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A synthetic biology approach to cellulose degradation

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Declaration:

I hereby declare that the research presented in this thesis is my own unless otherwise mentioned.

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2011

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Abstract:

Cellulose, the most abundant biopolymer on earth, is composed of β – 1,4 – linked glucose units, which in turn form a highly ordered crystalline structure that is insoluble and recalcitrant to degradation. It is the world's most attractive, abundant and renewable energy resource, representing the bioconversion of carbon dioxide into green plants. Cellulosic biomass, such as agricultural and forestry residues, waste paper and industrial waste can therefore be used as an inexpensive and abundantly available source of sugar for fermentation into fuel ethanol. The combustion of biofuels releases carbon dioxide which is thus recycled and hence the use of these fuels in transportation provides an alternative to fossil fuels, solving many environmental problems.

The ability to degrade crystalline cellulose seems to be restricted to a specialized group of microorganisms which includes for example *Clostridium*, *Cellulomonas*, *Cytophaga*, *Trichoderma* etc. Hence the aim of this project was to create BioBricks using different cellulases from cellulose degraders and express them in different expression hosts like *Escherichia coli*, *Bacillus subtilis*, *Citrobacter freundii* etc., using two different promoters, *spac* and *lac*.

It was observed that the expression of *Cytophaga hutchinsonii* cellulases (CHU_2103 and CHU_2802) and dehydrogenases (CHU_1944 and CHU_2315) was toxic to the *E. coli* host for some unknown reason. Therefore it was decided to use cellulases from *Cellulomonas fimi*, which are well characterized.

BioBricks of cellulases (*cenA* and *cex*) from *C. fimi* were introduced into different expression hosts. It was observed that under our experimental conditions *Citrobacter freundii* SBS197 gave the best results. Both P_{spac} and P_{lac} were functional in this organism with expression being higher when P_{spac} was used. When *E. coli* JM109 was used as an expression host, activity was only detected when the *lac* promoter was used to control the expression. Although the expression was higher when *E. coli* JM109 (containing P_{lac}) was used as an expression host, almost all of this activity was residing within the cells, whereas when *C. freundii* SBS197 was used as an expression host, considerable activity was detected in the surrounding medium, which is essential for cellulose degradation.

Growth curve studies were done to see if heterologous cellulases enable the host to use cellulosic substrates as a source of carbon. It was observed that *C. freundii* SBS197 expressing *cenA* and *cex* was able to use filter paper and Avicel as a source of carbon with maximum growth of up to 8.8×10^{8} cfu/ml and 1.2×10^{9} cfu/ml respectively. This was about 2 – 5 fold higher when compared to the control (vector and/or negative) strains. Filter paper completely disappeared within 3 – 4 days when *C. freundii* SBS197 was used. Slight degradation was observed when *E. coli* JM109 was used but there was no physical degradation seen when *B. subtilis* 168 was used as an expression host. Hence it was concluded that heterologous cellulases impart to *C. freundii* SBS197 with the ability to use cellulosic substrates as a source of carbon. The maximum growth obtained using these cultures is to our knowledge higher than what has been reported so far for recombinant organisms expressing heterologous cellulases using cellulosic substrates as a source of carbon.

List of abbreviations:

ACDP	Advisory Committee on Dangerous Pathogens
Amp	Ampicillin
BBF RFC	BioBricks Foundation Request For Comments
BLAST	Basic Local Alignment Search Tool
Carb	Carbenicillin
CBD	Carbohydrate binding domain
CBM	Carbohydrate binding module
CBO	Cellobiose oxidase
CBP	Consolidated bioprocessing
CBQ	Cellobiose:quinone oxidoreductase
CD	Catalytic domain
CMC	Carboxymethyl cellulose
Cml	Chloramphenicol
CMM	Chris minimal medium
DMM	Dubos mineral medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
GRAS	Generally Regarded As Safe
IPTG	Isopropyl-β-D-thio-galactoside
Kan	Kanamycin
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria Bertani medium
MU	Methylumbelliferone
MUC	Methylumbelliferyl cellobioside
NCBI	National Center for Biotechnology Information
ONPC	*O*-nitrophenyl-β-D-cellobioside
OPEC	Organization of Petroleum Exporting Countries
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PTM	Pre-transformation medium
RBB	Remazol Brilliant Blue
rbs	Ribosome binding site
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SHF	Separate hydrolysis and fermentation
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation
TAE	Tris-acetate-ethylenedinitrilotetraacetic acid
TM	Transformation medium
TSS	Transformation and storage solution
UV	Ultra violet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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1 INTRODUCTION:

The power of the sun is converted into a usable organic form – biomass, via photosynthesis (Glazer and Nikaido, 1995). Life on earth is hence dependent on this primary process of photosynthesis, resulting in production of plant biomass having cellulose as the major component (Lynd *et. al.,* 2002). With an estimated synthesis rate of 4×10^{10} tons/year, cellulose is by far the most abundant carbohydrate available in the biosphere (Beguin, 1990; Coughlan, 1985). It is basically a homopolymer of glucose constituting about 40 – 60% of the cell wall of woody plants (Li *et. al.,* 1997; Philips and Humphery, 1983). Plant biomass in addition to cellulose also consists of 25 – 50% hemicelluloses, 10 – 40% lignin and 2 – 20% pectin depending on whether the origin is hard wood, soft wood or grasses (Haan *et. al.,* 2007; Sun and Cheng, 2002) (Table 1.1). Hence the major polysaccharide present in plant biomass is the water insoluble cellulose, which contains the major fraction of fermentable sugars and is therefore the most abundant renewable bioresource available in nature (Haan *et. al.,* 2007; Zhang *et. al.,* 2006; Fujita *et. al.,* 2002). The degradation of cellulose by cellulolytic microorganisms results in the major carbon flow from fixed carbon sink to the atmosphere, hence the closure of the carbon cycle (Berner, 2003; Zhang *et. al.,* 2006). Thus in short, cellulose-utilizing microorganisms are responsible for one of the largest material flows in the biosphere (Lynd *et. al.,* 2002).

The use of cellulose for bioconversion to fuels and other commodity products has gained much interest in the last few decades probably because of its relatively low cost and plentiful supply (Li *et. al.,* 1997; Lynd *et. al.,* 2002; Philips and Humphery, 1983). Many environmental benefits would result from the replacement of petroleum based automotive fuels with renewable fuels from modern biomass. Coal and oil when used as fuels have a serious negative impact on the environment and intensive use of fossil fuels also increases global warming via greenhouse effect by adding carbon dioxide to the atmosphere (Glazer and Nikaido, 1995). The burning of coal also results in the emission of mercury. On the other hand when oil is burned it emits carbon monoxide and other air toxins all have serious impact on the quality of air. Fossil fuels contain sulphur as an impurity which up on combustion forms sulphur dioxide, a precursor to acid rain, which has a bad impact on buildings, man-made

materials, vegetation and human health. Hence an alternative to the use of fossil fuels are biofuels, which could help provide solutions to many environmental problems, as the carbon dioxide emitted by combustion of biofuels is recycled into the environment without any further increase in the carbon dioxide burden of the atmosphere (Fujita *et. al.,* 2002). Also the dramatic fluctuations in oil prices due to reduced oil output by OPEC (Organization of Petroleum-Exporting Countries) and occasional price hikes since 1973 have resulted in research focused on the development of technology to produce fuels and chemicals from renewable resources (Qureshi and Blaschek, 2001).

Hence lignocellulosic biomass, such as agricultural and forestry residues, waste paper and industrial waste is an attractive renewable feedstock for biofuels production (Fujita *et. al.,* 2002). Among lignocellulosic biomass cellulosic materials have gained much attention particularly because of their rather low cost and abundant supply (Lynd *et. al.,* 1999; Lynd *et. al.,* 2002). In addition as long as biomass use matches its regeneration no net increase in the atmospheric carbon dioxide would result from this source (Glazer and Nikaido, 1995).

The hindrance to the widespread utilization of this important resource is the general absence of suitable technology to overcome the recalcitrance of cellulosic biomass. This technological barrier can be overcome to a large extent by a promising strategy called 'consolidated bioprocessing' (CBP). This involves the production of cellulolytic enzymes, hydrolysis of biomass and fermentation of resulting sugars into the desired product in a single process step with a cellulolytic microorganism or consortium (Lynd *et. al.,* 1999; Lynd *et. al.,* 2002). Consolidated bioprocessing offers a very large cost reduction compared to the original conversion process involving discrete steps and different catalysts. These goals can largely be achieved if a microorganism or a consortium with the desired combination of substrate utilization and product formation properties can be developed.

Table 1.1: Cellulose, hemicelluloses and lignin content in different lignocellulosic materials (Sun and Cheng, 2002)

Lignocellulosic material	Cellulose (%)	Hemicelluloses (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste papers from chemical pulps	60-70	10-20	5-10
Primary waste water solids	8-15	NA	24-29
Swine waste	6.0	28	NA
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

1.1 STRUCTURE AND COMPOSITION OF LIGNOCELLULOSIC BIOMASS:

1.1.1 CELLULOSE:

Lignocellulose, which makes up to 50% of the earth's biomass, is a non-edible component of plant cell walls often produced as industrial and/or agricultural waste due to its rigid nature and stable structure (Sanchez and Cardona, 2008). The most abundant component of lignocellulosic biomass is cellulose which in addition to being a unifying feature of the plant cell wall is also produced in nature by some animals (like tunicates) and a few bacteria (Lynd *et. al.,* 1999; Lynd *et. al.,* 2002). However, in plants the cellulose fibres are embedded in a matrix of other structural polymers, hemicelluloses and lignin. These matrix interactions are a dominant structural feature limiting the rate and extent of utilization of biomass (Figure 1.1). However, cellulose under appropriate microbial and environmental conditions has the potential for complete hydrolysis and utilization (Lynd *et. al.,* 2002).

An exceptional feature of cellulose which is also relatively unusual in the polysaccharide world is its crystalline structure (Lynd *et. al.,* 2002; Brown and Saxena, 2000). Cellulose is basically a linear polymer of β – 1,4 – linked glucose units (Figure 1.2) which are self-assembled at the site of its synthesis. The β – 1,4 – linked *D*-glucose units are arranged in alternate orientation with respect to one another so that the repeating unit is cellobiose rather than glucose. Unlike starch, cellulose is a straight chain polymer with no coiling and the molecule has an extended, rather stiff rod-like conformation. Approximately 30 individual molecules of cellulose are assembled into larger units called elementary fibrils (protofibrils), which in turn are packed into still larger units called microfibrils (Lynd *et. al.,* 2002; Brown and Saxena, 2000) (Figure 1.3). The chains in the microfibrils are held together by hydrogen bonds giving them a high tensile strength. The microfibrils then associate into the well-known crystalline cellulose fibres.

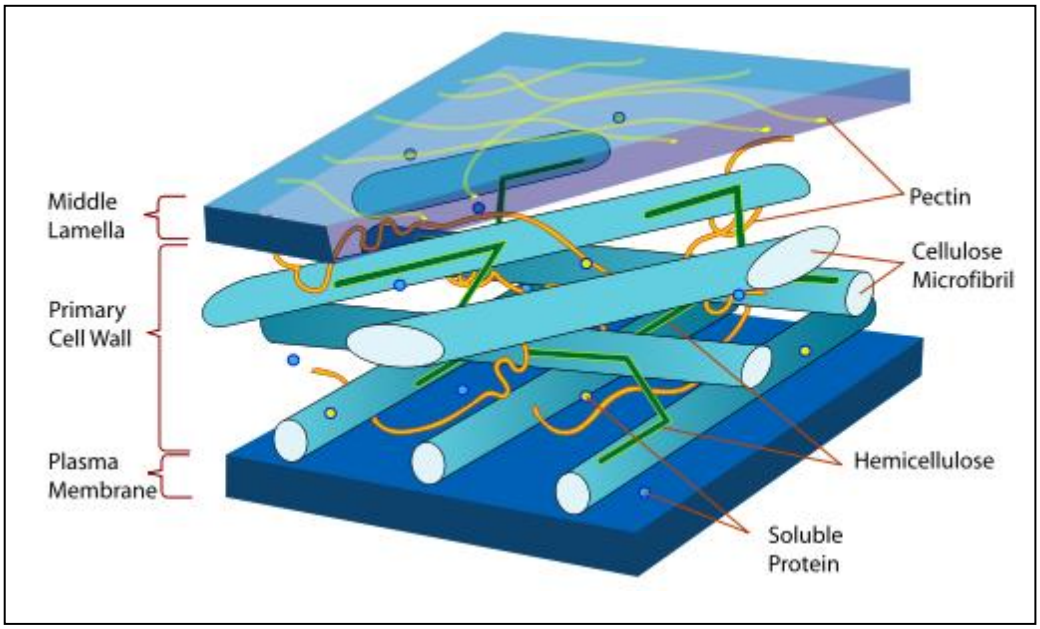


Figure 1.1: Arrangement of cellulose microfibrils in plant cell wall (www.en.wikipedia.org/wiki/File:Plant_cell_wall_diagram.svg).

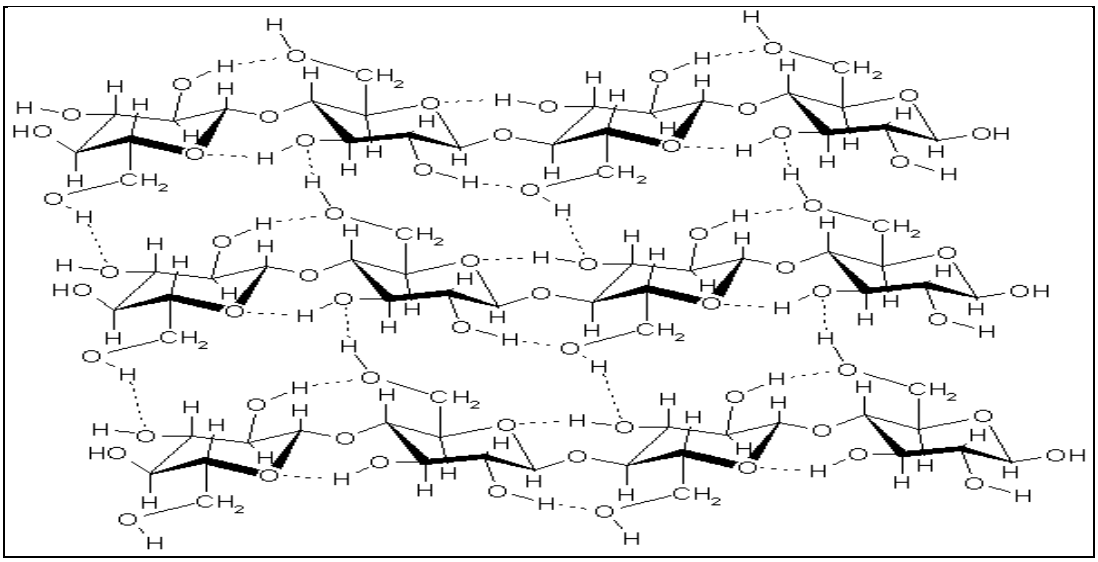


Figure 1.2: A strand of cellulose, showing the hydrogen bonds (dashed) within and between cellulose molecules (University of Cambridge).

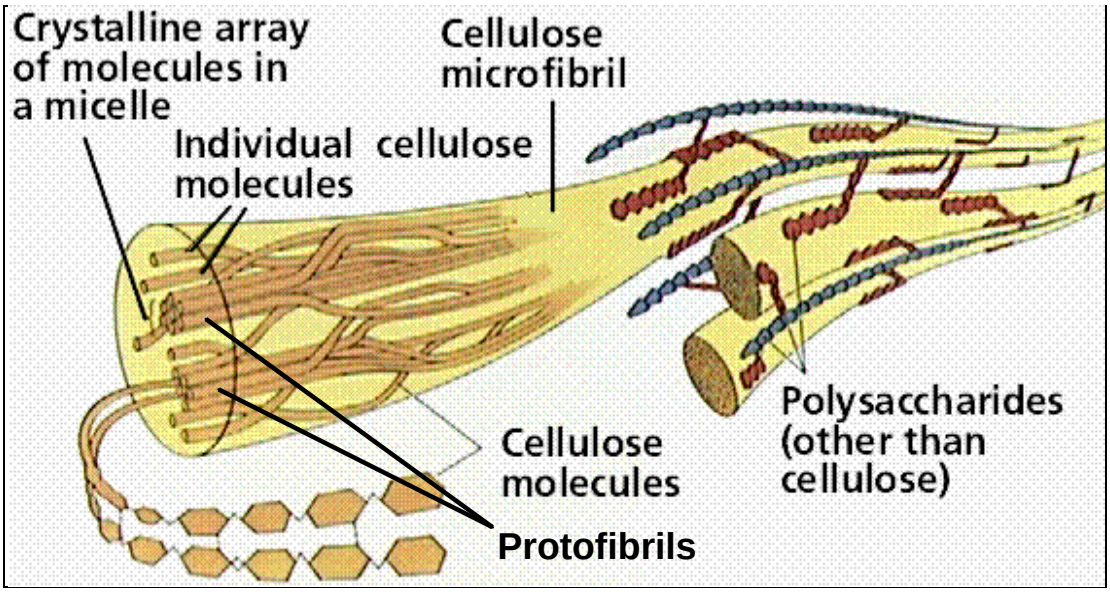


Figure 1.3: Organization of cellulose fibres (Farabee, 2000).

Although cellulose is said to have a crystalline structure, in nature these fibres are not purely crystalline. In the physical world, cellulose fibres range from purely crystalline to purely amorphous, with all degrees of order in between (Lynd *et. al.*, 2002; Marchessault and Howsmon, 1957). In addition, they also contain various irregularities such as kinks/twists or voids, surface micropores, large pits and capillaries etc., increasing the total surface area much larger than that of an ideally smooth fibre of the same dimensions (Lynd *et. al*., 2002; Blouin *et. al.*, 1970; Cowling, 1975; Fan *et. al.*, 1980).

1.1.2 OTHER PLANT POLYMERS:

Hemicelluloses are a group of polysaccharides of plant cell walls containing β-(1→4)-linked backbones composed of glucose, mannose and/or xylose (Scheller and Ulvskov, 2010). These are shorter molecules than cellulose (less than 200 sugar residues) and consist of a mixture of hexoses and pentoses such as galactose, mannose, xylose, arabinose etc., as well as sugar derivatives such as methyl glucuronic acid etc. (Glazer and Nikaido, 1995). Several hemicelluloses and their important characteristics are described in Table 1.2.

Table 1.2: Types of hemicelluloses and their characteristic features (Brett and Waldron, 1996)

Type of hemicellulose	Backbone	Side chain	Important features
Glucomannans	1,4-β-glucose and β-mannose.	Single galactose units	Some acetyl esters may be present on the hydroxyl groups of mannose.
Xylans	1,4-β-xylose units. Some xylose residues of backbone are substituted at C-2 by 4-*O*-methyl-α-D-glucuronic acid, at C-2 or C-3 by α-L-arabinose units and by acetyl esters on C-2 or C-3.	Sidechain containing arabinose as the major sugar are called arabinoxylan and glucuronic acid are called glucuronoxylan. Xylan having both arabinose and glucuronic acid are called glucuronoarabinoxylan.	An important feature of arabinoxylans of monocotyledonous plants is the presence of ferulic acid which may crosslink the arabinoxylans in the wall.
Arabinogalactan II	1,6-β-D-galactose residues. Highly branched at C-6.	β-1,3 linked arabinose units present on the outer chain. Glucuronic acid may also be present in small amounts.	A component of larches.
Mannans and galactomannans	β-1,4 linked mannose units	α -1,6 linked galactose residues.	Able to form very hard crystalline structure.
Glucuronomannan	Alternating sequence of α-1,4 linked mannose units and β-1,2 linked glucuronic acid residues.	Galactose units linked by β-1,6 bonds to mannose and arabinose residues linked by 1,3 bond to mannose.	
Xyloglucans	1,4-β-glucose	Xylose residues are attached to the backbone by α-1,6-bonds. Xylose are sometimes further substituted by fucose by α-1,2 bond, galactose by β-1,2 bond and rarely arabinose by 1,2 bond. Acetyl esters with a high proportion of galactose residues carrying acetyl groups may also be present.	
Callose	1,3-β-glucose units.		General name for β-1,3 glucan which is formed by variety of cells in response to wounding.
β-1,3 and β-1,4 glucans	Unbaranched glucan chains with the ratio of 1,3 to 1,4 linkage between 1:2 and 1:3. About two, three or four 1,4 linked residues are separated by single 1,3 linked residue.		Also known as mixed-linkage glucans.

Lignin is a complex three dimensional polymer of aromatic hydrocarbons and forms a matrix around the fibres. It is a random copolymer built up of phenylpropane (C9) units. It is found in the cell wall of higher plants where it fills up the spaces between cellulose and hemicellulose fibres of the cell wall. Lignols derived from *p*-hydroxycinamic acid (coniferyl, sinapyl and *p*-coumaryl alcohol) are the direct precursors of lignin. Based on the building blocks, lignin may be described as softwood, hardwood and grass lignin (Glazer and Nikaido, 1995). Softwood lignin mainly contains coniferyl and some *p*-coumaryl alcohol. About 46% of each coniferyl and sinapyl and about 8% of *p*-hydroxyphenylpropane units derived from *p*-coumaryl alcohol are present in hardwood lignin. Grass lignin contains coniferyl, sinapyl and *p*-hydroxyphenylpropane units. *p*-Coumaric acid is mainly esterified with the terminal hydroxyl of *p*-coumaryl alcohol side chains.

1.2 CELLULASE ENZYME SYSTEMS:

1.2.1 COMPONENT (CLASSES) OF CELLULASES:

Cellulose degrading microorganisms produce a number of cellulolytic enzymes that act together to convert crystalline cellulose to glucose (Leschine, 1995; Singh and Hayashi, 1995; Xie *et. al.,* 2007). For the complete hydrolysis of polymeric cellulose into its monomeric units three major types of enzymatic activities are required (Haan *et. al.,* 2007; Lynd *et. al.,* 2002; Teeri, 1997):

1. Endoglucanases
2. Exoglucanases (including cellodextrinases and cellobiohydrolases)
3. β – glucosidases

Endoglucanases produce random internal cuts within the amorphous region in the cellulose molecule yielding cello-oligosaccharides of various lengths and thereby generating new chain ends (Lynd *et. al.,* 2002; Teeri, 1997). Exoglucanases act processively on the reducing and/or non-reducing ends producing either glucose, cellobiose and/or cellooligosaccharides. These soluble cellodextrins and cellobiose are then hydrolyzed by β – glucosidases to glucose (Figure 1.4). All endoglucanases so far seems to have an open active site, as they are able to bind to the interior of long cellulose fibres (Teeri, 1997). This is in contrast to exocellulase which have their

active site in a tunnel and hence is consistent with their processive nature resulting in sequential release of cellobiose from the end of cellulose chain.

The three types of enzymes act in a coordinated manner to hydrolyse cellulose. The amorphous regions within the cellulose fibres are first attacked by endoglucanase creating sites for exoglucanases to proceed into the crystalline regions of the fibre (Beguin, 1990). Exoglucanases also tend to act on microcrystalline cellulose, to apparently peel cellulose chains off the microcrystalline structure. Lastly, β – glucosidases split cellobiose to glucose preventing the build-up of cellobiose which inhibits cellobiohydrolases.

The recalcitrant and insoluble nature of cellulose represents a challenge for cellulases; therefore the structure of most cellulases includes carbohydrate binding modules (CBM) in addition to catalytic modules (Lynd *et. al.*, 2002).

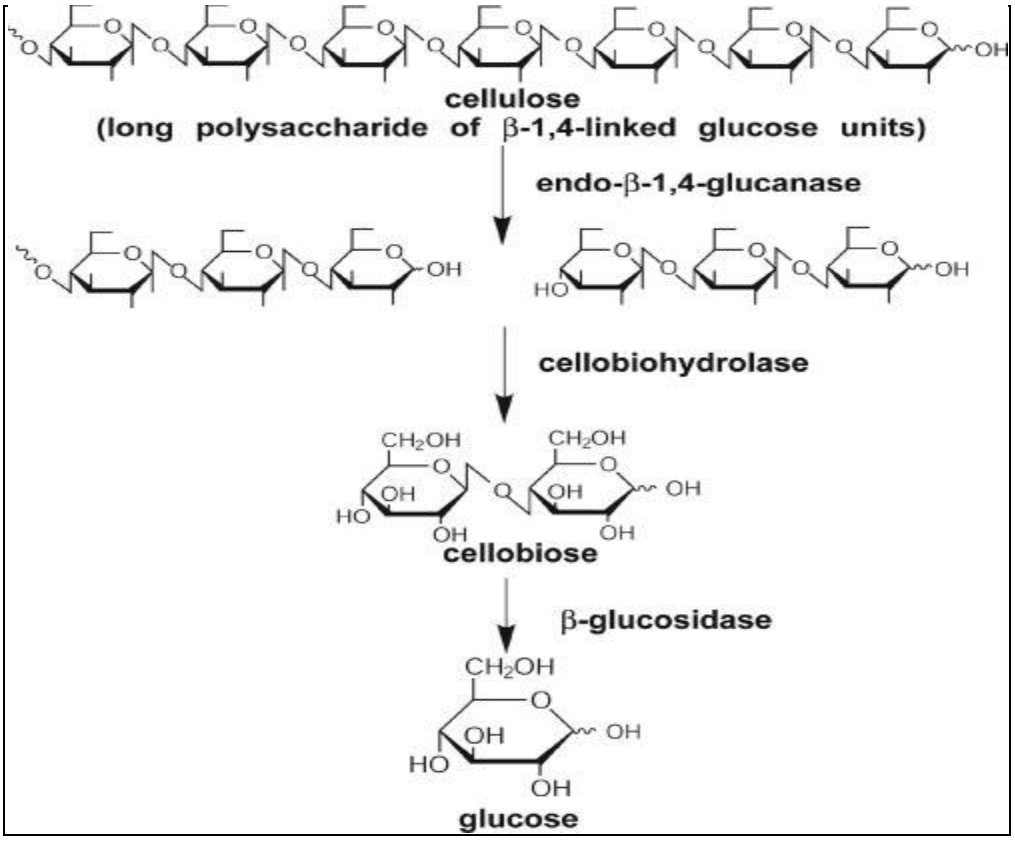


Figure 1.4: Different classes of Enzymes involved in cellulose breakdown by bacteria (Xie *et. al.*, 2007).

1.2.2 CARBOHYDRATE BINDING MODULES/DOMAINS:

In late 1940s, Reese and his associates proposed that the initial stage of microbial conversion of crystalline cellulose involves the action of an unknown non-hydrolytic component termed C_1 (now known as Carbohydrate Binding Module). This C_1 system makes the substrate more accessible to the hydrolytic component C_x (now known as Catalytic Module), by destabilizing the structure of cellulose (Din *et. al.,* 1994; Reese *et. al.,* 1950). Hence a CBM is defined as an adjoining amino acid sequence within a carbohydrate active enzyme with a distinct fold having carbohydrate binding activity. Hence to date, more than 300 CBM sequences in more than 50 different species have been identified which have been classified into 64 different families based on their amino acid sequences, structure and binding specificity (Shoseyov *et. al.,* 2006).

The CBMs exist as a single, double or triple domain in a protein and contains from 30 – 200 amino acids. The location of CBM in a polypeptide chain could be both, C- or N- terminal (Figure 1.5) and is sometimes also centrally positioned within the parental protein. The 3-D structure of several CBMs in complex with their ligands has been determined which provides insights into the underlying mechanism of CBM-ligand recognition and interaction (Shoseyov *et. al.,* 2006). The data from these structures hence indicates that CBMs from different families are structurally similar and that their carbohydrate binding capability could be accredited to several aromatic amino acids that comprise the hydrophobic surface. CBMs in hydrolytic proteins like cellulases are linked to the catalytic modules via relatively unstructured linker sequence rich in proline and threonine.

CBM is thought to function by bringing the biocatalyst into intimate and prolonged association with its recalcitrant substrate thereby increasing the rate of catalysis. They are thus considered important for the initiation and processivity of the enzymes particularly of exoglucanases (Lynd *et. al.,* 2002; Teeri *et. al.,* 1998; Reese *et. al.,* 1950). The removal of CBM from enzymatic subunit has found to dramatically decrease the activity of enzymatic subunit. In addition they also tend to play a role in sloughing off the cellulose fragments from cellulosic substrates enhancing hydrolysis.

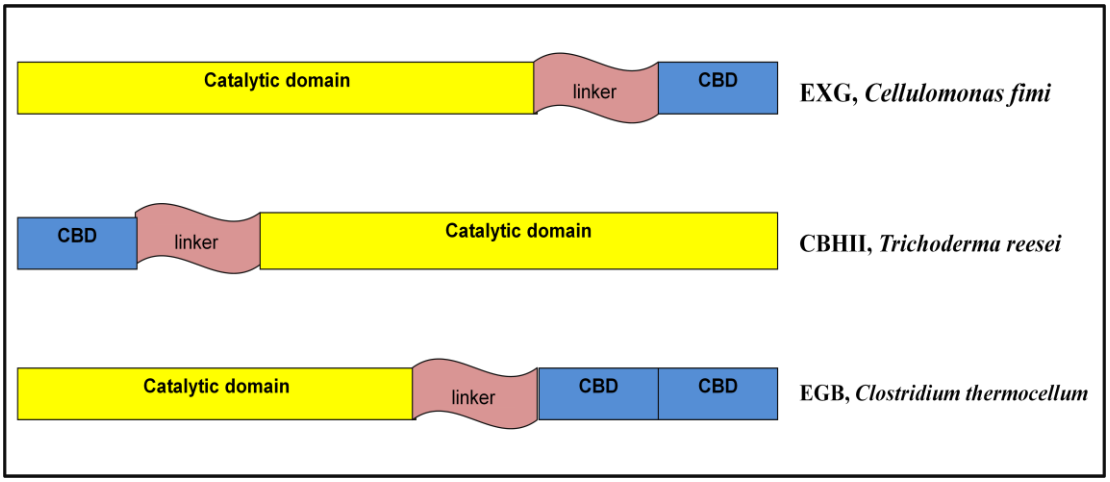


Figure 1.5: Schematic representation of multi-domain organization shown by non-complex cellulases. CBD – carbohydrate binding domain, EXG – exoglucanase, CBHII – cellobiohydrolase II, EGB – endoglucanase B.

1.2.3 CATALYTIC MODULE/DOMAIN:

The longest domain within cellulases corresponds to catalytic cores. Studies of gene deletion, proteolytic truncation etc., shows that they behave as an independent entities conferred with catalytic activity and defined specificity towards soluble model substrates (Beguin, 1990). Although the catalytic domains of cellulases exhibits considerable diversity they have been grouped into glycoside hydrolase families based on their amino acid sequence similarities. Glycoside hydrolases are a group of enzymes which hydrolyse the glycosidic bond between carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Lynd *et. al.*, 2002; http://www.cazy.org/Glycoside-Hydrolases.html). The enzymes in the same family contain similar basic fold based on the idea that a direct relationship exists between amino acid sequence and the folding of the protein. Hence based on this classification enzymes having different substrate specificities are sometimes found in the same family; for example, family 5 contains cellulase, xylanases and mannanases, indicating an evolutionary divergence to acquire new specificities. On the other hand enzymes with same substrate specificity are found in different families; for example, cellulases are found in 11 (5, 6, 7, 8, 9, 12, 44, 45, 48, 61 and 74) different families. This classification which contain more than 5000 glycoside hydrolase are grouped into 130 families.

The classification of glycoside hydrolase into structurally determined families provides valuable insights into the structural features of the enzymes, which are more informative than the substrate specificity which was the basis of old IUBMB classification. Hence the 3-D structure of one member of the family can be used to infer the structure of other members of the same family. This classification also defines the modules of the enzymes and thus resolves the contradiction about substrate specificity for multifunctional enzymes (Lynd *et. al.,* 2002). It also sheds the light on the evolution of the glycoside hydrolases; as for example, some families are deeply rooted evolutionarily, such as family 9, which contains cellulases of fungi, bacteria, animals and plants. However this is in contrast to family 7 which contains hydrolases of fungal origin only and family 8 which contains only bacterial hydrolases. In addition cellulases from several families and hence from several different folds are found in the same organism; for example, *Cellulomonas thermocellum* contains endoglucanase and exoglucanase from families 5, 8, 9 and 48. Cellulases are hence a complex group of enzymes that seems to have evolved through concurrence from a repertoire of basic folds. In addition, the extensive diversity within the cellulase families reflects the heterogeneity of cellulose and associated polysaccharide within plant material and a variety of environment where hydrolysis takes place.

1.2.4 TYPES OF CELLULASE SYSTEMS:

Cellulolytic microorganisms tend to adopt different strategies to hydrolyse cellulose effectively (Lynd *et. al.,* 2002; Tomme *et. al.,* 1995). They either produce free cellulsases (with or without CBMs) which do not form stable complexes and are thus called ‘noncomplexed’ systems, or ‘complexed’ cellulose systems, called ‘cellulosomes’ that often remain attached to the bacteria (Figure 1.6 and 1.7).

1.2.4.1 NONCOMPLEXED CELLULASE SYSTEMS:

Aerobic bacteria and fungi secrete soluble extracellular cellulolytic enzymes with or without CBMs that anchor them to the substrate (Wilson, 1992; Xie *et. al.,* 2007) (Figure 1.7). The enzymes attack cellulose resulting in the release of sugars which are eventually taken up by cells and metabolized. The ability of cellulolytic filamentous fungi (and actinomycete bacteria) to penetrate cellulosic materials through hyphae

enables them to release cellulases in confined cavities within cellulosic materials. These free cellulases therefore suffice for the efficient hydrolysis of cellulose under such conditions (Eriksson *et. al.,* 1990). In contrast anaerobic bacteria lack the ability of effective penetration of cellulosic substrates and thus have to find an alternative approach for cellulose degradation in order to gain first access (in the presence of other competing microorganisms) to the products of cellulose hydrolysis with the limited ATP available for the synthesis of cellulases. This could have resulted in the development of 'complexed cellulase' system which positions the anaerobic organism close to the site of hydrolysis.

1.2.4.2 COMPLEXED CELLULASE SYSTEM:

The various cellulase components in anaerobic bacteria are found in tight association forming multiprotein complexes called cellulosomes that often remain attached to bacterial cells (Beguin, 1990; Lynd *et. al.,* 2002) (Figure 1.6). They help anchor the bacteria to cellulose resulting in localized release of hydrolysis products which are taken up by the cells. Cellulosomes contain numerous kinds of cellulases and related enzyme subunits which are held together by a unique scaffoldin subunit. Scaffoldins are very large, modular polypeptides that hold the multimolecular complex together. They contain a carbohydrate binding domain (CBD), one or more conserved hydrophilic modules (the function of which is not known) and most importantly multiple copies of cohesin domains. The cellulosomal enzyme subunits are known to contain a dockerin domain, which mediates the integration of enzymes into the cellulosome complex. Dockerins of the enzymatic subunits are involved in a very stable type of binding interaction with the cohesins of the scaffoldin subunit. There is little or no specificity in the binding of various cohesins and the dockerins in the cellulosomes. The CBD helps in the recognition and binding of the scaffoldin subunit to the cellulosic substrate, hence, if the cellulosome is implanted in the cell surface, the CBD of scaffoldin results in the binding of the entire cell to its insoluble substrate, cellulose (Bayer *et. al.,* 1998).

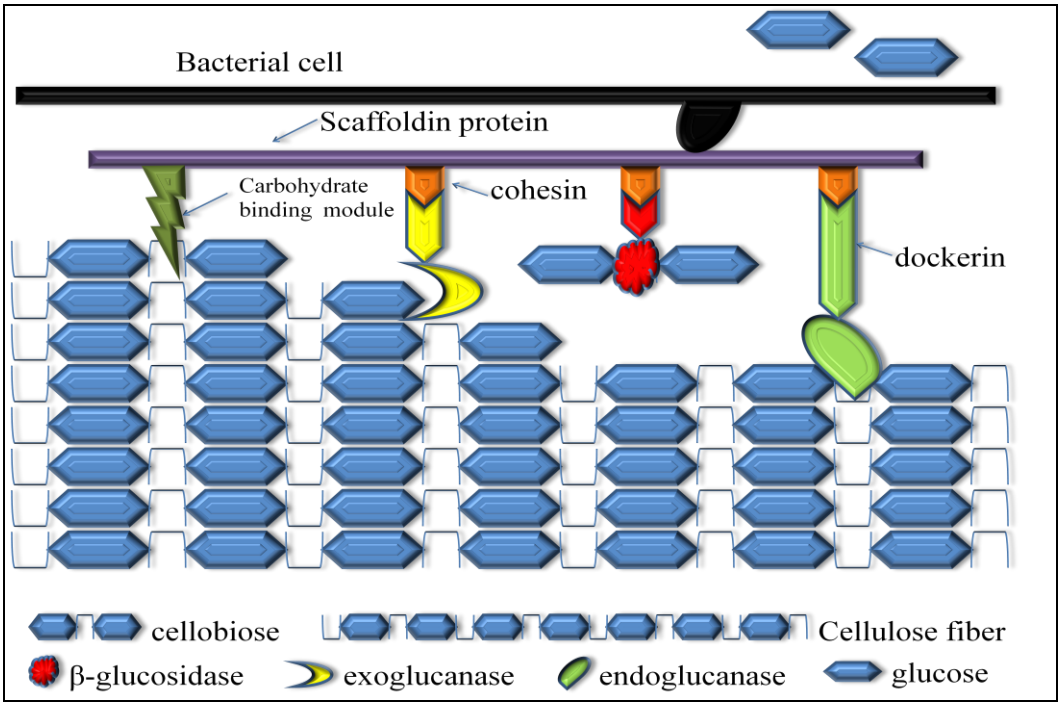


Figure 1.6: Hydrolysis of cellulose by complexed cellulase system.

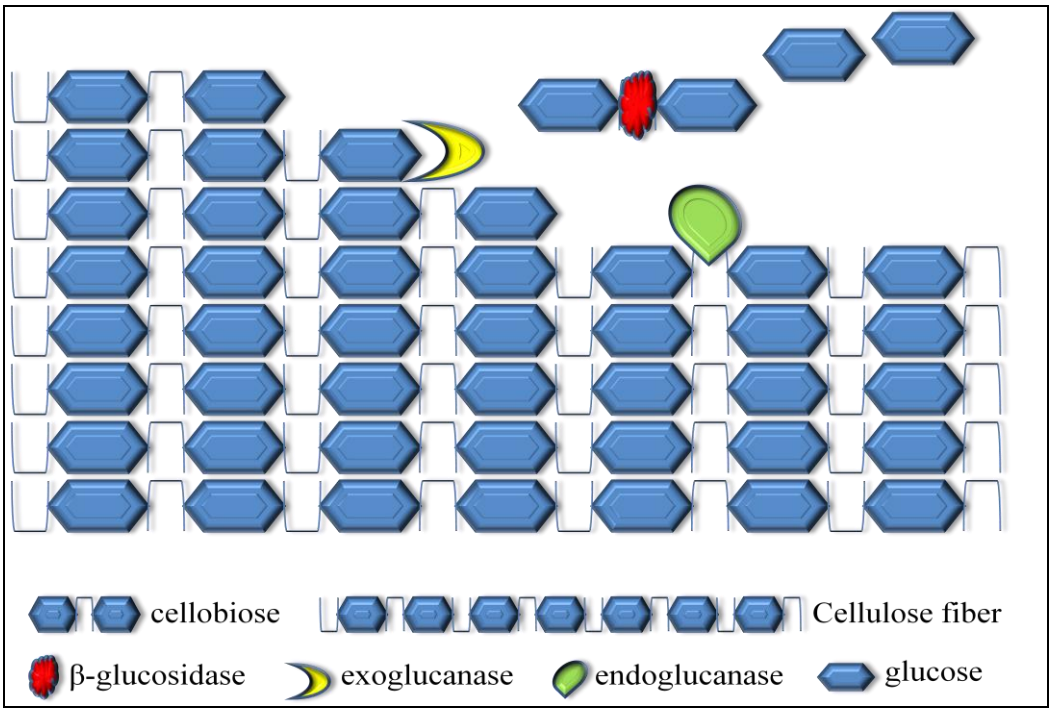


Figure 1.7: Hydrolysis of cellulose via noncomplexed cellulase system.

1.2.4.3 NON-HYDROLYTIC ENZYMES:

In addition to hydrolytic enzymes, occasional non-hydrolytic enzymes have also been cited in the literature to be involved in cellulose degradation (Eriksson *et. al.,* 1974; Li *et. al.,* 1996). These include oxidative enzymes such as cellobiose:quinone oxidoreductase (CBQ), cellobiose dehydrogenase and cellobiose oxidase (CBO) (Dekker, 1980; Eriksson *et. al.,* 1974; Westermark and Eriksson, 1974a and 1974b). These enzymes oxidize cellobiose and/or higher cellodextrin reducing ends to their corresponding lactones which can then be used by organisms as their source of carbon (Ayers *et. al.,* 1978; Li *et. al.,* 1996; Li *et. al.,* 1997).

After the discovery by Eriksson *et al.,* (1974) of oxidative enzymes as potential candidate involved in cellulose degradation research has mostly been focused on the enzymes from white rot fungi. To date very little is known about oxidative systems in bacteria (Li *et. al.,* 1996; Li *et. al.,* 1997).

The possible role played by these oxidizing enzymes in cellulose degradation may be to assuage of the inhibitory effect of cellobiose on cellobiohydrolase action, to regulate the synthesis of enzymes involved in cellulose degradation and/or in the metabolism of cellobiose itself (Li *et. al.,* 1997). In addition French (Edinburgh University, UK, personal communication) suggested that oxidases might also generate H_2O_2 required by lignin-degrading enzymes like peroxidases and laccases for the degradation of lignin.

1.3 SYNERGY AND COMPETITION AMONG CELLULASES:

The cellulase degradation system has been shown to have a higher collective activity than the sum of the activities of individual enzymes, a phenomenon called synergy (Din *et. al.,* 1994; Teeri, 1997). Four forms of synergism have been reported:

- Synergism between endoglucanase and exoglucanase, called endo-exo synergy
- Synergism between exoglucanases, called exo-exo synergy
- Synergism between exoglucanase and β – glucosidase to remove cellobiose that inhibits exoglucanase
- Synergism between catalytic and carbohydrate binding domains

Fujita *et. al.,* (2004) reported the synergistic hydrolysis of amorphous cellulose by a yeast strain co-displaying endoglucanase II (EGII) and cellobiohydrolase II (CBHII) from *Trichoderma reesei* and *Aspergillus aculeatus* β-glucosidase 1 (BGL1). They observed higher hydrolytic activity by the strain co-displaying EGII and CBHII (1.3 mM reducing sugar were released in 60 hours) on amorphous cellulose than the strain displaying only EGII (0.5 mM reducing sugars were released in 60 hours) with the main hydrolysis product been cellobiose. The co-display of BGL1 along with EGII and CBHII resulted in direct production of ethanol from amorphous cellulose. Ethanol was not produced from amorphous cellulose in the presence of only EGII and CBHII.

Zhou and Ingram (2000) observed synergism between two endoglucanases CelY and CelZ from *Erwinia chrysanthemi*. They observed about 1.8 fold synergy when the enzymes were used in combination. The synergy was due to the difference in substrate preference. CelY hydrolyzed CMC to fragments averaging 10.7 glucosyl units but was unable to hydrolyze cellotetraose and cellopentaose. On the other hand CelZ readily hydrolyzed soluble cellooligosaccharides and amorphous cellulose to produce cellobiose and cellotriose as major end product. Hydrolysis of CMC by CelZ resulted in fragments averaging 3.6 glucosyl units. In combination both enzymes hydrolyzed CMC to fragments averaging 2.3 glucosyl units. Synergy was also observed after the sequential addition of CelY and CelZ (after heat inactivation of CelY) showing that synergy does not require the simultaneous presence of both the enzymes. However no synergy was observed when CelZ was used as the first enzyme, hence showing that only CelY can act independently to modify the substrate to make it more accessible to CelZ.

By conventional definition, exocellulases releases cellobiose from non-reducing ends of cellulose chain, which does not explain exo-exo synergy and would hence compete for limited number of hydrolysis sites, instead of cooperating to give synergistic hydrolysis. Enzymatic hydrolysis would be insufficient if the enzymes only act at non-reducing ends, since half the chain ends would be unused. Hence this traditional model was questioned followed by the report describing two exoglucanases from *Aspergillus aculeatus*, one of which attacks from reducing end and the other from non-reducing end of cellulose chain. Similarly two different classes of cellobiohydrolases in *Trichoderma reesei*, CBHI (makes up 60% of total cellulolytic

proteins) acting from reducing end of the chain and CBHII (makes up 20% of total cellulolytic proteins) acting from non-reducing end can achieve complete solubilisation, although slow, of cellulose without the help of endoglucanases (Teeri, 1997). Hence these data suggests that the observed exo-exo synergy could be due to the interaction between non-reducing end attacking and reducing end attacking exocellulases.

Most cellulases are composed of catalytic and carbohydrate binding domains that function independently but act synergistically in the disruption and hydrolysis of cellulose fibres. The carbohydrate binding domain makes the substrate more accessible to hydrolytic domain by bringing the catalytic module in close proximity to boost hydrolysis (Lynd *et. al.,* 2002; Teeri *et. al.,* 1998; Reese *et. al.,* 1950). It is also considered to play a role in sloughing off the cellulose fragments from the surface of cellulose by splitting of the cross linkages.

It is also worth mentioning that cellulose degraders always seem to produce multiple enzymes of each class. As an example *Trichoderma reesei* cellulase system consists of two exoglucanases (CBHI and CBHII), five endoglucanases (EGI, EGII, EGIII, EGIV and EGV) and two β-glucosidases (BGLI and BGLII) (Nogawa *et. al.,* 2001). *Humicola insolens* produces at least seven cellulases including two cellobiohydrolases (CBHI and CBHII) and five endoglucanases (EGI, EGII, EGIII, EGV and EGVI) (Schulein,1997). *Thermobifida fusca* contains three endoglucanases (E1, E2 and E5), two exoglucanases (E3 and E6) and a cellulase with both endo-exo activity (E4) (Irwin *et. al.,* 1998; Lynd *et. al.,* 2002). The secretion of multiple cellulases of same class could be due to the heterogenous nature of their substrate. As cellulose structure varies from being purely crystalline to purely amorphous with all degrees of order in between, hence some of these enzymes are more effective towards one form of cellulose while others are more effective towards other forms. In addition, it also indicates that although each individual β-1,4,-glucosidic bond is chemically identical, the complex nature of the substrate and the environment in which they are present shows that they are not in identical context. Hence therefore one might expect to observe synergy between cellulases of same class as well as of different classes. The combination of these enzymes thus acts synergistically to hydrolyse cellulose. In addition to cellulase these organisms may also possess enzymes to degrade other

polysaccharides associated with cellulose such as hemicelluloses, presumably because their breakdown is required to gain access to cellulose fibres (Xie *et. al.,* 2007).

With regard to synergy between cellulases, synergy is observed in some cases but not in other. Anderson *et. al.,* (2008) reported the synergistic effect shown by a mixture of cel45A, cel6A (an endoglucanse and an exoglucanase respectively from *Humicola insolens*) and β-glucosidase from *Penicillium brasilianum* on amorphous cellulose. However on crystalline cellulose these enzymes seem to rather inhibit each other owing to the competition for binding sites on cellulose. Whereas some other studies (Valjamae *et. al.,* 1999; Zhang and Lynd, 2004) with different enzymes showed synergistic effect on crystalline cellulose but not on amorphous cellulose.

1.4 CELLULOLYTIC MICROORGANISMS:

While many organisms can degrade amorphous cellulose, soluble cellulose analogues like CMC and hemicellulose-like substrates, there are relatively few species of bacteria that can actually degrade crystalline cellulose. In fact the ability to degrade crystalline cellulose seems to be restricted to specialized cellulose-degrading microorganisms (Coughlan and Mayer, 1992). Cellulose being an abundant and relatively recalcitrant source of carbon for which there is very little competition, it is not surprising that certain microorganisms come to specialize in its degradation.

The efficient degraders of crystalline cellulose include anaerobic, spore forming, Gram positive bacteria of genus *Clostridium*, the aerobic Gram positive bacteria of the genus *Cellulomonas* with relatively high GC content and some of the streptomycetes, the anaerobic Gram negative bacteria including the *Fibrobacter* group, the aerobic Gram negative gliding bacteria *Cytophaga* and some myxobacteria, and a few other groups (French, 2009).

Fungal cellulose degraders belong to both ascomycetes and basidiomycetes. According to their ability to degrade lignin, they are traditionally classified as white rot fungi, brown rot fungi and soft rot fungi (Glazer and Nikaido, 1995). White rot fungi decompose most components of plant cell walls including lignin and cellulose, whereas, brown rot fungi can only degrade hemicelluloses and cellulose leaving behind brown shrunken lumps of partly modified lignin (Tanesaka *et. al.,* 1993). Soft rot fungi belong to ascomycetes and represent the second important group of wood

colonizing fungi. They cause soft-rot decay with considerable cellulose degradation compared to lignin. However a few studies have demonstrated lignin degradation as well (Lopez *et. al.,* 2007). Nevertheless there is still a lack of information about their capabilities and their potential degradation abilities still need to be explored.

1.4.1 *Cytophaga hutchinsonii:*

Cytophaga hutchinsonii, the type species of genus *Cytophaga*, is an aerobic Gram negative bacterium, abundantly found in soil and decaying plant material (Xie *et. al.,* 2007; Larkin, 1989). It is an actively cellulolytic bacterium which belongs to phylum *Bacteroidetes* (also known as the *Cytophaga-Flavobacterium-Bacteroides* group). It was been reported in earlier studies that only a few substrates are known to be utilized by *C. hutchinsonii* as sole carbon and energy source. Besides cellulose, its only known substrates are glucose and cellobiose. Cellodextrins were reported to be toxic for *Cytophagas*. However, Zhu *et. al.,* (2010) showed that *C. hutchinsonii* were able to grow using cellodextrins as a sole carbon source with longer cellodextrins been hydrolyzed extracellularly during growth. It was also able to use glucose and cellobiose efficiently which were previously reported to be a poor substrates for *Cytophaga* (Zhu, *et. al.,* 2010; Xie, *et. al.,* 2007).

Examination of the *C. hutchinsonii* genome sequence reveals the presence of clusters of genes responsible for degradation of cellulose (Xie *et. al.,* 2007). In addition to genes encoding probable β–endoglucanases, there are genes encoding possible β–glucosidases but no recognizable exoglucanases/cellobiohydrolases. In addition, most cellulases of *C. hutchinsonii* do not contain the carbohydrate binding module (CBM), hence proving the absence of any processive cellulases (Wilson, 2008; Louime *et. al.,* 2007). Cellulases generally consist of a catalytic domain (CD), a CBM and the linker joining the two. The major role of the CBM is to anchor the CD to its insoluble substrate and probably also to disrupt the structure of cellulose to make it more accessible to the CD. Since to date these features were considered mandatory for efficient degradation of cellulose by other cellulose degraders, *C. hutchinsonii* may therefore have a novel mechanism for cellulose degradation. One possible mechanism for cellulose digestion by these bacteria might be the one used by the starch degrading bacterium *Bacteroides thetaiotaomicron* for starch degradation (Wilson, 2008). In this model, cellulose is bound to a protein complex present in the outer membrane and the

individual molecules are transported into the periplasmic space, where they are degraded by cellulases. This mechanism excludes the requirement for processive cellulases, as individual cellulose molecules would be readily degraded by endoglucanases. This hypothesis also fulfils the unique requirement of these bacteria to be in direct contact with their insoluble substrate for efficient digestion (Wilson, 2008; Xie *et. al.,* 2007).

In earlier reports it was also mentioned that during cellulose digestion by *C. hutchinsonii* no reducing sugars like cellobiose and glucose accumulate in the medium (Stanier, 1942; Xie *et. al.,* 2007). These sugars do not accumulate even when the bacteria are incubated under anaerobic conditions, suggesting that it is not simply because of the efficient uptake and metabolism of these reducing compounds by the bacterium, since such anaerobic conditions should interfere with these processes. However Zhu *et. al.,* (2010) reported that when *C. hutchinsonii* were grown in the presence of cellulose small amounts of soluble sugars, less than 0.180 mg/ml, were accumulated in the medium during exponential growth phase. These include glucose, cellobiose and cellotriose. In addition a significant amount of longer cellodextrins (up to 4 glucose units) were also produced. These results suggest the presence of an efficient mechanism for cellulooligosaccharide production, which is tightly coupled to its assimilation. This may constitute an important part of digestion of cellulose in *C. hutchinsonii*. This is in agreement with the hypothesis that soluble oligomers derived from cellulose digestion by cell-surface-anchored endoglucanases are transported into the cells for further digestion (Xie *et. al.,* 2007; Zhu *et. al.,* 2010).

Louime *et. al.,* in 2007 did the biochemical characterization of endoglucanase gene (*cel9A*) from *C. hutchinsonii*. It belongs to family 9 of glycosyl hydrolase but lacks the CBD and linker. The enzyme was optimally active at 45°C and pH 5.0. It is a nonprocessive enzyme with endo-cellulase activity as suggested by the analysis of CMC and filter paper hydrolysis.

1.4.2 *Cellulomonas fimi:*

Cellulomonas fimi is a Gram positive, nonsporeforming, facultatively anaerobic actinomycete with high G+C content of 72 mol% and belongs to the coryneform group of bacteria (Greenberg *et. al.,* 1987; Stackebrandt and Kandler, 1979). The

organisms belonging to the genus *Cellulomonas* have unique capabilities of producing amylolytic, xylanolytic and cellulolytic enzymes. Their main habitat is soil where they tend to degrade crystalline cellulose (Chaudhary *et. al.,* 1997; Daly *et. al.,* 1983; Rodriguez *et. al.,* 1988; Langsford *et. al.,* 1984; Kumar *et. al.,*1995; Haggett *et. al.,* 1979).

Cellulases from *C. fimi* are very well characterized, including four endoglucanases (CenA, CenB, CenC and CenD), two exoglucanases (CbhA and CbhB) and an exoglucanase/xylanase (Cex) (Table 1.3).

Table 1.3: Cellulases from *Cellulomonas fimi*

Enzyme	Type	Domains	Charecteristics	Reference
CenA	Endoglucanase	GH6, CBM2	449 aa, 46.7kDa, first 31 N-terminal aa constitute leader peptide. C-terminal is crucial for activity and deletion of as little as 12 aa results in loss of activity. Trp 45, 81 and 99 in CBM play a role in carbohydrate binding and Asp252, 392 are essential for acid-base catalysis.	Wong *et. al.*, 1986; Damude *et. al.*, 1996
CenB	Endoglucanase	GH9, CBM3, FN3, CBM2	1,012 aa, 5 domains connected by proline and hydroxyamino acids rich linker. 607 N-terminal aa constitutes catalytic domain, 103 aa of C-terminal domain bind the domain to cellulose forming a 2nd cellulose binding site on the enzyme. The C-terminal 100 aa forms CBM and in between them are 3 identical domain of 98 aa each.	Meinke *et. al.*, 1991
CenC	Semiprocessive enzyme having endoglucanase and exoglucanase activity	GH9, CBM4_9, CBM4_9, cellulase_N, Ig	Has very distinctive structural and functional features. Contains 2 CBMs (N_1 and N_2) and 2 tandem C-terminus (C_1 and C_2) domains of unknown function suggesting some unusual properties.	Tomme *et. al.*, 1996
CenD	Endoglucanase	GH5, FN3, CBM2	747 aa, 75kDa, comprised of 4 domains separated by short linker peptide: 405 aa N-terminal catalytic domain, 2 repeats of 95 aa each and C-terminal 105 aa domain.	Meinke *et. al.*, 1993
CbhA	Exo-celobiohydrolase	GH6, FN3, CBM2	832 aa, composed of 5 domains: N-terminal catalytic domain, 3 repeats of 95 aa and C-terminal CBM.	Shen *et. al.*, 1995; Meinke *et. al.*, 1994
CbhB	Exo-cellobiohydrolase	GH48, FN3, CBM2	1,037 aa, 120kDa, initially thought to be an inverting endoglucanase, designated as CenE. Shen *et. al.*, in 1995 reported it to be an exocellobiohydrolase with weak but detectable endoglucanse activity. Comprised of 5 domains: N-terminal catalytic domain, 3 fibronectin type III repeats and C-terminal CBM of 643, 97 and 104 aa each.	Shen *et. al.*, 1995
Cex	Exoglucanse/Xylanase	GH10, CBM2	485 aa, 51.2kDa, N-terminal 41 aa constitutes signal peptide. Exoglucanase activity is based upon Glu274 residue which tends to cleave β-1,4 bonds to release cellobiose by nucleophillic attack.	O'Neill *et. al.*, 1986; Macleod *et. al.*, 1994

GH: Glycosyl hydrolase, CBM: Carbohydrate binding module, FN: Fibronectin type domain

1.5 COMMERCIAL PROCESSES FOR BIOMASS CONVERSION:

There are four biological events that occur during the biotechnological conversion of cellulosic biomass into fuels and other chemicals (Lynd *et. al.,* 2002). These include:

1. Cellulase production
2. Hydrolysis of cellulose and if present of other insoluble polysaccharides like hemicellulose
3. Fermentation of soluble hydrolysis products of cellulose
4. Fermentation of soluble hydrolysis products of hemicelluloses

The original conversion process, Separate Hydrolysis and Fermentation (SHF), involved four separate steps and four distinct biocatalysts (Lynd *et. al.,* 2002). An extension to this process is Simultaneous Saccharification and Fermentation (SSF) which consolidates hydrolysis and fermentation of cellulose hydrolysis products into one process step, while cellulase production and fermentation of hemicellulose hydrolysis products occur in two additional distinct steps. This reduces the complexity of the process while removing the issue of product inhibition as well (Lynd *et. al.,* 2002; French, 2009). A further advancement is Simultaneous Saccharification and Co-fermentation (SSCF) which involves two process steps: cellulase production and a second step in which hexoses and pentoses released by the hydrolysis of cellulose and hemicelluloses are fermented in the same process step. The rational final development is 'Consolidated Bioprocessing' (CBP), in which cellulase production, hydrolysis and fermentation of products of cellulose and hemicelluloses are all attained in a single process step.

1.6 PROCESSING OF CELLULOSIC BIOMASS – PRE-TREATMENT:

The hydrolysis of naturally occurring lignocellulosic materials by cellulolytic enzymes generally results in hydrolysis yield of less than 20% of theoretical (Lynd *et. al.,* 2002). Hence the cellulose hydrolysis process designed for biological conversion of cellulosic material almost always include a pre-treatment step, the properties of which are of central importance in defining the enzyme activities required for cellulose hydrolysis and product formation. The term 'pre-treatment' is widely used to

refer to a process step which converts biomass (which in its native form is recalcitrant to cellulase enzyme system) into a form which is susceptible to enzymatic hydrolysis.

In naturally occurring lignocellulosic materials cellulase enzymes are unable to gain access to most β – glucosidic bond as the pore size in heterogenous biomass matrix is very small. Associated hemicellulose and other structural polysaccharide are closely associated with cellulose in natural environment (Lynd *et. al.,* 2002). Carbohydrate rich microfibrils are also surrounded by lignin seal which also makes it difficult for cellulases to reach cellulose. Therefore in order to render lignocellulosic material susceptible to enzymatic hydrolysis both physical and chemical barriers must be overcome. Hence the effective lignocellulosic pre-treatment should result in increased porosity and solubilisation and/or redistribution of lignin.

The various widely used pre-treatment process include dilute acid, steam explosion, hydrothermal processes, organosolv processes, ammonia fiber explosion and strong alkali processes. Under appropriate conditions, all these processes have shown to retain nearly all of the cellulose material derived from herbaceous and/or hardwood – derived feedstocks. In addition they also allow theoretical yield of ethanol upon enzymatic hydrolysis. Table 1.4 summarizes the form in which cellulose, hemicellulose and lignin emerge from different pre-treatment processes and hence shows which enzyme activities would be required for further breakdown of the remaining biomass. Hence in most cases organism should be able to produce enzymes able to hydrolyse cellulose as most pre-treatment process only results in some depolymerisation and/or decrystallization of cellulose.

Table 1.4: Fate of biomass components after various pre-treatments (Lynd *et. al.*, 2002)

Pre-treatment process	Fate of biomass components		
	Cellulose	**Hemicellulose**	**Lignin**
Dilute-acid	Some depolymerization	80-100% solubilisation to monomeric units	Extensive redistribution but little or no solubilisation
Steam explosion	Some depolymerization	Mixture of monomers and oligomers resulting from 80-100% solubilisation	Extensive redistribution but little or no solubilisation
Hydrothermal processes	Some depolymerization	Oligomers resulting from 80-100% solubilisation	20-50% solubilisation
Organic solvents with water		Partial to nearly complete solubilisation	Partial to nearly complete solubilisation
Ammonia fiber explosion	Some decrystallization	Nearly 90% hydrolysis to oligomers with solubilisation from 0-60% depending on moisture	10-20% solubilisation
Sodium hydroxide pre-treatment	Conversion from type I to type II cellulose with substantial swelling	Often more than 50% solubilisation	Often more than 50% solubilisation
Lime pre-treatment		Up to 30% solubilisation under some but not all conditions	About 40% solubilisation

1.7 RECENT ADVANCES IN RECOMBINANT CELLULOLYTIC APPROACH:

Development of organisms with the goal of effective degradation of biomass via CBP begins with the expression of a functional cellulase system in a heterologous host. There have been many reports describing heterologous expression of cellulase genes, both for research purposes and with the intention of conferring the organism with the ability to degrade biomass effectively. However, the comparison of cellulase activity between different studies is rather difficult due to the complex nature of substrate and the difference in the assays used to detect the activity of cellulases. To date, mostly *Saccharomyces cerevisiae*, *Zymomonas mobilis* and enteric bacterial systems have been investigated as models for heterologous expression of cellulase, but it appears that expression of highly active enzymes has in many cases been challenging. While many reports describe the development of recombinant organisms with the ability to degrade amorphous cellulose and utilize the resulting sugars, effective degradation of crystalline cellulose still remains tricky (Lynd *et. al.,* 2002; French, 2009).

1.7.1 CELLULASE EXPRESSION IN BACTERIA:

1.7.1.1 *Zymomonas mobilis:*

Z. mobilis, a facultative anaerobic Gram negative ethanologenic bacterium, utilizes glucose through the Entner-Doudoroff pathway, in which 1 mol of glucose yields 2 moles of pyruvate and 1 mol of ATP. In order to remain competitive with this low energetic yield, the metabolism of *Z. mobilis* is greatly increased by elevated levels of glycolytic and ethanologenic enzymes, the PDC-pyruvate decarboxylase and the ADH-alcohol dehydrogenase. The resulting yield of ethanol by this organism is outstanding, about 97% of theoretical (Zaldivar *et. al.,* 2001; Panesar *et. al.,* 2006). In order to expand its substrate utilization spectrum for ethanol production from cellulosic biomass, several cellulase genes have been cloned and expressed in *Z. mobilis* with varying degrees of success. The endoglucanase gene (*eglX*) from *Pseudomonas fluorescens* var *cellulosa* has been introduced into *Z. mobilis* (Lejeune *et. al.,* 1988). The gene was expressed but the enzyme remained intracellular and no activity was detected in culture supernatant. The specific activity of endoglucanase in cell extracts was about 350 mU/mg. However the authors report this construction as

the initial step towards developing a cellulolytic *Z. mobilis* for which the cloning and expression of other cellulases and especially their secretion would be required.

An endoglucanase *celZ* from *Erwinia chrysanthemi* has also been cloned and expressed in *Z. mobilis*. The specific activity of this enzyme in *Z. mobilis* was comparable to that in *E. chrysanthemi* (3-4 IU/mg), but cellular localization was different. In *E. chrysanthemi* the enzyme was extracellular but in *Z. mobilis* the activity was cell associated during exponential phase. However after about 20 hours of growth 32% of the activity was detected in the medium (Brestic-Goachet *et. al.*, 1989). Hence they concluded that the cellulase gene was stable and expressed in *Z. mobilis*, but for the organism to degrade cellulose at least one β-glucosidase and a exoglucanase still needs to be cloned, and also these enzymes need to be secreted in the medium for the organism to use cellulosic substrate for growth.

In another report two cellulolytic enzymes, E1 and GH12 from *Acidothermus cellulolyticus*, were cloned and expressed in *Z. mobilis*. The proteins were successfully expressed as soluble, active enzymes with GH12 comprising as much as 4.6% of total cellular proteins. These enzymes resulted in minor changes in the growth rate of *Z. mobilis* with approximately 20% reduction in logarithmic growth rate when compared to the control strain (Linger *et. al.*, 2010). The enzymes seemed to be residing in the periplasmic space of *Z. mobilis*, but upon fusion with the native *Z. mobilis* secretion signal of the *phoC* gene (ZM0130) and that belonging to a hypothetical protein (ZM0331), significant levels of enzymes were secreted into the extracellular medium. However, the culture harbouring the Z331-E1 (enzyme E1 fused with ZM0331 secretion signal) construct had a major growth limitation. The authors also mentions the need to coexpress other classes of cellulases (at least one exoglucanase and β-glucosidase) along with the endoglucanases in order for the organism to degrade cellulosic biomass effectively.

Another CMCase gene from *Cellulomonas uda*, CB4, was introduced into *Z. mobilis* NRRL B-14023 on pZA22, containing the promoter of the chloramphenicol acetyltransferase. When this promoter was replaced by a native *Z. mobilis* promoter the enzyme activity reached 0.78 U/ml, about 6-fold higher than that from the chloramphenicol promoter (Misawa *et. al.*, 1988). The authors showed the intention of constructing a *Zymomonas* strain that can degrade and utilize cellulose

and this study was their first step towards expanding the substrate utilization range of this bacterium before being able to make a strain that can actually degrade cellulose.

Another cellulase gene that was successfully expressed in *Z. mobilis* is CMCase from *Acetobacter xylinum* (Okamoto, 1994). The gene was efficiently expressed in *Z. mobilis* with CMCase activity of 285 mU/mg of protein. Yanase *et. al.* (2005) later reported the introduction of the β-glucosidase gene from *Ruminococcus albus*, tagged with a 53 amino acid Tat signal peptide from *Z. mobilis* glucose-fructose oxidoreductase. This enabled 61% of β-glucosidase activity to be transported through the cytoplasmic membrane. The recombinant *Z. mobilis* was able to produce 10.7 g/l ethanol from 20 g/l cellobiose anaerobically. However the authors report the need for introduction of a more efficient secretion system in order to improve ethanol production from cellobiose and cellooligosaccharides.

1.7.1.2 ENTERIC BACTERIA:

Where enteric bacteria have been used as hosts, there have been reports of the introduction of cellulase genes into *E. coli* and its close relative *Klebsiella oxytoca* enabling them to generate ethanol with high yield. *E. coli* and several enteric bacteria possess a natural ability to convert hexoses, pentoses and uronic acids to pyruvate. This compound is further converted to a mixture of ethanol, lactate, acetate and formate. Hence to increase ethanol production in *E. coli* and *Klebsiella oxytoca* M5A1 carbon flux was redirected towards ethanol production by inserting *pdc* and *adhB* from *Z. mobilis* (Ohta *et. al.,* 1991; Ingram *et. al.,* 1987). These genes enabled the organisms to produce ethanol with high efficiency (about 34.6 g/l in *E. coli* and 45 g/l in *K. oxytoca*). In order for these bacteria to use lignocellulosic biomass for ethanol production several cellulases were introduced with varying degrees of success. Two *Erwinia chrysanthemi* endoglucanases, *celY* and *celZ*, have been successfully expressed in *E. coli* and *K. oxytoca* enabling them to ferment amorphous cellulose to ethanol without any supplemental cellulase (Zhou *et. al.,* 2001; Zhou and Ingram, 2001). Initial research demonstrated the production of a high level of CelZ as a periplasmic product by *E. coli* B. This enzyme was able to work with commercial cellulase of fungal origin to increase ethanol production. Several promoters, for example *gap* and *eno* (*E. coli* glycolytic promoters), were then tested with a low-copy-number vector, but found to be less effective when compared to the original *E.*

chrysanthemi celZ promoter. However, the expression of *celZ* in *E. coli* was increased 6-fold (with total enzyme activity being 6,600 IU/lit) by using a surrogate promoter from *Z. mobilis*. This construct resulted in large polar inclusion bodies in the periplasmic space of the *E. coli* recombinant strain. Further increase in production of active enzyme (total activity 13,000 IU/lit) and secretion of 60% of the CMCase activity into the culture medium was demonstrated by the addition of *out* genes which encode the type II secretion system from *E. chrysanthemi* (Zhou *et. al.,* 1999). This resulted in total cellulase production of about 4 to 6% of total cellular protein. A similar cloning strategy when used in *K. oxytoca* resulted in CMCase production of about 5% of total cellular protein (Zhou and Ingram, 1999). The recombinant strain was only partially able to depolymerise acid-swollen cellulose with the production of a small amount of ethanol (3.9 g/l, with 1.5 g/l already being present at the time of inoculation). However, the strains were barely able to depolymerise ball-milled cellulose, avicel and xylan. Subsequently Zhou and Ingram found strong synergy between two endoglucanases encoded by *celY* and *celZ* in the hydrolysis of CMC and acid swollen cellulose (Zhou and Ingram, 2000). They also found that the substrate preference for the two endoglucanases was quite different from one another. CelY was unable to hydrolyse cellotetraose and cellopentaose (soluble cellooligosaccharides) but hydrolysed CMC to fragments averaging 10.7 glucosyl units in length. CelZ in contrast hydrolysed cellotetraose, cellopentaose and amorphous cellulose producing cellobiose and cellotriose as the dominant end products. When CelZ was used, CMC was hydrolysed to fragments averaging 3.6 hexose units. In combination, the enzymes hydrolysed CMC to products having an average degree of polymerization of 2.3 glucosyl units. It was subsequently concluded that the this synergy was due to the sequential hydrolysis by two enzymes with CelY acting first, as the synergy between the two enzymes was based on the difference in substrate preference. This synergistic action of enzymes was also investigated against crystalline cellulose. The activity was very low when Avicel was used as a substrate, indicating that the enzymes were active only against the amorphous regions of cellulose. This avenue of investigation was taken one step forward by coexpressing CelY and CelZ in ethanologenic derivative (strain P2) of *K. oxytoca* M5A1, having the native ability to metabolize cellobiose (Zhou *et. al.,* 2001). In subsequent fermentation assays, Zhou *et al.,* concluded that the most important contribution was due to CelY rather than CelZ. However, the amount of ethanol produced using

Sigmacell 50 (a highly crystalline substrate) was very low. In the absence of Sigmacell 50 all strains (including the parent strain) were able to produce 0.22 g/l ethanol. In the presence of Sigmacell 50, the parent strain, the strain having functional *celZ*, the strain having functional *celY* and the strain having both *celY* and *celZ* produced 0.23, 0.28, 0.24 and 0.26 g/l ethanol respectively indicating the hydrolysis of only a small fraction of amorphous cellulose in the substrate. Most recently, Zhou and Ingram reported a derivative of *K. oxytoca* M5A1 containing chromosomally integrated copies of *pdc* and *adhB* from *Z. mobilis* for ethanol production and *celY* and *celZ* endoglucanases from *E. chrysanthemi* (Zhou and Ingram, 2001). This recombinant strain SZ21 was able to ferment 28.96 g/l amorphous cellulose to 11.3 g/l ethanol compared to the parent strain, which can produce 1.90 g/l ethanol with the same amount of amorphous cellulose. These values are however lower than that obtained with monomer sugars in pH-controlled fermentations with P2 parent strain. The hydrolysis of crystalline cellulose was not reported.

1.7.2 CELLULASE EXPRESSION IN YEAST:

When it comes to heterologous cellulase expression and production of ethanol and other commodity products in yeast, *S. cerevisiae* has gained most attention as it displays several traits useful for efficient fermentation. These include high ethanol yield and productivity, tolerance to high levels of ethanol as well as process hardiness, GRAS (Generally Regarded As Safe) status and tolerance to low pH. Under anaerobic conditions *S. cerevisiae* converts 1 mol of glucose to 2 moles of ethanol with the net production of 2 moles of ATP plus carbon dioxide via the glycolytic pathway (Zaldivar *et. al.*, 2001).

Genes encoding cellulases from various bacteria, fungi and plants have been cloned and expressed in *S. cerevisiae* with varying degrees of success. An endo-1,4-β-D-glucanase/carboxymethylcellulase (*cenA*) from *Cellulomonas fimi* was cloned and expressed in *S. cerevisiae* (Skipper *et. al.*, 1985; Lynd *et. al.*, 2002). The secretion of CMCase activity by the recombinant yeast was greatly increased when the leader sequence of yeast K1 toxin was inserted immediately upstream of the bacterial CMCase sequence. The authors reported the need for further incorporation of genes for extracellular exoglucanase and β-glucosidase to allow *S. cerevisiae* to utilize cellulose as a carbon source. An endo-1,3-1,4- β-glucanase (*beg1*) gene from

Bacillus subtilis has been cloned and expressed in *S. cerevisiae* under the control of its own secretion signal. However, it is not clear from their report whether the gene was transcribed from its own promoter or from a promoter sequence on the vector (Hinchliffe and Box, 1984; Lynd *et. al.,* 2002). The activity was rather low and could only be detected in cell extracts. Nevertheless the yield of endo-1,3-1,4- β-glucanase (Beg1) in recombinant yeast was subsequently increased 1,000 fold (from 34 U/ml of β-glucanase activity to 33,566 U/ml) by placing the gene under the control of the ADH1 promoter from *S. cerevisiae* instead of CYC1 promoter (Cantwell *et. al.,* 1986). The fact that no extracellular activity was detected in yeast culture supernatants was apparently due to the inability of yeast to process the protein to assist secretion. Even after fusing the gene to the $ADH1_p$-$MF\alpha1_s$ expression-secretion cassette, no extracellular enzyme activity was detected in the culture medium of yeast transformants (Van Rensburg *et. al.,* 1997; Van Rensburg *et. al.,* 1994; Lynd *et. al.,* 2002). This was in contrast with the fact that when a *Butyrivibrio fibrisolvens* endoglucanase gene (*end1*) was inserted into the same expression cassette ($ADH1_p$-$MF_\alpha1_s$-end1-$TRP5_T$), a high level of enzyme activity was detected in the culture medium of an *S. cerevisiae* lab strain as well as wine and distillers' yeast. The results also showed that Beg1 only hydrolyzed glucan and lichenan but not CMC whereas *end1* utilized all three substrates (Van Rensburg *et. al.,* 1997). However *beg1* recombinants produced a maximum of 16% breakdown of the glucan/congo red complex in 10 hours, whereas *end1* transformants generated 34% breakdown of the complex. The recombinants carrying *beg1* and *end1* produced only 37% breakdown of the complex which indicated that co-expression did not improve the breakdown of the complex significantly. Eventually Peterson *et. al.,* (1998) took cellulose degrading *S. cerevisiae* one step further by engineering the strain for the degradation of four polysaccharides (starch, pectin, cellulose and xylan). The engineered organism contained the α-amylase gene from *Lipomyces kononenkoae* (LKA1), pectate lyase from *Erwinia chrysanthemi* (PEL5), polygalacturonase gene from *Erwinia carotovora* (PEH1), endoglucanase (END1) from *Butyrivibrio fibrisolvens*, cellobiohydrolase (CBH1) from *Phanerochaete chrysosporium*, cellobiase (BGL1) from *Endomyces fibuligera*, exoglucanase (EXG1) from *S. cerevisiae* and endoxylanase (XYN4) from *Aspergillus niger*. The recombinant yeast was able to grow on starch, pectate and cellobiose as a sole source of carbon (approximately 10^8 cells/ml which is comparable to the positive control glucose) but only to some extent on partially amorphous

cellulose, glucan and lichenan (less than 10^7 cells/ml). It was not able to grow when Avicel was provided as a sole source of carbon.

The endo-β-1,3-glucanase gene (*bglH*) from *Bacillus circulans* has also been expressed in *S. cerevisiae* under the control of yeast GAL1 and SUC2 gene promoters (Nakajima *et. al.,* 1993; Lynd *et. al.,* 2002). The secretion of the enzyme was however directed by the SUC2-encoded leader peptide. The presence of the enzyme caused inhibition of growth and cell expansion. SUC2-mediated BglH expression resulted in decreased cell size and expansion of vacuoles probably due to the erosion of the β-1,3-glucan-containing cell wall of *S. cerevisiae* by exogenous enzyme causing the cells to undergo stress. This toxic effect was circumvented by prolonged incubation at low temperature (16°C for 15 days). When the β-glucosidase gene (*bgl*) from *B. circulans* and endo/exoglucanase (*cel*) from an unidentified *Bacillus* sp. (strain DO4) were inserted in between the yeast ADH1 promoter and PGK1 terminator sequences, a high level of expression with no noticeable inhibitory effect on the growth of transformants was observed (Cho and Yoo, 1999; Lynd *et. al.,* 2002). Cho *et al*. (1999) also reported the chromosomal integration of multiple copies of endo/exo-glucanase and β-glucosidase from *Bacillus sp. DO4* and *Bacillus circulans* respectively into *S. cerevisiae* DNA using the δ-sequences of the Ty1 retrotransposon as target site for homologous recombination.. The CMCase activity was around 300 U/l in the δ-integrated recombinant yeast and 125 U/l in a plasmid-based expression system, whereas, pNPGase activity was around 175 U/l in the plasmid based system and 575 U/l in the δ-integrated system. The δ-integrated yeast recombinants were able to produce approximately 4 g/l ethanol and nearly 6 g/l cell mass using cellodextrins as carbon source (Cho *et. al.,* 1999; Cho and Yoo, 1999; Lynd *et. al.,* 2002). However, the authors reported the need to enhance enzyme activities to increase the carbon flux to ethanol production. Cho and Yoo (1999) later also reported the significance of this strain in the SSF process, producing an ethanol concentration of 20.35 g/l after 12 hours from 50 g of microcrystalline cellulose/l (Avicel) when 17.2 FPU/g-cellulose of commercially available total cellulase and 14.2 IU/g-cellulose of commercial β-glucosidase was provided.

Genes from thermophilic bacteria have also been cloned and expressed in *S. cerevisiae*. A cellobiohydrolase from an unidentified thermophilic anaerobe was cloned and expressed in *S. cerevisiae* under the control of the SUC2 promoter and

invertase secretion signal. No enzyme activity was found in the culture medium indicating that the translocation of the hybrid protein was affected by the protein structure (Uozumi *et. al.,* 1993; Lynd *et. al.,* 2002). When the SUC2 promoter was replaced by the STA1 promoter and the invertase secretion signal was replaced by that from the glucoamylase secretion signal, almost 40% of the enzyme was secreted into the medium. Amendment of the amino acid at the cleavage site (by inserting a *Bam*HI or *Bgl*II DNA linker) resulted in 3.5-fold increase (from 13.1 U/l to 46 U/l) in the total cellobiohydrolase activity without affecting its secretion.

Cellulomonas fimi endoglucanase *cenA* and exoglucanase/xylanase *cex* have been successfully cloned and expressed in *S. cerevisiae* under the control of the yeast ADH1 and MEL1 promoters, giving it the ability to saccharify filter paper and pre-treated wood chips if a small amount of β–glucosidase was supplied in the medium (Wong *et. al.,* 1988). About 1 mg/ml of reducing sugars were released from filter paper after 30 hours when 4.3 units of *cex* and 27.6 units of *cenA* per ml were used along with 13 U/ml of commercial β-glucosidase. However in the absence of commercial enzyme only 0.2 mg/ml of reducing sugars were released. When pre-treated wood chips were used the co-expressed cellulases (8.1 units *cex* and 52.4 units *cenA*) released 22% total sugars in 3 days in the presence of 5.7 U/ml commercial β-glucosidase, whereas in the absence of commercial β-glucosidase only 2% of the total sugars were released, assuming that 100% total sugar was equivalent to 70 g sugar/100 g wood. The growth on cellulosic substrate as a sole carbon source and ethanol production was not reported.

Several plant glucanases have also been cloned and expressed in *S. cerevisiae*. The β-1,3-1,4-glucanase from barley was fused with the signal sequence of the mouse α-amylase, yeast PHO5-encoded phosphatase and SUC2-encoded invertase using the ADH1 and PGK1 promoters (Olsen and Thomsen, 1989). The amount of enzyme measured in the supernatant of yeast was 1-5 µg/l when the ADH1 promoter was used compared to 100 µg/l when the PGK promoter was used. The secretion was more efficient when invertase leader peptide was used. The recombinants harbouring invertase leader peptide secreted about 5 mg/l of β-glucosidase whereas, those harbouring yeast acid phosphatase signal peptide and mouse α-amylase signal peptide secreted 0.5 and 0.1 mg/l β-glucosidase into the medium. However, the growth on cellulosic substrates was not reported.

Nicotiana plumbaginifolia β-1,3-glucanase was also expressed in *S. cerevisiae* under the control of the GAL1 promoter and MFα1 signal sequence resulting in strong growth inhibition of *S. cerevisiae* by interfering with cell wall growth and the loss of up to 20% of some periplasmic enzymes (Demolder *et. al.*, 1993; Lynd *et. al.*, 2002).

Several cellulases from various fungi have also been expressed in *S. cerevisiase* with the aim of producing a yeast strain capable of directly fermenting cellulose to ethanol. Several cellulases from *Trichoderma reesei* and *Trichoderma longibrachiatum* were cloned and expressed in *S. cerevisiae*. These cellulases were efficiently secreted into the culture medium of the transformants but were highly glycosylated and heterogeneous in size. The hyperglycosylation resulted in a slight decrease in specific activity and the binding ability of recombinant CBHII compared to native enzyme. Yeast-made EGI and CBHII (gene originally from *T. Reesei*) were both active against β-1,3-1,4-glucan but inactive against β-1,3-1,6-glucan. CBHII had greater activity against crystalline cellulose whereas EGI was more active against amorphous cellulose. CBHII however did not show any activity against xylan but EGI showed even greater activity against unsubstituted hardwood xylan compared to amorphous cellulose (Lynd *et. al.*, 2002). Several other cellulases from various fungi like *P. chrysosporium, Agaricus bisporus, Penicillium janthinellium, Rhizopus oryzae, Ruminococcus flavifaciens, Aspergillus niger, Candida pelliculosa* were also cloned and expressed in *S. cerevisiae* with varing degrees of success (Lynd *et. al.*, 2002). Examples of some of them are presented in Table 1.5.

Fujita *et al*., reported the generation of a yeast strain expressing three cellulases (*T. reesei* endoglucanase II and cellobiohydrolase II together with *Aspergiluus aculeatus* β-glucosidase 1) as fusion proteins attached to the cell surface (Fujita *et. al.*, 2004). The strain was able to use 10 g/l amorphous cellulose (phosphoric acid swollen cellulose) as a sole source of carbon with the production of 2.9 g/l ethanol within 40 hours under their experimental conditions. However, degradation of crystalline cellulose was not reported.

In short, the attempts to transfer biomass-degrading capability by heterologous expression of one or more biomass-degrading genes/enzymes into an industrially useful organism have met to date with limited success. It could be due to the fact that effective biomass degradation requires synergistic action of a large number of

enzymes, the distinctive and cumulative actions of which are still poorly understood. Synthetic biology hence allows a new approach to decode the synergistic action of combinations of biomass-degrading enzymes in vivo by allowing any number of transgenes to combine in a modular, combinatorial way. It allows endless number of parts/genes to be pieced together to form complex systems, for example biomass-degrading systems, which can ultimately be transferred to industrially useful hosts.

Table 1.5: Fungal cellulases cloned in *S. cerevisiae*

Donor	Enzymes	Genes	Results and References
Trichoderma reesei	Cellobiohydrolase II and endoglucanase I	*cbh2, egl1*	Transformants were able to produce enzymes active against various substrates like barley-β-1,3-1,4-glucan, xylan, cellulose etc., but inactive against β-1,3-1,6-glucan (Bailey *et. al.,* 1993, Lynd *et. al.,* 2002).
Trichoderma reesei	Cellobiohydrolase I and cellobiohydrolase II	*cbh1, cbh2*	Both enzymes were able to degrade amorphous cellulose although the binding efficiency of CBHII was slightly less than the native enzyme. At concentration of 30 µg/ml of recombinant CBHII, only 50%-70% of the enzyme would bind to crystalline cellulose under conditions where 100% *T. reesei* enzyme would bind. This reduced binding was due to the hyperglycosylation of the yeast-made CBHII (Penttila *et. al.,* 1988; Lynd *et. al.,* 2002).
Phanerochaete chrysosporium	Cellobiohydrolase	*cbh1-4*	Successfully expressed under the control of PGK1 (phosphoglycerate kinase) promoter. Secretion was directed by its native signal sequence but activity was rather low in yeast (about 12.03 U/ml) (Rensburg *et. al.,* 1996)
Aspergillus aculeatus	FI-carboxymethyl cellulase		DNA was fused with GAP (glyceraldehyde-3-phosphate dehydrogenase) promoter resulting in enzyme production most of which resided in the cytoplasm of the yeast cells. Murai *et. al.,* later reported the genetic immobilization of FI-CMCase on the cell surface of *S. cerevisiae* by fusing it with the C-terminal half of yeast α-agglutinin, resulting in the enzyme activity detectable in cell pellet fraction. However the constructed strain reported here was not able to grow on cellulose as a sole carbon source (Ooi *et. al.,* 1994; Murai *et. al.,*1997, Lynd *et. al.,* 2002)

Donor	Enzymes	Genes	Results and References
Aspergillus aculeatus No. F-50	Cellobiohydrolase I and β-glucosidase 1	*cbh1, bgl1*	The active enzymes were efficiently secreted into the medium by the yeast transformants. When the combination of FI-CMCase, CBHI and BGL1 was used the specific activity against avicel was 11 mU/mg, which resulted in 59% hydrolysis of 2 mg/ml Avicel in 16 hours under their experimental conditions. Murai *et. al.*, latter also did genetic immobilization of β-Glucosidase along with FI-CMCase by fusing them with the C-terminal half of yeast α-agglutinin. The yeast transformant displaying cell-surface cellulases were able to grow on cellobiose and water soluble cellooligosaccharides as the sole source of carbon. However they were not able to grow on CMC or avicel as a sole source of carbon. (Takada *et. al.*, 1998; Murai *et. al.*, 1998).
Ruminococcus flavefaciens	cellodextrinase	*celA*	Gene was inserted between yeast expression-secretion ($ADH1_p$-$MF\alpha1_s$) cassette and yeast gene $TRP5_T$ terminator. The transformants were conferred with the ability to actively synthesize and secrete cellodextrinase. The major end-product of enzyme action on cellooligosaccharides was cellobiose (Rensburg *et. al.*, 1995)
Candida pelliculosa	β-glucosidase		80% of the enzyme produced by *S. cerevisiae* was located in periplasmic space from where it actively facilitated the growth of yeast on cellobiose. However no cellobiase activity could be detected in the cell extracts (Kohchi and Toh-e, 1986).
Saccharomycopsis fibuligera	β-glucosidase 1 and β-glucosidase 2	*bgl1, bgl2*	BGLI (cellobiase) hydrolyzed cellobiose efficiently whereas BGLII did not. BGLI recombinants were able to ferment cellobiose in 5 ml static culture, with an accumulation of 1% ethanol in the medium. Transformants carrying BGLII were not able to grow with cellobiose as a carbon source and hence no ethanol was produced (Machida *et. al.*, 1988).

Donor	Enzymes	Genes	Results and References
Kluyveromyces fragilis	β-glucosidase		High level expression of β-glucosidase was achieved by *S. cerevisiae* but the transformants failed to grow on cellobiose as a carbon source probably because the enzyme was intracellular and hence no activity was detected in the medium. This means cellobiose must enter the cells. As yeast strain used was not permeable to cellobiose hence it was concluded that growth could only be achieved if the strain able to take up cellobiose is used (Raynal and Guerineau, 1984).

1.8 SYNTHETIC BIOLOGY:

Synthetic biology is a new area of biological research which aims to design and engineer biologically based parts, devices and systems as well as redesigning existing, natural biological systems. It basically combines biology and engineering with the view of applying the engineering paradigm of systems design to biological systems to produce predictable and sturdy systems with unique functionalities. It revolves around the theme of constructing biological systems like artificial viral and bacterial genomes, assembly of artificial cell-like vesicles from non-living components and construction of artificial gene networks (French, 2009). The term 'synthetic biology' first appeared in the literature in 1980 to characterize bacteria that were genetically engineered using recombinant DNA technology. These bacteria were biological systems altered synthetically by human intervention, hence the term 'synthetic biology' (Hobom, 1980; Steven and Sismour, 2005). The basic approach of synthetic biology is to make the engineering of biology easy (Anderson, *et. al.*, 2010) by developing collections of reusable, standard biological parts. These standard biological parts, called 'BioBricks', are interchangeable and modular bricks of DNA, which allow rapid assembly of many engineered organisms by biological engineers (Voigt, 2006; Shetty, *et. al.*, 2008). These can be individual parts or combinations of different parts encoding a particular function and can be tested or characterized independently so that the parts or devices that do not work as expected can be identified, repaired or replaced as required (Shetty *et. al.*, 2008; Rosenfeld *et. al.*,

2005; Anderson *et. al.,* 2007; Yokobayashi *et. al.,* 2002). The bricks include promoters, ribosome-binding sites (RBS), coding sequences, transcriptional terminators etc (Anderson, *et. al.,* 2010). Hence, they can be defined as a natural nucleic acid sequence encoding a specific function which has been refined to conform to one or more defined standards (Shetty *et. al.,* 2008). The first widely accepted standard for the physical composition of biological parts was proposed by Knight (2003). This standard, now known as RFC 10, uses a combination of restriction sites that create compatible and incompatible sticky ends which can be further joined in any order to any desired number to create multi-gene systems, which can then eventually be combined to any desired degree. These BioBricks, which are physical DNA sequences on a plasmid, are stored and distributed by the Registry of Standard Biological Parts (http://partsregistry.org) as lyophilized DNA in 384-well plate format.

This BioBrick assembly method described by Knight (2003) allows the assembly of standard biological parts using single assembly chemistry. It involves a standardized sequence on BioBricks enabling standard assembly of two bricks using restriction enzyme digestion and ligation to create larger parts from smaller basic parts (Figure 1.8). Parts are flanked by standard sequence containing *XbaI* and *SpeI* restriction sites on their 5′ and 3′ ends respectively, digestion with which generates compatible sticky ends that can be ligated head-to-tail. The ligation creates scar sequence between the parts that is unaffected by further digestion with these enzymes as neither of the original sites is then present. The result is a new composite part with the same characteristics as the parent parts, which is still flanked with *XbaI* and *SpeI* sites at 5′ and 3′ ends respectively, hence can itself act as a BioBrick part and again can be assembled with any other BioBrick part into a larger unit.

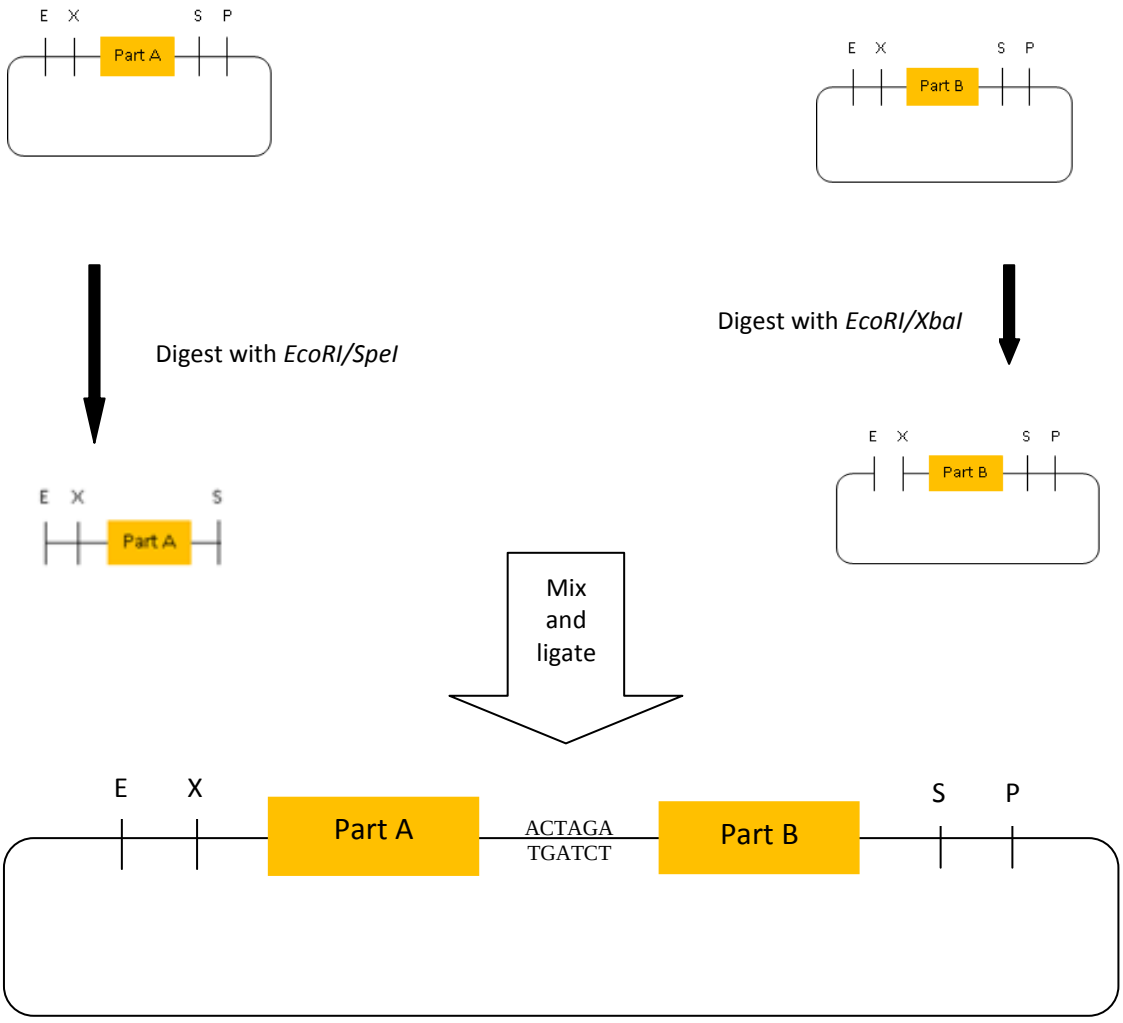


Figure 1.8: Knight's assembly standard for combining two BioBrick parts (Knight, 2003). BioBrick parts are flanked by standard sequences (*EcoRI*(E)/*XbaI*(X) and *SpeI*(S)/*PstI*(P)) on their 5′and 3′ends. Part A is digested using E/S and part B is digested using E/X. Digestion with X and S creates compatible sticky ends that can be ligated head-to-tail. The ligation creates scar sequence between the parts which is unaffected by further digestion with any of these enzymes as neither of the original site is then present. This results in a new composite part with the same characteristics as the parent parts, which is still flanked by the same restriction sites on both ends and hence can act as a BioBrick part and again can be assembled into any other BioBrick part into larger unit.

This kind of physical composition has two basic advantages (http://parts.mit.edu; Shetty *et. al.*, 2008):

1. Two biological engineers from different parts of the world can design parts conforming to the BioBrick assembly standard, and hence the parts will be physically composable via the standard.
2. In contrast to the traditional molecular cloning techniques the BioBrick assembly process is amenable to optimization and automation, since the engineers carry out the exact same operation every time.

The Registry of Standard Biological Parts currently maintains over 2,000 BioBrick standard biological parts (http://parts.mit.edu; Shetty *et. al.*, 2008). Every part has a BioBrick part number that act as a unique identifier of the part. The Registry also maintains information about each part such as its sequence, function and if possible user experience. The engineers can order parts from the Registry and assemble them in any order to construct many-component synthetic biological systems.

Knight's original assembly standard as well as some of its advantages and disadvantages is discussed below:

1.8.1 BBF RFC 10 'TOM KNIGHT'S ORIGINAL BIOBRICK ASSEMBLY STANDARD' (http://hdl.handle.net/1721.145138):

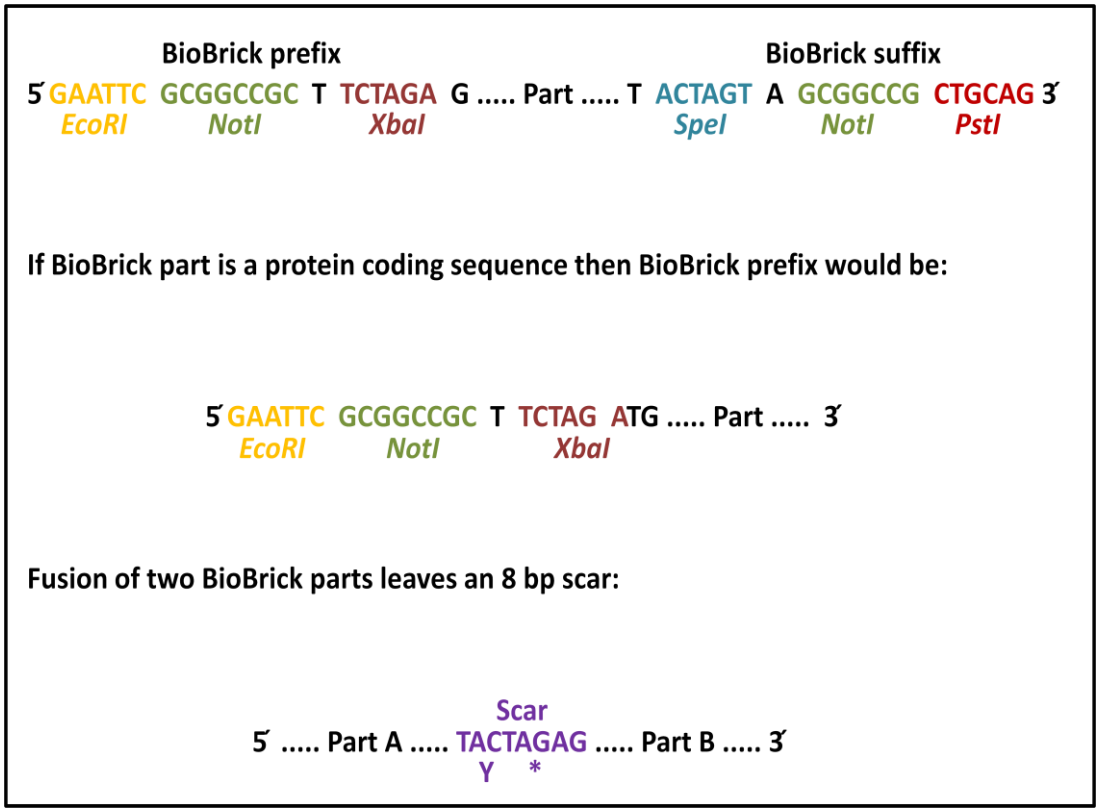


Figure 1.9: Important features of RFC 10. The fusion of BioBrick part A and part B resulted in 8 bp scar sequence which is not affected by *XbaI* and/or *SpeI* as neither of these sites exists anymore. Rather the scar sequence induces a frame shift and codes for tyrosine (Y) and a stop codon (*).

Advantages:

- It is the standard method used by most iGEM (international genetic engineering machine) teams.
- Most BioBricks in the MIT registry are in RFC 10 format.
- It is well tested and documented method.
- The start codon of the native protein can be preserved while using RBS parts.
- It is still growing with large sets of parts available.

Disadvantages:

- Fusion proteins cannot be made using this standard because of 8bp scar that produces a frame shift and codes for a stop codon.
- At the fused region a single mutation can ruin the setup.

Since the origin of this first BioBrick assembly standard several others have been proposed in order to improve the original standard which produces an 8-nucleotide scar that remains in between the parts when assembled together and so does not allow the creation of fusion proteins (http://openwetware.org/wiki/The_BioBricks_Foundation:RFC; Anderson *et. al.*, 2010). These problems can ideally be overcome by inserting a scar that contains a multiple of 3 nucleotides in such a way that no in-frame stop codon is present and frame shift is also eliminated (Anderson, *et. al.*, 2010). Hence several strategies and standards were then suggested in order to overcome these problems, for example Biofusion (RFC 23) standard, Fusion Part (RFC 25) standard.

In addition to BioBricks several other DNA assembly methods are now available. Important features of some of the latest assembly method are discussed below:

1.8.2 GIBSON ASSEMBLY:

Gibson assembly invented by Daniel Gibson in 2009 is a DNA assembly method which allows the joining of multiple DNA fragments in a single, isothermal reaction. Only a small number of components with very few manipulations are required to accomplish the task of DNA assembly using this method (Gibson *et. al.*, 2009). It can achieve the simultaneous assembly of more than 10 DNA fragments in a single assembly reaction. It uses a mixture of three enzymes (T5 exonuclease, DNA polymerase and DNA ligase) along with DNA fragments containing 20 – 40 base pair overlap with adjacent DNA fragment. Hence T5 exonuclease chews back DNA from 5' end resulting in single stranded regions on adjacent DNA that can anneal. DNA polymerase than incorporates nucleotides to fill in any gaps. Ligase finally covalently joins the DNA of adjacent segments, removing the nicks in the DNA.

1.8.3 GOLDEN GATE ASSEMBLY METHOD:

This method invented by Engler *et. al.,* (2008), relies upon the use of type IIs endonuclease, whose recognition sites are different from their cut sites. Digestion with type II restriction endonuclease results in 5′ or 3′ overhangs, which can be used for efficient assembly of multiple DNA fragments in a single ligation reaction. Hence with a proper design the two ligated fragments generate a product lacking the original restriction site without adding any nucleotide sequence to the final cloned product.

1.9 AIMS OF THE PROJECT:

This introduction has given a review of the advances achieved in the recombinant cellulolytic approach so far. The attempts to transfer biomass degrading capability to produce useful products by heterologous expression of one or a few enzymes have met to date with limited success. There are a number of issues that need to be resolved to generate an effective degradation system. The simplest explanation of the failure to achieve an effective cellulose degrading system could be the fact that it requires the synergistic action of a large number of enzymes. Synthetic biology, by allowing any number of transgenes to combine in a modular and combinatorial way, offers a new approach to uncover the synergistic action of a number of biomass degrading enzymes, which can ultimately be transferred into a useful host. Hence the aim of this project was to create BioBricks using cellulases from different cellulose degraders and then to introduce them into different expression hosts to see if they impart to the host organism the ability to use different cellulosic substrates as a source of carbon.

2 MATERIALS AND METHODS:

2.1 Bacterial cultures:

Genetic manipulation studies were carried out using *E. coli* JM109, *Bacillus subtilis* 168, *Citrobacter freundii* SBS197 and *Citrobacter freundii* NCIMB11490. *E. coli* BS(λDE3) was used as an expression host where the T7 promoter was used to express proteins. *Cytophaga hutchinsonii* ATCC 33406, a type species of the genus *Cytophaga* (a soil bacterium that rapidly degrades crystalline cellulose), obtained from DSMZ, and *Cellulomonas fimi* ATCC484 were used as a source of genes for cellulose degradation.

2.2 Media used:

Unless specified all media were sterilized by autoclaving at 121°C for 20 min and stored at room temperature. Whenever required media were solidified by adding 1 – 2% agar. See appendix for further details regarding recipes.

2.3 Chemicals and reagents:

All the chemicals and reagents apart from the ones listed below were obtained from Sigma.

- CMC – Fluka Analytical (21902)
- NaOH – A Fisher Scientific International Company (S/4880/53)
- Na-EDTA – A Fisher Scientific International Company (D/0700/53)
- H_3BO_3 – Fisons (B/3800/60)
- CH_3CO_2K – Fluka Biochemika (60035)
- 1 kb DNA ladder – New England Biolabs (N3232S)
- Sybr-Safe – Invitrogen (S33102)
- Gel Green – Biotium (41005)

2.4 Culture conditions:

E. coli, *Bacillus subtilis*, *Cellulomonas fimi* and *Citrobacter freundii* were grown in LB at 37°C with shaking for liquid cultures. *Cytophaga hutchinsonii* was grown at 30°C in DMM with shaking for liquid cultures. Where minimal medium was required

CMM was used for *Citrobacter freundii* and M9 was used for *E. coli* and *B. subtilis*. Glucose, filter paper, cellobiose and/or Avicel were used as a source of carbon. Glucose and cellobiose whenever used were filter sterilized and added to the medium after autoclaving of the mineral base.

2.5 Cloning vectors:

Plasmids used in this study are presented in table 2.1.

Table 2.1: List of plasmids used in this study

Plasmids	Antibiotics for selective pressure
pT7-7 (US Biochemical Corporation)	100 µg/ml Amp
pSB1A2 (Registry of Standard Biological Parts)	100 µg/ml Amp for *E. coli* and 80 µg/ml Carb for *C. freundii*
pTG262 (Shearman, *et. al.*, 1989)	10 µg/ml Cml for *E. coli* and 50 µg/ml for *B. subtilis*

2.6 Genes:

Genes used in this study are presented in table 2.2.

Table 2.2: List of genes used in this study

Genes	Source	Activity
CHU_2103	*C. hutchinsonii* ATCC 33406	Probable endoglucanase
CHU_2802	*C. hutchinsonii* ATCC 33406	Probable endoglucanase
CHU_1944	*C. hutchinsonii* ATCC 33406	Possible glucose/sorbosone dehydrogenase
CHU_2315	*C. hutchinsonii* ATCC 33406	Possible glucose/sorbosone dehydrogenase
lacI	*E. coli* B	Lac repressor
lacZ'α	*E. coli* B	Alpha complementation gene of β-galactosidase
ido	*Rhodococcus* ATCC 21145	Indole dioxygenase
cenA	*C. fimi* ATCC 484	Endoglucanase
cex	*C. fimi* ATCC 484	Exoglucanase

2.7 Promoters:

Promoters used in this are presented in table 2.3.

Table 2.3: List of promoters used in this study

Promoters	**Source**
lac	Promoter of *E. coli lac* operon (Reznikoff, 1992)
spac	hybrid promoter made using phage spo-1 promoter element and the *lac* operator (Yansura and Henner, 1984)
T7	Promoter Ø 10 (Moffatt and Studier, 1986)

2.8 Enzymes:

Restriction enzymes, ligases and Pfu/Taq polymerases were obtained from Promega and New England Biolabs and were used according to the manufacturer's instructions.

KOD Hot start DNA polymerase and RNAse A were obtained from Novagen and Sigma-Aldrich respectively and were used according to the manufacturer's instructions.

List of enzymes used is given below:

- Restriction enzymes: *NdeI, SalI, ClaI EcoRI, PstI, XbaI, SpeI* and *NotI*.
- T4 DNA ligase
- Pfu polymerase
- Taq polymerase
- KOD Hot start DNA polymerase
- RNAse A

2.9 Primers:

The primers used for gene amplification and introduction of restriction sites were all obtained from Sigma-Genosys. Primer sequences are shown in Table 2.4.

Table 2.4: Primers used for PCR amplification of genes

Plasmid	Backbone	Insert	Primer sequence	Restriction sites
pT7.7 + *ido*	pT7.7	*ido*	F= 5′ GGA GGA A**CA TAT G**GA CAT CAC CCG CAC C R= 5′ ACC GGG **ATC GAT** GCG AAG TTG	*NdeI, ClaI*
pT7.7 + *lacZ'*	pT7.7	*lacZ' α*	F= 5′ GGC AAT **CAT ATG** TTG CCC GTC TCA CTG G R= 5′ GAA **GAA TTC** ACT CCA GCC AGC TTT CC	*NdeI, EcoRI*
pT7.7 + CHU_2103	pT7.7 + *lacZ' α*	CHU_2103	F= 5′ TCT T**CA TAT G**AT TAA AAA AAT ATC CGT AG R= 5′ CAT **GTC GAC** CTG AAT GCA ATG CAA TAT GAG TTC	*NdeI, SalI*
pT7.7 + CHU_2802	pT7.7 + *lacZ' α*	CHU_2802	F= 5′ TTA G**CA TAT G**TT TAA GCG ACT CAT TC R= 5′ AAT **GTC GAC** TGA TGT TTT GTA CAG GAC C	*NdeI, SalI*
pT7.7 + CHU_1944	pT7.7 + *lacZ' α*	CHU_1944 (with signal sequence)	F= 5′ ATT T**CA TAT G**AA ACA ATT TTT ATT TAT CAG R= 5′ ATT T**GT CGA C**TG CTT ATT TCG CAA CGC TG	*NdeI, SalI*
pT7.7 + CHU_1944	pT7.7 + *lacZ' α*	CHU_1944 (without signal sequence)	F= 5′ ATT A**CA TAT G**TG TGC TGA AAA AAA AGA ACA GG R= 5′ ATT T**GT CGA C**TG CTT ATT TCG CAA CGC TG	*NdeI, SalI*

Plasmid	Backbone	Insert	Primer sequence	Restriction sites
pT7.7 + CHU_2315	pT7.7 + *lacZ' α*	CHU_2315 (with signal sequence)	F= 5′ AGC C**CA TAT G**AA AAG AAA CAC CGT TTT AC R= 5′ TGC T**GT CGA C**TT TTA TTT ATC AAT ATG ATA TAA TTT TCC	*NdeI, SalI*
pT7.7 + CHU_2315	pT7.7 + *lacZ' α*	CHU_2315 (without signal sequence)	F= 5′ GCT G**CA TAT G**TG TTC TAA AAA GAA AGA GGA TAC R= 5′ TGC T**GT CGA C**TT TTA TTT ATC AAT ATG ATA TAA TTT TCC	*NdeI, SalI*
pSB1A2 + P_{spac}	pSB1A2 + *lacZ'α*	P_{spac}	F= 5′ CTT C**GA ATT C**GC GGC CGC T**TC TAG A**GC CCA GTC CAG ACT ATT C R= 5′ CGC T**AC TAG T**AC TTA AGC TTA ATT GTT ATC CG	*EcoRI, XbaI, SpeI*
pSB1A2 + *lacI*	pSB1A2 + *lacZ'α*	*lacI*	F= 5′ CTT C**GA ATT C**GC GGC CGC T**TC TAG A**TG AAA CCA GTA ACG TTA TAC G R= 5′ CGC T**AC TAG T**AT TAT TAC TGC CCG CTT TCC AGT CG	*EcoRI, XbaI, SpeI*
pSB1A2 + *cenA*	pSB1A2 + *lacZ'α*	*cenA*	F= 5′ CGT **GAA TTC** GCG GCC GCT **TCT AGA** TGT CCA CCC GCA GAA CC R= 5′ CGT T**AC TAG T**AT TAT TAC CAC CTG GCG TTG C	*EcoRI, XbaI, SpeI*
pSB1A2 + *cex*	pSB1A2 + *lacZ'α*	*cex*	F= 5′ CGT **GAA TTC** GCG GCC GCT **TCT AGA** TGC CTA GGA CCA CGC C R= 5′ GCT **ACT AGT** ATT ATT AGC CGA CCG TGC AGG	*EcoRI, XbaI, SpeI*

2.10 DNA manipulation techniques:

2.10.1 Polymerase chain reaction:

PCR was used to amplify DNA fragments and introduce restriction sites to be used in cloning experiments. A standard reaction mixture is shown in Table 2.5, 2.6 and 2.7.

Table 2.5: Reaction mixture for PCR using Pfu polymerase

Reagents	Volume (µl)
Water	39.6*
10x reaction buffer	5
Forward primer (10 pmol/µl)	1
Reverse primer (10 pmol/µl)	1
dNTP mix (10 mM each)	1
Cell suspension**	2
Pfu polymerase	0.4
Total	50

Table 2.6: Reaction mixture for PCR using Taq polymerase

Reagents	Volume (µl)
Water	12*
5x reaction buffer	4
25 mM $MgCl_2$	1.2
Forward primer (50 pmol/µl)	0.5
Reverse primer (50 pmol/µl)	0.5
dNTP mix (10 mM each)	0.4
Cell suspension**	1
Taq polymerase	0.4
Total	20

Table 2.7: Reaction mixture for PCR using KOD Hot Start DNA Polymerase

Reagents	Volume (µl)
Water	32*
10x reaction buffer	5
25 mM $MgSO_4$	3
Forward primer (10 pmol/µl)	1.5
Reverse primer (10 pmol/µl)	1.5
dNTP mix (2 mM each)	5
Cell suspension**	1
KOD Hot Start DNA Polymerase	1
Total	50

The programme used for the Progene thermocycler is given in Table 2.8.

Table 2.8: Programme for Progene thermocycler

Parameters	Cycling conditions		
	Temperature (°C)	**Time**	**Cycles**
Initial denaturation	95	2 min	1
Denaturation using Taq and Pfu polymerase	95	30 sec*	30
Denaturation using KOD Hot Start DNA Polymerase	95	20 sec*	
Annealing using Taq and Pfu polymerase	Depending on T_m of the primer	30 sec	
Annealing using KOD Hot Start DNA Polymerase	Depending on T_m of the primer	10 sec	
Extension using Pfu polymerase	72	2 min/Kb	
Extension using Taq polymerase	72	1 min/Kb	
Extension using KOD polymerase	70	10 s/kb for <500 bp, 15 s/kb for 500-1000 bp, 20 s/kb for 1000-3000 bp and 25 s/kb for >3000 bp	
Final extension	72/70	10 min	1
Hold	4	99 hrs	1

*Whenever GC-rich DNA (like *cenA*, *cex* etc.) was amplified the denaturation time was extended up to 1 min (in each cycle) and 10 µl of 50% v/v glycerol was also added, after reducing the amount of water by same volume, to avoid the formation of secondary structures.

******Cell suspension was prepared by suspending a loop full culture from plate into 200 µl of sterile water.

2.10.2 Plasmid construction:

Standard protocols were used for plasmid construction. PCR-amplified genes with appropriate restriction sites were purified using a standard glass beads protocol based on US Bioclean (US Biochemical Corporation) and/or Qiagen gel extraction kit.

Once purified, vector and insert were digested using appropriate restriction enzymes and ligated together using T4 DNA ligase. The ligation mixture was then heat-treated at 65°C for 20 min and then used to transform *E. coli* JM109. The plasmids were eventually used to transform *E. coli* BS(DE3), *Bacillus subtilis* 168, *Citrobacter freundii* SBS197 and/or *Citrobacter freundii* NCIMB11490. The transformants were then screened for the presence of the gene of interest either by PCR and/or by plasmid extraction. Sequences were confirmed by sequencing the genes using Sanger method and Big Dye system (ABI).

2.10.3 DNA ligation:

To 10 µl of eluted DNA, 1.2 µl of 10x DNA ligase buffer and 1.2 µl of T4 DNA ligase was added and incubated at 16°C overnight. This was then heat treated at 65°C for 20 min to deactivate the ligase and then used to transform competent cells.

2.10.4 DNA purification:

DNA was purified using Qiagen gel extraction kit or silica beads by the protocol based on the US Bioclean (US Biochemical Corporation). To DNA solution 3x volumes of 6 M NaI was added followed by the addition of 5 µl of glass bead suspension, mixed and left on ice for 10 min. The mixture was spun briefly, supernatant was discarded followed by the addition of 250 µl of ice-cold wash buffer. This washing step was performed twice more followed by resuspending the pallet in elution buffer and incubating it in water bath at 55°C for 10 min, mixing again after 5 min. The glass beads were then spun out followed by transferring the supernatant containing DNA to a fresh tube.

2.10.5 Preparation of competent cells:

Competent *E. coli* JM109 and/or BS(λDE3) and *C. freundii* SBS197 and/or NCIMB11490 cells were prepared by the method of Chung *et. al.* (1989).

2.10.6 Transformation of *E. coli* JM109, *E. coli* BS(λDE3) *C. freundii* SBS197 and NCIMB11490:

Frozen competent cells (100 µl) were thawed on ice water slurry and transferred to ice. In the case of *C. freundii* SBS197, competent cells were prepared fresh and transformed immediately. After adding 5 µl of DNA, they were left on ice for 30 – 60 min. Heat shock was then given for about 90 sec at 42°C and the cells were immediately transferred to ice for another 90 sec. LB (900 µl) was then added and the tube was incubated at 37°C for 45 – 60 min. About 100 µl of cells were spread on LB plates with appropriate antibiotics. IPTG (90 µg/ml) and X-gal (40 µg/ml) were used when blue/white selection was required. The remaining cells were then centrifuged; supernatant was discarded except for 100 µl which was used to resuspend the cells in order to plate them on another plate of the same medium.

2.10.7 Transformation of *Bacillus subtilis* 168:

An overnight culture of *B. subtilis* 168 grown in LB medium was diluted to get a final $O.D_{600}$ of 0.25 (lightly inoculated) and 0.75 (heavily inoculated) in 5 ml PTM each. Cultures were then incubated at 37°C until they reached stationary phase (roughly five hours). The cells are motile and competent at this point and remain so for about 30 min. About 0.1 ml of each light and heavily inoculated culture were added to 1 ml of pre-warmed transformation medium followed by the addition of 5 µl of DNA. A negative control contains everything but no DNA. The mixture was then incubated at 37°C for 30 – 90 min with shaking. 100 µl of the mixture was then plated onto L agar plates containing about 10 µg/ml of chloramphenicol. The remaining culture was centrifuged and resuspended in 100 µl of supernatant and plated onto another L agar plates with 10 µg/ml chloramphenicol. Both the plates were incubated at 37°C for 2 days.

2.10.8 Plasmid extraction:

Plasmid DNA was extracted using the Qiagen miniprep kit. Whenever maxiprep was required (and sometimes for miniprep as well) the alkaline lysis method as described by Sambrook *et. al.*, (1989) was used.

2.10.9 Agarose gel electrophoresis:

A Bio-Rad minisub cell was used for gel electrophoresis. PCR products, restriction digests and clones were resolved on 0.8% agarose gels, electrophoresed in 0.5x TAE buffer at 100 V, 50 mA for 30 – 40 min. The gels were then stained using either ethidium bromide, SYBR Safe or Gel Green and destained (if required) in distilled water. They were eventually visualized under UV light. DNA ladder (5 µl of 1 kb) from New England Biolabs was used as DNA marker.

2.10.10 DNA sequencing:

For sequencing, 3 µl of DNA was mixed with 2 µl of sterile water and 1 µl of (5 pmol) specific primer. The sequencing reactions were performed by the Gene Pool, SBS sequencing service at the School of Biological Sciences, University of Edinburgh.

2.11 Protein analysis:

2.11.1 Protein extraction:

For preparing cell extracts BugBuster® HT Protein Extraction Reagent from Novagen was used according to the manufacturer's instructions.

2.11.2 Estimation of protein concentration:

The protein concentration was estimated by using the Coomassie Bradford assay kit from Pierce and was done according to the manufacturer's instructions.

2.11.3 SDS PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis):

For SDS PAGE, the Mini-Protean II electrophoresis system from Bio-Rad was used according to the manufacturer's instructions.

2.11.4 Silver staining of SDS gels:

Silver staining was performed according to the method of Gromova and Celis (2006) where the concentration of protein was very low and the bands were not visible by Coomassie stain.

2.12 Development of BioBricks:

Figures 2.1, 2.2 and 2.3 show the procedure used to construct BioBricks:

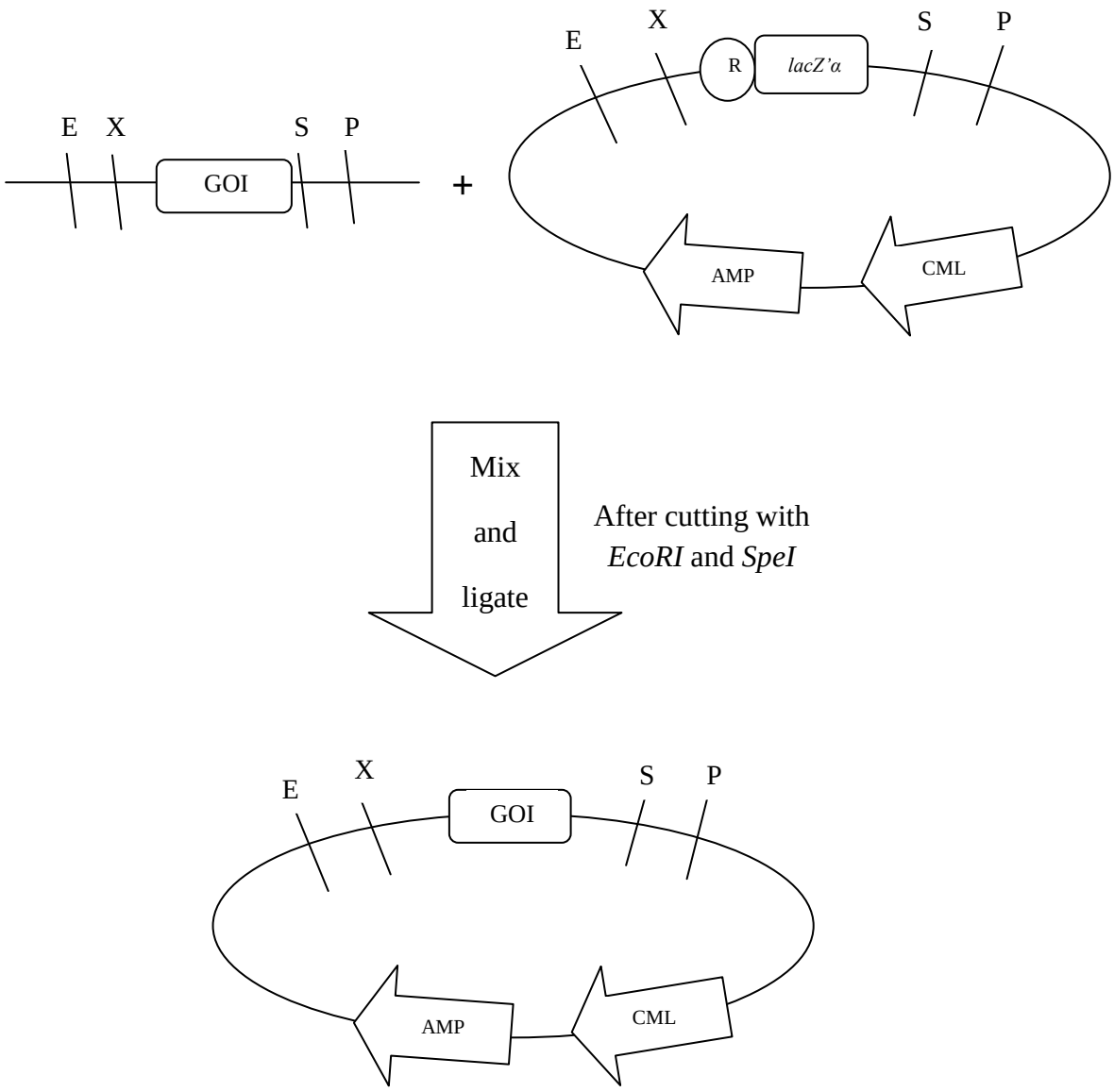


Figure 2.1: Procedure for inserting gene of interest (GOI) into a BioBrick vector. GOI and BioBrick vector flanked by *EcoRI*(E)/*XbaI*(X) and *SpeI*(S)/*PstI*(P) restriction sites on their 5' and 3' ends respectively is digested using E/S restriction enzymes, mixed and ligated using T_4 DNA ligase. This results in replacement of marker gene *lacZ'α* (alpha complementation gene of β-galactosidase) with our GOI. R – ribosome binding site, AMP – Ampicillin, CML – Chloramphinicol,

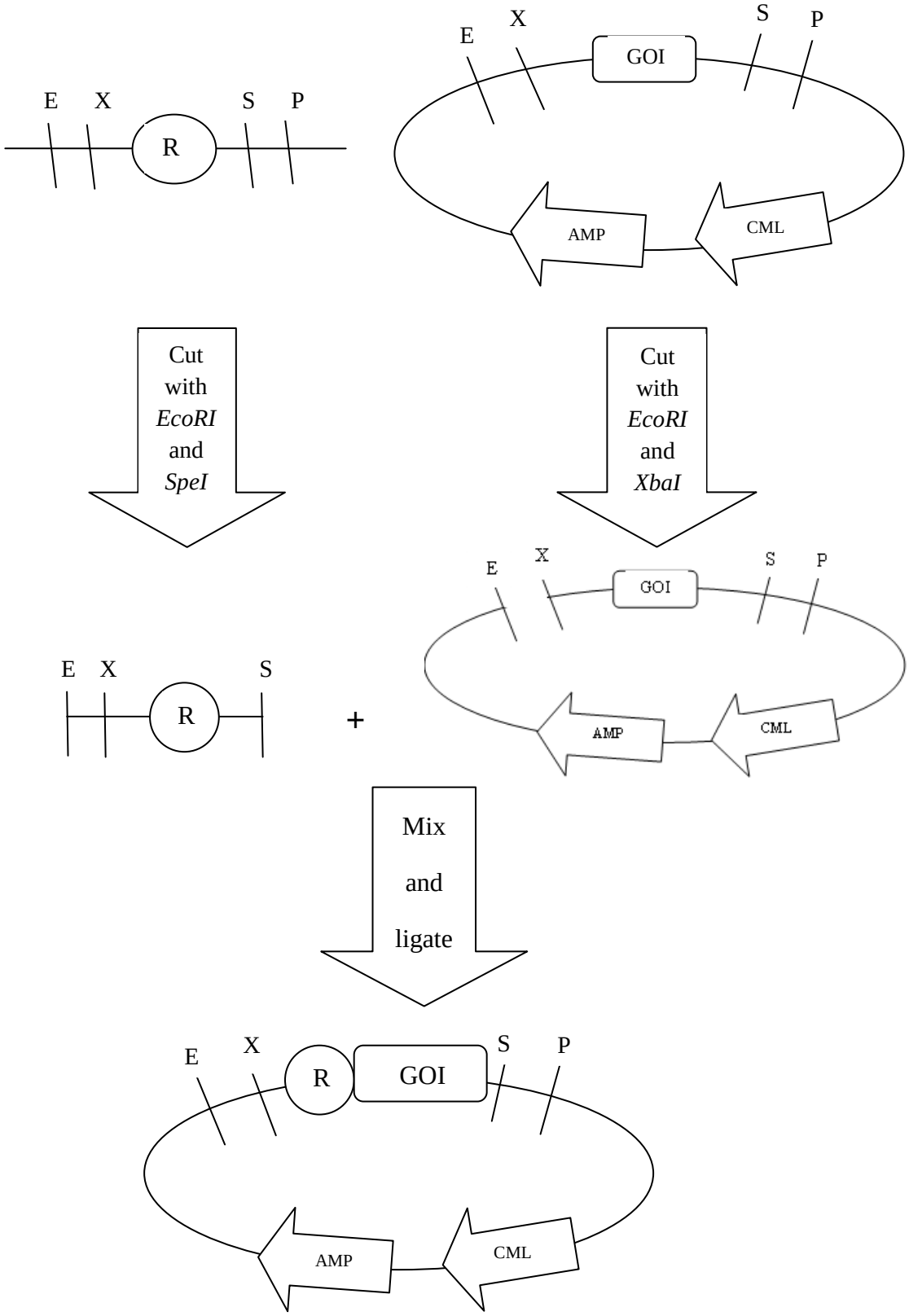


Figure 2.2: Procedure for adding a ribosome binding site (R) upstream of gene of interest (GOI). Ribosome binding site flanked by *EcoRI*(E)/*XbaI*(X) and *SpeI*(S)/*PstI*(P) on 5' and 3' end respectively is digested by E/S restriction enzymes. BioBrick vector with our GOI (flanked by same restriction sites on either side) is digested with E/X after which they are mixed and ligated using T_4 DNA ligase resulting in the addition of R upstream of GOI. AMP – Ampicillin, CML – Chloramphinicol,

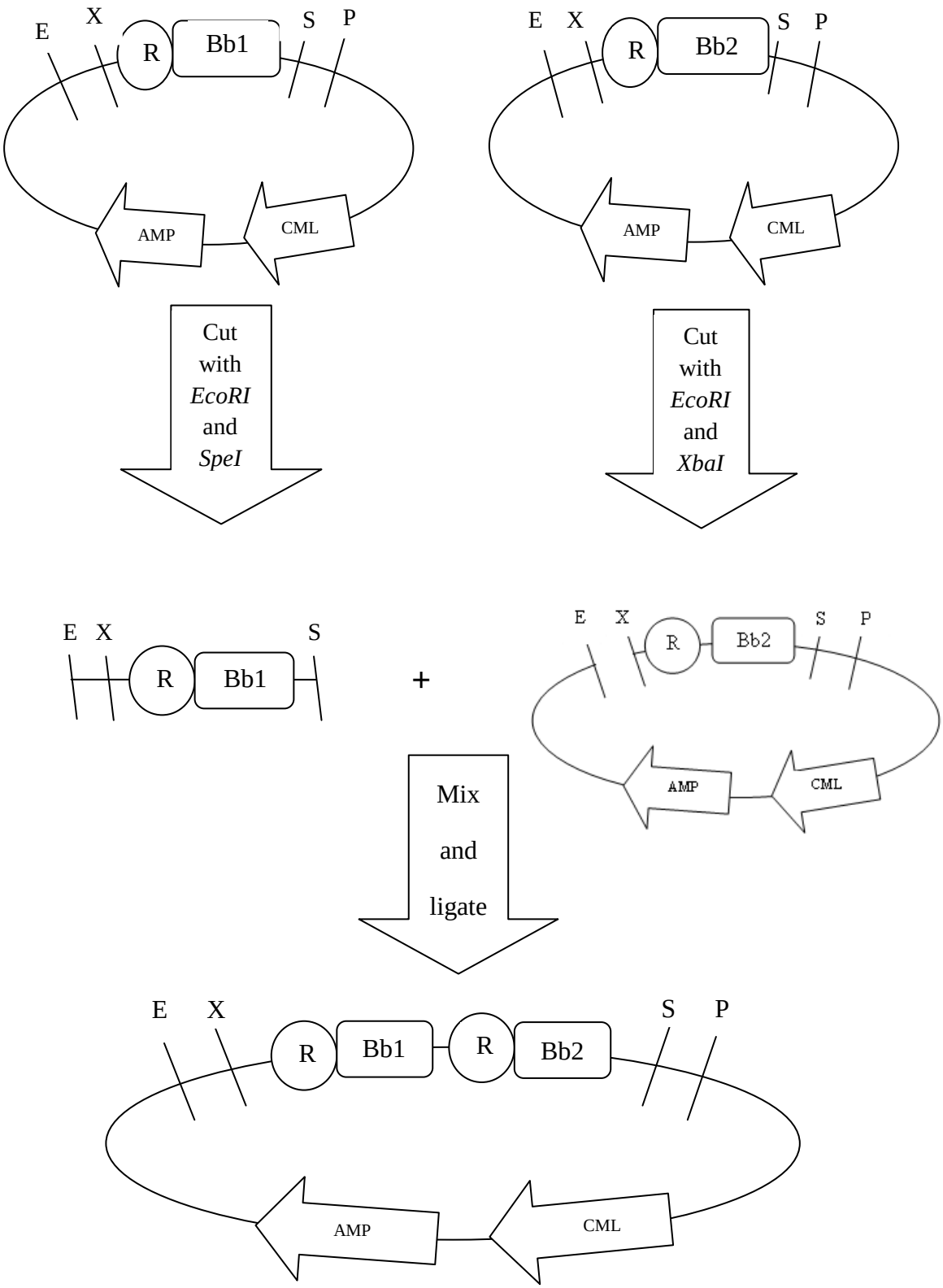


Figure 2.3: Procedure for combining two BioBricks. To insert BioBrick 1 (Bb1) upstream of BioBrick 2 (Bb2), Bb1 (flanked by *EcoRI*(E)/*XbaI*(X) and *SpeI*(S)/*PstI*(P) on 5' and 3' ends respectively) is digested with E/S and Bb2 (flanked by same restriction sites on either sides) is digested with E/X. Once digested they are mixed and ligated using T_4 DNA ligase resulting in the insertion of Bb1 upstream of Bb2. R – ribosome binding site, AMP – Ampicillin, CML – Chloramphinicol.

2.13 Assays:

2.13.1 Congo Red assay for qualitative detection of endoglucanase activity:

The assay method was similar to the one carried out by Teather and Wood (1982). Luria agar plates with 0.2% CMC were inoculated with particular strains and incubated at 37°C overnight. Next day the agar was flooded with 5 ml of 1 mg/ml Congo Red solution for 15 min. Excess Congo Red solution was poured off into a toxic waste bottle and 5 ml of 1 M NaCl was added and left for another 15 min. After 15 min, excess NaCl was poured off and results were observed.

2.13.2 MUC assay for qualitative detection of exoglucanase activity:

MUC (100 µl of 5 mg/ml) was spread on Luria agar plates and allowed to dry for at least 2 hours. Particular strains were then inoculated and incubated overnight at 37°C. The plates were then observed under long wave UV light (366 nm) to check for the production of fluorescence.

2.13.3 Assay for the qualitative detection of *xylE* activity:

To check the functionality of *spac* promoter *xylE* was added downstream to P_{spac}-*lacI* BioBrick. Few microliters (3 – 5) of 0.1 mM catechol was added on top of the colonies containing *xylE*. Development of yellow colour indicates positive result.

2.13.4 Assay for determining the specific activity of exoglucanase in cell extract:

The assay is used to determine the activity of exoglucanase using ONPC (*O*-nitrophenyl-β-D-cellobioside). Exoglucanase cleaves/hydrolyses ONPC producing *o*-nitrophenol which is yellow in colour. The activity can hence be measured by measuring the absorbance at 420 nm.

Cell extract (400 µl) was mixed with 440 µl of LacZ buffer and 160 µl of 4 mg/ml ONPC in phosphate buffer. The mixture was incubated in a water bath at 35°C. Samples (100 µl) were drawn every 30 min and mixed with 870 µl of LacZ buffer and

30 µl of 1 M Na_2CO_3. The absorbance was measured at 420 nm. LacZ buffer was used as a blank. See appendix for recipe regarding the composition of buffers.

2.13.5 Assay for quantitative determination of exoglucanase activity:

Methylumbelliferyl cellobioside (MUC) was used for quantitative analysis of exoglucanase activity. MUC in the presence of exoglucanase is broken down to methylumbelliferone and cellobioside. Methylumbelliferone in the presence of long wave UV light (366 nm) produces fluorescence. Overnight culture grown in suitable medium was diluted to get a final $O.D_{600}$ equal to 0.1 in the test medium. Samples (1 ml) were collected after several hours of growth. Cells were centrifuged, supernatant collected and cells resuspended in PBS and/or M9. MUC {5 µl (0.05 mM) of 5 mg/ml (10 mM)} was added to 1 ml supernatant/cell suspension and fluorescence was measured every 15 min for 45 – 90 min using a Modulus single tube multimode fluorometer (Turner BioSystems, BS040271) with UV filter. A calibration curve was prepared using different concentrations of 4-MU under the same assay conditions and was then used to determine the activity in test samples (see appendix for details).

2.13.6 Assay for quantitative determination of endoglucanase activity:

Dyed CM-Cellulose (Azo-CMC) from Megazyme was used for the quantitative analysis of endoglucanase activity according to manufacturer's protocol with slight modifications. Crude enzyme sample (500 µl) was mixed with 500 µl of Azo-CMC, vortexed and incubated at room temperature. Samples (300 µl) were collected at 0 min, 30 min and then finally at 60 min and added to 750 µl of 100% ethanol to stop the reaction. The mixture was vortexed and then centrifuged (15000 g) for 10 min to precipitate polysaccharides followed by reading the absorbance of the supernatant at 590 nm. A calibration curve was prepared using different concentrations of Remazol Brilliant Blue R under the same assay conditions and was eventually used to determine the enzyme activity in test samples (see appendix for details).

3 CELLULOSE DEGRADING SYSTEM OF *Cytophaga hutchinsonii*:

3.1 Background:

Cellulose, the polymer of β-1,4-linked glucose units, is the most abundant biopolymer on earth, but is insoluble and relatively recalcitrant to degradation. Since it is abundantly available it is not surprising for some microorganisms to specialize in its degradation as cellulose is a substrate for which there is relatively little competition.

The type species of genus *Cytophaga*, *Cytophaga hutchinsonii*, is a Gram negative cellulolytic soil bacterium that belongs to the phylum Bacteroidetes. Cellulose is its preferred substrate apart from which it also utilizes cellodextrins, cellobiose and glucose as the sole carbon and energy source. *C. hutchinsonii* cellulases were selected for several reasons. First, the genome of *C. hutchinsonii* was recently sequenced showing a large number of genes that were responsible for cellulose degradation (Xie *et. al.*, 2007). Second, studies in the past suggested that it may use a novel strategy for cellulose utilization as it's cellulases do not contain CBM plus the genome analysis does not show the presence of cellobiohydrolase (Xie *et. al.*, 2007; Wilson, 2008; Louime *et. al.*, 2007). These features to date were considered necessary for efficient utilization of cellulose. Third, it is a gliding bacterium whose gliding motility seems to facilitate cellulose digestion as it needs to be in direct contact with it's insoluble substrate for efficient digestion, and gliding also seems to align and move the cells along the fibre as they digest cellulose (Xie *et. al.*, 2007). Finally, genetic manipulation techniques are now available for this bacterium, which may provide new insights into cellulose utilization by *C. hutchinsonii*.

3.2 Cloning of endoglucanases:

Initially it was decided to clone the genes in BioBrick format as it allows endless number of parts to be pieced together to form complex systems and the number of stages in which parts are assembled is the log (base 2) of the length of the assembly (http://partsregistry.org). Therefore the initial thought was that once the cellulase BioBricks using genes from *C. hutchinsonii* are made, they would be expressed in

E. coli and their activity would be checked against different cellulosic substrates. BioBricks showing optimum activity against different substrates would then be combined together to build a complete biological system within a single organism which should then be able degrade different cellulosic substrates. In order for the genes to be cloned in the form of BioBricks, they needed to be inserted in BioBrick vectors using *EcoRI, PstI, XbaI* or *SpeI* as restriction enzymes. These are the standard enzymes used by BioBrick vectors, but unfortunately all the four genes CHU_1655, CHU_1842, CHU_2103 and CHU_2802 which were marked as probable cellulases upon genome examination of *C. hutchinsonii* (www.genome.jp) had either *EcoRI, PstI, XbaI* or *SpeI* restriction sites within the genes, except CHU_1401 which was annotated as a cellulase in the KEGG database but as a peptidase elsewhere (Table 3.1). It was therefore decided that this gene would not be used. Since all four genes contained forbidden restriction sites (Table 3.1) it was then decided to use pT7-7 (one of the series of pET vectors, Figure 3.1) as the cloning vector, *NdeI* and *SalI* as the restriction enzymes and finally to test the genes for activity. Successful results would have led to site directed mutagenesis of BioBrick restriction sites inside the genes and cloning of them into BioBrick vectors. We started by cloning CHU_2103 and CHU_2802. CHU_1655 and CHU_1842 were not used initially due to the fact that they had *NdeI* sites within the genes, so if we were to use *NdeI* for restriction digestion, it would have cut the gene internally. Hence, finally it was decided to use CHU_2103 and CHU_2802 (Table 3.1) initially, to test the procedures, before using a more complicated cloning strategy to clone the others.

Table 3.1: Probable endoglucanases of *C. hutchinsonii*

Gene	Family	protein length	Unique restriction sites within gene
CHU_1655	Glycoside hydrolase family 9	854 aa	*Kpn1, Nco1, Cla1, Nde1, BamH1, Sma1, EcoR1*
CHU_1842	Glycoside hydrolase family 5	551 aa	*EcoRV, Pst1, Kpn1, EcoR1, Nde1, Cla1*
CHU_2103	Glycoside hydrolase family 5	346 aa	*Kpn1, Pst1*
CHU_2802	Glycoside hydrolase family 16	345 aa	*EcoRV, EcoR1*
CHU_1401	Glutamylamino peptidase	350 aa	*EcoRV, Nde1*

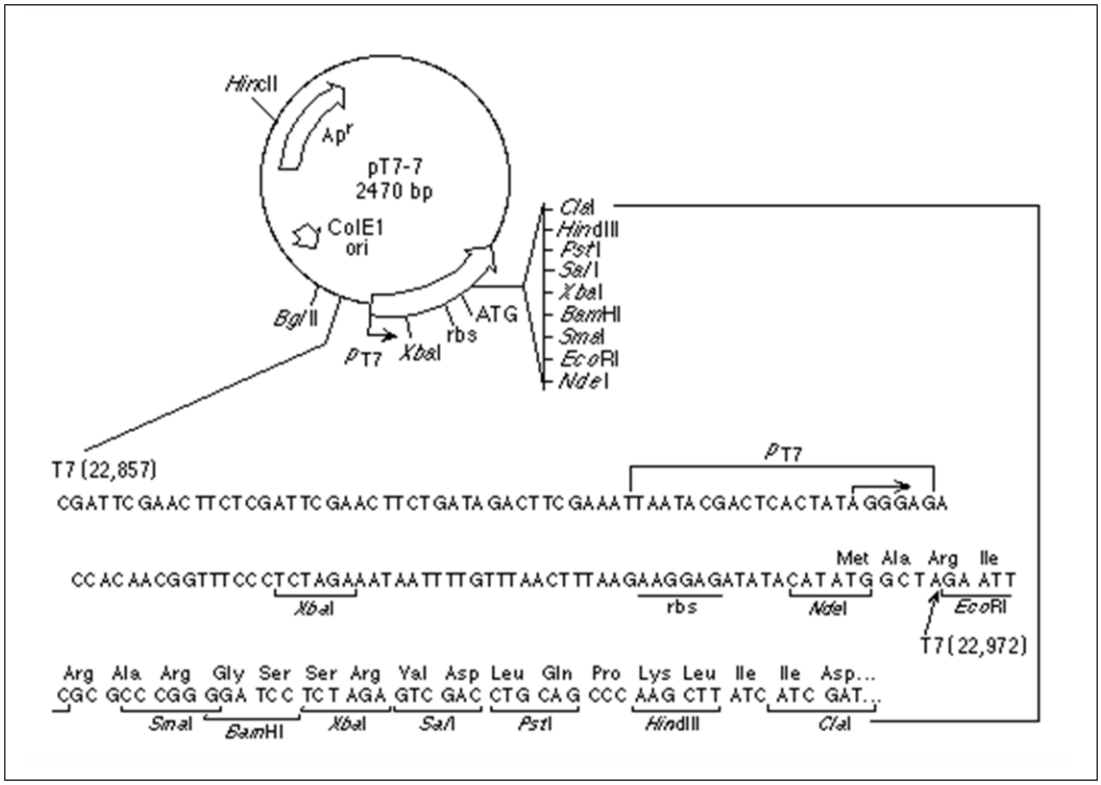


Figure 3.1: Diagram of pT7-7 showing the position of various multi-cloning restriction sites, ribosome binding site (rbs), T_7 polymerase (P_{T7}) and Col E1 origin of replication.

Several attempts were made to clone CHU_2103 and CHU_2802. Initially when pT7-7 was used as the cloning vector it did not contain a disruptable marker gene such as *lacZ'α*, therefore all of the transformants were screened for the presence of the gene of interest. Unfortunately, none of the transformants carried the desired insert.

An attempt was therefore made to introduce a marker gene into the plasmid pT7-7 that can be replaced. Initially we tried using the *ido* gene which encodes for indole dioxygenase (giving a black colour to the cell upon expression) from *Rhodococcus* ATCC 21145 (Heller *et. al.*, 2010). We succeeded in inserting it into pT7-7 but the problem faced was that the cells were turning black only after 3 – 4 days of incubation. Hence the *lacZ'α* gene was inserted into pT7-7 which was then eventually used for replacement by CHU_2103 and CHU_2802. The procedure for making black-white and blue-white selectable versions of pT7-7 and the procedure for replacement of the marker gene by our gene of interest is shown in Figures 3.2, 3.3 and 3.4 respectively.

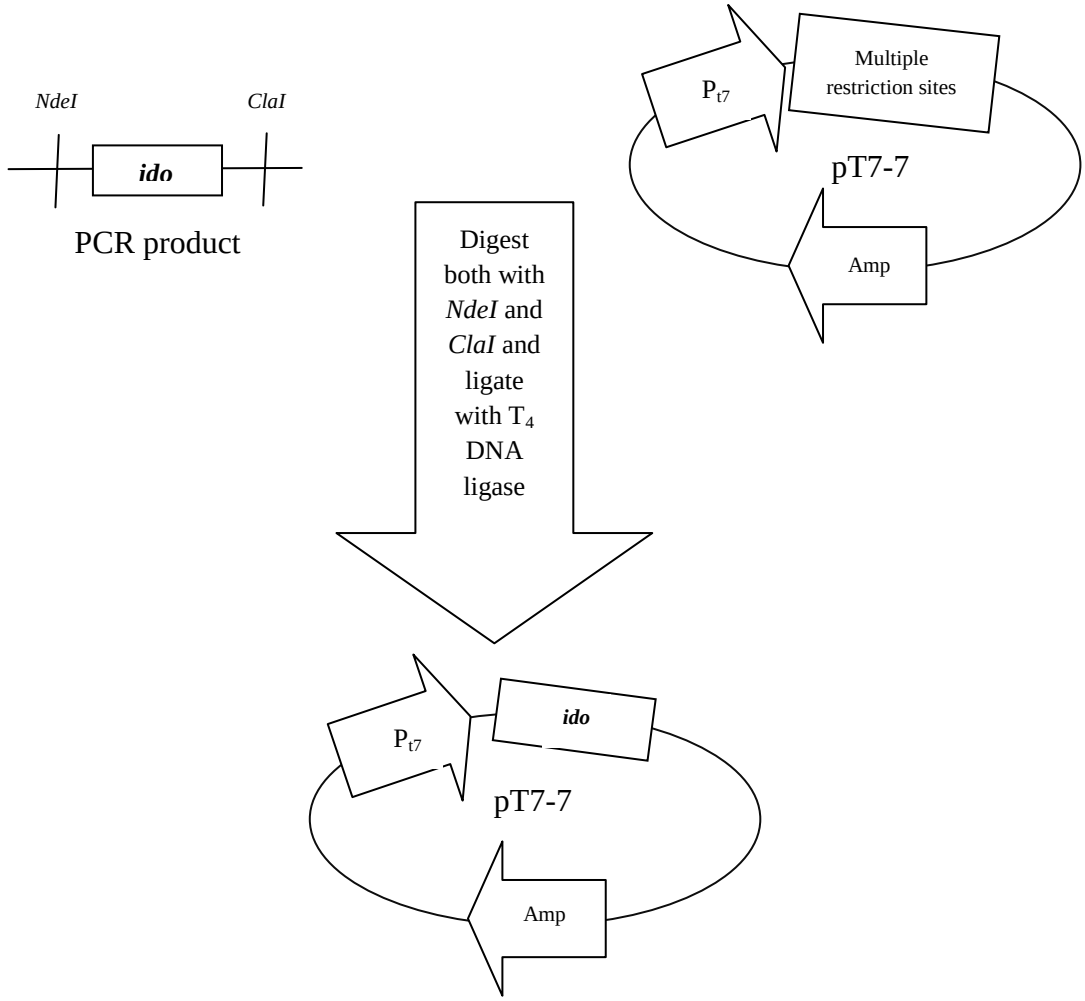


Figure 3.2: Diagram showing the construction of a black-white selectable version of pT7-7 using *ido*. PCR product (*ido* – Gene encoding for indole dioxygenase) and the vector pT7-7 were both digested with *NdeI/ClaI* and ligated with T_4 DNA ligase to make a black-white selectable version of pT7-7. P_{t7} – Promoter of T_7 polymerase, Amp – Ampicillin

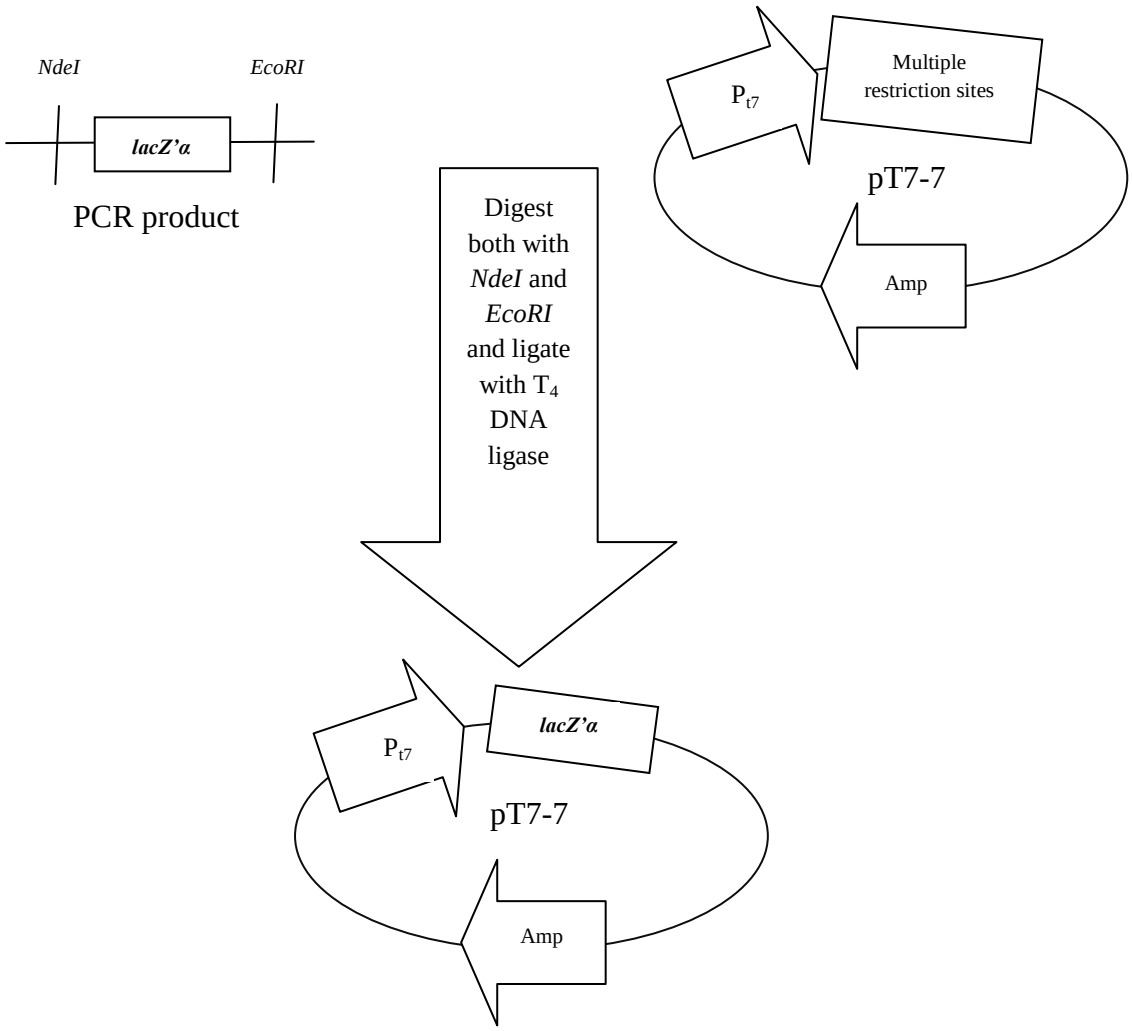


Figure 3.3: Diagram showing the construction of a blue-white selectable version of pT7-7 using *lacZ'α*. PCR product (*lacZ'α* – Alpha complementation gene of β-galactosidase) and the vector pT7-7 was digested with *NdeI/EcoRI* and ligated using T_4 DNA ligase to create a blue-white selectable version of pT7-7. P_{t7} – Promoter of T_7 polymerase, Amp – Ampicillin

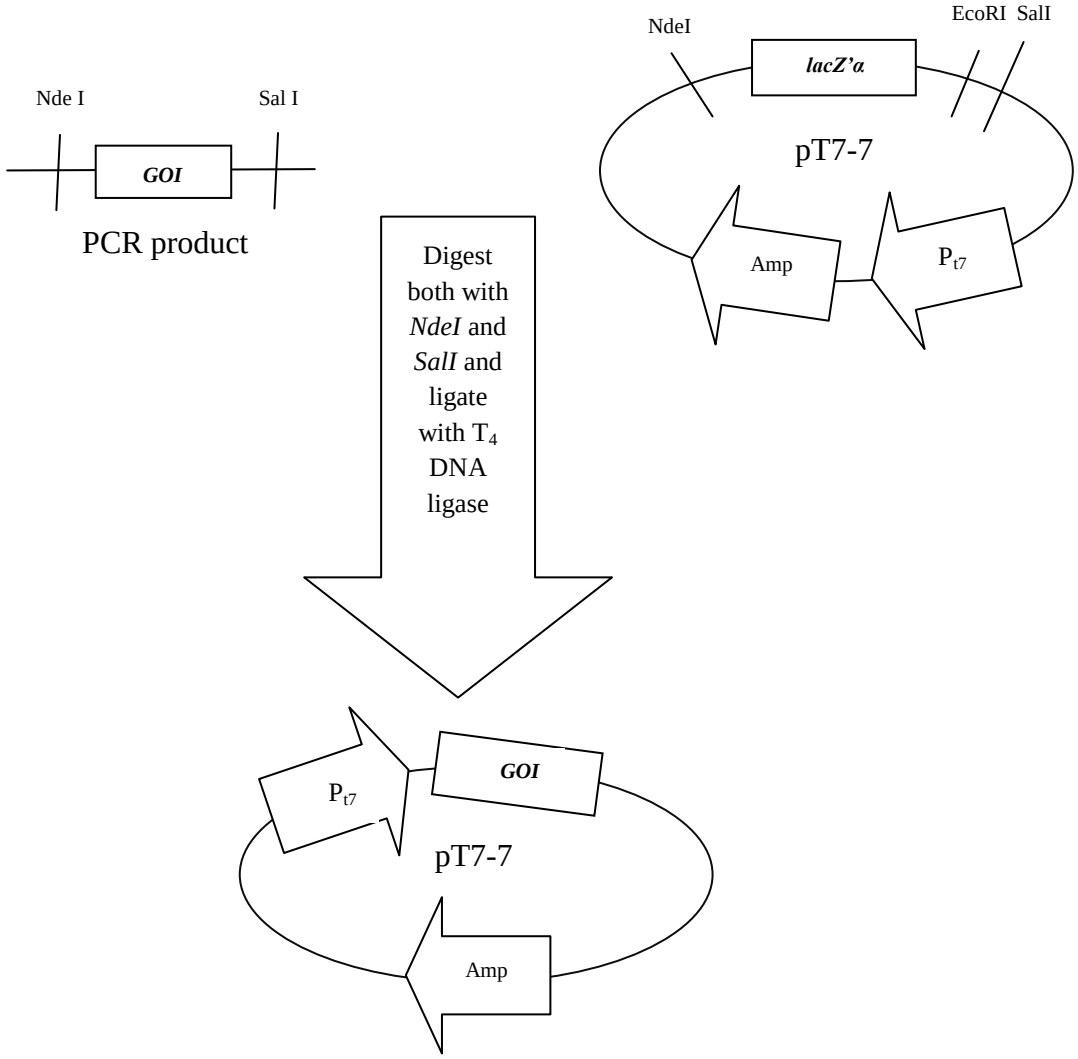


Figure 3.4: Diagram showing insertion of the gene of interest into pT7-7 by replacing the marker gene. PCR product (GOI – Gene of interest) and the vector pT7-7 were both digested with *NdeI/SalI* and ligated using T_4 DNA ligase resulting in the replacement of marker gene *lacZ'α* (Alpha complementation gene of β-galactosidase) by our gene of interest (GOI). P_{t7} – Promoter of T_7 polymerase, Amp – Ampicillin

The transformation was initially done using *E. coli* JM109 to get a good plasmid yield. Once the transformation was successful and the presence of the gene of interest was confirmed by sequencing, the plasmid harvested from *E. coli* JM109 (Figure 3.5) was then used to transform the expression host *E. coli* BS(λDE3) which was used for further experiments on protein expression and extraction.

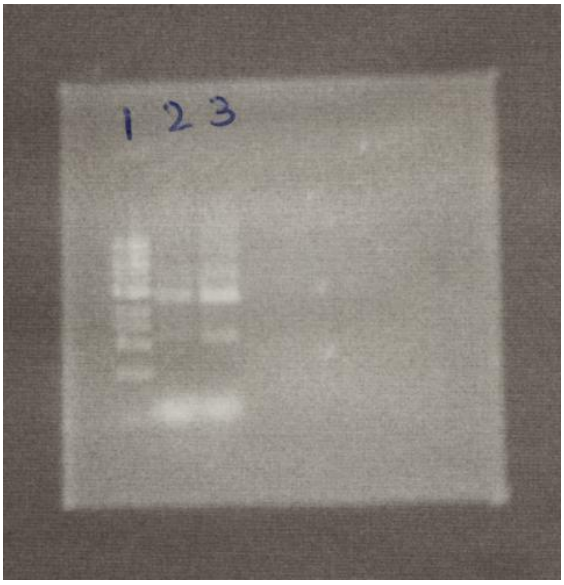


Figure 3.5: Gel picture showing plasmid harvested from *E. coli* JM109 and digested with *NdeI* and *SalI*. Lane 1 – 1 kb DNA ladder, lane 2 – pT7-7+2103, lane 3 – pT7-7+2802. Size of pT7-7 = 2.7 kb, 2103 = 1.1 kb, 2802 = 1.1 kb

3.2.1 Protein expression and extraction:

For the expression and extraction of proteins, 5 ml culture was grown overnight in LB containing 100 µg/ml ampicillin followed by its inoculation into 50 ml of fresh LB medium containing 100 µg/ml ampicillin and 90 µg/ml IPTG. The culture was grown for 4 hours at 37°C with shaking. Cell extracts was then prepared using BugBuster® Ht protein extraction reagent (Section 2.11.1). Expression of the proteins proved problematic. The cell extracts of the vector control showed higher protein concentrations than the ones containing our gene of interest, showing that the gene products might be affecting the growth (Figure 3.6).

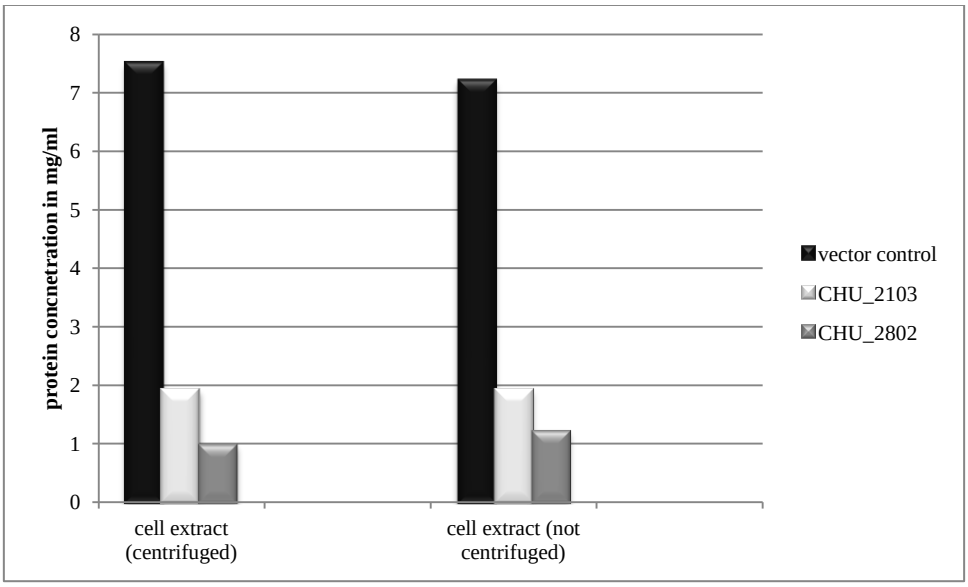


Figure 3.6: Concentration of total proteins in cell extracts.

An attempt was then made to determine whether the addition of IPTG for induction is making any difference. The culture was grown overnight in 5 ml LB with 100 µg/ml ampicillin. It was then inoculated into two sets of 50 ml fresh LB medium containing 100 µg/ml ampicillin. Once set of flask was induced using 90 µg/ml IPTG. The growth of the culture in the flasks with IPTG was much lower and hence the protein concentration was also lower except for the vector control, showing again that expression of the protein is deleterious to the cells (Figure 3.7).

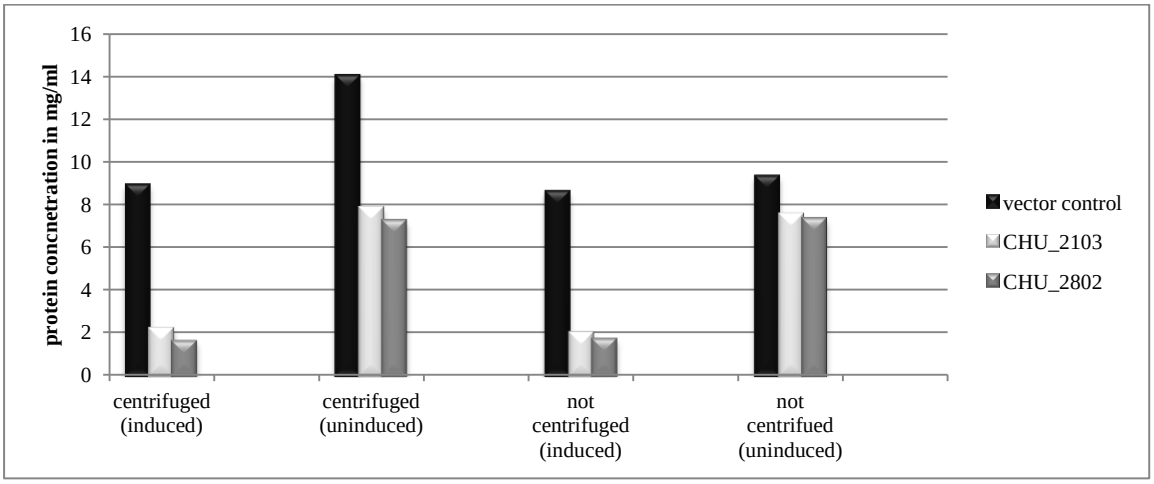


Figure 3.7: Effect of induction on protein concentration of cell extracts. One set of extract was centrifuged to remove cell debris.

In order to exclude the possibility that an unequal amount of inoculum is making this difference, the $O.D_{600}$ of the overnight culture was measured before inoculation, which was almost the same in all the bottles. It was then used to inoculate the flask such that the initial $O.D_{600}$ of all flasks becomes 0.1. Hence, a measured amount of inoculum (in terms of number of cells) was used to inoculate all the flasks. The results in this case were also consistent with the previous results, further confirming that the protein is deleterious to the cells (Figures 3.8 and 3.9). This experiment was repeated couple of times and each time same trend was observed.

SDS PAGE analysis of cell extracts was also done in order to see any distinct band in our induced clones. Unfortunately there was no band distinct in cell extracts of our constructs compared to vector control (data not shown). As the gene products were predicted to be extracellular, we also did the SDS PAGE analysis of supernatants in order to see any band distinct from the vector control. The results were the same in this case as well and there was no distinct band in the supernatant of recombinant organisms with our constructs compared to the vector control. The amount of proteins could not be determined in the supernatant as the concentration of protein in the supernatant was below the range of the assay used.

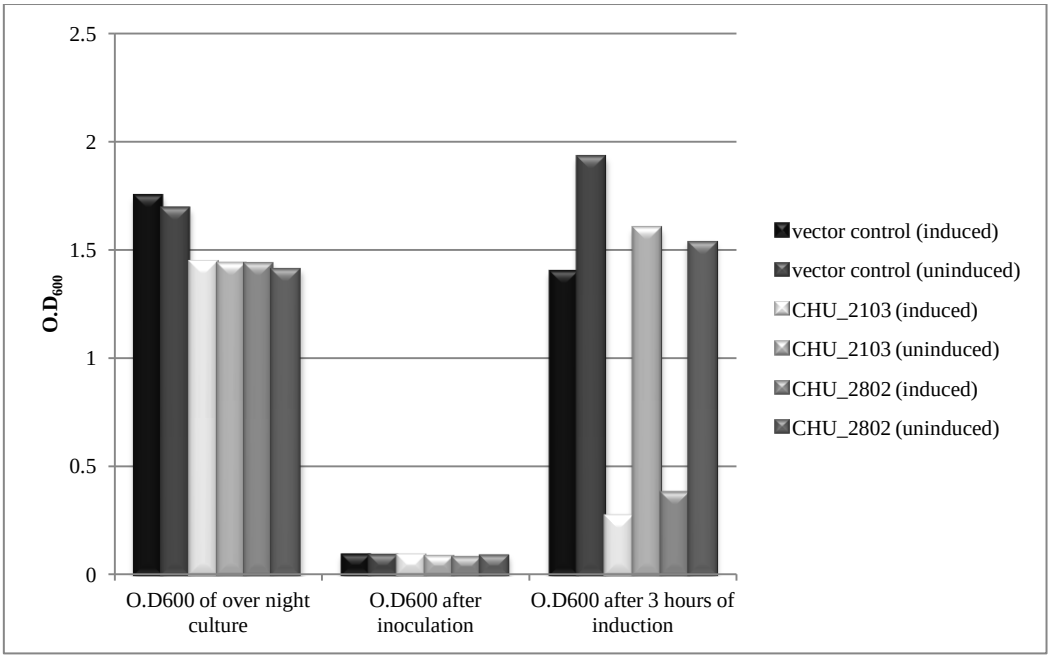


Figure 3.8: Effect of induction on growth of the culture.

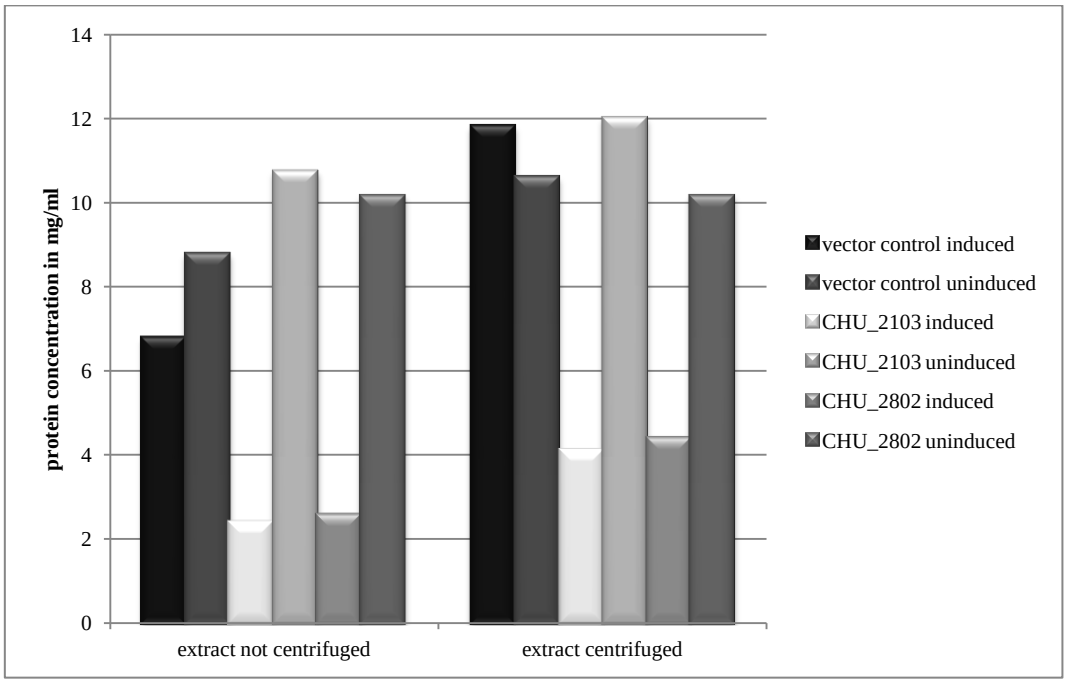


Figure 3.9: Comparison of protein concentration of induced and uninduced cultures.

3.3 Gluconate as carbon source:

It has been reported in the literature that in addition to hydrolytic enzymes, additional non-hydrolytic enzymes including oxidative enzymes (such as cellobiose-oxidizing enzymes) are also involved in cellulose degradation. They oxidize the reducing ends of the substrate to their corresponding lactones which are then utilized by the cells. Eriksson *et al.* (1974) was the first to observe that oxygen enhanced cellulose degradation by fungal cellulases and hypothesize that oxidative enzyme might be involved. This then led to the discovery of cellulose oxidizing enzymes in various fungi, but little attention was paid to the enzyme system in bacteria (Li *et. al.*, 1996). The cellulose system of *Cytophaga* was investigated by several workers, but none of them paid attention to detecting whether there is an oxidative system involved in *Cytophaga*, until Li *et al*., (1996, 1997) who first reported the presence of cellobiose oxidizing enzymes (cell bound cellobiose:quinone oxidoreductase and extracellular cellobiose oxidase) produced by *Cytophaga* sp. LX-7 isolated from the campus of Shandong University at Jinan, China.

In order to determine whether *C. hutchinsonii* also uses the oxidative system for cellulose degradation, a preliminary experiment was carried out by growing organism on gluconate (by putting small amount of gluconate on the centre of DMM plate containing no other carbon source) as the sole source of carbon. If *C. hutchinsonii* possesses the oxidizing enzyme converting cellobiose to its lactone and further to gluconate, then the organism may be able to grow on gluconate as sole carbon source. There was no growth on such media, indicating that the organism possibly is using some other mechanism for cellulose degradation.

3.3.1 Cloning of dehydrogenases:

BLAST (Basic Local Alignment Search Tool) searches however showed the presence of three genes CHU_1944, CHU_2315 and CHU_1221 in *C. hutchinsonii* with possible glucose/sorbosone dehydrogenase activity. Two of these genes, namely CHU_1944 and CHU_2315, were therefore cloned to test for dehydrogenase activity. The reason for initially using these two genes and not CHU_1221 was the fact that CHU_1221 was too big (4.9kb) and contained an *NdeI* site inside the gene. Several attempts were made to transform *E. coli* JM109 (by using various concentration of plasmid DNA (3 – 10 µl) and the plates with and without IPTG induction) but no transformants were obtained. We also tried cloning these genes without a signal peptide, in case for some reason the secretory versions of these genes were toxic for *E. coli* cells. We did not manage to get any transformants in this case either. Hence it was then finally concluded that the expressed protein was toxic for *E. coli* cells and no transformants were thus obtained which could be tested further for the activity.

3.4 Synergistic growth of *Cytophaga hutchinsonii* with *Escherichia coli* and/or *Bacillus subtilis*:

Since the cloning and expression of the *C. hutchinsonii* cellulose-degrading system in *E. coli* proved surprisingly challenging, we therefore decided to opt for a different strategy in which an active cellulose degrader is grown in mixed culture with an organism that can easily be genetically modified to produce useful products from the sugars released after cellulose degradation. Hence some preliminary growth curve experiments were performed. Two industrially important organisms, *E. coli* B and *B. subtilis* 168, were selected as organisms into which a recombinant product-

formation system can be introduced. *C. hutchinsonii* was used as a cellulose degrader. Mixed culture growth curve experiments were performed using 50 ml DMM medium with filter paper as a source of carbon. $O.D_{600}$ was measured every 24 hours and colony counts were performed at the end of growth/experiment to determine the number of *E. coli* and *B. subtilis* in the medium by plating them on plain LB agar plates. As *C. hutchinsonii* cannot grow on LB agar plates, the only organisms growing were either *E. coli* and/or *B. subtilis*. As *C. hutchinsonii* does not form reliable colonies on plates, we were not able to perform colony counts for this organism. The results are presented in Figure 3.10 and Table 3.2. As it was a preliminary experiment only single replicates were used. However the experiment has been repeated by other members in the lab and the results were reproducible.

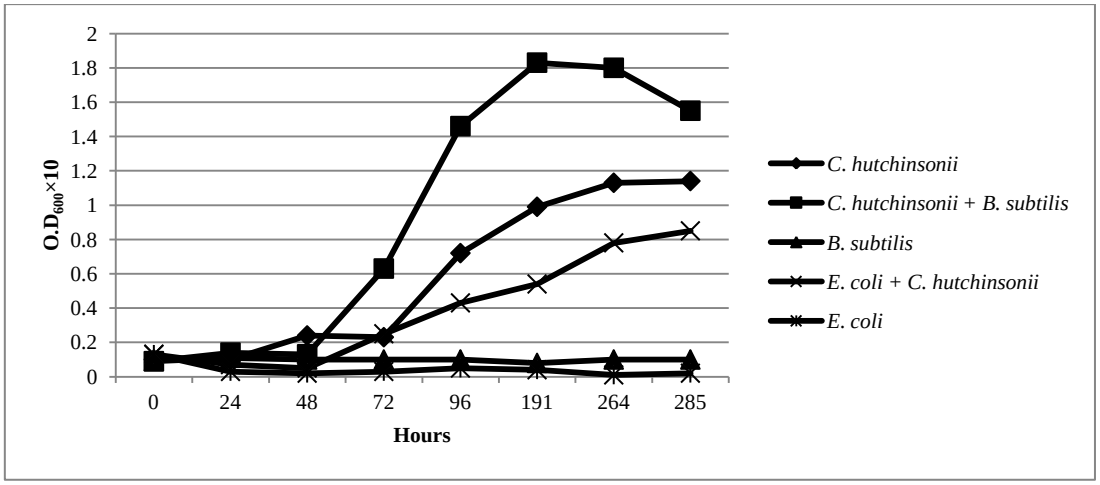


Figure 3.10: Growth curve of *E. coli*, *B. subtilis* and *C. hutchinsonii* growing individually and in mixed culture. $O.D_{600}$×10 – The culture was diluted 10 fold before measuring the optical density.

Table 3.2: Colony counts of *B. subtilis* and *E. coli* (at the end of experiment) when growing individually and in mixed culture with a cellulose degrader

Condition	**Cfu/ml (single replicate)**
C. hutchinsonii + *B. subtilis* 168	2×10^7
B. subtilis 168	3×10^4
C. hutchinsonii + *E. coli* B	4×10^6
E. coli B	2×10^4

The results showed a synergistic effect and a shorter lag phase when the organisms were growing together than when they were growing individually, suggesting that *C. hutchinsonii* cross feeds the two organisms and supports their growth with cellulose as a sole carbon source. However the growth was much better when *C. hutchinsonii* and *B. subtilis* 168 were growing together than when *C. hutchinsonii* and *E. coli* B were growing together. Also, in addition to providing *B. subtilis* with the source of carbon for growth, *C. hutchinsonii* apparently provides it with amino acids, as for *B. subtilis* 168, tryptophan is an essential amino acid and must be provided in the growth medium. We did not provide any source of amino acid in the growth medium, therefore it was concluded that *C. hutchinsonii* was also providing *B. subtilis* 168 with amino acids as well as with carbon. The results hence indicate that *B. subtilis* 168 is a better option than *E. coli* B for the introduction of a production system to be used in conjunction with *C. hutchinsonii* for the production of ethanol (or any other commodity product) from cellulosic substrates.

3.5 Discussion:

It was observed from the results that the expression of the genes from *C. hutchinsonii* was toxic for the *E. coli* host. Some possible reasons for *C. hutchinsonii* genes to be toxic for *E. coli* could be:

- Some of the codons used quite frequently by *C. hutchinsonii* (particular in CHU_2103, CHU_2802, CHU_1944 and CHU_2315 genes) are very rarely used by *E. coli* (Henaut and Danchin, 1996). Table 3.3 shows the percent codon usage of some codons commonly used in these genes in comparison to highly expressed genes of *E. coli* (Henaut and Danchin, 1996).
- The enzymes may well be clogging up the secretion system of *E. coli*, as almost all the cloned gene products (analysed by PSORT and LipoP programmes from Expasy website) were extracellular (www.expasy.org).
- It could also be due to the activity of the enzyme itself which somehow might be causing some inhibitory effect on the growth of the *E. coli* cells.

Hence after looking at all the results it was decided to construct a recombinant system initially using well studied cellulases from *Cellulomonas fimi*.

Table 3.3: Percent codon usage of commonly used codon (in CHU_2103, CHU_2802, CHU_1944 and CHU_2315 genes) by *C. hutchinsonii* compared to highly expressed genes of *E. coli*

Codons	***C. hutchinsonii***	***E. coli***
ata	17.71%	0.57%
aga	16.67%	0.62%
agg	1.85%	0.29%
cta	0.92%	0.83%
ccc	12.66%	1.63%
cga	7.41%	1.07%
cgg	11.11%	0.80%

The mixed culture growth curve experiments (although very preliminary) showed that *B. subtilis* 168 was growing in synergy with *C. hutchinsonii*. Hence if *C. hutchinsonii* genes were toxic for the expression host (*E. coli*) then the second option could be the introduction of the production system in *B. subtilis* 168 and use in mixed culture with *C. hutchinsonii* which will degrade cellulose and provide *B. subtilis* 168 with a source of carbon. *B. subtilis* 168 can then eventually convert it to ethanol or any other commodity product.

4 CELLULASES FROM *Cellulomonas fimi*:

4.1 Background:

Wong, *et. al* (1988) reported the hydrolysis of filter paper and pre-treated aspen wood chips by *Saccharomyces cerevisiae* transformed with two *Cellulomonas fimi* cellulases {CenA (an endoglucanase) and Cex (an exoglucanase/xylanase)} after the addition of a small amount of β-glucosidase. As the expression of genes in *E. coli* from *Cytophaga hutchinsonii* turned out to be unexpectedly difficult this gave us the idea of creating a bacterial version of the *cenA-cex* system which might potentially produce higher yields of secreted protein than *S. cerevisiae*, would have the added benefit of facilitated genetic manipulation and might overcome the need for supplemental β-glucosidase.

Escherichia coli is most commonly used for the purpose of genetic manipulation studies. Firstly, because it is very easy to work with. Secondly, genetic manipulation techniques for *E. coli* are well developed. Thirdly, a number of easily controllable promoter systems are available which regulate transcription, which has been important for the development of foreign protein expression systems. The *lac* repressor-operator system is one example of such a system in which the *lac* repressor binds to the *lac* operator, thereby preventing the binding of RNA polymerase to the promoter, hence repressing transcription. Upon binding of an inducer to the *lac* repressor, a conformational change occurs in the repressor which reduces its affinity for the operator resulting in induction of transcription. Such an inducible system can be used to control the transcription of other genes once they have been cloned downstream of inducible promoter. In this work genes from *C. fimi* (*cenA* and *cex*) were cloned downstream of P_{lac} (the promoter for the *lac* operon) so that the expression of these genes could be controlled using IPTG (an inducer of the *lac* operon). After all manipulation and construction was done in *E. coli* JM109, the system was also introduced into *Citrobacter freundii* SBS197 and NCIBM 11490 and *Bacillus subtilis* 168.

C. freundii was used for expression due to the following reasons:

1. It is an organism closely related to *E. coli*, for which reason the same plasmids can be used for transformation and expression of the gene of interest (in this case *cenA* and *cex*).
2. It can secrete proteins well into the medium, in contrast to *E. coli*, which lacks the Main Terminal Branch of the type 2 secretion system.
3. It is classified as a category 1 organism by ACDP (Advisory Committee on Dangerous Pathogens), which includes organisms that are not likely to cause any human diseases. Whereas, almost all of the close relatives of *E. coli* in addition to the wild type strains of *E. coli* itself, are included in category 2 which includes biological agents which can cause disease and are a hazard to employees but are not likely to spread to community and against which an effective treatment is usually available. Also when performing genetic modification experiments in category 2 organisms special permission is required from the Health and Safety Executive.
4. *C. freundii* in contrast to *E. coli* can assimilate cellobiose, the major breakdown product of cellulose degradation.

Similarly *B. subtilis* was used as an expression host because:

1. It is a naturally competent organism and can take up DNA from the environment and hence can easily be transformed if provided with suitable plasmid DNA in the medium.
2. Again compared to *E. coli*, *B. subtilis* can secrete proteins very well into the surrounding medium.
3. *B. subtilis* can form spores and survive for years under unfavourable conditions, which would be an advantage during transportation and storage.
4. It can degrade amorphous cellulose. This ability of *Bacillus* may work in synergy with the cloned cellulases.
5. It has a GRAS status i.e., it is generally regarded as a safe organism.

In order to express genes in *B. subtilis*, which is a Gram positive organism, the construct was introduced into pTG262 (a broad host range plasmid isolated from *Lactobacillus*) and transformed into *B. subtilis*. According to Yansura and Henner (1984), no system analogous to the *lac* operon exists in *B. subtilis*. The only transcriptionally controlled promoters recognised use σ factors, the proteins that bind to RNA polymerase to determine the recognition site for RNA initiation. Such a system would be hard to use for the controlled expression of another gene. However, Yansura and Henner (1984) reported the fact that the *E. coli lac* repressor-operator system can be used to control gene expression in *B. subtilis*. They reported the transfer of the *lac* repressor-operator system into *B. subtilis* and showed that the *lac* repressor and operator can function as transcriptional regulatory system in this organism. They constructed a hybrid promoter using phage SPO-1 promoter element and the *lac* operator, called *spac-I*. This system can be induced using IPTG. They concluded that *lac* repressor is expressed in a functional state in *B. subtilis* in order to control transcription from $P_{spac\text{-}I}$ and also that IPTG enters in sufficient amounts to derepress the promoters.

Hence, in this chapter two types of promoter, P_{lac} and P_{spac}, were used to express *cenA* and *cex* using IPTG as an inducer.

4.2 Cloning:

The genes *cenA* and *cex* were cloned in BioBrick format after mutating the forbidden restriction sites by the University of Edinburgh, iGEM team 2008. Ribosome binding site (BBa_J15001) was inserted upstream of *cenA* and *cex* followed by insertion of rbs-*cenA* and rbs-*cex* in pSB1A2 downstream to P_{lac}-*lacZ'α* by using the standard BioBrick assembly protocol (Knight, 2003) for combining two BioBricks by Dr. Chris French and Natasha Cain (Figure 4.1). All subsequent cloning steps were performed by the author.

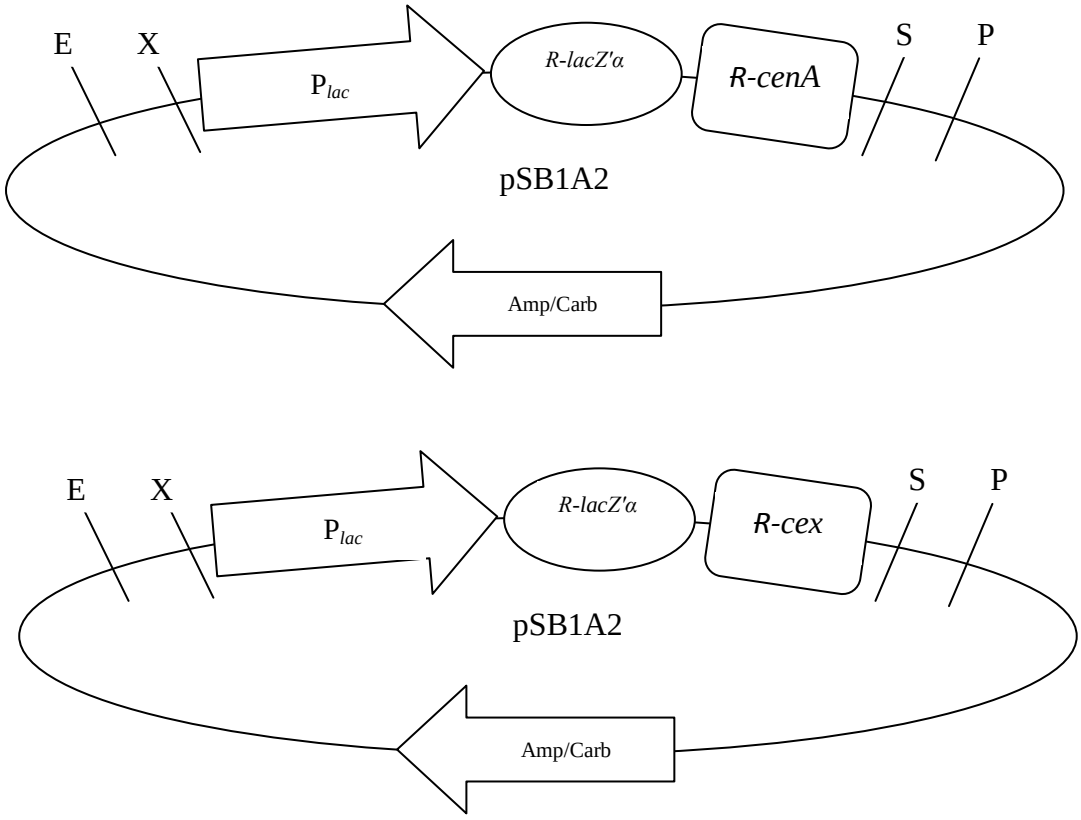


Figure 4.1: Diagram of pSB1A2 having *cenA* and *cex* downstream of P_{lac}-*lacZ'α*. E – EcoRI, X – XbaI, S – SpeI, P – PstI, P_{lac} – Promoter of *lac* operon, R-*lacZ'α* – Alpha complementation of β-galactosidase with ribosome bonding site, R-*cenA* – Endoglucanase (CenA) with ribosome binding site, R-*cex* – Exoglucanase (Cex) with ribosome binding site, Amp/Carb – Ampicillin and/or Carbenicillin. These plasmids were cloned by Dr. Chris French and Natasha Cian.

In order to express these genes in *B. subtilis* 168, the P_{spac} promoter was used. The gene encoding the *lac* repressor, *lacI*, was used to control expression of the gene. After cloning P_{spac} and *lacI* in BioBrick format, ribosome binding site (BBa_J15001) was added to the *lacI* BioBrick, followed by insertion of *lacI* downstream of P_{spac}. Next, rbs-*cenA* was inserted downstream of P_{spac}-*lacI* (Figure 4.2). The plasmid used during all this cloning procedure was pSB1A2.

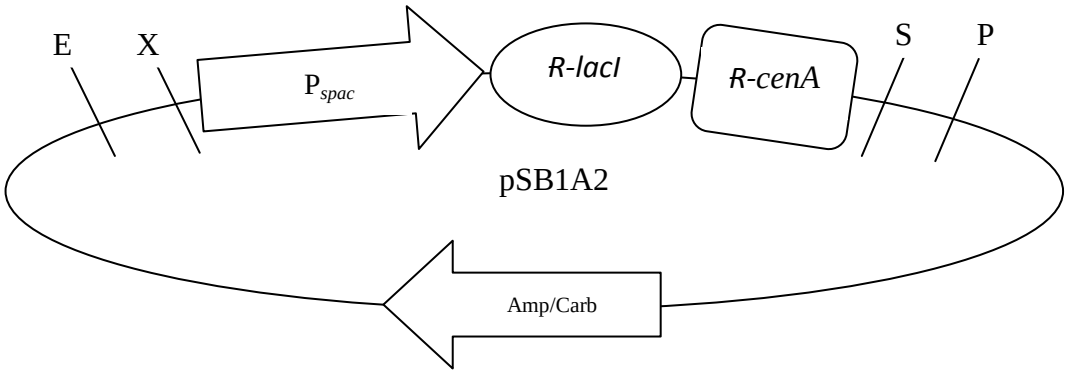


Figure 4.2: Diagram of pSB1A2 having *cenA* inserted downstream of P_{spac}-*lacI*. E – EcoRI, X – XbaI, S – SpeI, P – PstI, P_{spac} – Hybrid promoter made using phage spo-1 promoter element and the *lac* operator, R-*lacI* – *lac* repressor (repressor of *lac* operon) with ribosome bonding site, R-*cenA* – Endoglucanase (CenA) with ribosome binding site, Amp/Carb – Ampicillin and/or Carbenicillin

Once cloned, all these BioBricks were transformed into *E. coli* JM109, and the plasmids were extracted, sequenced and tested for activity.

4.2.1 Combining *cenA* and *cex* BioBricks:

The *cex* BioBrick was inserted downstream of the P_{lac}-*lacZ'α-cenA* BioBrick and P_{spac}-*lacI-cenA* BioBrick by using standard BioBrick protocols (Knight, 2003). Once combined they were transformed into *E. coli* JM109 and/or *C. freundii* SBS197 and NCIMB 11490 (Figure 4.3). Plasmids were extracted from *E. coli* JM109 (Figure 4.4), sequenced and tested for activity. However, due to difficulty in preparing plasmid DNA from *C. freundii*, the presence of plasmid in recombinant *C. freundii* was confirmed by colony PCR using *cenA/cex* primers to detect the presence of *cenA* and *cex* in the transformed cells (Figure 4.5). This confirms the presence of plasmid DNA in recombinant *C. freundii*.

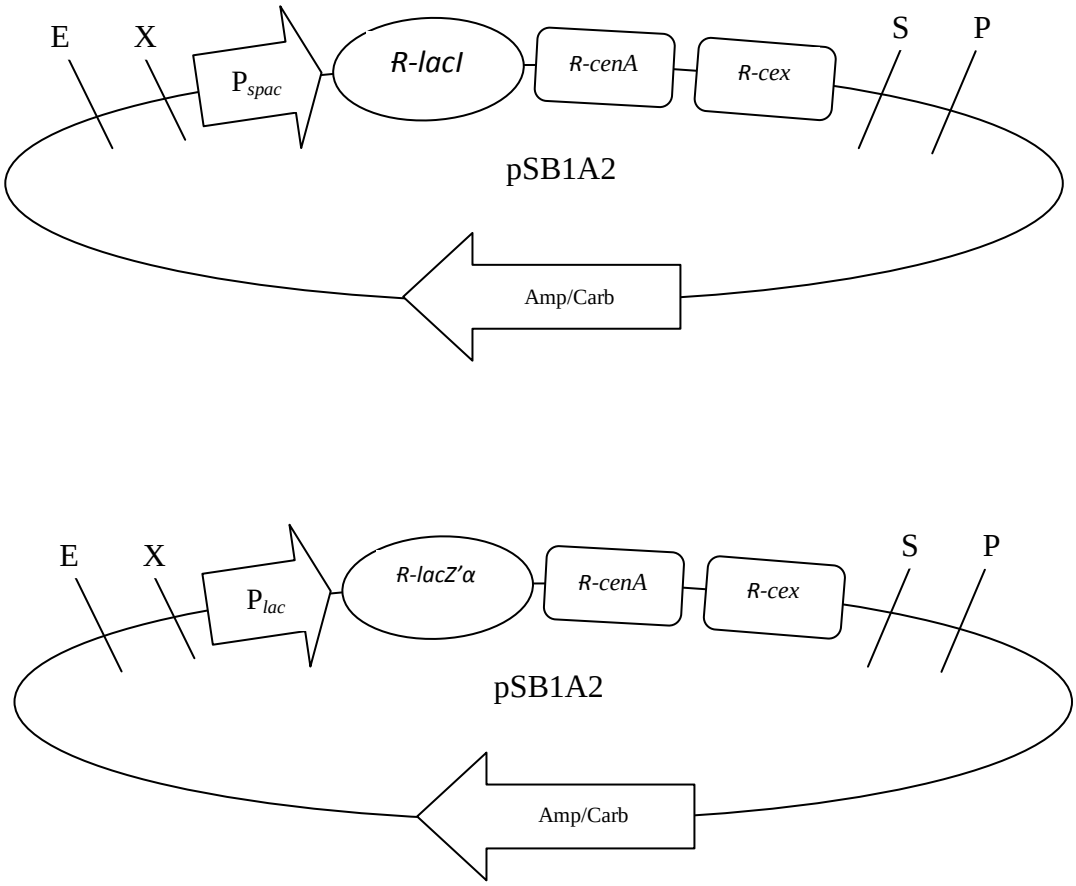


Figure 4.3: Diagram of pSB1A2 having *cenA* and *cex* downstream of P_{spac}-*lacI* and P_{lac}-*lacZ'α*. E – EcoRI, X – XbaI, S – SpeI, P – PstI, P_{spac} - Hybrid promoter made using phage spo-1 promoter element and the *lac* operator, R-*lacI* – *lac* repressor (repressor of *lac* operon) with ribosome bonding site, P_{lac} – Promoter of *lac* operon, R-*lacZ'α* – Alpha complementation of β-galactosidase with ribosome bonding site, R-*cenA* – Endoglucanase (CenA) with ribosome binding site, R-*cex* – Exoglucanase (Cex) with ribosome binding site, Amp/Carb – Ampicillin and/or Carbenicillin

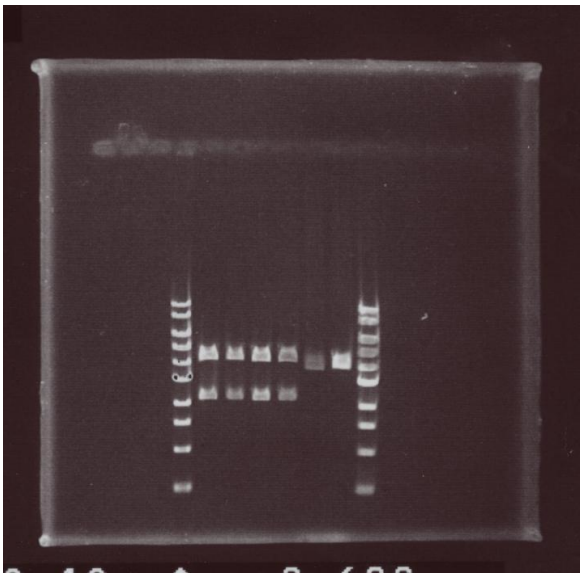


Figure 4.4: Gel picture showing plasmid harvested from *E. coli* JM109 and digested with *EcoRI* and *SpeI*. Lane 1 – 1 kb DNA ladder, lane 2 – pSB1A2+P_{spac}-*lacI-cenA-cex*. Size of pSB1A2 = 2 kb, P_{spac}-*lacI-cenA-cex* = 4.5 kb.

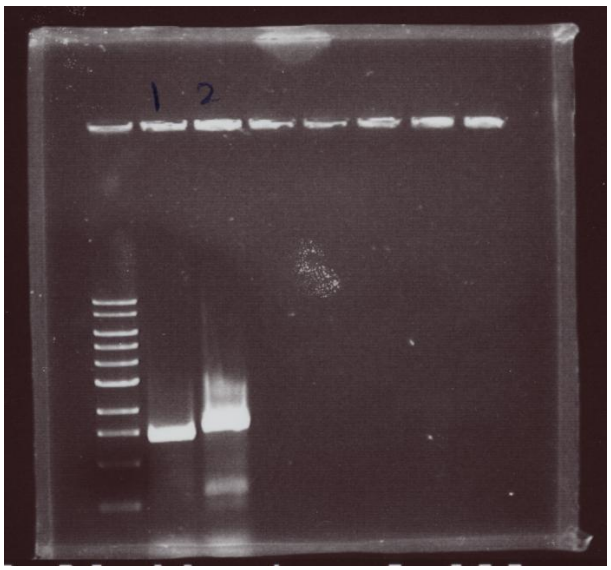


Figure 4.5: Gel picture showing the results of colony PCR using transformed and an untransformed *Citrobacter freundii* SBS197 using *cenA* and *cex* primers. Lane 1 – 1 kb DNA ladder, lane 2 – *cenA* from transformed *C. freundii* SBS197, lane 3 – *cex* from transformed *C. freundii* SBS197, lane 4 – *cenA* from untransformed *C. freundii* SBS197, lane 5 – *cex* from untransformed *C. freundii* SBS197. Size of *cenA* = 1.5 kb, *cex* = 1.5 kb.

Once P_{spac}-*lacI-cenA-cex* BioBrick was constructed in pSB1A2, it was transferred to pTG262 for expression in *B. subtilis* 168. P_{spac}-*lacI-cenA-cex* BioBrick and plasmid pTG262 were separately digested using *EcoRI* and *SpeI*. The digests were separated by agarose gel electrophoresis followed by the purification of desired fragments from the gel and ligation using T4 DNA ligase. Once ligated they were used to transform *B. subtilis* 168 (Figure 4.6). The presence of plasmid in recombinant *B. subtilis* was confirmed by colony PCR using *cenA/cex* primers to detect the presence of *cenA* and *cex* in the transformed cells (Figure 4.7). This hence confirms the presence of plasmid in recombinant *B. subtilis*.

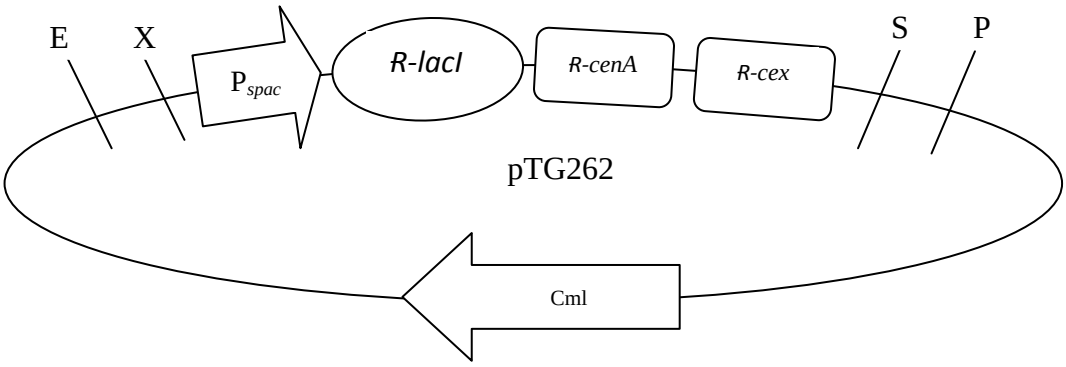


Figure 4.6: Diagram of pTG262 having *cenA* and *cex* downstream to P_{spac}-*lacI*. E – EcoRI, X – XbaI, S – SpeI, P – PstI, P_{spac} – Hybrid promoter made using phage spo-1 promoter element and the *lac* operator, R-*lacI* – *lac* repressor (repressor of *lac* operon) with ribosome bonding site, R-*cenA* – Endoglucanase (CenA) with ribosome binding site, R-*cex* – Exoglucanase (Cex) with ribosome binding site, Cml – Chloramphenicol

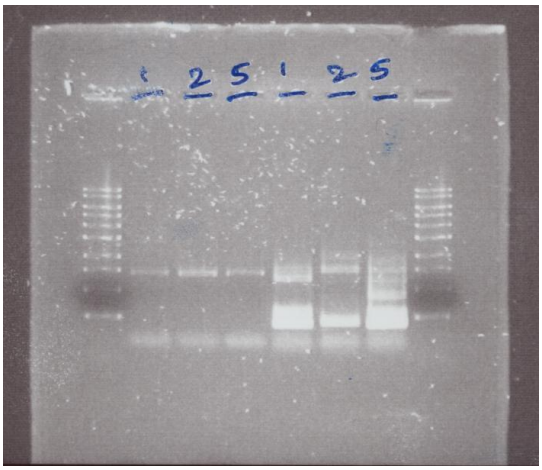


Figure 4.7: Gel picture showing the results of colony PCR using transformed *Bacillus subtilis* 168 using *cenA* and *cex* primers. Lane 1 – 1 kb DNA ladder, lane 3 – *cenA* from transformed *B. subtilis* 168, lane 6 – *cex* from transformed *B. subtilis* 168. Size of *cenA* = 1.5 kb, *cex* = 1.5 kb.

4.2.2 Expression of *xylE* (reporter gene) to check the utility of P_{spac}:

In order to check the functionality of P_{spac}, the reporter gene *xylE* was inserted downstream of P_{spac}-*lacI* BioBrick. XylE converts catechol to 2-hydroxy-*cis*-*cis*-muconic semialdehyde and hence a yellow colour is developed due to this reaction. The cultures were grown on LB agar plate containing 100 µg/ml ampicillin and 90 µg/ml IPTG. The functionality of promoter was determined (Section 2.13.3) by addition of few drops (3 – 5 µl) of 0.1 mM catechol on the colonies. The yellow colour of colonies after the addition of catechol showed that the promoter was functional and *xylE* was expressed resulting in the conversion of catechol to 2-hydroxy-*cis*-*cis*-muconic semialdehyde (Figure 4.8).

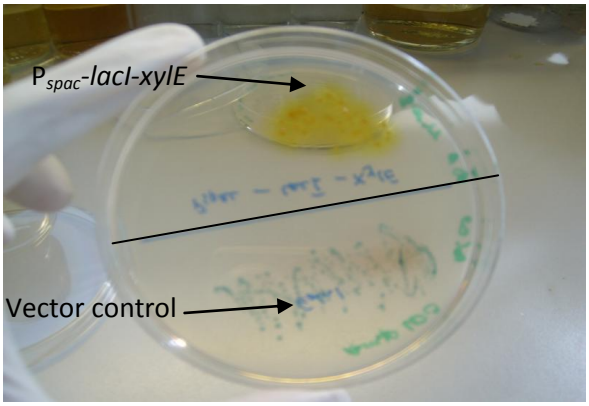


Figure 4.8: Picture showing yellow colour produced by *E. coli* JM109 expressing *xylE* from P_{spac}. The strains expressing *xylE* produced yellow colour upon addition of catechol. XylE when expressed from P_{spac} resulted in conversion of catechol to 2-hydroxy-*cis-cis*-muconic semialdehyde and hence a yellow colour was developed due to this reaction.

4.3 *cex* expression in *E. coli*:

To determine the expression of *cex*, a MUC assay was performed (Section 2.13.2). The cultures were grown on LB agar plates containing 100 µg/ml ampicillin plus 90 µg/ml IPTG. Methylumbelliferyl cellobioside (MUC) in the presence of exoglucanase is broken down into methylumbelliferone and cellobiose. Methylumbelliferone fluoresces under long wave length (λ=366 nm) ultra-violet light. Figure 4.9 shows the results from the qualitative MUC assay. The strains containing *cex* produced fluorescence when exposed to long wave UV light, whereas the strains lacking *cex* did not produce any fluorescence.

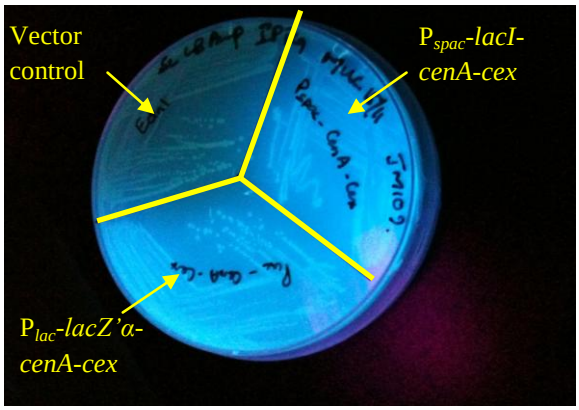
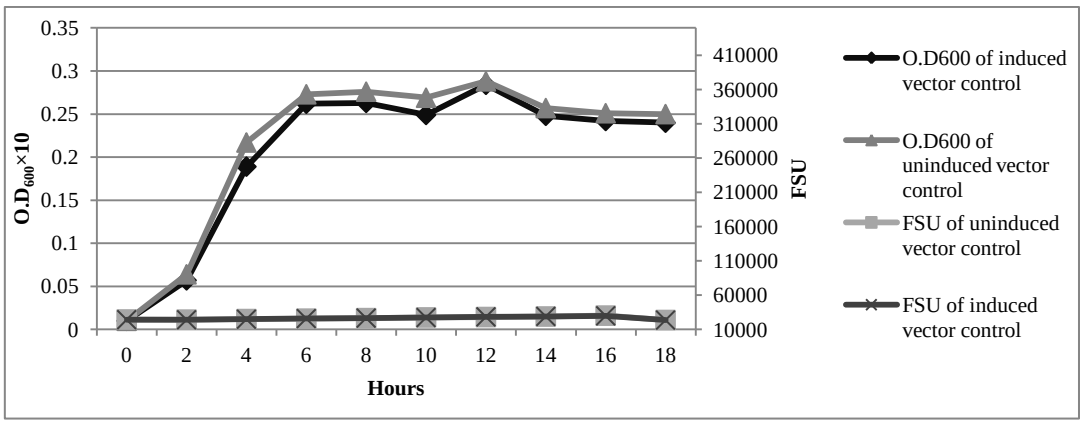


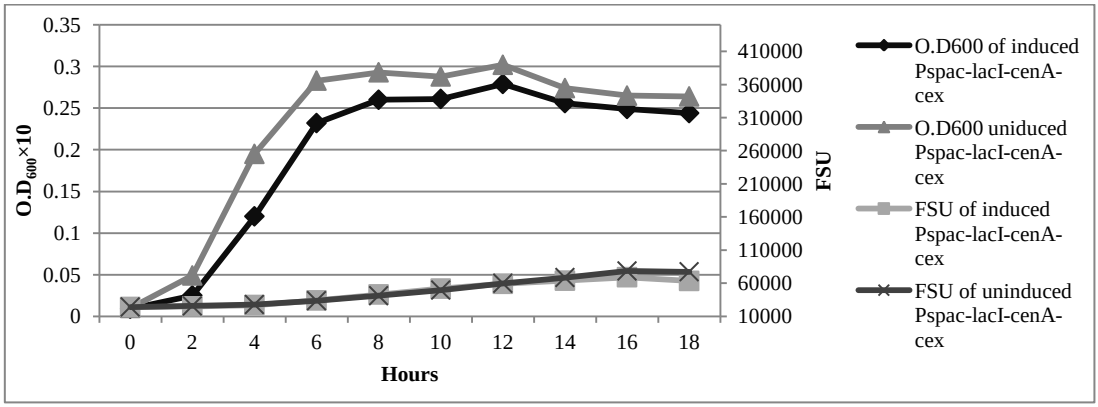
Figure 4.9: Picture showing fluorescence produced by *E. coli* JM109 expressing CEX. The strains expressing Cex produce fluorescence when exposed to long wave (λ=366 nm) ultra-violet light due to the breakdown of Methylumbelliferyl cellobioside into methylumbelliferone (a fluorescent product) and cellobiose, whereas the vector control did not produce any fluorescence.

In addition, *cex* expression was also checked in liquid cultures of *E. coli* along with the measurement of growth. An overnight culture was inoculated into 50 ml fresh LB medium (such that the final $O.D_{600}$ in the flasks becomes 0.1) along with 50 µl (0.05 mM) of 5 mg/ml MUC. One set of flasks was induced with IPTG (90 µg/ml). The $O.D_{600}$ and FSU was measured every two hours for up to 18 hours after which the experiment was stopped as the cultures have reached the stationary phase and no further increase in FSU was observed. Results are shown in Figures 4.10 and 4.11. In the vector control (induced and uninduced) there is hardly any increase in fluorescence (1.2 fold, about 5600-5660 FSU) with growth of the culture over time. In cultures with the *spac* promoter, the increase in fluorescence was about 3 fold (about 45000 and 54390 FSU in induced and uninduced cultures respectively) showing that induction doesn't really affect the expression in the case of P_{spac}. This could be due to the positioning of *lacI* (lac repressor) downstream of the inducible promoter which might have an effect on induction efficiency. However in cultures with the *lac* promoter, the increase in fluorescence with the growth of culture is about 16.5 fold (374180 FSU) in induced cultures and only 4 fold (82380 FSU) in uninduced cultures, indicating that the expression of the gene is strongly affected by induction. It is also observed (Figure 4.10) that because the induction increases enzyme production in P_{lac} cultures, it also has some inhibitory effect on growth of the culture. Hence, the maximum growth attained by induced P_{lac} cultures when measured by optical density at 600 nm was about 1.77, whereas in all the other cultures it was nearly 3.00.

A



B



C

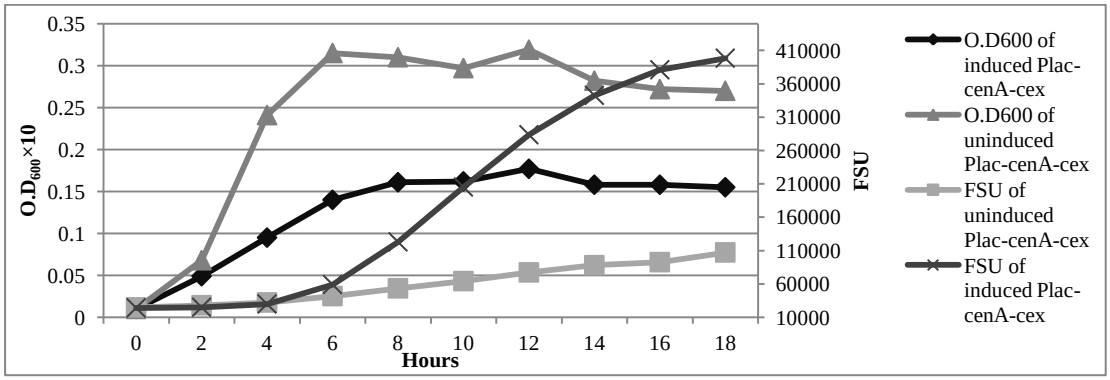


Figure 4.10: Graphs showing change in fluorescence produced with the growth by different strains of recombinant *E. coli* JM109.

A: vector control, B: P_{spac}-*lacI-cenA-cex*, C: P_{lac}-*cenA-cex*, $O.D_{600}$×10 – culture diluted 10 fold before measuring the optical density

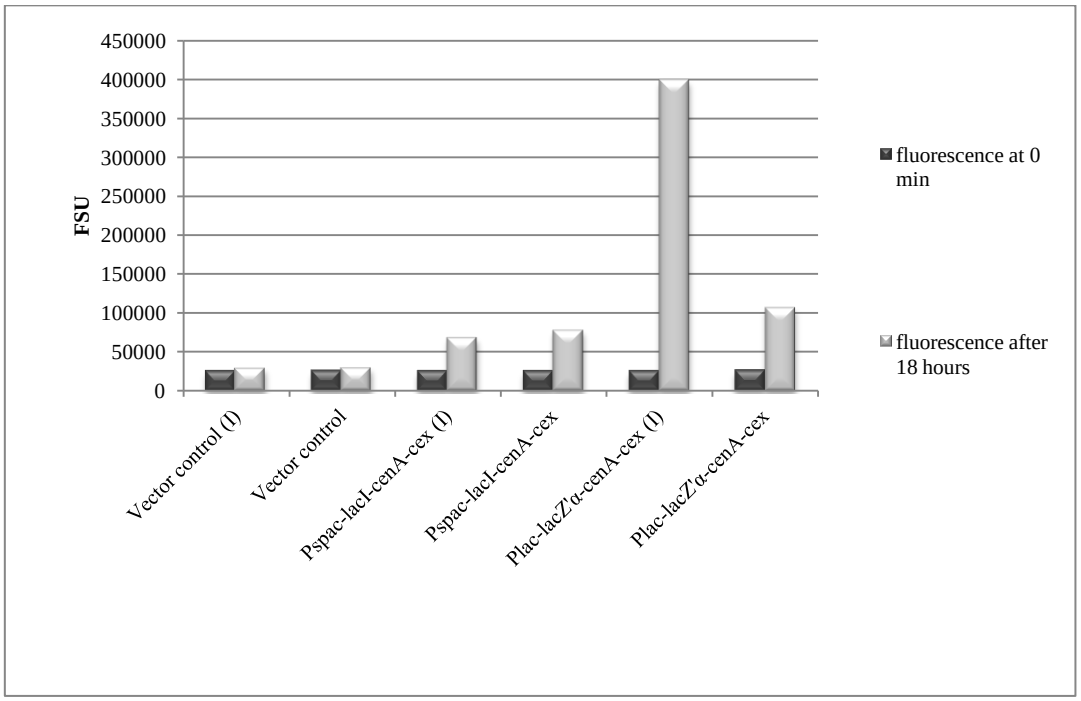


Figure 4.11: Graph showing fluorescence produced by different strains (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of recombinant *E. coli* JM109 at the start of the experiment and after 18 hours, (I): Induced with (90 µg/ml) IPTG

4.3.1 Quantitative determination of exoglucanase activity:

For quantitative analysis of *cex* activity (Section 2.13.5), cultures were grown in LB (induced with 90 µg/ml IPTG) and samples (1 ml) were collected during mid-exponential phase (4 hrs), at the end of exponential phase (8 hrs) and then after 24 hours. Cultures were centrifuged, supernatant collected and the cells were resuspended in PBS (1 ml). MUC {5 µl (0.05 mM) of 5 mg/ml (10 mM)} was then added to the supernatant as well as cell suspension and fluorescence was measured every 15 min using a UV filter for about 90 min. A calibration curve was prepared using different concentrations of 4-MU in LB as well as PBS and was then used to determine the number of moles of product formed per minute per ml under our experimental conditions. Figure 4.12 shows the number of moles of product (4-MU) formed per minute per ml.

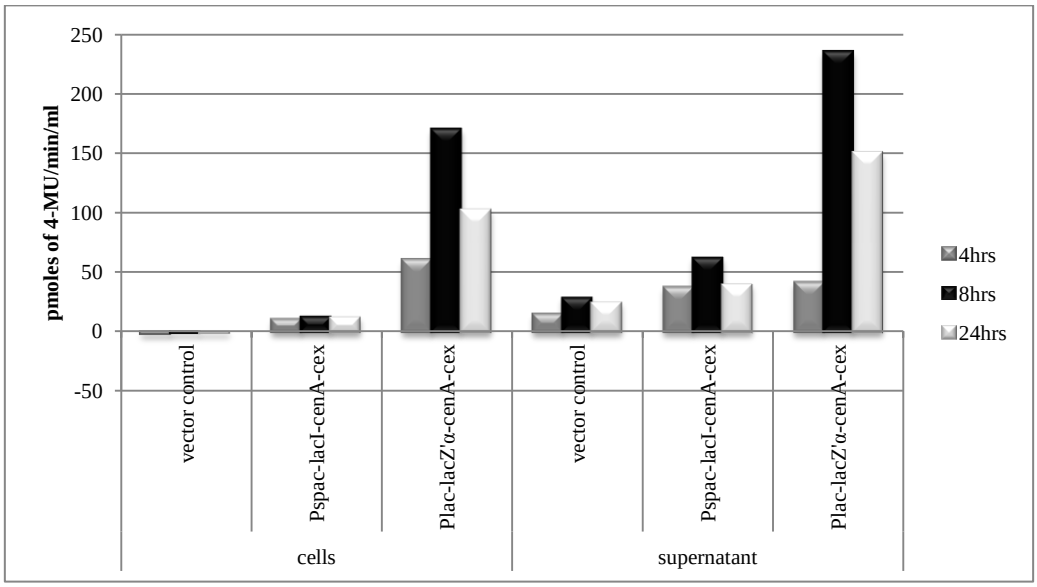


Figure 4.12: Graph showing number of moles of 4-MU formed/min/ml by different cultures (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of *E. coli* JM109 at different stages of growth when cells and culture supernatant were used as a source of enzyme (Cex).

The result shows that P_{lac} is a better promoter than P_{spac} for expression of genes in *E. coli* JM109 and the amount of product (4-MU) formed is highest at the end of exponential phase (8 hrs). Using cells as a source of enzyme, about 170 pmoles of product is formed per min per ml when P_{lac} was used compared to 13 pmoles/min/ml when P_{spac} was used showing about 13 fold difference in activity when the two different promoters were used. Whereas when supernatant was used as a source of enzyme, about 235 pmoles of 4-MU is formed/min/ml compared to 62 pmoles/min/ml when P_{lac} and P_{spac} were used respectively. This shows about 3.8 fold difference in product formation. The lower expression and hence the low product formation in case where P_{spac} was used could be due to the positioning of *lacI* downstream of P_{spac}, resulting in induction of repressor every time when the promoter was induced. In addition, because the cultures were grown in LB there was some background fluorescence (due to the yellow colour of LB medium itself) observed where supernatant was used as an enzyme source. Therefore it would be better to repeat the experiment using M9 in order to reduce the background fluorescence.

4.3.2 Cex assay in M9:

In order to decrease the effect of background fluorescence (caused by the yellow colour of LB medium) the assay (Section 2.13.5) was then performed in 50 ml M9 with 0.1% yeast extract and 31 mM glucose. The culture was grown overnight and then diluted to an extent that the final $O.D_{600}$ in the flasks becomes 0.1. Samples (1 ml) were then collected after 8 and 24 hours of growth. Cultures were centrifuged, supernatant collected and cells resuspended in the same M9 medium. MUC {5 µl (0.05 mM) of 5 mg/ml (10 mM)} was then added to the supernatant as well as cell suspension, and fluorescence was measured using a UV filter every 15 min for 45 min. Cex activity was determined using a calibration curve which was prepared using different concentrations of 4-MU in M9 medium with 0.1% yeast extract and 31 mM glucose. The experiment was performed in triplicate and results are presented as the average of three individual readings. Figures 4.13 and 4.14 shows the number of moles of 4-MU formed/min/ml under our experimental conditions.

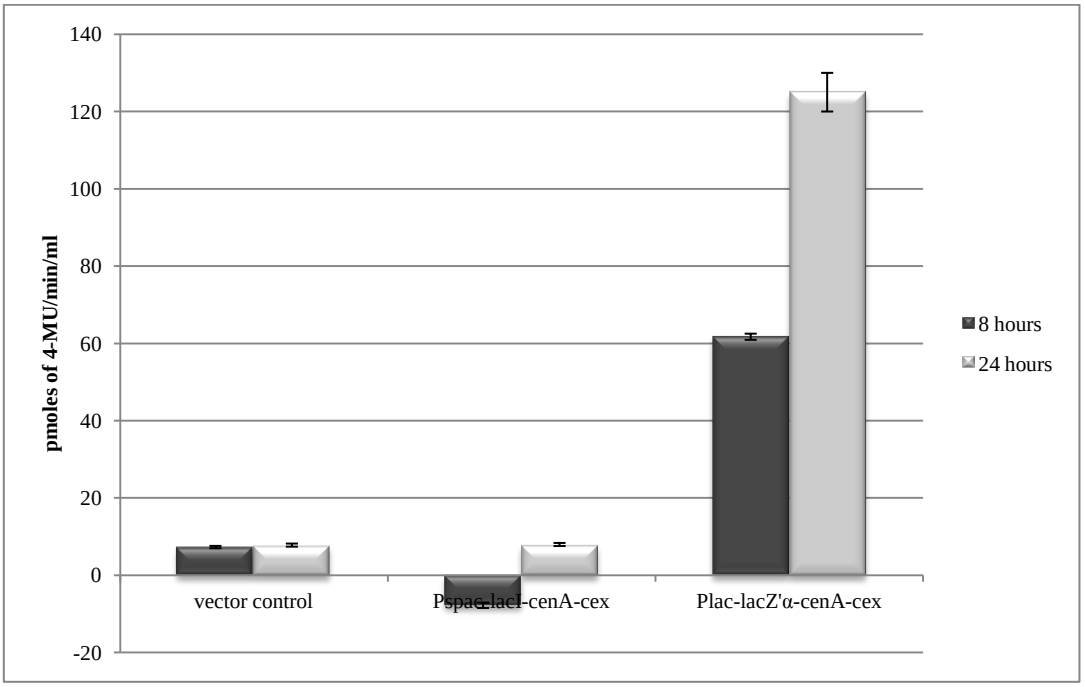


Figure 4.13: Graph showing number of moles of 4-MU formed/min/ml by different cultures (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of *E. coli* JM109 at different stages of growth when cells were used as a source of enzyme (Cex) (error bars indicate standard error).

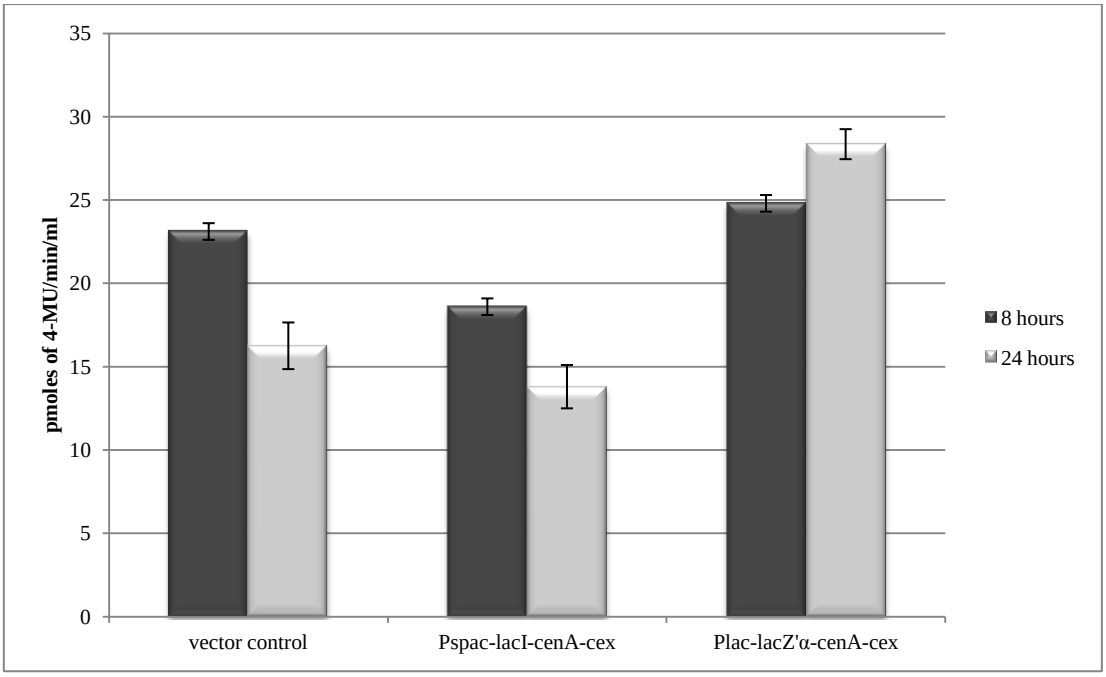


Figure 4.14: Graph showing number of moles of 4-MU formed/min/ml by different cultures (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of *E. coli* JM109 at different stages of growth when culture supernatant was used as a source of enzyme (Cex) (error bars indicate standard error).

The result shows that P_{lac} gives better activity than P_{spac} when *E. coli* JM109 is used for expression of genes using M9 medium. Again this could be due to the positioning of *lacI* downstream of P_{spac}. Similar results were observed when LB medium was used for the growth of cells. However in LB there was a small amount of activity observed with the *spac* promoter as well, whereas in M9 the activity of cultures having the *spac* promoter is near to that of the vector control, as seen in Figures 4.13 and 4.14. It was also observed that the number of moles of product formed is higher after 24 hours of growth, which was in contrast to what was observed when LB was used for growth, where the product formation was highest after 8 hours of growth and decreased slightly after 24 hours. This could be due to slow growth rate in M9 medium compared to LB medium as M9 is a minimal medium. When the activity was compared in supernatant and cells at 24 hours, it was seen that the activity was best in cells compared to supernatant. The amount of product formed in terms of pmoles/min/ml was 28 in supernatant of recombinant *E. coli* JM109 having P_{lac}-*cenA-cex* which was only about 1.8 fold higher compared to the vector control where the activity was 16 pmoles/min/ml. In contrast, pmoles of 4-MU formed/min/ml when cells having P_{lac}-*cenA-cex* were used as source of enzyme was 125 compared to 8 in

vector control. This shows about 15.6 fold increase in enzyme activity. Hence it was concluded that most of the activity was residing within the cells when *E. coli* JM109 was used for expression of genes. The results are shown in Figure 4.15. In addition when the activity is compared in LB and M9 medium, the activity is higher when the culture is grown in LB medium. This could be due to the fact that LB is complex/enriched medium whereas M9 is minimal medium containing just mineral salts. However the fact that the activity was very high in supernatant when the culture was grown in LB (235 pmoles/min/ml compared to 28 pmoles/min/ml) was due to the background fluorescence caused by the yellow colour of LB medium.

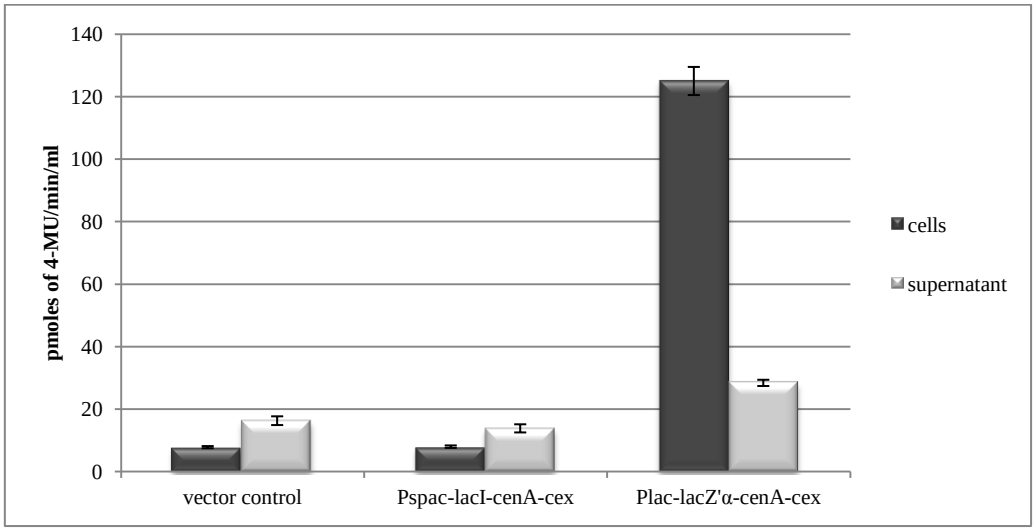


Figure 4.15: Comparison of enzyme activity in cells and supernatant of recombinant *E. coli* JM109 after 24 hours of growth (error bars indicate standard error).

4.4 c*enA* expression in *E. coli*:

In order to confirm if *cenA* was been transcribed and translated successfully in *E. coli*, Congo Red assay was performed (Section 2.13.1). The cultures were grown on LB agar plates containing 100 µg/ml ampicillin plus 90 µg/ml IPTG. The results are shown in Figure 4.16. The strains having *cenA* showed a zone of clearance around the colonies created by the hydrolysis of CMC into smaller fragments which were washed off during staining procedure, showing that *cenA* was successfully expressed by *E. coli* JM109. The negative control did not show any zone of clearance around the colonies.

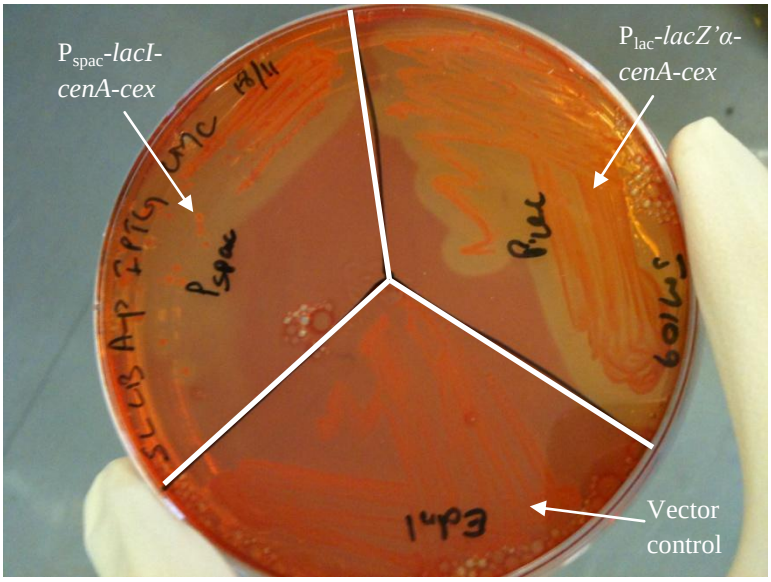


Figure 4.16: Picture showing halo formation (zone of clearance) around colonies produced by *E. coli* JM109 containing *cenA*. The strains expressing *cenA* produced a zone of clearance on LB-CMC plates after staining the plates with Congo Red followed by washing the stain with NaCl. As Congo Red only binds to long chain polysaccharides but not short chain which therefore are washed off during staining procedure resulting in halo formation.

4.4.1 Quantitative determination of endoglucanase activity:

For quantitative determination of endoglucanase activity (Section 2.13.6), recombinant *E. coli* JM109 were grown in M9 medium (with 0.1% yeast extract and 31 mM glucose) under induced conditions for 24 hours. Cells were then harvested and cell extract was prepared by using BugBuster® HT protein extraction reagent (Section 2.11.1). Endoglucanase assay to detect the activity of *cenA* in culture supernatant and cell extract was done using CM-cellulose dyed with Remazol Brilliant Blue (RBB). The culture supernatant and cell extract (150 µl) were mixed with 150 µl of substrate Azo-CMC and incubated. After suitable time intervals, the reaction was stopped by adding about 750 µl of 100% ethanol. The mixture was then centrifuged to remove precipitated polysaccharide, supernatant collected and the absorbance was measured at 590 nm. A calibration curve was prepared using different concentrations of RBB under the same assay conditions. The experiment was performed in duplicate and the results are presented as average of two individual sets of readings in Figure 4.17.

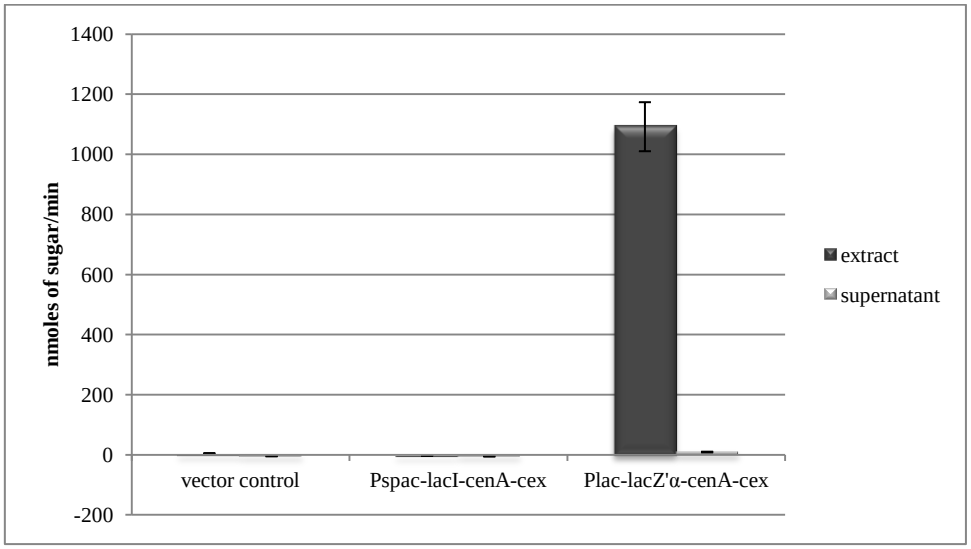


Figure 4.17: Graph showing nmoles of sugar residues released per min by recombinant *E. coli* JM109 producing CenA when cell extract and culture supernatant were used as a source of enzyme (error bars indicate standard error).

The result shows no activity in culture supernatant and/or extract when the *spac* promoter was used. Again this could be due to the positioning of *lacI* downstream of *spac* promoter resulting in induction of repressor when the promoter was induced. However there was considerable activity detected in cultures with the *lac* promoter, but most of it was residing inside the cell and was not secreted into the surrounding medium. Again P_{lac} was found to be a better promoter for expression in *E. coli* JM109. When cell extracts were used as a source of enzyme, about 1092 nmoles of sugar residues were released per min in cultures containing P_{lac}-*lacZ'α-cenA-cex*, about 273 fold higher compared to the vector control. However when culture supernatant was used, only 9 nmoles of sugar residues were released per min, again confirming that most of the activity resides inside the cell when *E. coli* JM109 is used as an expression host.

4.5 Determination of specific activity of Cex produced by *C. freundii* SBS197 and NCIMB11490 strains:

To determine the specific activity of Cex (Section 2.13.4), cell extracts of *C. freundii* SBS197 and NCIMB11490 were prepared. Overnight culture (such that the $O.D_{600}$ in the final flasks becomes 0.1) was inoculated into 50 ml fresh LB medium with one set of flasks being induced with 90 µg/ml IPTG and the other uninduced. The flasks were incubated at 37°C for 4 hours after which the cells were harvested and cell extract was prepared using BugBuster® HT protein extraction reagent (Section 2.11.1). The specific activity of Cex was then determined using ONPC. Cell extract (0.4 ml) was mixed with 4.4 ml of Lac Z buffer and 0.160 ml of 4 mg/ml ONPC and incubated at 35°C. Samples (0.1 ml) were collected at various time intervals and mixed with 0.870 ml of Lac Z buffer and 0.030 ml of 1 M Na_2CO_3 to stop the reaction. A_{420} was then measured. The protein concentration of the extract was determined using the Bradford protein determination kit (Section 2.11.2). Specific activity (defined as the amount of substrate the enzyme converts, per mg of protein in the enzyme preparations, per unit time) of Cex in each sample was then determined. Table 4.1 shows the specific activity of Cex in the cell extracts of different strains of *C. freundii*.

Table 4.1: Specific activity of Cex produced by different strains (vector control and P_{spac}-*lacI-cenA-cex*) of recombinant *C. freundii*

Specific activity of Cex (mU/mg) produced by *C. freundii*		
Type/condition	**SBS197**	**NCIMB**
Vector control (induced)	0.016	0.061
Vector control (uninduced)	0.017	0.045
P_{spac}-*lacI-cenA-cex* (induced)	0.070	0.078
P_{spac}-*lacI-cenA-cex* (uninduced)	0.037	0.049

The tubes with cell extracts of the SBSB197 strain showed yellow colour due to the release of *O*-nitrophenol, whereas the tubes with cell extracts from the NCIMB11490 strain did not turn yellow at all. These tubes rather became turbid (for some unknown reason as the cell debris was removed by centrifuging at the end) over time due to which the A_{420} for them was higher and hence the apparent specific activity became higher. Hence the results showed that SBS197 is a better strain than NCIMB11490 for Cex expression. Also there is about 2-4 fold increase in the specific activity in SBS197 strain having P_{spac}-*lacI-cenA-cex* compared to vector control under uninduced and induced conditions whereas the specific activity in NCIMB11490 strains was nearly the same as the vector control.

In addition there was a major difference in the transformation efficiency of these two strains using chemical (heat shock) method of transformation. With NCIMB11490 strain the transformation efficiency was really high (giving 100s of transformants), whereas the transformation efficiency of SBS197 was really low (only 1-10 transformants) using heat shock method. On the other hand although the transformation efficiency of SBS197 was really low, it was able to retain the plasmid for long period of time compared to NCIMB11490 as observed by less growth of NCIMB11490 strain on antibiotic plates over time. Hence it was therefore decided that SBS197 would be used for all the future experiments.

Since we got SBS197 from the teaching lab of the School of Biological Sciences, University of Edinburgh, we thought it was essential to do the 16S RNA sequencing in order to confirm the identity of the organism. Colony PCR was done to amplify the 16S rRNA gene, which was then sent for sequencing. After the arrival of the sequence, BLAST (Basic Local Alignment Search Tool) was done using NCBI nucleotide blast program. The results are presented in Table 4.2. All of the strongest matches to the database were to strains of *C. freundii*. Hence it confirmed the identity of the bacterium that we were using was *C. freundii*.

Table 4.2: Results showing the matches of the BLAST search of 16S RNA sequence of *C. freundii* SBS197

Organism	Strain	Similarity
Citrobacter	F3Boct.2	98%
Citrobacter freundii	BRN1	98%
Citrobacter freundii	JCM 24064	98%
Citrobacter freundii	JCM 24062	98%
Citrobacter freundii	MS 8	98%
Citrobacter	F3feb.27	98%
Citrobacter	F3feb.26	98%
Citrobacter	F2apr.41	98%
Citrobacter	F2apr.40	98%
Citrobacter	F2apr.36	98%

4.6 *cex* expression in *C. freundii* SBS197:

For qualitative exoglucanase tests an MUC assay was performed (Section 2.13.2). The cultures were grown on LB agar plates containing 80 µg/ml carbenicillin plus 90 µg/ml IPTG. Figure 4.18 shows the results from the MUC assay. The strains containing *cex* produced fluorescence when exposed to long wave UV light, whereas the strains lacking *cex* did not produce any fluorescence.

Expression of *cex* was also determined in liquid culture (in LB with and without (90 µg/ml) IPTG) of *C. freundii* SBS197. Overnight culture was inoculated into 50 ml fresh LB medium (such that the final $O.D_{600}$ becomes 0.1) along with (0.05 mM) of 5 mg/ml MUC. The $O.D_{600}$ and FSU was measured every two hours for up to 18 hours after which the cultures were in late stationary phase and the experiment was not run any further. The results (Figures 4.19 and 4.20) show that the cultures with P_{spac} produce less fluorescence compared to cultures with P_{lac} suggesting that P_{lac} is a better promoter in *C. freundii* SBS197. The increase in fluorescence with time is about 20-25 fold (381250 and 432150 FSU in uninduced and induced cultures respectively) in the case of P_{lac}, whereas it is about 15-16 fold (263140 and 291410 FSU in uninduced and induced cultures respectively) in the case of P_{spac} cultures. However there is a small amount of background fluorescence in the case of *C. freundii* SBS197, as the vector control also shows an increase in fluorescence with time (about 7-10 fold, 114500 and 162410 FSU). Also induction with IPTG does not have much effect on expression of the gene in the case of *C. freundii* SBS197 as there in not much difference in the fluorescence observed between induced and uninduced cultures in either P_{spac} and/or P_{lac} cultures.

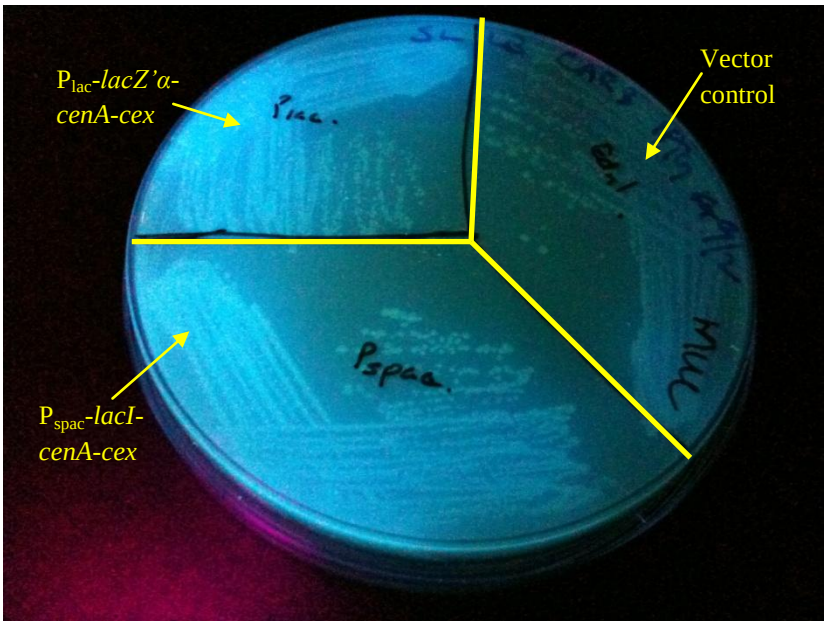


Figure 4.18: Picture showing fluorescence produced by *C. freundii* SBS197 expressing Cex. The strains expressing Cex produce fluorescence when exposed to long wave (λ=366 nm) ultra-violet light due to the breakdown of Methylumbelliferyl cellobioside into methylumbelliferone (a fluorescent product) and cellobiose, whereas the vector control did not produce any fluorescence.

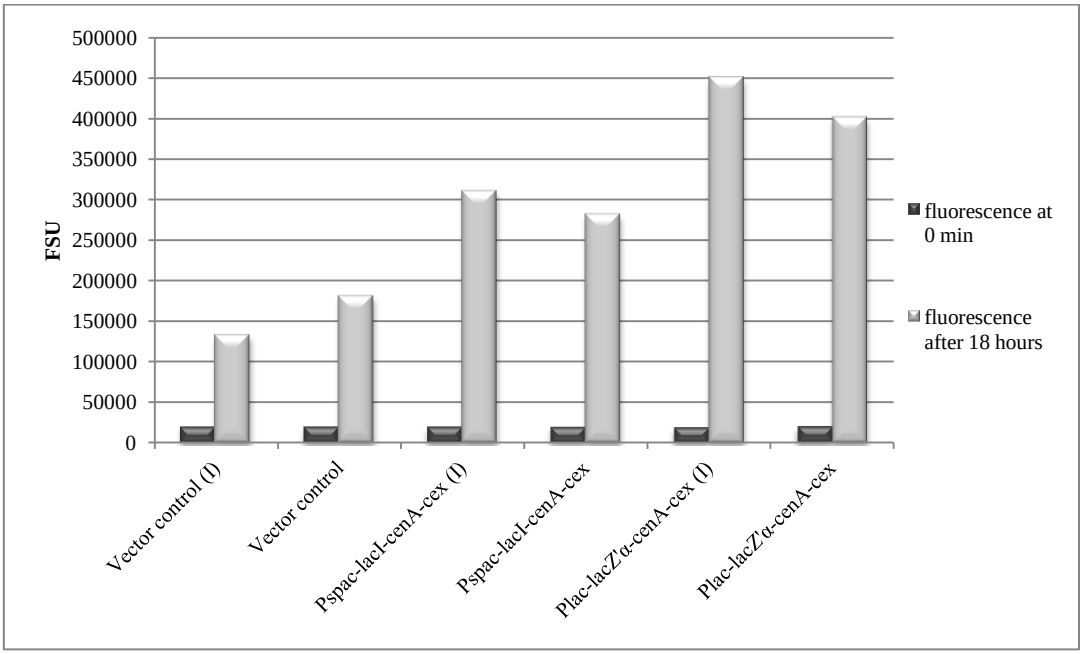
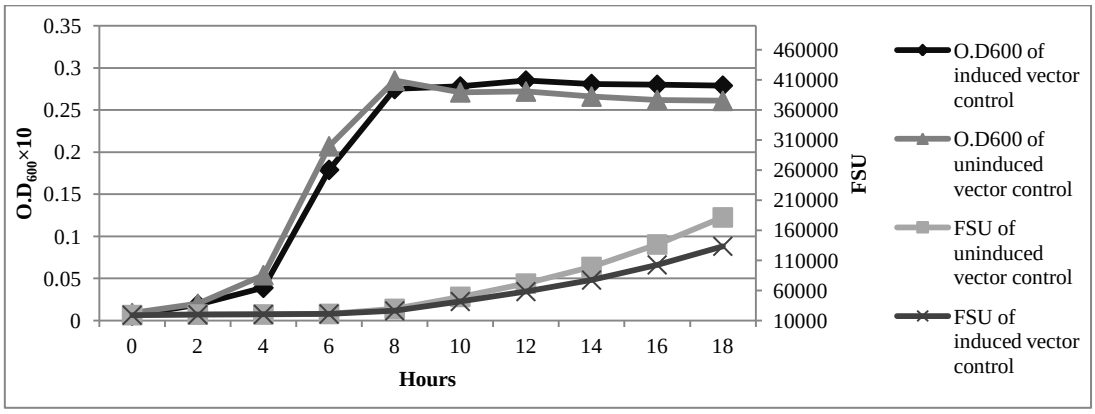
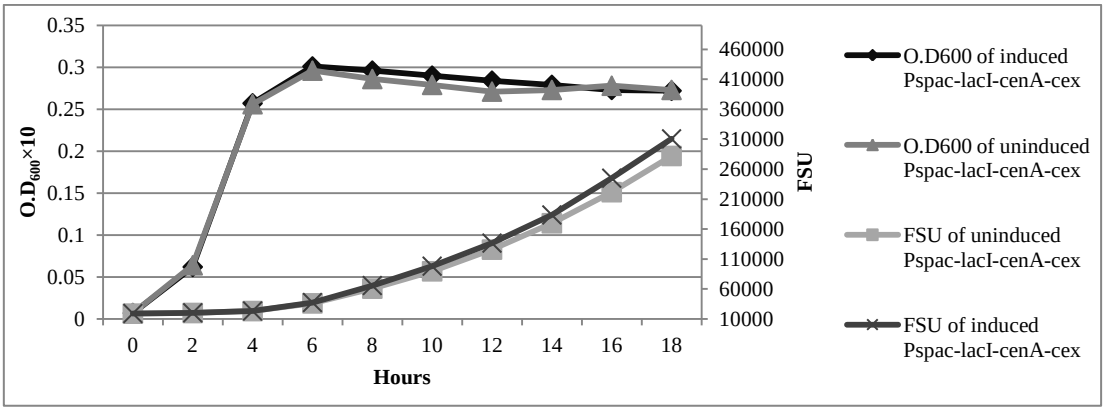


Figure 4.19: Graph showing fluorescence produced by different cultures (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of *C. freundii* SBS197 at the start of the experiment and after 18 hours.

A



B



C

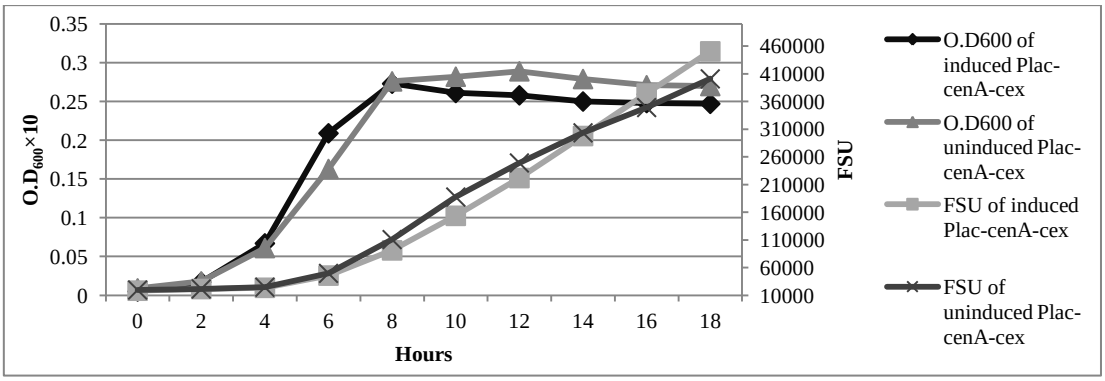


Figure 4.20: Graphs showing change in fluorescence with the growth of different strains (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of *C. freundii* SBS197.

A: vector control, B: P_{spac}-*lacI-cenA-cex*, C: P_{lac}-*cenA-cex*, $O.D_{600}$×10 – culture diluted 10 fold before measuring the optical density

4.6.1 Quantitative determination of exoglucanase activity:

For quantitative determination of Cex activity (Section 2.13.5), *C. freundii* SBS197 cultures were grown in LB (induced with 90 µg/ml IPTG). Samples (1 ml) were collected during mid-exponential phase (4 hrs), at the end of exponential phase (8 hrs) and then after 24 hours. Cultures were centrifuged, supernatant collected and the cells were resuspended in 1 ml PBS. MUC {5 µl (0.05 mM) of 5 mg/ml (10 mM)} was then added to the supernatant and to the cell suspension and fluorescence was measured every 15 min for 90 min using the UV filter. Activity was determined by using a calibration curve prepared using different concentrations of 4-MU in LB as well as PBS. Figures 4.21 and 4.22 shows the number of moles of product (4-MU) formed per minute per ml under our experimental conditions.

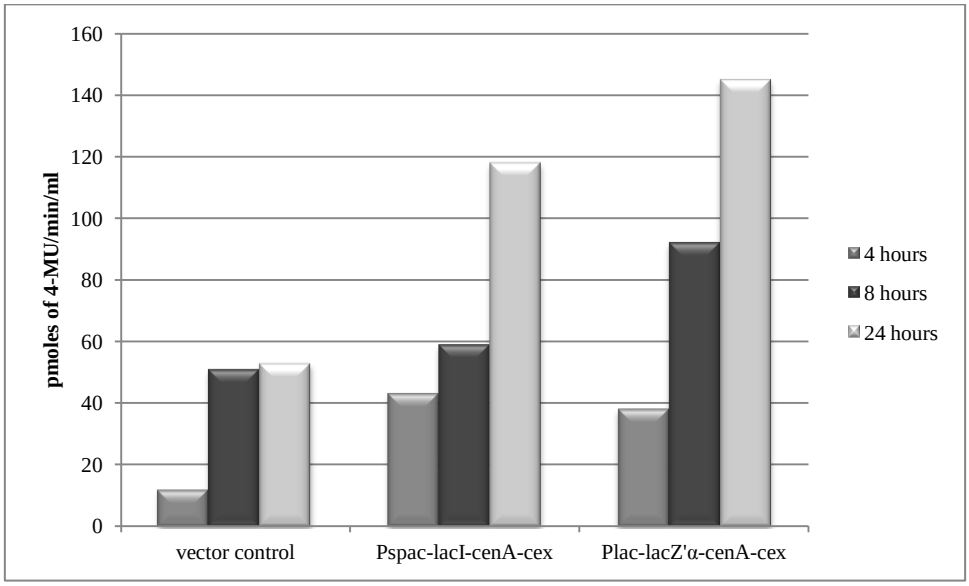


Figure 4.21: Graph showing number of moles of 4-MU formed/min/ml by different cultures (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of *C. freundii* SBS197 at different stages of growth when cells were used as a source of enzyme (Cex).

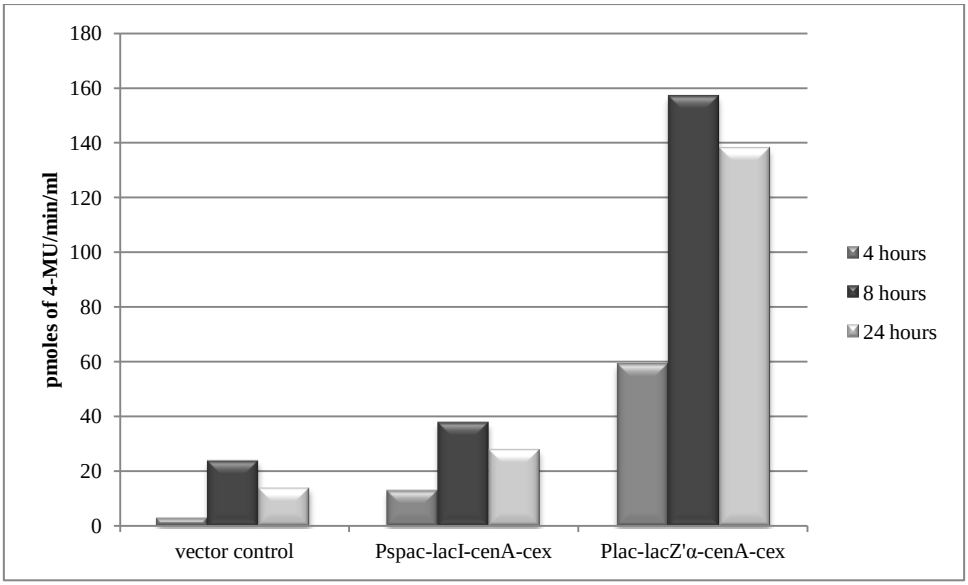


Figure 4.22: Graph showing number of moles of 4-MU formed/min/ml by different cultures (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of *C. freundii* SBS197 at different stages of growth when culture supernatant was used as a source of enzyme (Cex).

In the case where cells were used as a source of enzyme, the highest activity (in terms of the amount of product formed/min/ml) was obtained after 24 hours whereas when supernatant was used as a source of enzyme, the highest activity was seen at the end of exponential phase and the activity decreased slightly after 24 hours. P_{lac} was found to be a better promoter than P_{spac} for expression of the gene in *C. freundii* SBS197 grown in LB medium as evident from the number of moles of product formed per min per ml. When cells were used as a source of enzyme, 145 pmoles of 4-MU/min/ml were formed in the case of P_{lac} compared to 118 pmoles of 4-MU/min/ml when expression was controlled by P_{spac} in the samples collected after 24 hours of growth. Similarly, where supernatant was used as an enzyme source about 38 pmoles of product was formed per min per ml with 8 hours old culture when expression was controlled by P_{spac}, whereas about 157 pmoles of 4-MU were formed when P_{lac} was controlling the expression. There is a small amount of activity observed in the vector control as well in the case of *C. freundii* SBS197. However this activity was low compared to our constructs. In addition, where supernatant was used as an enzyme source, some background fluorescence (due to yellow colour of LB medium) was observed so it was decided to perform the experiment in M9 to reduce the background fluorescence due to the medium.

4.6.2 Cex assay in M9:

In order to reduce the background fluorescence (caused due to yellow colour of LB medium) observed in LB medium, the assay was performed after growing the organisms in 50 ml M9 medium containing 0.1% w/v yeast extract and 31 mM glucose. The overnight culture used as inoculum was diluted to an extent that the final $O.D_{600}$ in the flasks became 0.1. Samples (1 ml) were collected after 8 hours of growth and then again after 24 hours of growth. Cells were centrifuged, supernatant collected and cells resuspended in the same M9 medium used for growing the culture. MUC (5 µl of 5 mg/ml) was then added to give a working concentration of 0.05 mM. Fluorescence was measured every 15 min for 45 min using the UV filter. A calibration curve was prepared using different concentrations of 4-MU in the same medium used for growing cells and this was used to determine enzyme activity in test samples. Figures 4.23 and 4.24 show the pmoles of 4-MU formed/min/ml under our experimental conditions. The experiment was performed in triplicate and the results are presented as an average of three individual readings.

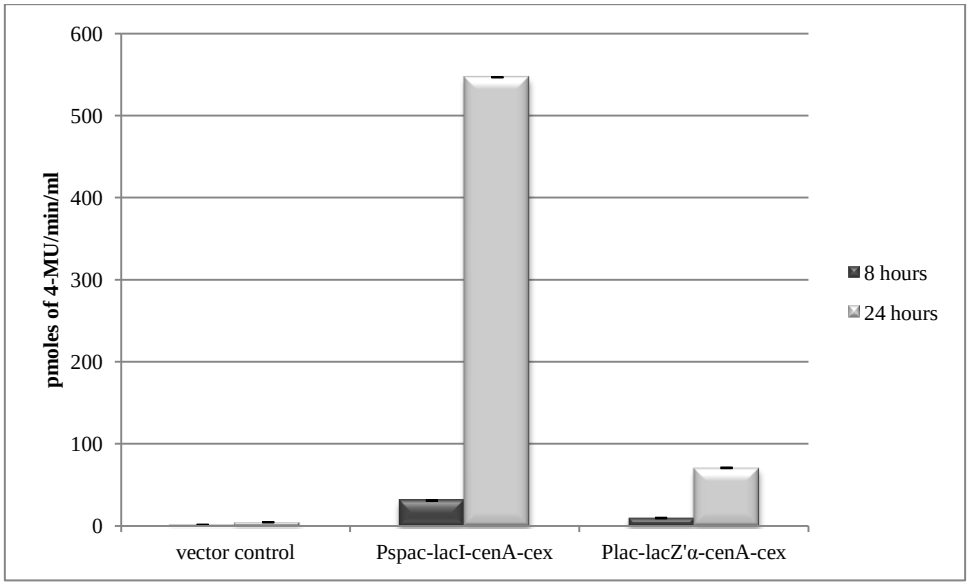


Figure 4.23: Graph showing number of moles of 4-MU formed/min/ml by different cultures (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of *C. freundii* SBS197 at different stages of growth when culture supernatant was used as a source of enzyme (Cex) (error bars indicate standard error).

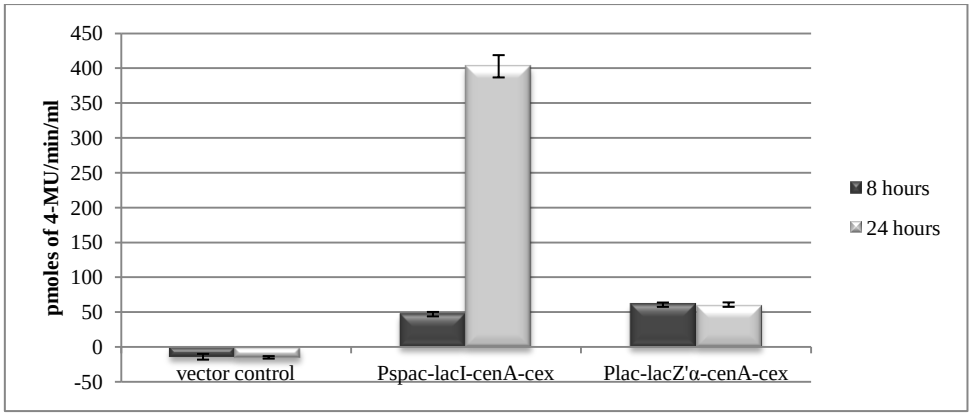


Figure 4.24: Graph showing number of moles of 4-MU formed/min/ml by different cultures (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of *C. freundii* SBS197 at different stages of growth when cells were used as a source of enzyme (Cex) (error bars indicate standard error).

It was observed from the results that the *spac* promoter gives much higher expression than the *lac* promoter when *C. freundii* SBS197 is grown in M9 medium. This was in contrast to what was observed when the cells were grown in LB medium where the *lac* promoter was performing slightly better than the *spac* promoter. When the *spac* promoter was used about 547 pmoles of 4-MU were formed per min per ml after 24 hours compared to 71 pmoles when the *lac* promoter was used and the source of enzyme was supernatant. This shows about 7.7 fold higher activity. Similarly when the source of enzyme was cells, about 403 and 61 pmoles of product were formed per min per ml by enzyme expressed by the *spac* and the *lac* promoters respectively. Again this is about 6.6 fold higher, indicating that P_{spac} is a better promoter for *cex* expression in *C. freundii* SBS197 when the cells are grown in minimal medium. The product formation was higher when the samples were collected after 24 hours of growth compared to 8 hours regardless of whether cells or supernatant was used as a source of enzyme. This was in contrast to what we found when the culture was grown in LB medium. Product formation was higher after 8 hours when supernatant was used as an enzyme source but decreased slightly when the samples were collected after 24 hours of growth (in case of supernatant only). In addition when activity was compared between cells and supernatant when the sample was collected after 24 hours of growth (as more activity was observed after 24 hours of growth), it was observed that activity was better when supernatant (P_{spac}-*lacI-cenA-cex* cultures) was used as a source of enzyme showing that a considerable amount of enzyme is secreted into the surrounding medium. The results are presented in Figure 4.25.

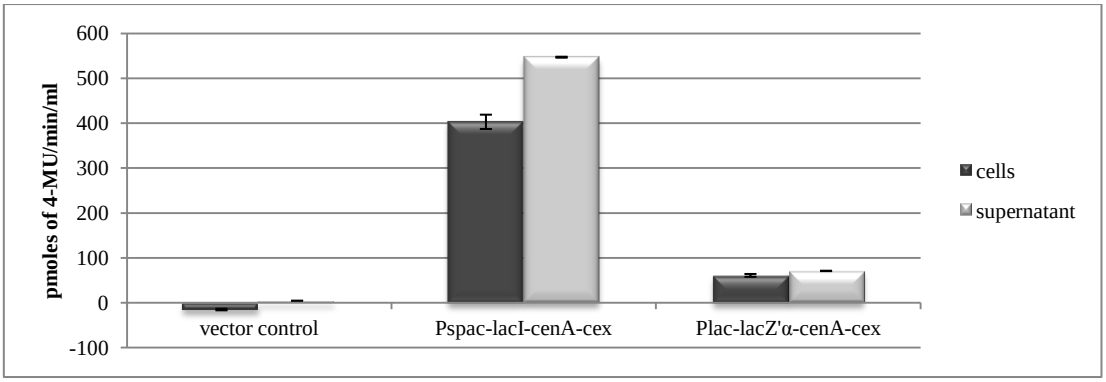


Figure 4.25: Comparison of enzyme activity in cells and supernatant of recombinant *C. freundii* SBS197 (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) after 24 hours of growth (error bars indicate standard error).

4.7 *cenA* expression in *C. freundii* SBS197:

In order to find out if *cenA* was been expressed successfully in *C. freundii* SBS197, Congo Red assay (Section 2.13.1) was performed. The cultures were grown on LB agar plates containing 80 µg/ml carbenicillin plus 90 µg/ml IPTG. The results are shown in Figure 4.26. The strains containing *cenA* showed a zone of clearance around the colonies created by hydrolysis of CMC showing that *cenA* was successfully transcribed and translated by *C. freundii* SBS197. The negative control did not show any zone of clearance around the colonies.

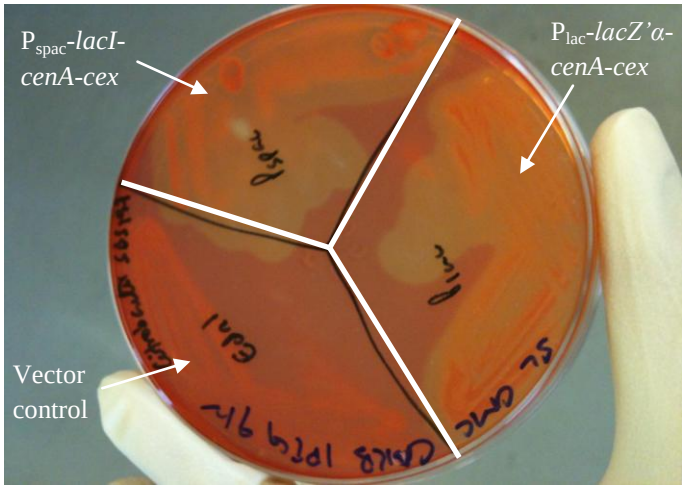


Figure 4.26: Picture showing halo formation (zone of clearance) around colonies produced by *C. freundii* SBS197 expressing CenA. The strains expressing *cenA* produced a zone of clearance on LB-CMC plates after staining the plates with Congo Red followed by washing the stain with NaCl. As Congo Red only binds to long chain polysaccharides but not short chain which therefore are washed off during staining procedure resulting in halo formation.

4.7.1 Quantitative determination of endoglucanase activity:

For quantitative measurement of CenA activity (Section 2.13.6) in *C. freundii* SBS197, the culture was grown in M9 (having 0.1% yeast extract and 31 mM glucose) under induced (90 µg/ml IPTG) conditions. After 24 hours, cell extract was prepared using BugBuster® HT protein extraction reagent (Section 2.11.1). The assay was done using CMC dyed with Remazol Brilliant Blue (RBB) by mixing 150 µl of culture supernatant/extract with 150 µl of Azo-CMC and incubating for certain time intervals after which the reaction was stopped by adding 750 µl of 100% ethanol. The mixture was centrifuged and absorbance of the supernatant was measured at 590 nm. A calibration curve was prepared using different concentrations of RBB under the same assay conditions and this was then used to determine the activity in the test samples. The experiment was performed in duplicate and the results are shown as the average of two individual sets of readings in Figure 4.27.

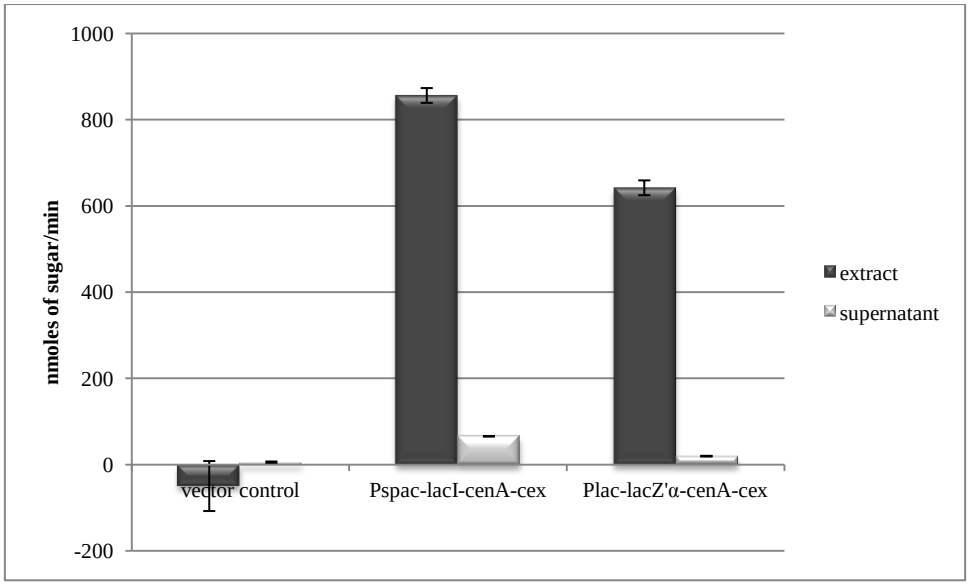


Figure 4.27: Graph showing number of moles of sugar released per unit time by different strains (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of recombinant *C. freundii* SBS197 using cell extract and supernatant as a source of enzyme (error bars indicate standard error).

The results show that *cenA* is expressed successfully under the control of both the *spac* and the *lac* promoters in *C. freundii* SBS197. However, activity was higher when the *spac* promoter was used than when the *lac* promoter was used. In addition measurable amount of activity is detected in culture supernatant in the case of *C. freundii* SBS197 and the secretion was higher when the *spac* promoter was used as shown in Figure 4.28. When the expression was controlled by the *spac* promoter, 66 nmoles of sugar molecules were released per min compared to 20 nmoles/min when *cenA* was expressed under the control of the *lac* promoter. Hence secretion is about 3.3 fold higher in cultures with the *spac* promoter than in cultures with the *lac* promoter. Similarly, higher activity was observed in the cell extracts of cultures with the *spac* promoter than with the *lac* promoter. When CenA was transcribed by P_{spac} 856 nmoles of sugar residues were released per min compared to 642 nmoles when it was transcribed by P_{lac}. This shows that although both *lac* and *spac* promoters are functional in *C. freundii* SBS197, expression as well as secretion is better when genes are under the control of the *spac* promoter than when they are under the control of the *lac* promoter.

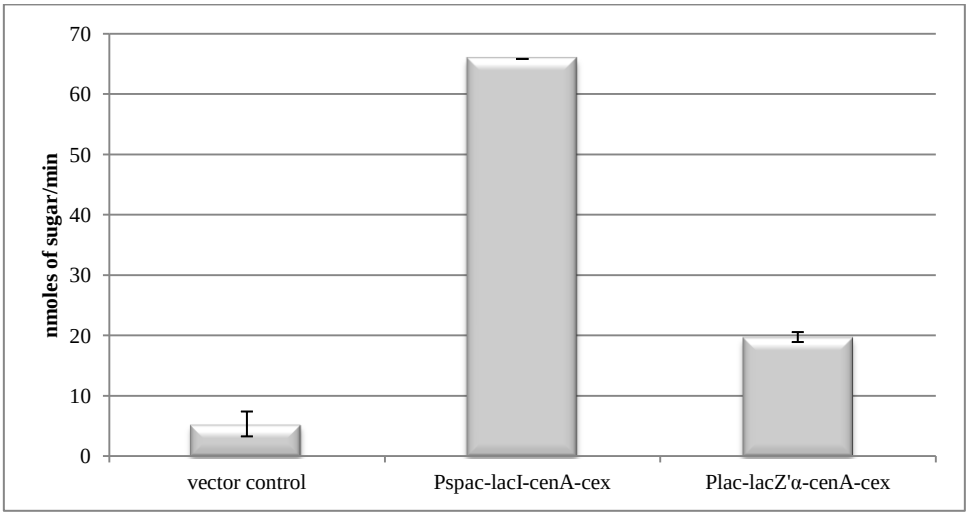


Figure 4.28: Comparison of secretion efficiency when *spac* and *lac* promoters were used for gene expression in *C. freundii* SBS197 (error bars indicate standard error)

4.8 *cex* expression in *B. subtilis*:

To determine the expression of *cex*, qualitative MUC assay (Section 2.13.2) was performed. The cultures were grown on LB agar plates containing 10 µg/ml chloramphenicol plus 90 µg/ml IPTG. The results from qualitative MUC assay showed that the strains containing *cex* produced fluorescence when exposed to long wave length (366 nm) UV light, as well as the strains lacking *cex* produced fluorescence.

4.9 *cenA* expression in *B. subtilis*:

In order to determine the expression of *cenA* in *B. subtilis*, Congo Red assay (Section 2.13.1) was performed. The cultures were grown on LB agar plates containing 10 µg/ml chloramphenicol plus 90 µg/ml IPTG. The results showed that the strain containing *cenA* as well as the control strain produced a zone of clearance around the colonies caused by the hydrolysis of CMC.

4.10 Discussion:

The above results showed that *C. freundii* SBS197 performs better than *E. coli* JM109 under our experimental conditions and when minimal medium was used. Both the *spac* and *lac* promoter worked well in *C. freundii* SBS197 although expression was higher in cultures with the *spac* promoter than in cultures with the *lac* promoter as shown in Figures 4.29 and 4.30, whereas in the case of *E. coli* JM109, activity was only detected when the genes were expressed under the control of the *lac* promoter. Enzyme activity was nearly the same as in the vector control when genes were under the control of the *spac* promoter, indicating that P_{spac} is not an effective promoter when *E. coli* JM109 is used as an expression host. Again this could be due to the positioning of *lacI* downstream of P_{spac}, which results in induction of repressor each time when the promoter was induced. Highest activity was observed when *E. coli* JM109 (containing P_{lac}-*lacZ'α-cenA-cex*) cell extracts were used to test the activity of CenA (Figure 4.30 and 4.31) showing that the expression is higher in *E. coli* JM109 compared to *C. freundii* SBS197, but the enzyme is not secreted into

the surrounding medium and most of it resides within the cell. In the case of *C. freundii* SBS197 extracts, although the activity is slightly less, a measurable amount of it is detected in the supernatant as well, showing that the protein is secreted well into the surrounding medium (Figure 4.31 and 4.32). Cellulose, being a large molecule, cannot enter the cells, and needs to be degraded outside the cell into smaller units which can then enter the cell, where it can be further metabolized. Therefore it is really important for at least endoglucanases (the enzymes that act first in cellulose degradation) to be released into the medium to act on cellulose. In *E. coli*, endoglucanase activity in the culture supernatant is very low, and almost the same as in the vector control (Figure 4.32), whereas in *C. freundii* SBS197 there is considerable activity detected in the culture supernatant especially of the cultures having *spac* promoter. Hence *C. freundii* SBS197 should be better in degradation of cellulosic materials compared to *E. coli* JM109.

The activity of CenA and Cex in recombinant *B. subtilis* 168 could not be determined as the control strain was also positive for the assays used. This is consistent with the fact that *B. subtilis* can degrade amorphous but not crystalline cellulose (Robson and Chambliss, 1984; Horikoshi *et. al.*, 1984).

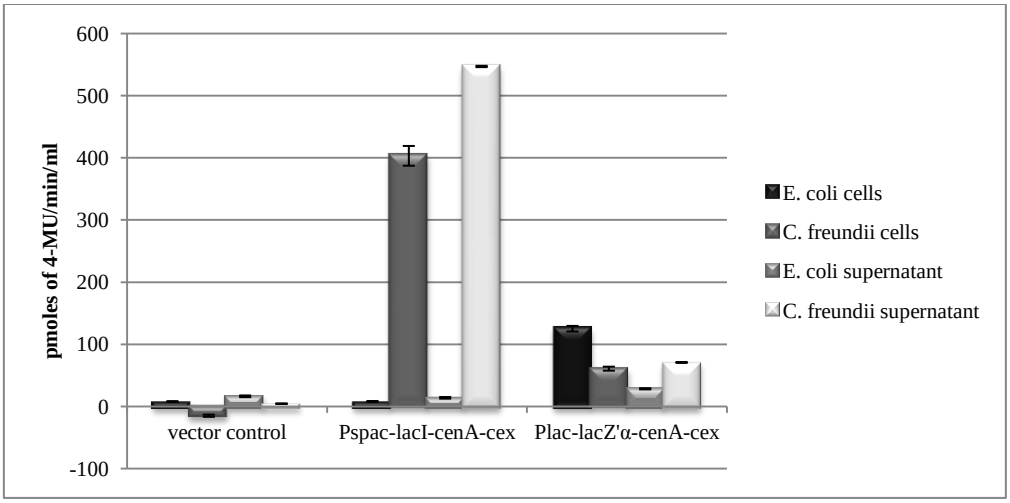


Figure 4.29: Comparison of the amount of 4-MU formed per min per ml by different strains (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of *E. coli* JM109 and *C. freundii* SBS197 during Cex assay when cells and supernatant were used as source of enzyme after 24 hours of growth (error bars indicate standard error).

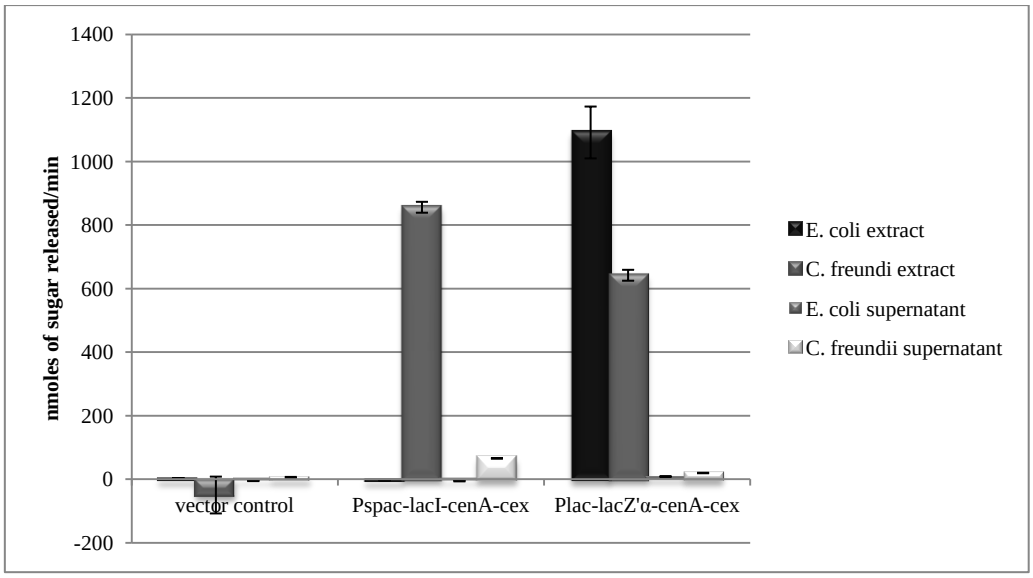


Figure 4.30: Comparison of the amount sugar residues released per min during CenA assay by different strains (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of *E. coli* JM109 and *C. freundii* SBS197 when cell extract and supernatant were used as source of enzyme (error bars indicate standard error).

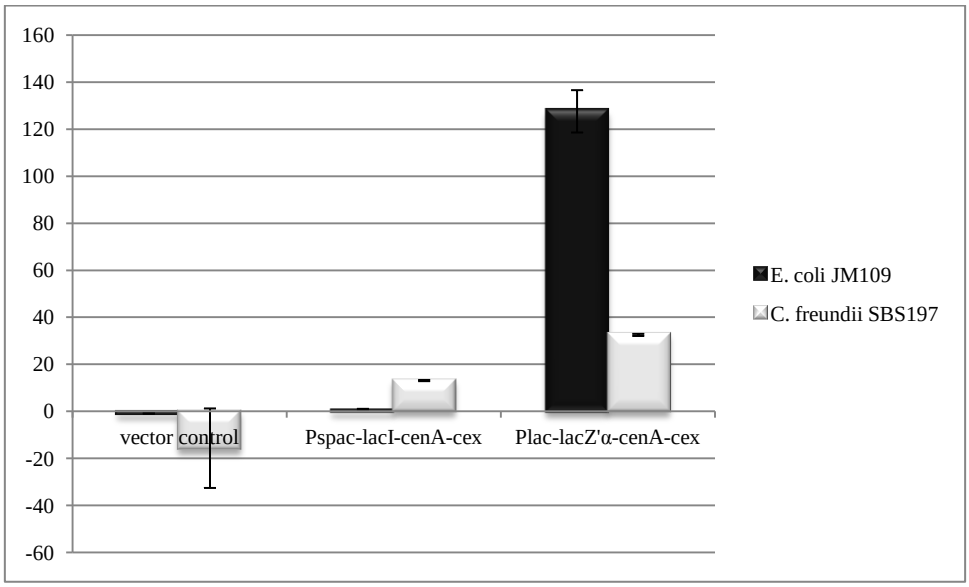


Figure 4.31: Ratio of cellular:supernatant activity of CenA in recombinant *E. coli* JM109 and *C. freundii* SBS197 (error bars indicate standard error).

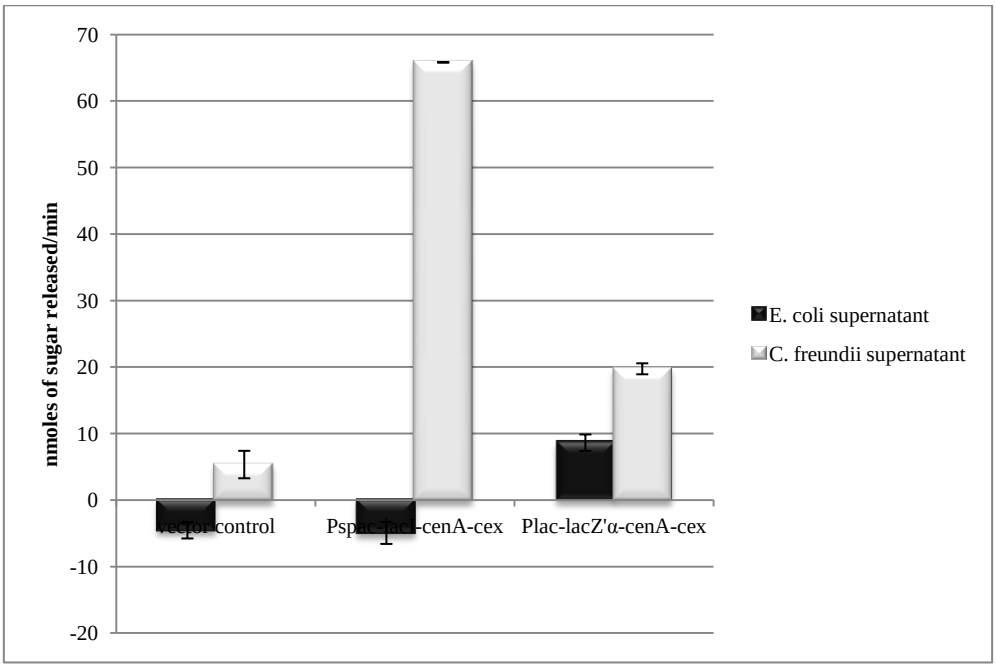


Figure 4.32: Comparison of the amount of endoglucanase activity detected in the culture supernatants of different strains (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*lacZ'α-cenA-cex*) of *E. coli* JM109 and *C. freundii* SBS197 (error bars indicate standard error).

5 ENGINEERING BACTERIA FOR CELLULOSE DEGRADATION:

5.1 Background:

Cellulose represents a large repository of polymerized sugar that could prove to be an inexpensive substrate for fermentation. Ideally cellulose can be hydrolysed releasing glucose which could be an appropriate source of carbon for the growth of microorganisms in order for them to be able to produce other commodity products. Hydrolysis of cellulose to its component sugars is difficult due to the nature of the substrate and therefore even though its an abundant source of glucose it is not an economically feasible source. Despite cellulose being a recalcitrant source of carbon there are organisms in nature that have come to specialize in its degradation. In addition considerable effort has also been made to generate strains that can degrade cellulose effectively.

To date studies of cellulose degradation processes in recombinant microorganisms have mainly focused on ethanol production with little or no attention being paid to the actual growth of degrading microorganism using cellulose as a sole source of carbon. There are a few papers describing the growth of genetically modified yeast, especially *S. cerevisiae,* on cellulosic substrates (Peterson *et. al.,* 1998; Murai *et. al.,* 1998) but to our knowledge there have been no reports describing growth of recombinant bacteria on such substrates. Murai *et. al.* (1998) engineered a cellooligosaccharide assimilating yeast. They reported that their yeast strain was able to use cellooligosaccharides as a sole carbon source and the maximum growth attained by their culture was about 9×10^6 cells/ml. However their transformants were not able to use Avicel (microcrystalline cellulose) as a carbon source at all. Similarly Peterson *et. al.* (1998) designed a polysaccharide-degrading yeast which was able to use starch, pectate and cellobiose but was only partially able to grow using amorphous cellulose (less than 10^7 cell/ml) and no growth was observed when avicel was used as a sole carbon source. We engineered a functional cellulose degrading system (using *cenA* and *cex* from *Cellulomonas fimi* as described in the previous chapter) in *Escherichia coli* JM109, *Citrobacter freundii* SBS197 and *Bacillus subtilis* 168. After confirming the presence and/or activity of *cenA* and *cex* in these transformants they were then

tested for their ability to degrade various cellulosic substrates and use them as a source of carbon for their growth.

5.2 Growth measurement of *C. freundii* SBS197:

5.2.1 Filter paper as a carbon source:

In order to find out whether *C. freundii* SBS197 can degrade filter paper and use it as a source of carbon we used 20 ml of CMM medium (containing 90 µg/ml IPTG and 80 µg/ml carbenicillin) with 100 mg filter paper (2 squares of 2×2 cm, Fords Gold Medal Blotting paper, equivalent to 31 mM glucose, considering it as pure cellulose) as a source of carbon. Yeast extract (0.1% w/v) was used to support initial growth in order for the organisms to reach sufficient number (to produce enzymes required for cellulose degradation) before they can start degrading filter paper. The overnight culture grown in LB medium was centrifuged, resuspended in the same CMM medium and then diluted so that the initial $O.D_{600}$ in the final flasks becomes 0.1. This is the amount of inoculum used in all other similar experiments. The growth was measured in terms of colony forming units per ml once every 24 hours. This method is only an estimate of the number of cells present, as colonies close to each other on the plate may merge, and that the neighbouring colonies may inhibit or stimulate growth. Hence countable plates were chosen from triplicate plating of three dilutions with the acceptable plates to be within 20% of the average as reported by Breed and Dotterrer (1916). Flasks with 31 mM filter sterilized-glucose and 15.5 mM cellobiose were used as positive controls. In the case where glucose was used as a carbon source the pH of the flasks dropped to about 4-4.5 or below after 24-48 hours and hence measurement of growth in terms of cfu/ml was not possible as after 24-48 hours the flasks were sterile, and therefore $O.D_{600}$ was measured. When growth was compared in the presence of glucose and cellobiose (in the form of $O.D_{600}$) it was observed that organisms grew much better in the presence of cellobiose than glucose (Figure 5.1) as pH did not drop below 6-6.5 when cellobiose was used as carbon source. Hence it was concluded that cellobiose can be used as a good positive control for *C. freundii* SBS197. The flasks without any carbon source apart from 0.1% yeast extract were used as a negative control and the flasks with 100 mg filter paper were used as a test to find out if the organisms were able to use filter paper as a sole carbon source. The results are shown in Figure 5.2. In preliminary tests one of the flasks accidently fell

off in the shaker due to which it was subjected to extreme physical agitation. It was observed that the filter paper degradation as well as colony counts were higher in that flask as compared to the ones that were simply mixed by rotating in the shaker. Therefore in order to find out that if any form of extreme physical force would help in the degradation of filter paper by microorganisms we vortexed one set of flasks for 60 seconds once every 24 hours. It was observed that physical agitation aids in filter paper degradation by microorganisms. The results are shown in Figures 5.3 and 5.4.

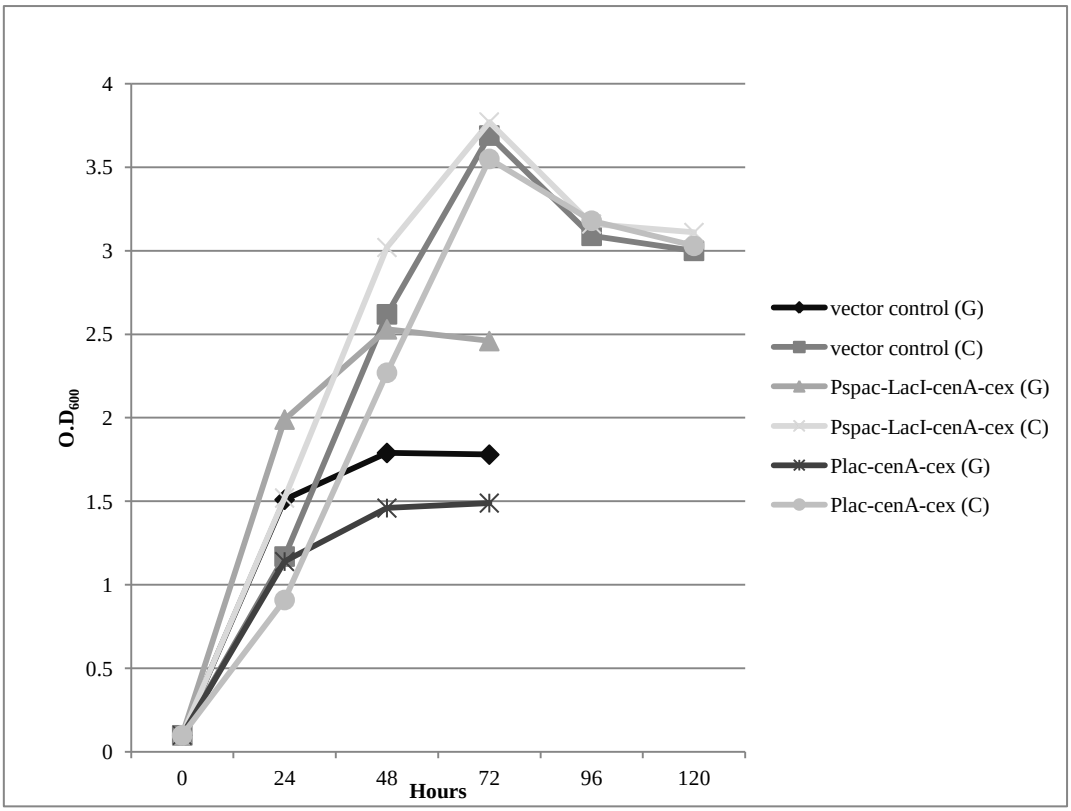
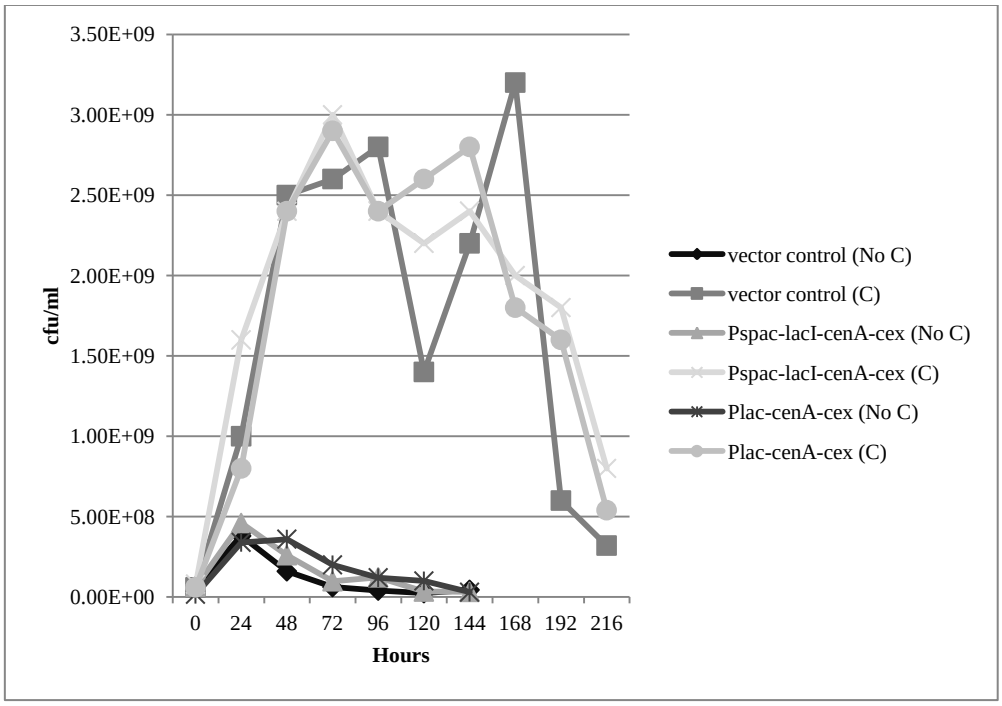


Figure 5.1: Graph comparing the growth of *C. freundii* SBS197 (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*cenA-cex*)when 31 mM glucose (G) and 15.5 mM cellobiose (C) was used as a source of carbon.

A



B

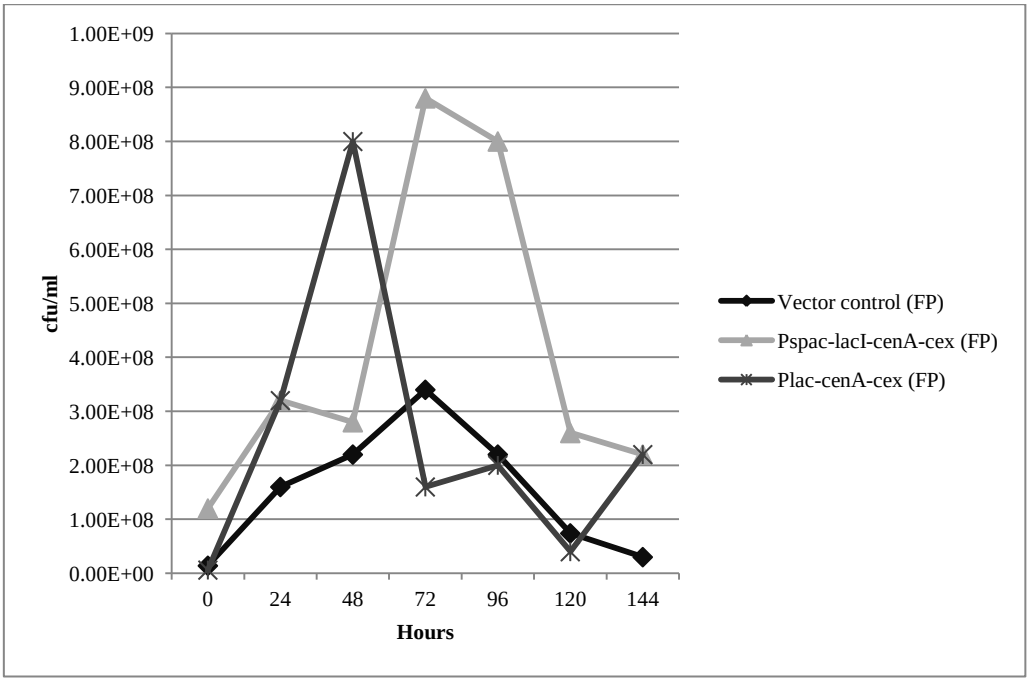
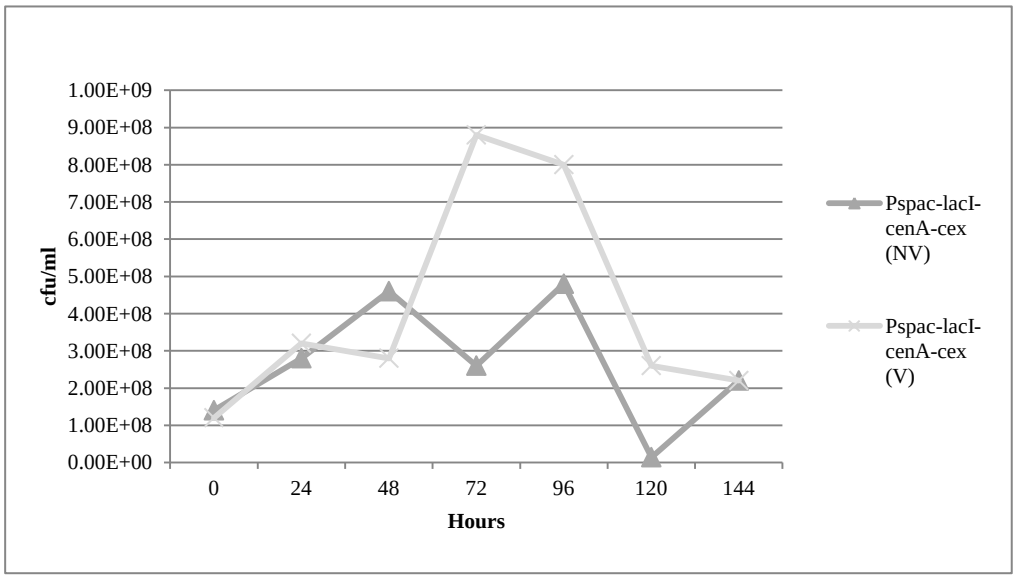


Figure 5.2: Graphs showing growth of *C. freundii* SBS197 (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*cenA-cex*) in the presence of different carbon sources.

A: Cellobiose (C, positive control), No carbon (No C, negative control) and B: filter paper (FP, test, vortexed)

A



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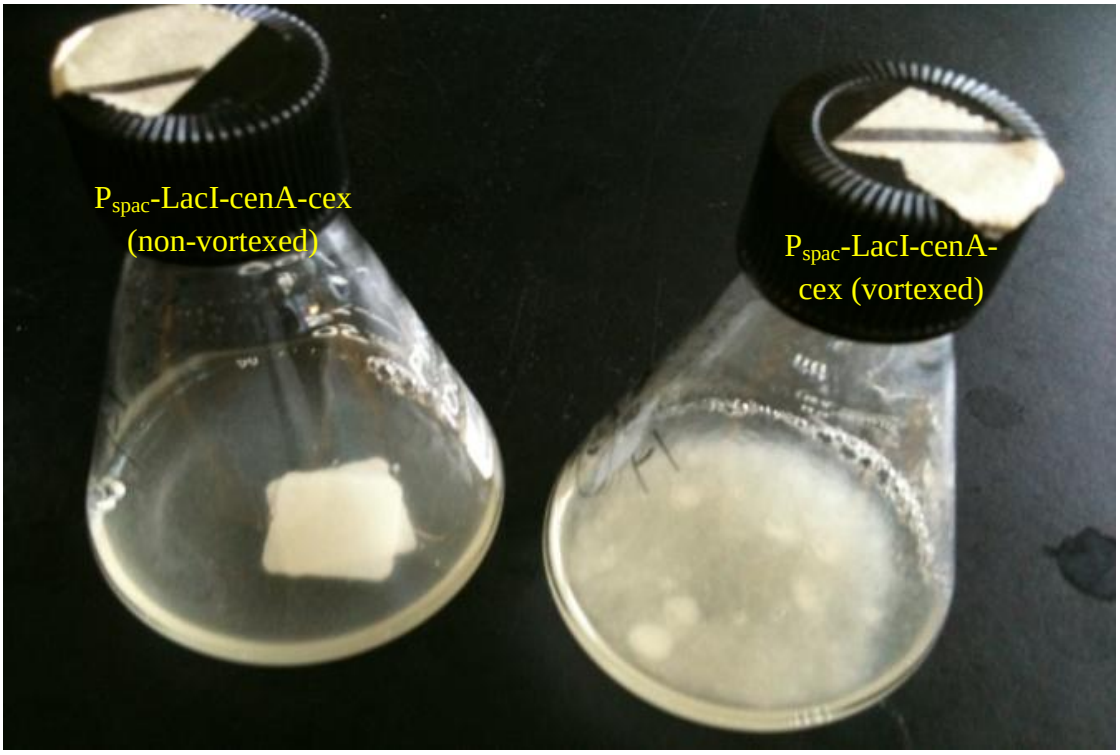
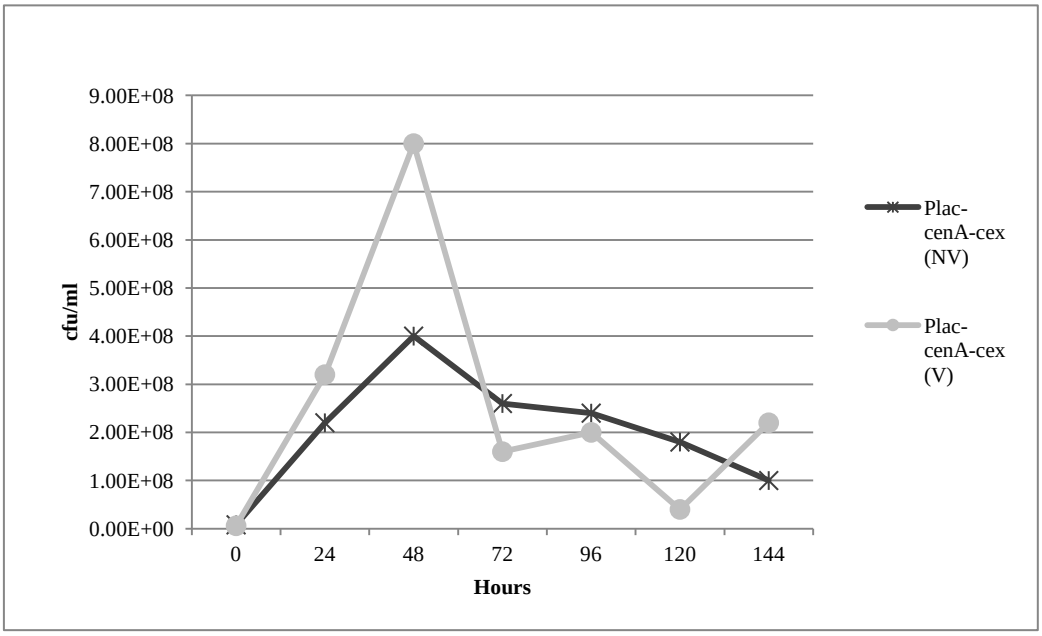


Figure 5.3: Difference in growth/filter paper degradation between vortexed (V) and non-vortexed (NV) cultures of *C. freundii* SBS197 after 48 hours of growth in cultures having *spac* promoter.

A: colony counts, B: destruction of filter paper squares

A



B

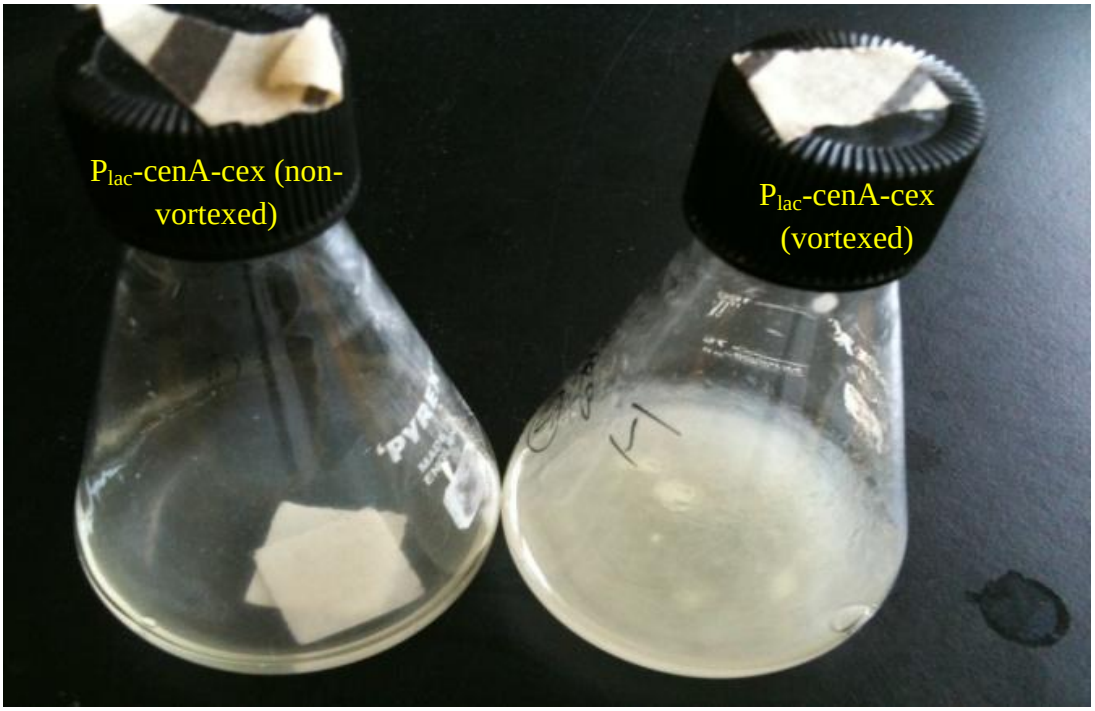


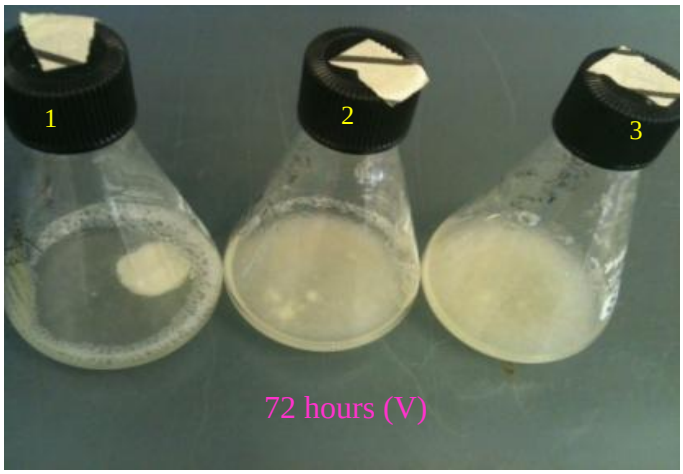
Figure 5.4: Difference in growth/filter paper degradation between vortexed (V) and non-vortexed (NV) cultures of *C. freundii* SBS197 after 48 hours of growth in cultures having *lac* promoter.

A: colony counts, B: destruction of filter paper squares

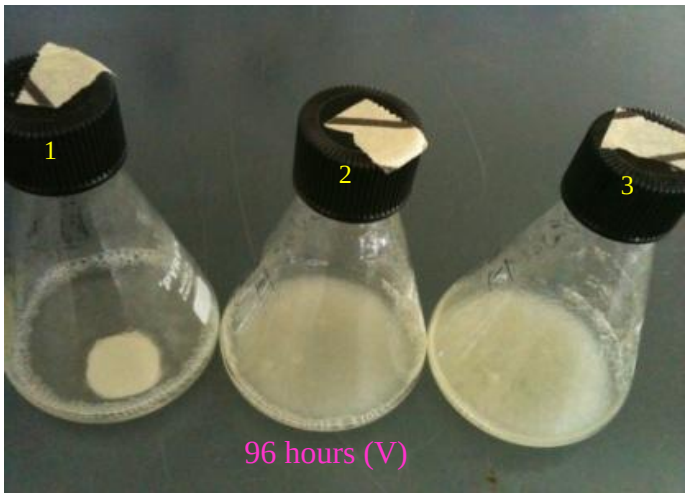
When the flasks were vortexed filter paper was completely destroyed (as observed by physical destruction) after 72 hours in cultures with P_{lac} and in 96 hours in cultures with P_{spac} as compared to non-vortexed flasks in which filter paper was not completely destroyed even after a week as shown in Figure 5.5. When maximum cfu/ml was plotted for each culture, the culture having P_{spac} (vortexed) was found to show the maximum growth followed by the cultures having P_{lac} (vortexed). The results are shown in Figure 5.6.

In addition the area under each curve was determined and plotted as cfu-hours/ml. It was observed that P_{spac}-*lacI-cenA-cex* (vortexed) covers the largest area followed by P_{spac}-*lacI -cenA-cex* (non-vortexed), P_{lac}-*lacZ'α-cenA-cex* (vortexed) and P_{lac}-*lacZ'α-cenA-cex* (non-vortexed). This shows that the cells after attaining maximum growth are maintained for longer in cultures having the *spac* promoter than the ones having the *lac* promoter (Figure 5.7). Hence these results shows that although filter paper takes a little longer to be completely degraded in cultures with P_{spac} than with P_{lac}, the cells achieve maximum count and are maintained for longer period of time in P_{spac} cultures than in P_{lac} cultures.

A



B



C

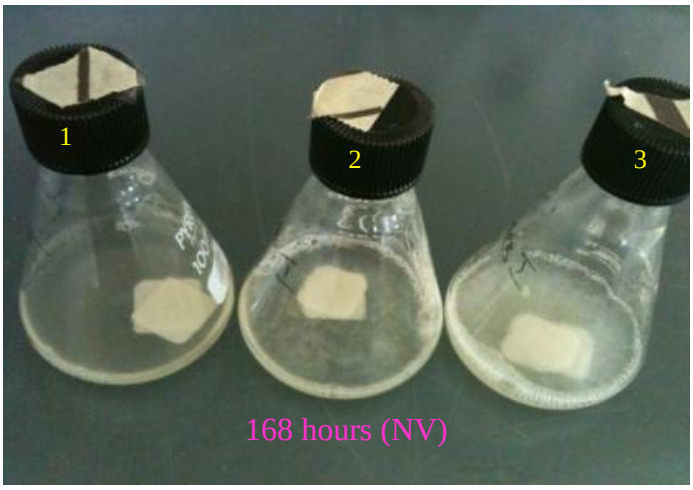


Figure 5.5: pictures showing degradation of filter paper at various time intervals.

1: vector control, 2: P_{spac}-lacI-cenA-cex, 3: P_{lac}-cenA-cex, V: vortexed culture, NV: non-vortexed culture

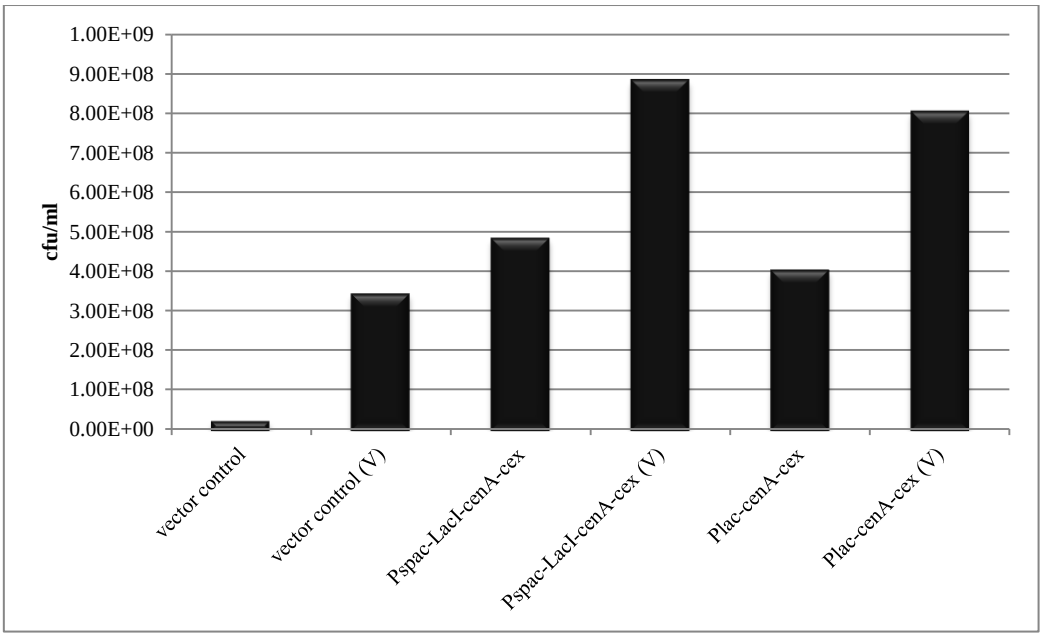


Figure 5.6: Graph showing maximum colony forming units per ml attained by each culture. V - vortexed

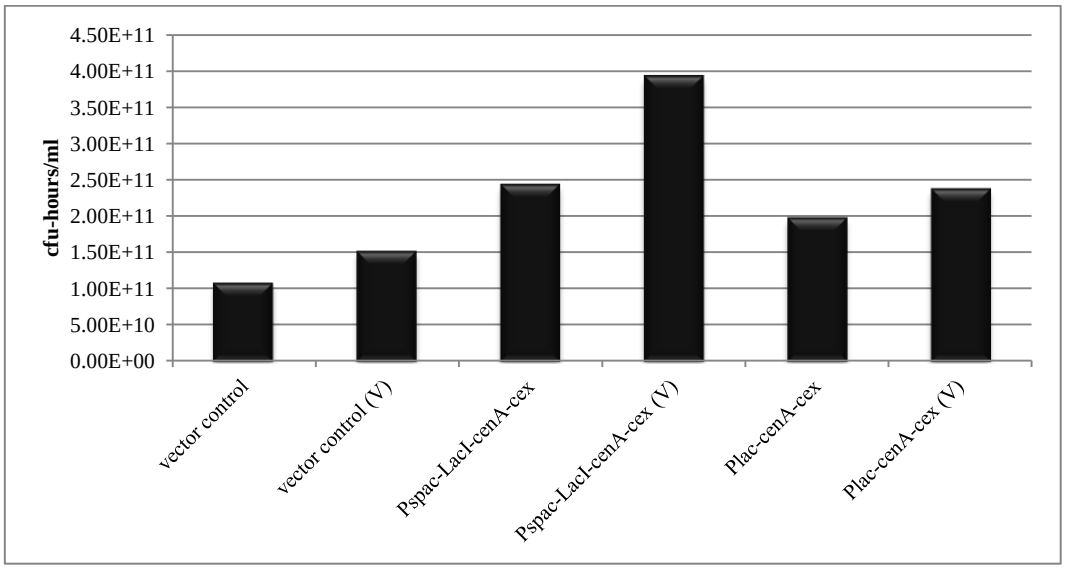


Figure 5.7: Graph showing maximum area covered under each curve for different strains (vector control, P_{spac}-*lacI-cenA-cex* and P_{lac}-*cenA-cex*) of *C. freundii* SBS197. V - vortexed

5.2.2 Avicel as a carbon source:

Avicel (microcrystalline cellulose) is a pure form of powdered cellulose derived from high quality wood pulp. To determine whether the organisms can degrade purified cellulose, 20 ml of CMM medium (containing 90 µg/ml IPTG, 80 µg/ml carbenicillin and 0.1% yeast extract) was used, with 100 mg Avicel as a source of carbon. The growth was measured as cfu/ml once every 24 hours. The results are shown in Figure 5.8.

In order to find out whether or not any form of physical force aids in the degradation when the cellulose is already in powdered form, one set of flasks were vortexed once every 24 hours for 60 seconds. It was observed that physical agitation does not aid in degradation and/or growth. This was in contrast to what we observed when filter paper was used as a carbon source. Hence it was concluded that when cellulose is in powdered form physical agitation has no effect on degradation of cellulose and/or on the growth of degrading microorganisms. The results are shown in Figure 5.9.

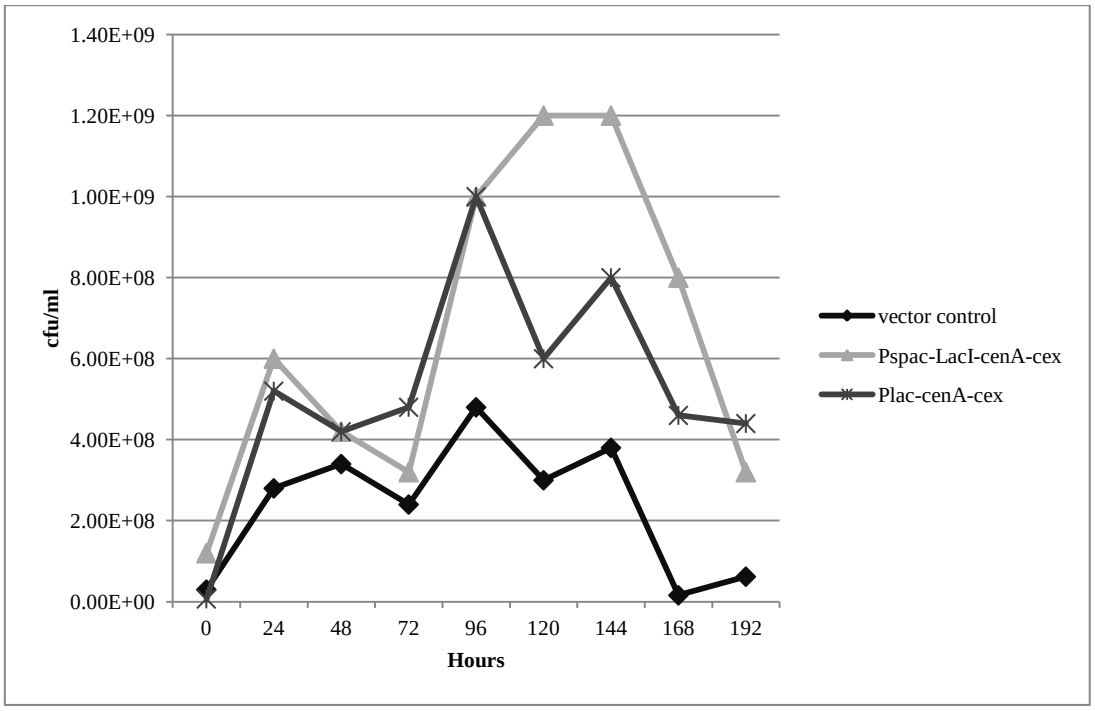
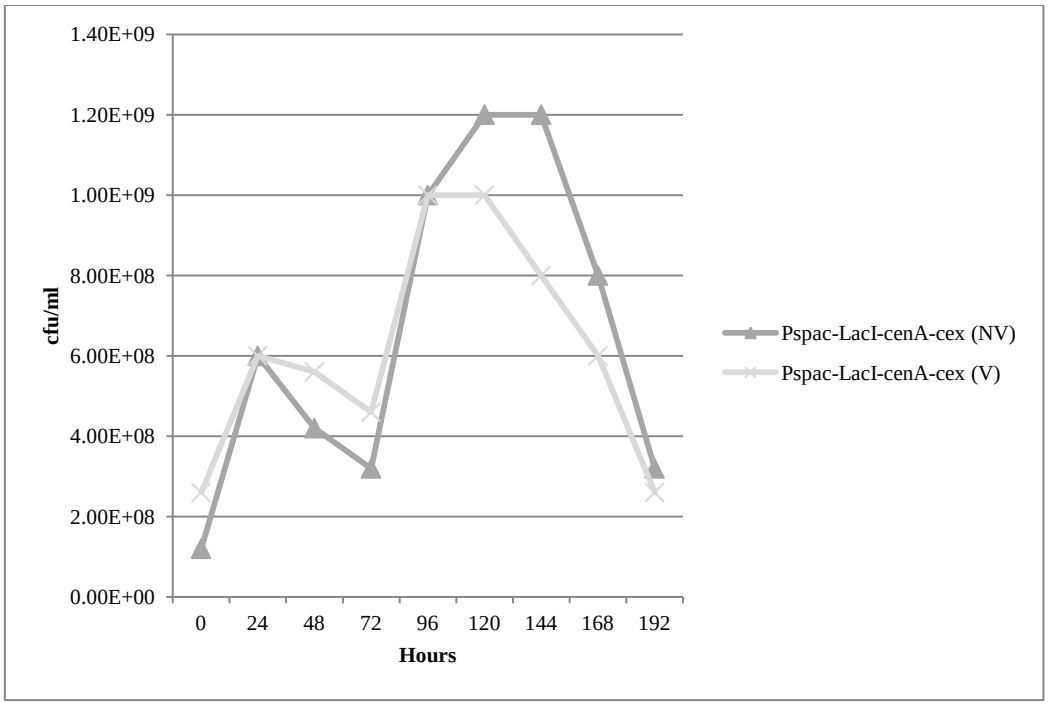


Figure 5.8: Graph showing growth of *C. freundii* SBS197 when 100 mg Avicel was used as a source of carbon.

A



B

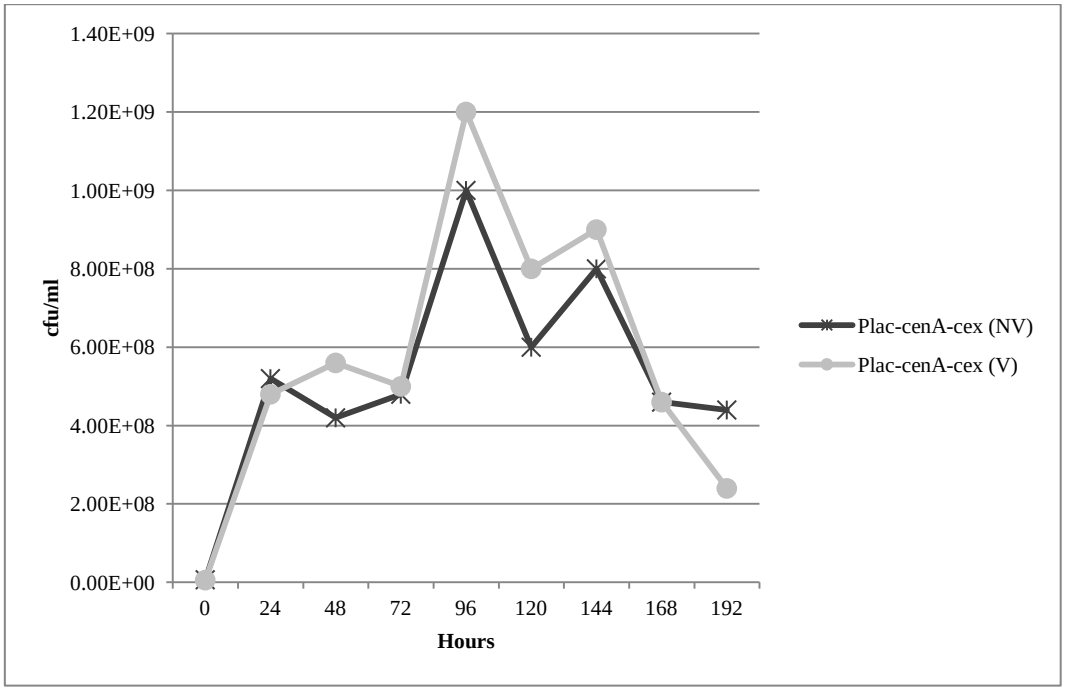


Figure 5.9: Difference in growth and/or biodegradation of avicel between vortexed (V) and non-vortexed (NV) cultures of *C. freundii* SBS197.

A: cultures expressing genes from the *spac* promoter, B: cultures expressing genes from the *lac* promoter.

As observed from the graph (Figure 5.8), the cultures expressing genes from the *spac* promoter show higher growth compared to cultures to the ones expressing from the *lac* promoter. Also when the area under each curve was determined and plotted as cfu-hours/ml, the cultures expressing from the *spac* promoter covers the maximum area showing that once achieving maximum growth they are maintained for longer before they start to die off (Figure 5.10). These results are consistent with what was observed when filter paper was used as a carbon source.

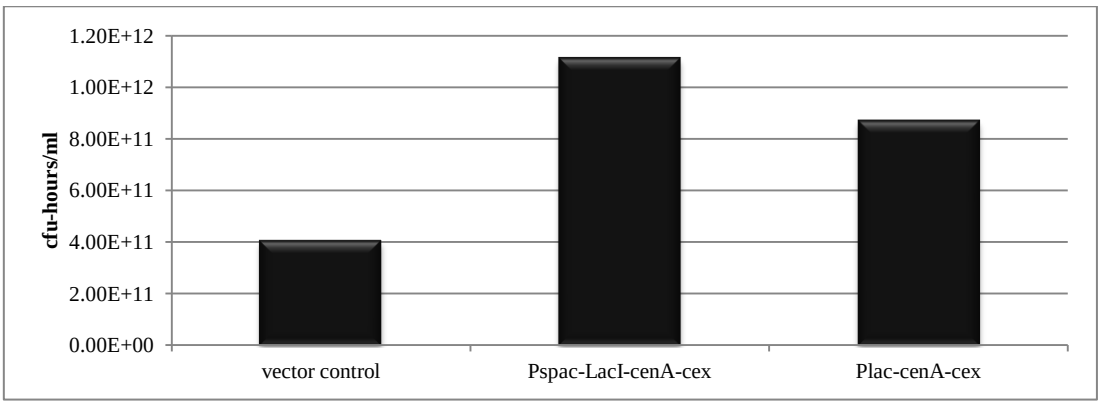


Figure 5.10: Graph showing maximum area covered under each curve for different organisms.

5.2.3 Comparison of growth with different carbon sources:

When growth was compared in the presence of different cellulosic substrates it was observed that the lag phase is much longer when Avicel is used as a source of carbon. The organisms start growing after about 72 hours in both P_{spac} as well as P_{lac} cultures. After reaching a certain population level (in the first 24 hours) by utilizing carbon from yeast extract provided in the medium they stay in lag phase for about another 48 hours after which they began to utilize Avicel and start growing again. In the case of filter paper there was no lag phase observed in P_{lac} cultures whereas there was a short lag phase in P_{spac} cultures. This could be due to the fact that Avicel is purely a crystalline form of cellulose whereas filter paper is a mixture of amorphous and crystalline cellulose which hence is much easier to break than purely crystalline cellulose. In addition paper may also contain a certain amount of xylan which would therefore be easier to hydrolyse as *cex* also has xylanase activity (Table 1.3). The results are shown in Figures 5.11 and 5.12.

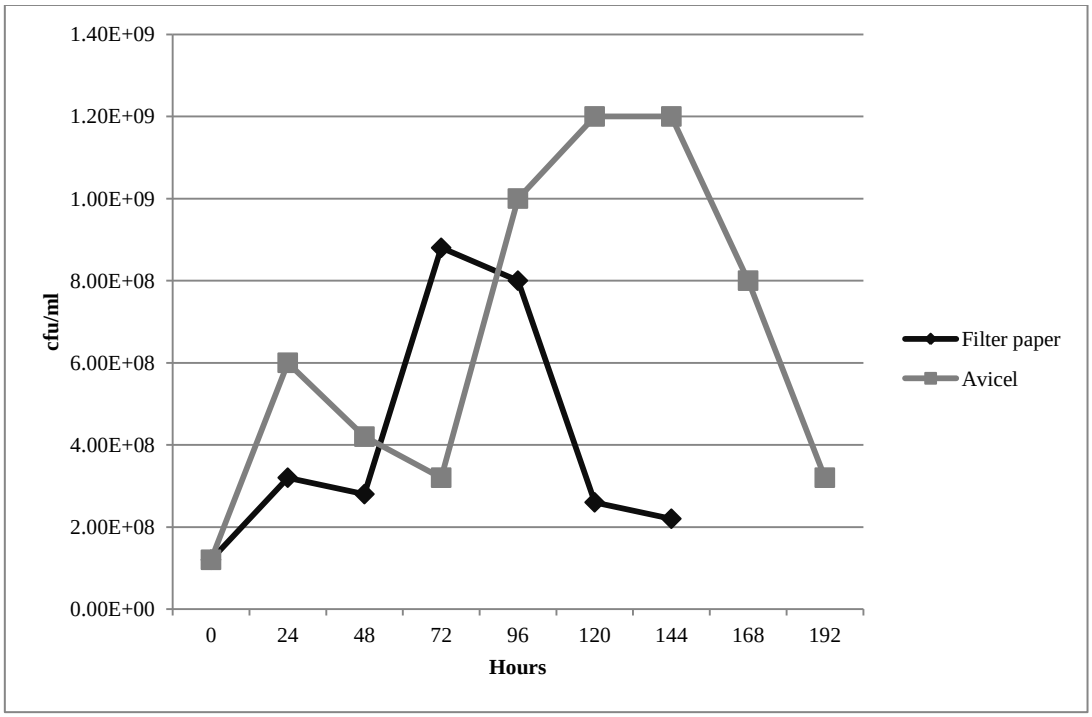


Figure 5.11: Graphs comparing the growth of *C. freundii* SBS197 containing *cenA-cex* in the presence of different carbon sources (Avicel and Filter paper) when P_{spac} was used as a promoter.

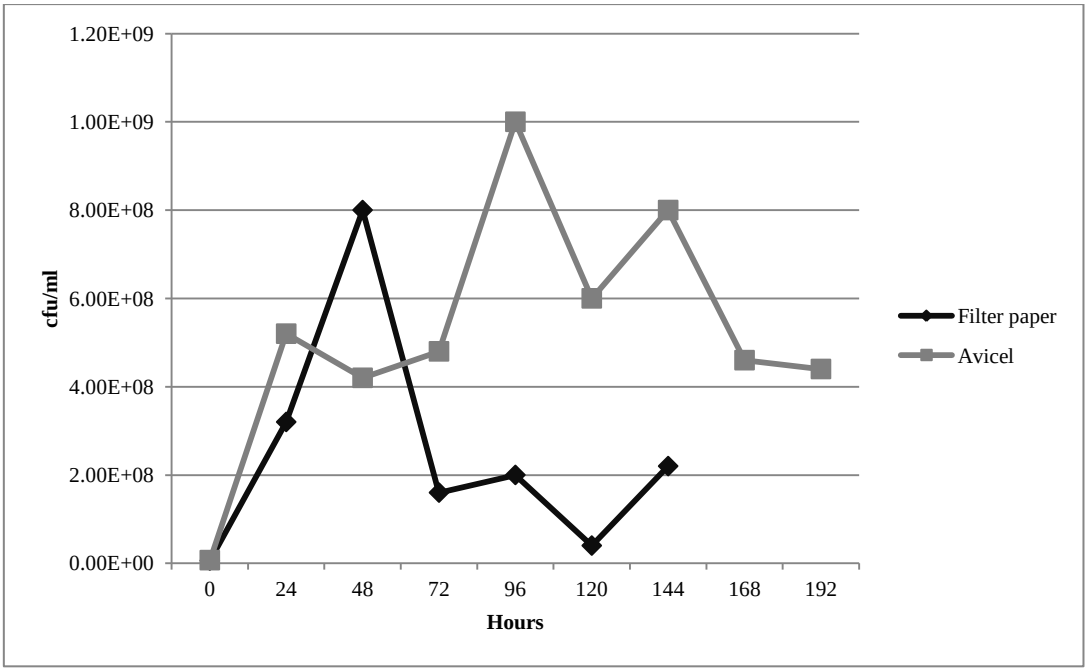


Figure 5.12: Graphs comparing the growth of *C. freundii* SBS197 containing *cenA-cex* in the presence of different carbon sources (Avicel and Filter paper) when P_{lac} was used as a promoter.

It was also seen that the cells grow better (in terms of achieving maximum colony count) in the presence of Avicel than in the presence of filter paper (Figure 5.13). This could be due to the fact that the filter paper used was not pure cellulose and had impurities which may have been preventing/inhibiting the growth of the cells. As an example Whatman Cellulose Filters contain trace amounts of aluminium, antimony, arsenic, barium, boron, bromine, lead, mercury etc., (http://www.whatman.com/CelluloseFilters.aspx) which could be inhibiting factors for the growth of microorganisms when filter papers are used as a source of carbon. Avicel on the other hand is a pure form of cellulose made from high quality wood pulp. Also filter paper is not 100% cellulose and hence therefore 100 mg filter paper will contain less sugar than the same amount of Avicel. In addition the surface area of Avicel (as it is in powdered form) is much larger compared to the surface area of filter paper, giving the organisms a better chance of being in contact with its substrate. Therefore, cells grown in the presence of Avicel attained higher counts then the cells grown in the presence of filter paper.

In addition when the area under the curve was determined for filter paper and Avicel and plotted as cfu-hours/ml it was observed that the cultures grown in the presence of Avicel cover larger area showing that after achieving maximum count they are maintained for longer when Avicel is provided as a source of carbon. The results are presented in Figure 5.14.

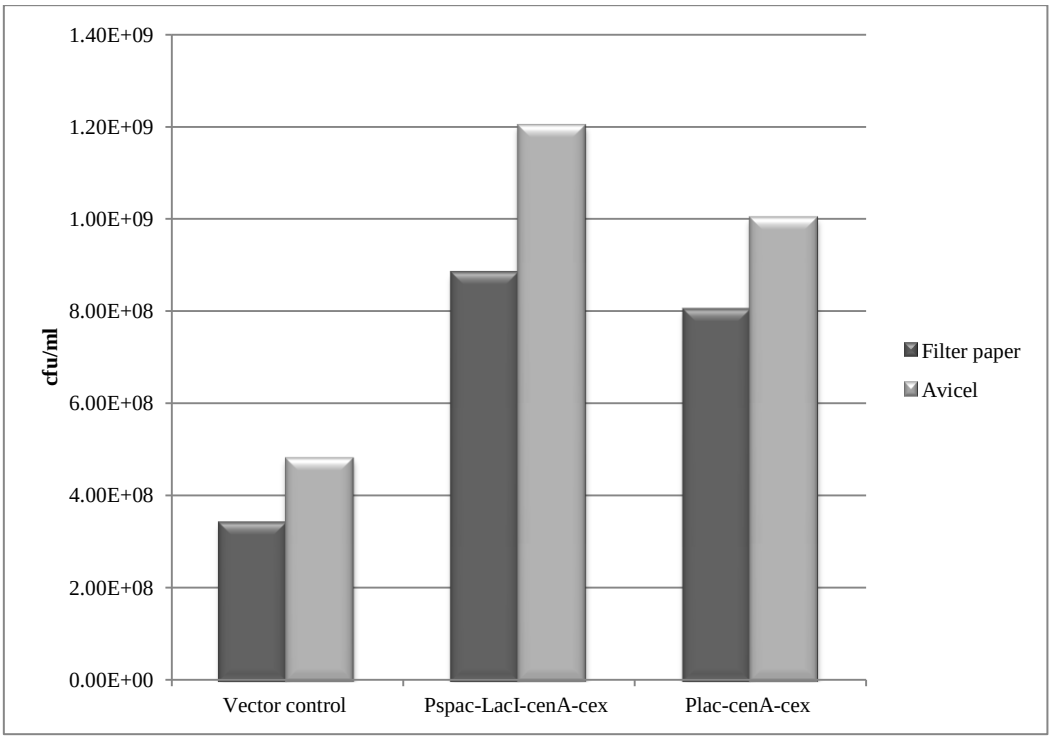


Figure 5.13: Graph showing maximum colony count attained by each culture in the presence of Avicel and filter paper.

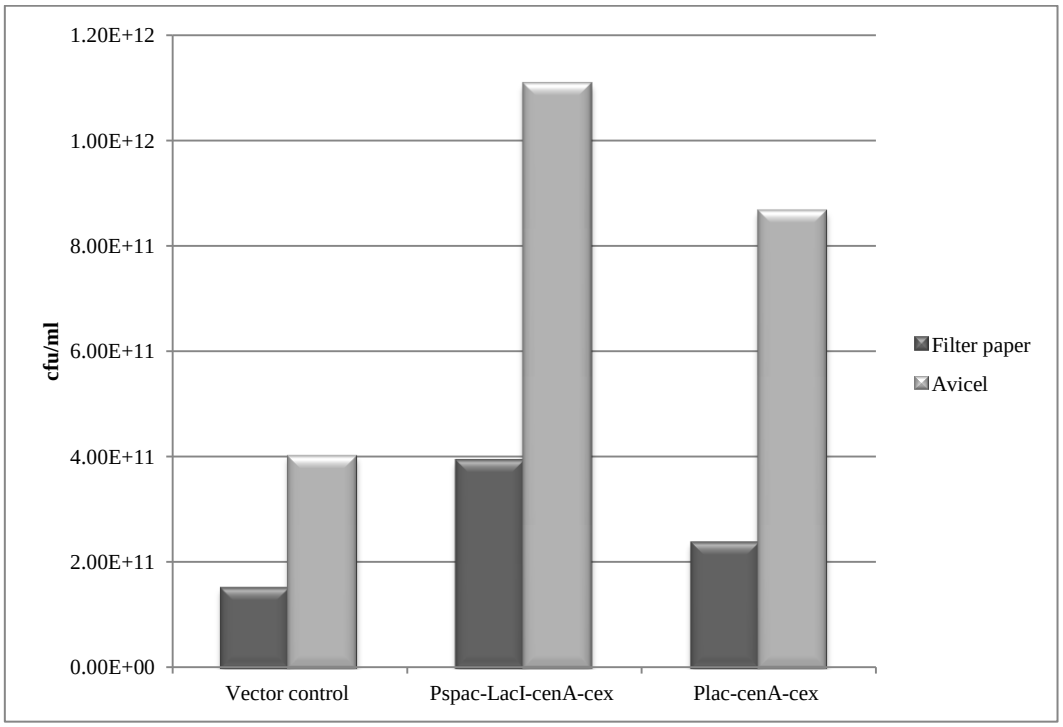


Figure 5.14: Graph showing maximum area covered under the curve by each culture when Avicel and filter paper was used as carbon sources.

5.3 Growth measurement of *B. subtilis* 168:

5.3.1 Filter paper as a carbon source:

In order to find out if *B. subtilis* 168 transformed with pTG262+P_{spac}-*lacI-cenA-cex* can degrade filter paper and use it as a source of carbon, 20 ml M9 medium (containing 90 µg/ml IPTG, 80 µg/ml carbenicillin and 0.1% yeast extract) with 100 mg filter paper (2 discs of Fords Gold Medal Blotting Paper 2×2 cm in size, equivalent to 31 mM glucose, considering filter paper as pure cellulose) was used as a source of carbon. M9 medium was used as it is a well-buffered medium compared to CMM and therefore pH drop wouldn't be a problem in such medium. Glucose (31 mM) and 15.5 mM cellobiose were used as positive controls. It was observed that the organisms are better able to use glucose than cellobiose. Also they tend to achieve maximum growth when glucose was provided as a carbon source in the medium rather than when cellobiose was used as a source of carbon. The results are presented in Figure 5.15. Hence it was decided to use glucose as a positive control, flasks with no carbon source (apart from 0.1% yeast extract) as a negative control and flasks with 100 mg filter paper as a test. The test flasks were vortexed once every 24 hours for 1 min. The results are shown in Figure 5.16. It was observed that organisms were not able to use filter paper as a source of carbon and the growth was much less than the positive control and nearly the same as the negative control when filter paper was provided as a source of carbon. These results were also observed when a bar graph was plotted with maximum cfu/ml. The maximum growth under the test condition (that is when filter paper was used as carbon source) was nearly the same as the negative control and much less than the growth obtained when glucose and cellobiose were used as carbon sources. Secondly there was not much difference in the maximum growth between the vector control and the strain transformed with *cenA* and *cex*. The results are presented in Figure 5.17.

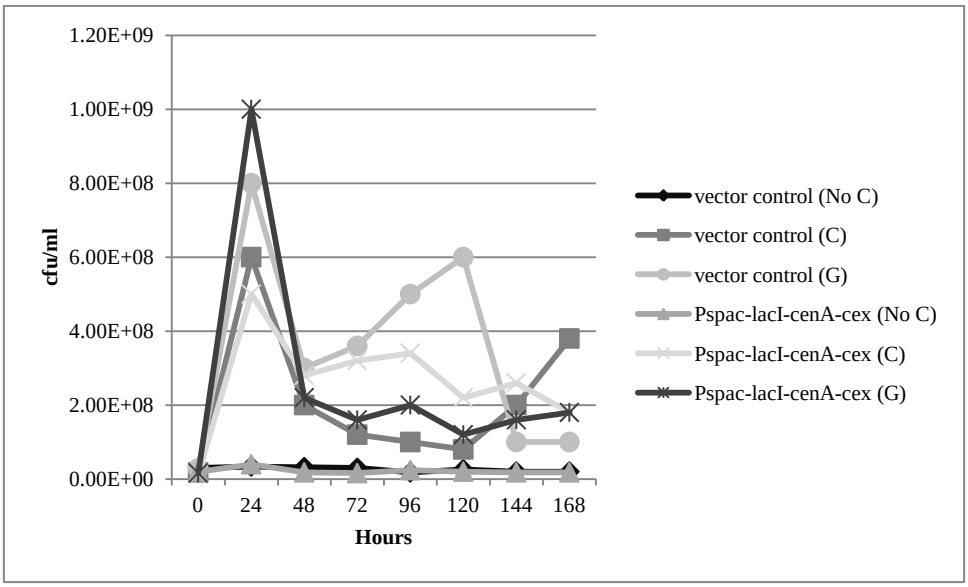


Figure 5.15: Graph showing growth of *B. subtilis* 168 transformed with pTG262 and pTG262+P_{spac}-*lacI-cenA-cex* without any carbon source (No C), in the presence of 31mM glucose (G) and 15.5 mM cellobiose (C).

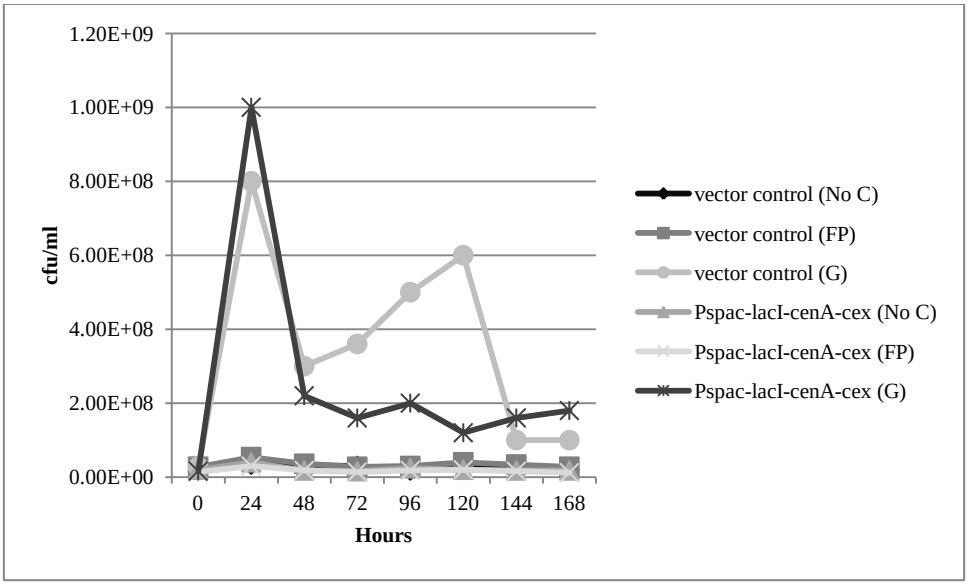


Figure 5.16: Graph showing growth of *B. subtilis* 168 transformed with pTG262 and pTG262+P_{spac}-*lacI-cenA-cex* without any carbon source (No C, negative control), in the presence of 31mM glucose (G, positive control) and 100 mg Filter paper (FP, test).

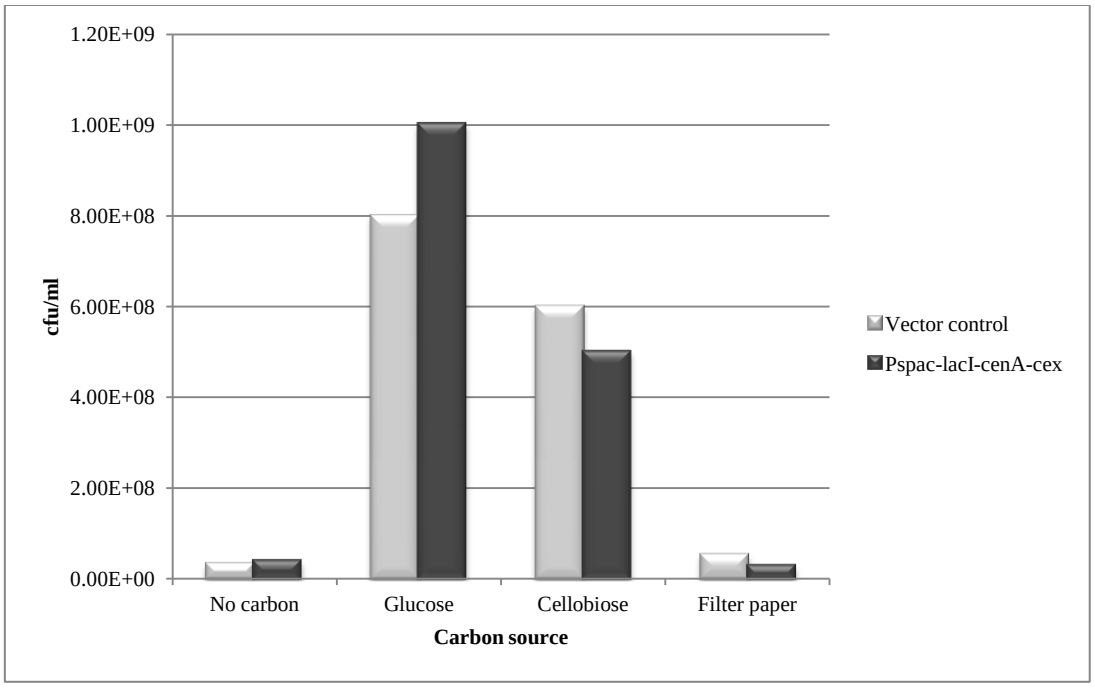


Figure 5.17: Graph showing maximum growth achieved by *B. subtilis* 168 transformed with pTG262 and pTG262+P_{spac}-*lacI-cenA-cex* when provided with different carbon sources.

5.3.2 Avicel as a carbon source:

In order to find out if *B. subtilis* 168 containing *cenA* and *cex* can use Avicel (highly purified crystalline cellulose) as a source of carbon, 20 ml M9 medium (containing 90 µg/ml IPTG, 80 µg/ml carbenicillin and 0.1% yeast extract) was used with 100 mg Avicel as a source of carbon. Again 31 mM glucose was used as a positive control and flasks without any carbon source (apart from 0.1% yeast extract) were used as negative control. The results showed that *B. subtilis* 168 transformed with pTG262+P_{spac}-*lacI-cenA-cex* was not able to use Avicel as carbon source. The growth was much less when compared to the positive control and nearly the same as the negative control as shown in Figures 5.18 and 5.19. Maximum cfu/ml obtained when Avicel was used was much lower than when glucose or cellobiose was used.

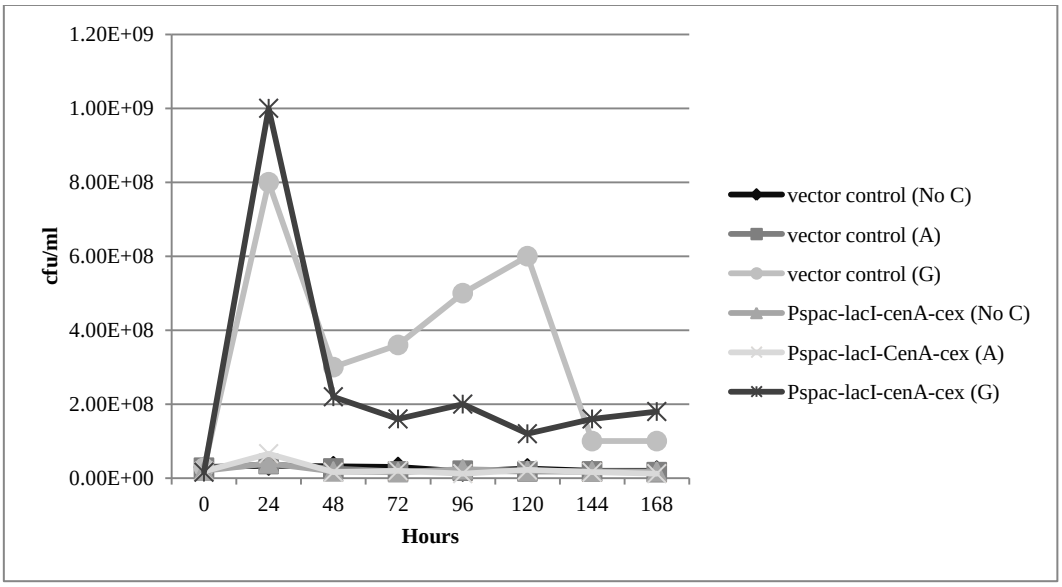


Figure 5.18: Graph showing growth of *B. subtilis* 168 transformed with pTG262 and pTG262+P_{spac}-*lacI-cenA-cex* without any carbon source (No C, negative control), in the presence of 31mM glucose (G, positive control) and 100 mg Avicel (A, test).

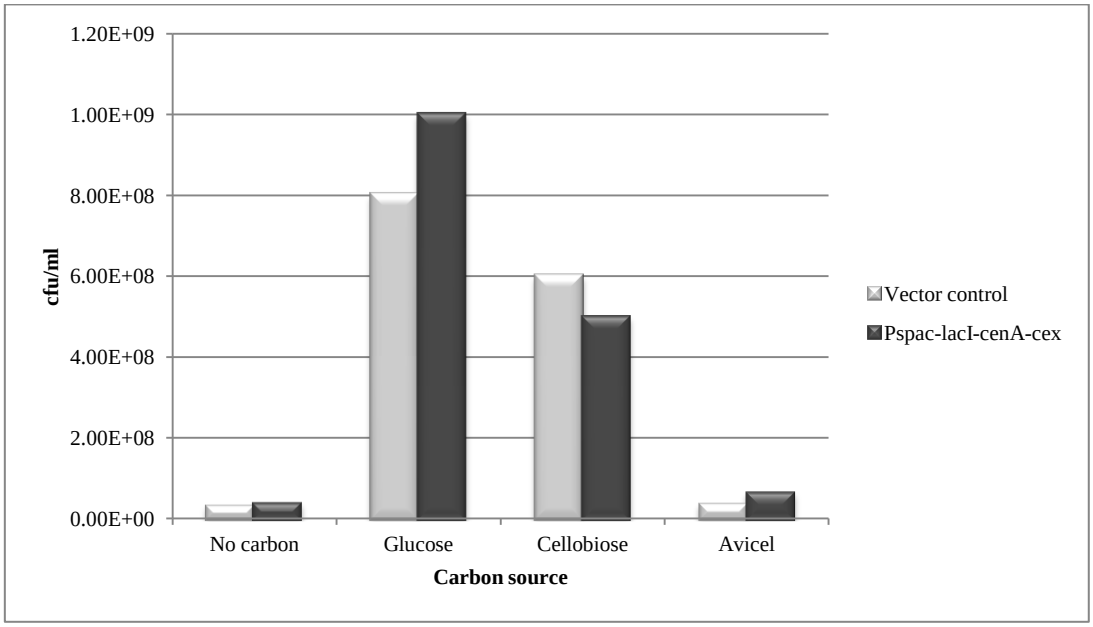


Figure 5.19: Graph showing maximum growth achieved by *B. subtilis* 168 transformed with pTG262 and pTG262+P_{spac}-*lacI-cenA-cex* when provided with different carbon sources.

5.3.3 Comparison of growth with different carbon sources:

It is evident from the results above that *B. subtilis* 168 transformed with cellulases from *C. fimi* (*cenA* and *cex*) cannot use cellulose in the form of filter paper or Avicel as a carbon source. The growth when filter paper and Avicel were used as carbon sources was very low and no physical destruction of filter paper was observed by vector control and/or the strain containing P_{spac}-*lacI-cenA-cex*. It was interesting to note that when area under each curve was determined and plotted as cfu-hours/ml, the peak for glucose and cellobiose was nearly the same. This shows that even though organisms grow better in the presence of glucose than in the presence of cellobiose, they tend to die off quicker, whereas they maintain themselves for longer in the presence of cellobiose. The results are shown in Figure 5.20.

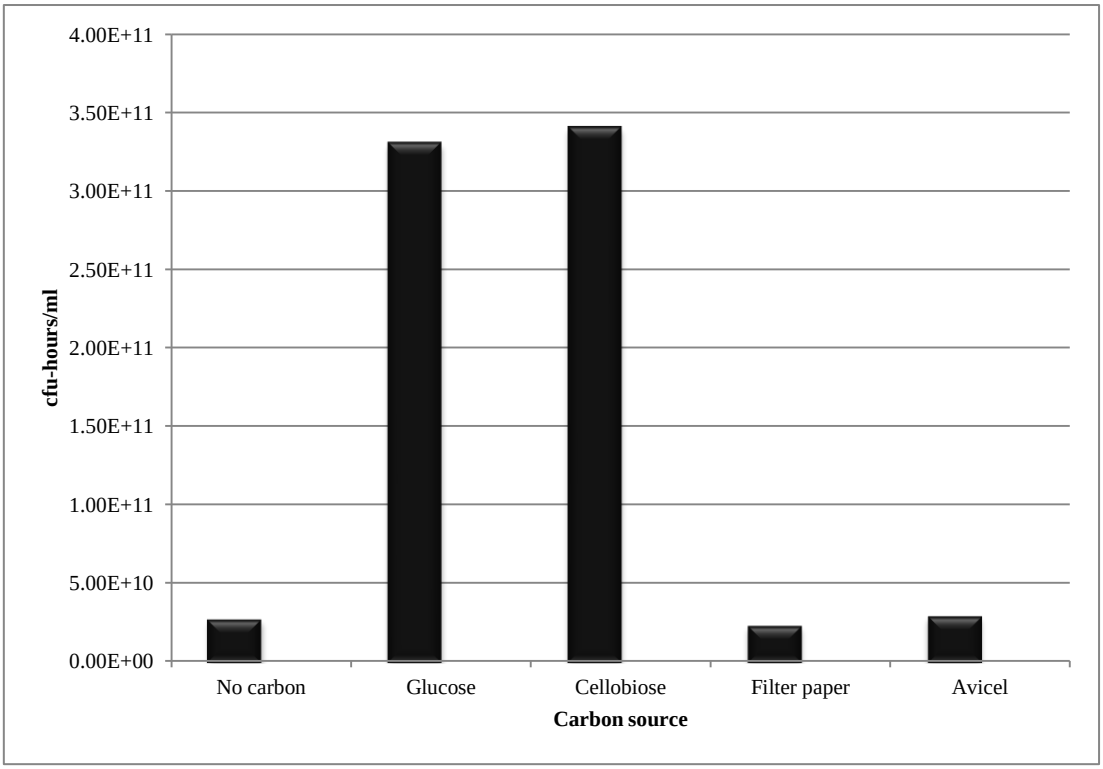


Figure 5.20: Graph showing maximum area covered under each curve for strain transformed with pTG262+P_{spac}-*lacI-cenA-cex* when provided with different carbon sources.

5.4 Growth measurements of *E. coli* JM109:

5.4.1 Filter paper as a carbon source:

In order to see if *E. coli* JM109 transformed with *cenA* and *cex* can use filter paper as a source of carbon growth curves were performed using 20 ml M9 medium (containing 90 µg/ml IPTG, 80 µg/ml carbenicillin and 0.1% w/v yeast extract) with 100 mg filter paper (Ford's Gold Medal Blotting Paper, 2 squares of 2×2 cm, equivalent to 31 mM glucose when filter paper is pure cellulose) as a source of carbon. Flasks with 31 mM glucose were used as a positive control and the flasks with no carbon source (apart from 1 g/l yeast extract) were used as a negative control. The flasks with filter paper were vortexed once every 24 hours for 60 seconds. The results are shown in Figures 5.21, 5.22 and 5.23.

The growth with filter paper of the strains transformed with *cenA* and *cex* was less than that of the vector control suggesting that the recombinant *E. coli* JM109 transformed with *cenA* and *cex* were not able to use filter paper as a source of carbon. However interestingly they were able to cause some breakdown of filter paper as evident from Figure 5.24.

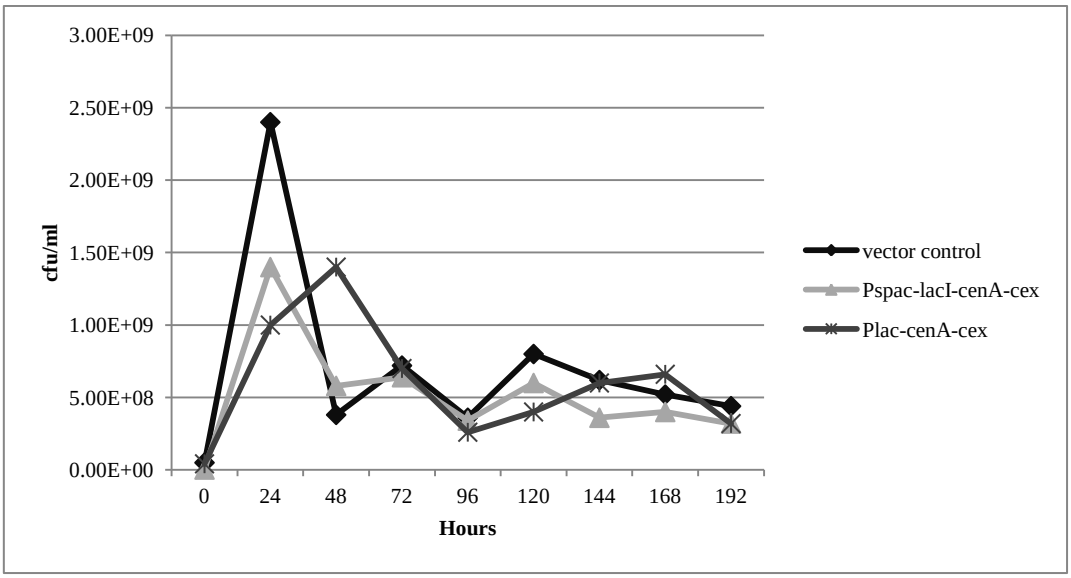


Figure 5.21: Graph showing growth of recombinant *E. coli* JM109 transformed with pSB1A2, pSB1A2+P_{spac}-*lacI-cenA-cex* and pSB1A2+P_{lac}-*cenA-cex* when filter paper was used as a carbon source.

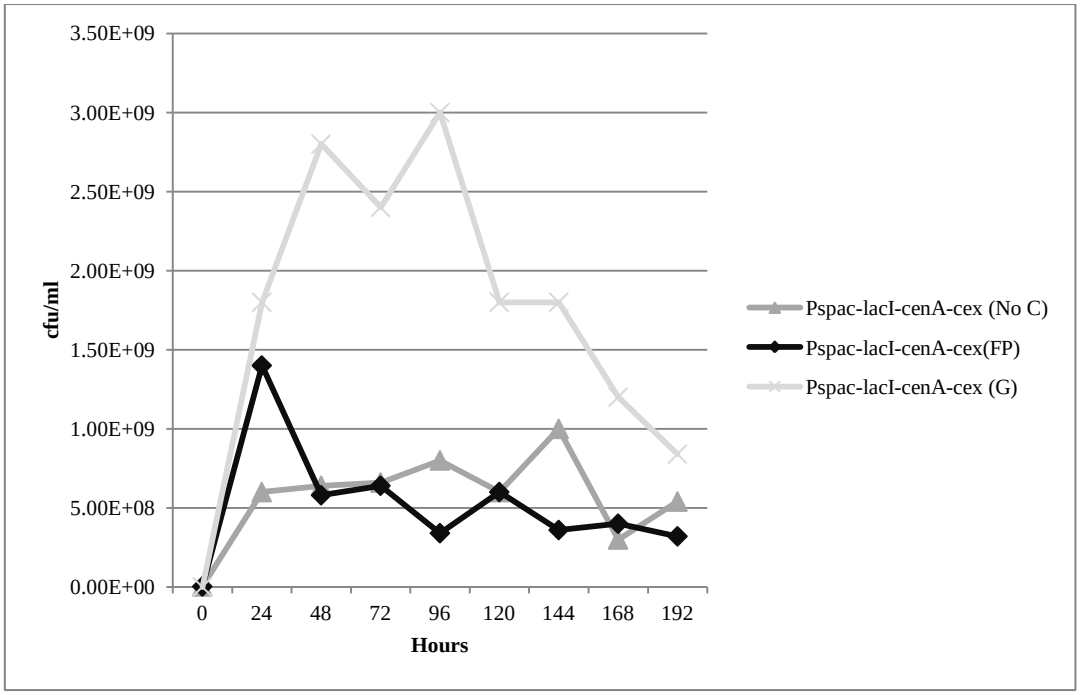


Figure 5.22: Graph showing growth of recombinant *E. coli* JM109 having P_{spac}-*lacI-cenA-cex* with different carbon sources. Glucose (G, positive control), No carbon (No C, negative control) and filter paper (FP, test).

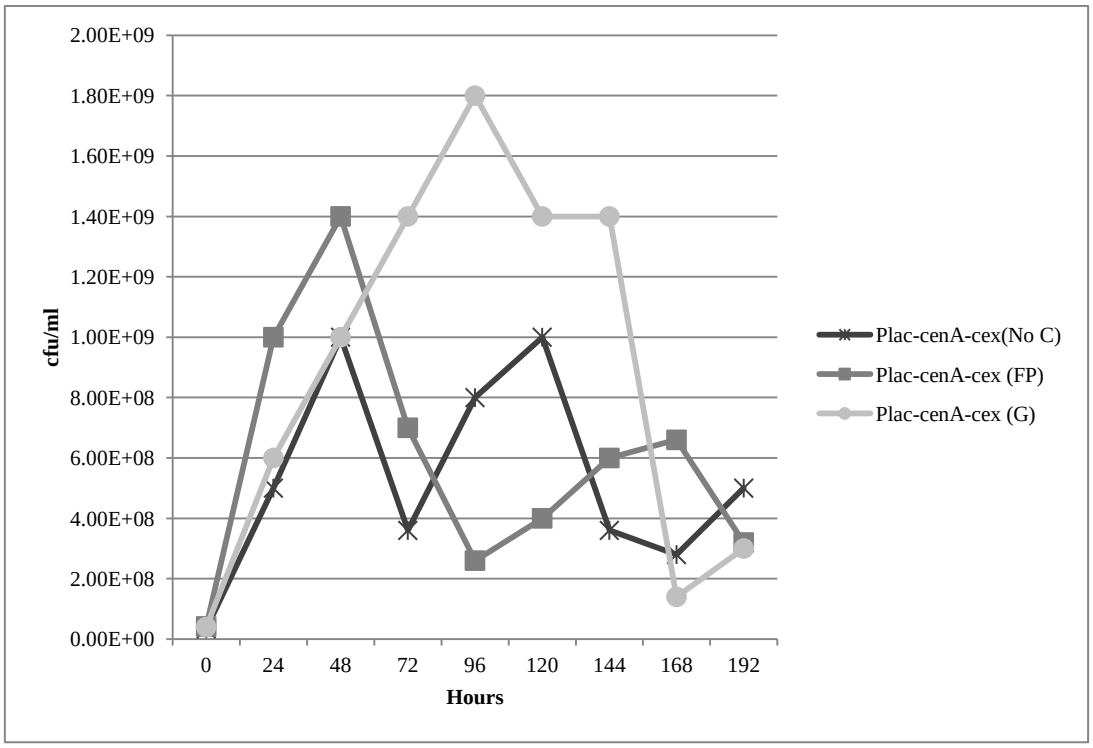


Figure 5.23: Graph showing growth of recombinant *E. coli* JM109 having P_{lac}-*cenA-cex* with different carbon sources. Glucose (G, positive control), No carbon (No C, negative control) and filter paper (FP, test).

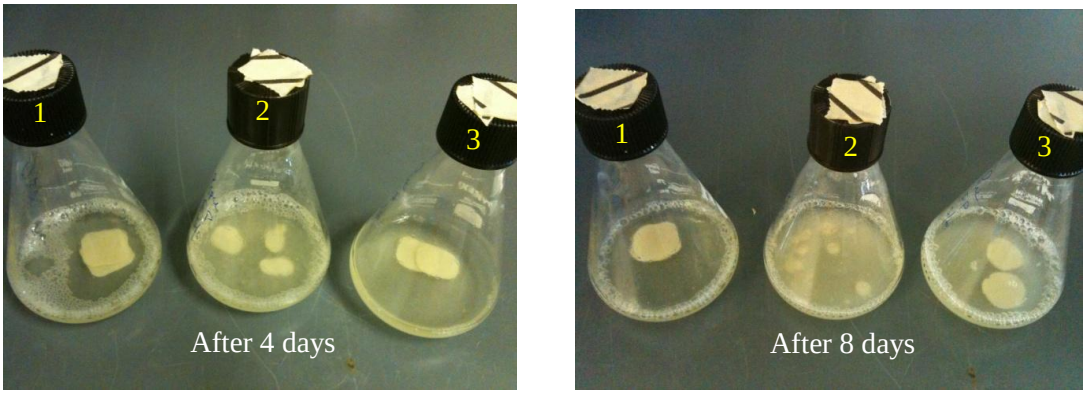


Figure 5.24: Picture showing breakdown of filter paper by recombinant *E. coli* JM109 having *cenA* and *cex* after 4 and 8 days.

1: vector control, 2: P_{spac}-*lacI-cenA-cex*, 3: P_{lac}-*cenA-cex*

The filter paper in the culture with the *spac* promoter was broken down into bits whereas the filter paper in the culture with the *lac* promoter became a bit smaller in size compared to vector control. This shows that recombinant *E. coli* JM109 is able to break down filter paper to some extent but not really able to use it as a source of carbon for growth, as the maximum growth attained by cultures containing *cenA* and *cex* is slightly less than the vector control and nearly the same as the negative control (Figure 5.25).

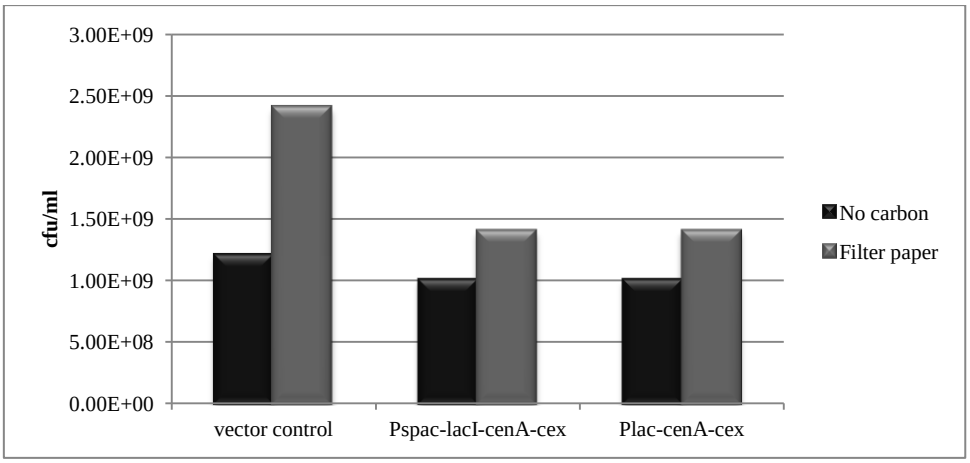


Figure 5.25: Comparison of maximum growth attained by different recombinant *E. coli* JM109 (vector control, P_{spac}-*lacI-cenA-cex*, P_{lac}-*cenA-cex*) when there was no carbon in the medium and when filter paper was used as a source of carbon.

5.4.2 Avicel as a carbon source:

To determine whether *E. coli* JM109 transformed with *cenA* and *cex* was able to use pure microcrystalline cellulose as a source of carbon for growth, 20 ml M9 medium (containing 90 µg/ml IPTG, 80 µg/ml carbenicillin and 0.1% w/v yeast extract) with 100 mg Avicel was used as a source of carbon. Again the flasks with 31 mM glucose were used as positive control and flasks without addition of any other carbohydrate was used as a negative control. The results showed the inability of recombinant *E. coli* JM109 to use Avicel as a carbon source for growth as the maximum growth attained by the cultures having *cenA* and *cex* was slightly less than the vector control. The results are presented in Figure 5.26.

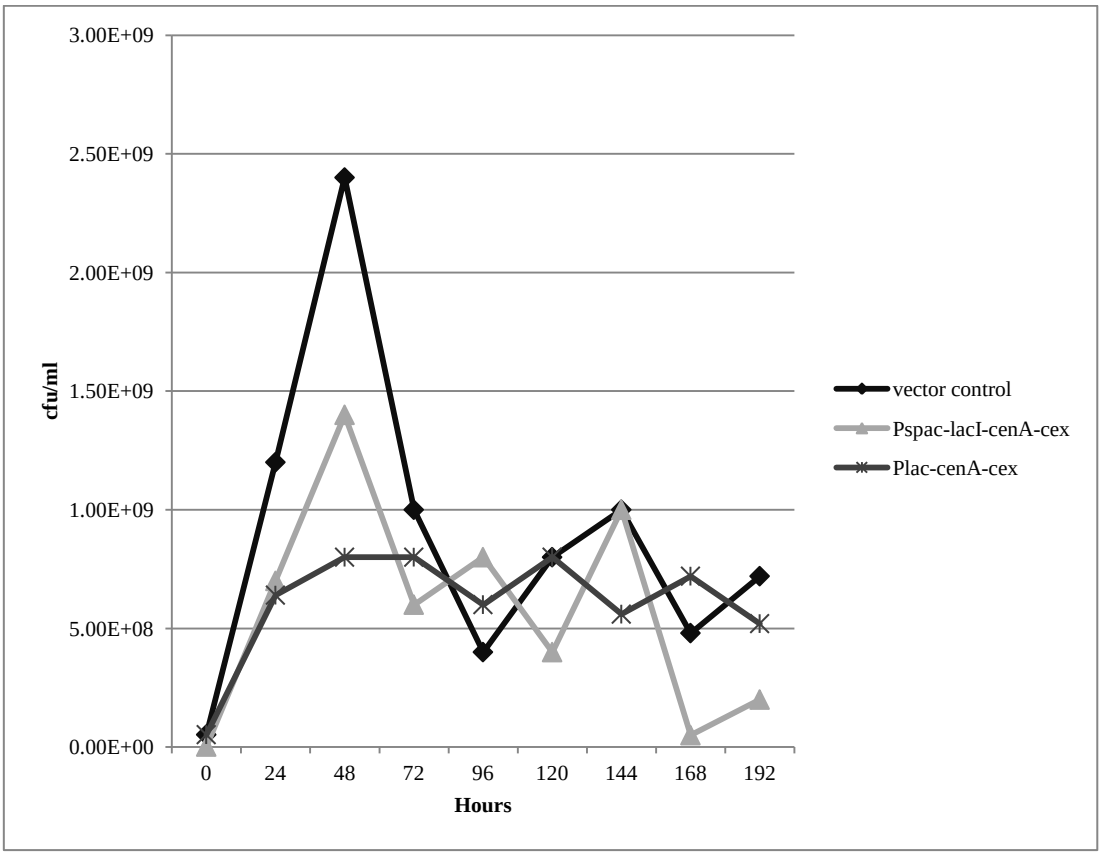


Figure 5.26: Graph showing growth of recombinant *E. coli* JM109 transformed with pSB1A2, pSB1A2+P_{spac}-*lacI-cenA-cex* and pSB1A2+P_{lac}-*cenA-cex* when Avicel was used as carbon source.

In addition the maximum growth by *E. coli* JM109 containing *cenA* and *cex* was nearly the same as the negative control and far less than the positive control as shown in Figure 5.27.

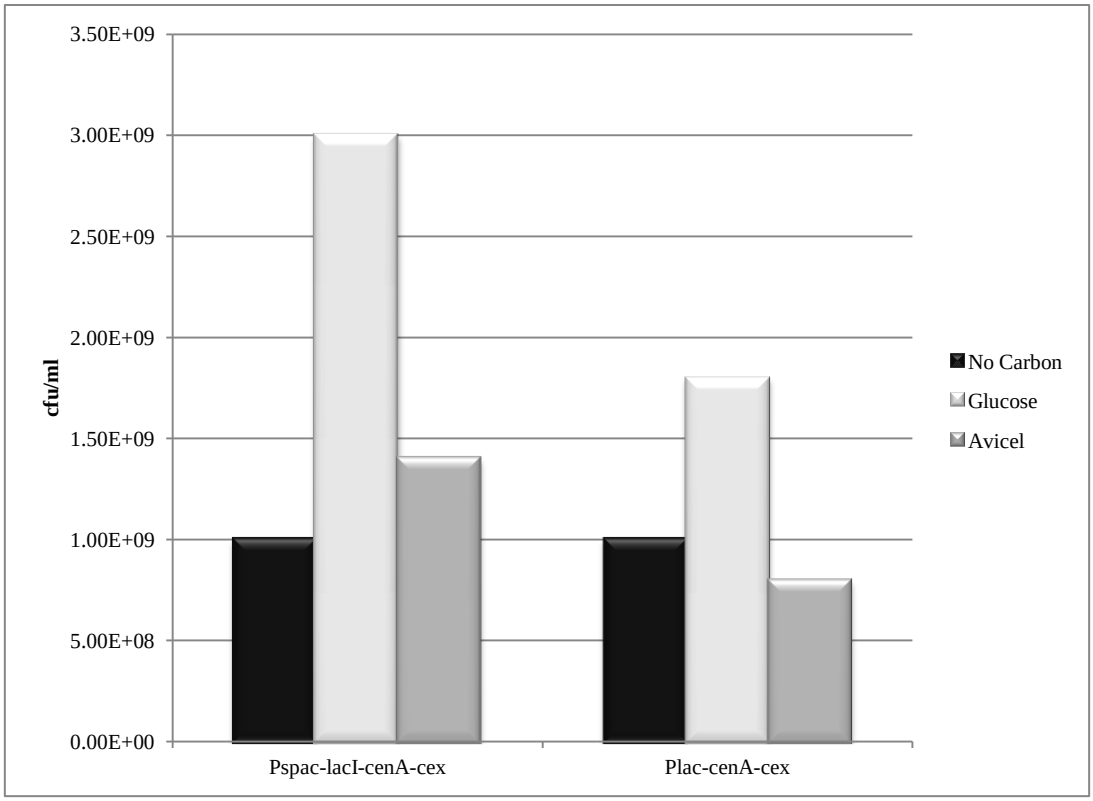


Figure 5.27: Maximum growth obtained by different recombinant *E. coli* JM109 (vector control, P_{spac}-*lacI-cenA-cex*, P_{lac}-*cenA-cex*) when different carbon sources were used. Glucose (G, positive control), No carbon (No C, negative control) and Avicel (A, test).

5.4.3 Comparison of growth with different carbon source:

When growth of different recombinant *E. coli* JM109 (vector control, P_{spac}-*lacI-cenA-cex*, P_{lac}-*cenA-cex*) was compared with different carbon sources it was observed that the growth was always better with glucose as a carbon source compared to filter paper and/or Avicel. With filter paper or Avicel growth was nearly the same as the negative control and less than the positive control. In addition the growth of recombinants having *cenA* and *cex* is also less than the vector control. The results are presented in Figure 5.28.

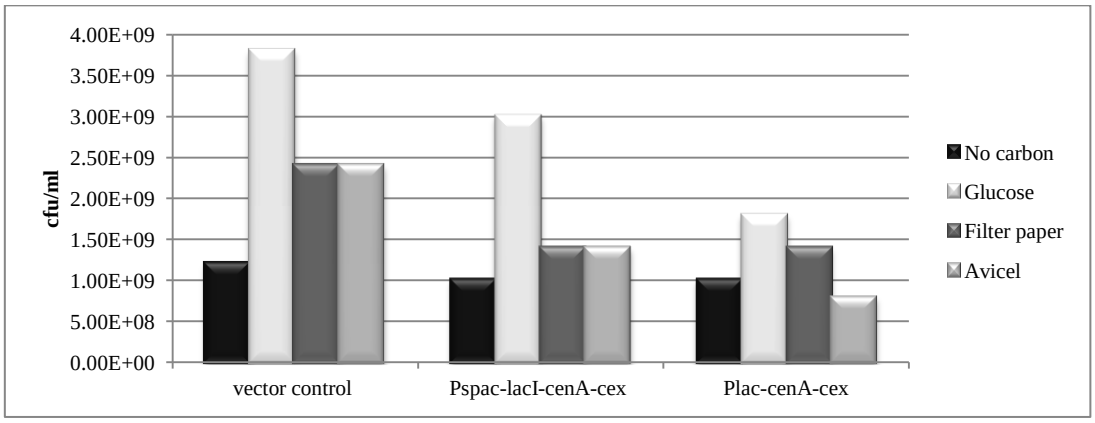


Figure 5.28: Comparison of growth of recombinant *E. coli* JM109 transformed with pSB1A2, pSB1A2+P_{spac}-*lacI-cenA-cex* and pSB1A2+P_{lac}-*cenA-cex* with different carbon sources.

When different strains were compared in the presence of glucose as a carbon source (as this was the carbon source that gave best growth) the growth was better in the vector control, suggesting that the expression of the genes *cenA* and *cex* might be causing some inhibition of growth in the test strains (Figure 5.29). However interestingly when the area under the curve was determined for each strain it was seen that the cultures with the *spac* promoter cover maximum area followed by the vector control and the cultures with the *lac* promoter. This shows that even though the maximum count in cultures with P_{spac} is slightly less than the vector control they are maintained for longer before they actually start to die. The results are shown in Figure 5.30. However, with all these results it is still difficult to draw a definite conclusion as the experiments were not performed in replicate and no statistical analysis was done.

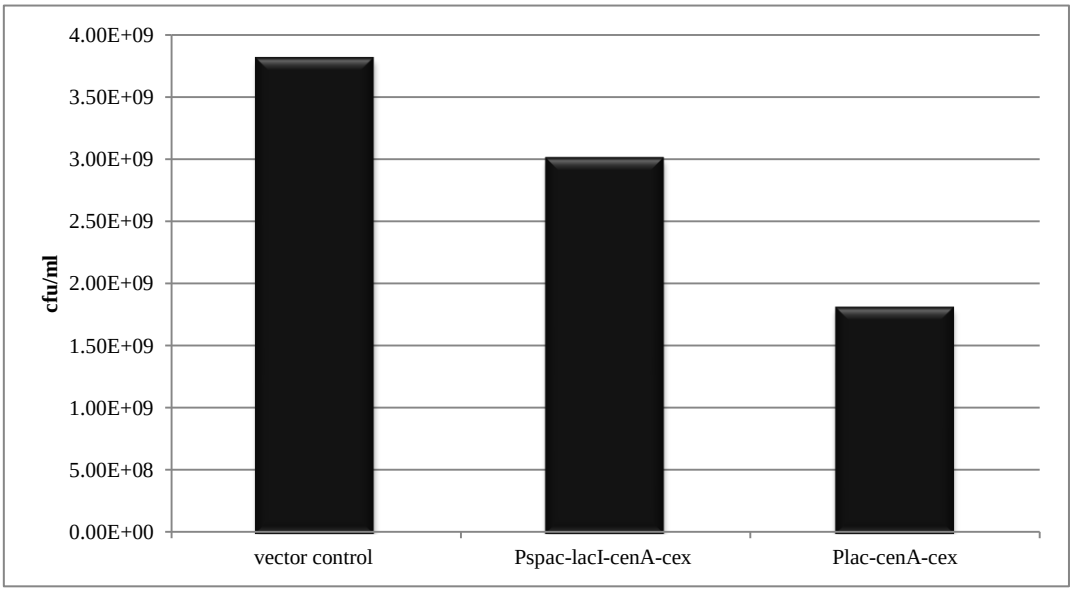


Figure 5.29: Maximum growth achieved by different *E. coli* recombinants (vector control, P_{spac}-*lacI-cenA-cex*, P_{lac}-*cenA-cex*) when glucose was used as a carbon source.

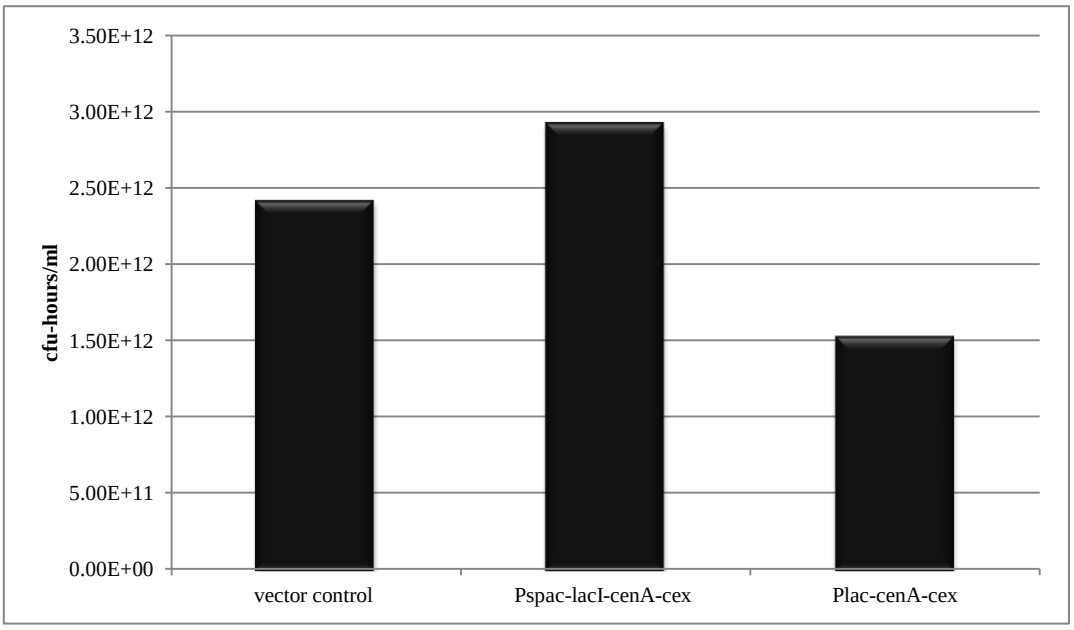


Figure 5.30: Maximum area covered under each curve by different recombinants of *E. coli* JM109 (vector control, P_{spac}-*lacI-cenA-cex*, P_{lac}-*cenA-cex*) when glucose was used as a source of carbon.

5.5 Discussion:

The above results showed that *C. freundii* SBS197 gave the best results under our experimental conditions. However, growth was maximum when *E. coli* JM109 were used but this growth was probably purely due to the yeast extract in the medium and not because of cellulosic substrates, as the vector control and the negative control also gave nearly the same growth. There was no physical breakdown of filter paper observed in the flask with vector control. However some breakdown of filter paper was observed in the flasks with our test strains of *E. coli* JM109 after 4-8 days, but they did not seem to be able to use filter paper as a carbon source because no additional growth was observed compared to the control strain. However, the filter paper only became slightly smaller in size compared to the vector control in cultures with the *lac* promoter, whereas it was broken down into pieces in cultures with the *spac* promoter. In contrast filter paper completely disappeared within 3-4 days when *C. freundii* SBS197 was used and the growth was much better (about 2 – 5 fold higher) in test strains compared to vector and/or negative control when Avicel/filter paper was used as a carbon source. The growth was much less when *B. subtilis* 168 was used, which was surprising as *B. subtilis* is known to degrade amorphous cellulose (Robson and Chambliss, 1984; Horikoshi *et. al.*, 1984) and can also secrete proteins very well into the medium compared to *E. coli*. The possible explanation for poor growth of *B. subtilis* 168 could be very low expression of the cellulase genes. We didn't manage to check the expression of *cenA* and *cex* in the case of *B. subtilis* 168 as it was naturally positive for the assays used.

The results hence show that *C. freundii* SBS197 is a better organism for cellulose degradation compared to *E. coli* JM109 and *B. subtilis* 168. The lower growth in *C. freundii* compared to *E. coli* might be overcome by providing more cellulosic substrate in the medium as it is possible that the amount of carbon provided in the medium in the form of cellulose was becoming limiting. Also changing the medium to M9 rather than CMM might give better growth as M9 is a well buffered medium compared to CMM and the tests with *E. coli* JM109 (which gave much higher growth) were performed in M9.

In all cases the cultures with P_{spac} seemed to be growing better as they attain maximum cell count and are maintained for longer compared to cultures with *lac* promoter. This is a bit surprising as in the case of *E. coli* JM109 the *lac* promoter gave much higher expression compared to the *spac* promoter. The expression level with the *spac* promoter in the case of *E. coli* JM109 (when grown in M9 medium) was much lower and near to the control strain but filter paper break down was more evident in cultures with P_{spac} than in cultures with P_{lac}. In the case of *C. freundii* SBS197, expression was much higher with the *spac* promoter than with the *lac* promoter and the enzyme was secreted well into the medium as confirmed by Cex assay, which again could also account for better breakdown of filter paper seen in *C. freundii* cultures.

As mentioned previously, Murai *et. al.* (1998) and Peterson *et. al.* (1998) engineered cellooligosaccharide and polysaccharide degrading strains of *S. cerevisiae*. None of these strains was able to use crystalline cellulose as carbon source. Our engineered *C. freundii* SBS197 can use Avicel and filter paper as a carbon source with maximum growth of up to 8.8×10^{8} cfu/ml and 1.2×10^{9} cfu/ml respectively, which is to our knowledge much better than what has been achieved so far using these substrates as carbon sources. However, the experiments with *C. freundii* need to be replicated in M9 medium so that statistical analysis can be done and a definitive conclusion can be drawn.

6 CONCLUDING REMARKS AND FUTURE PROSPECTS:

Cellulose, the major component of plant biomass, represents the conversion of solar power into organic matter via photosynthesis. Cellulolytic microorganisms that act on cellulose ensure the closure of the carbon cycle and the flow of carbon from fixed carbon sinks into the atmosphere. Due to the rising issues of global warming and green house effect related to the extensive use of fossil fuel, the search for alternative sources of fuel and energy has gained much attention during the last few decades. Hence the main aim of this project was to create BioBricks using cellulases from different cellulose degraders and then to check their activity against different cellulosic substrates. The ability of different host organisms to degrade crystalline cellulose and use it as a source of carbon was checked by performing growth curve experiments and measuring the growth as cfu/ml. To our knowledge this is the first report in which synthetic biology was used to create a novel heterologous cellulose degrader that could degrade crystalline cellulose and use it as a source of carbon for growth.

We started by cloning the probable cellulases from *Cytophaga hutchinsonii*, an active cellulolytic soil bacterium, belonging to the phylum Bacteroidetes (Xie *et. al.,* 2007; Larkin *et. al.,* 1989). Its genome analysis revealed the presence of a collection of genes presumed to be responsible for cellulose degradation, but it probably is using a mechanism different from other cellulose degraders as there were no processive cellulases nor cellobiohydrolases detected in its genome. The cloning of *C. hutchinsonii* cellulose degrading genes in an *Escherichia coli* host proved troublesome. In the case of dehydrogenases we were not able to get any transformants at all, whereas in the case of endoglucanases we did manage to obtain recombinant *E. coli* but the addition of IPTG for the induction of cloned genes resulted in severe growth limitation for some unknown reason. Linger *et. al.,* (2010) also reported the reduction in growth of recombinant *Zymomonas mobilis* transformed with two endoglucanases (E1 and GH12) from *Acidothermus cellulolyticus*. The effect on growth rate was detrimental when E1 was fused with the secretion signal of a hypothetical protein Z331 from *Z. mobilis*. Since the expression of E1 alone compared to Z331-E1 caused only minor limitation in growth, it was suggested that secretion of proteins was mainly responsible for this deleterious effect on growth. All four

cellulases (CHU_2130, CHU_2802, CHU_1944 and CHU_2315) from *C. hutchinsonii* were probably extracellular, as analysed by LipoP and PSORT programmes (www.expasy.org). Hence it may be due to the fact that the enzymes were clogging up the secretion system of *E. coli*. Therefore it would be beneficial to add native *E. coli* signal sequence in order for proper recognition of signal sequence and hence the secretion of proteins by the host organism. We also tried cloning CHU_1944 and CHU_2315 without the signal peptides, but unfortunately we were not able to get any recombinant *E. coli* cells at all. Therefore another possible reason could be an altered codon bias between *E. coli* and *C. hutchinsonii*, which may hinder the successful expression of cellulases in *E. coli*, since the SDS PAGE analysis also did not show any band distinct from the vector control. It has been reported in the literature previously that the differential codon bias between the host organism {*Acidothermus cellulolyticus*} and expression host {*E. coli* BL21(DE3)} could be a major obstacle for the successful expression of the heterologous proteins (Linger *et. al.*, 2010). Some of the codons used quite frequently by *C. hutchinsonii* (especially in the genes we tried to clone) (Table 3.3) are very rarely used by *E. coli* and hence this differential codon usage may have been responsible for detrimental effect on the expression. Hence one could use codon-enhanced strains or codon-optimized genes (via codon engineering), as these alternative methods have been reported in the literature to be useful to alleviate the detrimental effect of certain genes where altered codon usage between donor and the host organisms has been the issue (Linger *et. al.*, 2010).

As SDS-gel also did not show any protein band in the test sample that was distinct from vector control, it might would therefore be useful to use other more sensitive techniques for detection of expressed proteins like immunostaining (for example flow cytometry, immunoblotting, enzyme-linked immunosorbent assay etc.), immunoprecipitation (in which particular protein is precipitated out of the solution using an antibody that specifically binds to that protein) and/or immunoelectrophoresis etc., as these techniques are very sensitive and can detect very low quantity of expressed proteins.

As was observed from the result (Figure 3.6) that the addition of IPTG for induction of genes resulted in severe growth limitation, it might therefore be useful to add IPTG latter on during the growth (after considerable growth is obtained) in order to

minimize the toxic effect of expressed protein on growth and hence get some suitable level of expression. Nakajima *et. al.,* (1993) reported the introduction of *bglH* from *Bacillus circulans* into *Saccharomyces cerevisiae*. They also observed that the presence of the enzyme caused inhibition of growth and cell expansion. The expression of *bglH* resulted in decreased cell size and expansion of vacuoles probably due to the erosion of the β-1,3-glucan-containing cell wall of yeast. This toxic effect of the protein on yeast was overcome by incubating the organism at 16°C for 15 days (Nakajima *et. al.,* 1993; Lynd *et. al.,* 2002). Hence one could also try prolonged incubation of *E. coli* cells transformed with *C. hutchinsonii* genes at low temperature. This might circumvent the toxic effect of the gene products and we might be able to get some expression.

Synergy between different organisms in utilizing cellulosic biomass has been well reported in the literature (Ponce-Noyola and Torre, 1993; Rodriguez and Gallardo, 1993; Halsall and Gibson, 1985). Because the cloning and expression of *C. hutchinsonii* cellulases proved unexpectedly difficult, it was therefore decided to look for an alternative approach, in which a cellulose degrader is used in a mixed culture with an organism that could be genetically modified to introduce a production system. Hence growth curve experiments were performed with *Cytophaga hutchinsonii*/*Escherichia coli* and/or *Cytophaga hutchinsonii*/*Bacillus subtilis*. The results showed increased growth and a shorter lag phase when organisms were growing together (Figure 3.8) than when they were growing individually implying that there was a phenomenon of synergy observed when the organisms were growing together. *C. hutchinsonii* was cross feeding the two organisms, into which we could eventually introduce a (ethanol or any other commodity) production system for the formation of useful product from cellulose. However growth was much better when *C. hutchinsonii*/*B. subtilis* was used than when *C. hutchinsonii*/*E. coli* was used (Figure 3.8 and Table 3.2) indicating that the *B. subtilis* was a better option for the introduction of a production system which could then be used in a mixed culture with *C. hutchinsonii* for the production of useful products. These experiments were very preliminary but provide a useful line of investigation for further research and development.

Secondly, all *C. hutchinsonii* genes were initially cloned in pT7-7 due to the presence of forbidden restriction sites inside the genes required by the BioBrick vectors. pT7-7

is one of the series of pET vectors developed by Studier and Moffatt (1986) for high level expression of desired protein using bacteriophage T_7 RNA polymerase. As the plasmid contain Ø T_7 promoter, an S30 T_7 high-yield protein (cell free) expression system (from promega) could be used for the expression of *C. hutchinsonii* proteins. This system uses T_7 RNA polymerase for the expression of proteins directly from plasmid vectors containing a T_7 promoter and a ribosome binding site. Hence one can get about 500 µg/ml of protein in an hour (www.promega.com). This would not be appropriate for large scale production of the enzyme as it probably would be too expensive for that purpose, but would allow us to confirm the cellulase activity of these enzymes. In addition one could also try cloning these genes into newer versions of pET vectors which are designed with enhanced features to permit easier sub-cloning, detection and purification of target proteins. As for example many pET vectors encode a number of peptide tags which when fused with desired protein, facilitates detection and purification of target protein, whereas others increases biological activity by affecting solubility in the cytoplasm or export to the periplasm. The various fusion tags for pET vectors include T7-tag, S-tag, His-tag which helps in purification, whereas pelB/ompT, CBD_{cenA}-tag, CBD_{cex}-tag helps in protein export/folding.

Since the cloning and expression of *C. hutchinsonii* genes proved to be unexpectedly difficult, we therefore decided to clone the well-characterized equivalents from *Cellulomonas fimi*, a Gram positive bacterium of genus Cellulomonas (Greenberg *et. al.*, 1987; Stackbrandt and Kandler, 1979) with a very high G+C content (72 mol%). It is an active soil bacterium which degrades crystalline cellulose. *C. fimi* cellulases are very well characterized (Wong *et. al.*, 1986; Damude *et. al.*, 1996; Meinke *et. al.*, 1991; Meinke *et. al.*, 1993; Meinke *et. al.*, 1994; Tomme *et. al.*,1996; Shen *et. al.*, 1995; O'Neill *et. al.*, 1986; Macleod *et. al.*, 1994) and include four endoglucanase, two cellobiohydrolase and an exoglucanase/xylanase (Table 1.3).

We selected three different host organisms: *Escherichia coli*, *Citrobacter freundii* and *Bacillus subtilis* as an expression host for cloning *C. fimi* cellulases for several reasons. *E. coli* was chosen as being the most commonly used organism for genetic manipulation studies, as the genetic techniques for its manipulation are very well developed. It has a number of easily controllable promoter systems which can be used for the controlled expression of foreign genes. *C. freundii* and *B. subtilis* were

selected because they can both secrete proteins very well into the surrounding medium compared to *E. coli*. *B. subtilis* in addition has a GRAS status and *C. freundii* is a category 1 organism as classified by ACDP. *C. freundii* can assimilate cellobiose (a major breakdown product of cellulose), whereas *B. subtilis* can degrade amorphous cellulose. Because *C. freundii* is a close relative of *E. coli*, the same cloning vectors may be used for the expression of genes. On the other hand *B. subtilis* can form spores and survive unfavourable conditions for years. Hence once all manipulations were done in *E. coli*, the plasmids were extracted and incorporated in *B. subtilis* and *C. freundii* as well.

We opted for a synthetic biology approach, by creating different cellulase BioBricks and then combining them together, since it allows an endless number of genes to be joined together in the number of stages which is actually the log (base 2) of the length of assembly. As Wong *et. al.*, (1988) reported the degradation and hydrolysis of filter paper and pre-treated wood chips by *S. cerevisiae* transformed with *C. fimi cenA* and *cex* in the presence of small amount of β-glucosidase, we thought it might be beneficial to combine *cenA* and *cex* first to test the procedures and assay their activities against different cellulosic substrates. Therefore *cenA* and *cex* BioBricks (created by the University of Edinburgh, iGEM team 2008) were combined together and inserted downstream of the *lac* promoter (from the *lac* operon) and *spac* promoter (a hybrid promoter made by using the promoter element of phage SPO-1 and *lac* promoter) designed especially to make an inducible system that works in *B. subtilis*.

Once combined these systems were introduced into *E. coli*, *C. freundii* and/or *B. subtilis* and tested for the expression of *cenA* and *cex* activities. It was observed that *C. freundii* SBS197 gave the best performance under our experimental conditions. We were not able to check the expression in *B. subtilis* 168 as the control strain was already positive for the assay methods available, which is consistent with its ability to degrade amorphous cellulose (Robson and Chambliss, 1984; Horikoshi *et. al.*, 1984). In *E. coli*, only the *lac* promoter seemed to be active. The enzyme activity was nearly the same as the vector control in the case where the *spac* promoter was used to control expression. This could be due to the positioning of *lacI* downstream of P_{lac}, hence each time when the promoter was induced it resulted in expression of repressor as well. By contrast, in the case of *C. freundii*, both *lac* and *spac* promoters were active although the expression was better with *spac* promoter

than with *lac* promoter when cultures were grown in M9 medium. This was really surprising as the *spac* promoter was designed to be used in *B. subtilis* but it seemed to be performing very well and giving higher expression compared to the *lac* promoter in *C. freundii*. Although overall expression was higher in *E. coli* having the *lac* promoter, most of this enzyme activity was residing inside the cells and was not secreted into the medium. The importance of cellulases being secreted into the medium has been well reported in the literature (Linger *et. al.,* 2010; Lejeune *et. al.,* 1988; Yanase *et. al.,* 2005; Brestic-Goachet *et. al.,* 1989). Cellulolytic enzymes, especially endoglucanases, if remaining localized within the organism, are essentially wasted regarding the extracellular degradation of cellulosic biomass. Hence it is really important for at least the endoglucanase to be secreted into the surrounding medium for initial breakdown of cellulose fibres before they can enter the cell for further metabolism. In *C. freundii*, a considerable amount of activity was detected in the medium, showing that the protein is secreted well into the surrounding medium. This observation (that secretion was better in *C. freundii* SBS197 and almost no activity was detected in the supernatant of *E. coli* JM109) was not surprising as *E. coli* lacks the Main Terminal Branch of the type 2 secretion system and is mostly poor at secreting heterologous proteins (Linger *et. al.,* 2010; Makrides, 1996). Therefore although the expression was slightly less in *C. freundii* SBS197, we expected *C. freundii* SBS197 to give better performance compared to *E. coli* JM109 when it comes to the breakdown of cellulosic substrates. Hence growth curve experiments were performed with recombinant *E. coli* JM109, *C. freundii* SBS197 and *B. subtilis* 168 using cellulosic substrates as a source of carbon for growth. Various cellulosic materials like filter paper and Avicel were tested. As was expected, *C. freundii* SBS197 gave the best results under our experimental conditions.

Recombinant *C. freundii* SBS197 transformed with *cenA* and *cex* when grown in minimal medium gave about 2 – 5 fold higher growth compared to control strain. Filter paper was completely destroyed within 3 – 4 days and maximum growth was obtained when the expression was controlled by P_{spac} as compared to when it was controlled by P_{lac}. This is consistent with the fact that the expression and secretion of enzyme was better when in minimal medium the *spac* promoter was used.

However when the overall growth of different organisms was compared, it was observed that *E. coli* JM109 was giving higher growth compared to

C. freundii SBS197 and *B. subtilis* 168. However, this growth was probably due to yeast extract in the medium (which was added so that the organisms could achieve a certain level of growth to produce cellulases before they can actually be able to degrade cellulose) and not because of cellulosic substrates, as the vector and/or negative control also gave nearly the same growth. We did observe some physical breakdown of filter paper by recombinant *E. coli* JM109 (containing *cenA* and *cex*) after 4 – 8 days of incubation but *E. coli* did not seem to be able to use it as a source of carbon for growth because the growth in the vector control was greater than in the ones having *cenA*/*cex*, and there was no physical breakdown of filter paper observed in the flask with vector control. However, filter paper was broken down into pieces (but did not completely disappear) in *E. coli* cultures with the *spac* promoter (Figure 5.24) but only slightly decreased in size (compared to vector control) in cultures with the *lac* promoter after 4 – 8 days of incubation. Since most of the activity in cultures with the *lac* promoter was residing within the cells, it was not surprising that the filter paper only slightly decreased in size. However the fact that it was broken down into pieces in cultures having P_{spac} was surprising because when expression was checked in these cultures, the activity was nearly the same as in the vector control. This could be due to the fact that expression was checked only after 24 hours of growth and induction, whereas filter paper break down only became apparent after 4 – 8 days of incubation. Therefore it is possible that the expression of cellulases in *E. coli* with *spac* promoter is delayed due to some reason and the activity may well have been secreted after several days as observed by Van Rensburg *et. al.,* (1994). They observed delayed secretion of endoglucanase (*end1*) from *Butyrivibrio fibrisolvens* by *S. cerevisiae*. It may therefore be worth checking the expression after a few days of incubation with cellulosic substrates.

There was no physical degradation of filter paper observed in the case of recombinant *B. subtilis* 168 and growth was much less and the same as the negative and/or vector control. One possible reason could be very low expression of the genes, as we did not manage to check the expression of *cenA* and *cex* in this organism because of the lack of availability of suitable assays for which the control strain wouldn't be positive.

There have been few reports in the literature describing the growth of recombinant yeast (*S. cerevisiae*) on cellulosic substrates as a source of carbon (Peterson *et. al.,* 1998; Murai *et. al.,* 1998). Polysaccharide degrading yeast was engineered by

Peterson *et. al.* whereas Murai *et. al.* designed a cellooligosaccharide assimilating strain of yeast. Neither of these recombinant yeasts was able to utilize Avicel as a source of carbon for growth and the maximum growth reported by Murai *et. al.* for yeast using cellooligosaccharides was only about 9×10^6 cells/ml. Similarly the strain constructed by Peterson *et. al.* could only partially degrade amorphous cellulose and the growth attained was less than 10^7 cells/ml. Our engineered *C. freundii* SBS197 can use Avicel and filter paper as a carbon source with maximum growth of up to 1.2×10^9 cfu/ml and 8.8×10^8 cfu/ml respectively, which is to our knowledge much better than what has been reported so far using these substrates as a source of carbon for growth. However these experiments need to be repeated with replicates so that statistical analysis can be done and a definite conclusion can be drawn.

By looking at the overall results it was therefore concluded that *C. freundii* SBS197 is a better organism for the degradation of cellulosic substrates than *E. coli* JM109 and/or *B. subtilis* 168. However the low growth compared to *E. coli* may well be due to limitation of carbon, phosphate or nitrogen required for growth. Hence it would be an idea to check the growth by increasing the amount of each of these (carbon, phosphate and/or nitrogen) one by one. In addition the experiments for *E. coli* JM109 were performed in M9 medium and for *C. freundii* SBS197 CMM medium was used. CMM contains considerably less phosphate compared to M9 which may have some effect on the growth. In addition, another line of investigation in context with cellulose breakdown by *C. freundii* SBS197 could be the study of the degradation products of cellulose breakdown via HPLC (high-performance liquid chromatography). This technique would also allow us to reveal that if all the cellulose provided in the medium is been degraded and/or used, as in case of Avicel we cannot see any physical degradation due to the fact that it already is in powdered form. It would also be interesting to test other cellulosic substrates to see if the organism can use them as a source of carbon. In addition growth studies could also be performed using cells transformed with just *cenA* or just *cex* to see if the combined cellulases have some synergistic effect on growth and degradation.

Since the cellulase BioBricks developed to provide the organism with the ability to degrade cellulose, was successful and the organism have shown to grow using Avicel and filter paper as carbon source, this hence could be developed as a possible project in which BioBricks of other cellulases are developed and checked in combination

with the BioBricks designed so far against different cellulosic substrates. Hence a tool kit of exchangeable BioBricks parts could be made which are active against different cellulosic substrates. The modular interchangeable nature of BioBrick component could therefore help to generate a library of putative biomass degrading enzymes from different organisms and a parallel assembly process could be used to generate organisms expressing variety of different combinations of enzymes effective for degradation of different classes of biomass substrates.

The data presented in this report also shows that *C. freundii* SBS197 could prove to be an extremely valuable organism for the degradation of cellulosic substrate for its conversion into other commodity products by Consolidated Bioprocessing. It is a novel host strain not previously used for recombinant cellulose degradation and therefore shows a great potential for further investigation. We are currently incorporating a product formation system in *C. freundii* SBS917, by introducing *pdc* and *adhB* from *Z. mobilis* ZM4 {PET operon, previously used with *Escherichia coli* and *Klebsiella oxytoca* (Ohta *et. al.*, 1991; Ingram *et. al.*, 1987)} after *cenA* and *cex* BioBrick so that the organism can degrade cellulose and convert it into ethanol. Hence the ability of *C. freundii* SBS197 to express and secrete cellulases (using cellulase BioBricks) shown in this study and the capacity to degrade crystalline cellulosic substrates, shows that the infrastructure has been laid for further investigating in the establishment of *C. freundii* as a CBP organism.

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8 APPENDIX:

Media for *E. coli*:

Luria Bertani medium (Atlas, 1997)	
Bacto tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g
Distilled water	1000 ml

Media for *C. hutchinsonii*:

Dubos mineral medium (Atlas, 1997)	
$NaNO_3$	0.5 g
K_2HPO_4	1 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4.7H_2O$	10 mg
Distilled water	1000 ml

Media for *C. freundii*:

CMM medium (C. French, University of Edinburgh, personal communication)	
Na_2HPO_4	2 g
KH_2PO_4	1.32 g
NH_4Cl	1.07 g
Trace elements A	1 ml
Trace elements B	1 ml
Distilled water	1000 ml

Media for *E. coli* and *B. subtilis*:

M9 medium (Atlas, 1997)	
Na_2HPO_4	7 g
KH_2PO_4	3 g
NH_4Cl	1 g
NaCl	0.5 g
Trace elements A	1 ml
Trace elements B	1 ml
Distilled water	1000 ml

Trace elements A (C. French, University of Edinburgh, personal communication)	
$MgCl_2.6H_2O$	203.3 g
$CaCl_2$	11.76 g
Distilled water	1000 ml

Trace elements B (C. French, University of Edinburgh, personal communication)	
$FeSO_4.7H_2O$	22.4 g
$ZnSO_4.7H_2O$	5.76 g
$MnSO_4.4H_2O$	4.44 g
$CuSO_4.5H_2O$	1.00 g
$CoSO_4.H_2O$	0.62 g
H_3BO_3	0.248 g
HCl	20 ml
Distilled water	1000 ml

Do not autoclave; acidity should be sufficient to prevent contamination

Antibiotics:

Antibiotic	Stock solution (mg/ml)	Working solution (µg/ml)
Ampicillin	100	100
Carbenicillin	80	80
Chloramphenicol	50	10 (for *B. subtilis*), 40 (for *E. coli*)

Reagents and solutions:

20x TAE (Tris – acetate – EDTA buffer)	
Tris base	48.4 g
Sodium EDTA	3.72 g
Dissolve them both in about 450 ml of water and then add 11.4 ml glacial acetic acid. Make the final volume up to 500 ml with water.	

Agarose gel loading buffer	
50% v/v glycerol	0.6 ml
20x TAE	0.3 ml
10% w/v SDS	0.1 ml
Bromophenol blue	Few grains

1 kb DNA ladder	
Ladder stock solution	20 µl
Loading buffer	20 µl
Water	60 µl

Ethidium bromide	
10 mg/ml stock	20 µl
Water	100 ml

Gel Green	
10,000x stock	5 µl
Water	50 ml

Light sensitive

Sybr-Safe	
10,000x stock	5 µl
Water	50 ml

Store at 4°C

6M Sodium iodide for DNA purification	
NaI	18 g
Water	Make the final volume up to 20 ml

Store at 4°C

Glass bead suspension for DNA purification	
Silica gel (Sigma S-5631)	1 g
Suspend it in 10 ml 10 mM phosphate buffer, pH 7, with 0.9% w/v NaCl. Let the beads settle for about 2 hours. Discard the supernatant containing finest particles. Repeat this procedure once again. Transfer the remaining suspension to a 1.5 ml microcentrifuge tube, spin briefly, remove supernatant and resuspend the beads in 5 ml of 3 N NaI.	

Store at 4°C

EB (Elution buffer) for DNA purification	
Tris base	53 mg
Tris HCl	88 mg
Deionised water	100 ml

Sterilize by autoclaving

Wash buffer for DNA purification	
Tris base	24 mg
Tris HCl	127 mg
NaCl	293 mg
Na_2 EDTA	93 mg
Absolute ethanol	50 ml
Deionised water	50 ml

Store at -20°C

1x TSS (Transformation and storage solution) for *E. coli* and/or *C. freundii*	
LB broth	17 ml
40% w/v PEG 3350	5 ml
1M $MgCl_2$	1 ml
DMSO	1 ml

All components except for DMSO should be sterilized by autoclaving prior to mixing. Store at 4°C

PTM (pre-transformation medium) for *B. subtilis*	
5x Spizizen salts	2 ml
20% w/v glucose	0.5 ml
Solution P	0.10 ml
20% w/v Casamino acids	0.20 ml
Indole (11 mg/ml in DMSO)	10 µl
Sterile water	8 ml

TM (Transformation medium) for *B. subtilis*	
5x Spizizen salts	2 ml
20% w/v glucose	0.3 ml
20% w/v casamino acids	5 µl
Indole (11 mg/ml in DMSO)	10 µl
Sterile water	8 ml

5x Spizizen salts for *B. subtilis* transformation	
Ammonium sulphate	1 g
Dipotassium hydrogen phosphate	7 g
Potassium dihydrogen phosphate	3 g
Sodium citrate dihydrate	0.5 g
Magnesium sulphate	0.1 g
Deionised water	100 ml

Sterilize by autoclaving

Solution P for *B. subtilis* transformation	
$MgSO_4.7H_2O$	25 ml
$CaCl_2$	5 ml
$MnSO_4.4H_2O$	0.1 ml
Sterile water	70 ml

Solution 1 for plasmid DNA miniprep	
5mg/ml RNAse A	5 µl
Deionised water	1 ml

Solution 2 for plasmid DNA miniprep	
0.4M NaOH	0.5 ml
10% SDS	0.1 ml
Deionised water	0.4 ml

Solution 3 for plasmid DNA miniprep	
Potassium acetate	29.45 g
Dissolve it in about 80 ml water and then add 11.45ml glacial acetic acid. Make the final volume up to 100 ml with water.	

Store at 4°C

LacZ buffer for determining the specific activity of *cex* using ONPC	
Na_2HPO_4	8.52 g
NaH_2PO_4	5.51 g
KCl	0.75 g
$MgSO_4.7H_2O$	0.75 g
2-mercaptoethanol	2.7 ml
10% (w/v) SDS	0.5 ml
Distilled water	1000 ml

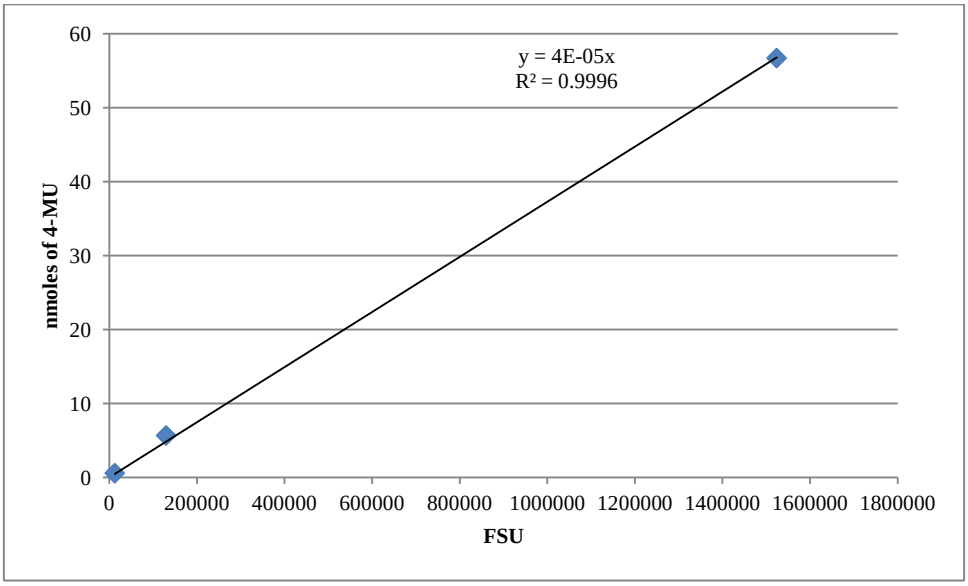
pH 7

Phosphate buffer for determining the specific activity of *cex* using ONPC	
$Na_2HPO_4.7H_2O$	1.61 g
NaH_2PO_4	0.55 g
Distilled water	100 ml

pH 7

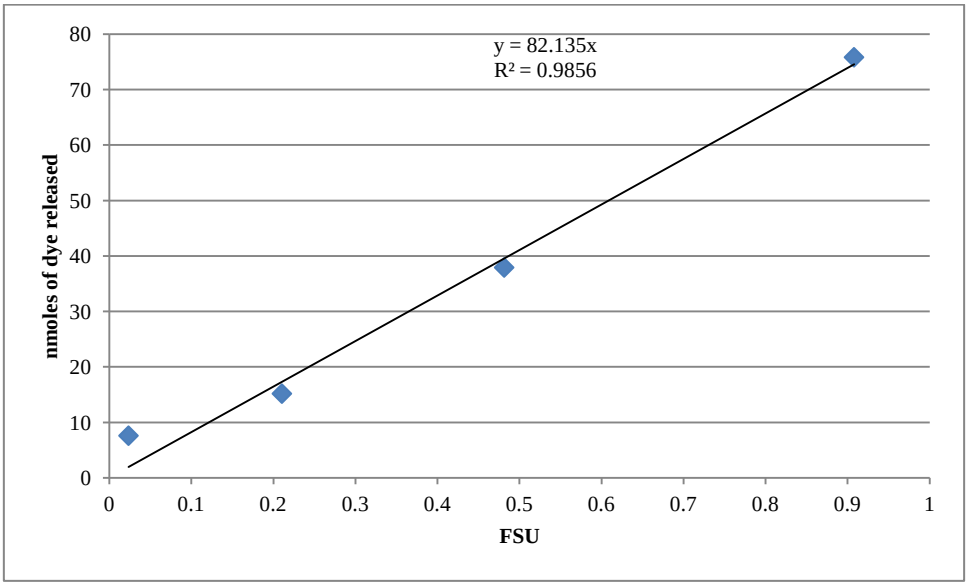
PBS	
KH_2PO_4	0.21 g
NaCl	9 g
Na_2HPO_4	0.726 g
Distilled water	1000 ml

Calibration curve for *cex* assay:

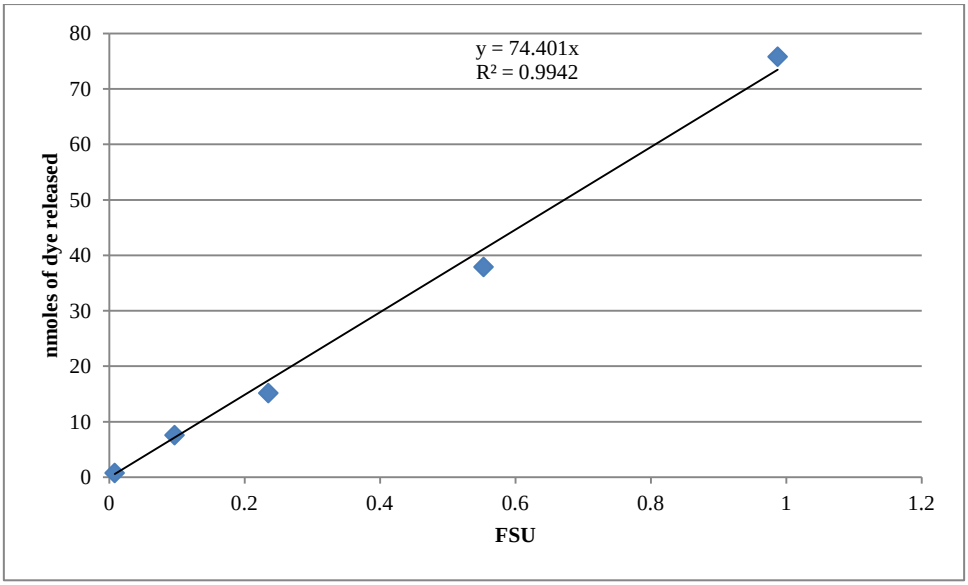


Standard curve for MUC (*cex* assay) prepared using different concentrations of 4-MU. Slope (nmoles of 4-MU/FSU) was determined using trendline option in Microsoft excel followed by the determination of reaction rates by multiplying the slope of the standard by the slope of the sample (FSU/time). y – slope of standard, R^2 – line of regression.

Calibration curve for *cenA* assay:



Standard curve for *cenA* assay prepared using different concentrations of RBB. Slope (nmoles of dye-brilliant blue/FSU) was determined using trendline option in Microsoft excel followed by the determination of reaction rates by multiplying the slope of the standard by the slope of the supernatant sample (FSU/time). this gives the nmoles of dye released/min. Since approximately 20 sugar residues were attached per dye molecule therefore this valus of then multiplied by 20 to get nmoles of sugar/min. y – slope of standard, R^2 – line of regression.



Standard curve for *cenA* assay prepared using different concentrations of RBB. Slope (nmoles of dye-brilliant blue/FSU) was determined using trendline option in Microsoft excel followed by the determination of reaction rates by multiplying the slope of the standard by the slope of the extract sample (FSU/time). this gives the nmoles of dye released/min. Since approximately 20 sugar residues were attached per dye molecule therefore this valus of then multiplied by 20 to get nmoles of sugar/min. y – slope of standard, R^2 – line of regression.