Microbial Biodegradation of Lignocellulose for Ethanol Production

Thesis

Microbial Biodegradation of Lignocellulose For Ethanol Production

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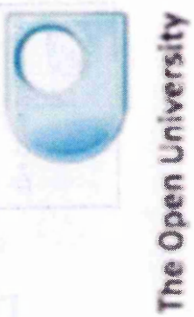
Thesis submitted for the degree of Doctor of Philosophy



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In accordance with

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Dedicated to my family...

Without you none of this would have ever been possible.

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ABSTRACT

Bioethanol can be produced from a range of substrates and it is considered to be renewable. Lignocellulose is one of the substrates that can be used for bioethanol production. Genetic engineering of a microorganism that can completely convert all the sugars of cellulosic material into ethanol is one of the important steps for the production process.

This study has two parts that aim to produce bioethanol by using genetically modified microorganisms. The first part of the study deals with bioethanol production from lignocellulosic material by microbial fermentation in two steps. The first step is the degradation of the lignocellulosic material by *Bacilli* to produce cellobiose, and the second step is to convert cellobiose into glucose by a β-glucosidase expressing recombinant yeast strain. As lignocellulosic material, wood powder from industrial waste was used and the degradation of the wood powder into glucose was confirmed by following the growth curve of the microorganisms and through appropriate enzymatic assays.

The second part of the study utilizes two novel technologies, Yeast Artificial Chromosome (YAC) and Bridge-Induced Translocation (BIT) to introduce new, multi-factorial genetic traits into a yeast strain, a process that would otherwise take several time-consuming and labor-intensive rounds of genetic engineering. After cloning exogenous cellulases onto a YAC vector, this was transformed into a *PEP4* mutant yeast upon which BIT technology was applied. This technology allowed the research to gain two advantages; one of them was to stabilize the YAC within the yeast genome and the other one was to have increased gene expression level consequent to the translocation event.

In conclusion, this work defines a successful microbial system that is able to efficiently utilize lignocellulosic material as a carbon source and a translocant yeast strain that has high level of cellulase activity.

LIST OF ABBREVIATIONS

ADH1	Alcohol dehydrogenase 1
ALP	Arginine transporter pathway
ARS	Autonomously replicating sequence
BGL	Beta-glucosidase
BIT	Bridge induced translocation
Bp	Base- pairs
CBH	Cellobiohydrolase
CPY	Carboxy peptidase Y
CYC1	Cytochrome C
ddH2O	Double distilled HO
EDTA	Ethylenediaminetetraacetic Acid
EGC	Endogluconase
ER	Endoplasmic reticulum
FLP	Flipase recombinase
FRT	Flipase recognition target
GTP	Guanosine triphosphate
HPLC-ELSD	High Performance Liquid Chromatography Evaporative Light Scattering Detectors
KBp	Kilo- Base pairs
LiAc	Lithium acetate
OST	Oligosaccharyl transferase
PEG	Polyethylene glycol

PGK1	Phosphoglycerate kinase 1
PHO5	Acid phosphatase
PMT	Protein O-mannosyl transferase
SPC	Signal peptidase complex
SRP	Signal recognition particle
SRPR	Signal recognition particle receptor
STIK	Specific targeted integration of kanamycin
TEF1	Translation Elongation Factor 1
Ubiq	Ubiquitin
YAC	Yeast artificial chromosome

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1. INTRODUCTION

1.1 YEAST AS A MODEL ORGANISM

Saccharomyces cerevisiae has been established over the years as a model eukaryotic organism. The origin of the name is coming from Latinized Greek where *Saccharomyces* means sugar-fungus; *Saccharo* stands for ''sugar" while *mycos* means ''fungus" and *cerevisiae* is Latin origin and means ''of beer''. It represents a typical eukaryote that offers the advantages of simplicity in cellular organization and easiness of manipulation via both classical genetics and modern recombinant DNA technology. The many features of this organism help in scientific studies.

For the scientific studies, yeast has a lot of advantageous to work with. *S. cerevisiae* does not require any complicated working conditions and is remarkably inexpensive compared to higher eukaryotic organisms. To work with, it does not need any special safety equipment and does not cause any infections or have toxicity for humans. It grows easily and rapidly at 30°C with a doubling time around 1,5- 2 hours.

The genetic system of the *S. cerevisiae* is very well defined and this helps a lot to scientific studies. Transforming with exogenous DNA can be done without any difficulty and this makes genetic engineering and genome manipulation easily efficient (Botstein, Chervitz, & Cherry, 1997; Merlini, Dudin, & Martin, 2013).

S. cerevisiae can be in haploid or in diploid form in nature and divides by budding. Meiotic division converts diploid strain to haploid strain similar to gametes and this division can be induced by starvation where there are no fermentable carbon sources (Byers, 1982). During the meiotic division, yeast produces asci containing four haploid spores that can

undergo vegetative growth or can subsequently mate. Haploid yeast cells appear in two mating types in nature as a mating type a or mating type alpha and they can stay either in stable haploid form or they can mate with the opposite mating type to produce diploid yeast cells (Herskowitz, 1988; Neiman, 2005). This feature of two forms of yeast life cycle is an additional aspect that makes yeast cells interesting to geneticists. Figure 1.1 shows the life cycle of the budding yeast.

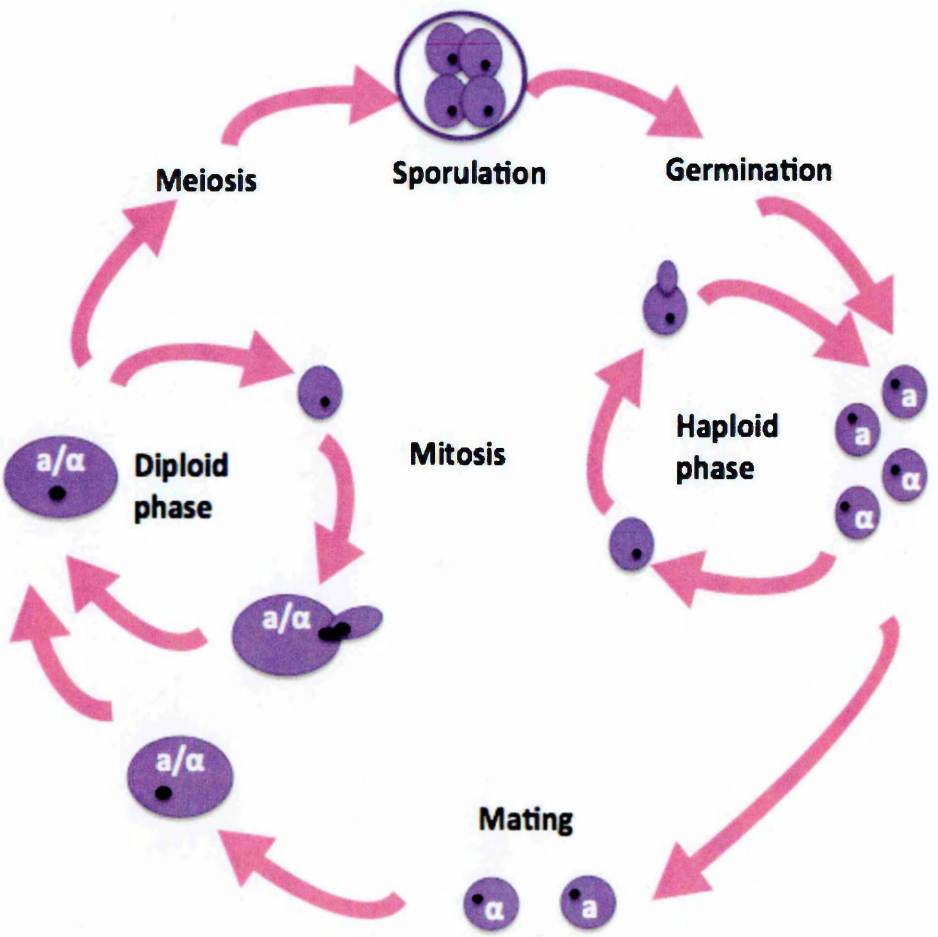

Figure 1.1: Yeast life cycle.

Budding yeast, *S. cerevisiae* has a small genome, which is around 12.5 Mb and contains 16 chromosomes ranging in size from 200 to 2000kb (A Goffeau et al., 1996; Mortimer &

Schild, 1985). Genome replication begins at the origin of replication locus and occurs bidirectionally. Yeast cells can have both mitotic and meiotic recombination. Mitotic recombination takes place in G1 phase before DNA synthesis where else meiotic recombination takes place in G2 after DNA synthesis. Due to the knowledge of the yeast recombination system, *S. cerevisiae* is a perfect model organism to study the recombination process in eukaryotic organisms (Herskowitz, 1988; Wagstaff, Klapholz, & Esposito, 1982).

The scientific community of yeast has a strong collaboration among itself. This helps us to go faster in yeast molecular biology and genetics field. All over the world, a large number of yeast mutants was collected and characterized. The first genetic map of *S. cerevisiae* was done in 1960s and by the help of it, research with yeast developed rapidly (Goffeau et al., 1996; Goffeau, 2000; Hawthorne & Mortimer, 1960; Mortimer & Schild, 1985). Yeast genome sequencing was completed in 1996 and this microorganism became the first eukaryote with the known DNA sequences (Mewes et al., 1997). Having the whole genome sequenced was a big achievement in science, able to allow and to make the progress smooth for the further experiments. Since the yeast genome sequenced, 5000 out of estimated 6000 yeast genes are well recognized (Goffeau, 2000; Peña-Castillo & Hughes, 2007) that make yeast a model organism in a wide variety of genetic analysis regarding to cell's behaviour, evolution, metabolism, DNA repair pathways and many others (Goffeau, 2000).

Respiration in yeast cells can be done in both ways; aerobic in the presence of oxygen and anaerobic in the absence of oxygen. Yeast cells are able to metabolize wide range of carbon sources depending on whether they are grown aerobically or anaerobically. When they have enough oxygen, they oxidize the sugars into carbon dioxide and water but if there is no oxygen, they grow anaerobically to convert the sugars to carbon dioxide and

ethanol (Gasmi et al., 2014; Murray, Haynes, & Tomita, 2011; Verduyn, Postma, Scheffers, & Van Dijken, 1992).

Yeast has some mutual features with other organisms. It possesses mitochondria to do aerobic respiration as eukaryotes. In addition to mitochondria, they have a nucleus with its own membrane, peroxisomes and a secretion system. Moreover, yeast contains also very large vacuoles, which are common in plants but missing in animals and it has a thick cell wall like in plant cells that generates periplasmic space between the wall and cell membrane (Banta, Robinson, Klionsky, & Emr, 1988; Wiederhold, Veenhoff, Poolman, & Slotboom, 2010). These features make it share the internal cell architecture with plants and animals.

All of the characteristics mentioned above, as well as numerous others, make yeast the organism of choice in genetic studies like; DNA repair mechanisms, recombination, cell behaviour, heterologous gene expression and heterologous protein production.

1.1.1 HETEROLOGOUS GENE EXPRESSION IN YEAST

Current heterologous gene expression researches involve either prokaryotic (*Escherichia coli*, *Bacillus subtilis*) or eukaryotic (yeast or various mammalian cell) hosts (Nielsen, Larsson, van Maris, & Pronk, 2013; Schmidt, 2004).

S. cerevisiae is one of the attractive hosts for the heterologous gene expressions for the production of foreign proteins. Before, *E. coli* was the main host for heterologous gene expressions but since it produces toxic and pyrogenic cell wall components and also mechanism of transcription, translation and post-translational process hold differences from eukaryotes, foreign protein production was having difficulties (Botstein & Davis, 1982). By the development of the exogenous DNA transformation into yeast (Hinnen, Hicks, & Fink, 1978), cloning and analyzing of foreigner genetic information in a eukaryotic organism became unproblematic. Since from this success, a wide range of

alternative systems for yeast transformation and different plasmid systems were developed (Bruschi, Comer, & Howe, 1987; Gietz, St Jean, Woods, & Schiestl, 1992; Ito, Fukuda, Murata, & Kimura, 1983). Within the past years, plasmid and yeast engineering is a key tool for the biotechnology industry. Development of recombinant yeast artificial chromosome is an important example of cloning and analysis of eukaryotic genes (Burke, Carle, & Olson, 1987). The most important advantages offered by this yeast technology are rapid and cheap growth, safety, most of the higher eukaryotic pathways are conserved and easy genome manipulations (Castelli, Mardon, Strike, Azad, & Macreadie, 1994).

When the yeast took an important place for the foreign protein production, it helped different biotechnology fields to be developed faster. By the help of the recombinant DNA technology, developing vaccines, therapeutic agents and bioactive compounds have received an extensive attention recently. Yeast has become the suitable alternative model organisms for the biotechnological compounds because of its simplicity, and being easily manipulated. Hence, yeast plasmid system that can be engineered and then transformed and selected in yeast, has had increased interest. Whole genome manipulation and cloning techniques do not just allow the analysis of the genetic sequence by itself, but also allow the analysis of its expression, function and purification (Kazemi Seresht, et al., 2012).

To express a gene in a host, easy way is to be in a plasmid that can replicate and stay in the host cells. Not always expressed heterologous proteins have the expected biological activity in the host cells because of the incorrect processing or different conformation. To keep the function of the gene product and expressing the gene enough to analyze, are the important steps in heterologous gene expression studies (Hou, Tyo, Liu, Petranovic, & Nielsen, 2012). Since the first aim is to analyze the expressed recombinant protein itself, expression of the gene must be easily manipulable and efficient. So that, bacteria or yeast have some advantages for the foreign protein production because of their being able to be transformed with plasmids efficiently and growing easily and fast.

When a protein get produced by direct expression without a signal sequence, it localizes to cytoplasm and it will have similar features whether produced in a prokaryotic or eukaryotic cell. Protein synthesis generally starts with AUG codon (methionine) except that some bacterial mRNAs also utilize some other initiator codons (Clements, Laz, & Sherman, 1988). Even though certain enzymes exist, in both eukaryotic and prokaryotic organisms that are able to modify the amino terminal ends of cytoplasmic proteins, the effect is probably minimum on biological activity. For example, in the expression of IFN-gamma in yeast, high-level expression resulted in the production of amino terminal analogues, which maintained the full biological activity (Fieschko et al., 2004). But still, amino terminal specificity should be considered when choosing a host for expression aims.

1.1.1.1 Yeast Vectors and Promoters

Most of the yeast vectors are based on the 2-micron plasmid and also able to replicate in bacteria. They contain sequences of promoter and terminator for the efficient transcription of the heterologous genes. Recent heterologous protein expression studies head to an increase in understanding of yeast plasmid system, especially increasing the strength of the promoter and stability of the vectors, which are now some of the restrictions that biotechnology is dealing with.

There is a wide range of expression vectors for the expression of foreign genes in yeast and they contain yeast transcription promoter sequence and transcriptional terminator sequence as well as some unique cloning sites for the insertion of the desired sequences to express. Yeast plasmids can be divided into groups like: YRp (yeast replication plasmids), YCp (yeast centromeric plasmids), YEp (yeast episomal plasmids), YIp (yeast integrating plasmids), YLp (yeast linear plasmids). YRp plasmids are able to replicate in yeast cells by having ARS (autonomous replication sequences) and it is highly unstable in the cells. YCp plasmids own both origin of replication and centromeres, which allow them to replicate in the cells and present low copy numbers from 1 to 2 copies per cells. YEp plasmids are

episomal vector that can replicate in the yeast cells and they contain 2-micron sequences, which allow them to be of high copy number. YIp plasmids are differed from the plasmids that mentioned above, they cannot replicate autonomously but they integrate to the genome with low homologous recombination frequency. Generally, they integrate as a single copy and integration is directed by the two copies of flanked sequences homolog to yeast genome. YLp plasmids contain sequences that function as telomeres and centromere. Copy number of the plasmid is 1 per cell and these artificial chromosomes are less stable compared to the natural chromosomes (Gunge, 1983; Lundblad, 2001; Romanos, Scorer, & Clare, 1992).

In this study, Yeast Artificial Chromosomes (YACs) were used for the strain improvement of the yeast cells for the bio-ethanol production. YACs are generally used for cloning and manipulation of large DNA inserts in yeast cells (up to 3 Mb pairs). Moreover they can be amplified in bacterial cells also because they are shuttle vectors. They contain centromere, telomeres, origin of replications and restriction sites for cloning experiments. As a chromosome YACs has to reach a certain size to be stable which is around 150 kb. This instability can be a restriction of the YAC cloning. YACs are important in biotechnology field since they are transportable to other organisms and they can carry big inserts. For these reasons, it is generally used for production of heterologous protein, recombination studies and analysis of foreign genomic sequences (Arnak, 2012; Burke et al., 1987).

Another important point for the heterologous gene expression is promoter strength. Strength of the promoter is particularly useful when the promoter is inducible and highly expressed. In yeast, heterologous proteins can be highly expressed compare to the total cell protein by playing with the promoter.

Heterologous gene expression in yeast can be driven by a large variety of promoters including *PGK*, phosphoglycerate kinase (Bitter & Egan, 1984), *ADH1*, alcohol dehydrogenase (Ammerer, 1983), and *PHO5*, acid phosphatase (Dumont et al., 1989).

These promoters are known as strong promoters to express high amount of proteins. For example *ADH1* promoter is able to have high amount of transcripts, which is almost 4-5% of the total mRNA (Romanos et al., 1992).

In some cases, the product of the heterologous gene itself or accumulation of it can be toxic for the host cell and this issue requires some control. Because of this reason some yeast promoter have been developed, which are able to being regulated according to the conditions. *GAL10* is one of them, which is able to express the gene inducibly rather than constitutively (Guarente, Yocum, & Gifford, 1982). By the help of the regulation system of this kind of promoters, transformed cells can be grown in culture till desired density reached and then expression of the genes can be induced. With this approach, inhibitory effects of expressed protein for the cell growth is easily avoided.

Evidently, more expressed gene products are related to the strength of the promoters. There is a possible problem with inducible promoters that display high expression levels. Since they are controlled, the inducer elements can be limiting in cells. Using a high copy number plasmid expression system to increase the gene expression level can be effected by these limits. This problem can be handled by elevating the internal level of the trans-activator protein in the cells through the genetic manipulation.

For the heterologous gene expression in yeast, not just the expression system but also the environment, where the expression is taking place has to be considered. In particular, which kind of nutrient is used for the growth of the host cells. Components of the media, carbon sources can affect the expression level of heterologous genes (Weinhandl, Winkler, Glieder, & Camattari, 2014).

In this study, different promoters were used for the expression of cellulase genes in yeast cells. *GAL10* inducible promoter was used for the expression of Bgl6 protein. This promoter is very well characterized and mostly used for the heterologous gene expression in yeast. However, there is a restriction in this process because gene expressions under this

promoter are inhibited in the presence of glucose. Glucose concentration has to be at a low level for the successful induction of the promoter (Matsuyama, Yamanishi, & Takahashi, 2011). For this reason, raffinose was used in the media with the addition of galactose for the induction experiments.

ADH1, TEF1 and *PGK1* strong constitutive promoters were also used for the expression of cellulase genes; *EGC1, CBH2* and *BGL5*. In contrast to inducible promoters, constitutive promoters does not require any special conditions or regulations for the expression of the proteins, as they are able to direct the transcription of the heterologous genes constantly as long as there is RNA polymerase available (Partow, Siewers, Bjørn, Nielsen, & Maury, 2010). *ADH1* is the promoter of alcohol dehydrogenase and it is widely used as constitutive yeast promoter (Ruohonen, Aalto, & Keränen, 1995). Phosphoglycerate kinase is under the control of *PGK1* promoter, which is used for heterologous gene expression studies as a constitutive yeast promoter (Castelli et al., 1994). *TEF1* promoter controls the expression of translation elongation factor EF1 alpha and known as constitutive yeast promoter (Gatignol, Dassain, & Tiraby, 1990).

External genetic information carried by yeast plasmids can be selected and maintained in selective media. There are several resistance marker genes against antibiotics or some toxic compounds and they are used in yeast for the selection of plasmids. However, using marker genes has some negative effects, as they need toxic compounds in the media and besides their toxic effects, they are also costly. Additionally, also the resistant strains can have negative effect by the antibiotics. For this reason new marker genes that are responsible of yeast auxotrophy became alternative to antibiotics (Pronk, 2002). Auxotrophy markers can be selected based on yeast genome according to its specific nutritional requirements. According to yeast auxotrophy markers, plasmids can be selected and maintained in minimal synthetic medium that is a mixture of carbon source, nitrogen source and a selection of vitamins and amino acids. One or more amino acids are deleted

from the synthetic media for selection of the plasmids. Cells that contain the mutation in a gene required for the growth of the cell in the synthetic media are maintained by the presence of the plasmids that express the complementation of the required gene product actively (Kaiser, Michaelis, & Mitchell, 1994a). *LEU2, URA3, HIS3* and *TRP1* are the ones that are used often as a marker for plasmid selections. Moreover, it has been already shown that auxotrophy markers have different fitness profiles (Ugolini, Tosato & Bruschi, 2002) and are affecting positively the expression level of heterologous genes. For this purpose, modulations of auxotrophy markers are important (Kazemi Seresht, Nørgaard, Palmqvist, Andersen, & Olsson, 2012).

1.1.2 THE SECRETORY PROCESS IN YEAST

High amount of recombinant protein can be produced just by direct expression system, but many of them also need a eukaryotic secretion pathway. There are some proteins getting secreted naturally like human tissue plasminogen activator (TPA) and bovine growth hormone (BGH). When these kinds of proteins express cytoplasmicaly, they become inactive because of not being cleaved properly (Schoner, Ellis, & Schoner, 1985). What is more is that, sometimes proteins require glycosylation like viral envelope proteins. All these requirements and special features play an important role in the analysis of recombinant proteins compare to the native proteins (Romanos et al., 1992; Schultza et al., 1987). For the similarity of secretion system, eukaryotic organisms are closer to yeast than the bacteria, which is helping to obtain a mature recombinant protein like the native one (Julius, Schekman, & Thorner, 1984).

Another advantage of using the secretory pathway for the heterologous proteins is that purification of the gene product can be done by having it exported into the extracellular location. There are quite a lot of yeast promoters that can be used efficiently in expression systems because of their being able to direct the gene product naturally into the secretory pathway. These include *MFa1* (Miyajima, Bond, Otsu, & Arai, 1985) and *SUC2* gene

(Smith, Duncan, & Moir, 1985) and some others. All of them have already shown to direct the heterologous products into the secretory pathway of yeast and successfully produce the active mature proteins. Additionally, It is also demonstrated that if the heterologous gene contains its own signal sequences and it is under the control of a direct expression yeast promoter like *PHO5* or *ADH1* (Jabbar, Sivasubramanian, & Nayak, 1985; Verbakel, Dekker, Rutgers, Pouwels, & Enger-valk, 1987), it can successfully direct the gene product into the secretory pathway.

There are different microorganisms that are used for the heterologous protein production for medical or biotechnological purposes. Bacteria are widely used for these purposes but they do not have post-translational modifications for the product of eukaryotic genes. Yeasts can produce the recombinant mature and active proteins since they are able to do post-translational modifications (Idiris, Tohda, Kumagai, & Takegawa, 2010; Mumberg, Müller, & Funk, 1995; Singhania, Patel, Sukumaran, Larroche, & Pandey, 2013). Figure 1.2 shows the secretory pathway of heterologous proteins in yeast.

Simplicity of genetic engineering, rapid growth, safety, being well characterized genetically and high density of recombinant protein production ability are the positive features that yeast has. Moreover, since *S. cerevisiae* is also able to secrete recombinant proteins in their native, biologically functional form, it became a chosen host organism for a wide range of foreigner proteins. Hepatitis B vaccine (Valenzuela, Medina, Rutter, Ammerer, & Hall, 1982), insulin (Thim, Hansen, & Sørensen, 1987) and human serum albumin (Okabayashi et al., 1991) are some examples of yeast expression and secretory system usage.

Directing the recombinant protein into the secretion pathway is carried out by the signal sequences, which are short peptides containing approximately 20 to 30 amino acids. There are different leader peptides that direct the secretions and secretion sequence from the *MFal* gene encoding alpha-factor is one of them that is used quite often for the purpose of

heterologous protein secretions. Alpha factor is able to provide high level of secreted protein by getting fused in to the frame with the heterologous gene sequence (Grant A Bitter, Chen, Banks, & Lai, 1984; Brake et al., 1984). Apart from yeast leader peptides, it is also possible to make use of the native secretion signal of the foreign protein, which can work on the secretion and production of mature proteins in yeast cells (Idiris et al., 2010).

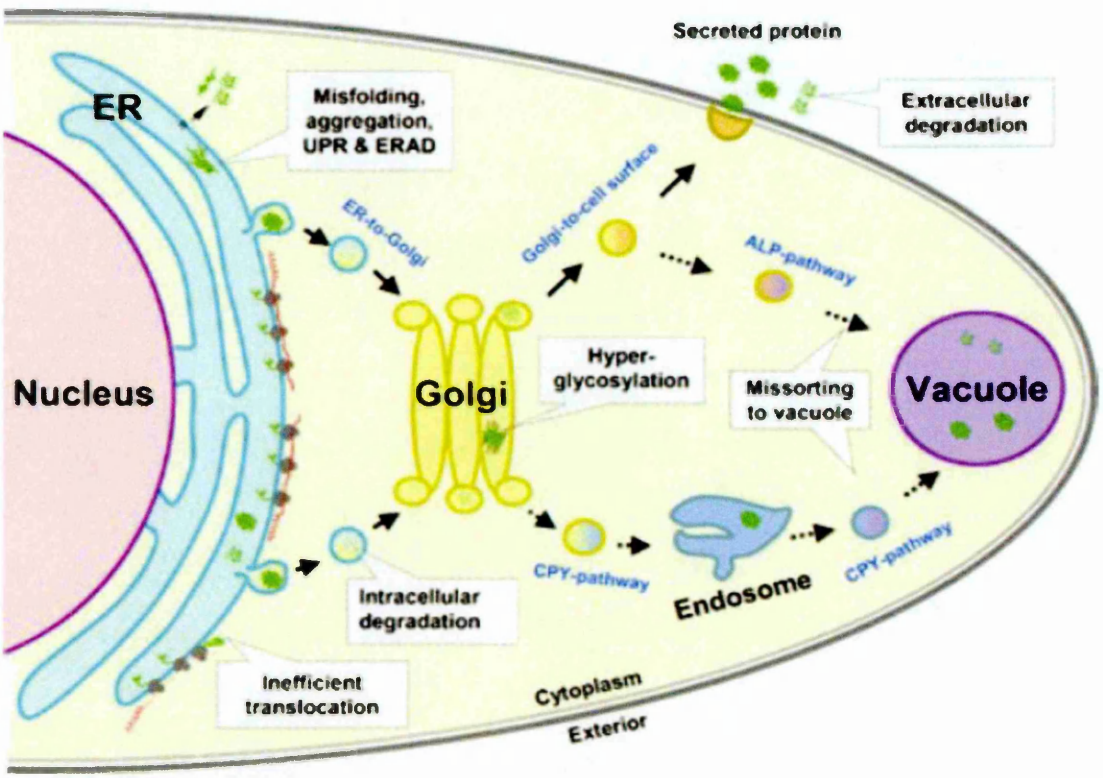

Figure 1.2: Secretory pathway of heterologous proteins in yeast (Adapted from Idiris, et al., 2010).

When the protein is expressed in the yeast cells, firstly it takes place in the cytoplasm of the cells and then secretion occurs similar to mammalian cells. Basically, proteins that will be secreted have a chain of transportation between cellular compartments. To start the secretion, first direction of the proteins is the endoplasmic reticulum (ER), after which is followed by the translocation from the ER to Golgi and then transport from Golgi to extracellular area.

Different secretory signal sequences have been used for heterologous protein secretion and *MFal* gene encoding alpha-factor is one of them that is used quite often (Brake et al., 1984; Ernst, 1986). The sequence of secretion signal is playing important role for the direction of secretory pathway by their hydrophobicity and interaction ability with the signal recognition particles (SRP). In *S. cerevisiae*, transfer from cytosol to ER can happen either via co-translational pathway or via post-translational pathway (Hou et al., 2012; Idiris et al., 2010; Shuster, 1991).

In co-translational pathway, the signal recognition particles (SRP) are ribonucleoproteins that bind to signal peptide and become a complex together. After that, this complex cooperates with the signal recognition particle receptor (SRPR), which is an important membrane protein related to endoplasmic reticulum (ER) and pre-protein is getting translocated into the lumen of ER. Moving into the lumen of ER requires some energy and this energy is provided by GTP hydrolysis (Shan & Walter, 2005).

Post-translational pathway is used when the secretory proteins show weak hydrophobic signal and they do not bind to SRP during their synthesis in cytosol. Because of this reason, translation takes place in cytosol where these proteins stay unfolded or folded but very lightly and they get stabilize by cytosolic chaperones. SRP independent of these proteins move to ER by the collaboration of Sec61 and Kar2p proteins (Plath, Mothes, Wilkinson, Stirling, & Rapoport, 1998).

When the protein translocates into the ER, several modifications appear for the process of folding and quality control of the proteins. Glycosylation, which can be done in two ways; N-linked, and O-linked, occurs during this translocation from ER to Golgi and it assists proteins for folding and checks the quality of the process. It also protects immature proteins from proteases. Through ER processing, misfolded proteins can be detected and retro-translocated to cytosol for their degradation by proteosome. Furthermore, properly

folded proteins continue the transportation between cellular compartments and move to the Golgi (Young & Robinson, 2014).

After ER process, proteins move to Golgi and last modifications of the proteins take place here, which allow protein to become mature with the correct functions. One of the modifications is cleavage. Most of the secreted proteins require proteolytic cleavage in order to be mature structurally and functionally (Diane Hopkins, Sato, Nakano, & Graham, 2000). There are different proteases, which are responsible for cleavage of proteins and they recognize different sequences. Cleavage of proteins makes them mature, active and arranges their conformation for the receptor. One of these proteases is Kex2 protein, it is an endopeptidase that locates in Golgi where it helps for the maturation of the secretion signal by recognizing the Lys-Arg and Arg-Arg pairs (Brenner & Fuller, 1992).

When proteins complete their Golgi process, they move into secretory vesicles by which they are transported from cytoplasm to extracellular environment. Some of the last modifications take place in the secretory vesicles. These vesicles interact with the cell membrane and release the secreted protein by exocytosis (Hou et al., 2012; Young & Robinson, 2014). Figure 1.3 basically shows all the steps for the protein secretion.

Therapeutical, environmental and food industry are the most common areas that yeast is used for recombinant protein production. Since yeast can be grown easily in large scale and it is generally recognized as safe (GRAS), yeast secretory systems is becoming more important for the biotechnological purposes (Young & Robinson, 2014).

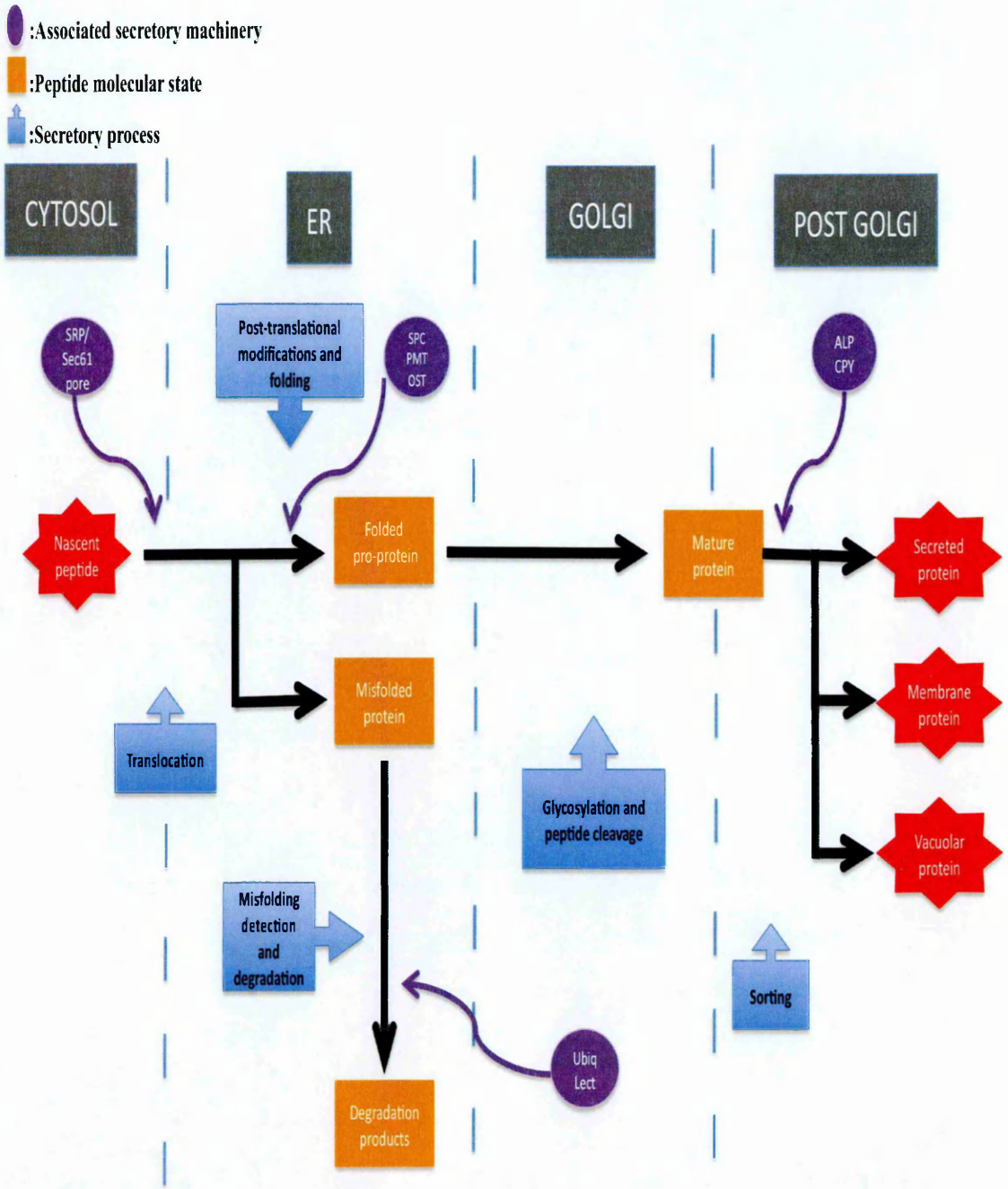

Figure 1.3: Overview of the secretory machinery (SRP, signal recognition particle; SPC, signal peptidase complex; PMT, protein O-mannosyl transferase; OST, oligosaccharyl transferase; Ubiq, ubiquitin; Lect, Lectin; ALP, arginine transporter pathway; CPY, carboxypeptidase Y pathway). (Modified from Hou et al., 2012).

1.1.2.1 Yeast Secretion Signal: Mating Pheromone Alpha Factor

Alpha factor is the product of mating type alpha cells, and it is naturally secreted from the cells. It contains a pro-sequence that directs the secretion and this signal can be used for the secretion of recombinant proteins (Bitter et al., 1984). As it is mentioned before, secretion of recombinant proteins in yeast can be directed either by native secretion signal of the peptides or by yeast signal. Most commonly used yeast signal sequences are; acid phosphatase, invertase and alpha factor (Romanos et al., 1992).

In this study, alpha factor was used as a secretion signal and cellulose degradation genes were cloned in frame with this signal under the strong constitutive promoters.

The alpha factor produces a precursor protein and it contains approximately 165 amino acids. Analysis of these sequences shows that the precursor contains an amino terminal hydrophobic signal sequence of 19 amino acids, a leader segment of 60 amino acids and four tandem copies of the mature alpha factor peptide preceded by a spacer of 6 to 8 amino acids. This leader peptide is expressed constitutively and is secreted from cytoplasm to extracellular environment in alpha mating type cells. Its expression depends on the mating type of the cells and because of this reason it is not produced in a mating type cells (Bitter et al., 1984; Caplan et al., 1991; Zsebo et al., 1986).

The alpha factor directs the polypeptide into secretory pathway and following the translocation into endoplasmic reticulum, where amino terminal of the signal sequence is removed. Moreover, this precursor molecule gets glycosylated and only glycosylated product is secreted. As it is mentioned before, there are different proteases, which are responsible for cleavage of proteins for their function and maturation. During the processing of the alpha factor, the KEX2 protease plays an important role on cleavage. It recognizes and cleaves after Lys-Arg and Arg-Arg pairs on the carboxyl side of the dibasic residues. These amino acids take place between alpha factor and heterologous protein and

later on they allow heterologous protein to be separated from leader sequence by Kex2 peptidase activity (Brenner & Fuller, 1992).

To sum up the importance of yeast in biotechnological science, it can be said that *S. cerevisiae* has been demonstrated as a good model organism for the foreign protein production and analysis. For biotechnological purposes, high level of heterologous protein can be obtained via yeast expression and secretion systems. Since yeast is able to direct the expression and secretion of foreign proteins correctly, it becomes preferred organism. There are still things to understand more and improve more for the heterologous protein production in yeast.

1.2 BRIDGE INDUCED TRANSLOCATION SYSTEM

The Bridge Induced Translocation (BIT) system was first developed in the Yeast Molecular Genetics Group at the ICGEB-Trieste. This Group started working on the field of chromosomal translocations since 2005 and developed a system to induce the chromosomal translocations by using *S. cerevisiae* as a model organism. The BIT technology development helped to study and to understand the mechanisms of non-reciprocal chromosomal translocations. The BIT system requires a DNA cassette with a selective marker (in this study kanamycin gene was used as a marker) that is flanked by two regions of homology to different chromosomal loci on the yeast genome and translocation is induced *in vivo* by the help of the homologous recombination (Tosato, Waghmare, & Bruschi, 2005). In figure 1.4 schematic representation of BIT system is shown.

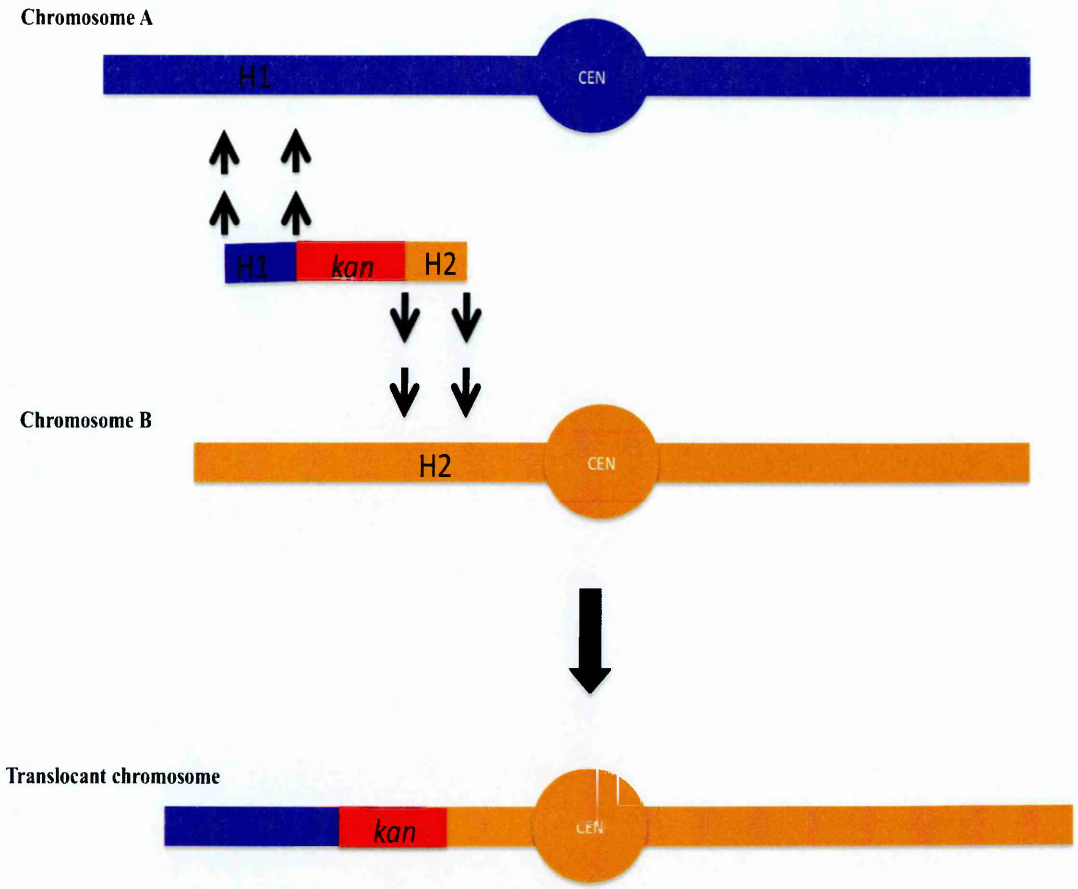

Figure 1.4: Schematic representation of BIT system. H1 and H2 represent the targeted homology sequences around the break point of translocation. As a selective marker kanamycin gene was used and translocant chromosome harboring the pieces from different chromosome can be obtained by the insertion of the cassette via homologous recombination mechanism.

BIT system creates several changes at both genomic and transcriptomic level in the yeast cells. In Yeast Molecular Genetics Group, lots of translocant yeast strains that carry different translocation background were produced and characterized to see the BIT system effects. It has also been shown that one of the important result after BIT event is that around the translocation break point, gene expression level is increasing in translocant yeast cells comparing to the wild type yeast cells (Nikitin et al., 2008). This feature of BIT event was

aimed and used in this thesis for the strain improvement of recombinant yeast strain for the bio-ethanol production.

As it is mentioned above, BIT event creates several changes in the yeast cells on their cellular morphology, physiology and genome re-organization. All of these BIT effects state that one single translocation event can result in a mixture of chained molecular events in the cells. It is also shown that regardless of the translocation origins, all translocant cells show differences of their phenotypes, ability to sporulate, regulation of gene expression and morphology. In another word, translocations that are produced by the integration of the same DNA cassette can cause different genomic alterations among the cells (Rossi, Noel, & Bruschi, 2010).

All of these results that occur after BIT event have been shown to be important for the manipulation of the genome. Since BIT technology does not require any previous genomic modification or engineering, it is simple and allows producing translocation between any desired points in the genome. All of these features make the BIT system suitable for the translocation studies compare to the other approaches developed until now. Apart from the advantages of the system, there are also some restrictions. Efficiency of the system requires to be improved, because obtaining a translocant chromosome possibility is not as high as it could be expected. Depending on the target regions' recombination aspect, frequency of obtaining translocants can fluctuate between 1-5% of transformants. For the same regions, the gene knock-out frequency can be around 80-90%, which is really much higher comparing to the BIT system. Furthermore, it has also been shown that most of the time, DNA cassette integrates either only in one of the targeted loci or somewhere in the genome randomly (ectopic integration). This low efficiency of BIT system is inhibiting the experimental procedure but on the other hand is opening other doors and is addressing a wide range of evolutionary concerns (Nikitin et al., 2008; Rossi et al., 2010; Tosato et al., 2012).

1.3 BIOETHANOL

Renewable and sustainable energies have become an important topic for the future energy technology development. Some of the renewable energies are produced by wind energy, solar energy or hydro energy but their contributions are currently very minimal to the world's energy necessity. Also all over the world, the main energy problem is rising because of oil reserves diminishing, global warming and expensive gasoline prices. Altogether, these facts have increased the attention for the production of bioethanol as an alternative fuel which has pros and cons for the environment (Chang et al., 2012; Kumari & Pramanik, 2012).

Some of the benefits of bioethanol are summarized as the following:

- Decreases air pollution

Bioethanol is very environmental friendly and clean comparing to other energy types. For example fossil fuels produce sulphur dioxide and nitric oxide, which cause air pollution. Also, high amount of sulphur dioxide can cause breathing problems.

- Biodegradable and renewable

Since it is produced from organic materials, it is extremely degradable compare to the petrol and it can be continuously replenished.

- Promotes being independent from fossil fuels

Another advantage of bioethanol is that it is an alternative energy production way to the fossil fuels. This kind of alternative energy development decreases the dependence on one kind of source.

- Development of agriculture

Bioethanol, which can be produced from crops or from crop remains, is able to increase the interest on agriculture and this interest positively affects the development of agriculture.

Apart from the benefits, unfortunately bioethanol also has some disadvantages that are summarized as the following:

-Energy yield

Compare to the traditional fuels, bioethanol has lower energy yield. Because of this reason, it requires to be produced larger amounts to provide the enough level of energy.

-Cost

Even though that low cost feedstock is used as a substrate, the cost of the bioethanol has to be reduced. Especially, pretreatment of the feedstock has to be improved in order to find out cheap methods.

-Restriction in its use

The vehicles need some engine modification in order to consume some type of bioethanol.

-Food field

To produce the crops for the bioethanol production, there is a field competition with food production. It is decreasing the food fields.

As it is mentioned above, bioethanol is a renewable fuel that can be produced from different biological sources and the production of bioethanol from cheap biomass, like waste of plant material, is important for fuel industry. All over the world, there are a lot of studies about bioethanol production from different biological sources and lignocellulosic biomass is one of them, which has numerous environmental and social benefits for bioethanol production (Lange & Solutions, 2007; Lee, 1997; Wyman, 1994).

In the beginning, bioethanol was produced from crops like sugar cane (juice) and corn (starch). This first attempt on bioethanol production was called first generation bioethanol. Even though bioethanol is clean and has some advantages, first generation bioethanol also brought forward many sustainability discussions. The reason for the discussions was that application of the first generation bioethanol was limiting the food production by

increasing the competition of crops in agricultural lands (Kricka, Fitzpatrick, & Bond, 2014).

All of these discussions induced the research on looking for alternatives feedstocks and second generation biofuels term came into the bioethanol world (Karp & Richter, 2011). Lignocellulosic materials found in agricultural remains and wood, which are abundant, renewable and cheap are generally used as a source for second generation biofuels (Kricka et al., 2014). For the production of bioethanol from lignocellulose, the process firstly requires the releasing of fermentable carbohydrate from biomass by using a set of enzymes and then these carbohydrate can undergo a saccharification and a subsequent conversion to ethanol by fermentation using yeast or bacteria (Lynd, Weimer, van Zyl, & Pretorius, 2002).

Conversion of lignocellulosic material into ethanol is not that easy, as there are some restrictions. The main limitation of lignocellulose degradation is its structure since it inhibits releasing of the sugars from biomass. As a result of increased interest on lignocellulosic-based bioethanol, necessity to develop technologies for making fermentable sugars easily reachable also increased. These lignocellulose deconstruction technologies are the key point for bioethanol production and that is why their cost, productivity, efficiency and sustainability are important for the whole process. This also explains the reason for strong interest in pre-treatment researches of biomass (Xu & Huang, 2014).

There are different ways to produce ethanol like by synthetic systems or by microorganisms. Among them, *S. cerevisiae* has more advantages and is an ideal model organism for bioethanol production studies because of its high ethanol productivity, high tolerance to ethanol, simplicity of genetic engineering and being a GRAS organism. For that reason, generating a recombinant yeast strain that can efficiently degrade the carbohydrate part of the biomass and produce ethanol out of it has drawn attention over

recent years (Chang et al., 2012; Fan, Zhang, Yu, Xue, & Tan, 2012; Kumari & Pramanik, 2012).

Since *S. cerevisiae* cannot degrade some particular sugars, some set of enzymes have to be expressed and secreted in yeast cells to make them able to utilize all sugars of biomass for the ethanol production. In this study cellulose was targeted as a sugar source for the ethanol production. Cellulose is one of the carbohydrate polymers that cannot be utilized by *S. cerevisiae* and degradation of the cellulose requires at least three enzymes namely endoglucanases (EGC), cellobiohydrolases (CBH) and β-glucosidases (BGL), for cellulose conversion into glucose (Bhat & Bhat, 1997). Detailed information about these enzymes will be mentioned later on but basically EGC and CBH are playing roles in the degradation of the cellulose chains to cellobiose and in the final step of enzymatic cellulose degradation, BGL hydrolyzes cellobiose molecules to glucose. In nature, there are some bacteria and fungi strains that have these cellulase enzymes and in this study we used *Pichia stipitis* and *Trichoderma reesei* as a source of the cellulase enzymes (Jeffries et al., 2007; Tsai, Goyal, & Chen, 2010).

1.3.1 LIGNOCELLULOSE STRUCTURE

As mentioned before, lignocellulosic biomass is important and preferred biomass for the production of second-generation biofuels. It is a main component of the plants and it contains carbohydrate (Cellulose and Hemicellulose) and aromatic compounds (Lignin). Carbohydrate parts of the lignocellulose are bound to lignin part via hydrogen and covalent bonds **(Figure 1.5).** Carbohydrate parts are the important compounds for the energy production but besides this principal advantage, structure of the lignocellulosic material is making process more complicated since the lignin part is tightly attached to the carbohydrates. For this reason, pre-treatment of the biomass is having a key role in releasing the fermentable sugars for microbial degradation and fermentation (Lee, 1997; Sun & Cheng, 2002).

Depending on the type and the age of the plants, structure and composition of the lignocellulose can be different. Due to the economical reasons, most suitable lignocellulose should contain high amount of fermentable sugars for the bioethanol production. Table 1.1 shows the percentages for the components of different lignocellulosic material (Sweeney & Xu, 2012).

Table 1.1: Composition of different lignocellulosic materials.

Lignocellulosic Material	**Lignin (%)**	**Cellulose (%)**	**Hemicellulose (%)**
Hardwood	18-25	45-55	24-40
Softwood	25-35	45-50	25-35
Grass	10-30	25-40	25-50

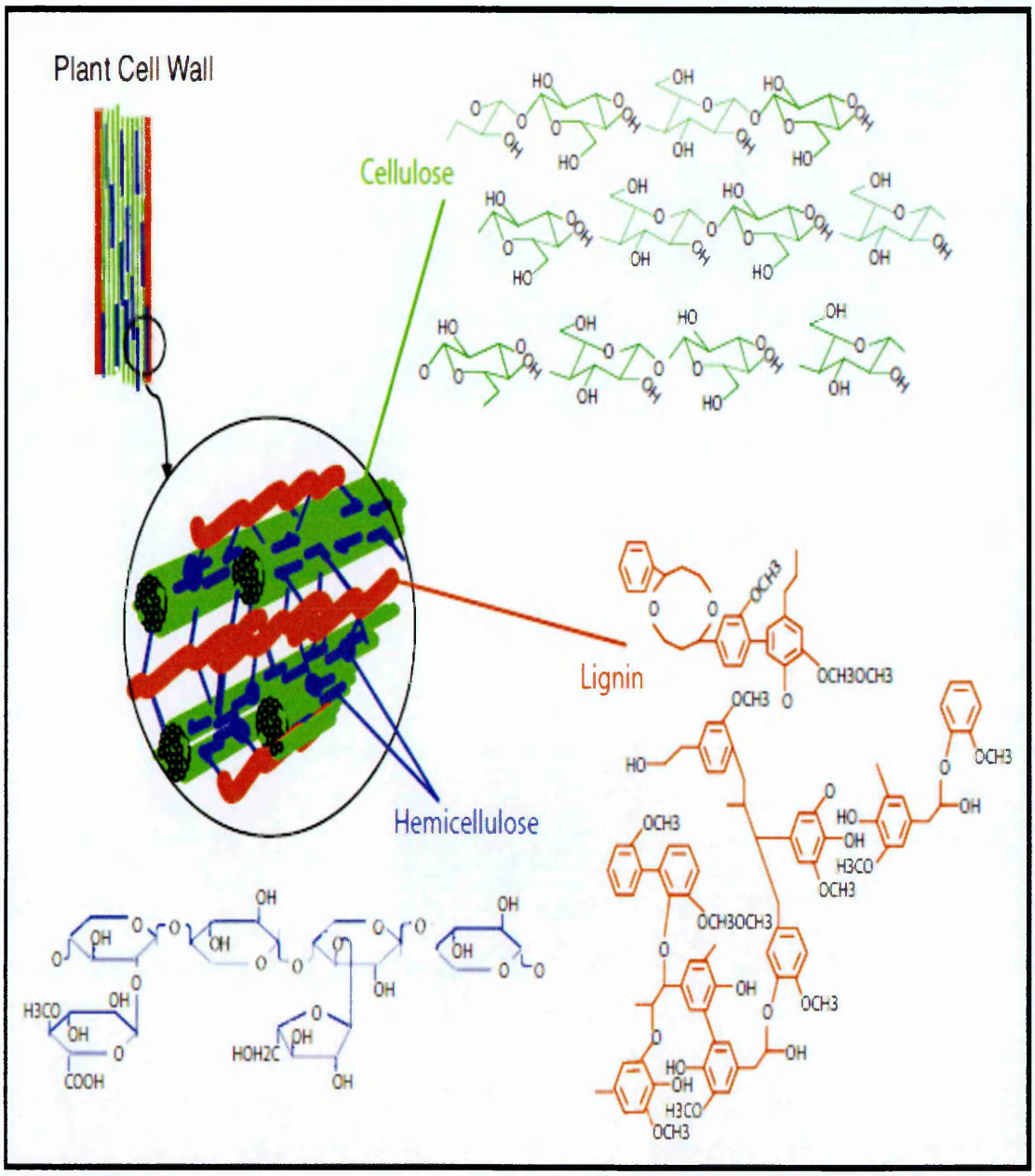

Figure 1.5: Composition and structure of lignocellulose (Adapted from http://biofuel.webgarden.com web page).

Cellulose component of lignocellulose is the target part in this thesis as a source for bioethanol production and more details about its structure and degradation will be given on the following section. The cellulose part is the biggest component of plant biomass that contains the most abundant polymers for the ethanol production. It is composed of D-glucose molecules bound to each other by β-(1,4)-glycosidic bonds (Mba Medie et al.,

2012). The cellulose molecules can contain the amorphous region and crystalline region. The difference of these regions is related to the order of microfibrils. In amorphous region, microfibrils are more disordered than the crystalline region. Hydrogen bonds and Van der Waals forces of each cellulose microfibrils are creating crystalline structure. That is why enzymatic degradation of the cellulose can be limited. Close association of cellulose, hemicellulose and lignin via complex and tight bonds decrease the accessibility of cellulase enzymes to cellulose (Beguin, 1990).

Hemicellulose is the other important carbohydrate part of lignocellulose for bioethanol production. Its structure is heterogeneous that contains branched and linear polymers of different sizes, which are the combination of different pentoses (D-xylose and L-arabinose), hexoses (D-mannose, D-glucose, and D-galactose), and sugar acids (4-*O*-methyl-glucuronic, D-galacturonic, and D-glucuronic acids). Hemicellulose is connected with cellulose by hydrogen bonds and with lignin by covalent bonds. This makes the structure of lignocellulose inflexible and as it is mentioned before, it requires the pre-treatment of the biomass to reach the hemicellulosic part. 20-35% of the lignocellulose is composed of hemicellulose sugar and xylose of hemicellulose is the main component that is generally used for microbial fermentation (Kricka et al., 2014; Kumari & Pramanik, 2012).

Apart from the carbohydrate parts of the lignocellulose, 10-25% of the lignocellulose is composed by lignin. Lignin is tightly branched aromatic polymer and connected to other parts via ether and carbon-carbon linkages. It has three phenolic components: p-coumaryl alcohol (p- hydroxyphenyl propanol), sinapyl alcohol (syringyl propanol) and coniferyl alcohol (guaiacyl propanol). These components polymerize to produce lignin complex and their amount diverge among different plants and wood tissues (Lee, 1997; Xu & Huang, 2014).

All these structural features have brought difficulties to deal with for the ethanol production. As a result of this barrier, different methodologies were developed that can efficiently overcome the structure of lignocellulose with sustainability and low cost (Mosier et al., 2005). These pre-treatment methods are necessary to make carbohydrates accessible for converting them into fermentable sugars. There are different ways to degrade lignocellulosic materials; chemically, physically and biologically (Kumar et al. , 2009; Lange & Solutions, 2007). Even though there are methods to deal with the lignocellulose structure, still they need to be improved for some parameters like; more efficiency, less time consuming and less cost.

1.3.1.1. Cellulose Structure

Cellulose is an ideal substrate for the bio-energy research and as it is mentioned above, it is the most abundant, renewable molecule on earth that provides the high amount of carbons. Cellulose is the component of plant cell walls that provides the structural stability. It can be found in nature in pure form like in cotton plants (95% cellulose) or it combines with other carbohydrates and lignin in the cell wall of plants. Lignocellulosic materials are the most common and important source of cellulose (Bayer et al., 1998).

Cellulose molecules are composed of linear polymeric chains of β-1,4-glycosidic linked D-glucopyranose units **(Figure 1.6)**. It has a polar structure by having one end containing a free semi-aldehyde group, which is a reducing end and the other end containing a free OH group, which is a non-reducing end. These free hydroxyl groups and oxygen atoms are responsible for the formation of hydrogen bonds that generate hydrogen bonds complex within the cellulose structure (Kricka et al., 2014).

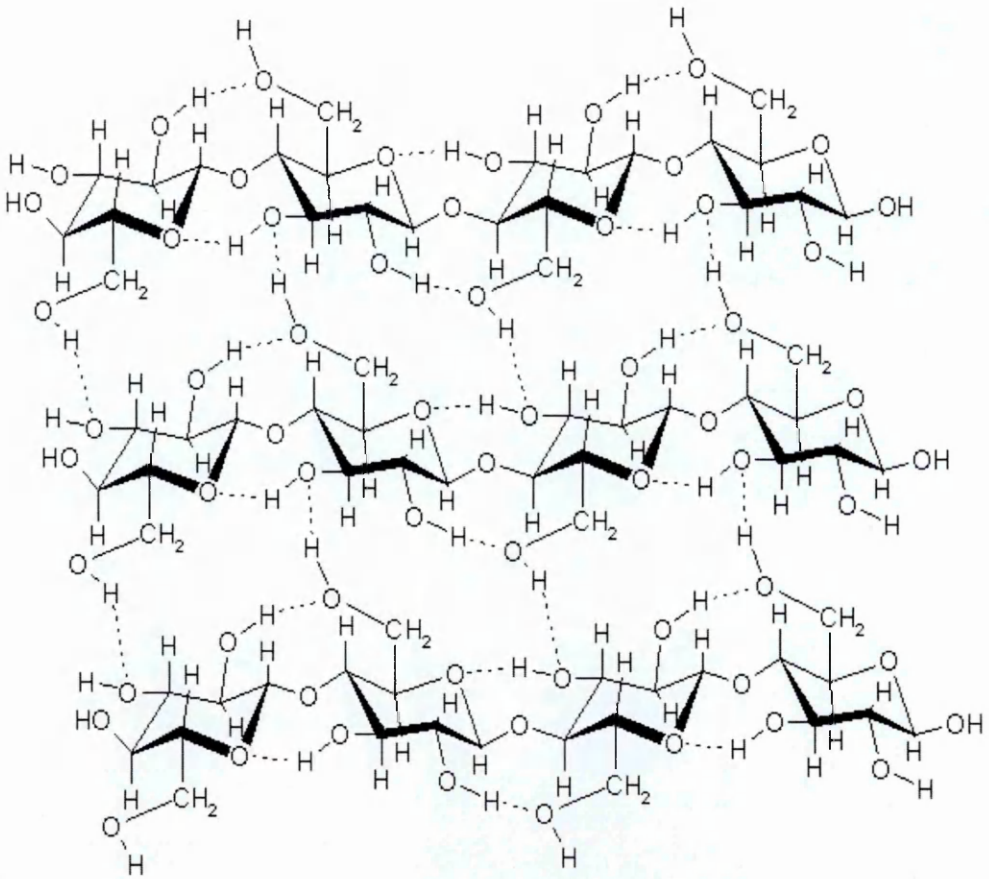

Figure 1.6: Cellulose structure (Adapted from http://www.doitpoms.ac.uk/tlplib/wood/index.php webpage).

Morphology of cellulose presents fibrillar elements. Cellulose molecules get together and create protofibrils and these protofibrils get attached to each other to produce microfibrils complex. These microfibrils are connected via hydrogen and Van der Waals bonds to form a stiff molecular structure, which is called macrofibril. These macrofibrils are not homogeneously distributed and ordered in entire cellulose molecules. That is why it has different regions called amorphous and crystalline structure. The amorphous region contains low order of macrofibrils, where else crystalline region contains high order of these macrofibrils. On top of these structural features, cellulose also contains a wide range of irregularities like bend or twist of fibrils, or different surface holes like micro and large pores.

In conclusion, cellulose fibres are generally establishing complex with other biopolymers especially hemicellulose and lignin. Even though these interactions vary according to plant type and age, they are an outstanding feature limiting the amount and efficiency of biomass utilization.

1.3.1.2. Enzymes Involves In Cellulose Degradation

Cellulose is one of the most abundant substrate on earth (Nielsen et al., 2013), and for that reason the enzymes can degrade cellulose, and the microorganisms possessing these enzymes are crucial for the world carbon cycle (Lynd et al., 2002). Microbial degradation of cellulose can take place in both aerobic and anaerobic conditions. Since cellulose structure is stiff and not easy to break, the complete enzymatic degradation of cellulose is restricted. In a classical cellulose degrading ecosystem, a wide range of cellulolytic bacteria and fungi strains cooperate to convert the cellulosic materials to fermentable sugars, mostly to glucose molecules, which can be transferred into the cell (Beguin, 1990).

Degradation of cellulose to glucose requires a set of enzymes to work in concert synergistically. These enzymes, which are involved in hydrolysis of cellulose via their complementary action, are endoglucanase (EGC), cellobiohydrolase (CBH) and β-glucosidase (BGL). Cellobiohydrolases and endoglucanases are playing role on hydrolysis of cellulose to cellobiose. The endoglucanases are responsible of cutting cellulose chains at amorphous sites to generate oligosaccharides fibrils by random cleavage of β-glucosidic bonds. Where else cellobiohydrolases are acting against crystalline structure of cellulose and remove cellobiose molecules from the reducing or non-reducing ends of the cellulose chains. The last step of cellulose degradation is the conversion of cellobiose into glucose and this step runs by BGL.

Synergism between cellulase enzymes is required for the efficient and complete degradation of cellulose (Fan et al., 2012; Matano, Hasunuma, & Kondo, 2012; Wen, Sun, & Zhao, 2010). As a result of this synergism, metabolisable carbon sources are provided for the microorganisms. A schematic view of degradation via these cellulase enzymes is shown in Figure 1.7.

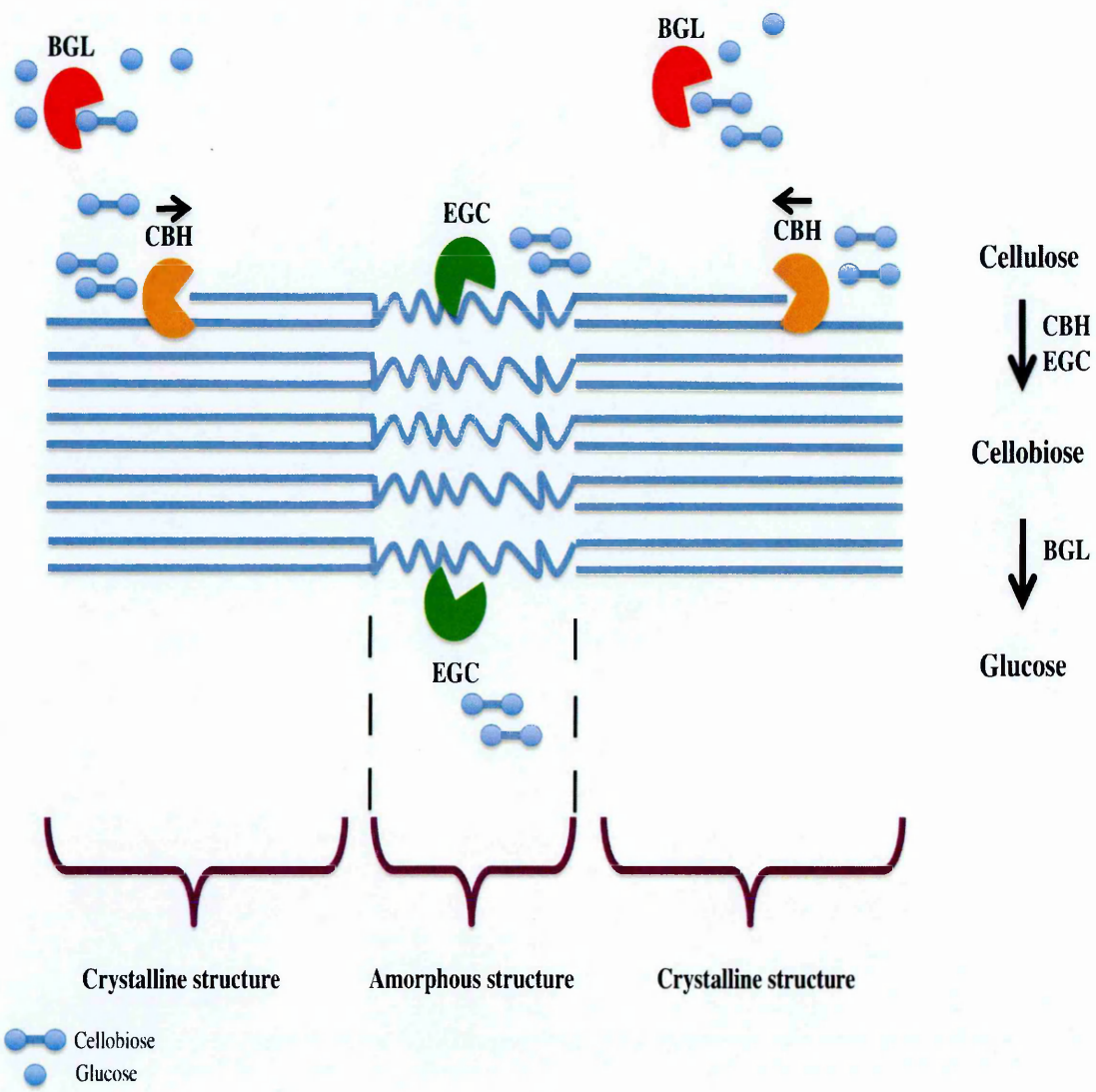

Figure 1.7: Schematic diagram that shows the hydrolysis of cellulose by cellulase enzymes.

1.4 *BACILLI* AND THEIR ROLE IN BIOETHANOL PRODUCTION

As it is mentioned in previous sections, lignocellulosic material needs pre-treatment to release the polysaccharides from the biomass and there are chemicals (enzymatic treatment, acid treatment, etc.), physical (applying pressure, applying heat, mechanical size reduction, etc.) and biological (microorganisms) pre-treatment methods for this purpose. The biological degradation of lignocellulose needs some enzymes for the separation of sugars (Lynd et al., 2002) and the microorganisms (some bacterial and fungal strains; *Pleurotus ostreatus, Phanerochaete chrysosporium, Postia placenta, Streptomyces cyaneus,* etc.) that are able to work in this degradation pathway can be found either free in the environment or as part of the digestive tract of animals (Shallom & Shoham, 2003).

The rumen is one of the environments where the lignocellulose can be degraded biologically by the help of bacteria (Akin & Benner, 1988). *Bacilli* are the rumen bacteria, which are gram-positive, rod-shaped microorganisms and they have been used for many important industrial applications. They have been used as a model microorganism for production of enzymes such as extracellular polysaccharide hydrolyzing enzymes (Bhat & Bhat, 1997), recombinant proteins, antibiotics, insecticides, amino acids (Arbige et al., 1993). One of the important task of *Bacilli* that was target in this study is their ability on catabolism of low molecular weight aromatic compounds, in particular ferulic acid and *p*-coumaric acid, which are the main phenolic compounds that bind to the complex lignin polymer for the separation of the hemicellulosic and cellulosic parts (Degrassi, Polverino De Laureto, & Bruschi, 1995; Zago et al., 1995).

In this study different *Bacilli* strains (*B. circulans, B. atrophaeus, B. licheniformis, B. macerans, B. pumilus* and *B. subtilis)* were used and they had been previously isolated from the rumen of the cow by our laboratory in collaboration with School of Veterinary Medicine, University of Udine.

1.5 AIM OF THE STUDY

This Ph.D. Thesis was meant to provide a novel view of lignocellulosic-based bioethanol production.

As it is mentioned in the Results section, this study divided in two chapters. First part of the research is interested in microbial combination system that is able to efficiently utilize the lignocellulosic waste as a carbon source. It aimed to study simultaneous saccharification and fermentation of wood waste to glucose molecules by using *Bacilli* and recombinant *S. cerevisiae* strains together.

Second part of the work was dealing with the two novel technologies for bioethanol production. Furthermore, it aimed to study the evolutionary effect of bridge induced translocation (BIT) technology to improve *S. cerevisiae* capacity to produce ethanol via the acquisition of an advantageous homoeostasis following the de-regulation of cellulose degradation genes carried on yeast artificial chromosome (YAC).

Covered fields involve in this thesis; (1) Production and secretion of cellulase genes in *S. cerevisiae,* (2) Using new techniques to improve yeast strains for bioethanol productions, (3) Pre-treatment of lignocellulosic material by *Bacilli* (4) Using wood waste directly for the microbial fermentation in the matter of cost and time effective process.

2. MATERIALS AND METHODS

2.1 ORGANISMS, PLASMIDS AND MEDIUMS

2.1.1 MICROBIAL STRAINS

For routine plasmid preparation the *E. coli* XL10-Gold (endA1 (glnV44 recA1 thi-1 gyrA96 relA1 lac Hte Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 tet^R F'[proAB $lacI^qZ\Delta M15$ Tn10(Tet^R Amy Cm^R)]) strain and the Stbl2 strain [(F^- *mcr*A Δ(*mcr*BC-hsdRMS-*mrr*) *rec*A1 *end*A1 *lon gyr*A96 *thi sup*E44 *rel*A1 λ^- Δ(*lac-pro*AB)] were used.

All strains of *Bacilli* (*B. circulans, B. atrophaeus, B. licheniformis, B. macerans, B. pumilus* and *B. subtilis)* used in this study had been previously isolated from rumen of the cow stomach by our laboratory in a collaboration with University of Udine.

Cellulose degradation genes were amplified from *Pichia stipitis* NBRC 10063 (CBS 6054) and *Trichoderma reesei* QM9414 strains.

S. cerevisiae BAP4 strain was used as a host for the cellulose degradation experiments. BAP4 strain is a diploid *pep4-3* mutant strain, it is produced by mating CBL1-20 strain: *a, [cir°], ura3-52, leu2-3,112, trp1-289, pep4-3* (Ludwig & Bruschi, 1991; Ludwig, Ugolini, & Bruschi, 1993) and *PEP4* knock-out YPH4 strain: a, *ura3-52, lys2-801, ade2-101* and *his3-A200* (prepared in this study).

2.1.2 PLASMIDS

pBLAST (Ludwig D.L. and Bruschi C.V., 1991), pFA6KanMX4, pGFKG, pVT100-U, pJL49 (Designed by Jean-Luc Parrou, CNRS Toulouse/France), pTEF and pYAC3 plasmids were used in this thesis (Appendix). Sequences of promoters and secretion signal were given below;

***ADH1* promoter:**

CAACTTCTTTTCTTTTTTTTTCTTTTCTCTCTCCCCCGTTGTTGTCTCACCATATCCGCAATGACAA
AAAAAATGATGGAAGACACTAAAGGAAAAAATTAACGACAAAGACAGCACCAACAGATGTCG
TTGTTCCAGAGCTGATGAGGGGTATCTTCGAACACACGAAACTTTTTCCTTCCTTCATTCACGCA
CACTACTCTCTAATGAGCAACGGTATACGGCCTTCCTTCCAGTTACTTGAATTTGAAATAAAAA
AAGTTTGCCGCTTTGCTATCAAGTATAAATAGACCTGCAATTATTAATCTTTTGTTTCCTCGTCA
TTGTTCTCGTTCCCTTTCTTCCTTGTTTCTTTTTCTGCACAATATTTCAAGCTATACCAAGCATAC
AATCAACT

***PGK1* promoter:**

TCTAGAGTCGAGATCTTGTTTTATATTTGTTGTAAAAAGTAGATAATTACTTCCTTGATGATCTG
TAAAAAAGAGAAAAAGAAAGCATCTAAGAACTTGAAAAACTACGAATTAGAAAAGACCAAAT
ATGTATTTCTTGCATTGACCAATTTATGCAAGTTTATATATATGTAAATGTAAGTTTCACGAGGT
TCTACTAAACTAAACCACCCCCTTGGTTAGAAGAAAAGAGTCGGGACT

***TEF1* promoter:**

ATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCG
TACCACTTCAAAACACCCAAGCACAGCATACTAAATTTCCCCTCTTTCTTCCTCTAGGGTGTCGT
TAATTACCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCGTCG
AAAAAGGCAATAAAAATTTTTATCACGTTTCTTTTTCTTGAAAATTTTTTTTTTTGATTTTTTTCT
CTTTCGATGACCTCCCATTGATATTTAAGTTAATAAACGGTCTTCAATTTCTCAAGTTTCAGTTTC
ATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGCTCATTAGAAAGAAAGCATAGCAATCTAA
TCTAAGTTTTCTAGAACTAGTAAAAGA

***GAL10* promoter:**

ACGGATTAGAAGCCGCCGAGCGGGTGACAGCCCTCCGAAGGAAGACTCTCCTCCGTGCGTCCTC
GTCTTCACCGGTCGCGTTCCTGAAACGCAGATGTGCCTCGCGCCGCACTGCTCCGAACAATAAA
GATTCTACAATACTAGCTTTTATGGTTATGAAGAGGAAAAATTGGCAGTAACCTGGCCCCACAA
ACCTTCAAATGAACGAATCAAATTAACAACCATAGGATGATAATGCGATTAGTTTTTTAGCCTT
ATTTCTGGGGTAATTAATCAGCGAAGCGATGATTTTTGATCTATTAACAGATATATAAATGCAA
AAACTGCATAACCACTTTAACTAATACTTTCAACATTTTCGGTTTGTATTACTTCTTATTCAAAT
GTAATAAAAGTATCAACAAAAAATTGTTAATATACCTCTATACTTTAACGTCAAGGAGAAAAAA
CC

Secretion signal (alpha factor):

ATGAGATTTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTC
AACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTTAGATT
TAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTT
ATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTTTGGATAAAAGGGAG
GCTGAAGCT

2.1.3 GROWTH MEDIA

All media were autoclaved with standard parameters (20min, 15 lb/sq.in. on liquid cycle). In the case when solid medium was required, the mixture was supplemented with 2% bacto- agar (214010, BD Biosciences) and pH of the solution was brought to 6,8- 7,2.

2.1.3.1. Yeast Media

The media preparations are as listed below and preparations were made according to literature with some modification (Kaiser, Michaelis, & Mitchell, 1994b). All media were autoclaved with standard parameters. Where solid agar medium was required, bacto-agar was added to 2% prior to sterilization. The plates were let dry for several hours prior to use. When necessary, either liquid or solid media was supplemented with 200μg/ml Geneticin G418 (Gibco, Rockville, USA.) to select for kanamycin resistant clones. YPD

was modified for some experiments and instead of glucose some other type of carbohydrates were used; with raffinose YPR, with cellobiose YPC and with carboxymethyl cellulose YPCMC were prepared.

For some selection conditions, synthetic media was prepared. Where dropout medium was desired, one or more of the components was misplaced from the supplements. For example, if uracil dropout medium was required, a supplement was made containing all the other components, but uracil.

YPD (yeast extract, dextrose, peptone)

2% Glucose (G8270, Sigma- Aldrich)

2% Bacto-peptone (211677, BD Biosciences)

1% Yeast extract (212750, BD Biosciences, Madison, USA)

Synthetic minimal and complete medium

2% Glucose (G8270, Sigma- Aldrich)

0.5% Ammonium sulphate

0.17% Yeast nitrogen base

0,2% Drop-out mix

Drop-out mix

0.5g Adenine	2g L-Tyrosine
2g L-Arginine	2g Uracil
2g L-Histidine	15g L-Threonine
10g L-Leucine	2g L-Phenylalanine
2g L-Lysine	2g L-Tryptophan
2g L-Methionine	2g Proline

2.1.3.2. Media For Bacteria

For the growth of all bacterial cultures the nutrient medium LB was used, as described by Sambrook, *et al.* (1989).

Ingredients:

1. 1% (w/v) Tryptone
2. 0,5% (w/v) Yeast Extract
3. 0,5% (w/v) NaCl

In order to obtain solid LB media, the pH was adjusted to value 6,8-7,2 and solution was supplemented with 2% (w/v) bacto-agar. Media was autoclaved under standard conditions (20 minutes with pressure 15 lb per square inch on liquid cycle). When media cooled below 55°C, as a selection for plasmids, antibiotics were added. Final concentration of antibiotics was 100μg/ml for ampicillin (194526, MpBio, France) and kanamycin disulphate (G4181, ForMedia Ltd., England). Prepared media was poured into plastic petri dishes immediately. Solidified and dried plates were closed by parafilm and stored for up to two weeks at 4°C.

For some experiments, LB poor media was modified and LB poor was prepared (0,5% NaCl, 0,5% tryptone, 0,25% yeast extract). For lignocellulosic degradation experiments, LB poor media was supplemented with wood powder. Wood powder was a waste product of wood industry (Lasole Company) moreover it was coming originally from fir and beech trees. First, wood powder was sterilized by heat (3 days at 80°C) and then mixed with LB poor for the *Bacilli* fermentation for seven days. *Bacilli* strains were used for the pre-treatment of wood powder. They degraded the wood to fermentable sugars for the yeast fermentation.

2.2 GENERAL MOLECULAR BIOLOGY TECHNIQUES

For routine plasmid preparations, the Wizard Plus Miniprep System was used (A1330, Promega, USA). For standard DNA gel extraction, Qiagen Gel Extraction Kit was used (28704, Qiagen, Germany). Due to limitations of the maximum DNA size possible to isolate with this kit (~10000 bp), QIAEX II Gel Extraction Kit was utilized when necessary (20021, Qiagen, Germany). For RNA extraction, Promega Total RNA Isolation System (Z3101, Promega, USA) was used.

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich.

2.2.1 OLIGONUCLEOTIDES

The sequences of the PCR primers were listed in Table 2.1 and were synthesized by Integrated DNA Technologies (Bologna/Italy). For PCR amplification, unless otherwise specified, the reaction was performed in a 50 µL of mixture consisting of 10ng of DNA template, 1x buffer, 0.2mM of each primer, 0.25mM dNTPs, and 2U of DNA polymerase. The typical PCR program consisted of an initial denaturation of 2 minutes at 95°C, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at appropriate temperature depends on the primers and extension at 72°C for 1 minute per 1kb with a final 10 minutes elongation at 72°C.

Table 2.1: Sequences of the primers used in this study.

Name of the Primer	**Sequence (5'-3')**
FwBGL5-Ext.	ACATCCCGTTTTGACGCTAC
RevBGL5-Ext.	GGGGGTTCGATTCCTATTGT
FwBgl5ecoR1	CCCGAATTCCCCATGGGTGTTCAAGAATTAGA

RevBgl5xho1	CCGCTCGAGCGGCTATAATCCCAACCAGTAGA
FwEGC1-Ext.	GATGAAACAACACGGTTACG
RevEGC1-Ext.	GATCTTTGATAAGGAGTAGGG
FwEGC1xho1	CCGCTCGAGCGGATGTCTACAGGATTCTTAACC
RevEGC1bamH1	GCCGGGATCCCGGTTAATTCTTGTAATCCTTCAAGA
FwCBH2ecoR1	CCCGAATTCGGGCAAGCTTGCTCAAGCGTCT
RevCBH2cla1	CCCATCGATGGGCAGGAACGATGGGTTTGCG
Fwalphafactor (fused to each gene with appropriate restriction sites)	ATGAGATTTCCTTCAATTTTTAC
Revalphafactor (fused to each gene with appropriate restriction sites)	CTTTTATCCAAAGATACCCCT
FwYackan	TGGCCCGCTTTATCAGAAGCCAGACATTAACGCTTCTG GAGAAACTCAACGAGCTGGACGCGGATGAACAGGCA GACATCGTCGACGGATCCCCGGGTTAA
RevPAD1kan	TTTCAGACAGGAATGCAAAGTCTGTAAAATACATCCA ATTTCTTGTCTGCAATTTATGTCATAACATGTTAAGCG TTTTCCGCGCGTTGGCCGATTCAT
BITControl a1	TCAGTAACCCGTATCGTGAG
BITControl b1	TGCGGTAAAGCTCATCAGCG
K1	ACAATCGATAGATTGTCGCAC
BITControl a2	GCGGTCTTGCCATGATATTC
BITControl b2	AACGGTAAACAGGAAAGGCG
K2	TCAGTCGTCACTCATGGTGAT
FwBGL6-I	TTACTGCTTGTAAAGCACAGG
RevBGL6-I	ACCAGATGATTGGTCTAGAAG
FwBgl6Pwo	ATGGGAGCACAAGAACTAGA

RevBgl6Pwo	CTACAACCCCAACCAGTAGA
FwBgl5rt	GGTTCCATCAACGGTCAGGT
RevBgl5rt	ATCGGCTGCAACAGACAAGT
FwEGC1rt	ACATGGGTCGCTGGTTTCAA
RevEGC1rt	CCATGGAGTAGGTGTTGGCA
FwCBH2rt	CGGGAACCGCTACGTATTCA
RevCBH2rt	AAGAGGGAACCTTTGCGACA
FwACT1rt	GAAATGCAAACCGCTGCTCA
RevACT1rt	TACCGGCAGATTCCAAACCC
FwPAD1rt	TAACCAGGGCATCAACTTCG
RevPAD1rt	AGCGTGGATGCCAAAGCAGT
FwEGC1construct-aat2	AAAAAGACGTCAAACCTGGTATCTTTATAGTCCTG
RevEGC1construct- snab1(sac2-asc1)	GGGCTACGTAGAGAGGCGCGCCAAGAAGAACCGCGG GAGAAGGTAGTCTAGTACCTCCTG
FwBGL5construct-asc1	GAGAGGCGCGCCGAGAGCTTTACACTTTATGCTTCCG
RevBGL5construct-sac2	GAGACCGCGGGAGAATGTGCTGCAAGGCGATTAA
FwCBH2construct-sac2	CGAGCCGCGGGCACGAGGGAACAAAAGCTGGAGCT
RevCBH2construct-pme1	CACGGTTTAAACCGAGGCGTAATACGACTCACTATAG
FwPEP4KO	ATGTTCAGCTTGAAAGCATTATTGCCATTGGCCTTGTT GTTGGTTAGGCGTATCACGAGGCCC
RevPEP4KO	TGTTCAGCTTGAAAGCATTATTGCCATTGGCCTTGTTG TTGGTTAGGCGTATCACGAGGCCC
FwcontrolPEP4	GAGAAGCCTACCACGTAAGG
RevcontrolPEP4	GACATTATGGGCAGCAGCATA

2.2.2 GEL ELECTROPHORESIS

Gels were prepared and run as described by Sambrook, *et al.* (1989). For the visualization of DNA plasmids and fragments of size 500 to 20,000 base pairs, a 0.8% agarose (16500500, Invitrogen, Carlsbad, USA) gel was prepared in 0.5xTBE buffer and melted in a microwave. The tray was assembled with the proper comb and moved into the gel tank. After the mixture had cooled to less than 60°C, the agarose was poured into a gel tray and left until it solidified. Once the gel solidified, it was placed into the horizontal gel apparatus and submerged with 0.5xTBE buffer. The DNA samples that had been treated with at least one-third volume of DNA gel running buffer were loaded into the wells. A known DNA base pair marker was typically placed in the first well for size identification. The gel was run at approximately 90 volts (65mA) for at least half an hour to ensure an optimal separation of the fragments. For smaller fragments, lower voltages were utilized. Afterwards, the gels were stained with ethidium bromide for 10-15 min. The DNA bands were visualized on a UV transilluminator at approximately 300nm. If the gel stained too intensely, it was destained in H_2O.

10X TBE (Running gel buffer)

1. 890mM Tris base (15504-020, Invitrogen)
2. 890mM Boric acid (B6768, Sigma- Aldrich)
3. 20mM EDTA (E9884, Sigma- Aldrich)

6x Loading buffer (STEB) for DNA

1. 100mM Tris base HCl, pH 7.5 (15504-020, Invitrogen)
2. 20% (w/v) Glycerol (49779, Sigma- Aldrich)
3. 1mM EDTA (E9884, Sigma- Aldrich)
4. A few grains of Bromophenol blue (B5525, Sigma- Aldrich)

2.2.3 DNA PRECIPITATION

DNA precipitation was used routinely as a method to purify and concentrate the DNA. Samples were transferred into the 2 ml eppendorf tube, and afterwards the 1/10 of the total mixture volume, 1M Sodium Acetate pH 5.2 (32319, Honeywell Riedel- de Haen, Selze, Germany) and 2.5 volumes of ice-cold ethanol (02860, Sigma Aldrich) were added. Reaction tubes were kept on dry ice for at least 20 minutes. Following that, samples were centrifuged for 15mins at 13000 rpm at 4 °C. Ethanol was removed and remaining pellet was washed with 250µl of 70% ice-cold ethanol. After 5 minutes 13000 rpm centrifugation at 4°C, ethanol was removed and tubes were left open in the room temperature approximately for 10 minutes for the evaporation of the remaining ethanol. Pellet was subsequently resuspended in water and stored at -20°C.

2.2.4 LITHIUM ACETATE YEAST TRANSFORMATION

Eurofan protocol based on the LiAc/ssDNA/PEG method (Ito et al., 1983) was followed for the transformation of the exogenous DNA inside the yeast nucleus.

A single yeast colony was picked and resuspended into 10 ml of YPD and incubated on a rotary shaker at 250 rpm and 30°C overnight. Following day, cell count from the overnight culture was determined by optical density measurements using GeneQuant Pro spectrophotometer (GE Healthcare, Buckinghamshire, England) and cells were diluted to reach $2x10^6$ cells/ml. Reinoculated culture was grown till the late exponential phase (number of cells was closed to $1x10^7$ cells/ml). The cells were washed once with 25 ml of sterile ddH_2O, once with 1 ml of 0.1M LiAc (517992-100G, Sigma- Aldrich). Then the cells resuspended in 250µl of 0.1 M LiAc to reach concentration of about $2x10^9$ cells/ ml and incubated at 30°C for 20 minutes. In the meantime, a 10µg/µl sheared herring sperm carrier DNA was denatured at 95°C for 10 minutes (D816, Promega) and straight away after denaturation placed on ice. Transforming DNA was added into 50µl of LiAc treated

cells with denatured herring sperm in concentration correlated to amount of DNA used. A negative control not including the transforming DNA was also set up and incubated for 20 minutes at 30 °C. After that 300µl of a mixture of 100mM LiAc and 40% w/v 3350 PEG (P4338, Sigma- Aldrich) were added to the reaction tube and incubated at 30°C for 20 minutes. Following the incubation, tubes were placed in circulating water bath set at 42°C and kept for another 20 minutes. When this heat-shock was terminated, tubes were centrifuged at 4000 rpm for 5 minutes and LiAc/PEG mixture was removed and cells were resuspended in 1 ml YPD and incubated at 30°C for 1 hour. Afterwards, cells were centrifuged at 4000 rpm for 5 minutes, resuspended in water, and plated on the selective media.

2.2.5 BACTERIAL COMPETENT CELL PREPARATION

A single bacterial colony was picked and inoculated into 10 ml of LB and incubated on a rotary shaker at 37°C overnight. Next day, 1 ml of the over night culture was inoculated into 40ml LB and let it grow till 0,3 OD reached (usually couple of hours). After that the cells were moved to 50 ml sterile falcon tubes and chilled on ice for 10 min. The cells were pelleted at 5000-6000 rpm at 4°C for 10 minutes and resuspended in 20ml of ice-cold 50mM $CaCl_2$. Resuspended cells were incubated on ice for 20min and pelleted again for 10 min (5000-6000 rpm, 4°C). The pellet was resuspended with 4 ml of ice-cold 50mM $CaCl_2$. 300µl aliquots of the cells were prepared by mixing 192µl of cells and 108µl of 50% glycerol and stored at -80°C.

2.2.6 BACTERIAL TRANSFORMATION

An aliquot of competent bacterial cells from -80°C was thawed on ice for 10 min. For each transformation, 100µl of bacteria cells were mixed with 100ng of DNA and left on ice for 30 min. For the heat shock, cells were transferred to circulating water bath preheated to 42°C for 90 seconds. At the end of the heat shock, 500µl LB medium was added to the

cells for recovery and the tubes were further incubated for 90 minutes at 37°C with shaking. In the last step, cells were spun down and pellets were resuspended in 200µl of ddH_2O. Water resuspended cells were plated on selective LB-agar plates and then plates were incubated overnight at 37°C.

2.2.7 COLONY PCR

After transformations, cells were checked by colony PCR approach to verify the positive transformants. PCR template derived directly from the colony growing on plates. Depending on the organism subjected to this analysis, cell destruction and release of the DNA is achieved differently.

For bacterial colony PCR, colonies were added directly in PCR mixture after heat pre-treatment. Their cell walls can be easily destroyed just by heating. Bacterial cells can be boiled in the microwave on full power for 5 minutes and after centrifugation, supernatant can be used as a PCR template.

For yeast cells, colonies (~ 1mm) were scraped with sterile toothpick and resuspended in 60U/ml zymolase solution (120491-1, Seikagaku Biobusiness Corporation, Japan). Tubes were incubated at 37 °C for 30 minutes and later centrifuged for 5 minutes at 2000 rpm. Zymolase was removed, cells were resuspended in sterile H_2O and kept at 95 °C for five minutes. In the meantime, PCR master mix was prepared. All reactions were done using GoTAQ polymerase (M31175, Promega).

After heating, 10µl of every sample was transferred into new PCR tube and 15µl of master mix was added. Programs used are presented in the respective sections. Afterwards, reaction samples were visualized in the agarose gel.

2.2.8 PLASMID CONSTRUCTION

Standard protocols were followed for DNA manipulations (Sambrook, Fritsch, & Maniatis, 1989). The enzymes for DNA cleavages and ligation were purchased from New England Biolabs (NEB) and used as recommended by the supplier. For sticky end ligations, DNA was added to the reaction at a molar insert to vector ratio of at least 3:1. For blunt end ligations, this ratio was sometimes increased to 10:1 or greater. Following overnight ligation incubation, the reactions were either added directly to competent *E. coli* for transformation, or an aliquot first analyzed by agarose gel electrophoresis, to visualize the formation of higher molecular weight ligation products. All the primers used for cloning were given in table 2.1.

2.2.9 DELETION OF *PEP4* GENE AND DETERMINATION OF THE *PEP4-3* MUTATION

YPH4 haploid strain used for *PEP4* gene (chromosome XVI) deletion to prepare a host strain for the heterologous gene expression. For the deletion, the STIK approach (Waghmare, Caputo, Radovic, & Bruschi, 2003) was applied by using pGFKG plasmid, in which kanamycin resistance genes is flanked by FRT sequences. To release the kanamycin gene from genome by the help of FLP-FRT recombination, cultures were incubated at 30°C for at least 10 generations to 2-3x10^8 cells/ml, where stationary phase was reached. At this time, serial dilutions were made, and cells plated onto several YPD plates to yield 100-300 cfu/plate. After three days growth, colonies were replica-plated onto selective medium (YPD+G418). Non-resistant colonies were chosen after pop-out of the kanamycin and used for the mating experiment to obtain diploid *pep4-3* mutant strain.

Determination of the presence of the *pep4-3* mutation in yeast strains was performed according to Jones (1977). The *pep4-3* mutant yeast was streaked onto a YPD agar plate and allowed to grow at 30°C for two days. The detection system involved overlaying the

yeast plate with a mixture of 3ml of 0.6% agarose, made with water and cooled to less than 50°C, and 2ml of 4mg/ml APE (N-acetyl-DL-phenylalanine-b-naphthyl ester) in water. After the overlay had solidified, the plate was allowed to set for 10 minutes and then flooded with 5ml of Fast Red stain at 5mg/ml. As soon as the plate was soaked, about 30 seconds, the stain was aspirated off, and the colony colour monitored. Those colonies derived from cells that are wild type *PEP4*, cleave the APE substrate, which reacts with the stain, and turns the colonies red. Those that are mutant, therefore remain white. Eventually, all colonies will become red, but the mutants can be readily distinguished after the initial staining period. A negative and a positive control strains were analyzed in parallel.

2.2.10 CHEF (CLAMPED HOMOGENOUS ELECTRIC FIELDS) GEL

Isolation and visualization of yeast chromosomes was performed as described by Bio-Rad for the CHEF-DRII system. Yeast cultures of selected strains were grown overnight in 10ml YPD medium to stationary phase. The cells were pelleted in a centrifuge at 3000rpm for 10 minutes. The supernatant was discarded and the cells resuspended in 180µl of 0.05M EDTA, pH 8.0 and 15µl of 2mg/ml Lyticase was added. During cell preparation, a 1.0% gel solution was prepared by melting Low Melt Preparative Gel Agarose in 0.125M EDTA, pH 7.5. The mix was allowed to cool to about 50°C. In fresh eppendorfs, 300µl of agarose solution was mixed with 180µl of cell/enzyme mix, then pipetted into individual mold chambers with a pipet. After cooling at 4°C for 20 minutes, the plugs were removed from the mold, with a clean spatula, and placed into individual falcon tubes. The plugs were then covered with LET buffer and incubated at 37°C overnight. Afterwards, the LET buffer was removed, and the plugs washed three times for 15 minutes each in 50mM EDTA, pH 8.0. NDS buffer was then added to the tubes, covering the plugs, and the tubes incubated overnight at 50°C. After this step, the plugs were washed 4 times for 1 hour each in 50mM EDTA, pH 8.0. The wash was then removed, the plugs were stored for prolonged periods at 4°C in the 50mM EDTA solution. The CHEF gel was cast using the provided

mold, with a 10 well comb. A 1.0% gel was prepared by chromosomal grade agarose (161-0137, Bio-Rad) in 0.5xTBE, in a volume of 100ml. After cooling to 50°C, the gel was poured, and allowed to solidify. The comb was then removed from the gel, and portions of the yeast plugs were placed into the wells such that they would occupy less than 90% of the volume of the wells. Typically, Bio-Rad yeast chromosomal marker plugs were placed in the outside wells for control. The sample wells were then filled in with a preparation of 1.0% low melting preparative grade agarose (9414, Sigma), cooled for 50°C. Care was taken to avoid trapping air bubbles. It was not necessary to fill in the wells that lacked sample plugs. After 15 minutes, the gel was ready for electrophoresis. The electrophoresis unit was placed in a 4°C cold room and the electrophoresis chamber filled with 2 litres of 0.5xTBE. The buffer was circulated by pump through a 4°C water bath to facilitate cooling of the buffer. The buffer should cover the gel by approximately 2mm. The flow rate of the buffer was adjusted to about 1L/min. The pulse unit was programmed to ramped switch time from 50 to 90 seconds for 22 hours. The voltage was set at 200V. After the run had been completed, the gel was stained with ethidium bromide and the bands visualized by ultraviolet light. For DNA hybridization, the gel was soaked for 15 minutes in 0.4N NaOH. Transfer to nylon membrane was accomplished via Southern blot, except that the transfer solvent was 0.4N NaOH. After at least 24 hours, the filter was removed and neutralized in 0.5M Tris, pH 7.0 for 5 minutes. Afterwards, it was rinsed briefly in 2xSSC, then dried and baked, as described for Southern transfer.

LET buffer	NDS buffer
0.5M EDTA (pH 8.0)	0.5M EDTA (pH 8.0)
0.01M Tris-Cl (pH 7.6)	0.01M Tris-Cl (pH 7.6)
7.5% β-Mercaptoethanol	1% Lauryl-sarcosine
	+ 1mg/ml Proteinase K

2.2.11 SOUTHERN BLOT AND HYBRIDIZATION

Southern blotting was employed, essentially as described by Sambrook, *et al.*, (1989) and based on the experimentation of Southern (1975). After EtBr staining and photography of the DNA gel, it was soaked for 30 minutes with gentle agitation in several volumes of denaturing solution (0.5N NaOH, 1.5M NaCl). After denaturation, the gel was briefly rinsed in water, then neutralized in several volumes of 1M Tris-Cl, pH 7.5, 1.5M NaCl for 30 minutes with gentle agitation. Afterwards, the gel was incubated for an additional 15 minutes in fresh neutralizing solution. During these incubations, several pieces of Whatman 3M filter paper, and a piece of nylon membrane (Hybond N+), was cut to the dimensions of the gel. Transfer was performed by capillarity. A Whatman filter paper wick was cut to the width of the gel, and long enough to span the length of the gel bridge and adequately touch the buffer in the tray. For most transfers, 10xSSC was used as the transfer buffer, and the reservoir was filled to just below the bottom of the gel bridge. The wick was completely soaked with buffer, and any air bubbles removed. The gel was then placed face down onto the wick. The nylon membrane was first wetted in water, prior to use, then placed on top of the gel. As before, trapped air bubbles were carefully removed. At least 5 sheets of Whatman filter paper cut to the size of the gel were then placed on top of the nylon filter. Several thicknesses of tissue papers were then cut to the size of the gel, and placed on top of the Whatman paper. A weight equivalent to 500g was then placed on the top of the blotting pads, and the transfer allowed to run at least 16 hours. Afterwards, the nylon filter was first floated on 6xSSC, and then submerged into the solution for 5 minutes. The nylon filter was then sandwiched between two fresh pieces of filter paper, and baked for 2 hours at 80°C. At this point, the filter was either used immediately or wrapped in aluminium foil, in the filter paper sandwich, and stored at room temperature. The agarose gel was restained with EtBr and visualized on a UV transilluminator to determine the degree of DNA transfer to the nylon filter.

20xSSC

175.3g NaCl

88.2g NaCitrate

pH to 7.0; bring to 1L with water

The hybridization procedure was performed essentially as described by Sambrook, *et al.* (1989). To prepare the nylon filter for hybridization, it was rewet in 6xSSC for 2 minutes, then placed into a plastic hybridization bag containing 0.2ml of pre-hybridization fluid for each square centimetre of filter. The bag was then sealed with a heat sealing unit, and incubated at 65°C, with occasional agitation, for at least 2 hours. Afterwards, one corner of the bag was cut with scissors, and the pre-hybridization fluid drained from the bag. Hybridization fluid with the probe (synthesized by using PCR DIG probe synthesis kit, Roche), pre-warmed to 65°C, was added into the bag at 50µl of fluid for each square centimetre of filter. The bag was then smoothed out to carefully remove trapped air bubbles from within the bag. The hybridization bag was then sealed into a second larger bag, to prevent contamination from possible leakage of radioactive fluid from within the hybridization bag. The hybridization was placed on a rocker platform at 65°C and incubated for at least 16 hours. After that, washing and detection steps were followed. One side of the bag was then completely opened, and hybridization solution was removed, and immediately placed into 200ml of 2xSSC, 0.5%SDS. The membrane was incubated in this solution for 5 minutes at room temperature, with gentle agitation, then the solution discarded into liquid waste. A second 200ml of 2xSSC, 0.1% SDS was then added, and the filter washed at room temperature for 15 minutes. This solution was then discarded, and the filter placed into 200ml of 0.1xSSC, 0.5%SDS. It was then incubated in this solution, with gentle agitation, for 30 minutes at 65°C. The membrane was then transferred to another 200ml of 0.1xSSC, 0.5% SDS and incubated for 30 minutes at 65°C. Afterwards the membrane was incubated 30 minutes in blocking buffer, 30 minutes in antibody

solution, 5 minutes in detection buffer, 5 minutes in CDP-Star Chemiluminescent Substrate and the hybridization was visualized by Kodak film.

Pre-hybridization solution	50x Denhardt's Solution
6xSSC	1% Ficoll
0.5% SDS	1% Polyvinylprrolidone
5x Denhardt's solution	1% Bovine serum albumin (Fraction V)
100µg/ml herring sperm DNA	

Hybridization solution	Blocking Buffer
6xSSC	12ml of 10x Blocking reagent
0.5% SDS	108ml of maleic acid buffer
100µg/ml herring sperm DNA	

Antibody solution	Detection Buffer
20ml Blocking buffer	0.1M Tris
2µl DIG antibody	0.1M NaCl

2.2.12 QUANTITATIVE PCR

Translocant strains were grown in YPD supplemented with 200µg/ml Geneticin G418 overnight. The following day the RNA was isolated using Promega Total RNA Isolation System (Z3101, Promega, USA) and the AMV Reverse Transcriptase (M501, Promega, USA) was used to produce cDNA on the RNA template according to the protocol provided by the manufacturer. Resulting cDNA was used as a template for amplification of *BGL5, EGC1, CBH2, PAD1* and *ACT1* gene (primers in the Table 2.1). PCR was carried out by using Rotor-Gene SYBR green kit (Qiagen) with the already provided PCR program

designed for the Rotor-Gene Cycler from Qiagen. *ACT1* mRNA level was chosen as a control. The final data were reported as normalized expression level of every gene analyzed with respect to its parental copy. The experiments were repeated at least three times and standard deviations were calculated.

2.2.13 DETERMINATION OF BETA-GLUCOSIDASE AND TOTAL CELLULASES ACTIVITY

To determine β-Glucosidase activity, the pNPG (p-Nitrophenyl-D-glucopyranoside) assay was performed by following what suggested in the literature (Jeon et al., 2009). The unit definition of the beta-glucosidase is the amount of enzyme, which catalyzes the formation of 1 μmol of p- Nitrophenol per minute at 37°C starting from PNPG (p-Nitrophenyl-D-glucopyranoside). The method of assay is based on the spectrophotometric determination at 405nm of p-Nitrophenol, which is formed during the reaction. Cells were centrifuged at 13,000 rpm for 4 min and the supernatant was incubated in 50mM acetate buffer with 5mM pNPG at pH 5.0, at 37°C for 15 min. The p-nitrophenol released from pNPG was detected at 405 nm after adding 2 ml of Na_2CO_3 0.2 M to stop the reaction. Appropriate blanks without enzyme or substrate were also run in parallel as control.

For cellulases activity assay, we used EnzChek Cellulase Substrate (E33953) from Invitrogen and followed the protocol as it is suggested by the supplier. EnzChek Cellulase Substrate is fluorescence-based cellulase substrate that was developed for simple and rapid quantitation of cellulase. Cellulase assay with this fluorescence substrate is highly sensitive, with a detection limit as low as 40μU/mL cellulase.

Different sample dilutions and standards were run in parallel and read the absorbance at 360nm.

2.2.14 CHROMOSOME STABILITY

Recombinant YAC and translocant YAC was checked by their stability in cell under non-selective conditions. At least three individual YAC-containing colonies were picked and used to inoculate separate vials containing 10ml of YPD to a density of approximately $2x10^5$ cells/ml. Cultures were incubated at 30°C for at least 10 generations to $2\text{-}3x10^8$ cells/ml, where stationary phase was reached. At this time, serial dilutions were made, and cells plated onto several YPD plates to yield 100-300 cfu/plate. After three days growth, colonies were replica-plated onto selective medium (SC-URA) and scored for growth as an indication of YAC-containing cells.

Stability was measured as a percentage of prototrophic cfu relative to total cfu plated. In total 2500 colonies were examined per each condition; recombinant YAC and translocant recombinant YAC containing cells.

2.2.15 ANTHRONE TEST

To estimate the amount of cellulose produced by *Bacilli,* we used the anthrone test (Bailey, 1958), which is a simple colorimetric method to measure the total amount of carbohydrates. The amount of total carbohydrates in the sample is estimated via reading the absorbance of the resulting solution against a glucose standard curve. The principle is based on the fact that, in the presence of sulphuric acid, glucose is converted into 5 - hydroxymethylfurfural and this reaction with anthrone results in a product that has the blue-green colour, whose maximum absorbance is read at 620nm.

All *Bacilli* strains were grown for 7 days in the LB poor media with or without wood powder. 1 ml from the media was mixed with 2 ml 75% H_2SO_4 and 4ml anthrone solution and let them boiling for 15 minutes at 100°C. Absorbance was read at 625nm. Appropriate controls were also run in parallel. Different concentrations of glucose solutions were used for the standard curve.

Anthrone solution:

0.1gr Anthrone

50ml 75% H_2SO_4

2.2.16 GLUCOSE DETERMINATION TEST

After 7 days of *Bacilli* fermentation (starting from $1x10^5$ cells/ml of *B. licheniformis* and $1x10^5$ cells/ml of *B. pumilus)* in LB poor media supplemented with the 4%wood powder, media was filtered to check the presence of cellobiose molecules by enzyme assay.

To estimate the amount of glucose molecules in the wood media after *Bacilli* fermentation, we used GO test (Sigma GAGO-20). The GO test protocol followed according to the manual to detect the glucose amount in the samples. For cellobiose detection, filtered mediums were treated with cellobiase (Biorad) according to the kit instruction for 2 hours at 37 °C and then GO test was applied to see whether or not glucose molecules were generated from the degradation of cellobiose. Appropriate controls were also run in parallel.

2.2.17 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

After 7 days of *Bacilli* fermentation (starting from $1x10^6$ cells/ml of *B. licheniformis* and $1x10^6$ cells/ml of *B. pumilus)* in LB poor media supplemented with the 4%wood powder, media was filtered to check the presence of glucose and cellobiose molecules by HPLC analysis.

HPLC-ELSD analysis was done by SPRIN Technologies Company, Trieste/Italy by using the conditions below:

Column: Supelco apHeraTM NH2 Polymer, 250 x 4.6 mm, 5 µm

Mobile phase: Acetonitrile:Water 75:25, isocratic

Flow rate: 1ml/min

Column temperature: 30°C

Drift tube temperature (ELSD): 60°C

Spray chamber temperature (ELSD): 45°C

Appropriate controls and standards were also run in parallel.

3. RESULTS

This chapter was divided in two parts; A and B. In Part A "Wood Powder Degradation By Microbial Fermentation" results and in Part B "Implementation Of The Bridge Induced Chromosome Translocation (BIT) Technology For Strain Improvement" results were shown.

PART A: WOOD POWDER DEGRADATION BY MICROORGANISMS

A.3.1 ANTHRONE TEST RESULTS

The various species of *Bacilli* (*B. circulans, B. atrophaues*, *B. licheniformis, B. macerans, B. pumilus* and *B. subtilis)* were characterized by the ribotyping analysis and the API test by a master student. The ribotyping analysis is a method to identify and classify different *Bacilli* species based on the differences in their 16S rRNA. Comparing the sequence of the 16S rRNA allows inferring the evolutionary relationships among organisms. The analytical profile index (API test) was used to confirm the species of *Bacilli,* based on their biochemical reactions.

After the characterization, different *Bacilli* strains were compared for their lignocellulosic material degradation ability. To do so, *Bacilli* fermentation was done in LB poor media supplemented with 1% wood powder for seven days and in the following term, media was analyzed by anthrone test to see the amount of the total carbohydrates released from the lignocellulosic substrate.

Anthrone test is a colorimetric method for qualitative and quantitative estimation of polysaccharides as well as monosaccharides. It is based on dehydration of carbohydrates to furfural derivatives by sulphuric acid. Furfurals react with anthrone to form green colour

and amount of the carbohydrates in the samples are estimated via reading the absorbance of the resulting solution against a glucose standard curve.

In figure 3.1, anthrone test results for the total carbohydrate production by different *Bacilli* strains were shown. These results show the value difference between samples that were fermented with *Bacilli* and the controls that were fermented without *Bacilli*. Figure 3.2 shows the standard curve of the glucose solutions for the estimation of carbohydrates in the samples.

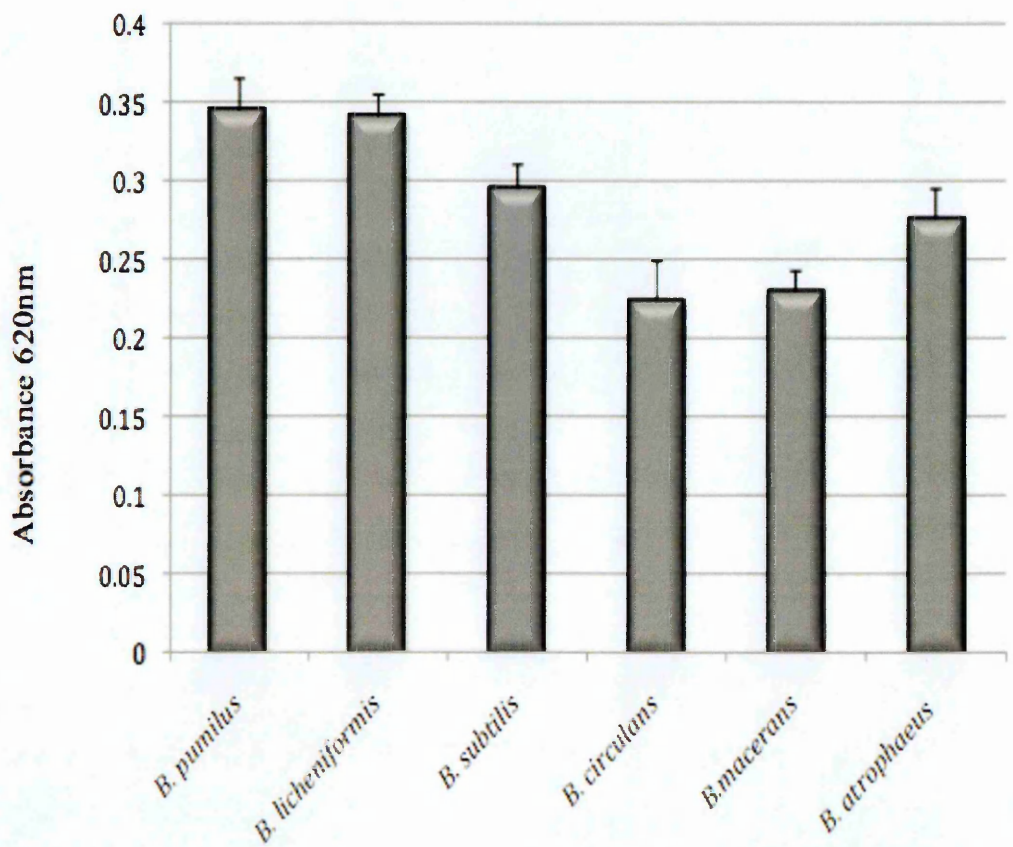

Figure 3.1: Graphic representation of the anthrone test for total carbohydrate estimation. Axis labels represents different *Bacilli* strains that grew in 1% wood media and Y values are the spectrophotometer measurements at 620nm. The error bars indicate the Standard Error.

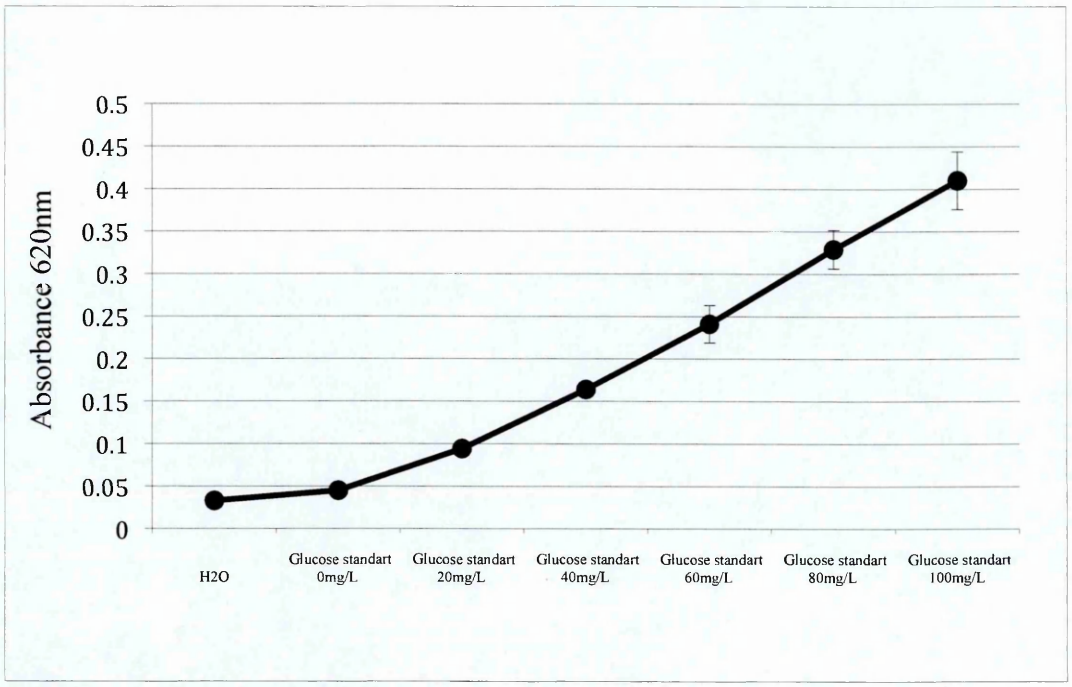

Figure 3.2: Glucose standard curve for the anthrone test with different concentrations of glucose.

According to the anthrone test results, *B. licheniformis* and *B. pumilus* have the best lignocellulose degradation ability in lignocellulosic media. They degraded more lignocellulosic material and produce more carbohydrates compare to the other species, therefore we selected these two *Bacilli* to work with.

To find out the optimal concentration for the wood degradation, *B. licheniformis* and *B. pumilus* were inoculated into LB poor media supplemented with the concentration of 1%, 2.5%, 4% and 6% wood powder **(Figure 3.3).**

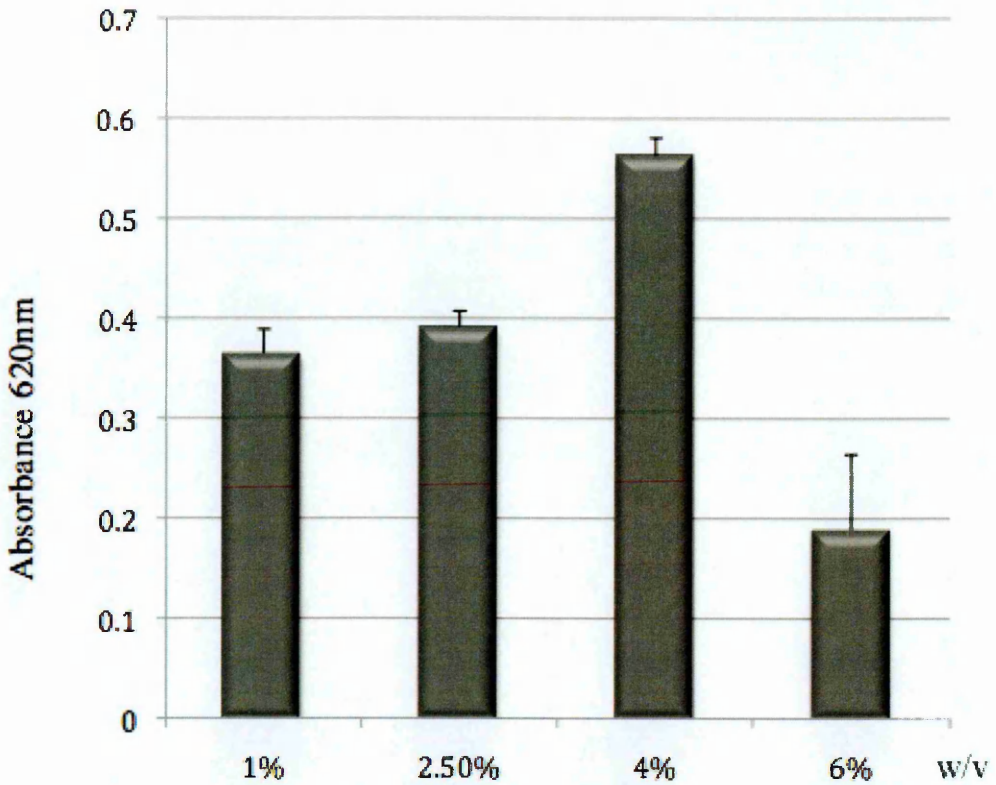

Figure 3.3: Graphic representation of the anthrone test to see optimum wood concentration for the degradation. Axis labels represent different concentration of wood powder in LB poor media and Y line shows the delta absorbance value of the fermentations with and without Bacilli. The error bars indicate the Standard Error.

Medium with 4% wood powder showed the highest amount of total carbohydrates after seven days of *Bacilli* fermentation compared to other concentrations. When the concentration over exceeded 4%, it was seen that *Bacilli* strains stopped growing. After these results, wood degradation experiments were done with the 4% wood media.

Figure 3.4 shows fluorescence microphotography of *B. licheniformis* and *B. pumilus.* These *Bacilli* were grown in different concentration of wood media for seven days and then they were checked under the fluorescence microscope.

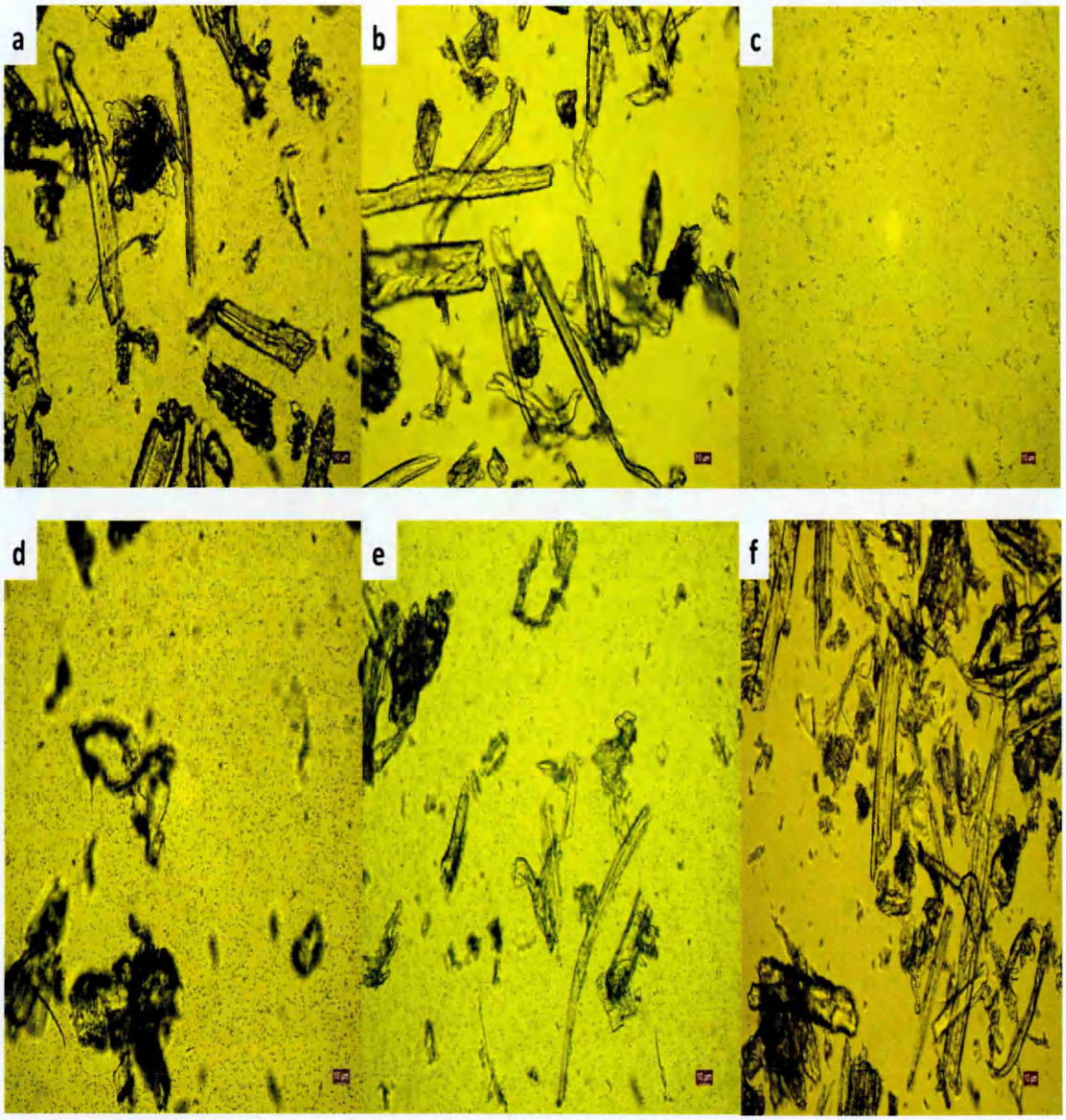

Figure 3.4: Fluorescence microphotography of *B. licheniformis* and *B. pumilus* strains in different concentration of wood media after 7 days (a; 4% wood media with *Bacilli*, b; 4% wood media without *Bacilli*, c; LB poor media with *Bacilli*, d; 1% wood media with *Bacilli*, e; 2,5% wood media with *Bacilli*, f; 6% wood media with *Bacilli*). Light microscopy of live cells was performed with a Leica DMBL photomicroscope equipped with a CCD computer-driven camera at 100x magnification.

It is showed also by fluorescence microphotography that of *B. licheniformis* and *B. pumilus* strains are not growing well when the wood concentration exceeded over 4% in the media.

A.3.2 SUGAR CONTENT DETERMINATION

After the confirmation of carbohydrate presence in the media after *Bacilli* fermentation, further analyses were done to understand the sugar content of the media. GO test (Sigma GAGO-20) and HPLC-ELSD method were used to analyze the media (LB poor media supplemented with 4% wood powder) after *Bacilli* fermentation.

A.3.2.1 GLUCOSE ASSAY

After seven days *Bacilli* fermentation in LB poor media supplemented with different concentration of wood powder, mediums were filtered for the analysis. To check the cellobiose presence, mediums were firstly treated by commercial cellobiase (Biorad) in order to release the glucose molecules for GO test. Determination of the glucose amount in the media was done according to the GO test manual (**Figure 3.5**).

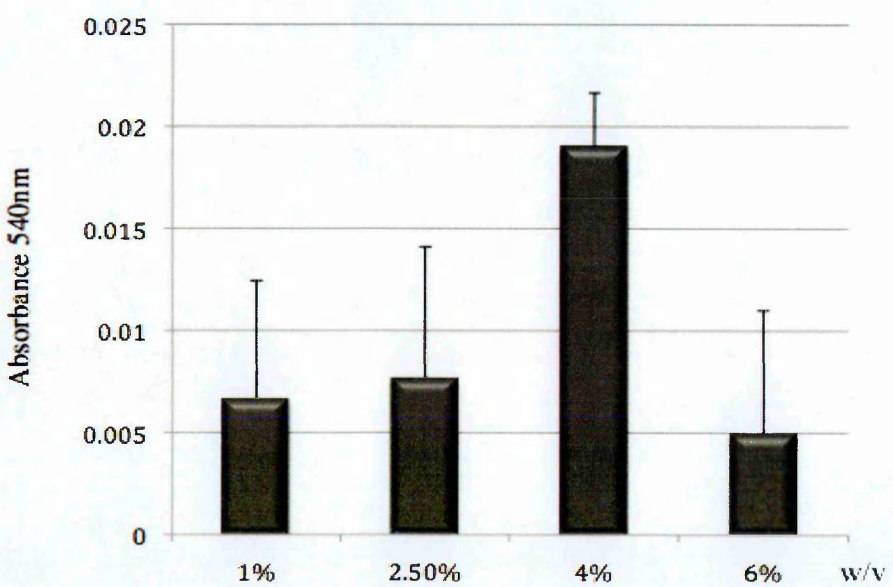

Figure 3.5: Graphic representation of cellobiose evaluation by applying glucose test to the samples that were treated with cellobiases. Absorbances of the samples are proportional to the glucose concentration. Axis labels represent different concentration of wood powder in LB poor media and Y values are the spectrophotometer value differences of *Bacilli* fermented wood mediums from the wood media without *Bacilli* fermentation. The error bars indicate the Standard Error.

After the cellobiase treatment, glucose molecules in the media were determined according to the GO test manual. In the presence of the glucose molecules, glucose oxidase is producing gluconic acid and hydrogen peroxide. Afterwards hydrogen peroxide reacts with o-dianisidine to form a colored product that can be read at 540nm. GO test results in figure 3.5 show the absorbance of the samples at 540nm that is proportional to the original glucose concentration, which means that during the lignocellulosic material degradation, some cellobiose and glucose molecules were produced.

GO test results showed that compared to other concentrations of wood powder, media supplemented with 4% wood powder contains more cellobiose and glucose molecules after *Bacilli* fermentation. To confirm these results further analysis was done.

A.3.2.2 SUGAR ANALYSIS BY HPLC

After glucose determination by GO test, further analysis was done by HPLC-ELSD method for glucose and cellobiose quantification. HPLC-ELSD analysis was done by SPRIN Technologies Company, Trieste/Italy with the conditions given in Material and Method section.

After seven days *Bacilli* fermentation in LB poor media supplemented with 4% wood powder, media was filtered for the analysis. Glucose and cellobiose solutions were used as positive controls and LB poor media supplemented with 4% wood powder without *Bacilli* fermentation was used as a negative control.

Figure 3.6 shows the analysis of media after *Bacilli* fermentation and figure 3.7 shows the analysis of media without *Bacilli* fermentation as a control. HPLC-ELSD analyses confirmed the results that were obtained by glucose test and these results gave as quantifications of the glucose and cellobiose contents.

Compound	Rt (min)	Area	Concentration (mg/ml)
Glucose	7.050	967768	0.049
Unknown	8.558	11845197	nd
Cellobiose	10.308	2413784	0.183

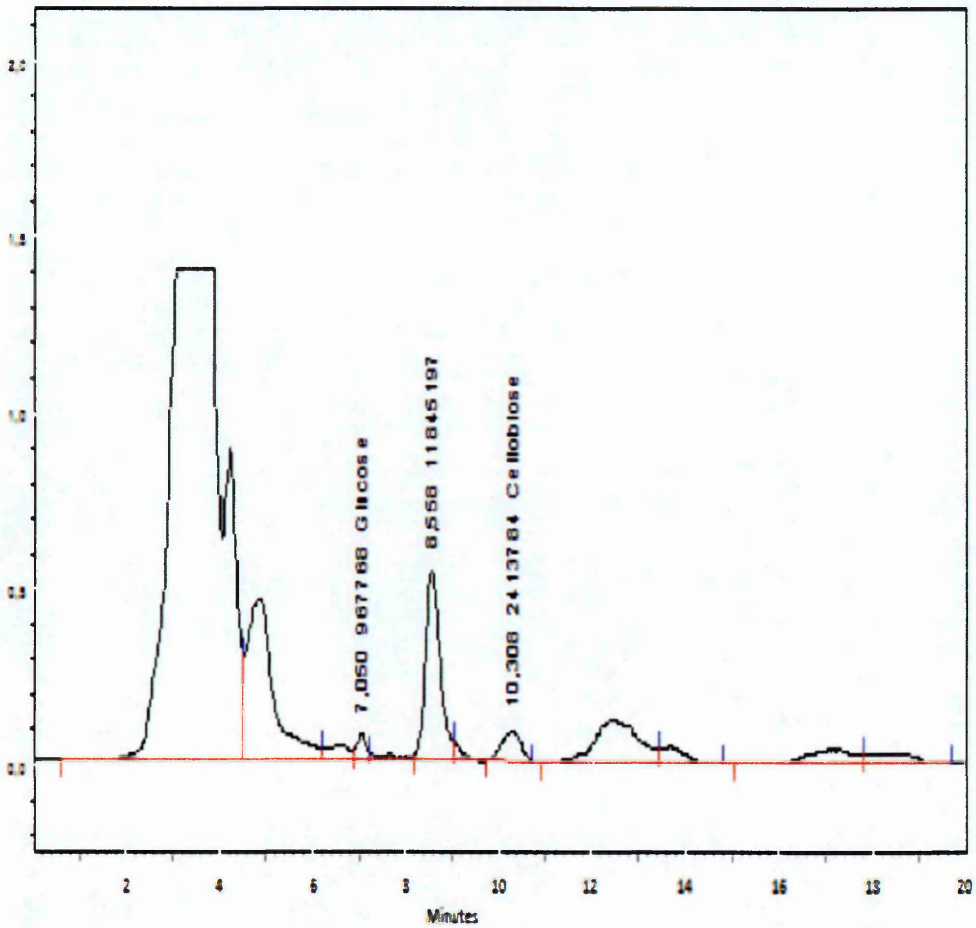

Figure 3.6: Graphic HPLC-ELSD analysis for glucose and cellobiose quantification of LB poor media supplemented with 4% wood powder after seven days of *Bacilli* fermentation.

No compounds of interest

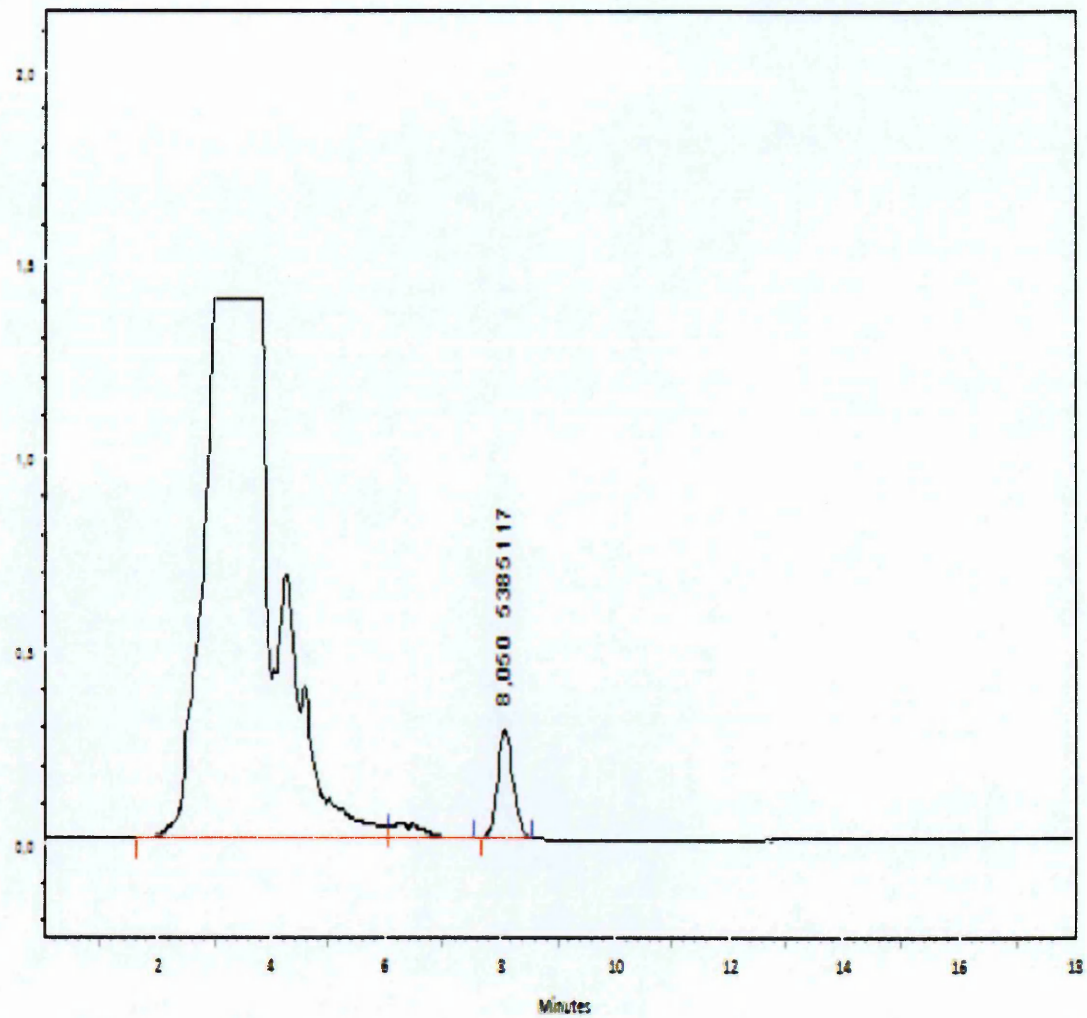

Figure 3.7: HPLC-ELSD analysis for glucose and cellobiose quantification as a control on a wood media (LB poor media supplemented with 4% wood powder) without *Bacilli* fermentation.

HPLC-ELSD results showed that after seven days of *Bacilli* fermentation there are 0,049mg/ml of glucose and 0,183mg/ml of cellobiose molecules in the media. In the control media, without *Bacilli* fermentation, no cellobiose and glucose molecules were found. Control media was also left in shaker for seven days to see whether or not shaking will release any sugars from wood powder.

Separation of the fermentable carbohydrates from the lignocellulosic material is the biggest barrier for the bio-ethanol production and by these results the degradation of the wood powder to oligosaccharides by *Bacilli* was demonstrated.

A.3.3 RECOMBINANT YEAST

After the *Bacilli* fermentation in wood media, some fermentable sugars were found out. To use cellobiose and glucose content of this media for the yeast fermentation, a recombinant yeast strain was constructed in Yeast Molecular Biology Laboratory in ICGEB/Trieste by Dr. Valentina Tosato.

Since *S. cerevisiae* is not naturally able to degrade the cellobiose to glucose, a cellobiase had to be expressed in *S. cerevisiae*. For this purpose, β -glucosidase gene (*BGL6*) from *P. stipitis* was extracted and cloned into pBLAST vector to be expressed and secreted in *S. cerevisiae*. Beta-glucosidases are responsible to hydrolyze cellodextrins and cellobiose into glucose to provide a straightforward metabolisable carbon source for the microorganisms.

Recombinant yeast strain that has best degradation ability among the other transformants was called 19s. By using this recombinant strain, yeast fermentation was done in the supernatant of *Bacilli* wood fermentation. Next experiments show the results of recombinant yeast's cellobiose degradation ability.

A.3.3.1 ENZYME ACTIVITY

The β-glucosidase activity was determined by using p-nitrophenyl-D-glucopyranoside substrate as it is described in Material and Method section. The unit definition of the β -glucosidase is the amount of enzyme that catalyzes the formation of 1 μmol of p-Nitrophenol per minute starting from PNPG (p-Nitrophenyl-D-glucopyranoside).

The method of assay is based on the spectrophotometric determination at 405nm of p-Nitrophenol, which is formed during the reaction. As a positive control β-glucosidase (Sigma cat no: G4511) and as a negative control wild type yeast strain were used (**Figure 3.8**).

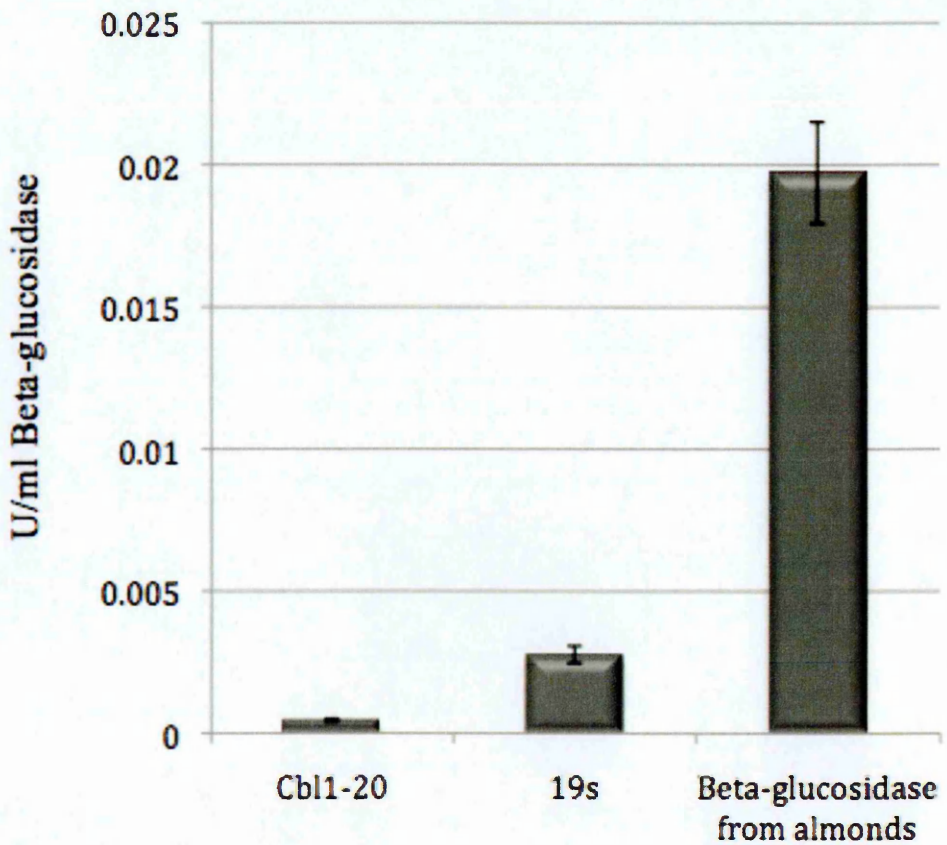

Figure 3.8: Graphic representation of Beta-glucosidase enzyme assay. Axis labels represent samples and Y line shows the amount of the enzyme U/ml. Beta-glucosidase from almonds was used as a positive control.

As it is shown in figure 3.8, there was no activity in the reference yeast strain harboring the empty plasmid pBLAST. Recombinant strain that is expressing Bgl6, is active 10-16 folds less than the pure β-glucosidase (Sigma cat no: G4511) that was used as a positive control.

This result proved that recombinant yeast strain is able to express and secrete the Bgl6 protein and this protein is active for the efficient cellobiose degradation to glucose monomers.

A.3.3.2 CELLOBIOSE FERMENTATION BY RECOMBINANT YEAST STRAIN

To prove the performances of the recombinant *S. cerevisiae* strains and demonstrate its functionality, cellobiose (Sigma, 99% cat no: 22150) was used as the sole carbon source.

The growth curve of CBL1-20 transformed with an empty vector and the recombinant 19s strains were checked in YPC media supplemented with 0.3% galactose for *Gal10* promoter induction. $1x10^5$ cells/ml of each strain were inoculated in YPC and followed their growth by counting the cells under the microscope. The introduced cells were washed twice before fermentation, suggesting that the growth in YPC could not be accomplished by the other sugars carried from previous cultures (**Figure 3.9**).

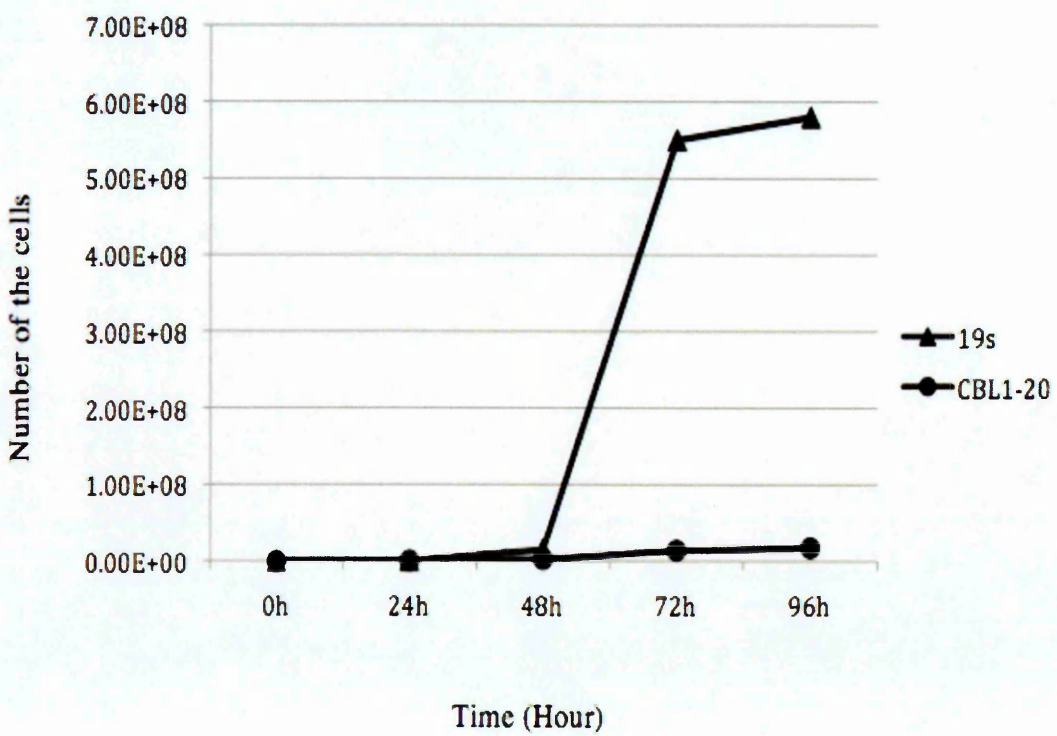

Figure 3.9: Growth curve of the 19s and CBL1-20 strains in YPC media. CBL1-20 is a wild type strain that carries empty pBLAST vector, 19s is a recombinant strain that carries pBLAST vector with *BGL6* gene. Axis labels shows the hours and Y values are the number of the cells counted at the time point.

As shown in figure 3.9, 19s recombinant strain grew much better than the wild type yeast strain in YPC media. Starting from an inoculum ($1x10^5$ cells/ml) of an exponentially growing culture in YPR, 19s reached a density of $5x10^8$ almost in 72 hours, while the control reached a density of $2.8\text{-}3.0x10^7$. The growth up to 10^7 of the control is due both to the presence of 0.3% galactose in the medium (that can be utilized as nutrient) and to the impurities of the cellobiose that can be contaminated with glucose (Sigma, 99% cat no: 22150).

After the confirmation of the recombinant *S. cerevisiae* strain's cellobiose degradation ability, fermentation was done by using the media of *Bacilli* fermentation. After *Bacilli* fermentation for seven days in LB poor media supplemented with 4% wood powder, the culture was subjected to 0,22um filtration and the supernatant was mixed with YP and 0.3% galactose for yeast fermentation.

$1x10^5$ cells/ml of 19s strain and CBL1-20 strain were inoculated. The growth curves of wild type and recombinant yeast strains were followed by counting cells under the microscope **(Figure 3.10)**.

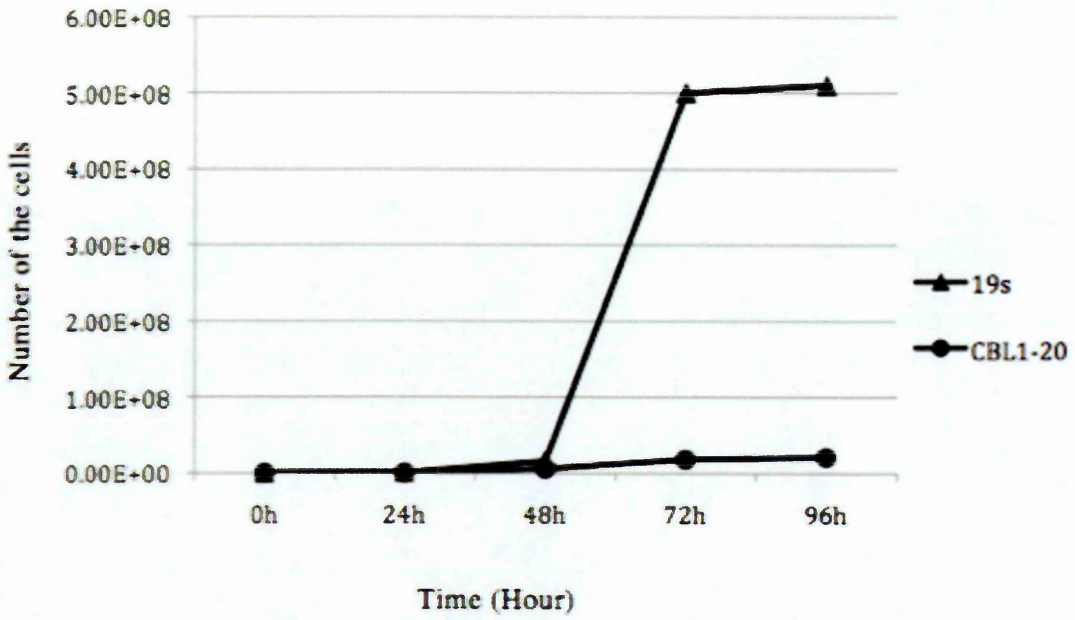

Figure 3.10: Growth curve of the 19s and CBL1-20 strains in the media, which is the supernatant of LB poor media supplemented with 4% wood powder after seven days *Bacilli* fermentation. CBL1-20 is a wild type strain that carries empty pBLAST vector, 19s is a recombinant strain that carries pBLAST vector with *BGL6* gene. Axis labels shows the hours and Y values are the number of the cells counted at the time point.

Growth of the yeast strains in the supernatant of bacterial fermentation of lignocellulosic material showed that the 19s strain is growing faster and better compared to the wild type strain.

There was a huge growth difference between recombinant and wild type yeast strains. When the yeast fermentation was done in parallel in media supplemented with commercially available cellobiose, more or less the same growth curve of the recombinant yeast strain was seen in both media. By this experiment, it is proved that recombinant yeast strain is able to degrade the cellobiose in both media; *Bacilli* pre-treated wood media and YPC.

This part of the research demonstrated that the sequential treatment of wood flour firstly by *Bacilli* and secondly by *S. cerevisiae* allows an efficient and economic conversion of biomass into glucose leading to foresee interesting perspectives in bio-ethanol production.

PART B: IMPLEMENTATION OF THE BRIDGE INDUCED CHROMOSOME TRANSLOCATION (BIT) TECHNOLOGY FOR STRAIN IMPROVEMENT

B.3.1 *PEP4-3* MUTANT DIPLOID YEAST STRAIN

S. cerevisiae BAP4 strain was used as a host for the cellulose degradation experiments. BAP4 strain is a diploid *pep4-3* mutant strain, it is produced by mating CBL1-20 strain: *a, [cir°], ura3-52, leu2-3,112, trp1-289, pep4-3* (Ludwig & Bruschi, 1991) and *PEP4* knock-out YPH4 strain: a, *ura3-52, lys2-801, ade2-101* and *his3-A200* (produced in this study).

The *pep4-3* mutation was added to the strain's background to potentially reduce the amount of proteolytic degradation of expressed recombinant protein. Vacuolar peptidases (proteinase-A, proteinase-B, and carboxypeptidase-Y) are the known proteases that are sensitive to the mutation (Zubenko, Park, & Jones, 1982).

To delete the *PEP4* gene in YPH4 strain, the STIK approach (Waghmare et al., 2003) was applied by using pGFKG plasmid, in which kanamycin resistance genes is flanked by FRT sequences.

Deletion cassette were amplified and purified (**Figure 3.11**) by ethanol precipitation and subsequently used for the transformation of YPH4 strain as described in the Material and Methods section.

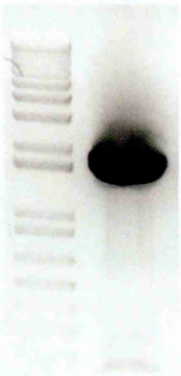

Figure 3.11: Agarose gel photograph of *PEP4* locus deletion cassette. Band represents a purified PCR product, which is around 1.6kb.

Positive G418 clones for the *PEP4* deletion were confirmed by colony PCR (**Figure 3.12**). Via FRT-FLP recombination system, selection marker was released by growing them under non-selective conditions. Almost 4000 colonies were checked for the kanamycin resistance loss (**Figure 3.13**). Non-resistant colonies were chosen after pop-out of the kanamycin and used for the mating experiment to obtain diploid *pep4-3* mutant strain.

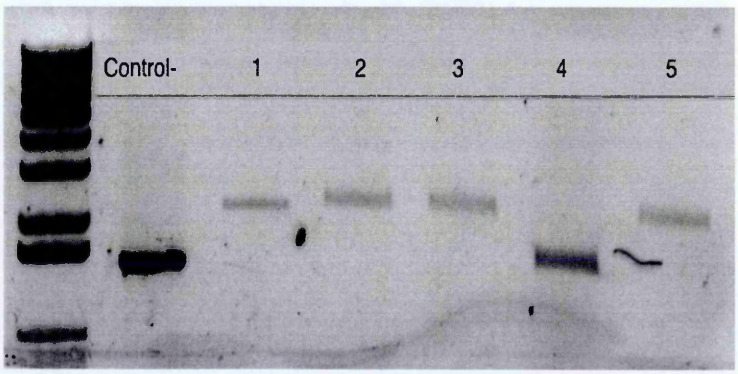

Figure 3.12: Verification of the deletion cassette integration by colony PCR. Control-, contains wild type strain, where *PEP4* gene is present. Lanes 1,2,3&5 contain positive clones carrying integration of knock-out cassette (around 2100bp band). Lane 4 does not contain correct integration, and it gives wild type band (around 1540bp band).

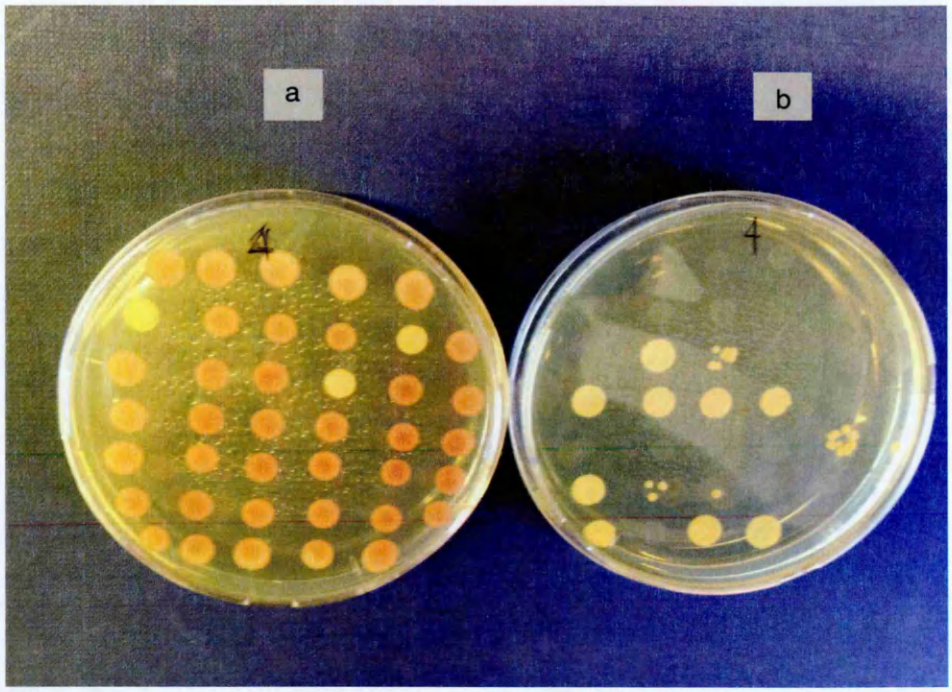

Figure 3.13: Pop-Out of kanamycin marker by FRT-FLP recombination in *pep4-3* mutant cells (a: cells in YPD plate, b: cells in YPD+G418 plate).

For the diploid *pep4-3* mutant strain construction, patched upon one another the two haploid *pep4-3* mutant strains were grown in YPD plate and replica plated on to dropout plates (SC -his –leu -lys -trp) by taking advantage of auxotrophies to inhibit growth of starting haploid strains. Resulting strain was also checked for the *pep4-3* mutation determination (Zubenko et al., 1982) by APE plate assay as described in the Material and Methods section. Those colonies derived from cells that are wild type *PEP4*, cleave the APE substrate, which reacts with the stain, and turns the colonies red. Those that are mutant, therefore remain white-yellow. Eventually, all colonies will become red, but the mutants can be readily distinguished after the initial staining period (**Figure 3.14**). By this test, engineered diploid strain was confirmed for the *pep4-3* mutation. Resulting strain was called BAP4 and used for the cellulose degradation experiments.

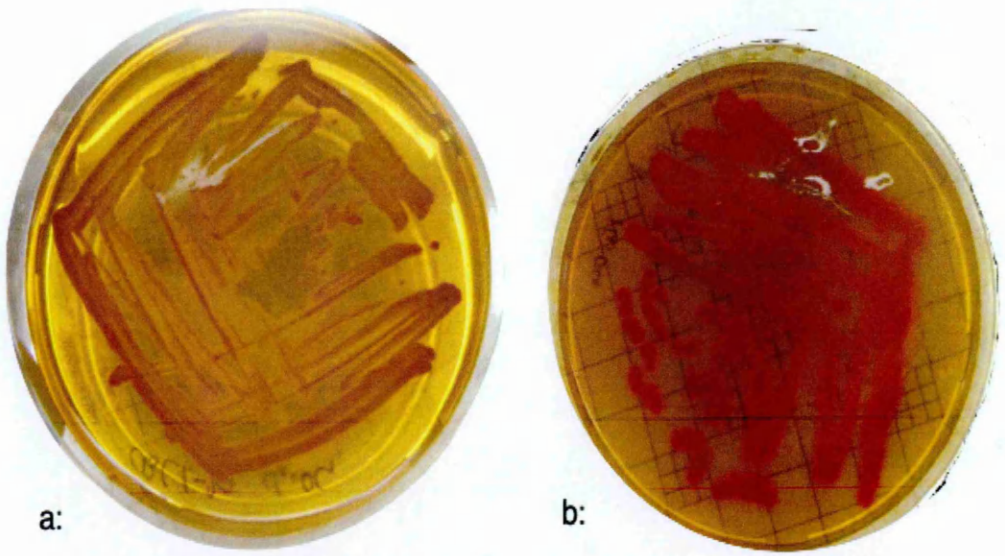

Figure 3.14: APE plate assay for *pep4-3* mutation determination (a: *PEP4* locus deleted diploid strain b: wild type strain). It can be seen that *PEP4* locus deleted strain stays white and wild type strain becomes red.

B.3.2 PLASMID CONSTRUCTION

MFa1 secretion signal sequence was amplified from pBLAST plasmid and cloned into pVT100-U, pJL49 and pTEF plasmids just after their promoter sequences. *MFa1* had been used effectively in previous reports to secrete a variety of heterologous gene products in yeast (Idiris et al., 2010; Romanos et al., 1992). As a component of the completed expression vectors, the *MFa1* signal sequence directs the cellulase genes into the secretory pathway, where later on it would be processed and secreted as mature protein (Ludwig & Bruschi, 1991; Ludwig et al., 1993).

B.3.2.1 CLONING OF *EGC1* GENE INTO pVT100-U PLASMID

To construct *EGC1* expressing yeast strain, this gene was amplified in two-step from *P. stipitis* NBRC 10063 (CBS 6054). This was necessary because of the strong similarities among the three *EGC* genes of *P. stipitis*. Both plasmid and *EGC1* gene were digested by the *Xho*1 and *Bam*H1 restriction enzymes in order to be in the frame with *MFa1*

sequences. Fragments were mixed, ligated and afterward transformed into *E. coli* XL10-Gold host. Positive transformants were confirmed by colony PCR, restriction enzyme analysis and sequences analysis.

B.3.2.2 CLONING OF *BGL5* GENE INTO pJL49 PLASMID

BGL5 gene was amplified from *Pichia stipitis* NBRC 10063 (CBS 6054) strain and cloned it into pJL49 vector to express in *S. cerevisiae*. *BGL5* was amplified in two-step by PCR because of the strong similarities among the seven *BGL* genes of *P. stipitis*. Both plasmid and *BGL5* gene were digested by the *Eco*R1 and *Xho*1 restriction enzymes in order to be in the frame with *MFa1* sequences. Fragments were mixed, ligated and afterward transformed into *E. coli* XL10-Gold host. Positive transformants were confirmed by colony PCR, restriction enzyme analysis and sequences analysis.

B.3.2.3 CLONING OF *CBH2* GENE INTO pTEF PLASMID

CBH2 cDNA (from *T. reesei* QM9414 strain) was provided by Dr. Sezerman from Yeditepe University/Istanbul. Both plasmid and *CBH2* cDNA were digested by the *Eco*R1 and *Cla*1 restriction enzymes in order to be in the frame with *MFa1* sequences. Fragments were mixed, ligated and afterward transformed into *E. coli* XL10-Gold host. Positive transformants were confirmed by colony PCR, restriction enzyme analysis and sequences analysis.

B.3.2.4 CLONING OF ALL CELLULASES GENES INTO pYAC3 PLASMID

Purpose of cloning all three cellulases genes into different plasmids was to obtain a full construct with the secretion signals and different strong constitutive promoters and terminators.

In order to linearize the circular YAC, it has to be cut by BamH1 restriction enzyme to release the telomeres and for this reason BamH1 sites has to be unique at the telomeres' junction. After cloning cellulases into plasmids, BamH1 sites were checked in the constructs to eliminate the problem of pYAC3 linearization. BamH1 sites (they were not inside the genes) in the constructs were deleted by Klenow fragments treatment and constructs were subsequently amplified with given primers in Material and Methods section in order to clone them into pYAC3 (**Figure 3.15**). Amplified constructs contain Promoter + Secretion Signal + *GENE* + Terminator and each construct called with the name of the gene that they contain.

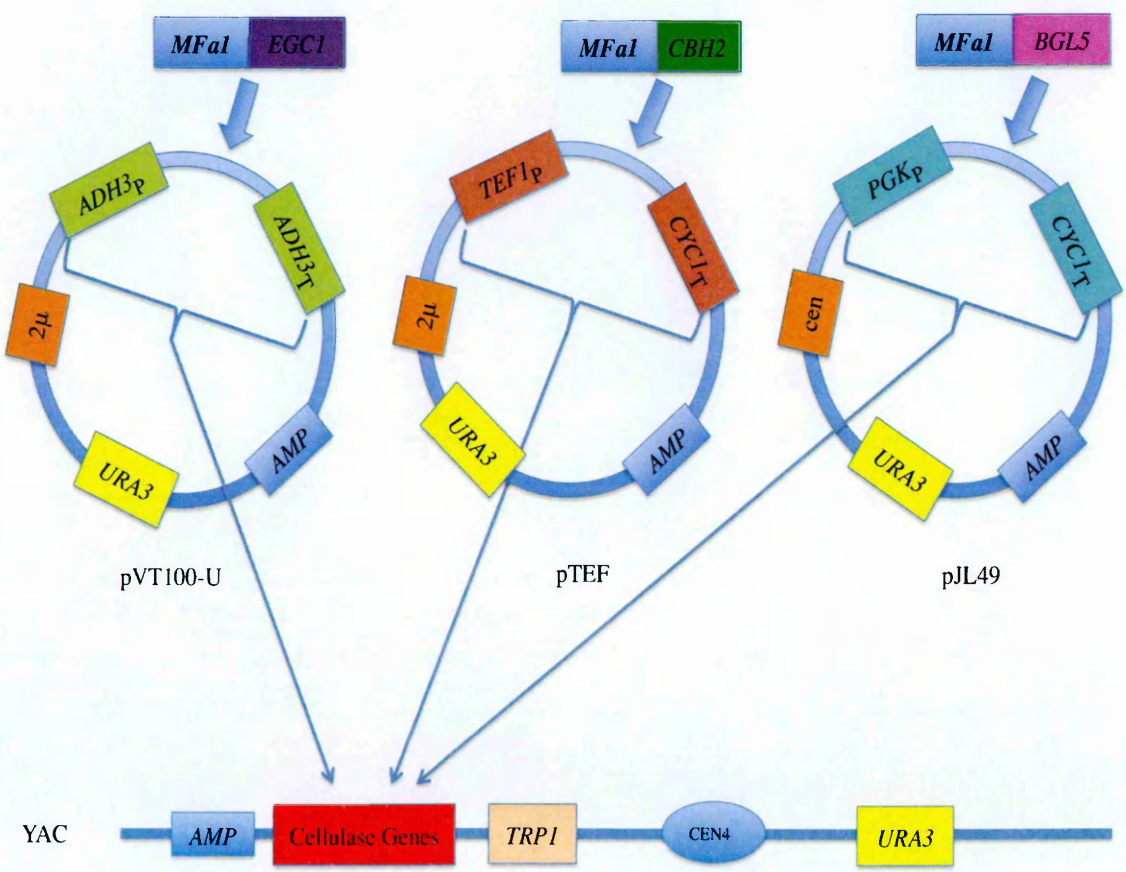

Figure 3.15: Cloning of cellulase genes first into the plasmids and then into the YAC.

Since pYAC3 didn't have enough option of restriction sites for cloning all three constructs, new restriction sites were added while cloning the constructs. Initially, *EGC1* construct was cloned between *Aat2* and *Pml1* restriction sites of pYAC3 and reverse primer of *EGC1* construct was designed in order to have the sequences of *Asc*1 and *Sac*2 restriction sites besides the *Pml*1 site. Then *BGL5* construct was cloned between new introduced restriction sites; *Asc*1 and *Sac*2. Later than *CBH2* construct was cloned between *Sac2* and *Pme*1 sites to conclude the pYAC3 cloning.

During pYAC3 cloning Stbl2 bacterial strain was used to eliminate the recombination of the similar sequences in the constructs. Positive transformants were confirmed by colony PCR, restriction enzyme analysis and sequences analysis.

Circular YAC with the cellulases genes was linearized by releasing telomeres via *Bam*H1 digestion and in the following term, linear YAC was extracted from agarose gel to transform into host yeast strain that has *pep4-3* mutation (**Figure 3.16**).

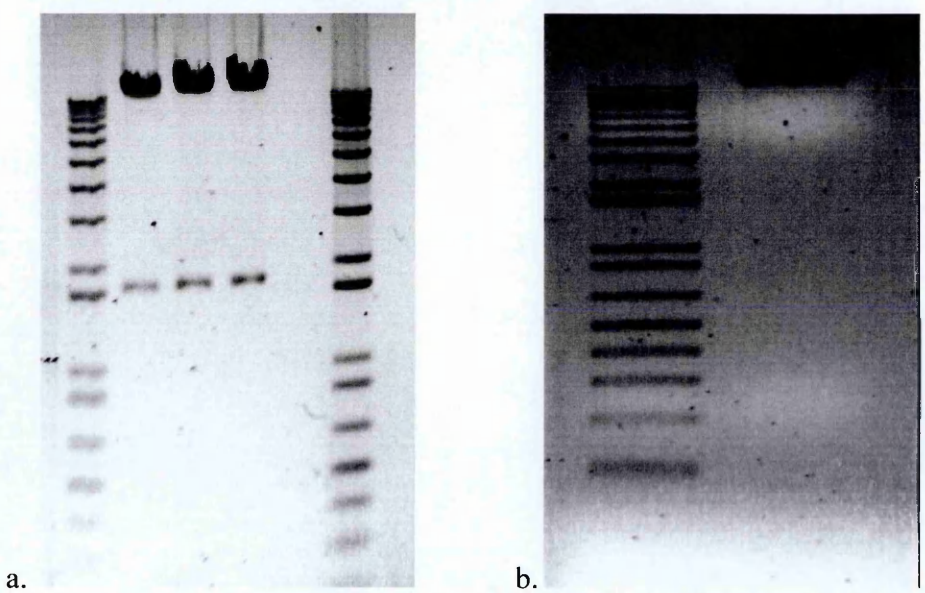

Figure 3.16: a) Agarose gel photograph of BamH1 restriction enzyme digestion of recombinant pYAC3. Lower band represents the *HIS3* gene of pYAC3 and higher band represents the linear artificial chromosome. b) Agarose gel photograph of the gel extraction of linear recombinant YAC (around 18kb). Band represents the linear recombinant artificial chromosome.

B.3.3 THE BRIDGE INDUCED TRANSLOCATION SYSTEM

Bridge Induced Translocation technology is allowing to obtain translocation between any desired points in yeast genome and it results in many different effects on cellular morphology, physiology and genome organization (Nikitin et al., 2008; Tosato et al., 2005).

In this study BIT technology was used for the recombinant yeast strain improvement for the bio-ethanol production.

B.3.3.1 TRANSLOCATION CASSETTE AMPLIFICATION

Translocation cassette was amplified by using pFAkanMX4 plasmid as a template and it contains 80bp homology to the desired pair of loci at both ends with kanamycin resistance gene in the middle (**Figure 3.17**). This cassette was designed in a way that it lacks homology to the yeast genome except the chosen homology regions, therefore homologous recombination will not occur with the endogenous DNA.

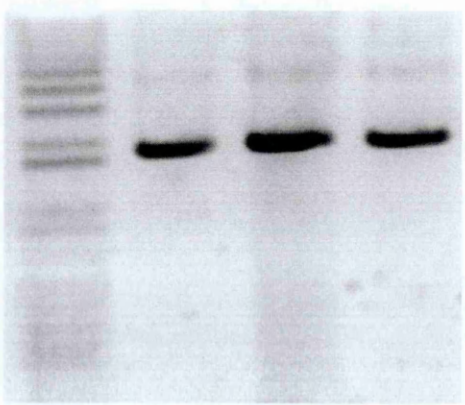

Figure 3.17: Agarose gel photograph of PCR based BIT cassettes (1.6kb).

B.3.3.2 BRIDGE INDUCED TRANSLOCATION IN THE *PEP4-3* MUTANT DIPLOID YEAST STRAIN

Exponentially growing *pep4-3* mutant diploid yeast strain was transformed with a PCR-amplified linear DNA cassette harbouring the KAN^R selectable marker.

BIT cassette carried at its ends two nucleotide sequences homologous to two separate genomic locations each located on a different chromosome. In this way, when the cassette is integrated, the selectable linear DNA fragment creates a molecular bridge between two unique, pre-selected genomic points.

With BIT method, the non-reciprocal translocation was applied between recombinant YAC and chromosome IV and homology regions were chosen close to the target genes; cellulases genes on YAC and *PAD1* gene on chromosome IV. Schematic representation of BIT method is described in Figure 3.18. The reason of choosing the break point in chromosome IV, close to *PAD1* gene, is that *PAD1* gene is known as Phenylacrylic acid decarboxylase that is responsible for resistance to some aromatic compounds and over expression of PAD1 gene results with high ethanol production, fast growth and high utilization of carbon sources (Larsson, Nilvebrant, & Jönsson, 2001). Because of this reason we wanted to use BIT as an advantage at this point to increase *PAD1* gene expression level (Nikitin et al., 2008).

BIT allowed us to gain two advantages; one of them is to make recombinant YAC stabilize into the resident yeast genome and the other one is to have increased gene expression levels of the heterologous genes.

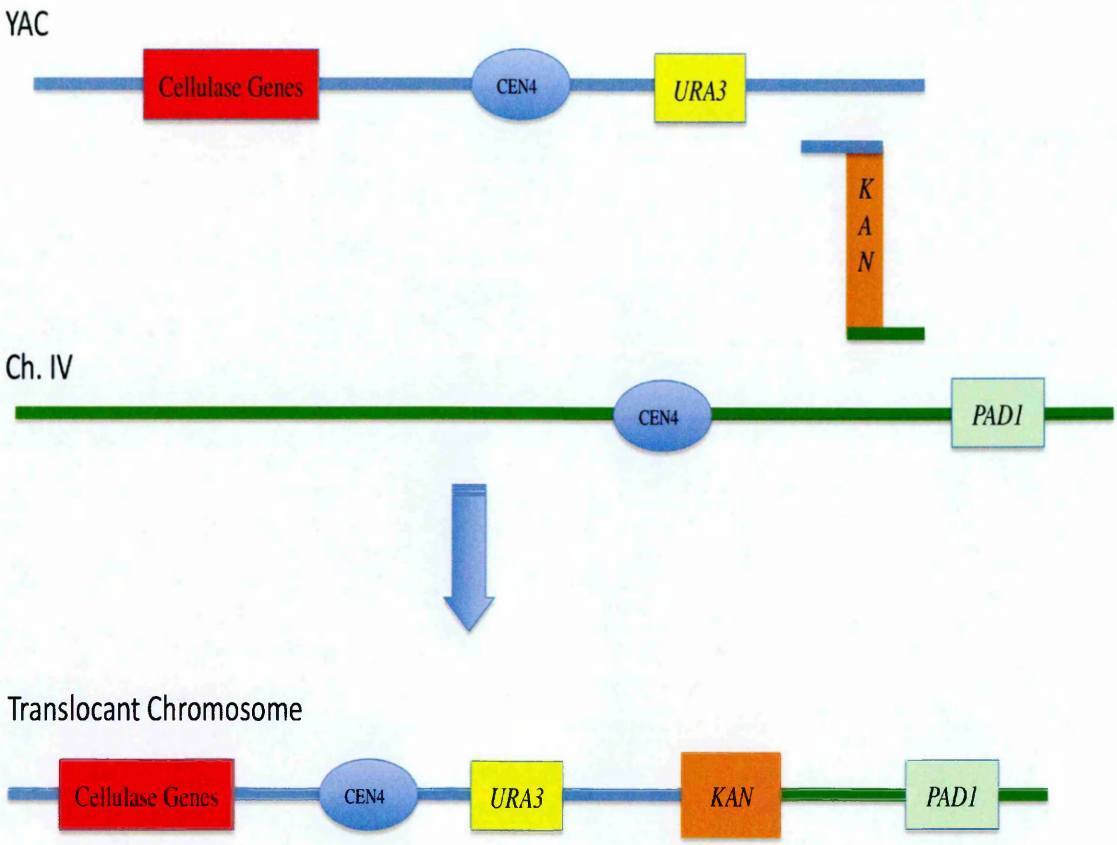

Figure 3.18: Schematic diagram illustrating BIT approach.

During the translocant cassette design, the most important thing was to pay attention to essential and haplo-insufficient genes for avoiding risk of creating unviable transformants. Since the expression level of genes around the translocation break point is increasing, neighbourhood of the break point should eliminate the genes encoding for polymerases, transcription factors, and other essential genes in order to prevent activating abnormal

situations for the cell. All chosen homology regions for translocation carry out these necessities.

After transformation of BIT cassette to the yeast cells, they were selected on YPD plates supplemented with G418 for further verification analysis.

B.3.3.3 BRIDGE-INDUCED TRANSLOCATION VERIFICATION

B.3.3.3.1 Colony PCR Results

Colony PCR was used to check the correct integration of the cassette for the translocation bridge. For each side of the cassette three sets of primers were designed in a way that after the amplification, the wild type was giving only one band and cassette integrated colony was given two bands.

Figure 3.19 shows the agarose gel picture of the colony PCR for checking the BIT cassette integration on the side of recombinant YAC. Three sets of primers were used; a1, b1 and k1. For the wild type cells, a1 and b1 primers amplified the fragment of 240nt size where else a1 and k1 primers amplified the fragment of 740nt size if there is an integration on the targeted side of YAC.

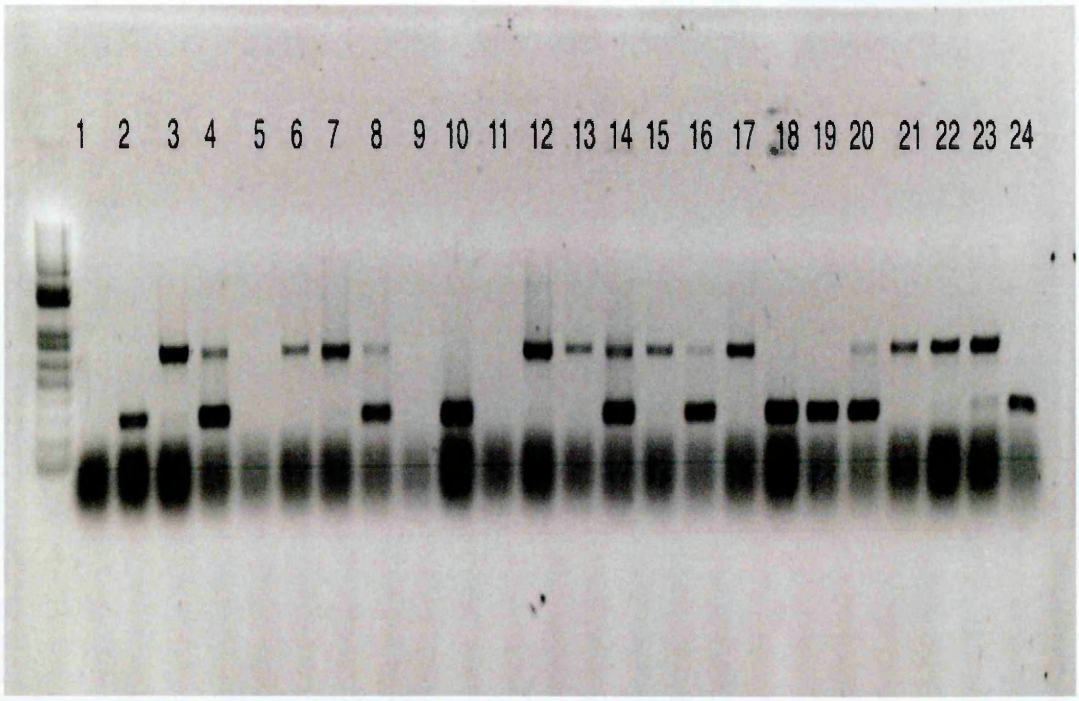

Figure 3.19: Agarose gel photograph of the colony PCR for checking the BIT cassette integration on the side of recombinant YAC. Upper bands (740bp) are the amplicon from the colonies that have the integration of the BIT cassette and lower bands (240bp) are PCR control bands.

Figure 3.20 shows the agarose gel picture of the colony PCR for checking the BIT cassette integration on the side of chromosome IV. Three sets of primers were used; a2, b2 and k2. For the wild type cells, a2 and b2 primers amplified the fragment of 453nt size where else a2 and k2 primers amplified the fragment of 850nt size if there is an integration on the targeted side of chromosome IV.

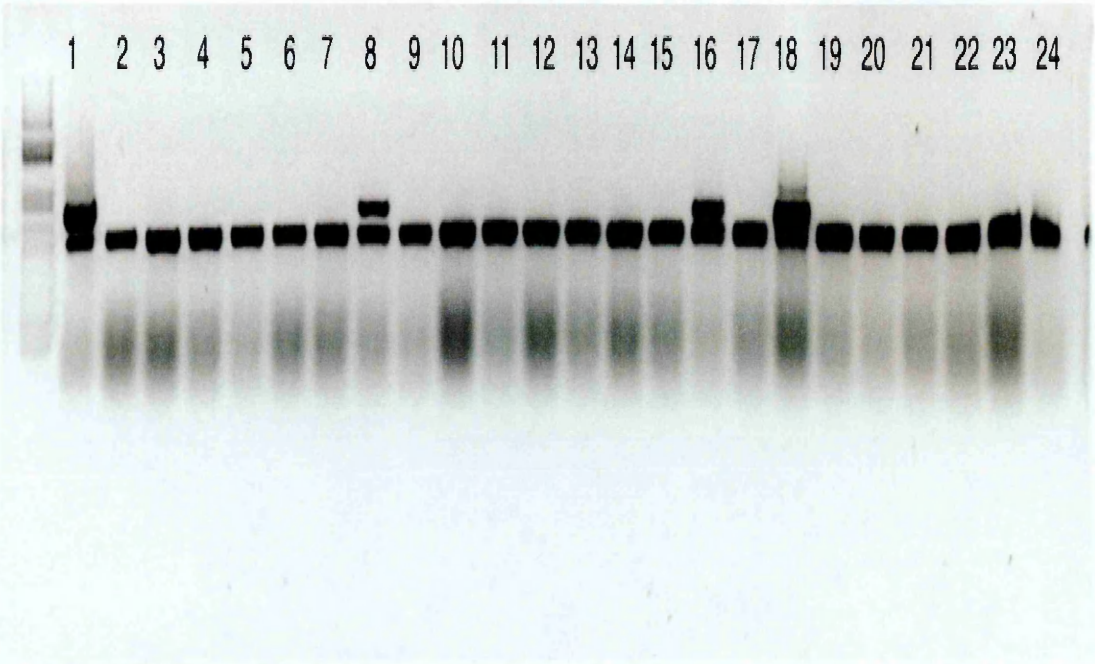

Figure 3.20: Agarose gel photograph of colony PCR for checking the BIT cassette integration on the side of chromosome IV. Upper bands (850bp) are the amplicon from the colonies that have the integration of the BIT cassette and lower bands (453bp) are PCR control bands.

As it is shown in the Figure 3.19 and Figure 3.20, colony 8 and colony 16 have the integration of the BIT cassette in both chromosome IV and YAC.

In total 98 colonies were obtained on G418 plates after the BIT transformation and all of them checked by colony PCR. From these results it is seen that BIT cassette integrated on the YAC side more than it integrated on the chromosome IV side (**Figure 3.21**).

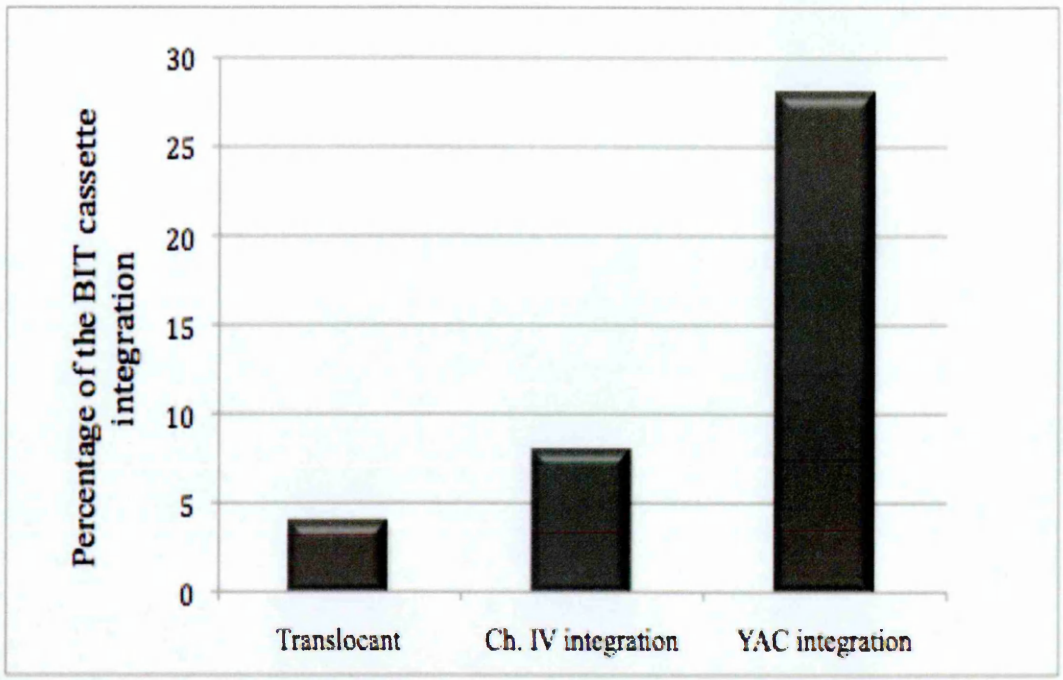

Figure 3.21: Graphic representation of the BIT cassette integration percentage. Axis labels represent different locations of integrations and Y line shows the percentage value of the integrations compared to all transformants.

After checking transformants by colony PCR to find out the colonies that have integration of cassette on both sides, bridge PCR was done for the further BIT confirmation. For the bridge PCR, primers a1 and a2 were used to amplify the bridge fragment between two genomic locations that were targeted on different chromosomes to create non-reciprocal translocation.

As it is shown in figure 3.22, colony number 8 and 16 were checked by bridge PCR and they had the amplification of translocation bridge fragment. After that these translocant strains were also confirmed by sequencing for the bridge part of the translocation.

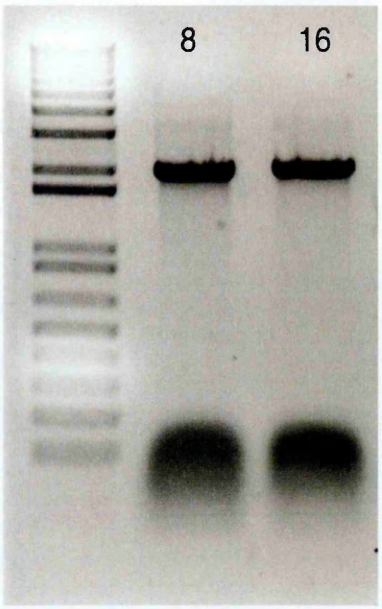

Figure 3.22: Agarose gel photograph of the bridge PCR (around 1.6kb).

These results show that chromosomal translocations are successfully induced by linear DNA integration between chromosome IV and YAC.

B.3.3.3.2 CHEF Gel Results

In order to determine the location of the chromosomes in the translocant and control strains, we employed pulsed-field gel electrophoresis. Chromosomes were prepared in agarose, as described in Materials and Methods, and run on a 1% agarose gel for 22 hours. Afterwards, the gel was stained with EtBr (Ethidium bromide) and photographed on a 302nm transilluminator.

Figure 3.23 shows the CHEF gel picture of translocant and control strains. Size of the translocant chromosome is very short, around 40kb. It contains around 19kb piece from recombinant YAC including YAC centromere and 21.5kb piece from chromosome IV.

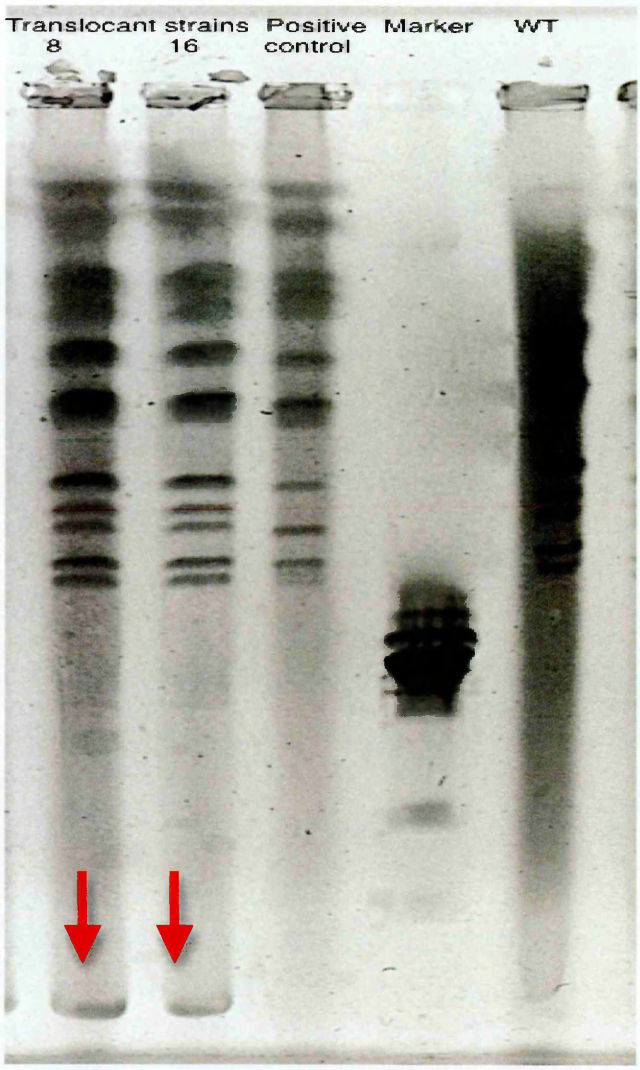

Figure 3.23: CHEF gel photograph of translocant and control strains. Arrows indicate translocant chromosomes. BAP4-bity and BAP4 strains were run in the gel. A yeast strain that contains kanamycin was used as a positive control for the *KAN* probe.

Visualizing of the chromosome separation was done by CHEF gel. Wild type yeast strain and a translocant yeast strain from the Yeast Molecular Genetics Laboratory Collection were used as controls. Since the translocant chromosome is really short, CHEF was set up for the Lambda DNA separation programme. With Lambda DNA separation programme, big chromosomes cannot be separated well; it is for the visualizing of the short chromosomes, that is why not all the chromosomes were visible on the gel.

Since there is a kanamycin resistance gene in the translocant chromosome, we did Southern Blot analysis by using kanamycin as a probe in order to visualize the location of translocant chromosome.

B.3.3.3.3 Southern Blot Results

After the separation of chromosomes by CHEF gel, the gel was transferred to nylon membrane utilizing the denaturing transfer system described in Materials and Methods. Afterwards, the nylon membrane was processed for DNA hybridization. The filter was allowed to hybridize with overnight, and was then washed, and processed for autoradiography (**Figure 3.24**).

Since BIT cassette contains a kanamycin resistant gene, we used kanamycin probe for the Southern blot analysis to visualize the location of translocation chromosome. By PCR, we confirmed two translocant strains and both of them used for the CHEF and Southern Blot analysis.

A translocant strain with the kanamycin marker was used as a positive control for the probe and the wild type strain (BAP4) was used as a negative control.

As it is shown in figure 3.24, there is no hybridization with the wild type chromosomes like it is expected and there are hybridizations with the positive control and translocant strains at the expected sizes.

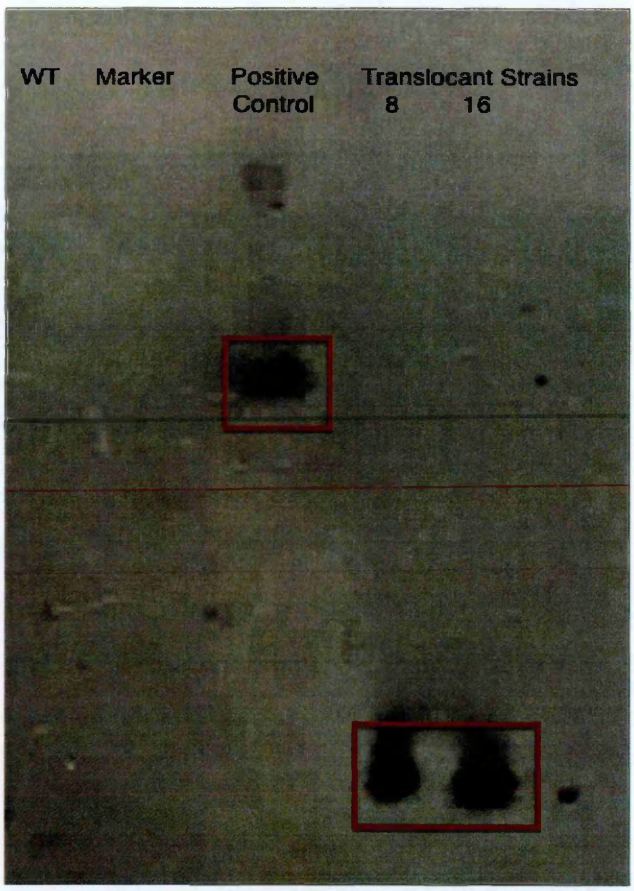

Figure 3.24: Southern Blot analysis of translocant and control strains. Bands represent the hybridization of the kanamycin marker, which indicates the translocant chromosome in the line of translocant strain 8 and 16. Appropriate controls were analyzed in parallel.

According to Southern blotting and PCR analysis, a new translocant chromosome, consisting of the left portion of chromosome YAC and the right portion of chromosome IV, was generated. These results demonstrate that chromosomal translocation was successfully induced by linear DNA integration between YAC and chromosome IV.

After translocation as it is mentioned before, there are too many changes in the cells. The morphology of wild type and translocant strains was compared by light and fluorescence microscopy. More or less 10% of the population in the translocant strains had an abnormal phenotype. In figure 3.25 microphotography of translocant strains was shown.

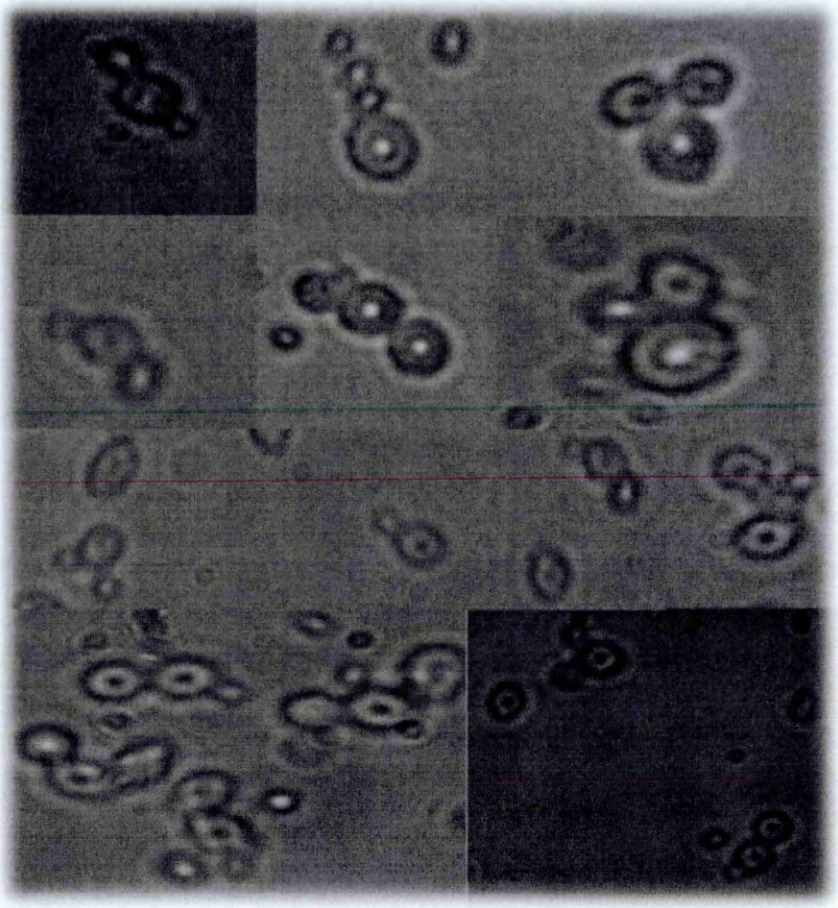

Figure 3.25: Microphotography of the translocant strains that shows the differences of their phenotypes. Light microscopy of live cells was performed with a Leica DMBL photomicroscope equipped with a CCD computer-driven camera at 100x magnification.

B.3.4 EXPRESSION PROFILING OF GENES LOCATED AT THE TRANSLOCATION BREAK POINTS

It has been previously shown that single translocation event creates too many different effects on cellular morphology, physiology and genome organization level. One of the important effect was that translocation results in different modifications of the gene expression levels around the translocation break point (Nikitin et al., 2008). In this study, this pattern had an important role for the recombinant gene expressions for the strain improvement. For this reason translocation break point was chosen in a way that it was

close to the genes that we were interested to increase their expression level. Features of all the recombinant S. cerevisiae strains are listed in Table 3.1.

Table 3.1: Features of recombinant yeast strains.

Recombinant Yeast Strains	**Relevant features**
BAP4	Parental strain, Diploid, *[cir°], pep4-3, ura3-52, leu2-3,112, trp1-289, lys2-801 ade2-101, his3-A200.*
BAP4-y3	BAP4 strain contains linear YAC3 that contains all three constructs (promoter-secretion signal-gene-terminator).
BAP4-ciry3	BAP4 strain contains circular YAC3 that contains all three cellulases constructs (promoter-secretion signal-gene-terminator).
BAP4-bity3	BAP4-y3 strain that has the translocation between recombinant YAC3 and chromosome 4 by BIT technology.

At first, the RNA was extracted, cDNA was produced by using AMV Reverse Transcriptase and quantitative PCR was run amplifying *BGL5, EGC1, CBH2, PAD1* genes and housekeeping *ACT1* gene was used for the normalization. BAP4, BAP4-y3, BAP4-y3cir and BAP4bity3 strains were used to compare the recombinant gene expression levels. The whole experiment was repeated three times. Fold change of gene expression levels are shown below (**Figure 3.26**). Student's *t*-test was used to determine significant differences between groups, where a p-value < 0.05 was considered statistically significant.

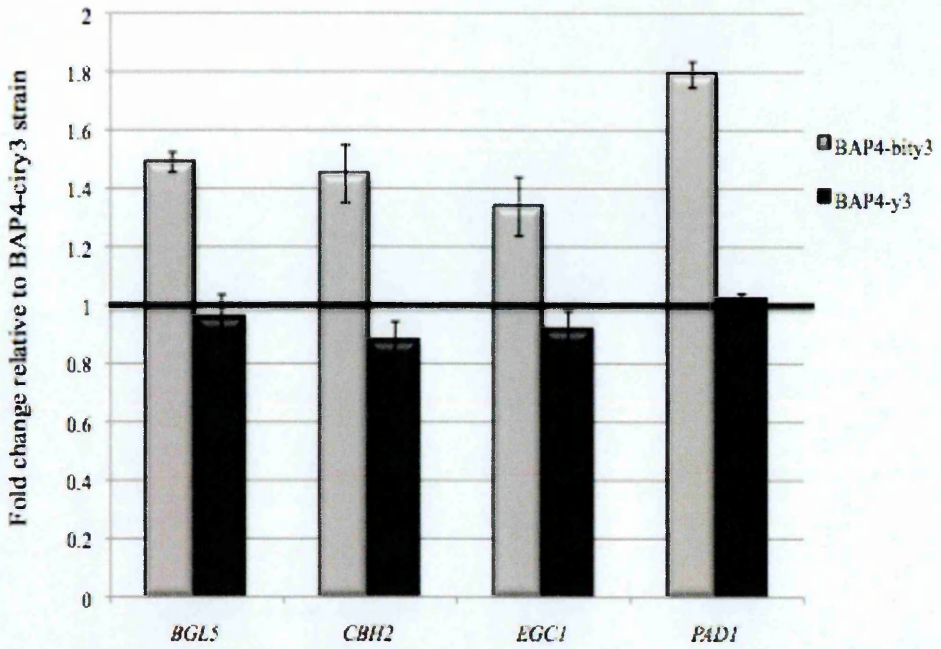

Figure 3.26: Graphic representation of the quantitative PCR gene expressions pattern for *BGL5, EGC1, CBH2* and *PAD1* genes on both sides of the translocations breakpoints. Fold change of their expression levels in BAP4bity3 and BAP4-y3 strains were compared to BAP4-ciry3 strain. *ACT1* gene expression level was used for normalisation.

As a result of quantitative PCR, it is showed that recombinant genes have different expression profile in BAP4bity3 and BAP4-y3 strains comparing to BAP4-ciry3 strain, which was used as a reference strain for the fold change of targeted genes. Linear and circular recombinant YAC carrier strains showed more or less the same expression profile and as expected, translocant BAP4bity3 strain showed increased gene expression level compared to other recombinant strains.

B.3.5 CELLULASE ENZYME ACTIVITY

To make the complete hydrolysis of cellulose to glucose, three different yeast strains were engineered to secrete cellulases. After obtaining the recombinant yeast strains with cellulases genes, we checked their enzyme activity for the cellulose degradation.

Complete hydrolysis of cellulose requires the synergistic reaction of *EGC*, *CBH*, and *BGL* (Lynd et al., 2002). Using the culture supernatant of each transformant strain, the enzyme activity was measured according to the standard assay procedure for cellulases by using EnzChek Cellulase Substrate from Invitrogen. If there were cellulases in media, it would react with the substrate and degradation outcome could be read at 360 nm spectrophotometrically.

With this method, we compared the linear BAP4-y3, BAP4-ciry3 and the BAP4bity3 strains for the cellulase amount in the media. Controls were run in parallel and no activity was detected in the reference yeast strain harbouring the empty YAC. Taken all together, all three recombinant enzymes were shown to be active in recombinant strains (BAP4-y3, BAP4-ciry3 and BAP4bity3) at different levels. After BIT application, enzyme activity and enzyme production were found to be increased **(Figure 3.27).**

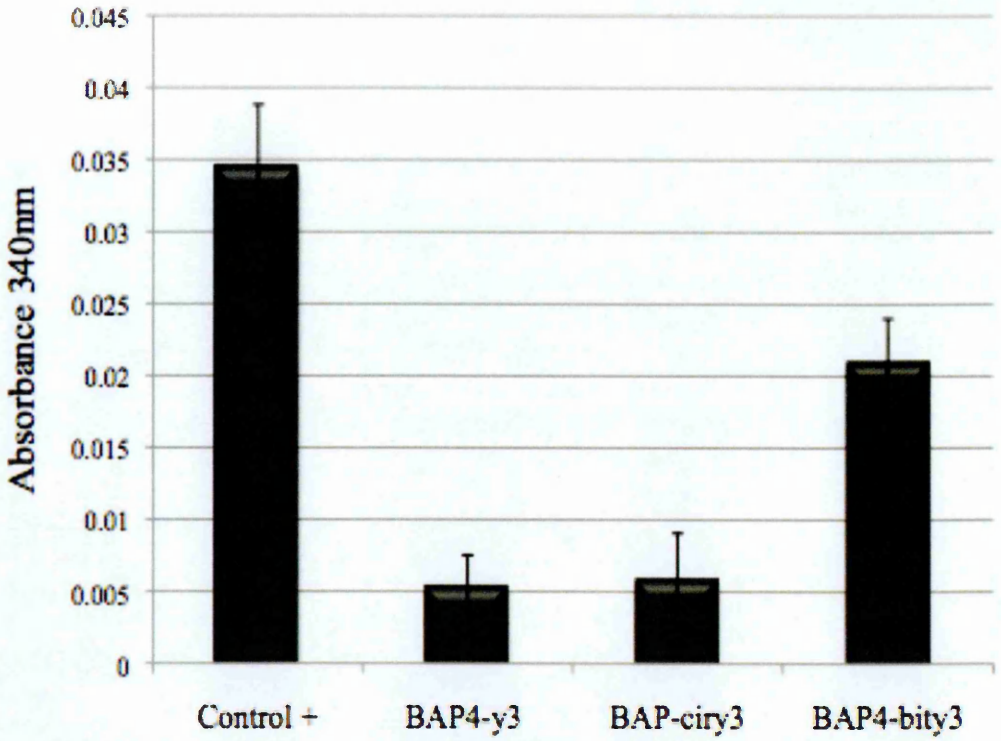

Figure 3.27: Graphic representation of cellulase assay. Axis labels represent different strains and Y line shows the absorbance value of the reaction. Control + is the commercial cellulase enzyme mixture (1ul from 700 units/g).

Control + is the commercial cellulase enzyme mixture. Student's *t*-test was used to determine significant differences between groups, where a p-value < 0.05 was considered statistically significant.

With these results, we saw that BIT technology can increase the heterologous enzyme activity. Since BIT increased the gene expression levels of recombinant genes, this assay also proved that expressed heterologous proteins were secreted and active in the media.

B.3.6 CHROMOSOME STABILITY

YACs are linear chromosome like shuttle vectors that can be amplified in bacterial cells and used for the cloning and manipulation of large deoxyribonucleic acid (DNA) inserts (up to 3Mb pairs) in the *S. cerevisiae*. They contain a centromere, telomeres, and replication initiation sites suitable for use in the yeast (Arnak, 2012).

YACs have large capacity for DNA fragments. This allows YACs to be used for engineering genetic determinants of new biochemical pathways for production of secondary metabolites and for heterologous protein expression. In yeast, YAC has to reach a certain size, which is more or less 150kb to be stable as a chromosome. The recombinant YAC that was produced in this study is more or less 21kb and after BIT application it became 40kb, which is still much smaller than 150kb.

Since the recombinant YAC was so small, it was checked for its stability in the yeast cells as a chromosome. We compared the linear YAC carrier strain (BAP4-y3) and the BIT applied YAC carrier strain (BAP4bity3) for the loss of recombinant YAC. We let BAP4-y3 and BAP4bity3 strains grow in non-selective media, where there is no pressure on YAC to be presence in the cells. Later on, we replica plated them into selective media to calculate the colony loss, which is a verification for the recombinant YAC lost. In total, we checked 2500 colonies for each recombinant strains and as a result we saw that BAP4bity3 strain had less chromosome loss than the BAP4-y3 strain **(Figure 3.28).**

Together with this result, it was demonstrated that BIT could make recombinant YAC stabilize into the resident yeast genome. Even though that translocant YAC was a bit larger than linear YAC, it was still far from 150kb size for being stable as a chromosome. Since the translocant chromosome is highly stable, this result proves that BIT is affecting the stability positively and size of the chromosome is not that important to be stable after BIT application.

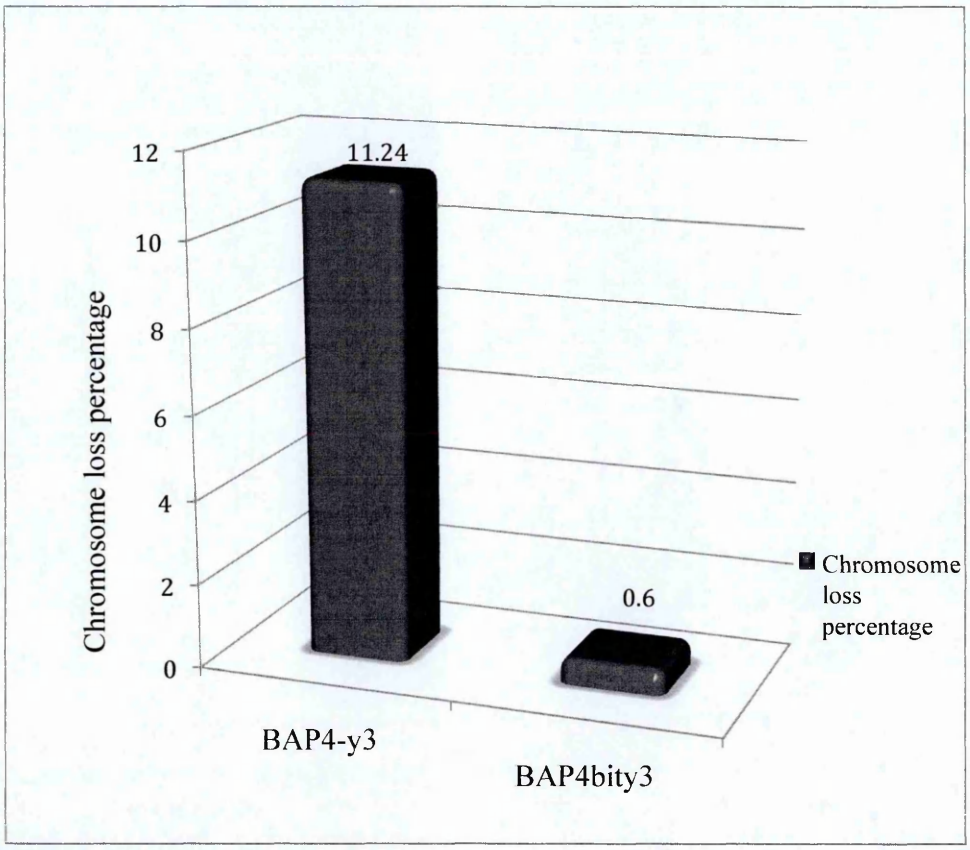

Figure 3.28: Graphic representation of recombinant chromosome loss. Axis labels represent different strains and Y line shows the percentage of the chromosome loss among the 2500 colonies.

B.3.7 DIRECT FERMENTATION OF THE CELLULOSIC SUBSTRATE

To prove the performances of the recombinant *S. cerevisiae* strains and demonstrate its functionality, CMC was used as the sole carbon source for cellulosic bio-ethanol production.

All recombinant strains were tested for their growth abilities in cellulosic medium. In parallel, a control was also run and insignificant growth was detected in the reference yeast strain harbouring the empty YAC. We inoculated $1x10^5$ cells/ml of each strain in YPCMC and followed their growth for a week by counting the cells under microscope. As shown in figure 3.29, both recombinant strains, BAP4-y3, BAP4-ciry3 and BAPbity3 grew better on cellulosic media compared to wild type yeast strain, with faster growth exhibited by BAP4bity3 strain as compared to BAP4-ciry3 and BAP4-y3 strains. Cellulose could not get through the cell wall, so it could not be hydrolyzed by intracellular cellulases. The introduced cells were washed twice before fermentation, suggesting that the growth in YPCMC could not be accomplished by the other sugars carried from previous cultures.

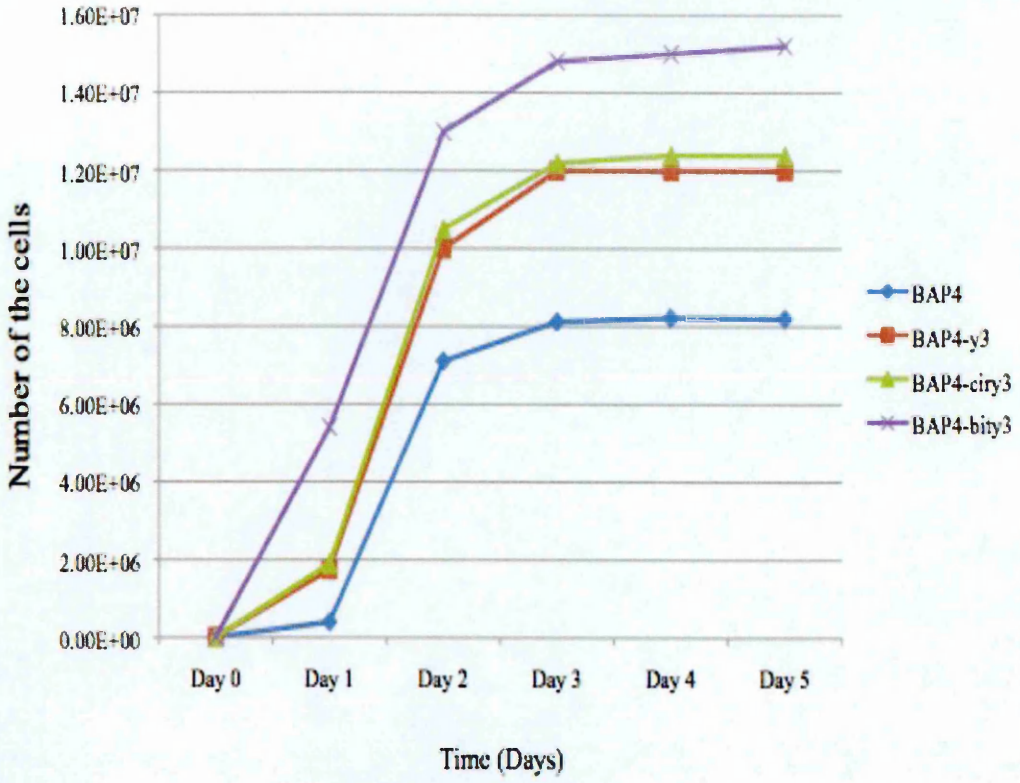

Figure 3.29: Growth curve of the BAP4, BAP4-y3 and BAP4bity3 strains in YPCMC media. Axis labels shows the days and Y values are the number of the cells counted at the time point. Appropriate controls were analyzed in parallel.

This BIT-YAC system is potentially applicable to any host that can undergo homologous recombination. Our study has demonstrated a success in the development of this yeast strain as a new host for heterologous protein production for cellulose degradation. BIT positive effect on strain improvement for cellulose degradation was once more proved with this growth curve analysis.

4. DISCUSSION

This chapter was divided in two parts; A and B. In Part A, discussion of "Wood Powder Degradation By Microbial Fermentation" and in Part B, discussion of "Implementation Of The Bridge Induced Chromosome Translocation (BIT) Technology For Strain Improvement" were shown.

Bioethanol is a renewable energy that can be produced from different biological sources and the production of bioethanol from cheap biomass, like waste plant biomass, is important for fuel industry. Around the world, there are a lot of studies about bioethanol production from different biological sources and lignocellulosic biomass is one of them, which has numerous environmental and social benefits for bioethanol production (Lange & Solutions, 2007; Lee, 1997; Wyman, 1994).

There has been an overwhelming interest in science to generate proteins by microbial organisms with the recombinant DNA technology. *S. cerevisiae* is one of the microbial organisms for the expression of heterologous genes. It gained considerable interest in recent years mainly due to its ability to correctly process and secrete proteins. *MFa1* signal sequence was used in this study for the secretion of foreign proteins. This sequence had been used in previous studies to secrete a wide range of heterologous proteins in yeast, like human interleukin-2 and murine granulocyte-macrophage colony stimulating factor (Idiris, Tohda, Kumagai, & Takegawa, 2010; Kazemi Seresht et al., 2012).

In this study, cellulosic material was used as a substrate for the bioethanol production. Since yeast cannot utilize the cellulosic materials, it has to express and secretes some set of recombinant enzymes. For this purpose, recombinant DNA technology was used to engineer the yeast cells to make them able to degrade the cellulosic sugars into the fermentable sugars.

PART A: WOOD POWDER DEGRADATION BY MICROBIAL FERMENTATION

The present research deals with the bioethanol production from the wood powder in two-step microbial fermentation. The first step is the utilization of lignocellulosic material by *Bacilli* to produce fermentable sugars and the second step is the conversion of fermentable sugars into glucose by recombinant enzyme expressing yeast strain to produce ethanol. *Bacilli* were able to produce cellobiose from wood powder and that's why yeast strain was engineered to express β-glucosidase to degrade cellobiose molecules.

The major challenge for the bioconversion of raw lignocellulosic materials to ethanol is the low efficiency of the degradation and the high cost of the process. Biological degradation of the lignocellulosic substrates to fermentable carbohydrates is one of the key steps for process to be cost effective and environmentally friendly. Several studies have been done to convert lignocellulose to sugars, but sugar yields are still low and the costs are high (Elkins, Raman, & Keller, 2010; Kumar et al., 2009; Sun & Cheng, 2002).

As a lignocellulosic material, the wood powder was used, which is waste of the wood industry and it was obtained from Lasole Company. To investigate the possible use of microorganisms as a candidate for bioethanol production from wood powder, *Bacilli* strains were extracted from the ruminal fluid of the cow and *S. cerevisiae* was engineered for the cellobiose degradation. Different *Bacilli* strains were characterized after extraction from the cow by Yeast Molecular Genetics Laboratory/ICGEB and compared by their lignocellulosic degradation ability.

Lignocellulose has the main components of plant biomass and contains cellulose, hemicellulose and lignin. Cellulose and hemicellulose parts contain sugar polymers that are important and economic for fuel productions. Ethanol production from these sugars has some restrictions to deal with. The most important difficulty to overcome is the structural

features of the lignocellulosic material (Mosier et al., 2005). As a result of this barrier, there is a need to develop new methodologies, which can efficiently separate the fermentable sugars from the lignocellulosic biomass with sustainability and low cost. For this reason, pre-treatment of lignocellulose is an important step to make cellulosic and hemicellulosic parts more accessible for converting them into ethanol. There are different ways to degrade lignocellulosic materials; chemical, physical and biological (Kumar et al., 2009; Lange & Solutions, 2007). They all not enough efficient for the pre-treatment but biological methods have advantage of being less expensive compare to other methods.

The lignocellulose degradation by microorganisms has great importance and significance for second-generation bio fuel technologies, the starting point of which is producing fermentable sugars (Lynd et al., 2002). Due to the complexity of lignocellulose structure, a wide range of enzymes is required for its degradation into monomer sugars. It has been shown previously that *Bacilli* species isolated from bovine ruminal fluid have the ability to degrade lignocellulosic material (Akin & Benner, 1988; Lee, 1997; Science & Russell, 1988). *Bacilli*, which are the rumen bacteria, have been used for many important industrial applications. They are responsible of the catabolism of some aromatic compounds (ferulic acid and *p*-coumaric acid), which are the most important phenolic compounds that bind the complex lignin polymer and keep lignin, hemicellulose and cellulose structures together (Degrassi, Polverino De Laureto, & Bruschi, 1995; Zago et al., 1995). In this study, we used different *Bacilli* strains for the degradation of the wood powder. Our laboratory isolated and characterized several new strains of *Bacilli* from bovine rumen, amongst which are, *B. licheniformis* and *B. pumilus* showed the highest level of activity for producing carbohydrates from lignocellulosic material, based on the results from the anthrone test. Furthermore, by using HPLC analysis and sugar assays, we detected cellobiose and glucose molecules in the fermentation broth, hereby allowing us to conclude that *B. licheniformis* and *B. pumilus* degraded the cellulose by separating it from the lignin in the wood powder.

Microbial degradation of wood powder from industrial waste is a unique work because of not using any physical or chemical pre-treatment method to lignocellulosic material that would otherwise consume too much time and increases the cost of the ethanol production. Separation of cellulosic carbohydrates from wood powder by *Bacilli* fermentation, which is important for the lignocellulose pre-treatment studies, provided fermentable sugars for recombinant yeast strain and this process easily leads the ethanol production. But unfortunately efficiency of microbial pre-treatment is low and this is the biggest disadvantage of the method. Other non-biological pre-treatments can increase the efficiency on the contrary they also increase cost of the process.

Saccharomyces cerevisiae is an ideal model organism for foreign protein expressions. The development of a recombinant yeast strain that is able to produce ethanol by fermenting cellulosic substrates has got attention of the science world over recent years (Den Haan, Mcbride, Grange, Lynd, & Van Zyl, 2007; Tsai, Goyal, & Chen, 2010; van Rooyen, Hahn-Hägerdal, La Grange, & van Zyl, 2005; Wen, Sun, & Zhao, 2010; You et al., 2012). Cellulose chains require the endo-exo synergy of endoglucanases (*EGC*), cellobiohydrolases (*CBH*) and β-glucosidases (*BGL*) to produce glucose for the glycolytic pathway for the production of ethanol (Kricka et al., 2014). There are some microorganisms that have these cellulase enzymes naturally for the cellulose degradation (Mba Medie et al., 2012). One of them is *Pichia stipitis* that has seven *BGL* genes responsible for cellobiose degradation pathway (Jeffries & Van Vleet, 2009). Since β-glucosidase hydrolyzes cellodextrins and cellobiose to glucose, it provides a straightforward metabolisable carbon source for the microorganisms (Beguin, 1990).

Cellobiose and cello-oligosaccharides, which are hydrolysed from cellulose, are the key substrates for ethanol production. The cellobiase enzyme, beta-glucosidase, that converts the cellobiose and cello-oligosaccharides into glucose has been shown to belong to the critical step in the sacchararification of cellulosic material (Lynd et al., 2002). *Pichia*

stipitis is generally used in hemicellulose and xylose degradation studies, and here we used it as a source of betaglucosidase for obtaining recombinant *S. cerevisiae,* which has several advantageous for heterologous protein expression due to its GRAS status. The *BGL6* gene was cloned from *P. stipitis* and fused to the *MFa1* signal sequence, which has been used previously to obtain secretion of heterologous gene products in yeast (Idiris, Tohda, Kumagai, & Takegawa, 2010; Romanos, Scorer, & Clare, 1992). As a component of the completed pBLAST expression vector, the *MFa1* signal sequence directed the beta-glucosidase into the secretory pathway, where it was processed and secreted as mature protein. With an enzyme activity assay and a XGAL plate-visualizing test, we confirmed that Bgl6 was secreted from the recombinant yeast strain, called 19s, and degraded the cellobiose molecules efficiently.

Cellobiose saccharification activity of the engineered *S. cerevisiae* has been demonstrated in filtered wood powder media after *Bacilli* fermentation, where the significant production of cellobiose molecules was confirmed by HPLC and sugar assays. Growth rate was far higher by recombinant yeast strain compared to the parental strain. When we tried in parallel the yeast fermentation in a media supplemented with commercially available cellobiose, we saw more or less the same growth curve of recombinant yeast strain in both media. Therefore, the cellobiose degradation ability of recombinant yeast strain was alike in the both media, *Bacilli* pre-treated wood media and YPC.

We succeeded obtaining a high performance of lignocellulose degradation by using a combination of *Bacilli* and recombinant budding yeast. As a lignocellulosic material wood powder was used, which is a waste from the wood industry. Wood powder is a dangerous product of wood milling for wood workers. Moreover, the only present utilization is burning the wood powder, which does not produce important return of energy. In this way, waste was used for the ethanol production and using *Bacilli* for the pre-treatment should allow to decrease the costs and time for the process compare to the physical and chemical pre-treatment methods (Chang et al., 2012; Matano, Hasunuma, & Kondo, 2012; You,

Zhang, & Zhang, 2012). This study proposed a big step in the use of wood powder for the production of bio-ethanol. To our knowledge this is the first study that shows the efficient degradation of wood powder by microorganisms without any chemical or physical pre-treatment methods.

In summary, wood fermentation was achieved in two steps; the first step was performed by *Bacilli* in poor LB media supplemented with 4% wood powder and then, in the second step, filtered wood powder media after *Bacilli* fermentation was used for the recombinant yeast fermentation. In every step proper control experiments were done in parallel. We successfully demonstrate simultaneous saccharification and fermentation of wood waste to glucose molecules by using *Bacilli* and recombinant yeast strain in combination. This study therefore provides the basis for efficient generation of bioethanol from waste wood powder products just in two microbial steps as an alternative to the usual wood based bioethanol production process that takes several time-consuming and labor-intensive rounds of techniques.

In conclusion, this work describes a successful microbial combination system that is able to efficiently utilize the lignocellulosic material as a carbon source under the same conditions and facilities in one bio-factory. These wood-fermenting recombinant microorganisms have great importance in strengthening bio-processing. The novelty of this study is to use directly an industrial waste as a substrate for the microbial fermentation, which is an indication for the environmental friendly process. These results will be beneficial in the process of bio-ethanol production by proper utilization of the waste and decreasing the ethanol cost.

PART B: IMPLEMENTATION OF THE BRIDGE INDUCED CHROMOSOME TRANSLOCATION (BIT) TECHNOLOGY FOR STRAIN IMPROVEMENT

The present research deals with implementation of the bridge-induced chromosome translocation (BIT) technology and yeast artificial chromosome (YAC) recombineering for strain improvement of bioethanol producing transgenic yeast. It is aimed to construct a YAC that carries cellulose degradation genes on it and then applying the BIT technology to stabilize the new genetic information into resident yeast genome and have increased expression levels of cellulases' genes.

Cellulose, the most abundant renewable carbon source, is the main part of the plant cell wall and it can be hydrolyzed enzymatically for the ethanol production. The main restrictions for the cellulose-based ethanol are the cost of the process and the efficiency of degradation. Therefore, engineering a microorganism that can convert all the sugars of cellulosic substrate to ethanol is an attractive approach in bio-fuel production studies (Lynd, Weimer, van Zyl, & Pretorius, 2002). In this study, BIT technology and YAC recombineering were used to engineer *S. cerevisiae* strains and the results showed that the recombinant yeast strains were able to hydrolyze cellulose to glucose. Especially BAP4bity3 strain was showing much better desired features as compared to BAP4-ciry3 and BAP4-y3 strains.

Three cellulase genes of fungal origins were successfully expressed in *S. cerevisiae*. All three genes were fused to the *MFa1* secretion signal sequence (Romanos, Scorer, & Clare, 1992) and expressed under constitutive promoters. To begin with, these three genes were cloned into different plasmids that contain different constitutive promoters to eliminate the recombination between the same promoters. Later the whole constructs were extracted (promoter + secretion signal + *GENE* + terminator) from plasmids and cloned into pYAC3

plasmid. This step is the first novel part of this research. Because recombinant YAC can be transformed to any other organisms and in this case it can be used as a carrier for cellulases.

Given the fact that *S. cerevisiae* has positive features like high ethanol productivity, high tolerance to ethanol and simplicity of genetic engineering, the development of a recombinant yeast strain that is able to produce ethanol by fermenting cellulosic substrates has drawn the attention of the scientific world over recent years (Chang et al., 2012; Fan et al., 2012). Since *S. cerevisiae* cannot degrade the cellulose molecules, at least three groups of enzymes had to be expressed, namely endoglucanases (*EGC*), cellobiohydrolases (*CBH*) and β-glucosidases (*BGL*), for cellulose conversion. Cellulose chains can be efficiently degraded to soluble cellobiose and cellooligosaccharides by the synergy of *EGC* and *CBH.* In the last step of the enzymatic cellulose degradation, cellobiose molecules get hydrolyzed to glucose by *BGL* (Kricka, Fitzpatrick, & Bond, 2014). There are bacteria and fungi strains that have some set of enzymes for cellulose degradation (Mba Medie, Davies, Drancourt, & Henrissat, 2012). In this study *Pichia stipitis* (for *BGL* and *EGC*) and *Trichoderma reesei* (for *CBH*) were used as a source for the cellulase genes.

Bioethanol production studies aim to achieve efficient degradation of biomass, to obtain high amount of ethanol, to simplify the process and to decrease the cost of the process (Elkins, Raman, & Keller, 2010). To improve the current bioethanol production processes, this research was designed to utilize two novel technologies (YAC recombineering and BIT) to introduce new, multi-factorial, complex genetic traits into yeast strains. In Yeast Molecular Laboratory/ICGEB, a new genomic system previously was developed, called bridge-induced translocation, BIT (Tosato *et al.*, 2005), which induces the chromosomal translocation between any desired two genomic loci in *S. cerevisiae,* by integration of a selectable DNA cassette and it results many different effects on cellular morphology, physiology and genome organization. Once the translocant strain was generated, many

differences were observed at both genomic and transcriptomic level compared to wild type strain (Nikitin et al., 2008). From this approach, the important result is that a single translocation event brings an accumulation of molecular events and these changes effects the genome instability (Nikitin et al., 2008; Rossi et al., 2010; Tosato et al., 2009). Some of the BIT effects have been demonstrated could also be important for the manipulation and stable maintenance of YACs, which can be used for production of secondary metabolites and for heterologous protein expression (Arnak, 2012; Nikitin, Tosato, Zavec, & Bruschi, 2008).

BIT technology was used for the strain improvement by the help of its effect on gene expression level. To do this, cloning of the yeast artificial chromosome was firstly done. YACs are linear chromosome-like shuttle vectors that can replicate in bacteria and employed for the cloning and manipulation of big DNA inserts in the *S. cerevisiae* (Arnak, 2012). After YAC engineering, BIT technology was used to improve the strain more. BIT system runs by the use of a PCR-derived DNA cassette that carries a selective marker and is flank by two homology region that is targeted to different locations in the genome (Tosato et al., 2005). YAC and chromosome IV were targeted for BIT event, and in the end a translocant chromosome was obtained, which contained YAC on the left side and chromosome IV on the right side.

In previous BIT studies, it was shown by the microarray analysis that most of the genes, which have different expression profile compare to wild type and are affected by the translocation events, are the ones that are responsible of metabolism and stress response (Nikitin et al., 2008). Therefore, translocant cells regain a new homeostasis by having many rearrangements on the level of gene expressions, which is different than the original wild type ones after induced BIT event. Therefore, application of appropriate selective pressure helped to choose the best cellulose utilization candidate from the other differently regulated translocants.

As a result of BIT application, some advantages were gained for the strain improvement. One of them was to make YAC stabilize into the yeast genome. To be stable as a chromosome, size of the YAC has to reach at least to 150kb (Arnak, 2012; Burke, Carle, & Olson, 1992). Recombinant YAC that was produced in this study as a carrier of cellulases genes was around 25kb and after BIT application it became 40kb. However, it was still really far from being 150kb for stability. When the stability of YAC was compared before and after BIT application, it was seen that BIT affected the stability of recombinant YAC positively and allowed it to gain more stability in the cells under non-selective conditions. Before the BIT application, almost 12% of the cells lost the recombinant YAC. Surprisingly, after the BIT application, this number decreased enormously and 0.6% of the cells lost the translocant recombinant YAC. This result showed that short chromosomes can be more steady by the BIT application and this novelty can help to recombinant DNA studies by increasing the stability of carrier artificial chromosomes.

The other advantage that was gained after BIT application was for the gene expression level. It was seen before that around the break point of translocation, gene expression level is increasing comparing to wild type (Nikitin et al., 2008). This effect was aimed to use as an advantage for the heterologous gene expression and the translocation break point was chosen close to the cellulase genes on YAC and *PAD1* gene on chromosome IV. The reason why translocation break point was chosen in chromosome 4, close to *PAD1* gene, is that *PAD1* gene is known as Phenylacrylic acid decarboxylase and its gene expression level was desired to be increased by the help of the BIT application. As it is already shown that overexpression of *PAD1* gene associates with the increased rate of carbon source utilization, increased rate of ethanol production and increased resistance to some aromatic compounds (Larsson, Nilvebrant, & Jönsson, 2001). Therefore, increased *PAD1* expression level could help to strain improvement of bioethanol producing yeast cells. After the BIT application, recombinant strains' gene expression profiles were compared

and it was seen that in BAP4bity3 strain, cellulases genes and *PAD1* gene had increased expression level compared to BAP4-y3 and BAP4-ciry3 strains. This unclear mechanism of BIT technology on gene expression level is a very useful tool for the strain improvement studies.

In bio-ethanol production studies, traditional cloning and expressing recombinant proteins are the main focus that most of the researchers are following (Chang et al., 2012; Fan et al., 2012). Moreover with traditional methods, strain cannot be improved more but in this study strain improvement was enhanced; two novel technologies (YAC recombineering and BIT) were utilized to introduce new functions into yeast strains to make it able to degrade the cellulosic substrate. Selection strategies were implemented to obtain novel genetic regulation that would achieve the final phenotype originally desired with the high cellulose degradation and high ethanol producing features. Therefore an efficient and economic conversion of cellulosic biomass into glucose is demonstrated in a GRAS microorganism, leading to interesting perspectives in bio-ethanol production.

This work describes the successful recombinant translocant yeast that is able to efficiently utilize cellulosic material as a carbon source with highly stable recombinant translocant chromosome and has high level of cellulases capacity. BIT-YAC system can also be used for the expression and production of other recombinant proteins for different purposes and for different kind of strain improvements.

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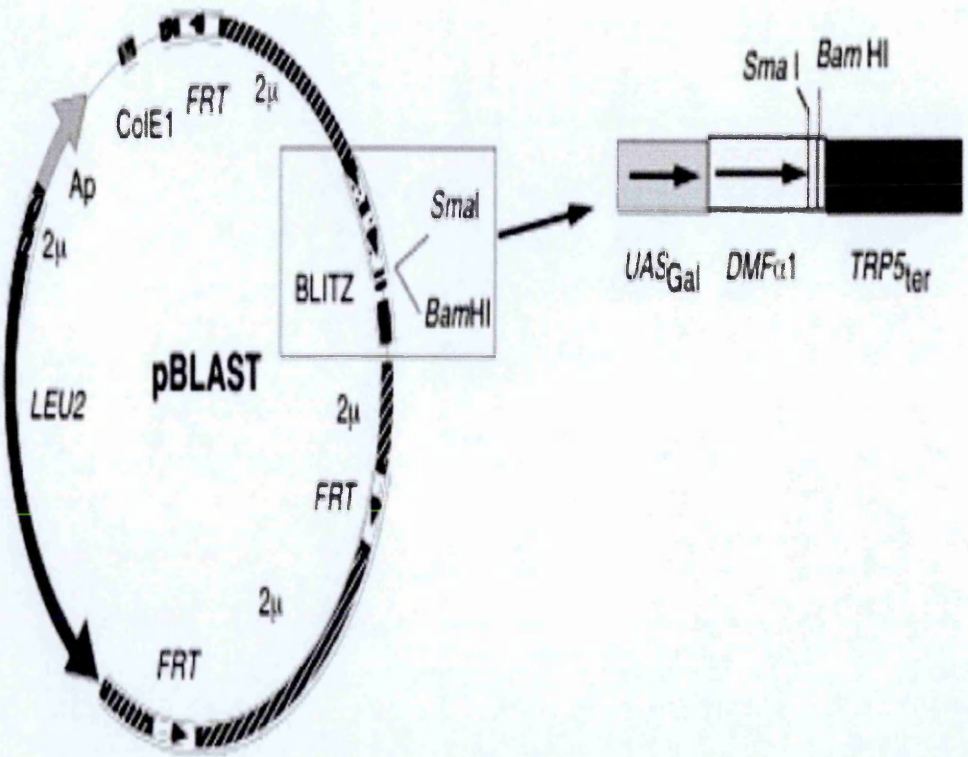
APPENDIX

MAPS OF THE PLASMIDS USED IN THIS THESIS

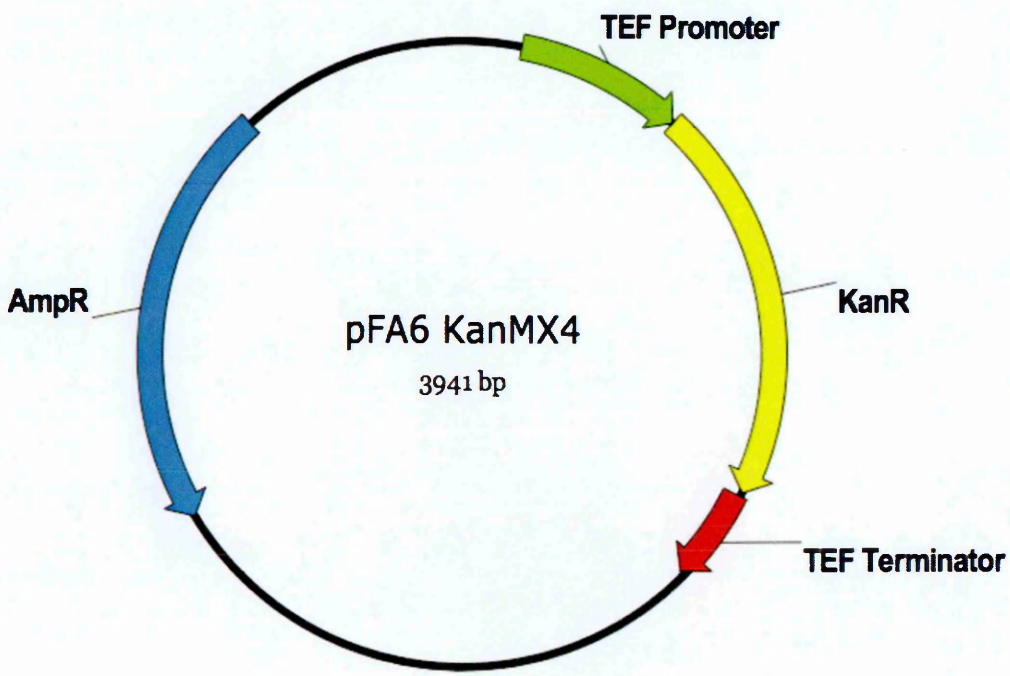
pBLAST

pBLAST plasmid contains *GAL10* inducible promoter, alpha factor secretion signal and sequences from 2 micron plasmid.



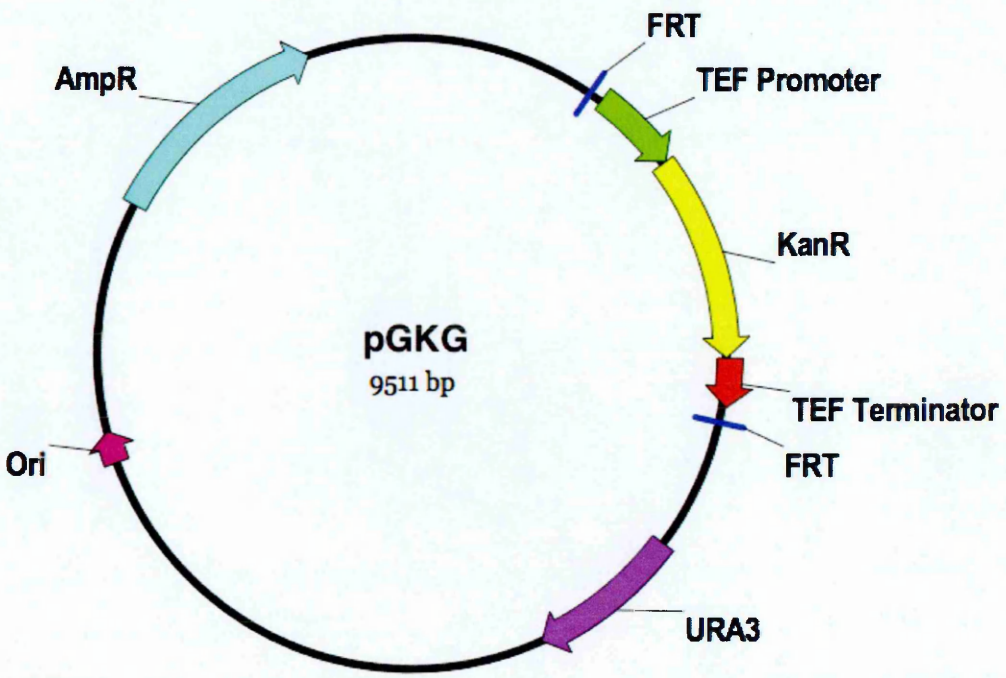
pFA-kanMX4

pFA6 plasmid with KanMX4 cassette conferring resistance to G418 drug. TEF promoter and terminator derived from filamentous fungus *Ashbya gossypii.*



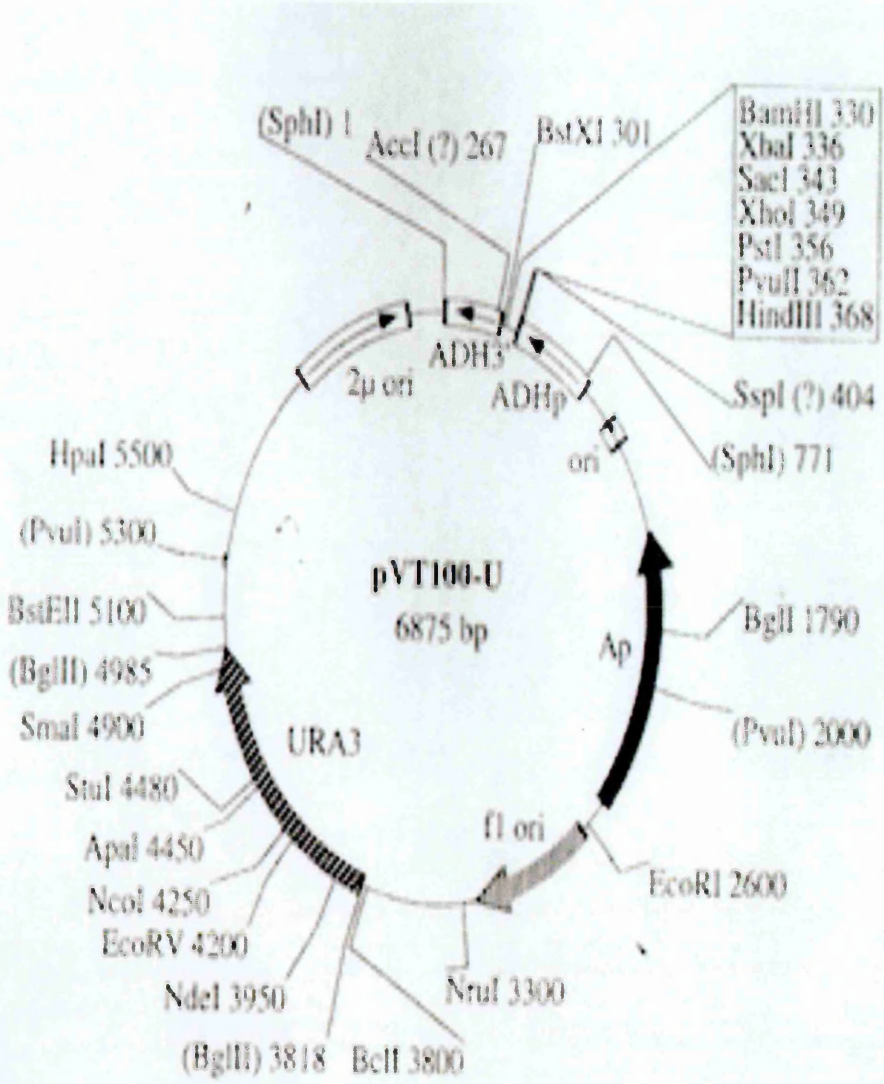
pGFKG

pGFKG is a derivate pGKG. It is constructed by inserting the *FLP* gene in the plasmid shown below. The *FLP* gene was cloned into the *BamHI* site upstream of the *kanMX4* gene and the *URA3Kl* gene respectively. Kanamycin cassette flanked by two flip- recognition targets



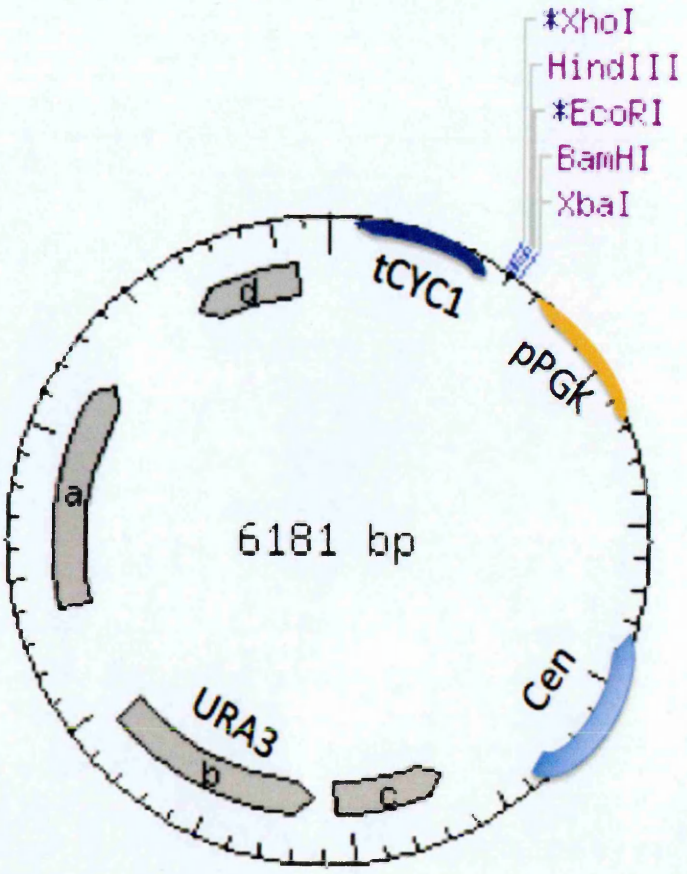
pVT100-U

pVT100-U contains ADH promoter and terminator and sequences from 2 micron plasmid. URA3 gene is used for the selection of the plasmid in auxotrophic yeast cells.



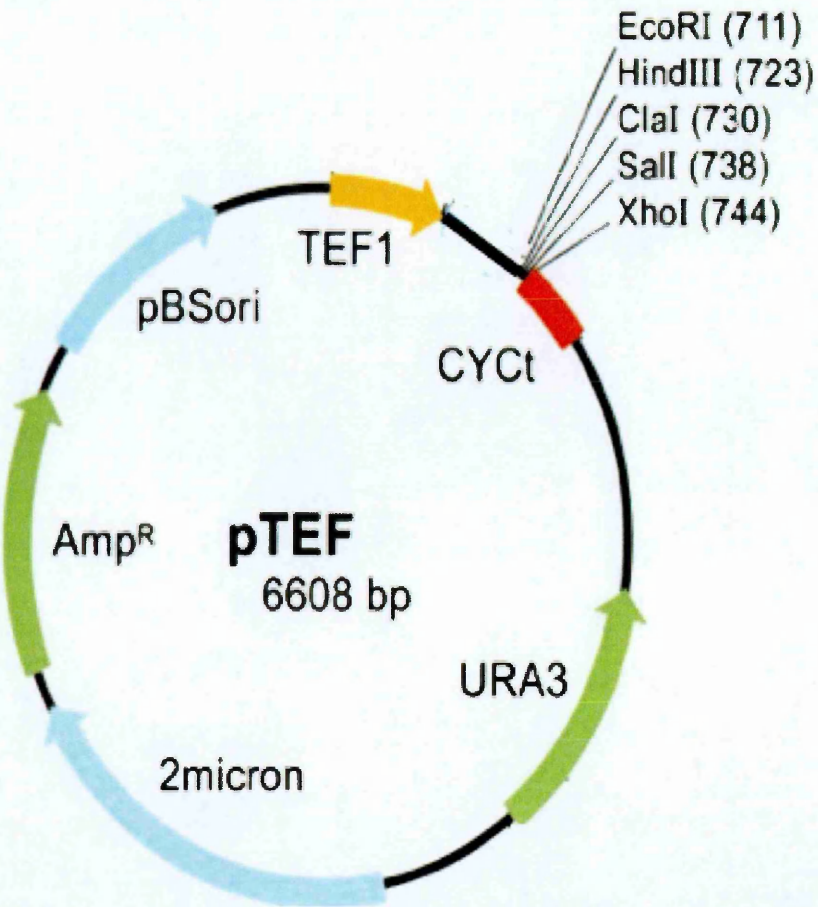
pJL49

pJL49 is a centromeric yeast plasmid that contains PGK promoter and CYC terminator. URA3 gene is used for the selection of plasmid in auxotrophic yeast cells.



pTEF

pTEF plasmid contains TEF promoter, CYC terminator and sequences from 2 micron plasmid. URA3 gene is used for the selection of plasmid in auxotrophic yeast cells.



pYAC3

pYAC3 is pYAC3 was constructed from pYAC2. It is an artificial chromosome and BamH1 digestion produces linear chromosome with the telomeres and removes HIS3 sequences. pYAC3 contains centromere 4 of budding yeast and allows cloning of big DNA inserts.