

CHITIN HYDROLYSIS AND *N*-ACETYLGLUCOSAMINE
UTILIZATION BY SOLVENTOGENIC CLOSTRIDIA

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ABSTRACT

Environment pollution and energy supply are among the huge problems which threaten the world, especially in industrialised countries. Several studies have considered how to exploit waste materials as renewable substrates for various industries to obtain different products. Some wastes from the aquatic food industry contain a considerable amount of the *N*-acetylglucosamine (NAG) polymer chitin, which has potential as a substrate for the solventogenic clostridia in the acetone-butanol-ethanol fermentation. Development of an effective process will, however, depend on a detailed understanding of the mechanism and control of chitin hydrolysis and NAG metabolism.

Clostridium beijerinckii NCIMB 8052 was shown to exhibit chitinase activity and to be able to grown on NAG. The predominant mechanism for uptake of sugars and sugar derivatives in the clostridia is the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). Extracts of *C.beijerinckii* grown on NAG exhibited a phosphotransferase activity for NAG which was also present in extracts of cells grown on glucose, consistent with the observation that glucose did not repress utilization of NAG in media containing both substrates. Genomic analysis has identified genes encoding a putative *nag-pts* that belongs to the glucose family of PTS permeases. Two divergent genes encode the IIA and IICB domains of the PTS, and are associated with a gene encoding a putative transcriptional antiterminator. These genes were found to be expressed in cells growing on NAG or glucose, but not glucitol. The role of the putative *nag-pts* genes in NAG uptake was confirmed by functional analysis. An artificial NAG operon was constructed in which the *nag-pts* genes were in series and expression of the operon in *Escherichia coli* mutants provided evidence for the ability of the PTS to transport and phosphorylate NAG and glucose, but not mannose.

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Posters

Phosphotransferase system for *N*-acetylglucosamine uptake in *Clostridium beijerinckii*, International Workshops on the Regulation of Metabolism, Genetics and Development of the Solvent- and Acid-forming Clostridia (12). (2012), Nottingham (UK).

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CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

In the last few years, both environmental pollution and energy supply have become huge problems which threaten the world, especially in the industrial countries, due to the accumulation of wastes from a range of sources. One of these wastes, produced by the aquatic food industry, consists of a large amount of chitin polymer. Several studies have considered how to exploit these materials for use as a renewable raw substrate to obtain different products for various industries (Demirbas, 2011). The biofuel industry is considered to be one of the attractive industries in this area, using biological fermentation as a renewable energy technology to produce chemical solvents from several raw materials. A large number of natural materials containing carbohydrate are being considered as suitable carbon sources for replacement of petroleum fuels. However, for the development of this industry, it is necessary to understand the nature of the microorganisms and the mechanism and utilization of sugars derived, in order to obtain effective microbial biocatalysts (Arantes and Saddler, 2010). Chitin, which is a homopolymer of *N*-acetylglucosamine, has considerable potential as a cheap carbon substrate for the solventogenic clostridia in the acetone-butanol-ethanol (ABE) industry.

1.1. Carbohydrates

Carbohydrates are the most abundant biomaterial in nature, they are an important energy source for both macroorganisms and microorganisms, and they play an important role as raw materials in various industries (Harvey and Ferrier, 2011). Chemically the carbohydrates and their sugar derivatives are an attractive raw material for several industries. For example, the food industry uses a huge amount of starch, mono and oligo-saccharides. In addition, carbohydrates are also used in the textile industries which are dependent on cellulose as a raw material. Moreover, some saccharides are important in medical industries, especially in the production of antibiotics, vitamin C and certain intravenous solutions. The basic structure of carbohydrate consists of carbon, hydrogen and oxygen atoms. Chemically the saccharides are classified into three main divisions, according to

many factors, such as their degree of polymerization, molecular size and type of bonds (α or β) (El Khadem, 1988).

- i. Monosaccharides and disaccharides contain a single sugar unit or two sugar units. Examples are glucose, fructose and galactose (monosaccharides), and sucrose, lactose and maltose (disaccharides). In addition there are sugar alcohols such as sorbitol, mannitol, lactitol, xylitol, erythritol and maltitol. Amino sugars are also considered as monosaccharides in which one hydroxyl group has been replaced by an amino group, for example in D-glucosamine, D-galactosamine, D-mannosamine, *N*-acetylglucosamine and *N*-acetylgalactosamine (Stoker, 2011; Cummings and Stephen, 2007).
- ii. Oligo-saccharides contain a chain from three to ten monosaccharide units, for example malto-oligosaccharides (α -glucans), including maltodextrins and non- α -glucans such as raffinose, stachyose, fructo- and galacto- oligosaccharides, polydextrose, and inulin. The oligosaccharides are commonly found in food and can be used in various applications, such as an enhancement to nutrition, and in the medical industry as an inhibitor of dental plaque formation (Cummings and Stephen, 2007; Hou, 2005)
- iii. Polysaccharides contain ten or more monosaccharides in a chain. In this group there is starch (amylose, amylopectin and modified starches), and non-starch polymers such as arabinoxylans, β -glucan, glucomannans, plant gums and hydrocolloids (Cummings and Stephen, 2007). Polysaccharides can be composed of a chain of one type of monosaccharide (homo-glycans, such as glycogen) or can be formed from multiple sugar constituents (hetero-glycans, such as murein, which can be found in the bacterial cell wall structure). Polysaccharides are found naturally in the structure of bacteria, plants and animals. Cellulose, which is the principal component in plant cell walls, is considered to be the most abundant molecule in nature. Chitin which is a polymer of *N*-acetylglucosamine is considered the second most abundant molecule after cellulose and it is a widespread component of plants and the cell walls of microorganisms such as fungi, and crustaceans (Koolman and Röhm, 2005).

1.2. Fermentation

In biotechnology, fermentation processing is known as a microbial bioconversion of sugar substrate (energy source) to produce desirable biochemical products which can be used commercially. Many microorganisms, such as *Clostridium* species, *Enterobacteriaceae* (*Escherichia coli*, *Klebsiella* and *Proteus* species), *Streptococcus faecalis*, *Staphylococcus aureus* and *Bacillus subtilis*, are capable of fermenting a variety of substrates anaerobically to produce a range of products (Boumba *et al.*, 2008). The development of fermentation technology usually depends on understanding the structure of the fermentable substrate and the relevant uptake mechanisms involved, along with the genetic characterization of the microorganisms (Lee, 2006). In biological fermentations microbial growth is dependent on many factors such as temperature, oxygen, pH and carbon source availability. Many microorganisms have the ability to take up one carbon source preferentially in the presence of others, and then utilize the other substrates only after the preferred one is expended (Bruckner and Titgemeyer, 2002).

This study is related to one of the largest industrial-scale fermentations known: the acetone-butanol-ethanol (ABE) fermentation. The ABE industry was successful in the early part of the 20th century, and then declined for economic reasons, due to the rise of the petroleum industry. However, recently, ABE fermentation is once again attracting attention as a renewable process, due to potential interest in butanol as a biofuel, as described later.

1.3. History and development of acetone-butanol-ethanol (ABE) fermentation

Butanol production by anaerobic bacteria was first observed in 1862 by French microbiologist Louis Pasteur when he isolated an anaerobic *Vibrion butyrique*. This strain was subsequently identified as *Clostridium butyricum* (Sauer *et al.*, 1993). At the beginning of the 20th century, butanol was recognised to be a precursor of butadiene which could be used in the production of synthetic rubber, as a substitute for natural rubber which was in high demand (Dürre, 1998). In 1909, the English company Strange and Graham Ltd. built the first factory for butanol production and employed W. H. Perkin, C. Weizmann, A. Fernbach, and M. Schoen, to establish the butanol industry. Weizmann isolated a variety of microorganisms between 1912 and 1914, one of which had the ability to degrade many kinds of starch substrate and

produce high yields of butanol and acetone. This strain was called BY, and was later renamed as *Clostridium acetobutylicum* (Awang *et al.*, 1988).

During the First World War, ABE fermentation played an important role, due to increasing demand from the British army for acetone for the manufacture of explosives, and that led to the large scale development of the fermentation process. Acetone, in that period, was used in the manufacture of cordite, a smokeless gunpowder used as a propellant for cartridges and shells (David and David, 1986).

At the end of the war the demand for acetone decreased. However, the automobile industry in the USA was increasing, and this also had a positive effect on the ABE fermentation industry, as butanol was used as a starting material to produce butyl acetate for use as a quick-drying paint for cars (Rose, 1961), and the butanol industry became distributed around the world. For instance, Peoria (Illinois) was the largest plant at that time. In the meantime, research focused on improving a new strain able to utilize maize mash as a raw material for the fermentation and a variety of strains were isolated from many sources, although none of them achieved the same result as the original Weizmann strain (Hastings, 1971). In the 1930s, the abundance of cheap molasses played a role in development of ABE fermentation technology as it became an alternative raw material, rather than maize mash. In 1932 molasses was used for the first time as a commercial substrate by Commercial Solvents Corporation (CSC) (Jones and Keis, 1995). Between 1936 and 1940 research made significant progress in this area and a new strain called *Clostridium saccharo-acetobutylicum* was isolated. This strain was capable of using molasses as a cheap raw material as a carbon source at a sugar concentration of about 6% with approximately 30% solvent yield. The strain prototype was recorded in the Northern Regional Research Laboratory (NRRL) culture collection in 1945 as NRRL B591. This was used as the main industrial strain for fermentation of molasses by CSC in the USA and new plants belonging to Commercial Solvents Great Britain (CS-GB) in Britain. In 1938 a new strain was patented by CSC and it was named *Clostridium saccharo-butyl-aceticum-liquefaciens*. This strain, lodged with NRRL in 1946 as NRRL B643, helped to improve the ABE fermentation process, giving higher yields of about 30-33% conversion to solvent from 6.5% sugar in a shorter fermentation time. Additional strains were isolated later that gave a higher concentration of solvents, among which was a strain named

Clostridium granulobacter acetobutylicum (Shaheen *et al.*, , 2000). A number of ABE fermentation factories were established around the world, for example in South Africa, Japan, Egypt, Russia, China, Brazil and Formosa (Taiwan) (Jones and Woods, 1986). The CSC decided to build a fermentation plant at Bromborough in the UK for ethanol and butanol production as a joint enterprise with the Distillers Corporation Ltd, and CS-GB plant as an operator. The Second World War led to further improvement in the acetone industry through isolation of strains capable of degrading molasses as a raw material to produce more solvent (Jones and Keis, 1995). However, the growth of the petrochemical industry in the middle of the last century contributed significantly to the demise of the ABE fermentation industry because of the orientation of the world towards oil products (Dürre, 2008). Acetone-butanol production plants closed down, particularly in the western world although, the fermentation process was continued in South Africa and Russia until the 1980s and in China and Egypt, until more recently (Zverlov *et al.*, 2006). In addition, there were some significant obstacles that affected the economics of the ABE fermentation, such as the high cost of substrate, low yield productivity, due to the toxicity of the solvents, and the cost of recovery of the products (Gapes, 2000). Since the 1980s, research has been focused on improvement of the ABE fermentation process by understanding the principles of physiological and genetic behaviour in clostridia, in addition to investigating the use of low cost fermentable materials such as agricultural and domestic organic waste (Gheshlaghi *et al.*, 2009). These and other challenges in re-establishing the ABE fermentation have been summarized by Green (2011), as shown in (Table 1).

Table 1: The barriers and proposed solutions for ABE fermentation, (*Green, 2011*)

<i>Problems</i>	<i>Solutions</i>
High feedstock cost significantly increases operating costs.	Transition towards cheaper (and more sustainable) feedstock such as wastes and agricultural residues.
Low butanol yield increases recovery costs. Low yields also reduce sugar loadings and increase water usage.	Develop improved microbes with improved solvent yields and/or develop methods for in situ product removal to alleviate end product tolerance.
Low butanol yield increases feedstock costs.	Develop improved microbes with higher butanol yields and/or develop microbes with higher butanol: solvent ratios.
Low volumetric solvent productivities increase capital and operating costs.	Develop continuous fermentation processes that reduce down-time and increase volumetric productivity.
Solvent recovery using conventional distillation is energy intensive and relatively expensive.	Develop low energy methods for solvent recovery and purification. Recovery can also be improved by improving the solvent yield.
High water usage is not sustainable and increases the cost of effluent treatment.	Recycle process water back through the fermentation.

1.4. Industrial applications of solvents (acetone, butanol and ethanol)

Acetone (Figure 1) is one of the commercial solvents used in resins and lacquers. It is also common in film coating compositions and can be used in chemical extraction applications to extract fats, oils and waxes from the natural material. In medical applications, uses of acetone include extracting B-vitamin complex, antibiotics, some enzymes and alkaloids (Cheremisinoff and Archer, 2003). Butanol is an important commercial liquid in many industries, such as production of plasticizers, lacquers, coatings, detergents and brake fluids (Kharkwal *et al.*, 2009). Ethanol is a minor product of the ABE fermentation and used in many industries such as the manufacturing and construction sectors, and also in the medical sector in some medicine ingredients and for sterilizing purposes (Clean Fuels Development Coalition, 2003).

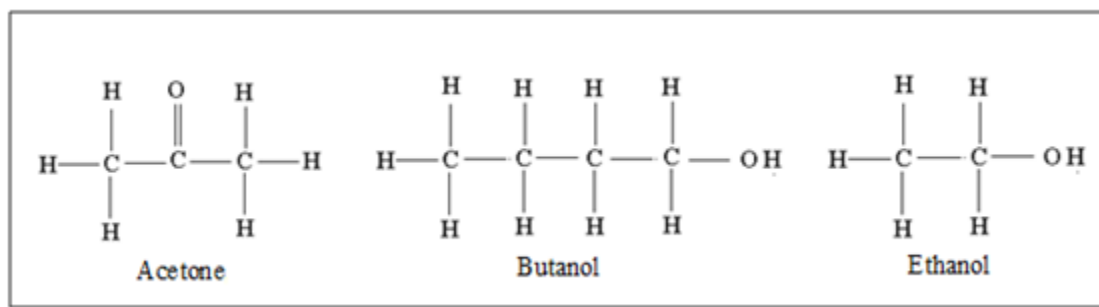


Fig 1: The chemical formulae of acetone, butanol and ethanol.

1.5. Biofuel

Biofuel is one of the sustainable energy sources derived from natural materials such as biomass and biological waste via biotechnology applications. Biofuel can be used for different purposes but is mainly used as a substitute for oil-based transportation fuels (Demirbas, 2009). Interest in the biofuel industry has increased recently because of economic considerations (the price of crude oil and limited fossil fuel reserves), and environmental aspects, such as carbon dioxide pollution and global warming (Dürre, 2008). Currently, the biofuel industry is receiving considerable attention as a renewable energy source, for example in the European Union (EU) (Demirbas, 2011). There are many types of biological energy sources,

including bioethanol, biodiesel, biogas, bio-oil, biohydrogen and vegetable oil, and most of them can be obtained from several different wastes. For example biodiesel, which is a clear yellow liquid and a suitable fuel for diesel engines, similar to petroleum diesel, can be obtained from various wastes such as animal fat (sheep tallow, beef tallow and poultry oil) or some vegetable oils (rapeseed oil, canola oil, soybean oil, sunflower oil, palm oil and used cooking oil). Biodiesel is considered to be much safer than petroleum diesel as it is nonflammable and nonexplosive. Environmentally, biodiesel also has lower toxicity during burning, when compared with petroleum diesel, and is more degradable compared to oil waste (Demirbas, 2009).

Bioethanol is also a renewable biofuel that can be used in transportation, after some modification to vehicle engines. Ethanol can also be used to blend with gasoline to increase the volume of the production. In general, since 2004, the production of biofuel has risen and the number of plants has also increased around the world (US, Europe, Brazil, China, India, Thailand, Canada, Russia, and South Africa) using different sources as fermentable raw materials, especially agriculture wastes or sugar and corn crops (Kecebas and Alkan 2009; Prabhakar and Elder; 2009; Hammerschlag, 2006). Bioethanol can be also produced from other polysaccharides, including lignocellulosic biomass, which is an abundant feedstock. However, the biodegradation of these structurally complex materials is one of the obstacles to producing large amounts of bioethanol at reasonable cost. Thus, the development of industrial microorganisms engineered to utilize these raw materials is considered to be one of the desirable scientific solutions (Cardona and Sanchez, 2007).

Biobutanol, which can be obtained from fermentation processing, has been described recently as a commercially attractive product for transportation purposes. Butanol burning releases more energy compared to ethanol. Moreover, there are a number of advantages which make bio-butanol a more attractive biofuel than bioethanol. For example, biobutanol has a lower vapor pressure and hygroscopy and in addition, can be combined with gasoline and biodiesel to be used without any modification of the engine. In the future, biobutanol could be used for aviation engines which may allow bio-butanol production to increase to 94 billion gallons per annum by 2020 (Kraemer *et al.*, 2010; Green, 2011; <http://www.butanol.com>).

The interest in the biofuel industry has led to a greater focus on developments to improve microbial performance, particularly with respect to the solventogenic clostridia.

1.6. Solventogenic clostridia

Clostridia are described morphologically as rod-shaped cells; Gram-positive staining (especially in the early stages of growth) and having cell walls consisting of a multilayer of peptidoglycan. The majority of clostridia are known as strictly anaerobic bacteria. The clostridia species are capable of forming heat-stable endospores with increased resistance against heat, radiation, dehydration and oxygen (Dürre, 2005). Clostridia are found in soils as a natural habitat and also in animal intestines (Carlos *et al.*, 2005). Clostridia contain low G + C DNA, and are members of the group referred to as firmicutes, together with many other genera such as *Bacillus*, *Staphylococcus*, *Streptococcus* and *Listeria* (Euzéby, 1997; Vos, 2009; Bahl and Dürre, 2001). For clostridia, the 16S rRNA sequence has become an accepted technique for defining species. Solvent forming strains including those mentioned earlier have been shown to belong to four species; *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharoperbutylacetonicum*, and *Clostridium saccharobutylicum* (Keis *et al.*, 2001). As shown first for *Clostridium acetobutylicum*, solvent production by clostridia occurs in two stages. Firstly, in the exponential growth phase, the principal products are acids, acetate and butyrate, along with hydrogen and carbon dioxide. In the second stage, solvent production is activated to produce acetone, butanol and ethanol, while the growth slows and the cells may eventually form endospores (Green, 2011). Some clostridial species produce isopropanol rather than acetone, including some strains of *C.beijerinckii*. The ability of many species of solventogenic clostridia to utilize a variety of carbohydrate sources leads to the possibility that a range of fermentable substrates can be used as raw materials for biofuel production.

Generally, in clostridia, the majority of sugars can be transported into the cell via a specific mechanism called the phosphoenolpyruvate (PEP) dependent phosphotransferase system (PTS) (Hutkins and Kashket, 1986; Mitchell *et al.*, 1991). The anaerobic metabolic routes for carbohydrate substrates are known, but

some aspects of regulation still have to be determined. *C. acetobutylicum* and *C. beijerinckii* strains utilize the glycolysis route of the Embden-Meyerhof-Parnas pathway (Rogers and Gottschalk, 1993), as shown in Figure 2. The metabolic production of acetone, butanol and ethanol in clostridia, therefore, starts with the uptake of the sugar substrate through the cell membrane, in many cases by the PTS then degradation to pyruvate. The enzyme ferredoxin-pyruvate oxidoreductase converts the pyruvate to acetyl-CoA with the release of hydrogen and CO₂. Then acetyl-CoA may be converted into several end-products. Butanol production is obtained by conversion of acetyl CoA to acetoacetyl-CoA, 3-hydroxybutyryl-CoA, crotonyl-CoA and butyryl-CoA. Then the butyryl-CoA is converted to butyraldehyde which, in turn, is reduced to butanol (Mitchell, 1998; Dürre, 1998).

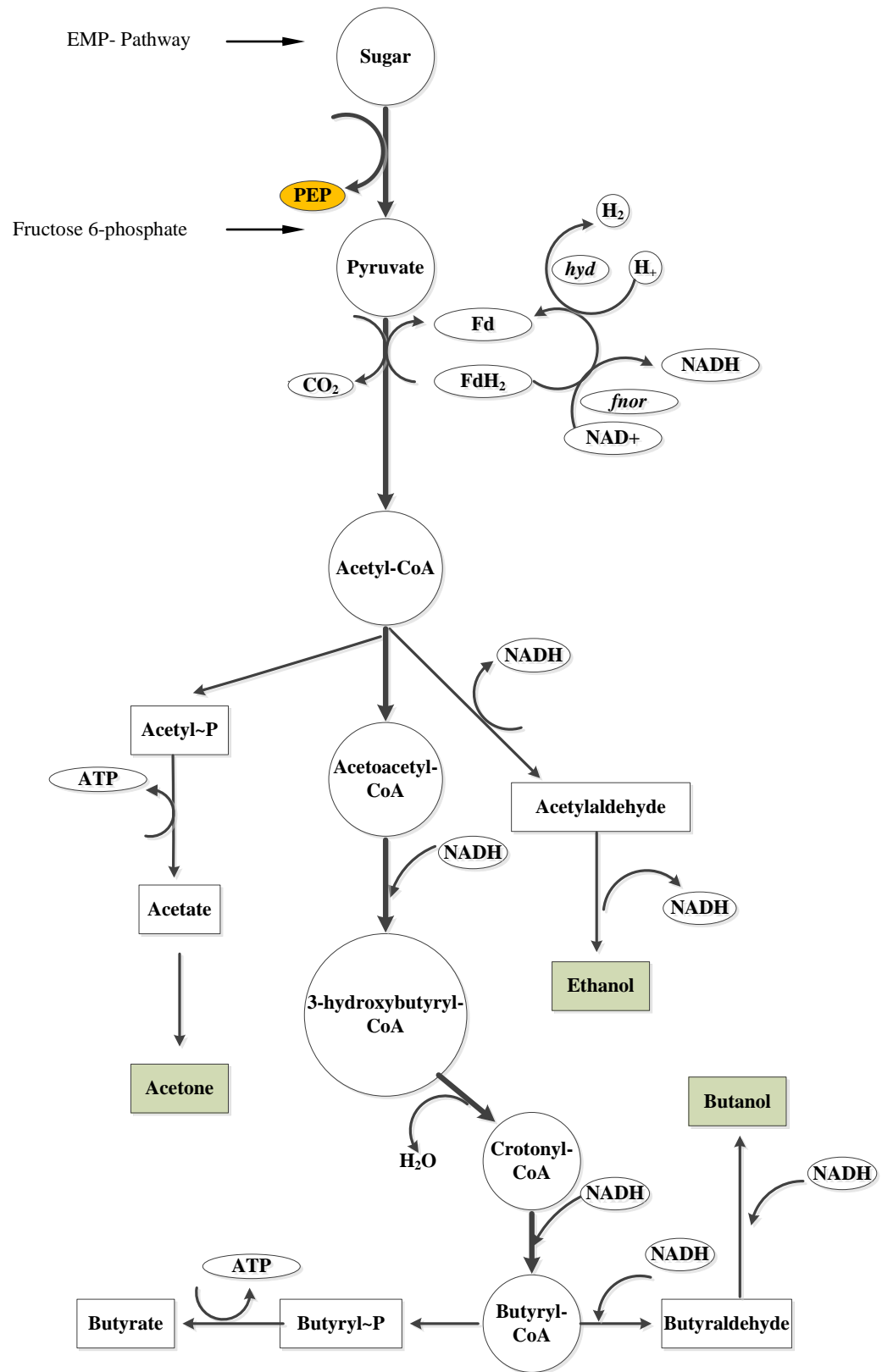


Fig 2: Metabolic route for carbohydrate fermentation by clostridia (Papoutsakis, 2008).

1.7. The phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS) in bacteria

The phosphotransferase system (PTS) was initially described in the bacterium *E. coli* as a transport system for sugar uptake. The PTS contains a number of proteins which have been described as Enzyme I, HPr (heat-stable, histidine-phosphorylatable protein) and Enzyme II (Saier, 2001). Kundig *et al.*, . (1964) described the phosphotransferase system in *E. coli* as a transport mechanism for several carbon sources such as glucose, mannose, and some amino sugars such as *N*-acetylmannosamine, glucosamine and *N*-acetylglucosamine. The PTS has subsequently been found in a range of bacteria, both Gram-positive and Gram-negative including clostridia (Deutscher *et al.*, 2006). The PEP dependent phosphotransferase system is one of the most important mechanisms for sugar uptake across the cell membrane in anaerobic bacteria, but is also found in some strictly aerobic genera (Romano *et al.*, 1979). The PTS involves a phosphoryl transfer chain (Figure 3), which functions to phosphorylate the substrate as it is translocated into the cell (Deutscher *et al.*, 2006). Practically, the PTS can be measured by detection of the PEP-dependent phosphorylation activity on the substrate, using cell-free extracts (Mitchell, 1998).

a) Enzyme I [EI]

Enzyme I has been reported as an autophosphorylated enzyme in the presence of Mg^{2+} , and this is considered to be the first step in operation of the transport system. Enzyme I is encoded by the *ptsI* gene in the *pts* operon in a variety of bacteria (Postma *et al.*, 1993). The phosphate is then transferred from Enzyme I to the general phosphocarrier protein HPr. Enzyme I has been purified from a variety of bacterial species such as *E. coli* (Dooijewaard *et al.*, 1979; Waygood and Steeves, 1980), *Salmonella typhimurium* (Weigel *et al.*, 1982) *Streptococcus faecalis* (Alpert *et al.*, 1985) and *Mycoplasma* (Ullah and Cirillo, 1976).

b) Phosphocarrier HPr protein

HPr is the second protein in the phosphoryl transfer chain of the phosphotransferase system. HPr has been described as a transfer protein which can pass the phosphoryl group from Enzyme I to Enzyme II (Singh *et al.*, 2008). This protein is small, approximately 90 amino acids with a molecular mass of 9 to 10 kDa. In the phosphotransfer chain, HPr is phosphorylated on the residue His 15 and then passes the phosphoryl group to Enzyme II (Postma *et al.*, 1993). The *ptsH* gene encoding HPr has been identified in many bacteria, and it appears that the expression of both genes *ptsI* and *ptsH* is increased in the presence of different PTS sugars (Tanaka *et al.*, 2008; Tangney *et al.*, 2003). It has been reported that the preference for glucose, which is considered the most effective sugar in the majority of these bacteria, can be due partly to an induction of the expression of these genes (De Reuse and Danchin, 1988; Stulke *et al.*, 1997; Viana *et al.*, 2000). Usually in bacteria, the *ptsI* and *ptsH* genes are linked together in an operon *ptsHI*, but this is not the case in clostridial species such as *Clostridium acetobutylicum*, *C. perfringens* and *C. tetani* (Tangney and Mitchell, 2004) nor in *C.beijerinckii* (Lee and Blaschek, 2001), or in *Mycoplasma capricolum* (Zhu *et al.*, 1993) and *Streptomyces coelicolor* (Parche *et al.*, 1999).

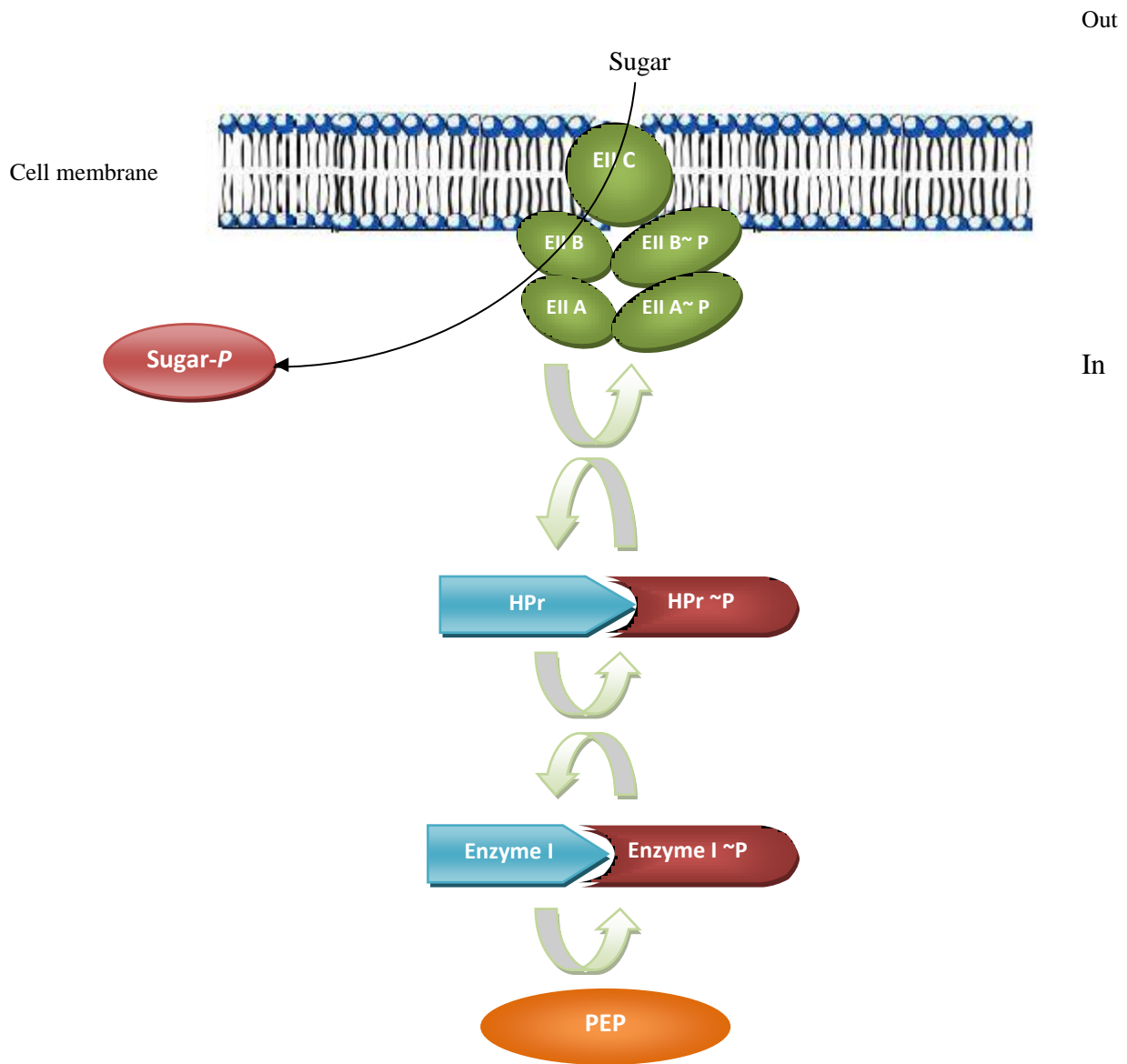


Fig 3: The organization of the phosphotransferase system (PTS) in bacteria, showing transfer of the phosphoryl group from the general Enzyme I and HPr proteins to Enzyme II-specific domains and then to the sugar substrate.

c) Enzyme II [EII]

The [EII] complex consists of different hydrophilic and hydrophobic domains EIIA, EIIB, EIIC and in some cases EIID. Each of these may be included in different polypeptides and distributed functionally on individual proteins, or found together in different combinations within one or more proteins. The complex accepts the phosphoryl group from the donor HPr, and phosphorylates the substrate as it is transported across the membrane into the cytoplasm (Deutscher *et al.*, 2006). The EIIA domain receives the phosphoryl group from the donor P~HPr on a histidyl residue [P~EIIA] and then passes the phosphoryl group to EIIB, on a histidyl residue in the case of the mannose family, or on a cysteyle residue in all other PTS families. The sugar can then be translocated and enters the cytoplasm through the membrane (Deutscher *et al.*, 2006). In addition, phosphotransferases belonging to the mannose family have the additional hydrophobic EIID domain (Saier *et al.*, 2005), which also contributes to the translocation of the substrate. In general, all EII domains (A, B and C) are required to make up a functional PTS, although in some PTS's an EIIA domain is absent and thus the EIIA domain must be provided from another system to complete the PTS chain. For example, in *B.subtilis* the phosphotransferases belonging to the glucose and sucrose families include nine pairs of EIICB domains, but only five EIIA domains are encoded in the genome. This indicates that an EIIA domain can functionally phosphorylate more than one EIIB domain in the glucose and sucrose families (Reizer *et al.*, 1999).

1.8. The PEP-phosphotransferase system in clostridia

It is reported that solventogenic clostridia strains depend on the PTS as a transport mechanism for uptake of many carbohydrates, as shown in Table 1 (Mitchell and Tangney, 2005). The PTS plays a key role in sugar utilization in *C.acetobutylicum* and *C.beijerinckii*, while it appears to be less important in others such as *C.thermocellum* (Mitchell, 1998). *In vitro*, studies have shown that the clostridial PTS is functionally equivalent to those in other bacteria. Therefore, the soluble cell extract of *C.beijerinckii* (formerly *C.acetobutylicum*) NCIMB 8052 has been shown to complement membranes from *E.coli*, *B.subtilis* and *C.pasteurianum*, or vice

versa, for glucose phosphorylation (Mitchell *et al.*, 1991). Also, EI and HPr from *C.beijerinckii* appeared to be of a similar size to these proteins from other bacteria (Mitchell *et al.*, 1991): approximately 63 kDa for enzyme I (Hu and Saier, 2002; Postma *et al.*, 1993) and 10 kDa for HPr (Postma *et al.*, 1993). Genome sequencing has further shown the relationships between the clostridial PTS and the PTS from other bacteria with regards to the Enzyme II domain structures. *C.acetobutylicum*, with genome size of 4.13 Mb, has 30 PTS genes, including 13 complete saccharolytic PT-systems (Barabote and Saier, 2005). In *C.acetobutylicum* ATCC 824, some of the PT- systems have been characterized with respect to their sugar substrates, such as the gene systems for the sucrose PTS and maltose PTS which were identified and named as enzyme II^{Scr} and II^{Mal} respectively (Tangney and Mitchell, 2000; Tangney *et al.*, 2001). In the same strain, PTS activity has also been recorded for cellobiose, fructose, glucose, lactose and mannitol (Hutkins and Kashket, 1986; Tangney and Mitchell, 2007; Yu *et al.*, 2007; Mitchell and Tangney, 2005). Currently, gene expression analysis is proving helpful in characterizing PTS activity during growth on a variety of substrates and providing more understanding of the gene functions relating to the PT- system and its domains (Servinsky *et al.*, 2010).

Table 1: Examples of transport mechanisms in solventogenic clostridia (Mitchell and Tangney, 2005).

Organism	PTS
<i>C.acetobutylicum</i>	glucose, mannitol, cellobiose, fructose, lactose, maltose, sucrose
<i>C.beijerinckii</i>	glucose, fructose, lactose, sucrose, glucitol, mannitol
<i>C.butyricum</i>	fructose
<i>C.thermocellum</i>	fructose, mannitol

1.9. *Clostridium beijerinckii*

This project is focused on *C.beijerinckii* NCIMB 8052 which was formerly known as *C.acetobutylicum* (Keis *et al.*, 2001). The potential industrial importance of *Clostridium beijerinckii* for utilizing several carbohydrate sources, including waste materials, and producing low cost solvents means that attention needs to be given to understanding the biochemistry and genetics of sugar uptake mechanisms, which can lead to improving industrial production of these solvents (Ezeji and Blaschek, 2008). Several studies have found that the phosphoenolpyruvate (PEP)-dependent phosphotransferase system plays a dominant role in the uptake of a variety of carbohydrates in the model strain *Clostridium beijerinckii* NCIMB 8052 (Saier and Reizer, 1992; Tangney *et al.*, 1998b).

The genome size of *Clostridium beijerinckii* NCIMB 8052 is 6.0 Mb, which is considerably larger than that of *Clostridium acetobutylicum* ATCC 824, by approximately 50% (Nolling *et al.*, 2001). The sequencing data of the *Clostridium beijerinckii* NCIMB 8052 genome was completed by the Joint Genome Institute (JGI), DOE, USA and published in 2007 (<http://genome.jgi-psf.org/clobe/clobe.home.html>), and it was noted that 47 sets of genes are involved in the PT-system although only 42 encode a complete PTS (Shi *et al.*, 2010). These include genes encoding members of all seven PTS families, as represented in Figure 4.

PTS activity has been demonstrated in *C. beijerinckii* for several sugar substrates (Tangney and Mitchell, 2005). The glucitol and sucrose systems have been characterised both genetically and biochemically (Tangney *et al.*, 1998a; Tangney *et al.*, 1998b; Reid *et al.*, 1999), but the function of the other system has not been identified. Shi *et al.* (2010) examined the nine systems belonging to the mannose/fructose/sorbose family and concluded that they were all fructose translocating systems. However, their conclusions were based only on sequence analysis of the IIB domains and no functional characterization of the systems was reported. Compared with other solventogenic bacteria, interest in *C. beijerinckii* has increased due to the capability of the strain to produce the highest concentration of butanol. The mutant strain of *C.beijerinckii* NCIMB 8052, known as *C.beijerinckii* BA101,

recorded the highest production of butanol, estimated at approximately 17-21g/L (Annous and Blaschek, 1991; Formanek *et al.*, 1997), and therefore provides several advantages in the butanol industry. For example, it has the capability to utilise several carbon sources and it has been experimentally established that it is suitable for genetic modification in order to increase the solvent production (Formanek *et al.*, 1997).

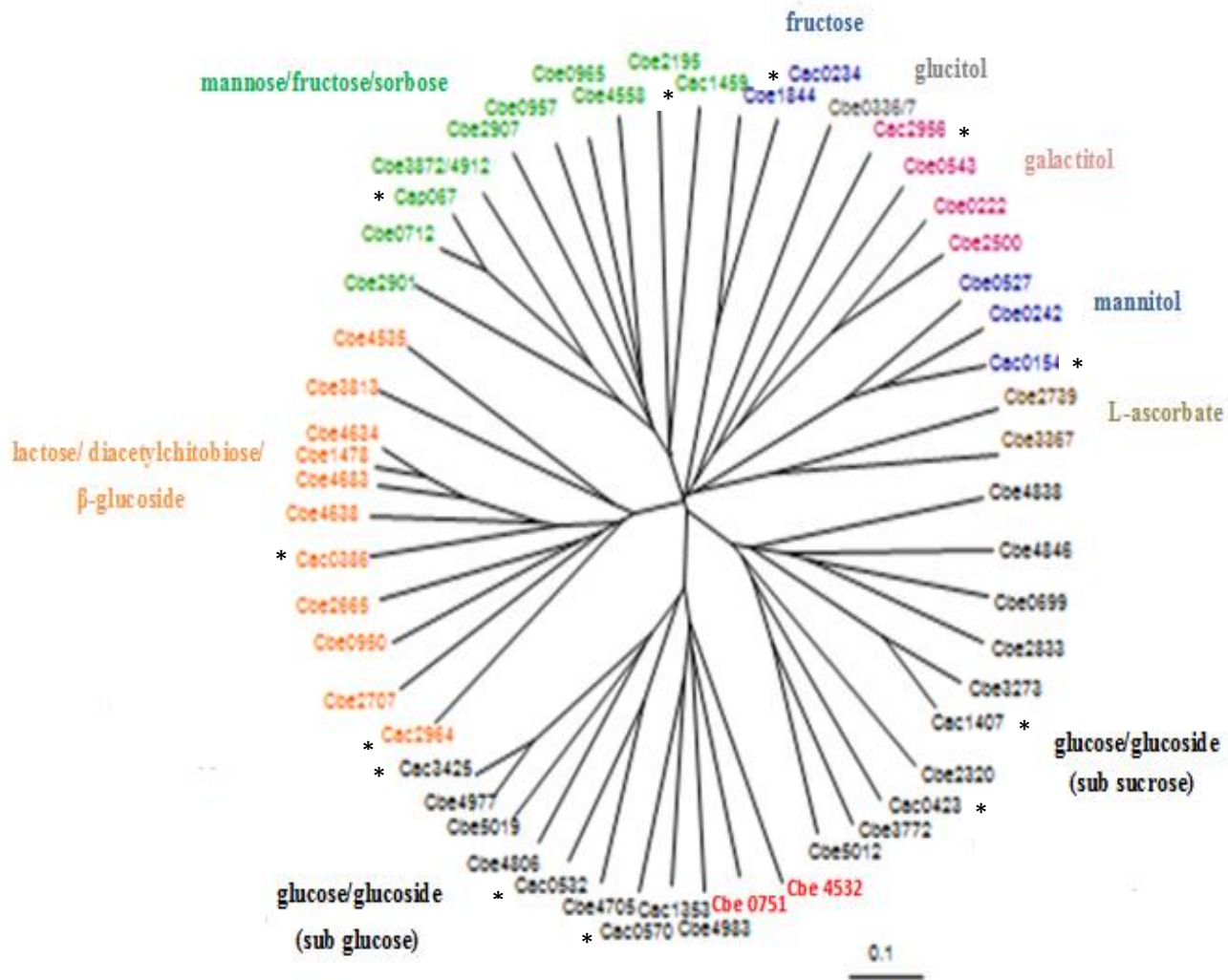


Fig 4: Phylogenetic tree of *C. beijerinckii* PTS IIC domains (Courtesy of W.J. Mitchell). The tree shows the IIC domains of the phosphotransferase of *C. beijerinckii* (Cbe) and *C. acetobutylicum* (*Cac), which are grouped in branches representing the different PTS families.

1.10. Carbon catabolite repression

Carbon catabolite repression (CCR) in Gram positive or Gram negative bacteria is known as a regulatory mechanism controlling carbohydrate uptake and metabolism. In general, bacteria show a sequential utilization of carbohydrates to select the preferred carbon substrate from a mixture of different carbon sources (Bruckner and Titgemeyer, 2002). For instance, in the case of the presence of different carbon sources in the medium, then the transport and metabolic system required for utilizing one of these substrates will often not be synthesised, due to repression by the favourable carbon source (frequently glucose) in the growth medium. This global regulation mechanism is dependent on specific proteins, such as the catabolite repression control (Crc) protein in *Pseudomonas* (Aranda-Olmedo *et al.*, 2005; Hester *et al.*, 2000) or the catabolite control protein (CcpA) in low-GC Gram-positive bacteria (Deutscher *et al.*, 2006).

In firmicutes, the principal global mechanism of CCR by which glucose represses the metabolism of other carbon sources is dependent on the phosphocarrier HPr protein which is considered as an important regulator of carbon catabolite mechanism in gram positive bacteria. The phosphorylation of the HPr protein does not only occur from PEP at His-15 during PTS activity, it can be also phosphorylated by an ATP-dependent protein kinase on a seryl residue (ser-46). When phosphorylated at ser 46, the HPr protein binds to the catabolite control protein CcpA which is a transcriptional regulator that is considered as a co-repressor in carbon catabolite repression (Stülke and Hillen, 2000). The CcpA/HPr (ser-P) complex then binds to specific sites known as catabolite responsive elements (*cre*'s) to control gene expression. Phosphorylation of the serine residue on HPr also inhibits phosphorylation at His-15 therefore controlling PTS activity, in conditions of high energy metabolism in firmicutes (Galiniér *et al.*, 1998, Reizer *et al.*, 1998, Kravanja *et al.*, 1999, Mijakovic *et al.*, 2002). In specific cases, HPr phosphorylated on His-15 can also play a role in CCR, for example, in regulation of the glycerol kinase enzyme in enterococci and other firmicutes (Charrier *et al.*, 1997).

These mechanisms of CCR have been studied in detail in some of the low-GC Gram-positive bacteria such as bacilli, staphylococci, and lactobacilli. For clostridia, it is commonly found that they can utilize different carbon sources in the growth media, and that the preferred carbon source (glucose) is utilized first. Although different systems show different characteristics with respect to regulation, genes concerned with utilization of mannitol, sucrose, maltose and lactose in *C. acetobutylicum* are associated with *cre* sequences, implying that they are regulated by a PTS-dependent mechanism (Behrens *et al.*, 2001; Tangney and Mitchell, 2005; Tangney *et al.*, 2001; Yu *et al.*, 2007). Also, HPr kinase activity has been shown in *C. acetobutylicum* as a sensor enzyme for catabolite repression, and the catabolite control protein (CcpA) was also identified in the same bacterium as a carbon regulation protein (Tangney *et al.*, 2003). Recently, it was observed that CcpA plays an important role in *C. acetobutylicum* as a repressor of xylose and arabinose metabolism (Ren *et al.*, 2012). It is clear that carbon catabolite repression plays an important role in clostridial cells during sugar utilization, and this regulation mechanism seems to be related to the PTS as is the case in other bacteria (Deutscher *et al.*, 2006; Tangney and Mitchell, 2005). It has been shown that *C. beijerinckii* displays a preference for glucose over other sugars (Tangney *et al.*, 1998a; Mitchell, 1996; Reid *et al.*, 1999). However, although it may be assumed that the mechanisms are similar, there have been no detailed studies of catabolite repression in this strain.

The PTS also plays an important role in the regulation of carbohydrate uptake and metabolism through the PTS regulation domains (PRDs), which are found as components of transcriptional regulators and antiterminators. Antiterminators have been found in both Gram positive and Gram negative bacteria, although DNA-binding transcriptional regulators appear to be present only in Gram-positives (Stulke *et al.*, 1998). Antiterminators bind to mRNA at a sequence known as a ribonucleic antiterminator (RAT), which overlaps with a transcriptional terminator upstream of the regulated gene(s). Binding to the RAT stabilizes its secondary structure, and prevents the terminator from forming, therefore allowing expression of the downstream genes. Antiterminators are involved in regulation of expression of genes encoding phosphotransferase systems, and their activity is controlled by

PTS-dependent phosphorylation. One PRD is phosphorylated by the EIIB domain of the associated PTS, and this inhibits the antiterminator activity. The second PRD is phosphorylated by HPr (His-P), and this activates the antiterminator (Figure 5). In the absence of substrate of the PTS, the inhibitory phosphorylation inactivates the antiterminator protein, the transcriptional terminator forms and gene expression is prevented (Langbein *et al.*, 1999). In the presence of the substrate, the phosphoryl group will transfer from EIIB to the substrate rather than the antiterminator PRD domain, resulting in activation of the antiterminator and expression of the controlled genes (Tortosa *et al.*, 2001). Induction therefore occurs in response to the substrate of the system. The second (activating) phosphorylation of the antiterminator can be prevented in the presence of a good carbon source such as glucose, since phosphate from HPr (His-P) will be used to support its uptake. The result will be repression of the expression of genes involved in uptake of the alternative sugar.

In Gram negative bacteria this mechanism was first reported in *E.coli* by demonstrating the PTS-dependent uptake of β -glucosides controlled by the *bgl* operon under the control of the antiterminator BglG (Schnetz *et al.*, 1987). Related antiterminator proteins such as SacT and LicT in *B.subtilis* have been found in Gram positive bacteria and they are collectively referred to as the BglG family (Stülke and Hillen, 2000). An antiterminator AbgG was also studied in *Clostridium longisporum* and shown to be involved in regulation of the *abgA* and *abgF* genes encoding a β -glucoside PTS and a hydrolase gene (Brown and Thomson, 1998). In *C.acetobutylicum* the proteins ScrT and GlcT belong to the antiterminator family and are associated with operons encoding the sucrose and glucose PTS respectively (Tangney and Mitchell, 2000; Mitchell and Tangney, 2005; Tangney and Mitchell, 2007).

Antiterminator

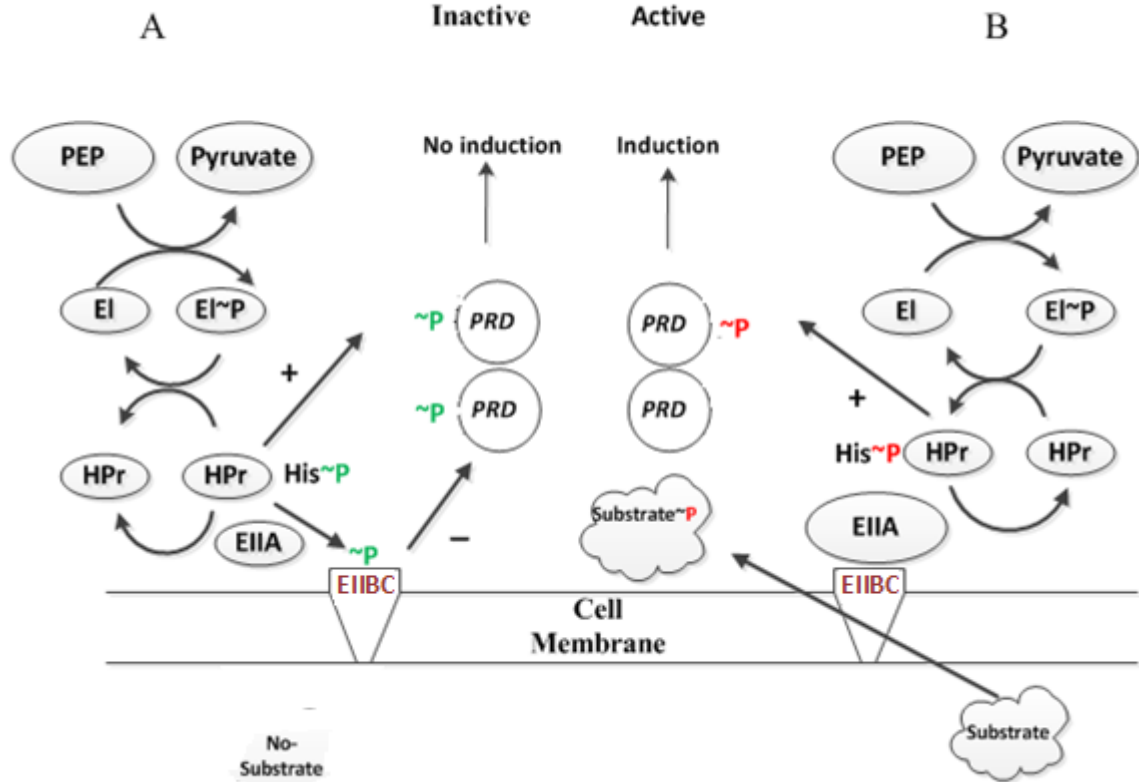


Fig 5: The mechanism of induction of genes dependent on an antiterminator containing two PRD domains (adapted from Mitchell and Tangney, 2005).

- A. In the absence of substrate of the associated PTS, the antiterminator is phosphorylated on both domains and is inactive. The regulated genes are therefore not induced.
- B. In the presence of the PTS substrate, phosphate from EIIB passes to the substrate, not to the antiterminator. The antiterminator is then active, and the regulated genes encoding the EIIA, EIIB and EIIC domains of the PTS are induced.

1.11. Waste as a sustainable resource

Nowadays, the hazard of waste is one of the huge problems facing natural life. Waste has been defined as any materials or substrates which lead to changes in the ecosystem. Generally, wastes are classified into many types, depending on the natural source or industries which generate them. This classification includes; municipal, hazardous, industrial, medical, universal, construction and demolition, radioactive, mining, and agricultural wastes. The hazards associated with wastes include human diseases, air pollution and groundwater contamination (Pichtel, 2005). Moving wastes elsewhere is not the final solution to avoid the risks. Thus, biotechnological applications have attracted considerable attention as a potentially effective solution for waste disposal while also providing economical energy sources and environmentally acceptable products (van Wyk, 2001). Agricultural waste is a cheap feedstock containing a high content of organic compounds which can be used anaerobically as fermentable substrates to produce biofuel (Blaschek *et al.*, 2010). Several clostridial strains show a high capability to utilize many kinds of agricultural residues to produce ethanol, acetone and butanol, as shown in Table 2. Genetic characterization and modification are required to understand and improve the production potential of clostridial strains, especially those strains which are amenable to modification, such as *Clostridium beijerinckii* (Milne *et al.*, 2011).

Table 2: Examples of agriculture wastes used as fermentable materials by some clostridia species.

Substrate	Strain	Reference
Maize	<i>Clostridium beijerinckii</i>	Mu <i>et al.</i> , , (2011)
Corn Stover	<i>Clostridium phytofermentans</i>	Jin <i>et al.</i> , , (2012)
Oil Palm	<i>Clostridium butyricum</i>	Ibrahim <i>et al.</i> , , (2012)
Wheat	<i>Clostridium beijerinckii</i>	Liu <i>et al.</i> , , (2010)
Cassava	<i>Clostridium saccharoperbutylacetonicum</i>	Thang <i>et al.</i> , , (2010)
Grain plant	<i>Clostridium acetobutylicum</i>	Ni and Sun, (2009)

1.12. Chitin as a cheap substrate

One of the most critical issues in the ABE fermentation industry is identification of a low cost substrate. A number of studies have investigated the use of various raw materials in the production of butanol, for instance, molasses, whey permeate, and corn (Ezeji and Blaschek, 2008; Ezeji *et al.*, 2007; Jones and Woods, 1986; Qureshi *et al.*, 2008). Recently, alternative polysaccharides have been evaluated as potential substrates for ABE fermentation; these include cellulose, xylan, starch and pectin, which are natural energy sources. Clostridia can utilize these materials by means of extracellular enzymes or enzyme complexes to degrade them to simple molecules that can be easily absorbed by the cell (Mitchell, 1998). The present study focuses on utilization of chitin as a cheap and abundant substrate in nature, with emphasis on utilization of the degradation product *N*-acetylglucosamine. Chitin (β -1, 4 linked *N*-acetylglucosamine) is the most widespread polymer on earth, after cellulose, and is found as a major component in insects, crustaceans, plants, and fungi (Chomphunuch *et al.*, 2010). Many industries, such as aquatic food and agriculture, lead to production of a huge amount of chitin waste which is estimated at 100 billion tons annually including crustaceans, insects and fungi (Tharanathan and Kittur, 2003). In 2005 the annual worldwide production of marine food (shrimp and prawns) was estimated at 6, 091, 896 tons and 40-50% of the total was discarded as waste. The total amount of chitin in this waste was around 40% in addition to other compounds such as calcium carbonate and lipid residues (Xu *et al.*, 2008). This is often considered to be a real burden on the environment because the chitin polymer is very difficult to degrade (Hayes *et al.*, 2008a, b). Several organisms can produce chitinase enzymes, including viruses (Hawtin *et al.*, 1995), bacteria (Chigaleïchik *et al.*, 1976) and fungi (Kuranda and Robbins, 1991). Chitinase enzymes (endo or exo chitinase) have been classified into two glycosyl hydrolase families (GH18 and GH19) according to amino acid sequence homology (Li and Greene, 2010). The importance of chitinase lies in its ability to biodegrade chitin into *N*-acetylglucosamine, which can be used as a carbon source by many bacteria (Dahiya *et al.*, 2006), including solventogenic bacteria.

1.13. *N*-acetylglucosamine uptake and metabolism

As mentioned before, *N*-acetylglucosamine is the monomer of chitin (Figure 6), and is therefore widespread as a component of bacterial cell walls, fungi and algae, and is also found in plants and animals. *N*-acetylglucosamine can be utilized as a good carbon source for growth of several bacteria (Cottrell and Kirchman, 2000, Bhatnagar and Sillanpaa, 2009).

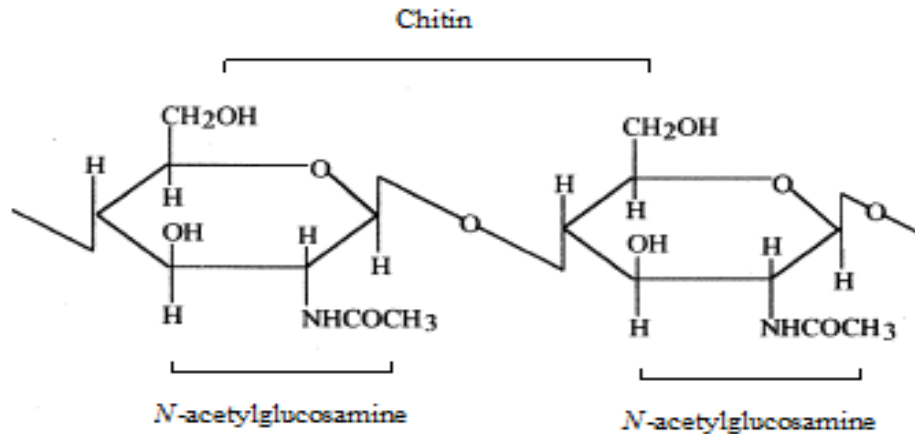


Fig 6: The chemical structure of chitin containing the *N*-acetylglucosamine monomer (Bhatnagar and Sillanpaa, 2009)

The mechanism of *N*-acetylglucosamine phosphorylation in *E.coli* was shown to be dependent on the PTS (Curtis and Epstein, 1975; White, 1970). The *nag* operon in *E.coli* (Figure 7) has been identified and includes the *nagE* gene encoding the PTS, and the *nag A*, *B*, *C* and *D* genes (Peri *et al.*, 1990, Plumbridge, 1989).

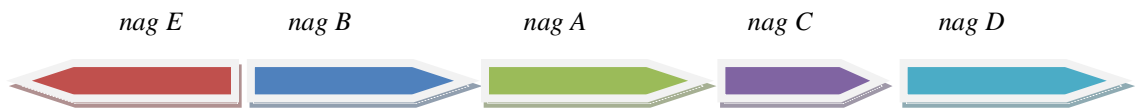


Fig 7: Organization of the *nag* operon in *E.coli* (Alvarez-Añorve *et al.*, 2005)

The product of each one of these genes has a specific function during *N*-acetylglucosamine utilization (Figure 8). The first step is uptake of NAG by the product of the *nagE* gene, a specific PTS-enzyme II containing B, C and A domains. The product of NAG uptake and phosphorylation, *N*-acetylglucosamine 6-phosphate, is then converted to glucosamine 6-phosphate by NagA and then

deaminated to fructose 6-phosphate by NagB. Fructose 6-P can then be metabolised by glycolysis (Jones-Mortimer and Kornberg, 1980; Lengeler, 1980). The *nagC* gene encodes a repressor (Plumbridge, 1996), but the function of the *nagD* gene has not yet been defined (Sohanpal *et al.*, 2004).

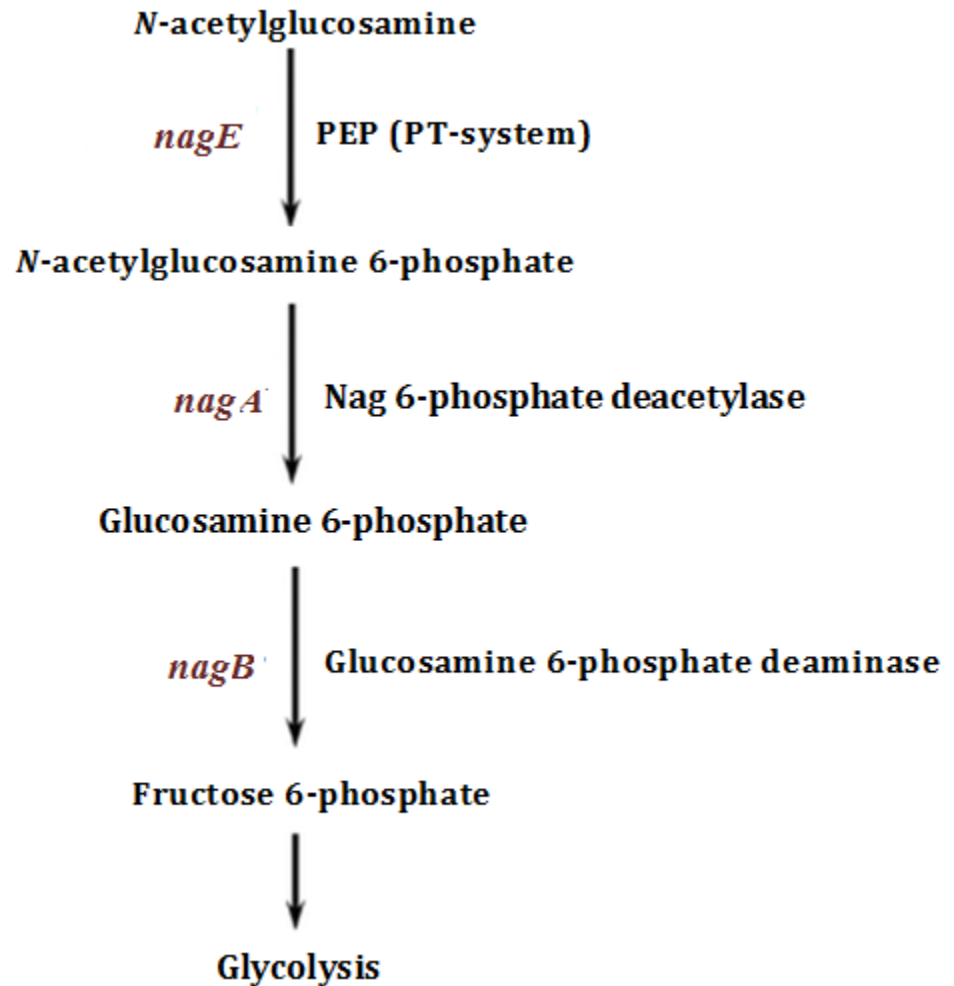


Fig 8: Metabolic route of *N*-acetylglucosamine by *E. coli* initiated by the PT-system (Plumbridge, 2001), after glycolysis step the route of *N*-acetylglucosamine metabolism involved the EMP-pathway as shown previously in Figure 2

A few studies have reported *N*-acetylglucosamine uptake by a phosphotransferase system in other bacteria including *Bacillus subtilis* (Bertram *et al.*, 2011; Mobley *et al.*, 1982), *Staphylococcus aureus* (Imada *et al.*, 1977), *Vibrio furnissii* (Bassler *et al.*, 1991), *Streptomyces olivaceoviridis* (Wang *et al.*, 2002) and *Caulobacter crescentus* (Eisenbeis *et al.*, 2008). *N*-acetylglucosamine metabolism was also studied by Alice *et al.*, (2003) in *Bacillus sphaericus*. This bacterium cannot use sugars as carbon sources for growth. However, an operon was identified which contained the genes encoding Enzyme I and HPr of the PTS along with enzymes involved in *N*-acetylglucosamine metabolism (Figure 9). Inactivating of the *ptsH* gene resulted in loss of the ability to grow on Nag, suggesting that uptake of this substrate was PTS-dependant, but the gene(s) encoding sugar-specific components of a Nag-PTS were not identified.

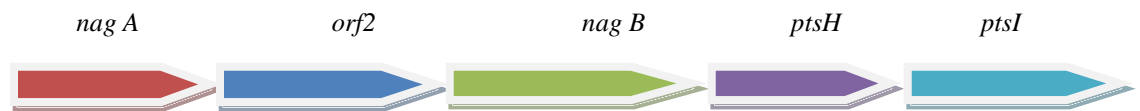


Fig 9: *nag* genes encoding Nag enzymes in *Bacillus sphaericus* (Alice *et al.*, 2003). The operon encodes the PTS proteins Enzyme I and HPr, and the enzyme NAG-6-phosphate deacetylase and glucosamine-6- phosphate deaminase. The function of *orf2* is unknown.

1.14. Aims of the Study

As mentioned before in 1.12, chitin-containing wastes could serve as cheap substrates for ABE fermentation. Effective exploitation of these materials in the fermentation will depend on an understanding of chitin hydrolysis and utilization of *N*-acetylglucosamine by the solventogenic clostridia. This study focuses on the *Clostridium beijerinckii* NCIMB 8052 strain and aims to investigate the ability of this bacterium to hydrolyse chitin and utilize *N*-acetylglucosamine and to understand the molecular basis of this ability. The study focused particularly on the physiology and genetics of the metabolism of the amino-sugar *N*-acetylglucosamine, by demonstrating the activity of a phosphotransferase system, identifying a putative gene system which is potentially involved with this activity and cloning the genes in order to verify their function.

CHAPTER2

MATERIALS AND METHODS

2. Materials and Methods

2.1 Bacterial strains

Clostridium beijerinckii NCIMB 8052 was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB), and *Clostridium acetobutylicum* ATCC 824 came from Dr. Philippe Soucaille in Toulouse. Spore suspension of both strains was stored at 4°C in sterile distilled water. The competent *Escherichia coli* cells (One Shot® TOP10) were obtained from Invitrogen, and the cells were stored at -70°C and used as the host strain for cloning experiments. The *E. coli* mutants used in this project for *N*-acetylglucosamine gene characterization were *E. coli* BW25113 (*nagE* mutant) from the Keio knockout collection (Baba *et al.*, 2006) and *E. coli* ZSC113 genotype (*gpt-2 mpt-2glk-7strA*) (Curtis and Epstein, 1975).

2.2 Buffers and solutions

The composition and description of buffers and solutions used in this project are either described within the specific sections or listed in Appendix 2.

2.3 Growth media and chemicals

Reinforced Clostridial Medium (RCM) was obtained from Oxoid. The synthetic Reinforced Clostridial Medium (sRCM) had the same composition as RCM, except that it did not contain soluble starch, glucose and agar, compared with the RCM medium. Clostridial Basal Medium (CBM) contained (per litre): carbon source 10 g; casein hydrolysate 4 g; MgSO₄·7H₂O 0.2 g; MnSO₄·4H₂O 10 mg; FeSO₄·7H₂O 10 mg; p-aminobenzoic acid 1 mg; thiamine HCl 1 mg; d-biotin 2 µg; KH₂PO₄ 0.5 g and K₂HPO₄ 0.5 g. The phosphates were sterilized separately as a ×10 concentrate and then added to the medium after autoclaving and cooling down. In the case of *N*-acetylglucosamine as the carbon source, it was made up as ×10 concentrate and sterilized by filtration and then added to sterile media immediately before inoculation.

E.coli strains were grown in Luria Broth (LB broth) medium which contained (per litre): tryptone 10 g; yeast extract 5 g and sodium chloride 5 g (Bertani, *et al.*, 1951). The fermentation phenotype was examined using Difco MacConkey agar base, which was sugar free and contained (per liter): peptone 17 g; proteose peptone 3 g; bile salts 1.5 g; sodium chloride 5 g; neutral red 0.03 g; crystal violet 0.001 g and agar 13.5 g. *N*-acetylglucosamine was added into the medium as required after filter sterilization, but glucose or mannose were added before autoclaving. The final pH was about 7.1. Where required, ampicillin or kanamycin was added to the media at a concentration of 50 µg/ml.

2.4 Preparation of clostridial starter cultures

A volume of 0.5-1 ml of spore suspension was placed in a heating block at 80°C for 10 min and then cooled and inoculated into a starter culture of 20 ml of RCM. The cells were incubated in strictly anaerobic conditions for 24 h at 37°C, under an atmosphere of N₂-H₂-CO₂ (80:10:10) in an anaerobic cabinet (Don Whitley Macs-MG-500 Anaerobic Station) (Yu *et al.*, 2007). After 24 h, samples were streaked onto a RCM plate and incubated in an aerobic atmosphere for 24 h. Gram stains were also carried out in order to confirm that the cultures were not contaminated.

2.5 Preparation of *Clostridium beijerinckii* NCIMB 8052 spores

C.beijerinckii NCIMB 8052 spores were grown in an overnight starter culture as described in section 2.4, and then inoculated into 500 ml of synthetic Reinforced Clostridial Medium (sRCM). The culture was incubated under anaerobic conditions at 37°C for approximately four weeks. The culture was transferred into two 250 ml centrifuge bottles under sterile conditions and then centrifuged at 12,000 g/15 min/4°C. The supernatants were removed, and the pellets were resuspended in 200 ml sterile distilled water and washed three times by centrifugation. Finally, the combined spore pellet was resuspended in approximately 100 ml of sterile distilled water and transferred into sterile bottles and stored at 4°C.

2.6. Preparation of colloidal chitin

Colloidal chitin was prepared according to Lingappa and Lockwood (1961) by taking 25 ml of concentrated hydrochloric acid and adding it to a conical flask containing 6.25 g of chitin and incubating at room temperature (23-25°C) for 1-2 hours. The mixture was transferred carefully in a chemical fume hood into a conical flask containing 500 ml distilled water at 4°C, and incubated overnight at room temperature (23-25°C). After that, the supernatant was removed and replaced by 50 mM potassium phosphate buffer (pH7) and then the suspension was centrifuged at 12000 g for 20 min in order to remove as much as possible of the hydrochloric acid. Resuspension and washing of the chitin was repeated three times. In the final step, the supernatant was removed and the colloidal chitin was washed again with distilled water, three times, under the same conditions. The resultant colloidal chitin obtained was filtered through 110 mm Whatman filter paper and then transferred into a sterile bottle containing 250 ml of 50 mM potassium phosphate buffer per litre (pH7) and stored at 4°C.

2.6.1. Chitinase assay

Initially chitinase activity was detected by streaking bacteria on CBM plates containing 2% (v/v) colloidal chitin and incubating at 37°C in the anaerobic cabinet for 48 h. The bacteria were also streaked on plates containing CM-chitin RBV solution (carboxy-methyl chitin Remazol brilliant violet) obtained from Loewe Biochemicals. The plates contained a 1:1 ratio of CM-chitin RBV and synthetic RCM and were incubated at 37°C in the anaerobic cabinet for 24h. Also, in other plates the chitinase enzyme activity was estimated by using a 1:1:1 ratio of CM-chitin RBV, 1% agarose in distilled water and 50 mM potassium phosphate buffer pH7. Cell culture supernatants were concentrated by placing them in dialysis membranes surrounded by polyethylene glycol PEG (Sigma), and incubating at 4°C. 100 µl of the 10 × concentrated culture supernatant (100 ml of supernatant concentrated to 10 ml) obtained from separate 100 ml CBM cultures containing 10 mM NAG, 10 mM glucose and 2% chitin, were placed in small wells on the plate, which was incubated aerobically at 37°C for 24 h. The chitinase enzyme which

used as a control was purified from *Streptomyces griseus* and obtained from Sigma (C6137-5UN).

In the same concentrated supernatant, chitinase activity was also estimated using silica gel thin-layer chromatography (TLC) (Tanaka *et al.*, 1999). A reaction mixture was prepared in an Eppendorf tube containing 100 µl of colloidal chitin and 100 µl of concentrated culture supernatant and was incubated with shaking at 37°C for 24 h. In this assay, the control contained 100 µl distilled water rather than crude extract. 1 µl of the reaction mixture was applied to a silica gel plate approximately 5×20 cm and then the plate was transferred to the TLC tank containing n-butanol-methanol, 25% ammonia solution and distilled water (5:4:2:1[vol/vol/vol/vol]), respectively. Sugars were detected by heating the plate using a dry heater at approximately 180°C for 3-5 min, until the bands appeared.

2.7. Growth and utilization of *N*-acetylglucosamine and glucose by *C.beijerinckii*

Utilization of *N*-acetylglucosamine and glucose by *C. beijerinckii* NCIMB 8052 was determined by growing cells on two types of growth media, sRCM and CBM. 1 ml of a starter culture (section 2.4) was inoculated into 100 ml of sRCM or CBM containing 10 g/L of *N*-acetylglucosamine or glucose, as an intermediate culture. The cells were then taken from the intermediate culture and inoculated into the experimental culture, the amount of cells depending on growth density (OD₆₀₀) in the medium. The experimental culture contained *N*-acetylglucosamine or glucose or both, depending on the experimental conditions. Growth was measured as optical density at 600 nm; when OD was ≥ 0.5, then the sample was diluted 1:1 (v/v) with 10% formalin before the measurement was taken.

The concentration of *N*-acetylglucosamine and glucose in the growth media was measured by taking 1 ml from the medium and centrifuging in a microfuge at 13,000 rpm for 10 min. The supernatant was then removed to another tube and stored at -20 °C until analysed. The technique used for analyzing the sample was high-performance liquid chromatography [HPLC] (Tanaka *et al.*, 2001); the columns used were a Dionex Carbopac PA-1 Guard column 4×80 mm, and Dionex

Carbopac PA-1column 4×250 mm. The instruments used were; Dionex PED (Pulsed Electrochemical Detector) with gold electrode, Dionex DX300 APG (Advanced Gradient Pump), Gilson 234 Autoinjector with 20µl loop, Dionex Eluent Degas module and Hewlett Packard Chemstation data handling (HP3365).

2.8. Preparation of cell-free extracts

From an overnight starter culture, cell-free extracts of *C.beijerinckii* were prepared from a 500 ml CBM culture containing 10 g/L *N*-acetylglucosamine or glucose. Cell samples were collected and harvested in the late exponential phase and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was removed and the cells were washed twice and resuspended in 50 mM potassium phosphate buffer (pH 7). The final cell pellet was resuspended in buffer containing 1 mM DTT and 5 mM MgCl₂ at a concentration of 4 ml/g. Preparation of the extract was carried out by two passages through a French pressure cell at 20,000 lbf in⁻² (138MPa). The extract was then transferred into Eppendorf tubes and centrifuged at 12,000 g for 15 min at 4°C to remove the cell debris. The supernatants, which were the crude cell-free extract were collected from each Eppendorf tube and combined in another tube, flash-frozen in liquid nitrogen, and stored at -70°C (Tangney and Mitchell, 2007). The same procedure was followed in order to prepare extracts of *E.coli* strains grown in LB medium containing *N*-acetylglucosamine.

The crude extract was further fractionated into soluble extract and the membrane extract by ultracentrifugation (BECKMAN TL-100) for two hours at 65,000 rpm (230,000 g) and 4°C. After the first centrifugation the supernatant was removed to another tube, while the pellet was washed with 50 mM potassium phosphate buffer (pH7) containing 5 mM of MgCl₂ and 1 mM DTT. The two fractions were centrifuged for another two hours under the same conditions as before. The supernatant, which formed the cytoplasm fraction, was placed into another tube and aliquoted and flash-frozen in liquid nitrogen, while the pellet membrane fraction was resuspended in 1/10 volume of the original crude extract and then flash-frozen in liquid nitrogen. All the samples were stored at -70°C.

2.9. Protein estimation in cell extracts

The protein concentration of extracts was determined colorimetrically ($OD_{310-390}$) using a microbiuret method, as described by Zamenhof (1957). Aliquots of 20 μ l of sample were mixed with 0.24 ml of 40% sodium hydroxide [NaOH]. The reaction mixture was incubated in a water bath for 15min. After incubation, 0.68 ml of distilled water was added and then vortexed for approximately 30sec. 63 μ l of 1% copper sulphate [$CuSO_4$] was added and mixed by vortexing. The absorbance was read at 310 and 390 nm against a water blank. For measuring the final concentration of protein in the sample, the difference of $OD_{310} - OD_{390}$ was used and related to a protein standard curve (Bovine serum albumin BSA), which was prepared each time the protein assay was carried out.

2.10. Assay of sugar phosphorylation by cell-free extracts

Sugar phosphorylation activity was assayed, as described by Mitchell *et al.*, (1991). The standard mixture in 1 ml volume contained 714 μ l of 50 mM potassium phosphate buffer (pH 7.0), 10 μ l of 0.2 M DTT, 5 μ l of 1M $MgCl_2$, 30 μ l of 0.4 M potassium fluoride [KF], 20 μ l of 50 mM phosphoenolpyruvate (PEP) or adenosine triphosphate (ATP) and 200 μ l of crude cell extract. The control consisted of the same reaction mixture without PEP or ATP. The mixture was incubated for 3-4 min in a water bath at 37°C, then 21 μ l of ^{14}C -NAG or ^{14}C -glucose (9.5mM, 1mCi/mole) was added to the reaction mixture. Samples (150 μ l) were taken at time intervals from the mixture, and added to 2 ml of 1% (w/v) $BaBr_2$ in 80% (v/v) ethanol solution, to precipitate the sugar phosphate. The precipitate was collected by filtration through a glass fibre disc (Whatman GF/F paper) and after filtration, the filter was washed with 5 ml of 80% ethanol and dried for 15 min under a heat lamp. After that, the discs were transferred into leak-proof scintillation tubes and 4 ml of scintillation fluid was added. The phosphorylated sugar was measured as radioactivity, by liquid scintillation counting in a 1900 CA Tri-Carb analyser. Sugar solutions used as inhibitors for some assays consisted of 0.1 M of non-radiolabelled *N*-acetylglucosamine, chitobiose or glucose, 100 μ l of which was added immediately before the labeled sugar was added to the reaction mixture. In some experiments, in

order to use both cell membrane and soluble extract, 20 µl of membrane fraction and 200 µl of cytoplasmic fraction were used in the reaction mixture, instead of crude cell extract. When these additions were made, the volume of 50 mM potassium phosphate buffer (pH 7.0) was reduced, so that the total volume remained at 1 ml. The blank of each experiment was prepared by taking 10.5 µl of the ¹⁴C-NAG or ¹⁴C-glucose and adding to the 490 µl of 50 mM phosphate buffer pH 7. An amount of 150 µl was taken and filtered as a normal sample, while 20 µl of the mixture was added directly on the filter paper. The result obtained from the scintillation counter for each sample was corrected by deducting the average of the filtered blanks and then converted to n moles using the equation:

$$\left(\frac{cpm-Blank}{cpm\ in\ the\ 20\mu l\ sample} \times 4 \right),$$

since the 20 µl contained 4 nmoles of labelled sugar. Where appropriate, the amount was converted to nmoles/mg protein by estimating the amount of protein in the extract, and therefore in each sample.

2.11. Bioinformatics analysis

Gene and protein sequence comparisons involving the *N*-acetylglucosamine PTS domains in *C.beijerinckii* (*Cbei4532*, *Cbei 4533*) and the putative antiterminator (*Cbei 4534*) were carried out using BLAST available at the National Center of Biotechnology Information (NCBI) (<http://blast.ncbi.nlm.nih.gov/Blast>).

2.12. Cloning of putative *N*-acetylglucosamine PTS genes.

2.12.1. Primers

Primers were designed (Table 2) for amplification of different genes encoding the components of the putative *C.beijerinckii* NAG-PTS (*Cbei4532*, *Cbei4533*) and associated regulatory gene (*Cbei4534*). The length of the primers was designed to be approximately 20 nucleotides. The primers were purchased from MWG Eurofins, and were dissolved in water at a concentration of 100 pmoles/µl and stored at -20°C.

Table 2: Primers used for cloning of *Cbei4532*, *Cbei4533*, and *Cbei4534*. Where indicated by underlining, primers included a restriction site for *Sall* or *XbaI*. T7-promoter, M13 Fwd and M13 rev primers were used for analysis of the gene orientation.

Name	5'-3' Sequence	GC (%)	Purpose and restriction sites	Lab reference number
<i>Cb4532</i> -Fwd	CATTTAGGGATATAACAATC	30.0%	Cloning	429
<i>Cb4532</i> -Rev	ACAGTCGACATATCATCAATCTCTTTTCC	37.9%	Cloning	431
<i>Cb4533</i> -Fwd	ACAGTCGACAAGGAAGTGACTGTTCCTG	50.0%	Cloning	428
<i>Cb4533</i> -Rev	ACATCTAGACTAGCAATGCATATAGAGAG	37.0%	Cloning	430
<i>Cb4534</i> -Fwd	TCAGATGATAAGTGATTTGC	35.0%	Cloning	516
T7-promoter	TAATACGACTCACTATAGGG	40.0%	Gene Orientation	371
M13-Fwd	GTAAAACGACGGCCAGTG	55.6%	Gene Orientation	373
M13-Rev	CAGGAAACAGCTATGACC	55.6%	Gene Orientation	374

2.12.2. PCR reaction

The whole gene system *Cbei4532*, *Cbei4533* and *Cbei4534* was amplified and cloned by using the StrataClone PCR cloning kit (Agilent), and in the PCR reaction the Easy-A High-Fidelity Strata gene (Agilent) was used. The genes *Cbei4532* and *Cbei4533* were also amplified and cloned separately, and the PCR reaction was carried out using *Pfu* DNA polymerase (Fermentas EP0571). 500 µl of the reaction buffer [5× buffer] was prepared by using: 250 µl [10× Easy-A reaction buffer or 10× *Pfu* buffer with MgSO₄]; 20 µl of 100 mM dNTP mix [dATP, dGTP, dTTP and dCTP, 5µl of each] and 230 µl of sterilized deionised water. The PCR reaction mixture (50 µl) contained: 36.5 µl of sterilized deionised water; 10 µl of *Pfu* 5× buffer; 1 µl of forward primer; 1 µl of reverse primer and 1 µl of *C.beijerinckii* NCIMB 8052 DNA template. The PCR mixture was heated at 95°C in a thermal cycler for 4min and then the reaction was started by adding 0.5 µl of the DNA polymerase enzyme. The program used in the PCR reaction consisted of 30 reaction cycles of 95°C for 1 min; annealing temperature for 1 min; 72°C for 3 min, and then a final extension at 72°C for 10 min. The melting temperature of the primers was calculated according to the primer sequences, using the formula $[4 \times (G+C) +$

2× (A+T)] and then the annealing temperature was calculated [T_m -5]. After the reaction was completed the PCR product was stored at 4°C.

2.12.3. Detection of the PCR product

The quality of the PCR product was examined by agarose gel electrophoresis. Bioline Agarose gel powder 1% (w/v), was dissolved in 1×Tris-acetate EDTA buffer [TAE buffer] by heating, and 1 µl of 10 mg/ml ethidium bromide was added to the 1% agarose (60-100 ml). 5 µl of sample was mixed with 2 µl of Fermentas 6× DNA loading buffer, containing: 10 mM Tris-HCl pH 7.6; 0.03% bromophenol blue; 0.03% cyanol FF; 60% glycerol and 60 mM EDTA, and loaded into the gel. 5 µl of hyperladder I (Bioline) was added alongside the samples. The gel was run by electrophoresis at 80 V for 1-2h under 1×TAE buffer and then the bands were detected under UV light.

2.12.4. Cloning of the *Cbei 4532* and *Cbei 4533* genes

The Fermentas Life Science JETTM PCR cloning kit K1232 was used to clone the *Cbei 4532* and *Cbei 4533* genes. The kit provided a cloning vector pJET1.2/blunt, designed to accept blunt-ended PCR products from 6 bp to 10 kb (Appendix 1). The pJET1.2 cloning protocol was carried out using 10µl 2× reaction buffer; 2 µl PCR product; 1 µl pJET1.2/blunt cloning vector (50 ng/µl) and 1 µl T4 DNA ligase, and then the total volume was made up to 20 µl by adding 6 µl water, nuclease-free, in a micro tube. The mixture was vortexed and centrifuged for 3-5 sec and then the ligation mixture was incubated at room temperature for 5 min. 2.5 µl of the mixture was transferred into a tube containing 40 µl *E.coli* TOP 10 competent cells (Invitrogen). After that the mixture was incubated on ice for 15 min. The tube was then transferred into a water bath at 42°C for 30 sec. After incubation, the tube was incubated again on ice for 2 min. 250 µl of SOC medium containing; 2% (w/v) tryptone; 0.5% (w/v) yeast extract; 8.6 mM NaCl; 2.5 mM KCl; 20 mM MgSO₄ and 20 mM glucose was added to the tube and then the mixture was incubated on a shaker (100 rpm) at 37°C for 1h. Samples of 20 µl to 50 µl were taken from the

mixture and spread onto LB agar medium containing 50 µg/ml ampicillin, and then incubated at 37°C overnight.

2.13. Cloning of the putative *nag* operon containing *Cbei 4532*, *Cbei 4533* and *Cbei 4534* genes

The StrataClone PCR cloning kit (Agilent) vector (Appendix 1) was used with the purpose of cloning all three genes *Cbei 4532*, *Cbei 4533* and *Cbei 4534*. The PCR reaction and detection was carried out as described in (2.12.2), (2.12.3) respectively, while the High Fidelity Easy-A was used in order to amplify the genes. The cloning protocol was carried out by adding into an Eppendorf tube 3 µl StrataClone cloning buffer, 2 µl of PCR product 50 ng and 1 µl StrataClone vector mix Amp/Kan. Then the reaction mixture was incubated at room temperature for 5 min then placed on ice. 1 µl from the reaction mixture was added to the cloning tube containing StrataClone SoloPack competent cells and mixed gently. The tube was then incubated on ice for 30 min and then the cells were heat shocked at 42°C for 90 sec. The mixture was replaced on ice again for 3 min. 800 µl of LB medium was added to the reaction mixture and incubated with shaking at 37 °C for 1 h. 50 µl and 100 µl were placed on separate LB agar plates containing 50 µg/ml ampicillin and incubated overnight at 37°C. The cells obtained after overnight incubation were screened for investigation purposes.

2.14. Screening of colonies for presence of insert

The colonies of *E.coli* obtained from the cloning stage were transferred to fresh LB plates containing the same concentration of ampicillin in a grid pattern and then incubated at 37°C overnight. The colonies were then screened by picking and resuspending them in Eppendorf tubes containing 20 µl of sterile deionised water and heating at 100°C for 10 min. The tubes were immediately transferred to ice for 3 min, and then centrifuged at 13,000 g for 3 min. The supernatant was used as a DNA template in a PCR screening reaction.

PCR screening was carried out as follows: 25 µl of 2× Biomix buffer containing ultra-stable *Taq* DNA polymerase (Bioline) was added into a micro tube with 1 µl of forward primer, 1 µl of reverse primer, 2 µl of DNA template and 21 µl of sterile deionized water to make the volume up to 50 µl. The PCR was carried out as described previously, in section 2.12.2.

2.15. Screening of colonies for orientation of the insert

The gene orientation was investigated in the same way as described for the PCR screening reaction, but the difference was in the primers used. Different primer combinations were used in separate reaction tubes. The reaction mixture contained 1 µl of a cloning primer, either forward or reverse, and 1 µl of either T7 promoter primer or M13-Fwd or M13-Rev primer, all of which anneal to the cloning vector. The pattern of products obtained was used to deduce the orientation of the cloned insert within the vector.

2.16. Preparation of miniprep plasmid

Plasmids were prepared by inoculating cells into 10 ml of LB broth containing 50µg/ml ampicillin and incubating on a shaker at 37°C, overnight. 4 ml of cells were centrifuged at 13,000 g for 10 min and the supernatant was removed carefully. The cell pellet was stored at -20 °C until required for plasmid purification.

Plasmids were purified using a Gene JET™ Plasmid Miniprep kit Fermentas K0501. The purification steps were carried out by resuspending the cell pellet in 250 µl of resuspension solution and then vortexing to make sure that all cells were resuspended completely. 250 µl of lysis solution was added into the tube and mixed gently by inverting the tube 4-6 times until the solution became clear. 350 µl of neutralization solution was added immediately and mixed thoroughly and carefully by inverting the tube 4-6 times. The mixture was then centrifuged at 13,000 g for 5 min to precipitate the cell debris and chromosomal DNA. After that, the supernatant was transferred carefully, by pipetting, into a Gene JET™ spin column and then the spin column was centrifuged at 13,000 g for 1min. The liquid in the collection tube was removed and then the column was replaced again into the collection tube. 500

µl of washing solution (diluted with ethanol) was added into the spin column, and centrifuged at 13,000 g for 1 min. After that, the liquid was removed and the column replaced back into the collection tube, and the washing step was repeated using 500 µl of wash solution. The wash solution was removed and then the column was centrifuged again for an additional 1min to remove the residual liquid.

The Gene JET™ spin column was finally transferred into a sterile Eppendorf tube and then 50 µl of elution buffer was added into the centre of the column. The tube was incubated at room temperature for 2 min and centrifuged for 2 min. The column was removed and the Eppendorf tube which contained the purified plasmid DNA was stored at -20°C.

2.17. Large-scale plasmid DNA preparation

To obtain a larger quantity of DNA, plasmids were purified using the Qiagen Midi-kit, which gives an expected DNA yield of up to 250 µg from 25-35 ml of culture. The protocol was followed by growing transformed *E.coli* cells in 10 ml of LB broth containing 50 µg/ml ampicillin at 37°C, by shaking overnight. 2 ml from the overnight culture was taken and inoculated into 100 ml of LB containing the same concentration of ampicillin and incubated overnight. 50 ml of overnight culture was centrifuged at 6000 rpm for 10 min, at 4°C. The supernatant was removed and then 4 ml of resuspension buffer P1 was added to the cell pellet and vortexed. After that, 4 ml of lysis buffer P2 was added and the mixture was incubated at room temperature for 5 min. After incubation, 4 ml of neutralization buffer P3 was added and the mixture was then incubated on ice for 15 min. The mixture was transferred to a centrifuge tube and centrifuged at 20,000 rpm for 30 min at 4 °C. The supernatant was transferred into a 15 ml centrifuge tube and centrifuged again at 7500 rpm for 10 min at 4°C. During the centrifugation time, 4 ml of the equilibration buffer QBT was added into a purification column and then, after it had passed through, the supernatant was added into the purification column in order to isolate the plasmid DNA. The column was then washed twice, by adding 10 ml of the washing buffer, QC. The column was transferred into a 15 ml centrifuge tube and 5 ml of elution buffer, QF, was added and the eluate collected. 3.5 ml of 100% isopropanol was added and then vortexed. The mixture was centrifuged at 6000 rpm

for 1h at 4 °C. The supernatant was removed gently and then 2 ml of 70% ethanol was added to the tube, and it was centrifuged again for 45 min at 6000 rpm at 4 °C. The supernatant obtained was removed carefully and 50 µl of deionized water was added to the tube and the pellet was dissolved by pipetting and then stored at -20 °C.

2.18. Determination of DNA concentration and preparation for sequencing

After the plasmid DNA was screened using gel electrophoresis, the DNA concentration was determined by taking 1µl of DNA sample in a sterile Eppendorf tube and adding 399 µl of 1× TE buffer; in this case the Dilution Factor [DF] was 400 [DF=Total volume/volume of DNA]. The absorbance of DNA was measured using a UV spectrometer at 260 nm, using TE as a blank. At this wave length, the concentration of DNA is given by: absorbance of 1.0 = 50 µg/ml of DNA. The original absorbance was calculated [Abs × DF] and the DNA concentration obtained by multiplying ×50. Then, according to the concentration, the sample was diluted to a final concentration of 50 ng/µl, using sterile deionized water, and then sent for sequencing to Beckman Coulter Genomics.

2.19. Transformation of plasmid into *E.coli* mutants (BW25113 *nagE* and ZSC113)

The *E.coli* BW25113 *nagE* mutant was inoculated into 10 ml LB broth medium containing 50 µg/ml kanamycin and then incubated overnight by shaking at 37°C. 1 ml of overnight culture was transferred into 100 ml of LB broth containing the same kanamycin concentration and then incubated on a shaker at 37°C for approximately 3 h until the OD₆₀₀ of cells reached 0.4-0.6. A volume of 50 ml of culture was incubated on ice for 10 min and then transferred into a centrifuge tube and centrifuged for 15 min at 4000 rpm at 4 °C. The supernatant was completely removed and 10 ml of 0.1 M CaCl₂ solution was added to the tube to resuspend the pellet and then the tube was placed on ice for 2-3 min. After that, the tube was centrifuged at 4000 g at 4 °C for 10 min and then the supernatant was completely removed. The pellet was resuspended in 2 ml 0.1 M CaCl₂ solution and placed on ice. 200 µl of the cell suspension was taken in an Eppendorf tube and 2 µl of plasmid was added. The mixture was mixed gently and then incubated on ice for 30

min. After that the cells were shocked by heating in a water bath at 42 °C for exactly 90 sec and then immediately placed on ice for 3 min. A volume of 800 µl of LB broth was added to the mixture and then incubated on a shaker at 37 °C for 1 h. After that, 100 µl and 50 µl of the cell suspension were spread onto separate LB plates containing 50 µg/ml each of ampicillin and kanamycin and the plates were incubated at 37 °C overnight.

2.20. Examination of the phenotype of *E.coli* strains

The phenotype characterization for the *nagE* mutant strain and transformants was monitored by the fermentation of *N*-acetylglucosamine [0.1% to 1%] on MacConkey agar plates with or without antibiotics. Control plates contained no sugar. Plates were dried in an oven at 50°C for 20 min before the cells were streaked on the plates and incubated at 37°C for up to 48 hours. In order to examine the phenotype of the ZSC113 mutant and transformants the same process was carried out but the *N*-acetylglucosamine was replaced with glucose or mannose.

2.21. Construction of an artificial *Cbei 4532 - Cbei 4533* operon

For digestion and ligation, the plasmids carrying *Cbei 4532* and *Cbei 4533* were purified, as mentioned in section 2.17, using the Qiagen Midi-kit. After the DNA sequence was confirmed, the protocol was followed, as described in the next sections. The strategy for construction of the artificial operon is summarised in Figure 9 of Appendix A.

2.21.1. Restriction digests

For DNA digestion FastDigest (Fermentas Life Science) enzymes *SaII*, *XbaI*, *XhoI*, *BglII* and *BamHI* were used to digest recombinant plasmids carrying the genes. For the *Cbei 4533* gene, *SaII* was used with either *XbaI* or *XhoI* in double digestion, and for the *Cbei 4532* gene, *SaII* and *XhoI* were used in double digestion. Digests were carried out in 20 µl volumes in Eppendorf tubes. Digests contained 2 µl of 10×

FastDigest green buffer, 2 μl of 1 $\mu\text{g}/\mu\text{l}$ DNA sample and 1 μl of each required FastDigest enzyme, and the volume was made up to 20 μl with nuclease-free water. The reaction mixture was gently mixed and centrifuged briefly before being incubated in a heating block at 37°C for 30 min. The 10 \times FastDigest green buffer can be used as an electrophoresis loading buffer; thus, all 20 μl of the reaction mixture were loaded directly onto an agarose gel and electrophoresed at 80V for 1h.

2.21.2. Gel extraction

The required restriction fragment of DNA was purified from the gel using the GeneJET Gel Extraction kit (Fermentas Life Sciences). A gel slice containing the DNA was cut out using a sterile razor blade and the gel slice was placed in a pre-weighed 1.5 ml sterile Eppendorf tube. The tube was weighed again after adding the gel slice and the difference in weight was recorded as the weight of the gel slice. A volume of 1:1 (volume: weight) of the binding buffer was added into the tube (e.g. 368 μl of binding buffer was added to 368 mg of 1% gel agarose slice). The gel mixture was incubated in a heating block at 60°C for 10 min and the tube was mixed by inversion every 2 min, to facilitate the melting process. After ensuring that the gel was completely dissolved, the gel solution obtained was transferred to the GeneJET purification column and centrifuged at 13,000 rpm for 1 min. The liquid was removed and the column was placed back into the same collection tube. 700 μl of washing buffer was added into the purification column and centrifuged for 1 min, the liquid was then removed and the column was replaced into the same collection tube. The empty purification column was centrifuged again for an additional 1 min to completely remove the residual washing buffer. Then the column was transferred into a sterile Eppendorf tube and 50 μl of elution buffer was added to the centre of the purification column membrane and centrifuged for 1 min. The purification column was removed and the purified DNA was stored at -20 °C.

2.21.3. Ligation of DNA fragments and isolation of recombinant plasmid

In the ligation procedure, linear DNA fragments were joined together using a Rapid DNA Ligation Kit (Fermentas Life Sciences). In an Eppendorf tube, 3 μl of

the linearized DNA was added, together with 1 µl vector (3:1 ratio of amount of DNA), 4 µl of 5 × rapid ligation buffer and 1 µl of T4 DNA ligase, and then the reaction mixture was made up to 20 µl with nuclease-free water. The mixture was vortexed briefly and then was incubated at room temperature for 5 min and stored at 4°C. The ligation mixture (5µl) was transformed into *E.coli* TOP 10 competent cells as described in section 2.12.4. After the transformed cells were isolated on LB plates containing 50 µg/ml ampicillin the plasmid was purified by the Midi-prep method, as mentioned previously in section 2.17, and screened by PCR to confirm the presence of the *Cbei 4532* and *Cbei 4533* genes.

2.21.4. Transfer of the artificial operon to pUC18 vector

Following characterization of the clone carrying both *Cbei 4532* and *Cbei 4533*, the plasmid was digested with *Xba*I and *Bgl*II section (2.21.1) and the required fragment was gel purified as described in section 2.21.2. The purified fragment was then ligated into PUC18 which had been digested with *Xba*I and *Bam*HI and gel purified. Isolated recombinant plasmid which contained both genes was transformed into *E.coli* TOP10 cells and the clones isolated on LB plates containing 50 µg/ml ampicillin. Plasmid was isolated from a selected clone and was purified using the large-scale plasmid purification kit (section 2.17), and used to transform *E.coli* mutants. Then the vector containing *Cbei 4532* and *Cbei 4533* genes was cloned into *E.coli* mutants as described in (2.12.4).

2.22. Ribonucleic acid [RNA] extraction

2.22.1. Isolation of RNA from growth cultures

C. beijerinckii NCIMB 8052 spores were inoculated into 20 ml of RCM starter culture and incubated anaerobically, overnight. 1 ml of the starter culture was inoculated into 100 ml of CBM containing one or more carbon source, at a concentration of 10 mM. The media were incubated anaerobically at 37°C, overnight. After the cell density (OD_{650nm}) had been measured, aliquots of this

culture were inoculated into another CBM culture containing the same sugar(s) and incubated under the same conditions. Samples for RNA isolation were taken during the exponential growth phase, (which was $OD_{600\text{ nm}}$ around 0.4 to 0.6). 750 μl of culture was placed into a 1.5 ml Eppendorf tube and then 750 μl of RNA stabilization reagent (Qiagen) was added and the mixture was incubated at room temperature for 10 min. The mixture was then centrifuged at 13,000 g for 3min. The supernatant was carefully removed with a sterile pipette tip and the pellet was immediately transferred into liquid nitrogen and then stored at -70°C .

2.22.2. RNA purification

The RNA was purified using the RNeasy-mini kit (Qiagen). The RNA purification protocol was carried out by adding 10 μl of β -mercaptoethanol to 1ml of RLT buffer, and the lysozyme buffer was prepared as 10 ml TE buffer (30mM Tris-HCl, 1mM EDTA, pH 8.0) containing 15 mg/ml lysozyme and 20 μl of Qiagen proteinase K. After the pellet had thawed on ice, the purification was initiated by adding 200 μl of lysis buffer to the cell pellet and carefully resuspending by pipetting several times. The mixture was incubated for 12 min at room temperature and during this incubation time, it was vortexed for 10 sec, every 2 min. 700 μl of RLT buffer containing mercaptoethanol was added and the tube was vortexed vigorously.

500 μl of ethanol [96%] was added to the mixture and mixed carefully by pipetting. 700 μl of the mixture was transferred into an RNeasy column tube and centrifuged for 15 sec at 11,000 rpm and the supernatant was removed. This step was repeated using the remaining 700 μl from the mixture and then the supernatant was removed again. 700 μl of RW1 solution was added to the column and centrifuged at 11,000 rpm for 15 sec, to wash the spin column membrane. The RNeasy column was then placed in an Eppendorf tube and 500 μl of RPE buffer was added and the column and the tube were centrifuged at 11,000 rpm for 15 sec. Then the liquid was removed and the collection tube was placed again into the tube and the previous step was repeated by centrifuging for 2 min. The RNeasy column was finally placed into a new 1.5 ml Eppendorf tube and 40 μl of RNase-free water was added directly to the column membrane and centrifuged at 11,000 rpm for 1 min, to elute the RNA.

Then the tube containing the RNA was transferred directly into liquid nitrogen and stored at -70°C.

2.22.3. Determination of RNA concentration

The RNA concentration was determined by taking 1 µl of the RNA sample in a sterile Eppendorf tube and adding 399 µl of 1× TE buffer; in this case the Dilution Factor [DF] was 400 [DF=Total volume/volume of RNA]. The absorbance of RNA was measured using a UV spectrometer at 260 nm. At this wavelength, the concentration of RNA is given by: absorbance of 1.0 = 40 µg/ml of RNA. The original absorbance was calculated [Abs × DF] and the RNA concentration obtained by multiplying ×40. TE buffer was used as a blank in the spectrophotometer.

2.23. Slot-blot hybridization

2.23.1. Preparation and examination of DIG-labeled probes

The digoxigenin used to label the DNA nucleotides was digoxigenin-11-dUTP (Roche Diagnostics GmbH) (Glick and Pasternak, 1998) Primers were designed for amplification of 250-350 bp of internal regions of the target genes *Cbei4532*, *Cbei4533*, *Cbei4534* and *Cbei0751*, as a control. From the gene sequences, the primer lengths were designed to be approximately 20 nucleotides, as shown in Table 3. The dig-labeled probes were produced by using Biomix (Bioline) for the PCR reaction. The reaction mixtures contained 25 µl of 2×buffer; 1 µl of forward primer; 1 µl of reverse primer; 2 µl of *C.beijerinckii* DNA template; 2 µl of digoxigenin-11-dUTP and 19 µl of sterile deionized water. After 30 cycles of PCR reaction, as described previously in section 2.12.2, the samples were loaded onto an agarose gel and electrophoresed at 80V for around 1 hour and then the bands were detected under UV light.

Table 3: Primer sequencing for hybridization probes

Name	5'-3' Sequence	GC (%)	Lab reference number
<i>Cb4532-Dig-Fwd</i>	CATTGCTTCAGCTTTTATGC	40%	506
<i>Cb4532-Dig-Rev</i>	ACAATAGCTTCACCAAATGC	40%	507
<i>Cb4533-Dig-Fwd</i>	TTAGTTGCACCTATAACTGG	40%	504
<i>Cb4533-Dig-Rev</i>	ATGTTTGTAACAAGGACTGG	40%	505
<i>Cb4534-Dig-Fwd</i>	GTTTCCGTAAACATGAATGG	40%	502
<i>Cb4534-Dig-Rev</i>	AGCTTACCTGAATTTCTAGC	40%	503
<i>Cb0751-Dig-Fwd</i>	ACAATGCTTGATACGTATGG	40%	510
<i>Cb0751-Dig-Rev</i>	TATGCAATGCTGCTGTACC	40%	511

The examination of dig-labeled probes was carried out by preparing five-fold serial dilutions (1:5) (v/v) of the probes. From each dilution, 1 μ l was transferred onto a nylon membrane as a dot. The membrane was fixed under UV light for 2 min. After that, the membrane was transferred into 10 ml blocking buffer solution and incubated for 45 min by shaking at room temperature. After incubation, the anti-dig fragment tube (Anti-Digoxigenin-AP Fab fragments – Roche Diagnostics GmbH) was centrifuged at 13,000 rpm for 5 min and then 1 μ l of anti-dig was added into the blocking buffer and incubated again for 30 min, under the same conditions. The membrane was then washed at room temperature with Dig-1 buffer (pH 8.5) three times for 15 min, with shaking. This was followed by a wash with Dig-4 buffer (pH 9.5) with shaking, for 15 min. The detection steps were then carried out by transferring the nylon membrane (approximately 4 \times 5 cm) to a plastic acetate sheet (cellulose acetate polymer film); 600 μ l of CDP star chemiluminescent substrate solution (Sigma) was added to wet the membrane completely, and then the membrane was covered by another plastic acetate sheet and the sheets, including the membrane, were placed in a X-ray cassette. After that, in the dark room, an X-ray film (Lumi-Film Chemiluminescent Detection Film 7.1 \times 9.4 inches, 18 \times 24 cm) was placed on top of the acetate sheets and left for 15 min. The film was then placed in developer solution (GBX developer/replenisher - Sigma) for 30 sec-1 min until the spots appeared. Then the X-ray film was washed with water for 1 min, and

finally, it was rinsed using fixer solution (GBX fixer/replenisher – Sigma) and then washed again with water.

2.23.2. Slot-Blotting

RNA was diluted to 50 ng/ μ l with sterile deionized water, and the samples were heated at 70°C for 10 min before being directly transferred onto ice and left for 3min. In the meantime, the blotting apparatus was prepared and cleaned using RNase Zap. Two pieces of Whatman paper were cut as a background for the blotting apparatus wells (Minifold II- Schleicher and Schuell, Inc. Keeno, N.H. SRC 072/0) and used to cover the bottom of the wells. A suitable size of nylon membrane, depending on the number of samples, was placed in the blotting apparatus and the membrane was washed with RNase-free water. Amounts of 10 μ l from 500 ng/ μ l RNA sample were loaded into the blotting apparatus wells, and after approximately 5 min the nylon membrane was removed and fixed under UV light for 1-2 min.

2.23.3. Hybridization

After the RNA had been fixed to the nylon membrane, the membrane was transferred to a hybridization tube containing 10 ml of hybridization buffer and then pre- incubated at 60°C. During this time, the probe was prepared by taking 1.5 μ l of the probe PCR product and adding it to 18.5 μ l of sterile deionized water, and then heating at 95°C for 10 min. After that, the tube was immediately placed on ice and left for 3 min and then centrifuged for 15 sec at 13,000 rpm. After 30min incubation, the total volume of the probe solution was added into the hybridization tube, which was then replaced into the incubator and left overnight. After incubation, the membrane was washed in two steps, using a high salt concentration buffer [2 \times SSC] and a low salt concentration buffer [0.2 \times SSC]. It was washed three times for 15 min at 60°C with 2 \times SSC, and then twice with 0.2 \times SSC for 15min, at 60°C. After this stringent washing, the membrane was placed in a blocking buffer and treated and analysed as described in section 2.23.1.

CHAPTER3

RESULTS

PART 1

CHITIN HYDROLYSIS AND *N*-ACETYLGLUCOSAMINE
UTILIZATION BY *CLOSTRIDIUM BEIJERINCKII*

3. RESULTS

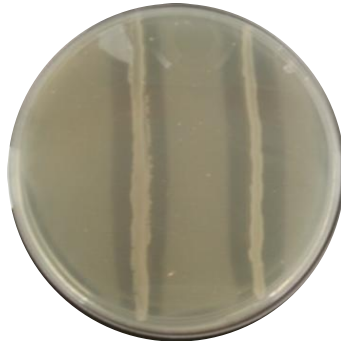
3.1. Chitinase assay

Sugars are known as an energy source for bacterial growth in their environment, in order to produce the requirements for all of the biosynthetic processes. However, the purpose in this experiment was to examine the ability of *Clostridium beijerinckii* to produce a chitinase enzyme which can degrade colloidal chitin. Cells were first streaked on plates containing 2% (v/v) of colloidal chitin in 100 ml CBM agar and incubated anaerobically at 37 °C for 48 h. In the same way, the cells were also streaked on plates containing CM-Chitin RBV solution (Carboxy-methyl Chitin Remazol Brilliant Violet) as colorimetric detection of chitinase activity.

As shown in Figure 1 (A, B), *C.beijerinckii* can produce chitinase and degrade colloidal chitin and CM-chitin RBV, as can be seen by the zones of hydrolysis surrounding the streaks of bacterial growth. Also, the chitinase activity of culture supernatants was examined on a plate containing CM-chitin RBV stain. The plate was divided into sectors, containing 100 µl of concentrated supernatant from culture grown on chitin, NAG or glucose, or chitinase enzyme, as a control. The results in Figure 2 show a clear zone around samples from chitin or NAG cultures, but not for the glucose culture supernatant, in comparison with the chitinase enzyme control. This indicates the potential capability of the strain for utilizing chitin by producing chitinase enzyme(s).

In another experiment, chitin hydrolysis by *C. beijerinckii* was determined using a Thin Layer Chromatography technique (TLC) as described in section 2.6.1. Thus, 1µl of samples of 2% chitin, 1mg/ml chitobiose and 1mg/ml NAG was spotted on the plate and used as markers. The same amount of sample (1 µl) was taken from each of the following: a mixture containing colloidal chitin with chitinase enzyme, as a control; colloidal chitin with concentrated culture supernatant obtained from a chitin growth culture, and colloidal chitin with non-concentrated supernatant. These samples were added to the TLC plate after incubating at 37 °C for 24h.

As shown in Figure 3, the chitobiose and *N*-acetylglucosamine were separated and moved to specific positions, depending on their chemical polarity. The chitinase enzyme from *S.griseus* had degraded chitin to chitobiose and *N*-acetylglucosamine. The concentrated *C.beijerinckii* culture supernatant showed the ability to degrade the colloidal chitin with some formation of NAG and chitobiose, but it also appeared that higher oligomers of Nag were present in the mixture. The non-concentrated supernatant sample also caused some degradation of the chitin, but not to the same extent as for the concentrated supernatant, since no chitobiose or Nag could be detected. Therefore, the results confirmed the earlier demonstration of the ability of *C. beijerinckii* to hydrolyse colloidal chitin.



(Fig 1. a)

Fig 1: (a) Chitin hydrolysis zone formed by *Clostridium beijerinckii* grown on CBM agar containing 2% colloidal chitin.



(Fig 1. b)

(b) Chitin hydrolysis zone formed by *Clostridium beijerinckii* grown on CM-Chitin RBV agar containing 2% colloidal chitin.

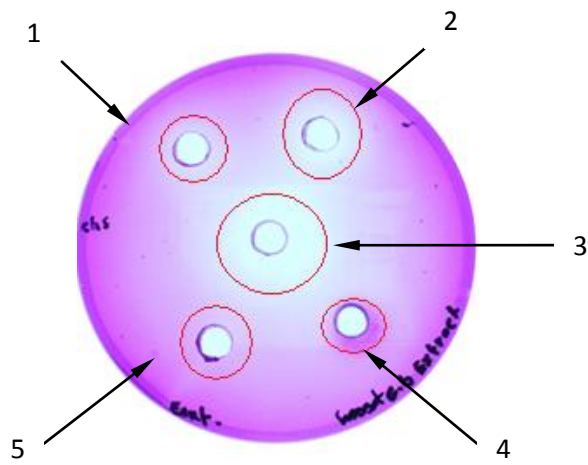


Fig 2: Hydrolysis of CM-RBV chitin by *C.beijerinckii* culture supernatants (1) concentrated supernatant from culture grown on NAG; (2) concentrated supernatant from culture grown on chitin; (3) chitinase enzyme from *Streptomyces griseus* as a control; (4) nothing was added as a control; (5) concentrated supernatant from culture grown on glucose.

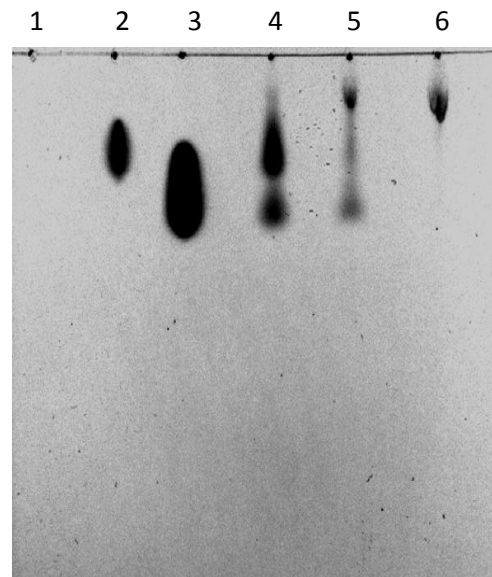


Fig 3: Analysis of chitin degradation by TLC. The plate contained (1) undegraded chitin, (2) chitobiose, (3) *N*-acetylglucosamine, (4) digest with chitinase enzyme from *Streptomyces griseus* as a control, (5) digest with concentrated cell supernatant from *C.beijerinckii* culture grown on chitin, (6) digest with non-concentrated cell supernatant from *C.beijerinckii* culture grown on chitin.

3.2. *N*-acetylglucosamine utilization by *Clostridium beijerinckii*.

After the capability of *C. beijerinckii* for hydrolysis of colloidal chitin had been demonstrated, in the following experiments the study was focused on the metabolism of the monomer of chitin (*N*-acetylglucosamine), with a view to assessing the potential for fermentation of chitin-containing waste materials for the production of butanol. The utilization of *N*-acetylglucosamine was studied in several stages, using different clostridial growth media. The effect of glucose on *N*-acetylglucosamine utilization was also studied, in order to investigate whether the two sugars can be co-utilized or whether one, which is usually glucose, may repress the utilization of the other (Mitchell, 1996). A measure of this effect can be obtained by following sugar consumption in the bacterial growth culture.

3.2.1. Utilization of *N*-acetylglucosamine by *C. beijerinckii* grown on Synthetic Reinforced Clostridial Medium [RCM] and Clostridial Basal Medium [CBM].

Initially, *N*-acetylglucosamine utilization by *C. beijerinckii* was followed by growing cells on both sRCM and CBM containing 30 mM and 15 mM *N*-acetylglucosamine respectively as a fermentable carbon source. The growth was followed by measuring the optical density of the culture at 650 nm and samples of the supernatant were collected for sugar analysis. Figures 4 and 5 respectively, show that growth in the two media was accompanied by *N*-acetylglucosamine utilization during the exponential phase, indicating that *N*-acetylglucosamine can be used as a carbon source by *C. beijerinckii* in both growth media, sRCM or CBM, and it was completely exhausted within 25 h.

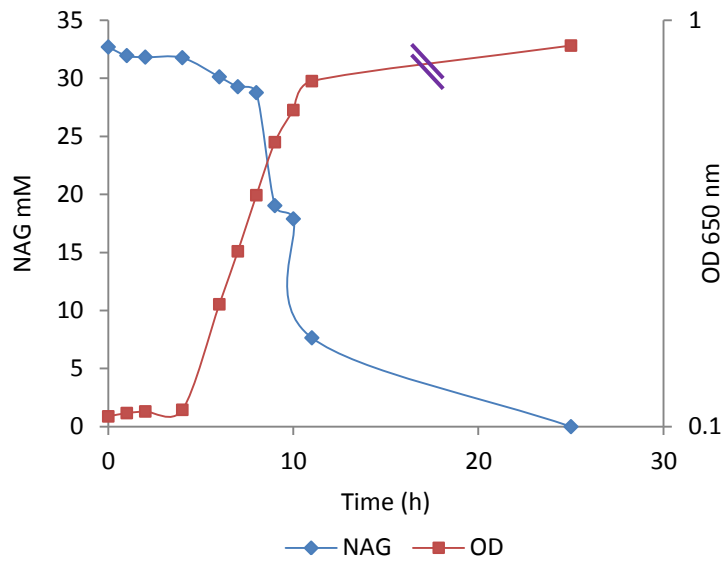


Fig 4: *N*-acetylglucosamine utilization by *Clostridium beijerinckii* in sRCM containing *N*-acetylglucosamine as a fermentable carbon source.

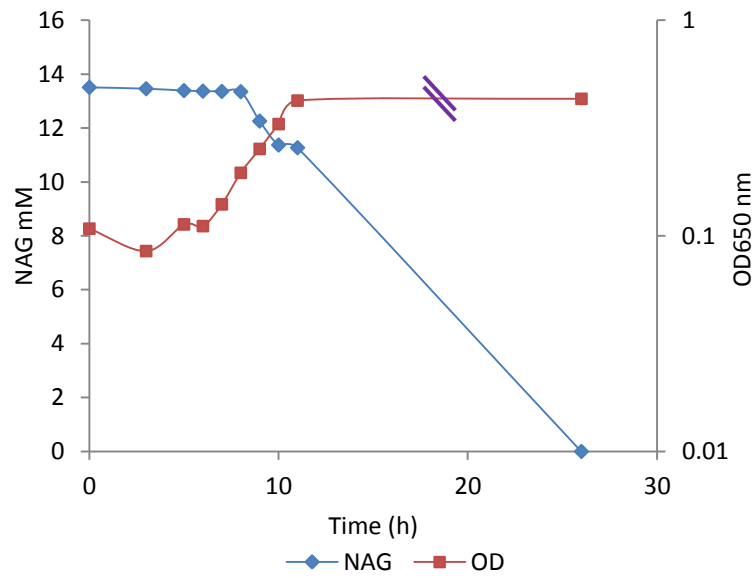


Fig 5: *N*-acetylglucosamine utilization by *Clostridium beijerinckii* in CBM containing *N*-acetylglucosamine as a fermentable carbon source.

3.2.2. The effect of the presence of glucose on *N*-acetylglucosamine utilization

As mentioned before, glucose is considered to be the preferred sugar by the majority of chemoheterotrophic microorganisms. Therefore, the effect of glucose on utilization of NAG was examined. Cells pre-grown in the presence of either glucose or NAG were washed with the same medium used for growth, but without sugar, before being inoculated into the experimental cultures containing both glucose and *N*-acetylglucosamine. The bacterial growth and sugar levels were determined, as described in the previous experiment.

In the experiment with sRCM culture containing 20 mM of *N*-acetylglucosamine and glucose, with cells pre-grown on either *N*-acetylglucosamine (Figure 6) or glucose (Figure 7), the data show that, during the exponential phase, a small amount of sugar utilization was associated with cell growth. However, in both cases, the cells appeared to utilize both *N*-acetylglucosamine and glucose together, at the same time, which indicates that no repression was effective on *N*-acetylglucosamine uptake in the presence of glucose.

The same result was obtained when 10 mM of *N*-acetylglucosamine and 5 mM of glucose were combined in a CBM culture with pre-growth on either *N*-acetylglucosamine (Figure 8) or glucose (Figure 9), in that the cells were clearly utilizing *N*-acetylglucosamine while glucose was present in the culture. Therefore, the presence of glucose did not prevent *N*-acetylglucosamine utilization, irrespective of the culture medium.

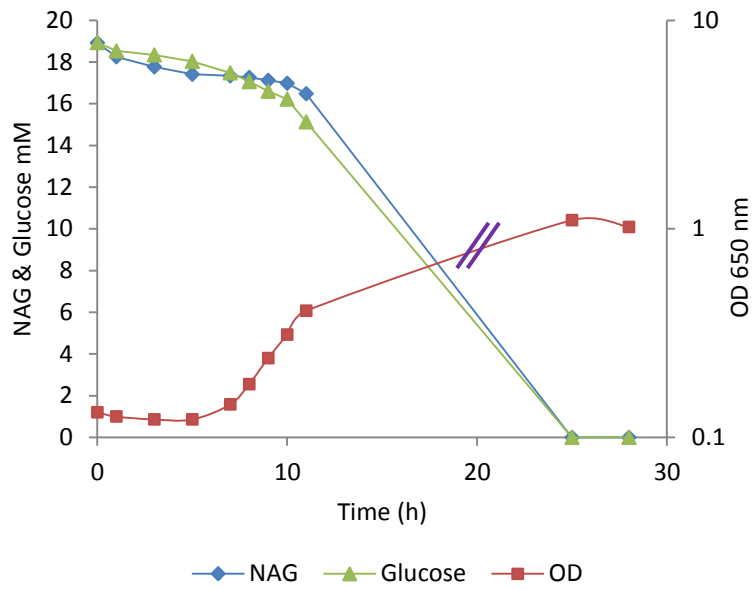


Fig 6: Growth of *Clostridium beijerinckii* in sRCM containing 20 mM *N*-acetylglucosamine and 20mM glucose: pre-grown on *N*-acetylglucosamine.

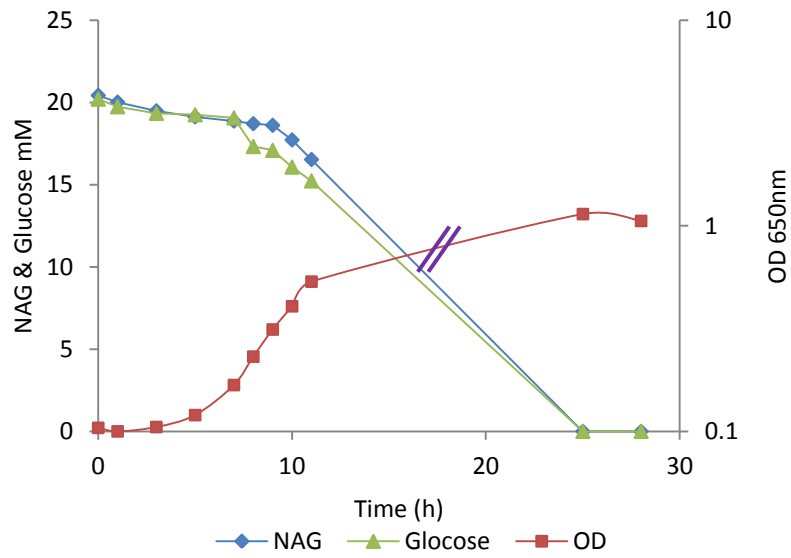


Fig 7: Growth of *Clostridium beijerinckii* in sRCM containing 20 mM *N*-acetylglucosamine and 20mM glucose: pre-grown on glucose.

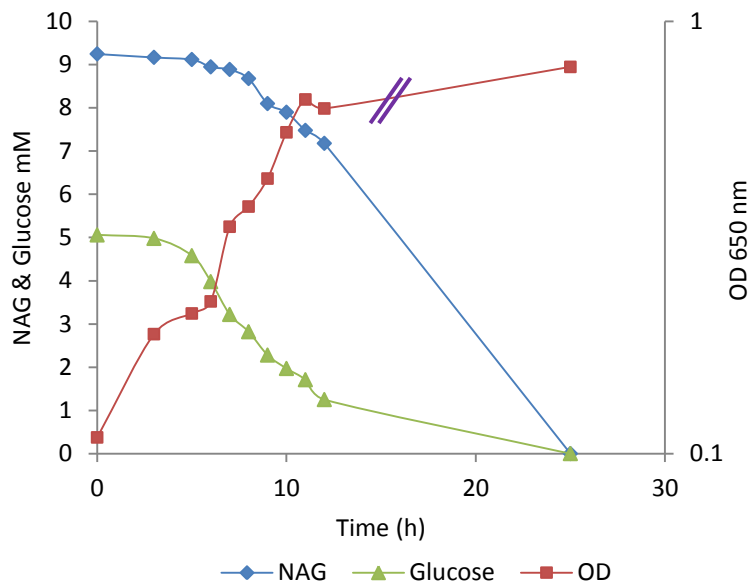


Fig 8: Growth of *Clostridium beijerinckii* in CBM containing 10 mM *N*-acetylglucosamine and 5 mM glucose: pre-grown on *N*-acetylglucosamine.

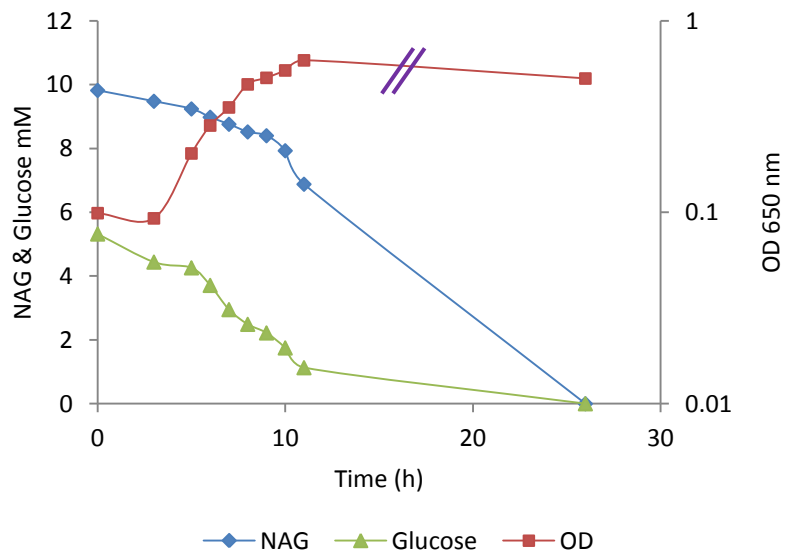


Fig 9: Growth of *Clostridium beijerinckii* in CBM containing 10 mM *N*-acetylglucosamine and 5 mM glucose: pre-grown on glucose.

PART 2

PHOSPHOTRANSFERASE SYSTEM FOR
N-ACETYLGLUCOSAMINE

3.3. Determination of *N*-acetylglucosamine PTS activity in *Clostridium beijerinckii* cells grown on *N*-acetylglucosamine.

It has been suggested that the uptake system of *N*-acetylglucosamine in *C. beijerinckii* is dependent on a PTS belonging to the glucose subfamily. As shown in section 1.9. (Figure 4) there are many PT-systems involved in sugar uptake in *C. beijerinckii*. The glucose subfamily is defined as one branch of these systems and it is likely that the NAG-PTS is a member of this branch. The previous results of the growth experiments showed that the enzymes required for *N*-acetylglucosamine utilization were expressed in both media tested, and also indicated that glucose did not prevent NAG utilization. In this experiment the PTS activity for *N*-acetylglucosamine was determined in extracts of cells grown on *N*-acetylglucosamine or glucose in the presence of phosphoenolpyruvate (PEP) or without it.

The initial results showed that, in the presence of PEP, *N*-acetylglucosamine was phosphorylated, while in the incubation without PEP, which was used as a control, no phosphorylation had occurred (Figure 10). Therefore, the extract of cells grown on *N*-acetylglucosamine showed *N*-acetylglucosamine PTS activity.

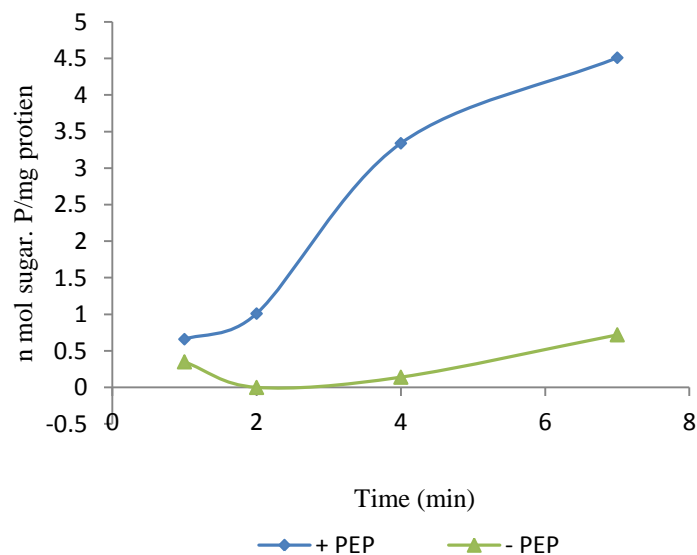


Fig 10: *N*-acetylglucosamine phosphorylation by extract of *C. beijerinckii* cells grown on *N*-acetylglucosamine with PEP, and without PEP, as a control.

In an additional experiment, under the same reaction conditions, the *N*-acetylglucosamine PTS activity was examined over a longer incubation time. It was shown that the additional time did not result in much increase in the amount of sugar phosphorylation occurring (Figure 11-A). Furthermore, this experiment also compared the activity of the *N*-acetylglucosamine PTS in a cell-free extract prepared from cells grown on glucose (Figure 11-B). Very little difference was shown in the phosphorylation activity for these extracts, demonstrating that the *N*-acetylglucosamine PTS is present in cells grown on either *N*-acetylglucosamine or glucose.

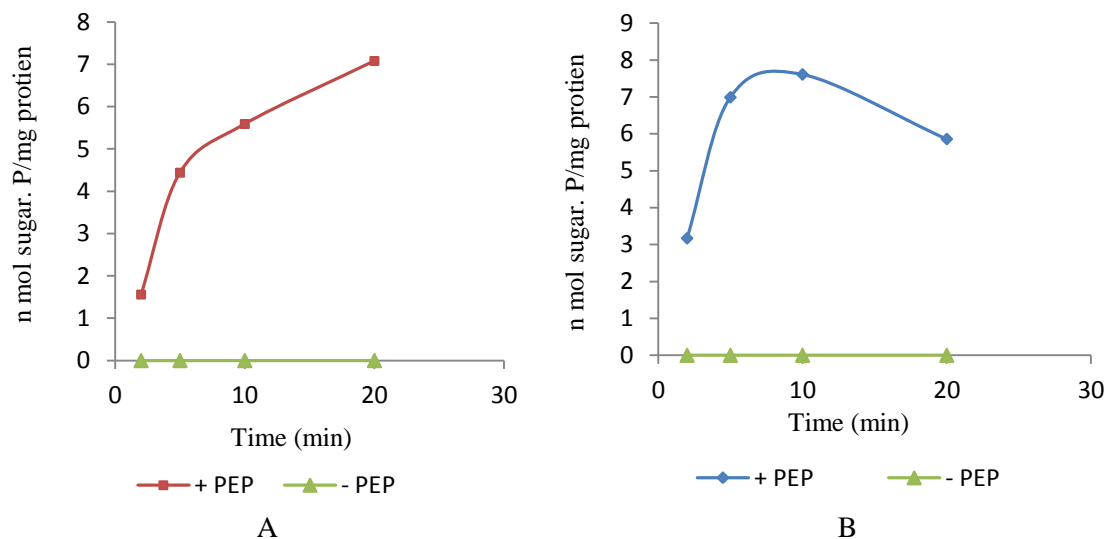


Fig11: Comparison of *N*-acetylglucosamine phosphorylation by extracts of *C.beijerinckii* cells grown on *N*-acetylglucosamine and glucose. (A) *N*-acetylglucosamine; (B) glucose. Experiments were done in the presence of PEP, and without PEP, as a control.

3.3.1. The effect of glucose and chitobiose on *N*-acetylglucosamine PTS activity

After demonstrating the presence of *N*-acetylglucosamine PTS activity and identifying the appropriate incubation time for assay of the system in cell-free extracts, the next experiment was designed to study the effect of glucose and chitobiose on *N*-acetylglucosamine phosphorylation. In the presence of 10 mM glucose, the *N*-acetylglucosamine PTS activity was strongly inhibited for both extracts, as shown in Figures 12 and 13.

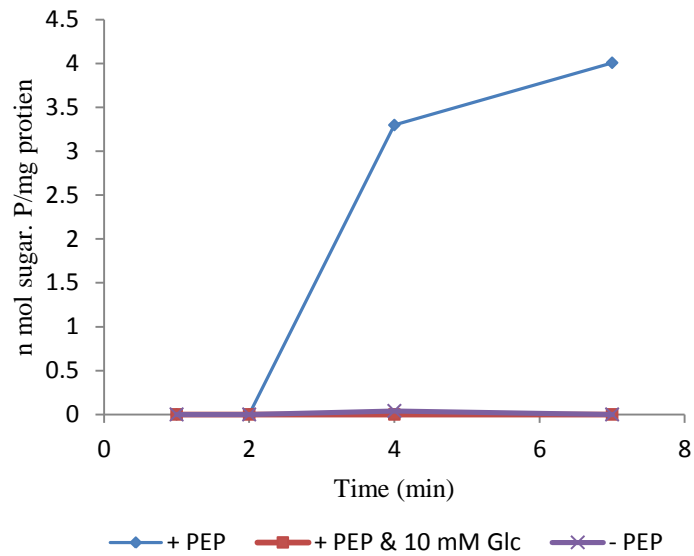


Fig 12: Effect of glucose on *N*-acetylglucosamine phosphorylation by an extract of *C.beijerinckii* cells grown on 10 mM *N*-acetylglucosamine. Experiments were done in the presence and absence of PEP and 10 mM glucose.

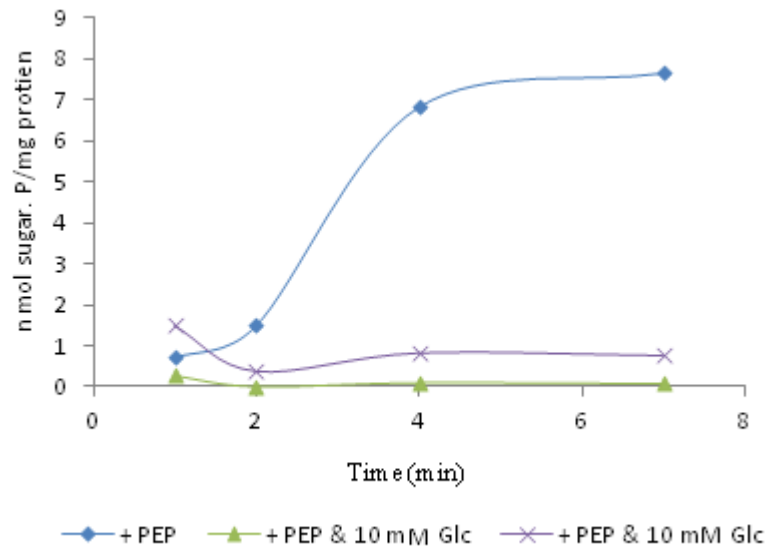


Fig 13: Effect of glucose on *N*-acetylglucosamine phosphorylation by an extract of *C.beijerinckii* cells grown on 10 mM glucose. Experiments were done in the presence and absence of PEP and 10 mM glucose.

On the other hand, a concentration of 1mM of glucose did not result in any inhibition of the *N*-acetylglucosamine phosphorylation in either of the cell-free extracts, as shown in Figures 14 and 15. Therefore, it appeared that the *N*-acetylglucosamine PTS may have a relatively low affinity for glucose. The effect of 10 mM chitobiose on *N*-acetylglucosamine phosphorylation was also studied (Figure 16) and the result was showed no inhibitory effect. It is likely that, if chitobiose is taken up by *C.beijerinckii* via a PTS, then this would occur via a system belonging to the lactose-diacetylchitobiose family, as shown in the phylogenetic tree in section 1.9. Since glucose, but not chitobiose, was shown to be an inhibitor of *N*-acetylglucosamine phosphorylation, it seems that the *N*-acetylglucosamine PTS belongs to the glucose subfamily.

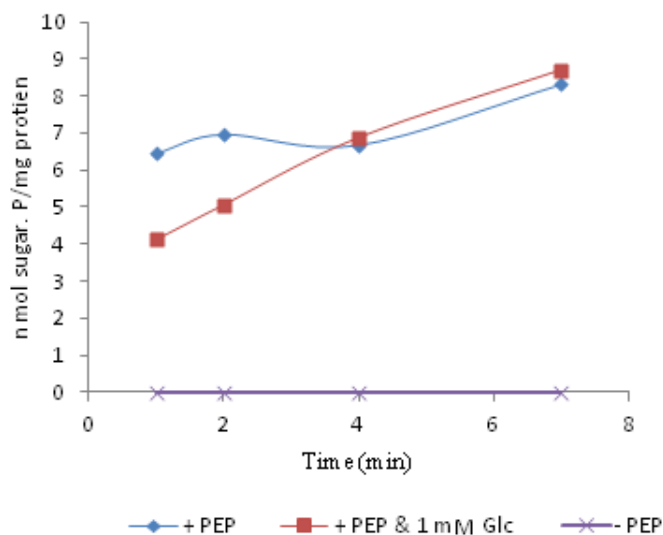


Fig 14: Effect of glucose on *N*-acetylglucosamine phosphorylation by an extract of *C.beijerinckii* cells grown on *N*-acetylglucosamine. Experiments were done in the presence and absence of PEP and 1 mM glucose.

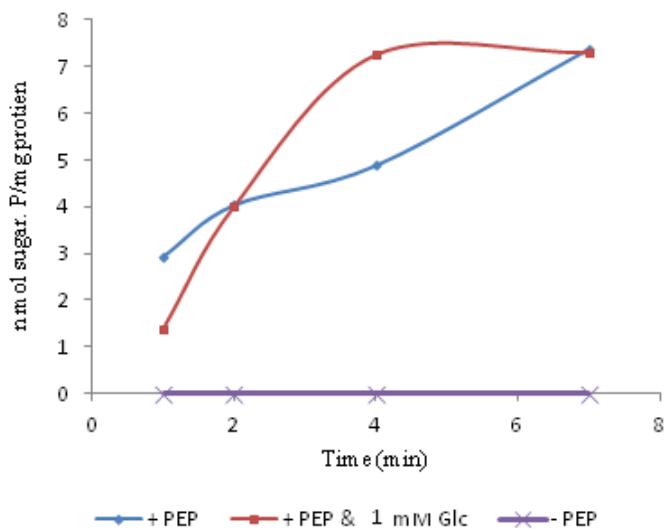


Fig 15: Effect of glucose on *N*-acetylglucosamine phosphorylation by an extract of *C.beijerinckii* cells grown on glucose. Experiments were done in the presence and absence of PEP and 1 mM glucose.

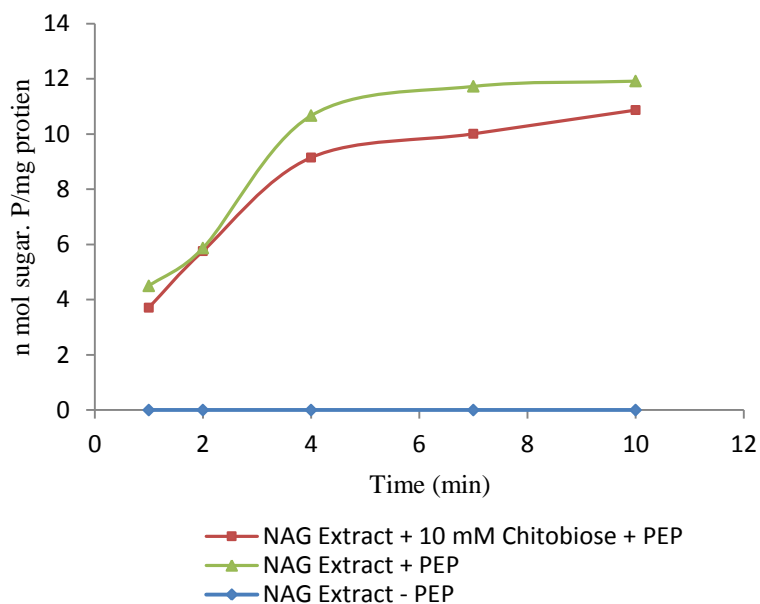


Fig 16: Effect of chitobiose on *N*-acetylglucosamine phosphorylation by an extract of *C.beijerinckii* cells grown on *N*-acetylglucosamine. Experiments were done in the presence and absence of PEP and 10 mM chitobiose.

3.3.2. ATP-dependent phosphorylation of *N*-acetylglucosamine and glucose

As mentioned above, extracts of *C.beijerinckii* grown on either *N*-acetylglucosamine or glucose showed PTS activity for *N*-acetylglucosamine. However, the cells may potentially use another uptake mechanism, such as an ATP-dependent phosphorylation mechanism in order to utilize the sugar by an alternative non-phosphotransferase route (Mitchell, 1998). ATP-dependent phosphorylation of *N*-acetylglucosamine activity was therefore measured using *N*-acetylglucosamine and glucose cell-free extracts (Figures 17 and 18, respectively). As shown, in the presence of ATP there was no phosphorylation of the amino sugar, while activity was observed in the presence of PEP, which again demonstrates the presence of the PTS. This result indicates that there is nothing in the extracts that can use ATP to phosphorylate the *N*-acetylglucosamine substrate, while in the same extracts phosphorylation could be supported by PEP.

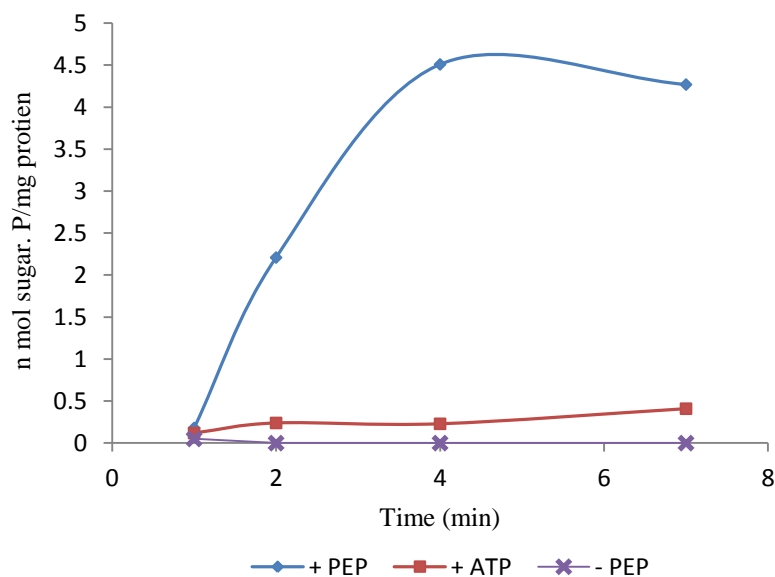


Fig 17: *N*-acetylglucosamine phosphorylation by an extract of *C.beijerinckii* cells grown on *N*-acetylglucosamine in the presence of ATP or PEP. Incubation without PEP or ATP was used as a control.

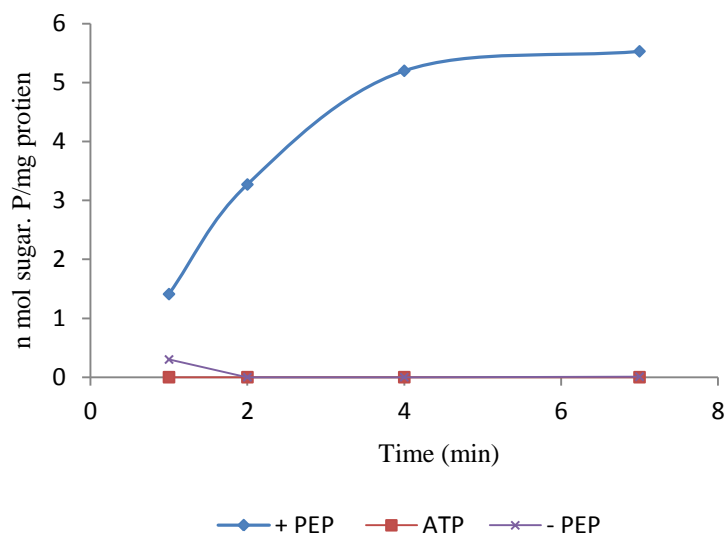


Fig 18: *N*-acetylglucosamine phosphorylation by an extract of *C.beijerinckii* cells grown on glucose in the presence of ATP or PEP. Incubation without PEP or ATP was used as a control.

3.3.3. Fractionation and reconstitution of the *N*-acetylglucosamine PTS

As indicated before, the PT-system in bacteria consists of a number of proteins which are required for sugar uptake and phosphorylation. The EI and HPr proteins are cytoplasmic proteins, and the Enzyme II complex consists of EIIA, EIIB and EIIC domains. The EIIC domain is considered as membrane protein and EIIA and EIIB domains may be contained within cytoplasmic proteins or may be joined to the EIIC domain as hydrophilic membrane-bound domains. To further investigate whether both soluble extract and membrane proteins are necessary for activity, in this experiment the cell fractions were prepared by growing the cells in CBM medium containing either *N*-acetylglucosamine or glucose and then the membranes and soluble fraction (cytoplasm) were separated. The *N*-acetylglucosamine PTS activity was measured using cell membranes only or soluble extract only (Figure 19), and the phosphorylation system was found to be inactivated, due to absence of a complete phosphoryl transfer chain. However, in the presence of both the soluble extract and cell membranes, the activity of the *N*-acetylglucosamine PTS was observed again, which indicates that a functional phosphoryl transfer chain had been reconstituted. The same experiment was carried out using extracts of cells grown on

glucose (Figure 20), and the same result was recorded. This clearly shows the functional correlation between a complete PTS phosphoryl transfer chain and *N*-acetylglucosamine phosphorylation activity.

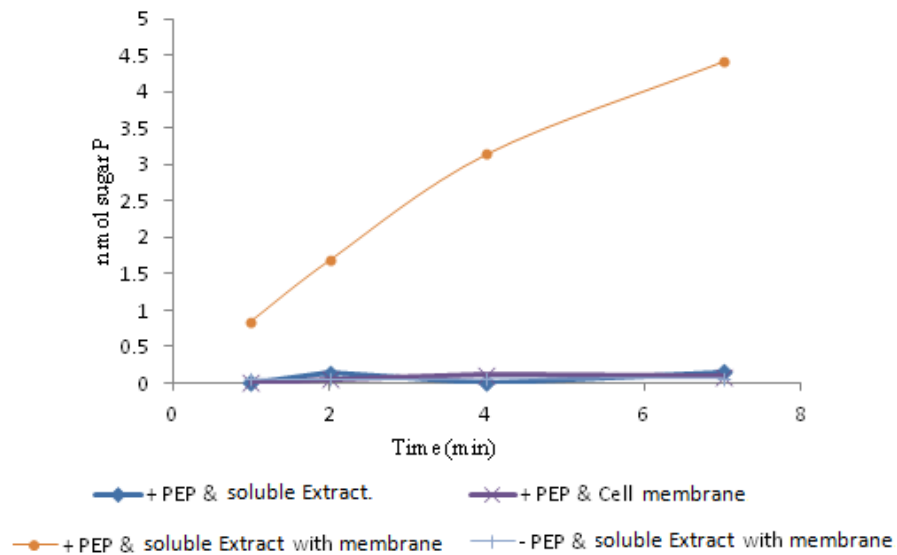


Fig 19: Fractionation and reconstitution of the PTS in extracts from *C.beijerinckii* cells grown on *N*-acetylglucosamine. Experiments were done using membrane only, soluble extract only or membrane with soluble extract, with PEP and without PEP as a control.

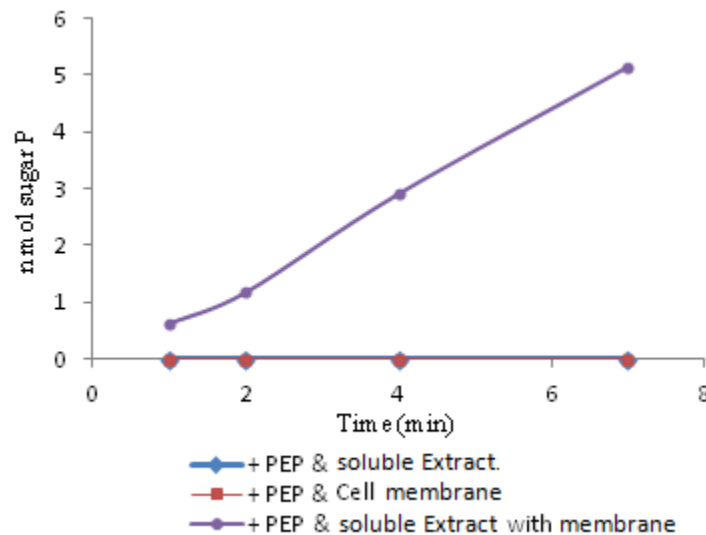


Fig 20: Fractionation and reconstitution of the PTS in extracts from *C.beijerinckii* cells grown on 10 mM glucose. Experiments were done using membrane only, soluble extract only or membrane with soluble extract.

3.3.4. *N*-acetylglucosamine phosphorylation by reconstituted PTS with membrane and soluble extract from cells grown on *N*-acetylglucosamine or glucose.

By following *N*-acetylglucosamine phosphorylation in extracts of cells grown on *N*-acetylglucosamine or glucose, the previous results obtained have demonstrated that both soluble and membrane proteins were required to form a complete chain during *N*-acetylglucosamine phosphorylation. Since extracts of cells grown on both *N*-acetylglucosamine and glucose showed the same behaviour, it was of interest to examine the effects of combining the membranes and soluble fractions of the different extracts. It is clear that the activity of *N*-acetylglucosamine phosphorylation was observed in the presence of both soluble extract from the glucose-grown cells and membranes from *N*-acetylglucosamine-grown cells (Figure 21). Also, the opposite experiment was carried out, using membranes from glucose grown cells and soluble extract from the *N*-acetylglucosamine grown cells. This combination also showed *N*-acetylglucosamine PTS activity (Figure 22). Therefore, the results indicate that the domains of the *N*-acetylglucosamine PTS are all present and that both the glucose soluble extract and membranes contained all the domains required for *N*-acetylglucosamine phosphorylation in combination with either membranes or soluble fraction of the *N*-acetylglucosamine extract, These domains maybe part of the Nag-PTS, or alternatively other PTS's expressed constitutively in *C.beijerinckii*.

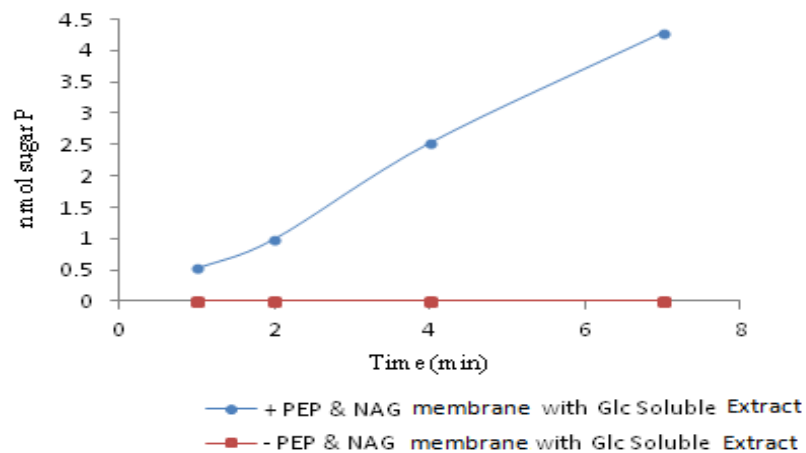


Fig 21: *N*-acetylglucosamine phosphorylation by reconstituted PTS with membranes from *N*-acetylglucosamine-grown cells and soluble extract from glucose-grown cells. Experiments were carried out with PEP and without PEP as a control.

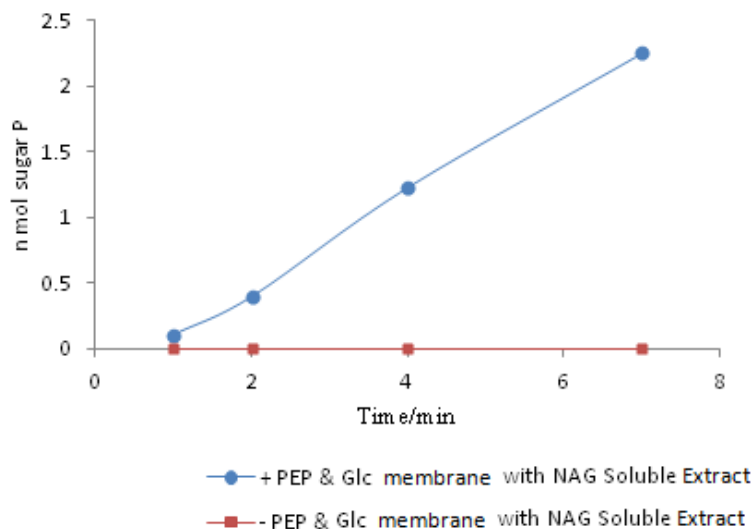


Fig 22: *N*-acetylglucosamine phosphorylation by reconstituted PTS with membranes from glucose-grown cells and soluble extract from *N*-acetylglucosamine-grown cells. Experiments were carried out with PEP and without PEP as a control.

3.3.5. The effect of *N*-acetylglucosamine on glucose phosphorylation by extracts of cells grown on glucose and *N*-acetylglucosamine.

The previous experiments were designed to demonstrate the existence of a *N*-acetylglucosamine PTS in *C.beijerinckii*, and to investigate its characteristics. The study was next extended to examine glucose PTS activity in *C.beijerinckii* extracts prepared from cells grown on *N*-acetylglucosamine or glucose. As shown in Figure 23, in the presence of PEP glucose was phosphorylated by extracts of cells grown on *N*-acetylglucosamine, while 10mM *N*-acetylglucosamine inhibited the activity. In the second experiment as shown in Figure 24, in which the extract of cells grown on glucose was used, similar activity was observed and 10 mM of *N*-acetylglucosamine again inhibited the glucose phosphorylation. These results were consistent with the inhibitory effect of glucose on *N*-acetylglucosamine phosphorylation for both cell-free extracts, and suggest a competitive relationship between *N*-acetylglucosamine and glucose.

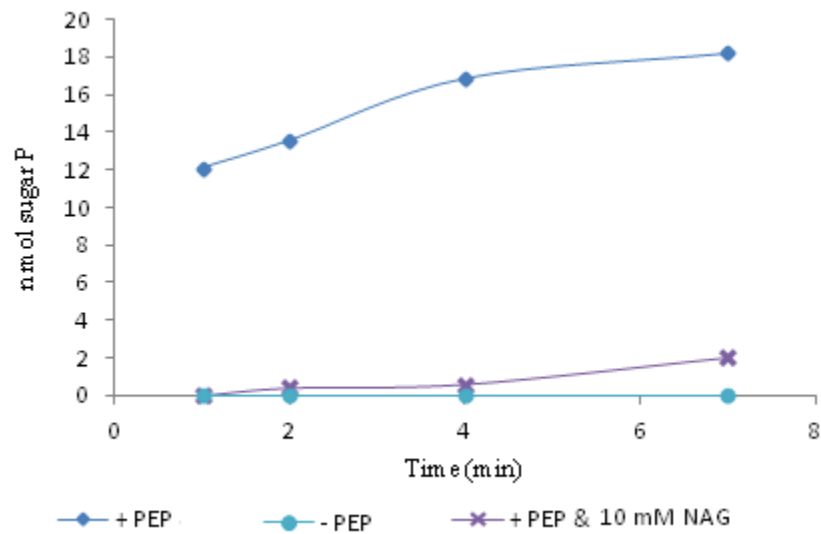


Fig 23: Effect of *N*-acetylglucosamine on glucose phosphorylation an extract of *C.beijerinckii* cells grown on *N*-acetylglucosamine. Experiments were done in the presence and absence of PEP and 10 mM *N*-acetylglucosamine.

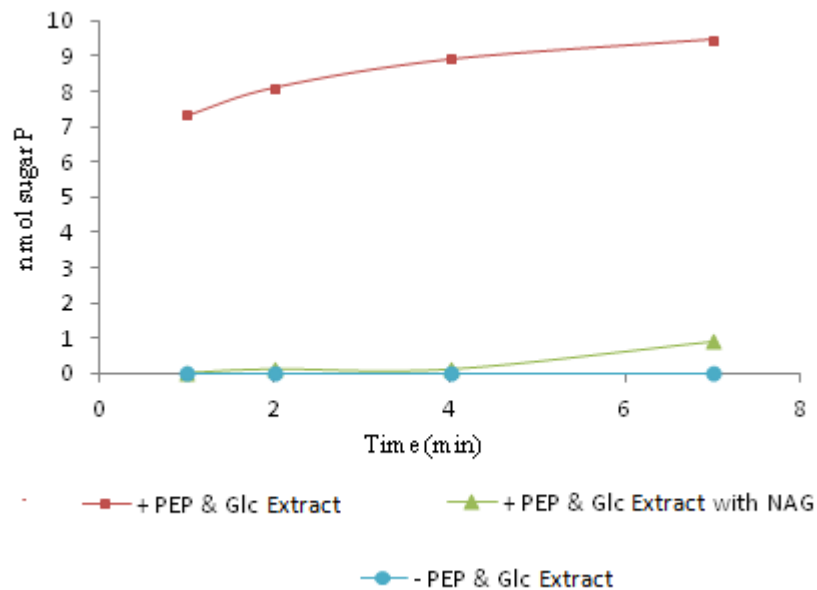


Fig 24: Effect of *N*-acetylglucosamine on glucose phosphorylation by a cell-free-extract of *C.beijerinckii* grown on glucose. Experiments were done in the presence and absence of PEP and 10 mM *N*-acetylglucosamine.

By comparing the results obtained for both *N*-acetylglucosamine phosphorylation and glucose phosphorylation, it is clear that *N*-acetylglucosamine can be phosphorylated by glucose extract and also glucose can be phosphorylated by *N*-acetylglucosamine extract. This may indicate that glucose and *N*-acetylglucosamine can be utilized by the same PT-system or by independent systems that are capable of phosphorylating both of them. Either possibility is consistent with the phylogenetic tree of PTS domains in Section 1.9, which suggested that the NAG- PTS belongs to the glucose subfamily.

In the next part of the research, bioinformatics data was collected in an attempt to identify the *N*-acetylglucosamine PTS in *C.beijerinckii* and to compare the proteins of this system with other proteins which have the same functional role either in Gram positive or Gram negative bacteria. Expression of genes encoding the putative *N*-acetylglucosamine PTS was then examined in cells growing on media containing different carbon sources.

PART 3

BIOINFORMATICS ANALYSIS

3.4. Identification of putative *nag-pts* genes in *C.beijerinckii*

The results described previously demonstrated that the PTS is used as a transport system for *N*-acetylglucosamine by *C.beijerinckii* cells. It was also observed that the system was able to phosphorylate glucose. However, it has been found that the *C.beijerinckii* strain has 42 PTS's three of which are in the phylogenetic glucose branch that includes systems known to be involved in uptake of *N*-acetylglucosamine in other bacteria.

To identify the similarity between the amino acid sequence of potential *N*-acetylglucosamine PTS proteins in *C.beijerinckii* and characterized NAG-PTS proteins from other bacteria, a bioinformatics analysis was carried out. This was done using the BLAST facility at the National Centre of Biotechnology Information (NCBI) database (Altschul *et al.*, 1997).

One PTS, encoded by the *Cbei 4532* (EIICB) and *Cbei 4533* (EIIA) genes, has been annotated as an *N*-acetylglucosamine PTS. The relationship between these proteins in *C.beijerinckii* and the NAG-PTS proteins in *B.subtilis* and *E.coli* was therefore investigated. As shown in Table 1, for the *Cbei 4532* the percentage identity of amino acids was 43% and 45% respectively when compared to the *E.coli* K12 NagE protein (EIICBA) and *B.subtilis* NagP (EIICB). For *Cbei 4533*, the percentage identity with NagE protein was 45%. Since *Cbei 4533* contains only the (EIIA) domain, an alignment with NagP is not possible. In addition, Figure 25a, b shows the protein sequence alignment between the *C.beijerinckii* NAG-PTS protein either the *Cbei 4532* (EIICB) or *Cbei 4533* (EIIA) compared with *E.coli* K12 NagE protein (EIICBA).

By comparison, the product of the *Cbei 0751* gene, which is a IICBA protein that also belongs to the glucose subfamily, showed 37% and 39% identity to NagE and NagP respectively. In addition, the third member of glucose subfamily encoded by *Cbei 4982* (IIA) and *Cbei 4983* (IICB), showed 38% and 35% identity when compared to the *E.coli* K12 NagE protein (EIICBA), while 33% identity showed between *Cbei 4983* (IICB) and *B.subtilis* NagP (EIICB).

Since the products of the *Cbei 4532* and *Cbei 4533* genes are most closely related to NagE and NagP, it seems that these genes are likely to be the *nag-pts* genes of *C.beijerinckii*. These genes are arranged divergently in the *C.beijerinckii* genome,

Table 1: Comparison of *N*-acetylglucosamine PTS proteins in *C.beijerinckii* with those in *E.coli* K12 and *B.subtilis*.

Gene name	Identity percentage	
	<i>E.coli</i> K12 Nag E	<i>B.subtilis</i> Nag P
<i>Cbei 4532</i>	43%	45%
<i>Cbei 4533</i>	45%	*
<i>Cbei 0751</i>	37%	39%
<i>Cbei 4982</i>	38%	*
<i>Cbei 4983</i>	35%	33%

* No comparison possible.

alongside a gene *Cbei 4534* that encodes a putative transcriptional antiterminator (Figure 25c). Comparison with other antiterminators showed between 30 and 35% identity with the LicT (Schnetz *et al.*, 1996) and SacY antiterminator proteins from *B.subtilis* (Idelson and Amster-Choder, 1998), and the BglG transcriptional antiterminator protein in *E.coli* K12 (Blattner *et al.*, 1997). These antiterminators are all associated with regulation of *pts* genes, so it appears that the *Cbei 4534* protein may be involved in regulation of the putative *nag-pts* genes in *C.beijerinckii*. Having identified likely *nag-pts* genes in *C.beijerinckii*, the next step would be to characterize these genes by following their expression under different growth conditions and by examining their function via cloning and complementation of an *E.coli* NAG-PTS mutant.

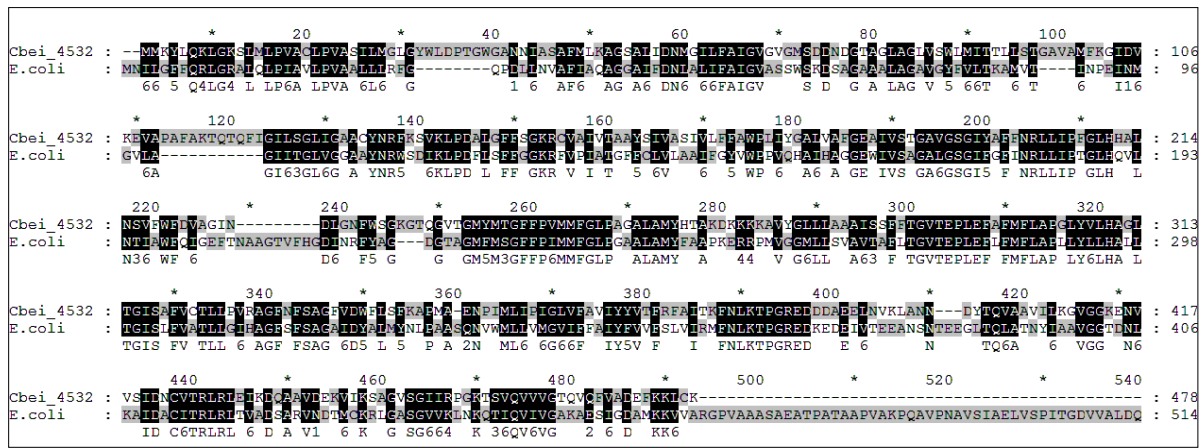


Fig 25a: Protein alignments between *C.beijerinikii* putative EIIBC domains and *E.coli* K12 NagE specific PTS enzyme: IIC, IIB, and IIA domains, the black shadow shows the similarity between amino acids in both proteins.

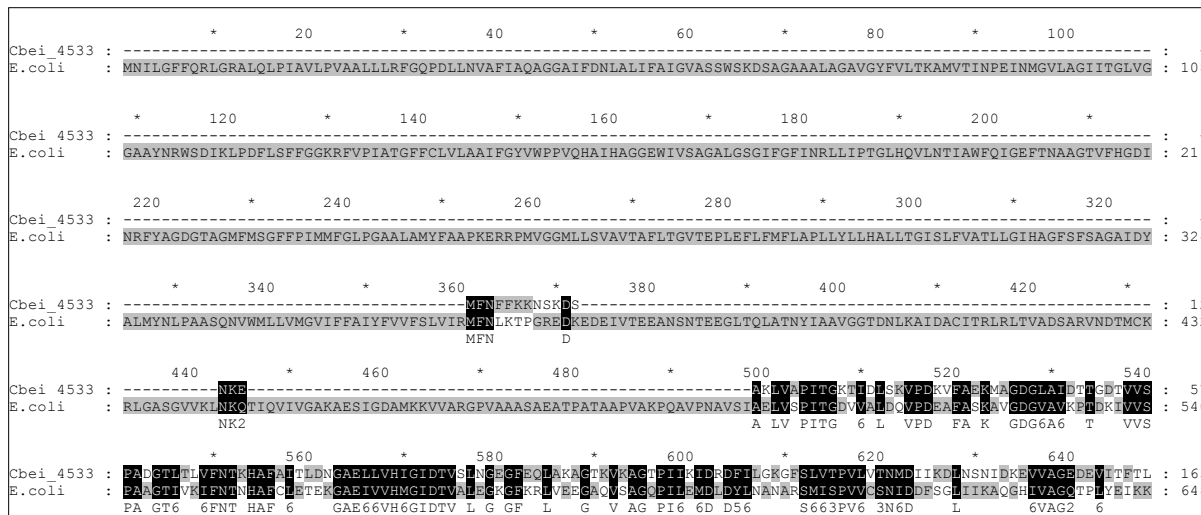


Fig 25b: Protein homologue alignments between *C.beijerinikii* putative EIIA domain and *E.coli* K12 NagE specific PTS enzyme: IIC, IIB, and IIA domains, the amino acids that are identical are shown in black shadow in both proteins.

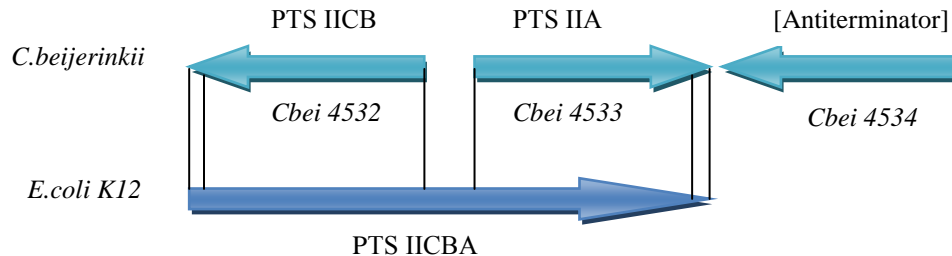


Fig 25c: The organization of genes related to the putative *N*-acetylglucosamine PT-system, *Cbei 4532*, *Cbei 4533* and *Cbei 4534*, compared with the specific NagE gene in *E.coli* K12. *Cbei 4534* encodes a putative anti-terminator protein which probably regulates expression of *Cbei 4532* and *Cbei 4533* (http://www.ncbi.nlm.nih.gov/gene?term=Cbei_4532).

PART 4

IDENTIFICATION AND EXPRESSION OF THE PUTATIVE
N-ACETYLGLUCOSAMINE GENES OF *C.BEIJERINCKII*

3.5. Slot-Blotting and Hybridization

According to the bioinformatics data obtained in the previous section (3.4), it was suggested that the PTS Enzymes IIBC and IIA encoded by *Cbei 4532* and *Cbei 4533* are involved in *N*-acetylglucosamine uptake in *C.beijerinckii*. In this section, a hybridization technique was used in order to examine the expression of these genes and of *Cbei 4534* and *Cbei 0751* in *C.beijerinckii*. The expression of these genes was examined by hybridization using labelled hybridization probes specific for each gene.

Cells were grown in CBM containing one of several sugar substrates. *N*-acetylglucosamine, glucose, both *N*-acetylglucosamine and glucose or glucitol, which is a substrate of a PTS that has been characterized and found to belong to another family of the PTS phylogenetic tree (Tangney *et al.*, 1998a). After the RNA was prepared from exponentially growing cells, 3 μ l of each RNA sample was screened by electrophoresis to make sure that it was of the required quality, as shown in Figure 26.

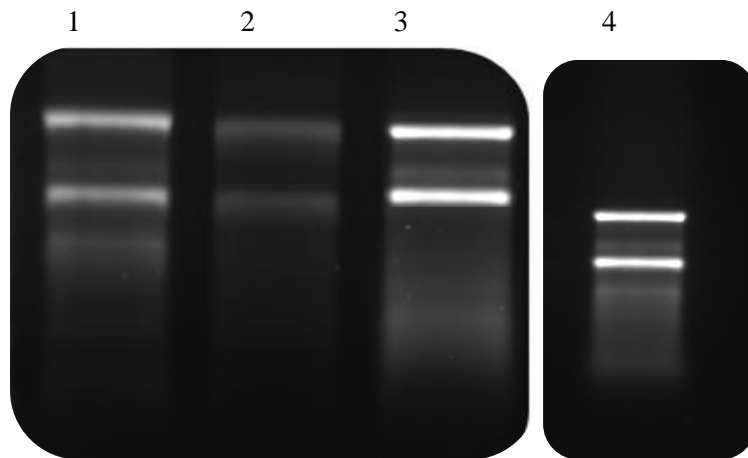


Fig 26: RNA purification from *C.beijerinckii* cells. Cultures were grown on CBM containing (1) *N*-acetylglucosamine, (2) glucose, (3) *N*-acetylglucosamine and glucose and (4) glucitol.

3.5.1. Preparation of Hybridization probes

Hybridization probes directed against the selected genes were prepared by designing internal primers for PCR amplification. The gene segments were then amplified by a PCR reaction, using *C.beijerinckii* DNA as template and digoxigenin to label the PCR products. The labelled samples were screened by gel electrophoresis with expected PCR product sizes of 420 bp for *Cbei 4532*, 350 bp for *Cbei 4533*, 500 bp for *Cbei 4534*, and 350 bp for *Cbei 0751*. The DIG-labelled PCR product appeared heavier than the unlabelled bands, indicating that the digoxigenin had been incorporated into the PCR amplified DNA products and thus that the probes should be suitable for hybridization (Figures 27 and 28).

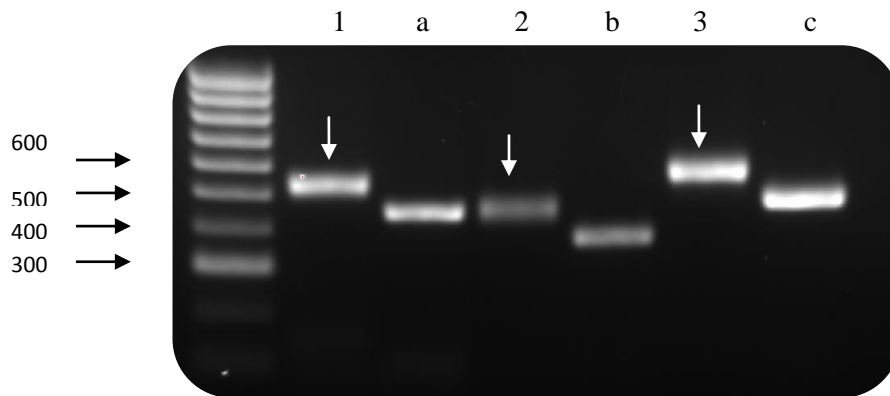


Fig 27: Gel electrophoresis screening of PCR-amplification of hybridization probes. (1) *Cbei 4532*, (2) *Cbei 4533* and (3) *Cbei 4534*. The labeled bands indicated by arrows were shown by bigger size compared with unlabelled bands (a, b, c).

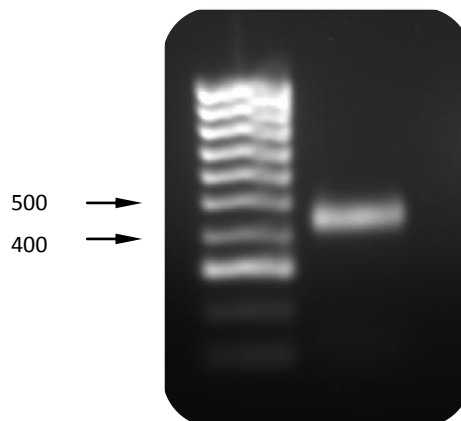


Fig 28: Gel electrophoresis screening for PCR-amplification of hybridization probe for *Cbei 0751*. The DIG-labelled PCR product is shown on the gel.

Further tests on the DIG-labelled probes were carried out. The probes were detected using dot blots on a nylon membrane by following the protocol indicated in Materials and Methods Sections 2.23.1. Five-fold serial dilutions were prepared for each probe in sterile deionized water, and 1µl samples were applied to the membrane. As demonstrated in Figure 29, the probes showed a high sensitivity starting from the original undiluted sample to the fourth dilution, which confirmed that the probes could be used in the hybridization experiments.

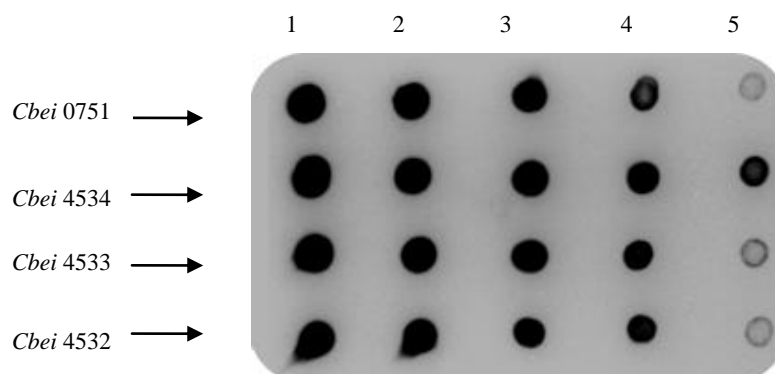


Fig 29: Dot blot of DIG-labeled probes for *Cbei 4532*, *Cbei 4533*, *Cbei 4534* and *Cbei 0751* at different concentrations. Starting from the highest concentration which is the first dilution (1) to the lowest concentration (5), each dilution was $\times 5$.

3.5.2. Expression of the *Cbei 4532*, *Cbei 4533* and *Cbei 4534* genes on a medium containing *N*-acetylglucosamine or glucose.

In order to detect the expression of the putative *N*-acetylglucosamine PTS genes, samples were taken from a culture growing on *N*-acetylglucosamine or glucose during the exponential phase. After purifying the RNA, the hybridization experiment was carried out for all *N*-acetylglucosamine PTS genes, using DIG-labeled DNA products (DNA probes) with the RNA samples. The experiment indicated that mRNA corresponding to the gene *Cbei 4532* was present in both the samples (Figure 30). Similarly, hybridization was also detected for the other genes *Cbei 4533* (Figure 31) and *Cbei 4534* (Figure 32). Thus, these results indicate that the *Cbei 4532*, *Cbei 4533* and *Cbei 4534* genes were induced during growth on *N*-acetylglucosamine or glucose. The results were also consistent with the results recorded in the PTS assays, which showed the presence of Nag PTS activity in cells

grown on both *N*-acetylglucosamine and glucose. Therefore, the next experiment was designed to examine the expression of these genes by isolating an RNA sample from a growth culture containing *N*-acetylglucosamine and glucose in the same medium.

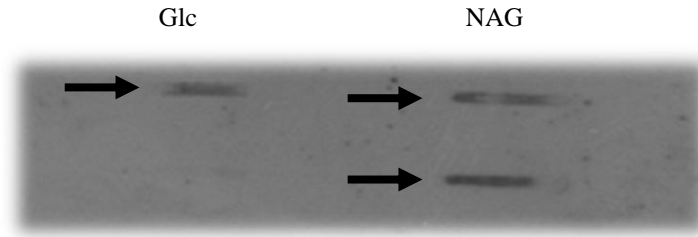


Fig 30: Slot blotting and hybridization of RNA sample, for *Cbei 4532*. Cells were grown on *N*-acetylglucosamine (Duplicate) or glucose in separate cultures. Each sample contained 500ng RNA applied to the membrane. samples were applied to the gel.

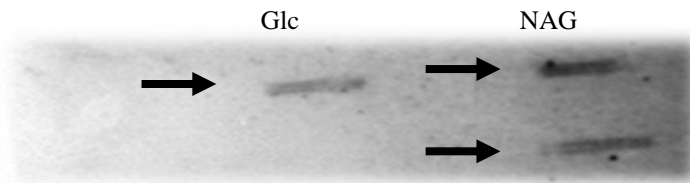


Fig 31: Slot blotting and hybridization of RNA sample, for *Cbei 4533*. Cells were grown on *N*-acetylglucosamine (Duplicate) or glucose in separate cultures. Each sample contained 500 ng RNA applied to the membrane.

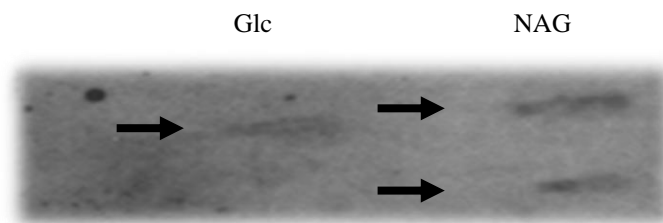


Fig 32: Slot blotting and hybridization of RNA sample, for *Cbei 4534*. Cells were grown on *N*-acetylglucosamine (Duplicate) or glucose in separate cultures. Each sample contained 500 ng RNA applied to the membrane.

3.5.3. Expression of the *Cbei 4532*, *Cbei 4533* and *Cbei 0751* genes in a medium containing *N*-acetylglucosamine, glucose, or *N*-acetylglucosamine with glucose.

According to the previous result, the genes *Cbei 4532*, *Cbei 4533* and *Cbei 4534* which encoded EIIBC, EIIA and an antiterminator were expressed in cultures growing either in a medium containing *N*-acetylglucosamine or a medium containing glucose. In the next experiment, the analysis was extended to cultures growing in a medium containing both glucose and *N*-acetylglucosamine, and expression of the *Cbei 0751* was also investigated. The *Cbei 0751* gene is believed to encode a protein of the glucose PTS, which may be involved in glucose uptake only, and using the *Cbei 0751* gene in this experiment as a control could potentially clarify its function. As already described in the previous section, the results shown in Figures 33 and 34 clearly showed that the genes *Cbei 4532* and *Cbei 4533* were expressed during growth on *N*-acetylglucosamine or glucose; in addition an apparent high degree of expression was shown in the sample from a culture containing both *N*-acetylglucosamine and glucose. The *Cbei 0751* gene was also expressed in the presence of glucose and *N*-acetylglucosamine with glucose, while no expression or very low expression was demonstrated in the *N*-acetylglucosamine sample, as shown in Figure 35. This implies that the PTS which it encodes cannot be involved in *N*-acetylglucosamine uptake.

These results obtained verified that the genes *Cbei 4532* and *Cbei 4533* were expressed in the presence of either *N*-acetylglucosamine or glucose, or both. Therefore, there is no evidence that their expression is repressed by glucose. This pattern of expression is consistent with the results obtained previously in the growth curves in which glucose and *N*-acetylglucosamine were utilized together, thus showing the apparent absence of repression of *N*-acetylglucosamine uptake by glucose.

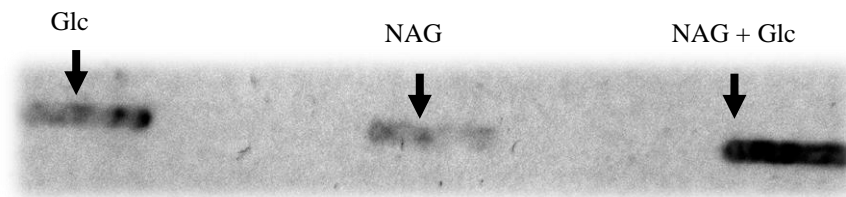


Fig 33: Slot blotting and hybridization of RNA sample, for *Cbei 4532*. Cells were grown on *N*-acetylglucosamine, glucose and *N*-acetylglucosamine with glucose in the same culture. Each sample contained 500 ng RNA applied to the membrane.

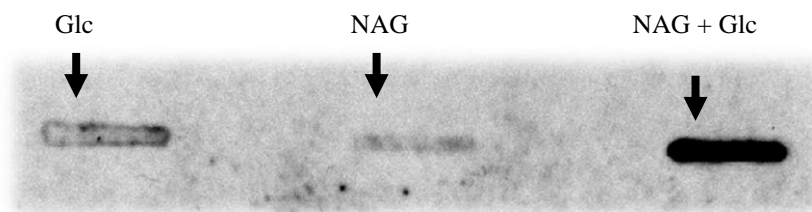


Fig 34: Slot blotting and hybridization of RNA sample, for *Cbei 4533*. Cells were grown on *N*-acetylglucosamine, glucose and *N*-acetylglucosamine with glucose in the same culture. Each sample contained 500 ng RNA applied to the membrane.

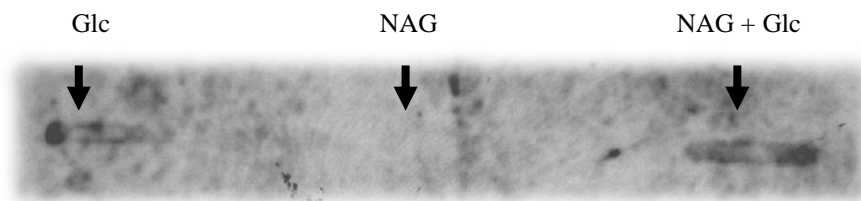


Fig 35: Slot blotting and hybridization of RNA sample, for *Cbei 0751*. Cells were grown on *N*-acetylglucosamine, glucose and *N*-acetylglucosamine with glucose in the same culture. Each sample contained 500 ng RNA applied to the membrane.

3.5.4. Expression of the *Cbei 4532*, and *Cbei 4533* genes in a medium containing *N*-acetylglucosamine, glucose, *N*-acetylglucosamine with glucose or glucitol.

The previous experiments confirmed that all the genes suggested as being related to *N*-acetylglucosamine utilization were expressed in the presence of *N*-acetylglucosamine. However, they were also expressed during growth in a culture containing glucose as the only carbon source, and in the presence of both sugars, suggesting that glucose did not repress expression of the genes. It was therefore of interest to examine expression during growth on an alternative substrate such as glucitol, which is a substrate of a PTS that belongs to another family of the PTS phylogenetic tree (Tangney *et al.*, 1998a). Thus, RNA prepared from a culture grown on glucitol was used in a series of hybridization experiments.

As shown in Figures 36 and 37, it is clear that, the *Cbei 4532* and *Cbei 4533* genes were expressed during growth in media containing *N*-acetylglucosamine and/or glucose. However, there was no expression of these genes during growth on glucitol.

Since bioinformatics and gene expression analysis of the *Cbei 4532* and *Cbei 4533* genes provided evidence that they are involved in *N*-acetylglucosamine uptake, the next step was to clone and characterize the genes in an *Escherichia coli* mutant (*nagE*) with an inactivated *N*-acetylglucosamine PTS (Baba *et al.*, 2006).

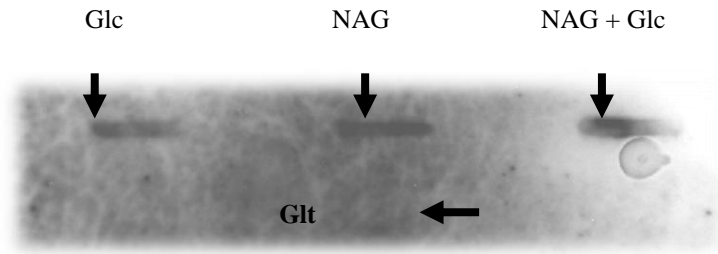


Fig 36: Slot blotting and hybridization of RNA sample, for *Cbei 4532*. Cells grown on *N*-acetylglucosamine, glucose, *N*-acetylglucosamine with glucose in the same growth culture, and glucitol as a control. Each sample contained 500 ng RNA applied to the membrane.

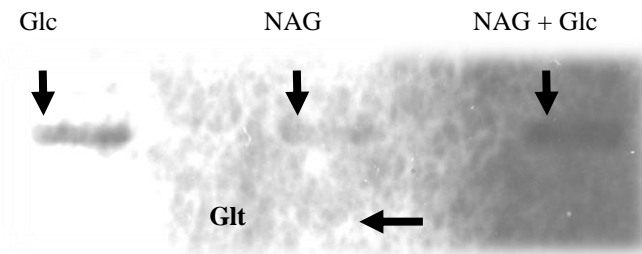


Fig 37: Slot blotting and hybridization of RNA sample, for *Cbei 4533*. Cells grown on *N*-acetylglucosamine, glucose, *N*-acetylglucosamine with glucose in the same growth culture, and glucitol as a control. Each sample contained 500 ng RNA applied to the membrane.

PART 5

CLONING AND CHARACTERIZATION OF THE PUTATIVE *N*-
ACETYLGLUCOSAMINE PTS GENES OF *C.BEIJERINCKII*

3.6. Cloning of the genes encoding the PT-System

As demonstrated in the previous part, the putative genes assumed to be involved in the *N*-acetylglucosamine PTS were expressed in the presence of *N*-acetylglucosamine or glucose but not glucitol, and it was also shown that the presence of both sugars in the same growth culture gave a high degree of expression for both genes, *Cbei 4532* and *Cbei 4533*. In order to verify the function of these genes, this part of the research was designed to clone and characterize them in a *nagE* mutant strain of *E.coli* with an inactivated *N*-acetylglucosamine phosphotransferase system.

In *Clostridium beijerinckii* NCIMB 8052 the completed genome is estimated to be 6,000, 632 bp. However, it was suggested that there are 42 PTSs encoded by this genome and the putative *N*-acetylglucosamine PT-system appears to be encoded by two genes in the opposite orientation in the genome (Figure 38).

The *Cbei 4532* gene (1437 bp) encodes a PTS protein including EIIC and EIIB domains, and the, *Cbei 4533* gene (489 bp), encodes a PTS EIIA domain. Therefore, it is expected that both genes *Cbei 4532* and *Cbei 4533* will be required to encode a functional PTS. These *pts* genes are adjacent to *Cbei 4534* encoding a putative antiterminator which is likely to be involved in regulation of their expression.



Fig 38: The organization of genes related to the putative *N*-acetylglucosamine PT-system, *Cbei 4532*, *Cbei 4533* and *Cbei 4534*. *Cbei 4534* encodes a putative anti-terminator protein which probably regulates expression of *Cbei 4532* and *Cbei 4533* (http://www.ncbi.nlm.nih.gov/gene?term=Cbei_4532).

3.6.1. Cloning of the putative *nag* operon (*Cbei 4532 – 4534*)

The first attempt at cloning was carried out by cloning the whole gene fragment expected to encode the *N*-acetylglucosamine PTS domains EIICB and EIIA and the putative transcriptional antiterminator. All domains (*Cbei 4532 – 4534*) were amplified together using primers *Cbei 4532*-Rev and *Cbei 4534*-Fwd, as described in Materials and Methods (Section 2.11.1), and then the PCR product (Figure 39) was cloned using the StrataClone vector (Agilent Technologies) as described in Appendix 1. The colonies obtained were screened by the methods described in section 2.13. As shown in Figure 40, three clones were isolated which contained the expected insert of size 3600 bp. Then the gene orientation was determined, by amplifying with *Cbei 4532*-Rev and either the M13-Rev primer or the M13-Fwd primer. For colony (1) a product was obtained with M13-Rev primer and the *Cbei 4532*-Rev primer indicating that the genes had been successfully cloned in the vector under the control of the *lac* promoter (Figure 41). However, no product was obtained with M13-Fwd primer and *Cbei 4532*-Rev as would be expected for this orientation. The other two clones did not show any products. Following that, the plasmid was purified using Miniprep plasmid purification, as described previously in Materials and Methods section 2.16.

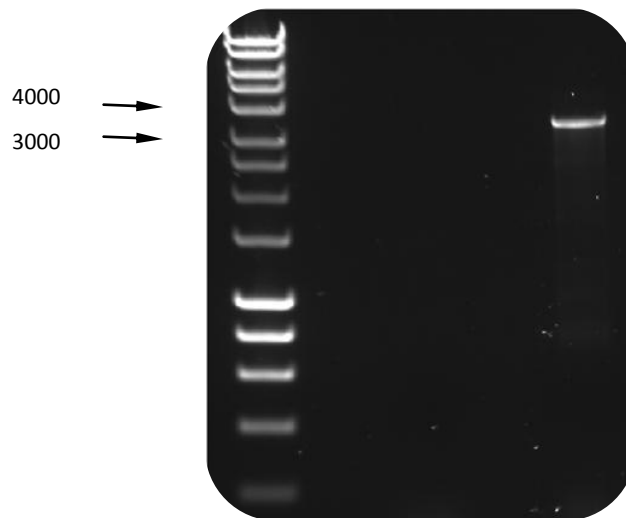


Fig 39: PCR product for the operon *Cbei 4532 - 4534* generated by Easy-A cloning enzyme with a product size of approximately 3600 bp.

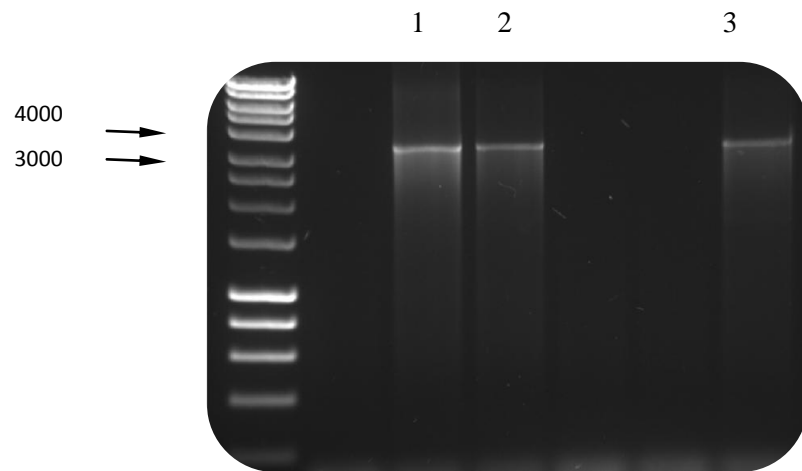


Fig 40: Screening of *E.coli* clones containing *Cbei 4532 - 4534*. Approximately 70 clones were screened and the figure shows only the three colonies which produced a PCR product of around 3600 bp in lanes 1, 2 and 3, while the negative samples do not show any bands in other lanes.

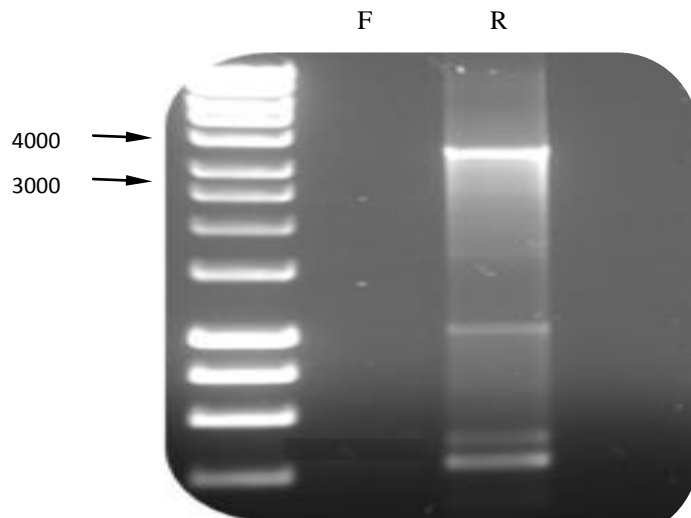


Fig 41: Determination of gene orientation of *Cbei 4532 – 4534* for colony 1. Orientation was determined by using *Cbei 4532*-Rev and M13-Rev primer (R) or M13-Fwd primer (F). No bands were shown with other clones.

3.6.2. Transformation into *nagE* mutant

The successful cloning of genes *Cbei 4532*, *4533* and *Cbei 4534* in one vector, which could encode all the domains required for the *N*-acetylglucosamine PTS and the transcriptional antiterminator, allowed for the possibility of screening for a functional PTS by complementation of a Nag-PTS mutant of *E.coli* for Nag fermentation. First, the *nagE* mutant was streaked onto MacConkey agar plates in order to determine its phenotype in the presence of different concentrations of *N*-acetylglucosamine. These plates contained 1%, 0.5%, 0.25%, 0.1% and no *N*-acetylglucosamine. Figure 42 shows that, at a concentration of 1%, the mutant fermented the *N*-acetylglucosamine, thus producing acid, which led to a decrease in the pH, shown by red/pink colonies. However, when the concentration of *N*-acetylglucosamine was decreased the cells could not utilize the lower concentration of *N*-acetylglucosamine. Under these conditions, the nitrogen sources in the medium were used, producing ammonia, which led to an increase in the pH of the medium and the colonies, appeared as dark yellow. From these results, it seems that the *nagE* mutant could utilize *N*-acetylglucosamine at the highest concentration tested (1%) while it was not capable of using the lower concentrations, 0.5% or below. Therefore, an *N*-acetylglucosamine concentration of 0.5% was chosen as the standard concentration for the complementation experiments.

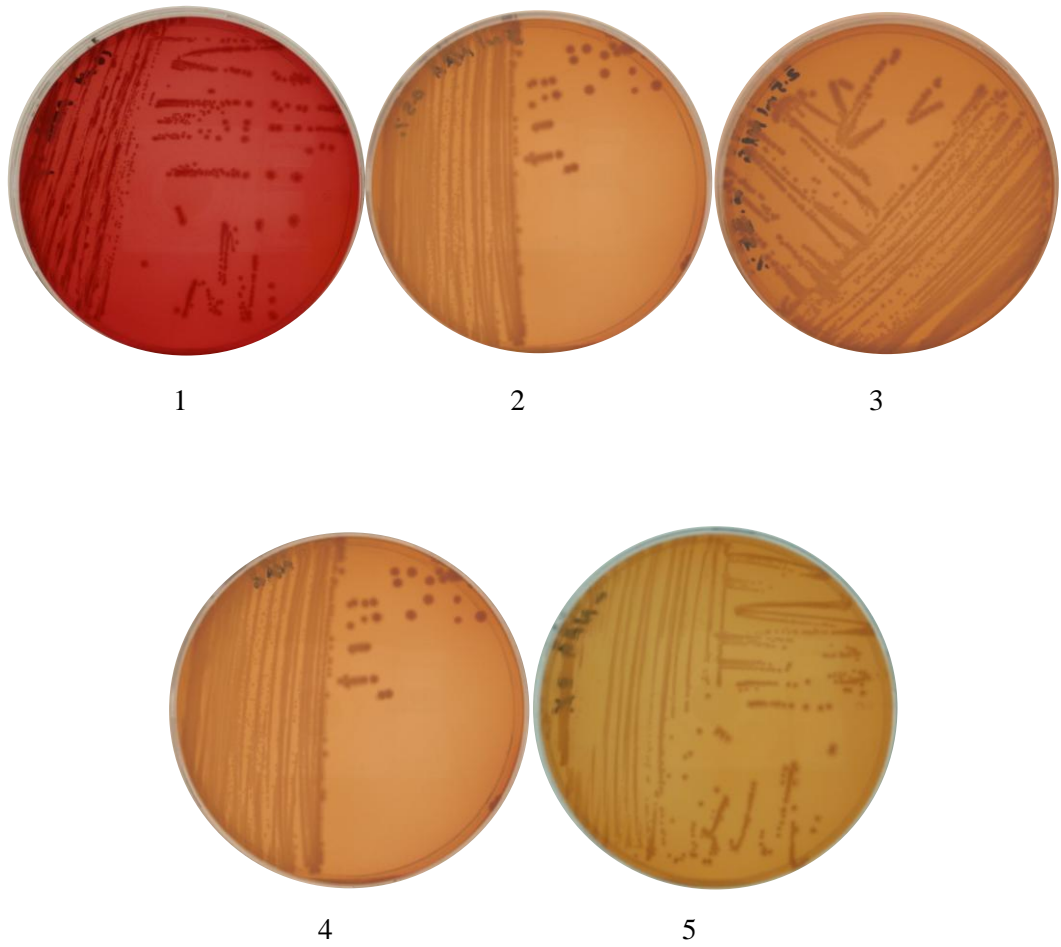


Fig 42: Fermentation of *N*-acetylglucosamine on MacConkey agar by the *E.coli nagE* mutant BW25113. The plates contained the following concentrations of NAG:

(1) 1% NAG. (2) 0.5% NAG. (3) 0.25% NAG. (4) 0.1% NAG. (5) No NAG.

Following transformation of the *nagE* mutant with plasmid isolated from colony 1, clones were isolated on LB agar containing ampicillin and then transferred on to MacConkey agar containing 0.5% *N*-acetylglucosamine. The results obtained showed that the cells could ferment the available *N*-acetylglucosamine, as shown in Figure 43, while no fermentation activity was shown in the plates containing 0.1% *N*-acetylglucosamine or without *N*-acetylglucosamine, as observed in Figures 44 and 45 respectively. In the meantime, since the untransformed cells could not grow in the presence of ampicillin, the transformed cells were compared with the untransformed mutant, on a plate without the antibiotic, and containing 0.5 % *N*-acetylglucosamine. High fermentation activity was shown by the transformed cells compared with the untransformed mutant (Figure 46), which clearly shows the effect of the putative *C.beijerinckii* *N*-acetylglucosamine PTS on the mutant. However, the transformed were able to ferment the *N*-acetylglucosamine for approximately 24 h and then lost the fermentation ability. The experiment was repeated by re-transforming *E.coli* cells using the same plasmid, and selecting again on LB plates containing ampicillin. These colonies were screened again on MacConkey agar containing 0.5% *N*-acetylglucosamine, and the same result of unstable fermentation was shown. The reason for this is unknown, but may be due to the mechanism of regulation of expression of the *nag-pts* genes, which are possibly under the control of an antiterminator which may not function properly in *E.coli*. Nevertheless, the result showed that the putative *C.beijerinckii* *nag-pts* genes potentially do code for an *N*-acetylglucosamine-PTS. Therefore, for further verification, in the next step, the genes *Cbei 4532* and *Cbei 4533* were cloned as an artificial operon which could be expressed in *E.coli* without the need for the putative antiterminator product of the *Cbei 4534* gene.

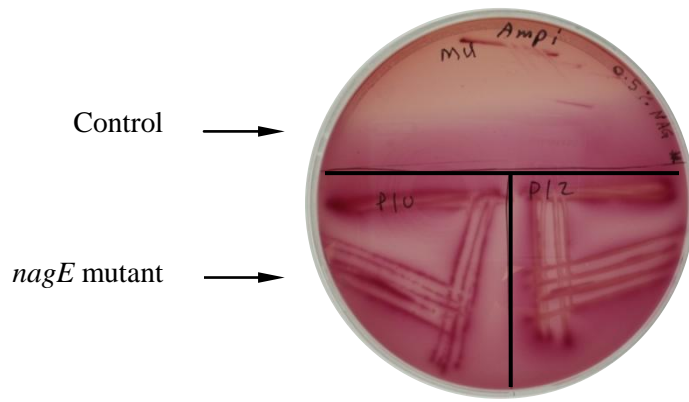


Fig 43: Fermentation of NAG by *E.coli nagE* mutant transformed by plasmid containing *Cbei 4532-4534*. The MacConkey agar contained 0.5% *N*-acetylglucosamine and 50 µg/ml ampicillin, untransformed cells as a control.

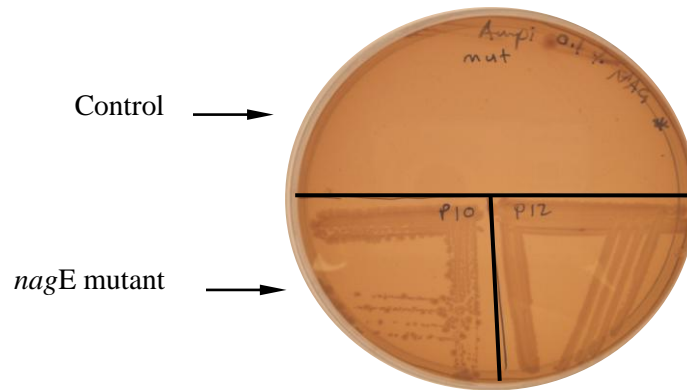


Fig 44: Fermentation of NAG by *E.coli nagE* mutant transformed by plasmid containing *Cbei 4532-4534*. The MacConkey agar contained 0.1% *N*-acetylglucosamine and 50 µg/ml ampicillin, untransformed cells as a control.

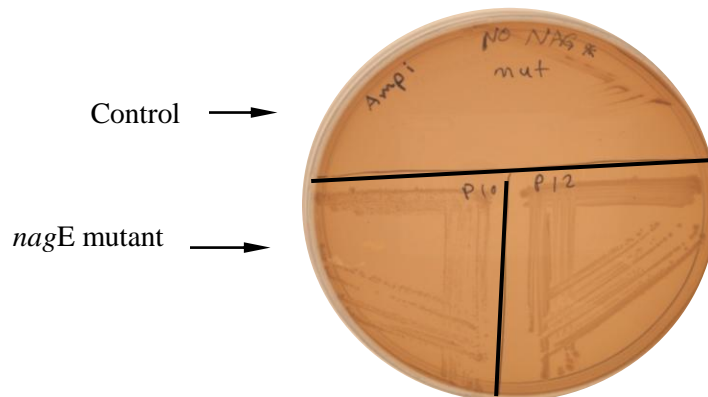


Fig 45: Culture of *E.coli nagE* mutant transformed by plasmid containing *Cbei 4532-4534* on MacConkey agar. The medium contained 50 µg/ml ampicillin but no *N*-acetylglucosamine, untransformed cells as a control.

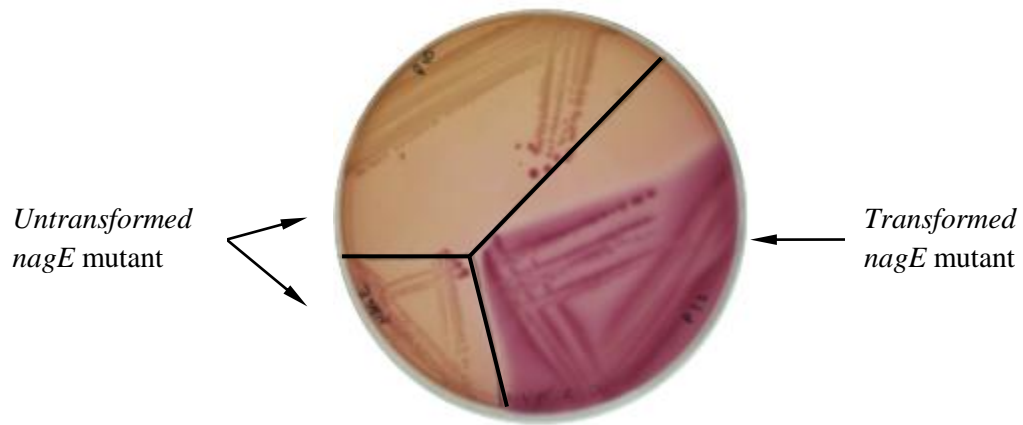


Fig 46: Fermentation of NAG by *E.coli nagE* mutant transformed by plasmid containing *Cbei 4532-4534*. The MacConkey agar contained 0.5% *N*-acetylglucosamine without ampicillin, untransformed cells as a control.

3.6.3. Cloning of the *Cbei 4532* gene

The previous experiment showed that the putative NAG-PTS operon, including genes encoding the domains EIICB, EIIA and the suspected transcriptional antiterminator, *Cbei 4534*, were cloned successfully under *lac* promoter control. Furthermore, by transforming the plasmid into a *nagE* mutant, it appeared that the mutant became capable of fermenting *N*-acetylglucosamine which suggested that the function of these genes was for utilizing *N*-acetylglucosamine. However, due to instability of the fermentation phenotype, another attempt to clone the *Cbei 4532* and *Cbei 4533* genes was carried out, but this time in a way that would result in the genes being in the same orientation. The strategy involved was to clone both genes in the same vector (pJET 1.2 blunt) separately, and then recombine them in a single vector in the same orientation. Both of genes would then form an artificial operon, which could be transferred to a vector in which they could be expressed. During the procedure, the *Cbei 4532* (EIICB) clone was tested by transforming into the *nagE* mutant and screening on MacConkey agar plates containing different concentrations of *N*-acetylglucosamine.

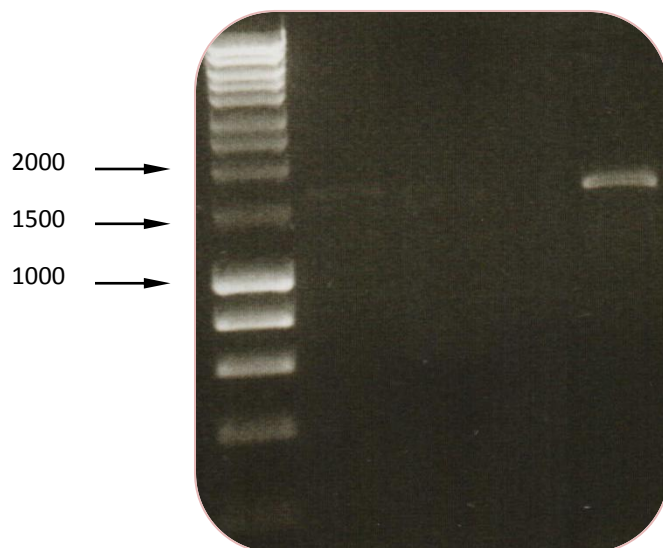


Fig 47: PCR product for *Cbei 4532* gene generated by *Pfu* polymerase by product size (1599 bp).

In order to clone the *Cbei 4532* gene, the pJet 1.2/blunt (Fermentas Life Science JET™ cloning kit K1232) vector was used. This vector has a size of 2,974 bp. A PCR product containing the *Cbei 4532* gene was generated by *Pfu* DNA polymerase, using primers *Cbei 4532*-Fwd and *Cbei 4532*-Rev, as described in the Materials and Methods chapter (2.11.1). The PCR amplification of the gene gave a product of the expected size 1599 bp, as shown in Figure 47. The gene was then cloned into the pJET 1.2/blunt vector and the mixture was transformed into *E.coli* TOP10, plated on LB plates containing 50µg/ml ampicillin and incubated overnight at 37°C. The colonies were transferred onto fresh LB agar plates containing the same amount of ampicillin, in order to obtain stock colonies and incubated again at 37 °C overnight. From the colonies grown, the DNA was extracted, as described in Materials and Methods, section 2.12.1, and screened by PCR screening, as shown in Figure 48. The screening result showed bands from different colonies (4 from 70 clones screened) with the same size as the cloned *Cbei 4532* gene, which indicated that the DNA fragment was successfully inserted into the pJet 1.2/blunt vector.

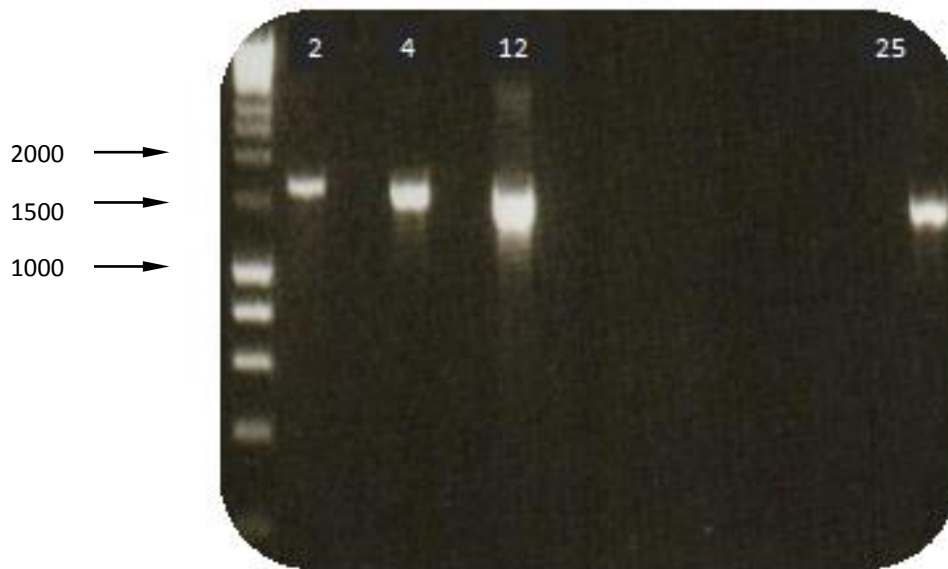


Fig 48: Screening of *E.coli* clones containing *Cbei 4532*. The figure shows the colonies screened and the cloned product of 1599 bp was obtained in lanes 2, 4, 12 and 25, while the negative samples do not shown any band in other lanes.

3.6.4. Determination of gene orientation of *Cbei 4532* clones

In order to find out the orientation of the *Cbei 4532* DNA fragment in the vector, PCR was performed as described in Materials and Methods, Section 2.14. The T7 promoter primer was used either with *Cbei 4532*-Fwd primer or with *Cbei 4532*-Rev primer. As shown in Figure 49 (a, b), the band that appeared was obtained from the PCR which contained *Cbei 4532*-Rev primer and T7 promoter primer, indicating that the *Cbei 4532* gene had been cloned under control of the T7 promoter in the vector. Since it would have been useful to obtain clones in which the *Cbei 4532* gene was under control of the *lac* promoter in the vector, in order to allow expression in the *nagE* mutant, the cloning procedure was repeated twice and more clones were screened. However, in all cases, the same result was obtained, and only clones with *Cbei 4532* under control of the T7 promoter were isolated.

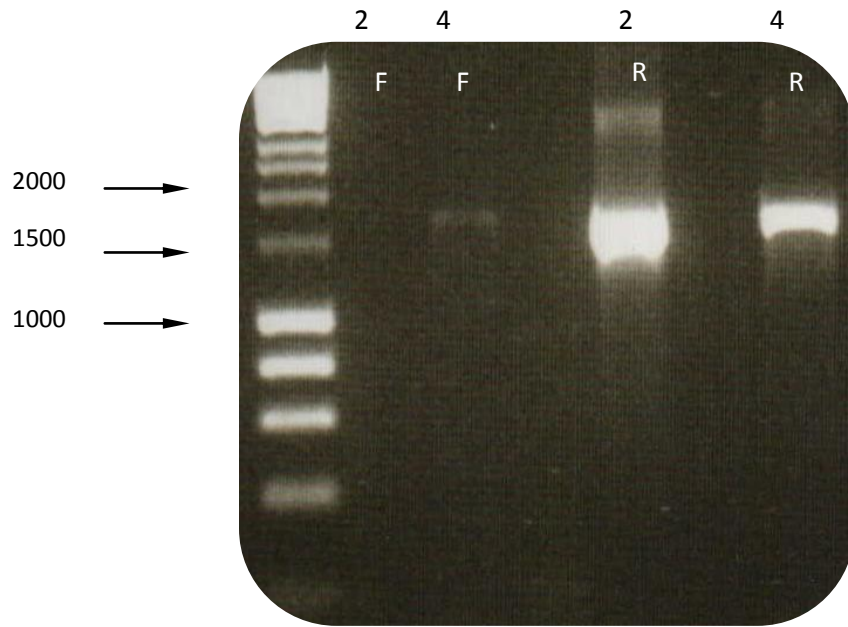


Fig 49 a: Determination of gene orientation of *Cbei 4532* clones (2, 4) determined by using *Cbei 4532*-Fwd (F) and *Cbei 4532*-Rev (R) with T7 promoter primer.

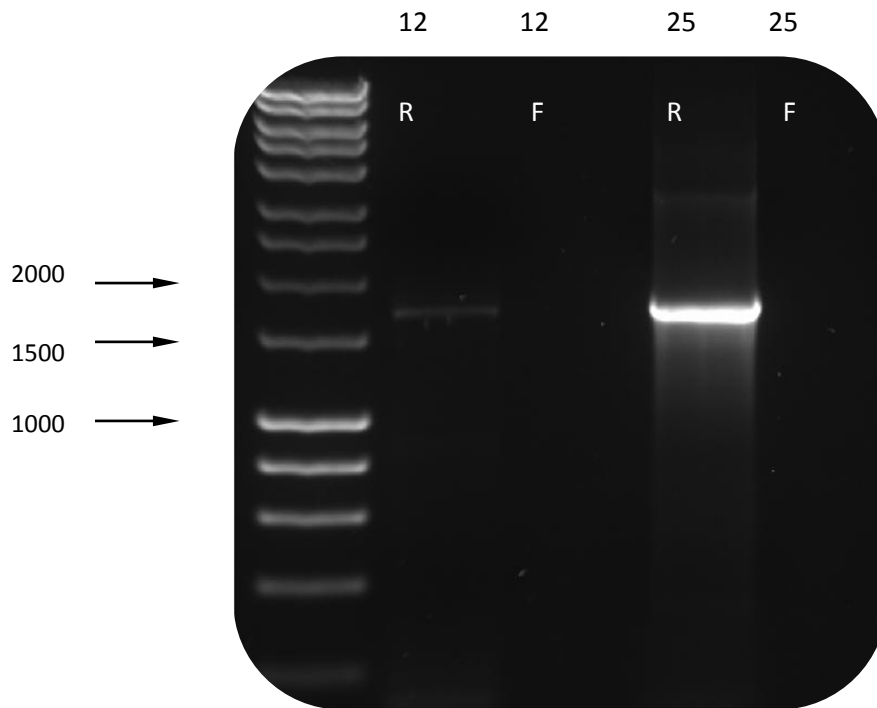


Fig 49 b: Determination of gene orientation of *Cbei 4532* clones (12, 25) determined by using *Cbei 4532*-Fwd (F) and *Cbei 4532*-Rev (R) primers with T7 promoter primer.

3.6.5. Plasmid purification of *Cbei 4532* clones

The plasmids from the recombinant cells obtained in the cloning stage were purified using the gene JET plasmid Miniprep kit, and then screened using gel electrophoresis, which indicated that the plasmid was purified successfully. Nevertheless, for sequencing purposes the plasmid was purified in a higher yield, using the Qiagen Midi-kit, as described in Materials and Methods, Section 2.17, and then screened, also using gel electrophoresis. Plasmids 2 and 25 were sent for sequencing to Beckman Coulter Genomics and the correctness of the sequence of the cloned gene was confirmed as 100% identity, as shown in Appendix 1.

3.6.6. Transformation of *Cbei 4532* plasmid into *nagE* mutant

After the *Cbei 4532* gene was cloned successfully, the plasmids 2 and 25 were transformed into the *E.coli nagE* mutant BW25113, and the fermentation phenotype with different concentrations of *N*-acetylglucosamine was examined as described previously, in section 3.6.2.

As shown in Figure 50, the mutant transformed with the *Cbei 4532* gene which provided the domains EIICB behaved in the same way as the mutant before transformation; that is fermentation positive phenotype was obtained at a concentration of 1% NAG and negative phenotype at lower concentrations. It was possible that the gene *Cbei 4532* was not expressed under the T7 promoter control. However, even if some leaky expression did take place, the protein encoded by *Cbei 4532* contains only the EIIC and EIIB domains of the PTS, and lacks a EIIA domain. Fermentation of *N*-acetylglucosamine could therefore be dependent on a compatible IIA domain being present in the *E.coli* strain.

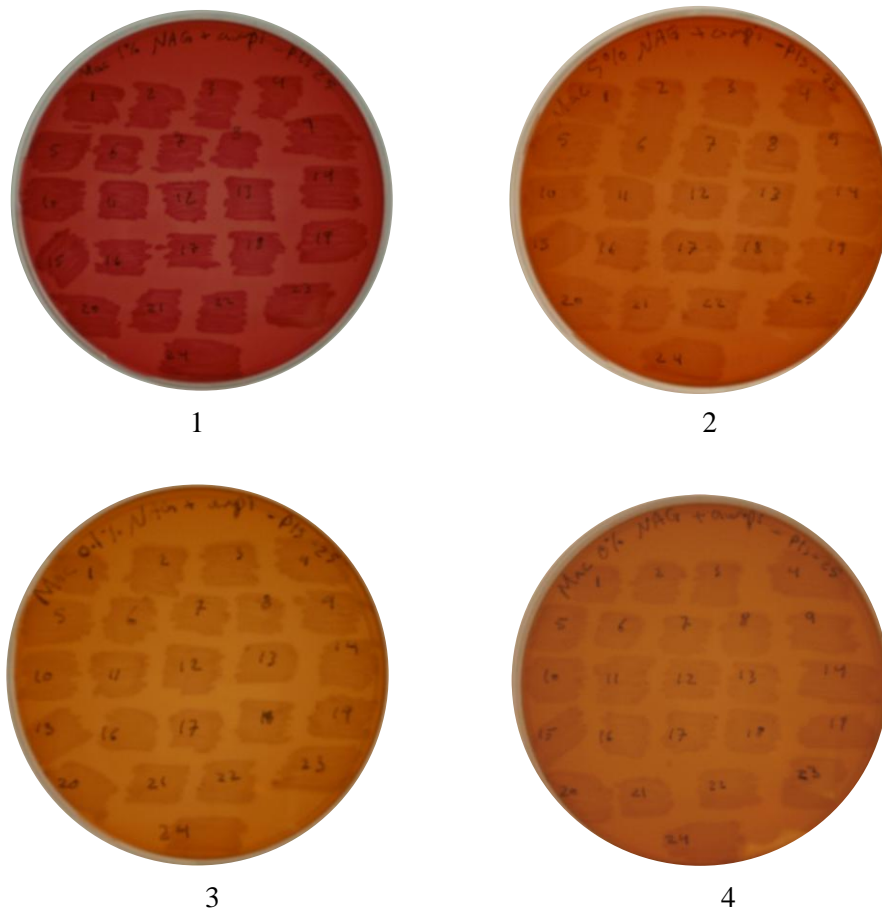


Fig 50: Fermentation of *N*-acetylglucosamine on MacConkey agar by an *E.coli nagE* mutant transformed with plasmid carrying the *Cbei 4532* gene. The plates contained the following concentration of NAG:

(1) 1% NAG. (2) 0.5% NAG. (3) 0.1% NAG. (4) No-NAG.

3.6.7. Cloning of the *Cbei 4533* gene

Since insertion of the *Cbei 4532* gene clone into the *E.coli nagE* mutant did not result in a positive fermentation phenotype, it was decided to proceed with cloning of the *Cbei 4533* gene.

The concept of this experiment was to clone the *Cbei 4533* gene and then join it to the *Cbei 4532* gene in order to design an artificial operon containing both genes in the same orientation, thus providing the required domains for PTS activity, EIICB and EIIA together. This artificial operon could then be tested for complementation of the *E.coli N*-acetylglucosamine mutant, as in the previous experiment.

The gene *Cbei 4533* was cloned using the same vector and procedure as described for *Cbei 4532*. The *Pfu* PCR product generated using primers *Cbei 4533*-Fwd and *Cbei 4533*-Rev had a size around 600 bp (expected size 649 bp), as shown in Figure 51. The PCR product was then cloned into TOP10 *E.coli* competent cells and the clones were screened for presence of the inserted gene (Figure 52), and then the gene orientation was determined using the T7 promoter primer with the *Cbei 4533* Fwd and *Cbei 4533* Rev primers (Figure 53). Two clones were found to be under T7 promoter control, since a PCR product was obtained with the reverse cloning primer and another two were under control of the *lac* promoter, since a PCR product was obtained with the forward cloning primer. Finally, plasmids were purified by the same protocols used previously for clones of *Cbei 4532* and screened by gel electrophoresis, and all four plasmids were sent for sequencing. All sequences gave 100% match to the sequences of the *Cbei 4533* gene, as shown in Appendix 1.

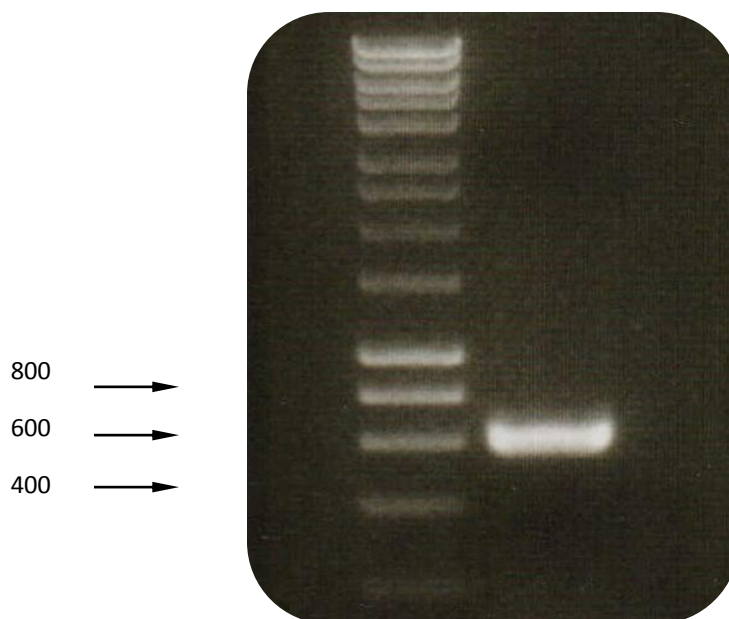


Fig 51: PCR product for *Cbei 4533* gene generated by *Pfu* polymerase.

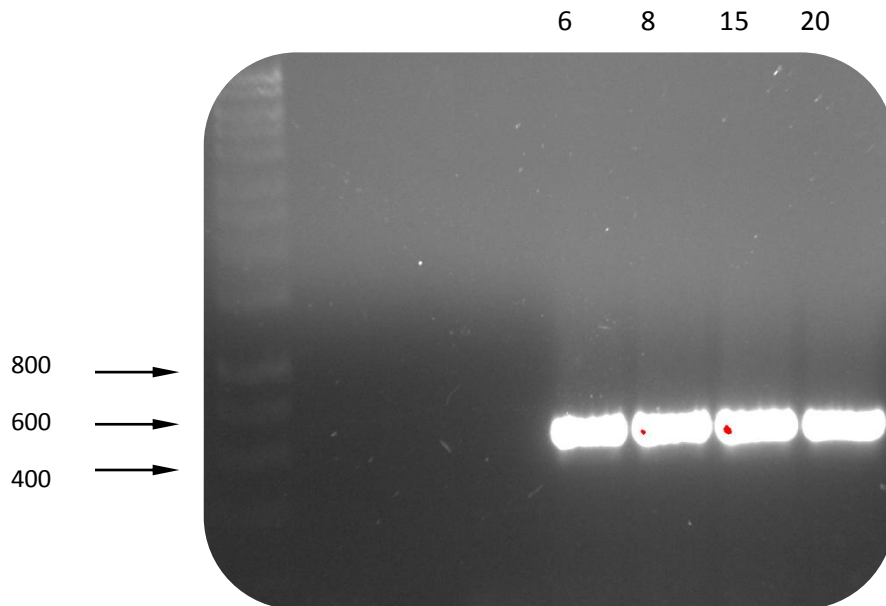


Fig 52: Screening of *E. coli* clones containing *Cbei 4533*. The figure shows 8 colonies screened and the cloned product was obtained in lanes 6, 8, 15 and 20, with product size around 600 bp, while the empty lanes contained unsuccessfully cloned samples.

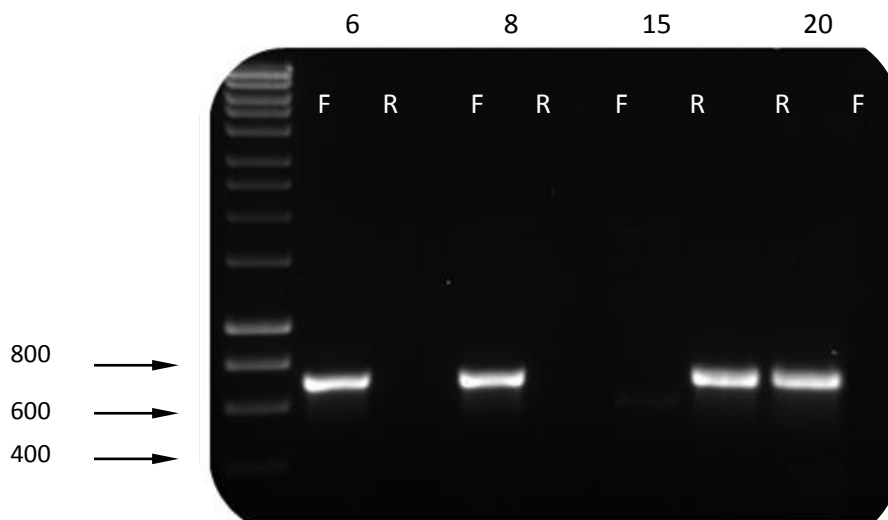


Fig 53: Determination of gene orientation of *Cbei 4533* clones (6, 8, 15, and 20) determined by using *Cbei 4533*-Fwd (F) and *Cbei 4533*-Rev (R) primers with T7 promoter primer.

3.7. Recombination of the *Cbei 4532* and *Cbei 4533* genes

As demonstrated in the previous experiments, the *Cbei 4532* and *Cbei 4533* genes were successfully cloned in the pJET 1.2 blunt vector. Following verification of the sequences of the cloned genes, the vectors containing the *Cbei 4532* and *Cbei 4533* genes were restriction digested as described in Material and Methods section 2.21.1. The vector carrying the *Cbei 4532* gene was double digested with *SalI* and *XhoI* to release the gene on a DNA fragment of around 1600 bp (Figure 54), which was purified as shown in Figure 55.

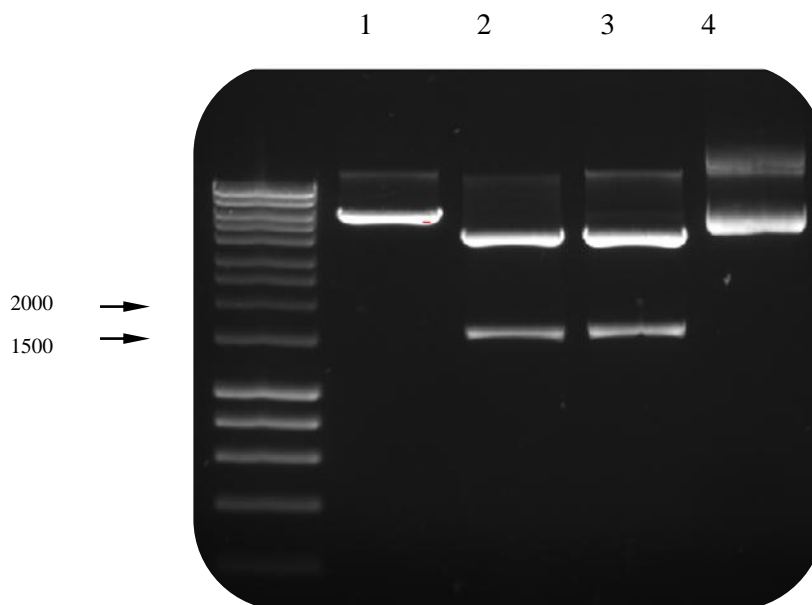


Figure 54: The *Cbei4532* DNA fragment restricted from the pJET 1.2 blunt vector using *SalI* and *XhoI*. Plasmid was digested with (1) *SalI*, (2, 3) *SalI* and *XhoI*. (4) Undigested pJET 1.2 blunt plasmid containing *Cbei4532* as a control.

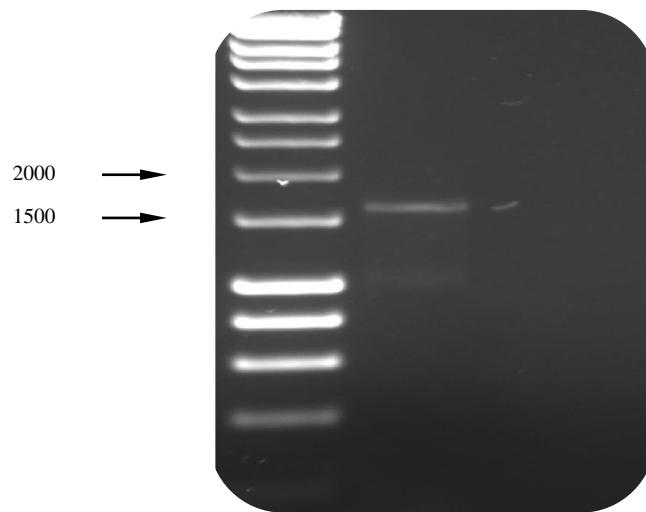


Figure 55: Purification of *Cbei4532* *SalI* - *XhoI* DNA fragment.

In a similar procedure, the pJET 1.2 blunt vector containing the *Cbei4533* gene was double – digested with *SalI* and *XhoI*. Then the fragment carrying *Cbei 4532* could be inserted. As shown in Figure 56, when the plasmid was cut with *SalI* and *XbaI*, a fragment of approximately 600 bp was released, confirming the presence of the *Cbei 4533* insert. Digestion with either *SalI* or *XhoI* (not shown) resulted in a single product of around 3500 bp, while the double digest produced a similar product as expected. After purifying the double – digested *Cbei 4533* vector, as shown in Figure 57, it was used for ligation with the DNA fragment containing *Cbei4532*, as described in Materials and Methods (2.21.3), to obtain a product carrying both *C.beijerinckii nag-pts* genes. A sample of the ligation mixture was analysed by agarose gel electrophoresis (Figure 58).

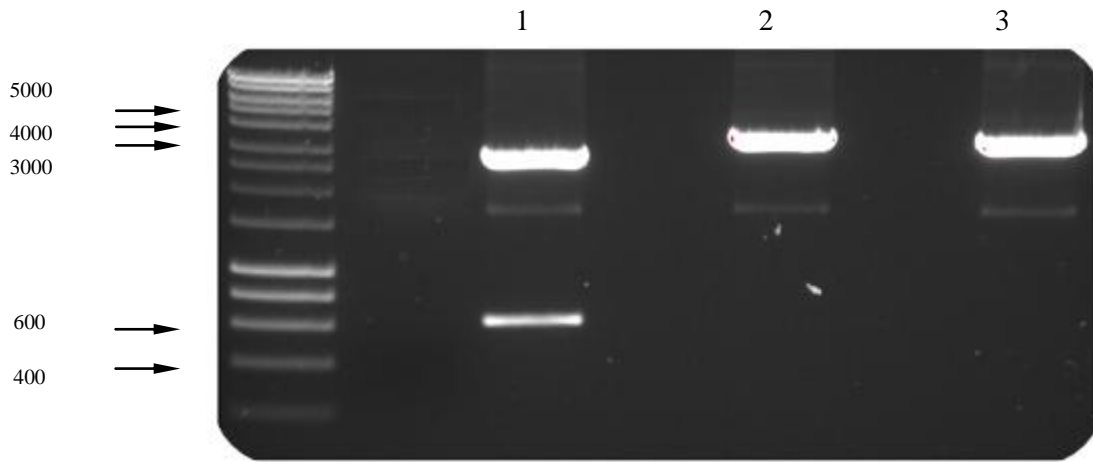


Figure 56: Restriction of the pJET1.2 blunt vector containing *Cbei4533* gene (1) *SalI* with *XbaI*, showing the *Cbei4533* gene fragment of approximately 600 bp, (2) *SalI* (3) *SalI* and *XhoI*.

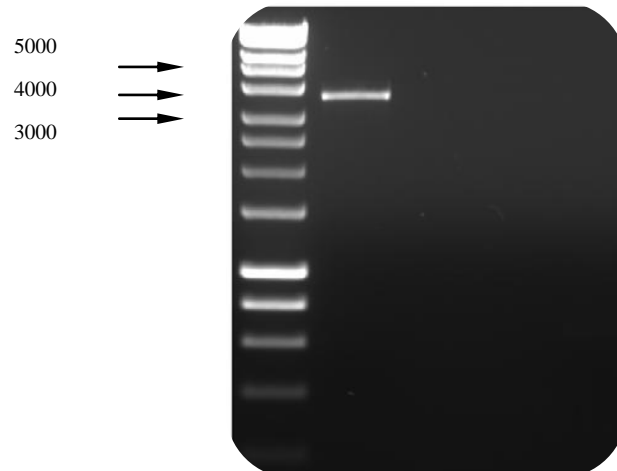


Figure 57: Purification of the pJET 1.2 blunt *Cbei4533* vector after double digestion with restriction enzymes *SalI* and *XhoI*.

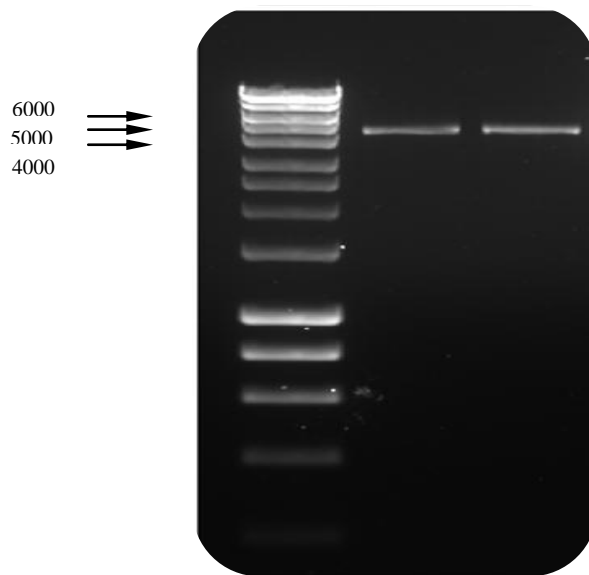


Figure 58: pJET 1.2 blunt vector, including the *Cbei4532* and *Cbei4533* genes after ligation.

The recombinant was transformed into *E.coli* TOP10 competent cells, and plasmid was purified from a single colony using the large-scale midiprep protocol, as described in Materials and Methods (2.17). To verify that the purified plasmid contained both genes, it was screened by PCR using different combinations of the gene specific forward and reverse primers. As shown in Figure 59, the products obtained were approximately 2100 bp for the whole fragment containing the *Cbei 4532* and *Cbei 4533* genes, 1600 bp for the *Cbei 4532* gene and 600 bp for the *Cbei 4533* gene.

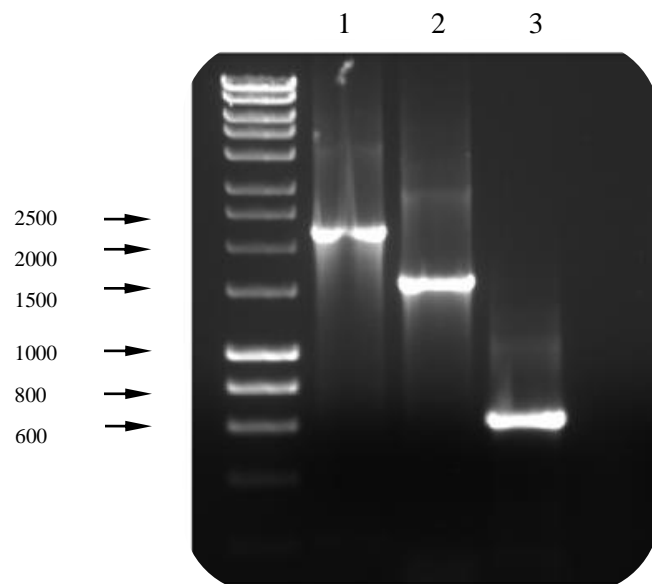


Figure 59: PCR analysis of pJET 1.2 blunt containing *Cbei4533* and *Cbei4532* genes. Primers used were (1) *Cbei4532*-Fwd and *Cbei4533*- Rev. (2) *Cbei4532*-Fwd and *Cbei4532*-Rev. (3) *Cbei4533*-Fwd and *Cbei4533*-Rev.

After the PCR screening had confirmed that the *Cbei4532* and *Cbei4533* genes were ligated together, the plasmid containing both genes was restricted by *Bgl*III and *Xba*I enzymes in order to cut the genes out in one fragment. As shown in Figure 60, the genes *Cbei 4532-4533* were removed in one fragment of approximate size 2100 bp. The fragment obtained was purified and concentrated, as shown in Figure 61.

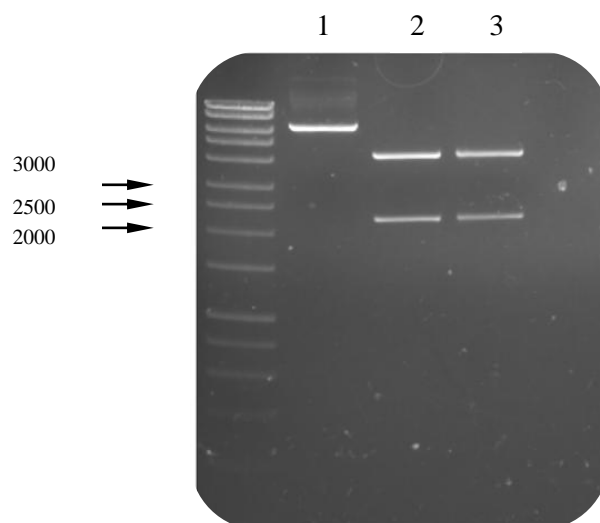


Figure 60: Removal of *Cbei4532* and *Cbei4533* from the pJET 1.2 blunt vector by restriction digestion. (1) Unrestrictied plasmid; (2, 3) *Bgl*III and *Xba*I.

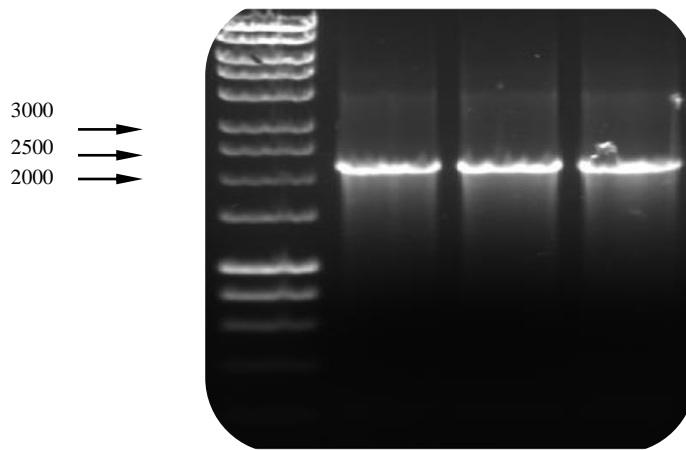


Figure 61: The purified fragment containing *Cbei4532* and *Cbei4533* after restriction from the pJET 1.2 blunt vector.

After the *Cbei 4532-4533* plasmid had been restricted by *Bgl*III and *Xba*I and purified, plasmid pUC18 was digested by *Xba*I and *Bam*HI enzymes, as shown in Figure 62, in order to allow insertion of the *Cbei 4532-4533* gene fragment with expression of the genes under control of the *lac* promoter. Following ligation, the mixture was transformed directly into *E.coli* TOP10 competent cells. As an additional step, the unmodified pUC18 vector was also transformed into *E.coli* TOP 10 competent cells in order to isolate the plasmid for use as a control in subsequent experiments. Plasmids were purified by the large-scale procedure and screened by gel electrophoresis, as shown in Figure 63.

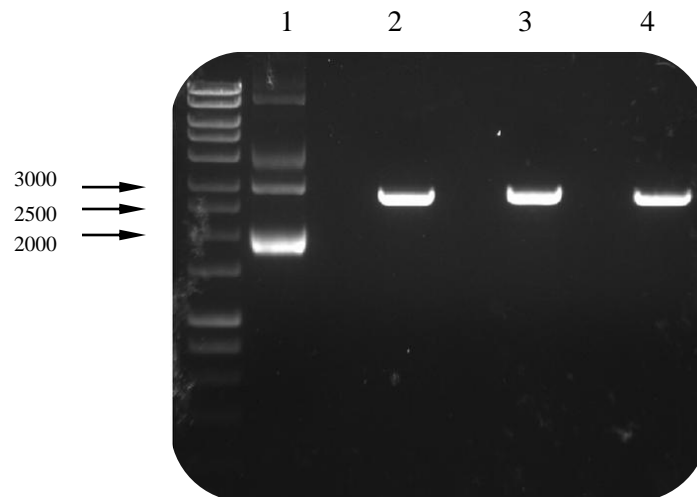


Figure 62: Restriction digestion of pUC18 plasmid. (1) Uncut plasmid. (2) After restriction by *Xba*I. (3) After restriction by *Bam*HI. (4) After restriction by *Xba*I and *Bam*HI.

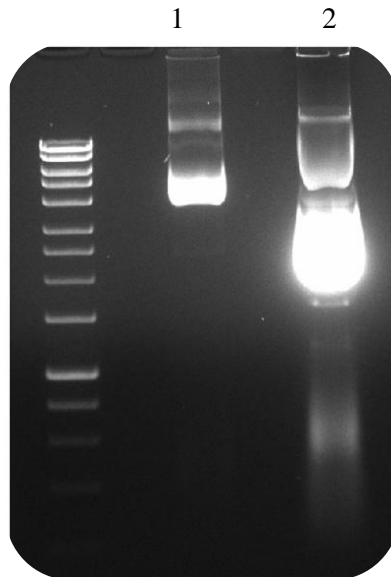


Figure 63: Preparation of plasmids from *E.coli* TOP10. (1) pUC18 vector. (2) pUC18 vector containing *Cbei4532* and *Cbei4533* genes.

3.8. Functional characterization of the *Cbei4532* and *Cbei4533* genes.

The pUC18 plasmid carrying the *Cbei4532* and *Cbei4533* genes and also non-recombinant pUC18 were transformed into the *E.coli nagE* mutant BW25113, as described in Materials and Methods (2.19), and plated on MacConkey agar containing 0.5% of *N*-acetylglucosamine and 50 µg/ml ampicillin. As shown in Figure 64, the transformed cells which contained *Cbei4532* and *Cbei4533* showed a positive fermentation phenotype, and this phenotype was stable for more than 48h at 37°C. On the other hand, cells transformed with the pUC18 vector without the *Cbei4532* and *Cbei4533* genes did not show any fermentation activity as demonstrated on the same plate. It is clear that these results reflected the function of these genes, *Cbei4532* and *Cbei4533*, to encode a *N*-acetylglucosamine PTS allowing uptake and phosphorylation of the amino sugar by the *nagE* mutant.

As a confirmation test, both the complemented and control strains were inoculated into LB broth medium containing 0.5% *N*-acetylglucosamine and 50 µg/ml ampicillin, and then growth and sugar utilization was followed, as described in Materials and Methods 2.6.3. The results shown in Figure 65 indicate that the cells containing the *Cbei 4532* and *Cbei 4533* genes were able to utilize the available *N*-

acetylglucosamine in the medium, while little or no utilization was recorded by the cells transformed with pUC18 only. This confirmed the conclusion based on the fermentation phenotype that the genes *Cbei4532* and *Cbei4533* encoded for *N*-acetylglucosamine uptake.

In addition, a PTS activity assay was carried out using extracts prepared from the recombinant and control *E.coli nagE* strains, as described in Materials and Methods 2.9. As the results show in Figure 66, PTS activity was observed in the extract of cells containing the *Cbei4532* and *Cbei4533* genes while no activity was seen in the extract of cells containing pUC18 only. These results were clearly compatible with the previous results demonstrating utilization of *N*-acetylglucosamine by the recombinant strain, and provided further evidence that the *Cbei4532* and *Cbei4533* genes encode a *N*-acetylglucosamine PTS.

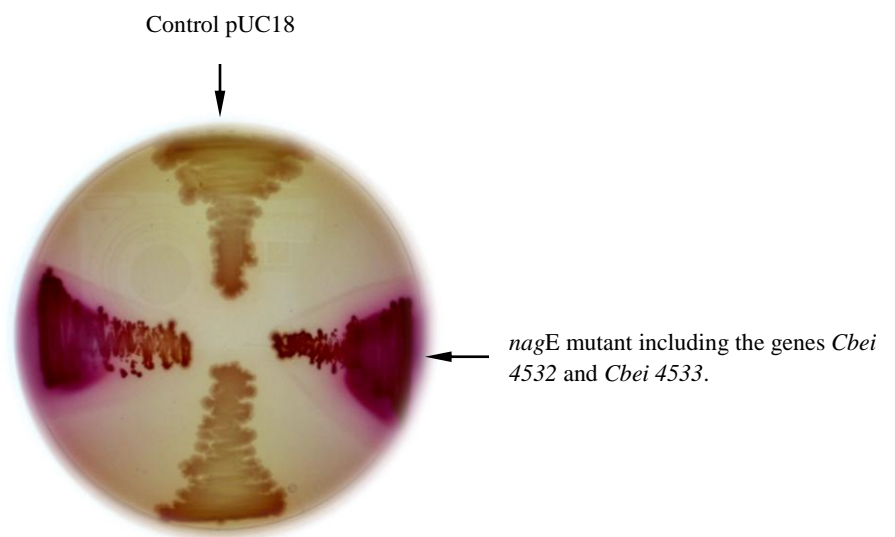


Figure 64: Fermentation phenotype of transformed *E.coli nagE* mutant on MacConkey agar containing 0.5% *N*-acetylglucosamine and 50 µg/ml ampicillin. Cells were transformed with pUC 18 carrying the *Cbei4532* and *Cbei4533* genes, and non-recombinant pUC 18 as a control.

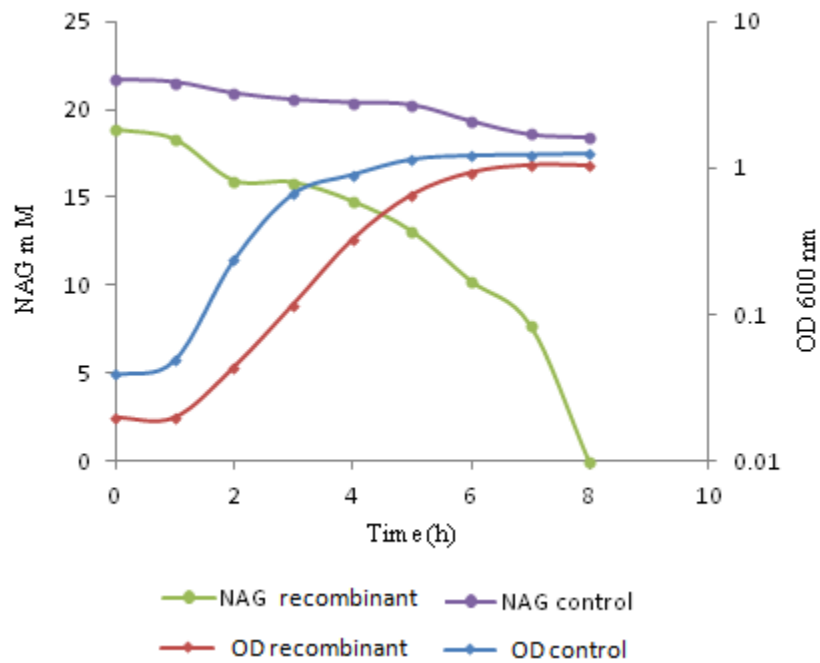


Figure 65: *N*-acetylglucosamine utilization by transformed *E.coli nagE* mutant in LB broth. Cells were transformed with recombinant pUC18 plasmid carrying the *Cbei4532* and *Cbei4533* genes (recombinant) and non-recombinant pUC18, as a control.

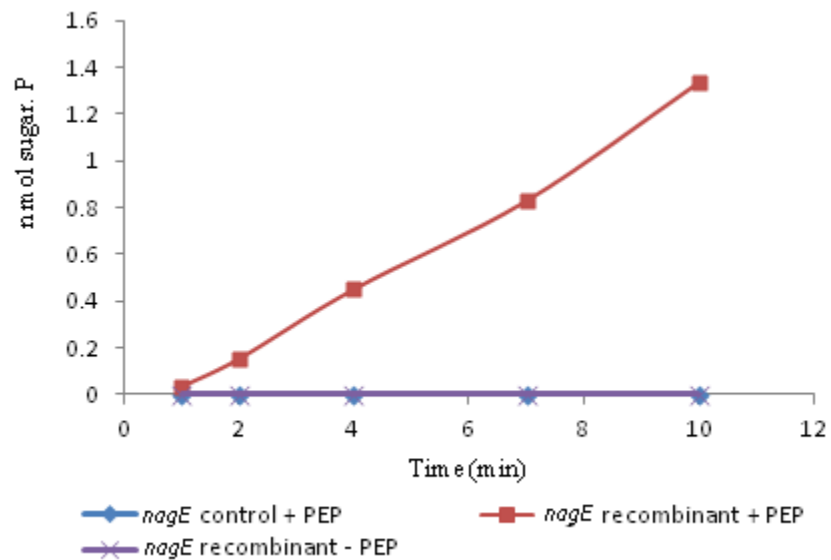


Figure 66: PTS activity assay for extracts of transformed *E.coli nagE* mutant. Cells were transformed by pUC18 plasmid carrying the *Cbei4532* and *Cbei4533* genes (recombinant) and non-recombinant pUC18 as a control. All assays contained PEP. No activity was observed for either extract in the absence of PEP.

3.9. Characterization of the *C.beijerinckii* N-acetylglucosamine PTS.

Cloning of the genes encoding the *C.beijerinckii* N-acetylglucosamine PTS allowed for further examination of the properties of the phosphotransferase system. Therefore, the recombinant pUC18 vector carrying *Cbei 4532* and *Cbei 4533* was transformed into *E.coli* mutant *ZSC113* with inactivated glucokinase (encoded by *glk*), and glucose and mannose phosphotransferase activities (encoded by *ptsG* and *ptsM* respectively) (Curtis and Epstein, 1975). The mutant is therefore unable to phosphorylate glucose or mannose, and gives a negative fermentation phenotype for these sugars on MacConkey agar. Transformants were selected and streaked on a MacConkey agar plate containing 0.5% glucose and 50 µg/ml ampicillin. The same transformed *E.coli* *ZSC113* mutant was also streaked on a MacConkey agar plate containing 0.5% mannose and 50 µg/ml ampicillin.

The result in Figure 67 shows considerable fermentation activity on glucose while no fermentation activity was observed when the mutant was transformed by the control pUC18 plasmid. On the other hand, no fermentation of mannose was observed for either strain (Figure 68). These results demonstrated that the PTS encoded by *Cbei 4532* and *Cbei 4533* can transport and phosphorylate glucose, but not mannose as a substrate.

In addition, a glucose PTS activity assay was also carried out using extracts prepared from the recombinant and control *E.coli* *ZSC113* strains, by the same method described in Materials and Methods 2.9. As shown by the results in Figure 69, glucose PTS activity was observed in the extract of cells containing the *Cbei4532* and *Cbei4533* genes, while no activity was seen in the extract of cells containing pUC18 only. It was therefore demonstrated that in addition to phosphorylating N-acetylglucosamine the PTS encoding by *Cbei4532* and *Cbei4533* was also capable of phosphorylating glucose.

To confirm that both N-acetylglucosamine and glucose were recognised by the same PTS, as had been observed in assays using *C.beijerinckii* crude extracts (chapter 3), the effect of 10 mM glucose on N-acetylglucosamine phosphorylation was examined

using the extract of the *nagE* recombinant strain (Figure 70). The inhibitory effect of glucose was consistent with this conclusion.

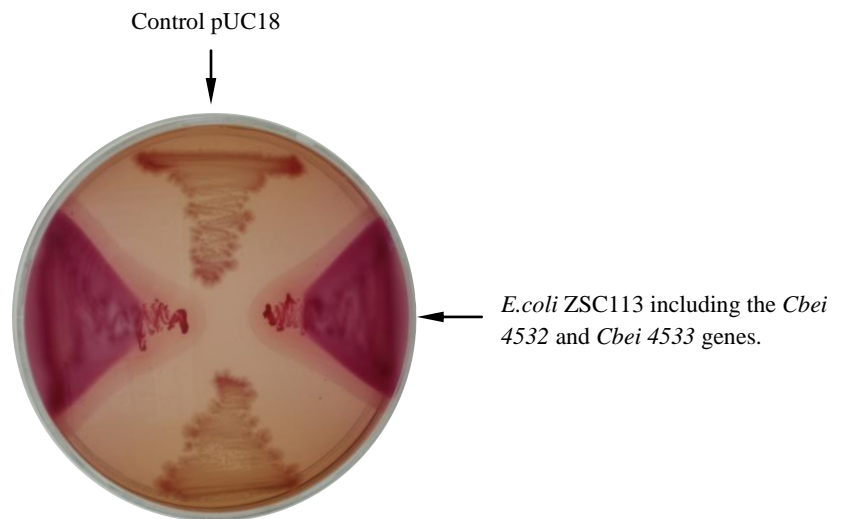


Figure 67: Fermentation phenotype of transformants of *E. coli* ZSC113 grown on MacConkey agar containing 0.5% glucose and 50µg/ml ampicillin. Cells were transformed with pUC18 carrying the *Cbei 4532* and *Cbei 4533* genes, and non-recombinant pUC18 as a control.

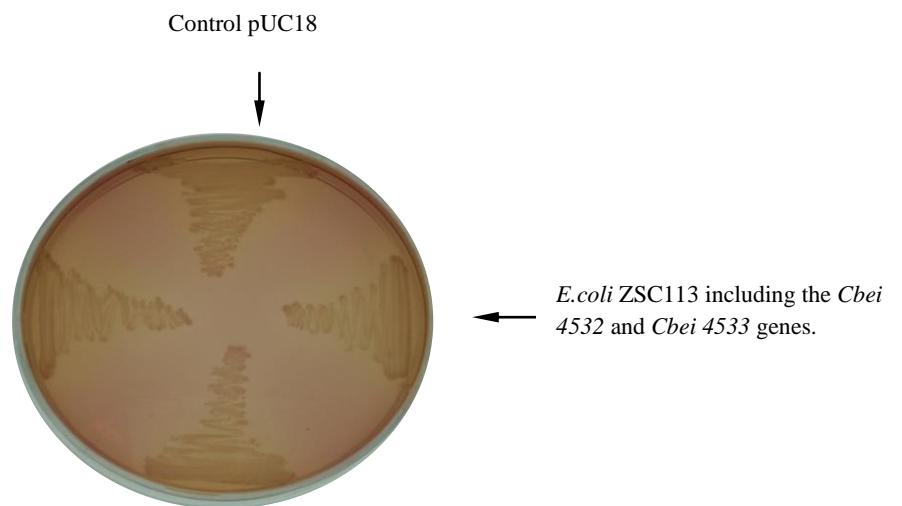


Figure 68: Fermentation phenotype of transformed of *E. coli* ZSC113 grown on MacConkey agar containing 0.5% mannose and 50µg/ml ampicillin. Cells were transformed with pUC18 plasmid carrying the *Cbei 4532* and *Cbei 4533* genes, and non-recombinant pUC18 as a control.

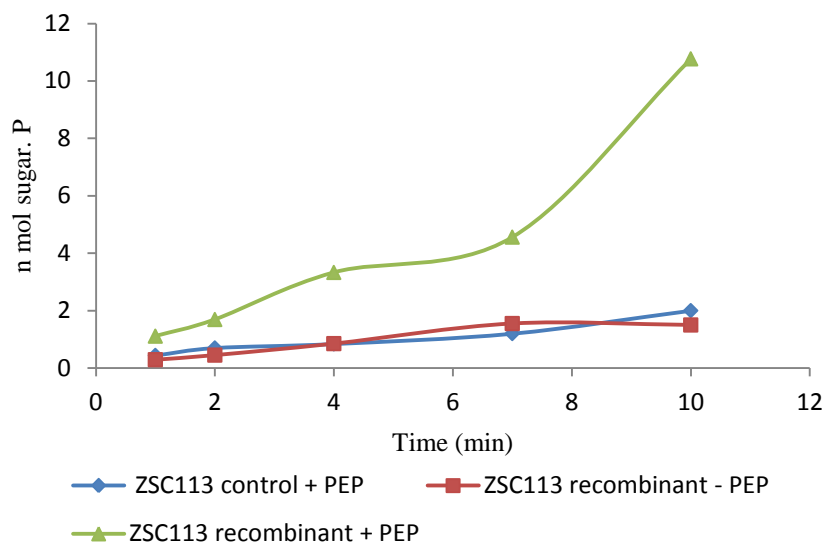


Fig 69: Glucose PTS activity assay for extracts of transformed *E.coli* ZSC113. Cells were transformed by pUC18 carrying the *Cbei4532* and *Cbei4533* genes (recombinant) and non-recombinant pUC18 as a control. No activity was observed for the control extract in the absence of PEP.

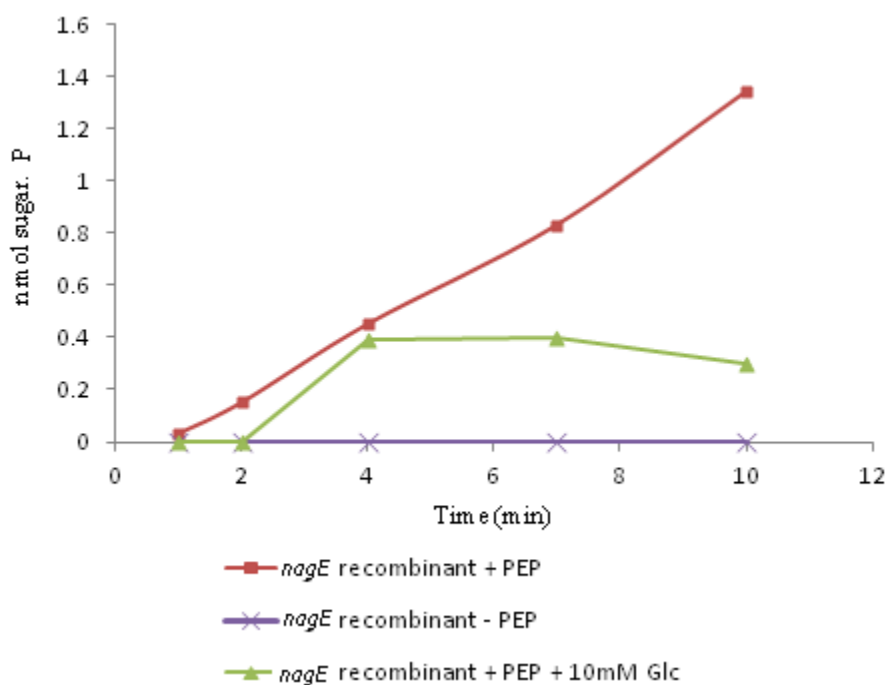


Fig 70: The inhibitory effect of 10 mM glucose on *N*-acetylglucosamine PTS activity in extract of an *E.coli* *nagE* recombinant. Cells were transformed by pUC18 carrying the *Cbei4532* and *Cbei4533* genes. *N*-acetylglucosamine PTS activity was assayed with and without adding 10 mM glucose. No activity was observed in the absence of PEP.

CHAPTER4

DISCUSSION

4. Discussion

Recently, biotechnology applications have supported many processes around the world, proving successful in several areas such as health, environmental and industrial applications. The biofuel industry has been considered an attractive branch of biotechnology, due to the environmental and economic advantages, and the fact that it uses renewable energy sources. The majority of waste products consist of several carbohydrates, which can be used as a sustainable fermentable carbon source for clostridia to produce the chemicals acetone and butanol. *N*-acetylglucosamine is a carbon source that is widely prevalent in environmental waste as a monomer of chitin. Understanding the molecular basis of chitin hydrolysis and utilization of *N*-acetylglucosamine and the factors which control these activities will give a clearer view of the potential for both *N*-acetylglucosamine and chitin utilization by solventogenic clostridia. This may lead to improving the ABE industry in the future by enabling it to use the biomass containing *N*-acetylglucosamine as a raw material.

A variety of transport systems have been identified for sugar transport in bacteria. However, the PTS is the only transport system which uses phosphoenolpyruvate (PEP) as an energy source, and is considered as an important mechanism of sugar uptake to support bacterial growth. The majority of solventogenic clostridia depend on the phosphotransferase system (PTS) for the utilization of different sugars including glucose, which is usually used as a preferred carbon source that can regulate the metabolism of other sugars. For example, it was shown by Tangney *et al.*, (2001) that when *C.acetobutylicum* ATCC 824 was grown in a culture containing maltose, the strain was capable of utilizing the sugar as a carbon source, accumulating it by the PT- transport system. However, the presence of glucose in the same growth culture led to inhibition of maltose uptake, and the cells started to utilize the maltose only after the preferred glucose was used up. The same regulatory pattern was also shown in *C.acetobutylicum* ATCC 824 for utilization of sucrose and lactose (Tangney and Mitchell, 2000; Yu *et al.*, 2007). The effect of glucose on utilization of some carbon sources by *C.beijerinckii* has also been

demonstrated. For example, it was reported that in a growth culture containing both glucose and glucitol, the presence of glucose led to prevention of glucitol uptake (Mitchell, 1996; Tangney *et al.*, 1998a). Glucose has therefore been shown to exert regulation and control over the metabolism of several alternative carbon sources in the solventogenic clostridia.

In this study, the results established showed clearly that *N*-acetylglucosamine can be utilized as a carbon source by *C.beijerinckii* cells depending on PTS activity. However, when the cells were grown in the presence of both *N*-acetylglucosamine and glucose, in the same growth culture, it was observed that glucose did not have an inhibitory effect, and that *N*-acetylglucosamine and glucose were utilized together, at the same time. Furthermore, the same results were obtained by using different concentrations of sugars or after pre-growing the cells in cultures containing either glucose or *N*-acetylglucosamine. In a parallel study, the same behaviour was observed for *C.acetobutylicum* cells growing in Reinforced Clostridial Medium (RCM) and Clostridial Basal Medium (CBM) containing *N*-acetylglucosamine and glucose as carbon sources. The *C.acetobutylicum* strain showed the same capability as *C.beijerinckii* to utilize *N*-acetylglucosamine only or *N*-acetylglucosamine and glucose in the same growth culture (Appendix 4). It is therefore clear that both these solventogenic clostridia are capable of co-utilizing *N*-acetylglucosamine and glucose when these are available in the same growth culture, which indicates that genes concerned with *N*-acetylglucosamine utilization must be expressed in the presence of glucose. The co-utilization of *N*-acetylglucosamine and glucose was also shown by Imada *et al.*, (1977) who grew *S.aureus* mutant 209P in separate cultures containing glucosamine with glucose and *N*-acetylglucosamine with glucose. The result showed that the glucosamine uptake was inhibited in presence of glucose, while no inhibition effect was reported for *N*-acetylglucosamine utilization.

Although PTS activity for *N*-acetylglucosamine has not been reported previously in the clostridia, *N*-acetylglucosamine phosphotransferase systems have been described in other bacteria such as *E.coli* (Plumbridge, 1990), *Bacillus subtilis* (Bertram *et al.*, 2011; Mobley *et al.*, 1982), *Staphylococcus aureus* (Imada *et al.*, 1977), *Streptomyces olivaceoviridis* (Wang *et al.*, 2002) and *Caulobacter*

crecenscentus (Eisenbeis *et al.*, 2008). Investigation of the mechanism of *N*-acetylglucosamine uptake by *C.beijerinckii* revealed the presence of a phosphotransferase system involved in its uptake and phosphorylation. The dependence of the phosphorylation of *N*-acetylglucosamine on phosphoenolpyruvate (PEP) was determined in an extract of cells grown on *N*-acetylglucosamine. It was shown that *N*-acetylglucosamine can be phosphorylated in the presence of PEP, which indicates that the transport mechanism of *N*-acetylglucosamine by *C.beijerinckii* involves PTS activity. Since no phosphorylation was observed in the presence of ATP, the PT-system is likely to be the only system for *N*-acetylglucosamine uptake, and *N*-acetylglucosamine can therefore be added to the list of sugars that are known substrates of the PTS in the clostridia. However, not all sugars are transported by the PTS as described in the Introduction (1.8). For example, Mitchell, (1996) studied galactose uptake and metabolism in *C.beijerinckii*, and found that phosphorylation was dependent on ATP rather than PEP. Similarly, a non-PTS route for uptake of xylose and arabinose is used by both *C.acetobutylicum* and *C.beijerinckii* (Servinsky *et al.*, 2010; Gu *et al.*, 2010).

Like other phosphotransferase systems, the *N*-acetylglucosamine PTS in *C.beijerinckii* was shown to require both soluble and membrane-bound proteins. These proteins were apparently present in extracts of cells grown on glucose, which were found to be able to phosphorylate *N*-acetylglucosamine. Therefore, it has been clearly shown that glucose does not repress synthesis of the *N*-acetylglucosamine PTS, consistent with the growth studies. The presence of unlabeled glucose in the *N*-acetylglucosamine phosphotransferase reaction mixture resulted in inhibition of the *N*-acetylglucosamine PTS activity, suggesting that the *N*-acetylglucosamine PTS could also recognize glucose as a substrate. PEP-dependent glucose phosphorylation activity was also found to be inhibited by unlabeled *N*-acetylglucosamine, which can be considered as further evidence for overlapping substrate specificity of the phosphotransferase(s) responsible for *N*-acetylglucosamine and glucose phosphorylation in *C.beijerinckii*. The results imply that the *N*-acetylglucosamine PTS of *C.beijerinckii* is a member of the glucose family rather than the lactose-diacetylchitobiose family which includes systems that

transport chitobiose, particularly since no inhibitory effect was seen on the *N*-acetylglucosamine PTS activity by adding unlabeled chitobiose. This is most likely because the *C.beijerinckii* *N*-acetylglucosamine PTS does not recognize chitobiose as a substrate. This result concurs with the study by Keyhani and Roseman (1997), which showed that chitobiose PTS also belongs to the lactose/chitobiose branch but not to glucose. Similar behaviour was also recently shown in a study of *Ralstonia eutropha*, in which the *N*-acetylglucosamine PTS can transport glucose across the cell membrane (Raberg *et al.*, 2012).

On the basis of bioinformatics analysis and sequence comparison, a set of three genes was identified as having a potential role in *N*-acetylglucosamine uptake by *C.beijerinckii*. *Cbei 4532* encodes for EIICB domains, *Cbei 4533* encodes for an EIIA domain and *Cbei 4534* encodes a putative transcriptional antiterminator of the BglG family that may be involved in controlling the expression of the other two genes. As reported in the results, these three genes were expressed during growth in the presence of *N*-acetylglucosamine or glucose, but not glucitol. Also, it was found that the *Cbei 4532* and *Cbei 4533* genes were expressed at an apparently high level during growth in a culture containing both *N*-acetylglucosamine and glucose, which might indicate that the presence of glucose in the growth culture containing *N*-acetylglucosamine played a role in stimulating the genes responsible for the *N*-acetylglucosamine PTS. This reflects an unusual situation for glucose, which, in clostridia, acts as an inhibitory regulator for other saccharides, such as lactose, maltose and sucrose.

In the second half of the project, the function of the putative *nag* genes (*Cbei 4532* and *Cbei 4533*) was investigated by cloning them into the *E.coli nagE* mutant BW25113, in which the *N*-acetylglucosamine PTS uptake system is inactivated due to a transposon insertion. This strain showed a negative fermentation phenotype on MacConkey agar containing *N*-acetylglucosamine at a concentration of 0.5% or lower, meaning that any *N*-acetylglucosamine PTS activity could be identified by complementation of the fermentation phenotype. As a first attempt, these genes were functionally characterized by cloning the entire putative *nag* operon (*Cbei 4532*, *Cbei 4533* and *Cbei 4534*). The recombinant cells showed fermentation activity but this appeared to be unstable and was limited to around 24h, at 37°C.

This could be because of the complex mechanisms of regulation of expression of the genes, which would be expected to depend on the status of the putative antiterminator *Cbei 4534*. Actually, at this stage, it is not clear whether the regulation would operate correctly in *E.coli*. Therefore, an alternative strategy was adopted, which involved cloning the *Cbei 4532* and *Cbei 4533* genes as an artificial operon under control of an *E.coli* promoter. The artificial operon containing *Cbei 4532* and *Cbei 4533* in the pUC18 vector showed a strong complementation of *nagE* BW25113 cells in the presence of 0.5% *N*-acetylglucosamine, and the fermentation phenotype was stable for more than 48h. The recombinant strain was also shown to be able to utilize *N*-acetylglucosamine when grown in LB broth, and extract showed PTS activity for *N*-acetylglucosamine. The *Cbei 4532* and *Cbei 4533* genes were also shown to complement a glucose and mannose negative *E.coli* mutant ZSC113 (Curtis and Epstein, 1975) for fermentation of glucose but not mannose. This demonstrated that the *N*-acetylglucosamine PTS of *C.beijerinckii* was also able to take up and phosphorylate glucose, but not mannose. However, in this study it was found that a gene encoding a putative glucose PTS (*Cbei 0751*) could not complemented BW25113 indicating that this PTS does not transport *N*-acetylglucosamine.

The divergent pattern of gene organization seen for *Cbei 4532* (EIICB) and *Cbei 4533* (EIIA) encoding the *N*-acetylglucosamine PTS in *C.beijerinckii* is unusual but not unique. A similar organization is found for the putative *N*-acetylglucosamine PTS genes in *C.acetobutylicum* (*Cac 1353* – *Cac 1354*). Although these genes have not been characterized in detail, the gene products show homology to the corresponding proteins in *C.beijerinckii*. *Cac 1353* and *Cbei 4532* show 45% identity, while *Cac 1354* and *Cbei 4533* show 53% identity. The arrangement of the *N*-acetylglucosamine PTS genes in the clostridia is different from that in other bacteria. In fact, a considerable diversity of *N*-acetylglucosamine PTS genes organization has been observed in different bacteria, as shown in Figure 71. In *E.coli* the *nagE* gene encodes all the PTS domains, EIICBA, is clustered with the genes *nagB*, which encodes glucosamine-6-phosphate deaminase and *nagA*, which encodes *N*-acetylglucosamine-6-phosphate deacetylase. On the basis of sequence analysis, the corresponding genes in *C.beijerinckii* are suggested to be *Cbei 4564*

and *Cbei 4562* respectively, while the *Cbei 4563* encodes as a GntR family transcriptional regulator. The bioinformatics analysis also showed that the putative *nagA* and *nagB* genes in *C.acetobutylicum* has the same function and encodes by *N*-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase, respectively. These genes could be regulated by the transcriptional antiterminator *Cac 0189*, as shown in Figure 71.

It was found that the expression of *nagE* and *nagAB* genes in *E.coli* is regulated by the product of *nagC*, as described in the Introduction (1.13) (Alvarez-Añorve *et al.*, 2005). In another study Bertram *et al.*, (2011) characterized the genes involved with the *N*-acetylglucosamine PTS in *B.subtilis* and it was shown that the *nagP* gene, encoding specific PTS EIIBC domains, was associated with *nagA* and *nagB* genes and the gene *nagR* which acts as a regulator gene for the *N*-acetylglucosamine PTS operon.

In a comparable recent study, the *N*-acetylglucosamine PTS of *Ralstonia eutropha* H16 was identified by Kaddor and Steinbüchel, (2011). In the mutant strain *Ralstonia eutropha* G⁺1, the *N*-acetylglucosamine PTS operon was characterized, and the genes identified were *nagF* encoding EI, HPr and EIIA, *nagE* encoding EIICB domains, *nagA* (*N*-acetylglucosamine-6-phosphate deacetylase), *nagB* (glucosamine-6-phosphate deaminase) and *zyfI* (glucose-6-phosphate dehydrogenase), while *nagR* encoded a regulator gene for the operon (Raberg *et al.*, 2012). Sequence analysis for the *Cbei 4532* (EIICB *N*-acetylglucosamine domains PTS) in *C.beijerinckii* and the corresponding proteins in other bacteria shown in figure 71, indicates a considerable amino acid identity in the range from 40% to 45%.

An interesting gene arrangement has been found in *B. sphaericus*, which has an operon containing the *ptsHI* genes which encode HPr and EI proteins, together with *nagA* and *nagB* genes encoding *N*-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase respectively, while no information was reported for genes encoding the PTS membrane domains in this strain (Alice *et al.*, 2003).

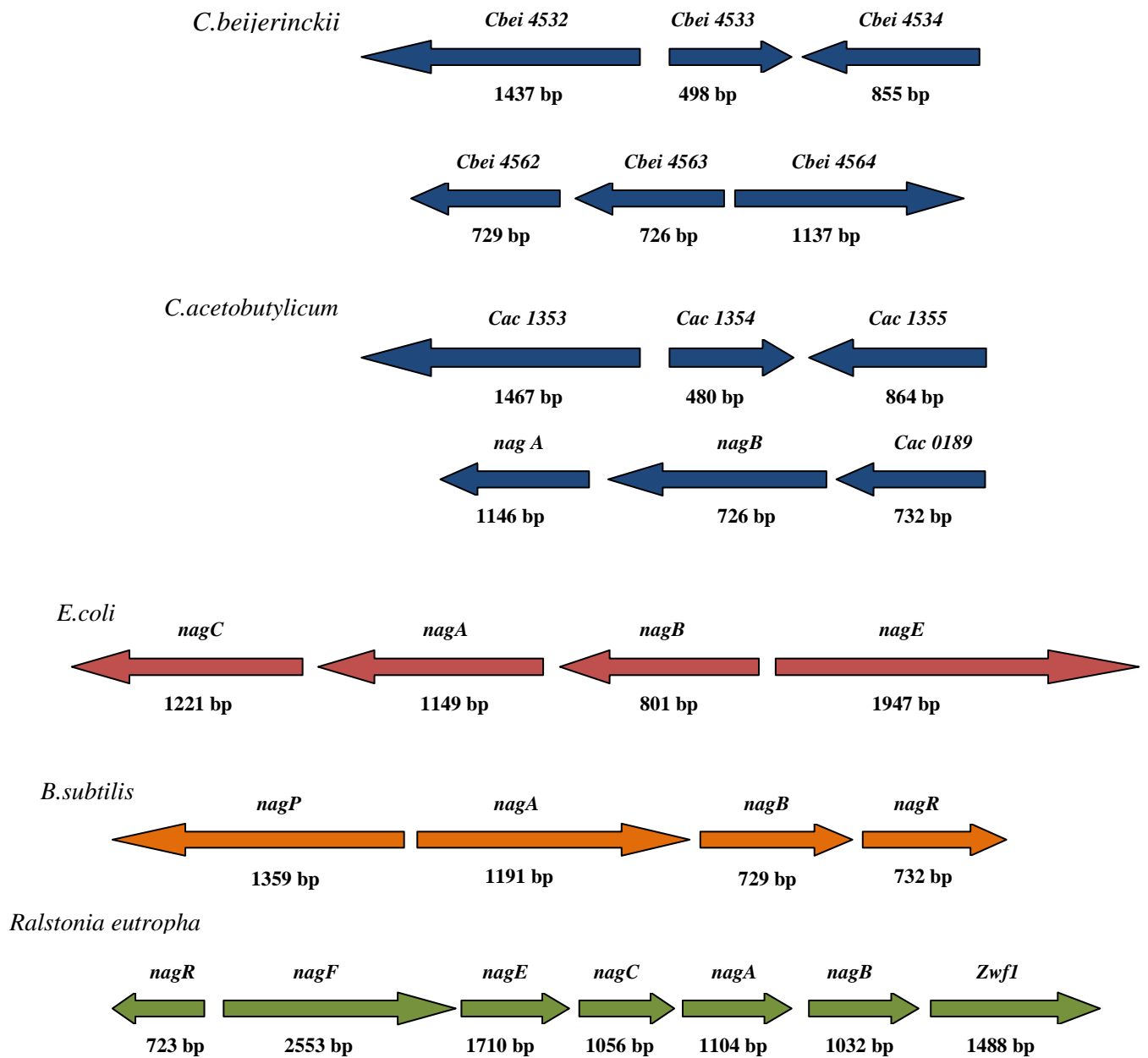


Fig 71: Comparison of the gene arrangement of the *N*-acetylglucosamine PTS in *C.beijerinckii* and different Gram-positive and Gram-negative bacteria; the putative genes in *C.acetobutylicum* ATCC 824, and the *nag* genes in *E.coli* K12, *B.subtilis* and *Ralstonia eutropha* G⁺1 are shown.

Induction of expression of the *C.beijerinckii* *N*-acetylglucosamine PTS genes is most likely controlled by a specific transcriptional mechanism involving putative antiterminator encoded by *Cbei 4534*. The regulatory signals for an antiterminator would be expected to be in the region between the *Cbei 4532* and *Cbei 4533* genes. Protein sequence analysis for *Cbei 4534* showed between 30-35% identity to the antiterminator proteins BglG in *E.coli* (Blattner *et al.*, 1997), and LicT (Schnetz *et al.*, 1996) and SacY (Idelson and Amster-Choder, 1998) in *B.subtilis*, and ScrT in *C.acetobutylicum* (Tangney and Mitchell., 2000). However, a greater identity of 51% is shown by *Cbei 4534* and the equivalent protein *Cac 1355* from *C.acetobutylicum* which is associated with the putative nag operon in that strain.

Although it is not clear at least at this stage why the *N*-acetylglucosamine PTS of *C.beijerinckii* should be induced during growth on both *N*-acetylglucosamine and glucose the fact that glucose is a substrate of the PTS provides an explanation for the mechanism by which the induction occurs. As described in the Introduction (1.10), antiterminator proteins are regulated by phosphorylation of PRD domains and are activated by dephosphorylation of a PRD as a result of uptake and phosphorylation of the substrate of the associated PTS. Therefore, if both *N*-acetylglucosamine and glucose are substrates of the system, it would be expected that both will cause it to be induced. Induction of expression of *Cbei 4532* and *Cbei 4533* on a culture of *C.beijerinckii* growing on glucose has also been observed by Wang *et al.*, . (2012). This study provided a global analysis of gene expression in *C.beijerinckii* using RNA sequencing technology, it was found that the *Cbei 4532* and *Cbei 4533* genes were the most highly expressed genes of the PTS glucose family under the culture conditions, particularly in the exponential phase. Interestingly, Servinsky *et al.*, (2010) also observed induction of the *Cac 1353* and *Cac 1354* genes in cells grown on glucose. However, in these studies no information on expression during growth on *N*-acetylglucosamine was provided for comparison.

In this study the result obtained by gene characterization confirmed that, in *C.beijerinckii*, the *Cbei 4532* and *Cbei 4533* genes encode a phosphotransferase system responsible for *N*-acetylglucosamine uptake, consisting of two proteins carrying EIICB and EIIA domains respectively. It was also observed that the *N*-

acetylglucosamine PTS system was able to support glucose phosphorylation and uptake confirming that the system belongs to the glucose subfamily as suggested at the beginning of this study. It is possible that other transport systems may have the ability to transport *N*-acetylglucosamine even if it is not their primary substrate. This possibility could potentially be investigated by constructing mutants using the ClosTron mutagenesis technology (Heap *et al.*, 2009; Heap *et al.*, 2010). If the *Cbei 4532* or *Cbei 4533* genes were knocked out, the *N*-acetylglucosamine PTS would be inactivated and if it is the only PTS for *N*-acetylglucosamine then the mutant should be unable to grow. Similarly, if the putative antiterminator product of *Cbei 4534* is required for expression of the *N*-acetylglucosamine PTS genes, inactivation of this gene should have the same effect. During this work, a ClosTron plasmid designed to inactivate *Cbei 4532* was introduced into *C.beijerinckii* cells, but no mutants were isolated within the time available. Further attempts to isolate mutants are justified in terms of understanding whether *C.beijerinckii* is absolutely dependent on the *Cbei 4532*, *Cbei 4533* and *Cbei 4534* genes for uptake and metabolism of *N*-acetylglucosamine.

While this study focused on *N*-acetylglucosamine uptake and metabolism, effective exploitation of chitin-containing biomass materials will depend in addition on an understanding of hydrolysis of chitin itself. In the course of the project it was shown that *C.beijerinckii* was capable of hydrolysing colloidal chitin and chitinase activity was shown to be present in culture fluid. Although studies of chitin degradation by solventogenic clostridia are rare, Reguera and Leschine, (2001) showed the capability of several clostridia species to produce chitinase enzyme, including *C.cellobioparum*, *C.cellulolyticum*, *C.longisporum*, *C.hungatei*, *C.populeti*, *C.cellulovorans* and *C.phytofermentans*, while no activity was observed for *C.hungatei*, *C.lentocellum*, *C.papyrosolvans*, *C.papyrosolvans* and *C.thermocellum* strains.

Factors controlling expression of chitinase activity by *C.beijerinckii* were not investigated in detail. However, the presence of chitinase activity in the concentrated supernatant obtained from cells grown on *N*-acetylglucosamine suggests that the presence of *N*-acetylglucosamine induced the chitinase gene(s) as

has been shown in both *Streptomyces thermoviolaceus* and *Streptomyces lividans* (Tsujiibo *et al.*, 1998). On the other hand, culture grown on glucose did not show chitinase activity. Repression of chitinase gene expression in the presence of glucose has been reported in *Streptomyces plicatus*, *S.thermoviolaceus* and *S. lividans* (Ni and Westpheling., 1997; Delic *et al.*, 1992; Tsujiibo *et al.*, 1998).

The *C.beijerinckii* NCIMB 8052 genome contains at least four genes which are predicted to be related to chitinase activity *Cbei 2826*, *Cbei 2830*, *Cbei 2831* and *Cbei 3353*. The function and expression of these genes could provide a clear future view for the regulation of the *N*-acetylglucosamine uptake and chitinase activity in this strain. Characterization of chitin hydrolysis and the mechanism of *N*-acetylglucosamine uptake together can potentially provide a full understanding of the potential of chitin as a fermentable substrate for the biofuel industry.

The environmental and economic aspects have created a widespread interest in several fields of science, due to their direct impact on communities. Several studies have been focused on different types of waste and their potential for use as cheap and sustainable substrate for the ABE industry. The wide abundance of chitin as a homopolymer of *N*-acetylglucosamine in natural wastes could provide an advantageous opportunity to exploit this biomass as cheap raw materials for several biotechnology industries, and the chitinase enzyme(s) might be considered as a secondary product beside solvents which could also be used in biological industries. It is particularly important that the chitin wastes can be fully reused as its persistence in the environment can cause pollution. The industrial and environmental concerns should be sufficient to justify continued investigation of chitin utilization by *C.beijerinckii* and related solventogenic clostridia.

APPENDIX A

GENE AND PROTEIN SEQUENCES

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TAGGTTTAGTATTTGCAGTAATCTATTATGTAACATTCGTTTTGCAATTACAAAATTTAATTTAAAGAC
ACCTGGTAGGGAAGATGATGATGCTGAAGAATAAATGTAACCTTGCGAATAATGATTATACTCAAGTG
GCAGCTGTAATATTAAGGGTGTGGAGGAAAAGAAAACGTAGTATCTATAGATAATTGTGTAAGTACTAGAT
TACGTTTAGAAATTAAGATCAAGCTGCTGTAGATGAAAAGTTATTAATCAGCAGGTGTATCTGGAAT
AATAAGACCAGGAAAACAAGCGTACAAGTTGTTGTAGGAACACAGGTGCAATTTGTAGCAGATGAATTT
AAAAAATTATGTAAGTAAATTTGAAGATCAATAATTAGATTAGGAAAATATCTGATTAATAAATTAAGA
ATATTTAAAATGTGGAAAAGAGATTGATGATATAAGAAATTAAGTTTTATATCAGACTCTTTTCCATTT
TTTTTGTATGCTTTAAGGCTTTTTTTTTAATTTACTTAAAGTTTATAATATCTAATGTATAAATTTATTTG
GTGGTGAATAATGGAATACATAAATGAGATTAATATAAATGAAGCAGTTATTCATATATTAGATAGTAA
TGGGGAAGAACCTATTTTAAACGAGTATAGTTTGAATTAATGAAGATATATATTTTTTCTTACATAAA
CATTTAGAAAAGTGTTTTAAAGGATGAAGAATTAATATGAAAAGTTAATCCAGAAAAGAAACATAGTTA
AAGAAGTATCC

4534 fwd primer

34-Dig fwd primer

*34-Dig rev primer

4534 fwd

4533 rev primer

33-Dig rev primer

4533 Rev

*33-Dig fwd primer

4533 fwd primer

4532 fwd primer

32-Dig fwd primer

32-Dig rev primer

4532 fwd

*DNA seq 2 rev

Seq 2 fwd

4532 rev primer

Figure1: Sequence of the *Cbei 4534*, *Cbei 4533* and *Cbei 4532* genes and annealing positions of primers used in this study. The sequence of the minus strand of the chromosome is shown, with *Cbei 4534* and *Cbei 4532* in forward orientation and *Cbei 4533* in reverse orientation.

- Amplification primers for gene cloning.
 - Primers for Dig-labelling and primers for sequencing.
 - *Cbei 4534* forward orientation
 - *Cbei 4533* reverse orientation
 - *Cbei 4532* forward orientation
- For some primers, indicated by (*), the primer sequence is the reverse complement of the sequence highlighted Primer 4532 Dig-rev was also used in sequencing of the gene.

Query	1	ATGATGAAGTATTTACAAAAATTAGGAAAATCCTTAATGCTTCCAGTAGCTTGTTTACCC	60
Sbjct	68	ATGATGAAGTATTTACAAAAATTAGGAAAATCCTTAATGCTTCCAGTAGCTTGTTTACCC	127
Query	61	GTTGCAAGTATTTTAATGGGTCTTGGTTATTGGCTTGACCCTACAGGCTGGGGAGCAAAT	120
Sbjct	128	GTTGCAAGTATTTTAATGGGTCTTGGTTATTGGCTTGACCCTACAGGCTGGGGAGCAAAT	187
Query	121	AACATTGCTTCAGCTTTTATGCTAAAAGCAGGTAGTGCTTTAATTGACAATATGGGAATT	180
Sbjct	188	AACATTGCTTCAGCTTTTATGCTAAAAGCAGGTAGTGCTTTAATTGACAATATGGGAATT	247
Query	181	TTGTTTGCAATTGGTGTAGGTGTAGGAATGTCAGATGATAATGACGGTACAGCAGGACTT	240
Sbjct	248	TTGTTTGCAATTGGTGTAGGTGTAGGAATGTCAGATGATAATGACGGTACAGCAGGACTT	307
Query	241	GCAGGACTTGTTTCATGGCTTATGATTACAACATTATTATCTACAGGAGCTGTAGCAATG	300
Sbjct	308	GCAGGACTTGTTTCATGGCTTATGATTACAACATTATTATCTACAGGAGCTGTAGCAATG	367
Query	301	TTTAAGGGAATTGATGTTAAAGAGGTAGCGCCAGCATTTCGCTAAAACCAACACAATTT	360
Sbjct	368	TTTAAGGGAATTGATGTTAAAGAGGTAGCGCCAGCATTTCGCTAAAACCAACACAATTT	427
Query	361	ATTGGTATTTTATCAGGTTTAATAGGTGCAGCCTGTTATAACAGATTTAAAAGTGTTAAA	420
Sbjct	428	ATTGGTATTTTATCAGGTTTAATAGGTGCAGCCTGTTATAACAGATTTAAAAGTGTTAAA	487
Query	421	TTACCAGATGCATTGGGATTCTTTAGTGGAAAAAGATGTGTGCGCAATTGTAACCGCAGCA	480
Sbjct	488	TTACCAGATGCATTGGGATTCTTTAGTGGAAAAAGATGTGTGCGCAATTGTAACCGCAGCA	547
Query	481	TATTCAATAGTTGCATCTATAGTATTATTCTTTGCATGGCCTCTTATTTATGGTGCATTA	540
Sbjct	548	TATTCAATAGTTGCATCTATAGTATTATTCTTTGCATGGCCTCTTATTTATGGTGCATTA	607
Query	541	GTAGCATTGGTGAAGCTATTGTATCAACAGGTGCAGTTGGATCTGGTATTTATGCATTC	600
Sbjct	608	GTAGCATTGGTGAAGCTATTGTATCAACAGGTGCAGTTGGATCTGGTATTTATGCATTC	667
Query	601	TTCAATAGATTATTAATACCATTTGGTCTTCACCATGCATTAAACTCAGTATTCTGGTTT	660
Sbjct	668	TTCAATAGATTATTAATACCATTTGGTCTTCACCATGCATTAAACTCAGTATTCTGGTTT	727
Query	661	GATGTAGCTGGAATTAACGACCTTGGTAACTTCTGGTCAGGTAAAGGAACACAAGGAGTA	720
Sbjct	728	GATGTAGCTGGAATTAACGACCTTGGTAACTTCTGGTCAGGTAAAGGAACACAAGGAGTA	787
Query	721	ACTGGTATGTATATGACTGGATTCTTCCCAGTAATGATGTTTGGATTACCAGCAGGAGCA	780
Sbjct	788	ACTGGTATGTATATGACTGGATTCTTCCCAGTAATGATGTTTGGATTACCAGCAGGAGCA	847
Query	781	TTAGCTATGTACCATACAGCTAAAGATAAGAAGAAGAAAGCGGTATATGGTTTATTATTA	840
Sbjct	848	TTAGCTATGTACCATACAGCTAAAGATAAGAAGAAGAAAGCGGTATATGGTTTATTATTA	907

Query	841	GCAGCAGCAATATCTTCATTCTTCACAGGTGTTACAGAACCATTAGAATTTGCATTTATG	900
Sbjct	908	GCAGCAGCAATATCTTCATTCTTCACAGGTGTTACAGAACCATTAGAATTTGCATTTATG	967
Query	901	TTCTTAGCTCCAGGTTTATATGTTCTTCATGCTGGATTAACAGGAATTTAGCATTGTA	960
Sbjct	968	TTCTTAGCTCCAGGTTTATATGTTCTTCATGCTGGATTAACAGGAATTTAGCATTGTA	1027
Query	961	TGTACATTATTACCAGTAAGAGCTGGATTTAACTTTAGTGTGGATTTGTAGATTGGTTC	1020
Sbjct	1028	TGTACATTATTACCAGTAAGAGCTGGATTTAACTTTAGTGTGGATTTGTAGATTGGTTC	1087
Query	1021	TTAAGTTTCAAAGCTCCTATGGCAGAAAATCCAATAATGTTAATCCCAATAGGTTTAGTA	1080
Sbjct	1088	TTAAGTTTCAAAGCTCCTATGGCAGAAAATCCAATAATGTTAATCCCAATAGGTTTAGTA	1147
Query	1081	TTTGCAGTAATCTATTATGTAACATTCCGTTTTGCAATTACAAAATTTAATTTAAAGACA	1140
Sbjct	1148	TTTGCAGTAATCTATTATGTAACATTCCGTTTTGCAATTACAAAATTTAATTTAAAGACA	1207
Query	1141	CCTGGTAGGGAAGATGATGATGCTGAAGAACTAAATGTAAAACCTGCGAATAATGATTAT	1200
Sbjct	1208	CCTGGTAGGGAAGATGATGATGCTGAAGAACTAAATGTAAAACCTGCGAATAATGATTAT	1267
Query	1201	ACTCAAGTGGCAGCTGTAATATTTAAAGGGTGTGGAGGAAAAGAAAACGTAGTATCTATA	1260
Sbjct	1268	ACTCAAGTGGCAGCTGTAATATTTAAAGGGTGTGGAGGAAAAGAAAACGTAGTATCTATA	1327
Query	1261	GATAATTGTGTAAGTAGATTACGTTTAGAAATTAAGATCAAGCTGCTGTAGATGAAAAA	1320
Sbjct	1328	GATAATTGTGTAAGTAGATTACGTTTAGAAATTAAGATCAAGCTGCTGTAGATGAAAAA	1387
Query	1321	GTTATTAAATCAGCAGGTGTATCTGGAATAATAAGACCAGGAAAAACAAGCGTACAAGTT	1380
Sbjct	1388	GTTATTAAATCAGCAGGTGTATCTGGAATAATAAGACCAGGAAAAACAAGCGTACAAGTT	1447
Query	1381	GTTGTAGGAACACAGGTGCAATTTGTAGCAGATGAATTTAAAAAATTATGTAAGTAA	1437
Sbjct	1448	GTTGTAGGAACACAGGTGCAATTTGTAGCAGATGAATTTAAAAAATTATGTAAGTAA	1504

Figure 2: Sequence alignment of *Cbei 4532* Fwd strand with the genome sequence. The sequence was derived from two reactions using the p.JET1.2 forward sequencing primer and primer seq2 Fwd.

(Query): The plasmid sequence.

(Sbjct): The genomic sequence.

Query	1	CATATCATCAATCTCTTTTCACATTTTTTAAATATTCTTAATTTATTAATCAGATAATTTT	60
Sbjct	1	CATATCATCAATCTCTTTTCACATTTTTTAAATATTCTTAATTTATTAATCAGATAATTTT	60
Query	61	CCTAATCTAATTATTGATCTTCAAATTTACTTACATAATTTTTTAAATTCATCTGCTACA	120
Sbjct	61	CCTAATCTAATTATTGATCTTCAAATTTACTTACATAATTTTTTAAATTCATCTGCTACA	120
Query	121	AATTGCACCTGTGTTCTACAACAACCTTGTACGCTTGTTTTCTGGTCTTATTATTCCA	180
Sbjct	121	AATTGCACCTGTGTTCTACAACAACCTTGTACGCTTGTTTTCTGGTCTTATTATTCCA	180
Query	181	GATACACCTGCTGATTTAATAACTTTTTTCATCTACAGCAGCTTGATCTTTAATTTCTAAA	240
Sbjct	181	GATACACCTGCTGATTTAATAACTTTTTTCATCTACAGCAGCTTGATCTTTAATTTCTAAA	240
Query	241	CGTAATCTAGTTACACAATTATCTATAGATACTACGTTTTCTTTTCTCCAACACCCTTT	300
Sbjct	241	CGTAATCTAGTTACACAATTATCTATAGATACTACGTTTTCTTTTCTCCAACACCCTTT	300
Query	301	AATATTACAGCTGCCACTTGAGTATAATCATTATTCGCAAGTTTACATTTAGTTCTTCA	360
Sbjct	301	AATATTACAGCTGCCACTTGAGTATAATCATTATTCGCAAGTTTACATTTAGTTCTTCA	360
Query	361	GCATCATCATCTTCCCTACCAGGTGTCTTTAAATTAATTTTGTAAATGCAAAACGGAAT	420
Sbjct	361	GCATCATCATCTTCCCTACCAGGTGTCTTTAAATTAATTTTGTAAATGCAAAACGGAAT	420
Query	421	GTTACATAATAGATTACTGCAAATACTAAACCTATTGGGATTAACATTATTGGATTTTCT	480
Sbjct	421	GTTACATAATAGATTACTGCAAATACTAAACCTATTGGGATTAACATTATTGGATTTTCT	480
Query	481	GCCATAGGAGCTTTGAAACTTAAGAACCAATCTACAAATCCAGCACTAAAGTTAAATCCA	540
Sbjct	481	GCCATAGGAGCTTTGAAACTTAAGAACCAATCTACAAATCCAGCACTAAAGTTAAATCCA	540
Query	541	GCTCTTACTGGTAATAATGTACATACAAATGCTGAAATTCCTGTAAATCCAGCATGAAGA	600
Sbjct	541	GCTCTTACTGGTAATAATGTACATACAAATGCTGAAATTCCTGTAAATCCAGCATGAAGA	600
Query	601	ACATATAAACCTGGAGCTAAGAACATAAAATGCAAATTCCTAATGGTTCTGTAACACCTGTG	660
Sbjct	601	ACATATAAACCTGGAGCTAAGAACATAAAATGCAAATTCCTAATGGTTCTGTAACACCTGTG	660
Query	661	AAGAATGAAGATATTGCTGCTGCTAATAATAAACCATATAACCGCTTTCTTCTTCTTATCT	720
Sbjct	661	AAGAATGAAGATATTGCTGCTGCTAATAATAAACCATATAACCGCTTTCTTCTTCTTATCT	720
Query	721	TTAGCTGTATGGTACATAGCTAATGCTCCTGCTGGTAATCCAAACATCATTACTGGGAAG	780
Sbjct	721	TTAGCTGTATGGTACATAGCTAATGCTCCTGCTGGTAATCCAAACATCATTACTGGGAAG	780
Query	781	AATCCAGTCATATACATAACCAGTTACTCCTTGTGTTTCTTTACCTGACCAGAAGTTACCA	840
Sbjct	781	AATCCAGTCATATACATAACCAGTTACTCCTTGTGTTTCTTTACCTGACCAGAAGTTACCA	840
Query	841	AGGTCGTTAATTCAGCTACATCAAACCAGAATACTGAGTTAATGCATGGTGAAGACCA	900
Sbjct	841	AGGTCGTTAATTCAGCTACATCAAACCAGAATACTGAGTTAATGCATGGTGAAGACCA	900

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Query  901  AATGGTATTAATAATCTATTGAAGAATGCATAAATACCAGATCCAACCTGCACCTGTTGAT  960
      |||
Sbjct  901  AATGGTATTAATAATCTATTGAAGAATGCATAAATACCAGATCCAACCTGCACCTGTTGAT  960

Query  961  ACAATAGCTTCACCAAATGCTACTAATGCACCATAAATAAGAGGCCATGCAAAGAATAAT  1020
      |||
Sbjct  961  ACAATAGCTTCACCAAATGCTACTAATGCACCATAAATAAGAGGCCATGCAAAGAATAAT  1020

Query  1021  ACTATAGATGCAACTATTGAATATGCTGCGGTTACAATTGCGACACATCTTTTTCCACTA  1080
      |||
Sbjct  1021  ACTATAGATGCAACTATTGAATATGCTGCGGTTACAATTGCGACACATCTTTTTCCACTA  1080

Query  1081  AAGAATCCCAATGCATCTGGNAATTTAACACTTTTAAATCTGTTATAACAGGCTGCACCT  1140
      |||
Sbjct  1081  AAGAATCCCAATGCATCTGGNAATTTAACACTTTTAAATCTGTTATAACAGGCTGCACCT  1140

Query  1141  ATTAACCTGATAAAAATACCAATAAATTGTGTTTGAGTTTTAGCGAATGCTGGCGCTACC  1200
      |||
Sbjct  1141  ATTAACCTGATAAAAATACCAATAAATTGTGTTTGAGTTTTAGCGAATGCTGGCGCTACC  1200

Query  1201  TCTTTAACATCAATTCCTTAAACATTGCTACAGCTCCTGTAGATAATAATGTTGTAATC  1260
      |||
Sbjct  1201  TCTTTAACATCAATTCCTTAAACATTGCTACAGCTCCTGTAGATAATAATGTTGTAATC  1260

Query  1261  ATAAGCCATGAAACAAGTCCTGCAAGTCCTGCTGTACCGTCATTATCATCTGACATTCT  1320
      |||
Sbjct  1261  ATAAGCCATGAAACAAGTCCTGCAAGTCCTGCTGTACCGTCATTATCATCTGACATTCT  1320

Query  1321  ACACCTACACCAATTGCAAACAAAATTCCTCATATTGTCAATTAAGCACTACCTGCTTTT  1380
      |||
Sbjct  1321  ACACCTACACCAATTGCAAACAAAATTCCTCATATTGTCAATTAAGCACTACCTGCTTTT  1380

Query  1381  AGCATAAAAGCTGAAGCAATGTTATTTGCTCCCCAGCCTGTAGGGTCAAGCCAATAACCA  1440
      |||
Sbjct  1381  AGCATAAAAGCTGAAGCAATGTTATTTGCTCCCCAGCCTGTAGGGTCAAGCCAATAACCA  1440

Query  1441  AGACCCATTAAAATACTTGCAACGGGTAAACAAGCTACTGGAAGCATTAAAGGATTTTCCT  1500
      |||
Sbjct  1441  AGACCCATTAAAATACTTGCAACGGGTAAACAAGCTACTGGAAGCATTAAAGGATTTTCCT  1500

Query  1501  AATTTTTGTAAATACTTCATCAT  1523
      |||
Sbjct  1501  AATTTTTGTAAATACTTCATCAT  1523

```

Figure 3: Sequence alignment of *Cbei 4532* reverse strand with the genome sequence. The sequence was derived from two reactions using the p.JET1.2 reverse sequencing primer and primer seq2 Rev.

(Query): The plasmid sequence.
(Sbjct): The genomic sequence.

```

Query 76  ATGTTTAATTTTTTTAAGAAAAATTCAAAGGACTCAAATAAAGAAGCAAATTAGTTGCA 135
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1    ATGTTTAATTTTTTTAAGAAAAATTCAAAGGACTCAAATAAAGAAGCAAATTAGTTGCA 60

Query 136 CCTATAACTGGTAAAACAATAGATTTATCAAAAGTACCAGATAAAGTTTTTGCAGAAAAA 195
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 61  CCTATAACTGGTAAAACAATAGATTTATCAAAAGTACCAGATAAAGTTTTTGCAGAAAAA 120

Query 196  ATGGCTGGAGACGGGCTTGCAATAGATACTACTGGAGATACAGTAGTTTCACCTGCTGAC 255
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 121  ATGGCTGGAGACGGGCTTGCAATAGATACTACTGGAGATACAGTAGTTTCACCTGCTGAC 180

Query 256  GGAACCTTAACATTAGTATTTAATACAAAGCATGCATTTGCAATTACTCTTGATAATGGA 315
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 181  GGAACCTTAACATTAGTATTTAATACAAAGCATGCATTTGCAATTACTCTTGATAATGGA 240

Query 316  GCTGAGTTATTAGTTCATATAGGTATCGATACTGTTTCTTTAAATGGTGAAGTTTTGAA 375
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 241  GCTGAGTTATTAGTTCATATAGGTATCGATACTGTTTCTTTAAATGGTGAAGTTTTGAA 300

Query 376  CAATTAGCTAAAGCTGGAACTAAAGTTAAAGCTGGTACTCCAATTATAAAAATAGATAGG 435
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 301  CAATTAGCTAAAGCTGGAACTAAAGTTAAAGCTGGTACTCCAATTATAAAAATAGATAGG 360

Query 436  GATTTTATTTTAGGAAAAGGTTTTTCTTTGGTCACTCCAGTCCTTGTTACAAACATGGAT 495
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 361  GATTTTATTTTAGGAAAAGGTTTTTCTTTGGTCACTCCAGTCCTTGTTACAAACATGGAT 420

Query 496  ATAATCAAGGATTTAAATTCAAATATTGATAAAGAAGTTGTTGCTGGAGAAGATGAAGTA 555
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 421  ATAATCAAGGATTTAAATTCAAATATTGATAAAGAAGTTGTTGCTGGAGAAGATGAAGTA 480

Query 556  ATTACTTTTACTTTATAA 573
          ||||||||||||||||||
Sbjct 481  ATTACTTTTACTTTATAA 498

```

Figure 4: Sequence alignment of *Cbei 4533* forward strand with the genome sequence. The sequence was derived from reactions using the p.JET1.2 forward sequencing primer and primer seq2 Rev.

(Query): The plasmid sequence.
(Sbjct): The genomic sequence.

```

Query 140 TTATAAAGTAAAAGTAATTACTTCATCTTCTCCAGCAACAACCTTCTTTATCAATATTTGA 199
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1 TTATAAAGTAAAAGTAATTACTTCATCTTCTCCAGCAACAACCTTCTTTATCAATATTTGA 60

Query 200 ATTTAAATCCTTGATTATATCCATGTTTGTAAACAAGGACTGGAGTGACCAAAGAAAAACC 259
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 61 ATTTAAATCCTTGATTATATCCATGTTTGTAAACAAGGACTGGAGTGACCAAAGAAAAACC 120

Query 260 TTTTCCTAAAATAAAAATCCCTATCTATTTTTATAATTGGAGTACCAGCTTTAACTTTAGT 319
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 121 TTTTCCTAAAATAAAAATCCCTATCTATTTTTATAATTGGAGTACCAGCTTTAACTTTAGT 180

Query 320 TCCAGCTTTAGCTAATTGTTCAAAACCTTCACCATTTAAAGAAACAGTATCGATACCTAT 379
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 181 TCCAGCTTTAGCTAATTGTTCAAAACCTTCACCATTTAAAGAAACAGTATCGATACCTAT 240

Query 380 ATGAACTAATAAECTCAGCTCCATTATCAAGAGTAATTGCAAATGCATGCTTTGTATTAAA 439
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 241 ATGAACTAATAAECTCAGCTCCATTATCAAGAGTAATTGCAAATGCATGCTTTGTATTAAA 300

Query 440 TACTAATGTTAAGGTTCCGTGAGCAGGTGAAACTACTGTATCTCCAGTAGTATCTATTGC 499
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 301 TACTAATGTTAAGGTTCCGTGAGCAGGTGAAACTACTGTATCTCCAGTAGTATCTATTGC 360

Query 500 AAGCCCGTCTCCAGCCATTTTTCTGCAAAAACCTTTATCTGGTACTTTTGATAAATCTAT 559
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 361 AAGCCCGTCTCCAGCCATTTTTCTGCAAAAACCTTTATCTGGTACTTTTGATAAATCTAT 420

Query 560 TGTTTTACCAGTTATAGGTGCAACTAATTTTGCTTCTTTATTTGAGTCCTTTGAATTTTT 619
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 421 TGTTTTACCAGTTATAGGTGCAACTAATTTTGCTTCTTTATTTGAGTCCTTTGAATTTTT 480

Query 620 CTTAAAAAATTAACAT 637
          |||||||
Sbjct 481 CTTAAAAAATTAACAT 498

```

Figure 5: Sequence alignment of *Cbei 4533* reverse strand with the genome sequence. The sequence was derived from tow reactions. The sequence was derived from reactions using the p.JET1.2 reverse sequencing primer and primer seq2 Rev.

(Query): The plasmid sequence.
(Sbjct): The genomic sequence.

PTS system *N*-acetylglucosamine-specific transporter subunit IIBC [*Clostridium beijerinckii* NCIMB 8052]

locus_tag "Cbei_4532"

```
1 mmkylqklgk slmlpvaclp vasilmgly wldptgwan gsalidnmg i niasafmlka
61 lfaigvgvgm sddndgtagl aglvswlmit tllstgavam fkgidvkeva pafaktqtqf
121 igilsgliga acynrfksvk lpdalggfsg krcvaivtaa ysivasivlf fawpliygal
181 vafgeaivst gavgsdiyaf fnrllipfgl hhalnsvfwf dvagindlgn fwsqkgtqgv
241 tgmymtgffp vmmfglpaga lamyhtakdk kkkavyglll aaaisffftg vteplefafm
301 flapglyvlh agltgisafv ctllpvragf nfsagfvdwf lsfkapmaen pimlipiglv
361 faviyyvtfr faitkfnlkt pgredddae lnvklanndy tqvaavilkv vggkenvsvi
421 dncvtrlrle ikdqaavdek viksagvsgi irpgktsvqv vvgqtqvqfva defkklck
```

PTS system glucose subfamily transporter subunit IIA [*Clostridium beijerinckii* NCIMB 8052]

locus_tag "Cbei_4533"

```
1 mfnffkknsk dsnkeaklva pitgktidls kvpdkvfaek magdglaidt tgdtvvsfad
61 gtltlvfntk hafaitldng aellvhigid tvslnggegfe qlakagtkvk agtppiikidr
121 dfilgkgfsl vtpvlvtmnd iikdlnsnid kevvagedev itftl
```

Transcriptional antiterminator BglG [*Clostridium beijerinckii* NCIMB 8052]

locus_tag "Cbei_4534"

```
1 mskiidpati iksynnyniv vnmngkeril fakgigfgrk sgdtiekgte vekifviede
61 dnlnrfkqvi envdeeffvl cekmisyvan elkenlderi hvalvdhlnf avkrlsdee
121 ienpflmeir alypqeysla ekvaevlqne kkvkipegei gfialhihsa rnsqklsnti
181 knthlinsii eyveakteik idktsldyar flthlrfaik rilddisien dfikeiksky
241 klsykvskgv akilvlklek kvdsdeiayl amhierfrka tikl
```

PTS system glucose subfamily transporter subunit IIA [*Clostridium beijerinckii* NCIMB 8052]

locus_tag "Cbei_0751"

```
1 mkdkvfgvlq rvgrsfmlpi ailpvaglfl gigesftnkt mldtygitgl igpgtfnal
61 lsvmndagni vfenlplifa igvaigmskk erevaalaag iaflimhasi gamikihggt
121 eallsgaste vlgiislqmg vfggiivglg taalhnryyk ielpqvlstf ggtrfvpiic
181 sivylivgil mfyiwppvqg aiykvgnivl assyagtwvy glmerllipf glhhvfylpf
241 wqtavggtaq vgdkvieaq niffaelgtp githfsvsat rfmsgkfpml ifglpgaala
301 mykcakpekr kavgglllsa altsmltgtp epieftflfv apvlygihcv laglaylmh
361 mlgvvgmmtf sggfidlflf gilqgnakts wilivivgiv yfvvyyllft flikklldkt
421 pgredsgevk lytrsdleak kndqnenade lsamicrglg gknnisdvdc cvtrlrctvh
481 nselvnegll kqtgasgifh kgvgvqimyg prvtviksnl edylvtapdk edtgyaiikk
541 esekdtekgi ekkvqgkvis tvilnspltg eakdlsevpd evfasrimgd gavvpsdgn
601 viapadgvis fvfpskhalg ltttdglell ihigidtvkl dkkafetyve egdkvqagdk
661 ilsfdlefik nnapsiaspc ictalnsnqk vrllktgdik agealiavda fe
```

Phosphotransferase system IIC component, possibly *N*-acetylglucosamine-specific [*Clostridium acetobutylicum* ATCC 824]

locus_tag "Cac_1353"

```
1 mgvtnkllaa cqklgkslmt piavlpaagl llrlgqpdll niswmmaagn gifnnlamif
61 aigiavgfae enngvaglsa avgyfvltntv atsfnkhidm gvlggiivgi lagnlynkyk
121 strlpdflgf fgrrlvpil tslcslvlgl isglvwpaiq nvinafgnsv shagvvsfi
181 ygllnrllip iglhhvntl fwfqfgtfks asgkivtgdI hrffaldkta gtymtgffpi
241 mmfalpaacl amisaakken rkkvsgmlIg iaftafltgV tepieflmf lapvlyvha
301 vltglsmait salgiksgft fsagfvdyim nfnistkpil livigilyai iyyflflfti
361 kkfnlptpgr mddlddddI dlddddld eepentpkik sspsknstle ekavgileai
421 gnknniqsld acvtrirltv kdgskvdepk lkkIgatgim klddknfqiv vgttadiat
481 hikeiikk
```

PTS system transporter subunit IIA [*Clostridium acetobutylicum* ATCC 824]

locus_tag "Cac_1354"

```
1 mlgffkknce liapasgkvl dlsevpdkvf seklvgdgva ielssdtiva pangelslIf
61 ktfhafgitl esgveilvhi gidtvklegk gfeplveqge tvkvgqpiik vdrefikeqg
121 fslitpvlit npelvtDiey isgidvdagn hklftyklk
```

BglG family transcriptional antiterminator [*Clostridium acetobutylicum* ATCC 824]

locus_tag "Cac_C1355"

```
1 mknstgdfki ikvlnnnvlf vlqnnkekil fergigfgkk iddlisadth vekvfsiene
61 nnsntfkqlv stvntniigi ceeiismisk elnenlneki hisltdhisf tlkrllsntd
121 iqnpflieta tlyktefela kkavkmleek tnieipedev gfialhihsa rkkgelsnti
181 kyafIstii efiedeldit idkhsidyar fithlrftie riinsspikn ellnaikhay
241 pesyklsski ckIledelhk kvvedetayl vmhieriknn tkgsilk
```

ScrT [*Clostridium acetobutylicum* ATCC 824]

locus "AF205034_1"

```
1 mvikkilnns avttiddatr iekvimgkgi afqkkpgdil neekiekifs ienqnenlkf
61 qsliseipie hikvseniis yakrklvkvf dehiyisltd hlsfaffrys kgikiknml
121 wdikriyke ynigmwavey ikgelgikmd edeagfialh iidaslnesm dntiniteii
181 dgilniikyf fsiefneddm sydrllthlk yfaqrvsrk naideeeksf leivktnyke
241 ayrcvgkiks fieknidyev kggeivyltl hvqrvisslr dk
```


Fused *N*-acetyl glucosamine specific PTS enzyme: IIC, IIB, and IIA components [*Escherichia coli* K-12 MG1655]

locus_tag "b0679 - nagE"

```
1 mnilgffqrl gralqlpiav lpvaalllrf gqpdllnvaf iaqaggaidf nlalifaigv
61 asswskdsag aaalagavgy fvltkamvti npeinmgvla giitglvgga aynrwsdikl
121 pdflsffggk rfvpiatgff clvlaaifgy vwppvqhah aggewivsag algsgifgfi
181 nrlliptglh qvlntiawfq igeftnaagt vfhgdirfgy agdgtagmfm sgffpimmfg
241 lpgaalamyf aapkerpmv ggmlsvavt afltgvtepl eflfmflapl lyllhalltg
301 islsvatllg ihagfsfsag aidyalmynl paasqnvwm lvmgviffai yfvvslvir
361 mfnlktpgre dkedevtee ansnteegt qlatnyiaav ggtdnlkaid acitrllrtv
421 adsarvndtm ckrlgasgvv klnkqtiqvi vgakaesigd amkkvvargp vaaasaeatp
481 ataapvakpq avpnavsiae lvspitgdvv aldqvpdeaf askavgdgva vkptdkivvs
541 paagtivkif ntnhafclet ekgaeivvhm gidtvalegk gfkrlveega qvsagqpil
601 mdldylnana rsmispvvc niddfsglii kaqghivagq tplyeikk
```

PTS system *N*-acetylglucosamine-specific transporter subunit IICB [*Bacillus subtilis* str.168] gene

locus_tag "BSU07700 - nagP"

```
1 mlsflqklgk sfmlpiavlp avgiilalgr edvfnipfvy qagtavfdhl plifaigia
61 giskdsngaa glsgaisylm ldaatktidk tnmavfvggi iagliagyty nrfdtklpe
121 ylgffsgrrl vpiltaiiti ilagifgvvw ppiqscinsf gewmlglggi gagifglfnr
181 lliplglhhv lnnifwfqfg eyngvtgdla rffakdptag tytgffpim mfglpaacla
241 mvvtakpskr katagmmigf altafitgit epiefafmfl spllyavhav ltblslfivn
301 wlgirsgfsf sagaidyvls ygiaekplll llvgicyaav yfivfyvlik alnlktpgre
361 dddvdevlde ntvqdvneni mlkglggken lqtidhcatr lrltvkdal vdeallkkag
421 akgvvksggq svqviigpvn efaaeelraa vk
```

Transcriptional antiterminator of the bgl operon [*Escherichia coli* str. K-12 substr. MG1655] gene "bglG"

ACCESSION NP_418179

```
1 mnmqitkiln nnvvvviddq qrekvvmgrg igfqkrager inssgiekey alsshelngr
61 lsellshipl evmatcdrii slaqerlgkl qdsiyisltd hcqfaikrfq qnvllpnpll
121 wdiqrlypke fqlgeealti idkrlgvqlp kdevgfiamh lvsaqmsgnm edvagvtqlm
181 remlqlikfq fslnyqeesl syqrlvthlk flswrileha sindsdslq qavkqnyppa
241 wqCacriaif iglqyqrkis paeimflain iervrkeh
```

Transcriptional antiterminator [*Bacillus subtilis*]

ACCESSION CAA82194

gene "Lic T"

```
1 mkiakvinnn visvneqgk elvvmgrgla fqkksyddvd eariekvftl dnkdvsefkf
61 tllydipiec mevseeiisy aklqlgkkl n dsiyvsldh infaiqrnqk gldiknallw
121 etkrlykdef aigkealvmv knktgvs lpe deagfialhi vnaelneemp niinitkvmq
181 eilsivkyhf kiefneeslh yyrfvthl kf faqrlfngth mesqddflld tvkekyhray
241 ectkkiqtyi ereyehklts dellyltihi ervvkqa
```

Transcriptional antiterminator [*Bacillus subtilis*]

ACCESSION YP_003868150

gene "sacY"

```
1 mkikrilnhn aivvkdqnee killgagiaf dkkkndivdp skiektfvrk dtadykqfee
61 iletlpedhi qiseqiisha ekelnikine rihvafsdhl sfaierslsg mviknpllne
121 ikvlypkefq iglwaralik eklgihipdd eigniamhih tarnagdm t qldittmir
181 diieieieql ainivedtis yerlvthlrf aqhikages iyeldaemid iikekfkdaf
241 lcalsigtfv kkeygfefpe kelcyiamhi qrfyqrsvar
```

PTS system glucose subfamily transporter subunit IIA [*Clostridium beijerinckii* NCIMB 8052]

locus_tag "Cbei_4982"

```
1 mfsklfkkn diviyspik kividsevpd pvfsdkimge giavipedni icspvngyva
61 qifktkhail lkssddleii ihigletvnl ngegfevlin egdevttgk likvdfefmk
121 nkgintiipv viinhadrni ikyfgdkqtg aeimkisiq
```

PTS system glucose-like transporter subunit IIB [*Clostridium beijerinckii* NCIMB 8052]

locus_tag "Cbei_4983"

```
1 mtntsyfskf qqlgkvlmtp ililpiagil mgigsaf lsv svmktmpflq mpffklffsl
61 lkssgsivfn nlpaifaisi tigyakkeg iaalsaf lgy mvmnvtmsal linlgkinpd
121 kllvgqsnil gvptvdtgvf ggiivgfliv ylhnyy nis lppvlsifsg tkfvpmvsii
181 gslilglals vvwpiqgil ielsilikns gaygsmygl aerallpfgl hhfvy l pfff
241 tslggsmeig gkvvegavni yqaqlatpge mfnidvtrfa mngkviesmf glpgaalamy
301 kcakperkkv iggfllaavi paffsgitep iefaf lfvap alygihaifa gtaylvtyll
361 qinipgsaaf ggpflsfifn gimqsdkgsh wifvpigvi yfclyyfsfk faikkwdlkt
421 pgreleedse eisvssnt iindivdal gknniks vda cftrlrsvn dmsmvkddni
481 wkklgangvv kvkdgvqviy g akadvyktq vrdllgme
```

phosphotransferase II ABC, *N*-acetylglucosamine-specific [*Ralstonia eutropha* H16]

gene "*nagE*"

locus_tag "*H16_A0312*"

```
1 mkmdllprvq rlgatlmlpi avlpvaglll rlgqpdvfdi klmaeagnav fanlallfai
61 gvavgfardn ngaaalagti gylvlttvk tidksldmgv lagivagava gglynryrnv
121 alppylgffs gkrfvpivot lcclllgivil ayawapvqag inaagawltt agsagalvfg
181 llnrlllvtg lhhlintlaw fvfgnyadpa tgaavsgdlh ryfagdpag lfmtgffpvm
241 mfglpaacla myhetpparr alvggmlfsm altsfltgit epiefsfmfl apvlyglhal
301 mtglsmalch aldirlgftf sagaidyvlv yglssrgwla iplglayalv yyglfrffir
361 rfnlltpgrd evvpvaaagg aaqpaagsva qqyvealggp anlvvvdact trlrlnvadi
421 gavseprlka lgvrgvlkrp pnvvqvvigp qaeqvagdir avlqqapqat avvaaapava
481 tqasvpaaga fdpawwidal ggaaniasvg vvaltrlrsv vrerarvrad hlagsqlmwi
541 gddtahiafg haadghaaaf eralqampt
```

Figure 6: Sequences of proteins used in alignments comparative in this study.

Hyperladder I



Hyperladder IV

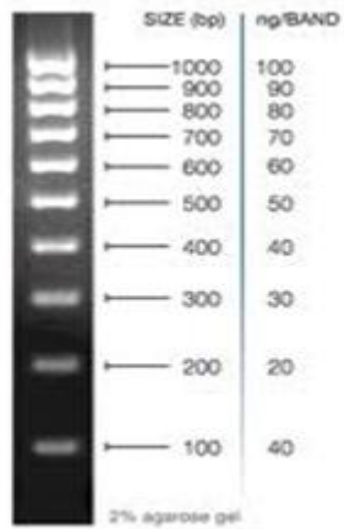


Figure 7: DNA profile of Bioline hyperladders.

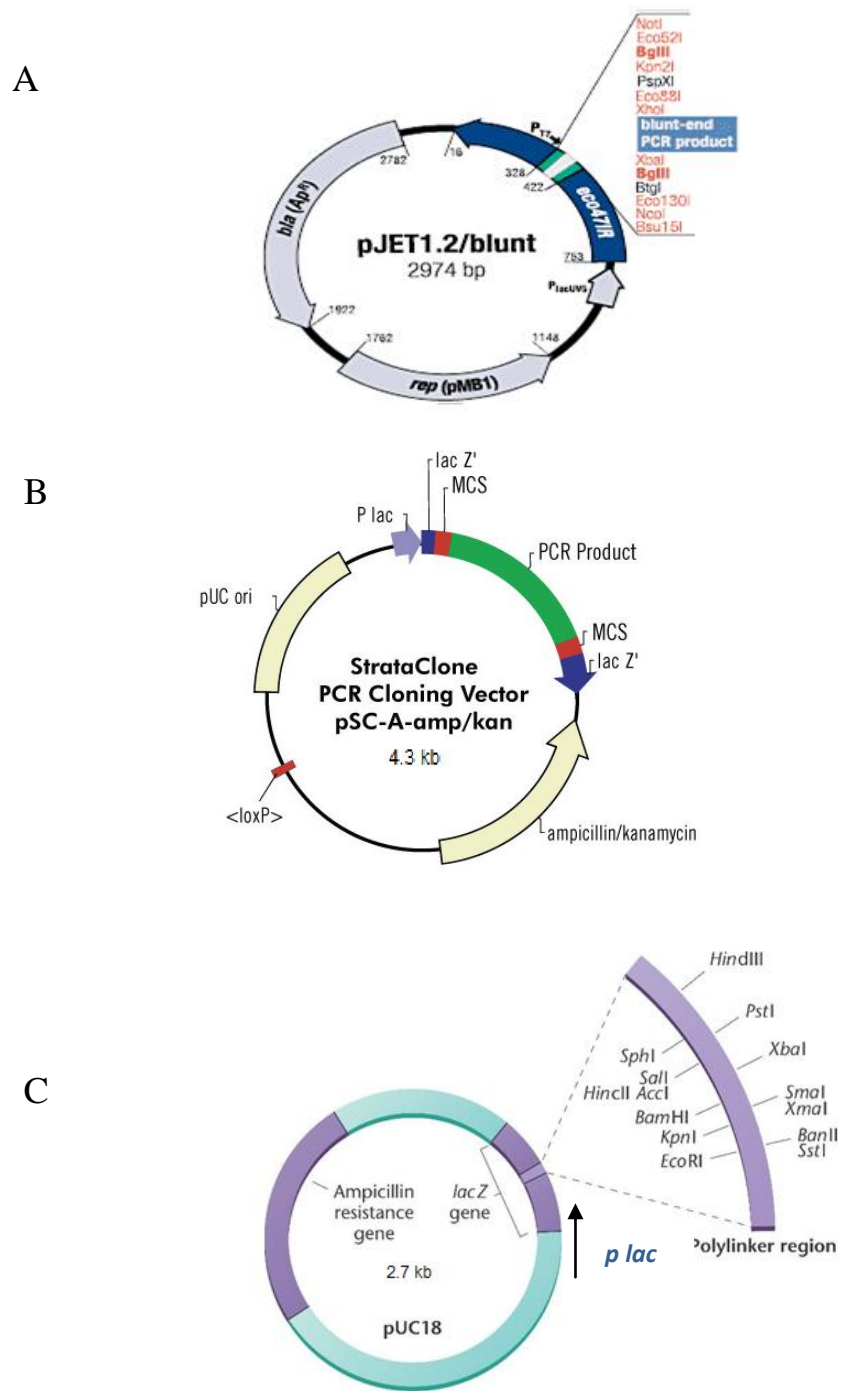


Figure 8: Vectors used in this study:

- A. pJet 1.2/blunt vector (Fermentas).
- B. StrataClone vector.
- C. pUC 18 vector.

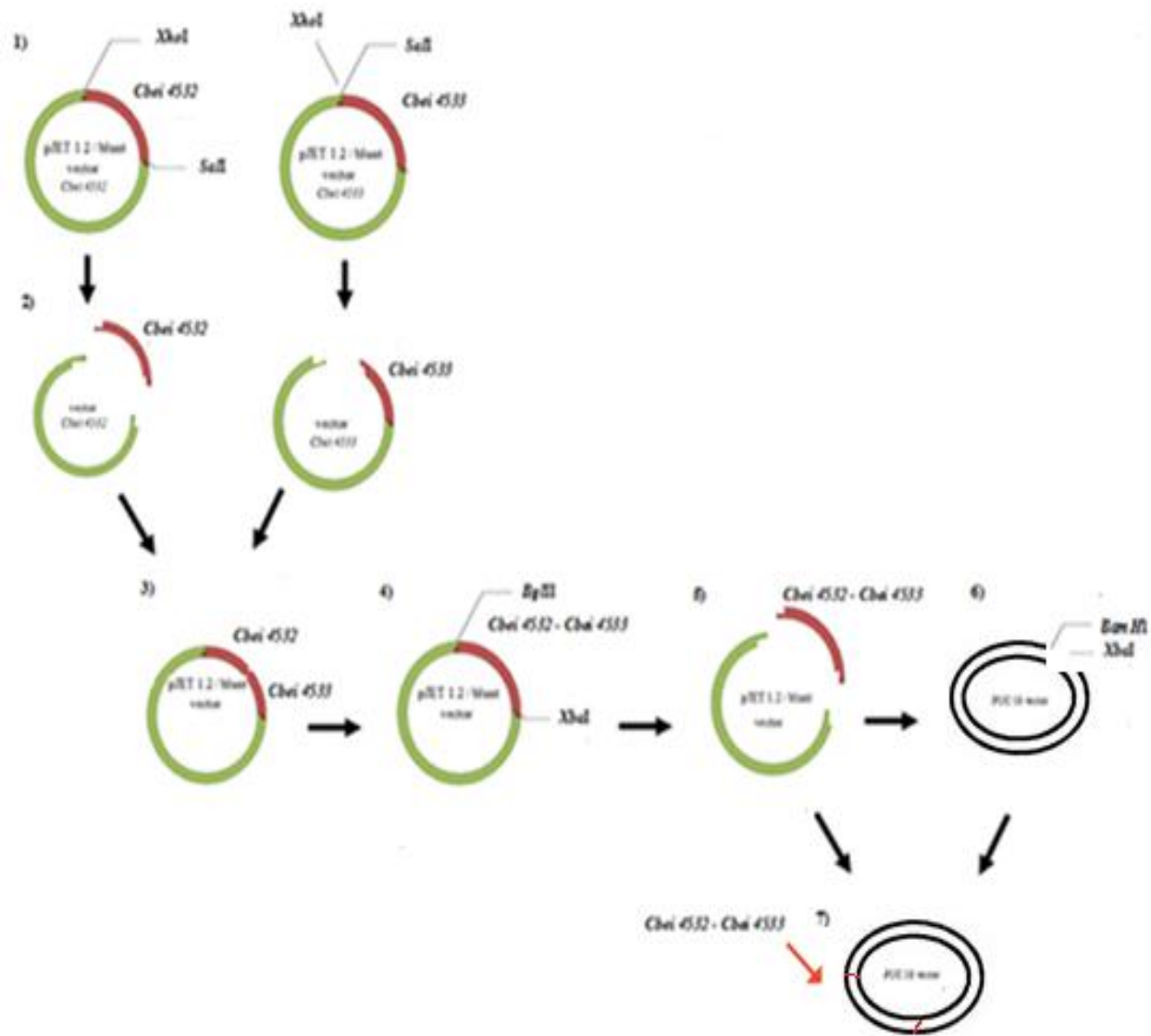


Figure 9: Restriction and digestion strategy for cloning of *Cbei 4532* and *Cbei 4533* as an artificial operon.

APPENDIX B

SOLUTIONS

1) 3, 5 Dinitrosalicylic acid solution (DNS) /250 ml:

- 3, 5 Dinitrosalicylic acid 0.25g.
- Sodium potassium tartrate (Rochelle salt) 75g.
- Sodium hydroxide (2M in 50 ml distilled water).

2) Phosphate buffer :

- 0.5M dipotassium hydrogen phosphate K_2HPO_4 .
- 0.5 M potassium dihydrogen phosphate KH_2PO_4 .
- Solutions were mixed to achieve pH 7.0

3) *Pfu* DNA polymerase (5×) buffer/500μl.

- 5μl of 100 mM Deoxyadenosine triphosphate (dATP).
- 5μl of 100 mM Deoxyguanosine triphosphate (dGTP).
- 5μl of 100 mM Deoxythymidine triphosphate (dTTP).
- 5μl of 100 mM Deoxycytidine triphosphate (dCTP).
- 250 μl (10×) *Pfu* buffer with $MgSO_4$.
- 230 μl Deionized water.

4) (20×) saline-sodium citrate (SSC) buffer/L

- 3M sodium chloride (NaCl)
- 0.3M sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$)
- 100μl Diethylpyrocarbonate (DEPC).

5) (2×) saline-sodium citrate (SSC) buffer/L

- 10 × diluted from 20× saline-sodium citrate (SSC) buffer.
- 1ml of 1% Sodium dodecyl sulfate (SDS).

6) (0.2×) saline-sodium citrate (SSC) buffer/L

- 100 × diluted from 20× saline-sodium citrate (SSC) buffer.
- 1ml of 1% sodium dodecyl sulfate (SDS).

7) Dig 1 buffer/L

- 0.1 M Tris HCl.
- 1M sodium chloride (NaCl).
- 0.2% Tween 80.
- pH 8.5

8) Dig 4 buffer/L

- 0.1 M Tris HCl.
- 1M sodium chloride (NaCl).
- 0.2% Tween 80.
- pH 9.5

9) Easy hybridization buffer/50 ml

- 6M Urea
- (6×) saline-sodium citrate (SSC) (15 ml of 20× SSC).
- 2.5 ml of 1% sodium dodecyl sulfate (SDS).
- 50mM Tris HCl.
- pH 7.5

10) Blocking buffer/100 ml

- 100 mM maleic acid.
- 1 M sodium chloride (NaCl).
- pH 8.
- 0.3% Tween 80.
- 0.5% casein.
- 0.1% Diethylpyrocarbonate (DEPC).

APPENDIX C

PROTEIN STANDARD CURVE

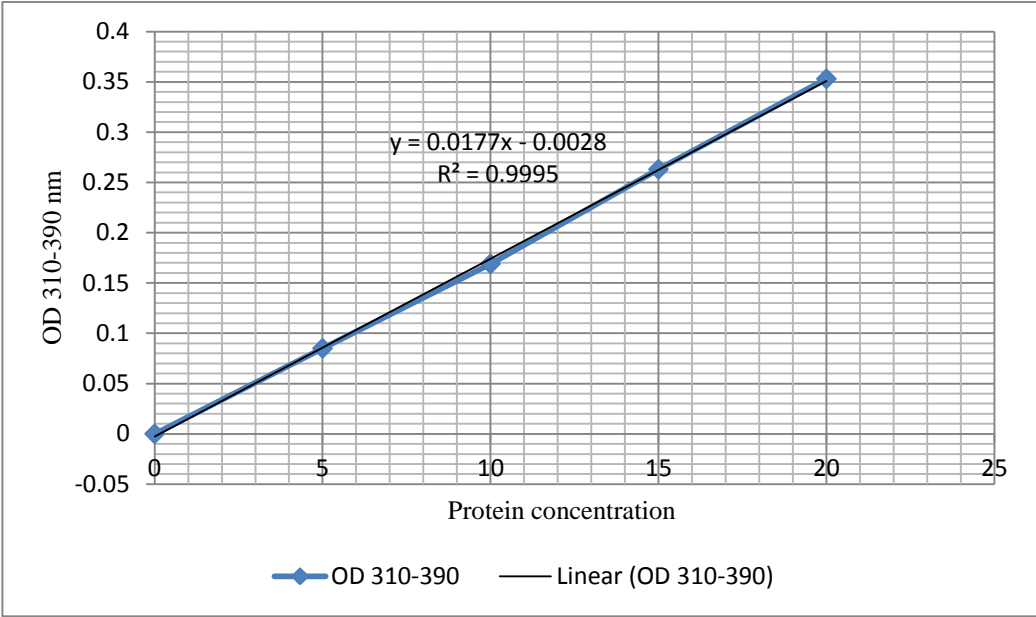


Figure 10: Protein standard curve

APPENDIX D

GROWTH CURVES

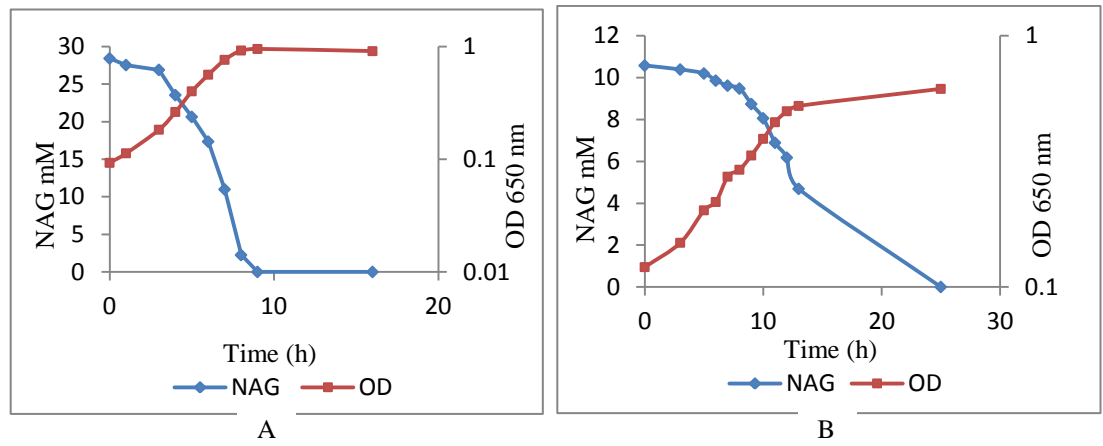


Figure 11: *N*-acetylglucosamine utilization by *Clostridium acetobutylicum* in RCM (A) and CBM (B), containing *N*-acetylglucosamine as a fermentable carbon source.

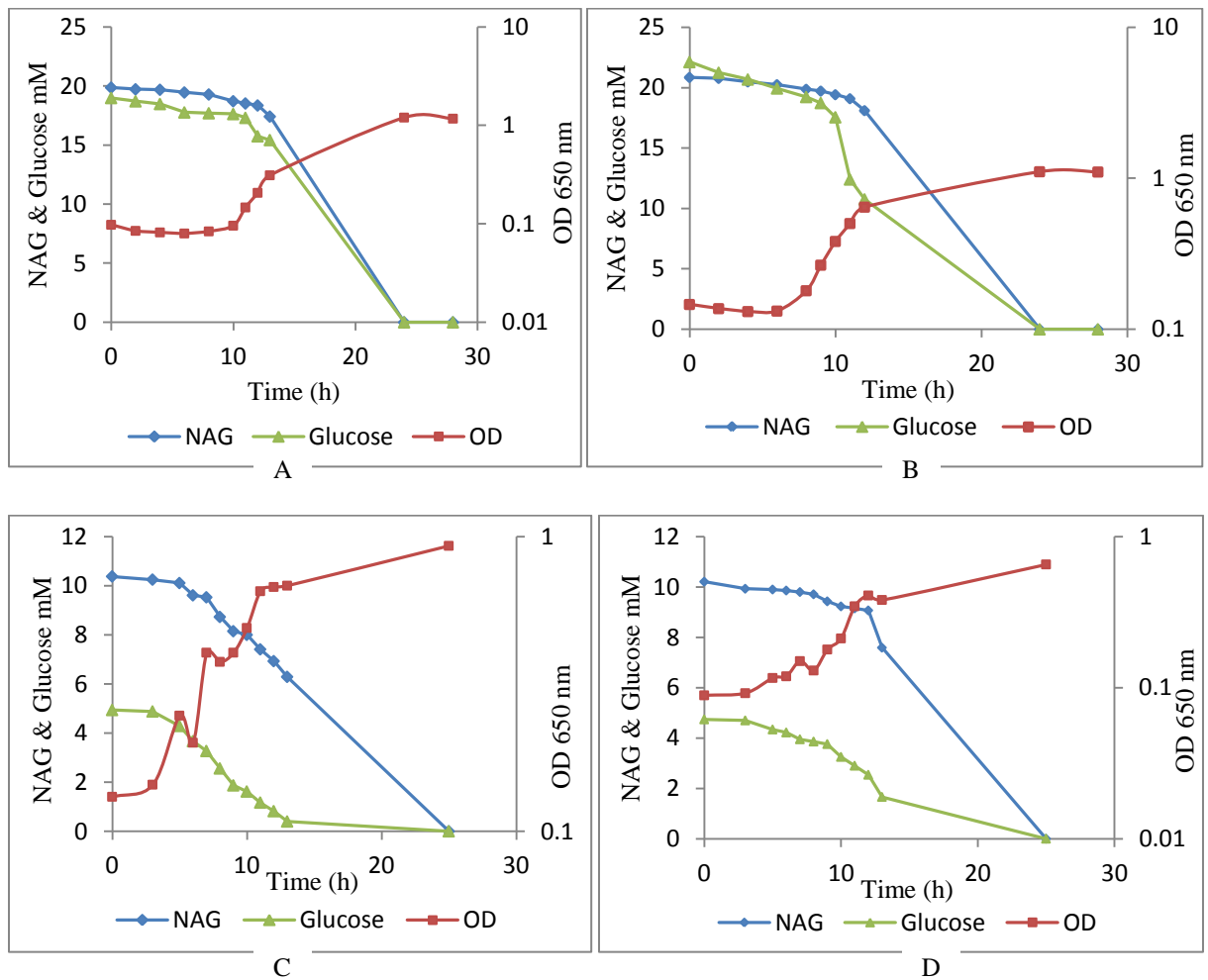


Figure 12: Growth and sugar utilization by *Clostridium acetobutylicum* in RCM (A,B) and CBM (C,D) containing *N*-acetylglucosamine and glucose: (A), (C) pre-grown on *N*-acetylglucosamine and (B), (D) pre-grown on glucose.

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