PREVENTING SLUGGISH OR STUCK FERMENTATIONS WHICH MAY BE CAUSED BY SUGAR UPTAKE DEFICIENCY IN SACCHAROMYCES CEREVISIAE WINE YEAST STRAINS BY USING GLUCOSE ISOMERASE

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submitted by **NAHİDE SERAY ÜNAL** in partial fulfillment of the requirements for the degree of **Master of Science in Food Engineering Department, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Serpil Şahin
Head of Department, **Food Engineering** _____

Prof. Dr. Haluk Hamamcı
Supervisor, **Food Engineering, METU** _____

Examining Committee Members:

Prof. Dr. Abdulkadir Halkman
Food Engineering, Ankara University _____

Prof. Dr. Haluk Hamamcı
Food Engineering, METU _____

Assoc. Prof. Dr. Deniz Çekmecelioğlu
Food Engineering, METU _____

Assoc. Prof. Dr. Yeşim Soyer
Food Engineering, METU _____

Prof. Dr. Pınar Çalık
Chemical Engineering, METU _____

Date: 05.09.2019

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.

Name, Surname: Nahide Seray Ünal

Signature:

ABSTRACT

PREVENTING SLUGGISH OR STUCK FERMENTATIONS WHICH MAY BE CAUSED BY SUGAR UPTAKE DEFICIENCY IN SACCHAROMYCES CEREVISIAE WINE YEAST STRAINS BY USING GLUCOSE ISOMERASE

Ünal, Nahide Seray
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Supervisor: Prof. Dr. Haluk Hamamcı

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The objective of this study was to prevent stuck fermentation, which may be caused by sugar uptake deficiency in yeast by using glucose isomerase, whose activity was tested in different media.

The unfermented grape juice contains almost equal amounts of glucose and fructose. After fermentation, amount of residual fructose is higher than that of glucose and the glucose/fructose discrepancy (GFD) occurs. One of the main reasons of GFD is that affinity of hexose transporters of yeast towards fructose decreases as the accumulation of ethanol increases. Due to formation of ethanol, fermentation slows or stops even if there is residual sugar in wine medium. This is called stuck fermentation. This is an undesirable situation since residual sugar causes unwanted sweetness and increases the risk of microbial spoilage. To prevent GFD, using glucose isomerase during or after wine fermentation may be a solution. The activity of this enzyme was tested in synthetic and original wine media to find out whether it can be a solution for stuck fermentation or not. Glucose formation, 0.5% w/v, from 1% w/v fructose occurred in synthetic medium containing 13% v/v ethanol and 1% v/v glycerol at pH 3.3 and at temperatures of 60ºC or 30ºC in approximately 48 hours. However, the glucose formation did not occur in synthetic medium if there was 0.3% w/v tartaric acid at pH

3.55 whereas glucose was formed at pH 6.33. In original wine medium, the glucose formation did not occur except that wine media with dilution factor of 20% v/v, 10% v/v and 5% v/v and at pH values equal or higher than 6 whether there was tartaric acid or not. Since dilution and increasing the pH of wine cannot be applicable, other ways to employ this enzyme should be tried.

Keywords: Glucose/Fructose Discrepancy, Stuck Fermentation, Glucose Isomerase, Fermentation, Yeast

ÖZ

SACCHAROMYCES CEREVISIAE ŞARAP MAYASINDA GÖRÜLEBİLEN ŞEKER ALIM EKSİKLİĞİ SONUCU OLUŞAN DURAKLAMIŞ VEYA DURMUŞ FERMENTASYONU GLİKOZ İZOMERAZ KULLANARAK ÖNLEMEK

Ünal, Nahide Seray
Yüksek Lisans, Gıda Mühendisliği
Tez Danışmanı: Prof. Dr. Haluk Hamamcı

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Bu çalışmanın amacı, mayada şeker alım eksikliği sonucu görülebilen duraklamış ya da durmuş fermentasyonu farklı ortamlarda aktivitesine bakılmış olan glikoz izomeraz kullanarak önlemeye çalışmaktır. Fermente olmamış üzüm yaklaşık eşit miktarda glikoz ve fruktoz içermektedir ve bu şekerlerin oranı yaklaşık olarak bire eşittir. Fermentasyondan sonra, artık olarak kalan fruktoz miktarı glikozdan daha fazladır ve bu glikoz/fruktoz farklılığını ortaya çıkarır. Bu glikoz/fruktoz farklılığının birçok sebebi olmasına rağmen, en temel sebeplerinden birisi de ortamda alkol biriktikçe mayanın heksoz taşıyıcılarının fruktoza olan eğiliminin azalmasıdır. Şarap ortamında şeker kalmış olmasına rağmen alkol oluşumundan dolayı fermentasyonun yavaşlamasına ya da durmasına duraklamış ya da durmuş fermentasyon denir. Kalan şeker istenmeyen tatlılığa sebep olduğu ve mikrobiyel bozulma riskini arttırdığı için şarap endüstrisi tarafından istenmeyen bir durumdur. Bu farklılığı önlemek için, fermentasyon esnasında ya da sonrasında fruktozu glikoza çeviren glikoz izomeraz kullanmak bir çözüm olabilir. Duran fermentasyona bir çözüm olup olmadığını görmek için enzimin aktivitesi sentetik ve orjinal şarap ortamlarında test edilmiştir. 13% h/h etanol, 1% h/h gliserol içeren sentetik ortamda pH değeri 3.3 iken 60^{o}C ve 30^{o}C sıcaklıklarda yaklaşık olarak 48 saatte 1% k/h fruktozdan 0.5% k/h glikoz

oluşumu gözlenmiştir. Fakat, 0.3% k/h tartarik asit içeren sentetik ortamda pH 3.55 iken glikoz oluşumu gözlenmezken pH 6.33 iken glikoz oluşumu gözlenmiştir. Orjinal şarap ortamında, 20%, 10% ve 5% h/h seyreltilmiş ve pH değeri 6 ve üzerinde olan ortamlar hariç, ortamda tartarik asit olsa da olmasa da glikoz oluşumu gözlenmemiştir. Şarabın pH değerini arttırmak ve şarabı seyreltmek uygulanabilir yöntemler olmadığı için durmuş fermentasyonu önlemek adına glikoz izomeraz kullanımı için farklı uygulamalar denenmesi gerektiği sonucuna varılmıştır..

Anahtar Kelimeler: Glikoz/Fruktoz Farklılığı, Duran Fermentasyon, Glikoz İzomeraz, Fermentasyon, Maya

To My Lovely Family & My All Time Supporters

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INTRODUCTION

1.1. Wine

For most of the societies, fermented foods and beverages are related with their economic and cultural situations. According to research of archeologists, the invention of fermentation technology is very old in history. Also, it is believed that people used fermentation technology to protect their foods and beverages against spoilage by microorganisms. In China, there was found an evidence of a fermented beverage at 7000 BC. Additionally, there was an evidence of wine production at 6000 BC in Iran and 3000 BC in Egypt. According to evidences, it is believed that fermentation technologies of foods and beverages were started in Mesopotamian society and spread all over the world. As an example, cultivating grapes and making wine from these grapes were first started in Mesopotamia and later seen in the Mediterranean region (Greece 2000 BC, Italy 1000 BC, Northern Europe 100 AD, America 1500 AD) (Legras, Merdinoglu, Cornuet, & Karst, 2007). As a result, it is seen that fermentation technology and its application on production of wine is very ancient and it is still a hot topic in biotechnology and some other areas.

There are some parameters to test if the grape fruit or juice can be fermentable or not. These are type of crop plants, environmental factors, maturity of grapes, productivity of soil and process conditions during wine making (Buescher et al., 2001). Instead of unfermented grape fruit, grape juice concentrate can be used for wine making because this way is cheaper, easier for transportation and grape juice concentrate has less risk of microbial spoilage than grape juice (Buescher et al., 2001).

Wine fermentation is a complicated biochemical process in which yeasts play an active role for production of ethanol, CO_2 and other metabolites from glucose and

fructose of grapes (Chen et al., 2018). During the analysis of wine after fermentation, wine components, sensorial properties, compositions of anthocyanin and volatiles, quality of aroma in wine, oenological tannin composition, amount of vinylphenolic pyranoanthocyanins, color stability are important (Chen et al., 2018). To improve these quality attributes of wine, using non-*Saccharomyces cerevisiae* strains like *Schizosaccharomyces* and *Lachancea thermotolerans* with *Saccharomyces cerevisiae* strains is an increasing demand for wine industry (Chen et al., 2018). These species have strong effects on anthocyanin content, aroma compounds and sensorial properties when used with *Saccharomyces* species, whereas the risk of spoilage, the production of undesirable components, the negative effect on sensorial features increase and the fermentation rate, the growth of cells decrease when non-*Saccharomyces* used alone (Chen et al., 2018).

In wine fermentation, the main objective is to control fermentation kinetics and produce the wine with best quality, which is very difficult to optimize, rather than achieving higher productivity or yield like in industrial fermentations. The quality of finished product is the most important parameter for wine industry. The amount of sugar during and after fermentation, the continued time of fermentation and the required energy amount for regulating the temperature of process are some of the technological parameters to control during fermentation. For example, too long or too fast fermentation durations are not desirable in wine industry since they have detrimental effect on finished product (Sablayrolles, 2009).

The most important controlling parameters on wine are yeast strain, temperature, nutrient addition such as nitrogen, oxygen, vitamin and mineral and using improved or mixed strains during fermentation (Sablayrolles, 2009). While controlling the fermentation kinetics and procedure, there are some legislation proposed by International Organization of Vine and Wine and the list is different in terms of the countries, for example it has more restrictions for France and Italy, and the varieties of wines. Acidification or deacidification of must, clarification of must or wine,

decreasing the alcohol content and supplementation of activators are allowed according to this list (Sablayrolles, 2009).

The most important compounds that affect the complicated sensorial attributes like aroma, taste and mouthfeel of wine are ethanol content, tannin amount and acidic environment. These compounds are adjustable before or during fermentation; tannin amount is related with bottling conditions and maceration (see 1.1.2) process, ethanol content is related with sugar concentration in grapes before fermentation and acidic environment of wine is related with tartaric acid concentration before fermentation (Frost, Harbertson, & Heymann, 2017).

One of the most important sensorial properties of wine is coming from aromatic compounds that are formed during fermentation and the total amount of them is between 0.8 and 1.2 g/L (Regodón Mateos, Pérez-Nevado, & Ramírez Fernández, 2006). The major volatile compounds are acetaldehyde, acetic acid, propanol, ethyl acetate, isobutanol, methylbutanol, whereas the minor ones are acetals, alcohols, phenolic, esters and so on. The amounts and quality of these major and minor volatiles depend on the environmental factors, enological conditions and the type and amount of yeasts used during process (Regodón Mateos et al., 2006).

One of the major volatile compounds is acetaldehyde, which is a very reactive component. It is formed during glycolysis pathway (see 1.2.2) of yeast from pyruvate and it is also an initiator for ethanol and acetates in wine. Finished wine products initially contain acetaldehyde below than an amount of 75 mg/L and during storage this amount decreases because of its reactivity. Like most of the compounds formed during fermentation, acetaldehyde formation also depends on must composition, pH and temperature, aeration conditions, SO_2 concentration and yeast strain. In wine, it is an undesired component at high concentrations because of the loss of organoleptic properties (Regodón Mateos et al., 2006).

After acetic acid (see 1.1.2), the second most important compound that affects the volatile acidity (made from compounds in types of acid found in wine and give

aromatic taste of wine) of wine and one of the major volatile component in wine is ethyl acetate. This component is produced by the reaction of enzymatic esterification of ethanol and acetic acid and its concentration increases with increasing aeration. If no aeration is applied to the must fermentation, the concentration of ethyl acetate is between 30 to 50 milligram per liter and if aeration is applied, the concentration of this compound increases to 60 to 110 milligram per liter. Taking into account the aromatic effect, the ethyl acetate concentrations below 70 mg/L is considered positive for wine and on the other hand higher than 150-200 mg/L is considered negative for wine. The wine yeast, *Saccharomyces cerevisiae*, is one of the lowest ethyl acetate producers among the wine yeast strains (Regodón Mateos et al.f, 2006).

The other major volatile compounds are fusel alcohols like methylbutanol, isobutanol, propanol, tyrosol, hexanol and phenylethanol (Regodón Mateos et al., 2006). During wine fermentation, fusel alcohols are formed by the formation of aromatic and aliphatic alcohols by yeasts. Fusel alcohols are formed via Ehrlich pathway, catabolism of amino acids. Like other volatile components, fusel alcohols at high concentrations are undesired due to off flavor effect (Hazelwood, Daran, Van Maris, Pronk, & Dickinson, 2008).

1.1.1. Fermentation

Biotechnology is a type of technology that deals with biological systems, living organisms to create or improve different products from them. For example, brewing and baking are the branches of biotechnology where yeast is used as a living organism and beer and bread are obtained as the desired products. These are the examples of traditional operations of biotechnology while modern biotechnology deals with advanced modifications of biological systems and living organisms. Nowadays, biotechnology includes many technologies like genetics, biochemistry, food, molecular biology, medicine, agriculture and so on (Department of Biotechnology and Food Science, n.d.).

As mentioned above, biotechnology is crucial for food industry for protection, production, nutritional enrichment and conservation applications of foods. In order to use biotechnology in food efficiently, it is important to try and apply new fermentation techniques.

Fermentation originally comes from the Latin word of "fermentum" which means "to ferment". Also, the science of fermentation is named as "zymology" and the first known zymologist in history was Louis Pasteur. Although the history of fermentation technology is very old, usage of fermentation technology for food and beverages for industrial purpose started in 16th century. From 16th century to 19th century, fermentation technology was used for preservation and flavor advancement; however, for last 2 centuries it has started to use for turning raw materials into edible foods and drinkable beverages with high stability, improved features and quality (Vilela, 2019).

In order to create innovative fermented food products, using biotechnological applications (utilizing living organisms for creating desired and innovative food and beverages) is widespread. Productions of cheese, vinegar or tea are the examples of solid-state fermentation whereas productions of wine, beer or yoghurt are the examples of submerged fermentation. All fermentation types depend on some external and internal factors; temperature, pH, composition of fermentation medium, dissolved gases, conditions of fermentation system and so on. These factors affect the rate of fermentation, the yield of fermentation products, sensorial properties of final product, nutritional value of the final product, the production of metabolites in media, physicochemical equilibrium of the system and health effect of final product (Vilela, 2019).

1.1.2. Wine Making Process (Vinification)

Wine processing is a series of unit operations that starts from crushing of grapes and ends with bottling of the wine. The processing of wine is different for red and white wine in accordance to presence of phenolic components.

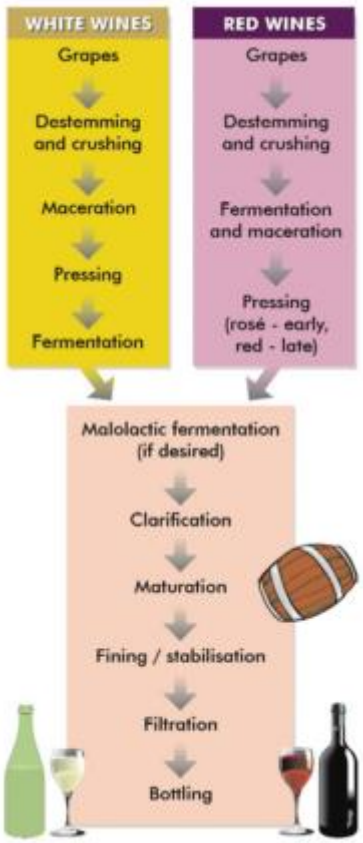

Figure 1.1. The main steps in wine production (Pretorius & Høj, 2005)

The wine processing basically consists of several steps: De-stemming, Crushing, Maceration, Pressing, Thermovinification, Filtration and Centrifugal Clarification, Fining, Membrane Filtration and Bottling (Soleas, Diamandis, & Goldberg, 1997).

De-stemming: To diminish the excessive uptake of lipids and phenols from grapes, external materials of grapes such as stems and leaves must be removed before crushing and after harvest immediately. Compared with seeds and skins, more astringency sensation and bitter taste can be produced by stem phenols. Therefore, if wine processing starts with red grapes that are low in phenol content, extraction of stem phenols can be applicable. Since leaves of grapes contain linoleic and linolenic acids, removing the leaves before crushing is important to avoid the inhibitory effect of

excessive formation of aldehydes and alcohols from oxidation reaction of linoleic and linolenic acids (Soleas et al., 1997).

Crushing: The spoilage of wine with unwanted microorganisms and the uncontrolled oxidation of wine are not desired in wine processing. Therefore, after de-stemming, the grapes must be crushed immediately to protect wine from microbial spoilage and to control the oxidation reactions. In crushing step, the grapes are pressed by passing the rollers or against a hollow wall to break the berries and collect the grape juice, the grape seeds and grape skins in the barrels (Soleas et al., 1997).

Maceration: Maceration process is defined as the amount of contact time between seeds, skins and stems and the fermenting must or wine for forming the phenolics and sensorial attributes of formed wine (Casassa & Harbertson, 2014). After crushing step, the enzymes in grape cells are released and the components that are bound in the skins, stems and seeds of grapes can be easily soluble through these enzymes. This step differs for red and white wine. For red wine, maceration step is applied to grape juice during the initial phase of fermentation; however, for white wine, limited maceration is applied to grape juice before the fermentation. During maceration process of white wines, the temperature and contact time are the parameters for formation and extraction of phenols from skin, seed and stems of grapes. On the other hand, during maceration process of red wines, the duration and conditions of process are the parameters for formation and extraction of phenols from skin, seed and stems of grapes. Maceration parameters affect the phenolic content, color, volatile compounds and aging properties of wine (Soleas et al., 1997).

Pressing: After maceration process, the pressing is applied to grape must. Different types and degrees of pressing change the characteristic of the final product. Horizontal, pneumatic and continuous screw pressing can be applicable for wine processing (Soleas et al., 1997).

Thermovinification: To accelerate the wine processing and to consume the final product (wine) earlier, thermovinification process is applicable. The color of red wines

is also enhanced by thermovinification process by heating the crushed and pressed grapes between the temperatures of 50 and 80ºC according to type of grapes (Soleas et al., 1997).

Filtration and Centrifugal Clarification: Before fermentation, the white grape juices must be clarified. Also, different types of materials that can be seen under microscope must be removed from the grape juices. This can be applied with 3 methods separately or together. First one is natural settling and racking process. The other one is filtration process with earth or pad. And the final one is centrifugal separation process also named as desludging or decanting (Soleas et al., 1997).

Fining: After filtration or clarification, there are still some unwanted components that must not be in fermentation media. By using a reactive and/or an adsorptive material, named as fining agents, the concentration of these unwanted components can be reduced or eliminated. Fining agents can be grouped under 7 headings: Proteins, Clays, Synthetic Polymers, Polysaccharides, Carbons, Silica Gels and Others. Gelatin, casein and albumin are the examples of proteins, bentonite is the example of clays, nylon is the example of synthetic polymers, gum arabic and agar are the examples of polysaccharides and activated carbon is the example of carbons (Soleas et al., 1997).

Membrane Filtration and Bottling: Membrane filtration is the final filtration step of wine processing and it is done with synthetic polymer membrane filters like cellulose acetate. In this step, the amounts of dissolved oxygen and carbon dioxide are calibrated. Before membrane filtration, wine is pre-filtered by filter cakes or pads. The types of these filters that are used in pre-filtration step differ from pore size and are adjustable according to desired flow direction (perpendicular to membrane or parallel to surface). The reason of pre-filtration is eliminating the larger particles like microorganisms and solutes like proteins and gums. After pre-filtration and membrane filtration, wine is ready for bottling in dark color bottles (Soleas et al., 1997).

1.2. *Saccharomyces cerevisiae*

Wine fermentation (basically conversion of grape sugar or grape must into ethanol or wine) is spontaneously occurred by yeast strains that are present on grape surface or winery equipment. By today's technology, to achieve complete fermentation, good oenological properties and high production yield, commercially produced yeast strains, mostly *Saccharomyces cerevisiae*, are used for wine fermentation as starter microorganisms (Bağder Elmacı et al., 2014). The reasons for choosing *Saccharomyces cerevisiae* are well adaptation to wine environment, high fermentation rate, resistance against high sugar, temperature, ethanol and sulfur dioxide concentrations, easily filtration after fermentation, rarely possession of killer phenotype, moderate glycerol production, low undesired component production (foam, acetaldehyde, SO_2, H_2S, higher alcohol) and dominant characteristic behaviour in wine environment (Bağder Elmacı et al., 2014).

Saccharomyces cerevisiae has generally round shape and is used as a eukaryotic cell in biological studies where Saccharo means "sugar fungus" and cerevisiae means "of beer". Its reproduction mechanism is budding and cells of these microorganisms take part in many types of fermentation process like baking. The growth form of yeast cells in haploid or diploid form and the doubling time is about 90 minutes. According to way of growth (aerobically or anaerobically) of cells, the types of consumed sugars by cells change; that is, the cells grown under aerobic conditions prefer galactose and fructose to ferment. For growth conditions, dihydrogen phosphate as a source of phosphorus and ammonia and urea as a source of nitrogen are required for all strains and sulfur and some materials like magnesium are required for optimum growth conditions. The advantages of using *S. cerevisiae* in biological and industrial applications are food-grade, low cost and mechanical strength (Salari & Salari, 2017).

1.2.1. Glycolysis Pathway

Glycolysis pathway (the word comes from glucose+lysis = glucose degradation) is a metabolic pathway, in which glucose is converted into pyruvate by releasing high energy molecules, ATP (adenosine triphosphate) and NADH (nicotinamide adenine dinucleotide hydride). As seen below and Figure 1.2, glycolysis pathway consists of series of reactions, which is a sequence of ten enzymatic reactions (Teusink et al., 2000):

1. Glucose + ATP^{4-} → Glucose-6-phosphate^{2-} + ADP^{3-} + H^{+}
2. Glucose-6-phosphate^{2-} → Fructose-6-phosphate^{2-}
3. Fructose-6-phosphate^{2-} + ATP^{4-} → Fructose-1,6-bisphosphate^{4-} + ADP^{3-} + H^{+}
4. Fructose-1,6-bisphosphate^{4-} → Dihydroxyacetone phosphate^{2-} + Glyceraldehyde-3-phosphate^{2-}
5. Dihydroxyacetone phosphate^{2-} → Glyceraldehyde-3-phosphate^{2-}
6. Glyceraldehyde-3-phosphate^{2-} + Pi^{2-} + NAD^{+} → 1,3-Bisphosphoglycerate^{4-} + NADH + H^{+}
7. 1,3-Bisphosphoglycerate^{4-} + ADP^{3-} → 3-Phosphoglycerate^{3-} + ATP^{4-}
8. 3-Phosphoglycerate^{3-} → 2-Phosphoglycerate^{3-}
9. 2-Phosphoglycerate^{3-} → Phosphoenolpyruvate^{3-} + H2O
10. Phosphoenolpyruvate^{3-} + ADP^{3-} + H^{+} → Pyruvate^{-} + ATP^{4-}

Like metabolism of glucose, fructose is also metabolized by yeast cells with very similar pathway and similar reactions. The reactions for fructose metabolism are as follows (Zimmermann & Entian, 1997):

1. Fructose + ATP^{4-} → Fructose-6-phosphate^{2-} + ADP^{3-} + H^{+}
2. Fructose-6-phosphate^{2-} + ATP^{4-} → Fructose-1,6-bisphosphate^{4-} + ADP^{3-} + H^{+}
3. Fructose-1,6-bisphosphate^{4-} → Dihydroxyacetone phosphate^{2-} + Glyceraldehyde-3-phosphate^{2-}
4. Dihydroxyacetone phosphate^{2-} → Glyceraldehyde-3-phosphate^{2-}

5. Glyceraldehyde-3-phosphate^{2-} + Pi^{2-} + NAD^{+} → 1,3-Bisphosphoglycerate^{4-} + NADH + H^{+}
6. 1,3-Bisphosphoglycerate^{4-} + ADP^{3-} → 3-Phosphoglycerate^{3-} + ATP^{4-}
7. 3-Phosphoglycerate^{3-} → 2-Phosphoglycerate^{3-}
8. 2-Phosphoglycerate^{3-} → Phosphoenolpyruvate^{3-} + H2O
9. Phosphoenolpyruvate^{3-} + ADP^{3-} + H^{+} → Pyruvate^{-} + ATP^{4-}

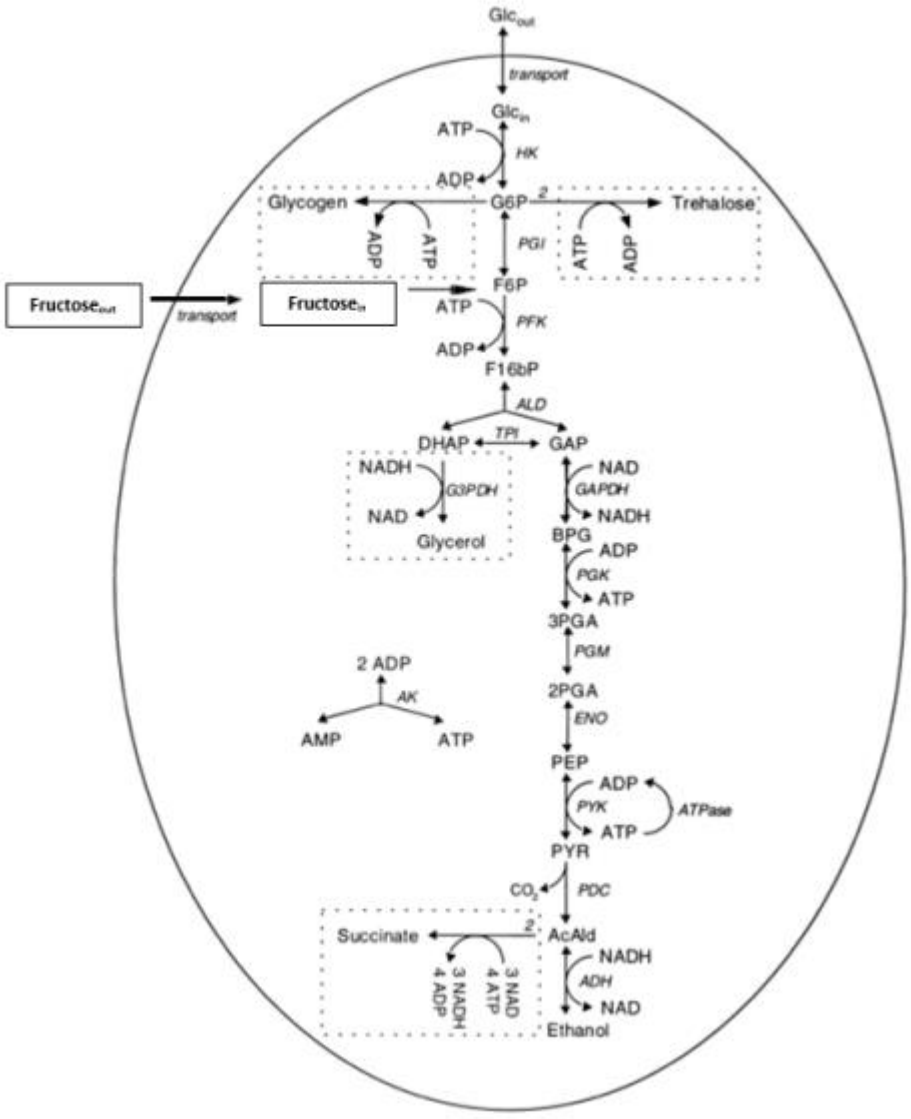

Figure 1.2. Schematic of glycolysis pathway (Teusink et al., 2000)

This pathway does not depend on oxygen availability in environment; thus, it can occur under oxygen free environment and also it occurs mostly in the cytosol parts of cells. There are some heterofermentative and homofermentative types of glycolysis pathways and the most common one is Embden-Meyerhof-Parnas pathway (EMP Pathway). The glycolysis pathway can be considered as two-stage: in first stage (Preparatory phase), ATP is consumed and in second stage (Pay-off phase), ATP is produced (Teusink et al., 2000).

After formation of pyruvate, if there is not sufficient oxygen in environment, lactate is formed in the cell. During this formation, NADH components are oxidized to NAD+ ions and these ions take part into glycolysis reactions. Excess lactate is removed from cells by diffusion. However, if there is sufficient oxygen in environment, pyruvate is converted into Acetyl CoA and Acetyl CoA enters the Kreb's Cycle (Citric Acid Cycle=Tricarboxylic Acid Cycle) to form carbon dioxide and water.

In some microorganisms (like yeasts), the ethanol formation occurs from pyruvate. With the help of pyruvate carboxylase, pyruvate is converted into acetaldehyde and with the help of alcohol dehydrogenase, acetaldehyde is converted into ethanol.

1.2.2. Hexose Transporters of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae strains derived from industrial wine has hexose transporters (*HXT* 1-7) that are responsible for wine fermentation. It is mentioned that there is no growth or fermentation when *HXT* 1-7 are deleted from the genes of this yeast (Luyten et al., 2002).

Hxt1 and Hxt3 are the low affinity carriers that have predominant role in wine fermentation. Hxt3 carrier has a major role when fermentation profile of yeast is considered. However, Hxt1 carrier is the most important carrier at the beginning of the fermentation. Hxt2, Hxt6 and Hxt7 are the high affinity carriers that have role in normal fermentation. Hxt2 is responsible for growth initiation and Hxt6 and 7 are responsible at the end of the wine fermentation (Luyten et al., 2002). That is, the hexose transporters or carriers are required for alcoholic wine fermentation.

Ethanol formation in wine medium cause change in the affinities of hexose transporters and the change in the affinities of hexose transporters cause stuck fermentation. In this study, stuck fermentation due to ethanol formation was discussed and the experiments were conducted to prevent stuck fermentation.

1.3. Sluggish or Stuck Fermentation

The hexose sugars, glucose and fructose, are the main reducing monosaccharides present in grapes or grape musts. Although their empirical formulas are same with each other, $C_6H_{12}O_6$, they have different physicochemical properties due to their different structure. The amounts of total sugars in grapes or grape musts change between 160 and 300 g/L which consist of almost equal amounts of glucose and fructose before fermentation (Tronchoni, Gamero, Arroyo-López, Barrio, & Querol, 2009). During wine fermentation, yeasts, especially *Saccharomyces cerevisiae*, co-ferment these monosaccharides and produce wine components; ethanol, glycerol, carbon dioxide and other metabolites (Luyten, Riou, & Blondin, 2002; Rodríguez-Sifuentes et al., 2014; Tronchoni et al., 2009). Since yeasts have glucophilic character, that is preference of fermenting glucose to fructose (Rodríguez-Sifuentes et al., 2014), the utilization rate of glucose is higher than that of fructose during fermentation (Tronchoni et al., 2009). The glucophilic character of yeasts may be due to transportation across the plasma membrane of yeast by hexose transporters or phosphorylation inside the cell of yeast by hexose kinases have different affinities through glucose and fructose (Tronchoni et al., 2009). These different utilization rates result in glucose/fructose discrepancy (GFD) and residual fructose amount higher than 2 g/L (Berthels, Cordero Otero, Bauer, Thevelein, & Pretorius, 2004) when fermentation process completed (Tronchoni et al., 2009). By winemakers, the total residual sugar content in wine must be between 2 and 4 g/L or less than 4 g/L to name a wine as complete or dry wine (Alexandre & Charpentier, 1998; Maisonnave, Sanchez, Moine, Dequin, & Galeote, 2013). However, higher than 2 g/L of fructose present in wine, due to glucose fructose discrepancy, is a problem for winemakers. Since the sweetness of fructose is approximately twice than that of glucose (Lee,

1979), it affects the final sweetness of wine and the wine fermentation results in higher sweetness, which is undesirable in wine industry. Also, high residual fructose increases the risk of microbial spoilage since heterofermentative lactic acid or acetic acid bacteria utilize this excess sugar and produce acetic acid, lactic acid or undesirable esters (Alexandre & Charpentier, 1998; Maisonnave et al., 2013; Zinnai, Venturi, Sanmartin, Quartacci, & Andrich, 2013) and decreases the ethanol yield in wine (Berthels et al., 2004; Rodríguez-Sifuentes et al., 2014; Tronchoni et al., 2009). This has been informed that sluggish (incomplete) or stuck (depleted) fermentation in literature (Berthels et al., 2004). Stuck fermentations decrease the quality and productivity of wine (Maisonnave et al., 2013).

Briefly, at the beginning of fermentation, different types of grapes contain almost equal amounts of glucose and fructose. Even if there is no alcohol in environment, the affinity of hexose transporters is higher for glucose than that of fructose. During wine fermentation, the ratio of glucose to fructose changes because the affinity of yeast hexose transporters is affected by ethanol formation. Since the difference between the affinity of hexose transporters increases with increasing alcohol, the affinity of hexose transporters increases for glucose and results with residual fructose (Mocke, 2013). This situation causes stuck fermentation and it is an undesirable situation for wine industry (Berthels, Cordero Otero, Bauer, Thevelein, & Pretorius, 2004).

1.3.1. Reasons of Stuck Fermentation

Although the exact reason of stuck or sluggish fermentations has not been determined yet, there are more than 15 reported reasons such as nitrogen deficiency (Alexandre & Charpentier, 1998; Berthels et al., 2004; Luyten et al., 2002; Maisonnave et al., 2013; Mendes-Ferreira, Mendes-Faia, & Leão, 2004; Tronchoni et al., 2009), limitation or excess amount of oxygen (Alexandre & Charpentier, 1998; Maisonnave et al., 2013), too much clarification (Alexandre & Charpentier, 1998; Maisonnave et al., 2013), formation of by-products due to fermentation (Maisonnave et al., 2013), high ethanol accumulation (Alexandre & Charpentier, 1998; Berthels et al., 2004;

Maisonnave et al., 2013; Tronchoni et al., 2009; Viana, Loureiro-Dias, & Prista, 2014), vitamin and mineral deficiency (Alexandre & Charpentier, 1998; Maisonnave et al., 2013), toxic residues for yeasts from fermentation (Alexandre & Charpentier, 1998; Maisonnave et al., 2013; Rodríguez-Sifuentes et al., 2014), deprivation of nutrients for yeasts (Zinnai et al., 2013), too high or low temperatures (Zinnai et al., 2013), environment with high acidity (Zinnai et al., 2013), the formation of inhibitors like phenols (Zinnai et al., 2013), change in the equation of ionic components (Zinnai et al., 2013), higher sulphite content (Alexandre & Charpentier, 1998) and so on.

1.3.2. Biochemical Facts of Stuck Fermentation

1.3.2.1. Ethanol Toxicity

Ethanol inhibits the growth and viability of yeast cells. Since ethanol shows the non-competitive inhibition (the inhibitor reduces the activity of the enzyme and binds equally well to the enzyme whether or not it has already bound the substrate), the catabolite metabolism of yeast cells are inactivated by ethanol (Alexandre & Charpentier, 1998; Luyten et al., 2002). As fermentation continues, the accumulation of ethanol increases. As a result of increasing of ethanol content in environment, some yeast species are unable to grow under high ethanol conditions and the utilization rates of hexose sugars show decreasing pattern (Rodríguez-Sifuentes et al., 2014). Although glucose and fructose are transported with same hexose transport proteins, the affinity of hexose transporters, especially for fructose, decreases as the accumulation of ethanol increases (Rodríguez-Sifuentes et al., 2014). The reason of decreasing affinity of fructose transporters with ethanol accumulation is shifting of equilibrium from fructopyranose (six-membered ring) to fructofuranose (five-membered ring) by ethanol (Rodríguez-Sifuentes et al., 2014).

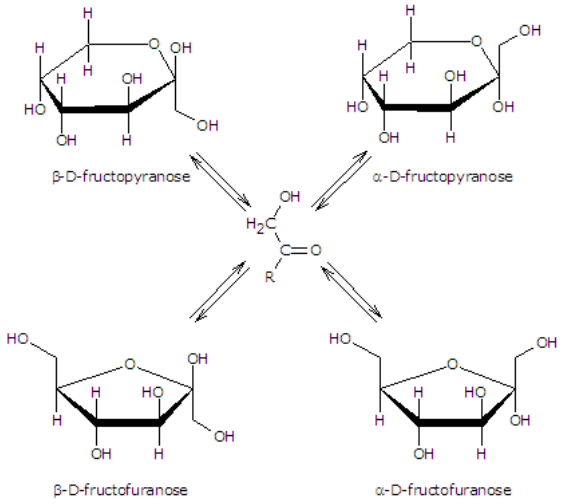

Figure 1.3. Ring structures of fructopyranose and fructofuranose

Since ethanol is a polar molecule, it increases the polarity of interior region when it enters the yeast cell and diminishes the hydrophobic properties. Also, ethanol formation causes change in plasma membrane permeability, organization of membrane compounds and therefore gives damage to yeast cells (Alexandre & Charpentier, 1998). Ethanol toxicity changes the membrane fluidity by changing the composition and chain length of lipids on membrane (Alexandre & Charpentier, 1998; Luyten et al., 2002).

1.3.2.2. Nitrogen Starvation

One of the known major causes of stuck fermentation is assimilable nitrogen starvation during fermentation (Berthels et al., 2004). According to grape type, region of grapes, maturity of grapes and process conditions, the available nitrogen amount changes between 60 to 2400 mg/L of grape juice. For wine fermentation, the requirement of assimilable nitrogen amount changes between 120 to 140 mg/L of grape juice for yeast cells (Alexandre & Charpentier, 1998). According to presence of oxygen and initial sugar content, assimilable nitrogen amount for yeast cells is changed. Also, growth, fermentation and utilization rates of yeasts decrease with

decreasing available nitrogen. Thus, the transportation of sugars by hexose transporters and the production of CO_2 by fermentation decrease (Alexandre & Charpentier, 1998). The depletion of nitrogen amount causes ceasing in the synthesis of proteins by inactivation of hexose sugar permeases (Luyten et al., 2002). According to Luyten et al., the inactivation of permeases is because of inducing of protein turnover during nitrogen starvation and degrading the proteins in cell vacuoles. Since hexose transporters are proteins, depletion in assimilable nitrogen amount causes the degradation of these transporters (Luyten et al., 2002; Mendes-Ferreira et al., 2004). After available nitrogen is depleted, inactivation of hexose transporters last 50 hours which can be protected by protein synthesis with ammonium supplying (Alexandre & Charpentier, 1998). Another aspect of presence of nitrogen or ammonia is its role of allosteric activator for phosphofructokinase which is responsible for signaling pathway in yeast metabolism. There is remaining sugar in environment due to nitrogen starvation, the pathway is induced by the existence of excess sugar and the production of CO_2 decreases with this mechanism because of the nitrogen starvation (Alexandre & Charpentier, 1998).

1.3.2.3. Oxygen Deficiency

The availability of oxygen in environment is an important parameter for the growth of yeast, synthesis of main components of cell membranes of yeasts; such as sterols and unsaturated fatty acids, providing cell viability and also decreasing the effect of ethanol toxicity (Bisson, 1999; Malherbe, Bauer, & Du Toit, 2007). For example, the unsaturated fatty acids are synthesized from saturated fatty acids by desaturase enzymes which are oxygen-dependent and if there is no oxygen in environment, the enzymes are inactivated because of oxygen deficiency (Alexandre & Charpentier, 1998). As amount of dissolved oxygen decreases, the production of toxic fatty acids and the effect of ethanol toxicity increases (Alexandre & Charpentier, 1998; Malherbe et al., 2007). The cell viability is related with the lipid composition in plasma membrane and as unsaturated fatty acids composition of membrane increase, the resistance of yeast cells against ethanol increases, too (Alexandre & Charpentier,

1998; Bisson, 1999). As mentioned before, the growth pattern and the viability of cells depend on the dissolved oxygen accumulation and if there is a deficiency of oxygen, the stuck or sluggish fermentation can occur (Malherbe et al., 2007). The oxidase enzymes such as polyphenol oxidase are naturally existing in grapes and inhibit the dissolved oxygen, therefore sulfur dioxide addition is essential to inhibit oxidases in grape must before fermentation (Alexandre & Charpentier, 1998; Bisson, 1999). Also, the aeration during fermentation can increase the availability of cells and the synthesis of lipids (Malherbe et al., 2007).

1.3.2.4. Vitamin and Mineral Deficiency

The vitamin content of environment before fermentation is very different from that of after fermentation, whereas the mineral content does not change too much during fermentation (Maisonnave et al., 2013). The viability and growth of cells are positively dependent on the amount of vitamins and minerals (Maisonnave et al., 2013). Moderate amounts of vitamins and minerals are required for wine fermentation. Vitamins like thiamine are directly used up by yeast cells, however vitamins like riboflavin are generated by cells (Maisonnave et al., 2013). Since thiamine is easily destroyed by sulfur dioxide, its bioavailability decreases with sulfur dioxide and it results with stuck fermentation (Alexandre & Charpentier, 1998). Also, high concentrations of minerals like $MgSO_4$ cause the formation of sulfate which has inhibitory effect on yeast (Maisonnave et al., 2013). For minerals, magnesium is the most important mineral for metabolism, growth and viability of yeast cells. Mg^{+2} can stabilize nucleic acids, proteins and lipids which are essential components for yeasts (Alexandre & Charpentier, 1998). Also, it plays an important role during wine fermentation since the activation of hexokinases, phosphofructokinases and alcohologenic enzymes are provided by magnesium ions during glycolysis and alcohol formation (Alexandre & Charpentier, 1998). The viable yeast cell amount decreases with decreasing available Mg^{+2} and this situation ends up with lower fermentation rate since Mg^{+2} has effect on the stability of cell membrane (Alexandre & Charpentier, 1998). Also, magnesium can inhibit the harmful effects of ethanol. Therefore, if

available magnesium concentration increases, the rates of growth, fermentation and ethanol production increase, too (Alexandre & Charpentier, 1998).

1.3.2.5. Higher Sulphite Content

Sulphite is widely used in the production of all types of wines to sterilize vessels, equipment and bottles and also protect must and wine against oxidation reactions. Most of the microorganisms, especially natural yeasts present on the surface of grapes, are very susceptible to sulphite depending on the pH and temperature of environment and the exposing time. At lower pH values; 3 to 3.5, which are the pH of wines, sulphite has more damaging activity on yeast cells. The amount of sulphite, which is added before fermentation, must be in the amount of killing non-beneficial microorganisms but not killing beneficial yeasts for fermentation. The high rate of sulphite transportation through the plasma membrane of yeasts by facilitate diffusion has killing effect on yeast cells by lowering the ATP in the cell, binding proteins and coenzymes and breaking the thiamine bonds. Therefore, yeasts can be killed with uncontrolled amount of sulphite content and this results with stuck fermentation (Alexandre & Charpentier, 1998).

1.3.3. Possible Ways to Prevent or Restart Stuck Fermentation

As mentioned above, there are some known reasons of stuck fermentation and also there are some possible ways and improvement methods against stuck fermentation such as nitrogen supplementation, controlling the oxygen amount, controlling the temperature of environment, selecting the yeast according to process, controlling nutrients for yeast growth and so on. Although many techniques and improvements are developed, stuck fermentation is already a major problem for wine industry since it causes product losses (Maisonnave et al., 2013). The detailed procedures of restarting or preventing stuck fermentation will be mentioned below.

As stated before, residual sugar can be present in the environment when stuck fermentation occurs. This residual sugar is consumed by lactic acid or acetic acid bacteria and undesirable acids or esters are produced. Adding sulphite in restricted

amounts to the spoiled environment is an important restarting procedure of stuck fermentation (Maisonnave et al., 2013). Using of yeast hulls before this restarting procedure is helpful for detracting spoiled wine from unwanted compounds such as fatty acids and alcohol (Maisonnave et al., 2013).

The reinoculation procedure (Rodríguez-Sifuentes et al., 2014), which is using a new yeast starter obtained from dried yeast, is a common method to restart stuck fermentation (Maisonnave et al., 2013). For example, *Torulaspora delbrueckii* is a *Saccharomyces* species and has fructophilic character, that is prefer fructose instead of glucose. By using this species, residual fructose in environment is utilized and restarting the stuck fermentation can be possible (Rodríguez-Sifuentes et al., 2014). This new yeast starter must have some properties like ability to utilize fructose in environment with ethanol, easy adaptation to high ethanol concentrations and ability to continue fermentation under these situations (Rodríguez-Sifuentes et al., 2014). By adding starter yeast, the fermentation can proceed, the residual sugar can be converted into CO_2 and ethanol and the undesirable sweetness of wine is diminished by lowering fructose concentration. Besides using a new yeast starter, non-*Saccharomyces* species can have more fructophilic character than *Saccharomyces* species and the residual fructose can be fermented by these species (Maisonnave et al., 2013). *Zygosaccharomyces bailii* is one of the popular non-*Saccharomyces* species for restarting procedure since it has higher preference for fructose and resistance against high ethanol concentration (Rodríguez-Sifuentes et al., 2014). According to literature, these two species; *Torulaspora delbrueckii* and *Zygosaccharomyces bailii* can survive in the environment containing 18 % v/v ethanol (Rodríguez-Sifuentes et al., 2014).

If there is oxygen starvation during fermentation, aeration of the must can protect the fermentation process against oxygen deprivation and it is helpful for protecting the must from stuck fermentation (Alexandre & Charpentier, 1998). By oxygen supplementation, the growth and viability of the yeast cells increase especially when oxygen is added at the end of the growth of cells (Alexandre & Charpentier, 1998). When aeration procedure is applied to the must, it is important that oxidase enzymes

are inhibited before by adding SO_2. If they are not inhibited, they react with the existence oxygen in environment and the dissolved oxygen content decreases (Alexandre & Charpentier, 1998).

Before wine fermentation, nitrogen supplementation can be done because of avoiding from nitrogen starvation during fermentation. Nitrogen supplementation is in the form of inorganic compounds like diammonium phosphate. However, it is important to obey legislations when nitrogen is added. The carcinogenic urea is produced by yeast when higher amounts of nitrogen added to environment (Mendes-Ferreira et al., 2004).

Because of the global warming and increased air temperature, grapes contain higher amounts of total sugar at harvest. Higher sugar content means that higher ethanol levels at the end of fermentation. Since high levels of ethanol have toxicity effect on yeast cells, there are some precautions to overcome this situation before fermentation. For example, using a strain of *Saccharomyces cerevisiae*, L2226, is a solution for grapes with higher sugar contents. This strain is more resistant to high levels of sugars and grapes can be inoculated with this *Saccharomyces cerevisiae* strain. By this way, the amount of residual sugar after fermentation can be reduced (Buescher et al., 2001).

There are 2 patents in the literature related with enzyme usage in grape juice or wine to process these media:

First one was named as “Reduced Stuck Alcoholic Fermentations in Wine Production”. In this method, glucose isomerase was used directly in grape juice containing high and low sugar contents or balanced and unbalanced sugar ratios before fermentation. As a result, more efficient alcoholic fermentation and wine with lower risk of stuck fermentation were achieved. The difference between this invention and this thesis is the time that glucose isomerase was added to the media, before fermentation and after fermentation. Also, the aim of this invention was to reduce the risk of stuck fermentation before grape juice fermentation however the aim of this thesis was to prevent the stuck fermentation after wine fermentation (Van Den Brink and Kristine, 2011).

The other patent was named as “Method for the Production of a Wine with Lower Content of Alcohol”. In this method, unfermented grape juice was treated with glucose oxidase and glucose isomerase to obtain wine with lower alcohol content. By lowering the alcohol content, the risk of unwanted stuck fermentation may inhibit by using these enzymes at the same time. Also, in this study, it was found that extra addition of glucose isomerase into environment helped to tackle negative effect of glucose oxidase on *Saccharomyces cerevisiae*. The difference between this invention and this thesis is the way that preventing stuck fermentation. In this invention, two types of enzymes were used and the aim was reducing the alcohol content of environment. In this thesis, one enzyme was used to prevent stuck fermentation directly into environment (Van Den Brink and Kristine, 2014).

Using of an enzyme in wine media for preventing or restarting the stuck fermentation was not studied before. Therefore, using glucose isomerase to prevent or restart the stuck fermentation was studied in this study as a novel approach.

1.4. Immobilized Glucose Isomerase

Enzymes are biocatalysts which are considered as significant alternative to chemical catalysts. The reasons for choosing biocatalysts are that their validity from regenerable sources, biodegradable properties, moderate operating conditions and selectivity of substrate and product. Enzymes also have the potential to produce pure products with different features. Their ranges of industrial applications and processes are very extensive such as food, animal feed, beverage, detergent, medicine and so on. The industrial applications of enzymes have three major categories; technical, food and animal feed. After technical enzymes, the food enzymes are the second largest industry and have different categories; dairy, starch, baking, juice, brewing, fat and oil, functional food industries. Food enzymes are basically enhancing the texture, flavor, color, aroma, nutritional value and appearance of the food products. The enzyme that was used in this study, immobilized glucose isomerase, is in the group of starch industry and its basic application is production of high fructose corn syrup

(Jemli, Ayadi-Zouari, Hlima, & Bejar, 2016). The enzyme in this study was the product of Novozymes and the group of this enzyme was Sweetzyme IT Extra (Novozymes, 2015).

D-xylose or D-glucose isomerase (EC 5.3.1.5) is one of the most important, valuable and high-tonnage enzymes in food industry. It is an intracellular bacterial enzyme and can be obtained from different species (Dhungel et al., 2007). It catalyzes the reversible isomerization reactions of D-xylose to D-xylulose or D-glucose to D-fructose. The reaction of conversion from D-xylose to D-xylulose is irrelevant with this study. The application of glucose isomerase for production of high fructose corn syrup is widely used in industry. Using of immobilized glucose isomerase is preferred and immobilization has a great importance in biotechnological applications (Ge et al., 1998).

The most attractive demand for enzyme industry is increasing the shelf life of enzymes by improving the enzyme productivity and advancing the novel applications. For this purpose, enzyme immobilization increases the usability of enzyme to its substrate. There are some advantages of using immobilized enzymes; lower pH drop after reaction, low color generation, higher productivity, easy to control, regenerable and reusable after process (Antri & Auterinen, 1986). Natural and synthetic supports are used to immobilize enzymes effectively and this application provides long term usage of enzymes. There are different techniques to immobilize an enzyme such as adsorption, covalent binding and entrapment methods (Datta, Christena, & Rajaram, 2013). Since the immobilization techniques enhance the activity, efficiency and stability of enzymes, the enzyme that was used in this study was glucose isomerase in the form of immobilized.

1.4.1. Isomerization Conditions

The activity, stability and productivity terms are important for determining the enzyme specifications. The rate of conversion of substrate to product is called enzyme activity. The retaining the activity of this enzyme during reaction is known as enzyme stability.

The effect of combination of activity and stability is called enzyme productivity and its measurement is the defined amount of product produced per defined amount of enzyme used at the end of its lifetime (Novozymes, 2015; Rahman, Hasan, Hussain, & Jahim, 2007). According to application sheet of Novozymes, temperature, pH, dry substance in feed, dextrose concentration in feed, produced fructose concentration, purity of feed, activation by Mg^{+2} and calcium ion content of feed are important parameters for using immobilized glucose isomerase (Novozymes, 2015).

According to extracted microorganisms, isolated environments and purification methods, the optimum substrate and enzyme concentrations, optimum pH and temperature values and optimum reaction time of same enzymes can differ. For example, if thermophilic microorganisms are used as a source, the extracted enzyme becomes thermostable. For glucose isomerase, most commercial ones are isolated from mesophilic microorganisms like *Bacillus*, *Streptomyces*, *Flavobacterium* spp and so on. Generally, glucose isomerases are not very thermostable, optimum temperature with maximum value of 60 ºC and also have lower activity at pH of 7. The glucose isomerase, which is thermostable and acid stable, is mostly preferred by the industrial applications. It provides shorter process time, higher product concentration, lower by-product concentration and higher stability of process for high fructose corn syrup production (Dhungel et al., 2007).

For the enzyme used in this project was supplied from Cargill, Bursa. It is an immobilized enzyme of Novozymes and it has application sheet. According to this sheet, the productivity is 11000 to 16000 kg fructose per kg of enzyme (400 IGIU/g). Although the more activity is obtained with the higher temperatures, its optimum temperature is 60 ºC since higher temperatures cause by-product formation. That is, stability and productivity decrease with increasing temperatures. On the other hand, these two important properties increase with decreasing temperatures, however, decreasing the temperatures cause microbial spoilage. In terms of pH, maximum stability is achieved between the values of 7.2 and 7.5 whereas maximum activity is achieved at pH above 8. However, higher pH values increase the risk of by-product

formation. All pH values in this sheet were measured at 25 ºC. In terms of initial dry substance content in feed, too high or too dilute feeds decrease the activity of the enzyme and increase the risk of microbial spoilage. Also, some components may either inhibit the activity of the enzyme such as calcium ions or block the active surface of the enzyme. Therefore, sterilization and purification of the feed are very important processes before the isomerization reaction. Sulfite can be added to feed with the amount not higher than 100 ppm. For example, dissolved oxygen in the feed increases the risk of by-product formation and the by-products may inhibit the activity of the enzyme, therefore it is important that there is no oxygen in environment. For this reason, cell culture flasks with closed caps were used for the experiments in this project. Since magnesium ions increase the stability and activity of the enzyme, $MgSO_4.7H_2O$ can be used as a cofactor. On the other hand, amount of calcium ions should not be higher than 1 ppm before isomerization (Novozymes, 2015).

In this study, the important parameters for immobilized glucose isomerase were pH, temperature, SO_2 content, calcium and magnesium content of feed. The operating temperatures and pH values were given in Figure 1.3 and 1.4 and the required amounts of SO_2, magnesium and calcium amounts were given in Table 1.1 below.

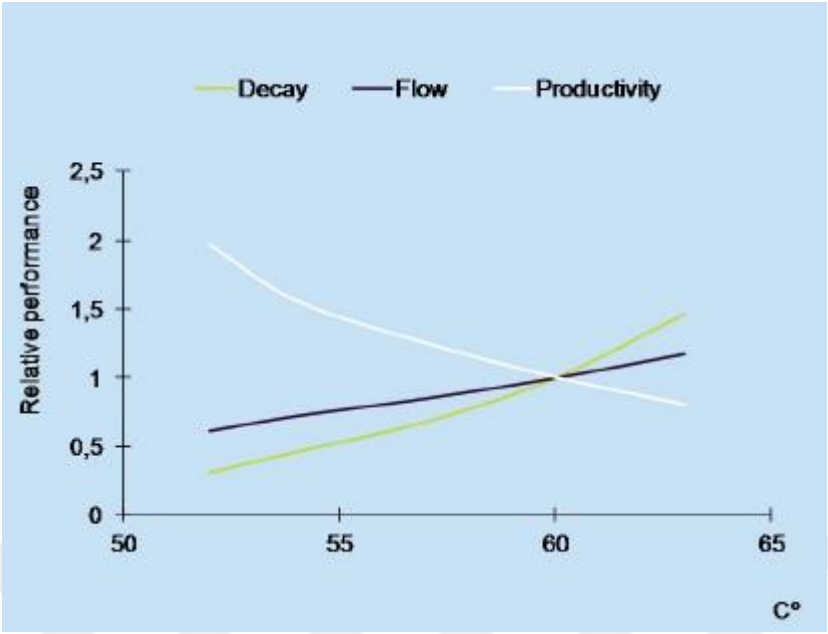

Figure 1.4. Effect of isomerization temperature on decay, flow, productivity and total performance (Novozymes, 2015)

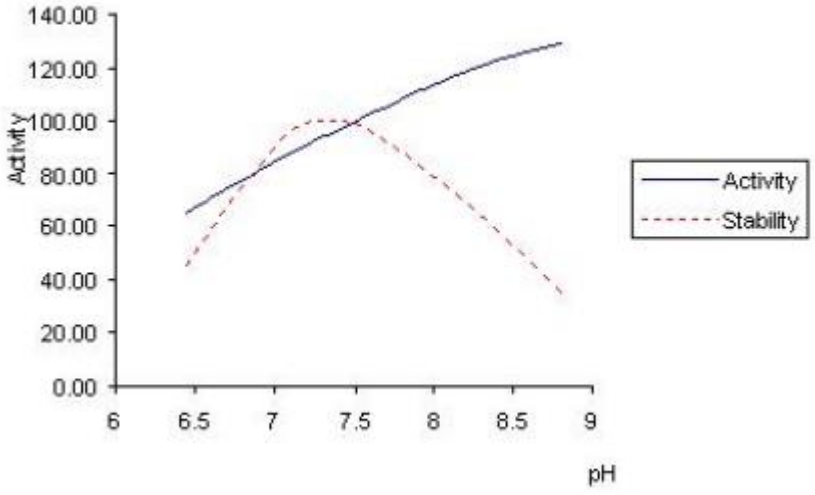

Figure 1.5. Effect of pH on activity and stability (Novozymes, 2015)

Table 1.1. Specification table for glucose isomerase used in this study (Novozymes, 2015)

Specifications	Values
Temperature	At 55-60 ºC
pH	7.5 (measured at 25 ºC)
SO_2	Approximately 100 ppm
Mg^{+2}	45 ppm
Ca^{+2}	Lower than 1 ppm

1.4.2. Cofactors and Inhibitors

Most of the glucose isomerases require metal ions; such as cobalt, magnesium or manganase as cofactors; starting the activity of the enzyme. Since presence of metals are needed, glucose isomerases are also named as metal-activated enzymes or metalloenzymes (Kasumi, Hayashi, & Tsumura, 1982). The required types and amounts of metal ions change according to source of enzymes. Since glucose isomerase that was used in this project is magnesium-activated enzyme from Novozyme, magnesium ion (Mg^{+2}) activates and stabilizes the enzyme. Before isomerization reaction, $MgSO_4.7H_2O$ or $MgSO_2$ must be added to the prepared solutions (Novozymes, 2015). The required amount for Mg^{+2} is decided according to calcium ion level in solution which is a strong competitive inhibitor for glucose isomerase (Ben Hlima, Aghajari, Ben Ali, Haser, & Bejar, 2012). For calcium ion levels at 1 ppm or lower, addition of 45 ppm magnesium ion is sufficient, however, the ratio between Mg^{+2} to Ca^{+2} must be at least 12 for higher concentrations of calcium ion (Chaplin & Bucke, 1992).

Ethylenediaminetetraacetate, EDTA, is a polyamine polycarboxylate chelator with the capacity of forming metal complexes with ions. EDTA forms Ca^{+2}-EDTA interactions by binding calcium with different enthalpy values (Griko, 1999). Therefore, EDTA was used in this study to hold the calcium ions in red wine by considering its ion retention capacity.

The procedures like using EDTA, ammonium lactate or ammonium acetate for separating calcium and magnesium ions are slower and not appropriate when compared with ion-exchange chromatography, which of cation-exchanger resin was preferred in this study. The rapid separation of calcium and magnesium ions from other metal ions are applicable when cation-exchanger is used. Also, determination of eluted metal ions are easier with automatic methods when cation-exchanger resin is used (Michael D. Arguello, 1977). Therefore, holding the calcium ions in red wine by cation exchange reaction was experimented.

1.5. Ion Exchangers

Ion exchange is a reversible process that provides interchanging of ions between a solid ion exchange material and a liquid used in process. After elution, there is no change in the nature of the solid material, therefore the solid material is reusable after suitable regeneration process. There are many applications of ion exchangers in many fields such as water softening, demineralization, waste treatment, nitrate removal, fermentation, sugar separations and purifications in HFCS and so on. For example, removing calcium from hard water and replacing it with sodium ions is widely used in water softening process. To regenerate the resin that is used for water softening, the calcium loaded resin is treated with sodium chloride solution and it can be used in any other water softening process. That is, ion exchange reactions are reversible with proper regeneration. The solid materials can be in the form of spheres, granules or bead form, the particle sizes change between 0.3 to 1.2 mm or they can be uniform as well (Dow Chemical Company, 1999). The working principle of ion exchange resins is that a solution is passed through a resin bed, cross-linked with the polymer of solid material and made interaction with the ion exchange sites of this solid. Both positively and negatively charged ions are exchanged and their names called cation and anion exchangers, respectively (Grant E., 1997).

1.5.1. Cation and Anion Exchangers

The atoms or molecules that have electric charge, either a net positive or negative charge, are called ions. If ions have a net positive charge, they are called cations, whereas if negative, they are called anions. For example, Mg^{+2}, Fe^{+3}, Ca^{+2} are some cations and Cl^{-}, SO_4^{-2}, CO_3^{-2} are some anions. To exchange cations and anions with hydrogen and hydroxyl groups, respectively, cation and anion ion exchangers are used. To hold cations, cation exchanger resins are used since they have negatively charged groups and attract positively charged ions. The cation exchange resins can be regenerated with HCl and the beads are charged with H^{+}. These positive ions attach with negatively charged ions in liquid solution and release H^{+} into the solution. There are two types of cation exchangers; weak and strong acid cation exchangers. For water softening process, strong acid cation exchangers are mostly used to hold Ca^{+2} in the water. Therefore, strong acid cation exchanger was used in this project to hold calcium and magnesium ions in the red wine and the results were measured with atomic absorption. On the other hand, to hold anions, anion exchanger resins are used since they have positively charged groups and attract negatively charged ions. The anion exchange resins can be regenerated with NaOH and the beads are charged with OH^{-}. These negative ions attach with positively charged ions in liquid solution and release OH^{-} into the solution. There are two types of anion exchangers; weak and strong acid anion exchangers. In the industry, weak acid anion exchangers are mostly preferred for holding the positively charged ions. Therefore, weak acid anion exchangers were used in this project to hold L- (+)-Tartaric acid in the red wine and the results were measured with HPLC. The beads of resins should be regenerated if all of the exchange sides of resins are consumed and they cannot hold ions anymore (PURETEC, 2014).

1.6. Objective of the Study

As mentioned before, there are some possible reasons that cause stuck fermentation like nitrogen, vitamin and mineral deficiencies, nutrient starvation, low and high pH values and high sulphite contents. One of the most important reason of stuck

fermentation is glucose/fructose discrepancy due to different reasons. The glucose/fructose discrepancy is the ability of the yeast in for using glucose faster than fructose.

The aim of this thesis was to find a remediation method for stuck fermentation that results from the glucose/fructose discrepancy due to the ethanol accumulation in wine medium. The utilization rate of glucose increases when compared to fructose utilization as the accumulation of ethanol increases. There are some possible ways to protect wine from stuck fermentation like nitrogen supplementation, controlling the oxygen amount, temperature of environment and content of nutrients, selecting the yeast according to process and controlling nutrients for yeast growth.

In this study, the use of enzyme, a method that has never been used before for preventing stuck fermentation, was tried. Therefore, using glucose isomerase as an enzyme in wine media can be a new solution as a prevention method against stuck fermentation. By using immobilized glucose isomerase, the residual fructose can be converted into glucose and yeast may continue to utilize glucose and fermentation process. Thus, undesirable sweetness of wine and the risk of microbial spoilage can be reduced.

In accordance with this purpose, immobilized glucose isomerase was tried in different wine like media (synthetic media) to test the activity of enzyme. To test the activity, following experiments were conducted with immobilized glucose isomerase. The effect of substrate type (glucose or fructose), temperature (30^{o}C and 60^{o}C), ethanol concentration (up to 15%), low pH values, glycerol content, sulphite content, presence of tannins and tartaric acid, fermentation components, calcium concentration, dilution factor and different pH values on isomerization reactions. These parameters were chosen according to the wine content and the enzyme was tested under the effect of wine content.

CHAPTER 2

MATERIALS AND METHODS

In this part, the materials that were used in the experiments and the methods that were applied for experiments were mentioned. For all experiments that were discussed in Chapter 3, the methods varied. Also, "synthetic media" term was defined in this chapter for different experiments that were in Chapter 3, too. This term means that media prepared with different wine components like ethanol, glycerol, tartaric acid, calcium and so on.

2.1. The effect of substrate type and temperature on the isomerization reaction of glucose isomerase

In these experiments, either glucose or fructose were used as substrates of reactions at an amount of 1 % w/v. The flasks were incubated in shakers at 60 ºC or 30 ºC at 150 rpm. Since the optimum activity temperature of immobilized glucose isomerase is 60 ºC (Novozymes, 2015), one of the temperature value of experiments was 60 ºC. Also, since the optimum growth condition of yeast is 30 ºC (Salari & Salari, 2017), the other temperature value of experiments was 30 ºC. Since Mg^{+2} is the activator of glucose isomerase and the required amount for activation is equal to 45 ppm, 0.06 g $MgSO_4.7H_2O$ was added into 100 mL solutions (Novozymes, 2015). The pH values of media were measured as 5.8 and 5.4 for glucose and fructose as substrates, respectively. The experiments were conducted with one gram of immobilized glucose isomerase added for 100 mL of solutions.

2.2. The effect of ethanol on the isomerization reaction of glucose isomerase

According to the information from Kavaklıdere Şarapları A. Ş., the dry wine products contain 9-15 % v/v ethanol. Therefore, to see the effect of ethanol on enzyme, synthetic media were prepared by adding 13 % v/v ethanol at 60 ºC and 30 ºC. As

mentioned before, fructose was added into the media at an amount of 1% w/v. The measured pH value for solution containing 1 % w/v fructose and 13 % v/v ethanol was equal to 5.5. In order that Mg^{+2} is the activator of glucose isomerase, 0.06 g $MgSO_4.7H_2O$ was added into 100 mL solutions according to reference (Novozymes, 2015). The prepared flasks were incubated in shakers at 150 rpm with one gram of immobilized enzyme added into 100 mL solutions.

For comparing wine and wine without ethanol, samples were prepared with 1 % fructose w/v and with two parallels for both. The flasks were incubated at 60 ºC for approximately 42 hours containing one gram of immobilized enzyme added for 100 mL solution. Since red wine already contains Mg^{+2} in it (Navarro-Alarcon et al., 2007), there was no addition of activator in these samples. The pH values of samples were determined as 3.6 and 2.8 for wine with alcohol and without alcohol, respectively.

2.3. The effect of low pH values on isomerization reaction in synthetic medium

Different pH values, which were 3.3, 3.6 and 4, were adjusted with 1:1 acetic acid in synthetic media containing 1 % w/v fructose. 0.06 g $MgSO_4.7H_2O$ was added to activate the enzyme into 100 mL solutions. The flasks were incubated at 60 ºC and 150 rpm for almost 150 hours with one gram of glucose isomerase per 100 mL solutions. The experiment was conducted with two parallels for each pH values.

2.4. The effect of glycerol in synthetic media on the isomerization reaction

The synthetic media were prepared with 1 % w/v fructose, 13 % v/v ethanol and different glycerol contents (1 %, 0.8 %, 0.6 %, 0.4 % and 0.1 % v/v). The flasks were incubated at 60 ºC for 47 hours containing one gram of immobilized enzyme in 100 mL solution. To increase the activity of the enzyme, 0.06 g $MgSO_4.7H_2O$ was added to each flask. In order to simulate the wine environment, the pH was adjusted to 3.3 with 1:1 acetic acid solution.

2.5. Effect of sulphite content on isomerization reaction

The synthetic medium contained 1 % w/v fructose as a substrate with different sulphite amounts; 0.01 %, 0.05 % and 0.1 % w/v. The flasks were incubated at 60 ºC and 150 rpm for 14.5 hours. As an activator, 0.06 g $MgSO_4.7H_2O$ was added into 100 mL solutions containing one gram of immobilized glucose isomerase. The pH values of solutions were adjusted to 3.5 with 1:1 acetic acid solution.

2.6. The effect of tannins in red wine on the isomerization reaction

In this experiment, one gram of fructose was added into 100 mL wine medium along with one gram of immobilized glucose isomerase. Samples were incubated in flasks at 60 ºC and 150 rpm for almost 70 hours. The pH values of solutions were 3.37, 3.05 and 3.5 for Mistik Red, Mistik White and Frontera Red, respectively. Since different types of wines have magnesium concentration 56.5 ppm on average (Joshi, Panesar & Kosseva, 2017) which is higher than 45 ppm, required amount for activation of glucose isomerase, there was no addition of activator due to wines already contain sufficient amounts of Mg^{+2} for activation of enzyme.

2.7. The effect of fermentation components on isomerization reaction

To obtain synthetic media with grape juice, 5 % w/v fructose, 13 % v/v ethanol and 0.8 % v/v glycerol were mixed with grape juice. Samples were incubated in flasks at 60 ºC and 150 rpm with one gram of immobilized enzyme in 100 mL solutions. Since grape juice already contained Mg^{+2} (Sousa et al., 2014), the enzyme did not require additional activator. The pH of home-made grape juice was 3.65 and 3.88 for synthetic media with home-made grape juice.

2.8. The effect of calcium content on isomerization reaction

In synthetic media, 1 % w/v fructose was used as a substrate, 13 % v/v ethanol and 0.8 % v/v glycerol were added to provide synthetic environment. The pH values of the media were adjusted to 3.6 with 1:1 acetic acid solution. For activator, 0.06 g $MgSO_4.7H_2O$ was added into 100 mL solution. Since 100 mL red wine contains 8 mg

calcium, same amount of calcium must be present in synthetic medium to provide same conditions. Therefore, 0.015 g Ca $(OH)_2$ was added into 100 mL synthetic medium. The flasks were incubated at 60 ºC and 150 rpm for almost 65 hours with one gram of immobilized glucose isomerase.

Another experiment was conducted by considering the ion retention capacity of EDTA to hold the calcium in home-made red wine media with alcohol content of 14 % v/v. Different concentrations of EDTA was added to red wine media containing 1 % w/v fructose as a substrate. The flasks were incubated again at 60 ºC and 150 rpm containing one gram of immobilized enzyme for 100 mL solutions. The pH of solutions with 0.1, 0.2, 0.6, 1 and 2 % w/v EDTA were 3.46, 3.43, 3.53, 3.53 and 3.6, respectively.

For cation exchanger experiment, to test the enzyme activity in red wine media containing little amounts of calcium, 1 % w/v fructose was added to samples 0, 4, 6 and 10 (Figure 3.16). Since magnesium is also a positive ion, it was thought that cation exchanger resin also held the magnesium ions. Therefore, 0.06 g $MgSO_4.7H_2O$ was added in 100 mL solutions for activator. The pH values of samples were 3.6 and they were incubated at 60 ºC and 150 rpm with one gram of enzyme in 100 mL of solutions for almost 80 hours.

2.9. The activity of glucose isomerase in dilute wine media

In the first experiment, home-made red wines were used containing 1 % w/v fructose (added after dilution) as a substrate. Wines were diluted with distilled water at 6 different concentrations; 90 %, 70 %, 50 %, 30 %, 20 % and 5 % v/v and also 100 % v/v red wine as a control. The prepared solutions were incubated at 60 ºC and 150 rpm with one gram of immobilized enzyme in 100 mL solutions for 42 hours. The pH values of solutions were 3.15, 3.15, 3.19, 3.3, 3.53, 3.59 and 4.45 for wines at concentrations of 100 %, 90 %, 70 %, 50 %, 30 %, 20 % and 5 % v/v, respectively.

Also, different brands and types of wines; Frontera, Chile, 2015 (alcohol: 12.5 % v/v), Doluca Mistik Red, Turkey, 2016 (alcohol: 14.0 % v/v) and Doluca Mistik White,

Turkey, 2016 (alcohol: 13.5 % v/v); at different concentrations; 100 %, 10 % and 5 % v/v, were used in another experiment. The samples containing 1 % w/v fructose as a substrate were incubated at 60 ºC and 150 rpm for 27 hours. The pH values of solutions were 3.5, 3.45 and 3.03 for 100 % v/v Frontera Red, Mistik Red and Mistik White, respectively.

2.10. The effect of tartaric acid on the enzyme activity

Synthetic environments with 1 % w/v fructose and 0.3 % w/v tartaric acid were prepared at pH values of 3.55 and 6.33 adjusted with 5M NaOH and 24 % w/v KOH solutions, respectively. Samples were incubated at 60 ºC and 150 rpm with one gram of immobilized enzyme. The flasks containing Mg^{+2} as activator were two parallels for both pH values.

For experiments, samples numbered as 0, 2, 5, 8, 9, 10 and 12 (Figure 3.22) were chosen with addition of 1 % w/v fructose. The reason of choosing these samples was to differentiate the pH effect from tartaric acid effect. The pH values of solutions; 0, 2, 5, 8, 9, 10 and 12, after fructose addition were 3.36, 9.85, 6.7, 4.8, 4.08, 3.83 and 3.66, respectively. The samples were incubated at 60 ºC and 150 rpm with one gram of immobilized enzyme in 100 mL of a solution.

2.11. The effect of pH on isomerization reactions in red wine medium

Home-made wines at different pH values; 4, 5, 6, 7 and 8, containing 1 % w/v fructose as a substrate were tested at temperatures of 60 ºC and 30 ºC for this experiment. The pH adjustments of samples were done with 5M NaOH solution. The samples were incubated at 150 rpm, 60 ºC and 30 ºC, for 70 hours with one gram of immobilized enzyme in 100 mL solutions.

2.12. The uptake rate of yeast under different ethanol concentrations

The Kalecik II strain of *Saccharomyces cerevisiae,* obtained from Ankara University, was grown in YPD medium. Then, for every samples in Figure 2.3, wet cell weight and dry cell weight measurements were conducted. The steps were as follows:

- The empty tubes (2 mL) were weighed. (1)
- 400, 700 and 1000 µL samples (for 20 mL, 100 mL and 1000 mL flasks) were put in empty tubes.
- The tubes with solutions were centrifuged (at 10^{o}C for 10 minutes).
- The supernatant parts of solutions were removed from samples.
- The remained parts were the tubes and biomass.
- The tubes were weighed again with biomass. (2)
- The difference between number (1) and number (2) was wet cell weight of samples.
- The tubes with wet cell weight were incubated in the oven at 100^{o}C with cap open.
- After about 24 hours, the wet biomasses were dried completely (the tubes were weighed at some hours and when the results stayed stable, it was meant that the wet biomass were dried completely).
- The tubes were weighed again with dry biomass. (3)
- The difference between number (1) and number (3) was dry cell weight of samples.
- The graphs were plotted as amount of wet or dry cell weight (g) versus sample amount (mL).
- As a result of the graph equations and R^2 values, the chosen flask was used in the experiments (The graphs are given in Chapter 3).

According to results of these experiments, the chosen flask was centrifuged at 10^{o}C for 10 minutes to separate the yeast cells from the supernatant.

To measure the uptake rate of yeast, 5 different media with the alcohol contents of 0, 1.4, 2.8, 7.1 and 14.5% v/v were prepared with different percentages of wine (100 % v/v 100 mM PPB, 10 % v/v red wine, 20 % v/v red wine, 50 % v/v red wine and 100 % v/v red wine were used with alcohol contents of 0 %, 1.4 %, 2.8 %, 7.1 % and 14.5 % v/v, respectively). In these different media, 2 % w/v glucose and fructose were added. The media were filtered through 0.45 µm filter paper to sterilize the

environment since autoclave sterilization could not be suitable for wine media, i.e. the wine could lose its characteristics under heat. The 100 mL samples with one gram of wet yeast were incubated in 250 mL flasks at 25 ºC for 20 hours. The graphs of uptake rates are given in Chapter 3.

For these experiments mentioned above, the materials and equipment used in this study were described below:

2.13. Grape Juice and Wine

In this study, different types of grape juices, red wines and white wines were used to test the enzyme activity and fermentation rate of yeast cells. Two types of grape juices were used during enzymatic reaction experiments. The industrial white and red grape juices that were obtained from the market were Kavaklıdere brand. The reason of choosing them was that they were well covered with metal cover, sterilized and additive-free. Therefore, they were suitable for the experiments. Also, the home-made grape juice was tested and used during the experiments. First, the red grapes were obtained from the market. Then, their stems were separated. After that, they were squeezed by pressing and the seeds of grapes were separated. After the juice from red grapes was obtained, it was filtered through the 0.45 µm filter paper with vacuum pump to sterilize the juice and to remove it from particles. Finally, the sterilized red grape juice was ready to analyze for measuring initial sugar content via HPLC. The results were given in the Chapter 3. For testing the red and white wines, different brands and types of wines were chosen. Frontera, Chile, 2015 (alcohol: 12.5 % v/v), Doluca Mistik Red, Turkey, 2016 (alcohol: 14 % v/v) and Doluca Mistik White, Turkey, 2016 (alcohol: 13.5 % v/v) were the market products for analyzing via HPLC and using during the experiments. Also, home-made red wines were used during the experiments of enzymatic reactions and the uptake rate of yeast. Two types of home-made wines were used during the experiments; produced in Biotechnology Laboratory (Öküzgözü and Boğazkere) and obtained from Bülent Yaltaöz (Kalecik Karası). The one that was produced at the Middle East Technical University, Food Engineering

Department, Biotechnology Laboratory was used for the enzymatic experiments. The detailed production of wine was given in Chapter 1. The other type was obtained from Bülent Yaltaöz, who is interested in wine-making, was used for the uptake rate of the yeast cells because of its low sulphite content which was lower than 7 ppm. The sulphite analyses of wines were conducted by Bülent Yaltaöz. The initial analyses of wines were conducted via HPLC and the results were given in Chapter 3.

2.14. Immobilized Glucose Isomerase

The enzyme that was used in this study was immobilized glucose isomerase and supplied from Cargill, Bursa. It was produced by Novozymes and the group of it was Sweetzyme IT Extra. The color of the enzyme was brown and it was in the form of granulated. Its approximate density value was 0.50 g/mL. Its typical activity range was above 400 IGIU/G (immobilized glucose isomerase unit per gram). Since it was used for high fructose corn syrup process in Cargill, glucose and fructose was released to the solution initially. Therefore, excessive hexose sugars were removed by washing the enzyme 4 times with distilled water and drying it at 60 ºC in closed petri dishes. During and after washing process, the water in the solution (enzyme+distilled water) was analyzed via HPLC to control the glucose and fructose contents in it. After washing the enzyme particles, the granulated particles were dried at 60 ºC in petri dishes for 1 day and then they were in the form of granulated spheres again as seen in Figure 1. The enzyme was stored at 4 ºC to avoid contamination.

Figure 2.1. Granulated spheres of immobilized glucose isomerase

Since the enzyme was in the form of immobilized rather than liquid enzyme, it can be regenerable and can be used again in the experiments after regeneration procedure applied. First, it must be sweetened off with distilled water. Then, it must be washed with 2 % w/v sodium hydroxide solution. After that, it must be washed with distilled water and buffer solution. Finally, the enzyme must be rinsed with distilled water again (Antri & Auterinen, 1986).

2.15. Atomic Absorption Spectroscopy

Atomic absorption spectroscopy is a common method to detect the metals and metalloids in liquid samples. It measures the concentrations of metals in the samples and it is easy to use for experimental analyses. Lithium, sodium, potassium, magnesium, calcium, cobalt, manganese, iron are some of the detectable metals by using atomic absorption. Free atoms or gas are generated in the atomizer and they can

absorb the radiation at given frequency. By atomic absorption, the absorption of ground state atoms in the gaseous state can be measured. The atoms make transitions to higher energy levels by absorbing the UV or visible light. The concentrations of metals or metalloids are determined from the absorption amount. Before the measurements, the standard samples of known concentrations must be prepared to calibrate the device (given in Appendix B). The atomic absorption unit is mostly composed of 5 parts: detector, hollow cathode lamp, nebulizer, atomizer (flame and graphite tube), monochromator. The schematic is given in Figure 2.2. It can be applicable to different industries such as food, pharmaceutical and environmental studies (Garcia & Baez, 1982).

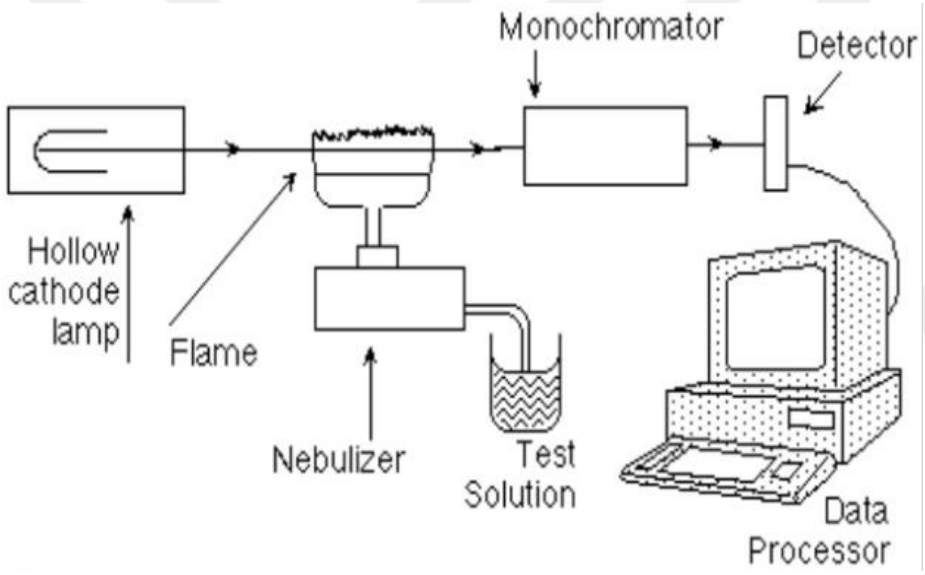

Figure 2.2. Schematic of atomic absorption spectroscopy (Atomic Absorption Spectroscopy Presentation by M. Sc. Suriti Sharma, 2012)

To measure calcium content of wine samples with analytical method, atomic absorption unit (Jarrell Ash) was used at Middle East Technical University, Chemical Engineering Department with the help of Selahattin Uysal. The results are given in Chapter 3.

Table 2.1. The parameters of atomic absorption and fame emission

	Atomic Absorption	**Fame Emission**
Primary wavelength	4227A	4227A
Optimum range	1 to 10 µg/mL	1 to 500 µg/mL
Spectral slit width	10 A	2 A
Hollow cathode (operating current, maximum current)	JA 45-450 Ca, Mg, Al (10 mA, 20 mA)	
Fuel	Acetylene 5 scfh	Acetylene 10 scfh
Oxidant	Air 10 scfh	Nitrous oxide 9.0 scfh
Aux. oxidant	Air 5.5 scfh	None
Sensitivity	0.07 µg/mL/1%A	
Detection limit	0.005 µg/mL	0.001 µg/mL
Secondary wavelengths with ranges	2399A 50 to 1000 µg/mL	2399A 50 to 1000 µg/mL

2.16. Yeast

In this study, The Kalecik II strain of *Saccharomyces cerevisiae,* obtained from Ankara University, was grown and used for determining the sugar uptake rate under different ethanol concentrations. In the article of Özçelik and others, 10 different types of yeast strains, which were all originated in Turkey, were tested for different characteristics. As a result, 5 different strains had the best technological properties for wine production and one of them was Kalecik II strain of *Saccharomyces cerevisiae* (Bağder Elmacı et al., 2014).

Fermentation experiments were carried out in the order of 20 mL, 100 mL and 1 L flasks. When cells were grown for 24 hours in one flask, they were transferred the larger flask. *Saccharomyces cerevisiae* cells were added to flasks from ready to use YPD (yeast peptone dextrose) broth. These broths were prepared by Ankara

University and stored under suitable laboratory conditions. For one 20 mL flask, 100 µL yeast cells were used as inoculum under aseptic conditions. pH and temperature of fermentation medium were set to 6.5 (100 mM PPB at pH 6.5) and 30ºC, respectively. Vitamin, mineral, nitrogen sources were added to flasks. The recipes for YPD environment, trace elements, vitamins and potassium phosphate buffer are given in Appendix Part "Ingredients of Used Solutions".

The schematic of the experiment is given in below figure:

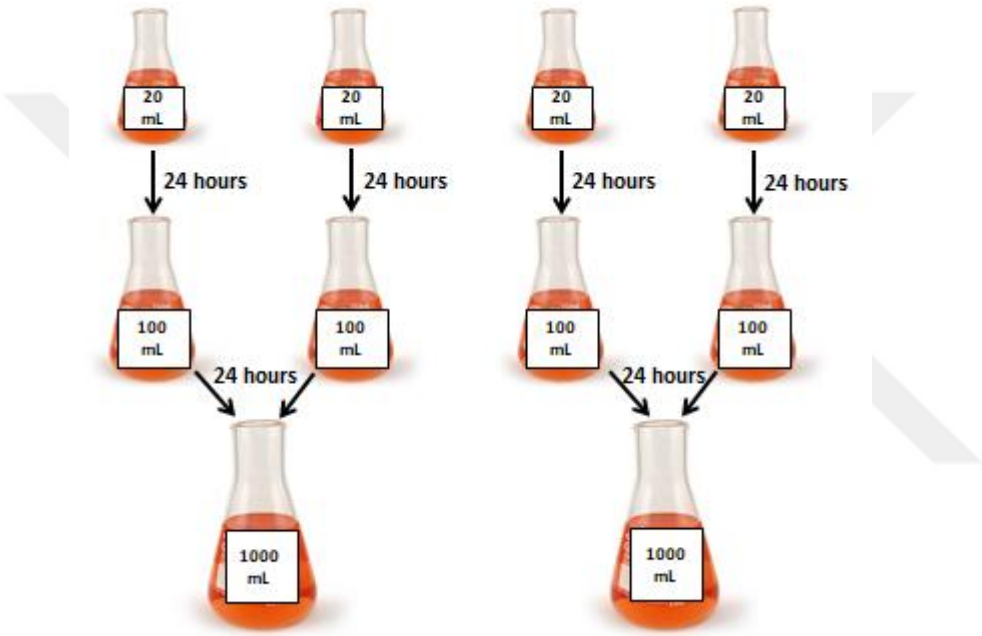

Figure 2.3. Schematic representation of yeast growth in laboratory conditions

Flasks were incubated at 30ºC for 24 hours. The samples were taken every 6 hours and checked under microscope for any contamination. Also, the absorbance values of samples were measured with UV spectrophotometer (UV 1202, Shimadzu, Japan) at 540 nm to express the optical density and concentration of cells in suspension.

2.17. Chemicals

D-(+)-Glucose monohydrate brand of Sigma Aldrich was used in this study. It was suitable for microbiological usage and HPLC grade. The purity of D-Glucose was ≥99%. It was used in fermentation media, synthetic media and wine.

D-Fructose brand of Serva was used in this study. It was suitable for microbiological usage and HPLC grade. The purity of D-Fructose was ≥99%. It was used in fermentation media, synthetic media and wine.

Sodium hydroxide brand of ISOLAB was used in this study. It was in the form of pure pellets. It was used for obtaining basic solution and increasing pH of wine and synthetic media.

Hydrochloric acid brand of Sigma-Aldrich was used in this study. It was in the form of liquid. It was used for obtaining acidic solution and decreasing pH of synthetic media.

To prepare Yeast-Peptone-Dextrose (YPD) medium for growing *Saccharomyces cerevisiae*, yeast extract, peptone from meat and glucose were used. Yeast extract brand of Merck was used in this study. It was in the granulated form. It was suitable for microbiological purpose. Peptone brand of Merck was used in this study. It was obtained from bovine meat. It was in the granulated form. It was suitable for microbiological purpose.

Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$) brand of Merck was used in this study. It was suitable for laboratory analysis. It was used for glucose isomerase activation.

Sulfur dioxide brand of Sigma-Aldrich was used in this study. It was suitable for microbiological usage and HPLC grade. The purity of sulfur dioxide was ≥99,98%. It was used for sterilization of wine media and equipment of wine making.

Ethanol brand of Merck was used in this study. It was absolute ethanol and suitable for classical laboratory analysis. The purity of ethanol was ≥99,9% and it was HPLC grade. It was used for obtaining synthetic media.

L-(+)-Tartaric acid brand of Merck was used in this study. It was in the form of powder and the purity of tartaric acid was ≥99%. It was used for obtaining synthetic media.

Glycerol brand of Merck was used in this study. It was in the form of liquid and the purity of glycerol was 85%. It was suitable for microbiological usage and HPLC grade. It was used for creating synthetic media.

Acetic acid brand of Merck was used in this study. It was in the form of liquid and anhydrous for analysis. The purity of acetic acid was 100%. It was used for adjusting pH of synthetic media.

Calcium hydroxide ($Ca(OH)_2$) brand of Merck was used in this study. The purity of it was ≥96%. It was used for creating synthetic media with calcium ions.

Ethylenediaminetetraacetic acid (EDTA) brand of Sigma-Aldrich was used in this study. The purity of EDTA was 99%. It was used for binding the cation (calcium) ions in wine environment.

2.18. Autoclave

Autoclave is a machine that provides a physical method for sterilization. The important parameters for autoclave cycle are steam, pressure and time. Since it operates at high temperature and pressure, the microorganisms and spores are killed with autoclave operations. It is used for sterilizing cell media and laboratory instruments like flasks and pipettes. Also, wastes in lab should be autoclaved before disposal to inactivate hazardous microorganisms. For effective operations, an autoclave must be operated at 121°C for at least 30 minutes by saturated steam at 15 psi pressure. The rate of exhaust is determined according to material type, for example, fast exhaust cycle for dry material and slow exhaust cycle for liquids and biological waste. Media solutions must be autoclaved in glass containers that filled 2/3 of the

containers. In this study, media solutions, pipettes, tips of pipettes, glass flasks and biological wastes were autoclaved at 121°C for 15 minutes. The autoclave machine in this study belongs to Middle East Technical University, Food Engineering Department and used in control of responsible staff.

2.19. HPLC (High Performance Liquid Chromatography)

In this study, the samples were analyzed to determine glucose, fructose, ethanol, glycerol, tartaric acid, malic acid and acetic acid concentrations by using HPLC in Middle East Technical University, Food Engineering Department, Biotechnology Laboratory (Agilent Technologies, USA). The column and detector type of this HPLC was Rezex™ RFQ-Fast Acid H+ (8%) LC Column, 100 x 7.8 mm (length and internal diameter, respectively) and refractive index detector, respectively. The temperature of refractive index detector and fast acid column was set to 30°C and 25°C, respectively. 0.05 M H_2SO_4 (prepared in distilled and sterilized water by adding 4.9 mL of H_2SO_4 for 1000 mL of water with constant stirring) was used as an eluent. For every sample, 10 µL of analyte was injected automatically with the flow rate of 0.6 mL/min.

CHAPTER 3

RESULTS AND DISCUSSION

The aim of this thesis was to prevent stuck fermentation that results from the glucose/fructose discrepancy (GFD) due to the ethanol presence in wine medium. The glucose/fructose discrepancy is the result of yeast's larger affinity for glucose than for fructose. This difference increases as the accumulation of ethanol increases. Ideally, by using immobilized glucose isomerase, the residual fructose can be converted into glucose, yeast may continue to utilize glucose and fermentation process. Thus, undesirable sweetness of wine and the risk of microbial spoilage can be reduced.

Since it is reported that the initial ratio of glucose to fructose is close to 1, different types of grape juices were analyzed and Figure 3.1 shows the initial amounts of glucose and fructose in home-made red, industrial red and industrial white grape juices. Industrial ones were Kavaklıdere brand and additive-free, home-made one was produced from red grapes by pressing and filtering in laboratory conditions. It can be easily seen in Figure 3.1 that the initial ratio of glucose to fructose is always close to 1.

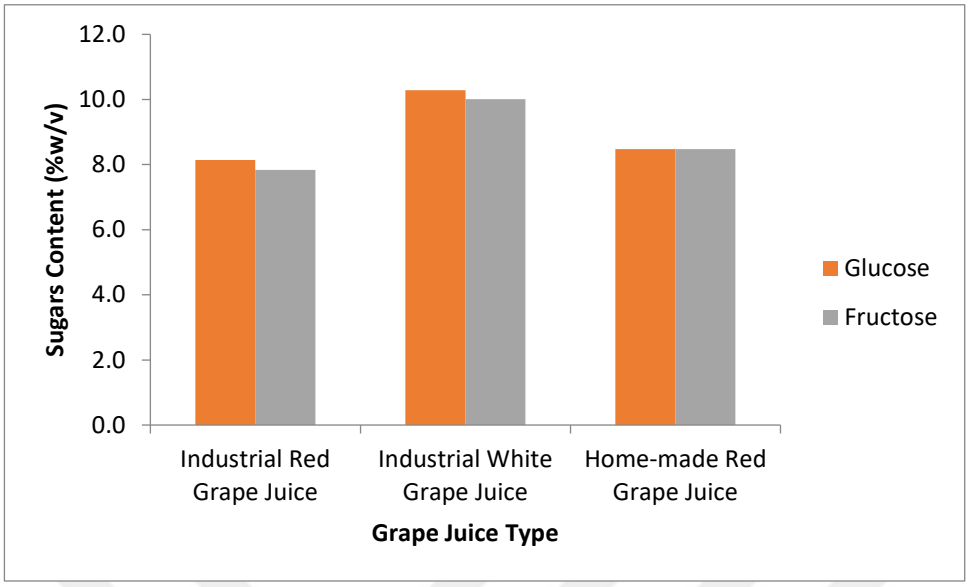

Figure 3.1. Glucose and fructose contents of different types of grape juices

Although, the ratio between glucose and fructose is equal to 1 at the beginning of wine fermentation, there is a residual of fructose at the end of wine fermentation. To determine and try to prevent stuck or sluggish fermentation in wine medium, some experiments were conducted and presented in the following sections.

3.1. The effect of substrate type and temperature on the isomerization reaction of glucose isomerase

In these experiments, either glucose or fructose were used as substrates of reactions. Since the optimum activity temperature of immobilized glucose isomerase is 60 ºC (Novozymes, 2015), one of the temperature value of experiments was 60 ºC. Also, since the optimum growth condition of yeast is 30 ºC (Salari & Salari, 2017), the other temperature value of experiments was 30 ºC.

In Figures 3.2 and 3.3, it was clearly seen that the equilibrium reaction occurred regardless of substrate type and temperature. According to the results of the experiments, the isomerization reactions were equilibrated at a faster rate when starting with glucose compared with fructose especially at 30 ºC. Another important

point resulting from experiments, the reactions occurred at faster rates when conducted at 60 ºC compared with 30 ºC. Also, an equilibrium point was reached after 5 hours at 60 ºC while after 9 hours at 30 ºC.

In reference to Novozyme specifications, the immobilized glucose isomerase can be a suitable enzyme within wide temperature range (Novozymes, 2015) that was also shown in these experiments.

As a result of these experiments, it can be understood that the enzyme is suitable for both substrate types at different temperatures.

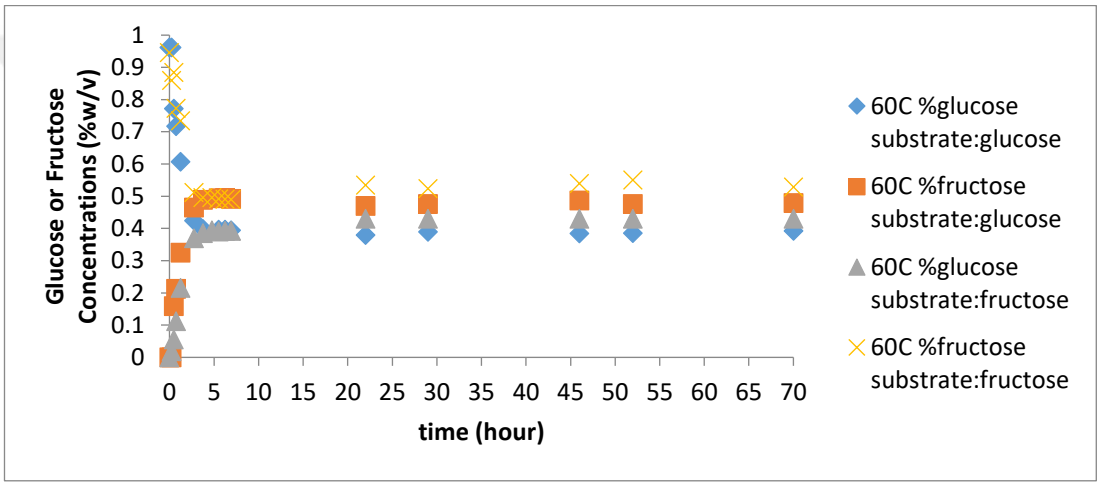

Figure 3.2. Glucose and fructose concentrations with respect to time at 60 ºC

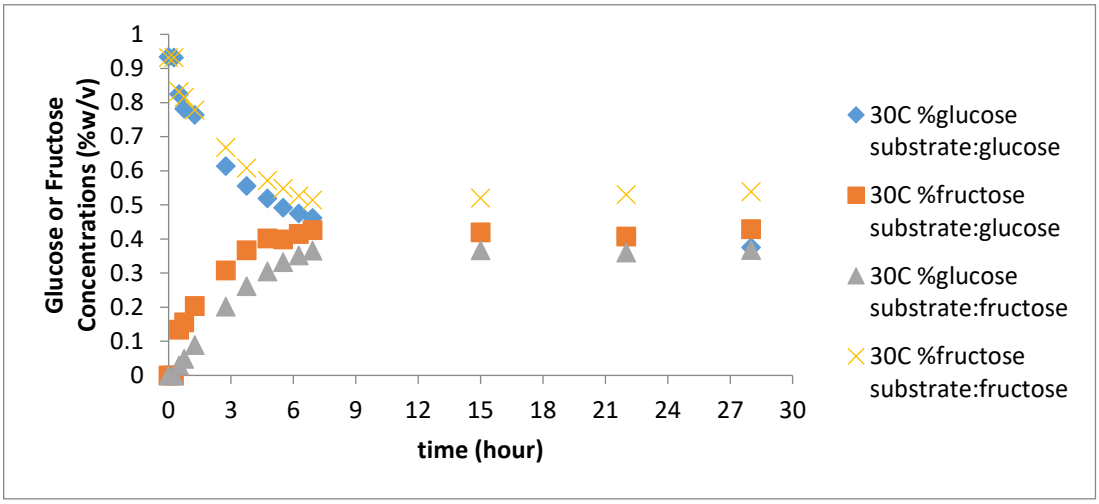

Figure 3.3. Glucose and fructose concentrations with respect to time at 30 ºC

3.2. The effect of ethanol on the isomerization reaction of glucose isomerase

One of the main compounds in wine is ethanol produced as a yeast metabolite (Luyten, Riou, & Blondin, 2002; Rodríguez-Sifuentes et al., 2014; Tronchoni, Gamero, Arroyo-López, Barrio, & Querol, 2009) and one of the reasons of stuck fermentation is production of ethanol in wine. Therefore, testing the effect of ethanol on the isomerization reaction was important.

In Figure 3.4, glucose formation and fructose depletion were shown at 60 ºC and 30 ºC. According to the literature, glucose concentration is expected to be higher than fructose concentration at temperatures below the optimum temperature of enzyme activity, however, fructose concentration is expected to be higher than glucose concentration at or above the optimum temperatures at the end of reaction (Schiffman et al., 2000). In accordance with this information, it was shown that glucose concentration was slightly higher than fructose concentration after 25 hours at 30 ºC. However, fructose concentration was slightly higher than glucose concentration after 25 hours at 60 ºC. As it was mentioned before, the reaction occurred more slowly at 30 ºC compared with 60 ºC by looking at the second data.

As a consequence of this experiment, it was interpreted that 13 % v/v ethanol did not inhibit the activity of the enzyme regardless of different temperature values.

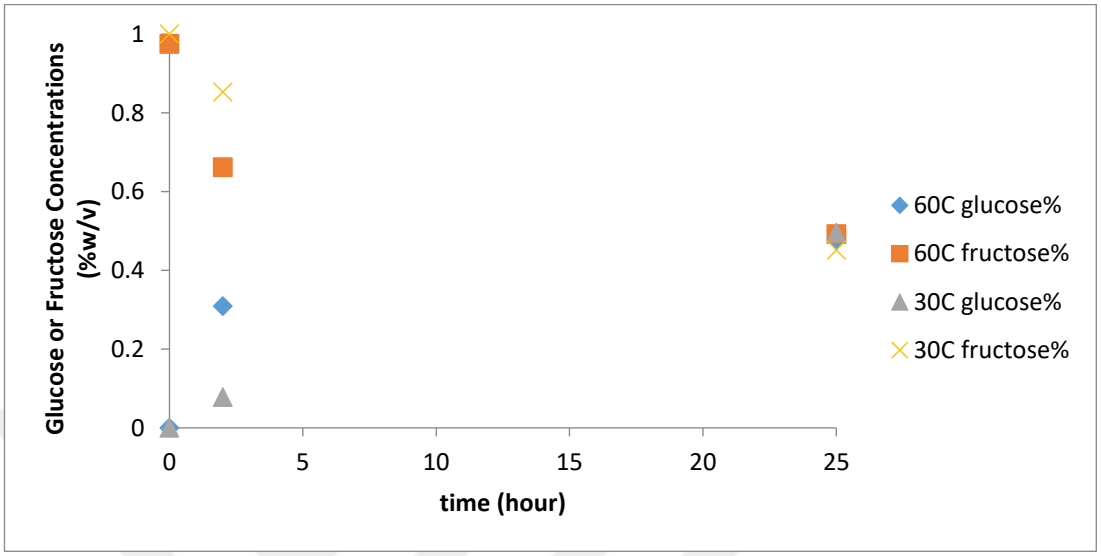

Figure 3.4. Glucose and fructose concentrations with respect to time at different temperatures

Since ethanol did not inhibit the isomerization reaction in synthetic environment, an experiment was conducted by vaporizing ethanol in red wine to show the effect of ethanol on red wine. The ethanol concentration was reduced approximately from 13% v/v to 0.3 % v/v by keeping wine at air temperature on the magnetic stirrer. The ethanol concentration was tested with HPLC day by day and after 3 days ethanol concentration decreased approximately to 0.3 % v/v. Red wine, Frontera, Chile, 2015, was used as a control in this experiment. At the end of 42 hours, glucose concentrations of four samples did not change as can be seen in Figure 3.5. This means that the enzyme did not isomerize fructose even if there was no ethanol in wine.

As a consequence, it was thought that an unknown component or low pH values or high calcium content of red wine (Navarro-Alarcon et al., 2007) could be inhibiting the isomerization reaction.

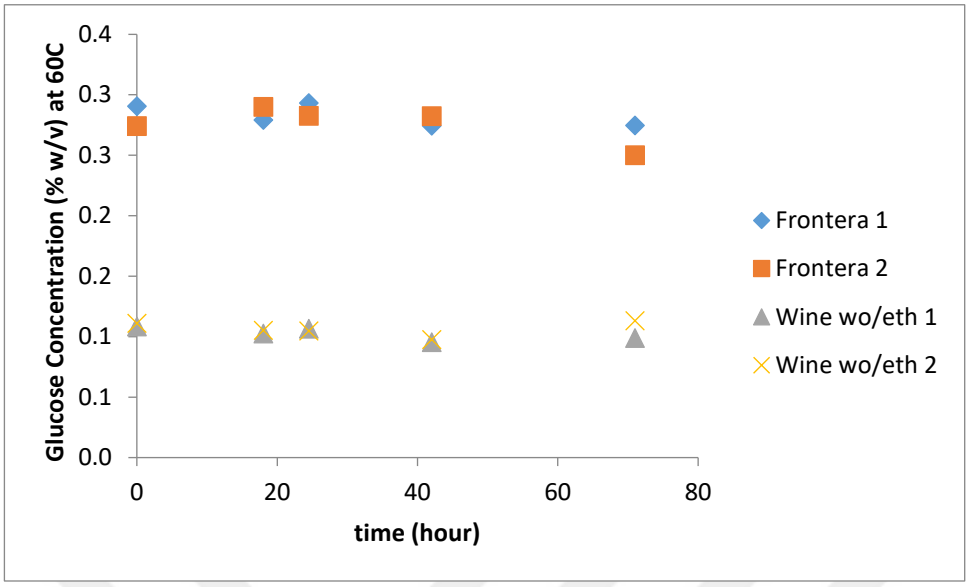

Figure 3.5. Glucose concentrations of wine with or without ethanol versus time

3.3. The effect of low pH values on isomerization reaction in synthetic medium

The pH values of red and white wines are between 3.5 to 4 and 3 to 4, respectively. Even though the graph of activity and stability of glucose isomerase shows that the pH range of this enzyme is very wide, enzyme activity had to be tested in acidic media.

As seen in Figure 3.6, it was noticed that fructose was converted to glucose regardless of the pH values. Also, it was clearly noticed that the reaction at pH 4 was the fastest one when compared to others whereas the slowest reaction was at pH 3.3 by looking at glucose formation rate. Therefore, it was thought that the reason of inhibition of enzyme in wine medium was not because of the low pH values. However, the effect of pH with alcohol had to be tested in different experiments.

In conclusion, the rate of reaction decreased proportionally with decreasing pH, the inhibition effect in wine medium was not due to acidic medium and the effect of ethanol with low pH values had to be tested.

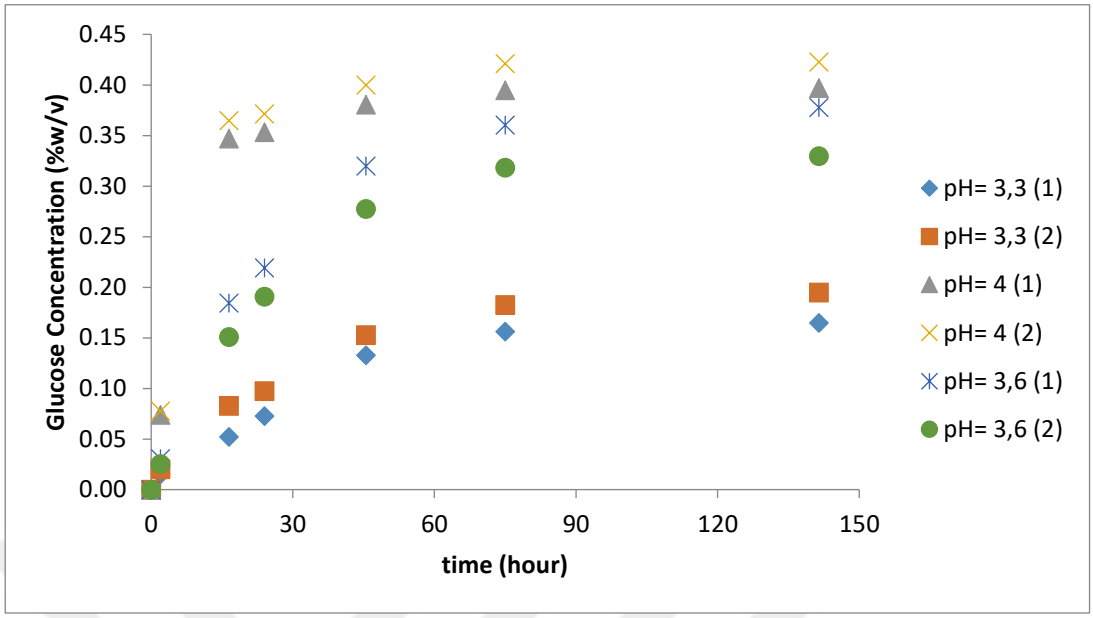

Figure 3.6. Change of glucose concentrations at different pH values without ethanol

Under the same conditions in Figure 3.6, the glucose was formed under same pH values with 13 % v/v ethanol in medium as seen in Figure 3.7. The ethanol effect experiments were conducted with the same amount of ethanol with experiments above, 13 % v/v. The rate of reaction was obviously slower at pH 3.3. When Figure 3.6 and Figure 3.7 were compared, almost same amounts of glucose were formed for pH values of 3.6 and 4, whereas the amount of glucose at pH 3.3 was clearly lower in ethanol medium. Also, the glucose amounts formed at pH 3.6 and 4 were equalized at about 45th hour with ethanol in the environment. The formed glucose amounts from 1 % w/v fructose were nearly 0.2 % w/v and 0.1 % w/v in medium without and with ethanol, respectively at pH 3.3.

As a consequence, glucose formation was detected in acidic media even in presence of ethanol. The rate of reaction at pH 3.3 was clearly slower than others regardless of the presence of ethanol.

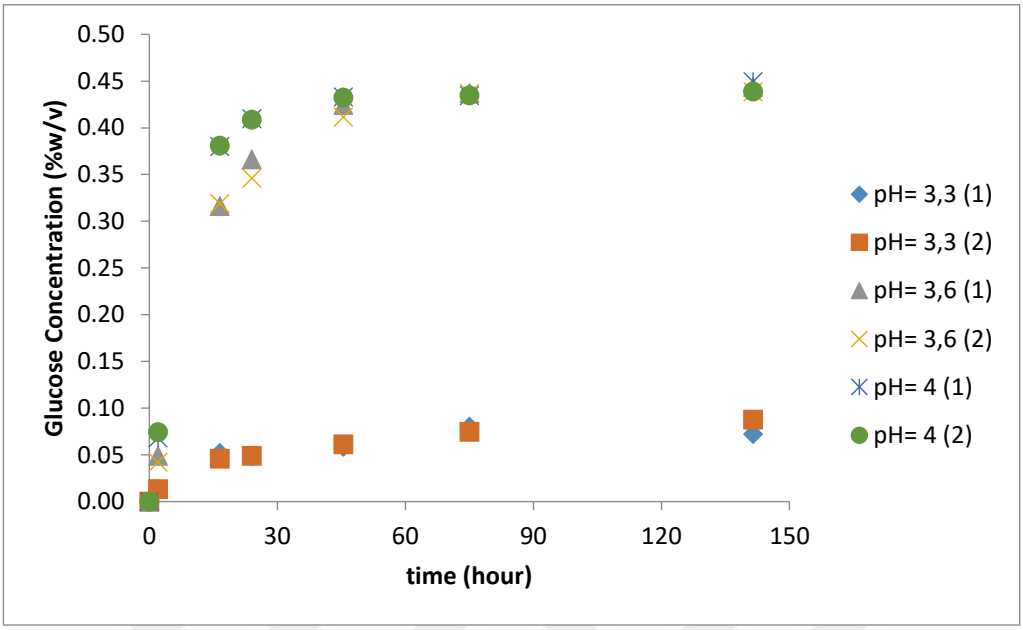

Figure 3.7. Change of glucose concentrations at different pH values with ethanol

3.4. The effect of glycerol in synthetic media on the isomerization reaction

One of the main components in wine is glycerol formed by yeast metabolism during fermentation process (Luyten et al., 2002; Rodríguez-Sifuentes et al., 2014; Tronchoni et al., 2009). Since polyols have inhibitory effects on enzymes (Kovalevsky et al., 2012), the effect of glycerol on isomerization reaction was tested. It was known from the literature that the glycerol contents of red and white wines are different and vary between 2 and 11 g/L depending on the yeast type, composition of grape and environmental conditions during fermentation (Remize, Cambon, Barnavon, & Dequin, 2003), which was also supported by the analyses of different wines as seen in Figure 3.8.

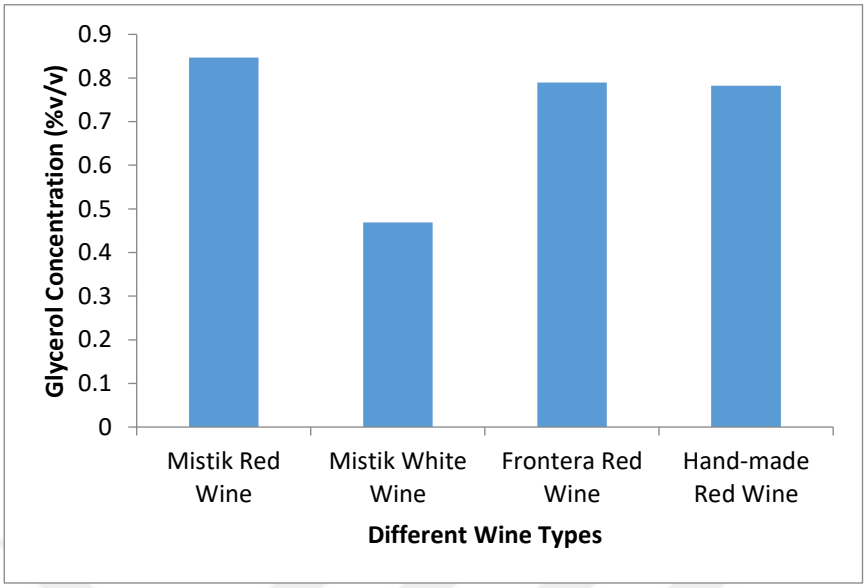

Figure 3.8. Glycerol contents of different types of wines

According to Figure 3.9, the isomerization reaction occurred in all synthetic media with different glycerol concentrations. The rates of reactions were close to each other for all glycerol concentrations until the glycerol concentration was approached to 1 % v/v. That is, the slowest reaction rate was obtained at the concentration of one percent of glycerol.

It was easily concluded that the existence of glycerol in synthetic media containing ethanol did not inhibit the isomerization reaction. Therefore, the reason of inhibition in red wine should be due to some other factors.

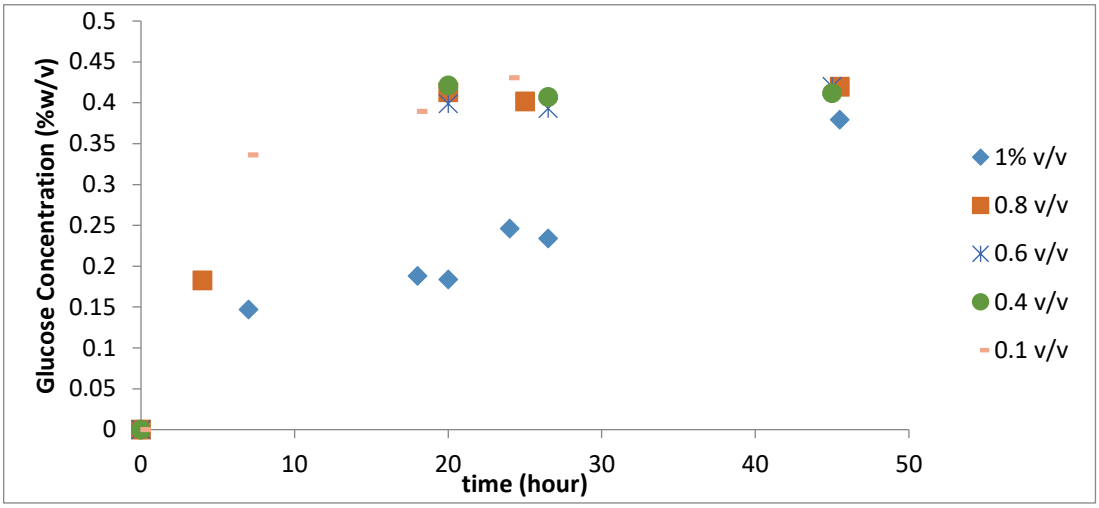

Figure 3.9. Change of glucose concentrations in synthetic media with different glycerol contents during 47 hours

3.5. Effect of sulphite content on isomerization reaction

In high fructose corn syrup industry, sulphite is added to feed syrup to protect against microbial contamination (Sperber, 2009). Also in wine industry, sulphite must be used to provide sterilization and dry wine products contain certain amounts of sulphite (Sperber, 2009). Although it is known that glucose isomerase is used with sulphite, the experiments were also conducted in synthetic media with sulphite.

As seen in Figure 3.10, after 14.5 hours of incubation, almost equal amounts of glucose were formed in all concentrations of sulphite. Therefore, even higher sulphite contents compared to those present in wine did not inhibit the activity of glucose isomerase.

As a consequence, other factors had to be tested to detect the inhibitory effect for glucose isomerase since the activity was not seen in red wine media as seen in Figure 3.5.

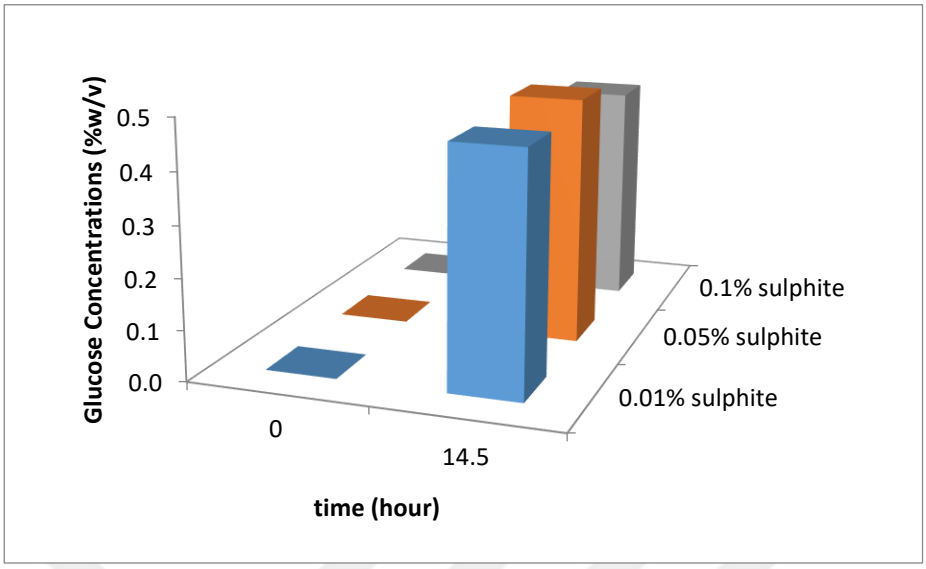

Figure 3.10. Forming of glucose in synthetic media with different sulphite concentrations

3.6. The effect of tannins in red wine on the isomerization reaction

During white wine process, grape skins are removed before fermentation, however during red wine process, grape skins are also present in initial fermentation steps. The specific components of red wine, tannins, originate mostly from grape skin. Therefore, while some types of tannins are present in red wine, white wine does not contain them (Keulder, 2006). Since the experiment in Figure 3.5 was only conducted with red wine, it was thought that tannins might have an inhibitory effect on the isomerization reaction. Therefore, the comparison of glucose isomerase activity in red and white wine media was made with following wine brands:

- Frontera, Chile, Red, 2015 (alcohol: 12.5 % v/v)
- Doluca Mistik Red, Turkey, 2016 (alcohol: 14 % v/v)
- Doluca Mistik White, Turkey, 2016 (alcohol: 13.5 % v/v)

As seen in Figure 3.11, glucose concentrations of all types of wines, either red or white, remained stable during 70 hours of incubation. Normally, enzyme activity is

seen in the first 2 or 3 hours of the experiments, in this case no activity was observed even after 70 hours.

As a consequence, it was concluded that tannins had no inhibitory effect on the isomerization reaction since white wine also showed a similar behavior.

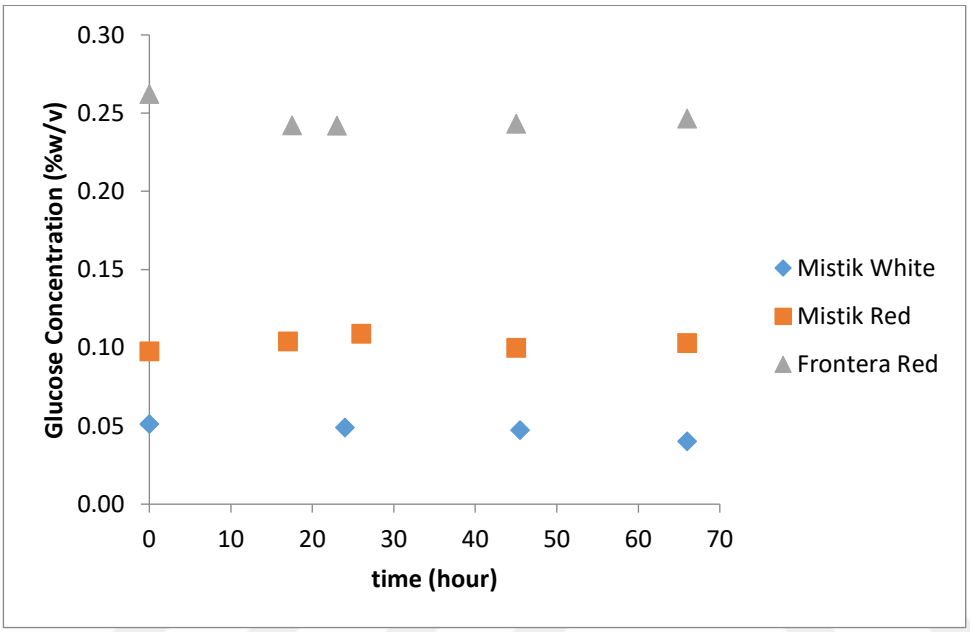

Figure 3.11. Glucose concentrations with respect to time in different wines

3.7. The effect of fermentation components on isomerization reaction

It was found that ethanol, glycerol, low pH, sulphite and tannins had no inhibitory effect on isomerization reaction and also glucose formation was not seen in wine media. Therefore, other parameters were tested for the inhibitory effect.

During wine fermentation, too many major and minor compounds are formed and affect the properties of wine products. One of these compounds formed during fermentation might have inhibited the enzyme. Therefore, grape must that had not undergone fermentation had to be examined to see whether inhibitory effect is because of fermentation process or not.

In this experiment, grape juice was obtained from red grapes by pressing and filtering in laboratory conditions. As shown in Figure 3.1, this red grape juice contains almost equal amounts of glucose and fructose before fermentation:

- Industrial red grape juice:
    - Glucose: 8.1 % w/v
    - Fructose: 7.8 % w/v
- Industrial white grape juice:
    - Glucose: 10.3 % w/v
    - Fructose: 10.0 % w/v
- Home-made red grape juice:
    - Glucose: 8.5 % w/v
    - Fructose: 8.5 % w/v

Therefore, to determine enzyme activity, 5 % w/v fructose was added into grape juice. As illustrated in Figure 3.12, glucose and fructose concentrations remained stable during 2.5 days. The fructose concentrations of samples were higher than that of glucose. Although samples were kept for 2.5 days, the enzyme did not isomerize the substrate in solutions.

As a result, since enzyme activity was not observed in grape must before fermentation, it was thought that a component that is present before fermentation would be inhibiting the enzyme, such as tartaric acid or malic acid (Volschenk & Van Vuuren, 2006).

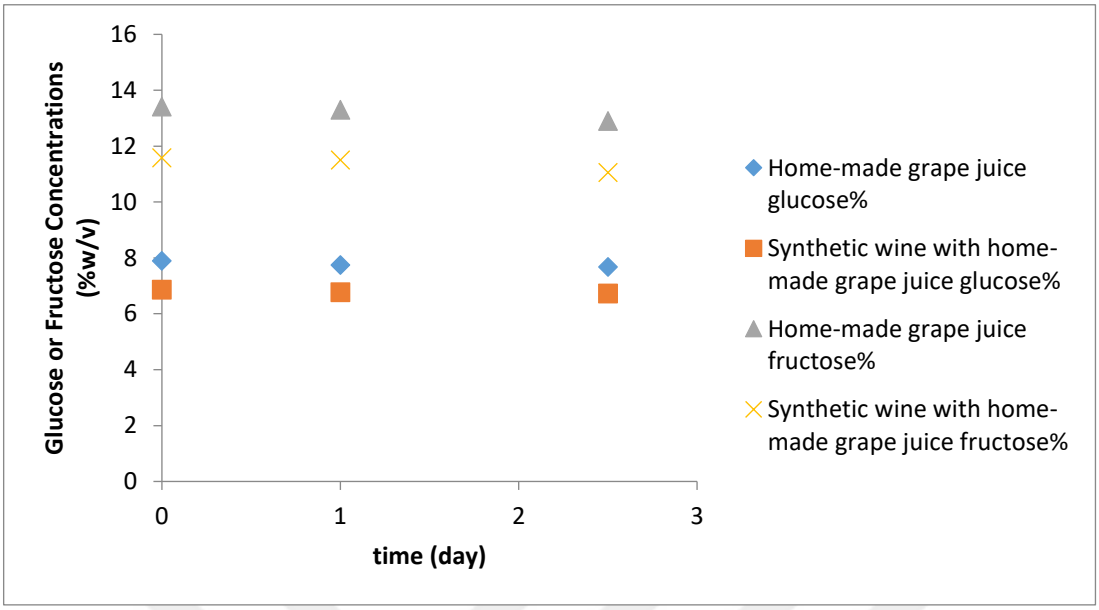

Figure 3.12. Glucose and fructose concentrations in home-made grape juice and synthetic media with home-made grape juice

3.8. The effect of calcium content on isomerization reaction

Magnesium is an activator, whereas calcium is an inhibitor for glucose isomerase (Ben Hlima, Aghajari, Ben Ali, Haser, & Bejar, 2012). Generally, red wine contains both calcium and magnesium as amounts of 80 ppm and 120 ppm, respectively (Cox, Eitenmiller, & Powers, 1977). The ratio of magnesium to calcium must be equal to 12 to provide activating conditions for glucose isomerase (Chaplin & Bucke, 1992). It was thought that the inhibition in wine medium was because of the calcium content. In the light of these information, experiments were conducted in synthetic media containing ethanol, glycerol and calcium, and red wine with increased magnesium content.

As seen in Figure 3.13, the conversion reaction occurred in synthetic medium without calcium, however there was no formation of glucose in the synthetic medium with calcium. As a result of this experiment, it was thought that calcium in wine may be inhibiting the glucose isomerase. Therefore, calcium effect in wine had to be reduced

with increasing magnesium in environment, sequestering it with EDTA or separating it from wine with cation exchanger.

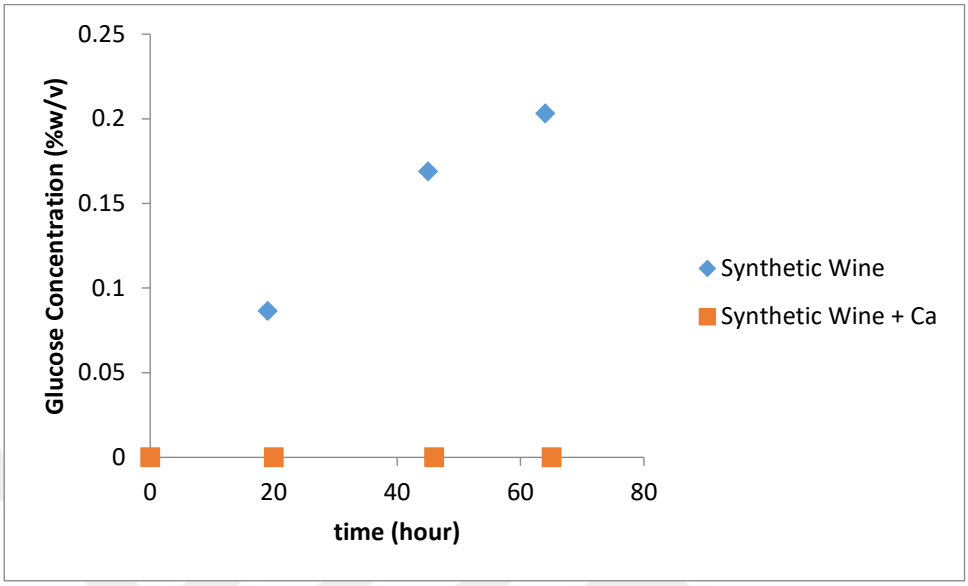

Figure 3.13. Glucose concentrations in synthetic media with and without calcium

Another experiment was conducted to evaluate the ratio between magnesium and calcium in red wine media. First of all, 1 % w/v fructose was added in red wine under different pH values; 3.28, 7.5 and 8.0. The flasks were incubated at 60 ºC and 150 rpm with one gram of enzyme in 100 mL solutions.

The ratio of magnesium to calcium must be equal to 12 according to the study of Chaplin and Bucke (1992). Since calcium content is equal to 80 ppm in red wine (Cox, Eitenmiller, & Powers, 1977), magnesium content must be equal to 960 ppm according to this information. Red wine already contains 120 ppm magnesium (Cox, Eitenmiller, & Powers, 1977), therefore 840 ppm magnesium must be added into red wine. This means that 84 mg of Mg^{+2} must be added into 100 g of solution. Therefore, 0.84 g of $MgSO_4.7H_2O$ must be added into 100 mL red wine.

Only magnesium content was increased according to literature for wine of pH 3.28. Only pH was adjusted with 5M NaOH for wine of pH 8.0. Both magnesium content

was increased according to literature and pH was adjusted with 5M NaOH for wine of pH 7.5.

By observing Figure 3.14, it was clearly seen that there was no formation of glucose in red wine medium even with increased magnesium content at pH 3.28. The glucose formation was observed in red wine media with increased pH with or without additional magnesium. However, the reaction rate was faster at pH 7.5 than at pH 8.0 since medium at pH 7.5 contained additional magnesium. The reactions reached equilibrium after about 50 hours.

As a result, it was concluded that the additional magnesium had no effect on low pH wine medium, however it speeded the reaction rate at high pH wine media. Therefore, the effect of pH on isomerization reaction in wine media must be considered.

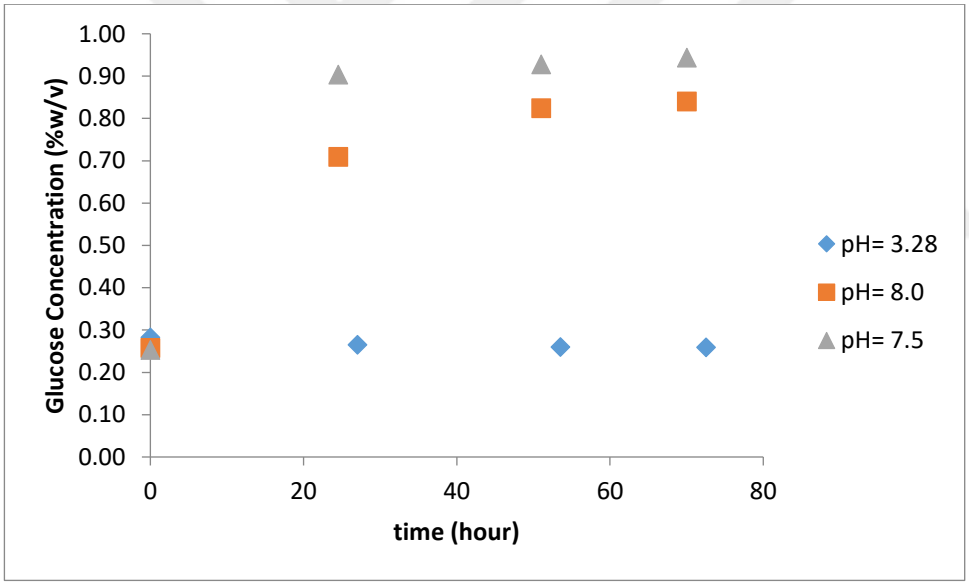

Figure 3.14. Glucose formations in different wine media; without or with additional magnesium and at different pH values

Another experiment was conducted by considering the ion retention capacity of EDTA to hold the calcium in home-made red wine media. It was known that it was not an applicable procedure for wine industry, however the effect of EDTA was tested for

isomerization reaction in wine media. As seen in Figure 3.15, there was no formation of glucose after 70 hours. Therefore, it was concluded that EDTA had no positive effect on isomerization reaction in red wine media.

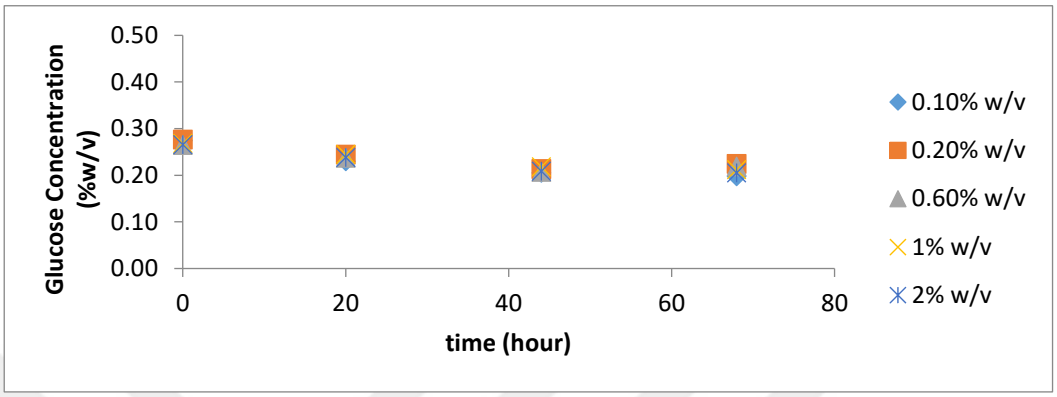

Figure 3.15. Glucose concentrations in red wine media with different EDTA concentrations

The last experiment for testing calcium effect in wine medium was using cation exchanger resin to hold calcium in the red wine. Since calcium is a positive ion, cation exchanger was used to hold positive ions. The analyzes of calcium amounts were done with atomic absorption.

In Figure 3.16, numbers of samples were named from 0 to 10. Number of 0 was the original wine sample and other numbers were wine samples that were eluted through the resin at a rate of 40 mL per sample. As can be seen in this figure, while sample 0 contained almost 13 ppm calcium in it, other samples contained much lesser amounts of calcium with respect to sample 0. From here, it was understood that cation exchanger resin could hold the calcium ions in red wine.

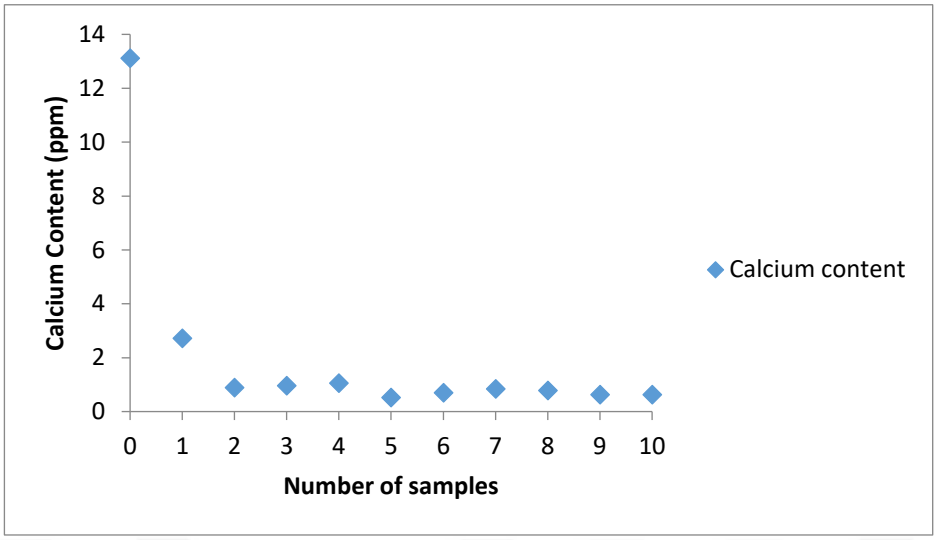

Figure 3.16. Calcium contents of samples passed from cation exchanger resin

To test the enzyme activity in red wine media containing little amounts of calcium, samples 0, 4, 6 and 10 were chosen. As seen in Figure 3.17, the glucose concentrations of samples 0, 4, 6 and 10 remained stable and isomerization reaction did not occur. Whereas calcium in synthetic medium inhibited the activity of glucose isomerase, there was also an inhibition effect in red wine even if there was no calcium content in environment.

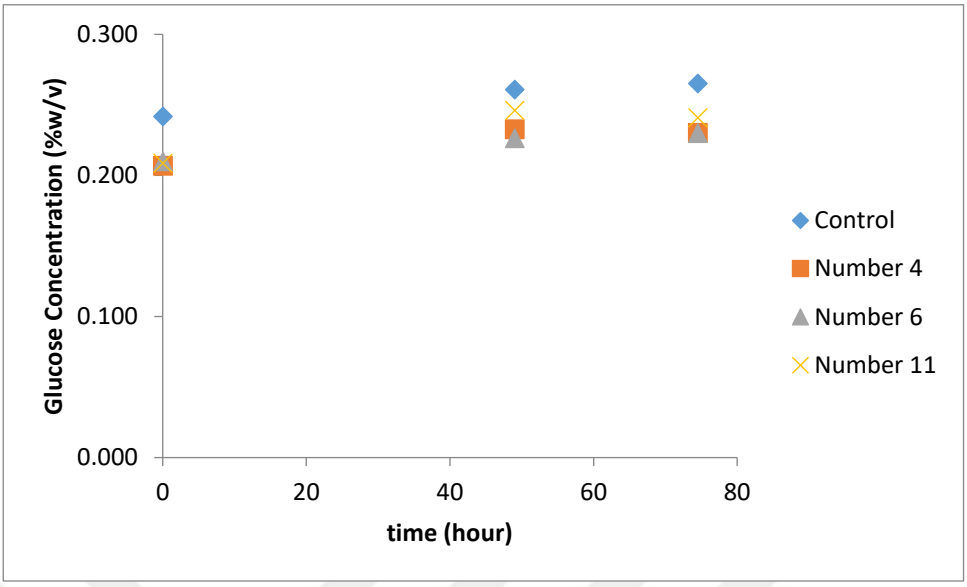

Figure 3.17. Glucose concentrations of red wine samples passed from cation exchanger resins according to time

3.9. The activity of glucose isomerase in dilute wine media

Since the effects of ethanol, glycerol, low pH, calcium, sulphite, tannins and fermentation components were tested on the isomerization reaction of glucose isomerase, the other experiments were conducted by diluting different wines by different factors. Although it was known that the physical and chemical properties of wine are altered by diluting the wine and it is not a desired situation in industry, these experiments were carried out in order to scientifically determine why the enzyme did not show activity in wine media.

In the first experiment, home-made red wines were diluted with distilled water at 6 different concentrations. As seen in Figure 3.18, except concentrations at 20 % and 5 % v/v, the glucose concentrations of samples remained stable. At concentrations of 5 % and 20 % v/v red wines, the glucose formation occurred during incubation.

Also, different brands and types of wines at different dilution concentrations were used in another experiment. As seen in Figure 3.19, the glucose formation occurred at

concentrations of 10 % and 5 % v/v regardless of the brand type. Also, almost equal amounts of glucose formed at same hours for different dilution factors and brand types.

As a result of these experiments, it was thought that a component or some components in wine coming from grapes may inhibit the isomerization reaction but this component or components may lose its or their effects in diluted wines but only at the levels of 5-fold dilutions.

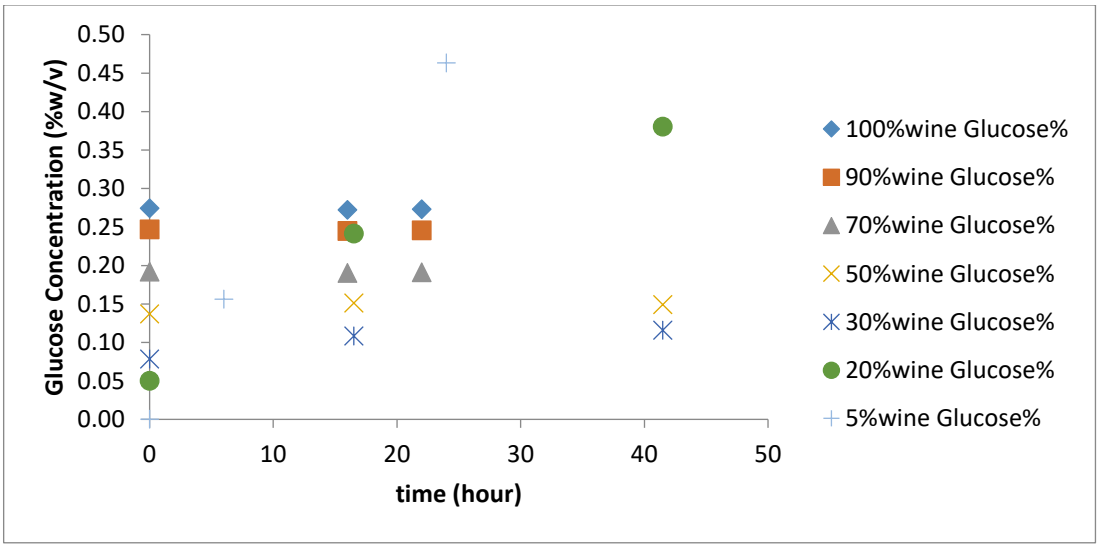

Figure 3.18. Glucose concentrations of home-made red wines at different concentrations

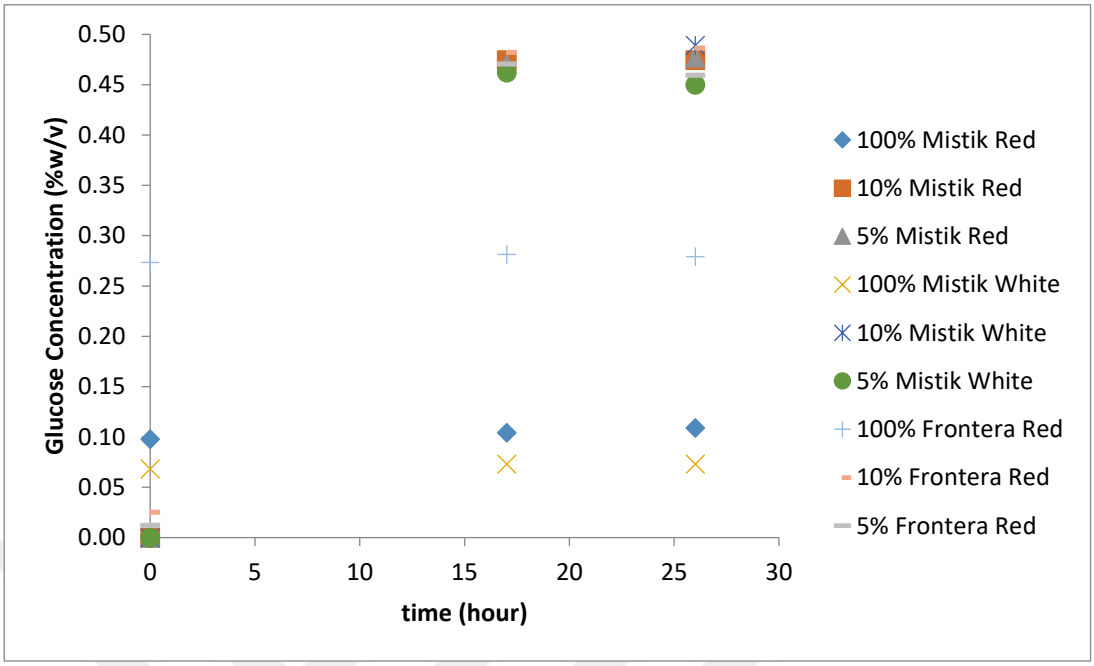

Figure 3.19. Glucose concentrations of different types of wines at different concentrations

3.10. The effect of tartaric acid on the enzyme activity

One of the main components in grape juice and wine is L- (+)-Tartaric acid and the amount of it is equal to 3 g/L (Viana, Loureiro-Dias, & Prista, 2014). Separating tartaric acid from wine is possible with anion exchanger since the working principle of anion exchanger resin is proper for this process (PURETEC, 2014). By holding negative charges in wine, the tartaric acid content of wine is reduced to zero. The experiments were conducted with synthetic media containing fructose and tartaric acid and red wine containing no tartaric acid.

As seen in Figure 3.20, fructose concentrations remained stable at 1 % w/v at pH 3.55 and also no glucose formation occurred in flasks after 150 hours. However, as seen in Figure 3.21, glucose formation and fructose consumption were observed at pH 6.33. The reaction reached equilibrium within 30 hours with higher fructose contents.

As a result, L- (+)-Tartaric acid inhibited the activity of glucose isomerase at pH 3.55, however if pH of the solution was 6.33, there was no inhibition with tartaric acid. Therefore, it was thought that if tartaric acid in wine is eliminated, the reaction would take place.

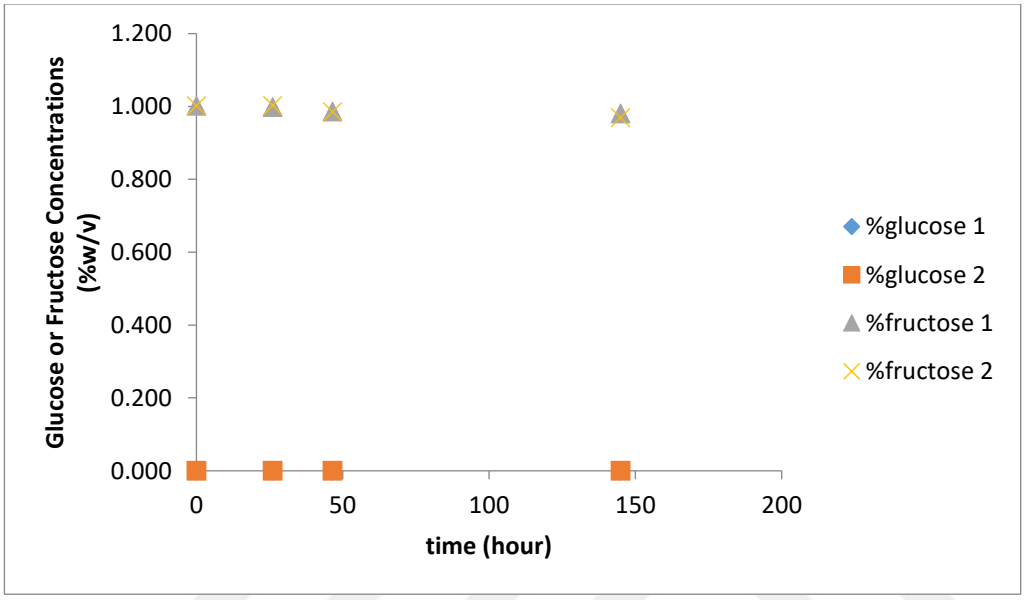

Figure 3.20. Glucose and fructose concentrations with tartaric acid at pH 3.55

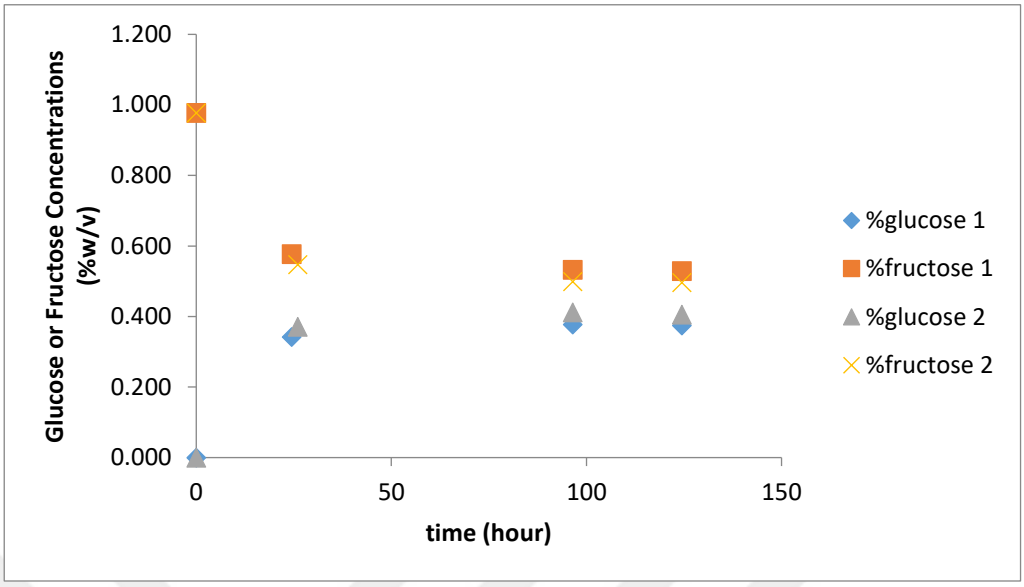

Figure 3.21. Glucose and fructose concentrations with tartaric acid at pH 6.33

To absorb the tartaric acid from home-made red wine samples, they were eluted through an anion exchanger resin. Anion exchanger was first washed with 1 % w/v NaOH solution to activate the resin to sequester negative ions in wine (PURETEC, 2014). Therefore, as indicated in Figure 3.22, first 9 samples passed through anion exchanger had higher pH values than the original sample. In original sample, number 0, the tartaric acid content was equal to 0.248 % w/v. Other samples from 1 to 13, each were 40 mL, did not contain any tartaric acid according to HPLC analyzes.

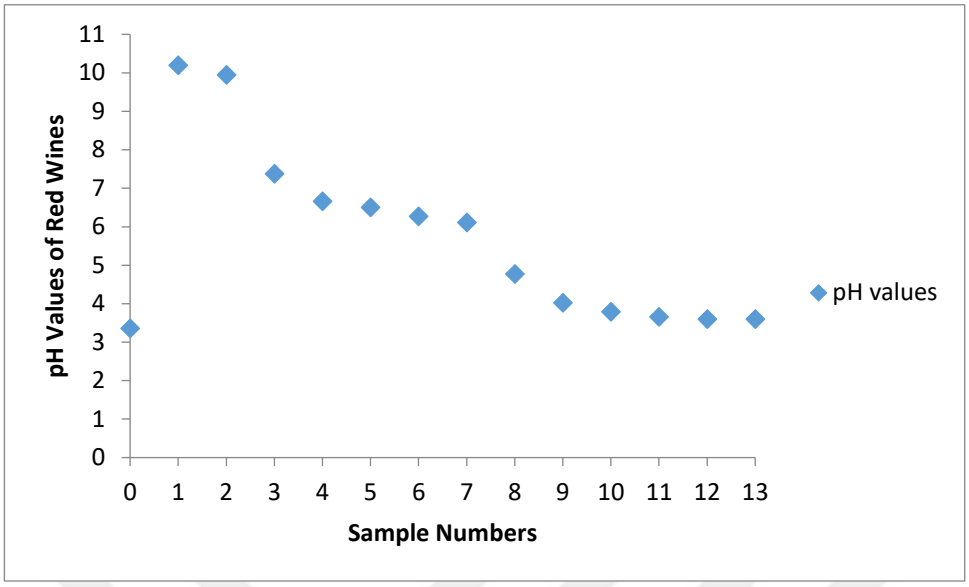

Figure 3.22. The pH values of red wine as eluted through anion exchanger

For experiments, samples numbered as 0, 2, 5, 8, 9, 10 and 12 were chosen. The reason of choosing these samples was to differentiate the pH effect from tartaric acid effect. As seen in Figure 3.23, the 100% glucose formation occurred at pH values of 9.85 and 6.7 and 50% glucose formation occurred at 4.8, that is sample numbers of 2, 5 and 8, respectively. Even if all samples did not contain tartaric acid, the conversion reaction occurred at only higher pH values. Therefore, it was thought that the tartaric acid had inhibitory effect at low pH values, under pH of approximately 5. As a result, it had to be examined that glucose isomerase could or not convert fructose to glucose in wine media containing tartaric acid at high pH.

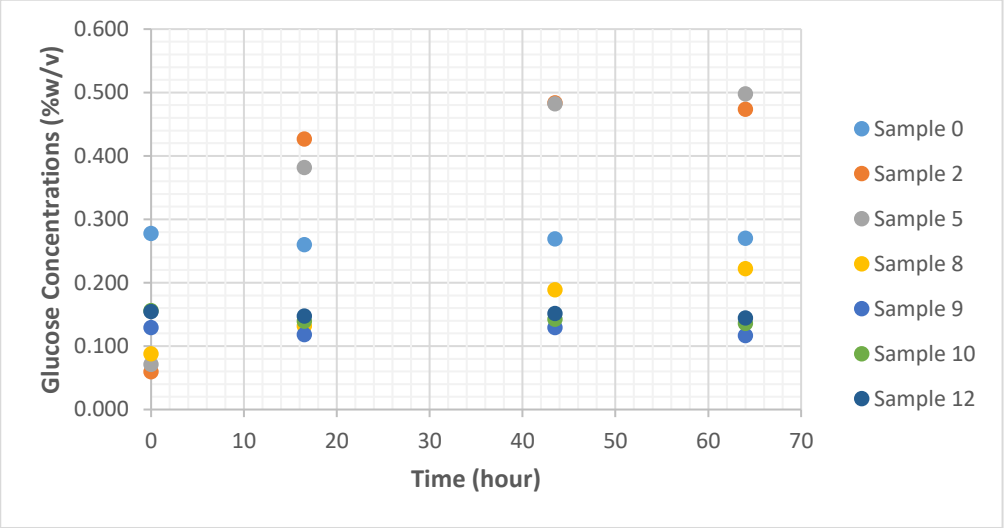

Figure 3.23. Glucose concentrations of wine samples without tartaric acid

3.11. The effect of pH on isomerization reactions in red wine medium

Although the activity pH range of glucose isomerase is between 2 and 10, the optimum pH is at 7.5 (Novozymes, 2015). The pH values of wine types are in this range but not at optimum pH values. While glucose formation occurred in synthetic environment at low pH values shown in results above, it did not occur in wine media. Although increasing the pH of wine is not an applicable process, it was tested as an experiment.

As seen in Figures 3.24 and 3.25, the glucose formation occurred at pH values of 6, 7 and 8 regardless of temperature. If temperature values were compared, the glucose concentration increased from 0.2 % to almost 0.9 % w/v for 60 ºC, however it was from 0.2 % to almost 0.4 % w/v for 30 ºC during 70 hours of incubation. If pH values were compared, the reaction rates were higher for pH 8, 7 and 6 in decreasing order at 60 ºC. However, the reaction rate was almost equal for pH 7 and 8 but lower for pH 6 at 30 ºC.

From the results of these experiments, the isomerization reactions occurred in red wine media at pH values higher than 5 regardless of temperature. Therefore, it was thought

that the pH of medium had to be suitable in order for the glucose isomerase to be active in wine media.

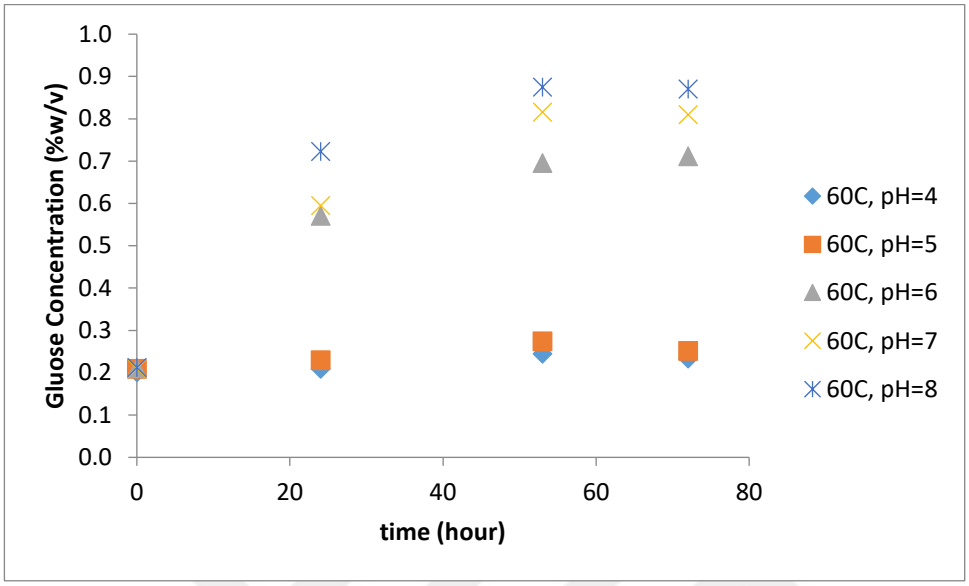

Figure 3.24. Glucose concentrations of home-made red wines at different pH values at 60 ℃

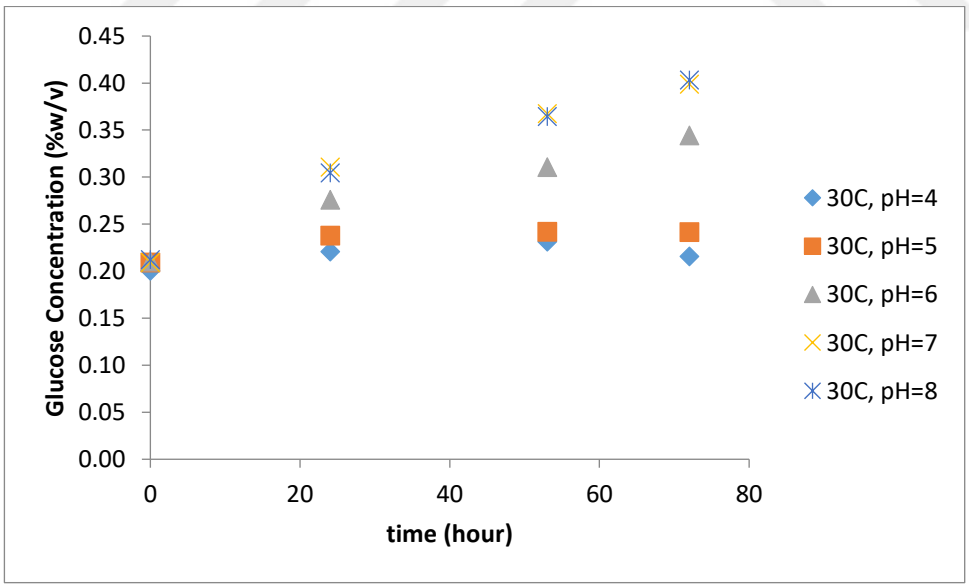

Figure 3.25. Glucose concentrations of home-made red wines at different pH values at 30 ℃

3.12. The uptake rate of yeast under different ethanol concentrations

It was known from the literature that uptake rate of *Saccharomyces cerevisiae* through fructose is always smaller than that of glucose. Also, the utilization rates of hexose sugars decrease as the accumulation of ethanol increases in media. The difference between glucose and fructose utilization rates increases as ethanol content increases, too (Zinnai, Venturi, Sanmartin, Quartacci, & Andrich, 2013).

First of all, wet cell weight and dry cell weight values were measured for different flasks mentioned in Figure 2.3 and the results are as follows:

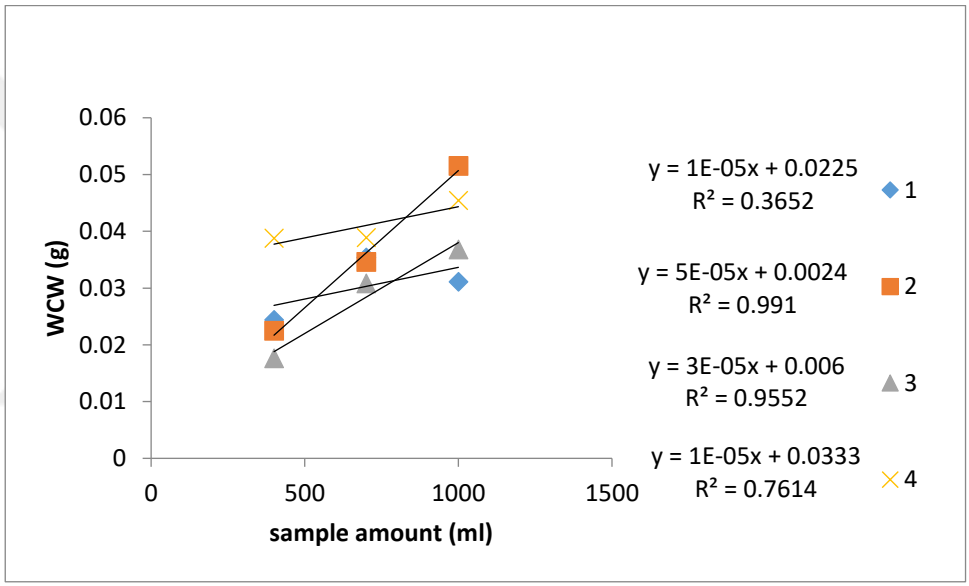

Figure 3.26. Wet Cell Weight of 20 mL flasks

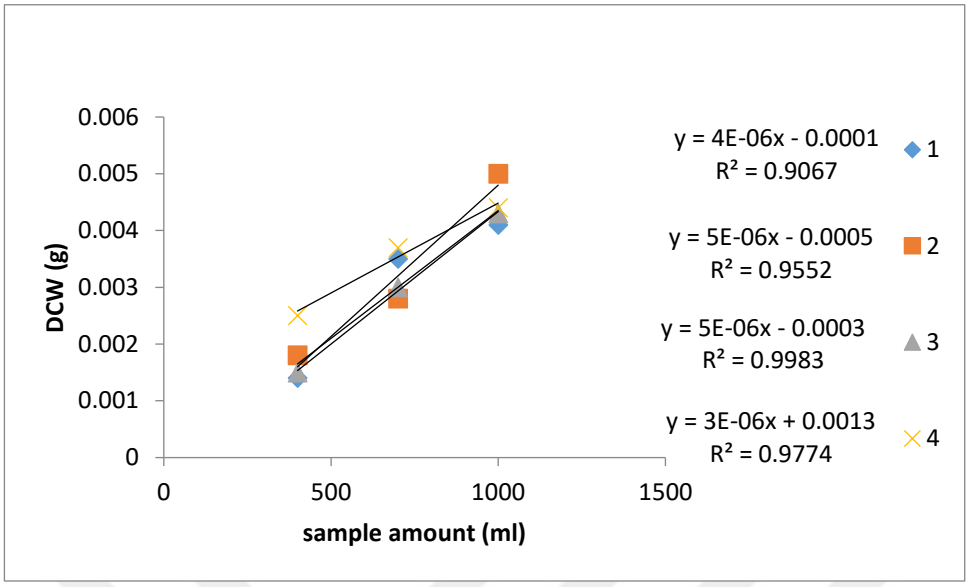

Figure 3.27. Dry Cell Weight of 20 mL flasks

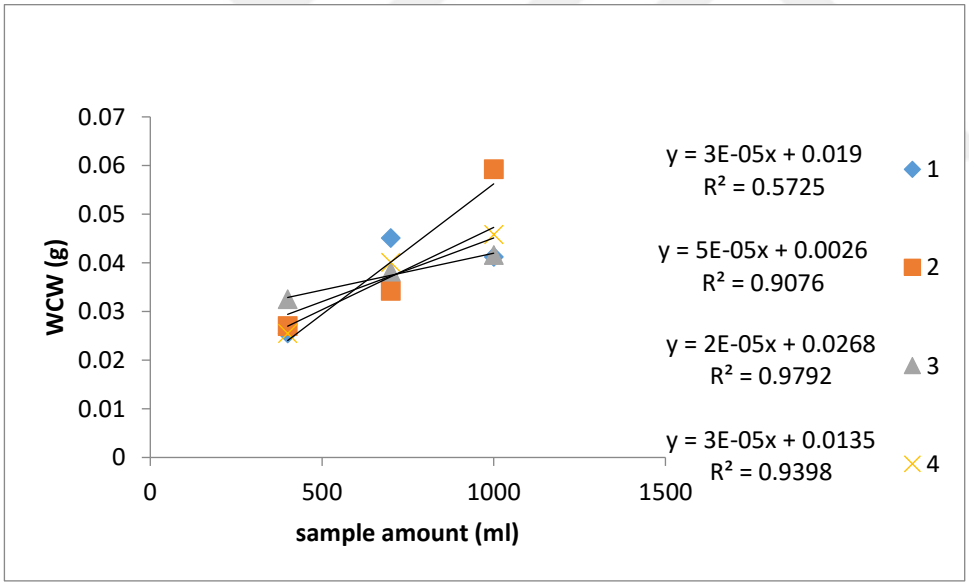

Figure 3.28. Wet Cell Weight of 100 mL flasks

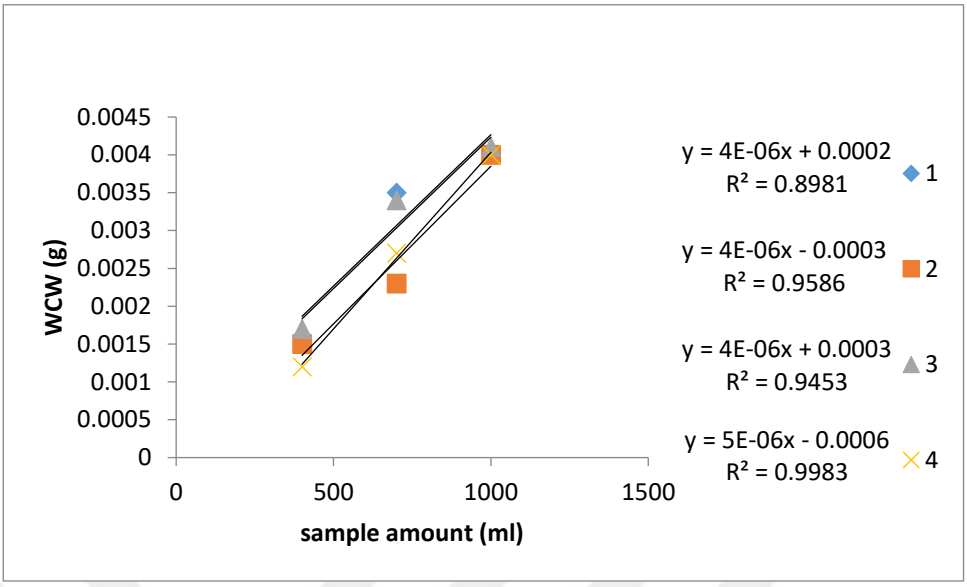

Figure 3.29. Dry Cell Weight of 100 mL flasks

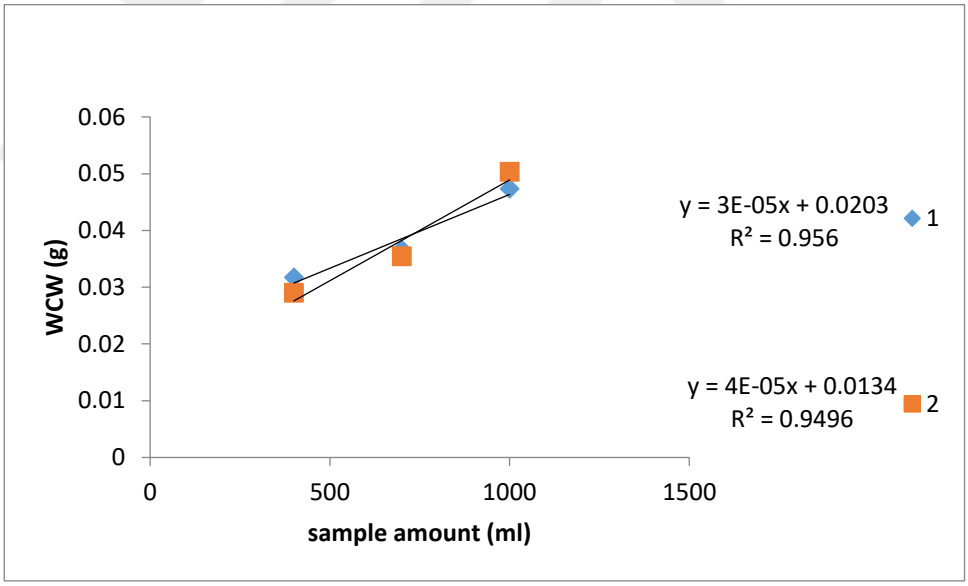

Figure 3.30. Wet Cell Weight of 1000 mL flasks

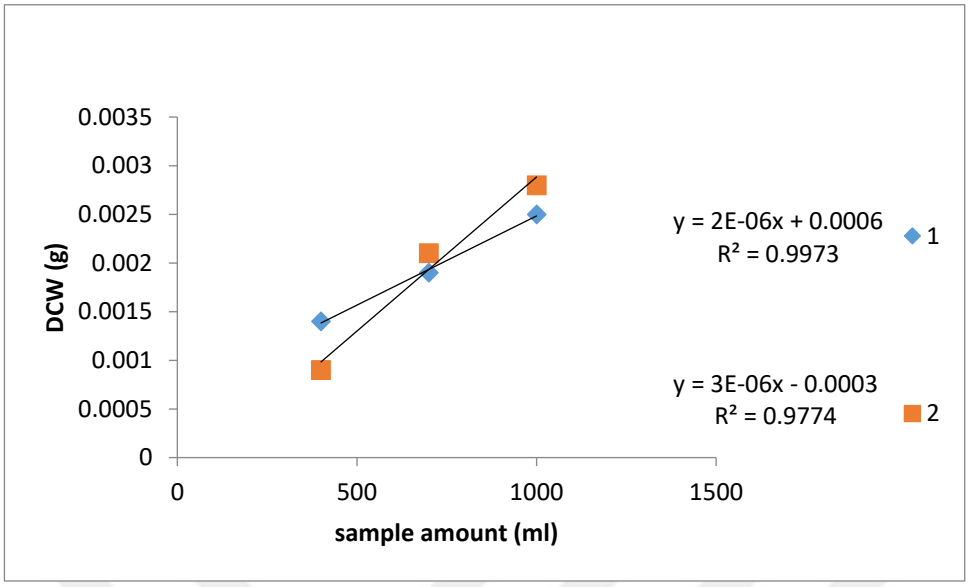

Figure 3.31. Dry Cell Weight of 1000 mL flasks

According to results, number 1 of 1000 mL flasks had better grown characteristics than others. To measure the uptake rate of yeast cells under different ethanol concentrations, 5 different environments with different alcohol contents were prepared with 100 mM PPB.

To provide media with different alcohol content, home-made red wine with SO_2 content lower than 7 ppm and alcohol content of 14.5 % v/v was chosen. The reason for choosing low SO_2 content was that the growth of *Saccharomyces cerevisiae* is repressed with SO_2 content higher than 10 ppm (Alexandre & Charpentier, 1998).

As seen in Figures 3.32-3.36, the consumption of fructose was always slower than that of glucose in different media. In media with alcohol contents of 0 %, 1.4 %, 2.8 % and 7.1 % v/v, glucose and fructose concentrations almost decreased to zero after 20 hours of incubation. However, in media with 14.5 % v/v alcohol, the glucose and fructose concentrations were equal to 1.1 % and 1.5 % w/v respectively after 20 hours. The reason of this situation was because of alcohol content in medium, there is glucose fructose discrepancy during uptake of yeast. Because of high ethanol concentration in

environment, the utilization of hexose sugars by yeast cells were slower than other media due to the affinity of hexose transporters are affected by the alcohol (Zinnai et al., 2013).

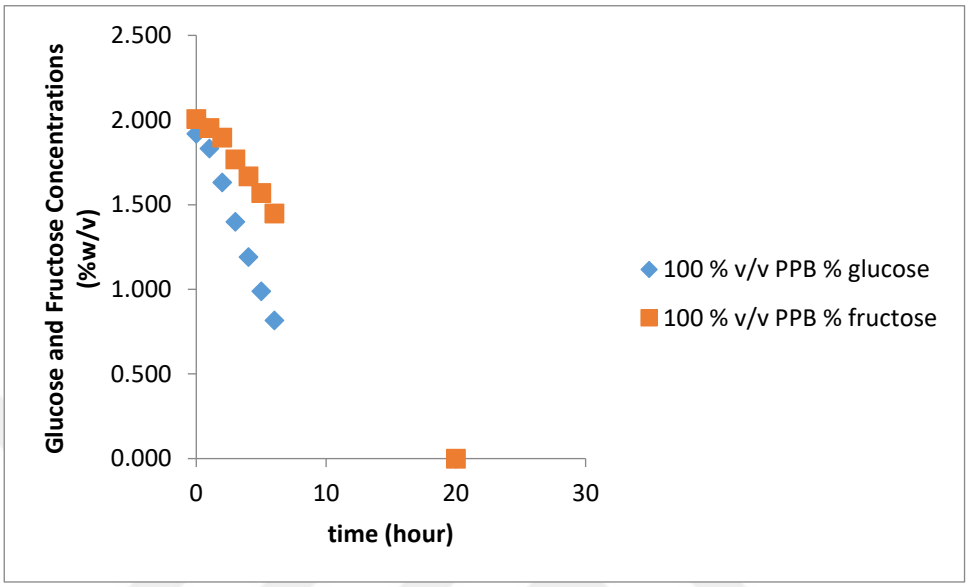

Figure 3.32. Glucose and fructose concentrations in 100 % v/v 100 mM PPB

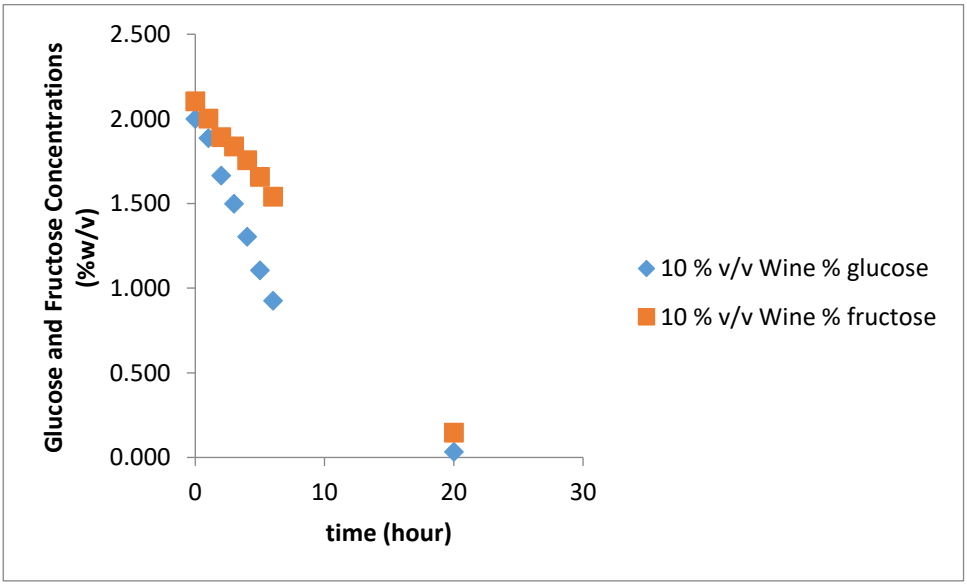

Figure 3.33. Glucose and fructose concentrations in 10 % v/v red wine

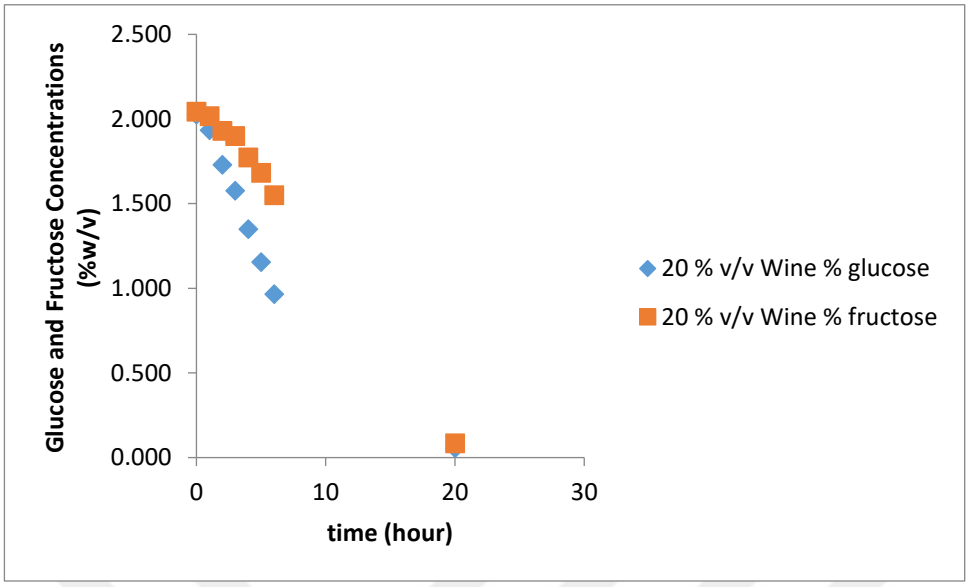

Figure 3.34. Glucose and fructose concentrations in 20 % v/v red wine

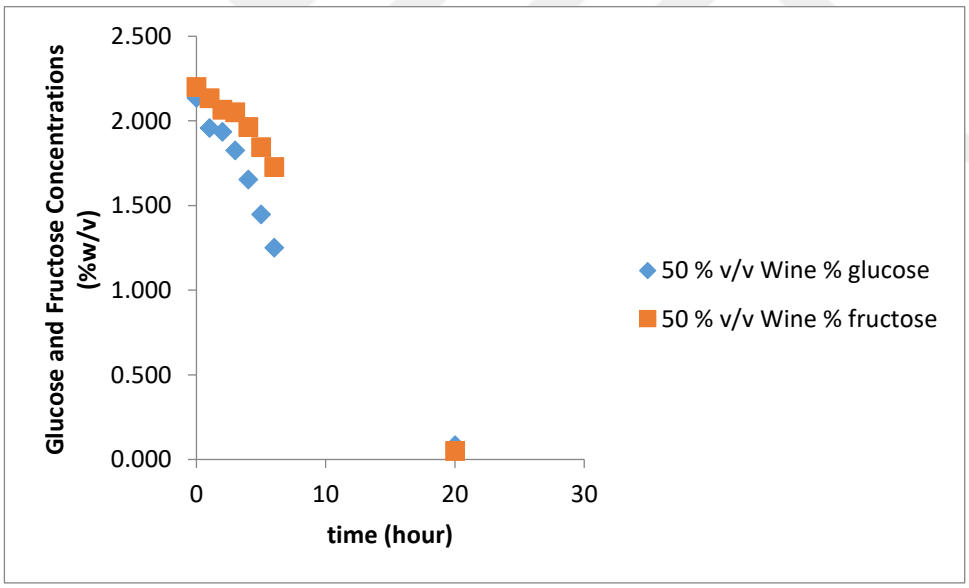

Figure 3.35. Glucose and fructose concentrations in 50 % v/v red wine

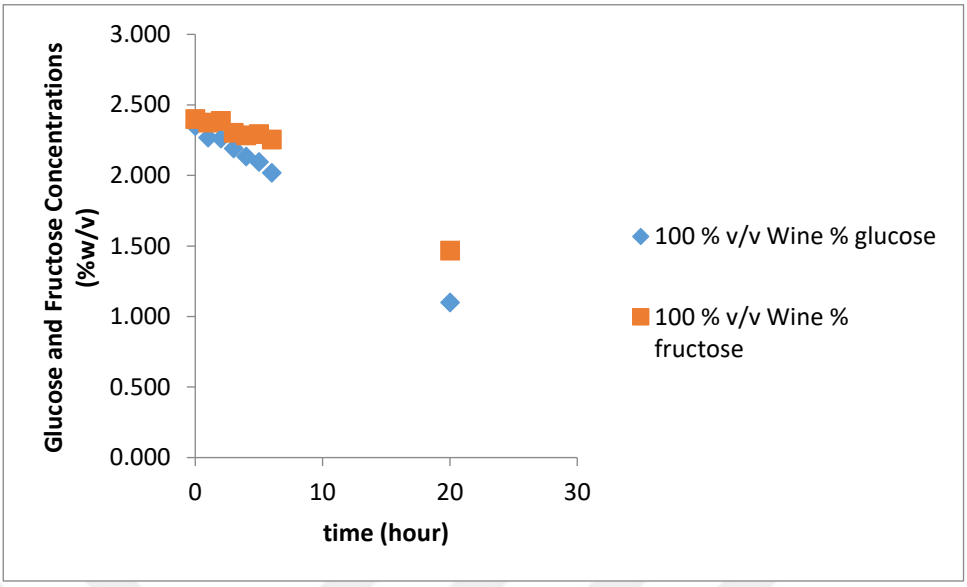

Figure 3.36. Glucose and fructose concentrations in 100 % v/v red wine

Also, by looking at Figures 3.37 and 3.38, it was easily seen that the consumption rates of glucose and fructose decreased as the alcohol content increased. Also, at ethanol concentration of 14.5 % v/v, the hexose sugars did not finish after 20 hours of incubation while others finished. The fastest consumption rate of hexose sugars occurred in medium with 100 % v/v 100 mM PPB. There was a clear difference between the consumption rates of glucose and fructose in media with 100 % v/v 100 mM PPB and red wine.

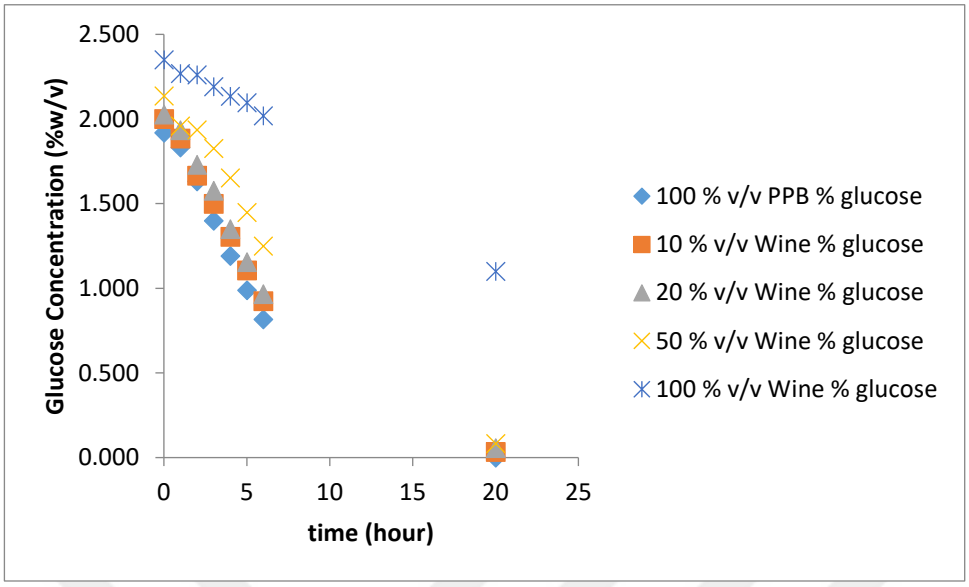

Figure 3.37. Glucose concentrations in different media

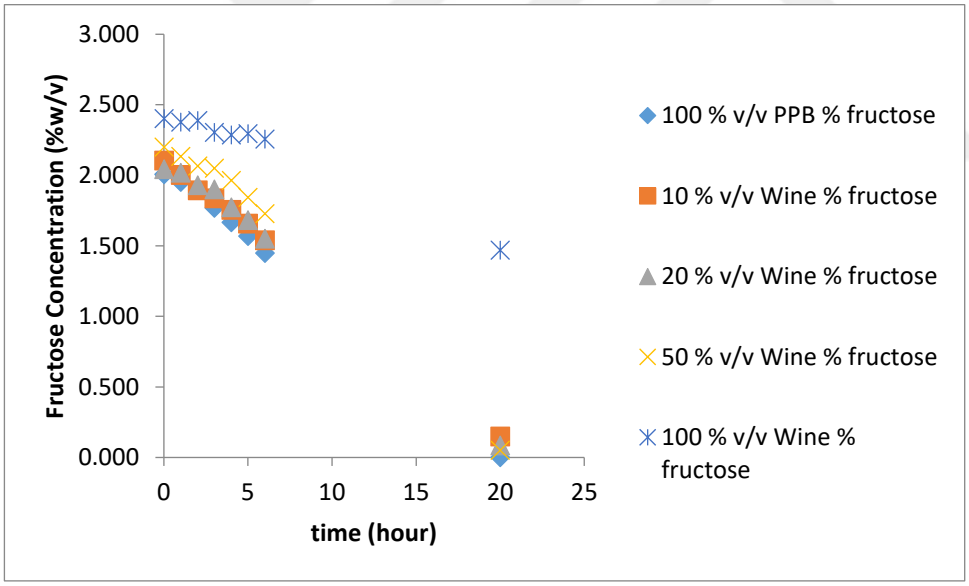

Figure 3.38. Fructose concentrations in different media

From the results of these experiments, the ethanol accumulation in media had slowing effect on the consumption rates of hexose sugars by yeast. The reason of that is the

affinity of hexose transporters through hexose sugars affected by ethanol accumulation (Rodríguez-Sifuentes et al., 2014). Also, since the affinity of hexose transporters through glucose is higher than that of glucose under same conditions (Rodríguez-Sifuentes et al., 2014; Tronchoni et al., 2009), the consumption rate of fructose was slower than that of glucose in all media.

CHAPTER 4

CONCLUSION AND RECOMMENDATION

In this study, the effects of different environmental and chemical factors on the activity of the enzyme glucose isomerase was tested with the final aim of using this enzyme for the conversion of fructose to glucose present in stuck wine fermentations.

Firstly, since temperature of the environment is important for wine making and the optimum temperature values of wine and glucose isomerase were different from each other, the enzyme was tested at different temperatures for converting glucose to fructose and vice versa. The results showed that the glucose or fructose were formed at different temperatures. Then, since ethanol is one of the major components of wine, glucose isomerase was tested under different ethanol concentrations in 1% w/v fructose solution, especially at ethanol concentration of wine media. Although the conversion reactions of glucose at different temperatures were occurred, the reaction was not seen in wine environment. After examining the effect of ethanol, the effect of acidic pH on glucose isomerase was tested in 1% w/v fructose solution without and with 13% v/v ethanol. Even if there was ethanol or not, glucose was formed at low pH. After that, since glycerol is also an important component for wine, glucose isomerase was tested in 1% w/v fructose, 13% v/v ethanol and acidic solution with different glycerol contents. Although reaction rate slowed down with increasing glycerol content, glucose was formed in all solutions with different glycerol concentrations. Since no inhibition effect on glucose isomerase was found with major components of wine, the effect of sulphite at different concentrations was examined in 1% w/v fructose solution at low pH. There was formation of glucose in synthetic media, too. After that, the effect of tannins was tested by comparing the formation of glucose in red and white wine environments. Since glucose was not formed in both media, it was thought that tannins did not affect the glucose isomerase reaction. Since

glucose isomerase was not active in wine media, the components formed after fermentation may inhibit the activity of glucose isomerase. Therefore, the experiment setup was performed to test grape juice versus red wine. There was also no glucose formation in grape juice like in wine media. It was thought that a component that is found in grape juice and present after fermentation may inhibit the glucose isomerase activity. It was known from the literature that calcium has inhibitory effect on glucose isomerase. Therefore, the activity of enzyme was tested in synthetic media with and without calcium in it. When there was calcium in environment, glucose was not formed due to inhibitory effect of calcium. Although, calcium in wine was separated with cation resin, glucose was also not formed from fructose in wine environment without calcium. To see that whether a component in wine may inhibit the isomerization reaction or not, red wine was diluted with water at different concentrations. The glucose formation occurred in wine media with dilution factor of 20% v/v, 10% v/v and 5% v/v. After all that experiments, the other major component of grape juice and wine, tartaric acid, was tested in synthetic media and red wine media. Synthetic media were prepared without and with tartaric acid and there was no isomerization reaction in medium with tartaric acid. Then, tartaric acid in wine was separated with anion exchanger, however, there was also no isomerization reaction in red wine. Finally, pH values of synthetic media and red wine media were increased to 4, 5, 6, 7 and 8. At pH 6 or more, the conversion from fructose to glucose was occurred even if there was tartaric acid or not.

To conclude, 0.5% w/v glucose formation from 1% w/v fructose occurred in synthetic medium containing 13% v/v ethanol and 1% v/v glycerol at pH 3.3 and at temperatures of 60ºC or 30ºC in approximately 48 hours. However, the glucose formation did not occur in synthetic medium if there was 0.3% w/v tartaric acid at pH 3.55 whereas glucose was formed at pH 6.33. In original wine medium with dilution effect and at pH values equal or higher than 6, glucose was formed from fructose whether there was tartaric acid or not. Since dilution and increasing the pH of wine cannot be applicable,

other ways to employ this enzyme to prevent stuck fermentation should be tried. Also, there are some recommendations about this study as mentioned below.

To use glucose isomerase for preventing stuck fermentation, some methods can be recommended. First of all, a membrane system can be used for separating acidic components like acetic acid from wine media. Thanks to this membrane system, the pH of wine can be increased, glucose formation from fructose may occur and stuck fermentation may be prevented by using glucose isomerase. Secondly, low pH resistant glucose isomerase can be chosen for this study. Thanks to this enzyme, glucose formation from fructose may occur in wine media and stuck fermentation may be prevented by using low pH resistant glucose isomerase. Finally, the yeast type that was used for wine making process had glucophilic character which prefer glucose against fructose. Instead of glucophilic character type of yeast, fructophilic character type of yeast can be chosen for wine process. By changing the characteristic of yeast, excessive fructose can be utilized by fructophilic character type of yeast and stuck fermentation may be prevented by different yeast type.

REFERENCES

Alexandre, H., & Charpentier, C. (1998). Biochemical aspects of stuck and sluggish fermentation in grape must. *Journal of Industrial Microbiology and Biotechnology*, *20*(1), 20–27. https://doi.org/10.1038/sj.jim.2900442

Antri, R. L., & Auterinen, A. L. (1986). A New Regenerable Immobilized Glucose Isomerase. *Starch/Starke*, *38*(4), 132–137. https://doi.org/10.1002/star.19860380408

Bağder Elmacı, S., Özçelik, F., Tokatlı, M., & Çakır, I. (2014). Technological properties of indigenous wine yeast strains isolated from wine production regions of Turkey. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *105*(5), 835–847. https://doi.org/10.1007/s10482-014-0138-z

Ben Hlima, H., Aghajari, N., Ben Ali, M., Haser, R., & Bejar, S. (2012). Engineered glucose isomerase from Streptomyces sp. SK is resistant to Ca 2+ inhibition and Co 2+ independent. *Journal of Industrial Microbiology and Biotechnology*, *39*(4), 537–546. https://doi.org/10.1007/s10295-011-1061-1

Berthels, N. J., Cordero Otero, R. R., Bauer, F. F., Thevelein, J. M., & Pretorius, I. S. (2004). Discrepancy in glucose and fructose utilisation during fermentation by Saccharomyces cerevisiae wine yeast strains. *FEMS Yeast Research*, *4*(7), 683–689. https://doi.org/10.1016/j.femsyr.2004.02.005

Bisson, L. F. (1999). Stuck and sluggish fermentations. *American Journal of Enology and Viticulture*, *50*(1), 107–119.

Buescher, W. A., Siler, C. E., Morris, J. R., Threlfall, R. T., Main, G. L., & Cone, G. C. (2001). High alcohol wine production from grape juice concentrates. *American Journal of Enology and Viticulture*.

Casassa, L. F., & Harbertson, J. F. (2014). Extraction, Evolution, and Sensory Impact of Phenolic Compounds During Red Wine Maceration. *Annual Review of Food Science and Technology*, *5*(1), 83–109. https://doi.org/10.1146/annurev-food-030713-092438

Chen, K., Escott, C., Loira, I., del Fresno, J. M., Morata, A., Tesfaye, W., … Benito, S. (2018). Use of non-Saccharomyces yeasts and oenological tannin in red winemaking: Influence on colour, aroma and sensorial properties of young wines. *Food Microbiology*, *69*, 51–63. https://doi.org/10.1016/j.fm.2017.07.018

Cox, R. J., Eitenmiller, R. R., & Powers, J. J. (1977). Mineral Content of Some California Wines. *Journal of Food Science*, *42*(3).

Datta, S., Christena, L. R., & Rajaram, Y. R. S. (2013). Enzyme immobilization: an overview on techniques and support materials. *3 Biotech*, *3*(1), 1–9. https://doi.org/10.1007/s13205-012-0071-7

Dhungel, B., Subedi, M., Tiwari, K. B., Shrestha, U. T., Pokhrel, S., & Agrawal, V. P. (2007). Thermostable glucose isomerase from psychrotolerant Streptomyces species. *International Journal of Life Sciences*, 6–10. Retrieved from http://www.nepjol.info/index.php/IJLS/article/viewArticle/2300

Dow Chemical Company. (1999). Dowex Ion Exchange Resins: Fundamentals of Ion Exchange. *Metal Finishing*, *97*(12), 69–70. https://doi.org/10.1016/S0026-0576(00)81191-5

Frost, S. C., Harbertson, J. F., & Heymann, H. (2017). A full factorial study on the effect of tannins, acidity, and ethanol on the temporal perception of taste and mouthfeel in red wine. *Food Quality and Preference*, *62*(May), 1–7. https://doi.org/10.1016/j.foodqual.2017.05.010

Garcia, R., & Baez, A. P. (1982). Atomic Absorption Spectrometry. *Techniques and Instrumentation in Analytical Chemistry*, *5*, 67–94. https://doi.org/10.1016/S0167-9244(08)70083-0

Ge, Y., Zhou, H., Kong, W., Tong, Y., Wang, S., & Li, W. (1998). Immobilization of glucose isomerase and its application in continuous production of high fructose syrup. *Applied Biochemistry and Biotechnology*, *69*(3), 203–215. https://doi.org/10.1007/BF02788814

Grant E., R. (1997). Ion exchange resins. *SRI International*, (December), 9–62. https://doi.org/10.1016/S0026-0576(00)81191-5

Griko, Y. V. (1999). Energetics of Ca2+-EDTA interactions: Calorimetric study. *Biophysical Chemistry*, *79*(2), 117–127. https://doi.org/10.1016/S0301-4622(99)00047-2

Hazelwood, L. A., Daran, J. M., Van Maris, A. J. A., Pronk, J. T., & Dickinson, J. R. (2008). The Ehrlich pathway for fusel alcohol production: A century of research on Saccharomyces cerevisiae metabolism. *Applied and Environmental Microbiology*, *74*(8), 2259–2266. https://doi.org/10.1128/AEM.02625-07

Jemli, S., Ayadi-Zouari, D., Hlima, H. Ben, & Bejar, S. (2016). Biocatalysts: Application and engineering for industrial purposes. *Critical Reviews in Biotechnology*, *36*(2), 246–258. https://doi.org/10.3109/07388551.2014.950550

Kasumi, T., Hayashi, K., & Tsumura, N. (1982). Roles of magnesium and cobalt in the reaction of glucose isomerase from Streptomyces griseofuscus S-41. *Agricultural and Biological Chemistry*, *46*(1), 21–30. https://doi.org/10.1080/00021369.1982.10865021

Keulder, D. B. (2006). The influence of commercial tannin additions on wine composition and quality. *Master Thessis*, (February), 101.

Kovalevsky, A., Hanson, B. L., Mason, S. A., Forsyth, V. T., Fisher, Z., Mustyakimov, M., Langan, P. (2012). Inhibition of d-xylose isomerase by polyols: Atomic details by joint X-ray/neutron crystallography. *Acta Crystallographica Section D: Biological Crystallography*, *68*(9), 1201–1206. https://doi.org/10.1107/S0907444912024808

Lee, C.-K. (1979). The chemistry and biochemistry of the sweetness of sugars. *Advances in Carbohydrate Chemistry and Biochemistry*, *45*, 199–350.

Legras, J.-L., Merdinoglu, D., Cornuet, J.-M., & Karst, F. (2007). Bread, beer and wine : Saccharomyces cerevisiae diversity reflects human history. *Molecular Ecology*, *16*, 2091–2102. https://doi.org/10.1111/j.1365-294X.2007.03266.x

Luyten, K., Riou, C., & Blondin, B. (2002). The hexose transporters of Saccharomyces cerevisiae play different roles during enological fermentation. *Yeast*, *19*(8), 713–726. https://doi.org/10.1002/yea.869

Maisonnave, P., Sanchez, I., Moine, V., Dequin, S., & Galeote, V. (2013). Stuck fermentation : Development of a synthetic stuck wine and study of a restart procedure. *International Journal of Food Microbiology*, *163*(2–3), 239–247. https://doi.org/10.1016/j.ijfoodmicro.2013.03.004

Malherbe, S., Bauer, F. F., & Du Toit, M. (2007). Understanding problem fermentations - A review. *South African Journal of Enology and Viticulture*, *28*(2), 169–186. https://doi.org/10.21548/28-2-1471

Mendes-Ferreira, A., Mendes-Faia, A., & Leão, C. (2004). Growth and fermentation patterns of Saccharomyces cerevisiae under different ammonium concentrations and its implications in winemaking industry. *Journal of Applied Microbiology*, *97*(3), 540–545. https://doi.org/10.1111/j.1365-2672.2004.02331.x

Michael D. Arguello, J. S. F. (1977). Ion-Exchange Separation and Determination of Calcium and Magnesium. *Analytical Chemistry*, *49*(11).

Mocke, L. (2013). Kinetic Modelling of Wine Fermentations: Why Does Yeast Prefer Glucose to Fructose? *University of Stellenbosch*, (March), 78.

Navarro-Alarcon, M., Velasco, C., Jodral, A., Terrés, C., Olalla, M., Lopez, H., & Lopez, M. C. (2007). Copper, zinc, calcium and magnesium content of alcoholic beverages and by-products from Spain: Nutritional supply. *Food Additives and Contaminants*, *24*(7), 685–694. https://doi.org/10.1080/02652030601185063

Novozymes. (2015). *Effective conversion of glucose to fructose for sweeteners*. *Effective Conversion of Glucose to Fructose for Sweeteners*.

Pretorius, I. S., & Høj, P. B. (2005). Grape and wine biotechnology: Challenges, opportunities and potential benefits. *Australian Journal of Grape and Wine Research*, *11*(2), 83–108. https://doi.org/10.1111/j.1755-0238.2005.tb00281.x

PURETEC. (2014). Basics of Deionized Water by Ion Exchange, 2.

Rahman, N. A., Hasan, M., Hussain, M. A., & Jahim, J. (2007). EFFECT OF pH , TEMPERATURE AND INITIAL GLUCOSE CONCENTRATION TO A PRODUCTION OF FRUCTOSE. In *Conference Paper*. Malaysia: Research Gate. Retrieved from https://www.researchgate.net/publication/228472125%0AEFFECT

Regodón Mateos, J. A., Pérez-Nevado, F., & Ramírez Fernández, M. (2006). Influence of Saccharomyces cerevisiae yeast strain on the major volatile compounds of wine. *Enzyme and Microbial Technology*, *40*(1), 151–157. https://doi.org/10.1016/j.enzmictec.2005.10.048

Remize, F., Cambon, B., Barnavon, L., & Dequin, S. (2003). Glycerol formation during wine fermentation is mainly linked to Gpd I p and is only partially controlled by the HOG pathway. *Yeast*, *20*(15), 1243–1253. https://doi.org/10.1002/yea.1041

Rodríguez-Sifuentes, L., Páez-Lerma, J. B., Rutiaga-Quiñones, O. M., Rojas-Contreras, J. A., Ruiz-Baca, E., Gutiérrez-Sánchez, G., Soto-Cruz, N. O. (2014). Identification of a yeast strain as a potential stuck wine fermentation restarter: A kinetic characterization. *CYTA - Journal of Food*, *12*(1), 1–8. https://doi.org/10.1080/19476337.2013.776637

Sablayrolles, J. M. (2009). Control of alcoholic fermentation in winemaking: Current situation and prospect. *Food Research International*, *42*(4), 418–424. https://doi.org/10.1016/j.foodres.2008.12.016

Salari, R., & Salari, R. (2017). Investigation of the Best Saccharomyces cerevisiae Growth Condition. *Electronic Physician*, *9*(1), 3592–3597. https://doi.org/10.19082/3592

Sanders, F. W. B., & Griffin, J. L. (2015). De novolipogenesis in the liver in health and disease: more than just a shunting yard for glucose. Biological Reviews, 91(2), 452–468. doi: 10.1111/brv.12178

Schiffman, S. S., Sattely-Miller, E. a, Graham, B. G., Bennett, J. L., Booth, B. J., Desai, N., & Bishay, I. (2000). Effect of temperature, pH, and ions on sweet taste. *Physiology & Behavior*, *68*(4), 469–481. https://doi.org/10.1016/S0031-9384(99)00205-X

Soleas, G. J., Diamandis, E. P., & Goldberg, D. M. (1997). Wine as a Biological Fluid: History, Production, and Role in Disease Prevention. *Journal of Clinical Laboratory Analysis*, *11*(5), 287–313. https://doi.org/10.1111/pme.12117

Sousa, E. C., Uchôa-Thomaz, A. M. A., Carioca, J. O. B., Morais, S. M. de, Lima, A. de, Martins, C. G., … Rodrigues, L. L. (2014). Chemical composition and bioactive compounds of grape pomace (Vitis vinifera L.), Benitaka variety, grown in the semiarid region of Northeast Brazil. *Food Science and Technology (Campinas)*, *34*(1), 135–142. https://doi.org/10.1590/S0101-20612014000100020

Sperber, W. (2009). *Compendium of the Microbiological Spoilage of Foods and Beverages*. https://doi.org/10.1007/978-1-4419-0826-1

Teusink, B., Passarge, J., Reijenga, C. A., Esgalhado, E., Weijden, C. C. Van Der, Schepper, M., Snoep, J. L. (2000). Can yeast glycolysis be understood in terms of in vitro kinetics of the constituent enzymes ? Testing biochemistry. *European*

Journal of Biochemistry, *267*(1), 5313–5329.

Tronchoni, J., Gamero, A., Arroyo-López, F. N., Barrio, E., & Querol, A. (2009). Differences in the glucose and fructose consumption profiles in diverse Saccharomyces wine species and their hybrids during grape juice fermentation. *International Journal of Food Microbiology*, *134*(3), 237–243. https://doi.org/10.1016/j.ijfoodmicro.2009.07.004

Van Den Brink, J., & Kristine, B. (2011). U.S. Patent No. US 20110020491A1. Washington, DC: U.S. Patent and Trademark Office.

Van Den Brink, J., & Kristine, B. (2014). U.S. Patent No. US 008765200B2. Washington, DC: U.S. Patent and Trademark Office.

Viana, T., Loureiro-Dias, M. C., & Prista, C. (2014). Efficient fermentation of an improved synthetic grape must by enological and laboratory strains of Saccharomyces cerevisiae. *AMB Express*, *4*(1), 16. https://doi.org/10.1186/s13568-014-0016-0

Vilela, A. (2019). The Importance of Yeasts on Fermentation Quality and Human Health-Promoting Compounds. *Fermentation*, *5*(2), 1–12. https://doi.org/10.3390/fermentation5020046

Volschenk, H., & Van Vuuren, H. J. J. (2006). Malic Acid in Wine : Origin , Function and Metabolism during Vinification. *S Afr J Enol Vitic*, *27*(2), 123–136.

Zimmermann, F. K., & Entian, K.-D. (1997). *Yeast sugar metabolism: biochemistry, genetics, biotechnology, and applications.* Lancaster, PA: Technomic Pub.

Zinnai, A., Venturi, F., Sanmartin, C., Quartacci, M. F., & Andrich, G. (2013). Kinetics of D-glucose and D-fructose conversion during the alcoholic fermentation promoted by Saccharomyces cerevisiae. *Seibutsu-Kogaku Kaishi*, *115*(1), 43–49. https://doi.org/10.1016/j.jbiosc.2012.08.008

APPENDICES

A. CHEMICALS USED AND PRODUCERS

Table A 1. Chemicals and Producers

Chemicals	Producers
Acetic Acid	Merck
Biotin	Sigma-Aldrich
Calcium Carbonate	Analar
Calcium Hydroxide	Merck
D-Fructose	Serva
D-Glucose Monohydrate	Sigma-Aldrich
EDTA	Sigma-Aldrich
Ethanol	Merck
Glycerol	Merck
Hydrochloric Acid	Sigma-Aldrich
Inositol	Fluka BioChemika
L-(+)-Tartaric Acid	Merck
Magnesium Sulfate Heptahydrate	Merck
Nicotinamide	Sigma-Aldrich
P-Aminobenzoic Acid	Sigma-Aldrich
Pantothenic Acid	Fluka BioChemika
Peptone from Meat	Merck
Potassium Hydroxide	Sigma-Aldrich
Potassium Phosphate Dibasic	Sigma-Aldrich
Potassium Phosphate Monobasic	Sigma-Aldrich
Potassium Sulfate	Sigma-Aldrich
Pyridoxine	Sigma-Aldrich
Riboflavin	Fluka BioChemika
Sodium Hydroxide	ISOLAB
Sulfur Dioxide	Sigma-Aldrich
Sulfuric Acid	Sigma-Aldrich
Thiamine	Sigma-Aldrich
Yeast Extract	Merck
Zinc Sulfate Heptahydrate	Merck

B. CALIBRATION CURVES

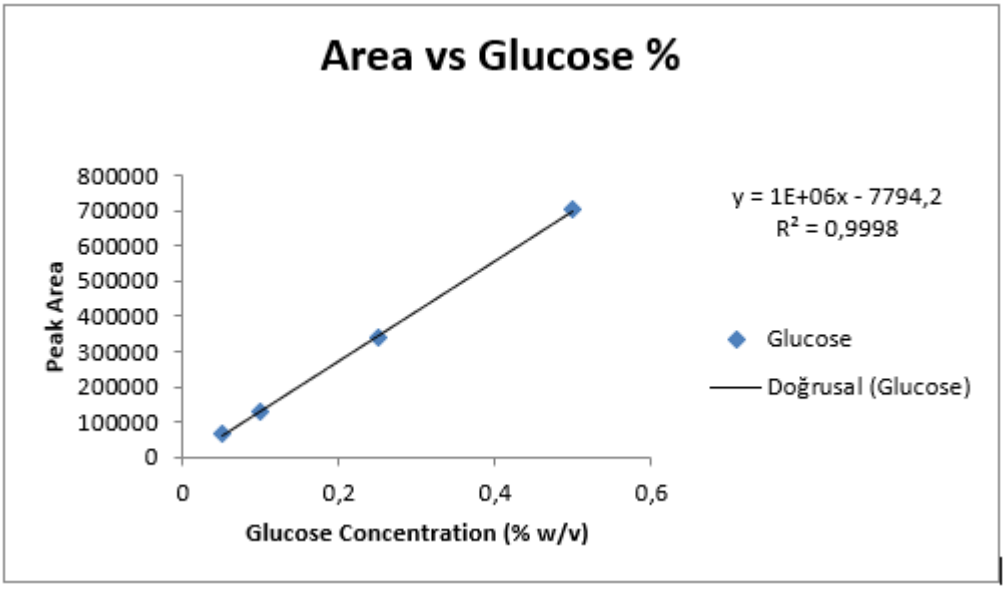

Figure B 1. Glucose calibration curve

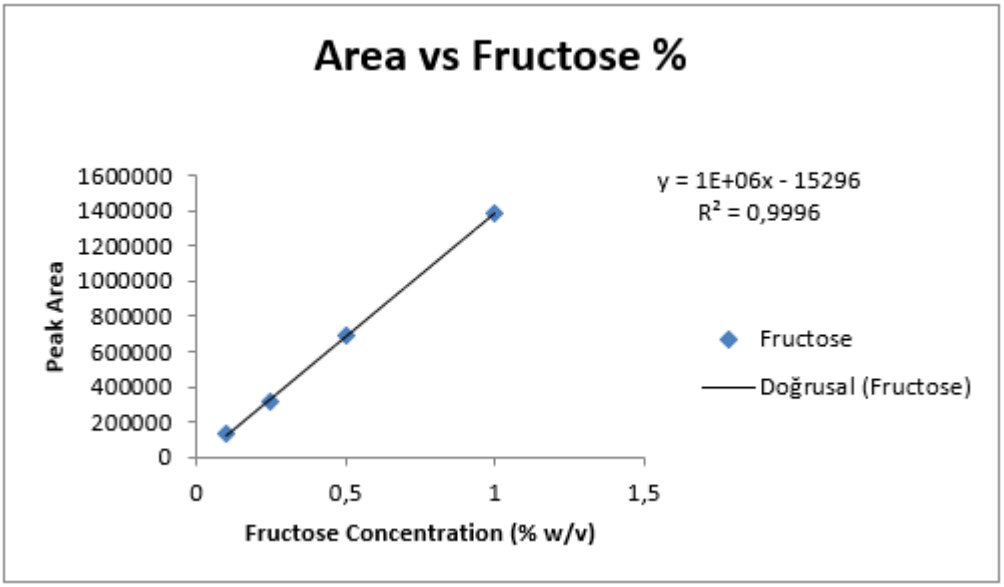

Figure B 2. Fructose calibration curve

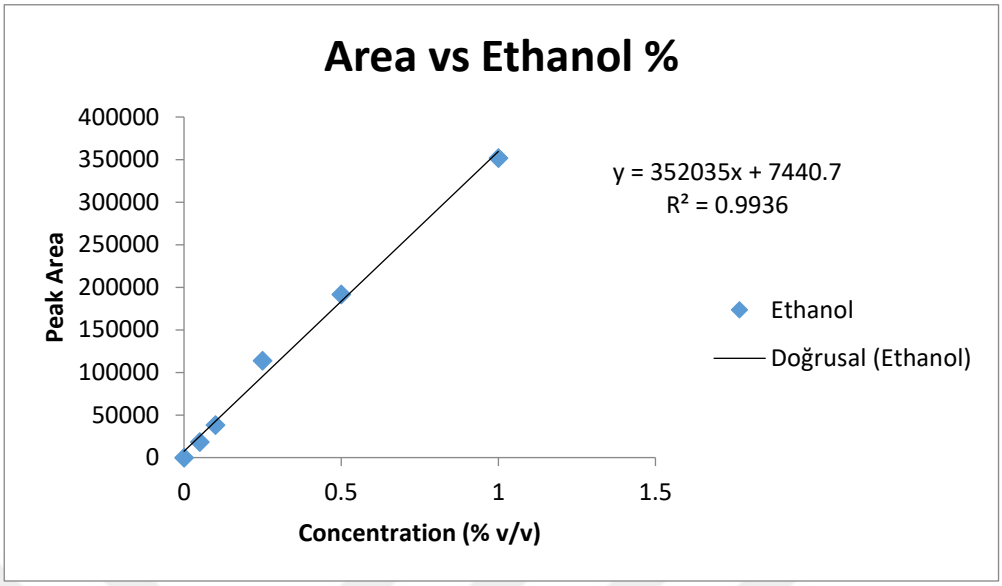

Figure B 3. Ethanol calibration curve

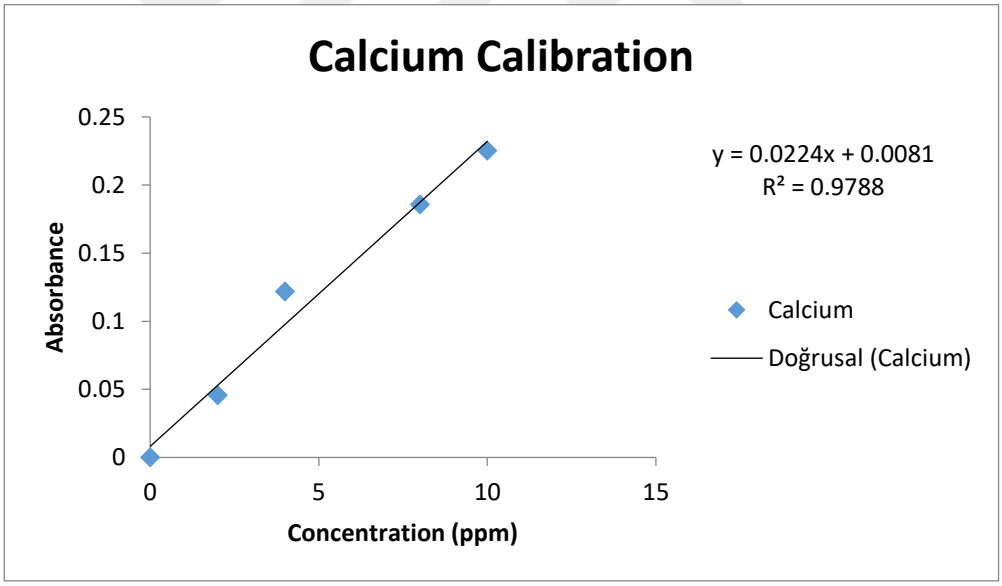

Figure B 4. Calcium calibration curve

C. INGREDIENTS OF USED SOLUTIONS

Table C 1. Ingredients of YPD environment

YPD Environment	
Peptone	20.0 g
Yeast Extract	10.0 g
Glucose	20.0 g
Complete to 1000 mL with distilled H_2O Sterilize by autoclave at 121 °C for 15 minutes	

Table C 2. Ingredients of trace elements

Trace Elements	
EDTA	1.5 g
$ZnSO_4$	0.45 g
$CoCl_2$	0.03 g
$MnCl_2$	0.1 g
$CuSO_4$	0.03 g
$CaCl_2$	0.45 g
$FeSO_4$	0.3 g
$NaMoO_4$	0.04 g
HBO_3	0.1 g
KCl	0.01 g
KI	0.01 g
Complete to 1000 mL with distilled H_2O Use 100 mL for 6 kg glucose	

Table C 3. Ingredients of minerals

Minerals	
$CuSO_4$	0.3 g
$ZnSO_4$	1.0 g
K_2SO_4	25.0 g
$MgSO_4$	25.0 g
Complete to 1000 mL with distilled H_2O Use 1000 mL for 6 kg glucose	

Table C 4. Ingredients of vitamins

Vitamins	
Thiamine (B1)	150 mg
Riboflavin (B2)	7.5 mg
Nicotinamide (B3)	240 mg
Pantothenic acid (B5)	570 mg
Pyridoxine (B6)	72 mg
Biotin (B7)	3 mg
PABA	33.6 mg
Inositol	8.64 g
Complete to 100 mL distilled H_2O Filtrate with 0.45 μm filter Use 100 mL for 6 kg glucose	

Table C 5. Ingredients of PPB

Potassium Phosphate Buffer (PPB) (100 mM at pH 6.5)	
Dibasic potassium phosphate (K_2HPO_4)	13.61 g
Potassium dihydrogen phosphate (KH_2PO_4)	17.42 g
Complete to 1000 mL with distilled H_2O for each Mix them until pH 6.5	