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DISSERTATION

MODIFIED DISACCHARIDES FOR NOVEL EXCIPIENTS SYNTHESIS

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I. SUMMARY

Aim of the work is the synthesis of azido derivatives of D-glucose and cellobiose by combined enzyme-catalyzed and organic-chemical methods. The target compounds envisioned are of interest as monomers for enzyme-catalyzed polymerization to afford azido-cellulose derivatives and as intermediates for perlon-type polymerization of carbohydrate-derived amino acids to give polyamide hydrogels. Furthermore, novel derivatives of glucosamine can be produced from the azido carbohydrates and are considered candidate chondroprotective agents.

Syntheses were performed using the phosphorolytic modes of the enzyme cellobiose phosphorylase (EC 2.4.1.20) from *Clostridium thermocellum*. Organic-chemical syntheses of 6-azido-6-deoxy-D-glucose, 6-azido-6-deoxy-cellobiose, 6'-azido-6'-deoxy-cellobiose and 2-azido-2-deoxy-cellobiose have been described in other dissertations or are known in the literature [Saber, 2009; Gabra 2009]. Cellobiose phosphorylase was initially prepared from fermented biomass of *Clostridium thermocellum*. A crude enzyme preparation from this organism was used for most substrate specificity studies. Purification of cellobiose phosphorylase from the crude extract was achieved via ultrafiltration, DEAE-cellulose and Sephacryl chromatographies. While enrichment of the appropriate band at 93 kDa could be demonstrated, the native enzyme is relatively unstable and contains an accompanying activity of cellodextrin phosphorylase. Therefore, a clone expressing cellobiose phosphorylase was developed in *Escherichia coli* using the appropriate cDNA from *Clostridium thermocellum*. For this purpose, the plain vector pET-26(+) was employed. Expression of cellobiose phosphorylase including a 6xHis tag is effected under promotion by the T7 promoter. Using native or cloned enzyme, the following conversions have been demonstrated: enzyme-catalyzed phosphorolytic cleavage of 6-azido-6-deoxy-cellobiose to afford α -D-glucose 1-phosphate and 6-azido-6-deoxy-D-glucose; and enzyme-catalyzed synthesis of 6-azido-6-deoxy-cellobiose from α -D-glucose 1-phosphate and 6-azido-6-deoxy-D-glucose. Phosphorolytic cleavage of 2-azido-2-deoxy-cellobiose has resulted in 2-azido-2-deoxy-D-glucose and α -D-glucose 1-phosphate. This protocol permits the production of 2-azido-2-deoxy-D-glucose by a combined organic-chemical and enzymatic reaction sequence without the need for chromatographic separation of the corresponding *manno*-isomer. Failure attended attempts to phosphorolytically cleave 6'-azido-6'-deoxy-cellobiose. In the case of 6'-azido-6'-deoxy-cellobiose, it is assumed that the reaction proceeds to a small extent, whereupon

the small amount of D-glucose formed would suffice to inhibit binding of the weak substrate 6'-azido-6'-deoxy-cellobiose and to block further cleavage. This interpretation is supported by the finding of a small amount of cellobiose, presumably formed from 2 molecules of D-glucose bound in both the α -D-glucose 1-phosphate and D-glucose binding sites.

Future activities suggested by the results of the present dissertation include enzyme immobilization to stabilize cellobiose phosphorylase and application of mutarotase to shift the anomeric configuration of D-glucose toward the β -anomer. Such studies are expected to enable the scale up of enzyme-catalyzed conversions, to facilitate the synthesis of 2-azido-2-deoxy-cellobiose from α -D-glucose 1-phosphate and 2-azido-2-deoxy-D-glucose and to enable the oxidative removal of D-glucose during phosphorolytic cleavage of 6'-azido-6'-deoxy-cellobiose. If successfully implemented, this latter conversion could produce 6-azido-6-deoxy- α -D-glucose 1-phosphate, a highly interesting intermediate for the synthesis of numerous carbohydrate mimetics.

II. ZUSAMMENFASSUNG

Ziel dieser Arbeit ist die Synthese von Azidoderivaten der D-Glukose bzw. Cellobiose mittels enzymkatalysierter und organisch-chemischer Methoden. Die erwarteten Syntheseprodukte sind von Interesse, da sie sowohl als Monomere für die enzymkatalysierte Polymerisation von Azidocellulose Derivaten wie auch als Zwischenprodukte für perlonartige Polymerisation von aus Kohlenhydraten hergeleiteten Aminosäuren, zur Herstellung von Polyamid-Hydrogelen, eingesetzt werden können. Darüber hinaus, können neue Glukosaminderivate aus Azido-Kohlenhydraten hergestellt werden, die als Kandidaten für chondroprotektive Mittel von Interesse sind.

Zur Durchführung der enzymkatalysierten Synthesen wurde das Enzym Cellobiose Phosphorylase aus dem Bakterium *Clostridium thermocellum* verwendet, welches allgemein phosphorolytische und synthetische Reaktionen mit den Substraten Cellobiose bzw. D-Glukose katalysiert.

Organisch-chemische Synthesen von 6-Azido-6-desoxy-D-glukose, 6-Azido-6-desoxy-cellobiose, 6'-Azido-6'-desoxy-cellobiose und 2-Azido-2-desoxy-cellobiose wurden in früheren Dissertationen beschrieben bzw. sind auch in Literatur bekannt [Saber, 2009; Gabra 2009]. Das Enzym Cellobiose Phosphorylase wurde ursprünglich aus fermentierter Biomasse des Bakteriums *Clostridium thermocellum* isoliert. Für die meisten substratspezifischen Untersuchungen wurde der Rohextrakt aus diesem Organismus verwendet. Die Aufreinigung von Cellobiose Phosphorylase aus dem Rohextrakt erfolgte über Ultrafiltration wie auch mit Hilfe von chromatographischen Methoden, DEAE-Cellulose und Sephacryl. Im Zuge der Anreicherung dieses Enzyms konnte eine entsprechende Bande bei 93 kDa gezeigt werden, wobei das Enzym relativ instabil ist und eine Zusatzaktivität des Enzyms Cellodextrin Phosphorylase aufweist. Daher wurden Klone von Cellobiose Phosphorylase in *E.coli* Zellen exprimiert, wobei die dazu entsprechende cDNA aus *Clostridium thermocellum* isoliert wurde. Zu diesem Zweck, wurde der Expressionsvektor pET-26(+) verwendet. Die Überexpression von Cellobiose Phosphorylase, welche einen Hexahistidinrest (6xHis tag) aufweist, wurde unter Einfluss von aktiviertem T7 Promotor induziert. Sowohl mit nativem als auch mit rekombinantem Enzym wurden folgende Reaktionen durchgeführt: enzymkatalysierte phosphorolytische Spaltung von 6-Azido-6-desoxy-cellobiose in α -D-Glukose-1-phosphat und 6-Azido-6-desoxy-D-glukose sowie enzymkatalysierte Synthese von 6-Azido-6-desoxy-cellobiose

aus α -D-Glukose-1-phosphat und 6-Azido-6-desoxy-D-glukose. Phosphorolytische Spaltung von 2-Azido-2-desoxy-cellobiose führt zur Synthese von 2-Azido-2-desoxy-D-glukose und α -D-Glukose-1-phosphat. Diese Methode ermöglicht die Herstellung von 2-Azido-2-desoxy-D-glukose im Zuge einer kombinierten organisch-chemischen und enzymatischen Reaktionsabfolge, ohne Einsatz von chromatographischen Verfahren zur Isolierung bzw. Abtrennung der entsprechenden *manno*-Isomeren. Bisher, ist es uns nicht gelungen die phosphorolytische Spaltung von 6'-Azido-6'-desoxy-cellobiose erfolgreich durchzuführen.

Im Falle von 6'-Azido-6'-desoxy-cellobiose wird vermutet, dass deren Spaltung zu einem geringen Anteil stattfindet, wobei eine geringe Menge an entstehender D-Glukose ausreichen würde, die Bindung des ohnehin schwach bindenden Substrats 6'-Azido-6'-desoxy-cellobiose zu hemmen; somit würde eine weitere Spaltung unterbleiben. Diese Hypothese wird durch den Fund geringerer Menge an Cellobiose gestützt, die vermutlich aus 2 Glukosemolekülen in den α -D-Glukose-1-phosphat- und D-Glukose-Bindungsstellen gebildet wurde.

Aufgrund der vorliegenden Resultate sind bestimmte zukünftige Aktivitäten naheliegend, wie z. B. die Enzymimmobilisierung zur Stabilisierung von Cellobiose Phosphorylase und Anwendung von Mutarotase um die Anomerenkonfiguration von D-Glukose in Richtung auf das β -Anomer zu verlagern. Im Rahmen solcher Untersuchungen wird erwartet, den entsprechenden Scale-up enzymkatalysierter Synthesen durchführen zu können, die Synthese von 2-Azido-2-desoxy-cellobiose aus α -D-Glukose-1-phosphat und 2-Azido-2-desoxy-D-glukose zu ermöglichen, sowie den oxidativen Abbau von D-Glukose während der phosphorolytischen Spaltung von 6'-Azido-6'-desoxy-cellobiose zu erleichtern. Wenn erfolgreich umgesetzt, könnte die durchgeführte Spaltung von 6'-Azido-6'-desoxy-cellobiose zur Bildung von 6-Azido-6-desoxy- α -D-glukose-1-phosphat führen, welches ein höchst interessantes Zwischenprodukt für die Synthese von zahlreichen kohlenhydratmimetischen Produkten darstellt.

III. INTRODUCTION

The long-range goal of studies on azido cellobiose derivatives is the synthesis of artificial aminopolysaccharides for use as novel excipients. Such studies are motivated by the many interesting features of chitosan as a model aminopolysaccharide excipient [Schwarz, *et al.*, 2003]. It is assumed that modified structures may be similarly useful, such as those containing amino functionality in positions other than C-2 of glucose, or those containing amino substitution in alternating glucose units. In the present dissertation, routes are being developed toward azido cellobiose analogs which might serve as monomers in enzyme-catalyzed polymerizations.

1. Principles of pharmaceutical excipients

Excipients perform important functions of guaranteeing the dosage, bioavailability and stability of the active principle in a pharmaceutical preparation. They ensure that a medicinal product has the weight, constituency and volume which are necessary for the correct administration of the active principle to the patient [Pifferi and Restani, 2003].

In 1957, excipients were defined as “the substance used as a medium for giving a medicament”, referring to the functions of an inert support of the active principle [Faber and Faber, 1957]. Again, in 1974 they have been described as “any more or less inert substance added to a prescription in order to confer a suitable consistency or form to the drug: a vehicle” [Saunders, 1974].

Nowadays, an excipient plays multiple roles in a modern pharmaceutical dosage form, suitable to be administered enterally, parenterally or topically. Modern pharmaceutical technology enables the stability, precision and accuracy of the dose, thus improving patient compliance; but it also requires verification of the physical state of the excipient, with the object of improving the bioavailability and consequently the efficacy and tolerability of the medicinal drug [Pifferi, 1996].

1.1 Origins and sources of excipients

Excipients, which may account for most of the weight of a medicinal product, can be of various origins such as animal (e. g. lactose, gelatin, stearic acid), plant/bacteria (e. g. starches, sugars, cellulose, alginates), mineral (e. g. calcium phosphate, silica) and synthesis (e. g. PEGs,

polysorbates). Concerning this matter, they belong to essential functional components of a modern pharmaceutical formulation, having also their own thermodynamic activity which can contribute to reactions leading to degradation or to interactions between the drug and the excipient (**Table 1**) [Crowley and Martini, 2001]. Different materials are used in the pharmaceutical industry to fulfill various functions such as diluents, disintegrants, colouring agents, sweeteners, etc. They belong to a chemically heterogeneous group of compounds “that go from simple molecules to complex mixtures of natural, semisynthetic or synthetic substances” [Pifferi and Restani, 2003].

Table 1

Chemical classification and roles of pharmaceutical excipients [Crowley and Martini, 2001]

<u>Chemical classification</u>	<u>Roles to enhance</u>
Water, alcohols	Compliance
Esters, ethers, carboxylic acids	Dose precision and accuracy
Glycerides and waxes	Stability
Carbohydrates (mono-, di- and polysaccharides)	Manufacturability
Hydrocarbons and halogen derivatives	Tolerability
Polymers (natural and synthetic)	Desaggregation
Minerals	Dissolution
Protein	Controlled release
Various: preservatives, dyes, sweeteners	Absorption

1.2 Physical-chemical requirements of excipient safety

Fundamental requirements of active principles as well as of excipients are quality and safety. The requirement of a drug’s therapeutic efficiency is substituted by that of functionality for excipients, defined as “the physical, physicochemical and biopharmaceutical properties” of the same [Pifferi and Restani, 2003].

Nevertheless, when dealing with pharmaceutical drugs, the most important requirement is safety, which comprises three important issues [Pifferi and Restani, 2003]:

Production, distribution and use

Toxicity

Pharmaceutical-excipient interactions

Principally, excipients should be subjected to the same toxicity studies as those requested for active principles. Adverse effects of any excipients can be generated by administration of them in a sufficiently high dose. Additionally, they also have to be free of immunotoxicity and allergenicity. Generally, such adverse effects are infrequent and mild, due to the fact that most excipients have been empirically selected because of their low toxicity. [Pifferi and Restani, 2003]

1.3 Excipients as stabilizers

Factors, that can cause the molecular transformation of drug substances, are used in order to determine the potential of excipients to prevent or retard the degradation, thus act as stabilizers. These include stresses during conversion to the dosage form (size reduction, compaction or sterilizing processes), environmental components (water vapour or sunlight) and interactions between adjacent molecules of a drug. [Crowley, 1999]

For the formulation of medicinal products, the capability to stabilize labile materials is required. In this context, stabilization using appropriate excipients for materials which were derived from biotechnological sources can be used in order to convert these materials into viable products. Categorization of excipients acting as stabilizers includes several examples which show the capability of enhancing product stability. These are [Crowley, 1999]:

- ➔ Use of suspending agents for the prevention of sedimentation
- ➔ Addition of a preservative for the prevention of microbial spoilage

1.4. Special interest of the aminopolysaccharide excipient, chitosan

Chitosan is a polysaccharide excipient that has engendered significant interest for a variety of experimental applications in recent years. Below, a description is provided of some aspects of chitosan as contained in the recent literature [Kumar *et al.*, 2004].

2. Pharmaceutical-technological uses of chitosan

Chitosan $[(1\rightarrow4)\text{-}2\text{-amino-}2\text{-deoxy-}\beta\text{-D-glucose}]_n$, an aminopolysaccharide [Kempe *et al.*, 2007], has been obtained from chitin by deacetylation [Madhumathi *et al.*, 2009]. Chitin is one of the most abundant natural polymers in the world and structurally resembles cellulose (**Figure 1**).

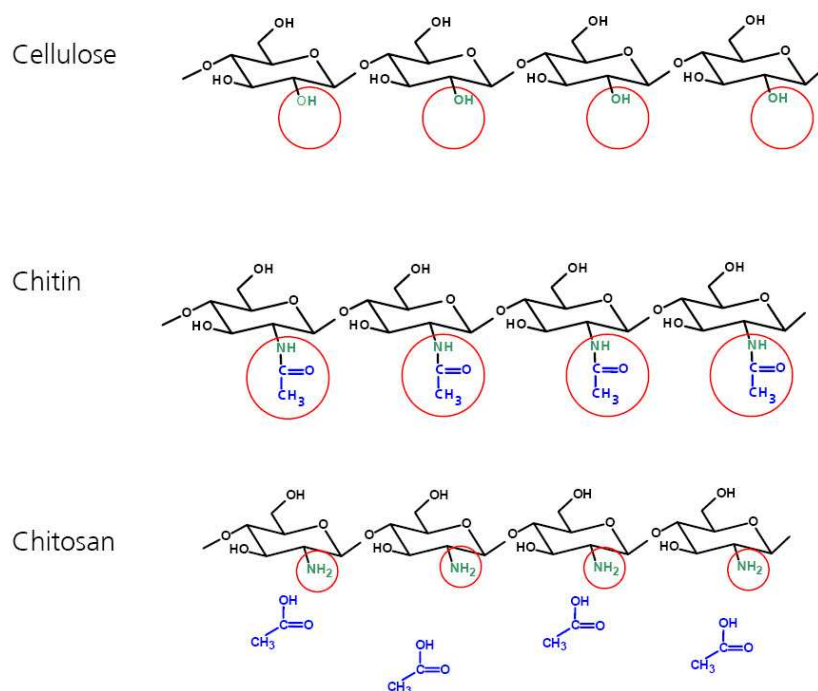


Figure 1 Conversion of cellulose into chitosan

(Source: <http://www.entec-emden.de/bilder/CelluloseChitinChitosan.jpg>)

Chitin-deacetylase catalyzes the conversion of chitin into chitosan (**Figure 2**). Considering that not all the $N\text{-acetyl}$ groups are removed during deacetylation, chitosan has also been defined as a copolymer of glucosamine and $N\text{-acetylglucosamine}$ connected by a $\beta(1\text{-}4)$ linkage.

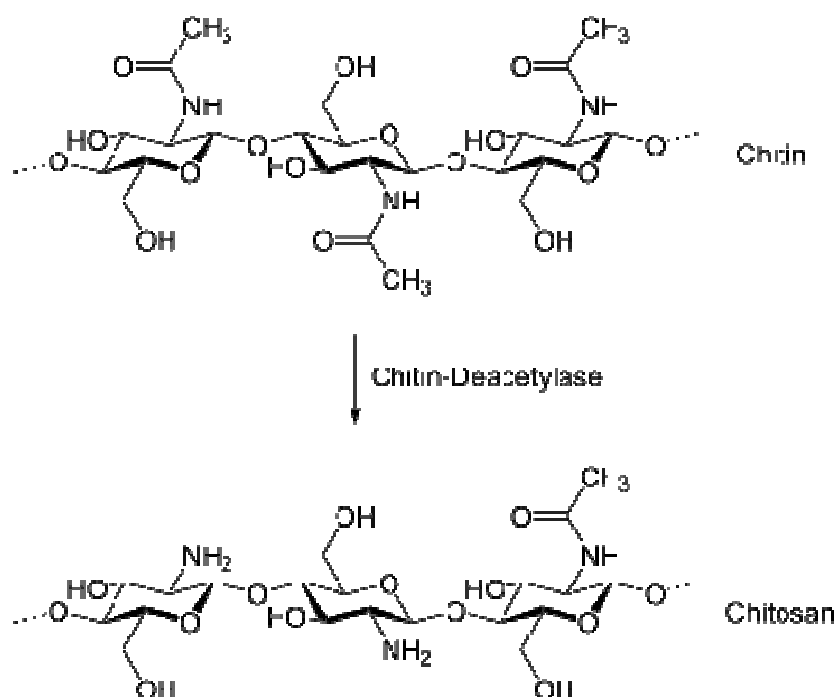


Figure 2 Role of chitin deacetylase

(Source:http://upload.wikimedia.org/wikipedia/commons/thumb/b/bb/Chitosan_Synthese.svg/400px-Chitosan_Synthese.svg.png)

It is so far the most frequently applied cationic biomimetic polymer [Madhumathi *et al.*, 2009], with experimental applications ranging from biomedical, food, drug and cosmetics. This natural biopolymer, derived from shells of crustaceans, arthropods and some fungi [Madhumathi *et al.*, 2009], has many favorable characteristics. It is nontoxic, biocompatible, and hydrophilic. Chitosan also has antibacterial properties and a remarkable affinity for many substances, thus making it interesting for use as an excipient for different research purposes, such as the formulation of controlled drug delivery systems.

2.1 General industrial use of chitosan

In view of the high molecular weight of chitosan (50-200kDa), its film forming ability and gelatin characteristics, this material has been extensively used in the industry, as a chelating agent for harmful metals, for the detoxification of hazardous waste, for the clarification of beverages and as a constituent of many food products.

Further advantages such as good oxygen permeability, slow enzymatic degradation and high water absorptivity make chitosan efficient in promoting wound healing, e.g. resulting in the inhibition of fibroplasia with enhanced tissue regeneration.

Lately, this amino-polysaccharide has also been introduced as a weight loss aid and cholesterol lowering agent. In this context, it has been suggested that the positively charged chitosan can bind to free fatty acids and bile salt components and disrupt lipid absorption. [He, 1998]

2.2 The use of chitosan in the pharmaceutical industry

Due to its potential in the development of controlled release drug delivery systems, chitosan has been extensively investigated in the pharmaceutical industry (**Table 2**) [He, 1998]. This is particularly due to its unique polymeric character and its gel and film forming properties. Such systems allow the control of the rate of drug administration, enabling the targeting of the drug to specific sites and prolonging the duration of therapeutic effects. In this context, it has been reported that chitosan has excellent properties as an excipient for direct tablets compression, where the addition of 50% chitosan resulted in rapid disintegration [Sawayanagi *et al.*, 1982]. When chitosan was added to tablets in a concentration higher than 5%, it was superior to corn starch and microcrystalline cellulose as a disintegrant. The efficiency was dependent on chitosan crystallinity, molecular weight, particle size and degree of deacetylation.

Chitosan and chitosan derivatives were used, in combination with other excipients, for the production of tablets with controlled release properties. The release rate of drug from these tablets was found to be directly related to the type and amount of chitosan used. Chitosan is not only able to promote the transmucosal absorption of small polar molecules, peptide and protein drugs, it is also able to enhance the immunological response to vaccines due to its absorption promoting abilities [Illum *et al.*, 1994].

Table 2

Various applications of chitosan as an excipient in pharmaceutical products [He, 1998]

<i>Conventional formulation</i>	Direct compression tablets Controlled release matrix tablets Wet granulation Gels Films Emulsions Wetting agents Coating agents
<i>Microspheres and microcapsules</i>	Use as controlled release implant systems for delivery of hormones over extended periods of time
<i>Novel applications</i>	Bioadhesion Transmucosal drug transport (for nasal and oral delivery of polar drugs to include peptides and proteins and for vaccine delivery)

Chitosan is commercially produced by treatment with alkali of crustacean shells and subsequent purification steps; it is so far the only aminopolysaccharide that is accessible in useful amounts from natural sources. Therefore, any artificial structural analogs would need to be synthesized, presumably using the capabilities of carbohydrate-related biotechnology. In the following, a number of examples are given of conversions that can be effected by methods of carbohydrate-related biotechnology.

2.3 Production and isolation of microbial polysaccharides

Sources of high molecular weight polysaccharides with thickener properties were originally plants, seeds and seaweeds. Later, the manufacture of these molecules from microorganisms became an important advance, resulting in polymers with constant properties [García-Ochoa *et al.*, 1999]. One of these biopolymers is xanthan gum, firstly commercialized in the 1960s [Kang and Pettitt, 1993]. Xanthan gum, which plays an important role in industrial gum applications, is a polysaccharide synthesized by *Xanthomonas sp.* Bacteria of *Xanthomonas* genus, which produce extracellular polysaccharides as bacterial capsules [Bradbury, 1984], have to be maintained in a viable form to be grown in a complex medium in order to build up an inoculum enabling the gum production by fermentation. Xanthan molecular structure (**Figure 3**) including

acetyl and pyruvyl constituents shows very high molecular weight of several millions of Daltons [Kennedy and Bradshaw, 1984]. Due to xanthan's properties, such as solubility in water, its high stability and thickener behavior, including the simplicity of its industrial manufacture make this polysaccharide a gum, which is frequently used for water rheological behavior modification in industries such as food, pharmaceuticals and cosmetics [García-Ochoa *et al.*, 1999].

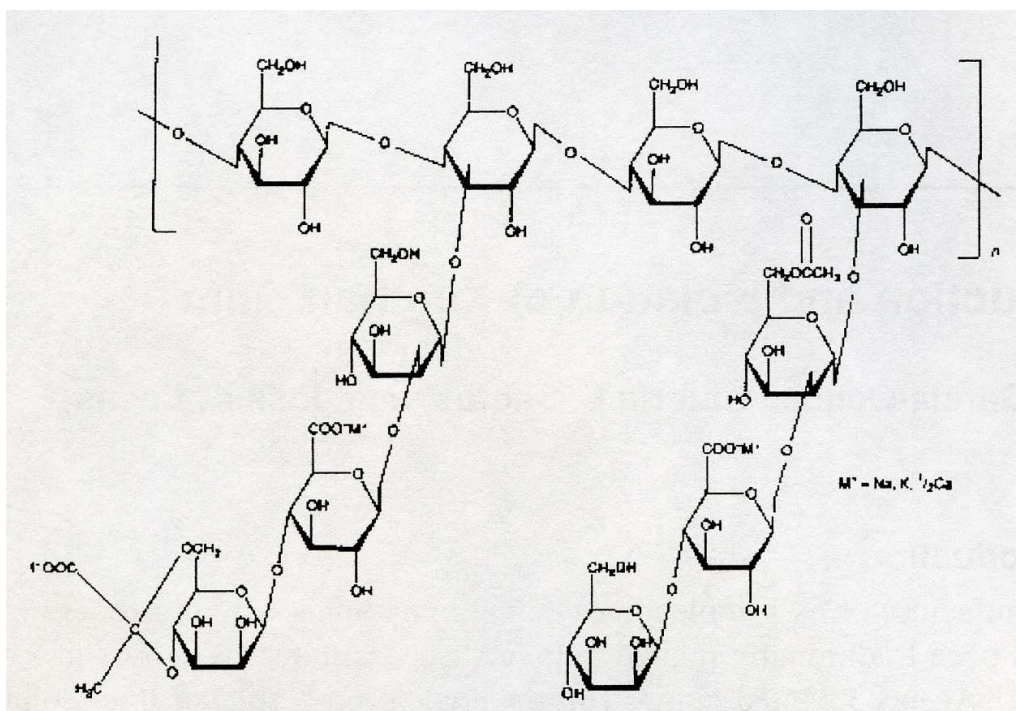


Figure 3 Xanthan molecule structure [García-Ochoa *et al.*, 1999]

Another group of polysaccharides are alginates, which occur as structural components or as capsular materials in the cell walls of the brown seaweeds and soil bacteria [Brivonese and Sutherland, 1989]. This polysaccharide represents a linear unbranched polymer containing β -(1 4)-linked D-mannuronic acid (M) and α -(1 4)-linked L-guluronic acid (G) residues (**Figure 4**).

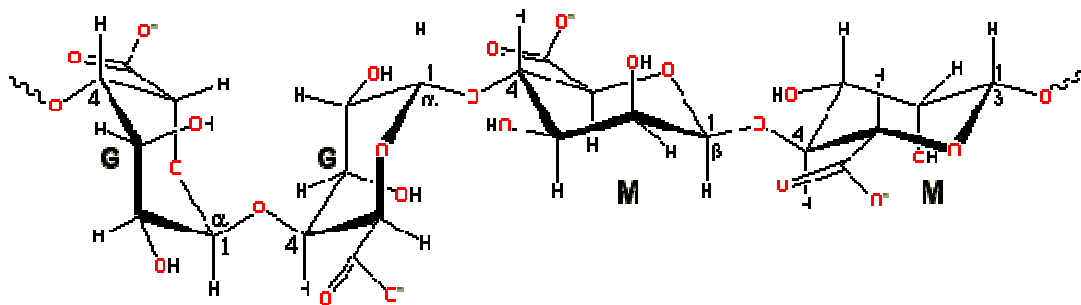


Figure 4 Alginate molecule structure

(Source: http://openwetware.org/wiki/IGEM:IMPERIAL/2009/Encapsulation/Phase2/Alginate_Properties)

Due to the fact, that only few species of brown algae are limited in abundance of commercial alginate production, there is recent interest in the bacterial production of alginate-like polymers [Kennedy *et al.*, 1992; Sutherland, 1994] which can be prepared e. g. from *Azotobacter vinelandii* [Parente *et al.*, 1998]. Regarding its applications, it is known, that ~30 000 metric tons of sodium alginates per year are currently used in the food, pharmaceutical, textile and paper industries as thickening, jellifying and stabilizing agents [Smidsrød and Draget, 1996].

Many fungi, like *Schizophyllum commune* ATCC 38548 [Kikumoto *et al.*, 1970], *Sclerotium rolfii* [Pilz *et al.*, 1991], *Sclerotium gluconicum* [Rau *et al.*, 1992] show the ability to secrete a neutral homogluconan, Schizophyllan (**Figure 5**). It consists of a backbone chain of 1,3- β -D-glucopyranose units with single 1,6-bonded β -D-glucopyranoses at about every third glucose molecule in the basic chain. The production of this polysaccharide is strongly coupled with growth and secretion under nitrogen starvation. It is a mucilage either loosely associated with the outer cell wall or released into a medium. Shear stress, which is caused by the agitator used during bioreactor cultivation as well as oxygen supply, can enhance the release of Schizophyllan gum from the cell wall [Rau, 1999]. Recent studies have shown that Schizophyllan can be used to form films almost impermeable to oxygen [Rau *et al.*, 1992] in food protection. Stimulation of the immune system by degraded glucans [Rau *et al.*, 1995] is a further application of this polysaccharide, and especially in Japan, it is used as cancer immunotherapeutic drug in combination with other chemotherapeutic compounds [Kishida *et al.*, 1992].

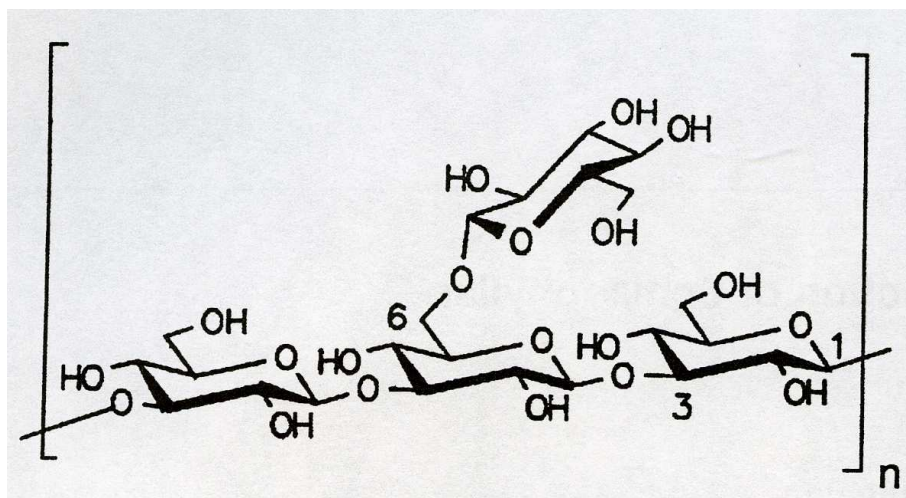


Figure 5 Primary molecule structure of Schizophyllan [Rau, 1999]

Enzyme-catalyzed conversions can take place after formation of the polysaccharides, e.g. epimerization reactions. This type of postpolymerization can be found in uronic acid containing polysaccharides from eukaryotes as well as from bacteria. So far, it has been shown that the treatment of alginate with the enzyme mannuronan-C-5 epimerase, will create guluronic acid residues in the chain and alter both the flexibility and the gel-forming and immunogenic properties of this polymer. To obtain epimerized alginate, it is necessary to express the appropriate epimerization enzymes recombinantly and purified, as these enzymes are not available commercially. [Ertesvåg and Skjåk-Bræk, 1999]

2.4 Production of oligosaccharides

Oligosaccharides currently produced for commercial market include maltodextrins, cyclomaltodextrin, fructooligosaccharides, galactooligosaccharides as well as soy oligosaccharides.

Enzymatic processes are involved in production of all agriculturally important oligosaccharides. In the case of production of certain fructooligosaccharides, microbial or plant transglycosylases may be responsible for this process. Also reversion reactions of glycosidases and their genetically engineered derivatives, glycosynthases, may be also used in oligosaccharide synthesis. [Eggleston and Côté, 2003]

One of the most important commercial enzyme processes for industries is the hydrolysis of starch to low molecular weight products via α -amylase catalysis [Marchal, 1999]. The degree of

hydrolysis is expressed as dextrose equivalent (DE), which is the percentage of reducing sugar calculated as dextrose on a dry-weight basis. In this context, starch hydrolysates with a DE above 20 are classified as glucose syrups. Maltodextrins have a DE between 3 to 20. Generally, the higher the DE value, the shorter the glucose chains and the higher the sweetness and the solubility. Hydrolyzed starches are commercially available at relatively low prices from major starch producers. These hydrolyzed products find applications in the food, paper and textile industries [Nigam and Singh, 1995].

Many oligosaccharides, such as fructo-, isomalto-, soybean- and galacto-oligosaccharides have wide use in bioindustries as so called functional sweeteners low in calories, preventing dental caries and having bifidogenic functionalities [Kohmoto *et al.*, 1992; Wada *et al.*, 1992; Kohmoto *et al.*, 1991; Tomomatsu, 1994; Yun, 1996]. Especially fructooligosaccharides (FOS) produced from sucrose have been used widely, due to their relatively easy mass production, their taste and physicochemical properties, which are similar to those of sucrose [Yun and Song, 1999]. Production yield of FOS by plant originated enzymes from asparagus, onion and Jerusalem artichoke [Allen and Bacon, 1956; Darbyshire and Henry, 1978; Satyanarayana, 1976; Chandorkar and Collins, 1974] is very low and mass production of enzyme is limited by seasonal conditions. Thus, industrial production of FOS uses several microbial fructosyltransferases (β -D-fructofuranosidases), produced by *Aspergillus* [Hirayama *et al.*, 1989; Hidaka *et al.*, 1987] and *Aureobasidium sp.* [Hayashi *et al.*, 1989; Yun *et al.*, 1992; Jung *et al.*, 1987]. Other beneficial health promoting properties of FOS are prevention of constipation, liver function, serum cholesterol and blood pressure, as well as its safety in diabetics [Yun and Song, 1999]. Fructooligosaccharides also possess property as functional food and prebiotics. These can be derived from inulin or synthesized from sucrose by use of fungal fructosyltransferases. Other linkages of fructooligosaccharides, such as β -(2 $\rightarrow$ 6), can be produced from sucrose by thermolysis or via action of levansucrase. Also other indigestible oligosaccharides have been investigated as potential prebiotics, including oligosaccharides of D-galactose, D-glucose, D-xylose, *N*-acetyl-D-glucosamine and combinations thereof. [Eggleston and Côté, 2003]

In spite all these physiological functionalities, most presently available oligosaccharide syrups have an important limitation, which is their total content of at most 60% in total dry weight [Yun *et al.*, 1994; Jung *et al.*, 1989]. Therefore, investigators have focused on process development of “high-content” commercial products [Yun *et al.*, 1994]. In this context, the use of dual enzyme

systems of fructosyltransferase and glucose oxidase has great potential in processing of high-content FOS syrup. Second mentioned enzyme, glucose oxidase is used for glucose removal, which accounts for 30% in total sugar and thus acts as a competitive inhibitor during enzymatic reaction in FOS production. In this regard, also inulin can be a promising source for oligosaccharide production as mentioned above. Inulooligosaccharides from inulin have similar chemical structure to fructooligosaccharides from sucrose. They are regarded as a soluble dietary fiber [Farnworth, 1993].

An alternative way for synthesis of a wide range of oligosaccharides is the use of glycosidases, which catalyze the condensation of underivatized sugars. This approach is preferred as both enzymes and substrates are inexpensive and readily available. This method has led to high yields of products by allowing the reactions to reach equilibrium. [Bucke *et al.*, 1999]

Optimization of oligosaccharide synthesis from cellobiose by use of dextranucrase from *Leuconostoc mesenteroides* B-512FMCM [Kim and Day, 2007] enables the production a series of cellobio-oligosaccharides. This dextranucrase synthesizes a dextran containing 95% α -(1 $\rightarrow$ 6) linear and 5% α -(1 $\rightarrow$ 3) branched linkages, thus being capable to transfer glucosyl units from sucrose onto the acceptor to produce oligosaccharides. In the study of Kim and Day [Kim and Day, 2007], the determination of optimal reaction conditions was performed, whereas reaction digests were prepared with various sucrose (100-800mM), cellobiose (50-300mM) and crude dextranucrase (13-67 U) concentrations in 20mM sodium citrate buffer (pH 3.2-6.0) at 20-50°C for 24h. An optimal production of cellobio-oligosaccharides synthesized by *Leuconostoc mesenteroides* B-512FMCM dextranucrase was obtained at pH 5.2, whereas the rate of cellobio-oligosaccharide production in dependence of temperature sharply decreased above 30°C and at 40°C was only 46% of the maximum production available. The effects of differing dextranucrase and sucrose as well as cellobiose concentration have shown, that the optimal conditions for the cellobio-oligosaccharide synthesis were as follows: sucrose = 289mM, cellobiose = 250mM, dextranucrase = 54 U, at pH of 5.2 and a temperature of 30°C, thus predicting a theoretical cellobio-oligosaccharide yield of 98.74%.

2.5 Bioavailability of cellobiose in humans

The indigestibility of oligosaccharides makes them good prebiotics due to the fact that they can be fermented by intestinal microbes. Enzymes capable of breaking down cellobiose are absent in

the human small intestine [Kim and Day, 2007]. Nevertheless, it was confirmed that orally ingested cellobiose was well fermented in human large intestine, and hence, reaches the colon undigested. In order to test cellobiose bioavailability, cellobiose tolerance tests and breath hydrogen excretion test were performed. [Nakamura *et al.*, 2004]

In conclusion of this study, it was shown, that after oral ingestion of 25 g of cellobiose, neither serum glucose nor insulin levels increased, whereas breath hydrogen gas excretion was remarkable. In contrast, after the same amount of glucose was ingested, almost all of this glucose was spontaneously absorbed in the small intestine and excretion of breath hydrogen gas was not detectable. These results demonstrate two facts about cellobiose metabolism in human:

- almost all orally ingested cellobiose has reached the large intestine with little digestion by enzymes
- cellobiose is not hydrolyzed by intestinal disaccharides and is readily fermented by intestinal microbes

These results support that cellobiose has a property as a prebiotics. [Nakamura *et al.*, 2004]

2.6 Novel sugars and sugar derivatives

There is a demand for ingredients that provide bulk and the correct texture to food having lower degree of sweetness than sucrose. One of these disaccharides is isomaltulose, which is about one third as sweet as sucrose but has similar sweetness profile to that of sucrose. It shows a lower rate of release of monosaccharides into the blood, resulting in diminished release of insulin compared to other sugars, creating consequently the possibility of applications in diabetic and sports foods as well as in drinks. [Cheetham and Bucke, 1999]

D-Mannitol, a sugar alcohol with many applications, can also be produced by bacteria such as heterofermentative lactic acid bacteria, e.g. *Lactobacillus brevis* and *Lactobacillus buchneri* respectively. Mannitol is about half as sweet as sucrose and it finds its largest application as a food additive (E421). It reduces the crystallization tendency of sugars and increases the shelf life of foodstuffs. As it is used widely in chewing gum, this sugar is used in the pharmaceutical formulation of chewable tablets and granulated powders. It does not interact with the active

components, and due to its sweet cool taste masks the unpleasant taste of many drugs. [Soetaert *et al.*, 1999]

Another approach for the production of useful compounds seems to be enzymatic glycosylation of sugars, vitamins and sugar alcohols, which presents a very effective biotransformation method [Suzuki and Uchida, 1999] by using glycosyltransferases (cyclomaltodextrin glucanotransferase [CGTase] and dextranucrase). CGTase can be isolated from *Bacillus macerans* and *Bacillus megaterium* [Kitahata *et al.*, 1992], whereas *Bacillus stearothermophilus* CGTase has much wider acceptor specificity than that previously reported [Kitahata *et al.*, 1978]. Enzymatic glycosylation enables the preparation of pharmacologically significant compounds with high stability against ultraviolet light, heating and air oxidation; these derivatives are nontoxic, are less bitter and have a stimulative tongue picking taste and water solubility [Suzuki, 1998; Suzuki and Uchida, 1991; Suzuki *et al.*, 1996].

In this context, an unusual and interesting class of oligosaccharides is those in which the glycosidic chain closes back upon itself, thus no reducing end is existing. The best known of these are the cyclomaltodextrins, also known as cyclodextrins or cycloamyloses, which are produced from starch or maltodextrins by the action of cyclomaltodextrin glucanotransferase. The presence of a hollow cavity in the middle of the macrocyclic ring is their most useful characteristic. The interior part of the cavity consists of a relatively hydrophobic surface, thus hydrophobic molecules can enter the space and form stable inclusion complexes. This helps to form water-soluble complexes of hydrophobic molecules, particularly drugs, as well as oil-based flavors and fragrances. [Eggleson and Côté, 2003]

2.7 Oligosaccharides from breakdown of polysaccharides

Polysaccharides can break down *in vivo* by exogenous digestive enzymes, particularly those of microbial origin. Generally, the enzyme-catalyzed degradation of polysaccharides to monosaccharides occurs through oligosaccharide intermediates. For instance, cellulolytic and amylolytic enzymes are capable to hydrolyze cellulose and starch, yielding series of glucooligosaccharides as the first step in their conversion to D-glucose. Nevertheless, the two most common enzymes involved are endohydrolases, which breaks down a long polysaccharide chain into short oligosaccharides, and exohydrolases, which enable hydrolysis of the oligosaccharide to monosaccharide. [Eggleson and Côté, 2003]

2.8 Enzyme-catalyzed polymerization of cellobiose units to form cellulose

Also cellulose, a homopolysaccharide of glucose units connected through β (1 $\rightarrow$ 4) glycosidic linkages, has become of great interest in many application fields as polymeric drugs and new biomaterials [Mark, 1980]. In the last several years, intensive attention has been given to this polysaccharide due to its application in the field of polymer science [Kobayashi *et al.*, 1995]. By addition of acetonitrile to an aqueous incubation mixture, a cellulase catalyst can promote the reverse reaction of the hydrolytic decomposition, whereby different degrees of polymerization lead to production of water insoluble synthetic cellulose. Also produced are water soluble cellooligomers [Kobayashi *et al.*, 1999]. The first successful in vitro synthesis of cellulose via a nonbiosynthetic pathway has been performed by utilizing cellulase as catalyst, where the enzymatic synthesis of cellulose can be achieved via polycondensation of β -cellobiosyl fluoride monomer [Kobayashi *et al.*, 1991; Lee *et al.*, 1994]. Additionally, highly pure samples of cellulose and its derivatives have been manufactured for use as functional macromolecules, specimens for basic crystallographic research as well as for enzyme substrates.

2.9 Polymerization of modified cellobiose units

The potential of enzyme-catalyzed polymerization of cellobiose monomers has been extended by Kobayashi and his associates to include the novel cellobiose derivatives, 6-O-methyl, 6'-O-methyl, and 6,6'-di-O-methyl- β -cellobiosyl fluorides. In a solvent mixture of acetonitrile and acetate buffer, 6-O-methylcellobiosyl fluoride was polymerized under catalysis by cellulase from *Trichoderma reesei* to afford an alternately 6-O-methylated cellulose derivative. The authors explained the stereoselective formation of the β -1,4-glycosidic bond by a mechanism involving formation of a glycosyl-enzyme intermediate with inversion at the anomeric center; this intermediate is then attacked by the 4-hydroxyl group of a bound glucose residue, again with inversion, to produce the β -1,4-glycosidic linkage. The 6'-O-methyl cellobiose fluoride was found less suitable for polymerization; incubation of this substrate under the polymerization conditions afforded a mixture of oligosaccharides from which a tetrasaccharide was isolated that contains the 6-O-methyl substitution on alternating glucose units. Synthesis of the methylated cellobiose derivatives was achieved by organic-chemical methods [Okamoto *et al.*, 1997]. In previous work in our laboratory, the 6-azido-6-deoxy- and 6'-azido-6'-deoxy-cellobiose derivatives have also been synthesized using organic-chemical methodology [Saber, 2009]. In

the present work, syntheses of azidocellobiose derivatives have been developed that combine organic-chemical methods with enzyme-catalyzed processes.

2.10. Enzyme-catalyzed modification of synthetic polymers

Recently, the first example of an enzyme-catalyzed modification of a synthetic polymer has been described. This process involves the introduction of epoxide (oxiran) groups into polymers containing olefinic double bonds [Jarvie *et al.*, 1999].

3. Enzymatic syntheses vs. chemical syntheses

Chemical synthesis of even the simplest oligosaccharides is complex and expensive, and requires the use of hazardous substances [Bucke, 1999; Anastas *et al.*, 2000]. On the contrary, enzymatic synthesis might provide a much more practical, generally inexpensive and environmentally benign approach for the development of new polymers [Bucke, 1999].

In the last decade, significant progress has been made in different research areas such as catalysis necessary to enable the design of safer chemicals and environmentally benign solvents [Anastas and Kirchhoff, 2002]. Catalytic reactions can reduce energy requirements [Manzer, 1994; Dijksam *et al.*, 2001; Mubofu *et al.*, 2001; Adams *et al.*, 1999; Dias *et al.*, 2001; Hoelderich, 2000; Murahashi *et al.*, 2000] and permit the use of less toxic reagents using e. g. hydrogen peroxide in the case of oxidation instead of traditional heavy metal catalysts [Collins *et al.*, 1998]. The manifestation of catalysts as a fundamental tool in pollution prevention has been finally recognized as a crucial factor for accomplishing a wide range of so called green chemistry goals [Anastas and Kirchhoff, 2002].

Green chemistry demonstrates a paradigm for achieving environmental and economic prosperity for a sustainable world. It consists of a set of principles (**Table 3**) [Collins, 1997] the implementation of which can reduce and eliminate the use as well as the generation of hazardous substances in the design, manufacture and application of chemical products.

Table 3

The 12 principles of green chemistry [Collins, 1997]

1. Prevention

It is better to prevent waste than to treat or clean up waste after it has been created.

2. Atom Economy

Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.

3. Less Hazardous Chemical Syntheses

Wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and the environment.

4. Designing Safer Chemicals

Chemical products should be designed to affect their desired function while minimizing their toxicity.

5. Safer Solvents and Auxiliaries

The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used.

6. Design for Energy Efficiency

Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.

7. Use of Renewable Feedstocks

A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.

8. Reduce Derivatives

Unnecessary derivatization (use of blocking groups, protection/ deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.

9. Catalysis

Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.

10. Design for Degradation

Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.

11. Real-time analysis for Pollution Prevention

Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.

12. Inherently Safer Chemistry for Accident Prevention

Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

3.1 Enzymes for chemical syntheses

New enzyme-catalyzed synthetic methods in organic chemistry have been recognized as practical alternatives to traditional, non-biological organic syntheses. These methods are in great demand by the pharmaceutical and chemical industries as they are able to satisfy stringent environmental constraints. [Koeller and Wong, 2001]

Most enzymes can be used as the sole catalyst in a reaction, in combination with other enzymes, or with non-biological reagents. Due to the chiral nature of enzymes a stereo- and regio-chemically defined reaction product can be obtained with remarkable rate acceleration. Oligosaccharides with complex structure are difficult to obtain by strictly chemical synthesis, but due to their great potential as carbohydrate drugs [Zopf and Roth, 1996], enzymatic syntheses are the most appropriate means of producing these. With regard to their construction, biocatalytic methods have aided evaluation of the biological significance of carbohydrates [Koeller and Wong, 2001].

In addition, not only the fact that enzymes can accept unnatural substances, they also can be genetically engineered and subsequently used to alter their stability, broaden their substrate specificity and increase their specific activity. Using recombinant DNA technologies both natural and engineered enzymes can be produced on a large scale in convenient host organisms. The application of enzyme-catalyzed syntheses represents a remarkable opportunity for the development of industrial chemical and pharmaceutical processes.

3.2 Practical parameters in enzymatic syntheses

Practically, there are several parameters affecting an enzymatic reaction (**Figure 6**).

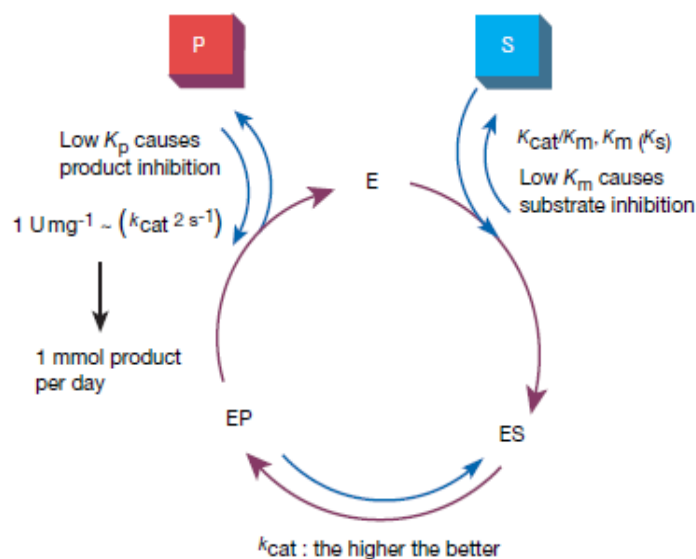


Figure 6 Practical parameters to be considered in enzymatic synthesis [Koeller and Wong, 2001]

The specific activity (quantified by k_{cat}), specificity (determined by the ratio k_{cat}/k_m) and stability of the enzyme are of particular importance. Also the degree of inhibition by substrate or product (determined by their affinity to the enzyme) is very important for the outcome of a reaction. In an optimal case, the enzyme used for the reaction would have high specific activity and stability, and at the same time minimal substrate and product inhibition. Much as the degree of substrate specificity provides a general synthetic utility, also the stereospecificity can be considered to be a most important parameter under consideration. Despite the fact that many enzymes have great synthetic use, became commercially available and show potential with regard to substrate specificity and stereoselectivity, they may be unpredictable with unnatural substrates. [Koeller and Wong, 2001]

Nevertheless, current researches in enzymatic synthesis are manifold (**Table 4**) [Koeller and Wong, 2001].

Table 4

Current research activities in enzymatic synthesis [Koeller and Wong, 2001]

- Development of new enzymatic reactions using designed substrates
- Use with organic solvents and co-solvents; one- and two-phase systems
- Overcoming substrate and product inhibition
- Modification of enzyme activity and semisynthetic enzymes
- Enzyme immobilization and stabilization
- Use of recombinant DNA for large-scale production of enzymes
- Mutagenesis and directed evolution for changes in enzymatic properties
- Exploration of new enzymes and enzymes from new species for synthetic use
- Scale-up in fine chemical and pharmaceutical synthesis
- Multienzyme systems for synthesis *in vitro* and *in vivo*
- Antibody catalysis

In summary, a strategic approach for the development of enzymatic catalysts for reactions of interest requires an appropriate selection of the enzyme for a given reaction. In the case of a known enzyme, it will be necessary to optimize the reaction conditions and improve the catalyst through directed protein engineering. If for the desired reaction no known enzyme exists, then non-biological methodology may be the method of choice. [Koeller and Wong, 2001]

Certain individual steps cannot be performed by biochemical means, such as the regiospecific introduction of the nitrogen function in the form of the azido group. Such steps need to be performed by conventional organic-chemical methods. [Koeller and Wong, 2001]

4. Carbohydrate processing phosphorolytic enzymes

Enzymes which are involved in the formation and cleavage of glycosyl linkages are categorized into three main classes (**Figure 7**) [Kitaoka and Hayashi, 2002]:

Hydrolytic enzymes

Phosphorolytic enzymes

Synthase enzymes (glycosyl-nucleotide glycosyltransferases)

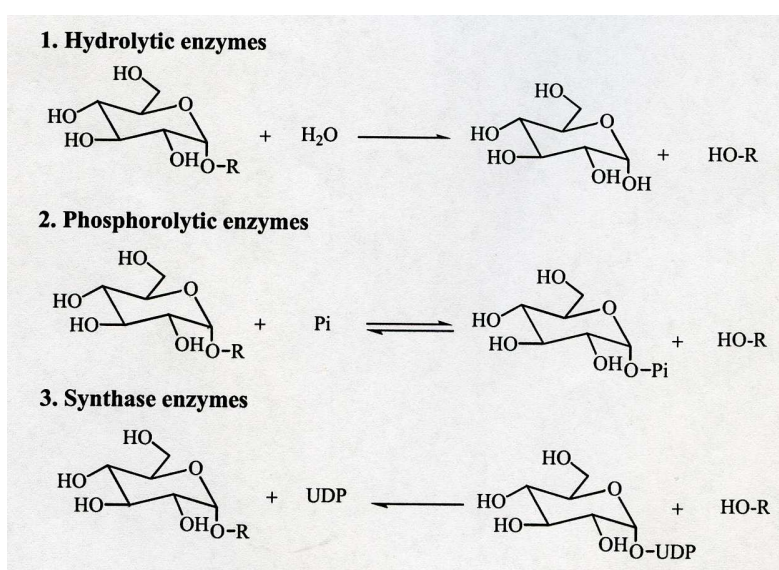


Figure 7 Three categories of enzymes [Kitaoka and Hayashi, 2002]

The most studied of the three classes of enzymes are hydrolytic enzymes which are frequently employed in the hydrolysis of polysaccharides and they are also very important industrially in the utilization of starch. Due to the existence of an abundance of water molecules in the reaction mixture of hydrolytic enzymes, these reactions appear to be substantially irreversible.

The synthase enzymes are biologically important due to their role of synthesizing carbohydrate chains *in vivo*. As most of the synthases are membrane-associated proteins and are relatively unstable, they do not find use in a practical sense in the *in vitro* synthesis of carbohydrate chains. Their reactions are irreversible in the synthetic direction due to the requirement for cleavage of the high-energy bond of the glycosyl-nucleotide (a phosphate diester linkage).

Phosphorolytic enzymes have properties which lie between those of the hydrolytic enzymes and the synthases. As the bond energy of the substrate, a glycosyl-phosphate, is not as high as that of a glycosyl-nucleotide, their reactions are reversible. Therefore, these enzymes can be involved in the practical synthesis of sugar chains, exploiting their reverse reactions. [Kitaoka and Hayashi, 2002]

4.1 Classification of phosphorolytic enzymes

These enzymes are named using a combination of “the name of the substrate” and “phosphorylase”. One of the most important features of the phosphorolytic enzymes are their regiospecificities, which are very strict and can be used in the synthesis of oligosaccharides with specific glycosyl linkages via their reverse reactions. Classification of these enzymes can be performed either by anomeric forms of the glycoside phosphorylated or by the anomeric forms of the glycosyl-1-phosphate produced. They also can be described in terms of the anomeric retention or inversion process they undergo in the reaction (**Table 5**). [Kitaoka and Hayashi, 2002]

Table 5

The phosphorolytic enzymes [Kitaoka and Hayashi, 2002]

EC	name	mechanism	substrate	product	d.p. substrate	family
2.4.1.1	(glycogen) phosphorylase	retention	Glc- α 1,4-Glc	α -Glc-1P	≥ 5 or 4	GT35
2.4.1.7	sucrose phosphorylase	retention	Glc- α 1,2-Fru	α -Glc-1P	2 only	GH13
2.4.1.8	maltose phosphorylase	inversion	Glc- α 1,4-Glc	β -Glc-1P	2 only	GH65
2.4.1.20	cellobiose phosphorylase	inversion	Glc- β 1,4-Glc	α -Glc-1P	2 only	GT36
2.4.1.30	laminaridextrin phosphorylase	inversion	Glc- β 1,3-Glc	α -Glc-1P	≥ 2	not cloned
2.4.1.31	laminaribiose phosphorylase	inversion	Glc- β 1,3-Glc	α -Glc-1P	≥ 2	not cloned
2.4.1.49	cellodextrin phosphorylase	inversion	Glc- β 1,4-Glc	α -Glc-1P	≥ 3	GT36
2.4.1.64	trehalose phosphorylase	inversion	Glc- α 1, α 1-Glc	β -Glc-1P	2 only	not identified
2.4.1.97	β 1,3glucan phosphorylase	inversion	Glc- β 1,3-Glc	α -Glc-1P	polymer	not cloned
2.4.1.?	trehalose phosphorylase	retention	Glc- α 1, α 1-Glc	α -Glc-1P	2 only	not identified
2.4.1.?	kojibiose phosphorylase	inversion	Glc- α 1,2-Glc	β -Glc-1P	≥ 2	not identified
2.4.1.?	β 1,3Gal-GlyNAc phosphorylase	inversion	Gal- β 1,3-GlyNAc	α -Gal-1P	2 only	not cloned
2.4.1.?	chitobiose phosphorylase	inversion	GlcNAc- β 1,4-GlcNAc	α -GlcNAc-1P	2 only	GT36

4.2 Phosphorolytic enzymes

Phosphorylases catalyze the reversible phosphorolysis of glucans [Graves and Wang, 1972]. They are grouped into several families of glycoside hydrolase (GH; families 13, 65, 94 and 112)

and glycosyl transferase (families 4 and 35). Among them, GH112 is a family of phosphorylases that react with β -galactosides, whereas most of the other families utilize glucosides as their substrates. [Nakajima *et al.*, 2010]

The GH112 family includes the enzyme D-galactosyl- β 1 $\rightarrow$ 3-N-acetyl-D-hexosamine phosphorylase (GalHexNAcP), which can be found in *Bifidobacterium bifidum*, *Clostridium perfringens* or *Bifidobacterium longum*. The GalHexNAcP enzymes such as galacto-N-biose/lacto-N-biose I phosphorylase, galacto-N-biose phosphorylase and lacto-N-biose I phosphorylase seem to be involved in metabolizing functional sugars derived from humans as GH112 proteins are distributed in pathogenic bacteria of humans. [Nakajima *et al.*, 2010]

The enzyme D-galactosyl- β 1 $\rightarrow$ 4-L-rhamnose phosphorylase (GalRhaP) is the second enzyme of the GH112 family, which was found in forest soil bacterium, *Clostridium phytofermentans*. This enzyme is capable to produce β 1 $\rightarrow$ 4-disaccharides from L-rhamnose derivatives as major acceptors. In contrast, β 1 $\rightarrow$ 3-disaccharides from D-glucose derivatives represent minor acceptors in the reverse reaction. [Nakajima *et al.*, 2010]

Another bacterium, *Opitutus terrae* PB90-1, which is a major anaerobic bacterium in paddy soil ecosystems, consists of a gene encoding GH112 protein (Oter_1377 protein). In the study of Nakajima *et al.* [Nakajima *et al.*, 2010] this protein showed phosphorolytic activity on D-galactosyl- β 1 $\rightarrow$ 4-L-rhamnose phosphorylase and a weak phosphorolytic activity on D-Gal- β 1 $\rightarrow$ 3-D-Glc, thus indicating identical specificity to that of D-galactosyl- β 1 $\rightarrow$ 4-L-rhamnose phosphorylase from *Clostridium phytofermentans*. Substrate specificity studies of various acceptors such as L-Rha, derivatives of L-Rha, and those of D-Glu, D-Xyl or D-Gal for this enzyme have shown its tendency to exhibit higher activity on L-rhamnose derivatives than on D-glucose derivatives, which is the same as that exhibited by D-galactosyl- β 1 $\rightarrow$ 4-L-rhamnose phosphorylase from *Clostridium phytofermentans*. This enzyme reveals an optimal activity at neutral pH (pH 6.0 – 7.0), which is also similar to the same enzyme from *Clostridium phytofermentans*. In conclusion, it was identified that the Oter_1377 protein acts as a D-galactosyl- β 1 $\rightarrow$ 4-L-rhamnose phosphorylase. It represents the second D-galactosyl- β 1 $\rightarrow$ 4-L-rhamnose phosphorylase, next to one from *Clostridium phytofermentans*.

In another study of Nakajima *et al.* [Nakajima *et al.*, 2009] three D-galactosyl- β 1 $\rightarrow$ 3-N-acetyl-D-hexosamine phosphorylase homologs (Cphy0577, Cphy1920 and Cphy3030) from an anerobic cellulolytic bacterium, *Clostridium phytofermentans* were characterized. In contrast to Cphy0577

and Cphy3030, which exhibit similar activity on galacto-*N*-biose and lacto-*N*-biose I, thus acting as D-galactosyl- β 1 $\rightarrow$ 3-*N*-acetyl-D-hexosamine phosphorylases, Cphy1920 protein phosphorylates neither galacto-*N*-biose nor lacto-*N*-biose I. It shows the highest activity with L-rhamnose as the acceptor substrate in the reverse reaction using α -D-galactose 1-phosphate as the donor. In conclusion, the reaction product was D-galactosyl- β 1 $\rightarrow$ 4-L-rhamnose, proposing D-galactosyl- β 1 $\rightarrow$ 4-L-rhamnose phosphorylase as the name for Cphy1920 protein.

As follows below, several of the known phosphorylases are briefly discussed according to the substrates whose cleavage they catalyze. All of the phosphorylases reported to date are classified based on their amino acid sequences (<http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>).

Sucrose phosphorylase (EC 2.4.1.7) is found in bacterial cells and is involved in the metabolism of extracellular sucrose. It catalyzes the reversible phosphorysis of sucrose into α -D-glucose 1-phosphate and fructose [Doudoroff, 1943]. The phosphorysis proceeds via a ping pong bi bi mechanism and the enzyme transfers α -glucoside in the absence of phosphate [Silverstein *et al.*, 1967]. With regard to reaction mechanism, sucrose phosphorylase can be considered to act as α -glucosyl transferase enabling the transfer of D-glucose to phosphate – quite different from the other phosphorytic enzymes.

Maltose phosphorylase (EC 2.4.1.8) is involved in the metabolism of extracellular maltose and can be found in bacterial cells. In a reversible reaction, this enzyme is able to phosphorylate maltose into β -D-glucose 1-phosphate and D-glucose [Fitting and Doudoroff, 1952].

The β -D-glucose 1-phosphate is subsequently converted into glucose-6-phosphate by the action of a β -specific phosphoglucomutase in order to facilitate entry into the glycolytic pathway [Ben-Zvi and Schramm, 1961]. The reaction mechanism follows a sequential bi bi mechanism; the enzyme recognizes the α -anomeric hydroxyl group at the reducing end of maltose, therefore catalyzing phosphorysis only of the α -anomer of the disaccharide [Tsumuraya *et al.*, 1990].

Cellodextrin phosphorylase (EC 2.4.1.49), only found in the cells of *Clostridia* and accompanying cellobiose phosphorylase [Reichenbecher *et al.*, 1997], catalyzes phosphorysis of cellooligosaccharides larger than cellotriose with generation of α -D-glucose 1-phosphate. The regiospecificity of this enzyme is identical with that of cellobiose phosphorylase whereas the

specificity with regard to the degree of polymerization of the acceptor is quite different [Kitaoka and Hayashi, 2002]. In the reverse reaction, cellodextrin phosphorylase does not use D-glucose as the acceptor but recognizes various aryl- β -glucosides and β -glucosyl disaccharides [Sheth and Alexander, 1969] as well as other β -D-glucosides such as cellobiose, 4-thiocellobiose, methyl- and phenyl- β -cellobioside [Samain *et al.*, 1995]. In the study of Shintate *et al.* [Shintate *et al.*, 2003] it was possible to show, that use of four acceptor substrates (cellobiose, β Glu-(1 $\rightarrow$ 4)-Xyl, β Xyl-(1 $\rightarrow$ 4)-Glu and xylobiose) and two donor substrates α -D-glucose 1-phosphate and α -D-xylose 1-phosphate demonstrated that cellodextrin phosphorylase can be employed in the synthesis of six possible hetero-trisaccharides as well as of fourteen possible hetero-tetrasaccharides consisting of β -(1 $\rightarrow$ 4)-linked D-glucose and D-xylose residues.

Trehalose phosphorylase (inversion type: EC 2.4.1.64) was first discovered in the cells of *Euglena gracilis* and has been implicated in the metabolism of intracellular trehalose, a storage disaccharide. The enzyme catalyzes the reversible phosphorolysis of trehalose to form β -D-glucose 1-phosphate and D-glucose [Marechal and Belocopitow, 1972]. The retention type of trehalose phosphorylase reversibly catalyzes the phosphorolysis of trehalose into α -D-glucose 1-phosphate and D-glucose [Kitamoto *et al.*, 1988]. This type of enzyme was isolated from various fungi. Its reaction mechanism was described as being a sequential bi bi process, different from that of sucrose phosphorylase [Eis *et al.*, 2001].

Cellobiose phosphorylase (EC 2.4.1.20) is present as an intracellular enzyme in bacterial cells that utilize cellulose [Yernool *et al.*, 2000]. It is involved in the catabolism of extracellular cellobiose [β -D-Glcp-(1 $\rightarrow$ 4)-D-Glc] [Nidetzky *et al.*, 2000] in the cytoplasm [Lou *et al.*, 1996]; cellobiose is formed by the action of cellulase. Cellobiose phosphorylase catalyzes the reversible phosphorolysis of cellobiose into α -D-glucose 1-phosphate and D-glucose [Sih and McBee, 1955]. In cellobiose synthesis activity, α -D-glucose 1-phosphate is used as the sugar donor and D-glucose as the sugar acceptor of cellobiose phosphorylase [Hidaka *et al.*, 2006]. The enzyme recognizes the β -anomeric hydroxyl group at the reducing end of the cellobiose moiety. The reaction mechanism for this enzyme is reported to be an ordered bi bi process [Kitaoka *et al.*, 1992]. With regard to acceptor specificity in the reverse reaction it was found that the enzyme

has a strict recognition of the hydroxyl groups of the D-glucose molecule at positions β -1, 3 and 4, but not of the hydroxyls at positions 2 and 6 [Kitaoka *et al.*, 1992].

Concerning the anomeric form of glycosidic linkages to be phosphorolyzed, sucrose, trehalose and maltose phosphorylases act on α -glycosides and cellobiose phosphorylase on a β -glycoside. Regarding the anomeric form of α -D-glucose 1-phosphate produced, the α -anomer is obtained by the action of sucrose and cellobiose phosphorylases and the β -anomer under catalysis by the maltose and trehalose phosphorylases. While operating by a phosphorolytic mechanism, only sucrose phosphorylase action results in retention of the anomeric configuration (α to α), while the action of the other enzymes results in inversion: maltose phosphorylase (α to β), cellobiose phosphorylase (β to α) and trehalose phosphorylase (α to β) [Kitaoka *et al.*, 1992].

4.3 Choice of cellulose-related enzymology

Chitosan and its hypothetical analogues are all structurally related to cellulose. Indeed, they may be considered cellulose derivatives containing amino groups in place of specific hydroxyl functions (such as substitution of OH-2 in the case of chitosan).

5. Fundamentals of cellulolytic microorganisms

5.1 Structure and composition of cellulosic biomass

Plant biomass represents the only foreseeable sustainable source of materials available to humanity [Lynd *et al.*, 1999]. As a major component of plant biomass, cellulose represents the world's most abundant biopolymer [Lynd *et al.*, 2002; Kim and Day, 2010]. One of the central technological strategies involves the use of cellulolytic enzymes for hydrolysis of biomass and fermentation of resulting sugars to desired products in a single process step via cellulolytic microorganisms [Lynd *et al.*, 2002]. This strategy offers very large cost reductions if an appropriate microorganism can be developed and a suitable combination of substrate utilization and product formation can be arranged [Lynd *et al.*, 1996].

Cellulose in its natural form and also as waste offers great potential as a source of energy and chemicals [Halliwell *et al.*, 1994]. The recognition that cellulosic wastes may be converted to glucose raised the commercial potential of cellulase enzymes tremendously, which consequently led to a remarkable progress in the past fifty years [Bhat and Bhat, 1997]:

- (I) Isolation of microorganisms producing cellulase [Henssen, 1957; Hungate, 1950; Orpin, 1975]
- (II) Improving the yield of cellulase by mutation, and protoplast fusion [Brown *et al.*, 1986; Durand *et al.*, 1988]
- (III) Purification and characterization of the cellulase components [Bhat *et al.*, 1993; Bhat *et al.*, 1989]
- (IV) Understanding the mechanism of cellulose degradation [Eriksson, 1978; Eriksson and Wood, 1985]
- (V) Cloning and expression of cellulase genes [Beguin and Aubert, 1993; Beguin *et al.*, 1985]
- (VI) Determining the 3-D structures of cellulase components [Alzari *et al.*, 1996]
- (VII) Understanding structure-function relationships in cellulases [Davies and Henrissat, 1995]
- (VIII) Demonstrating the industrial potential of cellulases [Coughlan, 1985; Mandels, 1985]

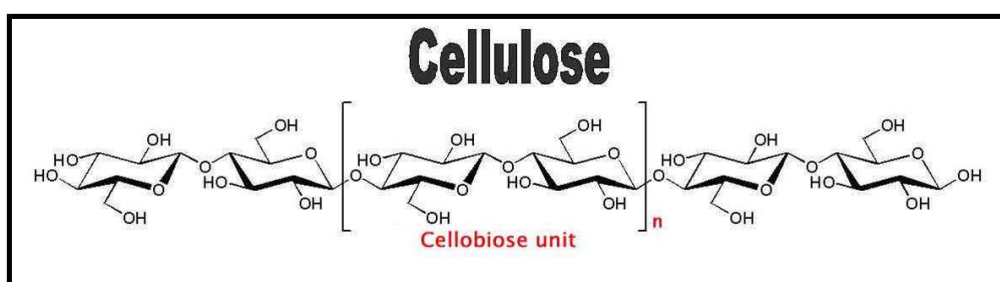


Figure 8 Cellulose

(Source: <http://sci.waikato.ac.nz/farm/images/cellulose%20structure%20RF.jpg>)

The basic molecular structure of the cellulose is a natural and linear homopolysaccharide, consisting of up to 10^4 D-glucose moieties which are arranged in fibrils. The fibrils are organized

into a “paracrystalline” state, therefore adding to the structural rigidity of cellulose. These glucose units are connected via β (1 $\rightarrow$ 4) glycosidic linkages (**Figure 8**).

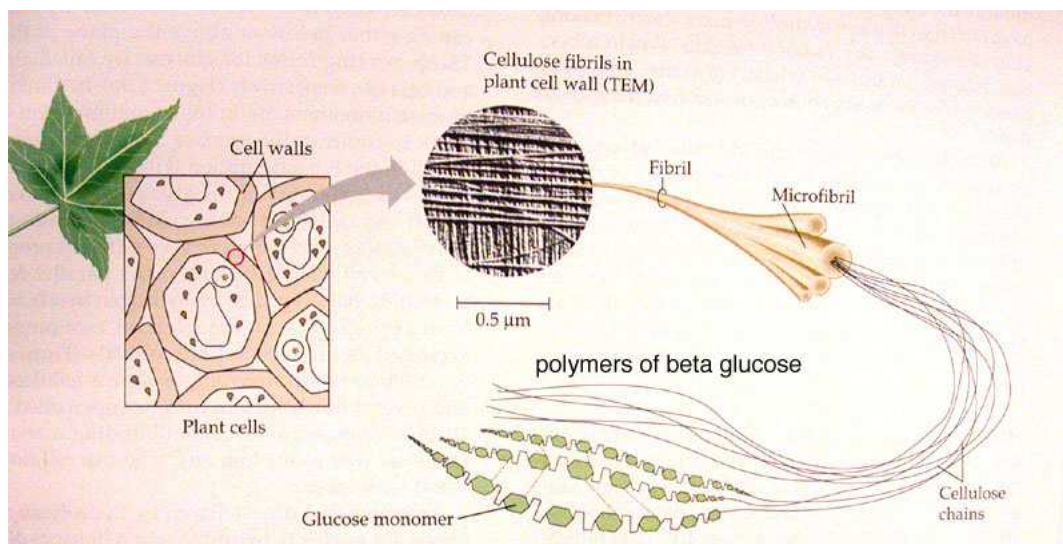


Figure 9 Cellulose in plants

(Source: <http://academic.brooklyn.cuny.edu/biology/bio4fv/page/struct-carbohydrates.html>)

Cellulose is found in nature in plant cell walls (**Figure 9**) and can also be produced by some animals (e. g. tunicates) and few bacteria [Lynd *et al.*, 2002]. Bacterial cellulose, which is mainly a protective coating, belongs to specific products of primary metabolism, whereas plant cellulose plays a structural role [Bielecki *et al.*, 2005].

Cellulose I and cellulose II are two major allomorphs. Cellulose I, the dominant form in nature [Nakamura *et al.*, 2004], consists of a microfibrillar crystalline array of linear β -1, 4-glucan chains. They all are oriented parallel to one another with the same polarity [Sanz *et al.*, 2005]. The extended chain formation of cellulose I enables the formation of microfibrils having extraordinary mechanical strength. Formation of cellulose II occurs from cellulose I through chemical treatment that alters the crystal structure. [Lee *et al.*, 1994]

Commercially available cellulose possesses, depending on its origin, a variation between 3000 and 10 000 β (1 $\rightarrow$ 4) glycosidically connected glucose units, with a general structural formula of $(C_6H_{10}O_5)_n$. Also the molecular masses vary between 500 000 and 1 600 000 Da [Bauer *et al.*, 2002]. Cellulose is to a large extent inert and insoluble in most organic solvents.

Although cellulose forms a distinct crystalline structure, cellulose fibers in nature are not purely crystalline [Marchessault and Howsom, 1957]. In addition to the crystalline and amorphous regions, cellulose fibers contain irregularities, such as kinks or twists of the microfibrils, large pits and capillaries [Cowling, 1975; Marchessault and Sundararajan, 1983]. The variable structural complexity of pure cellulose and the difficulty of working with insoluble substrates have led to the wide use of the highly soluble cellulose ether, carboxymethylcellulose (CMC). Thus, many organisms which cannot degrade cellulose have the ability to hydrolyze CMC via mixed β -glucan enzymes [Fields *et al.*, 1998].

5.2 Taxonomy, diversity and occurrence of cellulases

Notable thermophilic cellulolytic microorganisms with cellulase system are *Thermomonospora fusca*, *Thermoascus aurantiacus*, *Sporotrichum thermophile*, *Humicola insolens* and *Chaetomium thermophile* as well as other anaerobic bacteria such as *Acetivibrio cellulolyticus*, *Bacteroides succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. These microorganisms show the capability of fermenting a wide range of substrates with minimal risk of contamination by pathogens. In addition, cellulolytic thermophilic bacteria are of particular interest, due to their ability to produce thermostable cellulases showing a general stability under a variety of conditions including also somewhat more acidic and alkaline pH as well as temperature up to 90°C. [Lamed and Bayer, 1988]

The distribution of cellulolytic capability among organisms differing in oxygen sensitivity, temperature and salt tolerance is an acknowledgment to the wide availability of cellulose across natural habitats. [Lynd *et al.*, 2002]

In this context, a distinct difference in cellulolytic strategy exists between aerobic and anaerobic bacteria. Microorganisms which show the ability of producing complexed cellulase systems (cellulosomes) are mostly found in anaerobic environments. They exist in consortia with other microorganisms, both cellulolytic and noncellulolytic [Lynd *et al.*, 2002].

Utilization of cellulose by anaerobic bacteria is a challenging proposition from a bioenergetic perspective, due to the fact that the modest amount of ATP available from anaerobic catabolism needs to support microbial growth as well as cellulase production [Kirby, 1997]. In general, anaerobic cellulolytic species such as *Clostridium* grow preferably on cellulose and its hydrolytic products, but not on other polysaccharides or monosaccharides and oligosaccharides derived

from them. The fact that *Clostridium thermocellum* does not easily grow on glucose suggests the bacterial use of cellobiose in preference to glucose when both substrates are present [Ng and Zeikus, 1982].

Catabolism of cellulose involves enzymatic depolymerization of insoluble cellulose as well as cellular utilization of the hydrolytic products. Anaerobes degrade cellulose primarily via a highly potent, thermostable and complexed cellulase system which is also shown by the well characterized polycellulosome organelles of the thermophilic bacterium *Clostridium thermocellum* [Schwarz, 2001]. Thus, anaerobic bacteria show a tendency to adhere to cellulose (**Figure 10**) [Lynd *et al.*, 2002].

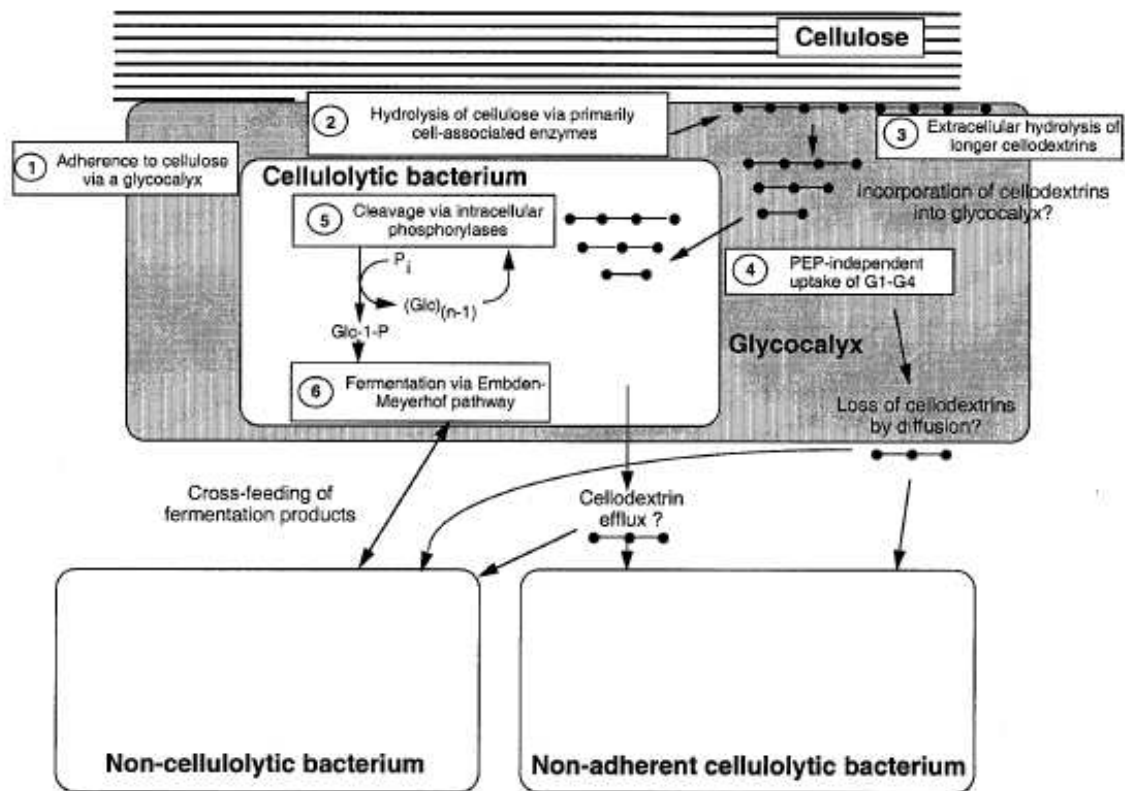


Figure 10 General strategy of cellulose hydrolysis and utilization by anaerobic bacteria [Lynd *et al.*, 2002]

Cellobiose, which has been used in this work, is a homoglucan disaccharide obtained by partial hydrolysis of cellulose, consisting of two D-glucopyranoses joined by a 1, 4- β -glycoside bond [Kim and Day, 2010]. 2D- and 3D-structures of this disaccharide are shown below (**Figure 11** and **Figure 12**).

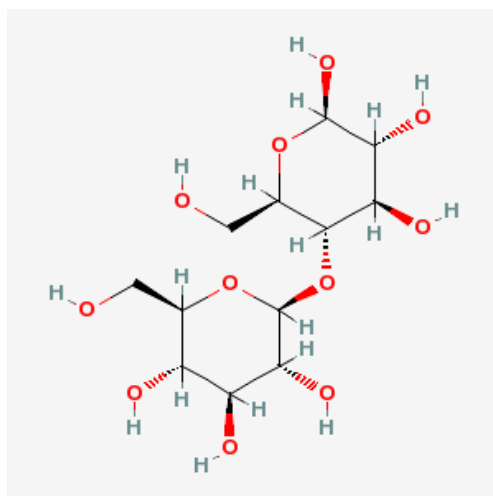


Figure 11 2D chemical structure of cellobiose

(Source: <http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=10712>)

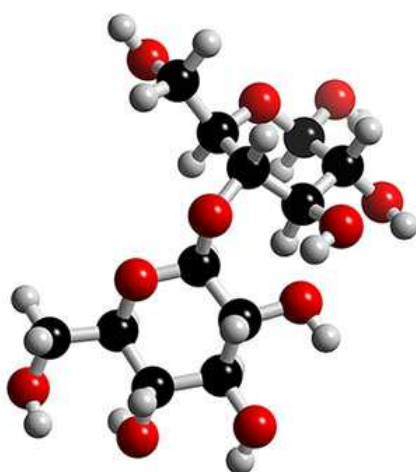


Figure 12 3D chemical structure of cellobiose

(Source: <http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=10712>)

Cellobiose offers significant potential as a source of renewable products for the cosmetics, food and pharmaceutical industries. It shows a higher prebiotic index (PI) than the currently favored prebiotic fructooligosaccharides [Sanz *et al.*, 2005]. Reduction of neutral fat in the liver and total cholesterol can be achieved by oral administration of cellobiose, indicating its potential drug use for prevention and treatment of life style-related diseases. Also, certain cellobio-oligosaccharides show potential as anti-fungal and anti-cancer agents [Kim, 2008].

5.3 Cellulose utilization by *Clostridium thermocellum*

The gram-positive thermophilic anaerobe *Clostridium thermocellum* [Beguín *et al.*, 1985], which was first obtained in stable pure culture over 50 years ago [McBee, 1954], secretes a thermostable cellulase complex, with the ability to hydrolyze cellulose and ferment the hydrolytic products cellobiose and D-glucose to ethanol, acetate and lactate [Duong *et al.*, 1983].

5.4 Different metabolism of D-glucose and cellobiose by *Clostridium thermocellum*

In most cellulolytic microorganisms examined, cellobiose has been cleaved into D-glucose via cellobiase [Halliwell, 1979]. However, cellulolytic bacteria such as *Clostridium thermocellum*, which ferments cellulose by extracellular cellulase [Ng *et al.*, 1981] can convert cellobiose into D-glucose (Glc) and α -D-glucose 1-phosphate (G-1-P) under catalysis of cellobiose phosphorylase.

Generally, cellulolytic anaerobes possess such intracellular enzymes, catalyzing a Pi-mediated (ATP-independent) phosphorolysis reaction [Lamed and Bayer, 1986]:



The latter mechanism causes the bacterium to prefer growth on cellobiose (versus glucose) as an energy source, probably due to conservation of the energy contained in the glycosidic linkage of cellobiose [Lamed and Bayer, 1986]. Therefore, this bacterium shows ability to produce glucose during the growth period in medium with a high concentration of cellobiose [Ng and Zeikus, 1982].

In strictly anaerobic, cellulolytic bacteria, G-1-P produced by the action of cellobiose phosphorylase is metabolized to glucose-6-phosphate, the entry point to the Embden-Meyerhoff pathway of sugar metabolism [Percival Zhang and Lynd, 2005]. The presence of intracellular cellobiose phosphorylase in *Clostridium thermocellum* suggests that cellobiose metabolism can occur by several processes:

- Extracytoplasmatic hydrolysis with subsequent uptake and catabolism
- Direct uptake of followed by intracellular phosphorolytic cleavage and catabolism
- Direct uptake of followed by intracellular hydrolytic cleavage and catabolism

5.5 Reactions of cellobiose phosphorylase (CeP)

The enzyme, cellobiose phosphorylase (cellobiose:orthophosphate α -D-glucosyltransferase) is widely distributed in cellobiose utilizing bacteria, both anaerobic and aerobic [Wannet *et al.*, 1998]. In anaerobic bacteria, this enzyme has been first described in *Clostridium thermocellum* [Sih and McBee, 1955], but it was also found in *Ruminococcus flavefaciens* [Ayers, 1958], *Cellvibrio gilvus* [Hulcher and King, 1958], *Clostridium stercorearium* [Reichenbecher *et al.*, 1997], *Cellulomonas uda* [Nidetzky *et al.*, 2000], *Thermotoga neapolitana* [Yernool *et al.*, 2000] and *Thermotoga maritima* [Rajashekhara *et al.*, 2002].

Purified cellobiose phosphorylase from *Cellvibrio gilvus* [Kitaoka *et al.*, 1992] has been investigated and it was found that the order of the substrate binding and the release of the products in the phosphorolytic reaction are as follows: initially cellobiose binds to the enzyme followed by phosphate, then D-glucose is released and, finally, α -D-glucose 1-phosphate was released to complete the reaction (**Figure 13**).

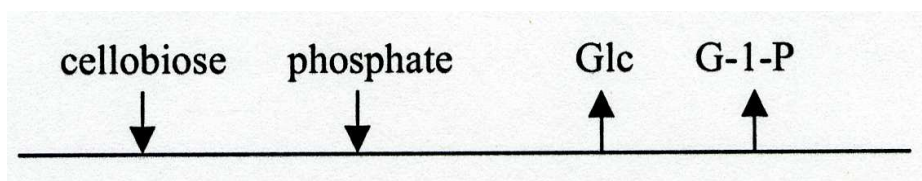


Figure 13 Ordered bi bi mechanism in the phosphorolysis catalyzed by cellobiose phosphorylase
[Kitaoka and Hayashi, 2002]

In this context, a conformational itinerary of the hydrolytic reaction pathway of cellobiose phosphorylase was described by use of computational analysis including docking studies when different conformers of D-glucose and cellobiose were used. It was concluded, that this reaction proceeds from 1S_3 as a phosphorolytic transition state conformer (= pre-TS conformer) for cellobiose via E_3 as a transition state conformer (= TS) for D-glucose, thus leading to G-1-P product which is in a 4C_1 conformation [Fushinobu *et al.*, 2008]. Therefore, it can be confirmed, that in phosphorolytic reaction of cellobiose phosphorylase, the sequence of product release follows at first D-glucose and then α -D-glucose 1-phosphate.

5.6 The azido group as a precursor to amino functionality in saccharides

The small size of the azido group ($-N_3$) suggests its use as a precursor to the amino group in certain enzymatic conversions that naturally process substrates containing only hydroxyl substituents. A number of examples for the substrate behavior of azido carbohydrate derivatives in relation to their physiological counterparts are given in Chapter 6 (p. 50). Thus, azido glucose derivatives can be expected to function in analogy to glucose derivatives, azido cellobiose derivatives in analogy to cellobiose. Hypothetically, azido cellulose derivatives may be formed according to a protocol that normally leads to formation of cellulose by polymerization of cellobiose monomers such as fluorides. Consideration of cellulose-related enzymology shows that enzymes are known and available which catalyze appropriate conversions with cellobiose and D-glucose, as well as the polymerization of cellobiose units to form cellulose.

5.7 Acceptor specificity of cellobiose phosphorylase

In a number of cases, the acceptor D-glucose can be replaced by C2 and C6 derivatives of D-glucose such as 2-deoxyglucose, 2-amino-2-deoxy-D-glucose, 6-deoxyglucose, D-mannose and D-xylose [Alexander, 1961; Alexander 1968], thus the enzyme can catalyze the formation of corresponding disaccharide analogs to cellobiose, although less effectively than D-glucose. Weak acceptor activity was also demonstrated for D-arabinose, L-fucose, L-galactose and D-altrose [Alexander, 1968]. This also indicates that the hydroxyl groups at C2 and C6 of the D-glucose molecule play more of a role in binding of D-glucose to the active site of the enzyme than in catalysis [Kitaoka *et al.*, 1992].

Derivatives at C1 such as methyl-D-glucosides cannot serve as acceptors; neither can epimers at C3 (D-allose), C4 (D-galactose) and C5 (L-idose). These results indicate that the D-glucose configuration at C3, C4 and C5 is strictly required (**Figure 14**) [Kitaoka and Hayashi, 2002; Kitaoka *et al.*, 1992].

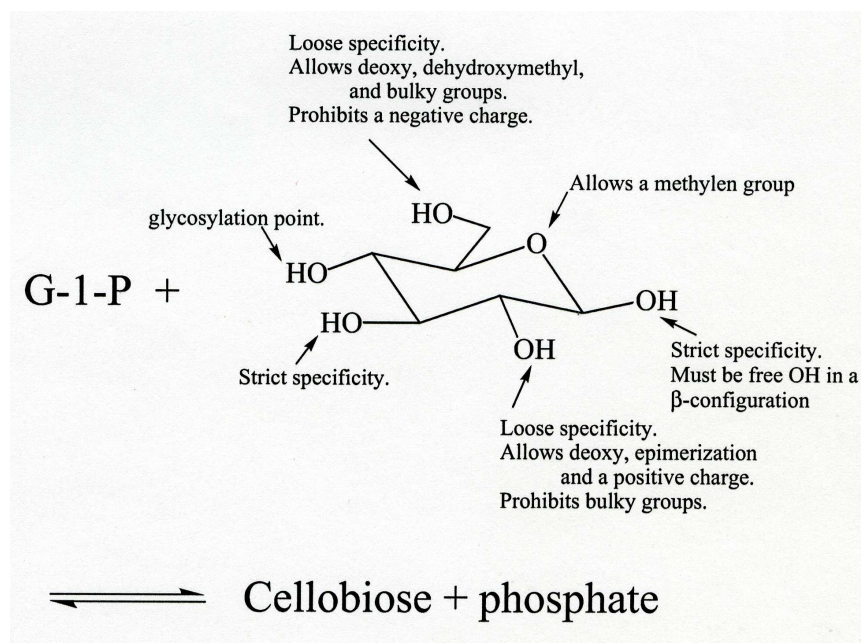


Figure 14 Summary of the acceptor specificity of cellobiose phosphorylase [Kitaoka and Hayashi, 2002]

Also, other experiments such as with the bacterium *Cellulomonas uda* [Nidetzky *et al.*, 2000] have been conducted in order to investigate the binding of various fluorinated analogs of α -D-glucose 1-phosphate or D-glucose to cellobiose phosphorylase. It has been shown that α -D-glucopyranosyl fluoride can function as a donor substrate in the synthetic mode of the cellobiose phosphorylase catalysis [Nidetzky *et al.*, 2004]. As the enzyme is unable to convert the reaction products (fluoride ion and cellobiose) back into substrates, the reverse reaction with α -D-glucopyranosyl fluoride was considered as “glycosynthase” type reaction, where the altered substrate triggers an increase in product yields.

In the reverse direction, it was shown that the reaction using β -glucose as the acceptor was significantly faster than that with α -glucose. In order to confirm this recognition pattern, two pseudo-glucoses, with the structure of a glucopyranose were examined as acceptors [Kitaoka *et al.*, 1993]. It was shown, that cellobiose phosphorylase was able to recognize only the pseudo- β -glucopyranose and not the pseudo- α -glucopyranose, indicating that this enzyme recognizes the β -anomeric hydroxyl group of the acceptor molecule (**Figure 15**) [Kitaoka and Hayashi, 2002]. However, it is not clear whether the cellobiose phosphorylase also utilizes α -D-glucose or not, because of the occurrence of mutarotation.

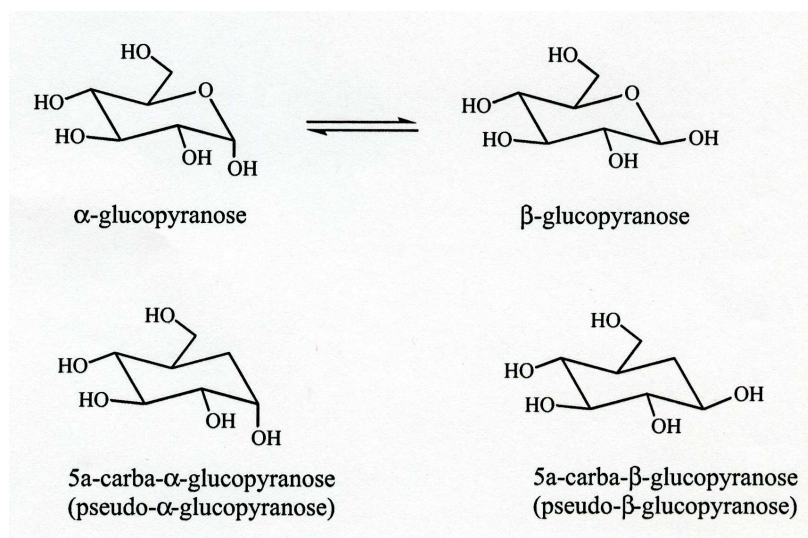


Figure 15 Structures of the pseudo-glucoses [Kitaoka and Hayashi, 2002]

In the reverse reaction, substrate inhibition was examined by the use of D-glucose, D-glucosamine and 6-deoxyglucose as the acceptors, with D-glucose displaying the strongest level of inhibition. The D-glucose substrate inhibits cellobiose phosphorylase activity in *Cellvibrio gilvus*, *Clostridium thermocellum* and *Thermotoga maritima* [Sallam *et al.*, 2006]. This phenomenon was probably caused by the competitive inhibition of the substrate D-glucose towards α -D-glucose 1-phosphate (**Figure 16**) [Kitaoka and Hayashi, 2002].

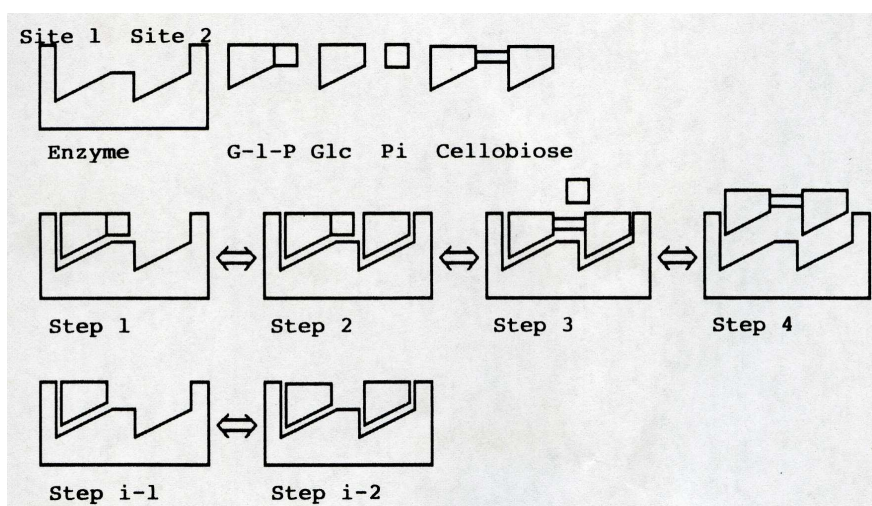


Figure 16 Mechanism of competitive substrate inhibition

As indicated in Figure 16, the synthetic reaction proceeds through the following four steps: in step 1, α -D-glucose 1-phosphate (G-1-P) binds to the enzyme at site 1, and in step 2, D-glucose binds (Glc) to the enzyme-G-1-P complex at site 2. In step 3, P_i is released from the enzyme-substrates complex, while in step 4, cellobiose is released from the enzyme-cellobiose complex. The inhibition involves the following two steps: in the step i-1, D-glucose binds at site 1 instead of G-1-P. In step i-2, another D-glucose molecule binds to the enzyme-glucose complex at site 2 to form a dead end complex [Kitaoka *et al.*, 1992].

5.8 Synthesis of oligosaccharides using cellobiose phosphorylase from *Cellvibrio gilvus*

Cellobiose phosphorylase from *Cellvibrio gilvus* is the most studied enzyme, consisting of 822 amino acid residues. In order to better understand the function of this enzyme in practical oligosaccharide synthesis, the determination of its three-dimensional structure is important. Therefore, X-ray crystallography experiments for the three-dimensional structure of two sugar-anion complexes (glucose-sulphate and glucose-phosphate) of *Cellvibrio gilvus* cellobiose phosphorylase have been conducted. It was possible to obtain two protein-sugar-anion complex structures, with SO_4 and PO_4 located at the anion binding site, and a glucose moiety located at subsite +1, taking a chair conformation. The binding affinity of subsite -1 for D-glucose is much weaker. Subsites -1 and +1 in the phosphorolytic reaction of cellobiose phosphorylase act as sugar-donor and sugar-acceptor sites in the synthetic reaction, respectively. It is also known that glycerol occasionally occupies sugar-binding subsites (**Figure 17 A/B**). [Hidaka *et al.*, 2006]

The sugar ring at subsite -1 may form a reaction intermediate with an oxocarbenium ion-like conformation during phosphorolysis [Hidaka *et al.*, 2004], the shift of the C-4, C-5 and C-6 positions are likely to be relatively small and thus the structure of the complex with glycerol does not conflict with the reaction mechanism.

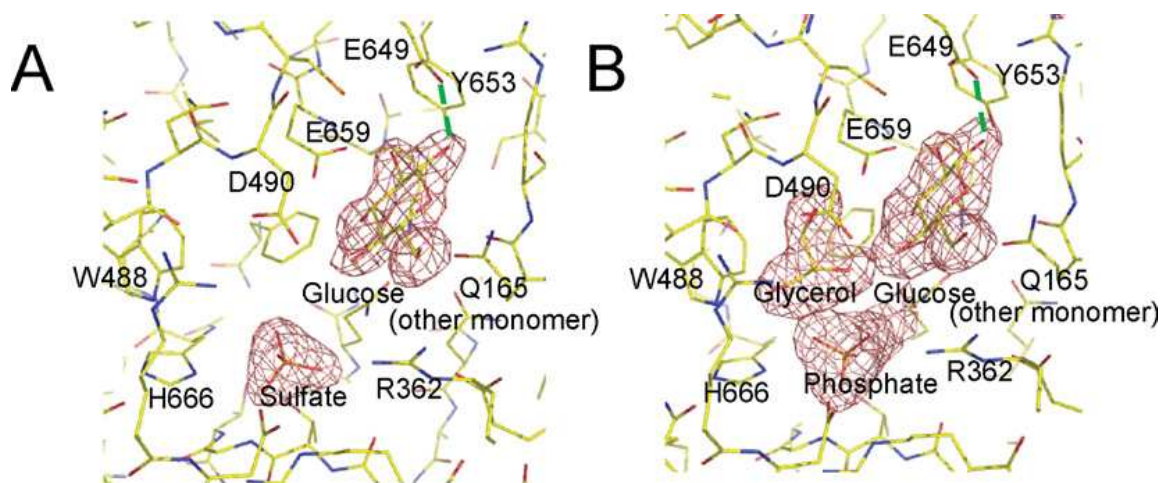


Figure 17 A/B Model of CBP-SO₄ and CBP-PO₄ [Hidaka *et al.*, 2006]

Among the highly conserved residues involved in anion recognition are Arg³⁵¹, His⁶⁶⁶, Thr⁷³¹, Gly⁷³² and Thr⁷³². The orientations of the bound SO₄ and PO₄ are different, in that one P-O bond points to His⁶⁶⁶, whereas one S-O bond points in the opposite direction (**Figure 18**) [Hidaka *et al.*, 2006].

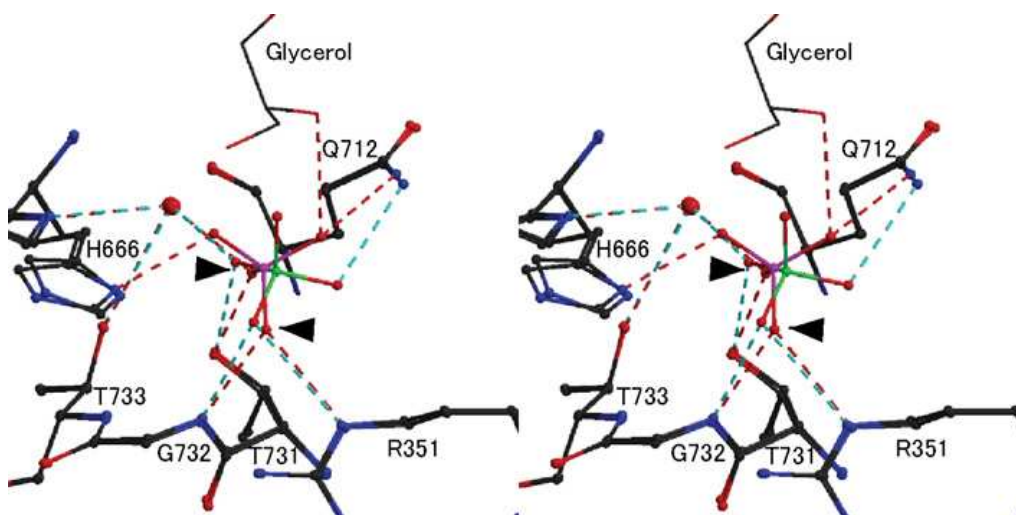


Figure 18 Stereo view of the anion binding site of CBP-SO₄ and CBP-PO₄ [Hidaka *et al.*, 2006]

In PO₄ recognition, three hydrogen bonds, those with the side-chain hydroxyl group of Thr⁷³¹, the main-chain NH group of Gly⁷³², and the N^εH group of Arg³⁵¹, bind to two of the four oxygen atoms of PO₄ (indicated by arrowheads in Figure 18). The other two oxygen atoms, positioned differently from those of SO₄, form two additional hydrogen bonds, one with the N^δ atom of

His⁶⁶⁶ and the other, which is held via Gln⁷¹² and glycerol, therefore appearing to be suitable for the direct attack on the C-1 atom of D-glucose at subsite -1. Using the structure of the cellobiose phosphorylase-PO₄ complex, it can be postulated that the reaction mechanism of inverting phosphorylase begins with direct nucleophilic attack by phosphate on the anomeric center [Hidaka *et al.*, 2004].

As already mentioned, the enzyme cellobiose phosphorylase is absolutely specific for the cleavage and synthesis of $\beta(1\rightarrow4)$ glucosidic bonds and it also exhibits a relaxed specificity with respect to the reducing sugar that has a function as a glucosyl acceptor in the synthetic reaction [Kitaoka *et al.*, 1992]. Using this relaxed substrate specificity, a number of novel β -1,4-glycosyl products can be synthesized as described below.

In this context, it was shown that disaccharides having a structure comprising of a “glucose bound by another saccharide at the position 6” could serve as the acceptor due to the loose specificity at position 6. Therefore, disaccharides such as isomaltose (Glc α 1-6Glc), gentiobiose (Glc β 1-6Glc) and melibiose (Gal α 1-6Glc) from *Cellvibrio gilvus* were examined as acceptors. Cellobiose phosphorylase was able to transfer a β -glucosyl residue at the reducing end to position 4 of the glucose moiety of the respective disaccharides to form the corresponding branched trisaccharides as expected. Thus, melibiose, gentiobiose, isomaltose and also the monosaccharide glucuronamide were found to react with cellobiose phosphorylase and glucose-1-phosphate giving β -D-Glcp-(1 $\rightarrow$ 4)-[α -D-Galp-(1 $\rightarrow$ 6)]-D-Glcp, β -D-Glcp-(1 $\rightarrow$ 4)-[β -D-Glcp-(1 $\rightarrow$ 6)]-D-Glcp, β -D-Glcp-(1 $\rightarrow$ 4)-[α -D-Glcp-(1 $\rightarrow$ 6)]-D-Glcp, β -D-Glcp-(1 $\rightarrow$ 4)-D-GlcUNp (**Figure 19**). [Percy *et al.*, 1998]

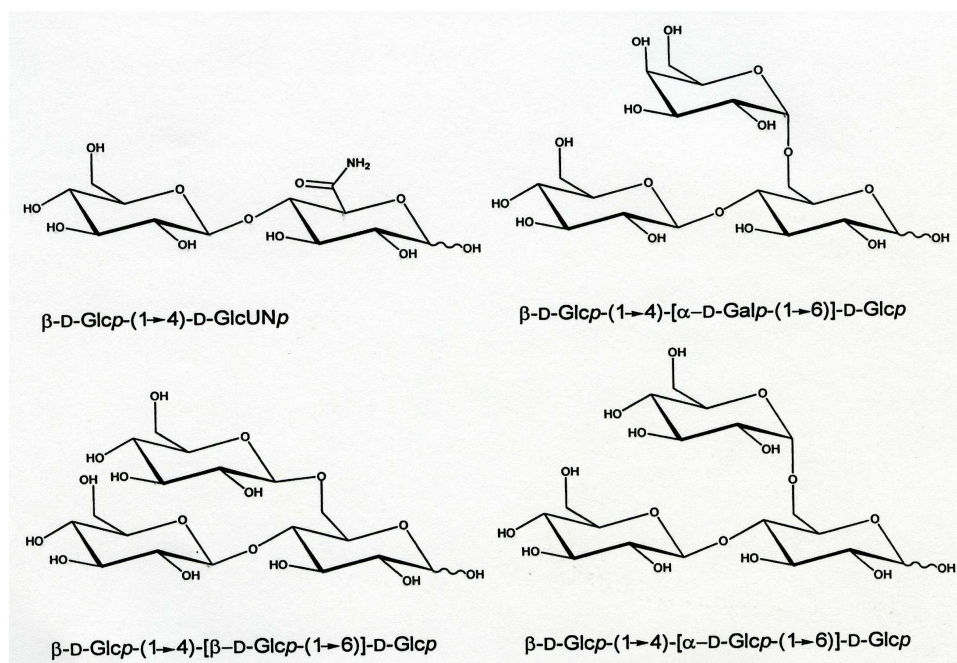


Figure 19 Synthesis of branched trisaccharides using cellobiose phosphorylase [Percy *et al.*, 1998]

In the presence of cellobiose phosphorylase from *Cellvibrio gilvus*, it was also possible to obtain other disaccharides, namely 4-*O*- β -D-glucopyranosyl-D-glucosamine and 4-*O*- β -D-glucopyranosyl-2-deoxy-D-glucose, which were successfully synthesized from equimolar amounts of D-glucosamine and α -D-glucose 1-phosphate, 2-deoxyglucose and α -D-glucose 1-phosphate respectively [Tariq *et al.*, 1995].

5.9 Reverse reaction of cellobiose phosphorylase by a three-enzyme system

In the reverse reaction of cellobiose phosphorylase, cellobiose is synthesized from α -D-glucose 1-phosphate and D-glucose. As a practical process, this reaction has limitations due to the fact that α -D-glucose 1-phosphate is prohibitively expensive. To lower costs, sucrose phosphorylase has been used to generate α -D-glucose 1-phosphate. This enzyme catalyzes the phosphorolytic cleavage of sucrose, a cheap substrate, into fructose and α -D-glucose 1-phosphate, the anomeric configuration of which is identical (α) with the analogous substrate/product of cellobiose phosphorylase. Therefore, using sucrose and D-glucose as raw materials with a catalytic amount of phosphate, a process can be developed for the production of cellobiose and fructose, catalyzed by the combined action of sucrose phosphorylase and cellobiose phosphorylase.

This reaction has still two problems: 1. the formation of the equimolar amount of fructose as a byproduct and 2. the competitive inhibition by D-glucose toward α -D-glucose 1-phosphate for both enzymes, causing a considerable decrease in the activities of the two enzymes. In order to solve this problem, xylose isomerase was added, an enzyme used industrially to produce high fructose corn syrup by the isomerization of glucose into fructose. In the reaction mixture, containing sucrose and the three enzymes (sucrose phosphorylase, xylose isomerase and cellobiose phosphorylase), the following sequence of reaction takes place: the initial phosphorolysis of sucrose into α -D-glucose 1-phosphate and fructose is catalyzed by sucrose phosphorylase. The fructose formed is converted into D-glucose by xylose isomerase and finally, cellobiose and phosphate are generated from the α -D-glucose 1-phosphate and the D-glucose by cellobiose phosphorylase. Since the phosphate (P_i) is recycled in the reaction of sucrose phosphorylase, it was predicted that sucrose would be converted into cellobiose in one step. As the system was investigated, it was found that cellobiose was formed from sucrose in the presence of a catalytic amount of phosphate as predicted. The yield of cellobiose was 76%, higher than the equilibrium yield using α -D-glucose 1-phosphate as the starting material (64%). This higher yield was attributed to the high energy of the β -fructofuranosyl linkage in the sucrose molecule. The three-enzyme system is applicable in the industrial production of cellobiose because of the high yield and the inexpensive raw material, sucrose. [Kitaoka and Hayashi, 2002]

Also starch seems to be an attractive alternative for the production of cellobiose due to its easy availability with low costs. This type of cellobiose production includes at first α -D-glucose 1-phosphate synthesis from starch by glucan phosphorylase. As the presence of P_i is not beneficial for the following cellobiose synthesis due to the application of cellobiose phosphorylase, selective removal of P_i is required which can be achieved via electrodialysis and by precipitation of it as magnesium ammonium phosphate by addition of ammonia water under alkaline conditions (pH = 8). Cellobiose synthesis takes place by incubation of separated α -D-glucose 1-phosphate and D-glucose using recombinant cellobiose phosphorylase from *Cellvibrio gilvus*. In comparison to above mentioned three-enzyme system, the yield from starch of 23.7% is low compared to cellobiose from sucrose (76%). Nevertheless, if the sugar which was obtained by glucan phosphorylase reaction, so called phosphorylase limit dextrin (PLD), with roughly 60%

yield, is converted to D-glucose and this one is used in cellobiose phosphorylase reaction, than the cellobiose yield from starch can be more than double. [Suzuki *et al.*, 2009]

5.10 Synthesis of 2^{II}-deoxycellobiose by use of cellobiose phosphorylase with D-glucal

In the study of Kitaoka *et al.* [Kitaoka *et al.* 2006] application of cellobiose phosphorylase from *Cellvibrio gilvus* has revealed, that in the reverse reaction, this enzyme catalyzes the production of heterodisaccharides such as 2-deoxy- β -D-arabino-hexopyranosyl-(1 $\rightarrow$ 4)-D-glucose (= 2^{II}-deoxycellobiose) from various acceptor sugars (D-glucose, D-xylose, D-mannose and 2-deoxy-D-arabino-hexose) when D-glucal is used as a donor substrate. It was confirmed, that the acceptor specificity for the D-glucal reaction was identical to that of the α -D-glucose 1-phosphate reaction, whereas the activity of D-glucal was approximately 500 times less than that of α -D-glucose 1-phosphate, using 10mM substrates. In addition, *Cellulomonas uda* cellobiose phosphorylase was found not to react with D-glucal at all [Nidetzky *et al.*, 2000].

6. Applications of azido sugars

Due to the fact that little is known about azido derivatives of D-arabinose [Shen, 1970; Hollenberg *et al.*, 1977; Buchanan and Clark, 1977] it appeared necessary to synthesize azido derivatives of D-arabinose. In this context, regiospecific syntheses of azidodeoxy and diazidodideoxy derivatives of methyl α -D-arabinofuranoside have been conducted with the aim to find inhibitors of Gram-negative bacterial lipopolysaccharide biosynthesis [Lee *et al.*, 1979]. Further studies have been conducted with adenine arabinoside analogs, such as 2'-azido- and 2'-amino-2'-deoxy- β -D-arabinofuranosyladenine [Lee *et al.*, 1979]. In this context, 9- β -D-Arabinofuranosyladenine (araA) is described as a purine nucleoside with activity against DNA viruses [Keeney and Buchanan, 1975] and experimental neoplasms [York and LePage, 1966; Koshiura and LePage, 1968]. This compound was also investigated for its ability to serve as a cancer chemotherapeutical agent in man [LePage *et al.*, 1973]. However, due to rapid deamination of araA by adenosine deaminase to 9- β -D-arabinofuranosylhypoxanthine, which has no anticancer activity, araA shows limited efficiency as antitumor agent [York and LePage, 1966; Brink and LePage, 1964]. The development of potential inhibitors for adenosine deaminase by structural modification of araA, thus prevent or minimize deamination, should

provide attempts to solve the problem of rapid inactivation [Montgomery and Hewson, 1957; Brockman *et al.*, 1977].

In another study [Lee *et al.*, 1979], it was possible to describe an approach for the modification of 1- β -D-arabinofuranosylcytosine to 2'-azido- and 2'-amino-2'-deoxy- β -D-arabinofuranosylcytosine which resulted in agents with antineoplastic activity and resistance to inactivation by deamination by cytidine deaminase.

In this context, arazide and aramine have been compared with araA as substrates for adenosine deaminase of cultured L1210 leukemia cells and it was shown, that both of these agents were inferior to araA. However, araA was 2.9 times better than arazide as a substrate for the enzyme from L1210 cells. Aramine was also less efficient as a substrate for adenosine deaminase than araA, but superior to arazide.

Similar studies were conducted comparing antineoplastic efficiency of arazide with that of araA against the P388 leukemia by use or absence of the adenosine deaminase inhibitor, 2'-deoxycoformycin [Lee *et al.*, 1981].

It was possible to show, that the antineoplastic activity of arazide in combination with adenosine deaminase inhibitor, 2'-deoxycoformycin is remarkable superior to that of araA and 2'-deoxycoformycin when administrated once daily for six days. This finding corresponds to relatively long lasting inhibition of DNA synthesis in P388 leukemia cells by the combination of arazide and 2'-deoxycoformycin.

Also studies on enzymatic synthesis of 9-azido-9-deoxy derivatives of N-acetylneuraminic acid (5-acetoamido-9-azido-3, 5, 9-trideoxy-D-glycerol-D-galacto-2-nonulosonic acid) have been conducted [Brossmer *et al.*, 1980].

The enzyme N-acetylneuraminate synthase from *Neisseria meningitidis* 60E was used to catalyze the conversion of phosphoenolpyruvate and 2-acetamido-6-azido-2, 6-dideoxy-D-mannose into 5-acetamido-9-azido-3, 5, 9-tri-deoxy-D-glycerol-D-galacto-2-nonulosonic acid. In this context, it was possible to use the commercially available [14 C] phosphoenolpyruvate as a cofactor to 5-acetoamido-9-azido-3, 5, 9-trideoxy-D-glycerol-D-galacto-2-nonulosonic acid (NeuAc 9-azide), which is a substrate of the reaction catalyzed by CTP:CMP-N-acetylneuramine cytidyltransferase (CMP-NeuAc synthetase). Therefore, the enzyme-catalyzed conversion of 2-acetamido-6-azido-2,6-dideoxy-D-mannose (ManNAc 6-azide) to NeuAc 9-azide can be used for the preparation of [14 C]- NeuAc 9-azide, which can be applied for studies of glycoconjugate biosynthesis.

IV. AIM OF THE WORK

The ultimate goal of the present work is to develop excipients alternative to chitosan, the only available and frequently applied cationic biomimetic polymer. Chitosan possesses several disadvantages such as the low solubility in water, the difficulty of its purification as well as the existence of traces of allergens. In order to overcome these disadvantages, it became of interest to develop alternative cationic polysaccharides with a broad spectrum of scientific applications. Among the methods available for the synthesis of artificial cellulose derivatives, the enzyme-catalyzed polymerization of cellobiose analogs is an attractive alternative. Therefore, the present dissertation aims at syntheses of cellobiose analogs bearing protected nitrogen functionality. The azido function can be considered as precursor to an amino group, and while amino groups are strongly basic and incompatible with enzyme-catalyzed processes, the azido function behaves similar to a hydroxyl group in many systems. Therefore, the present dissertation describes approaches to cellobiose analogs containing azido functions in positions 6, 6' and 2. Primarily, methods of combined organic-chemical and enzyme-catalyzed chemistry have been studied. However, a primary goal of the present dissertation was the use of green chemistry methods. Hence there is a strong emphasis of enzyme-catalyzed synthesis, for which a special source of the cloned enzyme cellobiose phosphorylase has been developed. The obtained products will have potential applications in pharmacy and medicine as pharmaceutical excipients and prebiotic dietary supplements. The cellobiose precursors could also serve as intermediates for syntheses of anti-inflammatory agents. Thus, these enzyme based approaches should open up innovative synthetic strategies, including improvements and scale-up of the syntheses of cellobiose analogs with azido substituents in the non-reducing glucose moiety (C-6') as well as in the reducing glucose moiety (C-2 and C-6). For preliminary work, crude as well as genetically recombinant cellobiose phosphorylase from *Clostridium thermocellum* strain NCIMB 10682 has been applied in this study.

In summary, the present work shows strategies for the preparation of precursors to azido cellulose derivatives. If successfully developed to a technical scale, such azido polysaccharides will be of value for biotechnological applications of both academic and industrial interest.

V. EQUIPMENTS

AnaeroGen[™] Oxoid AN0025A, Oxoid Limited, U.K.

Analytical balance, MC210P, 210 g max., Sartorius, Germany

Centrifugal Filter, Amicon Ultra, Ultracel-50K, UFC905008, Millipore, Ireland

Dialysis tubing cellulose membrane, D9402, Sigma Aldrich, Germany

Disposable Cuvettes, 222S, Sterilin, U.K.

Econo Column Pack A, 737-6601, BioRad, Germany

Electrophoresis chamber, PeqLab, Germany

Electrophoresis Power Supply-EPS 600, Pharmacia Biotech, Germany

Fraction Collector, LKB FRAC-100, Pharmacia, Germany

Glass chamber for TLC, DESAGA Heidelberg, Germany

Glass Pasteur Pipettes, 747720, Brand, Germany

GSA Rotor, Sorvall Instruments, Germany,

Heating oven, Heraeus Instruments, Germany

Hermle Z 323K, table centrifuge, Germany

Heto Power Dry, Freeze Dryer LL3000, Thermo Scientific, U.S.A.

Incubator, Memmert, Typ500, Germany

Laminar flow work bench, Waldner Electronics, FAZ3, Germany

Lightweight film processor, CP1000, AGFA

Micro Bio-Spin® Chromatography Columns, 732-6204, BioRad, Germany

Micropipettes 10 µl, 708709, Blau Brand, Germany

Microprocessor pH Meter, pH211, Hanna Instruments, Germany

MyCycler[™] Thermal Cycler, 170-9703, BioRad, Germany

Parafilm “M”, Laboratory Film, Chicago U.S.A.

Pipetman P10, F144802, Gilson, U.S.A

Pipetman P1000, F123602, Gilson, U.S.A

Plastic tubes 5 ml and 50 ml, Sterilin, U.K.

RC5C centrifuge, Sorvall Instruments, Germany

Safe Lock Tubes 1.5 ml, Eppendorf, Germany

Scotsman AF 100, ice machine

SS34 Rotor, Sorvall Instruments, Germany

Starsonic 60, degassing equipment, Liarre, Italy

TE 77 ECL Semi-Dry Transfer Unit, Amersham, U.K.

TLC Silica Gel 60 F₂₅₄, Aluminium sheets 10x20 cm, 1.05570.0001, Merck, Austria

U-1100 Spectrophotomer, Hitachi, Japan

Ultraturrax high performance disperser (Homogenizer), GLH-795 220V, OMNI International, U.S.A.

VARIOKLAV Typ 400, Germany

Vivaspin ultrafiltration spin columns 20 ml, Sartorius, Germany

Vortex, Heidolph, REAX top

VI. MATERIALS

1. Chemicals

Chemicals were prevalently purchased from BioRad, Glycon, Fermentas, Fluka, Merck, Metabion, Oxoid, Sigma Aldrich and Qiagen.

2. Bacterial strain

The Gram-positive strain of *Clostridium thermocellum* with accession number 10682, which has been used throughout this study, was purchased from NCIMB Ltd., Aberdeen, Scotland.

3. Mono- and Disaccharides

The following sugars have been used in this study:

2-Deoxy-D-glucose (D8375, Sigma Aldrich, Germany)

6-Deoxy-D-glucose (D9761, Sigma Aldrich, Germany)

D-(+) Xylose (X-3877, Sigma Aldrich, Germany)

D-(+)-Glucose (G7528, Sigma Aldrich, Germany)

Cellobiose (S96030, Glycon, Germany)

All azido sugars have been synthesized either chemically or enzymatically throughout this study.

4. Column chromatography materials

Column materials used in purification steps have been purchased:

Microgranular DEAE cellulose for anion exchange chromatography (4057050, DE52 Diethylaminoethyl cellulose, Whatman, U.K.)

Sephacryl® 200HR as suspension for gel filtration (S200HR, Sigma Aldrich, Germany), which was stored at 4°C in 20% EtOH

Silica gel 60 (0.063-0.200 mm) (1.07734.2500, Merck, Austria)

5. Buffers for column chromatography

Chemicals for buffers used in DEAE and Sephacryl purification step, were the following:

Trizma® base (T1503, Sigma Aldrich, Germany)

Sodium chloride extra pure (1.06400.1000, Merck, Austria)

6. Column material and buffer for Ni-NTA chromatography

Ni-NTA Agarose (30210, Qiagen, Austria)

Resuspension buffer pH 7.5:

2.3 g 50mM NaH₂PO₄*2H₂O, 0.055 g 1.4mM β-mercapthoethanol, 8.76 g 300mM NaCl, 0.68 g 20mM imidazole in 500 ml distilled H₂O

Elution buffer pH 7.5:

2.3 g 50mM NaH₂PO₄*2H₂O, 0.055 g 1.4mM β-mercapthoethanol, 8.76 g 300mM NaCl, 5.1 g 150mM imidazole in 500 ml distilled H₂O

7. Column material and buffer for Bio Gel

Bio Gel® P-2 Gel Fine (150-4114, BioRad, Germany)

8. Kits

Kits were preferably used in cloning steps.

InstaGene Matrix (732-6030, BioRad, Austria)

PCR Master Mix (2x) (K0171, Fermentas, Austria)

Gel Extraction Kit (28704, Qiagen, Austria)

High Fidelity PCR enzyme Mix (K0191, Fermentas, Austria)

GeneJet™ Plasmid MiniPrep Kit (K0502, Fermentas, Austria)

Plasmid Midi Kit (12143, Qiagen, Austria), including buffers:

Buffer P1 (resuspension buffer) 50mM Tris pH 8, 10mM EDTA, 100μg/ml RNase A

Buffer P2 (lysis buffer) 200mM NaOH, 1% SDS (w/v)

Buffer P3 (neutralization buffer) 3M potassium acetate pH 5.5

Buffer QBT (equilibration buffer) 750mM NaCl, 50mM MOPS pH 7, 15% isopropanol (v/v), 0.15% Triton® X-100 (v/v)

Buffer QC (wash buffer) 1M NaCl, 50mM MOPS pH 7, 15% isopropanol (v/v)

Buffer QF (elution buffer) 1.25M NaCl, 50mM Tris pH 8.5, 15% isopropanol (v/v)

QIAexpress® Anti-His HRP Conjugate Kits (34698, Qiagen, Austria)

Immun-Star Chemiluminescent Kits (170-5070, BioRad, Germany)

9. Culture medium for *Clostridium thermocellum* NCIMB 10682 cells

All ingredients have been purchased from Merck, except cellobiose (S96030, Glycon, Germany), microcrystalline cellulose, AVICEL Cellulose (Pharmatrans Sanaq, Germany) and yeast extract (LP0021, Oxoid, U.K.)

Cellobiose	12 g
AVICEL Cellulose	10 g
Yeast extract	2 g
(NH ₄)SO ₄	1.3 g
KH ₂ PO ₄	1.5 g
K ₂ HPO ₄ * 3 H ₂ O	2.9 g
MgCl * 6 H ₂ O	1 g
CaCl ₂	0.15 g
Distilled water	1000 ml

The pH of the prepared culture medium was adjusted to 7.8 with NaOH and the medium autoclaved at 121°C. Finally, freshly prepared and filter sterilized reductants solution (5ml/100ml culture medium), containing 0.5 g of L-cystein and 5 g of NaHCO₃ in 50 ml distilled water was added to the cool culture medium.

For the protection of cellobiose phosphorylase during sonication, PMSF (phenylmethylsulfonyl fluoride) has been used, which inhibits serine proteases such as trypsin and chymotrypsin (P7626, Sigma Aldrich, Germany).

10. Culture medium for BL21 (DE3) *E. coli* cells

The BL21 (DE3) competent cells were grown using 1 l of LB medium pH 7.5:

Bacteriological peptone	1 g
NaCl	10 g
Yeast extract	5 g

All ingredients were purchased as mentioned previously, except bacteriological peptone (097045/121, Lab M Limited, U.K.).

For the induction of expression of the *lac* operon in *Escherichia coli* BL21 (DE3) cells, IPTG (Isopropyl β -D-thiogalactoside) was used (I5502, Sigma Aldrich, Germany).

The antibiotic used in this study was kanamycin sulfate from *Streptomyces kanamyceticus* (K1377, Sigma Aldrich, Germany).

11. Buffers and solutions for SDS polyacrylamide gel electrophoresis (SDS PAGE)

Laemmli buffer:

0.3 g Tris, 0.8 g SDS, 2.35 g 20% glycerol, 0.05 g bromophenol blue in 10 ml distilled H₂O

4x separating buffer pH 8.8:

18 g Tris, 0.4 g SDS in 100 ml distilled H₂O

4x stacking buffer pH 6.8:

6 g Tris, 0.4 g SDS in 100 ml distilled H₂O

Anode buffer:

3 g Tris, 1 g SDS in 1000 ml distilled H₂O

Cathode buffer:

3 g Tris, 1 g SDS, 14.41 g glycine in 1000 ml distilled H₂O

Staining buffer:

PhastGel® Blue R (17-0518-01, Pharmacia Biotech, Sweden)

Destaining buffer:

75 ml acetic acid (99.8%), 208.3 ml ethanol (96%) in 1000 ml distilled H₂O

TEMED (N, N, N', N'-tetramethylethylenediamine) (35925, Serva, Austria)

APS (Ammonium peroxodisulphate) (1201.0100, Merck, Austria)

Dithiothreitol (43819, Fluka, Germany)

Precision Plus Protein™ standard, unstained (161-0363, BioRad, Germany)

12. Solutions for Lowry test

Reagent A, *D_C* Protein Assay (500-0113, BioRad, Germany)

Reagent B, *D_C* Protein Assay (500-0114, BioRad, Germany)

Chicken egg white albumin (A5503, Sigma Aldrich, Germany)

13. Solution for D-glucose test

The D-glucose test kit was purchased (10 716 251 035, r-biopharm/Boehringer Mannheim, Germany).

14. Buffers and solutions for western blot

Blotting buffer:

200 ml 20% methanol, 100 ml cathode buffer in 1000 ml distilled H₂O

TBST buffer:

87.7 g 1xTBS-buffer, 0.3% Tween20 in 1000 ml distilled H₂O

15. Plasmid vector and bacterial strains for cloning

pET-26b(+)plain vector and bacterial strains (chemocompetent DH5α *E.coli* and BL21 (DE3) *E. coli* cells) for cloning were kindly provided by the Department of Pathophysiology of the Medical University of Vienna.

16. Primers

All primers were designed using the software “Vector NTI” and were purchased from Metabion, Germany.

5′-CCA TGG AGT TCG GTT TTT TTG ATG AT-3′ (**CeP ATCC fwd**)

5′-ATA TCC CAC TCG AGC TTC AAC TTG TGA GTC T-3′ (**CeP ATCC rev**)

5′-GGA TTT GTA CAC CAG ATA CCC GCA AGA GCA-3′ (**CeP mut rem fwd**)

5′-AGA CGC AAA ACG CCA CGG AGA AGC TA-3′ (**CeP ATCC Nco rem fwd**)

5′-TAG CTT CTC CGT GGC GTT TTG CGT CT-3′ (**CeP ATCC Nco rem rev**)

5′-CGC CAA TCC TGA TAT AGT TCC TCC TTT CAG -3′ (**T7 seq fwd**)

5′-GAT GCG TCC GGC GTA GAG GAT CGA -3′ (**T7 seq rev**)

5′-GGC TCG GTT ATC TAT CAC AAG ACA GAG -3′ (**CeP ATCC seq rev**)

17. Restriction digest

FastDigest®NcoI (FD0573, Fermentas, Austria)

FastDigest®XhoI (FD0694, Fermentas, Austria)

FastDigest®Bsp1407I (ER0931, Fermentas, Austria)

18. Ligation

T4 ligase (EL0014, Fermentas, Germany)

Ligation buffer (K1422, Fermentas, Germany)

19. Other media

TBE buffer (5x):

53 g Tris, 27.5 g boric acid, 20 ml 0.5M EDTA pH 8 in 1000 ml distilled H₂O

SOC buffer:

20 g bacterial tryptophan, 5 g yeast extract, 0.5 g NaCl, 0.186 g KCl, 2.408 g MgSO₄, 3 g D-glucose in 1000 ml distilled H₂O

α-D-Glucose 1-phosphate disodium salt tetrahydrate (49230, Fluka, Germany)

Nuclease free water (R0581, Fermentas, Germany)

DNA ladder for agarose gel (SM1331, Fermentas, Germany)

VII. METHODS

1. Preparation of *Clostridium thermocellum* NCIMB 10682 glycerol stock

For the preparation of the glycerol stock, 5 ml of *Clostridium thermocellum* NCIMB 10682 cells (NCIMB Ltd.) were added to 10 ml sterile culture medium under sterile conditions. The cells were grown for 2 days under anaerobic conditions using AnaeroGen[®] Kits (Oxoid) at 60°C and subsequently centrifuged at 4°C and 8500rpm in a GSA rotor (Sorvall Instruments) for 10 min. The obtained precipitate was mixed with 5 ml of 20% glycerol solution and 5 ml of the sterile culture medium. The suspension was aliquoted in 1 ml cryo tubes and long term stored at -80°C.

2. Fermentation and harvest of *Clostridium thermocellum* NCIMB 10682 cells

2.1 Inoculation of the culture medium

2 ml glycerol stock of *Clostridium thermocellum* NCIMB 10682 cells were thawed at room temperature, vortexed and inoculated in 5 l of culture medium.

2.2 Incubation

The inoculated culture medium was incubated under anaerobic conditions at 60°C for 4 days in an anaerobic vessel, using an AnaeroGen[®] Kit in order to exclude oxygen from the culture medium.

After the 4th day, the bacteria were harvested by centrifugation at 4°C and 10 000rpm in a GSA rotor. Subsequently, the precipitate was collected and stored at -20°C for further applications.

3. Extraction of native cellobiose phosphorylase by sonication

3.1 Enzyme extraction

10 g of the precipitate was mixed with 40 ml of distilled water and 200 µl of 100mM PMSF (Sigma Aldrich) and sonicated by an ultraturrax high performance disperser (OMNI International) at 4°C for 6x10 min with 2 min breaks in-between the sonication phases. The purpose of the sonication is to break down the cell membranes and release protein complexes.

After the sonication step, the suspension was centrifuged at 4°C and 10 000rpm in a GSA rotor for 45 min in order to remove cell debris. Subsequently, the enzyme cellobiose phosphorylase is

found in the supernatant of *Clostridium thermocellum* NCIMB 10682, which was named the “enzyme crude extract”.

3.2 Ultrafiltration of the crude extract

The crude extract was ultrafiltered at 4°C and 3000rpm in a SS34-rotor (Sorvall Instruments) by the use of an Amicon® Ultra-15 centrifugal filter (Milipore) (**Figure 20**). This device enables fast ultrafiltration with the capability for high concentration factors and easy concentrate recovery from diluted and complex samples.

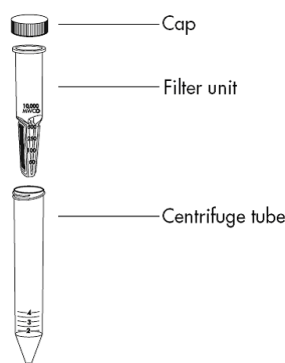


Figure 20 Amicon Ultra 15

Used Nominal Molecular Weight Limit (NMWL) is 50,000 NMWL. The concentrate is collected from the filter device sample reservoir using a pipettor, while the ultrafiltrate is collected in the provided centrifuge tube. Subsequently, the precipitate was collected and stored at -20°C for further applications.

4. Purification of native cellobiose phosphorylase by column chromatography techniques

The development of chromatography techniques and their availability with different selectivities provide a powerful combination for the purification of biomolecules. Most purification protocols require more than one step to achieve the desired level of product purity, whereas each step in this process will cause some loss of product. Therefore, it is desirable to reach the purity and yield of the target protein with the minimum number of steps, and the simplest possible design. Consequently, it is not always efficient to add one step to another until purity requirements are

fulfilled. Occasionally, purity of a target protein can be also achieved efficiently by simply adding or repeating chromatographic steps. [Protein Purification Handbook, 1999]

4.1 Ion exchange chromatography (IEC)

IEC, a binding technique independent of sample volume, offers different selectivities using either anion or cation exchangers. This purification technique is based on the reversible interaction between the charged protein and the oppositely charged chromatographic medium.

In anion exchange chromatography, negatively charged molecules are attracted to a positively charged solid support. Thus, the pH of the separation can be modified to alter the charge characteristics of the sample. [Protein Purification Handbook, 1999]

By increasing the salt concentration, the molecules with the weakest ionic interactions will be eluted earlier in the salt gradient, while those molecules that have a very strong ionic interaction require a higher salt concentration and they will elute later in the gradient. In this study, the chosen pH of the mobile phase buffer was higher than the isoelectric point (pI) of the cellobiose phosphorylase (pI = 5.63). The protein was bound to the anion exchanger, and formed a strong ionic interaction with the positively charged solid support. Thus, a high salt concentration was required for the elution of the protein.

4.2 Optimization of conditions for the purification of cellobiose phosphorylase

For the purification procedure for cellobiose phosphorylase from crude extracts of *Clostridium thermocellum* NCIMB 10682, implementation was attempted of similar methods and techniques as described in literature: particular protectants were used for maintaining enzyme activity, e.g., saturated protamine and ammonium sulfate solution [Alexander, 1968; Tanaka *et al.*, 1995] or dithiothreitol and 2-mercapthoethanol [Sasaki *et al.*, 1983] respectively. As these preliminary attempts completely failed due to great loss of protein activity, it became crucial to set up suitable conditions for the purification of the native cellobiose phosphorylase by determination of optimal concentrations for Tris buffer and NaCl, respectively, without any addition of above mentioned protectants.

4.2.1 Optimization of mobile phase buffer

In these preliminary experiments, minor sample volumes were applied and instead of a convenient column, 15 ml plastic tubes (Sterilin) were used to perform anion exchange chromatography. Total amounts of DE52 DEAE-Cellulose (Whatman) were 1.5 g and 3 g respectively. Both were compared in order to examine if the amount of column material employed influences the purification procedure. Initially, 20, 50 and 100mM of mobile phase buffer (= starting buffer) with a pH range between 5 and 7.5 were prepared. In order to equilibrate the column material, 10 ml of each buffer were added to defined amount of DE52 DEAE-Cellulose in a conical glass and stirred for 30 min at 4°C and 250rpm. After this equilibration step, DEAE-Cellulose was then transferred into a 15 ml plastic tube and washed 10 times with 10 ml of each starting buffer, using a table centrifuge (Hermle) for 2 min, at 4°C and 3000rpm. The supernatant of each sample was discarded, avoiding losses of the column material. After the washing step, 1.5 ml of the crude extract of *Clostridium thermocellum* NCIMB 10682 cells, which was sonicated and prepared as described previously, were added to equilibrated and washed DEAE-Cellulose. The suspension was vortexed and centrifuged for 20 min at 4°C and 1000rpm, using the table centrifuge. Subsequently, the obtained supernatant was transferred with a Pipetman P1000 (Gilson) into an Eppendorf tube and centrifuged for additional 15 min, at 4°C and 12 000rpm in order to remove traces of column material. 1 ml of each clear supernatant was incubated with 1.2 ml of cellobiose (0.1M) and 0.8 ml of phosphate buffer pH 7 (0.1M) for 12 h at 37°C in order to assay for the formation of D-glucose. Enzyme crude extract without any buffer treatment, only dissolved in distilled water, was similarly incubated and served as positive control. All samples were analyzed by Lowry test (BioRad) as well as by D-glucose test (r-biopharm) according to the working protocols described in chapters 6 (p.67) and 7 (p.68) respectively.

4.2.2 Optimization of elution buffer

In order to elute cellobiose phosphorylase from the DEAE-Cellulose, elution buffers with a salt concentration between 0.05 and 1200mM have been tested. Initially, 1.5 ml of each prepared elution buffer was applied onto previously treated DEAE-Cellulose, then vortexed and centrifuged for 15 min, at 4°C and 12 000rpm in order to precipitate traces of column material. 1 ml of each clear supernatant was incubated with 1.2 ml of cellobiose (0.1M) and 0.8 ml of

phosphate buffer pH 7 (0.1M) for 12 h at 37°C in order to determine the amount of D-glucose, which should be formed during the phosphorolytic reaction. Enzyme crude extract without any buffer treatment, only dissolved in distilled water, was analogously incubated and served as positive control. All results have been analyzed by Lowry test (BioRad) as well as by D-glucose test (r-biopharm) considering the working protocol as described in chapter 6 (p.67) and chapter 7 (p.68).

4.3 Sample and IEC column preparation

All solvents were degassed by use of Starsonic (Liarre) and all purification steps were conducted at 4°C in a cold room.

30 g of *Clostridium thermocellum* NCIMB 10682 cells (from 5 l of culture medium as described previously) were mixed with 120 ml of distilled H₂O and 600 µl of 100mM PMSF. After the enzyme was extracted and concentrated by use of the working protocol as described previously, the concentrated crude extract (= 6 ml) was lyophilized at -54°C (Thermo Scientific).

For the first purification step, DE52 DEAE-Cellulose was used as column material. For this purpose, 1 l of starting buffer 50mM TRIS pH 7 and 500 ml of elution buffer 600mM NaCl were prepared.

26 g of DE52 DEAE-Cellulose were suspended in 50 ml of the starting buffer and were applied to a column (27.5 cm x 1.7 cm, BioRad). Subsequently, the column was equilibrated with the same buffer in a total volume of 200 ml. The lyophilized sample was dissolved in a minimum volume of 2 ml starting buffer and then applied to the equilibrated column with a Pasteur pipette (Brand).

Firstly, 300 ml of 100mM NaCl and 300 ml of 50mM Tris pH 7 were used to produce a linear gradient in order to remove unspecific proteins from the column. Finally, cellobiose phosphorylase was eluted with a gradient produced from 300 ml of 600mM NaCl and 300 ml of 50mM TRIS pH 7, collecting 60 fractions in a total volume of 1 ml, using a fraction collector (Pharmacia).

For the determination of the specific activity, 400 µl of each fraction were incubated with 320 µl of phosphate buffer pH 7 (0.1M) and 480 µl of cellobiose (0.1M) for 12 h at 37°C in a heating oven (Memmert).

Subsequently, the phosphorolytic reaction of each sample was assayed by thin layer chromatography (= TLC) which was visualized by spraying with an anisaldehyde- H_2SO_4 solution and heating at 100°C in a heating oven (Heraeus Instruments). Active fractions were pooled together, dialyzed against 3.5 l of 50mM TRIS pH 7 and lyophilized at -54°C .

4.4 Sample and gel filtration (GF) column preparation

Also in this purification step, all solvents were degassed and all purification steps were performed at 4°C in a cold room.

A Sephacryl column (50 cm x 1.7 cm, BioRad) was filled with Sephacryl S-200HR (Sigma Aldrich) previously stored in 20% ethanol. In order to remove ethanol, the column was washed with 150 ml distilled H_2O and then equilibrated with 150 ml of 50mM TRIS pH 7. The lyophilized sample was dissolved in a minimum volume of 1.6 ml starting buffer and then applied to the equilibrated column with a Pasteur pipette. The enzyme was eluted with 50mM TRIS pH 7, collecting 95 fractions in a total volume of 850 μl .

For the determination of the specific activity, 200 μl of each fraction were incubated with 160 μl of phosphate buffer pH 7 (0.1M) and 240 μl of cellobiose (0.1M) for 12 h at 37°C .

After the TLC analysis, active fractions were pooled together, dialyzed against 3.5 l of 50mM TRIS pH 7 and finally lyophilized at -54°C . The purified enzyme was stored at -20°C .

The specific activity of cellobiose phosphorylase was determined by Lowry test and D-glucose test after each purification step.

5. Dialysis

In order to remove salt molecules or change the buffer of the target protein, dialysis, a timely but easy and inexpensive method, can be used. Dialysis tubing is a semi-permeable membrane with a specific range of pore sizes. In this study, dialysis tubing cellulose membrane (Sigma Aldrich) with cut off $\geq 12\text{ 000MW}$ was used.

The appropriate length of the tubing according to the volume of the crude extract was cut and boiled in distilled water for 15 min. Subsequently, the tubing was rinsed with cold water. One end of the tubing was pinched with a dialysis clamp and the protein solution was filled inside using a glass funnel. All air bubbles were removed, before the open end of the tubing was tied.

The membrane tubing was placed in a large beaker filled with dialysis buffer, at least 10 times the volume of the protein solution, and stirred over night at 4°C in a cold room.

6. Lowry test

6.1 Principle of the Lowry test

The Lowry test is a protein assay, which is based on the reaction of a protein with an alkaline copper tartrate solution (reagent A, BioRad) and the Folin reagent (reagent B, BioRad) to determine protein concentration. Under alkaline conditions, copper complexes with protein. When the Folin reagent (sodium 1, 2-naphthoquinone-4-sulfonate) is added, it binds to the protein. Bound reagent is slowly reduced and changes color from yellow to blue.

Following the standard assay protocol, 100 µl of the protein solution was used. 500 µl of reagent A were added, the whole vortexed and subsequently 4 ml of reagent B were mixed with the solution. The reaction reaches 90% of its maximum colour development within 15 min.

After the incubation time of 15 min at room temperature, the absorbance was read at 750 nm, using 1 ml of the sample in semi-micro disposable cuvettes (Sterilin).

6.2 Preparation of a standard curve

Typical protein assays are used to determine protein concentration by comparing the assay response of a sample to that of a standard whose concentration is known. Protein samples and protein standards are processed in the same manner by mixing them with assay reagents and using a spectrophotometer to measure the absorbances.

For the protein standard curve, a stock solution of chicken egg white albumin (Sigma Aldrich) was prepared (15 mg/10 ml) and the following dilutions were used:

Volume of distilled water	Volume of stock albumin solution	Final concentration
-	1 ml	1.5 mg/ml
0.2 ml	0.8 ml	1.2 mg/ml
0.4 ml	0.6 ml	0.9 mg/ml
0.667 ml	0.330 ml	0.5 mg/ml
0.867 ml	0.133 ml	0.2 mg/ml

Following the standard protocol as described above, the absorbance was read at 750 nm after 15 min of incubation, using 1 ml of the sample in semi-micro disposable cuvettes.

7. D-Glucose test

7.1 Determination of the enzyme activity for native and recombinant cellobiose phosphorylase

The D-glucose quantification test is used to determine the enzyme activity. Enzyme activity (in units U) describes the amount of enzyme (= cellobiose phosphorylase) that catalyzes the reaction of 1 μ mol of substrate (= cellobiose) per minute.

Specific activity defines the number of enzyme units per ml (= D-glucose test) by the concentration of protein in mg/ml (= Lowry test).

7.2 Principle of the D-glucose test

The test combination set contains two solutions:

- Solution 1, with approximately 7.2 g powder mixture, consisting of triethanolamine buffer pH approx. 7.6; NADP, approx. 110 mg; ATP approx. 260 mg and magnesium sulfate, which is dissolved in 45 ml redistilled water.
- Solution 2, with approximately 1.1 ml suspension, consisting of hexokinase, approx. 320 U; glucose-6-phosphate dehydrogenase, approx. 160 U.

D-Glucose is phosphorylated to D-glucose-6-phosphate (G-6-P) in the presence of the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate (ADP):



In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH):



The amount of NADPH formed in this reaction is stoichiometric to the amount of D-glucose. The increase in NADPH is calculated from the absorbance at 340 nm.

7.3 Instructions for performance of the D-glucose test

Generally, in order to determine the specific activity of cellobiose phosphorylase, enzyme crude extract or purified enzyme, was incubated with cellobiose as substrate for the phosphorolytic reaction in order to measure the amount of the released D-glucose.

For the incubation, which took place at 37°C for 12 h, the following components were used.

1.2 ml cellobiose (0.1M)

0.8 ml phosphate buffer pH 7 (0.1M)

1ml enzyme crude extract (0.62 U/20 mg) or

1ml purified enzyme (3 U/0.1 mg)

After 30 min, 50 µl of the incubated sample (*) was used to perform the D-glucose test by the following procedure:

Pipette into cuvettes	Blank	Sample
solution 1	500 µl	500 µl
sample solution*	-	50 µl
redistilled water	1000 µl	950 µl

The prepared solution was mixed and the absorbance (A_1) at 340 nm was read after 3 min. In order to start the reaction, the following components were added.

solution 2	10 µl	10 µl
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The prepared solutions were mixed and after 15 min the absorbance at 340 nm was read (A_2).

Finally, the absorbance differences ($A_2 - A_1$) were determined between blank and sample by subtraction the absorbance difference of the blank from the absorbance difference of the sample:

$$\Delta A = (A_2 - A_1)_{\text{sample}} - (A_2 - A_1)_{\text{blank}}$$

The measured absorbance differences should be, as a rule, at least 0.100 absorbance units to achieve sufficiently precise results.

7.4 Calculation of the D-glucose test

The final concentration [c] was calculated by the general equation:

$$[c] = \frac{V * MW}{\epsilon * d * v * 1000} * \Delta A \text{ [g/l]}$$

V = final volume [ml]

v = sample volume [ml]

MW = molecular weight of the substance to be assayed [g/mol]

d = light path [cm]

ϵ = extinction coefficient of NADPH at 340 nm = $6.3 \text{ [l * mmol}^{-1} * \text{cm}^{-1}]$

Therefore, it follows for D-glucose:

$$[c] = \frac{1.510 * 180.16}{6.3 * 1 * 0.050 * 1000} * \Delta A \text{ D-glucose} = \frac{272.04}{315} * \Delta A \text{ D-glucose [g D-glucose/l sample solution]}$$

8. Thin layer chromatography (TLC)

The term chromatography is derived from the Greek word *chromagraphein*, *chroma* = colour, *graphein* = write. [Kruiswijk, 2005]

There are several techniques to separate substances. All of the techniques depend upon the difference in distribution of the various compounds in the applied mixture between the mobile phase and the stationary phase. [Kruiswijk, 2005]

8.1 The stationary phase

Many different materials are capable of retaining both solvents and solutes. The two most commonly used as stationary phase (adsorbent) are silica gel (SiO_2) and alumina (Al_2O_3). In the adsorption processes on silica gel and alumina, not only electrostatic interactions are important but also hydrogen bonding plays an important role. [Kruiswijk, 2005]

Silica gel, also used in this study, has a good linear capacity and hardly any catalytic character that can lead to the decomposition of samples. It has a large specific surface area ($300\text{-}800 \text{ m}^2/\text{g}$) and a large pore volume ($> 0.7 \text{ ml/g}$). Vicinal and germinal hydroxyl groups are responsible for the adsorption process (**Figure 21**). [Kruiswijk, 2005]

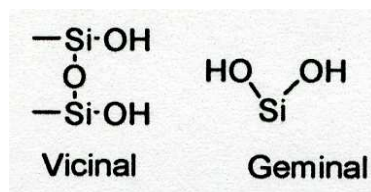


Figure 21 Vicinal and geminal hydroxyl groups [Kruiswijk, 2005]

On the surface of aluminium oxide acidic (Al^{3+}) and basic (O^{2-}) groups are present, therefore acidic compounds will be strongly adsorbed. The specific surface is smaller than for silica gel ($100\text{--}200 \text{ m}^2/\text{g}$) and the pore volume is also smaller ($0.2\text{--}0.3 \text{ ml/g}$). Alumina can catalytically decompose acidic compounds. Like silica gel, alumina may be regarded as a typical polar sorbent and the order of separation of compound classes in alumina and silica gel is generally similar. [Kruiswijk, 2005]

8.2 Elution solvents and speed coefficient

In most cases, the structure of the component is constant. This means that the composition of the elution solvent is the most important factor in adsorption chromatography. The elution solvent interacts with the component and with the adsorbent as well. Small changes in the composition of the elution solvent can have major effects in the separation. [Kruiswijk, 2005]

For TLC the speed at which the elution solvent is running is very important. The speed the elution solvent is running at is not constant and it decreases with the height of the solvent. In this study, 1-propanol-water solution was used in a ratio of 17:3.

8.3 Development and visualization of the TLC plate

After the TLC plate is spotted, it was transferred into a development tank, made of glass. This tank holds the solvent system. The baseline is not allowed to sink in the solvent system otherwise the compound will diffuse in the solvent. The solvent front should rise in a straight horizontal line until it reaches the top of the plate approximately 1 cm from the end. [Kruiswijk, 2005]

There are numerous reagents available to visualize TLC plates. Even when the material being analyzed is coloured, it is necessary to treat the TLC plate to visualize any non-coloured spots that might be present in the sample. The TLC plate can be dipped into a stock solution of an appropriate reagent or the plate can be sprayed with a diffuser. [Kruiswijk, 2005]

In this study, anisaldehyde-sulphuric acid spray (anisaldehyde-H₂SO₄ solution) was used, which consists of following substances:

90 ml absolute ethanol

5 ml 4-Methoxybenzaldehyde 98%

5 ml sulfuric acid

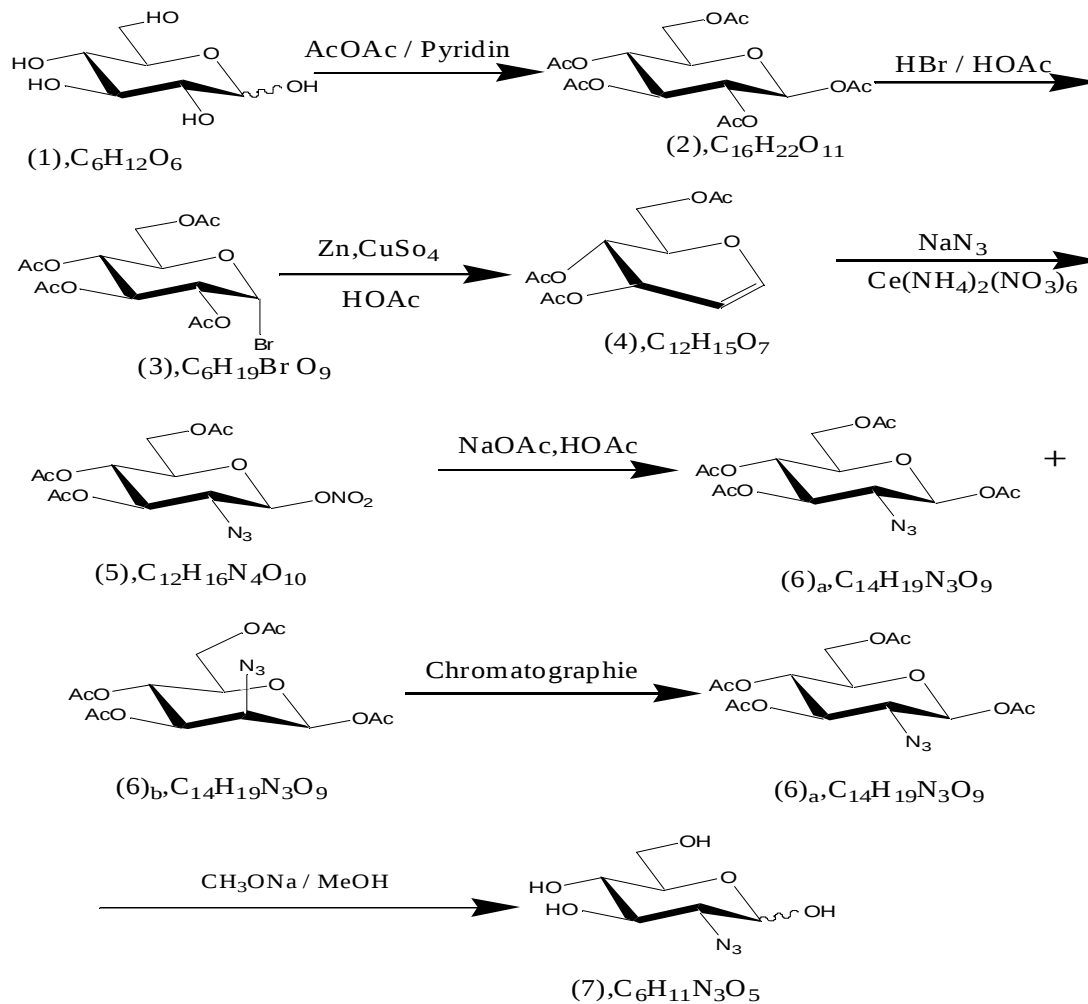
1 ml acetic acid 100%

9. Monitoring conversions of azido sugars by the use of TLC and the anisaldehyde-sulfuric acid spray reagent

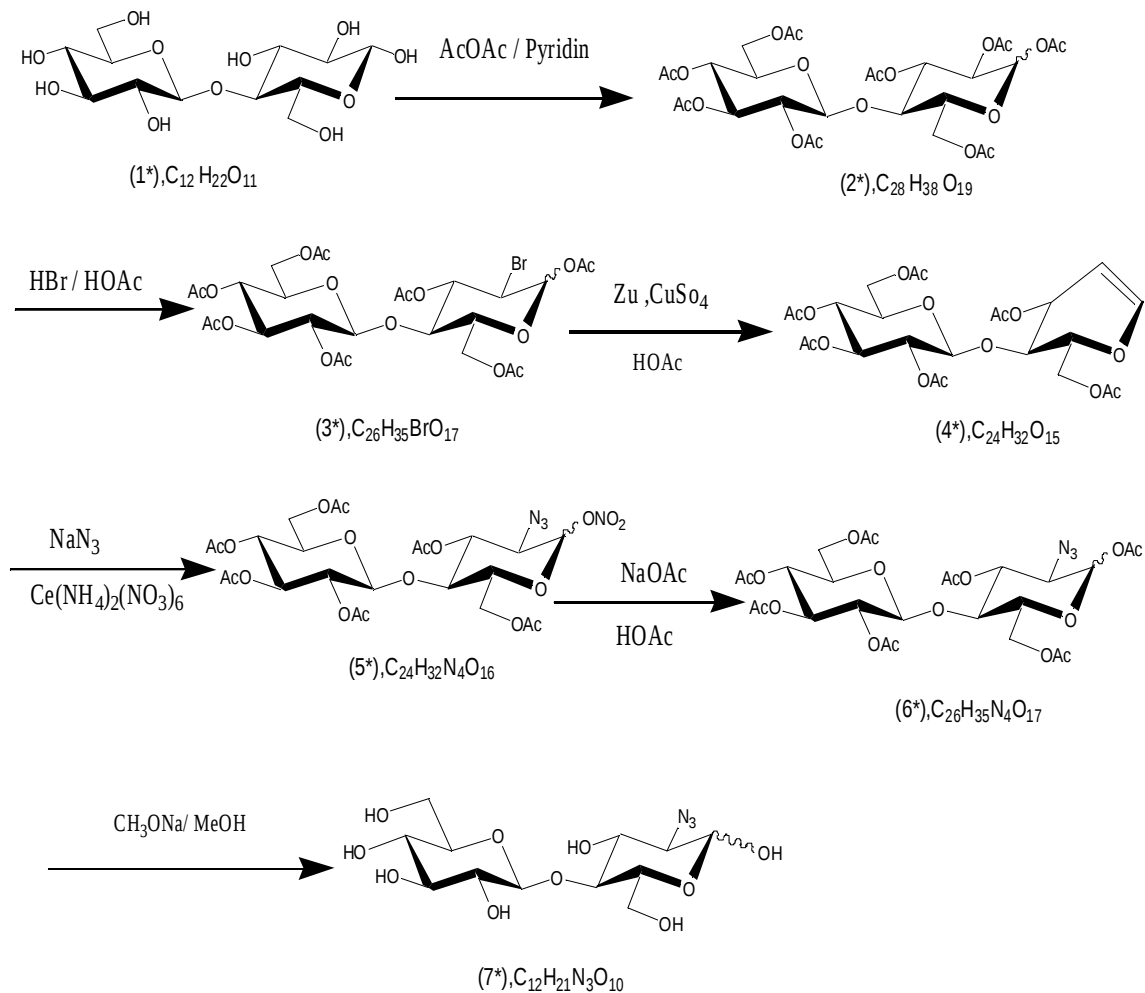
Anisaldehyde-sulphuric acid spray, which was used in this study, is a highly sensitive spray reagent used in thin layer chromatography. It enables the detection of carbohydrate derivatives and other natural products, producing different colorations depending on the types of sugar involved. This staining method greatly facilitates monitoring of reactions involving azido sugar derivatives [Schwarz, 2003].

In this study, chemically synthesized azido sugars 2-azido-2-deoxy-D-glucose, 2-azido-2-deoxy-cellobiose and 3-azido-3-deoxy-D-glucose were monitored by TLC with the anisaldehyde-H₂SO₄ spray reagent.

Synthesis of 2-azido-2-deoxy-D-glucose:

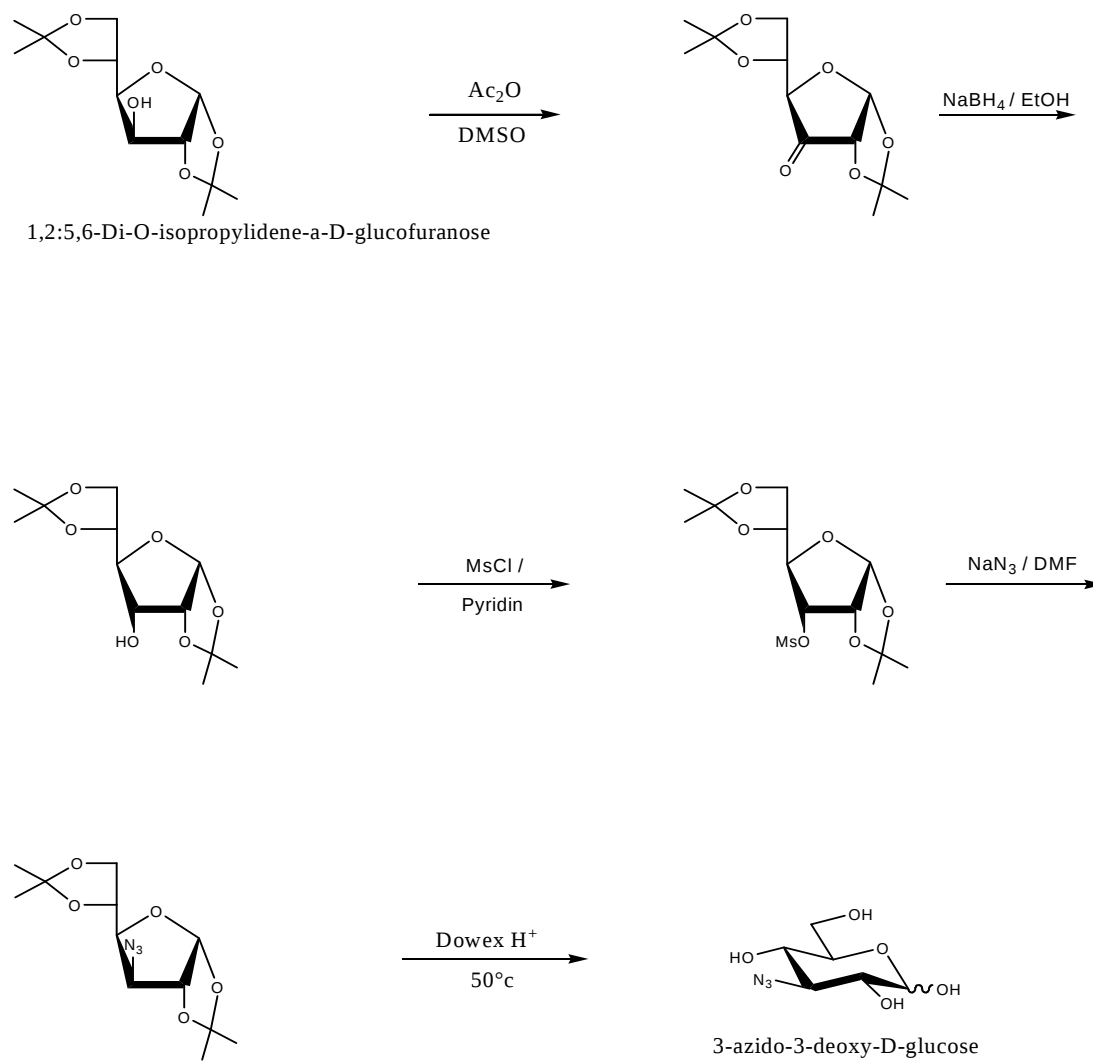


Synthesis of 2-azido-2-deoxy-cellobiose:



The chemical synthesis of 2-azido-2-deoxy-D-glucose and 2-azido-2-deoxy-cellobiose were performed within the framework of a diploma thesis [Gabra, 2009].

Synthesis of 3-azido-3-deoxy-D-glucose:



The chemical synthesis of 3-azido-3-deoxy-D-glucose was performed within the framework of a diploma thesis [Trinkl, 2005].

10. Preparation of the cellobiose phosphorylase genomic DNA for PCR

All cloning experiments have been performed under the supervision of Dr. Philipp Starkl at the Department of Pathophysiology of the Medical University of Vienna.

10.1 InstaGeneTM Matrix

In order to allow fast and easy preparation of PCR amplifiable DNA, InstaGene matrix (BioRad) has been used in this study, without use of intensive phenol/chloroform extraction steps. The protocol as described below was used for the preparation of the genomic DNA from the bacterium.

1 ml of the grown *Clostridium thermocellum* NCIMB10682 cells was resuspended in 1 ml of autoclaved, distilled water in a microfuge tube. The solution was centrifuged for 1 min at 10 000-12 000rpm. As the supernatant was removed, 200 µl of InstaGene matrix was added to the pellet and incubated at 56°C for 30 min. After the incubation, the suspension was vortexed and placed in a 100°C heat block. Finally, the sample was vortexed at 12 000rpm for 10 sec and spun at 12 000rpm for 3 min. 5 µl of the resulting supernatant per 25 µl PCR reaction was used, while 10 µl of the same supernatant was used for the determination of DNA concentration.

10.2 UV spectrophotometric measurement of DNA concentration

The most reliable way to evaluate DNA concentration is to use UV spectrophotometric measurements. DNA has absorbances in the region 230 to 320 nm, allowing measurement of the DNA concentration in this region. As DNA absorbs light most strongly at 260 nm, the absorbance value at this wavelength (called A₂₆₀) was used in this study to estimate the DNA concentration of the genomic DNA of *Clostridium thermocellum* NCIMB 10682.

Generally, the measured absorbance should have an OD between 0.05 and 0.3. A negative control or blank cuvette was filled with distilled water. As the tube has no DNA, the spectrophotometer was set the reference of zero. The sample with genomic DNA of *Clostridium thermocellum* was diluted hundredfold in distilled water (10 µl of the sample in 990 µl distilled water). 1 ml was placed in a semi-micro disposable cuvette and the OD was read at 260 nm.

The measured OD at A₂₆₀ was then multiplied by the dilution factor (100), using the relationship that an A₂₆₀ of 1.0 corresponds to 50 µg/ml pure DNA, thus giving the DNA concentration of the sample:

$$\text{Concentration (}\mu\text{g/ml)} = A_{260} \text{ reading} \times \text{dilution factor} \times 50 \mu\text{g/ml}$$

11. Amplification of the cellobiose phosphorylase genomic DNA

For the amplification step, the genomic DNA of *Clostridium thermocellum* NCIMB10682 was utilized as template DNA, using PCR Master Mix (Fermentas). This PCR Master Mix comprises a mixture of *Taq* DNA polymerase, PCR buffer, MgCl₂ and dNTPs. A pair of primers was designed based on the DNA sequence of *Clostridium thermocellum* ATCC27405. All solutions were thawed on ice, gently vortexed and briefly centrifuged.

For 25 µl of reaction volume the following protocol was used:

10 µl PCR Master Mix (2x)

5 µl genomic DNA

1 µl 5'-CCA TGG AGT TCG GTT TTT TTG ATG AT-3' 5 pM (**CeP YM4 ATCC fwd**)

1 µl 5'-ATA TCC CAC TCG AGC TTC AAC TTG TGA GTC T-3' 5 pM (**CeP ATCC rev**)

8 µl nuclease free H₂O

PCR, 40 cycles, was performed under these conditions: 95°C, 1 min for denaturation, (enzyme activation), 95°C, 30 sec for double strand melting, 52°C, 30 sec for primer annealing, 72°C, 3 and 5 min for elongation. PCR was performed in a thermal cycler (BioRad). After completing the PCR, the amplified DNA was stored at 4°C while an agarose gel was prepared. 1% of agarose (10 g) was added to 60 ml TBE buffer and heated until boiling in microwave. After the solution was cooled down to 40-50°C, 5 µl of ethidium bromide (1:1000) was added and the gel was finally poured in a gel chamber with 8 lane combs.

After the polymerization, end walls as well as combs were removed from gel chamber and the gel was placed into the electrophoresis chamber (PeqLab) filled with TBE buffer. 1/6 volume of loading dye was added to PCR samples. The whole volume was applied onto the gel with a DNA ladder (Fermentas) and the gel was run for 60 min at 120 V. Finally, the gel was analyzed under UV and the band was cut out.

11.1 PCR product extraction

For the extraction of the amplified DNA, a Gel Extraction Kit (Qiagen) was used, which provides an efficient and rapid extraction of DNA from agarose gels as well as from reaction mixtures. After the DNA fragment was excised from the agarose gel, this gel slice was weighed

in a colorless tube (~ 140 µg) and 420 µl of buffer QG have been added. The gel was incubated at 50°C for 10 min until the gel slice had completely dissolved and became yellow. 140 µl of isopropanol were added to the sample and mixed by inverting the tube several times. In order to bind DNA, the sample was applied to a MinElute column and centrifuged for 1 min. The flow through was discarded each time. The washing step was done by adding 750 µl of buffer PE to the MinElute column, which then was centrifuged for 1 min. The flow through was discarded each time. The MinElute column was placed into a clean 1.5 ml microcentrifuge tube and the DNA was eluted by adding 10 µl of buffer EB, which was centrifuged for 1 min. The eluted DNA was stored at -20°C.

11.2 High Fidelity PCR

The High Fidelity PCR was used due to its advantages over conventional PCR such as increased efficiency and range, high accuracy and greater yields. The High Fidelity PCR enzyme mix (Fermentas) contains a blend of a *Taq* DNA polymerase and *Pfu* DNA polymerase that exhibits a 3'→5' exonuclease activity. This blend increases the length and yield of amplification products with high DNA synthesis fidelity.

Due to the presence of the internal *Nco*I restriction site, the genomic DNA was amplified using two different forward primers as well as two different reverse primers, thus generating two fragments by removing the internal restriction site (*Nco*I).

20 µl of reaction volume for each PCR was prepared using the following protocol:

1. PCR:

0.1 µl High Fidelity PCR Enzyme Mix

1 µl genomic DNA

1 µl 5'-CCA TGG AGT TCG GTT TTT TTG ATG AT-3' 5 pM (**CeP YM4 ATCC fwd**)

1 µl 5'-TAG CTT CTC CGT GGC GTT TTG CGT CT-3' 5 pM (**CeP ATCC Nco rem rev**)

2 µl dNTPs (dATP, dCTP, dGTP, dTTP) 2mM

2 µl High Fidelity PCR buffer with MgCl₂ (10x)

13 µl nuclease free H₂O

2. PCR:

0.1 µl High Fidelity PCR Enzyme Mix

1 µl genomic DNA

1 µl 5'-AGA CGC AAA ACG CCA CGG AGA AGC TA-3' 5 pM (**CeP ATCC Nco rem fwd**)

1 µl 5'-ATA TCC CAC TCG AGC TTC AAC TTG TGA GTC T-3' 5 pM (**CeP ATCC rev**)

2 µl dNTPs (dATP, dCTP, dGTP, dTTP) 2mM

2 µl High Fidelity PCR buffer with MgCl₂ (10x)

13 µl nuclease free H₂O

PCR cycles were performed as described previously. After PCR, the amplified DNA was extracted by Gel Extraction Kit (Qiagen) as described above. 20 µl of DNA was eluted, whereas 5 µl of each fragment was used for the overlap extension PCR.

11.3 Overlap extension PCR

The two fragments obtained, were amplified in a second PCR, an overlap extension PCR (OE-PCR).

The overlap extension PCR is a variant of PCR which can produce polynucleotides from smaller fragments. Therefore, it preferentially amplifies one strand of the target DNA. The reaction mix is identical to previous PCR attempts, but with leaving out the primers.

20 µl of reaction volume for each OE-PCR was prepared using the following protocol:

0.1 µl High Fidelity PCR Enzyme Mix

5 µl fragment 1 (**CeP PCR ATCC rem Nco frag 1**)

5 µl fragment 2 (**CeP PCR ATCC rem Nco frag 2**)

2 µl dNTPs 2mM

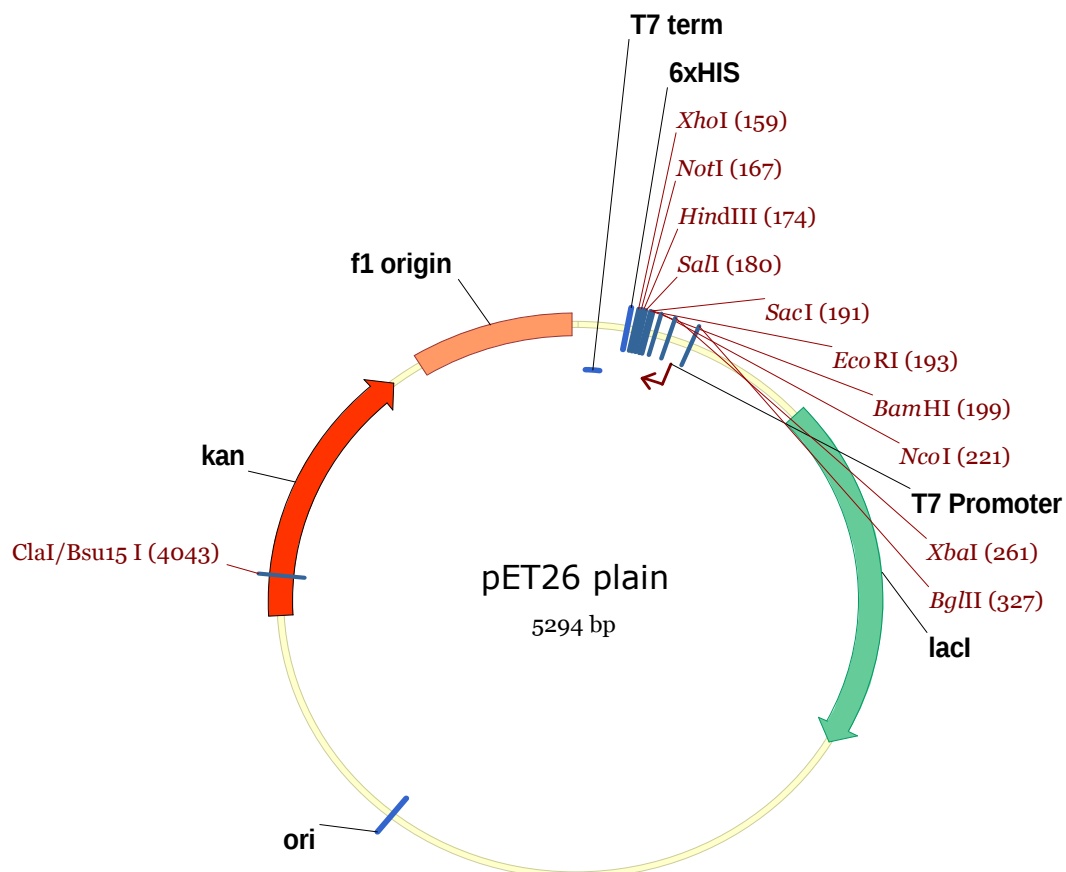
2 µl High Fidelity PCR buffer with MgCl₂ (10x)

5.9 µl nuclease free H₂O

The OE-PCR was performed using fragment 1 and 2 as templates with the short overlapping sequence of one fragment functioning as primer for the other fragment. The first 10 cycles were performed under following conditions: 95°C, 1 min for denaturation (enzyme activation), 95°C,

30 sec for double strand melting, 52°C, 30 sec for primer annealing and 72°C, 2 min for elongation. After 10 cycles, primers binding at the opposing ends of the full fragment were added (1 µl forward primer CeP YM4 ATCC fwd and 1 µl reverse primer CeP ATCC rev respectively) to the reaction mixtures. The PCR then continued under the same conditions as the High Fidelity PCR (40 cycles) as described above.

The obtained full coding sequence fragment of cellobiose phosphorylase, now without internal *NcoI* site, was cloned into the pET-26(+) plain vector (**Scheme 1**). In contrast to the original pET-26(+) vector, the plain variant does not contain the N-terminal pelB signal peptide for periplasmatic localization.



Scheme 1 pET-26b(+) vector

12. Preparation of pET26 vector and insert for the transformation into DH5α *E.coli*

12.1 Restriction digest

The vector should be linearized by digesting with one or more unique restriction enzymes to completion. Uncut vector in the prep will lead to a high background of *E. coli* transformants having no inserts in the vector. In this work, the vector was digested with two different restriction enzymes that have nearly adjacent recognition sites in the cloning region. This produces non-compatible sticky ends, thus reducing the background of vector recircularization without insert.

Using restriction enzyme sites, FastDigest®NcoI and FastDigest®XhoI (both Fermentas) for pET-26b(+) plain vector (Novagen) and cellobiose phosphorylase amplified gene without the internal NcoI site (= CeP) respectively, a restriction digest (20 µl) was conducted under the following conditions:

2 µl FastDigest buffer (10x)
1 µl FastDigest®NcoI 1 U
1 µl FastDigest®XhoI 1 U
5 µl pET26b plain vector (2 µg)
5 µl CeP without internal NcoI site (0.2 µg)
11 µl nuclease free H₂O

The samples were mixed and incubated at 37°C in a heat block for 15 min. Subsequently, 1% agarose gel was prepared as described previously. The restriction fragments were separated 1 h at 120 V and were subsequently extracted by use of a Gel Extraction Kit (Qiagen) as described previously.

12.2 Ligation

Through ligation by T4 ligase (Fermentas) of the digested cellobiose phosphorylase gene with the same sites of pET-26b(+) plain vector, a recombinant cellobiose phosphorylase protein harboring the 6xHis-tag at the C-terminus has to be expressed in *E.coli*.

20 µl of reaction volume for the ligation was prepared using the following protocol:

8.5 µl NcoI/XhoI digested fragment of CeP

8.5 µl NcoI/XhoI digested pET-26b(+) vector (2 µg)

2 µl ligation buffer (5x)

1 µl ligase T4

The sample was incubated at room temperature over night.

13 Transformation of the pET-26b(+) plain-CeP construct into chemocompetent DH5α *E.coli*

For the purpose of transformation, the DH5α strain of *E.coli* was used. This bacterial strain was made chemo-competent for transformation and was provided by the Department of Pathophysiology of the Medical University of Vienna.

A mixture of 7 µl ligation product and 50 µl of chemocompetent DH5α *E.coli* cells was incubated for 30 min on ice, and then exposed to heat shock at 42°C for 90 sec. The mixture was again incubated for 2 min on ice. 1 ml of SOC medium was added and the mixture was incubated at 37°C and 250rpm for 1 h. Subsequently, the cells were grown over night on kanamycin containing LB agar plates at 37°C.

13.1 PCR screening of the colonies and their minipreparation

In order to verify which chemocompetent DH5α *E.coli* cells contain the full pET-26b(+) plain-CeP construct, PCR screening was conducted. Firstly, single cell colonies were picked out with a sterile tip, transferred into an Eppendorf tube containing 50 µl of distilled water, and briefly vortexed.

The same tip was then used to inoculate 2 ml of LB medium containing antibiotics in 15 ml falcon tubes overnight at 37°C and 250rpm for the generation of glycerol stocks. The Eppendorf tube containing the water-bacteria suspension was incubated 5 min at 95°C, and subsequently spun down for 5 min at 12 000rpm. 5 µl of the supernatant were used for PCR as the template.

20 µl of reaction volume for the PCR was prepared using following protocol:

10 µl PCR Master Mix (2x)

5 µl bacterial lysate supernatant

1 µl 5'-CGC CAA TCC TGA TAT AGT TCC TCC TTT CAG -3' 5 pM (**T7 seq fwd**)

1 µl 5'-TAG CTT CTC CGT GGC GTT TTG CGT CT-3' 5 pM (**CeP ATCC Nco rem rev**)

8 µl nuclease free H₂O

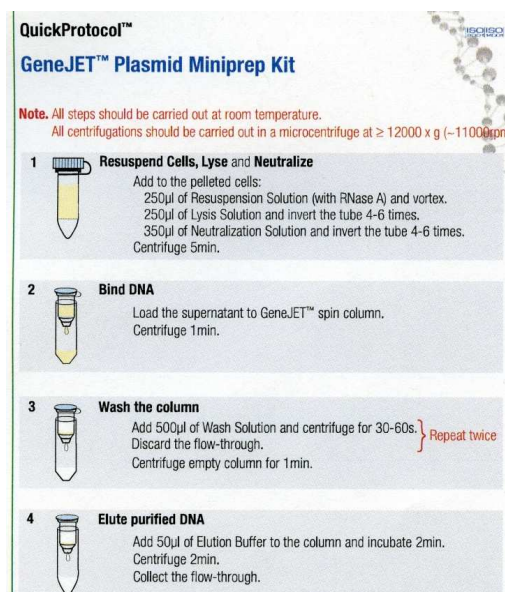
This primer combination was used to select for clones containing the successfully ligated construct as the forward primer (T7 seq fwd) binds on the pET26b plain sequence and the reverse primer (CeP ATCC Nco rem rev) binds within the cellobiose phosphorylase coding region.

PCR cycles were performed as described previously and the obtained product was analyzed by agarose gel electrophoresis as described previously. The bacterial clones (# 1, 2 and 6) which were tested positive by this PCR for successful transformation of pET26b plain CeP were inoculated for plasmid DNA isolation.

For this isolation of the plasmid DNA from recombinant *E.coli* cultures, a rapid, small-scale minipreparation was conducted. The extracted plasmid DNA is itself often called “miniprep”. Minipreps are used in the process of molecular cloning to analyze bacterial clones.

For the miniprep, the frozen glycerol stocks of positive tested clones were scratched with a sterile tip. The tip was subsequently released into a 15 ml tube containing 5 ml LB medium containing antibiotics and incubated over night at 37°C and 200rpm.

For the miniprep, a quick protocol from Fermentas (GeneJET™ Plasmid Miniprep Kit) was performed as shown below:



Source: http://www.cosmobio.co.jp/product/products_FER_20060307/GeneJETplasmidMiniprepKit1.pdf

Finally, a double restriction digest with *NcoI* and *XhoI* of eluted purified pET-26b(+)-CeP construct (clone 1, 2 and 6) was performed to confirm the presence of the cellobiose phosphorylase insert.

According to the results, DNA sequencing of clones 2 and 6 was conducted by the company Microsynth (<http://www.microsynth.ch>). For sequencing, 3 µl of each DNA template (= 0.8 µg), 4 µl of primer and 3 µl of distilled H₂O were mixed in 1.5 ml tubes (Sarstedt).

As the cellobiose phosphorylase gene (~ 2500bp length) is too big to be sequenced in a single run (Microsynth standard sequencing runs may sequence ~ 900bp at once), several sequencing steps resulting in overlapping sequenced fragments were conducted. The following primers were used:

5'-CGC CAA TCC TGA TAT AGT TCC TCC TTT CAG -3' 5 pM (**T7 seq fwd**)

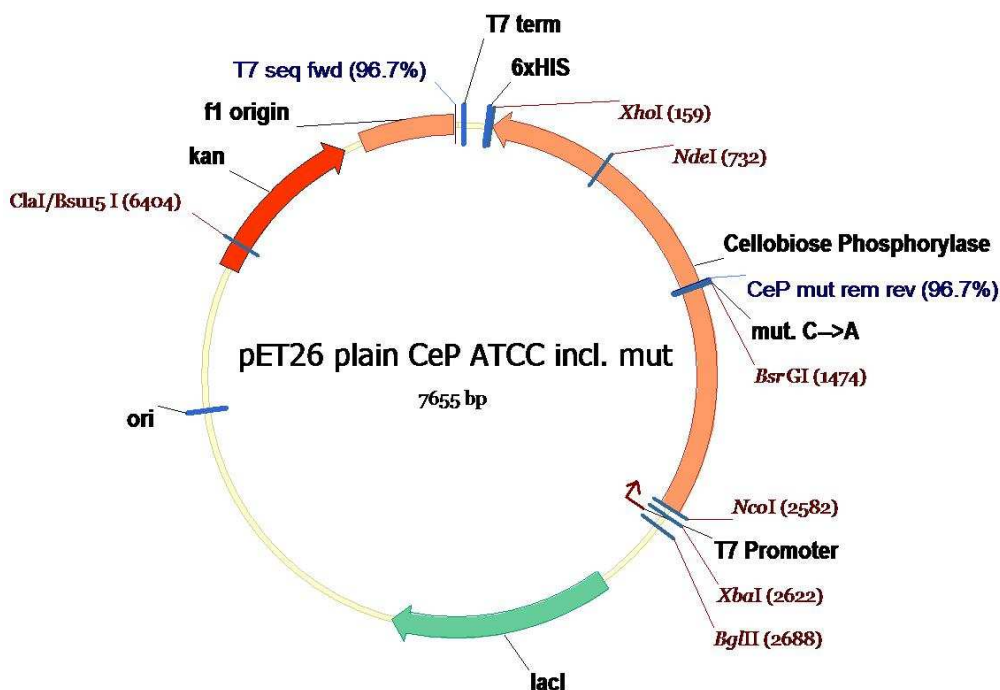
5'-TAG CTT CTC CGT GGC GTT TTG CGT CT-3' 5 pM (**CeP ATCC Nco rem rev**)

5'-GAT GCG TCC GGC GTA GAG GAT CGA -3' 5 pM (**T7 seq rev**)

5'-GGC TCG GTT ATC TAT CAC AAG ACA GAG -3' 5 pM (**CeP ATCC seq rev**)

14. Detection of a point mutation; removal of the mutation by PCR

Upon sequencing, it was found that a C→A point mutation existed within the insert at position 1461 of the vector (**Scheme 2**). This single nucleotide mutation would result in an alanine to glutamic acid missense mutation. In order to remove this mutation, a PCR technique was applied.



Scheme 2 pET-26b(+)-CeP construct containing mutation

For this PCR, a new primer was designed spanning the mutated region with the correct sequence. Importantly, this primer also spans the BsrGI restriction site which is in close proximity 5' of the mutation. The resulting PCR fragment of the cellulose phosphorylase 3' region can therefore be subsequently used for the replacement of the mutated vector version by directional cloning using XhoI and BsrGI restriction enzymes.

For 20 µl of reaction volume the following protocol was used:

10 µl PCR Master Mix (2x)

5 µl genomic DNA

1 µl 5'-GGA TTT GTA CAC CAG ATA CCC GCA AGA GCA-3' 5 pM (**CeP mut rem fwd**)

1 µl 5'-ATA TCC CAC TCG AGC TTC AAC TTG TGA GTC T-3' 5 pM (**CeP ATCC rev**)

3 µl nuclease free H₂O

PCR cycles were performed as described previously and the obtained product was extracted with the aid of a Gel Extraction Kit (Qiagen), also as described previously.

14.1 Restriction digest and ligation of CeP without mutation and pET-26b(+)-CeP construct with mutation

For the restriction digest of the amplified cellobiose phosphorylase gene without mutation as well as for the pET-26b(+)-CeP construct with mutation, restriction enzymes, FastDigest®Bsp1407I (= isoschizomer of BsrGI) and FastDigest®XhoI (both Fermentas) were used.

The restriction digest was conducted in a total reaction volume of 20 µl under the following conditions:

2 µl FastDigest buffer (10x)

1 µl FastDigest®Bsp1407I 1 U

1 µl FastDigest®XhoI 1 U

5 µl amplified cellobiose phosphorylase gene without mutation (0.2 µg)

11 µl nuclease free H₂O

2 µl FastDigest buffer (10x)

1 µl FastDigest®Bsp1407I 1 U

1 µl FastDigest®XhoI 1 U

5 µl pET-26b(+)-CeP construct with mutation

11 µl nuclease free H₂O

The sample was mixed and incubated at 37°C in a thermo block for 15 min. The enzymes were inactivated by heating for 5 min at 80°C. Subsequently, 1% agarose gel was prepared as described previously. The products were separated, analyzed under UV and extracted with the aid of a Gel Extraction Kit (Qiagen) as described previously.

Through ligation with T4 ligase (Fermentas), the mutated part of the cellobiose phosphorylase in pET-26b was replaced with the correct fragment.

A total of 20 µl of reaction volume for the ligation was prepared using the following protocol:

8.5 µl of the Bsp1407I/XhoI digested fragment of CeP without mutation

8.5 µl of the Bsp1407I/XhoI digested pET-26b(+)-CeP construct

2 µl ligation buffer (5x)

1 µl ligase T4

The ligation mixture was incubated at room temperature over night. Thereby, the mutation was replaced by insertion of the intact cellobiose phosphorylase gene. Subsequently, a restriction digest was performed as described above.

After the transformation of the pET-26b(+)-CeP construct into chemocompetent DH5α *E.coli* and subsequent miniprep, the correct sequence of the cellobiose phosphorylase gene was verified by DNA sequencing.

15. Midipreparation of the pET26b-CeP construct

After the bacterial cell growth, a bacterial pellet was prepared as described previously. 4 ml of the pellet were used for the midiprep preparation, following the Plasmid Midi Kit protocol (Qiagen):

4 ml each of buffer P1 and P2 were added to 4 ml of the bacterial pellet and mixed gently by inverting 4-6 times. The mixture was incubated at room temperature for 5 min. 4 ml of chilled buffer P3 were added and incubated again at room temperature for 5 min. As the lysate became less viscous, it was centrifuged at 4°C and 12 000rpm for 30 min. Meanwhile, Qiagen tip 100 was equilibrated by applying 4 ml of buffer QBT. After the centrifugation step, the supernatant

was applied to the Qiagen tip. After a washing step with 2x10 ml buffer QC, DNA was eluted with 5 ml of buffer QF.

DNA was precipitated by adding 3.5 ml of isopropanol at room temperature and then centrifuging at 4°C for 30 min. The supernatant was decanted and the DNA pellet was washed with 2 ml of 70% ethanol at room temperature and then centrifuged for 10 min. After another batch of ethanol had been added and decanted, the pellet was air dried for 5 to 10 min. The DNA was then dissolved in buffer TE and stored at -80°C.

16. Transformation of the pET26b-CeP construct into BL21 (DE3) *E. coli* cells

The BL21 (DE3) competent cells, which were provided by the Department of Pathophysiology of the Medical University of Vienna, are genomically modified for highly efficient recombinant protein production. Following a standard transformation protocol, frozen chemo-competent cells were thawed on ice. 100 µl of them were transferred into a chilled culture tube and 1 µl of purified pET26 plain-CeP plasmid DNA was added. The mixture was immediately placed on ice for 10 min. The cells were then exposed to heat shock for 45 sec in a 42°C water bath and then again placed on ice for 2 min. 900 µl of cold SOC medium was added and the cells were incubated for 60 min at 37°C with shaking at 200rpm. 100 µl cells were then spread onto pre-warmed, kanamycin containing plates under sterile conditions and were grown over night at 37°C. Four clones (clone 1, 2, 3 and 4) were picked randomly and inoculated in LB medium containing antibiotics. Recombinant protein production was tested next.

17. Overexpression of the recombinant cellobiose phosphorylase gene in BL21 (DE3) *E. coli*

The BL21 (DE3) competent cells belong to an all-purpose strain for high level protein expression and easy induction via isopropyl-1-thio- β -D-galactopyranoside (IPTG).

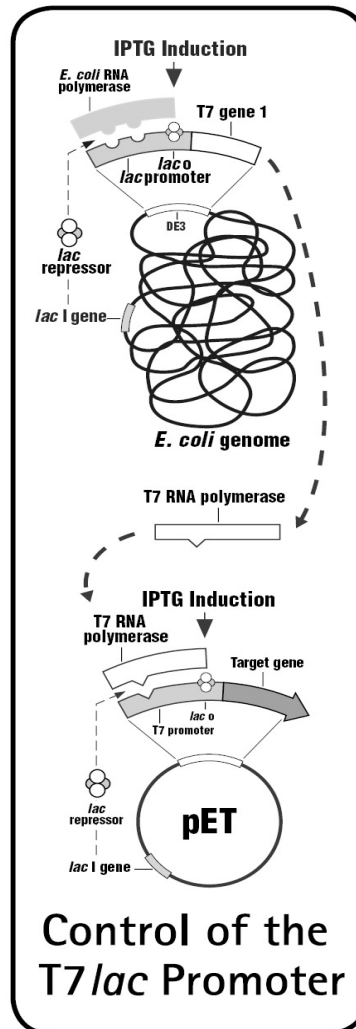


Figure 22 Expression of recombinant proteins in BL21 (DE3)

This strain, having the designation (DE3), is lysogenic for a λ prophage and contains an IPTG inducible T7 RNA polymerase. Prior to IPTG addition, the transcription of the T7 RNA polymerase and the gene of interest are inhibited by the Lac repressor. After IPTG addition, the inducer will bind specifically to the T7 promoter, initiating the transcription and translation of the T7 RNA polymerase and of the gene of interest (**Figure 22**).

To test the expression of cellobiose phosphorylase, 5 ml of LB medium containing 34 µg/ml kanamycin (Sigma Aldrich) was inoculated with four picked clones of BL21 (DE3) containing pET26b plain-CeP construct. The cells were grown at 37°C with shaking at 200rpm until the OD_{600nm} reached a value between 0.6-1. Subsequently, 1 ml was transferred into a fresh tube to be stimulated with 1mM IPTG over night. The following day, glycerol stocks were prepared from 3 ml of the unstimulated bacteria. 500 µl each of unstimulated and stimulated bacterial suspension were transferred into Eppendorf tubes and centrifuged for 1 min at 12 000rpm. The supernatant was removed and the pellet was subsequently resuspended with 50 µl of SDS-sample buffer containing β-mercaptoethanol. The lysate was heated for 5 min at 95°C, then cooled and homogenized using a syringe with a 0.19x40 needle. Next, the homogenized lysate was processed by SDS-PAGE for further analysis.

For the large scale production of recombinant cellobiose phosphorylase, 1 l of LB medium was prepared and autoclaved at 121°C. After cooling of the medium, 10 ml containing kanamycin were inoculated of the clone that had shown the highest level of protein production in the test. The cells were grown over night at 37°C and 250rpm. Next day, 1 ml of the overnight culture was separated as a glycerol stock. The remaining 9 ml were added to the rest of the LB medium (~990 ml) containing kanamycin and incubated at 37°C and 250rpm, to an OD_{600nm} between 0.6-1. In order to induce the expression, 1 ml of IPTG (1mM) was added to the cells which were grown for a further 12 h. Cells without IPTG induction were grown as well to serve as negative control for the western blot.

After 12 h, the cells were centrifuged at 4°C and 8000rpm for 15 min. The supernatant was discarded and the pellet of cells was snap frozen in liquid nitrogen and stored at -80°C for further experiments such as SDS polyacrylamide gel electrophoresis and western blot.

18. SDS polyacrylamide gel electrophoresis (SDS PAGE)

The standard electrophoretic technique used for the separation of proteins based on their size is polyacrylamide-gel electrophoresis (PAGE). After polymerization of monomeric acrylamide into polyacrylamide chains the polyacrylamide gels are formed. This process of polymerization is initiated by the addition of ammonium persulfate (APS), which in solution dissociates into

sulfate radicals, and tetramethylenediamine (TEMED), which catalyzes the formation of free radicals. Cross linking of polyacrylamide chains by *N*, *N*'-methylenebisacrylamide leads to a network of polyacrylamide chains that contain pores through which the proteins migrate. Sizes of proteins separated by SDS PAGE are dependent on the acrylamide concentration and the proportion of bisacrylamide in the total mixture [Lubura, 2003].

Acrylamide concentration %	Linear range of separation (kDa)
15	12-43
10	16-68
7.5	36-94
5	57-212

18.1 Separation of proteins by SDS-PAGE

In order to separate proteins in a polyacrylamide gel the most widely used method is the denaturing, discontinuous system with a separating gel and a stacking gel. The stacking gel has large pores and is prepared with a slightly acidic buffer (pH 6.8), whereas the separating gel has smaller pores to facilitate protein separation and has a higher pH (8.8).

For the preparation of both gels, the following solutions were used:

Separating Gel (10 ml, 12.5%) Stacking Gel (5 ml, 4%)

4.1 ml	0.65 ml	30.8% Acrylamide Stock Solution
2.5 ml		Separating gel buffer pH 8.8
	1.25 ml	Stacking gel buffer pH 6.8
46.9 µl	50 µl	10% APS
6.25 µl	5 µl	TEMED
3.4 ml	3 ml	distilled water

Protein samples were prepared as follows. 10 μ l of protein sample was heated for 10 min at 95°C in the presence of 10 μ l Laemmli buffer, which contains SDS and a reducing agent (DTT). SDS binds to the polypeptide chains giving a constant charge to mass ratio (**Figure 23**).

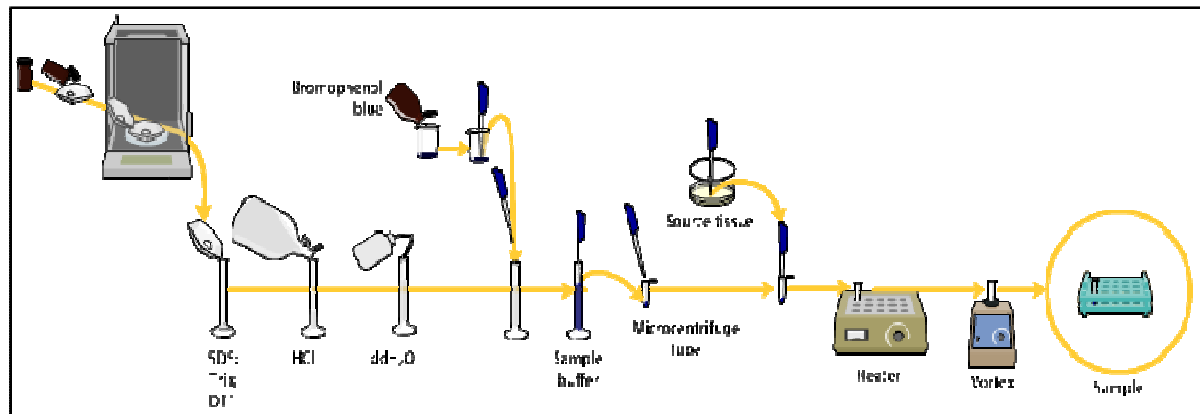


Figure 23 Preparation of the sample for SDS PAGE (Source: http://en.wikipedia.org/wiki/Western_blot)

Protein samples were loaded into wells in the stacking gel, an electric field is applied at constant current (125 V), and anions (chloride, SDS-coated proteins and glycine) begin to migrate toward the anode (**Figure 24**).

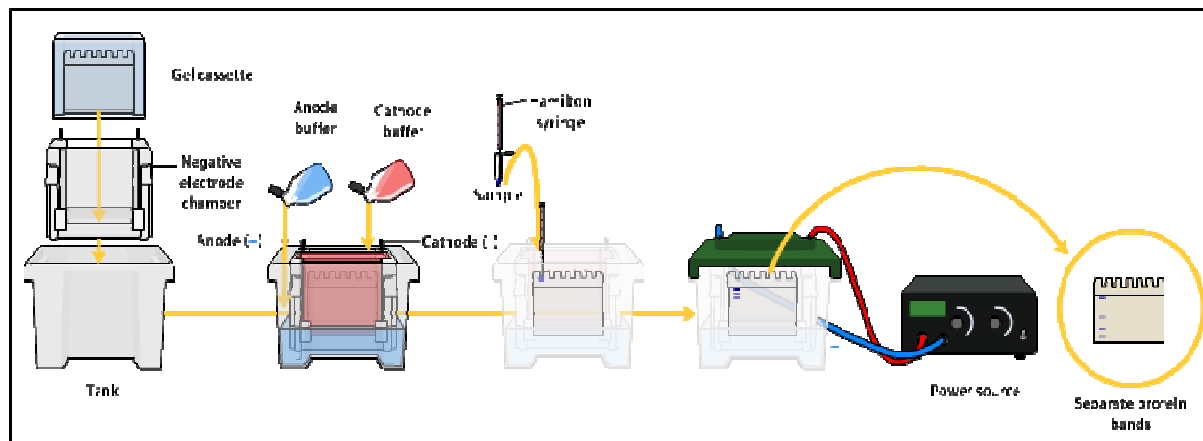


Figure 24 Procedure of SDS PAGE (Source: <http://en.wikipedia.org/wiki/SDS-PAGE>)

As the glycine ions have reached the stacking gel, their mobility is retarded as their net charge changes with the pH shift. Chloride ions and proteins remain mobile, thus continuing the movement toward the anode. As the retarded glycine ions and the faster-moving chloride ions

have created a zone of lower conductivity in the stacking gel between themselves, the voltage gradient increases, that allows proteins to migrate faster until they reach the zone of leading ions, where they concentrate, forming a tight band. When the proteins have reached the separating gel with much more smaller pores, their movement is slowed. Glycine ions regain their charge and pass the proteins, while the electric field becomes constant in the complete gel, allowing separation of proteins in the separating gel.

Due to the fact that proteins migrate in SDS PAGE as a function of their size, it is possible to determine their molecular weight by comparing their mobility with that of reference proteins. In this study, Precision Plus ProteinTM standard (BioRad) was used, designed to provide a ladder of convenient, consistent sizes (**Figure 25**).

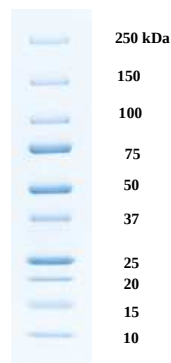


Figure 25 Unstained Precision Plus Protein standard

18.2 Coomassie blue staining

Coomassie dyes are a family of dyes commonly used to stain proteins in sodium dodecyl sulfate and blue native polyacrylamide gel electrophoresis gels. The gels are soaked in dye and excess stain is then eluted with a solvent ("destaining"). This treatment allows the visualization of protein bands. The gel usually contains a set of molecular weight markers (proteins of pre-determined weight) so that the molecular weight of a protein can be estimated in an unknown solution. [Lubura, 2003]

In this study, PhastGel Blue R (Pharmacia Biotech) was used. The stain stock solution is prepared using 1 tablet dissolved in 80 ml of distilled water. The solution was stirred for 10 min and 120 ml of methanol were added. The mixture was stirred until all of the dye had dissolved. The stock solution (0.2%) was stable for one to three weeks at 4°C.

After electrophoresis, the gels were removed from the SDS PAGE chamber and stained for 20 min in the stock stain solution by shaking at room temperature at 150rpm. Destaining of the gels was effected during 3x10 min by shaking at room temperature, in a stock destaining solution, consisting 99.8% of acetic acid (99.8 %) and 96% of ethanol, both dissolved in distilled H₂O.

19. Western blot

19.1 Transfer

In order to enable the antibody detection, the proteins separated on SDS PAGE were blotted from the gel onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes. PVDF membranes were first soaked in methanol and then placed on the top of the gel, and a stack of filter papers, soaked in TOWB buffer solution, was placed on top of the membrane. Electroblotting (protein transfer) uses an electric current in order to transfer the proteins from the SDS PAGE gel onto the PVDF membrane in a semi-dry transfer (Amersham). Thus, the proteins move from within the gel onto the membrane while maintaining the organization they had within the gel (50 minutes, at 80mA). As a result, proteins were exposed on a thin surface layer for detection (**Figure 26**). Protein binding is based upon hydrophobic interactions and charge interactions between the membrane and the protein.

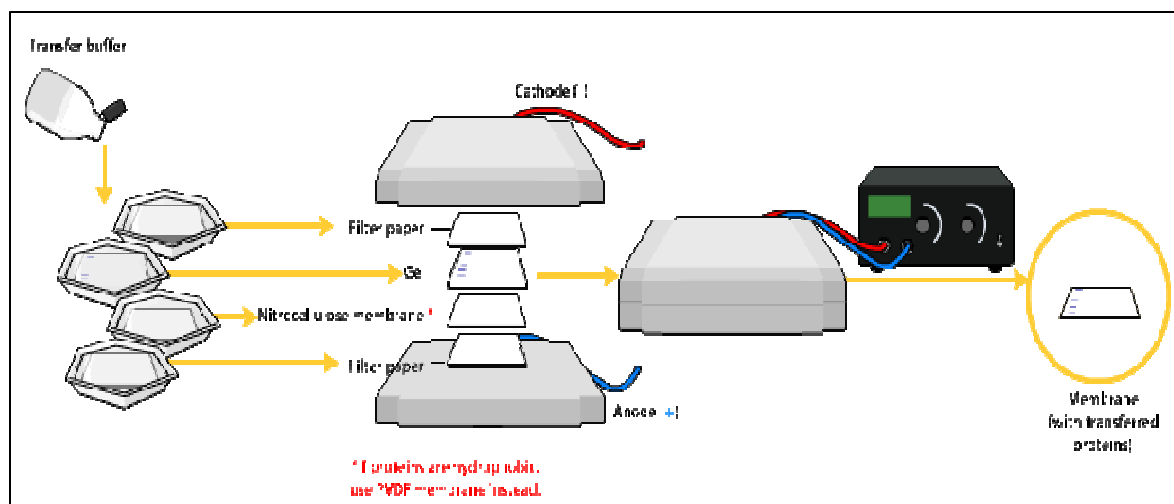


Figure 26 Western blot transfer (Source: <http://en.wikipedia.org/wiki/SDS-PAGE>)

19.2 Blocking

Due to the fact that the PVDF membrane is capable of binding protein, and both antibody and the target are proteins, it is important to prevent interactions between the membrane and the antibodies used for the detection of the recombinant enzyme cellobiose phosphorylase. In order to block non-specific binding, the membrane was placed in 5% of dry milk dissolved in 1xTBST buffer and incubated over night at 4°C. As a result, the milk proteins attached to the membrane in all places where the target protein, cellobiose phosphorylase was not attached.

19.3 Detection

For specific detection, it is necessary to choose an appropriate antibody. In the present study, an anti-His antibody was used. This antibody is linked to a reporter enzyme, which catalyzes the conversion of a colorless substrate into colored products.

After blocking of the PVDF membrane over night, it was washed 3 times for 10 min with TBST buffer by shaking. Subsequently, the PVDF membrane was incubated for 1 h at room temperature with shaking with a solution prepared by dissolving 2% of dry milk in 10 ml 1xTBST buffer and adding 5 µl of Penta-His antibody (1:1000, Qiagen). After 1 h the milk solution containing the antibody was removed from the membrane and the membrane washed 3 times for 10 min with 1xTBST buffer at room temperature with shaking. Finally, TBST buffer with 0.3% of Tween20 was added and the membrane was incubated by shaking at room temperature for 15 min.

Blot development was performed with a chemiluminescent detection method (CP1000, AGFA), using a chemiluminescent agent, which produces luminescence in proportion to the amount of protein. By placing a sensitive sheet of photographic film against the membrane, and due to the exposure to the light from the reaction, an image of the antibody bound on the blot is created.

20. Nickel-nitroltriacetic acid (Ni-NTA) metal ion affinity chromatography

The expression and purification of recombinant proteins facilitate production and detailed characterization of virtually any protein. Classical purification procedures can be employed, but in most cases recombinant DNA techniques permit the construction of fusion proteins in which specific affinity tags simplify the purification of the recombinant fusion proteins by employing

affinity chromatography methods. One of the methods, also used in this study, is based on Ni-NTA metal ion affinity chromatography matrices for biomolecules which were tagged with 6 consecutive histidine residues (6xHis tag). The advantage of the 6xHis tag is that, due to the immobilization of the protein on the metal-chelating surfaces, it simplifies many types of protein interaction studies. In addition, anti-his tag antibodies can be used for detection. [The QIAexpressionist, QIAGEN, 2003]

20.1 Preparation of cell lysate under native conditions

The harvested *Clostridium thermocellum* NCIMB 10682 cells were suspended in 10 ml of the resuspension buffer and sonicated on ice with the aid of an ultraturrax high performance disperser at 4°C for 6x10 min with 2 min breaks. The lysate was centrifuged at 8000rpm for 1 h to pellet the cellular debris and the supernatant was transferred to a fresh tube.

20.2 Preparation of the Ni-NTA column and purification procedure under native conditions

The decision to purify 6xHis-tagged proteins under native or denaturing conditions depends on the solubility of the protein and the need to retain biological activity for downstream applications. In this study, native conditions are used, due to the fact that the enzyme cellobiose phosphorylase is soluble (in the supernatant after lysis) and the protein activity has to be preserved.

For the preparation of the column, the Ni-NTA agarose (Qiagen) was resuspended in its bottle by inverting and gently tapping the bottle repeatedly. 400 µl of the resin was pipetted into a 1 ml micro Bio-Spin chromatography column (BioRad) and settled completely by gravity after 5 min. The resin was equilibrated (in total 5 times) by adding the resuspension buffer to the column, and removing the buffer each time by centrifugation for 1 min at 2000rpm and 4°C.

7 ml of the cell lysate, prepared under native conditions, were mixed with the Ni-NTA agarose in a 50 ml Falcon tube and incubated at room temperature for 3 h with shaking at 250rpm. The suspension was pipetted with a Pipetman P1000 in aliquots (each time 1 ml) to a 1 ml micro Bio-Spin chromatography column with the flow-through being collected.

Subsequently, the column was washed with 400 µl resuspension buffer 10 times collecting the flow through. The protein was eluted by use of 200 µl elution buffer for 10 times. Imidazole is used to elute tagged proteins bound to Ni ions attached to the surface of beads in the

chromatography column. An excess of imidazole is passed through the column, which displaces the His-tag from the nickel co-ordination complexes, freeing the His-tagged proteins. For each elution sample, a 1 ml Eppendorf tube was used for the collection of the protein by centrifugation at 2000rpm for 1 min. [The QIAexpressionist, QIAGEN, 2003]

The collected elution samples were analyzed by Lowry and D-glucose test and by western blot as described previously.

21. Growth of non competent BL21 (DE3) *E. coli* cells

In order to examine the possible existence of the enzyme phosphatase within BL21 (DE3) *E. coli* cells, which may influence the phosphorolytic reaction of cellobiose, these cells were grown at 37°C, then sonicated and the supernatants analyzed by Lowry test and by D-glucose test.

For the growth of these cells, 1 l of LB medium was used. At first, 500 µl of BL21 (DE3) *E. coli* stock solution was added to 10 ml LB medium with 10 µl kanamycin (34 mg/ml) and incubated over night at 37°C and 250rpm. The overnight culture was transferred to the remainder of the LB medium (1:100), with 1 ml kanamycin (1:1000) and incubated at 37°C and 200rpm. Growth was monitored spectrophotometrically by measuring OD_{600nm} periodically. The optimum OD was 0.6. 1 ml of IPTG (1mM) was added to the cells which were grown for a further 12 h. After 12 h, the cells were centrifuged at 4°C and 8000rpm for 15 min. Subsequently, the pellet was sonicated using an ultraturrax high performance disperser as described previously, the lysate ultrafiltered and the supernatant (= 1 ml) used for incubation, as shown below.

Substance	Concentration
cellobiose	1.2 ml 0.1M
phosphate buffer pH 7	0.8 ml 0.1M
BL21 (DE3) <i>E. coli</i>	1 ml

1 ml of the ultrafiltered crude extract from *Clostridium thermocellum* NCIMB 10682 was incubated with cellobiose as substrate; the D-glucose produced was used as positive control for the intracellular phosphorolysis.

Substance	Concentration
cellobiose	1.2 ml 0.1M
phosphate buffer pH 7	0.8 ml 0.1M
ultrafiltered crude extract	1 ml 1 U/0.8 mg

Both samples were incubated at 37°C, and analyzed by TLC method as described in Chapter 8 (p. 72) according to a set timetable (0, 2, 4, 20 and 24 h). The analysis of the positive control was performed according to the Lowry and D-glucose tests.

22. Substrate specificity studies of *Clostridium thermocellum* NCIMB 10682 purified cellobiose phosphorylase towards selected monosaccharides and cellobiose azidosaccharides

22.1 Synthetic reaction of native cellobiose phosphorylase towards monosaccharides

Monosaccharides D-xylose, D-glucose, 2-deoxyglucose and 6-deoxyglucose were incubated with purified cellobiose phosphorylase of *Clostridium thermocellum* NCIMB 10682 in the presence of α -D-glucose 1-phosphate at 37°C for 24 h.

Substance	Concentration
D-xylose	0.4 ml 0.1M
D-glucose	0.4 ml 0.1M
2-deoxyglucose	0.4 ml 0.1M
6-deoxyglucose	0.4 ml 0.1M
TRIS buffer pH 7	2 ml 0.1M
α -D-glucose 1-phosphate	0.6 ml 0.076M
purified cellobiose phosphorylase	1 ml 3 U/0.1 mg

1 ml of the purified cellobiose phosphorylase was incubated with cellobiose as substrate. The extent of reaction was used as positive control for the phosphorolysis.

Substance	Concentration
cellobiose	1.2 ml 0.1M
phosphate buffer pH 7	0.8 ml 0.1M
purified cellobiose phosphorylase	1 ml 3 U/0.1 mg

All samples were incubated at 37°C, and analyzed by TLC method as described in Chapter 8 (p. 72) according to the set timetable (0, 2, 4, 20 and 24 h). The analysis of the positive control was performed according to the Lowry and D-glucose tests.

22.2 Phosphorolytic reaction catalyzed by native cellobiose phosphorylase towards azidocellobiose derivatives

6-azido-6-deoxy-cellobiose, 6'-azido-6'-deoxy-cellobiose and 2-azido-2-deoxy-cellobiose were incubated with purified cellobiose phosphorylase of *Clostridium thermocellum* NCIMB 10682 at 37°C for 12 h.

Substance	Concentration
6-azido-6-deoxy-cellobiose	1.2 ml 0.1M
6'-azido-6'-deoxy-cellobiose	1.2 ml 0.1M
2-azido-2-deoxy-cellobiose	1.2 ml 0.1M
cellobiose	1.2 ml 0.1M
phosphate buffer pH 7	0.8 ml 0.1M
purified cellobiose phosphorylase	1 ml 3 U/0.1 mg

1 ml of the purified cellobiose phosphorylase was also incubated with cellobiose as substrate; the extent of reaction was used as positive control for the phosphorolysis under the same conditions as for azidocellobiose derivatives.

All samples were incubated at 37°C and analyzed by TLC method as described in Chapter 8 (p. 72) according to the set timetable (1, 3, 6, 8, 10 and 12 h); the analysis of the positive control was performed according to the Lowry and D-glucose tests.

22.3 Synthetic reaction catalyzed by native cellobiose phosphorylase towards 6-azido-6-deoxy-D-glucose

6-azido-6-deoxy-D-glucose was incubated with purified cellobiose phosphorylase of *Clostridium thermocellum* NCIMB 10682 in the presence of α -D-glucose 1-phosphate at 37°C for 12 h.

Substance	Concentration
6-azido-6-deoxy-D-glucose	0.8 ml 0.1M
TRIS buffer pH 7	4 ml 1M
α -D-glucose 1-phosphate	1.2 ml 0.076M
purified cellobiose phosphorylase	2 ml 3 U/0.1 mg

1 ml of the recombinant cellobiose phosphorylase was incubated with cellobiose as substrate; the extent of reaction was used as positive control for the phosphorolysis.

Substance	Concentration
cellobiose	1.2 ml 0.1 M
phosphate buffer pH 7	0.8 ml 0.1 M
purified cellobiose phosphorylase	1 ml 3 U/0.1mg

Both samples were incubated at 37°C and were analyzed by TLC method as described in Chapter 8 (p. 72) according to the set timetable (1, 3, 6, 8, 10 and 12 h); the analysis of the positive control was performed according to the Lowry and D-glucose tests.

22.4 Phosphorolytic reaction of recombinant cellobiose phosphorylase towards 2-azido-2-deoxy-cellobiose

2-azido-2-deoxy-cellobiose was also incubated with recombinant cellobiose phosphorylase at 37°C for 24 h. Previously, the enzyme was purified by use of Ni-NTA metal ion affinity chromatography as described in Chapter 20 (p. 97).

Substance	Concentration
2-azido-2-deoxy-cellobiose	1.2 ml 0.1 M
phosphate buffer pH 7	0.8 ml 0.1 M
recombinant cellobiose phosphorylase	2 ml 7.4 U/mg

1 ml of the recombinant cellobiose phosphorylase was incubated with cellobiose as substrate; the extent of reaction was used as positive control for the phosphorolysis.

Substance	Concentration
cellobiose	1.2 ml 0.1 M
phosphate buffer pH 7	0.8 ml 0.1 M
recombinant cellobiose phosphorylase	1 ml 7.4 U/mg

Both samples were incubated at 37°C and were analyzed by TLC method as described in Chapter 8 (p. 72) according to the set timetable (1, 3, 6, 12 and 24 h); the analysis of the positive control was performed according to the Lowry and D-glucose tests.

23. Lyophilization

In order to preserve perishable materials a dehydration process, so called freeze-drying (lyophilization) is used (<http://en.wikipedia.org/wiki/Lyophilization>).

Lyophilization is a very mild method to avoid destruction of the physical structure of substances such as pharmaceuticals. It involves freezing of materials, reducing of surrounding the pressure and the application of heat in order to allow frozen water to sublime. The freeze-drying process includes three stages: freezing, primary drying and secondary drying. As showed in the phase diagram below (**Figure 27**), the boundary between gas and liquid runs from the triple point to the critical point. Freeze-drying, indicated with blue arrow, brings the system around the triple point, therefore avoiding the direct liquid-gas transition as seen in ordinary drying, indicated by the green arrow.

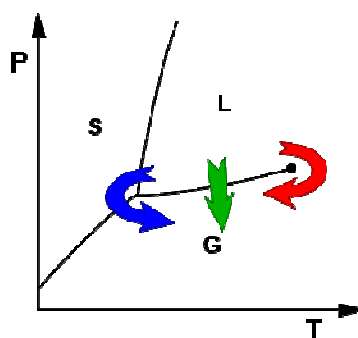


Figure 27 Phase diagram of freeze-drying process (Source: <http://en.wikipedia.org/wiki/Lyophilization>)

23.1 Applications of freeze-drying

By removing the water molecules from the material, the shelf life of the material can be prolonged and storage facilitated. In the pharmaceutical industry, products are often freeze-dried in order to make them more stable and easier to dissolve in water or a buffer for subsequent use as it was done while the purification procedure of native cellobiose phosphorylase. This method is also capable of concentrating substances with low molecular weights that are too small to be removed by a filtration membrane. The freeze-drying method has a long process time, because the exposure to too much heat can lead to melting or structural deformations of sensitive materials. Due to this fact, freeze-drying is used for materials which are heat sensitive, such as proteins, enzymes and microorganisms.

In this study, also azido sugars, synthesized by enzymatic catalysis of native and recombinant cellobiose phosphorylase were lyophilized in order to prolong their shelf life.

24. Bio-Gel® P Polyacrylamide Gel

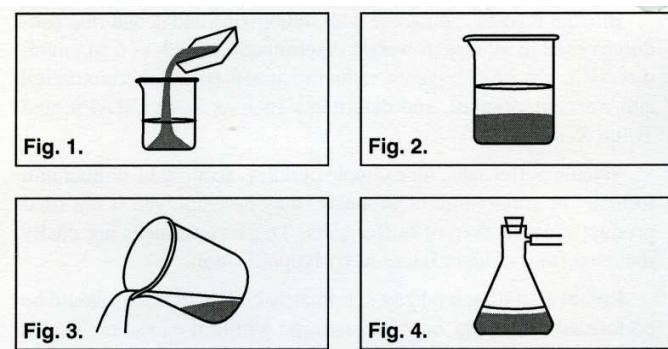
The Bio-Gel® P Polyacrylamide Gel [Bio Gel® P-2 Gel Fine Instruction Manual, 2000], provides an efficient, gentle gel filtration of sensitive compounds. The beads are extremely hydrophilic and consist of porous polyacrylamide beads, prepared by copolymerization of acrylamide and N, N'-methylene-bis-acrylamide. Its high resolution is assured by consistently narrow distribution of bead diameters and excellent molecular weight discrimination. Gel filtration is largely independent of sample concentration, whereas the volume of the sample relative to the bed volume is far more important. For analytical purposes the sample does not

have to be larger than 1-5% of the bed volume. The sample should be also clear and completely dissolved in running buffer, without solid contaminants.

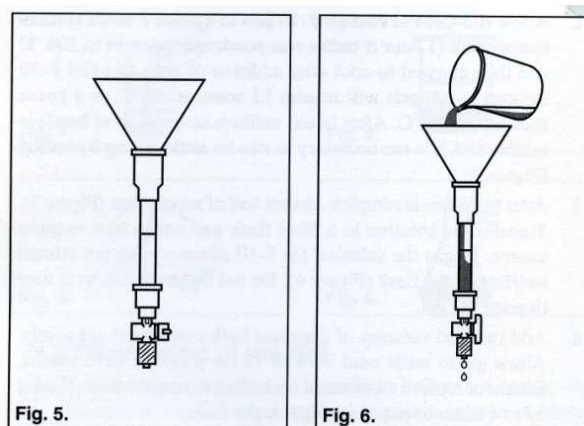
24.1 Preparation of the gel

The Bio-Gel[®] P Polyacrylamide Gel was used for the purification of the final product of appropriate azido sugars, which previously were obtained under catalysis of the enzyme, cellobiose phosphorylase. For this purpose the protocol provided by BioRad [Bio Gel[®] P-2 Gel Fine Instruction Manual, 2000] was used for the gel preparation (**Figure 28** [= 1-6]):

- I. Dry Bio-Gel P-2 Gel (fine) was added to distilled water in a beaker, the required amount of the gel being estimated from the volume of the column used (1.7 cm x 35 cm, BioRad; **Figure 1**)
- II. The Gel was hydrated at room temperature; stirring is not necessary (**Figure 2**)
- III. When the hydration was complete, half of the supernatant was decanted (**Figure 3**). The solution was transferred to a filter flask and degassed by application of vacuum for 5-10 min (**Figure 4**)



- IV. The used column was fixed (**Figure 5**) and the even slurry was poured into the column in a single, smooth movement, avoiding air bubbles. When 2-5 cm bed was formed, the column outlet was opened until the column was packed (**Figure 6**)



- V. Lyophilized samples of azido sugars were dissolved in the minimum amount of distilled water (200-300 μ l for ca. 5-10 mg) and loaded onto the upper bed surface with the aid of a Pasteur pipette. 500 μ l fractions were collected and TLC was performed in order to visualize the azido sugars in eluted fractions.

VIII. RESULTS

1. Optimization of conditions for the purification of native cellobiose phosphorylase

1.1 Optimization of the mobile phase buffer for DEAE cellulose chromatography

In order to obtain cellobiose phosphorylase from enzyme crude extract with high purity, conditions had to be set up which have enabled a simple and effective purification procedure. Mobile phase buffer (= starting buffer) with concentrations of 20 (= phosphate buffer), 50 (= Tris buffer) and 100mM (= Tris buffer) of pH 5-7.5 was tested. The optimal starting buffer should provide high binding capacity of cellobiose phosphorylase to the positively charged solid support (DE52 DEAE-Cellulose). For this purpose, the enzyme activity eluted during sample application should be minimized. In Table 6, the total yield of D-glucose which was obtained during the phosphorolytic reaction, catalyzed by cellobiose phosphorylase, is shown. Samples were eluted during sample application from DEAE cellulose when starting buffer with different concentrations was used (percentages indicated below). This indicates that pH = 7.5 and 50mM buffer constitute optimal conditions for binding of the enzyme to DEAE cellulose.

	20mM buffer % D-glucose	50mM buffer % D-glucose	100mM buffer % D-glucose
pH 5	20.8	85.5	46.8
pH 5.5	21.4	33	29
pH 6	21.4	22	19
pH 6.5	20	20.6	21.2
pH 7	20.4	3.3	4.2
pH 7.5	20.4	2.4	3.1
crude extract	100	100	100

Table 6 D-glucose yield in %

50 and 100mM Tris buffer of pH 7 and 7.5 enable strong binding to DEAE cellulose so that a residual (breakthrough) enzyme activity remains which ranges between 2.4 to 4.2% per 3 ml incubation volume. By contrast, the breakthrough activity was fivefold greater when 20mM phosphate buffer of pH 7 and 7.5 was used (20.4%).

Table 7 shows the total yield of D-glucose obtained during the phosphorolytic reaction of DEAE breakthrough samples when starting buffer with different concentrations and at different pH values was used; yields are indicated in μg per 3 ml incubation volume.

	20mM buffer	50mM buffer	100mM buffer
	μg D-glucose	μg D-glucose	μg D-glucose
pH 5	113	635	1160
pH 5.5	116	180	157
pH 6	116	117	103
pH 6.5	103	112	115
pH 7	111	18	23
pH 7.5	111	13	17
crude extract	543	543	543

Table 7 D-glucose yield in μg

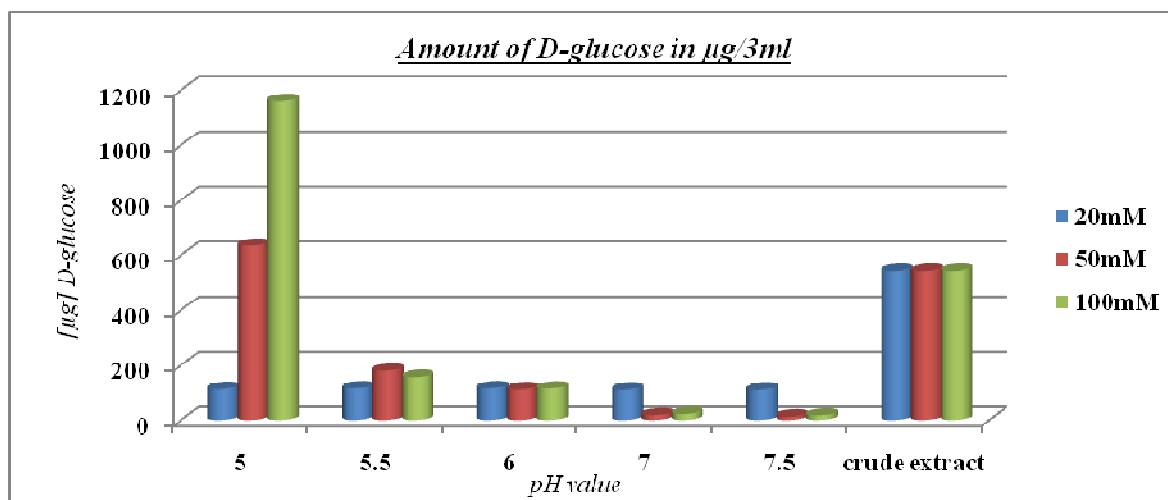


Figure 29 Diagram of D-glucose yield in μg

Both, Table 7 and Figure 29 show that the highest extent of breakthrough was observed with 100mM buffer within a pH range of 5, 5.5, 6 and 6.5. By comparison, there is almost no breakthrough activity for those samples at pH = 7 or 7.5 at 50 or 100mM buffer concentration.

These results were also expected, considering that in anion exchange chromatography, lowering the pH of the starting buffer proportional to the isoelectric point (= pI) of cellobiose phosphorylase (pI = 5.63) causes the molecule to become more protonated and hence positively charged; therefore, the enzyme protein will not bind to an anion exchanger. The protein no longer has the capability to form a strong ionic interaction with the positively charged solid support and is eluted earlier from the chromatography column.

With 50 and 100mM Tris buffer at pH 7 and 7.5, strong binding of cellobiose phosphorylase to the column material occurs; this is due to the fact that at pH values above the pI of cellobiose phosphorylase, the protein molecule has a net negative charge and thus binds to (positively charged) anion exchanger. This was also confirmed by the determination of a very low residual enzyme activity (D-glucose yield in a range between 13 to 23 µg per 3 ml incubation volume).

As clearly indicated by present results, the lowest extent of breakthrough (residual yield of D-glucose) was observed with samples dissolved in the following buffers:

- a) 50mM Tris pH 7**
- b) 50mM Tris pH 7.5**
- c) 100mM Tris pH 7**
- d) 100mM Tris pH 7.5**

All of the above mentioned Tris buffers can be considered as starting buffers for the purification of the native cellobiose phosphorylase.

The standard DEAE chromatography procedure used for purification of cellobiose phosphorylase demands a greater amount of column material than used in preliminary experiments. Considering this fact, it was necessary to test the binding capacity of cellobiose phosphorylase with greater amounts of DEAE-Cellulose. For this, double amount of Whatman DE52 DEAE-Cellulose was used than previously. The initial findings were shown to be reproducible as indicated in Table 8 when 1 g of Whatman DE52 DEAE-Cellulose was used and in Table 9 when 3 g of Whatman DE52 DEAE-Cellulose was used.

	50mM buffer	100mM buffer	50mM buffer	100mM buffer
	$\mu\text{g D-glucose}$	$\mu\text{g D-glucose}$	% D-glucose	% D-glucose
pH 7	50	54	11.5	12.4
pH 7.5	58	54	13	12.4
crude extract	436	436	436	436

Table 8 D-glucose yield in 1 g Whatman DE52 DEAE-Cellulose

	50mM buffer	100mM buffer	50mM buffer	100mM buffer
	$\mu\text{g D-glucose}$	$\mu\text{g D-glucose}$	% D-glucose	% D-glucose
pH 7	53	51	12	11.7
pH 7.5	50	51	11.5	11.7
crude extract	436	436	100	100

Table 9 D-glucose yield in 3 g Whatman DE52 DEAE-Cellulose

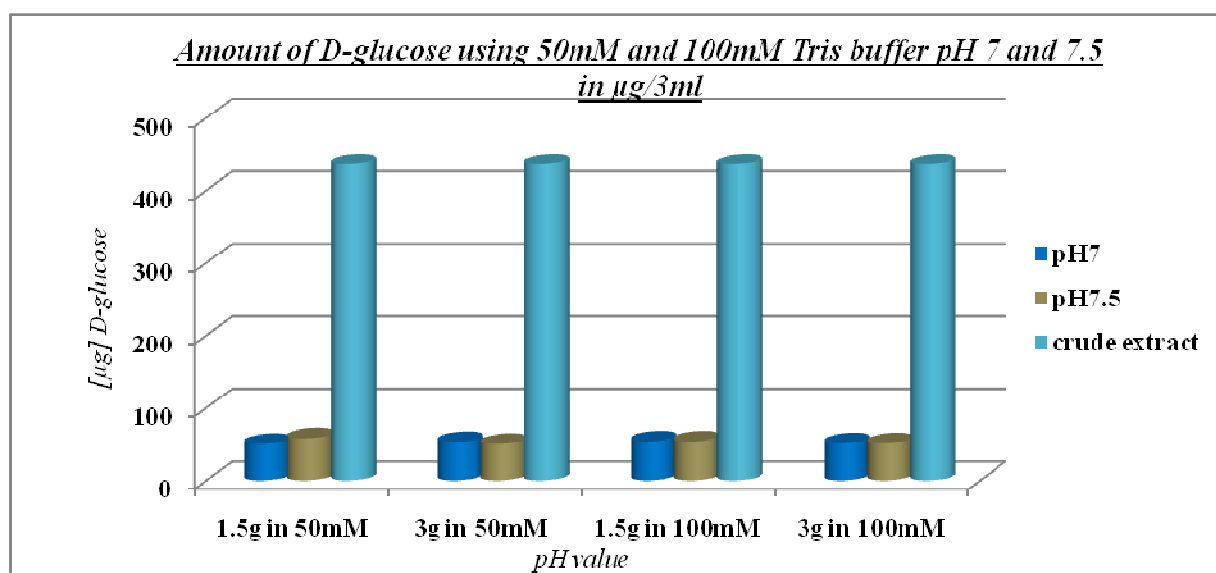


Figure 30 Diagram of D-glucose yield in 1 g and 3 g Whatman DE52 DEAE-Cellulose

All incubated samples are shown in Figure 30 and were also examined via TLC as shown in Figure 31. During these experiments, formation of a trisaccharide (orange arrow) was observed

whose appearance could be explained by an accompanying activity of cellodextrin phosphorylase. Conceivably, one glucose unit could be transferred from α -D-glucose 1-phosphate to cellobiose so as to form cellotriose. It is unlikely that cellotriose formation is catalyzed by cellobiose phosphorylase, as the cloned enzyme did not show this activity in our hands.

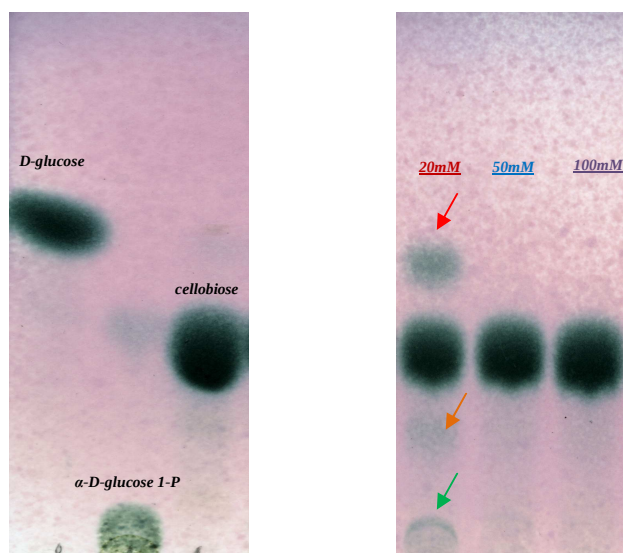


Figure 31 TLC with standards and incubated samples washed with 20mM, 50mM and 100mM Tris pH 7. Breakthrough activity is observed only with 20mM Tris buffer.

To choose a starting buffer with the lowest salt concentration yet giving the optimum protein binding capacity, 50mM Tris buffer pH 7 was chosen as the starting buffer rather than 100mM Tris pH 7.

1.2 Optimization of elution buffer

In order to optimize the salt concentration of the elution buffer, NaCl concentrations in a range of 0.05 and 1200mM were tested.

As shown in Figure 32, low salt concentration does not lead to an early elution of cellobiose phosphorylase, when using a salt gradient of the range between 0.05 and 100mM, indicated by a low yield of D-glucose during the phosphorolytic reaction, which ranges between 13 μ g per 3 ml incubation volume when applying 0.05mM NaCl and 90 μ g per 3 ml incubation volume when applying 100mM NaCl.

These results were also expected, considering that cellobiose phosphorylase shows a high binding capacity to the positively charged solid support as proven in the previous experiments for the determination of the optimized starting buffer. Due to its strong ionic interaction, a higher salt concentration is required for the elution of cellobiose phosphorylase.

In this context, when NaCl with higher concentration (150-1200mM) was applied, a significant difference in yield of D-glucose was observed. It ranges between 119 μg per 3 ml incubation volume when 150mM NaCl were used and 410 μg per 3 ml incubation volume when 600mM NaCl were used.

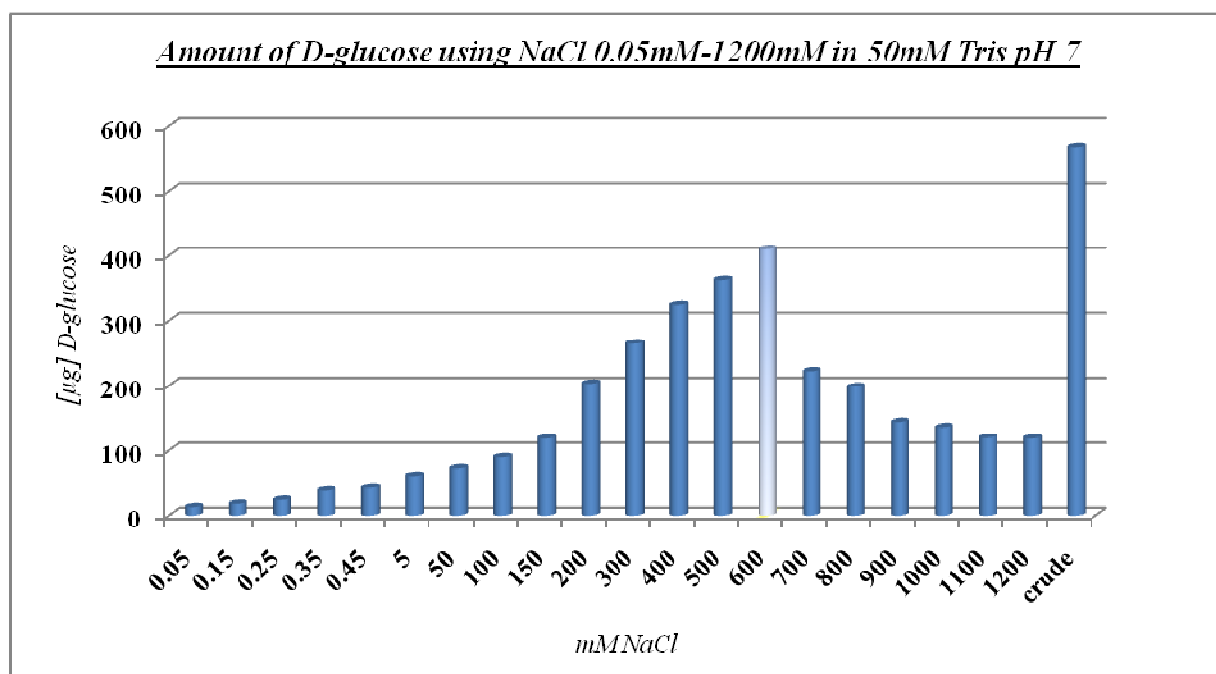


Figure 32 Diagram of D-glucose yield in 0.05mM-1200mM NaCl

In comparison to enzyme crude extract with a D-glucose yield of 568 μg per 3 ml incubation volume, 72% of D-glucose was detected during the phosphorolytic reaction when 600mM NaCl in the elution buffer was applied to the column.

By contrast, only 2.3% of D-glucose was detected during the phosphorolytic reaction when 0.05mM NaCl as elution buffer was applied, both shown in Tables 10 and 11.

	0.05mM NaCl	0.15mM NaCl	0.35 mM NaCl	50 mM NaCl	100 mM NaCl
% D-glucose	2.3	3.2	6.7	12.8	15.8

Table 10 Yield of D-glucose in 0.05-100mM NaCl (%)

	150 mM NaCl	400 mM NaCl	600 mM NaCl	800 mM NaCl	1200 mM NaCl
% D-glucose	35	57	72	21.4	30

Table 11 Yield of D-glucose in 200-1200mM NaCl (%)

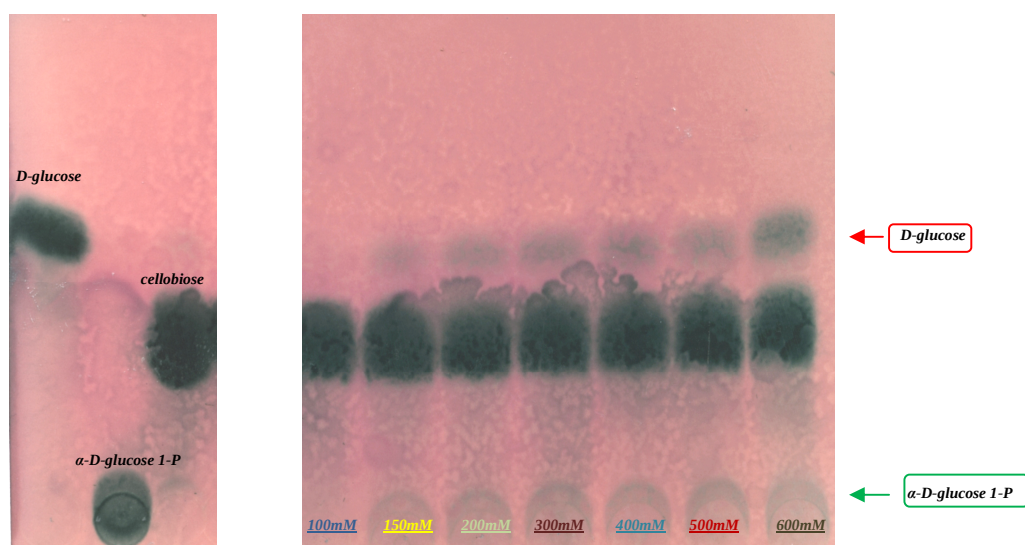


Figure 33 TLC with standards and incubated samples eluted with 100mM-600mM NaCl

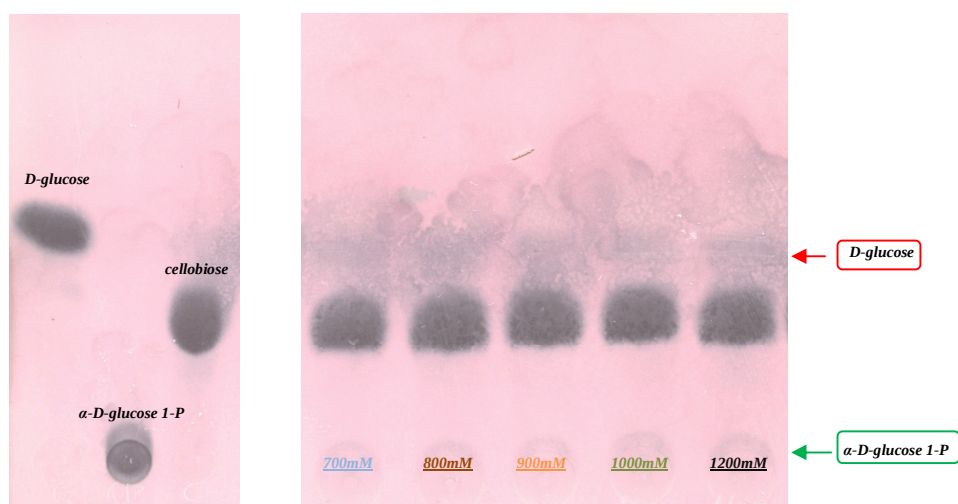


Figure 34 TLC with standards and incubated samples eluted with 700mM-1200mM NaCl

All incubated samples were also analyzed via TLC as shown in Figures 33 and 34. The chromatograms indicate the first evidence of D-glucose formation when 150mM NaCl was used, reaching the peak of the protein activity when the elution buffer containing 600mM NaCl was applied. Thus, the specific activity of cellobiose phosphorylase was proven by formation of D-glucose (red arrow) as well as of α -D-glucose 1-phosphate (green arrow). These results verify the conclusion of the above interpreted findings.

Considering these results, the elution buffer with 600mM NaCl concentration was chosen for the elution of native cellobiose phosphorylase from *Clostridium thermocellum* strain NCIMB 10682.

2. Purification of cellobiose phosphorylase under optimized conditions for IEC

The purification step by anion exchange chromatography using Whatman DE52 DEAE cellulose as column material was followed via TLC. Figure 35 shows a chromatogram of the formation of D-glucose and α -D-glucose 1-phosphate under catalysis by enzyme fractions eluted from DEAE cellulose.

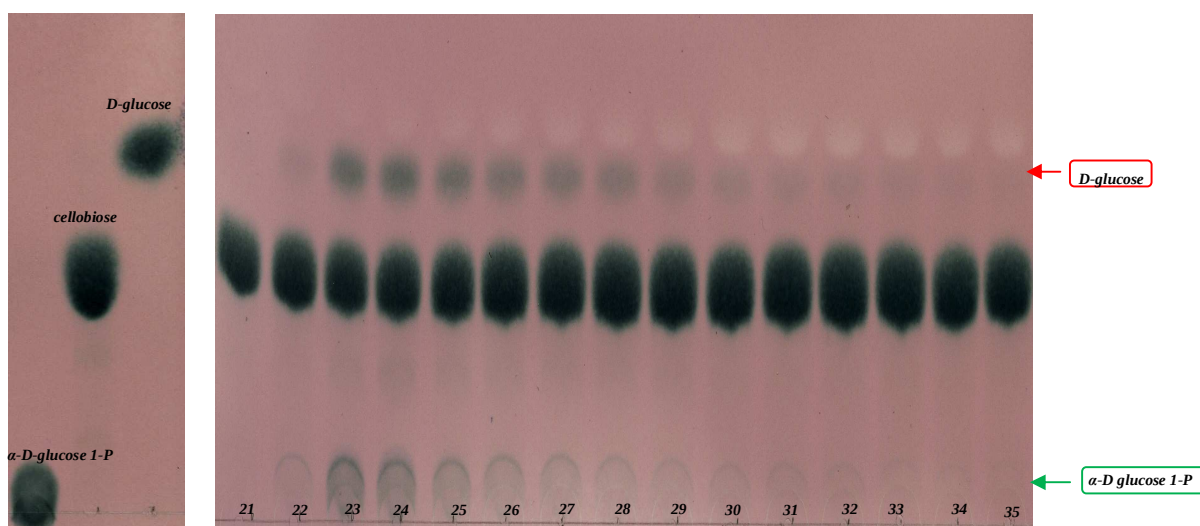


Figure 35 TLC with standards and fractions with enzyme activity

All fractions, even those showing the weakest protein activity were pooled together (fractions 22-35), dialyzed against 3.5 l 50mM Tris buffer pH 7 and lyophilized.

3. Gel filtration chromatography for cellobiose phosphorylase purification

The purification step by gel filtration chromatography (= Sephacryl S-200HR) was followed via TLC, which reveals a clear elution of cellobiose phosphorylase as indicated in Figures 36 and 37.

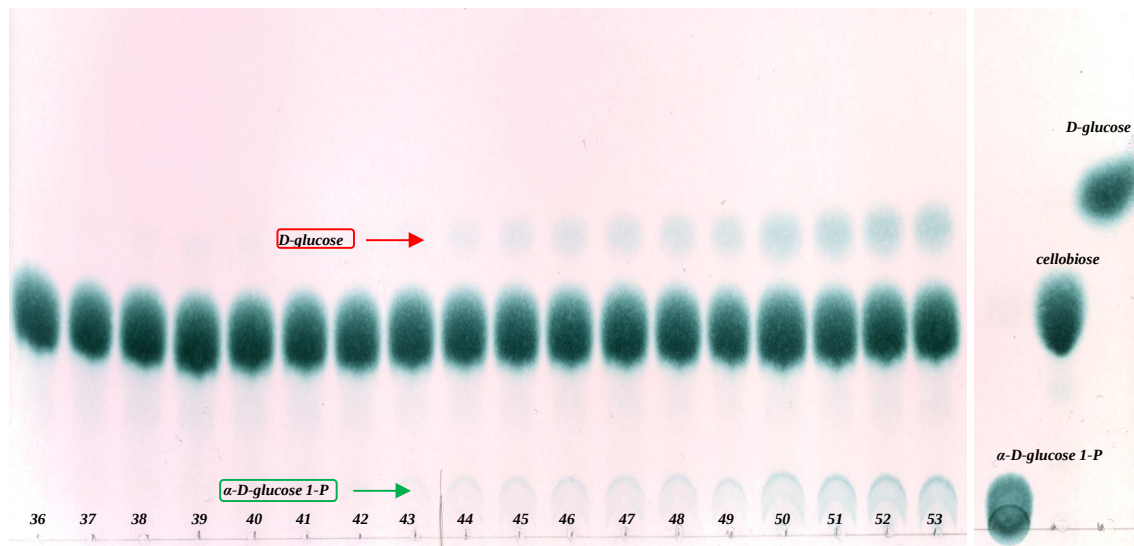


Figure 36 TLC with standards and fractions with enzyme activity

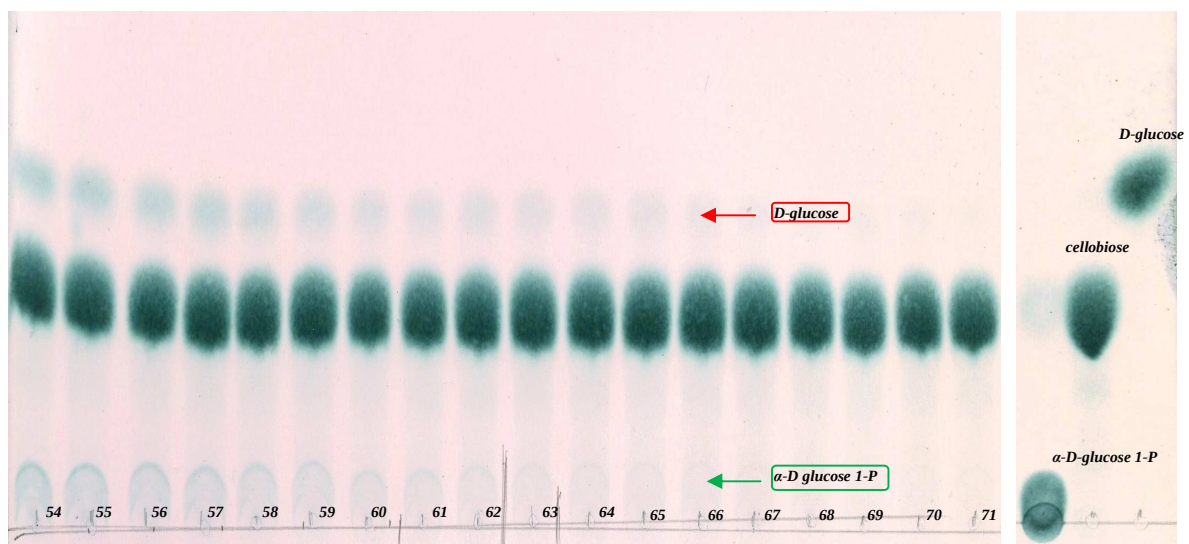


Figure 37 TLC with standards and fractions with enzyme activity

Both TLC's show the peak of activity within the fractions 50-59.

Fractions 44-65 were pooled together, dialyzed against 3.5 l 50mM Tris buffer pH 7 and lyophilized.

Table 12 summarizes the outcome of all purification steps conducted for cellobiose phosphorylase.

	Total volume [ml]	Glucose yield [$\mu\text{mol}/\text{min}$]	Total protein [mg]	Total activity [Units]	Specific activity [U/mg]	Purification - fold	Activity yield [%]
Crude extract	120	0.123	19.86	12.3	0.62	1	100
Ultrafiltration	6	0.157	0.83	0.8	1	1.6	6.5
Whatman DE52	1.6	0.237	0.13	0.316	2.43	4	2.6
Sephacryl S-200HR	1.2	0.245	0.09	0.245	3	5	2

Table 12 Purification of cellobiose phosphorylase from crude extract of *Clostridium thermocellum*

These three-step chromatography procedures reveal a fivefold greater enrichment of the cellobiose phosphorylase after the last purification step, the gel filtration chromatography using Sephacryl S-200HR as column material.

The rise of the cellobiose activity was also detected semiquantitatively by TLC, shown in Figure 38.

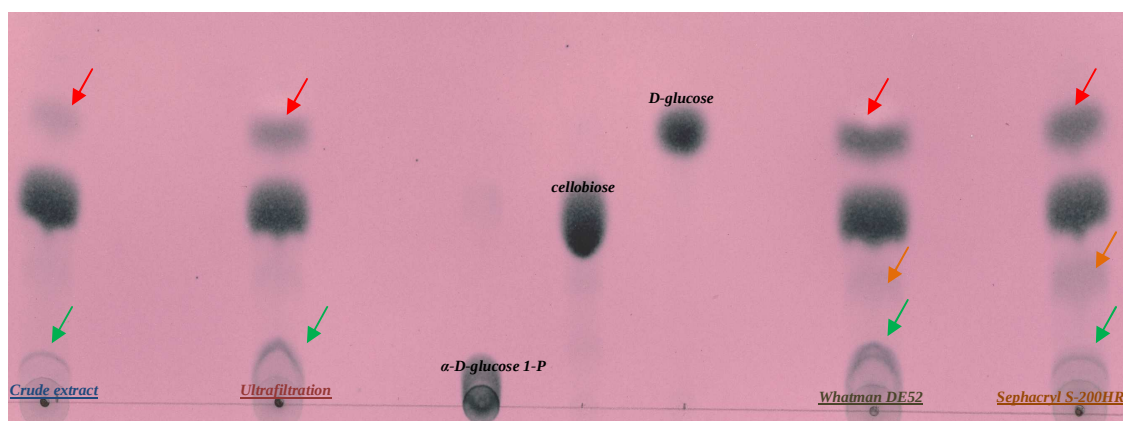


Figure 38 TLC with standards and incubated samples after three-step chromatography procedures

The TLC indicates the formation of D-glucose (red arrow) as well as of α -D-glucose 1-phosphate (green arrow) in all purification steps, revealing increasing activity of cellobiose phosphorylase. Also, a trisaccharide formation (orange arrow) was observed in samples after the anion exchange purification (Whatman DE52) step and gel filtration chromatography (Sephacryl S-200HR). This

indicates that the accompanying cellodextrin phosphorylase activity is not separated under the purification conditions employed.

On SDS PAGE analysis, the subsequent chromatographic steps resulted in the distinct appearance of the presumed band of cellobiose phosphorylase. An apparent molecular mass of ~93 kDa was determined for the purified cellobiose phosphorylase after gel filtration chromatography as indicated in Figure 39.

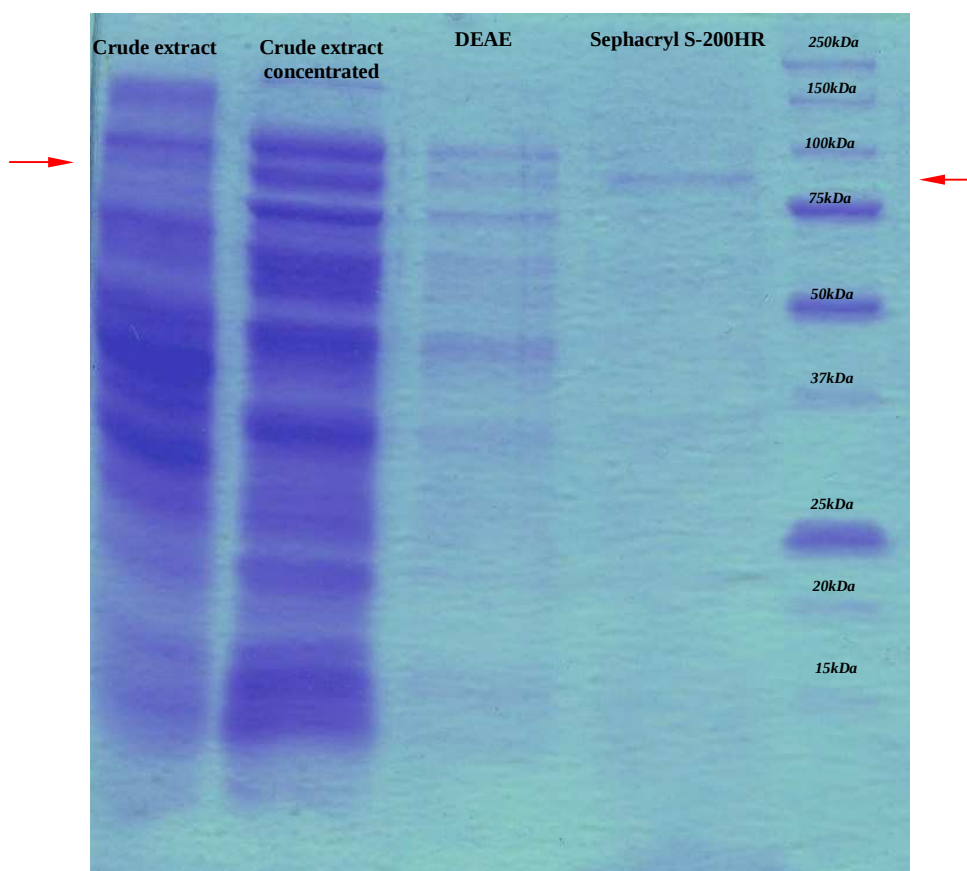


Figure 39 SDS PAGE of native cellobiose phosphorylase

4. Monitoring conversions of azido sugars by TLC and anisaldehyde-sulfuric acid spray reagent

4.1 Monitoring of the formation of 2-azido-2-deoxy-D-glucose

In synthetic studies of 2-azido-2-deoxy-D-glucose the reaction was monitored with TLC/anisaldehyde-H₂SO₄, as shown in Figure 40.

The TLC indicates the consumption of the dark green-staining starting material (lane 1) with formation of dark blue (lane 2) and yellow (lane 3) staining products respectively, followed by formation of light green products (line 4 and 5), existing as pairs of anomers.

The dark green-staining product was formed in 100% yield from glucose acetate, and was identified by NMR in previous studies [Gabra, 2009] as the expected *bromide* (= 1-bromo-1-deoxy-2, 3, 4, 6-tetra-O-acetyl-D-glucopyranose). The dark blue-staining product of R_F 0.41 in 4:1 acetic acid ethyl ester-hexane was identified by NMR as *glucal* (=3, 4, 6-tri-O-acetyl-D-glucal). The following product, yellow-staining, formed in 100% yield as shown in previous studies [Gabra, 2009], was identified as *nitro azide* (=3, 4, 6-tri-O-acetyl-2-azido-2-deoxy-1-nitro-D-gluco- and D-mannopyranose). The two pairs of products staining light green were identified as the mixture of *azido acetates* (= 1, 3, 4, 6-tetra-O-acetyl-2-azido-2-deoxy-D-glucopyranose). The first product (*gluco-azido acetate*) was formed in 44% yield and the second product (*manno-azido acetate*) in 45% yield respectively.

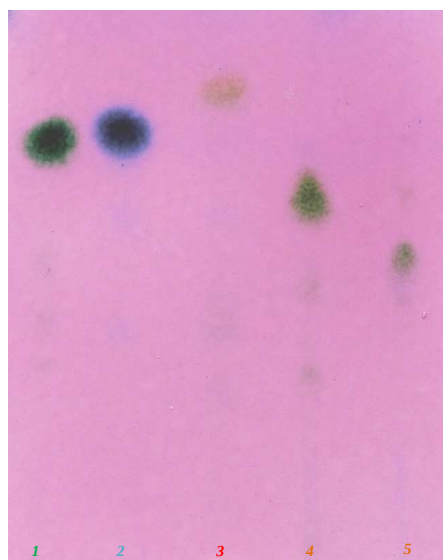


Figure 40 TLC of 2-azido-2-deoxy-D-glucose

- 1 1-Bromo-1-deoxy-2, 3, 4, 6-tetra-O-acetyl-D-glucopyranose
- 2 3, 4, 6-Tri-O-acetyl-D-glucal
- 3 3, 4, 6-Tri-O-acetyl-2-azido-2-deoxy-1-nitro-D-gluco- and D-mannopyranoses
- 4 and 5 D-gluco and D-manno-isomers of 1, 3, 4, 6-tetra-O-acetyl-2-azido-2-deoxy-D-hexopyranose

4.2 Monitoring of the formation of 2-azido-2-deoxy-cellobiose

In synthetic studies of 2-azido-2-deoxy-cellobiose, the reaction was monitored with TLC/anisaldehyde-H₂SO₄, as shown in Figure 41.

The TLC indicates the consumption of the dark green-staining cellobiose bromide (lane 1) with formation of dark gray cellobial (lane 2) and two pairs of products staining light green (lane 3), followed by formation of second light green products (line 4), all of them existing as pair of anomers.

The dark green-staining product was formed from cellobiose peracetate in 51% yield, and was identified by NMR in previous studies [Gabra, 2009] as the expected *bromide* (= α -cellobiosyl-bromide-hepta-acetate). The dark gray-staining product of R_F 0.54 in 3:2 ethyl acetic acid-toluene was identified by NMR as *cellobial* (= hexa-acetyl-D-cellobial). The crystalline, light green-staining product, formed in 40.8% yield as shown in previous studies [Gabra, 2009], was identified as the anomer mixtures of *nitro azide* (=2-azido-2-deoxy-1-nitro-cellobiosyl-hexa-acetate). The last two pairs of products staining light green were identified as the anomer mixtures of *azido acetate* (=2-azido-2-deoxy-cellobiosyl-hepta-acetate). The product was formed in 57% yield.

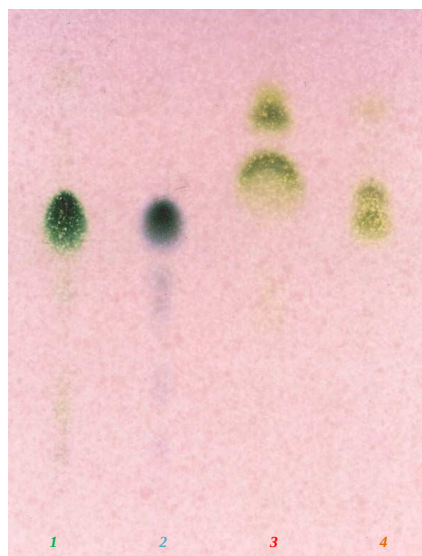


Figure 41 TLC of 2-azido-2-deoxy-cellobiose

- 1** *α -Cellobiosyl-bromide-hepta-acetate*
- 2** *Hexa-acetyl-D-cellobial*
- 3** *2-Azido-2-deoxy-1-nitro-cellobiosyl-hexa-acetate*
- 4** *2-Azido-2-deoxy-cellobiosyl-hepta-acetate*

4.3 Monitoring of the formation of 3-azido-3-deoxy-D-glucose

In synthetic studies of 3-azido-3-deoxy-D-glucose the reactions were monitored with TLC/anisaldehyde- H_2SO_4 , as shown in the Figure 42a.

The TLC indicates the consumption of the gray staining product (lane 1) with formation of gray green (lane 2) and dark blue-staining products (lane 3) respectively.

The gray staining product 1,2:5,6-di-O-isopropylidene- α -D-glucofuranose was purchased from GLYCON. The gray green-staining product of R_F 0.61 in 3:2 ethyl acetic acid-toluene [Trinkl, 2005] was identified as 1,2:5,6-di-O-isopropylidene- α -D-ribo-hexofuranos-3-ulose. The bright blue staining product, formed in 84% yield [Trinkl, 2005] was identified as 1,2:5,6-di-O-isopropylidene- α -D-allofuranose.

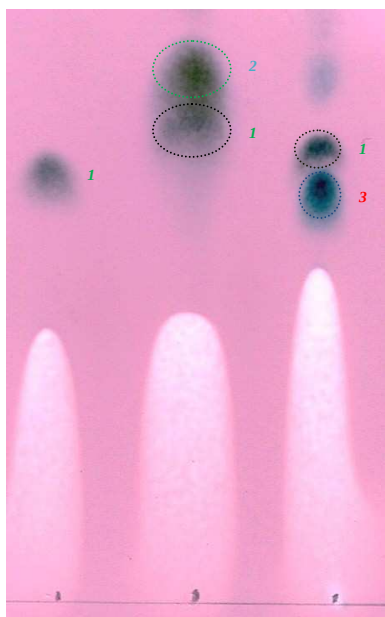


Figure 42a TLC of 3-azido-3-deoxy-D-glucose precursors

1 1,2:5,6-di-O-isopropylidene- α -D-glucofuranose

2 1,2:5,6-di-O-isopropylidene- α -D-ribo-hexofuranos-3-ulose

3 1,2:5,6-di-O-isopropylidene- α -D-allofuranose

1,2:5,6-di-O-isopropylidene- α -D-allofuranose was O-mesylated (methylsulfonyl chloride in pyridine) to afford 1,2:5,6-di-O-isopropylidene-3-O-methylsulfonyl- α -D-allofuranose. Treatment with sodium azide in dimethyl formamide at 170°C gave the desired 3-azido-3-deoxy-1,2:5,6-di-O-isopropylidene- α -D-glucofuranose [Brimacombe, *et al.*, 1967], from which 3-azido-3-deoxy-D-glucose (Figure 42b) was obtained by acid-catalyzed hydrolysis of the isopropylidene groups [Trinkl, 2005].

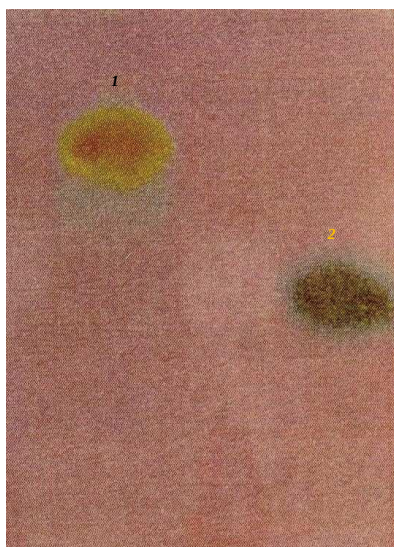


Figure 42b TLC of 3-azido-3-deoxy-D-glucose

1 3-Azido-3-deoxy-D-glucose

2 D-Glucose

4.4 Monitoring of the formation of 6-azido-6-deoxy-sugars

In synthetic studies of 6-azido-6-deoxy-sugars, the reactions were monitored with TLC/anisaldehyde- H_2SO_4 , as shown in Figure 43.

Azido sugar derivatives of glucose produce yellow to orange colorations with the anisaldehyde- H_2SO_4 spray reagent (6- N_3 Glucose). With 6-azido-6-deoxy-cellobiose and 6'-azido-6'-deoxy-cellobiose, yellow staining azido sugar residues and green staining glucose residues are combined in one molecule. The staining therefore results in the appearance of mixed colorations of yellowish green. The yellow contribution is more pronounced when the azide substitution is in the reducing moiety (6- N_3 Cellobiose) and somewhat weaker when the azido group is in the nonreducing moiety (6'- N_3 Cellobiose).

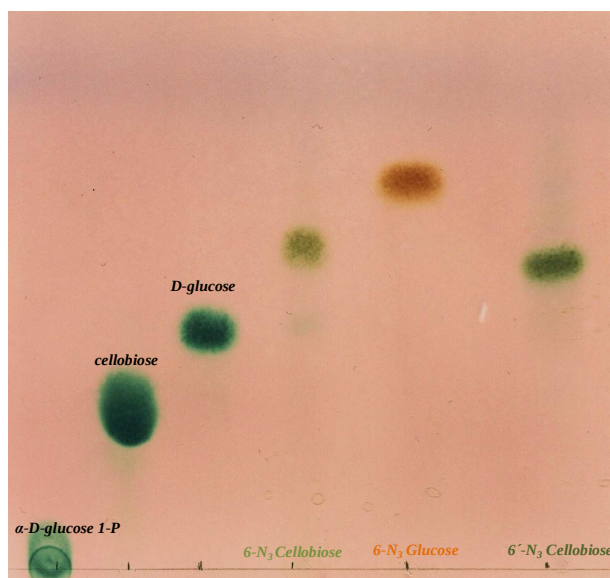


Figure 43 TLC of 6-azido-6-deoxy-sugars

4.5 Summary of azido sugars derivatives of cellobiose monitored by TLC

In synthetic studies of azido sugars the reaction was monitored with TLC/anisaldehyde- H_2SO_4 , as shown in Figure 44.

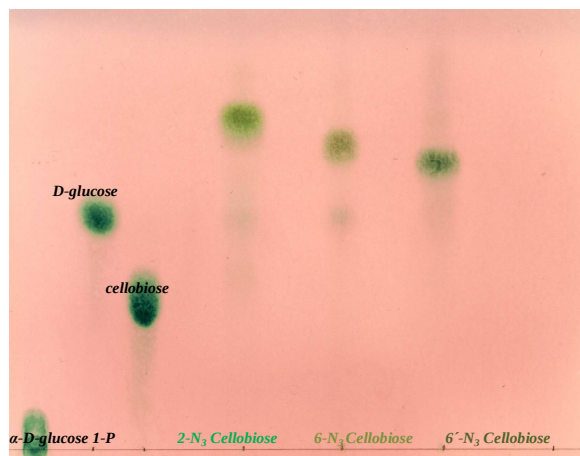
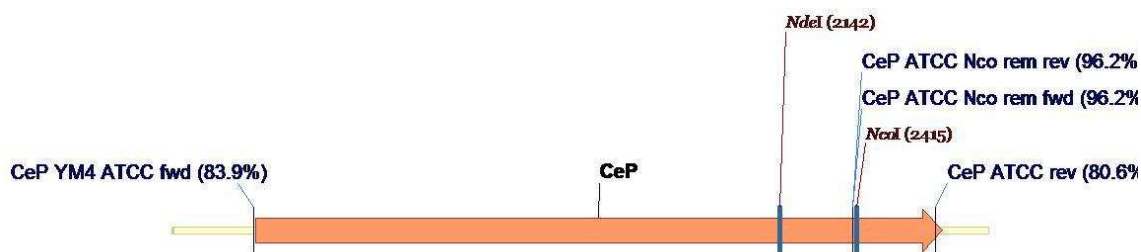


Figure 44 TLC of azido sugars

In conclusion, common hexose sugars and their ether, ester as well as acetal derivatives and alkyl glycosides stain in various grades of green colour, while deoxy sugars in bluish-gray. As distinct from other sugar types, azido sugars derivatives of glucose produce yellow to orange staining products and azido derivatives of cellobiose produce various shades of light green with the anisaldehyde- H_2SO_4 spray reagent.

5. Preparation of the cellobiose phosphorylase genomic DNA for PCR

Cellobiose phosphorylase gene sequence of the strain ATCC ID27405 was used as template for the design of primers needed for the polymerase chain reactions as indicated in Scheme 4.



Scheme 4 Cellobiose phosphorylase of *Clostridium thermocellum* ATCC ID27405

Genomic DNA was isolated from crude extract of *Clostridium thermocellum* NCIMB 10682 in a concentration of 156 $\mu\text{g/ml}$ as measured by UV spectrophotometry.

5.1 PCR procedures

5.1.1 Conventional PCR

Conventional PCR, conducted with a mixture of *Taq* DNA polymerase, PCR buffer, MgCl_2 and dNTPs reveals the presence of genomic DNA (2880bp), as indicated in Figure 45 (black arrow).

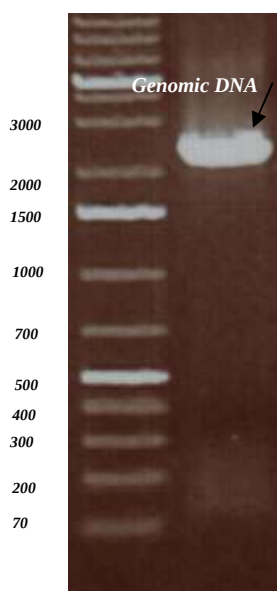
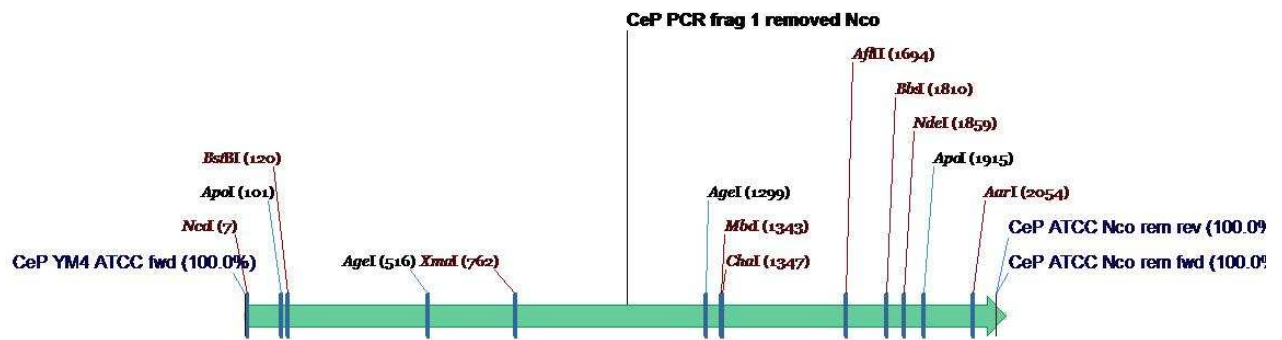


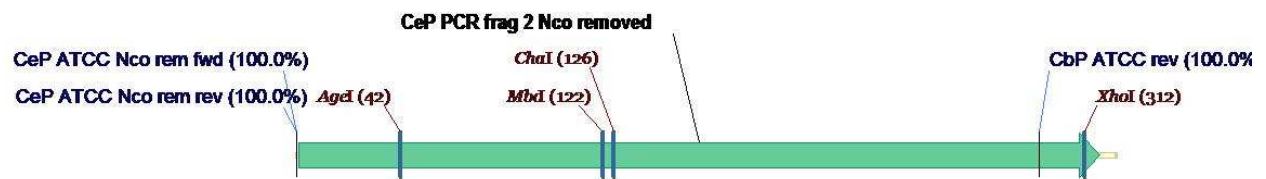
Figure 45 PCR of genomic DNA from *Clostridium thermocellum* NCIMB 10682

5.1.2 High fidelity PCR

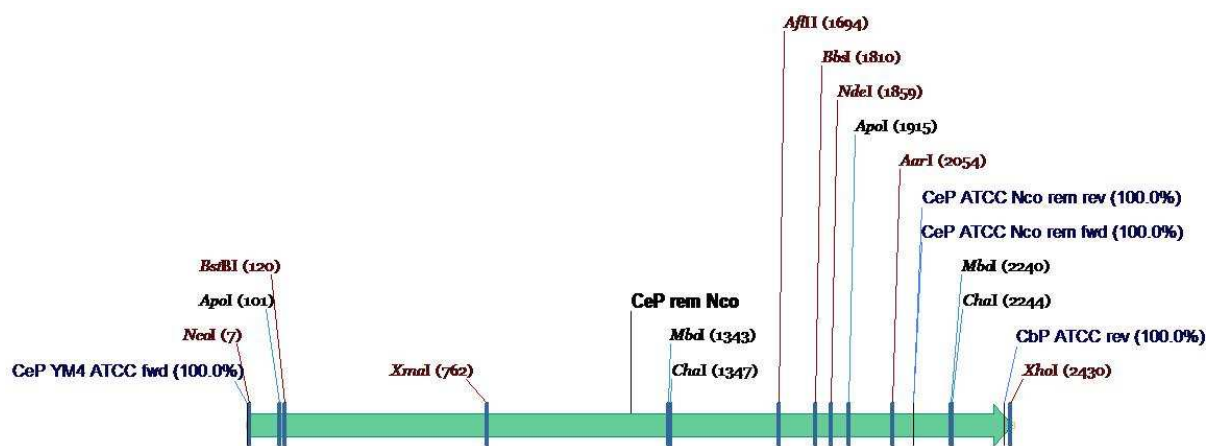
High fidelity PCR reveals formation of two fragments due to the removal of the internal restriction site (*Nco*I). Fragment 1 (2144bp) and fragment 2 (324bp) were generated as shown in Scheme 5-7 and in Figure 46, both fragments indicated with black arrows.



Scheme 5 Cellobiose phosphorylase fragment 1



Scheme 6 Cellobiose phosphorylase fragment 2



Scheme 7 Complete cellobiose phosphorylase sequence without internal *Nco*I

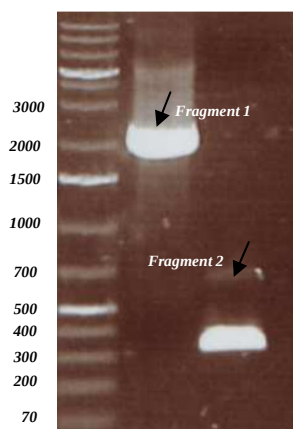


Figure 46 PCR of fragment 1 and 2

5.1.3 Overlap extension PCR

Overlap extension PCR reveals formation of the full length cellobiose phosphorylase gene of 2442bp as indicated in Figure 47 and in Scheme 8.

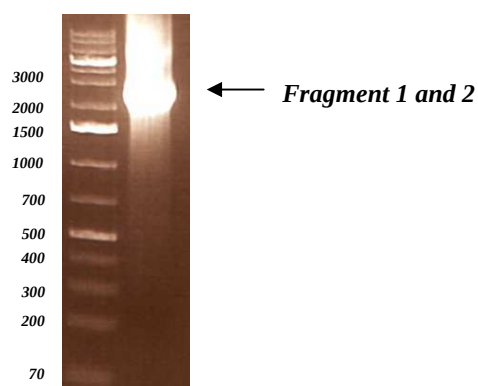
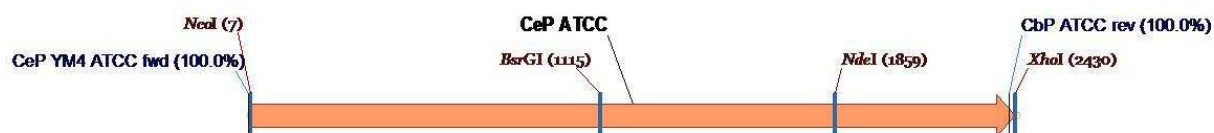


Figure 47 Full length sequence of CeP



Scheme 8 Full length sequence of cellobiose phosphorylase without internal restriction site (*NcoI*)

6. Preparation of pET26b-CeP construct

6.1 Restriction digest of pET-26b(+) vector and cellobiose phosphorylase gene

Figure 48 shows a restriction digest of pET-26b(+) plain vector (5294bp) and cellobiose phosphorylase gene (2442bp) respectively. After the ligation, the construct was transferred into chemocompetent DH5α *E.coli*.

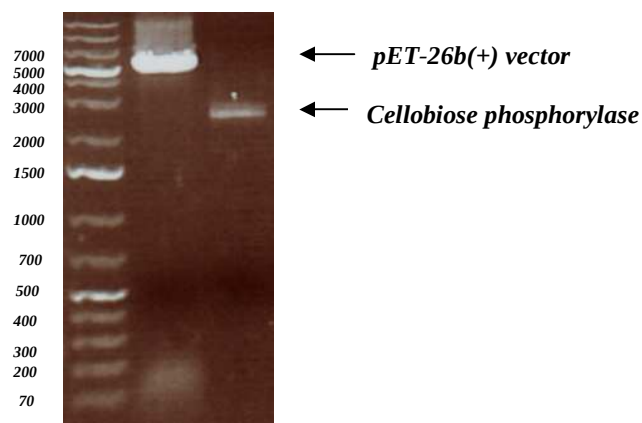


Figure 48 Restriction digest of pET-26b(+) vector and CeP

7. Transformation of the pET26b-CeP construct into chemocompetent DH5α *E.coli*

7.1 PCR screening of the colonies and their minipreparation

In order to verify which chemocompetent DH5α *E.coli* cells consist of pET-26b(+)-CeP construct, PCR screening of a short cellobiose phosphorylase gene fragment (~ 470bp) harboring the 6xHis tag of the vector was conducted. Colony clone 1 (red arrow), 2 (green arrow) and 6 (yellow arrow) were successfully screened as indicated in Figure 49.

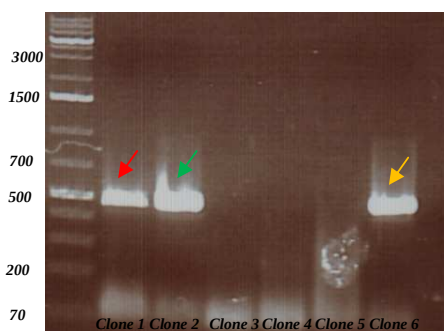


Figure 49 PCR screen of short CeP fragment

After miniprep of the plasmid DNA, a double restriction digest of the pET-26b(+)-CeP construct was performed, revealing both fragments in clone 2 and 6, as indicated in Figure 50.

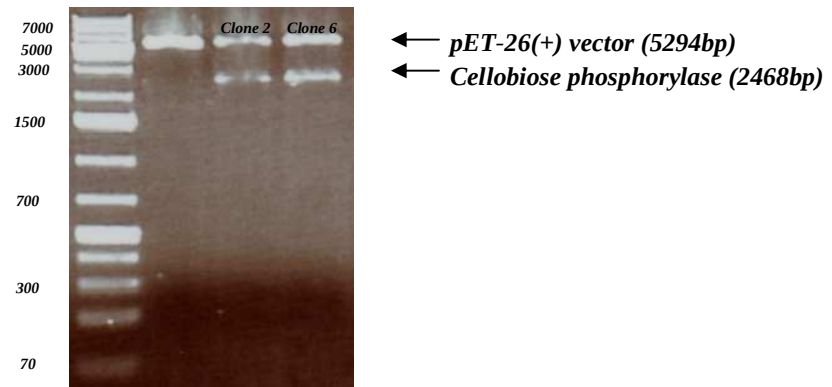
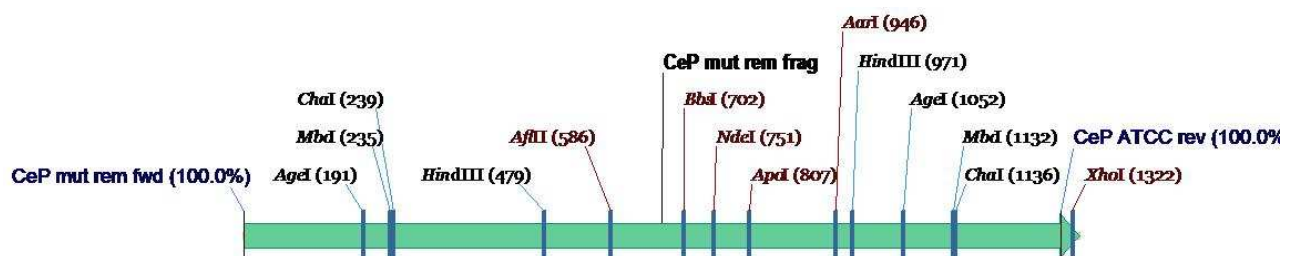
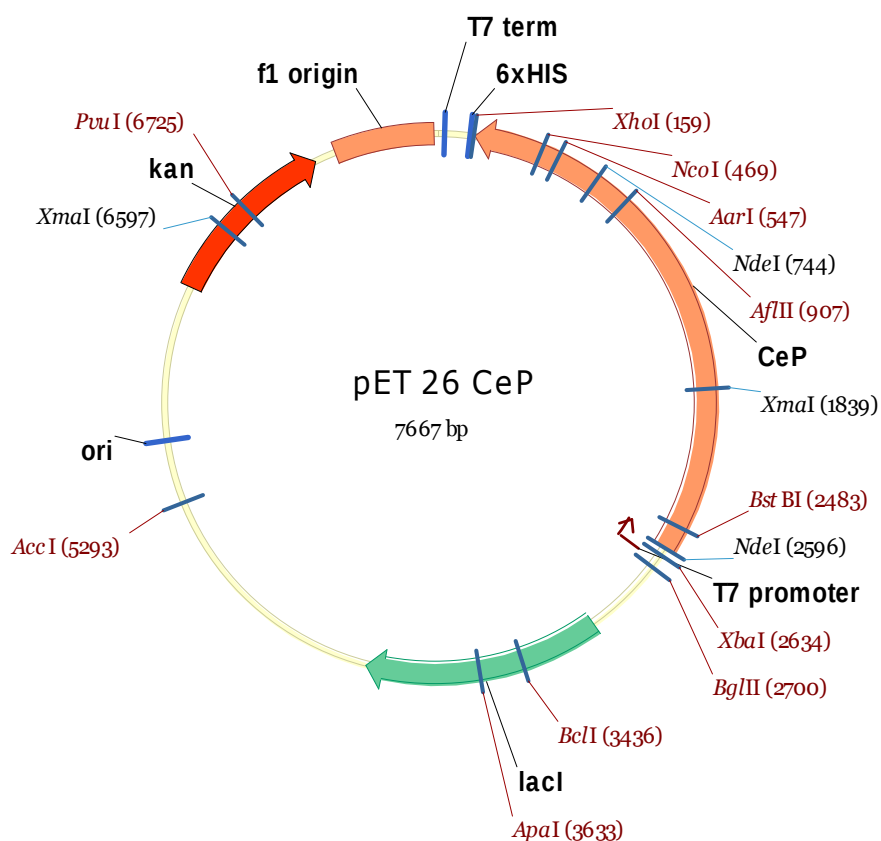


Figure 50 Double restriction digest of pET-26(+) vector and CeP

Due to the mutation within the pET26b-CeP construct, detected during the DNA sequencing, another step was needed to remove this mutation by PCR. The final product of this operation is shown in Schemes 9 and 10.



Scheme 9 Cellobiose phosphorylase gene without mutation (1334bp)



Scheme 10 pET-26b(+)-CeP construct without mutation

Subsequently, the cellobiose phosphorylase gene without mutation and the pET-26b(+)-CeP construct containing the mutation were digested. Ligation of both products proceeded over night. Subsequently, the final product was transferred into chemocompetent DH5α *E.coli* as described previously. Subsequent sequencing has revealed a pET-26b(+)-CeP construct without mutation.

8. Western blot of recombinant cellobiose phosphorylase transformed in BL21 (DE3) *E. coli* cells

After transformation of the pET-26b(+)-CeP construct into BL21 (DE3) *E. coli* cells, the cells were grown without and with IPTG induction respectively. Four clones were picked at random and western blot was performed. Figure 51 reveals the expression of the gene in all clones (1-4) induced by IPTG; the strongest signal was observed with clone 3 (red arrow).

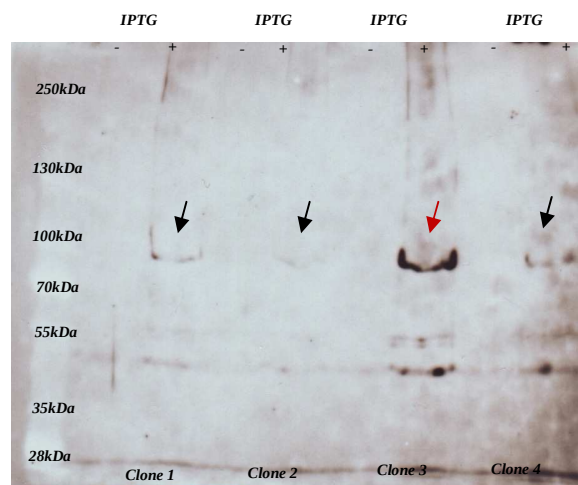


Figure 51 Western blot of 4 clones

9. Western blot of recombinant cellobiose phosphorylase after Ni-NTA metal ion affinity chromatography

Expression of the recombinant 6xHis tag cellobiose phosphorylase (= clone 3) induced by IPTG was followed by its purification via Ni-NTA metal ion affinity chromatography in order to obtain pure recombinant cellobiose phosphorylase. Figure 52 indicates its detection via SDS PAGE.

From 10 eluates collected, eluate 3, 4, 5 and 6 have shown the most homogenous enzyme preparation. Figure 53 indicates their detection via western blot.

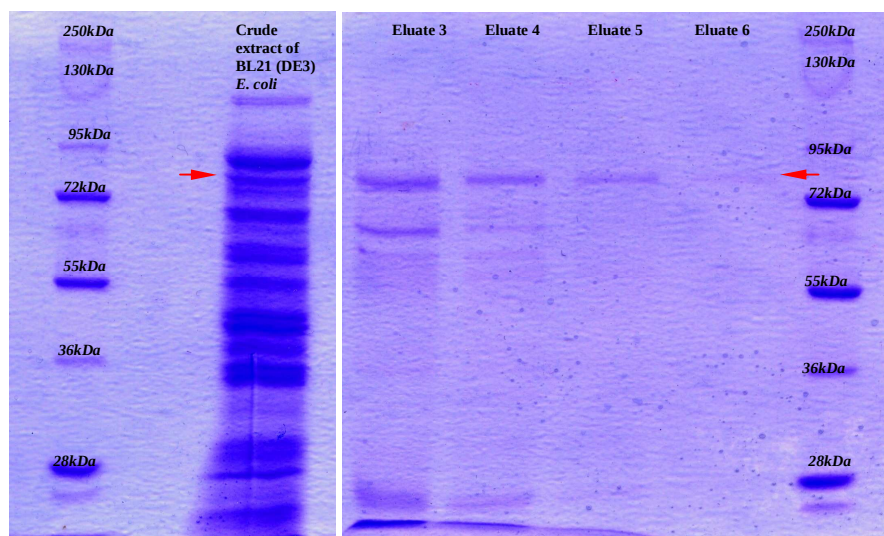


Figure 52 SDS PAGE of recombinant cellobiose phosphorylase after Ni-NTA

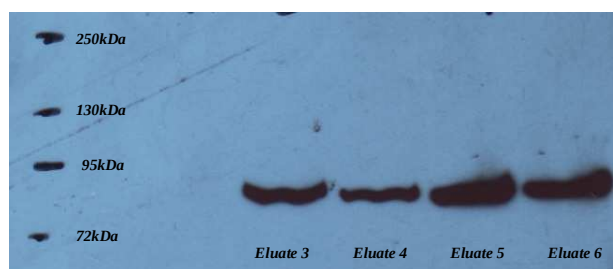


Figure 53 Western blot of recombinant CeP after Ni-NTA

The specific activity of the recombinant cellobiose phosphorylase was determined in the enzymatic synthesis with 2-azido-2-deoxy-cellobiose as substrate, as revealed in Chapter 10.6 (p.138).

In conclusion, the cellobiose phosphorylase gene of *Clostridium thermocellum* NCIMB 10682 was successfully cloned and the final result indicates a homogenous enzyme preparation with an apparent molecular mass of ~93kDa. This correlates well with the data described in the literature [Yernool *et al.*, 2000].

10. Enzymatic syntheses by catalysis of cellobiose phosphorylase

10.1 Synthetic reaction of monosaccharides catalyzed by native cellobiose phosphorylase

Figure 54 reveals the specificity of cellobiose phosphorylase when cellobiose is used as substrate for the phosphorolytic cleavage (forward) reaction, and when α -D-glucose 1-phosphate and D-glucose are used as substrates for the synthetic (reverse) reaction.

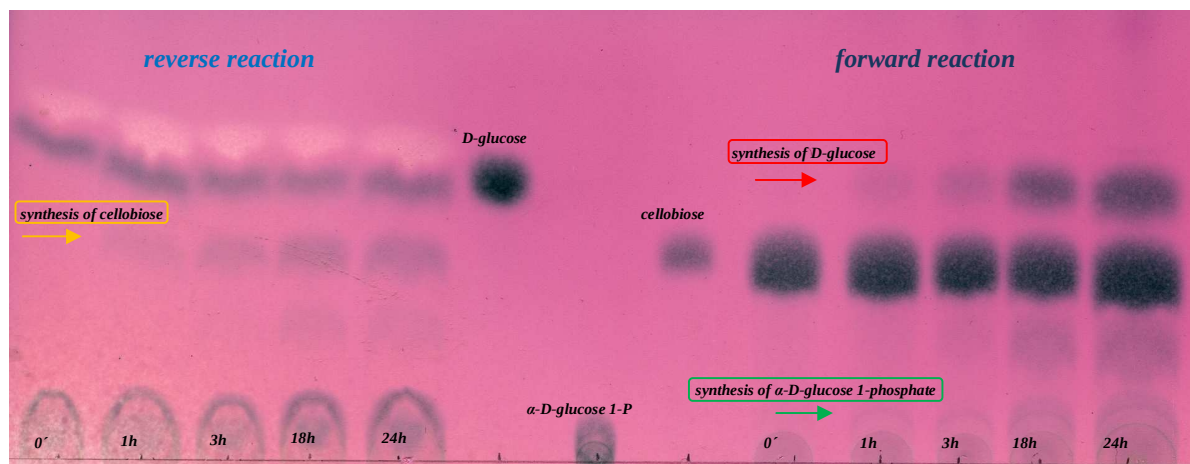


Figure 54 Catalytic reaction of cellobiose phosphorylase

As illustrated above, this enzyme catalyzes the conversion, by phosphorolytic cleavage, of cellobiose into α -D-glucose 1-phosphate (green arrow) and D-glucose (red arrow). In the reverse reaction, cellobiose phosphorylase catalyzes the synthesis of cellobiose (orange arrow), by transfer of a glucose residue from α -D-glucose 1-phosphate to the acceptor D-glucose.

The acceptor D-glucose may be replaced by analogs or derivatives of D-glucose such as 2-deoxyglucose, 6-deoxyglucose or D-xylose.

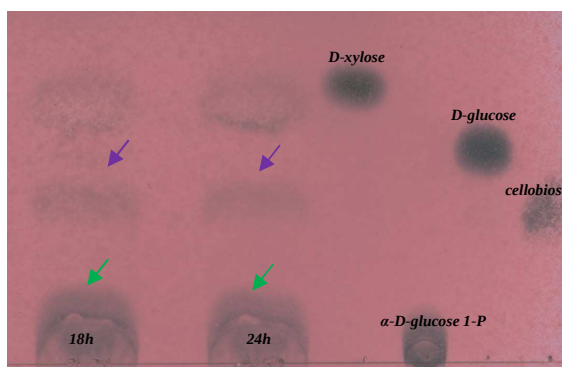


Figure 55 Synthetic reaction with D-xylose

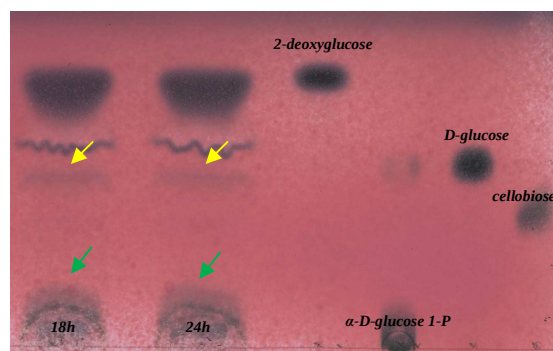


Figure 56 Synthetic reaction with 2-deoxyglucose

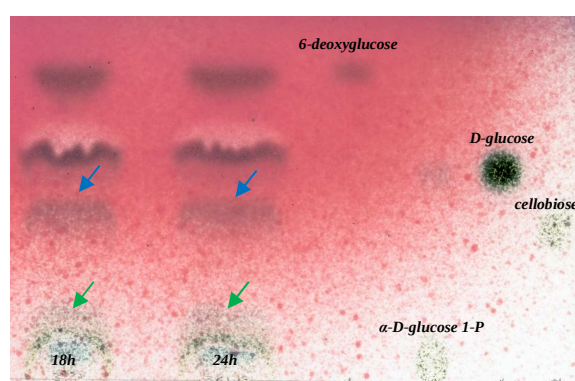


Figure 57 Synthetic reaction with 6-deoxyglucose

As indicated above, the replacement of D-glucose as acceptor by D-xylose (Figure 55), 2-deoxyglucose (Figure 56) or 6-deoxyglucose (Figure 57) results in the formation of the corresponding disaccharide analogs of cellobiose [Kim *et al.*, 2002; Percy *et al.*, 1998]. These analogs are indicated by purple, yellow and blue arrows respectively in the corresponding figures. In all samples, the strongest signal of formation was achieved after 18 and 24 h respectively.

10.2 Phosphorolytic reaction of native cellobiose phosphorylase towards 6-azido-6-deoxy-cellobiose

The phosphorolytic reaction of purified cellobiose phosphorylase was performed with 6-azido-6-deoxy-cellobiose.

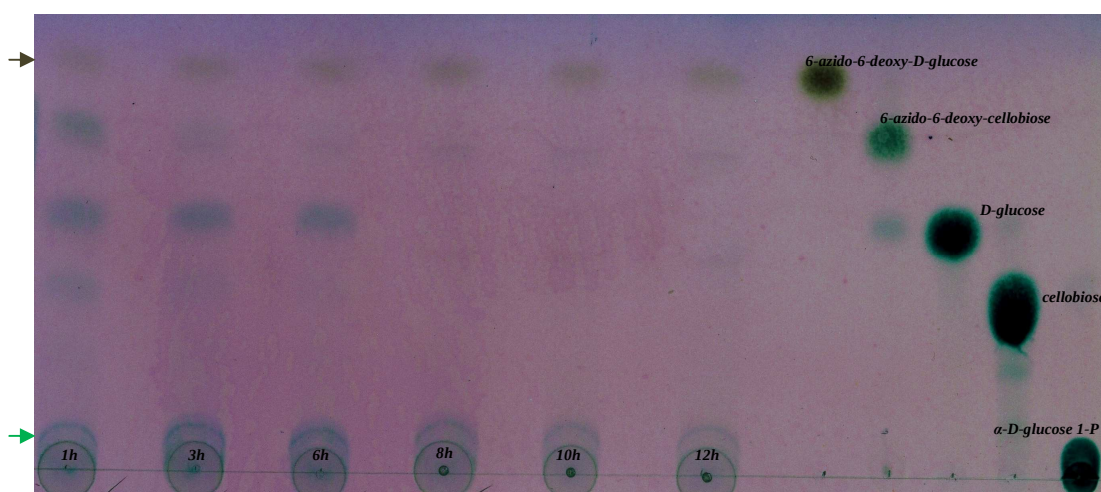


Figure 58 Phosphorolytic reaction by 6-azido-6-deoxy-cellobiose

Figure 58 clearly reveals the formation of 6-azido-6-deoxy-D-glucose (brown arrow) and α -D-glucose 1-phosphate (green arrow). Therefore, it can be stated that 6-azido-6-deoxy-cellobiose acts as an effective substrate for phosphorolytic action catalyzed by cellobiose phosphorylase. The D-glucose and cellobiose formed in this reaction can be explained by α -D-glucose 1-phosphate being hydrolyzed under the reaction conditions. The D-glucose formed may react with the remaining α -D-glucose 1-phosphate to form cellobiose.

10.3 Synthetic reaction of 6-azido-6-deoxy-D-glucose catalyzed by native cellobiose phosphorylase

The synthetic reaction of purified cellobiose phosphorylase was performed with 6-azido-6-deoxy-D-glucose. Figure 59 reveals the specificity of cellobiose phosphorylase when α -D-glucose 1-phosphate and 6-azido-6-deoxy-D-glucose (educts) are used as substrates for the synthetic (reverse) reaction to form 6-azido-6-deoxy-cellobiose (red arrow). Additionally, also a trisaccharide formation (green arrow) was observed, presumably due to the presence of cellodextrin phosphorylase [Reichenbecher *et al.*, 1997; Krishnareddy *et al.*, 2002].

Therefore, it can be stated that 6-azido-6-deoxy-D-glucose acts as an effective substrate for the synthetic reaction catalyzed by cellobiose phosphorylase.

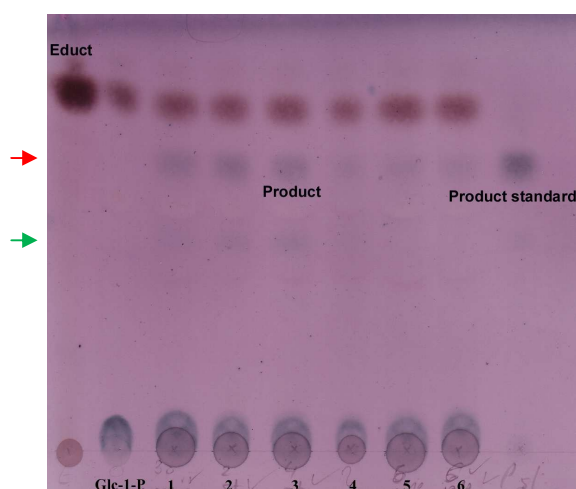


Figure 59 Synthetic reaction by 6-azido-6-deoxy-D-glucose

10.4 Phosphorolytic reaction of native cellobiose phosphorylase towards 6'-azido-6'-deoxy-cellobiose

For the preparation of precursors to azido cellobiose derivatives, catalytic reactions of cellobiose phosphorylase were performed with azido substituents in the non-reducing glucose moiety, namely 6'-azido-6'-deoxy-cellobiose.

6'-azido-6'-deoxy-cellobiose is not cleaved into monosaccharide derivatives by the action of cellobiose phosphorylase. Cleavage of 6'-azido-6'-deoxy-cellobiose under catalysis by cellobiose phosphorylase should afford 6-azido-6-deoxy-D-glucose 1-phosphate and D-glucose. It is assumed that the reaction proceeds to a small extent, whereupon the small amount of D-glucose formed would suffice to inhibit binding of the weak substrate 6'-azido-6'-deoxy-cellobiose and to block further cleavage. This interpretation is supported by the finding of a small amount of cellobiose, presumably formed from 2 molecules of glucose bound in both the α -D-glucose 1-phosphate and D-glucose binding sites.

10.5 Phosphorolytic reaction of native cellobiose phosphorylase towards 2-azido-2-deoxy-cellobiose

Another conversion of cellobiose analogs with azido substituents in the reducing glucose moiety was performed in the form of the phosphorolytic cleavage reaction with 2-azido-2-deoxy-cellobiose catalyzed by cellobiose phosphorylase.

For this purpose 2-azido-2-deoxy-cellobiose was synthesized by organic-chemical methods [Gabra, 2009]. To remove impurities, the product was chromatographed on Bio-Gel[®] P Polyacrylamide Gel. 70 fractions of 500 µl were collected. Complete purification of the eluates was not accomplished. Figure 60 indicates fractions 59-63 with the highest purification grade as shown on TLC. The final yield of the product was 23.5 mg (19%).

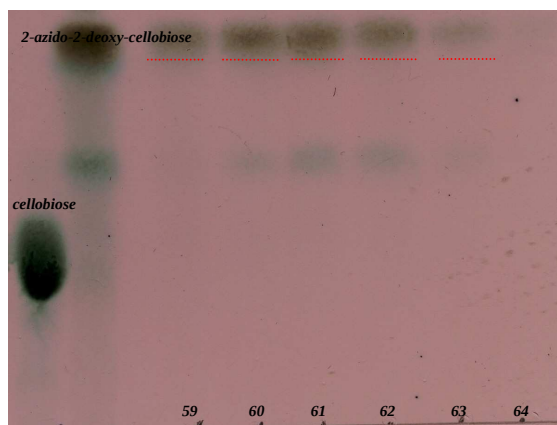


Figure 60 2-azido-2-deoxy-cellobiose after Bio Gel

Figure 61 indicates the phosphorolytic reaction of cellobiose phosphorylase with the pool of fraction 59-63 containing 2-azido-2-deoxy-cellobiose as substrate.

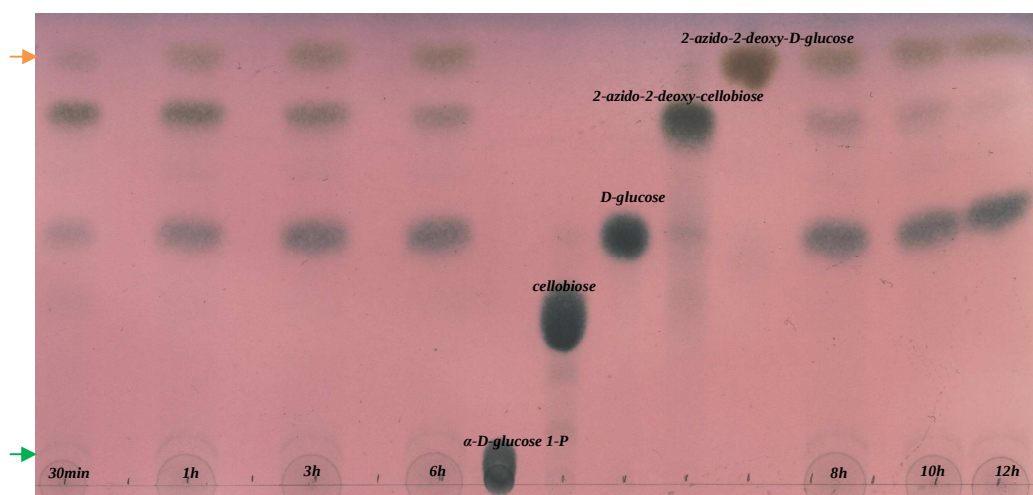


Figure 61 Cleavage reaction with 2-azido-2-deoxy-cellobiose

It clearly reveals the formation of 2-azido-2-deoxy-D-glucose (orange arrow) and α -D-glucose 1-phosphate (green arrow). Therefore, it can be stated that 2-azido-2-deoxy-cellobiose acts as an effective substrate for phosphorolytic action catalyzed by cellobiose phosphorylase.

Noticeable in this TLC is also formation of D-glucose, which yield was estimated by D-glucose test, revealing a D-glucose amount of 4159 μ l per 3 ml incubation volume after 24 h. In order to remove D-glucose from the desired product, active cellobiose phosphorylase was denatured. Figure 62 shows the denatured sample containing 2-azido-2-deoxy-D-glucose and D-glucose.

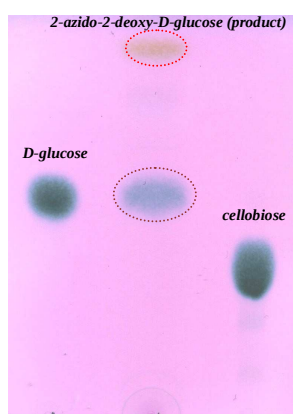


Figure 62 2-azido-2-deoxy-glucose sample after denaturation

After the sample was lyophilized, a purification procedure with silica gel 60 in 17:3 1-propanol-water solvent agent was performed. 60 fractions were collected; the highest grade of purification was indicated in fractions 36-43, as shown in Figure 63.

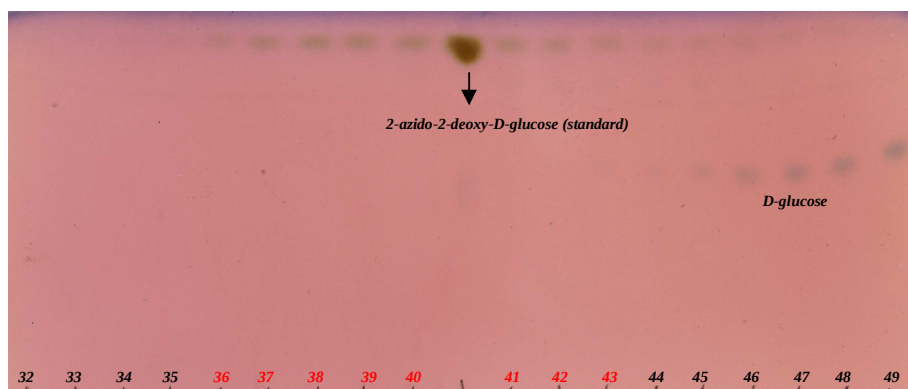


Figure 63 2-azido-2-deoxy-D-glucose after silica gel purification

The fractions 36-43 were lyophilized and stored at -20°C. The final yield of purified 2-azido-2-deoxy-D-glucose was 4.58 mg.

10.6 Phosphorolytic reaction of recombinant cellobiose phosphorylase towards 2-azido-2-deoxy-cellobiose

Phosphorolytic cleavage of 2-azido-2-deoxy-cellobiose was conducted by use of purified, recombinant cellobiose phosphorylase, as shown in figure 64.

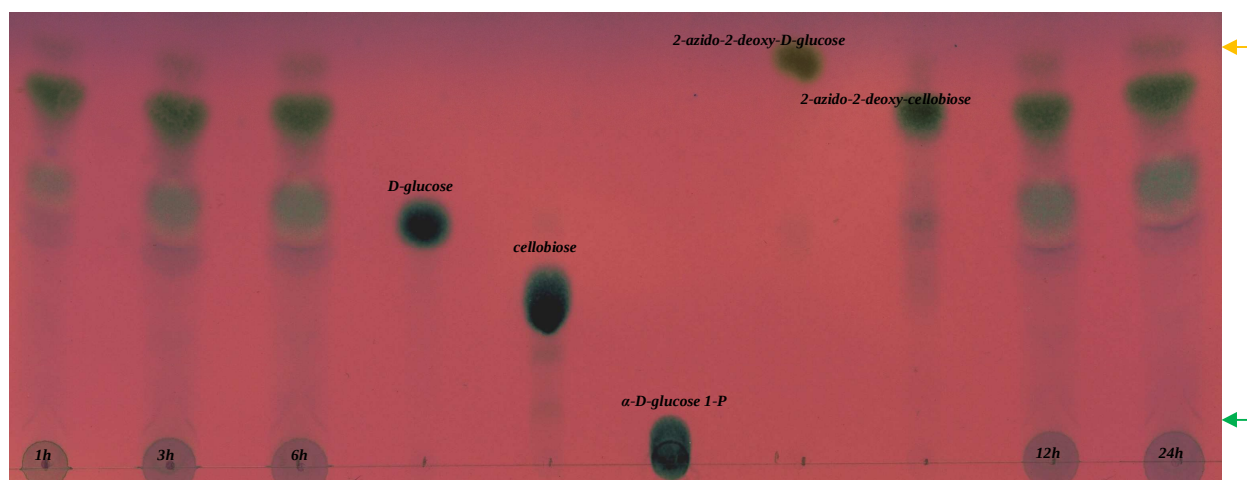


Figure 64 Phosphorolytic reaction of 2-azido-2-deoxy-cellobiose via recombinant CeP

In phosphorolytic reaction of recombinant cellobiose phosphorylase the formation of 2-azido-2-deoxy-D-glucose (yellow arrow) and α -D-glucose 1-phosphate (green arrow) respectively were observed. Therefore, it can definitely be stated that 2-azido-2-deoxy-cellobiose can act as a substrate.

2-azido-2-deoxy-D-glucose was purified by use of silica gel 60 in 17:3 1-propanol-water. A thin layer chromatogram of the pure product is shown in Figure 65.

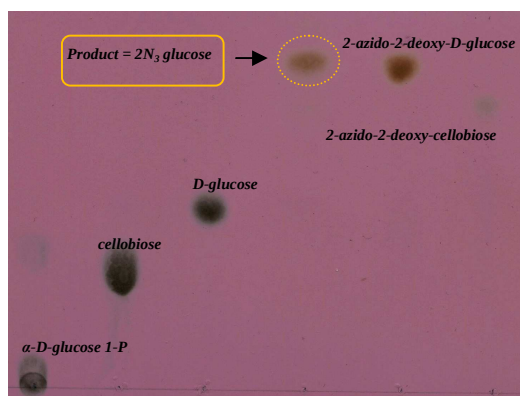


Figure 65 Purified 2-azido-2-deoxy-D-glucose

Non competent BL21 (DE3) *E. coli* cells have not shown formation of any secondary products when using cellobiose as substrate. Therefore, it can be stated, that these cells do not possess the enzyme phosphatase which might inhibit the function of the recombinant cellobiose phosphorylase.

By analogy to the incubation with 2-azido-2-deoxy-cellobiose, recombinant cellobiose phosphorylase from crude extract of BL21 (DE3) *E. coli* harboring the expression vector pET26(+)-CeP was incubated with cellobiose as substrate.

In this context, a specific cellobiose phosphorylase activity of 3.5 U/mg in a total protein volume of 7 ml was measured. The specific activity of purified recombinant cellobiose phosphorylase increased up to 7.4 U/mg in a total protein volume of 800 μ l, resulting in twofold purification after the Ni-NTA metal ion affinity chromatography.

For the same protein it was possible to show that incubation with cellobiose as substrate for the phosphorolytic reaction did not lead to formation of any trisaccharide as shown in Figure 66.

The most reasonable explanation for the formation of a trisaccharide during the incubation with native cellobiose phosphorylase might be catalysis by accompanying quantities of cellodextrin phosphorylase [Reichenbecher *et al.*, 1997; Krishnareddy *et al.*, 2002] as seen in Figure 67.

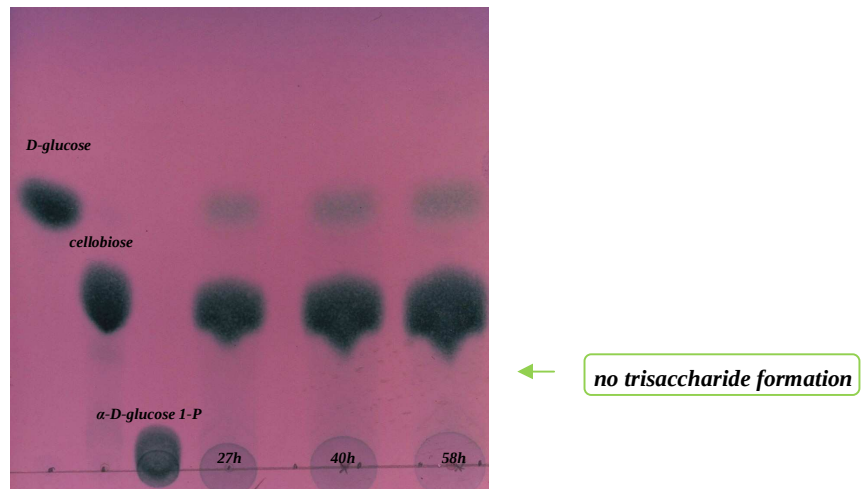


Figure 66 Phosphorolytic reaction of recombinant CeP

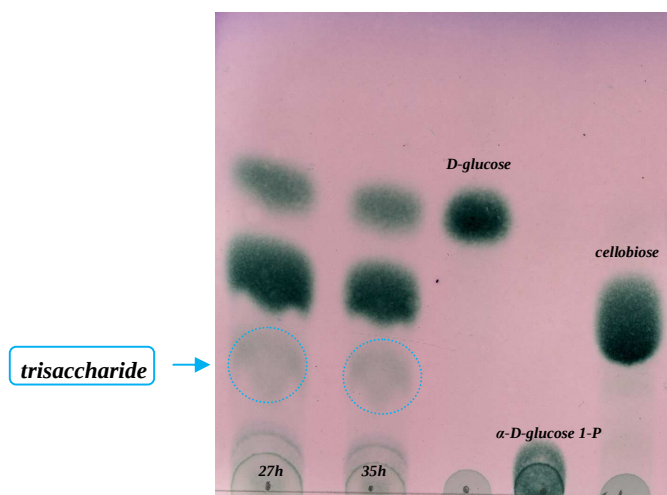


Figure 67 Phosphorolytic reaction of native CeP

IX. DISCUSSION

1. Biotechnological use of carbohydrates

Naturally occurring combinations of monosaccharides in form of glycans such as oligosaccharides and polysaccharides provide an enormous variety of structures and associated functions. For example, a monosaccharide such as D-glucose may function as glycosyl donor in four different ways (α -pyranose, β -pyranose, α -furanose and β -furanose) and as glycosyl acceptor at four different positions (2, 3, 4, 6 for the pyranose form and 2, 3, 5, 6 for the furanose form). Thus, a huge number of molecules are theoretically conceivable, based on only one monosaccharide. Considering the availability of at least a dozen commonly occurring monosaccharide constituents such as pentoses, hexoses, deoxy sugars and uronic acids, possible combinations become practically unlimited regarding numbers and biological functions. With the advent of techniques of molecular biology, tools have become available to construct not only modified polysaccharides, but also polysaccharides of new, artificial structures. Particularly in the field of aminopolysaccharides, there are few natural representatives; yet this field offers tempting opportunities of application. Therefore, there is considerable interest in developing new aminopolysaccharides by the combined techniques of organic chemistry and molecular biology.

2. Occurrence and biological significance of cellobiose phosphorylase

Cellobiose phosphorylase is a widely occurring enzyme among thermophilic cellulolytic bacteria such as *Thermomonospora fusca*, *Acetivibrio cellulolyticus*, *Bacteroides succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* [Lamed and Bayer, 1988]. The same enzyme was also detected in some mesophilic, filamentous fungi, such as *Trichoderma reesei* [Mandels and Reese, 1960] and *Trichoderma viride* [Reese and Maguire, 1971], *Sporotrichum thermophile*, *Humicola insolens* and *Chaetomium thermophile* [Lusis and Becker, 1973].

The objective of this study is focused on conversions with azido cellooligosaccharides catalyzed by cellobiose phosphorylase from the thermophilic cellulolytic bacterium, *Clostridium thermocellum* NCIMB 10682. In this context, intracellular phosphorolysis, catalyzed by this enzyme, seems to be a genetically advantageous process, which constitutes the primary route of cellooligosaccharide utilization in anaerobic environments and thus initiates the Embden-Meyerhoff-Parnas pathway [Sallam *et al.*, 2006]. The bacterium exhibits a high thermal stability

and possesses a thermostable cellulase system. This is distinct from fungal cellulases. Cellulase from *Clostridium thermocellum* NCIMB 10682 is highly active on crystalline cellulose, thus being able to completely solubilize crystalline sources of cellulose [Schafer and King, 1965]. Due to this fact, this bacterium was chosen as a source of cellobiose phosphorylase. It was grown under anaerobic conditions on a complex medium containing microcrystalline cellulose (AVICEL) as the only energy source and harvested in early stationary phase, which was reached after a growth period of 96 h. In contrast to other bacteria such as *Cellvibrio gilvus* or *Ruminococcus flavefaciens* which grow on glucose, this bacterium seems to be the only one using cellobiose in the culture medium, thus reaching the maximum growth rate [Swisher *et al.*, 1964]. This is attributed to a higher yield of energy per molecule of cellobiose utilized as compared with two free glucose molecules [Heale and Gupta, 1971].

Resulting cellobiose phosphorylase protein from 5 l of crude extract of *Clostridium thermocellum* NCIMB 10682 was 20 mg with a specific activity of 0.6 U/mg before and 0.83 mg with a specific activity of 1 U/mg after the ultrafiltration step. In comparison with other studies, cellobiose phosphorylase from *Cellvibrio gilvus* [Sasaki *et al.*, 1983] has been reported to have similar specific activity (determined after a two-step purification protocol, protamine sulfate and hydroxyapatite procedure) ranging between 0.1-1.3 U/mg. Both measurements were based on determination of α -D-glucose 1-phosphate (G-1-P). Specific activity of cellobiose phosphorylase from crude extracts of *Clostridium stercorarium* [Reichenbecher *et al.*, 1997] was 0.03-1 U/mg after Q-sepharose and phenyl-Sepharose.

3. Purification of native cellobiose phosphorylase

Treatment of crude extract with saturated protamine and ammonium sulfate solution [Alexander, 1968; Tanaka *et al.*, 1995] respectively, has failed in our hands to be an appropriate method for the purification of cellobiose phosphorylase from *Clostridium thermocellum* NCIMB 10682, leading to high grade precipitation of enzyme protein. However, also in anion exchange chromatography, the use of dithiothreitol or 2-mercapthoethanol [Sasaki *et al.*, 1983] in addition to 50mM Tris pH 7.5 during purification, has led to large losses in protein activity. Due to these findings, it became necessary to optimize conditions for the purification of native cellobiose phosphorylase. In the end, it was pointed out that a high enrichment of cellobiose phosphorylase

from crude extract was achieved when 50mM Tris pH 7 and DEAE cellulose chromatography with a linear gradient of NaCl from 100 to 600mM were applied. These findings correspond well to one described previously [Sasaki *et al.*, 1983], in which Tris buffer with the same concentration but of different pH (= 7.5) and a linear gradient of NaCl from 100mM up to 500mM were applied. In the present study, the above mentioned protectants were omitted as they were found to impair enzymatic activity.

However, cellobiose phosphorylase was sufficiently enriched in a series of three-step purification procedures. As demonstrated by SDS PAGE analysis, the subsequent chromatographic steps resulted in electrophoretic near-homogeneity of the cellobiose phosphorylase preparation after the last purification step. An apparent molecular mass of ~93 kDa was determined for the purified cellobiose phosphorylase. These findings correspond well with the molecular mass estimated using computation of the protein knowledgebase from UniProtKB, which was carried out on the complete sequence of 811 amino acids of the cellobiose phosphorylase gene of *Clostridium thermocellum* strain ATCC 27405, indicating a molecular weight of 92.8 kDa. Cellobiose phosphorylase of *Cellvibrio gilvus* [Sasaki *et al.*, 1983] has a molecular weight of 72 kDa, estimated after a three-step purification procedure. More closely similar results were reported by use of a two-step purification protocol for cellobiose phosphorylase of *Cellulomonas uda* [Nidetzky *et al.*, 2004], showing an estimate of 92 kDa for the molecular mass of this protein.

The enzyme cellobiose phosphorylase from *Clostridium thermocellum* NCIMB 10682 was purified 5-fold with a recovery of 2% and a specific activity of 3 U/mg. In comparison to these findings, cellobiose phosphorylase of *Clostridium stercorearium* [Reichenbecher *et al.*, 1997] with an estimated molecular weight of 92.7 kDa, reveals in a six-step purification procedure certain similarities, indicating a recovery of 5% and a specific activity of 4.7 U/mg respectively.

4. Cloning and expression of the recombinant cellobiose phosphorylase gene

Cellobiose phosphorylase gene of *Clostridium thermocellum* NCIMB 10682 was cloned in *Escherichia coli*. This gene encodes a protein with 811 amino acid residues. The 811bp fragment was cloned in the pET-26b(+) plasmid, previously digested with restriction enzymes of FastDigest®NcoI and FastDigest®XhoI, respectively. The resulting construct (= pET-26b(+)-

CeP) was transformed into chemocompetent DH5 α *E.coli* and expressed in BL21 (DE3) *E. coli* cells in order to yield the active recombinant enzyme containing a N-terminal metal affinity fusion peptide (6xHis tag), whose primary structure shares significant residue identity with different members of glycosyltransferase family 36.

In this context, five cellobiose phosphorylase genes have been cloned [Reichenbecher *et al.*, 1997; Liu *et al.*, 1998; Yernool *et al.*, 2000; Kim *et al.*, 2002; Nidetzky *et al.*, 2004] so far. Comparison of the amino acid sequence of cellobiose phosphorylase from *Clostridium thermocellum* NCIMB 10682 with those from *Clostridium stercorarium* (Accession number: Q59316), *Cellvibrio gilvus* (Accession number: O66264), *Thermotoga neapolitana* (Accession number: O52504), *Clostridium thermocellum* YM4 (Accession number: D1NLD9) and *Cellulomonas uda* (Accession number: Q7WTR6) reveals similarities of as high as 71, 63, 73, 92 and 61%, respectively.

The cellobiose phosphorylase gene of *Clostridium stercorarium* was cloned using oligonucleotide probes [Reichenbecher *et al.*, 1997], but so far these data have not been published.

According to other studies, cloning of cellobiose phosphorylase of *Cellvibrio gilvus* [Liu *et al.*, 1998] was conducted in a pUC18 plasmid and transformed into *E.coli* JM 109. The pUC18 plasmid was genetically engineered to include a gene for antibiotic resistance to ampicillin. In contrast, the pET-26b(+) plasmid used in this study, which is also suitable for bacterial expression, contains a gene coding for kanamycin instead of ampicillin resistance. Additionally, the pET-26b(+) plasmid provides a great advantage in contrast to pUC18 plasmid due to the presence of a N-terminal (His)₆ tag. Polyhistidine-tagging constitutes an effective and practical approach for the purification of 6xHis-tagged recombinant cellobiose phosphorylase as performed in the present study. For the efficient production of cellobiose phosphorylase of *Cellvibrio gilvus*, an overexpression system is under investigation [Liu *et al.*, 1998], while in the present study, BL21 (DE3) *E. coli* competent cells were used as the expression system for cellobiose phosphorylase of *Clostridium thermocellum* NCIMB 10682.

5. Characterization of the recombinant cellobiose phosphorylase

The characterization of the recombinant cellobiose phosphorylase from *Clostridium thermocellum* NCIMB 10682 showed the same catalytic features for azido analogs of cellobiose as the native enzyme. By contrast to the native enzyme, the recombinant enzyme is not accompanied by a cellodextrin phosphorylase activity; therefore, oligosaccharide side products are not found during conversions with the recombinant enzyme. The enzyme has been purified 2-fold, using only a one-step purification protocol, the Ni-NTA metal ion affinity chromatography. The specific activity of the purified recombinant enzyme was 7.4 U/mg towards cellobiose.

In this context, purification of the recombinant cellobiose phosphorylase of *Clostridium thermocellum* NCIMB 10682 resulted in a 2.5-fold greater increase in specific activity than the one shown for the native cellobiose phosphorylase of the same strain, which had a specific activity of 3 U/mg towards cellobiose after a three-step purification procedure. A possible explanation for this modestly increased specific activity of the recombinant cellobiose might be due to the application of imidazole (150mM) as elution buffer in Ni-NTA metal ion affinity chromatography, which can cause destabilization of the protein. So far, another report in the literature describing sensitivity of cellobiose phosphorylase towards imidazole was provided for the isolation of the recombinant enzyme of *Cellulomonas uda* [Nidetzky *et al.*, 2004] from an *E.coli* cell extract, which included a two-step purification protocol. The elution of the enzyme with 120mM of imidazole resulted in 2-fold purification, which corresponds to above described findings of recombinant cellobiose phosphorylase from *Clostridium thermocellum* NCIMB 10682.

In comparison to these findings, recombinant cellobiose phosphorylase from *Thermotoga neapolitana*, which enzyme elution included a linear gradient of 10-300mM imidazole, was enriched 17-fold [Yernool *et al.*, 2000]. The specific activity of the purified recombinant enzyme was estimated to be 11.8 U/mg. SDS PAGE analysis of the recombinant cellobiose phosphorylase of *Thermotoga neapolitana* showed a homogenous enzyme preparation with an estimated molecular weight of 93 kDa which well corresponds to the one determined for *Clostridium thermocellum* NCIMB 10682. Recombinant cellobiose phosphorylase of *Clostridium thermocellum* YM4 [Kim *et al.*, 2002], whose purification was carried out by three chromatographic methods, has also been reported to have a similar molecular mass of 92 kDa. A

similar estimate of 92 kDa was reported for the molecular mass of the recombinant cellobiose phosphorylase of *Cellulomonas uda* [Nidetzky *et al.*, 2004].

6. Synthesis of azidodeoxy derivatives of cellobiose catalyzed by native and recombinant cellobiose phosphorylase

In order to explore the feasibility of additional artificial basic cellulose derivatives as novel biomimetic polymers [Schwarz *et al.*, 2003], chemo-enzymatic syntheses of azidocellobiose analogs with native as well as with recombinant cellobiose phosphorylase of *Clostridium thermocellum* NCIMB 10682 were performed in this study. So far, not much was reported on azidodeoxy derivatives of cellobiose. However, the chemo-enzymatic method has shown to possess obvious advantages and the present investigations have proven that this methodology has much to offer in terms of enhanced regioselectivity over strictly chemical methods.

6-azido-6-deoxy-cellobiose and 6'-azido-6'-deoxy-cellobiose were synthesized from previously known cellobiose derivatives [Chernyak *et al.*, 1985; Saber, 2009]. For the preparation of precursors to azido cellobiose derivatives based on enzymatic synthesis, phosphorolytic reaction with purified cellobiose phosphorylase was performed with 6-azido-6-deoxy-cellobiose. This reaction has afforded 6-azido-6-deoxy-D-glucose and α -D-glucose 1-phosphate. In the reverse (synthetic) mode of phosphorylase catalysis, 6-azido-6-deoxy-cellobiose was formed from α -D-glucose 1-phosphate and 6-azido-6-deoxy-D-glucose [Saber, Lubura *et al.*, manuscript in preparation].

Catalytic reaction of purified cellobiose phosphorylase was performed with azido substituents in the non-reducing glucose moiety as well, namely 6'-azido-6'-deoxy-cellobiose. Cleavage of 6'-azido-6'-deoxy-cellobiose under catalysis by cellobiose phosphorylase from *C. thermocellum* was expected to afford 6-azido-6-deoxy-D-glucose 1-phosphate and D-glucose [Saber, Lubura *et al.*, manuscript in preparation]. Presumably, the substrate 6'-azido-6'-deoxy-cellobiose is initially cleaved, by phosphorolytic action, to a small extent with formation of D-glucose. The formed D-glucose may effectively compete with 6'-azido-6'-deoxy-cellobiose for binding to the active site of the enzyme, as D-glucose binds strongly to catalytic center of the enzyme cellobiose phosphorylase. Due to these circumstances, 6'-azido-6'-deoxy-cellobiose is at best a poor substrate of cellobiose phosphorylase from *Clostridium thermocellum* NCIMB 10682.

Another synthesis of cellobiose analogs with azido substituents in the reducing glucose moiety was performed with 2-azido-2-deoxy-cellobiose as substrate for cellobiose phosphorylase. Both, native purified and recombinant purified cellobiose phosphorylase, catalyzed the phosphorolytic cleavage of 2-azido-2-deoxy-cellobiose, thus affording 2-azido-2-deoxy-D-glucose and α -D-glucose 1-phosphate. This finding offers the opportunity to develop a simple synthesis of pure 2-azido-2-deoxy-D-glucose, as 2-azido-2-deoxy-cellobiose can be prepared from cellobial acetate in stereochemically homogeneous form, without formation of the *manno* isomer.

Hydrolysis of α -D-glucose 1-phosphate to afford D-glucose has been observed as a side reaction during conversions with azido analogues of cellobiose. As binding of the azido analogues would be considerably weaker than binding of cellobiose, α -D-glucose 1-phosphate would effectively compete for the binding site, with hydrolysis being a logical side reaction. The D-glucose formed could react with the remaining α -D-glucose 1-phosphate to form cellobiose, which is also observed in trace amounts.

7. Broad spectrum substrate specificity of native and recombinant cellobiose phosphorylase

In this study, the experiments with native and recombinant cellobiose phosphorylase of *Clostridium thermocellum* NCIMB 10682 have shown their capability to cleave cellobiose into α -D-glucose 1-phosphate and D-glucose by phosphorolytic reaction. These investigations correspond well with the findings of data published for cellobiose phosphorylase of *Cellvibrio gilvus* [Kitaoka *et al.*, 1992] and *Clostridium thermocellum* YM4 [Kim *et al.*, 2002]. The reaction of cellobiose phosphorylase follows a sequential bi bi mechanism, in which P_i binds to the enzyme before cellobiose and then α -D-glucose 1-phosphate is released after D-glucose. This mechanism was also described for cellobiose phosphorylase of *Cellulomonas uda* [Nidetzky *et al.*, 2000]. Cellobiose phosphorylase of *Clostridium stercorarium* [Reichenbecher *et al.*, 1997] also exhibits in phosphorolytic reaction a strict cleavage of cellobiose and has showed no activity with cellotriose, cellotetraose, cellopentaose and cellohexaose respectively.

In the reverse reaction, native and recombinant cellobiose phosphorylase of *Clostridium thermocellum* NCIMB 10682 catalyzes the synthesis of cellobiose by transfer of a glucose residue from α -D-glucose 1-phosphate to the acceptor D-glucose. In comparison to these findings, an extensive kinetic study of the synthetic reaction of the same enzyme from *Cellvibrio*

gilvus [Kitaoka *et al.*, 1992] was reported, revealing that the synthetic reaction follows four steps, in which α -D-glucose 1-phosphate binds to the enzyme before D-glucose. Consequently, this enzyme-substrate complex can release Pi as well as cellobiose.

In present study, it was also reported, that cellobiose phosphorylase of *Clostridium thermocellum* NCIMB 10682 is active on different derivatives of glucose acceptors, such as 2-deoxyglucose, 6-deoxyglucose and D-xylose as well, revealing the capability of the enzyme to catalyze the formation of corresponding disaccharide analogs of cellobiose in synthetic reaction. The same substrate specificity was also reported for cellobiose phosphorylase of *Clostridium thermocellum* YM4 [Kim *et al.*, 2002] and *Cellvibrio gilvus* [Percy *et al.*, 1998].

An explanation for the formation of trisaccharide in some experiments under catalysis by native cellobiose phosphorylase could be provided by the presence of cellodextrin phosphorylase. The finding of no trisaccharide formation during the incubation of recombinant cellobiose phosphorylase with cellobiose as the substrate is taken to indicate that cellobiose phosphorylase does not catalyze trisaccharide formation during the phosphorolytic reaction.

X. OUTLOOK

Artificial polysaccharides and biomimetic polymers

Polymers based on carbohydrates have emerged as an attractive area of polymer science and technology, due to a worldwide focus on sustainable materials. The enzyme-mediated syntheses of azidocellobiose analogs described in the present thesis represent a “green chemistry” approach in order to investigate potential uses of these azidocellobiose derivatives. In this context, the advantages associated with enzymatic catalyzed reactions, as proved in this study, are attributable to the avoidance of multi-step protection-deprotection procedures of the sugars. A more preferable and practical approach will be the use of recombinant cellobiose phosphorylase of *Clostridium thermocellum* NCIMB 10682 for the large scale production, possibly in immobilized form.

Applications of immobilized enzymes

Biocatalytic process economics can be enhanced by enzyme reuse and the improvement in enzyme stability afforded by immobilization [Brady and Jordaan, 2009; Brady *et al.*, 2008]. Enzyme immobilization with the aim of facilitating their use involves the reduction of production costs by enabling their efficient recycling [Cao, 2005] and it includes also their role as stable and reusable devices for analytical and medical applications or as selective adsorbents for purification of proteins and enzymes as well as effective devices for controlled release of protein drugs (**Figure 68**). Moreover, improved enzyme performance, such as increasing the activity, decreasing inhibitions, modulating stability and selectivity or improving the enzyme behavior in synthetic processes can often be achieved by enzyme immobilization [Mateo *et al.*, 2007]. Additional advantages involve the stabilization against harsh reaction conditions, due to the fact that they are deleterious to soluble enzyme preparations [Tischer and Kasche, 1999].

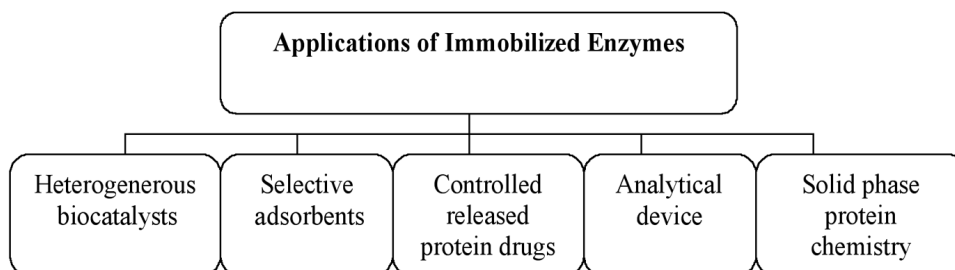


Figure 68 Range of application of immobilized enzymes [Cao, 2005]

An immobilized enzyme is defined as a composite consisting of two essential components: the non-catalytic structural component (carrier), designed to aid separation and reuse of the catalyst and therefore facilitate control of the process, and the catalytic functional component (enzyme), designed to convert the substrates of interest into the desired products. [Cao *et al.*, 2003]

Non-catalytic parameters are linked to the chemical and physical nature of the non-catalytic part, particularly the geometric parameters such as size, thickness, shape and length, whereas the catalytic parameters are related to the catalytic functions, such as activity, stability and selectivity, pH and temperature profiles (**Table 13**) [Cao *et al.*, 2003].

In practice, catalytic functions aim to maintain few side reactions, high tolerance of structural variation of the substrates and high durability of the catalyst. On the other hand, main objectives of non-catalytic functions are mainly to achieve easy separation of the immobilized enzymes from the reaction mixtures, flexibility of reactor design, and broad applicability in different reaction media and reaction systems [Cao, 2005].

Table 13

Criteria for robust immobilized enzymes [Cao *et al.*, 2003]

<u>Parameter</u>	<u>Requirement</u>	<u>Benefits</u>
<i>Non-catalytic function</i>	Suitable particle size and shape	Aids separation, easy control of the reaction
	Easy fabrication	Flexibility of reactor design
	High mechanical stability	No significant change of the internal structure
	High chemical stability	No decomposition of the solid catalysts, non-contamination
<i>Catalytic function</i>	High volume activity (U/g)	High productivity and space-time yield
	High selectivity	Fewer side reactions, easier downstream processing and separation of products, less pollution
	Broad substrate specificity	Tolerance of structural variation of the substrates
	Stability in organic solvents	Shift of reaction equilibrium with the organic solvents use
	Thermostability	Reduce reaction time by increasing temperature
	Operation stability	Cost-effective and lower cost-contribution for the product
	Conformational stability	Modulation of enzyme properties
<i>Immobilized enzyme</i>	Recyclability	Low-cost contribution of catalyst
	Broad applicability	Tolerance for process variation
	Reproducibility	Guarantees product quality
	Easy and quick design	Early insight into process development and avoidance of learning process

Economical and ecological considerations

Lower volume	Lower cost for the solid handling
Easy disposal	Less environmental concern
Rational design	Avoidance of laborious screening
Safety for use	Meeting the safety regulations

Intellectual property right

Innovative	Protection of intellectual property rights
Attractive	Licensing
Competitive	Strengthening marketing position

Numerous methods of immobilization on different materials have been developed, whereas main efforts have been put into the cross-linking of enzymes using either protein cross-linking or addition of inert materials approach [Silman and Katchalski, 1966]. Preferred method appears to be the binding to prefabricated carrier materials, but recently cross-linking enzyme crystals have become an interesting alternative.

Generally, the production of carrier-free immobilized enzymes includes directly different cross-linking enzyme approaches such as:

- a) Dissolved enzymes (Formation of cross-linked immobilized enzymes, CLEs)
- b) Crystalline enzymes (Formation of cross-linked enzyme crystals, CLECs)
- c) Physically aggregated enzymes (Formation of cross-linked enzyme aggregates, CLEAs)
- d) Spray-dried enzymes (Formation of cross-linked spray-dried enzymes, CSDEs)

Enzymes in carbohydrate synthesis

The ability of the recombinant cellobiose phosphorylase to perform only the synthetic reaction will be of practical importance, enabling syntheses of entirely new azido sugars. Overall, the enzyme catalyzed reactions constitute initial steps of a comprehensive concept to utilize the combined potentials of cellobiose analogs with azido substituents synthesis and biotechnology for preparations of alternative biomimetic materials on a large scale with a broad spectrum of scientific applications within academic and industrial research.

Characterization of azido polysaccharides

Characteristics of azido polysaccharides, which have been synthesized via organic-chemical and enzyme-catalyzed chemistry have not been thoroughly examined and need more in-depth investigations regarding their structure-property relationships. The extensive analyses of these artificial cellobiose derivatives could provide manifold insights into use of new matrix components with potential pharmacological and physiological applications. In a different type of application, these cellobiose derivatives in monomeric form could also serve as intermediates for syntheses of anti-inflammatory agents. They also might enhance or trigger desirable biological responses such as those applicable in cancer immunotherapy.

6-azido-6-deoxy-D-glucose 1-phosphate

Continuation of the present approach to provide facts regarding formation of 6-azido-6-deoxy-D-glucose 1-phosphate, which was not detected in the phosphorolytic reaction of cellobiose phosphorylase towards 6'-azido-6'-deoxy-cellobiose, will include application of the enzyme mutarotase (Aldose 1-epimerase). This enzyme possesses the ability to convert α -D-glucose into β -D-glucose, a substrate of glucose oxidase (EC 1.1.3.4). Oxidation of D-glucose would be expected to remove α -D-glucose, which strongly inhibits the action of cellobiose phosphorylase and effectively competes with 6'-azido-6'-deoxy-cellobiose, thus presumably inhibiting the formation of 6-azido-6-deoxy-D-glucose 1-phosphate.

Azido oligosaccharides formed under catalysis by cellodextrin phosphorylase

Another interesting aspect should include the analysis of azido trisaccharides, which formation was detected in native cellobiose phosphorylase phosphorolytic syntheses. The formation of these materials is probably catalyzed by cellodextrin phosphorylase, and so far their amounts are too small for efficient separation and structure elucidation.

XI. REFERENCES

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XII. CURRICULUM VITAE

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Publications

Paumann M., Lubura B., Regelsberger G., Feichtinger M., Köllensberger G., Jakopitsch C., Furtmüller P.G., Peschek G.A., Obinger C.

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6-Azido-6-deoxy- and 6'-azido-6'-deoxy- derivatives of cellobiose: syntheses and substrate studies with cellobiose phosphorylase from Clostridium thermocellum

Manuscript in preparation

Gabra N., Lubura B., Mulla D., Unger F.M.

A combined organic-chemical and enzyme-catalyzed synthesis of 2-azido-2-deoxy-D-glucose

Manuscript in preparation

Schwarz B., Saber Y., Trinkl M., Gabra N., Lubura B., Schmid W., Hedenetz A., Viernstein H., Unger F. M.

Monitoring carbohydrate conversions by the use of thin layer chromatography and the anisaldehyde-sulfuric acid spray reagent

Manuscript in preparation

Poster presentations

- Y. Saber, S. Salar-Behzadi, B. Lubura, S. Bogner, F. M. Unger, H. Viernstein
“Synthesis of 6-azido-6-deoxy- β -D-glucopyranose and β -D-glucopyranosyl-(1 $\rightarrow$ 4)-6-azido-6-deoxy- β -D-glucopyranose from D-glucose and cellobiose respectively and the enzymatic confirmation of these syntheses”
19.04. – 26.04.2007 Pharmaceutical Sciences World Congress and Pre-Satelite Meeting, Amsterdam, Netherlands

- Y. Saber, B. Lubura, S. Salar-Behzadi, H. Viernstein, F. M. Unger
„6-azido-6-deoxy-cellobiose: organic chemical and enzyme-catalyzed syntheses“
07.04. – 10.04.2008 6th World Meeting on Pharmaceutics and Biopharmaceutics and Pharmaceutical Technology, Barcelona, Spain

- Y. Saber, B. Lubura, H. Viernstein, F. M. Unger
„Carbohydrate derived azides as substrates for cellobiose phosphorylase from *Clostridium thermocellum*”
16.04. – 18.04.2009 21st Scientific Congress of the Austrian Pharmaceutical Society, Vienna, Austria

- B. Lubura, P. Starkl, E. Jensen-Jerolim, Y. Saber, S. Salar-Behzadi, H. Viernstein, F. M. Unger
„Substrate studies with the recombinant cellobiose phosphorylase from *Clostridium thermocellum* NCIMB 10682”
19.07. – 24.07.2009 15th European Carbohydrate Symposium, Vienna, Austria

- Y. Saber, B. Lubura, R. Löppert, R. Ludwig, H. Viernstein, F. M. Unger
„Carbohydrate derived azides for pharmaceutical technical applications“
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- B. Lubura, S. Salar-Behzadi, Y. Saber, H. Viernstein, F. M. Unger
„Enzyme-catalyzed approaches for the development of new biomimetic carbohydrate-derived excipients“
08.03. – 11.03.2010 7th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Valletta, Malta

- B. Lubura, N. Gabra, P. Starkl, H. Viernstein, F. M. Unger
„Enzyme-catalyzed synthesis of 2-azido-2-deoxy-D-glucose under catalysis by recombinant cellobiose phosphorylase”
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