SCREENING OF THE PUTATIVE HEXOKINASE GENES FROM *RHIZOPUS ORYZAE*

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SCREENING OF THE PUTATIVE HEXOKINASE GENES

FROM *Rhizopus oryzae*

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

SCREENING OF THE PUTATIVE HEXOKINASE GENES FROM *Rhizopus oryzae*

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Rhizopus oryzae is a filamentous fungus which can ferment sugar to ethanol, and lactic acid. Increasing demand of the world for use of renewable carbon sources has put this and similar organisms into a position where the biotechnology industries are more and more interested with these organisms. *R. oryzae* can grow on some renewable carbon sources which makes it a good candidate for production of ethanol as well as lactic acid.

The major use of lactic acid is in food industry and food- related application, which in the U.S., accounts for approximately 85% of demand. The rest (~15%) of the uses are for non-food industrial applications. *Rhizopus oryzae* produces only L-(+)-lactic acid form. Lactic acid is also produced by lactic acid bacteria which have at least 95 % yield on the basis of glucose converted into lactic acid (mainly lactobacillus species) and these bacteria produce both L –(+)-lactic acid and D-(-)-lactic acid form . L-(+)-lactic acid is preferred form of lactic acid since D-(-)-lactic acid can not been metabolized by human beings. Another advantage over lactic acid bacteria fermentations is that *R. oryzae* can grow in minimal medium which lowers the costs as well as it makes the downstream processing of lactic acid easier.

However; the only disadvantage of *R. oryzae* for production of lactic acid is the low yield compared to the carbon consumed ~70 % (for Lactic acid bacteria this value is ~95 %). In order to increase the lactic acid yield of *R. oryzae;* understanding the mechanism of lactic acid production and other pathways e.g., glycolysis and ethanol production pathway and the regulation of the key enzymes of these pathways and increasing the flux through the lactic acid production branch is important.

Glycolytic pathway lies at the centre of the energy metabolism and the intermediates of glycolytic pathway are channeled to different pathways like lactate production, ethanol production in different organisms which are industrially important. Glycolytic pathway is composed of 10 basic reactions that go from glucose to pyruvate. Hexokinase catalyse the first and irreversible step in glycolytic pathway and it is implicated as one of the major control point in this pathway.

In this study; the putative hexokinase genes of *R. oryzae* were screened. The probable hexokinase genes were found from the genome database of *R. oryzae* by doing a blast search using the known protein sequences of closely related *S. cerevisiae* hexokinases. Seven of our ten probable hexokinase genes were obtained by using PCR. Sequencing results of three of them proved that these genes have no introns meaning they were expressed in *R. oryzae*. In the complementation study done in hexokinaless mutant of *S. cerevisiae DFY632* for these genes, the growth of the transformant was observed on glucose or fructose containing media as a carbon source. The hexokinase activities of *RoHXKs* transformants determined and the inhibition effect of trehalose-6-phosphate on in *RoHXKs* transformants were investigated.

Keywords: *Rhizopus oryzae*, hexokinase, *Saccharomyces cerevisiae*, L (+) lactic acid, cloning, transformation

ÖZ

Rhizopus oryzae'DE OLASI HEKZOKİNAZ GENLERİNİN ARAŞTIRILMASI

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Rhizopus oryzae, şekeri etanol ve laktik asite fermente edebilen filamentli bir mantardır. Dünyada yenilenebilir karbon kaynaklarının kullanım ihtiyacının artması biyoteknoloji endüstrisinde bu ve benzer organizmaları daha ilgi çeker pozisyona getirmiştir. *R. oryzae*'nin bazı yenilenebilir karbon kaynakları üzerinde büyüyebilmesi onu laktik asit üretimi kadar ethanol üretiminde de iyi bir aday haline getirmektedir.

Gıda ve farmakoloji endüstrisinde laktik asit ve tuzları yaygın olarak kullanılmaktadırlar. Ticari olarak laktik asit çoğunlukla L (+) ve D (-) optik izomerlerinin karışımını üreten laktik asit bakterileri tarafından üretilmektedir. L (+) laktik asit laktik asitin tercih edilen formudur çünkü D (-) laktik asit insanlar tarafından metabolize edilememektedir. Son zamanlarda, parçalanabilen laktik asit polimerlerinin üretimi için hammadde olarak kullanılmak üzere laktik asitin saf L (+) formunun üretimine büyük bir ilgi bulunmaktadır. Bu açıdan %100 L (+) formunda laktik asit üreten *Rhizopus oryzae* özellikle ilgi çekmektedir. *R. oryzae*'nin laktik asit bakterilerine göre diğer bir avantajı minimal besiyerinde büyüyebilmesidir ki bu da laktik asit üretiminin proses maliyetini düşürmesinin yanı sıra aşağı akım prosesini kolaylaştırmaktadır.

Ancak *R.oryzae*'nin laktik asit üretimindeki tek dezavantajı karbon üretimi esas alındığında düşük verimliliktir (yaklaşık %70) . Laktik asit bakterilerinde bu oran yaklaşık %95'leri bulmaktadır.

R. oryzae'de laktik asit üretimini artırabilmek için; glikolitik izyolu, ethanol ve laktik asit üretimi yan yollarının mekanizmasını ve bu izyollarında yer alan anahtar enzimleri ve bu enzimlerin düzenlenmesini anlamak ve laktik asit üretimi ayırım noktasına akışı artırabilmek önemlidir.

Glikolitik izyolu enerji metabolizmasının merkezinde yer almakta ve bu izyolunun birçok ara metabolitleri laktat üretimi, ethanol üretimi gibi farklı izyollarına gitmektedir. Glikolitik izyolu glikozdan piruvata giden 10 temel basamaktan oluşmaktadır. Hekzokinaz glikolitik izyolunun geri dönüşümsüz olan ilk basamağını katalizlemektedir ki bu basamak izyolunun temel kontrol noktalarından biridir.

Bu çalışmada; *R. oryzae*'nin olası hekzokinaz genleri araştırılmıştır. *Saccharomyces cerevisiae*'da yapılan çalışmaların ışığında, *R. oryzae*'nin genomik veritabanında yer alan veriler kullanılarak olası hekzokinaz genleri saptanmıştır. Tespit edilen on adet olası *R.oryzae*'nin hekzokinaz geninden yedi tanesinin PCR bantları elde edilmiştir. Sekans analizi sonuçlarına gore bu genlerden üç adedinin intron içermediği ve *R. oryzae*'de sentezlendiği tespit edilmiştir. Klonlanan genlerin ekspresyonu hekzokinaz mutantı olan *S. cerevisiae* DFY632'de gerçekleştirilmiştir. Hekzokinaz mutantı olan *S. cerevisiae* DFY632'de gerçekleştrilen tamamlama çalışmalarında transformantların karbon kaynağı olarak glikoz ve fruktoz ortamlarda büyüdüğü tespit edilmiştir. Transformantlarda hekzokinaz aktivitesi saptanmış ve hekzokinaz üzerinde trehaloz-6-fosfatın inhibisyon aktivitesi araştırılmıştır.

Anahtar kelimeler: *Rhizopus oryzae*, hekzokinaz, *Saccharomyces cerevisiae*, L (+)-laktik asit, klonlama, transformasyon

To my family...

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amp	ampicilline
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
glk	glucokinase gene
GLK	glucokinase
hxk	hexokinase gene
HXK	hexokinase
PCR	polymerase chain reaction
PMSF	phenyl methyl sulfonyl flouride
R. oryzae	*Rhizopus oryzae*
S. cerevisiae	*Saccharomyces cerevisiae*
TAE	Tris acetate EDTA buffer
TE	Tris HCl EDTA buffer

CHAPTER 1

INTRODUCTION

Rhizopus oryzae, a filamentous fungus, can ferment sugar to ethanol, and lactic acid. A great demand for renewable carbon sources has put such organisms in a position that the industrial biotechnology firms are more interested with these organisms.

In lactic acid production; *R. oryzae* fermentation has important advantages over other fermentations on producing solely the L- (+) form of this acid. This form is the preferred form since it could be metabolised in the human body and growing in a chemically defined minimal medium which lowers the costs as well as it makes the downstream processing of the lactic acid easier. However, the production yield in *R. oryzae* is lower than that of other lactic acid producers.

Glycolytic pathway lies at the centre of the energy metabolism. The intermediates of glycolytic pathway are chanelled to different pathways like lactate production, ethanol production in different organisms which are industrially important. Glycolytic pathway is composed of ten basic reactions that go from glucose to pyruvate.

The main objectives of many biotechnological projects on the industrial organisms are understanding the physiology and revealing the regulatory metabolisms. Generally finding a way to increase the yield of an industrial product is the main aim of these studies. In this respect glycolysis is one of the main targets being the central metabolic pathway which lies at the heart of many other pathways leading to various products.

The carbon source utilization through glycolytic pathway can end with lactic acid or ethanol production. Therefore for increasing the yield of the desired end product, glycolytic pathway is the focus of our search. Hexokinase catalyses the first step of glycolytic pathway. As this step is also irreversible it is assumed as one of the major control points in this pathway.

In this study; the putative hexokinase genes of *R. oryzae* were screened. The data from genome database of *R. oryzae* was used to clone hexokinase genes of this organism. *Saccharomyces cerevisiae* was used as an expression host for the cloned genes from *R. oryzae.*

1.1. *Rhizopus oryzae*

Rhizopus oryzae belongs to the Kingtom Fungi, Division Eumycota, Subdivision Zygomycotina, Class Zygomycetes, Order Mucorales (http://www.ncbi.nlm.nih.gov). The principal characteristic that distinguishes class Zygomycetes is the production of zygospore which is a thick walled resting spore. Following the fusion of two gametangia zygosporangium is formed in which zygospore is developed. Another typical characteristic is the presence of a coenocytic mycelium (mycelium that lacks regular septation) which is a characteristic of the lower fungi. Asexual reproduction is usually by sporangiospores, growth starts by germination of the spore and followed by spread and penetration of the vegetative hyphae to the subsratum.

The synonyms of this fungus are: *R. tritici, R. thermosus, R. tamarii, R. suinus, R. peka, R. hangchow, R. formosaensis, R. formasaensis var.chylamydosporus, R. delemar, R. chiuniang, R. arrhizus, R. liquefaciens, R. javanicus Y. takeda, R. pseudochinensis* (http://www.ncbi.nlm.nih.gov).

1.1.1. *Rhizopus oryzae* in biotechnology

The application areas of *Rhizopus* in biotechnology include enzymes industry (e.g. glucoseamylase, lipase), organic acids (e.g. lactate, fumarate) and fermented food production (Skory, 2002).

Rhizopus oryzae was first described in 1936 as being able to convert glucose to great amounts of L form of the lactic acid (Lockwood et. al., 1936)

Lactic acid is the most abundant hydroxycarboxylic acid in nature. It is present in many foods, naturally and as a microbial fermentation product. Lactic acid is assumed as GRAS (generally recognized as safe) as a food additive by the Food and Drug Administration. However, D form of lactic acid can result in acidosis and decalcification thus it is harmful to human metabolism (Datta et al., 1995).

Although lactic acid is mainly used in food industry as a taste enhancer or preservative as an antimicrobial agent, recently it has been used by environmental interest like production of the nonchlorinated solvent ethyl lactate and the biodegradable plastic polylactic acid (Raber, 1998).

Lactic acid can be produced by fermentation or chemical synthesis but fermentation has received considerable interest recently, due to health and ecological concerns and the limited nature of petrochemical feedstocks. D and L forms are the two isomeric forms of the lactic acid. A mixture of D and L isomers of lactic acid is produced by the chemical synthesis.

Lactic acid is also produced by lactic acid bacteria which give at least 95 % yield on the basis of glucose converted into lactic acid (mainly lactobacillus species) and these bacteria produce both isomeric forms.

However; *R. oryzae* fermentations are preferred over these fermentations since it produces solely the L form of the lactic acid. This form is the preferred form of lactic acid since it could be metabolised in human body. Another advantage over lactic acid bacteria fermentations is that *R. oryzae* can grow in a chemically defined minimal medium which lowers the costs as well as it makes the downstream processing of the lactic acid easier. One disadvantage of *Rhizopus oryzae* for lactic acid synthesis is that productivity rates and yields are not as high as those obtained for Lactobacillus (Skory, 2003). Many studies, including cloning of the lactate dehydrogenase and pyruvate dehydrogenase genes, have been done for understanding the fermentative pathways better (Skory, 2000, 2003).

1.2. *Saccharomyces cerevisiae*

Yeasts are ascomycetous or basidiomycetous fungi which form sexual states that are not enclosed in a fruiting body. The subdivisions are based on aspects of yeasts' sexuality (Ascomycotina or Basidiomycotina) or lack of it (Deuteromycotina) and the lower taxonomic categories are based on various morphological, physiological and genetic characteristics (Walker, 1998).

The yeast *Saccharomyces cerevisiae* has played an important role in the evolution of biological sciences; in addition to its use in the production of leavening of dough and alcoholic beverages. *Saccharomyces cerevisiae* has become one of the model organisms for molecular genetics and cell biology with the release of the first complete nuclear genome sequence of a eukaryote (Dujon, 1996; Hieter et al., 1996; Johnston, 1996).

S. cerevisiae belongs to the Subdivision *Ascomycotina*, Family *Saccharomycetaceae*, Subfamily *Saccharomycetoideae*, Genera *Saccharomyces*, Species *cerevisiae* (Walker, 1998).

1.2.1. *Saccharomyces cerevisiae* in biotechnology

Yeasts are of major economic, social and health significance in human culture. In the nineteenth century, yeasts were first recognized as living organisms (fungi) responsible for fermenting sugar to ethanol in enology and in brewing. The brewing of beer probably represented the world's first biotechnology (Walker, 1998).

S.cerevisiae has been extensively studied in several areas of fundamental biological science and it serves as a valuable model for eukaryotic cell in such studies. Studies with yeast in following areas have contributed significantly to the advancement of biological knowledge; fermentation industry (e.g. brewing, bioethanol, fermentation products); food and chemical industries (e.g. savoury flavours, enzymes, baking, pigments, food acidulants, chemical reductions) health-care industries (pharmaceuticals, vaccines, probiotics, hormones, blood factors); biomedial research (e.g. cancer, AIDS, drug metabolism, genotoxicity screens, human genetic disorders); environmental technologies (e.g. bioremediation, waste utilization, crop protection, biosorption of metals) and fundemental biotechnological research (e.g. cell biology, genetics, biochemistry, molecular biology) (Walker, 1998).

Andre Goffeau and his co-workers started to Yeast Genome Project under the scientific programmes of European Commission in 1980s.

S. cerevisiae has also been a very important genetic model organism. Yeasts are the favorite hosts for genetic studies because of its various characteristics, like ability to grow on different medium, easy manipulation, non pathogenicity, and the absence of production of pyrogenic and allergenic substances (Walker, 1998).

1.3 Glycolysis

The sequence of enzyme-catalyzed reactions that oxidatively convert glucose to pyruvic acid is known as glycolysis. Glycolysis is the central pathway which is used to metabolise all the sugars aerobically and anaerobically. Pyruvate that is the end product of glycolysis is chanelled to different pathways like lactate production, ethanol production in different organisms which are industrially important.

Glycolysis converts glucose into pyruvate to synthesize ATP from ADP and Pi. The ten enzyme catalyzed reactions of glycolysis and its branch points are diagrammed in Figure 1.1 and Figure 1.2.

ATP is used in the early stages of glycolysis to synthesize phosphoryl compounds and is resynthesized in the later steps of the pathway. Thus, glycolysis may be considered to occur in two stages:

The first stage, (from 1st to 5th reaction), is a preparatory stage in which the hexose glucose is phosphorylated and converted to two molecules of the triose glyceraldehyde-3-phosphate. These reactions utilize two ATPs in a kind of energy invesment.

In the next stage, (from 6th to 10th); pyruvate is generated from the two molecules of glyceraldehyde-3-phosphate, with production of four ATPs

Thus, by glycolysis, net two ATPs per glucose are gained. Stage I consumes two ATPs; Stage II produces four ATPs. NAD^+ is the primary oxidizing agent of glycolytic pathway. For the constitutive flux of NAD^+ in the pathway, the NADH produced by this process must be continuosly reoxidized. (Voet&Voet, 2004).

Glycolytic pathway is an almost universal central pathway of glucose catabolism and it is found almost exclusively in all of the organisms. Since it is at the heart of complex metabolism, extensive studies are being done to better understand the action and regulation of this pathway.

Glycolysis is the widely studied pathway in plants, fungi, yeast and other eukaryotes. Understanding of regulation of this pathway is essential in the trying to partially control the metabolism and to increase the yield of the processes from biotechnological point of view. Biochemical characterisation of the pathway enzymes and expression analysis of the genes

coding for these enzymes are important to supply the data in order to be able to do metabolic engineering on the glycolytic pathway.

Hexokinase, pyruvate kinase and phosphofructokinase catalyse the reactions which are the the irreversible key regulatory points in glycolysis. Allosteric effectors or covalent modifications regulate the activities of these kinases. Additionally, transcription regulation affects the yield of these enzymes to fulfill changes in metabolic demands (Berg, 2002).

Overexpression studies of glycolytic enzymes displayed that there are strong controls on them by other factors (Ruijter, et. al.,1997; Schaaff et al.. 1989). For example increased trehalose-6-phosphate levels inhibits the hexokinase activity; there is strong regulation in phophofructokinase reaction by a nonglycolytic intermediate fructose-2,6-bisphosphate (Ruijter et. al. 1997).

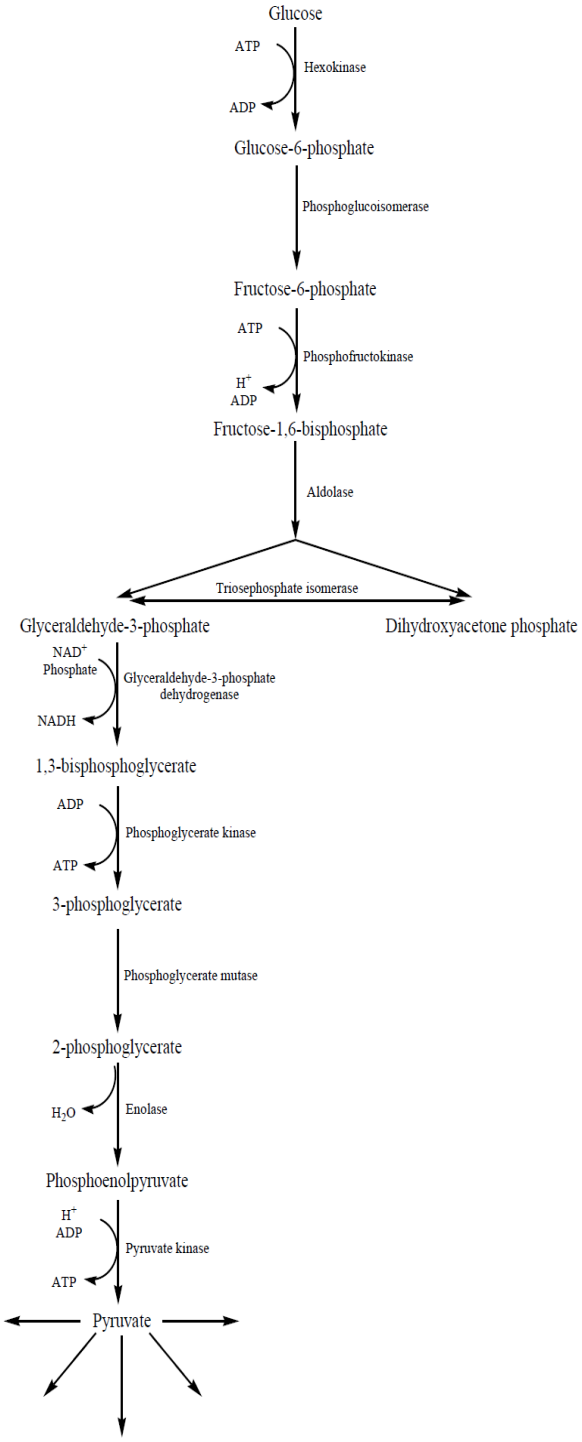

Figure 1.1 Scheme of the glycolytic pathway (Büyükkileci, 2007)

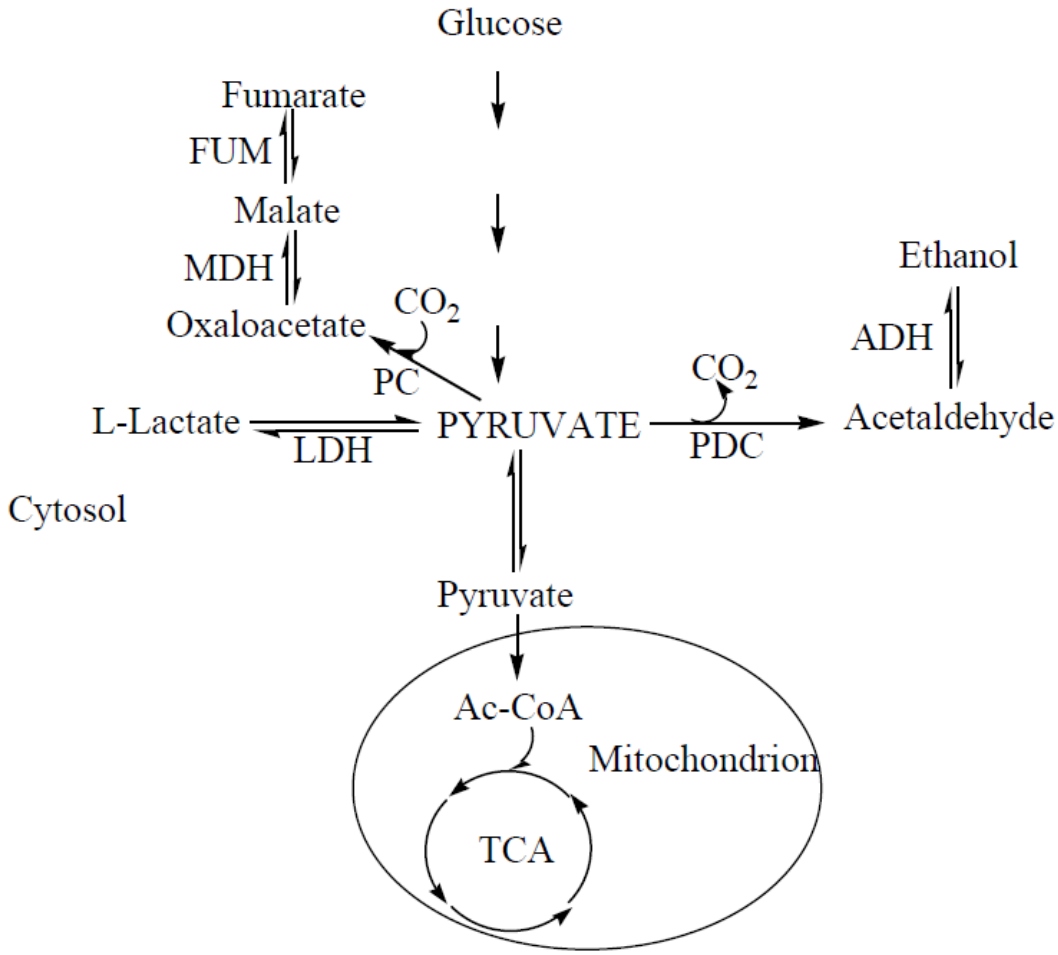

Figure 1.2 Pyruvate branch point of *Rhizopus oryzae* (Büyükkileci, 2007)

1.4. Hexokinase

1.4.1. Importance and role of hexokinase

Hexokinases play an important role in the sugar metabolism of living organism. Besides their catalytic activity, they have also important regulatory roles in glucose signalling, glucose repression, sugar uptake and regulation of some genes.

For the survival of organisms, adaptation to changes in the environment is important. The ability to grow in different conditions needs capacity to sense and response to changes in the environment. It was shown that hexokinases participate in glucose signalling in yeast, plants and mammals (De Winte et al. 1996; Efrat et al. 1994; German 1993; Jang&Shen 1994; Moore et al., 2003;). Also they have a role in the regulation of the transcription of many genes (Gancedo, 1998; Goffrini et.al., 1995, Ma et.al., 1989; Prior et.al.; 1993; Rose et.al.1991). It was found that hexokinases are important in the regulation of some genes in the yeast *S. cerevisiae* and *Kluyveromyces lactis* (Gancedo, 1998; Goffrini et.al., 1995; Maet. al., 1989; Prior et.al., 1993; Rose et. al., 1991).

In *Saccharomyces cerevisiae* hexokinases interact with sugar transporters and influence their kinetic characteristics (Bisson&Fraenkel, 1983) and hexokinase II participates in the transcriptional control of some genes repressed by glucose (Gancedo, 1992; Entian&Barnett, 1992).

In yeast, glucose is transported into cells via facilitated diffusion. It is mediated by proteins encoded by several *HXT* genes. For the transport of glucose in *S. cerevisiae*, two kinetically distinct transport systems have been reported. One of them is the low affitinity system (K_m=20 mM) and the other is the high affitinity transport system (K_m=1 mM) for glucose (Bisson et.al.1983; van Urk et.al.1989). Although high affinity system is repressed at high extracellular glucose concentrations; the low affinity transport system is constitutive (Bissonet. al., 1984). In addition, high affinity system requires presence of hexokinase in the cell (Bisson et. al., 1983). Fructose is also a subsrate for low affitinity (K_m=40 mM) and high affitinity (K_m=6 mM) transport systems. The *HXT* genes are regulated at the levels of expression and inactivation in response to growth conditions. It was reported that for the generation of the induction signal for expression of the *HXT* genes encoding for hexose transporters *HXK2* activity is required. (Ozcan et. al.; 1995).

Glucose repression (also known as carbon catabolite repression), which is the repression of transcription of a large majority of genes in the presence of glucose in the medium, adapts yeast cells for the fermentation of glucose, the preferred carbon source (Carlson,1998; Gancedo, 1998). Transcription of *SUC2*, *GAL* genes, *MAL* genes, *HXK1*, and genes encoding enzymes of the glyoxylate shunt, the tricarboxylic acid (TCA) cycle, gluconeogenesis; genes involved in respiration and other mitochondrial activities are all repressed in the presence of glucose. (Schuurmans et al., 2007). It was found that mutations in the *HXK2* gene blocked glucose repression of certain genes (Moreno& Herrero, 2002).

Studies indicated that, in *S. cerevisiae*, hexokinase II is a major factor in glucose repression (Entian, 1980;1997; Moreno & Herrero 2002). It was found that overexpression of hexokinase I but not glucokinase substitutes for isoenzyme II in glucose repression (Rose et al., 1991); and both hexokinases contribute to fructose repression (De Winde et al.,1996; Hohmann et al., 1999).

In *S. cerevisiae;* it was shown that, hexokinase II triggers glucose repression by leading to the binding of Mig1 repressor protein to several promoters (Gancedo, 1998; Ronne, 1995). In the presence of glucose, Hxk2 interacts with carbon catabolite repressor Mig1 and located into the nucleus. In the nucleus the Hxk2-Mig1complex seems to form a repressor complex and binds to the promoter of carbon catabolite repressible genes (Ahuatzi et. al., 2004, 2007)

A role of hexokinase in glucose repression was also reported for other yeasts, such as *Schwanniomyces occidentalis* (Rose,1995) and *Pachysolen tannophilus* (Wedlock&Thornton 1989), *Hansenula polymorpha* (Karp et al., 2004) It has been shown that *H. polymorpha* has two hexose kinases: a glucose-specific glucokinase and a hexokinase that phosphorylates both glucose and fructose and both enzymes can mediate glucose repression in *H.polymorpha* (Kramarenko et.al., 2000; Laht t al., 2002).

Among yeasts; the ability of a glucokinase to signal for glucose repression is exceptional. However, according to a paper by Flipphi et al., both hexokinase and glucokinase exhibit glucose-signaling ability in the filamentous fungus *Aspergillus nidulans* (Flipphi et. al, 2003).

1.4.2. Structure and physical properties of hexokinase

Hexose phosphorylation is the first step of glycolytic pathway. This first reaction, ATP-dependent phosphorylation of hexoses, is catalyzed by hexokinases (EC. 2.7.1.1). Although glucose is the most common subsrate; the phosphorylation of D-fructose, D-mannose and some other hexoses is catalyzed by hexokinase. Mg^{+2}-ATP complex is the second substrate for hexokinase, as for other kinases and an uncomplexed ATP is a potential competitive inhibitor for these enzymes. (Voet&Voet,2004).

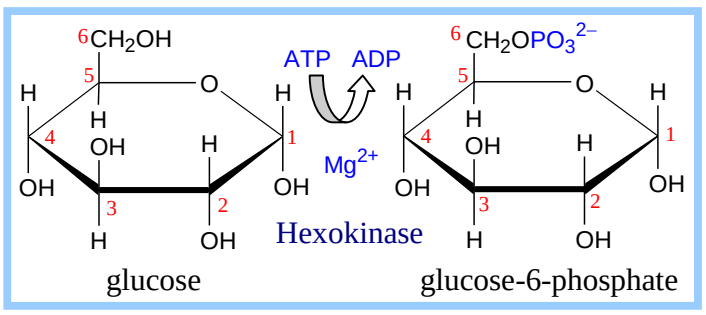

Figure 1.3 The hexokinase reaction (Voet&Voet, 2004)

Hexokinase has a random Bi Bi mechanism and it forms a ternary complex by glucose and Mg^{+2}-ATP before the occurance of the reaction. The Mg^{+2} ion, is thought to shield their negative charges, by complexing with the phosphate oxygen atoms, making the phosphorus atom more accessible for the nucleophilic attack of the C6-OH group of glucose (Voet & Voet,2004).

As known, the yeast hexokinases are exist as phosphoproteins in vitro (Fernandez et.al.,1986) and in vivo (Voet&Voet,2004); with the equilibrium of dimeric and monomeric form affected by phosphorylation. The in vivo phosphorylation site has been identified as Ser^{15} (Voet&Voet,2004).

There are many purification, cloning and characterization studies have been done for hexokinases and glukokinases of many organisms (Easterby&Rosemeyer,1972; Laht, 2002;Maitra,1970, ; Panneman et.al., 1996;1998; Rose ,1995; Ruiz-Amil & Sols, 1961; Steinböck et.al. ,1994). From the cloning studies of many eukaryotic hexokinase and glucokinase genes so far it was seen that , the kinetic and regulatory properties of the these enzymes vary considerably although they appear to have arisen from a common ancestor (Panneman et al.,1998)

Three isoenzymes with different subsrate specificities have been identified in the yeast *S. cerevisiae*:, hexokinase I , hexokinase II and glucokinase. These isozymes have different affinities for glucose and ATP and different specificities toward other sugars, such as fructose and mannose (Gancedo,1998;Lobo& Mailra,1977a). Hexokinase PI (also referred as hexokinase A) and hexokinase PII (also referred as hexokinase B) can both phosphorylate glucose, fructose and mannose as well, whereas glucokinase only accepts glucose and mannose as subsrates (Zimmermann&Entian,1997). Hexokinase II is the predominant hexokinase for *S. cerevisiae* growing on glucose and it is required for the catabolite repression, by glucose, of the expression of other genes (De Winde et.al.,1996; Entian,1997; Gancedo, 1998., Voet &Voet, 2004).

From many fungi, hexokinase and glucokinase purification and characterization have been done including those from *S. cerevisiae* (Herrero et.al.,1995&Maitra, P K. 1970;), *Aspergillus niger* (Panneman.et.al., 1996;1998) and *Hansenula polymorpha* (Helen Karp et.al., 2004). *Aspergillus nidulans, Hypocrea jecorina* (Flipphi et. al.,2003; Hartl&Seiboth, 2005).

Hexokinase catalyses the first and irreversible step of glycolysis (Figure 1.4). Phosphorylation of hexoses has been assumed as an important control point in glycolysis.

1.4.3. Regulation of hexokinase

The regulation of the hexose-phosphorylating enzymes varies among the species. For *S. cerevisia*, many allosteric regulators, of which ADP, ATP, trehalose-6-phosphate, have been described. (Zimmermann et.al., 1997). Negative cooperativity at low ATP concentration and activation by citrate, orthophosphate, malate and 3-phosphoglycerate was reported for yeast

hexokinase II (Kosow&Rose 1970, 1971). In *S. cerevisiae,* high physiological concentrations of ATP inhibit hexokinases I and II (Kopetzki&Entian, 1985).

Although the mammalian hexokinase IV and the yeast hexokinases I, hexokinase II and glucokinase are not inhibited; the mammalian hexokinases I-III are inhibited allosterically by glucose-6-phosphate (Colowick,1973; Gancedo,1998; Maitra,1970; Lobo.& Maitra, 1977a,b;Maitra and Lobo 1983*;* cited in Panneman et.al., 1996).

Trehalose-6 phosphate inhibits the *S. cerevisiae* hexokinase II (Blazquez.et al. 1993) and the inhibition of hexokinases by trehalose-6-phosphate appears to play an important role in the control of the glycolytic flux in *S. cerevisiae (*Blazquez et al. 1993; Thevelein.& Hohinann, 1995). Similar to yeast, trehalose-6-phosphate inhibition of hexokinase has also been revealed in *A. niger* (Arisan Atac et al., 1996 & Panneman et al.,1998)

The transcriptional regulations of the genes which encode hexose-phosphorylating enzymes are changes depending on the source and the amount of carbon (Herrero et al., 1995). It was displayed that the expression of hexokinase II is very high compared with that of hexokinase 1 or glucokinase, in glucose-grown *S. cerevisiae*, (Herrero, P. et al., 1995). It is indicated that in *S. cerevisiae*, mainly hexokinase II is crucial for glucose phosphorylation in vivo.

1.4.4. Studies carried out in METU Biotechnology Laboratory on *Rhizopus oryzae*

R. oryzae can grow on some renewable carbon sources which makes it a good candidate for production of ethanol as well as lactic acid.

Efficient production of organic acids, like lactic acid, using hexoses as a carbon source requires a high flux through glycolysis. Hexose phosphorylation has been assumed as one of the important control points in glycolytic pathway.

In our laboratuary; there are many studies have been done abut the investigation of the regulation of glycolytic pathway of *R. oryzae*

Some of these studies are about the cloning, purification and characterization of key enzymes in glycolytic pathway of *R. oryzae*. "Isolation and Preliminary Characterization of Hexokinase from *Rhizopus oryzae" (*Açar; 1999); "Partial Purification and Kinetic Characterization of L-lactate Dehydrogenase from *Rhizopus oryzae"* (Arslan; 2001); "Biochemical and Genetic Studies on The Pyruvate Branch Point Enzymes of *Rhizopus oryzae"* (Açar; 2004); "Expression of lactate dehydrogenase genes of Rhizopus oryzae in *Aspergillus niger"* (Acar et al; 2005); "Lactate and ethanol productions by *Rhizopus oryzae* ATCC 9363 and activities of related pyruvate branch point enzymes" (Büyükkileci et al.; 2006); "Purification and Characterisation of Two Isozymes of Pyruvate Decarboxylase from *Rhizopus oryzae" (*Açar et al.; 2007); "Purification And Kinetic Characterization of Hexokinase From *Rhizopus oryzae*." (Dedeoğlu; 2007); "Cloning and Characterization of Trehalose-6-Phosphate Syntase Gene from *Rhizopus oryzae"* (Özer Uyar; 2009) are the studies done about the enzymes in this pathway.

The other studies; "Effect of Different Stress Conditions on Trehalose Accumulation and Degradation in *Rhizopus oryzae"* (Özer Uyar ; 2002); "Investigation of Sugar Metabolism in *Rhizopus oryzae"* (Büyükkileci AO; 2007) ; "Effect of Different Stresses on Trehalose Levels in *Rhizopus oryzae"*(Özer Uyar et al.2010) are about the understanding of regulation of glycolytic pathway in *R. oryzae*.

1.5. Leloir's galactose pathway

Galactose is a monosaccharide sugar that is a C-4 epimer of glucose (Figure 1.3). The enzymes of glycolysis are unable to recognize the galactose configuration. The entire pathway converting galactose to glycolytic intemediate is known as Leloir's Pathway. It was elucidated by Luis Leloir.

The pathway involves four reactions (Figure 1.4): In the first reaction galactose is converted to galactose-1-phosphate by galactokinase. Then galactose-1-phosphate uridiylyltransferase transfers UDP-glucose's uridiylyl group to galactose-1 phosphate to yield glucose-1 phosphate and UDP-galactose. UDP galactose is converted to UDP glucose by UDP-galactose-4-epimerase. In the last reaction, Glucose-1-phosphate is converted to Glucose-6-phosphate by Phosphoglucomutase.

It was found that in *S. cerevisae* there are two phosphoglucomutase isoforms, Pgm1p and Pgm2p and about 80% of the total activity is provided by Pgm2p (Tsoi and Douglas, 1964).

The control of the gene expressions of this pathway was one of the first eukaryotic gene systems to be studied in detail (Timson, D.J. ; 2007) The genes *GAL1* (encoding galactokinase), *GAL2* (encoding galactose permease), *GAL7* (galactose-1-phosphate uridylyltransferase) and *GAL10* (uridine-diphosphoglucose 4-epimerase) all belong to the *GAL* regulon that is subject to tight transcriptional regulation.

In the presence of glucose, trancription of the *GAL* genes are repressed by the non-phosphorylated form of Mig1 (Johnston et al., 1994).

Only in the presence of galactose and the absence of glucose the rapid and high level activation is occured by the concerted actions of Gal4p, Gal80p, and Gal3p (Timson, 2007).

The interaction of galactose and ATP with Gal3, which then forms a complex with the negative regulator Gal80, induce the *GAL* genes (Platt & Reece, 1998).

This releases the positive transcriptional regulator Gal4 from Gal80 control and allows it to activate transcription of the *GAL1*, *GAL2*, *GAL7* and *GAL10* genes (Leuther & Johnston, 1992; Wu et al., 1996).

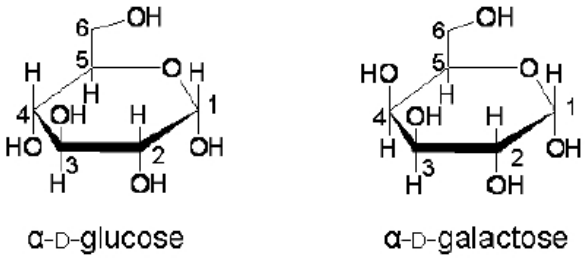

Figure 1.4 The pyranose ring stuructures of D-glucose and D-galactose (Timson, D.J. ;2007)

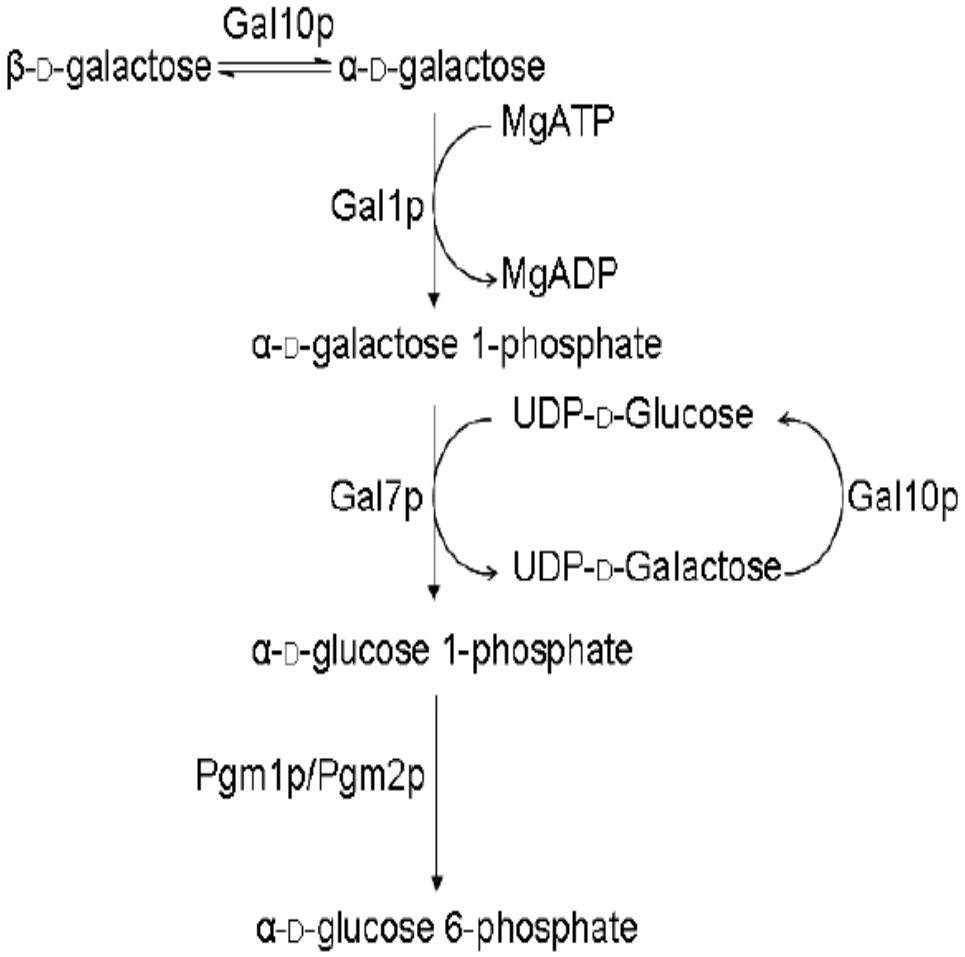

Figure 1.5 The Leloir pathway of galactose metabolism (Timson, D.J. ; 2007)

1.6. Aim of the study

The main objective of this study was to clone the putative hexokinase genes of *R. oryzae* and expression of them in *S. cerevisae*. In the light of studies done for *S. cerevisiae*, data from genome database of *R. oryzae* was used to clone hexokinase genes of this organism. Complementation studies were done with triple hexokinase mutant of *S. cerevisiae* DFY632 to identify the genes.

CHAPTER 2

MATERIALS AND METHODS

1. Materials

2.1.1. The strains

Rhizopus oryzae ATCC 9363 was purchased from American Type Culture Collection. *Eshericia coli* DH5α was used as competent strain for propagation of constructed plasmids. Hexokinaseless mutant of *S. cerevisiae* DFY632 was kindly provided by Prof. Dan G. Fraenkel and *S. cerevisiae* LBY was kindly provided by Prof. Dr.Linda Bisson. *Saccharomyces cerevisiae* CEN.PK 113.7D (*MATa, MAL2-8c, SUC2*) was obtained from Peter Kötter.

2.1.2. The plasmids

pGEM-T Easy vector Sytem (Promega) was used for the propagation of the gene fragments to be sequenced. The plasmid pFL-61 (Promochem ATCC number:77215) was used as the expression vector in *S. cerevisiae* transformations. The plasmids maps are given below:

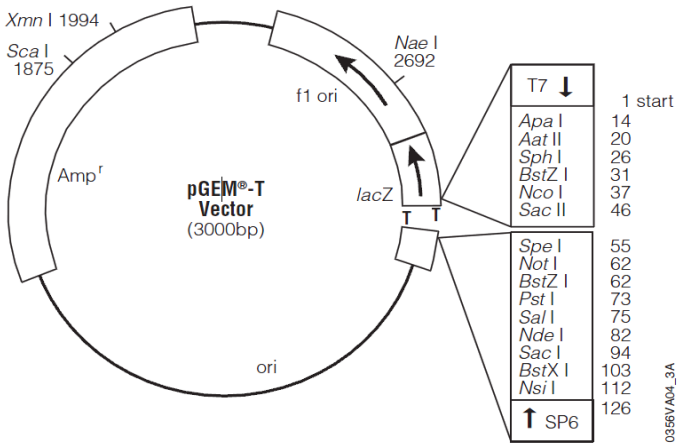

Figure 2.1 pGEM-T Easy Vector Map

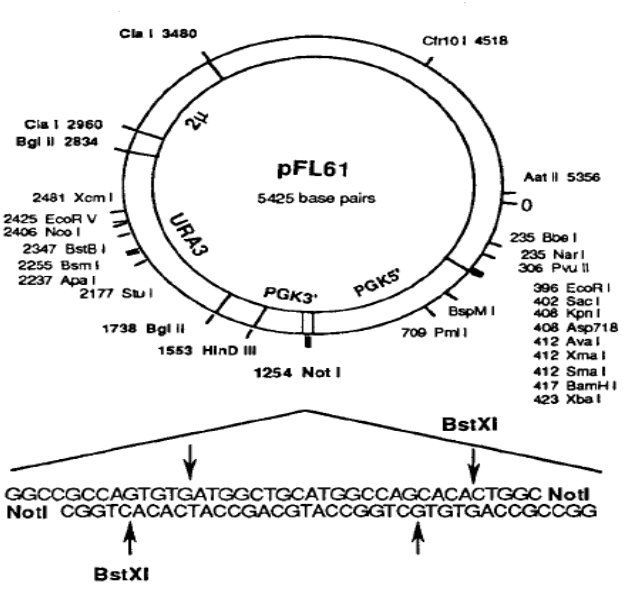

Figure 2.2 pFL61 Plasmid Map

2.1.3. The primers

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

2.1.4. The growth media

For the growth of *E.coli* DH5α the LB medium (Appendix A) was used. For *R. oryzae* ATCC 9363, *S. cerevisiae* DFY632, *Saccharomyces cerevisiae* CEN.PK 113.7D the growth media are given in Appendix B and C.

2.1.5. The chemicals

The chemicals used in all experiments were commercially avaliable from Sigma, Aldrich, Merck, İnvitrogen, Roche and Fluka.

2.2 Methods

2.2.1. Growth of organisms

2.2.1.1. Growth of *R. oryzae*

R. oryzae was sporulated on agar plates for 4-5 days at 30 ^{o}C. Spore concentration in the suspension was determined by counting the spores on hemocytometer for each inoculation. For liquid culture, *R. oryzae* was inoculated in the liquid medium 1/5 volume of flask and incubated in shaker incubator at 175 rpm and 35 ^{o}C.

2.2.1.2. Growth of *S. cerevisiae*

S. cerevisiae was grown on streaked agar plates for 4-5 days at 30 ^{o}C. After colony formation, the plates were stored at 4 ^{o}C. For each liquid preculture inoculation, a single colony was inoculated in the liquid medium1/5 volume of flask. Incubation was carried out at 30 ^{o}C and 160 rpm in shaker incubator.

2.2.1.3. Growth of *E. coli*

E.coli was grown on streaked LB agar plates for 4-5 days at 37 ^{o}C. After colony formation, the plates were stored at 4 ^{o}C. For each liquid LB preculture a colony was taken from a plate and inoculated in the 10 ml liquid medium. Incubation was carried out at 37 ^{o}C and 200 rpm in an incubator shaker.

2.2.2. PCR cloning of hexokinase

The basic steps of the cloning strategy are given in Figure 2.3.

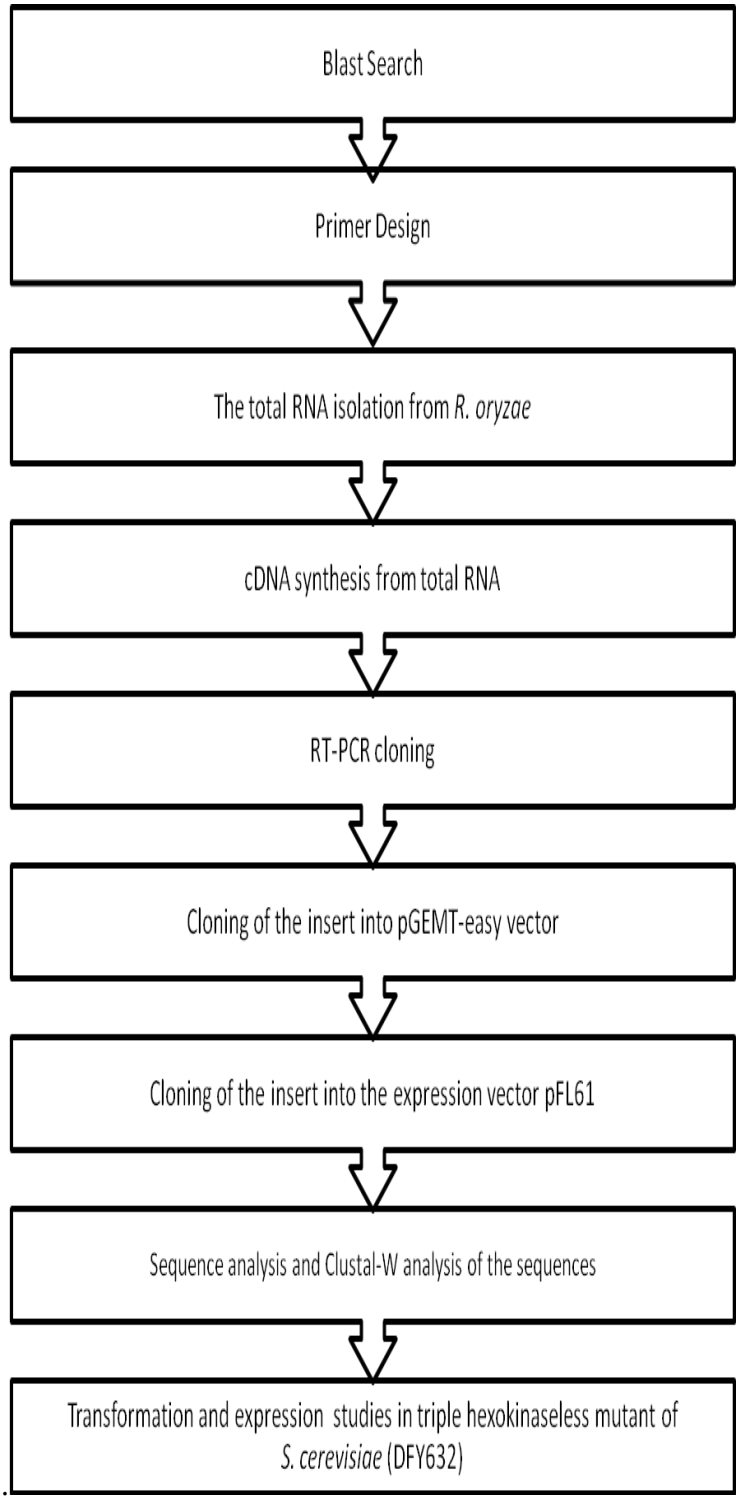

Figure 2.3 The Cloning Strategy

2.2.2.1. BLAST search

From NCBI site (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) the nucleotide sequence of hexokinases and glucokinase from *S.cerevisiae* was found. A blast search was done on the genome database of the *R.oryzae* by known nucleotide sequences of closely related *S. cerevisiae* hexokinases and glucokinase nucleotide sequences. According to the results, the sequences of the ten hypothetical proteins were documented from this site (http://www.broad.mit.edu/annotation /genome/rhizopus_oryzae/Home.html) to design primers.

2.2.2.2. Design of primers for PCR cloning

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

The primers used for cloning the hexokinase genes of the *R. oryzae* were designed based on the hypothetical sequences of *HXK* available in the *R. oryzae* database (http://broad.mit.edu/annotation/genome/rhizopus_oryzae/Multihome.html).

The primers used for PCR cloning of HXK from *R. oryzae* and regions of the sequence where these primers were based on are given in Table 3.2 The hypothetical proteins were named as 1-10 for convenience.

2.2.2.3. Total RNA isolation

The total RNA was isolated from *R. oryzae* according to the protocol optimized in our laboratory by Assoc. Prof. Dr. Leo H. de Graaff (TRIzol method). In order to obtain the mycelia for isolation of RNA, *R. oryzae* was grown in a 1/5 medium in 1L flask at 35 ^{o}C and 175 rpm for 24 hours. The mycelia were filtered with vacuum filtration and the pellet was dried between paper towels and wrapped up in a piece of aluminium foil and was immediately frozen in liquid nitrogen. Erlenmeyer, grinding balls, forceps and spatula were precooled in liquid nitrogen.

Approximately 0.5 g of frozen mycelium was submerged in liquid nitrogen in an Erlenmeyer together with the grinding balls. Excess liquid nitrogen was poured off, Erlenmeyer was capped and the assembly was quickly installed in the dismembrator. The mycelium was ground for 2 minutes at 2000 rpm. 1 ml of TRIzol reagent was added into an approximately 100 mg ground mycelium in a precooled 2 ml Eppendorf tube, and vortexed for 15minutes in a block shaker at room temperature, then centrifuged down for 5 minutes at room temperature at 12,000 rpm. 900µl of the supernatant was transferred to a new tube. 180µl Chloroform was added to the supernatant; the tube was shaken vigorously for 15 seconds and incubated at room temperature for 3 min, then centrifuged for 15 minutes at 4 ^{o}C at 12,000 rpm. The supernatant (450µl) was transferred into a 1.5 ml Eppendorf tube and 200 µl chloroform was added. The solution was mixed for 15 seconds and incubated at room temperature for 3 minutes, then centrifuged for 5 minutes at room temperature at 12,000 rpm. The supernatant (400-450µl) was transferred into a new 1.5 ml Eppendorf tube and 1

volume isopropanol was added, and mixed by inverting and incubated for 10 minutes at room temperature. The RNA was pelleted by centrifuging for 10 minutes at room temperature and at 12,000 rpm. The upper phase was discarded. One ml 75% ethanol was added and vortex mixed briefly to wash the pellet. The tubes were left for 3 minutes at room temperature and centrifuged for 5 minutes at room temperature at max speed. The supernatant was discarded and then centrifuged for an additional 15 seconds to collect the pellet. The tube were covered with aluminium foil and air dried for 10 min. DEPC-water (50µl) was added and incubated for 15 minutes at 65 ^{o}C to dissolve pellet.

The concentration of RNA was determined by measuring absorbance at 260 nm on a spectrophotometer (Shimadzu UV-1202) in 10 mM pH 8.0 Tris/HCl (one absorbance unit=40µg/ml RNA). The A_{260}/A_{280} ratio should be approximately 2.0, with ranges between 1.9 to 2.1 was assumed to be acceptable. The concentration of RNA should be >1.1 µg/µl. The RNA samples were sent to METU Central Laboratuvary in order to check the quality with Bioanalyzer.

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

2.2.2.4. cDNA synthesis

"Superscript^{TM} III First-Strand Synthesis System for RT PCR" supplied from Invitrogen was used for cDNA synthesis. RNA targets from 100 bp to 12 kb can be detected with this system. 1 pg to 5 µg of total RNA can be used as starting material. The total reaction mixture volume was 20 µl in a 0.2 ml sterile tube. Up to 5 µg total RNA, 1µl of 50 µM oligo$(dT)_{20}$ primer and 10 mM dNTP ; the mixture was incubated at 65 ^{o}C for 5 minutes for denaturation; then the mixture was placed on ice for at least 1 minutes; after that 2 µl of 10X RT Buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0,1 M DTT, 1µl of RNaseOUT^{TM} and 1 µl of Superscript^{TM} III RT were added into the mixture and it was incubated at 50 ^{o}C for 50 minutes for cDNA Synthesis, after the termination of reaction for 5 minutes at 85 ^{o}C; 1 µl of RNase H was added and the mixture was incubated at 37 ^{o}C for 20 min in order to remove RNA. cDNA synthesis reaction were used for PCR immediately or stored at -20 ^{o}C. "Eppendorf Master Cycler Personal" was used for heating the mixture.

2.2.2.5. Cloning of the hypothetical gene fragments of *RoHXK* by PCR

PCR mixture and the conditions were optimized with T*aq* polymerase in order to amplify the gene fragments of *RoHXK*. PCR amplification of the gene fragments of *RoHXK* was performed according to the optimized PCR conditions by proof reading polymerase (Invitrogen Accuprime Pfx Polymerase System) in order to minimize mismatches created by PCR. PCR were performed at "Eppendorf Master Cycler Personal".

2.2.2.6. Detection of PCR products on agarose gel

The amplification levels of PCR products were visualized on 1% agarose gel stained with ethidium bromide. "GeneRuler^{TM} Express DNA Ladder" supplied by Fermentas was used as molecular weight marker. After verifying that the product obtained was of the right size the PCR band was cut with a sharp scalpel and transferred into an Eppendorf.

2.2.2.7. Isolation of the gene fragments

The PCR products were isolated with QIAquick Gel Extraction Kit according to manufacturer's instructions. After the gel slice was weighed 3 volumes of Buffer QG was added to 1 volume of gel (100 mg -100 µl) and incubated at 50 ^{o}C for 10 minutes. The tube was mixed by vortexing every 2-3 minutes for dissolving the gel. The sample was applied to the QIAquick column placed in a provided 2 ml collection tube and centrifuged for 1 minute in order to bind the DNA. After the flow-through was discarded, 750 µl Buffer PE was added to wash and centrifuged for 1 minute. Then, the flow-through was discarded and the QIAquick column was centrifuged for a 1 minute at 12,000 rpm by microcentrifuge to remove residual wash buffer. QIAquick column was placed into a clean 1.5 ml microcentrifuge tube and 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 minute in order to elute DNA,

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

After the isolation of the gene fragments with Qiagen gel extraction kit (QIAquick). T/A cloning strategy were carried out in order to close these blunt ended fragments into pGEM-T Easy vector. 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, 0.5µl of 4 mM dATP, 2 µl of 25 mM $MgCl_2$, at 70 ^{o}C for 30 minutes in Eppendorf thermocycler. Taq polymerase adds an adenine base at the end of the fragments. Then 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 ^{o}C overnight.

5 µl of this mixture containing the plasmid with or without insert were used in transformation of *E. coli*. Fifty µl of component cells were used per transformation. Component cells were stored in -70 ^{o}C freezer. After cells were thawed, they were resuspended (pipette up and down gently with a yellow tip) and aliquots of 50 µl were transferred to each treatment tube. Five µl of DNA was added to sample tube and 1µl of control DNA (pGW635) was added to positive control tube. The DNA and cells were incubated on ice for 30 min. The cells were heat shocked by placing the tubes at 42 ^{o}C for 2 min. Half a ml of LB was added without antibiotics to each treatment tube.

After the tubes were incubated at 37 ^{o}C for 1 hour they were centrifuged for 5 min at 5000 rpm in minicentrifuge. The upper phase (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 ^{o}C. The plates may then be sealed and stored at 4 ^{o}C for about 1 month.

Blue/white selection was done for selection of transformants. The colonies containing the plasmid with insert appeared as white. These were picked and inoculated for minipreps. 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.

2.2.2.9. Plasmid DNA isolation

2.2.2.9.1. Plasmid DNA isolation by fast Mini-prep protocol

Five ml LB medium with ampicillin was inoculated (10µl from glycerol stock or a single colony from a plate) and incubated overnight at 37 ^{o}C. Two ml of culture was pipetted in a Eppendorf tube and centrifuged for 5 min at 5000 rpm by microcentrifuge (Mikro 200R). The upper phase was removed and then the cells were centrifuged for 1 minute for removing the rest of the supernatant. The pellet was resuspended in 100 µl buffer S1 for 5 min on a plate vortex. Buffer S1 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). After the pellet was resuspended; 100 µl buffer S2 which contains 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) was added and mixed manually. Then, 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 4 ^{o}C in centrifuge (Mikro 200R). 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 ^{o}C. After the upper phase was removed, 0.5 ml 70% ethanol was added to the pellet and centrifuged for 5 min at 14000 rpm at 4 ^{o}C. 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 ^{o}C.

2.2.2.9.2. Plasmid DNA purification by using 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. 2 µl overnight culture of *E. coli* in LB medium was centrifuged for 5 min at 5000 xg by microcentrifuge. Pelleted *E.coli* cells were resuspended in 250 µl Buffer P1 and transferred to a microcentrifuge tube. Then, 250 µl Buffer P2 was added and mixed thoroughly by inverting the tube 4-6 times. After 350 µl Buffer N3 was added and it was mixed immediately and thoroughly by inverting the tube 6-7 times. The mixture was centrifuged 10 min at 12,000 rpm. The upper phases were applied to the QIAprep spin column placed in a provided 2 ml collection tube. The column was centrifuged for 1 minute

and the flow-through was discarded. QIAprep spin column was washed with 0.75 ml Buffer PE and centrifuged for 1 minute. The flow-through was discarded and centrifuged for 1 minute to remove residual buffer. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl water was transferred to the center of each QIAprep spin column, after 1 minute standing it was centrifuged for 1 minutes.

2.2.2.10. Checking minipreps

Restriction digestions of minipreps were done with EcoRI or HindIII. Digestion mix contained 2 ml plasmid DNA, 2 µl EcoRI/HindIII buffer, 14 µl dH_2O, 2 µl EcoRI/HindIII. The mixture was incubated at 37 ^oC for 2-4 hours. The samples were run on 1% agarose gel.

2.2.2.11. Sequencing of the gene fragments

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

2.2.2.12. Multiple sequence aligment

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

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

The pGEMT-easy plasmids including insert DNA were isolated by using QIAprep Spin Miniprep kit from QIAGEN and digested with *NotI* enzyme. They were run on 1% agarose gel and then extracted from gel by using QIAGEN gel extraction kit. The expression vector (pFL61) was also digested with *NotI* and alkaline treated. Then, 0.1-0.2 µg of DNA were taken and ligated into 50 ng of pFL61 in the presence of 5 units T4 DNA ligase in a reaction volume of 10 µl overnight at 4^oC. After the ligation process completed, in order to propagate the plasmid, the vector with insert was transformed into *E. coli*. Then plasmid isolation was carried out. *E. coli* transformation and plasmid isolation were done as the protocol mentioned above.

2.2.2.14. Transformation of *S. cerevisiae*

For transformation of *S. cerevisiae* Li-acetate method was used. 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 30 ^oC and 140 rpm. When the OD_{600} of the culture was between 1-1.5, 9 ml of YEP-Gal media was inoculated with 1 ml from this culture. After the OD_{600} of the culture reached to 1-1.5, the cells were harvested at 1000 xg for 5 minutes and washed with sterile distilled water. After that the pellet was suspended in 1.5 ml TE buffer in which 1.5 ml 0.2 M lithium acetate was added. Then 0.5 ml cell suspension was transferred to tube (1.2x10.5cm) and incubated at 140 rpm and 30 ^oC for one hour. The cell suspension (400µl) was transferred to a 1.8 ml tube. After plasmid DNA solution was added (3-4 µg), the cell

suspension was incubated statically at 30°C for 30 min. 800 µl of 50% PEG-4000 (dissolved in water and sterilized autoclaving) was added, the tube was mixed thoroughly on a vortex and let stand at 30°C for 1 hour. The Eppendorf tube was immersed into a water bath at 42 °C and incubated for 5 minutes, then immediately cooled to room temperature. The cell suspension was washed with water at room temperature 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°C for 2 to 4 days.

2.2.3. Specific growth rate determination

Growth was followed by measurement of the optical density at 600 nm on a spectrophotometer (Shimadzu UV-1202). Culture samples were diluted in the same range such that the measured optical density was equal or lower than 0.600. Dilution was done by water and OD values were measured against water as blank.

Specific growth rate was calculated according to equation given below:

$$\frac{1}{x} * \frac{dx}{dt} = \mu$$

$$\frac{dx}{x} = \mu * dt$$

$$\ln(x_2/x_1) = \mu * (t_2 - t_1)$$

$$\mu = \ln(x_2/x_1) / (t_2 - t_1)$$

x: Concentration of cells (OD_{600})
t = time
µ = Specific growth rate (h^{-1})

The part that ln (x_2/x_1) value stayed at a stable level was taken as the exponential phase of the corresponding culture.

2.2.4. Dry cell weight determination

Dry cell weight was determined by using a standart curve (Alagöz, 2005). For dry cell weight determination preweighed nitrocellulose filters (pore size: 0.45m) were used. The medium was diluted to various OD_{600} values ranging from 0.1 to 0.6. After 15 ml each of these media was filtrated, the filters were washed with water and dried in a microwave oven for 20 min. The dried samples were weighed again and the difference between the preweighed filter and these samples give the dry cell weight of the the samples at different OD_{600} values. According to these results, OD versus dry cell weight standart graph was prepared.

2.2.5 Preparation of crude extract

Culture samples were harvested at late exponential phase by centrifugation (12,000 rpm for 30 minutes) in Sorvall centrifuge and washed with extraction buffer by centrifugated again at 12,000 rpm for 20 minutes. Wet yeast cells in 4 % of the extraction buffer were sonicated at 10μ amplitude (Soniprep 150) gently for 1 minute with 10 seconds intervals. The supernatant obtained by centrifugation of the cell homogenate at 12,000 rpm for 20 min at 4 ^{o}C in Sigma centrifuge was referred to as crude extract. All the steps were carried out at 0-4 ^{o}C.

2.2.6. Protein determination

Protein concentration was performed according to the Bradford Method's (1980). Bovine serum albumin (BSA) was used as a standard.The composition of the reagents and preparation of the standart curve is given in Appendix E. All experiments were carried out in three parallel runs.

2.2.7. Hexokinase assay

Enzyme activities were assayed spectrophotometrically by following NADPH formation at 340 nm. The cell of the spectrophotometer was kept at constant temperature (30 ^{o}C) by using a circulatory water bath.

Average OD340 change per minute was calculated and the unit of enzyme was calculated using this value and ε= 6.22 mM-1 cm-1 for NADPH according to equation given below.

$$\text{Enzyme activity}(\text{U.ml}^{-1}) = \frac{\Delta OD_{340}/\text{min}}{\varepsilon} \text{ x dilution factor(s) x } 10^{3}$$

One unit of enzyme activity was described as the amount of enzyme that catalyzes the conversion of 1 μmole of substrate per minute under assayed condition.

The specific activity of the enzyme was given as units per mg of protein.

Hexokinase enzyme activity was measured by a coupled enzyme assay system taken from Bergmeyer (1967) with some modifications.

In the assay glucose-6-phosphate formation was measured by coupling it with a further reaction in which glucose-6-phosphate was converted into 6- phosphogluconolactone in the presence of glucose-6-phosphate dehydrogenase which simultaneously catalyzes the reduction of $NADP^{+}$ to NADPH. Formation of NADPH was measured spectrophotometrically at 340 nm.

Reaction mixture was contained 100mM Tris-HCI pH 7.70, 1.7 mM EDTA, 10 mM MgCl2, 5mM ATP, 0.5mM $NADP^{+}$, 3.5 U/ml glucose-6-phosphate dehydrogenase, 10mM glucose.

Buffer, distilled water and enzyme were incubated at 30 ^{o}C before the reaction was started. The reaction was initiated by addition of $NADP^{+}$.

The reaction rate was measured as the increase in the absorbance of NADPH formed at 340 nm with 10 seconds intervals for 3 minutes in the spectrophotometer. The slope of this curve gave the reaction rate. One unit of hexokinase was defined as the amount of enzyme, which catalyzes the formation of 1 μmole of glucose-6-phosphate per minute under assayed conditions.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cloning of the putative gene fragments of *RoHXK* by PCR

In this study, three putative hexokinase genes of *R. oryzae* hexokinase genes were cloned. Data from genome database of *R. oryzae* was used to clone hexokinase genes of this organism.

From NCBI site (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) the nucleotide sequence of hexokinases and glucokinase from *S. cerevisiae* was found. A blast search was done on the genome database of the *R. oryzae* (http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae/Home.html) by known nucleotide sequences of closely related *S. cerevisiae* hexokinases and glucokinase nucleotide sequences.

3.1.1. BLAST search and identification of probable hexokinase genes of *R. oryzae*

According to the BLAST search results, ten probable genes were obtained (Table 3.1). The hypothetical proteins were named as 1-10 for convenience. The sequences of these hypothetical *Rohxk* were given in Appendix A.

3.1.2. Designed primers for PCR cloning

Primers for the both ends were designed for ten probable hexokinase genes. The primers used for PCR cloning of hexokinases from *R. oryzae* and regions of the sequence where these primers were based on, are given in Table 3.2.

Table 3.1 Probable genes and the sizes of the PCR products with and without introns, and theoretically calculated MW and pI of the hypothetical proteins

Annotation number	Contig no	Gene name	Length of ORF with introns (bp)	Length of ORF w/o introns (bp)	Length of protein product (aa)	MW of protein product (kDa)	pI of protein product
RO3G_01514.1	3.1	Hxk1	1600	1446	482	53.56	5.3
RO3G_13363.1	3.12	Hxk2	1580	1431	477	53.56	5.4
RO3G_02075.1	3.1	Hxk3	1616	1455	485	53.65	5.4
RO3G_05082.1	3.3	Hxk4	1458	1365	455	50.16	5.6
RO3G_11014.1	3.8	Hxk5	1268	1221	407	44.80	6.2
RO3G_05968.1	3.4	Hxk6	1518	1413	471	52.70	6.2
RO3G_03473.1	3.2	Hxk7	1491	1368	456	50.33	5.1
RO3G_10326.1	3.8	Hxk8	1363	1128	376	50.33	7.8
RO3G_04008.1	3.2	Hxk9	1349	1233	411	45.67	4.5
RO3G_15735.1	3.17	Hxk10	1372	1245	415	46.54	4.8

Table 3. 2 Primers used for PCR cloning of the *RoHXK* genes

The gene	The Sequence of Primer
ROhxk1_forward	ATGTTAAACAACAAAAAGAAGACACC
ROhxk1_backward	ATCAATCCGAGCCTTCTCTATTTAATTTC
ROhxk2_forward	ATGATTGTCACCCATAGCAATAATGATTC
ROhxk2_backward	TTAGCGCAAAGGGCCTTTAACTAATG
ROhxk3_forward	ATGCCTAGTAATAAGAAAGCACCTG
ROhxk3_backward	ATTTATGGCATAGCTACTTTTTGCTTTTC
ROhxk4_forward	ATGACTCAAACTGAATTTACAAAGGAAC
ROhxk4_backward	TTATTTTTTAGTAGCCATCATAGCAATG
ROhxk5_forward	ATGATTCCATCCTATGTCTCC
ROhxk5_backward	ATTTATTTGTGTAAAGCATTCTTTGTAG
ROhxk6_forward	ATGGGTTACGAACACCGTAAATC
ROhxk6_backward	ATTTTATTTTCTTTGAGTGATAGCATTC
ROhxk7_forward	ATGACAAAAGATATCTTTAATATTATTG
ROhxk7_backward	CTTATTCACACAACATGG
ROhxk8_forward	ATGAAAATTGGATTAAATAAGAACG
ROhxk8_backward	ACGTATTCACATATCTCTTACG
ROhxk9_forward	ATGACAATTGAAGAAGAGATTTTATTG
ROhxk9_backward	ATTTTTATTTACCGATAGCTAATCC
ROhxk10_forward	ATGACTACCAGAGAAGAG
ROhxk10_backward	ATGTTAACCTATAGTTATAAACC

3.1.3. Total RNA isolation and characterization

The total RNA was isolated from *R. oryzae* according to the TRIzol method. 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=40µg/ml RNA). The A_{260}/A_{280} ratio should be approximately 2.0, with ranges between1.9 to 2.1 was considered to be acceptable. The RNA concentration should be >1.1 µg/µl. The RNA quality was checked with Bioanalyzer. Some of analysis results were shown in Appendix F. The RNA integrity was also tested by Agarose Gel Electrophoresis. The RNA quality checked with BioanalyzerThe RNA concentration and A_{260}/A_{280} ratio of isolated RNA were given in Table 3.3. cDNA synthesis from total RNA was done with Oiagen cDNA Synthesis kit.

Table 3.3 Results of the RNA concentration and A_{260}/A_{280} ratio

Sample	OD260 nm	OD280 nm	OD260nm/OD280nm	Conc. (µg/ml)
1	**0.287**	**0.150**	**1.9133**	**3.2144**
2	0.190	0.098	1.9388	2.128

3.1.4. Optimized conditions of PCR

In order to amplify the gene fragments of *RoHXKs*, PCR mixture and the conditions were optimized. *Taq* DNA polymerase was used to obtain the PCR products. According to the T_m values of the primers the annealing temperature of the PCR was determined. Since the T_m values were in the same range (45-50 ^oC), the optimum PCR conditions were similar.Table 3.4 and 3.5 show the content of the optimized PCR mixtures and the PCR conditions.

Table 3.4 PCR mixture contents for the hypothetical gene fragments of *RoHXKs*

µl	*RoHXK1*	*RoHXK6*	*RoHXK7*	*RoHXK8*	*RoHXK10*
Buffer	2.5	2.5	2.5	2.5	2.5
dNTPs	1	1	1	1	1
$MgCl_2$	1.5	1.5	1.5	1.5	1.5
Taq polymerase	0.1	0.1	0.1	0.1	0.1
cDNA	2	2	2	2	2
Forward Primer	1.05	1.27	1.40	1.52	1.51
Reverse Primer	0.94	1.15	1.45	1.33	1.21
Water	15.91	15.48	15.05	15.18	15.18
Total	25	25	25	25	25

Table 3.5 PCR programme for amplification of the hypothetical gene fragments of *RoHXKs*

	RoHXK1	*RoHXK6*	*RoHXK7*	*RoHXK8*	*RoHXK10*	
Initial Denaturation	94 °C	94 °C	94 °C	94 °C	94 °C	30 sec
Denaturation	94 °C	94 °C	94 °C	94 °C	94 °C	30 sec
Annealing	50 °C	48 °C	49 °C	48 °C	47 °C	45 sec
Elongation	72 °C	72 °C	72 °C	72 °C	72 °C	90 sec
Total number of cycles	25	25	25	25	25	
Final Elongation	72 °C	72 °C	72 °C	72 °C	72 °C	120 sec
Cooling	4 °C	4 °C	4 °C	4 °C	4 °C	∞

3.1.5. Characterization of PCR product of probable genes

For *RoHXK1*, *RoHXK3*, *RoHXK6*, *RoHXK7*, *RoHXK8*, *RoHXK9* and *RoHXK10* from the ten probable genes PCR products were obtained with the primers designed for them and these were also replicated by using P*fx* polymerase. P*fx* polymerase has 3′ to 5′ proof reading activity and it corrects nucleotide-misincorporation errors. The PCR products were run on %1 of agarose gel (Figure 3.1).

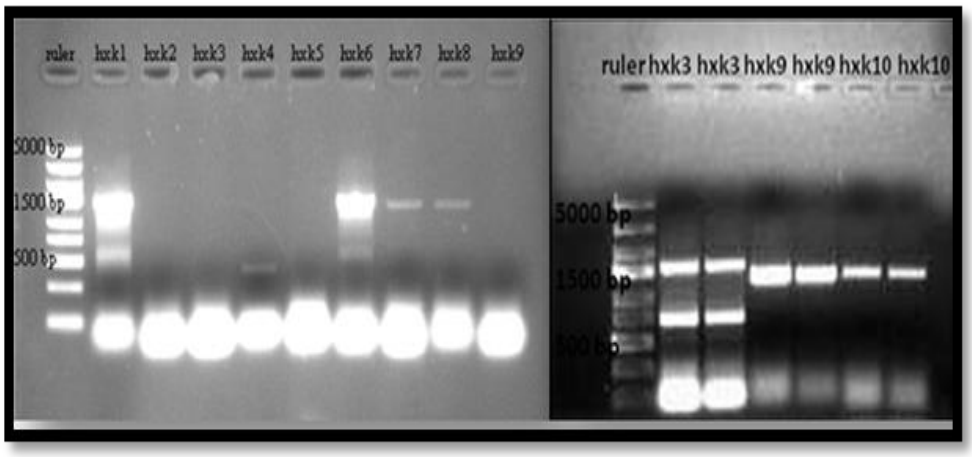

Figure 3.1 PCR results with ten different primer pairs

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

After verifying that the product obtained was of the right size the PCR band was cut from gel and gel extraction was done. The PCR products were isolated with QIAquick Gel Extraction Kit according to manufacturer's instructions.

Two vector systems were used for cloning of hexokinases of *R. oryzae*. One of them was pGEM®-T Easy shuttle vector and the other one was pFL61 expression vector.

After the isolation of the gene fragments T/A cloning strategy was carried out in order to close these blunt ended fragments into pGEM®-T Easy vector. Inserted vector was transformed to *E. coli* DH5α. After the incubation of transformed cell on LB agar+amp+X-gal overnight, blue/white selection was done for selection of transformants. The colonies containing the plasmid with insert appeared as white. These ones were picked and inoculated for minipreps.

3.1.7. Results of plasmid DNA isolation

The plasmids isolated from these colonies were cut with restriction digestion enzymes to check the transformation of pGEM®-T Easy vector and inserted hexokinase genes into *E. coli.*

3.1.8. Results of plasmid DNA isolation sequenced analysis of the gene fragments

To check the correction of the insert, sequence analysis was performed on the purified plasmids. The sequence analysis of plasmid carrying insert was performed with T7 and SP6 (Figure 3.2).

3.1.9. Multiple sequence aligment

Multiple sequence aligment was done by using Clustal W program (http://www.ebi.ac.uk/Tools/clustalw/index.html) in order to compare the sequence analysis results of the plasmid carrying gene insert.

At the end of the sequencing of three of them (*RoHXK1, RoHXK6, RoHXK7*), the results proved that the PCR products has no introns and they are expressed in *R. oryzae* (Figure 3.2).

3.1.10. Results of transformation of the insert into the expression vector:pFL61

In order to transfer the hexokinase genes from shuttle vector pGEM®-T Easy to expression vector pFL-61, the inserted gene should be taken from shuttle vector. The plasmids, isolated from white colonies selected with blue/white selection, 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.

The pGEM®-T Easy plasmids including insert DNA were isolated by using QIAprep Spin Miniprep kit from QIAGEN and digested with *NotI* enzyme. They were run on agarose gel

(1%) and extracted from gel by using gel extraction kit from QIAGEN. The expression vector (pFL61) was also digested with *NotI* and alkaline treated. After the ligation process completed, in order to propagate the plasmid, the vector with insert was transformed into *E. coli.*

In order to check the correction of the insert, sequence analysis was performed on the purified plasmids with the pFL61 primer A and pFL61 primer B (Figure 3.2).

Clustal W Multiple Sequence Aligments for pGEM®-T Easy +*RoHXK1* and hypothetical *RoHXK1*

```
CLUSTAL 2.1 multiple sequence alignment

RO3G_01514.1            ------------------------------------------------------------AT 2
PGEMT-HXK1              ACAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTAT 60
                                                                                   **

RO3G_01514.1            GTTAAACAACAAAAAGAAGACACCAAAAGCTTATGAAATTCATGAAATTGGAGGAACAGC 62
PGEMT-HXK1              GTTAAACAACAAAAAGAAGACACCAAAAGCTTATGAAATTCATGAAATTGGAGGAACAGC 120
                        ************************************************************

RO3G_01514.1            CGAACAAGAGGCGTTTATGAATGATATCATTGATCAGTTCACGATTGATTCAAGCCAACT 122
PGEMT-HXK1              TGAACAAGAGGCGTTTATGAAGGATATCATTGATCAGTTCACGATTGATTCAAGACAACT 180
                         ******************** ********************************* *****

RO3G_01514.1            TGTAGAAATACGTGATCATTTTATACAAGAAATGGAAAAGGGCTTGAACCAGGAAGGAGC 182
PGEMT-HXK1              TGTAGAAATACGTGATCATTTTATACAAGAAATGGAAAAGGGCTTGAATCATGAAGGAGC 240
                        ********************************************** ** ********

RO3G_01514.1            CACATTGGCCATGATTCCGTCTTATGTCGAAGGACGTTTGACTGGTAAAGAAGAAGGACG 242
PGEMT-HXK1              CACATTGGCCATGATTCCGTCTTATGTCGAAGGACGTTTGACTGGTAAAGAAGAAGGACG 300
                        ************************************************************

RO3G_01514.1            TTTTTTAGCTCTTGATTTGGGAGGAACGAACCTGAGGGTGGTTCTGGTTACACTAGAAGG 302
PGEMT-HXK1              TTTTTTGGCTCTTGATTTGGGAGGAACGAACCTGAGGGTGGTTCTGGTTACACTAGAAGG 360
                        ****** ***************************************************

RO3G_01514.1            GGATGGCAAGTTTCAAACTGTGTCTACAAAATCAAAAGTATCAGAGGAATTAAAGACAGG 362
PGEMT-HXK1              GGATGGCAAGTTTCAAACTGTGTCTACAAAATCAAAAGTATCAGAAGAATTAAAGACAGG 420
                        ********************************************** ***************

RO3G_01514.1            GCCTATGCGTAACTTGTGTGATTATATTGCCGATTGTGTTGACACGTTCTTGACAGAGCA 422
PGEMT-HXK1              TCCCATGCGTAACTTGTGTGATTACATTGCTGATTGTGTTGATACGTTCTTGACAGAGCA 480
                         ** ******************** ***** *********** *****************

RO3G_01514.1            TGGTCTTGAAAATCATGAAACGGAACTCAACCTGGGCTATACTTTCTCTTTTCCTATTCT 482
PGEMT-HXK1              TGGTCTTGAAAATCATGAAACGGAACTCAACCTGGGCTATACTTTCTCTTTTCCTATTCT 540
                        ************************************************************

RO3G_01514.1            TCAATCAAAGATCAATCGTGGTATACTCTCTACATGGACCAAAGGATTTTCTAGCTCGGG 542
PGEMT-HXK1              TCAATCAAAGATCAATCGTGGTATACTCTCTACATGGACAAAAGGATTTTCTAGCTCGGG 600
                        ************************************** ********************

RO3G_01514.1            TGCTGTGAACAAGGACCCTGTCCTATTACTTCAGGATGCCTTATTAAGAAAACATGTCCC 602
PGEMT-HXK1              TGCTGTGAACAAGGACCCTGTACTATTACTCCAGGATGCCTTATTAAGAAAACATGTCCC 660
                        ********************** ******** ******************************

RO3G_01514.1            AGTCAAGATTTCTGCACTTGTCAATGACACAGTCGGTACTCTTTTATCCAACGCATACAA 662
PGEMT-HXK1              AGTCAAGATTTCTGCACTTGTTAATGACACGGTCGGTACTCTTTTATCCAACGCATACAA 720
                        ********************** ******** ******************************

RO3G_01514.1            TAAACCTCACACCTTGGCTGGTCTCATCTTGGGCACAGGCGCTAATGGTGCTTATATCGA 722
PGEMT-HXK1              TAAACCTCACACCTTGGCTGGTCTCATCTTGGGTACAGGCGCTAATGGTGCTTATATCGA 780
                        ********************************** *************************

RO3G_01514.1            AAAGATGTCAAAGATTGGCAAATGGAAGGGAGGCAAGACAACAGCAGAAGAGATGATTAT 782
PGEMT-HXK1              AAAGATGTCAAAGATTG-TAAATGGA---GCAGCA-GACA-CAGCAGA-GAGATGATTAT 833
                        ****************  *******   *  *** **** ******* ***********

RO3G_01514.1            TAATATGGAGTTTGGTGCTTTTGATAATGAAAGACGTGTGCTTCCCTTGACACGTTTTGA 842
PGEMT-HXK1              TA-TATGGA-TTTGGTGCTTTTGATA-TGAAAGACGTGTGCTTCCCTTGACACGCTTTGA 890
                        ** ****** **************** *************************** *****

RO3G_01514.1            TAACAAACTTGATCGACAGTCCATTAATCCTCATGCTCAACTCTATGAAAAGATGATCTC 902
PGEMT-HXK1              TAACAAACTTGAT---------------------------------------------- 903
                        *************

RO3G_01514.1            AGGCATGTACCTGGGTGAAATCACTCGAAATGTGCTCATAGACATGATTGACCGTGAACT 962
PGEMT-HXK1              ------------------------------------------------------------

RO3G_01514.1            CTTGCTGAAACCACAAAACTTGGCCAAGGACATCTCTCGACACTGGTCCTTTGAGACAGC 1022
PGEMT-HXK1              ------------------------------------------------------------

RO3G_01514.1            CTTCATGTCAAGCATTGAAGAAGACAATTCTCCTGATTTACAACATACAAAGGAGACCTT 1082
PGEMT-HXK1              ------------------------------------------------------------
```

Percent Identity Matrix - created by Clustal2.1

1: RO3G_01514.1 100.00 97.40

2: PGEMT-HXK1 97.40 100.00

Clustal W Multiple Sequence Aligments for pGEM®-T Easy +*RoHXK6* and hypothetical *RoHXK6*

```
CLUSTAL 2.1 multiple sequence alignment


RO3G_05968.1            GGTCGTATCAGTACGCGTCAGCAGAAATATGTGATTCATGACCATCTCAAGAAGGGCTCG 360
PGEMT-HXK6              ------------------------------------------------------------

RO3G_05968.1            ATCAATGCGATGATTGATTTCTTGGTGGAGAGTGTGGATTCCTTTTTGAACTTTGTGGGT 420
PGEMT-HXK6              ------------------------------------------------------------

RO3G_05968.1            AAATATGATATCAAGCAACCACTGGCCCTTGGCTTCGTCTTGGCCTTTCCCTTGGAACAA 480
PGEMT-HXK6              ------------------------------------------------------------

RO3G_05968.1            ACCGCGTTGAATAAGGCCGTGGTGATCCAGTGGACCAAGGATTTCGAAATCACGGGTGCC 540
PGEMT-HXK6              ------------------------------------------------------------

RO3G_05968.1            AACAACAAGAACATTGCCGACCTGCTCCAGACCGGCTTCAACCGTCGTCATCTCAACGTG 600
PGEMT-HXK6              ------------------------------------------------------------

RO3G_05968.1            CATGTGGAGGCCGTGGTGAATGGTGCGGTCGGCTGTCTCTTGGCTCACAGCTACCGCAGT 660
PGEMT-HXK6              ------------------------------------------------------------

RO3G_05968.1            CTGGACACACTCGTGGCCTGTACGATCAGCACGGGTACCAACGCCGCCTACTGGGAAAAG 720
PGEMT-HXK6              ----------------------------------------ACGGCGGCTACTGGGAAAAG 20
                                                                *** ** **************

RO3G_05968.1            ACGAGCGAGCTGAAAAAGACCGAGGGCCAGACGGTTCCGAACGGGGATGGCGAGATGATT 780
PGEMT-HXK6              ACGAGCGAACTGAAAAAGACCGAGGGCCAGACGGCTCCGAATGGGGATGGCGAGATGATT 80
                        ********.************************ ****** ******************

RO3G_05968.1            GTGAATACGGAATGGGGAAGCTTTGGTGATCACAATCTCGATTTCCTTCCTCGTACGTTT 840
PGEMT-HXK6              GTGAATACCGAGTGGGGAAGCTTTGGTGATCACAATCTCGATTTCCTCCCTCGTACATTT 140
                        ******** **.********************************** ********.***

RO3G_05968.1            TATGATAACCGGGTCAATCGTCAGTCGGTCAATCCGGGTGTGCACGTCTTTGAGAAGATG 900
PGEMT-HXK6              TATGATAACCGGGTCAATCGTCAATCGGTCAATCCGGGTGTGCACGTCTTTGAAAAGATG 200
                        ************************.******************************.******

RO3G_05968.1            GTCTCTGGTCTCTATCTGGGTGAAATCGTCCGTCTGATCATGGTCGATTTCCTCGATCGT 960
PGEMT-HXK6              GTCTCTGGTCTCTATCTGGGTGAAATCGTTCGTCTGATCATGGTCGATTTTCTCGATCGT 260
                        ***************************** ******************** *********

RO3G_05968.1            CGTTTGATCTTTAACGGACAGTACACGACCGAACTCAACACGCCTTACTTTTTTGAATCT 1020
PGEMT-HXK6              CGTCTGATCTTTAATGGACAGTACACGACCGAACTCAACACGCCTTACTTTTTTGAATCT 320
                        *** ********** ************************************************

RO3G_05968.1            TCTTACATGAGTGCGATTCAATCGGATGATACGACTGAATTGGATGAGACCCGACATATT 1080
PGEMT-HXK6              TCTTACATGAGCGCGATTCAATCGGACGATTCGACTGAATTGGATGAGACAAGACATATT 380
                        ********** ************** ***:********************..********

RO3G_05968.1            TTGGAGTCCATCATGAACATTCCTTCGACTACTTTGAATGATCTTCAGATGGTCAAGATG 1140
PGEMT-HXK6              TTGGAGTCCATCATGAACATTCCTTCGACAACCTTGAATGATCTTCAGATGGTCAAGATG 440
                        ******************************:** ***************************

RO3G_05968.1            ATCTGTGACTTGGTCTCTCGACGTGCCGCTCGTCTGGTGGCTGCTGCGATCAGTGCGATC 1200
PGEMT-HXK6              ATTTGTGACTTGGTCTCTAGACGTGCCGCTCGTCTTGTGGCTGCTGCTATCAGTGCGATT 500
                        ** ***************.**************** *********** ***********

RO3G_05968.1            ATCGAAAAGAGAAACGCACTCGATCAAGGACTGACGATCAGTATGGAAGGCTCTGTGTAT 1260
PGEMT-HXK6              ATCGAAAAGAGAAACGCGCTGGATCAAGGGCTGACGATCAGTATGGAAGGCTCTGTTTAT 560
                        ******************.** ********.************************** ***

RO3G_05968.1            GAACATTTCCCTGATTTCCCAAGACGTGTGAATGATACACTCAAGAGTCTTTACAGTGAT 1320
PGEMT-HXK6              GAACATTTCCCTGATTTCCCAAGACGTGTGAATGATACACTCAAGAGTCTTTACAGTGAT 620
                        ************************************************************

RO3G_05968.1            CGTGTGGATCATATCAATATCGGCATCACCCGTGATGGACACGGCATCGGTGCTGCTTTG 1380
PGEMT-HXK6              CGTGTGGACCATATCAATATCGGAATCACTCGTGATGGACACGGTATCGGTGCTGCTTTG 680
                        ******** **************.***** ************** ***************
```

Percent Identity Matrix - created by Clustal2.1

1: RO3G_05968.1 100.00 94.95

2: PGEMT-HXK6 94.95 100.00

Clustal W Multiple Sequence Aligments for pGEM®-T Easy +*RoHXK7* and hypothetical *RoHXK7*

```
CLUSTAL 2.1 multiple sequence alignment

RO3G_03473.1            GGTGAAGGTGCAATGTTCTTTGACTGGATTGCAGATGCTGTTCGAGATCTCATCACTATT 360
PGEMT-hxk7              ------------------------------------------------------------

RO3G_03473.1            GAAGCTAAACACTTATTTACTCAAGACCAAGTGGAAGGTAAAGAAACTTTGGCTTTAGGT 420
PGEMT-hxk7              ------------------------------------------------------------

RO3G_03473.1            GTCTGTTGGAGTTTTCCTGTAGATCAGACTGCTGTTGACCGTGGTACTATTCTTAGAATG 480
PGEMT-hxk7              ------------------------------------------------------------

RO3G_03473.1            GGAAAAGGATTTACATTAAAGAATACGGAAGGGAATGATCTTGCTGATATGTTTCATGAG 540
PGEMT-hxk7              ------------------------------------------------------------

RO3G_03473.1            GCTTTCAAAAGAAAGAAACTGAATGTCAAGGTGACTGCTATATTAAATGATACAGTGGGT 600
PGEMT-hxk7              ------------------------------------------------------------

RO3G_03473.1            ACATTGGTTGCTCATGCTTATACAAATCCTGAATGCCGTATTGGTCTCATCTTTGCTACT 660
PGEMT-hxk7              -------------------------ATCCTGAATGCCGTATTGGCCTCAT-TTTGCTACT 34
                                                 ****************** ***** *********

RO3G_03473.1            GGTATCAACGCAGCTTATCCTGAAAAAGTATCTGCTATCACTAAATTAGATCCAGAGGTC 720
PGEMT-hxk7              GGTATCAACGCAGCTTATCCTGAAAAAGTATCTGCTATCACTAAATTAGCTCCAGAGGTC 94
                        ********************************************** **********

RO3G_03473.1            CGAAATAACTATACACCAACTACCGAAATGTTGATCAATACAGAAATTGACATTTTTGGT 780
PGEMT-hxk7              CGAAATAACTATACACCAAACACCGAAATGTTGATCAATACAGAAATTGACATTTTTGGT 154
                        ******************  ***************************************

RO3G_03473.1            ACAGAAGCCTATCTACCCCTCACTAAATATGATCTCGCACTCGATCTTTCACATAATCAG 840
PGEMT-hxk7              ACAGAAGCCTATCTACCTCTCACTAAATATGATCTCGCACTCGATCTTTCACATAATCAG 214
                        ***************** ******************************************

RO3G_03473.1            CCCAAATTTCAACTTTATGAAAAGATGCTATCAGGTGCCTACATGGGTGAACTCACACGG 900
PGEMT-hxk7              CCCAAATTCCAACTTTATGAAAAGATGCTCTCAGGTGCCTACATGGGTGAACTCACACGG 274
                        ******** ****************** ******************************

RO3G_03473.1            TTGATTGCTATGGACTTTATTGAGGCAGGCATCTTGTTTGGTGGTGAAATACCCAAGGGA 960
PGEMT-hxk7              TTGATTGCTGTGGACTTTATTGAGGCAGGCCTCTTGTTTGGTGGTGAAATGCCCAAGGGA 334
                        ********* ****************** ****************** *********

RO3G_03473.1            TTCAACCAACCTTGGTCCTTCCCAACCACTTACATGAGCGCATTAGAAAGCGATCAATCA 1020
PGEMT-hxk7              TTCAATCAACCCTGGTCGTTCCCAACAACTTACATGAGCGCACTCGAAAGCGATCAATCA 394
                        ***** ***** ***** ******** *************** * ***************

RO3G_03473.1            GAAACAAAAGAGGTCGGACAAAGAATCCTAACCGAATTTCCTACCAGCGAGAAGCCCACT 1080
PGEMT-hxk7              GAAACAAAAGAGGTCGGACAAAGAATCCTAACCGAATTTCCTACAAGCGAGAAGCCCACG 454
                        ******************************************** **************

RO3G_03473.1            TTGGATGATATCAATACCCTCACACGTATCTGTCGCATTGTCGCTGCTCGTTCTGCTTCC 1140
PGEMT-hxk7              TTGGATGATCTCAATACCCTCACACGTATCTGTCACATTGTCGCTGCTCGTTCTGCTTCC 514
                        ********* ************************ ************************

RO3G_03473.1            TTGGTAGCTGTGGCCATCATTTCTTTATTGGAACAACAGGGTCTGTCGGATCATATGGTT 1200
PGEMT-hxk7              TTGGTAGCTGTGGCCATCATTTCTTTATTGGAACAACAGGGTCTATCGGATCATATGGTG 574
                        ******************************************** **************

RO3G_03473.1            GTGGGTATCAATGGATCAACTTATGAATTTTATCCTCATATGGATCAACGTGTAAGACAA 1260
PGEMT-hxk7              GTGGGTATCAATGGATCAACCTATGAATTTTATCCTCATATGGATCAACGTGTAAGACAA 634
                        ******************* ****************************************

RO3G_03473.1            GCTTTAGATGAATGGTTTGGAAAAGAAATAAGTAGCAAGATTATACTTGAAGTTGCTTCT 1320
PGEMT-hxk7              GCTTTAGACGAATGGTTTGGAAAAGAGATGAGTAGCAAGATCATACTTGAAGTTGCTTCT 694
                        ******** ***************** ** ************ ******************

RO3G_03473.1            GAAGGAGGTAGTGTGGGTGGTGCTTTGATTGCCATGTTGTGTGAATAA------------ 1368
PGEMT-hxk7              GAAGGAGGTAGTGTGGGTGGTGCTTTGATTGCCATGTTGTGTGAATAAGAATCGAATTCC 754
                        ************************************************

RO3G_03473.1            ---------------------------------------------
PGEMT-hxk7              CGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGCCCAATCGG 800
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Percent Identity Matrix - created by Clustal2.1

1: RO3G_03473.1 100.00 96.36

2: PGEMT-hxk7 96.36 100.00

Clustal W Multiple Sequence Aligments for pFL61+*RoHXK1* and hypothetical *RoHXK1*

```
CLUSTAL 2.1 multiple sequence alignment

RO3G_01514.1            --------------------------------ATGTTAAACAACAAAAAGAAGACACCAA 28
PFL61-HXK1              AATTAATTGATCGCGGCCGCGGGAATTCGATTATGTTAAACAACAAAAAGAAGACACCAA 60
                                                        ****************************

RO3G_01514.1            AAGCTTATGAAATTCATGAAATTGGAGGAACAGCCGAACAAGAGGCGTTTATGAATGATA 88
PFL61-HXK1              AAGCTTATGAAATTCATGAAATTGGAGGAACAGCTGAACAAGAGGCGTTTATGAAGGATA 120
                        ********************************** ******************** ****

RO3G_01514.1            TCATTGATCAGTTCACGATTGATTCAAGCCAACTTGTAGAAATACGTGATCATTTTATAC 148
PFL61-HXK1              TCATTGATCAGTTCACGATTGATTCAAGACAACTTGTAGAAATACGTGATCATTTTATAC 180
                        **************************** ******************************

RO3G_01514.1            AAGAAATGGAAAAGGGCTTGAACCAGGAAGGAGCCACATTGGCCATGATTCCGTCTTATG 208
PFL61-HXK1              AAGAAATGGAAAAGGGCTTGAATCATGAAGGAGCCACATTGGCCATGATTCCGTCTTATG 240
                        ********************** ** **********************************

RO3G_01514.1            TCGAAGGACGTTTGACTGGTAAAGAAGAAGGACGTTTTTTAGCTCTTGATTTGGGAGGAA 268
PFL61-HXK1              TCGAAGGACGTTTGACTGGTAAAGAAGAAGGACGTTTTTTGGCTCTTGATTTGGGAGGAA 300
                        ****************************************** ******************

RO3G_01514.1            CGAACCTGAGGGTGGTTCTGGTTACACTAGAAGGGGATGGCAAGTTTCAAACTGTGTCTA 328
PFL61-HXK1              CGAACCTGAGGGTGGTTCTGGTTACACTAGAAGGGGATGGCAAGTTTCAAACTGTGTCTA 360
                        ************************************************************

RO3G_01514.1            CAAAATCAAAAGTATCAGAGGAATTAAAGACAGGGCCTATGCGTAACTTGTGTGATTATA 388
PFL61-HXK1              CAAAATCAAAAGTATCAGAAGAATTAAAGACAGGTCCCATGCGTAACTTGTGTGATTACA 420
                        ****************** ************** ** ******************** *

RO3G_01514.1            TTGCCGATTGTGTTGACACGTTCTTGACAGAGCATGGTCTTGAAAATCATGAAACGGAAC 448
PFL61-HXK1              TTGCTGATTGTGTTGATACGTTCTTGACAGAGCATGGTCTTGAAAATCATGAAACGGAAC 480
                        **** *********** ********************************************

RO3G_01514.1            TCAACCTGGGCTATACTTTCTCTTTTCCTATTCTTCAATCAAAGATCAATCGTGGTATAC 508
PFL61-HXK1              TCAACCTGGGCTATACTTTCTCTTTTCCTATTCTTCAATCAAAGATCAATCGTGGTATAC 540
                        ************************************************************

RO3G_01514.1            TCTCTACATGGACCAAAGGATTTTCTAGCTCGGGTGCTGTGAACAAGGACCCTGTCCTAT 568
PFL61-HXK1              TCTCTACATGGACAAAAGGATTTTCTAGCTCGGGTGCTGTGAACAAGGACCCTGTACTAT 600
                        ************* ****************************************** ****

RO3G_01514.1            TACTTCAGGATGCCTTATTAAGAAAACATGTCCCAGTCAAGATTTCTGCACTTGTCAATG 628
PFL61-HXK1              TACTCCAGGATGCCTTATTAAGAAAACATGTCCCAGTCAAGATTTCTGCACTTGTTAATG 660
                        **** ************************************************** ****

RO3G_01514.1            ACACAGTCGGTACTCTTTTATCCAACGCATACAATAAACCTCACACCTTGGCTGGTCTCA 688
PFL61-HXK1              ACACGGTCGGTACTCTTTTATCCAACGCATACAATAAACCTCACACCTTGGCTGGTCTCA 720
                        **** ************************************************************

RO3G_01514.1            TCTTGGGCACAGGCGCTAATGGTGCTTATATCGAAAAGATGTCAAAGATTGGCAAATGGA 748
PFL61-HXK1              TCTTGGGTACAGGCGCTAATGGTGCTTATATCGAAA-GATGTCAAAGATTGGTAAATGGA 779
                        ******* **************************** *************** *******

RO3G_01514.1            AGGGAGGCAAGACAACAGCAGAAGAGATGATTATTAATATGGAGTTTGGTGCTTTTGATA 808
PFL61-HXK1              CGG-AGGCAAGACAACAGCAGA-GAGATGATTATTA-TATGGAATTTGGTGCTTTTGATA 836
                         ** ****************** ************* ****** ****************

RO3G_01514.1            ATGAAAGACGTGTGCTTCCCTTGACACGTTTTGATAACAAACTTGATCGACAGTCCATTA 868
PFL61-HXK1              -TGAA-GACGTGTGCTTCCCTTGACACGCTTTGATA--CAACTTGATCGACAGTCCATTA 892
                         **** ********************** *******   **********************

RO3G_01514.1            ATCCTCATGCTCAACTCTATGAAAAGATGATCTCAGGCATGTACCTGGGTGAAATCACTC 928
PFL61-HXK1              -TCCTCATGCTCA-CTCTATGAAA-GATGATCTCAGGCATGTACCTGGGGTGAATCACTC 949
                         ************ ********** ************************   ********

RO3G_01514.1            GAAATGTGCTCATAGACAT-GATTGACCGTGAACTCTTGCTGAAACCACAAAACTTGGCC 987
PFL61-HXK1              GAA-TGTACTCTTA-ACATTGATGGATCGT------------------------------ 977
                        *** *** *** ** **** *** ** ***

RO3G_01514.1            AAGGACATCTCTCGACACTGGTCCTTTGAGACAGCCTTCATGTCAAGCATTGAAGAAGAC 1047
PFL61-HXK1              ------------------------------------------------------------

RO3G_01514.1            AATTCTCCTGATTTACAACATACAAAGGAGACCTTAGACAGCAATCTAAATTTGCATGAT 1107
PFL61-HXK1              ------------------------------------------------------------
```

Percent Identity Matrix - created by Clustal2.1

1: RO3G_01514.1 100.00 96.82

2: PFL61-HXK1 96.82 100.00

Clustal W Multiple Sequence Aligments for pFL61+*RoHXK6* and hypothetical *RoHXK6*

```
CLUSTAL 2.1 multiple sequence alignment


RO3G_05968.1            --------------------------------ATGGGTTACGAACACCGTAAATCATTTTC 29
pFL61-HXK6              AATTATTGATCGCGGCCGCGGGAATTCGATTATGGGTTACGAACACCGTAAATCATTTTC 60
                                                        ****************************

RO3G_05968.1            AAGTGGACCACTGGCGGGTGGTACGGATGACCAACATGCTGCTATGGAAGAATTAAAGGG 89
pFL61-HXK6              AAGTGGACCACTGGCTGGTGGTACGGATGATCAACATGCTGCTATGGAAGAATTAAAGGG 120
                        *************** ************** ****************************

RO3G_05968.1            TCACTTCAAGTTGACAACGGAACAACTCAAGACATTTCGTGATAATTTAATTCAAGAGAT 149
pFL61-HXK6              TCACTTCAAGTTGACGACTGAACAACTCAAAACATTTCGTGATAATCTAATTCAAGAGAT 180
                        *************** ** *********** *************** *************

RO3G_05968.1            GAACACTGGATTACAATCGCATGAATCCAATATGGCCATGTTACCCTCTTGGATCTTTAG 209
pFL61-HXK6              GAACACTGGCTTACAATCGCATGAATCCAACATGGCCATGTTACCCTCTTGGATCTTTAG 240
                        ********* ******************* ****************************

RO3G_05968.1            ACATCCTACCGGTCAAGAAACGGGTGAATATCTTGGTTTGGAAATTAGTGGTTCCAATGT 269
pFL61-HXK6              ACATCCTACCGGTCAAGAAACGGGTGAATATCTTGGTTTGGAAATTAGTGGTTCCAATGT 300
                        ************************************************************

RO3G_05968.1            CCGCATCTATCTCGTCAACTTGCACGGACAGGGTCGTATCAGTACGCGTCAGCAGAAATA 329
pFL61-HXK6              CCGAATCTATCTCGTCAATTTGCACGGACAGGGTCGTATCAGTACGCGTCAGCAGAAATA 360
                        *** ************** ****************************************

RO3G_05968.1            TGTGATTCATGACCATCTCAAGAAGGGCTCGATCAATGCGATGATTGATTTCTTGGTGGA 389
pFL61-HXK6              TGTGATTCATGACCATCTCAAGAAGGGTTCGATCACTGCGATGATTGATTTCTTGGTGGA 420
                        ************************** ******* ************************

RO3G_05968.1            GAGTGTGGATTCCTTTTTGAACTTTGTGGGTAAATATGATATCAAGCAACCACTGGCCCT 449
pFL61-HXK6              GAGTGTGGATTCCTTTTTGAACTTTGTGGGTAAATATGATATCAAGCAGCCTCTTGCCCT 480
                        ************************************************ ** ** *****

RO3G_05968.1            TGGCTTCGTCTTGGCCTTTCCCTTGGAACAAACCGCGTTGAATAAGGCCGTGGTGATCCA 509
pFL61-HXK6              TGGCTTCGTCTTGGCCTTTCCCTTGGAACAAACCGCGCTGAACAAGGCCGTGGTGATCCA 540
                        ************************************ **** ******************

RO3G_05968.1            GTGGACCAAGGATTTCGAAATCACGGGTGCCAACAACAAGAACATTGCCGACCTGCTCCA 569
pFL61-HXK6              GTGGACAAAGGATTTCGAAATCACGGGTGCCAGCAACAAGAACATTGCCGACCTCCTCCA 600
                        ****** ************************ ********************** *****

RO3G_05968.1            GACCGGCTTCAACCGTCGTCATCTCAACGTGCATGTGGAGGCCGTGGTGAATGGTGCGGT 629
pFL61-HXK6              GACCGGTTTCAACCGTCGTCATCTCAACGTCCATGTGGAAGCCGTGGTCAATGGTGCCGT 660
                        ****** ********************** ******** ******** ******** **

RO3G_05968.1            CGGCTGTCTCTTGGCTCACAGCTACCGCAGTCTGGACACACTCGTGGCCTGTACGATCAG 689
pFL61-HXK6              CGGCTGTCTCTTGGCTCACAGCTACCGCAGTCTGGACACACTCGTGGCCTGTACGATCAG 720
                        ************************************************************

RO3G_05968.1            CACGGGTACCAACGCCGCCTACTGGGAAAAGACGAGCGAGCTGAAAAAGACCGAGGGCCA 749
pFL61-HXK6              CACGGGTACCAACGCCGCCTACTGGGAAAAGACGAGCGAACTGAAAA-GACCGAGGGCCA 779
                        ************************************ ******* ************

RO3G_05968.1            GACGGTTCCGAACGGGGATGGCGAGATGATTGTGAATACGGAATGGGGAAGCTTTGGTGA 809
pFL61-HXK6              GACGGCTCCGAATGGGGATGGCGAGATGATTGTGAATACCGAGTGGGGAAGCTT--GTGA 837
                        ***** ****** *************************** ** ***********  ****

RO3G_05968.1            TCACAATCTCGATTTCCTTCCTCGTACGTTTTATGATAACCGGGTCAATCGTCAGTCGGT 869
pFL61-HXK6              TCACAATCTCGATTTC--TCCTCGTACATTT-ATGATA-CTGGGTCA-TCGTCA-TCGGT 891
                        ***************  ********* *** ****** * ****** ****** *****

RO3G_05968.1            CAATCCGGGTGTGCACGTCTTTGAGAAGATGGTCTCTGGTCTCTATCTGGGTGAAATCGT 929
pFL61-HXK6              CAATC-AGGTGTGC-CGTCTT--GAAAGATG-TCTCTAATCTCTATCTGGGTGAA-TCGA 945
                        *****  ******* ******    ****** *****  **************** ***

RO3G_05968.1            CCGTCTGATCATGGTCGATTTCCTCGATCGTCGTTTGATCTTTAACGGACAGTACACGAC 989
pFL61-HXK6              TCGTCTGATCATGGTCGATTT-------------------TTCA---------------- 970
                         ********************                  ** *

RO3G_05968.1            CGAACTCAACACGCCTTACTTTTTTGAATCTTCTTACATGAGTGCGATTCAATCGGATGA 1049
pFL61-HXK6              ------------------------------------------------------------


RO3G_05968.1            TACGACTGAATTGGATGAGACCCGACATATTTTGGAGTCCATCATGAACATTCCTTCGAC 1109
pFL61-HXK6              ------------------------------------------------------------
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Percent Identity Matrix - created by Clustal2.1

1: RO3G_05968.1 100.00 95.53

2: pFL61-HXK6 95.53 100.00

Clustal W Multiple Sequence Aligments for pFL61+*RoHXK7* and hypothetical *RoHXK7*

```
CLUSTAL 2.0.12 multiple sequence alignment

R03G_03473.1          ----------------------ATGACAAAAGATATCTTTAATATTATTGAAAAAGACTT 38
PFL61-HXK7            TAAAAAGCGGCGCGGGATCGATATGACAAAGAAT--CTTTAATATTATTGAAAAAGACTT 58
                                           ********  **  ************************

R03G_03473.1          TATACTTTCCAGACAGCAACTTGAACCTATCGTAAAAGGGTTTGATCAAGAGTATAAAAT 98
PFL61-HXK7            TATACTTTCCAGACTGCTCCCTCAACCTATCGTAAAAGGGTTTGATCAAGAATATAAAAT 118
                      ************** **  * * ***************************** ********

R03G_03473.1          GG-GGCTCAAGACTCCTTCCAAAGGCTTGGCTACCATGATTCCCTCATTTGTTACCAAGA 157
PFL61-HXK7            CGCGGCTCAAGACTCCTTC-AAAGGCTTGGCGACTATGATTCCCTCATTTGTTACCAAGA 177
                       * ***************** *********** ** *************************

R03G_03473.1          TGCCCAAGGGCAATGAGACCGGAACTTTTCTTTCCCTGGATATGGGTGGTACAAACTTAC 217
PFL61-HXK7            TGCCCAAGGGCAATGAGACCGGAACTTTTCTTTCCCTGGATATGGGTGGTACAAACTTAC 237
                      ************************************************************

R03G_03473.1          GTATTGCTGCTGTTGAGCTCAAGGGAGCTGGTAAAAGCACTGTTCATGAACTCAAGCGTT 277
PFL61-HXK7            GTATTGCTGCTGTTGAGCTCAAGGGAGCTGGTAAAAGCATTGTTCATGAACTCAAGCGCT 297
                      ************************************* ****************** *

R03G_03473.1          GTCCTTCCATAGAACTCAAGACAGGTGAAGGTGCAATGTTCTTTGACTGGATTGCAGATG 337
PFL61-HXK7            GTCCTTCCATAAAACTCAAGACAGGTGAAGGTGCAATGTTCTTTGACTGGATTGCAGATG 357
                      *********** ************************************************

R03G_03473.1          CTGTTCGAGATCTCATCACTATTGAAGCTAAACACTTATTTACTCAAGACCAAGTGGAAG 397
PFL61-HXK7            CTGTTCGAGATCTCATCACTATTGAAGCTAAACATTTATTTACTCAAAACCAAGTGGAAG 417
                      ********************************** ************ ************

R03G_03473.1          GTAAAGAAACTTTGGCTTTAGGTGTCTGTTGGAGTTTTCCTGTAGAGTAAAGTGTAAAAA 457
PFL61-HXK7            GTAAAGAAACTTTGGCTTTAGGTGTCTGTTGGAGTTTTCCTGTAGA-------------- 463
                      ********************************************

R03G_03473.1          AACAAAATAGCAAAGATCCCCCTTTTTTCTAAAAAAAACAACTATTTTTTTATAGTCAGA 517
PFL61-HXK7            ------------------------------------------------------------TAGA 467
                                                                                   ***

R03G_03473.1          CTGCTGTTGACCGTGGTACTATTCTTAGAATGGGAAAAGGATTTACATTAAAGAATACGG 577
PFL61-HXK7            CTGCTGTTGACCGTGGTACTATTCTCAGAATGCGAAAAGGGGTTACATTAAAGAACACGG 527
                      ************************* ****** *******  ************* ****

R03G_03473.1          AAGGGAATGATCTTGCTGATATGTTTCATGAGGCTTTCAAAAGAAAGGTAAATCAAAAAG 637
PFL61-HXK7            AAGGTAATGATCTTGCTGATATGTTTCATGAGGCTTTCAAAAGAAAG------------- 574
                      **** ****************************************

R03G_03473.1          AACAAAAAAAGATGATGTGACTAATGTAACAGATGGAATAGAAACTGAATGTCAAGGTGA 697
PFL61-HXK7            ------------------------------------------AAACTGAATGTCAAGGTGA 593
                                                                 ******************

R03G_03473.1          CTGCTATATTAAATGATACAGTGGGTACATTGGTTGCTCATGCTTATACAAATCCTGAAT 757
PFL61-HXK7            CTGCTATATTAAATGATACAGTGCGTACATTGGTTGCTCATGCTTATACGAATCCTGAAT 653
                      ********************** ************************ **********

R03G_03473.1          GCCGTATTGGTCTCATCTTTGCTACTGGTATCAACGCAGCTTATCCTGAAAAAGTATCTG 817
PFL61-HXK7            GCCGTATTGGCCTCATTTTTGCTACTGGTATCAACGCAGCTTATCCTGAAAGAGTATCTG 713
                      ********** ***** ********************************** ********

R03G_03473.1          CTATCACTAAATTAGATCCAGAGGTCCGAAATAACTATACACCAACTACCGAAATGTTGA 877
PFL61-HXK7            CTATCACTAAATTAGCTCCAGAGGTCCGAAATAACTATACACCAAACACCGAAATGTTGA 773
                      *************** ****************************  *************

R03G_03473.1          TCAATACAGAAATTGACATTTTTGGTACAGAAGCCTATCTACCCCTCACTAAATATGATC 937
PFL61-HXK7            TCAATACAGAAATTGACATTTTTGGTACGGAAGCCTATCTACCTCTCACTAAATATGATC 833
                      **************************** *************** ****************

R03G_03473.1          TCGCACTCGATCTTTCACATAATCAGCCCAAATTTCAACTTTATGAAAA-GATGCTATCA 996
PFL61-HXK7            TCGCACTCGATCTTTCACATATTCAGCCCAAATTCCAACTTTATGAAAAAGATGCTCTCC 893
                      ********************* ************ ************** ****** **

R03G_03473.1          GGT-GCCTACATGGG-TGAACTCACACGG--TTGATTGCTATGGA-CTTTATTGAGG-CA 1050
PFL61-HXK7            CGTTGCCTACATGGGATGAACTCCCCCGGGTTCAATTGCTGGGGAACTTTACTGAGGGCA 953
                       ** *********** ******* * ***  *  ******  *** ***** ***** **

R03G_03473.1          GGCATC-TTGTTTGGT-GGTGAAATACCC-AAGGGATTCAACCAA--CCTTGGTCCTTCC 1105
PFL61-HXK7            GGCCTCCTTGTTTATTTGGGGAAATGCCCCAAGGAATTCAATCAAACCCTGGGTCGTTCC 1013
                      *** ** ******  * ** ***** *** **** ****** ***  *** **** ****

R03G_03473.1          CAACCA-CTTACATGAG-CGCATTA-GAAAGCGATCAATCA--GAAACAAAAGAGG--TC 1158
PFL61-HXK7            CAACAATCTTACTTGGGGCGCACTCCAAAAGCGGTCTATCCCTAAAACAAAAGAGGGGCG 1073
```

Percent Identity Matrix - created by Clustal2.1

1: hxk7probable 100.00 92.25

2: HXK7-1-PFL61 92.25 100.00

Figure 3.2 Results of Clustal W Multiple Sequence Aligments

3.1.11.Transformation of *S. cerevisiae*

S. cerevisiae has long been a favoured organism for the study of genetics (Walker, 1998). It was the first fungus and eukaryote in which a sophisticated and well characterized transformation system was accomplished. The transformation of *S. cerevisiae* cells by foreign DNA was first described around 35 years ago. (Beggs, 1978; Hinnen et. Al, 1978)

For transformation of *S. cerevisiae* Li-acetate method was used. After the transformation procedure the cell suspension was spread on selection agar (-URA+galactose) and the agar plates were incubated at 30^{o}C for 2 to 4 days. Because the hexokinaless mutant cannot grow on glucose as carbon source; the transformed cells inoculated onto glucose and fructose medium in order to check the complementation of pFL61+*RoHXKs*. Transformant of *RoHXK1, RoHXK6* and *RoHXK7* could grow on glucose and fructose medium. As expected; *S. cerevisaea* DFY 632 hexokinaless mutant was unable to grow on glucose and fructose because of the absence of hexokinase. It could grow on galactose as a carbon source. In the absence of hexokinase, glucose can not be phosphorylated to enter the glycolysis however galactose can enter the glycolysis via Leloir's pathway.

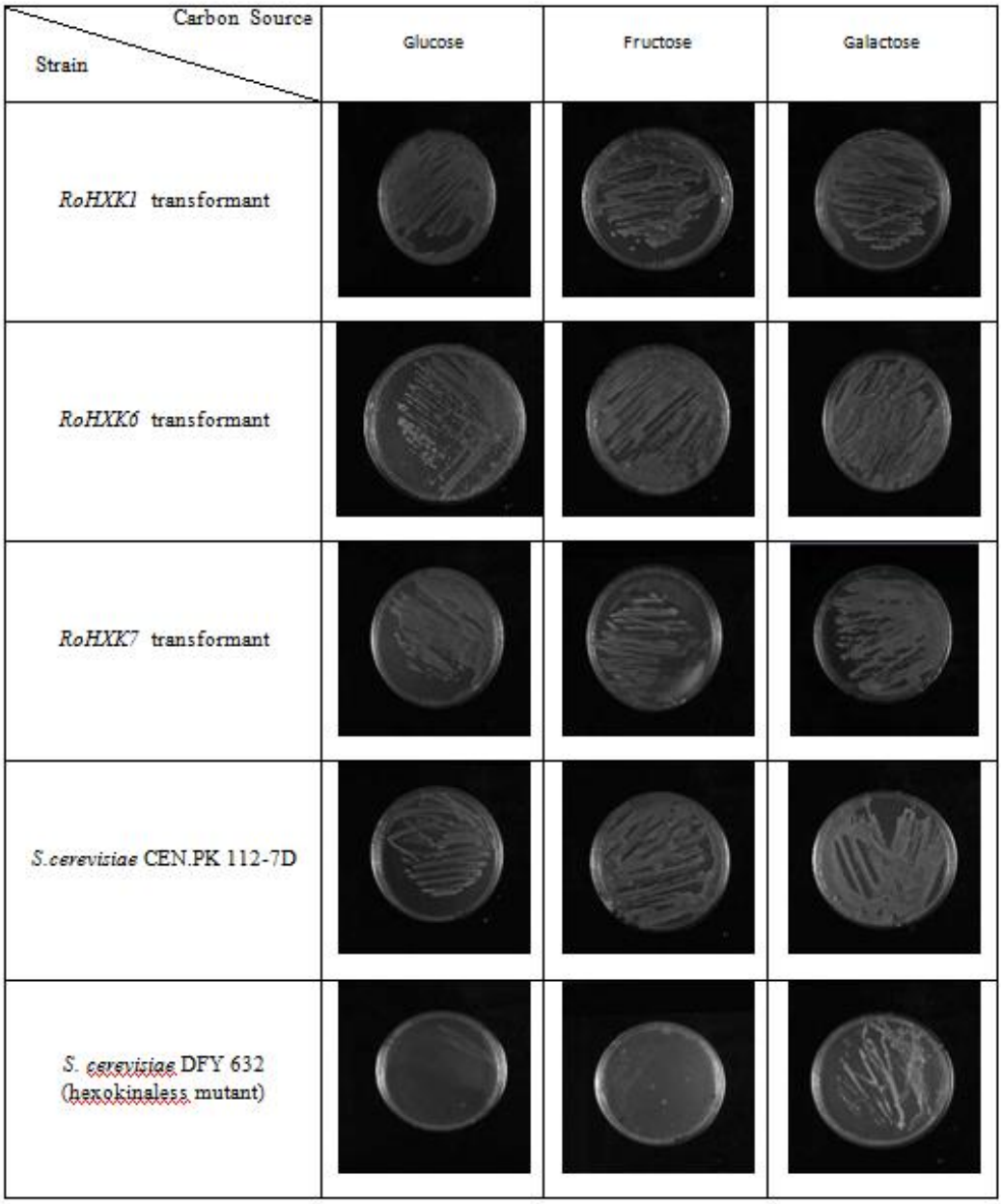

Figure 3.3. Growth profiles on agar plates (results of 4 days of growth)

3.2. Growth of Transformants

3.2.1. Specific growth rate determination

Complementation studies was done in order to check the complementation of pFL61+*RoHXK1,* pFL61+*RoHXK6* pFL61+*RoHXK7* and growth was observed on selective medium including glucose, fructose and galactose as carbon sources.

The main culture was grown at 30 ^{o}C and 160 rpm in 1 L Erlenmeyer flasks. Precultures were grown aerobically at 30 ^{o}C and 160 rpm in 250 ml Erlenmeyer flasks and inoculated to the fresh medium in the exponential phase. Figures 3.4, 3.6 and 3.8 show the OD_{600}-t graphs of main cultures of *S. cerevisiae* CEN.PK.112 and *RoHXK1* transformant*; RoHXK6* transformant; *RoHXK7* transformant in growth medium containing one of the following carbon sources; glucose, fructose or galactose. The specific growth rate-t graphs of *S. cerevisiae* in growth medium containing glucose, fructose or galactose as a carbon source are shown in Figure 3.5, 3.7 and 3.9 respectively. All experiments were carried out in two or three paralel runs.

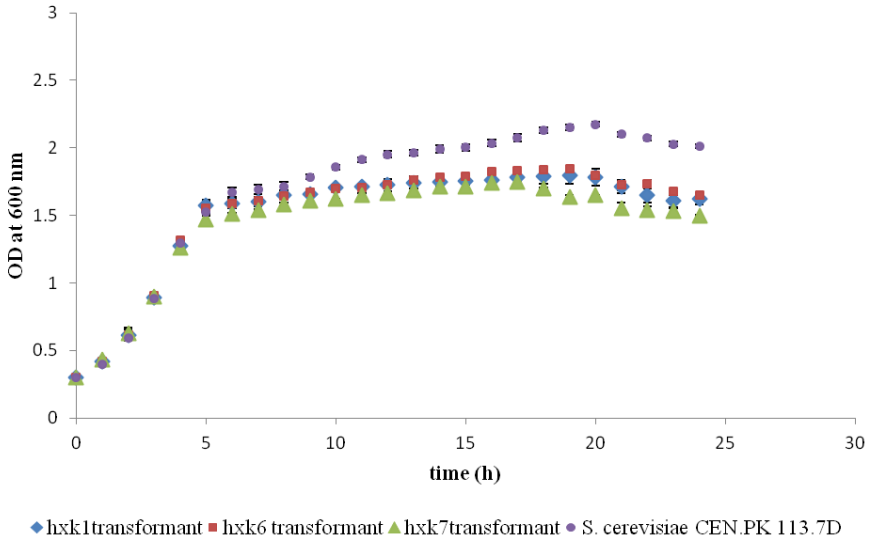

Figure 3.4 Growth of *S.cerevisiae* CEN.PK 112-7D; *RoHXK1*, *RoHXK6*, *RoHXK7* transformants of *S. cerevisiae* in growth medium containing 2% glucose as C source at 30 ^{o}C and 160 rpm

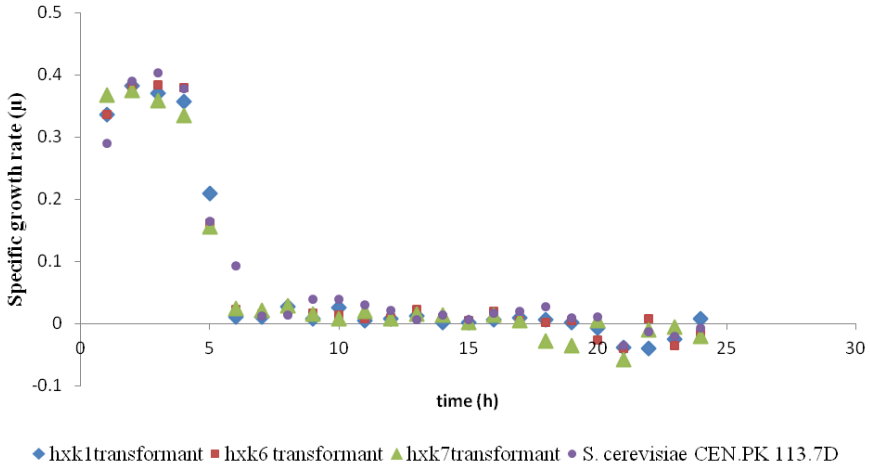

Figure 3.5 Specific growth rate (μ^{-1}) of *S.cerevisiae* CEN.PK 112-7D; *RoHXK1*, *RoHXK6*, *RoHXK7* transformants *of S. cerevisiae* in growth medium containing 2% glucose as C source at 30 ^{o}C and 160 rpm

As seen in OD_{600}-t graphs of main cultures of *S. cerevisiae* in growth medium containing glucose as carbon source in Figure 3.4; the OD_{600} values of *S. cerevisiae* CEN.PK 112-7D was slightly higher than transformants; the growth pattern of of transformant was similar and lower than *S. cerevisiae* CEN.PK 112-7D. As expected hexokinase mutant did not grow in growth medium containing glucose as carbon source. In glucose containing media; the maximum specific growth rate was 0.40 h^{-1} for *S. cerevisiae* CEN.PK 112-7D; 0.38 h^{-1} for *RoHXK1* and *RoHXK6* transformants of *S. cerevisiae*; 0.37 h^{-1} for *RoHXK7* transformant of *S. cerevisiae* as seen in Figure 3.5.

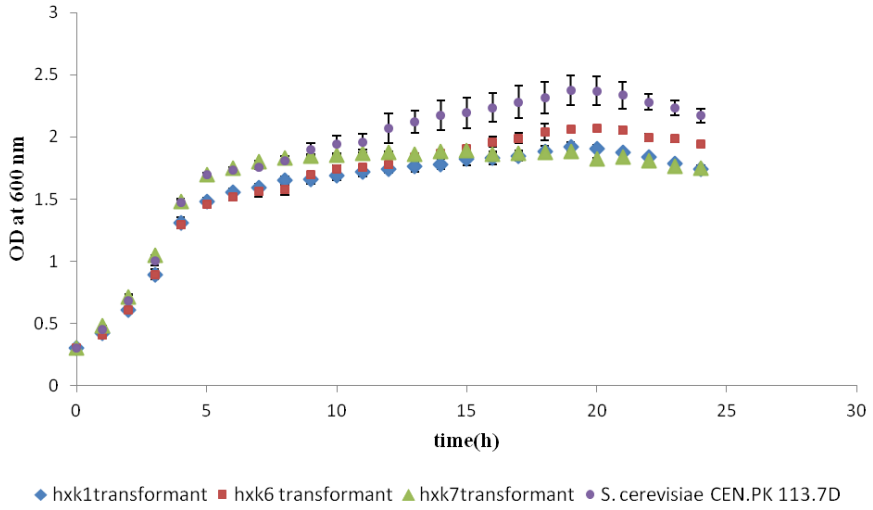

Figure 3.6 Growth of *S.cerevisiae* CEN.PK 112-7D; *RoHXK1*, *RoHXK6*, *RoHXK7* transformants of *S. cerevisiae* in growth medium containing 2% fructose as C source at 30 ^{o}C and 160 rpm

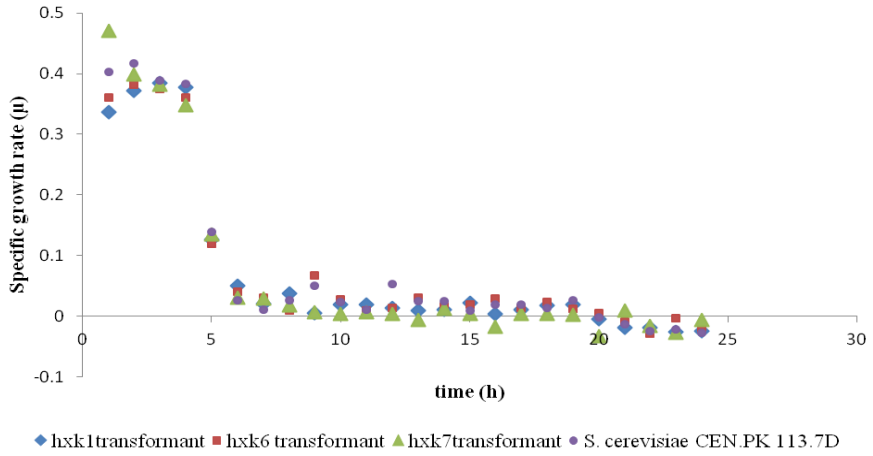

Figure 3.7 Specific growth rate (μ^{-1}) of *S.cerevisiae* CEN.PK 112-7D; *RoHXK1*, *RoHXK6*, *RoHXK7* transformants of *S. cerevisiae* in growth medium containing 2% fructose as C source at 30 ^{o}C and 160 rpm

In fructose containing media as a carbon source; the OD_{600} values of *S. cerevisiae* CEN.PK 112-7D was slightly higher than transformants as seen in Figure 3.6. Hexokinase mutant did not grow in fructose contaning medium as a carbon source as expected. The maximum specific growth rate was 0.41 h^{-1} for *S. cerevisiae* CEN.PK 112-7D; 0.39 h^{-1} for *RoHXK1* and *RoHXK7* transformants of *S. cerevisiae*; 0.38 h^{-1} for *RoHXK6* transformant of *S. cerevisiae* as seen in Figure 3.7.

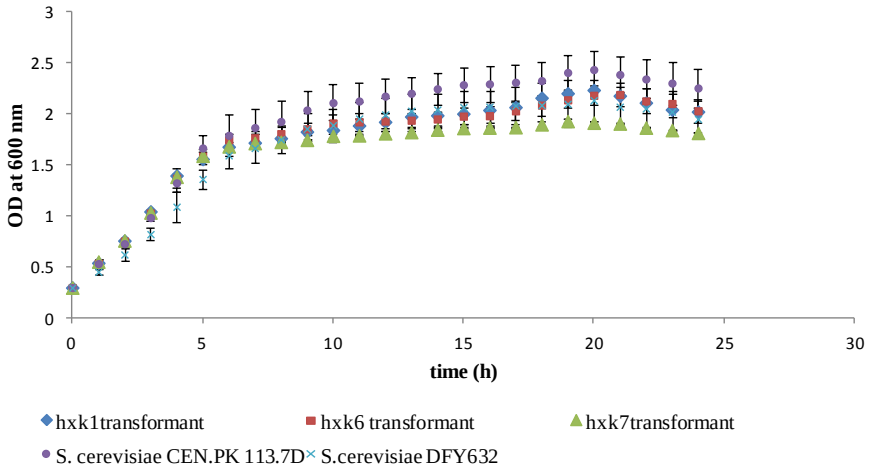

Figure 3.8 Growth of *S.cerevisiae* CEN.PK 112-7D; *RoHXK1*, *RoHXK6*, *RoHXK7* transformants of *S. cerevisiae* and hexokinase mutant in growth medium containing 2% galactose as C source at 30 ^{o}C and 160 rpm

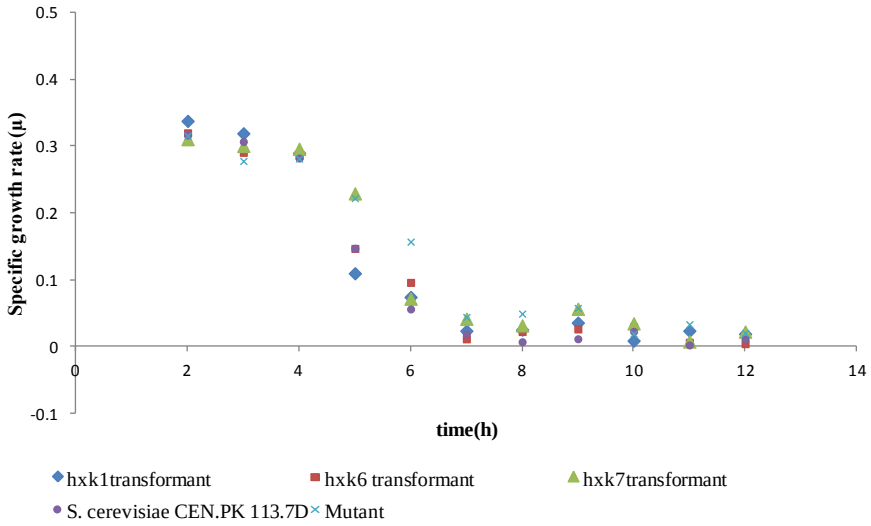

Figure 3.9 Specific growth rate (μ^{-1}) of *S.cerevisiae* CEN.PK 112-7D; *RoHXK1*, *RoHXK6*, *RoHXK7* transformants of *S. cerevisiae* in growth medium containing 2% galactose as C source at 30 ^{o}C and 160 rpm

As shown in Figure 3.8; in growth medium containing galactose as carbon source, the OD_{600} values of transformants were slightly lower than *S. cerevisiae* CEN.PK 112-7D and *S. cerevisiae* CEN.PK 112-7D reached the maximum OD_{600} values like in other carbon sources. Hexokinase mutant also grew in growth medium containing galactose as carbon source. The maximum specific growth rate was 0.31 h^{-1} for *S. cerevisiae* CEN.PK 112-7D; 0.33 h^{-1} for *RoHXK1* transformant of *S. cerevisiae* and 0.32 h^{-1} for *RoHXK6* transformant of *S. cerevisiae*; 0.31 h^{-1} for *RoHXK7* transformant of *S. cerevisiae*, 0.31 h^{-1} for hexokinase mutant of *S. cerevisiae* in galactose containing media as seen in Figure 3.9.

3.2.2. Dry cell weight determination

Dry cell weights were determined by using a standart curve which is shown in Appendix G (Alagöz Eda; 2005). The OD_{600} versus time graphs which are shown in Figure 3.4, 3.6, 3.8 were converted to dry cell weight versus time graphs (Figure 3.10, 3.11, 3.12).

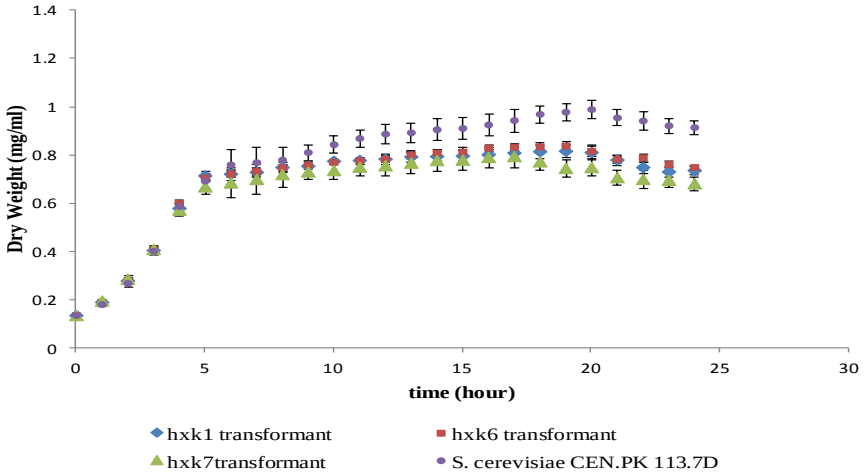

Figure 3.10 Dry cell weight of *S.cerevisiae* CEN.PK 112-7D; *RoHXK1*, *RoHXK6*, *RoHXK7* transformants of *S. cerevisiae* in growth medium containing 2% glucose as C source at 30 ^{o}C and 160 rpm

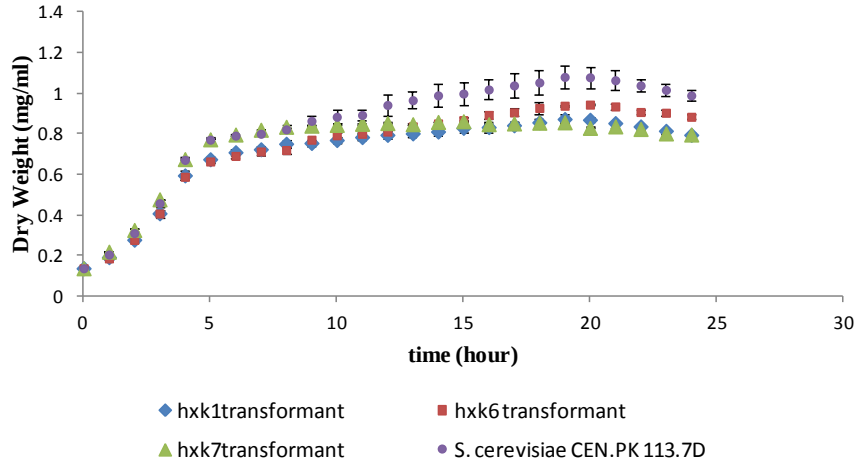

Figure 3.11 Dry cell weight of *S.cerevisiae* CEN.PK 112-7D; *RoHXK1*, *RoHXK6*, *RoHXK7* transformants of *S. cerevisiae* in growth medium containing fructose as C source at 30 ^{o}C and 160 rpm

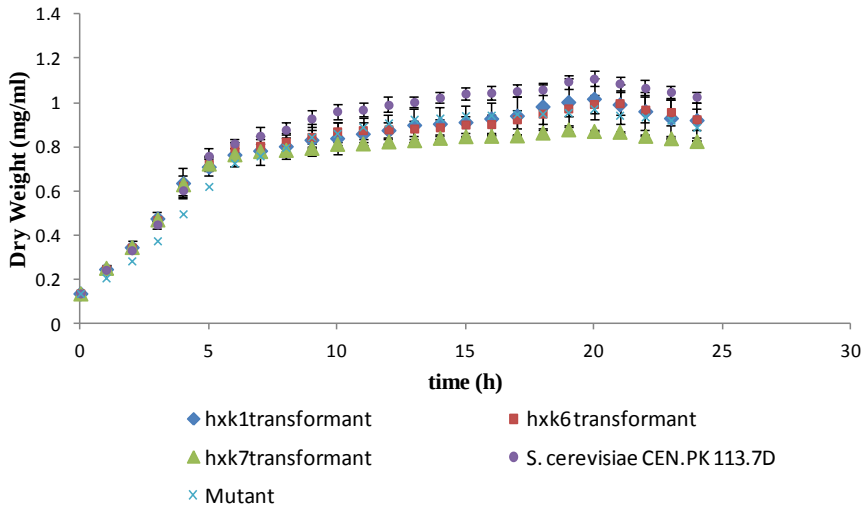

Figure 3.12 Dry cell weight of *S.cerevisiae* CEN.PK 112-7D; *RoHXK1, RoHXK6, RoHXK7* transformants *of S. cerevisiae* in growth medium containing 2% galactose as C source at 30 ^{o}C and 160 rpm

As shown in Figure 3.10, 3.11, 3.12; in glucose containing medium the maximum dry cell weight reached during growth is 0.990 mg/ml for *S. cerevisiae* CEN.PK 112-7D; 0.817 mg/ml for *RoHXK1* transformant of *S. cerevisiae* and 0.839 mg/ml for *RoHXK6* transformant of *S. cerevisiae*; 0.795 mg/ml for *RoHXK7* transformant of *S. cerevisiae.* The results in fructose containing medium were as following; 1.080 mg/ml for *S. cerevisiae* CEN.PK 112-7D; 0.872 mg/ml for *RoHXK1* transformant of *S. cerevisiae* and 0.943 mg/ml for *RoHXK6* transformant of *S. cerevisiae*; 0.856 mg/ml for *RoHXK7* transformant of *S. cerevisiae.* In galactose containing medium; the maximum dry cell weight was 1.109 mg/ml for *S. cerevisiae* CEN.PK 112-7D; 1.018 mg/ml for *RoHXK1* transformant of *S. cerevisiae* and 0.998 mg/ml for *RoHXK6* transformant of *S. cerevisiae*; 0.879 mg/ml for *RoHXK7* transformant of *S. cerevisiae* and 0.972 mg/ml for hexokinase mutant.

3.2.3. Sugar utilization and ethanol production by *S.cerevisiae* CEN.PK 112-7D and *RoHXK1, RoHXK6, RoHXK7* transformants of *S.cerevisiae*

HPLC was used for glucose and ethanol analyzes. Figure 3.13, 3.14, 3.15, and 3.16 the concentration of glucose, galactose and ethanol amounts during the growth of the *S.cerevisiae* CEN.PK 112-7D, *RoHXK1, RoHXK6, RoHXK7* transformants of *S.cerevisiae* are shown. As seen in figures in *S.cerevisiae* CEN.PK 112-7D culture; the remaining glucose concentration was lower and the ethanol formation was higher than *RoHXKs* transformants at the end of the 24th hour. In *RoHXK1, RoHXK6, RoHXK7* glucose and the ethanol concentration were similar to each other in media containing glucose, fructose and galactose as a carbon source.

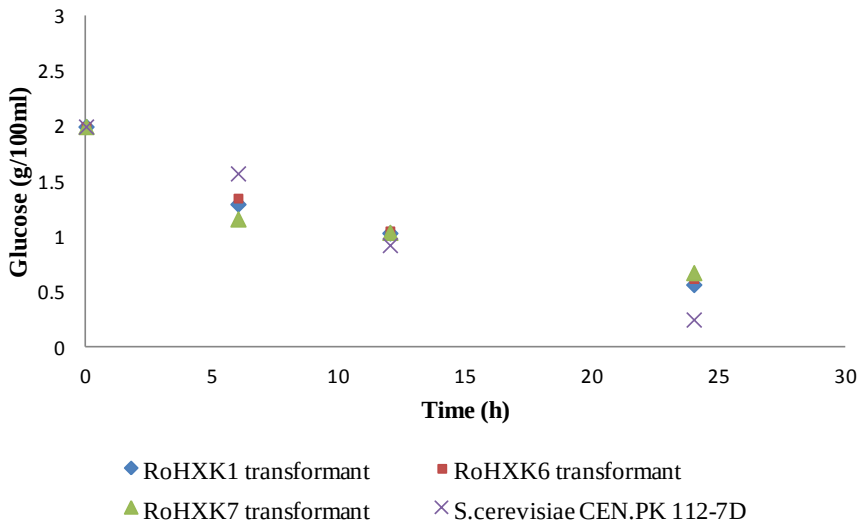

Figure 3.13 Glucose utilization by *S.cerevisiae* CEN.PK 112-7D; *RoHXK1*, *RoHXK6*, *RoHXK7* transformants of *S. cerevisiae* in growth medium containing glucose as C source at 30 ^{o}C and 160 rpm

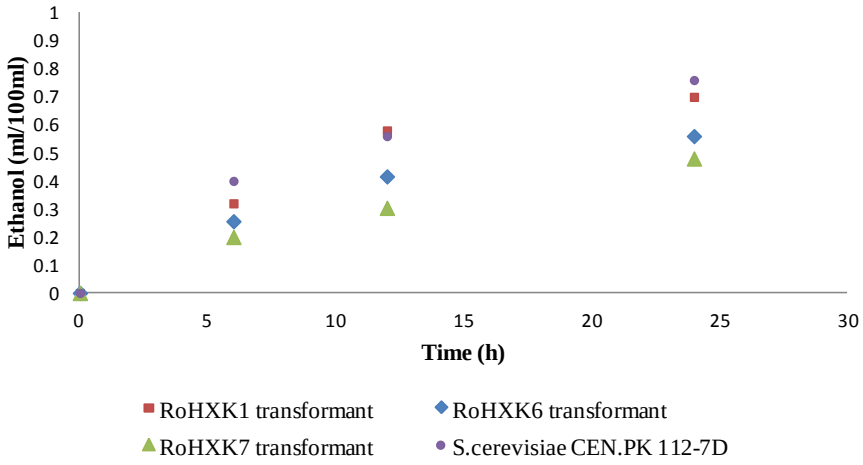

Figure 3.14 Ethanol production by *S.cerevisiae* CEN.PK 112-7D and *RoHXK1*, *RoHXK6*, *RoHXK7* transformants of *S.cerevisiae* in growth medium containing glucose as C source at 30 ^{o}C and 160 rpm

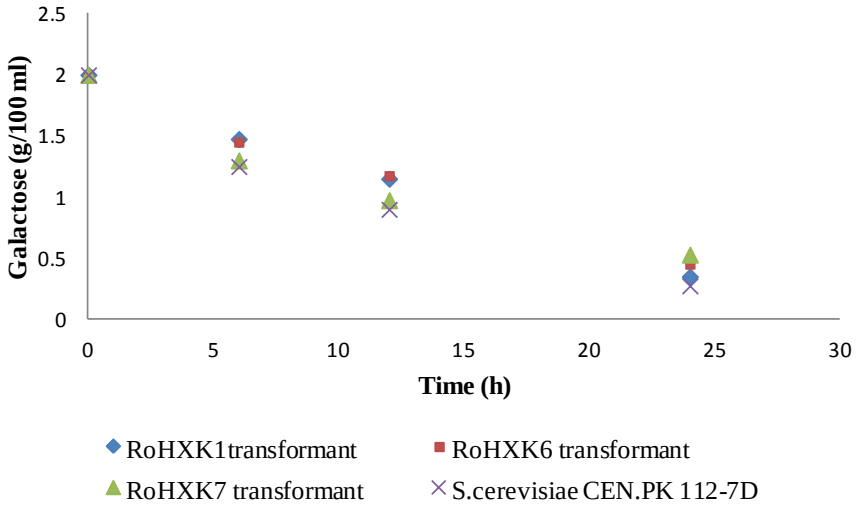

Figure 3.15 Galactose utilization by *S.cerevisiae* CEN.PK 112-7D and *RoHXK1*, *RoHXK6*, *RoHXK7* transformants of *S.cerevisiae* in growth medium containing galactose as C source at 30 ^{o}C and 160 rpm

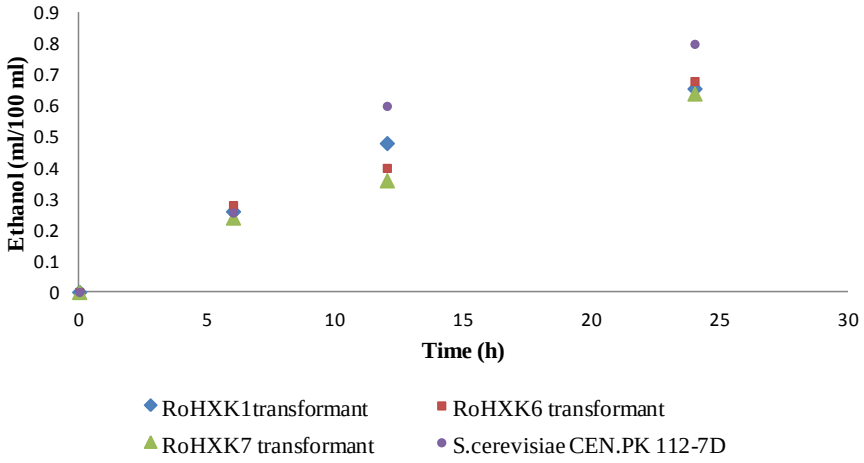

Figure 3.16 Ethanol production by *S.cerevisiae* CEN.PK 112-7D and *RoHXK1*, *RoHXK6*, *RoHXK7* transformants of *S.cerevisiae* in growth medium containing galactose as C source at 30 ^{o}C and 160 rpm

Table 3.6 The growth parameters of *S.cerevisiae* CEN.PK 112-7D, *RoHXK1, RoHXK6, RoHXK7* transformants, *S. cerevisiae* DFY 632 (hexokinaless mutant of *S.cerevisiae)* on different carbon sources

Carbon Source	Strain	$\mu_{max\ (h^{-1})}$	$(OD_{600})_{max}$	$(\text{Dry cell weight})_{max}$
Glucose	*S.cerevisiae* CEN.PK 112-7D	0.40	2.176	0.990
	RoHXK1 transformant	0.38	1.796	0.817
	RoHXK6 transformant	0.38	1.844	0.839
	RoHXK7 transformant	0.37	1,748	0.795
	S. cerevisiae DFY 632 (hexokinaless mutant)	-	-	-
Fructose	*S.cerevisiae* CEN.PK 112-7D	0.41	2.373	1.080
	RoHXK1 transformant	0.39	1.917	0.872
	RoHXK6 transformant	0.38	2.073	0.943
	RoHXK7 transformant	0.39	1.893	0.856
	S. cerevisiae DFY 632 (hexokinaless mutant)	-	-	-
Galactose	*S.cerevisiae* CEN.PK 112-7D	0.31	2.236	1.109
	RoHXK1 transformant	0.33	2.192	1.018
	RoHXK6 transformant	0.32	1.932	0.998
	RoHXK7 transformant	0.31	2.436	0.879
	S. cerevisiae DFY 632 (hexokinaless mutant)	0.31	2.136	0.972

As seen in Table 3.6; the μ_{max}; $(OD_{600})_{max}$ and (Dry cell weight)$_{max}$ values of *S. cerevisiae* CEN.PK 112-7D were slightly higher than *RoHXK1, RoHXK6, RoHXK7* transformants of *S. cerevisiae* in growth media containing glucose, fructose or galactose as carbon source. *A*t the end of the 24th hour; the remaining glucose concentration was lower and the ethanol production was higher than *RoHXKs* transformants. These results showed that *S. cerevisiae* CEN.PK 112-7D utilized the carbon sources more effective than *RoHXK1, RoHXK6* and *RoHXK7* transformants. According to these results it can be said that the sugar utilization activity of hexokinase of *S. cerevisiae* CEN.PK 112-7D is more than transformed hexokinase genes.

Another results from the values in Table 3.6; the μ_{max}; $(OD_{600})_{max}$ and (Dry cell weight)$_{max}$ values of *RoHXK1, RoHXK6, RoHXK7* transformants of *S. cerevisiae* were similar in each medium. The remaining glucose concentration and the ethanol production at the end of the 24th hour were also similar to each other.

In *S. cerevisiae*; according to the source and the amount of carbon, the transcriptional regulation of the genes that encode hexose-phosphorylating enzymes varies. (Herrero et al., 1995). The expression of hexokinase 2 is higher than that that of other hexoses-phosphorylated enzymes in glucose-grown *S. cerevisiae*, (Herrero, P. Et al., 1995 18). From this point of view, it is possible that hexokinase expressed from *RoHXK1, RoHXK6, RoHXK7* genes may transcript at differrent growth conditions in *Rhizopus oryzae*.

It was seen from Table 3.6; in galactose containing media μ_{max} (h^{-1}) was lower but $(OD_{600})_{max}$ and (Dry cell weight)$_{max}$ values were higher than values in glucose and fructose in all strains. The remaining sugar concentration was lower and the ethanol formation was higher than *RoHXKs* transformants at the end of the 24th hour. The lower specific growth rate in galactose medium can be due to the entire pathway of galactose in glycolysis. Galactose enters the glycolysis via Leloir's Pathway by converting glucose-6-phosphate. The pathway involves four reactions. Therefore the entry of galactose into glycolysis is slower than glucose or fructose. Also when the carbon source is utilized in a slower manner the conversion to biomass is more efficient.

3.3. The hexokinase activity of *S.cerevisiae* CEN.PK 112-7D and *RoHXK1, RoHXK6, RoHXK7* transformants

3.3.1. Optimization of sonication conditions for preparation of crude extract

The cell crude extracts were prepared as explained in Chapter 2. The cell distruption was performed with sonicator. The sonication time and the optimum amplitude were optimised. For optimization the same volume of sample (10 ml) was sonicated separately for 3, 5, 10, 15 and 20 minutes and crude extract were prepared by centrifugation of sonicated material. The specific activity of hexokinase was determined in these crude extracts.The results are shown in Figures. As seen from Figure 3.17 and Figure 3.18 there was an increase in specific activity with the time of sonication up to 15 minutes and the specific activity became decrease at 20 min. It could be cause the denaturation of the enzymes due to the heated up in time. The results showed that at 15 minutes and 80 amp the maximum specific activity of

hexokinase was obtained. According to optimized conditions, wet yeast cells in 4% of the extraction buffer were sonicated at 80 amp for 15 min with 0.5 cycle in this study.

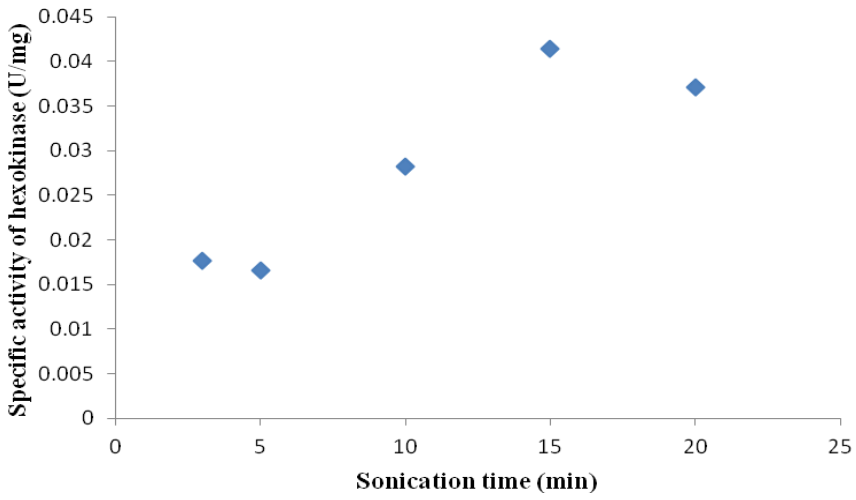

Figure 3.17 Specific activity of hexokinase (U/mg) of *S. cerevisiae* CEN.PK.112-7D

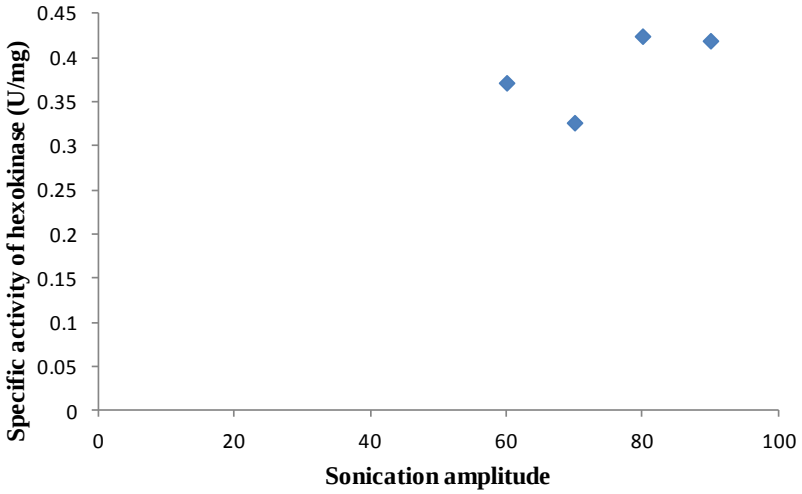

Figure 3.18 Specific activity of hexokinase (U/mg) of *S. cerevisiae* CEN.PK.112-7D

3.3.2. The hexokinase activity of *S.cerevisiae* CEN.PK 112-7D and *RoHXK1, RoHXK6, RoHXK7* transformants

Enzyme activities were assayed spectrophotometrically as described in "Materials and Methods". In Table 3.7. the specific activities of hexokinases from *S.cerevisiae* CEN.PK 112-7D, *RoHXK1* transformant, *RoHXK6* transformant, *RoHXK7* transformant are represented. As seen in Table 3.7 the specific activity of hexokinase of *S.cerevisiae* CEN.PK 112-7D were slightly higher than *RoHXK1* transformant, *RoHXK6* transformant, *RoHXK7* transformant. As shown Table 3.6; in growth media containing glucose, fructose or galactose as carbon source, the μ_{max}; $(OD_{600})_{max}$ and $(\text{Dry cell weight})_{max}$ values of *S.cerevisiae* CEN.PK 112-7D were slightly higher than *RoHXK1, RoHXK6, RoHXK7* transformants of *S.cerevisiae.* The remaining glucose concentration was lower and the ethanol formation at the end of the 24^{th} hour was higher than *RoHXKs* transformants.

As shown in Table 3.7, the hexokinase activities in fructose containing medium were higher than that of in glucose and galactose containing medium. In studies done with wild type and hxk2 mutant of S. cerevisiae (Diderich et al., 2000, 2002) it was also shown that a slightly higher specific phosphorylating activity of hexokinase was measured with fructose than with glucose which indicates the presence of hexokinase I. Therefore the higher specific activities in fructose medium that displayed in Table 3.7 may cause due to the increase in hexokinase I in *S. cerevisiae* CEN.PK.113.7D and also the kinetic properties of hexokinase enzymes of *RoHXKs* transformants may similar to the kinetic properties of yeast hexokinase I.

There are many purification, cloning and characterization studies have been done for hexokinases and glukokinases of many organisms (Easterby&Rosemeyer,1972; Laht, 2002;Maitra,1970, ; Panneman et.al., 1996;1998; Rose ,1995; Ruiz-Amil & Sols, 1961; Steinböck et.al. ,1994). It was seen from the cloning studies of many eukaryotic hexokinase and glucokinase genes so far the kinetic and regulatory properties of the these enzymes differ considerably although they appear to have arisen from a common ancestor (Panneman et al.,1998). According to the these results the cloned hexokinase genes of *R. oryzae* may probably have different kinetic and regulatory properties than *S. cerevisiae*. Further characterization and purification studies need be done for each of the cloned enzymes and kinetic and regulatory properties can be studied using these purified enzymes.

Table 3.7 The hexokinase activity of *S.cerevisiae* CEN.PK 112-7D and *RoHXK1, RoHXK6, RoHXK7* transformants

Organism	Glucose as carbon source Specific Act. (U/mg)	Fructose as carbon source Specific Act. (U/mg)	Galactose as carbon source Specific Act. (U/mg)
RoHXK1 transformant	**0.1517±0.005**	**0.2016±0.006**	**0.1069±0.010**
RoHXK6 transformant	**0.1134±0.008**	**0.2790±0.016**	**0.1200±0.021**
RoHXK7 transformant	**0.1301±0.001**	**0.2641±0.019**	**0.1832±0.014**
S. cerevisiae CEN.PK.113-7D	**0.2200±0.001**	**0.2801±0.023**	**0.2440±0.004**

3.4. The Inhibition of hexokinase by trehalose-6-phosphate

Trehalose-6-phosphate is the potent inhibitor of *Saccharomyces cerevisiae* and *Aspergillus niger* hexokinase while in both organism glucokinase are not affected by this intermediate metabolite.The effect of trehalose-6-phosphate on in Ro*hxk1* transformant; Ro*hxk6* transformant; Ro*hxk7* transformant were investigated by measuring the hexokinase activity in the presence and absence of trehalose-6-phosphate in crude extract. In order to show the inhibition effect of trehalose-6-phosphate commercial yeast hexokinase and crude extract of *S.cerevisiae* CEN.PK 112-7D were used as a positive control. Different concentrations of trehalose-6-phosphate were tested.

As seen in table; while the commercial hexokinase was inhibited by trehalose-6-phosphate; there was not any inhibition effect observed in hexokinase of transformants. Surprisingly; the inhibition effect of trehalose-6-phosphate could not be observed in hexokinase of *S. cerevisiae* CEN.PK.113-7D. According to these results; the effect of trehalose-6-phosphate on hexokinase couldn't be observed in crude extracts.

Trehalose-6-phosphate is one of the intermediate in trehalose synthesis. Trehalose is synthesized from UDP-glucose and glucose-6-phosphate via trehalose-6-phosphate. (Cabib and Leloir, 1958). Trehalose-6-phosphate is converted to trehalose by trehalose-6-phosphatase. Therefore it could be possible dephosphorylation of trehalose-6-phosphate to trehalose by trehalose-6-phosphatase in crude extract.

Table 3.8 Effect of Trehalose-6-phosphate on Hexokinase

	Trehalose-6-phosphate concentration	Activity (U/ml)
HXK commercial (Sigma Hexokinase from *Saccharomyces cerevisiae*)	w/o Trehalose-6-phoshate	0.812
	2µM Trehalose-6-phoshate	0.365
	6µM Trehalose-6-phoshate	0.201
S.cerevisiae CEN.PK 112-7D	w/o Trehalose-6-phoshate	1.202
	2µM Trehalose-6-phoshate	1.201
	6µM Trehalose-6-phoshate	1.181
	10µM Trehalose-6-phoshate	1.210
RoHXK1 transformant	w/o Trehalose-6-phoshate	0.320
	2µM Trehalose-6-phoshate	0.326
	6µM Trehalose-6-phoshate	0.317
	10µM Trehalose-6-phoshate	0.326
RoHXK6 transformant	w/o trehalose	0.246
	2µM	0.249
	6µM	0.244
	10µM	0.253
RoHXK7 transformant	w/o Trehalose-6-phoshate	0.303
	2µM Trehalose-6-phoshate	0.311
	6µM Trehalose-6-phoshate	0.300
	10µM Trehalose-6-phoshate	0.306

CHAPTER 4

CONCLUSIONS

Rhizopus oryzae is a filamentous fungus and it has some advantages as a candidate for production of lactic acid and ethanol like the ability of grow on some renewable carbon sources. However, in *R. oryzae* the production yields of these products are lower compared to alternative organisms. The carbon source utilization through glycolytic pathway can end with lactic acid or ethanol production. Therefore for increasing the yield of the desired end product, glycolytic pathway is the focus of our search. Hexokinase catalyses the first step of glycolytic pathway. As this step is also irreversible it is assumed as one of the major control points in this pathway.

In this study, the probable hexokinase genes were screened from the *R. oryzae* genome database by doing a blast search with the known protein sequences of closely related *S. cerevisiae* hexokinases and glucokinase. PCR bands of seven of the ten probable hexokinase genes were obtained. The sequence analysis showed that three of these obtained genes were expressed in *R. oryzae*. In the complementation studies in hexokinaless mutant, the growth of the transformants was monitored on glucose or fructose as a carbon source.

In growth media containing glucose, fructose or galactose as carbon source, the μ_{max}; $(OD_{600})_{max}$ and $(\text{Dry cell weight})_{max}$ values of *S.cerevisiae* CEN.PK 112-7D were slightly higher than *RoHXK1, RoHXK6, RoHXK7* transformants of *S.cerevisiae.* At the end of the 24^{th} hour in *S.cerevisiae* CEN.PK 112-7D culture the remaining glucose concentration was lower and the ethanol formation was higher than *RoHXKs* transformants.

The μ_{max}; $(OD_{600})_{max}$ and $(\text{Dry cell weight})_{max}$ values of *RoHXK1, RoHXK6, RoHXK7* transformants of *S.cerevisiae* were similar in each of the medium. The remaining glucose concentration and the ethanol formation at the end of the 24^{th} hour were also similar to each other.

In galactose containing media μ_{max} is lower but $(OD_{600})_{max}$ and $(\text{Dry cell weight})_{max}$ values were slightly higher than values in glucose and fructose in all strains. The remaining sugar concentration was lower and the ethanol formation at the end of the 24^{th} hour was higher than *RoHXKs* transformants.

The specific activity of hexokinase of *S.cerevisiae* CEN.PK 112-7D was slightly higher than *RoHXK1* transformant, *RoHXK6* transformant, *RoHXK7* transformant. In growth media containing glucose, fructose or galactose as carbon source, the μ_{max} ; $(OD_{600})_{max}$ and $(\text{Dry cell weight})_{max}$ values of *S.cerevisiae* CEN.PK 112-7D were slightly higher than *RoHXK1,*

RoHXK6, RoHXK7 transformants of *S.cerevisiae.* The remaining glucose concentration was lower and the ethanol formation at the end of the 24th hour was slightly higher than *RoHXKs* transformants.

The effect of trehalose-6-phosphate on in *RoHXK1* transformant; *RoHXK6* transformant; *RoHXK7* transformant were investigated by measuring the hexokinase activity in the presence and absence of trehalose-6-phosphate in crude extract. In order to show the inhibition effect of trehalose-6-phosphate commercial yeast hexokinase and crude extract of *S.cerevisiae* CEN.PK 112-7D were used as a positive control.While the commercial hexokinase was inhibited by trehalose-6-phosphate; there was not any inhibition effect observed in hexokinases of transformants. Surprisingly; the inhibition effect of trehalose-6-phosphate could not be observed in hexokinase of *S. cerevisiae* CEN.PK.113-7D. The effect of trehalose-6-phosphate on hexokinase could not be observed in crude extracts.

According these results the cloned hexokinase genes of *R. oryzae* may probably different kinetic and regulatory properties than *S. cerevisiae*. For the further characterization purification studies can be done for each of the cloned enzymes and kinetic and regulatory properties can be studied in purified enzymes.

REFERENCES

1. Açar Ş.; (1999); Isolation and Preliminary Characterization of Hexokinase from *Rhizopus oryzae*. Msc in Biotechnology,METU.

2. Açar Ş.; (2004); Biochemical and Genetic Studies on The Pyruvate Branch Point Enzymes of *Rhizopus oryzae*; PhD in Biotechnology,METU.

3. Acar Y, Trindade L, Yucel M, Hamamci H, De Graaff LH; (2005); Expression of lactate dehydrogenase genes of Rhizopus oryzae in *Aspergillus niger;* Journal of Biotechnology; 118/ 1/ S95-S96

4. Acar S, Yücel M, Hamamcı H.; (2007); Purification and characterisation of two isozymes of pyruvate decarboxylase from *Rhizopus oryzae*; Enzyme and Microbial Technology; 40/ 4/ 675-682 2007

5. Ahuatzi D, Herrero P, de la Cera T & Moreno F (2004) The glucose-regulated nuclear localization of hexokinase 2 in Saccharomyces cerevisiae is Mig1-dependent. J Biol Chem 279: 14440–14446.

6. Ahuatzi D, Riera A., Pelaez R., Herrero P & Moreno F (2007) Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution. J Biol Chem 282; 4485-4493

7. Arisan-Atac, I; Wolschek, M. F. & Kubicek, C. P. (1996). Trehalose-6-phosphate synthase A affects citrate accumulation by *Aspergillus niger* under conditions of high glycolytic flux. *FEMS Microbial Lett* 140, 77-83.

8. Arslan A.; (2001); Partial Purification and Kinetic Characterization of L-lactate Dehydrogenase from *Rhizopus oryzae*. Msc in Biotechnology,METU.

9. Behlke, J., K. Heidrich, M. Naumann, E. C. Muller, A. Otto, R. Reuter, and T. Kriegel. 1998. Hexokinase 2 from *Saccharomyces cerevisiae*: regulation of oligomeric structure by *in vivo* phosphorylation at serine-14. Biochemistry 37:11989–11995.

10. Biochemistry. 5th edition. Berg JM, Tymoczko JL, Stryer L. New York: W H Freeman; 2002.

11. Bisson L. F., Fraenkel D. G. (1983). Involvement of Kinases in Glucose and Fructose Uptake by Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 80, 1730-1734

12. Bisson L. F., Fraenkel D. G. (1984). Expression of kinase dependent glucose uptake in *Saccharomyces cerevisiae*. *J. Bacteriol*. 159:1013-1017

13. Blazquez. M. A., Lagumis. R.. Ganccdo, C. & Gancedo, J. M. (1993) Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases, *FEBS Lett. 329.* 51-54.

14. Büyükkileci AO, Hamamcı H, Yücel M; (2006); Lactate and ethanol productions by *Rhizopus oryzae* ATCC 9363 and activities of related pyruvate branch point enzymes; Journal of Bioscience and Bioengineering; 102/ 5/ 464-466

15. . Büyükkileci AO; (2007); Investigation of Sugar Metabolism in *Rhizopus oryzae;* PhD in Biotechnology; METU

16. Cardenas, M. L., A. Cornish-Bowden, and T. Ureta. 1998. Evolution and regulatory role of the hexokinases. Biochim. Biophys. Acta 1401:242–264.

17. Carlson, M. 1998. Regulation of glucose utilization in yeast. Curr. Opin. Genet. Dev. 8:560–564.

18. Colowick. S. P. (1973t The hexokinases, in *The enzymes* (Boyer, P. D.ed.) vol.9, pp. I-4!^. Academic Press. New York and London.

19. Datta R., Tsai S.P.,Bonsignore P.; Moon S.H.; Frank J.R; (1995). Technological and economic potential of poly(lactic acid) and lactic acid derivatives. Fems Microbiology Reviews 16 (1995) 221-231

20. Dedeoğlu, D., (2007); "Purification and kinetic characterization of hexokinase from *Rhizopus oryzae*.", MSc in Biotechnology, METU

21. De Winde JH, Crauwels M, Hohmann S, Thevelein JM, Winderickx J (1996) Differential requirement of the yeast sugar kinases for sugar sensing in establishing the catabolite-repressed state. Eur J Biochem 241:633–643

22. Diderich, J. A., Raamsdonk, L. M., Kruckeberg, A. L., Berden, J. A., Van Dam, K. (2001). Physiological Properties of Saccharomyces cerevisiae from Which Hexokinase II Has Been Deleted. *Appl. Environ. Microbiol.* 67: 1587-1593

23. Diderich, J. A., Raamsdonk, L. M., Kuiper A., Kruckeberg, A. L., Berden, J. A.,Joost Teixeira de Mattos M; Van Dam, K. (2002). Effects of a hexokinase II deletion on the Dynamics of glycolysis in continuous cultures of *Saccharomyces cerevisiae*; *FEMS Yeast Research*; 165-172

24. Easterby, J. S. & Rosemeyer, M. A. (1972); Purification and subunit interactions of yeast hexokinase, Eur. J. Biochem. 28, 241-252.

25. Efrat, S.; Tal, M. & Lodish, H. F. (1994) The pancreatic beta-cell glucose sensor. Trends Biochem. Sci. 19, 535-538

26. Entian K-D (1980) Genetic and biochemical evidence for hexokinase PII as a key enzyme involved in catabolite repression in yeast. Mol Gen Genet 178:633–637

27. Entian, K.D. and Barnett, J.A. (1992). Regulation of sugar utilization by *Saccharomyces cerevisiae*. Trends Biol. Sci. 17, 506-510

28. Entian K-D (1997) Sugar phosphorylation in yeast. In: Zimmermann FK, Entian K-D (eds) Yeast sugar metabolism, Technomic Publishing, Basel, pp 67–79

29. Fernandez, R.; Herrero, P., Fernandez, M. T., and Moreno, F. (1986) *J. Gen. Microbiol.* 132, 3467-3472.

30. Flipphi M, Vondervoort PJI van de, Rujter GJG, Visser J, Arst HN Jr, Felenbok B (2003) Onset of carbon catabolite repression in Aspergillus nidulans: parallel involvement of hexokinase and glucokinase in sugar signalling. J Biol Chem 278:11849–11857

31. Gancedo, J. M.. Clifton, D. & FraenkeL D. G. (1977) Yeast hexokinase mutants, ./. *Biol. Client. 252.* 4443-4444.

32. Gancedo,J.M.(1992).Carbon catabolite repression in yeast.Eur.J.Biochem206:297-313.

33. Gancedo, J. M. 1998. Yeast carbon catabolite repression. Microbiol. Mol. Biol. Rev. 62:334–361.

34. German, M. S. (1993). Glucose sensing in pancreatic islet beta cells: the key role of glucokinase and the glycolytic intermediates. *Proc. Natl Acad. Sci. U S A* 90, 1781–1785

35. Goffrini, P., Ficarelli, A. and Ferrero, I. (1995). Hexokinase activity is affected in mutants of *Kluyveromyces lactis* resistant to glucose repression. *Microbiology* 141, 441–447

36. Guerra, O.G., Rubio, I.G.S., da Silva Filho, C.G., Bertoni, R.A., dos Santos Govea, R.C., Vicente, E.J., A novel system of genetic transformation allows multiple integrations of a desired gene in *Saccharomyces cerevisiae* chromosomes. Journal of Microbiological Methods, 2006, 67: 437-445

37. Hartl, L. &Seiboth, B. (2005). Sequential gene deletion in Hypocrea jecorina using a single blaster casette. Curr Genet 48, 204-211.

38. Helen Karp H., Aiki JarvisteA, M. Kriegel T.M., Alamae T. (2004) Cloning and biochemical characterization of hexokinase from the methylotrophic yeast Hansenula polymorpha, Curr Genet. 44, 268-276.

39. Herrero, P., J. Galindez, N. Ruiz, C. Martinez-Campa, and F. Moreno. 1995. Transcriptional regulation of the *Saccharomyces cerevisiae HXK1, HXK2* and *GLK1* genes. Yeast 11:137–144.

40. Hohmann S, Winderickx J, Winde JH de, Valckx D, Cobbaert P, Luyten K, Meirsman C de, Ramos J, Thevelein JM (1999) Novel alleles of yeast hexokinase PII with distinct effects on catalytic activity and catabolite repression of SUC2. Microbiology 145:703–714

41. Jang, J. C. and Sheen, J. (1994). Sugar sensing in higher plants. *Plant Cell* 6, 1665–1669.

42. Kramarenko T, Karp H, Jarviste A, Alamae T. (2000) Sugar repression in the methylotrophic yeast Hansenula polymorpha studied by using hexokinase-negative, glucokinase-negative and double kinase-negative mutants. Folia Microbiol 45:521–529

43. Kruckeberg, A.L.; Bisson, L.F. (1990); The HXK2 Gene of Saccharomyces cerevisiae is required for high-affinity glucose transport; Molecular and Cellular Biology; 10; 5903-5913

44. Kopetzki, E. & Entian, K. D. (1985) Glucose repression and hexokinase enzymes in yeast. Isolation and characterization of a modified hexokinase PI1 isoenzyme, *Eur: J. Biochem. 146*, 657-662.

45. Kosow DP and Rose IA (1971) Activators of yeast hexokinase. *J Biol Chem* 246(8):2618-25

46. Kosow DP and Rose IA (1970) Product inhibition of the hexokinases. *J Biol Chem* 245(1):198-204

47. Ma, H., Bloom, L. M., Walsh, C. T. and Botstein, D. (1989). The residual enzymatic phosphorylation activity of hexokinase II mutants is correlated with glucose repression in *Saccharomyces cerevisiae*. *Mol.Cell. Biol.* 9, 5643–5649.

48. Maas R. H. W., Bakker R. R, Eggink G. and Ruud A. Weusthuis R A. (2006) Lactic acid production from xylose by the fungus *Rhizopus oryzae* Appl. Microbiol. And Biotech. 72, 861-868

49. Maitra, P K. (1970) A glucokinase from *Saccharomxces cerevisiae, .1. Biol. Chem. 245.* 2423-2431

50. Maitra, P. K. & Lobo, Z. (1*9S3)* Genetics of yeast glucokinase. *Genetics 105,* 501-515

51. Margulies, M., and W. Vishniac. (1961). Dissimilation of glucose by the MX strain of Rhizopus. J. Bacteriol. 81:1-9

52. Moore, B.; Zhou, L.; Rolland, F.; Hall, Q.; Cheng, W.-H.;Liu, Y.-X.; Hwang, I.; Jones, T. & Sheen, J. (2003) Role of the Arabidopsis glucose sensor HXK1 in nutrient, light, and hormonal signaling. *Science* 300, 332-336

53. Moreno F & Herrero P (2002) The hexokinase 2-dependent glucose signal transduction pathway of Saccharomyces cerevisiae. FEMS Microbiol Rev 26, 83–90

54. Moriya,H. and Johnston,M. (2004) Glucose sensing and signaling in Saccharomyces cerevisiae through the Rgt2 glucose sensor and casein kinase I. Proc. Natl Acad. Sci. USA, 101, 1572–1577.

55. 88.Özer Uyar G.E.; (2002); Effect of Different Stress Conditions on Trehalose Accumulation and Degradation in *Rhizopus oryzae* Msc in Biotechnology,METU.

56. Özer Uyar G.E.; (2009); Cloning and Characterization of Trehalose-6-Phosphate Syntase Gene from *Rhizopus oryzae*. PhD in Biotechnology; METU

57. Özer Uyar E. G.; Hamamcı H., and Türkel S.; (2010); Effect of Different Stresses on Trehalose Levels in *Rhizopus oryzae*. J. Basic Microbiol; 50, 368-372

58. Laht S, Karp H, Kotka P, Jarviste A, Alamae T (2002) Cloning and characterization of glucokinase from a methylotrophic yeast *Hansenula polymorpha*: different effects on glucose repression in *H. polymorpha* and *Saccharomyces cerevisiae*. Gene 296:195–203

59. Lobo. Z. & Mailra, P. K. (1977a) Physiological role of glucose phosphorylated enzymes in *Saccharomvces cerevisiae. Arch. Biochem. Biophys. 182.* 639-645.

60. Lobo. Z. & Maitra. *P.* K. (1977b) Genetics of yeast hexokinase. *Genetics 86.* 727-744.

61. Lockwood, L. B., G.E. Ward, and O.E. May. (1936). The physiology of Rhizopus oryzae. J. Agric. Res. 53:849-857

62. Teusink, B., M. C. Walsh, K. Van Dam, and H. V. Westerhoff. 1998. The danger of metabolic pathways with turbo design. Trends Biochem. Sci. 23:162–169

63. Thevelein. J. M. & Hohinann, S. (1995) Trehalose synthase: guard to the gate [if glycolysis in yeast? *Trends Biochem. Sii. 20,* 3 - 10.

64. Timson D.J. (2007); Galactose Metabolism in Saccharomyces cerevisiae ; Dynamic biochemistry, process biotechnology and molecular biology; *Global Science Books*; 63-73

65. Tsoi A, Douglas HC (1964); The effect of mutation of two forms of phosphoglucomutase in *Saccharomyces. Biochemica et Biophysşca Acta* 92; 513-520.

66. Ozcan, S., and M. Johnston. 1995. Three different regulatory mechanisms enable yeast hexose transporter (*HXT*) genes to be induced by different levels of glucose. Mol. Cell. Biol. 15:1564–1572.

67. Özer Uyar G.E.; (2009); Cloning and Characterization of Trehalose-6-Phosphate Syntase Gene from *Rhizopus oryzae*. PhD in Biotechnology; METU

68. Özer Uyar E. G.; Hamamcı H., and Türkel S.; (2010); Effect of Different Stresses on Trehalose Levels in *Rhizopus oryzae*. J. Basic Microbiol; 50, 368-372

69. Panneman. H., Ruijter G. J. G., Van Den Broeck. H.C., Drıever E.T. M. & Vısser J. (1996). Cloning and biochemical characterisation of an *Aspergillus niger* glucokinase Evidence for the presence of separate glucokinase and hexokinase enzymes. Eur. J. Biochem. 240, 518-525

70. Panneman. H., Ruijter G. J. G., Van Den Broeck. H.C. & Vısser J. (1998). Cloning and biochemical characterisation of an *Aspergillus niger* hexokinaseThe enzyme is strongly inhibited by physiological concentration of trehalose 6-phosphate. Eur. J. Biochem. 258, 223-232

71. Palomino A., Herrero P. & Moreno F. (2006). Tpk3 and Snf1 protein kinases regulate Rgt1 association with Saccharomyces cerevisiae HXK2 promoter. Nucleic Acids Research Vol. 34, No:5 1427-1438.

72. Petit, T., Bla´zquez, M. A. and Gancedo, C. (1996). *Schizosaccharomyces pombe* possesses an unusual and a conventional hexokinase: biochemical and molecular characterization of both hexokinases. *FEBS Lett.* 378, 185–189

73. Petit, T., Gancedo, C. (1999). Molecular Cloning and Charaterization of the Gene *HXK1* Encodind the Hexokinase from *Yarrowica lipolytica. Yeast 15, 1573-1584*

74. Prior, C., Mamessier, P., Fukuhara, H., Chen, X. J. and We´solowski-Louvel, M. (1993). The hexokinase gene is required for transcriptional regulation of the glucose transporter gene *RAG1* in *Kluyveromyces lactis*. *Mol. Cell. Biol.* 13, 3882–3889

75. Raamsdonk LM, Diderich JA, Kuiper A, van Gaalen M, Kruckeberg AL, Berden JA, Van Dam K & Kruckberg AL (2001) Co-consumption of sugars or ethanol and glucose in a Saccharomyces cerevisiae strain deleted in the HXK2 gene. Yeast 18: 1023–1033

76. Raber L. R. (1998). Green Chemistry here to stay. Chem. Eng. News 1998 (July):25-26

77. Randez-Gıl F., Sanz P., Entıan K. D. & Prieto J. A. (1998). Carbon Source-Dependent Phosphorylation of Hexokinase PII and Its Role in the Glucose-Signaling Response in Yeast Vol. 18, No:5, 2940-2948

78. Ronne, H., 1995. Glucose repression in fungi. Trends Gent. 11;12-17.

79. Rose, M., W. Albig, and K. D. Entian. 1991. Glucose repression in *Saccharomyces cerevisiae* is directly associated with hexose phosphorylation by hexokinases PI and PII. Eur. J. Biochem. 1991:511–518

80. Rose M (1995) Molecular and biochemical characterization of the hexokinase from the starch-utilizing yeast Schwanniomyces occidentalis. Curr Genet 27:330–338

81. Ruijter, G. J. G., Panneman, H. and Visser, J. (1997). "Overexpression of phosphofructokinase and pyruvate kinase in citric acid-producing Aspergillus niger." Biochimica Et Biophysica Acta-General Subjects 1334(2-3): 317-326.

82. Ruiz-Amil, M. & Sols A. (1961). Hexokinase aus Aspergillus oryzae, Biochem., Z. 334, 168-174.

83. Sanz, P., A. Nieto, and J. A. Prieto. 1996. Glucose repression may involve processes with different sugar kinase requirements. J. Bacteriol. 178, 4721– 4723.

84. Sauer U., et al. (1999). Metabolic flux ratio analysis of genetic and environmental modulations of Escherichia coli central carbon metabolism. J. Bacteriol. 181: 6679–6688

85. Schaaff, I., Heinisch, J. and Zimmermann, F. K. (1989). "Overproduction of glycolytic enzymes in yeast." Yeast 5(4): 285-290.

86. Schuurmans J. M., Boorsma A., Lascaris R., Hellingwerf K.J. & Teixeira de Mattos M.J. (2007) Physiologicaland transcriptional characterization of *Saccharomyces cerevisiae* strainswithmodi¢ed expression of catabolic regulators. FEMS Yeast res. 1-9

87. Steinböck, F., Choon, S., Held, I., Röhr, M., & Kubiteck, C. P., (1994). Characterization and regulatory properties of a single hexokinase from the citric acid accumulating fungus Aspergillus niger, Biochim. Biophy. Acta. 1200, 215-223.

88. Skory, C.D. (2000); Isolation and expression of lactate dehydrogenase genes from *Rhizopus oryzae*. Appl. Environ. Microbiol. 66, 2343-2348.

89. Skory, C.D. (2002). Homologous recombination and double-strand break repair in the transformation of Rhizopus oryzae. Mol. Genet. Genomics 268, 397-406.

90. Skory, C. D.(2003); Lactic acid production by *Rhizopus oryzae* transformants with modified lactate dehydrogenase activity. Appl. Mic.and Biotech.;

91. Skory, C.D. (2003); Induction of *Rhizopus oryzae* pyruvate decarboxylase genes. Current Microbiol. 47, 59-64

92. Van Urk, H., E.Postma, W.A. Scheffers, and J.P. van Dijken. (1989). Glucose transport in Crabtree-positive and Crabtree negative yeasts. *J. Gen. Microbiol*.135:2399-2406.

93. Voet, D., Voet, J. G., (2004). "Biochemistry". John Wiley & Sons., New York, Chapter 17 pp: 585

94. Vojtek, A. B., and D. G. Fraenkel. 1990. Phosphorylation of yeast hexokinases. Eur. J. Biochem. 190:371–375.

95. Wang C. W., Z. Lu, and G. T. Tsao. (1995). Lactic acid production by pellet-form *Rhizopus oryzae* in a submerged system. Appl. Biochem. Biotechnol. 51/52:57-71

96. Walker, M., G., Ed. (1998). "Yeast physiology and biotechnology". John Wiley & Sons Ltd. England.

97. Wedlock DN, Thornton RJ (1989) A hexokinase associated with catabolite repression in Pachysolen tannophilus. J Gen Microbiol 135:2013–2018

98. Yu, R. C., and Y. D. Hang. (1989). Kinetics of direct fermentation of agricultural commodities to L- (+)-lactic acid by *Rhizopus oryzae.* Biotechnol. Lett. 11:597-600

99. Zimmermann, F., K. and Entian, K., D., Ed. (1997). "Yeast sugar metabolism". Technomic publishing co. inc. USA.

APPENDIX A

GROWTH MEDIA FOR *ESCHERICHIA COLI* DH5A

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Chemical	g/L
Yeast Extract	5
Tryptophane	10
NaCl	10
Agar	15

Adjust the pH of the medium to 7.0 by addition of 5N NaOH.

APPENDIX B

GROWTH MEDIA FOR *Rhizopus oryzae* ATCC 9363

Chemical	Grams/100 ml
Ammonium sulfate	0.2
Potassium phosphate (monobasic)	0.065
Magnesium sulfate heptahydrate	0.025
Zinc sulfate heptahydrate	0.005
Glucose	0.5
Agar	2

APPENDIX C

GROWTH MEDIA FOR *Saccharomyces. cerevisiae* DFY632

Selective medium composition:

Chemical	Grams/100 ml
Casaminoacid	0.2
Ammonium sulfate	0.5
Yeast Nitrogen Base w/o amino acids	0.017
Tryptophane	0.008
Adenine	0.004
Carbon Source (Glucose, Fructose or Galactose)	2
Agar	2

- 0.005 gr/100ml Uracil was added for mutant cell culture.

APPENDIX D

COMPOSITION OF THE BRADFORD'S REAGENT AND STANDART CURVE PREPARATION TABLE

5X stock solution:

500 mg Brillant Blue G
250 ml 95 % Ethanol
500 ml 85 % Phosphoric acid

Complete the volume to 1000 ml with distilled water and store at 4 oC. To use dilute stock 1:4 with distilled water and filter through normal filter paper. Wait for at least 24 hours at 25 oC before use. Since the reagent is sensitive to light, store it in a dark bottle or cover the transparent bottle with aliminum foil.

Standart Curve Preparation:

Prepare a stock solution of 1 mg/ml BSA.

Tube#	BSA (µl)	dH2O (ml)	Bradford's reagent (ml)
1	0	500	5
2	5	495	5
3	10	490	5
4	15	485	5
5	20	480	5
6	25	475	5

After addition of reagents vortex and wait for 10 min. Read OD at 595 nm

APPENDIX E

SEQUENCES OF HYPOTHETICAL PROTEINS OF *R. oryzae* HEXOKINASE GENE

>RO3G_03473.1 HYPOTHETICAL PROTEIN (hxk1)

ATGTTAAACAACAAAAAGAAGACACCAAAAGCTTATGAAATTCATGAAATTGGAGGAACAGCCGAACA
AGAGGCGTTTATGAATGATATCATTGATCAGTTCACGATTGATTCAAGCCAACTTGTAGAAATACGTG
ATCATTTTATACAAGAAATGGAAAAGGGCTTGAACCAGGAAGGAGCCACATTGGCCATGATTCCGTCT
TATGTCGAAGGACGTTTGACTGGTAAATATGAGCAAATGATAGTAAAACTTTATCTGACGAGTTGATC
AGGTAAAGAAGAAGGACGTTTTTTAGCTCTTGATTTGGGAGGAACGAACCTGAGGGTGGTTCTGGTTA
CACTAGAAGGGGATGGCAAGTTTCAAACTGTGTCTACAAAATCAAAAGTATCAGAGGAATTAAAGACA
GGGCCTATGCGTAACTTGTGTGGTATGATCATTGTCACATCAATAAAGAAACACTCTTAATTATTTTT
TGTTATAGATTATATTGCCGATTGTGTTGACACGTTCTTGACAGAGCATGGTCTTGAAAATCATGAAA
CGGAACTCAACCTGGGCTATACTTTCTCTTTTCCTATTCTTCAATCAAAGGTATAAAAGAGTGATGCT
ATCTGAAGGGGGTGGTTTCTTATGCTTTGTTTAGATCAATCGTGGTATACTCTCTACATGGACCAAAG
GATTTTCTAGCTCGGGTGCTGTGAACAAGGACCCTGTCCTATTACTTCAGGATGCCTTATTAAGAAAA
CATGTCCCAGTCAAGATTTCTGCACTTGTCAATGACACAGTCGGTACTCTTTTATCCAACGCATACAA
TAAACCTCACACCTTGGCTGGTCTCATCTTGGGCACAGGCGCTAATGGTGCTTATATCGAAAAGATGT
CAAAGATTGGCAAATGGAAGGGAGGCAAGACAACAGCAGAAGAGATGATTATTAATATGGAGTTTGGT
GCTTTTGATAATGAAAGACGTGTGCTTCCCTTGACACGTTTTGATAACAAACTTGATCGACAGTCCAT
TAATCCTCATGCTCAACTCTATGAAAAGATGATCTCAGGCATGTACCTGGGTGAAATCACTCGAAATG
TGCTCATAGACATGATTGACCGTGAACTCTTGCTGAAACCACAAAACTTGGCCAAGGACATCTCTCGA
CACTGGTCCTTTGAGACAGCCTTCATGTCAAGCATTGAAGAAGACAATTCTCCTGATTTACAACATAC
AAAGGAGACCTTAGACAGCAATCTAAATTTGCATGATATAACCACAGTGGAGGCAAGGATGATTAAAA
AGGTCTGTGAGTTGGTAGGTAAACGTGCTGCTCGTCTGGCTGCGACCTCGATCTCAGCAATTGTCAAG
CATTGTGGTATGAGCGATGAAGGACATGATATAGGAATTGATGGTTCTCTTTATGAATTTTATCCTTC
TTTTGAAGAAAGACTTTATGAAGCTCTTCAAGAAATGATGCCTGAAGTTGAGAACATTCATGAAAAGA
TTCGTTTGGGACTTGCGAGAGATGGCAGCGGTGTTGGGGCTGCCCTTACAGCCTGTGTAGCTGCTCGT
ATGGAAGAGAAATTAAATAGAGAAGGCTCGGATTGA

> RO3G_04008.1 HYPOTHETICAL PROTEIN (hxk2)

ATGATTGTCACCCATAGCAATAATGATTCATCAGAAAACGCTGTACCTAAAATAAATGGTACAGAAGA
GCAACAAAAAGCGATTAAAGATCTTTTTCATCTATTTAATATCACCACAGCAACTGCACGAAATATCA
TACAAAGTTTCATACAAGAGATGCAAAAAGGACTTGATCGTGAAGGTGCCACGGGTAAGATTGTAAAC
CTTGACTGCTGTCTCTAGAAAACTAAAATTTTAAACCAGTACCGATGATTCCTAGTTTCGTCACCGGT
CGTCCTACAGGCCAGGAAACAGGAAGTTACCTTGCCTTGGACTTGGGAGGCACAAATCTTCGTGTATG
TGAATTTGAATTGAAAGGTGCAGGCCACTTTGAATTACGTCAACAAAAGTATGTGATTTCAGAAGAAC
TCAAAAAGGGCGATATGCGTCACTTGTTTGATTTTATTGCAGATTGTGTGGATAATTTTATTTTTGAA
TACCACACAACTCCCCAGACACTCTATTTAGGGTTCACGTTTTCTTTTCCTGTCAATCAGACAGCGAT
CGATAGAGGTACCTTGATGCATTGGACAAAAGGGTTTGCCTGTACGAATGCGATCAACAAAGACGTGG
TTATCATGCTTCAAGATGCTTTCATGCGTAAAAATCTTCAAGTTCATGTCGCTGCGGTAAGATATCTT
ATTCTATTGACATGTAATAACCTTTATTAGCTCGTAAATGACACTGTGGGAACATTGATGGCCCATGC
CTATCGTCATCCAGAAACAGCGATGGGTATCATTCTTGGCACAGGAACAAACGCTGGTAATAAAAAAC
AAGAGTAAATGAAAGACGTATAAAAATAAACTTGACTTATAGCTTATTATGAAAAACTCAAAAACATC
AAGAAATGGAAAGGAGGAGAACAAGCATTTGACGAAATGGTGATCAATATGGAATGGGGAGCCTTTGA
TTTAGAACGTCAAGTGCTGCCCTTTACTGTCTATGATAACAAACTAGACCGGGAATCGATCAATCCAC
GCGAGCAAACCTTTGAAAAGATGATCTCTGGCATGTATCTGGGCGAGATTGCACGTAATGCGATATTG
GAGTTAGTCGATCGTCGACTGTTGTTCTCTGGTGAATCCTCCACAGAACTCAACGCACAGTGGTCATT
CGAAACATCTTACATGTCCACGATCGTAGCTGACACCACAGACAATTTGGAAGACACTCGCTATATTT
TGGAGGAGAACCTTCAGCTGGAGAAGACAAGTTTGGTAGATCGACAGATGGTGCAGTTAATCTCGACC
TTTGTTGGACGTCGCGCTGCACGCCTTTCTGCTTGTGGTATCGCTGCCGTCTTGACATTTACAGATCA
TCTAGGAAAAGATGATACAATGATTGCTATCGATGGATCCGTCTATGAATTCTTTCCTCAATTTGAAA
CACACATGATGGAATTACTTGTAAACTTATTTGGCGTAGATGTCACAAAAAAGATTCAGTTTGCATTG
GCTCGAGATGGTTCTGGATTTGGTGCAGCGATGATTGCAATGATGGCACATAAAGCTTCATTAGTTAA
AGGCCCTTTGCGCTAA

> **RO3G_13363.1 HYPOTHETICAL PROTEIN (hxk3)**

ATGCCTAGTAATAAGAAAGCACCTGATGCTGTTGTAGTTAATGAAATTGGTGGAACACCCGAGCAAGA
AGCTTATATGAAAGACATCATCAATCAATTTACAATTAACTCTGAATACCTTGTAAAAATACGTGATC
ATTTTATGAGCGAAATGCAAAAAGGTCTTGACAAGGAAGGTGCAACCTTGGCCATGATCCCATCCTAT
GTTGAAGGACGTTTAACTGGTAATACATATAATAGCCATAGTTTGTATTTTAATATTAAATATTTTTT
AAAGGCAAGGAAGAAGGTAAATTTTTGGCTCTTGATTTGGGAGGAACCAATTTGAGAGTTGTACTTGT
TACTCTTGAAGGTGAAGGAAAATACAAGACAGTATCTTCTAAAGCTAGAGTCTCTGAGGAATTAAAGA
CGGGTCCAATGCGTAATTTGTGTGGTAAAGTGTAGAGATGAAGTCATCGATTGAACTTCCGATTAAGC
CAGATTTATATCATAGACTATATCGCCGAATGTGTGGATACTTTCTTAACAGAGCAGAACATTCAAGA
ACAAGAGATCGAACTTCACTTGGGTTATACTTTCTCTTTCCCCGTCCTTCAATCTAGAGTAAATATTA
AAATAAAGCATACAAATTGTAAATTAACACTTGTACAGATCAACCGTGGTATACTCACTACATGGACA
AAGGGATTTTCTTGTTCAGGTGCTGTCAATAAAGATCCCGTATTATTATTACAAGACGCATTATTAAG
AAAGCATGTTCCAGTTAAAGTCTCTGCGATCGTAAACGACACTGTTGGTACTCTTCTTTCAAACGCAT
ACAACAAGCCCGGTACCTTGGCTGGTCTCATTTTGGGTACAGGCGCTAATGGTGCTTACATTGAAAAG
ATGAAAAACATTGGCAAATGGAAGGGAGGCAAAACGACAGCAGATGAAATGGTGATCAACATGGAACT
TGGTGCCTTTGATAATGAACGTGTCGTACTTCCCTTGACTCGATTTGACAACAAATTGGATAGACAGT
CGATTAATCCTCATGCTCAAATTTACGAAAAGATGGTGTCGGGTATGTATCTTGGTGAAATCACCCGT
AATGTGTTGATTGAAATGATTGATCGTGAATTACTCTTGAAGCGTCCTTCACATAACTCCCTTGCTTG
GTCCAAGGATATTTCTCGACACTGGTCCTTTGAGACAGCCTTCATGTCAAATATTGAAGAAGACAGTT
CTTCCGATTTATCTTATACAAAGGAAATTTTGGATACCAACTTAAATTTAACCGACATCACCACTGTA
GAGGCTCGTATGATTAAAAAAGTCTGTGAGTTGGTAGGCAGACGTGCTGCTCGTTTGGCTGCTGTCTC
TATTTCAGCTATCGTCCAACATTGCGGTATTGGTGCTGAGGGCCATGATATTGGCATTGATGGTTCTC
TTTATGAATTCTATCCTTCCTTTGAAGATAGAATGTATGAAGCACTTGAGGAAATGATGCCTGAAGTA
GATAACCTTCGTCAAAAGATTCGTTTGGGTCTTGCAAGAGATGGCAGTGGCGTTGGTGCTGCCCTTAC
TGCTTGTGTGGCTGCTCAAATGGAAGAAAAGCAAAAAGTAGCTATGCCATAA

> **RO3G_05082.1 HYPOTHETICAL PROTEIN (hxk4)**

ATGACTCAAACTGAATTTACAAAGGAACAAAAGTTGGCAGTGGATGCCATTCTTGATCAATTCAAAGT
ATCAAGTGAGACCTTAAAGGCAATTTCTGAACAATTTGTGAAAGAAATGGAAAAGGGTCTGGACCATG
AAGGAGCTACGAGTAAGTAAGAAGAGGTAGAAACAAGAGATCTCATCATTTCACAGTTGCCATGATTC
CTTCTTTTGTATCCGGTCGCTGTACGGGGGATGAAAAAGGTCACTTTTTAGCCCTTGATTTAGGAGGC
ACGAATTTACGTGTTTGTCATGTGACCTTGCTCGGTGGAGGTGAATACACCATCCGTCAGCAAAAATA
TGTGATCTCTGAAGAGCACAAGGTGGGACCGATGCGTGTCCTGTGTGATTTTATTGCTGATTGTGTCG
ACAGTTTCCTGACTGAACACGGTACAAATGATGACAGTAATGCCCCTATCCAACTTGGTTATACTTTC
TCTTTTCCTGTGCTTCAAACAAGTAGTAATCGAGGCGTGCTCAAACAGTGGACAAAGGGATTTGCTTG
TTCAGGTGCTGTGGGTCGAGATGTGGCCATTCTCTTGCAAGATTCATTCCGTCGAAAGAACCTGAATG
TCAATATTGCTGCTGTAAGTTACCTTGTCCCACAAAATAAATGAAACCAACCCTTTTCAATAGATTGT
GAATGACACTGTGGGTACACTGATGGCTCATGCTTACAAGCATCCTGAAACAACCATGGGTGTCATCT
TGGGCACAGGTTTTAATGCATCTTATTATCAACACATGTCCAAGGTGAATAAGTGGAAAGGAGATCCA
GAGCAAGAGATGGTCATTAATATGGAATGTGGTGCATTTGATATCGAAAGAAGAGTGTTACCAATGAC
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GATGGTAACTCCTCAAAGGAACTCAATAAAATGTGGAGCTTTGAAACTGCCTACATGTCTACAATCGA
AGTGGATGACACACCCAACCTATCCGAAACAGCACACATCTTGGAATCTGTCCTTAATCTGTCACATA
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TTGGTGAGCAGGCTCGCTCTAAGGTTAGATTTGATTTAGCAAGAGATGGTTCAGGTCTTGGTGCTGCT
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> **RO3G_15735.1 HYPOTHETICAL PROTEIN (hxk5)**

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CTTTTCTTTCCCTGTCCTTCAGACTGGCATCAACCGCGGTGTGCTCAAGCAGTGGACCAAGGGATTTG
CTTGTTCAGGTGCTGTAGGTAGAGATGCTGTCATTCATCTCCAAGACTCTTTCCGTCGCAAGAACATC
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GTGAATGACACTGTGGGTACTCTGATGGCTCACGCTTACAAGCATCCTGAAACCACGATGGGTGTCAT
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AGGTGGCATGTATCTGGGTGAAATCACGCGTAATGCGCTCTTGGACTTGGTTGATCAGCAACTCTTAT
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CACAACCTTGGCCGATCGTCAAATCGTAAAACAAGTCTGTAACGCGGTCGGTACACGTGCTGCACGTA
TTGCCTGCTGTCATATTGCTGGCGTGATTATGCAAACGGGTAAAGTAGGCGAAGAGTGTGTGATCGCG
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CCTTGGTGAGCAGGCTCGTTCCAAGGTAAGATTTGAGCTTGCCAGAGATGGTTCCGGACTGGGTGCTG
CTATCATTGCCATGATGGCTACAAAGAATGCTTTACACAAATAA

> **RO3G_01514.1 HYPOTHETICAL PROTEIN (hxk6)**

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TAATTCAAGAGATGAACACTGGATTACAATCGCATGAATCCAATATGGCCATGTTACCCTCTTGGATC
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CAACTTGCACGGACAGGGTCGTATCAGTACGCGTCAGCAGAAATATGTGATTCATGACCATCTCAAGA
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GGGTACCAACGCCGCCTACTGGGAAAAGACGAGCGAGCTGAAAAAGACCGAGGGCCAGACGGTTCCGA
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CCTCGTACGTTTTATGATAACCGGGTCAATCGTCAGTCGGTCAATCCGGGTGTGCACGTCTTTGAGAA
GATGGTCTCTGGTCTCTATCTGGGTGAAATCGTCCGTCTGATCATGGTCGATTTCCTCGATCGTCGTT
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CTCGTCTGGTGGCTGCTGCGATCAGTGCGATCATCGAAAAGAGAAACGCACTCGATCAAGGACTGACG
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TGTATGAACATTTCCCTGATTTCCCAAGACGTGTGAATGATACACTCAAGAGTCTTTACAGTGATCGT
GTGGATCATATCAATATCGGCATCACCCGTGATGGACACGGCATCGGTGCTGCTTTGGCTGCTATGAT
TGCTATCACTCAAAGAAAATAA

> **RO3G_02075.1 HYPOTHETICAL PROTEIN (hxk7)**

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GCGTTGTCCTTCCATAGAACTCAAGACAGGTGAAGGTGCAATGTTCTTTGACTGGATTGCAGATGCTG
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CCCTTTTTTCTAAAAAAAACAACTATTTTTTTATAGTCAGACTGCTGTTGACCGTGGTACTATTCTTA
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TTCAAAAGAAAGGTAAATCAAAAAGAACAAAAAAAGATGATGTGACTAATGTAACAGATGGAATAGAA
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> **RO3G_10326.1 HYPOTHETICAL PROTEIN (hxk8)**

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CAAACTTCTTTTCGTAGACGTGAAATTCCTGTTGTGGTCAAAGCGGCTGTCAACGGAGCACGTAAGTA
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TGA

> **RO3G_11014.1 HYPOTHETICAL PROTEIN (hxk9)**

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TCTCTTATTTTATTTTATTAGGCTACCCTACAGGCAATGAAAAAGGGATTTATTTAGCACTCGAGATA
AGTGGCATCGATATTTACGTCTGTCAAGTGAAGTTAAAAGGAGAAGGAGGAAAACTAGCTATTAACCA
ATACCAATACAAAATACCCGATGATCTGACAGCAGGAGAAGACGTGACGGTCCTGATTGATTATGTAG
CTGATTGTGTAGCTGATTTTCAACAAAGAGTGGGCGCTAATCCCCACGAGACTTATTCGATGGCCATC
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TGACTTTCCTAATGCAATTGGGTGTGATGCGGTTGATCTGTTTGATCGACAGCTGAAGGCAAAAGGTT
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TATGTGAATGGGTAGGTAACCGTGCTGCATTTTTGCTTGGCGCAGGTATTGCTGGTGTTGTAAAATAT
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> **RO3G_05968.1 HYPOTHETICAL PROTEIN (hxk10)**

ATGACTACCAGAGAAGAGATTTTATACAAGGTTGAAAAGGCTTTTCAAGTGACTGAGGAACAACTCAA
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TAGAGATAAGTGGAGTTGATATCTATGTCTGTCAAGTGAAACTCAAAGGGGAAGGAGGAAAACTGGCT
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TTATGTCGCAGACTGTGTAGCTGATTTTCAACAAAGGGTCGGTGCTAACCCACACGAAACTTATTCGA
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TAAAGGACTGGCTATCAAAATTGTAGCTATTGCCAATGGTGAGTGATGGCCGTTTGACTACAAGTGAT
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CGGATTGGCGTTATTCACAGTGCTGGCACAAACTGCGCCTACTATGACAAGATGAAAAACATCAAACA
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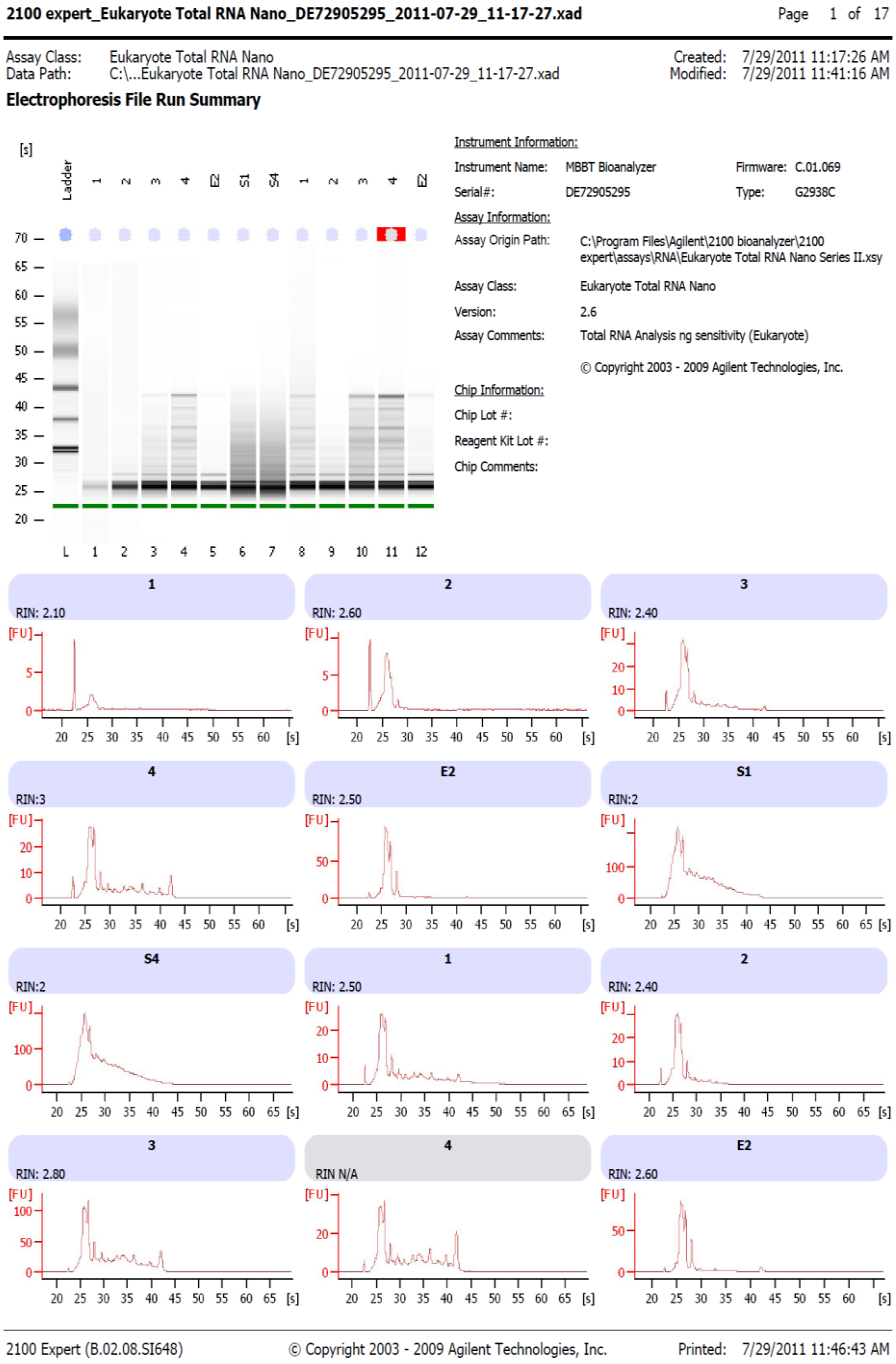
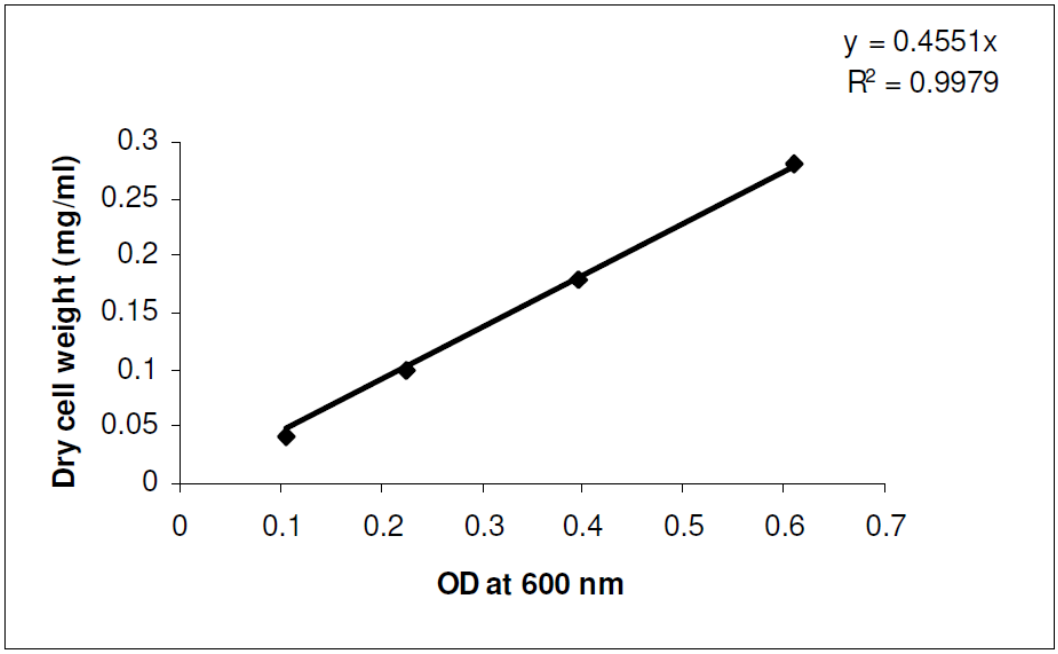
APPENDIX F

RESULTS OF BIOANALYZER



APPENDIX G

STANDART CURVE FOR DRY CELL WEİGHT DETERMINATION OF *S. cerevisiae*



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EDUCATION

Degree	Institution	Year of Graduation
MS	METU, Biotecnology	2005
BS	Ankara University, Biology	2001
High School	Fatih Sultan Mehmet High School	1997

WORK EXPERIENCE

Year	Place	Enrollment
2008-Present	T.C. Ministry of Environment and Forestry	Biologist
1999 August	Düzen Laboratory Group	Intern Student

FOREIGN LANGUAGES

English

PUBLICATIONS

Alagöz, E. , "Kinetic analysis of glucose-6-phosphate branch point in *Saccharomyces cerevisiae* ", METU, MSc in Biotechnology, September 2005

Alagöz, E., Açar, S., Yücel, M., Hamamcı, H., "Cloning and Expression Studies of Putative *Rhizopus oryzae* Hexokinase Genes in *Saccharomyces cerevisiae*" FEBS Journal 277 (Suppl. 1), 118; June 2010

CONFERENCE PRESENTATIONS

Alagöz, E., Açar, S., Yücel, M., Hamamcı, H., "Revealing The Hexokinase Step of Glycolysis In Lactic Acid Producer Fungus *Rhizopus oryzae*";15th European Congresson Biotechnology; 23-26 September 2012; İstanbul,TURKEY

Alagöz, E., Açar, S., Yücel, M., Hamamcı, H., "Cloning and Expression Studies of Putative *Rhizopus oryzae* Hexokinase Genes in *Saccharomyces cerevisiae*" Poster presentation in 35th FEBS Congress "Molecules of Life", 26 June –1 July, 2010, Gothenburg, SWEDEN

Alagöz, E., Açar, S., Yücel, M., Hamamcı, H., "Screening of the putative hexokinase genes from *Rhizopus oryzae* and their heterologous expression in *Saccharomyces cerevisiae"*;Poster presentation in National Biotechnology Congress, 27-30 September 2009, Ankara-TURKEY

Alagöz, E., Açar, S., Yücel, M., Hamamcı, H., "Kinetic analysis of glucose-6-phosphate branch point in *Saccharomyces cerevisiae* " Poster presentation in the symposium "14th National Biotechnology Congress", 31 August-2 September 2005, Eskişehir-TURKEY

FELLOWSHIPS AND SCHOLARSHIPS

2010	FEBS Bursary (registration and travel) for the 35th FEBS Congress "Molecules of Life" in Gothenburg
2007-2012	Ph.D. Programme Fellowship from TÜBİTAK (Scientific and Technological Research Council of Turkey -Scientist Supporting Department_BİDEB)
2002-2004	M.Sc. Programme Fellowship from TÜBİTAK (Scientific and Technological Research Council of Turkey –Scientist Training Group_BAYG)