Development of Eukaryotic (yeast) Expression System for Promoters Characterization and Recombinant Proteins Production

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2023

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A thesis submitted as a final year project as a requirement of MS In Industrial Biotechnology

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(NUST 2019ASAB)

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2023

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DECLARATION

I certify that this research work titled **"Development of Eukaryotic (yeast) Expression system for Promoters Characterization and Recombinant Proteins Production"** is my work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged/referred.

Saba Jahan

Saba Jahan

Master of Science in Industrial Biotechnology

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DEDICATION

Dedicated to,

My Loved ones and Friends

For all your support and care

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ACKNOWLEDGMENTS

All glory and praises be to Allah for the courage, patience and for his blessings, without his blessings, my efforts could not achieve anything.

I would like to extend thanks to my supervisor Dr. Hussnain Ahmed Janjua and my Co-supervisor Dr. Muhammad Saad Ahmed for allowing me to research. I express my deep gratitude for helping me at all stages of my project and thesis work.

Besides my supervisors, I would like to thank the rest of my examination committee, Dr. Fazal Adnan, Dr. Amjad Ali, and my external Dr. Wasim Sajjad from NUMS for guiding me throughout. my project, boosting my morale and giving valuable insights as a GEC member.

Special thanks to my seniors in Lab for valuable guidance and skills, and never-ending support and encouragement.

I would like to express my gratitude to Dr. Hazrat Ali for allowing me to complete my work at NIBGE.

My deepest thanks to my family and friends for their love and support throughout my life. Thank you for investing in me, spiritually as well as morally.

In the end, I am very thankful to the management and staff of Atta ur Rahman School of Applied Biosciences (ASAB), principal, Dr. Pro. Muhammad Asghar, Head of the department Dr. Amjad Ali, lab fellows and lab attendant Ms. Fozia of Virology-I lab for facilitating me with experimental work to complete my degree.

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ABBREVIATION

S. cerevisiae	*Saccharomyces cerevisiae*
TFs	Transcription factors
DNA	Deoxyribonucleic acid
TSS	Transcription start site
TBS	Transcription binding site
EGFP	Enhanced green fluorescent protein
PCR	Polymerase chain reaction
RT PCR	Reverse transcription PCR
E. coli	Escherichia coli
mRNA	Messenger RNA
DPE	Downstream promoter element
UAS	Upstream activating sequence.
URS	Upstream repressive sequences
TAs	Transcriptional activators
TRs	Transcriptional repressors
RBS	Ribosome-binding site
Ep-PCR	Error-prone PCR
EGFP	Enhanced Green fluorescent Protein

ABSTRACT

Promoter is a Crucial genetic element that plays major role in the expression of gens expression. It is a part of DNA located upstream of gene where relative enzymes bind to start transcription. Its structure, stability, and affinity with RNA polymerase influence the gene expression. The main focus of this research is UBC9_1 promoter of Saccharomyces *cerevisiae,* a unicellular eukaryotic model organism due to its known genetics and completely characterized physiology. Its promoters are widely characterized due to biotechnological usefulness because of fermentation capacity and production of bioethanol and other useful compounds. This study aims to check the expression of the EGFP reporter gene under the UBC9_1 promoter via yeast expression vector pCEV-G4-km to access its rate of expression with the control CYC1 promoter. Expression cassette designed, promoter and gene construct were synthesized and ligated into expression vector before CYC1 terminator, then transformed into *E. coli* BL21 to check the expression of the EGFP gene. Successful ligation of the construct into the vector and transformation is confirmed by the restriction digestion and colony PCR. After confirmation of transformation samples were sent for flow cytometry. Comparison of Side Scatted lin and Forward Scattered lin on Scatter plot of flow cytometry shows the greater expression of EGFP under UBC9_1 promoter as compared to control CYC1. Furthermore, analysis of the Florescence channel count shows greater Activity of EGFP expression under the UBC9_1 promoter. The geometric mean values of the UBC9_1 promoter are compared with the Control CYC1 promoter which also indicates a higher rate of expression of the UBC9_1 promoter.

1. INTRODUCTION

Yeasts are single-celled fungi with completely different properties from bacteria which are prokaryotes. 1t has cellular organization closely like higher organisms, hence are eukaryotes. The yeast species that has been mostly utilized by mankind is *Saccharomyces Cerevisiae* (Partow et al., 2010). This organism undergoes fermentation and useful yeasts used in baking food and textile industries. *S. cerevisiae* is single cell eukaryotic fungus, also known as bakers or brewer yeast. It is naturally present in the environment, mostly on the skin of grapes and other fruits but it can also be found in other habitats, including oak trees, desert soil, and flowers. (Sniegowski et al.,2010)

Reproduction in *S. cerevisiae* occurs by both methods. It uses asexual method to reproduce through budding also known as "budding yeast". Mostly all the yeast cells contain buds. Cell growth results in the growth of buds until they become mature. After this, they get separated from the paternal cell. On the other hand, *S. cerevisiae* sometimes undergoes sexual reproduction. Yeast cells consist of two mating types of cells. The mating of two different haploid yeast cells results in diploid cells which is important because it causes genetic differences in the population. Sometimes in stressful conditions, meiosis occurs in *S. cerevisiae*, and haploid spores are formed and get active environmental conditions. *S. cerevisiae* cells are mostly 5–10 μm in diameter. (Yeasts - an Overview | ScienceDirect Topics.,2003)

S. cerevisiae can grow in the presence or absence of oxygen and has a doubling time of approximately 90 min. It utilizes glucose according to the growth medium. If it grows in the presence of oxygen, it utilizes galactose and fructose. All yeast strains depend on nitrogen and phosphorus for growth and use ammonia and urea for nitrogen (Nandy & Srivastava, 2018). Dihydrogen phosphate is utilized to get phosphorus. They also require sulfur and metals such as magnesium for their normal growth. *S. cerevisiae* yeast cells are used as an efficient carrier because of their cheap characteristics. The

strength of the yeast's cell wall because of the beta-glucan and chitin ’ allows them to intake various active materials. (Salari & Salari, 2017)

All biological functions present in higher eukaryotic cells are also conserved in *S. Cerevisiae*, making it a model organism for research on eukaryotes in genetics and molecular biology. It is easy to culture in a lab and can also be genetically altered. It has a completely sequenced genome organized in 16 chromosomes containing nuclear genomic DNA of 12068 kilobases. Around 6000 genes are present, of which, most of them are protein-coding genes. (Parapouli et al., 2020)

Unlike *Escherichia coli* which is also a model organism, *S. Cerevisiae* due to its known genetic and physiological, is more industrially relevant for multiple industrial applications. *S. cerevisiae* has important characteristics, for industrial applications including its ability to produce several aromatics, volatile compounds, and resistance to high sugar quantity.

It has a variety of industrial applications in food, beverage, and biofuel production. Biotechnology is making great efforts to develop new methods in fermentation and production of ethanol, and many other biofuels. Therefore, *S. cerevisiae* cell factories have been used greatly biotech industry to produce many products such as ethanol, enzymes, and proteins for therapeutic purposes. (Morata & Loira, 2017)

The biotech industry requires a broad array of bioprocessing technologies, and modern genetic engineering tools, to improve the *S. cerevisiae* strain to enhance the manufacturing of useful industrial metabolites. Control over gene expression is crucial to increase the production of valuable products. Genes have a crucial role in the formation of these industrial compounds. however, expression of these genes can be controlled and regulated through the transcriptional regulatory elements.

The control regulation of gene expression is crucial to enhance the production of the desired products. Gene expression can be defined conversion of a gene into mRNA and then into protein. Gene

expression of downstream target genes is mainly controlled at the transcriptional level, largely because of the binding of regulatory proteins (TFs) to specific regions on DNA named Promotors. (Juven-Gershon & Kadonaga, 2010)

In eukaryotes DNA sequence present upstream of a gene is promoter. It contains a specific region for an enzyme, a start site of transcription, (TSS) where transcription begins and a location for many cis-regulatory elements. Eukaryotic promoters as compared to prokaryotes are extremely diverse and difficult to characterize(Tang et al., 2020).

The promoter region Contains three portions: core, proximal, and distal (Gao et al., 2020). The region located most proximal to the start codon is the core or main promoter and consists of the TATA box, a binding site for relevant enzymes, and a start site for transcription (TSS). Transcription of the coding strand begins after the RNA polymerase enzyme binds to the core part of the promoter. The proximal part is present above the core or main promoter. At this region general transcription factor binds also contain many primary regulatory elements. The distal is the final part located upstream of the proximal region and also has transcription binding site TBS and regulatory elements.

As the protein transcription level depends on promoters, it is important to identify the promoter strength based on their rate of gene expression under different conditions.

Quantitative characterization of promoters by the level of gene expression is a fundamental yet complex challenge. Characterizing biological parts has been done extensively in *Escherichia coli*. For example, hundreds of parts have been constructed and profiled as Bio Bricks(Xu et al., 2019). Protein expression regulation by the promoter is crucial to enhance the production of useful and valuable products.

1.1 Aims and Objective of Study

Promoter Characteristic is defined as a measurement of the transcriptional activity of promoter sequence under a gene. In this study, the UBC9_1 promoter from the *S. cerevisiae* genome was selected to analyze its expression The promoters, and reporter gene were synthesized, and a terminator from the pCEV-G4-km expression vector was used. The UBC9_1 promoter and EGFP reporter gene were assembled into the expression vector before the terminator. The sequence of the promoter was taken from the *S. Cerevisiae* database (http://rulai.cshl.edu/cgi-bin/SCPD/getgenelist,) and the EGFP gene from Snap Gene. The promoter and reporter gene construct were purified from gel and then transformed into plasmid PCEV-G4-Km using restriction digestion enzymes. After the construction of the expression vector, the constructed plasmid is transferred into *E. coli.* EGFP expression in *E. coli* under the UBC9_1 promoter was analyzed by flow cytometer.

2. LITERATURE REVIEW

2.1. Synthetic Biology

The designing of naturally occurring biological systems or the construction and joining of new biological parts for beneficial purposes is the major concern of synthetic biology. Synthetic biology is the combination of biological and engineering concepts. Synthetic biology is ‘‘the fusion of technology, engineering, and science to assist and accelerate the design, construction, or alteration of DNA information in living cells’’(Jin et al., 2019).The capability to construct or reconstruct biological parts and systems made hopeful to the proponent of synthetic biology as it can address great challenges of energy, food, environment, and medical-related problems and transform petrochemical technologies, impelling industrial revolution as viable bio-engineered processes (Shapira et al., 2017).

Production of different metabolites, chemicals, and fuels came true through synthetic (Xiong et al., 2018). The control of gene expression at various levels of gene regulation has enhanced the chance to effectively construct and regulate biological synthetic systems to achieve desired goals. Synthetic biology developed engineered bacteria that are capable of producing biofuel (PP et al., 2012). can sense heavy metals and construct immune cells that have the potential to identify and destroy cancer cells. All these efforts in synthetic biology including many others can have a great impact on the development of society.

2.2. Central dogma

The central dogma was first explained by Francis Crick. Genetic information stored in DNA is first replicated through a process replication, then transcribed into messenger RNA (mRNA) by transcription, and translation mRNA for the production of a particular amino acid. It predicts that a

particular sequence of amino acids cannot alter a sequence of nucleotides (mRNA or DNA). Alternatively, genetic information is in the form of nucleic acid converted into proteins (CRICK et al.,1970). In some cases, information encoded in mRNA is reverse transcribed into a new DNA process known as reverse transcription was predicted by Temin (HM, 1964)

2.3. Gene expression

In gene expression, the DNA instructions are translated into functional proteins. It has two key steps, transcription, and translation (Shabbir Hussain et al., 2016). During transcription, the coded information in the DNA is converted into small, transportable RNA messages. In translation these mRNA cassettes transport from the nucleus to the ribosomes where they translate to make specific proteins. It is a complex process that provides control points to the cell for regulating, the amount type, and production of protein (Alberts et al., 2002). Gene expression happens in slightly different ways in both prokaryotic and eukaryotic cells.

2.4. Gene expression in prokaryotes

Prokaryotes lack a defined nucleus, so their gene expression occurs within the cytoplasm and is regulated at the transcriptional level. Transcription process starts with the attachment of RNA polymerase enzyme to sequence located upstream of gene called promoter. Conversion of DNA into RNA regulated by promoter and regulatory protein Therefore, prokaryotic cells use a single type of RNA polymerase to undergo the transcription of all genes. RNA polymerase is a multi-subunit complex consisting of a core enzyme and regulatory subunit sigma factors, whereas the core enzyme undertakes the elongation of RNA (B. S & E et al., 2003).

Sigma factors have an important role in to start of RNA transcription. An extra level of control could be achieved by using different sigma factors. (MM et al.,1998)

TF is a protein required to regulate the transcription of a gene but is not part of RNA polymerase. When it binds to a specific sequence of DNA, the transcription process can start or stop.

In prokaryotes, proteins required for the same biochemical pathways or needed for specific functions are coded together called operons. Three types of regulatory elements regulate the operon's expression, including activator repressor, and inducer. The repressor and activator both proteins are produced by the cell. Activators increase the transcription of genes in response to external stimuli whereas repressors prevent the transcription according to the environmental signal. Inducers either can activate or stop transcription of the gene depending on the cell (Jacob & Monod, 1961)

2.5. Gene expression in eukaryotes

Gene expression is complex in eukaryotes as compared to prokaryotes. The structural difference in eukaryotic and prokaryotic genomes is the packaging of DNA into chromatin imparts further levels of complexity is the regulation of gene expression. Due to the lack of a defined nucleus, both processes happen in cytoplasm while in eukaryotes transcription process happens within the nucleus and newly synthesized RNA is then moved from cytoplasm to the nucleus where translation of the RNA into protein occurs by ribosomes. Multiple cis- and trans-elements are present in eukaryotes as corresponding regulatory machinery. The cis-regulatory elements are noncoding DNA present in all genomes (Narlikar & Ovcharenko, 2009)

Although gene expression is complex in eukaryotes some basic principles are the same as in prokaryotes The gene expression is controlled or regulated at the transcriptional level, but in eukaryotes, regulation occurs until the final step which is the development of active protein.

The transcription process in eukaryotes is regulated by proteins (transcription factors) that attach to specific sequences and regulate RNA polymerase activity. Generally, operons are absent in most of the eukaryotes instead every gene has Its promoter sequence and enhancer elements. The gene expression in prokaryotes does not have any restriction because RNA polymerase can bind to any promoter sequence without the presence of an activator or repressor. In eukaryotes “ground state” of DNA expression is different and restrictive because promoters in eukaryotes remain inactive without transcription factors. For example, RNA polymerase ll is unable to bind with the promoter if the absence in of transcription factors are not present (Struhl et al., 1984)

2.6. Promoter

A promoter is special part of DNA where the expression of a gene through the transcription process of a gene is started. Gene expression is regulated by the promoter. RNA polymerase enzyme binds on the promoter to initiate transcription. Promoter region present at the start of the gene or the 5' end of the TSS. A site is present for RNA polymerase enzyme to make a messenger RNA. Promoters are an important part of expression vectors as they regulate RNA polymerase binding and the regulatory proteins that start the transcription of DNA into mRNA which is translated into protein (V & B, 2016)

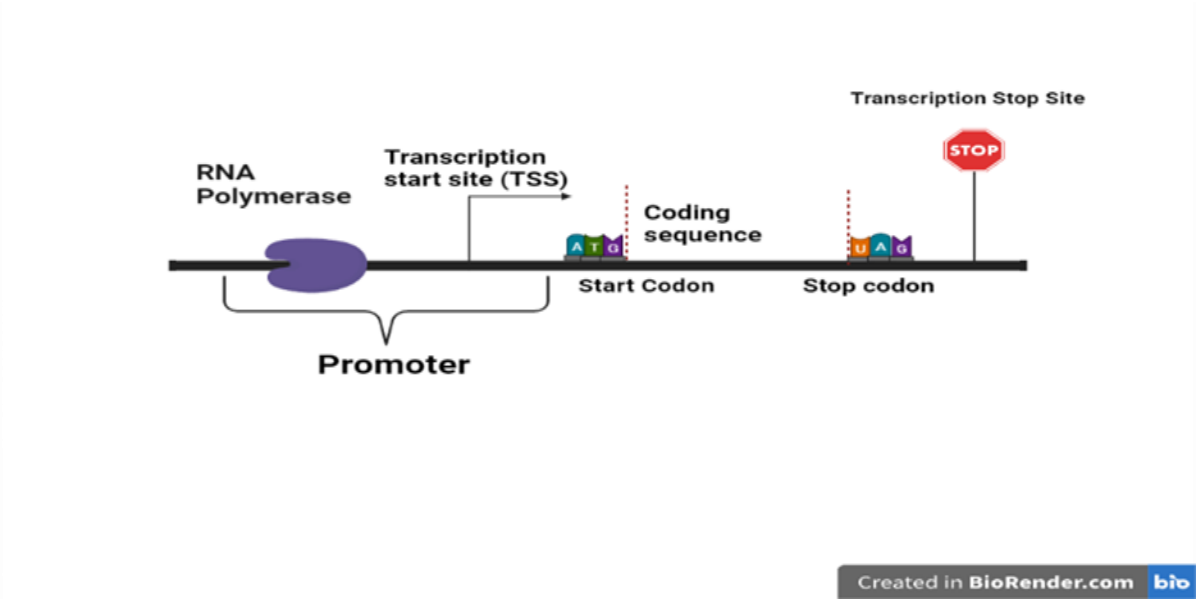

Figure 1 Basic Structure of a Promoter (Adapted from Tang et al., 2020)

2.7. Prokaryotic promoters

Promoters vary among the prokaryotes, but few sequences are conserved, that are present in all the promoters of the prokaryotes. Prokaryotic promoters comprise some specific elements present at multiple positions near the TSS. Most commonly there are two hexametric motifs or promoter consensus sequences near −10 and −35 positions relative upstream to TSS.

1: The -10-consensus, nucleotides known as the Pribnow box it has six consensus nucleotides TATAAT. It is crucial to initiate the transcription process in all prokaryotes.

2: The other consensus nucleotide sequence located at -35 usually has a sequence of six nucleotides TTGACA. Its presence also enhance the transcription rate.

When a promoter contains both consensus sequence TATAAT and TTGACA, it is usually a very efficient, constitutive promoter (Harley & Reynolds, 1987)

2.8. Eukaryotic promoter regions

The promoter region Contains three portions: core promoter, proximal promoter, and distal promoter. The region located most proximal to the start codon is the core or main promoter and consists of the TATA box, binding site for enzyme, and TSS. After RNA polymerase binds stably to core promoter transcription of the coding strand can start. The proximal region of promoter is located further upstream of the core promoter approximately 250 base pairs upstream from the TSS. At this region general transcription factor binds also contain many primary regulatory elements. The distal region of promoter is the last part located next to the Proximal promoter and also has transcription binding site TBS and regulatory elements.

2.9. Eukaryotic promoter elements

The eukaryotic promoters are difficult to characterize as they are extremely diverse. They generally have a more complex structure as compared to prokaryotes and they have multiple different consensus sequences, such as TATA, CCAAT, INR box, BRE, and GC. These sequence elements were identified by analysis of many promoters, and they show these consensus sequences. (B. P, 1990)

Core promoter consensus sequences have almost fixed locations near the TSS. For example, the TATA sequence is present at -30 bp above the TSS. TATA box is present in yeast and humans, but it is present only in some of the core promoters. The TATA sequence is recognized by the specific protein that binds to TATA sequence (Patikoglou et al., 1999).

2.10. Yeast Promoter

In yeast RNA polymerase ll-dependent promoters consist of three elements: the TATA box, the TSS, UAS, and URS (G et al., 2014). The TATA box is located above the TSS; its position is 40 to 120 bp upstream in different types of yeast and 25 to 30 bp upstream in eukaryotes. Many promoters in *S. cerevisiae* are TATA-less box and RNA polymerase II bound and recognized to them via other elements (H. S & ET, 2011).

In the upstream of core promoter and activator or repressor sequences (UAS/URS), there are specific transcription repressors or activators that recognize cis-acting elements. Gene expression starts by recruitment of the transcriptional machinery by Transcriptional activators. On the other hand, chromatin structure is maintained in a repressive state by transcriptional repressors by bounding to the cis-regulatory elements to stop transcription. UAS or URS are present approximately 100–1400bp upstream of the main or core promoter (JE et al., 1986).

2.11. Types of Promoters

Characterization of many promoters of yeast has been performed based on expression strength under different conditions. These promoters are useful tools and have many applications in metabolite engineering. In yeast, promoters are characterized based on strengths and classified into two types: inducible and constitutive promoters

2.12. Constitutive Promoter

Constitutive promoters usually show stable gene expression levels under different growth conditions. These promoters provide relatively stable transcription by the influence from the r or extracellular or intracellular for example carbon source or environmental factors. Research has been done to

perform the characterization of native promoters according to their rate of expression or strength in response to different factors to identify strong promotors (da Silva & Srikrishnan, 2012). In terms of relative activity several yeast promoters are well characterized.

A few examples of constitutive promoters, including PTDH3, PTEF1, PTPI1, and PPGK1 shows different strength in many studies due to different experimental conditions (P. S et al., 2010).

2.13. Inducible Promoter

Inducible promoters require certain stimuli to start transcription. Through inducible promoters genes express or repress in response to specific stimuli. The cis-regulatory elements have a great role in the function of promoters through transcriptional activator(s) and transcriptional repressor(s). The interaction of the regulatory proteins with the promoter and conformational changes depend on specific stimuli. In yeast, these kinds of stimuli or inducers, include carbon sources, such as galactose, sucrose, glucose, and ethanol acetate. these stimuli also include environmental factors, pH, temperature, and light. Amino acids, metabolites, hormones, and metal ions also act as stimuli. (K. S et al., 2015) Inducible promoters considered to be strong promoters include PGAL1, PGAL2, PGAL7, and PGAL10. All these promoters can be used to enhance the protein expression and production of valuable products (A et al., 2017).

2.14. Promoter Engineering Approaches

The precise control over the expression of genes demands multiple promoters with different levels of strength. Several methods have been used for the engineering of promoters, such as promoter replacement for the construction of endogenous promoters with required transcriptional strength, and library of promoters with different transcriptional strengths. (Blazeck & Alper, 2013). Multiple other methods, such as random mutagenesis, hybrid-promoter engineering, and saturated

mutagenesis, have been used for the engineering of special promoters. In recent years, promoter engineering methods have largely been employed in many strains that have great industrial importance, such as *E. coli,* and *S. Cerevisiae*.

2.15. Site-directed mutagenesis

The efficiency of a promoter is not guaranteed by the statistical consensus sequence, because the promoter is for physiological requirements instead of getting the highest level of transcription. The consensus regions have less highly conserved nucleotides around them which has an impact on the activity of promoters, so they could be utilized for the rapid construction of better promoters with different levels of strength i.e. strong or weak. For example, site mutation of the −10 element of C. glutamic dapA promoter from AGGTAACCT to TGGTATAAT resulted in improved activity of promoter. (V. P et al., 1999)

Although this method synthesizes specific strong promoters, it is not possible to rapidly synthesize large-scale promoter libraries with desired transcriptional strength by using this method.

2.16. Site-selective mutagenesis by error-prone PCR

The development of many promoter libraries and the identification of special sequences in promoters by this technique of random mutation have been applied through Site-selective mutagenesis of promoters. Error-prone PCR is a modern technique especially for site-selective mutagenesis, to get diverse DNA sequences and to build promoter libraries, which introduce random mutations into fragments (McCullum et al., 2010).

For example, In the library of *S. cerevisiae,* promoter TEF was made by error-prone -PCR method showed an activity between 8 and 120% of the native promoter (Nevoigt et al., 2006).

2.17. Hybrid Promoters

promoters are made by joining the DNA sequences that have an important role in transcription such as part of constitutive and inducible promoters. Hybrid promoter engineering can be made by joining upstream elements and core promoter regions belonging to different promoters (Portela et al., 2016).

Transcription efficiency can be improved and hybrid promoter libraries have been developed for the regulation of transcription in *S. Cerevisiae*. Hybrid promoters can be built by mixing parts of different promoters together, for example, core promoter elements of one promoter and UAS of another promoter can develop a new better promoter for gene expression (Blazeck et al., 2011). The tac promoter of the *E. coli* strain is built by joining the −10 region of the lacUV5 and − 35 region of the trp promoter (Boer et al., 1983). Therefore, hybrid promoter engineering is an efficient strategy to obtain stronger and better promoters for gene expression compared to native promoters.

3. MATERIALS AND METHODS

3.1. Materials

This part of the materials consists of all the online tools, software, equipment,chemicals, media enzymes, buffers oligonucleotides, plasmids and bacterial strains, used during the research work.

3.1.1. Online tools and software used

Eukaryotic Promoter Database:

The promoter sequence was retrieved from the Eukaryotic Promoter Database and through the search motif tool of EPD core promoter elements was checked.

DNAsmac

The sequence of promoters was also analyzed through DNAsmac.

NEBcutter V2.0

Restriction sites on plasmids and inserts were checked by NEBcutter V2.0

SnapGene

Snap gene was used to propose and foresee DNA cloning.

Primer3 Input (version 0.4.0

All primers were designed by Primer 3

Tm Calculator (Thermo Fisher scientific)

The melting and annealing temperature of primers was figured out by Tm Calculator (Thermo Fisher scientific)

Table 1 List of all online tools and software used in this study

Name	**Function**	**Link**
Eukaryotic Promoter Database	Sequence Retrieval, Search Motif Tool	https://epd.epfl.ch//index.php
DNAsmac	Sequence Analysis and modifications	
NEBcutter V2.0	Finds restriction sites in your plasmids/DNA sequences	http://nc2.neb.com/NEBcutter2/
SnapGene	Fast and easy to propose, foresee DNA cloning	https://www.snapgene.com/

Primer3 Input (version 0.4.0)	Designing of PCR primers	http://bioinfo.ut.ee/primer3-0.4.0/
Tm Calculator (Thermo Fisher scientific)	It allows us to figure out the melting and annealing temperature of primers	https://www.thermofisher.com/pk/en/home/brands/thermo-scientific/molecular-biology/molecularbiology-learning-center/molecular-biologyresource-library/thermo-scientific-web-tools/tmcalculator.html

3.1.2. Media for Microorganisms

Distilled water was used to prepare all the required solutions, buffers, and media. The media was prepared and autoclaved to maintain sterility. Autoclaving was carried out at 121ºC for 20 minutes. pH was adjusted at 7.0 unless otherwise mentioned.

LB media was used for the growth of all used bacteria consisting of tryptone, yeast extract, and sodium Chloride.

Table 2 LB Broth for Bacteria

Sr. No	Components	Quantity (g/L)
1	Sodium Chloride	10.0

2	Yeast Extract	5.0
3	Tryptone	10.0

LB agar was used to isolate bacterial colonies on an agar plate that consisted of tryptone, yeast extract, agar, and sodium chloride.

Table 3 LB Agar for Bacteria

Sr. No	Components	Quantity (g/L)
1	Sodium Chloride	10.0
2	Yeast Extract	5.0
3	Agar	10.0
4	Tryptone	10.0

YPD agar was used for the growth of yeast colonies on the agar plate. It consists of peptone, yeast extract, agar, and glucose.

Table 4 YPD Agar for Yeast

Sr. No	Components of media	Quantity of media(g/L)
1	Peptone	10.0
2	Yeast Extract	5.0
3	Agar	10.0
4	Glucose	10.0

YPD broth is used for the growth of yeast in a liquid medium for the isolation of DNA or plasmid. It consists of peptone, yeast extract, and glucose.

Table 5 YPD broth for Yeast

Sr. No	Components of media	Quantity of media (g/L)
1	Peptone	10.0
2	Yeast Extract	5.0
3	Glucose	10.0

3.1.3. Buffer used for Gel Electrophoresis TAE (1x)

TAE buffer was used in the preparation of the Gel and in the Gel electrophoresis Tank. It is prepared by Tris-HCl, Acetic Acid and EDTA.

Table 6 TAE (1x) Buffer

Sr. No	Components	Quantity
1	Tris-HCl	40 mM
2	Acetic acid	40 mM
3	EDTA	0.4 mM (pH 8.0)

3.1.4. Solutions for competent cell production

Competent cell of *E. coli* TOP10 and DH5α cell *E. coli* BL21 required in transformation is prepared by different solutions. This solution contains many chemicals listed in the table below.

Table 7 Composition of solutions for competent cells preparation

Solution	Components
Solution 1 (autoclaved)	• $MgCl_2$ (0.5M) • $CaCl_2$ (0.5M)
	ddH_2O
Solution 2 (autoclaved)	• $CaCl_2$ (0.5M) • Glycerol 50% • ddH_2O

3.1.5. Equipment used during this study

This table contains the list of all the equipment used in this study from different institutes during all experimental work.

Table 8 Equipment used during this study

Equipment	Manufacturer
Heat Block	Wealtec corp.
Thermal Cycler	Applied Biosystems
Nanodrop	Colibri
Concentrator Plus	Eppendorf
Ultraviolet Viewing cabinet	Extra Gene
Gel Electrophoresis Tank	Cleaver Scientific Ltd

Shaking Incubator	Jsr
Shaking Water Bath	Memmert
Tabletop Balance	ShiMADZu
Centrifuge Machine	Velp-Scientifica
Hot Plate	WTW inoLab
Microwave Oven	Hermle
pH Meter	Haier
Vortex Mixer	Esco
Spectrophotometer	Heidolph
Laminar Flow cabinet	Sigma
Microcentrifuge	Optima
Gel Dolphin	Weal Tech ELITE 300 Plus
Electroporator	Eppendorf
Doc Incubator	Memmert
PCR Purification Kit	Thermo Scientific™
Genomic DNA Purification Kit	Thermo Scientific™
Plasmid Miniprep Kit	Thermo Scientific™
Gel Purification kit	Thermo Scientific™
PCR Green Master Mix	Thermo Scientific™

3.1.6. Chemicals

All the chemicals were used during the different experiments to make different buffers and media for the growth of microorganisms.

Table 9 Chemicals used during this study

Chemicals	**Manufacturer**
Agar	Bioworld USA
Nutrient Broth	Lab M UK
Agarose	Lab M UK
Tryptone	Sigma Aldrich
Nuclease Free Water	Caisson labs
Yeast Extract	Lab M UK
Methanol	Chem Lab
Ethanol Absolute	Sigma Aldrich
Ethidium bromide	Sigma Aldrich
Peptone	Lab M UK
Sodium Chloride	BDH Laboratories

3.1.7. Molecular biology grade Enzymes use

RNase A and Proteinase K enzymes were used for DNA extraction and plasmid extraction. T4-DNA-Ligase enzyme was used for the joining of the insert into the vector. Sal1 and Kpn1 restriction enzymes were used for the restriction of vector and insert.

Table 10 List of Molecular biology grade Enzymes used

Enzymes	**Manufacturer**
RNase A	Thermo Scientific

Proteinase k	Thermo Scientific
T4-DNA-Ligase	Thermo scientific
Kpn1	Thermo Scientific
Sal1	Thermo scientific

3.1.8. Bacterial strains used

E. coli strain of TOP10 and DH5α cell was used for the cloning of the vectors. *E. coli* BL21strain is used for the expression of reporter genes under promoters.

Table 11 List of Bacterial strains used

Bacteria	**Description**	**Source/Reference**
TOP10 *E. coli*	Helper strain used for transformation	Molecular Virology lab, NIBGE Faisalabad
DH5α	Helper strain used for transformation	Molecular Virology lab, NIBGE Faisalabad
BL21	Helper strain used for transformation	Molecular Virology lab, NIBGE Faisalabad

3.1.9. Yeast Strain used during this study

Saccharomyces Cerevisiae strain was utilized for the PCR amplification of the CYC1 control promoter by PCR from its genomic DNA.

Table 12 Yeast Strain used during this study

Gene	Description	Source
Saccharomyces Cerevisiae	Yeast strain used contains UBC9_1 and CYC1 Promoter	IBD, NIBGE, Faisalabad

3.1.10. Molecular biology grade Markers used

1 kb and 100bp DNA ladder was used in gel electrophoresis to measure the size of DNA fragments.

Table 13 List of Molecular biology Markers used

Marker	Manufacturer
1kb DNA Ladder	Thermo Scientific
100bp DNA Ladder	Thermo Scientific

3.1.11. Antibiotics used in this study

Both the plasmids cloning vector and expression vector have antibiotic resistance marker for the ampicillin so ampicillin was used for selection.

Table 14 Antibiotics used during this study

Antibiotics	**Symbol**	**Stock solution (mg/ml)**	**Conc. used for *E. coli* (µg/ml)**	**Dissolution media**	**Manufacturer**
Ampicillin	amp	20 mg/ml	100µg/ml	75% EtOH	MBPs

3.1.12. Primer used during this study

Primer of qPCR for EGFP gene was designed and used for the confirmation of ligation of insert containing EGFP gene by colony PCR. Primers for the CYC1 control promoters were designed with Sal1 and Kpn1 restriction sites. and ordered from Molecular biology products.

Table 15 List of Primers used

Primers	**Sequence**	**Reference**
Forward primer of EGFP	5' CCACAACATCGAGGACGGCA 3'	This study

Reverse primer of EGFP	5' CGTCCATGCCGAGAGTGATCC 3'	This study
Forward primer of CYC1	5'GTCGACAAAAAGGAGGACGAAAC AAAAAGAATTC3'	This study
Reverse primer of CYC1	5'GTCGAC ATGCTGCAAAGGTCCTAATG GAATTC3'	This study

3.1.13. Plasmids used during this study

Two plasmid was during this study one for the cloning of synthesized promoter and gene and the second for the expression of reporter gene. The expression vector was ordered from Addgene whereas the cloning vector was ordered from Molecular Biology Products company.

Table 16 List of Plasmids used

Plasmids	**Description**	**Source/Reference**
pCEV-G4-Km	A eukaryotic expression system used for the expression of promoter	Lars Nielsen, Claudia Vickers (Addgene plasmid # 46819)
pTwist	The cloning vector used for the cloning of	(Molecular Biology Products pk)

	synthesized promoter and gene	

3.1.14. Gene used during this study

EGFP (Enhanced green fluorescent) protein is a reporter gene and it produces that can easily be measured by flow cytometry. This gene is used to check the expression of the promoter.

Table 17 Gene used during this study

Gene	Description	Source
EGFP	A reporter gene used to check the expression	(Sequence from Snap Gene)

3.2. Methodology for In-silico Analysis

3.2.1. Sequence retrieval Promoter:

The sequence of *UBC9-1* Promoter from + 1 upstream to -500 downstream of (Transcription Start Site) TSS was taken by *Eukaryotic Promoter Database* in FASTA format. Promoter motifs were checked through the search motif tool in the Eukaryotic Promoter Database.

3.2.2. Selection of reporter gene:

The EGFP (enhanced green fluorescent protein) gene was selected as a reporter gene to identify the strength of the promoter. Its sequence consists of 720 base pairs retrieved from Snap Gene.

3.2.3. Designing of expression Cassette:

Promoter and gene Sequence with suitable enzymes related to the vector were synthesized by Molecular Biology Product company. This construct consisted of the Sequence of *Sal1* enzyme *UCB9_1* promoter sequence, *EcoRI* enzyme sequence, eGFP gene sequence, and sequence of *Kpn1* enzyme.

3.2.4. Designing of primers for RT PCR:

For designing the primer some points were kept in mind;

- **GC content:** The GC content of primers should range between 40%-80%
- **Length:** The length should lie between 17-24 base pair
- **Product size:** The product size of amplicon for RT PCR should range from 70 to 200 base pairs.

Table 18 Sequence of oligonucleotides

Primers	Sequence	Reference
Forward primer of EGFP	5' CCACAACATCGAGGACGGCA 3'	This study
Reverse primer of EGFP	5' CGTCCATGCCGAGAGTGATCC 3'	This study

3.2.5. Designing of primers for CYC1 control:

For designing primers some points were kept in mind.

- **GC content:** The GC content of primers should range between 40%-80%
- **Length:** The length should lie between 17-24 base pair

- **Annealing Temperature:** The Annealing temperature of primers is from 55 to 58

- **Addition of restriction Sites:** Restriction sites should be added on both 5' and 3' for cloning into vector. Sal1 and Kpn1 restriction sites were added to this primers

Table 19 Sequence of oligonucleotides of control

Primers	Sequence	Reference
FP CYC1	5’GTCGACAAAAAGGAGGACGAAACAAAAAGAATTC3'	This study
RP CYC1	5’GTCGACATGCTGCAAAGGTCCTAATG GAATTC3'	This study

Table 3.16 Sequence of oligonucleotides of control

3.2.6. Selection of expression Vector (Plasmid):

For cloning of synthesized construct consisting of promoter and gene pTwist cloning vector was used. For the transformation and expression of the promoter and gene into the bacterial or yeast cells, the expression vector is required. For that purpose, plasmid vector pCEV-G4-km was ordered from Addgene. It contains the CYC1 terminator required for the expression cassette.

3.3. Wet Lab Methodology:

3.3.1. Growth of *Saccharomyces Cerevisiae*:

To grow *Saccharomyces Cerevisiae* strain YPD agar and YPD broth were prepared and autoclaved. *Saccharomyces Cerevisiae* strain was streaked into YPD agar plates for growth and then further inoculated into YPD broth. Plates were kept in a 37º C incubator overnight. The next day single colony was inoculated in YPD broth and kept at 37º C at 200 rmp for 12 hours. That was further used for DNA extraction.

3.3.2. Competent cell preparation of *E. coli* TOP10.

To prepare competent cells of TOP10 and BL21, a method described by (Chang et al., 2017) was used. Cells of BL21 strain were streaked on nutrient agar plates and placed overnight at 37°C to get separate colonies. The next day, isolated colonies from the Petri plate were taken and added to the conical flasks containing nutrient broth. Flasks were incubated for 12-16 hours in a shaking incubator for growth at a temperature of around 37°C by keeping the speed at 200 rpm to get growth. After this 1 ml of overnight or mother culture was taken and was subcultured in another flask having 50 ml of fresh nutrient broth. This culture was again incubated for an hour to two till its optical density OD600 reached 0.5 to 0.6. then it was added already ice-chilled with 50 ml falcons and was placed again on ice for 20 min. After this, falcons were centrifuged at 4°C at 4000 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in 20mL ice-cold 0.1M CaCl2 and kept on ice for 30 min. Again, centrifugation was done at 4°C at 4000 rpm for 10 min. The supernatant was again thrown, and 5ml ice-cold 0.1M CaCl2 was added to the pellet. The resuspended pellet was aliquoted as 50µl in already chilled microcentrifuge tubes and liquid N2 was used for snap freezing purposes. These freshly prepared cells were immediately used for the transformation process and the rest of the cells were stored at -80°C with 15% glycerol.

3.3.3. Transformation of Promoter gene Construct in TOP10 Cells by Heat Shock Method:

To transform the construct into the DH5α competent cells the heat-shock technique was used (Chang, Chau, Landas, & Pang, 2017). Competent cells were thawed on ice. The pTiwst cloning plasmid having Construct 1-2µl was mixed to 100 µl cell aliquot of competent cells. The sample was placed on ice for 30 min. After incubation on ice, the sample was placed in a 42ºC heat block for 1 minute and 20 seconds. After this 800ml pre-warmed LB medium without selection marker was added to the sample and incubated in a shaking incubator at 37ºC, 200 rpm, for 1 hour for outgrowth. All transformation mixture was spread on the plates having ampicillin as an antibiotic marker and the plates were placed in an incubator at 37ºC for 12-16 hours.

3.3.4. Expression Vector and Cloning Vector Isolation (Miniprep):

Expression Vector pCEV-G4-km and Cloning Vector pTwist contain promoter and gene construct were isolated from the cells of bacteris by Plasmid Miniprep Kit(Thermo Scientific™). The overnight grown cell was used in 1.5 ml autoclaved Eppendorf and centrifugation was performed at 13000 rpm for around 5 min. After centrifugation, the supernatant was discarded. More culture was added again if the pellet was not enough. The culture pellet was obtained after discarding the supernatant. To that pellet 300 µl of the resuspension buffer ice cold was added. RNase A enzyme was added to the resuspension solution before adding it to the pellet. The bacterial pellet was resuspended by pipetting or vortexing until the pellet of the cell completely dissolved. It was then incubated on a bench for 5 min. Then 250 ul of the Lysis buffer was added for the lysis of cells and mixed properly until solution became viscous and clear. For the neutralization of lysis solution, 350 ul of the neutralization buffer was added and mixed thoroughly. Centrifugation was carried out for 5 min to pellet debris and DNA. After that supernatant was added to column. Flow through was

discarded after Centrifugation for 1 min. Then 500ul of the Wash buffer was added to the column and centrifugation was carried out for 30-60 seconds and the flow-through was discarded. the column was placed into a new clean 1.5 ml centrifuge tube and 50 ul of the elution solution was added to the column membrane for the elution the plasmid DNA. After Incubation was carried out for 2 min at normal temperature and centrifugation step was performed for 2 min. The column was wasted and purified plasmid DNA after confirmation by Gel electrophoresis was placed at -20°C for other downstream processes.

3.3.5. Agarose gel electrophoresis:

For gel electrophoresis, 1% quantity of gel was prepared by dissolving 0.5 grams of gel in 50 ml of TAE buffer (Table 3.22) by boiling. After complete dissolution, the gel was cooled down for a few minutes and then ethidium bromide was added for stain. The gel was added into a taped gel tray which already had a comb. After polymerization, the gel try was transferred to the gel tank and it was filled with same TAE buffer used for making gel. Samples were mixed with 6x DNA loading dye. 1kb, 100bp ladders were used for reference. Electrophoresis was carried at 85V for 45 min.

3.3.6. Restriction Digestion of Vectors:

Restriction digestion of Expression Vector pCEV-G4-km and Cloning Vector pTwist contain promoter and gene contract was done with sal1 and Kpn1 enzymes. For that purpose, 2 µl of 10x BamH1 buffer was added, and plasmids having a concentration of (0.5-1 µg/µL) were added in quantity of 1 µl. Restriction enzymes Sal1 and Kpn1 were added in between the range of (0.5-2 µl) and 16 µl of NF water was added to make 20 µl reaction volume. After adding all the constituents, it was gently mixed in the vortex for a few seconds and placed in the incubator for up to 16 hours at 37°C. for confirmation of successful digestion samples were loaded in agarose gel and visualized by a UV illuminator.

3.3.7. Gel Purification of Digested Vector:

Before ligation, gel purification of digested expression Vector pCEV-G4-km and cloning vector pTwist containing promoter and gene construct was done which is also recommended for successful cloning. For that purpose, the digested vector sample was loaded into a larger well in the electrophoresis tank. The band from the gel having digested vector was cut out with the help of a clean blade and was put into 1.5 ml centrifuge tube. This tube was measured first when it was empty then it was measured with the gel mass and the gel weight was subtracted from the microcentrifuge tube weight. The gel weight was around 680mg, so 2.72 ml of dissolving buffer was mixed into the gel slice. The gel samples were vortexed from time to time until the gel was dissolved completely into the buffer. Upon complete dissolution, a sample was added to the column, the cap was closed, spun for 1 min, and the discarded flow through. The column was transferred into a tube and 200 µl of DNA buffer for a wash was added again centrifugation for 1 min was done and this process was repeated once again to ensure any leftover flow through. The column was placed in a clean centrifuge tube and 6 µl of buffer for elution was added to the center of the matrix after incubation for around 1 min centrifugation was done to elute DNA.

3.3.8. n-Butanol Precipitation:

For removal of different salt contaminations or other impurities from DNA/plasmids which can obstruct transformation or PCR reactions, samples were precipitated by n-butanol. The sample needed to be purified was first diluted with 50 µl ddH2O. After that, it was mixed with 500 µl n-butanol. The mixture was shaken vigorously and centrifugation was performed at around 12,000 rpm for 30 min at 4 °C. The supernatant was wasted, the pellet was cleaned with 70% ethanol and centrifugation was performed for 10 min. The resulting pellet was air-dried in Speed Vac and for further use, it was resuspended in 20 µl of ddH2O.

3.3.9. Ligation of gel purified pCEV-G4-km vector and UCB9_1(EGFP) insert:

To get a plasmid having promoter gene insert, ligation was done of already digested and gel purified pCEV-G4-km vector and UCB9_1(EGFP) construct. A ligation calculator was utilized to measure the concentration of vector and insert. Before these calculations' nanodrop was used and the concentrations were adjusted by the addition of nuclease-free water. Mostly insert to vector ratio is 1:3. So 4 µl UCB9_1(EGFP) inserts along with 10 µl of pCEV-G4-km vector were added to which 2 µl of T4 DNA ligase enzyme for ligation and 2 µl of 10X T4 ligase buffer was added. The reaction volume was raised to 20 µl by 2 µl of nuclease-free water addition to the mixture. The ligation mixture was kept at 16°C for overnight ligation. Ligases were deactivated at 65°C for 10 min before transformation for better results and kept at 4°C for further use.

3.3.10. Competent cell preparation of BL21

For expression of the EGFP gene under the UCB9_1 promoter competent cells of BL21 were prepared using a method described by (Chang et al., 2017) Cells of BL21 strain were streaked on nutrient agar plates and placed overnight at 37°C to get separate colonies. The next day, isolated colonies from the Petri plate were taken and added to the conical flasks containing nutrient broth. Flasks were incubated for 12-16 hours in a shaking incubator for growth at a temperature of around 37°C by keeping the speed at 200 rpm to get growth. After this 1 ml of overnight or mother culture was taken and was subcultured in another flask having 50 ml of fresh nutrient broth. This culture was again incubated for an hour to two till its optical density OD600 reached 0.5 to 0.6. then it was added already ice-chilled with 50 ml falcons and was placed again on ice for 20 min. After this, falcons were centrifuged at 4°C at 4000 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in 20mL ice-cold 0.1M CaCl2 and kept on ice for 30 min. Again, centrifugation was done at 4°C at 4000 rpm for 10 min. The supernatant was again thrown, and 5ml

ice-cold 0.1M CaCl2 was added in the pellet. The resuspended pellet was aliquoted as 50µl in already chilled microcentrifuge tubes and liquid N2 was used for snap freezing purposes. These freshly prepared cells were immediately used for transformation process and the rest of the cells were stored at -80°C with 15% glycerol.

3.3.11. Transformation of pCEV-G4-km+UCB9_1(EGFP) in BL21 cells:

To transform ligated vector i.e., pCEV-G4-km having insert i.e., UCB9_1(EGFP) into the freshly prepared competent cells of BL21 strain, the heat-shock technique was used (Chang, Chau, Landas, & Pang, 2017). Competent cells were thawed on ice. The ligated plasmid 1-5µl (10pg-100ng) was added to a 50µl cell aliquot of competent cells. The sample was placed on ice for around 30 min. After this, the sample was kept in a 42ºC water incubator for around 30 seconds. The cells were again kept on ice maximum for 2 min, to which 1ml pre-warmed LB medium was added and placed in a shaking incubator at 37ºC, 200 rpm, for 1 hour for outgrowth. 100µl transformation mixture was spread on the plates having ampicillin as an antibiotic selection marker and the plates were placed at 37ºC overnight or 12-16 hours.

3.3.12. Confirmation of Transformation by Colony PCR:

For the verification of successful ligation of pCEV-G4-km having UCB9_1(EGFP) colony PCR UCB9_1(EGFP) was done. For this purpose, EGFP primers for RT PCR were used.

Table 20 Sequence of oligonucleotides

Primers	Sequence	Reference
Forward primer of EGFP	5' CCACAACATCGAGGACGGCA 3'	This study
Reverse primer of EGFP	5' CGTCCATGCCGAGAGTGATCC 3'	This study

Steps of Colony PCR:

For Colony PCR, 20µl of NF water was taken in PCR tubes, and transformed colonies from the antibiotic plates were picked carefully and mixed with NF water in the PCR tubes until the solution got turbid. The tubes were spun in the mini spin. After spinning, the PCR tubes were placed in the thermocycler for 10 minutes at 95ºC followed by refrigerated centrifugation at 4ºC, 6000 rpm for 3 min. PCR mix was prepared to have a reaction volume of 15 µl which comprised of 7 µl of master mix, 0.6 of forward primer of PCR, and 0.6 µl of reverse primer along with 2µl of PCR water having transformed colonies, and NF water was added to complete the volume. PCR was performed using the profile i.e., 95ºC for approximately 5 min, succeeded by 35 cycles at 95ºC for 35 seconds, 55ºC for 35 seconds, and 72ºC for 30 seconds finally followed by an extension cycle at 72ºC for 10 min. Successful verification of amplification via colony PCR was assessed by gel electrophoresis.

3.3.13. Confirmation of restriction Digestion

For the confirmation of successful ligation of pCEV-G4-km having UCB9_1(EGFP), digestion of Expression vector pCEV-G4-km was done with sal1 and Kpn1 enzymes. For that purpose after extraction of plasmid DNA from transformed colonies, 2 µl of 10x BamH1 buffer was added, and plasmid having a concentration of (0.5-1 µg/µL) was added in quantity of 1 µl. Restriction enzymes

Sal1 and Kpn1 were added in between the range of (0.5-2 µl) and 16 µl of NF water was added to make 20 µl reaction volume. After adding all the constituents, it was gently mixed in the vortex for a few seconds and placed in the incubator for up to 16 hours at 37°C. For confirmation of successful digestion samples were loaded in agarose gel and visualized by UV illuminator.

4. RESULTS

4.1. *In Silico* gRNA Designing:

4.1.1. Sequence Retrieval of *UCB9_1* Promoter:

The sequence of *UBC9-1* Promoter from + 1 upstream to -500 downstream of gene TSS was taken by the *eukaryotic Promoter Database* in FASTA format. Promoter motifs were checked through the search motif tool in the Eukaryotic Promoter Database.

```
>FP000744 UBC9_1 :+U EU:NC; range -500 to 1.
GTACTTTAGCTGCCTCTTCTAGAGCACTAATCAGTTTATTAAAATCTTCTGTCTTCACAT
TATTCTCGTTACCGTTATTTTTCATCAAATTTGCGAACTCATTTTGCAAATCTACCATCA
TTTCTTTCAATTCTGGGTCCTCTTCAGATTCATTAGCTACCTGTACGCCATCACTGTCCT
TACTTTCGGCATTCTTCTCTTTGTTTTCACTATCGTTGTACACAGAACCCTTCGCTTGCA
CATCATCGGGCTCTGCTTCATCCAGTTTAGTGGGATCTTCATCTAAAAGGTCATCCAAAT
CATCAAAATTATCGTACTCGTTTTCATTCATTACTTCGTGTTGTATGTTTGGCATTTCTT
CTTTCCGTCAATACTTCGGTTCCCACAATTTGTAATTCTTTCTTCACTTTATATCTCTCA
GAAACCGCGTTTAACATCTGGAAATTAAAAATTATTCCTGTCTCCATAACAAACATTTAA
AAAAAGAAGAGAAATTTAGCAT
```

Figure 2 Sequence of UBC9_1 promoter: Sequence of promoter retrieved from EPD in FASTA format.

4.1.2. Sequence Retrieval EGFP reporter gene:

A synthetic sequence of enhanced green fluorescent gene that was used in this study as a reporter gene was taken by snap gene plasmids. It consists of a total of 720 base pairs.

```
LOCUS       egfp        720 bp    DNA     linear   UNA 12-Dec-2011
DEFINITION
FEATURES             Location/Qualifiers
ORIGIN
        1 ATGGTGAGCA AGGGCGAGGA GCTGTTCACC GGGGTGGTGC CCATCCTGGT CGAGCTGGAC
       61 GGCGACGTAA ACGGCCACAA GTTCAGCGTG TCCGGCGAGG GCGAGGGCGA TGCCACCTAC
      121 GGCAAGCTGA CCCTGAAGTT CATCTGCACC ACCGGCAAGC TGCCCGTGCC CTGGCCCACC
      181 CTCGTGACCA CCCTGACCTA CGGCGTGCAG TGCTTCAGCC GCTACCCCGA CCACATGAAG
      241 CAGCACGACT TCTTCAAGTC CGCCATGCCC GAAGGCTACG TCCAGGAGCG CACCATCTTC
      301 TTCAAGGACG ACGGCAACTA CAAGACCCGC GCCGAGGTGA AGTTCGAGGG CGACACCCTG
      361 GTGAACCGCA TCGAGCTGAA GGGCATCGAC TTCAAGGAGG ACGGCAACAT CCTGGGGCAC
      421 AAGCTGGAGT ACAACTACAA CAGCCACAAC GTCTATATCA TGGCCGACAA GCAGAAGAAC
      481 GGCATCAAGG TGAACTTCAA GATCCGCCAC AACATCGAGG ACGGCAGCGT GCAGCTCGCC
      541 GACCACTACC AGCAGAACAC CCCCATCGGC GACGGCCCCG TGCTGCTGCC CGACAACCAC
      601 TACCTGAGCA CCCAGTCCGC CCTGAGCAAA GACCCCAACG AGAAGCGCGA TCACATGGTC
      661 CTGCTGGAGT TCGTGACCGC CGCCGGGATC ACTCTCGGCA TGGACGAGCT GTACAAGTAA
//
```

Figure 3 Sequence of EGFP reporter gene: 7 Sequence of gene retrieved from snap gene plasmids.

4.1.3. Gene promoter Construct for expression cassette:

The construct consists of Sequence UBC9_1 promoter with EGFP gene and enzymes were designed and synthesized. The total size of the construct is 1238 bp. Promoter and gene Sequence with suitable enzymes related to the vector were synthesized by Molecular Biology Product company. This construct consisted of the Sequence of *Sal1* enzyme labelled in blue color in map, *UCB9_1* promoter sequence labelled in purple colour, *EcoRI* enzyme sequence (blue), EGFP gene sequence labelled in green on map , and sequence of *Kpn1* enzyme.

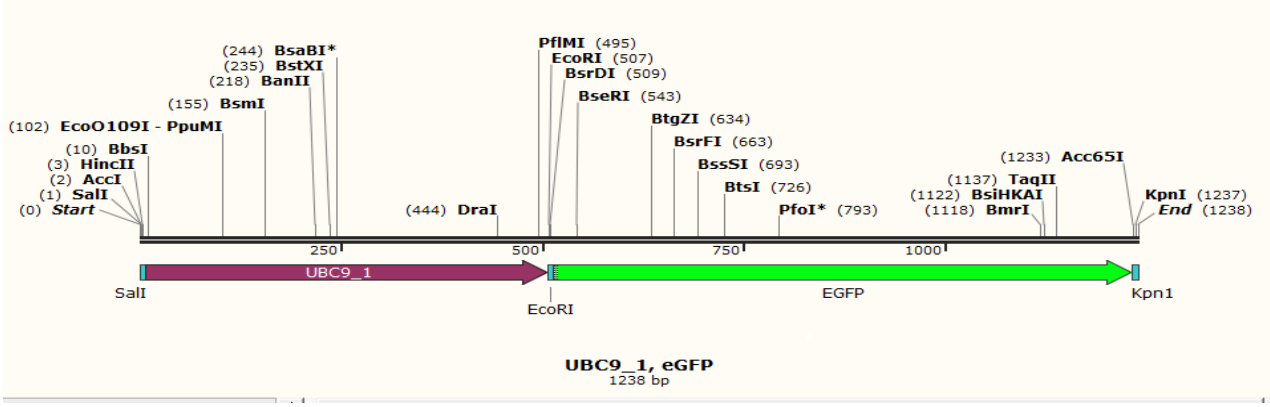

Figure 4 Map of Construct: UBC9_1, EGFP. Size1.2 kb

4.1.3. Map of plasmid for Expression of gene:

The Plasmid selected for expression of the gene under promoter was pCEV-G4-Km. The size of the vector is 6574-bp. For the transformation and expression of the promoter and gene into the bacterial or yeast cells, the expression vector is required. For that purpose, plasmid vector pCEV-G4-km was ordered from Addgene. It contains multiple cloning restriction sites, two replications of origin, both for yeast and bacteria, two antibiotic resistance genes ampicillin and kanamycin and the CYC1 terminator required for the expression cassette

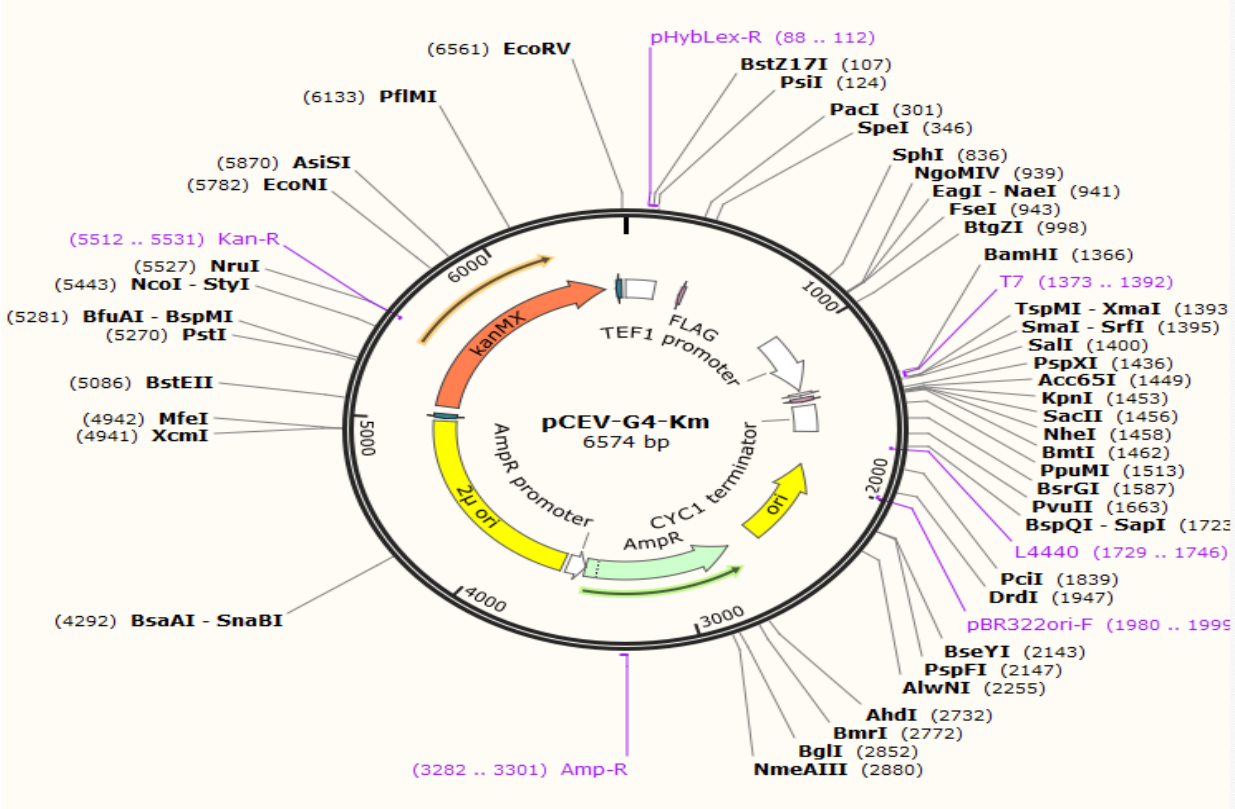

Figure 5 Map of Expression vector: pCEV-G4-Km

4.2. Recombinant Plasmid construction for the expression of EGFP gene:

The synthesized construct was provided in lyophilized form in the pTwist cloning vector by the Molecular biology products company. After adding nuclease-free water, a construct was transferred by the process of transformation into TOP10 competent cells. A single colony was taken and spread into an agar plate containing an ampicillin selection marker. For cloning plasmid extraction primary cultures were kept for growth in LB broth overnight in an incubator at 37ºC. The expression vector was provided in the form of agar stab from Addgene, pCEV-G4-km plasmid was streaked on nutrient agar plates having ampicillin antibiotics to get single colonies. Agar plates were kept at 4ºC and primary cultures were grown overnight in the incubator at 37ºC.

4.2.1: Transformation of synthesized UBC9_1, EGFP Construct in TOP10:

To transform UBC9_1, EGFP Construct in *E. coli* TOP10, competent cells were prepared as mentioned in 3.3.2 followed by heat shock transformation. Results showed the presence of colonies on the ampicillin-containing plate which were further used for isolation of plasmid.

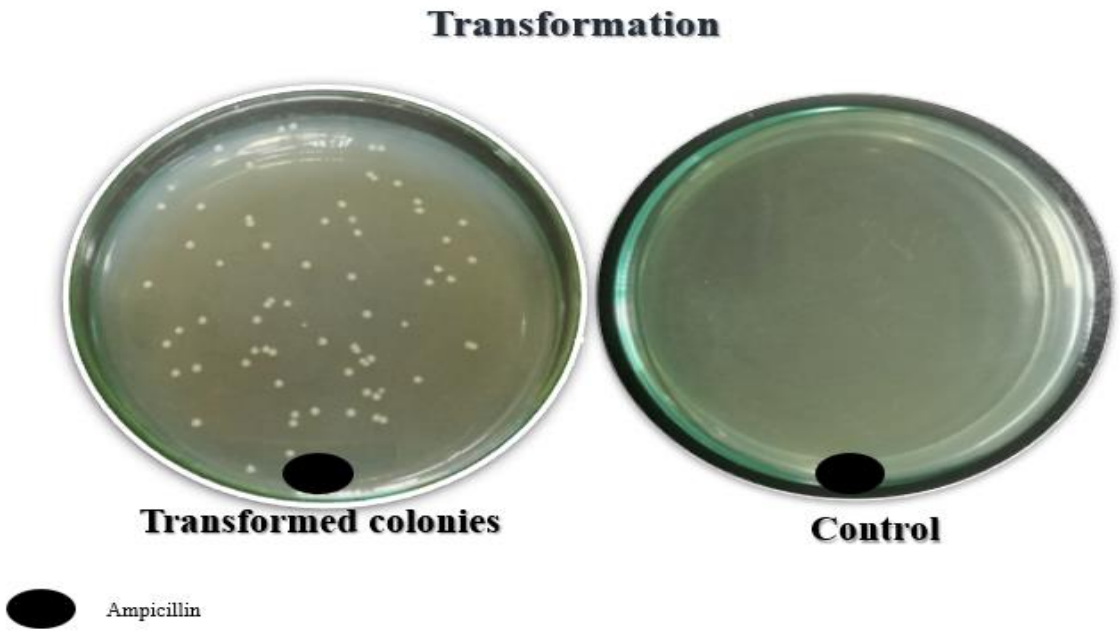

Figure 6 Results of transformation of plasmid contain insert in E. coli shows the transformed colonies on the ampicillin antibiotic plate since Twist cloning vector has ampicillin as its selection marker and control plate shows no colonies.

4.2.2. Confirmation of pTwist and pCEV-G4-Km Vectors:

Plasmid pCEV-G4-Km was ordered from Add gene and received in *E. coli* DH5α and UBC9_1, EGFP Construct was synthesized and received *in E. coli* TOP10. The protocol for plasmid extraction mentioned in section 3.3.4 was followed to extract plasmids from the given strain. For confirmation of plasmid extraction gel electrophoresis was performed, which showed bands of 3.4 and 6.5 respectively (Fig 7).

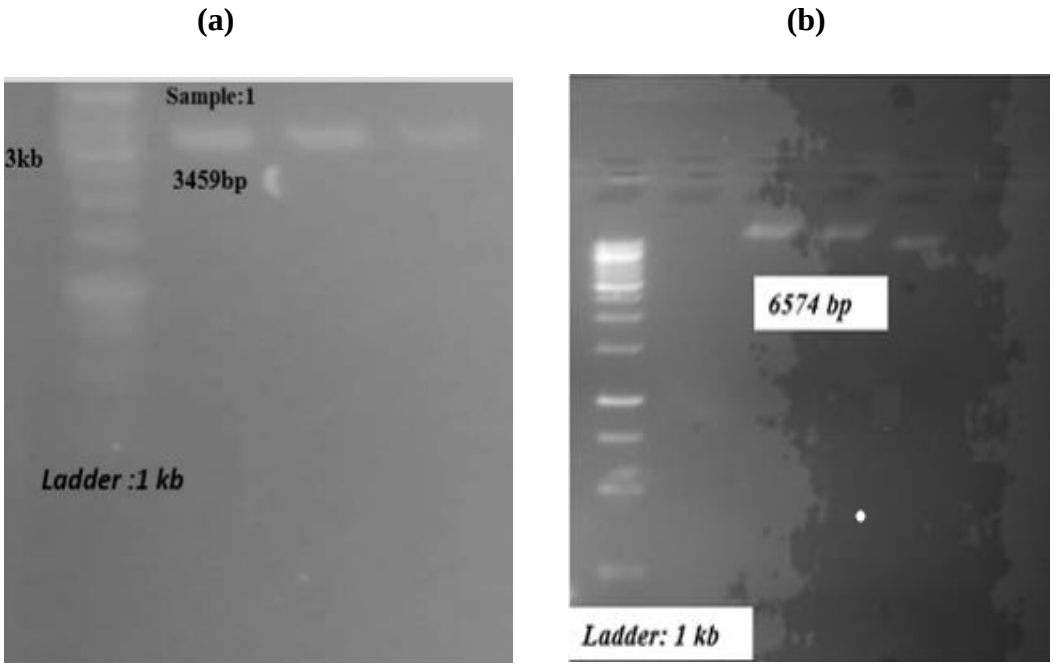

Figure 7 Confirmation of plasmids: (a) 3.4 kb circular plasmid pTwist contain insert can be seen on the agarose gel while (b) shows the image of gel having 6.5 kb bands of the pCEV-G4-Km plasmid

4.2.3. Confirmation restriction digestion of pTwist and pCEV-G4-Km Vectors:

Plasmid pCEV-G4-Km and pTwist having UBC9_1, EGFP Construct both were restricted with Sal1 and Kpn1 enzymes. Protocol for restriction digestion mentioned in section 3.3.2 was followed by ligation UBC9_1, EGFP insert into pCEV-G4-Km vector, and then transformation into *E. coli* BL21 cells. For confirmation restriction digestion of plasmids, gel electrophoresis was performed, in which successful restriction of both vectors can be seen. (Fig 7).

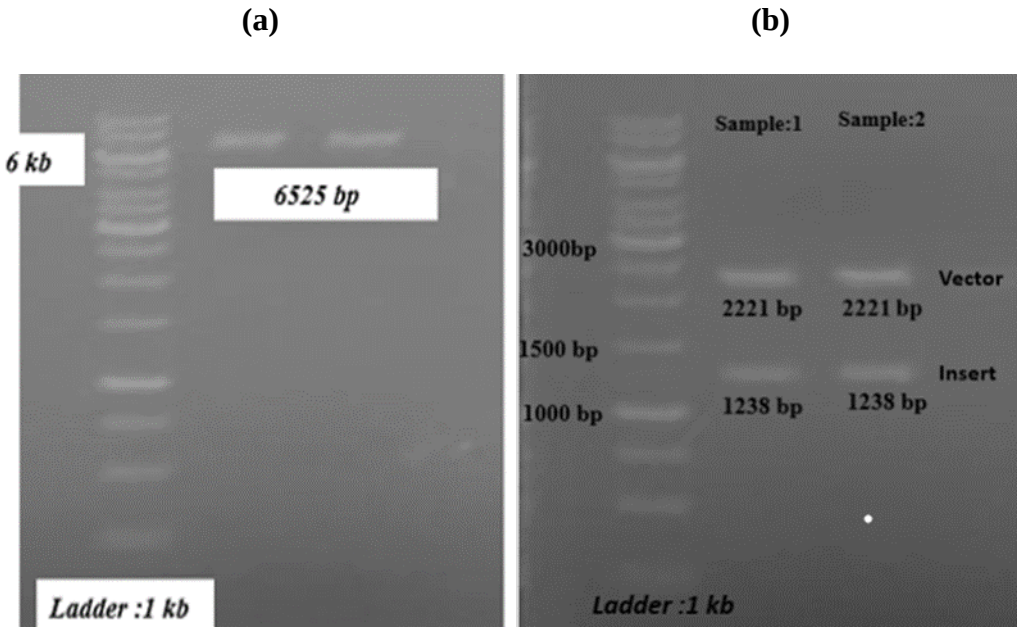

Figure 8 Confirmation of restriction digestion of plasmids: (a) 1.2 kb insert and 2.2 kb pTwist vector fragment can be seen on the agarose gel while (b) shows the image of gel having 6.5 kb bands of pCEV-G4-Km plasmid in which only 49 bp are removed.

4.2.4: Transformation of Ligated Vector:

To transform ligated UBC9_1, EGFP insert in pCEV-G4-Km vector in *E. coli* BL21, competent cells were prepared as mentioned 3.3.10 followed heat shock transformation. Results showed the presence of colonies on the ampicillin-containing plate.

Figure 9 Results of transformation of ligated insert in vector: One Plate shows the transformed colonies on the ampicillin antibiotic plate since pCEV- G4-Km has ampicillin as its selection marker. Control plate shows no colonies.

4.2.5 Confirmation of the growth of *Saccharomyces Cerevisiae*

To grow *Saccharomyces Cerevisiae* strain YPD agar and YPD broth were prepared and autoclaved. *Saccharomyces Cerevisiae* strain was streaked for growth into YPD agar plates and then further inoculated into YPD broth. Plates were kept in a 37º C incubator overnight. The next day single colony was inoculated in YPD broth and kept at 37º C at 200 rmp for 12 hours. That was further used for DNA extraction.

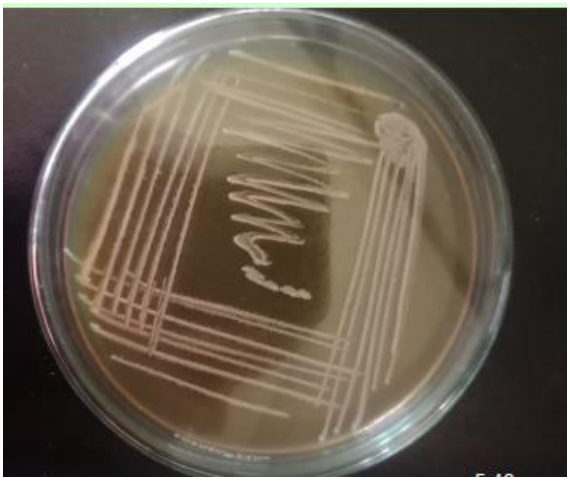

Figure 10 Result of Saccharomyces Cerevisiae strain on YPD agar plate. Plate shows clear white colonies of Saccharomyces Cerevisiae growth recipe of YPD agar media is given in 3.2.3 Table.

4.2.6 Confirmation of DNA extraction of *Saccharomyces Cerevisiae*

DNA of *Saccharomyces Cerevisiae* was extracted by using Thermo Scientific DNA extraction kit protocol motioned in section 3.3.4. DNA of *Saccharomyces Cerevisiae* was extracted for the amplification of CYC1 control promoter by PCR through genomic DNA.

Figure 11 Result of DNA extraction of S. Cerevisiae. Picture of gel shows bands of DNA of S. Cerevisiae. Size of DNA is 12 Mb.

4.2.7. Confirmation of PCR amplification of CYC1 Promoter

After the DNA extraction of *S. Cerevisiae* CYC1 control promoter is amplified by using genomic DNA by gradient PCR using 53, 56, 58, and 60 as annealing temperatures for primer. The primers utilized for PCR are mentioned in Table 3.16. Verification of amplification by colony PCR was done by gel electrophoresis.

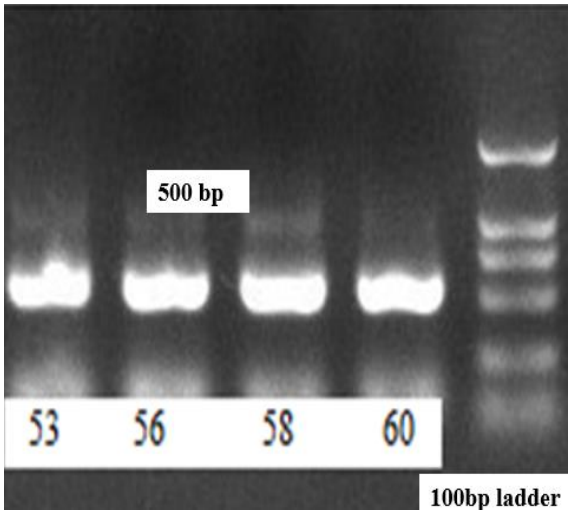

Figure 12 Result of PCR amplification of CYC1 control promoter of S. Cerevisiae. Gel picture shows clear band of amplified CYC1 control promoter. The size of amplified region of promoter is 500 bp.

4.2.8. Confirmation of ligation by Colony PCR

After the observation of colonies on antibiotic plates which indicated the successful transformation. These preliminary results were confirmed by colony PCR mentioned in section 3.3.12. Verification of amplification by colony PCR was done by gel electrophoresis. Genotypic confirmation by colony PCR confirmed successful ligation of construct into pCEV-G4-Km (Fig 12).

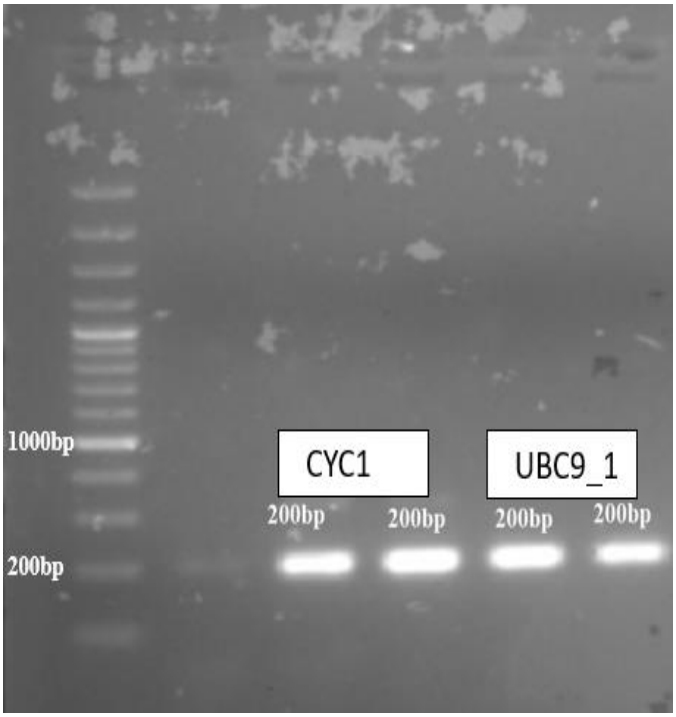

Figure 13 Confirmation via colony PCR: image of gel showing the EGFP amplicon size of 200 bp.

4.2.9. Confirmation of ligation by Restriction Digestion

After the observation of colonies on antibiotic plates and colony PCR which indicated the successful transformation. These preliminary results were further confirmed by restriction digestion mentioned in section 3.3.13. Verification of restriction digestion was done by gel electrophoresis. Restriction confirmation reconfirmed successful ligation of UBC9_1, EGFP constructs into pCEV-G4-Km (Fig 13).

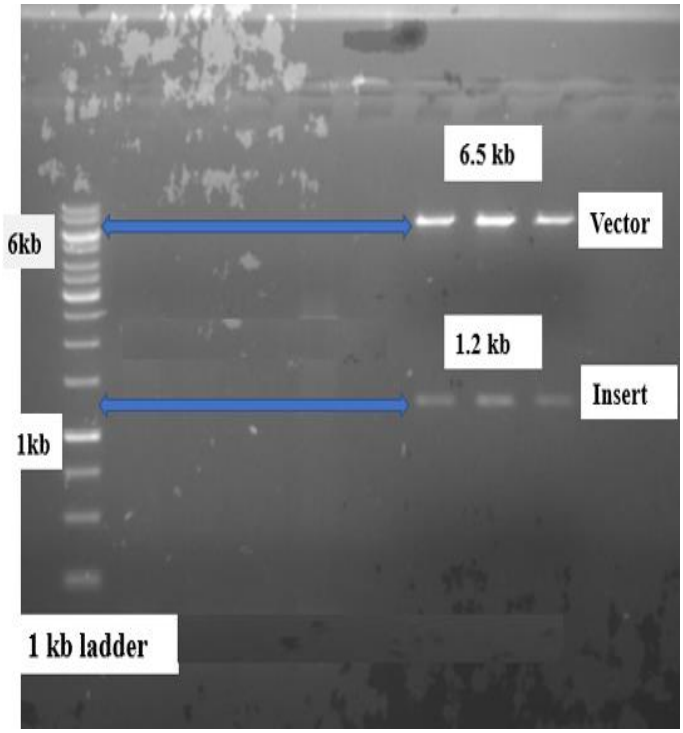

Figure 14 Confirmation via restriction digestion: the Image of a gel showing 1.2 kb insert and 6.5 kb vector fragments.

4.2.10: Results of Flow Cytometry

After confirmation of successful cloning of insert into the expression vector through colony PCR and restriction digestion sample proceeded for the flow cytometry. Comparison of Side Scatted and Forward Scattered lin of CYC1 control promoter with the Side Scatted and Forward Scattered lin of UBC9_1 promoter on Scatter plot of flow cytometry shows greater number of cells producing fluorescence toward the Forward Scattered lin of UBC9_1 promoter. This indicates that expression of EGFP under UBC9_1 promoter as compared to control CYC1 is greater.

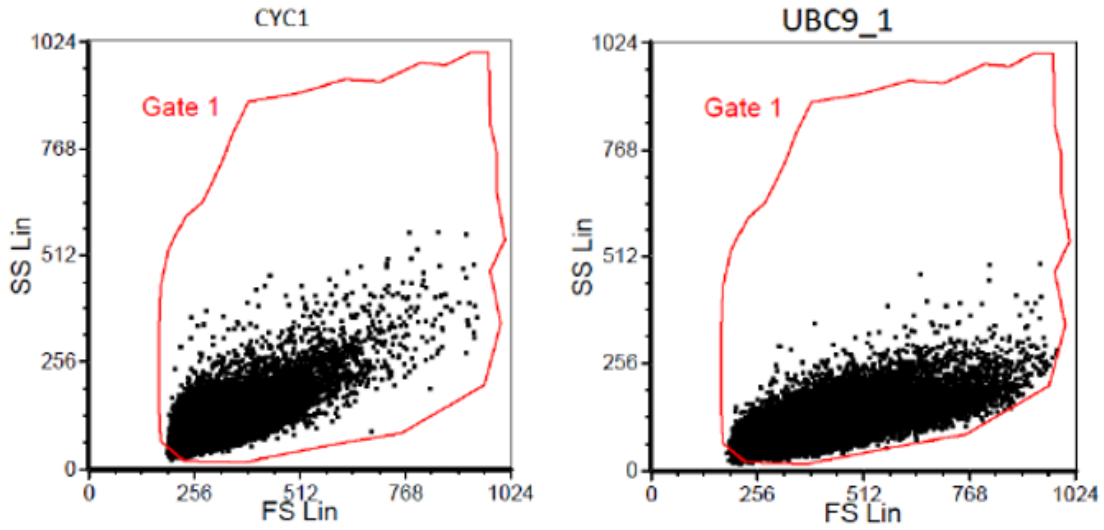

Figure 15 Comparison of Side Scatted lin and forward Scattered lin on Scatter plot of both promoters shows greater expression of EGFP under UBC9_1 promoter as compared to control CYC1.

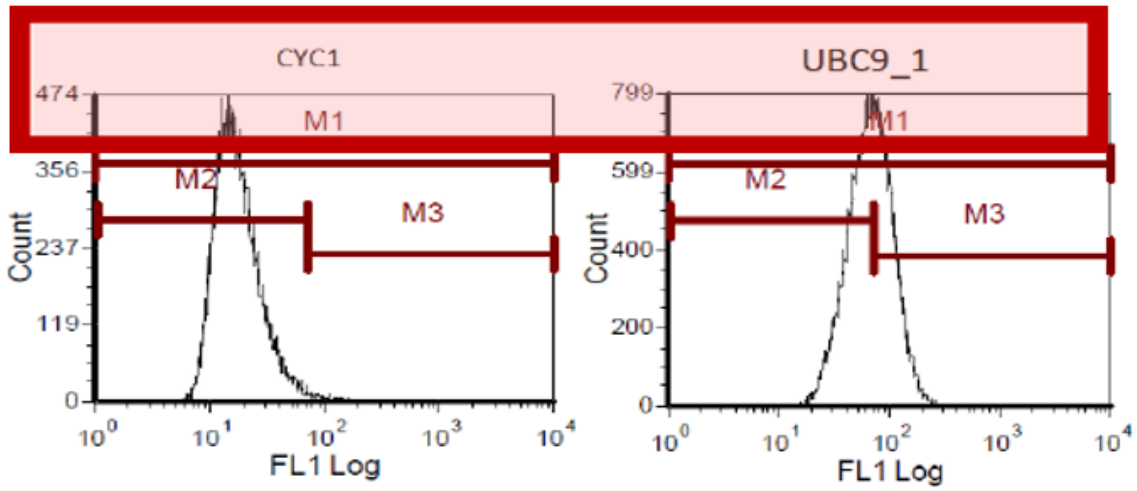

Figure 16 Comparison of Florescence channel count shows greater Activity of EGFP expression under UBC9_1 promoter.

After analyses of fluorescence channel count three geometric mean values of both CYC1 control and UBC9_1 promoter were compared. Two geometric mean values of UBC9_1 promoter were greater than the CYC1 control showing great expression of EGFP gene under UBC9_1 promoter.

Histogram #	Filename	Parameter	Low bound	High bound	# of Events	% of gated cells	Median	Geometric Mean	Arithmetic Mean	CV	Peak Value	Peak Channel
1	UBC9_1	FL1 Log	1.00	10000.00	100000	100.00	67.32	66.35	73.63	47.23	799.00	69.16
1	UBC9_1	FL1 Log	1.05	71.48	55031	55.03	50.48	47.65	49.65	26.61	799.00	69.16
1	UBC9_1	FL1 Log	71.48	10000.00	44969	44.97	94.75	99.50	102.97	29.31	798.00	72.99

Histogram #	Filename	Parameter	Low bound	High bound	# of Events	% of gated cells	Median	Geometric Mean	Arithmetic Mean	CV	Peak Value	Peak Channel
1	CYC1	FL1 Log	1.00	10000.00	50000	100.00	16.55	17.73	20.74	95.84	474.00	14.59
1	CYC1	FL1 Log	1.05	71.48	49282	98.56	16.40	17.25	19.09	50.92	474.00	14.59
1	CYC1	FL1 Log	71.48	10000.00	718	1.44	99.55	117.38	133.85	67.08	16.00	77.74

Figure 17 Comparison of Geometric mean values of UBC9_1 promoter is greater than Control CYC1 promoter shows greater activity of EGFP under UBC9_1 promoter.

5. DISCUSSION

Promoters are regulatory elements that control and regulate protein expression (Tang et al., 2020). Multiple tasks covered by natural *S. cerevisiae* promoters include multiple ranges of expression strength and beneficial tools for metabolic engineering(DeMarini et al., 2001).

In *S. cerevisiae,* endogenous promoters are characterized by the rate of expression and are mainly of two kinds. One is constitutive and the other is inducible or regulated promoters. The constitutive type of promoters shows constant transcription levels under the pressure of intracellular or extracellular stimuli (Da Silva & Srikrishnan, 2012). Some of commonly identified constitutive promoters include PPGK1, PTEF1, PTDH3, PTPI1, PENO2, and PCCW12, in various studies their strength varies due to the different experimental and sampling conditions (Partow et al., 2010).

Inducible promoters can start gene expression with or without certain molecules or stimuli. Through inducible promoters genes express or repress in response to specific stimuli. Some promoters that are identified as strong promoters are Galactose-inducible promoters, including, PGAL7 PGAL2, and PGAL1, and have been highly used to get higher protein expression levels and enhance production of desired products. (Apel et al., 2017)

In *S. Cerevisiae* promoter consists of three main regions including core promoter, UAS, and URS. Core promoter elements directly interact with transcription regulatory proteins to build the pre-initiation complex, TATA box, and TSS. However, TATA box is not present in all the promoters of *S. Cerevisiae* only 19% of them contain the TATA box. Previous studies show that promoters containing TATA boxe show higher transcription rates (Mogno et al., 2010). Interestingly some previous studies also show that TATA-less promoters also need transcription-binding proteins to build a pre-initiation complex just like TATA-containing promoters (Pugh & Tjian, n.d.).It is crucial

to understand the regulatory function and expression level of TATA fewer promoters so, the UBC9_1 TATA-less promoter was chosen for this study.

The main goal of this study was to identify the strength of the TATA-less UBC9_1 promoter of yeast as compared to the already reported and expressed CYC1 TATA box containing promoter of *Saccharomyces cerevisiae* (Partow et al., 2010).

Promoter activity and gene expression can be checked by using an in vivo reporter system. In this system, a gene that reports activity is usually linked to a regulatory sequence of DNA (promoter) and fluctuation in reporter gene activity predicts fluctuations in the regulatory biological processes (Jefferson et al., 1987). In this study, the EGFP gene was used to measure the strength or activity of the promoter. The EGFP gene encodes the enhanced green fluorescent protein, which produces green fluorescence when interacts with blue light in the presence of oxygen, without any substrate or cofactor, both in vitro and in vivo (Ma et al., 2001). The activity of EGFP is not dependent on cell type or location and remains stable under various conditions The EGFP fluorescence can be checked with minimal handling, without preparation of lysate, using a fluorescence microscope, or flow cytometry. To measure the rate of expression, the expression of cassettes is designed to consist of a UBC9 promoter, enhanced green fluorescent protein, and CYC1 terminator. This expression cassette is inserted into a yeast expression vector and expressed in *E. coli* BL21 cells.

Then, Egfp protein expression is measured by the flow cytometry. Flow cytometry is a valuable technique to quantitatively analyze fluorescent intensity. It is particularly effective as it can evaluate whether a protein of interest is expressed in a cell but also measure the amount of protein expressed by a single cell based on the intensity of fluorescence. The fluorescence intensity of each signal cell emitted by the reporter gene is quantified by flow cytometry and it also indicates footprints for the activity or strength of the promoter (Fischer et al., 2016).

Flow cytometry results were analyzed and comparison of Side Scatted lin and Farward Scattered lin on Scatter plot of flow cytometry shows greater expression of EGFP under UBC9_1 promoter as compared to control CYC1. Furthermore, analysis of Florescence channel count shows greater Activity of EGFP expression under UBC9_1 promoter. Geometric mean values of the UBC9_1 promoter are compared with the Control CYC1 promoter which also indicates a higher rate of expression of the UCB9_1 promoter.

This study can be useful to produce useful recombinant proteins by using this promoter, as the expression of proteins is regulated by the promoters.

6. CONCLUSION

The study conducted was to investigate the strength of UBC9_1 Promoter and the development of eukaryotic expression system. After successful integration of gene promoter constructs into the expression vector before the CYC1 terminator, expression of the enhanced green fluorescent protein under UBC9_1 promoter in E. coli BL21 expression cells is checked by flow cytometry. Comparison of Side Scatted Lin and Forward Scattered Lin on Scatter plot of flow cytometry shows the greater expression of EGFP under UBC9_1 promoter as compared to control CYC1. Furthermore, analysis of the Florescence channel count shows greater Activity of EGFP expression under UBC9_1 promoter. The geometric mean values of the UBC9_1 promoter are compared with the Control CYC1 promoter which also indicates a higher rate of expression of UBC9_1 promoter.

7. FUTURE PROSPECTS

Enhancing the production of metabolites is desirable in many industries like fermentation, fuel production, and the food industry. To know the strength of various promoters number of other promoters could be characterised by using this expression system and Identification of strong promotors and characterization of novel constitutive promotors further employed in pathway engineering for overproduction of metabolites and high-value compounds. Furthermore, the strength of the promoter in various conditions can be evaluated. Expression of the UBC9_1 promoter can also be done by the RT PCR. Promoters with different and predictable outcomes from transcription can be utilized in complex gene circuits, and discover new applications in industries. Other genes for useful compounds and enzymes can be expressed in this expression system. α-Amylases enzyme is demanding enzymes in textile industry can also be produced in the expression system

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