DESIGN AND CONSTRUCTION OF DOUBLE PROMOTER SYSTEMS AND THEIR USE IN PHARMACEUTICAL PROTEIN PRODUCTION IN *P. pastoris*

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DESIGN AND CONSTRUCTION OF DOUBLE PROMOTER SYSTEMS AND THEIR USE IN PHARMACEUTICAL PROTEIN PRODUCTION IN *P. pastoris*

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ABSTRACT

DESIGN AND CONSTRUCTION OF DOUBLE PROMOTER SYSTEMS AND THEIR USE IN PHARMACEUTICAL PROTEIN PRODUCTION IN *P. pastoris*

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Intracellular phenomena such as promoter strength, mRNA secondary structure, translation efficiency and codon preference, 5′-untranslated region processing, and protein turnover, have impacts directly on the expression of heterologous genes. Design of multi-promoter expression systems with constituent strong promoters and engineered promoter variants is a novel metabolic engineering strategy for increasing the promoter strength further, and tuning the expression for recombinant protein (r-protein) production with enhanced production and productivity in the yeast *P. pastoris*. Double-promoter expression systems (DPESs) carrying *enhanced green fluorescent protein* (*eGFP*) and *red fluorescent protein* (*mApple*) genes were designed and constructed for the synthesis of the heterologous model proteins mApple and eGFP in order to determine and justify the expression period of each promoter that can be either simultaneously, or by consecutively stimulating the changeover from one to another in a biphasic process or via successive-iterations in methanol-free

media, on glucose, glycerol or ethanol. A library of expression cassettes was designed with single naturally occurring promoters (NOPs) and novel-engineered promoter variants (NEPVs) having distinct regulatory properties: ***i)*** a NEPV of alcohol dehydrogenase1 gene (*ADH1*), ***ii)*** a NEPV of alcohol oxidase 1 gene (*AOX1*), and ***iii)*** P_{GAP}; and *P. pastoris* DPESs have been constructed as separate expression cassettes. Novel *P. pastoris* strains were constructed with the DPESs and tested in the fed-batch phase of the fermentation of the carbon sources 2% (v/v) ethanol, excess glucose, and excess glycerol. *P. pastoris* DPESs having double expression cassettes each having a different antibiotic resistance gene were constructed, denoted by; ***i)*** pADH2-Cat8-L2::*mApple* and pGAP::*eGFP*, ***ii)*** pADH2-Cat8-L2::*mApple* and pmAOX1::*eGFP*, ***iii)*** pADH2-Cat8-L2::*mApple* and pADH2-Cat8-L2::*mApple*, ***iv)*** pGAP::*eGFP* and pGAP::*eGFP* ***v)*** pmAOX1::*eGFP* and pmAOX1::*eGFP*. The proteins mApple and eGFP were expressed in the novel *P. pastoris* strains constructed with: ***i)*** pADH2-Cat8-L2::*mApple* and pGAP::*eGFP* consecutively and also simultaneously; ***ii)*** pADH2-Cat8-L2::*mApple* and pmAOX1::*eGFP* bifunctionally and simultaneously; ***iii)*** pADH2-Cat8-L2::*mApple* and pADH2-Cat8-L2::*mApple*; ***iv)*** GAP::*eGFP* and pGAP::*eGFP*; ***v)*** pmAOX1::*eGFP* and pmAOX1::*eGFP* simultaneously as identical-twin promoters. $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ DPES increased the production capacity on ethanol 2.1-fold compared to that with the single NEPVs $P_{ADH2\text{-}Cat8\text{-}L2}$ and P_{mAOX1}, respectively. With $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$, the expression increased to 1.3-fold on ethanol compared to that with identical-twin promoters. With simultaneously-operating $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ the expression was 1.6-fold higher than the consecutively-operating $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$

on ethanol. Strength of the DPESs were tested in fermentations for extracellular human growth hormone (rhGH) production. Secreted rhGH yields ($Y_{P/X}$, mg/g_{DW}) by novel *P. pastoris* strains constructed with $P_{ADH2\text{-}Cat8\text{-}L2}$, P_{mAOX1}, P_{GAP}, $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$, $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$, and $P_{ADH2\text{-}Cat8\text{-}L2+ADH2\text{-}Cat8\text{-}L2}$ were as 2.95, 3.00, 0.13, 4.86, 3.73 and 4.21 mg/g at t = 48 h of the fermentations on ethanol, respectively.

Keywords: *Pichia pastoris*, Double Promoter Expression Systems, Promoter Engineering, Naturally Occurring Promoter (NOP), Novel Engineered Promoter Variants (NEPV), Promoter Strength, Fed-Batch Fermentation

ÖZ

P. pastoris İKİLİ-PROMOTÖR SİSTEMLERİ TASARIMI VE OLUŞTURULMASI İLE FARMASÖTİK PROTEİN ÜRETİMİNE KATKISI

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Promotor gücü, mRNA sekonder yapısı, translasyon etkinliği ve kodon seçimi, 5′-çevrilmemiş- (untranslated-) bölge işleme ve protein devri gibi hücreiçi olaylar heterolog genlerin ekspresyonu üzerinde doğrudan etkilidir. Promotor kuvvetini artırmak için, kuvvetli promotorleri ve mühendislik promotor varyantlarını içeren çoklu-promotor ekspresyon sistemleri tasarımı, *Pichia pastoris*'te rekombinant protein (r-protein) üretimi ve verimliliği artırmak için yeni metabolik mühendislik stratejisidir. *mApple* ve *eGFP* genlerini taşıyan ikili-promotor ekspresyon sistemleri (DPES), iki-fazlı prosesin üretim fazında her promotorun eşanlı veya ardışık-tekrarlı birinden-diğerine geçen ekspresyon peryodlarını belirlemek ve kesinleştirmek için, heterolog proteinler mApple and eGFP sentezi için tasarlanmış ve oluşturulmuştur. Ekspresyon-kaset kütüphanesi, bir doğal promotor (NOP) ve iki mühendislik yapılmış yeni promotor varyantı (NEPV) i) alkol dehidrojenaz-1 geni (*ADH1*) için NEPV, ii) alkol oksidaz-1 geni (*AOX1*) için NEPV, ve iii) P_{GAP} (NOP), ile önce tasarlanmış; sonra *P. pastoris* ikili-promotor ekspresyon sistemleri (DPES) ayrı-ayrı ekspresyon kasetleri olarak tasarlanmış ve oluşturulmuştur. Oluşturulan yeni suşlar ile bifazik *P. pastoris* %2 etanol, aşırı glikoz ve aşırı gliserol fermentasyon koşullarında test edilmiştir. *P. pastoris* her biri farklı antibiyotik direnç genine sahip iki ekspresyon kasetli ikili-promotor ekspresyon sistemleri; i) pADH2-Cat8-L2::*mApple* ve

pGAP::*eGFP*, ii) pADH2-Cat8-L2::*mApple* ve pmAOX1::*eGFP*, iii) pADH2-Cat8-L2::*mApple* ve pADH2-Cat8-L2::*eGFP*, iv) pGAP::*eGFP* ve pGAP::*eGFP* v) pmAOX1::*eGFP* ve pmAOX1::*eGFP* tasarlanıp oluşturulmuştur. mApple ve eGFP proteinleri : i) pADH2-Cat8-L2::*mApple* ve pGAP::*eGFP* ardışık / eşanlı; ii) pADH2-Cat8-L2::*mApple* ve pmAOX1::*eGFP* bifonksiyonel ve eşanlı; iii) pADH2-Cat8-L2::*mApple* ve pADH2-Cat8-L2::*mApple*; iv) pGAP::*eGFP* ve pGAP::*eGFP*; v) pmAOX1::*eGFP* ve pmAOX1::*eGFP* ikiz-promotorlarla eşanlı, oluşturulan yeni *P. pastoris* suşlarında ekspres edilmiştir. Oluşturulan DPES $P_{mADH+mAOX1}$ ve tek-promotorlu $P_{ADH2\text{-}Cat8\text{-}L2}$ ve P_{mAOX1} ekspresyon sistemleriyle kıyaslandığında floresans protein üretimi 2.1-kat artmıştır. Eşzamanlı-çalışan $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ ile etanoldeki ekspresyon $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$ ile ardışık-çalışan DPES'e göre 1.6-kat artmıştır. DPES'lerin gücü, hücre-dışı rekombinant insan büyüme hormunu üretimi için yapılan fermentasyonlarda denenmiştir. Fermentasyon prosesinin t = 48 st'inda, $P_{ADH2\text{-}Cat8\text{-}L2}$, P_{mAOX1}, P_{GAP}, $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$, $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$, ve $P_{ADH2\text{-}Cat8\text{-}L2+ADH2\text{-}Cat8\text{-}L2}$ ile oluşturulan *P. pastoris* hücrelerinden biyoreaktör üretim ortamına aktarılan rhGH verimleri, sırasıyla, 2.95, 3.00, 0.13, 4.86 3.73 ve 4.21mg/g'dır.

Anahtar Kelimeler: *Pichia pastoris*, İkili-Promotor Ekspresyon Sistemleri, Promotor Mühendisliği, Doğal Promotor, Mühendislik Yapılmış Yeni Promotor Varyantı, Promotor Gücü, Yarı-Kesikli Fermentasyon

To My Lovely Family

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C	Concentration in the medium	g L-1 or mol m-3
min.	Minute	
OD600	Optical density at 600 nm	
SD	Standard deviation	
t	Cultivation time h	
X	Cell amount (mass) g	
Y	Yield g g^{-1}	
α-MF	*Saccharomyces cerevisiae* mating factor	
max	Refers to maximum amount of a parameter	
P/s	Refers to product over substrate	
P/x	Refers to product over biomass	
x/s	Refers to biomass over substrate	
ADH	Alcohol dehydrogenase	
AOX	Alcohol oxidase	
ARG4	Argininosuccinate lyase	
BLAST	Basic local alignment search tool	
BMGY	Buffered minimal glycerol complex medium	
bp	Base pair	
BP	Base plasmid	
DNA	Deoxyribonucleic acid	
EDTA	Ethylene diamine tetra acetic acid	

GRAS	Generally regarded as safe
GTF	General transcription factor
LB	Luria broth (lysogeny broth)
PCR	Polymerase chain reaction
PTM	Post-translational modification
PTM1	Pichia trace minerals medium
rDNA	Recombinant DNA
rhGH	Recombinant human growth hormone
RNA	Ribonucleic acid
RT	Room temperature
SD	Standard deviation
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TCA	Tricarboxylic acid
TE	Tris EDTA
TEF	Translation elongation factor
TF	Transcription factor
TFBS	Transcription factor binding site(s)

CHAPTER 1

INTRODUCTION

P. pastoris has gained attention as a host for recombinant protein (r-protein) production since it allows high level expression with naturally occurring promoters (NOPs) and engineered novel promoter variants (NEPVs), both of which operate in methanol-free media besides the toxic methanol. In addition, these systems are used in high cell density fermentations, hence simplifying downstream purification and regulatory documentation (Gasser, Steiger and Mattanovich, 2015)(Gasser *et al.*, 2013)(Massahi and Çalık, 2018). Efficient and tunable promoter selection is crucial for r-protein production as it directly relates to transcription of the target gene, which is the first step of protein synthesis, where in *P. pastoris* these are often derived from carbon-source utilization pathways. Several inducible and constitutive *P. pastoris* promoters have been identified with distinct properties and strengths (Çalık et al., 2015a)(Massahi and Çalık, 2018). Further promoter engineering and transcription engineering strategies have been developed and applied to understand the functioning of promoters and regulation behind the TFs and to improve the expression strength of promoters.

P. pastoris alcohol dehydrogenase 2 (*ADH2*) gene encodes alcohol dehydrogenase 2 enzyme which catalyzes the first reaction in the ethanol utilization pathway (EUT). *P. pastoris ADH2* promoter (P_{ADH2}) is induced by ethanol (Cregg and Tolstorukov, 2012).

ADH2 (PAS chr2-1 0472) functional assessment discovered that it is the only gene involve in ethanol utilization in *P. pastoris*, However knock-out forms of the ADH2 strain still produce ethanol (Karaoglan, Karaoglan and Inan, 2016c). P_{ADH2} expression capacity in recombinant xylanase production was evaluated and-compared with P_{AOX1} and P_{GAP} (Karaoglan, Karaoglan and Inan, 2016a)(Karaoglan, Karaoglan, & Inan, 2016b) . For the enhancement of heterologous protein production in *P. pastoris*, novel metabolic engineering strategies need to be investigated such as design of recombinant system with different NEPVs and NOPs. Designing double-promoter expression system (DPES) is a promising approach to increase r-protein production. Guan et al. (2016) reported that DPES significantly increase the production capacity of target protein by enhancing the transcriptional activity. DPES can be defined as the combinatorial use of two promoters that control the transcription of same gene or different genes. Therefore, it needs to be designed to maintain higher specific product formation rate within the production domain (Yang *et al.*, 2013). Development and optimization of fermentation conditions is essential in order to fulfill both promoters requirements as each promoter has unique expression conditions to reach their maximum potential (Öztürk, Ergün and Çalık, 2017). Double promoter expression systems enhance specific product formation to the upper limit of production domain. Thus, to improve productivity and production, transcription activity of both promoters should be satisfied either simultaneously or consecutively by shifting from one promoter to other promoter. Based on operational mechanism, double promoter expression systems can be grouped into two classes. The first is consecutively (CNT-

) operating double promoter system and the other is simultaneously (SMT-) operating double promoter system. Simultaneously operating double promoter system can be classified as; i) identical twin promoter system constructed by the same promoter ii) non-identical twin promoter system constructed by a promoter and its variant iii) bifunctional double promoter system constructed by two different promoters (Öztürk, Ergün and Çalık, 2017).

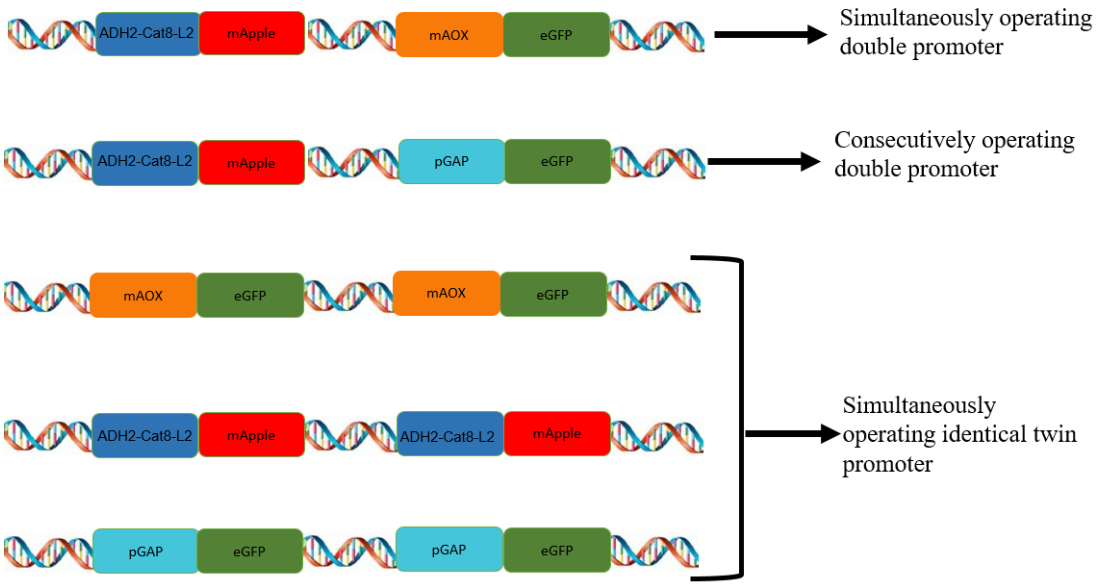


Figure 1. 1 a. Simultaneously (SMT-) operating bifunctional double promoter b. Consecutively (CNT-) operating bifunctional double promoter c. Simultaneously (SMT-) operating identical twin promoter d. Simultaneously (SMT-) operating identical twin promoter e. Simultaneously (SMT-) operating identical twin promoter

Among the different approaches for construction of eukaryotic double promoter expression systems, *P. pastoris* double promoter expression systems were constructed through sequential transfection with bipartite vectors including two expression cassettes (Öztürk, Ergün and Çalık, 2017).

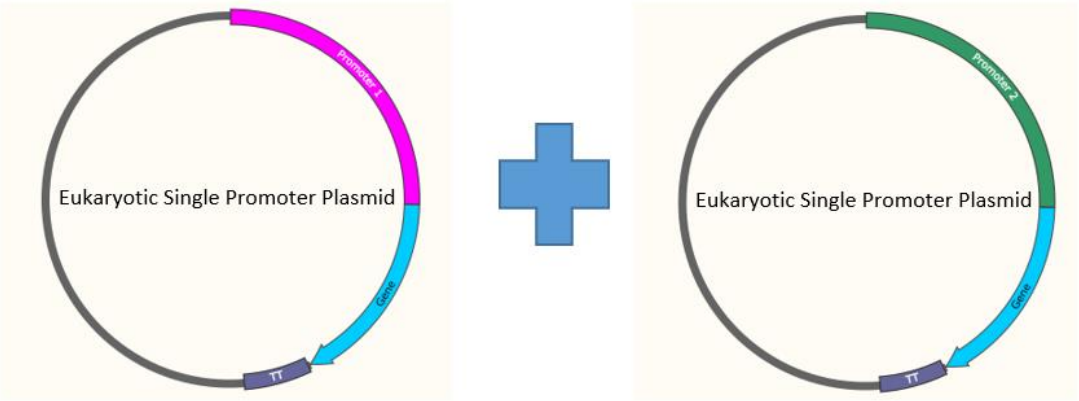


Figure 1. 2 Design of two single promoter plasmids for sequential transfection in eukaryotic microorganisms

In this MSc Thesis, DPESs were designed and constructed using a strong NOP and the superior NEPVs which designed in Industrial Biotechnology and Metabolic Engineering Laboratory at METU (Ergün *et al.*, 2019) for extracellular r-protein production. To determine and identify individual and simultaneous expression strengths of the NEPVs P_{mAOX1}, $P_{ADH2\text{-}Cat8\text{-}L2}$, and P_{GAP}, two reporter proteins were used. For the reliable comparison of the single- and double- promoter expression systems, *P. pastoris* strains carrying single- and multi-copy reporter genes which identified by qPCR analyses, were also constructed. Fermentations in shake-bioreactors were conducted to evaluate the potential of double-promoter expression systems compared to *P. pastoris* cells that either produce eGFP with P_{GAP}/P_{mAOX1}, or mApple with $P_{ADH2\text{-}Cat8\text{-}L2}$. Enhanced-green-fluorescent-protein (eGFP) and red-fluorescent-protein (mApple) were cultivated as intracellular reporter gene products, and fluorescent protein synthesis was measured quantitatively with fluorescence spectrophotometer. The strength of the DPESs also was tested in fermentations for extracellular human growth hormone (rhGH) production.

CHAPTER 2

LITERATURE SURVEY

2.1. Target Proteins

Red fluorescent protein (mApple) and green fluorescent protein are widely used as reporter gene in double promoter expression systems (DPESs). Recombinant human growth hormone (rhGH) is a therapeutic recombinant protein produced extracellularly that is used to further confirm the strength of DPESs in r-protein production.

2.1.1. Human growth Hormone

Recombinant human growth hormone (rhGH) is pharmaceutically important therapeutic protein having a molar mass 22 kDA and 191 amino acid residues linked by disulfide bridges in two peptide loops and primary structure of rhGH can be seein in Figure 2.1. Human growth hormone is used to treat diseases such as dwarfism, injuries, bone fractures, burns and bleeding ulcers (Tritos and Mantzoros, 1998) and can be identified as one of the most vital hormones of the body since it plays wide range of role in biological functions (Kim *et al.*, 2013). Besides other features, hGH affect human metabolism including inhibition of glucose metabolism, involved in lipolysis and protein synthesis. The first human growth hormone was isolated from cadavers' pituitary glands extracts in the 1950s. Recombinant hGH was first express in *E. coli* in 1979 (Goeddel *et al.*, 1979). Today, rhGH is still expressed by bacteria but, due to presence of over expressed protein as aggregates production is very costly and even exceed $ 20,000 annually (Cunha *et al.*, 2011), different microorganism is used to produce rhGH.

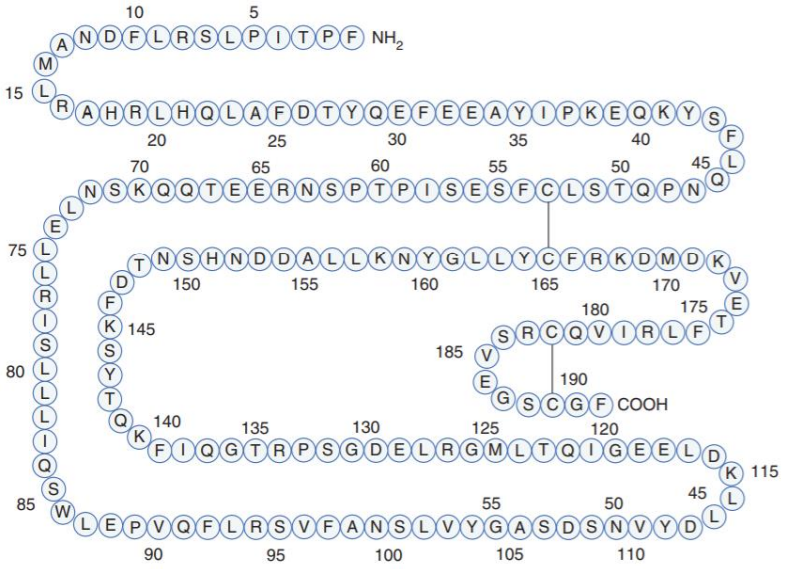


Figure 2. 1 Primary structure of recombinant human growth hormone

hGH have different isoforms such as disulfide-linked dimers, oligomers, 20 kDa monomer and other modified forms (Lewis, 2008). Different gene products, different mRNA splicing and posttranslational modifications cause occurrence of the 20 kDa monomer, dimers, oligomers, and other modified forms. Expression levels of these isoforms are usually less than 22 kDa hGH (Baumann, 2009).

2.1.2. Fluorescence Proteins

Many marine organisms undergo chemiluminescent or fluorescence processes to produce light. In the early 1960's Osamu Shimomura's identified the molecular basis for the glow of the jellyfish. Later, Shimomura and Frank Johnson developed a method to extract the emitted light from Aequorea Victoria (Shimomura, H. Johnson and Saiga, 1962). Characteristic of fluorescence proteins were tabulated in Table 2.1.

Table 2. 1 *Fluorescence characteristic of mApple and eGFP*

Protein (acronym)	Ex/nm	Em/nm	Quantum yield	Quaternary structure
eGFP	488	509	0.60	Monomer
mApple	568	592	0.49	Monomer

All fluorescent proteins (FPs) are approximately 25 kD in size, which can be considered as large compared to organic fluorophores such as fluorescein. Despite their relatively large size, FPs are beneficial for many applications, i.e. for live-cell and whole-animal imaging. In addition, FPs can also be fused to their protein targets, so that they are expressed in a 1:1 ratio with the target molecule which makes them ideal for quantitative imaging (Knobel *et al.*, 2009). FP is composed of rigid β-barrel structure that surround a central α-helix (Ormo *et al.*, 1996). Due to fluorescent proteins unique β-barrel structure, fluorescent properties can significantly change with mutations of residues throughout the whole protein. The most important effect of mutations is the wide range of different emission and absorption spectra, which can greatly contribute to enhance the application field of these proteins (Shaner, Patterson, and Davidson 2011).

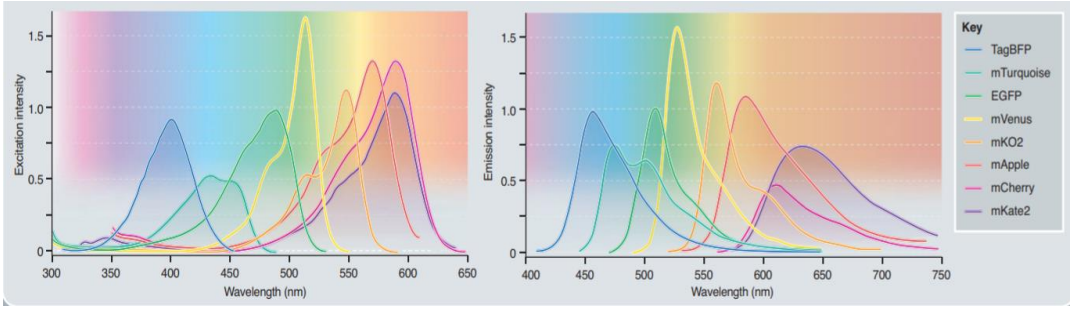


Figure 2. 2 Excitation and Emission spectra of different fluorescent proteins

FPs are highly practical as reporters for gene-expression studies in cultured cells. Some criteria should be taken into consideration while choosing FPs. First of all, FP should be expressed efficiently and non-toxicity in the chosen cell culture, and its brightness should be high enough to exceed auto fluorescence. Second, FP should have sufficient photo stability in order to be detected after experiment. Third for the more than one labeling experiment, the pair of FPs used should have minimal crossover in the excitation and emission spectra. Finally, FPs should be insensitive to environmental effects that cause interpretation of quantitative experimental results (Shaner, Steinbach and Tsien, 2005). Furthermore, quantum yield, protein stability and photo stability are the other key parameters that should be considered while choosing FPs. Combination of light absorbance and fluorescence quantum yield is brightness of FP. Some FPs turn over quickly or change color over time. Higher the protein stability longer the change over time. Photo stability can be defined as how fast the probe photo bleaches.

2.1.2.1. Red Fluorescent Protein (mApple)

The red fluorescent protein (mRFP) cloned from *Discosoma* greatly contributes the biotechnology and cell biology since it has distinct spectra and pairs well with eGFP (Shaner *et al.*, 2008). For multicolor tracking extending the spectrum from red to green would provide distinct label as RFP has longer excitation and emission wavelength than GFP (Mizuno *et al.*, 2001). eGFP and mApple displayed a moderate level of sensitivity compared with the other fluorescent proteins

2.1.2.2. Enhanced Green Fluorescent Protein (eGFP)

The GFP chromophore is formed spontaneously via self-catalyzed protein folding and intramolecular rearrangement. Rather than molecular oxygen, this spontaneous reaction occurs without requirement for cofactors and external enzyme components (Chalfie and Kain, S.R. (Agilent, 2007). Moreover, GFP fluorescent protein (FP) is

encoded by the primary amino acid sequence. For the first time GFP enabled to the labeling specific protein without antibody-labeled fluorescent tags in living organism (Patterson *et al.*, 1997). GFP is used in *P.pastoris* for several aims including tracking proteins (Heiss *et al.*, 2013), monitoring organelles (Gasser *et al.*, 2013) and screening different promoter and its variants (Ruth *et al.*, 2010).

2.2. Selection of the host microorganism

Designing optimal recombinant expression system mainly requires selection of suitable expression host organism (Macauley-Patrick *et al.*, 2005). For the high yield and high-quality recombinant protein stress responses of the host should be investigated to overcome the problems about the production of foreign proteins. Moreover, suitable host should grow rapidly on minimal cheap media at moderate conditions, allow post-transitional modifications and easy scale-up. One important aspect is the byproduct formation. An ideal host can able to keep the byproduct formation at minimum level as much as possible (Ferrer-Miralles *et al.*, 2009). For each foreign gene that is expressed in host organism, genetic and fermentation conditions should be considered as there is no single system optimum for all proteins (Çelik and Çalik, 2012).

For the production of both eukaryotic and prokaryotic proteins, bacterial systems can be suitable vehicles like *Escherichia coli*. (*E. coli*). *E. coli* is mostly preferable in cloning experiments in research as it allows easy genetic manipulations (Ferrer-Miralles *et al.*, 2009). High amount production was achieved in fed-batch fermentation system due to fat and easy growth on minimal medium (Demain and Vaishnav 2009). Most eukaryotic proteins do not require post-translational modifications (PTMs) which makes this system the cheapest, easiest and quickest expression host for structurally simple proteins (Corchero et al. 2012). However, for S-S rich and large protein expression requirement of PTMs will result in difficulties (Daly and Hearn, 2005). S-S bond formation, phosphorylation, and proteolytic cleavage are the key steps for functional protein production and lack of the required machinery for PTMs

may result in inactive, unstable or insoluble protein formation (Ferrer-Miralles *et al.*, 2009). However, high purification steps of product is needed like urea solubilization of inclusion bodies and acid precipitation (Patra *et al.*, 2000). Moreover, another important drawback of *E. coli* is the lack of general secretory system that results in inefficient protein secretion (Corchero et al. 2012). Another shortcoming is proteolytic cleavage and it can be overcome by deletion of several proteases (Graumann and Premstaller, 2006).

B. subtilis is another common bacterial host that is involved in recombinant protein production. It is gram negative bacteria that has powerful secretion capacity (Schmidt, 2004). Although it is able to secrete the molecules into the fermentation medium, availability of rich genetic data and being cost friendly microorganism, proteolytic degradation is observed after production. Besides, main disadvantages of *B. subtilis* is the huge amount of protease production that results in cleavage of the protein (Demain and Vaishnav 2011).

Eukaryotic microorganism categories are fungi, mammalian cells, insect cells, transgenic animals, plants and yeast.

Yeast is an excellent model for studying the gene expression, promoter strength and protein production due to its simplicity as a single-celled organism with short and well-defined promoter regions, genetic manipulation simplicity and availability of rich functional genomic data (Tirosh, 2009).Yeast like S. cerevisiae or *P. pastoris* quite different from their bacterial similitude due to their intrinsic complexity of eukaryotic transcription. Consequently, yeast promoters requested hundreds of base pairs to reach high transcriptional capacity. Minimal core elements can be made possible to establish high-strength promoters with less base pairs (Redden, 2015).

As expression host yeasts can be classified in two groups: Crabtree positive (respiro-fermenting) and Crabtree negative (respiring). Crabtree positive microorganism can produce ethanol under aerobic conditions as these phenotypes can performed respiration and fermentation under aerobic condition simultaneously. In the presence of oxygen Crabtree positive yeast such as *S. cerevisiae, Vanderwaltozyma polyspora,*

Torulaspora franciscae, Lachancea waltii, Lachancea kluyverii can exhibit alcoholic fermentation until low glucose concentration (Hagman, Säll and Piškur, 2014). Crabnegative yeast such as *Yarrowia lipolytica*, *Candida albicans*, *Eremothecium coryli* and *P. pastoris* can prefer respiratory growth instead of fermentative growth.

Yeasts are classified as methylotrophic and non-methylotrophic yeasts. Non-methylotrophic yeast: *S. Cerevisiae, P. stipitis, K. Lactis, Y. Lipolytica* and *S. occidentalis* can consume wide range of carbon and energy source (Porro et al., 2005). Methylotrophic yeasts have the methanol assimilating capabilities thus methanol can be used as sole carbon source by these yeast: *Candida*, *Torulopsis, Pichia*, and *Hansenula* (Gellissen *et al.*, 1992) (Hollenberg and Gellissen, 1997).

Table 2. 2 *Comparison of host microorganism for recombinant protein production (Demain AL, 2009) (Vogl, 2013) (Berlec A, 2013)*

	Higher eukaryotes	**Yeast**	**Bacteria**
Cultivation	Slow growth rates expensive complex growth media	Fast and robust growth, defined minimal media	Fast growth, defined minimal media
Contamination	Risk of viral contamination	Little risk of viral DNAs	Possible phage infections
Ease of genetic manipulation	Moderate	Simple	Simple
Post transitional modification(PTMs)	Yes	Require additional steps	None
Protein yields and secretory capacities	High yields, highly efficient secretion, high specific productivity	High yields, secretory capacities depending on the species	High expression capacities, secretion mostly inefficient, extensive purification and downstream processing required

Table 2. 3 *Most commonly used species as production host and its characteristics (Mattanovich D, 2012) (Martinez JL, 2012)*

	Insect cell	**Pichia Pastoris**	**Saccharomyces cerevisiae**	**Escherichia coli**
Scaling up	Complex	Easy	Easy	Easy
Expression level	Low	Moderate	Moderate	High
Secretion	Secretion to extracellular environment	Secretion to extracellular environment	Secretion to extracellular environment	Secretion to periplasm
Cell growth	Slow	Rapid	Rapid	Rapid

2.2.1. *Pichia pastoris*

P. pastoris was first isolated from exudate of chestnut tree and known as *Zygosachharomyces pastori* in France by Guilliermond and Phaff named as *P. pastoris* (Phaff et al., 1956). In mid-20th century its methanol utilization ability as sole carbon and energy source was discovered (Ogata, Nishikawa and Ohsugi, 1969). *P. pastoris*, however, was reclassified and re-named as *Komagataella* based on ribosomal gene sequence data. Despite that in the literature *P. pastoris* is still known as established name (Cregg et al. 1985).

Methylotrophic yeasts *P. pastoris* is gained great attention as host microorganism for recombinant protein production (Gasser B P. R., 2013). Since it enables high level expression both intracellularly and extracellularly and purification procedure of desired product is relatively simpler compare to bacteria. Moreover, *P. pastoris* is able to reach high cell densities in minimal and inexpensive media.

P. pastoris has gained GRAS status and, thus, its legal to use in food-related products. *P. pastoris* has been used to produce more than 700 proteins ranging from bacteria to human from 2000 to 2010 (Li *et al.*, 2010).

The notable difference between *P. pastoris* and *E. coli* is the disulfide bond production ability, glycosylation and extracellular protein production in *P. pastoris* (Demain and Vaishnav 2009). Furthermore, *P. pastoris* can produce correctly folded consequently functional protein since like all the other eukaryotic organism it conducts proteolytic processing. *P. pastoris* use simple, defined and inexpensive media compare to mammalian and insect cells thus the procedure is more economical. The major advantages of *P. pastoris* over *S. cerevisiae* is the shorter N-glycan chain length. In addition, in comparison to other non-conventional yeasts and *S. cerevisiae* the secretion pathway of *P. pastoris* is more aligned to eukaryotes (Corchero et al. 2012). *P. pastoris* has less complex and cheaper downstream purification procedure as secretion of endogenous protein to extracellular media is low (Li *et al.*, 2007). Central carbon mechanism of *P. pastoris* is illustrated in Figure 2.3.

Table 2. 4 *Advantages and disadvantages of P. pastoris*

Advantages	**Disadvantages**
Rapid growth	High proteolytic activity
Low purification cost	Requirement of bioreactor for high level production
Easy scale up	Long cultivation time
High cell density	Recombinant protein degradation with protease
High expression level	
High yield	
Clean and simple medium	
Genetically stable strains	
Large pH range	
Strong constitutive P*GAP*	
Strong, tightly-regulated P*AOX1*	

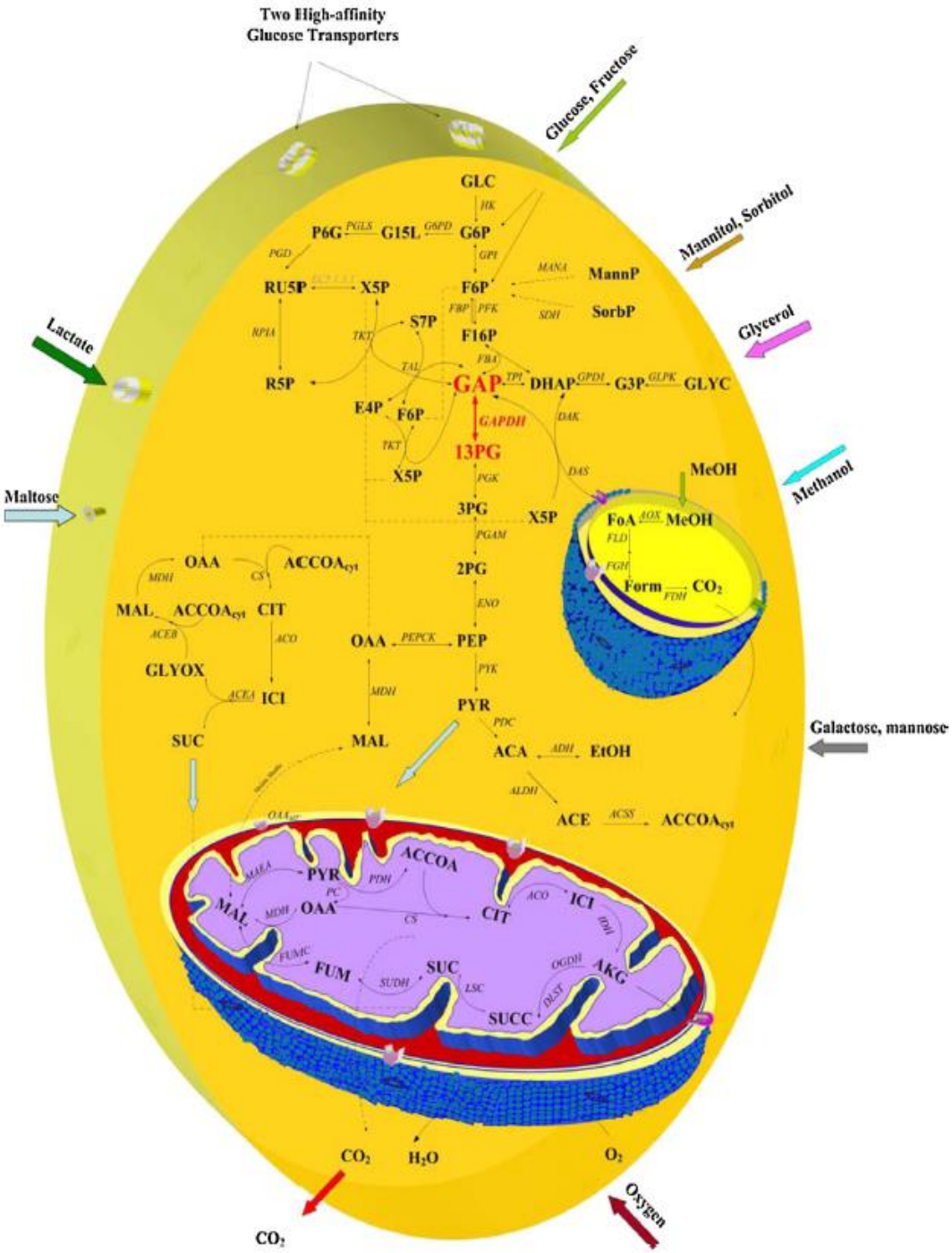


Figure 2. 3 Central carbon mechanism of *P. pastoris*

The complete genome sequence data of different *P. pastoris* strains such as GS115 (auxotrophic for HIS4) (De Schutter et al. 2009), *DSMZ* 70382 (Mattanovich et al. 2009) and original SCP production strain CBS7435(Sturmberger et al. 2016) (Valli et al. 2016) were published. These trials open the way to a greater insight into the genetics of the yeast, and accelerate advances of the genetic toolbox.

2.2.1.1. *P. pastoris* transfection

Electroporation or a process of generating spheroplanes or complete cell techniques, such as lithium chloride (or lithium acetate) and polyethylene glycol may also be used to *P. pastoris* transfection (Nel et al., 2009). Lithium chloride and electroporation techniques are currently the prevalent techniques of transfection. The hosts have episomal and integrative vectors in the main; i.e. the newly introduced (recombinant) DNA , after transformation, either integrate itself into the host genome (chromosomal DNA) or remain separate and can autonomously replicate as a circular episomal plasmid (Nel et al., 2009). Like *S. cerevisiae* there's a tendency to recombinate genomic DNA homologously with introduced DNA in *P. pastoris* that lead to either single crossover or gene replacement events. In order to have more stable transfection, the activity is selectively performed through chromosomal integration with the assistance of integrative vectors (Sreekrishna *et al.*, 1997). Before *P. pastoris* transfection, *E. coli* transformation has been performed to increase plasmid concentration. Therefore, all vectors should be designed as *E. coli* / *P. pastoris* shuttle vectors (Cereghino and Cregg, 2000)

2.3. Eukaryotic Protein synthesis

Initiation of protein synthesis involves binding of 40s and 60s ribosome to messenger RNA (mRNA) and this complex assemble an 80s ribosome at the initiation codon. Bringing together an 80s ribosome with a mRNA and initiator methionyl tRNA (Met-tRNAi) makes codon- anticodon base-pair interactions at the start of the open-reading frame (ORF) (Merrick and Pavitt, 2018)(Starck *et al.*, 2012). Initiation process in eukaryotes can be categorized in three (Figure 2.4) *i)* formation of 43s preinitiation complex by combination of MettRNAi and multiple initiation factors with the 40s ribosome; *ii)* binding of this complex to RNA *iii)* combination of an 80s ribosome with 60s ribosomal subunit edition. 40s ribosome released during the early stage of initiation as 40s ribosomal subunits are then reused to catalyze further initiation steps (Pain, 1996).

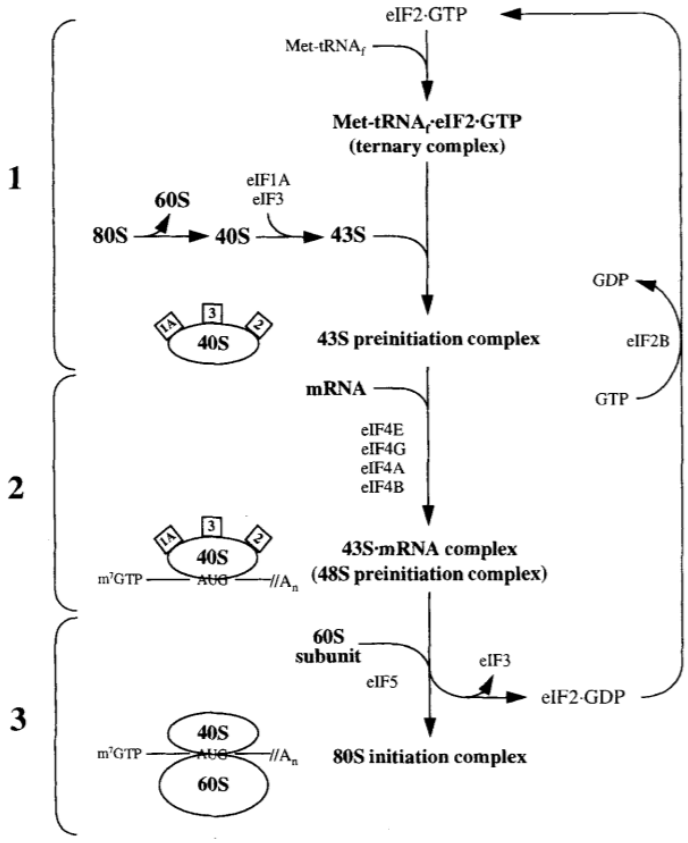


Figure 2. 4 Initiation of protein synthesis mechanism

Several initiation factors are involved in the binding of the 40s ribosome to mRNA consequently 40s ribosomal subunit control both the overall rate of translation and the relative rates of utilization of different mRNA molecules. The binding of 40s ribosomal subunit is at the 5' end of the mRNAs then it migrates in a 5'to 3'direction towards the initiation codon for most of the eukaryotic. Hairpin loops and secondary structures formation are observed by the sequence analysis of mRNA. The 43s preinitiation complex binding to secondary structure region in 5' UTR (untranslated region) of mRNA retard the protein synthesis especially near to 5' end (Kozak, 1991).

2.4. Promoters

Strong promoters are important vehicles for recombinant protein production since efficient transcription is a key factor for gene expression. Promoters play crucial role in transcription. Promoters are regions of DNA located upstream of the transcriptional start site of a gene that serve as a binding site for the RNA polymerase complex and other transcription factors. Multiple transcription factor-binding sites can regulate transcription of the downstream gene. The binding of certain transcription factors such as activators boost transcription of the downstream gene, while binding of other transcription factors such as repressors prevents transcription of the downstream gene (Goodrich, 2001) (Gasser B S. M., 2015) (Jacobs PP, 2010). Proper initiation and regulation of transcription can be achieved by the DNA elements that is present in promoter region. Promoters contains two types of DNA elements: core promoter elements and regulatory elements. Core promoter elements bind to the RNA polymerase and other transcription factors while regulatory elements bind transcriptional activators and repressors. For many RNA polymerase holoenzymes, consensus sequences of core promoters have been inferred. Although each promoter has unique sequence (Keaveney, 1998).

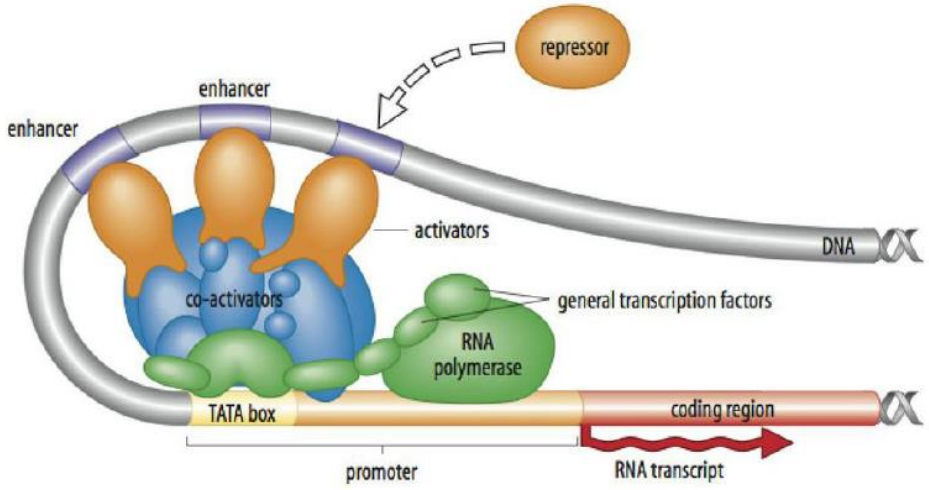


Figure 2. 5 Schematic representation of initiation of transcription

The expression strength capacity has been linked to various characteristic of gene promoter such as number of binding sites for transcription.

The TATA box, which is an omnipresent core promoter element, is bound by the transcription pre-initiation complex. Transcription can be defined in two steps: first, transcription factors are enrolled with pre-initiation complex (PIC) and accumulated at the core promoter with RNA polymerase; second, gene transcription is begun with the release of the polymerase from the pre-initiation complex. Presence of the transcription factors necessary for the initiate transcription, the reporter gene is expressed in the cell via selected promoter. Consequently, promoters, enhancers, and repressors are play critical role in regulation, the duration and strength of a reporter gene expression (Blake *et al.*, 2006) (Lodish, 1995) (Blake WJ, 2006) (K, 1996). Transcription of a particular gene can be defined in two steps: i) pre-initiation complex is captured by TF and accumulate at the core promoter region in the presence of RNA polymerase. ii) RNA polymerase released, and transcription of gene started. RNA polymerase release from PIC can be recycled and called as re-initiation (Geiger *et al.*, 1996) (Weideman *et al.*, 1997).

Promoters can be categorized inducible or constitutive. Several inducible or constitutive promoters are available with distinct properties and strengths (Çalik *et al.*, 2015b). Inducible promoters are induced to express the reporter gene (Gasser, Steiger and Mattanovich, 2015).

2.4.1. Inducible promoters

Alcohol oxidase I (AOX1) and formaldehyde dehydrogenase (FLD1) are the common strong and inducible promoters of the metabolic pathway that are involved in recombinant protein production in *P. pastoris* (Tschopp, 1987) (Shen, 1998). AOX1 catalyzes the methanol metabolism and convert methanol to formaldehyde. As a side product oxygen and hydrogen peroxide are released as reaction takes place in the peroxisomes (Cereghino and Cregg, 2000). However, these promoters need methanol as inducer which is toxic for the cell. Consequently, there is huge requirement for strong regulated promoters rather than methanol inducible promoters.

Table 2. 5 *Inducible promoters of P. pastoris*

Gene	Gene product	Regulation
AOX1	Alcohol oxidase 1	Induction by methanol
AOX2	Alcohol oxidase 2	Induction by methanol
AOD	Alternative oxidase	Expression on glucose but not on methanol or upon glucose depletion
ADH1	Alcohol dehydrogenase	Repression by glucose and methanol, induction by glycerol and ethanol
ADH3	Protein involved in Ethanol utilization	Induced by ethanol
PHO89 or NSP	(putative) Sodium-coupled phosphate symporter	Induction by phosphate limitation
FLD1	Formaldehyde dehydrogenase	Induction by methanol and methylamine
THI11	Protein involved in thiamine biosynthesis	Complete repression by Thiamine

2.4.2. Constitutive promoters

Continuous protein production can be achieved by providing continuous transcription of the target gene with constitutive promoters (Çalik *et al.*, 2015a). Glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter is common constitutive promoter which shows high level expression (Waterham, 1997). Moreover, constitutive promoters are able to overcome the disadvantages that results in the usage of inducible promoters like methanol usage. High level expression capacity has been achieved with methanol-induced tightly regulated pAOX1. In *P. pastoris* even high level of methanol usage as main carbon source is toxic for the cell and pAOX is repressed by glucose, glycerol or ethanol. Strong and constitutive GAP promoter is mainly used to eliminate methanol usage.

Table 2. 6 *Constitutive promoters of P. pastoris*

Gene	Gene product	Regulation
GAP	Glyceraldehyde 3-Phosphate dehydrogenase	Expression on glucose, to a lesser extent on glycerol and methanol
TEF1	Translation elongation factor 1 α	Strong growth association
PET9	ADP/ATP carrier of the inner mitochondrial membrane	
SDH	Sorbitol dehydrogenase	
KAR2	ER resident chaperone	
ENO1	Enolase	

2.4.3. Double promoter expression system

Instead of using single promoter system, using double promoter expression system (DPES) is promising advanced to increase heterologous protein production as DPES can maintain a higher specific product formation rate within the production domain. Therefore, the ideal transcription performance must either be achieved by meeting simultaneously the requirements of both promoters or by using consecutively activities, which are carried out by switching from one to the other during a biphanistic system or by successive iterations between the promoters to increase production and productivity (Öztürk, Ergün and Çalık, 2017). Both promoters' requirement needs to be balanced in order to obtained maximum potential via fine-tuning the culture as each promoter has unique expression conditions (He *et al.*, 2015).

P. pastoris double promoter expression systems have been used for several reasons such as enhancing target protein activity, yield and stability through chaperons' co-expression (Inan *et al.*, 2006) (Gasser et al. 2008), catalyzing posttranslational modifications via enzymes co-expression (Vuorela, 1997)(Toman *et al.*, 2000), promoter transcriptional capacity enhancement for r-protein production through

overexpression or deletion of TFs (Ata *et al.*, 2017) and new metabolic pathway construction (Pereira et al. 2004) (Westfall *et al.*, 2012).

In prokaryotic microorganisms, like bacteria double promoter vectors are transformed in a single step. In eukaryotic microorganisms, however, like *P. pastoris* there are three different methods illustrated in figure 2.6. First, sequential transfection with single promoter expression cassettes, second, transfection with bipartite vectors including two promoters in one expression cassettes located successively, and the last, transfection with bidirectional promoter plasmids.

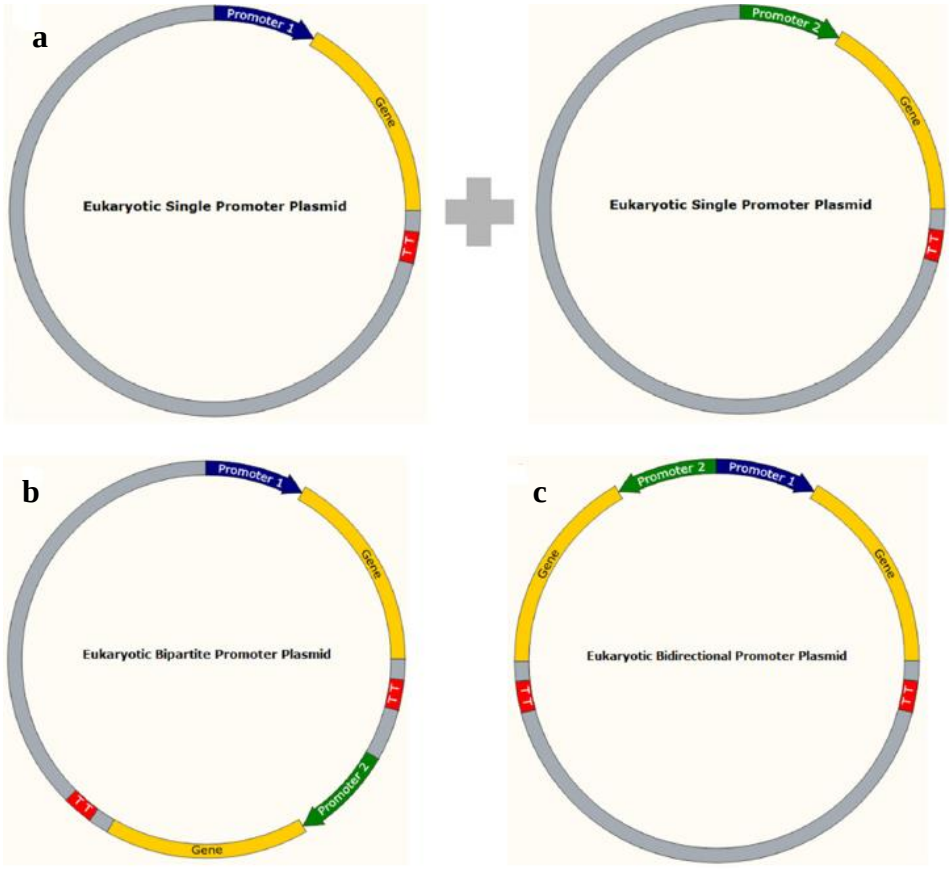


Figure 2. 6 a. Design of two single promoter plasmids for sequential transfection in eukaryotic microorganisms, b. Design of a bipartite promoter plasmid in eukaryotic microorganisms c. Design of a bidirectional promoter plasmid in eukaryotic microorganisms.

2.5. Promoter Engineering

Promoter engineering methods focus on the regulation of promoter transcriptional capacity and based on promoter architecture in order to create variable range of promoter library via mutation on promoters' DNA sequence. The nature of eukaryotic promoters has resulted in the fusion of various combinations of upstream activator and key promoters that reveal hybrid promoters. The upstream activation sequence and key promoter components at the most basic level have TFBSs that regulate the general function of the promoter. Randomized mutagenesis of the promoter introduces changes in TFBS nucleotide sequences, hence changing the capacity of the promoter. Since mutations on TFBSs are more effective at changing the binding effectiveness of particular TFs, weaker or stronger promoter variations than initial promoters have been developed with a random mutagenesis strategy.

2.5.1. Transcriptional Engineering

Transcription engineering is an instrument that enables regulating an intracellular response pathway to be altered through engineering of several parts, including transcription variables, such as promoters or regulators. In addition to promoter engineering methods, transcription factors play a critical part in the cell activation and determination of the specificity of transcription; therefore, TFs can also be an engineering goal. In this regard *P. pastoris* cells, the P*GAP-V* expression levels range from 35 to 310% of the wild-type P*GAP* driven expression in wild-type *P. pastoris* and were improved via over-expression or deletion of the TF genes (Ata et al. 2017).

2.5.2. Modification of TFBSs

Changes to TFBSs can merely be described in a promoter sequence as the rational and systemic engineering of TFBSs. Basically, the modifications (deletion, duplicating, altering) of TFBSs are the basis of the techniques as they regulate the transcriptional strength of the promoter. Synthetic promoter engineering uses TFBSs to develop new

promoter architectures as a modular genetic component. Finally, the addition, abrogation or alteration of TFBSs and their genetic phenotype can rely on all promoter engineering techniques to modulate transcriptional strength of promoters. With the sharp spike of understanding of TFBSs and their respective TFs, the rational building of promoter libraries with different regulatory features will be feasible. Changing TFBSs by adding or deleting putative TFBSs to the *P. pastoris* AOX1 promoter, and the transcriptional capacity of a P_{AOX1} built promoter library ranges from 6 to 160% of natural promoter activity (Hartner et al. 2008).

Recently the *P. pastoris* GAP promoter was altered through its putative TFBSs, whereas the repressor motives were removed and putative activator binding sites were duplicated. The strengths of the P_{GAP} library were 82% to 190% of wild type P_{GAP} (Ata et al. 2017).

2.5.3. Recombinant DNA technology

Basically, in rDNA technology, the gene of interest that codes for desired protein is isolated from the DNA (e.g. human DNA) and linked with an appropriate vector, usually a plasmid, for recombinant / chimeric DNA molecule (Figure 2.7) through genetic engineering methods by employing appropriate enzymatic restrictions. The recombinant DNA molecule is implemented by an appropriate technique such as $CaCl_2$ or Electroporation into a host organism that does not naturally have a gene of interest. Then, by selecting a plasmid marker i.e., antibiotic gene that was naturally available in the plasmid or designed in it, the transformed host cells are selected properly in the selective media. The colonies selected can be used as platforms to express them. The new plasmid should be isolated from the host and analyzed to validate the recombination procedure.

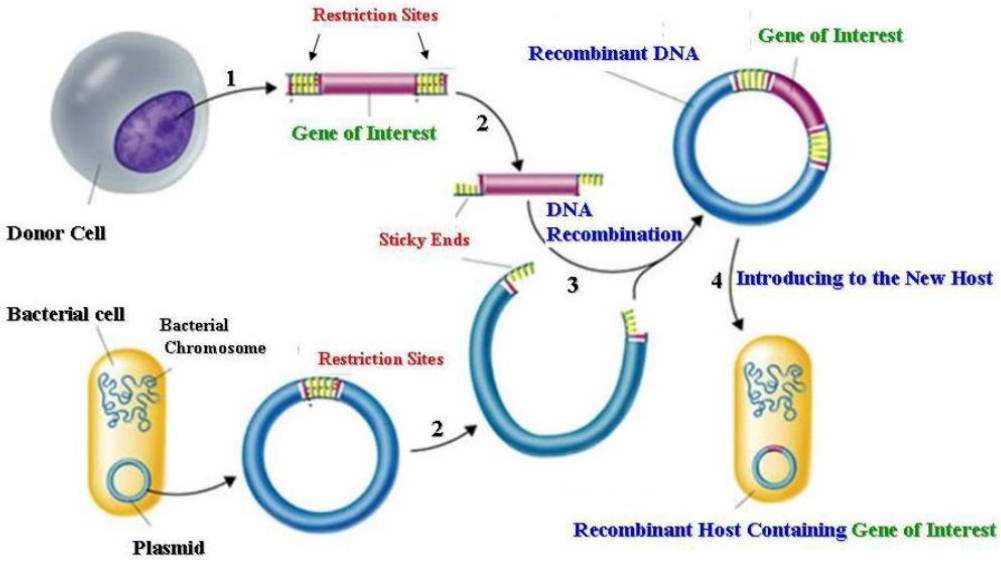


Figure 2. 7 Schematic representation of recombinant DNA technology

Besides, if only the new plasmid is isolated and then introduced to the second host (main production host). In general, a bacterial cell was used just for cloning. The chosen (right) colonies are used in the preparation of the required recombinant product under specified bioprocess circumstances following the conversion of the fresh host.

On the other hand, if a primary host, generally bacterial cell, is only used for cloning, the new plasmid, i.e. the primary producing host, should be isolated and placed in the second host. The selected colonies are used to produce the required recombinant product under specified bioprocess conditions after the fresh host is transformed. The nucleotide sequence of the newly designed plasmid should receive a special attention during rDNA procedure and the checking of the new plasmid sequence will therefore be one of the validating stage following plasmid isolation in order to avoid any unwanted frame shift or transcription mutation that could affect the amino acid transcript sequence. The conservation of structural integrity in the product is a significant problem in recombinant therapeutic expression. Any modification or replacement of the amino acid sequence may lead to inefficiencies, immunity or negative impacts.

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals, DNA Ladders, Kits and Enzymes

All major chemicals used in this work are supplied from Sigma-Aldrich and Merck Millipore. All the DNA ladders and the kits used in plasmid isolation, PCR purification and gel extraction are supplied from Thermoscientific (Thermo Fisher, USA). All the restriction enzymes are supplied from Thermoscientific and NEB.

3.2. Strains, Plasmids, Primers and Maintenance

E. coli DH5α (Invitrogen, USA) strain was used for the purpose of amplification of the constructed plasmid and cloning. *P. pastoris* X-33 (Invitrogen, Carlsbad, Ca, USA) strain were used as expression strain for cloning experiments. pGAPZαA (Invitrogen) plasmid were chosen as parent plasmid in order to construct predesigned plasmid. Schematic representation of pGAPZαA base plasmid can be seen in Figure 3.1.

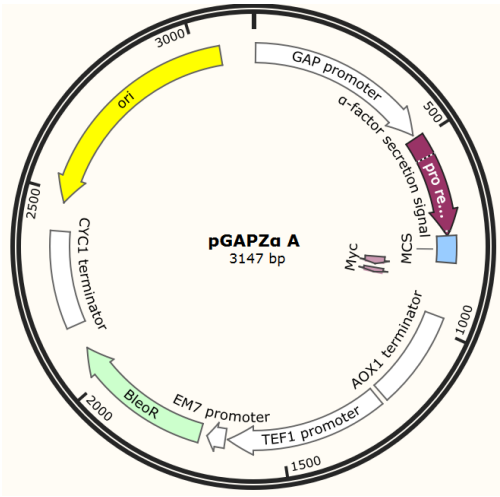


Figure 3. 1 Schematic representation of pGAPZαA

For long term storage all microorganism strains were kept as stocks in - 80°C with Microbank^{TM} and 25% glycerol stock solution. Primer stocks (100µM) are synthesis by Oligomer (Ankara). In this study primers stocks (100 µM) were diluted to 10 µM working concentration. All primer stocks and diluted primers 10µM were kept at -20°C. The primers designed for construction of recombinant plasmids are listed in Table 3.1.

Table 3. 1: *Primers designed and used in this study. Italic characters represent to restriction enzyme recognition sites*

Primers	Sequence
Forward opt cat_PADH2	CAG*ATGCAT*TCCTTTTTACCACC CAAGTGC
Reverse opt cat_PADH2	GCCCTTGCTCACCATTTTCGTAAAGTAAATAAGATA AAAGCTAGTAGC
Forward mApple	GCTACTAGCTTTTATCTTATTTACTTTACG AAAATGGTGAGCAAGGGC
Reverse mApple	CT*GGTACC*TTACTTGTACAGCTCGTCATGC
Forward NTC	CAA*GGATCC*GACATGGAGGCCCAG
Reverse NTC	GCC*ACATGT*CAGTATAGCGACCAGCATTC
Forward ADHoptcat Hgh	TTAGGATCC*GGCGCGCC*TTCCTTTTTACC ACCCAAG
Forward modAOX	CAG*ATGCAT*AACATCCAAAGACGAAAGG
Reverse modAOX	CAGCAGTAAAAATTGAAGGAAATCTCATCGT TTCGAATAATTAGTTG
Forward α Factor HGh	CAACTAATTATTCGAAACGATGAGATTTCCTTC AATTTTTACTGCTG
Reverse HGh	CAA*TCTAGA*CTAGAAGCCACAGCTG
Forward-P*AOX1*	CTC*AGATCT*AACATCCAAAGACGAAAGG
Reverse-eGFP	CC*GGTACC*TCACTTGTACAGCTCGTCCAT
Forw mAOX1-AddAdr2	GACCCCACATTTTTTTTTTGACCCCACATGTT CCCCAAATGGCC
Rev mAOX1-AddAdr2	TGGGGTCAAAAAAAAAATGTGGGGTCGCCC TCATCTGGAGTGATG
Forward P*AOX1*	CTC*AGATCT*AACATCCAAAGACGAAAGG
Reverse P*AOX1*	CTG*AGCACTGC*ACGCCGTAGGT

Table 3. 2 *Strains and plasmids constructed*

Microorganism	Plasmid	Comment	Source
***E. coli* DH5α**	pPICZα-A::*eGFP*	Backbone plasmid	Plasmid was synthesized and stored in *E. coli* DH5α
***E. coli* DH5α**	pmAOX1::*eGFP*	Plasmid carrying $P_{AOX1/Adr1\text{-}L3/Cat8\text{-}L3}$ with *eGFP*	Plasmid was synthesized and stored in *E. coli* DH5α
***E. coli* DH5α**	pADH2-Cat8-L2::*eGFP*	Plasmid carrying $P_{ADH2\text{-}Cat8\text{-}L2}$ with *eGFP*	Plasmid was synthesized and stored in *E. coli* DH5α
***E. coli* DH5α**	pAOX::*eGFP*	Plasmid carrying native P*AOX1* with *eGFP*	Plasmid was synthesized and stored in *E. coli* DH5α
***E. coli* DH5α**	pAOX1-Cat8-2::*eGFP*	Plasmid carrying P*AOX1-Cat8-2* with *eGFP*	Plasmid was synthesized and stored in *E. coli* DH5α
***E. coli* DH5α**	pGAP::*eGFP*	Basal plasmid carrying native PGAP	Plasmid was synthesized and stored in *E. coli* DH5α

Table 3.2 (Continued)

***E. coli* DH5α**	pGAP::*hGH*	Plasmid carrying *hgh* fused to α-factor under P*GAP*	Plasmid was synthesized and stored in *E. coli* DH5α
***E. coli* DH5α**	pmApple	Plasmid carrying the mApple gene	Gift from Mukhopadhyay's lab
***E. coli* DH5α**	pNATMX6	Plasmid carrying the NTC antibiotic resistance gene	Plasmid was synthesized and stored in *E. coli* DH5α
***E. coli* DH5α**	pADH2-Cat8-L2::*mApple*	Plasmid carrying $P_{ADH2\text{-}Cat8\text{-}L2}$ with *mApple* (selection marker: zeocin)	Constructed in this study
***E. coli* DH5α**	pADH2-Cat8-L2::*mApple*	Plasmid $P_{ADH2\text{-}Cat8\text{-}L2}$ with *mApple* (selection marker: NTC)	Constructed in this study
***E. coli* DH5α**	pADH2-Cat8-L2::*hGH*	Plasmid carrying $P_{ADH2\text{-}Cat8\text{-}L2}$ with *hGH* (selection marker: NTC) with its native secretion signal	Constructed in this study
***E. coli* DH5α**	pmAOX1::*hGH*	Plasmid carrying $P_{AOX1/Adr1\text{-}L3/Cat8\text{-}L3}$ with *hGH* with its native secretion signal	Constructed in this study

Table 3.2 (Continued)

***E. coli* DH5α**	peAOX:: *eGFP*	Plasmid carrying P*mAOX1/ACA1 deleted* with eGFP	Constructed in this study
***E. coli* DH5α**	pGAP::mApple	Plasmid carrying P*GAP* with mApple	Constructed in this study
***P. pastoris* X-33**	pADH2-Cat8-L2::*mApple*	*P. pastoris* strain used for screening $P_{ADH2\text{-}Cat8\text{-}L2}$ (single copy)	Constructed in this study
***P. pastoris* X-33**	pADH2-Cat8-L2::*mApple*	*P. pastoris* strain used for screening $P_{ADH2\text{-}Cat8\text{-}L2}$ (two copy)	Constructed in this study
***P. pastoris* X-33**	pmAOX1::*eGFP*	*P. pastoris* strain used for screening $P_{AOX1/Adr1\text{-}L3/Cat8\text{-}L3}$ (single copy)	Constructed in this study
***P. pastoris* X-33**	pmAOX1::*eGFP*	*P. pastoris* strain used for screening $P_{AOX1/Adr1\text{-}L3/Cat8\text{-}L3}$ (two copy)	Constructed in this study
***P. pastoris* X-33**	pGAP:: *eGFP*	*P. pastoris* strain used for screening P*GAP* (single copy)	Constructed in this study
***P. pastoris* X-33**	pGAP:: *eGFP*	*P. pastoris* strain used for screening P*GAP* (two copy)	Constructed in this study
***P. pastoris* X-33**	pADH2-Cat8-L2::*mApple* + pGAP:: *eGFP*	*P. pastoris* strain used for screening $P_{ADH2\text{-}Cat8\text{-}L2}$ (parent plasmid)and P*GAP* at the same time	Constructed in this study

Table 3.2 (Continued)

***P. pastoris* X-33**	pADH2-Cat8-L2::*mApple* + pmAOX1:: *eGFP*	*P. pastoris* strain used for screening $P_{ADH2\text{-}Cat8\text{-}L2}$ (parent plasmid) and P*mAOX1* at the same time	Constructed this study	in
***P. pastoris* X-33**	pADH2-Cat8-L2::*mApple* + pGAP:: *eGFP*	*P. pastoris* strain used for screening $P_{ADH2\text{-}Cat8\text{-}L2}$ and P*GAP* (parent plasmid) at the same time	Constructed this study	in
***P. pastoris* X-33**	pADH2-Cat8-L2::*mApple* + pmAOX1:: *eGFP*	*P. pastoris* strain used for screening $P_{ADH2\text{-}Cat8\text{-}L2}$ and $P_{AOX1/Adr1\text{-}L3/Cat8\text{-}L3}$ (parent plasmid) at the same time	Constructed this study	in
***P. pastoris* X-33**	pADH2-Cat8-L2::*hGH*	*P. pastoris* strain used for screening $P_{ADH2\text{-}Cat8\text{-}L2}$ for hGH production	Constructed this study	in
***P. pastoris* X-33**	pmAOX1::*hGH*	*P. pastoris* strain used for screening $P_{AOX1/Adr1\text{-}L3/Cat8\text{-}L3}$ for hGH production	Constructed this study	in
***P. pastoris* X-33**	pADH2-Cat8-L2::*hGH* + pGAP::*hGH*	*P. pastoris* strain used for screening $P_{ADH2\text{-}Cat8\text{-}L2}$ and P*GAP* for hGH production	Constructed this study	in

Table 3.2 (Continued)

***P. pastoris* X-33**	pADH2-Cat8-L2::*hGH* + pmAOX1::*hGH*	*P. pastoris* strain used for screening $P_{ADH2\text{-}Cat8\text{-}L2}$ and $P_{AOX1/Adr1\text{-}L3/Cat8\text{-}L3}$ for hGH	Constructed in this study
***P. pastoris* X-33**	peAOX:: *eGFP*	*P. pastoris* strain used for screening P*eAOX*	Constructed in this study

3.3. Growth Media

3.3.1. Solid Medium

Escherichia coli strains were inoculated on LB agar medium at 37°C and 24 h with proper antibiotics (Table 3.3). For the *E. coli* strains with pADH2-Cat8-L2*::mApple* 50µg/ml nourseothricin (NTC), and for pGAP::*eGFP* or pmAOX1::*eGFP* 25 µg/ml Zeocin^{tm} is added into the medium for antibiotic resistance. For inoculation of *E. coli* wild type DH5α strain LB medium without an antibiotic is used. *Pichia pastoris* strains were inoculated on YPD agar medium at 30°C for 48 h (Table 3.4). For the inoculation of *P. pastoris* strains with pADH2-Cat8-L2::*mApple* 50µg/ml nourseothricin (NTC), and for the strains with pGAP*::eGFP* or pmAOX1::*eGFP* 25 µg/ml Zeocin^{tm} is added into YPD agar. Inoculation of *P. pastoris* X-33 strains YPD agar medium is used without an antibiotic.

Table 3. 3 *LB agar medium composition for E. coli*

Compound	**Concentration (g/L)**
Tryptone	10
Yeast extract	5
NaCl	5
Agar	15

Table 3. 4 *YPD agar medium composition for P. pastoris*

Compound	**Concentration (g/L)**
Peptone	20
Yeast extract	10
D-glucose (dextrose)	20
Agar	20

3.3.2. Precultivation Medium

P. pastoris cells grown on the solid medium were inoculated into precultivation medium (BMGY or YP) in either 12 deep-well-plates or shake bioreactors. Compositions of BMGY and YP media are presented in Tables 3.5 and 3.6.

Table 3. 5 *BMGY composition for precultivation of P.pastoris*

Compound	**Concentration(g/L)**
Yeast extract	10g
Peptone	20g
Glycerol	10 ml
Yeast Nitrogen Base (YNB ,w/o amino acids)	4.08g
Potassium phosphate buffer (pH=6.0)	0.1M
Ammonium sulfate	12g
Biotin	0.0004g
Chloramphenicol (34 mg/ml)	1ml

Table 3. 6 *YP composition for precultivation of P.pastoris*

Compound	Concentration(g/L)
Yeast extract	10g
Peptone	20g

3.3.3. Fermentation Media

After precultivation, the cells were harvested and inoculated into five fermentation media with different carbon sources (Table 3.9) at an initial cell concentration at OD_{600} = 1. For the screening of Aca 2 TFBS modification of mAOX1 promoter, five different carbon sources were used as 2% (v/v) ethanol, 1% (v/v) methanol, limited glucose, excess glycerol, excess glucose (Table 3.9). Initial OD_{600} for ethanol methanol and limited glucose was 1 while initial OD_{600} for excess glucose and glycerol was 0.1

Table 3. 7 *Defined cultivation medium for screening*

Component	Concentration (g/L)
(NH4)2HPO4	4.95
MgSO4.7H2O	14.9
CaCl2.2H2O	1.17
PTM Trace Element Solution	1.47 mL
Biotin (0.2 g/L)	2 mL
Potassium phosphate buffer (pH=6)	0.1 M
Carbon source	Carbon-source and its concentration presented in Table 3.9

Table 3. 8 *ASMV6 defined cultivation-base-medium for screening*

Component	**Concentration (g/L)**
$(NH_4)_2HPO_4$	6.3
$(NH_4)_2SO_4$	0.8
$MgSO_4.7H_2O$	0.49
KCl	2.64
$CaCl_2.2H_2O$	0.0535
Citric acid monohydrate	22
PTM Trace Element Solution	1.47 mL
NH_4OH (25%)	20 mL
Biotin (0.2 g/L)	2 mL
KOH solid	Adjust pH 6.4-6.6
Carbon source	Carbon-source and its concentration presented in Table 3.9

Table 3. 9 *Carbon sources* used in the cultivations

Cultivation/Fermentation denoted as:	**Carbon Source**	**Substrate-initial concentration**
Excess Glucose	Glucose	20 g/L
Excess Glycerol	Glycerol	20 g/L
Limited Glucose	Glucose	2 g/L
Ethanol	Ethanol	1% (v/v) or 2% (v/v)
Methanol	Methanol	2% (v/v)

3.4. Genetic Engineering Techniques

3.4.1. Plasmid Isolation

Plasmid isolation was carried out from *E. coli* by GeneJET Plasmid Miniprep Kit (ThermoFisher, USA) according to the manufacturer's instructions. *E. coli* cells were cultivated overnight at 200 rpm in 10 ml LB medium with Zeocin™ or Nourseothricin (NTC) at 37°C. The standard protocol for plasmid isolation is as follows:

Cultures were harvested by centrifugation at 6800g for 5 minutes. Supernatant is discarded and pellet is suspended with 250 μL resuspension solution and re-suspended cells were transferred to microcentrifuge tube. Thereafter, 250 μL Lysis solution added and completely mixed. After that, immediately 350 μL Neutralization solution is added, after complete mixing cells were centrifuge for 5 min. Following the washing and elution steps, purified plasmid DNA is stored at -20 °C.

3.4.2. PCR Purification

PCR product purification was performed by GeneJET PCR Purification Kit (ThermoFisher, USA) according to manufacturer's protocol.

3.4.3. Agarose Gel Electrophoresis

Agarose-gel electrophoresis is conducted for the control of isolated plasmid and PCR product, visualization and extraction of digested DNA fragments. Purified DNA fragment was visualized according to size of the DNA fragment after agarose gel electrophoresis (AGE). For AGE Mini-sub® Cell GT Cell system (Bio-Rad, CA, USA). The standard protocol for AGE can be report as follows:

1. 50X TAE buffer is diluted to 1X buffer before each usage.
2. Appropriate amount of agarose is dissolved (depending on the size of the fragment) in 1X TAE buffer to achieve required resolution. For the visualization of DNA fragment 8 g/L agarose was dissolved, and for the gel extraction 12 g/L agarose was dissolved in 1X buffer.
3. The solution is heated until bubbles are appeared (boiling) and solution become clear.
4. The solution is left for cooling to prevent denaturation of EtBr. EtBr (Sigma) is added for 0.4 μg/mL final concentration (1.75 μL EtBr for 50 ml 1X TAE).
5. The combs are placed in the plastic trays to create wells. The gel is poured into the plastic trays and left for complete drying for 30 minutes.
6. 1 μL 6X DNA gel-loading dye was used with 1-5 μL DNA sample which can be diluted with sterilized water if necessary.
7. The DNA solution is mixed by pipetting and loaded into wells.
8. 5 μL DNA marker (Thermoscientific) is also loaded into the DNA solutions in wells according to size of the DNA fragments.
9. The lid is attached carefully to the gel-electrophoresis tank and the electrical leads are connected.
10. Gel-electrophoresis is carried out at 90 V for 30-80 minutes according to the DNA fragment size and gel concentration.
11. DNA bands can be visualized under UV-light with ethidium bromide filter. Hamamatsu Digital CCD Camera was used for visualization and saving the images.

3.4.4. Extraction of DNA Fragments

DNA samples were run on the agarose gel in order to separate craved DNA fragments. The DNA fragments are extracted from the gel by sterile razor blade under UV light and placed in Eppendorf tubes. In order to not to harm the DNA fragment extraction should be done carefully and quickly. Purification of single DNA was conducted by Gel Extraction Kit (ThermoFisher, USA).

3.4.5. Gel Elution

Gel elution was conducted with GeneJet Gel Extraction Kit (ThermoFisher, USA). Gel extraction kit was used according to the manufacturer's protocols. The main protocols can be summarized as follows:

1. Agarose gel with DNA fragment is sliced not to exceed 400 mg and placed into 1.5 mL Eppendorf tubes.
2. Binding buffer is added based on the weight of the agarose gel. For 400 mg agarose gel 400 ml binding buffer should be added.
3. Eppendorf tube is incubated for 10 minutes at ~ 65°C in order to dissolve the gel completely.
4. The dissolved solution is transferred to the GeneJet purification column and centrifuged at 14000 g for 1 minute.
5. Discard the flow through.
6. 700 µL Wash buffer is added and centrifuged for another 1 minute at 14000 g
7. Empty purification column is centrifuged for 2 minutes to avoid ethanol residual. (presence of ethanol may harm to DNA sample)
8. The column is transferred to new clean 1.5 mL Eppendorf tube to get rid of any ethanol residual.
9. 25-50 µL elution buffer or filtered ultra-pure water is added to column and centrifuged at 1400 g for 1 minute.
10. Purified DNA fragment stored at -20°C.

All centrifugations were carried out at the room temperature.

3.4.6. Genomic DNA Isolation

Genomic DNA isolation was performed by Wizard Genomic DNA purification Kit (Promega, USA) from *P. pastoris.* Manufacturer's instructor was followed. *P. pastoris* cells were cultivated overnight in YPD medium with Zeocin^{TM} or Nourseothricin (NTC) at 30°C and 200 rpm. 1 ml cultures were harvested by centrifugation at 14000 g for 2 minutes. Supernatants are removed and pellets were suspended for genomic DNA isolation. Isolated DNAs are stored at -20°C.

3.4.7. Transformation to *E. coli*

The plasmids designed and constructed were transformed to the competent *E. coli* DH5α cells. Competent *E. coli* DH5α cells were prepared with calcium chloride ($CaCl_2$) method as described in Sambrook and Russell (2001). For this purpose, first *E. coli* DH5α cells were inoculated on LB Agar medium (Sigma) (10 g/L tripton, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar). After 16-20 h incubation, a single colony will be selected and transferred to LB broth (10 g/L tripton, 5 g /L yeast extract, 5 g/L NaCl). Cells were incubated in the 250 mL Erlenmeyer flask at 37°C at 200 rpm until OD_{600} reaches 0.35-0.40 at 200rpm and then cells stored on ice for 10 min. The cell pellet obtained by centrifugation at 4°C, 2700 g for 10 min was re-suspended by adding 30 mL of 80 mM $MgCl_2$-20 mM $CaCl_2$ solution and gentle swirling. After centrifugation at 4°C, 2700 g for 10 min, supernatant is removed and cells were re-suspend with 2 ml of 0.1 M $CaCl_2$ solution by pipetting and competent cells were prepared. A maximum 50 ng of 7.5 µl DNA is added into the 50 µL competent cells (50 µL competent cell is used for each transformation) and stored on ice for 30 min. Then, the cells were exposed to heat-shock without shaking for exactly 90 seconds in a water bath at 42°C and left for 5 minutes on ice. 900 µL LB medium was added to the tubes and incubated for 1 hour at 37°C in shaker incubator with agitation of 200 rpm. Thereafter, cells were streaked on selective LB Agar medium containing 25 µL/100 ml Zeocin or 50 µL/100 ml Nourseothricin. In the antibiotic containing medium, at least 10 single colonies will be selected from each cell strain carrying the

different gene that proliferates after 16-20 h incubation at 37°C and the first verification was performed with colony PCR. At least 4 clones were selected from confirmed strains by colony PCR and plasmids were isolated by Plasmid MiniPrep Kit (Thermoscientific) according to the manufacturer's recommendations. The amplification of the desired DNA segments from isolated plasmids was conducted with polymerase chain reaction (PCR). Size of the DNA fragments were further confirmed by agarose gel electrophoresis. DNA fragments were sent to central laboratory for sequencing to definite verification purpose. Microbank stocks of the cells were prepared and stored at -80°C. These cells were isolated in accordance with the manufacturer's instructions with MiniPrep Plasmid Isolation Kit (Thermoscientific) to be used in later were stocked at -20°C.

3.4.8. Transfection to *P. pastoris*

Transfection to *P. pastoris* wild type X-33 cells was performed by lithium chloride (LiCl) method (Invitrogen, 2000) with linearized plasmids. *P. pastoris* wild type X-33 from glycerol stock was streaked onto YPD agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 20 g/L agar) plate and incubated for 48 hours at 30°C. After incubation single colony was selected and inoculated 50 mL YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) and incubated up to OD_{600} reaches 0.8-1.0 at 30°C and 200 rpm for approximately 14-16 hours. Then cell pellets were harvested by centrifugation at 4000 g for 5 min at room temperature. The cell pellets were washed with 25 ml sterile water and centrifuged at 1500 g for 10 min at room temperature. Supernatant was removed and cell pellet was resuspended in 1 mL 100 mM filter sterilized LiCl, transferred to a 1.5 mL Eppendorf tube and centrifuged at maximum speed for 15 seconds. The cell pellet was resuspended in 400 µl 100 mM LiCl and 50 µl cell suspension was dispense for each transfection. LiCl was removed by pipette after centrifugation at a maximum speed for 15 s. For each transformation of the cell 240 µl of 50% PEG, 36 µl 1 M LiCl, 25 µl 2 mg/ml single stranded DNA and linearized 5-10 µg plasmid DNA in a maximum of 50 µl of purified water were

added in a given order and vortex vigorously until complete mixing of cell pellet approximately 1 min The tubes were then incubated at 30°C for 30 minutes without stirring and the heat-shock in water-bath at 42°C for 25 min was applied. Cells were pelleted by centrifuging at 6000-8000 rpm for 15 seconds, transformation solution was removed and pellet was gently resuspended in 1 mL YPD and incubated at 30°C with shaking. After 3 hours of incubation, 25-100 µL of the medium was spread on YPD agar plates containing appropriate antibiotics and incubated for 2-3 days at 30°C.

The putative transfectants were chosen and checked by applying the colony PCR method to determine whether the transferred gene is integrated. The PCR reaction was carried out after 5 minutes of initial cell denaturation at 95°C. According to the results of the colony PCR, at least 8 clones from each strain that carry the gene of interest were chosen and inoculated on individual selective YPD media and incubated at N = 200 rpm, T = 30°C for 12-16 h. 25% glycerol stock was prepared and stored at -80°C for further investigations from replicating recombinant cells.

3.4.9. Total RNA Isolation

3.4.9.1. Roche Total RNA Isolation

a. Resuspend the cell (OD_{600} = 5/mL) pellet in 200 µl PBS
b. Add 5 µl lyticase (2 µg/µl) and gently mix by tipping and incubate for 20 min at 37°C by shaking at 75 rpm.
c. Add 400 µl Lysis/Binding Buffer and vortex for 15 s
d. Transfer the sample to High Pure Filter Tube (max 700 µl) and centrifuge at 8000 g for 15 seconds. Discard the flow through
e. Add 90 µl DNase I Incubation Buffer and 10 µl DNase I mix onto upper reservoir of the Filter tube (For each sample mix 90 µl DNase I Incubation Buffer and 10 µl DNase I into RNase free tube then add)
f. Incubate the tubes at room temperature (15 to 25°C) for 15-20 min
g. Add 500 µl Wash Buffer I to the Filter Tube and centrifuge for 15 s at 8000 g and discard the flow through

h. Add 500 µl Wash Buffer II to the Filter Tube and centrifuge for 15 s at 8000 g and discard the flowthrough
i. Add 200 µl Wash Buffer II to the Filter Tube and centrifuge for 2 min at 14000 g to remove any residual of Wash Buffer.

   ! Extra centrifugation can be applied for 1 min to ensure the removal
j. Discard the collection tube and insert Filter Tube into a clean, RNase free sterile 1.5 ml microcentrifuge tube.
k. Add 30-100 µl Elution Buffer and centrifuge at 8000 g for 1 min
l. Store the eluted RNA at –80°C for later analysis

3.4.9.2. TRI REAGENT Total RNA Isolation Protocol

1. Sample preparation
   a. Add 1 mL of TRI REAGENT to max 200 mg wet cell weight of yeast, then approximately 500 µL glass beads added in Eppendorf
   b. Agitate the cell in tissuelyzer at maximum speed 30 Hz for 1 min and stop the machine 20 seconds to prevent heat accumulation (apply for 5 times)
   c. Add 200 µL chloroform for 1 mL TRI REAGENT and vortex vigorously for 15 seconds
   d. Let the sample stay at room temperature for 10 min then, centrifuge at 14000 g for 15 min at 4°C to separates phases (observed 3 phases: colorless aqueous phase containing RNA (upper), DNA containing interphase (middle) and protein containing red phase (bottom))

2. RNA Isolation
   a. Transfer the upper aqueous phase to RNAse free tube (Do not transfer DNA interphase or red protein phase) and add 500 µL isopropanol for 1 mL TRI REAGENT
   b. Let the sample stay at room temperature for 5 min
   c. Centrifuge at 14000 g for 10 min at 4°C. (RNA precipitation can be visible as pellet on the bottom of the tube)

d. Discard the supernatant and add 1 mL 75% (cold) ethanol to wash the pellet
e. Briefly vortex the sample
f. Centrifuge at 14000 g for 5 min at 4°C

3. RNA Solubilization
   a. Let the RNA pellet dry briefly by air-drying (Take care not to dry RNA completely. Do not dry RNA pellet under vacuum)
   b. Add 25-50 µL nuclease free water and dissolved the pellet by pipetting
   c. Incubate the sample at 65°C for 5-10 min
4. Additional Phenol Removal
   a. Add 1 µL glycogen (molecular biology grade), 3M NaAc 1/10 volume (molecular biology grade) and 2 ½ volume 100% ethanol
   b. Incubate at -20 ° C overnight
   c. Centrifuge at 14000 g at 4°C for 20 min
   d. Add 400 µL 70% Ethanol. Wash the pellet by pipetting or vortexing
   e. Centrifuge at 14000 g for 10 min at 4°C
   f. Discard the ethanol (Do not let ethanol remain)
   g. Add 20-25 µL RNase free water
   h. Incubate the sample at 65°C for 10 min

3.4.9.3. QIAGEN Total RNA Isolation

1. Sample preparation
   a. Harvest the cells by centrifuging at 4500 x g for 5 min at 4°C. Decant the supernatant
   b. Add 600 Buffer RLT, and briefly vortex the cell pellet. Add the sample to the 500-600 µl acid-washed glass beads

      Note: Ensure that β-ME is added to Buffer RLT before use
   c. Ribolyze the cell in tissuelyzer at maximum speed 30 Hz for 1 min and stop the machine 20 seconds to prevent heat accumulation (apply for 5 times)

d. Let the beads to settle at room temperature and transfer the lysate (usually 350 µl) to a RNase free microcentrifuge tube (not supplied) then centrifuge for 2 min at full speed and transfer the supernatant to a new RNase free microcentrifuge tube (not supplied).

2. RNA Isolation
   a. Add 1 volume of 70% ethanol to supernatant (~ 350 µl) and mix well by pipetting.
   b. Transfer the sample (~700 µl) to a RNeasy spin column collection tube (supplied) and centrifuge at 8000-10000 g for 15 s. Discard the flow-through
   c. Add 350 µl Buffer RW1 to the RNeasy spin column and centrifuge at 8000-10000 g for 15 s to wash the spin column membrane. Discard the flow-through.
   d. Mix 10 µl DNase I (supplied with Roche RNA isolation kit) and 90 µl DNase I incubation buffer for each isolation in new microcentrifuge tube and mix by gently inverting the tube (Do not vortex)
   e. Add the DNase I incubation mix (100 µl) directly to the RNeasy spin column membrane, and let the sample stand for 15 min at room temperature (20–30°C)
   f. Add 350 µl Buffer RW1 to the RNeasy spin column and centrifuge at 8000-10000 g for 15 s. Discard the flow-through.
   g. Add 500 µl Buffer RPE to the RNeasy spin column centrifuge at 8000-10000 g for 15 s to wash the spin column membrane. Discard the flow-through.
   h. Add 500 µl Buffer RPE to the RNeasy spin column and centrifuge at 8000-10000 g for 2 min

      Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube and centrifuge at full speed for 1 min

3. RNA Solubilization
    a. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane let the sample stand for 2 min at room temperature and centrifuge for 1 min 8000-10000 g to elute the RNA.
    b. Repeat the previous step using eluate and reuse the collection tube (if high RNA concentration is required)

3.5. Construction of Strains and Plasmids

3.5.1. Construction of Recombinant Plasmids pADH2-Cat8-L2::*mApple*

3.5.1.1. Primer design

Primers were designed by using Oligo analyzer 3.1 (http://eu.idtdna.com/calc/analyzer). Melting points (Tm), possible homo and hetero dimer formation ΔG values is important for primer design. These primers are supplied from Sentegen (Ankara, Turkey). Restriction sites were determined with SnapGene Viewer and Restriction Mapper. These primers and restriction sites were used to amplify the target gene and insert the pGAPZαA vector.

3.5.1.2. Amplification of the target DNA

Polymerase chain reaction (PCR) was conducted to amplify target DNA fragments. The primers designed were used for the PCR experiments. Annealing temperature and extension time were optimized according to designed primers and PCR reactions were carried out in thermocycler (Techne®, Flexigene and TC-3000X). *Modified alcohol dehydrogenase 2* promoter was amplified from pADH2-Cat8-L2::*eGFP* plasmid. mApple is special gift from Associate Professor Tuli Mukhopadhyay. PCR thermocyclic operation condition and the reaction composition for both $P_{ADH2\text{-}Cat8\text{-}L2}$ and mApple were tabulated in Table 3.10 and Table 3.11.

Table 3. 10 *Thermocyclic PCR operation condition for mApple and* $P_{ADH2\text{-}Cat8\text{-}L2}$

Steps	**Temperature**	**Time**	**Cycle**
Initial Denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	30
Annealing	65°C	30 sec	30
Extension	72°C	40 sec	30
Final Extension	72°C	2 min	1
Hold	4°C	∞	-

Table 3. 11 *PCR reaction composition for mApple and* $P_{ADH\text{-}Cat8\text{-}L2}$

Component	**50 µL Reaction**
5X Q5 Reaction Buffer	10 µL
5mM dNTPs	2 µL
10 µM Forward Primer	2.5 µL
10 µM Reverse Primer	2.5 µL
Template DNA	2 µL
Q5 High-Fidelity DNA Polymerase	0.5 µL
H_2O	Up to 50 µL

$P_{ADH2\text{-}Cat8\text{-}L2}$ fragment was fused via fusion PCR to reporter protein mApple (red fluorescent protein) in a way that there was no additional amino acid between them. Then, $P_{ADH2\text{-}Cat8\text{-}L2}$ - mApple fragment (Figure 3.2) was inserted to pGAPZαA base plasmid. PCR thermos-cyclic conditions and reaction compositions for overlap extension is available in Table 3.12 and Table 3.13.

Table 3. 12 *Thermo-cyclic PCR condition for construction of ADH-Cat8-L2–mApple*

Steps	**Temperature**	**Time**	**Cycle**
Initial Denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	30
Annealing	68°C	30 sec	30
Extension	72°C	60 sec	30
Final Extension	72°C	2 min	1
Hold	4°C	∞	-

Table 3. 13 *Fusion PCR condition for construction of ADH2-Cat8-L2–mApple*

Component	**50 µL Reaction**
5X Q5 Reaction Buffer	10 µL
5mM dNTPs	2 µL
10 µM Forward Primer	2.5 µL
10 µM Reverse Primer	2.5 µL
Template 1 (ADH2-Cat8-L2)	2 µL
Template 2 (mApple)	2 µL
Q5 High-Fidelity DNA Polymerase	0.5 µL
H_2O	Up to 50 µL

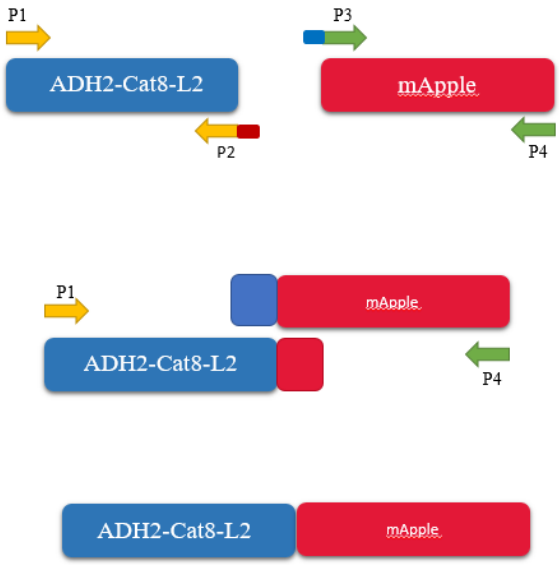


Figure 3. 2 Construction of ADH2-Cat8-L2 - mApple

Polymerase chain reaction was used for amplification of ADH2-Cat8-L2 and mApple by using predesigned primers. $P_{ADH2\text{-}Cat8\text{-}L2}$ was amplified by forward-PADH2-**v** (P1) and reverse-PADH2-**v** (P2) primers while mApple was amplified by forward mApple (P3) and reverse mApple (P4). P2 and P3 have overlapping sequence which make possible to fuse two fragments ($P_{ADH2\text{-}Cat8\text{-}L2}$ and mApple) in a second PCR. In the second PCR two fragment was fused to each other and final $P_{ADH2\text{-}Cat8\text{-}L2}$ - mApple fragment was constructed.

3.5.1.3. Digestion reactions and purification of PCR products

PCR purification Kit (ThermoScientific) was used to purify amplified DNA fragments obtained by PCR. Purification of PCR reaction products and byproducts is essential prior the next experiment to eliminate meddling agents. For the construction of the designed plasmid, digestion of the base plasmid (pGAPZαA) and PCR product (ADH2-Cat8-L2 – mApple fragment) is needed. Two digestion enzymes were selected previously while designing the primers which were *Nsi*I and *Kpn*I. To increase the enzyme activity, an appropriate buffer solution should be added into the reaction medium to maintain the optimum H^+ ion concentration (pH). Since *Kpn*I and *Nsi*I are incompatible to each other, sequential digestion is required instead of double

digestion. First, the single digestion reaction catalyzed with *Kpn*I was started in the reaction medium containing pGAPZαA and SOE fragment. The single digested products were purified with the PCR purification Kit (ThermoFisher, USA); thereafter, the single digestion reaction with *Nsi*I was catalyzed. The digestion reactions were carried out at 37°C for 4 hours with each enzyme. The residence time for each digestion reaction, so called the digestion time, was optimized considering the complete digestion and over-digestion.

Table 3. 14 *Single digestion reaction with KpnI for pGAPZαA and SOE*

Component	**Amount**
10X Buffer KpnI	2 µL
DNA (0,5-1 µg)	16 µL
KpnI	1 µL
H_2O	Up to 20 µL

DNA concentration is 55 ng/µL

Table 3. 15 *Single digestion reaction with NsiI for pGAPZαA and SOE*

Component	**Amount**
10X Cut smart Buffer	2 µL
DNA (0,5-1 µg)	16 µL
NsiI HF (Neb)	1 µL
H_2O	Up to 20 µL

Inactivation procedure was conducted after sequential digestion at 80°C for 20 min in water bath. After sequential digestion of both DNA fragment and vector, they run on the agarose gel and double digestion was verified. Both desired segment of vector and double digested insert were extracted from the gel and purified by gel extraction kit (Thermoscientific) explained in section 3.4.5.

3.5.1.4. Ligation reaction of the Plasmid and PCR product

Ligation of the pGAPZαA (vector) and ADH2-Cat8-L2 –mApple fragment (insert) was achieved by T4 DNA Ligase enzyme. 20 µL reaction volume was sufficient for the construction of desired DNA fragments. Ligation reaction was conducted by overnight incubation at 16°C. Component of ligation reaction and constructed plasmid are available in Table 3.16 and Figure 3.3, respectively.

Table 3. 16 *Ligation reaction composition of pGAPZαA and ADH2-Cat8-L2 –mApple*

Component	**20 µL Reaction**
T4 DNA Ligation Buffer	2 µL
Vector DNA (pGAPZαA)	2 µL
Insert DNA (ADH2-Cat8-L2 –mApple)	15 µL
T4 DNA Ligase	1 µL
H_2O	Up to 20 µL

The ratio of the DNA vector to insert was optimized as 3:1. The quantity of insert and vector in the reaction mixture was calculated, as follows:

$$50\ ng\ vektor\ x\ \frac{\text{Size of insert (bp)}}{\text{Size of vector (bp)}}\ x\ molar\ ratio = amount\ of\ insert\ (ng) \qquad (3.1)$$

The heat inactivation of T4 DNA ligase was performed at 65°C for 10 minutes to terminate the reaction; the ligation reaction mixture was stored at -20°C until *E. coli* transformation.

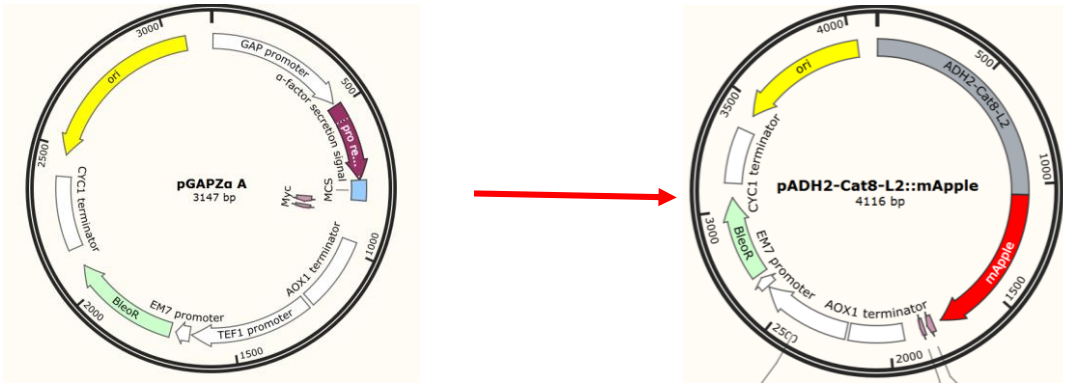


Figure 3. 3 Constructed DNA sequence *(*pADH2-Cat8-L2::*mApple)* from pGAPZα

The transformation of wild *E. coli* DH5α with calcium chloride method was conducted after ligation. The transformation mixture has been inoculated into medium-containing selective marker (zeocin). After 16-18 h incubation period at 37°C, selected colonies were streaked on a fresh LB agar media containing 25 µg/µl Zeocin. After verification of inserted plasmid with colony PCR, four colonies were selected among the positive colonies and plasmid isolation was conducted using the Plasmid Isolating Kit. Insert sequence was controlled by gene sequencing.

For construction of double promoter with different expression cassette, two different selection markers are needed. Commercially PGAPZα carry the Zeocin antibiotic resistance gene as selection marker. Constructed pADH2-Cat8-L2::*mApple* plasmid antibiotic resistance gene was replaced with Nourseothricin (NTC) with TEF promoter and terminator (NATMX6).

NTC antibiotic resistance gene were amplified by Q5 High Fidelity DNA Polymerase (Thermoscientific). PCR mixture composition and thermocyclic conditions are given, as follows:

Table 3.17 *Thermocyclic PCR operation condition for NATMX6 cassette*

Steps	**Temperature**	**Time**	**Cycle**
Initial Denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	30
Annealing	68°C	30 sec	30
Extension	72°C	60 sec	40
Final Extension	72°C	2 min	10
Hold	4°C	∞	-

Table 3.18 *PCR reaction composition for NATMX6 cassette*

Component	**50 µL Reaction**
5X Q5 Reaction Buffer	10 µL
5mM dNTPs	2 µL
10 µM Forward Primer	2.5 µL
10 µM Reverse Primer	2.5 µL
Template DNA	2 µL
Q5 High-Fidelity DNA Polymerase	0.5 µL
H_2O	Up to 50 µL

Amplified NATMX6 cassette and vector plasmid pADH2-Cat8-L2*::mApple* were double digested by *BamH*I and *Pci*I REs (Table 3.19 and 3.20). Double digestion reaction conditions are given, as follows:

Table 3.19 *Double digestion reaction of pADH2-Cat8-L2::mApple* with *KpnI* and *BamHI*

Component	**Amount**
10X Tango Buffer	2 µL
DNA (0,5-1 µg)	10 µL
BamHI	1 µL
PciI	1 µL
H_2O	Up to 20 µL

DNA concentration is 100 ng/µ

Table 3.20 *Double digestion reaction of the insert with KpnI and BamHI*

Component	**Amount**
10X Tango Buffer	2 µL
PCR reaction mixture (0.1-0.5 µg)	15 µL
*BamH*I	1.5 µL
*Pci*I	1.5 µL
H_2O	Up to 20 µL

Insert concentration is 20 ng/µL

*BamH*I and *Pci*I RE were used to double digest both DNA fragment amplified with PCR and the vector. The mixture was incubated at 37°C in water bath overnight. After overnight digestion REs were inactivated at 65°C for 20 minutes in water bath. After double digestion of both insert end vector, amplified DNA fragments and double digested vector were purified using PCR purification kit (Thermoscientific) to eliminate meddling agents and obtain intended segments of vector.

Table 3.21 *Ligation reaction composition*

Component	**20 µL Reaction**
T4 DNA Ligation Buffer	2 µL
Vector DNA (pADH2-Cat8-L2::*mApple*)	2.5 µL
Insert DNA (NATMX6)	12.5 µL
T4 DNA Ligase	1 µL
H_2O	Up to 20 µL

- Incubate at 16°C for 16 h
- Inactivate at 65°C for 10 min

Schematic algorithm of the metabolic engineering strategy used for each construction beginning from primer design is illustrated in Figure 3.4.

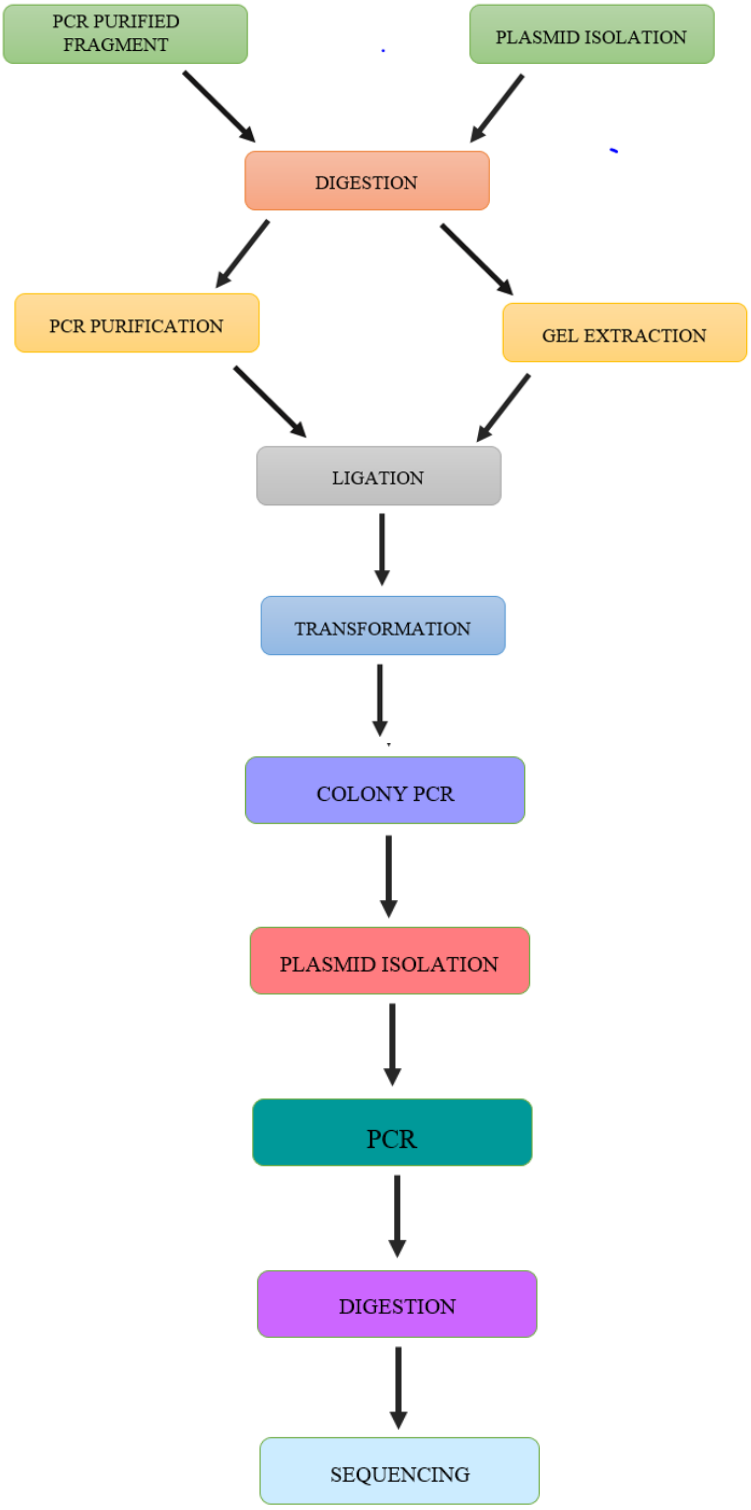


Figure 3.4 Schematic algorithm of the designed metabolic engineering strategy for each construction

3.5.2. Construction of expression cassettes pADH2-Cat8-L2::*hGH* and pmAOX1:: *hGH*

For recombinant human growth hormone (rhGH) production and secretion, fusion PCR was employed to fuse α-factor signal sequence between the NEPVs or NOP and rhGH. For extracellular rhGH production, hGH were cloned by two-step overlap extension PCR without adding any additional nucleotides. Addition of nucleotide including RE sites between promoter, α-factor signal sequence and hGH were prevented as it can decrease the expression capacity of targeted gene. P*mAOX1*, α-factor signal sequence and *hGH* were amplified with Q5 DNA Polymerase (Neb, USA) as indicated in Table 3.10 and Table 3.11. For rhGH production, pGAPZzα-A vector and insert (pmAOX1:: *α-factor::hGH*) were double digested with the REs *Nsi*I and *Xba*I (Table 3.22 and 3.23), and then gel extracted with gel purification kit.

Table 3.22 *Double digestion reaction of pGAPZαA with NsiI and XbaI*

Component	**Amount**
10X Cut smart Buffer	2 µL
Plasmid DNA (0.5-0-1 µg)	10 µL
*Nsi*I	1 µL
*Xba*I	1 µL
H_2O	Up to 20 µL

DNA concentration is 100 ng/µL

Table 3. 23 *Double digestion reaction of the insert with NsiI and XbaI*

Component	**Amount**
10X Cut smart Buffer	2 µL
SOE product (0.1-0.5 µg)	12 µL
*Nsi*I	1.5 µL
*Xba*I	1.5 µL
H_2O	Up to 20 µL

Insert concentration is 40 ng/µL

- Incubate at 37°C for overnight
- Inactivate at 65°C for 10 min

The T4 DNA Ligase (ThermoScientific) was bonded to purified insert and vector genes by using a 3:1 ligation ratio (Table 3.24). After ligation, a chemical technique centered on calcium chloride was used for transformation of wild type *E. coli* DH5α cells. Inoculation of transformation blend was performed into the specific NTC containing media.

Table 3.24 *Ligation reaction compositions*

Component	**Amount**
T4 DNA Ligation Buffer	2 µL
Vector DNA	50 ng
Insert DNA	3:1 molar ratio
T4 DNA Ligase	1 µL
Nuclease-free water	up to 20 µL

3.5.3. Construction of Recombinant Plasmids P*eAOX1*

P. pastoris modified *alcohol oxidase 1* (*AOX1*) promoter's TFBSs will be manipulated to boost its regulatory mechanism on ethanol induction. pAOX1 variant (eAOX) were designed based on putative TFBS deletion. ACA2 TFBS (Table 3.25) were deleted.

Table 3. 25 *TFBS used to modify P$_{mAOX1}$*

TFBS	**Source**
Aca2	
GCCTATTGTAGACGTCAACCC	MatInspector

An extra primer set was also used to create fast and efficient P$_{AOX1}$-*eGFP* variant (peAOX). As the RE site between promoter and reporter gene might influence transcriptional activities, the base plasmid was designed with Forward and Reverse

AOX-eGFP primers by OE-PCR. Complementary fragments were first purified by GeneJetPCR (Thermoscientific) purification kit and then used as a template for two steps of the OE-PCR. In the 2nd step of the OE-PCR forward AOX1, reverse eGFP and purified fragment with supplementary ends were used. Amplification was performed with Q5 DNA Polymerase as explained before, but the elongation time was increased to 1 min 20 sec. Amplicons from OE-PCR were run on agarose gel electrophoresis and bands were in the anticipated place. Insert and base vector pGAPZα were double digested with *Bgl*II and *Kpn*I RE. T4 DNA Ligase (Thermo Scientific), by using a 3:1 ligation ratio (Table 3.24), was used to link purified insert and vector. The ligation product was used to transform the *E. coli* DH5α cells. As mentioned in Sambrook and Russell (2001), competent *E. coli* DH5α cells were developed using a calcium chloride technique.

3.5.4. Construction of *P. pastoris* strains carrying $P_{ADH2\text{-}Cat8\text{-}L2}$, P_{GAP}, P_{mAOX1} and P_{eAOX}

Verified pADH2-Cat8-L2*::mApple,* pmAOX1::*eGFP,* pGAP::*eGFP,* pADH2-Cat8-L2::*hGH,* pmAOX1::*hGH* and peAOX::*eGFP* plasmids were linearized with the aim of stimulating plasmid integration into the *P. pastoris* genome. Linearization was performed by incubating the reaction mixture containing plasmids at 37°C for 2-6 h depending on the quantity of DNA. Linearization of plasmids were performed by *BamH*I RE (Table 3.26) and purification of the linearized product was performed as described in section 3.4.2. Samples were eluted with 0.2 μm filter-sterilized pure water. Linearized plasmids were transformed to of wild type *P. pastoris* X-33 cell or recombinant *P. pastoris* cells by LiCl method as described in section 3.4.8. For each construct randomly 24 colonies were picked and inoculated new petri dishes containing proper antibiotic. Integration of expression cassettes into wild type *P. pastoris* X33 has been confirmed through colony PCR. For each structure at least 8 positive clones were chosen and used for additional testing steps.

Table 3. 26 *Linearization of plasmid for P. pastoris transfection*

Component	**Amount**
10X Buffer *BamH*I	2 µL
DNA (0,5-1µg)	15 µL
*BamH*I	1 µL
H_2O	Up to 20 µL

3.6. Determination of gene-copy number

The gene copy number is determined by quantitative polymerase chain reaction. In order to compare promoters' expression strength properly gene-copy numbers should be equal.

To determine *mApple*, *eGFP* and *hGH* gene copy number relative quantification was conducted. As a reference gene argininosuccinate lyase gene was used and quantified parallel with the unknown *mApple*, *eGFP* and *hGH* in the genomic DNA as *ARG4* is a single copy gene in *P. pastoris.* Firstly, primers for *mApple*, *eGFP*, *hGH* and *ARG4* were designed using Snap-gene viewer and Oligo Analyzer 3.1 (Table 3.27). Then, genomic DNA of *P. pastoris* strains were isolated with Wizard® Genomic DNA Purification Kit (Promega) according to instructions of manufacturer.

Table 3.27 *Primers for mApple, eGFP, hGH, and the standard ARG4 genes standard*

Name	**Sequence (5'-3')**	**Length (bp)**	**Tm (ºC)**	**GC Content**	**Size of Amplicon (bp)**
mApple-F	GCTACTAGCTTTTATCTTATTTACT TTACGAAA ATGGTGAGCAAGGGC	36	73	38	711
mApple-R	CTGGTACCTTACTTGTACAGCTCG TCATGC	30	72	50	711
mApple-qPCR-F	GAGGTCAAGACCACCTACAA	20	64	50	105
mApple-qPCR-R	CTGTTCCACGATGGTGTAGT	20	64	50	105
eGFP-F	GGAATTCATGGTGAGCAAGGGCG AGGAG	28	64.4	57.1	735
eGFP-R	CCGGTACCTCACTTGTACAGCTCG TCCAT	29	64.3	52.2	735
egfp-qPCR-F	GGA CGA CGG CAA CTA CAA GA	20	63.8	55	185
egfp-qPCR-R	CCT TGATGC CGTTCTTCTGC	20	63.3	55	185
hGH-F	GCCTTTGACACCTACCAGGA	20	67	55	582
hGH-R	ACACCAGGCTGTTGGCGAAG	20	71	60	582
hGH-qPCR-F	GCCTTTGACACCTACCAGGA	20	67	55	238
hGH-qPCR-R	ACACCAGGCTGTTGGCGAAG	20	71	60	238
ARG4-Std-F	GTTT ACA CTG AGG GCC TGG A	20	56.8	55	1259
ARG-Std-R	GACTCTAGCTTTTCATTCAGTGC	23	53.8	43.5	1259
ARG-F	GGTGAGTTGATTGGTCGTGG	20	62.7	55	185
ARG-R	CCGGGCATCAAGACGTCTAT	20	63.6	55	185

ARG4 represents the single copies of the genome and the *mApple*, *eGFP* and *hGH* assayed copies are normalized by *ARG4* gene. The relative gene copy number can be calculated as described below:

$$Copy\ number = \frac{Assayed\ copy\ quantity\ of\ target\ gene}{Assayed\ copy\ quantity\ of\ ARG4} \quad (3.2)$$

In order to determine gene-copy number in genomic DNA standard curve is necessary for the absolute quantification. Since there are four genes that should be measured and standards curves should be obtained. *P. pastoris* X33 genome, pADH2-Cat8-L2::*mApple* pmAOX1::*eGFP* and pGAP::*hGH* plasmids were isolated, respectively. For determination of gene copy number two sets of primers are used namely inner and outer primers. The outer primers amplify a template (standard DNA) and inner primers can amplify a part of standard DNA. PCR was performed with ARG Standard primers (Arg-Std-R and Arg-Std-F) using *P. pastoris* X33 genome as template and mApple standard primers (mApple-F and mApple-R), eGFP standard primers (eGFP-F and eGFP-R) and hGH standard primers (hGH-F and hGH-R) using pADH2-Cat8-L2::*mApple* pmAOX1::*eGFP* and PGAP::*hGH* plasmids respectively as a template PCR products were purified with GeneJet PCR Purification Kit (Fermentas).
According to Bioinformatics online DNA molecular weight calculator (http://www.bioinformatics.org/sms2/dna_mw.html)

ARG4 Standard Amplicon Molecular Weight: 777867.71 Da

mApple Standard Amplicon Molecular Weight: 457351.56 Da

eGFP Standard Amplicon Molecular Weight: 454273.33 Da

hGH Standard Amplicon Molecular Weight: 177296.52 Da
10^3, 10^4, 10^5, 10^6 copy quantities were used to draw standard curve for *mApple*, *eGFP*, *hGH* and *ARG4* standard genes. 9 µl diluted genomic DNA samples (2 ng/µl) were used as template with LightCycler® 480 SYBR Green I Master (Roche) for each qPCR. Roche LightCycler ® 480 Instrument II was used to conduct qPCR.

Table 3.28 *Composition of qPCR mixture*

Compound	**Amount**
Master Mix 2X conc	10 µl
Forward Primer (10µM)	0.5 µl
Reverse Primer (10µM)	0.5 µl
Template	9 µl

$$Copy\ quantity = \frac{amount\ of\ sample\ (ng) * 6.022 * 10^{23}}{DNA\ molecular\ weight * 10^{9}} \quad (3.3)$$

- $6.022*10^{23}$ Avagadro number
- 10^{9}, to convert molar mass into ng

Table 3.29 *Thermal-cycler operation-profile for the qPCR experiments*

Denaturation	**Amplification**	**Melting**	**Cooling**
1 Cycle	45 Cycles	1 Cycle	1 Cycle
	Denaturation:		
	95°C for 10 sec	Melting:	Keeping:
Hold:	Annealing:	50°C to 99°C	40°C for 30 sec
95°C for 10 min	55°C for 5 sec	Continuous with slope of 1°C/s	
	Elongation:		
	72°C for 10 sec		

3.7. Screening Conditions

3.7.1. mApple and eGFP syntheses with selected r-*P. pastoris* strains

Intracellular mApple and eGFP syntheses and comparison of constructed double promoters as well as that of single and that of multi-copies were carried out 12-deep well plates. Selected colonies were grown in 2 mL YP medium containing 25 µg/µL zeocin or 25 µg/µL for 18-20 h and then cells were transferred to both minimal production medium (Table 3.7) and ASMv6 (Table 3.8) production medium with selected carbon source. Screening of constructed double promoters' expression systems and their single copies for mApple and eGFP production in all carbon sources was carried out at an initial OD_{600} of 1 to production medium supplemented 20 g/L glucose or glycerol, limited glucose 1% (v/v) methanol and 2% (v/v) ethanol. The cells were harvested at 4500g for 5 min at t = 22-24 h of the cultivation. Then, diluted or concentrated to OD_{600} of 8 and directly used for mApple and eGFP production measurement with fluorescence spectrophotometer (Agilent technologies). Screening experiments were conducted in duplicate and the schematic representation is given in Figure 3.5.

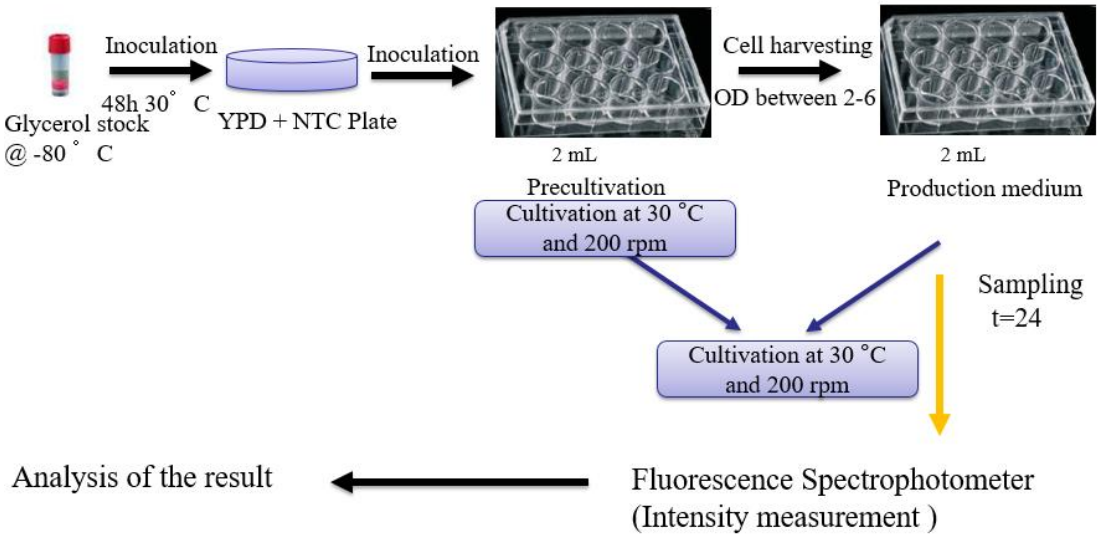


Figure 3.5 Schematic representation of screening experiments for mApple and eGFP synthesis

3.7.2. Extracellular rhGH production with *P. pastoris* strains

Extracellular rhGH production as third model protein was carried out in shake-flask air-filtered bioreactors. The selected single-copy and double-copy strains of *P. pastoris* were grown in 50 mL YPD medium with Zeocin concentration of 25 µg/mL or NTC concentration of 50 µg/mL at 30°C and 200 rpm for 18-20 h. Fermentation were initiated at OD_{600} of 1 in production medium (Table 3.7). The cells were harvested at t = 48 h at 4500 g for 5 min and supernatants were collected for rhGH determination by SDS-PAGE analyses whereas cell growth was measured by wet cell weight measurement. rhGH production capacity of the selected double promoter expression systems were tested in 2% ethanol. Screening experiments were conducted in duplicate and the schematic representation is given in Figure 3.6.

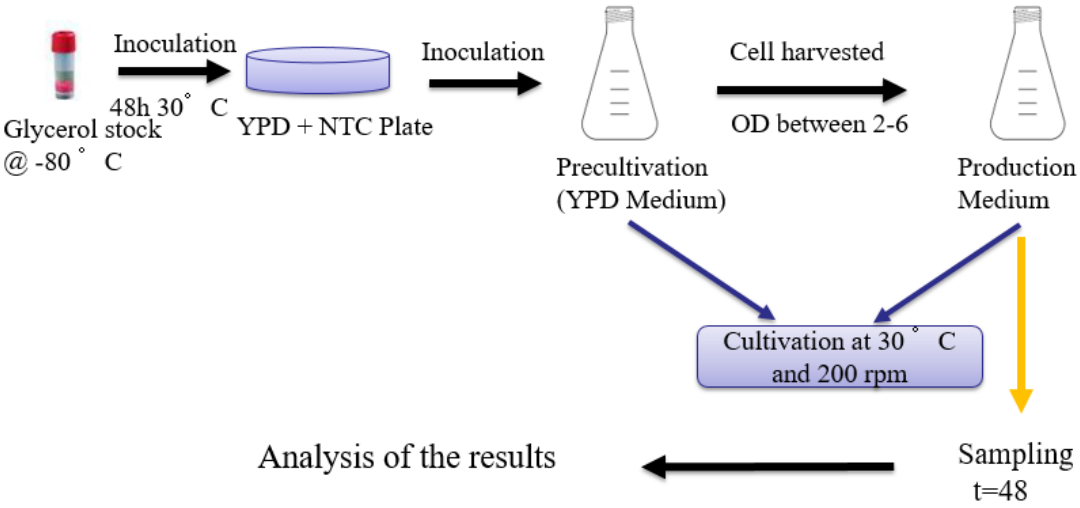


Figure 3.6 Schematic representation of the experiments for hGH production

3.8. Analyses

3.8.1. Cell concentration

Wet cell concentration (g/L) was calculated related to OD_{600} of the samples that were collected during the exponential phase and following equation was used to determine cell concentration.

$$C_X = 0.24 \times OD_{600} \times Dilution\ factor \quad (3.4)$$

3.8.2. mApple and eGFP syntheses

Fluorescence Spectrophotometer (Agilent technologies) was used for the measurement of mApple and eGFP by using 96-well microtiter-plates (Thermoscientific). Red fluorescent protein (mApple) measurements were performed at excitation and emission wavelength of 568/592 nm whereas green fluorescent protein (eGFP) was measured at excitation and emission wavelength of 488/509. The cell suspensions were diluted or concentrated to OD_{600} = 8 and mixed by pipetting with multi-pipette. For mApple and eGFP fluorescence measurement the background signal of wild type *P. pastoris* X-33 was removed for normalization. mApple and eGFP production were given in arbitrary unit (a.u.) based on the intensity measurement by fluorescence spectrophotometer

3.8.3. rhGH Production

Extracellular rhGH concentration was measured by SDS-PAGE by silver staining. 13 µL of supernatant with 5 µL loading dye (4X fold) and 2 µL DTT were treated at 95°C for 5 minutes then immediately chilled on ice for 5 min to determine rhGH levels. 15 µL samples and 2 µL of maintained marker were run for roughly 40-50 min at a steady voltage at 200 V constantly. The gel was incubated at room temperature with shaking in the fixer for at least 1 hour (maximum 16 hours).

The fundamental steps of the silver staining procedure include: i) fixation to dispose of the intervening compounds, ii) rinsing to improve the transparency and susceptibility of staining, iii) impregnation with a solution of silver chloride, iv)

rinsing and advancement to produce a golden picture, v) rinsing and stopping the growth to avoid the formation of unnecessary context and further handling.

Table 3.30 *Silver staining procedure*

Steps	**Solution**	**Time**
Fixing	Fixer solution	min 1 hour
Washing	50% EtOH	3 x 20 min
Rinsing	Water	3 x 20 sec
Pretreatment	Pretreatment solution	1 min
Rinsing	Water	3 x 20 sec
Impregnation	Silver nitrate solution	20 min
Rinsing	Water	3 x 20 sec
Developing	Developing solution	~ 5 min
Stop	Stop solution	∞

All solutions were freshly prepared and compositions are given in Appendix D

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Novel *P. pastoris* strains with double-promoter expression system architectures

Two reporter proteins were used to determine and differentiate individual and simultaneous strengths of the novel-engineered promoter variants (NEPVs) P_{mAOX1} and $P_{ADH2\text{-}Cat8\text{-}L2}$, and the naturally occurring promoter (NOP) P_{GAP}. *P. pastoris* strains carrying single-copy and multi-copy reporter genes determined by the qPCR analysis were constructed to make clear comparisons of the double-promotor expression systems (DPESs) with their constituent systems (Figure 4.1 and 4.2). Fermentations in shake-bioreactors were performed to evaluate the strength of novel *P. pastoris* cells constructed with DPESs, producing either eGFP under P_{GAP} / P_{mAOX1} or mApple under $P_{ADH2\text{-}Cat8\text{-}L2}$. Enhanced green fluorescent protein (eGFP) and red fluorescent protein (mApple) were encoded by the reporter genes *eGFP* and *mApple* in the expression cassettes, and the intracellular fluorescent protein syntheses were quantitatively measured using fluorescent spectrophotometer. DPES strength was also tested in fermentations for extracellular human growth hormone (rhGH) production. In order to demonstrate the strength of: ***i)*** each DPES explicitly, DPESs were compared with the DPESs having identical twin promoter architectures; and ***ii)*** each constituent NEPV or NOP separately, two different fluorescent protein genes were used where each encodes a characteristic model protein with the NEPV in the related DPES; furthermore, ***iii)*** DPESs were also compared with that the corresponding single-promoter expression systems (SPESs). A DPES was designed with the constituent NEPVs $P_{ADH2\text{-}Cat8\text{-}L2}$ and P_{mAOX1} as a simultaneously- (SMT-) operating DPES induced by ethanol, while $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$ was designed as a consecutively- (CST-) operating DPES. If P_{GAP} is used in a CST- operating DPES which preferably uptake glucose and

also contributes to the cell growth, consecutive iterations of the carbon sources glucose and ethanol is required in fed-batch phase of the fermentations by switching from one substrate to the other. Where with P_{GAP}, ethanol is also produced as a byproduct on glucose, and in turn by interrupting glucose-feeding and shifting to ethanol-fed batch culture shifts the control of the bioprocess to the control of the second constituent $P_{ADH2\text{-}Cat8\text{-}L2}$, which is induced by the accumulated- and also the continuously-fed-ethanol, that proceeds in successive-cycles.

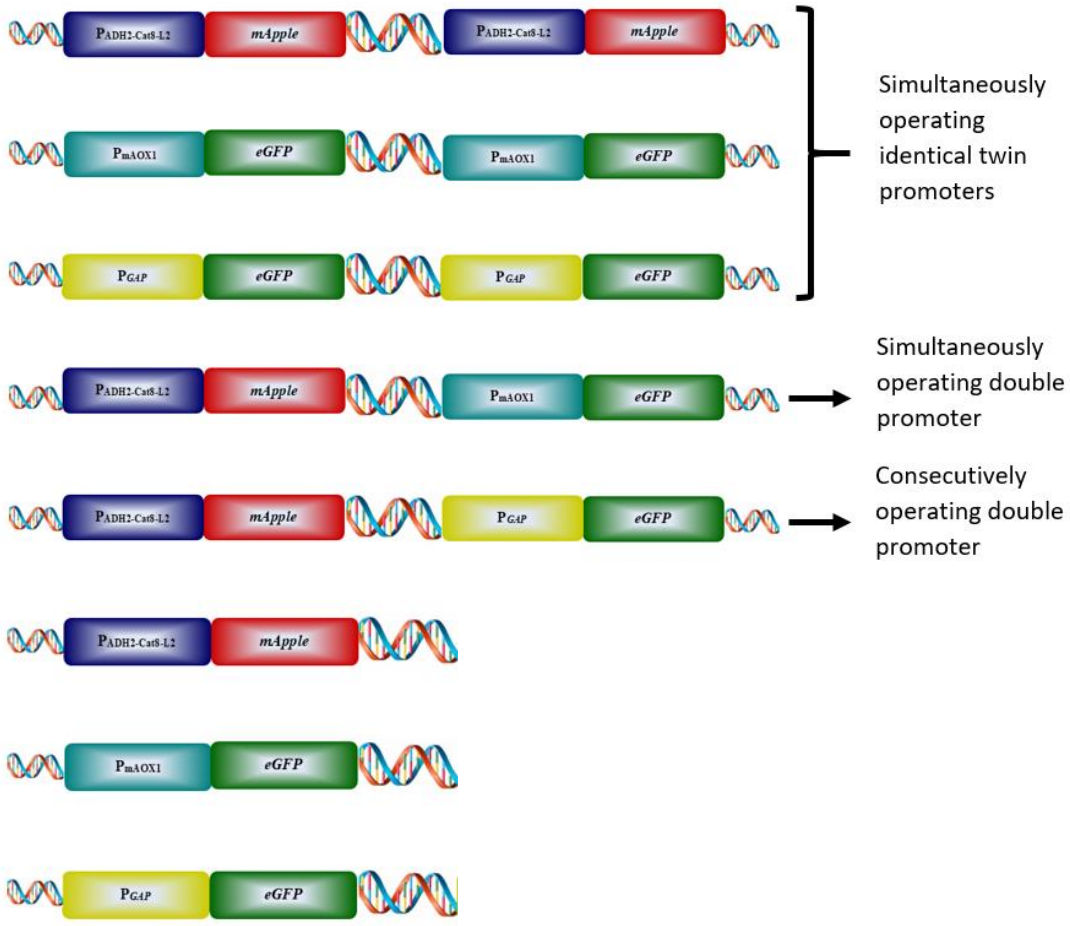


Figure 4.1 Double promoter expression system architectures constructed with fluorescent protein genes

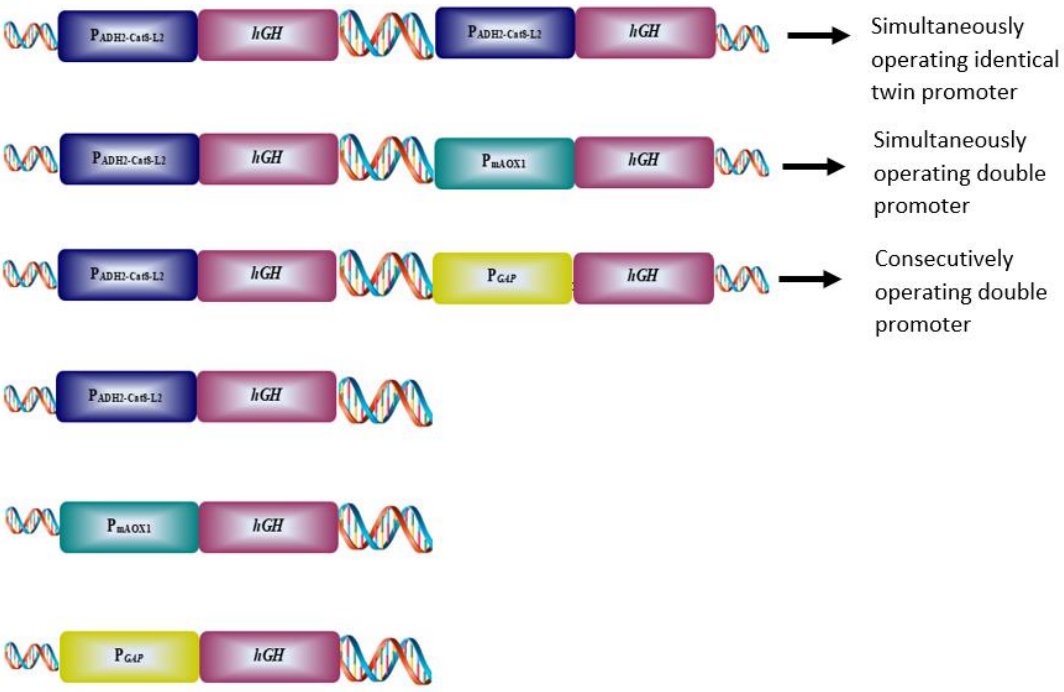


Figure 4. 2 Double promoter expression system architectures constructed with human growth hormone gene (*hGH*)

Table 4. 1 *Minimal set of SPESs constructed to cover regulatory profiles*

SPESs	Regulation	Strength
$P_{ADH2\text{-}Cat8\text{-}L2}$	Inducible	Strong on ethanol
P_{mAOX1}	Inducible	Strong on ethanol
P_{GAP}	Constitutive	Strong on glucose, weak on ethanol

Table 4. 2 *Minimal set of DPESs constructed to cover regulatory profiles*

DPESs	Regulation	Strength
$P_{ADH2\text{-}Cat8\text{-}L2}$ - $P_{ADH2\text{-}Cat8\text{-}L2}$	Inducible – Inducible	Strong on ethanol
P_{mAOX1} - P_{mAOX1}	Inducible – Inducible	Strong on ethanol
P_{GAP} - P_{GAP}	Constitutive - Constitutive	Strong on glucose, weak on ethanol
$P_{ADH2\text{-}Cat8\text{-}L2}$ - P_{mAOX1}	Inducible – Inducible	Strong on ethanol – Strong on ethanol
$P_{ADH2\text{-}Cat8\text{-}L2}$ - P_{GAP}	Inducible - Constitutive	Strong on ethanol - Strong on glucose, weak on ethanol

Double promoter vectors are transformed by sequential transfection with single promoter expression cassettes. To construct DPESs with two different expression cassettes two different antibiotic resistance gene were used: Zeocin and NTC (Figure 4.3).

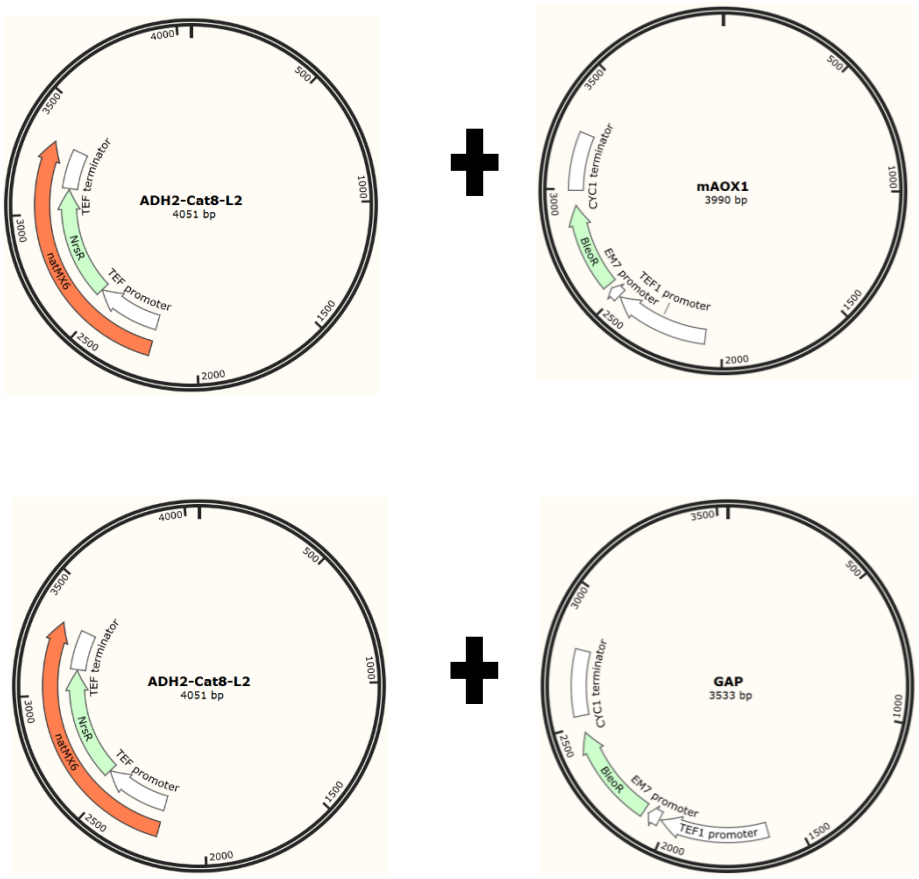
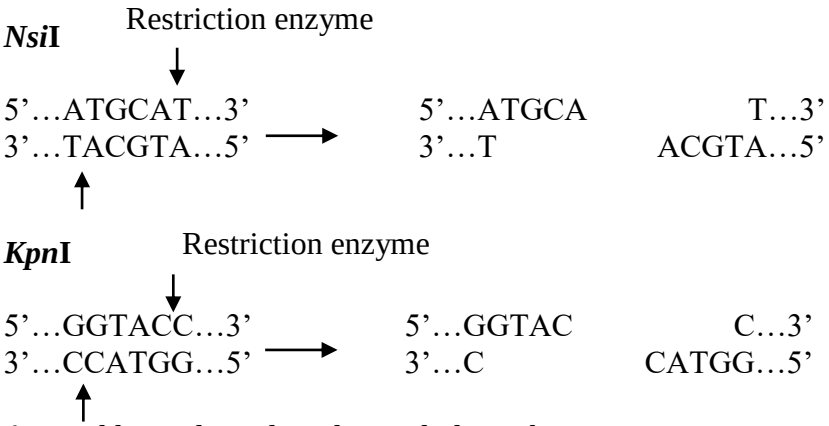


Figure 4. 3 Design of two single promoter plasmids for sequential transfection in *P. pastoris*

4.2. Recombinant plasmids constructed with DPES and SPES in *P. pastoris*

The plasmid backbone was designed using the selected restrictive enzymes, i.e. *Nsi*I and *Kpn*I, to insert *ADH2-Cat8-L2* and *mApple* sequence into the base-vector pGAPZα-A. Optimum PCR annealing temperature and extension time were determined (section 3.5.1.2). *E. coli* DH5α transformation of the constructed plasmids was performed by calcium chloride method (section 3.4.7). Least eight clones were selected and the plasmids were isolated for the analysis. The insertion of constructed plasmids was controlled by the PCR using the isolated plasmid as template, and by DNA sequencing (METU Central Laboratory). Glycerol stocks were prepared and stored at - 80°C for the positive colonies

Recombinant pADH2-Cat8-L2*::mApple* was constructed by using pGAPZα-A as the base vector. Noncutters of $P_{ADH2\text{-}Cat8\text{-}L2}$, *mApple* and unique cutter of pGAPZα-A were determined by SnapGene Viewer and Restriction mapper. *Nsi*I and *Kpn*I were selected as the restriction sites.



Any additional nucleotide, including the restriction enzyme sites, was avoided between the promoter and *mApple* gene sequences during constructing the recombinant plasmids. To eliminate the complexity of the secretion process and examine the individual effects of the promoter capacity on r-protein production, the DPESs constructed with $P_{ADH2\text{-}Cat8\text{-}L2}$ produce *mApple* intracellularly.

A high-fidelity Q5 DNA polymerase (NEB) was used to amplify insert $P_{ADH2\text{-}Cat8\text{-}L2}$ (1047 bp) and *mApple* (711 bp). The PCR mixture content and thermal-cycler conditions were presented in Tables 3.10 and 3.11. As a result of OE-PCR, the gene fragments of the expected size were amplified and in order to increase the PCR efficiency, different annealing temperatures were tested systematically and shown in Figure 4.4.

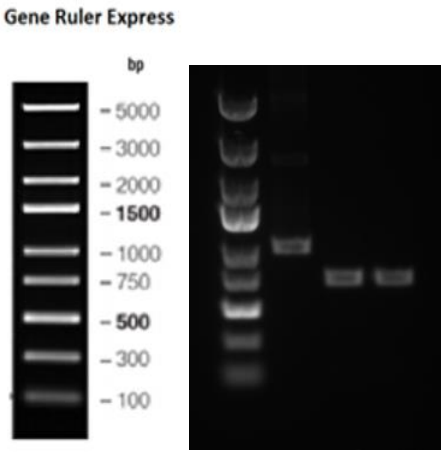


Figure 4.4 Agarose gel electrophoresis image of the genes amplified with different primer combinations after the first step of PCR. 1: Generuler Express DNA ladder (Fermentas) 2: Forward_ADHoptcat and Reverse_ADHoptcat 3: forward_α-mApple and reverse_mApple at 62°C 4: forward_α-mApple and reverse_mApple at 65°C

Annealing temperature for amplification of ADH2-Cat8-L2 was optimized as 65°C whereas annealing temperature of mApple was optimized as 62°C. The amplified $P_{ADH2\text{-}Cat8\text{-}L2}$ and *mApple* fragments that have complementary ends were purified by using the PCR Purification kit and then used as a template for an overlap extension PCR (SOE-PCR) with Q5 DNA Polymerase, as explained in Tables 3.12 and 3.13.

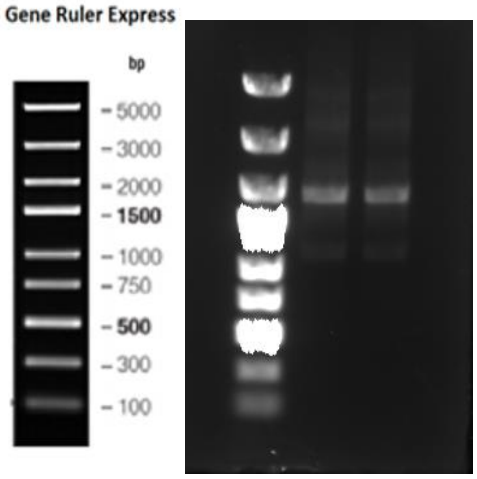


Figure 4.5 Agarose gel electrophoresis image of genes amplified with different primer combinations after the first step of OE-PCR.;1: Generuler Express DNA ladder 2 & 3: Forward_ADHoptcat and Reverse_mApple

The amplified insert of $P_{ADH2\text{-}Cat8\text{-}L2\text{-}mApple}$ and the base-plasmid pGAPαA were sequentially digested by *Nsi*I and *Kpn*I REs. As *Nsi*I and *Kpn*I does not work with 100% efficiency in a single buffer, both insert and vector were digested with *Nsi*I and purified with the PCR purification kit. Then, they were digested with *Kpn*I (Figure 4.6), and gel extracted. Double-digestion reaction mixture compositions and conditions were given in Tables 3.14 and 3.15.

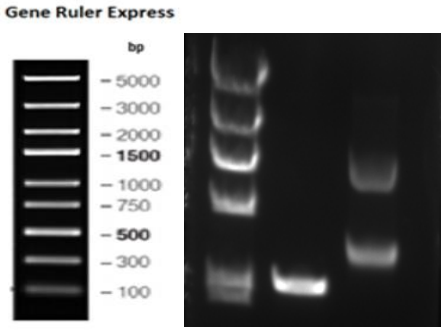


Figure 4.6 1: GeneRuler DNA ladder (Fermentas), 2: *Nsi*I and *Kpn*I REs digested insert, 3: pGAPZα-A *Nsi*I and *Kpn*I digested with REs

After the sequential digestion of the insert DNA and vector, the bands were cut from the gel and purified with the gel elution kit. The double-digested and purified insert and vector DNA fragments were ligated with T4 DNA Ligase (Thermo Scientific) as described in section 3.5.1.4.

After the ligation reaction, transformation to wild type *E. coli* DH5α cells were conducted by the calcium chloride method; and the mixture was inoculated into the selective medium containing zeocin. Single colonies were chosen and inoculated in a fresh zeocin containing LB-agar medium after 16-18 hour of incubation at 37°C. Among the putative transformants, four colonies were randomly chosen and the plasmid isolation was carried out. Colony PCR with *ADH2-Cat8-L2* forward and *mApple* reverse primers was conducted with *Taq* DNA Polymerase.

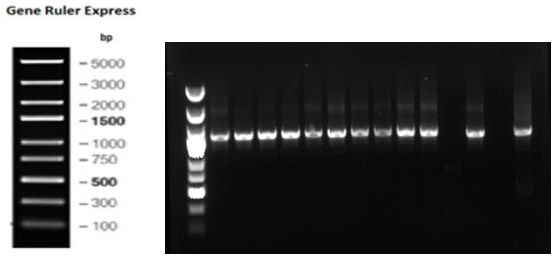


Figure 4.7 Agarose gel image of colony PCR control of potential recombinant plasmids. 1: Generuler Express DNA ladder, 2-13: Potential plasmids carrying the $P_{ADH2\text{-}Cat8\text{-}L2}$ 14: Negative control for PCR control of the insert gene 15: Positive control for PCR control of the insert gene

Plasmid isolation of putative transformants were carried out with 4 different positive clones. In order to further verify the insertion of desired plasmid, PCR with *ADH2-Cat8-L2* forward and *mApple* reverse primers using the isolated plasmids as template. The PCR mixture and the thermal conditions were presented Tables 4.3 and 4.4.

Two verified colonies were sequenced (The Central Laboratory of METU). NCBI BLAST was employed to verify the sequence analyses based on the anticipated components availability of the designed plasmids. The nucleotide sequence was used in all the isolated plasmids of the selected transforming colonies, and Microbank stocks from *E. coli* strains were prepared and stored at -80° C. Appendix B provides examples of the sequence analyses results of the pADH2-Cat8-L2::*mApple*.

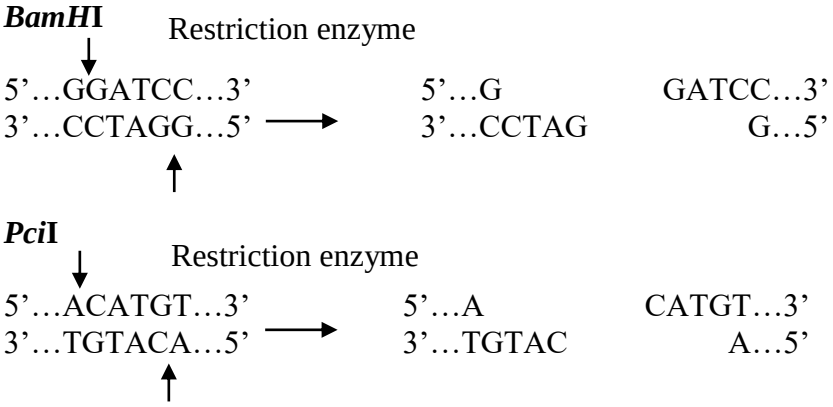
Table 4.3 *Thermocyclic PCR operation condition for the colony PCR with Taq DNA Polymerase*

Steps	**Temperature**	**Time**	**Cycle**
Initial Denaturation	95°C	30 sec	1
Denaturation	95°C	10 sec	30
Annealing	60°C	30 sec	30
Extension	68°C	1.5 min	30
Final Extension	68°C	5 min	1
Hold	4°C	∞	-

Table 4.4 *PCR reaction composition for the colony PCR*

Component	**50 µL Reaction**
10X Standard Taq reaction Buffer	5 µL
5mM dNTPs	2 µL
10 µM Forward Primer	1 µL
10 µM Reverse Primer	1 µL
Template DNA	4 µL
Q5 High-Fidelity DNA Polymerase	0.25 µL
H_2O	Up to 50 µL

In order to construct DPESs with two different expression cassettes, two different antibiotic resistance genes were used: Zeocin and NTC. The commercial plasmid pGAPZα carries a Zeocin antibiotic resistance-gene. The antibiotic resistance-gene of the constructed and sequenced pADH2-Cat8-L2*::mApple* plasmid was replaced with the NTC *gene*. RE sites to digest zeocin in pADH2-Cat8-L2::*mApple* and the noncutter of NTC were determined by SnapGene Viewer and Restriction mapper. *BamH*I and *Pci*I were selected as the restriction sites; and, the digestion reactions catalyzed by *BamH*I and *Pci*I provided the restriction digestion in 5‟ and 3‟ ends, as follows:



Zeocin is deleted from the constructed plasmid with its own promoter (TEF) and terminator (CYC1), while NTC is inserted into the plasmid with TEF promoter and TEF terminator, and the cassette is denoted as NATMX6 (1120 bp).

NATMX6 was amplified with NTC forward and NTC reverse primers, and the PCR compositions and thermocyclic conditions are given in Tables 3.17 and 3.18.

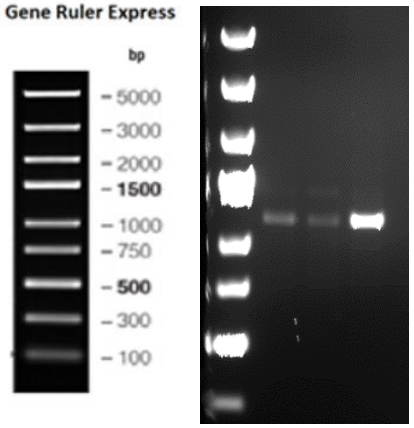


Figure 4.8 Agarose gel electrophoresis image of genes amplified with different primer combinations after the first step of PCR. 1: Generuler Express DNA ladder (Fermentas) 2: Forward_NTC and Reverse_NTC at 62°C 3: forward_Pα-mApple and reverse_mApple at 62°C Forward_NTC and Reverse_NTC at 65°C 4: Forward_NTC and Reverse_NTC at 68°C

Double-digestion reactions of the sequenced pADH2-Cat8-L2::*mApple* and NATMX6 cassette were conducted with *BamH*I and *Pci*I to excrete Zeocin antibiotic resistance-gene, and for the integration of NATMX6 cassette into the pADH2-Cat8-L2::*mApple*. *BamH*I and *Pci*I double digestions of the parent-plasmid and the insert were conducted as outlined in Tables 3.19 and 3.20. Figure 4.9 shows the result of the double digestion reaction. The intended parts were cut and extracted by gel extraction kit, as previously explained.

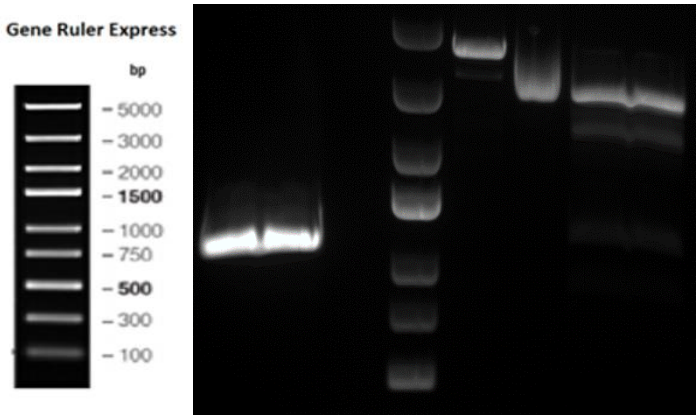


Figure 4.9 Agarose gel electrophoresis image of 1: double digested insert BamH*I and* Pci*I.*, 2: Generuler ready to use (Thermoscientific), 3: Linearize plasmid, 4: circular plasmid, 5: double digested vector with BamH*I and* Pci*I.*

T4 DNA ligase enzyme, as outlined in chapter 3.5.1.4 and Table 3.16, was linked to the double-digested insert and vector at corresponding ends, i.e., with a respective digested end of the same restriction enzymes, *BamH*I and *Pci*I. The ratio of the insert NATMX6 to pADH2-Cat8-L2::*mApple* that is the backbone for the two separate sections (equation 3.1) was 3:1.

As described in section 3.4.7, the ligation reaction results were used to transform *E. coli* DH5α cells by a calcium chloride method. The colonies should be tested to determine the positive-seeming false colonies after the conversion. Among thousands of colonies a few colonies were picked randomly and inoculated for the verification and analysis on selective LB-agar containing NTC.

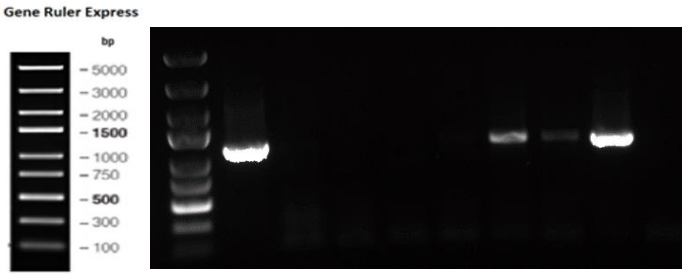


Figure 4.10 Agarose gel image of colony PCR control of potential recombinant plasmids. 1: Generuler Express DNA ladder, 2-13: Potential plasmids carrying the NATMX6 14: Negative control for PCR control of the insert gene

The first step of the confirmation analysis was the colony PCR with NTC forward and NTC reverse primers (Table 3.1). Tables 4.3 and 4.4 show the PCR reaction mixture and the PCR thermocyclic conditions, respectively. The distance between the two primers in the base plasmid (pADH2-Cat8-L2::*mApple*) is ca. 1120 bp. The result of the PCR of the selected putative transformants should be ca. 1120 bp if NATMX6 cassette is inserted and excluding the Zeocin antibiotic resistance gene with *BamH*I and *Pci*I cleavage sites. The confirmed four colonies were isolated by the plasmid isolation kit (section 3.4.1); and to further confirm the insertion of the plasmid, PCR was performed on the four of the isolated plasmids with NTC forward and NTC reverse primers (Figure 4.11).

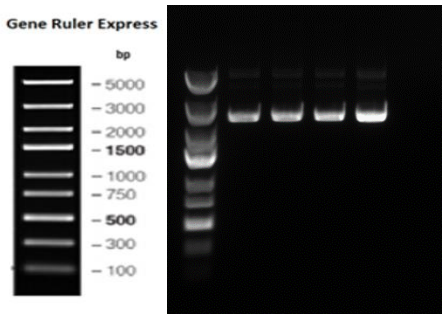


Figure 4.11 Agarose gel image of potential recombinant plasmids. 1: Generuler Express DNA ladder, 2-5: Potential plasmids carrying the NATMX6 cassette (1120 bp)

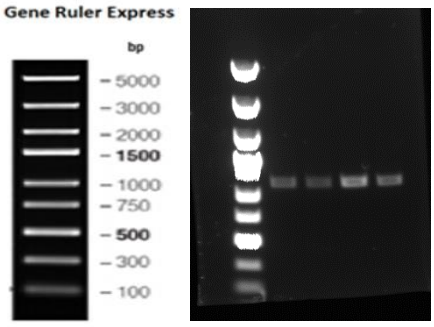


Figure 4.12 Agarose gel image of control PCR for potential recombinant plasmids. 1: Generuler Express DNA ladder, 2-5: Control PCR with NTC forward and reverse (NATMX6 cassette 1120 bp)

As can be seen in Figures 4.11 and 4.12, the isolated plasmids are in the expected range in the agarose gel (1120bp). For the four isolated plasmid PCR was conducted and the results revealed the proper ligation and transformation. The isolated two plasmids were sequenced. Appendix B confirmed the results of both colonies were confirmed based on BLAST analysis and one of them was used for subsequent *P. pastoris* transfection

Transformation of *P. pastoris*

The cloned plasmids were transfected to *P. pastoris* X-33 and the recombinant cells by genomic integration following the development of base plasmid (pADH2-Cat8-L2::*mApple*). The plasmids were first linearized then purified and used for transfection after linearization with *BamH*I. After the development of single-colony cultures, plasmid isolation was conducted. Table 3.26 provide the digestion-reaction solution for the plasmids containing $P_{ADH2\text{-}Cat8\text{-}L2}$ and *mApple* gene. After the digestion, digested and linearized plasmid was extracted from the gel and purified with gel purification kit where ultrapure water was used for elution instead of elution buffer.

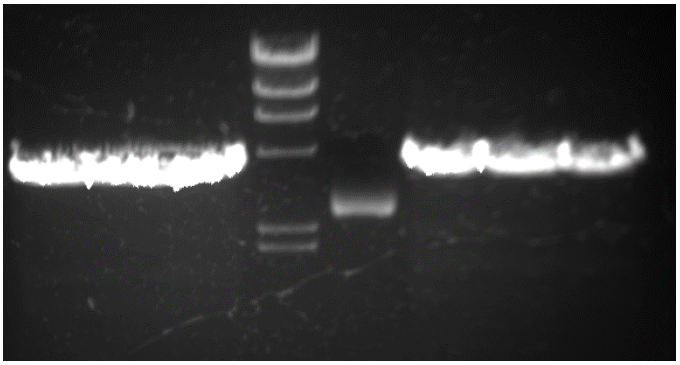


Figure 4.13 Agarose gel electrophoresis image of 1: Linearized plasmid (pADH2-Cat8-L2::*mApple*) with *BamH*I., 2: Generuler ready to use (Thermoscientific), 3: circular plasmid, 4: Linearized plasmid (pADH2-Cat8-L2::*mApple*) with *BamH*I

The gel-electrophoresis results revealed that the digestion reaction was successfully completed. Wild type X-33 and recombinant (pGAP::*eGFP* and pmAOX1::*eGFP*) *P. pastoris* transfections were performed with the linearized plasmids by the lithium chloride method (section 3.4.8). After approximately 48 hours, the putative colonies

were verified. For the each plasmid, 16 colonies were selected. The randomly selected 16 colonies were grown on YPD agar plate containing NTC for 24 hours at 30°C after that used for further verification of the colony PCR experiments. After *P. pastoris* transfection, insertion of the plasmid was controlled with colony PCR, where instead of the genomic DNA the *P. pastoris* cells boiled at 95°C at 5 min were used as the template.

4.2.1. Screening of constructed DPESs and SPESs

Following the conversion of recombinant and wild-type X-33 *P. pastoris* cells, least eight clones were analyzed for the each construct; and the clones representing the entire population were selected for further analysis. In order to select the true colonies representing the entire population screening experiments were conducted in duplicate of each strain in 12-deep well plates at 30°C, 200 rpm for 22 h in the production medium. The cells were grown in the precultivation medium (YP) for16-18 h, and then were harvested at 4500g and 4°C for 5 min and inoculated into production media at initial OD_{600}= 1.

P. pastoris cells that carry intracellular mApple expression cassettes constructed with $P_{ADH2\text{-}Cat8\text{-}L2}$ demonstrated similar cell-growth except the colonies-1 and 6 on 1% (v/v) ethanol. The results are presented in Table 4.5 and Figure 4.14.

Table 4.5 *Optical cell density (OD_{600}) values of the strains constructed with $P_{ADH2\text{-}Cat8\text{-}L2}$ at t=22 h of the fermentation on ethanol (1% v/v)*

Colonies	**OD_{600}**
ADH2-Cat8-L2 1	3.1
ADH2-Cat8-L2 2	4.7
ADH2-Cat8-L2 3	5.3
ADH2-Cat8-L2 4	5.1
ADH2-Cat8-L2 5	3.8
ADH2-Cat8-L2 6	5.8
ADH2-Cat8-L2 7	10.7
ADH2-Cat8-L2 8	6.5
ADH2-Cat8-L2 9	3.4
ADH2-Cat8-L2 10	4.1
ADH2-Cat8-L2 11	5.3
ADH2-Cat8-L2 12	5.0
X-33	5.4

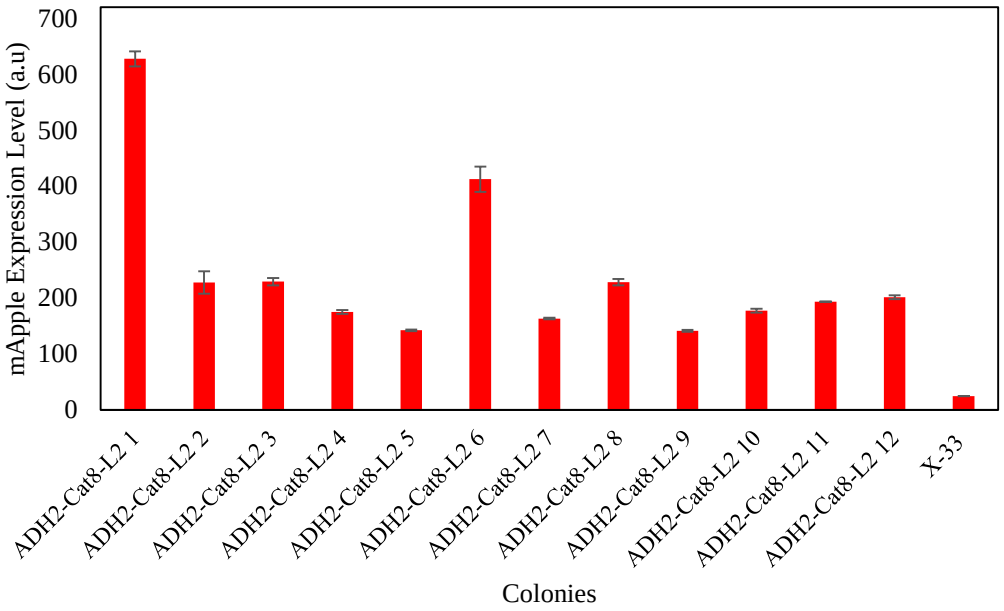


Figure 4.14 mApple expression levels of *P. pastoris* strains carrying pADH2-Cat8-L2::*mApple* on 1% (v/v) ethanol at t = 22 h of the fermentation. Error bars represent the standard deviation (±)

The constructed pADH2-Cat8-L2::*mApple* plasmid was transfected to the recombinant *P. pastoris* cells carrying pGAP::*eGFP* to construct the DPES with two different expression cassettes. To detect the true colony that represents the entire population, 12 colonies and their single copies were screened on 1% (v/v) ethanol fed medium. In the construction of the DPESs two fluorescent proteins were used to identify the operation of the constituent NEPV(s) ($P_{ADH2\text{-}Cat8\text{-}L2}$ and/or P_{mAOX1}) or NOP (P_{GAP}) and the operation period, separately. Therefore, when we excite and emit mApple, $P_{ADH2\text{-}Cat8\text{-}L2}$ expression is determined and measured quantitatively, or/and when we excite eGFP P_{GAP} expression is confirmed and measured quantitatively. While constructing pADH2-Cat8-L2::*mApple* + pGAP::*eGFP*, recombinant pGAP::*eGFP P. pastoris* was used as the parent strain. As the integration site in the *AOX1* locus varies, pADH2-Cat8-L2::*mApple* plasmid transfection to *P. pastoris* cells by targeted integration to *AOX1* locus resulted in changing expression performances of the biological replicates. Moreover, increasing the gene copy number could also cause fluctuations in the expression among the biological replicates.

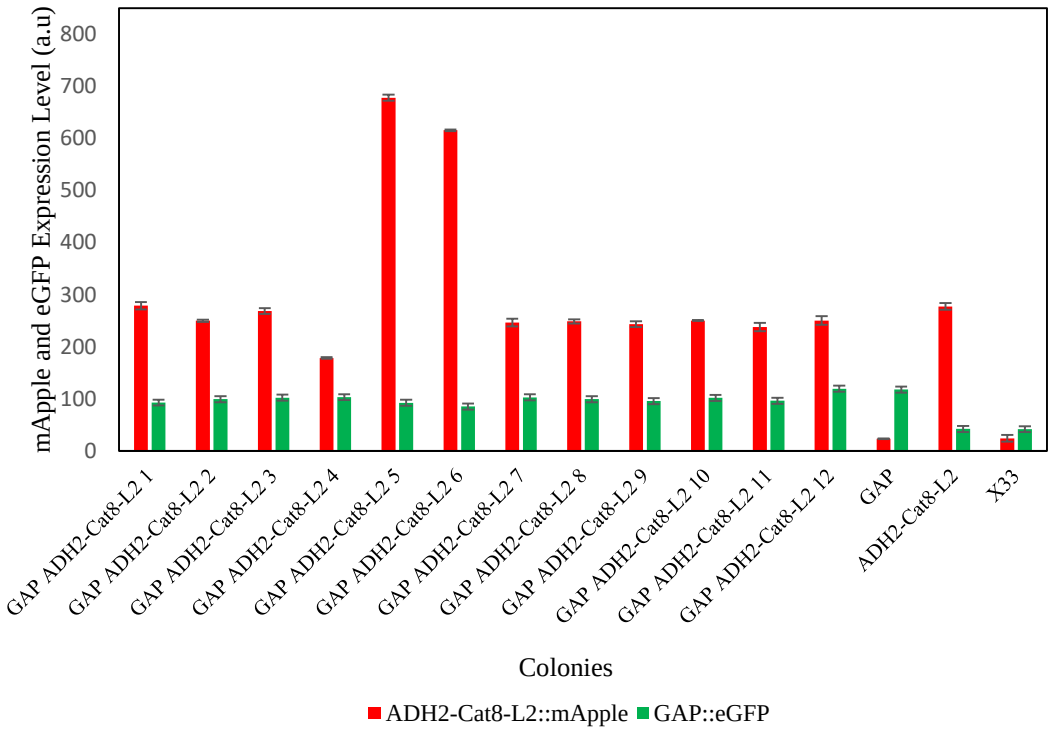


Figure 4.15 mApple and eGFP expression levels of *P. pastoris* strains carrying the DPES pADH2-Cat8-L2*::*mApple + *pGAP::*eGFP on 1% (v/v) ethanol at t=22h of the fermentation. mApple and eGFP represent the expression levels of the constituent NEPV ADH2-Cat8-L2 and the NOP GAP, respectively. Error bars represent the standard deviation (±)

Based on screening results of pADH2-Cat8-L2::*mApple* + pGAP::*eGFP* DPES on 1% (v/v) ethanol, eGFP expression level with *GAP* promotor did not change among the colonies in the DPES as it is the parent-plasmid. Moreover, its expression capacity in the DPES were slightly (12%) decreased compared with the SPES pGAP::*eGFP*. Biological replicates reached almost similar cell concentrations on 1% (v/v) ethanol; and the mApple expressions demonstrated similar patterns except with the colonies-5 and 6. The difference in the expressions in colony- 5 and 6 probably resulted in due to increased gene-copy number. The differences (± 4 %) in the expressions of the colonies compared with the SPES pADH2-Cat8-L2::*mApple* might be because of the variations in the integration site in the *AOX1* locus.

The constructed pADH2-Cat8-L2::*mApple* plasmid was transfected to the recombinant *P. pastoris* cells carrying pmAOX1::*eGFP* cassette. For statistical reliability at least eight clones were analyzed and the clones representing the whole population was selected for further analysis. When we excite and emit mApple, $P_{ADH2\text{-}Cat8\text{-}L2}$ expression capacity was determined, whereas excitation and emission of eGFP determine the expression capacity of P_{mAOX1} in the DPES. While constructing pADH2-Cat8-L2::*mApple* + pmAOX1::*eGFP*, recombinant *P. pastoris* cells carrying pmAOX1::*eGFP* was used as the parent plasmid.

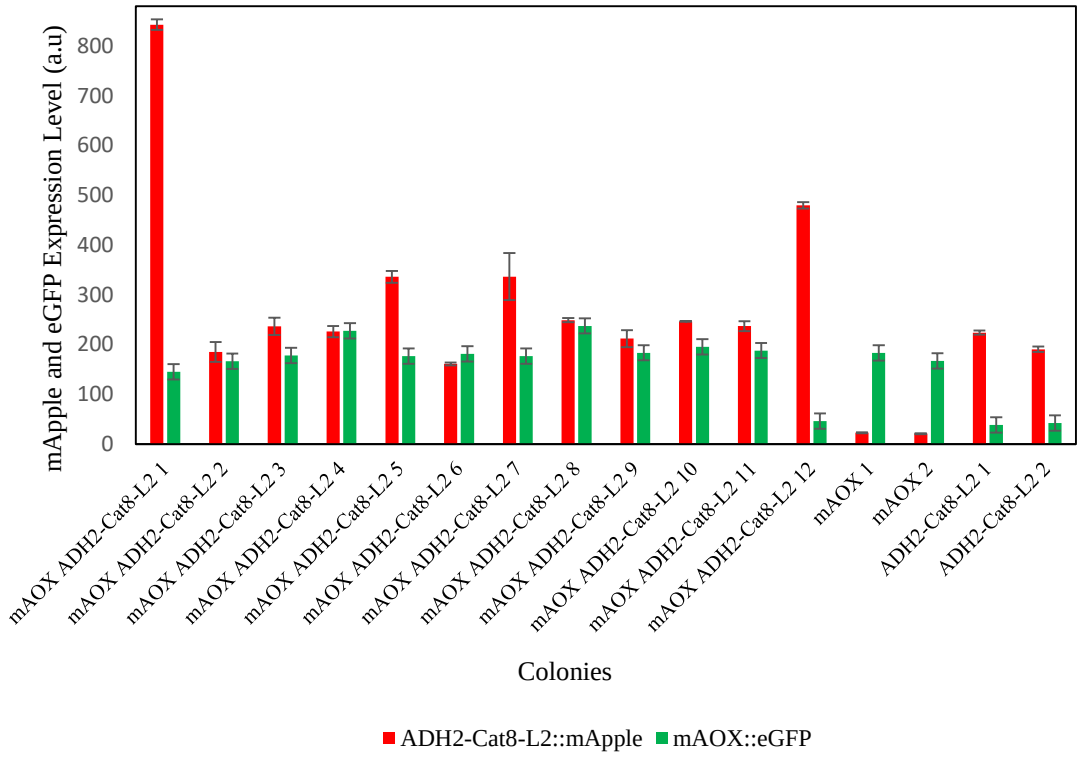


Figure 4.16 mApple and eGFP expression levels of *P. pastoris* strains carrying the DPES pADH2-Cat8-L2::*mApple* + pmAOX1::*eGFP DPES* on 1% (v/v) ethanol at t=22h of the fermentation. mApple and eGFP represent the expression levels of the constituent NEPVs ADH2-Cat8-L2 and mAOX1, respectively. Error bars represent the standard deviation (±)

Based on screening results of the DPES pADH2-Cat8-L2::*mApple* + pmAOX1::*eGFP* on 1% (v/v) ethanol, eGFP expression level of the colonies with the

NEPV mAOX1 did not change. Biological replicates reached to a similar cell concentration under 1% (v/v) ethanol condition, as expected; however, mApple expression levels exhibited distinct patterns. The variation in expression levels with $P_{ADH2\text{-}Cat8\text{-}L2}$ was resulted in either gene copy number or integration site difference. Moreover, ADH2-Cat8-L2 and mAOX1 performed in harmony and strong pair of NEPVs for the DPES, and exhibited increased expression levels compared to the corresponding SPESs. In order to clarify integration site influence in *P. pastoris*, a DPES was constructed by using pADH2-Cat8-L2::*mApple* as the parent plasmid. Constructed and linearized pGAP::*eGFP* and pmAOX1::*eGFP* plasmids were transfected to the recombinant *P. pastoris* cells carrying pADH2-Cat8-L2::*mApple* for construction of the DPESs with two different expression cassettes.

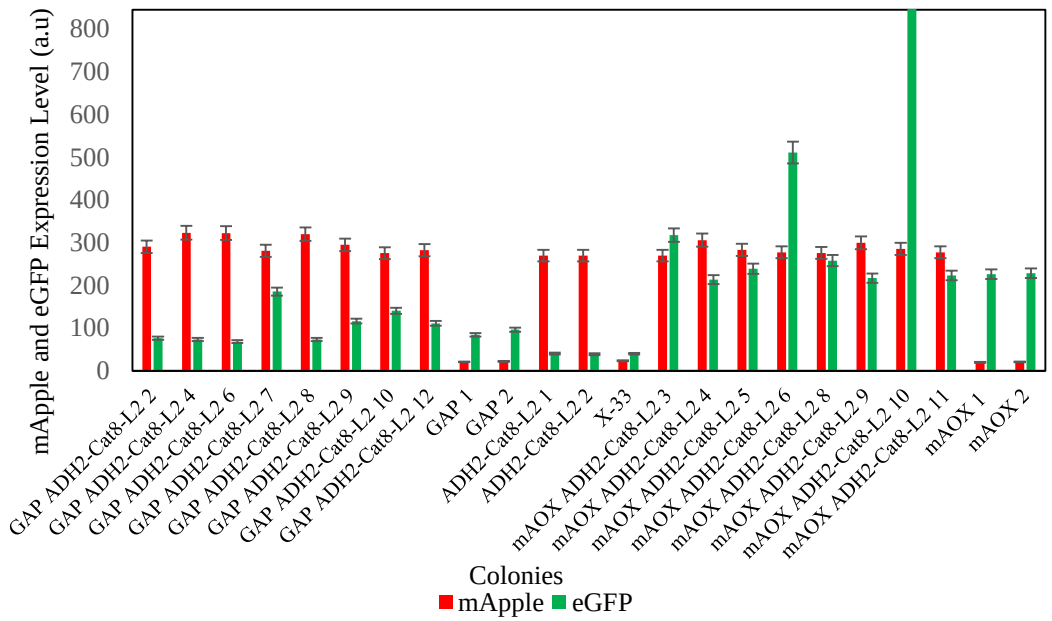


Figure 4.17 mApple and eGFP expression levels of *P. pastoris* strains carrying the DPES pADH2-Cat8-L2::*mApple* + pmAOX1::*eGFP* or pADH2-Cat8-L2::*mApple* + pGAP::*eGFP* on 1% (v/v) ethanol at t=22h of the fermentations. mApple represent the expression level of the constituent NEPV ADH2-Cat8-L2; while, eGFP represent the expression level of the NEPV mAOX1 in the former DPES, or of the NOP GAP in the latter DPES. Error bars represent the standard deviation (±).

The results demonstrated in Figure 4.17 reveal that changing the parent strain significantly affected the expression. Expression level with $P_{ADH2\text{-}Cat8\text{-}L2}$ did not change among the biological replicates as it is the parent-plasmid. Its expression strength in the DPESs did not exhibit distinct patterns compared with the SPES $P_{ADH2\text{-}Cat8\text{-}L2}$. Whereas, integrated plasmids pGAP::*eGFP* and pmAOX1::*eGFP* showed distinguished expression performances either due to the variations in the integration site in the *AOX1* locus or change in the gene-copy number. For further analysis, both *P. pastoris* strains that have different parent strains were used to eliminate the misleading effect of integration site.

Table 4.6 *Optical cell density (OD_{600}) values of DPESs at t = 22 h of the fermentation on ethanol (1% v/v)*

Colonies	**OD_{600}**	**Colonies**	**OD_{600}**
mAOX1 ADH2-Cat8-L2 3	7.1	GAP ADH2-Cat8-L2 2	10.1
mAOX1 ADH2-Cat8-L2 4	7.2	GAP ADH2-Cat8-L2 4	8.8
mAOX1 ADH2-Cat8-L2 5	8.1	GAP ADH2-Cat8-L2 6	8.3
mAOX1 ADH2-Cat8-L2 6	7.2	GAP ADH2-Cat8-L2 7	11.0
mAOX1 ADH2-Cat8-L2 8	7.3	GAP ADH2-Cat8-L2 8	4.0
mAOX1 ADH2-Cat8-L2 9	6.0	GAP ADH2-Cat8-L2 9	7.8
mAOX1 ADH2-Cat8-L2 10	5.8	GAP ADH2-Cat8-L2 10	9.5
mAOX1 ADH2-Cat8-L2 11	5.2	GAP ADH2-Cat8-L2 12	9.1
mAOX1-1	9.2	GAP 1	17.2
mAOX1- 2	7.4	GAP 2	19.5
ADH2-Cat8-L2 2	5.2	ADH2-Cat8-L2 1	8.2

4.2.2. The cell growth and variations in concentrations with the cultivation time

The cell growth of the *P. pastoris* strains were investigated. The cells were grown on 50 mL YP medium for 18-20 h in air-filtered 250 mL shake- bioreactors at 30ºC and 200 rpm; and were harvested at 4500 g for 10 min at 4ºC and inoculated into the fermentation media (Table 3.7) at initial OD_{600} = 1. Three different carbon sources which are excess glucose (Glu), 2% (v/v) ethanol (E), and 1% (v/v) methanol (M) were used in the fermentations.

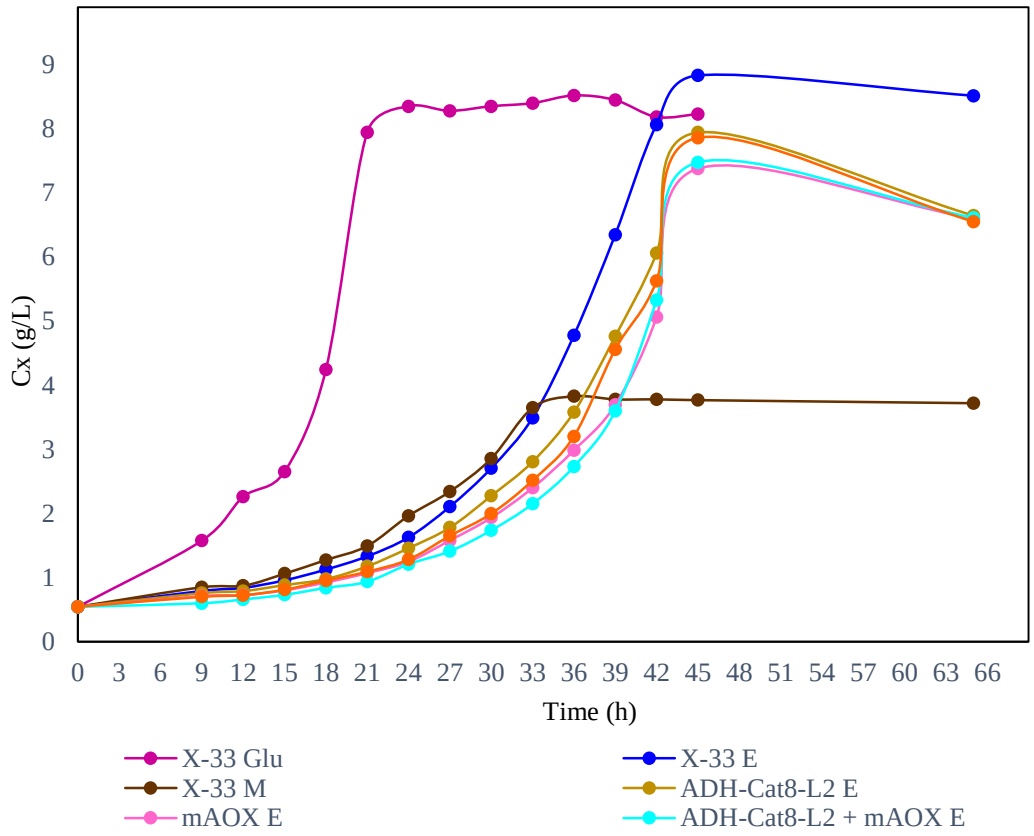


Figure 4.18 Variations in the cell concentrations of *P. pastoris* strains with the cultivation time on the carbon sources: Glu: excess glucose, E: 2% (v/v) ethanol and M: 1% (v/v) methanol.

4.2.3. *mApple* and *eGFP* gene-copy number determination in selected strains

In order to determine the gene-copy numbers of *mApple* and *eGFP*, quantitative PCR was used. Wizard ® Genomic DNA purification kit (Promega) was used to isolate *P. pastoris* genomic DNA. Single-copy and double-copy transfectants of the novel *P. pastoris* strains were determined (Section 3.6). As the housekeeping gene, *argininosuccinate lyase* (*ARG4*) was used since *P. pastoris* has only one copy of *ARG4* gene. In order to have a standard curve for the completion of the qPCR experiment and an absolute quantification, *ARG4* and *hGH* were amplified. The outer primer couples for the amplification of these standard genes were shown in Table 3.27. Tables 4.7 and 4.8 are presenting the optimal PCR combination and PCR conditions for the amplification of standards (*ARG4*, *mApple* and *eGFP*).

Table 4. 7 *PCR mixture for amplification of the standards*

Component	**50 µL Reaction**
10X *Taq* Reaction Buffer	5 µL
5mM dNTPs	2 µL
10 µM Forward Primer	1 µL
10 µM Reverse Primer	1 µL
Template DNA (Genomic DNA or plasmid)	2 µL
Taq DNA Polymerase	0.5 µL
H_2O	Up to 50 µL

For standard DNA preparation ARG4 was isolated from *P. pastoris* genome with ARG4-Std Forward and ARG4-Std Reverse outer primers. mApple and eGFP amplified from isolated plasmid with their outer primers.

Table 4. 8 *PCR mixture for amplification of the standards*

Steps	**Temperature**	**Time**	**Cycle**
Initial Denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	30
Annealing	68°C	30 sec	30
Extension	72°C	60 sec	30
Final Extension	72°C	2 min	1
Hold	4°C	∞	-

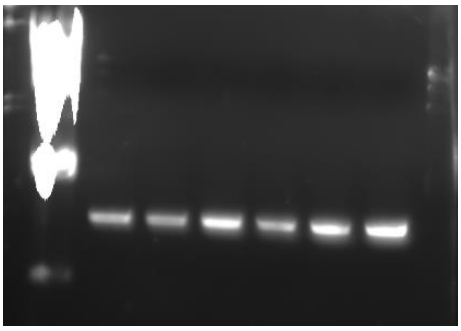


Figure 4.19 1: Lambda DNA 2-7: Isolated ARG4 gene standard sample (expected length of 1259 bp) by using *P. pastoris* genome as template.

The standard samples presented in Figure 4.19 were prepared as explained in Section 3.6.

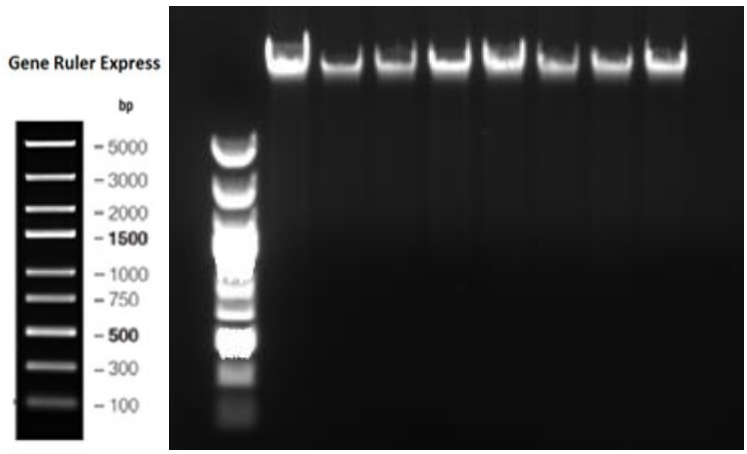


Figure 4.20 1: GeneRuler ready to use 2-9: Isolated genomes for selected colonies

The resulting PCR fragments were however purified with gel extraction in order to obtain a complete sample without any residual. The quality of the amplified genomes (Figure 4.20) was tested with nanodrop after gel extraction kit and results were represented in Table 4.9.

Table 4.9 *Nanodrop results of isolated genomic DNA samples from P. pastoris strains*

The cells constructed with:	**Concentration(ng/µl)**	**260/280**	**260/230**
ADH2-Cat8-L2::*mApple* 1	398.6	1.99	1.23
ADH2-Cat8-L2::*mApple* 2	792.9	2.0	1.44
ADH2-Cat8-L2::*mApple* 6	1153.4	1.99	1.55
ADH2-Cat8-L2::*mApple* + GAP::*eGFP* 1	1093.2	1.97	1.59
ADH2-Cat8-L2::*mApple* + GAP::*eGFP* 2	982.2	2.0	1.77
ADH2-Cat8-L2::*mApple* + mAOX1::*eGFP* 4	977.6	2.0	1.64
ADH2-Cat8-L2::*mApple* + mAOX1::*eGFP* 5	563.5	2.03	1.52
mAOX1::*eGFP* 1	1069.8	2.09	1.64
mAOX1::*eGFP* 2	917	2.02	1.71
GAP::*eGFP* 4	1061.9	1.98	1.48
GAP::*eGFP* 1	973	1.88	1.67
ARG STD	115.6	1.85	1.43
mApple STD	44.7	1.81	1.49

$$Copy\ quantity = \frac{amount\ of\ sample\ (ng) * 6.022 * 10^{23}}{DNA\ molecular\ weight * 10^{9}} \qquad (4.1)$$

Arg Standard Copy Quantity= $115.6*6.022*10^{23}/777867.71*10^{9}=8.94*10^{10}$

mApple Standard Copy Quantity= $44.7*6.022*10^{23}/457351.56*10^{9}=5.88*10^{10}$

The genes-copy quantities of *ARG4* and *mApple* were calculated by equation 4.1 as $8.94x10^{10}$ and $5.88x10^{10}$, respectively. Isolated genomic DNA samples were diluted to a concentration of ca. 2 ng/μL, whereas the standards of *ARG4* and *mApple* were prepared at different gene-copy-number/μL (10^3, 10^4, 10^5 and 10^6/μL) values by dilution in series, in order to obtain standard-calibration curve.

Table 4. 10 *Experimental results for the standard-curves of ARG4 and mApple*

Gene	**Cp**	**Gene-copy number**	**Standard-curve data**
mApple 10^3	32.28	5.32E3	5.30E3
mApple 10^4	27.47	5.19E4	5.30E4
mApple 10^5	23.05	5.30E5	5.30E5
mApple 10^6	18.88	5.30E6	5.30E6
Arg 10^3	25.83	8.05E3	8.05E3
Arg 10^4	21.42	8.23E4	8.05E4
Arg 10^5	18.14	7.71E5	8.05E5
Arg 10^6	14.67	8.22E6	8.05E6

Table 4. 11 *Standard-curve data for ARG4 and mApple*

	mApple	***Arg***
Error	0.00232	0.00572
Efficiency	1.736	1.978
Slope	-4.174	-3.375
Yintercept	46.95	38.01
Link	530,000	82,260

With ARG4 inner primers, mApple inner primers and eGFP inner primers isolated genome of each colony was analyzed and relative-gene-copy-numbers were determined for *mApple* and *eGFP* compared to *ARG4*.

Table 4. 12 *Relative quantification results for selective P. pastoris cells*

The cells constructed with:	Advanced Relative Quantification	Basic Relative Quantification	Target Cp	Reference Cp
ADH2-Cat8-L2::*mApple* 1	3.806	4.509	25.75	23.10
ADH2-Cat8-L2::*mApple* 2	1.042	1.084	28.86	24.28
ADH2-Cat8-L2::*mApple* 6	2.150	2.176	26.26	22.79
ADH2-Cat8-L2::*mApple* + GAP::*eGFP* 1	1.313	1.459	26.89	22.71
ADH2-Cat8-L2::*mApple* + GAP::*eGFP* 2	1.033	0.088	27.09	22.56
ADH2-Cat8-L2::*mApple* + mAOX1::*eGFP* 4	1.050	1.059	27.06	22.56
ADH2-Cat8-L2::*mApple* + mAOX1::*eGFP* 5	2.021	2.128	24.75	21.19
mAOX1::*eGFP* 1	0.945	1.038	21.71	22.53
mAOX1::*eGFP* 2	2.498	1.926	20.45	22.72
GAP::*eGFP* 4	1.814	1.794	21.09	22.68
GAP::*eGFP* 1	1	1	20.94	22.57

According to measurements, it was revealed that the copy numbers were lower than unity which makes the inference difficult. The reasons of this problem might be any kind of mistakes particularly in the difference between the lengths of the amplified fragments, namely the target gene and the reference gene (*ARG4*). Additionally, concentration measurement during standard sample preparation might cause an error in gene-copy-number determination. As a conclusion, advanced relative quantification and basic relative quantification results were given as normalized data according to

the pGAP::*eGFP* 1 strain since the gene-copy-number of this strain was verified as unity previously.

4.2.4. Performance of novel *P. pastoris* strains constructed with DPESs

The novel *P. pastoris* strains constructed with DPESs carrying *mApple* and *eGFP* fluorescent reporter protein genes enhanced the recombinant protein synthesis by demonstrating the operation periods of each constituent NEPV or NOP clearly whereupon their contribution to the protein expression single-handedly; furthermore, their cell growth characteristics are also good for the fermentations for high production with increased productivity.

The ethanol concentration was used as 2% (v/v) according to the study by Ergün et al. (2019). The expression of Green and Red Fluorescent proteins (FP) (Figure 4.21 and 4.22) demonstrated the strength of the DPESs. Expression levels (relative fluorescent units) were normalized since specific quantum yields and sampling settings were depending on the FP. mApple fluorescent protein expression was normalized to that of with $P_{ADH2\text{-}Cat8\text{-}L2}$ (%)E whereas eGFP FP was normalized to that of with P_{mAOX1} (%)E. All DPESs and SPESs were tested in two production media, that is the minimal production medium which was previously used in our research group, and the other is the ASMV6 base-production medium used (Table 3.8) with the three carbon sources ethanol, glucose, and glycerol (Table 3.9) separately, to identify potential regulation of the promoters and the results are presented in Figure 4.20 and 4.21.

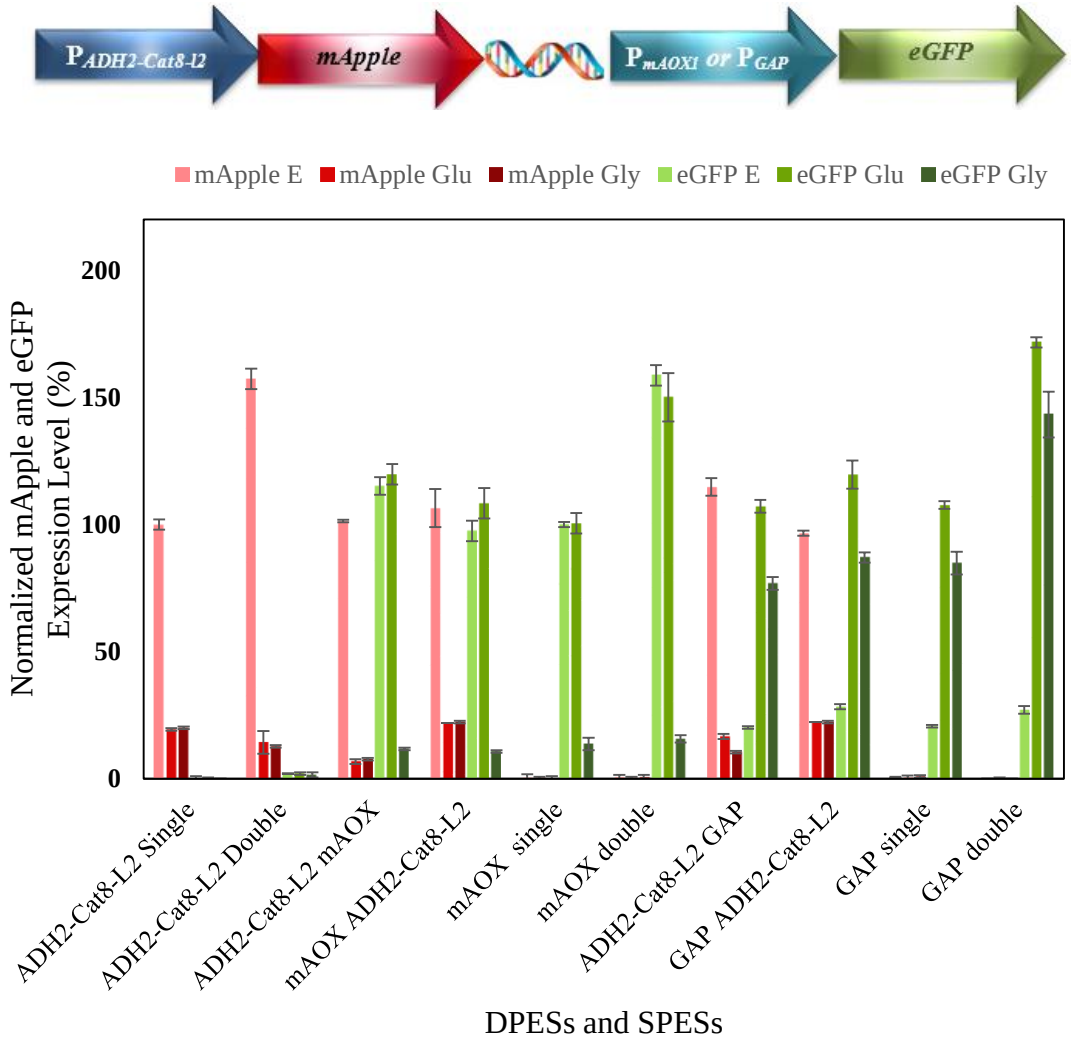


Figure 4.21 Normalized mApple and eGFP expression levels of novel *P. pastoris* strains with DPESs and SPESs at t=24h of the fermentation processes on minimal production medium, on the carbon sources: E: 2% (v/v) ethanol, Glu: excess glucose, Gly: excess glycerol. mApple represent the expression strength with ADH2-Cat8-L2 while eGFP represents the expressions with either mAOX1 or GAP. Normalized mApple expression levels were given relative to $P_{ADH2\text{-}Cat8\text{-}L2}$ (%)E and normalized eGFP expression levels were given relative to P_{mAOX1} (%)E. Error bars represent the standard deviation (±).

Table 4. 13 *Normalized mApple and eGFP expression levels of novel P. pastoris strains with DPESs and SPESs at t=24h of the fermentation processes on minimal production medium, on the carbon sources: E: 2% (v/v) ethanol, Glu: excess glucose, Gly: excess glycerol. mApple represent the expression strength with ADH2-Cat8-L2 while eGFP represents the expressions with either mAOX1 or GAP. Normalized mApple expression levels were given relative to* $P_{ADH2\text{-}Cat8\text{-}L2}$ *(%)E and normalized eGFP expression levels were given relative to* P_{mAOX1} *(%)E.*

	E		**Glu**		**Gly**	
	mApple	**eGFP**	**mApple**	**eGFP**	**mApple**	**eGFP**
ADH2-Cat8-L2 Single	100±2	0±1	19±1	0±1	20±1	0±0
ADH2-Cat8-L2 Double	157±4	2±0	14±4	2±1	13±1	2±1
ADH2-Cat8-L2 mAOX1	101±1	115±4	7±1	120±4	8±1	12±1
mAOX1 ADH2-Cat8-L2	106±8	98±4	22±0	108±6	22±1	11±1
mAOX1 single	0±2	100±1	0±1	100±4	0±1	14±3
mAOX1 double	0±1	159±4	0±0	150±10	1±1	16±2
ADH2-Cat8-L2 GAP	115±4	20±1	17±1	107±2	10±1	77±3
GAP ADH2-Cat8-L2	97±4	28±1	22±0	120±6	22±1	87±2
GAP single	1±0	21±1	0±1	108±2	1±1	85±5
GAP double	0±0	27±2	0±1	172±2	0±0	143±9

Table 4.14 *Optical cell density (*OD_{600}*) values of the DPESs and SPESs on three carbon sources at t = 24 h of fermentation*

Cells	**Ethanol%2 (v/v)**	**Excess Glucose**	**Excess Glycerol**
ADH2-Cat8-L2 single	7.0	24.7	27.5
ADH2-Cat8-L2 double	4.0	23.9	29.1
ADH2-Cat8-L2 GAP	14.5	37.6	47.9
ADH2-Cat8-L2 mAOX1	5.8	26.1	27.1
mAOX1 single	6.9	28.0	26.5
mAOX1 double	5.7	25.6	30.8
GAP double	7.9	26.3	29.1
GAP single	16.1	35.5	40.5
GAP ADH2-Cat8-L2	6.9	24.8	28.4
mAOX ADH2-Cat8-L2	6.3	23.8	26.0
X-33	7.7	26.5	26.4

The expression strength of the five DPESs: $P_{ADH2\text{-}Cat8\text{-}L2+mAOX}$, $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$, $P_{ADH2\text{-}Cat8\text{-}L2+\ ADH2\text{-}Cat8\text{-}L2}$, $P_{mAOX+mAOX}$ and $P_{GAP+GAP}$ compared with each other and with the three SPESs: $P_{ADH2\text{-}Cat8\text{-}L2}$, P_{mAOX} and P_{GAP}. While constructing DPESs, both recombinant *P. pastoris* strains constructed with pGAP/mAOX1::*eGFP* and pADH2-Cat8-L2::*mApple* were used as the parent strains. Since integration site in the AOX1 locus varies, plasmid transfection to *P. pastoris* cells by targeted integration to AOX1 locus resulted in changing expression performances of the biological replicates. Based on the screening results, the mApple synthesis levels of $P_{ADH2\text{-}Cat8\text{-}L2+mAOX}$ and $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$ with ethanol, glucose, and glycerol induction did not result an increase compared to the SPES $P_{ADH2\text{-}Cat8\text{-}L2}$. Accordingly, the regulation pattern of $P_{ADH2\text{-}Cat8\text{-}L2}$ was same in DPESs and SPES, and $P_{ADH2\text{-}Cat8\text{-}L2}$ was still repressed by glucose and glycerol and activated in the presence of ethanol. As P_{mAOX1} was also activated in the presence of ethanol, $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ is a promising DPES for methanol-free production in *P. pastoris* with DPESs. In this context, $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ was designed as a SMT-operating DPES since both promoters' requirements could be satisfied at the same time with ethanol induction. According to the results, $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ demonstrated a synergetic effect and increased the expression level of r-proteins 2.1-fold compared to single $P_{ADH2\text{-}Cat8\text{-}L2}$ and P_{mAOX1} on ethanol. However, the efficiency of the constructed DPES should be compared to the performance of the corresponding identical twin promoter systems. SMT-operating $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ increased the production capacity 1.3-fold in comparison to both identical twin promoters, $P_{ADH2\text{-}Cat8\text{-}L2+ADH2\text{-}Cat8\text{-}L2}$ and $P_{mAOX1+mAOX1}$ on ethanol. As core promoters were the basic region required for transcription initiation and bound by general TFs and RNA polymerase II, TF limitation was prevented by using different promoters in DPES architectures instead of identical twin promoters. In DPESs with identical twin promoters, both promoters required same TFs thus when their capacities reached to the plateau due to TF limitation in the cell, product formation rate decreases to a lower value than that of SMT-operating double promoter systems. This effect can be obvious with the increase in cultivation time. However, as eGFP was toxic for the cell, the

process was terminated at a certain cultivation time due to the accumulation of the intracellular reporter protein in the cell.

$P_{ADH2\text{-}Cat8\text{-}L2+GAP}$ was designed as a CNT-operating DPES since the constituent promoters' requirements can be satisfied by encouraging biphasic processes or consecutive iterations to switch from one promoter to the other. GAP is a strong constitutive promoter and ADH2-Cat8-L2 is a strong inducible promoter in the presence of ethanol or methanol. $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$ resulted in a high expression level on ethanol only due to the contribution of ADH2-Cat8-L2 promoter while the phenomenon was also observed on glucose only due to the contribution of GAP promoter. According to the results, the CNT-operating DPES architectures allow successive transcription with each constituent promoter. When $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$ was designed, the aim was to operate strong constitutive GAP promoter with glucose as the major carbon source while the induction of the second promoter $P_{ADH2\text{-}Cat8\text{-}L2}$ was started by the release of by-product ethanol. However, based on mApple expression level with $P_{ADH2\text{-}Cat8\text{-}L2}$ in DPES was repressed by glucose and could not be induced by the byproduct ethanol. Constitutive expression of *eGFP* under the control of GAP promoter's stress on the cell could potentially be suppressed *mApple* expression by $P_{ADH2\text{-}Cat8\text{-}L2}$. With $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$, 1.3-, 6.2-, and 4.8-fold higher expression levels were achieved compared to single $P_{ADH2\text{-}Cat8\text{-}L2}$, single P_{GAP}, and identical twin $P_{GAP+GAP}$, respectively; whereas 1.2-fold decrease was detected in comparison to identical twin $P_{ADH2\text{-}Cat8\text{-}L2+ADH2\text{-}Cat8\text{-}L2}$ on ethanol. The high performance of the strain constructed with $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$ on ethanol relative to the strain constructed with P_{GAP} can be attributed to $P_{ADH2\text{-}Cat8\text{-}L2}$. SMT-operating $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ performed 1.6-fold higher expression than that of CST-operating $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$ in minimal production medium containing ethanol. Therefore, $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ was determined as the superior DPES for methanol-free r-protein production processes. To further confirm the expression strengths and behaviors of the DPESs and constituent SPESs, ASMV6 was used as an alternative defined base-medium with three different carbon

sources separately, i.e., ethanol, glucose and glycerol. The results are represented in Figure 4.22.

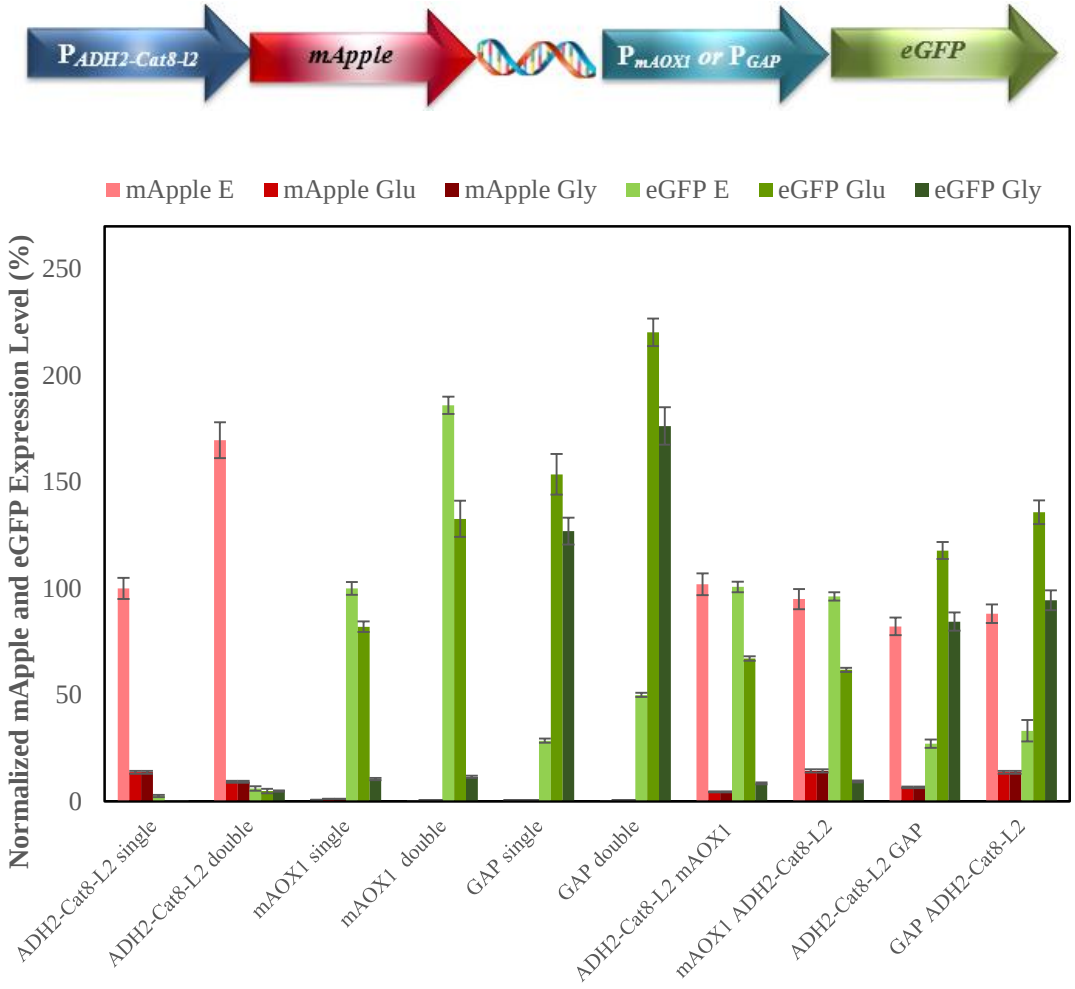


Figure 4. 22 Normalized mApple and eGFP expression levels of novel *P. pastoris* strains with DPESs and SPESs at t = 24 h of the fermentation processes on ASMV6 base-production media, on the carbon sources: E: 2% (v/v) ethanol, Glu: excess glucose, Gly: excess glycerol. mApple represent the expression strength with ADH2-Cat8-L2 while eGFP represents the expressions with either mAOX1 or GAP. Normalized mApple expression levels were given relative to $P_{ADH2\text{-}Cat8\text{-}L2}$ (%)E and normalized eGFP expression levels were given relative to P_{mAOX1} (%)E. Error bars represent the standard deviation (±).

Table 4. 15 *Normalized mApple and eGFP expression levels of novel P. pastoris strains with DPESs and SPESs at t = 24 h of the fermentation processes on ASMV6 base-production media, on the carbon sources: E: 2% (v/v) ethanol, Glu: excess glucose, Gly: excess glycerol. mApple represent the expression strength with ADH2-Cat8-L2 while eGFP represents the expressions with either mAOX1 or GAP. Normalized mApple expression levels were given relative to* $P_{ADH2\text{-}Cat8\text{-}L2}$ *(%)E and normalized eGFP expression levels were given relative to* P_{mAOX1} *(%)E.*

	E		**Glu**		**Gly**	
	mApple	**eGFP**	**mApple**	**eGFP**	**mApple**	**eGFP**
ADH2-Cat8-L2 Single	100±5	3±1	14±1	0±0	14±1	0±0
ADH2-Cat8-L2 Double	170±4	6±1	9±1	5±1	9±1	5±1
mAOX1 single	1±1	100±3	1±1	82±2	1±1	10±2
mAOX1 double	0±0	186±4	0±1	133±8	0±1	12±2
GAP single	0±1	29±1	1±1	154±10	1±1	127±8
GAP double	0±1	50±1	0±1	220±7	0±0	176±8
ADH2-Cat8-L2 mAOX1	102±2	101±2	4±1	67±1	4±1	8±1
mAOX1 ADH2-Cat8-L2	95±10	96±2	14±1	62±1	14±1	9±2
ADH2-Cat8-L2 GAP	82±4	27±2	7±0	118±4	7±0	84±4
GAP ADH2-Cat8-L2	88±11	33±10	14±0	136±6	14±1	94±2

DPESs and SPESs expression levels and the cell-growth of the strains were similar in ASMV6-based defined medium and in the previously used minimal medium. $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ increased the production level 2-fold compared to both single $P_{ADH2\text{-}Cat8\text{-}L2}$ and single P_{mAOX1}, whereas the expression of the DPES was 1.2- and 1.1-fold higher than that of identical twin $P_{ADH2\text{-}Cat8\text{-}L2+ADH2\text{-}Cat8\text{-}L2}$ and $P_{mAOX1+mAOX1}$, respectively on ethanol. Under the control of $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$, the heterologous gene expression increased 1.3-, 4.4- and 2.6-fold compared to single $P_{ADH2\text{-}Cat8\text{-}L2,}$ single P_{GAP}, and identical twin $P_{GAP+GAP}$, respectively, whereas 1.25-fold decrease was detected compared with identical twin $P_{ADH2\text{-}Cat8\text{-}L2+ ADH2\text{-}Cat8\text{-}L2}$ on ethanol. The expression level with SMT-operating $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ was 1.5-fold higher than that of CST-operating $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$ in ASMV6-based defined medium containing ethanol.

When P_{mAOX1} was used for the heterologous gene expression, eGFP was detected in the presence of glucose where the NEPV was strong as P_{GAP}. The DPESs containing P_{mAOX1} showed similar r-protein production performance in either ethanol or glucose. $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1,}$ $P_{mAOX1+mAOX1}$ and P_{mAOX1}-controlled *eGFP* expression levels reached to the production levels attained with P_{GAP} on glucose. In order to verify P_{mAOX1} expression strength on glucose, eight colonies for each DPES and SPES were screened to show that the performance of P_{mAOX1} on glucose was not colony specific. Nevertheless, repression effect was not observed and eGFP was produced under the control of P_{mAOX1} on glucose in all selected colonies.

According to the results of Ergün et al. (2019), P_{mAOX1} did not demonstrate an activity in glucose containing medium when it was compared with the production level obtained in ethanol containing medium. It should be noted that Ergün et. al. (2019) reported the r-protein production performance of P_{mAOX1} on glucose at the initial cell concentration OD_{600} = 0.1. However, in this study, while comparing expression strength of the DPESs and the constituent SPESs, OD_{600} = 1 was used on all carbon sources. Therefore, the change in the initial cell concentration significantly affected the r-protein production. As glucose consumption was higher at higher initial cell concentration, glucose depleted during the fermentation. Hence, higher initial cell concentration caused to drive the system to limited glucose condition. The variations in cell concentration and *eGFP* expression level with the cultivation time for wild-type and recombinant *P. pastoris* strains carrying either P_{mAOX1} or P_{AOX1} were displayed in Figures 4.23 and 4.24.

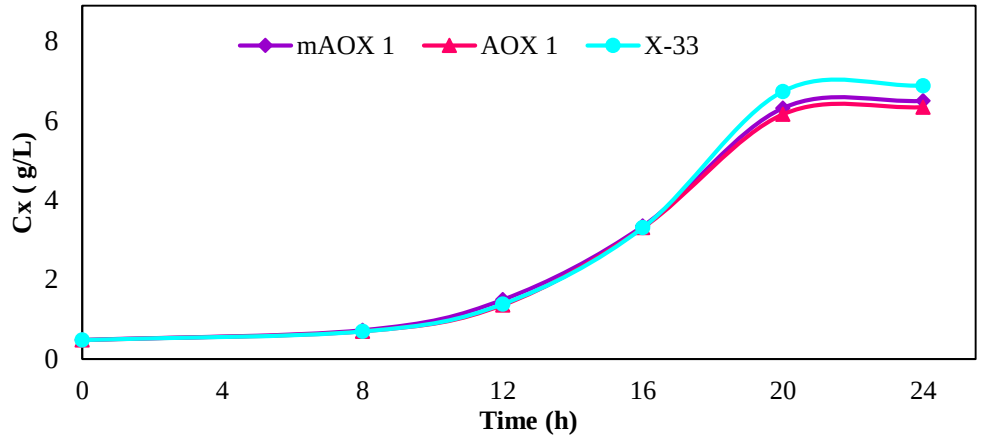


Figure 4.23 Variations in the cell concentrations of *P. pastoris* strains constructed with pmAOX1::*eGFP or* pAOX1::*eGFP*, and wild type X-33 , with the cultivation time at the substrate condition excess glucose

The cells were grown in 2 mL YP medium for 18-20 h in 12-deep well plate at T = 30ºC and N = 200 rpm. Thereafter, the cells were harvested by centrifugation at 4500 g and 4ºC for 5 min and inoculated in 2 mL production medium (Table 3.7) containing excess glucose (20 g/L) with an initial OD_{600} of 1. According to the results, there were no difference between the cell growth profiles of the wild-type and recombinant *P. pastoris* strains carrying either P_{mAOX1} or P_{AOX1}.

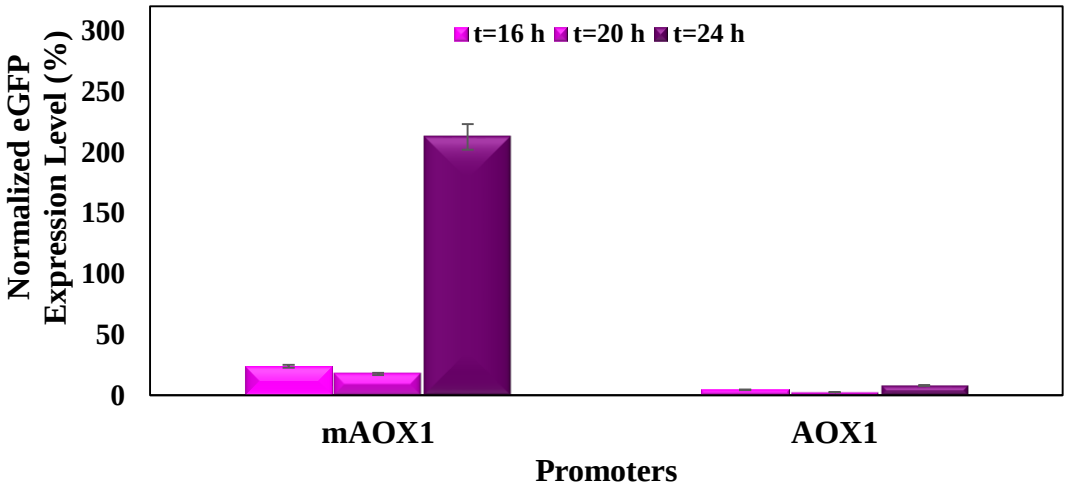


Figure 4.24 eGFP expression levels of novel *P. pastoris* strains. Error bars represent the standard deviation (±)

In order to determine the operation period of P_{mAOX1} and its expression capacity, samples were taken at certain time intervals and eGFP intensities were measured. In the lag phase and throughout the exponential phase mAOX1 and AOX1 promoters were repressed by glucose as expected. The major eGFP synthesis under the control of P_{mAOX1} was observed in the stationary phase of fermentation nearly at t = 24 h. Thus, P_{mAOX1} could produce the target protein, when glucose became limited. While repression effect of glucose on mAOX1 promoter was eliminated, this variant promoter could also start to use the by-product ethanol as the carbon source to produce eGFP. P_{mAOX1} behaved like a derepressed promoter, meaning expression started once glucose in the media was depleted and was further strongly induced by ethanol. Hence, in the recombinant strain carrying SMT-operating $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ and growing on glucose, *eGFP* gene was at first repressed, partially activated in the derepressed phase and then fully activated when glucose depleted on by-product ethanol (Figure 21, 22 and 24). This finding indicated that apart from the characteristics and strengths of the promoters, their regulatory profiles were critical and could be readily optimized using diverse DPESs library.

4.3. Construction of the rhGH producing plasmids and strains

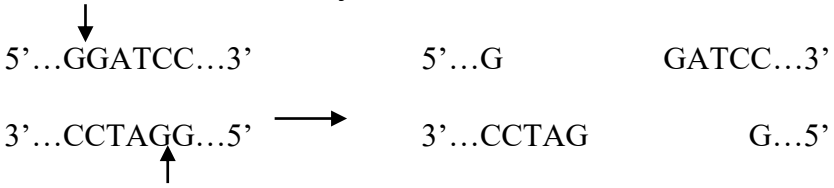
4.3.1. Construction of pADH2-Cat8-L2::*hGH*

The double promoter systems were intended to be formed by two separate expression cassettes. Two different antibiotic resistance genes: Zeocin and Nourseothricin (NTC) were used to construct recombinant plasmids to insert two different expression cassettes with sequential transfection. To properly select positive colonies after *P. pastoris* transfection, the antibiotic resistance gene of an expression cassette must be different for each of the double promoter systems. For this purpose, the antibiotic resistance gene of plasmid pADH2-Cat8-L2::*hGH* was replaced with the NTC gene. Commercial plasmid pGAPZαA carries A zeocin antibiotic resistance gene.

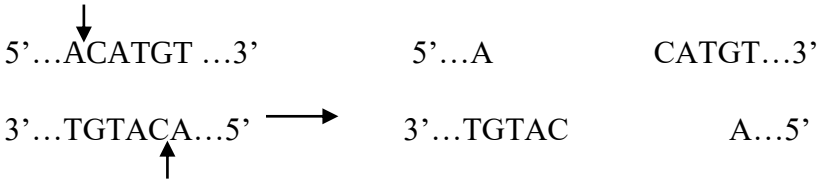
SnapGene viewer and the restriction mapper were used to determine the restriction enzymes (RE) that did not digest the Nourseothricin resistance gene and should digest the plasmid pADH2-Cat8-L2::*hGH* in one position. The Zeocin antibiotic resistance

gene is intended to be cleaved together with its promoter and terminator. There is a *BamH*I RE recognition site at the 5 ’end of the TEF promoter and *Pci*I RE at the end of the CYC1 terminator.

***BamH*I** Restriction enzyme



***Pci*I** Restriction enzyme



In the first step of PCR, NATMX6 (TEF promoter, NTC, TEF terminator) was amplified together with forward NTC primer and reverse NTC primers. In our study, amplification of all genes required for cloning was performed with Q5 DNA Polymerase enzyme (NEB). PCR mixture content and thermal cycling conditions are given in Table 3.12 and 3.13, respectively. For the optimum PCR conditions three different annealing temperatures (62, 65 and 68°C) were tried. Among the three different temperature amplification efficiency of target DNA sequence was higher at 68° C. The amplified NTC antibiotic resistance gene was purified using PCR purification kit (Thermoscientific) prior to double digestion according to manufacturer’s instructions. Purified DNA fragments and vector pADH2-Cat8-L2::*hGH* were double digested with *BamH*I and *Pci*I REs as described in Table 3. 19.

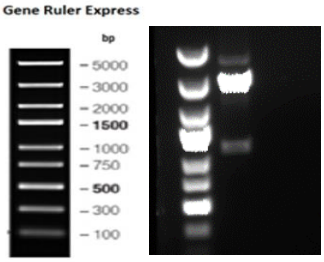


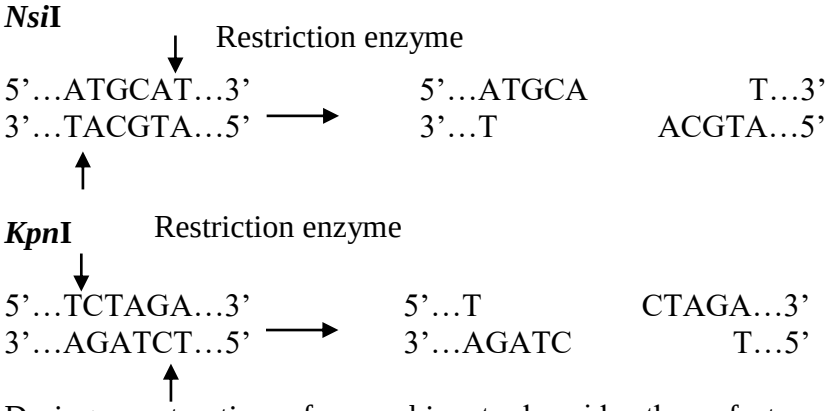
Figure 4. 25 1: GeneRular DNA ladder (Fermentas), 2: *BamH*I and *Pci*I digested vector pADH2-Cat8-L2::*hGH*

The double digested vector and the gene to be integrated were ligated with the T4 DNA Ligase enzyme (Fermentas) using the ratio of the insert NATMX6 to pADH2-Cat8-L2::*hGH* was 3: 1 (Table 3.25). The size of the vector digest by *BamH*I and *Pci*I is 3056 bp, the size of the insert to be integrated digested by *BamH*I and *Pci*I is 1120 bp. After ligation reaction, *E. coli* transformation was performed with calcium chloride method.

The first step of confirmation analysis was the colony PCR with NTC forward and NTC reverse primers. The distance between two primers in the base plasmid is ca. 1120 bp. Four of the confirmed colonies were isolated by plasmid isolation kit (section 3.4.1). Integration of insert was further confirmed by PCR using isolated plasmid as a template. Two colonies were sequenced. Sequencing results were analyzed by BLAST. Microbank stocks were prepared for confirmed *E. coli* strains and stored for *Pichia* transfection at -80°C.

4.3.2. Construction of pmAOX1::*hGH*

In this study, the P_{mAOX1} and α-factor signal sequence were cloned in front of the *human growth hormone* (*hGH*) gene. SnapGene viewer and Restriction mapper 3 were used to identify Restriction enzymes (RE) that do not digest P_{mAOX1}, α-factor signal and *hGH* and digest the base-vector (pGAPZα-A) from one position. The plasmid pGAPZα-A has one *Nsi*I RE recognition site at the 5 ’end of P_{GAP}; *Xba*I RE was selected to cut the plasmid from the multiple cloning site.



During construction of recombinant plasmids, the α-factor signal sequence was inserted between the promoter gene and human growth hormone to observe extracellular production. It was desirable that no nucleotide, including RE recognition sites, entered between the promoter, α-factor and gene sequence, so that all sequences were amplified by polymerase chain reaction (PCR) and combined with OE-PCR. The *Nsi*I RE recognition sites was added to the 5 'end of the P_{mAOX1} genes while *Xba*I RE was added to 5 'end of the *hGH*.

In the first step of PCR, forward mAOX1 and reverse mAOX1 primers were combined with AOX promoter variant, Forward α-factor-hGH primer and reverse hGH primer were combined together with α-factor and *hGH* genes (Figure 4.26). PCR mixture content and thermal cycling conditions are given in Tables 3.10 and 3.11 respectively.

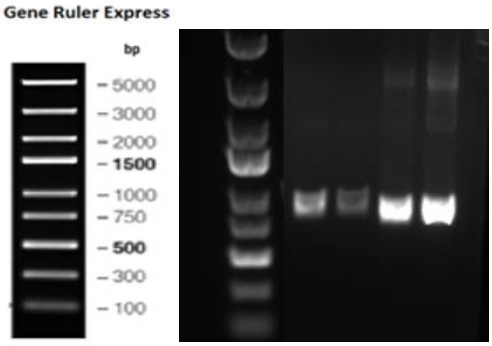


Figure 4. 26 Agarose gel electrophoresis image of the genes amplified with different primer combinations after the first step of OE-PCR. 1: Generuler Express DNA ladder (Fermentas) 2: Forward_mAOX1 and Reverse_mAOX1 at 66 °C 3: Forward_mAOX1 and Reverse_mAOX1 at 68°C 4: forward_α-factor-hGH and reverse_hGH at 66°C 5: forward_α-factor-hGH and reverse_hGH at 68°C

PCR efficiency was increased with different annealing temperatures for both amplifications of P_{mAOX1} and *α-factor-hGH* gene sequences. The annealing temperature was optimized as 66°C for both fragments. Amplified fragments (P_{mAOX1} and *α-factor-hGH)* were purified with PCR purification kit to eliminate the reagents that could decrease the SOE-PCR efficiency.

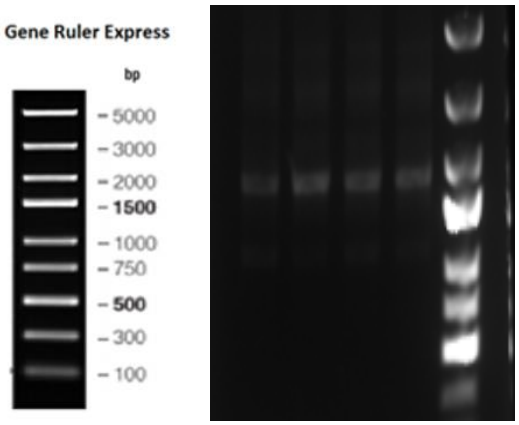


Figure 4. 27 Agarose gel electrophoresis image of genes amplified with different primer combinations after the second step of OE-PCR.; 1 & 4: Forward_mAOX 1and Reverse_hGH 5: Generuler Express DNA ladder

Purified amplicons containing overlapping compatible ends were used as template with Forward mAOX1 and reverse hGH primers for step 2 of OE-PCR.

Gene fragments of the expected sizes are amplified by OE-PCR and shown in Figure 4.27. P_{mAOX1} (940 bp) and α-factor-*hGH* (849 bp), and then used as a template for a SOE-PCR. Then both insert and vector pGAPZαA were double digested by *Nsi*I and *Xba*I on both sides for cloning as described in Table 3.22 and 3.23.

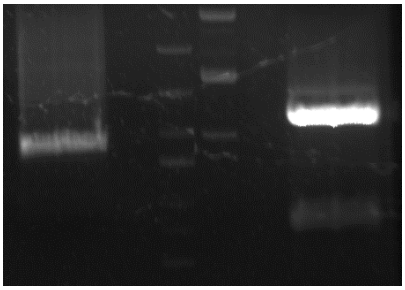


Figure 4. 28 1: *Nsi*I and *Xba*I REs double digested insert 2: GeneRuler DNA ladder (Fermentas), 4: *Nsi*I and *Xba*I double digested vector pGAPZαA

Double digested vector (2324 bp) and the insert gene (1789 bp) to be integrated were extracted from the agarose gel and purified with GeneJet Gel Extraction Kit (Thermoscientific) according to the manufacturer's recommendations.

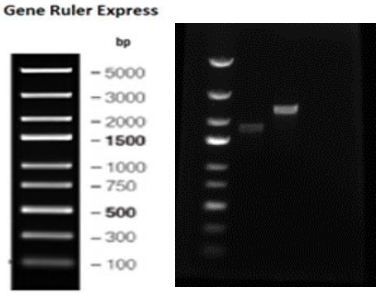


Figure 4. 29 Agarose gel electrophoresis image of the purified fragments after gel elution 1: GeneRuler DNA ladder (Fermentas), 2: Double digested insert by *Nsi*I and *Xba*I RE 3: Double digested vector (pGAPZα-A) by *Nsi*I and *Xba*I RE

After concentration measurement of the double digested insert and vector (Figure 4.29) ligation reaction was performed with T4 DNA ligase insert to vector ratio as 3:1 (chapter 3.5.1.4). After ligation reaction *E. coli* transformation was conducted by the calcium chloride method.

The first step of confirmation was colony PCR with mAOX1 forward and hGH reverse primers (Table 3.1). The distance between two primers in the base plasmid (pmAOX1::*hGH*) is ca. 1789 bp. The result of the PCR of the chosen putative transformants should be ca. 1789 bp if cassette is inserted. The results of the colony PCR can be seen in Figure 4.30. Four of the confirmed colonies were isolated by

plasmid isolation kit (section 3.4.1) and to further confirm the insertion of desired plasmid PCR was performed using the isolated plasmid as a template.

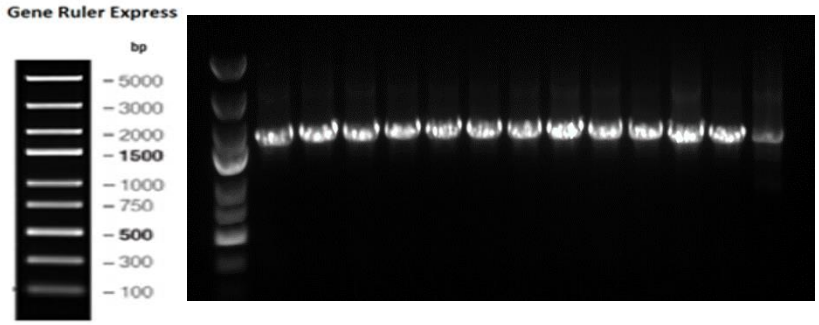


Figure 4. 30 Agarose gel image of colony PCR control of potential recombinant plasmids. 1: Generuler Express DNA ladder, 2-13: Potential plasmids carrying the pmAOX1::*hGH* 14: Control for PCR control of the insert gene 15: Negative Control for PCR control of the insert gene

After verification with PCR, isolated plasmids were sequenced. The desired nucleotide sequence based on BLAST analyzes was available to all isolated plasmids from a given putative transformants. Microbank stocks were prepared for confirmed *E. coli* strains and stored for *Pichia* transfection at -80°C.

P. pastoris strains with these expression cassettes were developed following the construction and selection of actual plasmids. As outlined in sections 3.4.8, *P. pastoris* transformation conducted. Eight clones were chosen for screening from each construct.

4.3.3. *hGH* gene copy number determination

Gene-copy number of *hGH* strains was measured by qPCR and as described previously in Section 3.6, the single-copy and double-copy transfectants of the novel *P. pastoris* strains were determined. As a housekeeping gene, *argininosuccinate lyase* (*ARG4*) was used since *P. pastoris* has only one copy of *ARG4* gene. In order to have a standard curve for the completion of the qPCR experiment and an absolute quantification, *ARG4* and *hGH* were amplified. The outer primer couples for the amplification of these standard genes were shown in Table 3.27.

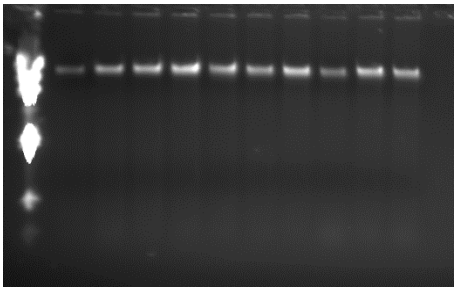


Figure 4. 31 M: GeneRuler™ 100bp plus DNA ladder 2-11: Isolated genome for selected colonies

For standard DNA preparation *ARG4* was isolated from *P. pastoris* genome with ARG4-Std Forward and ARG4-Std Reverse outer primers (Figure 4.31). *hGH* amplified from isolated plasmid with their outer primers.

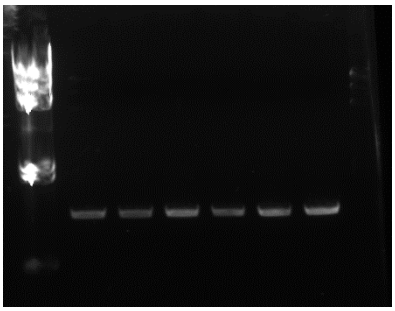


Figure 4.32 M: Marker 2-7: *ARG4* standard gene (expected length of 1259 bp) by using *P. pastoris* genome as the template.

Table 4. 16 *Nanodrop results of isolated genomic DNA samples from P. pastoris strains*

The cells constructed with:	**Concentration (ng/µl)**	**260/280**	**260/230**
ADH2-Cat8-L2::*hGH* 3	1764.6	1.96	1.24
ADH2-Cat8-L2::*hGH* 6	1520.2	2.02	1.37
ADH2-Cat8-L2::*hGH* 8	1036.2	2.04	1.51
mAOX1::*hGH* 3	1123.4	1.97	1.39
mAOX1::*hGH* 12	2119.8	2.07	1.60
ADH2-Cat8-L2::*hGH* + mAOX1::hGH 1	976.8	2.0	1.43
ADH2-Cat8-L2::*hGH* + mAOX1::hGH 8	2763.6	2.07	2.00
ADH2-Cat8-L2::*hGH* + GAP::hGH 1	1047.0	2.00	1.44
ADH2-Cat8-L2::*hGH* + GAP::*hGH* 5	1743.6	1.99	1.37
GAP::*hGH*	1381.4	2.05	1.57
ARG STD	121.8	1.86	1.27
hGH STD	146.9	1.88	1.13

ARG4 and *hGH* genes copy quantity were calculated as $9.43x10^{10}$ and $2.49x10^{11}$ respectively by equation 4.1. Isolated genomic DNA samples were diluted to a concentration of ca. 2 ng/µl, whereas the standards of *ARG4* and *mApple* were prepared at different gene-copy-number/µl (10^3, 10^4, 10^5 and 10^6/µl) values by dilution in series, in order to obtain standard-calibration curve.

Table 4. 17 *Standard curve data for hGH*

	hGH
Error	0.0171
Efficiency	2.021
Slope	-3.272
Yintercept	42.65
Link	42,600

Table 4. 18 *Relative quantification results for P. pastoris cells*

The cells constructed with:	**Advanced Relative Quantification**	**Basic Relative Quantification**	**Target Cp**	**Reference Cp**
ADH2-Cat8-L2::*hGH* 3	2.331	2.363	16.43	18.58
ADH2-Cat8-L2::*hGH* 6	2.299	2.945	15.88	18.35
ADH2-Cat8-L2::*hGH* 8	1.114	0.944	17.26	18.09
mAOX1::*hGH* 3	1.519	1.712	15.31	16.99
mAOX1::*hGH* 12	4.747	5.125	14.97	18.24
ADH2-Cat8-L2::*hGH* + mAOX1::hGH 1	4.376	4.261	13.99	17.58
ADH2-Cat8-L2::*hGH* + mAOX1::hGH 8	9.978	9.617	13.46	17.99
ADH2-Cat8-L2::*hGH* + GAP::hGH 1	4.716	4.304	15.21	18.22
ADH2-Cat8-L2::*hGH* + GAP::*hGH* 5	4.385	4.279	14.46	18.09
GAP::*hGH*	1	1	16.49	17.40

With ARG4 inner primers and hGH inner primers isolated genome of each colony was analyzed and relative the copy quantity was obtained for *hGH* compare to *ARG4*. According to measurements, it was revealed that the copy numbers were higher than one which resulted in misleading inference. The reasons of this problem might be any kind of mistakes particularly in the difference between the lengths of the amplified fragments, namely the target gene and the reference gene (*ARG4*). Additionally, concentration measurement during standard sample preparation might cause an error in gene-copy-number determination. As a conclusion, advanced relative quantification and basic relative quantification results were given as normalized data according to the pGAP::*eGFP* 1 strain since the gene-copy-number of this strain was verified as unity previously.

4.3.4. Extracellular human growth hormone production

In the production of extracellular hGH, the strengths of two DPESs, $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$ and $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$, were investigated and compared to that of the constituent SPESs. The *hGH* gene was cloned under the control of the NEPVs $P_{ADH2\text{-}Cat8\text{-}L2}$ and P_{mAOX1} with α-factor signal sequence. The engineered recombinant plasmids were incorporated into AOX1 gene termination locus to be free of the impacts of genomic integration locations. In order to obtain a reliable data, eight clones per construct were investigated. Final screening for each strain representing the entire population was conducted with two clones for DPESs. The production of extracellular hGH was carried out in 250 mL Erlenmeyer flask at T = 30°C and N = 200 rpm for 48 hours. The precultivation was conducted at T = 30°C and N = 200 rpm for 18-20 h in YPD medium; and then the cells were harvested by centrifugation at 4500 g and 4ºC for 5 min. The fermentation was started with an initial OD_{600} of 1 in a 50 mL minimal production medium (Table 3.7) containing 2% (v/v) ethanol. The samples were withdrawn at t = 48 h for analysis and supernatants were kept at –80°C

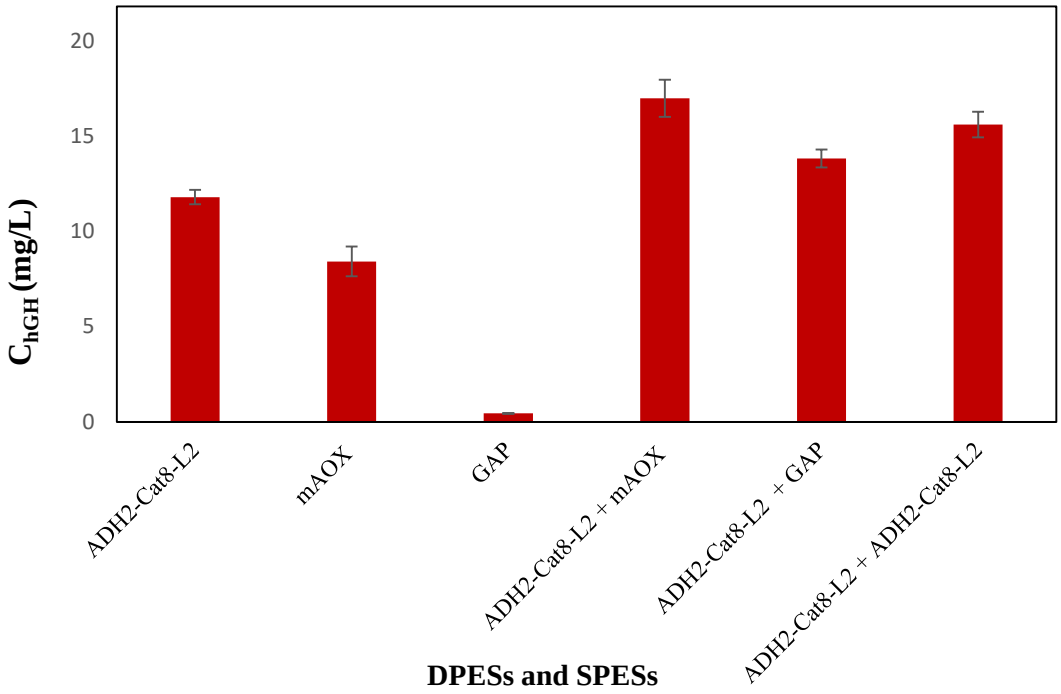


Figure 4.33 Extracellular human growth hormone (hGH) concentration of *P. pastoris* strains constructed with DPESs and SPESs on 2% (v/v) ethanol at t = 48 h of the fermentation. Error bars represent the standard deviation of cultivations (±).

Table 4. 19 *Extracellular human growth hormone (hGH) concentration of P. pastoris strains carrying DPESs and DPESs on 2% (v/v) ethanol at t = 48 h of the fermentation. StDev: Standard deviation among three different concentration measurement*

Cells	C_{hGH} (mg/L)	StDev
ADH2-Cat8-L2	11.8	0.38
mAOX1	8.4	0.78
GAP	0.4	0.03
ADH2-Cat8-L2+ mAOX1	17.0	0.98
ADH2-Cat8-L2 + GAP	13.8	0.48
ADH2-Cat8-L2 + ADH2-Cat8-L2	15.6	0.67

The average extracellular hGH yield per gram wet cell weight for recombinant *P. pastoris* strains carrying $P_{ADH2\text{-}Cat8\text{-}L2}$, P_{mAOX1}, P_{GAP}, $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ and $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$ $P_{ADH2\text{-}Cat8\text{-}L2+ADH2\text{-}Cat8\text{-}L2}$ were calculated as 2.95 mg/g, 3 mg/g, 0.13 mg/g, 4.86 mg/g 3.73 mg/g, and 4.21 mg/g, respectively. Hence, $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ enhanced the hGH production 1.4-, 2- and 1.2-fold than that of the single promoters $P_{ADH2\text{-}Cat8\text{-}L2}$ and P_{mAOX1}, and SMT-operating identical twin promoters $P_{ADH2\text{-}Cat8\text{-}L2}{}^{+}{}_{ADH2\text{-}Cat8\text{-}L2}$, respectively.

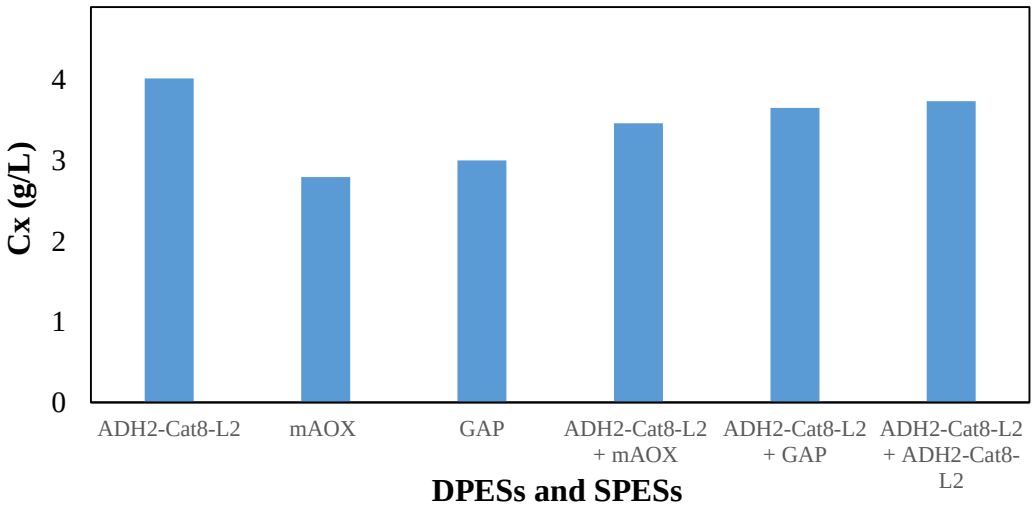


Figure 4. 34 Final cell concentrations (g/L) of *P. pastoris* strains constructed with $P_{ADH2\text{-}Cat8\text{-}L2}$, P_{mAOX1}, P_{GAP}, $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ and $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$ on 2% (v/v) ethanol at t = 48 h of the fermentation.

Table 4. 20 *Optical cell density (OD_{600}) values of P. pastoris strains carrying DPESs and SPESs s on 2 (v/v) ethanol producing hGH at t = 48 h of fermentation*

Cells	**OD_{600}**
ADH2-Cat8-L2	16.7
mAOX1	11.6
GAP	12.5
ADH2-Cat8-L2+ mAOX1	14.4
ADH2-Cat8-L2 + GAP	15.2
ADH2-Cat8-L2 + ADH2-Cat8-L2	15.3

4.4. Design and performance of Aca2 TFBS modification of mAOX1 promoter

4.4.1. Design of Aca2 TFBS modification

The objective was to develop bioprocesses with methanol-free expression systems in *P. pastoris* under the control of *alcohol oxidase 1* promoter. The baseline of the engineering of P_{AOX1} was based on the regulations of ADH2 promoters in *S. cerevisiae* and *P. pastoris*. In the light of the expertise of these ethanol-regulated promoters, P_{AOX1} architecture was developed to control the gene expression under this regulatory component not only with methanol but also with ethanol although ethanol was a natural repressor of the P_{AOX1} (Ergün et al. 2019).

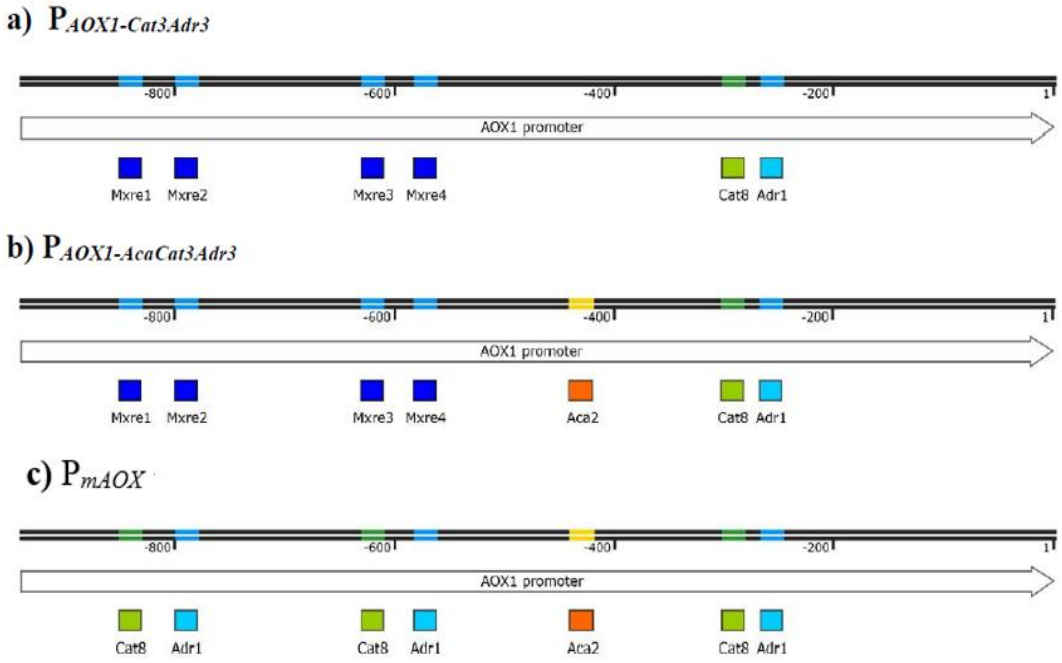
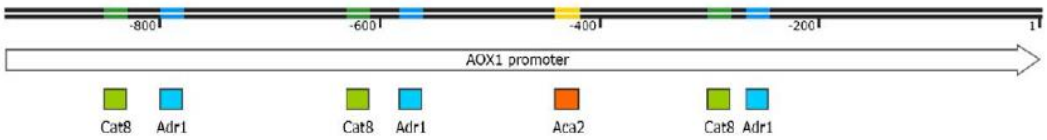


Figure 4. 35 Design of the promoter architectures of the NEPVs: **a)** $P_{AOX1\text{-}Cat3Adr3}$, **b)** $P_{AOX1\text{-}AcaCat3Adr3}$, **c)** P_{mAOX1}

Aca2 binding site integration to AOX promoter provided 33% increase in r-protein production in methanol whereas it did not affect the promoter activity in ethanol. The capacity of P_{ADH2} in ethanol could not also be improved by Aca2 binding site integration. The activity of the AOX NEPV in ethanol decreased from 85% to 60%, when Aca2 binding motive was incorporated into $P_{AOX1\text{-}Cat3Adr3}$ variation, while methanol induction activity was risen from 133% to 165%. Ergün et al. (2019) constructed mAOX1 promoter by applying seven distinct modifications. Among all of P_{AOX1} variants, the highest production capacity was reported for P_{mAOX1}, which reached to 130% in ethanol growing cells and 197% in methanol growing cells compared to the wild-type AOX1 promoter activity. All in all, the results showed that Aca2 engineering in P_{AOX1} was mostly feasible. Moreover, deletion of Aca2 binding site would increase the promoter strength in ethanol growing cells while it would have an adverse effect on methanol growing cells. In the most promising P_{AOX1} variant, i.e., P_{mAOX1}, Aca2 TFBS was deleted.

a) P_{mAOX1}



b) P_{eAOX}

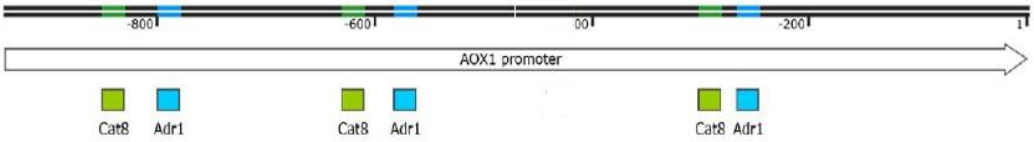


Figure 4. 36 Design of the promoter architectures of the NEPVs: **a)** P_{mAOX1}, **b)** P_{eAOX}

Recombinant peAOX*::eGFP* were constructed by using pGAPZα-A as a base vector as a restriction site *Bgl*II and *Kpn*I were used.

***Bgl*II**

5'…AGATCT…3' → 5'…A GATCT…3'
3'…TCTAGA…5' → 3'…TCTAG A…5'

***Kpn*I**

5'…GGTACC…3' → 5'…GGTAC C…3'
3'…CCATGG…5' → 3'…C CATGG…5'

First 500 bp of P_{mAOX1} was amplified with forward AOX and reverse mAOX1-AddAdr2 as a template mAOX1 plasmid is used. In order to prevent any nucleotide addition between promoter and *eGFP*, the last 440 nucleotides of mAOX1 and eGFP were added to the 3' of the designed promoters and synthesized together.

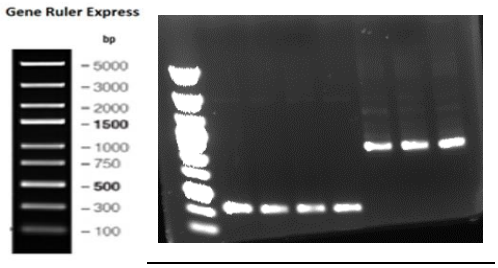


Figure 4. 37 Agarose gel electrophoresis image of genes amplified with different temperatures after the first step of OE-PCR. 1: Generuler Express DNA ladder (Fermentas) 2-5: Forward AOX Reverse and mAOX1-AddAdr2 68°C 6-8: Forward mAOX1-AddAdr2 Reverse eGFP 68°C

Amplified two fragments that have complementary ends were purified with PCR purification kit and used as a template for a second SOE-PCR at 68°C. Forward AOX and reverse eGFP primers were used in SOE PCR as shown in the Figure 4. 38.

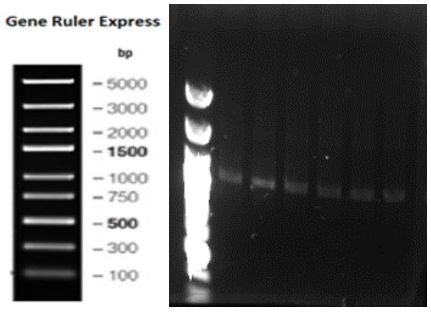


Figure 4. 38 Agarose gel electrophoresis image of genes amplified with different primer combinations after the first step of OE-PCR.;1: Generuler Express DNA ladder 2 & 7: Forward AOX and Reverse eGFP

Amplified insert and vector plasmid were double digested with *Bgl*II and *Kpn*I as explained in section 3.5.1.3.

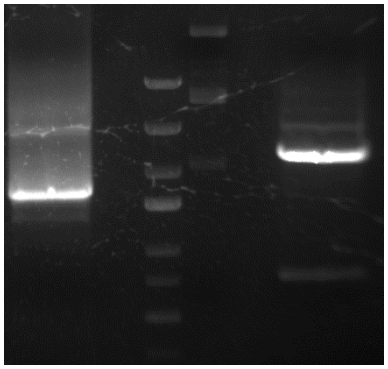


Figure 4. 39 1: *Bgl*II and *Kpn*I REs double digested insert 2: GeneRuler DNA ladder (Fermentas), 4: *Nsi*I and *Kpn*I REs double digested vector pGAPZαA

The double digested vector and the gene to be integrated (Figure 4.39) were ligated with the T4 DNA Ligase enzyme (Fermentas) using the vector to insert ratio 3: 1 as described in section 3.5.1.4 The size of the vector cut by *Bgl*II and *Kpn*I RE is 2350 bp, the size of the insert to be integrated cut by *Bgl*II and *Kpn*I RE is 1639 bp.

Ligation products were used for the transformation of *E. coli* DH5α cells. Recombinant plasmids prepared by genetic engineering methods were cloned into *E. coli* DH5α cells by using calcium chloride method to increase plasmid concentration before *Pichia* transfection (Sambrook and Russell, 2001). Twelve colonies formed after the transformation were selected and their first confirmation was applied by colony PCR (Figure 4.40). Four clones were selected from the strains identified as carrying the gene of interest by the colony PCR and their plasmids were isolated by the Plasmid MiniPrep Kit (Fermentas) according to the manufacturer's recommendations, and the size of the recombinant plasmids (3989 bp) containing the correct genes was determined by agarose gel electrophoresis.

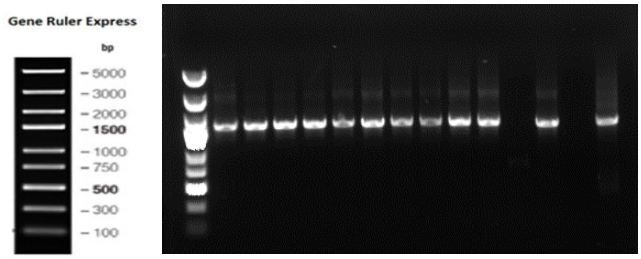


Figure 4. 40 Agarose gel image of colony PCR control of potential recombinant plasmids. 1: Generuler Express DNA ladder, 2-13: Potential plasmids carrying the eAOX::*eGFP* gene 14: Negative Control for PCR control of the insert gene 15: Positive for PCR control of the insert gene

After control experiments, the plasmids were sequenced for final verification. Microbank stocks of the cells which were confirmed by sequencing were prepared and plasmids were isolated from these cells with MiniPrep Plasmid Isolation Kit (Fermentas) for use in the later stages of the project and stored at -20°C.

4.4.2. Performance of Aca 2 TFBS modification

After wild-type *P. pastoris* X33 transformation, at least eight clones were investigated for each construct to make a more reliable deduction. Clones representing the entire population were selected for further evaluation. As the specific mApple and eGFP production values of the constructed novel strains were based on the cell volume-related fluorescence intensity (Hohenblum et al. 2003), the constant cell concentrations were used in mApple and eGFP fluorescence calculations.

Final screening experiments were conducted using three biological replicates in 12-deep well plates, at T = 30°C and N = 200 rpm for 20 hours to measure the transcriptional capacity of $P_{AOX\text{-}v}$. Precultivation was conducted in YP medium for 16-18 hours. Then, cells were harvested by centrifugation at 4500 g and 4°C for 5 min and inoculated to ASMV6 base-production medium at a pre-determined initial cell concentration for each carbon source, i.e., OD_{600} = 1 for ethanol, methanol and limited glucose, OD_{600} = 0.1 for excess glucose and excess glycerol. *P. pastoris* cells carrying intracellular eGFP cassettes under the control of NEPVs of P_{AOX1} displayed similar cell growth patterns in the same carbon source.

According to the pre-screening results, eGFP production level obtained with P_{eAOX} did not exhibit any differences in comparison to P_{mAOX1}-controlled expression. *P. pastoris* strains carrying P_{eAOX} and P_{mAOX1} with *eGFP* gene were compared in fermentations where five carbon sources, i.e., E: 2% alcohol (v/v), M: 1% methanol (v/v), X: limited glucose, D: excess glucose, G: excess glycerol, were used in order to assess the regulation and expression of P_{eAOX}.

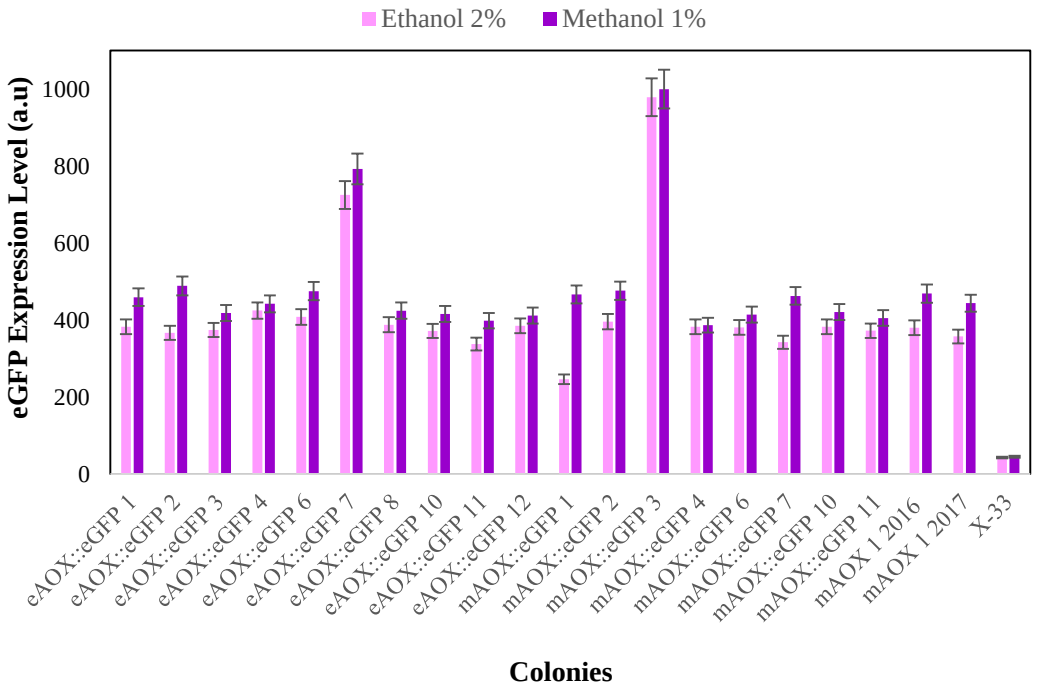


Figure 4. 41 Normalized eGFP synthesis capacity of novel $P_{AOX1\text{-}v}$ *P. pastoris* strains at the cultivation time of t = 20 h. Carbon sources: E: 2% (v/v) ethanol, M: 1% (v/v) methanol, Error bars represent the standard deviation (±)

Screening results demonstrated that P_{eAOX} had the same gene expression pattern with P_{mAOX1}. P_{eAOX} exhibited an increase in promoter strength in response to 1% (v/v) methanol and 2% (v/v) ethanol compared to P_{AOX1}, and it was still repressed by excess glucose and excess glycerol. At limited glucose condition, it exhibited slightly higher expression than P_{AOX1}. There was no significant effect of Aca2 binding site deletion in any of five carbon sources, except a slight decrease in r-protein production compared to P_{mAOX1} in limited glucose condition.

P. pastoris Aca1 also displayed as an activator in the MUT pathway, even though its homologous Aca2 in *S. cerevisiae* was identified as ethanol and glycerol activator in metabolic pathways (Garcia-Gimeno and Struhl 2000). Although the Aca2 binding site was not reported for $P_{AOX1,}$ it was available in P_{ADH2} according to literature. Ergün et al. (2019) proved that Aca2 binding site designs applied on P_{AOX1} could be an efficient promoter engineering approach. Because of the methylotrophic nature of *P. pastoris*, it was concluded that *P. pastoris* Aca2 opens new doors while closing some others.

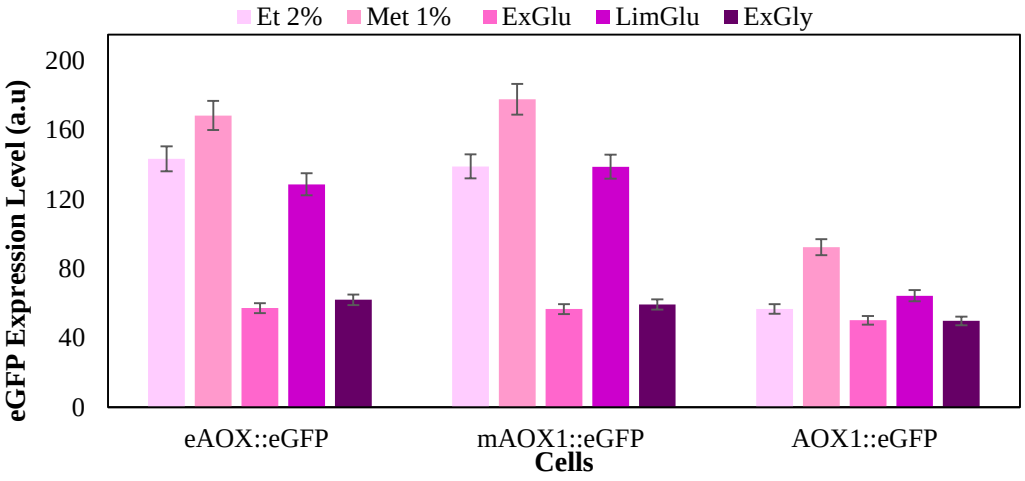


Figure 4. 42 Normalized eGFP synthesis capacity of novel $P_{AOX1\text{-}v}$ *P. pastoris* strains at the cultivation time of t = 20 h. Carbon sources: E: 2% (v/v) ethanol, M: 1% (v/v) methanol, LimGlu: limited glucose, ExGlu: excess glucose, ExGly: excess glycerol Error bars represent the standard deviation (±)

The results revealed that P_{eAOX} constructed by engineering of P_{mAOX1} could not enhance r-protein production significantly, however the expression strength of P_{eAOX} on ethanol was increased to 196% in comparison to methanol induced case.

4.5. RNA isolation optimization

For high reproducibility of the quantitative polymerase chain reaction (qPCR), microarray and next generation sequencing (NGS) results, the starting quality of RNA is essential for obtaining both *P. pastoris* and all other organisms. It was particularly crucial to isolate RNA from yeast cells of high quality because they were abundant in polysaccharides and proteins. The total RNA isolation technique was optimized through modifications of pretreatment application. For this purpose, three different RNA isolation method: commercially available kit (Roche), TRI reagent-based method and commercially available kit (Qiagen) were compared. The quality; concentration and integrity, of the RNA samples were measured by nanodrop and Bioanalyzer.

RNA qualities are critical to obtain the extremely reproducible results of qPCR, microarrays and NGS for all species, as well as *P. pastoris*, with the typical $OD_{260/280}$ (A260/280) between 1.7 to 2.3. Different RNA isolation techniques require additional step for extracting the yeast cell wall including commercial kits. Many of these techniques use phenol warm acid, physical shear forces or enzymatic lysis to break very robust cell walls (Schmitt, Brown and Trumpower, 1990).

P. pastoris cells were growth on YP medium with shaking at 200 rpm and 30°C for 16-18 hours. Then, cells were harvested and inoculated in production medium with OD_{600} of 1. At the mid-logarithmic phase t = 24-27 h for ethanol fed culture, cells were collected by centrifugation at 4500 g for 5 min at 4°C and directly use for RNA isolation.

10^8 cells with 1 mg/ml enzymatic lysis were used for Protocol A and 5 x 10^8 cells with 2 mg/ml Lyticase were used for Protocol B. In both protocols RNAs were extracted by High Pure RNA Isolation Kit (Roche). Protocol C composed of approximately 10^9 cells with mechanic cell disruption at 30 Hz 1 min and 20 second cold treatment (5 times) with the addition of 1 ml TRI reagent. In Protocol D, complete RNA was

produced in accordance with the manufacturer's orders using Total RNA isolation Kit (Qiagen) with mechanic cell disruption.

Table 4. 21 *Pretreatments used for RNA isolation protocols*

Protocol	Yeast cell number/ml	Cell disruption technique	Lyticase (mg/ml)	Mechanical
A	2 OD	Enzymatic	1	_
B	5 OD	Enzymatic	2	_
C	8 OD	Mechanic	_	1 min 30 Hz 20 sec ice treatment (x5)
D	8 OD	Mechanic	_	1 min 30 Hz 20 sec ice treatment (x5)

4.5.1. mRNA isolation with Roche-kit

In this section RNA extraction assay was applied with commercially available High Pure RNA Isolation Kit (Roche) with two different pre-treatment applications. After 27h for ethanol fed systems, cells were harvested at 4500g and 4°C for 5 min. The concentration of *P. pastoris* cell was $1x10^8$ (that is the maximum amount that protocol recommend) and the enzyme was 1 µg/µl. The amount of enzyme and amount of cells should be optimized to increase RNA yields and prevent RNA degradation. If too many cells are used, the cell wall cannot be completely disrupted. Likewise, if enzyme concentration is too high, proteins can be disrupted and RNA can be contaminated. Total RNA was isolated as described in the section 3.4.10.

Table 4. 22 *Nanodrop results for isolated RNAs for protocol A*

Samples	Concentration (µg/µl)	260/280
1	11.61	1.78
2	18.73	1.88
3	12.77	1.87

According to nanodrop results RNA concentration was low for further analysis like NGS. As elution volume of RNA was 50 µl, RNA amount is approximately 580 µg. This could indicate initial cell amount was not sufficient or in the elution step we could lose some RNA. However, RNA quality for all three samples were checked by bioanalyzer.

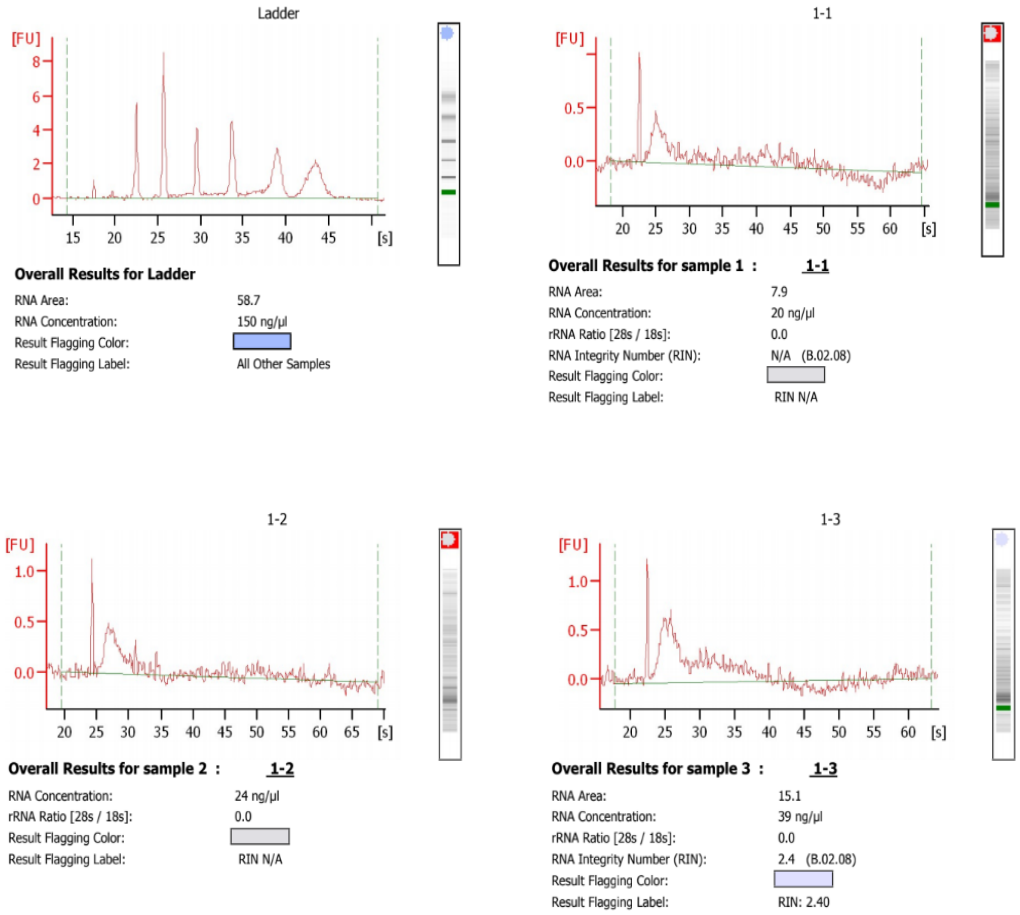


Figure 4. 43 Bioanalyzer results for isolated RNAs for protocol A

Based on bioanlyzer results, all the RNA for three samples were degraded and gel images of the RNA were smear. 18S and 28S bands are not explicit so RNA integrity number (RIN) cannot be calculated. To increase RNA concentration and eliminate RNA degradation, protocol was optimized. First, initial cell amount was increase to

$5x10^8$ consequently enzyme amount was also increase to 4 µg/µl. Then, extra elution step was added by using elute and the same collection tube to increase RNA yield and the elution volume was decrease from 50µl to 35µl. After the modifications on protocols RNA qualities and quantities were measured and presented in Table 4.23 and Figure 4.44.

Table 4. 23 *Nanodrop results for isolated RNAs for protocol B*

Samples	**Concentrations**	**260/280**
1	76.83	1.83
2	88.12	1.89
3	67.53	1.87

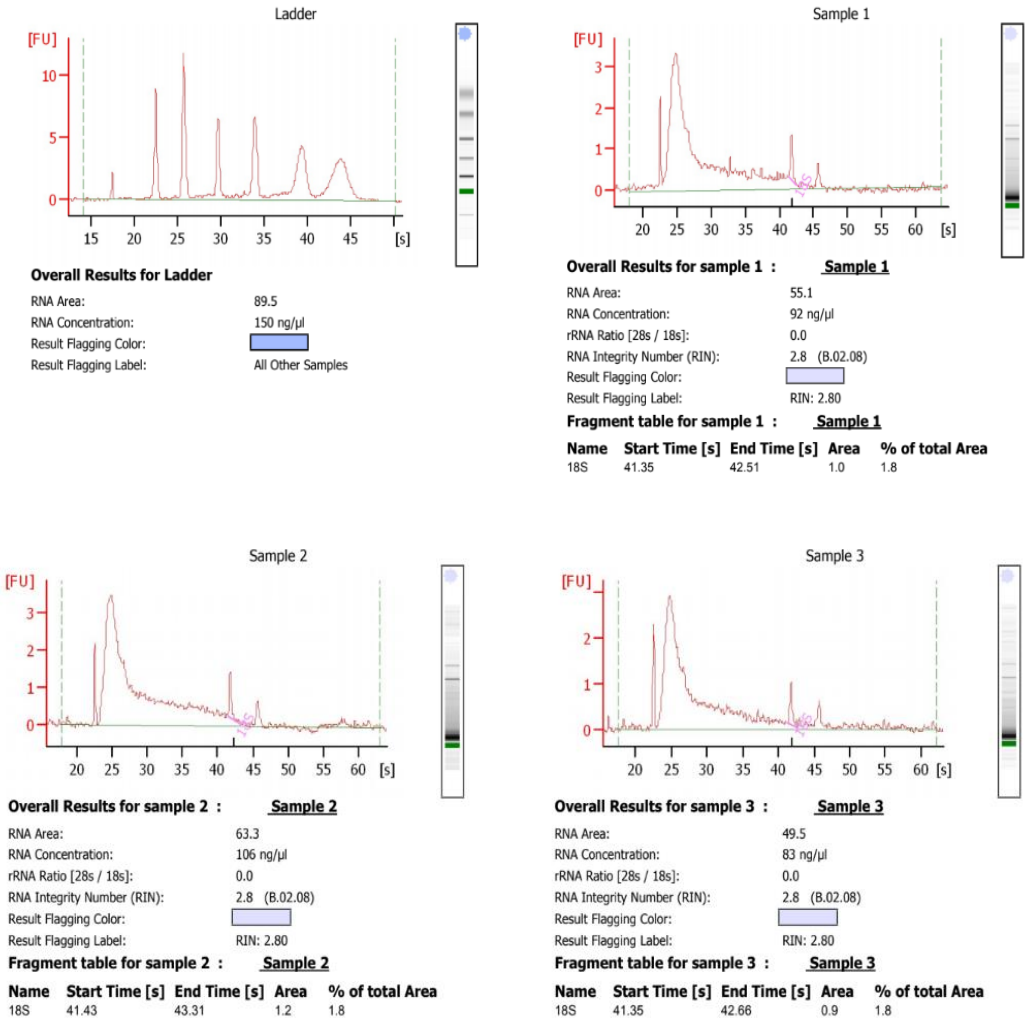


Figure 4. 44 Bioanalyzer results for isolated RNAs for protocol B

RNA isolation experiments were conducted with 3 different samples. Even if the ratio 260/280 are around 1.83 in nanodrop, the profile is not really correct. It is possible that the presence of alcohol may be at the origin of the high absorption at 250 nm so 260/280 ratio seems to be high. Moreover, although 18S can be detect both in gel image and the bioanalyzer results, 28S was partially degraded. Another reason may be that the cell wall is not completely digested. The enzyme concentration may be low or the amount of cells may be too high to lysate.

4.5.2. RNA isolation by TRI-AGENT method

Optimization of enzymatic lysis is expensive and time consuming. Also TRI reagent-based method was more efficient then and simpler then Roche Kit for yeast (Remziye Yılmaz, 2012). In this protocol mechanical cell disruption technique was applied to totally digest the cell yeast cell wall by tissue-lyzer. TRI reagent-based protocol was also recommend the mechanical cell disruption. At max, 100 mg wet cell weight was used. RNA was isolated as explained in section 3.4.11, nanodrop and bioanalyzer results were in Table 4.24 and Figure 4.45, respectively.

Table 4. 24 *Nanodrop results for isolated RNAs for protocol C*

Samples	**Concentrations**	**260/280**
1	246.12	1.83
2	294.58	1.78
3	211.47	1.88

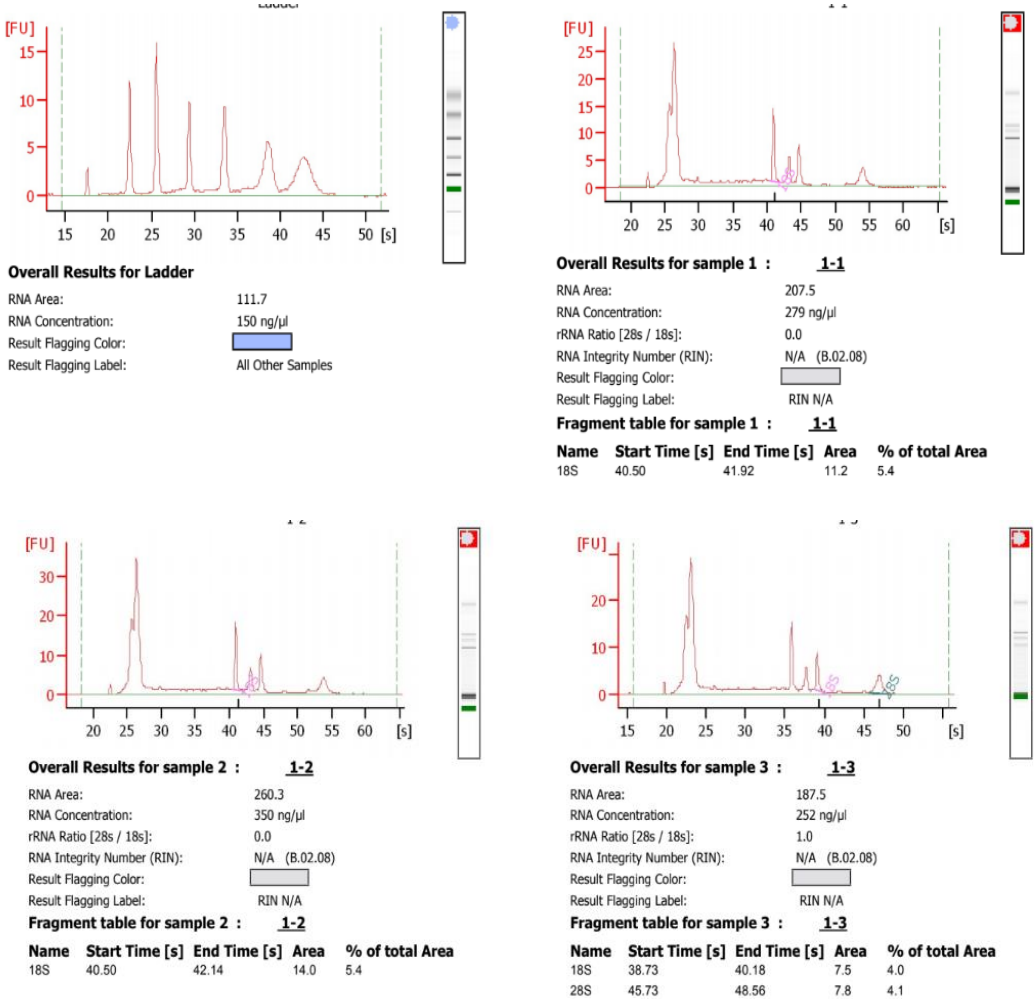


Figure 4. 45 Bioanalyzer results for isolated RNAs for protocol C

Even if the ratio 260/280 is 1.85 to nanodrop, the profile is not really correct. It is possible that the presence of phenol may be at the origin of the high absorption at 270nm and this effect the 260/280 ratio. Main disadvantage of this protocol is the residue of phenol consequently phenol should be carefully removed from the system. Based on bioanalyzer results and gel image, eventough both 18S and 28S RNA were detectable, partial RNA degradation is observed and 5S RNA amount is relatively higher compare to 18S and 28S. Therefore, RNA integrity number (RIN) could not be calculated

4.5.3. mRNA isolation with Qiagen kit

Last protocol for isolating the total RNA was to use Qiagen total RNA isolation Kit. For, total RNA isolation, cells were growth in 12-deep well plates at 30°C, 200 rpm. Cells were harvested at the mid-logarithmic phase this corresponds to 27 h for ethanol and methanol fed system and 18 h for glucose fed system. The precultivation was performed in 2 mL YP at 30°C and 200 rpm for 16-18 h. After that cells were harvested at 4500 g for 5 min at 4°C and inoculate in the production medium (Table 3.7) with OD600 of 1. As a carbon source 1% (v/v) methanol or 2% (v/v) ethanol or 20 g/L glucose was introduced in production medium. The cells were harvested and RNA isolation protocol was applied as explained in section 3.4.12 and nanodrop results are presented in Table 4.25.

Table 4. 25 *Nanodrop results for Total RNA samples*

Sample name	**Nucleic acid (ng/µl)**	**260/280**	**260/230**
1	112.9	2.04	1.01
2	102.9	2.12	0.87
3	170.8	2.09	1.33
4	86.5	2.06	0.79
5	117.6	2.08	1.55
6	115.2	2.07	1.03
7	63.8	2.07	1.02
8	82.4	2.12	0.93
9	103.8	2.11	1.55
10	125.2	2.04	0.49
11	126.2	2.07	1.31
12	160.6	2.12	1.59
13	96	2.1	1.29
14	75.9	2.11	0.76
15	71.8	2.09	1.55
16	65.2	2.12	1.66
17	81.7	2.11	1.22
18	87.1	2.22	1.3
19	68.9	2.08	1.51
20	142.6	2.08	1.16
21	131.5	2.07	1.13
22	113.8	2.08	1.24
23	142.4	2.1	0.39
24	119.5	2.14	1.01

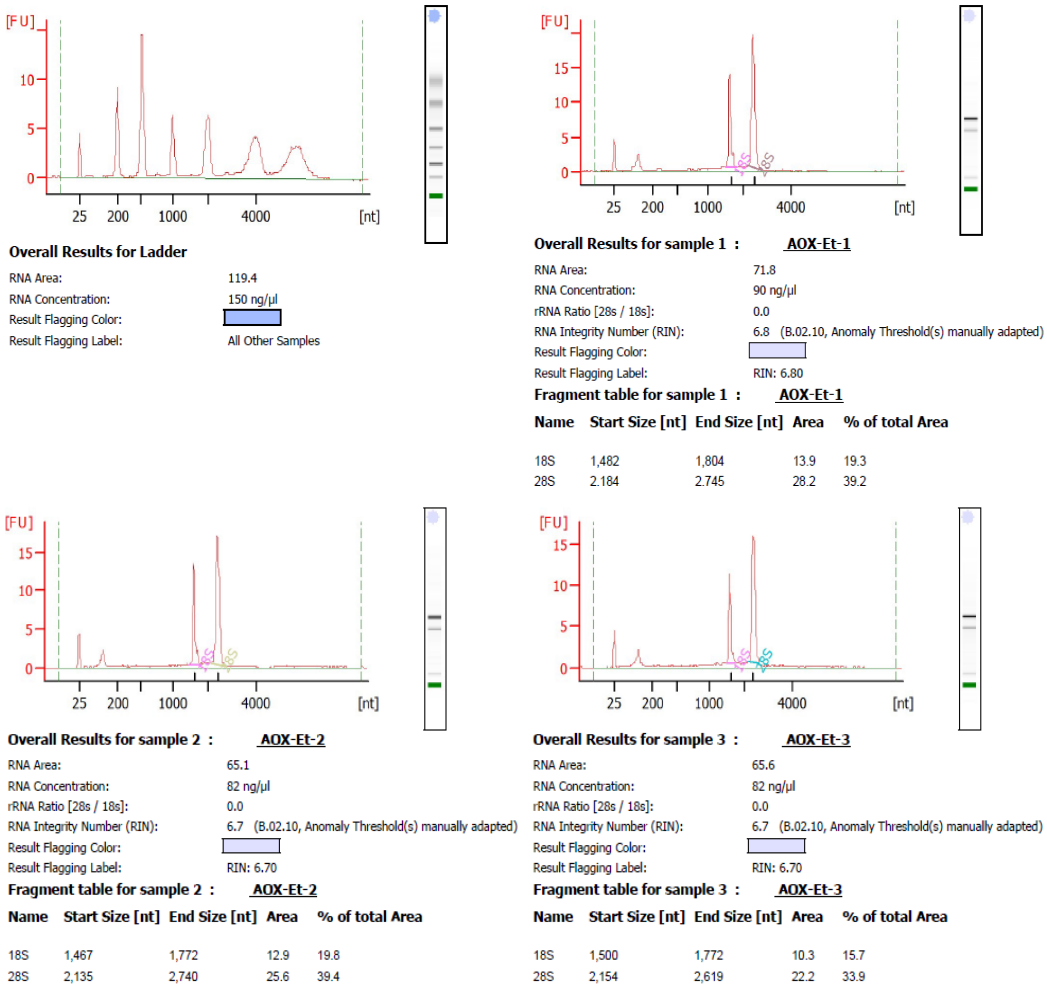


Figure 4. 46 Bioanalyzer results for isolated RNAs for protocol D

In the last protocol, total RNA was isolated successfully. Both RNA concentration and 260/280 are in desired range for the further analysis. In the bioanalyzer graph and the gel images 28S and 28S peaks and bands were clearly seen. Consequently, RNA integrity number can be calculated as (approximately) 7. Also, there is no background throughout the graph. One minor problem of this protocol is to presence of ethanol. The 260/230 ratio is low, normally it should be the 1.8-2.0 range. However, for further analysis, i.e. NGS, high fidelity (quality) enzyme is used so presence of alcohol does not affect the efficiency of further reactions. All bioanalyzer results are presented in the Appendix E.

CHAPTER 5

CONCLUSIONS

The objective of this MSc Thesis is to enhance productivity and design of multi-promoter expression systems with a NOP and two NEPVs in the yeast *Pichia pastoris*. The DPESs were designed and constructed for the synthesis of the heterologous model proteins mApple and eGFP in order to determine and maintain the operation time of the syntheses with each constituent promoter, which can be either: ***(i)*** simultaneously (SMT-) operating system to enhance the transcriptional activity with two constituents activated under similar conditions within the production domain, or ***(ii)*** by consecutively (CNT-) operating system to stimulate the changeover from one to another in a biphasic process or via successive-iterations to extend the expression period with two constituents activated under different conditions. To this end, five DPESs: ***i)*** pADH2-Cat8-L2::*mApple* and pGAP::*eGFP*, ***ii)*** pADH2-Cat8-L2::*mApple* and pmAOX1::*eGFP*, ***iii)*** pADH2-Cat8-L2::*mApple* and pADH2-Cat8-L2::*mApple*, ***iv)*** pGAP::*eGFP* and pGAP::*eGFP*, and ***v)*** pmAOX1::*eGFP* and pmAOX1::*eGFP*, were constructed. Among the designed and constructed five DPESs: ***i)*** pADH2-Cat8-L2::*mApple* and pGAP::*eGFP* is the CNT- and SMT- operating, ***ii)*** pADH2-Cat8-L2::*mApple* and pmAOX1::*eGFP* is the SMT- operating ; and the following three DPESs with twin- constituents, ***iii)*** pADH2-Cat8-L2::*mApple* and pADH2-Cat8-L2::*mApple*, ***iv)*** pGAP::*eGFP* and pGAP::*eGFP*, and ***v)*** pmAOX1::*eGFP* and pmAOX1::*eGFP* are SMT- operating systems. The expression systems were also tested in recombinant human growth hormone (rhGH) production under the SMT- and CNT- operated DPES, besides with the expression systems constructed with the constituent NEPVs and the NOP in order to verify its applicability for the production of extracellular r-proteins. Besides the main research program on novel metabolic

engineering strategies for designing and construction of DPESs, the NEPV P_{mAOX1} was designed for deregulated *AOX1* expression in methanol-free media, on ethanol. Aca2 transcription factor binding site (TFBS) was also used in the hybrid-promoter architecture of P_{mAOX1}. With the novel *P. pastoris* strains constructed with the DPES $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ the intracellular fluorescent protein syntheses was increased 2.1-fold, and the extracellular rhGH production was increased 1.5-fold, compared to that with the NEPV $P_{ADH2\text{-}Cat8\text{-}L2}$ while it reached 2.1-fold higher intracellular fluorescent protein synthesis and 2-fold higher extracellular rhGH production than with the NEPV P_{mAOX}, on ethanol., Whereas, with the DPES $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ 1.3-fold higher intracellular fluorescent protein synthesis was obtained compared to their constituent twin-DPESs on ethanol. On ethanol, with the SMT- operating $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$, the expression strength was 1.6 fold higher than that with the CNT- operated $P_{ADH2\text{-}Cat8\text{-}L2+GAP}$. Overall, for methanol-free r-protein production, the DPES $P_{ADH2\text{-}Cat8\text{-}L2+mAOX1}$ with its enhanced strength compared to its constituent NEPVs, furthermore also compared to the NOPs P_{AOX1} on methanol and P_{ADH2} on ethanol, is the most successful and strong promoter system. When P_{mAOX1} was used for the heterologous gene expression, eGFP was detected in the presence of glucose where this variant promoter was strong as the commonly used promoter P_{GAP} in *P. pastoris*. The DPESs containing P_{mAOX1} showed similar r-protein production performance on either ethanol or glucose. $P_{ADH2\text{-}Cat8\text{-}L2+mAOX}$, $P_{mAOX1+mAOX1}$ and P_{mAOX1}-controlled *eGFP* expression levels reached to the production levels attained with P_{GAP} on glucose. The major eGFP synthesis under the control of P_{mAOX1} was observed in the stationary phase of fermentation nearly at t = 24 h. Thus, P_{mAOX1} could produce the target protein, when glucose became limited. In order to support the research results related with the NEPVs of *AOX1* promoter, the NEPV P_{mAOX1} was redesigned by single TFBS modification. On ethanol the NEPV P_{eAOX} did not exhibit significant intracellular eGFP synthesis compare to P_{mAOX1} yet, however intracellular eGFP synthesis increased to 196 % compare to P_{AOX1} on methanol. Finally, the total RNA isolation technique was optimized through modifications of pretreatment application to obtain the extremely reproducible results of qPCR, microarrays or NGS for *P. pastoris*.

Among the four total RNA isolation techniques, mRNA isolation with Qiagen kit was the most promising one.

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APPENDICES

A. Plasmid and Gene Sequences

TEF promoter NTC TEF Terminator

GACATGGAGGCCCAGAATACCCTCCTTGACAGTCTTGACGTGCGCAGCT
CAGGGGCATGATGTGACTGTCGCCCGTACATTTAGCCCATACATCCCCAT
GTATAATCATTTGCATCCATACATTTTGATGGCCGCACGGCGCGAAGCAA
AAATTACGGCTCCTCGCTGCAGACCTGCGAGCAGGGAAACGCTCCCCTC
ACAGACGCGTTGAATTGTCCCCACGCCGCGCCCCTGTAGAGAAATATAA
AAGGTTAGGATTTGCCACTGAGGTTCTTCTTTCATATACTTCCTTTTAAAA
TCTTGCTAGGATACAGTTCTCACATCACATCCGAACATAAACAACCATG*G*
GTACCACTCTTGACGACACGGCTTACCGGTACCGCACCAGTGTCCCGGGGGA
CGCCGAGGCCATCGAGGCACTGGATGGGTCCTTCACCACCGACACCGTTTTT
CGCGTCACCGCCACCGGGGACGGCTTCACCCTGCGGGAGGTGCCGGTGGA
CCCGCCCCTGACCAAGGTGTTCCCCGACGACGAATCGGACGACGAATCGGA
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ACGACGGCGACCTGGCGGGCTTCGTGGTCGTCTCGTACTCCGGCTGGAACC
GCCGGCTGACCGTCGAGGACATCGAGGTCGCCCCGGAGCACCGGGGGCAC
GGGGTCGGGCGCGCGTTGATGGGGCTCGCGACGGAGTTCGCCCGCGAGCG
GGGCGCCGGGCACCTCTGGCTGGAGGTCACCAACGTCAACGCACCGGCGAT
CCACGCGTACCGGCGGATGGGGTTCACCCTCTGCGGCCTGGACACCGCCCT
GTACGACGGCACCGCCTCGGACGGCGAGCAGGCGCTCTACATGAGCATGCC
*CTGCCCCTAATCAGT*ACTGACAATAAAAAGATTCTTGTTTTCAAGAACTTG
TCATTTGTATAGTTTTTTTATATTGTAGTTGTTCTATTTTAATCAAATGTTA
GCGTGATTTATATTTTTTTTCGCCTCGACATCATCTGCCCAGATGCGAAGT
TAAGTGCGCAGAAAGTAATATCATGCGTCAATCGTATGTGAATGCTGGT
CGCTATACTG

Arg Standard Amplicon Size: 1259 bp

>XM_002490002.1 Komagataella phaffii GS115 Argininosuccinate lyase, catalyzes the final step in the arginine biosynthesis pathway partial mRNA

ATGTCGAATCAAGAAGAAGGACTTAAACTGTGGGGTGGCAGGTTTACTGGGGCTACTGA
CCCCTTGATGGATTTGTATAACGCTTCCTTACCTTACGACAAGAAAATGTACAAGGTGGA
TTTAGAAGGAACAAAAGTTTACACTGAGGGCCTGGAGAAAATTAATTTGCTAACTAAAG
ACGAACTAAGTGAGATTCATCGTGGTCTCAAATTGATTGAAGCAGAGTGGGCAGAAGGG
AAGTTTGTTGAGAAGCCAGGGGATGAGGATATTCACACTGCTAATGAACGTCGCTTGGG
TGAGTTGATTGGTCGTGGAATCTCTGGTAAGGTTCATACCGGAAGGTCTAGAAATGATCA
AGTTGCCACTGATATGCGGTTGTATGTCAGAGACAATCTAACTCAGTTGGCTGACTATCT
GAAGCAGTTCATTCAAGTAATCATCAAGAGAGCTGAACAGGAAATAGACGTCTTGATGC
CCGGTTATACTCACTTGCAAAGAGCTCAACCAATCAGATGGTCTCACTGGTTGAGCATGT
ATGCTACCTATTTCACTGAAGATTATGAGAGACTGAATCAAATCGTTAAAAGGTTGAAC
AAATCCCCATTGGGAGCTGGAGCTTTGGCTGGTCATCCTTATGGAATTGATCGTGAATAC
ATTGCTGAGAGATTAGGGTTTGATTCTGTTATTGGTAATTCTTTGGCCGCTGTTTCAGACA
GAGATTTTGTAGTCGAAACCATGTTCTGGTCTTCGTTGTTTATGAATCATATTTCTCGATT
CTCAGAAGATTTGATCATTTACTCCACTGGAGAGTTTGGATTTATCAAGTTGGCAGATGC
TTATTCTACTGGATCTTCTCTGATGCCTACAAAAAAAAACCCAGACTCTTTGGAGTTATT
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CATTCCGTCAACCTATAACAAAGATATGCAAGAGGATAAGGAGCCTTTATTTGATACTCT
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EGFP Std Amplicon Size: 735 bp

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> **P*AOX1***

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AATTGCGACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAAC
GACAACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACG

> **P*AOX1/Cat8-L3/Adr1-L3***

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GGATACACTAGCAGCAGACCGTTGCAAACGCAGGACCTCCACTCCTCTT
CTCCTCAACACCCACTTTTGCCATCGAAAAACCAGCCCAGTTATTGGGCT
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TCTCTCTATCGCTTCTGAACCCCGGTGCACCTGTGCCGAAAC**ATATTCCG**
TTCGTCCGAATCTTTTTGGATGATTA**ACCCCAATACATTTTGGGGT**TGC
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> P_{mAOX1}

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CGCAGTCTCTCTATCGCTTCTGAACCCCGGTGCACCTGTGCCGAAAC**ATA**
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P_{eAOX1}

AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCC
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B. Nucleotide Sequences and Plasmids

ADH-Forward

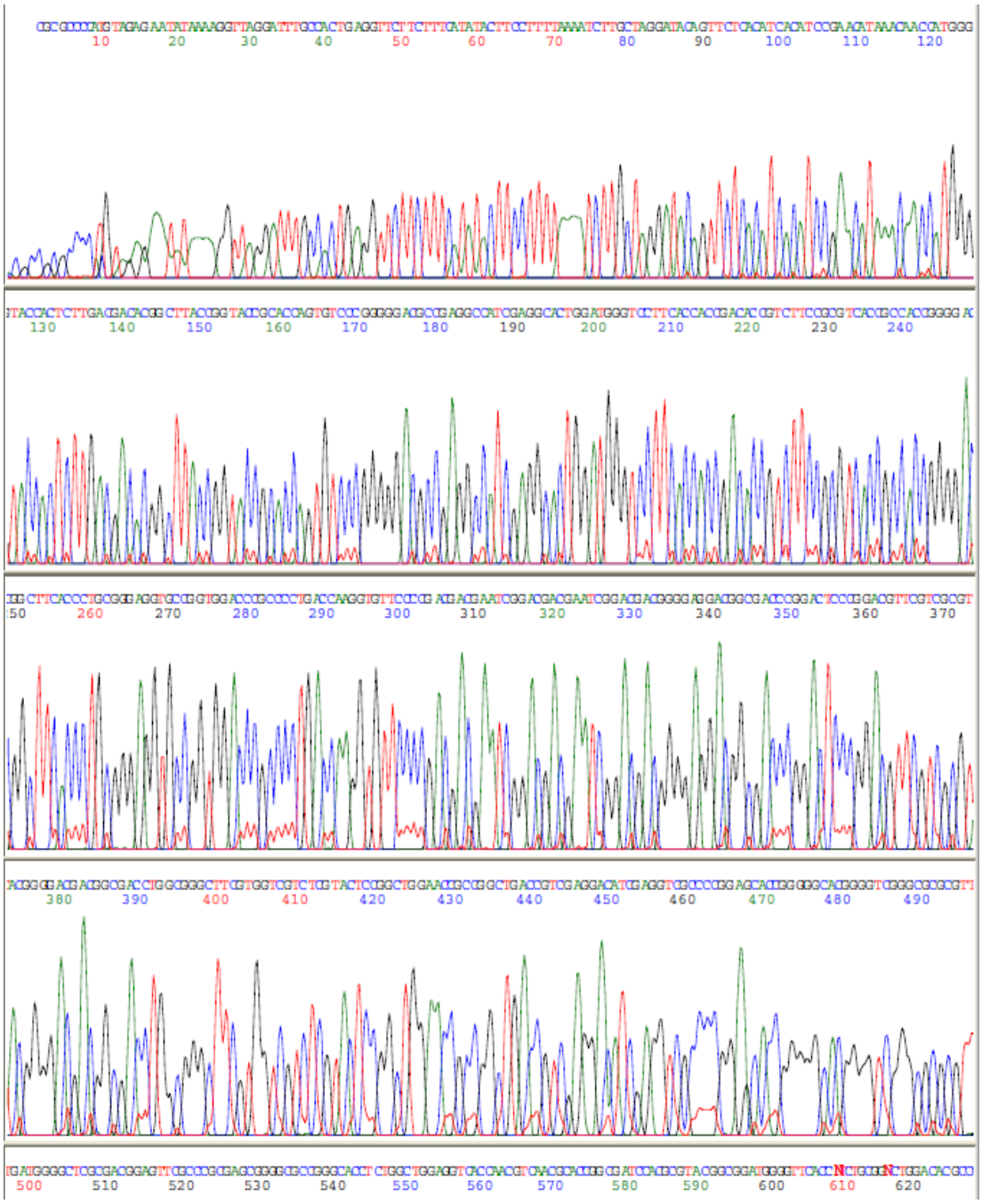
ADH-Middle

mApple Reverse

mApple Middle

NTC Forward

NTC Middle



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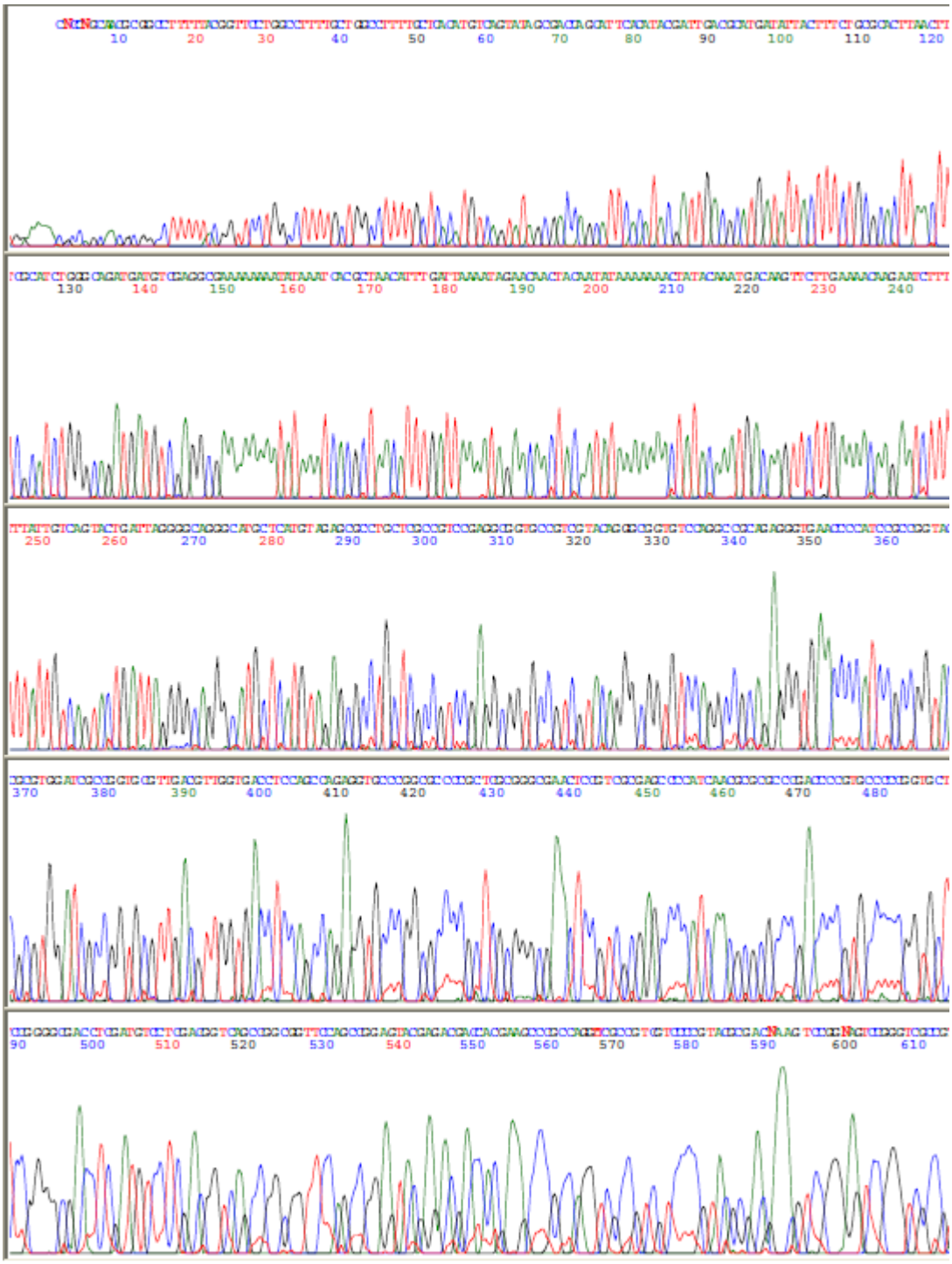

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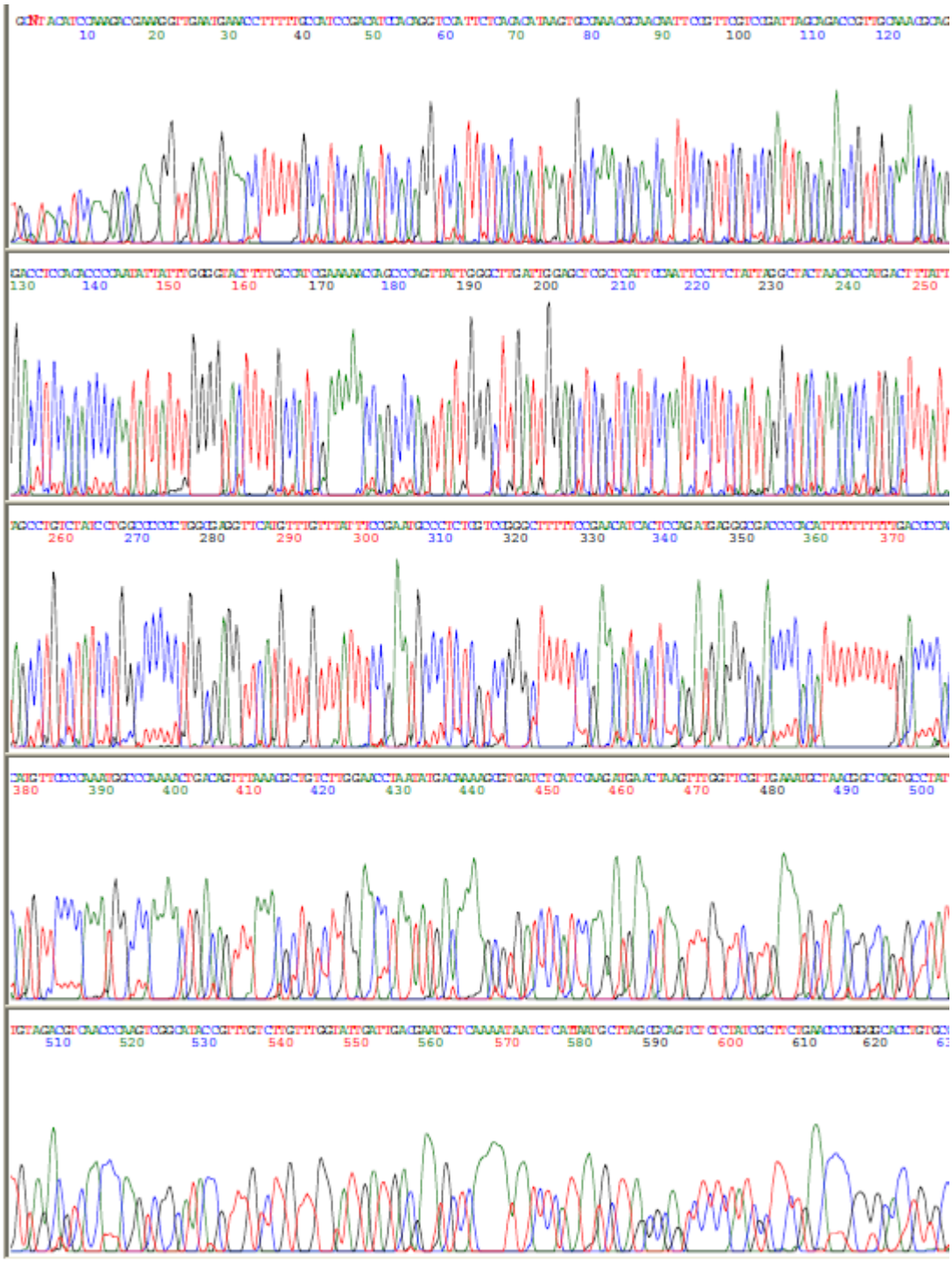
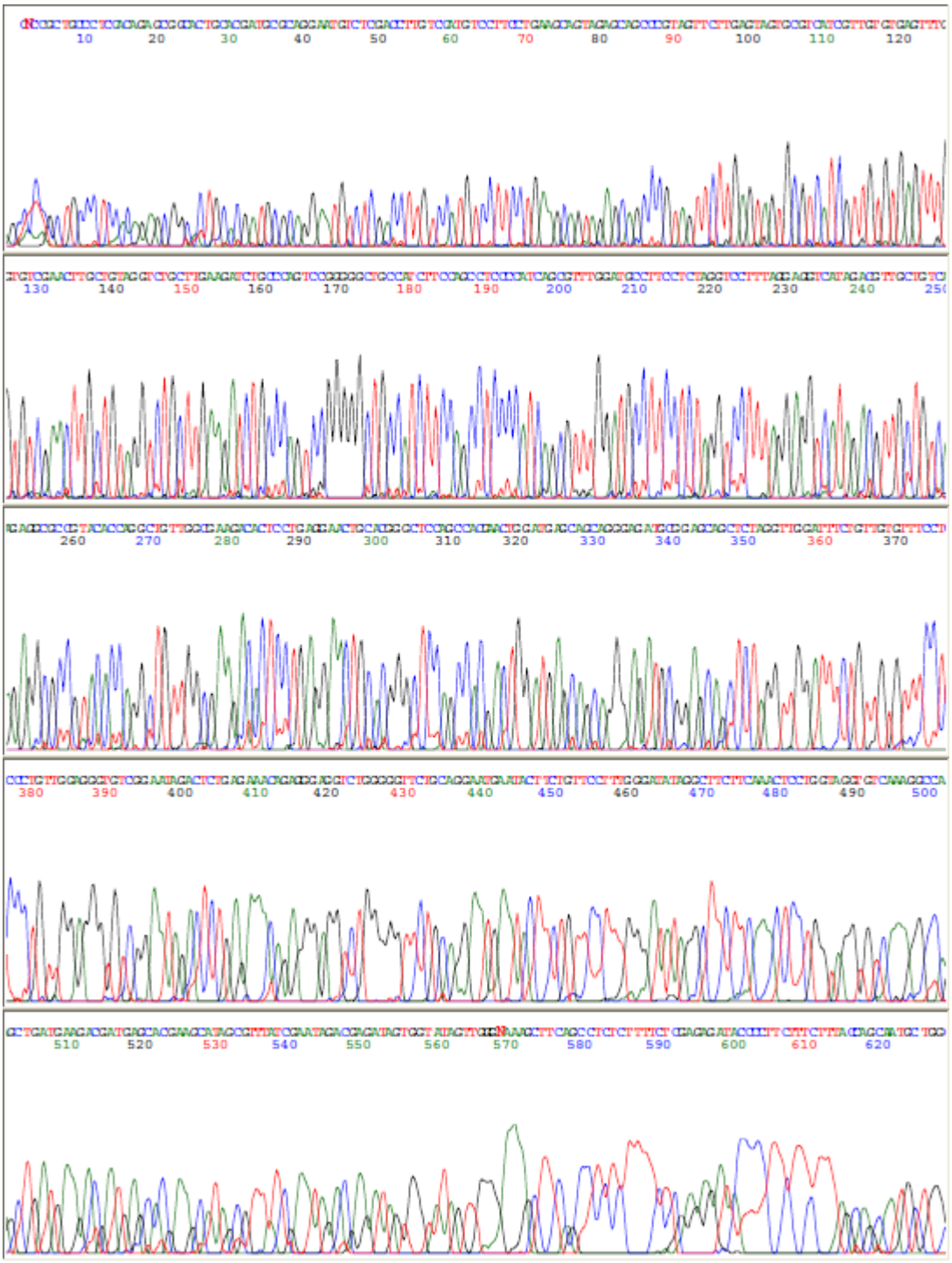


hGH Reverse



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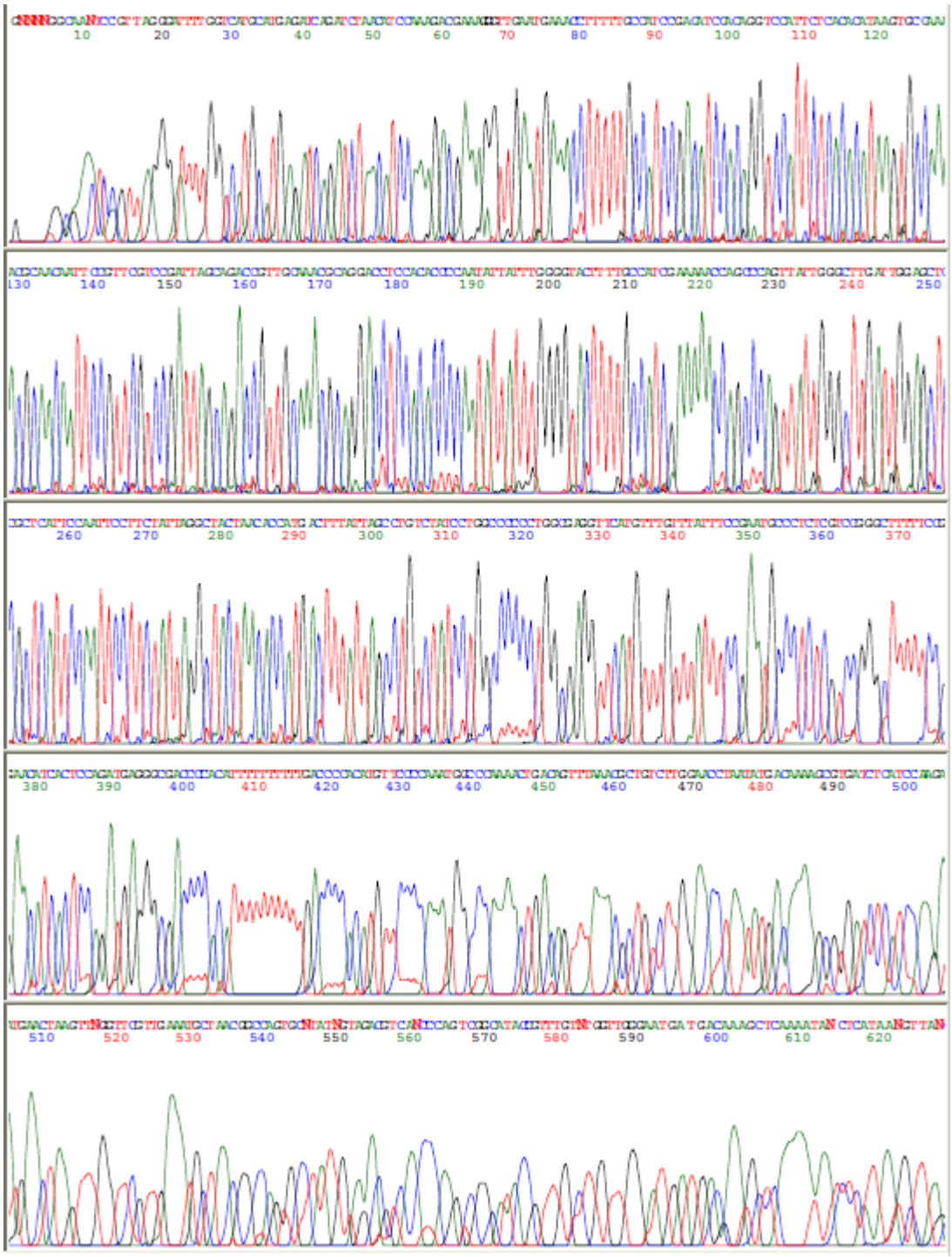

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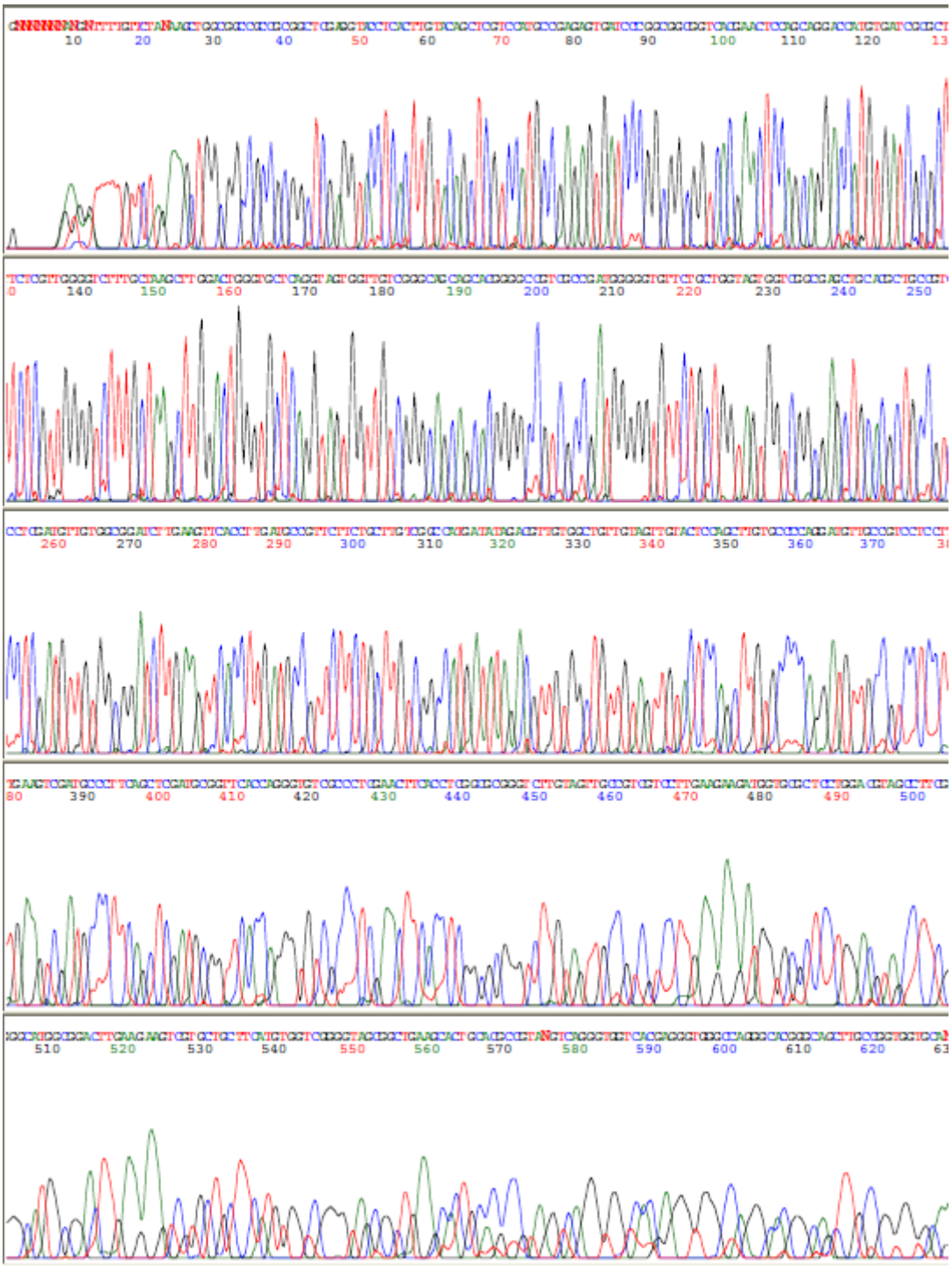


C. Thermodynamic Properties of Designed Primers

Forward opt cat_PADH2

SEQUENCE	5'- CAG ATG CAT TCC TTT TTA CCA CCC AAG TGC -3'
COMPLEMENT	5'- GCA CTT GGG TGG TAA AAA GGA ATG CAT CTG -3'
LENGTH	30
GC CONTENT	46.7 %
MELT TEMP	61.6 °C
MOLECULAR WEIGHT	9076.9 g/mole
EXTINCTION COEFFICIENT	274500 L/(mole·cm)
nmole/OD_{260}:	3.64
µg/OD_{260}:	33.07

Reverse opt cat_PADH2

SEQUENCE	5'- GCC CTT GCT CAC CAT TTT CGT AAA GTA AAT AAG ATA AAA GCT AGT AGC -3'
COMPLEMENT	5'- GCT ACT AGC TTT TAT CTT ATT TAC TTT ACG AAA ATG GTG AGC AAG GGC -3'
LENGTH	48
GC CONTENT	37.5 %
MELT TEMP	63.5 °C
MOLECULAR WEIGHT	14742.6 g/mole
EXTINCTION COEFFICIENT	476200 L/(mole·cm)
nmole/OD_{260}:	2.1
µg/OD_{260}:	30.96

Forward mApple

SEQUENCE	5'- GCT ACT AGC TTT TAT CTT ATT TAC TTT ACG AAA ATG GTG AGC AAG GGC -3'
COMPLEMENT	5'- GCC CTT GCT CAC CAT TTT CGT AAA GTA AAT AAG ATA AAA GCT AGT AGC -3'
LENGTH	48
GC CONTENT	37.5 %
MELT TEMP	63.5 °C
MOLECULAR WEIGHT	14786.6 g/mole
EXTINCTION COEFFICIENT	463000 L/(mole·cm)
nmole/OD_{260}:	2.16
µg/OD_{260}:	31.94

Reverse mApple

SEQUENCE	5'- CTG GTA CCT TAC TTG TAC AGC TCG TCA TGC -3'
COMPLEMENT	5'- GCA TGA CGA GCT GTA CAA GTA AGG TAC CAG -3'
LENGTH	30
GC CONTENT	50 %
MELT TEMP	61.6 °C
MOLECULAR WEIGHT	9123.9 g/mole
EXTINCTION COEFFICIENT	272400 L/(mole·cm)
nmole/OD_{260}:	3.67
µg/OD_{260}:	33.49

Forward NTC

SEQUENCE	5'- CAA GGA TCC GAC ATG GAG GCC CAG -3'
COMPLEMENT	5'- CTG GGC CTC CAT GTC GGA TCC TTG -3'
LENGTH	24
GC CONTENT	62.5 %
MELT TEMP	63.5 °C
MOLECULAR WEIGHT	7396.8 g/mole
EXTINCTION COEFFICIENT	237700 L/(mole·cm)
nmole/OD_{260}:	4.21
µg/OD_{260}:	31.12

Reverse NTC

SEQUENCE	5'- GCC ACA TGT CAG TAT AGC GAC CAG CAT TC -3'
COMPLEMENT	5'- GAA TGC TGG TCG CTA TAC TGA CAT GTG GC -3'
LENGTH	29
GC CONTENT	51.7 %
MELT TEMP	62.4 °C
MOLECULAR WEIGHT	8846.8 g/mole
EXTINCTION COEFFICIENT	279900 L/(mole·cm)
nmole/OD_{260}:	3.57
µg/OD_{260}:	31.61

Forward ADHoptcat hGH

SEQUENCE	5'- TTA GGA TCC GGC GCG CCT TCC TTT TTA CCA CCC AAG -3'
COMPLEMENT	5'- CTT GGG TGG TAA AAA GGA AGG CGC GCC GGA TCC TAA -3'
LENGTH	36
GC CONTENT	55.6 %
MELT TEMP	68.8 °C
MOLECULAR WEIGHT	10923.1 g/mole
EXTINCTION COEFFICIENT	322300 L/(mole·cm)
nmole/OD_{260}:	3.1
µg/OD_{260}:	33.89

Forward modAOX

SEQUENCE	5'- CAG ATG CAT AAC ATC CAA AGA CGA AAG G -3'
COMPLEMENT	5'- CCT TTC GTC TTT GGA TGT TAT GCA TCT G -3'
LENGTH	28
GC CONTENT	42.9 %
MELT TEMP	58 °C
MOLECULAR WEIGHT	8632.7 g/mole
EXTINCTION COEFFICIENT	292800 L/(mole·cm)
nmole/OD_{260}:	3.42
µg/OD_{260}:	29.48

Reverse modAOX

SEQUENCE	5'- CAG ATG CAT AAC ATC CAA AGA CGA AAG G -3'
COMPLEMENT	5'- CCT TTC GTC TTT GGA TGT TAT GCA TCT G -3'
LENGTH	28
GC CONTENT	42.9 %
MELT TEMP	58 °C
MOLECULAR WEIGHT	8632.7 g/mole
EXTINCTION COEFFICIENT	292800 L/(mole·cm)
nmole/OD_{260}:	3.42
µg/OD_{260}:	29.48

Forward α Factor HGh

SEQUENCE	5'- CAA CTA ATT ATT CGA AAC GAT GAG ATT TCC TTC AAT TTT TAC TGC TG -3'
COMPLEMENT	5'- CAG CAG TAA AAA TTG AAG GAA ATC TCA TCG TTT CGA ATA ATT AGT TG -3'
LENGTH	47
GC CONTENT	31.9 %
MELT TEMP	61.4 °C
MOLECULAR WEIGHT	14376.4 g/mole
EXTINCTION COEFFICIENT	449900 L/(mole·cm)
nmole/OD_{260}:	2.22
µg/OD_{260}:	31.95

Reverse HGh

SEQUENCE	5'- CAA TCT AGA CTA GAA GCC ACA GCT G -3'
COMPLEMENT	5'- CAG CTG TGG CTT CTA GTC TAG ATT G -3'
LENGTH	25
GC CONTENT	48 %
MELT TEMP	56.9 °C
MOLECULAR WEIGHT	7644 g/mole
EXTINCTION COEFFICIENT	247600 L/(mole·cm)
nmole/OD_{260}:	4.04
µg/OD_{260}:	30.87

Forward-P_{AOX1}

SEQUENCE	5'- CTC AGA TCT AAC ATC CAA AGA CGA AAG G -3'
COMPLEMENT	5'- CCT TTC GTC TTT GGA TGT TAG ATC TGA G -3'
LENGTH	28
GC CONTENT	42.9 %
MELT TEMP	57 °C
MOLECULAR WEIGHT	8583.7 g/mole
EXTINCTION COEFFICIENT	285800 L/(mole·cm)
nmole/OD_{260}:	3.5
µg/OD_{260}:	30.03

Reverse-eGFP

SEQUENCE	5'- CCG GTA CCT CAC TTG TAC AGC TCG TCC AT -3'
COMPLEMENT	5'- ATG GAC GAG CTG TAC AAG TGA GGT ACC GG -3'
LENGTH	29
GC CONTENT	55.2 %
MELT TEMP	64.3 °C
MOLECULAR WEIGHT	8764.7 g/mole
EXTINCTION COEFFICIENT	261400 L/(mole·cm)
nmole/OD_{260}:	3.83
µg/OD_{260}:	33.53

Forw mAOX1-AddAdr2

SEQUENCE	5'- GAC CCC ACA TTT TTT TTT TGA CCC CAC ATG TTC CCC AAA TGG CC -3'
COMPLEMENT	5'- GGC CAT TTG GGG AAC ATG TGG GGT CAA AAA AAA AAT GTG GGG TC -3'
LENGTH	44
GC CONTENT	47.7 %
MELT TEMP	68.2 °C
MOLECULAR WEIGHT	13288.6 g/mole
EXTINCTION COEFFICIENT	393400 L/(mole·cm)
nmole/OD_{260}:	2.54
µg/OD_{260}:	33.78

Rev mAOX1-AddAdr2

SEQUENCE	5'- TGG GGT CAA AAA AAA AAT GTG GGG TCG CCC TCA TCT GGA GTG ATG -3'
COMPLEMENT	5'- CAT CAC TCC AGA TGA GGG CGA CCC CAC ATT TTT TTT TTG ACC CCA -3'
LENGTH	45
GC CONTENT	48.9 %
MELT TEMP	68.8 °C
MOLECULAR WEIGHT	14014.1 g/mole
EXTINCTION COEFFICIENT	446700 L/(mole·cm)
nmole/OD_{260}:	2.24
µg/OD_{260}:	31.37

Forward P_{AOX1}

SEQUENCE	5'- CTC AGA TCT AAC ATC CAA AGA CGA AAG G -3'
COMPLEMENT	5'- CCT TTC GTC TTT GGA TGT TAG ATC TGA G -3'
LENGTH	28
GC CONTENT	42.9 %
MELT TEMP	57 °C
MOLECULAR WEIGHT	8583.7 g/mole
EXTINCTION COEFFICIENT	285800 L/(mole·cm)
nmole/OD_{260}:	3.5
µg/OD_{260}:	30.03

Reverse P*AOX1*

SEQUENCE	5'- CTG AGC ACT GCA CGC CGT AGG T -3'
COMPLEMENT	5'- ACC TAC GGC GTG CAG TGC TCA G -3'
LENGTH	22
GC CONTENT	63.6 %
MELT TEMP	63.9 °C
MOLECULAR WEIGHT	6736.4 g/mole
EXTINCTION COEFFICIENT	204200 L/(mole·cm)
nmole/OD_{260}:	4.9
µg/OD_{260}:	32.99

D. Buffers and Stock Solutions

1X TAE Agarose Gel Electrophoresis Buffer	40 mM Tris, 20 mM acetic acid, 1 mM EDTA.
1 M Tris-Cl (pH 8.0)	Dissolve 12.1 g Tris base 80 mL dH2O and adjust the pH to 8.0 by adding concentrated HCl. Make up the volume up to 100 mL. Autoclave and store at room temperature.
0.5 M Ethylendiaminetetra acetic acit EDTA (pH:8.0)	Dissolve 18.61 g EDTA in 80 ml distilled water. Adjust pH to 8.0 and bring final volume up to 100 mL. Autoclave and store at room temperature.
TE Buffer	Mix 1 mL 1M Tris-HCl (pH 8.0) and 0.5 mL of 0.5M EDTA, and complete to 100mL with dH_2O.

Calcium chloride Transformation Solutions

$MgCl_2$-$CaCl_2$ Solution	Dissolve 8.13 g $MgCl_2.6H_2O$ with 1.1099 g $CaCl_2$ in 500 mL dH_2O. Filter-sterilize and store at +4°C.
0.1 M $CaCl_2$ Solution	Dissolve 11.1 g $CaCl_2$ in 1 L dH_2O. Filter-sterilize and store at +4°C.

Lithium Chloride Transfection Solutions

1M LiCl	Dissolve 4.24 g of LiCl in distilled water and filter sterilize. Dilute with sterile water when needed.
PEG	Dissolve 50 % polyethylene glycol (PEG-3350) in distilled water and filter sterilize. Store in tightly capped bottle.
Single-stranded DNA	2 mg/mL denaturated, fragmented salmon sperm DNA in TE (pH 8.0) buffer, store at -20°C.

SDS-PAGE Solutions

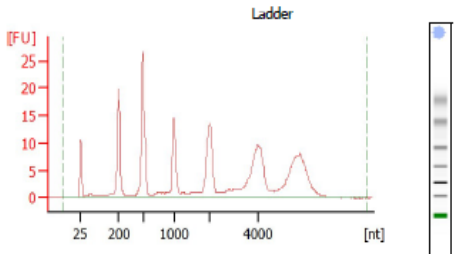
10%(w/v) APS (Ammonium PerSulfate)	Add 0.1g APS to 1 mL dH_2O, freshly prepared.
4X Sample Loading Buffer for SDS-PAGE	200 mM Tris-HCl, pH 6.8; 40% glycerol; 6% SDS; 0.013% Bromophenol blue. Distributed into microcentrifuge tubes and stored at -20°C.
5X SDS-PAGE Running Buffer	15 g Tris Base, 72 g glycine, 5 g SDS, dH_2O to 1 liter. The buffer can be stored at 2-8°C.
1X SDS-PAGE Running Buffer	Diluted from 5X buffer solution prior to use and can be used three times.
Fixer Solution	Mix 100 mL methanol and 24 mL acetic acid with100 µL 37% formaldehyde and complete to 250 mL with distilled water.
Pretreatment Solution	Dissolve 0.05 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$) in 200 mL distilled water by mixing with a glass rod. Take 2 mL and set aside for further use in developing solution preparation.
Silver Nitrate Solution	Dissolve 0.2 g silver nitrate in 100 mL distilled water and add 75 µL 37% formaldehyde.

Developing Solution	Dissolve 2.25 g potassium carbonate in 100 mL distilled water. Add 2 mL from pretreatment solution and 75 µL 37% formaldehyde.
Stop Solution	Mix 50 mL methanol with 12 mL acetic acid and complete to 100 mL with distilled water.

Genomic DNA Isolation Solutions

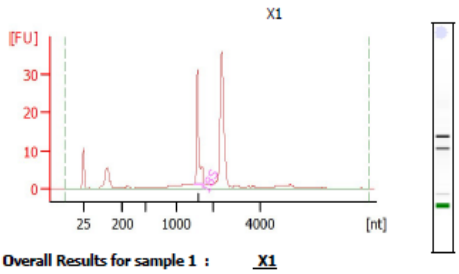
Yeast Lysis Buffer for Genomic DNA Isolation	Dissolve 2 g of Triton-X 100, 1 g of SDS, 5.84 g of NaCl, 0.1 moles of Tris-Cl (pH=8.0) and 0.338 g of Na2EDTA in 1 L of dH_2O.
3 M Sodium Acetate	Add 24.6 g sodium acetate anhydrous (CH_3COONa) to 100 mL dH_2O store at room temperature.

E. Nanodrop Results of Total RNA Isolation



Overall Results for Ladder

RNA Area:	275.8
RNA Concentration:	150 ng/µl
Result Flagging Color:	
Result Flagging Label:	All Other Samples

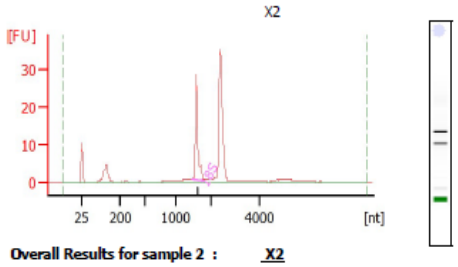


Overall Results for sample 1 : X1

RNA Area:	151.1
RNA Concentration:	82 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	6.6 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.60

Fragment table for sample 1 : X1

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,477	1,769	30.6	20.3

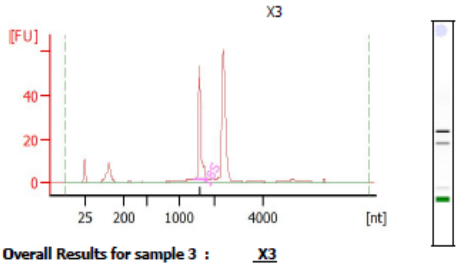


Overall Results for sample 2 : X2

RNA Area:	134.2
RNA Concentration:	73 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	6.9 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.90

Fragment table for sample 2 : X2

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,464	1,780	27.6	20.5

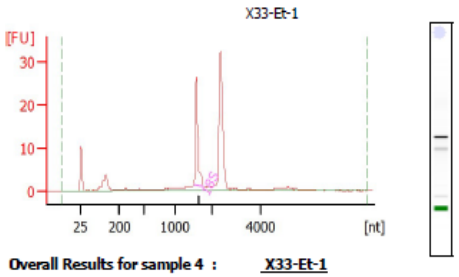


Overall Results for sample 3 : X3

RNA Area:	233.6
RNA Concentration:	127 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	6.8 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.80

Fragment table for sample 3 : X3

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,408	1,782	53.0	22.7

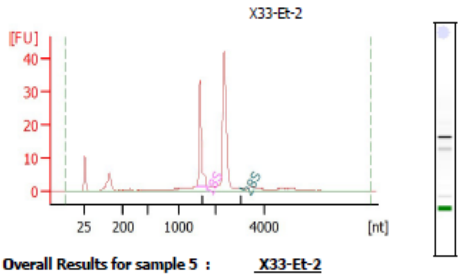


Overall Results for sample 4 : **X33-Et-1**

RNA Area:	129.9
RNA Concentration:	71 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	6.8 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.80

Fragment table for sample 4 : **X33-Et-1**

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,516	1,764	24.3	18.7

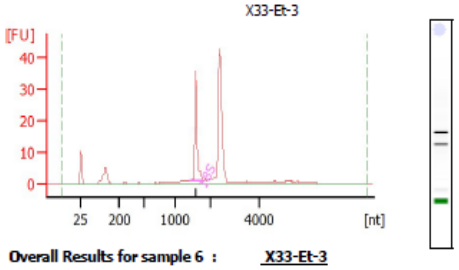


Overall Results for sample 5 : **X33-Et-2**

RNA Area:	166.3
RNA Concentration:	90 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	6.8 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.80

Fragment table for sample 5 : **X33-Et-2**

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,465	1,789	31.7	19.1
28S	2,968	3,132	0.1	0.1

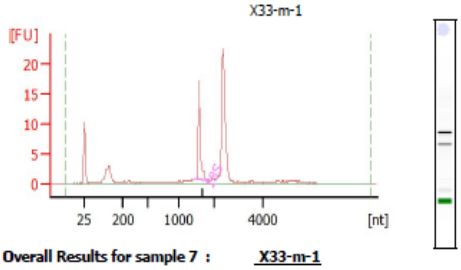


Overall Results for sample 6 : **X33-Et-3**

RNA Area:	160.5
RNA Concentration:	87 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	6.8 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.80

Fragment table for sample 6 : **X33-Et-3**

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,404	1,780	32.6	20.3

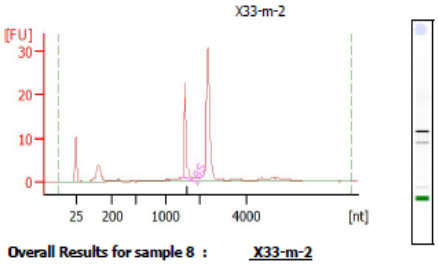


Overall Results for sample 7 : **X33-m-1**

RNA Area:	86.5
RNA Concentration:	47 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	7 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN:7

Fragment table for sample 7 : **X33-m-1**

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,464	1,857	15.5	17.9

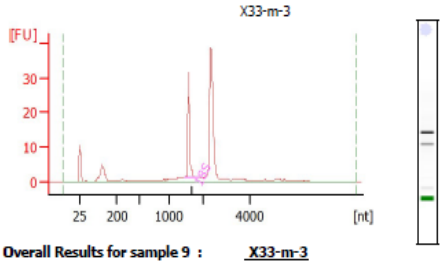


Overall Results for sample 8 : X33-m-2

RNA Area:	116.3
RNA Concentration:	63 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	7 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN:7

Fragment table for sample 8 : X33-m-2

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,493	1,759	19.9	17.1

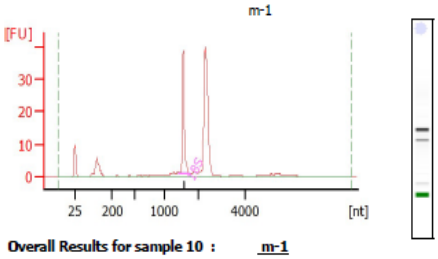


Overall Results for sample 9 : X33-m-3

RNA Area:	156.4
RNA Concentration:	85 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	6.9 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.90

Fragment table for sample 9 : X33-m-3

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,429	1,843	28.5	18.2

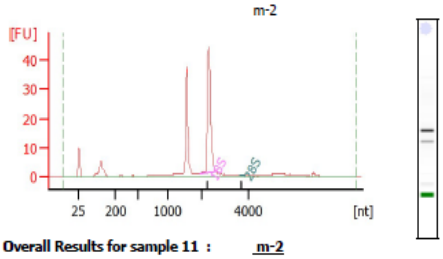


Overall Results for sample 10 : m-1

RNA Area:	166.1
RNA Concentration:	90 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	6.6 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.60

Fragment table for sample 10 : m-1

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,435	1,730	36.0	21.7

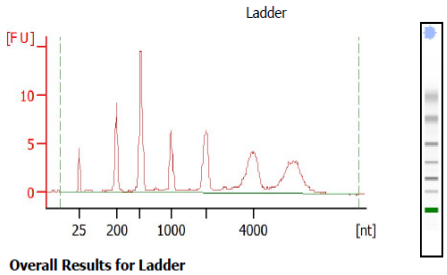


Overall Results for sample 11 : m-2

RNA Area:	169.3
RNA Concentration:	92 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	6.8 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.80

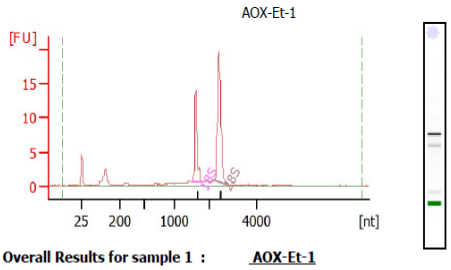
Fragment table for sample 11 : m-2

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,962	2,591	64.0	37.8
28S	3,652	3,820	0.1	0.0



Overall Results for Ladder

RNA Area:	119.4
RNA Concentration:	150 ng/µl
Result Flagging Color:	
Result Flagging Label:	All Other Samples

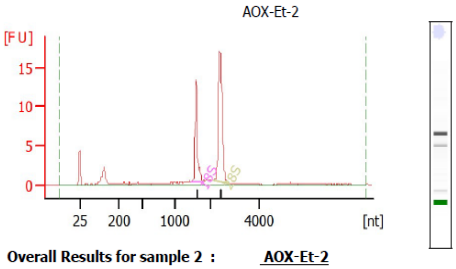


Overall Results for sample 1 : AOX-Et-1

RNA Area:	71.8
RNA Concentration:	90 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	6.8 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.80

Fragment table for sample 1 : AOX-Et-1

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,482	1,804	13.9	19.3
28S	2,184	2,745	28.2	39.2

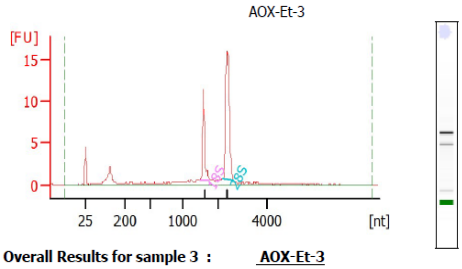


Overall Results for sample 2 : AOX-Et-2

RNA Area:	65.1
RNA Concentration:	82 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	6.7 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.70

Fragment table for sample 2 : AOX-Et-2

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,467	1,772	12.9	19.8
28S	2,135	2,740	25.6	39.4



Overall Results for sample 3 : AOX-Et-3

RNA Area:	65.6
RNA Concentration:	82 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	6.7 (B.02.10, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.70

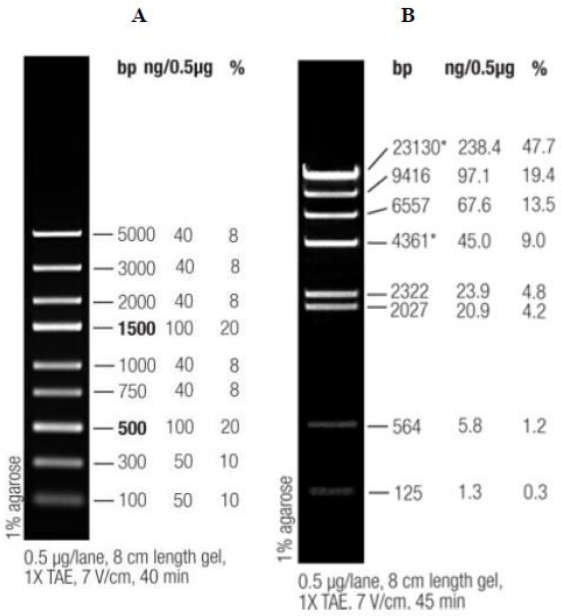
Fragment table for sample 3 : AOX-Et-3

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,500	1,772	10.3	15.7
28S	2,154	2,619	22.2	33.9

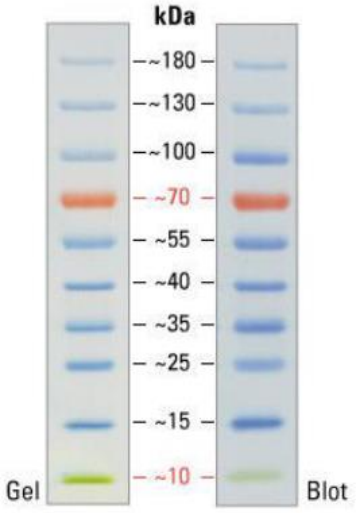
Nanodrop results for Total RNA isolation with Qiagen Kit

Sample ID	User name	Date and Time	Nucleic Acid	Unit	260/280	260/230	Sample Type
35-19_X1	nanodrop	04/06/2019 14:17:35	112,9	ng/µl	2,04	1,01	RNA
35-19_X2	nanodrop	04/06/2019 14:21:17	102,9	ng/µl	2,12	0,87	RNA
35-19_X3	nanodrop	04/06/2019 14:22:18	170,8	ng/µl	2,09	1,33	RNA
35-19_X33-Et_1	nanodrop	04/06/2019 14:23:26	86,5	ng/µl	2,06	0,79	RNA
35-19_X33-Et_2	nanodrop	04/06/2019 14:24:00	117,6	ng/µl	2,08	1,55	RNA
35-19_X33-Et_3	nanodrop	04/06/2019 14:24:43	115,2	ng/µl	2,07	1,03	RNA
35-19_X33-m_1	nanodrop	04/06/2019 14:25:53	83,8	ng/µl	2,07	1,02	RNA
35-19_X33-m_2	nanodrop	04/06/2019 14:26:24	82,4	ng/µl	2,12	0,93	RNA
35-19_X33-m_3	nanodrop	04/06/2019 14:26:53	103,8	ng/µl	2,11	1,55	RNA
35-19_m_1	nanodrop	04/06/2019 14:28:11	125,2	ng/µl	2,04	0,49	RNA
35-19_m_2	nanodrop	04/06/2019 14:28:40	128,6	ng/µl	2,07	1,31	RNA
35-19_m_3	nanodrop	04/06/2019 14:29:19	160,6	ng/µl	2,12	1,59	RNA
35-19_AOX-Et_1	nanodrop	04/06/2019 14:29:58	96	ng/µl	2,1	1,29	RNA
35-19_AOX-Et_2	nanodrop	04/06/2019 14:30:29	75,9	ng/µl	2,11	0,76	RNA
35-19_AOX-Et_3	nanodrop	04/06/2019 14:31:00	71,8	ng/µl	2,09	1,55	RNA
35-19_AOX-m_1	nanodrop	04/06/2019 14:31:37	85,2	ng/µl	2,12	1,06	RNA
35-19_AOX-m_2	nanodrop	04/06/2019 14:32:09	81,7	ng/µl	2,11	1,22	RNA
35-19_AOX-m_3	nanodrop	04/06/2019 14:32:48	87,1	ng/µl	2,22	1,3	RNA
35-19_ssh-E_1	nanodrop	04/06/2019 14:33:41	88,9	ng/µl	2,08	1,51	RNA
35-19_ssh-E_2	nanodrop	04/06/2019 14:34:13	142,6	ng/µl	2,08	1,16	RNA
35-19_ssh-E_3	nanodrop	04/06/2019 14:34:44	131,5	ng/µl	2,07	1,13	RNA
35-19_ssh-AOX-E_1	nanodrop	04/06/2019 14:35:45	113,8	ng/µl	2,08	1,24	RNA
35-19_ssh-AOX-E_2	nanodrop	04/06/2019 14:36:15	142,4	ng/µl	2,1	0,39	RNA
35-19_ssh-AOX-E_3	nanodrop	04/06/2019 14:36:56	119,5	ng/µl	2,14	1,01	RNA

F. Molecular Weight Markers



Molecular weight markers utilized during agarose gel electrophoresis. A: GeneRuler™ Express DNA Ladder, ready-to-use (Fermentas), B: Lambda DNA/HindIII Marker (Fermentas).



SDS-PAGE band profile of the PageRuler™ Prestained Protein Ladder (Fermentas).