CLONING AND CHARACTERIZATION OF TREHALOSE-6-PHOSPHATE SYNTHASE GENE FROM *Rhizopus oryzae*

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

GÜLSÜM EBRU ÖZER UYAR

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

SEPTEMBER 2009

Approval of the thesis

CLONING AND CHARACTERIZATION OF TREHALOSE-6-PHOSPHATE SYNTHASE GENE FROM *Rhizopus oryzae*

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ABSTRACT

CLONING AND CHARACTERIZATION OF TREHALOSE-6-PHOSPHATE SYNTHASE GENE FROM *Rhizopus oryzae*

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September 2009, 99 pages

In many organisms, trehalose protects against several environmental stresses, such as heat, desiccation and salt, probably by stabilizing protein structures and lipid membranes. Trehalose-6-phosphate synthase 1 (TPS1) is a subunit of trehalose synthase complex in fungi; it plays a key role in the biosynthesis of trehalose.

In this study, a *TPS1* gene fragment in *R. oryzae* was cloned successfully by PCR with primers designed according to eight hypothetical proteins found from BLAST search which was performed by using *S. cerevisiae TPS1* gene template. The full length of *R. oryzae TPS1* gene (designated *RoTPS1*) was attained by RT-PCR with primers specific to the 3′ and 5′end of the *RoTPS1* cDNA. The *RoTPS1*

cDNA was composed of 2505 bps encoding a protein of 834 amino acids with a molecular mass of 93.8 kDa. The amino acid sequence has relatively high homology with the *TPS1*s of several other organisms.

RoTPS1 was further characterized by transformation into *S. cerevisiae tps1* mutant. In galactose media, the growth curves of wild type, *tps1* mutant and transformant *S. cerevisiae* cells had a comparable pattern in general, *tps1* mutant reached to a higher maximum cell concentration compared to the others and wild type had a slightly lower specific growth rate compared to the *tps1* mutant and transformed cells. Trehalose levels of transformant and wild type cells were increased up to 37 mg/gdw in the stationary phase.

Keywords: *R. oryzae*, Trehalose, Trehalose-6-Phosphate Synthase, Cloning, Complementation

ÖZ

R. oryzae DAN TREHALOZ-6-FOSFAT SENTAZ GENİNİN KLONLANMASI VE KARAKTERİZASYONU

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Eylül 2009, 99 sayfa

Trehaloz bir çok organizmada sıcaklık, kuraklık, tuz gibi çevresel strese karşı muhtemelen protein yapısını ve lipid membranları stabilize ederek korur. Trehaloz-6-fosfat sentaz 1 mantarlarda trehaloz sentaz birleşiğinin bir alt birimidir ve trehaloz biyosentezinde anahtar görevi vardır.

Bu çalışmada *S. cerevisiae TPS1* gen şablonu ile yapılan BLAST araması sonucu bulunan sekiz hipotetik proteine gore tasarlanan primerler kullanılarak *R.oryzae*'den bir gen parçası PZR ile başarılı olarak klonlandı. *RoTPS1* cDNA'nın 3′ ve 5′ uçlarına özel primerler kullanılarak yapılan RT-PZR ile *R. oryzae TPS1* geninin (*RoTPS1* olarak gösterildi) tam uzunluğuna ulaşıldı. *RoTPS1* cDNA, moleküler ağırlığı 93.8 kDa olan 834 amino asitli bir proteini kodlayan 2505 baz çiftinden oluştu. Amino asit dizisinin diğer bazı organizmaların *TPS1*'leriyle göreceli olarak yüksek homolojiye sahip olduğu görüldü.

RoTPS1'in *S. cerevisiae tps1* mutantına transformasyonu yapılarak daha ileri karakterizasyonu yapılmıştır. Galaktozlu besiyerinde yaban tür, *tps1* mutantı ve transformant *S. cerevisiae*'nin büyüme eğrileri genel olarak benzerdir, *tps1* mutantı diğerlerine kıyasla daha yüksek maksimum hücre konsantrasyonuna erişmiştir, *tps1* mutantı ve transformant hücrelerle karşılaştırıldığında yaban tür hafifçe daha düşük spesifik büyüme hızına sahiptir.Transformant ve yaban tür hücrelerin trehaloz seviyeleri durağan fazda 37mg/gkha düzeyine kadar çıkmıştır.

Anahtar Kelimeler: *R. oryzae*, Trehaloz, Trehaloz-6-Fosfat Sentaz, Klonlama, Komplementasyon

To my family and my dear husband Başar Uyar

ACKNOWLEDGEMENTS

First of all I would like to express my deepest gratitude to Prof. Dr. Haluk Hamamcı and Prof. Dr. Meral Yücel for their invaluable guidance and supervision. I wish to thank for their understanding and support.

I am also appreciative of the valuable critics and advices provided by the thesis commitee members Prof. Dr. Hüseyin Avni Öktem and Prof. Dr. Sezai Türkel. I am grateful to Prof. Dr. Sezai Türkel for providing the wild type and mutant yeast strains that were used in this thesis.

I also wish to extend my great appreciation to Dr. Şeyda Açar and Dr. Beray Ünsal for their advices and suggestions, without their assistance this would not have become a reality. I would like to thank to Eda Alagöz for being ever ready to provide me with valuable suggestions and assistance in organizing and conducting experiments.

I wish to thank to all my labmates, Ceylan Şahin Büyükkileci, Ali Oğuz Büyükkileci, Gül Sarıbay, Bekir Gökçen Mazı, Ayşem Batur and Gökhan Duruksu who have been more than friends to me throughout my research work.

I also wish to thank to all my workmates in Gülhane Military Medical Academy Medical Biochemistry Emergency Laboratory for their understanding and encouragements.

My thanks are also extended to my friends Dr. Tamay Şeker, Dr. Demet Çetin, Demet Ataman, Songül Okur and others whose names couldnot be written here for their understanding throughout my thesis.

I want to send all my love to my family, and wish to thank them for their constant support and encouragement throughout all these years.

...and the last but never the least I want to send my love to my dear husband Başar Uyar for his love, patience and unconditional support.

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LIST OF SYMBOLS AND ABBREVIATIONS

A	Absorbance
ADP	Adenosine diphosphate
amp	Ampicilline
AMP	Adenosine monophosphate
ATH	Acidic trehalase gene
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
cAMP	Cyclic adenine monophosphate
CIF	Catabolite inactivation of fructose- 1,6-bisphosphatase gene
DEPC	Diethylpyrocarbonate
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
G1P	Glucose 1 phosphate
G6P	Glucose 6 phosphate
GGS1	General glucose sensing gene
GLK	Glucokinase
GRAS	Generally Recognized As Safe
HCl	Hydrochloric acid
HXK	Hexokinase gene
LB	Luria Bertani
Mg^{2+}	Magnesium ion
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NTH	Neutral trehalase gene

OD	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PKG	Phosphoglycerate kinase
R. oryzae	*Rhizopus oryzae*
RNA	Ribonucleic acid
RT	Reverse transcriptase
S. cerevisiae	*Saccahromyces cerevisiae*
SM	Selective media
T6P	Trehalose 6 phosphate
T6PP	Trehalose-6-phosphate phosphatase
T6PS	Trehalose-6-phosphate synthase
TAE	Tris acetate EDTA buffer
TCA	Trichloroacetic acid
TCA	Tricarboxylic acid
TE	Tris HCl EDTA buffer
TPS1	Trehalose 6 phosphate synthase gene
TPS2	Trehalose 6 phosphate phosphatase gene
TPS3	Trehalose synthase complex regulatory gene
TSL1	Trehalose synthase complex regulatory gene
UDPG	Uridine di phosphoglucose
URA	Uracil
WT	Wild type
X-Gal	5-bromo-4-chloro-3-indolyl b-D-galactopyronoside
μ_{max}	Maximum specific growth rate, (1/h)

CHAPTER 1

INTRODUCTION

Trehalose is a non-reducing disaccharide widely found in nature. In 1958, Cabib and Leloir [1] identified for the first time two enzymatic activities leading to the formation of trehalose from UDP-glucose (UDP-Glu) and glucose-6-phosphate (G6P). The two enzymes catalyzing these reactions, trehalose-6-phosphate synthase (T6PS) and trehalose-6-phosphate phosphatase (T6PP) were thereafter shown to form a multifunctional protein complex [2, 3].

In this study, trehalose-6-phosphate synthase, which is the first enzyme of the trehalose metabolism, in an industrially important microorganism *Rhizopus oryzae,* was investigated.

Saccharomyces cerevisiae, which is another industrially important microorganism, was used as an expression host for the cloned gene from *R. oryzae*.

The primary metabolism of industrial microorganisms has been studied for long time and most biochemical pathways and reaction networks have been elucidated to improve the yield of the microbial and cellular processes. This large pool of biochemical information, together with data from proteomics, metabolomics and genomics underpins the strategies for design of experiments and choice of targets for manipulations. These targets are often located in the primary metabolic pathways, such as glycolysis, pentose phosphate pathway, the TCA cycle and amino acid biosynthesis and mostly at major branch points within these pathways. Glycolysis is a pathway of central importance for most living organisms, ranging from prokaryotes to man and leads to various products (Figure 1.1).

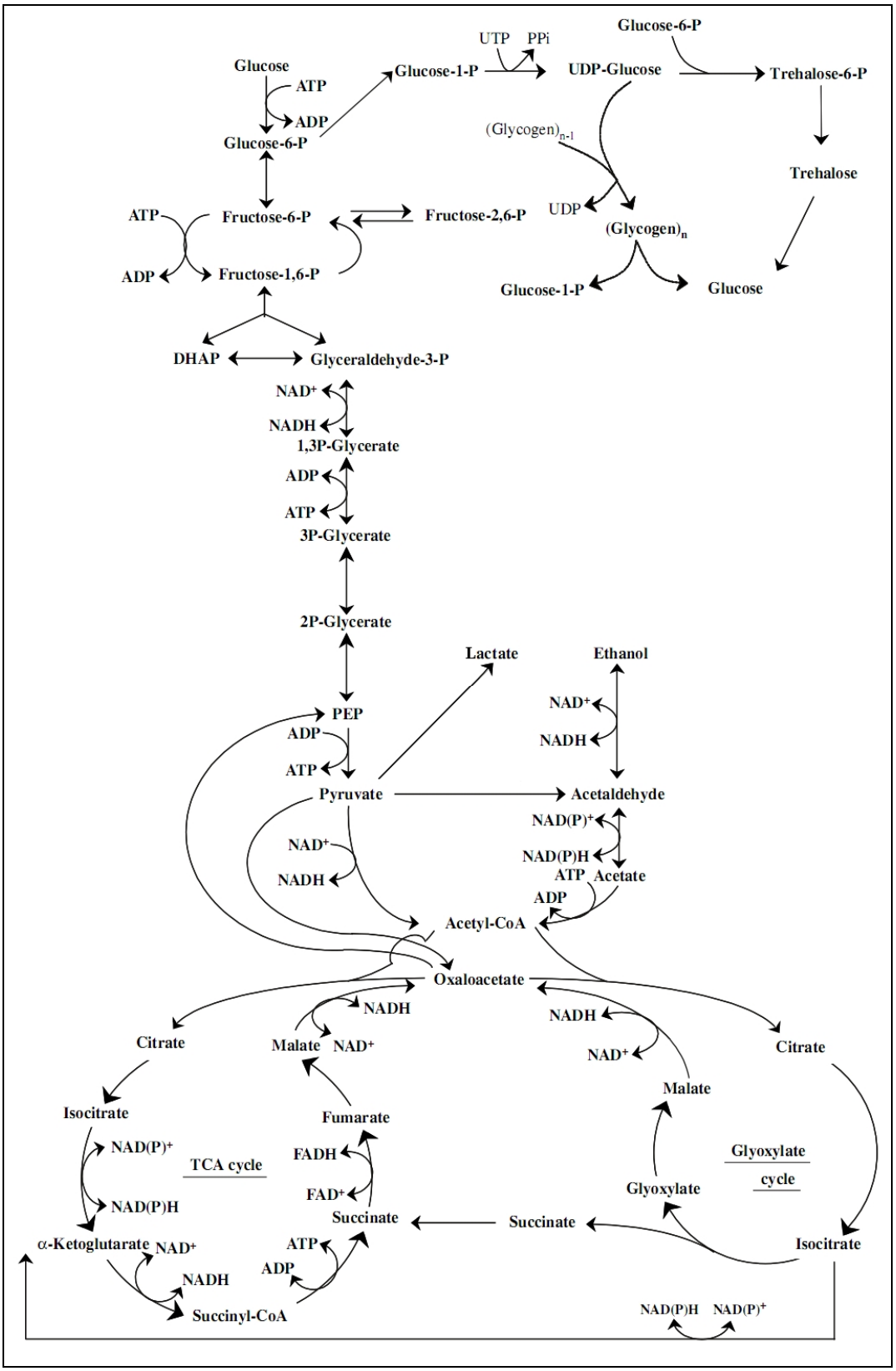

Figure 1.1. The glycolytic, storage carbohydrate and TCA cycle metabolic pathways [4].

Glycolysis is the widely studied pathway in plants, fungi, yeast and other eukaryotes. Most of the studies have been focused on increasing the flux rate by overexpressing one or more enzymes of the glycolytic pathway [5]. Others study metabolic engineering which use analytical methods to identify the regulation of the glycolysis and to quantify the flux at different physiological conditions [6, 7].

Biochemical characterization of the enzymes catalyzing the pathways and expression analysis of the genes coding for these enzymes are important in this sense to supply the data in order to be able to do metabolic engineering on the glycolytic pathway.

Trehalose metabolism is a short side-branch of glycolysis.

The subject organisms, the trehalose metabolism and the underlying information found in the literature are explained in this chapter.

1.1. The microorganisms

1.1.1. *Rhizopus oryzae*

Rhizopus group is important in the production of fermented food products. Traditionally, classification and identification of these species has been done according to their morphological and physiological features. *R. oryzae* belongs to the Kingdom Fungi, Division Eumycota, Subdivision Zygomycotina, Class Zygomycetes, Order Mucorales. The principal characteristic is the production of a thick-walled resting spores called zygospores that distinguish the class Zygomycete. Taxonomically *R. oryzae* belongs to a completely different phylum in the Fungi, and its strategy for acidifying the environment also appears to be distinct.

Rhizopus species produce a variety of industrial products by submerged fermentation and biotransformation including enzymes (lipases, proteases, glucoamylase and celluloytic enzymes), organic acids (lactic acid, fumaric acid) and steroids, terpenoids and alkoloids, pesticides and herbicides, antibiotics [8].

The fungal production of L(+)-lactic acid by a surface culture of *Rhizopus* species was reported early in the last century [9]. However, the first report of an

efficient submerged fermentation for the fungal production of L(+)-lactic acid was in 1936 [10, 11]. This was the era in which the efficiencies of submerged fungal fermentations first became widely recognized. Ward *et al*. described a fermentation process utilizing the Zygomycete genera, *Rhizopus* and *Actinomucor* in general, and *R. oryzae* (syn. *arrhizus*) specifically, which resulted in 63–69% yields of L(+)-lactic acid from chemically defined media containing 15% glucose [11]. Lactic acid can be synthesized chemically, but such synthesis results in a mixture of D and L isomers. The products of microbiological fermentations depend on the organism used and also may include a mixture of the two isomers or individual isomers in a stereospecific form. The desired stereospecificity of the product depends on the intended use; however, L(+)- lactic acid is the form desired for most applications [12].

R. oryzae imports glucose and exports lactate, an acid that is not a component or by-product of the citric acid cycle. Lactate is produced by the organism aerobically, and the commercial process requires agitation and aeration just as the other fungal organic acid processes do.

In aqueous solution, lactic acid dimerizes to form lactide, an intermediate for the biodegradable plastic, polylactic acid. Use of lactic acid in biodegradable plastics is expected to gather momentum, given the rising demand for environmentally friendly packaging. Use of lactic acid bacteria in anti microbial compounds, food additives, flavoring agents, and as a substitute for hazardous solvents in industrial applications, will all help ensure steady consumption into the future. Given the low toxicity and biodegradability advantages of lactic acid as a raw material, demand for lactic acid in biodegradable polymers is forecast to post healthy gains. The lactic acid industry is additionally expected to benefit significantly from the backlash arising out of growing environmental pollution caused by plastic disposal, rising oil prices and greater consumer interest towards the use of greener products [13].

Until recently, lactic acid was used primarily in the food industry as a preservative, flavor enhancer, and acidulant. A non-food application for lactic acid is the manufacture of the biodegradable solvent, ethyl lactate. A significant

commercial source of lactic acid is a bioprocess employing the Zygomycete fungus *Rhizopus oryzae*. The ability to produce large amounts of lactic acid appears to be restricted to the phylum Zygomycota [14].

Considerable progress has been made in understanding the physiology and biochemistry of acid production by *R. oryzae*. Early studies demonstrated that *R. oryzae* produced L-lactate via glycolysis with the concomitant production of ethanol and carbon dioxide [15-17]. These studies also showed that lactic acid yield was increased and ethanol formation decreased under aerobic conditions, while the opposite was true under low oxygen conditions.

In addition to producing L-lactate, *Rhizopus* spp. are also the best of the identified fungal sources for fumarate production. The nutritional and physical requirements of *R. oryzae* leading to maximum yields of fumarate have been examined. Like other fungal fermentations accumulating high concentrations of organic acids, high carbohydrate concentrations, and high carbon to nitrogen ratios are conducive to high fumaric acid yields with minimal biomass accumulation [18]. L-Malic acid production has been observed in *R. oryzae* [18]. Generally, L-malate accumulation in *R. oryzae* is minor compared to L-lactate or fumarate. Figure 1.2 shows the critical pathways for organic acid synthesis in *R. oryzae.*

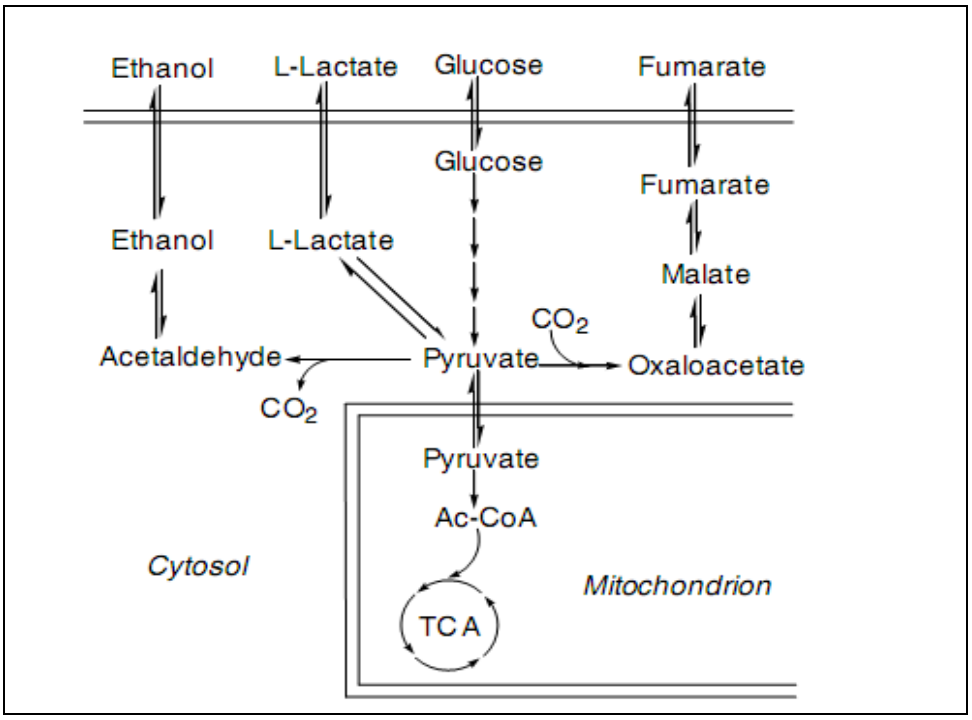

Figure 1.2. Critical pathways for organic acid synthesis in *R. oryzae*.

1.1.2. *Saccharomyces cerevisiae*

Yeast are unicellular fungi. The precise classification is a field that uses the characteristics of the cell, ascospore and colony. Physiological characteristics are also used to identify species. One of the more well known characteristics is the ability to ferment sugars for the production of ethanol. Budding yeasts are true fungi of the phylum *Ascomycetes*, class *Saccharomycetes* (also called *Hemiascomycetes*). The true yeasts are separated into one main order *Saccharomycetales*.

The most well-known and commercially significant yeasts are the related species and strains of *S. cerevisiae* which has played a central role in the evolution of microbiology, biochemistry and genetics. These organisms have long been utilized to ferment the sugars of rice, wheat, barley, and corn to produce alcoholic beverages and in the baking industry to expand, or raise, dough. *S. cerevisiae* is commonly used as baker's yeast and for some types of fermentation [19].

S. cerevisiae has also been a very important genetic model organism. Particularly, it is a very useful organism due to the huge amount of.genetic, molecular and cellular data currently available [20, 21]. These microorganisms have been classified as GRAS (Generally Recognized As Safe) by the U.S. Food and Drug Administration (FDA). The combination of diverse characteristics, such as their easy manipulation, ability to grow on different carbon sources, non-pathogenicity, and the absence of production of pyrogenic or allergenic substances, like those present in bacterial cell walls, has made yeasts the preferred hosts for genes encoding heterologous proteins and other substances of biotechnological interest [22]. The first successful genetic transformation of *S. cerevisiae*, 49 years ago [23], opened new possibilities for the insertion of exogenous genes into this organism which has been used in genetic studies as a host for "foreign gene expression" to produce proteins of technical and medical interest and even as a model organism in pharmaceutical development for many decades, and many different yeast transformation protocols have since been described [24, 25].

Since it is very small and unicellular, large numbers of the yeast can be grown in culture in a very small amount of space, in much the same way that bacteria can be grown. *S. cerevisiae* was the first eukaryote to have its entire genome sequenced [26].

1.2. Trehalose

1.2.1. Structure and physical properties

Trehalose (α, α-trehalose) is a disaccharide formed by a 1,1 linkage of two D-glucose molecules. It is a non-reducing sugar that is not easily hydrolyzed by acid, and the glycosidic bond is not cleaved by α-glucosidase. The molecular formula and weight are $C_{12}H_{22}O_{11}$ and 342.31 daltons, respectively. When purified it is usually found in the dihydrate form, which is the typical commercial product. Physical properties that make trehalose unique are its high degree of optical rotation and its melting behavior. Trehalose first melts at 97°C. Additional heat

drives off the water of crystallization until the material resolidifies at 130°C, and then the anhydrous trehalose melts at 203°C. The combination of the molecular structure and physicochemical properties of trehalose result in a very stable disaccharide. Although α,β (neotrehalose) and β,β (isotrehalose) isomers of trehalose have been synthesized, they are rarely found in nature. The α, α form is the isomer commonly referred to as trehalose (α, α -trehalose, α-D-glucopyranosyl α-D-glucopyranoside, mushroom sugar, mycose), and is widespread throughout the plant and animal kingdoms (cited in [27]). It is also a unique disaccharide in that it doesn't have a free aldehyde group (or hemiacetal hydroxide) and therefore doesn't react with free amino groups in a non-enzymic glycation reaction [28]. The structure of trehalose is given in Figure 1.3.

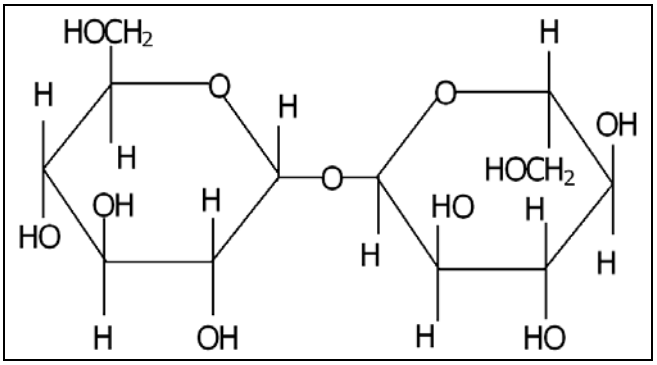

Figure 1.3. The structure of trehalose (α-D-glucopyranosyl-α-L-glucopyronoside)

1.2.2. Importance and role

This naturally occurring disaccharide is widespread throughout the biological world. Elbein summarized the distribution of 1,1-trehalose in over 80

species representing plants, algae, fungi, yeasts, bacteria, insects and other invertebrates [29].

Trehalose is a non-reducing disaccharide. Nonreducing disaccharides play a unique role in the biosphere. They provide a soluble energy source in the form of a stable molecule that can also function as a protectant compound. Nonreducing disaccharides are used as a translocated energy source by all organisms except vertebrates. Trehalose is the main blood sugar of arthropods, fueling flight in insects. Trehalose is also found at high levels in fungi, bacteria, and archaea.

Genetic and metabolic studies led to the proposal that trehalose plays two distinct functions in living cells. On the one hand, it acts as a stress protectant of proteins and biological membranes against adverse conditions. On the other hand, it may play a role as a storage carbohydrate in the yeast *S. cerevisiae*.

Trehalose is now being recognized as a crucial defense mechanism that stabilizes proteins and biological membranes under a variety of stress conditions, including increased temperature, hydrostatic pressure, desiccation, nutrient starvation, osmotic or oxidative stress, and exposure to toxic chemicals [30-40]. The protective function can be interpreted in terms of water replacement hypothesis [41] or the glass transition hypothesis [42].

Trehalose can withstand heating at 100°C between pH 3.5–10 for 24 h. Trehalose protects proteins and membranes from denaturation by replacing water as it hydrogen bonds to polar residues. During desiccation trehalose forms an amorphous glass structure that limits molecular motion, preventing protein aggregation and free radical diffusion [43].

The α,α-1,1 configuration is crucial for the ability of trehalose to preserve lipid bilayer structure in the absence of water [44]. Even close analogs such as α,β-trehalose or sucrose do not show this property. As a nonreducing sugar trehalose shows no Maillard reaction with amino compounds and does not cause browning during cooking. Based on its unique properties of stabilizing molecules, its mild sweetness, high solubility, low hygroscopicity and, last but not least, a price that has become affordable through genetic modifications of microorganisms, trehalose has become an important target for biotechnology [45, 46].

Trehalose masks unpleasant tastes and odors in food and has its own sweet clean taste, with approximately half the sweetness to taste as sucrose; trehalose is an important ingredient in foodstuffs and drinks as a stabilizer and preservative of even fresh food [45, 46].

Trehalose inhibits the polyglutamine-induced protein aggregation found in Huntington disease in experiments conducted on a mouse model of this illness [47]. This has led to the marketing of trehalose as a health food. Trehalose has been produced on an industrial scale only since the mid 1990s [46].

Trehalose has been studied extensively in the baker's yeast, *S. cerevisiae*, where it was originally thought to serve as a carbohydrate reservoir like glycogen [48] The trehalose system is also important for the control of glucose influx during the cellular response to adverse conditions [49, 50], and the gene of one of its production enzymes (*TPS1*) shows strong homology with *GGS1*, a gene that is associated with a glucose-sensing complex and with transport of glucose into the cell [49].

1.2.3. Biosynthesis

At least five different pathways for the biological synthesis of trehalose have been reported (Figure 1.4) [46, 51]. The best known and most widely distributed pathway is a two-step process from G6P and UDPG. Glucose is converted into glucose-6-phosphate (G6P) which, together with uridine diphosphate glucose (UDPG), leads to the formation of trehalose-6-phosphate (T6P) and subsequently trehalose. Glucose is taken up from the medium and immediately phosphorylated and further metabolized, with the consequence that glucose transport into the cell is the most influential step of trehalose production [52].

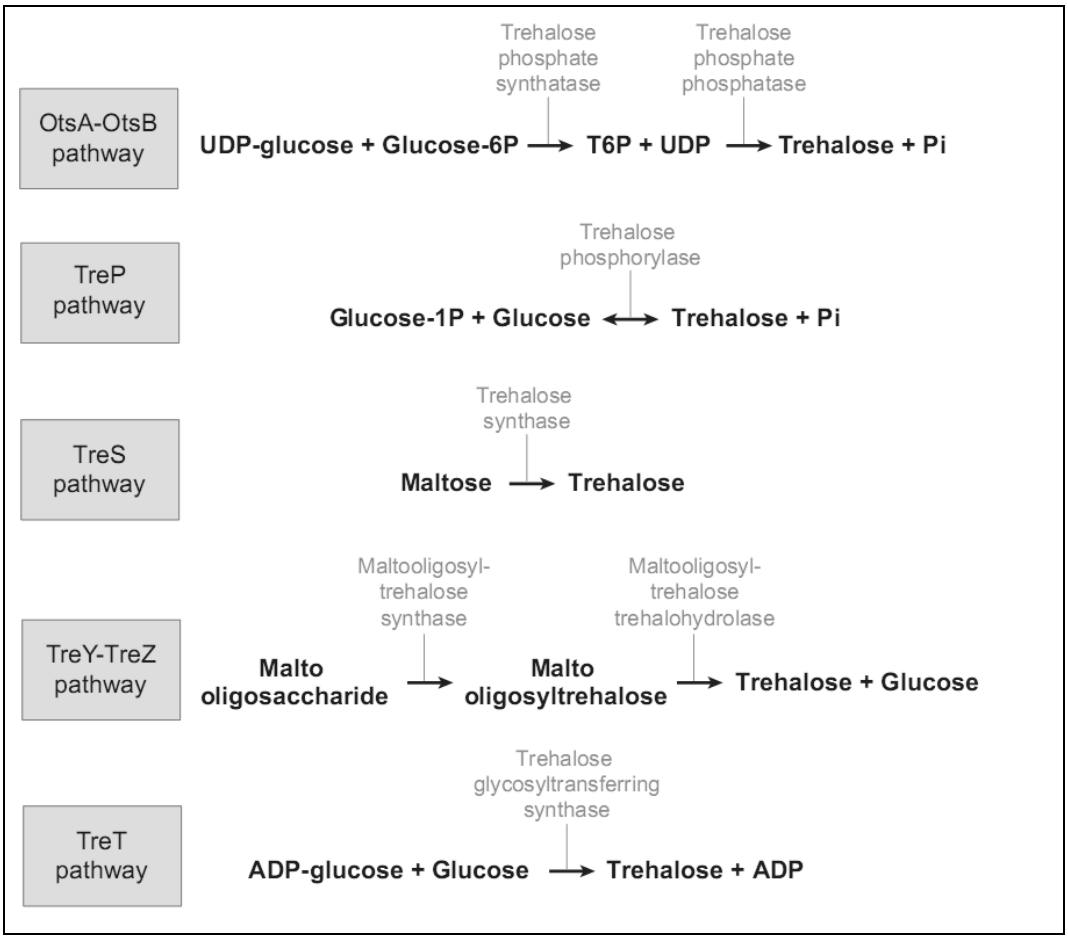

Figure 1.4. Trehalose biosynthesis pathways [46].

1.2.3.1. Enzymes in biosynthesis of trehalose

Trehalose is synthesized by a multienzyme complex in yeast (Figure 1.5 and Figure 1.6). This complex contains four subunits. Trehalose-6-phosphate synthase (T6PS) (EC 2.4.1.15) encoded by *TPS1* and trehalose-6-phosphate phosphatase (T6PP) (EC 3.1.3.12) encoded by *TPS2* carry the catalytic activity of trehalose synthesis. Two regulatory subunits tsl1 and tps3 encoded by the *TSL1* and *TPS3* genes seem to be important for the integrity of the trehalose synthase complex and to play a purely regulatory role without obvious enzymatic activity [39, 49, 53-55]. The enzymes, catalysing the synthesis of trehalose are localized in the yeast cytosol.

Genes involved in the trehalose cycle and degrees of their up-regulation are shown in Figure 1.7.

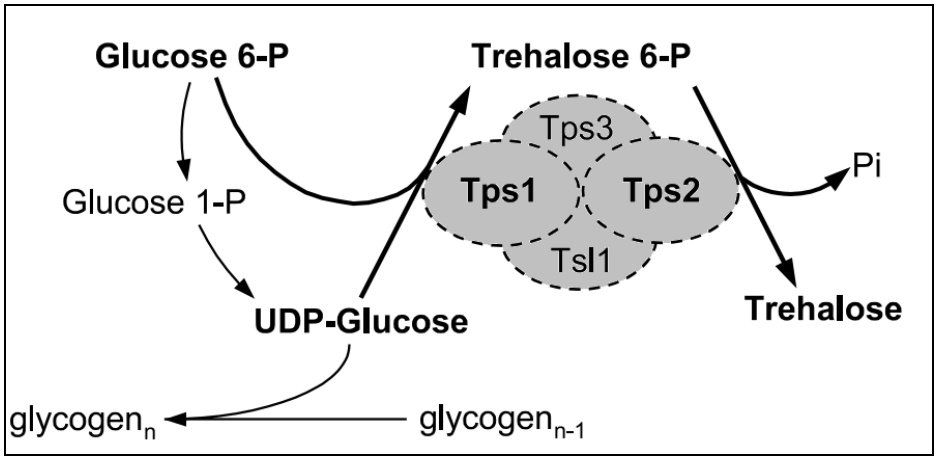

Figure 1.5. The multienzyme complex of trehalose synthesis pathway in yeasts [54].

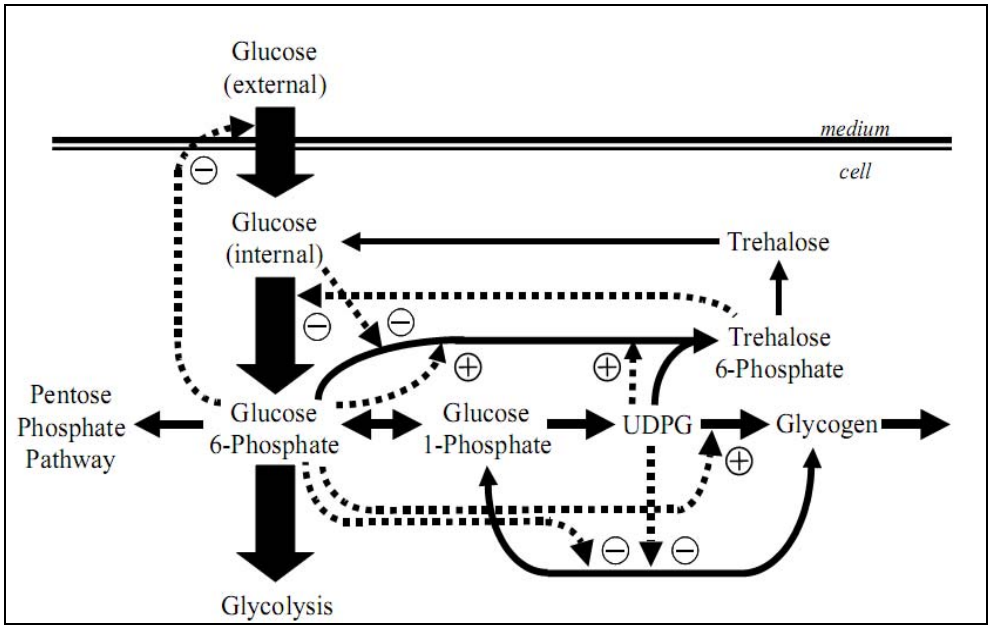

Figure 1.6. Diagram of trehalose cycle in *S. cerevisiae*. Solid arrows show flow of material, with thickness indicating the magnitude of flux. Dotted arrows represent inhibitory (-) and activating (+) signals [56].

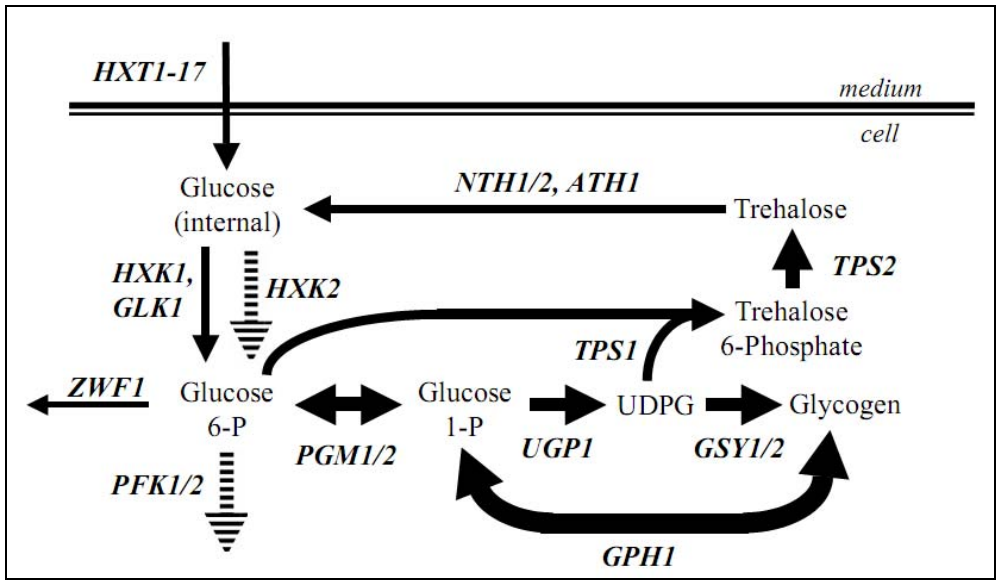

Figure 1.7. Genes involved in the trehalose cycle and degrees of their up-regulation 10–20 min after moderate heat shock (37°C). Thickness of solid arrows indicates the magnitude of up-regulation. Striped arrows indicate that these steps are unaltered over baseline [56].

1.2.3.1.1 Trehalose-6-phosphate synthase

T6PS catalyzes the transfer of glucose from UDPG to glucose-6-phosphate (G6P) to form T6P and uridine diphosphate (UDP), and trehalose phosphate phosphatase (TPP) dephosphorylates T6P to form trehalose and inorganic phosphate. This pathway has been extensively studied in *S. cerevisiae* and *E. coli*.

$$\text{UDP-glucose} + \text{glucose-6-phosphate} \xrightarrow{\text{T6PS}} \text{trehalose-6-phosphate} \qquad \text{(Eq. 1)}$$

DeSilva-Udawatta and Cannon [57] found that T6PS synthesizes trehalose 6-phosphate inside and outside of the TPS complex. In *E. coli* the trehalose-6-phosphate synthase was not part of a complex, it was independent of the phosphatase activity [58].

1.2.3.1.1.1. Importance of trehalose-6-phosphate synthase

T6PS has an additional function, apart from its catalytic function, in restricting glucose influx into glycolysis.

In *S. cerevisiae, TPS1* has an important role in the regulation of glycolysis [49]. Control of the glycolytic flux in *S. cerevisiae* has been considered to occur mainly at the level of phosphofructokinase and pyruvate kinase. Phosphofructokinase is regulated through activation by fructose-2,6-bisphosphate, phosphate (P) and AMP, and inhibition by ATP, while pyruvate kinase activity is modulated by its activation by fructose-l,6-bisphosphate. However, since phosphofructokinase does not catalyze the first irreversible step in the utilization of glucose, its regulation is not sufficient to control the rate of glucose utilization, and therefore some mechanism should exist to regulate the rate of glucose transport, phosphorylation, or both. The need for a regulation of the first steps of yeast glycolysis is illustrated by the pattern of ambulation of metabolites in certain yeast mutants upon addition of glucose [59-61]. Yeast strains carrying the mutations *fdpl* [59] or *cifl* [61] do not grow on glucose, although the glycolytic enzymes are operative. These mutations turned out to be allelic [62] , and strains bearing them become depleted of ATP upon addition of glucose and accumulate fructose-l,6-bisphosphate up to 20 mM [59, 61, 62], suggesting that the rate of the first glycolytic steps exceeds the capacity of the glycolytic pathway.

The sequence of the *CIF1* gene encodes the small subunit of the trehalose-6-P synthase/trehalose-6-P phosphatase complex [53]. Gonzales *et al.* [62] showed that the *TPS1* gene is identical to the yeast *CIF1* gene. *CIF1* is essential for catabolite inactivation of fructose- 1,6-bisphosphatase, an enzyme required for the normal growth of *S. cerevisiae* on glucose [63]. A plausible explanation for the growth behaviour and the metabolic defects of *cifl* strains could be that either trehalose or trehalose-6-P play a role in the regulation of the yeast glycolytic flux. Cansado *et al.* [64] showed that mutants from *S. cerevisiae* impaired on trehalose-6-P synthesis did not grow on glucose and on other readily fermentable carbon sources. Moreover, they showed accumulation of glycolytic intermediates, rapid depletion of ATP and lack of cAMP increase upon glucose addition. It appears that

TPS1 controls the flux of glucose into glycolysis thereby preventing a stall. Teusink *et al.* [65] have proposed that glycolysis operates by an autocatalytic (or turbo) principle and ATP is used to drive the catabolism of glucose before it is replenished. Therefore when the glucose concentration increases in the cell, glycolysis tends to use ATP faster, causing metabolism to turn off.

The mechanism by which *TPS1* controls glycolysis in yeast has not been fully elucidated but the main target is thought to be the initial step, which is catalysed by hexokinase. Blazquez *et al*. [66] showed that T6P competitively inhibits hexokinase II from *S. cerevisiae* at physiological concentrations, but the strongest inhibition was observed upon hexokinase II, which is the most abundant isoenzyme of hexokinase during growth of *S. cerevisiae* on glucose [67]. During exponential growth on glucose, hexokinase I and glucokinase are down-regulated, whereas hexokinase II is induced [68].

1.2.3.1.1.2. Regulation of trehalose-6-phosphate synthase

The T6P synthase reaction is irreversible, at least in bacteria [69]. Trehalose-6-P synthase activity is modulated by several factors. Its gene, *TPS1*, is repressed by glucose [70], and the level of glucose repression determines the concentration and state of activation of the trehalose production complex [71]. By contrast, the phosphorylated substrates of the process, G6P and UDPG, induce trehalose production [2, 69]. Van Vaeck *et al.* [72] showed that the addition of glucose to cells of a *tps1*Δ strain results in hyperaccumulation of the initial sugar phosphates in glycolysis, especially fructose 1,6-bisphosphate (Fru1,6bisP), and a rapid depletion of ATP and P_i. In particular the Fru1,6bisP level was still much lower than that in the *tps1*Δ strain. In addition, the ATP level decreased more than in the wild-type strain but it also recovered very fast. These results indicate that a low Tre6P level is enough to control glucose influx into glycolysis and in particular that most of the glucose-induced increase in Tre6P is not required to limit glucose influx. The Tps1 protein itself is sufficient to sustain growth on glucose [72].

At temperatures below 35ºC, it was inhibited by physiological concentrations of phosphate and activated by fructose-6-P [2]. At 50°C, both the inhibition by phosphate and the activation by fructose-6-P were strongly reduced, and trehalose-6-P synthase intrinsically was more active [3].

Fructose-6-P was found to be a strong activator of Tre6P synthase [73], whilst inorganic P, inhibited Tre6P synthase and activated Tre6P phosphatase [2, 73]. Furthermore, evidence was presented that both Tre6P synthase and Tre6P phosphatase were subjected to catabolite inactivation and repression during growth on glucose [74]. In addition, substrate availability [75] and post-translational modification [76, 77] may regulate activities of these enzymes.

1.2.3.1.2 Trehalose-6-phosphate phosphatase

There are many studies about T6PS and T6PP, but researches involved T6PP solely are less comparing with T6PS.

$$\text{Trehalose-6-phosphate} \xrightarrow{\text{T6PP}} \text{Trehalose} + P_i \qquad \text{(Eq. 2)}$$

De Virgilio *et al.* [78] showed that *TPS2* encodes the subunit with trehalose-6-phosphatase activity. Disruption of *TPS2* causes excessive accumulation of trehalose-6-phosphate with heat shock or at the entrance to the stationary phase in normal wild-type cells in vivo [78]. Therefore, the *S. cerevisiae tps2* disruption mutant, which lacks *TPS2* activity, cannot proliferate at elevated temperature [78]. Trehalose-6-phosphate phosphatase is highly specific for trehalose-6-P and its activity is dependent on Mg^{2+} concentration and is activated by phosphate [79].

T6PP activity was not influenced by the levels of cAMP, ATP, or cAMP-dependent protein kinase. These investigations have led to a reconsideration of the data on the regulation of the activity of trehalose synthetase by means of ATP-dependent phosphorylation and dephosphorylation [77].

Studies have shown that numerous forms of stresses induce trehalose synthesis by regulation of trehalose-synthesizing genes, *TPS1* and *TPS2*, at the

transcriptional level. For example, the transcripts of *TPS1* and/or *TPS2* were increased by heat shock [78, 80-82] , cold or ethanol stres [82, 83], salt stress [55], nutrient starvation and other osmotic stresses [80] in *S. cerevisiae*, Schizosaccharomyces or other microorganisms. However in addition to transcriptional control of trehalose-synthesizing genes (*tpsA*) upon various stresses, there is also a report that trehalose accumulation in response to heat shock is not at transcriptional level but probably due to post-translational control in *Aspergillus nidulans* [84].

1.2.4. Degradation of Trehalose

Due to its potential industrial applications, much work has been devoted to the biochemical and genetic control of trehalose metabolism in yeast [49, 79]. In the yeast *S. cerevisiae*, the intracellular level of trehalose is the result of a well-regulated balance between enzymatic synthesis and degradation. Hydrolysis of trehalose can be carried out by two enzymatic systems: a neutral trehalase encoded by *NTH1* [85] and an acid trehalase encoded by *ATH1* [86]. A third gene, namely, *NTH2*, a paralog of *NTH1* (77% identity at the protein level) exists in the yeast genome, but no trehalase activity has been associated with its product so far. Moreover, only Nth1p is known to catalyze the intracellular mobilization of trehalose [87], and the apparent lack of Ath1p to carry out this function could be attributed to its exclusion from the cytosolic compartment [88].

Trehalose and trehalase have been shown to be important as modulators of growth in filamentous fungi in adverse environmental injuries [84, 89, 90]. High activity of trehalase in conditions of heat shock can be related to its synthesis. It has been demonstrated that heat shock in some organisms leads to induction and expression of genes for trehalase synthesis [91, 92]. Under heat shock, the increase of activity of trehalase is positively correlated with amounts of intracellular glucose content into mycelium. Thus, the activity of trehalase in the mycelium can be a physiological condition that permits the fungus to recover growth after exposition to thermal stress. The in vitro assays showed that the content of glucose

in cells depended on neutral trehalase, suggesting this enzyme acts in the control of the glycolytic flux [49, 56].

1.2.5. Trehalose as a stress protectant

One of the greatest challenges faced by living organisms including fungi is the constant fluctuation of biotic and abiotic factors in the environment e.g. temperature, pH, level of oxygen, water potential and the concentration of toxic compounds. The attempt of an organism to maintain homeostasis during stress involves a variety of different cellular reactions more or less specific to the stress factor. The fungal stress response often involves the production of various protective substances [93] and these will typically form complexes with essential enzymes keeping them functional.

One of the protective compounds known to be accumulated during stress is the sugar, trehalose [94]. Trehalose has been detected in a variety of organisms and is known to serve numerous functions [95]. In fungi, the role of trehalose has been subject to great debate but large amounts of evidence has established the disaccharide as a protectant of cellular structures during stress [96]. In yeast, the investigations of arguments for trehalose as a stress protectant are numerous. The survival of *S. cerevisiae* grown in 3 M NaCl correlated significantly with intracellular levels of trehalose, and mutants unable to produce trehalose were more sensitive to the severe salt stress treatment [37]. With regard to heat stress, the mesophilic fungus *Chaetomium brasiliense* and the thermophilic *Chaetomium thermophilum var. thermophilum* were subjected to temperatures 9 ºC above optima, and they both accumulated cytosolic trehalose [97]. Hottiger et al. [71] showed that at 27°C, cells of *S. cerevisiae* growing exponentially on glucose contained only traces of trehalose. However, they accumulated large amounts of this disaccharide when they were subjected to a heat shock. The threshold temperature for trehalose accumulation was 33 to 35°C. While 35°C was still comparatively ineffective, the trehalose pool rapidly expanded at temperatures between 37 and 45°C. At 50°C, trehalose accumulation ceased and cells died.

The accumulation of trehalose has also been seen in cells of *S. cerevisiae* grown in lack of carbon, nitrogen, phosphor or sulphate [48] and in cells subjected to desiccation or freezing [96].

Trehalose is also abundant in chemostat cultures maintained at low dilution rates [98] and in batch cultures during adaptation to new carbon sources or transition to the stationary phase [48, 99]. Under some of these conditions, trehalose can account for up to 23% of the dry weight of the cells [48].

Oxidative stress is also a critical situation for maintaining the stability of the native conformation of proteins, probably because the oxidative modifications of certain residues lead to protein unfolding [82]. Benaroudj and his colleagues [36] have indicated that trehalose, functioning as a ROS scavenger, also protects yeast cells and cellular proteins from damages caused by H_2O_2.

1.2.6. Cloning of trehalose-6-phosphate synthase

In recent years there has been a growing interest in trehalose metabolism since this non-reducing disaccharide can act as a stabilizer and protectant of proteins and membranes against heat, cold, desiccation, freezing, hypoxia and oxidative stresses in various organisms. The gene encoding T6PS protein has been cloned and sequenced in some species of bacteria, fungi and plant. The results of selected studies are given in Table 1.1.

Table 1.1. The results of trehalose-6-phosphate synthase gene cloning studies.

	Organism	Gene	Amino acid length	Molecular Weight	Ref.
Bacteria	*E. coli*	*OTSA*	474	54.7 kDa	[58]
	Thermus aquaticus	*TaTPS1*	963	105 kDa	[100]
	Mycobacterium smegmatis	*TreS*	593	71 kDa	[101]
	Thermobifida fusca	*TfTPS1*	610	66 kDa	[102]
Fungi	*S. cerevisiae*	*ScTPS1*	495	56 kDa	[53]
	Zygosaccharomyces rouxii	*ZrTPS1*	492	56 kDa	[63]
	Candida albicans	*CaTPS1*	478	54 kDa	[103]
	Aspergillus nidulans	*TpsA*	1054	115 kDa	[94]
Plant	*Selaginella lepidophylla*	*SlTPS1*	994	109-kDa	[104]
	Arabidopsis thaliana	*AtTPS1*	942	NA*	[105]
	Triticum aestivum	*TaTPS*	859	96.7 kDa	[106]
	Ginkgo biloba	*GbTPS*	868	97.9 kDa	[107]

* NA: Not available

In addition, there has been also a growing interest in trehalose metabolism as a means of stress tolerance engineering recently, especially in crops (Table 1.2.). Because, in the plant kingdom, most species do not seem to accumulate detectable levels of trehalose, exception for the highly desiccation-tolerant 'resurrection' plants. Increasing trehalose accumulation in crop plants could improve drought and salinity tolerance. Transgenic plants have been developed with trehalose biosynthetic genes. Developing new cultivars with improved abiotic stress tolerance can have a significant impact on global food production.

Table 1.2. Expression of trehalose biosynthetic genes in transgenic plants [108].

Origin	Gene used	Target	Prominent effects
E. coli	*otsA, otsB*	Tobacco	Improved growth under stress conditions, morphological alterations
Yeast	*TPS1*	Tobacco	Stunted growth, lancet-shaped leaves, reduced sucrose content and improved drought tolerance
E. coli	*TPS*	Potato	No trehalose levels detected
E. coli and yeast	*TPS*	Tobacco	Enhanced rate of photosynthesis
E. coli and yeast	*TPP*	Tobacco	Reduced photosynthesis
E. coli	*otsA, otsB*	Rice	Sustained plant growth, less photo-oxidative damage, favorable mineral balance (under salt, drought and low temperature stress) and more trehalose. Increased stress tolerance

1.3. Aim of the study

R. oryzae was used for industrial production of lactic acid, yet little is known about the genetics of this fungus.

Lactic acid production is controlled by the glycolytic pathway and the trehalose metabolism affects the glycolytic pathway.

The molecular researches of trehalose biosynthesis and metabolization have been carried on actively because of its important roles in stress protection and glycolytic pathway. Trehalose protects against several environmental stresses, such as heat, desiccation, and salt, probably by stabilizing protein structures and lipid membranes. Trehalose synthesis in yeast is mediated by a complex of trehalose-6-phosphate synthase (TPS1) and trehalose-6-phosphate phosphatase (TPS2).

The main objective of this study was PCR-cloning of trehalose-6-phophate synthase gene of *R. oryzae* and expression of the gene in *S. cerevisae* which was used as an expression host. The gene was functionally identified by its

complementation of the *tps1* yeast deletion mutant which was unable to grow on glucose medium.

Trehalose synthesis in the transformed cells were shown. Tools of bioinformatics were used to evaluate the data obtained. The schematic representation of the overall experimental planning for the study was given in Figure 1.8.

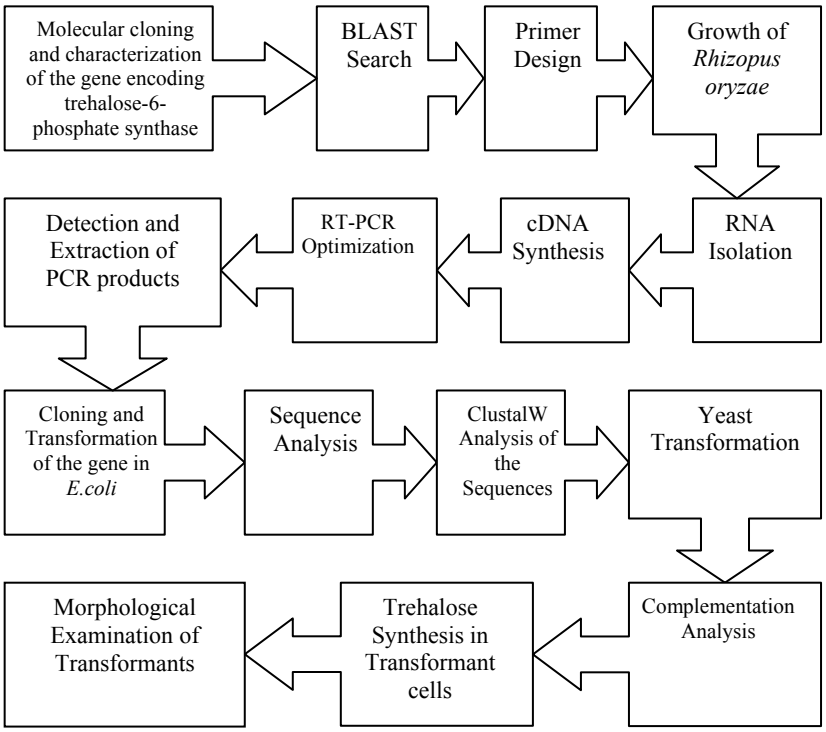

Figure 1.8. Scope of the study.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. The chemicals

The chemicals used in all experiments were all of analytical grade and were purchased from Sigma, Aldrich, Merck, Oxoid, Fluka, Fermentas and Invitrogen.

2.1.2. The strains

Rhizopus oryzae ATCC 9363 was purchased from American Type Culture Collection. *Escherichia coli* DH5α was used as competent strain for propagation of the constructed plasmids.

S. cerevisiae strains were isogenic to W303-1A (wild type) with relevant genotype Mat a leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC and YSH 290 tps1Δ: : TRP1; they were supplied by Prof. Dr. Sezai TÜRKEL, Uludağ University, Department of Biology, Bursa.

2.1.3. The growth media

For growth of *E. coli* DH5α the LB medium (Appendix D) was used. For *R. oryzae* and *S. cereviisae* the growth media are given in Appendix D.

2.1.4. The plasmids

pGEM®-T Easy vector system (Promega) was used for the propagation of the gene fragments to be sequenced. The plasmid pFL61 (Promochem, ATCC number: 77215) was used as the expression vector in *S. cerevisae* transformations. The plasmid maps were given in Appendix H.

2.1.5. The primers

The primers used for generating the *tps1* and *hxk* gene fragments were synthesized by Iontek, İstanbul. The primers (SP6 and T7) used for sequencing experiments were supplied by Refgen, Ankara.

2.2. Methods

2.2.1. Growth of organisms

2.2.1.1. *R. oryzae*

R. oryzae was sporulated on streaked agar plates for 4-5 days at 30 °C. After sporulation, the plates were stored at 4 °C until preparation of spore suspension. For each inoculation, spore concentration in the suspension was determined by counting the spores on hemocytometer. For liquid culture, *R. oryzae* was inoculated in the liquid medium and incubated in shaker- incubator at 35 °C and 175 rpm.

2.2.1.2. *S. cerevisiae*

S. cerevisiae was grown on streaked agar plates for 4-5 days at 30 °C. After colony formation, the plates were stored at 4 °C. For each liquid preculture inoculation, a colony was taken from a plate by toothpick and inoculated in the liquid medium. The incubation of the liquid medium was done in shaker- incubator at 30 °C and 140 rpm.

2.2.1.3. *E. coli*

E. coli was grown on streaked LB agar plates for 4-5 days at 37 °C. After colony formation, the plates were stored at 4 °C. For each liquid LB preculture inoculation, a colony was taken from a plate by toothpick and inoculated in the liquid medium. The incubation of the liquid medium was done in shaker- incubator at 37 °C and 200 rpm.

2.2.2. PCR cloning of trehalose-6-phosphate synthase

2.2.2.1. BLAST Search

From NCBI site (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) the nucleotide sequence of T6PS from *S. cerevisiae* was found to be:
>gi|4626|emb|X68214.1| *S. cerevisiae* TPS1 mRNA for alpha,alpha-trehalose-phosphate synthase

```
TTCTTGAACAAGCACGCAGCTAAGTAAGCAACAAAGCAGGCTAACAAACTAGGTACTCACATACAGACTT
ATTAAGACATAGACCTATGACTACGGATAACGCTAAGGCGCAACTGACCTCGTCTTCAGGGGGTAACATT
ATTGTGGTGTCCAACAGGCTTCCCGTGACAATCACTAAAAACAGCAGTACGGGACAGTACGAGTACGCAA
TGTCGTCCGGAGGGCTGGTCACGGCGTTGGAAGGGTTGAAGAAGACGTACACTTTCAAGTGGTTCGGATG
GCCTGGGCTAGAGATTCCTGACGATGAGATGGATCAGGTGAGGAAGGACTTGCTGGAAAAGTTTAATGCC
GTACCCATCTTCCTGAGCGATGAAATCGCAGACTTACTCTACAACTGGTTCAGTAATTCTATTCTATGGC
CGTTATTCCATTACCATCCTGGTGAGATCAATTTCGACGAGAATGCGTGGTTGGCATACAACGAGGCAAA
CCAGACGTTCACCAACGAGATTGCTAAGACTATGAACCATAACGATTTAATCTGGGTGCATGATTACCAT
TTGATGTTGGTTCCGGAAATGTTGAGAGTCAAGATTCACGAGAAGCAACTGCAAAACGTTAAGGTCGGGT
GGTTCCTGCACACACCATTCCCTTCGAGTGAAATTTACAGAATCTTACCTGTCAGACAAGAGATTTTGAA
GGGTGTTTTGAGTTGTGATTTAGTCGGGTTCCACTCATACGATTATGCAAGACATTTCTTGTCTTCCGTG
CAAAGAGTGCTTAACGTGAACACATTGCCTAATGGGGTGGAATACCAGGGCAGATTCGTTAACGTAGGGG
CCTTCCCTATCGGTATCGACGTGGACAAGTTCACCGATGGGTTGAAAAAGGAATCCGTACAAAAGAGAAT
CCAACAATTGAAGGAAACTTTCAAGGGCTGCAAGATCTTAGTTGGTGTCGACAGGCTGGATTACATCAAA
GGTGTGCCTCAGAAGTTGCACGCCATGGAAGTGTTTCTGAACGAGCATCCAGAATGGAGGGGCAAGGTTG
TTCTGGTACAGGTTGCAGTGCCAAGTCGTGGAGATGTGGAAGAGTACCAATATTTAAGATCTGTGGTCAA
TGAGTTGGTCGGTAGAATCAACGGTCAGTTCGGTACTGTGGAATTCGTCCCCATCCATTTCATGCACAAG
TCTATACCATTTGAAGAGCTGATTTCGTTATATGCTGTGAGCGATGTTTGTTTGGTCTCGTCCACCCGTG
ATGGTATGAACTTGGTTTCCTACGAATATATTGCTTGCCAAGAAGAAAAGAAAGGTTCCTTAATCCTGAG
TGAGTTCACAGGTGCCGCACAATCCTTGAATGGTGCTATTATTGTAAATCCTTGGAACACCGATGATCTT
TCTGATGCCATCAACGAGGCCTTGACTTTGCCCGATGTAAAGAAAGAAGTTAACTGGGAAAAACTTTACA
AATACATCTCTAAATACACTTCTGCCTTCTGGGGTGAAAATTTCGTCCATGAATTATACAGTACATCATC
AAGCTCAACAAGCTCCTCTGCCACCAAAAACTGATGAACCCGATGCAAATGAGACGATCGTCTATTCCTG
GTCCGGTTTTCTCTGCCCTCTCTTCTATTCACTTTTTTTATACTTTATATAAAATTATATAAATGACATA
ACTGAAACGCCACACGTCCTCTCCTAAAAAAAAAAAAAA
```

This sequence was compared with *R. oryzae* database to find the hyphothetical sequences of *TPS1* mRNA for alpha, alpha-trehalose-phosphate

synthase since genome of *R. oryzae* is known completely. According to the results, the sequences of the eight hyphothetical proteins were documented from this site (http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae/Home.html) to design primers.

2.2.2.1.1 Design of primers for PCR cloning

Oligoanalyser 3.1 (Integrated DNA Technologies, Inc.) primer design program (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) was used to design the primers.

The primers used for cloning of the trehalose-6-phosphate synthase gene of *R. oryzae* was designed based on the hyphothetical sequences of *TPS1* available in the *R. oryzae* database (http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae/MultiHome.html).

The primers used for PCR cloning of *TPS1* from *R. oryzae* and the regions of the sequence where these primers were based on are given in Table 2.1. The hypothetical proteins were named as 1-8 in paranthesis for convenience.

Table 2.1. Primers used for PCR cloning of the *RoTPS1* gene.

	The gene		**The sequence of the primer 5'→3'**	**Base region for primer**	**Size of the product**
(1)	*R. oryzae TPS1* RO3G_14824.1	Start	ATGTCAAGCGATAATAATGATAAGAACC	1-28 bp	2058
		End	TTAGTTTATGTGCATGCGTACTC	2030-2058 bp	
(2)	*R. oryzae TPS1*RO3G_14320.1	Start	ATGTCTTCTATTGTCAACAAGATAAAAG	1-28 bp	2403
		End	TTATTTTTGTGTAAGCAAAGCTAAAAG	2376-2403 bp	
(3)	*R. oryzae TPS1* RO3G_14593.1	Start	ATGCCATCATTAACAGCTGAAAAAGTAG	1-28 bp	2505
		End	CTATTTTTCTTCTTGTGCCATCAGACTC	2477-2505 bp	
(4)	*R. oryzae TPS1*RO3G_11196.1	Start	ATGCCCTCATTAACAGCTGAAAAGGTAG	1-28 bp	2505
		End	TTACTGTTTGTCTGCTTCAGCCATAACG	2477-2505 bp	
(5)	*R. oryzae TPS1* RO3G_07383	Start	ATGAGCTCATTAACTGCTGAAAATG	1-25 bp	3621
		End	TTACTTTCCAAACTGACTTTGATAG	3596-3621 bp	
(6)	*R. oryzae TPS1* RO3G_09908	Start	ATGCAACAAAAGACAACAAATGGTC	1-25 bp	2157
		End	TCACGACTGAGACATAATCTGAAG	2131-2157 bp	
(7)	*R. oryzae TPS1*RO3G_13130	Start	ATGACAGGAGAAGATACTGTGC	1-22 bp	2310
		End	TTATTGAGACAGGATCTGTAACGAATC	2283-2310 bp	
(8)	*R. oryzae TPS1* RO3G_03240	Start	ATGGCGCGAATTACGGAAAAAAG	1-23 bp	2370
		End	CTAGAATAATTGTTCAAGAGTAGACAAAAC	2340-2370 bp	
	R. oryzae HXK	Start	ATGTTAAACAACAAAAAGAAGACACC	1-26 bp	
		End	ATCAATCCGAGCCTTCTCTATTTAATTTC		
	pFL61	A	GCGTAAAGGATGGGGAAAGAGAAAAG		
		B	CAGATCATCAAGGAAGTAATTATCTAC		

2.2.2.2. Total RNA isolation

The total RNA from *R. oryzae* was isolated according to the protocol optimized in our laboratory by Dr.Leo H. de Graaff (TRIzol method). To obtain the mycelia for isolation of RNA, *R. oryzae* was grown in a 400 ml medium in 1L flask. The spores were inoculated in a concentration of 10^5 spores/ml and were grown at 35ºC and 175 rpm for 24 hours, then one hour heat stress was applied by increasing the incubation temperature to 45ºC. The mycelia were filtered with suction and the pellet was dried between paper towels and was wrapped up in a piece of aluminum foil and was immediately frozen in liquid nitrogen.

Shake flask, grinding balls, forceps and spatula were precooled in liquid nitrogen.

Approximately 0.5 g of frozen mycelium was weighed and submerged in liquid nitrogen in shake flask together with the grinding balls. Excess liquid nitrogen was poored off, shake flask was capped and the assembly was quickly installed in the dismembrator. The mycelium was ground for 2 minutes at 2000 rpm. The shake flask assembly was taken out of the dismembrator and opened. Approximately 100 mg ground mycelium was transferred to a precooled 2 ml Eppendorf tube, 1 ml of TRIzol reagent was added and vortexed for 15 min in a block shaker at room temperature, then spinned down for 5 min at room temperature at max speed. 900 µL of the supernatant was transferred to a new tube. Chloroform (180 µL) was added to the supernatant, the tube was shaken vigorously for 15 seconds and incubated at room temperature for 3 min, then spinned down for 15 min at 4 ^{o}C at max speed. Upper phase (450 µl) was taken into a new 1.5 ml tube and 200 µl chloroform was added. The solution was shaken vigorously for 15 seconds and incubated at room temperature for 3 min, then spinned for 5 min at room temperature at max speed. The upper phase (400-450 µL) was taken and transferred into a new 1.5 ml Eppendorf tube and 1 volume isopropanol was added, and mixed by inverting several times and incubated for 10 min at room temperature. The RNA was pelleted by spinning for 10 min at room temperature and at max speed. The supernatant was discarded. One mL 75% ethanol was added

and vortexed briefly to wash the pellet. The tubes were left for 3 min at room temperature and spinned down for 5 min at room temperature at max speed. The supernatant was discarded and then spinned down for an additional 15 sec to collect the pellet at the bottom of the tube and any visible liquid was removed. The tubes were covered with aluminum foil and air dried for 10 min. DEPC-water (50 μL) was added and incubated for 15 min at 65^{o}C to dissolve pellet.

During this protocol, several precautions were taken when working with RNA:

RNases can be introduced accidentally into the RNA preparation at any point in the isolation procedure through improper technique. Because RNase activity is difficult to inhibit, it is essential to prevent its introduction.

Since skin often contains bacteria and molds that can contaminate a RNA preparation and be a source of RNases, disposable gloves were worn always.

Sterile, disposable plastic ware and automatic pipettes reserved for RNA work was used to prevent cross-contamination with RNases from shared equipment. In the presence of TRIzol Reagent, RNA is protected from RNase contamination. Downstream sample handling requires that non-disposable glassware or plasticware be RNase-free.

In order to obtain RNase-free materials, tips, Eppendorf tubes and glassware were autoclaved for 25 min at 120 $^{\circ}$C and dried for overnight at 100 $^{\circ}$C. Chloroform, 75% ethanol, isopropanol and DEPC-water were prepared in sterile bottles and used for RNA work only.

In order to prepare DEPC-water, 0.1 % (v/v) diethylpyrocarbonate was dissolved (under vigerously shaking) in water and incubated overnight at room temperature in fumehood, then autoclaved for 25 min at 120 $^{\circ}$C.

The RNA concentration was determined by measuring absorbance at 260 nm on a spectrophotometer in 10 mM Tris/HCl pH 8.0 (one absorbance unit = 40 μg/mL RNA). The A_{260}/A_{280} ratio should be approximately 2.0, with ranges between 1.9 to 2.1 is considered to be acceptable. The RNA concentration should be >1.1 μg/μl

The integrity of the RNA was tested by agarose gel electrophoresis. For this RNase-free 1% agarose gels in TAE was prepared. The samples were diluted to approximately 100-150 ng/µl before loading the gel. The RNA sample was loaded in RNase-free DNA loading buffer and run for approximately 30 minute at 75V. The rRNA bands should be clear without any obvious smearing patterns.

2.2.2.3. cDNA synthesis

"Transcriptor First cDNA Synthesis Kit" supplied from Roche was used for cDNA synthesis. In a sterile PCR tube, the template-primer mixture for one 20 µl reaction was prepared by adding the components in the order of; isolated total RNA in final concentration of 1µg total RNA, anchored oligo$(dT)_{18}$, 50 pmol/µl in final concentration of 2.5 µM, and PCR grade water to make total volume 13 µl. This template-primer mixture was denatured by heating the mixture for 10 minutes at 65°C in a thermal block cycler with a heated lid. After denaturation, the rest of the components of the RT mix was added in the order of; 4 µl transcriptor reverse transcriptase reaction buffer, 0.5 µl protector RNase inhibitor (40 U/µl), 2µl deoxynucleotide Mix, 10 mM each and 0.5 µl transcriptor reverse transcriptase (20 U/µl) to make final volume of 20 µl. The RT mix was incubated at 55 °C for 30 minutes, the reaction was stopped by placing the tube on ice. This protocol is traditional for cDNA synthesis and it is given as schematically in Figure 2.1.

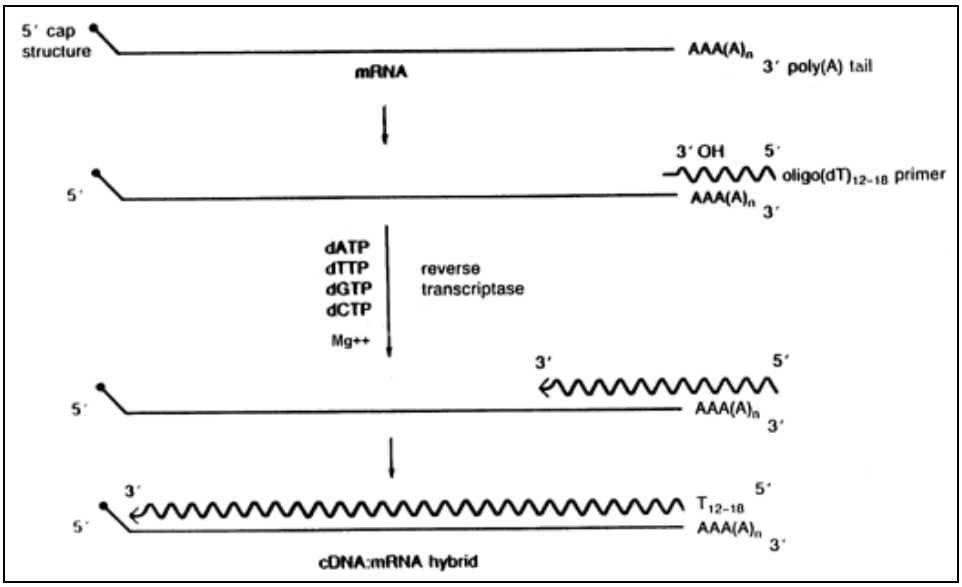

Figure 2.1. Synthesis of the first strand of cDNA using an oligo (dT) primer and reverse transcriptase

2.2.2.4. Cloning of the hypothetical gene fragments of *RoTPS1* by PCR

In order to amplify the gene fragments of *RoTPS1*, PCR mixture and the conditions were optimized. Invitrogen Accuprime Taq DNA Polymerase System was used to obtain the PCR products. Table 2.2 and 2.3 show the content of the PCR mixtures and the PCR condition for the first trial to get PCR products respectively.

Table 2.2. PCR mixture content for the hypothetical gene fragments of *RoTPS1*.

µl	Control	RO_14824.1	RO_14593.1	RO_09908	RO_03240
Buffer	2.5	2.5	2.5	2.5	2.5
Taq Polymerase	0.5	0.5	0.5	0.5	0.5
cDNA	1	2	2	2	2
Forward Primer	1.05	0.67	0.95	0.63	0.93
Reverse Primer	0.94	0.7	0.67	0.96	0.65
Water	19.01	18.63	18.38	18.41	18.42
Total	25	25	25	25	25

Table 2.3. PCR programme for amplification of the hypothetical gene fragments of *RoTPS1*.

Initial Denaturation	94 ºC	30 sec
Denaturation during cycling	94 ºC	30 sec
Annealing	52 ºC	60 sec
Elongation	68 ºC	150 sec
Total number of cycles	25	
Final Elongation	68 ºC	120 sec
Cooling	4 ºC	∞

PCR were performed at "Eppendorf Master Cycler Personal". After optimization of the PCR conditions, PCR amplification of the gene fragments of *RoTPS1* was performed by proof reading polymerase (Invitrogen Accuprime *Pfx* Polymerase System) in order to minimize mismatches created by PCR.

After verifying that the product obtained was of the right size the band was cut with a sharp scalpel and transferred into an Eppendorf tube.

2.2.2.5. Detection of PCR products on agarose gel

The expression levels of PCR products were visualized on 1% agarose gel stained with ethidium bromide.

2.2.2.6. Isolation of the gene fragments

The gene fragments were isolated by QIAquick Gel Extraction Kit Protocol supplied by QIAGEN. According to this protocol, the DNA fragment was excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a colorless tube. 3 volumes of Buffer QG was added to 1 volume of gel (100 mg ~ 100 µl). The gel slice was incubated at 50°C for 10 min until the gel slice has completely dissolved. The tube was mixed by vortexing every 2–3 min for dissolving the gel. A QIAquick spin column was placed in a provided 2 ml

collection tube. The sample was applied to the QIAquick column, and centrifuge for 1 min to bind DNA. The flow-through was discarded and QIAquick column was placed back in the same collection tube. Buffer PE (0.75 ml) was added to QIAquick column to wash and centrifuged for 1 min. The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 min at 13000 rpm by microcentrifuge. QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. To elute DNA, 30 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the center of the QIAquick membrane and the column was centrifuged for 1 min.

2.2.2.7. Cloning the gene fragments in pGEM®-T Easy vector and expression in *E. coli*

The *Pfx* polymerase, which was used to generate the gene does not add an adenine base unlike *Taq* polymerase. The fragments obtained by PCR were run on a 1% agarose gel and were then cut out by use of a sharp scalpel and were extracted from gel slices by use of Qiagen gel extraction kit (QIAquick). In order to clone these blunt ended fragments into pGEM®-T Easy vector, which uses a T/A cloning strategy, A-tailing procedure was carried out. 5.5 µl of the PCR fragment was incubated in the presence of 1 µl Taq Polymerase (Fermentas), 1 µl 10x buffer, 0.5 µl of 4 mM dATP, 2 µl of 25 mM $MgCl_2$, at 70 °C for 30 minutes in Eppendorf thermocycler. *Taq* polymerase adds an adenine base at the end of the fragments. Later 3 µl of these fragments were taken and ligated into 1 µl of pGEM®-T Easy vector in the presence of 1 µl of T4 DNA ligase and 5 µl of 2x Rapid Ligation buffer in a reaction volume of 10 µl. The ligation was carried out at room temperature for 3 hours or at 4 °C overnight. Five µl of this mixture containing the plasmid with or without insert were used in transformation of *E. coli*.

In order to make transformation of *E. coli* DH5α, 50 µl of competent cells which were stored in -70 ºC freezer were thawed on ice (approximately 20 minutes). When cells were thawed, they were resuspended (pipetted up and down gently with a yellow tip) and aliquots of 50 µl were transferred to each treatment tube. 5 µl of DNA was added to sample tube and 1 µl of control DNA (pGW635)

was added to positive control tube. The DNA and cells were incubated on ice for 30 minutes. The cells were heat shocked by placing the tubes at 42 ºC for 2 minutes. Half a ml of LB was added without antibiotics to each treatment tubes.

The tubes were incubated at 37 ºC for 1 hour. The tubes were centrifuged for 5 minutes at 4000rpm in minifuge, the supernatant (400 µl) was poured off and the cells in remaining supernatant (100 µl) were resuspended prior to plating on LB plates containing ampicilline and X-Gal.

The plates were incubated overnight at 37 ºC. The plates may then be sealed and stored at 4 ºC for about 1 month.

Although several colonies were grown on selective media not all of them contained the plasmid with insert. The colonies containing the plasmid with insert appear as white or pale blue colonies which were picked and inoculated for minipreps (2.2.2.9). The plasmids isolated from these colonies were tested for presence of an insert by digesting 0.1-0.5 µg DNA from each with *NotI* enzyme and run on a 1 % agarose gel.

2.2.2.8. Plasmid DNA isolation

2.2.2.8.1. Plasmid DNA isolation by fast Mini-prep protocol

Five ml LB medium was inoculated (10 µl from glycerol stock or a single colony from a plate) and incubated overnight at 37 ^{o}C. 2 ml of medium was pipetted in an eppendorf tube and centrifuged for 5 min at 5000 rpm by microcentrifuge. Supernatant was removed and the cells were centrifuged for 1 min, the rest of the supernatant was carefully removed. The pellet was resuspended in 100 µl buffer S1 (5 min on a plate vortex), which contains 50 mM Tris/HCl (pH 8.0), 10 mM EDTA and 400 µg/ml DNase-free RNase A (RNase A solution was made DNase-free by boiling for 5 min and was added after sterilization of buffer S1. The RNase containing buffer was stored at 4 ^{o}C). 100 µl buffer S2 was added and mixed manually. Buffer S2 contained 200 mM NaOH and 1 % SDS, they were freshly prepared by mixing equal volumes of 0.4 M NaOH and 2 % SDS and they were stored at room temperature. 100 µl buffer S3 was added, mixed manually and

left on ice for 5 min. Buffer S3 contained K-acetate buffer pH 5.2, prepared by mixing 60 ml of 5 M K-acetate, 11.5 ml acetic acid and 28.5 ml water. The mixture was centrifuged for 15 min at 14000 rpm at 4ºC in centrifuge. The supernatant was transferred to a 1.5 ml Eppendorf tube and 200 µl isopropanol was added, then mixed manually and centrifuged for 15 min at 14000 rpm at 4 ºC. The supernatant was removed, 0.5 ml 70% ethanol was added to the pellet and centrifuged for 5 min at 14000 rpm at 4 ºC. The supernatant was removed and the pellet was briefly dried under vacuum. The pellet was dissolved in 20 µl sterile water. They were stored at 4 ºC.

2.2.2.8.2. Plasmid DNA Purification Using the QIAprep Spin Miniprep kit

Plasmids isolated by the fast mini-prep method were checked and if the bands obtained were good then they were isolated again from the culture by QIAprep kit to obtain more purified plasmids. Two µl overnight cultures of *E. coli* in LB medium was centrifuged for 5 minutes at 5000g.by microcentrifuge Pelleted bacterial cells were resuspended in 250 µl Buffer P1 and transferred to a microcentrifuge tube. 250 µl Buffer P2 was added and mixed thoroughly by inverting the tube 4–6 times. Add 350 µl Buffer N3 was added and mixed immediately and thoroughly by inverting the tube 4–6 times. The mixture was centrifuged 10 min at 13,000 rpm in the microcentrifuge. The supernatants were applied to the QIAprep spin column placed in a provided 2 ml collection tube by decanting or pipetting. The column was centrifuged for 30–60 s and the flow-through was discarded. QIAprep spin column was washed by adding 0.75 ml Buffer PE and centrifuged for 30–60 s. The flow-through was discarded and centrifuged for an additional 1 min to remove residual wash buffer. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl water was added to the center of each QIAprep spin colum, after 1 minute standing it was centrifuged for 1 min.

2.2.2.9. Checking Minipreps

Restriction digestion of minipreps were done with *EcoRI* or *HindIII*. Digestion mix contained 2 µl plasmid DNA, 2 µl *EcoRI*/*HindIII* buffer,14 µl dH_2O, 2 µl *EcoRI/ HindIII*. The mixture was incubated at 37°C for 2 hours. The samples were run on agarose gel (1%).

2.2.2.10. Sequencing of the gene fragments

The clones containing the inserts with the expected size were sequenced by using SP6 and T7 primers by Refgen, Ankara. In order to obtain the whole sequence of the insert, another set of primers were used.

2.2.2.11. Multiple sequence alignment

ClustalW program (http://www.ebi.ac.uk/Tools/clustalw/index.html) was used for multiple sequence alignment.

2.2.2.12. Transformation of the insert into the expression vector: pFL61

The plasmids were isolated by using QIAprep Spin Miniprep kit from QIAGEN and they were digested with two digestion enzymes *BauI* and *NotI*. They extracted from gel by using gel extraction kit from QIAGEN. After inserts extracted from gel, they were ligated with pFL61 which was digested with *NotI* and alkaline treated. When ligation process completed, the vector with insert was transformed into *E. coli* according to the protocol previously mentioned here. Then plasmid isolation was carried out.

2.2.2.13. Transformation of *S. cerevisiae*

For transformation of *S. cerevisiae,* 10 ml YEP-Gal medium (in 50 ml flask) was inoculated with a yeast colony from YEP-Gal plate and the cells were grown overnight at 30ºC and 140 rpm. When the OD_{600} of the culture was between 1-1.5,

1 ml was inoculated in 9 ml of YEP-Gal media. When the OD of the culture reached to 1-1.5 (this should take 3 to 4 hours) the cells were harvested at 1000xg for 5 min, washed once with sterile distilled water, suspended in 1.5 ml TE buffer and 1.5 ml 0.2 M lithium acetate was added. Then 0.5 ml of cell suspension was transferred to a test tube (1.2 x 10.5 cm) and incubated at 30 ºC 140 rpm for 1 hour. The cell suspension (400 µl) was transferred to a 1.8 ml Eppendorf tube, plasmid DNA solution was added (3-4 µg) and incubated statically at 30ºC for 30 min. 800 µl of 50% PEG-4000 (dissolved in water and sterilised by autoclaving) was added, the tube was mixed thoroughly on a vortex mixer and let stand at 30 ºC for 1 hour. The Eppendorf tube was immersed into a water bath at 42 ºC and incubated for 5 minutes, then immediately cooled to room temperature. The tube was washed once with water at RT and suspended in 150 µl of distilled water. The cell suspension (75 µl) was spread on selection agar (-URA+galactose) and the agar plates were incubated at 30 ºC for 2 to 4 days.

2.2.2.14. DNA Isolation

Isolation of the DNA of transformed yeasts were carried out by using "Epicentre Biotechnologies MasterPure™ Yeast DNA Purification Kit".

According to the kit protocol, a single yeast colony (2 mm in diameter) from an agar plate was scraped and transferred to a microcentrifuge tube containing 300 ml of Yeast Cell Lysis Solution. The Yeast Cell Lysis Solution was mixed thoroughly to ensure uniform composition before dispensing. The cells were suspended by either vortex mixing. The suspended cells were incubated at 65 ºC for 15 minutes. The samples were placed on ice for 5 minutes. 150 µl of MPC Protein Precipitation Reagent was added to the samples and the samples were mixed by vortex for 10 seconds. Cellular debris were pelleted by centrifugation in a microcentrifuge for 10 minutes at 10,000 rpm. The supernatant was transferred to a clean microcentrifuge tube and 500 µl of isopropanol was added. The tube was mixed thoroughly by inversion.

The DNA was pelleted by centrifugation in a microcentrifuge for 10 minutes at 13,000 rpm by microcentrifuge.

The supernatant was removed by pipeting and discarded. The pellet containing the DNA was washed with 0.5 ml of 70% ethanol. The ethanol was removed carefully by pipetting and discarded. The DNA pellet was centrifuged briefly and any remaining ethanol was removed. The DNA was suspend in 35 ml of TE Buffer and stored at 4 ºC.

2.2.3. Trehalose determination

The trehalose level of yeast cells were determined by trichloroacetic acid (TCA) extraction method [48]. All the procedures were carried out at 4°C. The 4 ml culture sample were quenched in 2 ml cold methanol, immediately vortexed and centrifugated at 4°C, 10000 rpm, 5 minutes in Sigma cold centrifuge. The pellets were washed with equal volume of cold distilled water and after 1 ml cold TCA addition they were left for extraction through 1 hour at 4°C by vortexing in 15 minute intervals. The extraction solution was centriguated and pellets were washed with 1 ml cold distilled water and supernatants were combined and stored at 4°C for trehalose determination.

The amount of trehalose extracted was determined by Anthrone method [48, 109]. The sample (400 µl) was cooled on ice in glass tubes and 2 ml cold Anthrone solution was added with rapid stirring and the tubes were kept in ice. After 5 minutes chilling, the tubes were transferred to a boiling water bath for 10 minutes to destroy the carbohydrate bonds with hot sulfuric acid. The tubes again were chilled in ice for 5 minutes. Transferring to the room temperature, the optical density was read at 625 nm with respect to a blank containing distilled water instead of the extract. The composition of Anthrone solution and trehalose standard curve used in calculation were given in Appendix A and B, respectively.

2.2.4. Dry weight measurements

The sample was taken from the culture and centrifugated by refrigerated table top centrifuge at 10000 rpm for 5 minutes. The supernatant was discarded, distilled water was added to wash the pellets and the solution was centrifugated again at 10000 rpm for 5 minutes. After discarding the supernatant the pellet was dried in pre-weighed aluminum caps at 90°C. After 24 hours the caps were weighed again and the dry weight of the pellet was calculated.

2.2.5. Specific growth rate determination

The cell growth data obtained were used for growth rate determination. Natural logarithm of dry cell weight versus time were drawn and two points were selected in logarithmic growth period. The maximum specific growth rate was calculated according to the formula:

$\mu_{max} = (\ln X_2 - \ln X_1)/(t_2 - t_1)$ (Eq. 3)

where;

μ_{max} : maximum specific growth rate

$\ln X_2$: cell concentration at t_2 (gdw/l)

$\ln X_1$: cell concentration at t_1 (gdw/l)

t_2 : second time point (h)

t_1 : first time point (h)

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cloning of trehalose-6-phosphate synthase gene of *R. oryzae*

In this study, *R. oryzae TPS1* (*RoTPS1*) was cloned using PCR with hypothetical *RoTPS1* gene primers (Table 2.1). The *R. oryzae* genome has been known and the primers were designed to be homologous to *R. oryzae* gene sequences. The hypothetical proteins for trehalose-6-P synthase gene were found from the *R. oryzae* genome database (http://www.broadinstitute.org/annotation/genome/rhizopus_oryzae/BlastOutput.html). The output of the *R. oryzae* hypothetical *RoTPS1* BLAST search showed that there are eight hypothetical *RoTPS1*.

BLAST search was made by using the input entry "gi|4626|emb|X68214.1| *S. cerevisiae TPS1* mRNA for alpha, alpha-trehalose-phosphate synthase" found from NCBI site (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The results of the BLAST search were given in Table 3.1. The sequences of these hypothetical *RoTPS1* were given in Appendix G.

Table 3.1. The output of the hypothetical *RoTPS1* BLAST search.

Target	**Score (Bits)**	**Alignment Length**	**Identities**	**Positives**
R. oryzae RO3G_14824.3	660.603	480	308	392
R. oryzae RO3G_14320.3	348.206	468	183	279
R. oryzae RO3G_14593.3	342.813	473	186	277
R. oryzae RO3G_11196.3	342.428	472	184	273
R. oryzae RO3G_07383.3	338.576	453	181	270
R. oryzae RO3G_09908.3	335.495	416	168	256
R. oryzae RO3G_13130.3	332.413	483	177	271
R. oryzae RO3G_03240.3	306.99	439	161	250

The total RNA isolation from *R. oryzae* was made by Trizol method before PCR cloning. *R. oryzae* was grown for 24 hours and one hour heat stress applied to induce trehalose synthesis. After RNA isolation, cDNA synthesis was performed to make RT-PCR. In RT-PCR, however, RNA strand is first reverse transcribed into its DNA complement (complementary DNA, or cDNA) using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional PCR. RT-PCR is very useful in the cloning of eukaryotic genes in prokaryotes. Due to the fact that most eukaryotic genes contain introns which are present in the genome but not in the mature mRNA, the cDNA generated from a RT-PCR reaction is the exact (without regard to the error prone nature of reverse transcriptases) DNA sequence which would be directly translated into protein after transcription. When these genes are expressed in prokaryotic cells for the sake of protein production/purification, the RNA produced directly from transcription need not undergo splicing as the transcript contains only exons (prokaryotes, such as *E. coli*, lack the mRNA splicing mechanism of eukaryotes).

The quantity of the isolated RNA was found to be 1.88 µg/µl. It was calculated according to the formula:

Quantity (µg/µl) = (Absorbance unit x 40 x Dilution factor)/1000 (Eq. 4)

Where;

1 OD_{260} (1 absorbance unit) = 40 µg/ml RNA

The absorbance was measured by the spectrophotometer at 260 nm in 10mM Tris/HCl pH 8.0.

The purity of the isolated RNA sample was determined by measuring the ratio of A_{260}/A_{280} and was found to be 2.05. The integrity of the RNA samples was tested by 1% agarose gel electrophoresis (Figure 3.1).

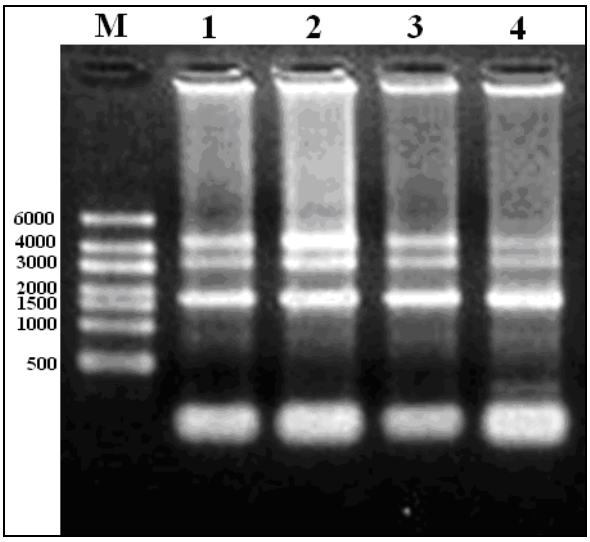

Figure 3.1. The 1 % of agarose gel electrophoresis results of RNA samples isolated from 24 hours grown and one hour heat stress applied *R. oryzae* cultures. M: Marker (2µl), 1-4: Samples (3µl sample + 2µl loading dye).

The PCR optimization studies were performed for all hypothetical proteins by using "Invitrogen AccuPrime Taq DNA Polymerase System". The annealing temperature of the PCR was determined according to the Tm values of the primers. Therefore hypothetical proteins were grouped based on the Tm values of the primers. Since Tm values of the primers for TPS1 RO3G_14824 (2058bp), TPS1 RO3G_14593 (2505bp), TPS1 RO3G_09908 (2157bp) and TPS1 RO3G_ 03240

(2370bp) were in the same range, the genes were amplified under the same PCR conditions.

The PCR products were run on 1% of agarose gel (Figure 3.2). As can be seen from the agarose gel electrophoresis result, the yield of PCR products were high, and a second PCR was performed by using "Invitrogen AccuPrime Pfx DNA Polymerase System". Pfx DNA Polymerase has 3'→5' proof reading activity; when an errant nucleotide is incorporated and forms a mismatch with the template, it is removed by a 3′ to 5′ exonuclease proof-reading activity associated with this polymerase. The yields of "Invitrogen AccuPrime Pfx DNA Polymerase System" were low compared to the "Invitrogen AccuPrime Taq DNA Polymerase System" due to its proof reading activity.

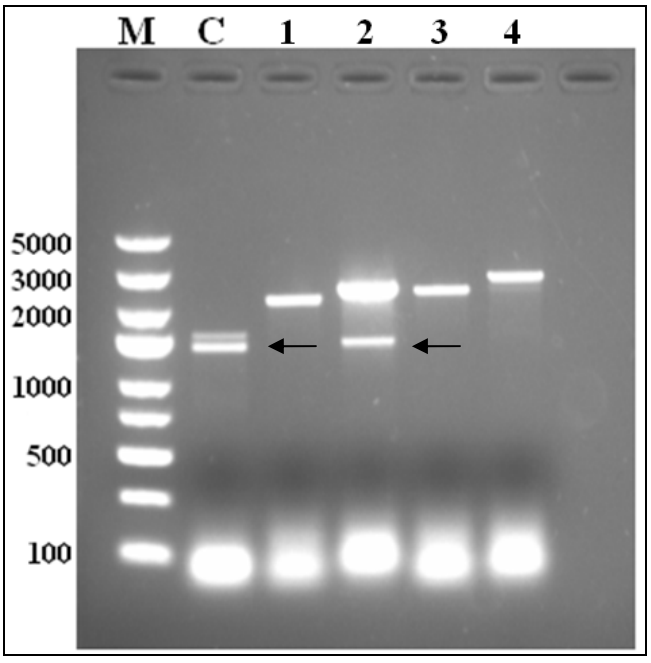

Figure 3.2. The 1 % of agarose gel electrophoresis results of PCR products amplified by using the primer. M: Molecular weight marker (given in Appendix), C: Control (Hexokinase I), 1: TPS1 RO3G_14824 (2058bp), 2: TPS1 RO3G_14593 (2505bp), 3: TPS1 RO3G_09908 (2157bp), 4: TPS1 RO3G_ 03240 (2370bp). The extra bands (←) in the lanes C and 2 were due to primer contamination.

After Pfx DNA Polymerase PCR, the amplification product of *TPS1* RO3G_14593 (2505bp) had the best yield compared to the others, and this band was cut from the gel to perform gel extraction.

Two vector systems were used for cloning of *R. oryzae* trehalose-6-phosphate synthase gene. One of them was shuttle vector: pGEM®-T Easy and the other was expression vector: pFL61. The plasmid maps was given in Appendix H.

In order to construct the shuttle vector, the DNA extracted from the gel was directly ligated by T/A cloning kits.

Inserted vector was transformed to *E. coli* DH5α. After the incubation of the transformed cells on LB agar + X-Gal, white colonies were taken and inoculated in LB liquid media at 37°C, 200rpm for overnight. Plasmid isolation was made from the grown cultures according to the "Fast Mini-Prep Protocol". Plasmid isolation was checked by agarose gel electrophoresis and given in Figure 3.3.

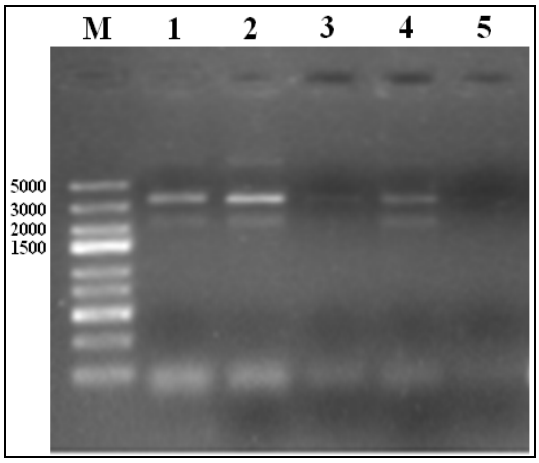

Figure 3.3. The 1 % of agarose gel electrophoresis results of plasmid samples. M: Marker, 1-5: Intact plasmids.

According to the Figure 3.3, the cultures of samples 1, 2 and 4 were used for plasmid isolation by "Plasmid DNA Purification QIAprep Kit". The purified plasmid isolation products obtained were cut with the restriction digestion enzyme

HindIII to check the transformation of pGEM®-T Easy vector + *RoTPS1* into *E. coli*. The products of restriction digestion were shown in Figure 3.4.

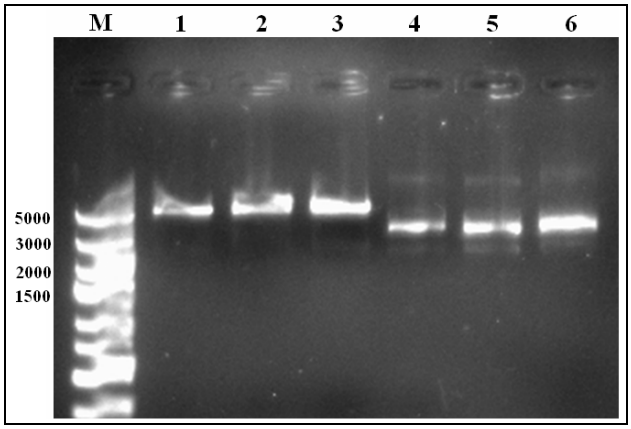

Figure 3.4. The 1 % of agarose gel electrophoresis results of plasmid samples. M: Marker, 1-3: Samples cut with *HindIII*, 4-6: Intact plasmids.

Sequence analysis was performed on the purified plasmids to check if the correct insert is in the plasmid.

In order to analyze all of the *RoTPS1* sequence, five of the internal primers were designed. Those primers were given in Table 3.2.

Table 3.2. Designed internal primers of *R. oryzae* RO3G_14593.3: hypothetical protein.

***R. oryzae* RO3G_14593.3**	**The sequence of the primer 5'→3'**	**Base region**
IP1	GGAAAAGGGACAGATTGTGCCAG	435-458
IP2	GAGTGGTTTCCTGGGTGCGAATC	792-815
IP3	CACGACACCTACCTTTGGTGATAAC	1158-1183
IP4	GACACGCCACAGTCAACTCTACAAC	1528-1558
IP5	GATCAGTGGACGAATGCGTTGGAAGAC	1939-1966

The sequence analysis of plasmid (PGEM®-T Easy vector + *RoTPS1*) was performed with SP6, T7 and the designed internal primers. The sequence analysis results were tabulated in Table 3.3.

In order to compare the sequence analysis results of the plasmid with *R. oryzae* RO3G_14593: hypothetical protein, multiple sequence alignment was carried out by using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html).

Table 3.3. Multiple sequence alignment Clustal 2.0.8. for PGEM®-T Easy vector + *RoTPS1* and hypothetical *RoTPS1*

```
Plasmid           MPSLTAEKVADYLPLAGKMPQISGRIINVTHQIPYHISRSAPSKIVTEAPSPQLPEDVSG 60
Hypothetical      MPSLTAEKVADYLPLAGKMPQISGRIINVTHQIPYHISRSAPSKIVTEAPSPQLPEDVSG 60
                  ************************************************************

Plasmid           APVSKLARHHHRRGTLRAKFHAAEWTVTQSRGHLALHAGLQSLREGYETIQIGWTGPIKD 120
Hypothetical      APVSKLARHHHRRGTLRAKFHAAEWTITQSRGHLALHAGLQSLREGYETIQIGWTGPIKD 120
                  ************************:***********************************

Plasmid           KATKAVLSSEDLAEEDIAKLEGLLMEKGQIVPVFLDSKSRGHYEGYCKEVLWPLFHYLVW 180
Hypothetical      KATKAVLSSEDLAEEDVAKLEGLLMEKGQIVPVFLDSKSRGHYEGYCKEVLWPLFHYLVW 180
                  ****************:*******************************************

Plasmid           SDHAGGLSEKQYWEDYVSVNQQFAQRIADHYRPGDIIFINDYHLLLVPEMLREMIPDAPI 240
Hypothetical      SDHAGGLSEKQYWEDYVAVNQQFAKRIADHYRPGDIIFINDYHLLLVPEMLREMIPDAPI 240
                  *****************:******:***********************************

Plasmid           GLFLHAPFPVQKSFVAQLVKRSVAFWVR----ISIQTYSYARHFIGACTRVLGCESTQTG 296
Hypothetical      GLFLHAPFPSSEIFRCLTTRKEILSGFLGANLVGFQTYSYARHFIGACTRVLGCESTQTG 300
                  ********* .: * .  .::.:   .     :.:************************

Plasmid           VNVNGHIVSVGTFPIGIDAKRVNQFRQEPGVAAKMKAIRAMYADKKIIIGRDKLDSTKGV 356
Hypothetical      VNVNGHIVSVGTFPIGIDAKRVNQFRQEPGVAAKMKAIRAMYADKKIIIGRDKLDSTKGV 360
                  ************************************************************

Plasmid           VQKLHAFEKFLRDYPEWRKKVVLIQVTTPTFGDNSKLESKVSELVIHINCLYGSLEFTPV 416
Hypothetical      VQKLHAFEKFLRDYPEWRKKVVLIQVTTPTFGDNSKLESKVSELVSHINSLYGSLEFTPV 420
                  ****************************************** ***.**********

Plasmid           HHYYQDVDRDEYYALLSVADVGLITSLRDGMNTTSFEYILCQQEQHDSIILSEFTGTAGS 476
Hypothetical      HHYYQDVDRDEYYALLSVADVGLITSLRDGMNTTSFEYILCQQEQHGSIILSEFTGTAGS 480
                  ********************************************.*************

Plasmid           LGADLYREPIRYRWVVGQRHPS--SRSCKKATRHGHFTTMLTIG-----ASHLLNRMAEQ 529
Hypothetical      LGAAFIVNPFDIAGVARAIHEALVLSAEEKATRHSQLYNYVVEHSAAYWAKSFAKQLAES 540
                  *** :  :*:    *.   * :    : :*****.:: . :.       *. : :::**.

Plasmid           HTYLQTELQTCALLYISIGFHDRLQGLPKASMFFLRPQLTADRLCAEQTPKPLARRCNYL 589
Hypothetical      TQNFRLQSHTAPPLDIDR-FTTAFRSAKKRLMFFDYDGTLTPIVSVPTDAKPSQEMLEYL 599
                     :: : :*.. * *.  *   ::.  *  ***      :  :..   .**  .  :**

Plasmid           PAAFAIDSRTHVWLNLIQIKACLK--YGRNQNLGYTPSMAILETLNG---WAMGKVINHW 644
Hypothetical      -QALCNDPKNHVWVVSGRDQACLEDWLGGIKNLGLSAEHGCFWKAAGSDQWTN-ALEDTD 657
                    *:. *.:.***:   : :***:   *  :*** :.. . : .  *   *:   : :

Plasmid           HGLEKDVTVIFDYYTERTEGSFVEHKKSSITWHYRMADEDYGLFQAKECQNHLENSVVSK 704
Hypothetical      MDWKKDVTEIFDYYTERTEGSFVEHKKSSITWHYRMADEDYGLFQAKECQNHLENSVVSK 717
                   . :**** **************************************************

Plasmid           RPVEILVGKKNLEVRPMMINKGEVVKRILTLTPDADFIVCAGDDKTDEDMFRTLSATYFA 764
Hypothetical      RPVEILVGKKNLEVRPMMINKGEVVKRILTLTPDADFIVCAGDDKTDEDMFRTLSATYFA 777
                  ************************************************************

Plasmid           RYQEQLANTGGEASWEEAKSTLYSITIGPPKKSMANWRVEAPSDVIHLLSLMAQEEK 821
Hypothetical      RYQEQLANTGGEASWEETKSTLYSITIGPPKKSMANWRVEAPSDVIHLLSLMAQEEK 834
                  ****************:*************************************
```

In order to transfer the *RoTPS1* gene from shuttle vector pGEM®-T Easy to the expression vector pFL61, the inserted gene should be taken from the shuttle vector. The stock culture of *E. coli* containing vector pGEM®-T Easy + *RoTPS1*

was inoculated at 37 ºC, 200 rpm for overnight and the plasmid isolation was made. After plasmid isolation, the plasmids were cut from their *NotI* site to obtain the inserted gene. However, inserted gene and pGEM®-T Easy have closer base pairs (2505 and 3015, respectively), and the two bands that appear in the gel were too close to each other and it was not possible to cut the inserted gene from the gel appropriately. In order to overcome this problem, double digestion was carried out at *BauI* and *NotI* sites. The schematic representation of the double digestion of pGEM®-T Easy vector was shown in Figure 3.5.

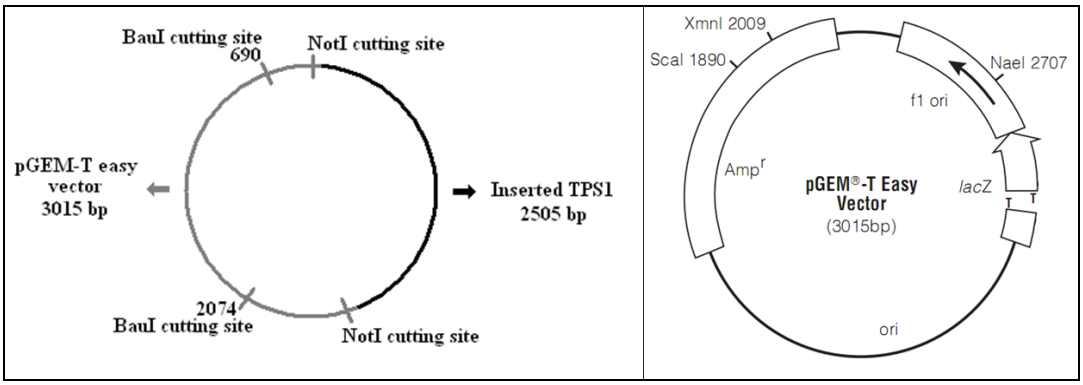

Figure 3.5. The schematic representation of the double digestion of pGEM®-T Easy vector.

Since *BauI* cut the plasmid at 690 and 2074, there were two bands on the agarose gel one of them was 1384 bp and the other was 4136 bp including interested gene. Double digestion results were shown in agarose gel (Figure 3.6).

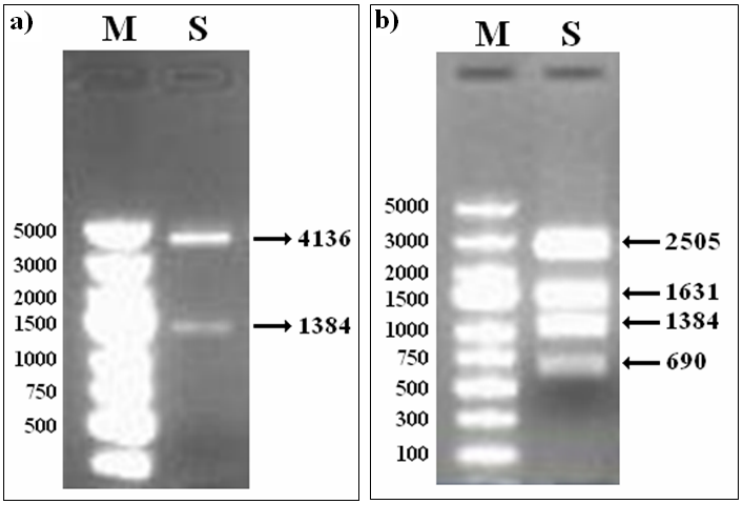

Figure 3.6. The 1 % of agarose gel electrophoresis results of double digestion products; a) *BauI* digestion, b) *NotI* digestion. M: Marker (3µl + 2µl loading dye), S: Sample (3µl + 2µl loading dye).

RoTPS1 gene was extracted from the gel by using the gel extraction kit (QIAGEN). Before the ligation of the *RoTPS1* gene with expression vector pFL61, pFL61 was digested with *NotI* restriction enzyme to linearize the vector and was treated with alkaline phosphatase which removes the terminal 5' phosphate from DNA molecules to prevent self ligation.

The ligation procedure was carried out to ligate the expression vector pFL61 and *RoTPS1* extracted from the shuttle vector pGEM®-T Easy. The ligated vector was transformed into *E. coli* DH5α. After the incubation of the transformed cells on LB agar, the colonies were taken randomly by toothpick and inoculated in LB liquid media at 37°C 200 rpm for overnight. Plasmid isolation was made from the grown cultures according to the "Plasmid DNA Purification QIAprep Kit".

Plasmid isolation products were cut by *HindIII* digestion enzyme to check whether plasmids contain insert or not. *HindIII* digestion products were run on agarose gel, the resulting gel photograph was given in Figure 3.7.

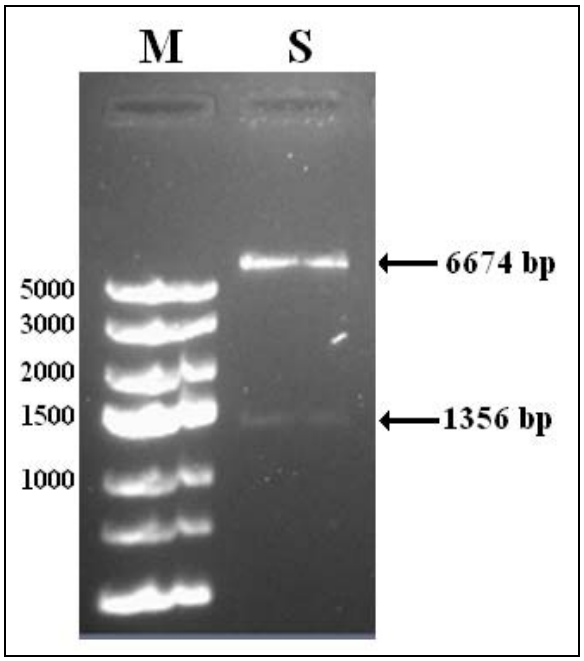

Figure 3.7. The 1 % of agarose gel electrophoresis results of *HindIII* digestion product of the vector pFL61. M: Marker [GeneRuler™ Express DNA Ladder (Fermentas)] (3µl + 2µl loading dye), S: Sample (3µl + 2µl loading dye).

The cutpoints of *HindIII* on the vector and *RoTPS1* gene were 1553 and 1057, respectively. Therefore, there were two bands (6674 bp and 1356 bp) on the gel after *HindIII* digestion showing that the plasmid isolation products cut by *HindIII* digestion enzyme contain plasmid insert.

Although two bands were seen on the gel, it must be shown that the insert sticked to the vector in the right direction because sticky ends of the vector and insert contain *NotI* site.

In order to check if the orientation of the insert was correct in the plasmid, sequence analysis was performed on the purified plasmid with the pFL61 primer A (GCGTAAAGGATGGGGAAAGAGAAAAG). The sequence analysis results were tabulated in Table 3.4.

Table 3.4. Multiple sequence alignment Clustal 2.0.8 for pFL61 vector + *RoTPS1* and hypothetical *RoTPS1*.

```
PFL61vector       ATGATCGCGGCCGCGGGAATTCGATTATGCCATCATTAACAGCTGAAAAAGTAGCCGACT 60
Hypothetical      -------------------------ATGCCATCATTAACAGCTGAAAAAGTAGCCGACT 34
                                           **********************************

PFL61vector       ATTTACCTTTGGCAGGCAAGATGCCACAGATCTCTGGCCGTATCATTAACGTGACCCATC 120
Hypothetical      ATTTACCTTTGGCAGGCAAGATGCCACAGATCTCTGGTCGTATCATTAACGTGACCCATC 94
                  ************************************ ********************

PFL61vector       AAATTCCGTATCATATCTCAAGATCGGCTCCTTCCAAGATTGTCACTGAGGCTCCCTCAC 180
Hypothetical      AAATTCCGTATCATATCTCAAGATCGGCTCCTTCCAAGATTGTCACCGAGGCTCCTTCGC 154
                  ********************************************** ******** ** *

PFL61vector       CTCAACTCCCAGAGGATGTCTCTGGAGCACCGGTGTCCAAGCTCGCCCGTCATCATCACC 240
Hypothetical      CTCAACTCCCAGAGGATGTCTCTGGAGCACCGGTGTCCAAGCTCGCCCGTCATCATCACC 214
                  ************************************************************

PFL61vector       GCCGTGGCACTTTGAGAGCTAAATTTCATGCGGCCGAATGGACCGTCACTCAGAGCAGAG 300
Hypothetical      GCCGTGGCACGTTGAGAGCTAAATTTCATGCGGCCGAATGGACCATCACCCAGAGCAGAG 274
                  ********** ********************************** **** **********

PFL61vector       GCCATCTTGCTTTGCATGCTGGCTTGCAAAGCCTGAGAGAAGGATACGAAACGATCCAGA 360
Hypothetical      GCCATCTCGCTTTGCATGCTGGCTTGCAAAGCCTGAGAGAAGGATACGAAACGATCCAGA 334
                  ******* ************************************************

PFL61vector       TCGGATGGACGGGACCGATCAAAGACAAGGCGACAAAGGCCGTATTGTCTTCAGAAGATT 420
Hypothetical      TCGGATGGACAGGCCCGATCAAAGACAAGGCAACAAAGGCCGTACTATCTTCAGAAGACT 394
                  ********** ** ***************** ************ * *********** *

PFL61vector       TGGCAGAGGAAGACATTGCTAAACTGGAAGGTCTCTTGATGGAAAAGGGACAGATTGTAC 480
Hypothetical      TGGCAGAGGAAGACGTGGCTAAACTGGAAGGTCTCTTGATGGAAAAGGGACAGATTGTGC 454
                  ************** * ************************************** *

PFL61vector       CCGTCTTTTTGGATAGCAAGTCGCGTGGTCATTATGAAGGTTATTGTAAAGAAGTACTTT 540
Hypothetical      CAGTCTTTTTGGATAGCAAGTCGCGTGGTCATTATGAAGGTTATTGTAAAGAAGTACTTT 514
                  * ************************************************************

PFL61vector       GGCCATTGTTCCACTATCTGGTCTGGTCAGATCATGCGGGCGGTTTGTCCGAAAAGCAAT 600
Hypothetical      GGCCTTTGTTCCACTATCTGGTCTGGTCGGATCACGCGGGCGGTTTGTCCGAAAAGCAAT 574
                  **** ********************** ***** ************************

PFL61vector       ATTGGGAAGATTATGTGTCTGTGAACCAGCAGTTTGCTCAAAGGATCGCTGATCATTATC 660
Hypothetical      ATTGGGAAGATTATGTGGCTGTGAACCAGCAGTTTGCTAAAAGAATTGCTGATCATTATC 634
                  ***************** ******************** **** ** *************

PFL61vector       GTCCGGGTGATATAATCTTTATCAATGATTATCATCTCCTGCTTGTTCCTGAAATGTTGC 720
Hypothetical      GCCCGGGTGATATAATCTTTATCAATGATTATCATCTCCTGCTTGTTCCTGAAATGTTGC 694
                  * ************************************************************

PFL61vector       GTGAGATGATTCCTGATGCCACCATCGGTCTCTTTCTTCATGCTCCTTTCCCCAGTTCAG 780
Hypothetical      GTGAGATGATTCCTGATGCCCCCATCGGTCTCTTTCTTCATGCTCCTTTCCCTAGTTCAG 754
                  ******************** ******************************** *******

PFL61vector       AAATCTTTCGTTGCCTGACAACTCGTAA-GAGATCTTGAGTGGCTTTCTGGGTGCCAATC 839
Hypothetical      AAATCTTTCGTTGCCTGACAACTCGAAAAGAGATCTTGAGTGGTTTCCTGGGTGCGAATC 814
                  ************************** ** ************** ** ******** ****

PFL61vector       TAGTCGGATTCCAA-CTTATTCTTATGCTCGACACTTTATCGGCGCATGCACTCGGGTCC 898
Hypothetical      TAGTCGGATTCCAAACTTATTCTTATGCTCGACACTTTATCGGCGCATGCACTCGGGTCC 874
                  ************** ********************************************

PFL61vector       TTGGTTG-GAATCGACACAGACGGGTGTGACATGTGA-TGGTCTATTGTAGTCTGTCGG- 955
Hypothetical      TGGGTTGTGAATCGACACAGACAGGTGTGA-ATGTGAATGGACATATAGTGTCTGTCGGG 933
                  * ***** ************** ******* ****** *** *   *   *********

PFL61vector       ACGTTTC-GATCGG-ATCGACGCCA-GCGAGTCA-TCAGTTCCTCAGAG---CTGGGGTG 1008
Hypothetical      ACGTTTCCGATTGGTATCGACGCCAAGCGAGTCAATCAATTTCGTCAAGAGCCTGGGGTG 993
                  ******* *** ** ********** ******** *** ** *    **   ********
```

Table 3.4.continued

```
PFL61vector       GCCGC--AGATGAAGC--ATCCA--TCATG-ATGCG-ACAGAAATCTCATC----GTCGT 1056
Hypothetical      GCCGCCAAGATGAAGGCGATCCGAGCAATGTATGCGGACAAGAAGATCATCATCGGTCGT 1053
                  *****  ********   ****     *** ***** ***  **  *****    *****

PFL61vector       GACA-GCTT-ATTCAGC--AGGGC-TAGTC---AAACTAC-CGCGTTTGAAA-GTTTTGC 1106
Hypothetical      GACAAGCTTGATTCGACCAAGGGTGTGGTTCAAAAACTGCATGCGTTTGAAAAGTTTTTG 1113
                  **** **** ****  *  ****  * **    ***** *  ********** *****

PFL61vector       TGTA-----TCAAGATGGCAAGAA----TGGTGCTGAT-CAGTCCG----ACCTACTTC- 1151
Hypothetical      CGTGATTATCCAGAATGGCGAAAGAAAGTGGTGCTGATTCAGGTCACGACACCTACCTTT 1173
                   **       **  ***** * *     ********** ***  *     ****** *

PFL61vector       ----GTGAATCCAATT---GATCAAG---------------------------------- 1170
Hypothetical      GGTGATAACTCCAAATTGGAATCGAAGGTGTCTGAGCTTGTCAGCCATATCAACAGCCTC 1233
                       * * ***** *    *** *

PFL61vector       ------------------------------------------------------------
Hypothetical      TATGGCTCACTCGAGTTTACTCCTGTCCATCACTATTACCAAGACGTGGATCGTGACGAG 1293


PFL61vector       ------------------------------------------------------------
Hypothetical      TACTATGCTTTGCTGTCTGTGGCTGACGTTGGCTTGATCACTAGCTTAAGAGATGGCATG 1353


PFL61vector       ------------------------------------------------------------
Hypothetical      AACACCACTTCATTTGAATACATCCTCTGTCAGCAAGAACAGCATGGTTCCATCATCCTA 1413


PFL61vector       ------------------------------------------------------------
Hypothetical      TCTGAATTCACCGGCACAGCAGGTTCACTGGGTGCGGCCTTTATCGTGAATCCATTCGAC 1473


PFL61vector       ------------------------------------------------------------
Hypothetical      ATCGCTGGTGTGGCCAGGGCGATCCATGAGGCTCTCGTTCTCAGTGCTGAAGAAAAGGCG 1533


PFL61vector       ------------------------------------------------------------
Hypothetical      ACACGCCACAGTCAACTCTACAACTATGTCGTCGAACACTCTGCGGCCTATTGGGCAAAG 1593


PFL61vector       ------------------------------------------------------------
Hypothetical      TCATTTGCCAAACAGCTGGCCGAGAGCACACAAAACTTCAGACTGCAATCACACACTGCA 1653


PFL61vector       ------------------------------------------------------------
Hypothetical      CCTCCTCTGGATATCGATCGGTTCACGACCGCCTTCAGATCTGCCAAGAAGCGTCTGATG 1713


PFL61vector       ------------------------------------------------------------
Hypothetical      TTCTTTGACTATGACGGCACACTGACGCCGATCGTCTCTGTGCCGACGGACGCCAAACCT 1773


PFL61vector       ------------------------------------------------------------
Hypothetical      TCACAAGAGATGCTAGAATACCTTCAAGCGCTTTGCAATGATCCAAAGAACCATGTCTGG 1833


PFL61vector       ------------------------------------------------------------
Hypothetical      GTTGTGTCTGGTCGAGATCAGGCCTGTCTGGAGGATTGGCTGGGCGGGATCAAGAACCTT 1893


PFL61vector       ------------------------------------------------------------
Hypothetical      GGGCTCAGCGCAGAGCATGGCTGCTTCTGGAAAGCGGCCGGTTCGGATCAGTGGACGAAT 1953


PFL61vector       ------------------------------------------------------------
Hypothetical      GCGTTGGAAGACACTGACATGGACTGGAAAAAGGATGTGACAGAGATCTTTGATTATTAC 2013
```

3.2. Transformation of *Saccharomyces cerevisiae*

Yeast has been extensively used as a model system for the study of a broad variety of eukaryotic processes. Yeast can be used to clone and functional characterize genes from other organisms. Since yeast is expressing all genes necessary for basic functions in eukaryotic cells [110], it can be used as a tool to isolate genes from other organisms which share similar functions by complementation with a cDNA library under the control of a yeast promoter. In the literature numerous examples have been reported for the identification of functional homologous proteins from different organisms using specific yeast mutants [111, 112]

In order to isolate the *R. oryzae* homologue of *TPS1*, *S. cerevisiae tps1* mutant was transformed with *RoTPS1* under the control of the yeast PGK1 promoter and checked for complementation of its growth defect in glucose.

As stated, mutants deficient in *TPS1* function are unable to grow on glucose or other rapidly fermentable carbon sources, but they can grow on galactose, mannose, and nonfermentable sugars. This is due to an inability to sense and regulate glucose influx during glycolysis because trehalose-6-phosphate, which functions as a feedback inhibitor of hexokinase, is absent in the *tps1* mutant. The other possible cause is the excessive accumulation of sugar phosphate and a concomitant depletion of ATP and phosphate. *RoTPS1* complemented the *tps1* mutant *S. cerevisiae*, allowing it to grow on medium containing glucose as the carbon source. The *tps1* mutant *S. cerevisiae* recovered the ability to ferment glucose after being transformed with pFL61. In order to complement the yeast *tps1* mutant with the *RoTPS1* gene, cells that were transformed with pFL61+ *RoTPS1* were selected on selective medium which had galactose as the carbon source and no uracil. Because the *S. cerevisiae tps1* mutant can not grow on glucose, transformed yeast cells were inoculated to glucose media to check whether the transformation was successful or not. Figure 3.8 shows the complementation of *RoTPS1* with *tps1* mutant *S. cerevisiae.* The volume of inoculations were the same for all the plates, but since the transformant inoculums contained smaller number of cells compared to mutant and wild type inoculums, small number of transformant

colonies were observed in glucose media compared to grown plates of mutant and wild type cells after the incubation period.

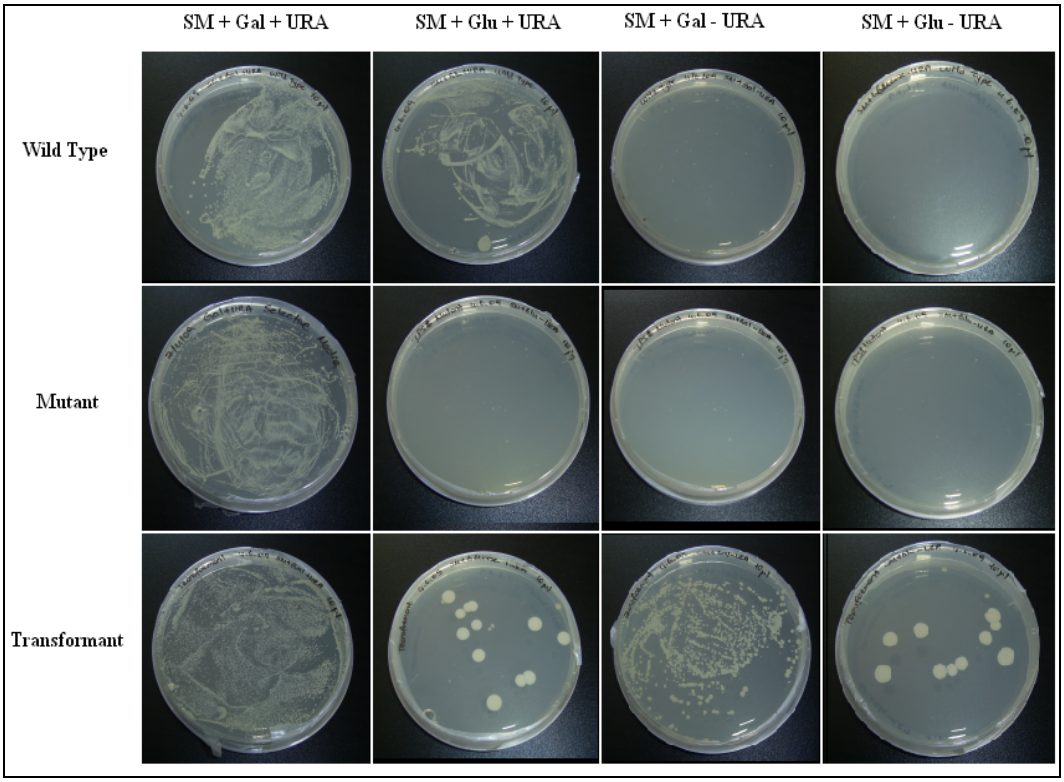

Figure 3.8. Complementation of mutant *tps1 S. cerevisiae* (SM: Selective Medium, Gal: Galactose, URA: Uracil).

Furthermore, expression of pFL61 restored the capacity to synthesize trehalose in the *tps1* deletion mutant. These results indicate that plasmid pFL61 encodes a protein involved in trehalose synthesis.

The expression of *TPS1* gene is different among different organisms under normal conditions. In *S. cerevisiae* [53], *TPS1* is expressed at very low levels and increases dramatically with heat shock. However in *Zygosaccharomyces rouxii* [62], it is highly and constitutively expressed, and fluctuates slightly after heat shock. The gene was cloned successfully with the RNA extracted from *R. oryzae*

after heat shock in this study. The result suggested that the expression pattern of *TPS1* in *R. oryzae* was probably the same as that in *S. cerevisiae*. In fact, the conclusion was confirmed by the previous studies [113].

In wild type yeast, trehalose synthesis is enhanced during the stationary phase as a result of the activation of the transcription of *tps1* and *tps2*. However, in the transformants, trehalose synthesis did not reach the values of the wild type strain during the stationary phase.

Strains wild type, mutant and transformant were grown in minimal medium containing 2% galactose. While the wild type strain accumulated trehalose preferentially in the stationary phase the *tps1* mutant was unable to synthesize trehalose in either phase of growth transformation of the tps1 mutant with plasmid pFL61 allowed accumulation of trehalose on both phases of growth as expected for the constitutive expression of the *RoTPS1* from the yeast PGK1 promoter.

The transformation process was further confirmed by PCR using *R. oryzae TPS1* primers. After complementation analysis of yeast *tps1*, one colony was taken from the plate which was labeled as 'Transformant + Gal – URA' and DNA isolation was done. After the isolation, PCR cloning was carried out by using *RoTPS1* primers. The gel photograph of the fragment amplified by the primers was given in Figure 3.9.

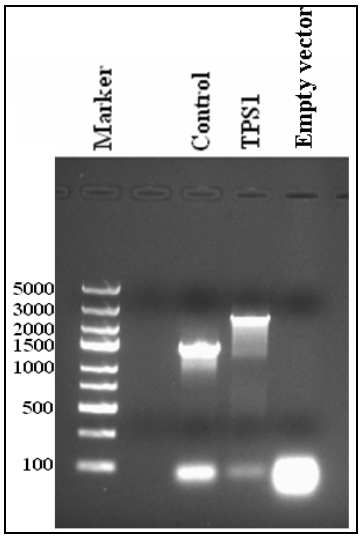

Figure 3.9. The 1 % of agarose gel result showing the PCR screening for *tps1* transformants.

3.3. Sequence analysis of *RoTPS1*

Sequence analysis of *RoTPS1* was isolated by PCR using primers designed according to eight hypothetical proteins found from BLAST search which was performed by using *S. cerevisiae TPS1* gene template. The recombinant *RoTPS1* enzyme was expressed in *tps1* mutant *S. cerevisiae. RoTPS1* cDNA consists of 2505 bps, which encodes a polypeptide of 834 amino acids with a molecular mass of 97.8 kDa. Based on BLAST analysis of the predicted *RoTPS1* amino acid sequence, *RoTPS1* exhibits amino acid sequence homology to a number of trehalose-P synthases from several sources, including ***Arabidopsis*** *thaliana*, *Selaginella lepidophylla*, *Saccharomyces cerevisiae, Aspergillus nigulans*.

Fig.3.10 shows the predicted amino acid sequence of *RoTPS1* and its sequence alignment with those of homologous ORFs from several other organisms. As indicated by Clustal W alignment, the alignment shows several regions of the protein with very high homology in the middle of the sequences, whereas less similarity at C-terminal portions. Certainly, further experimental investigations are required to elucidate the structure details of *RoTPS1*.

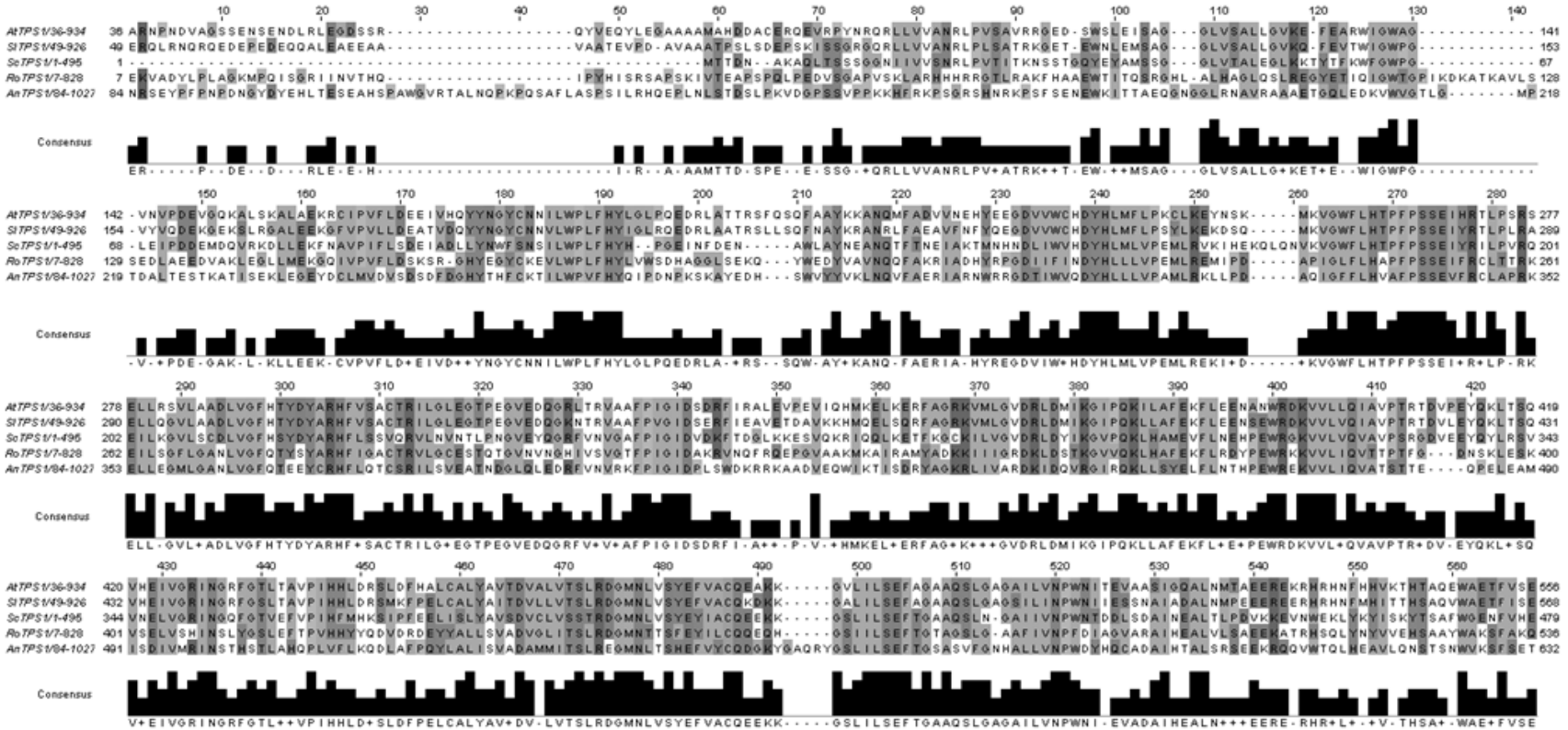

Figure 3.10. Amino acid sequence comparison between the predicted *RoTPS1* amino acid sequence and several organisms *TPS1*s. Identical residues are starred. Sequences are from: 1 *Arabidopsis thaliana*, 2 *Selaginella lepidophylla*, 3 *Saccharomyces cerevisiae* 4 *Rhizopus oryzae*, 5 *Aspergillus nigulans*.

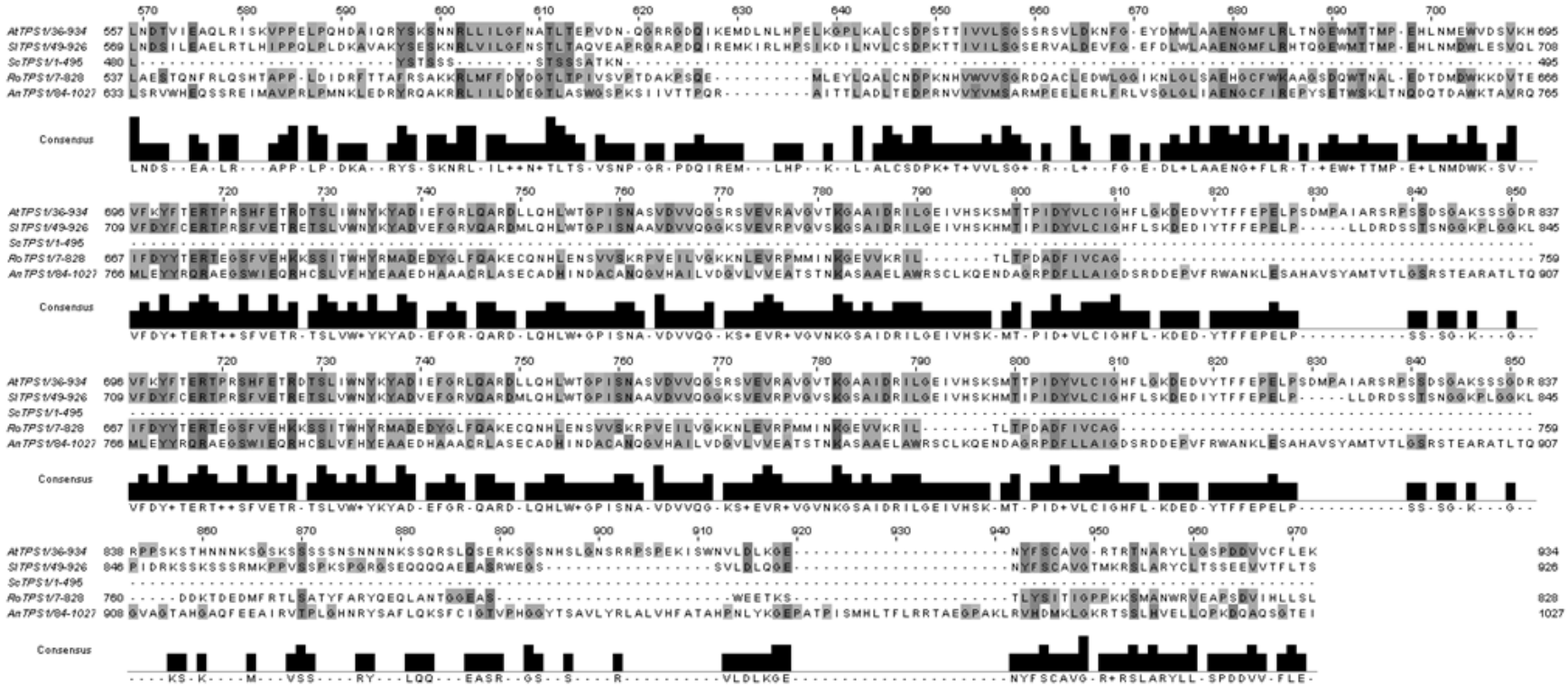

Figure 3.10. Continued.

3.4. Comparison of the growth and trehalose production of wild type, *tps1* mutant and transformed *S. cerevisiae* cells

3.4.1. Growth and trehalose production in galactose media

Wild type, *tps1* mutant and transformed *S. cerevisiae* cells were inoculated in selective medium containing galactose as the C source. The averages of the growth and trehalose data obtained from up to 5 replicated flasks were calculated and plotted in Figure 3.11 and 3.12, respectively.

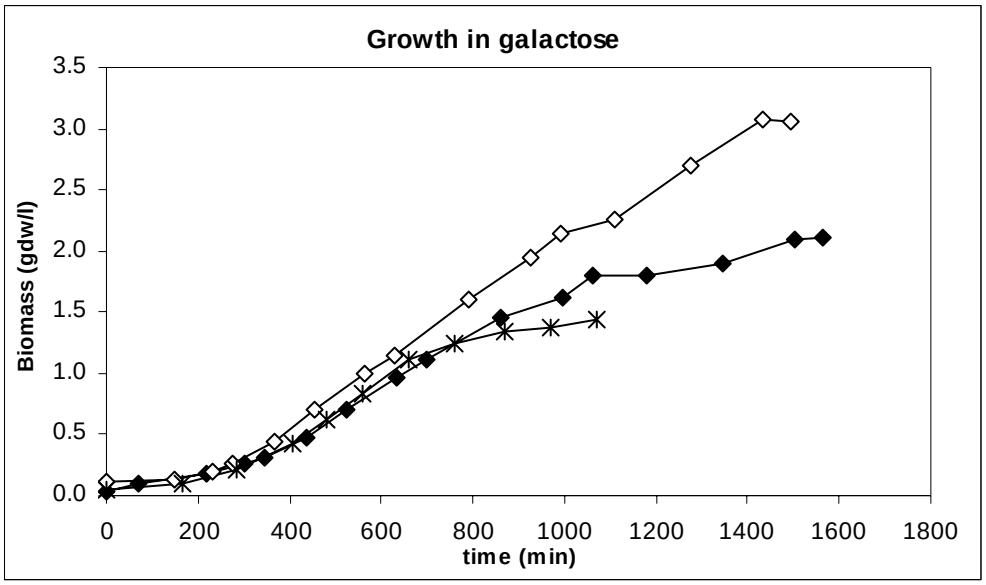

Figure 3.11. Cell growth curve of wild type (♦), *tps1* mutant (◊) and transformed (*) *S. cerevisiae* cells grown in selective medium containing galactose as the carbon source, selective medium also contained uracil for wild type and *tps1* mutant cells.

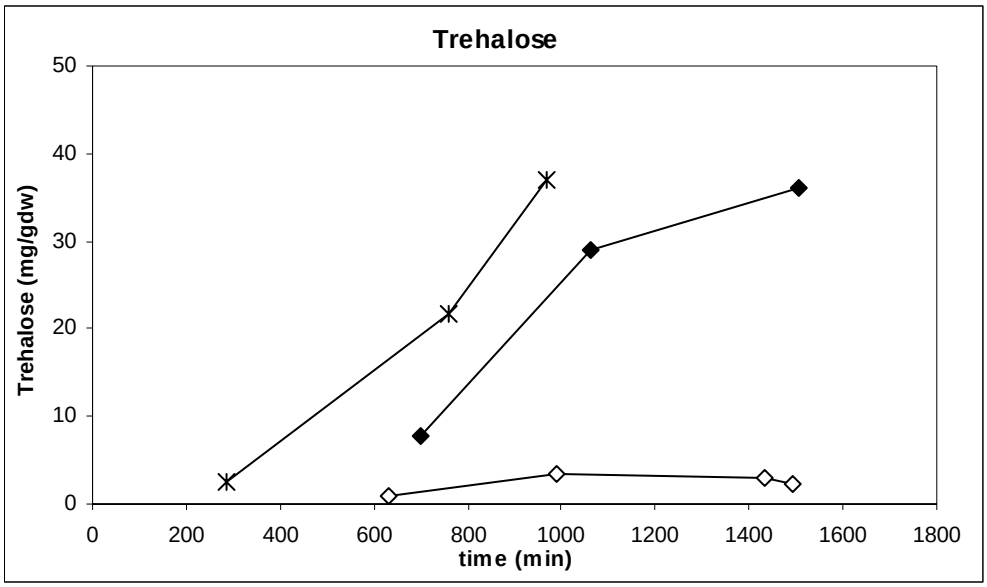

Figure 3.12. Trehalose amounts of wild type (♦), *tps1* mutant (◊) and transformed (*) *S. cerevisiae* cells grown in selective medium containing galactose as the C source.

The growth curves had a comparable pattern in general, *tps1* mutant reached to a higher maximum cell concentration compared to the others. Trehalose levels of transformant and wild type cells were increased up to 37 mg/gdw in the stationary phase, a negligible amount of trehalose was detected in mutant cells (3 mg/gdw) which is possibly due to the error caused by the method used for trehalose determination which is highly sensitive to the carbohydrate residues.

3.4.2. Growth and trehalose production in glucose media

Wild type, *tps1* mutant and transformed *S. cerevisiae* cells were inoculated in selective medium containing glucose as the C source. As expected, *tps1* mutant did not grow in glucose medium. The averages of the growth and trehalose data for wild type and transformed cells were calculated and plotted in Figure 3.13 and 3.14, respectively.

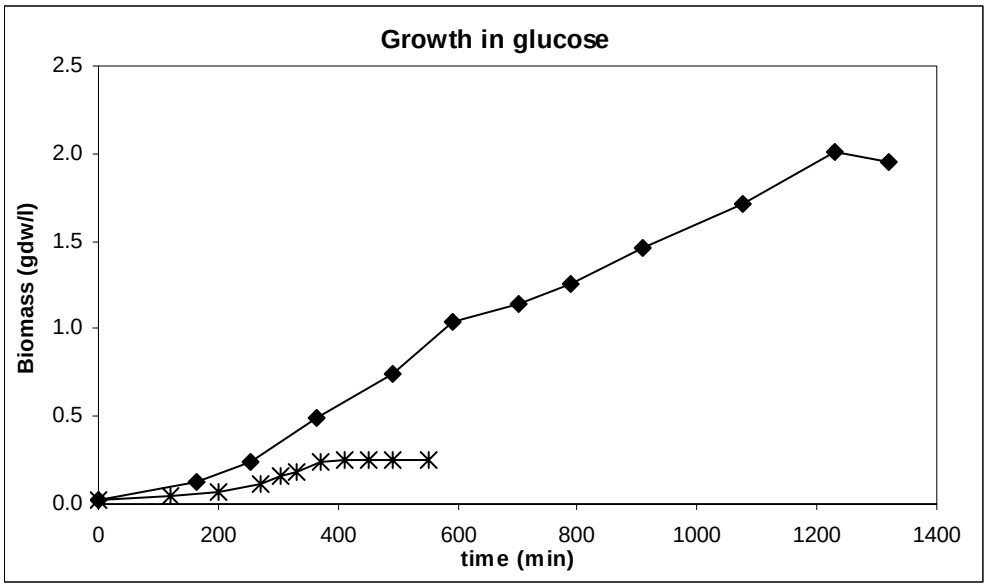

Figure 3.13. Cell growth curve of wild type (♦) and transformed (*) *S. cerevisiae* cells grown in selective medium containing glucose as the C source, selective medium also contained uracil for wild type and *tps1* mutant cells.

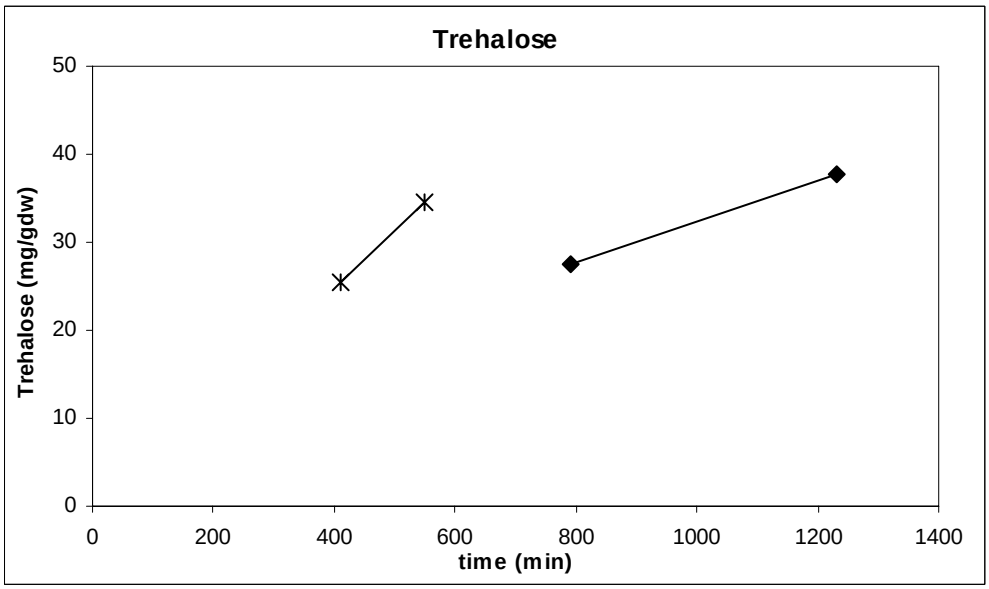

Figure 3.14. Trehalose amounts of wild type (♦) and transformed (*) *S. cerevisiae* cells grown in selective medium containing glucose as the C source.

Growth curve of transformant cells was different than the growth curve of wild type cells; the maximum cell concentration of transformants did not exceed 0.26 gdw/l where wild type cells reached up to 2.00 gdw/l. On the other hand, trehalose levels of transformant and wild type cells were comparable; they increased up to 38 mg/gdw and 35 mg/gdw in the stationary phase, for wild type and transformant cells, respectively.

One possible reason of the low transformant cell concentration could be the overexpression of *RoTPS1* because *RoTPS1* constitutively expressed under PGK promoter which leads to the continuous production of T6P. T6PP and trehalase might not be working fast enough resulting in the accumulation of T6P which caused the organism to enter into the stationary phase early. Another possible reason might be *RoTPS1*, *TPS2* and regulatory subunits of trehalose synthase complex of *S. cerevisiae* not working properly altogether thus trehalose mobilization could not be achieved.

Overall, the results showed that the transformant is capable of producing trehalose, to the same extent than the wild type cells in both glucose and galactose media.

3.4.3. Specific growth rate determination

In order to determine and compare the maximum specific growth rate (μ_{max}) for wild type, mutant and transformant cells, cell concentration versus time were drawn in logarithmic scale (Figure 3.15).

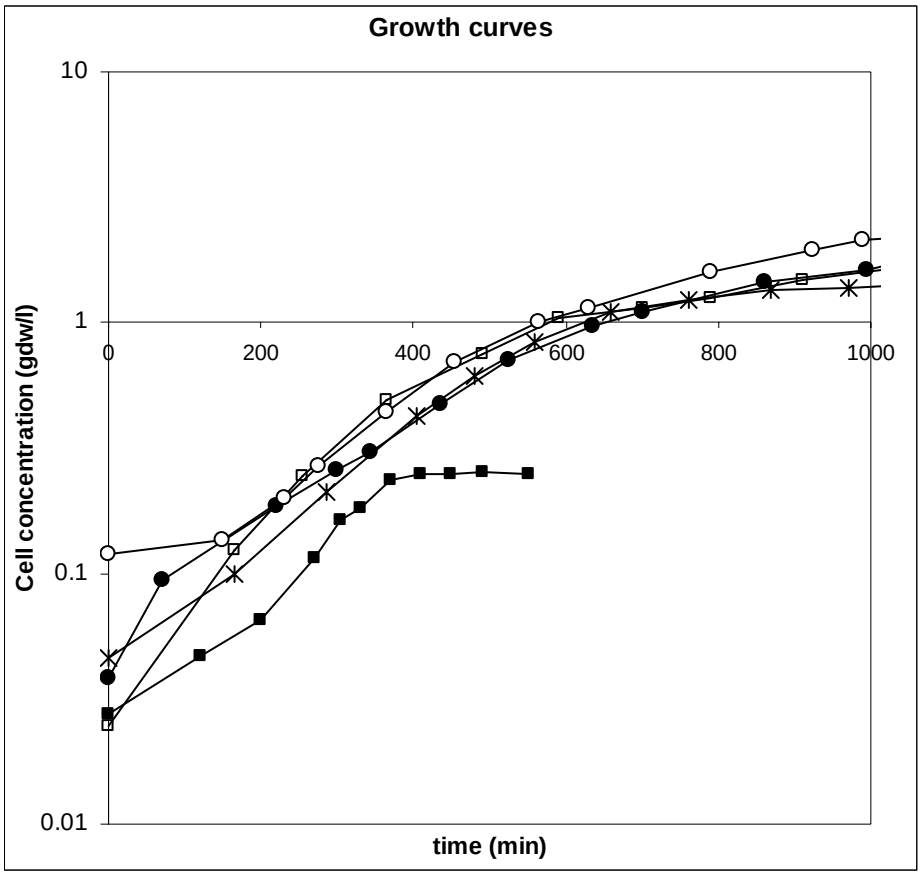

Figure 3.15. Growth curves in logarithmic scale. (●) wild type in galactose media, (○) tps1 mutant in galactose media, (*) transformant in galactose media, (■) transformant in glucose media, (□) wild type in glucose media.

Maximum specific growth rates were calculated as described in Materials and Methods Section, the values obtained were given in Table 3.5.

Table 3.5. Maximum specific growth rates of the strains used.

Strain	**C source in medium**	**μ_{max}**
Wild type	Galactose	0.27
tps1 mutant	Galactose	0.32
Transformant	Galactose	0.32
Wild type	Glucose	0.41
tps1 mutant	Glucose	0
Transformant	Glucose	0.45

In glucose media, the specific growth rates were comparable; 0.41/h for wild type and 0.45/h for transformant cells. In galactose media, wild type had a slightly lower specific growth rate (0.27/h) compared to the *tps1* mutant (0.32/h) and transformed cells (0.32/h).

CHAPTER 4

CONCLUSIONS

Trehalose-6-phosphate synthase 1 (*TPS1*) is a subunit of trehalose synthase complex in fungi. In this study, gene encoding TPS1 was isolated from *Rhizopus oryzae* (designated as *RoTPS1*). *R. oryzae* has been used for industrial production of L-lactic acid, lactic acid production is controlled by the glycolytic pathway and the trehalose metabolism affects the glycolytic pathway. Full-length *RoTPS1* cDNA was composed of 2505 bps encoding a protein of 834 amino acids with a molecular mass of 97.8 kDa.

Moreover *RoTPS1* was cloned into *Saccharomyces cerevisiae* and it was functionally identified by its complementation of the *tps1* yeast deletion mutants, which are unable to grow on glucose medium.

In glucose media, the maximum cell concentration of transformants did not exceed 0.26 gdw/l where wild type cells reached up to 2.00 gdw/l. However the specific growth rates and trehalose levels of transformant and wild type cells were in the same range.

It was also shown that the transformant cells were capable of producing trehalose, to the same extent as in the wild type cells in both glucose and galactose containing media.

It was suggested that the possible reasons of the low transformant cell concentration might be the overexpression of *RoTPS1* resulting in the accumulation of T6P which caused the organism to enter into the stationary phase early or *RoTPS1*, *TPS2* and regulatory subunits of trehalose synthase complex of *S. cerevisiae* not working properly altogether.

Overall, it was concluded that the transformant was capable of producing trehalose, to the same extent than the wild type cells in both glucose and galactose media.

The efforts will be useful for understanding the role of the gene and enzyme in *R. oryzae*. The expression system will provide sufficient amounts of recombinant TPS1 for future structural characterization of the protein and use for further investigation of *RoTPS1*'s function. With the expression system, *TPS1* function could be improved and the improvement would be helpful to promote the stress resistance of *R. oryzae*.

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APPENDIX A

COMPOSITION OF ANTHRONE SOLUTION

Components:
75% (v/v) H_2SO_4
Anthrone
96% (v/v) Ethanol

Preparation: 200 mg anthrone and 5 ml ethanol were dissolved in acid solution up to the 100 ml volume at 4°C and kept in cold during the experiment. Solution was prepared daily.

APPENDIX B

TREHALOSE STANDART CURVE

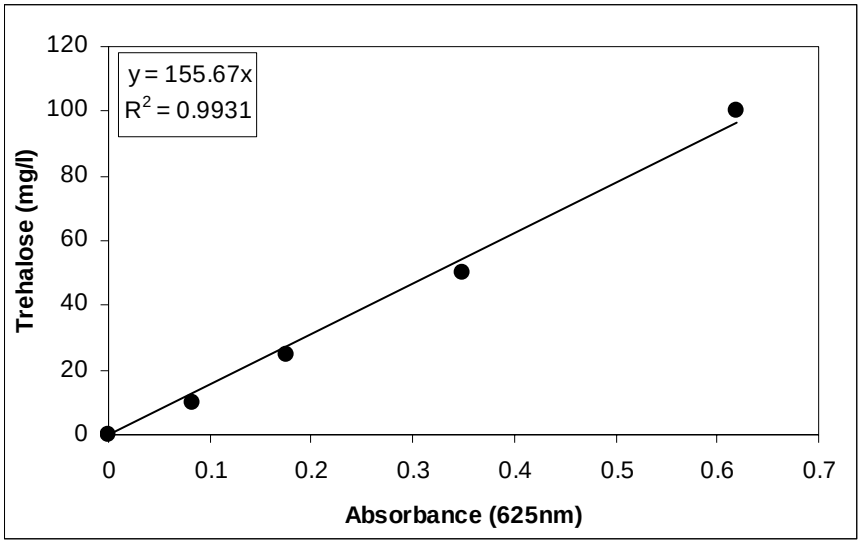

Figure B.1. Standard curve for trehalose.

APPENDIX C

DRY WEIGHT CALIBRATION CURVES

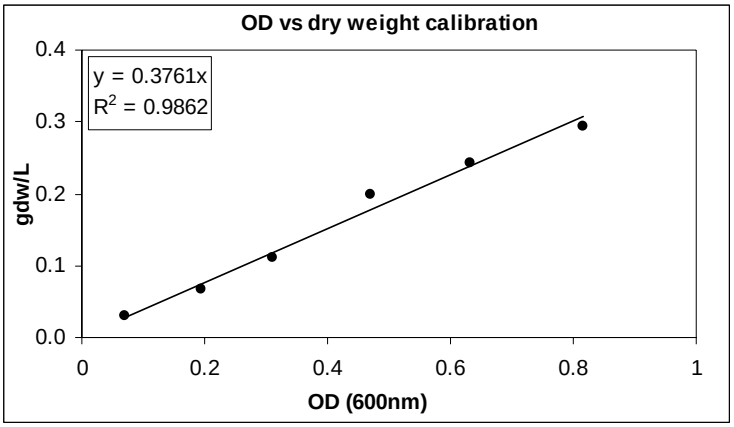

Figure C.1. Dry weight vs. OD600 Standart curve for wild type.

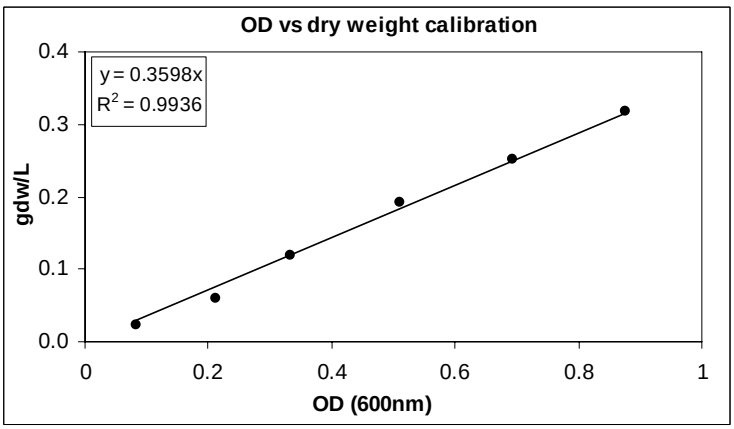

Figure C.2. Dry weight vs. OD600 Standart curve for *tps1* strain.

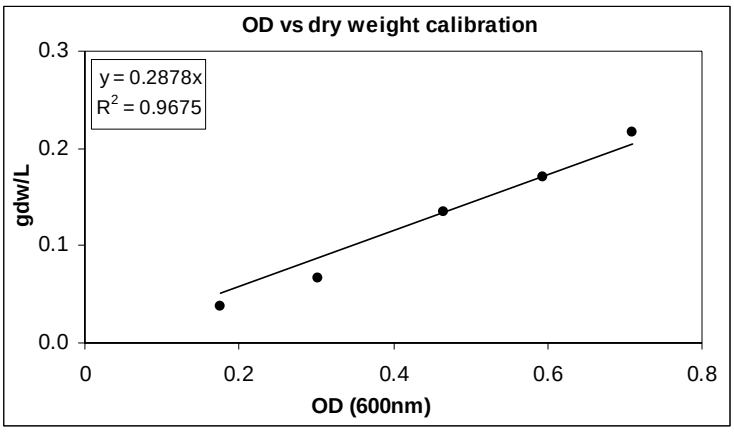

Figure C.3. Dry weight vs. OD600 Standart curve for transformant.

APPENDIX D

GROWTH MEDIA COMPOSITIONS

Growth media for *Rhizopus oryzae*

Chemicals	**Grams/100 ml**	**Final concentration**
Potassium phosphate (KH_2PO_4)	0.065	5 mM
Ammonium sulfate $(NH_4)_2SO_4$	0.2	15 mM
Magnesium sulfate ($MgSO_4.7H_2O$)	0.025	1 mM
Zinc sulfate ($ZnSO_4.7H_2O$)	0.005	0.2 µM
Glucose (anhydrous)	2	112 mM
Agar	2	2 % (w/v)

The above composition is used for solid media but it can also be used as liquid defined media by exclusion of agar and addition of variable concentration of glucose.

Growth media for *Escherichia coli* DH5α

Luria Bertani (LB) medium composition :

Chemicals	**g/L**
Yeast extract	5
Tryptone	10
NaCl	10
Agar	15

Adjust the pH of the medium to 7.0 by addition of 5 N NaOH (~0.2 ml).

Growth media for *Saccharomyces cerevisiae*

Selective media composition:

Chemicals	**Grams/100 ml**
Casamino acid	0.2
Ammonium sulfate $(NH_4)_2SO_4$	0.5
Yeast Nitrogen Base without amino acids	0.017
Tryptophane	0.008
Adenine	0.004
Uracil*	0.005
Carbon source (Glucose or Galactose)	2
Agar	2

* Uracil was added for mutant and wild type yeast cell cultures

APPENDIX E

PREPARATION OF COMPETENT CELL *E. coli* DH5α

Materials:

K-Mes transformation buffer:

in 100 ml:

6 ml 1 M $CaCl_2$

0.5 ml 1 M $MgCl_2$

2 ml 1 M K-Mes (pH 6 adjusted with KOH)

0.5 ml 1 M $MnCl_2$

Storage buffer:

K-Mes transformation buffer + 15 % glycerol

Protocol:

1 Inoculate DH5α on a LB plate and incubate overnight at 37 °C

2 Inoculate100 ml LB, 10 mM $MgSO_4$ (in 500 ml erlenmeyer flask) with 10 colonies from the plate and incubate until OD_{600} ~ 0.5

3 Cool the culture on ice and centrifuge 8 min at 3000 rpm using pre-cooled buckets Resuspent the cells in 40 ml cold K-Mes transformation buffer and put on ice for 30 min

4 Centrifuge 8 min at 3000 rpm

5 Resuspend the cells in 10 ml K-Mes transformation buffer, 15% glycerol

6 Aliquot 200 µl portions of the competent cells in 1.5 ml eppendorf tubes and freeze immediately in liquid nitrogen

7 Store at -70^{o}C.

APPENDIX F

PREPARATION AND STORAGE OF *E. coli* GLYCEROL STOCKS

1. Inoculate 5 ml LB medium containing the appropriate antibiotic with the relevant bacterial strain and grow overnight.
2. Label two vials for bacterial stock storage well and add 0.5 ml sterile 50% glycerol
3. Transfer 1 ml of the overnight culture to each vial and mix well
4. Immediately transfer the vials to the -70 freezer and update the collection database
5. Sterilize the materials used and the bacterial culture left

Using a strain from a glycerol stock:

1. To take a strain from the stock use wooden toothpicks
2. Take an inoculum from the stock and transfer to liquid LB containing the appropriate antibiotic and grow overnight.
3. Return the stock to the freezer as quickly as possible

APPENDIX G

HYPOTHETICAL PROTEINS OF *R. oryzae* TREHALOSE-6-P SYNTHASE GENE

TPS(1)--RO3G_14824.1 hypothetical protein (Transcript:RO3T_14823)

(2058 bp)

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TPS(2)--RO3G_14320.1 hypothetical protein (Transcript:RO3T_14319) (2403 bp)

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TPS(4)--RO3G_11196.1 hypothetical protein (Transcript:RO3T_11195) (2505 bp)

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TPS(5)--RO3G_07383: hypothetical protein

(3621 bp)

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TPS(6)--RO3G_09908: hypothetical protein

(2157 bp)

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TPS(7)--RO3G_13130: hypothetical protein
(2310 bp)

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TPS(8)--RO3G_03240: hypothetical protein

(2370 bp)

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TTCAAATTGCGCTCTCGACTTCTGAACAAAACGAATTGCGTGCTCATATCTCTGATGTGGTATCCAGGGTTAATTTCAAA
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ATATACCAAATGTAGATGCAGTTTTGTCTACTCTTGAACAATTATTCTAG

APPENDIX H

PLASMID MAPS

pGEM®-T Easy Vector sequence reference points:
T7 RNA polymerase transcription initiation site 1
multiple cloning region 10–128
SP6 RNA polymerase promoter (–17 to +3) 139–158
SP6 RNA polymerase transcription initiation site 141
pUC/M13 Reverse Sequencing Primer binding site 176–197
lacZ start codon 180
lac operator 200–216
β-lactamase coding region 1337–2197
phage f1 region 2380–2835
lac operon sequences 2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site 2949–2972
T7 RNA polymerase promoter (–17 to +3) 2999–3
Inserts can be sequenced using the SP6 Promoter Primer ,T7 Promoter Primer, pUC/M13 Forward Primer , or pUC/M13 Reverse Primer .
A single digest with *BstZI* , *EcoRI* or *NotI* will release inserts cloned into the pGEM®-T Easy Vector. Double digests can also be used to release inserts.

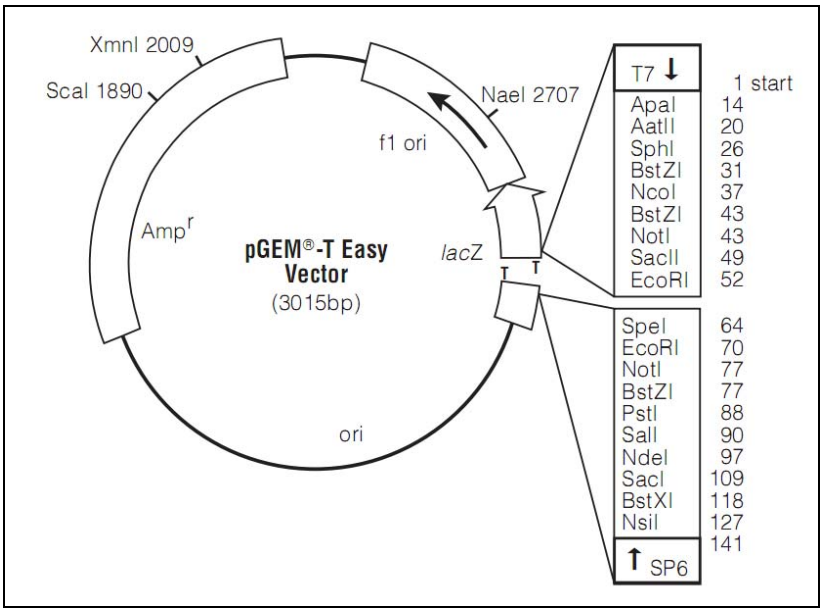

Figure H.1. pGEM®-T Easy Vector map

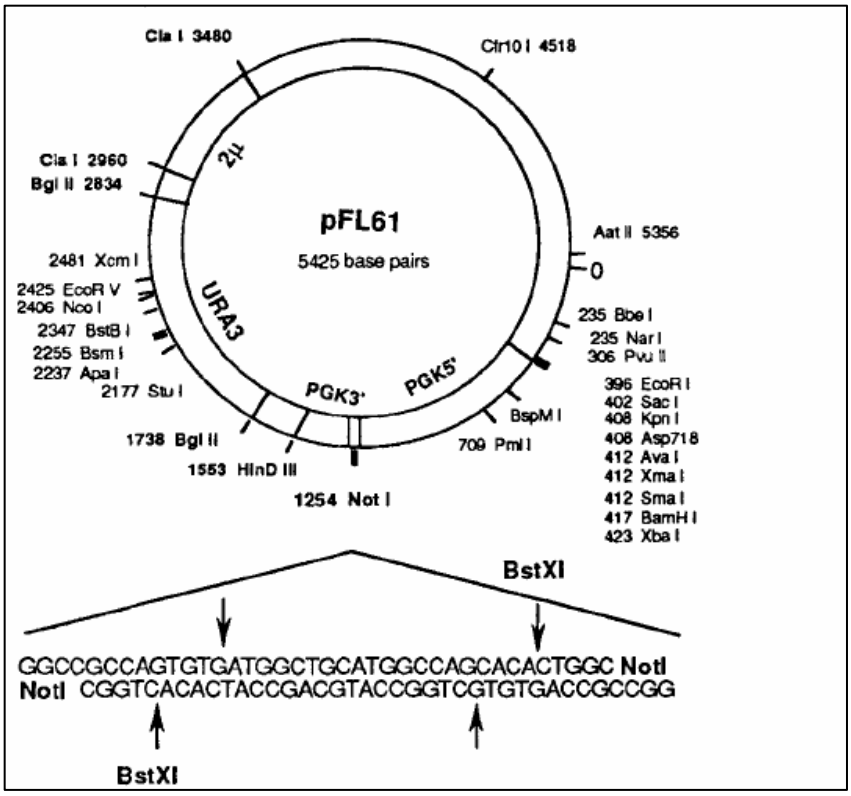

Figure H.2. pFL61 plasmid map

APPENDIX I

PREPARATION OF AMPICILLINE STOCK SOLUTION AND X-GAL

For 50 mg/ml stock solution of ampicilline mix 2.5g ampicilline with 50 ml distilled water. Final concentration of ampicilline in LB or LB agar should be 50 µg/ml.

For preparation of 2% X-gal (5-bromo-4-chloro-3-indolyl b-D-galactopyronoside), 200 mg of X-Gal was dissolved in 10 ml of dimethylformamide (DMF). They are stored at -20°C. For LB ampicilline plates, about 40µg/ml of X-Gal is necessary. Therefore 50 µl of X-Gal stock was spreaded to each LB ampicilline plate.

APPENDIX J

DNA MARKERS

GENEMARK DNA Marker

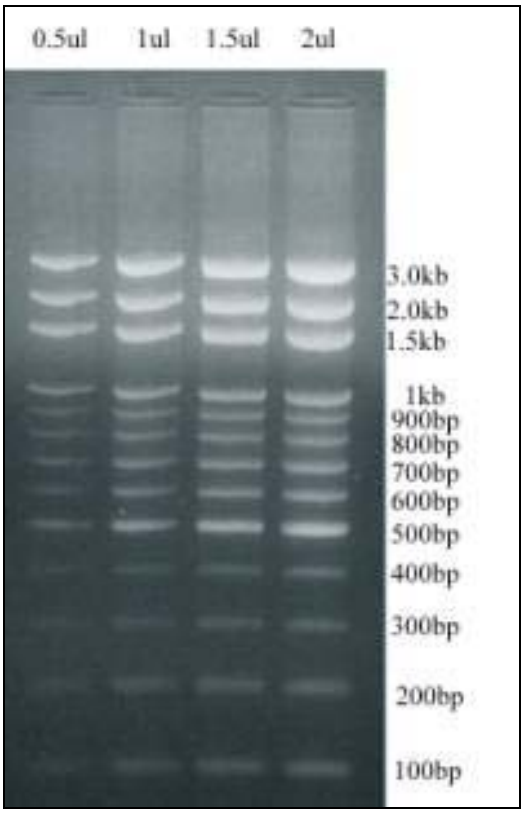

Figure J.1. 2% Agarose gel electrophoresis results of GENEMARK DNA Marker loaded at different amounts (Lanes 1: 0.5 µl, 2: 1 µl, 3: 1.5 µl, 4: 2 µl; from left to right) in 0.5X TAE buffer.

Gen100 DNA ladder is consisted of the following 13 DNA fragments:100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500. 2000, 3000 bp .The intensity of the 500, 1000, 1500, 2000, 3000 bp band has been increased to yield an internal reference indicator.

GeneRuler™ Express DNA Ladder (Fermentas)

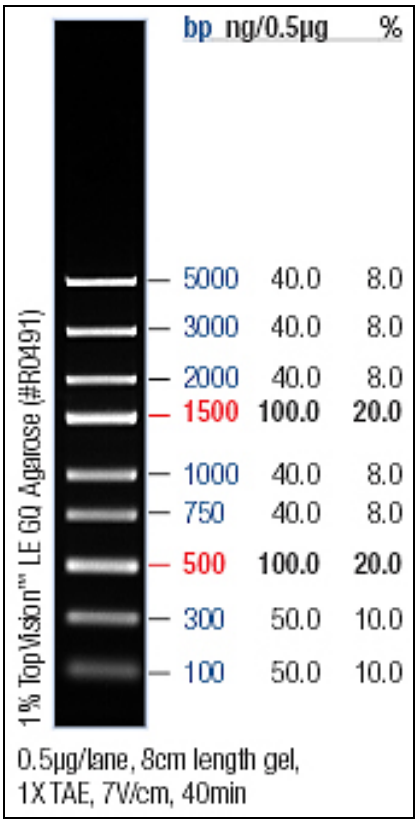

Figure J.2. 1% Agarose gel electrophoresis results of GeneRuler™ Express DNA Ladder in 1X TAE buffer.

Range

9 fragments (in bp): 5000, 3000, 2000, 1500, 1000, 750, 500, 300, 100.

CURRICULUM VITAE

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1997 July	Ankara Medicine Faculty Hepatology Institute	Intern Student

FOREIGN LANGUAGES

English