An Investigation of the Transferase Activity of Cellulase from *Trichoderma Reesei*

By

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To my family,

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Abstract

A study of the transglycosylation reactions catalysed by a multi-enzyme complex, cellulase from *Trichoderma reesei,* was undertaken. An activated substrate donor, p-nitrophenyl β-D-cellobioside (PNPC), and various mono- and disaccharide acceptors were tested in the studies which were performed under kinetically controlled conditions.

Surprisingly, three main transfer products were obtained as opposed to the single product cited in the literature for the cellulase catalysed reaction. Two were identified as the N-(p-nitrophenyl)-β-D-cellobioside (a β-(1-4) linked disaccharide) and the N-(p-nitrophenyl)-β-D-gentiobiosylamine (a β-(1-6) linked disaccharide). A number of experimental parameters were varied and their effects on the yield of the transglycosylation reaction were determined. The variables investigated included: increasing of substrate concentration, increasing the acceptor concentration, varying the pH and the temperature of the reaction. The effect of the addition of a co-solvent (ACN, t-butanol, dioxane or acetone) was also studied. The reactions were found to be stereospecific but not regioselective. The latter was found to vary with the substrate concentration: at low concentrations (< 1.5 mM), the β-(1-6) linked disaccharide was the preferred transfer product whereas at higher concentrations, the β-(1-4) linked disaccharide was favoured. Increasing the acceptor concentration was found to increase the transglycosylation yield (6% to 19%) whereas the addition of co-solvent resulted in a decrease. These results are discussed in relation to the components of the complex which are responsible for the production of the various transfer products. Interestingly, the use of p-nitrophenyl 1-thio-β-D-glucopyranoside as an acceptor proved to give a higher yield of the transfer products (~40 %) showing the importance of the acceptor structure in the transglycosylation reaction

Moreover, the transglycosylation studies were also undertaken in the presence of the β-glucosidase inhibitor, 1,5-glucono-δ-lactone. This resulted in the formation of a single product, the β-(1-4) linked disaccharide and therefore β-glucosidase was the only cellulase component responsible for producing the β-(1-6) transfer product. A difference in the degree of orientation of the acceptor between the different enzyme components of the cellulase complex was then suggested.

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List of Abbreviations

Ac	Acetyl
ACN	Acetonitrile
Bn	Benzyl
Bz	Benzoyl
CBH	Cellobiohydrolase
CE	Capillary electrophoresis
COSY	Correlation spectroscopy
DMAP	4-N,N-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
EG	Endoglucanase
FT-IR	Fourier transform infra-red spectroscopy
HPLC	High pressure liquid chromatography
MS	Mass spectrometry
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NGP	Neighbouring group participation
NMR	Nuclear magnetic resonance
ppm	Part per million
THF	Tetrahydrofuran
Tr	Triphenylmethyl

Chapter 1

Introduction

1. Carbohydrate Overview

Carbohydrates represent the most abundant biomolecules on earth and, each year, photosynthesis converts more than a hundred billion tonnes of carbon dioxide and water into cellulose and other plant products[1]. They are also synthesised and derivatised by various mechanisms inside cells to produce a large number of biologically active compounds.

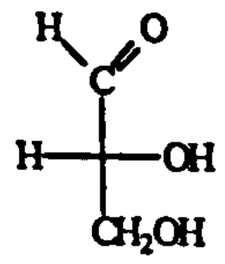
1.1. Carbohydrate Chemistry

The basis of our understanding of the organic chemistry of carbohydrates was laid in Germany, at the end of the 1800s, by Emil Fischer. He was awarded the second Nobel Prize for Chemistry in 1902 for the elucidation of the structure of glucose and its isomers[2].

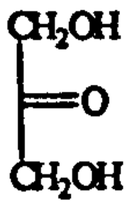
In their simplest form, carbohydrates are considered as polyhydroxy- aldehydes or ketones. The simple empirical formula of carbohydrates, e.g. glucose and its isomers, is $C_n(H_2O)_n$ and hence they are sometimes referred to as 'hydrate of carbon'. However, many compounds considered as carbohydrates contain nitrogen, sulfur, and phosphorus atoms; the name carbohydrate is a generic name used to cover a broad variety of molecules.

1.1.1. Carbohydrate families

Carbohydrates may be divided into three major classes: monosaccharides, oligosaccharides and polysaccharides. Monosaccharides are the simplest sugars, and comprise of a single polyhydroxy- aldehyde or ketone unit. They are divided into two different families, the aldose and the ketose families (Figure 1).



Glyceraldehyde
an aldose



Dihydroxyacetone
a ketose

Figure 1 : Examples of aldose and ketose structures

The -ose suffix is used to designate a carbohydrate and the aldo- and keto- prefixes indicate the nature of the carbonyl group. The number of carbon atoms in the monosaccharide is given by using tri-, tetr-, pent-, hex-, etc... as the base name. For example, glyceraldehyde is an aldotriose, dihydroxyacetone is a ketotriose. Oligosaccharides consist of short chains of monosaccharide units joined together by glycosidic linkages. Polysaccharides are comprised of long chains of monosaccharides.

1.1.2. Cyclisation and mutarotation

Monosaccharides with five or more carbon atoms in the backbone occur in solution as cyclic hemiacetals. The presence of both a carbonyl and multiple alcohol functions in the molecule allows nucleophilic attack of hydroxyl groups on the carbonyl to yield a more thermodynamically favoured cyclic product. Two diastereoisomers result from the cyclisation and are called **anomers.** In D-glucose, ring closure occurs by preferential nucleophilic attack of the hydroxyl oxygen at C-5 on the carbonyl carbon atom, C-1, forming a new chiral centre, called the **anomeric centre,** within a pyranose ring (Figure 2). Two different stereoisomers can thus be formed according to which face of the carbonyl, Re or Si, the addition takes place at. In the case of glucose, these two anomeric forms are denoted as α- and β-anomers; the α-anomer having the hydroxyl group trans to the methylene substituent of the pyranose ring whereas the β-anomer has the opposite configuration (cis).

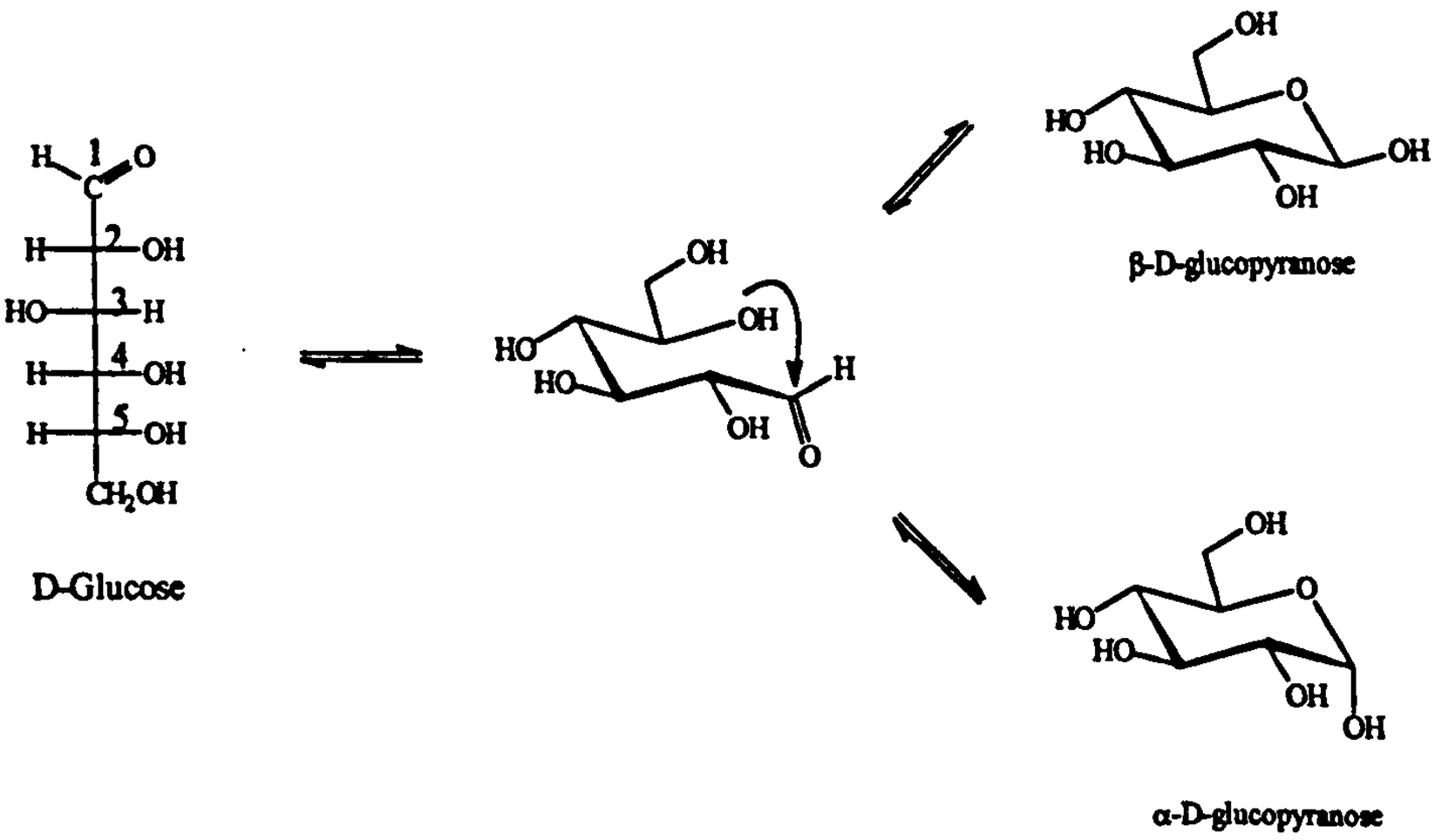


Figure 2 : Cyclisation of D-glucose and mutarotation

The formation of a cyclic structure gives rise to a number of properties associated with the anomeric effect and the aglycon orientation at C-1.

1.1.3. Anomeric effect

Although bulky substituents favour equatorial orientation, allowing minimum 1,3-diaxial interactions, electronegative groups attached to the anomeric centre tend to favour axial positions[2,3]. This preference, first identified by Edward and Lemieux, results from the requirement for a synperiplanar arrangement, a preferential axial orientation, of the lone pair of the ring oxygen where it overlaps with the antibonding orbital, σ*, of the axial C-X bonds. This results in the shortening of the ring O-C_1 bond and lengthening of the C_1-X bond compared to the equatorial anomer.

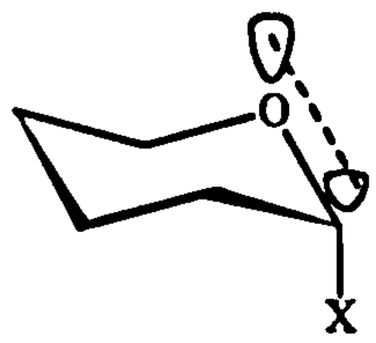


Figure 3 : Anomeric effect

The preference for axial orientation of electronegative substituents is also favoured by the greater electrostatic repulsion between the ring oxygen's lone pair and the dipole of the C_1-X bond, when located in an equatorial position. The ratio of α- and β-anomers thus depends on the electronegativity of X and the overall dipole of the molecule, which will be influenced by the environment of the molecule. A polar solvent will tend to favour β-anomer due to the greater solvation of the larger dipole of the molecule i.e. compared to that of the α-anomer. A reverse anomeric effect can be observed, resulting in an equatorial orientation of the substituent when a positively charged nitrogen group is found directly attached to C-1 (Figure 4).

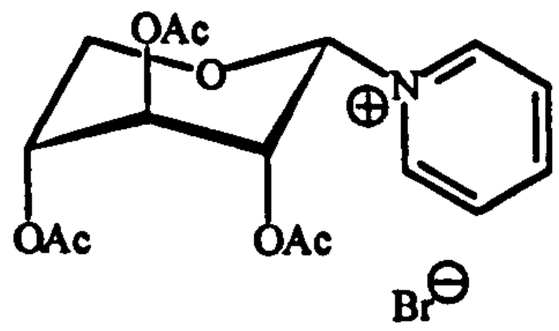
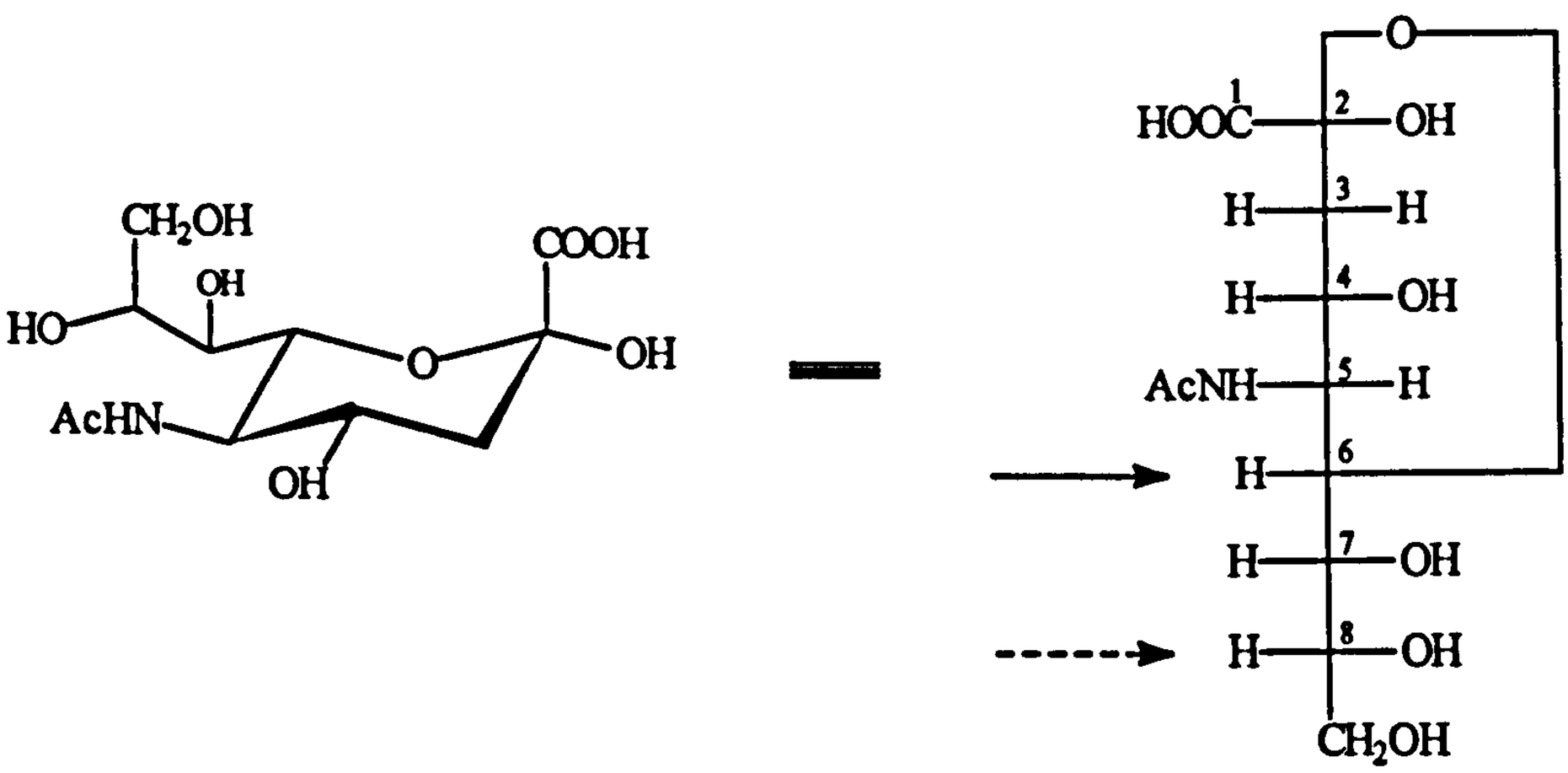


Figure 4 : Reverse anomeric effect

1.2. Carbohydrate Nomenclature

1.2.1. Anomeric configuration

The anomeric configuration is determined by first identifying the anomeric reference atom e.g. the highest numbered chiral carbon atom contained within the heterocyclic ring (Figure 5) e.g. C-5 for glucopyranose and C-4 for furanose. In the α-anomer, the exocyclic oxygen atom at the anomeric centre is formally cis, as viewed in the Fischer projection diagram, to the oxygen attached to the anomeric reference atom. In the β-anomer, these oxygens are formally trans[4].



⟶ Anomeric reference atom

⋯⋯▸ Configurational atom

Figure 5 : 5-acetamido-3,5-dideoxy-D-glycero-D-galacto-2-β-nonulosonic acid (N-acetylneuraminic acid)

1.2.2. Sugar configuration

The configuration of a monosaccharide is defined as D- or L- according to the configuration at the highest-numbered centre of chirality[4], the 'configurational atom' (Figure 5). Thus, if the hydroxyl group attached to the configurational atom projects to the right in the Fischer projection, the sugar belongs to the D-series.

1.3. Importance of Carbohydrates

1.3.1. Biologically active carbohydrates

Over the course of the last decade, the scientific community has become increasingly aware of the biological importance of carbohydrates[5]. *In vivo*, carbohydrates are found as an energy source, as a means of specific biological recognition, and they play important roles in a large number of biologically active events such as metastasis[6], fertility[7], etc... Oligosaccharides are usually found covalently attached to lipids (glycolipids) or proteins (glycoproteins[8]). These so-called glycoconjugates are often used as cell surface receptors (Figure 6). Cell surface receptors are used as blood group determinants, for adhesion processes, as binding sites for antibodies or proteins, for interaction with pathogens, bacteria and viruses and for initiation of the immune system defences[9].

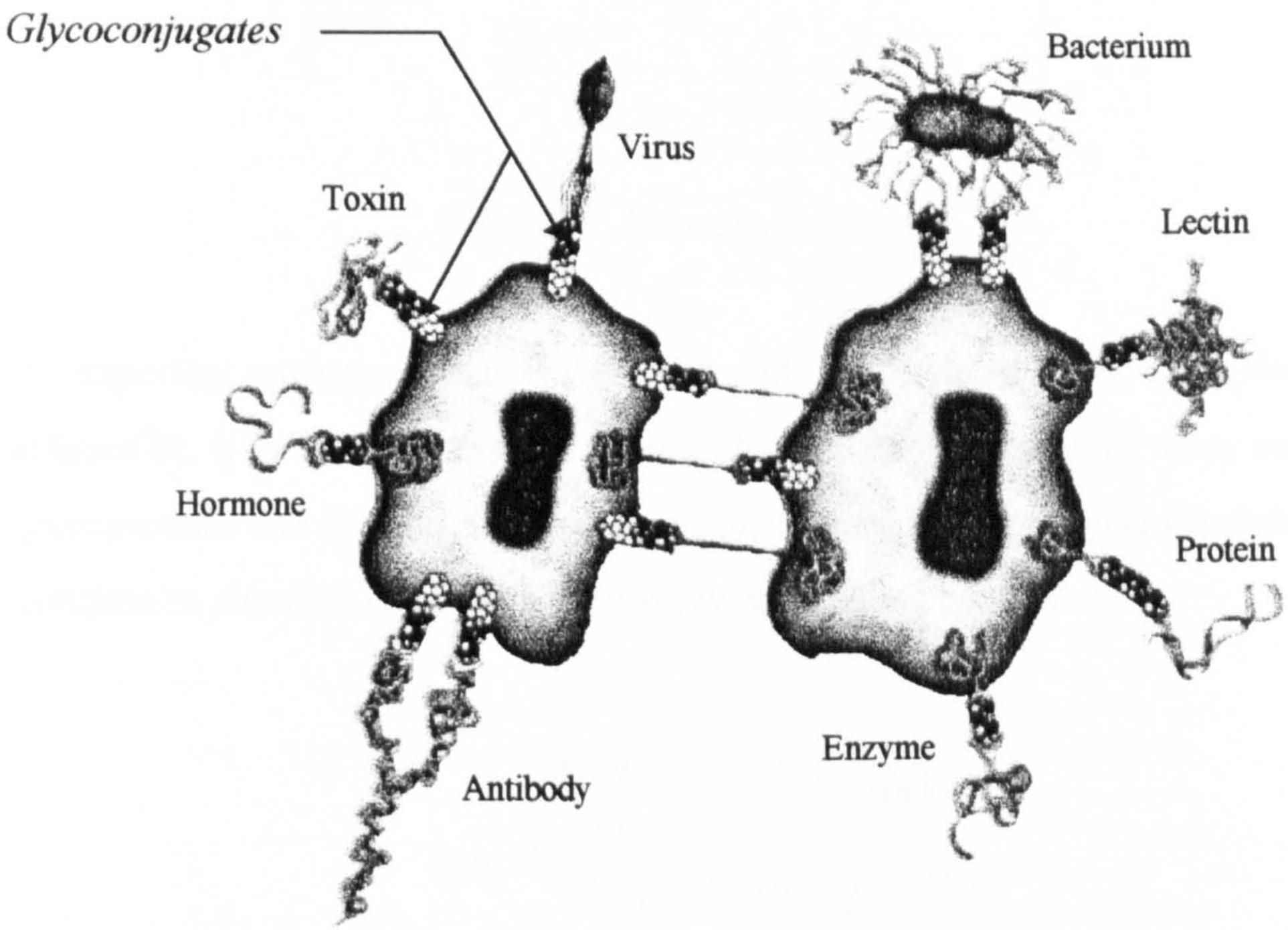


Figure 6 : Cell surface carbohydrates.

The wide range of applications of biologically active carbohydrate molecules has recently led to a classification according to their therapeutic activity[10]. Six main

groups have been defined including antibiotics, anticonvulsant, antiviral, antidiabetic, antitumor and anticoagulant agents as well as other minor groups[11].

Heparin is an example of an anticoagulant, it is a polysaccharide involved within the blood circulation system, with anticoagulant properties due to its specific affinity for the protease antithrombin which inhibits the proteolytic enzymes involved in blood clotting[12] (Figure 7).

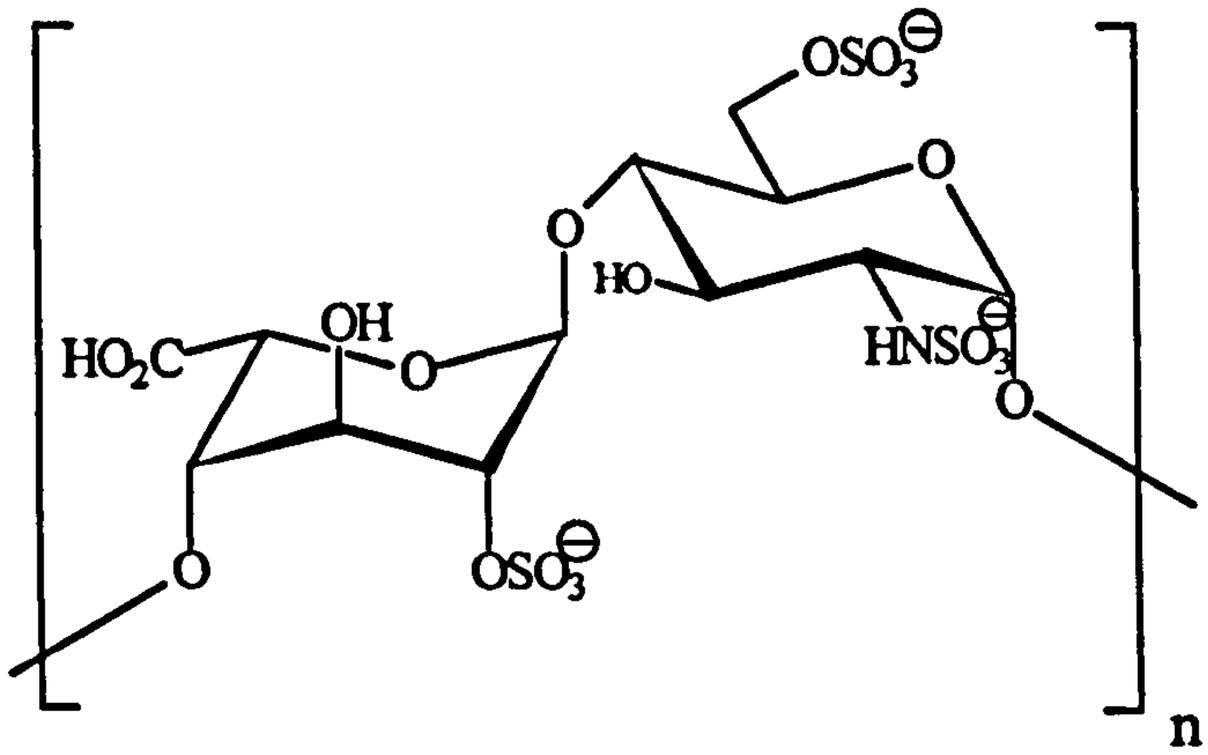


Figure 7 : Heparin structure

An important monosaccharide is the sialic acid: N-acetylneuraminic acid (NeuAc) (Figure 8), it is found at the end of the oligosaccharide chains of many soluble glycoproteins and its presence or absence determines whether a given protein will continue to circulate in the blood stream or be removed by the liver[13].

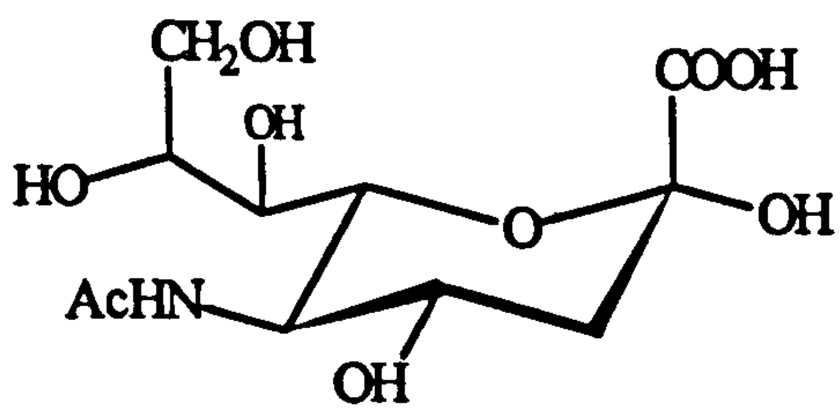


Figure 8 : N-acetylneuraminic acid

1.3.2. Structural and energy storage carbohydrates

Carbohydrates also have an important structural role and are used for energy storage.

Cellulose, for example, forms the principal structural constituent of cell walls in plants and wood[14]. It is an unbranched homopolysaccharide composed of D-glucose residues linked together by β-(1-4) glycosidic bonds to form linear polymeric chains containing greater than 10,000 glucose residues. The most stable conformation for the polymer is that in which each chair is turned 180° relative to the preceding subunit, yielding a straight extended chain. The individual chains adhere to each other along their lengths by hydrogen bonding and Van der Waals forces (Figure 9) and provide a rigid structure.

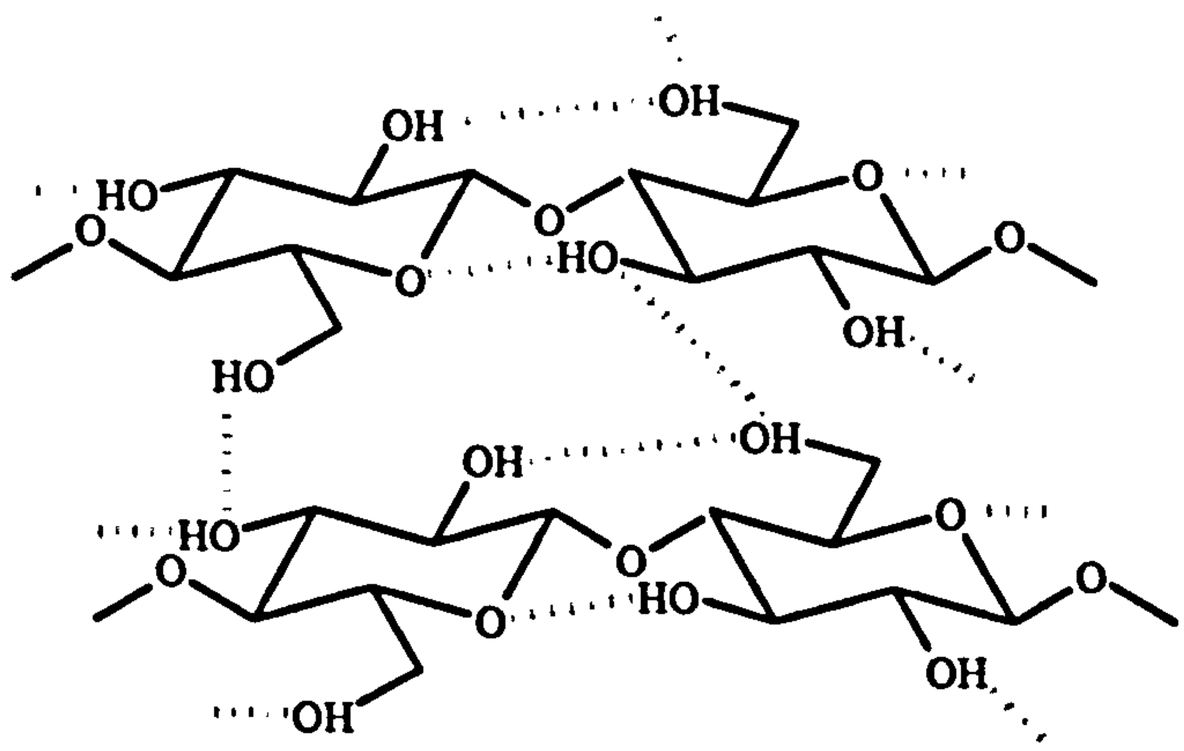


Figure 9 : Cellulose structure

Cellulose has commercial applications in a wide range of areas such as paper, insulating tiles and cardboard. In nature, cellulose can also be used as an energy source as part of the diet of ruminants.

Chitin, a derivatised cellulose, is another structural homopolysaccharide. It is composed of N-acetyl-D-glucosamine residues connected via β-(1-4) linkage and represents the principal component of the exoskeletons of arthropods[1].

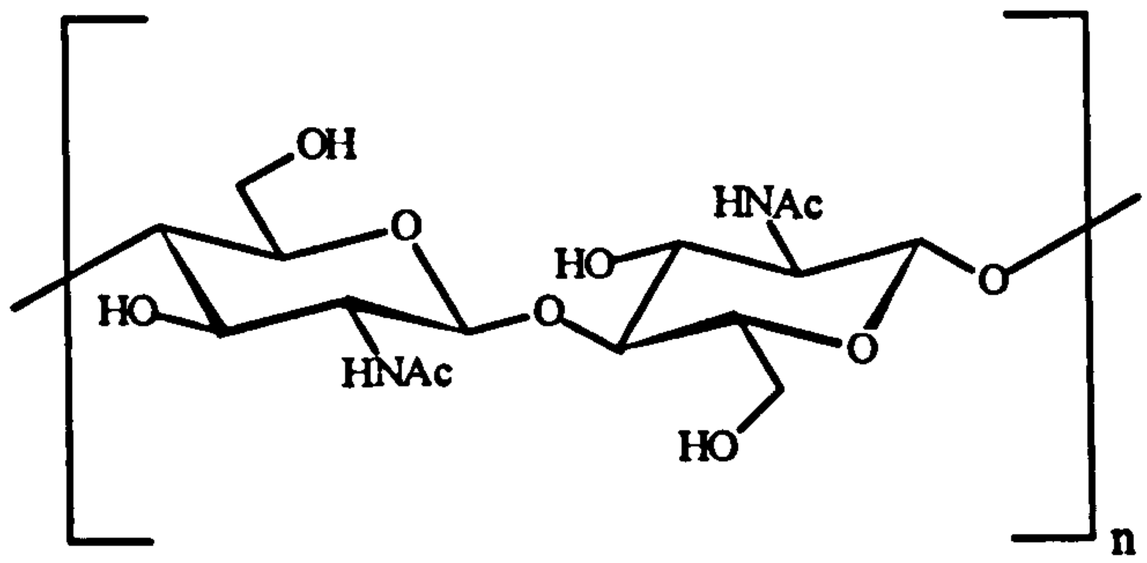
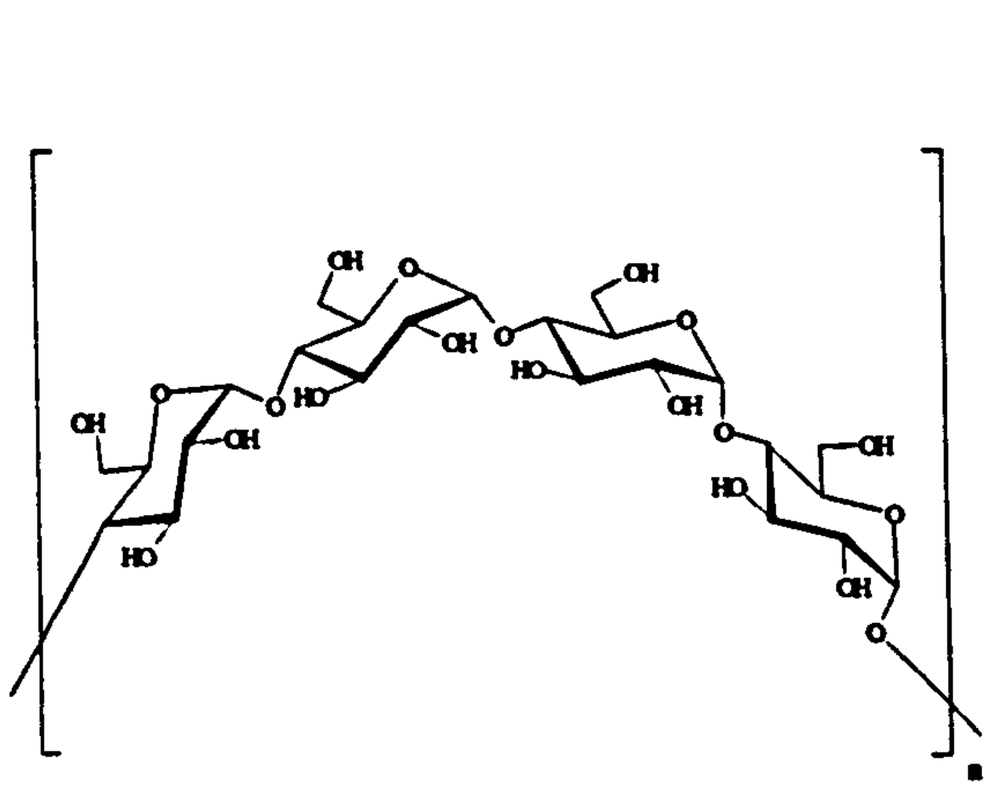
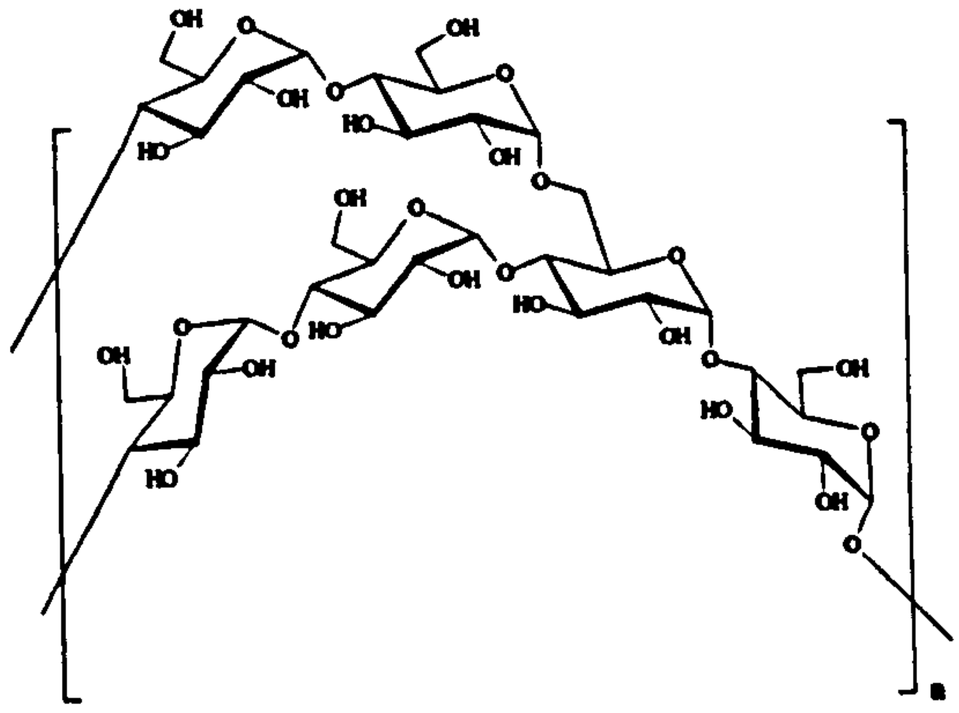


Figure 10 : The polysaccharide chitin

The food storage polysaccharides, starch and glycogen, are important energy sources. Whilst starch serves as an energy reserve in plants, glycogen fulfils that role in animal cells. Starch consists of two structurally different α-glucans, amylose (α-(1-4) glucan) and amylopectin (α-(1-4) and α-(1-6) glucan) (Figure 11). Glycogen is similar to amylopectin in that it is a polysaccharide composed of α-(1-4) and α-(1-6) glucan, the major difference being that glycogen has a higher proportion of branching linkages.



Amylose



Amylopectin

Figure 11 : Amylose and amylopectin structure
(α-(1-6) ramifications in amylopectin occur every 24 to 30 glucose residues)

2. Oligosaccharide Synthesis

The slow progress in the preparation of biologically active oligosaccharides arises from the difficulty of their chemical synthesis. Indeed, the synthesis of carbohydrates is often compared to the synthesis of proteins. The presence of multiple hydroxyl functions and the variation of configuration of the glycosidic linkage (α- or β-) allow a large number of possible products. In contrast to peptide synthesis, where only one type of linkage is formed, the synthesis of saccharides yields many isomers. This is highlighted by comparison of the number of possible isomers obtained by joining peptides and monosaccharides in Table 1.

Product	Composition	Number of peptide isomers	Number of saccharide isomers
monomer	Z	1	1
dimer	Z_2	1	11
trimer	Z_3	1	120
tetramer	Z_4	1	1424
pentamer	Z_5	1	17872

Table 1 : Isomeric possibilities for biopolymers
(saccharide referred to hexopyranoses)

Carbohydrate synthesis must therefore be performed with strict control of the stereochemistry and the regioselectivity of reactions. Their synthesis normally requires a minimum of four different steps involving protection, activation, coupling and deprotection.

2.1. Protecting Groups

The different reactivities of the different hydroxyl groups present in a molecule (primary or secondary alcohol) as well as that of substituents attached at the anomeric centre and around the pyranose ring can be exploited so that a particular glycoside may be formed using appropriate protecting groups. The following examples describe the most common protecting groups used in carbohydrate synthesis (Figure 12).

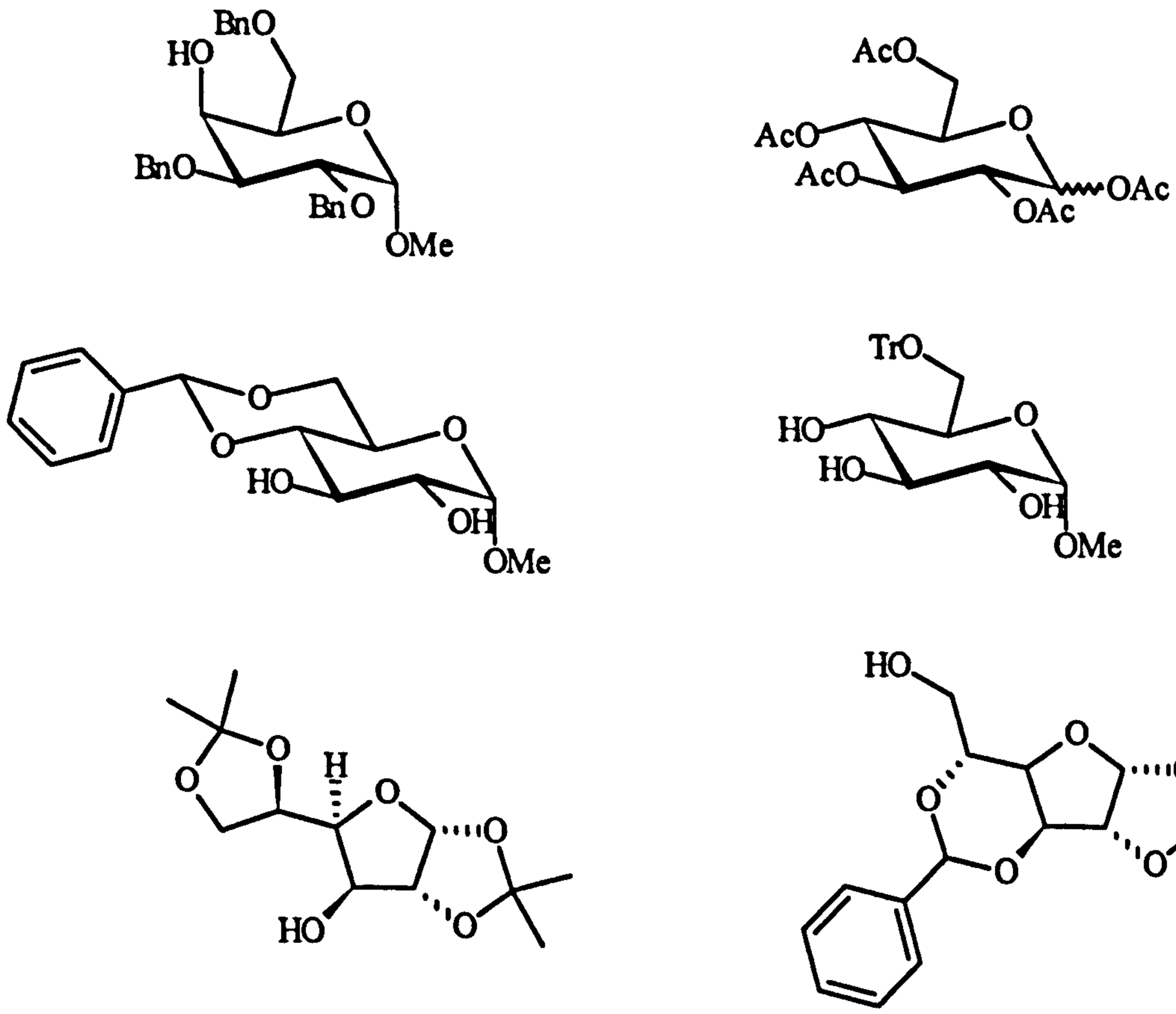
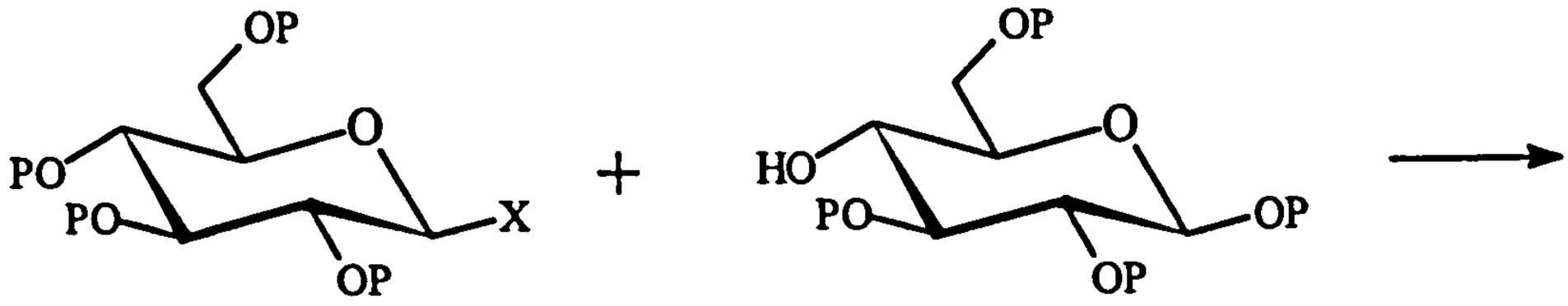


Figure 12 : Common protecting groups used in carbohydrate chemistry[15]

New protecting groups have recently been introduced as a strategy to limit the number of protecting group manipulation steps required, thereby reducing the total number of steps involved in a synthesis. Ley[16,17] and co-workers have recently introduced dispiroketals as protecting groups for trans-1,2-diols; existing acetal protecting groups were only applicable to cis-1,2-diols.

2.2. Coupling Methods

The coupling reaction between two sugars involves the production of a glycosylating agent, a glycosyl donor, activated at a single centre, e.g. at C-1, and protected at all other positions, and a glycosyl acceptor which is selectively protected at all but one hydroxyl group (Scheme 1). The coupling reaction must occur with chemoselectivity and with stereocontrol so as to provide the required linkage.



Activated Glycosyl Donor Glycosyl Acceptor

Scheme 1

Four classical methods are commonly used in the laboratory to synthesise new glycosides[18] :

- The Koenigs-Knorr method which uses an activated glycosyl halide as a glycosyl donor and requires the use of heavy metal salts (usually silver salts) to catalyse the reaction
- The Fischer-Helferich method where an acid is added to the reaction mixture and the reaction proceeds via acetal formation
- The direct 1-O-Alkylation (base activation) which consists of the formation of the alkoxide and coupling with the activated donor
- The trichloroacetimidate method (base-acid activation) involves the formation of a glycosyl trichloroacetimidate donor, using basic conditions, followed by a nucleophilic displacement under mild acid conditions.

All these methods have advantages and disadvantages, and the stability of the protecting and activating groups under the reaction conditions must be taken into consideration when deciding which approach to use. New methods have recently

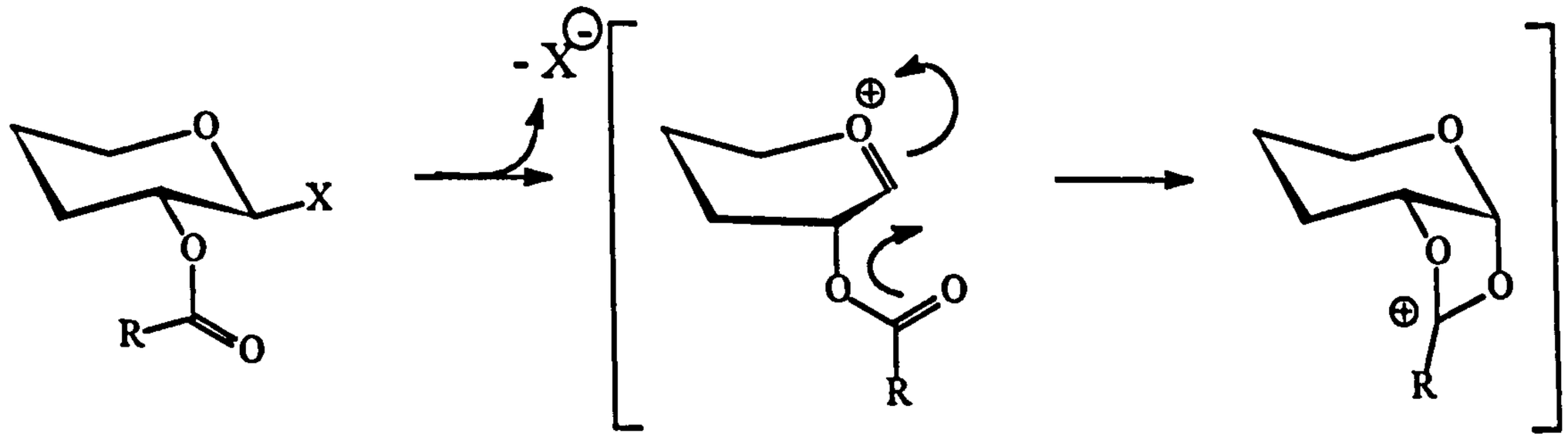
been developed using glycals[19], thioglycosides[20] and pentenyl glycosides[21] which again reduce the necessity to protect as many hydroxyl groups.

2.3. Stereoselective Glycosylation

The stereoselectivity of glycosylation reaction is often dependent on the nature of the group located next to the anomeric centre. Indeed, the presence of a participating group at C-2 favours production of a 1,2-trans glycosidic linkage whereas the type of linkage obtained with a non-participating group at C-2 is dependent on the reaction conditions (e.g. solvent, temperature, promoter). The structure of the glycosides (donor and acceptor) also influences the final ratio of anomers, α- and β-, produced.

2.3.1. Neighbouring group participation

The neighbouring group participation (NGP) of the substituent attached at C-2 (typically a 2-O-acyl function) results from a possible stabilisation of the oxenium ion intermediate. Intramolecular nucleophilic attack of the NGP on the anomeric centre gives a more stable acetoxonium ion intermediate (Scheme 2).



Scheme 2

The anomeric centre can then be attacked by a nucleophile and displacement of the acetate yields a 1,2-trans glycoside. A glucosyl donor will lead to a β-link whereas a mannosyl donor will result in an α-mannoside. The possibility of nucleophilic addition at the dioxolane ring reduces the reaction yield as an orthoester is formed.

2.3.2. Reaction conditions

The stereocontrol of a glycosylation reaction which involves a glycoside donor having a non-participating protecting group at C-2 depends on the reaction conditions. Temperature, promoters and solvents have been shown to have an important role on the outcome of the stereochemistry of the glycosylation reaction. As an example, *in situ* anomerisation provides a recently developed method for the formation of α-glycosidic linkages. Promoters such as tetrabutylammonium bromide has been shown to catalyse the fast equilibration between α- and β-glycosyl halide in apolar solvents. The latter isomer being the most kinetically favourable to nucleophilic attack of the glycosyl acceptor, a resulting α-glycosyl is formed in more or less good yield[22] (Figure 13).

Figure 13 : *In situ* anomerisation

Glycosylation with inversion of configuration provides another method for controlling the stereoselectivity of the reaction. In this strategy, axial glycosyl halides are used in the presence of insoluble silver salts and form an equatorial glycosidic linkage (Figure 14).

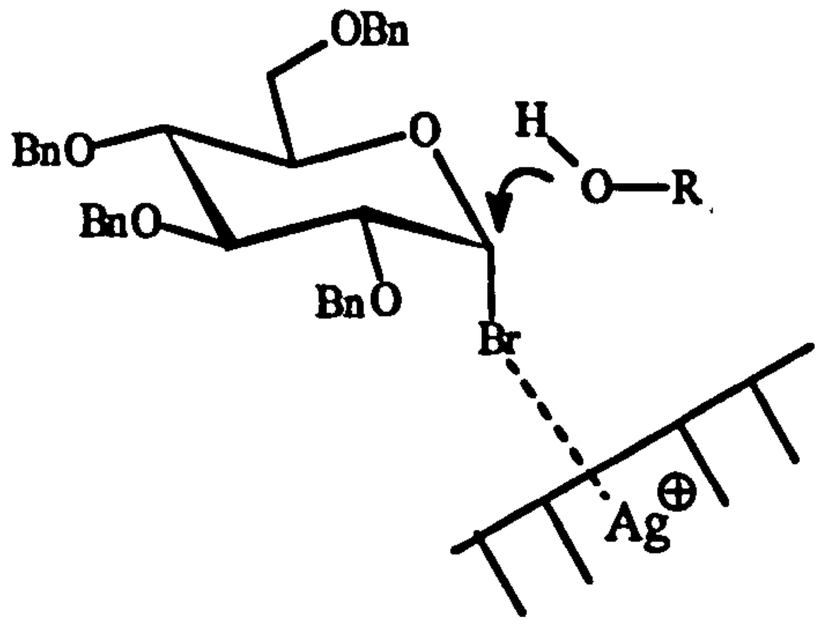


Figure 14 : S_N2 displacement

2.4. Regioselective Glycosylation

Regioselective glycosylation results from the preference of one substituent to react under a set of conditions over another similar one present in the same molecule. The difference of reactivity between the hydroxyl groups present in glycosides is frequently the result of their environmental positions (next to electron withdrawing or electron donating groups), their spatial arrangements (axial being less reactive than equatorial) or the reaction conditions (e.g. promoter). An example of regioselective glycosylation[23] is illustrated on Figure 15:

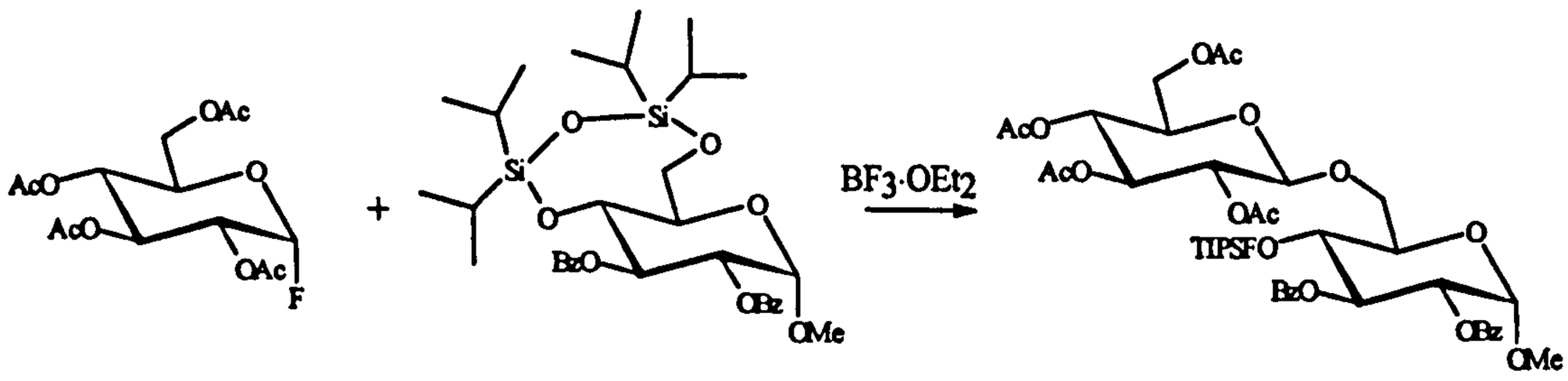


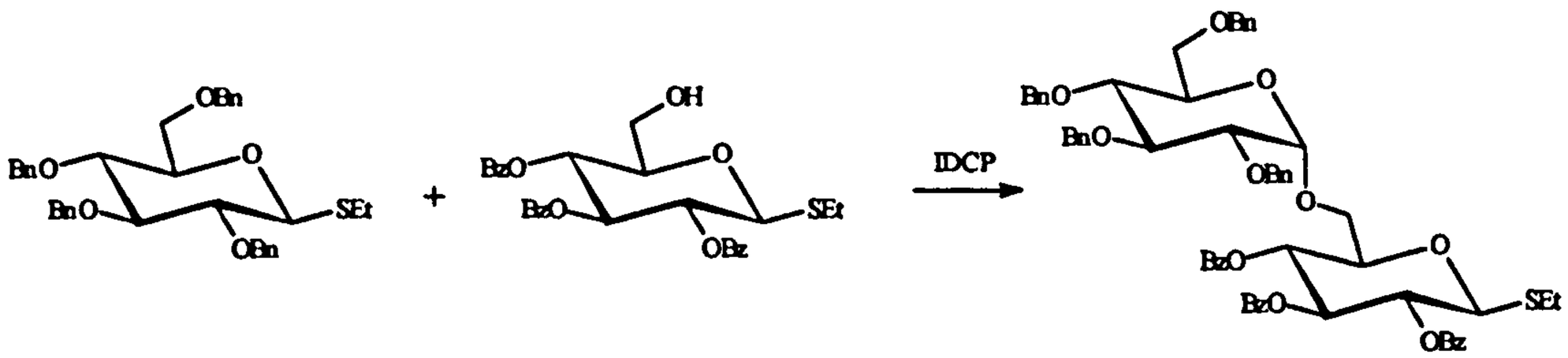
Figure 15 : Regioselective glycosylation

2.5. Chemoselectivity

Chemoselective glycosylation has been proven to be an effective method which can be used to reduce the number of steps required in protecting group manipulations. The presence of an electron donating or electron withdrawing group at C-2 has been exploited to achieve selective coupling. Commonly, esters are used as electron withdrawing groups which deactivate the anomeric centre (disarmed glycoside), whereas ethers are used as electron donating groups and activate the anomeric centre (armed glycoside)[24].

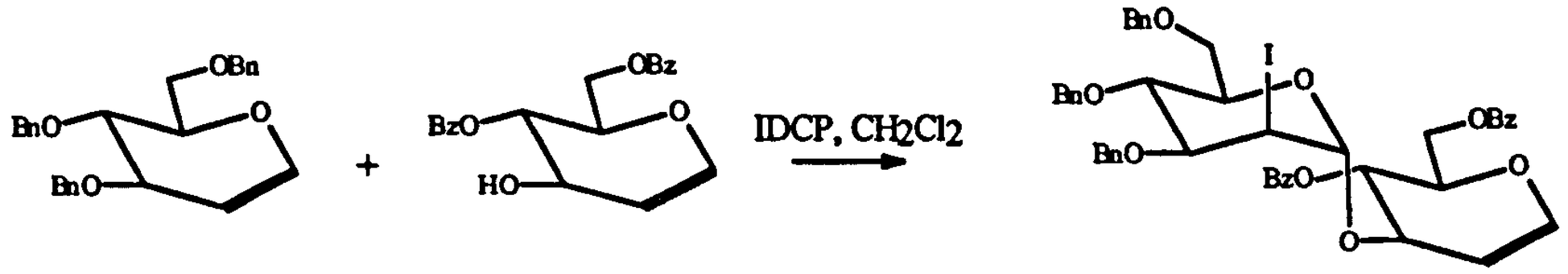
Chemoselective glycosylation can thus be achieved using a glycoside donor and a glycoside acceptor which both have a similar anomeric group but different C-2 protecting group. Examples of chemoselective glycosylations using thioglycosides (a) and glycals[19] (b) are illustrated below, Figure 16:

(a)



Armed thioglucosyl donor Disarmed thioglucosyl donor 84 %

(b)



Donor glycal Acceptor glycal 76 %

Figure 16 : Chemoselective glycosylations

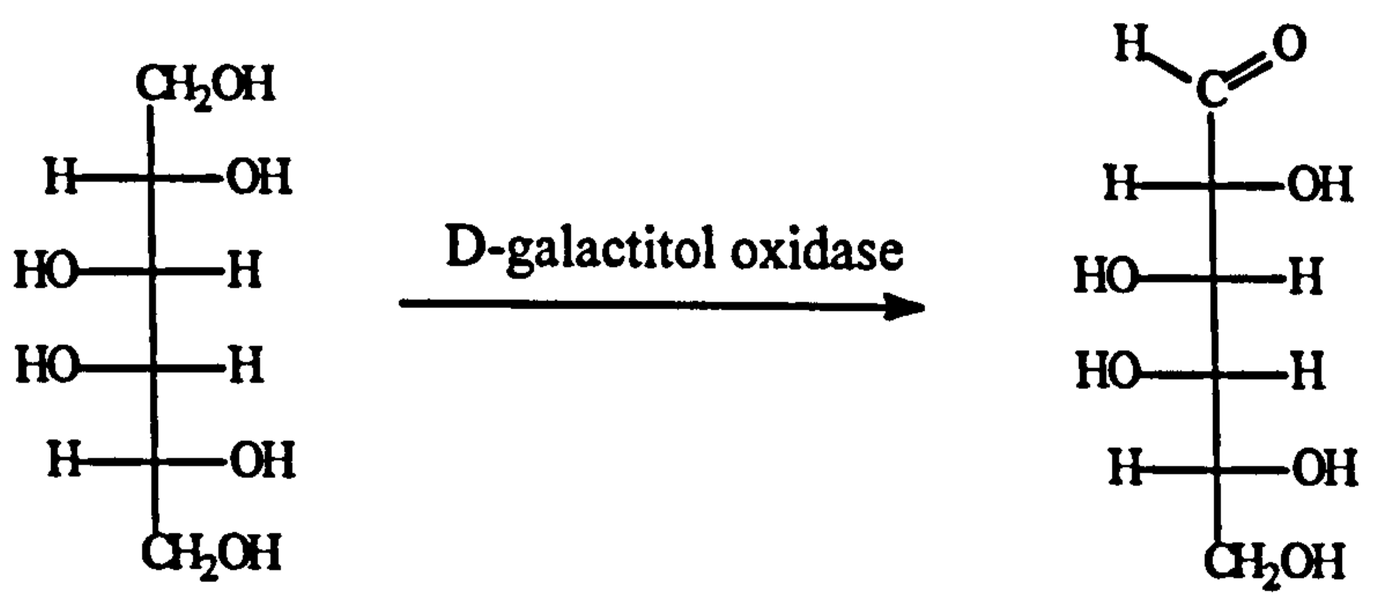
3. Enzymes in Carbohydrate Synthesis

An alternative strategy, one which simplifies oligosaccharide synthesis would be to employ enzymes. Indeed, the use of enzymes in organic synthesis is becoming more widespread and it is frequently possible to find an enzyme capable of realising a similar reaction to the chemical synthesis. Enzymes have the advantages of being stereoselective and regiospecific when compared to their chemical reaction analogues. Enzymatic reactions are usually performed in mild conditions and require a minimum number of steps. The requirement for selective activation, protection and deprotection steps are thus eliminated. Due to the complexity of carbohydrate synthesis, the role of enzymes in the synthesis of carbohydrates has been explored[25]. Six main groups of enzymes can be utilised covering most types of organic reactions.

3.1. Enzymes Used in Carbohydrate Synthesis

3.1.1. Oxidoreductases

Oxidoreductases are enzymes catalysing oxidation-reduction reactions, e.g. oxidation of a hydroxyl methylene unit to a carbonyl or the reduction of ethylene to ethane. A good example of the use of an oxidation reaction is the enzyme-catalysed oxidation of galactitol by D-galactose oxidase (Figure 17).



Galactitol D-galactose

Figure 17 : Oxidation with D-galactitol oxidase

Another important example is that involved in the first step of the Payoff phase of glycolysis[1]. This involves the oxidation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate and is described on Figure 18.

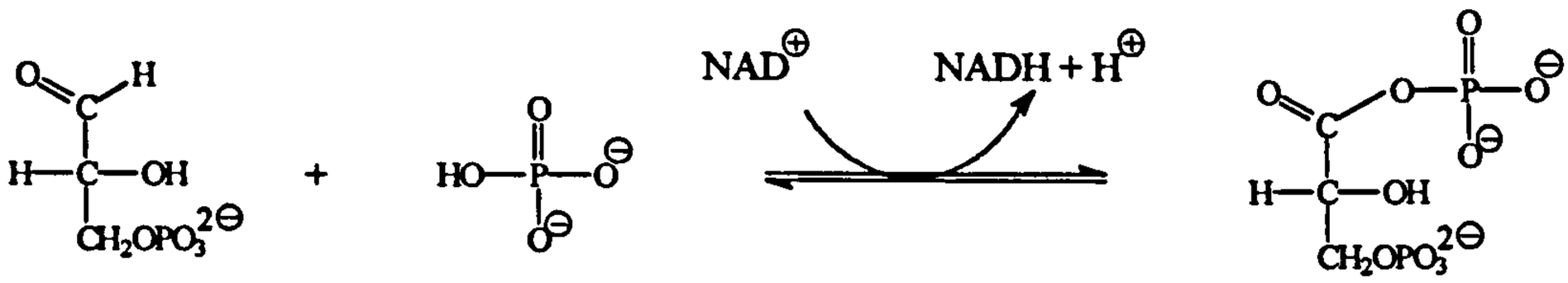
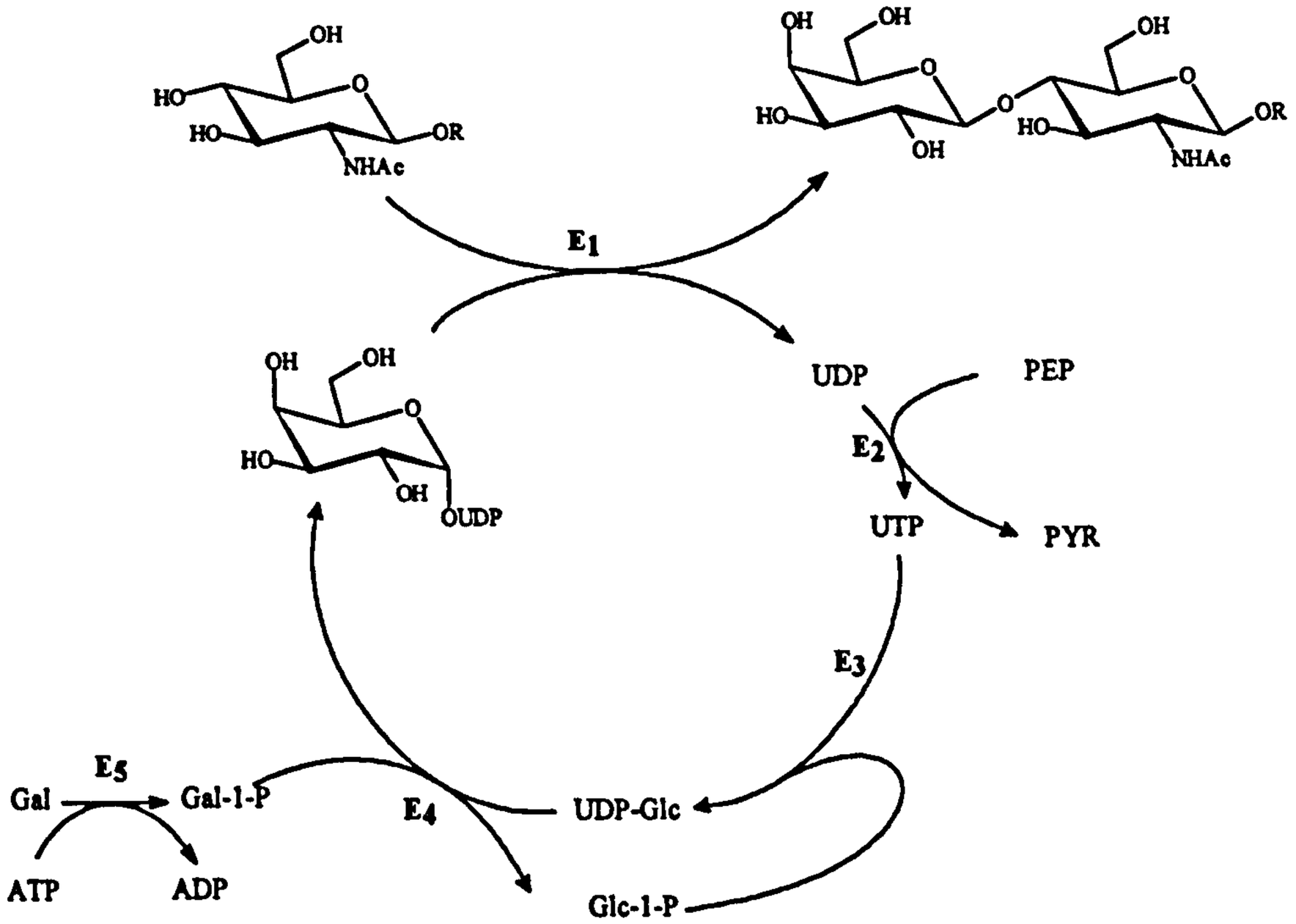


Figure 18 : Oxidation of glyceraldehyde-3-phosphate

3.1.2. Transferases

Transferases are enzymes catalysing the transfer of functionality from one group to another, e.g. acyl, glycoside, and phosphoryl groups amongst others. A good example of the use of a transferase is in the synthesis of disaccharides from monosaccharides using galactosyltransferase[26] (Figure 19).



E_1 : β-(1-4)-galactosyltransferase,
E_2 : pyruvate kinase,
E_3 : UDP-Glc pyrophosphorylase,
E_4 : Galactose-1-phosphate uridyltransferase,
E_5 : galactokinase.

UDP : uridine diphosphate
UTP : uridine triphosphate
PEP : phosphoenolpyruvate
PYR : pyruvate

Figure 19 : Galactosyltransferase-catalysing glycosylation with *in situ* regeneration of the co-factor

Although the reaction does not require protection steps, the main disadvantage of glycosyl transferases is the requirement of a co-factor (either in a 1:1 ratio or as part of a regenerating process). The use of co-factors represents an increase in the number of steps involved in the biosynthesis.

The regioselective transfer of an acyl group using enzymes is an important reaction used in chemical synthesis which provides the product without the use of multiple protection and deprotection steps. Examples of regioselective acylation[27] are shown in Figure 20.

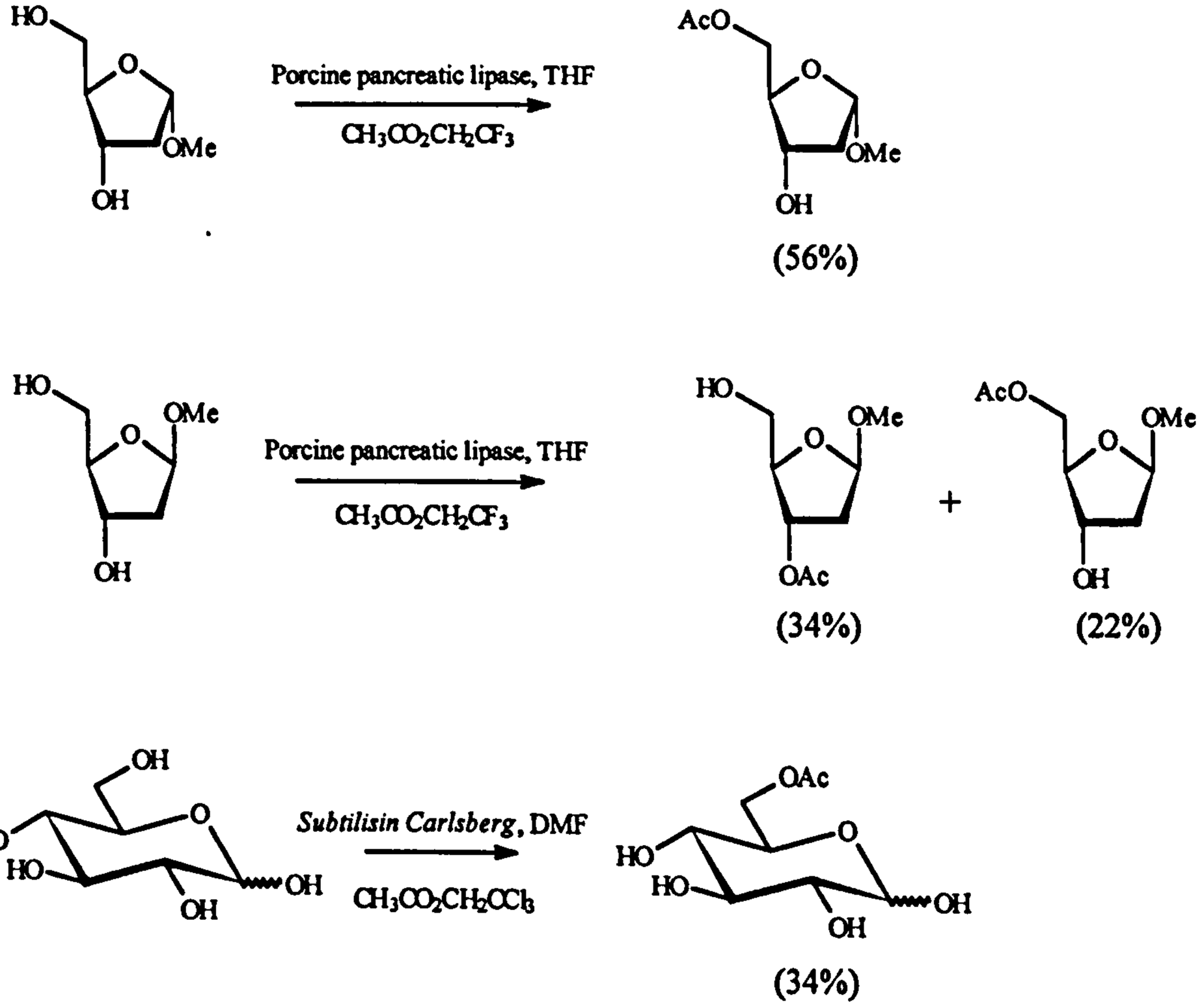


Figure 20 : Examples of enzymatic acylation reaction

3.1.3. Hydrolases

Hydrolases are enzymes that catalyse the specific hydrolysis of a functional group. A wide variety of hydrolases are found *in vivo*: glycosidases, esterases, peptidases, etc. Esterases are often used in chemical synthesis for the selective deprotection of multi-ester compounds. Their selective action is often dependent on the source of enzyme[28] and is illustrated in Figure 21.

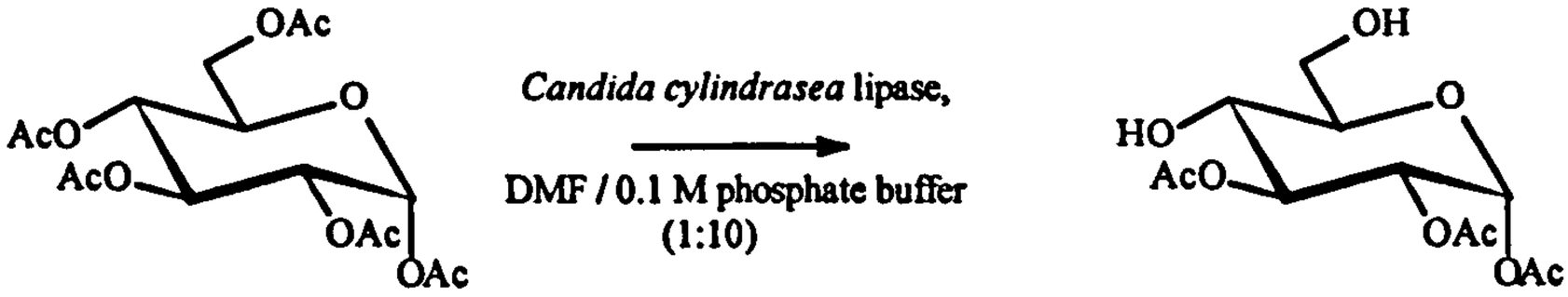


Figure 21 : Selective ester protecting group removal

3.1.4. Isomerases

Isomerases are enzymes which catalyse the isomerisation of their substrates. This may involve cis-trans isomerisation, racemisation or double bond migration. An example of the use of an isomerase is found in the glycolysis pathway in the human body and includes the interconversion of 3-phosphoglycerate to 2-phosphoglycerate (Figure 22).

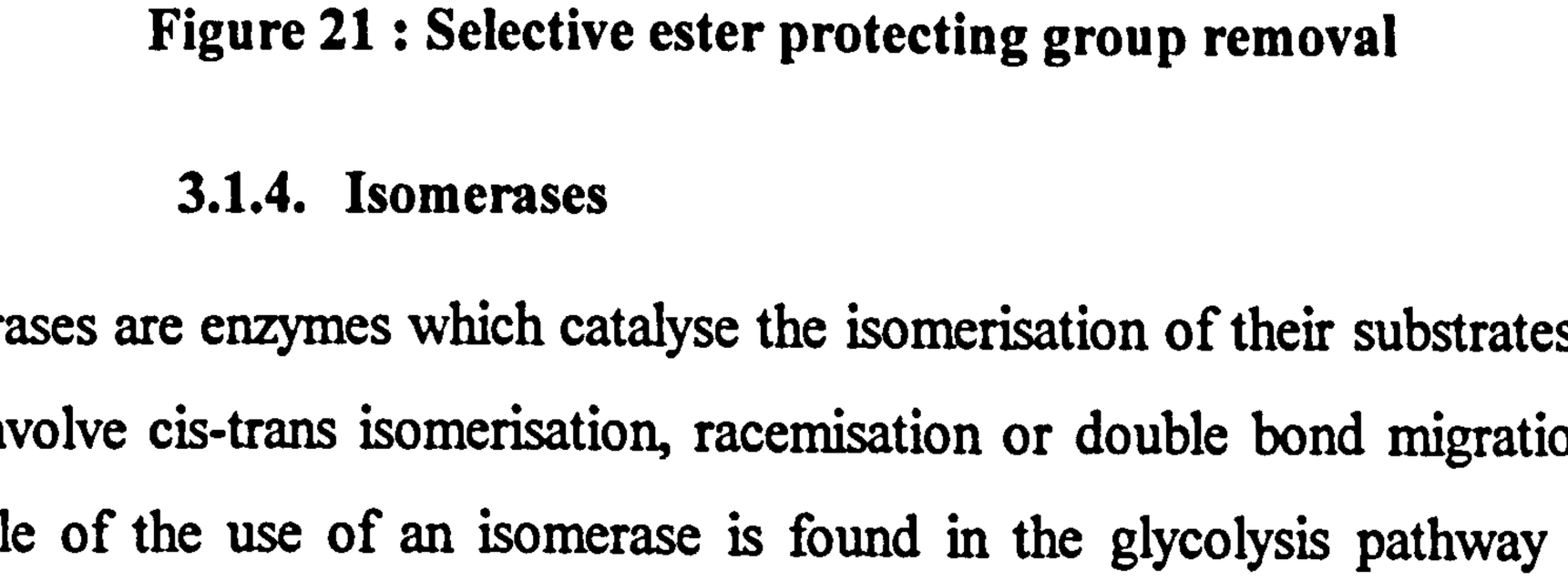


Figure 22 : Isomerase-catalysed reaction

Ligases and lyases are two other groups of enzyme participating in the formation of new oligosaccharide bonds. Ligases are enzymes which catalyse the formation of C-O, C-S, C-N, C-C and phosphate ester bonds, whereas lyases are enzymes which catalyse the additions, usually of HX to double bonds such as C=C, C=N, and C=O, and the reverse processes.

Although, enzymes are generally regarded as substrate specific, some of them have been shown to accept a variety of substrates, catalysing the reaction in the same stereospecific manner.

3.2. Enzyme-catalysed Oligosaccharide Synthesis

3.2.1. Glycosyl transferases

3.2.1.1. The Leloir Pathway enzymes

The Leloir Pathway enzymes catalyse the synthesis of oligosaccharides via a three-step mechanism: activation, transfer, and modification (Scheme 3).

The initial activation step involves the production of an 'energy-containing' sugar by formation of a phosphate ester linkage (sugar-1-P). The sugar phosphate then reacts with a nucleoside triphosphate to form an activated nucleoside diphosphate sugar (NDP-sugar). Subsequent transfer and modifications give rise to the desired compound.

$$\text{sugar} + P_i \longrightarrow\longrightarrow \text{sugar-1-P} \longrightarrow\longrightarrow \text{NDP-sugar} + PP_i$$

Scheme 3

The activated sugar nucleotide is then transferred to a hydroxyl of an acceptor monosaccharide. The transfer reaction being catalysed by a specific transferase enzyme e.g. in the synthesis of sialyl Lewis^x, UDP-galactose is transferred to the 4-hydroxyl of N-acetylglucosamine, the reaction being catalysed by β-(1-4)-galactosyltransferase (See Figure 19). The total enzymatic synthesis of sialyl Lewis^x using glycosyl transferases was recently published by C. Wong *et al*[29]. It involves three main transfer reactions such as those described above with additional co-factor regeneration steps. Sialyl Lewis^x (Figure 23) is an important tetrasaccharide involved in cell adhesion processes.

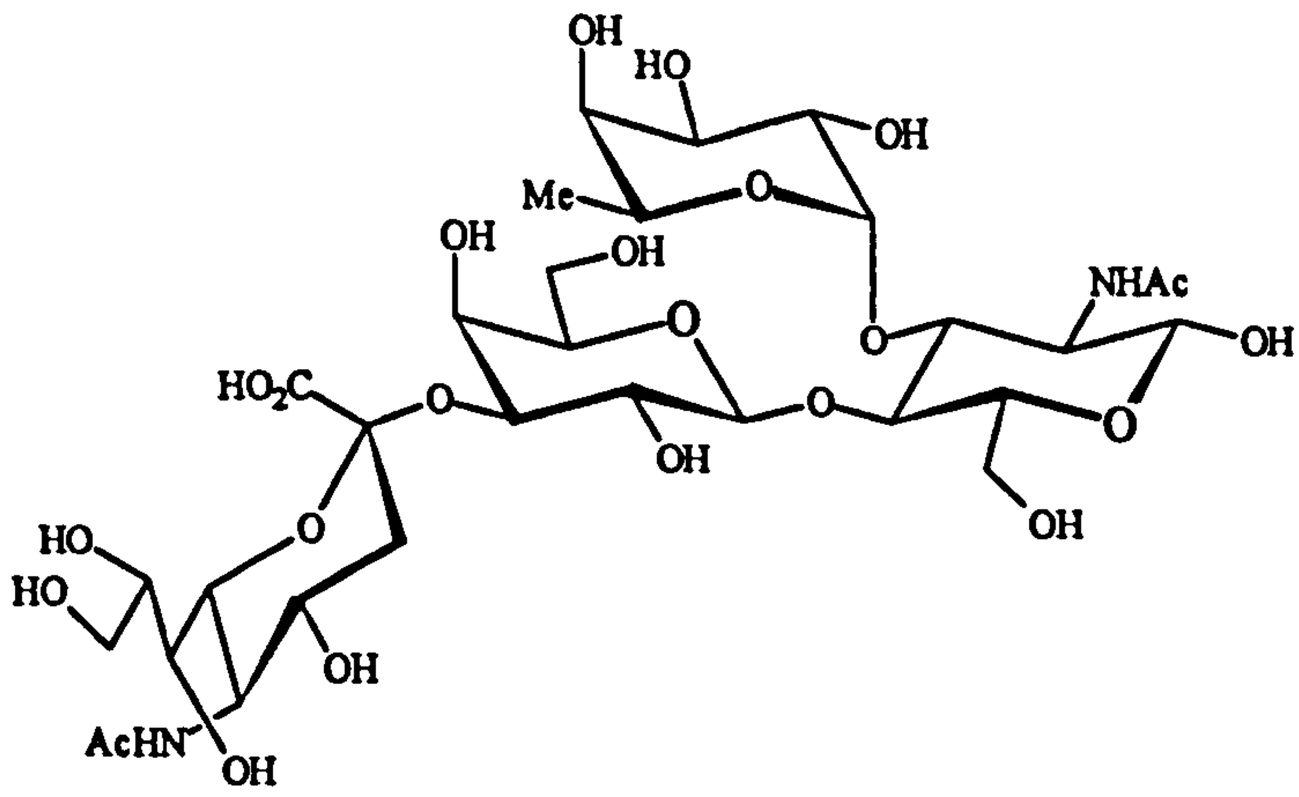


Figure 23 : Sialyl Lewis^x

3.2.1.2. The non-Leloir pathway enzymes

The non-Leloir pathway enzymes utilise glycosyl phosphates as activated substrate donors rather than sugar nucleotides, in the synthesis of oligosaccharides. A typical example is the biosynthesis of sucrose[30] (Figure 24).

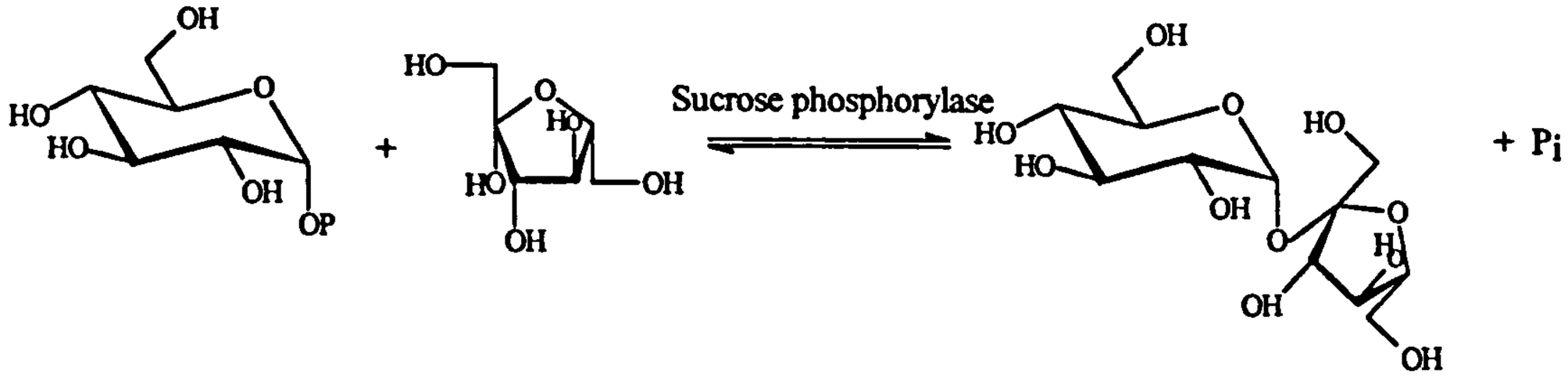


Figure 24 : Biosynthesis of sucrose

The non-Leloir pathway enzymes still require co-factor during the reaction.

3.2.1.3. Characteristics of glycosyl transferases

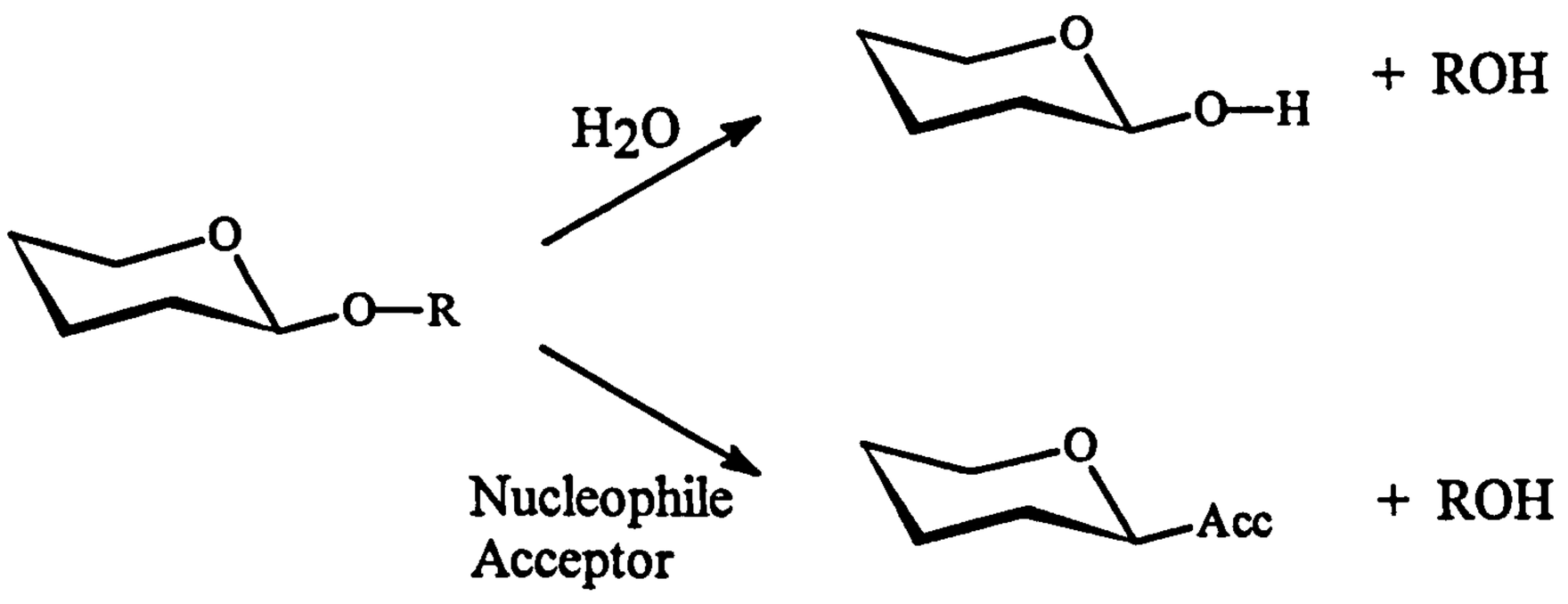
Glycosyl transferases have often been considered to be very specific towards the substrate, the acceptor and the type of linkage produced and have led to the widely used 'one enzyme one bond' concept.

The use of glycosyl transferases for the routine synthesis of oligosaccharides is hampered by the poor reaction yields and the necessity to include co-factor regeneration steps, each of which requires the use of additional enzymes. As glycosyl transferases are difficult to isolate and are generally present in low concentrations and frequently membrane-bound, their use has been restricted.

An alternative source of enzymes are the hydrolytic glycosidases.

3.2.2. Glycosidases

Glycosidases or glycosyl hydrolases are enzymes that normally catalyse the hydrolysis of glycosidic linkages *in vivo*, but have been used *in vitro* to synthesise oligosaccharides. Glycosidases can be considered to catalyse the **transfer** of a glycosyl unit to water and in the presence of other nucleophiles, new glycosidic linkages can be formed. This ability to form new glycosidic linkages is often referred to as the transferase activity of the enzyme (Scheme 4).



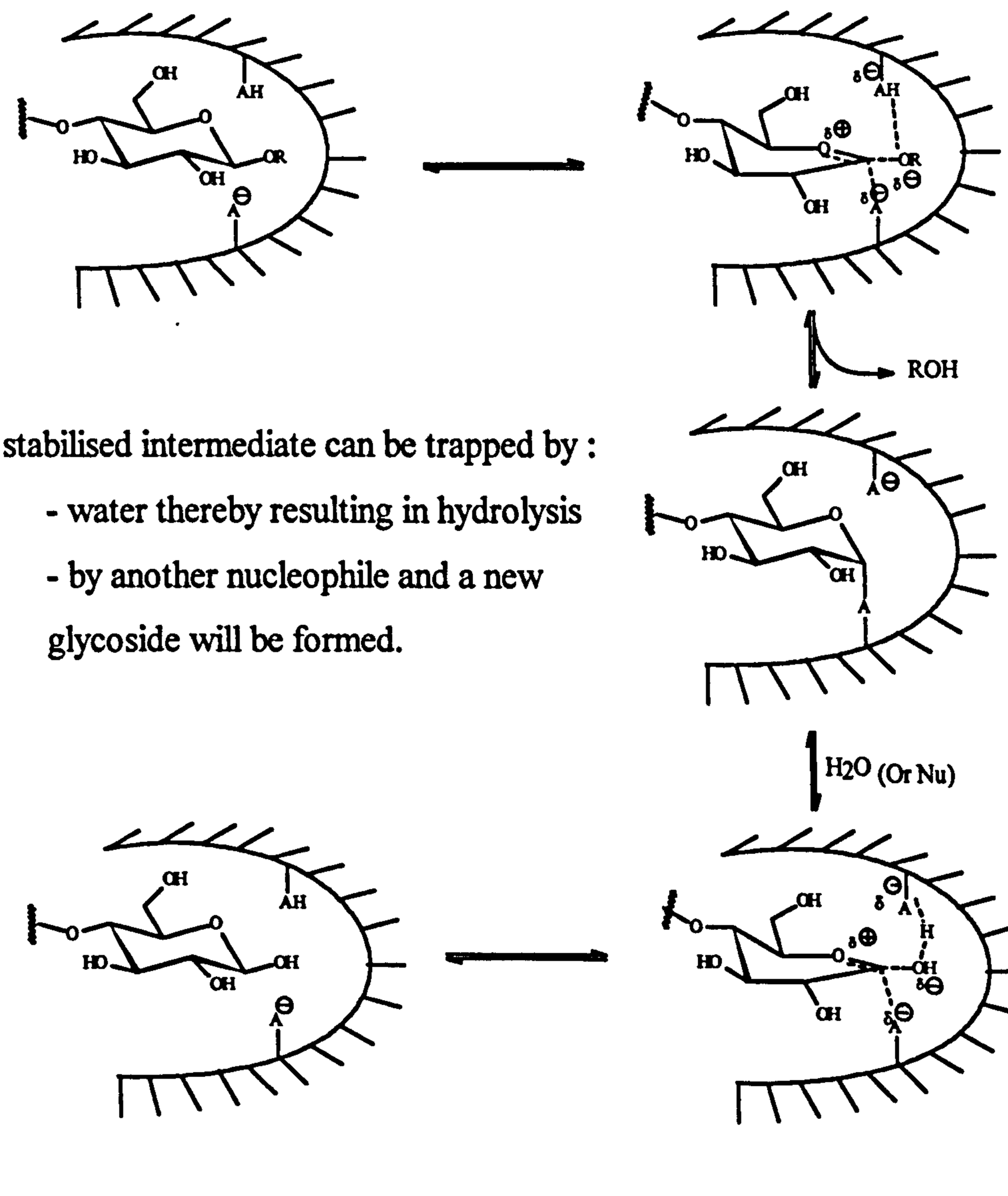
Scheme 4 : Enzymatic synthesis of oligosaccharides

In general, glycosidases demonstrate high specificity for the glycosyl moiety and the orientation of the glycosidic linkage (equatorial or axial) but little if any specificity for the aglycon[31]. Two different synthetic strategies have been adopted for oligosaccharide synthesis using glycosidases:

- the direct glycosylation or reverse hydrolysis method
- the transglycosylation method or kinetically-controlled synthesis.

3.2.2.1. Glycosyl hydrolase mechanisms

To understand why glycosidases are able to catalyse transfer it is important to understand the mechanism by which they catalyse hydrolysis/transfer. Glycosidases hydrolyse their substrates with either retention or inversion of configuration at the anomeric centre. Their mechanism of hydrolysis is often compared to the mode of action of lysozyme which involves a general acid/base catalysis and proceeds via an oxenium intermediate which is stabilised by a carboxylate. Recent studies[32,33,34] have shown that the formation of an enzyme-bound intermediate is preferred rather than the formation of an oxenium ion which is stabilised by the enzyme. Koshland[35] proposed a double displacement mechanism for retaining enzymes (Figure 25) whereas a single displacement mechanism was suggested for the inverting enzymes (Figure 26).



The stabilised intermediate can be trapped by :

- water thereby resulting in hydrolysis
- by another nucleophile and a new glycoside will be formed.

Figure 25 : Double displacement mechanism

Typically, the double displacement mechanism involves initial proton donation from a carboxylic acid to the glycosidic oxygen of the substrate which facilitates the departure of the aglycon. Attack of a basic residue located on the opposite side of the acid residue produces an enzyme-bound intermediate. The incoming nucleophilic molecule of water can then react with the glycosyl-enzyme intermediate. A proton is abstracted from the water molecule to regenerate the acid catalyst. If a nucleophile other than water is used, the transfer of this nucleophile produces a new glycoside, i.e. a transglycosylation product.

Inverting enzymes hydrolyse glycosides via a concerted single displacement mechanism where the glycoside is hydrolysed by the intervention of a water molecule[35] located between the base residue and the substrate (Figure 26).

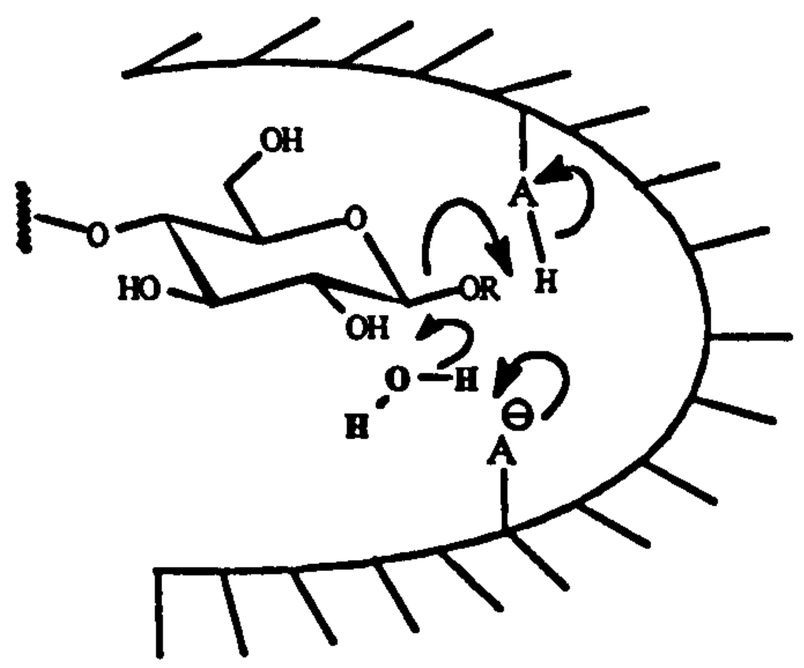
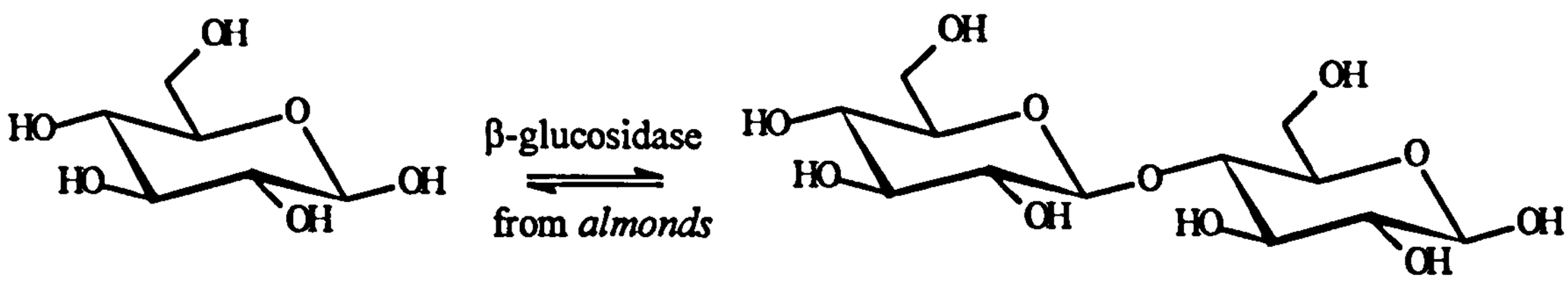


Figure 26 : Single displacement mechanism

The main differences observed between the retaining and inverting enzymes are the relative distance between the two catalytic species. In retaining β-glycosidases, the distance between the catalytic species is about 5.3 Å, shielding of one face from the nucleophilic attack, whereas in inverting enzymes the distance reaches 9.5 Å[36]. Transglycosylation reactions can thus only be performed with retaining enzymes as the limited space found between the two catalytic species in inverting enzymes does not allow the presence of a molecule other than the substrate and a molecule of water.

3.2.2.2. Reverse hydrolysis or equilibrium controlled synthesis

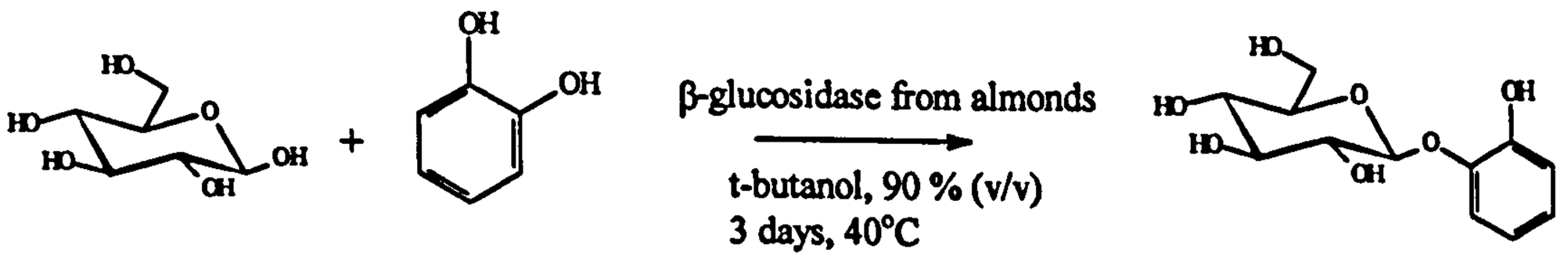
This method uses high concentrations of monosaccharide reactants. Although the equilibrium of the reaction favours the hydrolysis products, the high concentrations of the monosaccharides allow the formation of some oligosaccharides. As the process is controlled by the reaction equilibrium, the yields obtained during reverse hydrolysis are often very low. Different types of acceptor have been used with some success such as alcohols[37], glycosides, etc.
The synthesis of disaccharides derived from glucose was achieved using 90% w/v of glucose as the glucoside donor (Figure 27). The equilibrium controlled synthesis gave rise to a mixture of disaccharides containing different glycosidic linkages (such as β-(1-2), β-(1-3), β-(1-4) and β-(1-6) linkages)[38].



Glucose, 90% w/v

Figure 27 : Synthesis of glucose disaccharides by reverse hydrolysis

Reaction conditions can be altered to favour the formation of the transglycosylation product, by adding a co-solvent to the reaction mixture and thus disturbing the reaction equilibrium[39]. Yields have been increased from 3.5 % to 18 % using 90 % t-butanol as a co-solvent[40] in the following reaction:

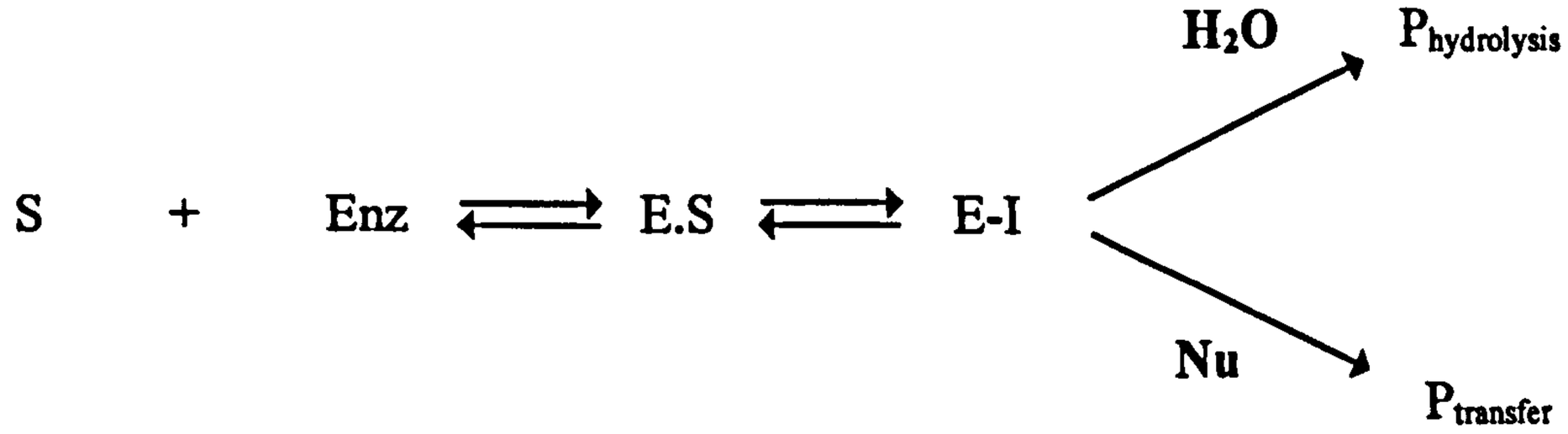


$+ H_2O$

Figure 28 : Transglycosylation in the presence of a co-solvent

3.2.2.3. Transglycosylation or kinetically-controlled synthesis

A kinetically-controlled synthesis involves an activated substrate donor which reacts to form a stabilised enzyme-bound intermediate; subsequent attack on this intermediate, by a nucleophile other than water, produces a new glycoside. Kinetically-controlled transglycosylation relies on the rate of formation of the product being greater than its rate of subsequent hydrolysis and on the more rapid trapping of the intermediate by the glycosyl acceptor than by water (Scheme 5).



Scheme 5 : Kinetically-controlled synthesis

As the thermodynamically-controlled hydrolysis process remains favoured the reaction must be monitored so that it can be stopped as the maximum yield of product is reached, thereby avoiding its subsequent hydrolysis. A recent example of a kinetically-controlled reaction developed by Crout[41] and co-workers is the synthesis of the linear B type 2 trisaccharide α-D-Gal-(1-3)-β-D-Gal-(1-4)-β-D-GlcNAcSEt which plays an important role in xenograft rejection in xenotransplantation (Figure 29). The transfer of a p-nitrophenyl activated β-D-galactoside to the 4-hydroxyl of ethyl 2-N-acetyl-2-deoxy-1-thio-β-D-glucosamine was selectively catalysed by β-galactosidase from *Bacillus circulans*. In a reaction catalysed by α-galactoside from *Aspergillus oryzae*, the resulting lactoside is then transferred to p-nitrophenyl α-D-galactoside with formation of the α-(1-3) glycosidic linkage (ratio 1 : 0.9 with α-(1-6) link) thereby forming the required trisaccharide structure.

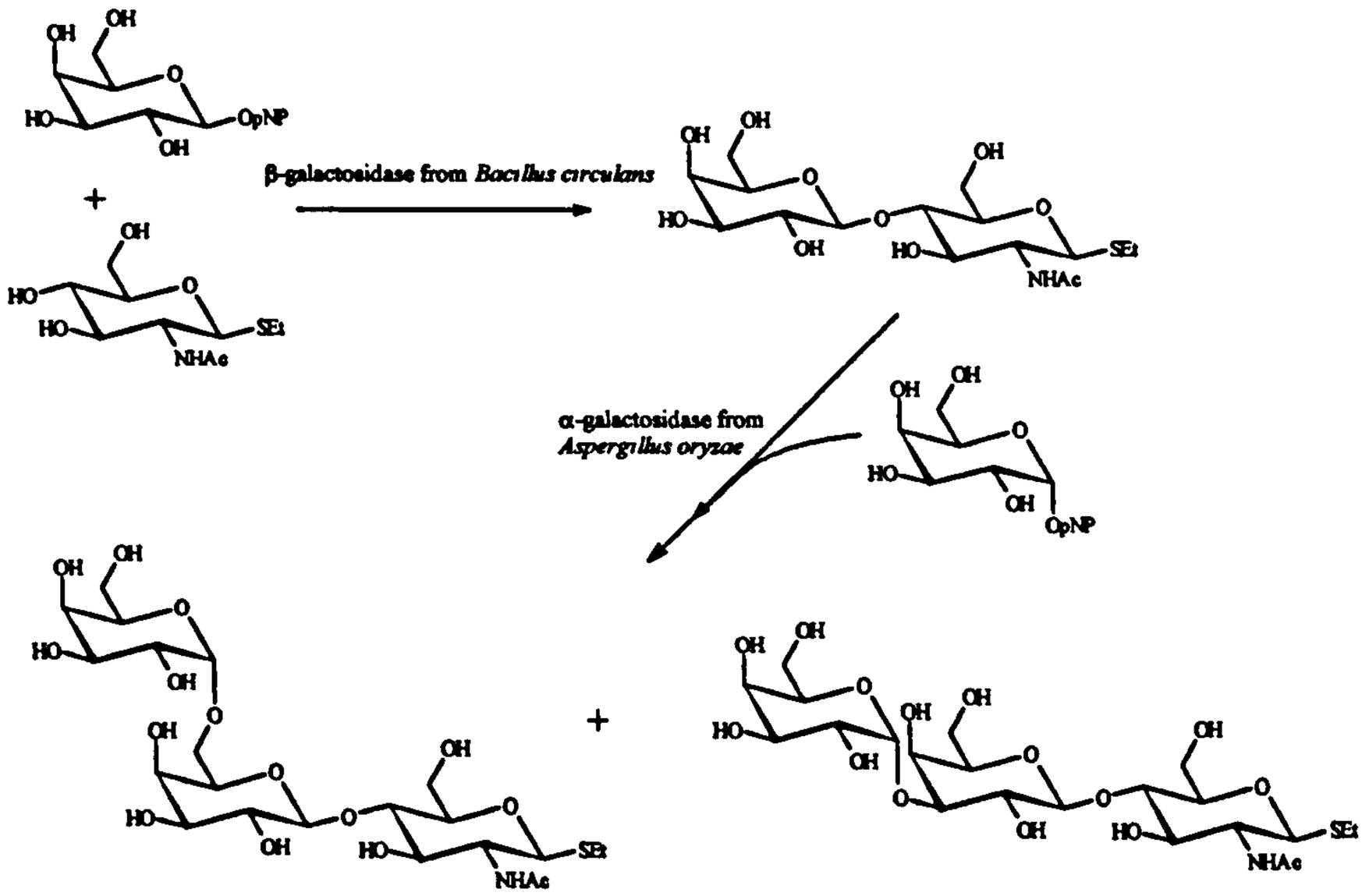


Figure 29 : Biosynthesis of two trisaccharides in two enzymatic steps

The diverse properties displayed by various enzymes can thus be studied to achieve regio- and stereo- selective glycosylation and thus provide an important role in chemical synthesis allowing the elimination of multiple protection and deprotection steps.

Many attempts have been made to increase the yield of transglycosylation reactions. Methods developed have included the addition of a co-solvent, the continuous slow addition of substrate[42] and use of different types of acceptor such as alcohols[43], glycopeptides[44] and glycosides[45].

3.2.2.4. Characteristics of transfer reactions catalysed by glycosidases

Many glycosidases are commercially available as are their natural and activated substrates. Glycosidases are usually highly selective towards the formation of one configuration at the anomeric centre (α- or β-), but show low regioselectivity in the type of glycosidic linkages formed (1-4, 1-6, etc.).

The yield of the reactions depends on various parameters such as:

- the source of enzymes
- the acceptor configuration and the structure of the aglycon moiety
- the reaction time (due to subsequent product hydrolysis in kinetically-controlled reactions)
- the reaction conditions, e.g. temperature and the presence of a co-solvent
- the substrate and acceptor concentrations.

The regioselectivity obtained often depends on the enzyme source[46]. The different regioselectivity of α-galactosidase[47] from different sources is illustrated in Figure 30.

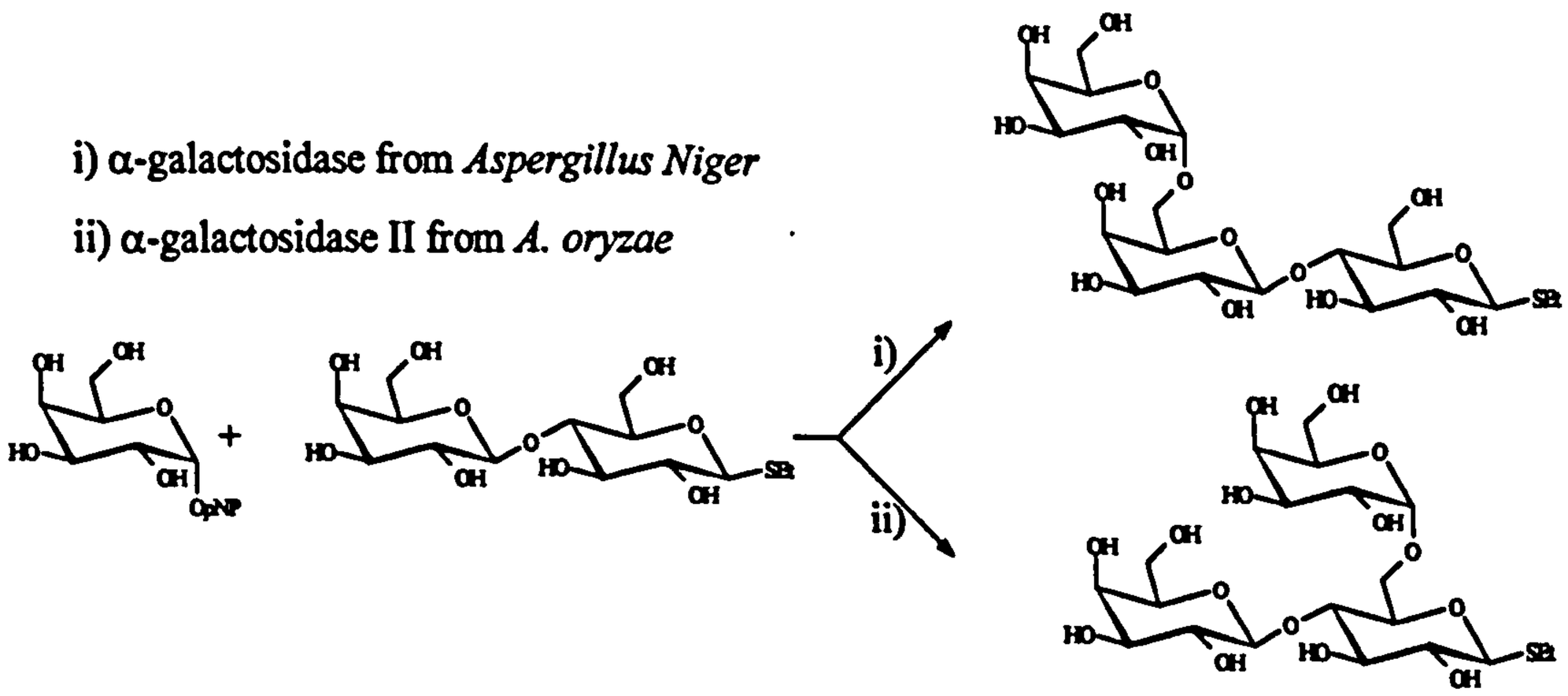


Figure 30 : Regioselectivity obtained with different α-galactosidase

It has also been reported that the structure and the anomeric configuration of the acceptor aglycon greatly influence the regioselectivity of the reaction[48]. The synthesis of disaccharide derivatives with α-galactosidase predominantly results in the formation of an α-(1-3) disaccharide when the α-anomer of methyl α-D-galactose is used as an acceptor whereas the α-(1-6) compound is the major product when the β-anomer is used (Figure 31).

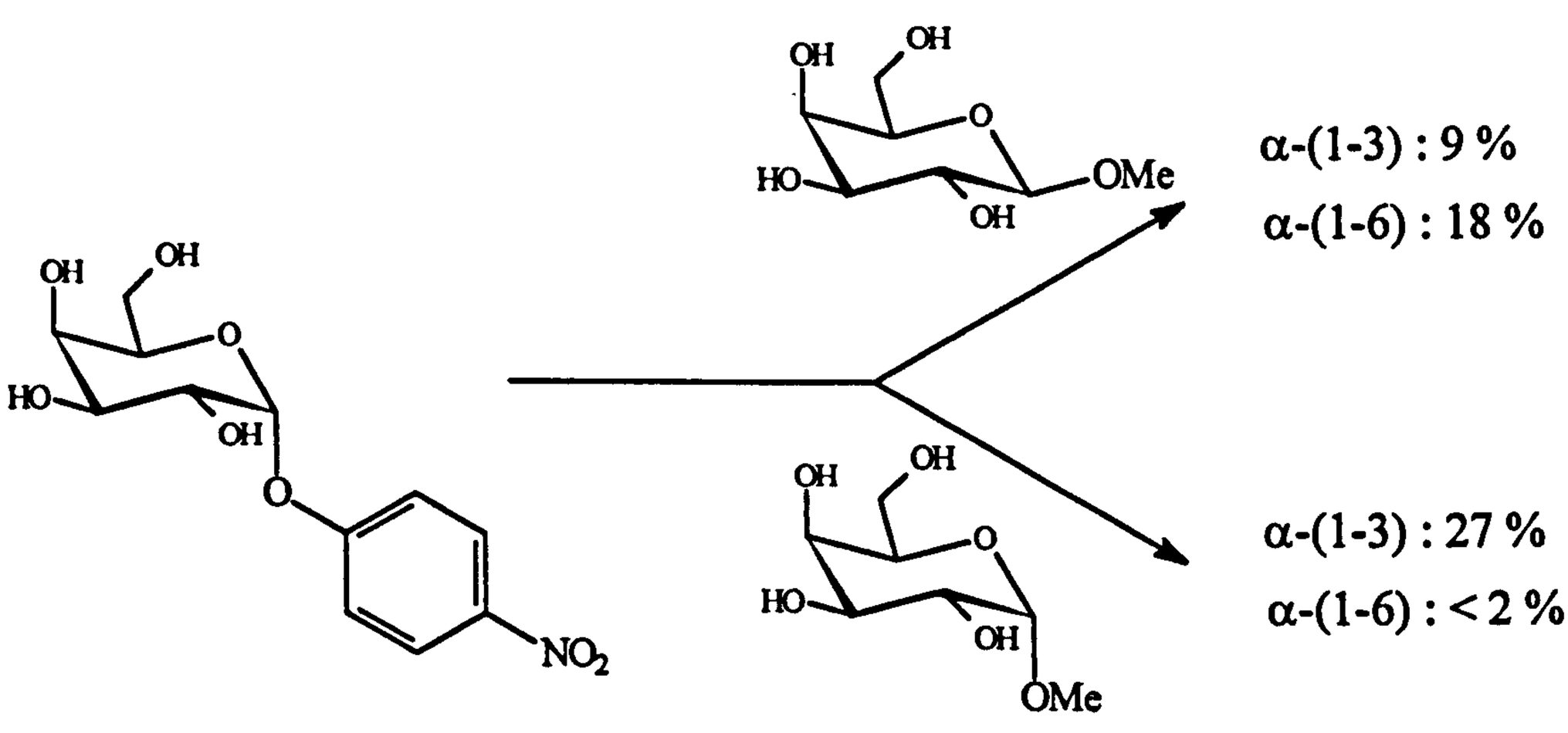


Figure 31 : Effect of the acceptor anomeric configuration

Other studies have revealed the influence of the nature of the structure of the aglycon of the acceptor. In the case of α-galactosidase, it was found that there is a preference for a hydrophobic aglycon. This was interpreted to be a direct result of the higher affinity of the hydrophobic structure of the active site of α-galactosidase for the hydrophobic aglycon of the acceptor[48].

4. Aim of the project

The aim of this project was to undertake a detailed study of the transferase activity of the multi-enzyme complex cellulase from *Trichoderma reesei,* ATC 26921. Previously, Kobayashi *et al.*[49] have shown that the cellulase from *Trichoderma viride* may be successfully used as a glycosyl transferase, under specific conditions, to synthesise the homopolysaccharide cellulose.

Earlier studies at Huddersfield[50] have shown the ability of the enzyme complex to transfer the activated substrate donor p-nitrophenyl β-D-glucopyranoside [1] to the glucoside acceptor N-(p-nitrophenyl)-β-D-glucopyranosylamine. p-Nitrophenyl β-D-glucopyranoside is a specific substrate for one group of enzymes present in the cellulase complex, the β-glucosidases. The reaction was performed under kinetically-controlled conditions and the formation of three different transglycosylation products, two of which were identified as the β-(1-4) and β-(1-6) linked disaccharides ([3], [4]), was reported (Figure 32).

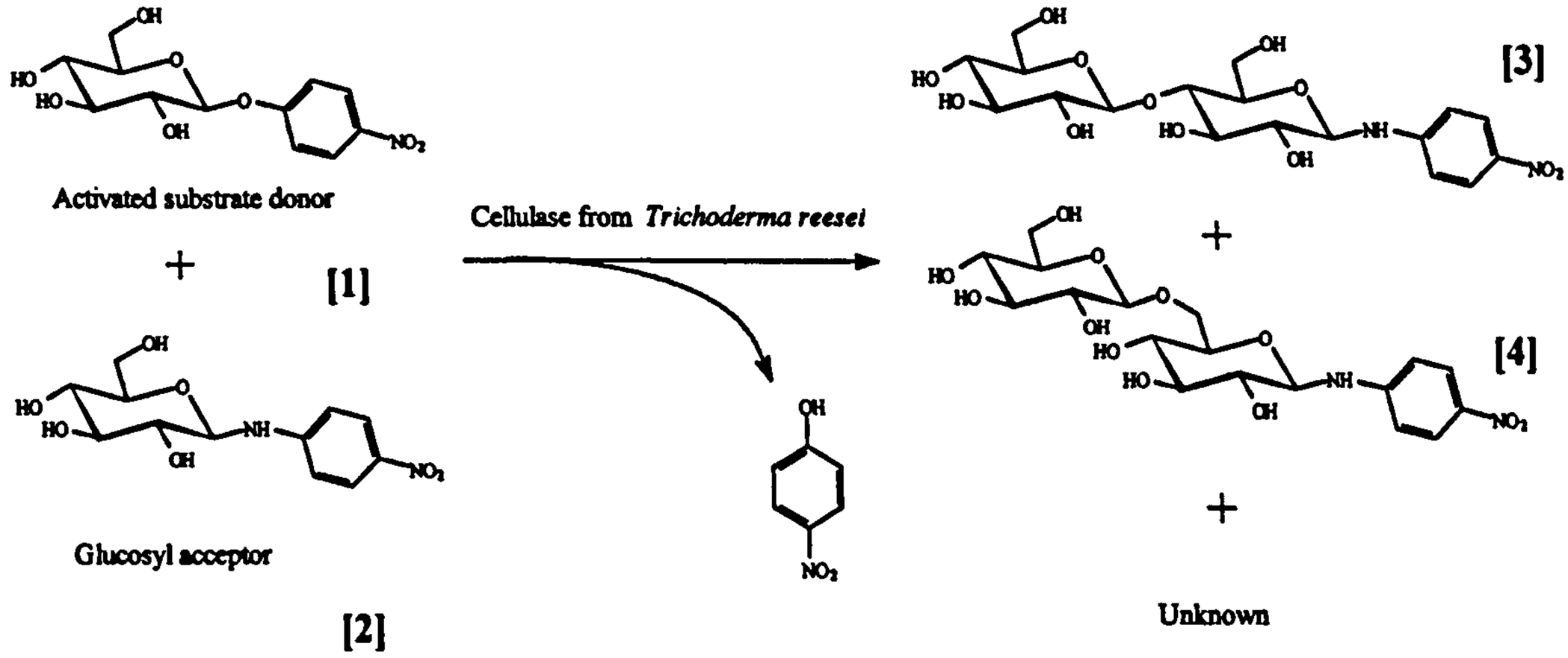


Figure 32 : Transglycosylation reaction using cellulase from *Trichoderma reesei*

The aim of the current project was to investigate the transferase activity of the other components of the enzyme complex (endo- and exo- acting enzymes) utilising disaccharide derivatives as substrate donors. Chemically synthesised mono- and disaccharide glycosyl acceptors are used in the transglycosylation

reaction which were performed under kinetically-controlled conditions. Different products are expected and will be identified by comparison with the chemically synthesised analogues. An investigation to determine the reaction conditions required to maximise the yield of each transglycosylation product is reported. The effect of pH, temperature and addition of a co-solvent were studied. The stereo- and regioselectivity of the reaction will be analysed and a mechanistic scheme explaining the transglycosylation pathway will be deduced. The role of the crude complex and that of the purified enzymes, in the catalysis of the transglycosylation reaction will be explored using specific inhibitors.

5. Description of the enzyme components of Cellulase from *Trichoderma reesei*

Cellulases are enzyme complexes responsible for the hydrolysis of the homopolysaccharide water insoluble cellulose, its natural substrate. As described in section 1.3.2., cellulose is an unbranched homopolysaccharide of D-glucose residues linked together by β-(1-4) glycosidic bonds to form linear polymeric chains of over 10,000 glucose residues.

Cellulases have various functions in Nature[51], and their main roles are:

- as morphogenic agents which weaken the cellulose-rich cell walls in preparation for growth, or abscissions such as dropping of leaves, flowers or seeds or in the ripening process
- invasive agents used by some plant pathogens to facilitate penetration into the plant
- digestive agents which permit the cellulose to be used as a carbon source and, in so doing, may make other non-cellulosic plant tissue accessible to degradative enzymes.

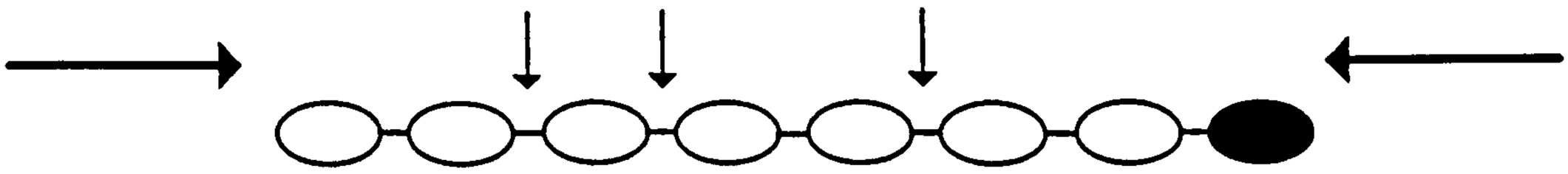
The most widely studied cellulases are of fungal origin. *Trichoderma* species are the most potent cellulase producers of the group and the *Trichoderma reesei* cellulase systems are those that have received considerable attention due to their economical potential (especially in the bioconversion processes of cellulose to ethanol)[52,53,54]. This potential rests in the fact that cellulase is a complete, extracellular enzyme system capable of catalysing the hydrolysis of crystalline cellulose. The rigid structure of cellulose requires the properties of several groups of enzyme to lead to its total hydrolysis.

5.1. Enzyme Groups

Cellulases are multi-enzyme complexes composed of three different classes of glycosidases acting synergistically[55] to undertake the total hydrolysis of cellulose. Each of these enzymes has multiple glucosyl binding sites which increase their affinity for their substrates. These different classes of enzyme are defined according to their regiochemical and substrate specificities[56] and are called endoglucanases, exoglucanases and β-glucosidases.

5.1.1. Endoglucanases

Endoglucanases are enzymes which hydrolyse glycosidic linkages randomly along a polysaccharide chain.



They are generally found to be inactive against crystalline cellulose but hydrolyse amorphous cellulose and soluble derivatives such as carboxymethyl-cellulose (CMC) (See Figure 33).

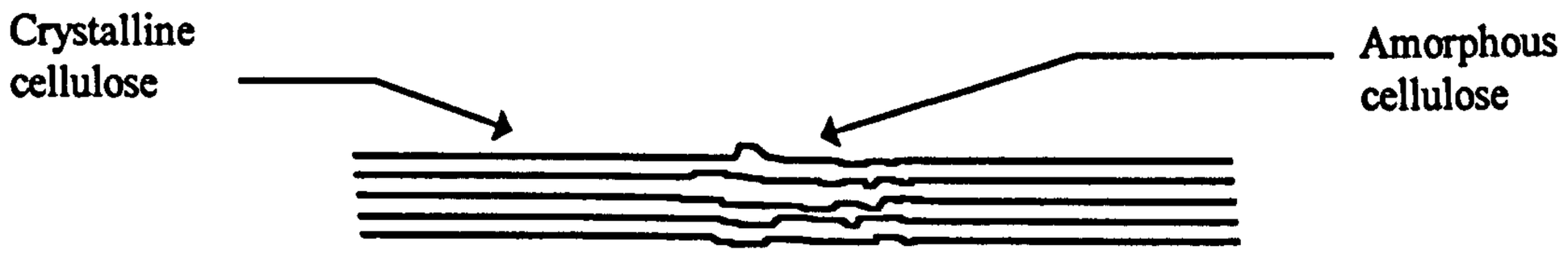


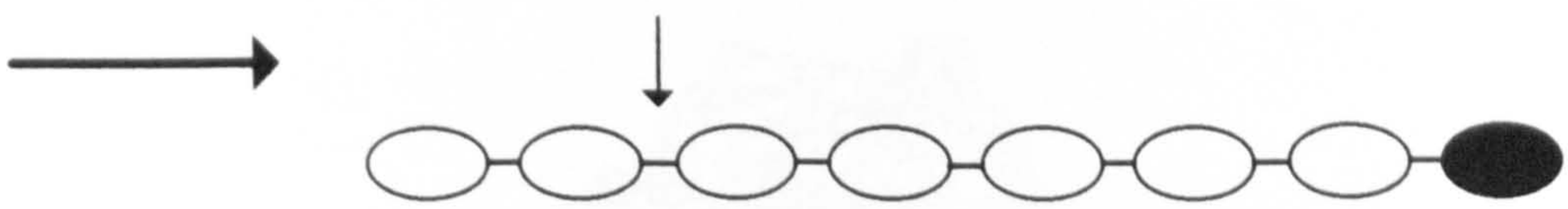
Figure 33 : Cellulose structure

Their random activity is attributed to the topology of their active site which is defined as an 'open cleft' structure[57] (Figure 34) which allows the random attachment of the enzyme to the saccharide and thus a random hydrolysis.

Figure 34 : Endoglucanase active site structure

5.1.2. Exoglucanases

Exoglucanases are defined as enzymes that catalyse the hydrolysis of glycosidic linkages from one end of the poly- and oligosaccharide chains. They are specific for the anomeric configuration of the substrate and the oligosaccharide residues released, but not for the aglycon that is linked to the sugar.



The structure of the active site of exoglucanases appears as a tunnel shape[58] which restrains the enzyme to hydrolyse the substrate from the extremities (Figure 35).

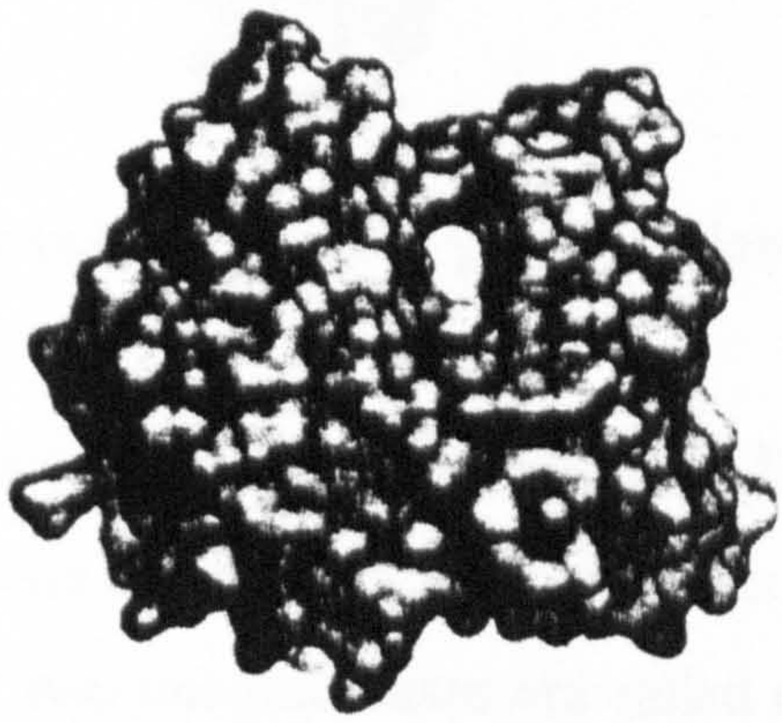


Figure 35 : Exoglucanase active site structure

This tunnel structure allows the enzymes to release the product while remaining bound to the polysaccharide chain and allows an efficient hydrolysis of the

substrate. The directionality of the enzyme motion along the chain is not unique and differs within the group, e.g. cellobiohydrolase II (*Trichoderma reesei*) hydrolyses its substrate from the non-reducing ends whereas the reverse process occurs with cellobiohydrolase I.

5.1.3. β-Glucosidases

β-glucosidases are enzymes which act on small β-glycosides. They hydrolyse cellobiose, cellobiose derivatives and glucose derivatives from the non-reducing end. Two classifications have resulted from their preferred activity either on cellobiose (cellobiases) or aryl glycosides (aryl β-glucosidases)[59]. The topology of the active site depicts a pocket or crater which is involved in the recognition of the substrate from the non-reducing end. A typical example is represented by Figure 36 :

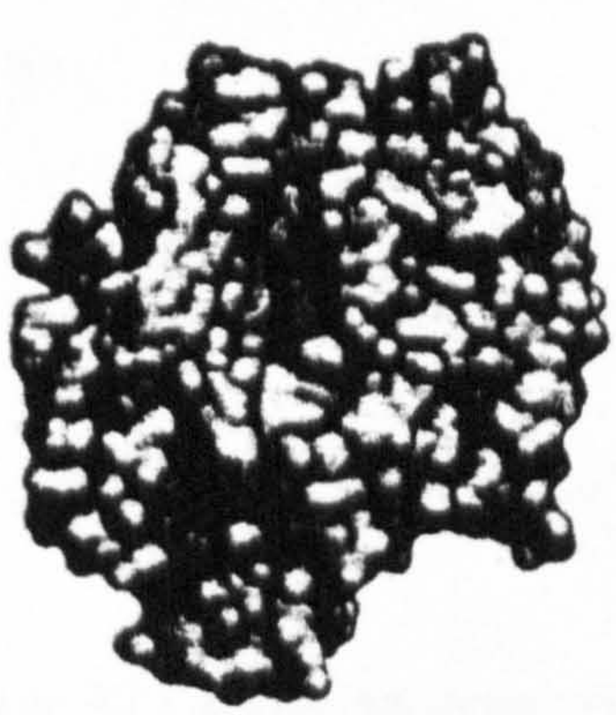


Figure 36 : The pocket structure of glucoamylase from *A. awamori*

Two main endoglucanases and two exoglucanases have been isolated from *Trichoderma reesei* cellulase and they are referred to as EG I, EG II, CBH I and CBH II respectively. The two exoglucanases are called cellobiohydrolases (CBH) due to their ability to catalyse the release, in the main, of cellobiose residues from the polysaccharide chains.

5.2. Catalytic Activities

The topology of the active site is in part responsible for the catalytic activity of each enzyme component of the cellulase complex.

5.2.1. Exoglucanases

As described earlier, the tunnel-like structure of the cellobiohydrolases is thought to explain part of their mechanism i.e. where only the ends of the cellulose chains are subject to hydrolysis. However, some differences present inside the same group of enzymes have led Henrissat *et al.*[60] to a new classification based on the structure-function relationship of an enzyme towards the substrate. Indeed, CBH I and CBH II are described as exoglucanase, but belonging to different families of glycosidases. Whereas CBH I hydrolyses its substrate from the reducing end[61,62], CBH II was found to act from the opposite direction[63]. This complementary action is essential as it enhances the rate of hydrolysis of cellulose. It is also referred to as an exo-exo synergism (Figure 37).

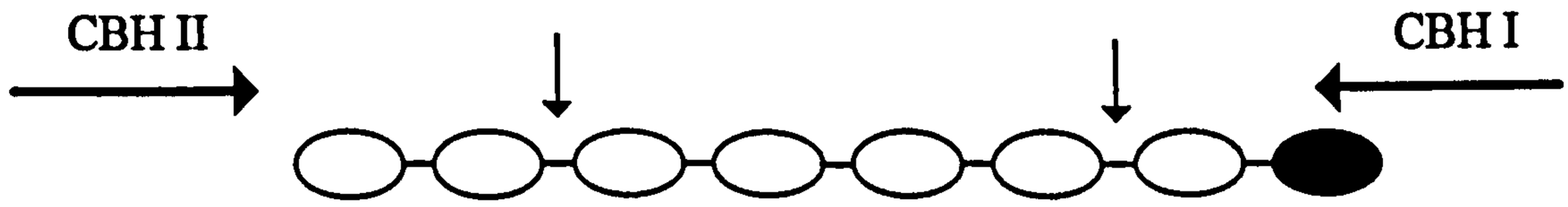


Figure 37 : Exo-exo synergism

Another major difference was found between CBH I and CBH II. The tunnel structure of CBH I was established to be 40 Å long whereas that of CBH II is only 20 Å long[58]. It is currently thought that a single glucan chain is fed into the tunnel structure of CBH I from one end, and is then thread through the entire length of the tunnel. The hydrolysis then takes place at one end of the tunnel releasing cellobiose as the main product. The remaining glucan chain remains inside the tunnel of the enzyme and, without dissociation of the enzyme-substrate complex, is continually hydrolysed in the same **processive** mode of action[64].

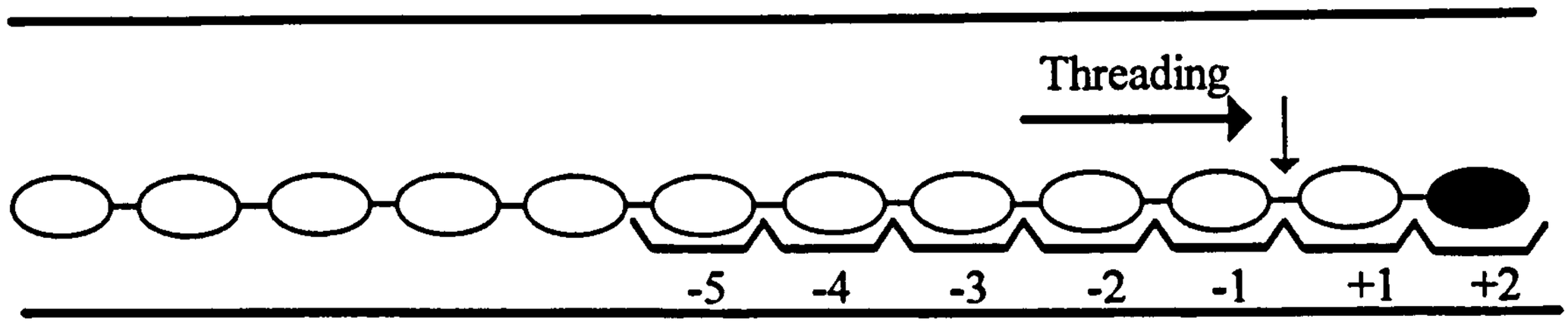


Figure 38 : CBH I catalytic domain schematic

CBH I can thus progress along the chain liberating more and more cellobiose from the reducing end. It could also weaken the interactions between neighbouring chains and allow easier access of CBH II to the cellulolytic chain. The short active site tunnel will probably allow continuous attachment and separation of CBH II with the polysaccharide.

Another difference between the two cellobiohydrolases comprising the cellulase from *Trichoderma reesei* is the outcome of the product configuration. Indeed, CBH I is a retaining enzyme whereas CBH II is an inverting enzyme[65]. Although cellobiohydrolase liberates mainly cellobiose residues from the chain ends, CBH I also hydrolyses other linkages of oligosaccharides liberating glucose or cellotriose[61].

5.2.2. Endoglucanases

The open cleft structure of endoglucanases allows the random attachment of the enzyme along the cellulolytic chains and a random hydrolysis creating new ends for the exo-acting enzyme. The latter can then hydrolyse the surface saccharide chain leaving new chains available for the endoglucanases. This endo-exo synergism increases the rate of cellulose hydrolysis (Figure 39).

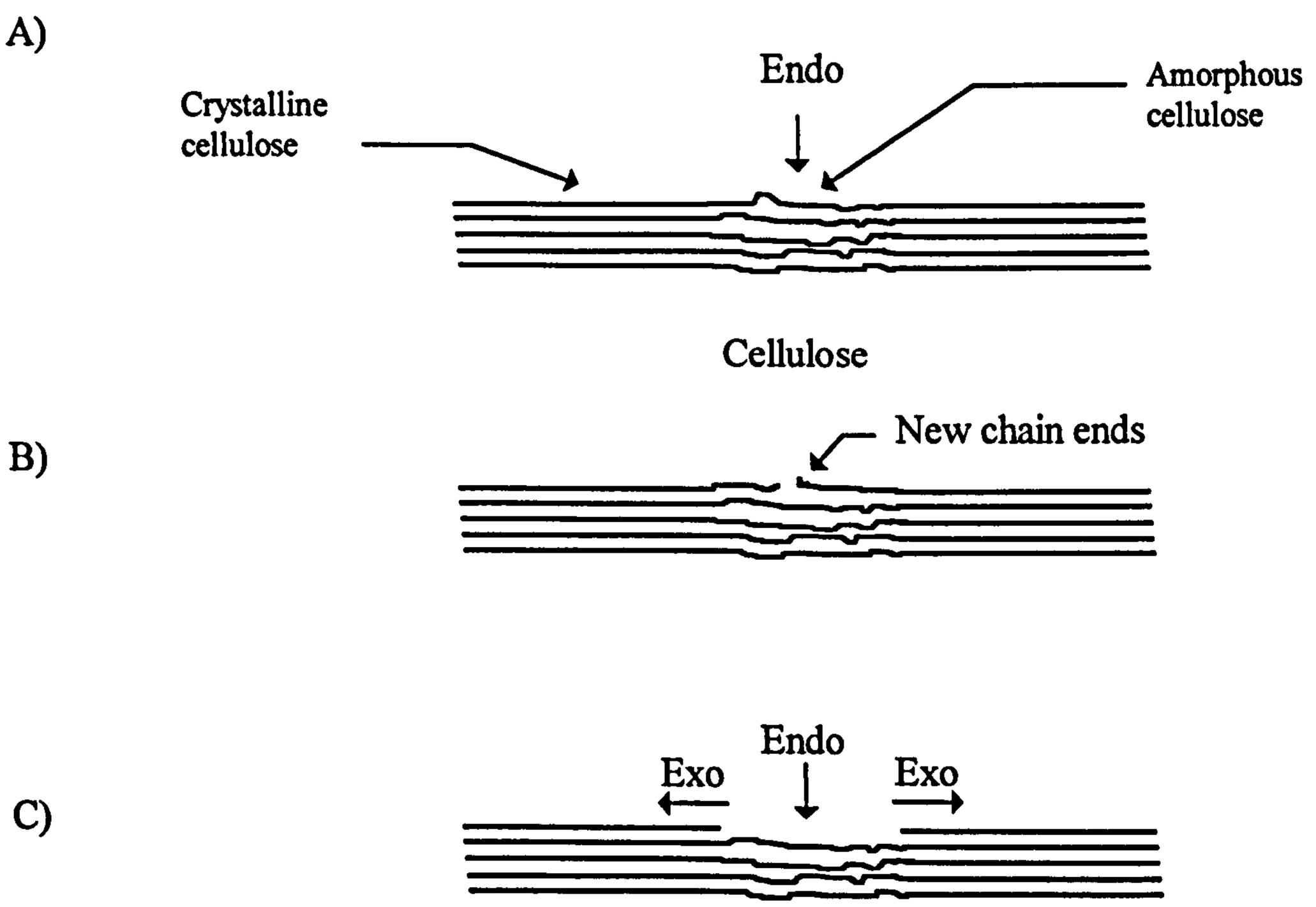


Figure 39 : Endo-exo synergism

Both endoglucanases from *Trichoderma reesei* hydrolyse their substrate with retention of configuration[66]. EG I has been shown to hydrolyse small oligosaccharides and has been demonstrated to possess transfer activity[67]. The amino acid sequence of endoglucanase I shows many similarities with the amino acid sequence of cellobiohydrolase I (>45%)[68] the difference being the additional loops in CBH I giving rise to a tunnel-like structure for the latter. EG II catalyses the hydrolysis of long chain oligosaccharides with a minimum requirement for four glucose residues.

5.2.3. Binding sites

The catalytic sites of the different enzymes comprising the cellulase from *Trichoderma reesei* are composed of multiple binding sites for glucose residues, allowing multiple substrate-enzyme interactions (Table 2). The relative binding affinities of the substrate in the enzyme active site have been shown to depend on the oligosaccharide chain length and the substrate concentration[69].

Enzyme	Number of glucose binding sites
β-glucosidase	3 (CS between 1 and 2)
CBH I	≥ 7 (CS between 2 and 3)
CBH II	6 (CS between 2 and 3)
EG I	≥ 5 (CS between 2 and 3)
EG II	≥ 5 (CS between 3 and 4)

CS : catalytic site.

Table 2

5.2.4. Cellulose binding domain (CBD)

Structural studies have revealed similarities in the enzyme composing the cellulase multi-enzyme complex. A 'tadpole structure'[70] has been described for the endo- and exo-acting enzymes[71], the tadpole containing two distinct functional domains: a catalytic domain connected via a highly glycosylated flexible hinge region to an additional cellulose binding domain. The latter is thought to promote the adsorption onto cellulose and thus increases the affinity of the enzymes for the polysaccharide substrate allowing multiple catalytic cycles without desorption of the CBD[72]. The binding is thought to be reversible to allow movement of the enzyme along the chain.

A model structure of the CBD of CBH I is illustrated in Figure 40, with the linker and the catalytic site attached to cellulose. The release of a cellobioside residue can also be observed.

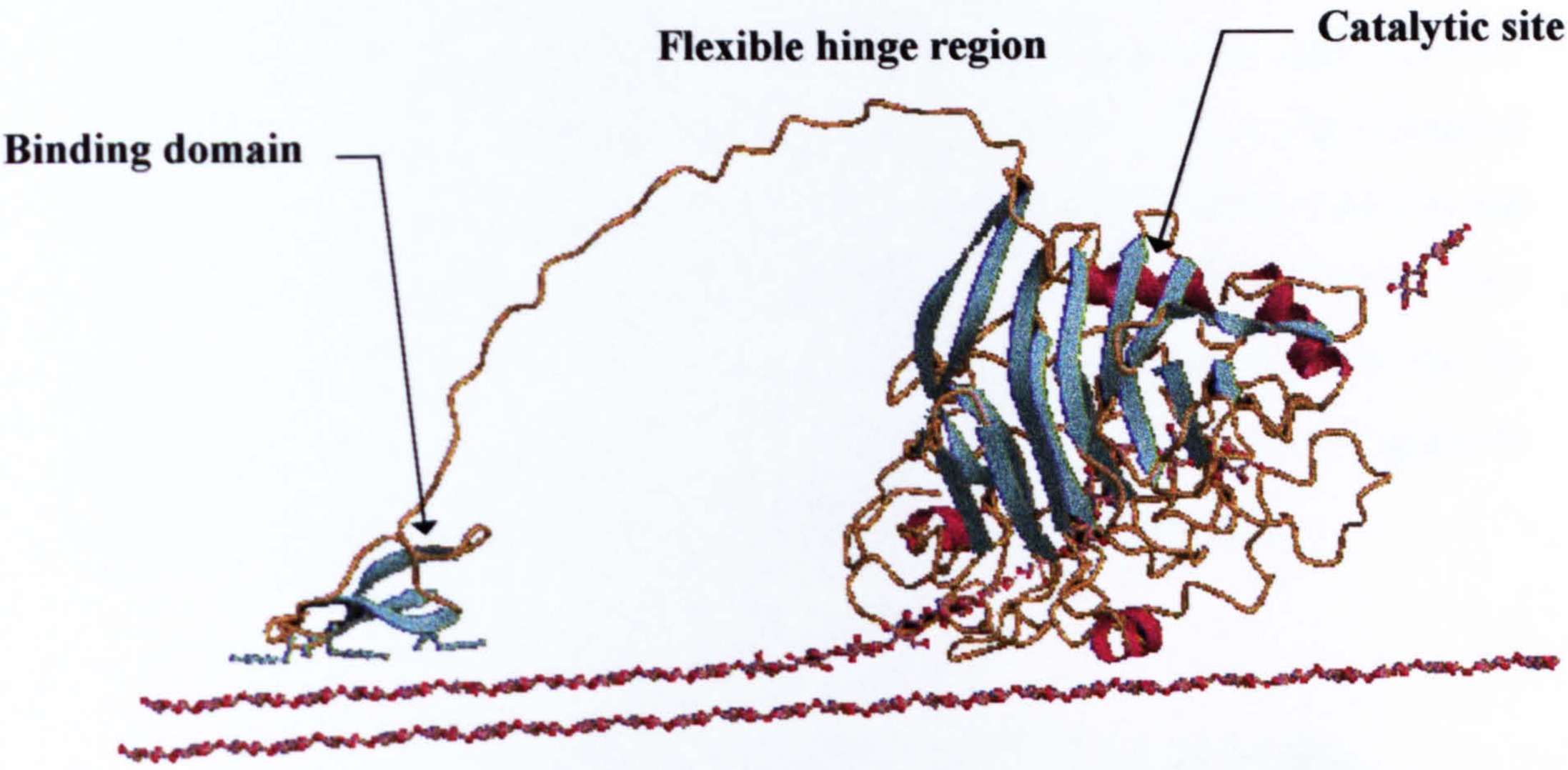


Figure 40 : CBH I model structure

The cellulose binding domain has been shown to be shaped like a wedge with one hydrophilic face allowing interaction with the substrate chains and destabilisation of the rigid structure of cellulose. Indeed, J. Knowles *et al.*[58] have shown that the CBDs reduce the interactions between the polysaccharide chains, destabilising the structure of the substrate and thus facilitating the attack on cellulose.

5.3. Cellulose Hydrolysis

Although, the complete mechanism of the hydrolysis of cellulose is not yet fully understood, it is possible to comprehend the importance of each of the different enzyme components present in the system. The research on the mechanism of cellulose hydrolysis started in the 1950s with the C_1-C_x concept of Reese[73] *et al.* C_1 was described as a desaggregating enzyme (now called endoglucanases) and C_x (exoglucanases) was the hydrolytic enzymes. The hydrolysis of cellulose starts

with the **adsorption** of the enzyme and the **random** attack of the endo-acting enzymes upon the water-insoluble amorphous polysaccharide, cellulose. This initial attack produces new reducing and non-reducing ends available for the complementary or synergistic action of the exo-acting enzymes releasing, in the main, cellobiose residues. These combined actions (endo-exo and exo-exo synergisms)[74] expose the underlying chains to further endo-action. The enhanced rate of hydrolysis caused by the action of the enzyme components results in the conversion of the insoluble polysaccharide chains to soluble polysaccharide ones and finally to cellobiose. The last enzyme involved in the process is the β-glucosidase which catalyses the hydrolysis of cellobiose and glucose. Figure 41 represents a schematic picture of the cellulose hydrolysis mechanism.

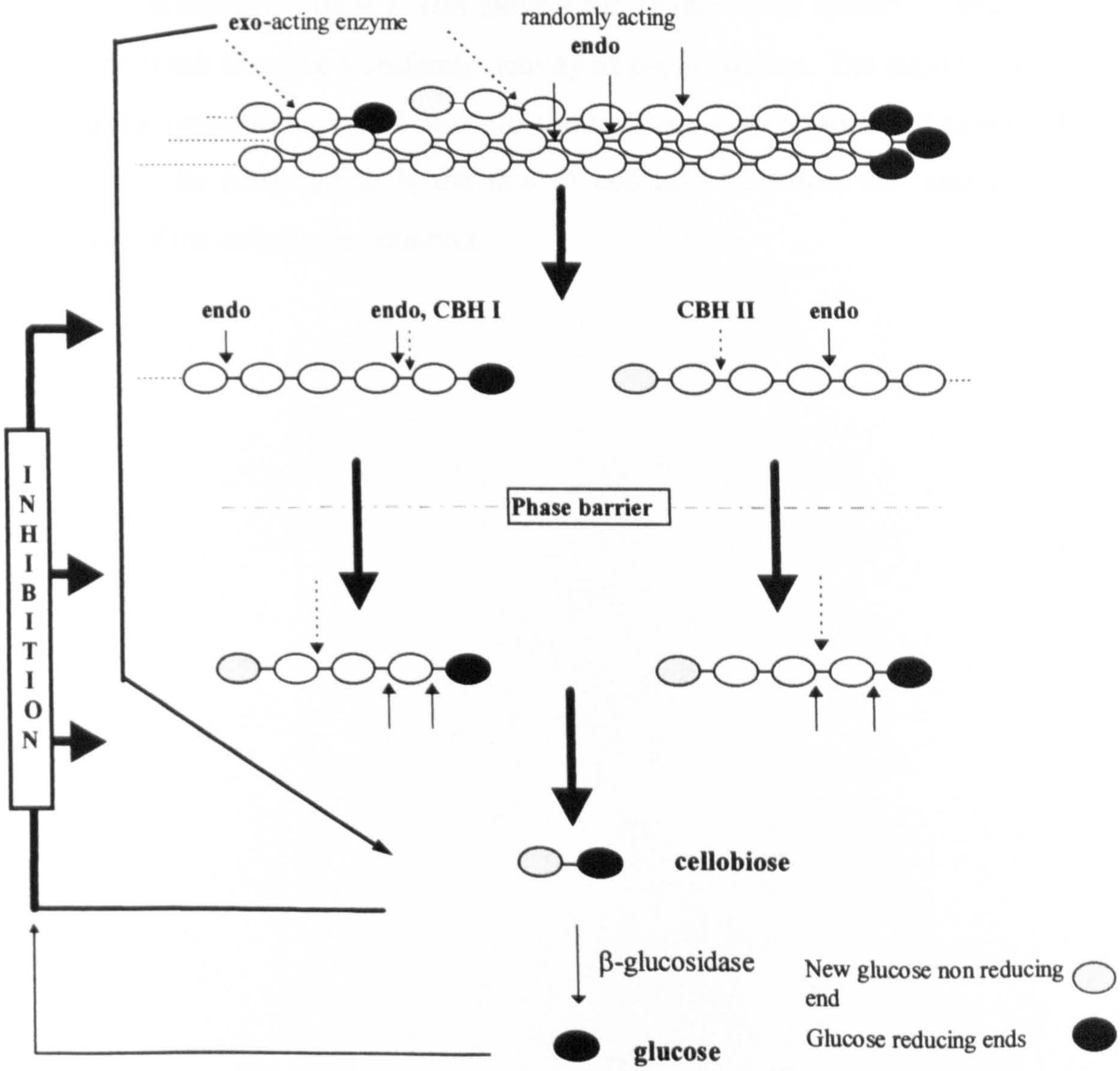


Figure 41 : Schematic cellulose hydrolysis mechanism

The degradation of the soluble polysaccharide chains leads to cellobiose, a disaccharide which is only hydrolysed by the action of β-glucosidase. This final step is then rate-limiting and depends upon the requirement of the organisms for glucose as a source of energy. Cellobiose is itself an inhibitor of the exo-acting enzymes, and thus participates in the regulation process involved in the hydrolysis of cellulose[75]. It has been shown that the hydrolysis of smaller oligosaccharides proceeds via transglycosylation as the products provide new higher substrates for the endo- and exo-acting enzymes and thus increase the rate of hydrolysis. EG I, CBH I, and β-glucosidase have been shown to be capable of transglycosylation[76]. The production of cellulase in *Trichoderma reesei* has been shown to be induced by different compounds. Among them sophorose (2-O-β-glucopyranosyl-β-glucose) represents the most potent inducer for *Trichoderma* species[77,78] and was proven to result from the transferase activity of β-glucosidase. The importance of this enzyme representing only 1 % of the total protein concentration is thus related not only to its participation in the rate of cellulose hydrolysis but also in the induction of the cellulase production.

Chapter 2

Experimental

1. Synthesis

1.1. Materials

1.1.1. Instruments

^{1}H and ^{13}C nmr spectra were recorded using a Brüker AC-270 NMR spectrometer. Electrospray mass spectrometry was carried out using a VG (Fisons Instruments) QUATTRO (II) SQ mass spectrometer. The melting points were determined using a Gallenkamp melting point apparatus. Infra-red spectra were recorded with a Perkin Elmer Paragon 1000 Series FT-IR.

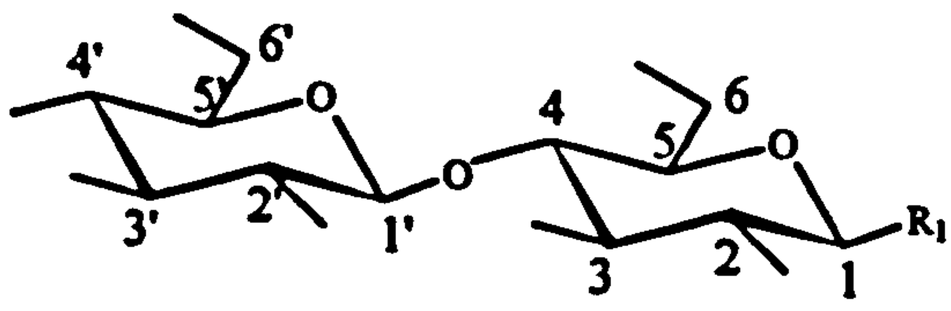
1.1.2. Materials

Most chemicals were bought from Sigma Co (St. Louis, USA), or Fluka Co and used without further purification. Solvents were purchased from Fisher.

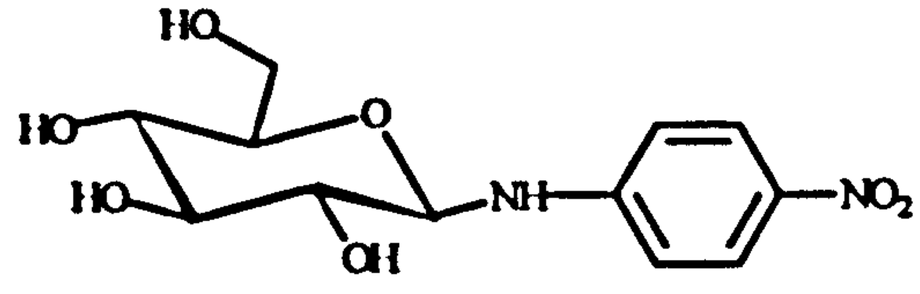
Pyridine was dried over potassium hydroxide and chloroform over solid sodium (x 2). Column chromatography was performed on silica gel 60 (70-230 mesh ASTM), Merck.

1.1.3. NMR assignment

In assigning signals in ^{1}H-nmr and ^{13}C-nmr spectra, protons and carbons of the pyranose ring are numbered conventionally, 1 through 6, from the anomeric centre to the reducing end and 1' to 6' for the non-reducing end sugar. COSY spectra were obtained for most compounds and C-H correlations were obtained when difficulties in assigning the signals were encountered.



1.2. N-(p-nitrophenyl)-β-D-glucopyranosylamine



(1)

α-D-(+)-Glucose (10.0 g, 5.5 mmol) and p-nitroaniline (9.0 g, 6.5 mmol) were dissolved in methanol (150 ml). After adding three drops of conc. HCl as a catalyst, the reaction mixture was refluxed for 4 to 5 hours and then left to cool down to room temperature. A bright yellow precipitate appeared as the temperature decreased. The precipitate was filtered and recrystallised from hot methanol. Yield: 5.10 g (31.5 %), m. pt. : 185 °C. (Lit.[50] m.pt. : 189°C).

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 3400-3100 (O-H and N-H stretching vibrations), 1601 and 1500 (aromatic C-C stretching vibrations, doublet due to conjugation with - NO_2), 1551 and 1349 (C-NO_2), 1460 to 1200 (O-H and C-H deformation), 1175 to 1020 (C-O stretching).

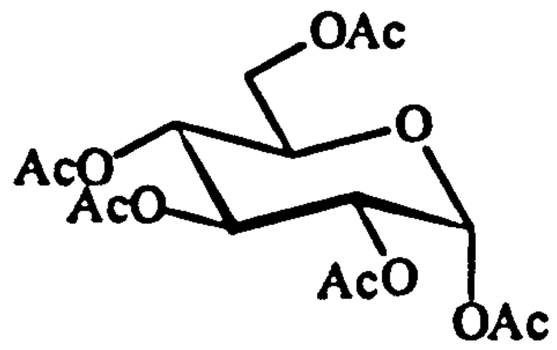
^{1}H-nmr (D_6-DMSO, 270 MHz, ppm) : δ 8.03 (d, 2 H, Ar-H, J 9 Hz), 7.78 (d, 1 H, NH, J 8 Hz), 6.81 (d, 2 H, Ar-H, J 9 Hz), 5.08 (d, 1 H, OH-3, J 5 Hz), 5.04 (d, 1 H, OH-2, J 5 Hz), 4.98 (d, 1 H, OH-4, J 5 Hz), 4.49 (m, 2 H, H-1, OH-6), 3.66 (dd, 1 H, $H\text{-}6_a$, J 5 Hz, J 10 Hz), 3.46 (m, 1 H, $H\text{-}6_b$), 3.29 (m, 1, H-5), 3.24 (m, 1, H-2), 3.17 (m, 2, H-3, H-4).

^{1}H-nmr (D_6-DMSO+D_2O, 270 MHz, ppm) : δ 8.00 (d, 2 H, Ar-H, J 9 Hz), 6.79 (d, 2 H, Ar-H, J 9 Hz), 4.50 (d, 1 H, H-1, J 9 Hz), 3.54 (m, 1 H, H-6), 3.42 (m, 1 H, H-6), 3.30 (m, 1 H, H-5), 3.28 (t, 1 H, H-2, J 9 Hz), 3.20 (t, 1 H, H-3, J 9 Hz), 3.13 (t, 1 H, H-4, J 9 Hz).

^{13}C-NMR (D_6-DMSO, 270 MHz, ppm) : δ 153.77 (ArC-NO_2), 137.21 (ArC-NH), 125.92 (2 x ArC-H), 112.40 (2 x ArC-H), 83.52 (C-1), 77.71 (C-5), 77.65 (C-2), 72.89 (C-3), 69.96 (C-4), 60.81 (CH_2).

m/z (EI+, MeOH:H_2O (7:3) + NH_4OAc 10 mM, 40°C) : 301 ($[M + H]^+$).

1.3. 1,2,3,4,6-Penta-O-acetyl-α-D-glucopyranoside (2)



(2)

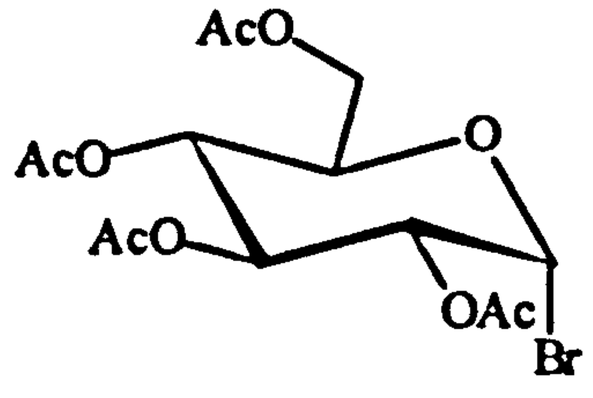
α-D-(+)-glucose (5.0 g, 28.0 mmol) was dissolved in a mixture of dry pyridine and acetic anhydride (2:1, v/v, 90 ml) under a nitrogen atmosphere. A catalytic amount of DMAP was then added to the solution and the reaction was stirred at room temperature for 24 hours. The mixture was then poured into a beaker of crushed ice (~ 400 ml) and stirred for approximately one hour. A white precipitate was then filtered off, and washed thoroughly with cold water. Crystals of **2** were obtained by recrystallisation from hot methylated spirits. Yield : 9.5 g (87 %), m. pt. : 110°C. (Lit.[79] m.pt. : 110°C).

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 1741 (C=O stretching vibration), 1350-1200 (C-H deformation), 1238 (C-O stretching vibration of ester), 1120 to 1020 (C-O stretching).

^{1}H-nmr ($CDCl_3$, 270 MHz, ppm) : δ 6.26 (d, 1 H, H-1, J 4 Hz), 5.41 (t, 1 H, H-3, J 10 Hz), 5.08 (t, 1 H, H-4, J 10 Hz), 5.03 (dd, 1 H, H-2, J 4 Hz, J 10 Hz), 4.20 (dd, 1 H, H-6, J 4 Hz, J 12 Hz), 4.04 (m, 2 H, H-6, H-5), 2.12, 2.03, 1.98, 1.97, 1.96 (5s, 15 H, 5 x CH_3).

^{13}C-nmr ($CDCl_3$, 270 MHz, ppm) : δ 170.48, 170.09, 169.53, 169.27, 168.63 (5 x C=O), 88.90 (C-1), 69.66, 69.66, 69.02, 67.70 (C-2, C-3, C-4, C-5), 61.29 (CH_2), 20.75, 20.56, 20.54, 20.44, 20.32 (5 x CH_3).

1.4. 2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide (3)



(3)

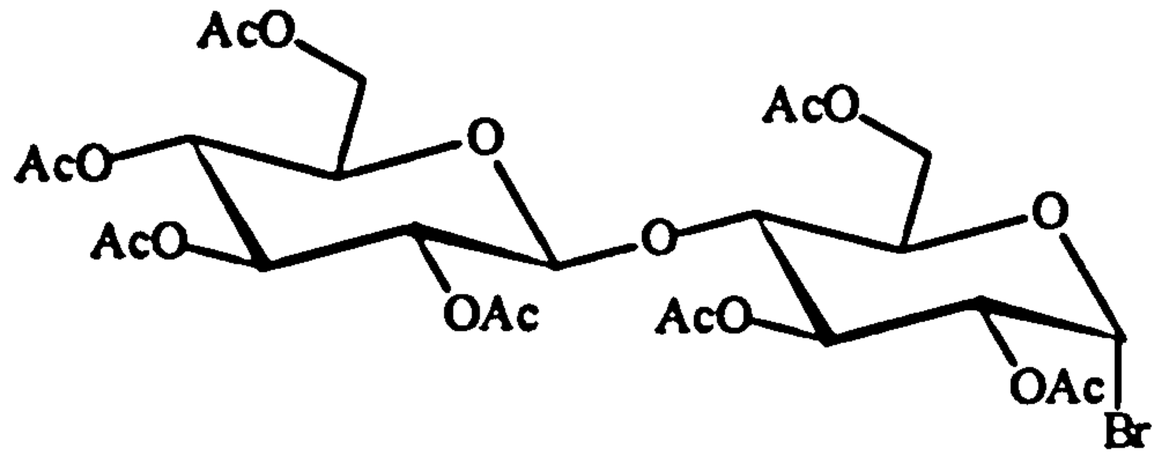
A solution of HBr in glacial acetic acid (45 %, 20 ml) was added to 1,2,3,4,6-penta-O-acetyl-α-D-glucopyranoside **2** (10.0 g, 26.0 mmol) contained in a conical flask fitted with a ground glass stopper. The reaction was left to stand at room temperature for 1 hour. The solution was diluted with chloroform (30 ml) and poured into a 500 ml beaker of crushed ice and stirred for 1 hour. The organic phase was separated and washed successively with a saturated solution of sodium bicarbonate (15 ml x 2) and a saturated solution of sodium chloride (15 ml x 2). The organic phase was finally dried over anhydrous magnesium sulphate and filtered. The chloroform was then evaporated. 2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide **3** was recrystallised from boiling ether with slow addition of hexane to yield white crystals. Yield : 9.5 g (86 %), m. pt.: 89°C. (Lit.[79] m.pt. : 89°C).

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 1742 (C=O stretching vibration), 1350-1200 (C-H deformation), 1246 (C-O stretching vibration of ester), 1120 to 1020 (C-O stretching).

^{1}H nmr ($CDCl_3$, 270 MHz, ppm) : δ 6.58 (d, 1 H, H-1, J 4 Hz), 5.53 (t, 1 H, H-3, J 10 Hz), 5.12 (t, 1 H, H-4, J 10 Hz), 4.80 (dd, 1 H, H-2, J 4 Hz, J 10 Hz), 4.27 (m, 2 H, H-5, H-6), 4.07 (m, 1 H, H-6), 2.07, 2.06, 2.02, 2.00 (4s, 12 H, 4 x CH_3).

^{13}C nmr ($CDCl_3$, 270 MHz, ppm) : δ 170.45, 169.80, 169.74, 169.41 (4 x C=O), 86.51 (C-1), 72.07, 70.53, 70.09, 67.09 (C-2, C-3, C-4, C-5), 60.88 (CH_2), 20.61, 20.61, 20.51, 20.51 (4 x CH_3).

1.5. 2,3,6-Tri-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-α-D-glucopyranosyl bromide (4)



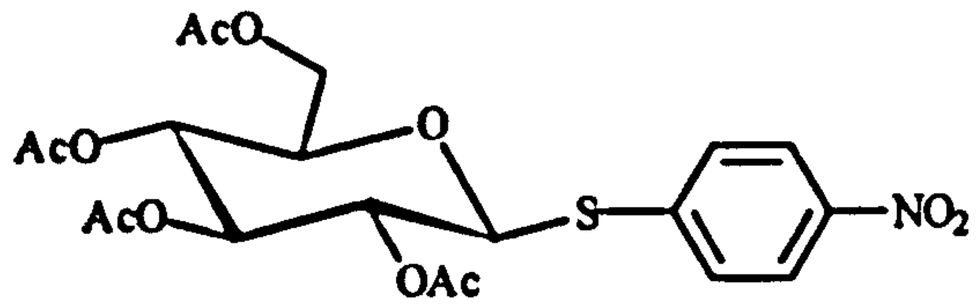
(4)

α-D-(+)-Cellobiose octaacetate (5.03 g, 7.41 mmol) was diluted in a solution of 45 % HBr in glacial acetic acid (20 ml). The same procedure as 1.4. was used to obtain the final compound (4). Yield : 4.11 g (80 %), m. pt. : 158-160°C.

^{1}H-nmr ($CDCl_3$, 270 MHz, ppm) : δ 6.50 (d, 1 H, H-1, J 4 Hz), 5.51 (t, 1 H, H-2', J 9 Hz), 5.13 (t, 1 H, H-3, J 10 Hz), 5.06 (t, 1 H, H-3', J 9 Hz), 4.92 (t, 1 H, H-4', J 9 Hz), 4.74 (dd, 1 H, H-2, J 4 Hz, J 10 Hz), 4.52 (d, 1 H, H-1', J 9 Hz), 4.49 (m, 1 H, H-6), 4.35 (m, 1 H, H-6'), 4.17 (m, 1 H, H-5), 4.13 (m, 1 H, H-6), 4.02 (m, 1 H, H-6'), 3.82 (t, 1 H, H-4, J 10 Hz), 3.65 (m, 1 H, H-5'), 2.07, 2.07, 2.03, 2.03, 2.12, 1.99, 1.67 (7 s, 21 H, 7 x CH_3).

^{13}C-nmr ($CDCl_3$, 270 MHz, ppm) : δ 170.47, 170.24, 170.09, 170.09, 169.28, 169.28, 168.96 (7 x C=O), 100.56 (C-1'), 86.40 (C-1), 75.21, 72.99, 72.93, 72.03, 71.58, 70.75, 69.40 (C-2, C-2', C-3, C-3', C-4, C-5, C-5'), 67.75 (C-4'), 61.58, 60.91 (2 x CH_2), 20.80, 20.80, 20.67, 20.67, 20.54, 20.54, 20.54 (7 x CH_3).

1.6. p-Nitrophenyl 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (5)



(5)

2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide **3** (4.02 g, 10.0 mmol) was added to a solution of p-nitrothiophenol (1.65 g, 11.0 mmol) and sodium hydroxide (4 M, 2.5 ml, 11.0 mmol) in acetone (200 ml). After refluxing for 3 to 4 hours, the acetone was removed using a rotatory evaporator. The mixture was then diluted with chloroform (30 ml). The organic phase was then separated and washed successively with a saturated solution of sodium bicarbonate (10 ml x 3) and a saturated solution of sodium chloride (10 ml x 3). The solution was finally dried over anhydrous magnesium sulphate, filtered and evaporated under reduced pressure. The crude product **5** was recrystallised from chloroform with slow addition of ether. Yield : 2.13 g (44 %), m. pt. : 181°C. (Lit.[80] m.pt. : 182-184°C).

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 1741 (C=O stretching vibration), 1598 and 1577 (aromatic C-C stretching vibrations, doublet due to conjugation with $-NO_2$), 1519 and 1343 (asym. and sym. NO_2 stretching), 1460 to 1200 (O-H and C-H deformation of carbohydrate), 1265-1230 (C-O stretching vibration of ester), 1120 to 1020 (C-O sym. stretching).

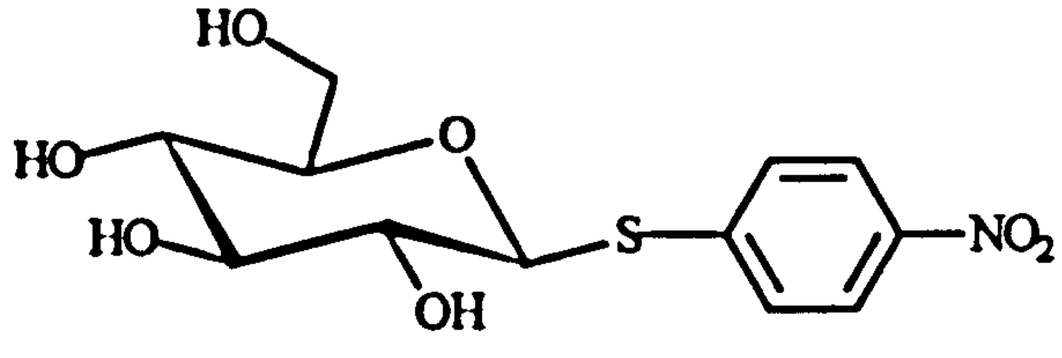
^{1}H nmr ($CDCl_3$, 270 MHz, ppm) : δ 8.11 (d, 2 H, Ar-H, J 9 Hz), 7.54 (d, 2 H, Ar-H, J 9 Hz), 5.24 (t, 1 H, H-3, J 9 Hz), 5.04 (t, 1 H, H-4, J 9 Hz), 5.00 (t, 1 H, H-2, J 9 Hz), 4.85 (d, 1 H, H-1, J 9 Hz), 4.16 (m, 2 H, H-$6_{a,b}$), 3.80 (m, 1 H, H-5), 2.06, 2.04, 1.99, 1.96 (4s, 12 H, CH_3).

^{13}C nmr ($CDCl_3$, 270 MHz, ppm) : δ 170.32, 169.94, 169.26, 169.12 (4 x C=O), 146.66 (ArC-NO_2), 141.69 (ArC-S), 130.81 (2 x ArC-H), 123.77 (2 x ArC-H), 84.17

(C-1), 75.95, 73.46, 69.45, 67.87 (C-2, C-3, C-4, C-5), 61.94 (CH_2), 20.64, 20.54, 20.44, 20.44 (4 x CH_3).

m/z (EI+, MeOH:H_2O (1:1), 40°C) : 508 ($[M + Na]^+$).

1.7. p-Nitrophenyl 1-thio-β-D-glucopyranoside (6)



(6)

Compound **5** (2.10 g, 4.3 mmol) was added to a solution of methanol (60 ml) containing a catalytic amount of sodium methoxide. The reaction mixture was left overnight at room temperature and the solvent was evaporated. The compound was purified using silica gel column chromatography (Chloroform : Methanol, 9.5:0.5, v/v). Yield : 1.30 g (95 %), m. pt : 129-130°C.

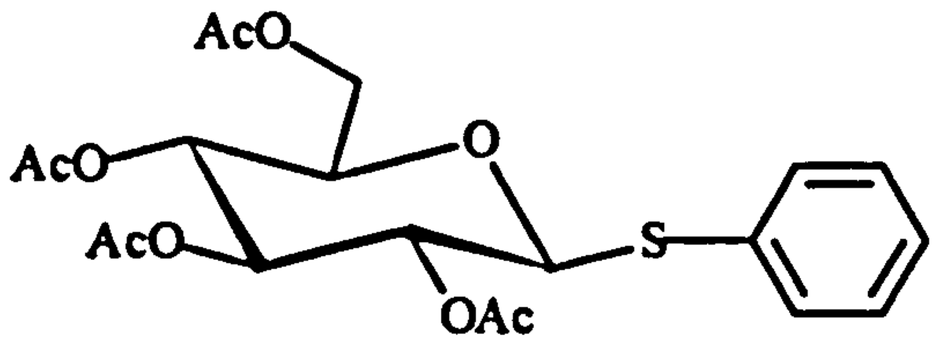
IR-FT (Nujol mull, cm^{-1}) : υ_{max} 3500-3100 (O-H), 1595 and 1578 (aromatic C-C stretching vibrations, doublet due to conjugation with -NO_2), 1507 and 1345 (C-NO_2), 1460 to 1200 (O-H and C-H deformation), 1175 to 1020 (C-O stretching).

^{1}H nmr (D_6-DMSO, 270 MHz, ppm) : δ 8.11 (d, 2 H, Ar-H, J 9 Hz), 7.61 (d, 2 H, Ar-H, J 9 Hz), 5.53 (d, 1 H, OH-3, J 6 Hz), 5.22 (d, 1 H, OH-4, J 5 Hz), 5.10 (d, 1 H, OH-2, J 5 Hz), 4.92 (d, 1 H, H-1, J 10 Hz), 4.62 (t, 1 H, OH-6, J 6 Hz), 3.70 (m, 1 H, H-6), 3.47 (m, 1 H, H-6), 3.4 (m, 1 H, H-5), 3.28 (m, 1 H, H-4), 3.16 (m, 2 H, H-2, H-3).

^{13}C nmr (D_6-DMSO, 270 MHz, ppm) : δ 146.21 (ArC-NO_2), 144.91 (ArC-S), 127.73 (2 x ArC-H), 123.82 (2 x ArC-H), 85.10 (C-1), 81.07, 78.14, 72.44, 69.66 (C-2, C-3, C-4, C-5), 60.83 (CH_2).

m/z (EI+, MeOH:H_2O (1:1), 40°C) : 340 ($[M + Na]^+$).

1.8. Phenyl 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (7)



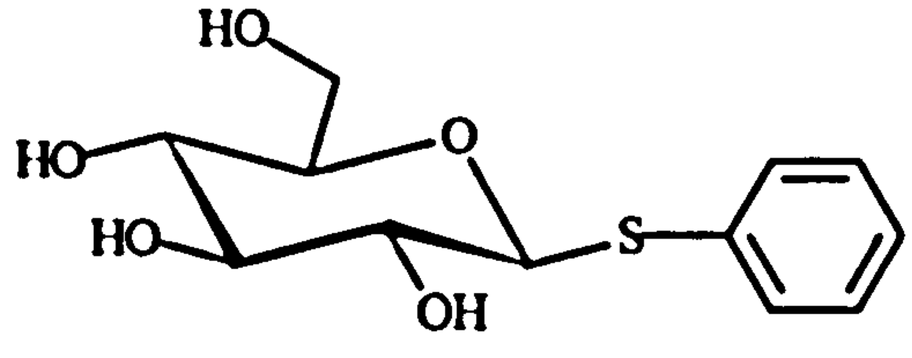
(7)

Bromo 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside **3** (1.64 g, 4.0 mmol) was added to a solution of sodium thiophenolate (0.55 g, 4.0 mmol) in anhydrous acetone (100 ml). The mixture was then refluxed for 2 hours after which time the acetone was then evaporated off. The mixture was then diluted with chloroform (30 ml). The organic phase was separated and washed successively with a saturated solution of sodium bicarbonate (10 ml x 3) and a saturated solution of sodium chloride (10 ml x 3). The solvent was removed under reduced pressure (25°C) and the crude product was purified using silica gel column chromatography (Petroleum ether : Ethyl acetate, 3:1, v/v). Compound **7** was obtained as white crystals. Yield : 1.21 g (69 %), m. pt. : 116°C. (Lit.[81] m.pt. : 116-117°C).

^{1}H nmr ($CDCl_3$, 270 MHz, ppm): δ 7.48 (m, 2, Ar-H), 7.30 (m, 3, Ar-H), 5.21 (t, 1 H, H-3, J 9 Hz), 5.01 (t, 1 H, H-4, J 9 Hz), 4.96 (t, 1 H, H-2, J 9 Hz), 4.69 (d, 1 H, H-1, J 10 Hz), 4.17 (m, 2 H, H-$6_{a,b}$), 3.71 (m, 1 H, H-5), 2.07, 2.06, 2.00, 1.97 (4s, 12 H, CH_3).

^{13}C nmr ($CDCl_3$, 270 MHz, ppm) : δ 170.07, 169.61, 169.41, 169.14 (4 x C=O), 132.27 (Ar-C), 130.94 (2 x ArC-H), 129.17 (2 x ArC-H), 127.71 (ArC-H), 83.27 (C-1), 74.28, 72.98, 69.59, 66.03 (C-2, C-3, C-4, C-5), 61.93 (CH_2), 20.56, 20.45, 20.45, 20.32 (4 x CH_3).

1.9. Phenyl 1-thio-β-D-glucopyranoside (8)



(8)

Phenyl 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside 7 (0.85 g, 1.9 mmol) was added to a solution of methanol (100 ml) containing a catalytic amount of sodium methoxide. The reaction was left overnight at room temperature. The solvent was then evaporated. Compound 8 was obtained as white crystals from recrystallisation from hot methanol. Yield: 0.45 g (86 %), m. pt. : 129-130°C.

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 3500-3200 (O-H stretching), 1460 to 1200 (O-H and C-H deformation), 1120 to 1020 (C-O stretching).

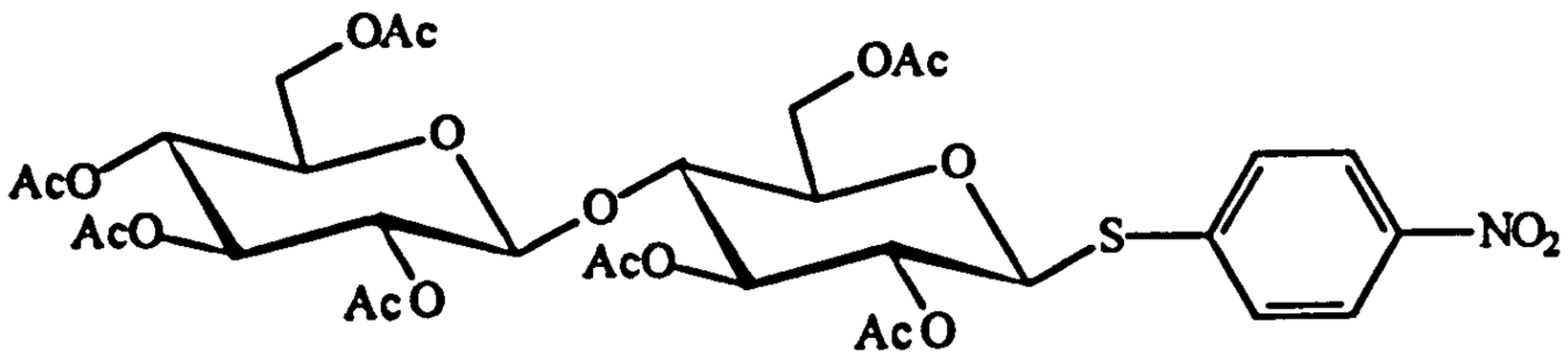
^{1}H nmr ($CDCl_3$, 270 MHz, ppm) : δ 7.46 (m, 2 H, Ar-H), 7.28 (m, 3 H, Ar-H), 5.29 (d, 1 H, OH-2, J 6 Hz), 5.10 (d, 1 H, OH-3, J 5 Hz), 4.99 (d, 1 H, OH-4, J 5 Hz), 4.61 (d, 1 H, H-1, J 10 Hz), 4.57 (t, 1 H, OH-6, J 6 Hz), 3.69 (m, 1 H, H-6), 3.44 (m, 1 H, H-6), 3.23 (m, 1 H, H-5), 3.20 (m, 1 H, H-3), 3.11 (m, 1 H, H-4), 3.04 (m, 1 H, H-2).

^{1}H nmr ($CDCl_3$+D_2O, 270 MHz, ppm) : δ 7.46 (m, 2 H, Ar-H), 7.28 (m, 3 H, Ar-H), 4.59 (d, 1 H, H-1, J 9 Hz), 3.67 (m, 1 H, H-6), 3.42 (m, 1 H, H-6), 3.23 (m, 2 H, H-5, H-4), 3.11 (t, 1 H, H-3, J 9 Hz), 3.04 (t, 1 H, H-2, J 9 Hz).

^{13}C nmr ($CDCl_3$, 270 MHz, ppm) : δ 135.09 (ArC-S), 129.51 (2 x ArC-H), 128.86 (2 x ArC-H), 126.30 (ArC-H), 87.06 (C-1), 80.98, 78.20, 72.40 (C-2, C-3, C-5), 69.76 (C-4), 61.01 (CH_2).

m/z (EI+, MeOH:H_2O (1:1), 40°C) : 839 ($[3M + Na]^+$), 567 ($[2M + Na]^+$), 295 ($[M + Na]^+$).

1.10. p-Nitrophenyl 2,3,6-tri-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-1-thio-β-D-glucopyranoside (9)



(9)

2,3,6-Tri-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-α-D-glucopyranosyl bromide **4** (4.1 g, 5.9 mmol) was added to a solution of p-nitrothiophenol (1.1 g, 7.1 mmol) in acetone (100 ml). The same procedure as described in part 1.6. was used. Compound 9 was obtained as white crystals by recrystallisation from chloroform and ether. Yield: 4.0 g (88 %).

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 1747 (C=O stretching vibration), 1596 and 1578 (aromatic C-C stretching vibrations, doublet due to conjugation with $-NO_2$), 1519 and 1345 (asym. and sym. NO_2 stretching), 1350 to 1200 (C-H deformations), 1230 (C-O stretching vibration of ester), 1120 to 1020 (C-O sym. stretching).

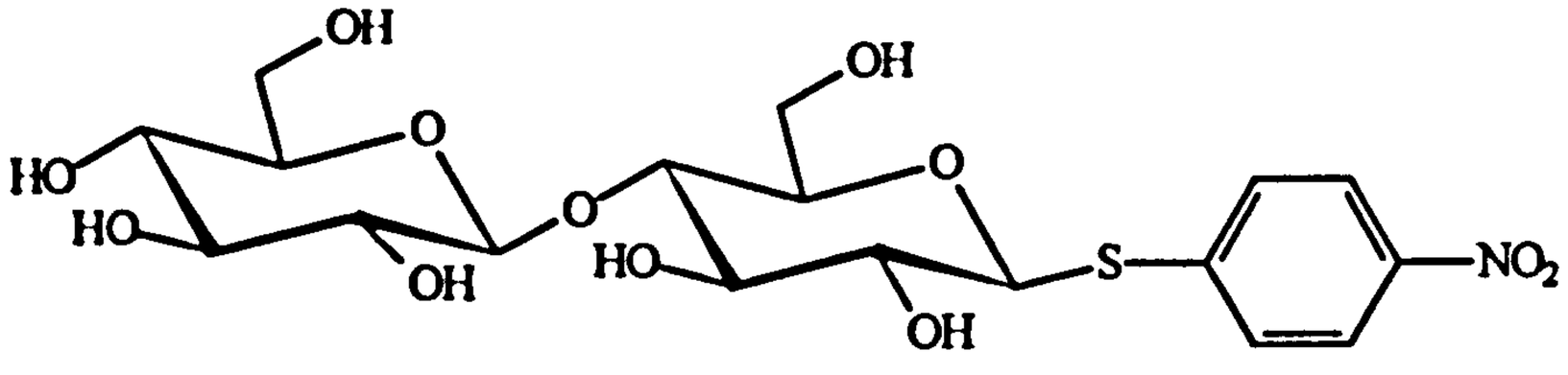
^{1}H nmr ($CDCl_3$, 270 MHz, ppm) : δ 8.18 (d, 2 H, Ar-CH, J 9 Hz), 7.55 (d, 2 H, Ar-CH, J 9 Hz), 5.22 (t, 1 H, H-3, J 10 Hz), 5.14 (t, 1 H, H-3', J 9 Hz), 5.06 (t, 1 H, H-4', J 9 Hz), 4.96 (t, 1 H, H-2, J 10 Hz), 4.92 (t, 1 H, H-2', J 8 Hz), 4.80 (d, 1 H, H-1, J 10 Hz), 4.56 (m, 1 H, H-6), 4.49 (d, 1 H, H-1', J 8 Hz), 4.37 (m, 1 H, H-6'), 4.10 (m, 1 H, H-6), 4.02 (m, 1 H, H-6'), 3.75 (t, 1 H, H-4, J 10 Hz), 3.72 (m, 1 H, H-5), 3.65 (m, 1 H, H-5'), 2.15, 2.07, 2.06, 2.03, 2.01, 2.00, 1.97 (7s, 21 H, 7 x CH_3).

^{13}C-nmr ($CDCl_3$, 270 MHz, ppm) : δ 170.37, 170.11, 170.03, 169.58, 169.39, 169.22, 168.98 (7 x C=O), 146.61 (ArC-NO_2), 141.81 (ArC-S), 130.74 (2 x ArC-H), 123.72 (2 x ArC-H), 100.70 (C-1'), 83.96 (C-1), 76.67, 76.03, 73.16, 72.71, 71.68,

71.44, 69.64, 67.54, 65.72 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 61.82 (CH_2), 61.36 (CH_2), 20.74, 20.55, 20.55, 20.42, 20.42, 20.35, 20.35 (7 x CH_3) .

m/z (EI+, MeOH:H_2O (1:1), 40°C) : 796 ($[M + Na]^+$).

1.11. p-Nitrophenyl 4-O-β-D-glucopyranosyl-1-thio-β-D-glucopyranoside (10)



(10)

Compound 9 (3.0 g, 3.88 mmol) was added to a solution of methanol (100 ml) containing a catalytic amount of sodium methoxide. The reaction was left overnight at room temperature and the solvent was then evaporated. Compound **10** was finally purified using silica gel chromatography (at first chloroform (100 %) then chloroform: methanol, 9.5 : 0.5, v/v). Yield: 1.67 g (90 %).

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 3450-3200 (O-H), 1592 and 1569 (aromatic C-C stretching vibrations, doublet due to conjugation with $-NO_2$), 1506 and 1345 ($C-NO_2$), 1460 to 1200 (O-H and C-H deformation), 1175 to 1020 (C-O stretching).

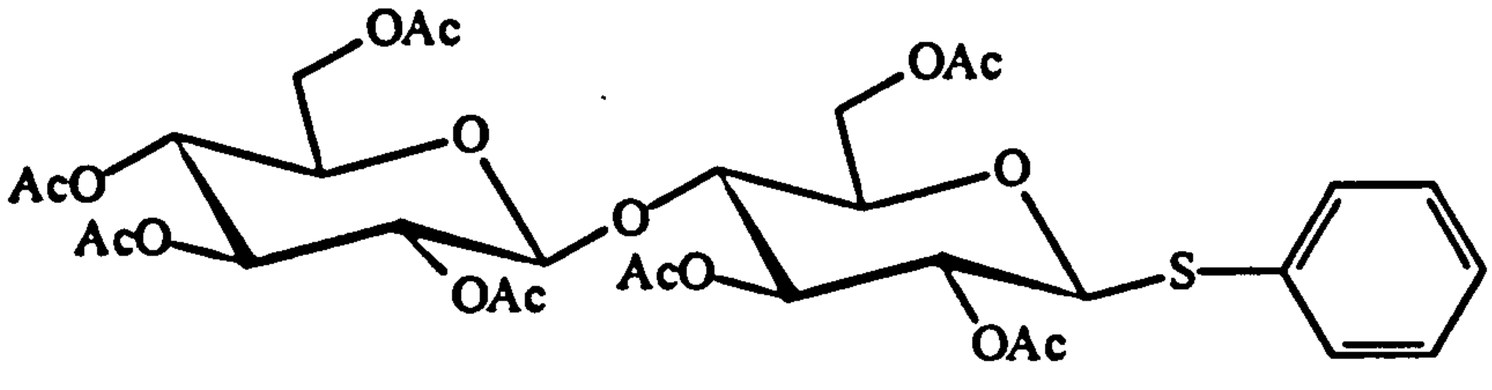
^{1}H nmr (D_6-DMSO, 270 MHz, ppm) : δ 8.13 (d, 2 H, Ar-H, J 9 Hz), 7.62 (d, 2 H, Ar-H, J 9 Hz), 5.1 (bs, OHs), 5.02 (d, 1 H, H-1, J 10 Hz), 4.7 (bs, OHs), 4.29 (d, 1 H, H-1', J 8 Hz), 3.66 (m, Hs), 3.45 (m, Hs, H-3, H-4), 3.23 (m, H-2, H-3'), 3.05 (m, 2 Hs, H-2').

^{1}H nmr (D_6-DMSO+D_2O, 270 MHz, ppm) : δ 8.12 (d, 2 H, Ar-H, J 9 Hz), 7.58 (d, 2 H, Ar-H, J 9 Hz), 4.97 (d, 1 H, H-1, J 10 Hz), 4.28 (d, 1 H, H-1', J 8 Hz), 3.66 (m, Hs), 3.45 (m, H), 3.23 (m, H), 3.05 (m, 2 H, H).

^{13}C nmr (D_6-DMSO, 270 MHz, ppm) : δ 145.76 ($ArC-NO_2$), 144.97 (ArC-S), 127.68 (2 x ArC-H), 123.81 (2 x ArC-H), 103.09 (C-1'), 84.56 (C-1), 79.63, 78.90, 76.85, 76.48, 76.31, 73.30. 72.20, 70.05 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 61.07, 60.06 (2 x CH_2).

m/z (EI+, MeOH:H_2O (7:3), 40°C) : 502 ($[M + Na]^+$).

1.12. Phenyl 2,3,6-tri-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-1-thio-β-D-glucopyranoside (11)



(11)

2,3,6-Tri-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-α-D-glucopyranosyl bromide **4** (4.0 g, 5.7 mmol) was added to a solution of sodium thiophenolate (0.93 g, 7.0 mmol) in anhydrous acetone (150 ml). The mixture was refluxed for 150 min. The acetone was then evaporated and the crude product was diluted with chloroform (30 ml). The organic phase was then washed successively with water and a saturated solution of sodium chloride (15 ml x 2). The organic phase was dried over anhydrous magnesium sulphate and filtered. After evaporation of the solvent, the product was purified using column chromatography (ethyl acetate : hexane, 1:1, v/v). Compound **11** was obtained as white crystals. Yield : 3.30 g (79 %), m. pt. : 214°C.

IR-FT (nujol mull, cm^{-1}) : υ_{max} 1742 (C=O stretching vibration), 1350 to 1200 (C-H deformations), 1232 (C-O-C stretching vibration of ester), 1120 to 1020 (C-O sym. stretching).

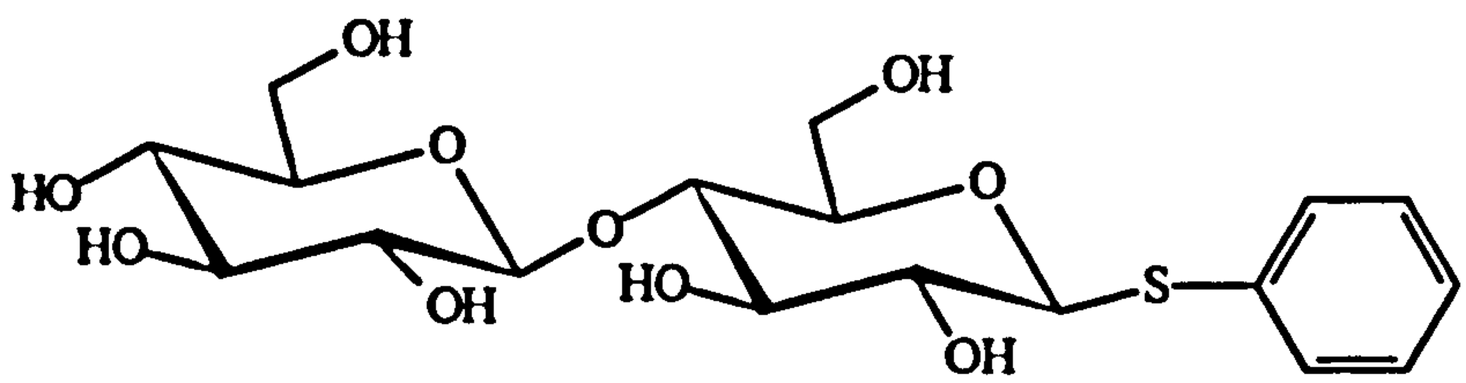
^{1}H nmr ($CDCl_3$, 270 MHz, ppm) : δ 7.42 (m, 2 H, Ar-H), 7.25 (m, 3 H, Ar-H), 5.17 (t, 1 H, H-3, J 9 Hz), 5.11 (t, 1 H, H-3', J 9 Hz), 5.03 (t, 1 H, H-4', J 9 Hz), 4.90 (t, 1 H, H-2', J 8 Hz), 4.86 (t, 1 H, H-2, J 9 Hz), 4.64 (d, 1 H, H-1, J 10 Hz), 4.53 (m, 1 H, H-6), 4.47 (d, 1 H, H-1', J 8 Hz), 4.34 (m, 1 H, H-6'), 4.08 (m, 1 H, H-6), 3.99 (m, 1 H, H-6'), 3.70 (t, 1 H, H-4, J 9 Hz), 3.61 (m, 2 H, H-5', H-5), 2.02 (7s, 21 H, CH_3).

^{13}C nmr ($CDCl_3$, 270 MHz, ppm) : δ 170.47, 170.21, 1702.21, 169.74, 169.53, 169.29, 169.02 (7 x C=O), 133.03 (2 x ArC-H), 131.70 (ArC-S), 128.87 (2 x ArC-H), 128.30 (ArC-H), 100.74 (C-1'), 85.47 (C-1), 76.32, 73.54, 72.87, 71.92, 71.53,

70.09, 67.67 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 61.96, 61.47 (2 x CH_2), 20.83, 20.76, 20.64, 20.53, 20.53, 20.53, 20.53 (7 x CH_3) .

m/z (EI+, ACN, 40°C) : 751 ($[M + Na]^+$).

1.13. Phenyl 4-O-β-D-glucopyranosyl-1-thio-β-D-glucopyranoside (12)



(12)

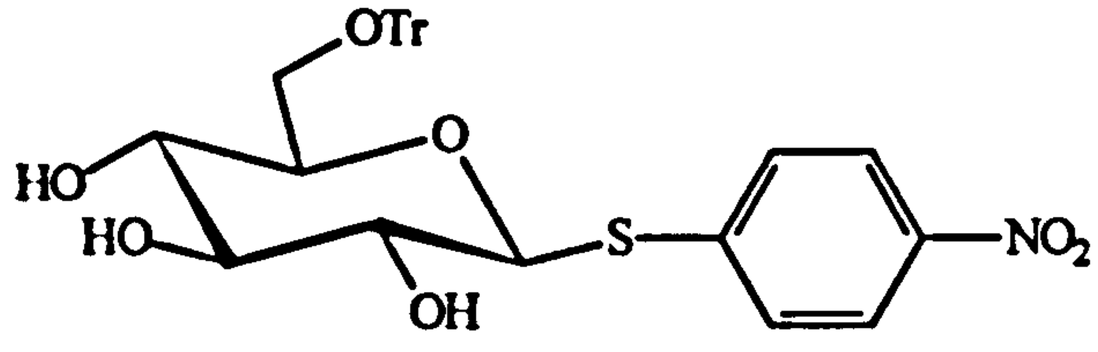
Compound **12** was obtained from the deacetylation of **11** (3.0 g, 4.1 mmol) following the same procedure as described in 1.7. Yield: 1.09 g (80 %).

^{1}H nmr ($CDCl_3$, 270 MHz, ppm) : δ 7.46 (m, 2 H, Ar-H), 7.25 (m, 3 H, Ar-H), 5.50 (d, 1 H, OH-2, J 6 Hz), 5.27 (bs, 1 H, OH-2'), 5.05 (bs, 2 H, OHs), 4.82 (s, 1 H, OH), 4.68 (d, 1 H, H-1, J 10 Hz), 4.67 (m, 2 H, OHs), 4.28 (d, 1 H, H-1', J 8 Hz), 3.72 (m, 3 H, Hs), 3.10 (m, 6 H, Hs).

^{1}H nmr ($CDCl_3$+D_2O, 270 MHz, ppm): δ 7.31 (m, 2 H, Ar-H), 7.16 (m, 3 H, Ar-H), 4.52 (d, 1 H, H-1, J 10 Hz), 4.15 (d, 1 H, H-1', J 8 Hz), 3.72 (m, 3 H, Hs), 3.10 (m, 6 H, Hs).

^{13}C nmr ($CDCl_3$, ppm) : δ 134.52 (ArC-S), 130.07 (2 x ArC-H), 129.00 (2 x ArC-H), 126.67 (ArC-H), 103.12 (C-1'), 86.64 (C-1), 79.88, 78.90, 76.67, 76.54, 76.43, 73.38, 72.23, 70.12 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 61.14, 60.37 (2 x CH_2).

1.14. p-Nitrophenyl 6-O-trityl-1-thio-β-D-glucopyranoside (13)



(13)

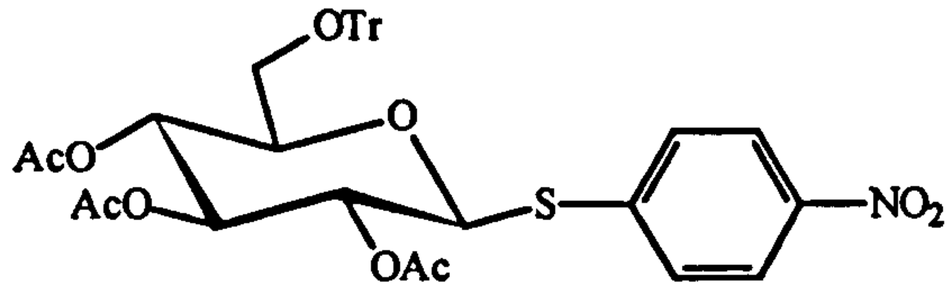
p-Nitrophenyl 1-thio-β-D-glucopyranoside (6.0 g, 18.9 mmol) and triphenylmethyl-chloride (7.9 g, 28.0 mmol) were dissolved in dry dimethylformamide (100 ml). A catalytic amount of DMAP was added to the solution. The reaction was then stirred at room temperature for 12 hours under a nitrogen atmosphere. The compound precipitated out of the solution. It was then filtered and redissolved in chloroform (100 ml). The organic phase was successively washed with water (50 ml x 2) and a saturated solution of sodium chloride (50 ml x 2). The solution was dried over anhydrous sodium sulphate and the chloroform was removed under reduced pressure (< 40°C). Compound **13** was finally purified using silica gel column chromatography (methanol : chloroform, 20 : 80, v/v). Yield: 9.12 g (86 %), m. pt. 85°C.

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 3450-3100 (O-H), 1597 and 1577 (aromatic C-C stretching vibrations, doublet due to conjugation with $-NO_2$), 1490 and 1340 (asym. and sym. NO_2 stretching), 1460 to 1200 (O-H and C-H deformations), 1120 to 1020 (C-O sym. stretching).

^{1}H nmr ($CDCl_3$, 270 MHz, ppm) : δ 8.10 (d, 2 H, Ar-H, J 9 Hz), 7.80 (d, 2 H, Ar-H, J 9 Hz), 7.39 (m, 6 H, Ar-H), 7.25 (m, 9 H, Ar-H), 5.66 (d, 1 H, OH, J 6 Hz), 5.30 (d, 1 H, OH, J 5 Hz), 5.16 (d, 1 H, H-1, J 9 Hz), 5.11 (d, 1 H, OH, J 5 Hz), 3.29 (m, 3 Hs), 3.06 (m, 2 Hs).

^{1}H nmr (d_6-DMSO + D_2O shake, 270 MHz, ppm) : δ 8.05 (d, 2 H, Ar-H, J 9 Hz), 7.75 (d, 2 H, Ar-H, J 9 Hz), 7.24 (m, 6 H, Ar-H), 7.09 (m, 9 H, Ar-H), 5.10 (d, 1 H, H-1, J 9 Hz), 3.31 (m, 3 Hs), 3.04 (m, 2 Hs).

1.15. p-Nitrophenyl 2,3,4-tri-O-acetyl-6-O-triphenylmethyl-1-thio-β-D-glucopyranoside (14)



(14)

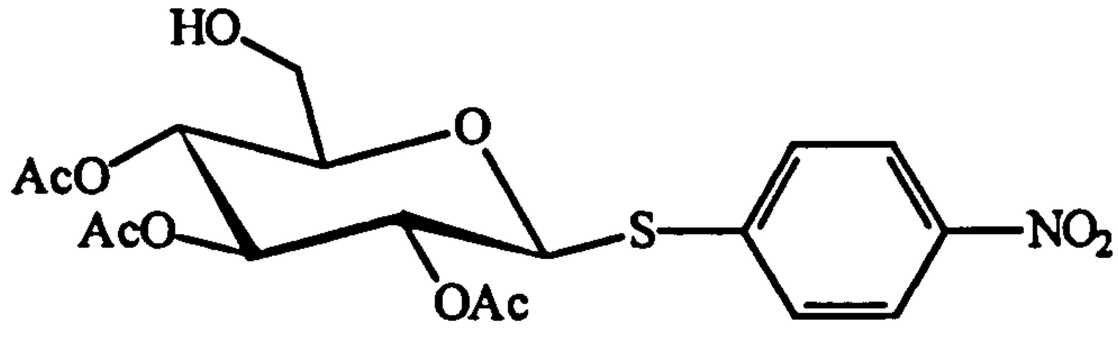
p-Nitrophenyl 6-O-trityl-1-thio-β-D-glucopyranoside (8.5 g, 15.2 mmol) and a catalytic amount of DMAP were dissolved in a mixture of pyridine (20 ml) and acetic anhydride (7.15 ml). The solution was stirred for 24 hours at room temperature under a nitrogen atmosphere. The mixture was then poured into a beaker of crushed ice-water (500 ml) for 1 hour. The precipitate formed was filtered off and washed thoroughly with cold water. The precipitate was then redissolved in chloroform (30 ml), washed with a saturated solution of sodium chloride (15 ml x 2) and dried over anhydrous sodium sulphate. The solvent was removed under reduced pressure and compound **14** was recrystallised from ethyl acetate. Yield: 7.32 g, (70 %), m. pt. : 180-181°C.

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 1746 (C=O stretching vibration), 1598 and 1584 (aromatic C-C stretching vibrations, doublet due to conjugation with -NO_2), 1520 and 1343 (asym. and sym. NO_2 stretching), 1211 (C-O-C stretching vibration of ester), 1120 to 1020 (C-O sym. stretching).

^{1}H-nmr ($CDCl_3$, 270 MHz, ppm) : δ 8.07 (d, 2 H, Ar-H, J 9 Hz), 7.69 (d, 2 H, Ar-H, J 9 Hz), 7.41 (m, 6 H, Tr), 7.27 (m, 9 H, Tr), 5.22 (t, 1 H, H-3, J 10 Hz), 5.07 (t, 1 H, H-2, J 10 Hz), 5.05 (t, 1 H, H-4, J 10 Hz), 4.86 (d, 1 H, H-1, J 10 Hz), 3.65 (m, 1 H, H-5), 3.25 (m, 2 H, CH_2), 2.07, 1.97, 1.74 (3s, 9 H, 3 x CH_3).

^{13}C-nmr ($CDCl_3$, 270 MHz, ppm) : δ 170.15, 169.32, 169.00 (3 x C=O), 146.80 (ArC-NO_2), 143.32 (3 x ArC, Tr), 142.44 (ArC-S), 130.57 (2 x ArC-H), 128.61 (6 x ArC-H), 127.88 (6 x ArC-H), 127.26 (3 x ArC-H), 124.00 (2 x ArC-H), 87.03 (C-Ph_3), 84.35 (C-1), 77.87, 73.84, 69.72, 68.20 (C-2, C-3, C-4, C-5), 62.11 (CH_2), 20.68, 20.58, 20.33 (3 x CH_3).

1.16. p-Nitrophenyl 2,3,4-tri-O-acetyl-1-thio-β-D-glucopyranoside (15)



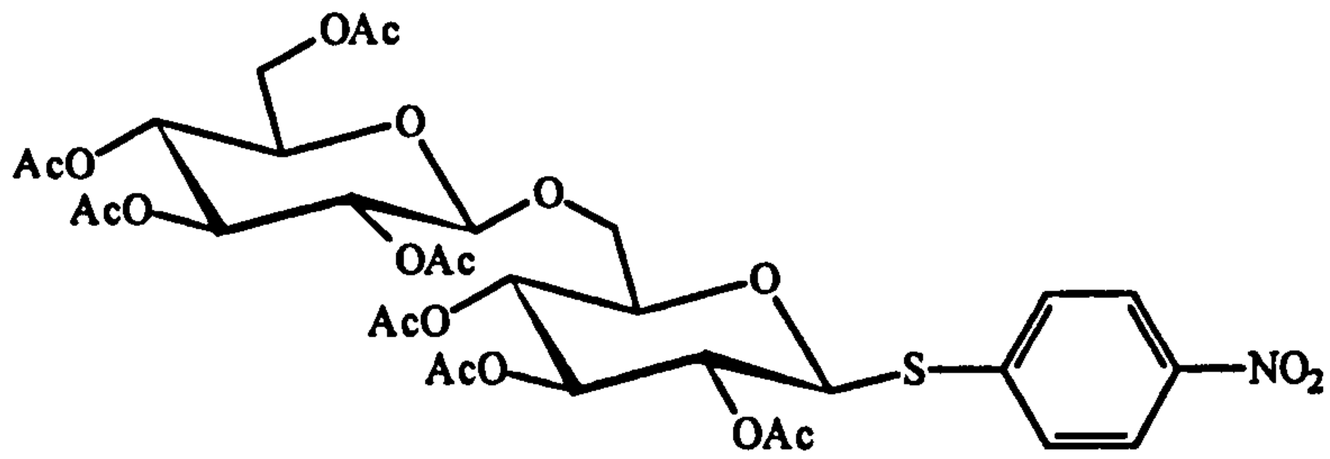
(15)

p-Nitrophenyl 2,3,4-tri-O-acetyl-6-O-trityl-1-thio-β-D-glucopyranoside (5.43 g, 7.9 mmol) was dissolved in a mixture of diethyl ether and formic acid (250 ml, 1:1, v/v) and stirred for 30 minutes at room temperature. The solution was then diluted with diethyl ether (200 ml) and washed successively with water (50 ml x 3), a saturated solution of sodium bicarbonate (50 ml x 3) and a saturated solution of sodium chloride (50 ml x 3). The solution was dried over anhydrous sodium sulphate, filtered and the diethyl ether was evaporated under reduced pressure. Compound **15** was finally dissolved in chloroform and obtained by titration with petroleum ether. Yield: 2.42 g (69 %), m. pt. : 132-134°C.

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 3400 (O-H), 1752 (C=O stretching vibration), 1598 and 1578 (aromatic C-C stretching vibrations, doublet due to conjugation with -NO_2), 1520 and 1340 (asym. and sym. NO_2 stretching), 1211 (C-O-C stretching vibration of ester), 1120 to 1020 (C-O sym. stretching).

^{1}H-nmr ($CDCl_3$ + D_2O shake, 270 MHz, ppm) : δ 8.14 (d, 2 H, Ar-H, J 9 Hz), 7.54 (d, 2 H, Ar-H, J 9 Hz), 5.31 (t, 1 H, H-3, J 10 Hz), 5.03 (t, 1 H, H-2, J 10 Hz), 5.02 (t, 1 H, H-4, J 10 Hz), 4.90 (d, 1 H, H-1, J 10 Hz), 3.77 (m, 1 H, H-5), 3.62 (m, 2 H, CH_2), 2.06, 2.05, 2.00 (3s, 9 H, 3 x CH_3).

1.17. p-Nitrophenyl 2,3,4-tri-O-acetyl-6-O-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-1-thio-β-D-glucopyranoside (16)



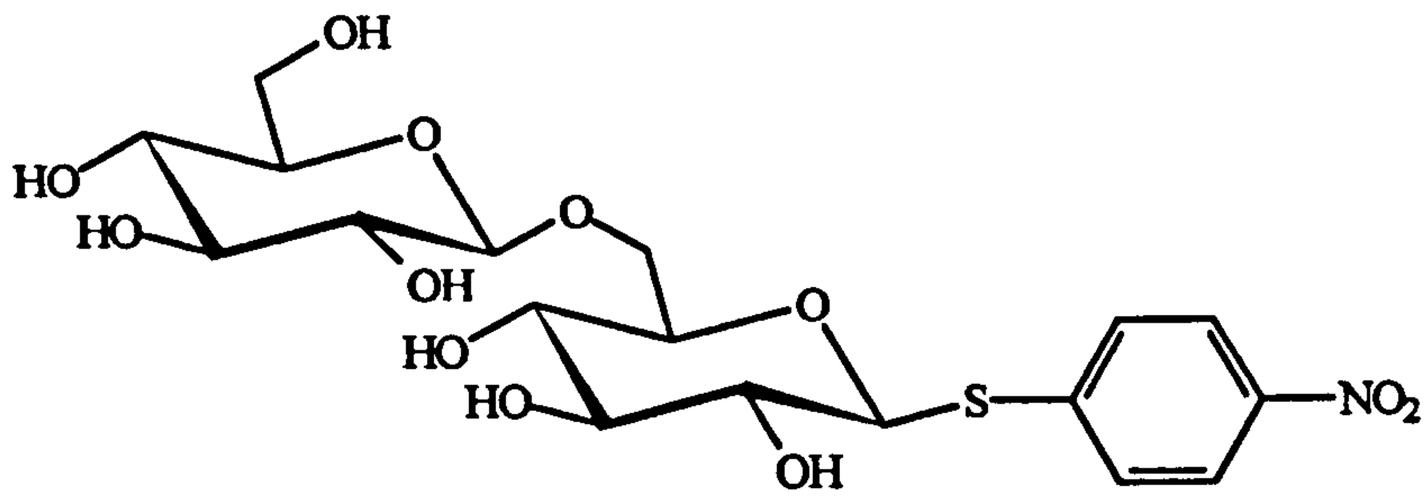
(16)

p-Nitrophenyl 2,3,4-tri-O-acetyl-β-D-glucopyranoside (1.54 g, 3.5 mmol) was dissolved in a dry solution of chloroform containing 4A molecular sieves. Silver carbonate (2.40 g, 8.7 mmol) and a catalytic amount of silver perchlorate were added to the solution. The mixture was stirred under a nitrogen atmosphere for 15 minutes. 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide was then slowly added to the solution. The solution was stirred at room temperature for a further 12 hours. Finally, the mixture was filtered, washed successively with water (2 x 50 ml) and a saturated solution of sodium chloride (2 x 50 ml), dried over anhydrous sodium sulphate and filtered. Compound **16** was finally purified by silica gel column chromatography (diethyl ether : chloroform, 1 : 1, v/v). Yield: 0.48 g (18 %), m. pt. : 175-177°C.

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 1743 (C=O stretching vibration), 1598 and 1578 (aromatic C-C stretching vibrations, doublet due to conjugation with $-NO_2$), 1522 and 1340 (asym. and sym. NO_2 stretching), 1211 (C-O-C stretching vibration of ester), 1120 to 1020 (C-O sym. stretching).

No nmr analysis.

1.18. p-Nitrophenyl 6-O-β-D-glucopyranosyl-1-thio-β-D-glucopyranoside (17)

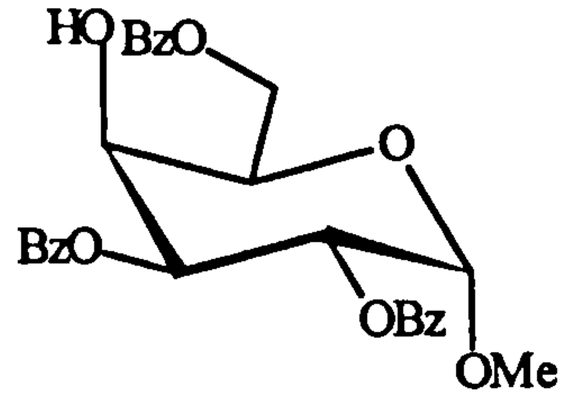


(17)

p-Nitrophenyl 2,3,4-tri-O-acetyl-6-O-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-1-thio-β-D-glucopyranoside (30 mg, 0.039 mmol) was dissolved in dry methanol (20 ml) containing a catalytic amount of sodium hydroxide. Compound **17** was obtained as a pale yellow powder. Yield : 17.60 mg (96 %), m.pt. : 75°C.

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 3278 (O-H stretching vibration, broad), 1595 and 1578 (aromatic C-C stretching vibrations, doublet due to conjugation with $-NO_2$), 1508 and 1340 (asym. and sym. NO_2 stretching), 1140 to 1020 (C-O sym. stretching).

1.19. Methyl 2,3,6-tri-O-benzoyl-α-D-galactopyranoside (18)



(18)

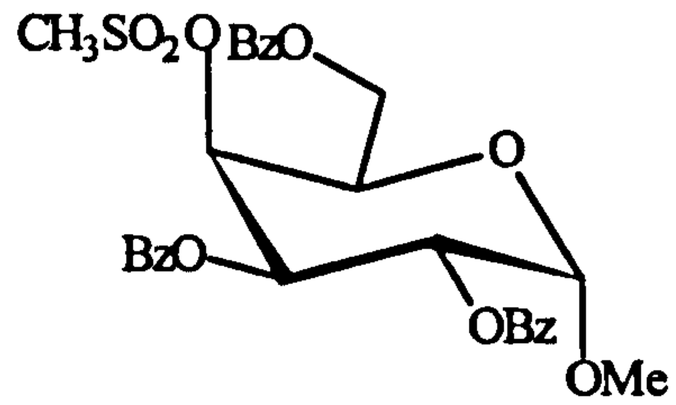
Methyl α-D-galactopyranoside monohydrate (5.0 g, 23.6 mmol) was dissolved in dry pyridine (150 ml) in a round bottomed flask. The solution was then cooled to -30°C. Benzoyl chloride (12 ml, 103.4 mmol) was added dropwise (20 minutes with exclusion of moisture). The solution was maintained at -30°C for two hours and was then allowed to reach room temperature. After stirring for a further 48 hours, the pyridine was evaporated under reduced pressure and the residue was dissolved in chloroform (30 ml). The solution was washed with dilute hydrochloric acid (2 M) (10 ml x 3) to remove the excess of pyridine, a solution of saturated aqueous sodium bicarbonate (15 ml x 2), water (10 ml x 3), and was then dried over anhydrous sodium sulphate. The salt was filtered off and removal of the solvent gave a white foamy compound. Tlc (chloroform : methanol, 24:1, v/v) indicated that at least five compounds were present. The major component was recovered by recrystallisation from absolute ethanol and purified further by a second recrystallisation. Yield: 5.17 g (52 %), m. pt. : 139°C. (Lit.[82] m.pt. : 139-140°C).

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 3500 (-OH), 1724 (C=O), 1602 and 1585 (aromatic C-C stretching vibrations, doublet due to conjugation with C=O), 1271 and 1113 (asym. and sym. C-O-C stretching vibrations of benzoates), 1350-1200 (C-H deformations), 1175 to 1020 (C-O sym. stretching).

^{1}H nmr ($CDCl_3$, 270 MHz, ppm) : δ 7.95 (m, 6 H, Ar-H), 7.45 (m, 9 H, Ar-H), 5.72 (dd, 1 H, H-3, J 3 Hz, J 11 Hz), 5.66 (dd, 1 H, H-2, J 3 Hz, J 11 Hz), 5.17 (d, 1 H, H-1, J 3 Hz), 4.81 (bs, 1 H, OH), 4.63 (dd, 1 H, H-6, J 6 Hz, J 11 Hz), 4.53 (dd, 1 H, H-5, J 7 Hz, J 11 Hz), 4.37 (m, 1 H, H-4), 4.31 (m, 1 H, H-6), 3.41 (s, 3 H, CH_3).

^{13}C nmr ($CDCl_3$, 270 MHz, ppm) : δ 166.46, 166.07, 165.74 (3 x C=O), 133.35, 133.23, 133.23 (3 x ArC-H), 129.80, 129.74, 129.65 (6 x ArC-H), 129.27 (3 x ArC), 128.42, 128.42, 128.34 (6 x ArC-H), 97.46 (C-1), 70.77, 68.66, 68.13, 67.68 (C-2, C-3, C-4, C-5), 63.42 (CH_2), 55.44 (CH_3O-).

1.20. Methyl 2,3,6-tri-O-benzoyl-4-O-methylsulphonyl-α-D-galactopyranoside (19)



(19)

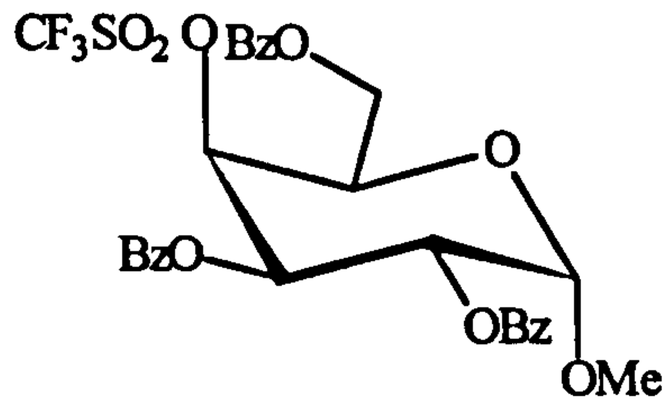
Methanesulphonyl chloride (1.1 ml, 14.2 mmol) was added to a solution of methyl 2,3,6-tri-O-benzoyl-α-D-galactopyranoside (2.19 g, 4.3 mmol) in dry pyridine (50 ml). The solution was kept at room temperature for 18 hours. The excess of methanesulphonyl chloride was decomposed by the addition of water (0.2 ml). The resulting reaction mixture was added to chloroform (26 ml) and the organic phase was washed with water (15 ml x 2) and a saturated aqueous solution of sodium bicarbonate (10 ml x 3). The solution was then dried with anhydrous sodium sulphate and the salt was filtered off. A brown syrup was obtained after evaporation of the solvent. Compound **19** was purified by column chromatography (chloroform : methanol, 50:1, v/v). Yield: 1.76 g (70 %), m. pt. : 142°C. (Lit.[83] m.pt. : 141-142°C).

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 1723 (C=O), 1601 and 1584 (aromatic C-C stretching vibrations, doublet due to conjugation with C=O), 1365 and 1177 ($-SO_2-O$), 1270 and 1111 (asym. and sym. C-O-C stretching vibrations of benzoates), 1350-1200 (C-H deformations), 1175 to 1020 (C-O sym. stretching), 781 and 711 (monosubstituted benzene ring).

^{1}H nmr ($CDCl_3$, 270 MHz, ppm) : δ 8.01 (m, 6 H, Ar-H), 7.46 (m, 9 H, Ar-H), 5.86 (dd, 1 H, H-3, J 3 Hz, J 11 Hz), 5.63 (dd, 1 H, H-2, J 3 Hz, J 11 Hz), 5.50 (bd, 1 H, H-4, J 3 Hz), 5.23 (d, 1 H, H-1, J 3 Hz), 4.68 (dd, 1 H, $H-6_a$, J 6 Hz, J 11 Hz), 4.53 (m, 1 H, H-5), 4.46 (dd, 1 H, $H-6_b$, J 7 Hz, J 11 Hz), 3.47 (s, 3 H, CH_3O-), 3.09 (s, 3 H, CH_3SO_2-).

^{13}C nmr ($CDCl_3$, 270 MHz, ppm) : δ 165.96, 165.96, 165.72 (3 x C=O), 133.51, 133.51, 133.39 (3 x ArC-H), 129.85 (4 x ArC-H), 129.78 (2 x ArC-H), 129.36 (ArC), 129.02 (2 x ArC), 128.54 (4 x ArC-H), 128.48 (2 x ArC-H), 97.53 (C-1), 76.30, 68.38, 68.14, 66.33 (C-2, C-3, C-4, C-5), 62.07 (CH_2), 55.65 (CH_3O-), 38.93 (CH_3SO_2-).

1.21. Methyl 2,3,6-tri-O-benzoyl-4-O-trifluoromethane-sulphonyl-α-D-galactopyranoside (20)



(20)

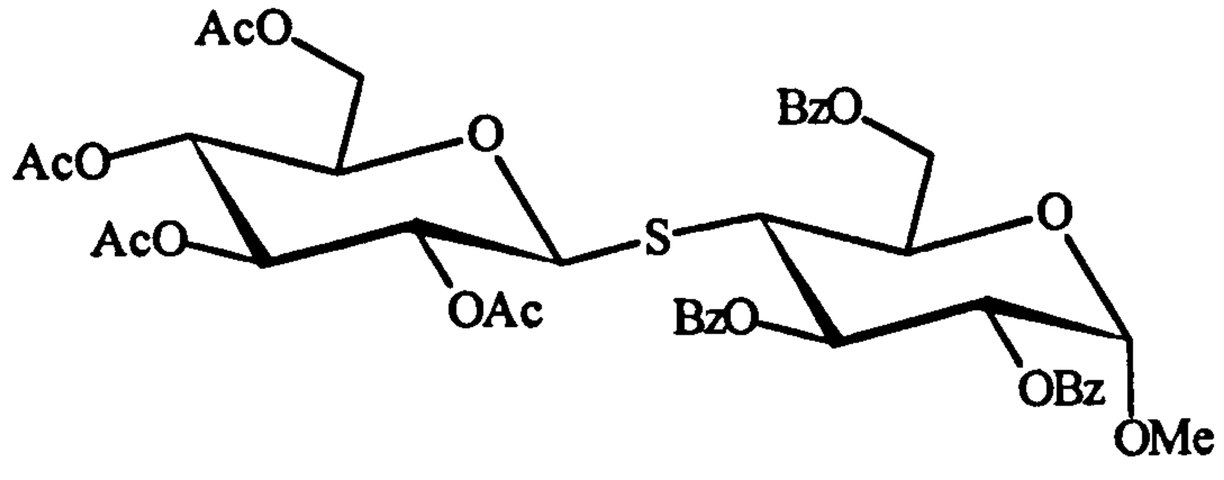
Trifluoromethanesulphonic anhydride (3.7 ml, 22 mmol) was added to a solution of methyl 2,3,6-tri-O-benzoyl-α-D-galactopyranoside (4.60 g, 9.0 mmol) in dry dichloromethane-pyridine (2.5:1, v/v, 35 ml), cooled to 0°C. The solution was kept at 0°C for 30 minutes and was then allowed to warm up to room temperature. After 90 minutes, t.l.c. on silica showed complete conversion of the starting material into the less polar triflate ester. The mixture was dissolved in chloroform (90 ml) and washed with a ice cold solution of potassium hydrogen sulphate (10% w/v, 90 ml x 2), a saturated aqueous solution of bicarbonate (30 ml x 3) and with water (30 ml x 3). The solution was then dried with anhydrous sodium sulphate and the salt was filtered off. A brown syrup was obtained after evaporation of the solvent. Compound **20** was purified by column chromatography (chloroform : methanol 49:1). Yield: 5.63 g (97 %), m. pt. : 137°C. (Lit.[84] m.pt. : 137-138°C).

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 1730 (C=O), 1601 and 1583 (aromatic C-C stretching vibrations, doublet due to conjugation with C=O), 1415 and 1213 (-SO_2-O-), 1288, 1264 (CF_3-), 1272 and 1108 (asym. and sym. C-O-C stretching vibrations of benzoates), 1350-1200 (C-H deformations), 1175 to 1020 (C-O sym. stretching), 713 (monosubstituted benzene ring).

^{1}H nmr ($CDCl_3$, 270 MHz, ppm) : δ 8.01 (m, 6 H, Ar-H), 7.46 (m, 9 H, Ar-H), 5.94 (dd, 1 H, H-3, J 3 Hz, J 11 Hz), 5.58 (m, 2 H, H-4, H-2), 5.28 (d, 1 H, H-1, J 3 Hz), 4.68 (dd, 1 H, H-6, J 7 Hz, J 11 Hz), 4.58 (t, 1 H, H-5, J 7 Hz), 4.34 (dd, 1 H, H-6, J 7 Hz, J 11 Hz), 3.46 (s, 3 H, CH_3O-).

^{13}C nmr ($CDCl_3$, 270 MHz, ppm) : δ 165.79, 165.63, 165.63 (3 x C=O), 133.74 (ArC-H), 133.52 (ArC-H), 133.52 (ArC-H), 130.06 (2 x ArC-H), 129.82 (2 x ArC-H), 129.67 (2 x ArC-H) , 128.99, 128.82, 128.82 (3 x ArC), 128.82 (2 x ArC-H), 128.46 (2 x ArC-H), 128.30 (2 x ArC-H), 97.36 (C-1), 82.85 (C-4), 68.17, 67.39, 65.72 (C-2, C-3, C-5), 61.35 (CH_2), 55.99 (CH_3O).

1.22. Methyl 2,3,6-tri-O-benzoyl-4-S-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-4-thio-α-D-glucopyranoside (21)



(21)

Method 1

The monohydrate of the sodium salt of 1-thio-β-D-glucopyranose (0.66 g, 2.8 mmol) was added to a solution of N', N'', N'''-hexamethylphosphoramide (10 ml) containing methyl 2,3,6-tri-O-benzoyl-4-O-methanesulphonyl-α-D-galactopyranoside (1.72 g, 2.9 mmol). After 21 hours stirring at room temperature, acetic anhydride-pyridine (2:1, v/v, 30 ml) and a catalytic amount of DMAP were added and the solution was left for 48 hours. The solvent was evaporated, and dichloromethane (20 ml) was added. This mixture was then successively washed with an ice cold aqueous solution of 10 % potassium hydrogen sulphate (10 ml x 3), saturated sodium bicarbonate (10 ml x 3), and with ice cold water (10 ml x 3). The aqueous washings were then re-extracted with dichloromethane. The organic solution was dried over anhydrous sodium sulphate and evaporated under reduced pressure to give a dark solid which was purified by silica gel column chromatography (chloroform : ethyl acetate, 9:1, v/v). Tlc showed that the compound was not pure, recrystallisation from ethyl acetate/hexane and chloroform/hexane gave an oil. No product was isolated from this method.

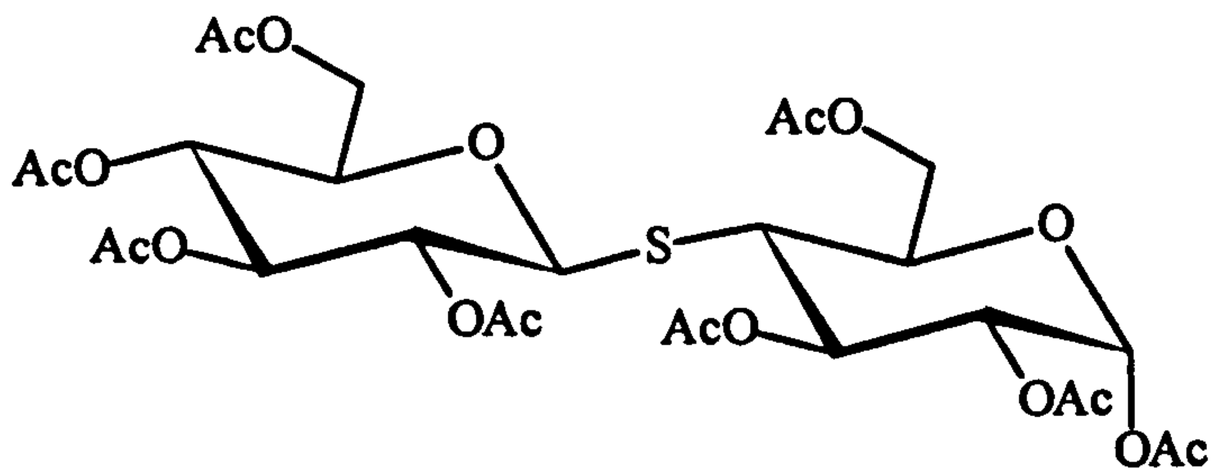
Method 2

The hydrated sodium salt of 1-thio-β-D-glucose (0.96 g, 4.1 mmol) was added to a solution of N', N'', N'''-hexamethylphosphoramide (16 ml) containing methyl 2,3,6-tri-O-benzoyl-4-O-trifluoromethylsulphonyl-α-D-galactopyranoside (2.5 g, 3.9 mmol). After 4 hours stirring at room temperature, a mixture of acetic anhydride and pyridine (1:1, v/v, 13 ml) was added and the solution was left to stand overnight. After diluting with dichloromethane, the organic phase was successively washed with an ice cold aqueous solution of 10% potassium hydrogen sulphate, saturated sodium bicarbonate, and with ice cold water. The aqueous washings were then re-extracted with dichloromethane. The organic solution was dried over anhydrous sodium sulphate and evaporated under reduced pressure. The solid was purified by column chromatography (ethyl acetate : hexane : dichloromethane, 5:5:1, v/v). Yield: 2.21 g (66 %), m. pt. : 188-189°C. (Lit.[85] m.pt. : 185-188°C).

^{1}H-nmr ($CDCl_3$, 270 MHz, ppm) : δ 8.02 (m, 6 H, Ar-H), 7.42 (m, 9 H, Ar-H), 5.99 (dd, 1 H, H-3, J 10 Hz, J 11 Hz), 5.23 (dd, 1 H, H-2, J 4 Hz, J 10 Hz), 5.19 (t, 1 H, H-3', J 9 Hz), 5.17 (d, 1 H, H-1, J 4 Hz), 5.00 (t, 1 H, H-4', J 9 Hz), 4.96 (d, 1 H, H-1', J 10 Hz), 4.90 (dd, 1 H, H-2', J 8 Hz, J 10 Hz), 4.81 (m, 2 H, CH_2), 4.42 (m, 1 H, H-5), 4.12 (m, 2 H, CH_2'), 3.73 (m, 1 H, H-5'), 3.45 (s, 3 H, CH_3O-), 3.29 (t, 1 H, H-4, J 11 Hz), 2.0 (4s, 3 H, CH_3CO-).

^{13}C-nmr ($CDCl_3$, 270 MHz, ppm) : δ 170.45, 170.00, 169.32, 169.32 (Ac, 4 x C=O), 166.06, 165.78, 165.58 (Bz, 3 x C=O), 133.36 (ArC-H), 133.36 (ArC-H), 133.21 (ArC-H), 129.88 (2 x ArC-H), 129.80 (2 x ArC-H), 129.62 (2 x ArC-H), 129.16 (ArC-), 128.96 (ArC-), 128.51 (2 x ArC-H), 128.42 (2 x ArC-H), 128.36 (2 x ArC-H), 97.23 (C-1), 81.40 (C-1'), 75.44 (C-5'), 73.70 (C-3'), 73.26 (C-2), 69.71 (C-2'), 69.37 (C-5), 68.07 (C-4'), 67.42 (C-3), 63.91 (CH_2), 61.61 (CH_2'), 55.66 (CH_3O-), 46.39 (C-4), 20.65, 20.55, 20.55, 19.96 (Ac, 4 x CH_3).

1.23. 1,2,3,6-Tetra-O-acetyl-4-S-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-4-thio-α-D-glucopyranoside (22)

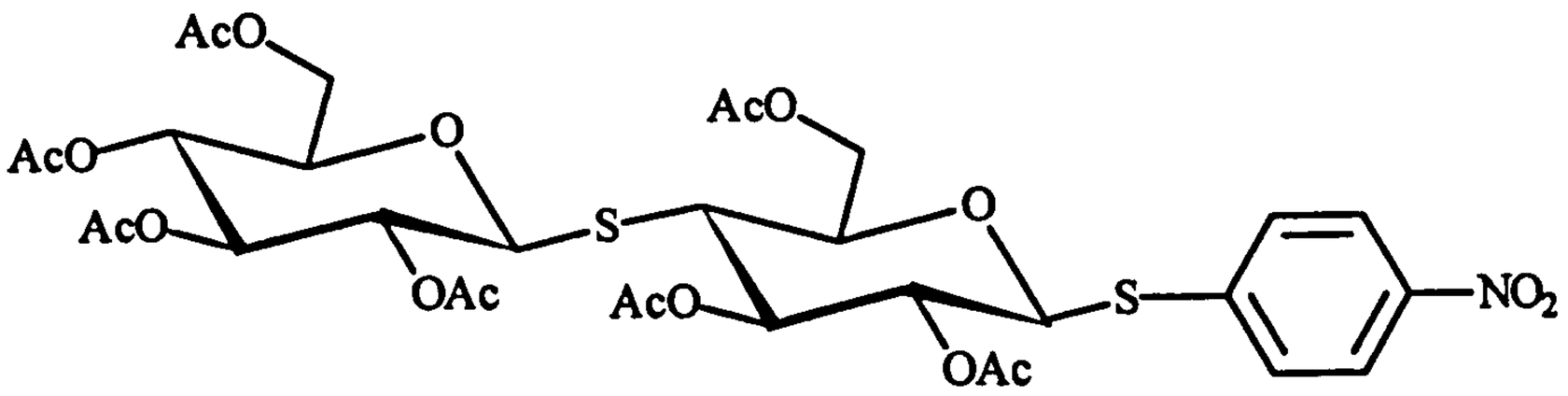


(22)

Compound **21** (1.60 g, 1.88 mmol) was dissolved in methanol (100 ml) and treated with a solution of sodium methoxide in methanol (1 M, 2.3 ml). After neutralisation with Amberlite IR-120(H+) cation exchange resin, filtration, and concentration to dryness, the residue was acetylated at room temperature with a mixture of pyridine and acetic anhydride (1:1, v/v, 30 ml) for 36 hours. After reaction, the solvent was evaporated under reduced pressure and the solid re-dissolved in dichloromethane. Successive washings with ice-cold aqueous solutions of 10 % potassium hydrogen sulphate (10 ml x 3), saturated sodium bicarbonate (10 ml x 3), and with ice-cold water gave a crude compound which was then treated with 7:300:700 (v/v) H_2SO_4-acetic acid-acetic anhydride (147 ml) for 24 hours at room temperature. After coevaporation with toluene in the presence of sodium acetate and the same previous work up, column chromatography (ethyl acetate-hexane, 3:2, v/v) of the mixture gave **22**, contaminated with its β-anomer. Yield: 1.05 g (80 %), m. pt. : 168°C. (Lit.[85] m.pt.: 175-177°C).

^{1}H-nmr ($CDCl_3$, 270 MHz, ppm) : δ 6.4 and 5.6 (d, 1 H, H-1, α- and β-anomer, 3:1 ratio), 5.4 (dd, 1 H, H-3), 5.2 (t, 1 H, H-3'), 5.1 (dd, 1 H, H-2), 4.9 (m, 2 H, H-2', H-4'), 4.7 (d, 1 H, H-1'), 4.4 (m, 2 H, CH_2), 4.2 (m, 1 H, H-5), 4.2 (m, 2 H, CH_2'), 3.7 (m, 1 H, H-5'), 3.0 (t, 1 H, H-4), 2.0 (7s, 24 H, CH_3).

1.24. p-Nitrophenyl 2,3,6-tri-O-acetyl-4-S-(2',3',4', 6'-tetra-O-acetyl-β-D-glucopyranosyl)-1,4-dithio-β-D-glucopyranoside (23)



(23)

HBr in glacial acetic acid (45 %, 14 ml) was added to a cold solution of acetylated **22** (1.05 g, 1.51 mmol) in dichloromethane (30 ml) and the mixture was stirred for 2 hours at 0°C. After successive washings with ice-cold aqueous solutions of 10 % potassium hydrogen sulphate (10 ml x 3), saturated hydrogen bicarbonate (10 ml x 3), and ice-cold water, the organic phase was evaporated and the crude bromide was used without further purification. This was then dissolved in acetone (40 ml). p-Nitrothiophenol (0.99 g, 6.4 mmol) and an aqueous solution (28 ml) of potassium carbonate (0.88 g, 6.4 mmol) were added. The mixture was stirred overnight at room temperature, and then evaporated. After the usual work up, column chromatography (ethyl acetate-hexane, 1:1, v/v) of the residue gave **23** which crystallised from ether. Yield: 0.81 g (68 %), m. pt. : 83-84°C.

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 1748 (C=O stretching vibration), 1597 and 1578 (aromatic C-C stretching vibrations, doublet due to conjugation with $-NO_2$), 1519 and 1343 (asym. and sym. NO_2 stretching), 1231 (C-O-C stretching vibration of ester), 1120 to 1020 (C-O sym. stretching).

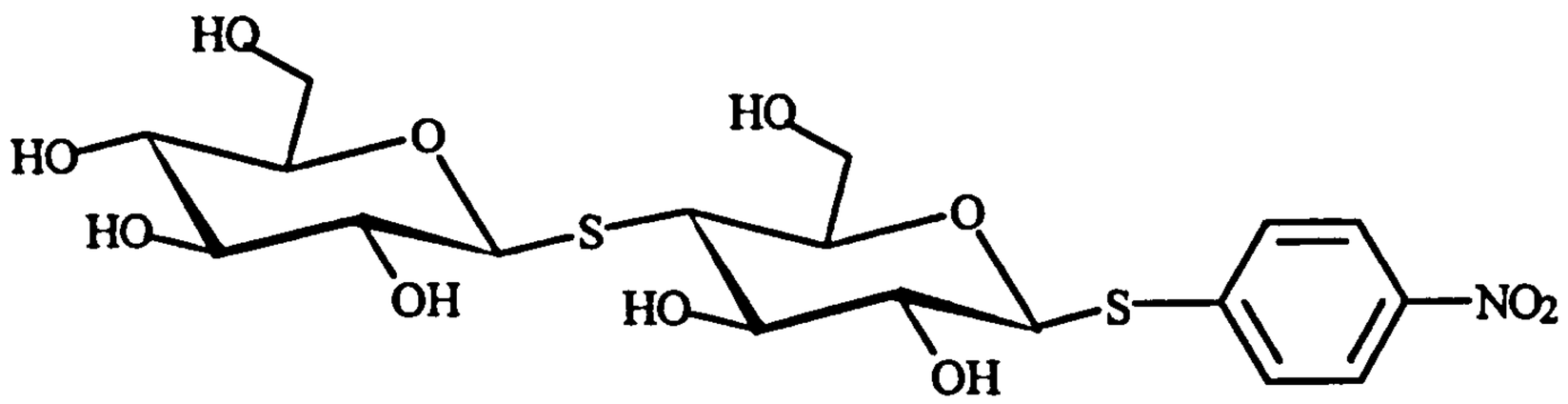
^{1}H-nmr ($CDCl_3$, 270 MHz, ppm) : δ 8.10 (d, 2 H, Ar-H, J 9 Hz), 7.58 (d, 2 H, Ar-H, J 9 Hz), 5.20 (dd, 1 H, H-3, J 9 Hz, J 11 Hz), 5.16 (t, 1 H, H-3', J 9 Hz), 5.02 (t, 1 H, H-4', J 10 Hz), 4.95 (t, 1 H, H-2, J 9 Hz), 4.91 (t, 1 H, H-2', J 9 Hz), 4.82 (d, 1 H, H-1, J 10 Hz), 4.72 (d, 1 H, H-1', J 10 Hz), 4.66 (dd, 1 H, H-6_a, J 2 Hz, J 12 Hz), 4.35

(dd, 1 H, H-6_b, J 5 Hz, J 12 Hz), 4.22 (dd, 1 H, H-6_a', J 2 Hz, J 12 Hz), 4.10 (m, 1 H, H-6'), 3.93 (m, 1 H, H-5), 3.70 (m, 1 H, H-5'), 2.94 (t, 1 H, H-4, J 11 Hz), 2.08, 2.07, 2.04, 2.02, 2.02, 2.00, 1.98 (7s, 21 H, 7 x CH_3).

^{13}C-nmr ($CDCl_3$, 270 MHz, ppm) : δ 170.29, 170.16, 170.01, 169.86, 169.33, 169.33, 169.16 (7 x C=O), 146.98 (ArC-NO_2), 141.61 (ArC-S), 131.23 (2 x ArC-H), 123.78 (2 x ArC-H), 84.10 (C-1), 81.71 (C-1'), 78.26 (C-5), 75.70 (C-5'), 73.51 (C-3'), 70.98 (C-3), 70.89 (C-2), 70.15 (C-4'), 66.05 (C-2'), 63.50 (CH_2), 61.95 (CH_2'), 46.05 (C-4), 20.83, 20.67, 20.54, 20.54, 20.46, 20.46, 20.46 (7 x CH_3).

m/z (EI+, MeOH:H_2O (1:1), 40°C) : 789 ($[M + Na]^+$).

1.25. p-Nitrophenyl 4-S-β-D-glucopyranosyl-1,4-dithio-β-D-glucopyranoside (24)



(24)

Compound **23** (0.30 g, 0.38 mmol) was deacetylated in a solution containing a catalytic amount of sodium methoxide in methanol as described in 1.7. Compound **24** was obtained as an orange powder. Yield: 0.12 g (81 %), m. pt. (decomp.) : 134°C.

IR-FT (Nujol mull, cm^{-1}) : υ_{max} 3400-3100 (O-H stretching vibrations), 1600 and 1576 (aromatic C-C stretching vibrations, doublet due to conjugation with $-NO_2$), 1500 and 1350 (C-NO_2), 1460 to 1200 (O-H and C-H deformation), 1175 to 1020 (C-O stretching).

^{1}H-nmr (D_6-DMSO + D_2O, 270 MHz, ppm) : δ 8.12 (d, 2 H, Ar-H, J 9 Hz), 7.58 (d, 2 H, Ar-H, J 9 Hz), 4.99 (d, 1 H, H-1, J 10 Hz), 4.46 (d, 1 H, H-1', J 9 Hz), 3.75 (m, 4 H, H-5, H-5', CH_2'), 3.44 (m, 3 H, H-3, CH_2), 3.21 (m, 3, H-2, H-4', H-3'), 3.07 (t, 1 H, H-2', J 9 Hz) 2.72 (t, 1 H, H-4, J 11 Hz).

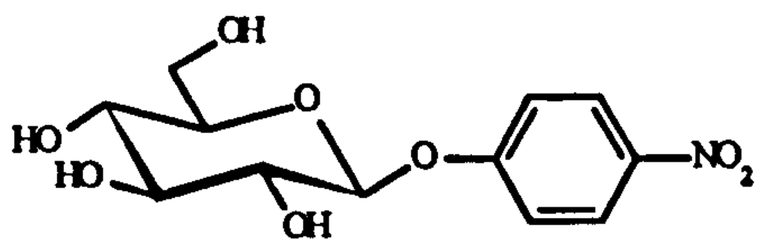
^{13}C-nmr (D_6-DMSO, 270 MHz, ppm) : δ 146.14 (ArC-NO_2), 144.83 (ArC-S), 127.59 (2 x ArC-H), 123.80 (2 x ArC-H), 84.53 (C-1), 83.21 (C-1'), 80.72, 80.72, 77.69, 74.66, 73.38, 72.59, 69.96 (C-2, C-2', C-3, C-3', C-4', C-5, C-5'), 61.21, 61.09 (2 x CH_2), 45.60 (C-4).

m/z (EI+, MeOH:H_2O (1:1), 40°C) : 1013 ($[2M + Na]^+$), 518 ($[M + Na]^+$).

2. Enzyme assays

2.1. Materials

p-Nitrophenyl β-D-glucopyranoside **(25)**, p-nitrophenyl β-D-cellobioside **(26)**, p-nitrophenyl β-D-lactopyranoside **(27)**, crude cellulase from *Trichoderma reesei*, β-glucosidase from almond and p-nitrophenol were purchased from Sigma-Aldrich Company Ltd and were used as supplied unless otherwise stated.



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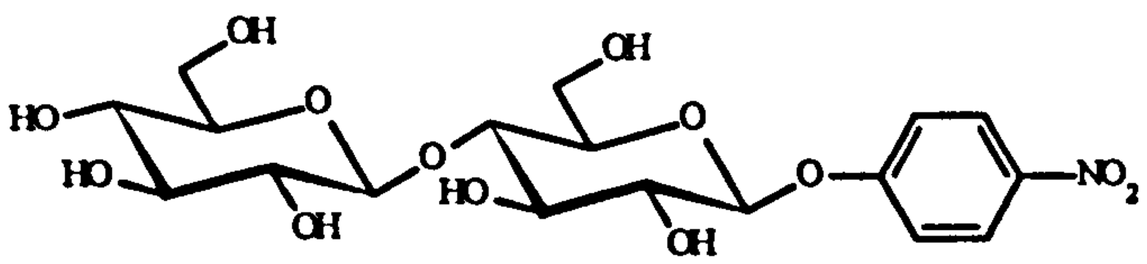

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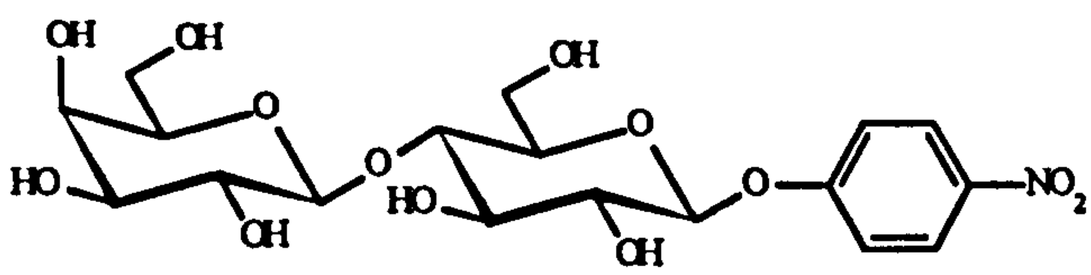


27

The acceptors used during the experiment and the expected transglycosylation products were chemically synthesised except the N-(p-nitrophenyl)-β-D-cellobiosylamine and the N-(p-nitrophenyl)-β-D-gentiobiosylamine which were kindly synthesised and supplied by John V. Baum, research student at the University of Huddersfield.

2.2. Equipment

2.2.1. *High performance liquid chromatography*

The substrate hydrolysis and transglycosylation reaction studies were monitored using a Beckman liquid chromatograph which comprised of a 127 pump system and a 116 UV detector. A 507e autosampler was recently acquired and coupled to the HPLC. The HPLC was connected to a computer running the "Summit" integration package.

HPLC standard method : Flow rate : 1.2 $ml.min^{-1}$,

Mobile phase : ACN 87.5 %, H_2O 12.5 %.

Column : Supelco LC-NH_2, 25 cm x 4.6 mm.

From 0 to 11.5 minutes : Wavelength : 300 nm, Range 0.5,

From 11.5 to 30 minutes : Wavelength : 360 nm for amine derivatives, 320 nm for thiol-derivatives, Range 0.05.

Retention times

p-nitrophenyl 1-thio-β-D-glucopyranoside	:	4.2 min
p-nitrophenyl β-D-glucopyranoside	:	4.3 min
N-(p-nitrophenyl)-β-D-glucopyranosylamine	:	4.4 min
p-nitrophenol	:	7.8 min
unknown (thio)	:	9.4 min
p-nitrophenyl 1-thio-β-D-cellobioside	:	9.5 min
p-nitrophenyl β-D-cellobioside	:	11.0 min
unknown (amine)	:	11.9 min
N-(p-nitrophenyl)-β-D-cellobiosylamine	:	12.3 min
p-nitrophenyl 1-thio-β-D-gentiobioside	:	15.4 min
p-nitrophenyl β-D-gentiobioside	:	16.2 min
N-(p-nitrophenyl)-β-D-gentiobiosylamine	:	26.6 min
Trisaccharides	:	>40 min

The p-nitrophenyl 1-thio-β-D-cellobioside transfer product was not analysed due to its retention time being too close to that of PNPC. Two successive changes of wavelength and range would have been inappropriate as would be required in order to measure either amount. The use of another column could have allowed this measurement.

2.2.2. *UV-vis spectrophotometry*

A Cary 1E UV-vis spectrophotometer (Varian) was used to follow reaction rates in the absence of acceptor. The release of p-nitrophenol was monitored at 365 nm and at 30°C unless otherwise specified.

2.2.3. *Capillary zone electrophoresis*

A Dionex capillary electrophoresis system, fitted with a 67 cm, 75 μm i.d., "uncoated", fused silica capillary was used for the different experiments. $Na_2H_2PO_4$ was used as a buffer (pH 9.2, 0.03 M) during the experiment. A UV-vis detector was used to identify and quantify protein components. The capillary zone electrophoresis has an IDE-interface connected to an external computer which allowed the storage, manipulation and analysis of the collected data.

2.3. Other equipment

Reaction systems were maintained at a constant temperature with a Beckman water bath or with the Peltier temperature controller attached to the Cary UV-vis spectrophotometer.

All pH measurements were recorded with a Russell CMAWL-series pH electrode connected to a Beckman pH meter.

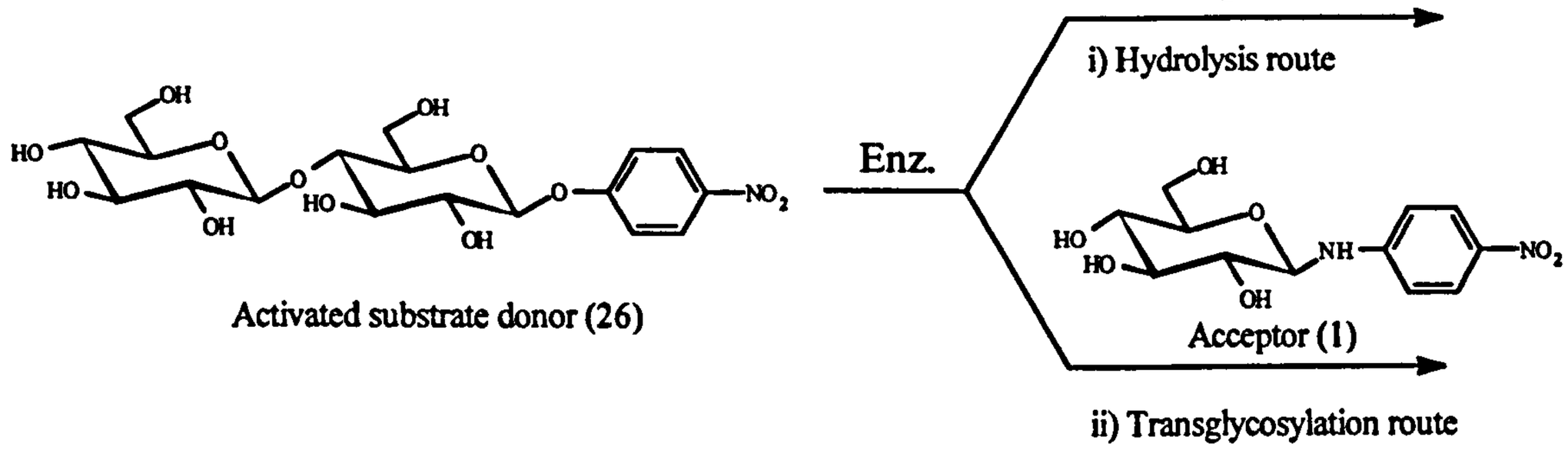
Solution volumes were measured with SGE micro-syringes or Gilson autopipettes. Analysis was performed on samples removed from the reaction system at noted time intervals and diluted in ethanol[86] (50 μl sample + 450 μl ethanol).

<u>NOTE</u> :

- *All quantities that follow are in microliters unless otherwise specified.*
- *All reactions were maintained at 30 ℃ and were monitored by HPLC unless otherwise specified.*

2.4. β-glucosidase, from almond, assays

The hydrolysis and transglycosylation reaction were studied using p-nitrophenyl β-D-cellobioside (26) as a substrate donor.



Scheme 6

A stock solution was prepared for the substrate, the acceptor and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.01 M,

N-(p-nitrophenyl)-β-D-glucopyranosylamine (1) : 0.02 M,

β-glucosidase from almond : 1.0 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

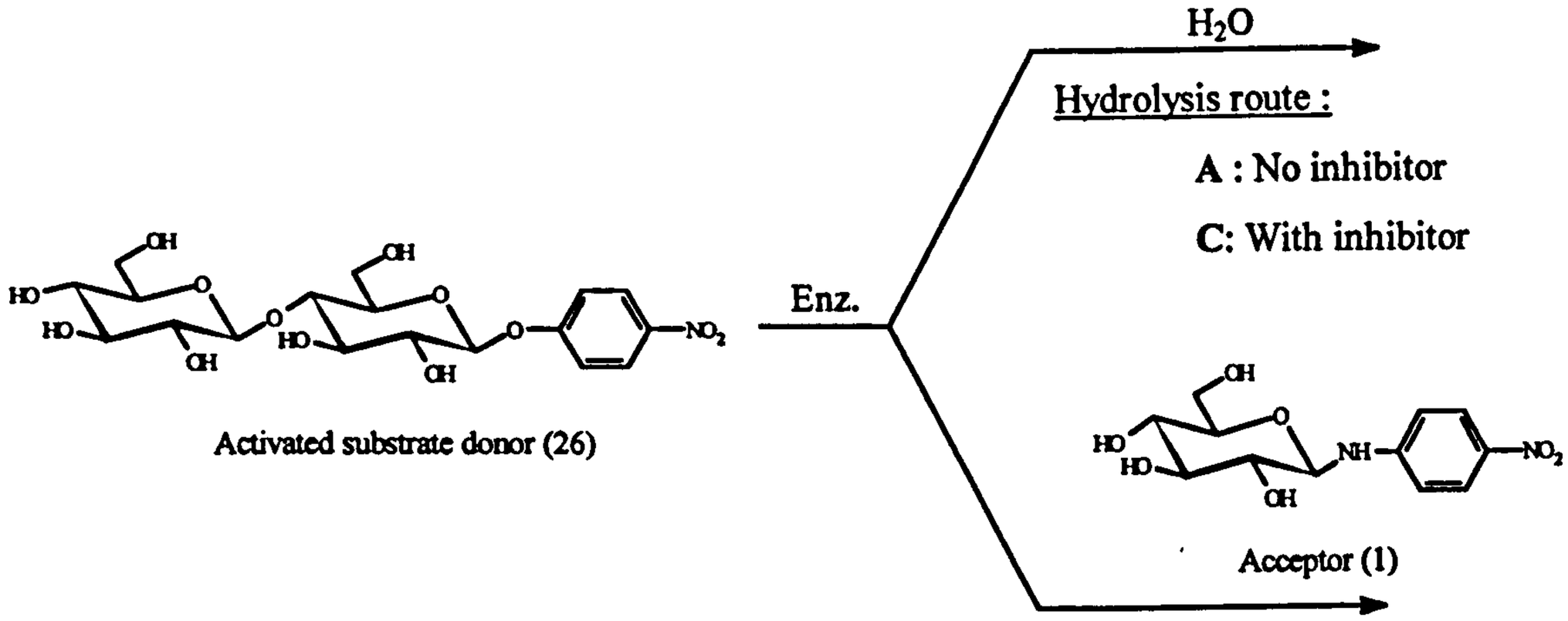
The reaction systems were prepared as follows :

	Substrate	Acceptor	Enzyme	Buffer	H_2O
A	100	--	150	350	1900
B	100	1250	250	250	650

A represents the hydrolysis of the p-nitrophenyl β-D-cellobioside on its own and **B** in the presence of the glucopyranosylamine acceptor for the transglycosylation study.

2.5. Cellulase, from *Trichoderma reesei*, assays

2.5.1. p-Nitrophenyl β-D-cellobioside as the substrate donor



Scheme 7

A stock solution was prepared for the substrates and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.01 M,

N-(p-nitrophenyl)-β-D-glucopyranosylamine : 0.02 M,

1,5-glucono-δ-lactone : 0.08 M,

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

	Substrate	Acceptor	Inhibitor	Enzyme	Buffer	H_2O
A	100	--	--	250	250	1900
B	100	1250	--	250	250	650
C	100	--	650	250	250	1250
D	100	1250	650	250	250	--

C and **D** are the same experiments as **A** and **B** but include the presence of a β-glucosidase inhibitor (1,5-glucono-δ-lactone)[87].

2.5.2. p-Nitrophenyl β-D-cellobioside : K_m determination

A stock solution was prepared for the substrate and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.01 M, (*=0.02 M),

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

The reaction systems were prepared as follows :

	Substrate	Enzyme	Buffer	H_2O	[Substrate]
A	5	250	250	1995	**0.02**
B	10	250	250	1990	**0.04**
C	15	250	250	1985	**0.06**
D	20	250	250	1980	**0.08**
E	37.5	250	250	1962.5	**0.15**
F	45	250	250	1955	**0.18**
G	60	250	250	1940	**0.24**
H	100	250	250	1900	**0.40**
I	200	250	250	1800	**0.80**
J	487.5	250	250	1512.5	**1.95**
K	1000	250	250	1000	**4.00**
L	1550	250	250	450	**6.20**
M*	1025	250	250	975	**8.20**
N*	1275	250	250	725	**10.20**
O*	1562	250	250	438	**12.50**

2.5.3. Acceptor inhibition studies

A stock solution was prepared for the substrate, the acceptor and the enzyme :

p-nitrophenyl β-D-cellobioside : 5.0 mM

Acceptor : N-(p-nitrophenyl)-β-D-glucopyranosylamine (1)

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

- **[A] = 2.00 mM**

The reaction systems were prepared as follows :

	Substrate	Acceptor	Enzyme	Buffer	H_2O	**[Substrate]**
A	10	250	250	250	1740	**0.020**
B	12	250	250	250	1738	**0.023**
C	14	250	250	250	1736	**0.028**
D	16	250	250	250	1734	**0.032**
E	20	250	250	250	1730	**0.040**
F	30	250	250	250	1720	**0.060**
G	62	250	250	250	1688	**0.125**
H	80	250	250	250	1670	**0.160**
I	100	250	250	250	1650	**0.200**
J	125	250	250	250	1625	**0.250**
K	200	250	250	250	1550	**0.400**
L	400	250	250	250	1350	**0.800**
M	600	250	250	250	1150	**1.200**

- **[A] = 6.00 mM**

The reaction systems were prepared as follows :

	Substrate	Acceptor	Enzyme	Buffer	H_2O	[Substrate]
A	10	750	250	250	1240	**0.020**
B	12	750	250	250	1238	**0.023**
C	14	750	250	250	1236	**0.028**
D	16	750	250	250	1234	**0.032**
E	20	750	250	250	1230	**0.040**
F	30	750	250	250	1220	**0.060**
G	62	750	250	250	1188	**0.125**
H	80	750	250	250	1170	**0.160**
I	100	750	250	250	1150	**0.200**
J	125	750	250	250	1125	**0.250**
K	200	750	250	250	1050	**0.400**
L	400	750	250	250	850	**0.800**
M	600	750	250	250	650	**1.200**

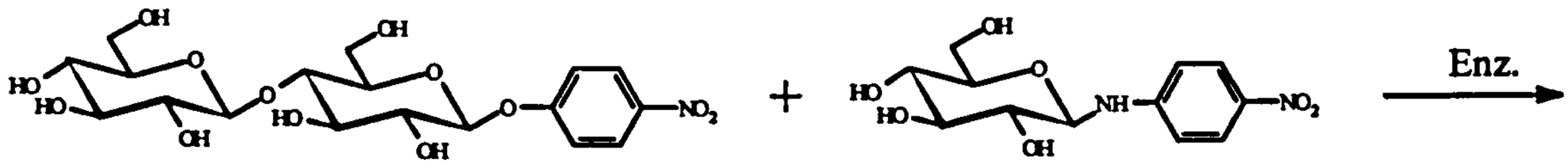
- **[A] = 10.00 mM**

The reaction systems were prepared as follows :

	Substrate	Acceptor	Enzyme	Buffer	H_2O	**[Substrate]**
A	10	1250	250	250	740	**0.020**
B	16	1250	250	250	734	**0.032**
C	20	1250	250	250	730	**0.040**
D	62	1250	250	250	688	**0.125**
E	80	1250	250	250	670	**0.160**
F	200	1250	250	250	550	**0.400**
G	400	1250	250	250	350	**0.800**
H	600	1250	250	250	150	**1.200**

2.5.4. Substrate concentration studies

The following reactions were performed to study the effect of the substrate concentration on the transglycosylation reaction yield. The substrate concentration was varied while the acceptor concentration remained constant.



Activated substrate donor (26) Acceptor (1)

Scheme 8

A stock solution was prepared for the substrate, the acceptor and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.0125 M (or 0.0143 M)*

N-(p-nitrophenyl)-β-glucopyranosylamine : 0.025 M,

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

The reaction systems were prepared as follows :

	Substrate	Acceptor	Enzyme	Buffer	H_2O	[Substrate]
A	20	1000	250	250	980	**0.10**
B	85	1000	250	250	915	**0.425**
C	220	1000	250	250	780	**1.10**
D	370	1000	250	250	630	**1.85**
E	460	1000	250	250	540	**2.30**
F	500	1000	250	250	500	**2.50**
G	600	1000	250	250	400	**3.00**
H*	920	1000	250	250	80	**4.60**
I*	1000	1000	250	250	-	**5.00**

2.5.5. Acceptor concentration studies

The following reactions were performed to study the effect of the acceptor concentration on the transglycosylation reaction yield. This time, the acceptor concentration was varied while the substrate concentration was kept constant (See scheme 8). Different acceptors were tested and their relative effect will be discussed later.

A stock solution was prepared for the substrate, the acceptor and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.0154 M,

N-(p-nitrophenyl)-β-glucopyranosylamine : 0.040 M in 10% acetonitrile,

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer (10% ACN), pH 4.50, 0.25 M.

The reaction systems were prepared as follows :

	Substrate	Acceptor	Enzyme	Buffer	ACN	H_2O	[Acceptor]
A	200	157	125	125	84.3	558.7	**5.02**
B	200	313	125	125	68.7	418.2	**10.00**
C	200	650	125	125	35	115	**20.80**
D	200	10.40 mg	125	125	100	700	**27.70**
E	200	15.02 mg	125	125	100	700	**40.00**
F	200	18.03 mg	125	125	100	700	**48.10**

Acetonitrile (10 %) was added to the system to allow higher acceptor concentrations to be achieved.

2.5.6. Temperature studies

A stock solution was prepared for the substrate, the acceptor and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.01 M,

N-(p-nitrophenyl)-β-glucopyranosylamine : 0.02 M,

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

Two systems were analysed at the same temperature to observe :

(A) the effect of the temperature on the rate of hydrolysis of the substrate donor

(B) the transglycosylation products yield.

	Substrate	**Acceptor**	**Enzyme**	**Buffer**	**H_2O**
A	100	-	250	250	1900
B	100	1250	250	250	650
Conc. (/mM)	**0.40**	**10.00**	**0.23 $mg.ml^{-1}$**	**50.00**	**-**

The reaction systems were monitored at 365 nm (using a UV-vis spectrophotometer or an HPLC attached to a UV detector) at 10, 20, 30, 40, 50 and 60 °C.

2.5.7. Co-solvent studies

A stock solution was prepared for the substrate, the acceptor and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.01 M,

N-(p-nitrophenyl)-β-glucopyranosylamine : 0.0209 M,

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer (+ corresponding % co-solvent present in the reaction), pH 4.50, 0.25 M.

The reaction systems were prepared as follows :

	Substrate	Acceptor	Co-solvent	Enzyme	Buffer	H_2O
A	100	1200	200 (10%)	250	250	500
B	100	1200	450 (20%)	250	250	250
C	100	1200	700 (30%)	250	250	0
Conc. (/mM)	**0.40**	**10.00**	**--**	**0.23 $mg.ml^{-1}$**	**50.00**	**--**

These reactions were carried out using ACN, t-butanol, dioxane and acetone as co-solvents.

2.5.8. pH rate profiles

2.5.8.1. No acceptor, no co-solvent

A stock solution was prepared for the substrate and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.01 M,

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in buffer (0.25 M),

(Lot 95H0087, 6.6 units/mg solid).

The reaction system was prepared as follows :

	Substrate	**Enzyme**	**Buffer**	**H_2O**
A	100	250	250	1900
Conc. (/mM)	**0.40**	**0.23 $mg.ml^{-1}$**	**50.00**	**--**

The reaction was carried out at the following pHs :

pH 2.24, 2.78 (glycine buffer, pK_a 2.34), 3.01, 3.12, 3.43, 3.66 (formic acid buffer, pK_a 3.77), 3.96, 4.01, 4.27, 4.50, 4.97, 5.44 (acetate buffer, pK_a 4.76), 5.77, 6.10, 6.41, 6.79, 6.88 (MES buffer, pK_a 6.15), 7.06, 7.45 (MOPS buffer, pK_a 7.25).

2.5.8.2. No acceptor, in 24 % acetonitrile

A stock solution was prepared for the substrate and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.01 M,

Crude cellulase from *T. reesei* : 6.3 $mg.ml^{-1}$ in buffer (0.25 M, 30 % ACN).

(Lot 74H0590, 4.8 units/mg solid)

The reaction system was prepared as follows :

	Substrate	**Enzyme**	**ACN**	**H_2O**
A	100	500	500	1900
Conc. (/mM)	**0.40**	**1.26 $mg.ml^{-1}$**	**24 %**	**--**

The reaction was carried out at the following pHs :

pH 3.60 (formic acid buffer, pK_a 3.77), 3.97, 4.07, 4.51, 4.90, 5.40 (acetate buffer, pK_a 4.76), 5.93, 6.35, 6.44, 6.55 (MES buffer, pK_a 6.15).

2.5.8.3. *With acceptor, no co-solvent*

A stock solution was prepared for the substrate, the acceptor and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.01 M,

N-(p-nitrophenyl)-β-D-glucopyranosylamine : 0.02 M,

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in buffer (0.25 M).

(Lot 95H0087, 6.6 units/mg solid).

The reaction system was prepared as follows :

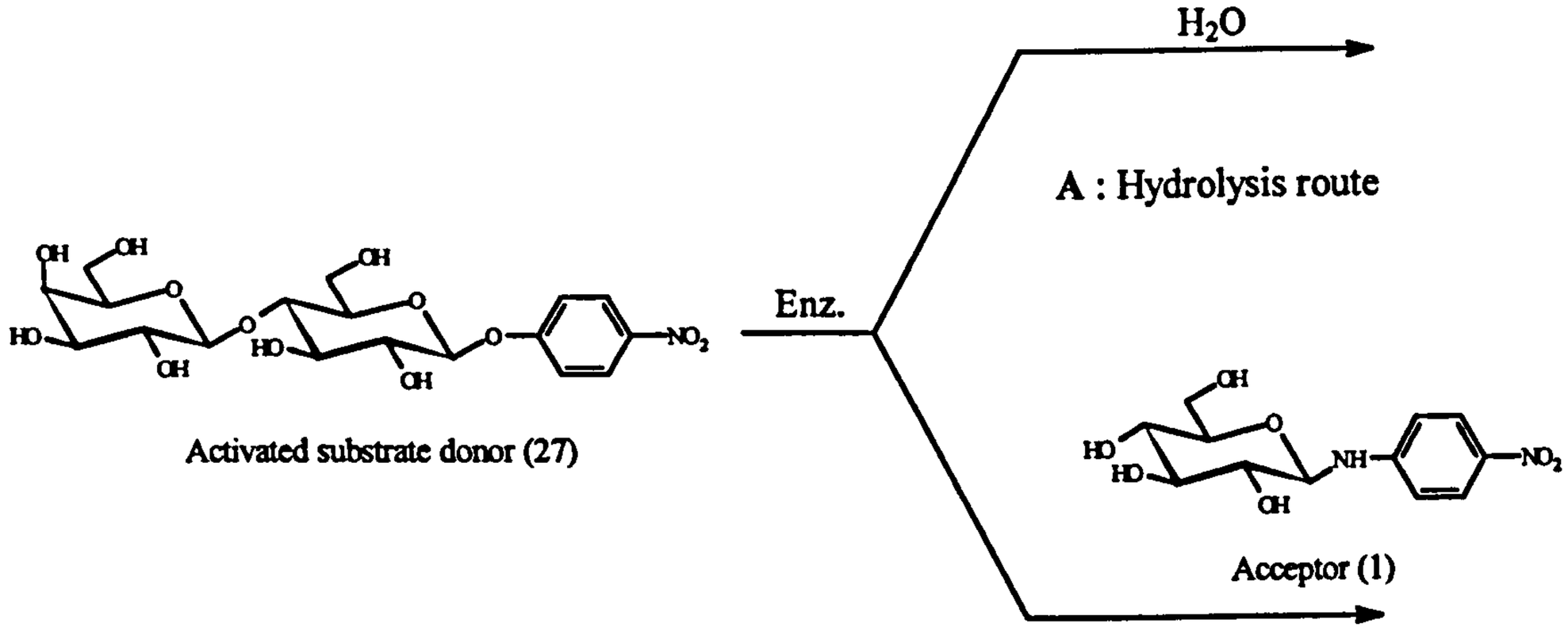
	Substrate	**Acceptor**	**Enzyme**	**Buffer**	**H_2O**
A	100	1250	250	250	1900
Conc. (/mM)	**0.40**	**10.00**	**0.23 $mg.ml^{-1}$**	**50.00**	**--**

The reaction was carried out at the following pHs :

pH 2.24, 2.38 (glycine buffer, pK_a 2.34), 2.54, 2.90, 3.11, 3.45 (formic acid buffer, pK_a 3.77), 3.85, 4.25, 4.55, 4.85, 5.25 (acetate buffer, pK_a 4.76), 5.65, 6.00, 6.29, 6.56 (MES buffer, pK_a 6.15), 6.87 (MOPS buffer, pK_a 7.25).

2.5.9. p-Nitrophenyl β-D-lactopyranoside as the substrate donor

2.5.9.1. Hydrolysis and Transglycosylation studies



Scheme 9

A stock solution was prepared for the substrates and the enzyme :

p-nitrophenyl β-D-lactopyranoside : 0.01 M,

N-(p-nitrophenyl)-β-D-glucopyranosylamine : 0.02 M,

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

The reaction systems were prepared as follows :

	Substrate	Acceptor	Enzyme	Buffer	H_2O
A	100	--	250	250	1900
B	100	1250	250	250	650

A represents the hydrolysis of the p-nitrophenyl β-D-lactopyranoside on its own, B in the presence of the glucopyranosylamine acceptor for the transglycosylation study.

2.5.9.2. K_m determination

A stock solution was prepared for the substrate and the enzyme :

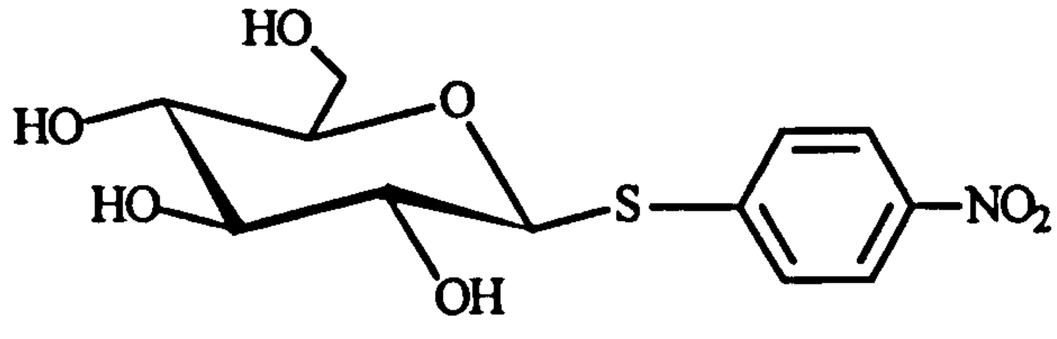
p-nitrophenyl β-D-lactopyranoside : 0.01 M,

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

The reaction systems were prepared as follows :

	Substrate	Enzyme	Buffer	H_2O	[Substrate]
A	5	250	250	1995	**0.02**
B	10	250	250	1990	**0.04**
C	20	250	250	1980	**0.08**
D	30	250	250	1970	**0.12**
E	50	250	250	1950	**0.20**
F	100	250	250	1900	**0.40**
G	150	250	250	1850	**0.60**
H	200	250	250	1800	**0.80**
I	400	250	250	1600	**1.60**
J	625	250	250	1375	**2.50**
K	625	125	125	375	**5.00**
L	937.5	125	125	62.5	**7.50**

2.5.10. p-Nitrophenyl 1-thio-β-D-glucopyranoside (6) as a new acceptor



(6)

2.5.10.1. *Transglycosylation studies*

A stock solution was prepared for the substrate and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.01 M

p-nitrophenyl 1-thio-β-glucopyranoside : 0.02 M

1,5-Glucono-δ-lactone : 0.08 M

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

The reaction systems were prepared as follows :

	Substrate	Acceptor	Enzyme	Buffer	Inhibitor	H_2O
A	100	1250	250	250	--	650
B	100	1250	250	250	650	--

2.5.10.2. Acceptor inhibition studies

A stock solution was prepared for the substrate, the acceptor and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.005 M

p-nitrophenyl 1-thio-β-glucopyranoside : 0.02 M

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

- **[A] = 2.00 mM**

The reaction systems were prepared as follows :

	Substrate	Acceptor	Enzyme	Buffer	H_2O	[Substrate]
A	10	250	250	250	1740	**0.02**
B	15	250	250	250	1736	**0.03**
C	20	250	250	250	1730	**0.04**
D	30	250	250	250	1720	**0.06**
E	40	250	250	250	1688	**0.08**
F	70	250	250	250	1670	**0.14**
G	100	250	250	250	1650	**0.20**
H	200	250	250	250	1550	**0.40**

- **[A] = 6.00 mM**

The reaction systems were prepared as follows :

	Substrate	Acceptor	Enzyme	Buffer	H_2O	[Substrate]
A	10	750	250	250	1240	**0.020**
B	14	750	250	250	1236	**0.028**
C	20	750	250	250	1230	**0.040**
D	100	750	250	250	1150	**0.200**
E	200	750	250	250	1050	**0.400**
F	400	750	250	250	850	**0.800**
G	600	750	250	250	650	**1.200**

2.5.10.3. *Acceptor concentration studies*

A stock solution was prepared for the substrate, the acceptor and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.01 M

p-nitrophenyl 1-thio-β-glucopyranoside : 0.02 M (*0.03 M)

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

The reaction systems were prepared as follows :

	Substrate	Acceptor	Enzyme	Buffer	H_2O	[Acceptor]
A	100	250	250	250	1650	**2.0**
B	100	750	250	250	1150	**6.0**
C	100	1250	250	250	650	**10.0**
D	100	1900	250	250	--	**15.2**
E*	100	1900	250	250	--	**22.8**

2.5.10.4. K_m determination : p-Nitrophenyl 1-thio-β-D-cellobioside

A stock solution was prepared for the substrate and the enzyme :

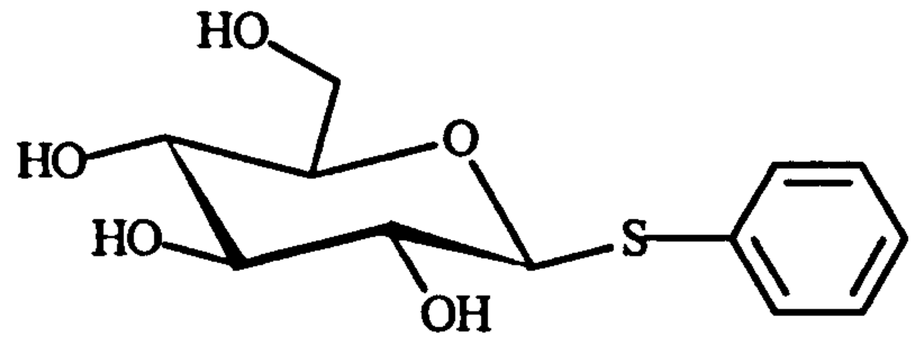
p-nitrophenyl 1-thio-β-D-cellobioside : 0.005 M,

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

The reaction systems were prepared as follows :

	Substrate	Enzyme	Buffer	H_2O	[Substrate]
A	30	250	250	1970	**0.06**
B	40	250	250	1960	**0.08**
C	50	250	250	1950	**0.10**
D	60	250	250	1940	**0.12**
E	70	250	250	1930	**0.14**
F	80	250	250	1920	**0.16**
G	90	250	250	1910	**0.18**
H	100	250	250	1900	**0.20**

2.5.11. Phenyl 1-thio-β-D-glucopyranoside (8) as a new acceptor



(8)

A stock solution was prepared for the substrate and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.01 M

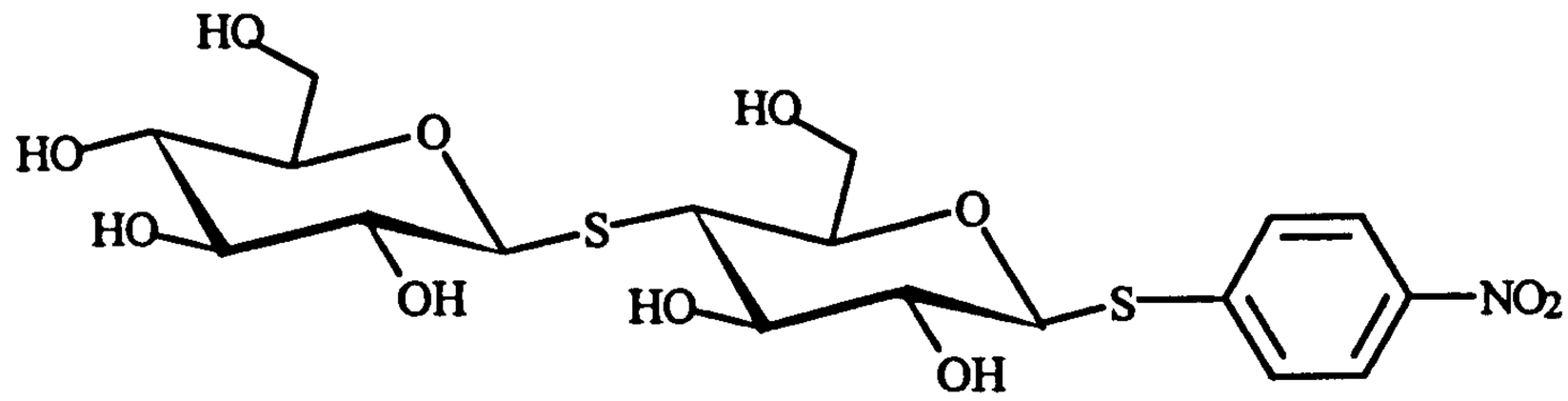
phenyl 1-thio-β-glucopyranoside : 0.02 M

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

The reaction system was prepared as follows :

	Substrate	Acceptor	Enzyme	Buffer	H_2O
A	100	1250	250	250	650

2.5.12. p-Nitrophenyl 4-S-(β-D-glucopyranosyl)-1,4-dithio-β-D-glucopyranoside (24) as a disaccharide acceptor



(24)

A stock solution was prepared for the substrate, the acceptor and the enzyme :

p-nitrophenyl β-D-cellobioside : 0.01 M

p-nitrophenyl 4-S-(β-D-glucopyranosyl)-1,4-dithio-β-D-glucopyranoside : 0.02 M

Crude cellulase from *T. reesei* : 2.3 $mg.ml^{-1}$ in sodium acetate buffer, pH 4.50, 0.25 M.

The reaction systems were prepared as follows :

	Substrate	Acceptor	Enzyme	Buffer	ACN	H_2O
A	100	--	250	250	400	1500
B	100	500	250	250	400	1000

3. Enzyme Purification

3.1. Ion Exchange Chromatography

The enzyme components were separated as described by Pettersson et al.[88] using DEAE-Sepharose CL-6B anion exchange chromatography from Sigma, Lot 66H0884, Wet Bead size 45-165 μm). The crude cellulase from *Trichoderma reesei* (410 mg, Sigma, Lot 95H0087) was loaded on the top of the column, where a linear ionic strength gradient (0.01 M to 0.50 M) was used to elute the different enzyme components. Ammonium acetate was used as a buffer of pH 5.0 and the flow rate determined to be 0.24 $ml.min^{-1}$. The column was set-up in a cold room at 4°C together with a fraction collector and a peristaltic pump. Each fraction tube (8 ml) was then analysed by UV spectroscopy to determine the protein concentration absorption at 280 nm.

3.2. Determination of the Enzyme Components

Specific enzyme tests were performed using the fractions collected at the maximum UV-absorbance of a peak.

Test	Active Enzyme
CMCase activity	Endo-acting enzymes
Avicelase activity	Exo-acting enzymes
p-nitrophenyl β-D-glucopyranoside	β-glucosidase enzymes

CMCase activity : The reaction mixture consisted of a 0.5% solution of carboxymethyl cellulose (CMC), 0.7 DS, in 0.05M sodium acetate buffer, pH 5.0 and 50 μl of enzyme solution. The incubation conditions were 40°C for 10 minutes. Reducing sugars liberated were determined using 3,5-dinitrosalicylic acid (DNS).

Avicelase activity : The reaction mixture consisted of a 1% Avicel (crystalline cellulose) in 0.05M sodium acetate buffer, pH 5.0 and 100 µl of enzyme solution. The incubation conditions were 40°C for 90 minutes. Reducing sugars liberated were determined using 3,5-dinitrosalicylic acid (DNS).

3.3. Gel and capillary zone electrophoresis

SDS-PAGE gel electrophoresis (Pre-cast electrophoresis gel, BDH, 43619 5U, Electrograd SDS-PAGE gel SDS-620, 82 x 30 x 1 mm) and capillary zone electrophoresis were used to analyse the purity of the each fraction.

3.3.1. SDS-PAGE electrophoresis procedure

Enzyme solutions (25 µl) were first treated with β-mercaptoethanol at 95°C for 5 minutes. Sucrose was added to increase the enzyme solution density and bromophenol blue was used as a marker. A small amount of the enzyme solution (5 µl) was applied to the top of the gel. A standard solution consisting of phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin (Pharmacia Biotech, Lot 7020446011) was used to provide a calibration measurement. The electrophoresis was run in a tris-HCl buffer at 200 V. The gel were then removed and placed into Coomasie blue dye for 15 minutes and finally de-stained in 15 % acetic acid.

3.3.2. Capillary zone electrophoresis

Capillary electrophoresis was used as described by Khaled[89] *et al*..The method involved the use of a silica column with a $Na_2H_2PO_4$ buffer solution (pH 9.2, 0.03 M) and a gravity injection of 50 mm for 10 seconds. 20,000 Volts were applied and the sample were detected with a UV-detector at 280 nm.

3.4. β-glucosidase activity test

p-Nitrophenyl β-D-glucopyranoside activity : The reaction mixture consisted of a 0.4 mM p-nitrophenyl β-D-glucopyranoside in 0.02 M sodium acetate buffer, pH 4.5 and 250 µl of enzyme solution. The incubation conditions were 30°C and 10 hours. The reaction was followed by HPLC using a UV detector at 300 nm. The same reaction system with added 1,5-glucono-δ-lactone was repeated whenever β-glucosidase activity was found.

3.5. Transglycosylation studies

p-Nitrophenyl β-D-cellobioside activity : The reaction mixture consisted of a 0.4 mM p-nitrophenyl β-D-cellobioside in 0.02 M sodium acetate buffer, pH 4.5 and 250 µl of enzyme solution. The incubation conditions were 30°C and 10 hours. The reaction was followed by HPLC using a UV detector at 300 nm.

Transglycosidase activity : The reaction mixture consisted of 0.4 mM p-nitrophenyl β-D-cellobioside, and 6.0 mM N-(p-nitrophenyl)-β-D-glucopyranosylamine in 0.02 M sodium acetate buffer, pH 4.5 and 250 µl of enzyme solution. The incubation conditions were 30°C and 10 hours. The reaction was followed by HPLC using a UV detector at 300 nm and 360 nm to observe any transglycosylation products.

3.6. Cellobiohydrolase Activity Test

Similar reaction systems as 3.5 with added cellobiose (2.0 mM) (strong inhibitor of cellobiohydrolases) were used to detect the presence of CBHs.

Chapter 3

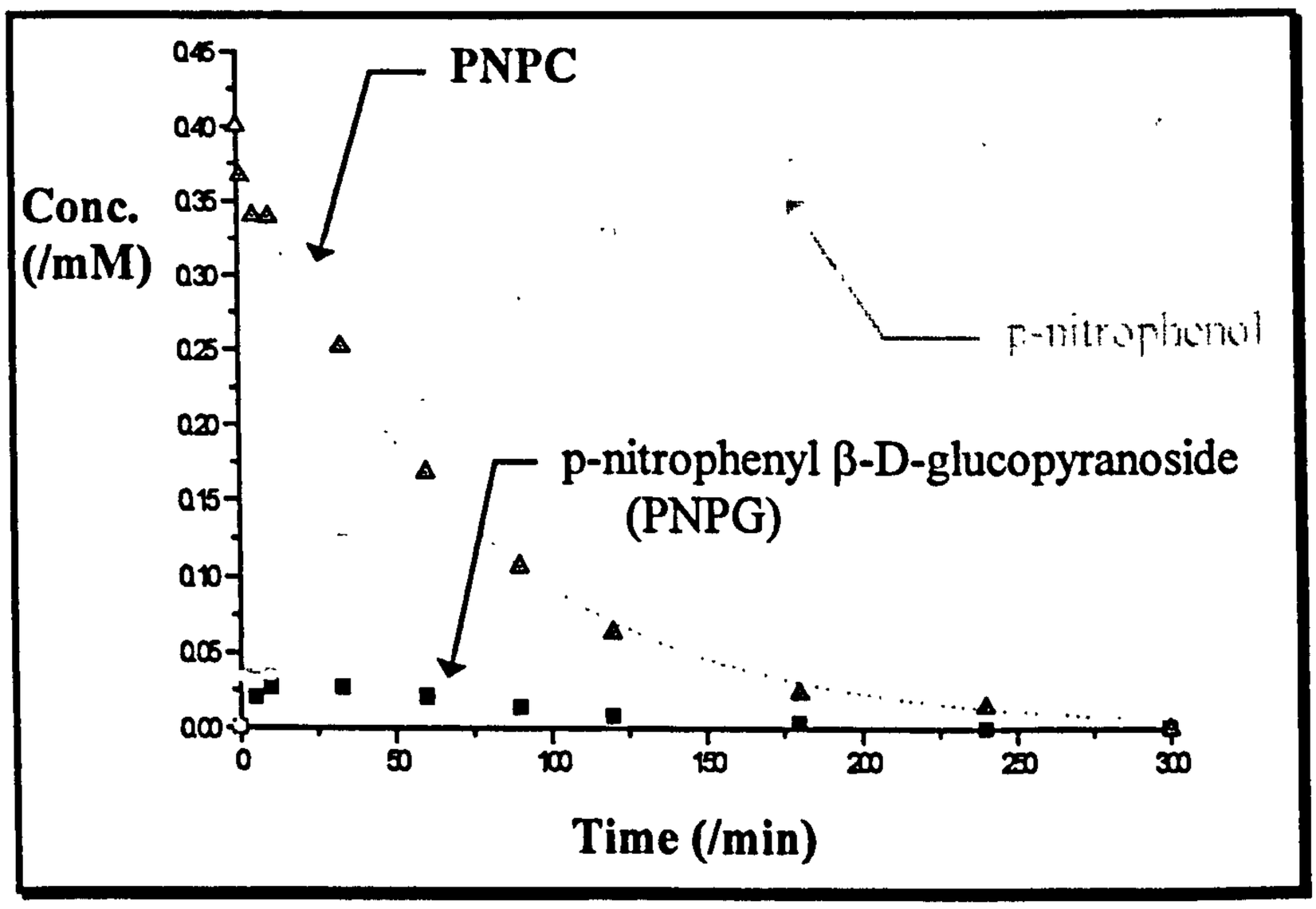
Results

1. β-Glucosidase from almond

1.1. Hydrolysis of p-nitrophenyl β-D-cellobioside (PNPC)

• *Reaction conditions*

p-nitrophenyl β-D-cellobioside (PNPC) :	0.40 mM
β-glucosidase from almond :	0.06 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, 0.05 M



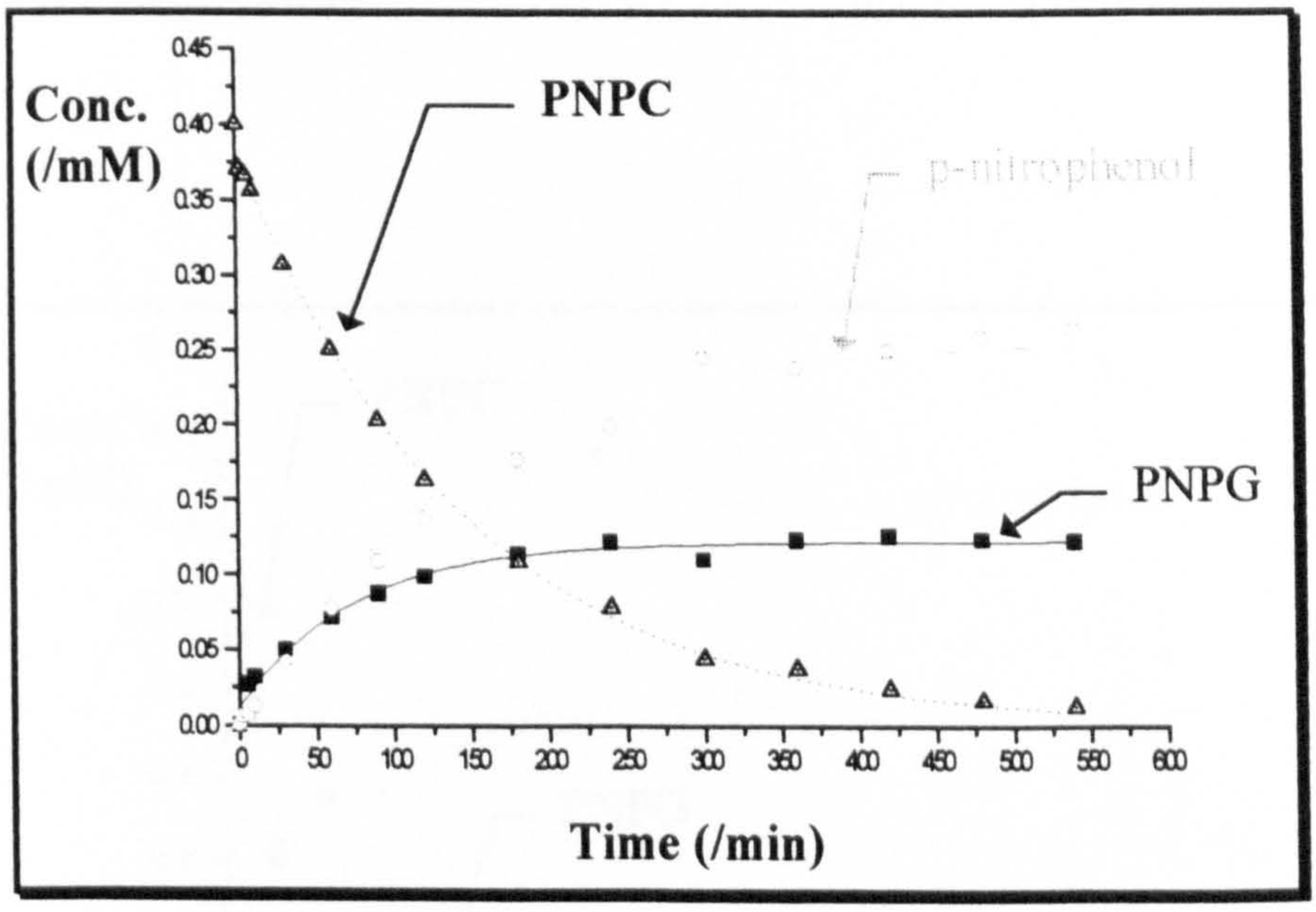
1.2. Transglycosylation studies

- *Reaction conditions*

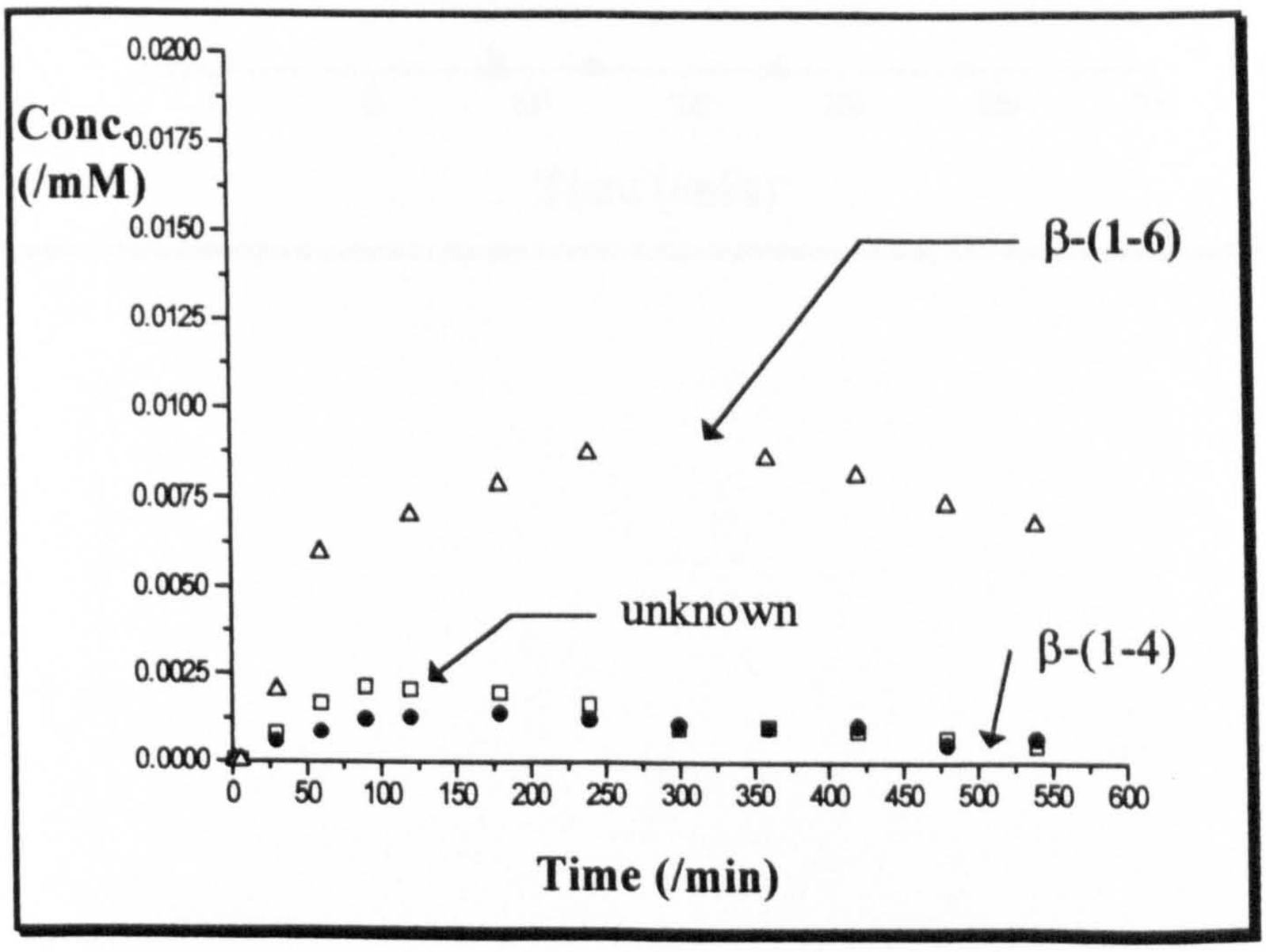
Substrate : p-nitrophenyl β-D-cellobioside :	0.4 mM
Acceptor : N-(p-nitrophenyl)-β-D-glucopyranosylamine :	10.0 mM
β-glucosidase from Almond :	0.10 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, 0.25 M

Transglycosylation reaction

Substrate hydrolysis



Transglycosylation products

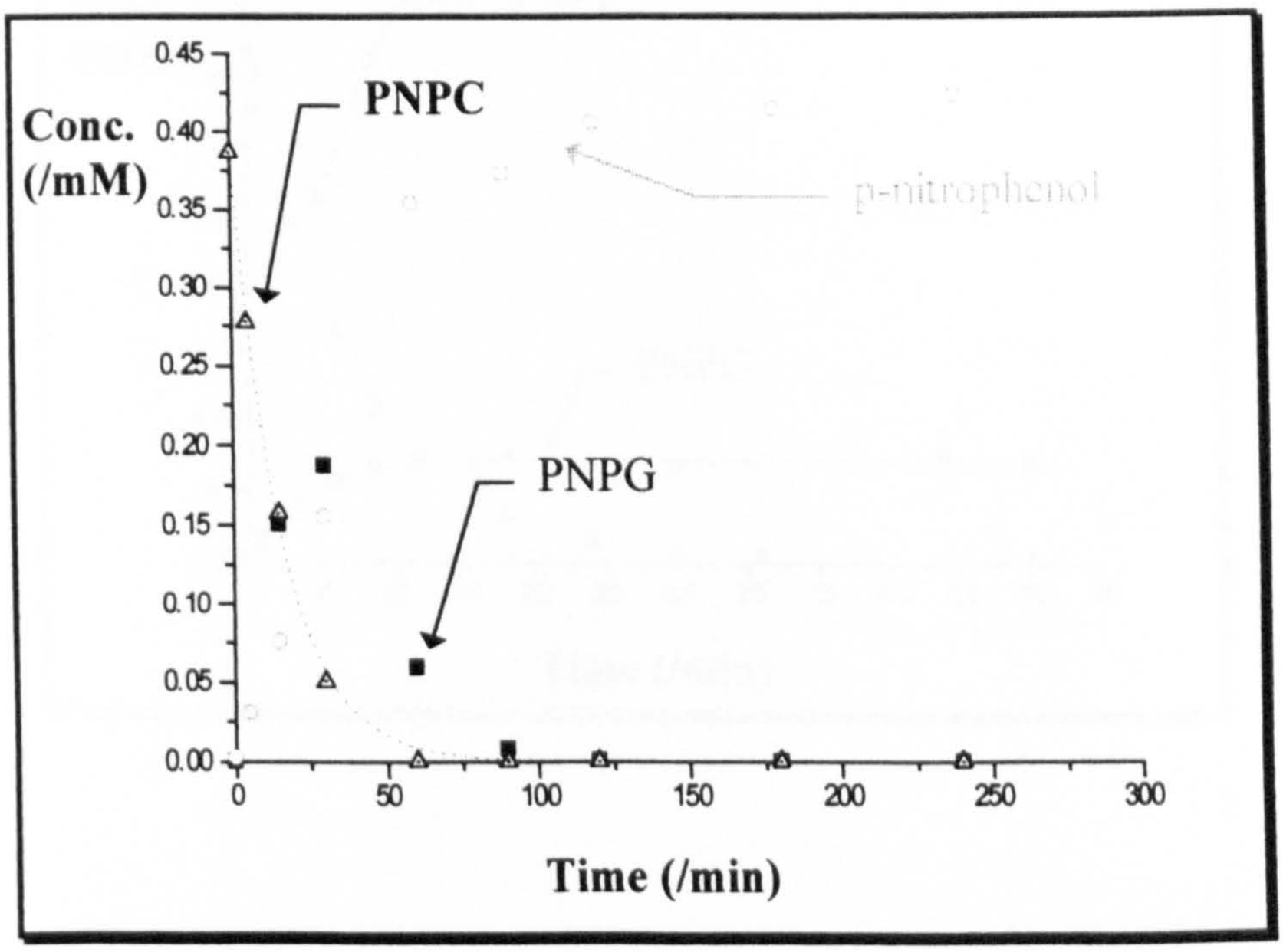


2. Crude cellulase from *Trichoderma reesei*

2.1. Hydrolysis of p-nitrophenyl β-D-cellobioside

- *Reaction conditions*

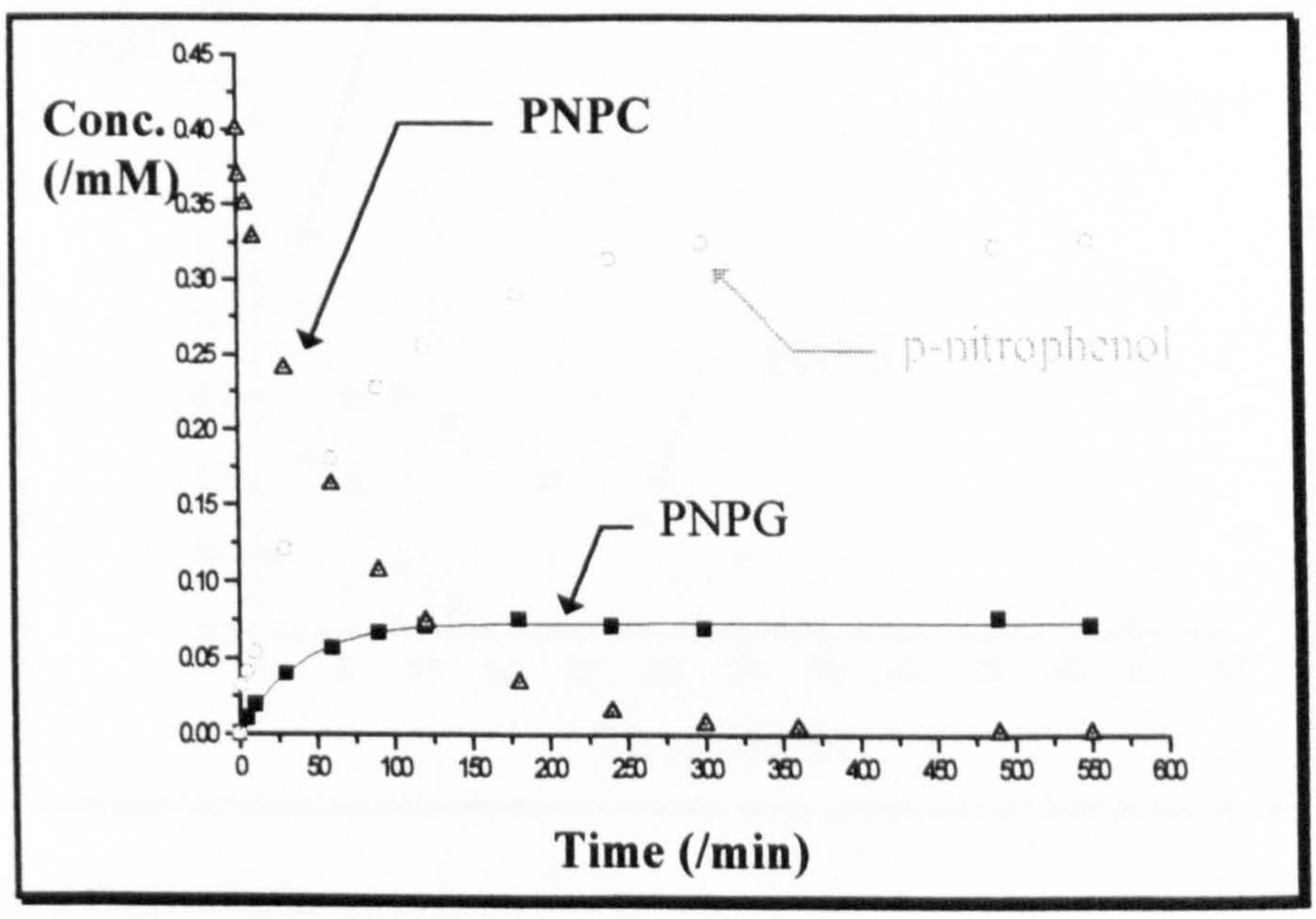
p-nitrophenyl β-D-cellobioside :	0.40 mM
Crude cellulase from *Trichoderma reesei* :	0.23 mg.ml^{-1}
Sodium acetate buffer :	pH 4.5, 0.05 M



2.2. Hydrolysis of PNPC in the presence of β-glucosidase inhibitor: 1,5-glucono-δ-lactone

- *Reaction conditions*

p-nitrophenyl β-D-cellobioside :	0.40 mM
1,5-Glucono-δ-lactone :	20.80 mM
Crude cellulase from *Trichoderma reesei* :	0.23 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, I = 0.05 M



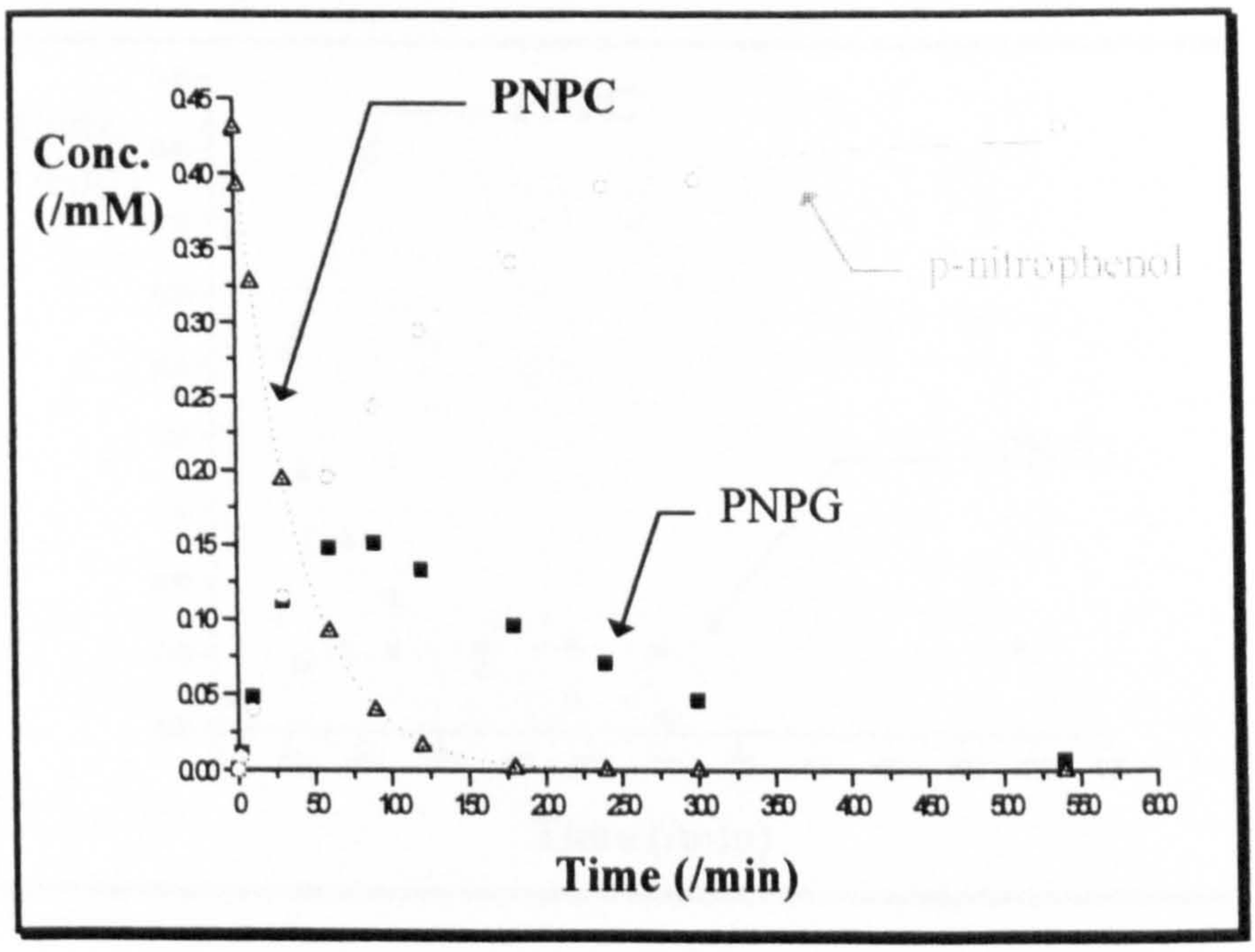
2.3. Transglycosylation studies

- *Reaction conditions*

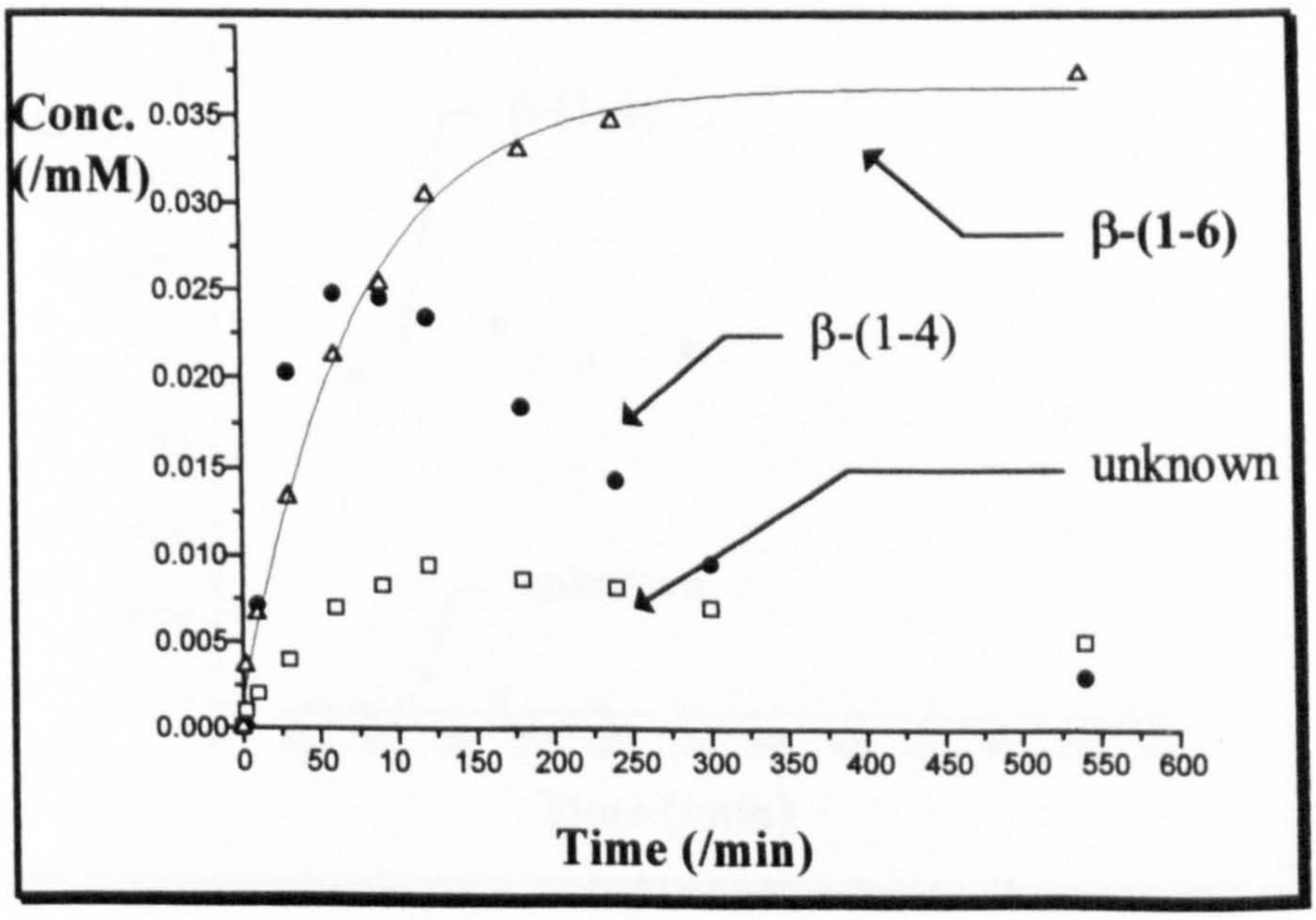
Substrate : p-nitrophenyl β-D-cellobioside :	0.40 mM
Acceptor : N-(p-nitrophenyl)-β-D-glucopyranosylamine :	10.00 mM
Crude cellulase from *Trichoderma reesei* :	0.23 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, I = 0.05 M

Transglycosylation reaction

Substrate hydrolysis



Transglycosylation products



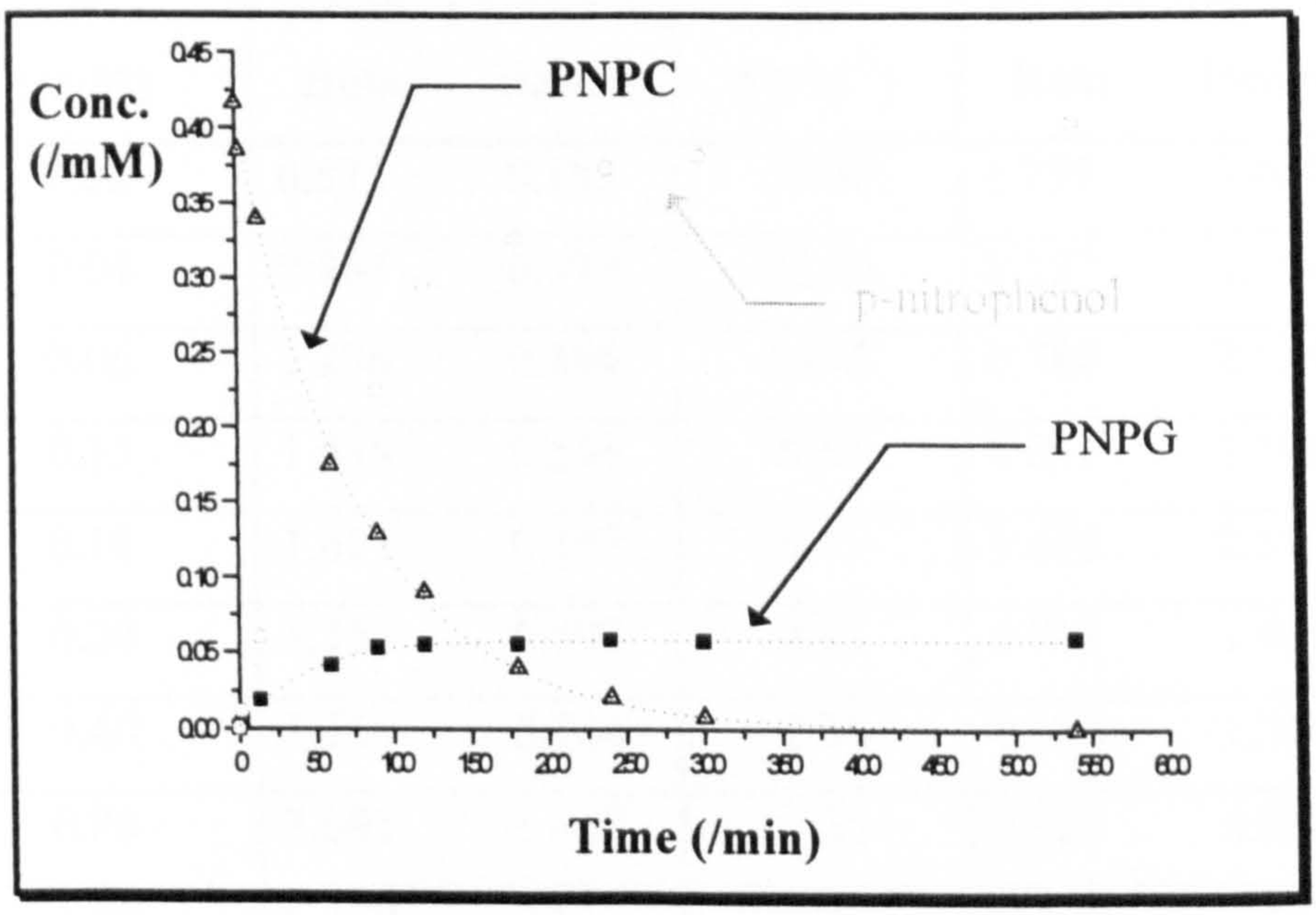
2.4. Transglycosylation with 1,5-glucono-δ-lactone

- *Reaction conditions*

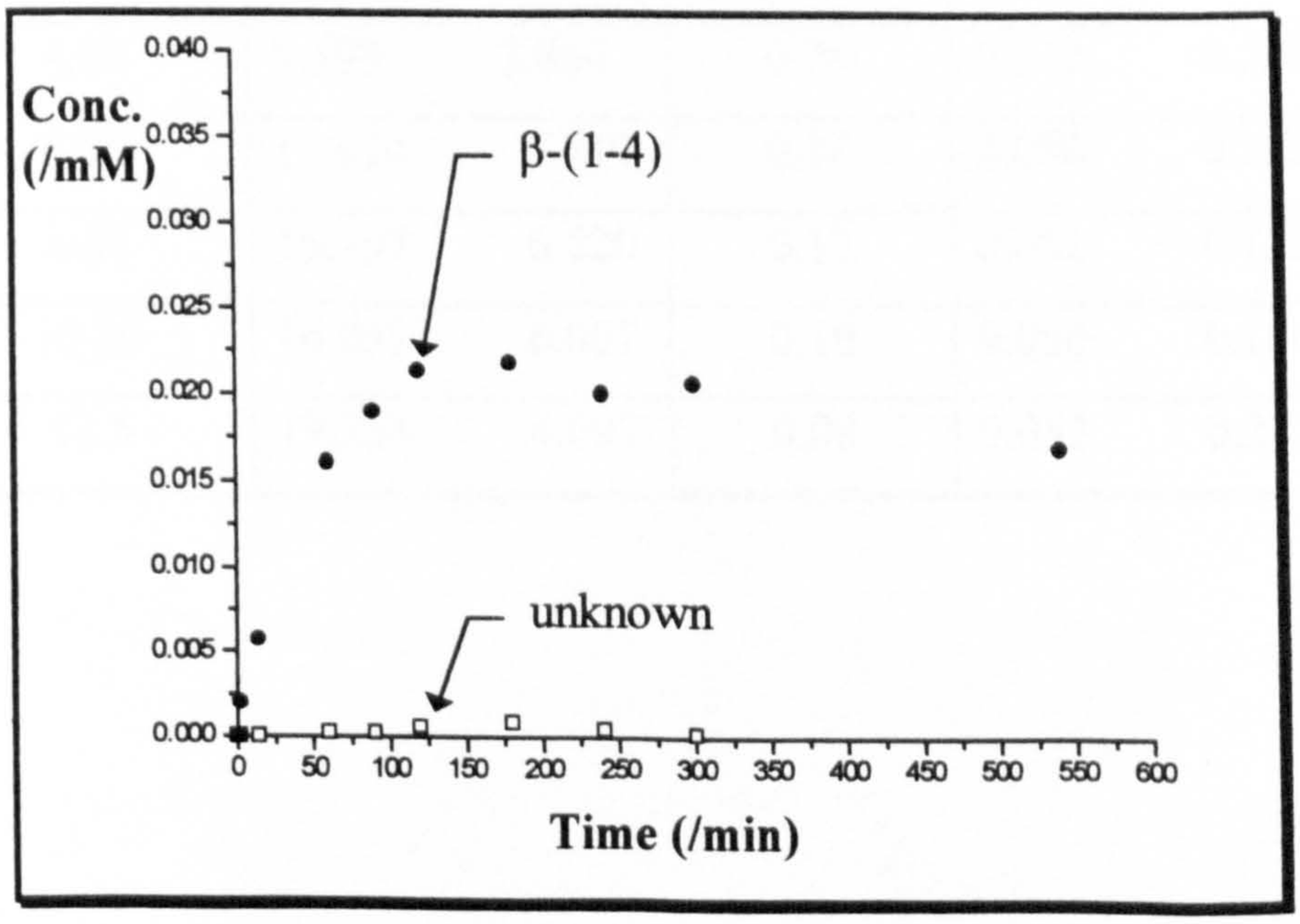
p-nitrophenyl β-D-cellobioside :	0.40 mM
N-(p-nitrophenyl)-β-D-glucopyranosylamine :	10.00 mM
Crude cellulase from *Trichoderma reesei* :	0.23 $mg.ml^{-1}$
1,5-Glucono-δ-lactone :	20.80 mM
Sodium acetate buffer :	pH 4.5, 0.05M

Transglycosylation

Substrate hydrolysis



Transglycosylation products



2.5. p-Nitrophenyl β-D-cellobioside : K_m determination

- Reaction conditions

Substrate : p-nitrophenyl β-D-cellobioside : Variable

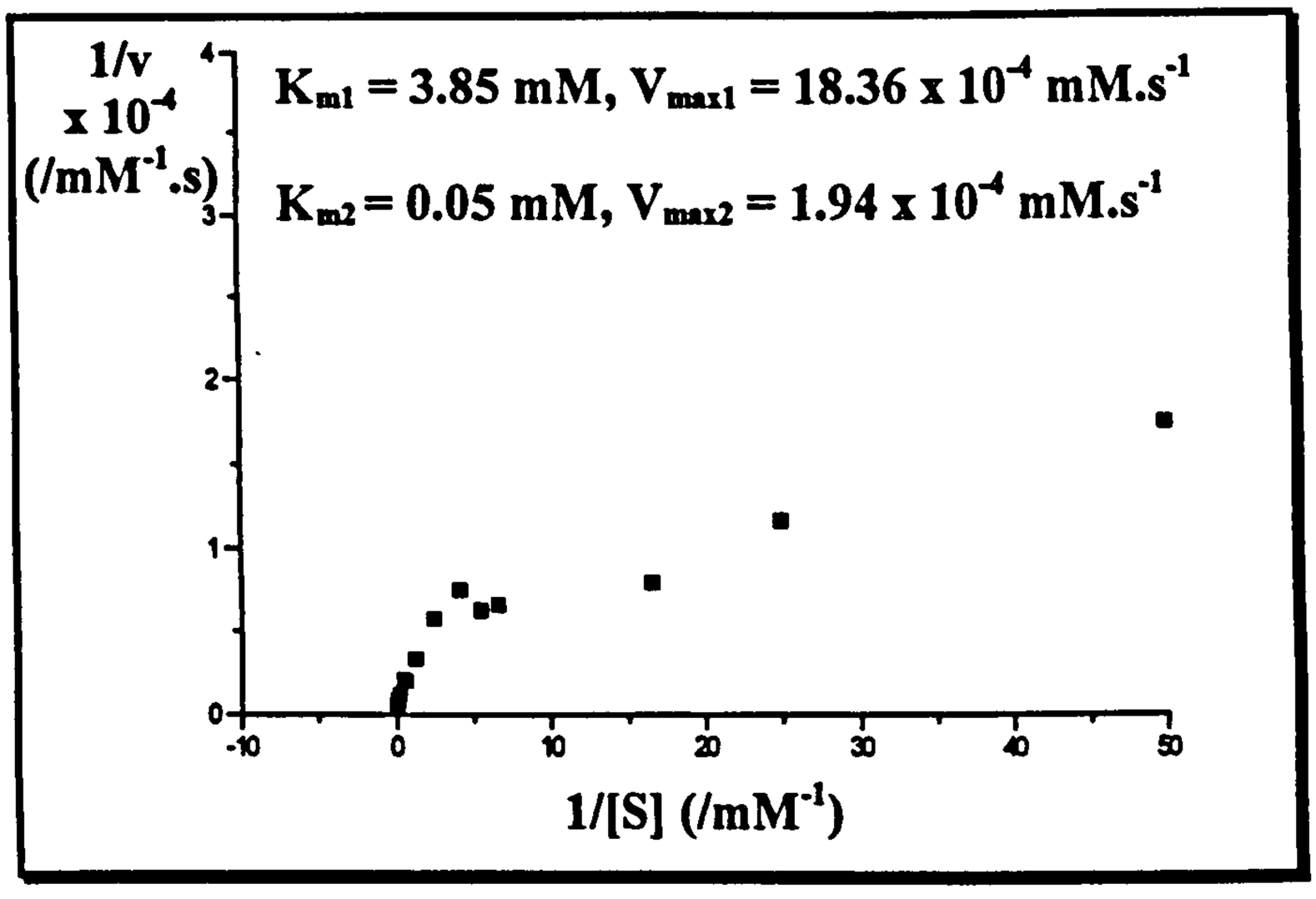
Crude cellulase from *Trichoderma reesei* : 0.23 $mg.ml^{-1}$

Sodium acetate buffer : pH 4.5, I = 0.05 M

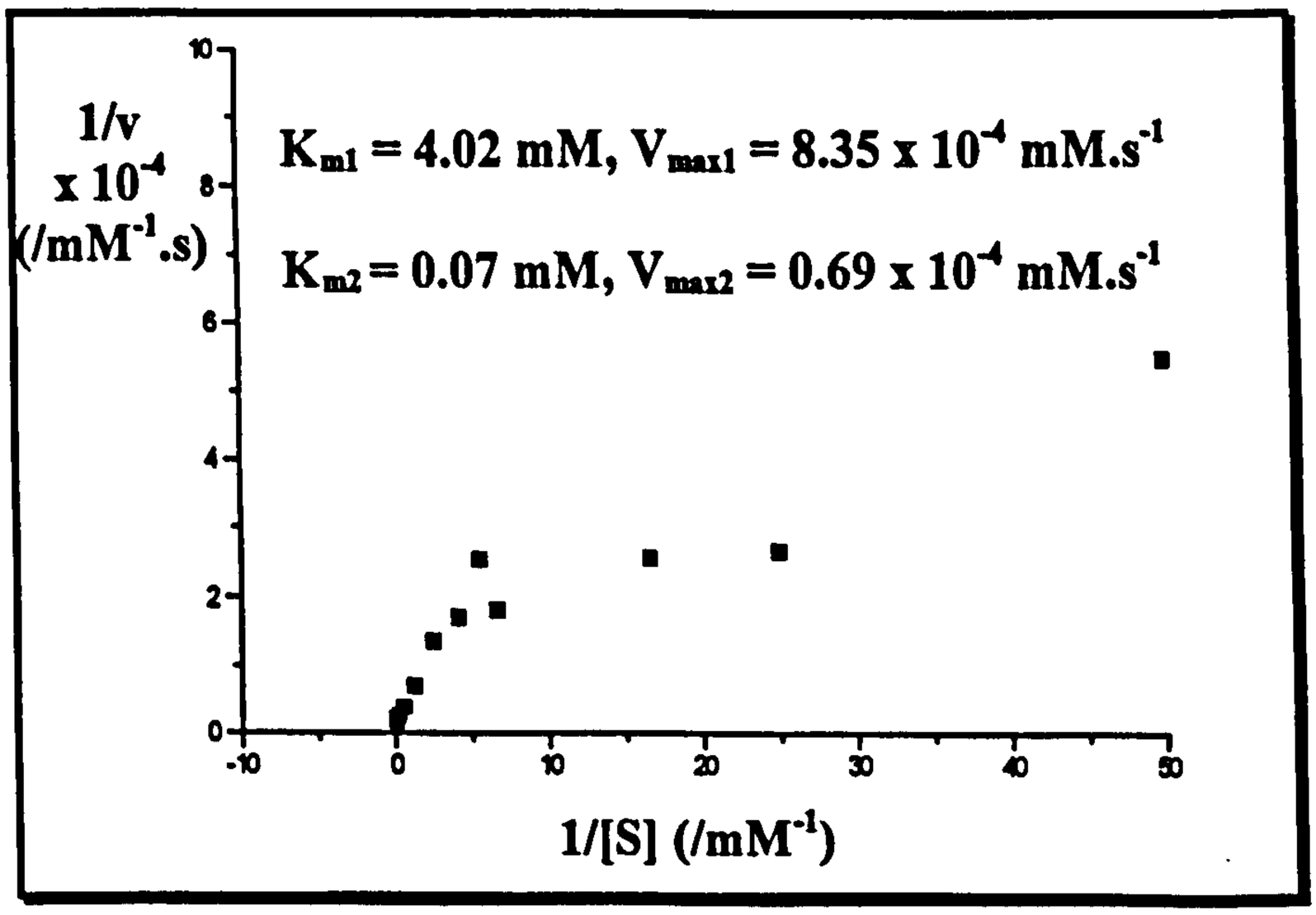
S (/mM)	v x 10^{-4} (/mM.s^{-1}) Holo.	Hetero.	1/S (/mM^{-1})	1/v x 10^4 (/mM^{-1}.s) Holo.	Hetero.
0.02	0.575	0.183	50.00	1.739	5.464
0.04	0.864	0.379	25.00	1.157	2.638
0.06	1.276	0.394	16.67	0.784	2.538
0.15	1.535	0.556	6.67	0.651	1.798
0.18	1.615	0.397	5.55	0.619	2.519
0.24	1.350	0.592	4.17	0.741	1.689
0.40	1.766	0.744	2.50	0.566	1.344
0.80	3.095	1.447	1.25	0.323	0.691
1.60	5.109	2.612	0.62	0.196	0.382
1.95	4.978	2.702	0.51	0.201	0.370
4.00	8.398	3.864	0.25	0.119	0.259
6.20	11.624	5.219	0.16	0.086	0.192
8.20	16.197	6.520	0.12	0.062	0.153
10.20	16.652	6.007	0.10	0.060	0.166
12.5	19.258	4.697	0.08	0.052	0.213

Lineweaver-Burk plots

A) Initial rate of PNPG formation as a function of the substrate concentration



B) Initial rate of p-nitrophenol formation as a function of the substrate concentration



2.6. Acceptor Inhibition Studies

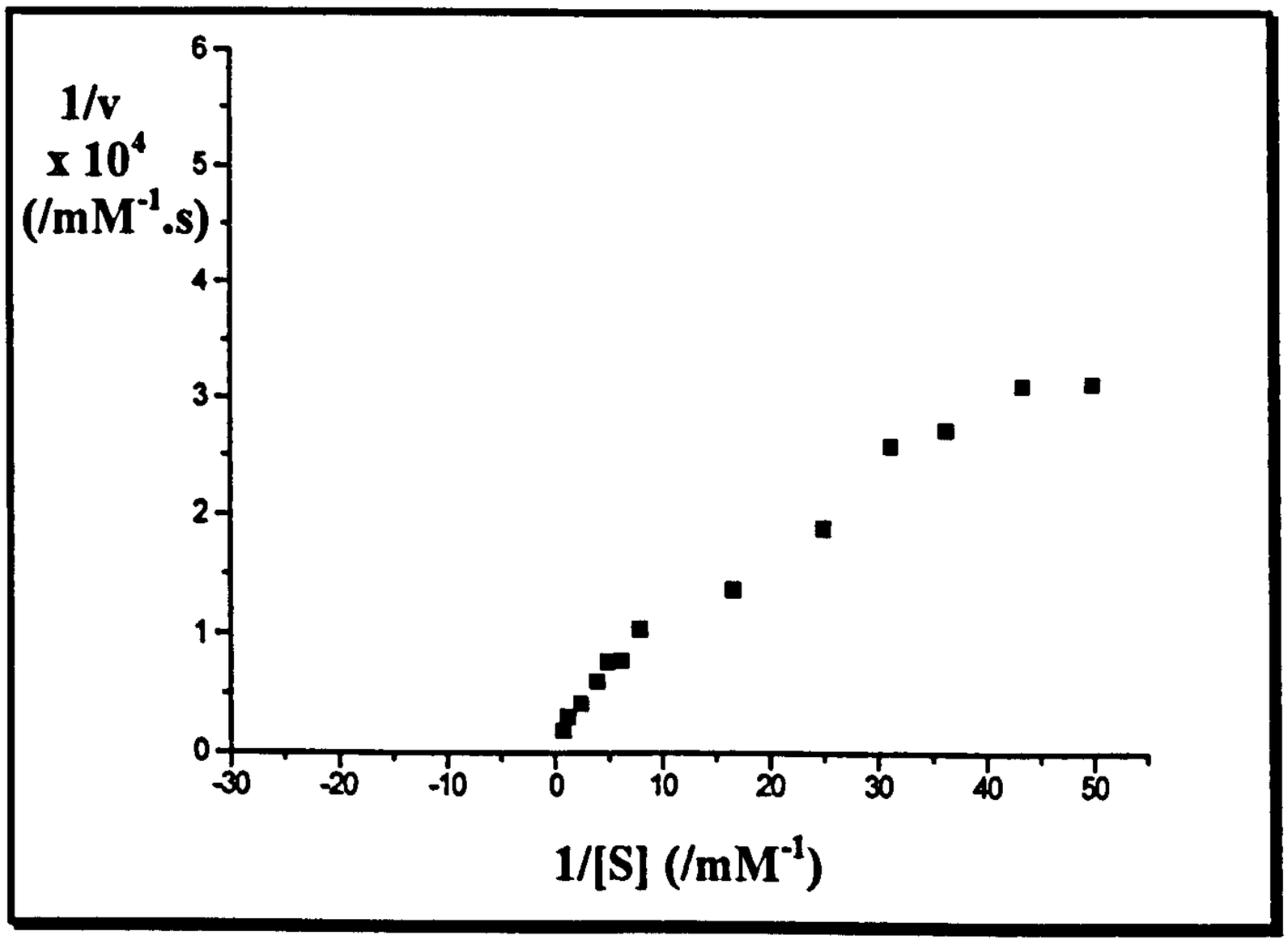
- Reaction conditions

p-nitrophenyl β-D-cellobioside :	0.4 mM
N-(p-nitrophenyl)-β-D-glucopyranosylamine :	*variable*
Crude cellulase from *Trichoderma reesei* :	0.23 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, I = 0.05 M

2.6.1. N-(p-nitrophenyl)-β-D-glucopyranosylamine : 2.00 mM

[S] (/mM)	v x 10^{-4} (/$mM.s^{-1}$)	1/[S] (/mM^{-1})	1/v x 10^4 (/$mM^{-1}.s$)
0.02	0.32	50	3.12
0.023	0.32	43.48	3.12
0.0275	0.37	36.36	2.70
0.032	0.39	31.25	2.56
0.04	0.53	25.00	1.89
0.06	0.73	16.67	1.37
0.125	0.97	8.00	1.03
0.16	1.29	6.25	0.77
0.2	1.31	5.00	0.76
0.25	1.67	4.00	0.60
0.4	2.45	2.50	0.41
0.8	3.42	1.25	0.29
1.2	5.48	0.83	0.18

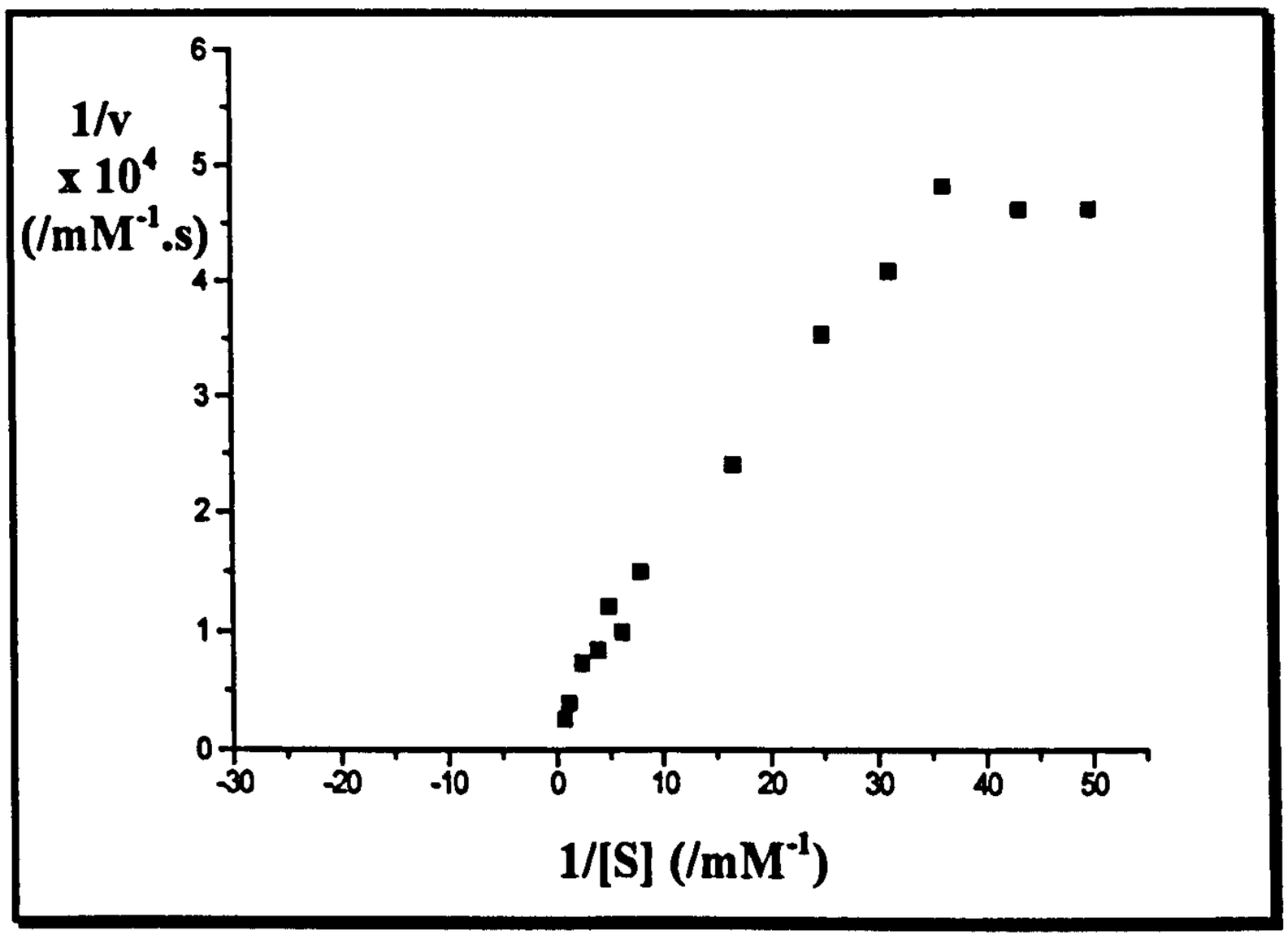
Lineweaver-Burk plot



2.6.2. N-(p-nitrophenyl)-β-D-glucopyranosylamine : 6.00 mM

[S] (/mM)	v x 10^{-4} (/mM.s^{-1})	1/[S] (/mM^{-1})	1/v x 10^{4} (/mM^{-1}.s)
0.02	0.22	50.00	4.54
0.023	0.22	43.48	4.54
0.0275	0.21	36.36	4.76
0.032	0.24	31.25	4.17
0.04	0.28	25.00	3.57
0.06	0.42	16.67	2.38
0.125	0.67	8.00	1.49
0.16	1.01	6.25	0.99
0.2	0.83	5.00	1.20
0.25	1.20	4.00	0.83
0.4	1.38	2.50	0.72
0.8	2.60	1.25	0.38
1.2	3.87	0.83	0.26

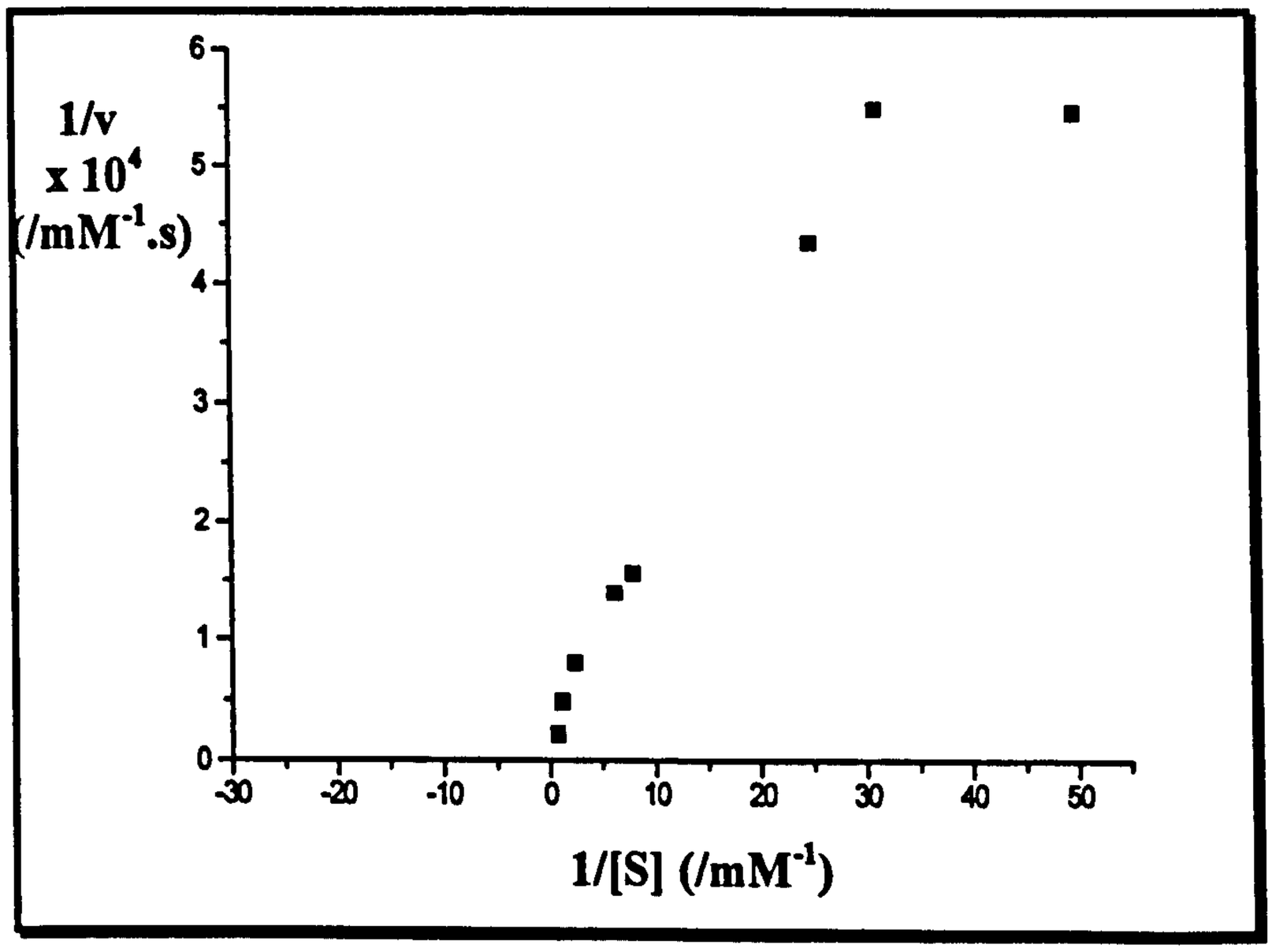
Lineweaver-Burk plot



2.6.3. N-(p-nitrophenyl)-β-D-glucopyranosylamine : 10.00 mM

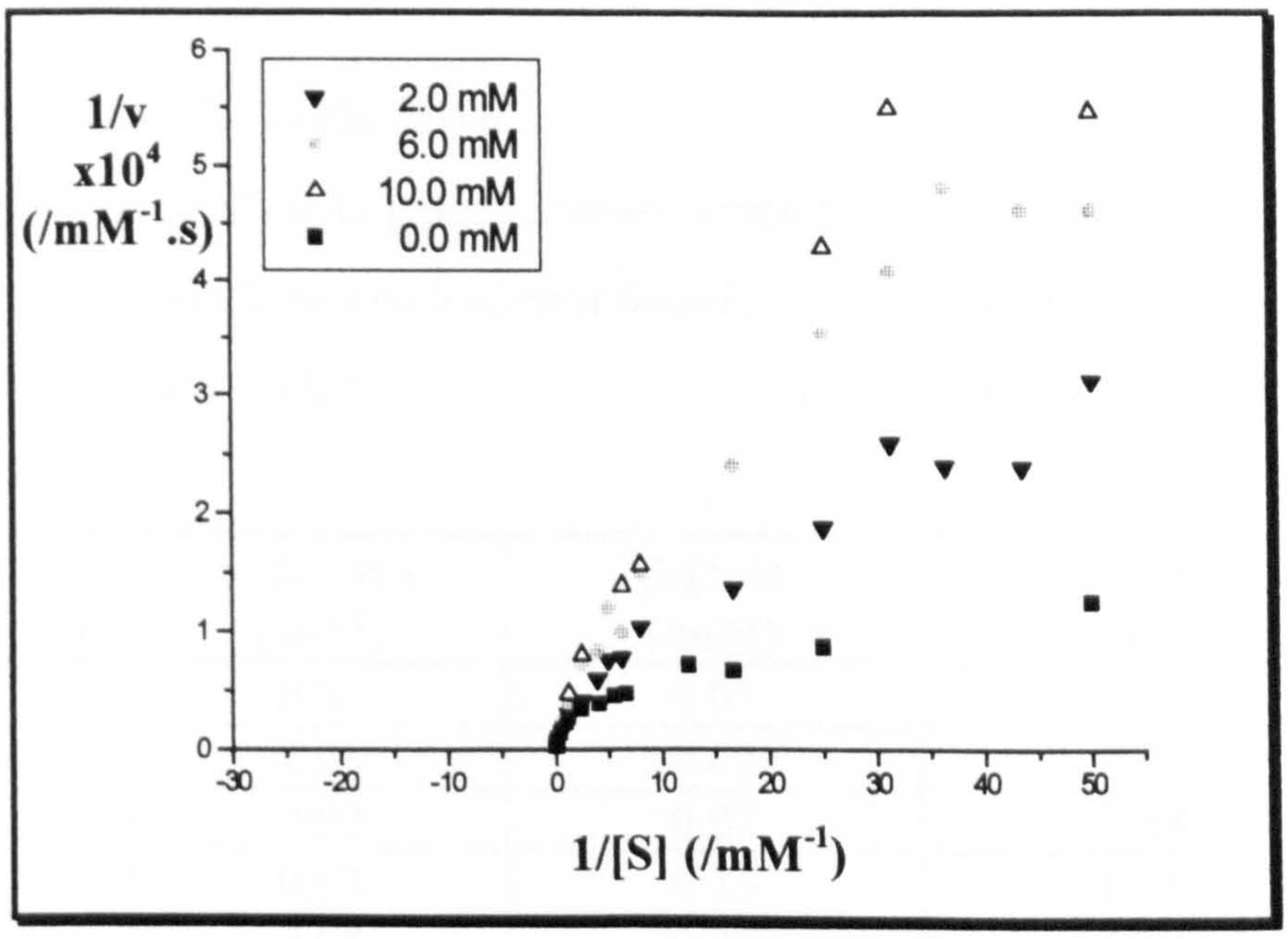
[S] (/mM)	v x 10^{-4} (/mM.s^{-1})	1/[S] (/mM^{-1})	1/v x 10^{4} (/mM^{-1}.s)
0.02	0.18	50.00	5.55
0.032	0.18	31.25	5.55
0.04	0.23	25.00	4.35
0.125	0.64	8.00	1.56
0.16	0.72	6.25	1.39
0.40	1.25	2.50	0.80
0.80	2.10	1.25	0.48
1.20	4.75	0.83	0.21

Lineweaver-Burk plot

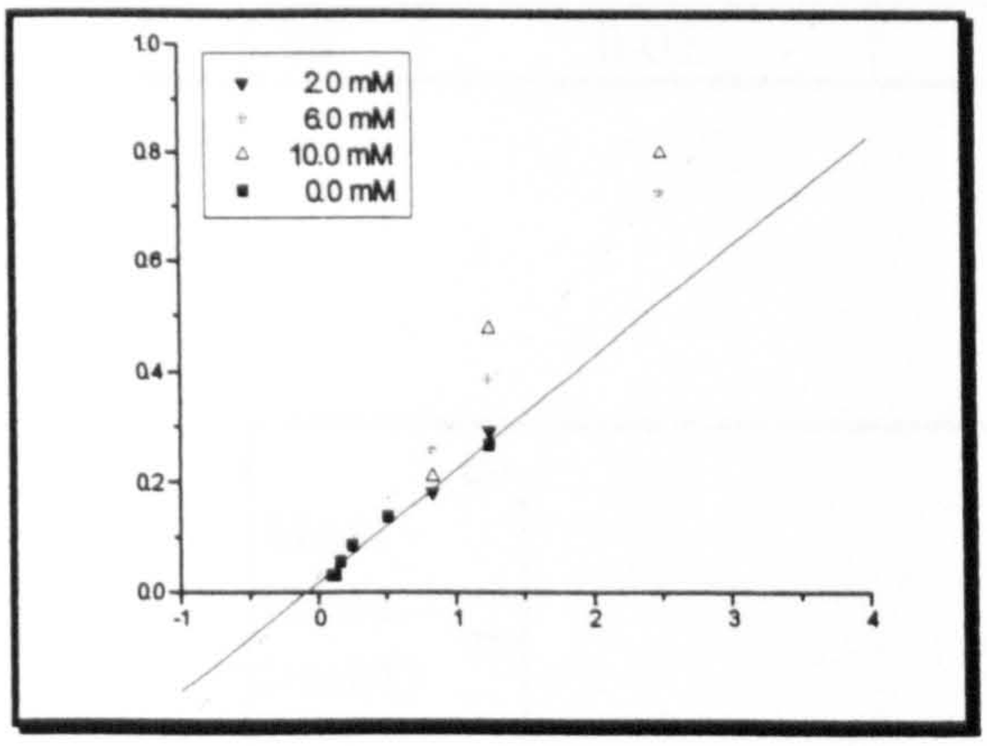


Lineweaver-Burk plots

for N-(p-nitrophenyl)-β-D-glucopyranosylamine

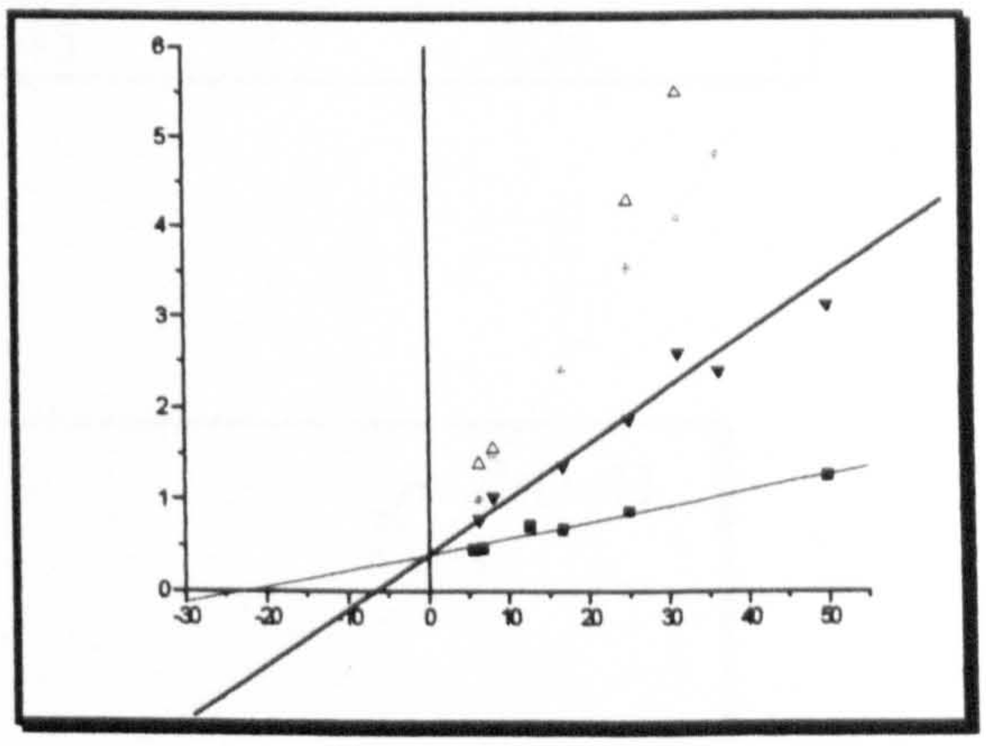


High substrate concentration



Inhibition type ?

Low substrate concentration



Competitive inhibitor

$(K_i)_{av}$ = 1.4 mM

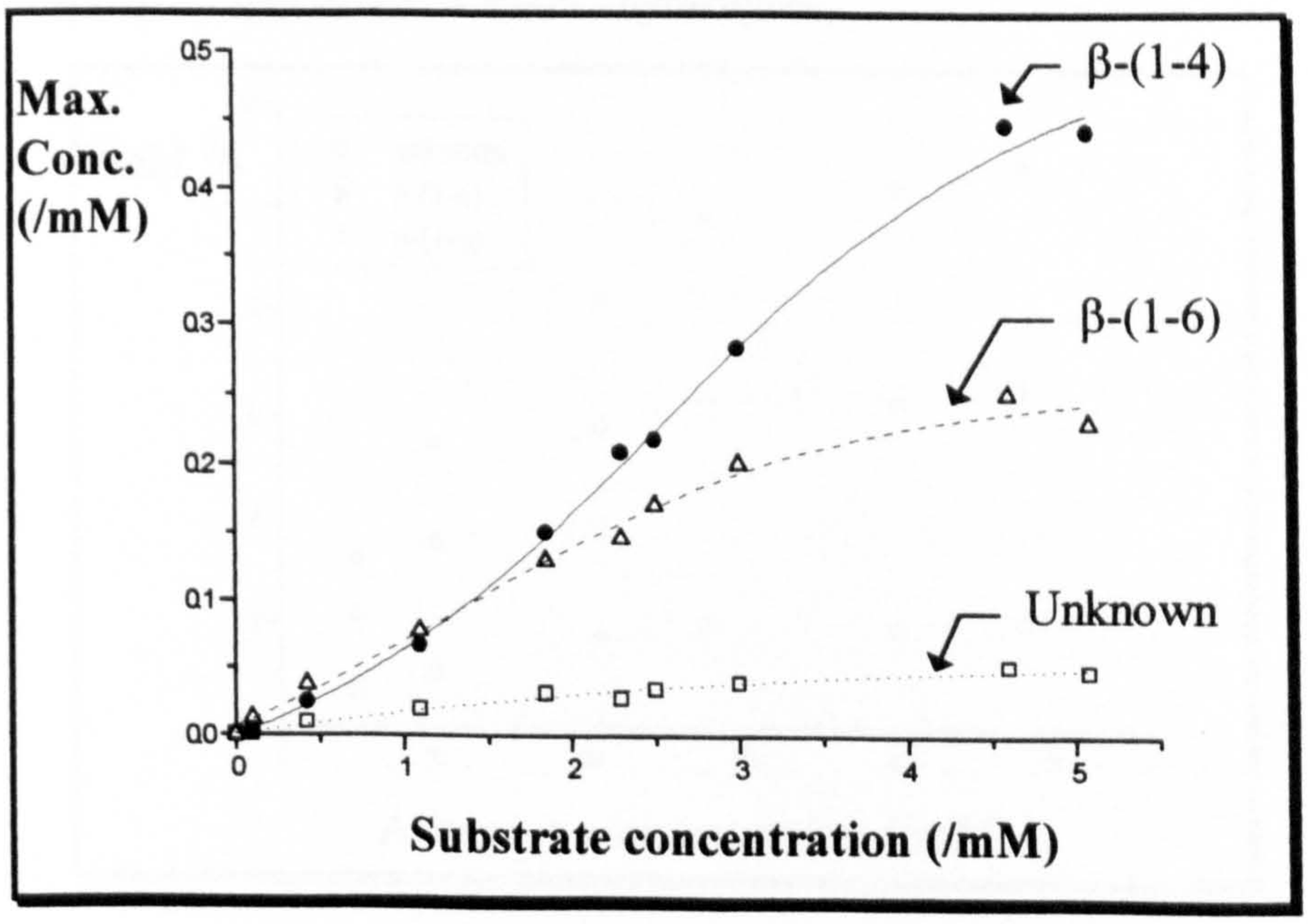
with $(K_m)_{app} = K_m (1+ [I]/K_i)$

2.7. Substrate Concentration Studies- Effect on transglycosylation products yield

- Reaction conditions

p-nitrophenyl β-D-cellobioside :	*variable*
N-(p-nitrophenyl)-β-D-glucopyranosylamine :	10.00 mM
Crude cellulase from *Trichoderma reesei* :	0.23 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, 0.05 M

PNPC (/mM)	unknown (/mM)	β-(1-4) (/mM)	β-(1-6) (/mM)
0.10	0.00	0.01	0.01
0.425	0.01	0.02	0.04
1.10	0.02	0.07	0.08
1.85	0.03	0.15	0.13
2.30	0.03	0.21	0.15
2.50	0.03	0.22	0.17
3.00	0.04	0.28	0.20
4.60	0.05	0.45	0.25
5.08	0.05	0.45	0.26



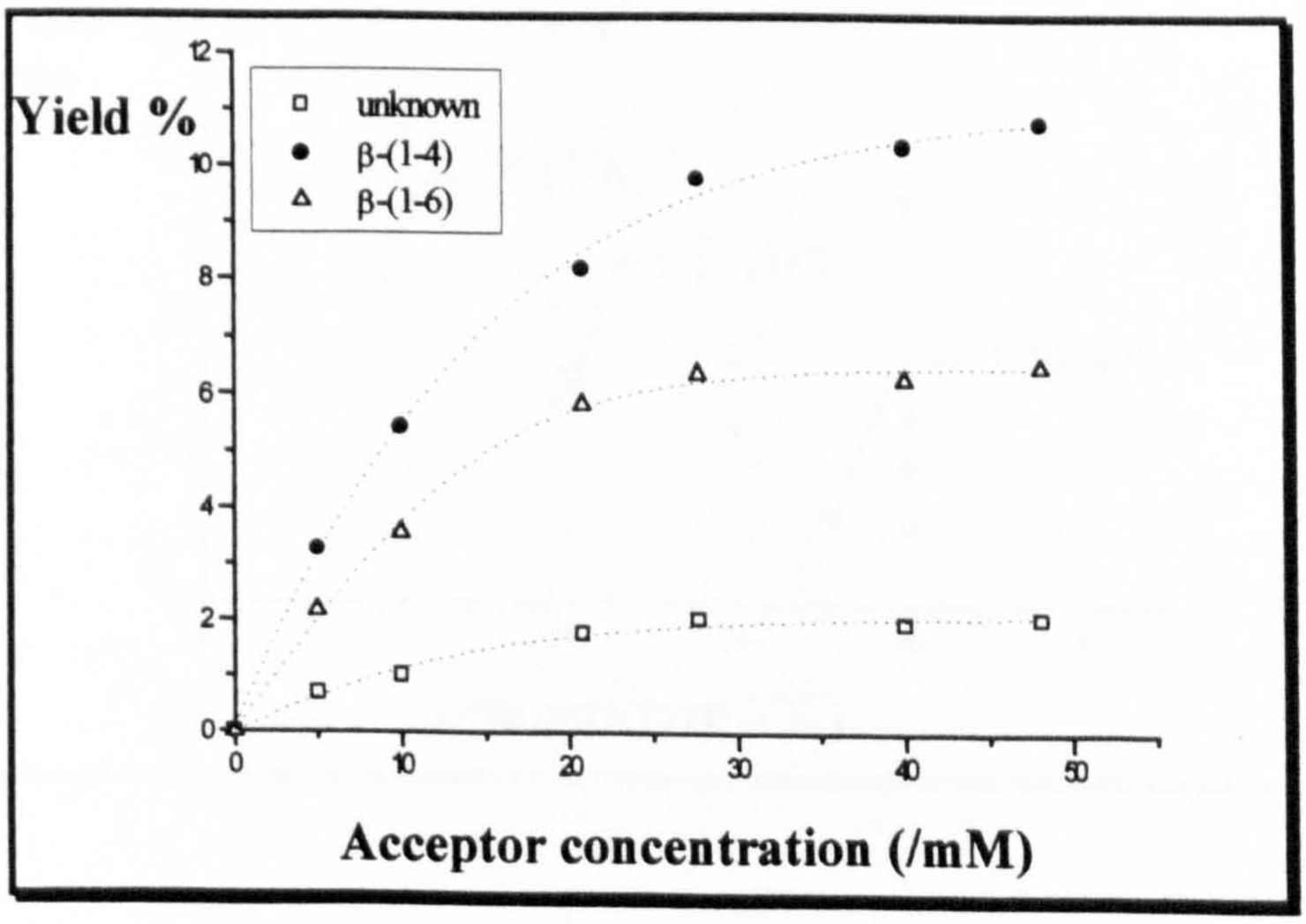
2.8. Acceptor Concentration Studies- Effect on transglycosylation products yield

- Reaction conditions

p-nitrophenyl β-D-cellobioside :	2.50 mM
N-(p-nitrophenyl)-β-D-glucopyranosylamine :	*variable*
Crude cellulase from *Trichoderma reesei* :	0.23 $mg.ml^{-1}$
Acetonitrile :	10 %
Sodium acetate buffer :	pH 4.5 (10% ACN), 0.05M

Acceptor (/mM)	**unknown (/mM)**	**β-(1-4) (/mM)**	**β-(1-6) (/mM)**
5.0	0.03	0.08	0.15
10.0	0.04	0.13	0.22
20.8	0.05	0.21	0.22
27.7	0.06	0.25	0.25
40.0	0.05	0.25	0.21
48.1	0.05	0.26	0.17

Yields correspond to that at 75 % PNPC hydrolysis.



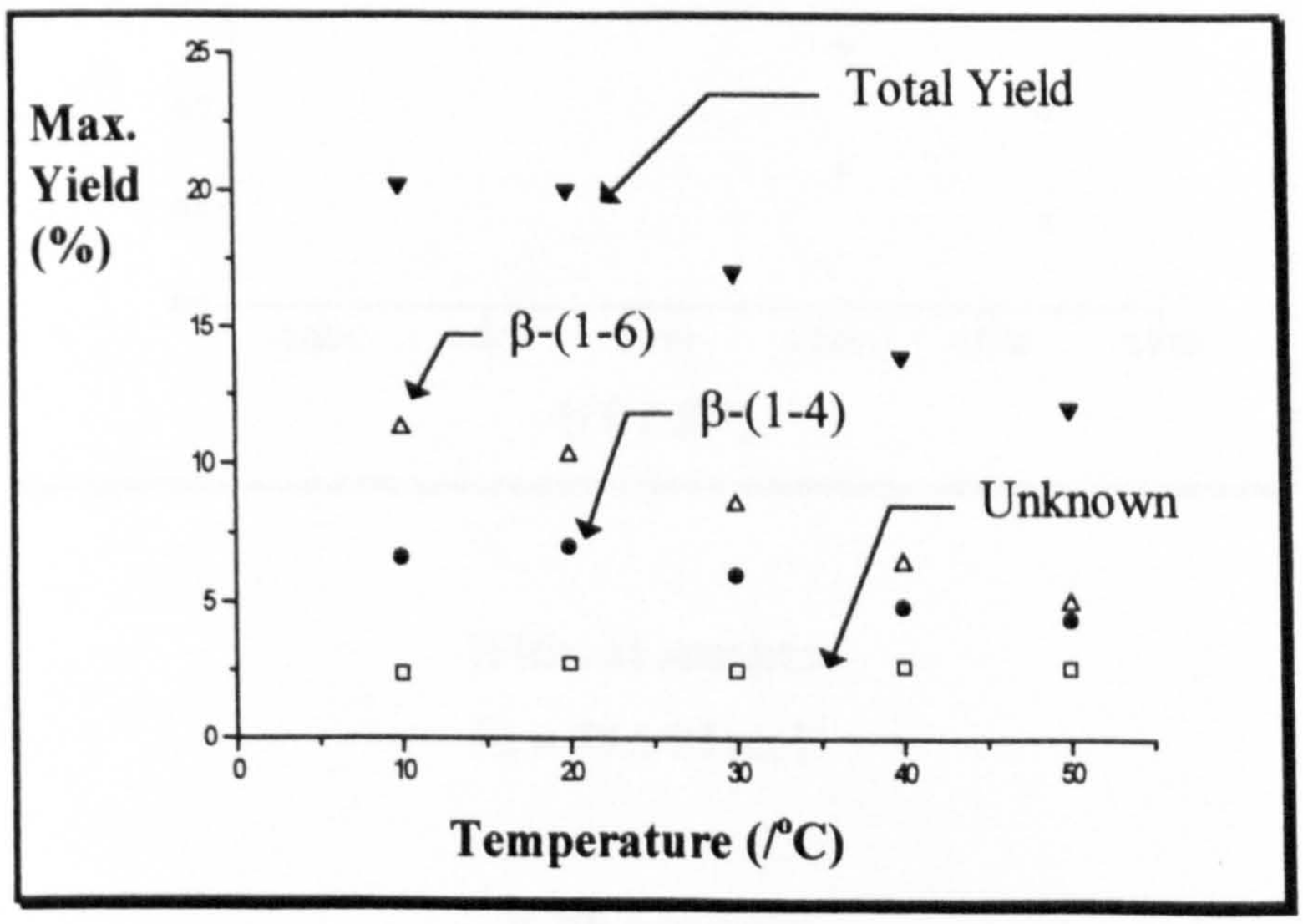
2.9. Temperature Studies

- Reaction conditions

p-nitrophenyl β-D-cellobioside :	0.40 mM,
N-(p-nitrophenyl)-β-D-glucopyranosylamine :	10.00 mM,
Crude cellulase from *Trichoderma reesei* :	0.42 $mg.ml^{-1}$,
Sodium acetate buffer :	pH 4.5, 0.1 M,
Temperature :	*variable.*

Temperature (/°C)	% Yield unknown	% Yield β-(1-4)	% Yield β-(1-6)	Total % Yield
10	2.3	6.6	11.2	20.2
20	2.7	7.0	10.2	20.0
30	2.5	6.0	8.5	17.0
40	2.7	4.8	6.4	13.9
50	2.6	4.4	5.0	12.0

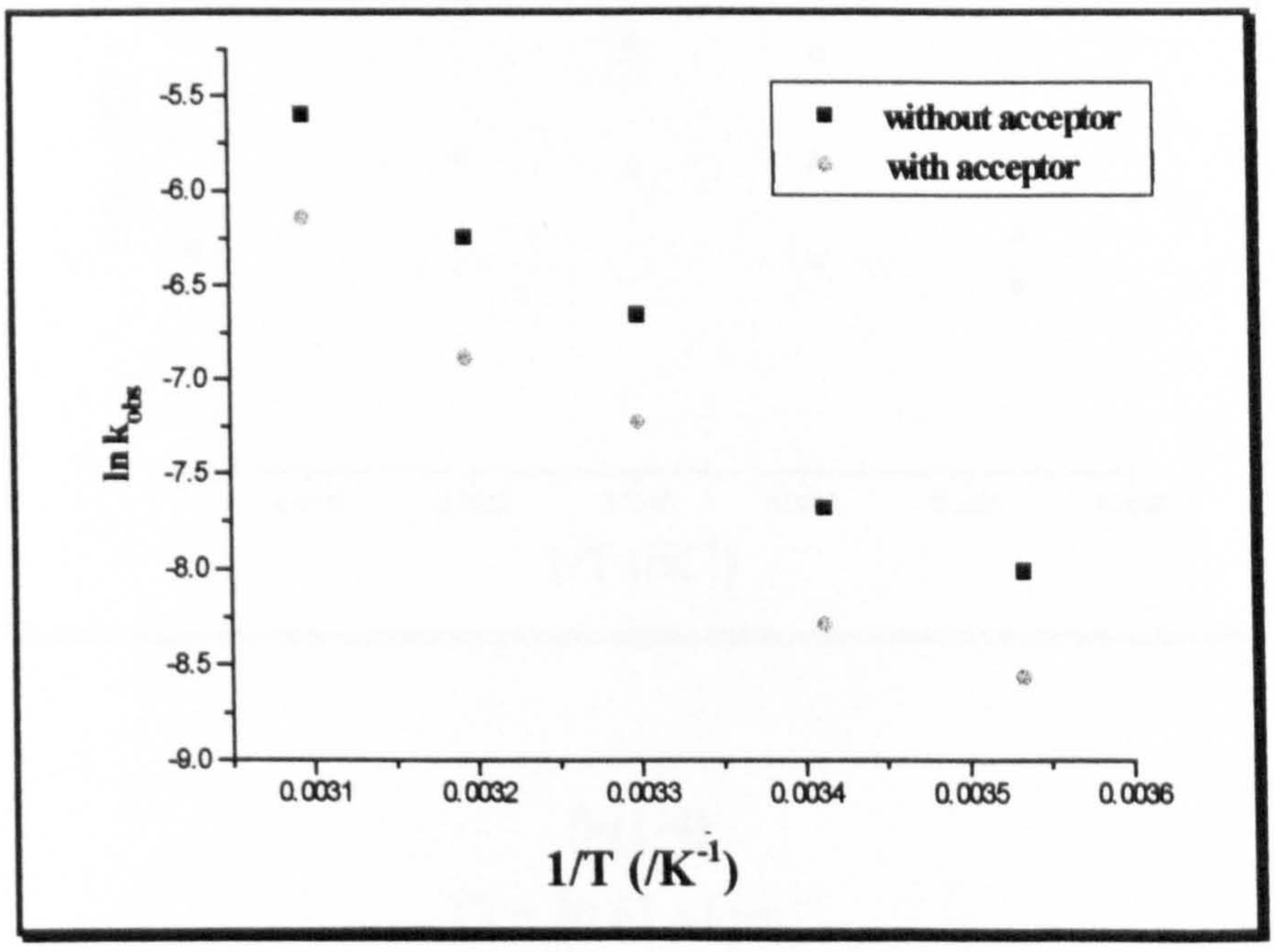
% Maximum yield for the individual transfer product



i) Activation energy for hydrolysis

T (/K)	k_{obs} x 10^{-4} ($/s^{-1}$)	k_{obs} x 10^{-4} ($/s^{-1}$)	1/T ($/K^{-1}$)	ln k_{obs}, no acceptor	ln k_{obs}, with acceptor
50°C (323)	36.91	21.39	0.0031	-5.60	-6.15
40 °C (313)	19.25	10.22	0.0032	-6.25	-6.89
30 °C (303)	12.84	7.22	0.0033	-6.66	-7.23
20 °C (293)	4.62	2.46	0.0034	-7.68	-8.29
10°C (283)	3.30	1.93	0.0035	-8.02	-8.57

$$\ln k_{obs} = \ln A - E_a/RT$$



Without acceptor

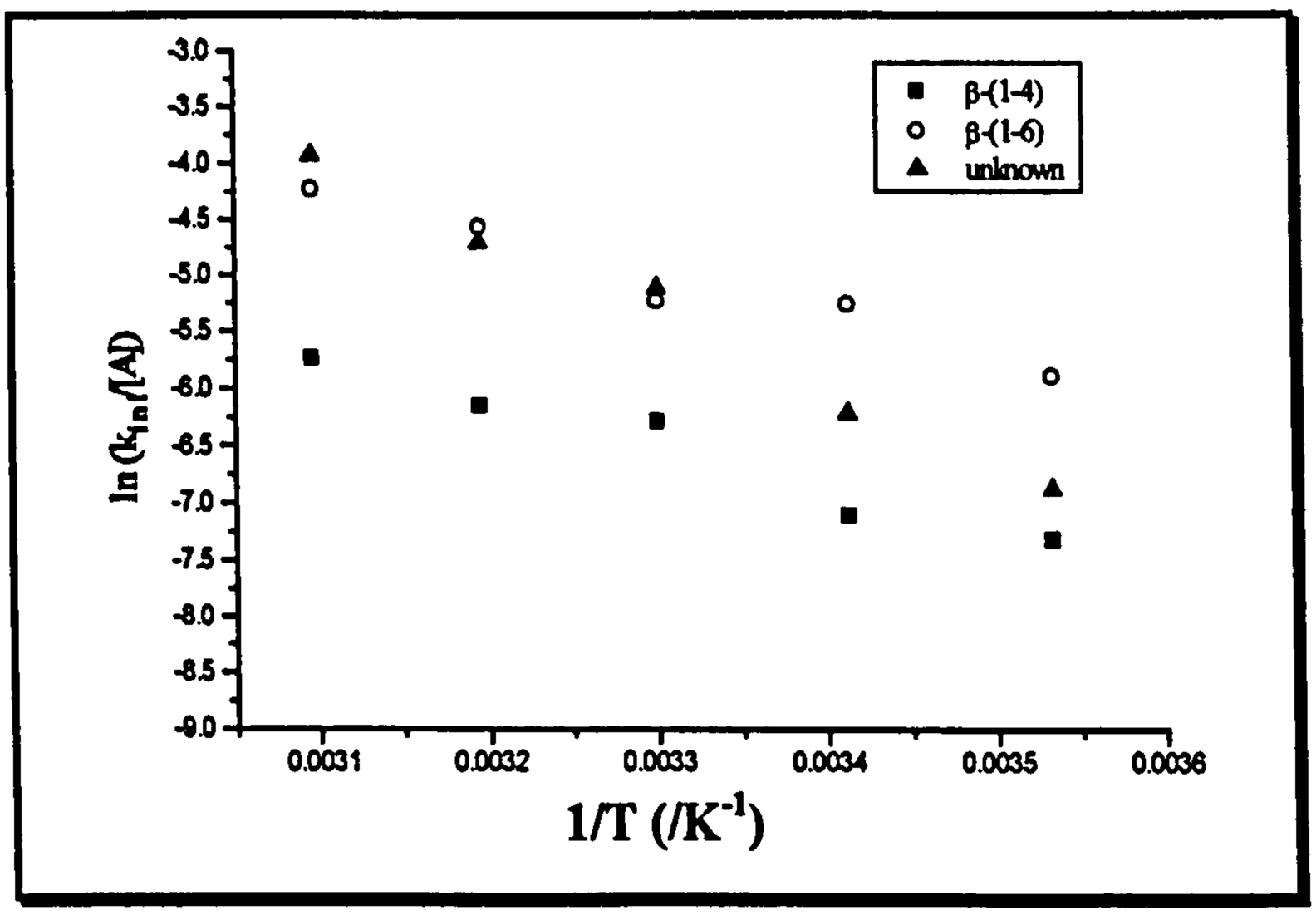
$E_a = 49.1$ kJ.mol^{-1}

With acceptor

$E_a = 54.2$ kJ.mol^{-1}

ii) Activation energy for transglycosylation

T (/°C)	k_{ini}/[A] (/s^{-1}) β-(1-4)	k_{ini}/[A] (/s^{-1}) β-(1-6)	k_{ini}/[A] (/s^{-1}) unknown	1/T (/K^{-1})	ln k_{ini} β-(1-4)	ln k_{ini} β-(1-6)	ln k_{ini} unknown
10	0.066	0.028	0.010	0.00353	-7.32	-5.89	-6.88
20	0.082	0.052	0.020	0.00341	-7.11	-5.26	-6.22
30	0.183	0.053	0.060	0.00300	-6.30	-5.23	-5.12
40	0.212	0.103	0.090	0.00319	-6.16	-4.57	-4.71
50	0.321	0.146	0.196	0.00310	-5.74	-4.23	-3.93



β-(1-4)

$E_a = 30.61$ kJ.mol^{-1}

β-(1-6)

$E_a = 29.83$ kJ.mol^{-1}

unknown

$E_a = 55.20$ kJ.mol^{-1}

2.10. Co-solvent Studies

- Reaction conditions

p-nitrophenyl β-D-cellobioside : 0.40 mM

N-(p-nitrophenyl)-β-D-glucopyranosylamine : 10.00 mM

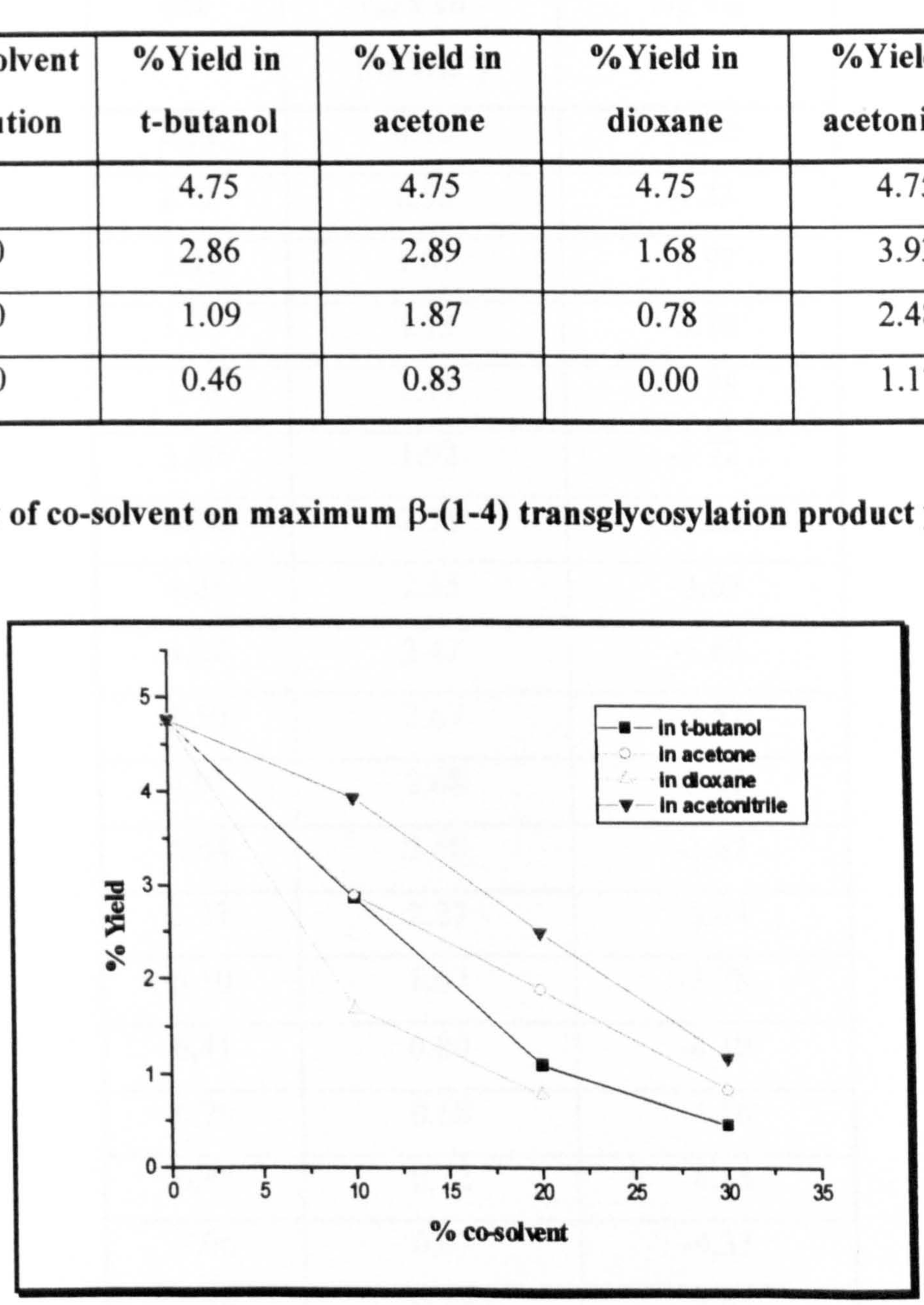
Crude cellulase from *Trichoderma reesei* : 0.23 $mg.ml^{-1}$

Sodium acetate buffer : pH 4.5, 0.05 M

Co-solvent : *t-butanol, acetone, acetonitrile, or dioxane.*

% co-solvent in solution	%Yield in t-butanol	%Yield in acetone	%Yield in dioxane	%Yield in acetonitrile
0	4.75	4.75	4.75	4.75
10	2.86	2.89	1.68	3.93
20	1.09	1.87	0.78	2.48
30	0.46	0.83	0.00	1.17

Effect of co-solvent on maximum β-(1-4) transglycosylation product yield



2.11. pH Rates Profiles

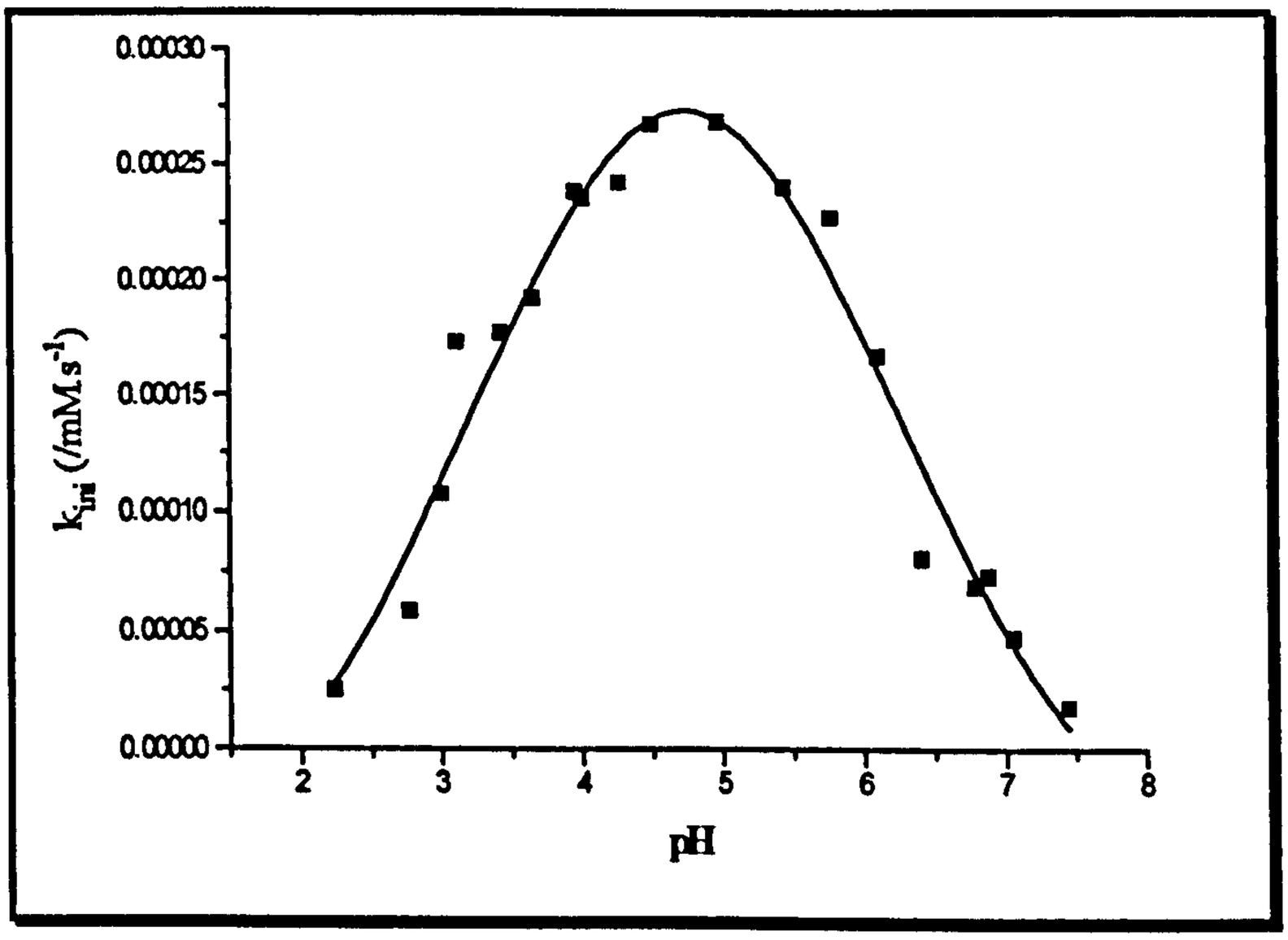
2.11.1. No co-solvent, no acceptor

• Reaction conditions

p-nitrophenyl β-D-cellobioside : 0.40 mM,
Crude cellulase from *Trichoderma reesei* : 0.23 $mg.ml^{-1}$
(Lot 95H0087, 6.6 units/mg)
Buffer : pH variable, 0.05M,

pH	$v_{ini} \times 10^{-4}$ ($/mM.s^{-1}$)	log v_{ini}
2.24	0.30	-4.52
2.78	0.58	-4.23
3.01	1.07	-3.97
3.12	1.73	-3.76
3.43	1.77	-3.75
3.66	1.92	-3.72
3.96	2.38	-3.62
4.01	2.35	-3.63
4.27	2.42	-3.62
4.50	2.67	-3.57
4.97	2.68	-3.57
5.44	2.40	-3.62
5.77	2.27	-3.64
6.10	1.67	-3.78
6.41	0.80	-4.10
6.79	0.68	-4.16
6.88	0.72	-4.14
7.06	0.47	-4.33
7.45	0.18	-4.75

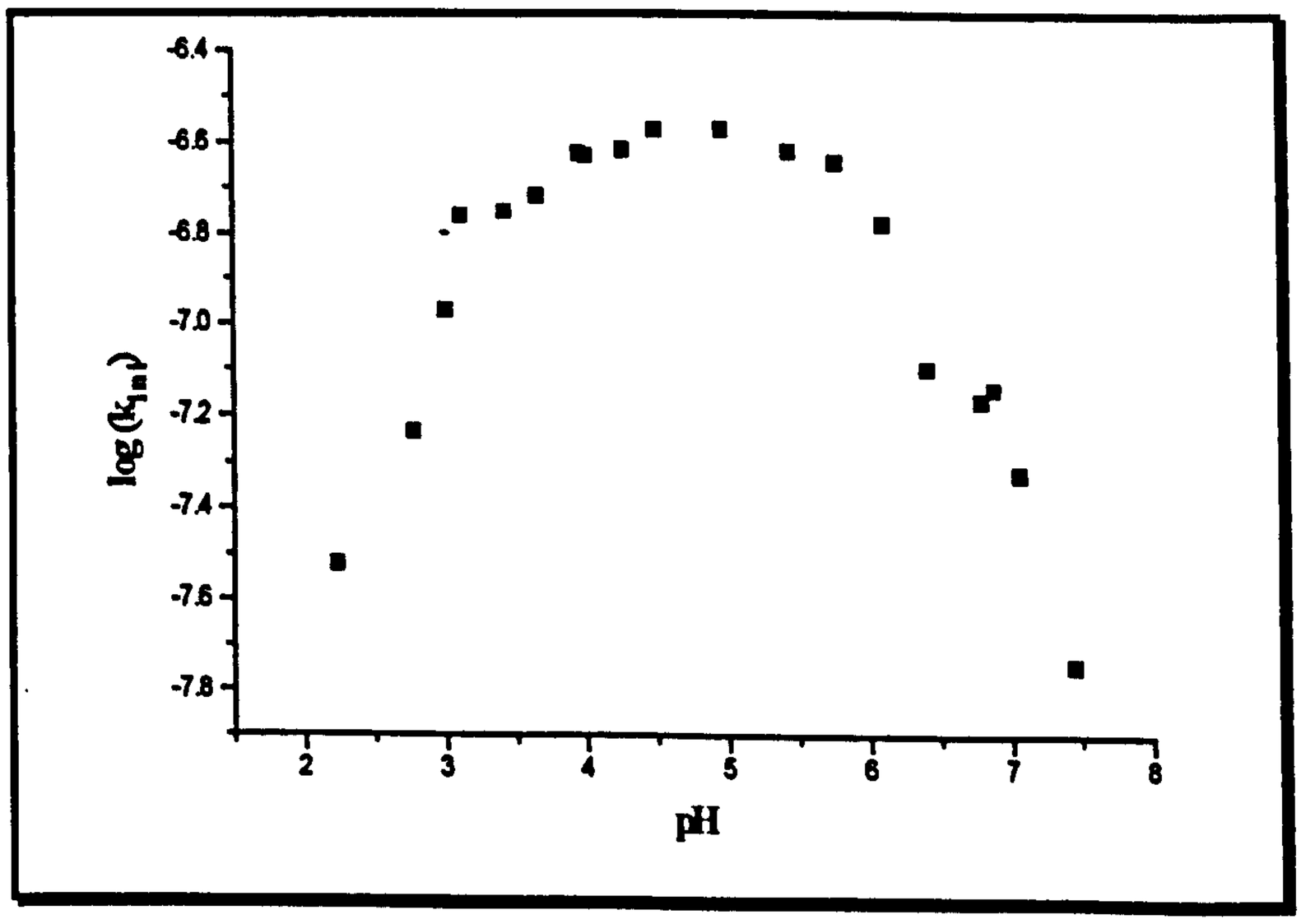
pH rate profile



The selective fitting of the data to the equation for two overlapping sigmoidal dependent rate constants, using Enzfitter, gave the apparent pK_as values as:

$pK_{a1} = 3.2$

$pK_{a2} = 6.2$



2.11.2. pH rate profile in 24% acetonitrile

- Reaction conditions

p-nitrophenyl β-D-cellobioside : 0.40 mM

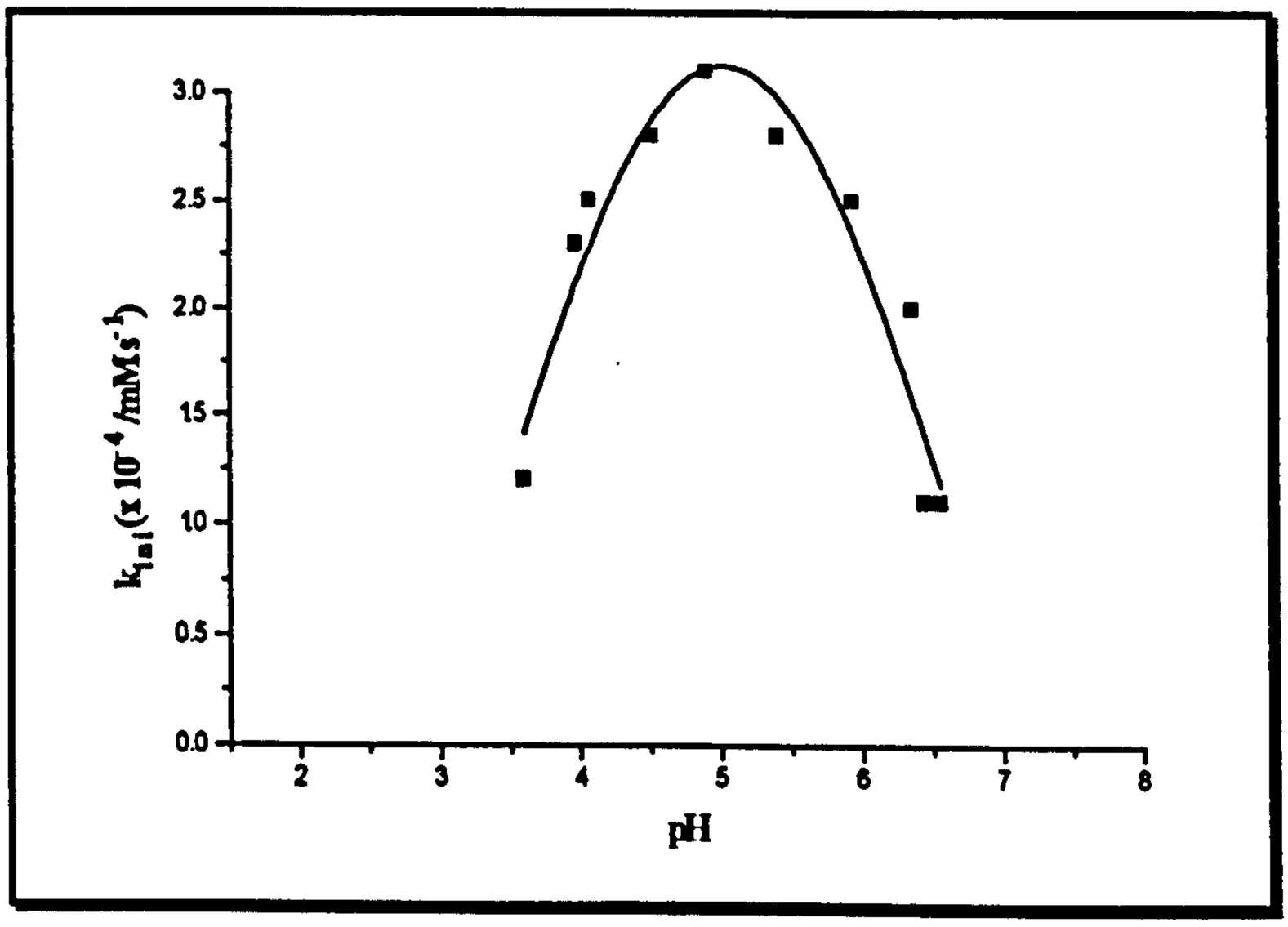
Crude cellulase from *Trichoderma reesei* : 1.26 $mg.ml^{-1}$

(Lot 74H0590, 4.8 units/mg)

Buffer : pH variable, 0.05 M

pH	$v_{ini} \times 10^{-4}$ ($/mM.s^{-1}$)	log v_{ini}
3.6	1.20	-3.93
3.97	2.30	-3.63
4.07	2.50	-3.60
4.51	2.80	-3.55
4.90	3.10	-3.51
5.40	2.80	-3.56
5.93	2.50	-3.61
6.35	2.00	-3.70
6.44	1.10	-3.94
6.55	1.10	-3.95

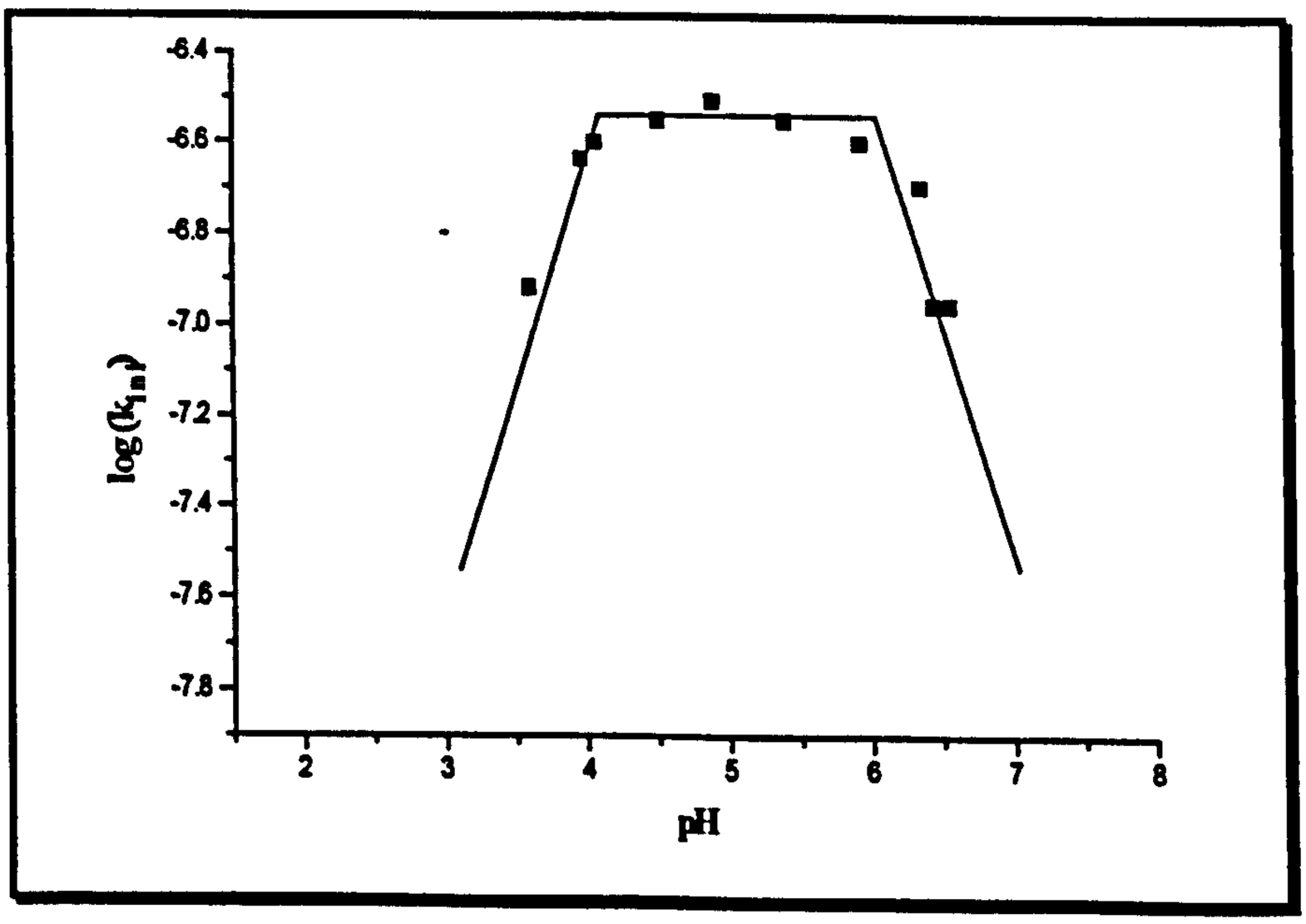
pH rate profile with 24% acetonitrile



The apparent pK_a values were established as :

$pK_{a1} = 3.7$

$pK_{a2} = 6.3$



2.11.3. pH rate profile with acceptor and no co-solvent

- Reaction conditions

p-nitrophenyl β-D-cellobioside : 0.40 mM

N-(p-nitrophenyl)-β-D-glucopyranosylamine : 10.00 mM

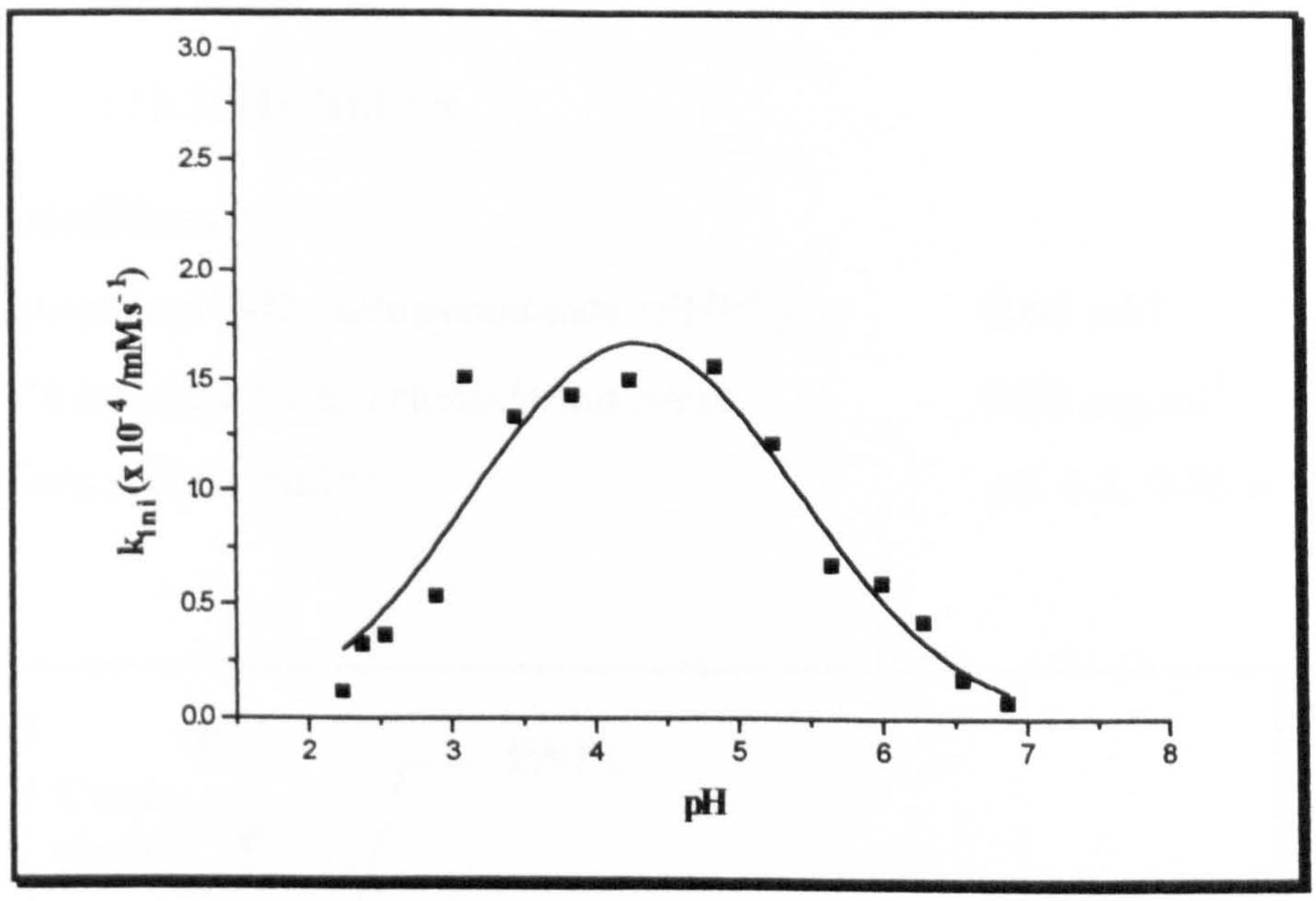
Crude cellulase from *Trichoderma reesei* : 0.23 $mg.ml^{-1}$

(Lot 95H0087, 6.6 units/mg)

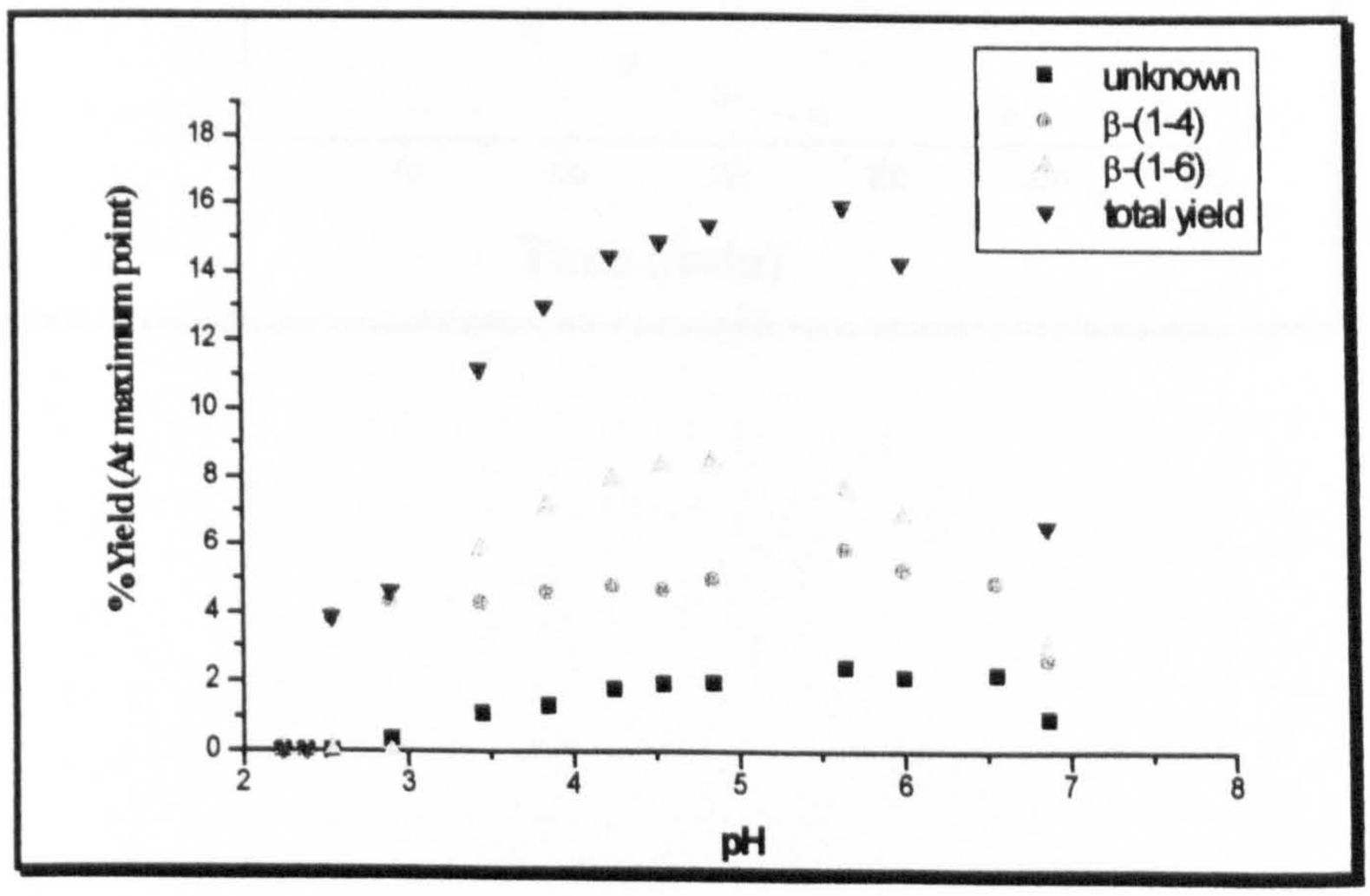
Buffer : pH variable, 0.05 M

pH	v_{ini} x 10^{-4} ($/mM.s^{-1}$)	log v_{ini}
2.24	0.11	-4.94
2.38	0.32	-4.49
2.54	0.36	-4.44
2.90	0.53	-4.28
3.11	1.51	-3.82
3.45	1.33	-3.87
3.85	1.43	-3.84
4.25	1.50	-3.82
4.85	1.56	-3.80
5.25	1.21	-3.92
5.65	0.67	-4.18
6.00	0.59	-4.22
6.29	0.42	-4.38
6.56	0.17	-4.78
6.87	0.07	-5.16

pH rate profile with 10.0 mM acceptor



Variation of %yield with pH



2.12. p-Nitrophenyl β-D-lactopyranoside as a new substrate donor

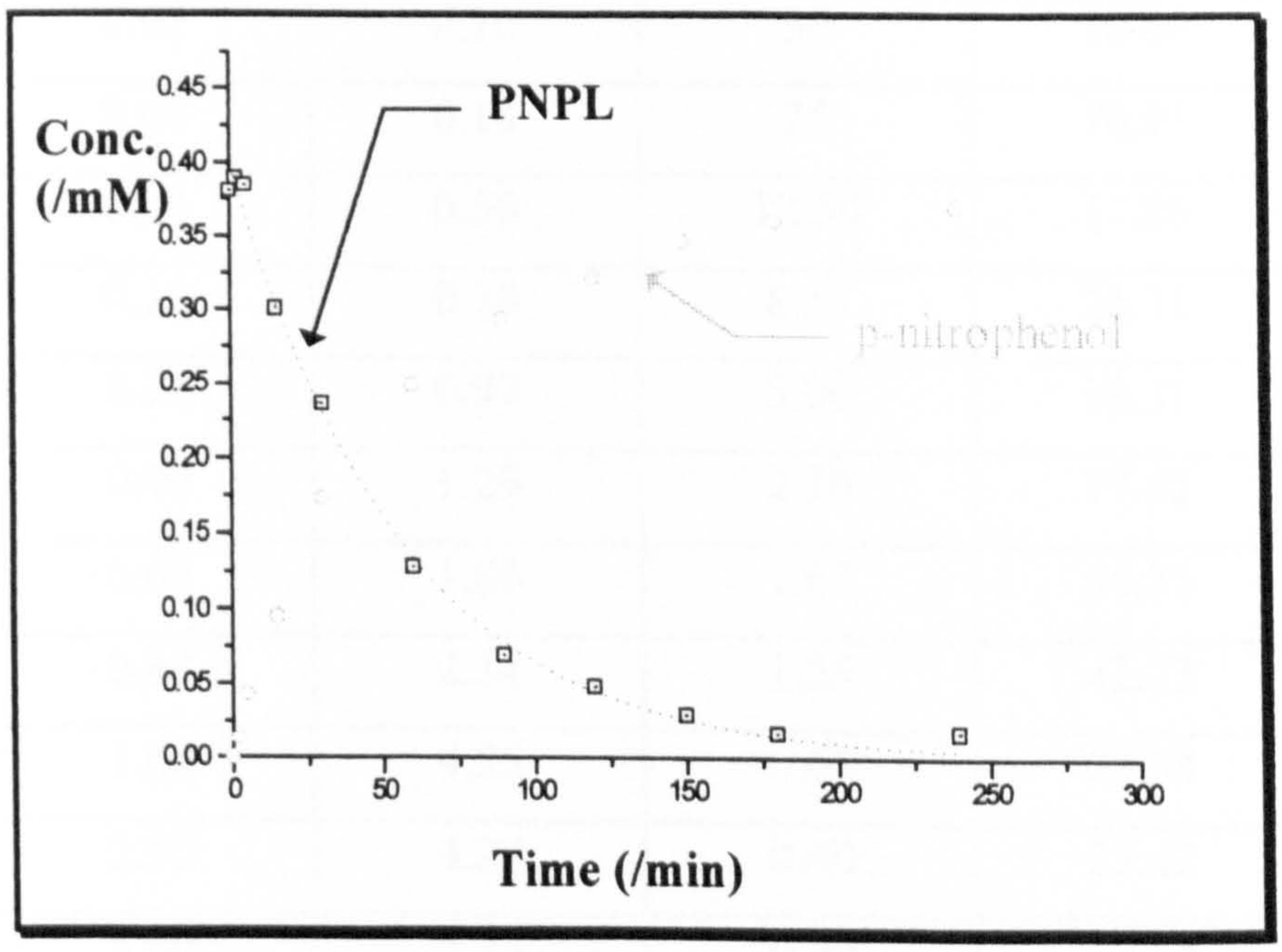
2.12.1. Hydrolysis

- Reaction conditions

p-nitrophenyl β-D-lactopyranoside (PNPL): 0.40 mM

Crude cellulase from *Trichoderma reesei* : 0.23 $mg.ml^{-1}$

Sodium acetate buffer : pH 4.5, 0.05 M



2.12.2. K_m determination

- Reaction conditions

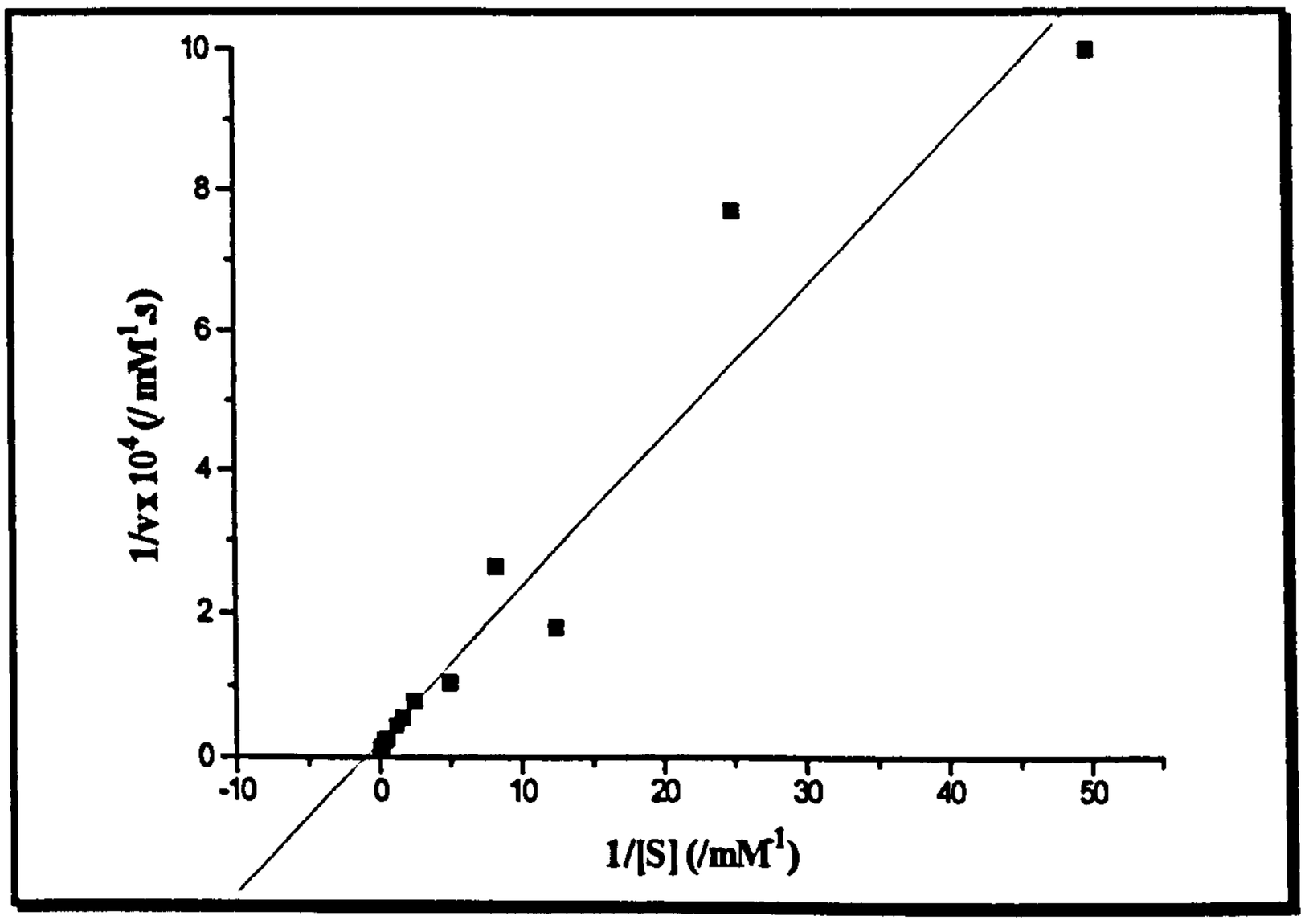
Substrate : p-nitrophenyl β-D-lactopyranoside : variable

Crude cellulase from *Trichoderma reesei* : 0.23 $mg.ml^{-1}$

Sodium acetate buffer : pH 4.5, 0.05 M

S (/mM)	v x 10^{-4} ($/mM.s^{-1}$)	1/S ($/mM^{-1}$)	1/v x 10^4 ($/mM^{-1}.s$)
0.02	0.10	50	10.00
0.04	0.13	25	76.92
0.08	0.56	12.50	17.86
0.12	0.38	8.33	26.31
0.20	0.97	5.00	10.31
0.40	1.29	2.50	77.52
0.60	1.84	1.67	54.35
0.80	2.34	1.25	42.73
1.60	4.25	0.62	23.53
2.50	4.27	0.40	23.42
5.00	5.25	0.20	19.05
7.50	7.75	0.13	12.90

Lineweaver-Burk plot



$$K_m = 1 \text{ mM},$$

$$V_{max} = 4.5 \times 10^{-4} \text{ mM.s}^{-1}$$

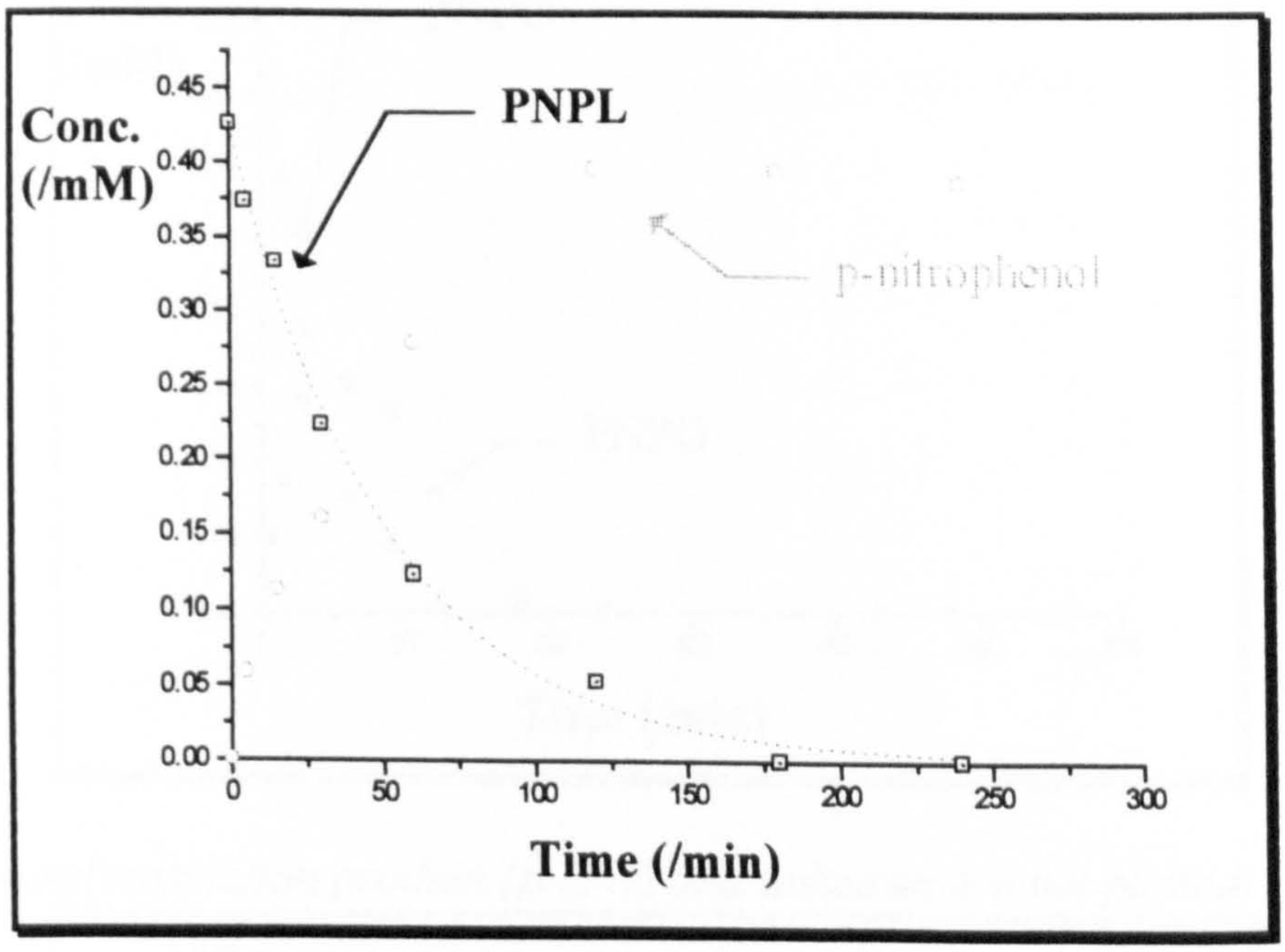
2.12.3. Transglycosylation studies

• *Reaction conditions*

p-nitrophenyl β-D-lactopyranoside :	0.40 mM
N-(p-nitrophenyl)-β-D-glucopyranosylamine :	2.00 mM
Crude cellulase from *Trichoderma reesei* :	0.23 mg.ml^{-1}
Sodium acetate buffer :	pH 4.5, 0.05 M

Transglycosylation

Substrate hydrolysis



Low concentration of two trisaccharides were observed.

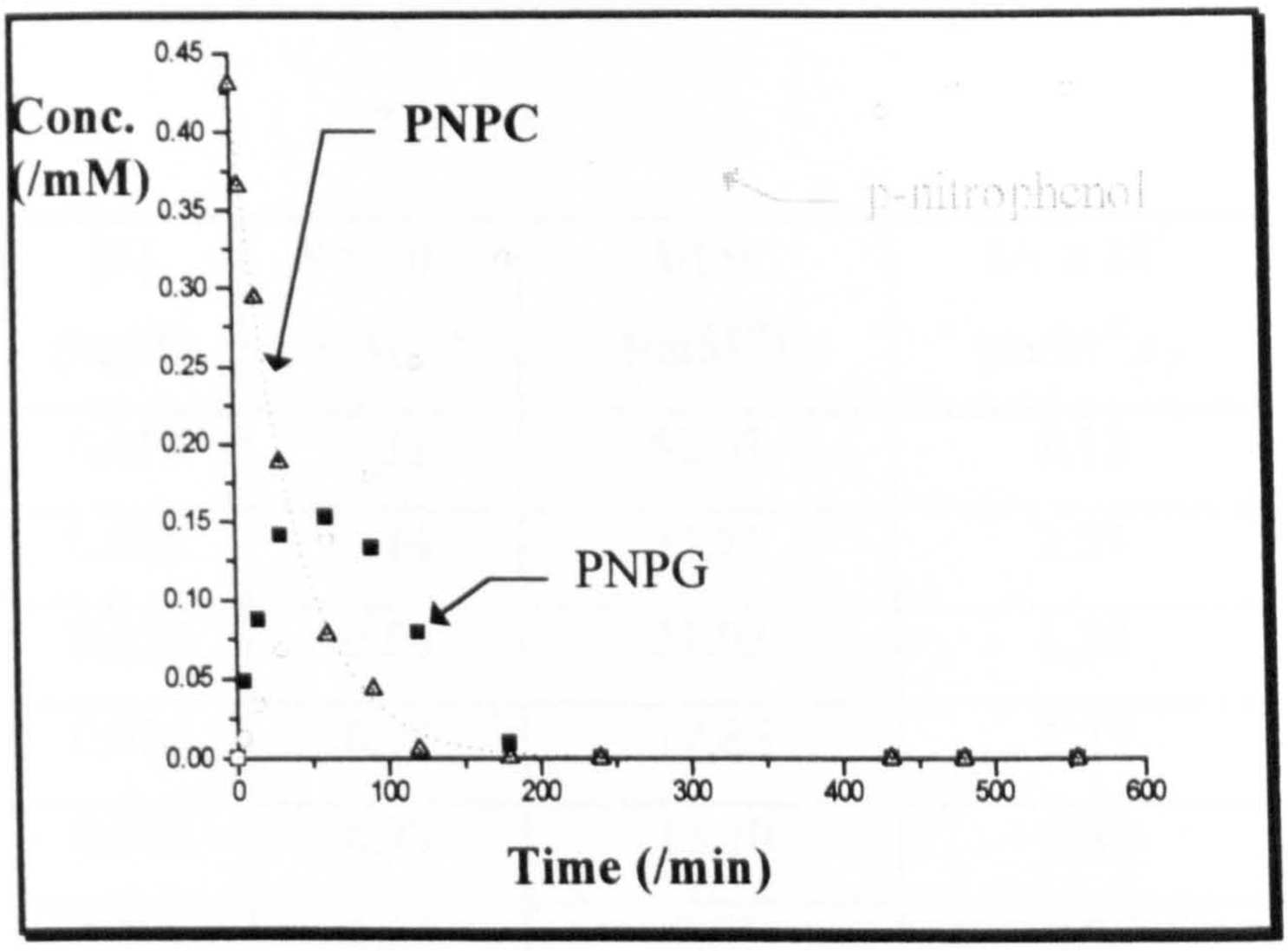
2.13. p-Nitrophenyl 1-thio-β-D-glucopyranoside as a new acceptor

2.13.1. Transglycosylation study

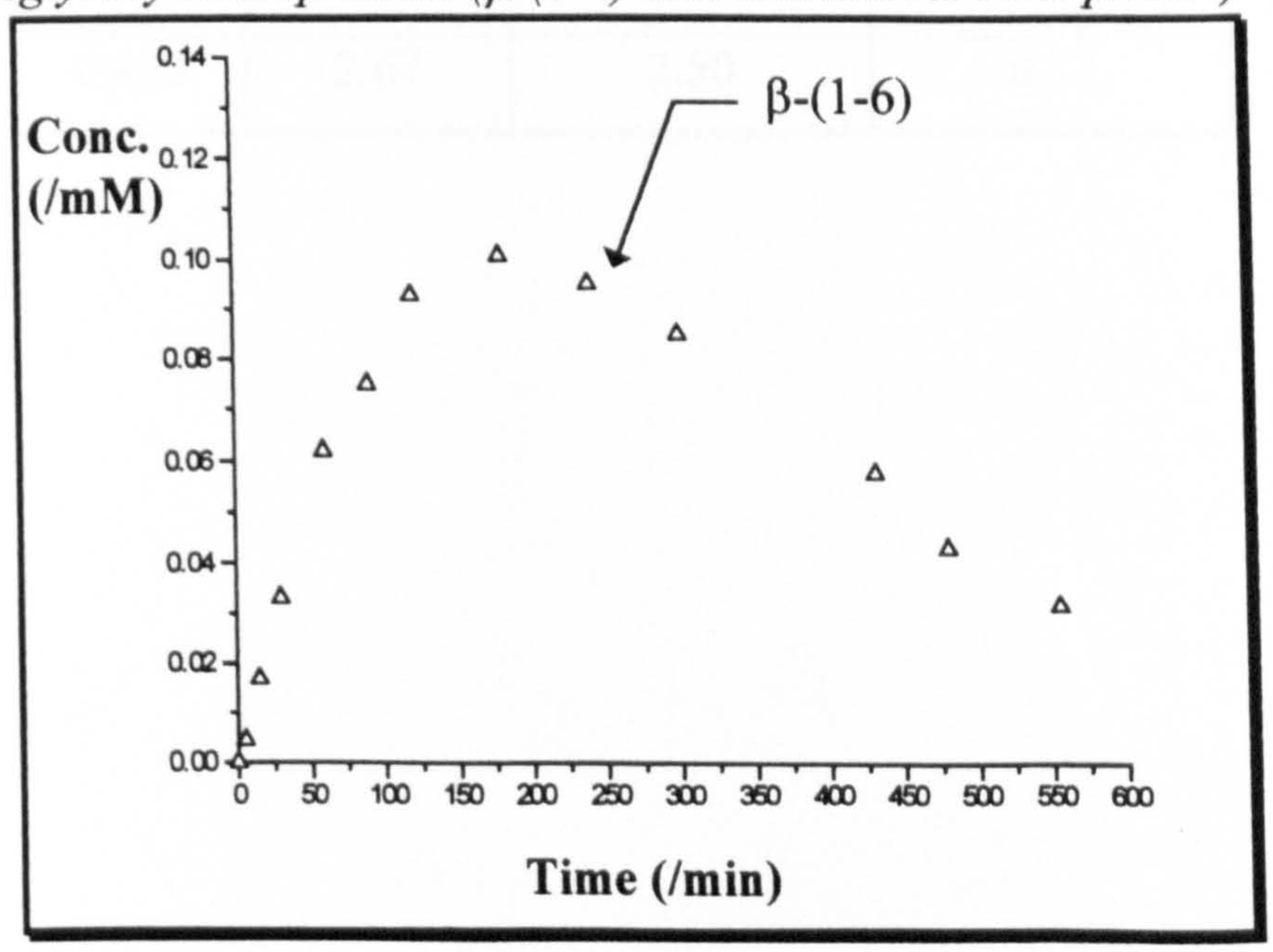
- *Reaction conditions*

p-nitrophenyl β-D-cellobioside :	0.40 mM
p-nitrophenyl 1-thio-β-D-glucopyranoside :	10.00 mM
Crude cellulase from *Trichoderma reesei* :	0.23 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, 0.05 M

Hydrolysis of the substrate



Transglycosylation product (β-(1-4) and unknown are not plotted)



2.13.2. Acceptor inhibition studies

- Reaction conditions

p-nitrophenyl β-D-cellobioside : *variable*

p-nitrophenyl 1-thio-β-D-glucopyranoside : *as indicated*

Crude cellulase from *Trichoderma reesei* : 0.23 $mg.ml^{-1}$

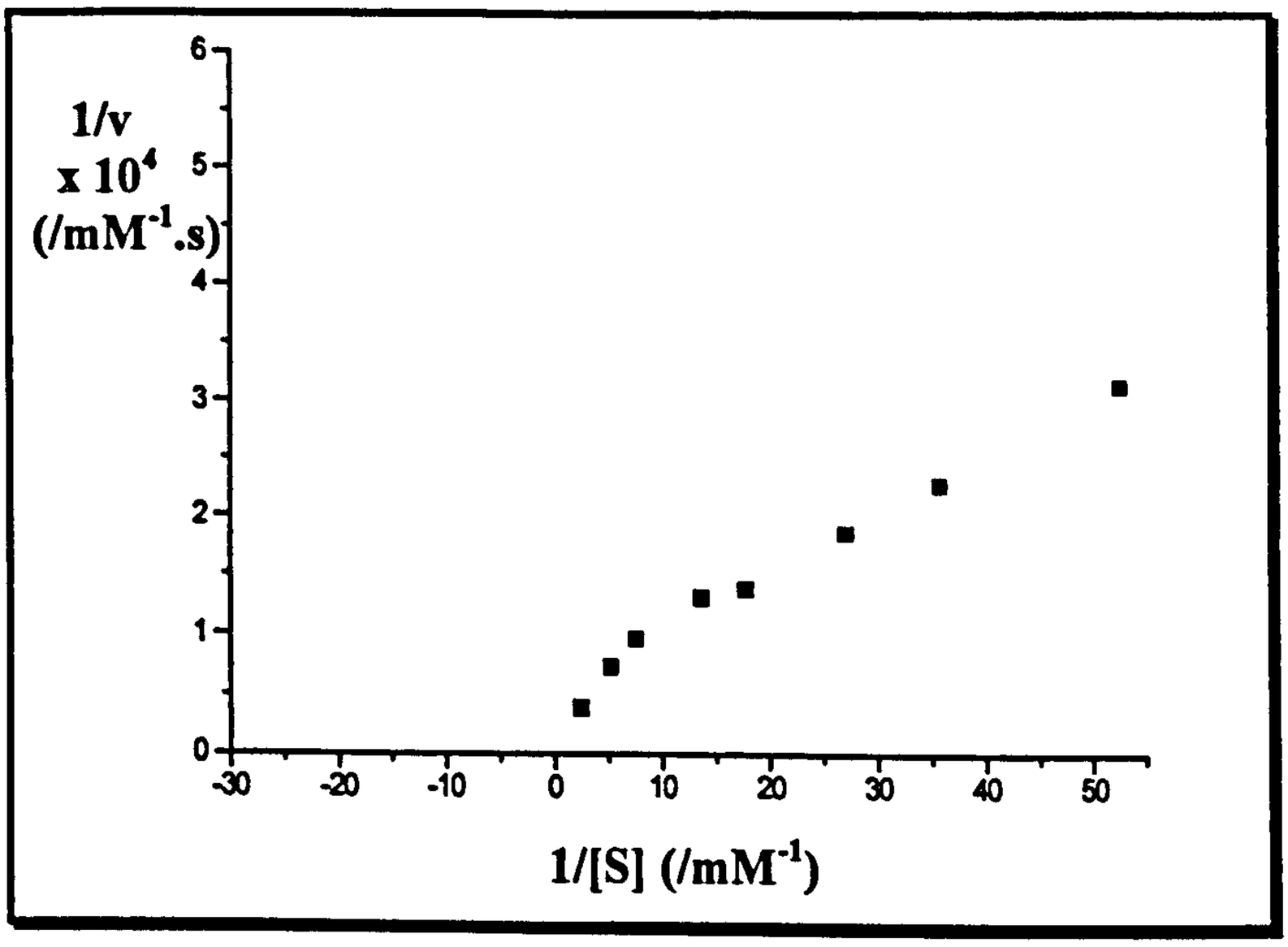
Sodium acetate buffer : pH 4.5, I = 0.05 M

2.13.2.1. p-nitrophenyl 1-thio-β-D-glucopyranoside :

2.00 mM

[S] (/mM)	v x 10^{-4} (/$mM.s^{-1}$)	1/[S] (/mM^{-1})	1/v x 10^{4} (/$mM^{-1}.s$)
0.019	0.32	52.63	3.12
0.028	0.44	35.71	2.27
0.037	0.54	27.03	1.85
0.056	0.73	17.86	1.37
0.073	0.77	13.70	1.30
0.131	1.05	7.63	0.95
0.190	1.39	5.26	0.72
0.400	2.67	2.50	0.37

Lineweaver-Burk plot

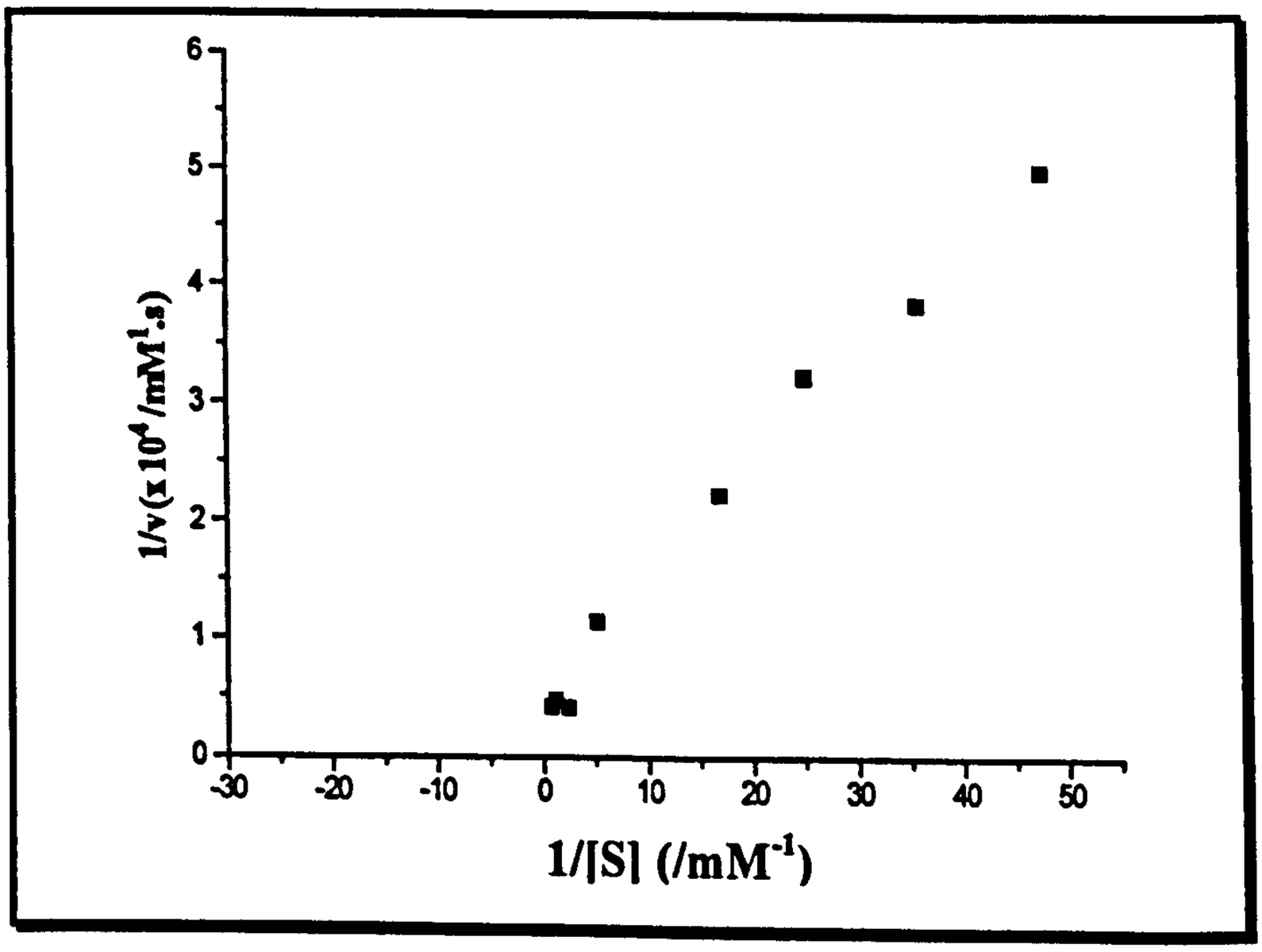


2.13.2.2. p-nitrophenyl 1-thio-β-D-glucopyranoside :

6.00 mM

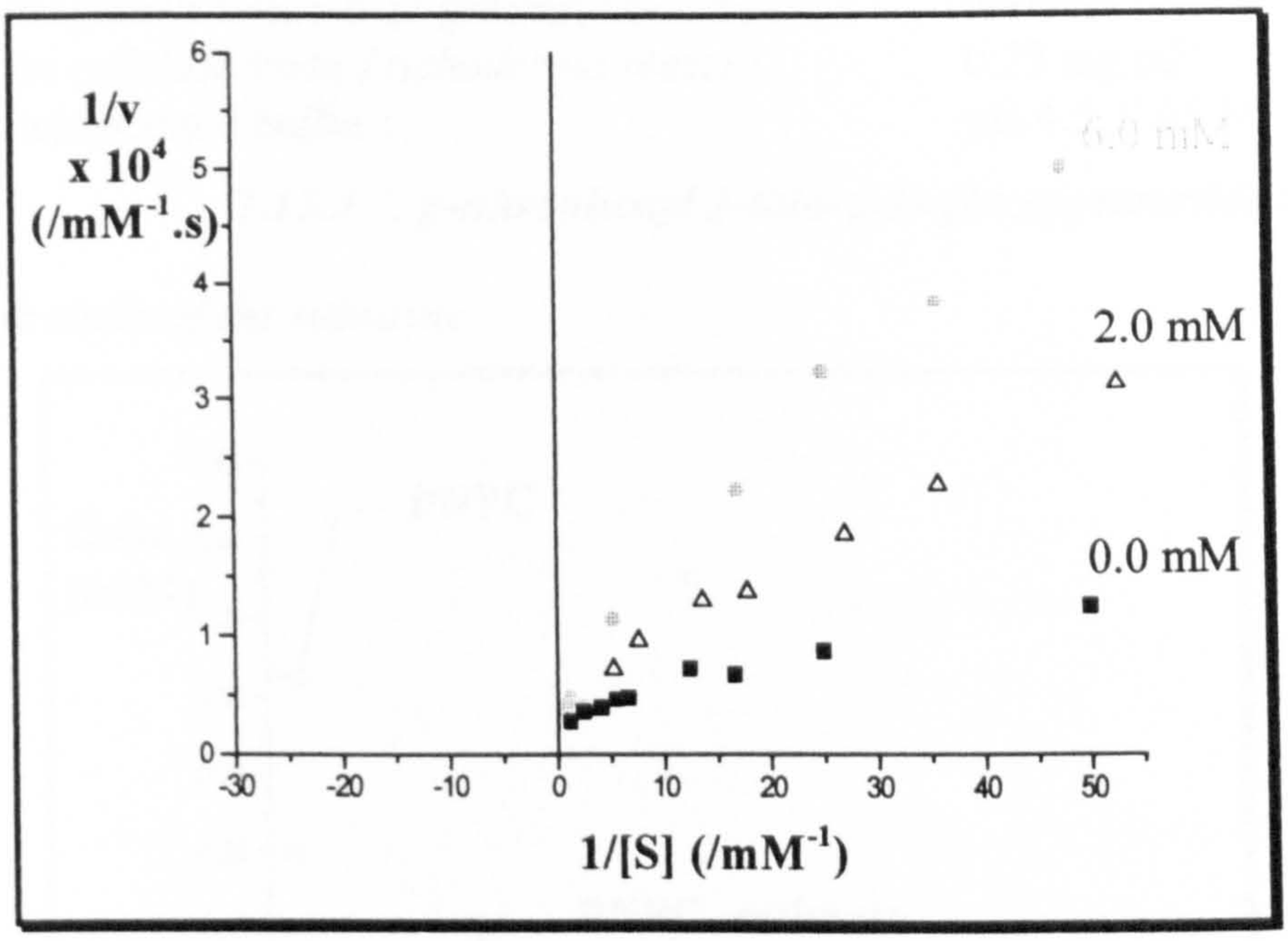
[S] (/mM)	v x 10^{-4} (/mM.s^{-1})	1/[S] (/mM^{-1})	1/v x 10^4 (/mM^{-1}.s)
0.021	0.20	47.62	5.00
0.028	0.26	35.71	3.85
0.040	0.31	25.00	3.22
0.059	0.45	16.95	2.22
0.190	0.88	5.26	1.14
0.400	2.50	2.5	0.40
0.750	2.15	1.33	0.46
1.140	2.44	0.88	0.41

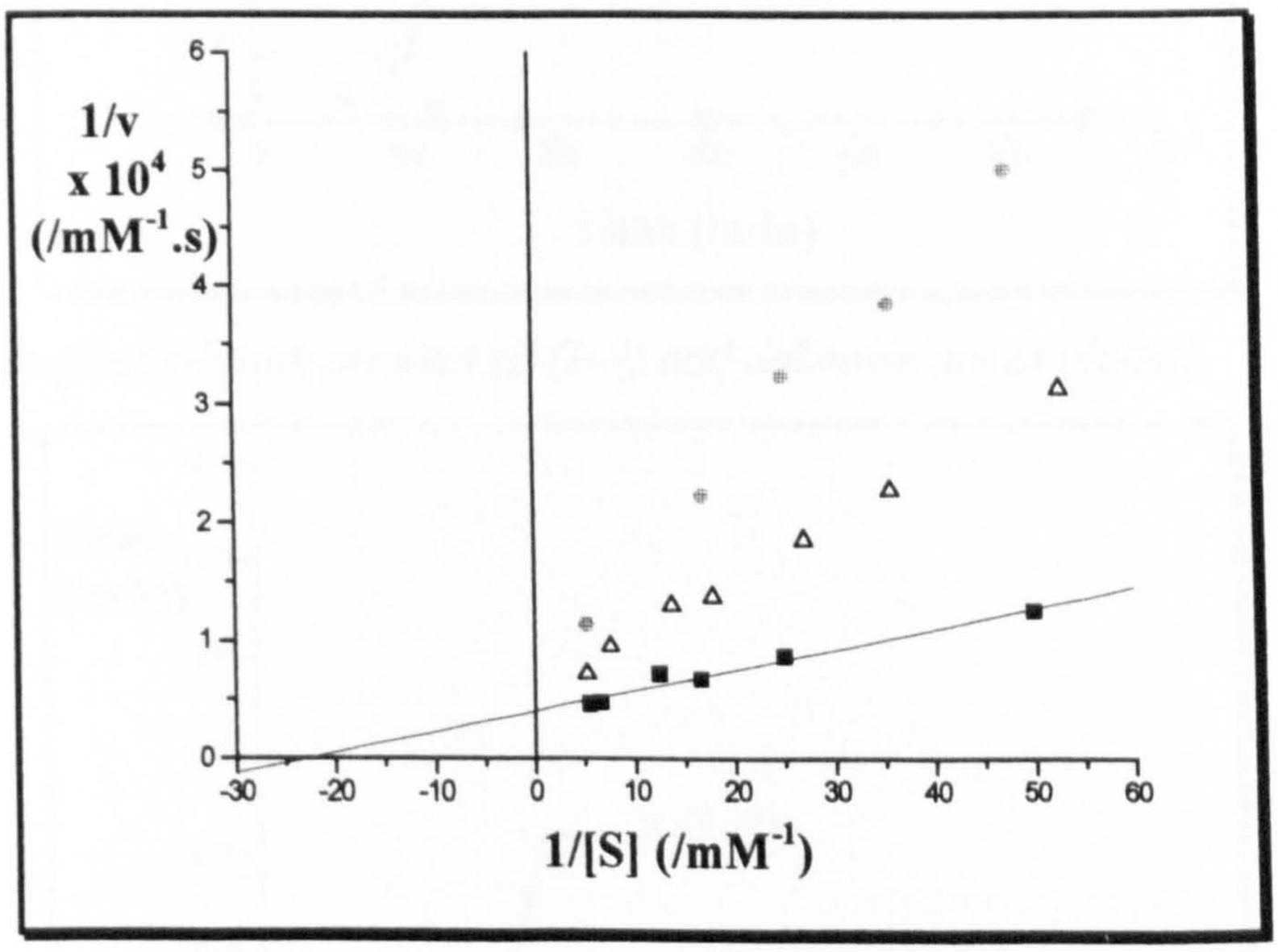
Lineweaver-Burk Plot



Lineweaver-Burk plot for

p-nitrophenyl 1-thio-β-D-glucopyranoside





Competitive inhibitor

K_i= 1.5 mM

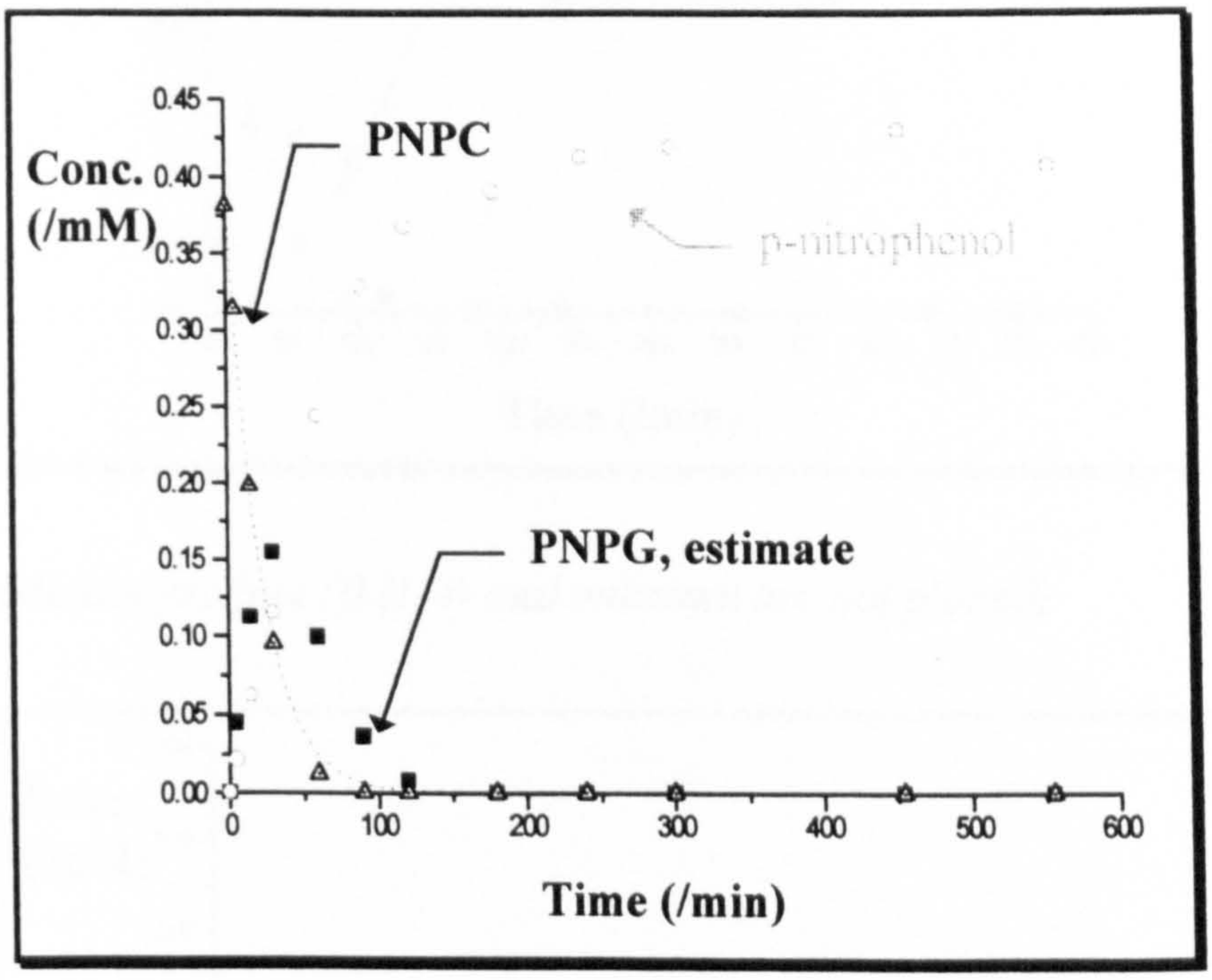
2.13.3. Variation of acceptor concentration

- *Reaction conditions*

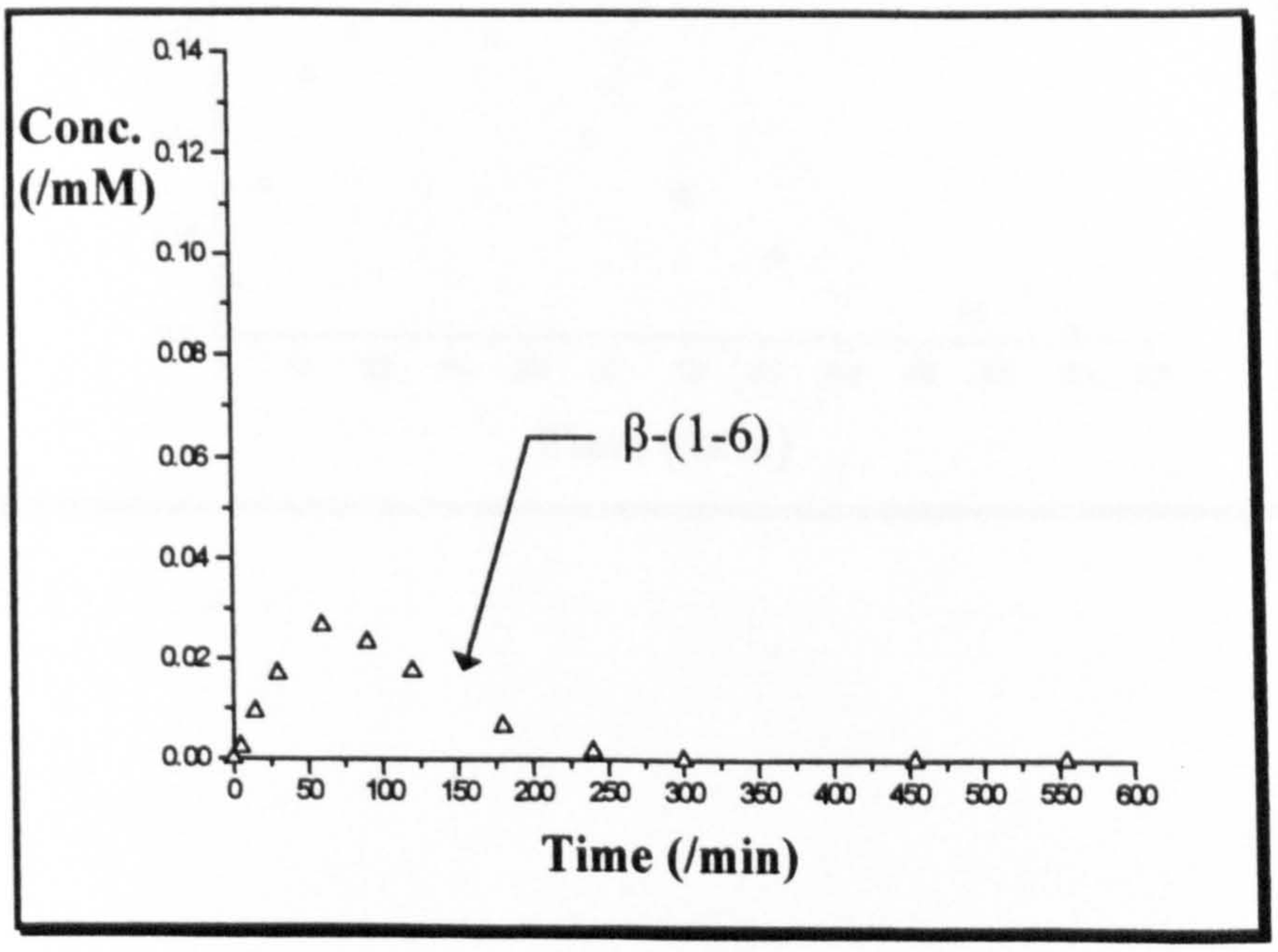
p-nitrophenyl β-D-cellobioside :	0.40 mM
p-nitrophenyl 1-thio-β-D-glucopyranoside :	*variable*
Crude cellulase from *Trichoderma reesei* :	0.23 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, 0.05 M

2.13.3.1. p-nitrophenyl 1-thio-β-D-glucopyranoside : 2.0 mM

Hydrolysis of the substrate

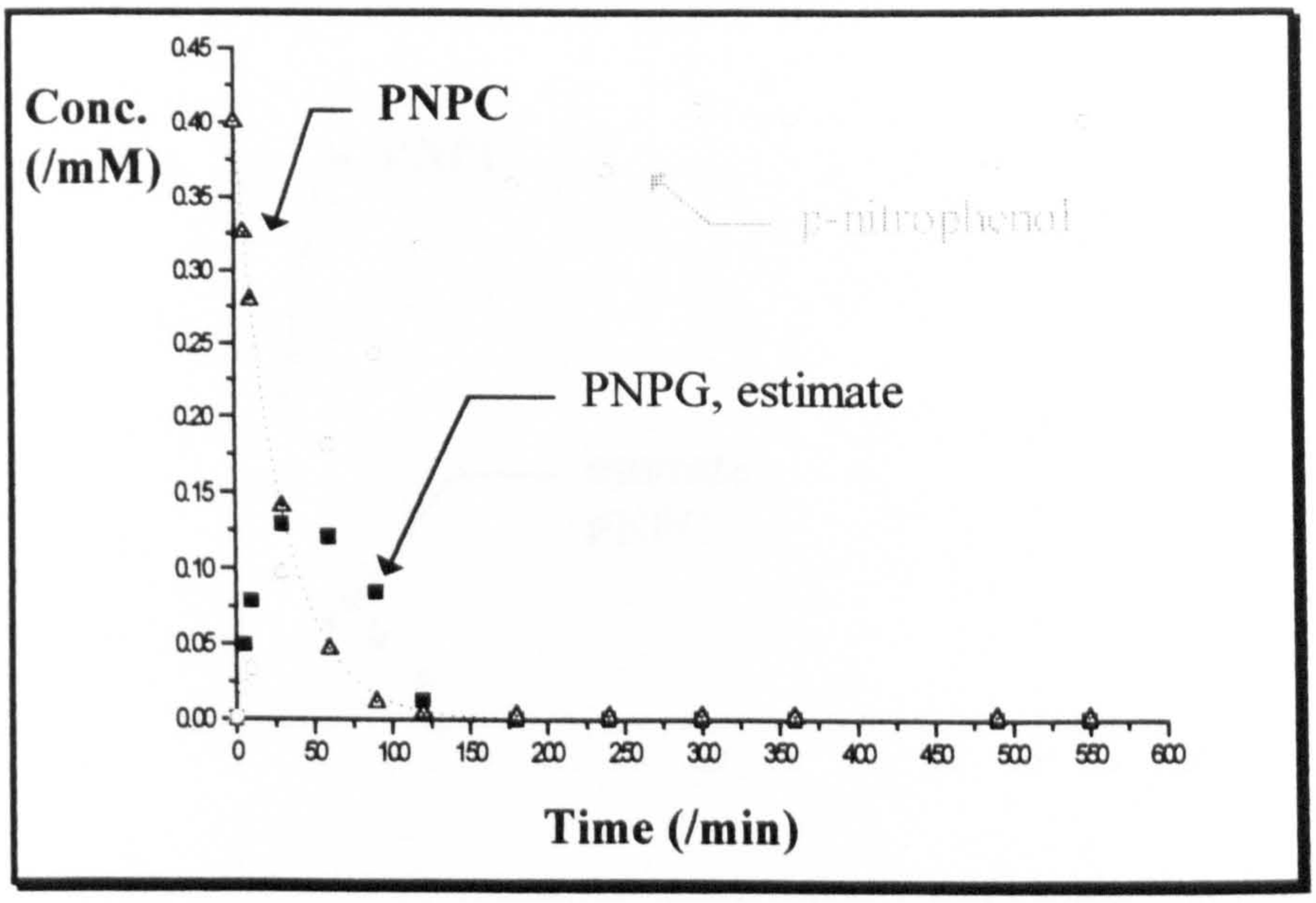


Transglycosylation product (β-(1-4) and unknown are ot plotted)

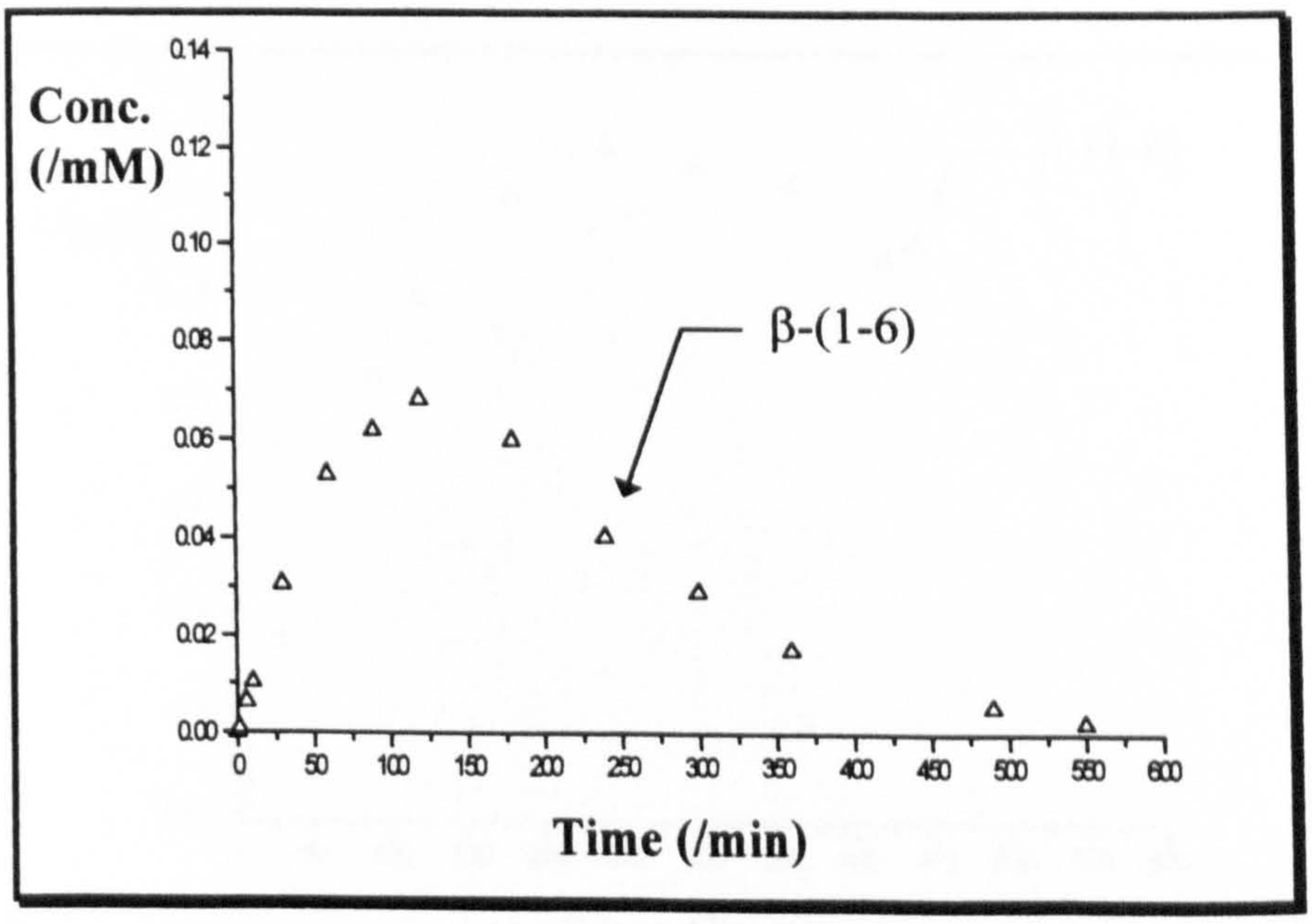


2.13.3.2. p-nitrophenyl 1-thio-β-D-glucopyranoside : 6.0 mM,

Hydrolysis of the substrate

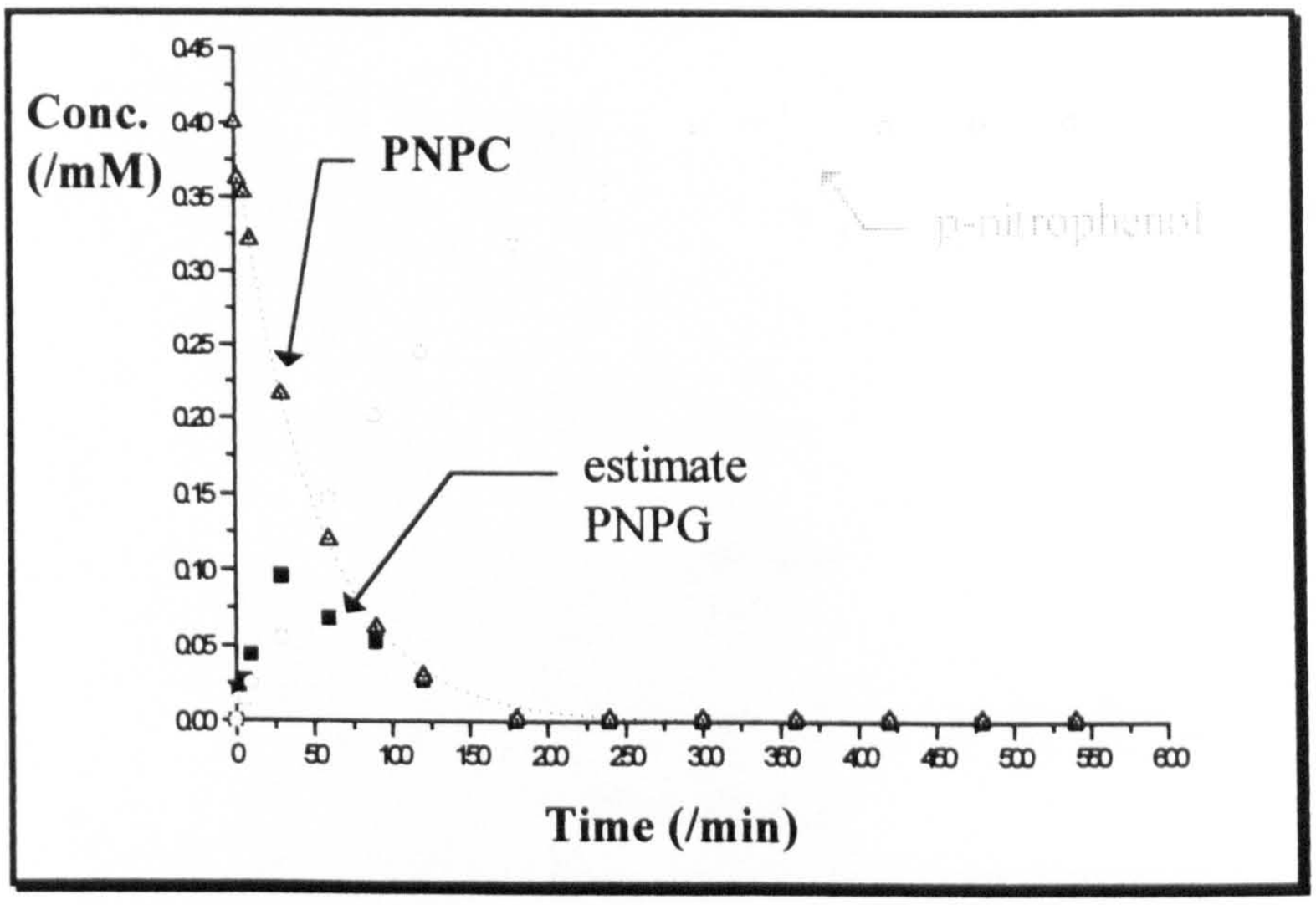


Transglycosylation product (β-(1-4) and unknown are not plotted)

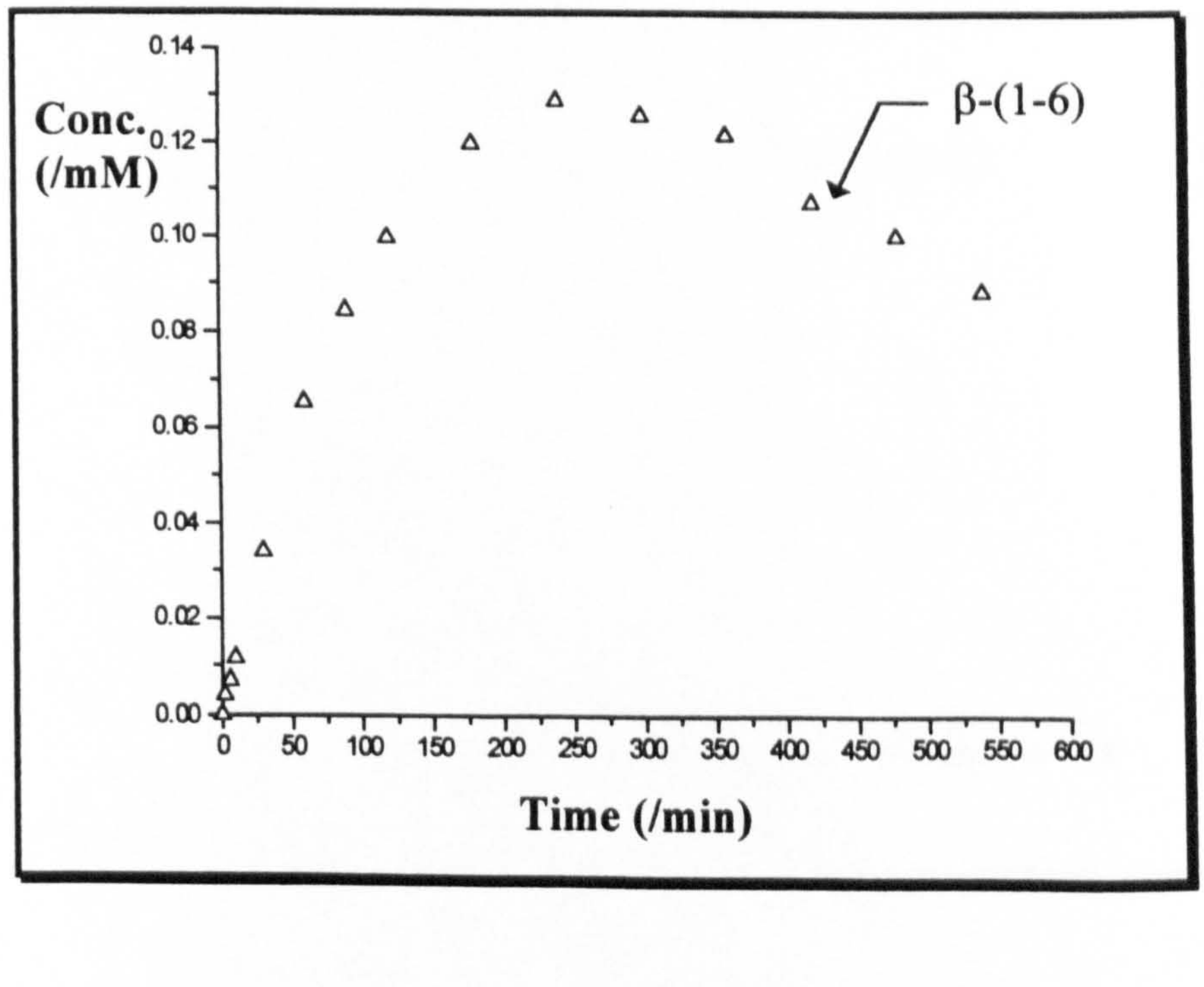


2.13.3.3. p-nitrophenyl 1-thio-β-D-glucopyranoside: 15.2 mM,

Hydrolysis of the substrate

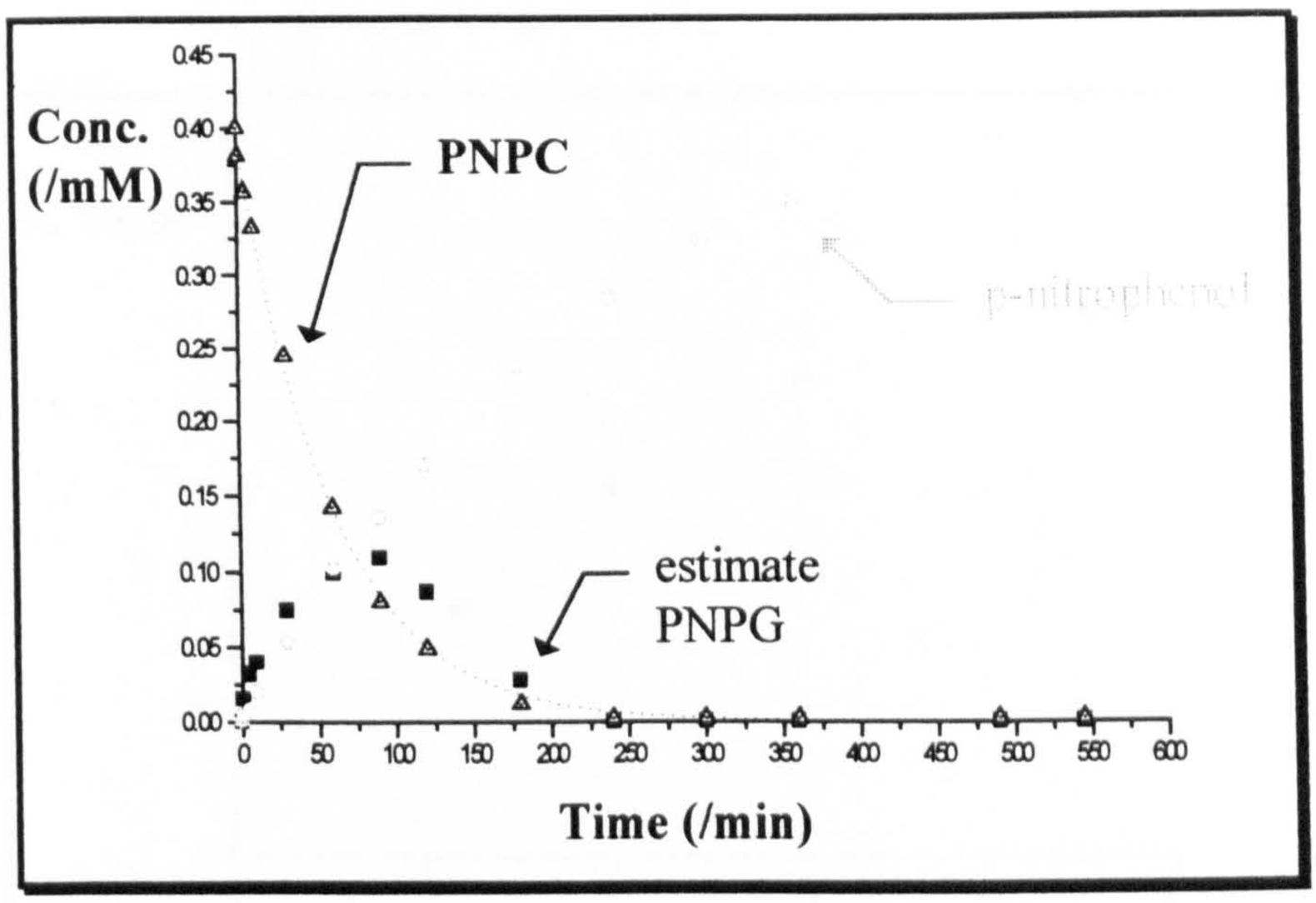


Transglycosylation product (β-(1-4) and unknown are not plotted)

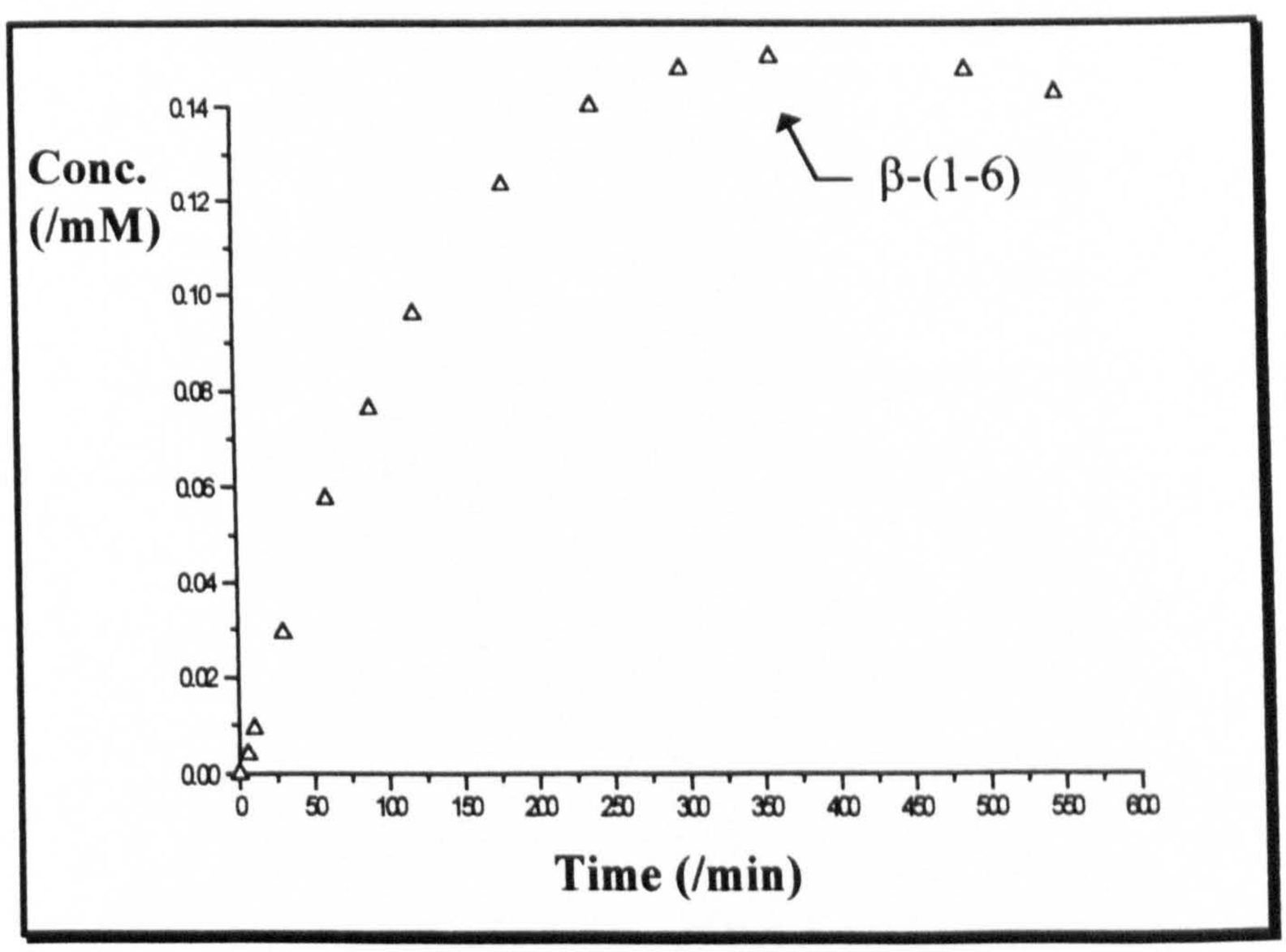


2.13.3.4. p-nitrophenyl 1-thio-β-D-glucopyranoside : 22.8 mM,

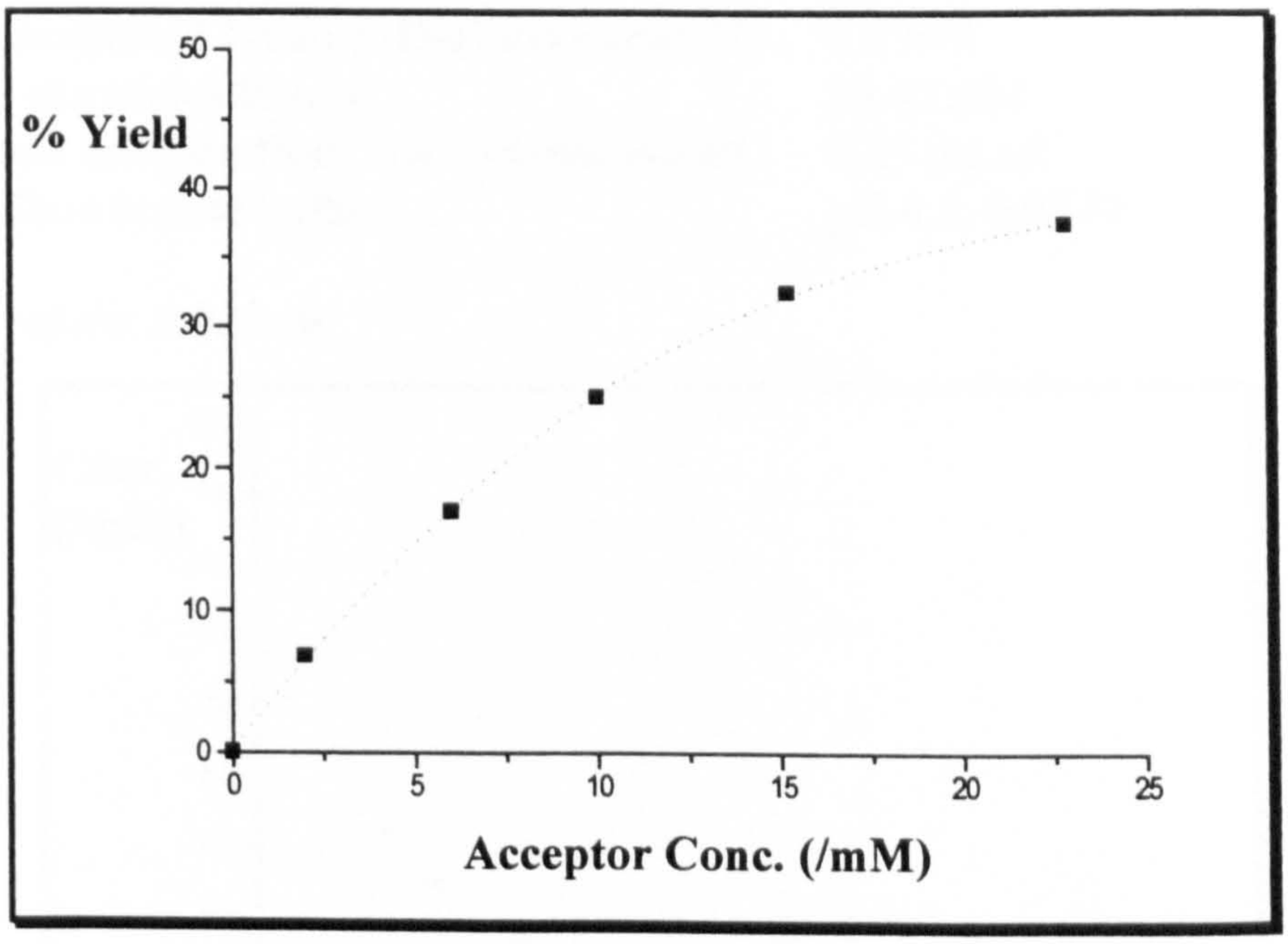
Hydrolysis of the substrate



Transglycosylation products (β-(1-4) and unknown are not plotted)



Overall effect of increasing the acceptor concentration on the % yield of β-(1-6) linked disaccharide transfer product

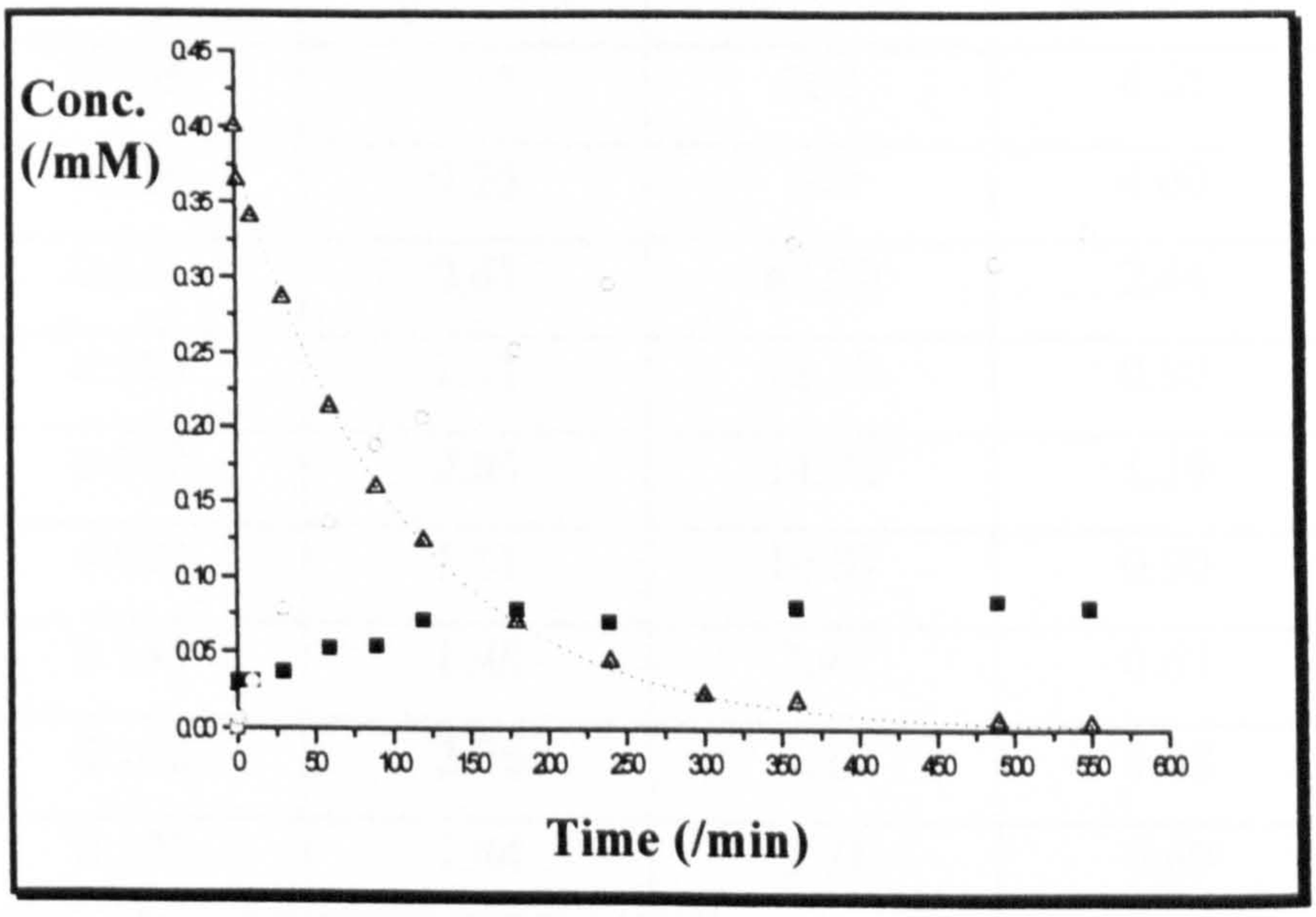


2.13.4. Transglycosylation studies in the presence of 1,5-glucono-δ-lactone

- Reaction conditions

p-nitrophenyl β-D-cellobioside :	0.4 mM
p-nitrophenyl 1-thio-β-D-glucopyranoside :	6.0 mM
1,5-glucono-δ-lactone :	20.80 mM
Crude cellulase from *Trichoderma reesei* :	0.23 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, 0.05 M

Hydrolysis of the substrate



Transglycosylation product : β-(1-4) and unknown were produced but no β-(1-6) product formation was observed.

2.13.5. p-Nitrophenyl 1-thio-β-D-cellobioside : K_m determination

- Reaction conditions

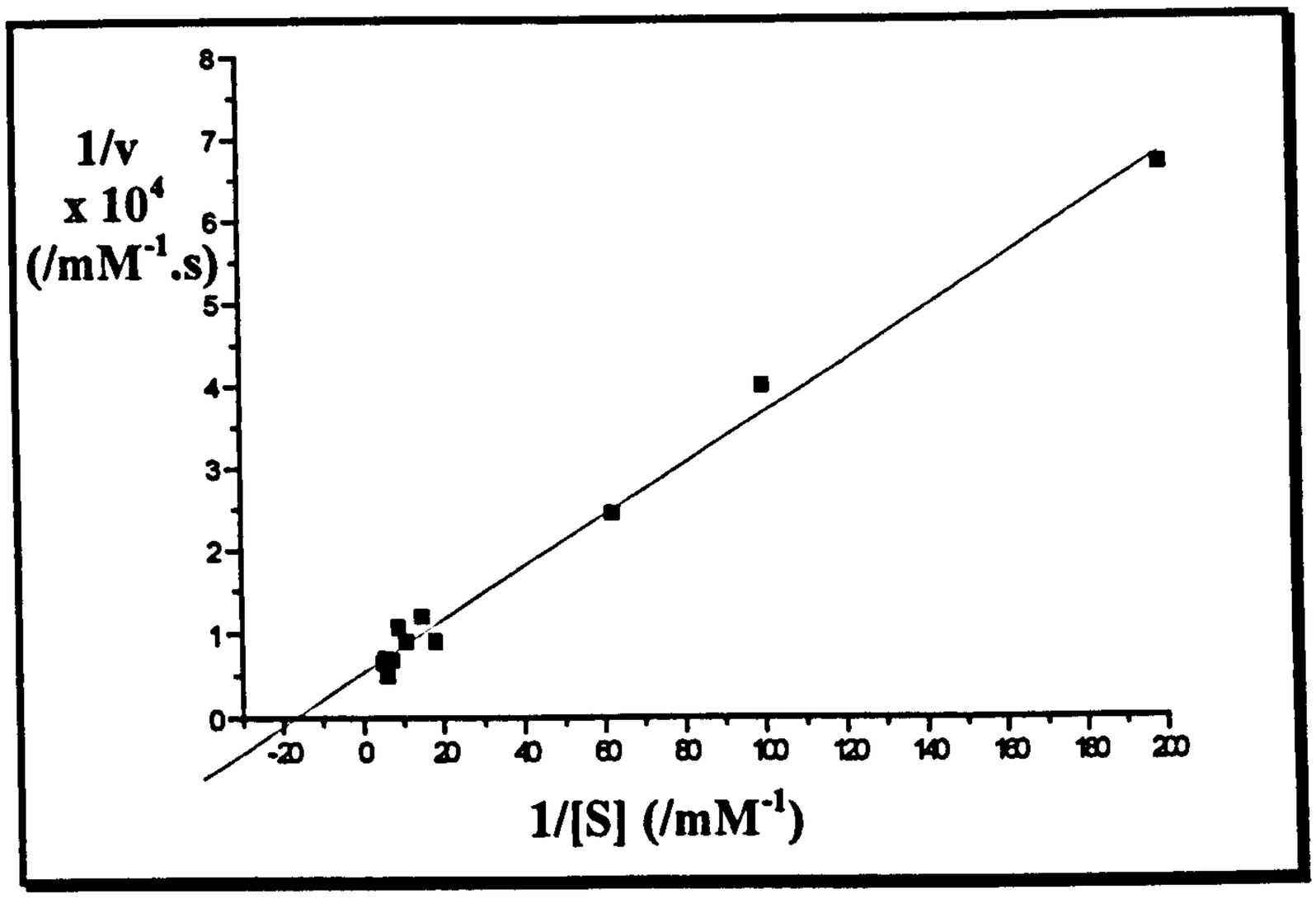
Substrate : p-nitrophenyl 1-thio-β-D-cellobioside

Crude cellulase from *Trichoderma reesei* : 0.23 $mg.ml^{-1}$

Sodium acetate buffer : pH 4.5, 0.05 M

S (/mM)	v x 10^{-4} (/mM.s^{-1})	1/S (/mM^{-1})	1/v x 10^4 (/mM^{-1}.s)
0.005	0.15	200	6.67
0.010	0.25	100	4.00
0.016	0.41	62.50	2.44
0.055	1.11	18.18	0.90
0.067	0.84	14.92	1.19
0.092	1.11	10.87	0.90
0.135	1.48	7.41	0.67
0.160	2.06	6.25	0.48
0.175	1.44	5.71	0.69
0.190	1.54	5.26	0.65

Lineweaver-Burk plot



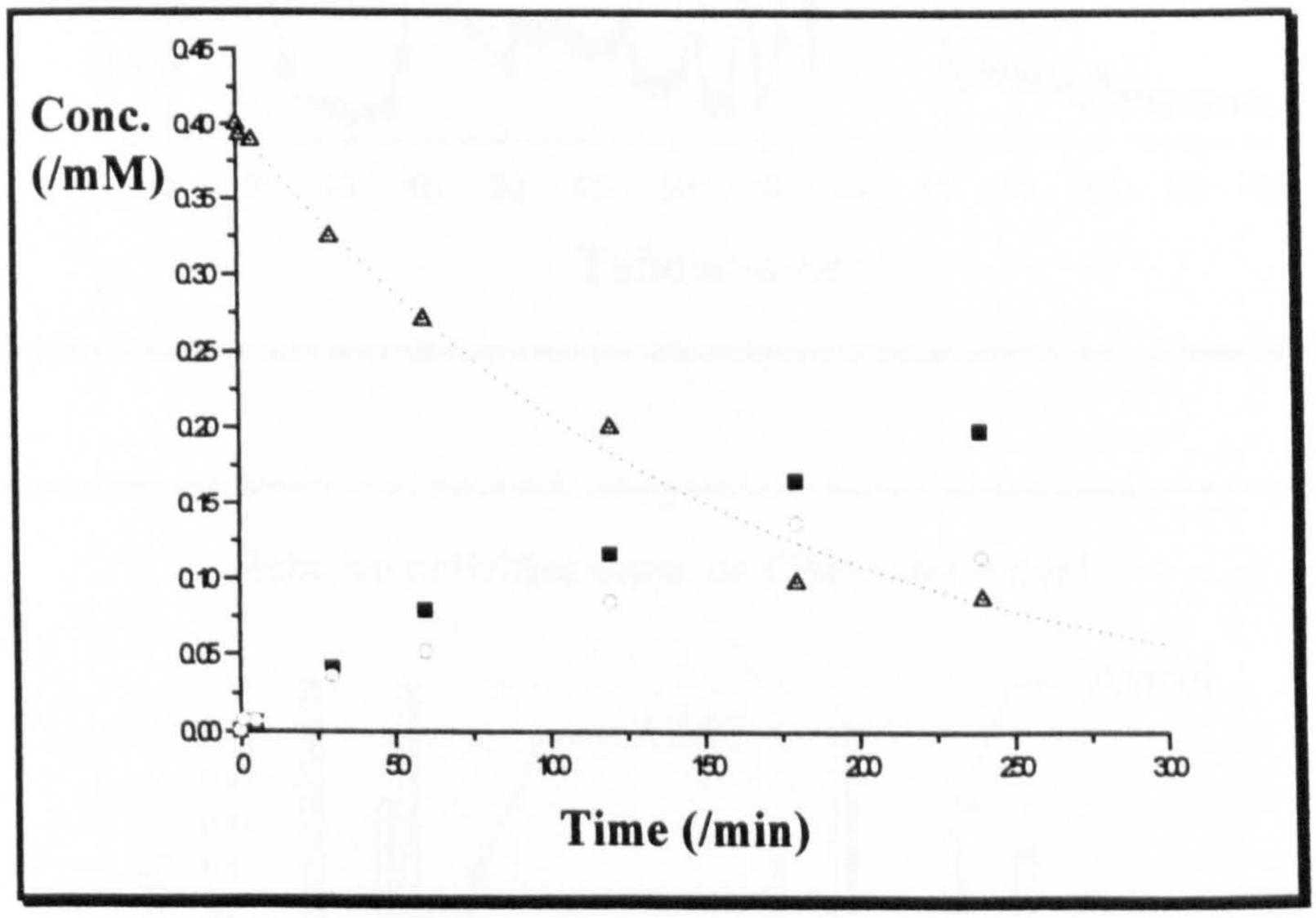
$K_m = 0.1$ mM, $V_{max} = 1.88$ mM.s^{-1}.

2.14. p-Nitrophenyl 4-S-β-D-glucopyranosyl-1,4-dithio-β-D-glucopyranoside as a disaccharide acceptor

- Reaction conditions

p-nitrophenyl β-D-cellobioside :	0.4 mM
disaccharide acceptor :	4.0 mM
Crude cellulase from *Trichoderma reesei* :	0.23 $mg.ml^{-1}$
% ACN :	20 %
Sodium acetate buffer :	pH 4.5 (20 % ACN), 0.05 M

Hydrolysis of the Substrate

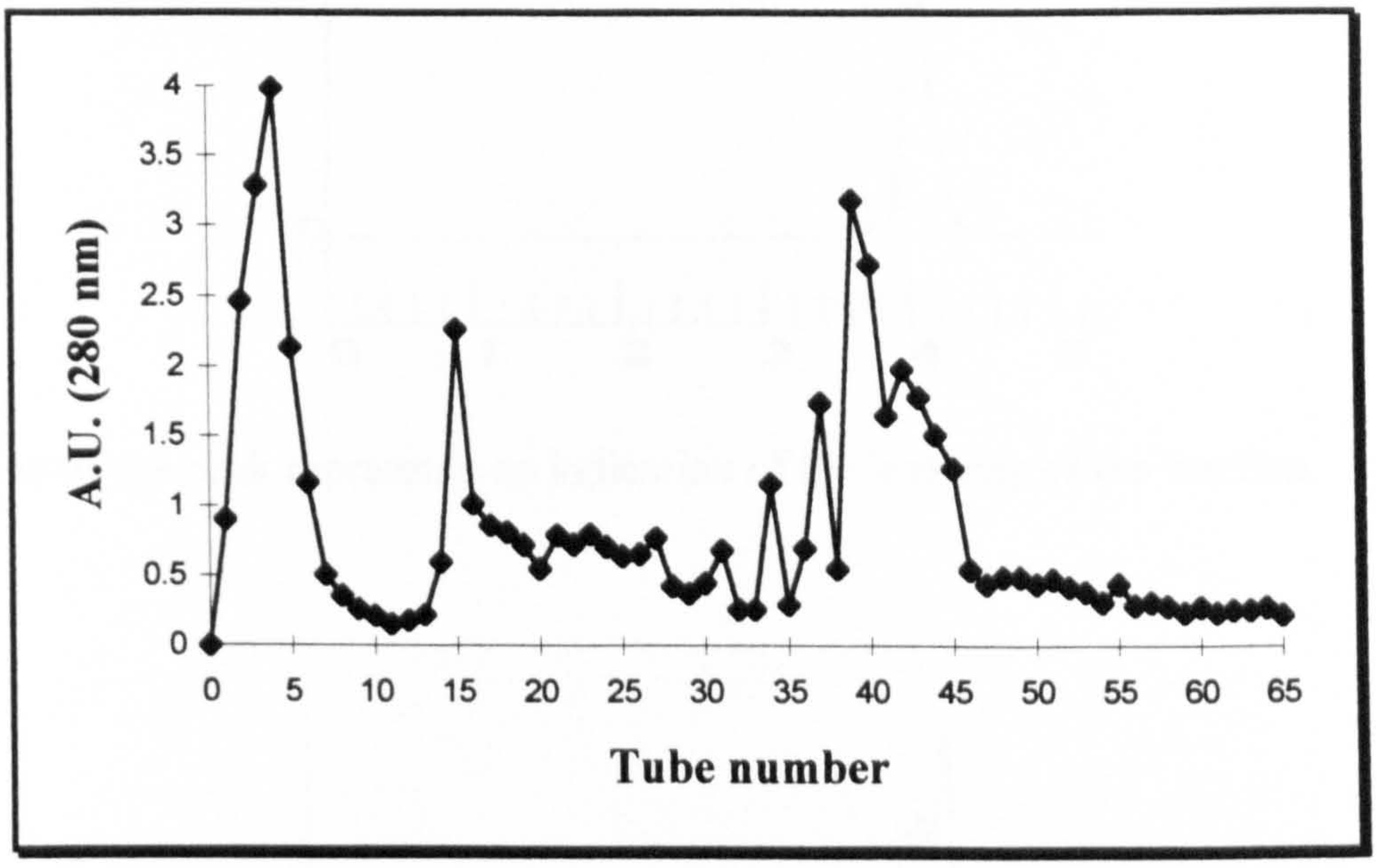


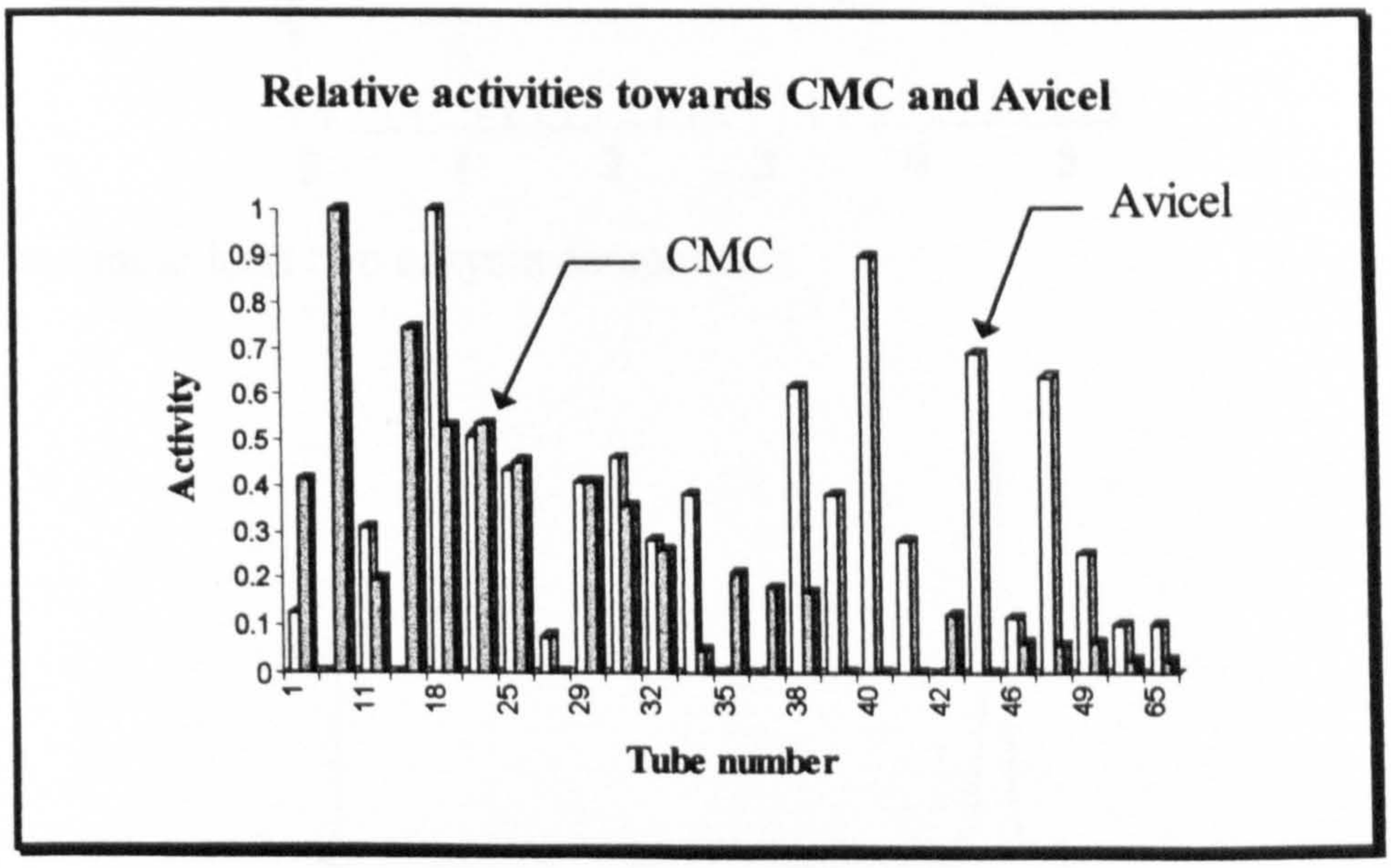
Transglycosylation products : **None detected**

3. Purification and Identification of The Enzyme Components of Cellulase from *Trichoderma reesei*

3.1. Anion-Exchange Chromatography

Protein absorbance at 280 nm in each tube collected

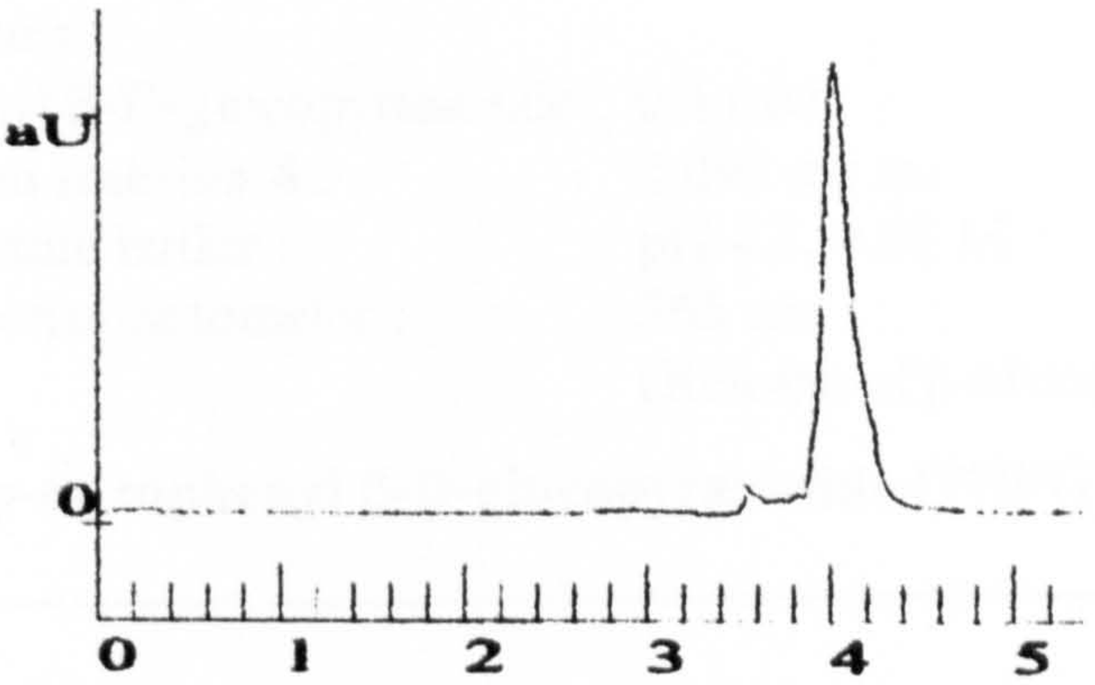




3.2. Electrophoresis analysis

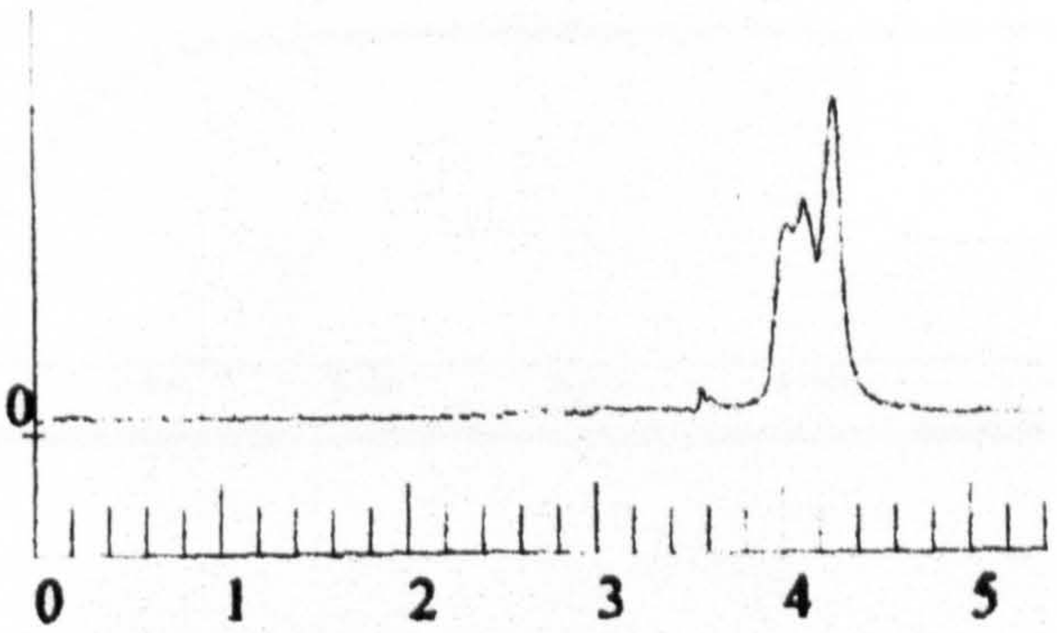
Three main fractions were analysed by capillary electrophoresis following a literature method[91].

Peak 1 :



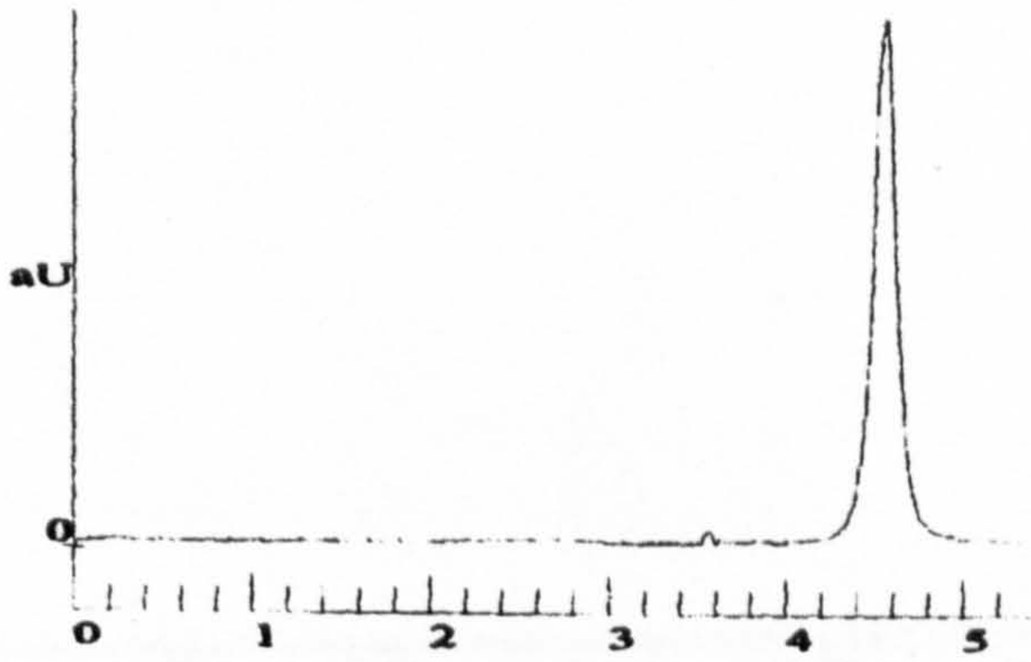
The shape of the peak represents an indication of the impurity of the fraction.

Peak 2 :



Peak 2 contains at least two enzyme components.

Peak 3 :



Peak 3 seems to be a single enzyme component.

3.3. Peak 1 analysis

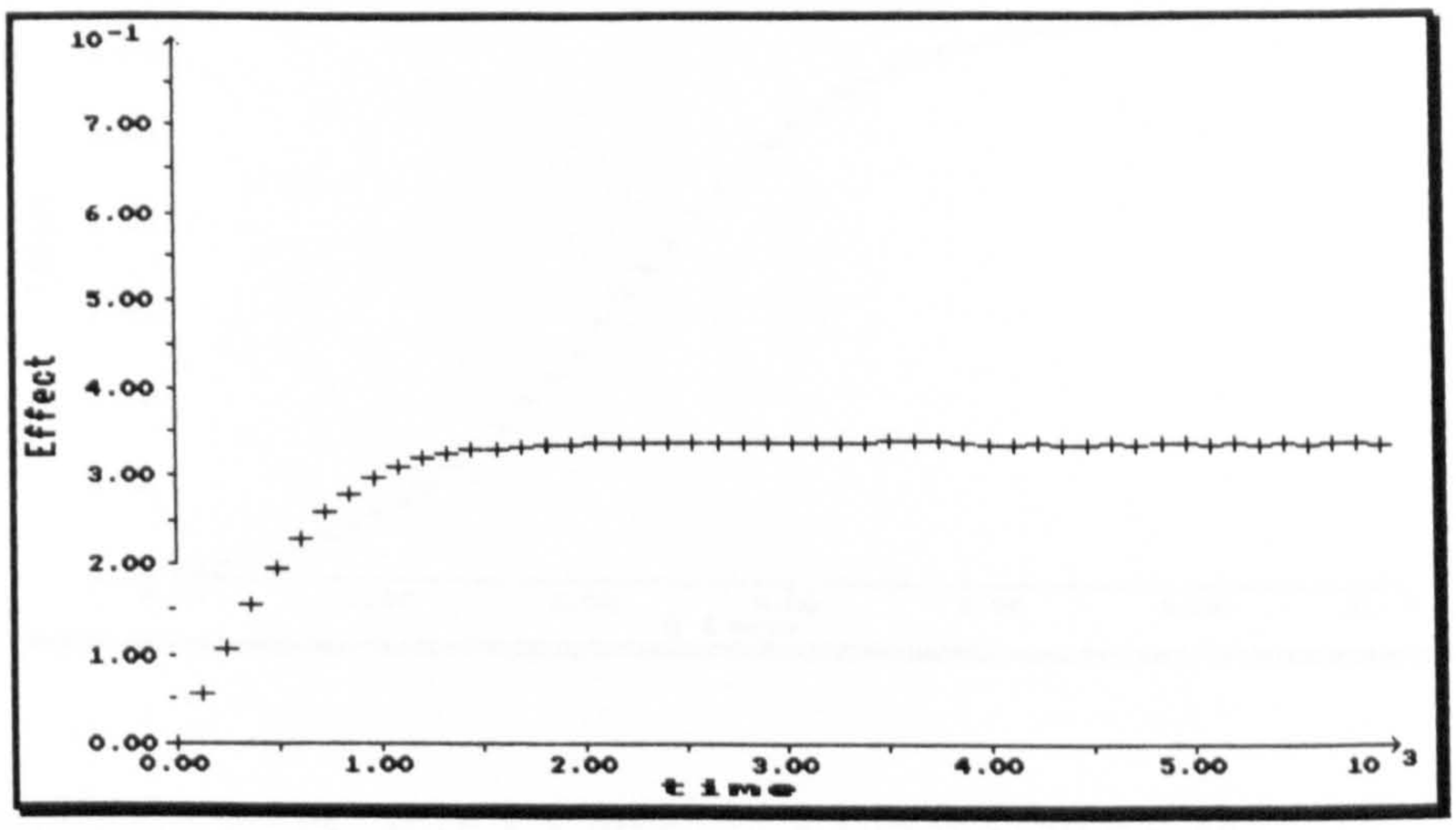
3.3.1. β-glucosidase activity

Substrate : p-nitrophenyl β-D-glucopyranoside

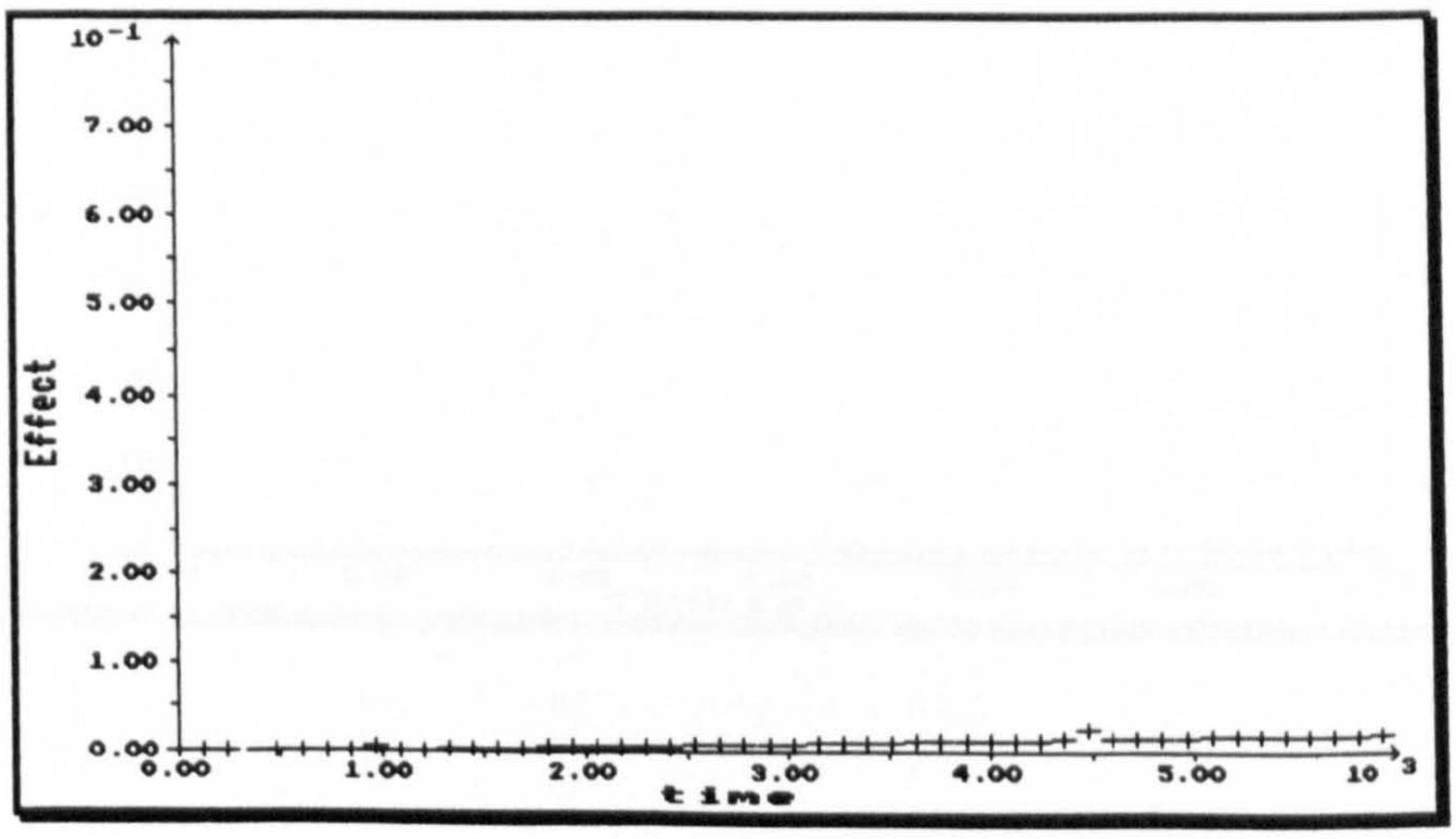
- *Reaction conditions*

p-nitrophenyl β-D-glucopyranoside :	0.4 mM
Sample from **fraction 4** :	0.092 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, 0.05 M
UV-vis spectrophotometer :	365 nm (Release of p-nitrophenol)

A. Hydrolysis of p-nitrophenyl β-D-glucopyranoside (PNPG)



B. Hydrolysis of PNPG with 1,5-glucono-δ-lactone (20.8 mM)

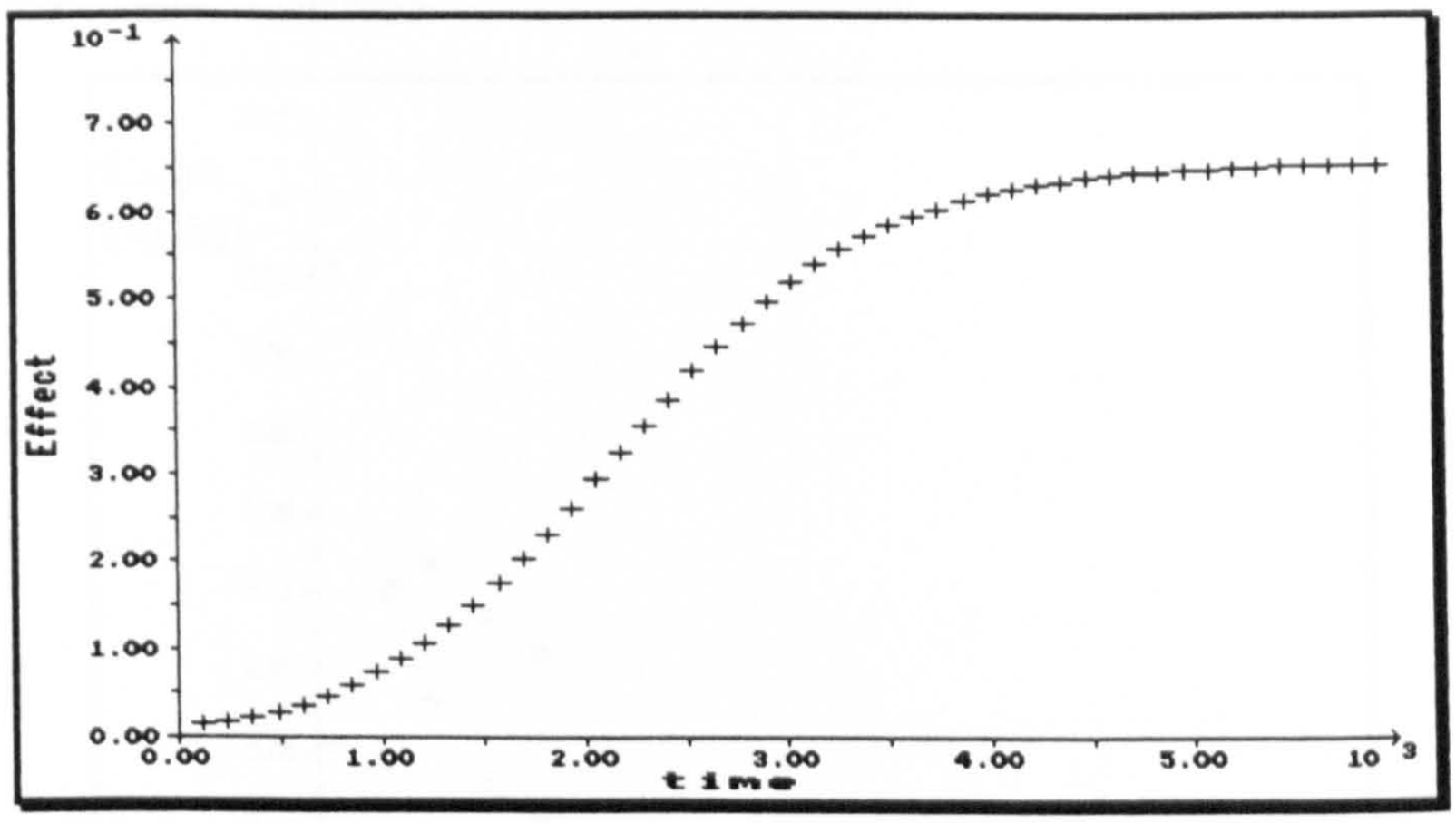


Substrate : p-nitrophenyl β-D-cellobioside

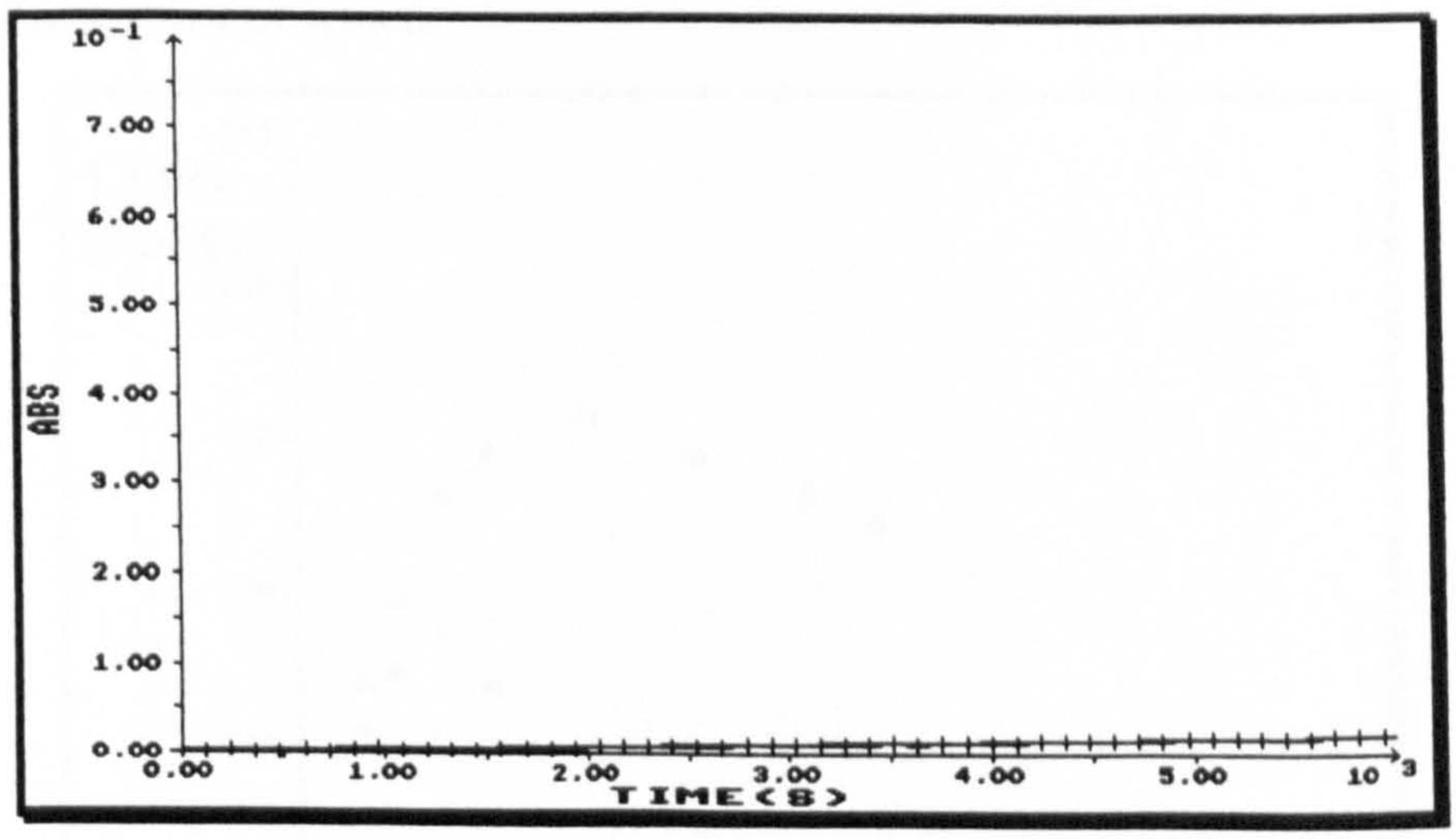
- *Reaction conditions*

p-nitrophenyl β-D-cellobioside :	0.4 mM
Sample from **fraction 4** :	0.092 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, I = 0.05 M
UV-vis spectrophotometer :	365 nm (Release of p-nitrophenol)

A. Hydrolysis of p-nitrophenyl β-D-cellobioside (PNPC)



B. Hydrolysis of PNPC with 1,5-glucono-δ-lactone (20.8 mM)



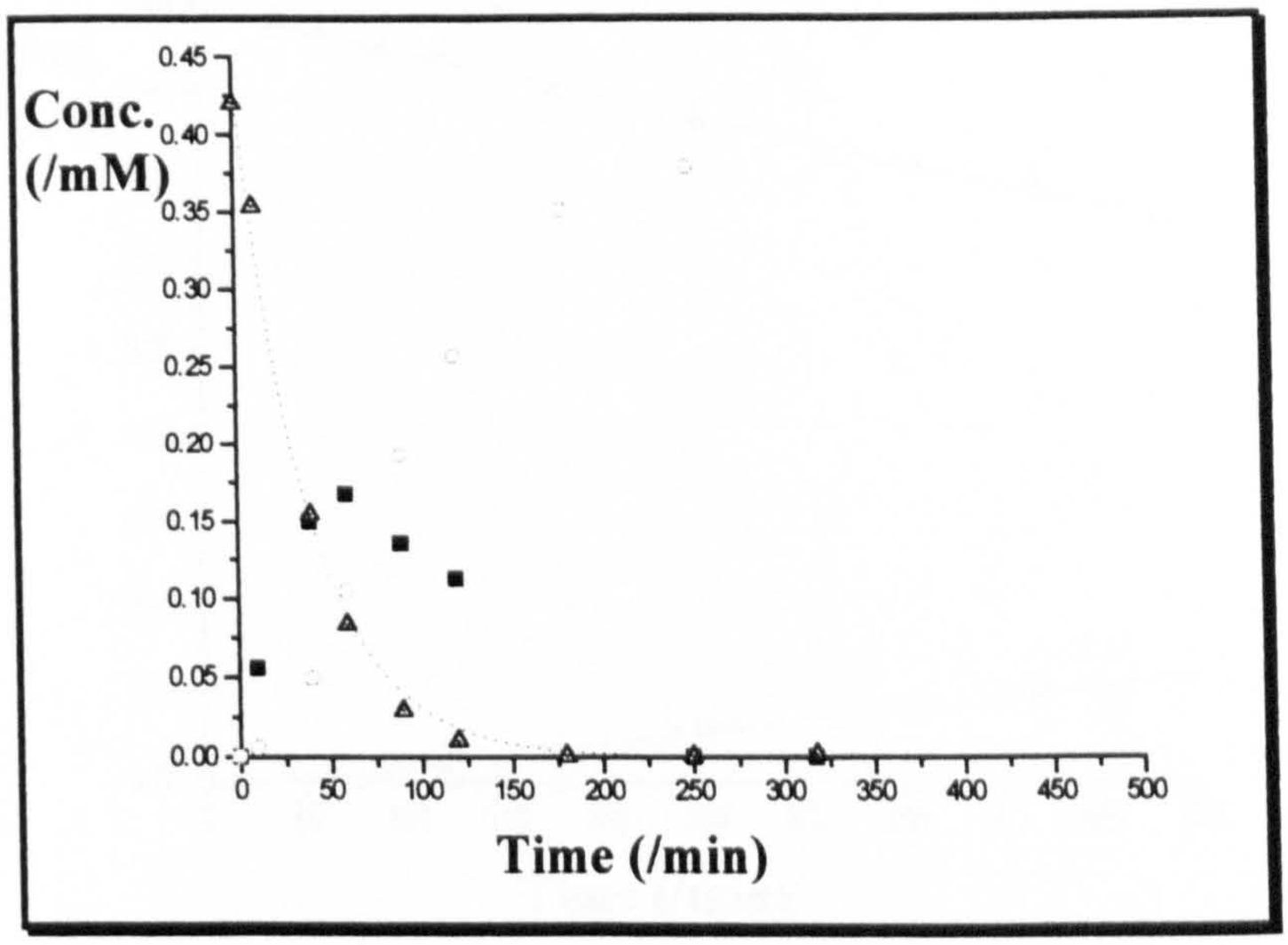
3.3.2. Transglycosylation reaction

Substrate : p-nitrophenyl β-D-cellobioside

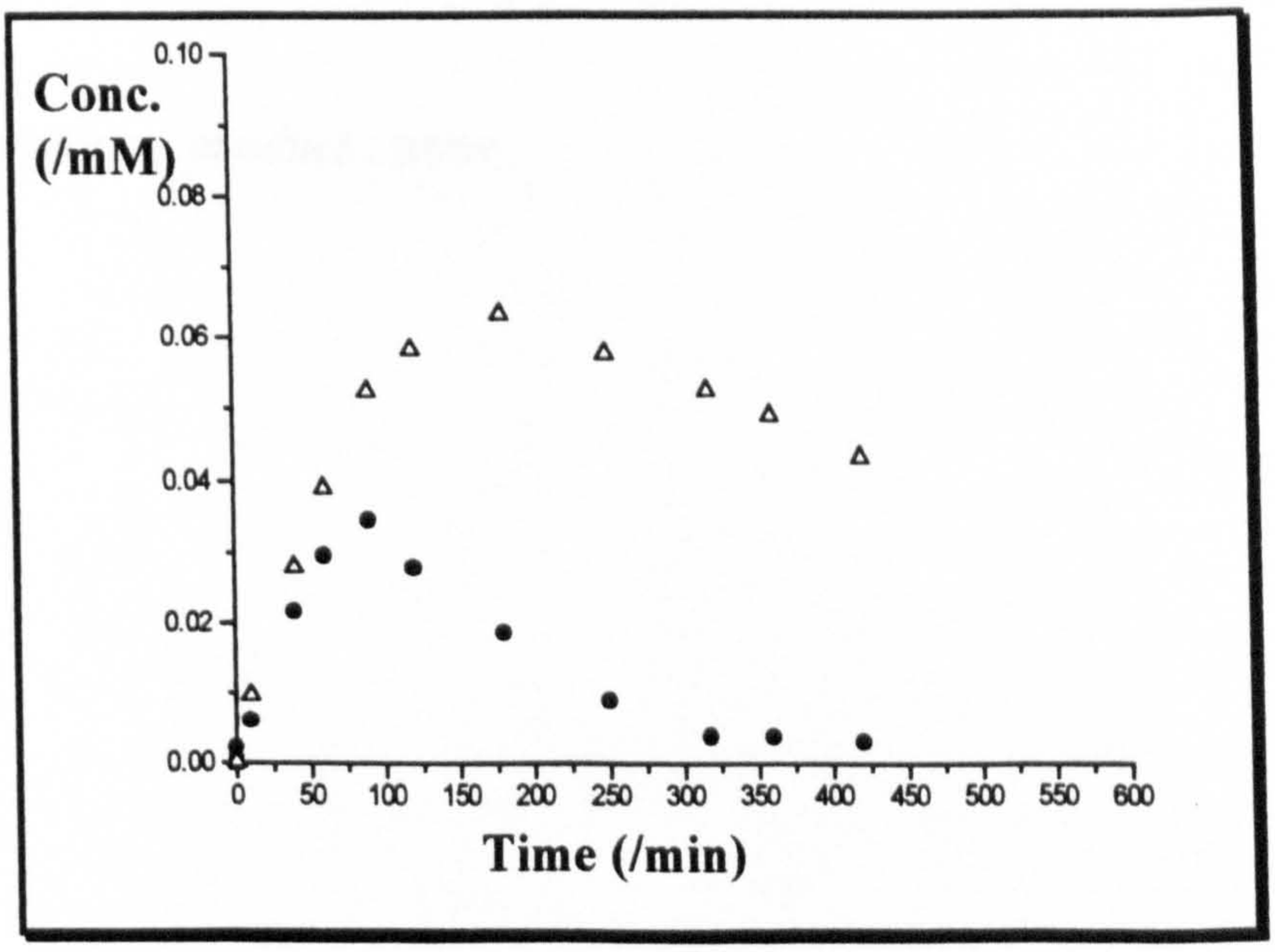
- *Reaction conditions*

p-nitrophenyl β-D-cellobioside :	0.4 mM
N-(p-nitrophenyl)-β-D-glucopyranosylamine :	**6.0 mM**
Sample from **fraction 4** :	0.092 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, I = 0.05 M
HPLC analysis, UV-vis:	300 and 365 nm

Hydrolysis of the substrate



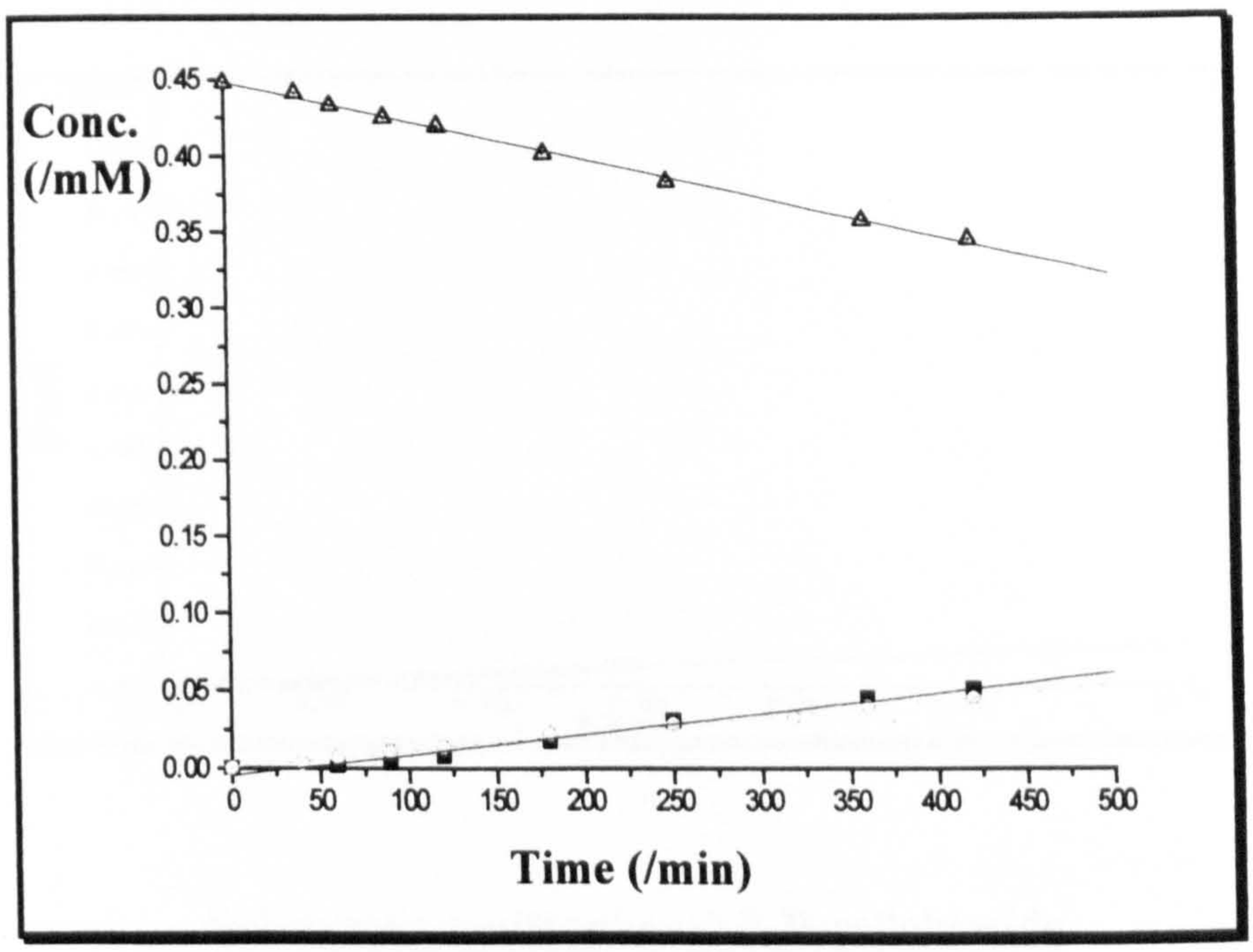
Transglycosylation products



- *Reaction conditions*

p-nitrophenyl β-D-cellobioside :	0.4 mM
N-(p-nitrophenyl)-β-D-glucopyranosylamine :	**6.0 mM**
1,5-Glucono-δ-lactone :	**20.8 mM**
Sample from **fraction 4** :	0.092 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, I = 0.05 M
HPLC analysis, UV-vis:	300 and 365 nm

Hydrolysis of the substrate



Transglycosylation product : **none**

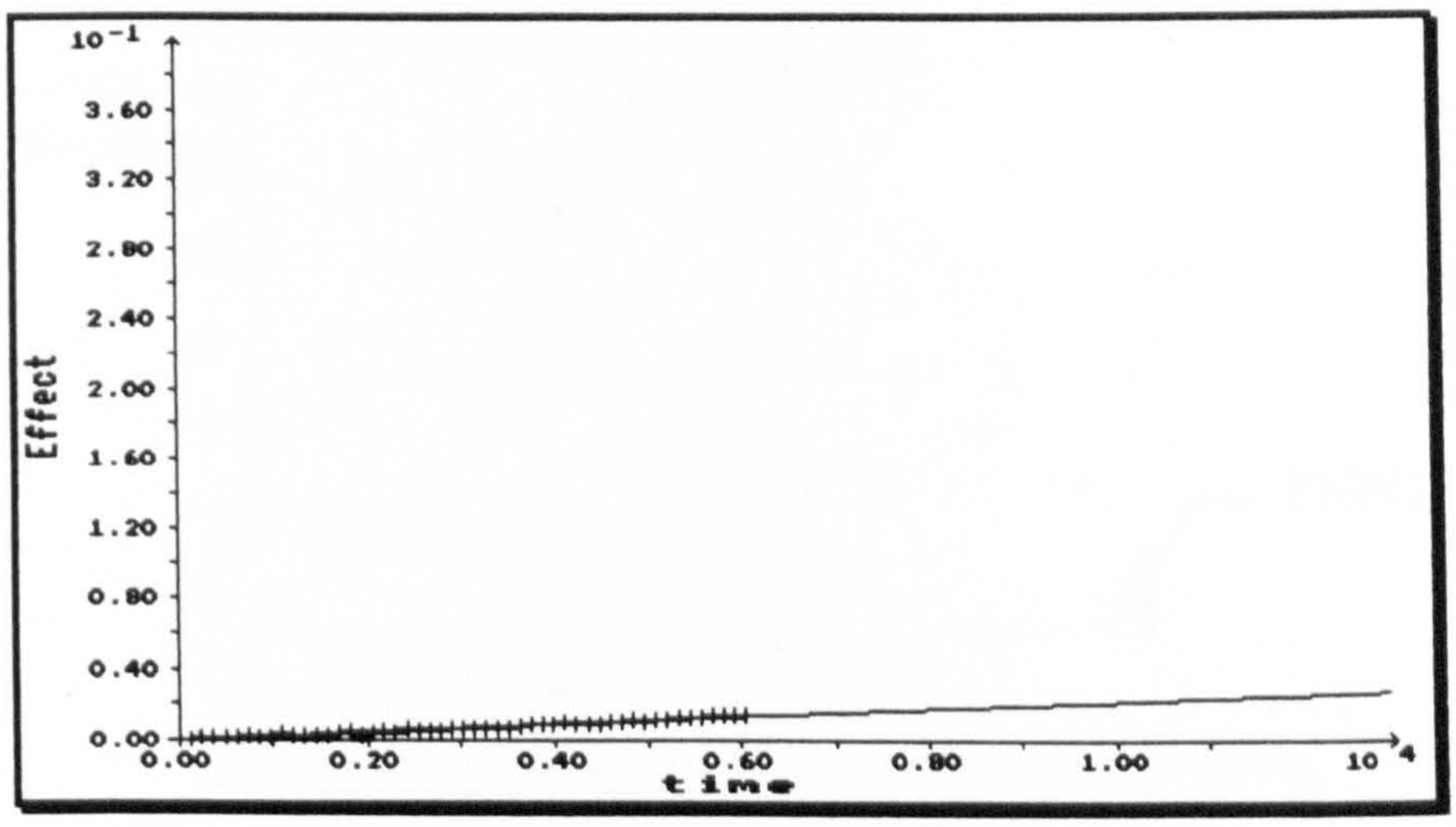
3.4. Peak 2 analysis

3.4.1. β-glucosidase activity

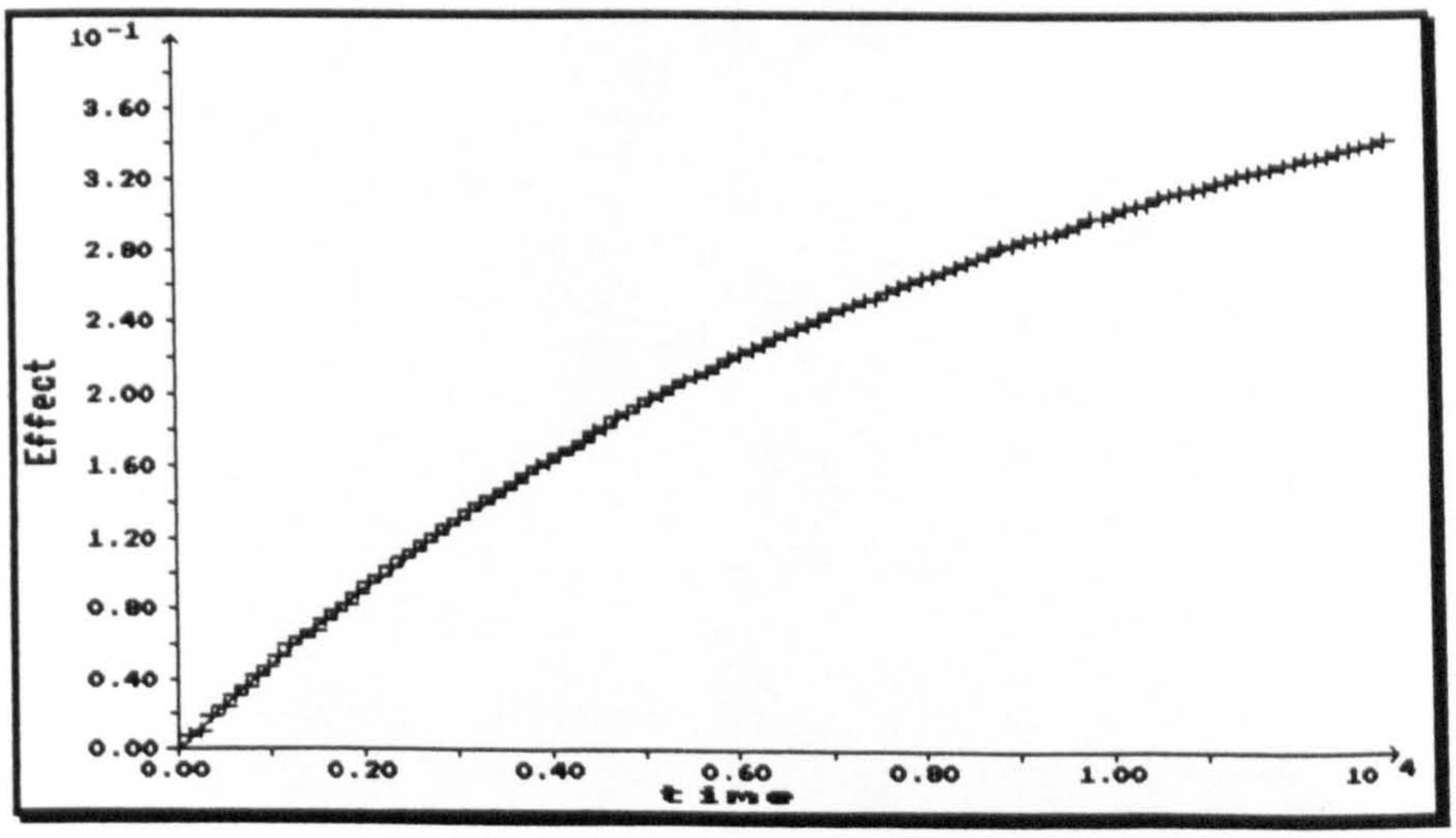
- *Reaction conditions*

Substrate :	0.4 mM
Sample from **fraction 14-16** :	0.23 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, I = 0.05 M
UV-vis spectrophotometer :	365 nm (Release of p-nitrophenol)

<u>Substrate : p-nitrophenyl β-D-glucopyranoside</u>



<u>Substrate : p-nitrophenyl β-D-cellobioside</u>



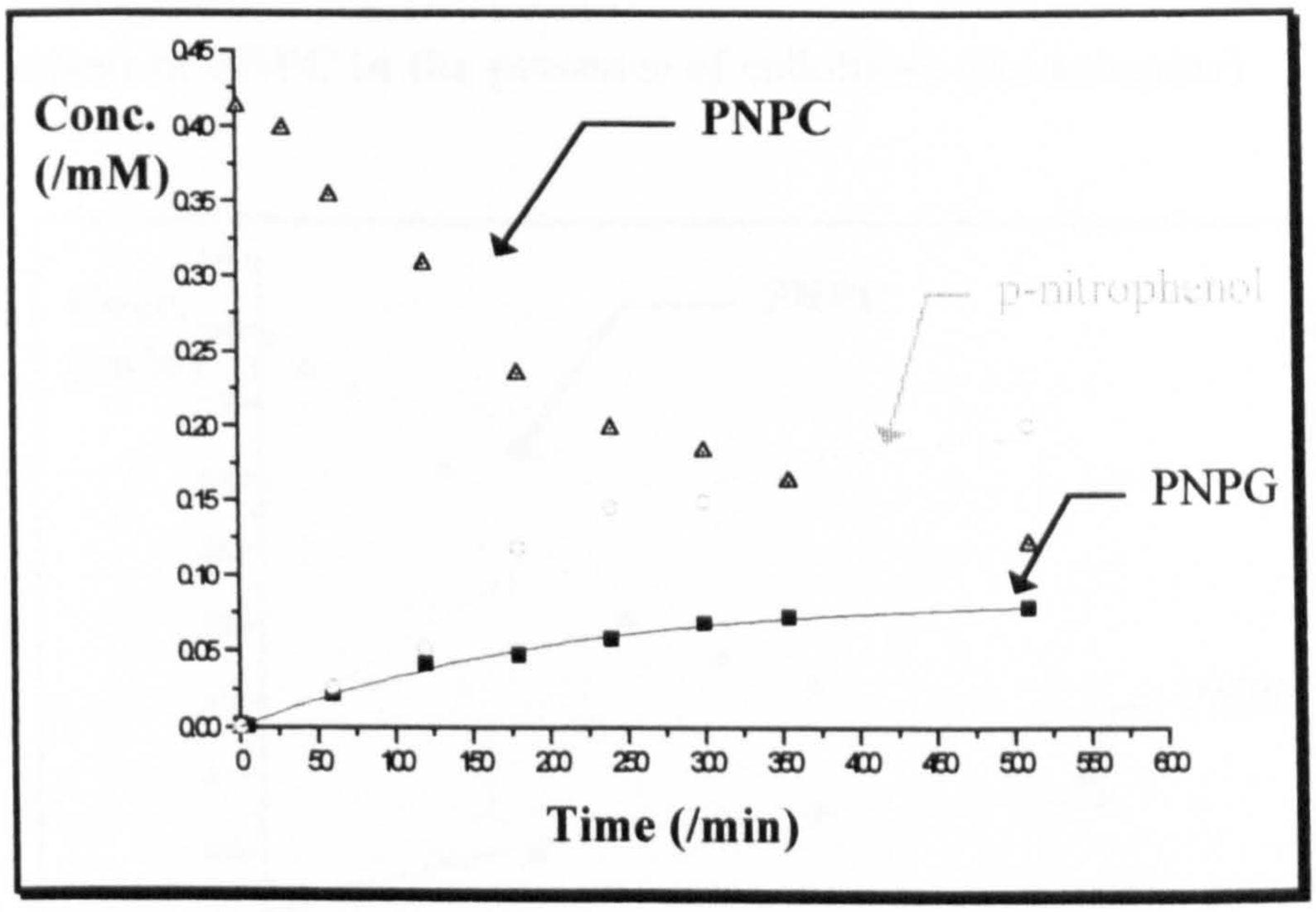
3.4.2. Transglycosylation reaction

Substrate : p-nitrophenyl β-D-cellobioside

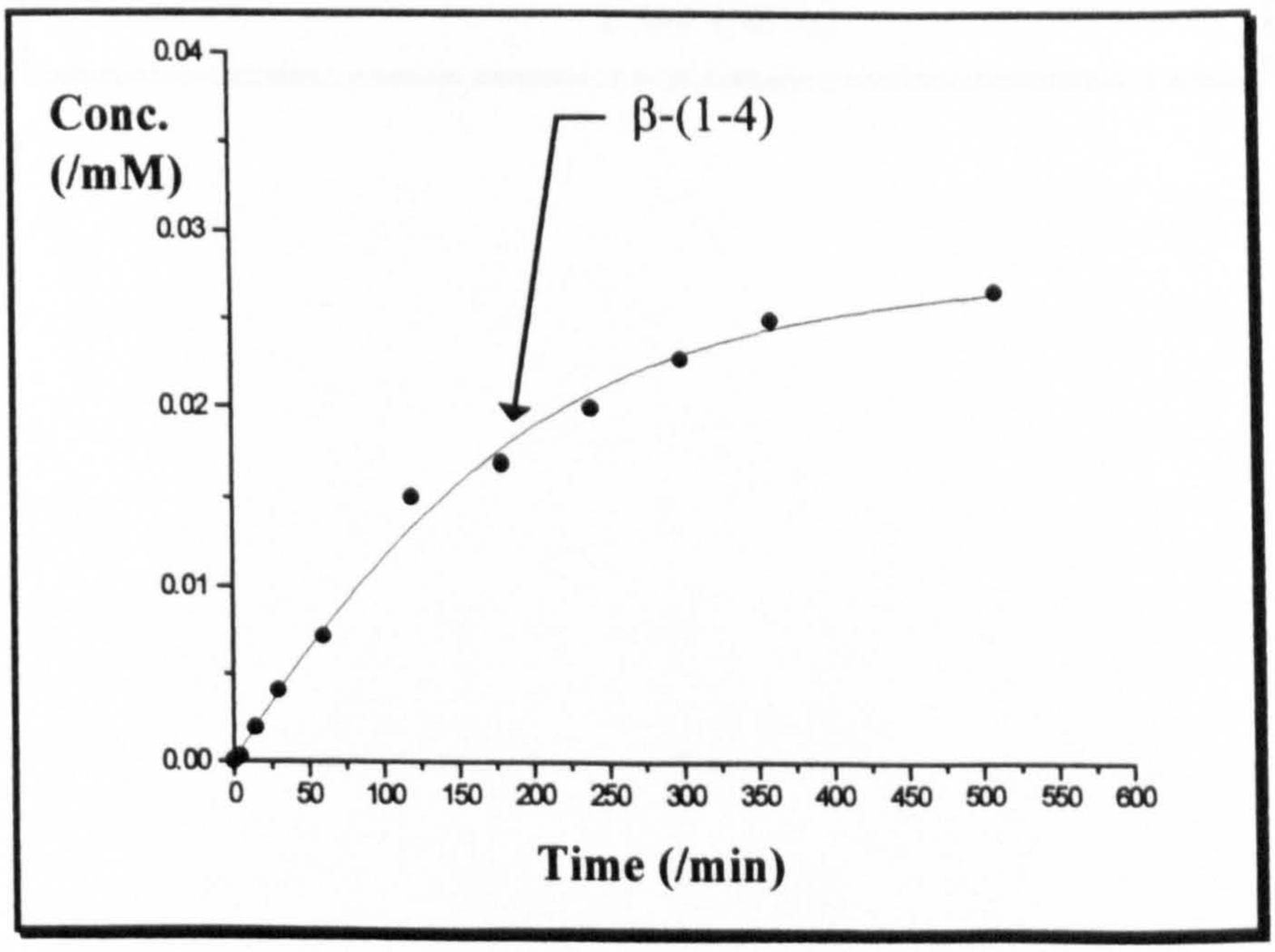
- *Reaction conditions*

p-nitrophenyl β-D-cellobioside :	0.4 mM,
Sample from **fraction 14-16** :	0.092 $mg.ml^{-1}$
N-(p-nitrophenyl)-β-D-glucopyranosylamine :	**6.0 mM**
Sodium acetate buffer :	pH 4.5, I = 0.05 M
HPLC analysis, UV-vis:	300 and 365 nm

Substrate hydrolysis



Transglycosylation product



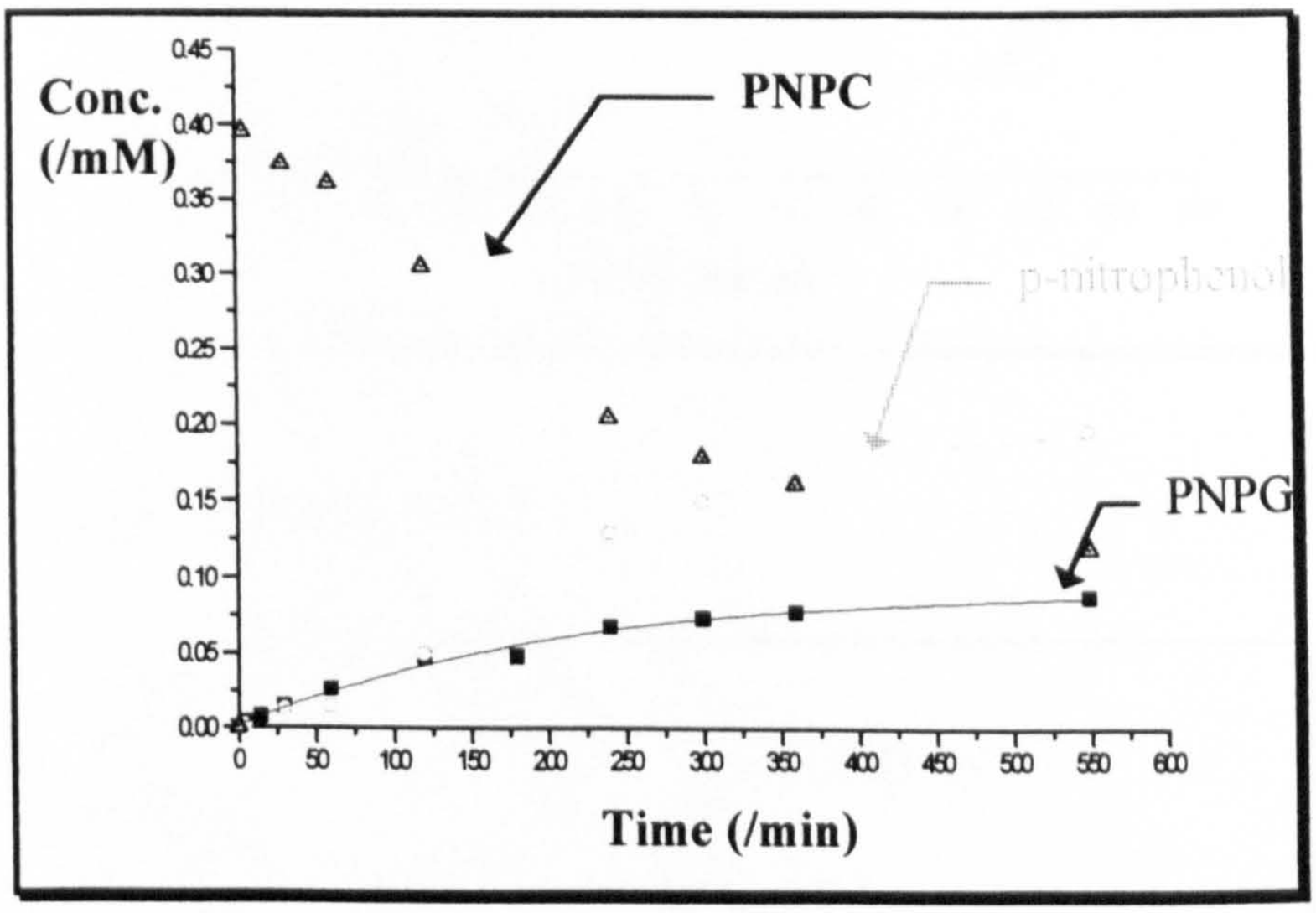
3.4.3. Cellobiohydrolase activity

Substrate : p-nitrophenyl β-D-cellobioside

- *Reaction conditions*

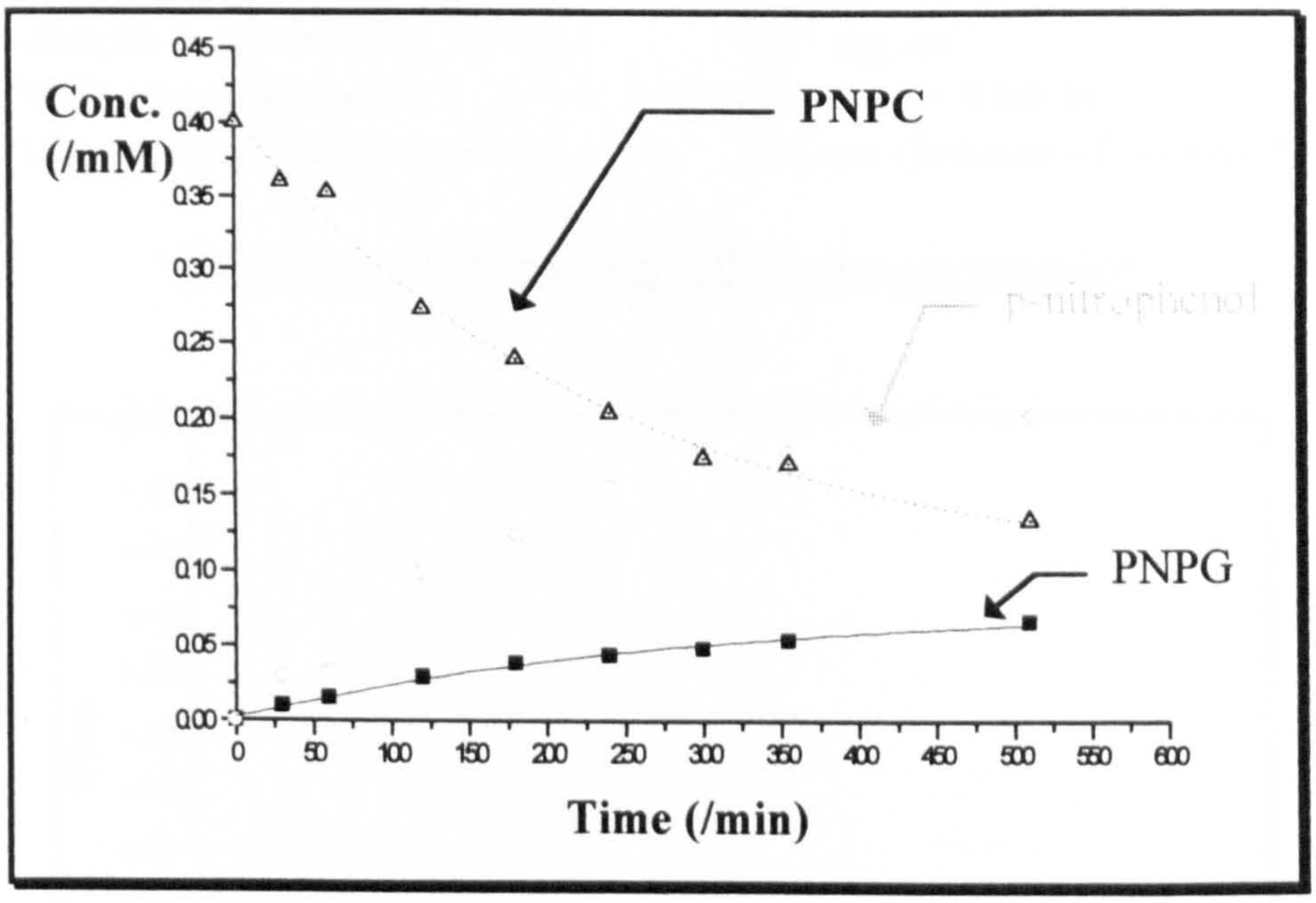
(p-nitrophenyl)-β-D-cellobioside :	0.4 mM
Cellobiose :	**2.0 mM**
Sample from **fraction 14-16** :	0.092 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, I = 0.05 M
HPLC analysis, UV-vis:	300 and 365 nm

A. Hydrolysis of PNPC in the presence of cellobiose (No acceptor)

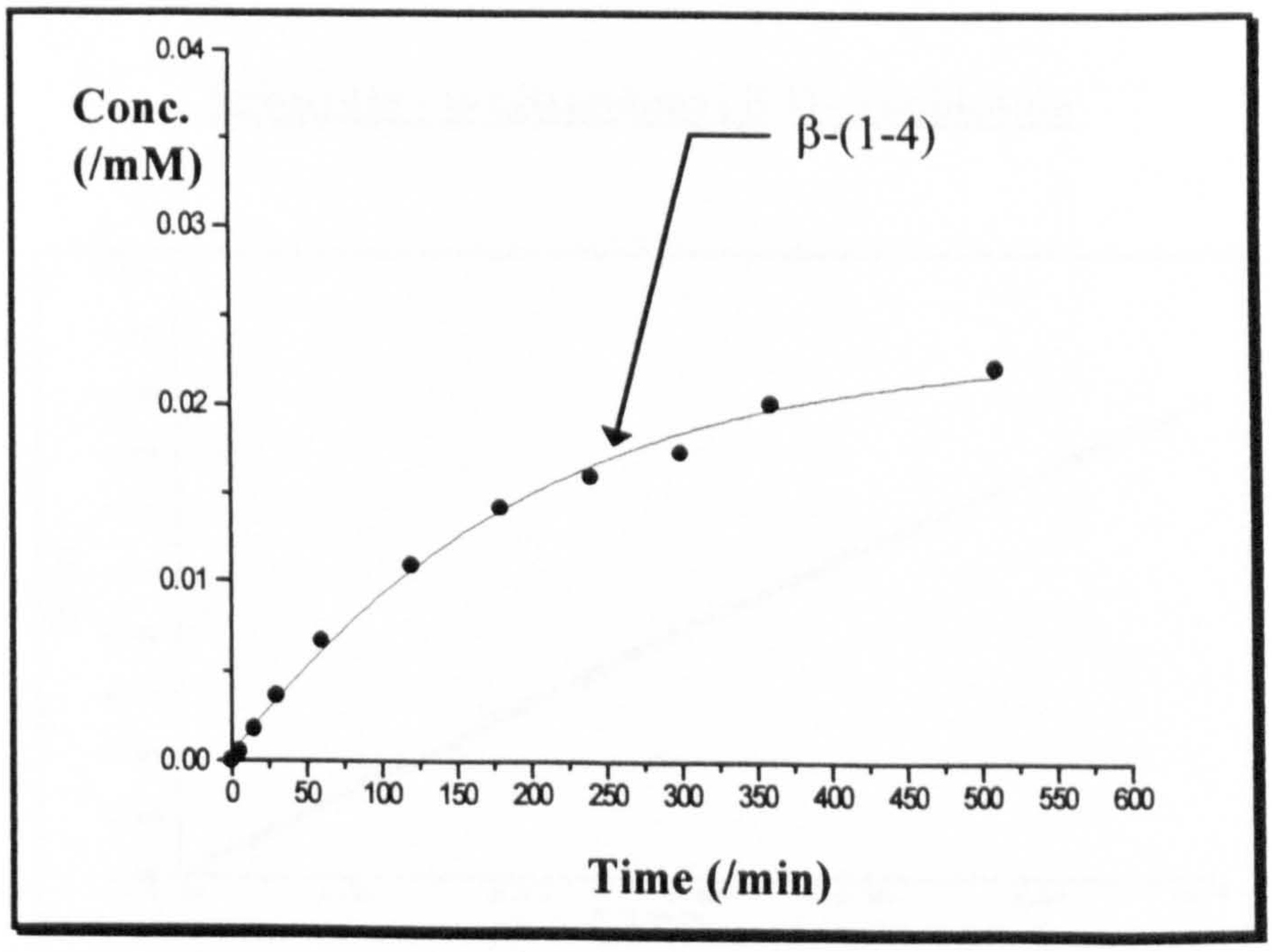


B. Transglycosylation using N-(p-nitrophenyl)-β-D-glucopyranosylamine acceptor (6.0 mM) as an acceptor in the presence of cellobiose

Substrate hydrolysis



Transglycosylation product



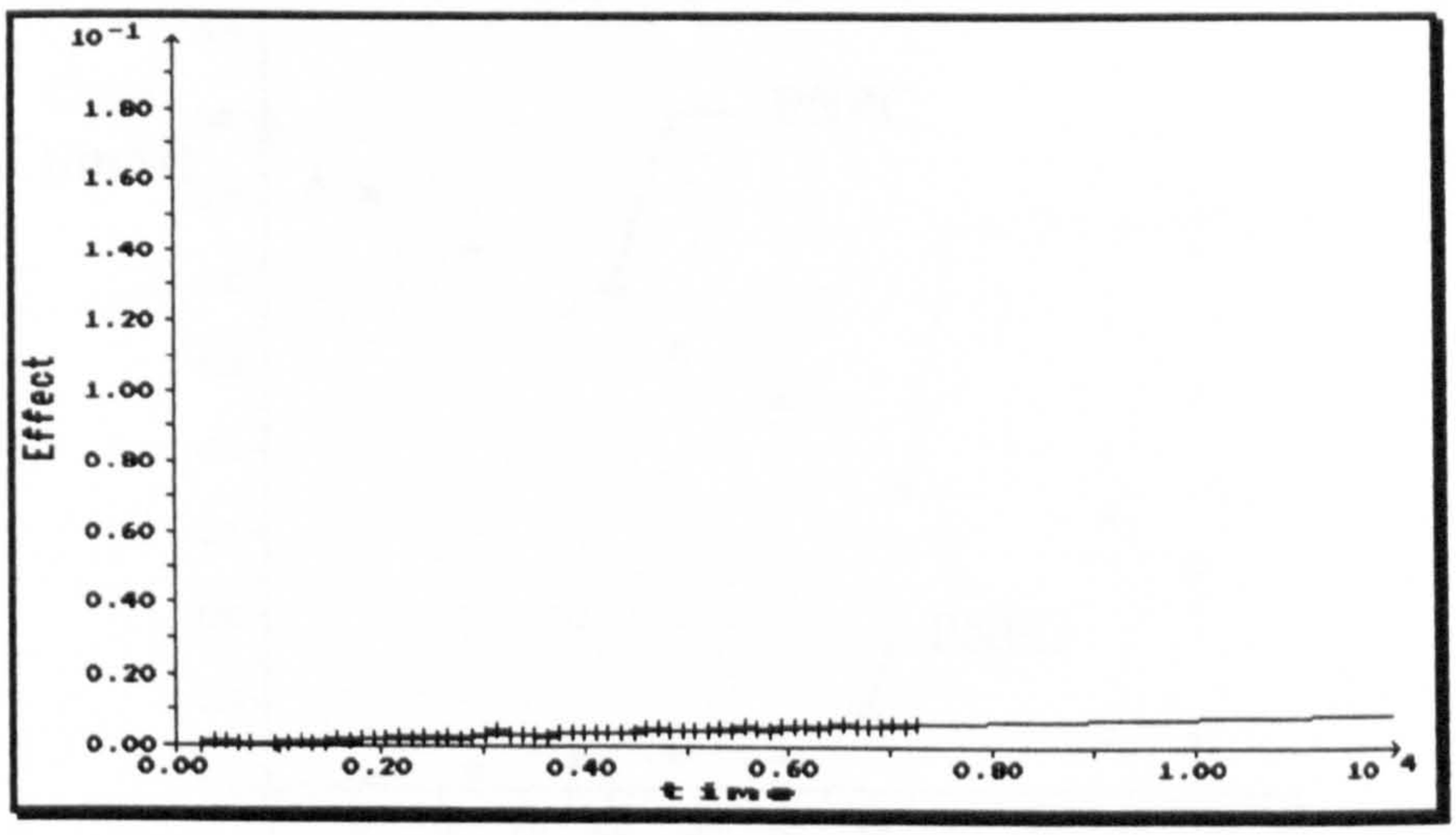
3.5. Peak 3 analysis

3.5.1. β-glucosidase activity

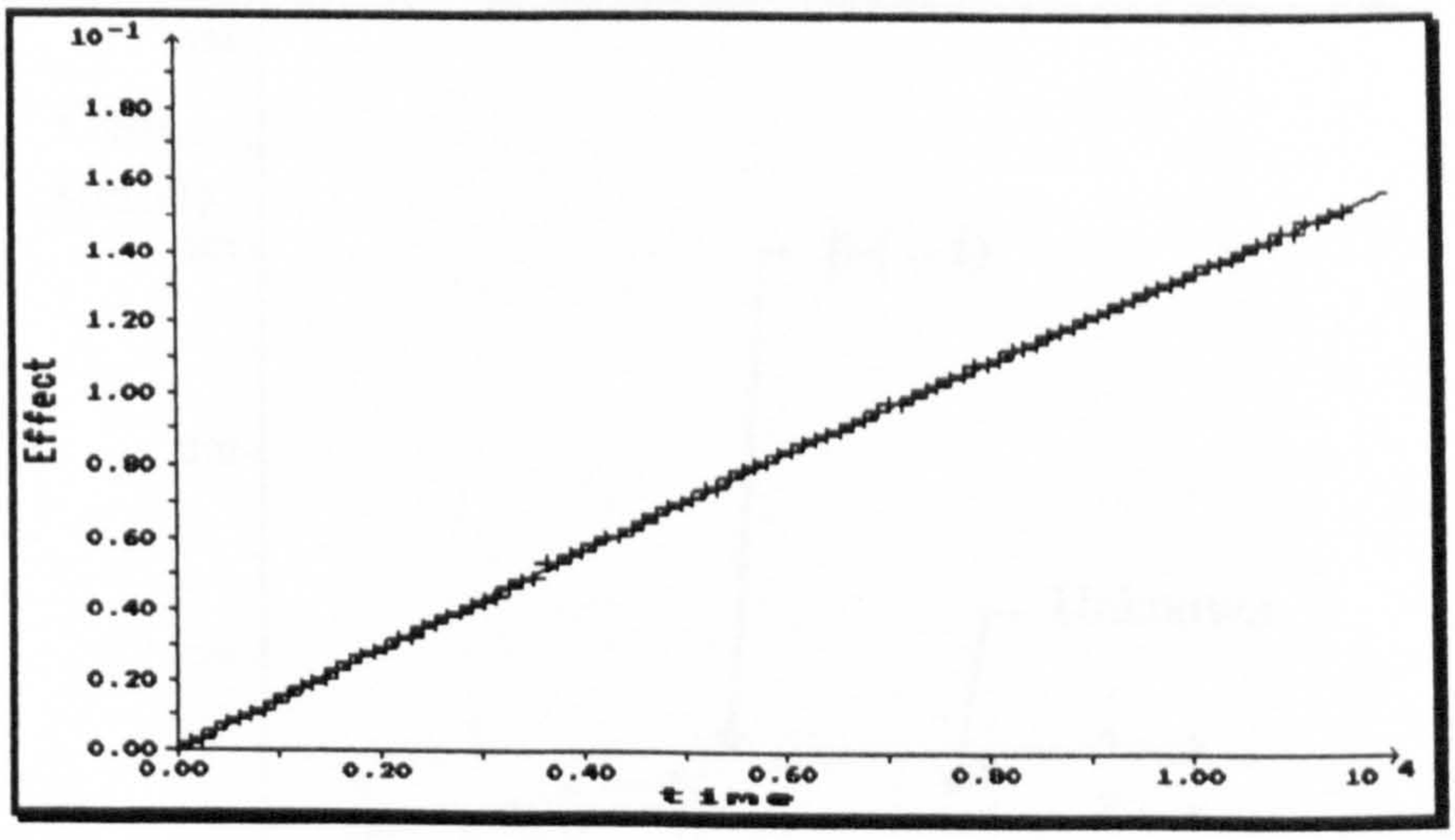
- *Reaction conditions*

Substrate :	0.4 mM
Sample from **fraction 39-40** :	0.23 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, I = 0.05 M
UV-vis spectrophotometer :	365 nm (Release of p-nitrophenol)

Substrate : p-nitrophenyl β-D-glucopyranoside



Substrate : p-nitrophenyl β-D-cellobioside



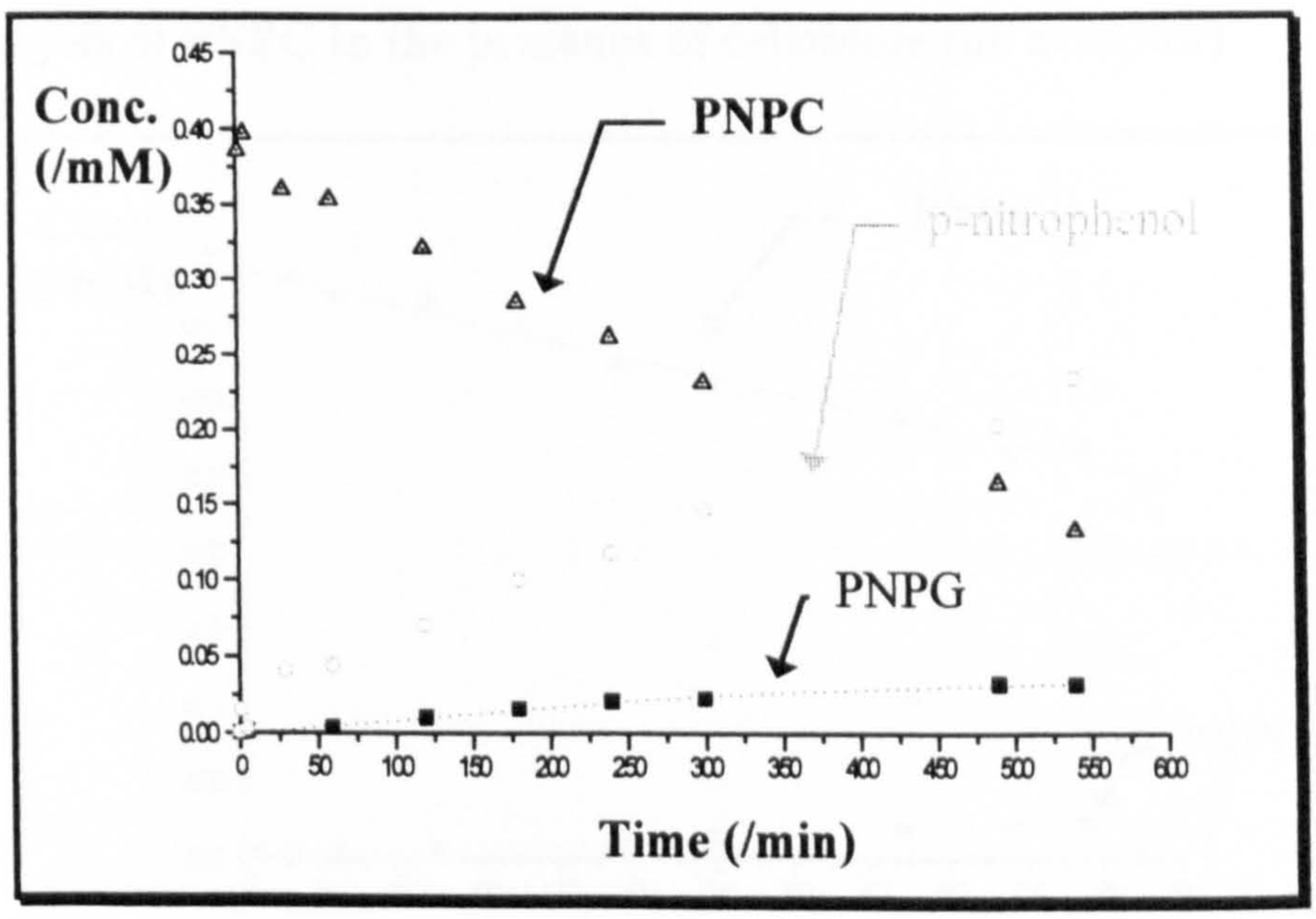
3.5.2. Transglycosylation reaction

Substrate : p-nitrophenyl β-D-cellobioside

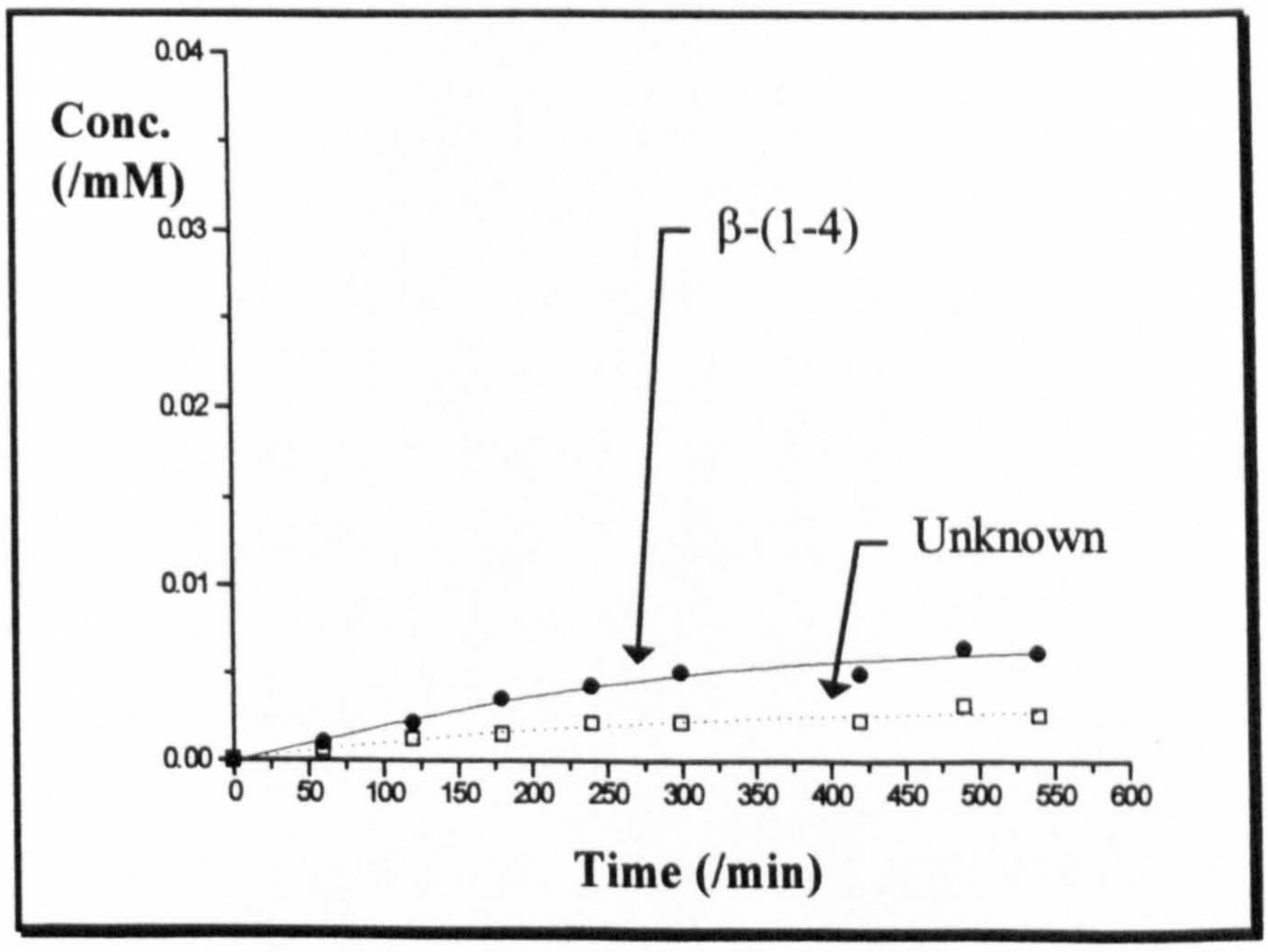
- *Reaction conditions*

p-nitrophenyl β-D-cellobioside :	0.4 mM
N-(p-nitrophenyl)-β-D-glucopyranosylamine :	**6.0 mM**
Sample from **fraction 39-40** :	0.092 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, I = 0.05 M
HPLC analysis, UV-vis:	300 and 365 nm

Substrate hydrolysis



Transglycosylation products



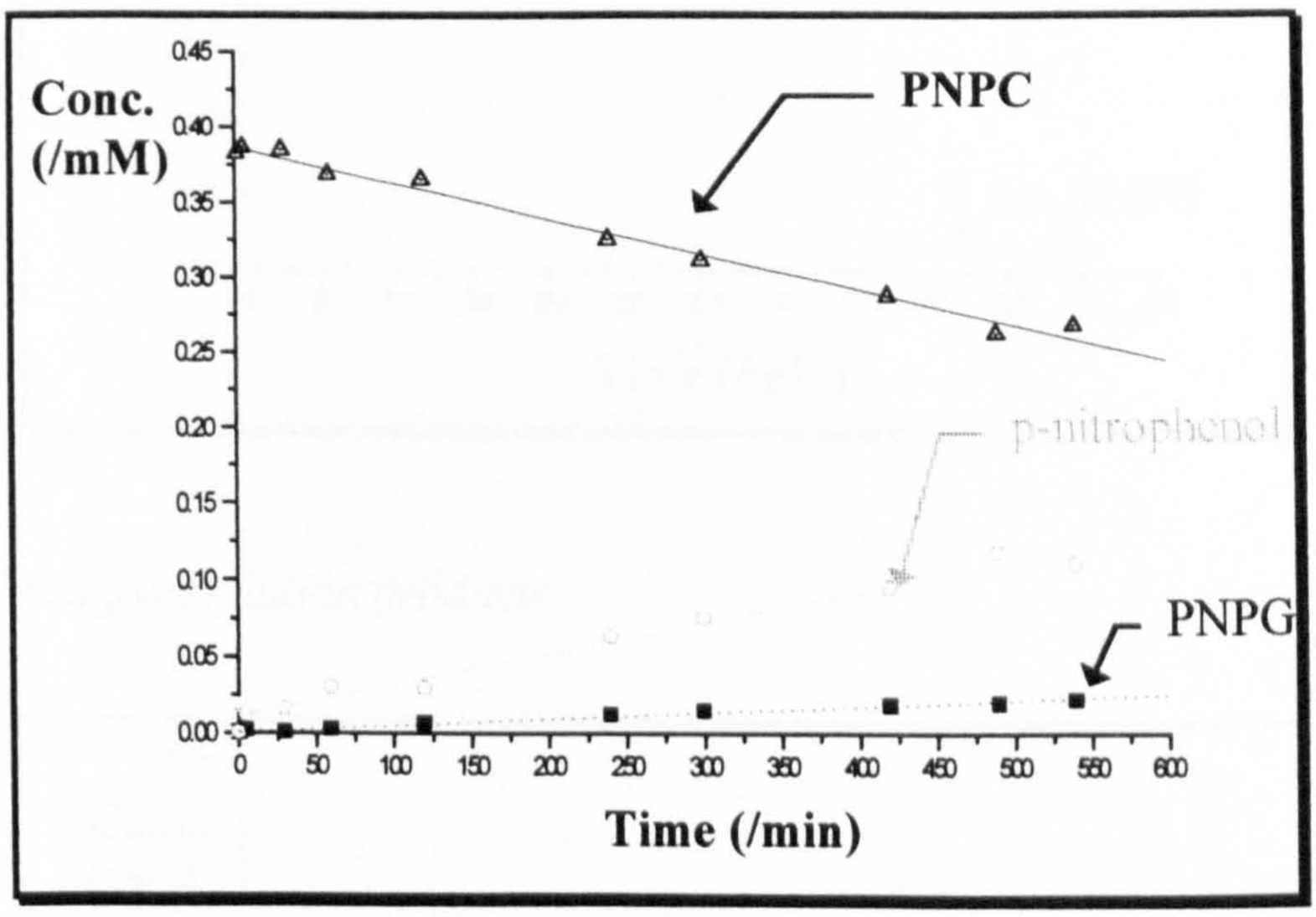
3.5.3. Cellobiohydrolase activity

Substrate : p-nitrophenyl β-D-cellobioside

- *Reaction conditions*

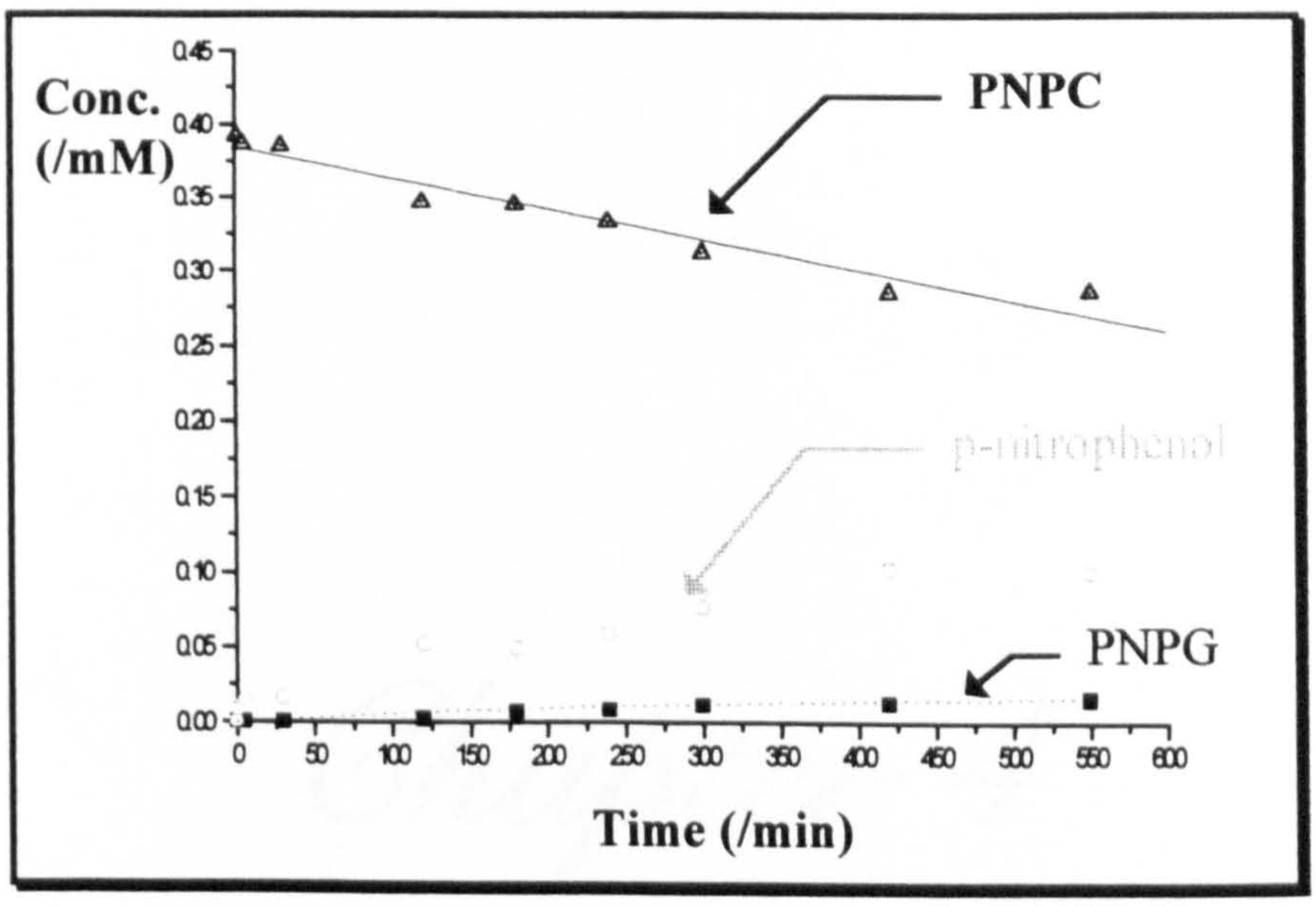
p-nitrophenyl β-D-cellobioside :	0.4 mM
Cellobiose :	**2.0 mM**
Sample from **fraction 39-40** :	0.23 $mg.ml^{-1}$
Sodium acetate buffer :	pH 4.5, I = 0.05 M
HPLC analysis, UV-vis:	300 and 365 nm

A. Hydrolysis of PNPC in the presence of cellobiose (no acceptor)

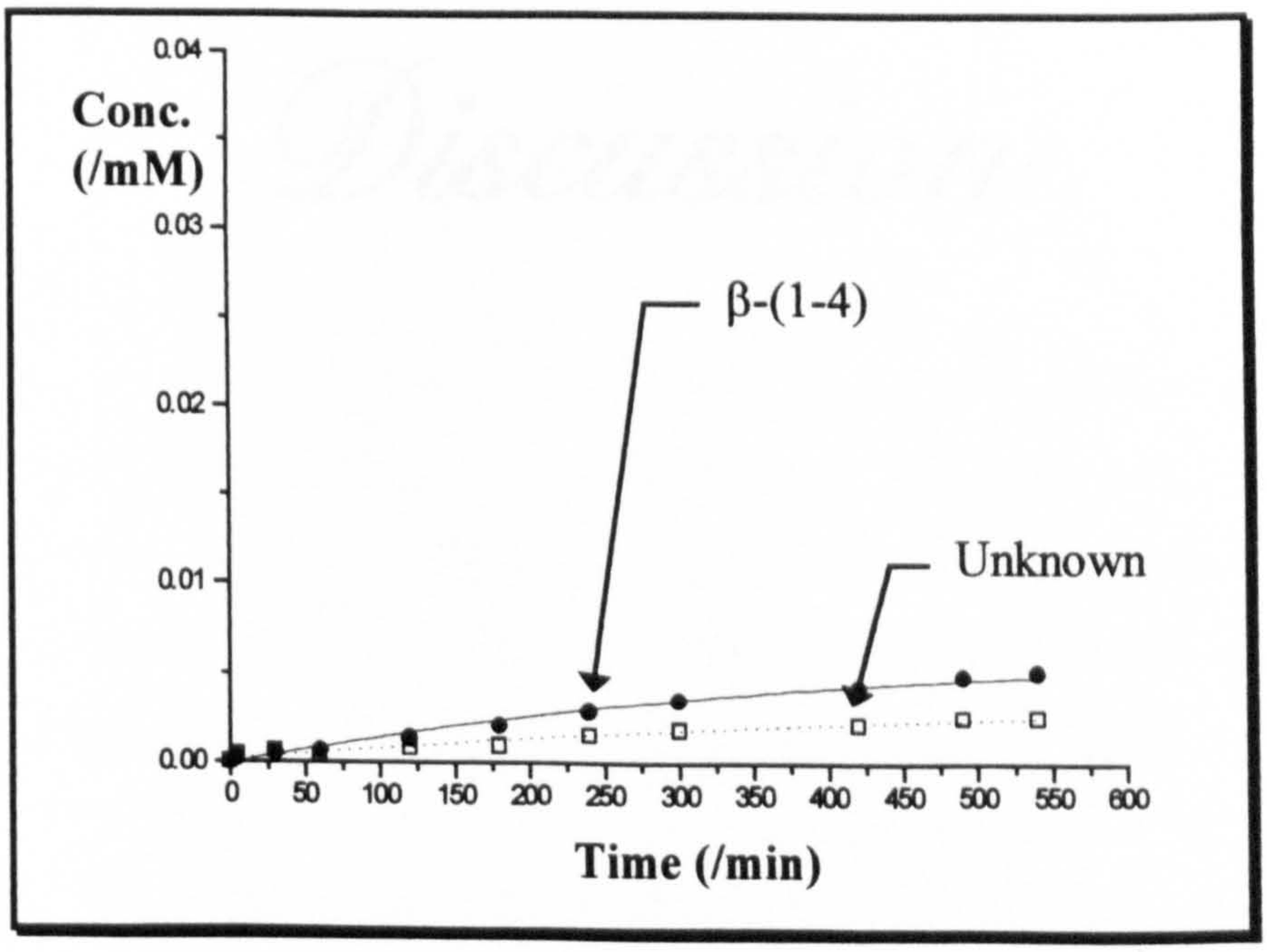


B. Transglycosylation using N-(p-nitrophenyl)-β-D-glucopyranosylamine acceptor (6.0 mM) as an acceptor in the presence of cellobiose

Substrate hydrolysis



Transglycosylation products

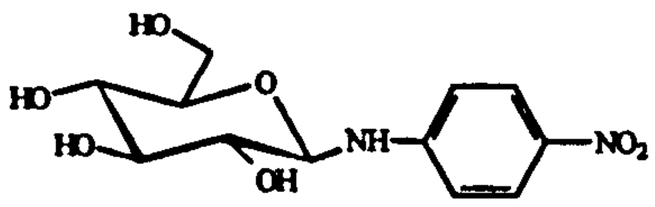


Chapter 4

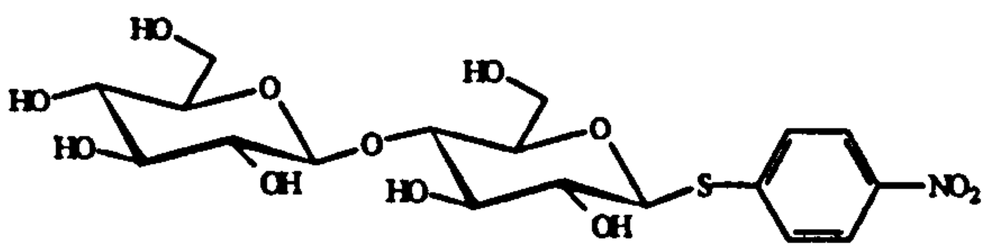
Discussion

1. Synthesis

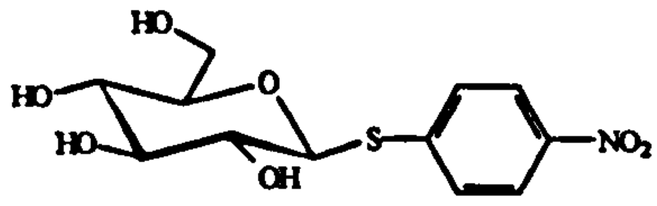
In order to undertake a study of the transferase activity of the various components of the cellulase complex, it was necessary to synthesise a range of glycosyl acceptor molecules. The preparation of the various glycosyl acceptors (compounds 1, 6, 8 & 24) and anticipated transfer products (10, 12 & 17) was therefore undertaken.



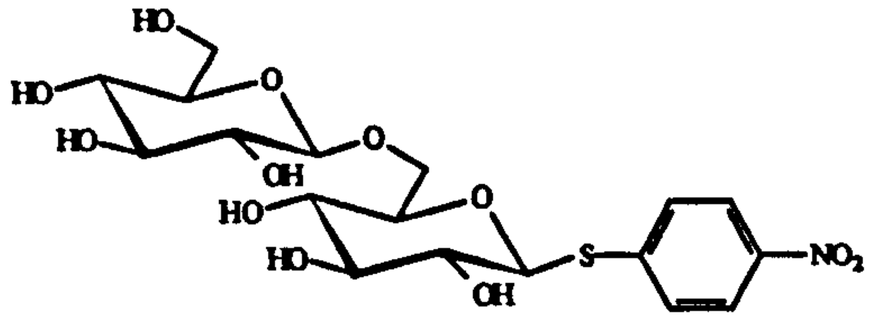
(1)



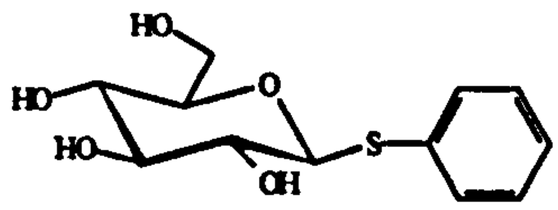
(10)



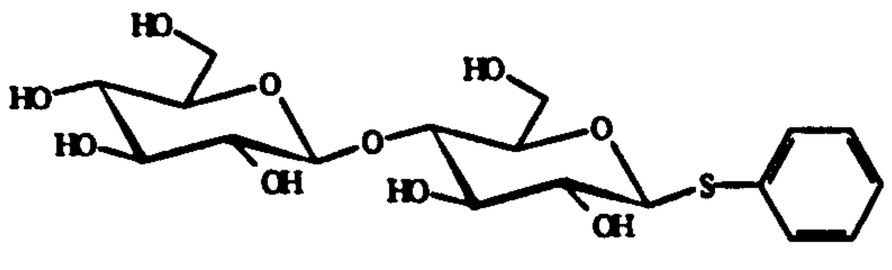
(6)



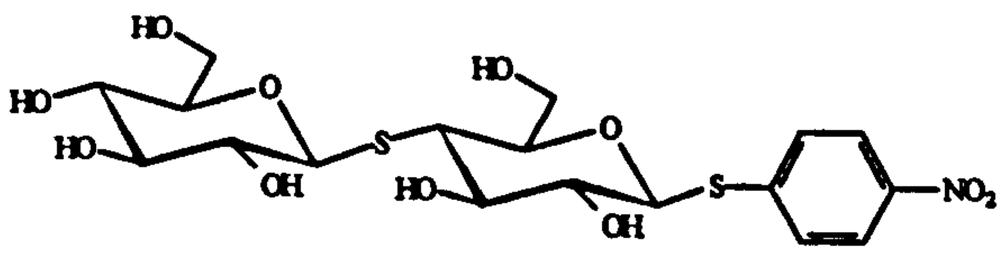
(17)



(8)



(12)



(24)

The synthesis of the acceptor and transfer products has been previously reported in the literature and will not be discussed in detail here other than to say that lengthy protection, activation and deprotection methods were required to provide products in low overall yields. The glycosyl donors were activated as their corresponding halides and reacted directly with aglycon nucleophiles. The synthesis of the dithio disaccharide acceptor **(24)** required at least 6 different steps and gave an overall yield of 14.7 %. The coupling between the glucosyl donor and galactosyl acceptor was achieved using the methyl 2,3,6-tri-O-benzoyl-4-O-trifluoromethylsulphonyl-α-D-galactopyranoside **(20)**, no product was recovered

from the coupling using methyl 2,3,6-tri-O-benzoyl-4-O-methylsulphonyl-α-D-galactopyranoside **(19)** (Figure 42).

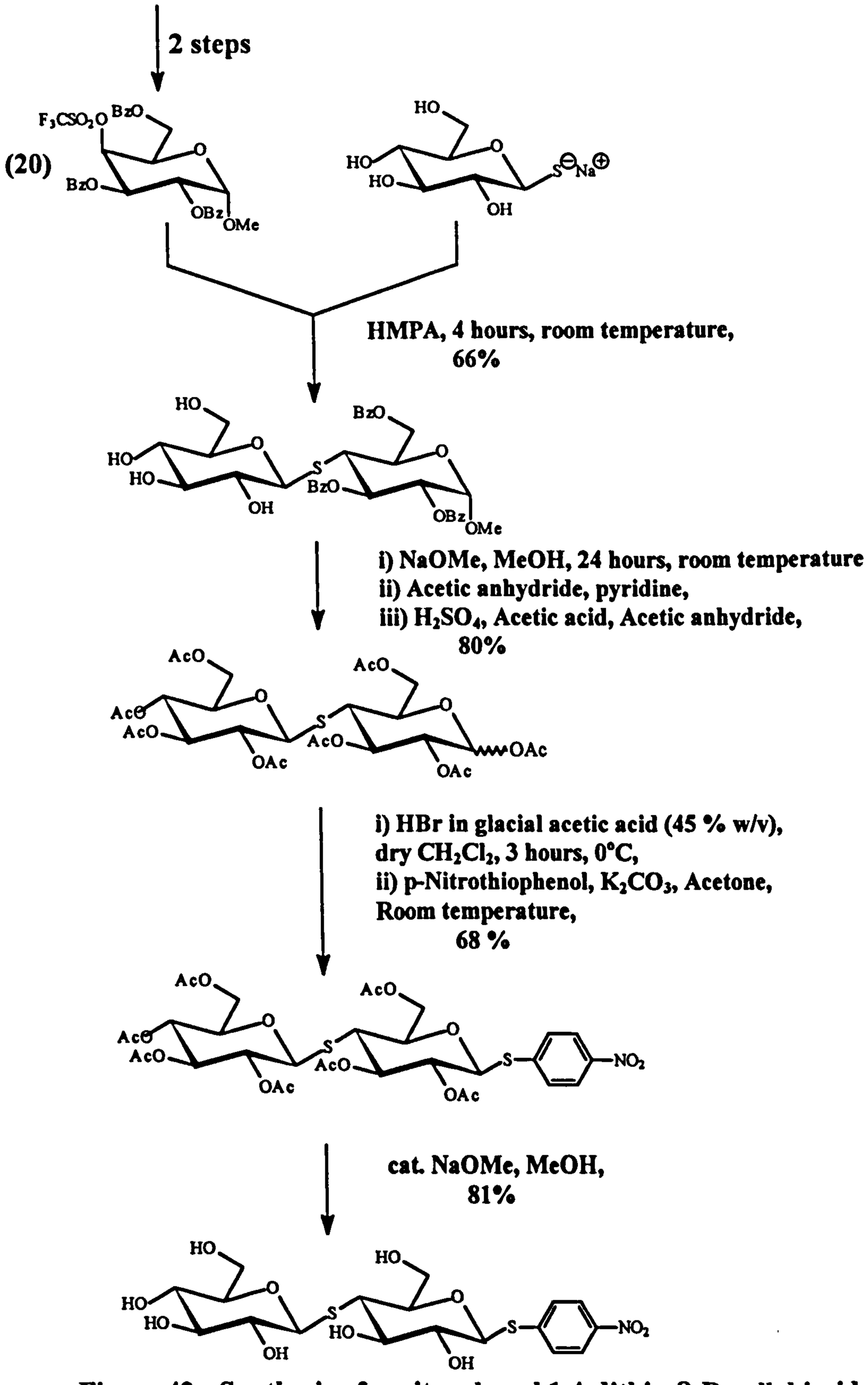


Figure 42 : Synthesis of p-nitrophenyl 1,4-dithio-β-D-cellobioside

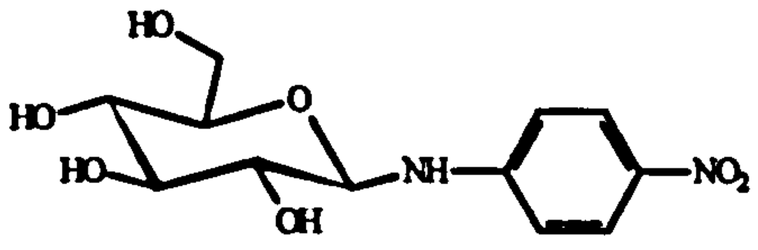
The anomeric configuration and the purity of all the compounds prepared in the project were confirmed by nmr experiments.

2. Studies Of The Transglycosylation Reactions

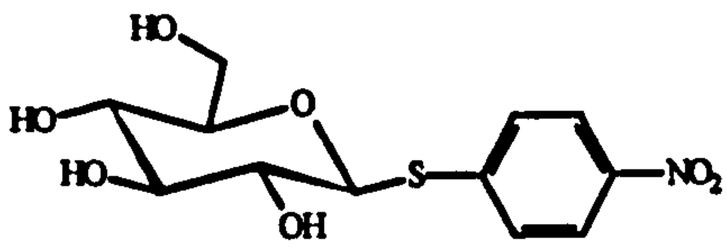
The total hydrolysis of the polysaccharide cellulose by *Trichoderma reesei* relies on the presence of a fully functional cellulase complex. The fungi *Trichoderma reesei* has a constitutive cellulase complex which initiates hydrolysis of cellulose. However, as cellulose is consumed, compounds produced from the catabolism mechanism induces production of further enzymes[78]. It has been postulated that the compounds inducing cellulase activity are transfer products derived directly, or indirectly, from the action of the components of the cellulase complex. Prior to this work, there have been no detailed kinetic studies of the transfer activity of the whole cellulase complex. The transferase activity of the β-glucosidase has been studied in isolation[50]. The aim of this project is to study the transferase activity of β-glucosidase and the other components of the cellulase complex in order to maximise the transferase activity of the whole enzyme.

2.1. Choice of Acceptors

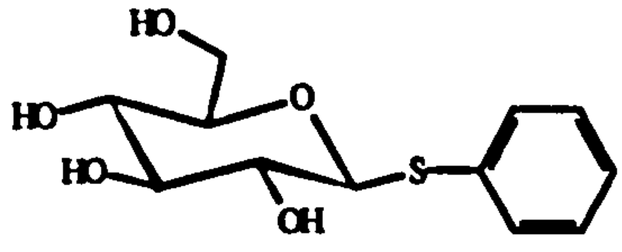
The following compounds were prepared and tested to determine their suitability for use as glycoside acceptors, in the transfer reactions catalysed by the cellulase complex:



(1)



(6)



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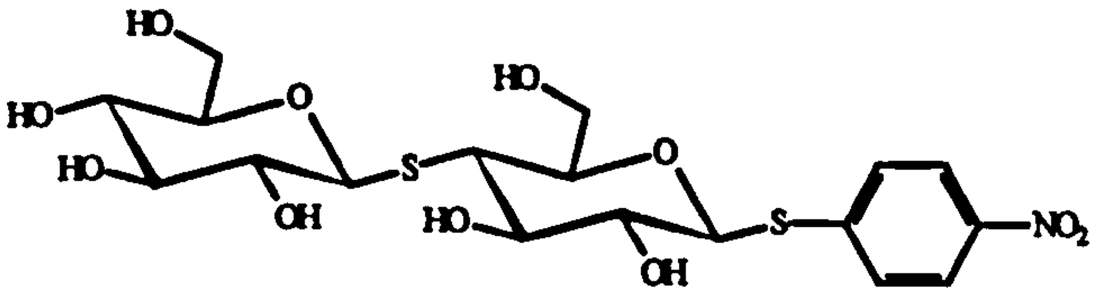


(24)

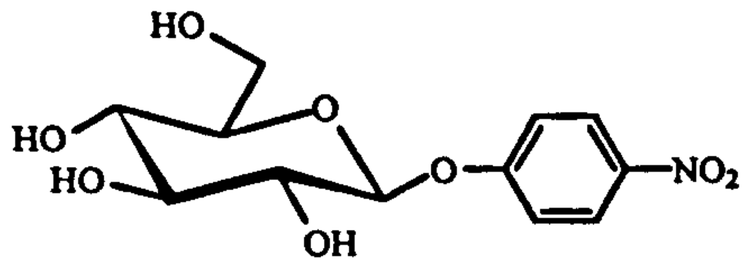
To be a suitable acceptor molecule, the acceptor must be recognised and preferentially interact with the glycosyl binding sites of the enzymes of the complex. They must be stable to hydrolysis and should show a low or moderate affinity for the substrate binding site of the enzymes. It has been shown that the aromatic glycosyl amines are ideal acceptor molecules[50]. The aromatic amines are able to interact with a binding pocket of the β-glucosidase of cellulase and participate in transfer reactions. The reduced basicity of the aromatic amine prevents strong interaction with the catalytic aspartate groups reducing the extent to which they interact with the substrate binding sites especially in comparison to that of the known β-glucosidase inhibitors the alkyl amines[90].

It has also been reported that thioglycosides, mono- and disaccharides, can be used as affinity ligands for the selective purification of the different components of the cellulase complex[91] suggesting different binding interactions with the different enzymes of the complex. Thioglycosides are known inhibitors of glycosidases and are stable to their hydrolytic activity[92].

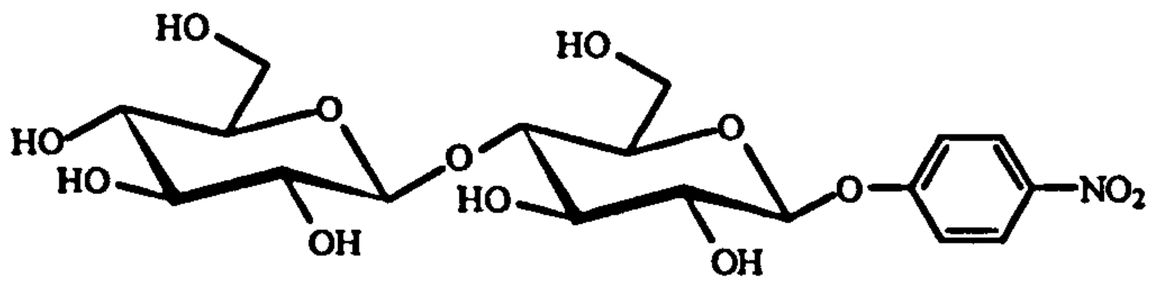
With these results in mind, it was proposed that the aromatic amine glycosides and thioglycosides should be tested to see if they were suitable acceptors for use in studying the transferase activity of cellulase from *Trichoderma reesei*.

2.2. Choice Of Substrate

In the initial studies, N-(p-nitrophenyl)-β-D-glucopyranosylamine **(1)** was used as an acceptor and a new substrate p-nitrophenyl β-D-cellobioside **(26)** was used as an activated disaccharide donor. Previous studies, at the University of Huddersfield[50], have used p-nitrophenyl β-D-glucopyranoside **(25)** as an activated monosaccharide donor.



(25)



(26)

The monosaccharide is a specific substrate for the β-glucosidase component of the complex. It showed transferase activity, forming three different transglycosylation products. Two of them were identified using chemically synthesised analogues as the resulting β-(1-4) and β-(1-6) disaccharides. The identity of the third compound is still under investigation. It was decided to use a disaccharide substrate in an attempt to establish if other components of the cellulase complex were able to catalyse transfer reactions and if so, to determine the nature of the transfer products obtained. As was suggested in the introduction, as the number of glycosyl units in the substrate is increased, the component of the complex catalysing the turnover of the substrate will change, those components of the complex having a larger number of glycosyl binding pockets will have a higher affinity for the substrate.

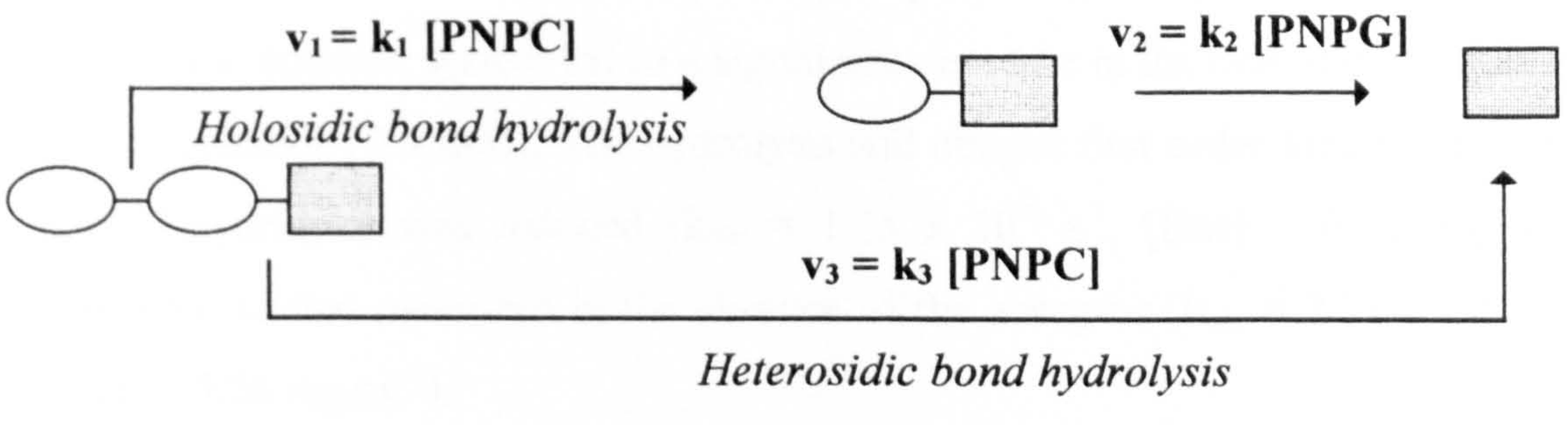
2.3. β-glucosidase from almond

In order to study the transferase activity of the cellulase complex as a whole unit, it was necessary to determine which of the components of the complex were capable of hydrolysing the disaccharide glycosyl donor, p-nitrophenyl β-D-cellobioside (PNPC). First, it was decided to determine whether the disaccharide is a substrate for β-glucosidase by studying its hydrolysis in the presence of the β-glucosidase from almond. It was also necessary to see if the β-glucosidase was able to catalyse transfer reactions. The transglycosylation reaction was then studied using N-(p-nitrophenyl)-β-D-glucopyranosylamine as an acceptor. Previous studies have shown that β-glucosidase from almond can be used as a glycosyl transferase *in vitro*[93].

2.3.1. Hydrolysis of p-nitrophenyl β-D-cellobioside catalysed by the β-glucosidase from almond

The hydrolysis of the disaccharide, p-nitrophenyl β-D-cellobioside, was shown to be catalysed by the β-glucosidase from almond and so as such is a moderate substrate for this enzyme. The reaction system was studied using a concentration of substrate (0.4 mM) which would subsequently be used in studies of the transfer reactions under kinetically controlled conditions. At this concentration, the disappearance of the substrate obeyed first order kinetics, saturation kinetics were not observed. The first order rate constant measured, k_{obs}, was determined (k_{obs}= 2.31 x 10^{-4} s^{-1}, [Enz]= 0.06 $mg.ml^{-1}$). The HPLC chromatograph showed the formation of both p-nitrophenyl β-D-glucopyranoside (PNPG) and p-nitrophenol at the beginning of the reaction, resulting from the hydrolysis of both, holosidic and heterosidic, linkages present in the substrate (See Figure 43).

The hydrolysis pathway can be considered as having two competing steps:



: p-nitrophenyl β-D-cellobioside (PNPC)

: glucosyl residue

: p-nitrophenyl

Figure 43 : Hydrolysis of PNPC by β-glucosidase

The monosaccharide PNPG appears as an intermediate, it is itself a substrate for the β-glucosidase. Under the conditions of the reaction, the concentration of the PNPG reaches a maximum concentration (0.026 mM after 25 minutes) before being hydrolysed further by the enzyme.

It is also important to notice the absence of self-transfer products under the conditions of the reaction.

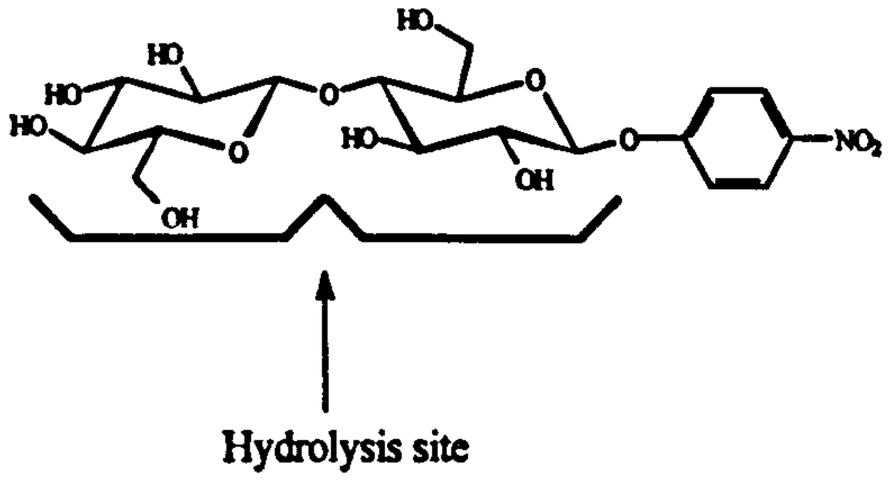
2.3.2. Transglycosylation studies

The addition of the acceptor N-(p-nitrophenyl)-β-D-glucopyranosylamine (10.0 mM) to the reaction system led to a significant decrease in the rate of hydrolysis of the disaccharide (0.4 mM). The hydrolysis still obeyed first order kinetics, but the rate of hydrolysis was reduced (k_{obs} = 1.15 x 10^{-4} s^{-1}, [Enz] = 0.10 $mg.ml^{-1}$) compared to that measured in the absence of the acceptor (k_{obs} = 2.31 x 10^{-4} s^{-1}, [Enz] = 0.06 $mg.ml^{-1}$).

By comparison with the reaction system containing no acceptor, both the rates of p-nitrophenol and PNPG production were much slower. In the presence of the acceptor, the rate of formation of PNPG is greater than that of p-nitrophenol whereas the opposite was observed in its absence.

The shift in relative rates of bond cleavage from favouring heterosidic cleavage to holosidic cleavage may be due to a number of different mechanisms. The inclusion of a high concentration of an acceptor molecule may influence the binding characteristics of the substrate within the active site of a single enzyme (Figure 44).

With acceptor:



Without acceptor:

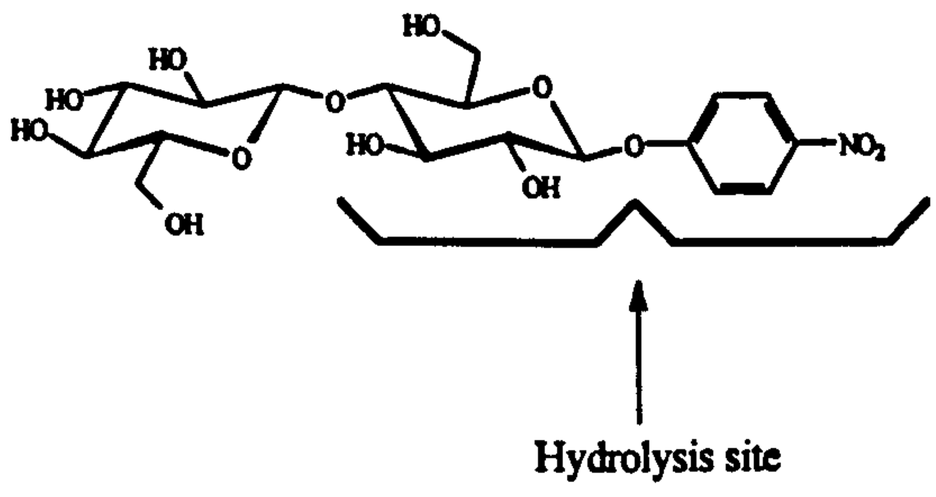


Figure 44 : Preferred hydrolysis site with and without acceptor

Initial binding of the acceptor may also exclude the substrate from specific binding pockets (See later discussion of the substituted enzyme mechanism).

A similar shift in the pattern of bond cleavage frequencies, on increasing substrate concentration, has been observed for several purified components of the cellulase complex[60].

In addition to observing a reduction in the rate of hydrolysis of the substrate, transfer products were identified. The transglycosylation reaction was found to be stereospecific but not regioselective. Three different transfer products were formed, two have been identified by comparison with chemically synthesised analogues, the main component being N-(p-nitrophenyl)-β-D-gentiobiosylamine (2.3 %), a β-(1-6) linked disaccharide and the N-(p-nitrophenyl)-β-D-cellobiosylamine (0.3 %), a β-(1-4) linked disaccharide (see Figure 45). The identity of the third compound (0.5 %) is still being investigated. By comparison of retention times, it is suggested that the unknown is also a disaccharide and that it also possesses a β-link as it is subsequently hydrolysed by cellulase.

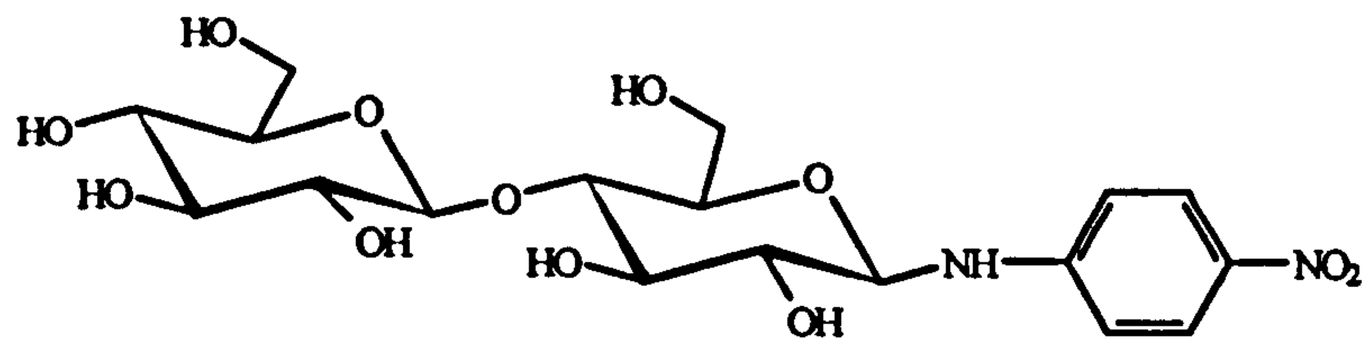


Figure 45 : Transglycosylation products

No trisaccharide transfer products were observed.

The observation of the transferase activity of the β-glucosidase suggests that, under the conditions of the reaction (using an acceptor concentration of 0.01 M), it is possible to use kinetic control to isolate glycoside products. As was mentioned in the introduction, kinetic control relies on the use of an activated substrate to quickly provide an enzyme bound intermediate. The latter is subsequently attacked by the acceptor nucleophile to provide disaccharide products which under the conditions of the reaction are only moderate substrates for the enzyme. Indeed, even with the large ratio of the two nucleophiles ([A] :

[H_2O], 1 : 5500) present in the system, it is possible to observe nucleophilic attack of the acceptor on the enzyme intermediate. The rate limiting step in the turnover of activated substrates is thought to be the attack of the nucleophile on the enzyme intermediate[94]. To observe transfer products, given the much greater concentration of water over that of the glycosyl nucleophile, the rate constant for transfer, $k_{transfer}$, must be much greater than the rate constant for hydrolysis (Figure 46).

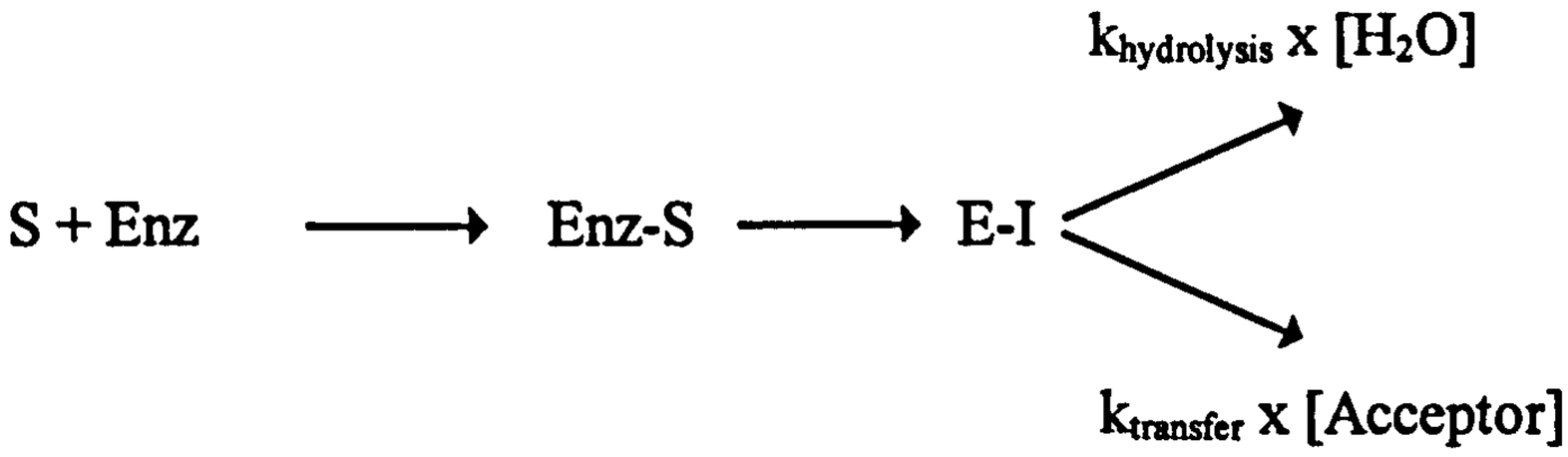


Figure 46 : Hydrolysis versus transglycosylation pathways

The transglycosylation yield will thus depend on the relative rates of transfer and hydrolysis. In order to maximise the amount of transfer, it is necessary to monitor the system throughout the reaction.

The low regioselectivity obtained during the transfer reaction suggests a loose attachment for the acceptor in the active site. This is possible if β-glucosidase is considered to be an aryl glucosidase and this would involve the hydrophobic interaction of the nitrophenyl group within a hydrophobic pocket present in the active site of the enzyme[95], rather than the binding of a glucose moiety. The orientation of the acceptor towards the glucosyl-enzyme intermediate will be controlled by the aglycon allowing a greater freedom of movement for the glucose moiety. The transglycosylation products can then be formed by the nucleophilic attack of the different hydroxyl groups onto the enzyme intermediate (Figure 47).

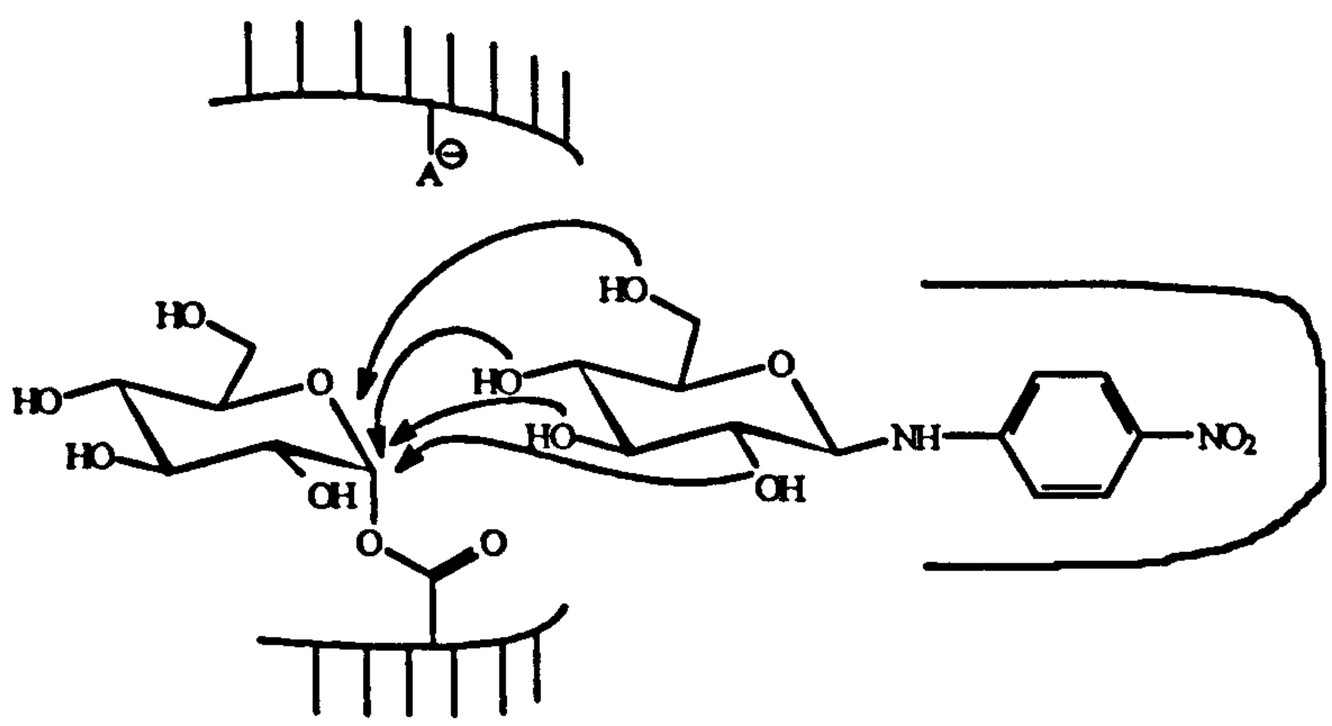


Figure 47: Formation of transglycosylation products

Taking the partition of the intermediate between the two nucleophiles, it is possible to estimate the relative ratio for the rate constants for hydrolysis and transfer:

$$\frac{k_{hydrolysis} \times [H_2O]}{k_{transfer} \times [Acceptor]} = \frac{4.39 \times 10^{-5}}{21.53 \times 10^{-7}}$$

$$\frac{k_{hydrolysis}}{k_{transfer}} = \frac{4.39 \times 10^{-5}}{21.53 \times 10^{-7}} \times \frac{0.01}{55.55}$$

$$\frac{k_{hydrolysis}}{k_{transfer}} = 3.67 \times 10^{-3}$$

$$\boxed{k_{transfer} = 272 \times k_{hydrolysis}}$$

The rate constant obtained for transfer is approximately two hundred and seventy times larger than that for hydrolysis, this value can then be compared to a similar value quoted in the literature for the transferase activity of β-glucosidase from *Aspergillus foetidus*[96]. Indeed, the transfer of both glucose and cellobiose to a glucose-enzyme bound intermediate was studied and the rate constant for the

transfer reaction was found to be equal to about 250 times that of the hydrolysis. The value obtained here for the glucosyl acceptor is similar to that reported in the literature[98]. The large ratio can be explained by the effective concentration of the hydroxyl groups of a glycoside inside the active site compared to that of water and to the binding of the acceptor in the enzyme active site leading to a higher local concentration of the acceptor compared to that of water[97] which favours transglycosylation.

The transfer products were produced at different rates: the β-(1-6) being formed the fastest (v_{ini} = 13.9 x 10^{-7} mM.s^{-1}), followed by the unknown (v_{ini} = 4.2 x 10^{-7} mM.s^{-1}) and the slowest being the β-(1-4) (v_{ini} = 3.5 x 10^{-7} mM.s^{-1}) ([Enz] = 0.01 mg.ml^{-1}). The amount of each product formed was very low, the maximum being that for the β-(1-6) (0.0087 mM, 2.3 %), followed by the unknown (0.0021 mM, 0.5 %) and finally the lowest concentration being that for the β-(1-4) (0.0012 mM, 0.3 %) giving rise to a total yield of only 3.1 %. The transfer products are themselves substrates for the enzyme and were slowly hydrolysed. This latter result is surprising considering the apparently similar structures of the disaccharide substrate, PNPC, and the transfer products, especially the β-(1-4) linked product. The result is also important as it explains why, in the current system, it is possible to observe transferase activity under the conditions of kinetic control whilst using a moderately activated substrate i.e. cleavage of the holosidic bond. The reduced rate of hydrolysis of the transfer products must be a consequence of a difference in the geometrical arrangements of the aromatic aglycon (see later discussion).

2.4. Crude cellulase from *Trichoderma reesei*

The transferase activity of cellulase has been studied by different research groups[98,99]. These earlier studies with the multi-enzyme complex cellulase have shown the capacity of the complex for producing higher oligosaccharides. Indeed, Kobayashi *et al.*[98,100,101] have used purified cellulase from *Trichoderma viride* to synthesise cellulose (DP ≥ 22) from the activated substrate β-D-cellobiosyl fluoride. The reaction was performed in an acetonitrile/acetate buffer mixture and gave rise to an overall yield of transfer products of 64 % (Figure 48).

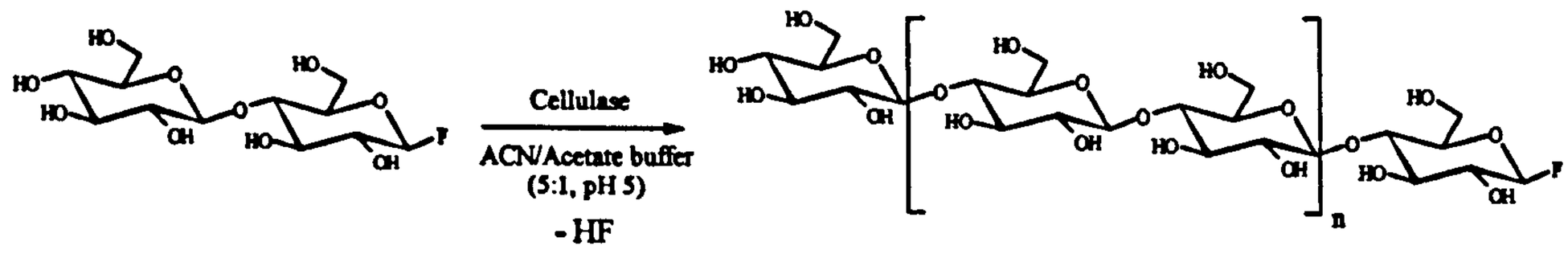


Figure 48 : Biosynthesis of cellulose

Oligosaccharides have also been synthesised by transfer reaction using cellulase as a biocatalyst. Karthaus *et al.*[102] have used different monosaccharide, disaccharide and trisaccharide glycosyl acceptors with β-D-lactosyl and β-D-cellobiosyl fluoride substrates. Examples are given in the following figure :

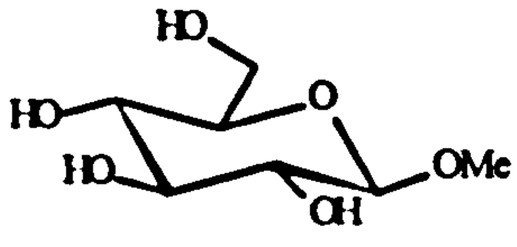
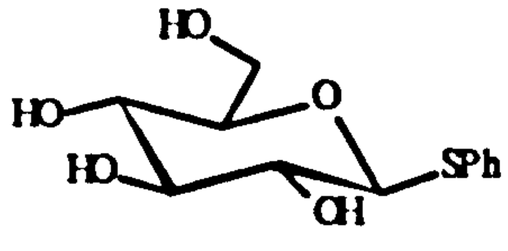
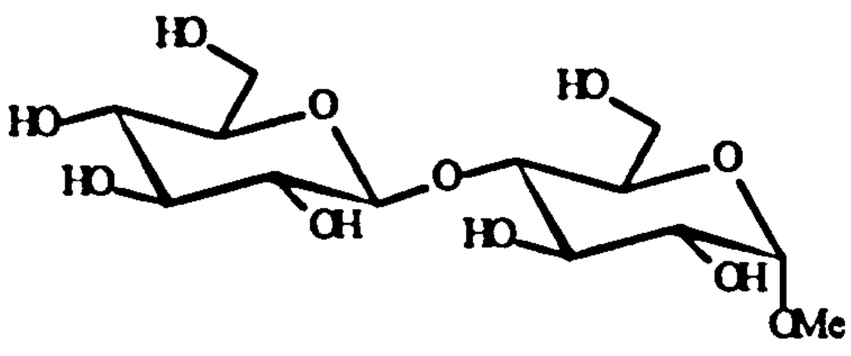
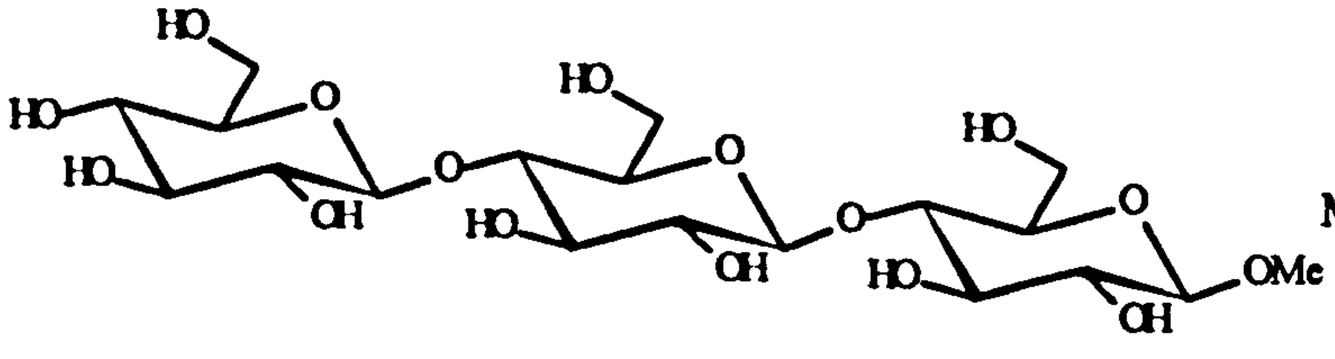
Acceptor	Conditions	Yield of transfer
	ACN / Acetate buffer (1:2)	51 %
	Acetate buffer	36 %
	Acetate buffer	60 %
	MeOH / Acetate buffer (4:1), 27 %	

Figure 49 : Examples of glycosyl Acceptors used with lactosyl fluoride and cellulase from *Trichoderma viride*

M. Vaheri *et al.*[103] have demonstrated the transferase activity of the crude cellulase from *Trichoderma reesei* during the hydrolysis of cellulose. The transglycosylation products formed were identified as sophorose (β-(1-2) linked disaccharide), laminaribiose (β-(1-3) linked disaccharide) and gentiobiose (β-(1-6) linked disaccharide) (Figure 50).

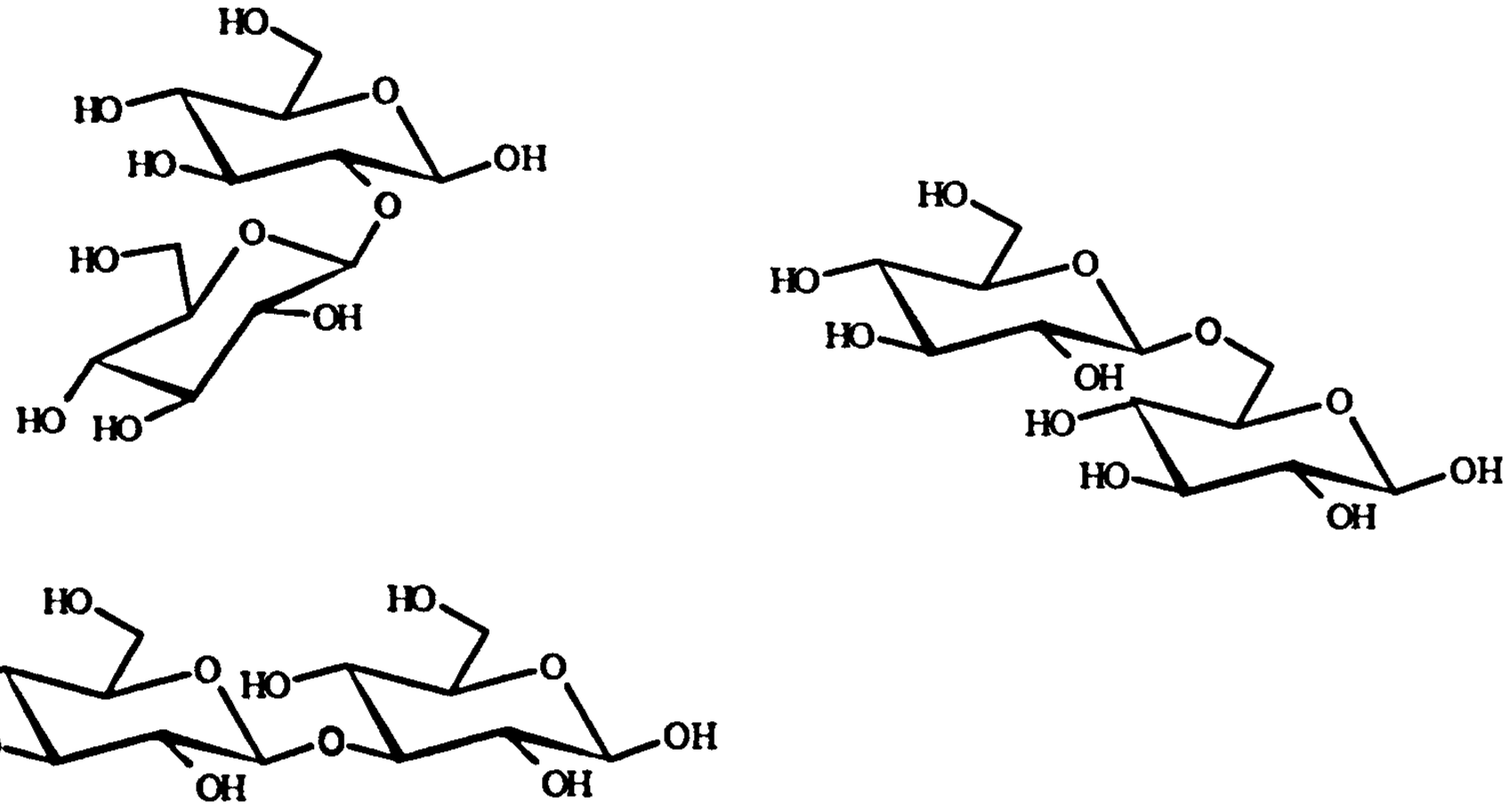


Figure 50 : Transglycosylation from cellulose hydrolysis with cellulase from *Trichoderma reesei*

In this previous study, the different transfer products were not quantified and no attempt to maximise the transferase activity was undertaken.

2.4.1. Study Of The Cellulase Catalysed Transfer

In the first experiments which have just been described, it was shown that the disaccharide was a substrate for the β-glucosidase and that β-glucosidase was able to catalyse transfer reactions using the disaccharide as a substrate. This initial result was disappointing as it was hoped, that with the use of a disaccharide substrate, it would have been possible to monitor the transferase activity of the other components of the cellulase complex. Fortunately, these early results do not necessarily preclude studies of the transfer activity of the intact complex. Specific inhibitors for the β-glucosidase activity of cellulase complexes are known, both 1,5-glucono-δ-lactone and 1-deoxynojirimycin are very specific inhibitors of β-glucosidases[104]. With this in mind, the cellulase catalysed hydrolysis and transfer reactions of the disaccharide substrate were investigated.

In order to study the transglycosylation reaction catalysed by the cellulase from *Trichoderma reesei*, it is important to understand the mechanism of the hydrolysis of the activated substrate donor. The hydrolysis of p-nitrophenyl β-D-cellobioside was investigated in an attempt to identify the mode of action of cellulase on this disaccharide and to determine the relative frequency at which the holosidic and heterosidic bonds were cleaved.

2.4.1.1. Hydrolysis of p-nitrophenyl β-D-cellobioside

p-Nitrophenyl β-D-cellobioside was found to be hydrolysed very efficiently by the cellulase from *Trichoderma reesei* (rate k_{obs} = 4.56 x 10^{-3} s^{-1} /mg of enzyme). Again, both the holosidic and heterosidic bonds are cleaved and a similar scheme for the hydrolysis pathway, to that presented for the reaction catalysed by β-glucosidase from almond emulsin, can be drawn (Figure 51).

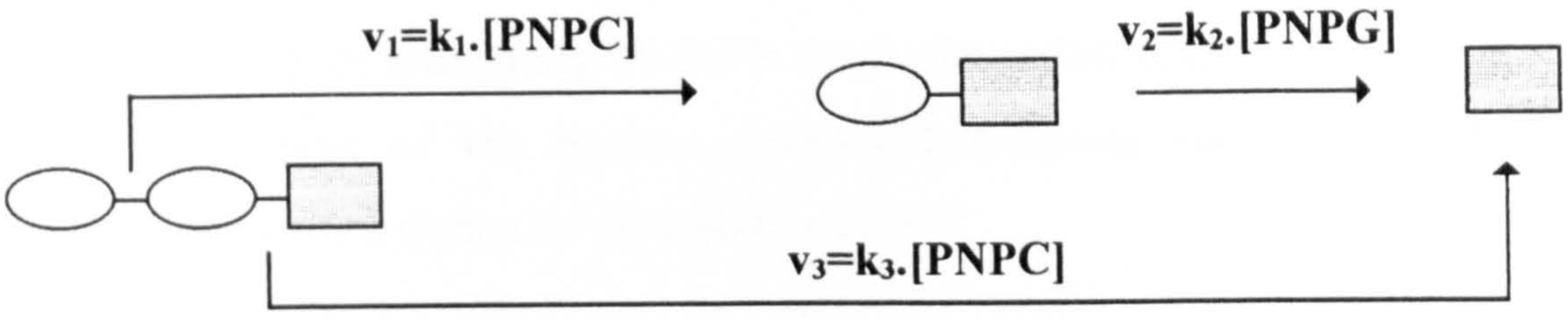


Figure 51 : Hydrolysis of PNPC by cellulase from *Trichoderma reesei*

Some important differences were identified. With the cellulase complex, the amount of PNPG released reaches nearly 50 % of the total substrate concentration ([PNPG] = 0.19 mM, t = 29 min) and the initial rate of production was found to be $v_1 = 7.26 \times 10^{-4}$ mM.s^{-1} /mg of enzyme. p-Nitrophenol is released slowly at first ($v_3 = 3.49 \times 10^{-4}$ mM.s^{-1} /mg of enzyme), but as the reaction proceeds the rate of production increases, this results in the observation of a lag phase in the rate of production of p-nitrophenol (Figure 52).

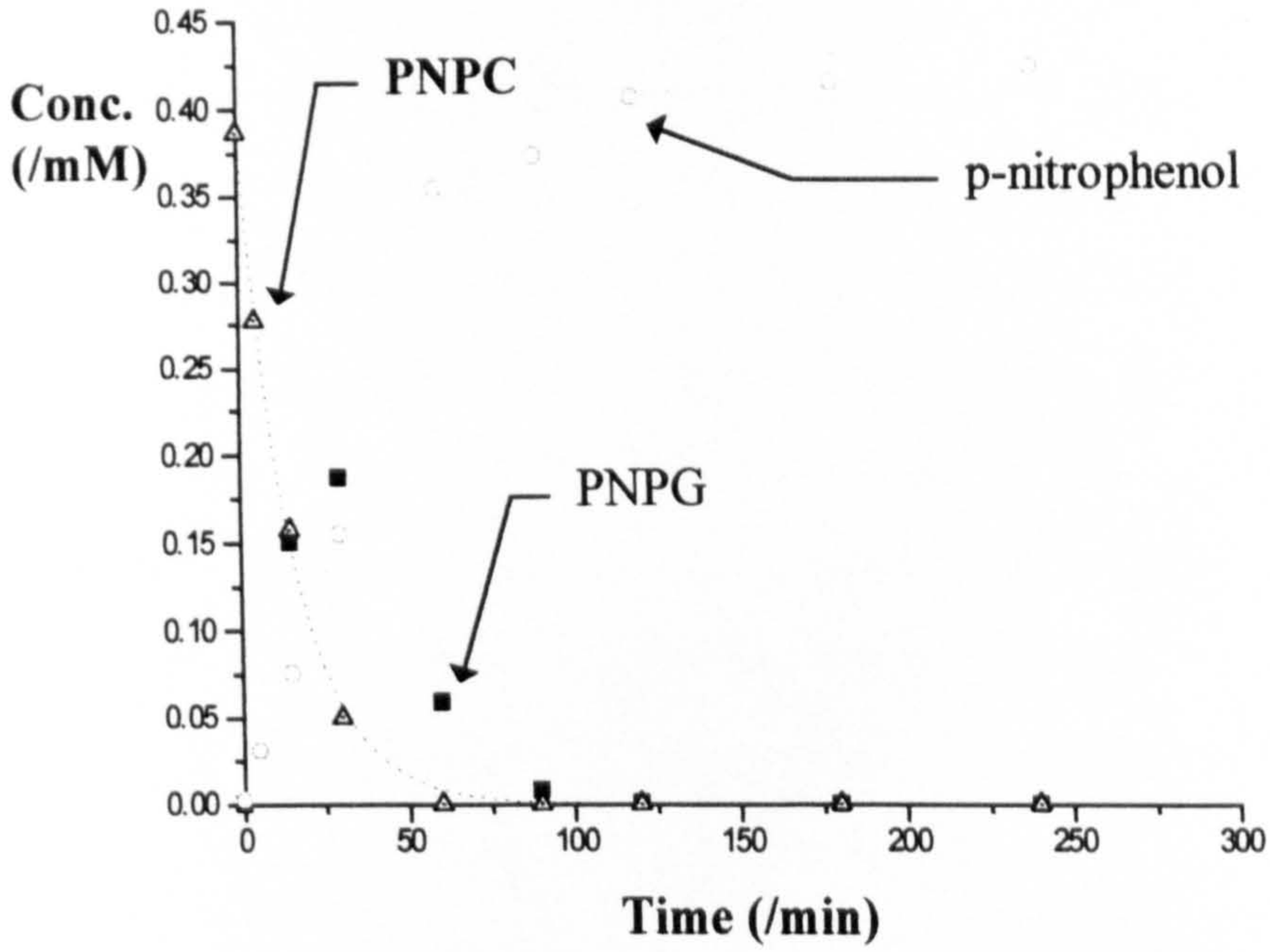
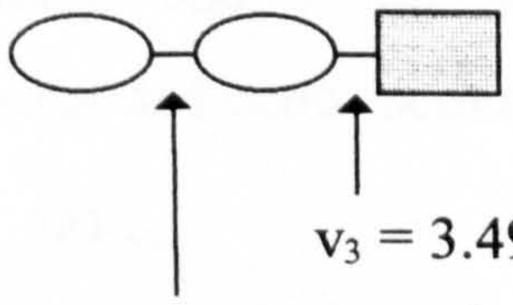


Figure 52 : PNPC hydrolysis by cellulase from *Trichoderma reesei*

The lag phase results from the preferential hydrolysis of PNPC at the holosidic bond at first releasing another substrate for the complex, PNPG. The rate of p-nitrophenol production then increases due to the introduction of a second process: the hydrolysis of PNPC at the heterosidic bond ($v_3 = 3.49 \times 10^{-4}$ mM.s^{-1} /mg of

enzyme) plus the subsequent hydrolysis of PNPG. The only component of the complex capable of hydrolysing PNPG is the β-glucosidase and thus appears as an active component of the enzyme complex hydrolysing the monosaccharide intermediate formed during the hydrolysis of PNPC.



$v_3 = 3.49 \times 10^{-4}$ mM.s^{-1}

$v_1 = 7.26 \times 10^{-4}$ mM.s^{-1}

It is also important to notice that the sum of the product concentrations matches the amount of substrate lost indicating that there are no self-transfer products formed during the hydrolysis reaction.

2.4.1.2. The Trichoderma reesei catalysed hydrolysis in the presence of β-glucosidase inhibitor : 1,5-glucono-δ-lactone

As was suggested earlier, it has been reported that 1,5-glucono-δ-lactone is a specific inhibitor of β-glucosidase and at the same time does not have a measurable effect on either the exo- and endo- activity of cellulase[105].

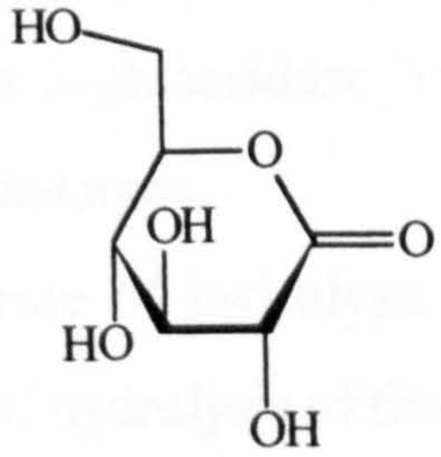


Figure 53 : 1,5-glucono-δ-lactone, an inhibitor of β-glucosidase

The addition of 1,5-glucono-δ-lactone to the reaction system suppresses the β-glucosidase activity and allows the study of the other enzymes present in the cellulase complex. It has been shown that PNPG is a specific substrate for β-glucosidases[105], so that when the β-glucosidase is inhibited PNPG hydrolysis is stopped. The study of PNPC catalysed hydrolysis with cellulase from *Trichoderma reesei* in the presence of 1,5-glucono-δ-lactone shows the production of PNPG which reaches a maximum concentration ([PNPG] = 0.075 mM, 19 %) and then remains at the maximum throughout the study. The addition of 1,5-glucono-δ-lactone removes v_2 (see scheme in Figure 51) and thus provides a new pathway (See Figure 54) for the substrate hydrolysis.

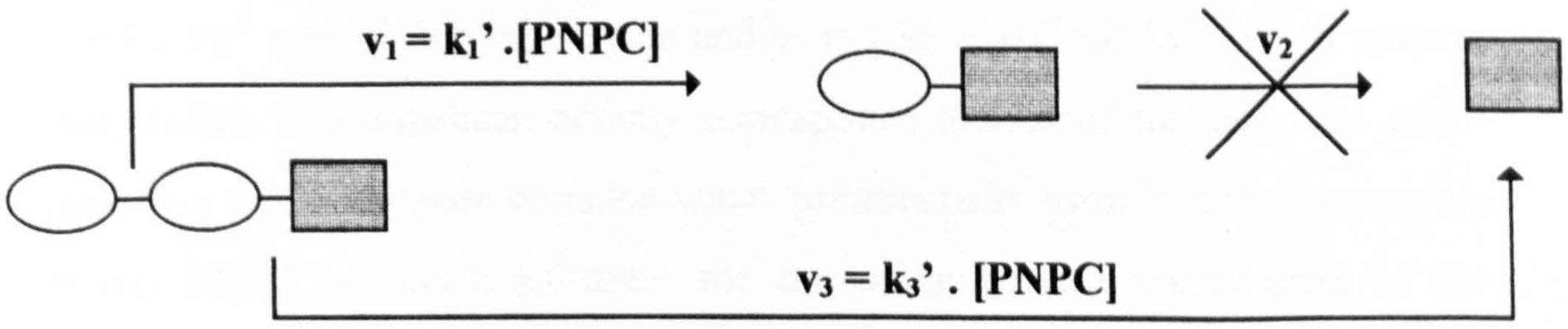


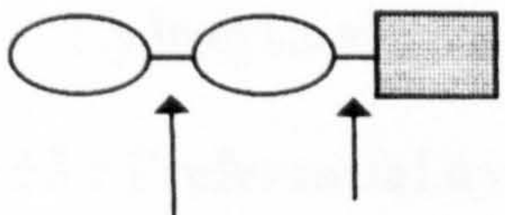
Figure 54 : Inhibition of β-glucosidase

A decrease in the rate of PNPC hydrolysis corresponding to a loss of the β-glucosidase activity (k_{obs} = 1.09 x 10^{-3} s^{-1} /mg of enzyme) was observed. This corresponded to a four fold decrease in activity.

These results when considered alongside with the measured rates for hydrolysis of PNPC in the presence of β-glucosidase (k_{obs} = 4.56 x 10^{-3} s^{-1} /mg of enzyme) suggest that PNPC is also a very good substrate for the β-glucosidase in the cellulase of *Trichoderma reesei* ($k_{estimate} = k_{\text{no inhibitor}} - k_{\text{with inhibitor}}$ = 3.47 x 10^{-3} s^{-1} / mg of enzyme complex) and that β-glucosidase is capable of hydrolysing PNPC at both, holosidic and heterosidic, linkages.

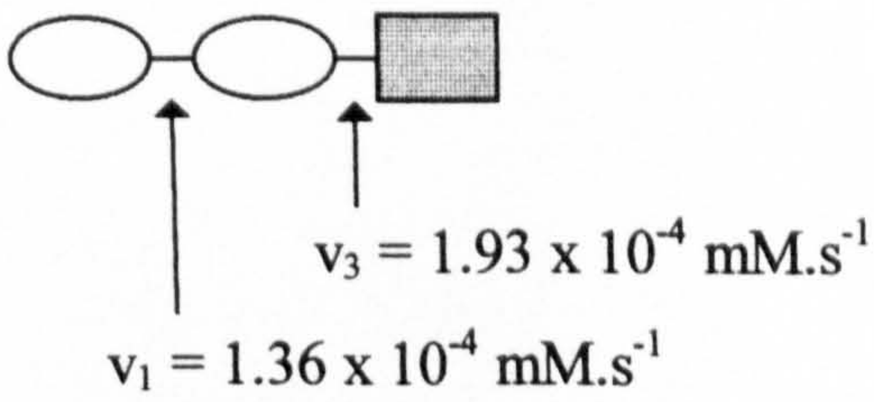
The observed decrease in the rate of hydrolysis of PNPC was reflected, in the main, in a reduction in the rate of hydrolysis of the holosidic bond.

No inhibitor :



Ratio : ~2:1

With inhibitor :



Ratio : ~0.6:1

The initial rate of production of p-nitrophenol is now larger than that of PNPG (v_3 = 1.93 x 10^{-4} mM.s^{-1} /mg of enzyme and v_1 = 1.36 x 10^{-4} mM.s^{-1} /mg of enzyme respectively). The remaining activity corresponds to that of the exo- and endo-glucanases of the cellulase complex which preferentially hydrolyse the heterosidic linkage. PNPC is thus a substrate for the other enzyme components of the cellulase complex and is hydrolysed at both the holosidic and heterosidic bonds.

The role of the exo- and endo- acting enzymes in the transfer reaction can thus be studied by removing the action of β-glucosidase with 1,5-glucono-δ-lactone. The large decrease observed in the rate of hydrolysis of the holosidic bond reflects the preferential activity of the β-glucosidase component at this position.

This last result provide information of the substrate positioning inside the β-glucosidase active site and could thus be represented as follows:

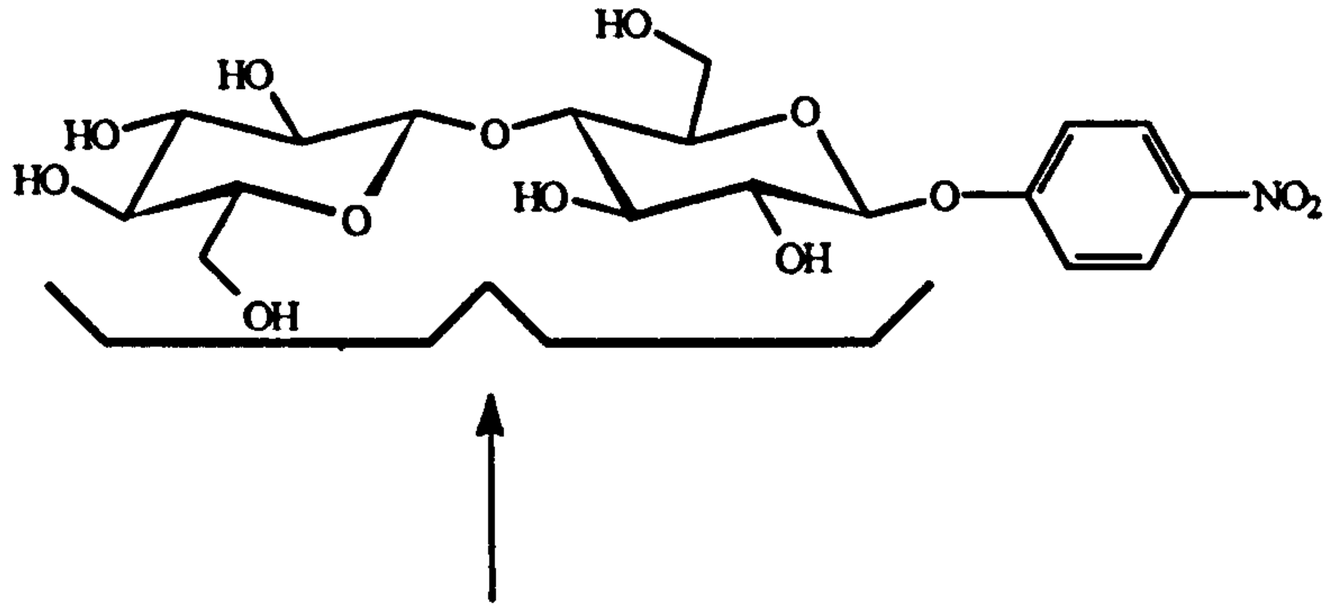


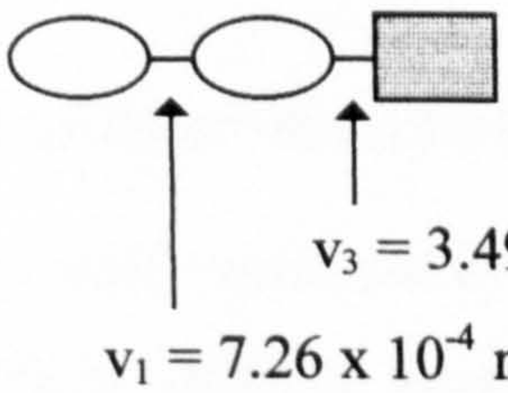
Figure 55 : Preferential hydrolysis site inside β-glucosidase active site

2.4.1.3. Transglycosylation studies

The addition of N-(p-nitrophenyl)-β-D-glucopyranosylamine (10.0 mM) as a glycoside acceptor led to a reduction in the initial rate of PNPC hydrolysis (k_{obs} = 1.79 x 10^{-3} s^{-1} compared to k_{obs} = 4.56 x 10^{-3} s^{-1} without acceptor). The rate of hydrolysis was reduced by more than 50 % and the acceptor was considered to be acting as an inhibitor.

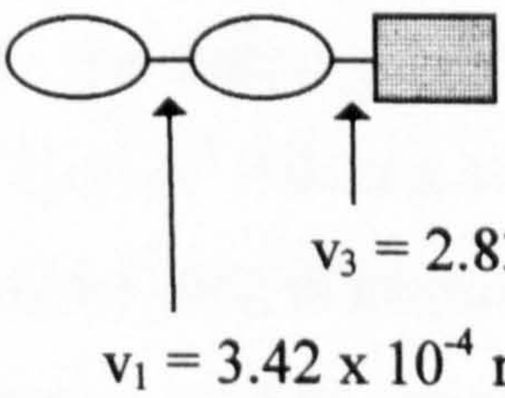
At the acceptor concentration used, both glycosidic bonds are hydrolysed at a slower rate, no real preference for the holosidic or the heterosidic linkages hydrolysis was observed (v_1 = 3.42 x 10^{-4} mM.s^{-1} and v_3 = 2.83 x 10^{-4} mM.s^{-1} /mg of enzyme respectively) whereas a preferential hydrolysis occurred at the holosidic bond in the absence of the acceptor. As was the case when 1,5-glucono-δ-lactone was added the initial rate of hydrolysis of the holosidic bond is affected to a greater extent than that of the heterosidic bond.

Without acceptor



Ratio : ~2:1

With the acceptor, 10.0 mM



Ratio : ~1:1

It is tentatively suggested that the acceptor has an affinity for the substrate site of the enzymes but as it affects preferentially the hydrolysis of the holosidic bond, the binding of the acceptors could influence β-glucosidase more than the other components of the system.

Again, three different transglycosylation products were formed and two of them have been identified as the β-(1-4) cellobiosylamine and the β-(1-6) gentiobiosylamine by comparison with chemically synthesised analogues. The third compound has the same retention time as that of the unknown obtained with β-glucosidase from almond emulsin.

The formation of disaccharide transfer products suggests that a glucosyl-enzyme intermediate is formed and that the intermediate is more rapidly trapped by the acceptor (0.01 M) rather than a molecule of water (~ 55.55 M). The disaccharide synthesis is again operating under the conditions of kinetic control with $k_{transfer} > k_{hydrolysis}$:

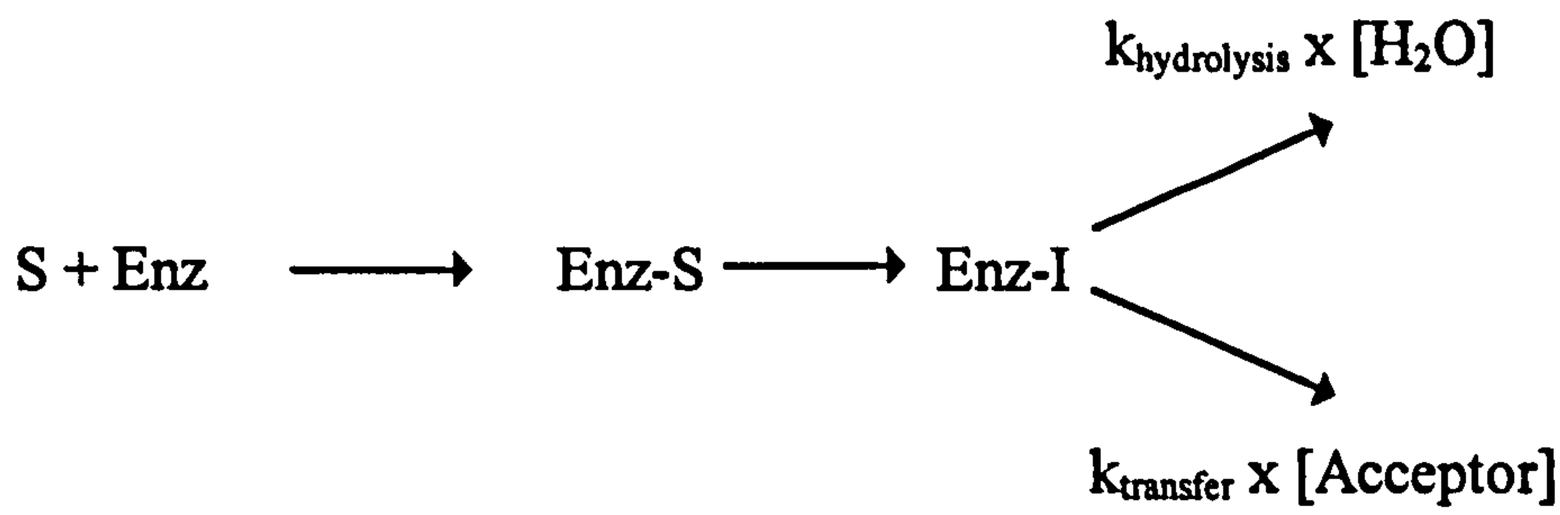


Figure 56 : Hydrolysis versus transglycosylation pathways

By comparison with the reaction catalysed by the β-glucosidase from almond, the same transfer products were obtained. However, they did not follow the same concentration versus time profiles. Indeed, whereas the β-(1-6) was produced with the largest initial rate in the almond system, in the present system the β-(1-4) disaccharide is produced at the fastest rate ($v^4 = 0.48 \times 10^{-4}$ mM.s^{-1} /mg of enzyme) followed by the β-(1-6) ($v^6 = 0.35 \times 10^{-4}$ mM.s^{-1} /mg of enzyme) and the unknown ($v^u = 0.09 \times 10^{-4}$ mM.s^{-1} /mg of enzyme).

The β-(1-4) linked disaccharide product resembles the natural substrate of the enzyme and was hydrolysed at the fastest rate. Indeed, *in vivo*, cellulase hydrolyses the β-(1-4) linked polysaccharide cellulose.

The β-(1-6) remains the major compound (0.037 mM, 9 % yield), followed by the β-(1-4) (0.025 mM, 6 % yield) and the unknown (0.009 mM, 2 % yield) corresponding to a total transglycosylation yield of 17 %. The β-(1-6) linked

disaccharide was the only product still being produced after complete PNPC hydrolysis. In fact, the intermediate hydrolysis compound, PNPG, was still present in the solution, it was suggested that PNPG was the most likely source of the additional β-(1-6) product. Under the conditions of the reaction, no trisaccharide products were observed. As the β-glucosidase is the only enzyme capable of hydrolysing PNPG, it was proposed that β-glucosidase was involved in this remaining transglycosylation activity.

Measuring the partition of the intermediate between the two nucleophiles, it is again possible to estimate the ratio of the rate constants for hydrolysis and transfer:

$$\frac{k_{hydrolysis} \times [H_2O]}{k_{transfer} \times [Acceptor]} = \frac{1.57 \times 10^{-4}}{2.84 \times 10^{-5}}$$

$$\frac{k_{hydrolysis}}{k_{transfer}} = \frac{1.57 \times 10^{-4}}{2.84 \times 10^{-5}} \times \frac{0.01}{55.55}$$

$$\frac{k_{hydrolysis}}{k_{transfer}} = 9.95 \times 10^{-4}$$

$$\boxed{k_{transfer} = 1005 \times k_{hydrolysis}}$$

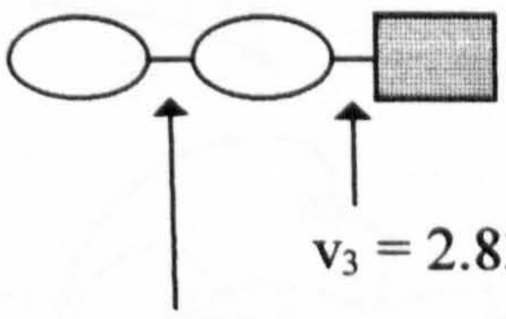
It was thus estimated that the rate constant obtained for transfer is more than 1000 times larger than that for hydrolysis. This represents a significant increase compared to the ratio obtained with β-glucosidase from almond and that obtained in the literature for β-glucosidase from *Aspergillus foetidus*[96].

This large difference could be explained by a difference in the acceptor binding in the active site of the endo- and exo- enzymes catalysing transfer reactions.

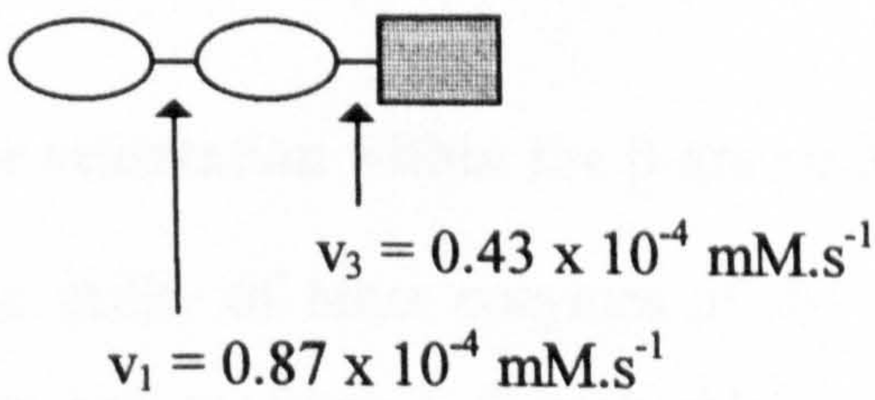
2.4.1.4. Transglycosylation in the presence of the β-glucosidase inhibitor: 1,5-glucono-δ-lactone

The addition of 1,5-glucono-δ-lactone to the transglycosylation reaction system resulted in the inhibition of the β-glucosidase. Indeed, PNPG, a specific substrate for β-glucosidase, was formed but not hydrolysed, reaching a maximum concentration of 0.06 mM. Again, the rate of PNPC hydrolysis decreased. The initial rates of production for p-nitrophenol (v_3 = 0.43 x 10^{-4} mM.s^{-1} /mg of enzyme) and PNPG (v_1 = 0.87 x 10^{-4} mM.s^{-1} /mg of enzyme) gave a total rate of 1.30 x 10^{-4} mM.s^{-1}/mg of enzyme. The initial rate of hydrolysis of PNPC (v_{hyd} = 1.74 x 10^{-4} mM.s^{-1} /mg of enzyme) suggests that a fraction of the disaccharide is converted to transfer products. At the end of the reaction, the combined concentrations of the products, p-nitrophenol and PNPG, matched the amount of substrate hydrolysed.

With acceptor (10.0 mM), no inhibitor :



With acceptor (10.0 mM) and inhibitor (20.8 mM) :



The addition of 1,5-glucono-δ-lactone resulted in a decrease in the number of transglycosylation products obtained from the reaction system. In the presence of 1,5-glucono-δ-lactone, the β-(1-4) linked disaccharide was the only product obtained in any significant concentration ([β-(1-4)] = 0.022 mM, 5 %). Trace

amounts of the unknown could be observed but no β-(1-6) linked disaccharide was obtained.

The β-(1-4) transfer product was hydrolysed very slowly which suggested that the product is not a very good substrate for the endo- or exo-acting enzymes present in the system. Again, this is rather an unexpected observation as it was anticipated that the hydrolysis of the holosidic linkage of the product would resemble that of the substrate, a molecule having a very similar structure to the product.

The removal of β-glucosidase activity provides an opportunity to view the transferase activity of the other enzyme components of the cellulase from *Trichoderma reesei* as well as that of β-glucosidase. Indeed, the absence of β-(1-6) disaccharide product reveals that β-glucosidase is the only enzyme capable of producing such a linkage, revealing a lesser degree of orientation of the acceptor within its active site compared to that in the other enzyme components of the multi-enzyme complex (Figure 57).

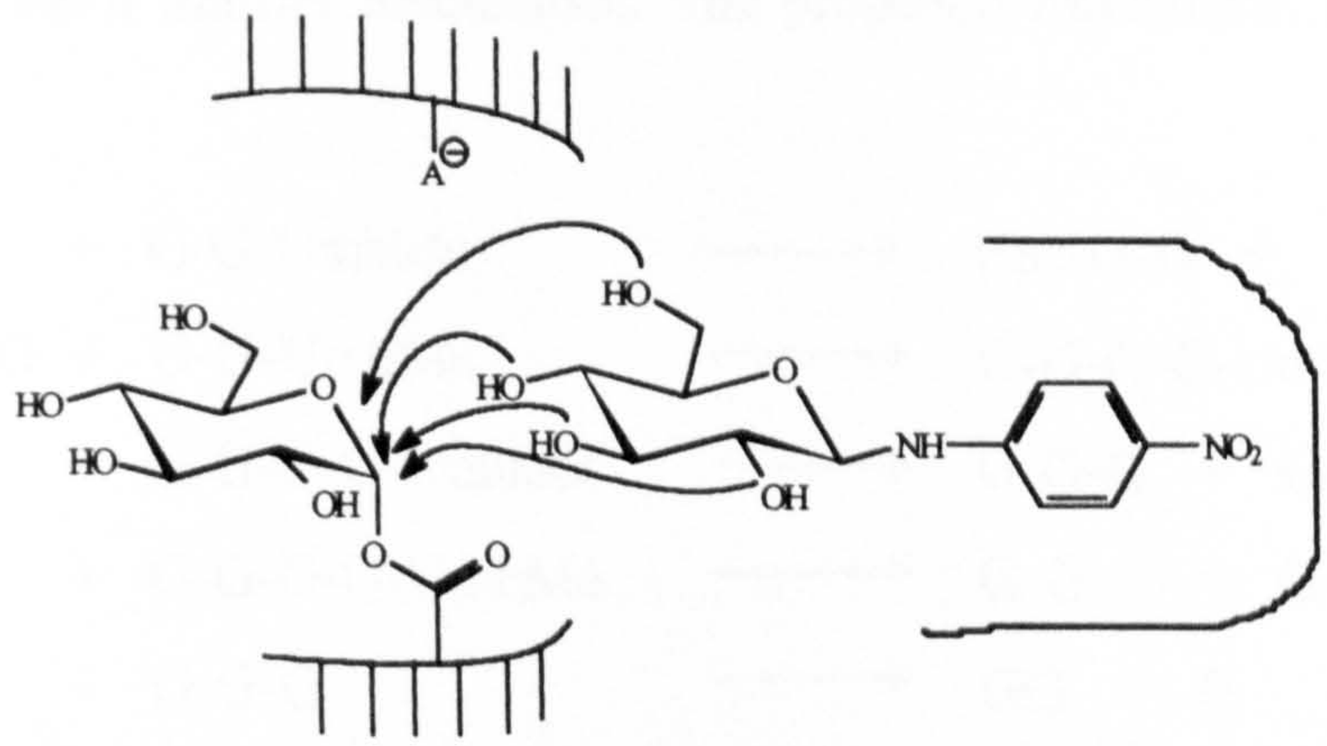


Figure 57 : Acceptor orientation within the β-glucosidase active site

The results also show the ability of other enzymes of the complex to catalyse glycosyl transfer reactions and to form a disaccharide product. No traces of trisaccharide derivatives were observed during the experiment. The latter result suggests that the β-(1-4) linked disaccharide product is formed from a monosaccharide-enzyme bound intermediate. CBH I and EG I have been shown to transfer glycosyl groups to acceptors[106]. However, the transglycosylation mechanism observed for EG I has been shown to occur via multiple reaction steps

requiring the intermediate synthesis of higher oligosaccharides. Claeyssens *et al.*[107] have shown that EG I is unable to catalyse the hydrolysis of the holosidic bond in a disaccharide derivative such as 2',4'-dinitrophenyl 1-thio-β-D-cellobioside when present on its own (Figure 58).

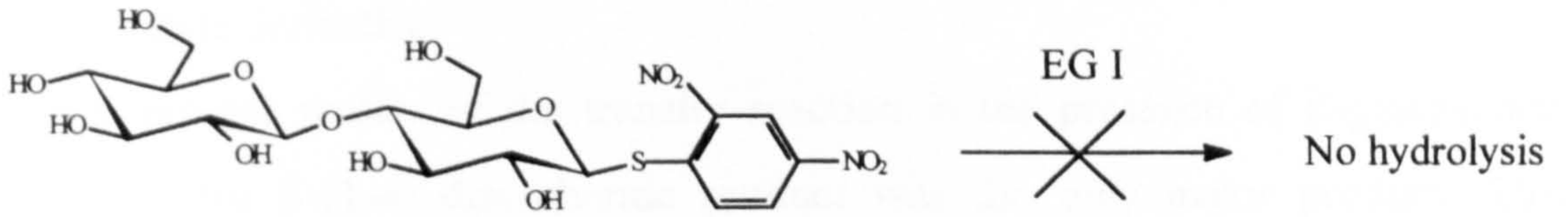


Figure 58 : Absence of hydrolysis of a disaccharide derivative by EG I

The addition of cellotriose to the reaction system led to a rapid formation of the corresponding 1-thio-β-D-glucopyranoside, a mechanism involving self transfer was then suggested. In the EG I catalysed hydrolysis of 4-methylumbelliferyl β-D-cellobioside, higher oligosaccharides were produced, again providing support for the hydrolysis via a transfer mechanism. The proposed mechanism is outlined in Figure 59.

Enz	+	G-G-UmbMe	⟶	Enz-G-G	+	UmbMe
Enz-GG	+	G-G-UmbMe	⟶	G-G-G-G-UmbMe	+	Enz
Enz	+	G-G-G-G-UmbMe	⟶	G-G-G	+	G-UmbMe
Enz	+	G-G-G-G-UmbMe	⟶	G-G	+	G-G-UmbMe
Enz	+	G-G-G	⟶	GG	+	G

Figure 59 : Self transfer mechanism of EG I

According to this mechanism, EG I would not be capable of transferring the glucopyranosylamine acceptor to a glucosyl intermediate to form a disaccharide transfer product.

CBH I has also shown transferase activity and remains the best candidate for the synthesis of transfer products. Previous work has illustrated that other components are not capable of catalysing transfer reactions: CBH II hydrolyses its substrate with inversion of configuration and thus is not capable of transfer

reactions; EG II acts on longer oligosaccharide substrates (at least 3 contiguous glucosyl moieties).

CBH I hydrolyses its substrate from the reducing end and in the hydrolysis of PNPC is capable of producing a monosaccharide-enzyme bound intermediate which could subsequently be attacked by the glycoside acceptor forming a disaccharide derivative.

In the present studies of the transfer reaction in the presence of β-glucosidase inhibitor, the β-(1-4) disaccharide product was the only major product. This suggests a greater degree of orientation of the acceptor within the enzyme catalysing the transfer compared to that of β-glucosidase catalysed reaction. The glucose moiety of the glucoside acceptor could bind into one of the glucosyl binding site available in the active site of the second enzyme, this would restrain its freedom inside the active site and favour the nucleophilic attack of the 4-hydroxyl group on the acceptor (Figure 60). This is similar to Kobayashis model described earlier.

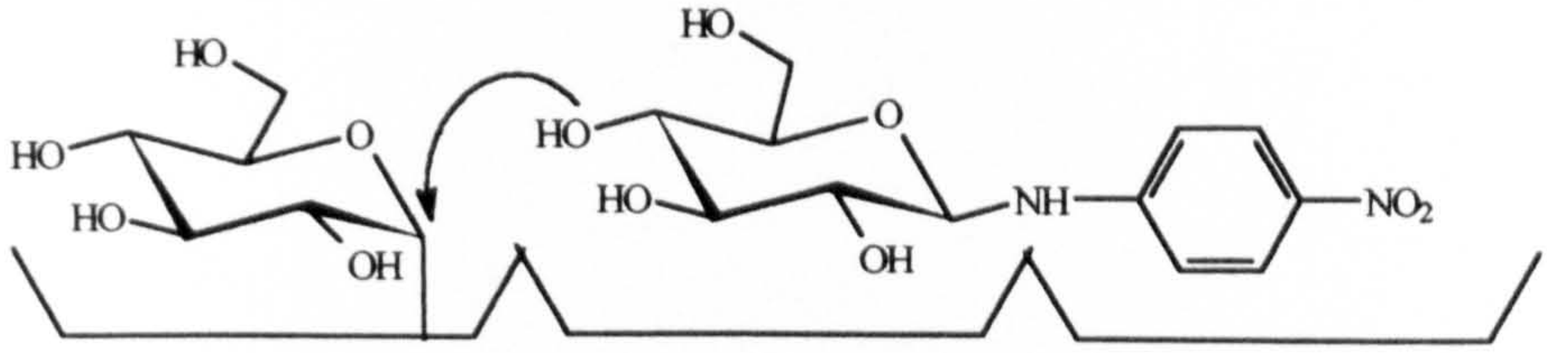


Figure 60 : Orientated acceptor in the enzyme active site

Again, by determining the partition of the intermediate between the two nucleophiles, it is possible to estimate the ratio of the rate constants for hydrolysis and transfer:

$$\frac{k_{hydrolysis} \times [H_2O]}{k_{transfer} \times [Acceptor]} = \frac{2.17 \times 10^{-4}}{8.33 \times 10^{-6}}$$

$$\frac{k_{hydrolysis}}{k_{transfer}} = \frac{2.17 \times 10^{-4}}{8.33 \times 10^{-6}} \times \frac{0.01}{55.55}$$

$$\frac{k_{hydrolysis}}{k_{transfer}} = 4.69 \times 10^{-3}$$

$$\mathbf{k_{transfer} = 213 \times k_{hydrolysis}}$$

It was thus estimated that the rate constant obtained for transfer is now, in the presence of the inhibitor, approximately two hundred times larger than that for hydrolysis. This number is then comparable to that obtained in the literature (obtained for the transfer of glucose and cellobiose) but is much lower than that obtained in the presence of β-glucosidase ($k_{transfer} = 1005 \times k_{hydrolysis}$).

2.4.2. Determination of the kinetic parameters

A detailed kinetic scheme for the hydrolysis and transfer reactions would be complex. By introducing several simplifications to the overall scheme, it is possible to determine some apparent kinetic constants relating to the relative binding of the substrates (monosaccharide, disaccharide) and the acceptors. The transferase activity of glycosyl hydrolase is an example of a substituted enzyme mechanism. Initially, the free enzyme E_β binds the substrate (S) to form a complex $E_\beta S$ which then transfers glucose to the active site aspartate group with the loss of the activated leaving group. This results in a substituted enzyme E_β-G. In a true substituted enzyme mechanism, it is necessary then to consider binding of the 'acceptor', which in the case of the present studies is either water or a p-nitrophenyl β-D-glucoside to form a new enzyme complex, E-GA. The latter reacts to provide the reaction product and to liberate the free enzyme:

$$E\beta + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\beta \cdot S \xrightarrow[k_2]{} E\beta\text{-}G \overset{[A]}{\rightleftharpoons} E\beta\text{-}G \cdot A \longrightarrow$$

The rate of the reaction, assuming steady state approximation, follows the equation:

$$v = \frac{V \,.\, [S] \,.\, [A]}{K_{mS} \,.\, [A] + K_{mA} \,.\, [S] + [A] \,.\, [S]}$$

In most reactions involving transfer, typically hydrolysis, the concentration of the acceptor is kept constant and the kinetics simplify to standard Michaelis-Menten kinetics. In all the comparisons made in the discussion, either the acceptor concentration or the substrate concentration remains constant, in which case single substrate kinetics should be observed. Rearrangement of the rate expression for a substituted enzyme mechanism (by dividing by (K_m + A)) illustrates why this should be so :

$$v = \frac{V \cdot [S] \cdot [A]}{K_{mS} \cdot [A] + \{K_{mA} + [A]\} \cdot [S]}$$

$$v = \frac{\frac{V}{K_{mA} + [A]} \cdot [S] \cdot [A]}{\frac{K_{mS} \cdot [A]}{K_{mA} + [A]} + [S]}$$

If a constant value is taken for the acceptor concentration [A] and the substrate concentration varied then the apparent kinetic parameters are :

$$(K_m)_{app} = \frac{K_{mS} \cdot [A]}{K_{mA} + [A]} \qquad (V_{max})_{app} = \frac{V}{K_{mA} + [A]}$$

At high acceptor concentration i.e. $[A] >> K_{mA}$, the apparent parameters reduce to the standard Michaelis Menten constants, K_{mS} and V_{max}. Similarly, if a constant value for the substrate concentration is chosen and the acceptor concentration is varied then $(K_m)_{app}$ is equivalent to the binding constant for the acceptor.

2.4.2.1. K_m determination of p-nitrophenyl β-D-cellobioside

In an attempt to determine which of the cellulase components were catalysing the hydrolysis of the substrate, a detailed study of the kinetics of the substrate hydrolysis was investigated. As the concentration of substrate increases, the successive saturation of the different components of the complex can be observed; in doing so the fraction of the substrate being hydrolysed by the different components of the complex is altered.

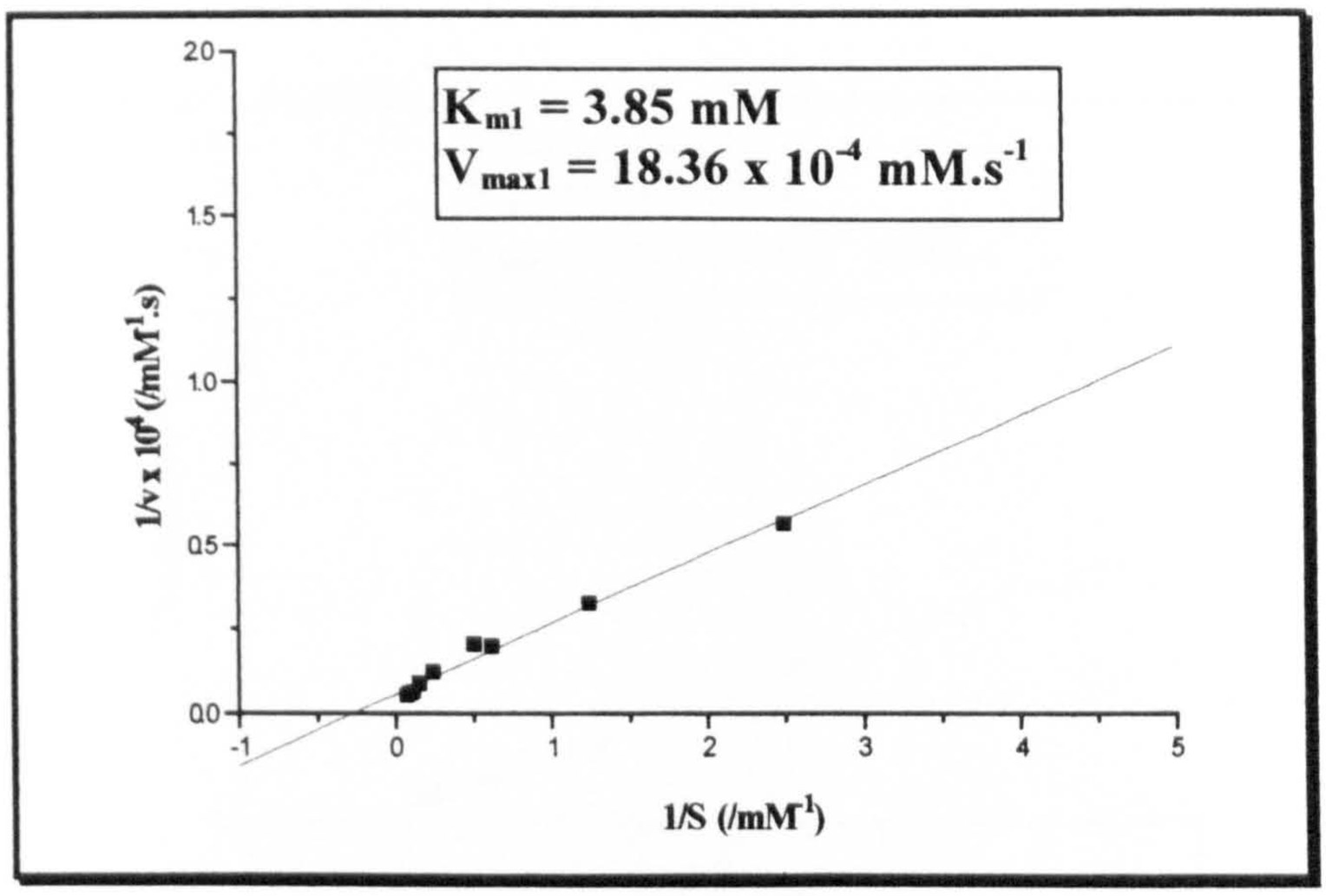
In reality, the system under study is too complex for accurate determination of kinetic parameters; the system contains a minimum of five different enzymes and an additional complexity arises from the number of different possible substrate binding modes for each enzyme. This complicates the situation further; as the

substrate concentration increases, there will be an interplay between the different possible binding modes. This is reflected in the pattern of changing frequencies of bond cleavage catalysed by a pure enzyme upon changing the concentration of the substrate.

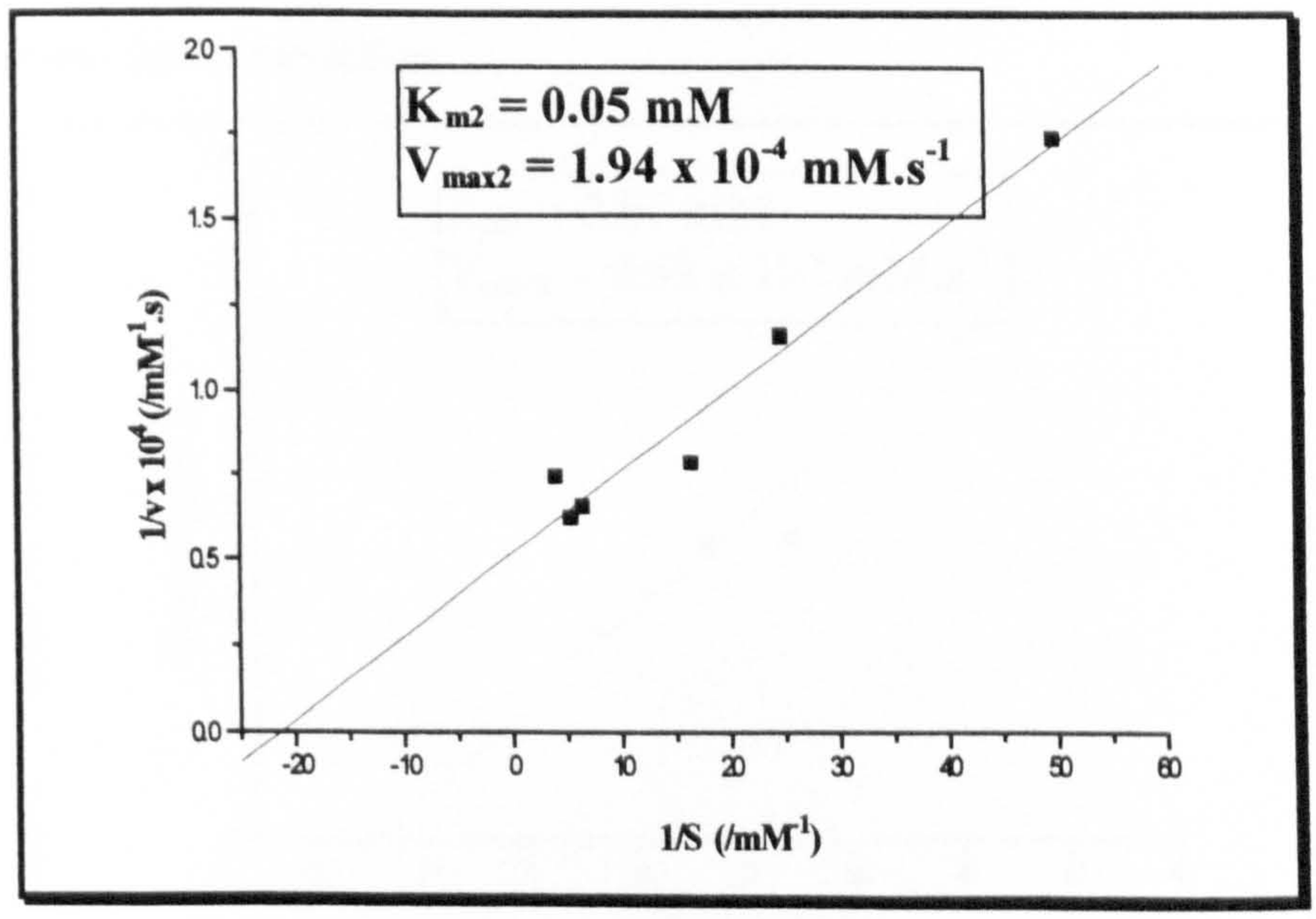
If a simple model is considered, i.e. the presence of multiple enzymes having a single mode of binding leading to the release of p-nitrophenol and p-nitrophenyl β-D-glucopyranoside, then it is possible to obtain some indication of the number of enzymes acting on the substrate.

The determination of the kinetic parameters was achieved using the initial rate of formation of p-nitrophenol and p-nitrophenyl β-D-glucopyranoside (PNPG). As expected, the Lineweaver-Burk plots obtained from the rate of formation of p-nitrophenol and PNPG as a function of the initial p-nitrophenyl β-D-cellobioside concentration gave rise to a curved plot which can be considered to be composed of at least two different linear regions suggesting that at least two enzymes are able to hydrolyse the substrate. The apparent values measured for K_m from the two linear regions obtained from the Lineweaver-Burk plot for PNPG formation, plot A, were $K_{m1} = 3.85$ mM and $K_{m2} = 0.05$ mM (Figure 61). The apparent values measured for K_m from the two linear regions obtained from the Lineweaver-Burk plot for the formation of p-nitrophenol, plot B, were $K_{m1}' = 4.02$ mM and $K_{m2}' = 0.07$ mM (Figure 62). The two sets of results show a reasonable level of agreement.

High substrate concentrations



Low substrate concentrations



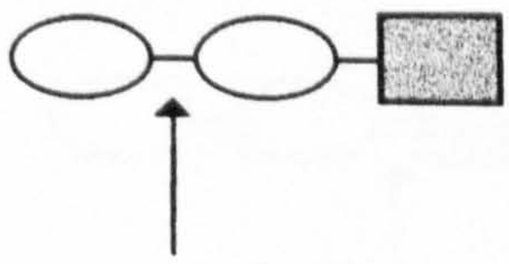
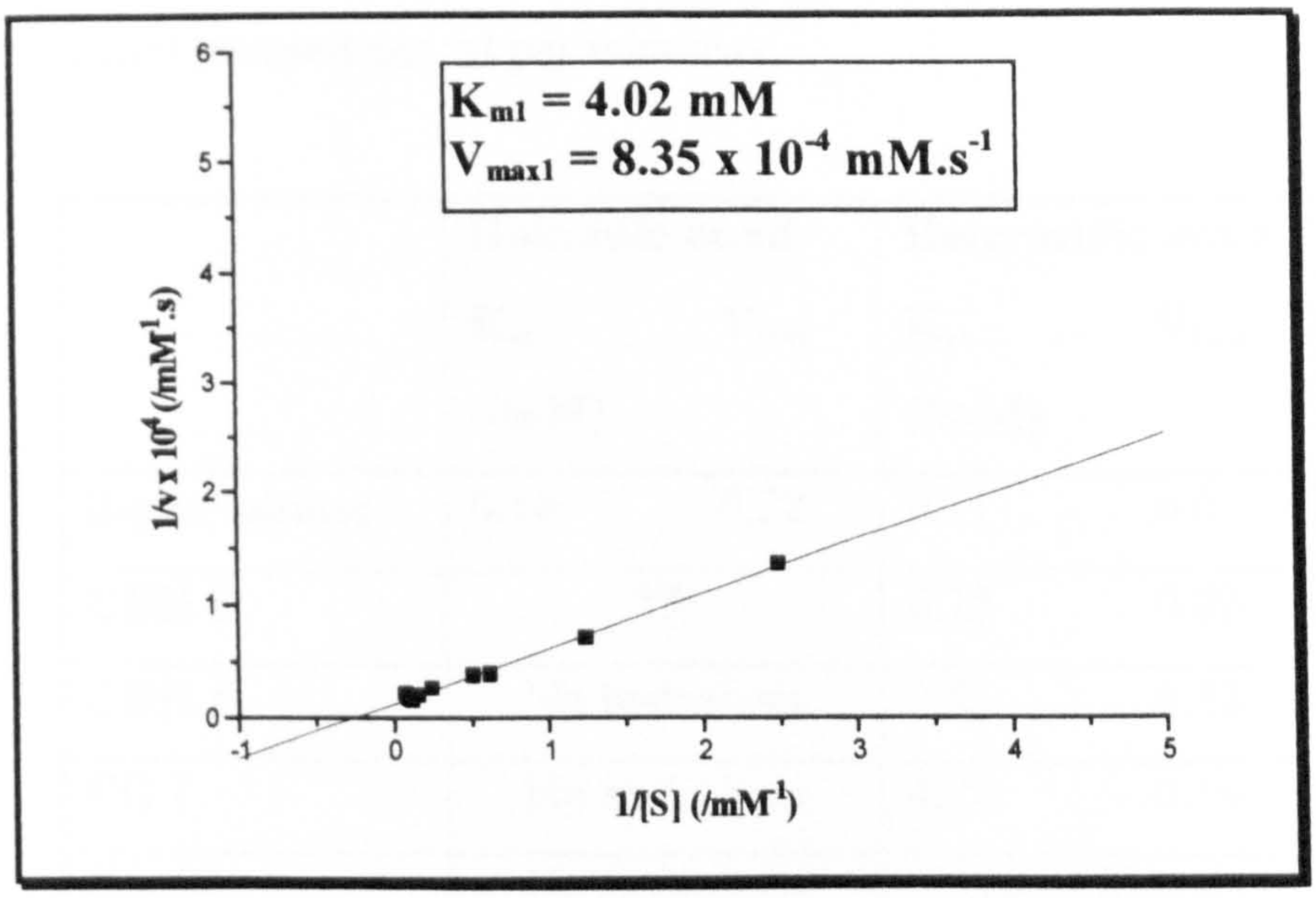
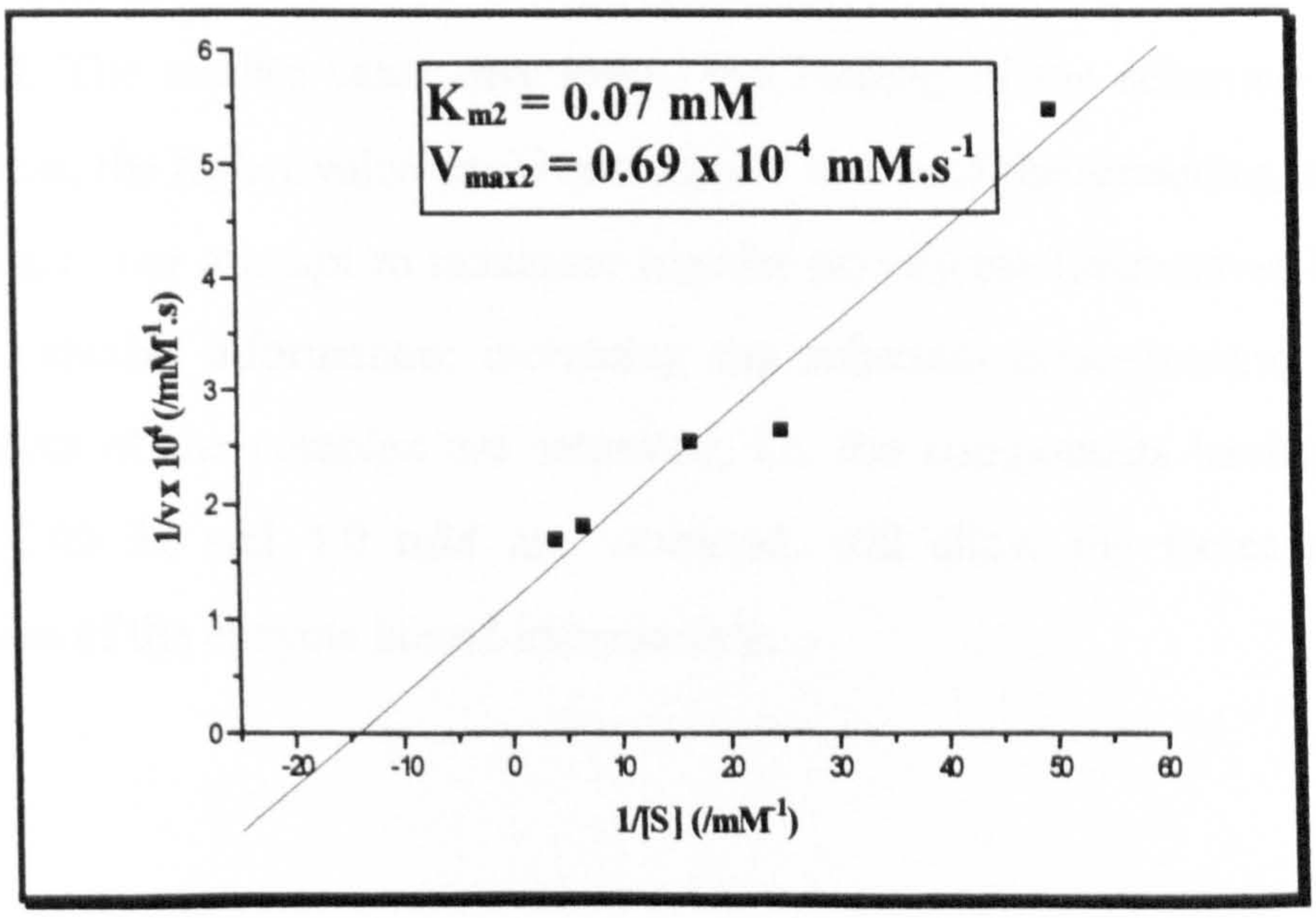


Figure 61 : Lineweaver-Burk plots for K_m determination of holosidic bond hydrolysis of PNPC

High substrate concentrations



Low substrate concentrations



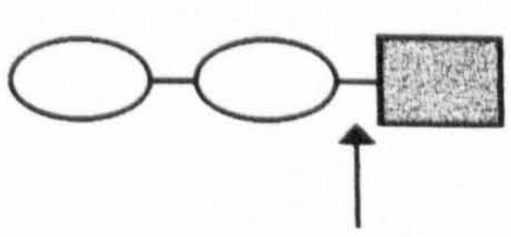


Figure 62 : Lineweaver-Burk plots for K_m determination of heterosidic bond hydrolysis of PNPC

The apparent K_m values can be compared to those published in the literature[105] from the isolated enzyme components (V_{max} corresponds to the number of µmole of p-nitrophenol released per ml per minutes)

	Holosidic bond		**Heterosidic bond**	
	K_m (/mM)	**V_{max}**	**K_m (/mM)**	**V_{max}**
β-glucosidase	0.16	0.28	0.11	0.07
CBH I	???		0.25	0.02
CBH II	No hydrolysis		2.51	0.12
EG I	No hydrolysis		4.20	0.19
EG II	No hydrolysis		No hydrolysis	

It was impossible to make any firm predictions about the origin of the K_m values measured. The smaller value may reflect the binding of the substrate by the β-glucosidase, the higher value could correspond to any of the remaining enzymes.

In relation to our attempt to maximise transfer activity the Lineweaver-Burk plots provide valuable information; increasing the substrate concentration until both components of the complex are saturated, i.e. the components having apparent K_ms of 0.05 M and 4.0 mM are saturated, will allow the increased rate of production of the enzyme bound intermediate.

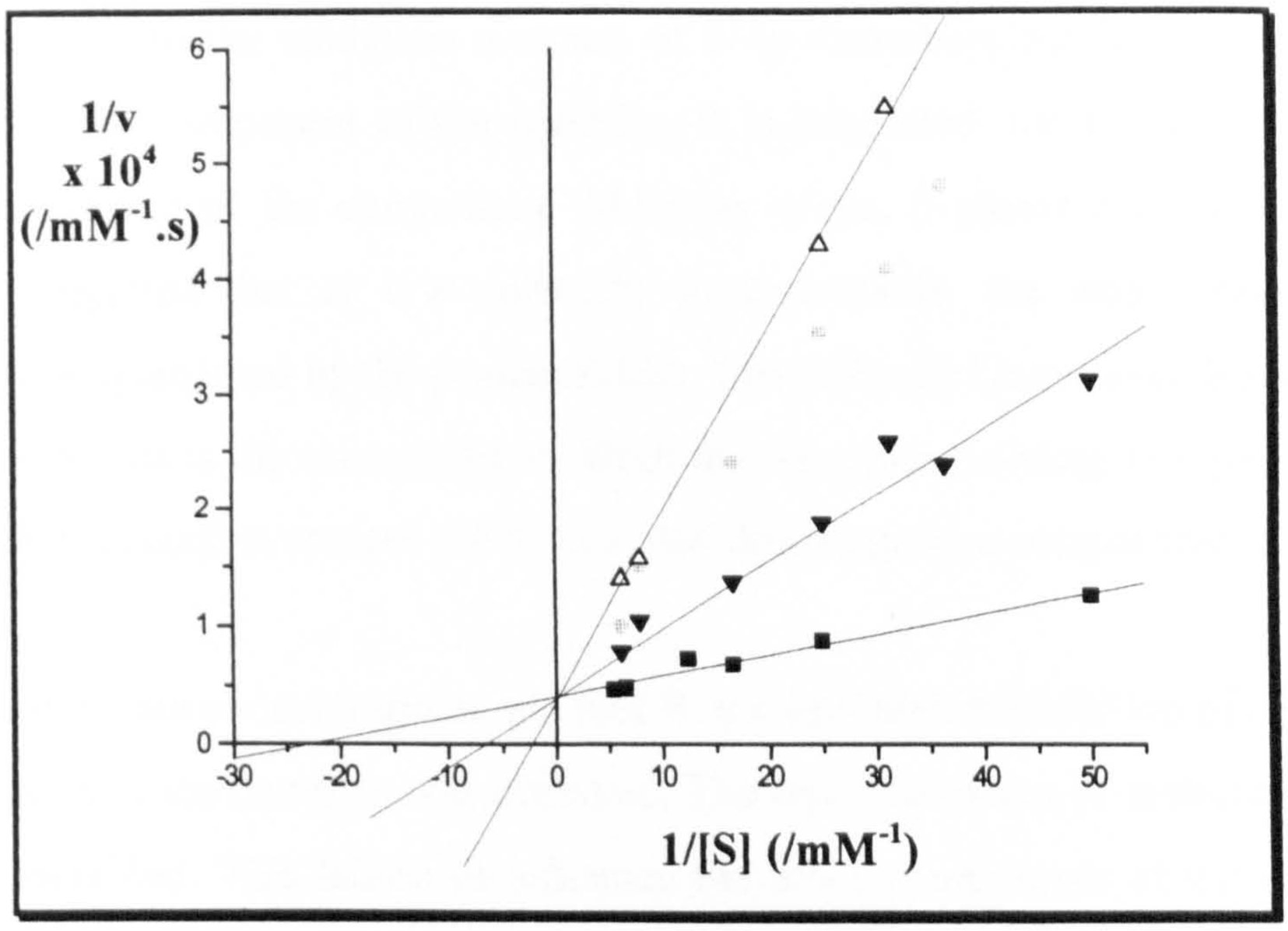
2.4.2.2. Acceptor Inhibition

An alternative method of determining which components of the complex are active in the transfer reaction would be to selectively inhibit the action of one of the enzyme components. The addition of 1,5-glucono-δ-lactone inhibits the β-glucosidase activity and consequently it was shown that the β-glucosidase component of the cellulase complex catalyses the formation of the β-(1-6) and the unknown transfer products.

The acceptor also inhibits the activity of the complex. An attempt was made to establish if the acceptor was capable of inhibiting, selectively, components of the cellulase complex. N-(p-nitrophenyl)-β-D-glucopyranosylamine reduces the rate of hydrolysis of PNPC. Using a Lineweaver-Burk plot obtained for different concentrations of acceptor, it is possible to obtain an approximate value of K_i. Again, two different straight lines were obtained for each acceptor concentration tested.

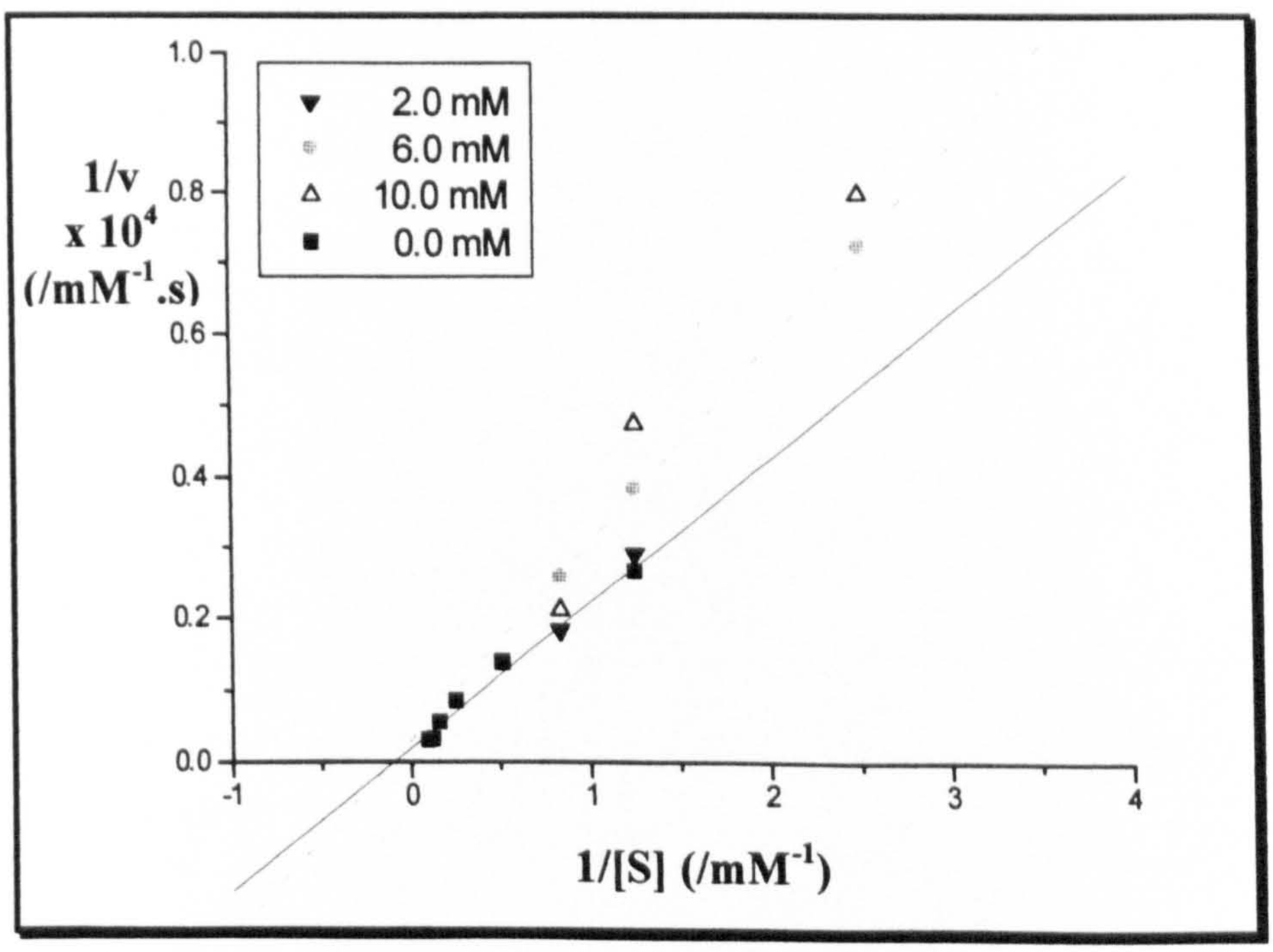
Plotting the Lineweaver-Burk data at both low and high substrate concentrations allows us to observe the differential inhibition of the two cellulase components (Figure 63).

Low substrate concentrations



Plot A : Competitive inhibition, $(K_i)_{av}$= 1.4 mM

High substrate concentrations



Plot B: Inhibition type ?

Figure 63 : Lineweaver-Burk plots at low and high substrate concentrations

At low substrate concentrations, a K_i value of 1.4 mM was obtained corresponding to the inhibition constant of N-(p-nitrophenyl)-β-D-glucosylamine for one of the component of the complex. It is suggested that this is more than likely a measure of the competitive inhibition of the β-glucosidase. The earlier results suggested that at low substrate concentrations, the major fraction of substrate is hydrolysed by the β-glucosidase. The different Lineweaver-Burk plots, cross the Y axis at the same point at which the line corresponding to a plot where no inhibitor is present crosses the Y axis, and this suggests a competitive inhibition process.

At high substrate concentrations, see plot B, a more modest inhibition of the other components of the complex was observed. The type and extent of inhibition could not be identified. This failure to influence the other components of the complex suggests that no real advantage, different to that obtained by adding 1,5-glucono-δ-lactone, could be achieved by inhibiting components of the complex.

2.4.3. Substrate concentration studies

As the initial rate studies indicated that the fraction of substrate hydrolysed by the different components of the complex varied with the substrate concentration, it was considered important to study the effect of altering the substrate concentration on the yield and regioselectivity of the transglycosylation reaction. S. Shoda *et al.*[108] have shown that increasing the substrate donor concentration leads to an increase in the transglycosylation product yield.

In transglycosylation reactions, using the acceptor N-(p-nitrophenyl)-β-D-glucopyranosylamine (10.0 mM), the substrate concentration was varied in an attempt to increase the transglycosylation yield. The concentration was increased from 0.10 mM to 5.08 mM, and the total concentration of the transglycosylation products increased from 0.02 mM to 0.76 mM (Figure 64).

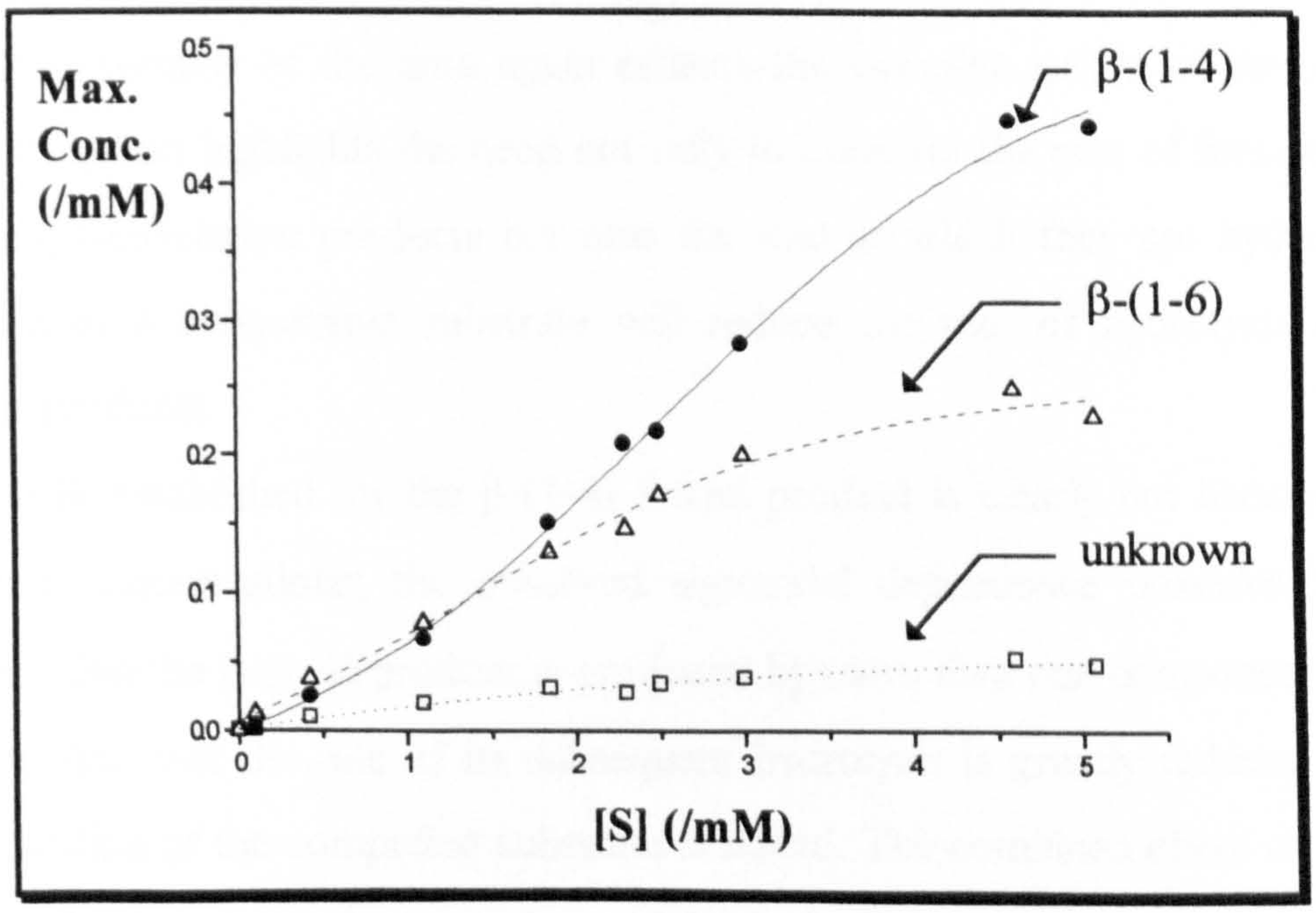


Figure 64 : Effect of Substrate Concentration on the Production of Transglycosylation products

From the graph, it can be observed that the change in the amount of transglycosylation product formed varies according to the type of linkages produced. A change in regioselectivity could be observed. At low substrate concentration (below 1.5 mM), the β-(1-6) linked disaccharide was the preferred

product (10% yield), whereas at highest concentrations, the β-(1-4) transfer product was the major compound (10% yield). As expected, the yield obtained for the unknown and the β-(1-6) linked product increased linearly until a fixed substrate concentration, approximately 2.5 M, when the rate of increase slowed and a saturation phenomenon was observed. The substrate concentration at which the total amount of transglycosylation products formed reaches a maximum, approximately 5 mM, is very much larger, several orders of magnitude, than the K_m value determined for β-glucosidase. This could correspond to an apparent value, K_{mapp} which would be measured for a β-glucosidase competitively inhibited by the glycoside at concentration of the acceptor :

$$K_{mapp} = K_m (1 + I/K_i)$$

$$K_{mapp} = 0.05 (1 + 10.0/1.4)$$

$$\mathbf{K_{mapp} = 0.41\ mM}$$

The inconsistency of the data again reflects the complex nature of the system under study and highlights the need not only to consider the rate of formation of the transglycosylation products but also the rate at which they are hydrolysed. Addition of a competitive substrate will reduce the rate of hydrolysis of the transfer products.

The profile established for the β-(1-4) linked product is clearly not linear at low substrate concentrations; the observed sigmoidal dependence provides further evidence that the β-(1-4) product is produced by more than one component of the complex and that the rate of its subsequent hydrolysis is greatly reduced as the concentration of the competing substrate is added. The combined effect of adding additional substrate on the formation and the hydrolysis of the transfer products is to favour production of a greater proportion of transfer products and to increase production of the β-(1-4) product. These results may provide an explanation for the exclusive formation of β-(1-4) linked products during Kobayashi's transglycosylation reactions. The high substrate concentrations used in his studies and a pretreatment of the cellulase complex which may have removed some or all

the β-glucosidase activity could account for the only production of β-(1-4) linkages.

At high substrate concentrations, self transfer products were now observed, p-nitrophenyl β-D-gentiobioside was identified by comparison with an authentic sample. Indeed, the large substrate concentration used provides new acceptors to the reaction system, both the substrate and substrate hydrolysis products are able to act as acceptors (Figure 65).

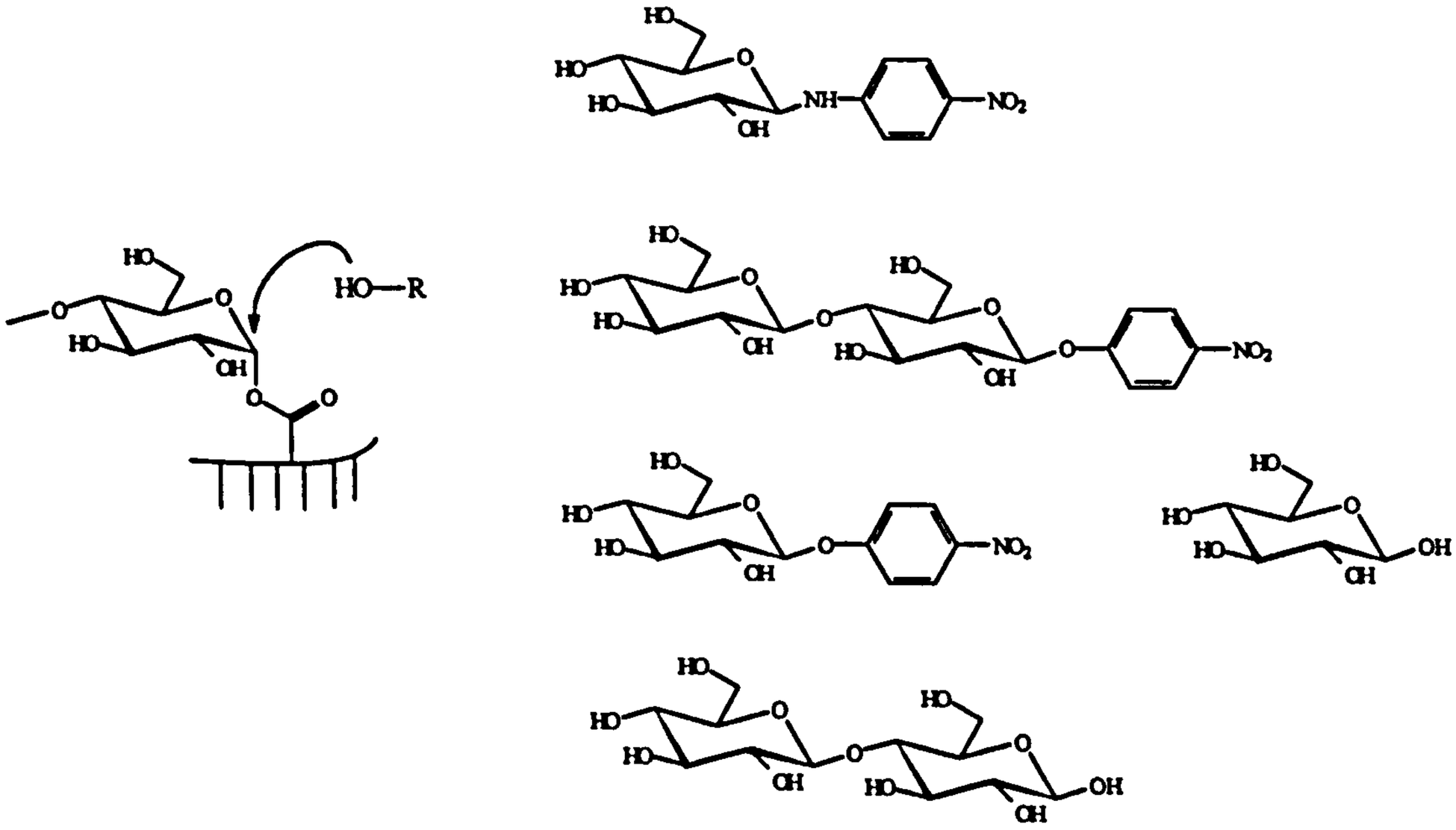


Figure 65 : Transglycosylation in presence of a high substrate concentration

2.4.4. Acceptor concentration studies

A similar experiment to that outlined in 2.4.3. was performed in which the effect of increasing the acceptor concentration was studied, the acceptor concentration was increased from 5.00 to 48.10 mM. To reach high acceptor concentrations, the reactions were performed in 10 % acetonitrile solution in order to aid solubility. The experiment was carried out with a substrate concentration of 2.50 mM, this concentration was chosen to minimise the production of self-transfer products. As can be observed from the graph, the total yield of transglycosylation products released increased from 6 % to 19 % with an increase in the acceptor concentration.

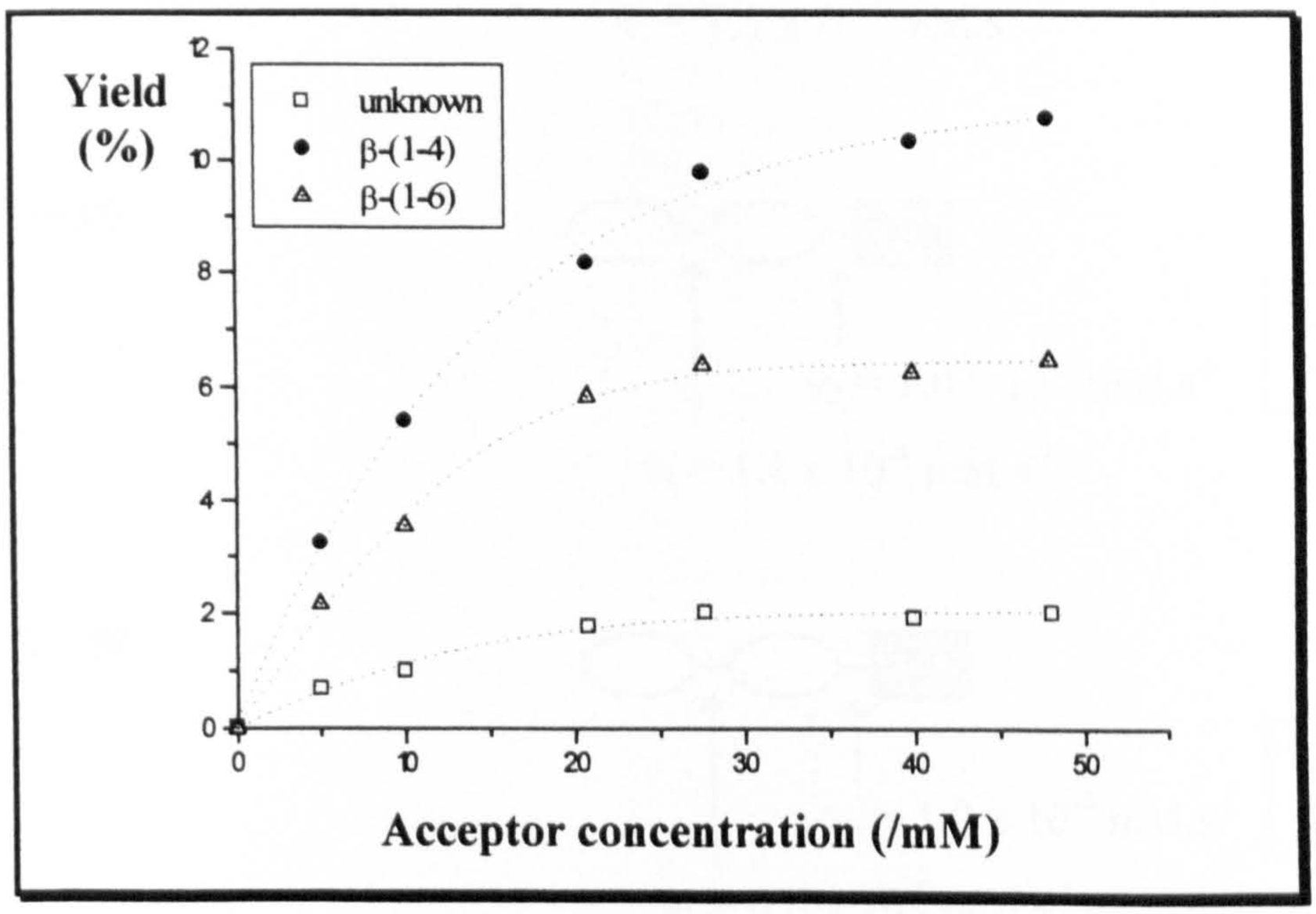


Figure 66 : Effect of Acceptor Concentration on the Production of Transglycosylation Products at 75 % conversion of PNPC

The yield of the unknown and the β-(1-6) reached a maximum value at an acceptor concentration of ~ 25.0 mM which can be explained by a saturation of the β-glucosidase, whereas the yield of the β-(1-4) disaccharide did not reach a maximum at the acceptor concentration of 50 mM. These results showed again the involvement of different enzyme components in the formation of the β-(1-4) linked disaccharide product.

The non productive binding and subsequent inhibition of the β-glucosidase also influences the relative frequency of cleavage of the holosidic and heterosidic linkages of the disaccharide substrate (Figure 67). Indeed, as the acceptor concentration increases, the rate of hydrolysis of the holosidic bond decreases (the preferred site of cleavage for β-glucosidase) whereas that of the holosidic linkage remains constant.

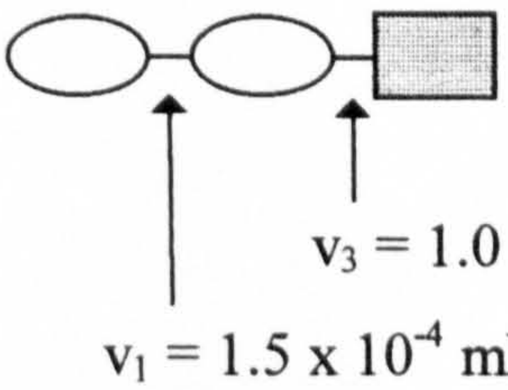
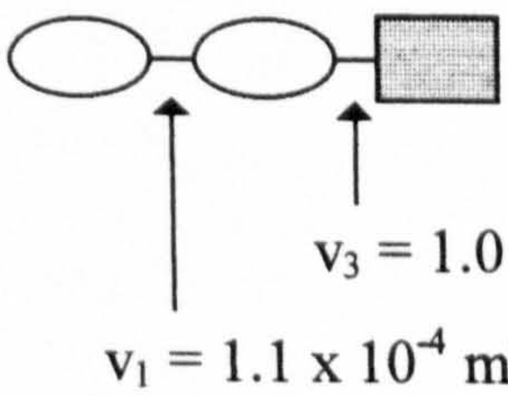
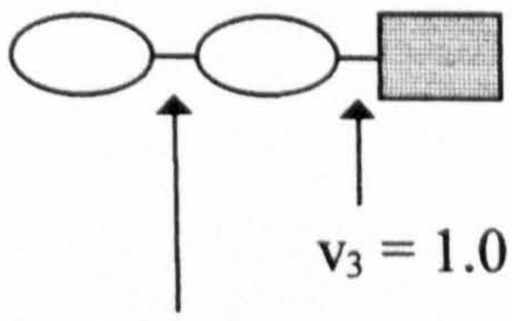
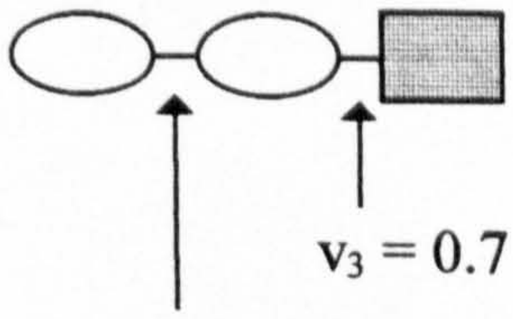
Acceptor Conc. (/mM)	Hydrolysis rate	
5.00		**RATIO 1.5 : 1**
10.00		**RATIO ~1 : 1**
20.80		**RATIO 0.6 : 1**
40.00		**RATIO 0.4 : 1**

Figure 67 : Frequency of bond cleavage at different acceptor concentrations

Using a substituted enzyme mechanism, two different explanations for the observed saturation are possible. The first relates simply to the acceptor concentration exceeding the apparent K_m for the acceptor binding site. The second relates to an increased occupation of the substrate binding site, non-productive binding, by the acceptor. In reality, both effects will be additive and, when combined with the effect that increasing the acceptor concentration has on transfer product hydrolysis, will lead to the observed maximum in the transfer reaction.

2.4.5. Temperature studies

The next variable to be adjusted in an attempt to increase the yield of the transglycosylation reaction was the reaction temperature. The effect of the temperature was studied in order to observe if there was any increase or decrease in the transglycosylation products yield with increase in the temperature of the reaction system. Cellulase complexes have been shown to be stable at high temperature, being still active at 60°C[109]. The study revealed an increase in the transglycosylation yield with decreasing temperature. At high temperature, 50°C, the total yield of transfer products was 12.0 %, whilst at low temperature, 10°C, the total transglycosylation yield was considerably higher and equal to 20.2 % which represents an increase of more than 60 %. By looking at the yields of the individual transglycosylation products, it is possible to observe a different effect of the temperature on the production of β-(1-4), β-(1-6), and unknown linkages. The production of β-(1-6) decreases by ~ 50 % when the temperature is raised from 10°C to 50°C, whilst the production of β-(1-4) decreases by ~ 30 %, and the production of the unknown remains, to a first approximation, constant throughout the reaction (See Figure 68).

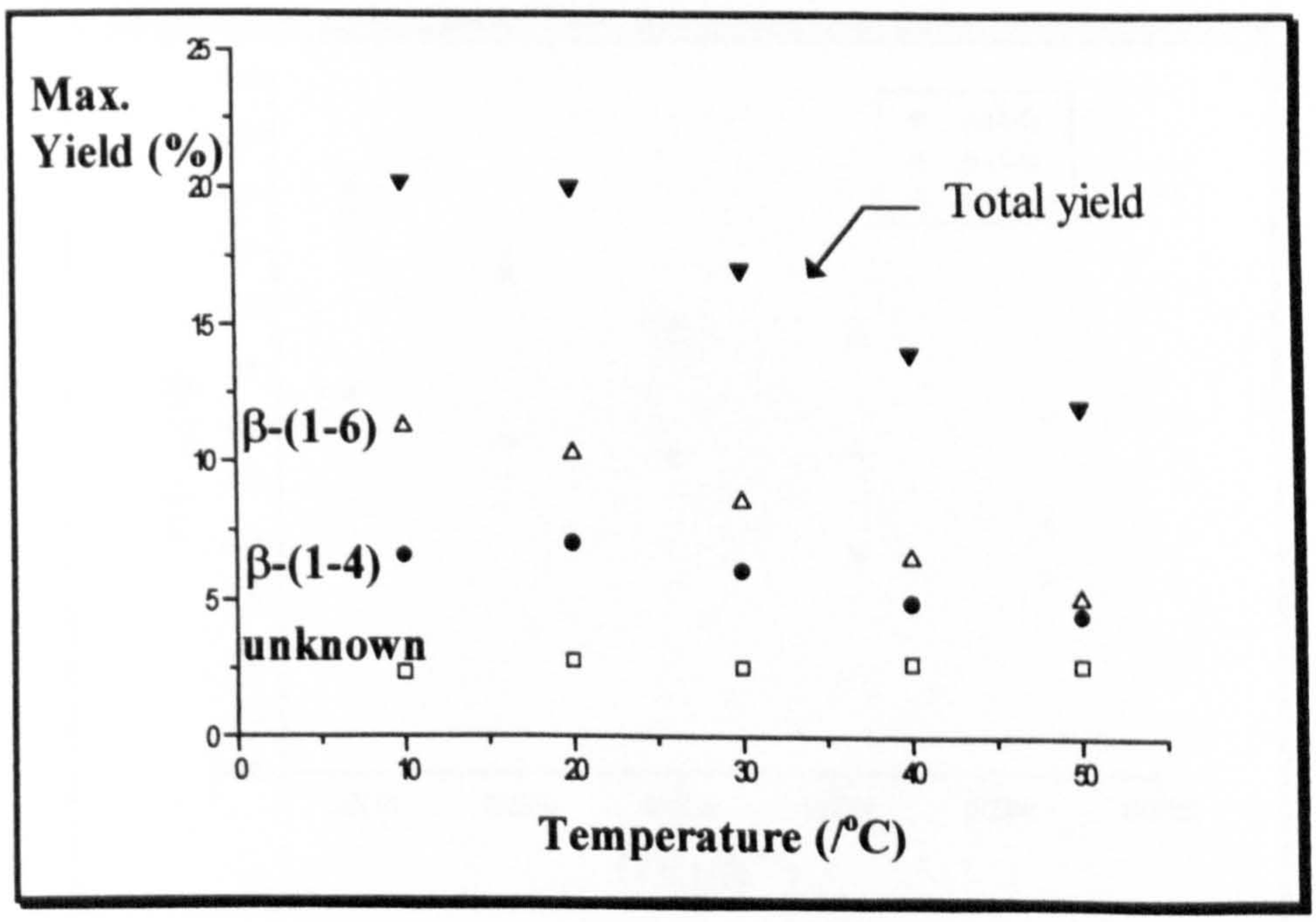
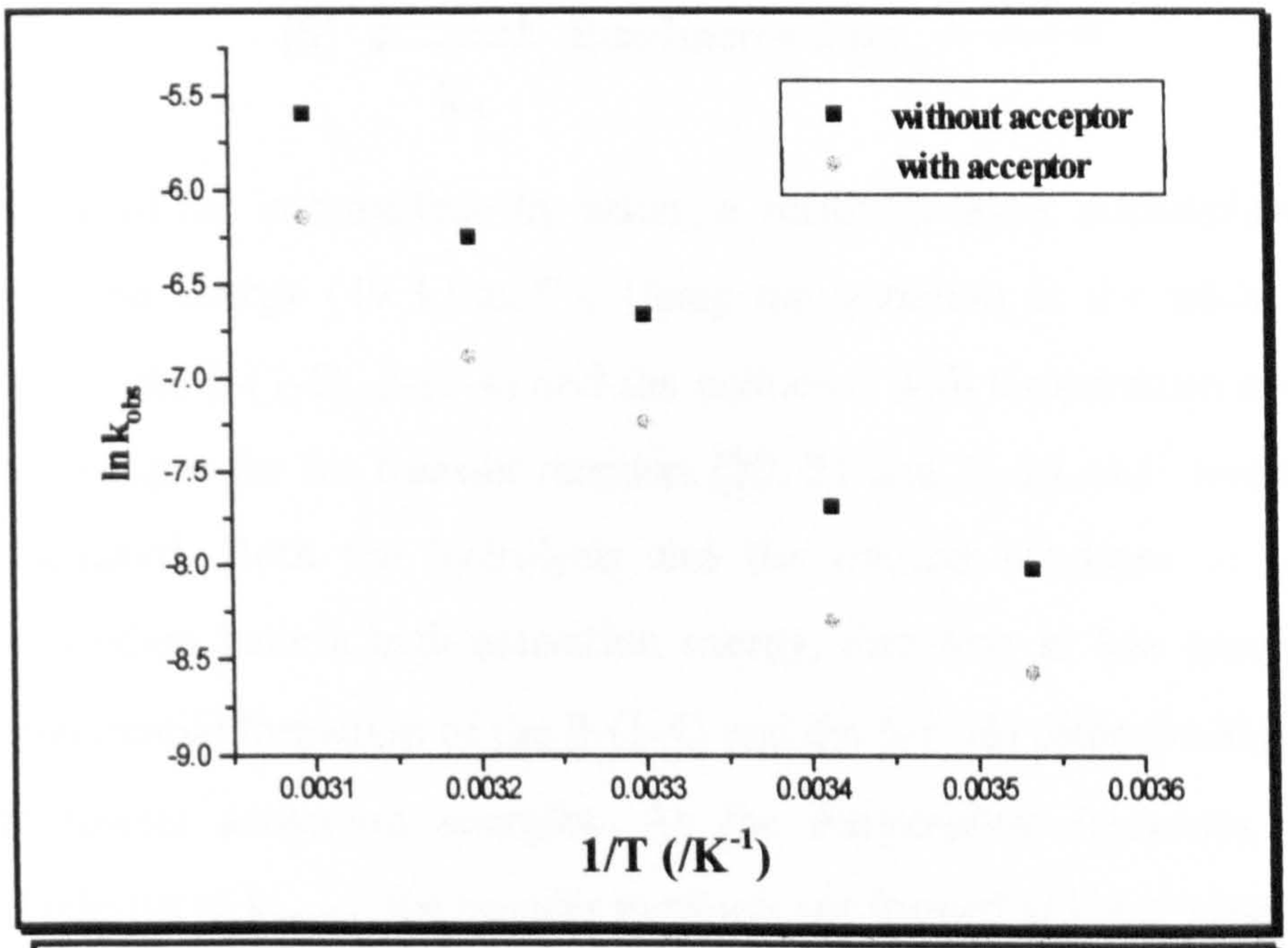


Figure 68 : Effect of varying the temperature on the production of the transglycosylation products

The results imply that transfer is favoured at low temperature and this indicates that the hydrolysis route has a higher activation energy than that of the transglycosylation pathway. A crude estimate of the relative sizes of the activation energy can be obtained from the Arrhenius plots (See Figure 69) with:

$$k = A \,.\, \exp(-E_a / RT)$$



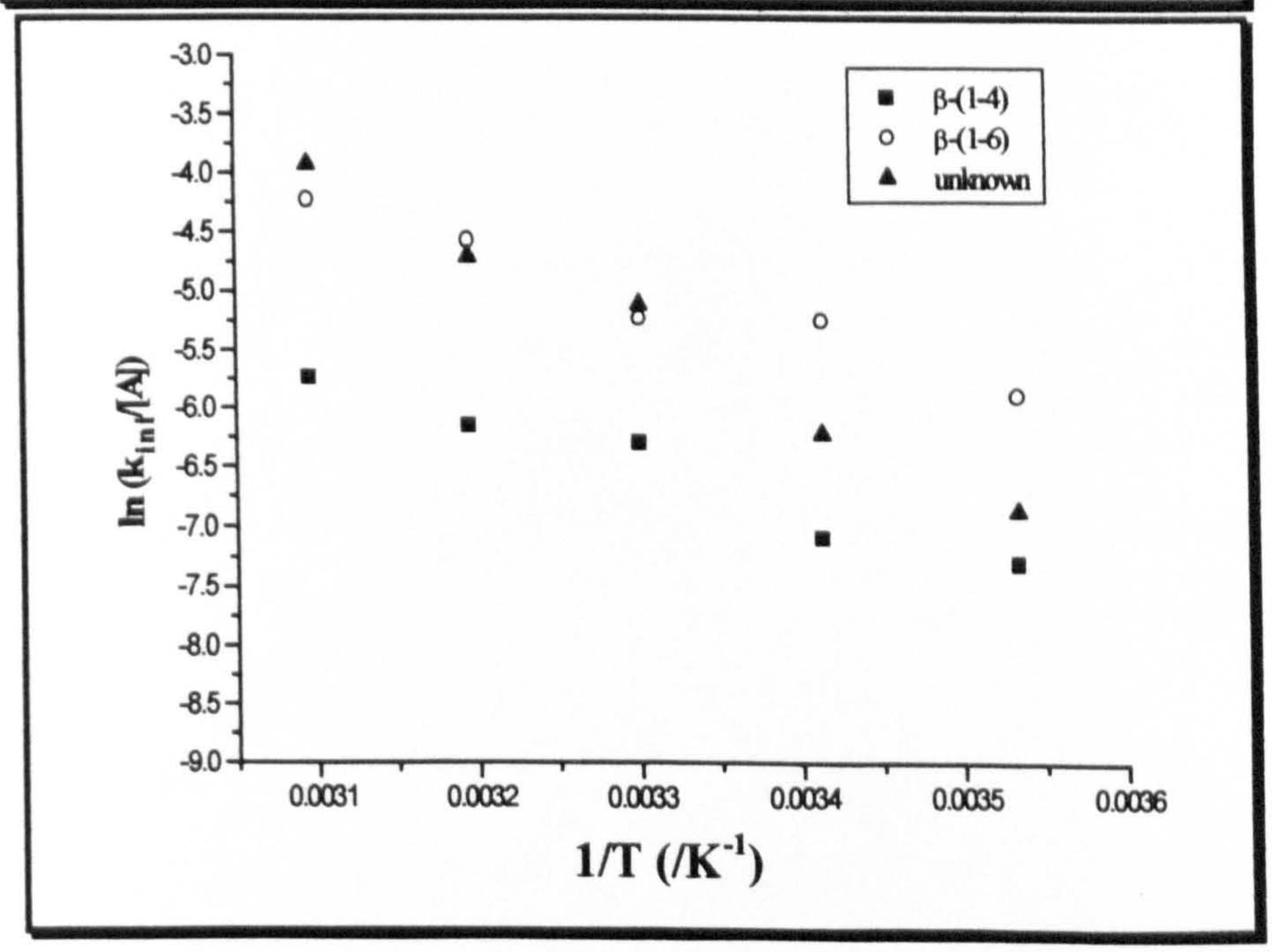


Figure 69 : Arrhenius plots for hydrolysis and transfer product formation

The activation energy corresponding to the hydrolysis of PNPC, determined in the absence of the acceptor, was calculated to be 49 kJ.mol^{-1}. This value was similar to that published in the literature for the hydrolysis of cellobiose by β-glucosidase, an enzyme component of the cellulase from *Aspergillus niger* (45 kJ.mol^{-1})[110]. For the hydrolysis of activated substrates, there is little variation in k_{cat} suggesting that the addition of the nucleophile is the rate determining step.

$$[S] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Enz-Intermediate} \xrightarrow{k_{cat}}$$

The trapping of the intermediate by water, a relatively weak nucleophile, has a large activation energy (49 kJ.mol^{-1}). Using the variation in the initial rate of production of the β-(1-6), β-(1-4) and the unknown with temperature allows the activation energies for the transfer reaction (30, 31 and 55 kJ.mol^{-1} respectively) to be calculated. Both the hydrolysis and the transfer reactions to give the unknown product have a high activation energy, therefore at low temperatures there is preferential formation of the β-(1-4) and the β-(1-6) disaccharides as they have the lowest activation energies. As the temperature increases, $k_{hydrolysis}$ increases relative to $k_{transfer}$, the transfer products are formed in lower yields.

2.4.6. Co-solvent studies

In a further attempt to favour the transglycosylation route, a co-solvent was added to the system in order to decrease the competing activity of water and to thus favour the formation of transfer products. Many research groups have shown that the addition of a co-solvent increased the yield of transglycosylation products[111,112,113].

The addition of co-solvent to the reaction system resulted in a decrease in the rate of hydrolysis of PNPC (Figure 70). A similar result has been reported for the cellulase catalysed hydrolysis of PNPG[49].

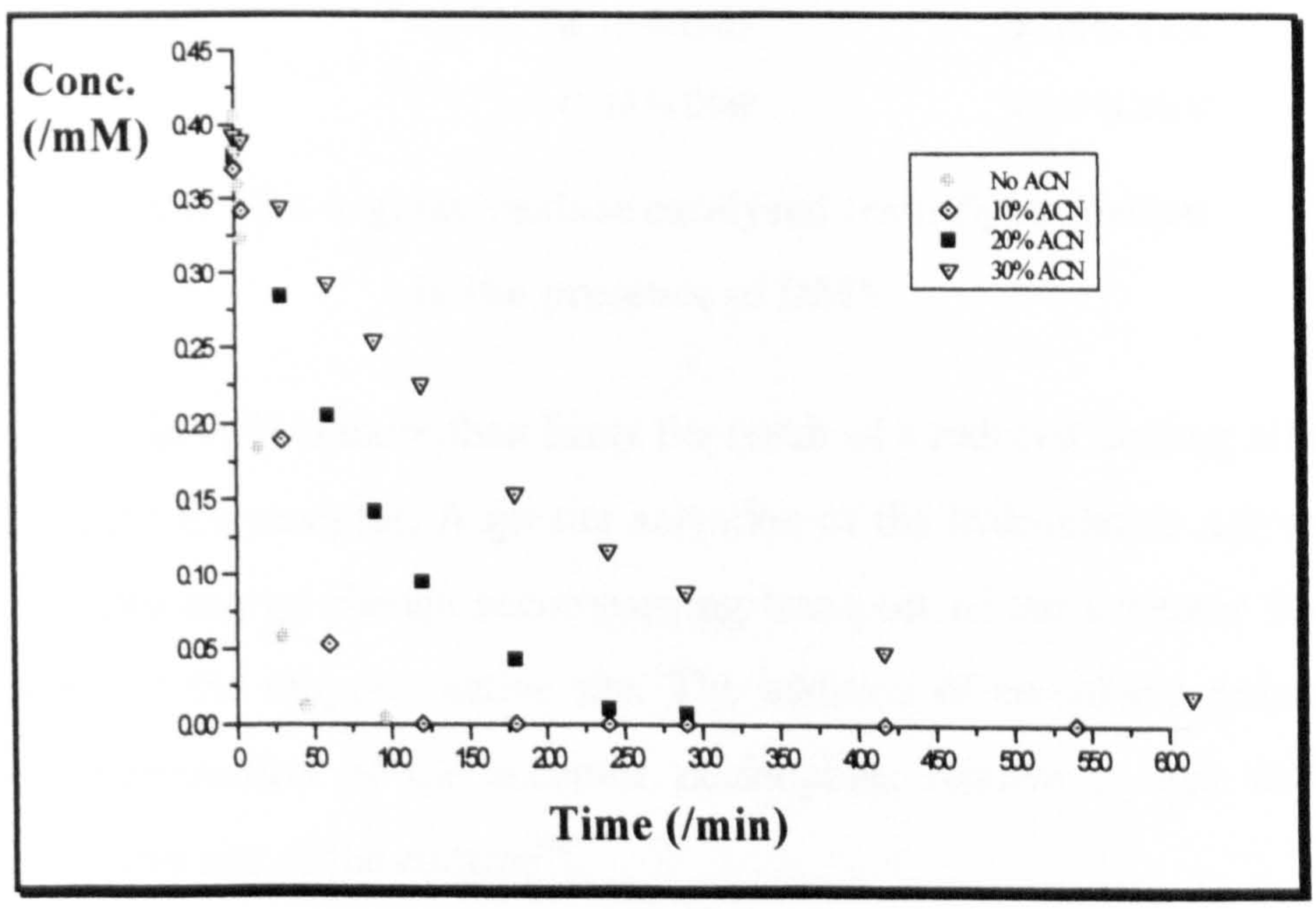
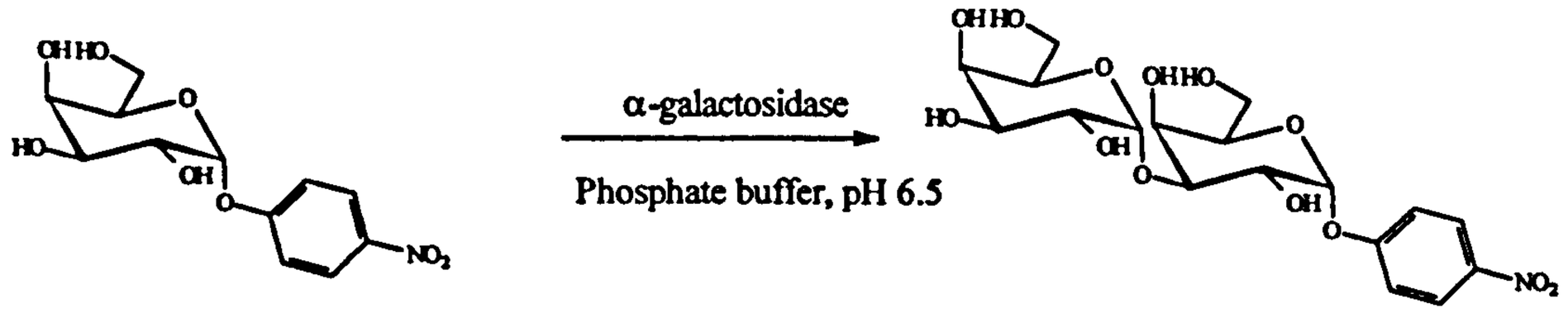


Figure 70 : Effect of acetonitrile on the rate of hydrolysis of PNPC

It has been suggested that the reduction in rate is due to a reduction in the effective water concentration and is also due to a preferred partitioning of the amphipatic substrate to the bulk solvent.

A range of different co-solvents was tested (t-butanol, acetone, acetonitrile and dioxane). In each case, the addition of co-solvent resulted in a decrease in the maximum yield of the transglycosylation products. In changing the solvent

composition from an aqueous system to one containing a thirty per cent co-solvent by volume, the yield of the transfer products decreased by a factor of four.
A similar result was also observed by Nilsson[114] who used DMF as co-solvent in a transglycosylation reaction involving α-galactosidase (Figure 71).



A. No-co-solvent	A. 32 % Yield
B. 10 % DMF	B. 18 % Yield
C. 30 % DMF	C. 10 % Yield

Figure 71 : α-galactosidase catalysed transglycosylation in the presence of DMF

The reduction in yield is more than likely the result of a reduced binding affinity of the enzyme for the acceptor. A greater solvation of the hydrophobic aglycon will reduce the free energy change accompanying transport of the acceptor from the bulk solvent to the enzymes active site. The addition of co-solvent reduces the effective concentration of the acceptor nucleophile, relative to that of water, within the active site of the enzyme[49].
In our experiment, an increasing solubility of the acceptor was noticed when the co-solvent was added to a solution confirming the enhanced solvation of the substrate. The binding of the substrate and the acceptor into the enzyme active site is believed to be generally that between the hydrophobic component of the acceptor and hydrophobic tryptophan residues[115] in the active site. Addition of co-solvent will increase the observed K_m by lowering the affinity of the substrate/acceptor for the active site relative to that in a completely aqueous reaction medium. The hydrophobic acceptor will favour the reaction medium on the addition of co-solvent.

2.4.7. pH rate profiles

The next variable to be modified in an attempt to increase the yield of the transglycosylation reaction was the pH of the reaction medium. Variation of the activity of the enzyme expressed as V_{max}/K_m as a function of pH was determined and the results are plotted in Figure 72. It is surprising, considering the complexity of the cellulase enzyme complex, that the plot of activity as a function of the pH of the reaction system should be so closely related to that of a single enzyme and provide a good bell-shaped curve.

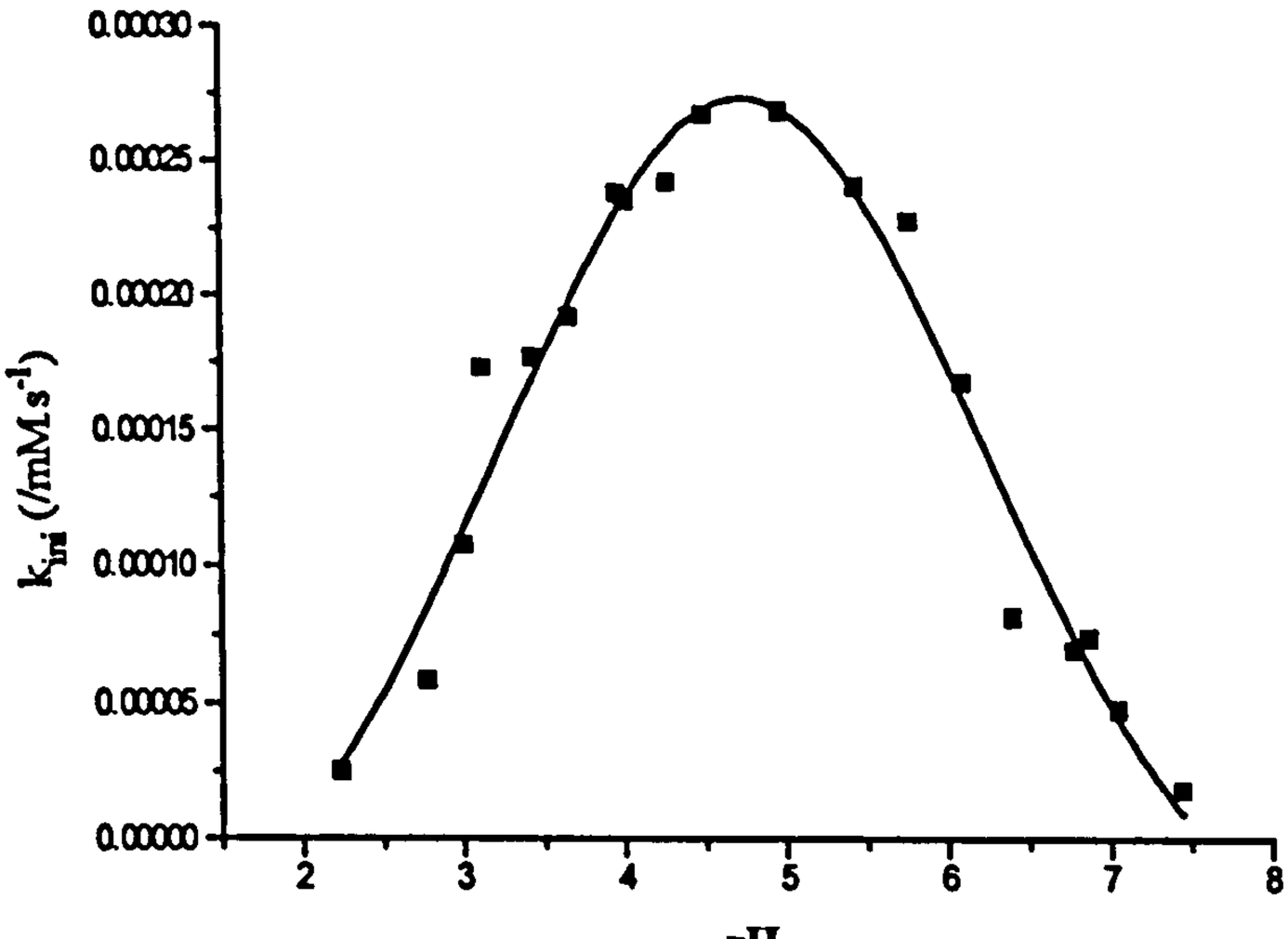
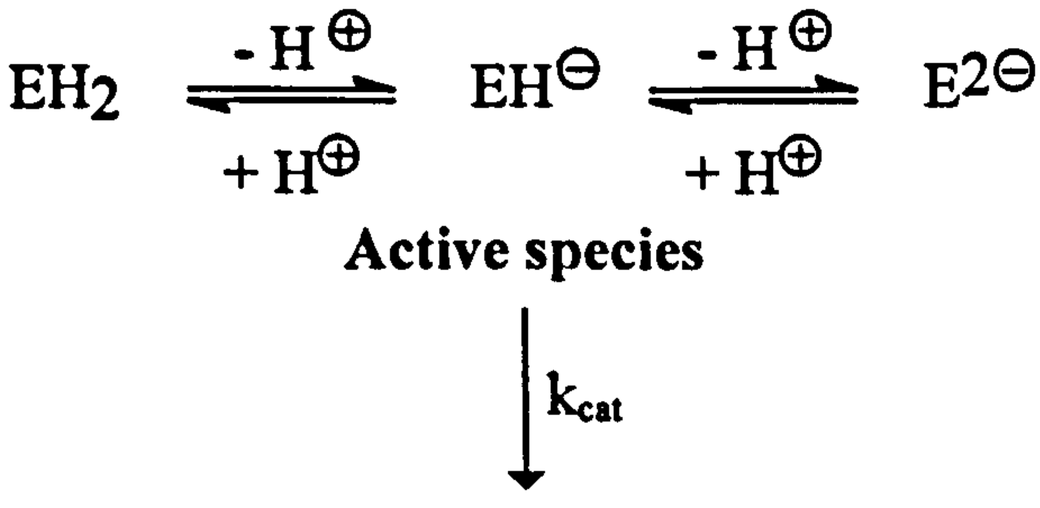


Figure 72 : pH rate profile of cellulase

The bell-shaped pH vs rate profile suggests the presence of two catalytic species inside the active site of the enzymes. A bell shaped curve with limits of zero is indication of a single catalytical active form, EH⁻, one of the catalytic residues is required in its basic form whilst the other is required in its acidic form. Fitting of the data to a double sigmoidal curve allowed the determination of the apparent pK_as of the 'active sites' residues. Considering the cellulase complex as a single dibasic acid enzyme, the rate of the reaction can be considered to vary as a function of the concentration of protons in the solution:



The rate of the reaction is proportional to the fraction of active enzyme present in the system. The rate is found to vary as follows :

$$V_{max} = (V_{max})_m \times \frac{1}{1 + \frac{[H^{\oplus}]}{K_{a1}} + \frac{K_{a2}}{[H^{\oplus}]}}$$

$$K_{a1} = \frac{[EH^{\ominus}] \times [H^{\oplus}]}{[EH_2]} \quad \text{and} \quad K_{a2} = \frac{[E^{2\ominus}] \times [H^{\oplus}]}{[EH^{\ominus}]}$$

The pK_as were determined to be equal to 3.2 and 6.2. The pH rate profile obtained for an exoglucanase present in cellulase from *Trichoderma viride* revealed a maximum activity between the pHs 4.0 and 5.8[116]. The pK_as are typical of those observed for glycosidases in which the active site contains a catalytically active aspartic acid and aspartate group.

For comparison, a similar plot was constructed for a hydrolysis reaction in a medium including co-solvent (24 % acetonitrile) and again a bell shaped plot was obtained with a small shift in the pK_a observed for the aspartate residue (3.7 and 6.3). The shift observed is indicative of this anionic residue being destabilised in the organic solvent.

Another pH rate profile can be constructed in which the yield of the individual transfer products is plotted as a function of the pH. The plot was drawn to see if there was any change in the regioselectivity on changing the pH of the reaction medium (Figure 73).

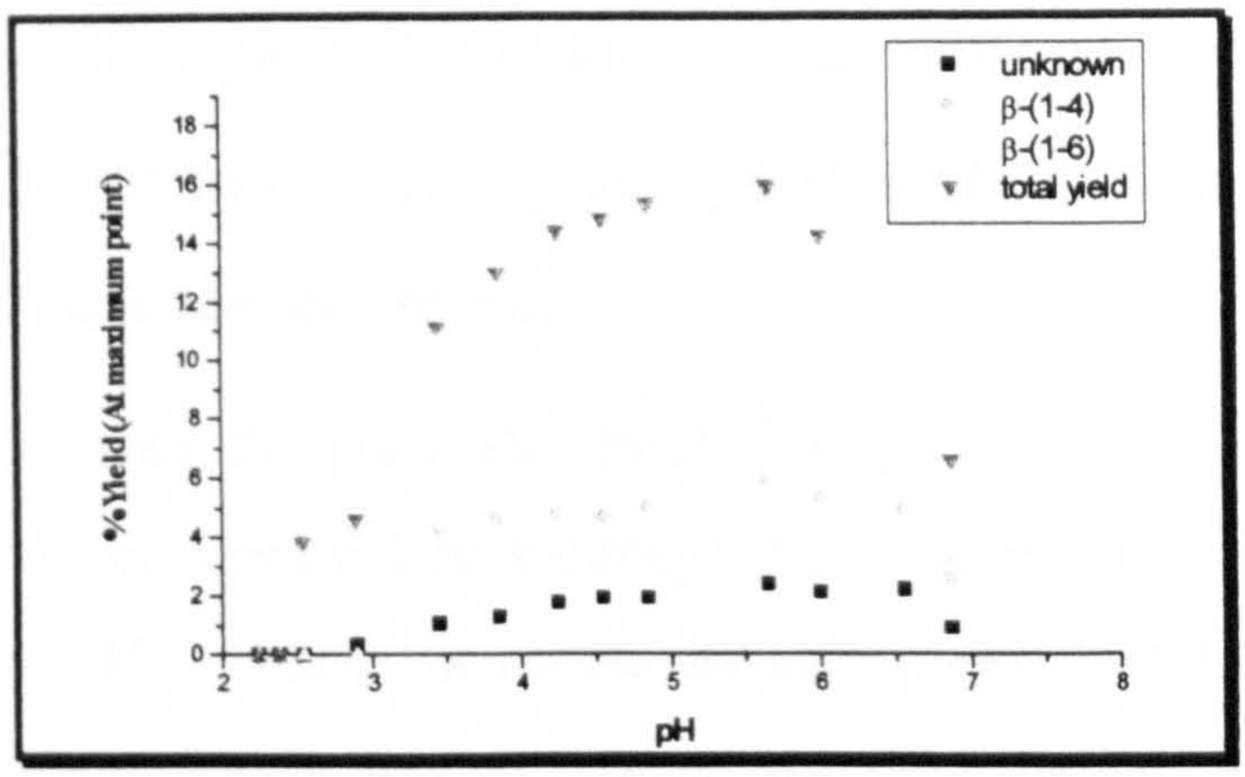


Figure 73 : Regioselectivity as a function of pH

As can be observed from the graph, the profiles obtained for the β-(1-6) and the unknown disaccharides produce a bell shape curve which must represent the pH rate profile of the β-glucosidase and has a maximum activity between pH 3.5 and pH 6. The yield decreases rapidly outside this range of pHs. The measured pK_as are close to those reported for the purified β-glucosidase from *Trichoderma reesei*[117]. The β-(1-4) disaccharide was produced over a much broader range of pHs ($2.5 < pH < 6.5$) and again this suggests that an additional enzyme is capable of catalysing transfer reactions to produce the β-(1-4) product.

2.5. p-Nitrophenyl β-D-lactopyranoside as a new substrate donor in the transglycosylation study of cellulase from *Trichoderma reesei*

Our observations with the substrate, PNPC, suggest that a minimum of two components of the complex catalyse transglycosylation reactions. From previous work at Huddersfield and using the results presented here, it has been conclusively proven that β-glucosidase is able to catalyse transfer reactions. In addition, this investigation suggests that a second enzyme component is also able to produce the β-(1-4) linked disaccharide. Cellulase from *Trichoderma reesei* is mainly composed of cellobiohydrolases, CBH I represents 60 % of the total protein concentration and CBH II representing 10 %[118]. Endoglucanases constitute 28 % of the total protein concentration and finally β-glucosidase 1 %. The residual 1 % represents oxidative enzymes. Evidence from our earlier results suggests that the most likely component which is catalysing the transfer is CBH I. Indeed, CBH II is an inverting enzyme and thus can not act as a transferase and EG II acts on longer oligosaccharides (at least 3 glucosyl moieties).

EG I has been reported to catalyse transglycosylation reaction via a self-transfer mechanism but is unable to directly hydrolyse glycosidic linkage adjacent to the non-reducing end of substrates. The formation of transglycosylation products from EG I must involve the intermediate formation of trisaccharide products:

$$\text{Glu-O-Glu-OPNP} + \underline{\text{Enz}} \longrightarrow \underline{\text{Enz}}\text{-O-Glu-O-Glu} + \text{PNP}$$

$$\underline{\text{Enz}}\text{-O-Glu-O-Glu} + \text{Glu-NH-PNP} \longrightarrow \textbf{(Glu-O)}_2\textbf{-Glu-NH-PNP} + \underline{\text{Enz}}$$

$$(\text{Glu-O})_2\text{-Glu-NH-PNP} + \underline{\text{Enz}} \longrightarrow \text{Glu-O-Glu-OH} + \text{Glu-NH-PNP}$$

EG I should be considered as a cellobiosyl transferase forming a cellobiosyl linked substituted enzyme. Indeed, both CBH I and EG I have cellobiosyl transferase activity and should produce trisaccharide products; however, in the present study, no trisaccharide products were observed.

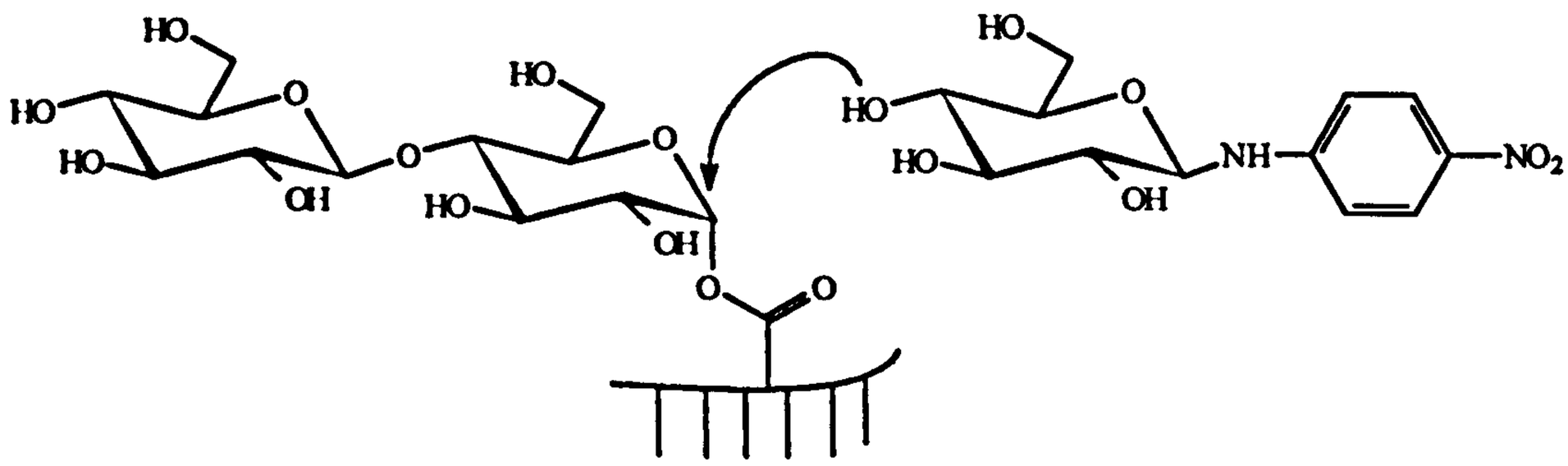


Figure 74 : Trisaccharide formation

Previous workers have used lactosyl fluoride as an activated substrate donor in transfer reactions[102] with cellulase. It was proven to be an efficient substrate and has been used in transglycosylation reactions with a diverse range of acceptors.

Pettersson *et al.*[105] have shown that p-nitrophenyl β-D-lactopyranoside (C4'-epimer of PNPC) is a good substrate for CBH I (K_m = 1.23 mM) and a poor substrate for EG I (K_m = 6.1 mM) and is not effectively hydrolysed by the other components of the complex. Lactopyranosides are not substrates for β-glucosidase[105].

In addition, p-nitrophenyl β-D-lactopyranoside (PNPL) has been shown to be only hydrolysed at the agluconic bond. It was thus decided to study the possible formation of trisaccharide transfer products.

PNPL was used as a substrate and was shown to be hydrolysed exclusively at the agluconic bond releasing p-nitrophenol and lactose. A Lineweaver-Burk plot was constructed to determine which of the enzymes present in the cellulase complex were catalysing the substrate hydrolysis. A range of substrate concentrations was studied (0.02 to 7.50 mM) surrounding that used in the transfer reaction. The results are scattered and the data is of relatively poor quality. However, an estimate for the apparent K_m was determined (K_m = 1 mM) and the value recorded was close to that of CBH I.

The transglycosylation studies using N-(p-nitrophenyl)-β-D-glucopyranosylamine as an acceptor did not show any significant production of transfer products. No

disaccharide products were formed but traces of two trisaccharides could be observed (Figure 75).

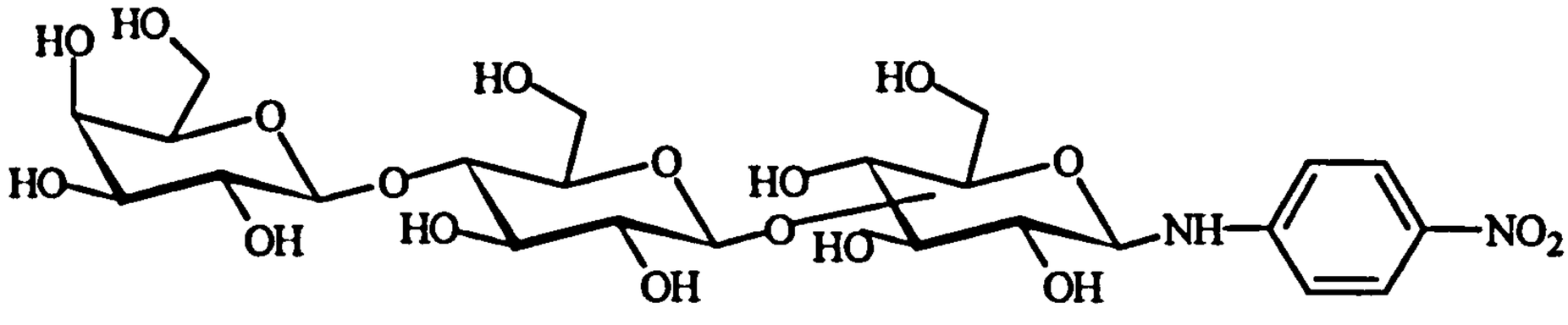


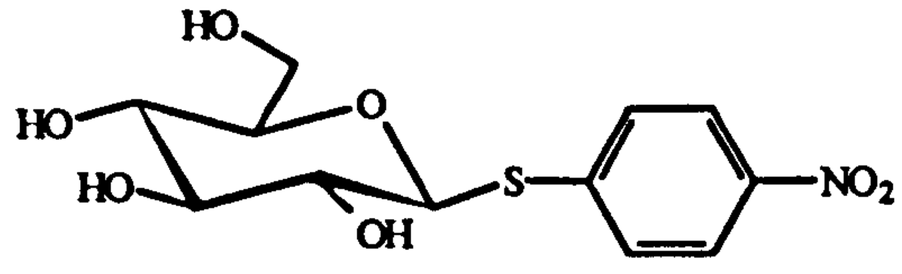
Figure 75 : Expected transglycosylation product

The possible presence of trisaccharides (on the basis of much higher retention times) suggests that both components (CBH I and EG I) may be responsible for the transfer activity. However, the measured K_m suggests that, under the conditions of the experiment, the enzyme catalysing the transfer reaction is CBH I.

2.6. Variation of the acceptor structure: New acceptor molecules

Our earlier studies have indicated that there is little opportunity to influence the yield by varying the reaction conditions. In an alternative approach, it was decided to study the effect of the nature of the acceptor structure on the yield of the transfer reaction. Thioglycosides have been used as suitable acceptors in transglycosylation reactions[119].

2.6.1. Acceptor: p-nitrophenyl 1-thio-β-D-glucopyranoside

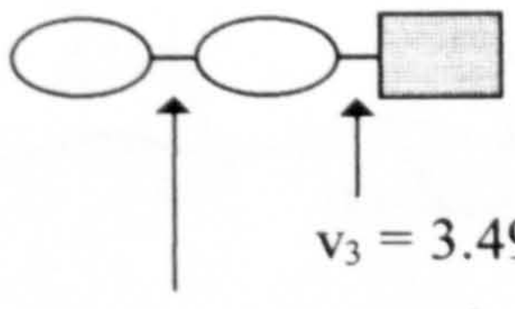


(6)

2.6.1.1. Transglycosylation studies

The addition of p-nitrophenyl 1-thio-β-D-glucopyranoside (10.0 mM) as a new glycoside acceptor led to a decrease in the hydrolysis rate of PNPC (k_{obs} = 1.96 x 10^{-3} s^{-1} /mg of enzyme), a reduction which corresponds approximately to the reduction in the rate observed when the N-(p-nitrophenyl)-β-D-glucopyranosylamine (10.0 mM) was tested as an acceptor. However, the decrease in the rate of hydrolysis of the heterosidic link was much greater with the new acceptor. As the reaction at the heterosidic link can not directly provide disaccharide products, this initial observation was very promising. Indeed, the heterosidic linkage hydrolysis decreased from 3.49 x 10^{-4} $mM.s^{-1}$, without inhibitor, to 1.56 x 10^{-4} $mM.s^{-1}$ in the presence of the thioglycoside, a decrease of 50 %.

No inhibitor

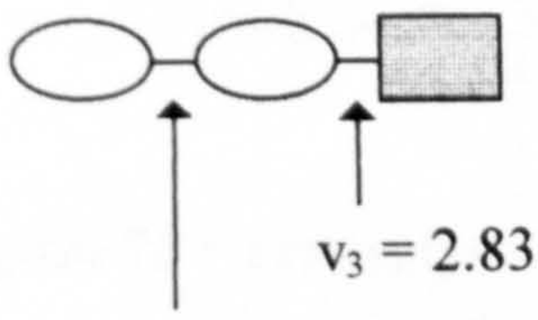


$v_3 = 3.49 \times 10^{-4}$ mM.s^{-1}

$v_1 = 7.26 \times 10^{-4}$ mM.s^{-1}

Ratio ~2:1

With N-(p-nitrophenyl)-β-D-glucopyranosylamine, 10.0 mM

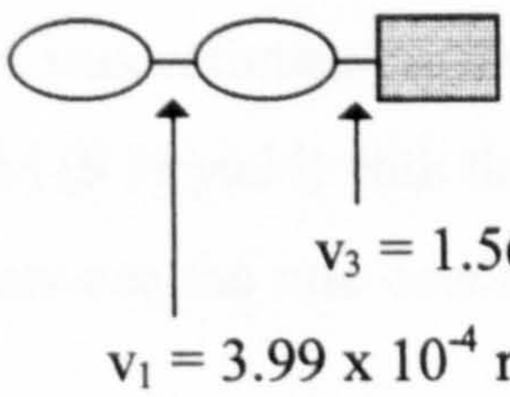


$v_3 = 2.83 \times 10^{-4}$ mM.s^{-1}

$v_1 = 3.42 \times 10^{-4}$ mM.s^{-1}

Ratio ~1:1

With p-nitrophenyl 1-thio-β-D-glucopyranoside, 10.0 mM



$v_3 = 1.56 \times 10^{-4}$ mM.s^{-1}

$v_1 = 3.99 \times 10^{-4}$ mM.s^{-1}

Ratio 2.5 : 1

Three transglycosylation products were formed during the reaction, two of them were identified as the β-(1-4) and the β-(1-6) disaccharides (Figure 76). Further evidence for the identity of the β-(1-6) product was obtained by the addition of 1,5-glucono-δ-lactone which resulted in the loss of the β-(1-6) product. The addition of the inhibitor to the current system results in the observation of one main transfer product, the β-(1-4) disaccharide.

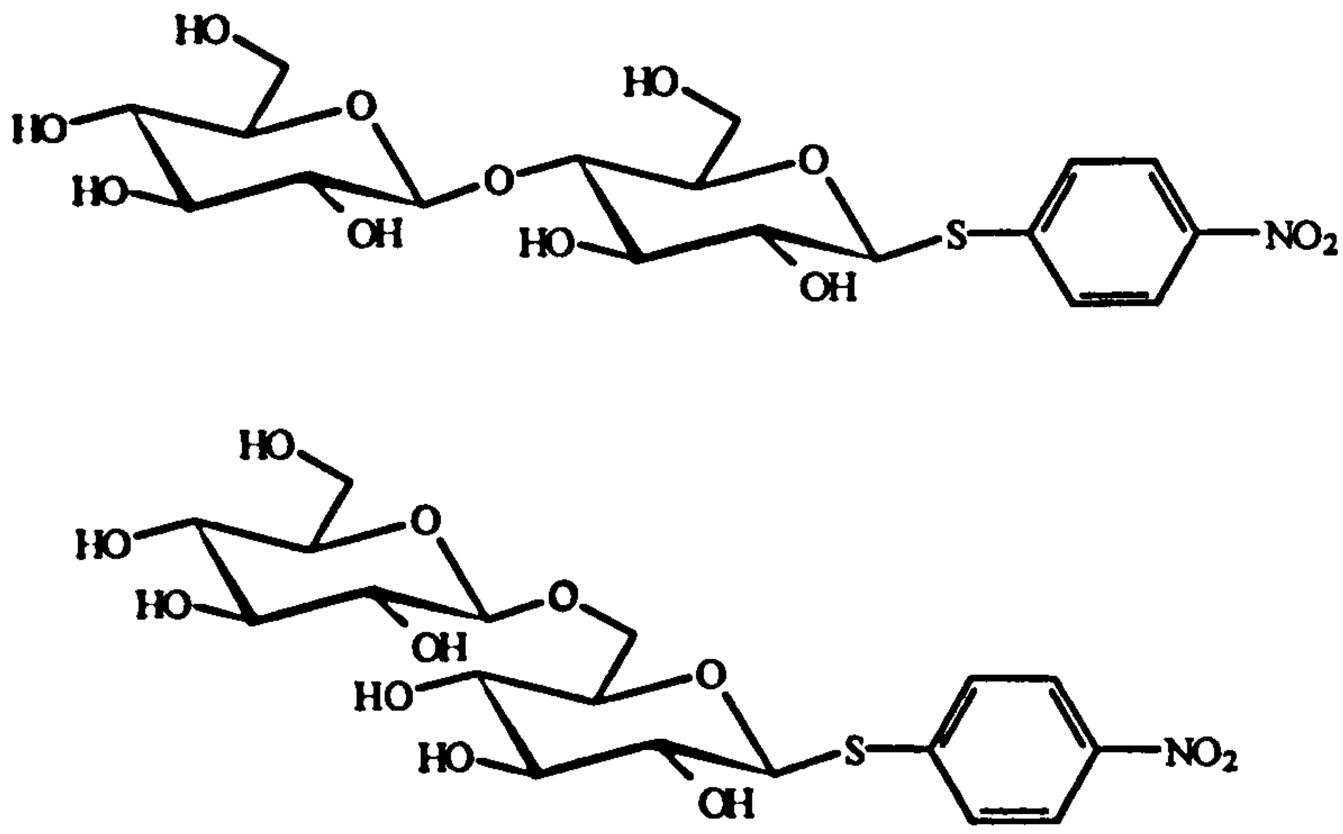


Figure 76 : Transglycosylation products

The amount of β-(1-6) produced was significantly larger than that obtained using the glucopyranosylamine acceptor **(1)**. Indeed, using a similar acceptor concentration (10.0 mM), the amount of β-(1-6) produced from the reaction with the thioglycoside acceptor was estimated at 0.10 mM corresponding to a 25 % yield compared to 0.04 mM (9 % yield) with the glucopyranosylamine.

Again, the relative ratio between the rate constants for transfer and hydrolysis can be calculated :

$$\frac{k_{hydrolysis} \times [H_2O]}{k_{transfer} \times [Acceptor]} = \frac{1.85 \times 10^{-4}}{1.85 \times 10^{-5}}$$

$$\frac{k_{hydrolysis}}{k_{transfer}} = \frac{1.85 \times 10^{-4}}{1.85 \times 10^{-5}} \times \frac{0.01}{55.55}$$

$$\frac{k_{hydrolysis}}{k_{transfer}} = 1.80 \times 10^{-3}$$

$$\boxed{k_{transfer} = 555 \times k_{hydrolysis}}$$

The value for the relative ratio is now a minimum value as the initial rates of formation of the other transglycosylation products were not considered; this value is higher than that obtained for the glycopyranosylamine acceptor. Indeed, the result can be compared to that obtained with the glucopyranosylamine acceptor for the formation of the β-(1-6) transfer product ($k_{transfer}$ = 113 x $k_{hydrolysis}$). The higher solubility observed for the thioglucoside compared to that of the glucopyranosylamine acceptor implied a greater solvent stabilisation of the former. This increase in stabilisation combined with the enhancement observed in the rate of transfer led to the conclusion that an increase in the acceptor concentration in the acceptor binding site occurs i.e. a higher affinity of the enzyme for the thioglucoside. It was proposed that the binding of the thioglucoside occurs with binding of both, the glucose moiety and the aglycon within the acceptor binding pocket of at least one component of the complex.

p-Nitrophenyl 1-thio-β-D-glucopyranoside was thus assumed to be a better acceptor to be used in transfer reaction than the glucopyranosylamine probably due to its spatial orientation closer to that of the natural substrate. This would provide an increase in the binding affinity of the enzyme with the acceptor and increase the probability of forming the transfer products. The resulting transglycosylation products would however have greater affinity with the enzyme compared to the transfer products formed when the glucopyranosylamine acceptor was used.

Increasing the concentration of the acceptor led to an increase in the transglycosylation product yield until saturation was reached. The maximum concentration of the product obtained for the β-(1-6) transfer product was approximately 0.16 mM, corresponding to 40 % yield ([S] = 0.4 mM) (Figure 77).

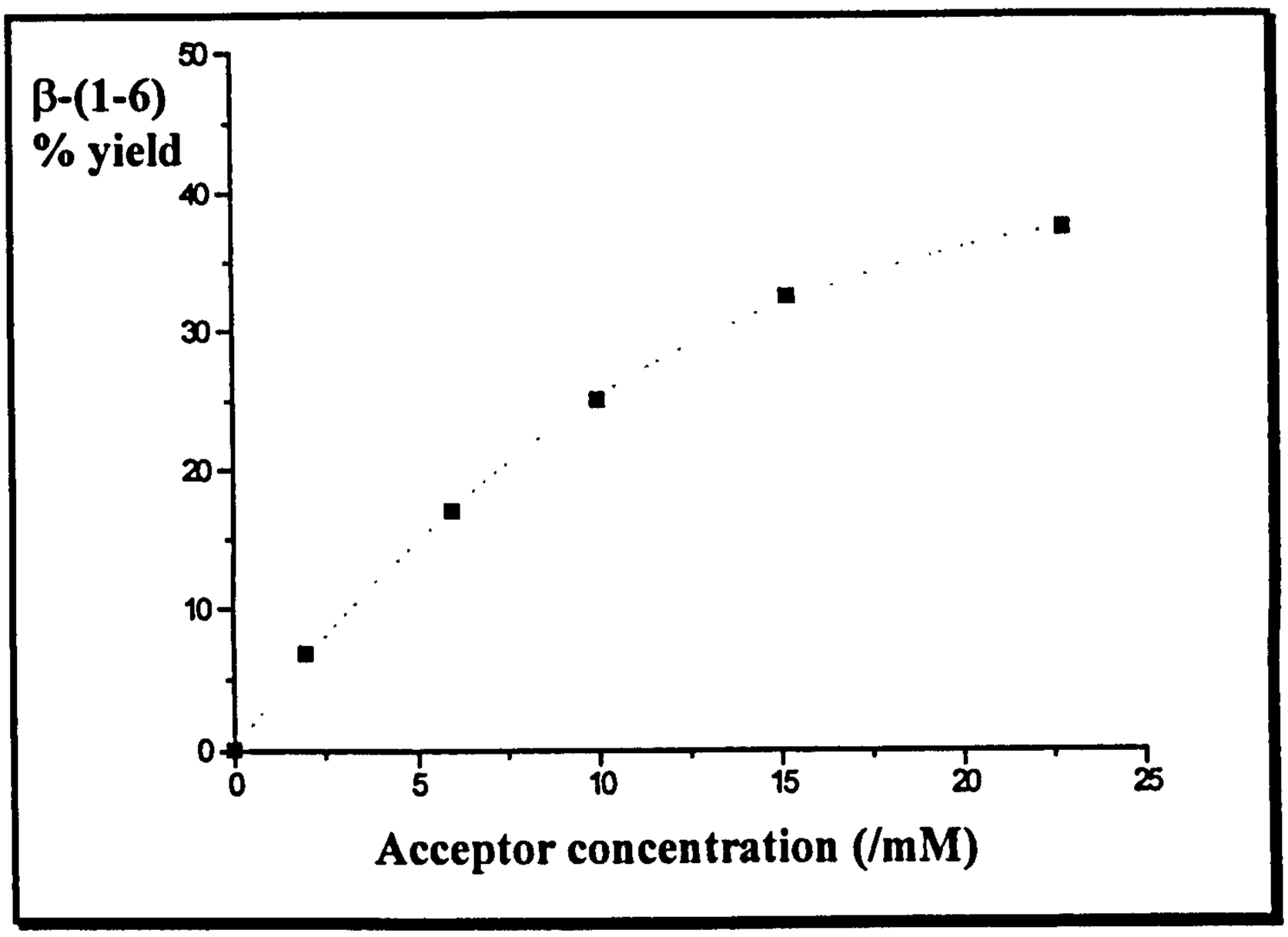


Figure 77 : Variation of yield with acceptor concentration

By comparison with the N-(p-nitrophenyl)-β-D-glucopyranosylamine acceptor where the saturation limit was obtained at [A] = 25.0 mM and the maximum β-(1-6) product yield was 6 % using a substrate concentration of 2.5 mM, it is possible to observe that the point at which saturation is reached (about 25 mM) is the same. The difference relies on the amount of acceptor transferred: under the same reaction conditions ([S] = 0.4 mM, [A] = 10.0 mM), the β-(1-6) transglycosylation product formed was 0.1 mM using the thioglucoside as an acceptor and 0.04 mM using the glucopyranosylamine as an acceptor. This again could suggest an increase in affinity of the former for the enzyme active site.

It was proposed to determine the extent to which the acceptor inhibited the cellulase complex. The inhibition studies showed that p-nitrophenyl 1-thio-β-D-glucopyranoside acted as a competitive inhibitor (K_i = 1.5 mM) at low concentration of substrate. This value was much higher than expected and is comparable with that obtained for the glucopyranosylamine acceptor. The thioglucoside, being more similar in structure to the substrate, was expected to have a greater affinity with the substrate binding site of the enzyme (see later discussion concerning the 1,4-dithiodisaccharide).

2.6.1.2.Determination of the hydrolytic reactivity of the transfer product : p-nitrophenyl 1-thio-β-D-cellobioside

The transfer products, both 1,6-linked and 1,4-linked disaccharides, will themselves be substrates for the components of the cellulase complex. p-Nitrophenyl 1-thio-β-D-cellobioside is one of the compounds formed during the transglycosylation reaction when p-nitrophenyl 1-thio-β-D-glucopyranoside is used as the acceptor and PNPC as the substrate donor. The rate of hydrolysis of this substrate was studied and the apparent Michaelis-Menten constant, K_m, was determined (K_m = 0.1 mM). The measured apparent Michaelis-Menten constant is very similar to that determined for PNPC.

p-Nitrophenyl 1-thio-β-D-cellobioside is only hydrolysed by cellulase at the holosidic bond and thus represents a potential activated substrate donor to be used in future transglycosylation reaction work. The hydrolysis would thus exclusively occur at the holosidic bond and provide a glucosyl-enzyme intermediate susceptible to nucleophilic attack by the acceptor (Figure 78).

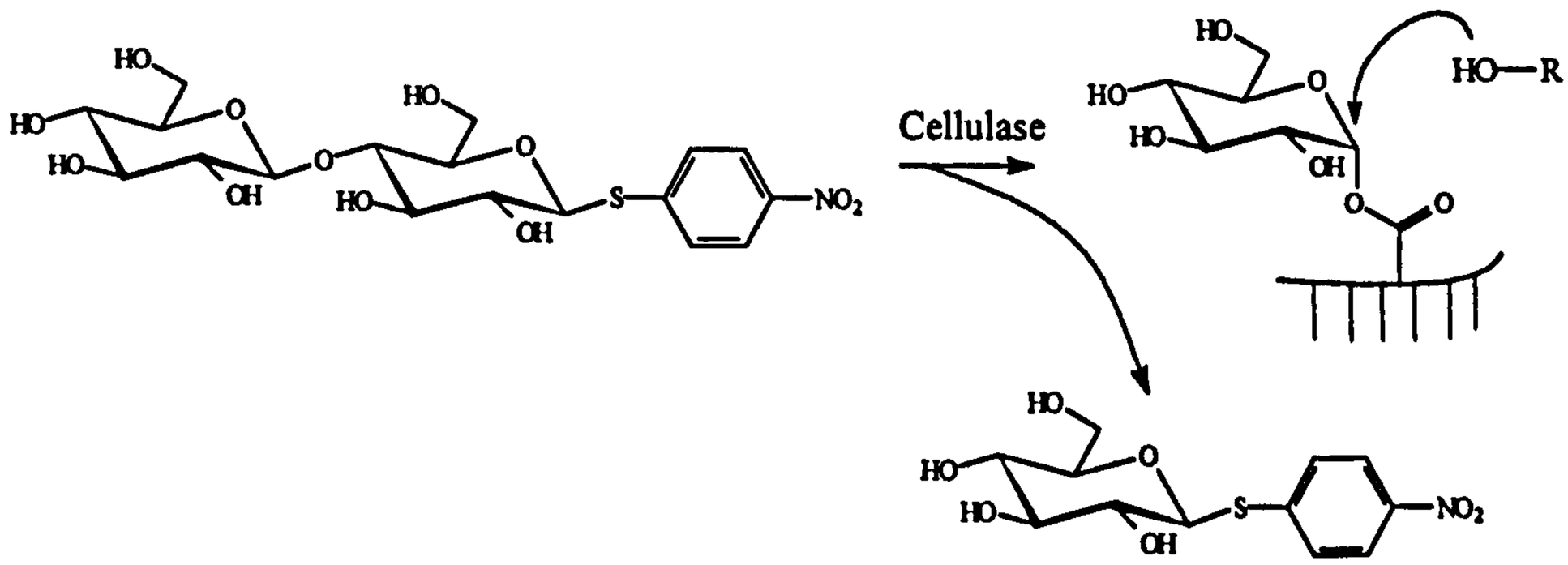


Figure 78 : p-Nitrophenyl 1-thio-β-D-cellobioside used as a substrate donor in transglycosylation reactions

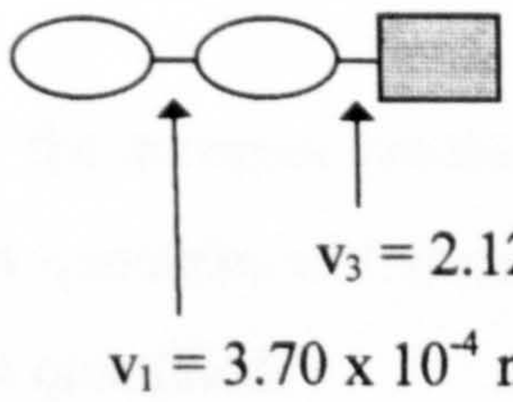
This reaction has not been studied as part of this project but could be investigated in future projects (see discussion in the conclusion).

2.6.2. Acceptor: p-nitrophenyl 1,4-dithio-β-D-cellobioside

p-Nitrophenyl 1,4-dithio-β-D-cellobioside was tested as a disaccharide acceptor using PNPC as a substrate. Previous results (2.6.1.1) have demonstrated the possible greater affinity of the enzyme for the p-nitrophenyl 1-thio-β-D-glucopyranoside compared to the glucopyranosylamine acceptor. It was thus expected that the inclusion of an additional glucose residue would increase the binding affinity of the compound into the acceptor site of those components of the complex having multiple glucosyl binding sites and thus would increase the probability of transfer reactions.

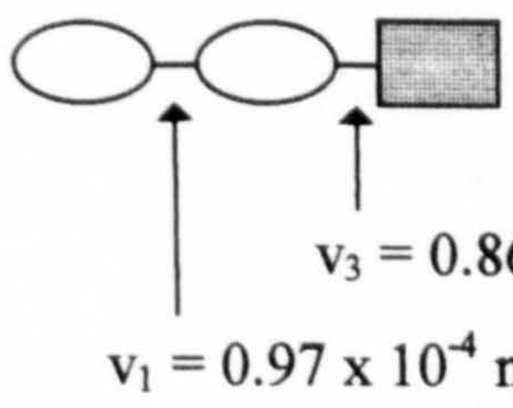
The transglycosylation reaction was performed with added co-solvent, the added co-solvent being required to increase the solubility of the disaccharide acceptor. The hydrolysis of PNPC followed first order kinetics and comparison of the rates, in a medium containing 20 % acetonitrile in the presence and absence of the disaccharide acceptor, showed marked inhibition of the substrate hydrolysis. The rate of PNPC hydrolysis, k_{obs}, was found to be 4.33×10^{-4} s^{-1} with acceptor and 9.13×10^{-4} s^{-1} without acceptor.

Without acceptor, 20 % ACN :



Ratio ~2:1

With acceptor (4.0 mM), 20 % ACN :

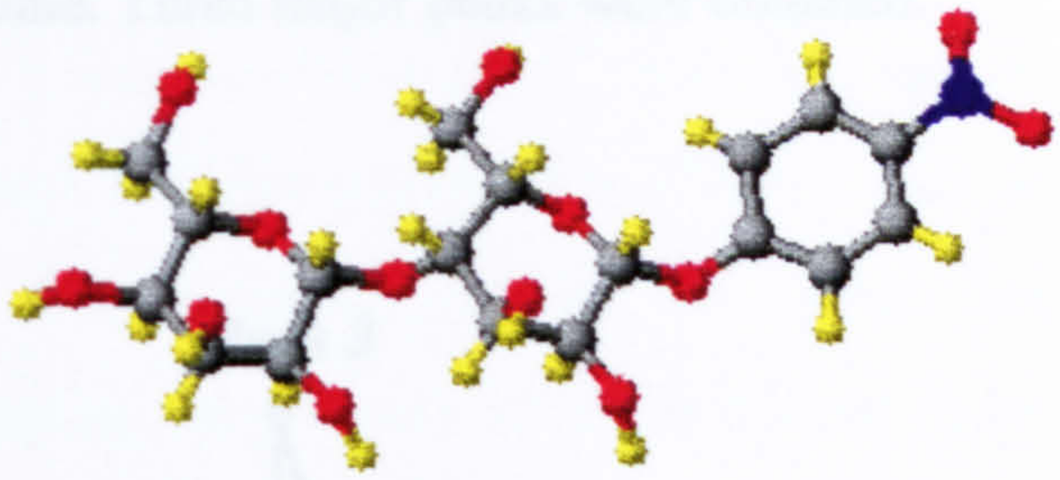


Ratio ~1:1

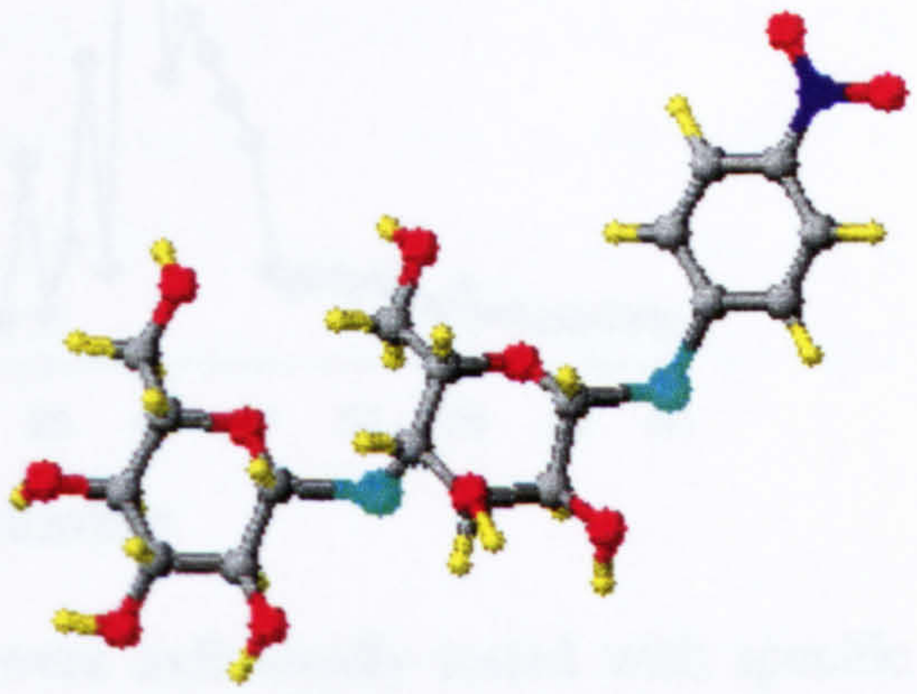
Both rates were affected (holosidic and heterosidic bond cleavages) and no transfer products were formed during the reaction.

It was then suggested that the structure of the dithio-disaccharide acceptor was too closely related to that of the substrate (PNPC). This will therefore lead to a competition from both molecules for the enzyme binding sites.

PNPC



p-nitrophenyl 1,4-dithio-β-D-cellobioside

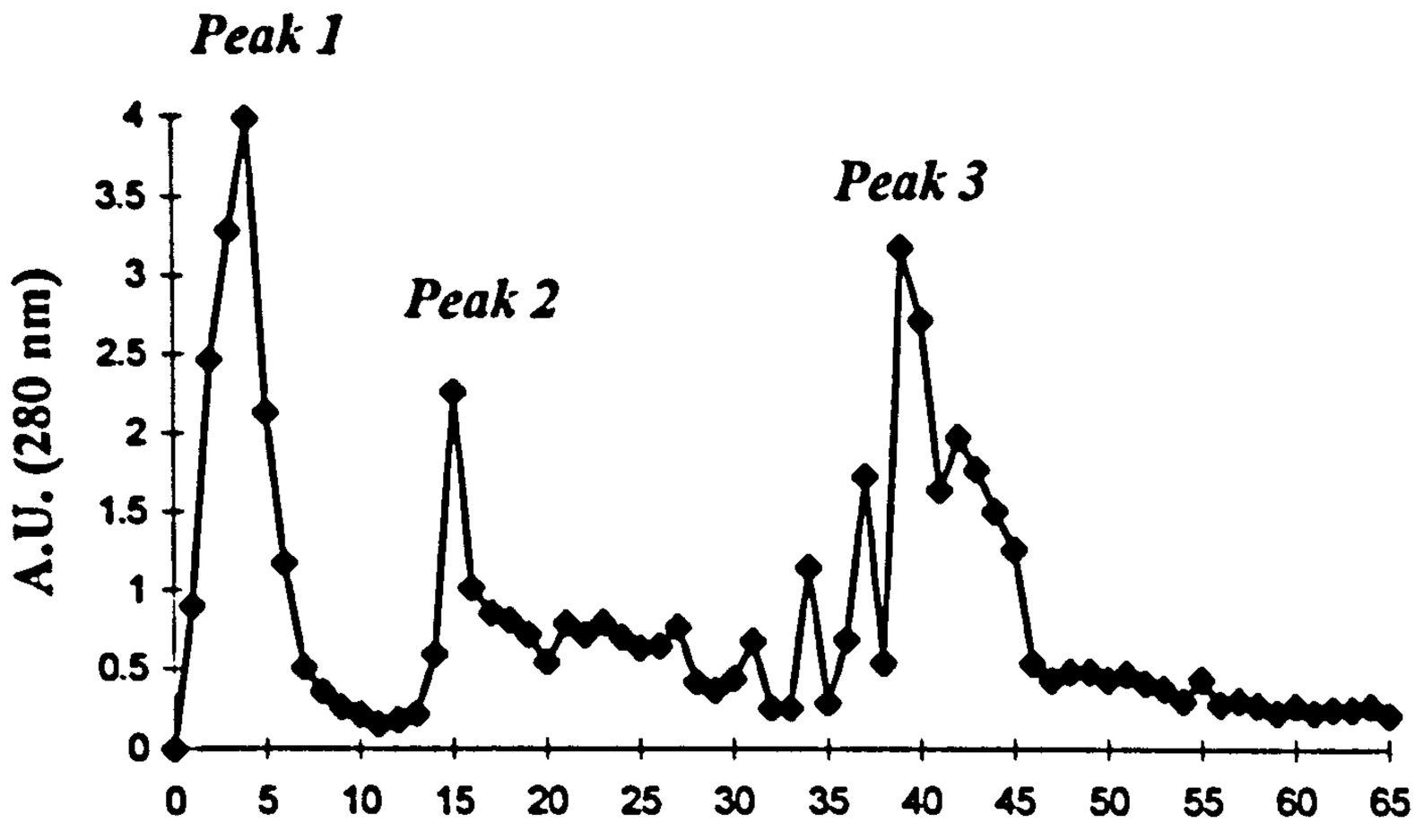


The low solubility of the acceptor precluded any realistic hope of undertaking inhibition studies. Low quantities of trisaccharide transfer products were detected by HPLC but were not quantified.

3. Enzyme Purification

3.1. Anion exchange chromatography and analysis

The crude cellulase from *Trichoderma reesei* was partially purified on a DEAE-Sepharose anion exchange chromatography column according to a method published in the literature[120]. The collected tubes were analysed by UV (280 nm) to obtained a protein concentration profile. Three major peaks were obtained.



The tubes having the highest absorbances were individually tested with specific enzyme substrates : carboxylmethylcellulose (CMC) for endoglucanase activity and Avicel (highly crystalline cellulose) for exoglucanase activity.

3.2. Peak 1 analysis

The polysaccharide test showed a high activity towards CMC (endo-acting enzyme) but no activity towards avicel (exo-acting enzymes) which suggests that an endo-acting enzyme is one of the components present in peak 1. The sample was then analysed by SDS-PAGE and capillary electrophoresis which showed the presence of more than one component present in this fraction.

The third test performed with peak 1 was to determine if there was any β-glucosidase activity using p-nitrophenyl β-D-glucopyranoside (PNPG) and p-nitrophenyl β-D-cellobioside (PNPC). The results showed that the fraction had activity towards both substrates suggesting the presence of β-glucosidase in the reaction system. This was established by addition of 1,5-glucono-δ-lactone which resulted in a complete loss of catalytic activity in both systems. The absence of PNPC hydrolysis indicates that the endo-acting enzyme present in the system is EG II, known to only hydrolyse cellotrioside and higher oligosaccharides[121]. It was thus concluded that peak 1 contained β-glucosidase and EG II activities. The β-glucosidase activity is pictured in the following figure :

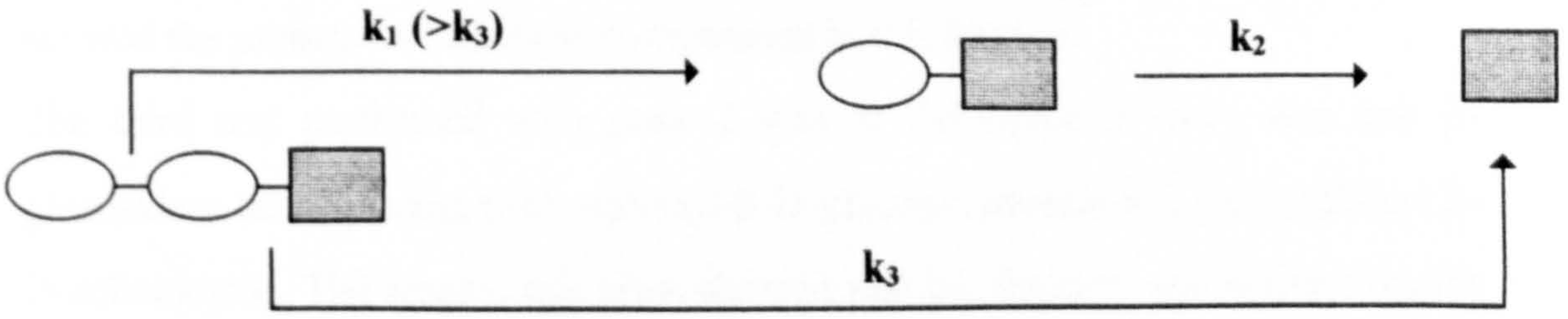


Figure 79 : β-glucosidase activity from *Trichoderma reesei*

The hydrolysis of the substrate PNPC led to the observation of a lag phase plot where at the beginning of the reaction a small amount of p-nitrophenol is produced but as the reaction proceeds the rate of release becomes a lot greater. These results suggest that the hydrolysis of PNPC occurs preferentially at the

holosidic linkage ($k_1 > k_3$) producing PNPG which itself is a substrate for the enzyme.

The reaction profile for the transglycosylation reaction using N-(p-nitrophenyl)-β-D-glucopyranosylamine as an acceptor, shows the production of three compounds identical to those produced with the crude enzyme. The addition of 1,5-glucono-δ-lactone to the reaction system resulted in the absence of transglycosylation product. These results showed that β-glucosidase is an enzyme showing transferase activity and catalysing the formation of all three transfer products formed with the crude cellulase. It is also possible to conclude that the residual transfer activity observed in the transglycosylation studies is not due to the EG II enzyme.

3.3. Peak 2 analysis

The same tests were repeated for peak 2. The polysaccharide tests showed a high activity towards CMC (endo-acting enzyme) but no activity towards avicel (exo-acting enzymes) which suggests that an endo-acting enzyme is one of the component present in peak 2. The SDS-PAGE and capillary electrophoresis showed the presence of multiple components in this fraction.

The third test performed with peak 2 was to determine if there was any β-glucosidase activity using p-nitrophenyl β-D-glucopyranoside and p-nitrophenyl β-D-cellobioside. The results, this time, showed that the fraction was active towards the disaccharide but not towards the monosaccharide substrate suggesting that β-glucosidase was not present in the reaction system and hence in this fraction. The hydrolysis scheme drawn from these results is pictured in Figure 80 :

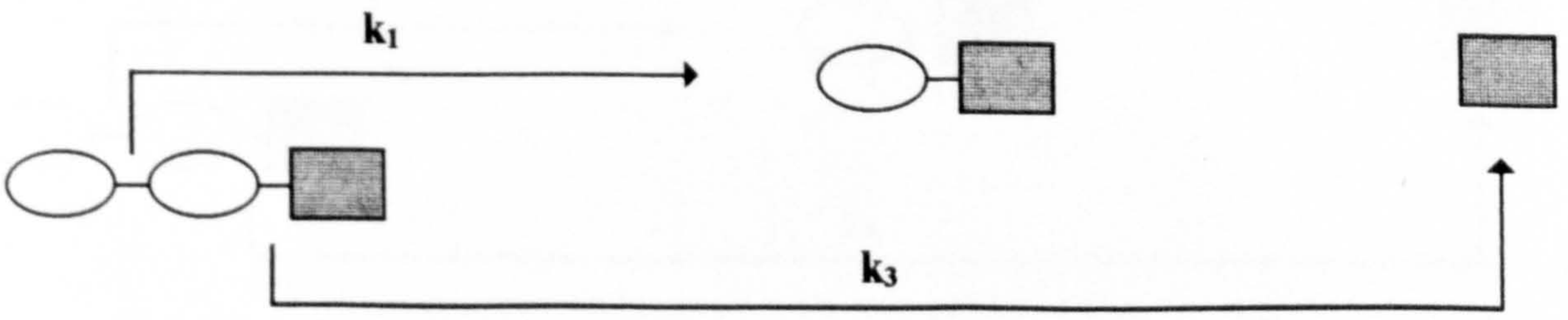


Figure 80 : Hydrolysis scheme for peak 2 from *Trichoderma reesei*

Further information was provided when the acceptor was added to the reaction system. Indeed, the transglycosylation reaction led to the formation of a single compound, the β-(1-4) disaccharide. The rate of hydrolysis was not reduced by the addition of the acceptor. This result suggests that this fraction is capable of transferase activity. The addition of cellobiose, a potent inhibitor of cellobiohydrolases, did not reduce the rate of hydrolysis which suggested the absence or low concentration of an exoglucanase in the system. Indeed, the results did not showed any inhibition in the rate of PNPC hydrolysis ($k_{obs} = 0.45 \times 10^{-4}$ s^{-1}) and the β-(1-4) transfer product was still formed. From these results, it is difficult to conclude which enzyme is taking part in the strict regio- and stereo- selective transglycosylation reaction. Further purification of the fraction is required to identify the 'transferase' enzyme.

3.4. Peak 3 analysis

The polysaccharide tests showed a high activity towards Avicel (exo-acting enzyme) but no activity towards CMC (endo-acting enzyme) which suggested that an exo-acting enzyme is one of the component present in peak 3. The sample was then analysed by SDS-PAGE and capillary electrophoresis which showed the presence of one enzyme component.

The third test performed was to determine if there was any β-glucosidase activity in the sample using p-nitrophenyl β-D-glucopyranoside and p-nitrophenyl β-D-cellobioside as substrates. The results showed activity towards the disaccharide but no activity towards the monosaccharide derivative suggesting the absence of β-glucosidase in the reaction system. The hydrolysis scheme drawn from these results is pictured in Figure 81:

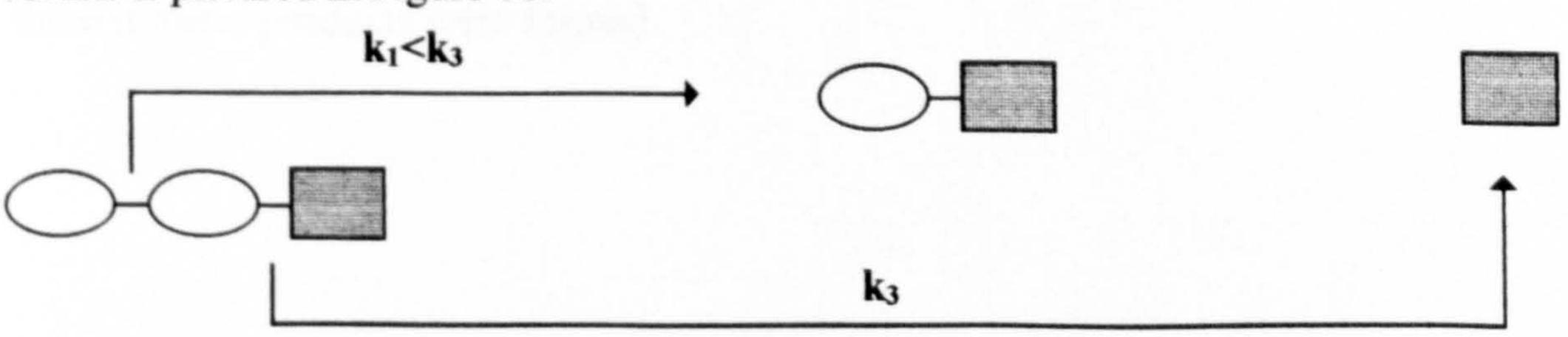
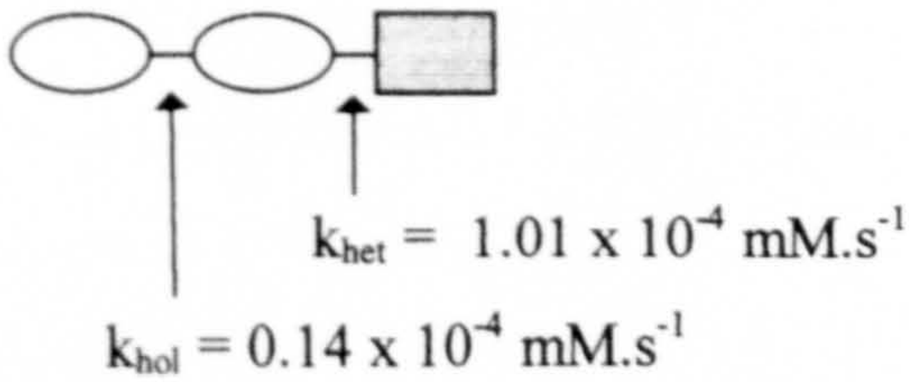


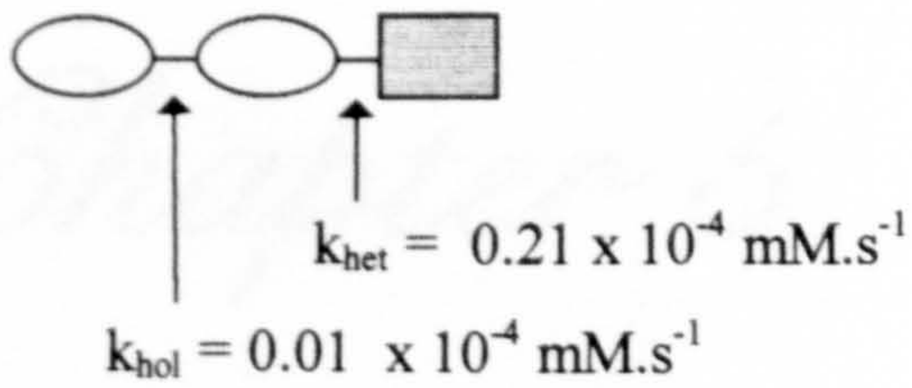
Figure 81 : Hydrolysis scheme for peak 3 from *Trichoderma reesei*

Further information was provided when cellobiose was added to the reaction system. Indeed, the addition of the cellobiose (inhibitor of CBHs) led to a dramatic decrease in the rate of PNPC hydrolysis.

Transglycosylation <u>without</u> cellobiose :



Transglycosylation <u>with</u> cellobiose :



The addition of the glucopyranosylamine acceptor to the system led to the formation of two compounds, the β-(1-4) disaccharide, being the major component, and the unknown. Traces of two trisaccharides were observed after longer reaction times reaching a maximum concentration at 90 minutes and then being slowly hydrolysed. The rate of hydrolysis was not reduced by the addition of the acceptor.

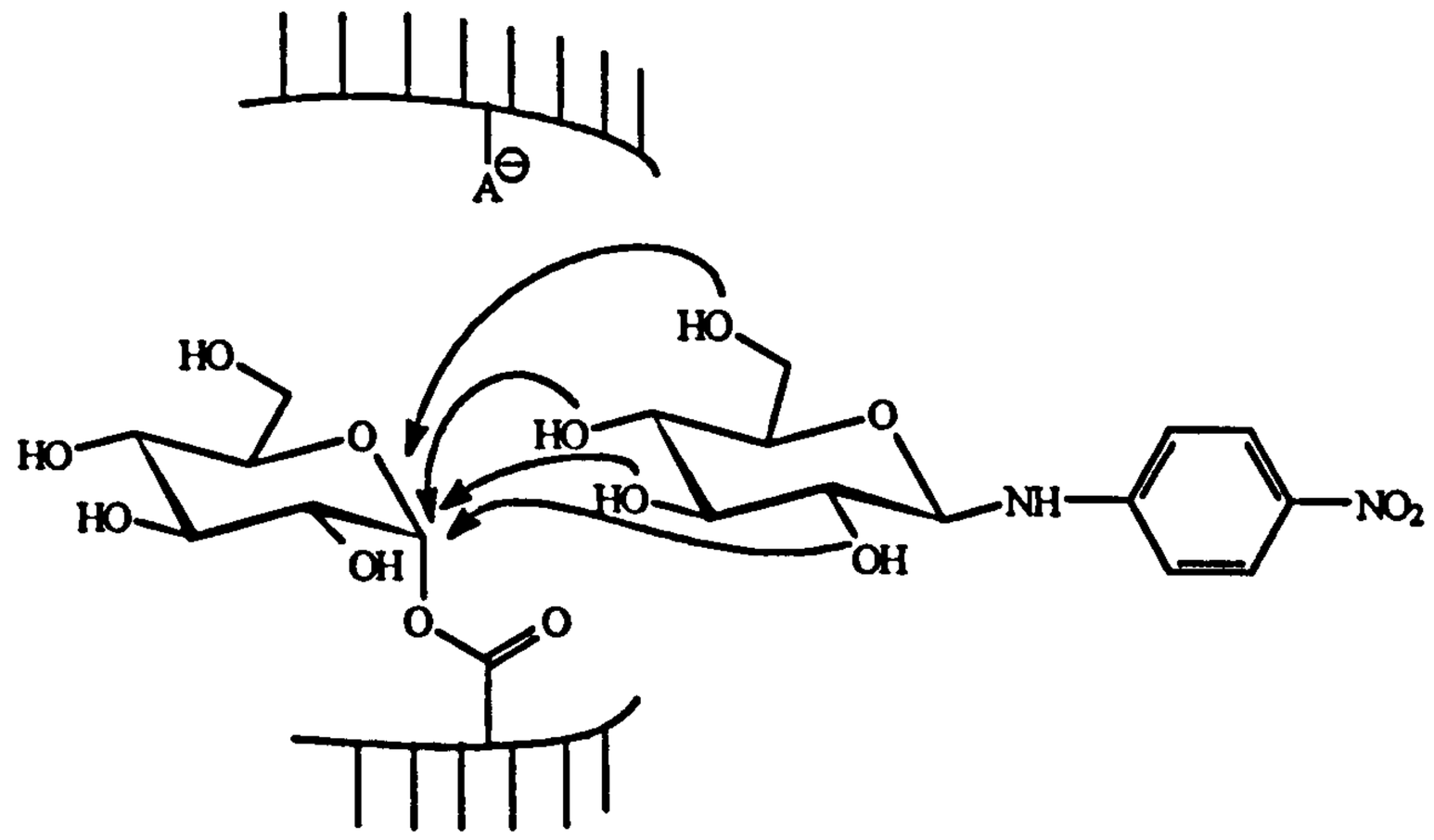
These results suggest the presence of CBH I, an exo-acting enzyme showing transferase activity. The transfer products formed suggests a greater degree of orientation of the acceptor inside the enzyme active site as only two out of the three transfer products were formed

Chapter 5

Conclusion

1. Conclusion

The use of the multi-enzyme complex, cellulase from *Trichoderma reesei*, as a glycosyl transferase was investigated. The transglycosylation reactions were found to be stereoselective (β-link only) and showed low regioselectivity. Three major transglycosylation products were formed using p-nitrophenyl β-D-cellobioside as an activated substrate donor and N-(p-nitrophenyl)-β-D-glucopyranosylamine or p-nitrophenyl 1-thio-β-D-glucopyranoside as a glucoside acceptor. Two of the transfer products were identified, by direct comparison with chemically synthesised analogues, as being the corresponding β-(1-4) and β-(1-6) linked disaccharides. The remaining product is probably the corresponding β-(1-2) or β-(1-3) linked disaccharide. Disaccharide derivatives were thus produced resulting from the nucleophilic attack of the hydroxyl of the acceptor onto a glucosyl bound intermediate.



The regioselectivity of the reaction varied with the β-(1-4) transfer product being the major component at the beginning of the reaction whereas the β-(1-6) linked disaccharide was the major one at the end. Indeed, the transfer products are themselves substrates for the enzyme components and the yield of each product will depend on their relative rate of formation and rate of hydrolysis. Monitoring the reaction system could thus allow a limited control of the regioselectivity.

The variations of reaction conditions, medium and substrate/acceptor, were studied. The increase in the acceptor concentration was found to be the only variable to increase the transglycosylation yield (the transglycosylation yield was increased from 6% to 19% with the glucopyranosylamine acceptor). Indeed, an increase in the substrate concentration was reported to successively saturate the enzyme components of the cellulase complex (β-glucosidase at first followed by CBH I, CBH II and EG I being the last) and to favour the hydrolysis pathway.

The structure of the acceptor was varied and p-nitrophenyl 1-thio-β-D-glucopyranoside was used as a monosaccharide acceptor. The transglycosylation studies showed an improvement in the affinity of the enzymes for the acceptor resulting in a higher transglycosylation yield (~40%). It was suggested that the structure of the acceptor was responsible for the increase in the affinity of the enzyme for the acceptor.

p-Nitrophenyl 1,4-dithio-β-D-cellobioside was also tested as an acceptor. Traces of trisaccharides were observed but the reaction did not lead to an increase in the yield but instead led to a significant decrease. This was explained by a possible stronger interaction of the disaccharide acceptor (low K_i) with the substrate binding sites of the enzymes, reducing the rate of hydrolysis of PNPC.

Among the three classes of enzymes comprising the complex, it was clearly established (using the specific inhibitor 1,5-glucono-δ-lactone, studies of the pH dependence and using partially purified enzyme) that the major transferase activity was catalysed by the β-glucosidase component. The latter was demonstrated to be exclusively responsible for the formation of the β-(1-6) transfer product. The multiple products observed in the β-glucosidase catalysed transfer reaction has been attributed to a greater degree of freedom of the acceptor in this enzyme than for the other components. The loose binding of the acceptor inside the acceptor binding pocket of the active site of β-glucosidase thus allows the formation of the three transfer products. The transfer reaction was also shown to be catalysed by another enzyme in the cellulase complex and is suggested to be the exoglucanase,

CBH I. The addition of 1,5-glucono-δ-lactone to the reaction system led to the formation of one major compound, the β-(1-4) linked disaccharide. This suggests a greater degree of orientation in the enzyme active site of CBH I. It is proposed that hydrophobic interactions between the phenyl ring of the acceptor and a hydrophobic pocket inside the β-glucosidase active site would allow the greater freedom observed.

2. Future work

2.1. The Role of the Substrate

During this project, p-nitrophenyl β-D-cellobioside was used as an activated substrate donor. The formation of disaccharide transfer products suggested that a glucosyl-bound intermediate was responsible for their production. It would be interesting to investigate the formation of cellobiosyl substituted enzymes and to investigate the production of trisaccharides. Formation of a cellobiosyl-enzyme intermediate could be investigated using cellobiosyl fluoride as a substrate.

2.2. The role of the Acceptor

N-(p-Nitrophenyl)-β-D-glucopyranosylamine was found to be a weak acceptor but the use of p-nitrophenyl 1-thio-β-D-glucopyranoside increased significantly the affinity of the enzyme for the acceptor. The role of the acceptor structure was found to play an important role in the transglycosylation reactions and future work would thus involve a study of the structure of the aglycon moiety. Variation of the acceptor hydrophilicity could be achieved by adding a polar function to the phenyl ring for example.

p-Nitrophenyl 1-thio-β-D-cellobioside was chemically synthesised during the project and represents also a potential acceptor. A similar inhibition study to that performed with N-(p-nitrophenyl)-β-D-glucopyranosylamine would determine if the acceptor could selectively inhibit one of the enzyme components.

2.3. Role of the Enzyme

Future work would be based on the role of the different enzyme components present in the cellulase complex.

Isolated enzyme components would allow a precise study of each enzyme role in the transglycosylation reactions. Purified enzyme components could be obtained from ion chromatography but the advantage of the recently developed recombinant technique would allow the recovery of a single pure enzyme.

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