MAKING USE OF SYNTHETIC BIOLOGY TOOLBOX FOR INDUSTRIAL BIOTECHNOLOGY APPLICATIONS: THE CASE OF ENZYME PRODUCTION

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ABSTRACT

MAKING USE OF SYNTHETIC BIOLOGY TOOLBOX FOR INDUSTRIAL BIOTECHNOLOGY APPLICATIONS: THE CASE OF ENZYME PRODUCTION

Microorganisms have long been used in industrial biotechnology. However, since yield and diversity are limited by the natural capacity of the microorganism, it cannot adequately respond to the increasing demand. At this point, recombinant DNA technology offers alternative solutions. Compared to recombinant DNA technology methods, synthetic biology, which enables use of standardized parts and protocols, has been widely used in industrial biotechnology. The focus of synthetic biology is not only the integrated design of DNA fragments such as the promoter, coding region, and metabolic chemical reactions such as the pathway, but also the design of the microorganism itself, namely "host design". These designs aim to eliminate the challenges in the over-production of natural products, heterologous production, keeping the product stable for a longer time or utilization of alternative carbon sources in production.

This thesis includes synthetic biology applications for strain development studies in industrially important filamentous fungi and yeast. In the first chapter, one of the well-studied filamentous fungi, *Aspergillus brasiliensis*, was used as the host and rendered protease-deficient. This strain ensured that the activity of natural or heterologous enzymes remained stable without degradation. Furthermore, promoter engineering studies were carried out on carbon catabolite repression. Glucoamylase promoter was selected and base addition/deletion was performed on the sequence to remove carbon catabolite repression. 3 different promoter variants were obtained and the variant that continued to exhibit activity in the presence of xylose was then used as promoter for heterologous protein expression in the protease-deficient strain. As a conclusion, heterologous protein production was carried out in the presence of an alternative lignocellulosic carbon source, enzyme activity was maintained during fermentation and simultaneous utilization of different carbon sources was achieved in this strain. In the second chapter, it was aimed to develop a CRISPR/Cas9 system to be used in gene disruption studies in *Saccharomyces cerevisiae*. In this context, beta-isopropylmalate dehydrogenase gene, *LEU2*, was targeted, related sgRNA was designed and a CRISPR plasmid was constructed using Golden Gate assembly. As a result, construction and utilization of a functional CRISPR/Cas9 system in yeast was confirmed.

ÖZET

ENDÜSTRİYEL BİYOTEKNOLOJİ UYGULAMALARI İÇİN SENTETİK BİYOLOJİ GEREÇ KUTUSUNUN KULLANIMI: ENZİM ÜRETİMİ

Mikroorganizmalar endüstriyel açıdan önemli enzimlerin üretimi için uzun yıllardır kullanılmaktadır. Ancak verim ve çeşitlilik mikroorganizmanın doğal kapasitesiyle sınırlı olduğundan artan ihtiyaca yeterince cevap verememektedir. Bu noktada rekombinant DNA teknolojisi alternatif çözümler sunmaktadır. Rekombinant DNA teknolojisi yöntemlerine kıyasla, standardize edilmiş parçaların ve protokollerin kullanımını mümkün hale getiren sentetik biyoloji yaklaşımı ise endüstriyel biyoteknoloji alanında yaygın olarak kullanılmaya başlamıştır. Sentetik biyolojinin odağında sadece promotör, kodlayıcı bölge gibi DNA parçalarının ve yolak gibi metabolik kimyasal reaksiyonların bütünleşik tasarımı değil, aynı zamanda mikroorganizmanın kendisinin de tasarlanması yani "konakçı tasarımı" da bulunmaktadır. Bu tasarımlar, hücrelerin doğal ürünlerini daha fazla üretme, heterolog üretim, ürünü daha uzun süre stabil tutma ya da alternatif karbon kaynaklarının üretimde kullanımı çalışmalarındaki zorlukların giderilmesini amaçlamaktadır.

Bu tez, endüstriyel olarak anlamlı fungusta ve mayada suş geliştirme çalışmalarına yönelik sentetik biyoloji uygulamalarını içermektedir. İlk bölümde, bilinen filamentli funguslardan *Aspergillus brasiliensis* konakçı olarak kullanılmış ve proteaz-eksik hale getirilmiştir. Oluşturulan suş, doğal ya da heterolog ürettiği enzimlerin daha uzun süre bozunmaya uğramadan stabil kalmasını sağlamıştır. Bunun yanı sıra, karbon katabolit represyon alanında promotör mühendisliği çalışmaları yapılmış, glukoamilaz promotörü üzerinde karbon katabolit represyonu giderecek şekilde baz ekleme/silme gerçekleştirilmiştir. 3 farklı promotör elde edilmiş ve ksiloz varlığında aktivite göstermeye devam eden varyant, proteaz-eksik suşta heterolog enzim ifadesi için promotör olarak kullanılmıştır. Sonuçta, lignoselülozik bir karbon kaynağı varlığında heterolog enzim üretimi gerçekleştirilmiş, enzimin aktivitesi fermantasyon boyunca sabit tutulmuş ve bu suşta farklı karbon kaynaklarının eşzamanlı kullanımı sağlanmıştır. İkinci bölümde, *Saccharomyces cerevisiae* mayasında gen silme çalışmaları için CRISPR/Cas9 sistemi geliştirilmesi amaçlanmıştır. Bu bağlamda, beta-izopropilmalat dehidrojenaz geni, *LEU2*, hedeflenmiş, ilgili kılavuz RNA tasarlanmış, Golden Gate montajı ile bir CRISPR plazmidi oluşturulmuştur. Sonuçta, mayada işlevsel bir CRISPR/Cas9 sisteminin oluşturulması ve kullanımı doğrulanmıştır.

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LIST OF SYMBOLS/ABBREVIATIONS

α	Alpha
Δ	Delta
μ	Micro
Ω	Omega (Ohm)
Amp	Ampicillin
Bp	Base pair
BSA	Bovine serum albumin
Cam	Chloramphenicol
CCR	Carbon catabolite repression
CM	Complete medium
Cre	Catabolite responsive element
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR ribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
Ds	Double-strand
EC	Enzyme commission
FBB	Fungal backbone
GFP	Green fluorescent protein
Gla	Glucoamylase
GG	Golden Gate
GRAS	Generally recognized as safe
HDR	Homology directed repair
HF	High fidelity
HmB	Hygromycin B
Kan	Kanamycin
LB	Luria Bertani broth
MMS	Minimal medium sorbitol
MPD	Malt extract peptone dextrose
NHEJ	Non homologous end joining

OEPCR	Overlap extension polymerase chain reaction
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEG	Polyethylene glycol
RNA	Ribonucleic acid
sgRNA	Single guide ribonucleic acid
SOC	Super optimal broth with catabolite repression
SMA	Skim milk agar
WT	Wild type
Xyn	Xylanase
YBB	Yeast backbone
YPD	Yeast extract peptone dextrose
YPDS	Yeast extract peptone dextrose sorbitol
Zeo	Zeocin

CHAPTER I: IMPROVING *ASPERGILLUS BRASILIENSIS* FOR HETEROLOGOUS PROTEIN PRODUCTION

1. INTRODUCTION

Microorganisms have long been used as cell factories for the production of numerous industrially important biomolecules within the context of industrial biotechnology [1,2]. One such class of commercially important biomolecules consists of enzymes, which are nowadays accepted as indispensable tools for sustainable bio-economy in several fields. World demand for microbial enzymes including amylase, lipase, protease, phytase is expected to reach 9.6 billion dollar by 2024. In order to respond to the increased demand, research has focused on the production of microbial enzymes in high yield and quantity that exceed the natural capacity of microorganisms. Although improving the fermentation conditions is an option to increase production capacity, recombinant DNA technology offers more effective solutions such as heterologous protein production in this regard.

Synthetic biology is an emerging interdisciplinary branch of biology focusing on the design and construction of so-called “biological parts, tools, and systems” typically from scratch not seldomly on the redesign of existing biological systems for novel functions or improved performance. The key factor into this is considered to be the assembly and possible synergy as well as antagonism of the designed biological parts in particular for successful application of the concepts to living systems.

The use of synthetic biology toolbox for heterologous enzyme production generally requires engineering of not only the product, the gene or the pathway but also often the microorganisms as well [3]. This engineering process may be carried out in several ways of cloning including traditional (e.g. using restriction enzymes and expression vectors) and modern cloning techniques (Gateway Recombination, Ligation Independent Cloning, Gibson Assembly, Golden Gate Assembly).

Modern cloning techniques allow fast, effective and accurate production of desired product via novel molecular cloning systems [4]. Despite significant improvements in applications of modern cloning techniques, limited molecular tools, plasmid instability problems, specific sequence-based requirements and the secretion pathway properties of host organisms are still bottlenecks for heterologous production of microbial enzymes in industrial biotechnology context [5].

1.1. SYNTHETIC BIOLOGY

Synthetic biology revolutionized biotechnology especially through engineered organisms carrying novel features, normally absent in the corresponding host [6]. The key idea in this approach is the design of standardized biological parts and their assembly using standardized protocols. Synthetic biology considers genes, transcription factors, ribosome binding sites, proteins, terminators as functional parts and the cell as a machinery [7]. Correct assembly of designed biological parts is as important as designing and standardization of those parts especially in industrial application of synthetic biology.

The idea of integrating biology and engineering leads to development of new expression systems based on assembly of DNA parts up to the size of whole bacterial genome [8–12]. However, this DNA assembly concept is not just a process of bringing functional DNA parts together, rather combining different functional DNA parts in a proper way. Standardization of assembly in synthetic biology is performed via special sequences namely prefixes and suffixes. These regions act as junction points for an easy, fast and less laborious assembly. Consequently, complexity is decreased and integrated circuits are built.

Assembly methods developed over the past few years mainly use homologous recombination mechanism both in vivo and in vitro, so that the necessity of containing restriction enzyme recognition sites in the fragments to be assemble is eliminated. Alternatively, new assembly methods have been introduced that they allow the simultaneous and directional integration of multiple DNA fragments into a plasmid backbone via Type IIS restriction enzymes and T4 ligase.

As one of the assembly methods, Golden Gate assembly uses BsaI, BsmBI and BbsI Type IIS restriction enzymes for cloning of multiple DNA fragments into a plasmid backbone. Type IIS restriction enzymes cut the DNA outside of their recognition sites and recognition sites are eliminated from the final product. This feature allows simultaneous restriction digestion and ligation, resulting in one-pot irreversible cloning of the parts. Additionally, non-palindromic overhangs generated after restriction digestion are utilized to ensure scar-free assembly of DNA parts.

1.2. HOST DESIGN

Microorganisms are the powerful biological factories for the production of valuable products. Since microbes can rapidly grow using a large portfolio of carbon sources, scaling up process of microbial cultures is desired to produce industrially important products in large quantities [13]. Hence, production of vitamins, organic acids, ethanol, antibiotics and therapeutic agents via microorganisms is economically highly attractive [14]. However, the yield and the diversity of products are limited due to the natural capacity of the microbial metabolism. To overcome this limitation, novel tools such as heterologous protein production, genome reduction etc. have been discovered and used for improving and optimizing microbes, especially for their use within industrial biotechnology context [15].

Nowadays it is possible to engineer even the entire organism owing to the improvements in synthetic biology tools [16]. Host design means making changes on the host genome in order to generate improved strains via mutations, genetic recombination, and the modern DNA engineering techniques (Figure 1.1. and 1.2.). The most common way to additionally produce of novel structures is genome reduction that relies on knockouts of non-essential genes for the microorganisms. In addition, genome reduction studies not only reveal the role of these non-essential genes but also provide information about essential genes for the microorganisms. In other words, reduction and engineering of microbial genome serve as a guide for designing and improving of cell factories to determine the essential genes for sustained growth and production of metabolites [17–19].

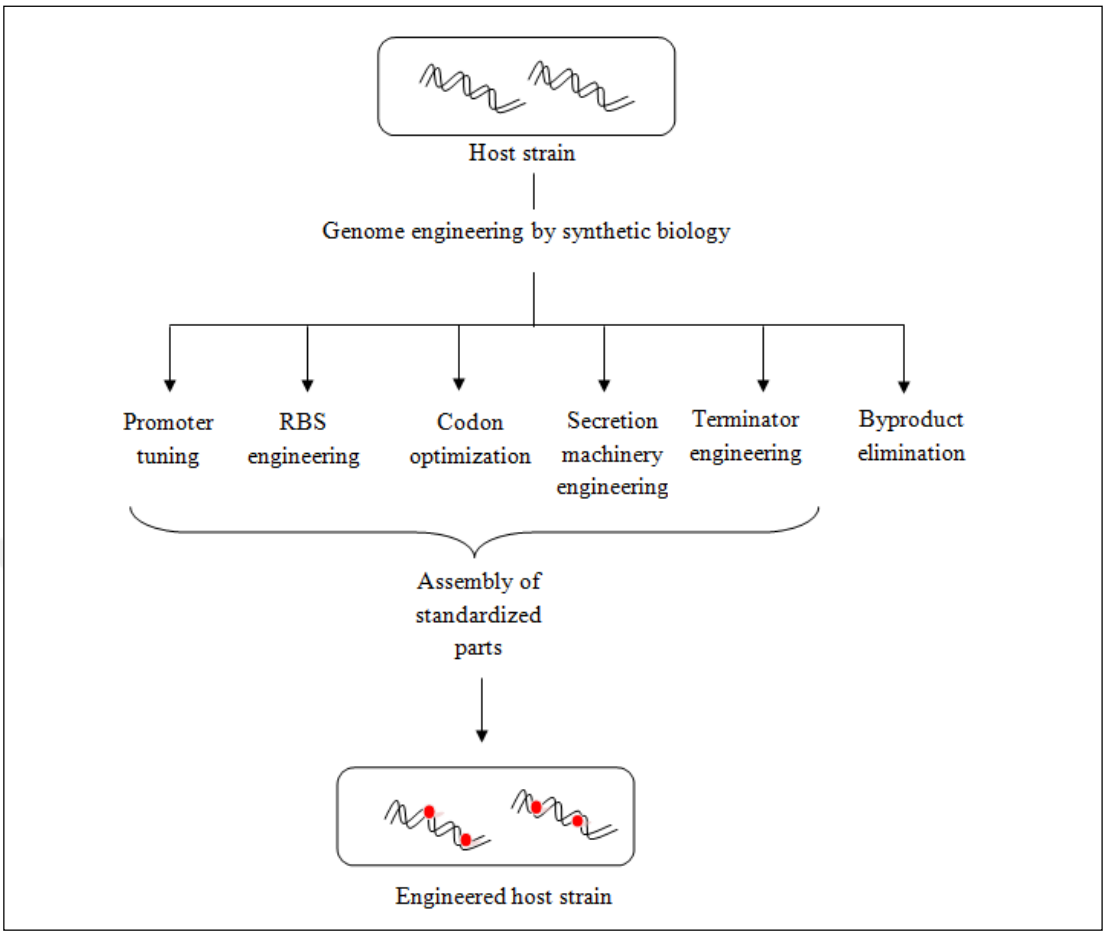

Figure 1.1. Illustration of the host design process

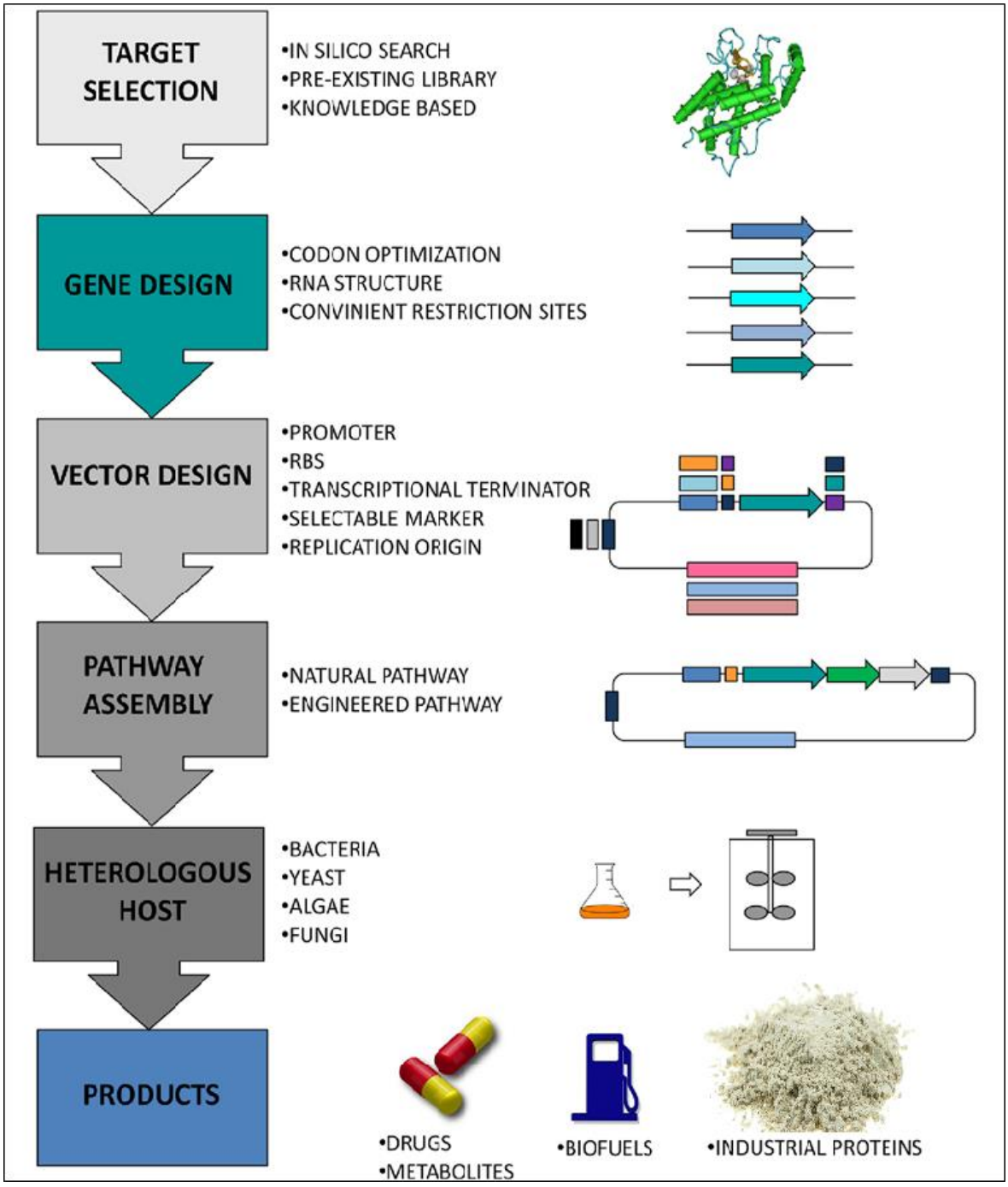

Figure 1.2. Illustration of a host design workflow using synthetic biology [20]

Genome engineering studies have demonstrated that the product of a non-essential gene can inhibit the production of an enzyme or a metabolite since it consumes energy, building blocks and the other common molecules. Therefore, better understanding of the role of each gene takes part in important metabolic pathways and eliminating of non-essential

genes are the key points of high yield production of valuable products via optimization of energy balances and adjusting the production pathway efficiency [21].

Construction of protein and RNA scaffolds for pathway engineering in synthetic cell factories, simple inactivation of chromosomal genes by the PCR-mediated gene replacement [22], multiplex genome engineering and accelerated evolution (MAGE) techniques, assembly methods such as Gibson Assembly, Golden Gate Assembly etc, and CRISPR-Cas9 system [23] are included in the scope of genome reduction strategies. Moreover, discovery and improvement of new computer-aided design tools such as CRISPOR, CHOPCHOP used for guide RNA design for CRISPR studies are important for the systematic design of metabolic production pathways. Thus, the results and effects of regulations in gene expression levels on microbial metabolism can be predicted and simulated using mathematical models, and also designing of optimal metabolic components for production of desired products and debottlenecking of pathways *in silico* can be possible [24,25]. Ultimately, developing the design processes and synthetic biology tools will help engineering of microorganisms to be much more effective.

1.2.1. Industrially Important Host Organisms

A key step in recombinant protein production is the selection of host organisms for production. Upon high level expression of heterologous proteins, toxic or inhibitory effects on the host are expected. Therefore, host selection should be considered together with the expression system selection.

Several commercially important hosts have been used in the past for heterologous protein production. Some of those are chosen because of the already available genomic portfolio and available toolboxes, some due to favorable fermentation performance, some other due to favorable secretion system and some others due to favorable energetics. The hosts that are abundantly used within industrial biotechnology context can be listed as: *Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae, Pichia pastoris, Yarrowia lipolytica* and *Aspergillus* sp.

Escherichia coli is a frequently used prokaryotic host organism for recombinant protein production through its well-known genetic structure, fast growth kinetics, and its ability to

reach high cell density on inexpensive substrates [26]. However, this growth profile may change due to metabolic burden caused by production of a recombinant protein in the cell [27].

Many different expression systems, mutant strains, cloning vectors have been developed for recombinant protein production by *E. coli*. Despite being a workhorse organism, *E. coli* is not an appropriate host for production of extracellular proteins because it keeps the proteins in periplasm and does not typically secrete out of the cell. Moreover, post-translational modifications such as folding, glycosylation, and phosphorylation etc. of proteins are as important as their extracellular secretion, especially for eukaryotic proteins. As a bacterium, *E. coli* does not have sufficient post-translational modification mechanisms, thus, recombinant proteins produced in *E. coli* cannot acquire their correct tertiary structure which leads to the formation of inclusion bodies in bacteria and even degradation of proteins by proteases [28]. Although several of these drawbacks can be overcome by using alternative strains for production, glycosylation still remains as a challenge for production in *E. coli*.

Bacillus subtilis is used as a host organism in heterologous protein production due to being GRAS, having a high secretion capacity for extracellular proteins, no significant codon bias, allowing genetic manipulations and being suitable for large-scale fermentation [29]. Many cloning vectors, expression systems, and genome engineering tools have been developed for *B. subtilis* since its complete genome sequence is known [30,31]. Secretion of proteins into the culture medium is one of the most important advantages in case of industrial applications. Most of the proteins secreted by *B. subtilis* contain N-terminal signal sequences which promote their release from the cell via an appropriate secretion mechanism. This means that production of proteins may be performed at high levels and yields by engineering of both signal peptides and secretion mechanism elements. Despite having great advantages, heterologous protein production may be affected by some limitations such as high protease production and secretion capacity of *B. subtilis* which strongly decreases the yield of recombinant protein production [32]. To overcome this challenge, many different protease-deficient strains have been developed to maintain the stability of recombinant proteins [33,34].

Saccharomyces cerevisiae is a eukayotic model organism frequently used as a host for heterologous protein production due to its well-known genetic structure, ability of

performing post-translational modifications [35], GRAS (Generally Regarded As Safe) status, ability of growing on high sugar and ethanol substrates and tolerance to broad range pH which are the key features in terms of performance of industrial fermentations [36,37]. Following the integration of knowledge about its complete genome sequence [38] and physiology, development of new strains, selection markers, promoters, expression systems, vectors, signal sequences, transformation methods and various genetic elements has been rapidly accomplished. However, low product yields for most of the heterologous proteins, metabolic burden caused by expression of foreign gene products, poor plasmid stability, and more importantly, poor secretion into the culture medium are the most crucial limitations for recombinant protein production in *S. cerevisiae*. Using strong promoters, integration plasmids and improved strains may partly provide solutions but poor secretion ability has led to the search for new organisms to be used as host for the production and efficient secretion of recombinant proteins.

Pichia pastoris is a methylotrophic yeast used especially for the production of single-cell proteins [39]. The ability to grow on simple defined media and reach high cell densities, ease of scaling-up process with high yields and capability of efficient production of both intracellular and extracellular proteins make *P. pastoris* a good candidate for production of heterologous proteins. *P. pastoris* uses methanol as a sole carbon source, therefore new expression systems have been developed for high yield production of recombinant proteins by using vectors contain methanol-inducible promoters which are strong and tightly regulated [40]. *Pichia* expression vectors can be integrated into genome, thus expression and production of desired protein can be performed successfully at higher yields. In addition, several mutant strains with different auxotrophic properties and protein deficiencies [41], signal peptides, glycosylation mechanisms [42], secretion machineries have been developed for allowing production of different recombinant proteins.

Yarrowia lipolytica is an oleaginous yeast which has been recently used for recombinant protein production due to the availability of relevant genetic tools, and its ability to utilize almost all of renewable substrates and grow at low pH values which prevents bacterial contamination. Basic genetic elements and tools such as promoters, expression systems, vectors, selection markers, signaling sequences and transformation methods have been developed to be used for metabolic engineering of *Y. lipolytica*. However, introducing foreign genes into *Y. lipolytica* via different methods such as Lithium acetate, PEG

mediated transformation still needs to be overcome. In order to decrease the time and effort, the novel DNA assembly methods such as Golden Gate Assembly [43] are mainly used, thus, recombinant protein production is feasible for industrial applications. Moreover, for targeted and markerless genome engineering studies, CRISPR-Cas9 system, is generally applied with an average success rate since making modifications on genome of oleaginous yeasts is harder than *S. cerevisiae* due to their poor homologous recombination mechanism and their tendency to non-homologous end-joining [44]. In conclusion, genetic tools for genome and metabolic engineering, heterologous and homologous synthesis pathways, strategies for expression systems and strain development still require more attention and research for *Y. lipolytica*.

Aspergillus sp. is widely used as a eukaryotic host for microbial production of industrially important proteins, especially enzymes, pharmaceuticals and organic acids. Its GRAS status and ability to grow on a large number of different substrates under several growth conditions render *Aspergillus* a popular producer for industrially important products. Additionally, its advanced oxidation, hydroxylation and demethylation machineries enable use of *Aspergillus* for bioremediation [45]. *Aspergillus* has an advanced secretion mechanism which provides secretion of a wide range of enzymes into the culture medium, therefore, production of industrial products by *Aspergillus* via both solid state and submerged fermentations is more cost effective in terms of downstream processes [46,47]. Despite being advantageous, *Aspergillus* sp. produce several types of proteases in high quantity and this proteolytic activity decreases the amount of heterologous proteins produced from *Aspergillus*. Moreover, its complex, unstable, unpredictable and inconsistent structure makes *Aspergillus* a challenging host for homologous and heterologous protein production. Additionally, its morphology directly affects the protein production efficiency. Therefore, performing genetic manipulations and generation of expression systems for heterologous protein production in *Aspergillus* still remains as a challenge and requires several optimization steps.

1.2.2. Host Design and the “Protease Problem”

Proteases (EC 3.4.X.X) are hydrolases responsible for degradation of proteins into small polypeptides and amino acids. They have high demand and significance with a wide range

of applications in textile, food, feed, detergent industries. Despite their popularity, they often can be problematic in both homologous and heterologous protein production since proteins produced are degraded after a certain period of time by proteases, resulting in decreased protein production [48,49].

In general, using strong constitutive/inducible promoters, strong signal peptides, codon optimization are the main molecular biology strategies during design of a successful heterologous protein production experiment. However, properties of the host strain have a significant impact on yield and quality of the heterologous protein. In particular, host strains with high protease activity can be challenging for this purpose. To overcome this bottleneck, decreasing or eliminating protease activity in the host strain is a common strategy [48,50,51].

Proteolytic activity of a cell can be manipulated by adjustment in fermentation conditions such as utilization of primary carbon and nitrogen sources at different concentrations, different temperatures and pH levels [52]. However, these adjustments are always temporary, need further improvement in molecular level and can be unfavorable for the fermentation conditions [48]. Therefore, various industrial hosts such as *E. coli, B. subtilis, S. cerevisiae, P. pastoris, A. oryzae, A. niger, A. fumigatus* have been engineered to reduce their protease activity [48,51,53–57].

Aspergillus sp. has a large portfolio of extracellular and intracellular proteases. Acid proteases constitute a significant portion of protease content of *Aspergillus* sp. Other types are aspartyl, serine carboxypeptidase and subtisilin type serine proteases. This portfolio limits utilization of *Aspergillus* sp. in heterologous protein production. Different strategies have been applied to decrease proteolytic activity of *Aspergillus* sp. such as UV mutagenesis, single, double or multiple disruptions of individual protease genes etc. For instance, gene replacement and UV mutagenesis techniques have been applied to *A. niger* and resulted in a protease deficient mutant [58]. In another research, *A. awamori* strain with a 20 per cent residual protease activity has been generated by disruption of the major acid protease gene, *pepA* [59]. As a result of the studies in 1997, it is reported that strains with mutations in different protease genes have revealed their role. In the study, deletion of *pepA* and *pepB* genes caused 84 per cent and 6 per cent reduction of extracellular protease activity. Additionally, deletion of *pepE* gene resulted in 32 per cent residual intracellular protease activity [48].

These solutions remained inadequate to obtain improved heterologous protein production. Punt and his colleagues pointed out that a strain generated via UV mutagenesis exhibited lower extracellular protease than the strain lacking *pepA* gene generated via gene replacement [58]. This result showed that the UV exposure caused a mutation in a regulatory gene that is responsible for expression of all protease genes. In the light of this idea, Punt *et al.* conducted a study to characterize this gene and reported that it encodes a protease regulatory gene, *prtT*, which is conserved among *Aspergillus* sp. [60]. Based on these outcomes, the disruption of *prtT* gene has been suggested as a one-stop solution to the protease problem and further can lead a decrease in degradation of heterologous proteins produced from Δ*prtT* host strains [61].

1.2.3. Regulation of Protease Production in *Aspergillus*

Experiments with deletion mutants revealed that there is a complex regulation system for protease expression in fungi [62–64]. It has been reported that proteases are repressed in the presence of primary nitrogen sources such as ammonia and amino acids, known as nitrogen catabolite repression (NCR) [65]. This repression effect is relieved once preferred nitrogen source is depleted in the medium. Following derepression, expression of protease genes is induced to start utilization of alternative nitrogen sources. NCR is regulated by a transcription factor, AreA, in *A. nidulans, A. oryzae, A. fumigatus* and similar regulation by a transcription factor is occurred in other yeasts and fungi such as *C. albicans, N. crassa, P. chrysogenum.* In the presence of a primary nitrogen source, this transcriptional activator remains in cytoplasm and cannot stimulate protease expression due to NCR. Furthermore, deletion of AreA causes strong reduction in protease expression [66].

Similar to NCR, utilization of carbon sourcess for protease production is tightly regulated by a certain transcription factor, CreA, in yeasts and fungi. Availability of a preferred carbon source has a repressing effect on expression of proteases which is known as carbon catabolite repression (CCR) [67]. In the presence of a primary carbon source such as glucose, CreA binds to a specific motif on promoter regions of protease genes and represses their expression. Once glucose is completely consumed, derepression causes release of CreA from its binding site on promoter region and protease genes are expressed. An external transcription factor, XprG, in *A. nidulans* plays a key role in this activation

response. Deletion studies for CreA showed that there is an increased protease expression in Δ*CreA* mutants [63]. On the other hand, Δ*XprG* mutants showed dramatical decrease in protease expression even in the absence of a carbon source [68]. Both CreA and XprG have homologues among yeasts and fungi [69–72].

Environmental sulfur level also regulates expression of proteases and this regulation is mediated by several transcription factors. Low levels or depletion of inorganic sulphates stimulate expression of protease genes to reach alternative sulfur sources such as cysteine and methionine released by degradation of proteins [73]. Transcription factors involved in sulfur catabolite repression have homologues in yeasts and fungi; however regulation mechanisms vary [62].

Addition of an inducer, a protease substrate in this case, in growth medium can be a signal for activation of protease genes in several species. Specifically, in *A. niger*, expression of *pepA* gene evidently increased in response to starvation in the medium supplemented with BSA, casein or elastin [74]. Conversely, no similar response has been observed in *A. nidulans* due to the lack of prtT regulatory protein. Presence of a pathway involved in transcriptional regulation, stimulation of secretion more than production, positive feedback mechanism through residues released after degradation of proteins are reasonable ideas to explain the mechanism behind such regulation through an inducer since it is yet unknown [64,75,76].

Fungi, especially *Aspergillus* sp. have a broad range of proteases such as acid, neutral and alkaline and expression of the genes encoding proteases are affected by environmental pH directly. Regulation of protease genes depending on pH is mediated through a transcription factor, PacC, along with the other transcription factors involved in signal transduction in fungi [77]. Alkali pH leads to activation of PacC and PacC activates the genes expressed under alkali conditions by binding to their promoter regions. Acidic pH inactivates PacC thus its repressive effect on the genes expressed under acidic conditions is relieved. Homolog transcription factors for pH regulation are present in yeasts. For instance, expression of alkaline proteases is induced at alkaline pH while acid proteases are inactive at that pH levels in *A. nidulans* [78]. Similar observations exist for protease expression in *A. oryzae* [79]. Interestingly, Jarai and Buxton suggested that pH is the decisive factor for regulation of protease genes rather than nutrient starvation and presence of an inducer protein. In their study, they have observed that expression of *pepA* gene in *A. niger* has

been only induced at pH 3, not at alkaline pH in which absence of repression and presence of an inducer protein [74].

Temperature is often kept constant during cultivation and production stages of fermentation. However protein production studies have shown that adjustments in growth and production temperature have a significant impact on expression of the protein. There are numerous studies reporting that different proteases from different microorganisms have higher production capacities when they are cultivated at 30°C compared to those at 45°C [80–82]. Generally, protease activity in fungi is high at low growth temperatures [83,84]. For instance, expression from the promoter of *pepA* in *A. oryzae* is regulated by temperature and it is decreased as the temperature increase from 30°C to 42°C [85]. However, low cultivation temperatures are not favorable for all species due to slow growth and decreased biomass. As an alternative, researchers have applied heat shock or temperature shift methods at the production level during a fermentation and reported increased protein production [86].

Regulation of protease production at molecular level is mediated by a transcriptional factor, prtT, in fungi. This protease-specific transcription factor binds a putative motif (5'-CCGHCGG-3') in the promoter regions of protease genes and induces the gene expression [60]. It has been reported that deletion of *prtT* gene has led to decreased proteolytic activity in various fungi [60,87,88] while overexpression of *prtT* gene has resulted in increased proteolytic activity compared to wild type strain in *A. niger* [89]. Moreover, stability of the heterologous protein produced by a Δ*prtT* mutant has been also investigated and longer storage time for the heterologous protein due to the reduced proteolytic activity in extracellular medium has been reported [61]. In conclusion, protease production can be manipulated by changes in environmental conditions and regulated by prtT protein.

1.3. CARBON CATABOLITE REPRESSION

Microorganisms utilize primary carbon sources when they are readily available with other carbon sources in the environment. Presence of a primary carbon source provides a signal for repression of the genes which are involved in consumption of secondary carbon sources. This mechanism is named carbon catabolite repression (CCR) and allows microorganisms to adapt their environment, even affects virulence and interactions

between cells [90]. Carbon catabolite repression has been studied for years in different microorganisms such as *E. coli, S. cerevisiae and A. nidulans* as model organisms for bacteria, yeasts and filamentous fungi, respectively [91–94]. The most common example of CCR occurs in *E. coli* through lac operon. In this system, presence of glucose blocks transcription of lac operon genes. Once glucose is depleted in the enviroment, lactose metabolism is turned on by transcription of beta-galactosidase gene from lac operon [95].

CCR functions through special regulatory proteins named as "catabolite responsive elements (cre)". Among these, CreA is the major transcription factor responsible for repression of the target genes during CCR in filamentous fungi. CreA basically binds a DNA motif on the upstream region (promoter) of the target gene and attenuates its expression. The mechanism behind this regulation is explained in detailed in the next section.

The transcription factor corresponding to CreA in *S. cerevisiae* is Mig1. Although they act in the same manner, both regulation of these two elements and CCR mechanisms in yeast and filamentous fungi have dissimilarities. For instance, monosaccharides apart from glucose such as xylose and arabinose can be used to regulate CCR by CreA in filamentous fungi when they are supplied to the medium in sufficient amounts while glucose regulates CCR through Mig1 in yeast [96].

1.3.1. Regulatory Components of CCR in Filamentous Fungi

CreA is the key player in CCR, however, its activity during this process is strictly regulated by other catabolite responsive elements such as CreB, CreC, CreD and Snf1.

As a transcriptional regulator, CreA, is present in almost all filamentous fungi such as *Aspergillus* spp., *Trichoderma* spp, *Fusarium* spp., *Neurospora crassa* etc. A region of 42 amino acids in this 427 amino acid long protein is highly conserved among filamentous fungi. It has been suggested that this region may have a crucial role for growth on carbon, nitrogen and lipid sources [97].

CreA requires a specific DNA motif, 5'-SYGGRG-3', for binding to the upstream region of the target gene. When glucose is available in the medium, this provides a signal for CreA to repress the genes involved in the metabolism of the other carbon sources by

binding this motif in their upstream region. Following the complete consumption of glucose, CCR is relieved to allow utilization of the other carbon sources and CreA loses the repressing effect on the target genes. This mechanism regulates metabolism of arabinan, xylan, ethanol and proline in *A. nidulans* [97].

Functionality and the cellular localization of CreA are determined by phosphorylation and ubiquitination processes (Figure 1.3.). Activation of CreA occurs in a two-step process consists of ubiquitination and deubiquitination. CreD is a regulatory protein in carbon metabolism and responsible for ubiquitination of CreA. During CCR, CreB and CreC proteins form a complex to remove ubiquitin tag from CreA to activate its transcription. Upon deubiquitination, CreA is rescued from degradation in proteasomes and represses the target genes to perform CCR.

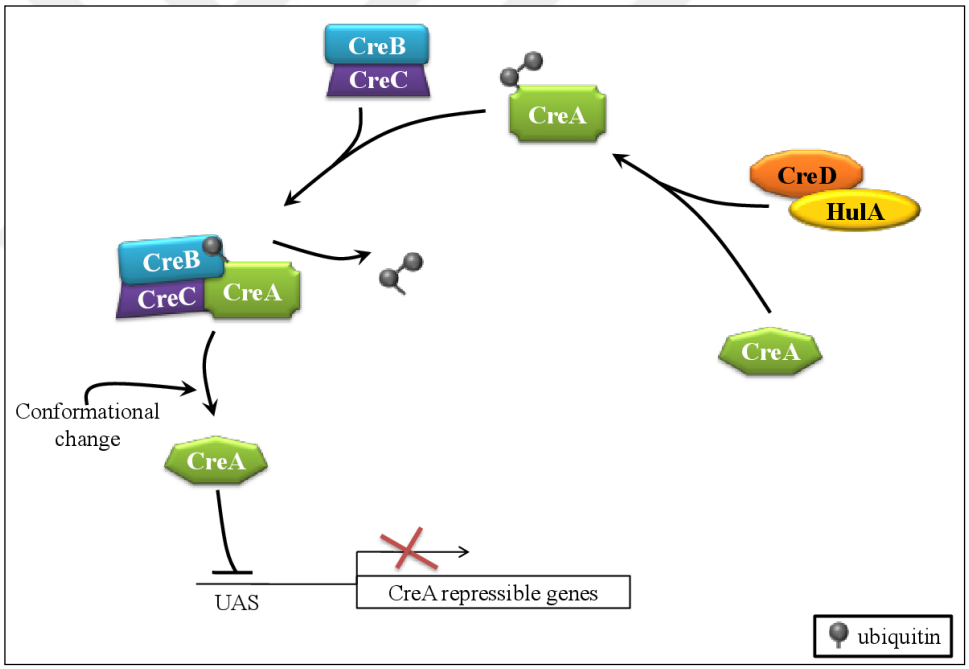

Figure 1.3. Regulation of CreA protein [98]

Localization of CreA has a crucial role in regulation of CCR. Phosphorylation is the main process that uses SnfA, a kinase to determine whether CCR will be active or not. In the presence of glucose, SnfA is an inactive protein and cannot phosphorylate CreA. Dephosphorylated CreA is located in nucleus to activate CCR. When a secondary carbon source such as cellulose is supplied, SnfA becomes active and phosphorylates CreA, thus, CreA goes into the cytoplasm and CCR is turned off.

CCR is tightly regulated by transcription factors below, thus deletion or mutations of these regulatory proteins directly affect CCR. Numerous studies around this theory are available in literature; however, CreA draws the attention most. Based on the idea that deletion of CreA (or its homologues) would directly repress CCR in filamentous fungi, plenty of researches have reported abnormal colony morphology, slow growth, inability to grow in liquid media, or even lethality in different species [99–103]. As a specific example, Ichinose *et al.* reported that deletion of *CreA* and *CreB* genes (both separately and simultaneously) caused defects in morphology and growth in broth in *A. oryzae*, but these mutants successfully formed clear zones on agar plates containing starch and glucose, indicating relieved CCR [104].

To overcome morphology and growth issues, researchers focused on CreA binding sites in promoter regions of target genes, rather than eliminating CreA from the microorganism. In the study on utilization of ethanol in *A. nidulans*, *alcA* gene encoding alcohol dehydrogenase and its regulatory protein, alcR were investigated for CreA binding sites in their promoter regions. Outcomes revealed that deletion of putative binding sites allowed expression of these genes in the presence of glucose due to the relief of CCR [105]. In another study with a different approach, two putative CreA binding site motif in the promoter of α-glucuronidase gene, *aguA*, were changed from 5'-SYGGRG-3' to 5'-SYAARG-3'. They considered changes in mRNA levels and found a significant increase in expression levels in these mutants [106]. Similar to the first example, deletion of CreA binding sites in promoter regions of β-glucosidase gene, *bgl2*, in *A. niger* and penicillin encoding genes, *pcbAB-pcbC* in *P. chrysogenum* has led to the relief of CCR and consequently increased protein expression [107,108].

1.4. INDUSTRIAL ENZYME PRODUCTION

Enzymes are the major biocatalysts for biochemical reactions since they are specific to the reaction or the substrate and are able to work at the mild temperature and pH values, consequently require less energy for functioning [109]. Specificity of enzymes also means that they do not produce byproducts and thus purification of desired product needs less effort and costs. On the other hand, enzymes may be involved as a biocatalyst in a wide range of industrial processes such as detergent, food, feed and textile processing [110].

Selection of a suitable organism is a key point in the enzyme production process. This selection depends on not only the enzyme but also the industry in which the enzyme to be used for. For instance, GRAS organisms are mostly preferred for the production of enzymes used in food or medical applications. Moreover, most of the proteases and the amylases are produced from *Bacillus* spp. which is one of the model organisms used for industrial protein production. Nowadays, enzymes produced from microbial sources are widely used for industrial purposes than animal and plant-derived enzymes [111]. Although, this popularity is due to extracellular secretion of microbial proteins (enzymes in this case) which makes purification process of enzymes more effortless to be obtained, actually, secretion of enzymes to the culture medium strongly depends on the type of microbial sources.

In addition to the homologous production of enzymes, recombinant DNA technology may help for production of desired enzyme when it is difficult to cultivate the source organism. Instead of searching a strategy to cultivate the source organism and produce the enzyme, it would be more cost-effective to transfer the corresponding gene to an easily cultured host organism. With the help of genetic engineering methods it is now possible to express a gene encoding an enzyme in a host regardless of source organism [112]. In fact, some genetic modifications lead production of desired enzyme at a higher yield than natural ones. In addition, these genetic modifications are not restricted with heterologous gene expression, strain improvement have become one of the powerful approaches to enhance the production capacity of microorganisms. Previously, strain improvement studies cover genetic manipulations and their consequences in the microorganisms, however, discovery of new technologies enhanced strain engineering studies by enabling metabolic pathway engineering, genome reduction an secretion machinery engineering [21].

1.4.1. Promoter Tuning for Heterologous Enzyme Production

Promoters are the main parts found in the upstream regions of the genes that initiate gene expression and control the expression via several regulatory proteins. Thus, selection of the promoter is a key parameter for a successful expression of a heterologous protein in the host.

Promoters function under the control of various regulatory factors such as enhancers, boundary elements (insulators), and silencers and they harbor binding sites for RNA polymerase and transcription factors. Every transcription process is controlled through regulatory factors and this control mechanism also works depending on the type of the promoter which may be inducible, constitutive and stress-dependent.

Transcription begins with the binding of RNA polymerase and the other regulatory proteins to the promoter region. Initiation of transcription occurs through different mechanisms in prokaryotes and eukaryotes. Generally, in bacteria, presence of the associated sigma factor is enough for RNA polymerase to bind the promoter region while in eukaryotes several transcription factors should be present for eukaryote-specific RNA polymerase binding to the promoter region [113].

There are three main regions inside a promoter in general: core promoter, proximal promoter, and distal promoter (Figure 1.4.). Core promoter region is the essential part of a promoter since it has RNA binding site, TATA box, and transcription start site. Engineering of these parts using traditional techniques such as mutagenesis, chimeric design, and tuning of nucleosome architecture can be applied for determination of roles of these parts in transcription [114]. Additionally, *de novo* design of optimized promoters is more utilized to increase the transcription, consequently expression of gene products.

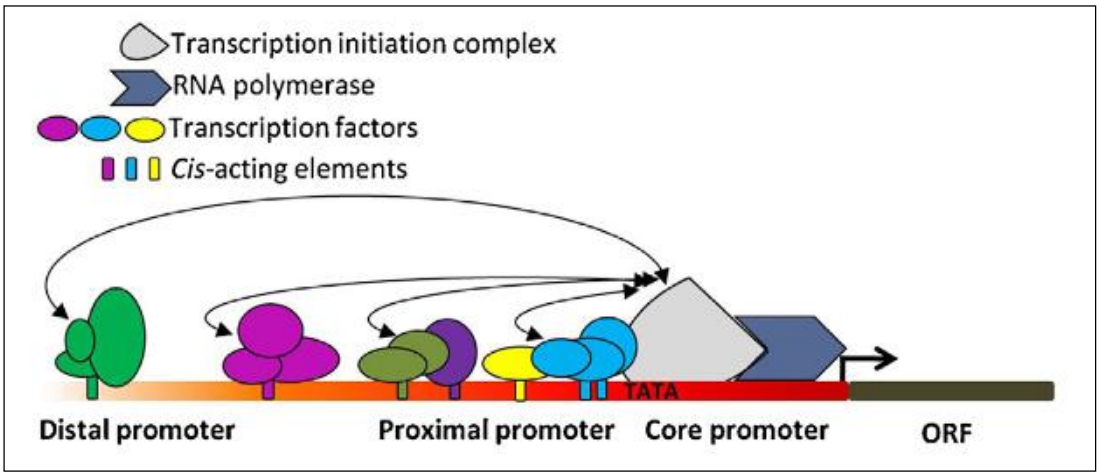

Figure 1.4. Main regions of a promoter sequence [115]

1.4.2. Glucoamylase and Gla Promoter

Glucoamylase (Gla) is one of the most important industrial enzymes generally used in food and beverage industry. Glucoamylase acts on non-reducing ends of starch or maltose and releases glucose molecules from these ends. It is produced by human, animals, plants and microorganisms.

Fungal glucoamylases are popular since they are one of the most abundant extracellular enzymes in fungi especially in *Aspergillus* species [116–121]. Popularity of Gla renders it an attractive target for molecular studies, especially for heterologous protein production. There are several researches on utilization of its promoter and signal peptide sequence for heterologous protein expression studies [122–125]. In 1990, Fowler *et al.* proposed that the region between -214 and the translational start site is the minimum promoter sequence required for the expression from Gla promoter. They also found out that the region between -562 and -318 harbors significant sequences for high level expression from the promoter [126]. In 1994, multiple copies of Gla promoter were fused with *GUS* gene from *E. coli* and decreased expression levels were obtained as the copy number increased due to the titration effect. It was also revealed that the region between -571 and start codon comprises binding sites for regulatory proteins while the region between -810 and -321 from start codon comprises binding sites for CreA [127]. Fan *et al.* supported the conclusions above by reporting that the region between -408 and -513 is required for high level expression of *GlaA* gene and the region between -809 and -513 may consist of transcription factor binding sites responsible for starch utilization [128].

Deletion studies showed that Gla promoter consists of two certain parts which are between -489 and -414; and -390 and -345. These parts both are active and their synergistic effect tightly regulates initiation of transcription and high level expression from the promoter. Transcription factor binding sites located at above positions on promoter region have a significant effect on expression of *Gla* gene and still requires further comprehensive investigation [129].

1.4.3. Carbon Catabolite Repression Effect on Gla Promoter

Glucoamylase production from Gla promoter is induced in the presence of starch in culture medium. In addition to starch, maltose and glucose act as inducers for protein expression from Gla promoter in *A. niger* [130] while the presence of glucose represses glucoamylase production in *A. oryzae* [124].

It has been widely studied and reported that xylose is a strong repressor of Gla promoter and this effect is attributed to carbon catabolite repression of xylose [126,131,132]. In the presence of xylose, CreA binds to a specific motif on Gla promoter and repress protein expression from the promoter. Repressing effect of xylose may be overcome by adding maltose or glucose to the pregrown culture on xylose to the detectable levels [126,133].

As stated in carbon catabolite repression section, deletion of putative CreA binding sites in promoter regions allowed expression from these promoters in the presence of a repressor due to the relief of CCR [105]. Therefore, it is more favorable to tune promoter region to reveal CCR effect when a repressing agent is used as the sole carbon source since CCR may inhibit the uptake of the inducer carbon source as it is added to the pregrown culture [132].

1.5. AIM OF THE STUDY

Increasing demand for microbial enzymes calls for exploiting advanced molecular biology tools within the context of industrial biotechnology. Synthetic biology is an emerging interdisciplinary branch of biology and covers engineering of not only the product, the gene or the pathway but also often the microorganisms as well (host design). Basically, it comprises design and use of standardized biological parts such as promoters, coding sequences and their standardized assembly protocols to develop novel and robust expression systems.

This study first focused on generation of a protease deficient *Aspergillus* strain to be used as a host for heterologous protein production since decreased protease activity assures long-term stabilization of the product produced. The major protease regulatory transcription factor, prtT, was targeted for this purpose. Using homologous recombination

machinery, *GFP* was integrated into the locus of *prtT* in genome via gene replacement. Secondly, it was aimed to enable utilization of alternative substrates in protein production studies via promoter engineering. Glucoamylase promoter was engineered to obtain three different promoter variants to release repressor effect of xylose on this promoter. Further, utilization of engineered promoters on heterologous protein production by protease deficient *Aspergillus* strain was investigated. Finally, co-consumption of carbon sources by strains carrying native and engineered promoters was evaluated.

2. MATERIALS

2.1. STRAINS AND PLASMIDS

A. brasiliensis ATCC16404 strain was used as the final recipient host in this study. *E. coli* DH5α strain was used for plasmid maintenance and propagation. Parts for construction of expression plasmids were taken from plasmids present in MoClo Yeast Toolkit from Addgene (Addgene kit # 1000000061).

2.2. CHEMICALS

All media components, salts and antibiotics were purchased from Sigma Aldrich. Dsoligos and primers were synthesized by SenteBioLab, Ankara. Synthetic promoters were synthesized by Twist Bioscience, USA. BsmBI (Catalogue No. R0580L) and BsaI-HFv2 (Catalogue No. R3733L) restriction enzymes and T4 DNA Ligase (Catalogue No. M0202M) were purchased from New England Biolabs (NEBs).

dNTPs were purchased from Invitrogen (Catalogue No. 10297018). Phusion DNA polymerase was purchased from NEBs (Catalogue No. M0530L). GeneRuler DNA Ladder Mix was purchased from Thermo Scientific (Catalogue No. SM0333). Machery-Nagel Nucleospin Gel and PCR Clean-up kit (Catalogue No. 740609.50) was used for DNA purification from both PCR tubes and agarose gel. Machery-Nagel Nucleospin Plasmid kit (Catalogue No. 740588.50) was used for isolation of constructs from *E. coli*.

Lysing enzymes from *Tricoderma harzanium* (Catalogue No. L1412) was purchased from Sigma Aldrich. Miracloth (Catalogue No. 475855-1R) was supplied from Calbiochem, Merck. PEG4000 (Catalogue No. 8.07490) was purchased from Merck.

2.3. EQUIPMENT

Biorad T100 Thermocycler was used for PCR studies and enzymatic incubations during plasmid construction. Biorad Gene Pulser Xcell Electroporation System was used for DNA transfer via electroporation.

Shaking incubations were performed in New Brunswick Scientific Innova Incubator Shakers. Cell growth and color development during assays were monitored using Genesys 10UV-VIS Spectrophotometer from Thermo Scientific.

Centrifuges (Eppendorf 5424), waterbaths (Grant SUB Aqua), laminar flow safety cabinet (GreenLab) were routinely used during the experiments.

3. METHOD

The overall workflow for this part of the thesis is provided below (Figure 3.1.). The subsequent subsections describes each step in detail.

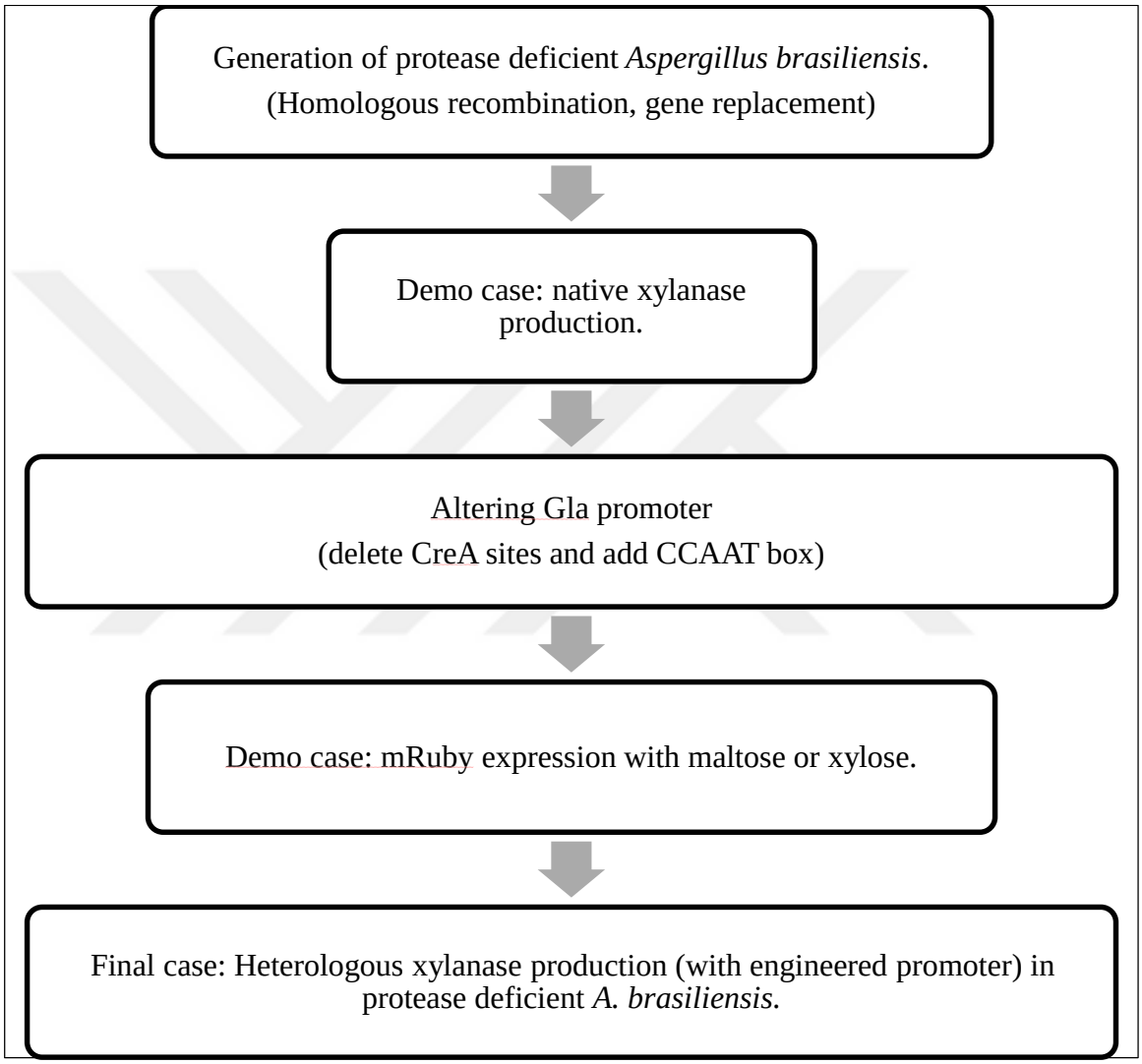

Figure 3.1. Flow chart of synthetic biology applications in *A. brasiliensis* within this thesis

3.1. GENERATION OF ΔPRTT STRAIN

3.1.1. Construction of Deletion Cassette using Overlap PCR

PrtT deletion cassette was constructed by fusion of three parts via two-step overlap PCR (OEPCR). First part consists of 1500 base upstream from the first base of the *prtT* gene, the second part is GFP expression unit (promoter-*GFP* gene-terminator) and the third part consists of 1500 base downstream from the last base of the *prtT* gene. Each part was amplified via conventional PCR. Primers used in this study are listed in Table 3.1. R1_GFP, F2_GFP, R2_GFP and F3_GFP are chimeric primers that carry part specific sequences to provide homology between parts. PCR protocol and conditions are shown in Table 3.2. and 3.3.

Table 3.1. Primers used in this study

Primer	**Sequence**	**Product**
F1	GATGTCTGCGTCAAAGGCCATA	5' upstream part
R1_GFP	ATCACGTTTCACTTTCGGTCTCCCAATTGATGGGCAGGCAATC	5' upstream part
F2_GFP	GATTGCCTGCCCATCAATTGGGAGACCGAAAGTGAAACGTGAT	GFP unit
R2_GFP	TTCCCAGCTCCATTACAACTAGTATAAACGCAGAAAGGCCCACC	GFP unit
F3_GFP	GGTGGGCCTTTCTGCGTTTATACTAGTTGTAATGGAGCTGGGAA	3' downstream part
R3	TCAGGTGCTTATTGCGATAATG	3' downstream part

Amplified parts (5' upstream, GFP and 3' downstream) were used in two-step overlap PCR. In the first step (OEPCR 1.1 and 1.2), three parts were fused using homologous sequences between them during 15 cycles. 5 µl of resulting PCR product was checked on agarose gel and 10 µl from the rest was used as template in second step OEPCR (OEPCR 2.1 and 2.2). Protocols and conditions are shown in Table 3.4.-3.6. Purified deletion cassette was transformed into *A. brasiliensis* via PEG mediated transformation.

Table 3.2. Conventional PCR protocol for amplification of the parts

Reagent	**5' upstream**	**GFP**	**3' downstream**
Template	150 ng (*A. brasiliensis* gDNA)	50 ng (GFP plasmid)	150 ng (*A. brasiliensis* gDNA)
5X HF buffer	4 µl	4 µl	4ul
$MgCl_2$ (50 mM)	0	0	0.4 µl
dNTP (10 mM)	0.4 µl	0.4 µl	0.4 µl
Primer 1 (10 uM)	1 µl (F1)	1 µl (F2_GFP)	1 µl (F3_GFP)
Primer 2 (10 uM)	1 µl (R1_GFP)	1 µl (R2_GFP)	1 µl (R3)
Phusion DNA polymerase (100U/ul)	0.2 µl	0.2 µl	0.2 µl
Nuclease free water	10.4 µl	12.9 µl	10 µl
Total	20 µl	20 µl	20 µl

Table 3.3. Thermocycling conditions for conventional PCR

Step		**Temperature**	**Time**
Initial denaturation		98°C	3 min
Denaturation Annealing Elongation	31 cycles	98°C	10 sec
		55°C	30 sec
		72°C	1 min
Final extension		72°C	10 min
Hold		4°C	∞

Table 3.4. First step of overlap PCR protocol for construction of deletion cassette

Reagent	**OEPCR 1.1 & 1.2**
5' upstream	3.16 µl (23 ng/ul)
GFP	1.85 µl (27 ng/ul)
3' downstream	5.35 µl (14 ng/ul)
5X HF buffer	5 µl
$MgCl_2$ (50 mM)	0
dNTP (10 mM)	0.5 µl
Phusion DNA polymerase (100U/ul)	0.25 µl
Nuclease free water	8.64 µl
Total	25 µl

Table 3.5. Second step of overlap PCR protocol for construction of deletion cassette

Reagent	**OEPCR 2**
Template	10 µl from OEPCR 1.1 & 1.2
5X HF buffer	10 µl
$MgCl_2$ (50 mM)	1 µl
dNTP (10 mM)	1 µl
F1 (10 µM)	2.5 µl
R3 (10 µM)	2.5 µl
Phusion DNA polymerase (100U/µl)	0.5 µl
Nuclease free water	22.5 µl
Total	50 µl

Table 3.6. Thermocycling conditions for two-step overlap PCR

	OEPCR 1.1		**OEPCR 1.2**			**OEPCR 2.1&2.2**		
Step	**Temp.**	**Time**	**Temp.**	**Time**		**Temp.**	**Time**	
Initial denaturation	98°C	3 min	98°C	3 min		98°C	3 min	
Denaturation	98°C	10 sec	98°C	10 sec	15 cycles	98°C	10 sec	30 cycles
Annealing	72°C	30 sec	55°C	30 sec		55°C	30 sec	
Elongation	72°C	3 min	72°C	3 min		72°C	1 min	
Final extension	72°C	10 min	72°C	10 min		72°C	10 min	
Hold	4°C	∞	4°C	∞		4°C	∞	

3.1.2. Skim Milk Plate Assay for Qualitative Protease Activity

Wild type and mutant cells were grown in complete medium (CM) containing 20 g/L glucose, 10 g/L peptone, 1 g/L KH_2PO_4 and 3 g/L yeast extract, pH 4.0 for protease production. Skim milk agar (SMA) plates containing 5 per cent skim milk powder, 1.5 per cent agar and 0.25 per cent (v/v) triton X-100 were used for qualitative analysis of protease activity.

Resulting cells were grown at 30°C, 150 rpm for 96 hours for protease production. Samples were taken every 24 h, separated from conidia via centrifugation and used for quantitative protease assay. 20 µl of supernatant was spotted on SMA plates and incubated at 37°C for five days.

3.1.3. Quantitative Protease Activity

Extracellular protease activity was measured as described by Kamaruddin *et al.* with a slight modification [61]. 250 µl of culture supernatant was mixed with 67 µl of three per cent bovine serum albumin in sodium acetate (pH 4.0) and incubated at 37°C for 30 min. Reaction was stopped by adding 500 µl of 10 per cent trichloroacetic acid. Following the incubation at 4°C for 30 min, tubes were centrifuged at 10000 g for 10 min. 250 µl of supernatant was mixed with 625 µl of 0.5 M $NaCO_3$ and 125 µl Folin and Ciocalteu's reagent (1:3 diluted). This solution was incubated at 37°C for 30 min and color development was measured at 660 nm. Blank samples were treated same as the test samples but they were boiled at 100°C for 15 min prior to addition of the substrate. One unit of protease activity is defined as the amount of enzyme required for release of 1 µmol of L-tyrosine per minute under the reaction conditions [134].

3.1.4. Native Xylanase Activity in Wild Type and Protease Deficient Strain

Wild type and Δ*prtT* strains were grown in CM medium at 30°C, 150 rpm, overnight. 1 ml of grown spores were used for inoculation of 100 ml production medium containing 1 g/L xylan as a sole carbon source, 0.05 g/L $MgSO_4$, 0.005 g/L $CaCl_2$, 0.005 g/L $NaNO_3$, 0.009 g/L $FeSO_4$, 0.002 g/L $ZnSO_4$, 0.012 g/L $MnSO_4$, 0.23 g/L KCl, 0.23 g/L KH_2PO_4 and 7

g/L peptone [135]. Xylanase production was inspected for 7 days and extracellular broth was used for xylanase activity measurement.

3.1.5. Xylanase Activity Assay

500 µl of enzyme sample was mixed with 500 µl of 0.6 per cent xylan from beechwood in 50 mM sodium acetate buffer pH 5.0. Mixture was incubated at 50°C for 5 minutes. Reaction was stopped by adding 1 ml of DNS solution into tubes and incubation at 99°C for 5 minutes. After incubation the final volume was brougt to 5 ml by adding distilled water. Tubes were incubated at room temperature for 30 minutes and absorbance was measured at 540 nm. Blank solutions were treated same as the enzyme sample but enzyme solution was added to the reaction after DNS solution. Xylose at various concentrations was used as standard and one unit of enzyme activity is defined as the amount of enzyme required for release of 1 µmol of xylose per minute under reaction conditions [136].

3.2. DESIGN OF GLA PROMOTER VARIANTS

Native Gla promoter was engineered to obtain three different variants (Figure 3.2.). PGlaV1 was obtained by deleting two out of four CreA binding motifs which are located at -95 and -282 from translation start site on native Gla promoter. This type is expected to be less affected than native Gla promoter by CCR. PGlaV2 was designed by deleting all putative CreA binding motifs which are located -95, -282, -449 and -682 from translation start site on native Gla promoter. CCR is expected to have minimum effect on this variant. PGlaV3 was derived from PGlaV2 and generated to contain three more copies of CCAAT motif right next to the original location of CCAAT box. PGlaV3 is expected to have increased mRNA levels compared to PGlaV2. Promoter variants was designed as Golden Gate (GG) compatible parts and sent for *de novo* synthesis.

TGCCATTGGCGGAGGACTCCGGATGGTCAGGGACCTACTAGCCTTATGAAATTCATGATGCATGTGTTTGGCCTCGGCAAATAT
ATACTATAGGATAATGTTAGTGACTAGTAGCAATATTAATGAAGAATATATGTGTGACCACGCGTCGGACCTGCATTATACGGA
GTAGTTTCCCGTTAGTCATATTTGCCGCCGTTATAGGGGTCGTCATAGCAGCCAATCAAGCCACCACGCACGACCCGGGGACGGC
GAATCCCCGGTAATTGAATAAAATTGCATCCCCGGCCAGTGAGGCCAGCGATTGGACACCTCTGCCAGGTCCAGGACCATTCTG
CAGCGATGGTTGATTAATTGCAATTTCCCCCGGGCCGGCCCGACACCGCGATAGGCTGGTTTTTCCACACCACCGGAGATTCGT
CGCCTGGTGCCTCGTCCGTTCACCAGCTGAAGTGGCGAGATGTCTCTGCAGGAATTCAAGCTAGATGCTAAGCGACGTACGGA
GTAGCATTGCAATCTATCTCGATGCATGTGCTTCATCCCTCTTGCATACCCCTCGTGCAGATGTGGTTTGGTTATAAATTGAAGT
GGTTGGTCCGGGGGTCCGTGAGGGGTTGAATTGTTTTCCTCCTCATCTCAGACGCGACTGCAGTGATTTGAGCTTCATCCCCAG
CATCGTTCATCTTCAGCA

Figure 3.2. Gla promoter sequence. CreA binding motifs on the promoter were highlighted as yellow and CCAAT box is highlighted as green

3.3. CONSTRUCTION OF FUNGAL EXPRESSION PLASMIDS WITH GLA PROMOTER VARIANTS

3.3.1. Cloning of Native Gla and AMA1 Parts into Entry Vector

Native Gla promoter (NPgla, ~700 bp) and fungal origin of replication part (AMA1, ~3000 bp) were amplified to be assembled via GG method. Therefore, a PCR was set up with genomic DNA (gDNA) of *A. brasiliensis*, AMA1 plasmid and specific chimeric primers. These primers consist of two parts: one part forms compatible regions for GG assembly (highlighted as green) and second part contains gene specific sequences (highlighted as yellow) (Table 3.7.).

Table 3.7. Chimeric primers used to amplify NPgla and AMA1 sequences

Primer name	**Primer Sequence**
NPgla_GGF	GCATCGTCTCATCGGTCTCAAACGTGATGCCATTGGCGGAGGACT
NPgla_GGR	ATGCCGTCTCAGGTCTCACATATGCTGAAGATGAACGATGCTGGG
AMA1_GGF	GCATCGTCTCATCGGTCTCAGAGTTACTGTTTTGTGATAGCACG
AMA1_GGR	ATGCCGTCTCAGGTCTCATCGGTACTGTTTTGTGATAGCACG

A gradient PCR was set up using 60 ng DNA template, 1X Taq Phusion High Fidelity Buffer, 0.2 mM dNTPs, 0.5 uM primers and 0.5 units of Phusion Polymerase. Reaction volume was brought up to 25 µl using nuclease free water. Reaction was started with an

initial denaturation at 98°C for 3 min and followed by 31 cycles of denaturation at 98°C for 10 sec, annealing at 65-55°C for 30 sec and elongation at 72°C for 4 min. A final elongation step was carried out at 72°C for 10 min. A small amount of products were used for gel electrophoresis and the rest were cleaned up from the tubes.

NPgla and AMA1 products were cloned into GG entry vector, pYTK001, via compatible regions to be used in further assembly reactions. Reagents and conditions are shown in Table 3.8.

Table 3.8. GG assembly reaction using BsmBI restriction enzyme and T4 DNA ligase

Reagent	**Amount**	**Temperature**	**Time**	
NPgla/AMA1 product	10 ng/40 ng	42°C	90 sec	25 cycles
pYTK001 plasmid	200 ng	16°C	3 min	
T4 DNA ligase buffer	1X	50°C	5 min	
T4 DNA ligase	1 µl	80°C	10 min	
BsmBI	1 µl	4°C	∞	
Nuclease free water	up to 20 µl			

5 µl of the reaction were mixed with 50 µl of *E. coli* DH5α competent cells. Mixture was incubated on ice for 30 min. Then, heat shock was applied by incubating at 42°C for 50 sec and then on ice for 2 min. 945 µl of SOC medium was added to the tubes and cells were grown at 37°C, 150 rpm for 90 min. Following the incubation, cells were spread on LB+chloramphenicol (cam) plates and placed in an incubator overnight.

Several white colonies from LB+cam plates were transferred into 5 ml LB+cam medium and grown overnight. Cultures were used for plasmid isolation and the plasmids were named as pTYK001+NPgla and pYTK001+AMA1 (Figure 3.3.).

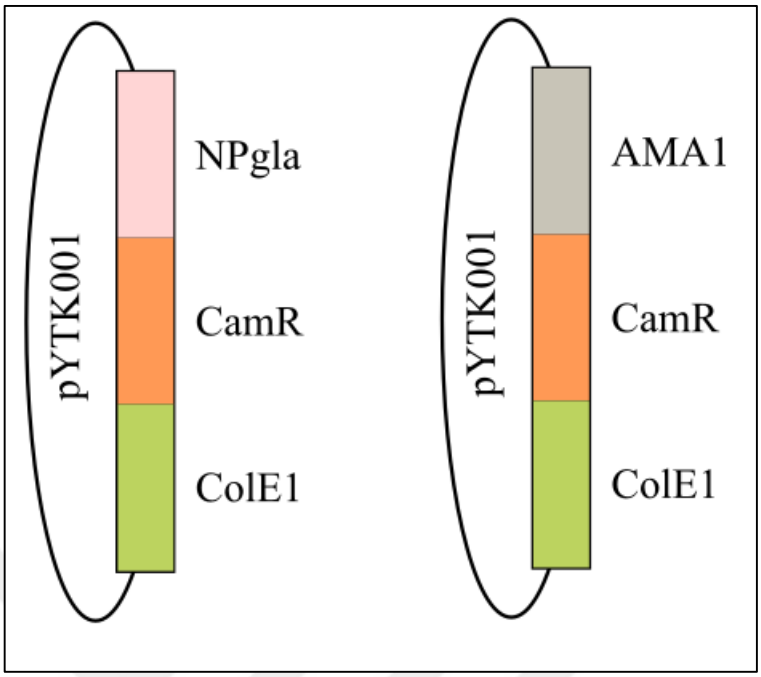

Figure 3.3. pYTK001+NPgla and pYTK001+AMA1 plasmid maps

3.3.2. Cloning of Gla Promoter Variants into Entry Vector

Gla promoter variants were amplified using synthetic DNA products as template with the primers in Table 3.9. These primers consist of two parts: one part forms compatible regions for GG assembly (highlighted as green) and second part contains gene specific sequences (highlighted as yellow). PGlaV1 was amplified using Less_ccr_F and Less_ccr_R, PGlaV2 was amplified using No_ccr_F and Less_ccr_R, PGlaV3 was amplified using No_ccr_F and Less_ccr_R.

Table 3.9. Primers used to amplify Gla promoter variants

Primer name	**Primer Sequence**
Less_ccr_F	GCATCGTCTCATCGGTCTCAAACGTGCCATTGGCGGAGGACTCCG
Less_ccr_R	ATGCCGTCTCAGGTCTCACATATGCTGAAGATGAACGATGCTG
No_ccr_F	GCATCGTCTCATCGGTCTCAAACGTGCCATTGGACTCCGGATGGT

A PCR was set up using 50 ng template DNA, 1X Taq Phusion High Fidelity Buffer, 0.2 mM dNTPs, 0.5 uM primers and 0.5 units of Phusion Polymerase. Reaction volume was brought up to 25 µl using nuclease free water. Reaction was started with an initial denaturation at 98°C for 3 min and followed by 31 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec and elongation at 72°C for 1 min. A final elongation step was carried out at 72°C for 10 min. Products were run on agarose gel and extracted from gel.

Purified promoter parts were separately cloned into GG entry vector, pYTK001, via compatible regions to be used in further assembly reactions. Reagents and conditions are shown in Table 3.10.

Table 3.10. GG assembly reaction using BsmBI restriction enzyme and T4 DNA ligase

Reagent	**Amount**	**Temperature**	**Time**	
PGlaV1/PGlaV2/PGlaV3	3.3 µl (50 ng)	42°C	90 sec	25 cycles
pYTK001 plasmid	1 µl (200 ng)	16°C	3 min	
T4 DNA ligase buffer	2 µl (1X)	50°C	5 min	
T4 ligase	1 µl	80°C	10 min	
BsmBI	1 µl	4°C	∞	
Nuclease free water	up to 20 µl			

50 µl of *E. coli* DH5α competent cells were transformed with 5 µl of GG reaction by heat shock. Transformants were grown at 37°C, 150 rpm for 90 min. Following the incubation, cells were spread on LB+cam plates and placed in an incubator overnight.

Several white colonies from LB+cam plates were transferred into 5 ml LB+cam medium and grown overnight. Cultures were used for plasmid isolation and the plasmids were named as pTYK001+PGlaV1, pTYK001+PGlaV2 and pTYK001+PGlaV3.

3.3.3. Construction of Fungal Backbone (FBB) Plasmid

Construction of FBB plasmid requires assembly of six parts into a backbone plasmid (pYTK084). These parts contain connectors from pYTK008 and 073, GFP expression cassette from pYTK047, hygromycin resistance gene cassette from pYTK079, fungal replication origin (AMA1) from pYTK001+AMA1 and bacterial replication origin-selection marker part from pYTK084. Once the resulting construct is formed, it can be used as a fungal expression plasmid backbone since it provides fungal replication origin, AMA1, and fungal antibiotic selection marker, hygromycin. In this construct, GFP enables quick scanning of correct clones in further studies since GFP will be replaced by gene of interest when it is successfully assembled into backbone. Reaction conditions and reagents are shown in Table 3.11.

Table 3.11. GG assembly for FBB plasmid construction

Reagent	**Amount**	**Temperature**	**Time**
pYTK008	100 ng	37°C	3 hours
pYTK047	100 ng	25°C	1 hour
pYTK073	100 ng	65°C	20 min
pYTK079	100 ng		
pYTK001+AMA1	100 ng		
pYTK084	100 ng		
T4 DNA ligase buffer	1X		
T4 ligase	1 µl		
BsaI	1 µl		
Nuclease free water	up to 10 µl		

5 µl of reaction was transformed into 50 µl of competent *E. coli* DH5α cells via heat shock. Following 90 min incubation cells were spread on LB+kan plates and grown overnight at 37°C. Since pYTK084 plasmid harbors red fluorescence gene, it allows green/red selection after this assembly reaction. Successfully constructed plasmids led to green colonies due to the GFP while undigested pYTK084 plasmids led to red colonies.

Couple of green colonies were grown in LB+kan liquid media overnight and used for plasmid isolation to obtain FBB plasmid (Figure 3.4.).

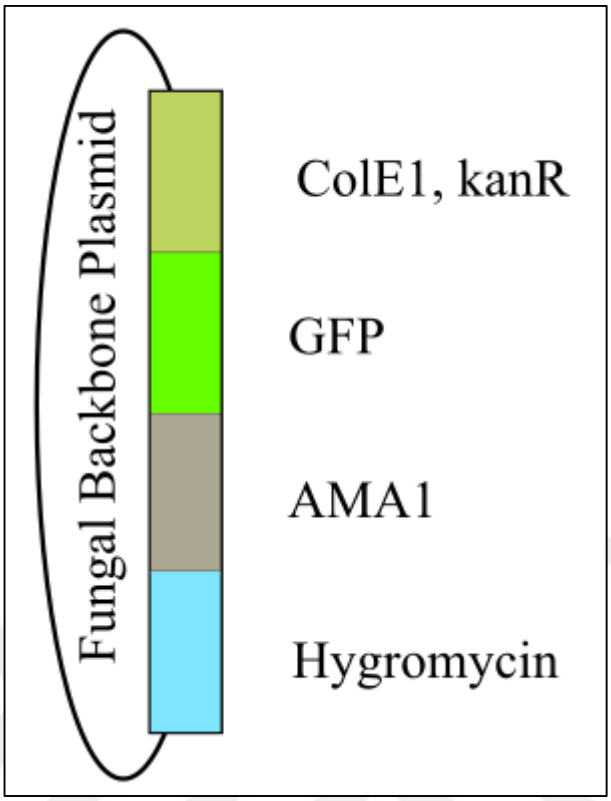

Figure 3.4. Fungal backbone (FBB) plasmid map

3.3.4. Assembly of PGlaVx, mRuby and Terminator Parts into FBB

NPgla, PGlaV1, PGlaV2 and PGlaV3 promoters from pYTK001+NPgla, pTYK001+PGlaV1, pTYK001+PGlaV2 and pTYK001+PGlaV3 respectively, mRuby gene from pYTK034 and TPGK1 terminator from pYTK054 plasmids were cloned into FBB to express mRuby under native and engineered Gla promoters in fungi. Reaction conditions and reagents are shown in Table 3.12.

Table 3.12. GG assembly for mRuby expressing FBB plasmid construction

Reagent	**Amount**	**Temperature**	**Time**
pYTK001+NPgla	100 ng	37°C	2 hours
pYTK034	100 ng	25°C	1 hour
pYTK054	100 ng	65°C	20 min
FBB	100 ng		
T4 DNA Ligase Buffer	1X		
T4 Ligase	1 µl		
BsaI	1 µl		
Nuclease free water	up to 20 µl		

5 µl of reaction was transformed into 50 µl of competent *E. coli* DH5α cells via heat shock. Cells were incubated at 37°C and 150 rpm for 90 min and, then spread on LB+kan plates and grown overnight at 37°C. Since FBB plasmid harbors GFP, it allows green/white selection after this assembly reaction. Successfully constructed plasmids led to white colonies while undigested FBB plasmids led to green colonies.

Couple of white colonies was grown in LB+kan liquid media overnight and used for plasmid isolation to obtain NPgla-mRuby-FBB, PGlaV1-mRuby-FBB, PGlaV2-mRuby-FBB and PGlaV3-mRuby-FBB plasmids (Figure 3.5.). Purified plasmids were sent to sequencing with primers listed in Table 3.13.

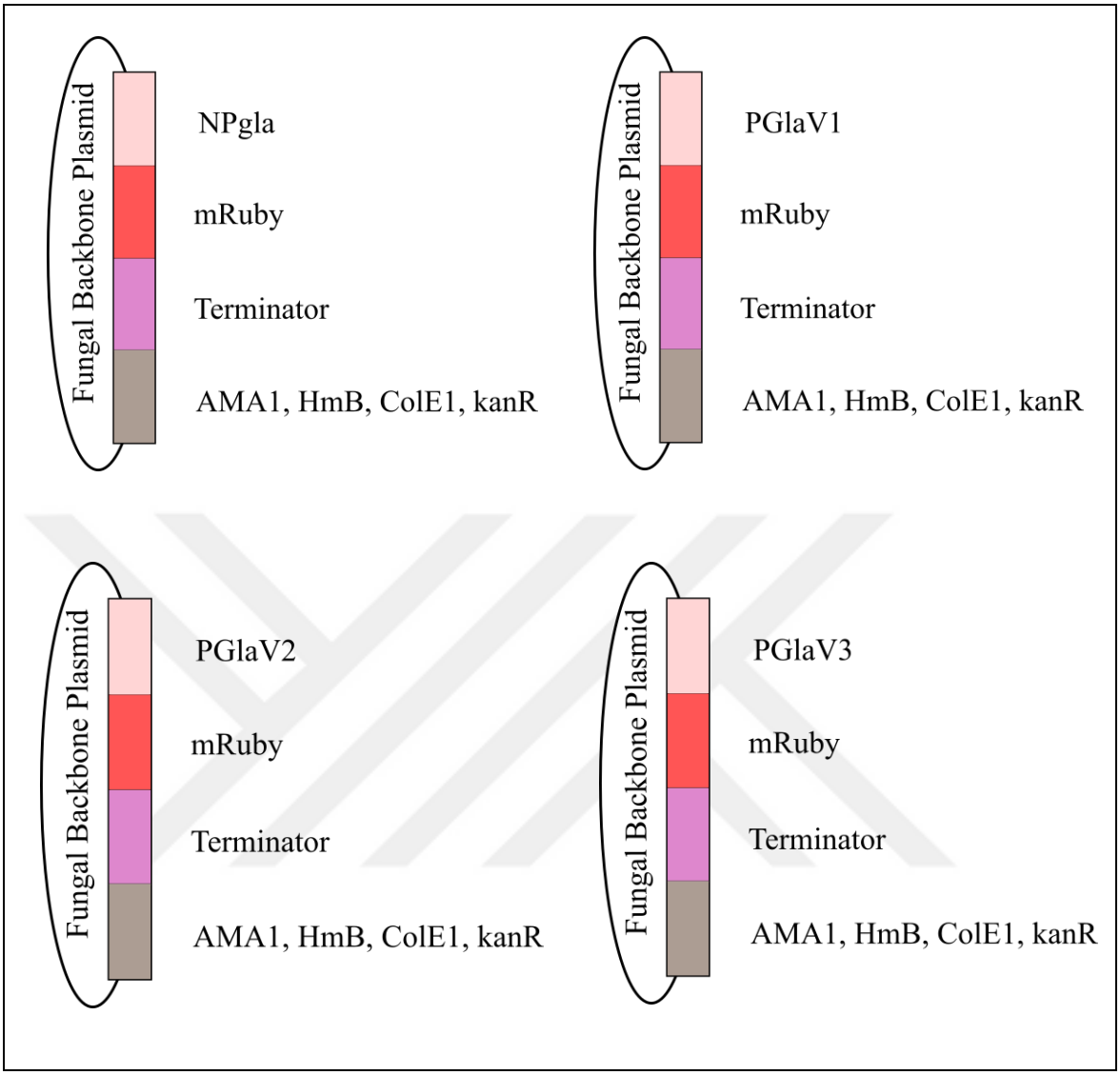

Figure 3.5. NPgla FBB, PGlaV1 FBB, PGlaV2 FBB and PGlaV3 FBB plasmid maps

Table 3.13. Primers used for sequencing of FBB and NPgla-mRuby-FBB plasmids

Primer Name	**Plasmid Name**	**Sequence to be obtained**
sgRNA_ColE1	FBB	GFP
YBB_GFP	FBB	Hygromycin cassette
YBB_TTEF	FBB	AMA1
NPgla_F	NPgla-mRuby-FBB	Native Gla promoter
NPgla_R	NPgla-mRuby-FBB	Native Gla promoter
Cas_TPGK	NPgla-mRuby-FBB	Hygromycin cassette

3.3.5. Construction of Expression Plasmids with Xylanase Gene under Selected Promoter Variants

Xylanase gene was amplified using *Bacillus subtilis* genomic DNA as the template. Primers used in this reaction were shown in Table 3.14. These primers consist of two parts: one part forms compatible regions for GG assembly (highlighted as green) and the second part contains gene specific sequences (highlighted as yellow).

Table 3.14. Chimeric primers used to amplify xylanase gene

Primer name	**Primer Sequence**
Xyn_GG_F	GCATCGTCTCATCGGTCTCATATGATGTTTAAGTTTAAAAAG
Xyn _GG_R	ATGCCGTCTCAGGTCTCAGGATTTACCACACTGTTACGTT

A PCR was set up using 100 ng DNA template, 1X Taq Phusion High Fidelity Buffer, 0.2 mM dNTPs, 0.5 uM primers and 0.5 units of Phusion Polymerase. Reaction volume was brought up to 25 µl using nuclease free water. Reaction was started with an initial denaturation at 98°C for 3 min and followed by 31 cycles of denaturation at 98°C for 10 sec, annealing at 50°C for 30 sec and elongation at 72°C for 4 min. A final elongation step was carried out at 72°C for 10 min. A small amount of products were used for gel electrophoresis and the rest were cleaned up from the tubes.

Resulting PCR product was cloned into GG entry vector, pYTK001, via compatible regions to be used in further assembly reactions. Reagents and conditions are shown in Table 3.8. 5 µl of the reaction were mixed with 50 µl of *E. coli* DH5α competent cells. Heat shock method was used for transformation. 945 µl of SOC medium was added to the transformation tubes and cells were grown at 37°C, 150 rpm for 90 min. Following the incubation, cells were spread on LB+cam plates and placed in an incubator overnight.

Several white colonies from LB+cam plates were transferred into 5 ml LB+cam medium and grown overnight. Plasmids were isolated from the cells from this culture and named as pTYK001+xyn.

In order to express xylanase gene in a protease deficient strain, two different promoter variants were chosen. NPgla and PGlaV2 promoters were used as promoters to drive xylanase expression in two separate expression plasmids. Accordingly, 100 ng of pTYK001+xyn plasmid, 100 ng of pTYK001+NPgla or pTYK001+PGlaV2 plasmids, 100 ng of pYTK054, and 100 ng of FBB plasmid were mixed and a GG reaction with conditions given in Table 3.12. was set.

5 µl of reaction was transformed into 50 µl of competent *E. coli* DH5α cells via heat shock. Following 90 min incubation cells were spread on LB+kan plates and grown overnight at 37°C. Since FBB plasmid harbors GFP, it allows green/white selection after this assembly reaction. Successfully constructed plasmids led to white colonies while undigested FBB plasmids led to green colonies.

Couple of white colonies were grown in LB+kan liquid media overnight and used for plasmid isolation to obtain NPgla-xyn-FBB and PGlaV2-xyn-FBB plasmids.

3.4. TRANSFORMATION OF ASPERGILLUS BRASILIENSIS

3.4.1. PEG-mediated Transformation of *A. brasiliensis* Protoplasts

Expression plasmids were transferred into *A. brasiliensis* using PEG-mediated protoplast transformation. PEG-mediated transformation was performed following to Arentshort *et al.* [137].

Mycelia grown on PDA plates were harvested, transferred into MPD (malt extract-peptone-dextrose) medium and incubated overnight at 30°C, 150 rpm. 0.5 g wet weight was transferred into 10 ml SMC solution containing 1.33 M sorbitol, 50 mM $CaCl_2.2H_2O$, 20 mM MES, pH 5.6 and 200 mg of lysing enzymes from *Trichoderma harzanium*. Protoplasting solution was incubated horizontally at 37°C, 75 rpm for ~4 hours. Protoplast formation was checked every 30 min under microscope to observe weakening of the cell wall. At the end of 4-hour incubation, protoplasts were collected through a sterile miracloth, centrifuge at 2000 g for 10 min. Supernatant was discarded and pellet was gently dissolved in 1 ml STC solution containing 1.33 M sorbitol, 50 mM $CaCl_2.2H_2O$ and 10 mM Tris-HCl, pH 7.5. Another centrifugation step was carried out at 3000 g for 5 min,

supernatant was discarded and pellet was dissolved in 1 ml STC solution. This step was repeated and final pellet was dissolved in 1 ml STC.

100 µl of protoplast and ~1 ug of plasmids were mixed in a 50 ml falcon tube and incubated at room temperature for 20 min. Following incubation, 250-250-850 µl of freshly prepared 60 per cent PEG4000 solution were added sequentially. Tubes were incubated at room temperature for 20 min. After incubation, 5 ml of STC were added and tubes were centrifuged at 3000 g for 5 min. Supernatant was discarded, 2 ml of RCM containing 2 g/L yeast extract, 2 g/L peptone, 20 g/L glucose, 1M sorbitol were added and protoplasts were incubated overnight at 30°C, 150 rpm. Next day, grown spores were spread on MMS+hygromycin plates until colonies appeared. MMS medium contain 325.2 g/L sucrose, 12 g/L agar, 1X ASPA+N, 2 ml/L 1 M $MgSO_4$ and 1 ml/L 1000x trace element solution. ASPA+N is prepared as 50x and contains 297.5 g/L $NaNO_3$, 26.1 g/L KCl, 74.8 g/L KH_2PO_4, pH 5.5. 1000x trace element solution contains 10 g/L EDTA, 4.4 g/L $ZnSO_4.7H_2O$, 1.01 g/L $MnCl_2.4H_2O$, 0.32 g/L $CoCl_2.6H_2O$, 0.315 g/L $CuSO_4.5H_2O$, 0.22 g/L $(NH_4)_6Mo_7O_{24}.4H_2O$, 1.11 g/L $CaCl_2$ and 1.0 g/L $FeSO_4.7H_2O$, pH 4.0.

3.4.2. Electroporation of *A. brasiliensis* Protoplasts

A plate of *A. brasiliensis* cells were transferred into 100 ml MPD (malt extract-peptone-dextrose) medium. Cells were incubated at 30°C, 150 rpm overnight. Next day, cells were harvested through sterile miracloth. 0.5 mg wet weight was transferred into 10 ml SMC solution containing 200 mg lysing enzymes. Protoplasting solution was incubated at 37°C, 70 rpm for 3hours, horizontally. After incubation 10 ml of ice cold HEPES-mannitol buffer (1 mM HEPES, 50 mM mannitol, pH 7.0) was added into solution. Protoplasts were harvested into 50 ml falcon tube through sterile miracloth. Filtrate was centrifuge at 4000 rpm for 10 min. Pellet was washed two times with 20 ml of ice cold HEPES-mannitol buffer. After second wash, protoplasts were suspended in 1 ml of ice cold electroporation buffer (10 mM Tris-HCl pH 7.5, 270 mM sucrose, 1 mM lithium acetate).

150 µl of protoplast solution was mixed with 20-50 µl plasmid. Protoplast-plasmid mixture was incubated on ice for 30 min and then transferred into ice cold 0.2 cm cuvettes. Electroporation was performed under the condition of 1500 V, 25 uF, 400 Ω for approximately 6 ms. After electroporation 1 ml of RCM was added into cuvettes and this

solution was transferred into 15 ml falcon tubes. On the other hand, 150 µl of protoplast solution was mixed with 1 ml of RCM to check protoplast viability. Tubes were incubated on ice for 15 min and then placed in a shaker at 30°C for overnight incubation. Next day, grown spores were spread on MMS+hygromycin plates until colonies appeared.

3.5. TEST OF PROMOTER VARIANTS FOR MRUBY EXPRESSION IN DIFFERENT CARBON SOURCES

Expression plasmids harbor native and engineered Gla promoters and mRuby reporter gene were transferred into *A. brasiliensis*. Transformants were transferred into 50 ml of a chemical medium contains maltose or xylose as a sole carbon source. Chemical medium was prepared according to Verduyn *et al.* and comprised carbon source 20 g/L, $(NH_4)_2SO_4$ 5 g/L, KH_2PO_4 3 g/L, $MgSO_4.7H_2O$ 0.5 g/L, 1000x vitamin solution 1 ml/L and 100x trace elements 10 ml/L, pH 5.0 [138].

Growth was inspected for 3 days and samples were analyzed for protein and fluorescence measurements. At the end of the 3rd day, cells were harvested, separated from culture broth and ground into a fine powder using liquid nitrogen. Cell lysates were diluted using distilled water and centrifuged. Supernatants and pellets were used for fluorescence measurements for mRuby.

3.5.1. Total Protein Determination

Bradford was used for protein concentration analysis from extracellular broth and supernatants of disrupted cell lysates. According to the instructions, 5 ul of sample was mixed with 250 ul of Bradford reagent. Mixture was incubated at room temperature at least 5 minutes and blue color development was measured at 595 nm in a microplate reader.

3.5.2. Fluorescence Measurement for mRuby

Samples from extracellular broth, supernatants and pellets of cell lysates were used for detection of mRuby expression from recombinant cells. Fluorescent signal was measured using a microplate reader at the excitation/emission wavelengths of 559/600 nm.

3.6. GENERATION OF HETEROLOGOUS XYLANASE EXPRESSING HOST

Fungal expression plasmids harboring bacterial xylanase gene (*xyn*) under NPgla and PGlaV2 promoters were transferred into protease deficient *A. brasiliensis* strain via electroporation. Following transformation, cells were plated on MMS+HmB agar and incubated until colonies appeared.

Transformants were grown in xylan medium detailed in section 3.1.4 and inspected for 7 days. Xylanase activity measurements were performed according to the assay in section 3.1.5.

4. RESULTS AND DISCUSSION

4.1. GENERATION OF PROTEASE DEFICIENT ΔPRTT STRAIN

4.1.1. Construction of Deletion Cassette using Overlap PCR

Deletion of *prtT* gene was performed using a deletion cassette which contains green fluorescent protein (*GFP*) gene as a reporter for replacement. First, a conventional PCR was set up to obtain each part harboring homologous sequences to each other. In this step, primers were designed and checked to bind specifically to the targeted sites, however non-specific bands were also obtained during PCR (Figure 4.1.). Increasing annealing temperature leaded loss of all bands, so 55°C was chosen for the optimum annealing temperature for amplification of each product.

In Figure 4.1., marker is indicated as M and it is followed by 5' upstream, 3' downstream and GFP PCR products as duplicate PCR bands, respectively. Since PCR for amplification of 3' downstream part was failed in the first run, another reaction was set up under the same conditions and the products were obtained. 1500 bp PCR products were amplified for 5' upstream and 3' downstream parts and a 1000 bp PCR product was amplified for GFP. GFP was amplified as a transcriptional unit (promoter, coding sequence and terminator) to avoid any frame shift.

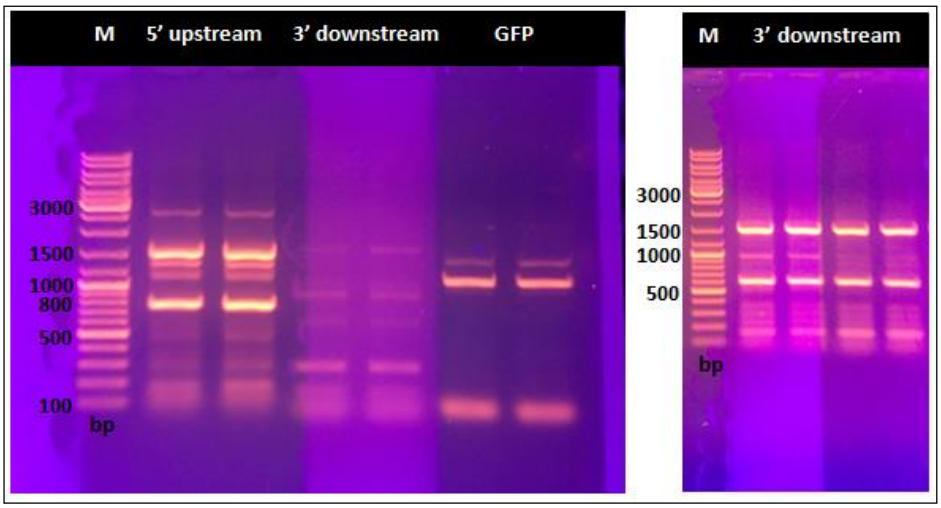

Figure 4.1. Conventional PCR products on one per cent agarose gel. M: marker. The first well of the gel on the left contains DNA ladder (marker). Two wells following the marker contain PCR products of 5' upstream region. Two wells following the 5' upstream PCR products contain PCR products of 3' downstream region. Two wells following the 3' downstream PCR products contain PCR products of GFP transcriptional unit. The first well of the gel on the right contains DNA ladder (marker). Four wells following the marker contain PCR products of 3' downstream region

Corresponding bands for each part were excised from agarose gel, purified and used for the first step of OEPCR. In this step, parts annealed each other via homologous sequences during a 15-cycle PCR. This reaction generally leads to a faint band at the expected position on the gel. Figure 4.2. shows PCR products belong to the deletion cassette which is 4000 bp in length. In Figure 4.2., OEPCR 1.1 and 1.2 indicate utilization of two different annealing temperature to increase fusion efficiency without non-specific product formation. Nevertheless, both annealing temperatures (72°C and 55°C, respectively) gave similar results.

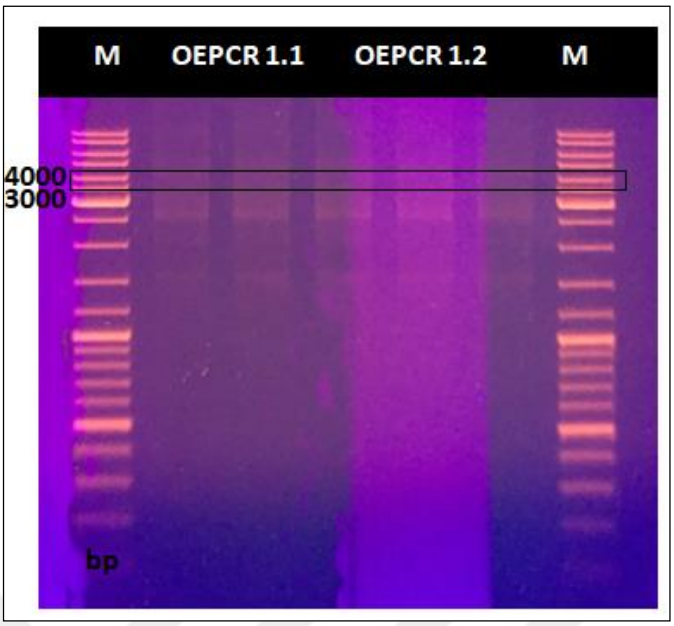

Figure 4.2. Products from first step of overlap PCR (OEPCR 1). M: marker. The first well contains DNA ladder (marker), following two wells contain OEPCR 1.1 products and the following three wells contain OEPCR 1.2 products

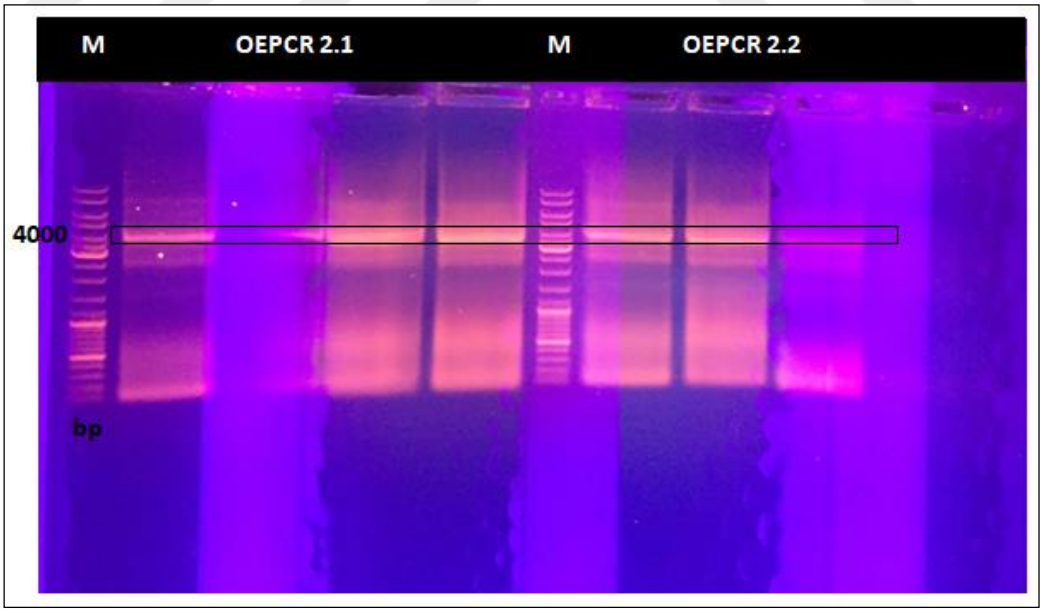

Figure 4.3. Products from second step of overlap PCR (OEPCR 2). M: marker. The first well contains DNA ladder (marker) and the following four wells contain OEPCR 2.1 products. The sixth well contains DNA ladder (marker) and the following three wells contain OEPCR 2.2 products

Resulting PCR product was directly used as a template for second step of OEPCR without any purification. A sharp band was yielded at the position of 4000 bp on agarose gel, indicating successful amplification of the deletion cassette (Figure 4.3.). Smearing on agarose gel is expected after overlap PCR and caused by high concentration of input DNA from the first reaction [139].

4.1.2. Protease Activity of Wild Type and ΔprtT Strains

Deletion cassette was transferred into *A. brasiliensis* by PEG mediated transformation of protoplasts. After transformation, protoplasts were spread on PDA plates and incubated at 30°C. Correct transformants harboring integrated *GFP* gene were confirmed using a fluorescence microscope (Figure 4.4.).

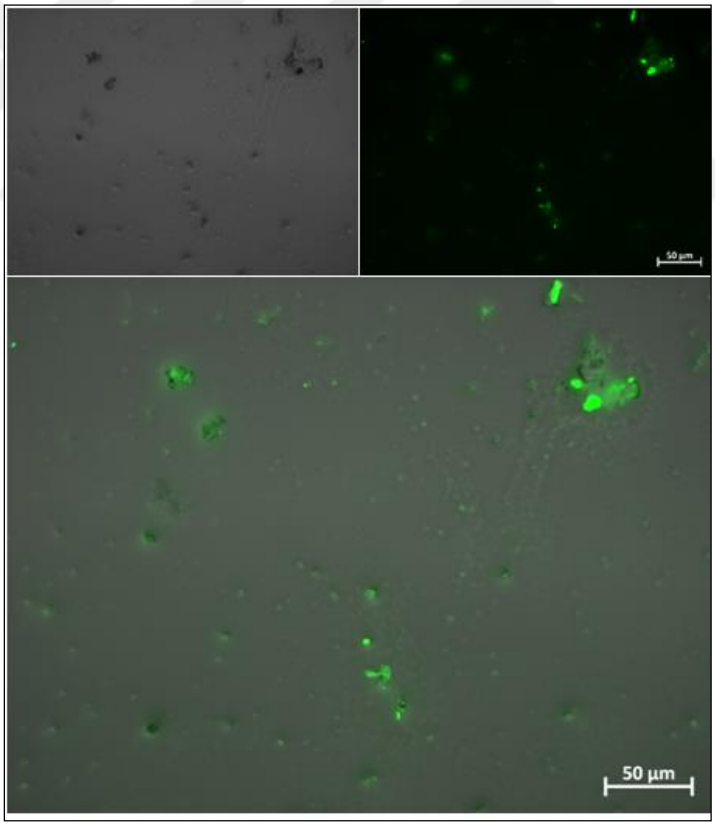

Figure 4.4. GFP expressing protease deficient *A. brasiliensis* cells (20x). Image at the top-left belongs to the protease deficient cells without any excitation, image at the top-right belongs to the same cells with excited at 488 nm and the final image at the bottom was a merged image of protease deficient cells which express GFP

GFP expressing cells were then further tested for the protease deficiency. Mutant and wild type *A. brasiliensis* spores were scraped from PDA plates and transferred into complete medium for protease production. Initial amount and size of mutant and wild type spores were identical as ~10^6 spores/mL. Samples from each culture broth were taken periodically and tested for protease activity. Firstly, samples were spotted on SMA plates for a qualitative comparison. After five days of incubation at 37°C degree a large clear zone around the wild type sample was obtained while there was no halo formation around the mutant sample (Figure 4.5.). Since SMA plate results indicate the decreased proteolytic activity via deletion of *prtT* gene, supernatant samples were analyzed for a further quantitative comparison using a protease activity assay.

According to the protease activity results of samples taken at 48th and 96th hour there is a difference between WT and Δ*prtT* strains (Figure 4.6.). Specifically at 48th hour wild type strain has a protease activity while Δ*prtT* strain gives no activity which validates SMA plate assay results. Figure 4.6. also indicates the loss of protease activity for wilt-type strain at 96th hour of fermentation.

Since *prtT* is the main regulatory gene for proteases in most fungi deletion of *prtT* leads a significant decrease in proteolyic activity of cells [57,60,87,88]. Decreased protease activity results obtained from Δ*prtT* strain correspond to the literature.

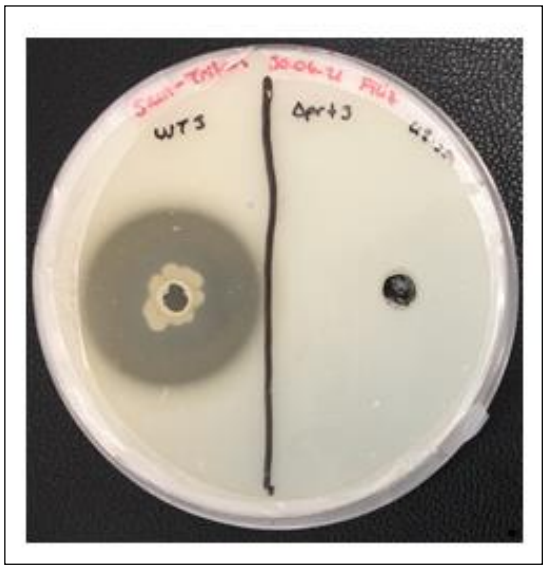

Figure 4.5. Skim milk agar plate results of wild type and Δ*prtT* strains. Wild type is on the left side and the Δ*prtT* is on the right side of the plate

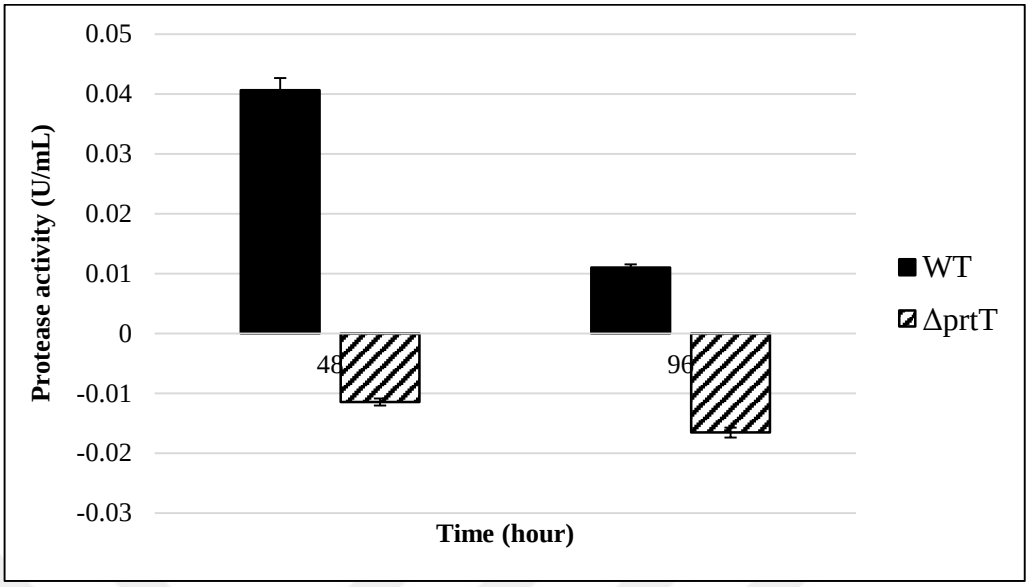

Figure 4.6. Protease activity results of wild type and Δ*prtT* strains

4.1.3. Native Xylanase Activity in Wild Type and Protease Deficient Strain

Wild type and Δ*prtT* strains were grown in xylan containing medium to demonstrate the effect of host originated proteases on protein production. Enzyme production was inspected for 7 days and extracellular broth was used as the enzyme sample for xylanase activity measurement (Figure 4.7.).

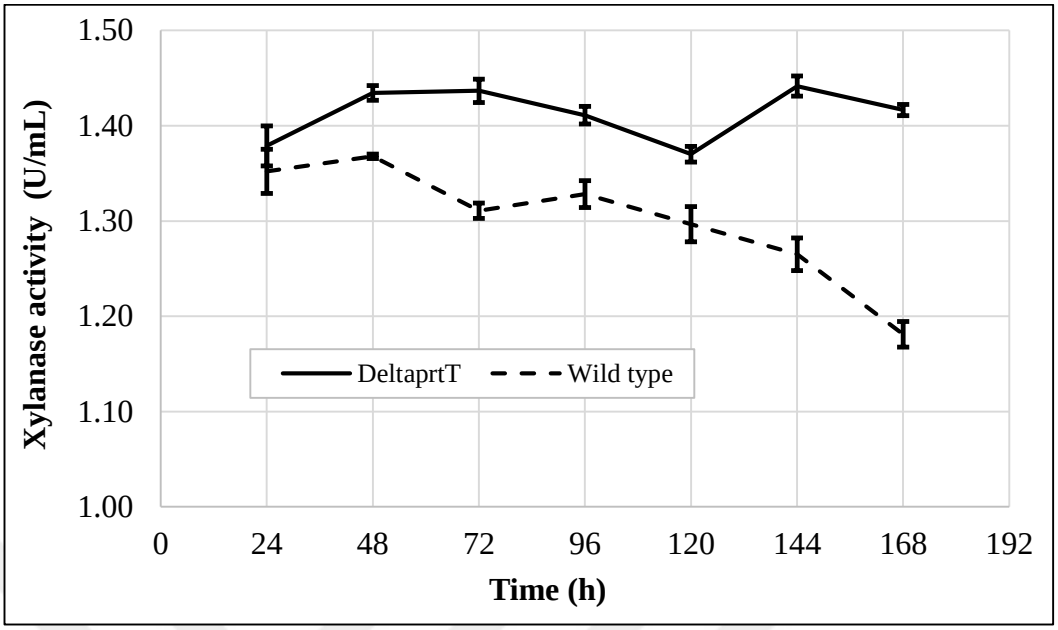

Figure 4.7. Xylanase activity of wild type and Δ*prtT* strains in xylan medium

Xylanase activity reached a maximum at 48th hour of the fermentation of both strains. However, a gradual decrease in activity was observed after 48th hour in wild type strain while the enzymatic activity remained constant until 168th hour of fermentation for Δ*prtT* strain. It is clear to observe that there is a positive effect of decreased protease activity on heterologous enzyme production as a sustained activity was obtained after 7 days fermentation of protease deficient strain, Δ*prtT*.

The highest xylanase activity of wild type strain was obtained as 1.36 U/mL at 48th hour of fermentation. Burlacu *et al.* reported the highest xylanase activity of *A. brasiliensis* to be 3.05 U/mL at 4th day of fermentation in xylan medium [136]. They used 50 ml of production medium containing 5 g/L xylan as the sole carbon source and monitored xylanase activity for 7 days. This difference in activities may be caused by different xylan concentrations used since it was 1 g/L in this thesis. In another study, Costa-Ferreira reported a xylanase activity of 6.44 U/mL from an *A. niger* isolate in the presence of 10 g/L xylan as the sole carbon source. They also investigated the effect of increased amount of xylan on increased xylanase production and reported 66 U/mL activity in the presence of 40 g/L xylan [140].

4.2. CONSTRUCTION OF FUNGAL EXPRESSION PLASMID WITH DIFFERENT PROMOTERS AND MRUBY

Native Gla promoter was engineered to obtain three different promoter variants to investigate carbon catabolite repression effect on Gla promoter. Native promoter and the variants (PGlaVx) were cloned into a fungal backbone plasmid to express a reporter gene, mRuby, in the presence of different carbon sources.

4.2.1. Amplification of NPgla, AMA1 and Promoter Variants

Since Gla is naturally present in *A. brasiliensis* native Gla promoter was obtained from genomic DNA of *A. brasiliensis.* Gla promoter products which are 700 bp in length were obtained as expected (Figure 4.8.).

AMA1 is a fungal replication origin that frequently takes part in fungal expression plasmids as it can improve transformation efficiency in terms of yielded colonies per µg DNA [141]. Therefore, AMA1 part was amplified from a commercial fungal plasmid to construct a fungal expression plasmid using Golden Gate assembly. Primers were specifically designed to amplify full length AMA1 sequence, however only a 3000 bp part of it was obtained successfully (Figure 4.8.).

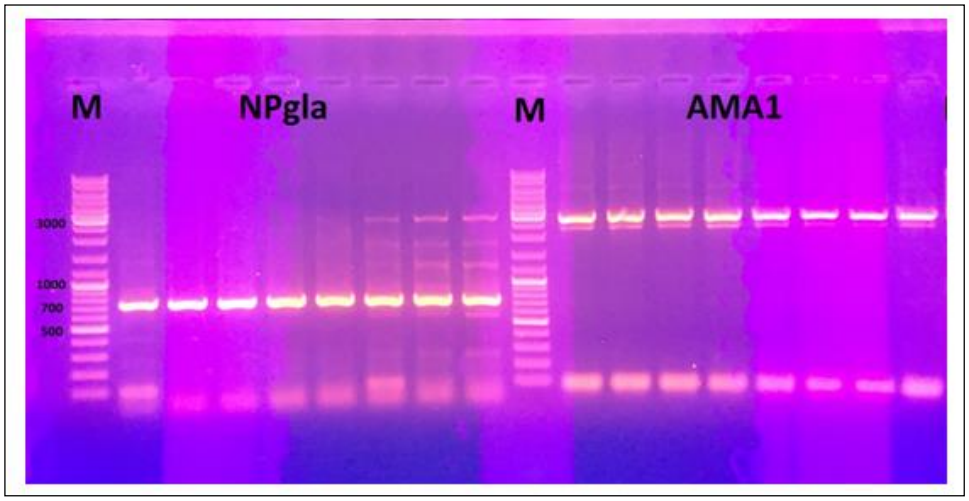

Figure 4.8. NPgla and AMA1 products (~700 bp and ~3000 bp, respectively). M: Marker. NPgla: Native Gla promoter. AMA1: fungal replication origin, AMA1. Each PCR band after the marker corresponds to an annealing temprature ranging between 65-55°C

Full length of AMA1 is 5200 bp and this sequence consists of an inverted duplication of a palindromic sequence (Figure 4.9.). This structure causes difficulties in amplification of full size of the part by PCR or in sequencing. Therefore studies have been performed on using half of AMA1 part for plasmid construction and it has been found that the truncated form of AMA1 is still effective in terms of plasmid replication [142–144].

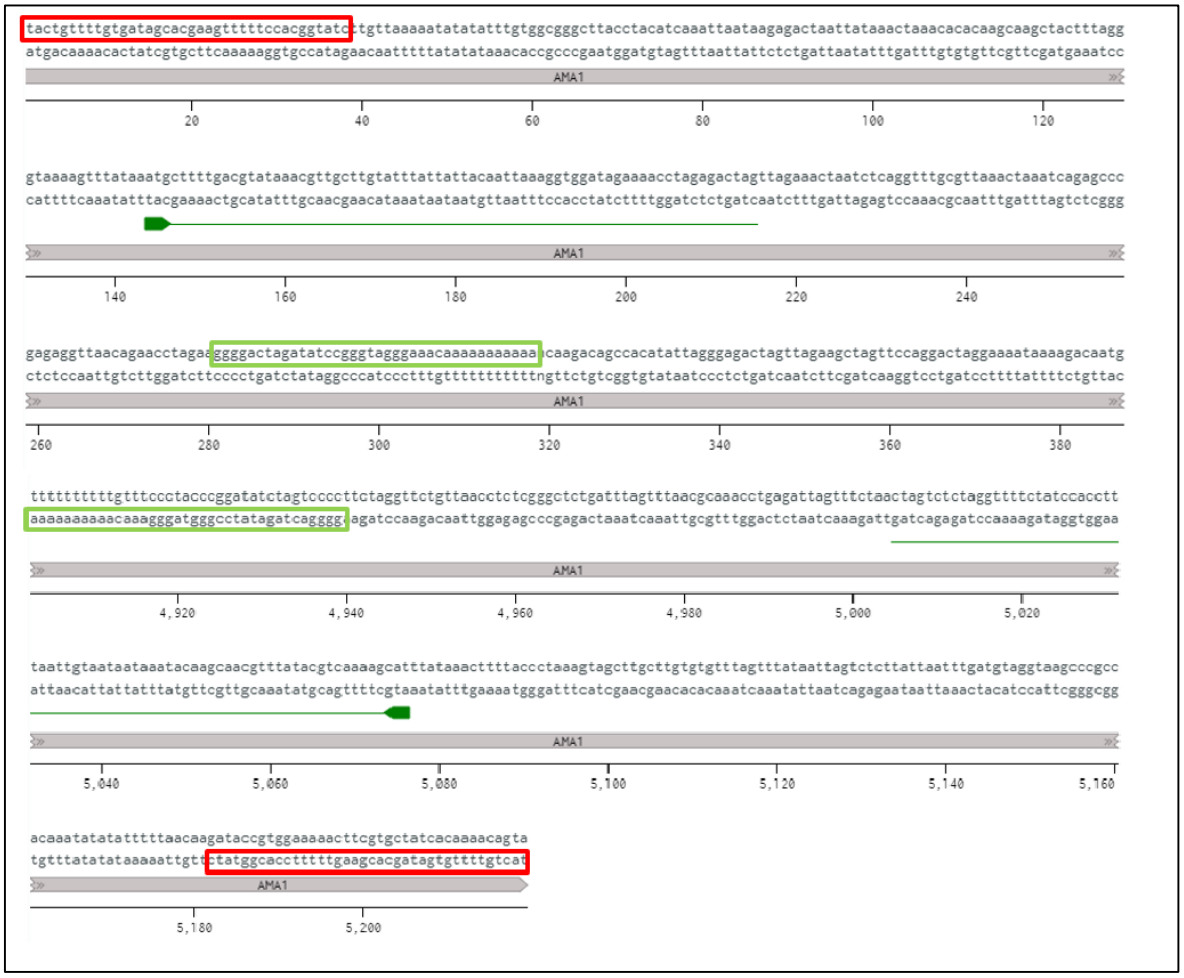

Figure 4.9. Partial AMA1 sequence with palindromic repeats. Boxes in the same color represent palindromic bases

Engineered Gla promoter variants were obtained as synthetic gene fragments via commercial provider. Using these fragments as templates each promoter variant was PCR amplified to have Golden Gate-compatible flanks at 5’ and 3’ ends. Since deletion and insertion of bases is limited to small numbers, all of the variants have identical length which is around 700 bp (Figure 4.10.).

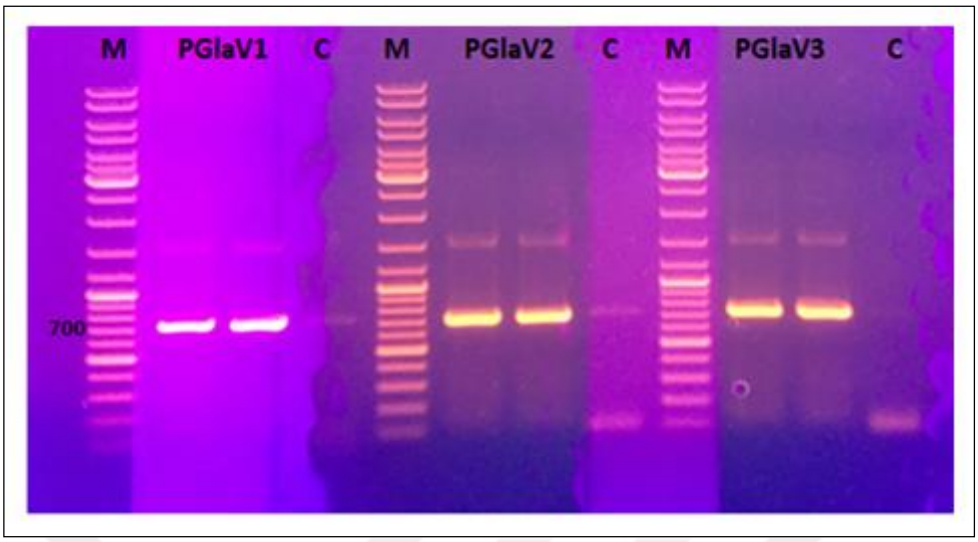

Figure 4.10. PGlaV1, PGlaV2 and PGlaV3 products (~700 bp). M: marker. C: PCR control. Two wells after the first marker contain PCR products for PGlaV1, two wells after the second marker contain PCR products for PGlaV2 and the two wells after the third marker contain PCR products for PGlaV3

4.2.2. Cloning of Promoter and AMA1 Parts into Entry Vector

PCR amplified promoter parts and AMA1 sequence were cloned into an entry vector, pYTK001, provided in Yeast Toolkit. Cloning of PCR products into a small vector ensures safe storage of DNA parts which will be used in further cloning steps. Additionally, providing a part in a plasmid increases the efficiency of restriction digestion and ligation, especially for reactions in which digestion and ligation of multiple parts occur simultaneously.

In this step, pYTK001 plasmid harbors GFP transcriptional unit thus it gives green fluorescence when the plasmid is intact. Once GG assembly was performed successfully *GFP* gene is disrupted and green/white selection is possible as colonies with the cloned sequence will be white.

4.2.3. Construction of Fungal Backbone Plasmid

Construction of a backbone plasmid enables quick transfer of transcriptional unit parts (promoter, coding sequence, terminator) between plasmids while keeping other plasmid parts (antibiotic resistance gene, replication origin etc.) constant. Here, fungal backbone (FBB) plasmid was constructed to clone different promoter parts for expression of a reporter gene. FBB plasmid basically contains connectors for correct assembly, GFP expression cassette for further cloning selection, hygromycin resistance gene for antibiotic selection in fungi, various antibiotic resistance genes for selection in bacteria, bacterial replication origin for replication in bacteria, and a fungal replication origin AMA1 for replication in fungi.

In the final backbone plasmid, *GFP* serves as a reporter gene in which green colonies indicates correct assembly of fungal backbone plasmid. In the following steps, GFP expression cassette will be replaced with a transcriptional unit when it is successfully cloned into the backbone, yielding non-green colonies.

4.2.4. Construction of mRuby Expressing FBB Plasmids

Promoter parts, mRuby gene and TPGK1 terminator were cloned in FBB plasmid to construct mRuby expressing plasmids with different promoter variants. Since FBB plasmid contains GFP, cloning of promoter, mRuby and terminator sequences disrupted GFP expression, resulting in pink colonies due to the mRuby expression (Figure 4.11.).

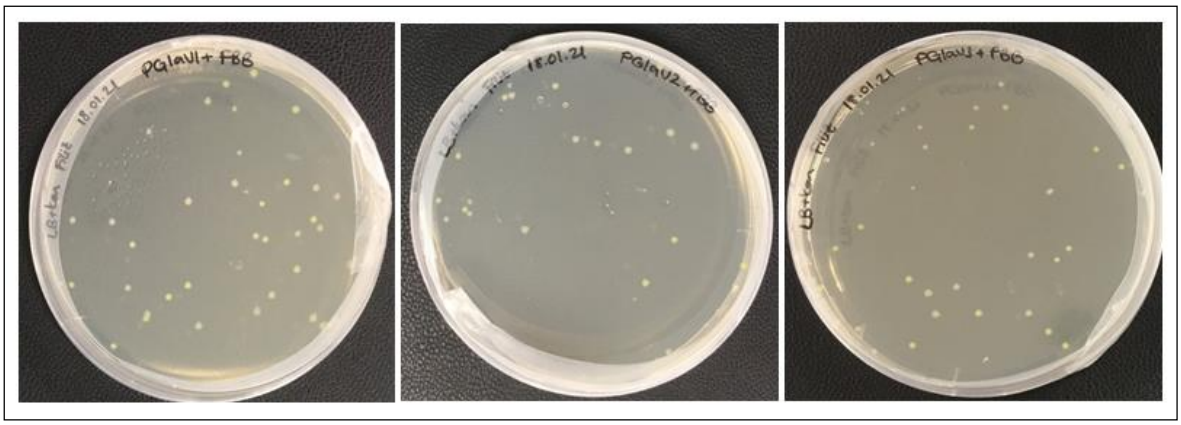

Figure 4.11. Colonies with Gla promoter variants (PGlaV1, PGlaV2, PGlaV3), mRuby and FBB plasmid

Construction of fungal expression plasmids with mRuby and different promoters is a successful example of performing genetic manipulations on standardized parts and their one-pot assembly using standardized protocols.

4.3. TEST OF PROMOTER VARIANTS IN THE PRESENCE OF DIFFERENT CARBON SOURCES

Native Gla promoter and the engineered promoter variants were cloned into FBB plasmid along with mRuby gene. These expression plasmids were transferred into *A. brasiliensis* and transformants were grown in the presence of different carbon sources to investigate for their ability to overcome the repressive effect of carbon sources. Additionally, transformants were analyzed for their ability for co-consumption of primary and secondary carbon sources to demonstrate the relief of carbon catabolite repression.

Transformation of *A. brasiliensis* with different expression plasmids were performed both PEG-mediated protoplast transformation and electroporation. PEG-mediated transformation is a well established protocol used for transformation of different fungi species, however protocol requires attention since protoplasts are sensitive to environmental changes. Moreover, PEG concentration and application duration can be toxic to the protoplasts [144]. Electroporation is another commonly used transformation method applied to several species from bacteria to plant cells. This method is

straigthforward and mostly does not require a protoplast preparation step for transformation [145]. Therefore fungal expression plasmids carrying different Gla promoter variants and mRuby gene were transformed into *A. brasiliensis* via electroporation (Figure 4.12.).

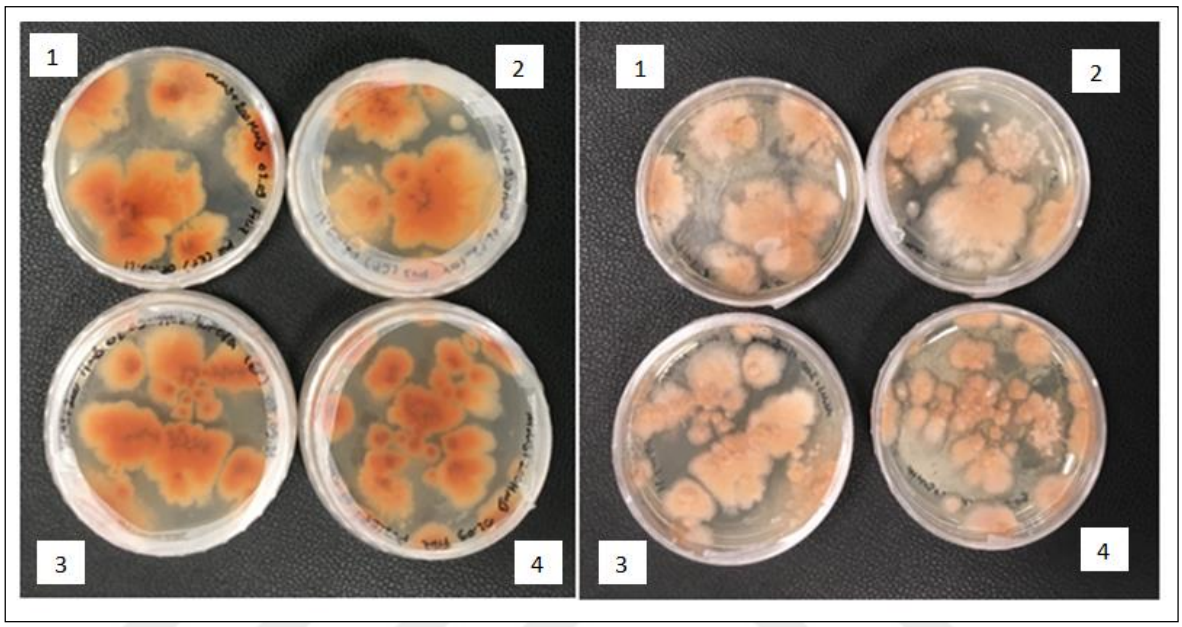

Figure 4.12. *A. brasiliensis* cells after transformation with fungal expression plasmids containing mRuby under the control of different Gla promoter variants. Image on the left and image on the right are a back and a front view of the plates of cell expressing mRuby, respectively. Colonies in plate number 1 contain NPgla+mRuby, in number 2 contain PGlaV1+mRuby, in number 3 contain PGlaV2+mRuby and in number 4 contain PGlaV3+mRuby expression plasmids

During transformation experiments, a commercial fungal plasmid (pFC332) was used as positive control. Figure 4.13. shows *A. brasiliensis* colonies transformed with pFC332. As a negative control, transformation mixture without plasmid was prepared. This control yielded zero colony on the selective media after transformation. Cell viability was additionally checked by spreading a small aliquot on MM agar without antibiotic after protoplast preparation (Figure 4.14.).

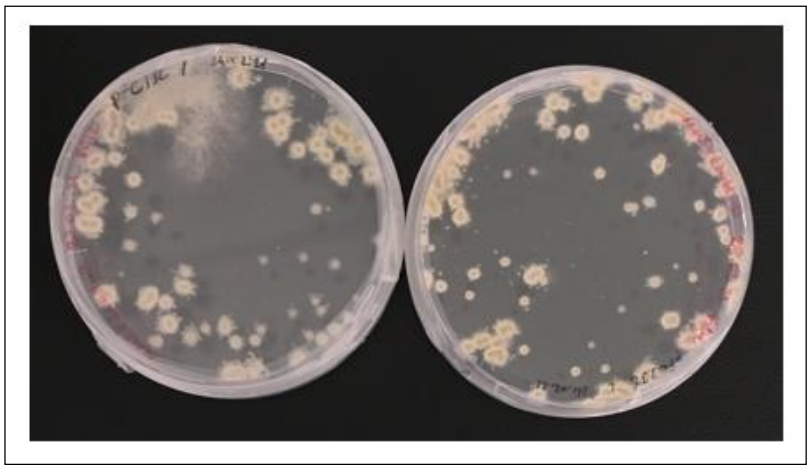

Figure 4.13. *A. brasiliensis* colonies after transformation with pFC332 plasmid as positive control in duplicate

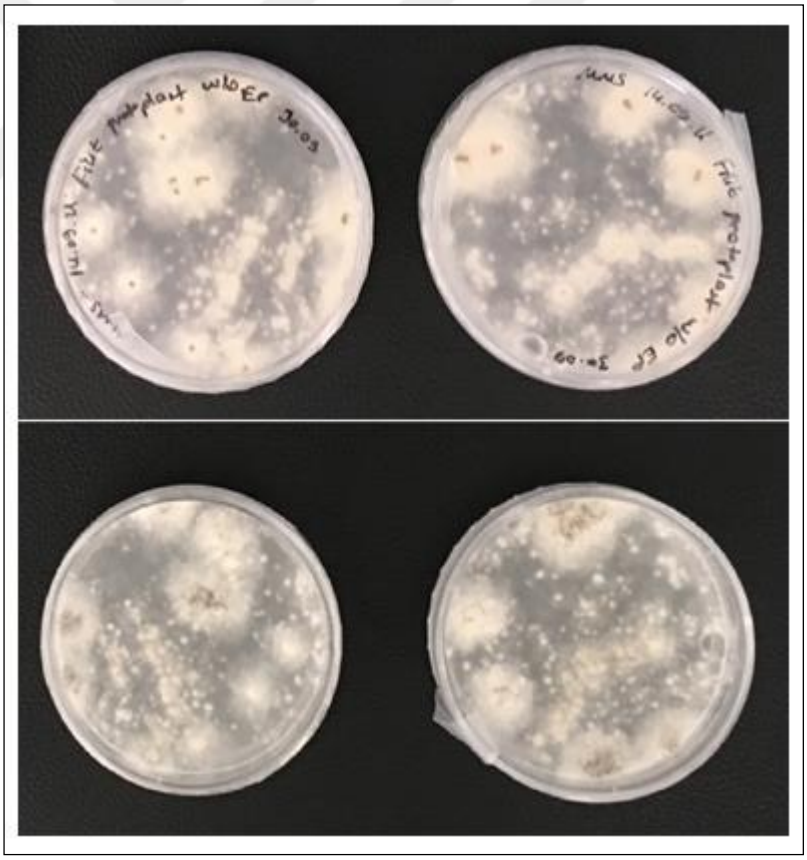

Figure 4.14. Viable *A. brasiliensis* protoplasts prior to transformation. Back view is on the top and the front view is at the bottom

A. brasiliensis cells with mRuby plasmids were grown in a chemical medium containing 20 g/L carbon source which is maltose or xylose to investigate carbon source effect on promoter variants. First, cells including wild type were grown in the presence of maltose as a sole carbon source for three days. This incubation time was found to be sufficient for protein production and the diameter of a conidia in the culture reached 6 mm approximately. Cultures were grown as triplicate and at the end of incubation culture broth was separated from conidia using filter paper. Final wet weight of culture was measured (Table 4.1.).

Table 4.1. Wet weight values of cells grown in maltose medium

Strain	**Wet weight 1 (g)**	**Wet weight 2 (g)**	**Wet weight 3 (g)**
WT	5.49	4	3.6
NPgla	3	3	3
PGlaV1	4.34	3.49	4.89
PGlaV2	5.75	4.3	3.41
PGlaV3	5.34	3.85	5.4

Since promoter variants were designed without a signal peptide, mRuby is expected to be produced as an intracellular protein. Thus resulting conidia from each erlen was ground in liquid nitrogen into a fine powder. Powder was transferred into a tube and diluted with water to separate soluble intracellular proteins from disrupted cell lysates. Supernatant after centrifugation contained total intracellular protein including mRuby. Fluorescence measurement was performed using a microplate reader and normalized using wet weight values of each erlen to point out a specific activity (Figure 4.15.).

Secondly, cells including wild type were grown in the presence of xylose as a sole carbon source. In this medium, conidial diameter of cells except wild type, reached 6 mm after six days (Figure 4.16.). Cultures were grown as triplicate and at the end of incubation culture broth was separated from conidia using filter paper. Final wet weight of culture was measured (Table 4.2.).

Table 4.2. Wet weight values of cells grown in xylose medium

Strain	**Wet weight 1 (g)**	**Wet weight 2 (g)**	**Wet weight 3 (g)**
WT	3.42	5.64	2.34
NPgla	6.1	4.12	7.99
PGlaV1	0.85	3.82	3.22
PGlaV2	1	-	1.87
PGlaV3	5.04	5.93	5.39

Similar to maltose case, mRuby is expected to be produced as an intracellular protein. Thus resulting conidia from each erlen was ground in liquid nitrogen, disrupted cell lysate was separated from intracellular proteins and fluorescence measurement was carried out using a microplate reader and normalized using wet weight values of each erlen to point out a specific activity (Figure 4.15.).

Maltose is known to be a strong inducer of Gla promoter, thus expression of mRuby from native and the other promoter variants was obtained as expected. However, native Gla promoter exhibited the highest mRuby levels among promoter variants and increasing expression was obtained from PGlaV1 to PGlaV3. PGlaV3 has an increased number of transactivator binding site (CCAAT box) and it was expected to obtain increased expression in this strain compared to PGlaV1 and PGlaV2.

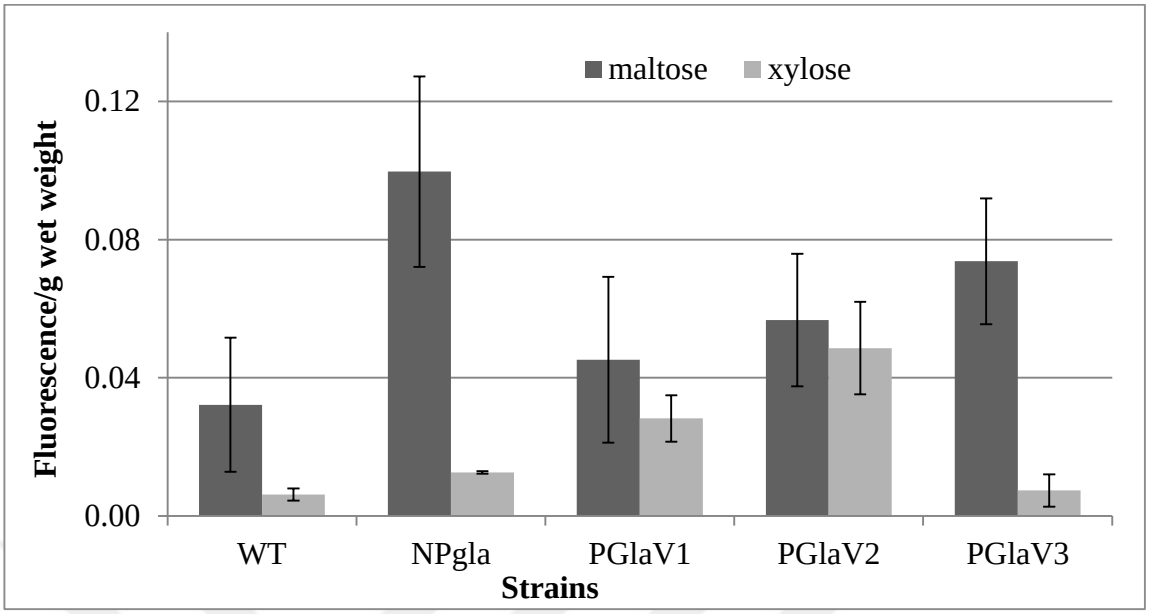

Figure 4.15. mRuby/g wet weight ratios of each strain grown in maltose or xylose medium. Values are the mean of three replicates

Xylose represses glucoamylase production from Gla promoter by binding a specific motif in promoter sequence. Therefore a repressed expression in the strain carrying native Gla promoter was expected. At the same time, an increased expression from PGlaV1 to PGlaV3 was expected since putative CreA binding sites were removed systematically from the Gla promoter sequence. As expected, NPgla is significantly repressed and provided a lower mRuby fluorescence in the presence of xylose compared to the levels in the presence of maltose as native promoter harbors four putative CreA binding sites. In PGlaV1 two out of four CreA binding sites were deleted and consequently an increased mRuby expression was obtained compared to those from NPgla promoter. In a similar manner, PGlaV2 was designed to have no putative CreA binding sites, thus obtained a higher expression level compared to NPgla and PGlaV1 as expected. PGlaV3 was designed as an improved version of PGlaV2 as it contains four copies of a transcriptional activator sequence (CCAAT box) and expected to have the highest mRuby expression among the other promoter variants. However, in the presence of xylose, a significant decrease in expression of mRuby under the control of PGlaV3 was observed compared to the highest expression promoted by PGlaV3 in the presence of maltose.

CCAAT box is known as a cis-acting element and it occurs in the promoter regions of approximately 30 per cent of the genes in eukaryotes. Binding a corresponding activator protein to CCAAT box in a promoter leads activation of expression of downstream gene. CCAAT binding protein known as a complex consists of multiple subunits and plays a role in regulation of primary/secondary metabolism, stress response, and virulence in fungi [146].

Xingguo *et al.* reported the presence of a separate regions containing CCAAT box inside in Gla promoter in *A. niger* T21 strain. They investigated the impact of these regions on Gla promoter activity and found out that the mutations in these regions or changes their relative orientations severely affected the protein expression from Gla promoter. Moreover, they revealed that multiplexing of one of those regions in its original site on Gla promoter caused a decrease in expression of the reporter gene under the control of Gla promoter. Accordingly they suggested a titration effect caused by multiplexing of a transcription binding site, CCAAT box in this case [129]. In this study, multiplexing CCAAT sequence in its original site did not caused a titration effect in the presence of maltose as the sole carbon source.

In *Aspergillus* species, AngHapC protein is responsible for activation of certain genes by binding CCAAT boxes in their promoter regions [147]. AngHapC protein is a homolog of *S. cerevisiae* Hap4 protein which is a transcriptional activator that binds to CCAAT box. Bolotin-Fukuhara investigated the promoter region of Hap4 protein to enlighten its transcriptional regulation mechanism. According to the results there is a binding site in the promoter region for Mig1 protein which is the key protein for regulation of carbon catabolite repression. Thus, it has been concluded that expression of *Hap4* gene is abolished or very weak in the presence of a primary carbon source, glucose in this case [148]. In the light of this idea, it was concluded that, CCAAT-binding protein, AngHapC, was expressed in very low levels, thus multiplexing of CCAAT box in PGlaV3 promoter caused a titration effect, resulting in decreased promoter activity in the presence of xylose.

To further investigate titration effect of CCAAT box in the presence of a repressor, promoters should be designed as they have incremental copies of CCAAT sequence and tested for their activity. Moreover, strain with PGlaV3 promoter may be grown in the presence of maltose first and then xylose may be added to the pre grown culture to observe the decrease in gene expression.

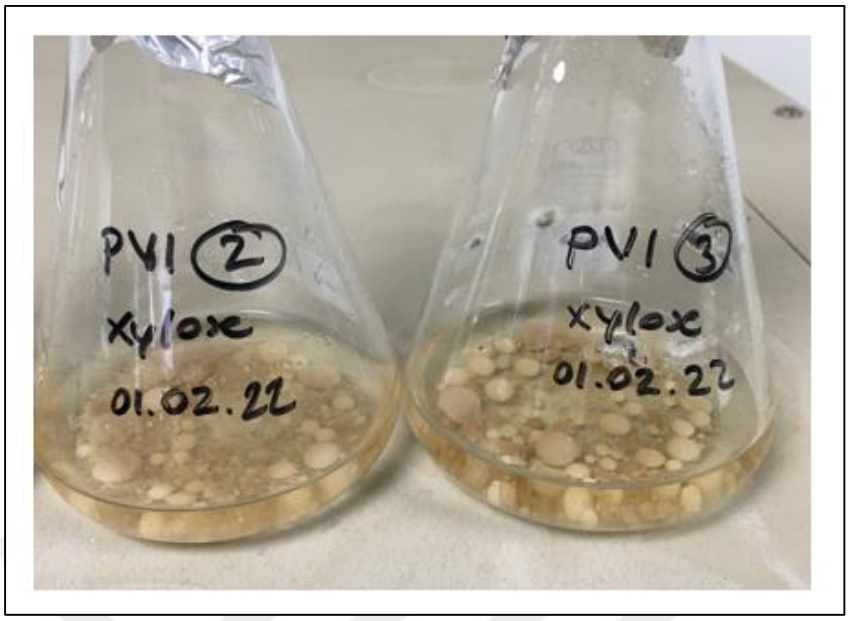

Figure 4.16. 6-day old *A. brasiliensis* grown in xylose medium

4.4. TEST OF SELECTED PROMOTER VARIANTS FOR RELIEF OF CARBON CATABOLITE REPRESSION

Xylose may inhibit uptake of primary carbon sources as a consequence of carbon catabolite repression. Thus, xylose repression on Gla promoter was first removed. According to the results of Figure 4.15., PGlaV2 promoter is a promising candidate to investigate carbon catabolite repression. NPgla promoter was also investigated to compare derepression of CCR to the one in PGlaV2 strain. Verduyn medium was prepared containing 10 g/L xylose and 10 g/L maltose. Strains with NPgla and PGlaV2 promoters were grown in this media for 120 hours. Utilization of sugars was analyzed using HPLC.

Strains exhibited a different xylose consumption pattern. It was gradually utilized by PGlaV2 throughout the fermentation while a decrease in xylose concentration was apparently seen after 24 hour in NPgla. Similarly, PGlaV2 strain started to consume maltose from the beginning of the fermentation while NPgla strain started to consume maltose after 72 hours of fermentation in which xylose is depleted (Figure 4.17.). It can be concluded that, **simultaneous utilization of maltose and xylose was achieved in the PGlaV2 strain, pointing/indicating relieved carbon catabolite repression of xylose on this promoter.**

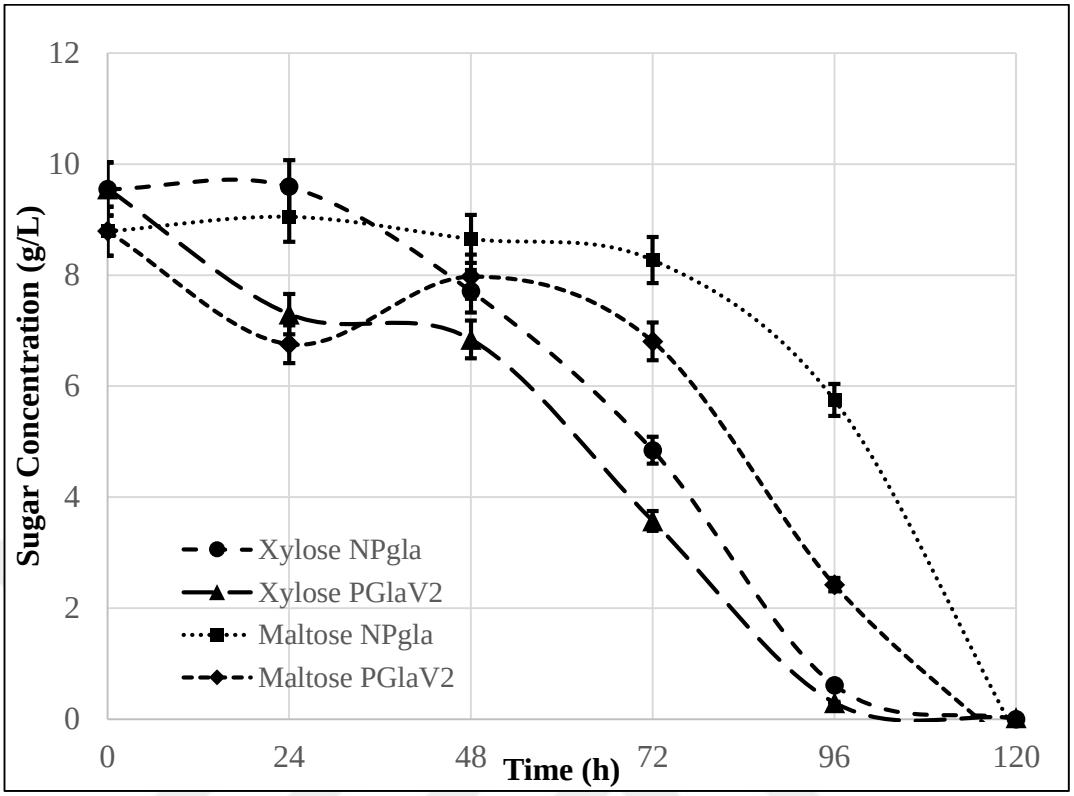

Figure 4.17. Consumption of xylose and maltose by NPgla and PGlaV2 strains

4.5. HETEROLOGOUS XYLANASE ACTIVITY OF NPGLA+XYN AND PGLAV2+XYN STRAINS

A. brasiliensis Δ*prtT* strains harboring bacterial xylanase gene under NPgla and PGlaV2 promoters were generated, cultured on agar and shown in Figure 4.18. Transformants were grown in xylan medium and extracellular broth was used as the enzyme sample for xylanase activity measurement.

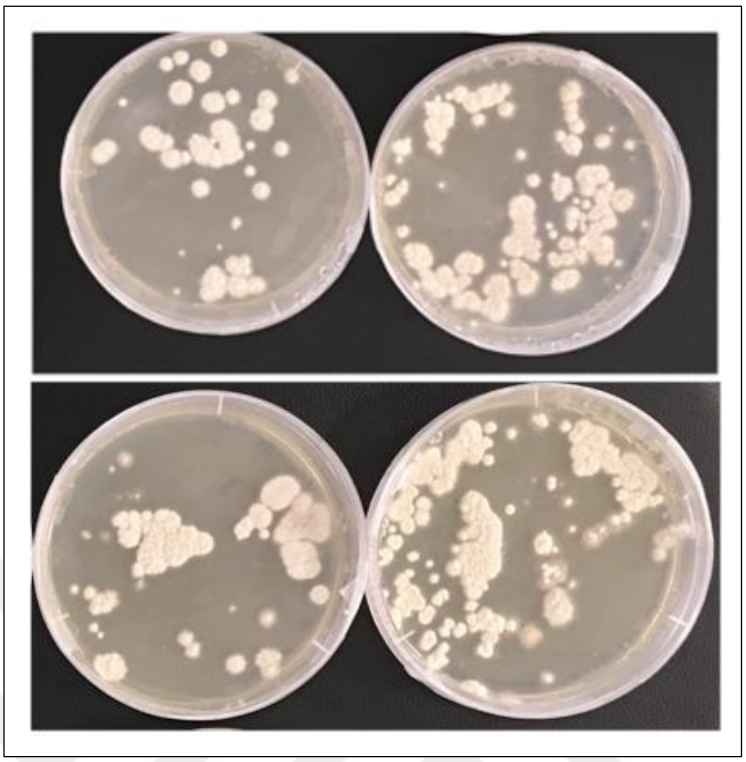

Figure 4.18. *A. brasiliensis* Δ*prtT* strains with NPgla+xyn plasmid (top) and PGlaV2+xyn plasmid (bottom)

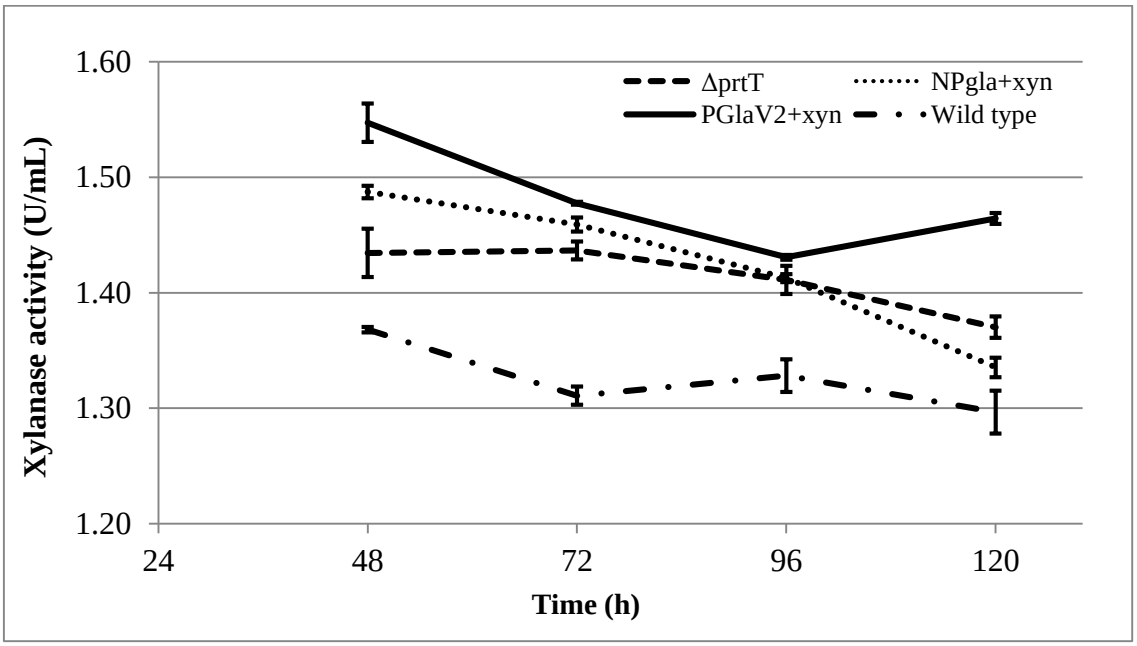

Figure 4.19. Xylanase activity of Δ*prtT*, NPgla+xyn, PGlaV2+xyn and wild type strains

Heterologous xylanase production from NPgla+xyn and PGlaV2+xyn strains was compared to the previous homologous xylanase production levels from wild type and Δ*prtT* strains (Figure 4.19.). The highest xylanase activity was measured as 1.55 U/mL produced from PGlaV2+xyn strain at 48th hour of fermentation. The second highest activity was recorded as 1.48 U/mL produced from NPgla+xyn strain at 48th hour of fermentation. These results were compared to those which was 1.43 U/mL and 1.36 U/mL produced from Δ*prtT* and wild type strains, respectively.

In conclusion, heterologous xylanase expression was achieved in protease-deficient strain and a highest activity was obtained from the strain that contain PGlaV2 promoter to drive expression of the heterologous xylanase.

CHAPTER II: CRISPR/CAS9 APPLICATION IN *SACCHAROMYCES CEREVISIAE*

5. INTRODUCTION

5.1. SACCHAROMYCES CEREVISIAE AS A HOST

Saccharomyces cerevisiae is the most frequently used eukaryotic model organism in microbiological studies. Its GRAS status renders this yeast an appropriate microorganism for utilizing in several industries such as food, beverage, textile, etc. Since *S. cerevisiae* can grow in mild conditions in simple, affordable media to high cell densities, working with this yeast is broadly cost effective in terms of upstream and downstream processes.

Although it is known that *S. cerevisiae* is widely used for fermentation studies it is also a suitable host for molecular work. A remarkable, well studied, well established toolbox exists to allow heterologous protein production, strain design and mutation studies to be performed in *S. cerevisiae*. With the development of molecular techniques, a great portfolio of functional parts and techniques has emerged [149,150]. Highly stable plasmids were constructed for efficient production of heterologous proteins in yeast with the discovery of different promoters, terminators, chromosomal replication regions and selection markers. These plasmids enable adjusting protein production rate by providing high copy or low copy number options.

Advanced homologous recombination machinery of *S. cerevisiae* allows targeted integration of a DNA sequence into its genome. Integration occurs through homologous regions between chromosome and integrating DNA which can be a linear cassette or a plasmid. Expression from integrated part is generally more stable than ones from replicating plasmids [151].

Promoters are key parts in heterologous protein production since they are responsible for the initiation and regulation of the expression. There are several types of promoters explored and improved which can be classified as strong, weak, constitutive, inducible and their use in protein production in yeast is well studied [152–157].

Secretion of proteins out of the cell is generally preferable due to the decreased downstream processes, consequently the cost. However, it is quite inefficient to utilize *S. cerevisiae* as a host for production of secreted proteins since it can secrete only 0.5 per cent of its own proteins into extracellular space. This bottleneck can be overcome by using

appropriate signal peptides to address proteins to endoplasmic reticulum but folding still remains as a problem at this stage.

Posttranslational modifications are important for certain proteins since their activity is highly dependent on additional residues. Especially pharmaceutical proteins require specific glycosylation process to become active. *S. cerevisiae* can be a great host for production of pharmaceuticals due to its improved posttranslational machinery; however types and stages of glycosylation vary between eukaryotes. On the other hand, hyperglycosylation can be problematic since it may inhibit interactions between proteins.

To sum up, *S. cerevisiae* has significant advantages compared to other model organisms despite its drawbacks. It is safe, practical and applicable to use this yeast not only in fermentation studies but also in genome engineering research.

5.2. CRISPR/CAS9 SYSTEM

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system is based on the adaptive immune system of prokaryotes. This system has a wide range application area for genome editing studies of many species. Briefly, the system includes an endonuclease named as Cas9 and a synthetic chimeric guide RNA (sgRNA) molecule. Guide RNA molecule guides Cas9 enzyme to the target gene, so Cas9 creates a double-stranded break at the target sequence. Subsequently, this double-strand break may be fixed in two ways: nonhomologous end joining (NHEJ) and homology-directed repair (HDR). As a result of NHEJ repair in-del mutagenesis occurs, while homology-directed repair can be carried out by a DNA repair template which is simultaneously provided with the sgRNA [158].

The effective CRISPR/Cas9 system for genome-editing studies has been successfully reported in yeasts and some fungi including *Saccharomyces cerevisiae* [158,159], *Yarrowia lipolytica* [44], *Pichia pastoris* [160], *Schizosaccharomyces pombe* [161], *Candida albicans* [162,163], *Aspergillus strains* [164,165], *Penicillium chrysogenum* [166], *A. fumigatus* [167]. According to the results of the studies on different strains of *Saccharomyces cerevisiae*, owing to its homologous recombination capacity, the success rate has been reached to the 99 per cent for single/multiple gene disruptions depends on the

type of the sources for Cas9 and sgRNA [158,159]. Several studies have revealed the success of both the efficient NHEJ-generated gene deletions and HR-mediated gene deletions in *Yarrowia lipolytica* which is well-known oleaginous yeast [44]. Conversely, as an important recombinant protein producer *Pichia pastoris* has been failed in CRISPR/Cas9 genome editing studies due to its poor homologous recombinant capability [160].

5.3. CRISPR/CAS9 SYSTEM COMPONENTS

5.3.1. Cas9

Cas9 is a 158 kDa endonuclease containing 1388 amino acids. Cas9 protein consists of six domains including RECI, RECII, bridge helix (BH), PAM interacting (PI), HNH and RuvC. RECI, the biggest domain, is responsible for the binding of guide RNA along with the RECII and BH domains. PI acts in recognition of the three-nucleotide (5'-NGG-3') PAM motif, which enables the Cas9 to recognize target DNA. HNH domain is responsible for cleavage of the target DNA strand while RuvC is responsible for cleavage of the complementary strand.

Cas9 from *Streptococcus pyogenes* is the most common Cas9 endonuclease used in CRISPR studies. It is possible to use natural Cas9 protein as well as the codon optimized version for different host organisms [159,168–176].

Optimum expression of Cas9 protein is crucial for efficient genome editing as well as for avoiding any negative effect on growth [168–170,172,176]. Despite reports on Cas9 expression from strong/weak promoters, as an integrated cassette or an expression plasmid with different copy numbers, constitutive promoters with variable strength is routinely used for expression of Cas9 in CRISPR studies [159,168,177,178,169–176].

5.3.2. Single Guide RNA

Single guide RNA is a specific RNA molecule that recognizes the target DNA sequence and directs Cas9 to this region. It consists of two non-coding RNA namely CRISPR RNA

(crRNA) and trans-activating CRISPR RNA (tracrRNA). crRNA is typically 20 bp in length and it complements the target DNA. tracrRNA is a 80 bp RNA that acts as a scaffold for binding to Cas9. While crRNA is designed to be specific to the target DNA, tracrRNA is a constant sequence and does not change depending on the target site. As a result of the combination of crRNA and tracrRNA fragments, "single guide RNA" is formed and becomes a single molecule. sgRNA can be synthesized *in vitro* or the related DNA sequence can be designed and delivered into the cell to be expressed by *in vivo* transcription.

Expression vectors with high copy numbers, suitable promoter and terminator sequences are frequently used for in vivo transcription of sgRNA. It is crucial that the selected promoter should be capable of performing post transcriptional modifications required for maturation of the sgRNA. Therefore, RNA polymerase III promoters which are responsible for synthesis of small nuclear RNAs such as 5S rRNA, tRNA, U6 and U3 snRNA are widely used for sgRNA expression. However, utilization of these promoters in CRISPR/Cas9 studies is limited despite their successful applications. For instance they provide ubiquitous expression, so they cannot be used for sgRNA expression in specific tissues and cells. On the other hand these promoters are constitutive and not tunable which is unfavorable in certain applications.

Alternatively, utilization of ribozymes along with RNA polymerase promoters is getting more attention in literature. The idea behind this method is to add ribozymes that cut the RNA molecule from specific regions to the ends of the sgRNA, to ensure that the relevant modifications are performed appropriately following the expression. It should be noted that the ribozyme cutting sites should be located at just before the first base and right after the last base of the sgRNA. For this purpose, Hammerhead (HH) and Hepatitis delta virus (HDV) ribozymes are commonly used owing to their small size and strong nuclease activity.

5.4. REPAIR IN CRISPR/CAS SYSTEM

DNA damages are known as natural events occurring during replication or causing by environmental factors. It is highly important to repair DNA damages properly, double-strand DNA breaks in particular, since they cause severe mutations or even death if they

remain un/misrepaired [179]. Repair of DNA damages occurs in two mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ) (Figure 5.1.). Most of microorganisms tend to use NHEJ for repair of double-strand breaks while HR is the dominant repair mechanism in yeast, *S. cerevisiae*. CRISPR/Cas9 studies can benefit from these repair mechanisms to make the desired changes in genome.

Several proteins are involved in HR mechanism and these proteins are highly conserved among eukaryotes [179]. In yeast, if a double-strand break occurs, 5' ends of DNA are truncated and this process called "resection". Replication protein A (RPA) binds the resulting single stranded ends and this is the first step of HR mechanism. In the meantime, binding of RPA protects unstable single stranded ends from additional damages. Following, Rad51 protein displaces RPA and a search for a template DNA begins. When a template is found Rad51 mediates binding of single stranded ends to the one strand of the template. This structure is known as D-loop and resolving of this loop occurs in two pathways which are double-strand break repair (DSBR) and synthesis dependent strand annealing (SDSA) [180].

In DSBR a Holliday junction is formed during repair and repaired DNA sequences result in mostly crossover and rarely non-crossover. In SDSA one of the damaged strands is repaired using a template DNA, and then repaired strand is used for the repair of the other damaged strand. Thus, SDSA results in production of non-crossover recombinant DNA strands [181].

Non-homologous end joining repairs DNA damages by direct ligation of the ends around the break without a donor DNA. Unlike HR, NHEJ is active throughout the entire cell cycle. This mechanism requires a nuclease for resection of the damaged DNA, a polymerase for elongation of single stranded DNA and a ligase to combine elongated ends. NHEJ mediated repair begins with the binding of the Ku protein to the DNA ends around the break, prior to binding of the above enzymes. Ku:DNA complex serves as a scaffold for binding of the nuclease, polymerase and ligase enzymes. Artemis, a nuclease in NHEJ, is phosphorylated by PKcs kinase and Artemis:DNA:PKcs complex cleave the DNA. Cleaved DNA parts are elongated by Mu or Lambda polymerases and the gaps are ligated by XLF:XRCC4:DNA ligase IV complex. Thus, the double-strand break is repaired [182].

In the resection step, Artemis:DNA:PKcs complex may remove a 25-bp sequence from the damaged region. If removed sequence is not filled with new bases by DNA polymerase and the gap is filled by DNA ligase, NHEJ results in a deletion. On the other hand, addition of extra bases to the resected region and elongation of these ends may lead a repair with a new sequence, resulting in frame shift, indel etc. Thus, NHEJ mediated repair is an error prone mechanism [183].

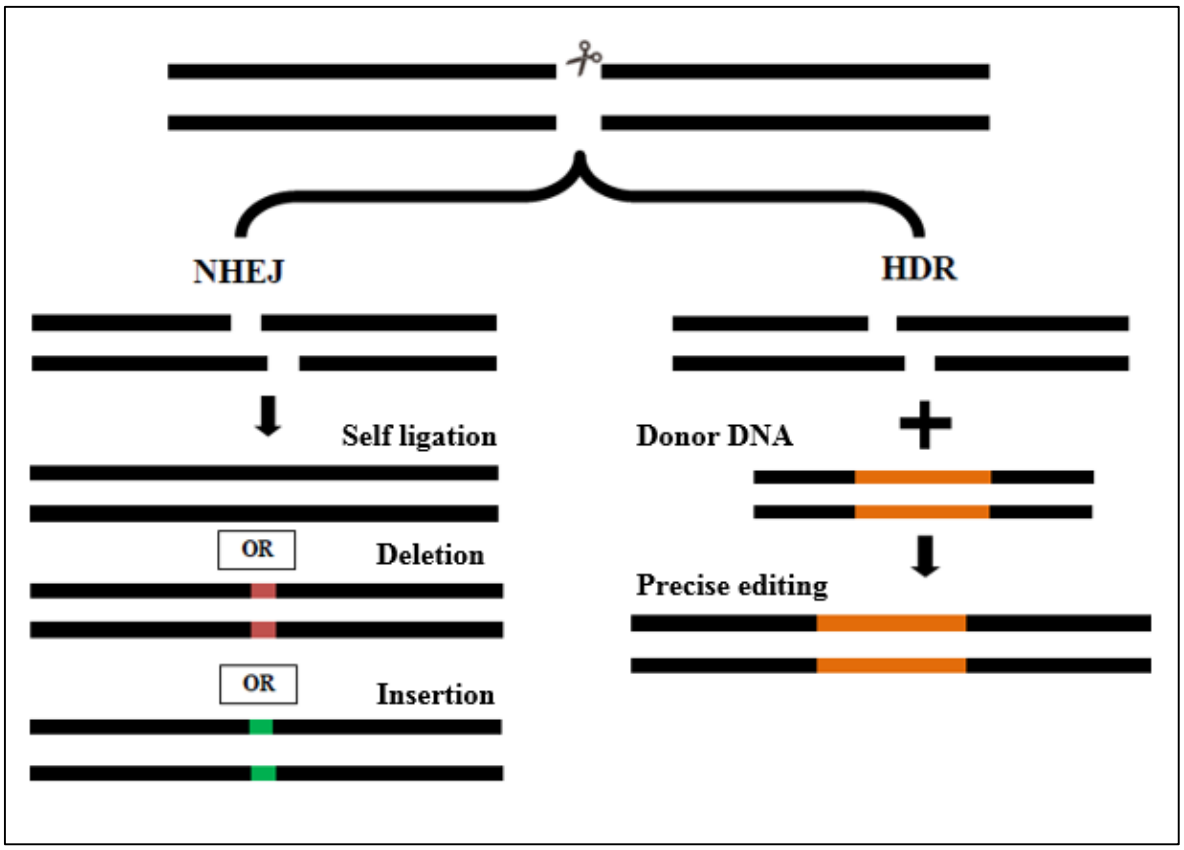

Figure 5.1. Illustration of NHEJ and HDR mediated repair

5.5. LEU2: BETA-ISOPROPYLMALATE DEHYDROGENASE

LEU2 gene encodes beta-isopropylmalate dehydrogenase enzyme that catalyzes conversion of 3-isopropylmalate to 4-methyl-2-oxopentanoate in L-leucine biosynthesis pathway. This pathway-specific step is the third step in the biosynthesis pathway and disruption of *LEU2* gene causes leucine auxotrophy.

5.6. AIM OF THE STUDY

Saccharomyces cerevisiae is an industrial workhorse with a broad range of applications in different areas. Well-documented genome information, well-established fermentation techniques and molecular methods render this yeast as a valuable model organism for studies of eukaryotic metabolism. Especially synthetic biology applications in *S. cerevisiae* are gradually increasing with the discovery of novel biological parts such as promoters, coding sequences, signal peptides, secretion machinery components etc. Besides, state of art technologies for genome engineering studies emerge for gene characterization and genome reduction studies.

This study aims to establish a CRISPR/Cas9 system for multiplex genome editing studies in *S. cerevisiae*. In this system, a guide RNA is designed specifically for the gene of interest, and cloned into an expression plasmid. A Cas9 expression plasmid is constructed using an appropriate promoter, *Cas9* gene and a terminator part included in MoClo Yeast Toolkit. Following, gRNA and Cas9 expression cassettes are cloned into a yeast backbone plasmid constructed during this study. The final construct, yeast CRISPR plasmid, is transferred into *S. cerevisiae* along with a repair template. Repaired transformants are analyzed for their loss of gene function. *LEU2* gene was used as gene of interest for system development.

6. MATERIALS

6.1. STRAINS AND PLASMIDS

S. cerevisiae CEN.PK113-7D is used as the host strain in this study. Parts for construction of expression plasmids were from Addgene MoClo Yeast Toolkit (Kit # 1000000061).

6.2. CHEMICALS

All media components, salts and antibiotics were purchased from Sigma Aldrich. dsoligos and primers were synthesized by SenteBioLab, Ankara. BsmBI (Catalogue No. R0580L) and BsaI-HFv2 (Catalogue No. R3733L) restriction enzymes and T4 DNA Ligase (Catalogue No. M0202M) were purchased from New England Biolabs (NEBs).

dNTPs were purchased from Invitrogen (Catalogue No. 10297018). Phusion DNA polymerase was puchased from NEBs (Catalogue No. M0530L). GeneRuler DNA Ladder Mix was purchased from Thermo Scientific (Catalogue No. SM0333). Machery-Nagel Nucleospin Gel and PCR Clean-up kit (Catalogue No. 740609.50) was used for DNA purification from both PCR tubes and agarose gel. Machery-Nagel Nucleospin Plasmid kit (Catalogue No. 740588.50) was used for isolation of constructs from *E. coli*.

6.3. EQUIPMENT

Biorad T100 Thermocycler was used for PCR studies and enzymatic incubations during plasmid construction. Biorad Gene Pulser Xcell Electroporation System was used for DNA transfer via electroporation.

Shaking incubations were performed in New Brunswick Scientific Innova Incubator Shakers. Cell growth and color development during assays were monitored using Genesys 10UV-VIS Spectrophotometer from Thermo Scientific.

Centrifuges (Eppendorf 5424), waterbaths (Grant SUB Aqua), laminar flow safety cabinet (GreenLab) were routinely used during the experiments.

7. METHODS

The overall construction workflow was provided in Figure 7.1.

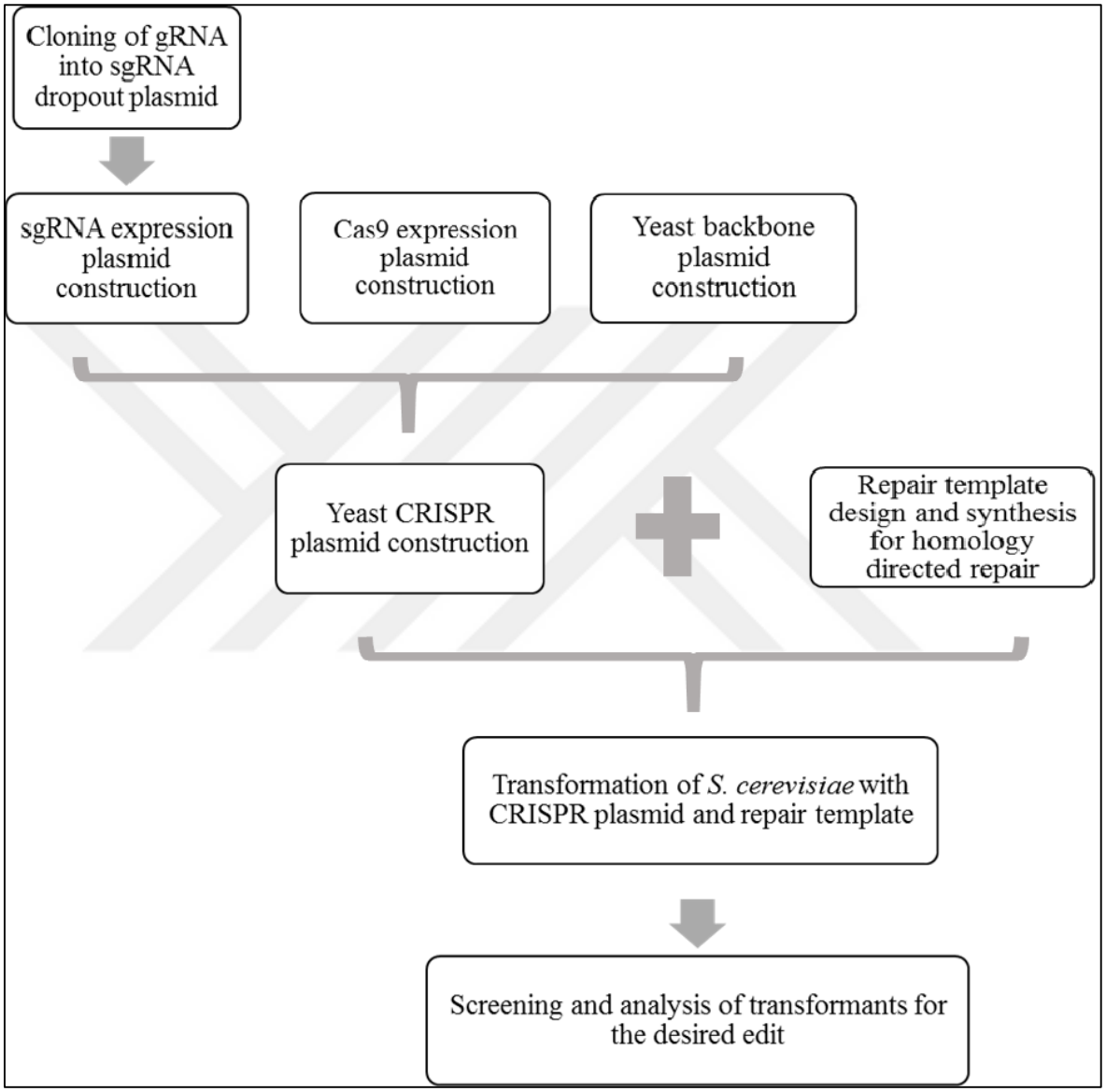

Figure 7.1. Flow chart of construction of CRISPR/Cas9 system for yeast

7.1. CONSTRUCTION OF CRISPR/CAS9 PLASMIDS FOR DISRUPTION OF LEU2 GENE IN YEAST

7.1.1. Construction of sgRNA Expression Cassette Plasmid

Two annealing oligonucleotides were ordered to target *LEU2* gene in *S. cerevisiae* (Figure 7.2.). These oligonucleotides act as crRNA.

Figure 7.2. Annealing oligonucleotides to target *LEU2* gene

In a 20 µl reaction, these oligos (100 µM) were mixed at a ratio of 1:1 to be annealed each other. Reaction was carried out in a thermocycler as an initial denaturation at 95°C for 5 min, annealing at 50°C for 15 min and 25°C for 15 min.

A digestion and ligation reaction was set with BsmBI restriction enzyme and T4 DNA ligase to clone annealed oligonucleotides into pYTK050 (sgRNA dropout plasmid). Conditions and reagents are listed in Table 7.1.

Table 7.1. BsmBI/T4 DNA ligase reaction conditions

Components	**Amount**	**Reaction Conditions**	
dsoligo	2 µl (50 µM)	42°C, 1.5 min	25 cycles
pYTK050	1 µl (200 ng)	16°C, 3 min	
BsmBI	1 µl	50°C, 5 min	
T4 DNA ligase	1 µl	80°C, 10 min	
10X T4 DNA ligase buffer	2 µl		
Nuclease free water	13 µl		
Total	20 µl		

2 µl of this reaction was directly transformed to 50 µl of *E. coli* DH5α competent cells by heat shock. Transformed cells were spread on LB+cam (25 µg/ml) plates. pYTK050 harbors *GFP* gene, thus cloning into this plasmid allows green/white screening. Couple of white colonies were transferred into LB+cam broth to be used for plasmid isolation. Resulting plasmid was named as **pYTK050+gRNA_*LEU2*** (Figure 7.3.).

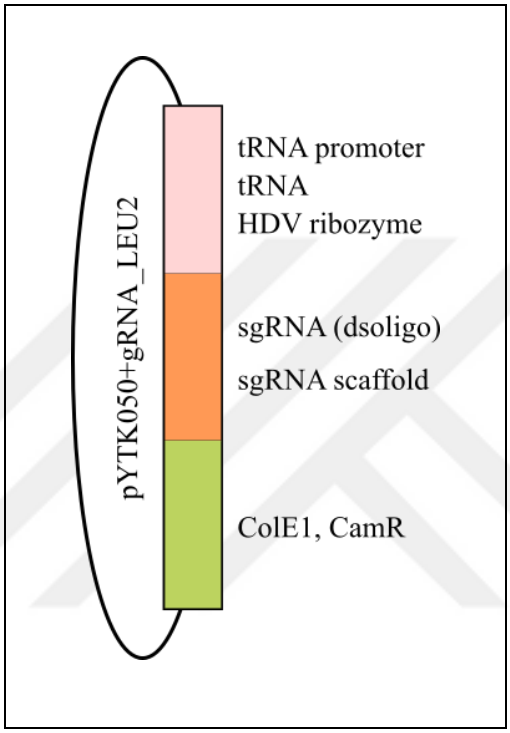

Figure 7.3. pYTK050+gRNA_*LEU2* plasmid map

Another digestion and ligation reaction was set with BsaI-HFv2 restriction enzyme, T4 DNA ligase, pYTK002, pYTK50+gRNA_*LEU2*, pYTK068 and pYTK095 plasmids to construct a bacterial **sgRNA expression cassette plasmid** with connector sequences. Reaction conditions and reagents are listed in Table 7.2.

Table 7.2. BsaI-HFv2/T4 DNA ligase reaction conditions

Components	**Amount**	**Reaction Conditions**	
pYTK002	100 ng	37°C, 5 min	30 cycles
pYTK50+gRNA_*LEU2*	100 ng	16°C, 5 min	
pYTK068	100 ng	55°C, 15 min	
pYTK095	100 ng	85°C, 20 min	
BsaI-HFv2	1.5 µl		
T4 DNA ligase	0.5 µl		
10X T4 DNA ligase buffer	2 µl		
Nuclease free water	12.75 µl		
Total	20 µl		

5 µl of this reaction was directly transformed to 50 µl of *E. coli* DH5α competent cells by heat shock. Transformed cells were spread on LB+amp (100 µg/ml) plates.

pYTK095 plasmid harbors *GFP* gene, thus cloning into this plasmid allows green/white screening. Couple of white colonies were transferred into LB+amp broth to be used for plasmid isolation. Resulting plasmid was named as **sgRNA_*LEU2* expression cassette plasmid** (Figure 7.4.).

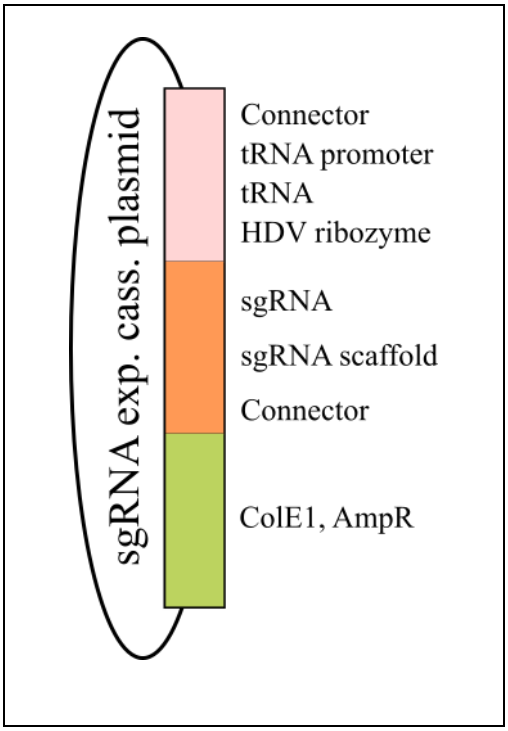

Figure 7.4. sgRNA_*LEU2* expression cassette plasmid map

7.1.2. Construction of Cas9 Expression Cassette Plasmid

A digestion and ligation reaction was set with BsaI-HFv2 restriction enzyme, T4 DNA ligase, pYTK004, pYTK011, pYTK036, pYTK054, pYTK072 and pYTK095 plasmids to construct a bacterial **Cas9 expression cassette plasmid.** Reaction conditions and reagents are listed in Table 7.3.

Table 7.3. BsaI-HFv2/T4 DNA ligase reaction conditions

Components	**Amount**	**Reaction Conditions**	
pYTK004	100 ng	37°C, 5 min	30 cycles
pYTK011	100 ng	16°C, 5 min	
pYTK036	100 ng	55°C, 15 min	
pYTK054	100 ng	85°C, 20 min	
pYTK072	100 ng		
pYTK095	100 ng		
BsaI-HFv2	1.5 µl		
T4 DNA ligase	0.5 µl		
10X T4 DNA ligase buffer	2 µl		
Nuclease free water	12.75 µl		
Total	20 µl		

5 ul of this reaction was directly transformed to 50 µl of *E. coli* DH5α competent cells by heat shock. Transformed cells were spread on LB+amp (100 µg/ml) plates. Couple of white colonies were transferred into LB+amp broth to be used for plasmid isolation. Resulting plasmid was named as **Cas9 expression cassette plasmid** (Figure 7.5.).

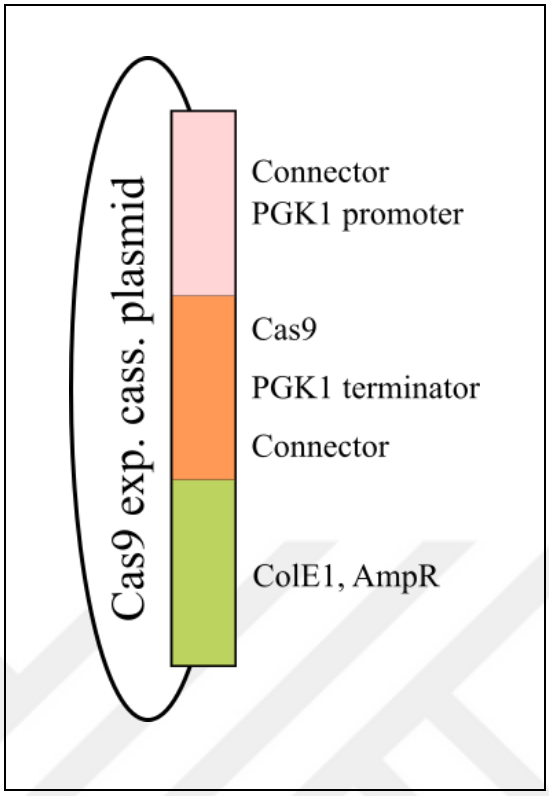

Figure 7.5. Cas9 expression cassette plasmid map

7.1.3. Construction of Yeast Backbone (YBB) Plasmid

A digestion and ligation reaction was set with BsaI-HFv2 restriction enzyme, T4 DNA ligase, pYTK008, pYTK047, pYTK073, pYTK079, pYTK081 and pYTK084 plasmids to construct a **yeast backbone plasmid**.

100 ng of each plasmid, 2 µl of 10X T4 DNA ligase buffer and 1 µl of BsaI-HFv2 were mixed in a total volume of 19 µl reaction. Tubes were incubated in a thermocycler at 37°C for 1 hour followed by a heat inactivation at 65°C for 20 min. At the end of the incubation, tubes were taken and 1 µl of T4 DNA ligase was added to each. Tubes were incubated in a thermocycler set at 25°C for 1 hour followed by 65°C for 20 min. 5 µl of this reaction was directly transformed to 50 µl of *E. coli* DH5α competent cells by heat shock. Transformed cells were spread on LB+kan (50 µg/ml) plates.

Couple of green colonies were transferred into LB+kan broth to be used for plasmid isolation. Resulting plasmid was named as **yeast backbone plasmid** (Figure 7.6.).

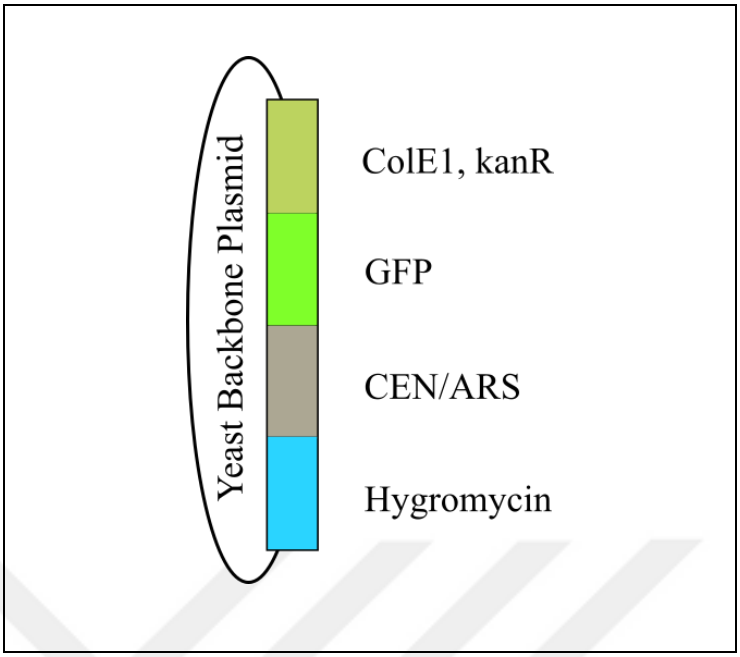

Figure 7.6. Yeast backbone (YBB) plasmid map

7.1.4. Construction of Yeast CRISPR Plasmid

sgRNA_*LEU2* expression cassette and **Cas9 expression cassette** were assembled into **yeast backbone plasmid** using BsmBI and T4 DNA ligase in order to construct a **yeast CRISPR plasmid**. Reaction components and conditions were listed in Table 7.4.

Table 7.4. BsmBI/T4 DNA ligase reaction conditions

Components	**Amount**	**Reaction Conditions**	
sgRNA_*LEU2* expression plasmid	100 ng	42°C, 5 min	30 cycles
Cas9 expression plasmid	100 ng	16°C, 5 min	
Yeast backbone plasmid	100 ng	50°C, 5 min	
BsmBI	1 µl	80°C, 10 min	
T4 DNA ligase	1 µl		
10X T4 DNA ligase buffer	2 µl		
Nuclease free water	13 µl		
Total	20 µl		

5 µl of this reaction was directly transformed to 50 µl of *E. coli* DH5α competent cells by heat shock. Transformed cells were spread on LB+kan (50 µg/ml) plates. Couple of white colonies were transferred into LB+kan broth to be used for plasmid isolation. Resulting plasmid was named as **yeast CRISPR plasmid** (Figure 7.7.).

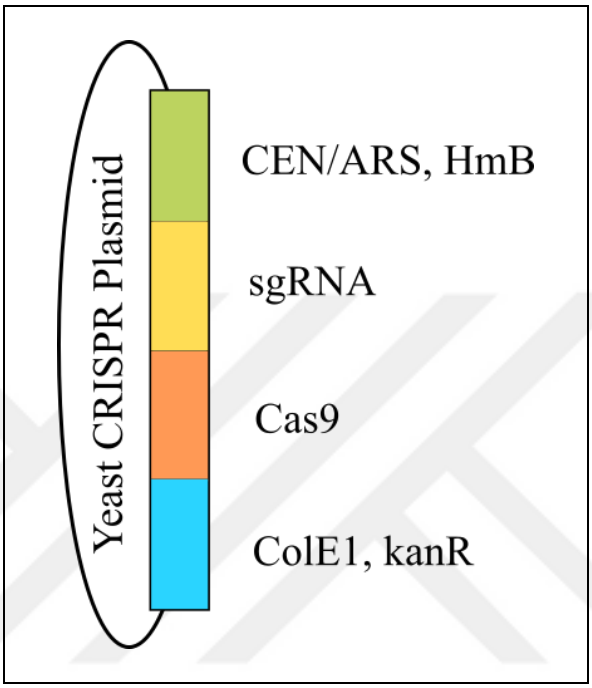

Figure 7.7. Yeast CRISPR plasmid (YCP) map

7.1.5. Construction of Repair Template for Homology Directed Repair

Repair template was designed as it introduces an in-frame stop codon in *LEU2* gene which leads to loss of gRNA sequence (Figure 7.8.). For this, a 80 bp primer was designed containing 60 bp homology arms to ensure homology directed repair and 20 bp sequence to introduce a stop codon (Table 7.5). 20 bp sequence is underlined in the table.

Table 7.5. Primer pair for repair template synthesis for *LEU2* gene

Primer name	**Sequence**
Rep_temp_F	CTAACTTTTCTTACCTTTTACATTTCAGCAATATATATATATATATTTCAAGGATATACCTAAAACTTGCGCTCAATTCC
Rep_temp_R	TCAGAAATAGCTTTAAGAACCTTAATGGCTTCGGCTGTGATTTCTTGACCAACGTGGTCAGGAATTGAGCGCAAGTTTTA

Using a standard PCR protocol, forward and reverse primers first annealed each other and then were utilized as the template in the presence of 0.4 mM dNTP, 2.5 mM $MgCl_2$, 1X Taq buffer and 1.25 U Taq polymerase. Resulting 140 bp PCR product was used as repair template. Reaction was performed with an initial denaturation of 95°C for 3 min, followed by 34 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and a final extension of 72°C for 10 min. Following, PCR products were extracted from gel using Macherey-Nagel Gel and PCR Clean-up kit.

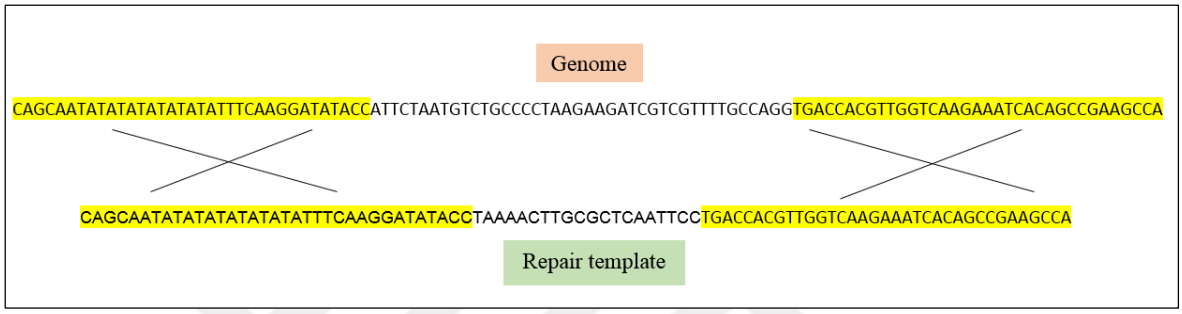

Figure 7.8. Repair template design to target *LEU2* gene via HDR

7.2. TRANSFORMATION WITH YEAST CRISPR PLASMID AND REPAIR TEMPLATE

40 µl of electrocompetent *S. cerevisiae* cells were mixed with 15 µl of purified repair template (~3 µg) and 3 µl of YCP (~1 µg) in an eppendorf tube. Mixture was transferred into 0.2 cm gapped ice-cold electroporation cuvettes and incubated on ice for 5 min. Cells were electroporated under 25 µF, 200 Ω, 1.5 kV. 1 ml of 1 M ice-cold sorbitol was immediately added to the cuvettes and cells were recovered by pipetting up and down. This solution was transferred into a 15 ml test tube and incubated for 2 h at 30°C, 150 rpm. After incubation, cells were spread on YPDS (YPD+sorbitol) agar plates containing 250 µg/ml hygromycin and then incubated at 30°C until colonies appear.

7.3. GROWTH OF TRANSFORMANTS IN CHEMICAL MEDIUM

Transformants grown on YPDS+HmB plates were transferred into Verduyn medium supplemented with or without leucine for further selection. Leucine concentration in Verduyn medium was adjusted to be 400 mg/L. Cells were grown at 30°C, 150 rpm.

7.4. VERIFICATION OF GENE DISRUPTION VIA CONVENTIONAL PCR

Culture grown in Verduyn medium supplemented with leucine was used as template for conventional PCR. First, 10 µl of culture was mixed with 5U/µl lyticase to lyse the cell membrane. This mixture was incubated at 30°C for 30 min. PCR was set up using 5 µl of lyzed mixture, 1X Taq buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 1.2 µM primers and 1.25 units of Taq Polymerase. Reaction volume was brought to 25 µl using nuclease free water. Reaction was started with an initial denaturation at 95°C for 5 min, followed by 34 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 45 sec and elongation at 72°C for 1 min. A final elongation step was carried out at 72°C for 10 min. A small amount of products were used for gel electrophoresis and the rest were cleaned up from the tubes.

8. RESULTS AND DISCUSSION

8.1. CONSTRUCTION OF CRISPR/CAS9 PLASMIDS FOR YEAST

Functional CRISPR/Cas9 system requires successful expression of each component in the cell. Therefore, promoter selection for accurate expressions of sgRNA and Cas9, and construction of relevant plasmids to maintain their activities are the main parameters in this system. Constitutive promoters with variable strength are commonly used for successful Cas9 expression while selection of the proper promoter for sgRNA expression is still under debate.

In the present study, expression of sgRNA for *LEU2* was driven by phenylalanine tRNA promoter included in sgRNA dropout plasmid (pYTK050) in MoClo Yeast Toolkit. pYTK050 also contains HDV ribozyme, tracrRNA and SNR52 terminator sequences required for construction of a functional sgRNA expression plasmid.

Maturation and nuclear localization of sgRNA is crucial for directing Cas9 to the target sequence [184]. These parameters are directly related to selection of promoter for sgRNA expression. Generally, RNA polymerase II or III promoters are widely used for efficient sgRNA expression with a few limitations. In *Pichia pastoris*, sgRNA expression was driven by RNA polymerase II promoters, however this caused multiplexing studies more challenging [160]. They suggested that when a sgRNA is properly fused to a downstream of tRNA sequence, knockout efficiency can be increased up to 95 per cent. As another example, SNR52 RNA polymerase III promoters were successfully used for sgRNA expression in *S. cerevisiae* since it allows maturation of sgRNA due to its native cut sites. However, synthetic promoters have been developed in this study via fusion of polymerase promoters and HDV ribozyme or a tRNA to obtain more efficient promoter activity [44]. It is known that tRNAs are highly conserved among microorganisms and their structural processes are performed precisely in the cell. Besides, it has been reported that utilization of tRNA gene and its cognate tRNA promoter can increase editing efficiency up to 93 per cent [184]. In line with the above ideas, Song *et al.* reported an example of using tRNA promoter and the tRNA gene together to express sgRNA in filamentous fungi. They claimed that carbon source independent transcription of tRNAs along with the constitutive expression and self-splicing ability render tRNA promoters a significant candidate for gene

editing studies in fungi and plants [185]. In this part of thesis, the phenylalanine promoter was fused to phenylalanine tRNA and HDV ribozyme sequences for efficient expression and improved process of downstream sgRNA. Since it is a modular assembly system, sgRNA dropout plasmid, pYTK050, has the promoter, tRNA, HDV and tracrRNA sequences, so only the crRNA sequence is synthesized every time the target loci changes. Thus, construction of a sgRNA expression plasmid becomes more cost effective and less time consuming.

Yeast CRISPR plasmid was successfully constructed as it contains *Cas9* gene and sgRNA for *LEU2* simultaneously. CRISPR plasmid was transformed into *S. cerevisiae* along with a related repair template which contains yeast homology regions and the desired edit. Repair template for *LEU2* was designed to introduce a stop codon within *LEU2* gene. As a result, disrupted gene sequence, consequently loss of function was expected (Figure 8.1.).

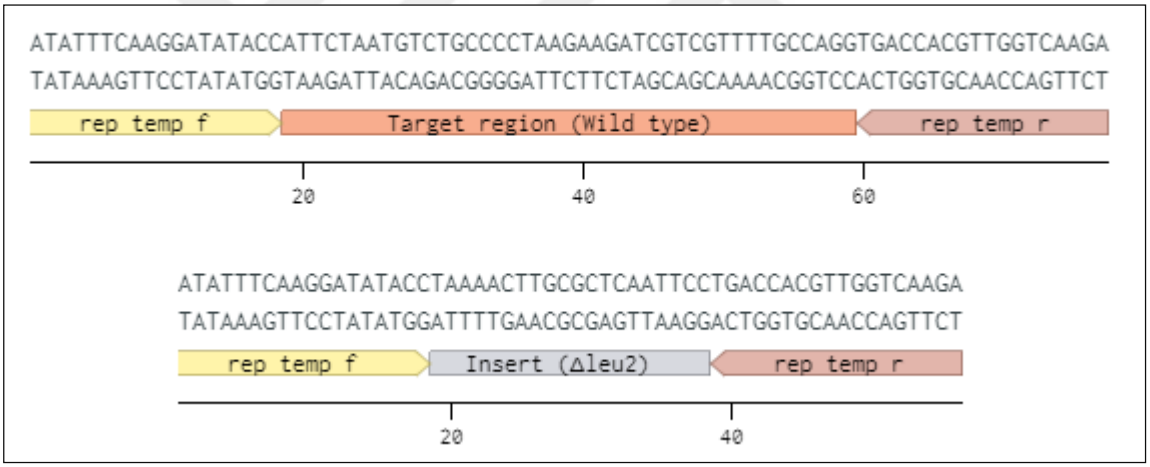

Figure 8.1. Sequence difference between wild type (top) and Δ*leu2* (bottom) strains. "Target region (Wild type)" annotation in the sequence at the top indicates the intact *LEU2* gene and "Insert Δ*leu2*" annotation in the sequence at the bottom indicates the insert used to introduce a stop codon in *LEU2* gene

8.2. VERIFICATION OF GENE DISRUPTION IN TRANSFORMANTS

Electroporated cells were spread on YPDS agar supplemented with hygromycin for *LEU2*. Here, hygromycin was used for selection of CRISPR plasmid. Figure 8.2. shows repaired transformants (Δ*leu2*) that contain yeast CRISPR plasmid.

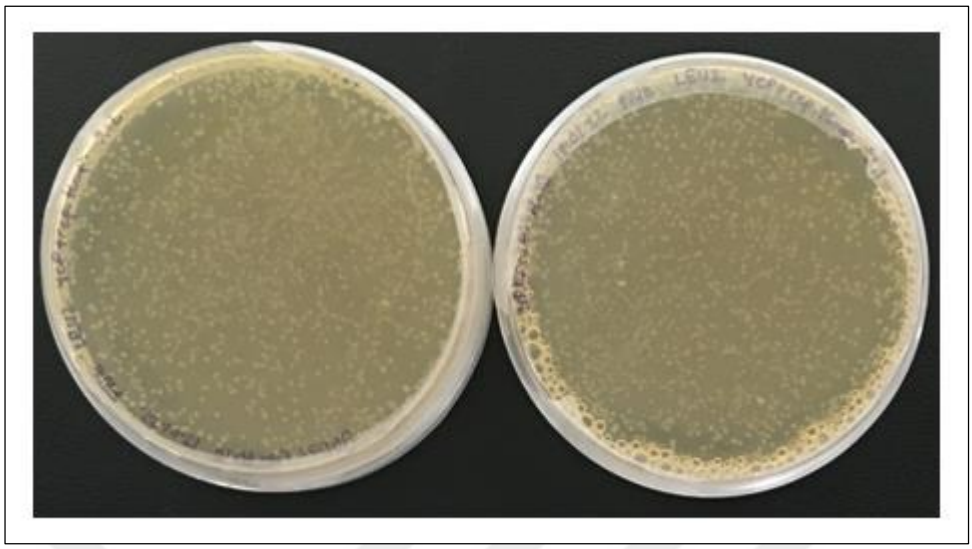

Figure 8.2. Repaired *S. cerevisiae* (Δ*leu2*) after transformation with CRISPR plasmid and repair template to disrupt *LEU2* gene

It is known that *S. cerevisiae* repairs damaged region via homologous recombination in the presence of a donor DNA since double-strand breaks occurred in any circumstances are toxic to the cells. Therefore even antibiotic selection for CRISPR plasmid may prove the repair event. However, it was decided to confirm successful repair by DNA sequencing for Δ*leu2* strains. For this, colonies grown on YPDS+HmB plates were randomly selected, grown in Verduyn and Verduyn+leu medium separately. Since Verduyn medium is a chemically defined medium in which wild type yeast can easily grow, transformants cannot grow unless they have an intact *LEU2* gene. Therefore, transformants whose *LEU2* gene was disrupted by an early stop codon sequence can only grow in Verduyn medium supplemented with leucine.

25 colonies was randomly selected and inoculated into Verduyn and Verduyn+leu medium. At the meantime, wild type yeast was grown in the same media and reached an OD_{600} values of 1.2 overnight. Five transformants grown in Verduyn+leu but Verduyn and two out of five reached an OD_{600} of 1.2 after 48 hours (Figure 8.3.). Three out of five reached an OD_{600} of 0.5 after 48 hours.

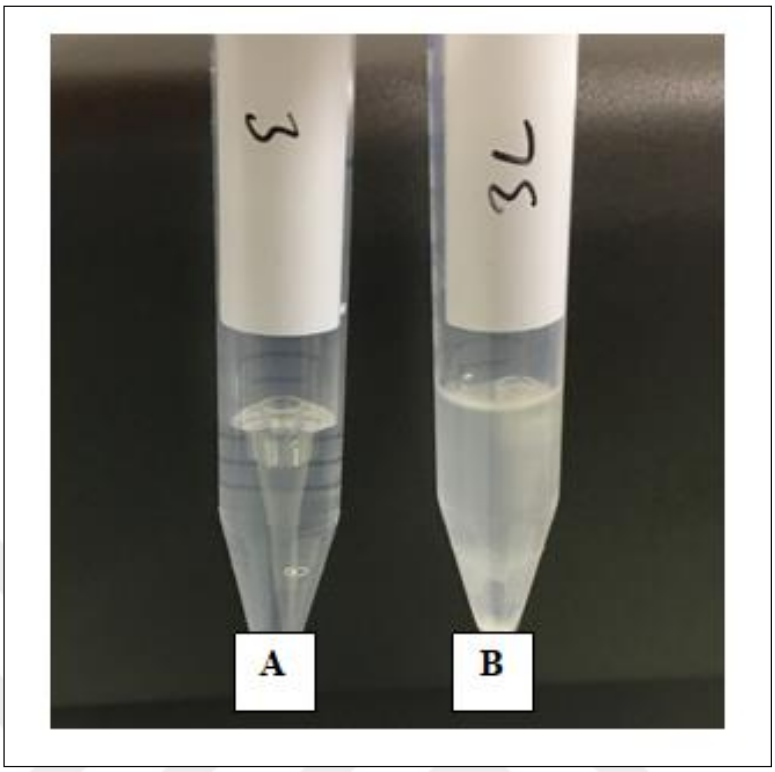

Figure 8.3. Culture grown in Verduyn (A) and Verduyn+leu (B) medium

Figure 8.3. represents a physiological confirmation of loss of *LEU2* gene since the colony cannot grow in the absence of leucine. Physiologically confirmed colonies need to be further confirmed.

9. CONCLUSIONS

Standardization of functional parts such as promoter, coding sequence, terminator, selection markers etc. and their precise assembly using standard protocols revolutionized heterologous protein expression in microorganisms.

In this thesis, a successful application of modular assembly systems in fungi was demonstrated. In this context, glucoamylase promoter was engineered to investigate carbon catabolite repression effect on this promoter. Putative binding motifs of catabolite responsive element, CreA, was identified on native Gla promoter and different promoters were derived from the native promoter by altering CreA binding motifs. Promoter variants were tested for their activity to express a heterologous protein in the presence of a repressing carbon source (xylose) and their ability to consume different carbon sources (xylose and maltose) simultaneously. A promoter variant that is relieved from the repressive effect of xylose and can be utilize xylose and maltose simultaneously was determined. This variant was used for expression of heterologous xylanase in protease-deficient fungi, as a result, sustained heterologous xylanase production under the control of an engineered Gla promoter was achieved. This study points out the effect of motif sequences present in a promoter region on gene expression. Integrative evaluation of host-gene-promoter relation was also demonstrated.

The “plug and play” strategy of modular assembly systems allows quick transfer of functional parts between plasmids. In this context, a backbone plasmid is constructed as it contains standard parts such as replication origin, selection marker, multiple cloning sites etc. This backbone is further used to construct various expression plasmids by cloning different transcriptional unit parts such as promoter, coding sequence and terminator while keeping the main backbone parts stable. Thus, it is possible to construct an expression vector successfully in a few days with less effort.

Additionally, a functional CRISPR/Cas9 system was established and applied for gene disruption in yeast. This study is a successful illustration of next generation techniques in the synthetic biology context.

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