ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

CONSTRUCTION OF EXPRESSION VECTORS FOR THE HETEROLOGOUS PRODUCTION OF Kpkt KILLER TOXIN IN *Saccharomyces cerevisiae* AND *Pichia pastoris* CELLS

M.Sc. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

Kpkt KATİL TOKSİNİNİN *Saccharomyces cerevisiae* VE *Pichia pastoris* HÜCRELERİNDE HETEROLOG ÜRETİMİ İÇİN EKSPRESYON VEKTÖRLERİNİN OLUŞTURULMASI

YÜKSEK LİSANS TEZİ

Murat ÜSTÜN
(521121144)

Moleküler Biyoloji, Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji, Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Zeynep Petek ÇAKAR

OCAK 2015

Murat Üstün, a **M.Sc.** student of ITU **Graduate School of Science, Engineering and Technology** student ID 521121144, successfully defended the **thesis** entitled "**Construction of Expression Vectors for the Heterologous Production of Kpkt Killer Toxin in *Saccharomyces cerevisiae* and *Pichia pastoris* Cells**", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Zeynep Petek ÇAKAR** Istanbul Technical University	.....
Co-advisor :	**Assoc. Prof.Dr. Ilaria Maria MANNAZZU** Università degli Studi di Sassari	.....
Jury Members :	**Assoc. Prof. Dr. Fatma Neşe KÖK** Istanbul Technical University	.....
	Assoc. Prof. Dr. Nevin GÜL-KARAGÜLER Istanbul Technical University	.....
	Prof. Dr. Nazlı ARDA Istanbul University	.....

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To my family,

FOREWORD

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Murat ÜSTÜN
(Molecular Biologist)

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ABBREVIATIONS

µg	**:** microgram
µL	**:** microliter
µM	**:** micromolar
AOX	:Alcohol Oxidase
CPB	**:** Citrate-Phosphate Buffer
DHA	:Dihydroxyacetone
DHAS	:Dihydroxyacetone Synthase
DNA	**:** Deoxyribonucleic Acid
dNTP	**:** Deoxynucleoside Triphosphates
EDTA	**:** Ethylenediaminetetraacetic Acid
et al.	**:** And others
g	**:** gram
GAP	:Glyceraldehyde 3-phosphate
h	**:** hour
ITS	:Internal Transcribed Spacer
KT	**:** Killer Toxin
KTR	**:** Killer Toxin Receptor
L	**:** liter
LB	**:** Luria Broth
LiAc	:Lithium Acetate
M	**:** molar
MD	**:** Minimal Dextrose
mg	**:** milligram
min	**:** minute
mL	**:** milliliter
mM	**:** millimolar
MM	**:** Minimal Methanol
Mut^{+}	:Methanol Utilization Plus
Mut^{S}	:Methanol Utilization Slow
ng	**:** nanogram
OD	**:** Optical Density
PCR	**:** Polymerase Chain Reaction
PEG	**:** Polyethylene Glycol
PMSF	**:** Phenylmethylsulfonyl Fluoride
SDS	**:** Sodium dodecyl sulfate
TAE	**:** Tris-Acetate-EDTA
TE	**:** Tris-EDTA
YMM	**:** Yeast Minimal Medium
YNB	**:** Yeast Nitrogen Base
YPD	**:** Yeast Extract-Peptone-Dextrose

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CONSTRUCTION OF EXPRESSION VECTORS FOR THE HETEROLOGOUS PRODUCTION OF Kpkt KILLER TOXIN IN *Saccharomyces cerevisiae* AND *Pichia pastoris* CELLS

SUMMARY

In nature, microorganisms process several defense mechanisms to survive under limited nutritional conditions. Killer yeasts have one of the defense mechanisms. They produce toxins called killer toxins (KTs) against sensitive microorganisms. Killer toxins are produced by killer yeasts into extracellular environment and they show their activities by binding to specific compounds called killer toxin receptors (KTRs). Killer toxins can be produced as either proteins or glycoproteins. While killer yeasts have self-immune system to their own KTs, their cytocidal activities usually process in a two-step mechanism on sensitive strains. In the first step, they bind the receptors on cell-wall of the sensitive strains. These receptors are known as primary KTRs. Secondary KTRs found on cell membrane are the second targets of KTs. They bind to these receptors to kill the sensitive strains to enter the cell. Different action mechanisms have been presented for killer toxins and they depend on the genetic determinants as well as physical-chemical properties of KTs.

Kpkt is one of the killer toxins produced by *Tetrapisispora phaffii* (formerly known as *Kluyveromyces phaffii*). Kpkt killer toxin is active against wine spoilage yeasts such as *Kloeckera apiculata* and *Hanseniaspora uvarum*. Therefore, Kpkt has a potential to be used in wine fermentation since it maintains its zymocidal activity for more than 14 days in wine. Kpkt is a glycoprotein and it has a β-glucanase activity. It shows its activity by hydrolyzing β-1,3- and β-1,6- glucans on cell-walls of the sensitive strains. Kpkt killer toxin is encoded by *TpBGL2* gene in *T. phaffii*.

The aim of the study was to construct expression vectors for heterologous production in *Saccharomyces cerevisiae* and *Pichia pastoris*. For this purpose, pYES2.1/V5-His-TOPO® vector was utilized for both extracellular and intracellular production in *S. cerevisiae* as well as pPIC9 vector was utilized for extracellular production in *P. pastoris*.

In the first part of the study, suitable yeast strains resistant to Kpkt were selected for heterologous production of Kpkt. *S. cerevisiae* YPH501 and BY4741 along with *P. pastoris* GS115 strains were subjected to killer plate assay using YPD plates containing citrate-phosphate buffer (pH 4.6). *P. pastoris* GS115 appeared as a suitable strain after this plate assay. Then, *S. cerevisiae* YPH501 and BY4741 were grown in galactose containing media to observe their growth profiles in galactose containing media since pYES2.1/V5-His-TOPO® vector harbors GAL1 promoter which is active only in the presence of galactose. Finally, BY4741 was selected as a suitable strain for transformation.

In this study, three different vectors were constructed: i) *TpBGL2* gene in pYES2.1/V5-His-TOPO® under the control of GAL1 promoter; ii) *TpBGL2* gene in pYES2.1/V5-His-TOPO® under the control of GAL1 promoter and downstream the α sequence; iii) *TpBGL2* gene in pPIC9 under the control of AOX1 promoter and downstream the α sequence. To construct them, first DNA extraction was carried out from *T. phaffii*. Next, DNA quality was checked by PCR amplifying with *ITS1* and *ITS4* universal primers.

First, *TpBGL2* gene was amplified using *BGL2F* and *BGL2R* primers from *T. phaffii* total DNA in order to ligate it directly to pYES2.1/V5-His-TOPO® vector to construct the first vector (*TpBGL2* gene in pYES2.1/V5-His-TOPO® under the control of GAL1 promoter) for intracellular expression in *S. cerevisiae* BY4741. After ligation, vectors were transformed into *Escherichia coli* cells and vectors were extracted from *E. coli* cells through minipreparation. The resulting vectors were further analyzed to investigate both the presence and the orientation of *TpBGL2* gene in the vectors by constructing restriction patterns with *Mly*I and *Xba*I enzymes as well as by performing sequence analysis. In addition, PCR amplification was carried out using *GAL1F* and *BGL2R* primers. All results confirmed that the insert was in frame with GAL1 promoter.

To construct the second and the third vectors, first *TpBGL2* was amplified from *T. phaffii* total DNA with *FW2* and *RV2* primers having *Eco*RI and *Not*I restriction sites, respectively. *TpBGL2* gene flanked by *Eco*RI and *Not*I restriction sites was ligated into pGEM-T Easy Vector by TA cloning. After transformation and cloning in *E. coli* cells, extracted primers were digested by *Eco*RI to confirm the presence of the insert. Next, both empty pPIC9 vectors and pGEM-T Easy vectors having *TpBGL2* gene flanked by *Eco*RI and *Not*I restriction sites were digested by *Eco*RI and *Not*I enzymes. Then, they were ligated to construct the third vector (*TpBGL2* gene in pPIC9 under the control of AOX1 promoter and downstream the α sequence). Both sequence analysis and PCR amplification with *AOX1-BGL2R* primer set confirmed that the insert was in frame with AOX1 promoter and α sequence. To construct the second vector (*TpBGL2* gene in pYES2.1/V5-His-TOPO® under the control of *GAL1* promoter and downstream the α sequence), *TpBGL2* gene downstream the α sequence was amplified using *ALFA* and *BGL2R* primers and the amplicon was ligated into pYES2.1/V5-His-TOPO® vector. However, sequence analysis showed that the insert was in wrong orientation and no further studies were carried out with this vector.

pYES2.1/V5-His-TOPO® + *TpBGL2* vector was transformed into *S. cerevisiae* BY4741 strain for intracellular production of Kpkt killer toxin and the presence of the insert was confirmed by PCR analysis in *S. cerevisiae* transformants. The selection was carried out on YNB selective medium without uracil plates. One of the six transformants called T1 was cultivated in 2% (w/v) galactose and 2% (w/v) raffinose containing buffered yeast minimal medium. After 24 h and 48 h, sampling was carried out for crude extraction and for taking supernatant from the culture. Next, well plate assay was performed to investigate killer activity on buffered YPD plates spread by sensitive *S. cerevisiae* DBVPG 6500 strain to Kpkt killer toxin. T1 was also cultivated in 0.2% (w/v) galactose and 2% (w/v) raffinose containing buffered yeast minimal medium for 48 h and it was transferred into 2% (w/v) galactose containing minimal medium. After 24 h incubation, well plate assay was performed again for both crude extract and the supernatant of the culture. In addition, T1 was cultivated in 2% sucrose (w/v) containing buffered selective minimal

medium and it was transferred into 2% (w/v) galactose containing buffered selective medium after 48 h of growth. Well plate assay was performed again after 24 h of growth in galactose containing medium. All studies showed that Kpkt killer toxin did not perform its killing activity when it was expressed intracellularly.

Finally, the third vector (PIC9 + *TpBGL2*) was transformed into *P. pastoris* GS115 cells for extracellular expression of Kpkt in *P. pastoris*. To do that, *P. pastoris* cells were prepared to obtain competent cells for transformation which was carried out through electroporation. For transformation, linearized plasmids obtained by digesting with either *Sac*I or *Bgl*II enzyme were utilized along with linearized empty pPIC9 vectors with the same enzymes. Selection was carried on minimal dextrose plates. Next, the transformants were grown on both minimal dextrose and minimal methanol plates to determine putative Mut^+ (Methanol utilization plus) or Mut^S (Methanol utilization slow) phenotypes of the transformants since only Mut^+ phenotypes can grow on methanol containing medium if the insertion results in functional *AOX1* gene encoding alcohol oxidase, which is the main enzyme in methanol utilization pathway. Finally, transformants were subjected to PCR amplification using *AOX1* and *AOXR* primers to investigate whether they have inserts. The evaluation of the killer activity of Kpkt killer toxin in *P. pastoris* is in progress.

Kpkt KATİL TOKSİNİNİN *Saccharomyces cerevisiae* VE *Pichia pastoris* HÜCRELERİNDE HETEROLOG ÜRETİMİ İÇİN EKSPRESYON VEKTÖRLERİNİN OLUŞTURULMASI

ÖZET

Mikroorganizmalar, doğada sınırlı besin koşullarında hayatta kalabilmek için diğer mikroorganizmalarla mücadele halindedirler. Bu yüzden, farklı savunma mekanizmaları geliştirerek diğer türlere karşı üstünlük kurmaya çalışırlar. Katil mayalar, bu savunma mekanizmalarından birine sahiptirler. Bu özel maya türleri protein ya da glikoprotein şeklinde katil toksinler sentezlerler. Bu ürettikleri katil toksinleri hücre dışı ortama çıkararak bu toksinlere karşı dirençli olmayan mikroorganizmaların ölmesine neden olurlar. Katil toksinler aktivitelerini ancak o toksine karşı dirençli olmayan mikroorganizmalarda bulunan spesifik reseptörlere bağlanarak gösterirler. Bu reseptörler katil toksin reseptörleri olarak adlandırılır. Katil mayalar, kendi ürettikleri toksinlere karşı dirençlidir. Fakat dirençli olmayan mikroorganizmalarda genellikle iki aşamalı bir mekanizmayla sitosidal etkilerini gösterirler. İlk olarak dirençli olmayan mikroorganizmaların hücre duvarındaki çeşitli bileşenlere bağlanarak hücreyi öldürebilirler veya hücre membranına geçerler. Hücre duvarında bulunan bu reseptörler birincil katil toksin reseptörleri olarak adlandırılır. İkinci aşamada ise hücre membranı üzerindeki bileşenlere bağlanıp sitosidal etkilerini gösterebilecekleri gibi hücre içine geçerek de hücrelerin ölümüne neden olabilirler. Hücre membranı üzerinde bulunan bu reseptörler ise ikincil katil toksin reseptörleri olarak bilinir. Katil toksinler farklı etki mekanizmalarına sahiptir ve bu etki mekanizmaları genetik belirleyicilere ve katil toksinlerin fiziko-kimyasal özelliklerine bağlıdır. Son yıllarda yapılan çalışmalarda, katil toksinlerin endüstriyel ve terapötik alandaki potansiyel kullanım alanları gösterildiğinden, bu toksinler önemli bir yere sahip olmaya başlamıştır. Bu toksinlerin gıda koruyucusu, alkol fermentasyonunda biyo-kontrol ajanı, kemoterapötik ve antibakteriyel ajanlar olarak kullanımı ileriki yıllarda hedeflenmektedir.

Kpkt katil toksini son yıllarda endüstriyel bir potansiyele sahip olması nedeniyle önem kazanmıştır. Kpkt katil toksini *Tetrapisispora phaffii* (önceden bilinen adıyla *Kluyveromyces phaffii)* tarafından üretilmektedir. Bu toksin, şarapların bozulmasına neden olan mayalar üzerinde etkili olduğu için alkol fermentasyonunda potansiyel bir kullanım alanına sahiptir. Kpkt, özellikle *Kloeckera apiculata* ve *Hanseniaspora uvarum* türleri üzerinde ölümcül etkiye sahiptir. Ayrıca şarap fermentasyonu sırasında 14 günden fazla süreyle etkisini gösterebildiğinden değerli bir toksindir. Kpkt toksini, bir glikoproteindir ve β-glukanaz aktivitesine sahiptir. Sitosidal aktivitesini ise bu toksine dirençli olmayan mayaların hücre duvarlarında bulunan β-1,3- ve β-1,6- glukanları hidrolize ederek gösterir. Kpkt, *T. phaffii* tarafından *TpBGL2* geni ile ifade edilir.

Bu çalışmanın amacı, Kpkt katil toksinini heterolog olarak *Saccharomyces cerevisiae* ve *Pichia pastoris*'te üretebilmek için ekspresyon vektörlerinin oluşturulmasıdır. Bu amaçla iki farklı ekspresyon vektörü kullanılmıştır. pYES2.1/V5-His-TOPO® vektörü Kpkt katil toksininin *S. cerevisiae*'de hücre içi ve hücre dışı üretimi için kullanılmışken, pPIC9 vektörü bu toksinin *P. pastoris*'te hücre dışı üretimi amaçlanarak kullanılmıştır.

Çalışmanın ilk bölümünde, Kpkt'nin heterolog üretimi için bu toksine karşı dirençli uygun *S. cerevisiae* ve *P. pastoris* suşları seçilmiştir. Bunun için *S. cerevisiae* BY4741 ve YPH501 suşları ile *P. pastoris* GS115 suşları tercih edilmiştir. Tüm suşlar, sitrat-fosfat tampon çözeltisi (pH 4.6) içeren katı besiyerlerine ayrı ayrı ekilerek Kpkt'ye karşı dirençleri gözlemlenmiştir. *P. pastoris* GS115 suşu bu toksine karşı güçlü bir direnç gösterdiğinden sonraki çalışmalarda heterolog üretim için tercih edilmiştir. *S. cerevisiae* BY4741 ve YPH501 suşları ise galaktoz içeren besiyerlerinde ayrı ayrı büyütülerek büyüme profilleri gözlemlenmiştir. Daha sonraki aşamada transformasyon için pYES2.1/V5-His-TOPO® vektörü kullanıldığından ve bu vektör sadece galaktoz varlığında indüklenen GAL1 promotörüne sahip olduğundan, galaktoz varlığında üreyebilen suş seçilmeye çalışılmıştır. Sonuç olarak, BY4741 transformasyon için uygun *S. cerevisiae* suşu olarak seçilmiştir.

Bu çalışmada üç farklı vektör dizayn edilmiştir: i) GAL1 promotörü kontrolünde *TpBGL2* genini içeren pYES2.1/V5-His-TOPO® vektörü; ii) GAL1 promotörü kontrolünde ve α sekansı sonrası *TpBGL2* geni içeren pYES2.1/V5-His-TOPO® vektörü; iii) AOX1 promotörü kontrolünde ve α sekansı sonrası *TpBGL2* geni içeren pPIC9 vektörü.

Bu vektörleri oluşturmak için ilk olarak *T. phaffii*'nin total DNA'sı izole edilmiştir. Daha sonra, elde edilen total DNA'nın kalitesini belirlemek amacıyla evrensel primerler olan *ITS1* ve *ITS4* kullanılarak polimeraz zincir reaksiyonu (PZR) ile bu primerlere spesifik olan kısım çoğaltılmıştır ve elde edilen total DNA sonraki PZR amplifikasyonları için kullanılmıştır.

S. cerevisiae'da Kpkt'nin hücre içi üretimi amacıyla ilk vektörü (GAL1 promotörü kontrolünde *TpBGL2* genini içeren pYES2.1/V5-His-TOPO® vektörü) oluşturmak için, *T. phaffii* total DNA'sından *TpBGL2* geni *BGL2F* ve *BGL2R* primerleri kullanılarak PZR ile çoğaltılmıştır. Daha sonra bu gen pYES2.1/V5-His-TOPO vektörüne ligasyon ile yerleştirilmiştir. Ligasyondan sonra vektörler ilk olarak *Escherichia coli* hücrelerine klonlanmıştır ve vektörler *E. coli* hücrelerinden izole edilmiştir. Elde edilen vektörlerde *TpBGL2* geninin hem varlığını belirlemek hem de düzgün oryantasyonda yerleştiğini anlamak amacıyla ilk olarak *Mly*I ve *Xba*I enzimleri ile kesilmiştir. Buna ek olarak, vektörler *GAL1* ve *BGL2R* primerleri ile çoğaltılmıştır ve sekanslama analizinin sonuçlarıyla birleştirilerek *TpBGL2* geninin vektörlere doğru oryantasyonla girdiği doğrulanmıştır.

İkinci ve üçüncü vektörleri oluşturmak için ise ilk olarak *TpBGL2* geni 5' ucundan ve 3' ucundan sırasıyla *Eco*RI ve *Not*I restriksiyon kesim bölgelerine sahip olacak şekilde *FW2* ve *RV2* primerleriyle PZR ile çoğaltılmıştır. *Eco*RI ve *Not*I restriksiyon kesim bölgeleri içeren *TpBGL2* geni ilk olarak pGEM-T vektörüne ligasyon ile yerleştirilmiştir ve *E. coli* hücrelerinde klonlanmıştır. Ardından vektörler izole edilmiştir ve *TpBGL2* geninin vektörlerdeki varlığını belirlemek amacıyla *Eco*RI ile restriksiyon kesimi yapılmıştır. Daha sonra, hem boş pPIC9 vektörleri hem de *TpBGL2* geni içeren pGEM-T vektörü *Eco*RI ve *Not*I enzimleri kullanılarak kesilmiştir. Restriksiyon ürünleri ligasyon ile birleştirilmiştir ve üçüncü vektör

(AOX1 promotörü kontrolünde ve α sekansı sonrası *TpBGL2* geni içeren pPIC9 vektörü) elde edilmiştir. Bu vektörde *TpBGL2* geninin doğru oryantasyonda olduğunu belirlemek amacıyla PZR kullanılarak *AOX1* ve *BGL2R* primerleri ile amplifikasyon yapılmıştır. Sekanslama sonuçları da hedef genin doğru oryantasyonla vektör içerisine girdiğini doğrulamıştır. Ardından ikinci vektörü (GAL1 promotörü kontrolünde ve α sekansı sonrası *TpBGL2* geni içeren pYES2.1/V5-His-TOPO® vektörü) oluşturmak için *TpBGL2* geni α sekansı ile birlikte *ALFA* ve *BGL2R* primerleri kullanılarak PZR ile çoğaltılmıştır. Elde edilen PZR ürünleri pYES2.1/V5-His-TOPO® vektörüne klonlanmıştır. Daha sonra, vektör içerisindeki oryantasyonunu belirlemek amacıyla sekanslama analizi yapılmıştır. Sekanslama sonucunda insert'in vektör içerisinde yanlış oryantasyonda olduğu belirlenmiştir. Bu yüzden, sonraki çalışmalarda bu vektör kullanılmamıştır ve maya hücrelerine transformasyon için elde edilen üç vektörden diğer ikisi kullanılmıştır.

Kpkt'nin hücre içi üretimi için tasarlanan pYES2.1/V5-His-TOPO® + *TpBGL2* vektörü *S. cerevisiae* BY4741 suşuna transforme edilmiştir. *TpBGL2* geninin *S. cerevisiae* transformantlarındaki varlığı total DNA ekstraksiyonundan sonra PZR ile doğrulanmıştır. Seleksiyon işlemi urasil içermeyen minimal katı besiyerinde yapılmıştır. Elde edilen altı *S. cerevisiae* transformantından bir tanesi (T1) Kpkt'nin aktivitesini incelemek amacıyla kullanılmıştır. Bu amaçla, T1 ilk olarak %2 galaktoz ve %2 rafinoz içeren tampon çözeltili (pH 4.6) minimal besiyerinde büyütülmüştür. Hem 24 saat sonra hem de 48 sonra örnek alımı yapılmıştır ve hücrelerin hem kaba özütü hem de üst fazı (süpernatantı) elde edilmiştir. Bu örnekler *S. cerevisiae* DBVPG 6500 (Kpkt'ye karşı hassas suş) ile ekilmiş katı besiyerinde oluşturulan kuyucuklara aktarılmıştır ve Kpkt katil toksininin varlığı hassas suşa karşı aktivitesine bakılarak belirlenmiştir. İkinci olarak T1, %0.2 galaktoz ve %2 rafinoz içeren minimal besiyerinde büyütülüp %2 galaktoz içeren minimal besiyerine aktarılmıştır. Tekrar örnekleme yapılıp katı besiyerinde aktivitesine bakılmıştır. Son olarak T1 %2 sükroz içeren seçici besiyerinde inkübe edildikten sonra, %2 galaktoz içeren minimal besiyerine aktarılmıştır ve hem kaba özüt için hem de üst faz için örnekleme yapılarak katı besiyerinde Kpkt'ye hassas suş üzerindeki aktivitesine bakılmıştır. Tüm büyüme stratejileri sonucunda yapılan testlerde, Kpkt'nin hücre içi üretimi durumunda sitosidal aktivitesini gösteremediği belirlenmiştir.

Son olarak, Kpkt'nin *P. pastoris'te* hücre dışı üretimi amacıyla üçüncü vektör (PIC9 + *TpBGL2*) elektroporasyon ile *P. pastoris* GS115 suşuna transforme edilmiştir. İlk olarak *P. pastoris* hücreleri kompetan hücre elde etmek amacıyla hazırlanmıştır. Daha sonra, hem insert içeren hem de boş pPIC9 vektörleri *Sac*I ya da *Bgl*II enzimi kullanılarak lineerize edilmiştir ve lineerize edilen vektörler transformasyonda kullanılmıştır. Seleksiyon minimal dekstroz besiyerinde yapılmıştır. Ardından elde edilen transformantlar hem minimal dekstroz hem de minimal metanol katı besiyerlerinde ayrı ayrı üretilmiştir ve Mut^+ (metanol kullanabilen) ile Mut^S (metanol kullanımı yavaş) fenotiplere aday transformantlar belirlenmiştir. Alkol oksidaz enzimini ifade eden *AOX1* geni transformasyon sonrası fonksiyonel olması durumunda Mut^+ fenotipi gözlemleneceğinden fenotip testi yapılmıştır ve ek olarak PZR amplifikasyonu yapılarak insert içeren transformantlar belirlenmiştir. Kpkt katil toksininin *P. pastoris*'te üretildikten sonraki sitosidal aktivitesi ile ilgili çalışmalar devam etmektedir.

1. INTRODUCTION

1.1 General Information about *Saccharomyces cerevisiae*

Yeasts classified in kingdom fungi are known as eukaryotic unicellular organisms. *Saccharomyces cerevisiae* is one of the members of this kingdom as a species of class *Saccharomycetes*. It has been utilized as a main organism in baking and alcoholic beverage industry due to its fermentative properties for long years (Url-1). Scanning electron microscope image of *S. cerevisiae* is shown in Figure 1.1 (Alberts, 2008).

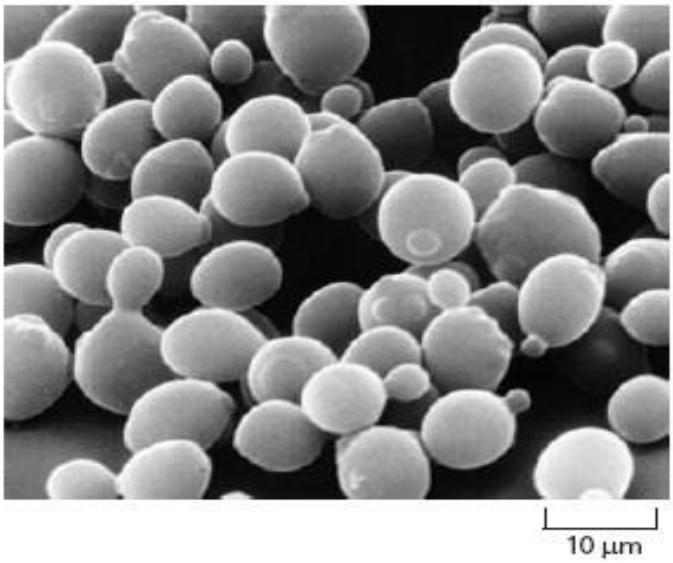

Figure 1.1 : Scanning electron microscope (SEM) image of *S. cerevisiae* (Alberts, 2008).

S. cerevisiae has been preferred as a common model organism in biological research. Since it is classified as a eukaryotic organism, some similarities exist between mammalian cells and *S. cerevisiae* cells at organelle and macromolecular level. This provides an opportunity to identify homologous proteins between those two different cell types (Costa and Moradas-Ferreira, 2001). In addition to this, *S. cerevisiae* has also been commonly used as a model eukaryotic organism to overcome experimental and ethical limitations caused by mammalian cells/organisms. Therefore, it is utilized

as a model organism in different biological processes such as heterologous protein expression, aging studies, signal transduction, gene expression regulation analysis and cell cycle identification (Karathia *et al.,* 2011).

The whole genome of *S. cerevisiae* was sequenced in middle of 1990's (Goffeau *et al.,* 1996) and *S. cerevisiae* has become a more popular suitable model eukaryotic organism for genetic engineering studies since it is easily cultured and it grows fast. Studies have shown that *S. cerevisiae* possesses 16 chromosomes comprised by ~ 12.5 mega base pairs of DNA. There are approximately 6000 open reading frames (ORFs) in *S. cerevisiae* genome (Goffeau *et al.,* 1996 and Link and Olson, 1991). *S. cerevisiae* cells divide and proliferate by budding which produces a daughter cell asymmetrically on the surface of its mother cell. After forming a bud on the surface of the mother cell, nuclear division and cell wall formation occur to produce a daughter cell. (Shcheprova *et al.,* 2008). Budding known as mitotic phase of the yeast cells is seen on both haploid (n) and diploid (2n) forms. Diploid state of the cells is formed by mating of two types of haploid phases: 'a' and 'alpha' (α). Haploid types are determined by *MATa* or *MATα* alleles locating on *MAT* locus referring to mating type which is found on the chromosome III. Haploid forms could be produced through meiosis by diploid forms of the cells (Herskowitz, 1988). In the haploid phase, two types of mating factors are secreted. They are called a-factor and α-factor which are secreted by a and α cells, respectively. These factors are hormones which work on the opposite cell type (Anderegg *et al.,* 1988) (Figure 1.2, Herskowitz, 1988).

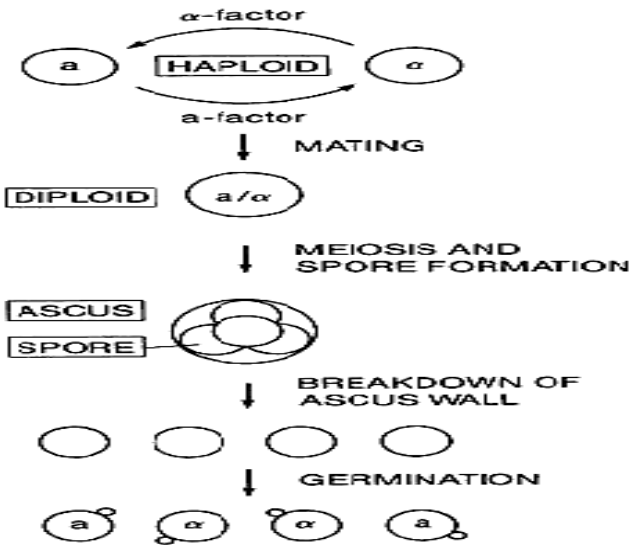

Figure 1.2 : Life cycle of *S. cerevisiae* (Herskowitz, 1988)

1.2 Pichia pastoris and Methanol Metabolism

The yeast *Pichia pastoris* is ascribed to the class of *Saccharomycetes*. It is widely used for heterologous expression of recombinant proteins in molecular biology studies and medical studies all around the world since it provides some advantages for eukaryotic protein expression such as protein folding and posttranslational modifications (Cregg *et al*., 2000). *P. pastoris* is an important organism especially for producing biopharmaceuticals due to its protein folding ability. Both heterologous glycosylated and non-glycosylated proteins can be expressed in *P. pastoris* such as serum albumin and some vaccines (Watanabe *et al*., 2001, Hardy *et al*., 2000).

Recent studies have reported that the GS115 strain of *P. pastoris* have a genome of 9.43 Mbp of DNA comprising 4 chromosomes. In this genome, there are 5,313 protein-coding genes which encompass 80% of the total genome. This indicates that it has dense genome compared to those of other unicellular eukaryotes (De Schutter *et al*., 2009).

P. pastoris is a methylotrophic yeast which means that it grows in culture media containing methanol as the sole carbon source (Veenhuis *et al*., 1983). The first step in methanol assimilation pathway (Figure 1.3) (Cereghino and Cregg, 2000) is catalyzed by the enzyme alcohol oxidase (AOX) which oxidizes methanol to produce formaldehyde and hydrogen peroxide. Hydrogen peroxide is then degraded by catalase in peroxisomes with the production of oxygen and water. Formaldehyde is turned into formate and CO_2 through oxidation carried out by two cytoplasmic dehydrogenases. These reactions provide cells with energy for growing. Formaldehyde is also condensed with xylulose 5- monophosphate to produce cellular constituents by means of a cyclic pathway. The product of this reaction catalyzed by dihydroxyacetone synthase (DHAS) is glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone (DHA). They are utilized in the cyclic pathway to regenerate xylulose 5-monophosphate. One net molecule of GAP is produced in every three cycle. The production of AOX and DHAS is induced by methanol and repressed by other carbon sources (Couderc and Baratti, 1980; Roggenkamp *et al*., 1984).

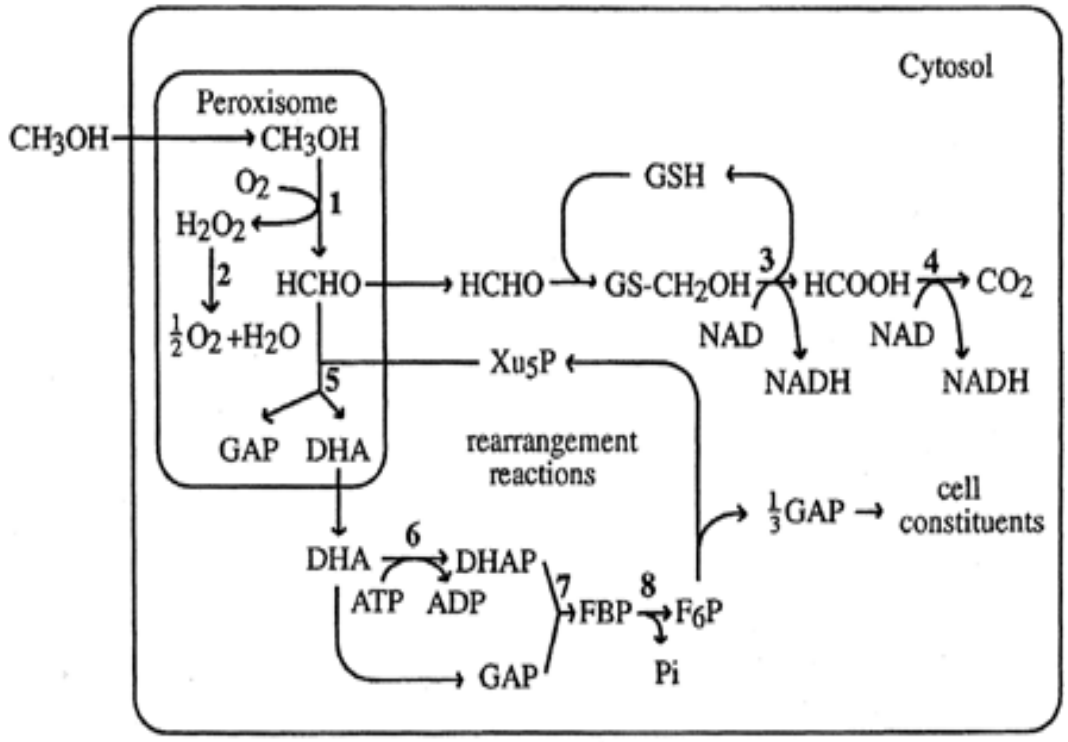

Figure 1.3 : Methanol utilization pathway in *P. pastoris*; 1: alcohol oxidase, 2: catalase, 3: formaldehyde dehydrogenase, 4: formate dehydrogenase, 5: dihydroxyacetone synthase, 6: dihydroxyacetone kinase, 7: fructose 1,6-biphosphate aldolase, 8: fructose 1,6-bisphosphatase (Cereghino and Cregg, 2006)

1.3 Expression Systems Used in This Study

The vectors utilized were pYES2.1/V5-His-TOPO® and pPIC9 for heterologous expression of Kpkt killer toxin in *S. cerevisiae* and *P. pastoris*, respectively.

pYES2.1/V5-His-TOPO® vector utilizes TOPO® cloning (also known as TA Cloning) to ligate fresh PCR product amplified by Taq polymerase. Since Taq polymerase adds a single deoxyadenosine (A) to the 3´ ends of PCR products, it is directly ligated into the vector containing overhanging 3´ deoxythymidine (T) residues. TA Cloning is catalyzed by topoisomerase I from *Vaccinia* virus. The enzyme cuts phosphodiester backbone after 5′-CCCTT by binding to duplex DNA (Shuman, 1991). The energy after cutting phosphodiester backbone is further utilized to form covalent bond between a tyrosyl residue (Tyr-274) of topoisomerase I and the 3′ phosphate of the cut strand. The bond between DNA (vector) and the enzyme is then attacked by the 5′ hydroxyl of the insert (PCR product) and the enzyme is released by reversing the reaction (Shuman, 1994).

In this vector, GAL1 promoter controls the transcription of the gene of interest. Even though glucose is the main energy source of *S. cerevisiae* since glycolysis pathway utilizes glucose to produce energy, galactose can be utilized as an alternative energy source when glucose is not available. Galactose activates the gene encoding the enzymes for both transporting galactose into the cell and converting it to glucose-6-phosphate (G6P) which is an intermediate of glycolysis. Galactokinase is the first enzyme used in galactose pathway and it is encoded by *GAL1* gene under the control of GAL1 promoter. GAL1 promoter has negative and positive regulatory sites and it is regulated by repressor proteins and transcription factors. In the absence of glucose and the presence of galactose, Gal4p, which is a zinc-finger transcription factor binds to the positive regulatory site and transcription of the gene under the control of GAL1 promoter is activated. In the presence of glucose, repressor protein Gal80p binds to Gal4p and transcription is repressed (catabolite repression) as shown in Figure 1.4 (Url-2). In the presence of raffinose, transcription occurs, but it cannot induce transcription as much as galactose (Johnston, 1987).

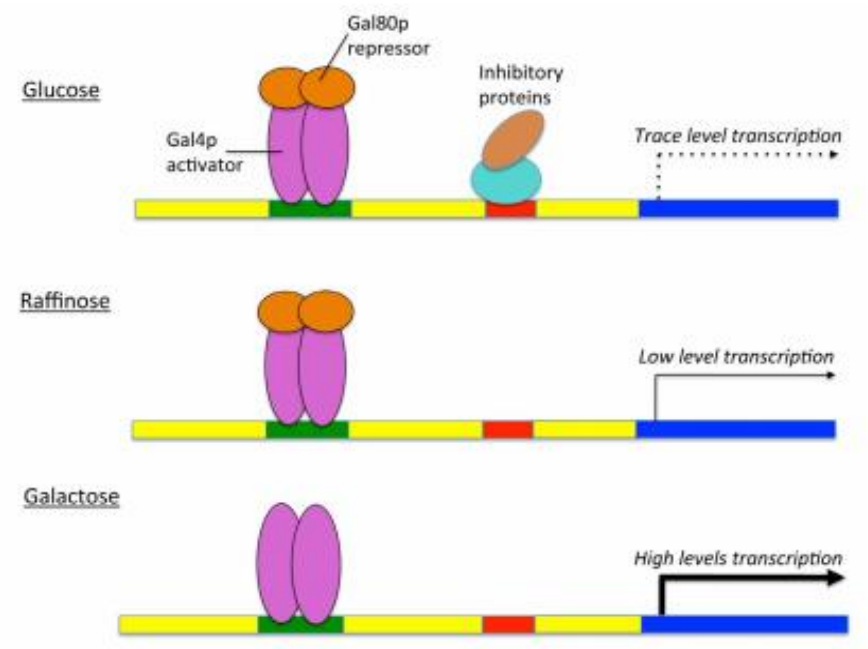

Figure 1.4 : Regulation of GAL1 promoter (Url-2).

In pPIC9, the expression of the gene of interest is under the control of the promoter of *AOX1* gene called AOX1 promoter. *AOX1* gene codes for alcohol oxidase, which is the main enzyme in methanol utilization pathway as described in 1.2. Like GAL1 promoter, AOX1 promoter is regulated by carbon sources. In particular, AOX1

promoter is switched on by methanol. In methanol containing media, Aox1p constitutes more than 30% of the total soluble proteins in the cell. On glucose containing media, AOX1 promoter is repressed and methanol utilization is carried out by AOX2 enzyme, which is another alcohol oxidase in *P. pastoris*. Since growth on methanol is much slower with AOX2 enzyme than that with AOX1, inactivation of AOX1 promoter and loss of AOX1 enzyme results in a phenotype called Mut^S (Methanol utilization slow). Wild type phenotype of *P. pastoris*, which can utilize methanol is called Mut+ (Methanol utilization plus) (Ellis *et al*., 1985; Koutz *et al*., 1989; Tschopp *et al*., 1987).

1.4 Killer Toxins Produced by Yeasts

In nature, microorganisms fight with each other under limited nutritional conditions to survive through different defense mechanisms processed in the cells (Magliani *et al*., 2008). In recent years, killer yeasts producing killer toxins (KTs) have been identified in nature. Killer phenomenon was firstly described by Bevan and Makover (1963). These authors observed that *S. cerevisiae* strains were able to produce extracellular proteins active against sensitive strains of the same species having specific cell wall killer toxin receptors (KTRs). The yeasts producing KTs have self-immune system to their own toxins. KTs are secreted as proteins or glycoproteins by yeasts and they show their cytocidal activity on sensitive strains in a two-step mechanism. Firstly, they bind to KTRs on cell-wall of the sensitive strains. They can either bind to secondary KTRs on cell membrane or enter the cell to kill it. There are different mechanisms to recognize and kill the sensitive strains and there are different genetic determinants along with different physical-chemical properties of KTs (Magliani *et al*., 2008).

Different cell-wall components can act as primary KTRs for KTs produced by different yeast strains. Recent studies showed that β-1,6-glucan is the main target for killer toxins K1 and K2 produced by *S. cerevisiae* (Hutchins and Bussey, 1983) and for the killer toxin of *Hanseniaspora uvarum* (Radler *et al*., 1990). Mannoproteins are also identified as KTRs for killer toxins KT28 produced by *S. cerevisiae* (Schmitt and Radler, 1987) and for *Zygosaccharomyces bailii* killer toxin (zygocin) (Radler *et al*., 1993). In addition, chitin is known as another KTR for killer toxin of

Kluyveromyces lactis (Takita and Castilho-Valavicius, 1993). β-1,3-Glucan has also known as KTR for *Hansenula mrakii* LKB 169 killer toxin (Kasahara *et al*., 1994).

Rather little is known on secondary KTRs, Breining *et al*. (2002) showed that Kre1p protein is a secondary KTR for killer toxin K1 of *S. cerevisiae*. Kre1p is a glycosyl phosphatidyl inositol (GPI)-anchored and is responsible for synthetizing β-1,6-glucan. Cwp2p and its GPI-anchored precursor are the other membrane receptors identified specific to killer toxin PMKT, which is known as channel-forming killer toxin produced by *Pichia membranifaciens* (Santos *et al*., 2007).

The mechanism of action of a killer toxin may vary depending on the interactions between killer toxins and their target sensitive strains. In addition to PMKT; K1, K2, and zygocin are channel-forming killer toxins which damage cell membrane by producing cation-selective ion channels on the membrane. While some of the KTs interact with proteins involved in cell cycle, some of them block proteins responsible for chromosomal DNA synthesis (Magliani *et al*., 2008). For instance, Klkt stops cell cycle permanently at the G_1 phase (Butler *et al*., 1991) whereas K28 causes cell-cycle arrest at the G_1/S phase permanently (Schmitt and Breinig, 2006). Some other KTs such as *Pichia acaciae* and *W. robertsiae* KTs arrest cell-cycle at the S phase permanently to kill the sensitive strains (Klassen *et al*., 2004).

Killer toxins have a number of potential applications. They can be used in ecological, fermentation and medical studies. They can be used as chemotherapeutic agents, food preservatives, biocontrol agents against food and wine spoilage yeasts, bacteria, fungi (Bajaj and Sharma, 2010; Hernandez *et al*., 2008). In a recent study, Bajaj *et al*. (2013) identified a novel killer toxin produced by *Pichia kudriavzevii* RY55. This killer toxin has an antibacterial activity on some human pathogens such as *Escherichia coli, Enterococcus faecalis, Klebsiella sp., Staphylococcus aureus, Pseudomonas aeruginosa* and *Pseudomonas alcaligenes*. Similarly, a mosquito-associated strain ascribed to the species *Wickerhamomyces anomalus* was identified as killer yeast, which produces killer toxin having antimicrobial activity. Therefore, it is a promising bioagent for the symbiotic control of malaria (Cappelli *et al*, 2014). In another study, killer toxin HMK produced by *Williopsis mrakii* was presented as a potential food preservative against silage spoilage and yoghurt spoilage by other yeasts due to high thermal stability, high pH stability, and broad spectrum of the killer toxin (Lowes *et al*., 2000). Killer toxins also have a potential to be used in

biotyping of important microorganisms utilized in medical studies and in industry (Buzzini *et al*., 2007).

1.5 Kpkt Killer Toxin Produced by *Tetrapisispora phaffii*

Tetrapisispora phaffii which was formerly known as *Kluyveromyces phaffii* (Ueada-Nishimura and Mikata, 1999) was first identified by Van der Walt (1963). It was first classified as *Fabospora* genera. It was then assigned to *Kluyveromyces* genus by Lodder (1970). Finally, it was named *Tetrapisispora phaffii* according to phylogenetic analyses showing its close relationships with *Tetrapisispora iriomotensis*, *Tetrapisispora nanseiensis* and *Tetrapisispora arboricola* (Ueada-Nishimura and Mikata, 1999). *T. phaffii* cells reproduce by bipolar budding and they are known as homothallic and diploid. The whole genome of *T. phaffii* was sequenced in recent years showing that it has 16 chromosomes with 5250 protein coding genes (Gordon *et al*, 2011).

Tetrapisispora phaffii cells produce a killer toxin known as Kpkt killer toxin, which is especially active against wine spoilage yeasts such as *Kloeckera apiculata* and *Hanseniaspora uvarum* (Ciani and Fatichenti, 2001). This property makes Kpkt killer toxin a valuable product in wine fermentation industry considering that it maintains its zymocidal activity for more than 14 days in wine. In addition, during this period it blocks the proliferation of sensitive spoilage yeasts by repressing their metabolic activity. Thus, Kpkt killer toxin is a potential biocompound to be used in pre-fermentative process of alcoholic fermentation (Comitini *et al*., 2010; Oro *et al*., 2014).

Gene disruption and genetic analyses have recently showed that Kpkt killer toxin is encoded by *TpBGL2* gene in *T. phaffii* (Oro *et al*., 2014). The gene shows great similarity with *BGL2* gene, which codes for Bgl2p protein with a molecular mass of 29 kDa in *S. cerevisiae*. Bgl2p protein is known as a glucan transferase, which is a cell wall protein (Mrsa *et al*., 1993; Goldman *et al*., 1995; Sarthy *et al*., 1997).

Molecular characterization of Kpkt has showed that it is a glycoprotein with a molecular mass of 33 kDa. It shows a β-glucanase activity (Comitini *et al*., 2004). Accordingly, Kpkt killer toxin shows its cytocidal activity by hydrolyzing β-glucans on the cell wall of the sensitive strains. Unlike other killer toxins such as K1 and K2

of *S. cerevisiae* (Novotna *et al*., 2004), Kpkt killer toxin does not have any effect on cell membrane (Comitini *et al.*, 2009). The primary receptors of Kpkt killer toxin on the cell wall of sensitive strains are β-1,3- and β-1,6- glucans. Thus, it shows its killing activity by recognizing these components on the cell wall (Comitini *et al*., 2004).

1.6 Aim of the Study

Killer yeast cells and their killer toxins could be used to fight against spoilage yeasts in the winemaking industry. Kpkt killer toxin, which is produced by *Tetrapisispora phaffii* (previously classified as *Kluyveromyces phaffii*), is a glycoprotein of 33kDa and it is a promising killer toxin for wine spoilage yeasts. The aim of this study is to clone *TpBGL2* gene encoding Kpkt killer toxin protein into two different expression vectors in view of the implementation of a biotechnological process for the heterologous production of Kpkt toxin in *Saccharomyces cerevisiae* and *Pichia pastoris*. To do that, *TpBGL2* gene was cloned in pYES2.1/V5-His-TOPO® and in pPIC9 vectors and the recombinant plasmids obtained were transformed in *S. cerevisiae* and *P. pastoris*, respectively.

2. MATERIALS AND METHODS

2.1 Materials and Laboratory Equipment

2.1.1 Microorganisms used in the study

The microorganisms used were: *Tetrapisispora phaffii* CBS4417 natural producer of Kpkt; *S. cerevisiae* DBVPG 6500 killer toxin sensitive strain; *S. cerevisiae* BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and *S. cerevisiae* YPH501 (MATa/MATα ura3-52/ura3-52 lys2-801_amber/lys2-801_amber ade2-101_ochre/ade2-101_ochre trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1), laboratory strains to be used as hosts for the heterologous production of Kpkt; *Pichia pastoris* GS115, laboratory strain to be used as host for the heterologous production of Kpkt; chemically competent TOP10 One Shot® *Escherichia coli* (provided by pYES2.1/V5-His-TOPO® TA Expression Kit) for molecular biology procedures, chemically competent *Escherichia coli* JM109 (Promega, USA) for molecular biology procedures.

2.1.2 Media compositions

2.1.2.1 Yeast extract peptone dextrose (YPD) medium

Table 2.1 : Ingredients of YPD medium.

Component	Concentration (w/v)
Yeast extract	1.00 %
Peptone	2.00 %
Dextrose	2.00 %
Agar (only for solid media)	2.00 %

2.1.2.2 Super optimal broth with catabolite repression (SOC) medium

Table 2.2 : SOC medium ingredients.

Component	Concentration (w/v)
Yeast extract	0.50 %
Tryptone	2.00 %
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	20 mM

2.1.2.3 Yeast minimal medium (YMM) with raffinose and galactose

Table 2.3 : Ingredients of YMM with raffinose and galactose.

Component	Concentration (w/v)
Galactose	2.00 % or 0.2 %
YNB without amino acids and without ammonium sulphate	0.17 %
Ammonium sulphate	0.50 %
Raffinose (Filter sterilized)	2 %
Adenine Arginine Cysteine Leucine Lysine Threonine Tryptophan Uracil	0.01% (For each one)
Aspartic Acid Histidine Isoleucine Methionine Phenylalanine Proline Serine Tyrosine Valine	0.005 % (For each one)
Agar (only for solid media)	2.00 %

2.1.2.4 Luria broth (LB) medium

Table 2.4 : LB medium content.

Component	Concentration (w/v)
Yeast extract	0.50 %
Tryptone	1.00 %
NaCl	1.00 %
Agar (only for solid media)	2.00 %

2.1.2.5 Yeast nitrogen base (YNB) selective medium without uracil

Table 2.5 : Contents of YNB selective medium without uracil.

Component	Concentration (w/v)
Carbon source (Glucose, Galactose, Raffinose, Sucrose)	2.00 %
YNB without amino acids and without ammonium sulfate	0.17 %
Ammonium sulfate	0.50 %
Adenine Arginine Cysteine Leucine Lysine Threonine Tryptophan	0.01% (For each one)
Aspartic Acid Histidine Isoleucine Methionine Phenylalanine Proline Serine Tyrosine Valine	0.005 % (For each one)
Agar (only for solid media)	2.00 %

2.1.2.6 Minimal dextrose (MD) medium

Table 2.6 : Minimal dextrose (MD) medium contents.

Component	Concentration (w/v)
Yeast nitrogen base	1.34 %
Biotin	$4x10^{-5}$ %
Glucose	2.00 %
Agar (only for solid media)	2.00 %

2.1.2.7 Minimal methanol (MM) medium

Table 2.7 : The ingredients of minimal methanol (MM) medium.

Component	Concentration (w/v)
Yeast nitrogen base	1.34 %
Biotin	$4x10^{-5}$ %
Methanol	0.50 %
Agar (only for solid media)	2.00 %

2.1.3 Chemicals, laboratory equipment, solutions, buffers, enzymes, kits, vectors and primers

The list of chemicals and enzymes used in this study is presented in Table 2.8.

Table 2.8 : Chemicals used in this study.

Chemical	Supplier (Country)
Acetic Acid Glacial	Carlo Erba Reagents (Italy)
Adenine	Sigma (USA)
Agar	Oxoid (England)
Agarose	Sigma (USA)
Albumin from Bovine Serum	Sigma (USA)
Ammonium sulfate	Fluka (Germany)
Ampicillin	Sigma (USA)
Bacto Yeast-Extract	Microbiol (Italy)
Bradford Reagent	Sigma (USA)
Citric Acid Monohydrate	MERCK (Germany)
D(+) Glucose Anhydrous	Carlo Erba Reagents (Italy)
D(+)-Galactose	Fluka (Germany)
Disodium Phosphate	Sigma (USA)
dNTPS	Invitrogen (USA)

Table 2.8 (contd.) : Chemicals used in this study.

Chemical	Supplier (Country)
D-Sorbitol	Sigma (USA)
Ethanol	Carlo Erba Reagents (Italy)
Ethylenediaminetetraacetic Acid Disodium Salt Dihydrate, EDTA	Carlo Erba Reagents (Italy)
Gel Loading Dye Purple 6X	Biolabs (USA)
Glycerol	Carlo Erba Reagents (Italy)
Hydrogen Chloride	Carlo Erba Reagents (Italy)
Isopropanol	Carlo Erba Reagents (Italy)
L (+)-Aspartic Acid	MERCK (Germany)
L(-)-Phenylalanine	MERCK (Germany)
L(-)-Tyrosine	MERCK (Germany)
L(+)-Lysine Monochlorylhydrate	MERCK (Germany)
L-Arginine Monohydrochloride	MERCK (Germany)
L-Cysteine	Carlo Erba Reagents (Italy)
L-Histidine	Sigma (USA)
L-Isoleucine	Sigma (USA)
Lithium Acetate	Sigma (USA)
L-Leucine	Sigma (USA)
L-Methionine	Sigma (USA)
L-Proline	Sigma (USA)
L-Serine	Sigma (USA)
L-Threonine	Sigma (USA)
L-Tryptophane	Sigma (USA)
L-Valine	Sigma (USA)
Magnesium Chloride	Carlo Erba Reagents (Italy)
Magnesium Sulfate	Carlo Erba Reagents (Italy)
PEG4000	Sigma (USA)
Peptone Bacteriological	Microbiol (Italy)
Phenylmethanesulfonyl Fluoride	Sigma ALDRICH (USA)
Potassium Acetate	Carlo Erba Reagents (Italy)
Potassium Chloride	MERCK (Germany)
Potassium Phosphate	Carlo Erba Reagents (Italy)
Raffinose	Acros-Organics (USA)
Salmone Testes DNA	Sigma (USA)
Sodium Chloride	Microbiol (Italy)
Sodium dodecyl sulfate (SDS)	Sigma (USA)
Sodium Hydroxide (NaOH)	Carlo Erba Reagents (Italy)
Sucrose	Sigma (USA)
SYBR Safe DNA Gel Stain	Invitrogen (USA)
Tryptone	Microbiol Diagnostici (Italy)
Tris Hydrochloride	Sigma (USA)
Yeast Nitrogen Base Without Amino Acid And Ammonium Sulphate	Sigma ALDRICH (USA)

Solutions and buffers used in this study are listed in Table 2.9**.**

Table 2.9 : Solutions and buffers used in this study.

Solution / Buffer	Content / Concentration
10X Cut Smart Buffer	Provided by Biolabs (USA)
10X PCR Reaction Buffer	Provided by Invitrogen (USA)
10X TE Buffer pH 7.5	100 mM Tris, 10 mM EDTA
1X TE Buffer	10 mM Tris, 1mM EDTA
2X Rapid Ligation DNA Ligase Buffer	Provided by Promega (USA)
Citrate-Phosphate Buffer (CPB)	2X (53.4% 'v/v' of 0.1M citric acid, 46.6% 'v/v' of 0.2 M Na_2HPO_4)
DNA Extraction Buffer	0.1 M Tris (pH 8), 50mM EDTA (pH 8), 1% SDS
EDTA (pH 8)	0.5 M
Lithium Acetate (LiAc)Buffer	0.1 M LiAc
NaOH	10 M
PEG 4000 Buffer	40% 'w/v' of PEG4000, 100 mM LiAc, 1X TE (pH 7.5)
Salmone sperm DNA	10 mg/mL
Sodium dodecyl sulfate (SDS)	10% 'w/v'
Sodium dodecyl sulfate (SDS)	10% 'w/v'
Solution I	25 mM Tris.Cl (pH8), 10 mM EDTA (pH 8), 50 mM glucose
Solution II	0.2 M NaOH, 1%SDS
Solution III	60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid, 28.5 mL of water
Tris-Acetate-EDTA (TAE) Buffer	50X (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.4)
Tris-EDTA (TE) Buffer	10 mM Tris, 1mM EDTA
TrisHCl (pH 8)	1M

The laboratory equipment used during the study is listed in Table 2.10.

Table 2.10 : The list of the laboratory equipment used in this study.

Equipment	Supplier (Country)
Autoclaves	Nüve OT 90L (Turkey)
Balance	Sartorius CP2201S (Germany)
Benchtop Centrifuge	Eppendorf 5417R (Germany)
Deep Freezers and Refrigerators	-80°C Forma Scientific 8523 (USA) -20 °C Ariston (Italy) +4°C Ariston 409 (Italy)
Electrophoresis System	Biorad MiniSubCell GT (USA)
Electroporation Cuvettes (0.2 cm)	Sigma ALDRICH (USA)
Electroporation Machine	BioRad Gene Pulser II (USA)
Glas-bead, Acid Washed	Sigma (USA)
Incubators (37^{o}C, 50^{o}C)	Inter-Continental (Italy)
Laminar Flow	Gelaire BSB6A (Italy)
Light Microscope	Olympus CH30 (USA)
Magnetic Stirrer	Bibby Scientific HC502 (UK)
Microcentrifuge Tubes (1.5 mL)	Eppendorf (Germany)
Microfilter	Sartorius Minisart (Germany)
Micropipettes	Biohit Proline Plus 1000 µL, 200 µL, 100 µL, 20 µL, 10 µL (Finland)
Microplate Reader	BMG Labtech Spectrostar (Germany)
Microwave Oven	Haier HR-6755GT (China)
Orbital Shaker Incubators	Lab Companion SI-600R (USA)
PCR Tubes (0.2 mL)	Eppendorf (Germany)
pH Meter	XS Instruments pH510 (Italy)
Plastic Pipettes	LP Italiana Spa (Italy)
Power Supply for Electrophoresis	BioRad (USA)
Syringe	Becton Dickinson (Ireland)
Thermal Cycler	BioRad MyCyler™ 580BRU9452 (USA)
Ultracentrifuge	Hettich Rotanta 460 S (Germany)
Ultrapure Water System	Merck Millipore TANKMPK01 (Germany)
UV Transilluminator	Biorad Chemidoc XRS (USA)
UV-Visible Spectrophotometer	BioRad Smartspec Plus (USA)
Vortex Mixer	Velp Scientifica (Europe)
Water Bath	Julabo F12 (Germany)

The kits and the enzymes as well as the vectors used in the study are listed in Table 2.11.

Table 2.11 : List of the kits and the enzymes used in this study.

Kits, Enzymes and Vectors	Supplier (Country)
*Bgl*II	Invitrogen (USA)
*Eco*RI High Fidelity	Invitrogen (USA)
*Mly*I	Invitrogen (USA)
*Not*I High Fidelity	Invitrogen (USA)
pGEM ® T-Easy Vector	Promega (USA)
pPIC9 Expression Kit	Invitrogen (USA)
pUC19 Vector	Invitrogen (USA)
pYES2.1/V5-His-TOPO® TA Expression Kit	Promega (USA)
Ribonuclease A from Bovine Pancreas	Sigma (USA)
*Sac*I	Invitrogen (USA)
T4 DNA Ligase	Promega (USA)
Taq DNA Polymerase	Invitrogen (USA)
Wizard® SV Gel and PCR Clean-Up System	Promega (USA)
*Xba*I	Invitrogen (USA)

Primers used for PCR analyses are listed in Table 2.12.

Table 2.12 : Primers used in the study.

Primer	Sequence (5'- 3')
ALFA	ATGAGATTTCCTTCAATTTTTACTG
AOX1	GACTGGTTCCAATTGACAAGC
AOXR	GCAAATGGCATTCTGACATCC
BGL2F	ATGCGTTTTTCTACGTTCGTTT
BGL2R	AAATTTAAGAGAAGTTACAATCCAAAGA
FW2	ATAATCTTGAATTCATGCGTTTTTCTACGTTCG
GAL1F	TATACCTCTATACTTTAACGTCAAGGAG
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC
RV2	TTTATCTAGCGGCCGCAAATTTAAGAGAAGTTA

2.2 Methods

2.2.1 Killer plate assay

Killer activity was evaluated by plate assay as described by Rosini (1983) on yeast extract-peptone-dextrose (YPD) plates (Table 2.1) added with citrate-phosphate buffer pH 4.6 (CPB) (Table 2.9) prepared by mixing 2X YPD medium with the same volume of 2X CPB. 200 µL of cell suspensions of the putative sensitive strain (OD_{600} of 0.2) prepared in sterile distilled water were spread onto YPD plates (pH 4.6). *T. phaffii* and DBVPG 6500 streaked on plate were utilized as positive and negative controls of killer activity, respectively. Killer activity of recombinant strains was evaluated by well test assay. Briefly, small wells were cut on YPD (pH 4.6) plate and 100 µL of the sample to be tested (supernatant or crude cell extract) was loaded into the wells. *T. phaffii* and DBVPG 6500 were streaked as positive and negative controls of killer activity, respectively.

2.2.2 Growth of *S. cerevisiae* strains in galactose containing medium

S. cerevisiae BY4741 and YPH501 were grown in minimal media containing either 2% galactose or 0.2% galactose in addition to 2% raffinose (Table 2.3). Briefly, the two yeasts were inoculated in 20 mL of growth medium in 100 mL flasks and incubated at 25^{o}C under shaking (150 rpm) for 4 days. BY4741 was inoculated into 2% and 0.2% galactose media with an initial OD_{600nm} of 0.116 and 0.196, respectively, while YPH501 was inoculated into 2% and 0.2% galactose media with an initial OD_{600nm} of 0.030 and 0.040, respectively. Yeast growth was monitored by measuring optical density at 600 nm at the indicated times using a spectrophotometer.

2.2.3 DNA extraction and PCR amplification

2.2.3.1 Yeast total DNA extraction

DNA extraction was carried out with yeast cells grown both on YPD plate at 25^{o}C and in 15 mL of YPD medium (Table 1) overnight at 30^{o}C with shaking at 150 rpm. Cells sampled from plate (1/8 of the whole plate for each sample) or from 15 mL of YPD liquid medium were washed in 1 mL of distilled water, centrifuged in a benchtop centrifuge at maximum speed for 5 min and re-suspended in 300 µL of

DNA Extraction Buffer (Table 2.9). Glass beads (a bead size of 452-600 μm) were added to the tubes (about 1/3 of the volume of the mixture), cells were broken by two cycles of 1 min vortexing and 1 min on ice treatment and the tubes were maintained in boiling water for 10 min. Next, 20 μL of 1M TrisHCl, 15 μL of 0.5 M EDTA, 50 μL of 10% SDS and 200 μL of 5 M potassium acetate were added and the mixture was incubated for 30 min on ice. The tubes were centrifuged at maximum speed for 10 min and the supernatants were transferred into clean tubes. DNA was then precipitated with ice-cold isopropanol at room temperature for 5 min and the tubes were centrifuged as above. The DNA pellet was washed in 0.5 mL of 70% (v/v) ethanol, and the supernatant was discarded after centrifugation at maximum speed for 5 min. The DNA pellet was air-dried, dissolved in 50 μL of TE buffer, kept at 30^{o}C for 15 min and stored at -20^{o}C.

When needed, DNA quality was checked by PCR amplifying the extracted DNA with internal transcribed spacer (ITS). PCR was carried out in 100 μL reaction mixture made of 1 μL of DNA, 1X Taq polymerase buffer, 1.5 mM magnesium chloride ($MgCl_{2)}$, 0.1 mM dNTPs, 0.05 μM of *ITS1* and 0.05 μM of *ITS4* primers (Table 2.12) as well as 0.01 units/ μL Taq polymerase enzyme. Reaction conditions were: 95^{o}C for 5 min followed by 35 cycles of 94^{o}C for 1 min, 55.5^{o}C for 45 sec, and 72^{o}C for 2 min and final extension at 72^{o}C for 10 min. Ten μL of each PCR product mixed with 2 μL of loading dye was loaded into 1% agarose gel containing 7.5 μL of SYBR® Safe DNA Gel Stain. One μL of marker (1 Kb, Euroclone) was loaded onto agarose gel. The samples were run at 100 volts for 60 minutes in 1X TAE buffer and visualized using a Chemidoc XRS (Biorad Laboratories).

2.2.3.2 Small scale preparation of plasmid DNA

Minipreparation of plasmid DNA was carried out as described by Sambrook *et al.* (1989). Briefly, *E. coli* colonies of transformants were picked from plates, grown overnight at 37^{o}C with vigorous shaking in 1 mL of LB medium with ampicillin and centrifuged at 10,000 rpm for 30 seconds at 4^{o}C. The pellet was resuspended in ice-cold 100 μL of Solution I (Table 2.9). Next, 200 μL of freshly prepared Solution II (Table 2.9) was added individually into the tubes and stored on ice after inverting the tubes rapidly by five times. After that, 150 μL of ice-cold Solution III (Table 2.9) was added and mixed by inverting the tubes. The tubes were stored on ice for 3-5

min and centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant was transferred to fresh tubes and DNA was precipitated with 2 volumes of ethanol at room temperature. The mixtures were vortexed, kept at room temperature for 2 min and centrifuged again at 10,000 rpm for 5 min at 4°C. The supernatant was discarded, the DNA pellets were washed in 1 mL of 70% (v/v) ethanol at 4°C, centrifuged as above and the DNA pellets were air dried after discarding the supernatant. Finally, 50 µL of TE (pH 8) containing RNase (20 µg/mL) was used to dissolve DNA.

2.2.3.3 Amplification of *TpBGL2* gene

The sequences of the primers utilized for *TpBGL2* gene amplification are reported in Table 2.12. In most cases, gradient PCR was set up to individuate the optimal annealing temperature for each set of primer. For the amplification of *TpBGL2* with primers *BGL2F* and *BGL2R*, gradient PCR was carried out using annealing temperatures gradually increasing from 56°C to 60°C. PCR reaction was carried out in 25 µL reaction buffer containing 1X Taq polymerase buffer, 2.5 µM $MgCl_2$, 0.2 µM dNTPs, 0.5 µM of each primer and 0.04 units/ µL Taq polymerase. Reaction conditions were 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56-60°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. Gel electrophoresis was performed as described in Section 2.2.3.1.

For TA cloning in pYES2.1/V5-His-TOPO® vector, the amplification of *TpBGL2* gene was carried out at the annealing temperature of 57.4°C and with a final extension to 20 min in order to ensure 3' adenylation of the PCR product. Ten µL of PCR product was mixed with 2 µL of loading dye and loaded onto 1% agarose gel containing 1.5 µL of SYBR® Safe DNA Gel Stain. The samples and 1µL of marker (1 Kb) were run at 70 volts for 60 min in 1X TAE buffer and the gel was visualized as mentioned above.

For the amplification of *TpBGL2* with primers *FW2* and *RV2* that carry *Eco*RI and *Not*I restriction sites, respectively, gradient PCR was carried out under the same conditions mentioned above, with annealing temperature ranging from 65°C to 69°C. Gel electrophoresis was performed as described above. For *TpBGL2* gene cloning into pGEM, PCR reaction was carried out at 65.3°C with 20-min final extension. Gel electrophoresis was carried out as described above.

2.2.4 Cloning methods

TpBGL2 gene was cloned in different plasmid vectors. The following paragraphs will describe the methods utilized for the production of recombinant plasmids.

2.2.4.1 Cloning *TpBGL2* into pYES2.1/V5-His-TOPO® vector

TpBGL2 cloning into pYES2.1/V5-His-TOPO® vector was carried out according to the instructions reported in pYES2.1/V5-His-TOPO® TA Expression Kit manual, with some modifications. Briefly, both 1 µL and 3 µL of fresh PCR products obtained with *BGL2F* and *BGL2R* primers were mixed with 1 µL of salt solution and 1 µL of TOPO® vector (both provided by the kit), separately. Sterile water was added to each tube to a final volume of 5 µL. The reaction mixture was incubated at room temperature for 30 min and placed on ice. Next, 2 µL of reaction mixture from each tube was added into two different vials of chemically competent TOP10 One Shot® *Escherichia coli* cells (provided by pYES2.1/V5-His-TOPO® TA Expression Kit) along with 1 µL of pUC19 vector in a different tube as a positive control. Cells were incubated on ice for 30 min, heat-shocked at 42^{o}C for 30 seconds and transferred on ice. After adding 250 µL of SOC medium (provided by the kit, Table 2.2), the tubes were incubated at 37^{o}C with shaking (200 rpm) for 1 h. Next, both 20 µL and 200 µL of competent cells transformed with the ligation mixtures were spread onto LB plates (Table 2.4) containing 100 µg/mL ampicillin. Cells transformed with pUC19 were diluted 1:10 in LB medium. Twenty five µL and 100 µL of the diluted mixture were plated on LB plates with 100 µg/mL ampicillin. Fifty colonies were picked from the plates, inoculated into 500 µL of LB + ampicillin and grown overnight. Cells were centrifuged and 500 µL of LB medium with ampicillin was added along with 500 µL of 8% (v/v) glycerol for storage at -80^{o}C.

Restriction digestion of the recombinant plasmids was carried out as follows: 8 µL of plasmid DNA was added with 0.6 U/µL of *Mly*I, 2 U/ µL of *Xba*I and 1X NEBuffer 2.1 in a final volume of 25 µL and incubated overnight at 37^{o}C. Fifteen µL of this mixture was mixed with 3 µL of loading dye and loaded onto 1.5% agarose gel with 6 µL of SYBR® Safe DNA Gel Stain. One µL of marker (both 100 bp and 1Kb) was also loaded. The gel was run at 100 V for 2 h and was visualized as indicated in Section 2.2.3.1.

The orientation of the inserts in the recombinant plasmids was assessed by PCR utilizing primers *GAL1F* (Table 2.12) which is specific to GAL1 Forward priming site in GAL1 promoter sequence within the vector used for cloning (Figure 2.1) and *BGL2R* reverse primer. For the amplification of *TpBGL2* with these primers, gradient PCR was carried out using different annealing temperatures gradually increasing from 55°C to 60°C in reaction mixture containing 1X Taq polymerase buffer, 2.5 µM $MgCl_2$, 0.2 µM dNTPs, 0.5 µM of each primer and 0.04 units/µL Taq polymerase. Reaction conditions were 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55-60°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. Gel electrophoresis was carried out in 1.5% agarose gel containing 6 µL of SYBR® Safe DNA Gel Stain at 70 Volts for 60 min in 1X TAE buffer. One µL of DNA size marker (1 Kb) was used and the gel was visualized as indicated in Section 2.2.3.1.

Recombinant plasmid was sequenced by using *GAL1F* primer and *BGL2R* primer. Reaction mixtures were prepared as follows: 700 ng of plasmid DNA and 0.64 µL of 10 µM primer were dried at 65°C for 8 min and sent for sequencing (www.bmrgenomis.it).

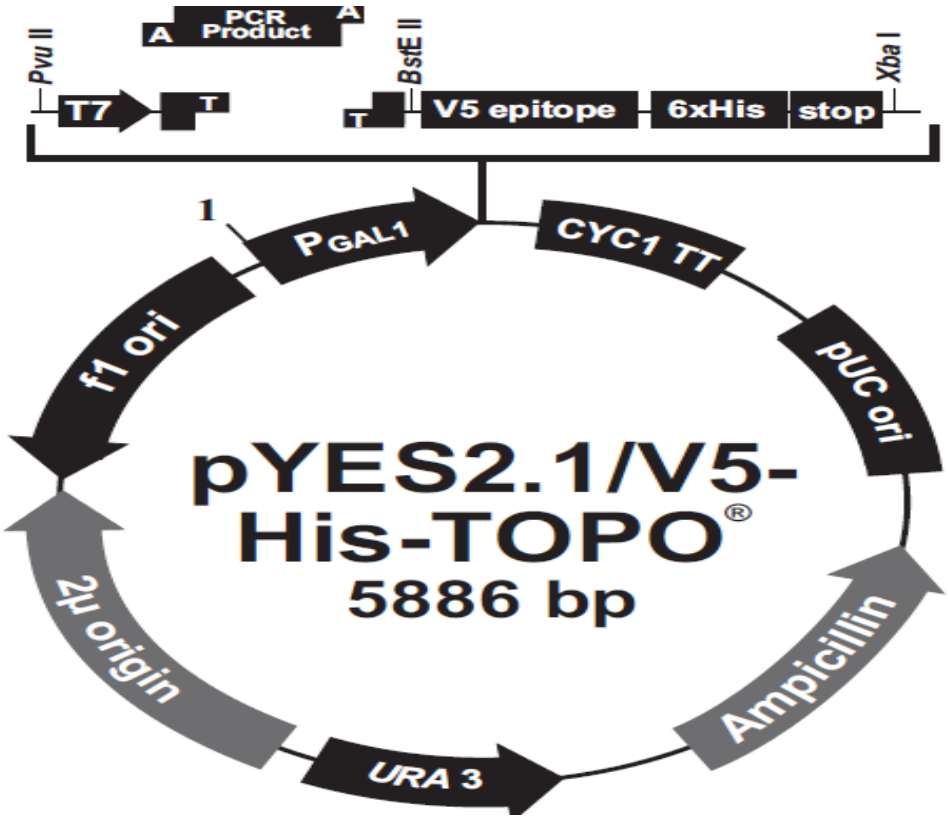

Figure 2.1 : pYES2.1/V5-His-TOPO® vector.

2.2.4.2 Cloning *TpBGL2* into pGEM®-T Easy Vector

TpBGL2 gene amplified with *FW2* and *RV2* primers, carrying *Eco*RI and *Not*I restriction sites, respectively, was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) according to the supplier instructions. Purified PCR product was ligated into pGEM®-T Easy Vector. Ligation mixture was prepared as described in the manual of pGEM®-T Easy Vector Systems (Promega, USA). Briefly, 50 ng of PCR product was mixed with 1 µL of pGEM®-T Easy Vector, 1 µL of T4 DNA Ligase and 5 µL of 2X Rapid Ligation Buffer (both provided by the kit), and nuclease-free water in a total volume of 10 µL reaction mixture. In this ligation, 3:1 ratio was applied as insert:vector ratio. Ligation mixture was incubated at 4°C overnight. Next day, ligation mixture was used for transformation of *E. coli* JM109 competent cells. The transformation was carried out according to the protocol provided by the supplier of the pGEM®-T Easy Vector. Briefly, 2 µL of ligation mixture was added to 50 µL of *E. coli* cells just after thawing on ice. Cells were placed on ice for 20 min and heat-shocked for 45-50 seconds in water bath at 42°C. The tubes were then returned to ice for 2 minutes. After adding 950 µL of SOC medium, samples were incubated at 37°C for 1.5 hours with shaking at 150 rpm. Hundred µL of the sample was plated onto LB plates containing 100 µg/mL ampicillin and the plates were incubated at 37°C overnight.

Recombinant plasmids were prepared by Miniprep as described in 2.2.3.2 and PCR amplified with *FW2* and *RV2* primers to check for insert orientation. PCR and gel electrophoresis were carried out as described in 2.2.3.1.

2.2.4.3 Cloning *TpBGL2* into pPIC9

pGEM-T Easy Vector+*TpBGL2* and pPIC9 were subjected to double restriction with *Eco*RI and *Not*I enzymes. High Fidelity enzymes (New England Biolabs) were used according to the instruction of the supplier. Analytical digestions carried out in a final volume of 25 µl and incubated at 37°C were followed by preparative digestion. Briefly, 50 µL of pGEM-T Easy vector+*TpBGL2* (1020 ng/µL) was mixed with 4 µL of *Eco*RI (20,000 units/mL) and 4 µL of *Not*I (20,000 units/mL) in a total volume of 70 µL of reaction mixture using 7 µL of 10X buffer (provided with the enzymes). For pPIC9 vector, 40 µL of DNA was subjected to double digestion in a total volume of 60 µL containing 4 µL of *Eco*RI (20,000 units/mL) and 4 µL of *Not*I (20,000

units/mL), and 6 µL of 10X buffer (provided with the enzymes). The mixtures were incubated at 37^{o}C for 2.5 h. After incubation, the entire volumes of restriction products were run on 1% agarose gel containing 7.5 µL of SYBR® Safe DNA Gel Stain for 1.5 h at 100 V. One kb of marker was used. The gel image was visualized with the Chemidoc XRS and the restriction bands containing the gene of interest and the linearized vector were cut. DNA embedded in the gel was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). Purified samples were run on agarose gel for quality check. For this purpose, 5 µL of samples of both vector and insert were loaded into 1% agarose gel containing 5 µL of SYBR® Safe DNA Gel Stain and run for 1 h at 100 V. After having visualized the gel image with the Chemidoc XRS, the ligation mixture was prepared as follows. Twelve µL of vector (3.62 ng/µL), 5 µL of the insert (3.5 ng/µL), 1 µL of T4 DNA ligase (1–3 u/µL) and 2 µL of buffer (provided with the enzyme) were mixed in a total volume of 20 µL of reaction mix. The whole mixture was incubated at room temperature for 2.5 hours for an efficient ligation and transformed in *E. coli* as follows. Briefly, 10 µL of the ligation mix was added to 50 µL of *E. coli* competent cells and the tubes were flicked several times. For negative control, 10 µL of sterile water was added onto 50 µL of *E. coli* competent cells. Cells were incubated on ice for 30 min, heat-shocked for 15-20 sec at 42^{o}C without shaking in a water bath and were placed on ice for 2 min. After adding 450 µL of SOC medium, cells were incubated at 37^{o}C for 60 min with shaking. At the final step, two 200 µL of the mixture and one 100 µL of the mixture were plated onto LB plates containing 100 µg/mL ampicillin separately and the plates were incubated at 37^{o}C overnight.

The colonies of the transformants were grown in LB+amplicillin and subjected to miniprep of DNA as already described. The recombinant clones were subjected to PCR amplification with primers *AOX1* and *BGL2R* (Table 2.12) to check the correct orientation of the fragment. *AOX1* primer anneals to a site in pPIC9 vector upstream of the insert, *BGL2R* primer (Table 2.12) anneals to 3' end of the gene of interest. PCR conditions and gel electrophoresis were applied as described in Section 2.2.3.1. The annealing temperature for PCR reaction was 55.5°C.

In order to make sure that the insert was in the correct orientation in pPIC9 vector, one of the recombinant plasmids was sent for sequencing. On this purpose, 0.64 µL of 10 µM *AOX1* primer was mixed with 700 ng of pPIC9 vector containing the

insert. The sample was then dried at 65°C for 8 min and sent for sequencing (www.bmrgenomics.it).

2.2.4.4 Cloning α+*TpBGL2* cassette in pYES2.1/V5-His-TOPO® vector

For the amplification of α+*TpBGL2* cassette, *ALFA* primer and *BGL2R* primer were utilized (Table 2.12). *ALFA* primer anneals to the 5' end of the alpha leader secretion site that is upstream *TpBGL2* gene in pPIC9+*TpBGL2* recombinant plasmid; *BGL2R* anneals to the 3' end of *TpBGL2* gene. Firstly, gradient PCR was utilized to determine the best annealing temperature for this primer couple. PCR conditions and gel electrophoresis were carried out as described in Section 2.2.3.1. Annealing temperature was gradually increased from 58°C to 61°C.

For TA cloning of the α+*TpBGL2* cassette, the annealing temperature was set at 60.4°C. PCR conditions were the same as described in Section 2.2.3.1 with a final elongation step of 20 min. The amplicon obtained was visualized on agarose gel and utilized for cloning in pYES2.1/V5-His-TOPO® Vector. Three µL of amplicon and 1 µL of vector were ligated as indicated in the manual of the pYES2.1/V5-His-TOPO® TA Expression Kit and the ligation mixture was transformed in chemically competent TOP10 One Shot® *E. coli*. For the positive control, 1 µL of pUC19 (provided by the kit) was utilized.

In the final step of the transformation, 200 µL, 20 µL and 80 µL of sample transformed with pYES2.1/V5-His-TOPO® +α+*TpBGL2*, and 100 µL and 25 µL of 1:10 dilution of the positive control, were plated onto LB + ampicillin. After overnight incubation of the plates at 37°C, 24 colonies were picked. The pYES2.1/V5-His-TOPO® Vectors having *TpBGL2* gene with alpha leader secretion site were extracted from the clones by miniprep as described previously.

In order to determine the orientation of the insert, one of the recombinant clones was picked for sequencing analysis. For this purpose, the recombinant vector was PCR amplified using *GAL1F* and *BGL2R* primers at the annealing temperature of 60.4°C. PCR conditions and the gel electrophoresis conditions were as described previously. Gel electrophoresis was carried out using 0.8% agarose gel containing 1.5 µL of SYBR® Safe DNA Gel Stain. Ten µL of each sample was added with 2 µL of loading dye and run at 70 V for 60 min along with 2.5 µL of marker (1 kb). After PCR, samples were purified using Wizard® SV Gel and PCR Clean-Up System

(Promega, USA). Finally, 1 µL, 2 µL and 3 µL of one of the purified PCR products at a concentration of 118 ng/µL, as well as 2 µL, 4 µL and 6 µL of another purified PCR product at a concentration of 48 ng/µL were sent for sequencing.

2.2.5 Transformation of recombinant plasmids in yeast

Two recombinant plasmids, namely pYES2.1/V5-His-TOPO® +*TpBGL2* and pPIC9+*TpBGL2* were transformed in *S. cerevisiae* and *P. pastoris*, respectively, as follows:

2.2.5.1 Transformation of pYES2.1/V5-His-TOPO® +*TpBGL2* in *S. cerevisiae*

S. cerevisiae BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) cells were grown overnight at 30°C, 150 rpm in 20 mL of YPD medium, inoculated into 50 ml of YPD medium with an initial OD_{600} of 0.2 and incubated at 30°C, 150 rpm until OD_{600} of the culture reached 0.4-0.7. Cells were harvested at 4000 rpm for 5 min, washed with 10 mL of sterile water, resuspended in 0.5 mL of sterile water and aliquoted into 5 centrifuge tubes with a volume of 0.1 mL. The cells were centrifuged at 4000 rpm for 5 min and each pellet was resuspended in 7 µL of boiled salmon sperm DNA, 10 µL of 10X TE (pH 7.5) and 10 µL of lithium acetate buffer pH 7.5. Next, 1 and 2 µL of plasmid DNA (1290,3 ng/ µL) were added into the tubes separately while one of the tubes contained 2 µL of distilled water instead of DNA as a negative control and water was then added to a final volume of 100 µL. Freshly prepared 600 µL of PEG 4000 Buffer (Table 2.9) was added into the tubes separately. The mixtures were incubated at 30°C for 1 h and at 42°C for 15 min. Cells were centrifuged at 4000 rpm for 30 seconds, washed with 1 mL of 1X TE and resuspended in 100 µL of 1X TE after centrifugation. Finally, all mixtures were plated down into YNB selective media without uracil, and with glucose as the sole carbon source (Table 2.5).

Six *S. cerevisiae* transformants were grown in YNB selective medium without uracil for 2 days at 25°C. Total DNA extraction was performed as reported in Section 2.2.3.1. *TpBGL2* gene was then PCR amplified by using *BGL2F* and *BGL2R* primers, as described previously. Gel electrophoresis was carried out by loading 10 µL of PCR product and 2 µL of loading dye in 1% agarose gel containing 7.5 µL of SYBR® Safe DNA Gel Stain. The samples and 1µL of marker (1 Kb) were run at 100 volts for 60 min in 1X TAE buffer and the gel was visualized in Chemidoc XRS.

2.2.5.2 Transformation of pPIC9+*TpBGL2* in *P. pastoris*

Preparation of competent cells

Transformation of *P. pastoris* GS115 cells was carried out as indicated on the manual of Pichia Expression kit. Briefly, cells were grown overnight in 5 mL of YPD in 50 ml flasks at 30°C. 250 μL of this overnight culture were inoculated into 250 mL of YPD in a 1 L flask and incubated overnight as above. When the OD_{600} of 1.3 was reached, cells were centrifuged at 1500 g for 5 min at 4°C and resuspended in 250 μL of ice-cold sterile water, centrifuged again as above, and resuspended in 125 μL of ice-cold water, centrifuged once again as above and resuspended in 10 mL of ice-cold sorbitol. Cells were centrifuged as above and resuspended in 500 μL of ice-cold sorbitol and 80 μL aliquots of competent cells were prepared.

Preparation of linearized pPIC9+*TpBGL2*

Plasmids to be transformed into GS115 were linearized as indicated in the manual of Pichia Expression Kit. In particular, aliquots of 6 μg of pPIC9+*TpBGL2* were restricted in a final volume of 200 μL containing 1X restriction buffer, 70 Units of *Sac*I or 50 Units of *Bgl*II restriction enzymes 200 ng of BSA. The reactions were carried out at 37°C for 4 h. In the last 30 min, 2 μg RNase was added. Reaction mixtures were extracted with the same volume of phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol and resuspended in 15 μL TE (pH 7.6). DNA concentration was determined spectrophotometrically. In parallel, pPIC9 was linearized with the same enzymes to obtain negative controls of recombinant protein expression.

Electroporation of GS115

Competent cells were added with 2.5 μg or 150 μg of linearized plasmids in 10 μL of TE buffer, transferred to ice-cold electroporation cuvettes (0.2 cm), kept on ice for 5 minutes and pulsed (1500 V, 200 Ampere, 25 μFaraday). Immediately 1 ml of ice-cold 1 M sorbitol was added, the cuvette content was transferred to a microcentrifuge tube and aliquots of 200 or 800 μL were spread on minimal dextrose (MD) plates and incubated at 30°C.

Phenotype test for the transformants

After transformation, 25 transformants were picked for each transformation and they were grown on both MD and minimal methanol (MM) plates for 3 days at 30°C, to

test for Mut^+ or Mut^S phenotypes of the transformants. After determining putative Mut+ or MutS phenotypes, they were subjected to PCR analysis with *AOX1* and *AOXR* primers (Table 2.12) for screening of the clones. For this purpose, a single colony was picked and resuspended in 10 µL of water for each clone. Five µL of a 5 U/µL solution of lyticase was added individually and they were incubated at 30°C for 10 minutes. Next, the cells were frozen at -80°C for 10 min and 5 µL of cell lysate was mixed with 5 µL of 10X reaction buffer, 5 µL of 25 mM $MgCl_2$, 1 µL of 25 mM dNTPs, 1 µL of both *AOX1* (10 pmol/µL) and *AOXR* (10 pmol/µL) primers, and 27 µL of sterile water. The mixture was placed in the thermocycler and incubated at 95°C for 5 min. Then, 5 µL of a 0.16 U/µL solution of Taq polymerase (0.8 units) was added and PCR was performed. Reaction conditions were: 30 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min and final extension at 72°C for 7 min. Finally, 10 µL of aliquot was analyzed using gel electrophoresis, as described in Section 2.2.3.1.

2.2.6 Evaluation of killer activity in *S. cerevisiae* recombinant strains

2.2.6.1 Induction of killer protein production in galactose containing media

S. cerevisiae BY4741 transformed with pYES2.1/V5-His-TOPO® +*TpBGL2* was picked for further analyses. In order to determine whether the protein was expressed as an intracellular or extracellular protein, and to evaluate the killer activity of the protein, cells were precultured in three different media before induction in galactose-containing medium.

Briefly**,** the transformant was inoculated into 20 mL of buffered (CPB pH 4.6) yeast minimal medium without uracil containing: (i) 2% galactose and 2% raffinose; (ii) 0.2% galactose and 2% raffinose; (iii) 2% sucrose as carbon sources (Table 2.5) in 100 mL shaking flasks at 25°C, 150 rpm for 48 h until OD_{600} value of the culture reached ~15.

Cells grown in 0.2% galactose - 2% raffinose as well as 2% sucrose media were harvested by centrifugation at 10,000 rpm for 10 min and washed with 1 mL of water and transferred into 20 mL of YNB selective media without uracil, and with 2% galactose as the sole carbon source (Table 2.5). They were then incubated at 25°C, 150 rpm for 24 h.

2.2.6.2 Evaluation of killer activity

After 48 h of growth in minimal medium with 2% galactose and 2% raffinose, crude extracts were prepared by washing the pellet of the whole culture twice in 1 mL water after centrifugation at 10,621 g for 10 min. Next, the pellet was resuspended with 200 µL of potassium phosphate buffer (50mM, pH7 containing 1% of 18 mg/mL phenylmethylsulfonyl fluoride (PMSF) with 6 mM EDTA). Acid washed glass beads were added half the volume of the suspension and the cells were broken down with 8 cycles of vortex for 30 sec and incubation on ice for 30 sec. Finally, supernatant was transferred into a fresh tube after centrifugation at 20,817 g for 10 min as a crude extract of the culture.

Crude extraction was also performed for the cultures in YNB selective media without uracil and, with 2% galactose as the sole carbon source after 24-hour growth which was the following step of growth in both 0.2% galactose-2% raffinose and 2% sucrose media after washing the cells. However, for this time crude extraction was carried out using CPB pH 4.6 containing 1% of 18 mg/mL PMSF with 6mM EDTA in addition to the extraction with potassium phosphate buffer, as described previously.

3. RESULTS

3.1 Selection of Suitable Yeast Strains for Heterologous Expression of *TpBGL2*

The first step of the experiments was the selection of *S. cerevisiae* and *P. pastoris* laboratory strains insensitive to Kpkt. For this purpose, *S. cerevisiae* BY4741, YPH501 and *P. pastoris* GS115 were tested for Kpkt sensitivity by means of the well plate assay. In these experiments, *S. cerevisiae* DBVPG 6500 was utilized not only as the reference strain for killer toxin sensitivity, but also as a negative control of killer activity. *T. phaffii* CBS 4417 was used as the positive control of killer activity. As expected, *T. phaffii* showed killer activity on the universal sensitive strain DBVPG 6500. BY4741 and YPH501 showed slight sensitivity to the killer toxin produced by *T. phaffii*. On the contrary, *P. pastoris* GS115 was resistant to Kpkt. Thus *P. pastoris* GS115 was chosen as a suitable strain for the heterologous production of Kpkt (Figure 3.1).

However, in spite of their slight sensitivity to Kpkt toxin, the two *S. cerevisiae* strains were subjected to further experiments. Since in vector pYES2.1/V5-His-TOPO® the heterologous gene is cloned under the control of GAL1 promoter, growth kinetics of BY4741 and YPH501 were analyzed in growth media containing either 2% or 0.2% galactose. *S. cerevisiae* strains grow poorly when galactose is the sole carbon source, thus 2% raffinose was added to the growth medium. As reported in Table 3.1, the OD_{600nm} of BY4741 was higher than that of YPH501 at all indicated times, thus suggesting that BY4741 grows better than YPH501 in galactose-containing media.

On the basis of the results obtained (Figure 3.1, Table 3.1), BY4741 was selected to attempt the heterologous production of Kpkt in *S. cerevisiae* and a medium containing 2% galactose and 2% raffinose was chosen to induce GAL1 promoter in recombinant strains of *S. cerevisiae* BY4741.

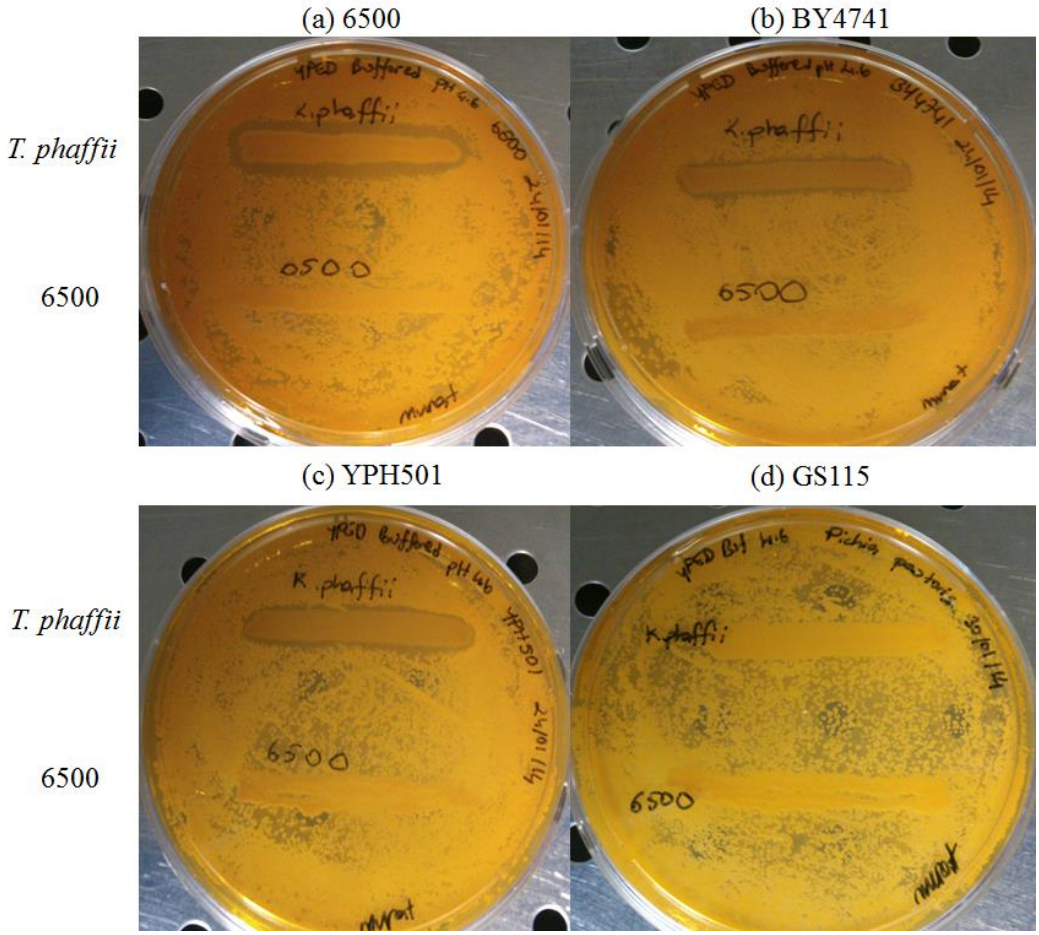

Figure 3.1 : Killer plate assay. *T. phaffii* killer activity was evaluated against *S. cerevisiae* strains DBVPG DBVPG 6500 (a), BY4741 (b) and YPH501 (c) as well as against *P. pastoris* GS115 (d). DBVPG 6500 was utilized as the positive control of Kpkt sensitivity, and as a negative control of killer activity.

Table 3.1 : OD_{600} of BY471 and YPH501 in different galactose-containing media.

Incubation hour	BY4741		YPH501	
	2% galactose	0.2% galactose	2% galactose	0.2% galactose
0	0.11	0.19	0.12	0.14
20	3.06	2.98	1.60	2.01
96	12.40	16.55	12.00	13.65

3.2 Total DNA Extraction of *T. phaffii* CBS4417 and PCR amplification of *TpBGL2* gene

T. phaffii total DNA, extracted as reported in Section 2.2.3.1, was first PCR amplified with the universal primers *ITS1* and *ITS4*. These reactions produced amplicons of the expected size (475 bp) in samples named P2, P3, L and PC (Figure 3.2), thus confirming the good quality of these DNAs. PC sample DNA showed the

brightest bands for all annealing temperatures of the amplification carried out by using *ITS1* and *ITS4* primers. Hence, it was decided to utilize PC DNA for further experiments on the basis of the results obtained. To determine the best amplification conditions, a gradient of annealing temperature from 56°C to 60°C was set up for primer *BGL2F* and *BGL2R* which amplify the entire *TpBGL2* gene. A gradient of annealing temperature from 65°C to 69°C was set up for primers *FW2* and *RV2* which produce *TpBGL2* gene flanked by *Eco*RI and *Not*I restriction sites. An annealing temperature of 57.4°C was utilized for the amplifications with *BGL2F* and *BGL2R* primers, while the amplifications with *FW2* and *RV2* primers was carried out at 65.3°C (data not shown).

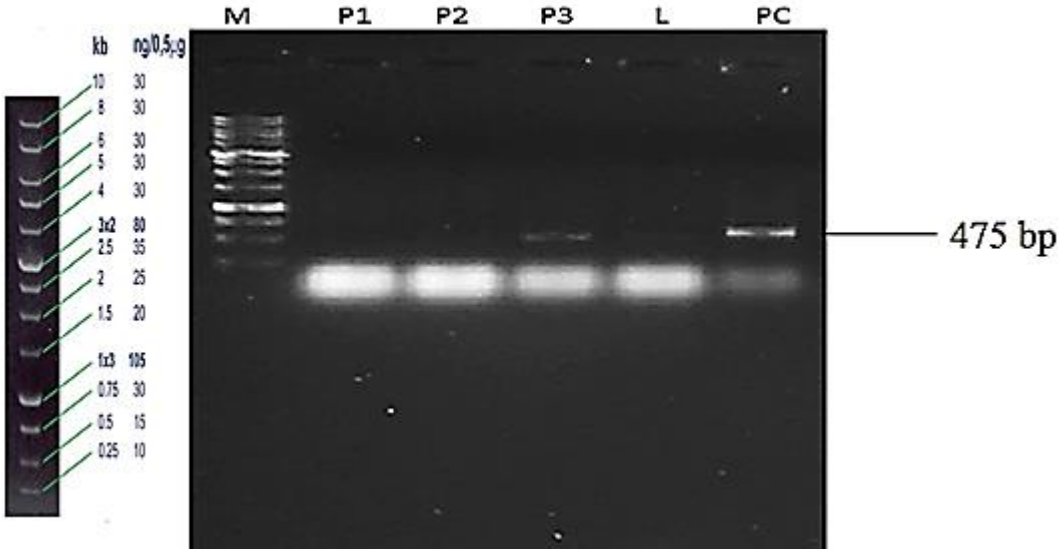

Figure 3.2: PCR amplification of total DNA of *T. phaffii* with *ITS1* and *ITS4* primers. M: Marker (SHARPMASS™ 1-DNA LADDER); P1, P2, P3, L, PC: Different samples of total DNA of T. phaffii.

3.3 Construction of the Expression Cassettes

The cloning strategies used were aimed at obtaining three different constructs (Figure 3.3): i) *TpBGL2* gene in pYES2.1/V5-His-TOPO® under the control of GAL1 promoter; ii) *TpBGL2* gene in pYES2.1/V5-His-TOPO® under the control of GAL1 promoter and downstream the α sequence; iii) *TpBGL2* gene in pPIC9 under the control of AOX1 promoter and downstream the α sequence. Due to the slight sensitivity to Kpkt shown by BY4741 in the case of *S. cerevisiae*, both intracellular

and extracellular productions of Kpkt were attempted. For *P. pastoris*, the extracellular production of Kpkt was preferred.

To obtain the three constructs, fresh PCR products obtained by using primers *BGL2F* and *BGL2R* (Figure 3.4), and *FW2* and *RV2* (Figure 3.5) were ligated into pYES2.1/V5-His-TOPO® and in pGEM-T Easy Vector, respectively, and transformed into *E. coli* cells.

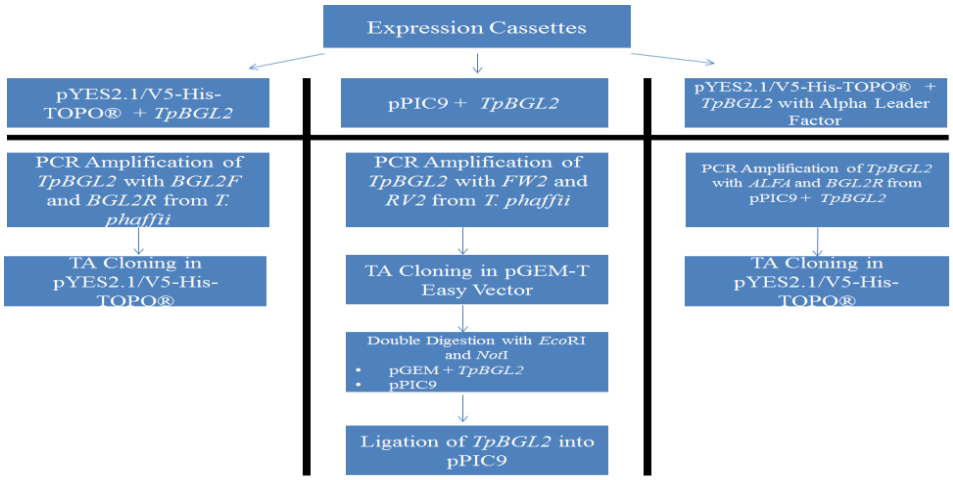

Figure 3.3 : Summary for the construction of three different expression cassettes used in the study.

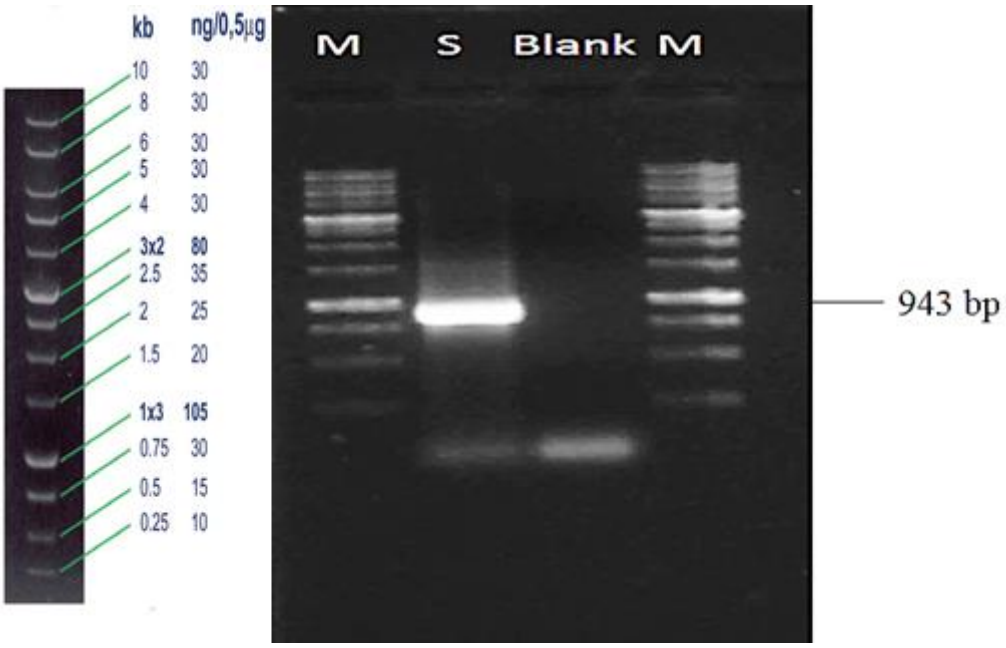

Figure 3.4 : PCR amplification of *TpBGL2* with primers *BGL2F* and *BGL2R* (M:Marker (SHARPMASS™ 1 - DNA LADDER), S: Sample).

The amplification of *TpBGL2* gene with *BGL2F* and *BGL2R* gave a band of 943 bp (Figure 3.4) that was cloned into pYES2.1/V5-His-TOPO® under the control of GAL1 promoter.

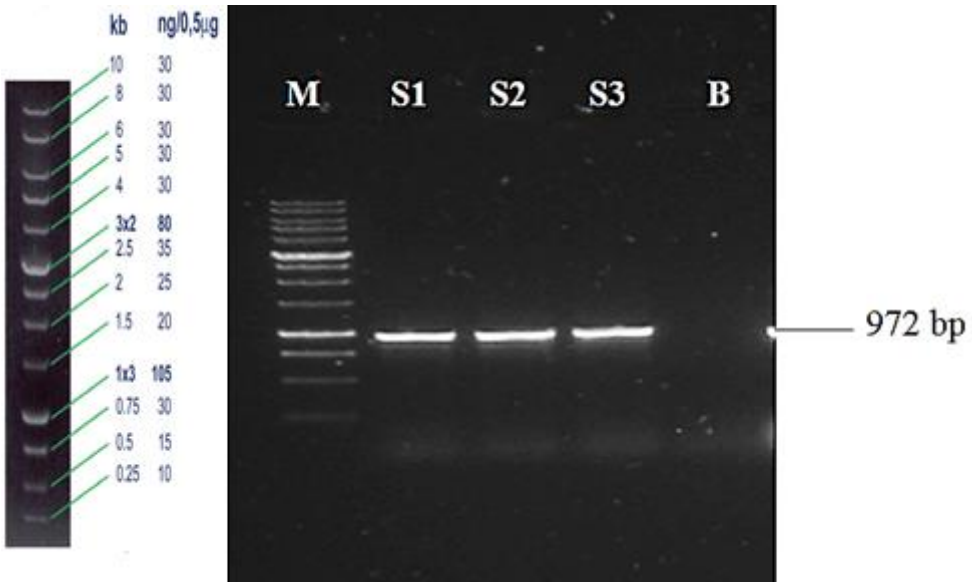

Figure 3.5 : PCR amplification of *TpBGL2* with primers *FW2* and *RV2* (M: Marker (SHARPMASS™ 1 - DNA LADDER), S1, S2, and S3: Labelling of the samples, B: Blank).

3.3.1 Cloning *TpBGL2* into pYES2.1/V5-His-TOPO®

Twenty-six colonies resulting from the transformation of pYES2.1/V5-His-TOPO® + *TpBGL2* ligation mixture into *E. coli* were subjected to minipreparation of DNA and the resulting plasmids were subjected to further analyses to evaluate both the presence and the orientation of *TpBGL2* gene within the expression plasmid. At first, a restriction analysis of the recombinant plasmids was performed using *Mly*I and *Xba*I enzymes. The exact size of the bands in case of correct orientation of the insert within the vector was determined using NEBcutter V2.0 software (Table 3.2). According to this table, correct restriction patterns were observed in clones named 11 and 24 (Figure 3.6). PCR analysis of the two clones was carried out with *GAL1F* and *BGL2R* primers. To determine the best annealing temperature for *GAL1F* and *BGL2R* primers, a gradient PCR was set up. All annealing temperatures utilized produced a 1040 bp amplicon, thus confirming that *TpBGL2* gene was cloned in pYES2.1/V5-His-TOPO® with the correct orientation (Figure 3.7). This result was further confirmed by analyzing the sequence of Sample 11 (see Section Appendices). Thus, Sample 11 was used for transformation into *S. cerevisiae* BY4741 cells.

Table 3.2 : Virtual *Mly*I and *Xba*I restriction patterns of pYES2.1/V5-His-TOPO® plasmids containing *TpBGL2* with correct and with reverse orientation.

Restriction Fragment	Length (bp) of the fragments in case of correct orientation	Length (bp) of the fragments in case of reverse orientation
Restriction Fragment 1	2964	2964
Restriction Fragment 2	662	1091
Restriction Fragment 3	554	662
Restriction Fragment 4	502	502
Restriction Fragment 5	486	486
Restriction Fragment 6	437	437
Restriction Fragment 7	342	342
Restriction Fragment 8	318	305
Restriction Fragment 9	305	38
Restriction Fragment 10	219	7
Restriction Fragment 11	38	-
Restriction Fragment 12	7	-

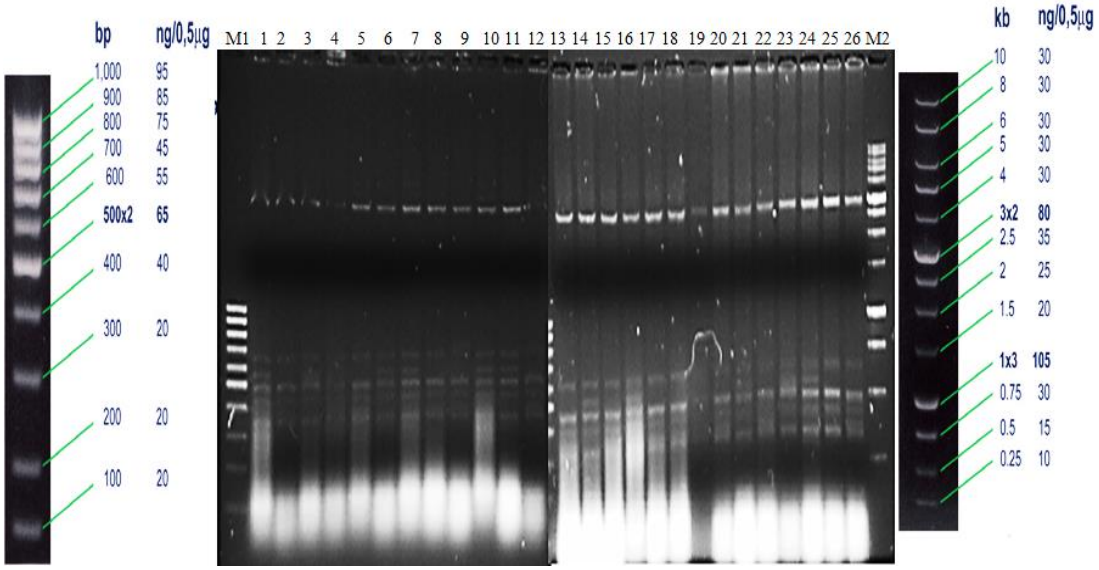

Figure 3.6 : *Mly*I and *Xba*I restriction analysis of 26 clones [M1: Marker 100bp (SHARPMASS™ 100 - DNA LADDER), M2: Marker 1Kb (SHARPMASS™ 1 - DNA LADDER)]. The numbers on the wells indicate different clones.

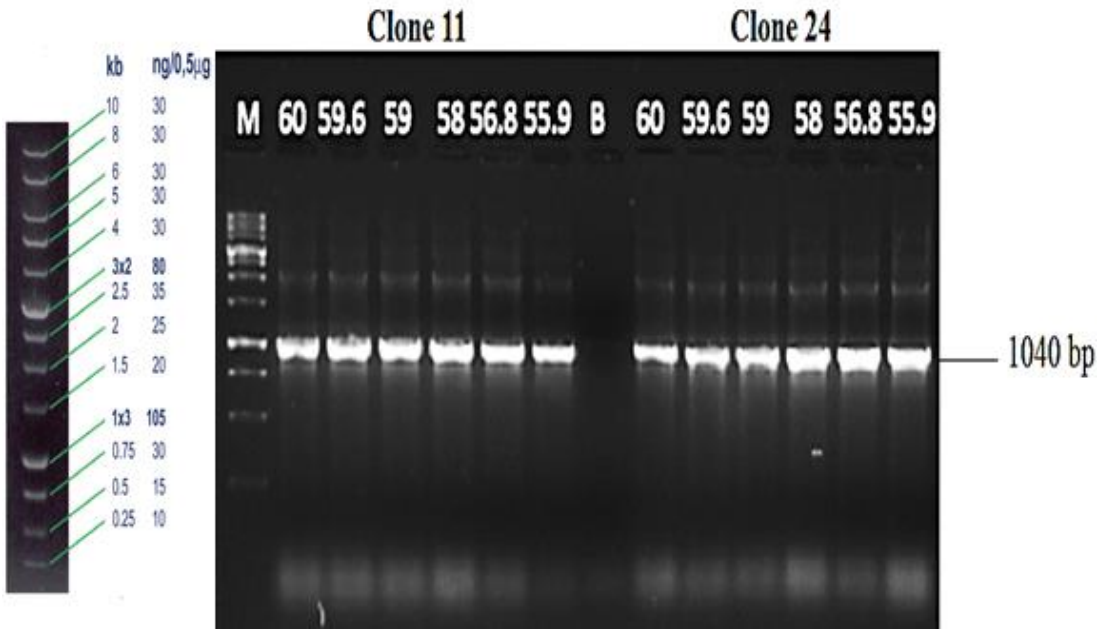

Figure 3.7 : PCR amplification of Clone 11 (left) and Clone 24 (right) with primers *GAL1F* and *BGL2R*. The numbers indicate the annealing temperature for that sample [M: Marker (SHARPMASS™ 1 - DNA LADDER)].

3.3.2 Cloning *TpBGL2* in pPIC9 downstream α sequence

TpBGL2 was amplified using *FW2* and RV primers. These primers, designed on *TpBGL2* gene, contain *Eco*RI (*FW2*) and *Not*I (RV) restriction sites. By using these primers on *T. phaffii* total DNA, an amplicon that contains the target gene flanked by *Eco*RI and *Not*I restriction sites at 5' end and 3' end of the gene, respectively, was obtained. The amplicon of the expected size of 972 bp (Figure 3.5) was cloned into pGEM-T Easy Vector by TA cloning. Before cloning, all fresh PCR products were purified via Wizard® SV Gel and PCR Clean-Up System.

Twelve white colonies resulting from the transformation of the ligation mixture pGEM-T Easy Vector + *TpBGL2* into *E. coli* were subjected to miniprep of plasmid DNA and five clones were subjected to restriction with *Eco*RI (Figure 3.8). Since pGEM-T Easy Vector has *Eco*RI restriction site both upstream and downstream of the insert, a single digest with *Eco*RI was expected to produce two bands, one corresponding to *TpBGL2* (974 bp) and the other one corresponding to the vector (2996 bp). Four of the clones showed this restriction pattern (Figure 3.8) and C6 was chosen for double digestion and ligation in pPIC9 vector since the DNA

concentration of C6 was higher than the other clones (data not shown). Figure 3.9 shows the restrictions of 10 miniprep products of empty pPIC9 vectors. The miniprep in lane 7 (labelled as "8") showed high quality DNA thus it was used for subsequent ligation of *TpBGL2* into pPIC9.

After having confirmed the insertion of *TpBGL2* gene in pGEM-T Easy Vector, the recombinant plasmid was subjected to double restriction with *Eco*RI and *Not*I and the fragment obtained was ligated into pPIC9 restricted with the same enzymes. The ligation mixture was transformed into *E. coli*. Twenty-two colonies selected on LB+ampicillin were subjected to miniprep and PCR amplified with *AOX1* and *BGL2R* primers to identify the clones carrying *TpBGL2* gene with the correct orientation (Figure 3.10). Eleven clones produced amplicons of 1310 bp which is the expected size in case of correct orientation of the insert in pPIC9 vector. The amplicon produced by clone 19 was sequenced with primer *AOX1* and sequence analyses confirmed that the *TpBGL2* gene was cloned into pPIC9 under the control of AOX1 promoter in frame with the α sequence (see Section Appendices).

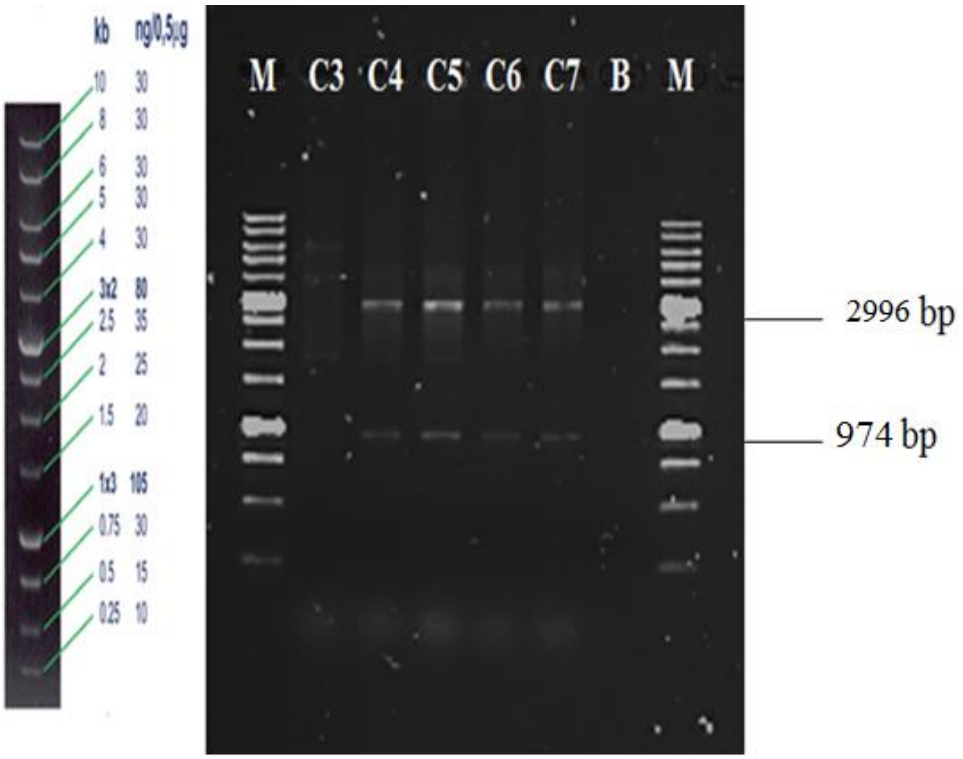

Figure 3.8 : *Eco*RI restriction digestion of clones C3, C4, C5, C6, C7 transformed with pGEM T Easy Vector+ *TpBGL2*. M: Marker (SHARPMASS™ 1 - DNA LADDER), B: Blank.

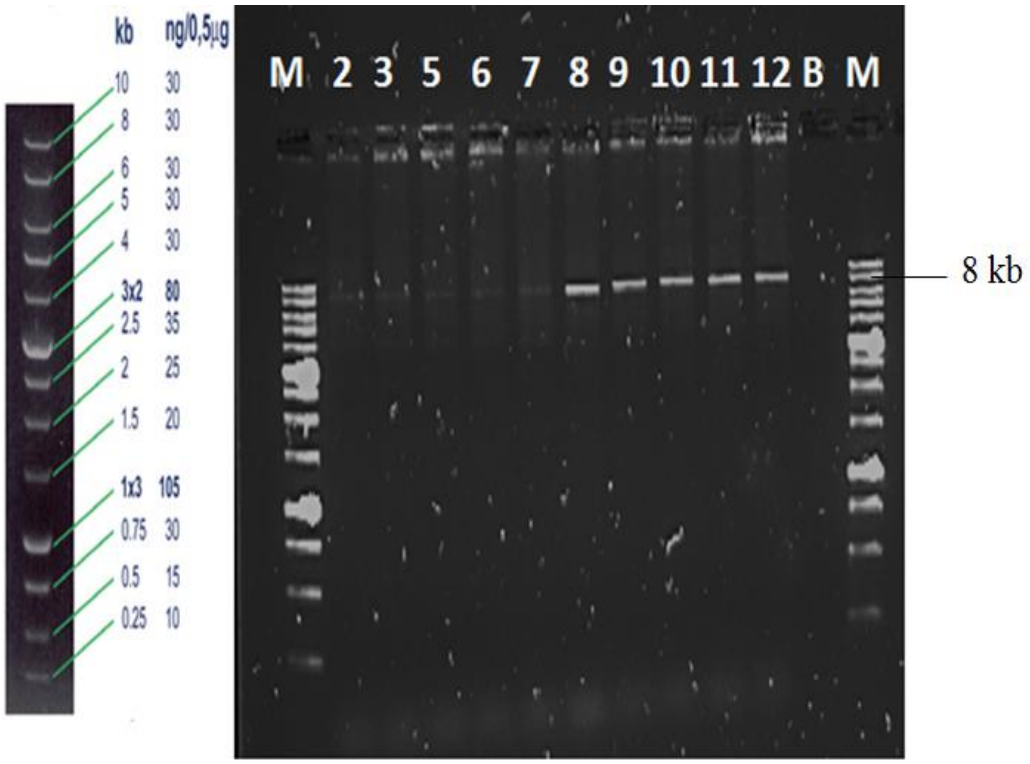

Figure 3.9 : Restriction digestion of pPIC9 vectors with *Eco*RI. Numbers indicate the labelling of the pPIC9 vectors (M: Marker (SHARPMASS™ 1 - DNA LADDER), B: Blank).

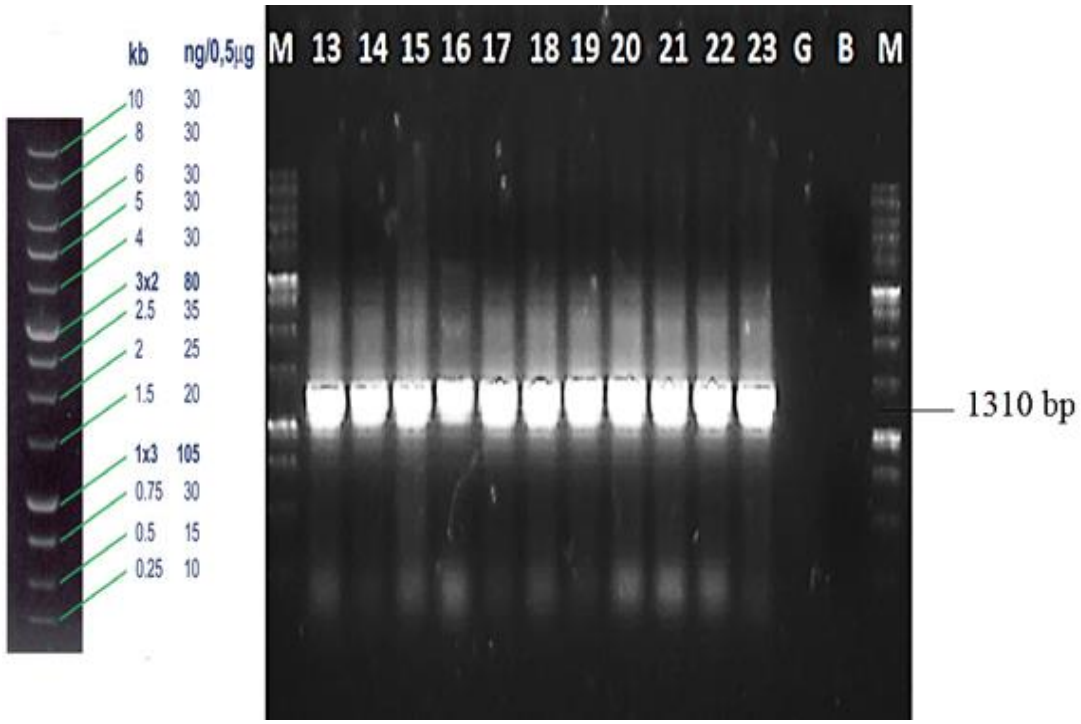

Figure 3.10 : PCR amplification of pPIC9 + *TpBGL2* clones with primers *AOX1* and *BGL2R*. Numbers indicate different clones (M: Marker (SHARPMASS™ 1 - DNA LADDER), B: Blank, G: Gap).

3.3.3 Cloning *TpBGL2* in pYES2.1/V5-His-TOPO® downstream α sequence

After having confirmed that *TpBGL2* was cloned in frame with α sequence in pPIC9+*TpBGL2*, this recombinant vector was utilized as the DNA template for PCR amplification of the cassette α+*TpBGL2* using *ALFA* and *BGL2R* primers. Fresh PCR product of the expected size (1222 bp) (Figure 3.11) was ligated in pYES2.1/V5-His-TOPO® Vector to obtain the third construct in which *TpBGL2* gene is in pYES2.1/V5-His-TOPO® under the control of GAL1 promoter and downstream the α sequence. The ligation mixture was transformed into competent *E. coli* cells. Twenty-four white colonies were picked for minipreparation of DNA and for sequence analysis to determine the orientation of the insert in pYES2.1/V5-His-TOPO®. Sequence analysis showed that *TpBGL2* was cloned downstream α sequence but in reverse orientation (data not shown). Thus, no further experiments were carried out using this clone.

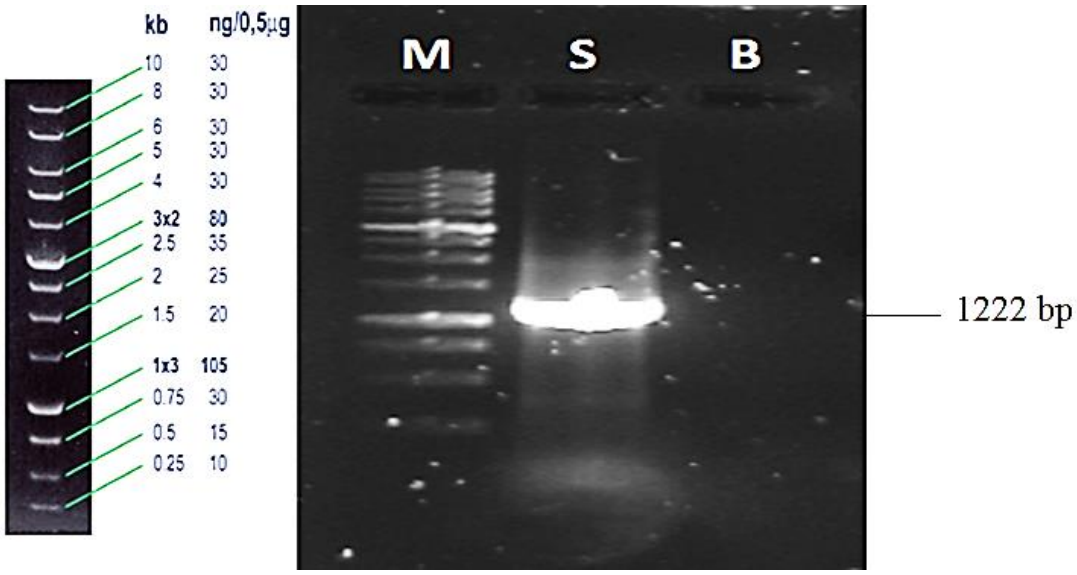

Figure 3.11 : PCR amplification of *TpBGL2* from pPIC9 + *TpBGL2* using *ALFA* and *BGL2R* primers (M: Marker (SHARPMASS™ 1 - DNA LADDER), B: Blank, S: Sample).

3.4 Transformation of the Recombinant Plasmids in Yeast

Two of the three desired recombinant vectors were obtained: pYES2.1/V5-His-TOPO® + *TpBGL2* and pPIC9 + *TpBGL2*. These two vectors were therefore transformed in *S. cerevisiae* BY4741 and *P. pastoris* GS115, respectively, to enable the heterologous production of Kpkt in the two hosts.

3.4.1 Transformation of pYES2.1/V5-His-TOPO®+*TPBGL2* in *S. cerevisiae* and evaluation of recombinant killer toxin production

After cloning *TpBGL2* in pYES2.1/V5-His-TOPO® Vector and confirming that the insert had the correct orientation, the recombinant vector, lacking the α sequence, was utilized for transformation of *S. cerevisiae* BY4741. Six BY4741 transformants were selected by plating onto YNB selective medium without uracil and with glucose as the sole carbon source. In order to confirm that all transformants had the insert, PCR was carried out with primers *BGL2F* and *BGL2R* following total DNA extraction. All transformants produced a clear band of 943 bp corresponding to *TpBGL2* gene (Figure 3.12).

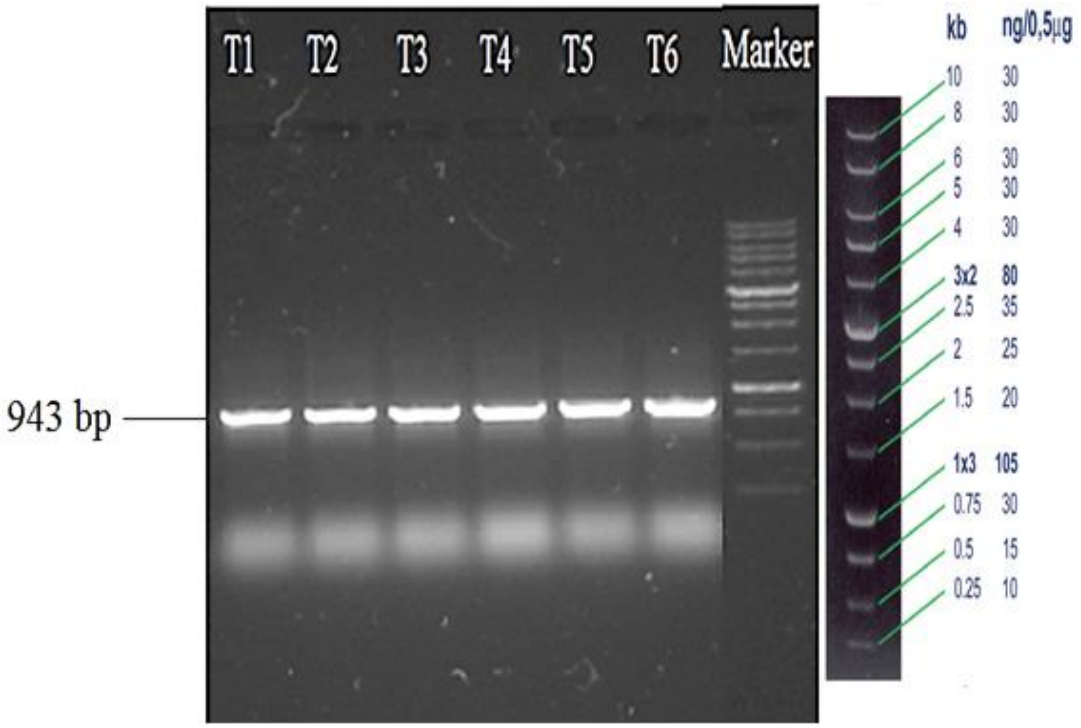

Figure 3.12 : PCR analysis of the six *S. cerevisiae* transformants **(**Marker: SHARPMASS™ 1 - DNA LADDER).

Transformant T1 (from now on T1) was picked for further experiments. In order to induce the transcription of *TpBGL2* and to obtain the heterologous expression of Kpkt, T1 was cultivated as follows: in the first strategy, T1 was grown in buffered yeast minimal medium with 2% galactose and 2% raffinose until reaching OD_{600} ~15. After 24h and 48h, supernatants and crude extracts were assayed for killer activity as described previously (Figure 3.13).

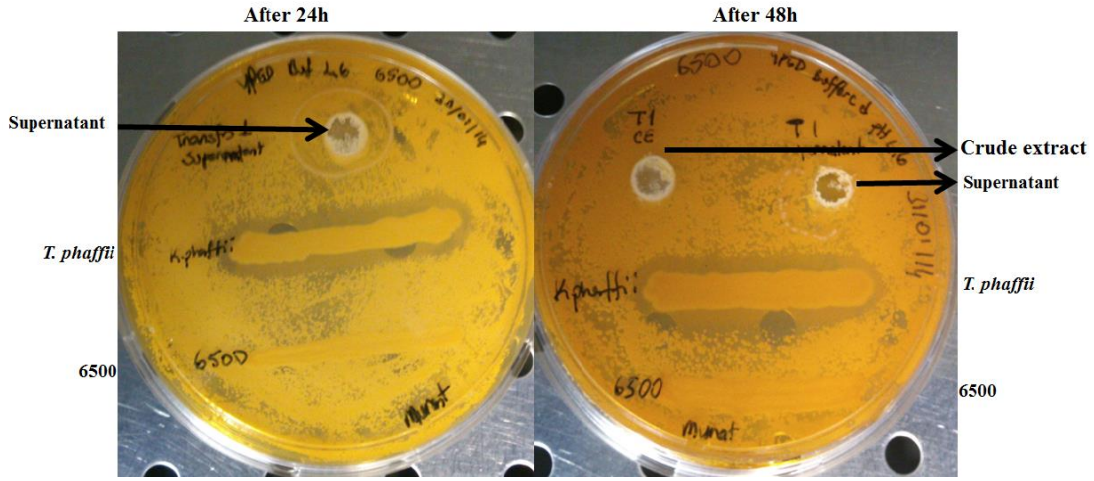

Figure 3.13 : Killer plate assay for the first growth strategy. DBVPG 6500 was spread and used as a sensitive strain.

In the second growth strategy, T1 was grown in buffered 0.2% galactose 2% raffinose YNB selective medium and cells were transferred after 48 h into buffered 2% galactose YNB selective medium. The culture was incubated for 24 h. Crude extract was obtained from the whole culture by using both potassium phosphate buffer and citrate phosphate buffer. Finally, killer plate assay was performed as described (Figure 3.14). However, no clear zones were observed around wells containing crude extract or supernatant, thus indicating that Kpkt was not expressed properly.

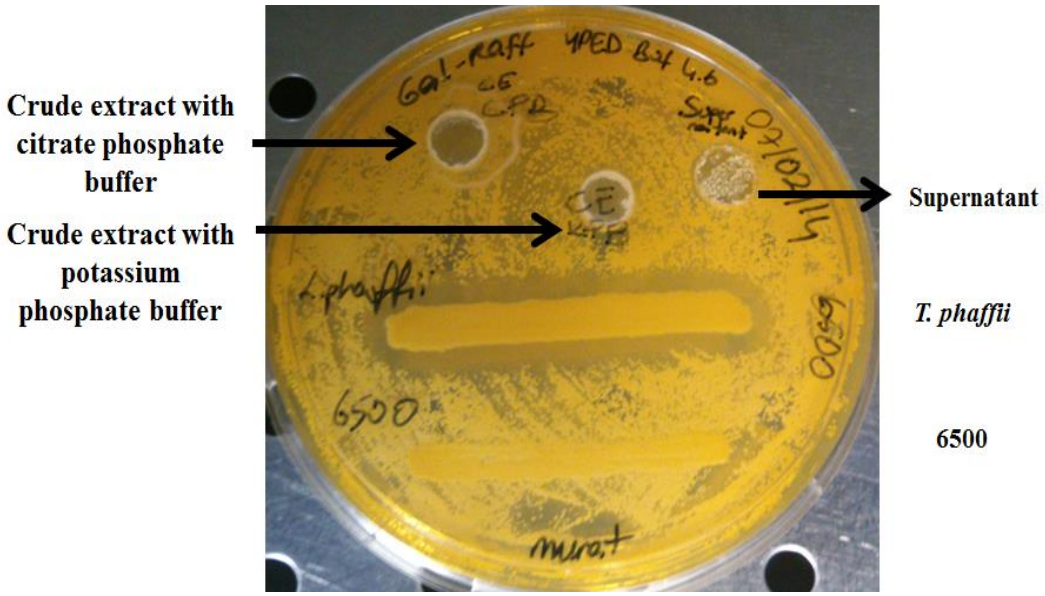

Figure 3.14 : Plate killer assay for the second growth strategy. DBVPG 6500 was spread and used as a sensitive strain.

In the third growth strategy, T1 was grown in buffered 2% sucrose YNB selective medium for 48 h and the cells were transferred into buffered 2% galactose YNB selective medium. After 24 h incubation, crude extract and supernatants obtained as before were subjected to killer test assay. Unfortunately, no clear zone was observed around any wells (Figure 3.15). This also indicated that growth strategy chosen for T1 did not induce heterologous Kpkt expression in T1 properly.

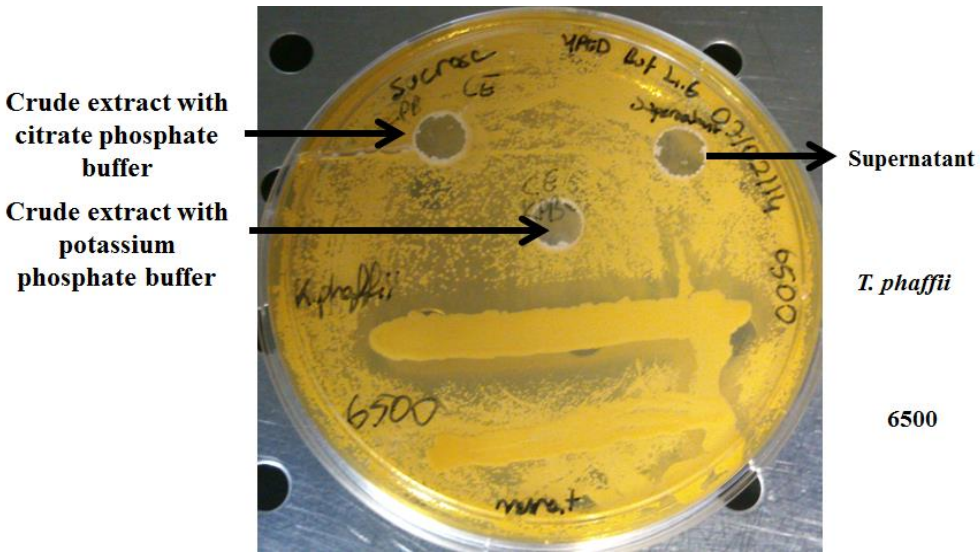

Figure 3.15 : Plate killer assay for the third growth strategy. DBVPG 6500 was spread and used as a sensitive strain.

3.4.2 Transformation of pPIC9+*TPBGL2* in *P. pastoris*

After cloning *TpBGL2* in pPIC9 and confirming that the insert was in frame with the α sequence, the recombinant plasmid was prepared for transformation in GS115 for the heterologous expression of Kpkt killer toxin in *P. pastoris*. Plasmid pPIC9+*TpBGL2* was linearized with either *Sac*I or *Bgl*II restriction enzyme in order to obtain His^+ Mut^+ and His^+ Mut^S transformants, respectively. In parallel, the empty pPIC9 was restricted with the same enzymes to obtain His^+ Mut^+ and His^+ Mut^S negative control of expression. The rationale behind this strategy is that the transformation of linearized DNA results in the stable integration of one or more copies of the vectors in the genome of *P. pastoris*, mainly for single crossing over (Cregg *et al.*, 1985; Cregg *et al.*, 1989). Since pPIC9 contains HIS4 gene, all the transformants became prototrophic for this amino acid (phenotype His^+). Moreover, they may have Mut^+ phenotype in case the integration event results in a functional *AOX1* gene, or Mut^S phenotype in case the integration event results in a

nonfunctional *AOX1* gene. Thus, *P. pastoris* GS115 was transformed with pPIC9 and pPIC9+*TpBGL2* linearized with *Sac*I and with pPIC9 and pPIC9+*TpBGL2* linearized with *Bgl*II restriction enzymes. Transformation was carried out by electroporation as indicated in Section 2.2.5.2. Each of the four transformations produced a number of transformants ranging from 50-100. These were grown on minimal dextrose (MD) medium plates. About 25 transformants for each transformation were selected and tested for Mut+ or Mut^S phenotype by growing them on MD and MM media. Mut^S transformants are expected to grow slowly on methanol containing MM medium. On the basis of this plate assay, 28 transformants presumptively representative of the two phenotypes were selected (Table 3.3).

Table 3.3 : *P. pastoris* transformants selected on the basis of growth assay on MD and MM media.

Vector	Putative Mut^S Transformant Number	Putative Mut+ Transformant Number
pPIC9+*TPBGL2* *Sac*I	52, 54, 57, 102	38, 46, 50, 103, 108, 112
pPIC9+*TPBGL2* *Bgl*II	15, 16, 20, 90	9, 10, 12, 17, 19, 85
pPIC9 *Sac*I	24	29, 36
pPIC9 *Bgl*II	5, 73, 119	6, 71

PCR analysis of the transformants carried out with primers *AOX1* and *AOXR* showed that all of them present 2 bands, one corresponding to the *AOX1* gene and the other one to *TpBGL2* gene. Thus, no Mut^S was obtained with the sole exception of transformant 24. This presents 1 band of 492 bp produced by pPIC9 linearized with *Sac*I (Figure 3.16).

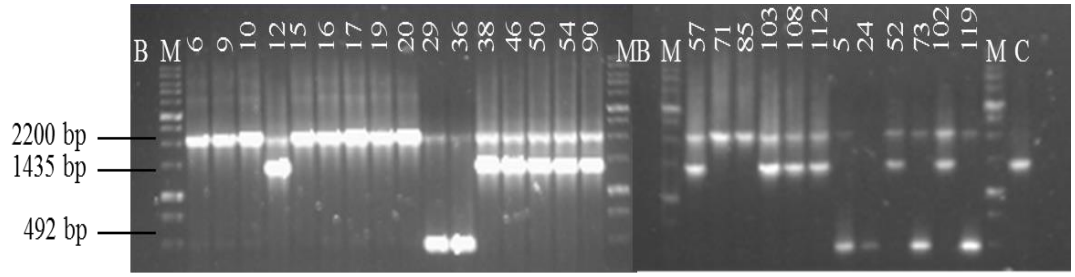

Figure 3.16 : PCR amplification of the total DNA extracted from *P. pastoris* transformants. B: blank; C: pPIC9+*TpBGL2*; M DNA Ladder 1 Kb, Euroclone. Band of 2200 bp refers to a functional AOX1 gene; band of 1435 bp derives from the integration of *TpBGL2* gene; band of 492 bp derive from the integration of pPIC9.

4. DISCUSSION AND CONCLUSIONS

This study was aimed at the heterologous expression of Kpkt killer from *T. phaffii* in *S. cerevisiae* and *P. pastoris* using pYES2.1/V5-His-TOPO® expression system and pPIC9 expression vector for *S. cerevisiae* BY4741 and *P. pastoris* GS115 strains, respectively. *S. cerevisiae* and *P. pastoris* are generally considered as suitable hosts for heterologous expression due to their advantages compared to other microorganisms. First of all, both microorganisms are capable of post-translational modifications and protein folding in order to produce recombinant protein in a native form (Cregg *et al*., 2000; Buckholz and Gleeson, 1991). Another advantage is that they grow very fast due to their short doubling time, thus producing proteins at high amounts in a short time. In addition, the whole genomes of both microorganisms have been sequenced (Goffeau *et al*., 1996; De Schutter *et al*., 2009). Thus, *S. cerevisiae* and *P. pastoris* are good candidates as host strains for heterologous expression of proteins with some modifications.

pYES2.1/V5-His-TOPO® vector harboring GAL1 promoter upstream of ORF was utilized for heterologous expression of Kpkt killer toxin in *S. cerevisiae*. Catabolite repression effect is observed in GAL1 promoter function unless there is galactose in the environment as the sole carbon source (Johnston, 1987). Even though *S. cerevisiae* grows slowly in galactose containing medium, GAL1 promoter is preferred in most commercial expression vectors since the protein of interest could be expressed in high amounts in the presence of galactose, compared to other host proteins. Hence, purification of the protein of interest could be easier as well as lethal protein expression could be repressed in glucose-containing medium, if the protein of interest has lethal characteristics (Primrose et al., 2001).

In the first part of the study, a suitable *S. cerevisiae* host strain was selected for heterologous expression of Kpkt. *S. cerevisiae* BY4741 strain appeared as a good candidate since it can grow in galactose-containing medium (Table 3.1), although being slightly sensitive to Kpkt killer toxin (Figure 3.1). For this purpose, it was

decided to construct two different expression vectors for heterologous expression: i) pYES2.1/V5-His-TOPO® under the control of GAL1 promoter; ii) pYES2.1/V5-His-TOPO® under the control of GAL1 promoter and downstream the α sequence. The first one was used for intracellular heterologous expression of Kpkt, whereas the second one was used for extracellular protein expression, since *S. cerevisiae* strains showed a slight sensitivity for the killer toxin in extracellular environment.

P. pastoris GS115 strain that showed a strong resistance to the killer toxin (Figure 3.1) was utilized for extracellular heterologous expression of the killer toxin.

Solely two of the three constructs were obtained.

TA cloning system proved very useful for the construction of pYES2.1/V5-His-TOPO® + *TpBGL2*, as confirmed by sequence analysis. On the contrary, the construction of pYES2.1/V5-His-TOPO® + α+ *TpBGL2* was unsuccessful as TA cloning led to the insertion of the α+ *TpBGL2* cassette with wrong orientation. The two step cloning utilized for the insertion of *TpBGL2* gene in pPIC9 led to the construction of the recombinant vector pPIC9+*TpBGL2*, in which the heterologous gene is under the control of AOX1 promoter and in frame with the α leader sequence of secretion.

pYES2.1/V5-His-TOPO® + *TpBGL2* was transformed into BY471 *S. cerevisiae* strain for intracellular expression of Kpkt killer toxin. Once it was shown that the vector was successfully transformed into BY4741 (Figure 3.12), killer plate assay was performed against *S. cerevisiae* 6500 sensitive strain for the killer toxin. Thus, crude extract and the supernatant of *S. cerevisiae* BY4741 transformant grown in different galactose-containing media were analyzed to investigate the activity of the killer toxin expressed (Figure 3.14, 3.15, 3.16). However, no killer activity was observed in recombinant *S. cerevisiae*. This might be due to the fact that the killer toxin is a secreted protein that may not have been correctly processed intracellularly. In future studies, the construction of pYES2.1/V5-His-TOPO® + α+ *TpBGL2* will be made to assess whether *S. cerevisiae* may be a useful host for the extracellular expression of this heterologous protein. In addition, Western Blot analysis will be performed to investigate whether the heterologous protein is expressed intracellularly, although being not active.

For extracellular expression of Kpkt killer toxin in *P. pastoris*, GS115 strain pPIC9+*TpBGL2* was utilized. Once it was observed that the gene was in the frame with AOX1 promoter as well as the α sequence, the vector was transformed into *P. pastoris* GS115. The results showed that it was successfully transformed into *P. pastoris* (Figure 3.16). However, further studies have yet to be performed to investigate the killer activity of the killer toxin produced by *P. pastoris*. The evaluation of heterologous Kpkt production is in progress.

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APPENDICES

APPENDIX A: Sequence Analysis Results

After obtaining pYES2.1/V5-His-TOPO® + *TpBGL2* expression cassette, sequence analysis was carried out using *GAL1F* primer in order to determine the orientation of the insert within the vector. Approximately 1015 bp of the cassette was sequenced. The analyzed sequence showed 96.8% identity with the expected result in case of the correct orientation of the insert in the vector (Figure A.1). The sequenced analyzed showed alignment to the sequence from the 429th bp to the 1459th bp of the expected result. This indicates that the insert was in frame with GAL1 promoter.

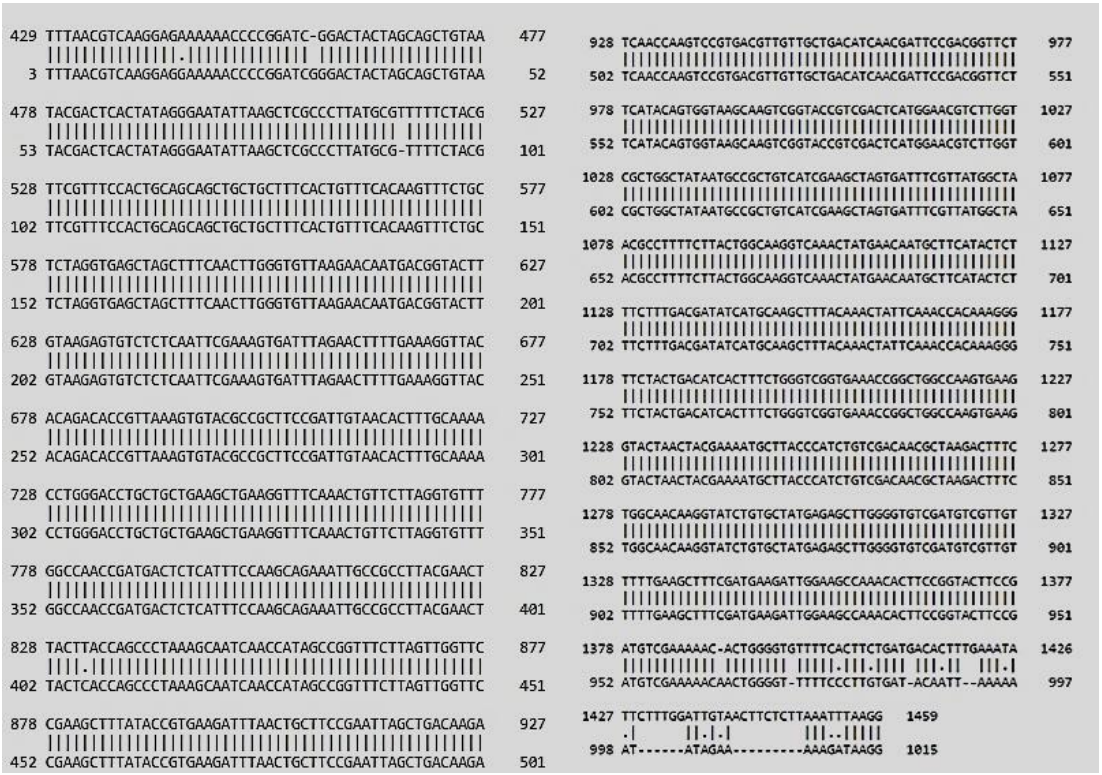

Figure A.1 : Sequence analysis result for pYES2.1/V5-His-TOPO® + *TpBGL2* expression cassette. Upper sequence shows the expected result, while the lower sequence presents the analyzed sequence.

In addition, pPIC9 + *TpBGL2* expression cassette was sequenced in order to determine the correct orientation of the insert in the vector using *AOX1* primer. Approximately 723 bp of the expression cassette was analyzed and it was shown that the analyzed sequence showed 96.6% identity with the expected result in case the insert was in the correct orientation in the vector (Figure A.2). The alignment between the analyzed sequence and the expected result started from the 868th bp of the expected result and lasted at the 1602nd bp of the expected result. This result indicated that the insert was in frame with both AOX1 promoter and alpha leader secretion site in the vector.

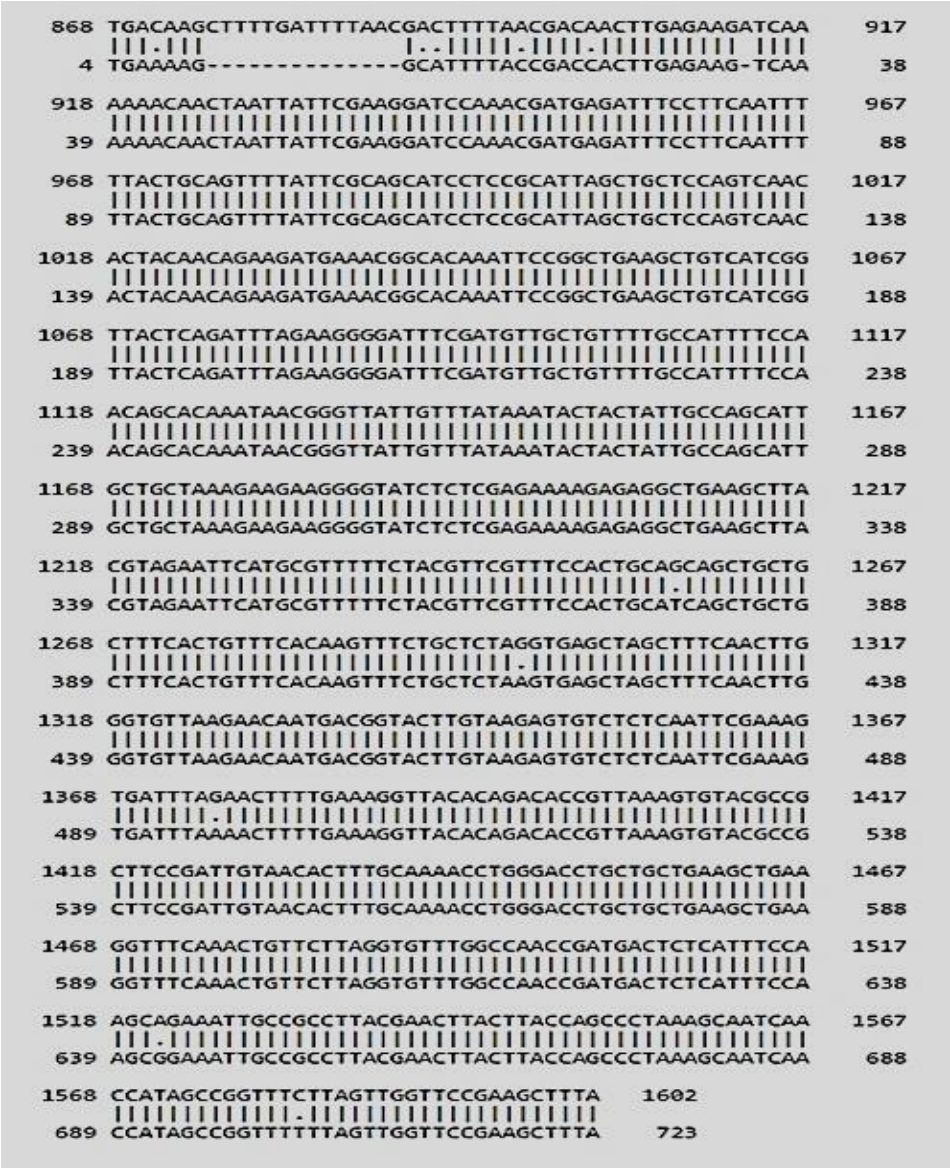

Figure A.2 : Sequence analysis result for pPIC9 + *TpBGL2* expression cassette. Upper sequence shows the expected result, while the lower sequence presents the analyzed sequence.

CURRICULUM VITAE

Name Surname:	**Murat ÜSTÜN**
Place and Date of Birth:	**İzmir – 30.12.1988**
Address:	**Manavkuyu Mah. 279 Sok. No:13/3 Bayraklı / İZMİR**
E-Mail:	**ustunmu@gmail.com**
B.Sc.:	**Molecular Biology and Genetics**
	Istanbul Technical University (2007-2012)
M.Sc.:	**Molecular Biology, Genetics and Biotechnology**
	Istanbul Technical University (2012-2015 expected)

Presentations:

▪ Yılmaz, Ü., Holyavkin, C., **Üstün, M.**, Şahin, D., Çakar, Z. P., (**2012)**. Evolutionary engineering and molecular characterization of freeze-thaw resistant *Saccharomyces cerevisiae*. 1st International Congress of the Molecular Biology Association of Turkey. Abstract Book p.106, poster no: 82. 23-24 November, 2012 Istanbul, Turkey (Poster Presentation)