the equilibrium approach, Ajisaka, Nishida and Fujimoto (1987b) used immobilized β-galactosidase to synthesize lactulose and allolactulose from mixtures of galactose and fructose, and *N*-acetyllactosamine and *N*-acetylallolactosamine from mixtures of galactose and *N*-acetylglucosamine.

These workers immobilized the *E. coli* enzyme on either Eupergit C or Sepharose 4-B, and syntheses were carried out by circulating solutions containing 10% (w/v) galactose and either 30% (w/v) *N*-acetylglucosamine or 50% (w/v) fructose through columns of immobilized enzyme. The immobilized enzyme columns were connected in series with a column of activated carbon to remove disaccharide products (Abe, Hayashi and Katagawa, 1983; *see Figure 11*), and thus shift the equilibrium in the direction of condensation and increase yield. Product oligosaccharides can then be eluted from the column by washing with 10–50% (v/v) ethanol. This system gave yields of 0·7–0·9% *N*-acetyllactosamine, and 7·0–9·1% *N*-acetylallolactosamine per 24 h of recirculation; and the reaction was repeated four times to give total yields of 3·2% *N*-acetyllactosamine and 31·6% *N*-acetylallolactosamine. Ajisaka, Fujimoto and Nishida (1988) have extended these results to include glucose as an acceptor, and examined the influence of immobilization on product spectrum. They found that the immobilized enzyme-activated carbon system increased yields when glucose and *N*-acetylglucosamine were used as acceptors, but not when fructose was used. Immobilization also had a small influence on the product spectrum but it is not clear how significant from the results presented.

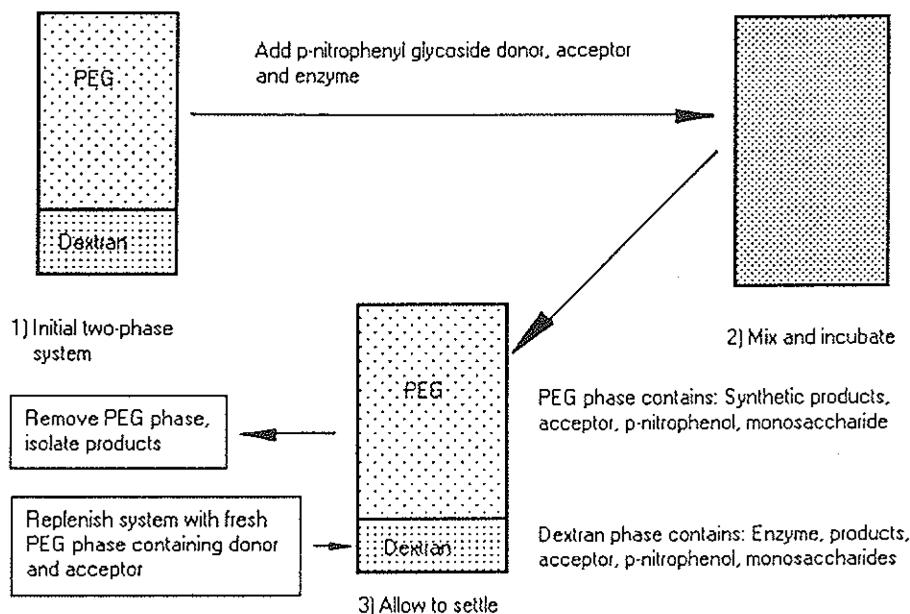


Figure 10. Synthesis of oligosaccharides in aqueous two-phase systems.

These same workers (Ajisaka and Fujimoto, 1989) immobilized α-galactosidase from *M. vinacea* and used it in the same continuous synthesis method. From a solution of 10% (w/v) galactose and 50% (w/v) sucrose, this system yielded a single trisaccharide identified as raffinose. This contrasted with a mixture of raffinose and planteose (3:2) obtained from the batch reaction. A similar selectivity was found with immobilized β-galactosidase (*E. coli*) which yielded only isoraffinose as compared to a mixture of isoraffinose

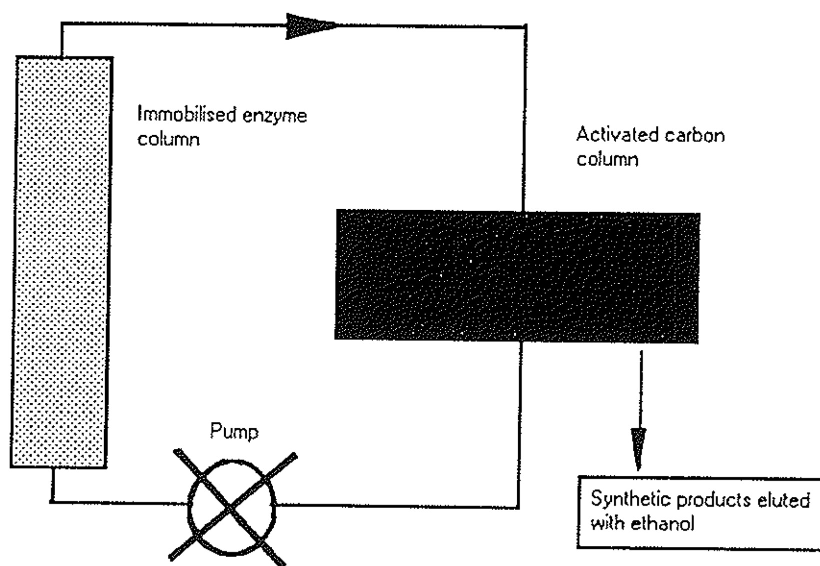


Figure 11. Continuous synthesis of oligosaccharides using immobilized enzymes.

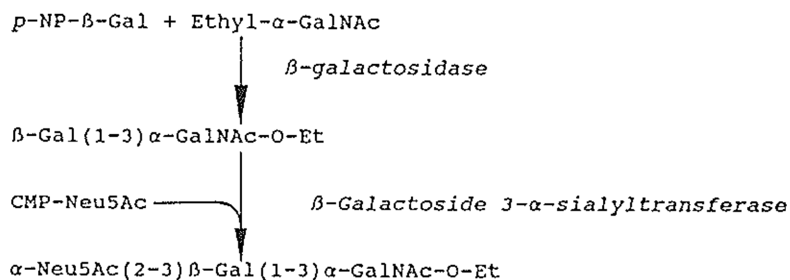


Figure 12. Example of integrated synthesis approach using glycosidase reversal followed by natural glycosyl transferase. Adapted from Nilsson (1989).

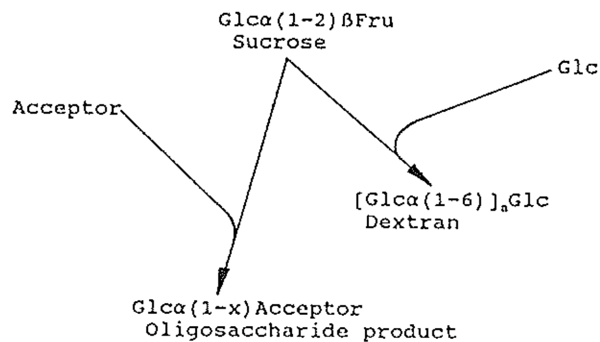


Figure 13. Synthesis of dextran and acceptor products by dextransucrase.

and D-glc*p*α(1–2)-[D-gal*p*β(1–6)]β-D-fru*f* obtained from the batch reaction. The authors explained this regioselectivity as being due to the immediate binding of trisaccharide products to the activated carbon column. As a result, the reaction never comes to equilibrium as in the case of the batch reaction, and the product spectrum is thus a consequence of differences in activation energies of formation.

Immobilized enzymes have also been used to synthesize oligosaccharides by the kinetic approach. Larsson *et al.* (1987) immobilized β-galactosidase on Sephadex CL-4B and used the preparation to hydrolyse lactose in the presence of *N*-acetylgalactosamine, resulting in the synthesis of D-galβ(1–6)-D-gal*N*Ac.

Mozaffar, Nakanishi and Matsuno (1988) used a commercial preparation of *Bacillus circulans* β-galactosidase, immobilized on porous silica gel by cross-linkage with glutaraldehyde, to synthesize a range of oligosaccharides from disaccharides to a pentasaccharide during lactose hydrolysis.

Cellulose beads have also been successfully used as support matrix for immobilized β-galactosidase (Kéry *et al.*, 1991). These workers used *p*-nitrophenyl-β-galactoside as glycosyl donor and methyl-α-galactoside as acceptor, and synthesized a mixture of 6- and 3-linked disaccharides and trisaccharides.

ADVANTAGES AND DISADVANTAGES OF GLYCOSIDASES IN REVERSE: FUTURE PROSPECTS

The major advantages of this approach to synthesis are that glycosidase enzymes are often easily purified, stable and many are readily and cheaply available, and the enzyme preparations used need not be completely pure (Rastall *et al.*, 1992a). The reactions are very easy to set up, requiring only an incubator, and many linkage isomers can be formed in one reaction. These isomers do, however, pose a separation problem.

The biggest disadvantage with glycosidase reversal is that it is difficult to regulate the proportions of the various product oligosaccharides in the reaction mixture using the equilibrium approach, and the kinetic approach (which gives more control over the products formed) gives small quantities of products due to their low solubility in water. Further research into the use of organic solvents may reduce these disadvantages to some extent. To date, most enzymatic synthesis work with glycosidases has focused on the exo-enzymes which have rather loose specificity; it will be instructive to consider endo-enzymes and exo-enzymes possessing more strict specificities.

In the future it may prove worthwhile to modify, by protein engineering, the active sites of glycosidase enzymes to reduce the number of linkage isomers formed, and the acceptor binding sites to introduce acceptor specificity. This would produce a useful and robust synthetic catalyst. This development must, however, wait for a much more detailed understanding of the way in which various acceptors interact with the enzyme.

Integrated syntheses

A useful approach to the synthesis of oligosaccharides is the integration of two or more methods of synthesis. This has been achieved (Nilsson, 1989) by using β-galactosidase to transfer galactose from *p*- or *o*-nitrophenyl-β-D-galactosides to ethyl-α-D-*N*-acetylgalactosaminide, bromoethyl-β-D-*N*-acetylgalactosaminide, or methyl-β-D-*N*-acetylgalactosaminide to synthesize acceptors for β-D-galactoside 3-α-sialyltransferase (*see Figure 12*). The linking together of different enzymatic synthesis methods has not yet been fully exploited but should have great potential; glycosidases and sucrose-dependent glycosyl transferases (see below) provide short oligosaccharides for further glycosylation by nucleotide-dependent glycosyl transferases or by chemical methods.

The use of sucrose-dependent glycosyltransferases in oligosaccharide synthesis

INTRODUCTION

Sucrose-dependent glycosyltransferases form a class of microbial enzymes with potentially wide applications in enzymic synthesis of oligosaccharides. Several such enzymes are known and some are very well characterized (see below). These enzymes are attractive catalysts as they are easy to obtain from microbial culture. In fact, in many cases, it may not be necessary to purify the enzyme at all, as the culture supernatant is usually very rich in enzyme activity.

DEXTRAN SUCRASE

Dextran sucrase is an enzyme capable of synthesizing α(1–6) linked glucan polymers (dextrans). This is achieved by the hydrolysis of sucrose and the subsequent transfer of the glucosyl residue to glucose to form polymeric dextran, or to an acceptor molecule to form oligosaccharides (*Figure 13*). A great deal of information is known about these enzymes, most of all about the enzyme from *Leuconostoc mesenteroides* (for example, Robyt and Eklund, 1982, 1983).

Many carbohydrates have been investigated for their ability to act as acceptors with the *L. mesenteroides* enzyme (Robyt and Eklund, 1983; *see Table 5*). Where the structures of the product oligosaccharides have been determined, 1–6 linkages have most frequently been found. Notable exceptions are 2-α-glucosylcellobiose, 2-α-glucosyllactose, and 2-α-glucosylraffinose (Robyt and Eklund, 1982). These results have been explained by Robyt and Eklund (1982) in terms of the hydrogen bonding pattern between the acceptor and the glucosyl–enzyme complex.

In addition to the primary products many of the products were themselves acceptors, forming series of α(1–6) linked oligosaccharides terminating with the primary product. This has been observed (in order of decreasing affinity) with maltose, isomaltose, nigerose, methyl α-glucoside, D-glucose, turanose, methyl β-glucoside, cellobiose and L-sorbose (Robyt and Eklund, 1982).

Table 5. Acceptor molecules identified with *L. mesenteroides* dextran sucrase

Acceptor sugar	Yield*
D-Glucose	6·9
D-Mannose	2·2
D-Galactose	1·3
D-Fructose	4·8
L-Sorbose	1·4
D-Xylose	0·4
Methyl α-glucoside	23·4
Methyl β-glucoside	8·0
1,5-Anhydro-D-glucitol	9·1
α, α-Trehalose	0·0
α, β-Trehalose	ND
β, β-Trehalose	ND
Maltose	48·0
Nigerose	20·5
Cellobiose	5·4
Isomaltose	43·0
Isomaltulose	ND
Gentiobiose	ND
Melibiose	ND
Sophorose	ND
Kojibiose	ND
Laminaribiose	3·2
Turanose	5·5
Raffinose	3·3
Lactose	8·1
Lactulose	ND
Leucrose	ND
Theanderose	ND

* Yields are expressed as the percentage incorporation of the acceptor supplied. Dextran, isomaltose, leucrose, and free glucose were also produced in all cases in varying amounts.
ND, not determined.
Table adapted from Robyt and Eklund (1982, 1983).

ALTERNANSUCRASE

Alternansucrase is an enzyme isolated from *Leuconostoc mesenteroides* which synthesizes a glucan polysaccharide containing alternating α(1–6) and α(1–3) linkages (Côté and Robyt, 1982) using sucrose as a glycosyl donor. These workers found that this enzyme can synthesize a range of oligosaccharides containing α(1–6) and α(1–3) linkages (*Table 6*).

The primary products listed in *Table 6* are also acceptors and they form series of oligosaccharides containing α(1–6) and α(1–3) bonds. it was found that α(1–3) bonds were only formed if the non-reducing glucose of the acceptor is linked by an α(1–6) bond.

STREPTOCOCCUS GLUCOSYL TRANSFERASES

Oral streptococci such as *Streptococcus mutans* are known to produce glucan polysaccharides involved in adhesion to dental surfaces. Two polymers are produced: a water-insoluble mutan, an α(1–3) linked glucan containing 7% α(1–6) linkages; and a water-soluble dextran containing α(1–6) linkages with

Table 6. Acceptor molecules identified with *L. mesenteroides* alternansucrase

Acceptor sugar	Products	Yield*(%)
Maltose	Panose and higher dp oligomers	84
Nigerose	6^2-α-Glucosylnigerose and higher oligomers	80
Isomaltose	Isomaltotriose, 3^2-α-Glucosylisomaltose, and higher oligomers	41
D-Glucose	Isomaltose	29
Methyl α-glucoside	Methyl α-isomaltoside	49
Methyl β-glucoside	Methyl β-isomaltoside	19

* Yields are percentage incorporation of glucose into products. Alternan polysaccharide and leucrose were also synthesized in all cases in varying amounts.
Data adapted from Côté and Robyt (1982).

27% α(1–3) linked branches (Shimomura, Tsumori and Mukawa, 1982; Fukui *et al.*, 1982). These polymers are synthesized by two glucosyl transferases called GTF-I, and GTF-S, synthesizing mutan and dextran, respectively (Hare, Svensson and Walker, 1978; Robyt and Martin, 1983).

These enzymes transfer glucose from sucrose and certain other glucose oligosaccharides to acceptor sugars to form the polysaccharide. They can, however, also transfer glucose to acceptor sugars to give rise to oligosaccharides. These reactions have been studied to some extent, as they inhibit the formation of the polysaccharide, and hence reduce adhesion to dental surfaces. Fu and Robyt (1991) have studied the transfer reaction with maltodextrins as acceptors. They found that, with maltose as acceptor, the enzymes both gave rise to panose as primary product. This then acted as acceptor to give rise to a series of oligosaccharides with α(1–6) linkages. Incubation of GTF-I with maltotriose gave rise to four tetrasaccharides containing α(1–6) and α(1–3) linkages on the reducing and non-reducing terminal glucoses. GTF-S, however, gave rise to two tetrasaccharides containing α(1–6) linkages on the reducing and non-reducing terminals, and then a series of α(1–6) linked oligosaccharides. This pattern was found for maltopentaose, maltohexaose and maltoheptaose.

CYCLODEXTRIN-GLUCOSYLTRANSFERASE (CGTase)

CGTase is an enzyme catalysing the breakdown of starch with the concomitant synthesis of cyclic α(1–4) linked oligosaccharides (cyclodextrins) of six, seven or eight sugars. The enzymes are produced by *Bacillus macerans*, *B. megaterium*, *B. stearothermophilus*, *B. circulans*, *B. ohbensis*, Alkalophilic *Bacillus* 38-2, *Micrococcus* sp. and *Klebsiella pneumoniae*.

Cyclodextrins have proved to have wide applications in a variety of industries due to their ability to produce inclusion complexes. For a review of

cyclodextrins, their applications, and the enzymes that produce them, see Bender (1986).

Due to the industrial importance of these molecules, much attention has been focused on the transfer reactions catalysed by CGTases. Despite this interest, relatively little has been discovered about the ability of these enzymes to synthesize hetero-oligosaccharides. Kitahata, Okada and Fukui (1978) examined the acceptor specificity of the *B. megaterium* and *B. macerans* enzymes using 22 sugars and sugar alcohols as acceptors (*see Table 7*). These workers found that L-sorbose, D-glucose, phenyl-α-glucoside, phenyl-β-glucoside, methyl-α-glucoside and methyl-β-glucoside were all good acceptors. 6-Deoxy-D-glucose and D-xylose were slightly weaker acceptors; 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose were weak acceptors; and D-galactose, D-glucuronic acid, L-arabinose, D-ribose,

Table 7. Acceptor specificity of *Bacillus* CGTase

Acceptors	Non-acceptors
D-Glucose	D-Fructose
L-Sorbose	D-Mannose
D-Xylose	D-Glucosamine
D-Galactose	*N*-Acetyl-D-glucosamine
2-Deoxy-D-glucose	L-Rhamnose
3-*O*-Methyl-D-glucose	D-Allose
6-Deoxy-D-glucose	D-Quinovose
Methyl-α-glucoside	D-Glucuronic acid
Methyl-β-glucoside	L-Arabinose
Phenyl-α-glucose	D-Ribose
Phenyl-β-glucoside	α-D-Glucose-1-phosphate
Maltose	D-Glucose-6-phosphate
Nigerose	Trehalose
Sophorose	2-Deoxy-D-*arabino*hexose
Sucrose	*myo*-Inositol
Maltulose	Glucitol
Palatinose	Xylitol
Cellobiose	Glycerol
p-Nitrophenylmaltoside	
6^2-α-Maltosylmaltose	
6^3-α-Glucosylmaltotriose	

Data collected from Kitahata, Okada and Fukui (1978), and Vetter *et al.* (1992).

D-mannose, D-glucosamine, *N*-acetyl-D-glucosamine, L-rhamnose, sorbitol, xylitol, glycerol and D-fructose were either non-acceptors or very weak acceptors (D-galactose). On the basis of the acceptor specificity, the authors concluded that, in order to be a good acceptor, the sugar concerned must have the same orientation of hydroxyl groups about C-2, C-3 and C-4 as glucose. The glucosyl group is transferred to C-3 hydroxyl of L-sorbose, to C-4 hydroxyl of D-glucose and D-xylose (Kitahata and Okada, 1976) and to C-4 hydroxyl of 2-deoxy-D-glucose (Kitahata, Okada and Fukui, 1978). The products of the weak reaction with galactose as acceptor were found to be α(1–1), α(1–3), α(1–2) and α(1–4) linkages in the ratio of 26:10:1, respectively, grouping α(1–2) and α(1–4) (Kitahata, Okada and Misaki, 1979).

More recently, Vetter *et al.* (1992), have used CGTase to synthesize linear

and branched oligosaccharides. These workers found that the enzyme transferred maltohexaosyl residues from cyclomaltohexaoside (α-cyclodextrin) to a range of acceptors (*see Table 7*). In an interesting example of the use of multiple enzymes to achieve synthesis of specific products, they used maltohexaosyl transfer to cellopentaose to generate an undecasaccharide, then used glucoamylase to digest five residues from the non-reducing end to liberate 4^5-α-glucosylcellopentaose.

Concluding remarks

There are plenty of references in the literature to suggest that enzymatic approaches have a significant contribution to make to the field of oligosaccharide synthesis. Enzymatic syntheses can be performed using a variety of approaches with a variety of enzymes. Glycosidases are perhaps best suited to the synthesis of small oligosaccharide building blocks, although the wide acceptor specificity of these enzymes means that they are capable of synthesizing novel oligosaccharides of unknown, possibly exploitable, biological activities. The potential development of oligosaccharide 'blocking factors' to prevent biological interactions involving carbohydrates will, hopefully, be facilitated by the use of glycosidases in reverse.

The microbial sucrose-dependent glucosyl- and fructosyl-transferases are a very promising group of enzymes that have yet to have their full synthetic potential defined. These enzymes also have loose acceptor specificities, and they would be particularly suited to adding glucose or fructose residues to the products of glycosidase reversal.

Natural glycosyl transferase enzymes are very attractive synthetic reagents due to their innate specificity. These enzymes are perhaps best suited to the specific addition of sugar residues to building blocks synthesized using other enzymes or by chemical protocols.

It is to be expected that the techniques of genetic recombination and protein engineering will have great impact in the field of enzymatic oligosaccharide synthesis. Gene cloning technology will, hopefully, make available a wider range of glycosyl transferases to use in synthetic regimes. The development of more stable glycosyl transferases by protein engineering will vastly increase the utility of these enzymes in synthesis. Protein engineering techniques may conceivably be applied to the development of glycosidases with more defined acceptor specificities. These enzymes naturally tend to be rather thermostable, are widely available in nature, and are not difficult to purify. Genetically modified glycosidases would be very attractive synthetic catalysts.

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