BOLU ABANT IZZET BAYSAL UNIVERSITY

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

THE EFFECT OF DIFFERENT COFFEE TYPES ADDED TO YEAST GROWTH MEDIUM ON THE MAIN ENZYMES INVOLVED IN BREAD STALING

MASTER OF SCIENCE

ULVİYE PALA

BOLU, JULY 2019

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APPROVAL OF THE THESIS

THE EFFECT OF DIFFERENT COFFEE TYPES ADDED TO YEAST GROWTH MEDIUM ON THE MAIN ENZYMES INVOLVED IN BREAD STALING submitted by **ULVİYE PALA** and defended before the below named jury in partial fulfillment of the requirements for the degree of **Master of Science** in **Department of Biology, The Graduate School of Natural and Applied Sciences of Bolu Abant Izzet Baysal University in 29. 07. 2019** by

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Prof. Dr. Ömer ÖZYURT .....

Director of Graduate School of Natural and Applied Sciences

To my family and my husband

DECLARATION

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.

ULVİYE PALA

ABSTRACT

THE EFFECT OF DIFFERENT COFFEE TYPES ADDED TO YEAST GROWTH MEDIUM ON THE MAIN ENZYMES INVOLVED IN BREAD STALING
MSC THESIS
ULVIYE PALA
BOLU ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: PROF.DR. SEYHUN YURDUGÜL))

BOLU, JULY 2019

According to different studies, there is a strict interaction between the yeasts and the coffee fermentations. A study carried out reports on the indigenous espresso yeasts and their potential use as starter cultures during fermentation.

The use of coffee wastes is also important in providing better yields in industrial enzyme production such as proteases, cellulases, invertases, and alpha-amylase. Coffee is very important from the economical point of view, since numerous countries benefit from its development, handling, exchanging, and offers a market that employs a huge number of individuals. In coffee planting and processing nations, innovation strategies of treating coffee wastes are critically important to avoid its harm to the environment. Distinctive yeast species are viably utilized in multi-arrange biorefineries with the possibility to change over waste created at harvest preparing activities, for example, coffee pulping units, to significant biofuel and bioproduct generation utilizing transformation advances through biochemistry and thermochemistry, after the processing of coffee. In this study; the role of different coffees in *Saccharomyces cerevisiae* yeast growth medium to provide better yields in industrial enzyme production secreted by the aforementioned organism including proteases, cellulases, invertases, and alpha-amylases were analyzed. Its contribution to bread quality by examining water retention, oil retention, and total suspended solids, was investigated by adding partially purified extracts obtained from growth medium of yeast to the bread made from flour obtained from a variety of wheat (*Triticum aestivum* L.) It was found that the different coffees in yeast growth medium provide better protease secretion yield when compared to other enzymes.

KEYWORDS: Bread, Coffee, *Saccharomyces cerevisiae*, Protease, Invertase, Cellulose, Amylase

ÖZET

MAYA BÜYÜME ORTAMINA EKLENEN FARKLI KAHVE ÇEŞİTLERİNİN EKMEK BAYATLAMASINDA ROL ÜSTLENEN BAŞLICA ENZİMLER ÜZERİNE ETKİSİ
YÜKSEK LISANS TEZI
ULVIYE PALA
BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ
BIYOLOJI ANABILIM DALI
(TEZ DANIŞMANI: PROF.DR. SEYHUN YURDUGÜL)

BOLU, TEMMUZ - 2019

Yapılan farklı çalışmalara göre kahve fermantasyonu ve mayalar arasında kuvvetli bir etkileşim bulunmaktadır. Hedef tanımlama için kullanılan yerli kahve mayaları ve bu mayaların başlangıç kültürleri deneysel olarak fermantasyon aşamasında rapor edilmiştir. Kahve atıklarının kullanımı proteaz, selülaz, invertaz ve alfa amilaz gibi endüstriyel enzim üretiminde daha iyi verim sağlamak açısından önemlidir.

Kahve sadece kültürden ziyade, tüketilen birçok ülkede farklı amaçlar doğrultusunda ülke ekonomisine katkı sağlamaktadır. Kahve kültürünün oluşturduğu üretim sahaları ve tüketim alanları kafeler, kahve ekim işlemleri, işlenmesi, ticari olarak satışı ve pazarlaması gibi birçok alanda milyonlarca insana iş desteği sağlamaktadır.

Kahve ekim ve işleme işlemleri gerçekleşen ülkelerde, çevreyle ilgili hasarın önlenebilmesi adına teknolojik gelişmeler ışığında bilimsel katkılar ile kahve işlemi sonucunda meydana gelen atığın geri dönüşüme tabi tutularak tekrar değerlendirilmesi kritik öneme sahiptir. Değerli biyoyakıtlar ve biyoürünler gibi biyokimyasal ve termokimyasal dönüşüm teknolojilerini kullanan farklı maya türleri, kahve işlemden geçtikten sonra, işlem sonrası oluşan atıkların geri dönüşüm potansiyeli bulunan ve birçok alanda kullanılabilecek ve farklı aşamalardan oluşan biyo-ürünlerde etkin ve etkili bir şekilde değerlendirilebilir.

Bu çalışmada; *Saccharomyces cerevisiae* tarafından salgılanan proteazlar, selülazlar, invertazlar ve alfa-amilazlar gibi enzimlerin, endüstriyel enzim üretiminde daha iyi verim sağlamak için farklı kahve türleri eklenen maya büyüme ortamındaki rolü incelenmiştir. *Triticum aestivum L.* çeşidi olan ekmeklik buğdaydan elde edilen undan yapılan ekmeklere, bahsedilen büyüme ortamından elde edilmiş olan, kısmi saflaştırılmış bu enzimleri içerdiği düşünülen özütlerin eklenmesi ile ekmek kalitesine olan katkısı; mikrobiyal analiz, su tutma, yağ tutma ve kuru ağırlık gibi özelliklerin incelenmesi ile araştırılmıştır. Maya büyüme ortamındaki farklı kahvelerin, diğer enzimler ile karşılaştırıldığında proteaz enziminin daha iyi bir verimle salgılandığı bulunmuştur.

ANAHTAR KELİMELER: Ekmek, Kahve, *Saccharomyces cerevisiae,* Proteaz, invertaz, Selülaz, Amilaz

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LIST OF ABBREVIATIONS AND SYMBOLS

µg/mL : Microgram Per Milliliter

CMC : Carboxymethylcellulose

CO2 : Carbondioxide

DNS : Dinitrosalicylic Acid

HCl : Hydrogen chloride

I-KI : Iodine-Potassium Iodide

NaCl : Sodium chloride

nmol/mL : Nanomoles Per Milliliter

NU/mL : Normal Unit Per Milliliter

PCA : Plate Count Agar

S. cerevisiae : *Saccharomyces cerevisiae*

TCA : Tricarboxylic Acid Cycle

TMAB : Total Mesophilic Aerobic Bacteria

U/mL : Unit per Milliliter

ACKNOWLEDGEMENTS

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1. INTRODUCTION

1.1. Yeast

One of the former biochemical activities in the world is the bread making by fermentation of yeast. Saccharomyces cerevisiae (or baker's yeast) is the main dough raising agent by providing carbon dioxide in bread and other similar items. Fermentative activity is considered to be important in the bread making by improving surface, taste, flavor, structure, and molding. The effect of yeast modification and substitution by unique yeast strains in the bread- making process has gained considerable attention.

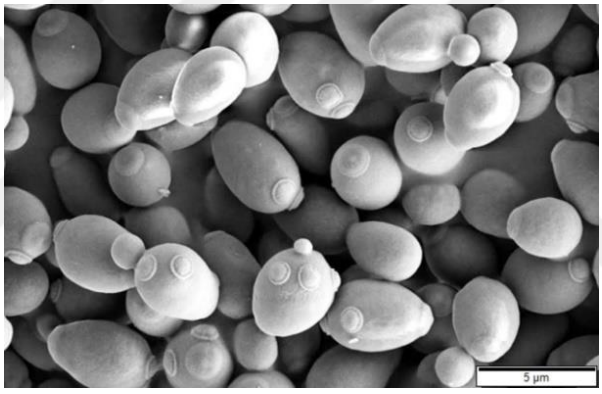

Figure 1.1: Yeast (*Saccharomycerevisiae*)
(https://en.wikipedia.org/wiki/Saccharomyces_cerevisiae)

During the last years, scientists are focused on the harvest period of S.cerevisiae at various physiological stages or the substitution of *S.cerevisiae* by lager yeasts. Brewer's yeast strains are famous to reach the best of flavor-related metabolite formation depending upon their metabolic activity. On the contrary, baker's yeast takes the responsibility of fast fermentative activity and uniform dough raising because of CO2 generation. Studies performed by Heitmann et al. (2015) confirmed the utilization of various S. cerevisiae strains and showed a great variation to the widely applied yeast in wheat bread. It was additionally discovered that quality parameters including the texture, structure and the aroma profile of bread by S. cerevisiae can be improved.

1.2. Bread And Crumb Texture Made up of Pores

Fermented bakery items contain a wide variety of food, including bread, buns, and cakes; are the most consumed ones throughout the world in the last 150 years. Although food items display identical chemical structure, diverse mechanical characteristics and sensorial properties are displayed relying upon their cell structure. Hence, the mechanical and sensory characteristics of the bread crumb that may be effective on consumer behavior are mainly associated with quality parameters of fermented bakery items. A single crumb particle was termed in previous studies as the texture of crumb of a bakery item subjected to leavening is cut into slices that can be mainly observed as a two-stage soft cellular solid, including the solid part, clearly identifiable by the cell wall structure and a fluidized part composed of air(Rathnayak et al., 2018). According to materials science, strong cell components can be distinguished mainly as open or closed cell foam (Rathnayak et al., 2018). Open cell structure containing food items composed of pores are associated with one another through an interconnected system that has been made up of pores that is respectively softer than the closed cell foam type. The cell foam is defined as closed cell structures that doesn't have interconnected pores bears greater compressive force because of their dense structures. A thorough investigation of various slices can show noteworthy differences in the cell properties even for a single sample with respect to the complex mechanical characteristics of the crumb. Therefore, a wide range of research has been performed since many years to be informed about characteristics and structure of the crumb, related to the mechanical and sensorial nature of the last bakery item (Rathnayak et al., 2018). The dough components, the conditions of the baking process, the activities of the yeast, fermentation temperature and gas bubble formation are the crucial parameters for obtaining a porous crumb structure. Flour, water, the agents of fermentative activity (either a leavening chemical substance e.g. $NaHCO_3$ or a living organism e.g. yeast), table salt, sugar, and shortening are essential agents that are mainly utilized for fermented bakery items. The processing of bread contains the following steps: kneading, fermentation of dough, proofing, and baking; which would allow the components of bread to turn into a well organized porous network. Both water and flour are the most important components that can influence the textural properties of the bread. Wheat flour, which was made up of two important proteins, called gliadin and glutenin, is the most intensively

utilized flour type for yeast and bakery - items. Viscoelastic gluten network is organized, when these two proteins join together, thus, this can keep the carbon dioxide not only at the fermentation, but also during baking as well, and when rehydration of this flour takes place, the yeast (*S. cerevisiae*) starts to consume glucose and CO_2 formation is seen.

The carbohydrate, mainly starch, may play a role as a tenderizing agent, sweetening agent and an extra fermentable substrate. Moreover, it displays a moisture retaining behavior; particular to the bakery products and it can lead to starch gelatinization and protein denaturation temperatures, that can improve an increase of the volume of the loaf while heating (Navaratne et al., 2018). The carbohydrate sources are utilized by the yeast cells without oxygen during fermentation (since dough formation is claimed to be anaerobic) to generate energy, as well as ethanol and carbon dioxide as the final products following a sequential flow chart, including various enzymes. The fermentation is significant, because of the generation of flavor substances.

The carbon dioxide produced may partially dissolve in the liquid phase, and afterward, a diffusion to the nuclei is seen while the dough was mixed, depending to the concentration difference of gas that leads to the changes of the dough structure, cause a modification in porous nature of the crumb. When carbon dioxide is made ineffective to the nuclei during the fluid phase, this nuclei may enlarge into gas cells and the density of the dough can be decreased when a small increase of pressure takes place.

The sugar fermentation process is defined as the Krebs cycle in scientific publications. All main carbohydrates can be metabolized by *S.cerevisiae* in dough during fermentation, examples include glucose, fructose, saccharose (sucrose), and maltose. Glucose and fructose are subjected to leavening right away. Just after the consumption of fructose and glucose takes place, the sucrose is degraded into glucose and fructose by the help of the amylase. This conversion takes place quickly, and following a couple of minutes of mixing the dough, whole sucrose has been degraded to glucose and fructose.

Maltoses can be also degraded into two glucose molecules by the enzymes of the yeast called ‘maltase’. The summarized reaction of dough fermentation can be pointed out as in the equation given below:

$$C_6H_{12}O_6 \longrightarrow 2C_2H_2OH + 2CO_2 + 234kJ$$

The total carbon dioxide quantity formed during carbohydrate fermentation corresponds to about 70% of the theoretical amount, shown by the equation above, moreover, the energy requirement for the yeast during reproduction within the dough is maintained by this much sugar. The expansion capacity of the dough is mainly provided by viscoelasticity during fermentation. The viscous part of the dough mass permit gas cells to expand to equalize the pressure, while the elastic part of the dough guarantees the suitable strength to avoid the dough from overexpansion and collapsing.

Heat and mass transfer occur at the same time during baking, leading to a physicochemical and textural change like water evaporation increase in volume starch gelatinization, and protein denaturation, providing a porous network. Generally, the protein denaturation and starch gelatinization take place at a temperature range between 60-85°C and lead to a transition from the dough to the crumb, thermal expansion of vapor takes place with the elevation of the temperature inside the oven and the saturation pressure of the water inside the dough gets higher. This leads to a volume increase of the loaf. As indicated by Hayman et al. (1998) the expansion of the loaf depends upon the increase in the volume of the product, during the first few minutes of baking, allowing to form a capable strain inside the dough that can compress the heat-related cellular structure formation of the outside texture of the bread.

Because of that; the cells residing at the external part can be elongated with their long axes parallel to the crust planes. Likewise, carbon dioxide has a significant role in the expanded bubble formation during baking, by its release from the dough, when the walls of the bubbles start to break by pressure, leads to a stable and porous structure, and open to the outside of the bread.

1.2.1. Bread Stailing

Bread staling is a complex period that is composed of aroma loss, changes in mouthfeel, crumb hardening, and crumbliness formation. It is a term which indicates decreasing consumer acceptance of bakery products caused by changes in crumb other than those resulting from spoilage organisms. It is usually characterized by loss of aroma, changes in mouthfeel, development of crumbliness, as well as toughening of the crust, firming of the crumb, loss of moisture and flavor and loss in product freshness (Besbes et al.,2016) .

The major cause of crust staling is believed to be the moisture redistribution. Water becomes adsorbed from the air or immigrates to the crust from the crumb, causing hydration of crust ingredient, resulting in a soft, leathery texture and is generally less offensive than is the crumb staling (Primo-Martin et. al, 2006). Crumb staling is more complex, more significant and less understood; the firmness of bread varies with position within a loaf, with maximum firmness occurring in the central portion of the crumb (Gray and Bemiller, 2003).

The crumb is a complex solid matrix composed mainly of gluten, starch, and water (Bollaín et al., 2005). Therefore, bread staling occur in the starch-protein matrix of bread crumb as a result of physical changes. Retrogradation is a process by which starch amylopectin returns to more regular state after gelatinization (Katina et al, 2006), therefore the main cause of bread staling is considered as retrogradation (Xie et al, 2004). The other affecting factor may be the proteins of wheat flour. Other ingredients of dough such as pentosans, lipids (D' Appolonia and Morad, 1981); emulsifiers, sugars and enzymes also affect crumb softness of fresh bread and shelf-life (Katina et al., 2006). Modifications occur in both the crumb and the crust of the bread. Researchers have probably used the largest extent of increase in crumb firmness in following bread staling. The other alteration, loss of flavor, decrease in water capacity of absorption, amount of soluble starch crystallinity and opacity, and changes in x-ray diffraction patterns have also been listed (D'Appolonia and Morad, 1981).

1.2.2. Starch In Bread Stailing

Starch is a glucose polymer that is linked to one another through the C1 oxygen, by a glycosidic bond and this glycosidic bond is stable at high pH but it can hydrolyze at low pH. Two types of glucose polymers are present in starch. These are amylose and amylopectin. When amylopectin is present, the regulation of starch granules is designed as amorphous, where amylase is present and crystalline.

While amylopectin can solve in water, amylose and starch granules are insoluble in cold water. When water and starch solution is heated, the granules began to a swelling process which is irreversible and called gelatinization. During this process, amylose leaches out the granule and causes an increase in the viscosity of the slurry. The continued rise in temperature direct to maximum swelling finally granules break apart and resulting in a complete viscous colloidal dispersion (van der Maarel et al., 2002).

During the transformation of dough to bread in bread making process, the increased temperature causes gelatinization of starch content of bread at the microscopic level. At the macroscopic level, baking includes the solidification of dough and change from a foam type system with gas cells to an open pore system. On cooling and aging of bread, rearrangements in starch structure cause a series of changes which are referred to as crystallization and gelation. This transformation is generally called as the starch retrogradation and this is accepted as the major cause of bread crumb firming which is referred to bread staling (Hug-Iten et al., 1999). During retrogradation, the starch is subjected to a transition from dissolved and dissociated state to associated stage and retrogradation is caused by the amylose content of the starch rather than amylopectin content, which has a highly branched organization that is prone to less retrogradation (Van Der Maarel et a.l, 2002).

1.2.3. Proteins In Bread Staling

It has been proposed that protein content is a crucial factor in the rate of bread staling, that protein (gluten) decreases the firming rate of bread while staling, the firming rate is not affected, and is required for firming, which means that staling relies upon starch – gluten interaction (Gray and Bemiller, 2003). Gluten is a mixture

of proteins that leads to a big network during dough baking. This system holds the gas in microstructures during dough proofing and baking. The yeast will raise the bread and bakery products by the strength of this gluten network and this is found to be important (URL 1). By heat application, the gluten is converted from a gel to a coagel by polymerization, which implies that the gel loses its cohesiveness. The starch granules which forms a network by partial gelatinization is the main component of the semi-rigid structure of the bread (Hug-Iten et al., 1999). During bread staling, the recrystallization of amylopectin results in increase in rigidity of the starch granule and a decrease in the flexible gluten matrix. All these modifications may have a role in leading to the firming of the crumb (Valjakka et al., 1994, Zobel and Kulp, 1996, Keskin, 2003).

1.2.4. Coffee

Studies have proved a high level of free radicals accumulation, that include oxygen and nitrogen reaction products in body fluids such as hydroperoxyl radical, superoxide anion, hydroxyl radical. Free radicals can result in aging and numerous disease by causing a major pathological change in the human body. The increasing of free radicals in cells is called oxidative stress. Antioxidant therapy is important for reducing oxidative stress with using up of natural antioxidants that include vegetables, fruits, berries, vegetable oils, honey, tea, coffee, cocoa, juices, wine, sprouted grains, and other foods. Coffee has high antioxidative activity, that can inhibit the oxidation also which has significant beneficial health effects in the daily diet of different people. Many investigations showed that the antioxidative properties of coffee are mainly due to chlorogenic, ferulic, caffeic, and n-coumaric acids. In coffees which are cooked by dry heat in an oven, synthesized melanoidins (brown pigments) have a strong antioxidant effect. During the roasting process, phenylalanines are formed that indicate excessive antioxidant activity (Yashin et al., 2013).

Guatemalan coffee is one of the most notorious coffees in the world. It is grown in Guatemala, a South American country. Atitlan and Antigua varieties are the most famous, providing a floral acidity, leaving spice and chocolate notes as a mouthfeel. Between December and April it is harvested and grown between 1200-

1700 meters above the sea level, and Guatemala is among the ten important coffee-producing countries (URL2 and URL3).

Kenya coffee is another important coffee variety consumed in many parts of the world, offering a distinct bright taste as well as complex fruit and berry tones. It is grown between 1700-1800 meters, harvested between November-December and providing bergamot, berry and lemongrass flavor with its fresh or floral aroma (URL4 and URL5).

Terebinth (*Pistacia terebinthus* L.) belongs to Anacardiaceae, mostly present in the south and western parts of the countries in Mediterranean basin including Turkey, is characterized as a turpentine tree fruit, is among a member of twenty *Pistacia* species. Turpentine tree exists in the shrubs and is mainly grown in pine forests or on hillsides. Its fruit is called çögre, çitlenbik, çıtlak, çedene, sakız ağacı, yabani fıstık, melengiç and menengiç (terebinth) in the folk language (Baytop, 1994). 100 g of terebinth fruits contain approximately 594 kcal, 20.8 g protein, 51.6 g fat, 16.4 g carbohydrate, 2 g crude fiber, 500 mg P, 136 mg Ca, 7.3 mg Fe, 1.02 mg K,158 mg Mg, 66 IU of vitamin A, 0.62 mg vitamin B1, 1.45 mg vitamin B2 0.4 mg vitamin B6,7 mg vitamin C and 5.2 mg vitamin E (Özcan, 2004). It is believed to cure different important disorders such as cough, eczema, asthma, diarrhea, ulcers, and arthritis, providing that it is mostly used in alternative medicine as a side treatment agent (Bonsignore et al., 1998). In addition, it finds its use in manufacturing cookie and bakery products as well as cooking oils. Moreover, fruits are used in the production of 'bıttım' and turpentine soap and also in different seasonings and spices. Also, terebinth fruits are processed in roasted form as the terebinth coffee providing an extremely attractive color and smell to the consumer. In addition, this coffee is among the intensively preferred traditional coffees in Turkey and is generally cooked in milk (Matthäus and Özcan, 2006).

Black cumin seeds are favorable for use in coffee, tea, salads, and bread. Seeds are shown to be effective against therapy for asthma hypertension, diabetes, inflammation, cough, bronchitis, and influenza. According to an investigation on antioxidant activity (Burits and Bucar, 2000; Luther et al., 2007); and antimicrobial activity, the results confirmed that black cumin oil is rich in terms of linoleic and

oleic acids as well as sterols and tocols (Hassanien, M. F.R et al.,2014) Black cumin seed coffee, native to Turkey has been reported to be produced around Denizli and

Burdur provinces, made by roasted black cumin seed and chickpea, mixed by using a mortar and pestle; and ground by a grinder to obtain its final powder form (URL6).

It is believed to help cure some diseases as a side therapeutic agent, including asthma, heart diseases, certain cancers, mostly by its antioxidative, antiallergic and antimicrobial properties (URL7)

Carobs have been intensively used in food, pharmaceutical, and cosmetic industries. A considerable amount of sugar and a comparatively low level of fats and protein have been present in carob pulp. But lower sugar and more fat content have been found in seeds by comparison with the pulp. According to an investigation, carob pods have been suggested as an alternative source of mineral because of having a high content of minerals such as calcium, phosphorus, and potassium (Ayaz et al., 2007). Also, carobs have antioxidant activity because of the content of polyphenols and another group of compounds which is important for health(Ioannis, 2018). In the Meditteranean basin, including Turkey, Greece, Turkish Republic of North Cyprus and Greek Administration of Cyprus, carob fruits are used in ground form as coffee. It is said to be caffeine free and naturally sweet (URL8) antioxidative, treating diarrhea and expectorant (URL9) side treatment agent in asthma, as well as containing calcium and potassium (URL10).

1.3. The role of enzymes in bread making process

Enzymes used in bakery products are mainly related with bread staling and help extension of sensory properties of these items throughout their shelf life, by acting on main functional flour components (Gimenez et al., 2005). As a result, different types of enzymes, such as amylases, xylanases, lipases, proteases are preferred in bakery items.

1.3.1. α-Amylases

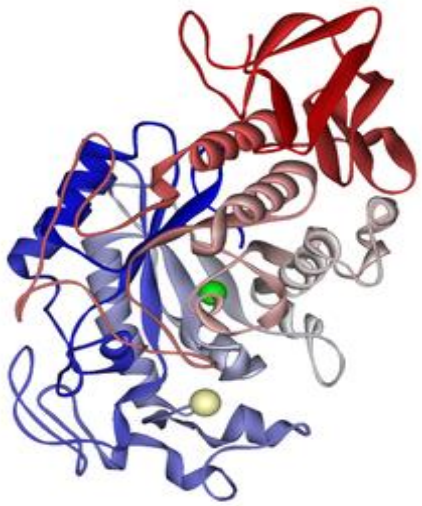

Figure 1.2. Alpha-amylase, (α-amylase)
(https://en.wikipedia.org/wiki/Alpha-amylase)

Amylases are significant for improving dough-handling properties and enhancing bread quality in the bread making industry. Therefore the addition of α-amylase not only showed a substantial anti-staling effect but also improved the elasticity of bread crumb (Kim et al.,2006).

The general point of adding α-amylases to flour helps generating substances available for fermentation, mainly maltose in the dough. In the other hand some other changes, such as increased bread volume, improved crumb grain, crust color, and flavor may also exist (Sahlström & Brăthen, 1997).

The influence of α-amylase on the staling bakery products was originally thought to result from a modification to the structure of the starch. Since α-amylase is an endoenzyme that hydrolyzes the α-1,4-glucosidic bonds in a random fashion along the chain, it hydrolyzes amylopectin into oligosaccharides that are composed of two to six glucose units. This hydrolytic reaction, at the same time, results in a rapid decrease in viscosity, but a few monosaccharide generation. Thus a mixture of amylase and amylopectin will be hydrolyzed into a mixture of dextrins, maltose, glucose and oligosaccharides (deMan, 2004). Depending on the suggestions in literature dextrin production by α-amylases persist in the baked bread which may give a different dextrin distribution that in control of loaf volumes.

The α-amylases which are commercially used in bakery items are originated from cereals, fungi, and bacteria. These enzymes differ each other in their temperature optima, the fungal enzymes having the lowest (50-60°C) and bacterial the highest (70-80°C) (Hamer, 1992 in Sahlström & Bråthen, 1997). In general, fungal α-amylases are used in the baking industry, because of their thermostability and lower risk for overdose. In fact, bacterial α-amylases should be carefully dosed for the reason that overproduction of dextrins may be observed. This would result in a sticky bread crumb (Zobel and Kulp, 1996; Poutanen, 1997; in Keskin, 2003).

1.3.2. Cellulases

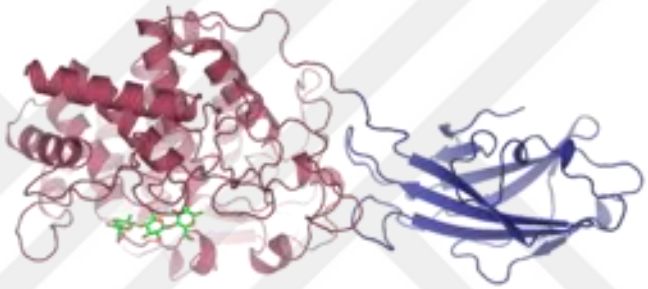

Figure 1.3. Cellulase produced from *Thermomonospora fusca* (https://en.wikipedia.org/wiki/Cellulase)

Cellulose is comprised of glucose units connected by β-1,4-glycosidic bonds in a linear arrangement. The distinction of bond structure and the highly ordered crystalline type of the compound among starch and cellulose increase the resistance of cellulose to digestion and hydrolysis. The enzymes involved in the hydrolysis of cellulose are listed as endoglucanases, exoglucanases, and β-glucosidases. While cellulase is endoglucanase that hydrolyzes cellulose arbitrarily, produce oligosaccharides, cellobiose, and glucose, exoglucanase hydrolyzes β-1,4-D-glycosidic linkages in cellulose discharging release cellobiose from the nonreducing end (Haki and Rakshit, 2003). Cellulases/hemicellulases cut non-starch polysaccharides present in the flour. This influences the water maintenance and water binding capacity, viscosity, and proofing (rising) capacity of not only the dough but also the texture, aroma, taste, and freshness of the bread. In general, the utilization of cellulases/hemicellulases gives an improved oven spring to the dough

and an improved bread volume, grain structure and anti-staling properties to the bakery item.

1.3.3. Proteases

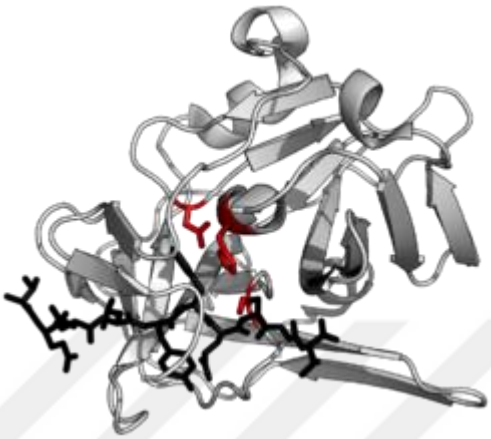

Figure 1.4. The structure of Protease (https://en.wikipedia.org/wiki/Protease)

In general, proteases break down the gluten protein which makes the dough softer and extensible. The purpose of adding proteases to bread is to improve flavor profiles, flow characteristics, machining properties, gas retention, and mixing time (Gray and Bemiller., 2003). Also, protein has a significant effect on bread staling mechanism; the activity of proteases may affect this mechanism via some modifications in gluten network. These modifications can be explained as the cleavage of peptide bonds by releasing water which has been bound to gluten; thus viscosity of dough is reduced.

1.3.4. Invertase

Invertase is an enzyme that catalyzes the hydrolysis (breakdown) from sucrose to fructose and glucose that is an S-bD-fructofuranosidase (systematic name: beta-fructofuranosidase) and is usually derived from *Saccharomyces cerevisiae* and other microorganisms such as bees, which use it to make honey from nectar. One of the invertase's different applications is that can make the manufacture of inverted sugar. Inverted sugar is sweeter than sucrose and it is important in industry and suitable to use in biotechnology. The best activity is observed between 3.0 and 5.0 in terms of pH and temperature between 40° and 60°C. When invertase-S has applied at a rate of 0.6% in a solution of sucrose 40% w/w at 40°C, invertase-S reverses 80% of sucrose approximately after 4 h. and 20 min. When invertase is added to banana juice for assessing its sweetness potential by Cardoso et al. (1998), an increase in juice viscosity is observed in addition to an increase in sweetness. Some microorganisms have an important role in potential applications in various industrial sectors. Certain filamentous fungi are one of the good producers of invertase. The filamentous fungus *Rhizopus spp*. in wheat bran medium is cultivated by Gould et al. (2003) and invertase obtained was characterized in polyacrylamide gel. Commonly known *Aspergillus casiellus* is another potentially producing fungus invertase. *Aspergillus casiellus* was inoculated in soybean meal medium, after 72 hours its raw extract, containing invertase was isolated (Novak et al., 2010).

2. AIM AND SCOPE OF THE STUDY

The aim and scope of the study are to understand the possible action of the different coffee grains on the growth and the yield of enzyme secretion of *Saccharomyces cerevisiae* before its use on bread making, therefore the retardation of bread staling is expected by the use of these enzymes.

3. MATERIALS AND THE METHOD

3.1 Materials

3.1.1. Coffee

Five different coffee types (Guatemala, Kenya, Terebinth Berry, Cumin and Carob Coffees) were purchased from a local herb market, in the granulated form (Egyptian Bazaar (Mısır Çarşısı), Eminönü, Istanbul, Turkey).

3.1.2. Microorganism

Yeast was given as a generous gift from Pakmaya A.Ş. Cumayeri, Düzce, Turkey.

3.1.3. Flour

Ziyabey-98 wheat originated flour (Efsane, Turkey), purchased from a local market, was used for bread making.

3.2. Method

3.2.1. Microbiological analysis

1 g, from five different types of each coffee was mixed with an initial population of 10 mL of 1.5x 10^8 CFU/mL yeast solution added to 89 mL yeast extract broth, in Erlenmeyer flasks (Sigma-Aldrich, St.Louis, USA) and incubated for a period of 24-48 hours at 30° C in an incubator (Nüve, Turkey). A control flask was prepared with 1 mL distilled water instead of one gram from each coffee and grown at the same organism, growth medium volume, time and temperature conditions. The yeast content was initially adjusted to 1.5×10^8 CFU/mL by an Mc

Farland densitometer (Biosan, Latvia) and then centrifuged at 4000 rpm for fifteen minutes and microfiltered through 0.45 μ filters (Sartorius Stedim Minisart 16555-K). The microfiltered extract in treatment groups was used in bread making as 0.1, 0.2 and 0.4 mLs.

3.2.2. The microbial counts

Three different parts of bread were transferred into each treatment group by a sterile tweezer as one gram, and put into the test tube which contained 9 mL of distilled water and mixed thoroughly by a vortex. Subsequently, 100 μL from this mixture was inoculated onto the plate count and yeast extract agars for the initial enumeration (0^{th} day). After the inoculation, the Petri plates were kept for a period of 48 hours at 37° C in the incubator. Serial dilutions were made in five different test tubes and 10^4 and 10^5 dilutions were used during the enumerations. The mesophilic aerobic bacteria (MAB) counts were carried out on the PCA (plate count agar)(Merck, Darmstadt, Germany). All the microbiological analysis were done in the appropriate agars in triplicates. The analysis was repeated at the 4^{th} and 8^{th} days.

3.2.3. Bread Making

For this purpose, 300 g flour was mixed with 190 mL natural spring water (Erikli) and 1.8 mL yeast (Pakmaya) at ambient temperature. No additives and salt were used. The loaves were adjusted to 25 ± 0.2 g including the microfiltered extract in treatment groups was used in bread making as 0.1, 0.2 and 0.4 mLs with respect to control, containing no treatment group. The loaves are baked at 170° C in a conventional oven (Arçelik) and the bread was kept in a refrigerator at $+4^{\circ}$ C in order to detect its shelf life.

3.2.4. Water holding capacity

Water holding capacity was determined according to the method of Gould and others (1989). Three grams of bread sample (dried in an incubator) was weighed into a centrifuge tube, completed to 30 mL by distilled water and mixed via a vortex

(Vortex V1 Plus, Boeco, Germany) for 30 seconds. The example was allowed to hydrate for 2 h at room temperature.

Subsequent centrifugation followed this process, by a bench top centrifuge at 2800 rpm for 10 min. The supernatant was discarded and the hydrated sample was weighed. Results were expressed as :

$$\text{Water holding capacity} = \frac{\text{Weight of hydrated sample(g)} - \text{the weight of dry}}{\text{Capacity}\left(\frac{\text{g}}{\text{g}}\right)\text{weight of the dry sample(g)}}$$

3.2.5. Oil holding capacity

Oil holding capacity was determined according to the method of Caprez and others (1986). Three grams of bread (dried in an incubator) was weighed and completed to 30 mL by corn oil, and mixed by a vortex blender (Vortex V1 Plus, Boeco, Hamburg, Germany) for thirty seconds. The sample was kept at a period of one hour at room temperature. This was followed by centrifugation at 2800 rpm for ten minutes. The supernatant was discarded and the pellet was weighed. Results were expressed as:

The results were expressed as :

$$\text{Oil holding capacity} = \frac{(\text{Residue} + \text{Filter})(\text{mg}) - \text{Filter (mg)}}{\text{Sample volume(mL)}} \text{ x } 1000 \text{ (mL/L)}$$

3.2.6. Total Suspended Solids

In order to measure the total suspended solids content of bread, an amount of 25 g bread sample (dried in an incubator) was weighed. The sample was first weighed and placed into a glass beaker. Firstly 500 mL distilled water was added to the beaker, mixed and boiled approximately for 5 – 10 minutes. A filter from the base was placed at first, then carefully removed and dried at least one hour at 103-105^{O}C. The dried filter paper was weighed. A 0,45 µm membrane filter was placed into glass funnel. All traces of water was removed from the filter and hot water was

subsequently added to filter paper after the filtration of water. The filter part was removed from the membrane filter paper and dried in an incubator at 103-105 ºC for one hour.

3.3. Enzyme assays

3.3.1. Invertase

In order to measure the invertase activity, 0.1 mL yeast cell suspension, 0.2 mL 10 mM sodium acetate buffer (Merck) (pH 4.6) and 0.7 mL of sucrose solution were mixed to reach a total volume of one mL, having a final concentration of 0.4 M. The reaction mixture was then incubated for 30 min. at 35° C. The reaction was then stopped by adding 0.1 M Na_2HPO_4 solution and subsequently transferred to a boiling water bath for 10 min. 0.02 mL of this mixture was diluted by an appropriate amount of distilled water to increase volume to 0.50 mL. The absorbance was recorded at 560 nm (Gajjar et al., 1997)

3.3.2. Amylase

For assay of the enzymic activity in the usual aqueous medium, 1.0 mL yeast cell suspension in 0.1 M sodium acetate buffer (pH 4.8) and 1.0 mL starch solution composed of 10 mg/mL in 0.15 M NaCl was incubated at $^{\circ}$C for 30 min. The reaction was stopped by adding 1 mL of 6N HCl. In order to estimate the unhydrolyzed starch, 1 mL was transferred to a 25 mL volumetric flask and 15 mL water was added followed by 0.5 mL I-KI solution (0.2% 12 in 2% KI). Volume was made up to 25 mL with water and absorbance was read at 660 nm (Gajjar et al.,1997)

3.3.3. Protease

The protease activity was measured essentially by Anson-Hagiwara's method (Shimogaki et al., 1991) To the reaction mixture (3 mL) containing 0.6% casein and 10 mM borax-NaOH buffer (pH 10.5), 0.5 mL of enzyme solution was added at 35°C. After incubation for 10 min., the reaction was stopped by the addition of 3.2 mL of a stopper solution consisting of 0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid (TCA mixture). The reaction mixture was kept at 35°C for 10 min. following a subsequent filtration and the absorbance of the filtrate was measured at 275 nm.

3.3.4. Cellulase

The cellulolytic activity of anaerobic rumen fungi was determined by the dinitrosalicylic acid method (DNS). For this purpose, the isolates were incubated at 39°C four days in M10 medium containing carboxymethylcellulose (CMC) at an incubator. After the incubation period, 1 mL of medium was centrifuged at 4000 g for 15 minutes and 0.5 mL of the supernatant was used as an enzyme complex containing solution and placed into a glassware test tube. The enzyme solution was mixed with 1.8 mL 50 mM Citrate-Phosphate buffer (pH=6.8); and 10 mg AVICEL (MERCK) and the reaction was stopped by adding 3 mL DNS to the enzyme and buffer solution, incubation was done in a water bath at 50 °C for 30 minutes. The absorbance was determined by a spectrophotometer at O.D. 550 nm (Willis et al., 2010)

3.3.5. Standard Preparation In Enzyme Assays

The activities of the enzymes were determined according to the appropriate standard curves (Appendix 1,2,3,4). In order to determine the activity of protease, tyrosine standards, ranging from a concentration of 0-110 µg/mL were prepared and the absorbance was read at 275 nm. To determine the activity of invertase, ranging from a concentration of 0-10 nmol/mL glucose standards were prepared and the absorbance was read at 275 nm. Cellulase activity was determined according to the standards of pure cellulase (Sigma Aldrich) ranging from 0-0.35 U/mL. Alpha-

amylase activity was determined according to the standards of pure amylase ranging 0-2.5 U/mL. 3.4. Texture analysis of bread

The texture analysis of bread was performed by texture analyzer (T.A: HD plus) and parameters including hardness, fracturability, (g) adhesiveness, (g.sec), springiness, cohesiveness, gumminess, chewiness, resilience was measured.

3.4. The statistical analysis

The data were demonstrated as means and analyzed by the SPSS program. One-way ANOVA-Tukey test was used to detect the significance between treatment groups, dilutions and process times of each treatment group. The significant level was set to $p<0.05$ at the beginning of the study.

4. RESULTS AND DISCUSSION

Among the five coffees tested (Guatemala, carob, black cumin, Kenya and terebinth berry) in this study, the yeast grown in Guatemala coffee containing medium was found to show a high protease activity, so a solution that is believed to have a protease enzyme activity was obtained from this yeast, grown in the medium mentioned above by centrifugation and microfiltration reported in Section 3.2.1. was added to bread reported in Section 3.2.3. and the microbial quality of these loaves was investigated. The microbial analysis was carried out by enumerating the total mesophilic aerobic bacteria, yeast and mold by using appropriate agars. The possible differences and similarity between the groups, dilutions, and concentration were analyzed by ANOVA-Tukey test (A.5). According to A.5. a significant difference was found between the groups, the dilutions, the groups-concentrations and groups-dilutions and concentrations of control, 0.1, 0.2 and 0.4 mL enzyme containing bread($p<0.05$). No significant difference was found between the others ($p>0.05$) According to the same table, the concentration showed no effect on the microbial analysis ($p>0.05$). In this study, results showed that groups and dilution are important factors for microbial analysis. Days are set as a determinant for significant alteration in microbiological analysis.

A.6 indicated the differences and similarities between groups.In our studies, the shelf life of bread, kept in sealed transparent nylon bags, was found to be eight days in the refrigerator, since total mesophilic aerobic bacteria and yeast were started to reproduce in eight days' time according to the results. A.6. showed the comparison between the initial and the eight days' growth of yeast and total mesophilic aerobic bacteria and it was found that between the initial (0) and eight-day counts of yeast a significant difference was found($p<0.05$). In addition, between the fourth and eight-day counts of yeast, a significant difference was found as well ($p<0.05$). These results pointed out that, rather than the concentrations of enzymes and the dilutions, the counts obtained at 0, 4 and 8^{th} days between yeast-total mesophilic aerobic counts showed a significant difference($p<0.05$).

A.7. showed a multiple comparison, an increase of yeast and total mesophilic bacteria was observed in eight days' time according to the results. If the results of the

TMAB-0 days' is compared with the other groups, significant differences are found between yeast 8 days' and TMAB-8 days' ($p<0.05$). Four-day counts of total mesophilic aerobic bacteria have significant differences in respect to eight days of yeast and total mesophilic bacteria ($p<0.05$). In addition, most differences were observed on 8^{th} days between groups of 0^{th} and 4^{th} days of yeast and total mesophilic bacteria. According to results, considerable differences were not observed among these groups.

According to ANOVA-Tukey test (A.8), when the groups were compared within each other, a significant range of 0 and 4 days of yeast and total mesophilic bacteria has appeared. A similarity between 8 days' counts of yeast and total mesophilic bacteria were resembled each other according to the significance range. Consequently, TMAB and yeast reproduction level in 8^{th} days was more than 0^{th} and 4^{th} days. But according to the results, when compared with the total mesophilic aerobic bacteria, the counts of yeasts were found to be higher.

Coffees, other than the green beans variety, serving as different substrates were used during fermentation by yeast starters in order to convert their aromatic characteristics and volatility in different studies. Research carried out by Lee et al. implied that the scientists tried to change the volatility and aroma profile of roasted coffee through the fermentation of green coffee beans by using a different aerobic yeast species, *Yarrowia lipolytica*, assessing the impacts of *Y. lipolytica* fermentation on the volatile and nonvolatile characteristics of green coffee beans. After fermentation, the number of carbohydrates was decreased by 1.2- folds while the amino and phenolic content by 1.3-folds, demonstrating that the fermentation of *Y. lipolytica* of green coffee beans leads to the change of the aromatic compounds and volatility profile of green coffee beans (Lee et al., 2017).

In our study the experiment was based on the microbial and physicochemical assays of bread, so no taste panel was carried out to understand the contribution of coffees to bread taste by yeast fermentation. The yeast can serve as a starter culture, not only to ferment the coffees but also they can induce the enzyme activity which may influence the flavor and retardation of staling of bread.

The worldwide distribution of microorganisms can display its effect on human activities. The best example is the yeasts, which are involved in the winemaking process. The selection of feasible fermentation products lead to the formation of particular strains of *Saccharomyces cerevisiae* that were transported along with grapevines. Today, *S. cerevisiae* strains, present in vineyards worldwide, are hereditarily identical and they show a common ancestor that pursued the way of human migration like wine, coffee, and cacao related to microbial fermentation and have been scattered by human beings to worldwide. Human-related fermentation and relocation may have influenced the circulation of yeast associated with the production of cacao and chocolate (Ludlow et al., 2016). Yeasts related to cocoa and coffee beans are hereditarily distinguishable. These populations have been formed through the relocation and blending of populations related to vineyards, trees in America, and the ancestral seat of these species in Far East Asia (Goddard, 2016).

According to these studies, in our research the yeast can perform well, growing together in the medium containing different types of coffees. This is mainly related to the increase in the enzyme activity induced very after the confluent growth of yeast in the growth medium. Likewise, the studies conducted in our laboratory, novel strains may be isolated and this may promote the research and development studies carried out by yeast and bakery product production plants.

The dynamic of *Saccharomyces cerevisiae*, *Pichia kluyveri*, and *Hanseniaspora uvarum* during the native and starter-added fermentations of cocoa fermentation and their impact on sensory properties of chocolate were analyzed. Yeast populations were analyzed by qPCR. *S. cerevisiae* was found to be dominant during native (normal 5.4 logs cell/g) and starter yeast culture added (normal 7.2 logs cell/g) fermentations. The growth of *H. uvarum* was found to be prevented by the other two yeasts, as it demonstrated a population similarity (roughly 4.0 logs cell/g) even in the starter yeast culture added experiments. The carbohydrate concentration was quickly decreased (68% and 42%) in the starter added group with respect to the control at 24 h. Ethanol content was increased in the starter yeast culture added (83 g/kg at 48 h) than in the control (4.6 g/kg at 96 h) fermentation. No any significant preference was reported by consumer groups for the chocolate ($p < 0.5$). Therefore, according to the consumer attributes, notable differences were present between the

flavor traits, on the contrast the starter culture added group containing coffees were defined as strong and sour. This is the first run of qPCR, it has been utilized to evaluate the dynamic of yeasts during the complex fermentation of cocoa beans. The addition of starter culture yeasts facilitated this process. *S. cerevisiae* and *P. kluyveri* lead to the formation of coffee, sour and bitter flavors to the yeast starter culture added chocolate (Batista et al., 2015). This study inspired us to use the different coffee types in bread making since yeast is the dominant organism in bread making.

Actually, the utilization of coffee waste can be a different choice for the suitable growth of yeast leading to an appropriate growth medium component, revealed by (Perez-Sarinana et al., 2015). This might be understood from the bioethanol production demonstrating that the waste can also help fermentation essential for a maximized yeast efficiency. In order to provide ideal conditions for bioethanol generation, coffee mucilage (CM) was added as the substrate to *Saccharomyces cerevisiae* NRRL Y-2034 growth medium. CM is a waste product obtained during coffee fermentation, principally made up of simple carbohydrates; the product yield and efficiency process were investigated with respect to the fermentation, as well as the pH, temperature, and the initial carbohydrate fermentation. Providing the predicted ideal operational conditions resulted in the most elevated bioethanol production: pH 5.1, temperature 32 degrees C, and initial carbohydrate concentration of 61.8 g/L. The estimated amount of bioethanol was 15.02 g/L, and the experimental amount formed was 16.29 g/L +/ - 0.39 g/L, with a bioethanol yield of 0.27 g/L and a production process of 0.34 g/Lh. Glycerol was found to be the main side product of the *S. cerevisiae* fermentation. The yeast culture added fermentation process requires optimization to completely exploit the capability of the strains and increase the production of bioethanol (Perez-Sarinana et al.,2015).

Another study of yeast interaction with coffee fermentation was proposed by Pereira et al. (2014) was also in line with our study, allowing the promotion of yeast growth when used with coffee pulp as well. This promotion is best explained with increasing the enzyme activity, especially with pectinases. On contrast, our study showed an increase in protease activity, indicating the difference of coffee types between the studies. No detailed study was carried out about the ingredient comparison of the well-known world coffee types. Since the protease activity is

increased, either yeast grown in Guatemala coffee containing medium or some amount of this coffee may be added to the dough while bread making to observe the gliadin and glutenin reduction in another extensive study, in order to provide a potential for observing these results on gluten, therefore celiac patients as well.

The coffee fermentation is characterized by the presence of different microorganisms belonging to the groups of bacteria, fungi, and yeast by another research group. A study carried out by Silva et al. (2013) was focused on the selection of pectinolytic microorganisms isolated from coffee fermentations and evaluate their performance on coffee pulp culture medium. These studies are in line with our study, as since the enzyme activities may have a strict dependence on the coffee fermentations of yeast.

Wheat proteins can be balanced with supplementation of dried yeast; however, the dramatic increase in protein biological value was accompanied by the impairment of dough or pasta quality. These negative findings might be the result of high proteolytic enzyme activity of yeast. To confirm this hypothesis, the effect of baker's yeast proteinases(proteases) on gluten at two incubation temperatures was studied at 30° C and 50° C respectively after 10, 30 and 60 minutes of incubation time. The reaction was followed up by determination of protein solubility. Results showed a significant increase in protein solubility at both temperatures. The effect of yeast on S-S bond content was determined from dough samples after 30, 60 and 90 min. incubation. After 60 min. significant increase of S-S bonds occurred, this drastically decreased after the extension of leavening time. As the main proteinase activities of baker's yeast are influenced by propagation parameters (glucose content and aeration intensity) while high proteolytic level causes gluten degradation, this should be taken into consideration in the yeast production (Halasz and Barath, 2006). In our study protease enzyme activity was found to be the highest in Guatemala coffee when compared with other types of coffee. For this purpose, this variety was selected in the bread making process.

Different wheat bran based fiber components that had treated with chemicals or enzymes were tested at 10, 15, or 20% flour substitution levels in a sponge and dough formulated bread (containing 2% added gluten) under commercial baking conditions. Mixing and baking characteristics were identified. The fiber compounds

were found to be ranging between 52 to 78%, expressed in neutral detergent fibers (dry weight basis). A commercial wheat fiber ingredient and white wheat bran were served as controls. The water holding capacity, a proximate examination of the fiber components and the effect of hydrogen peroxide treatment on the color of the treated bran components were also analyzed. The components, with the most noteworthy neutral detergent fiber content, were obtained via an alpha-amylase treatment or a blend of alpha-amylase and protease treatments. Protease treatment yielded components with a relatively poor heating characteristic. The bread containing bran treated with either alpha-amylase or alpha-amylase/calcium oxide had the best crumb grain score of the fiber components that were experimentally analyzed (Rasco et al., 1991). In our studies, 0.2 mL solution which is believed to indicate protease activity, was found to provide a soft bread texture with respect to 0.4 mL solution and the control.

According to the research carried out by Renzetti and Arendt(2009), brown rice batters were treated by protease enzyme and its effect was analyzed on the textural and baking characteristics of bread. When the enzyme was added to bread, not only a significant specific volume increase ($p < 0.05$), but also a decreased crumb hardness and chewiness was observed. The fundamental rheological and viscometric analysis indicated that the hydrolytic action of the enzyme on dough proteins increased initial viscosity, while no change in the phase angle value was observed. The paste viscosity and breakdown were significantly decreased when flour pasting characteristics were investigated ($p < 0.05$). When the nature of the proteins of the batters was considered, the protease showed its effect by induction of the release of low molecular weight proteins from macromolecular protein complexes. As a result, the performance of bread making is found to be strongly related with the lowered resistance to deformation of batters not only at proofing period but also at the initial phases of baking, moreover, an increase in paste stability and the maintenance of elastic

Table 4.1. The results of the enzyme activities of the treatment groups (Protease, amylase, cellulase, invertase)

Treatment groups	Protease activity (µg/mL)	Amylase activity (NU/mL)	Cellulase activity (U/mL)	Invertase activity (nmol/mL)
Control	125.7	3.461538462	0.00125	0.01
Guatemala coffee	134.4	3.583638584	0.0005	0.04
Kenya coffee	123.5	3.705738706	0.0005	0.08
Carob coffee	125	3.583638584	0.01625	0.04
Black cumin	125.4	3.583638584	0.000025	0.04
Terebinth berry coffee	121.2	3.705738706	0.000625	0.04

When the enzyme activity was measured. protease was found to be slightly higher in Guatemala -added extract with respect to control. but on the other hand. a slight decrease was observed in terebinth berry. Kenya coffees, and black cumin coffee. In a study conducted by Kandasamy et al. (2016), new sources of the substrate from agro-industrial waste for protease production by Bacillus spp. was investigated on coffee pulp waste (CPW) substrate. A maximum amount of protease production (920 U/mL) was observed after 60 h of incubation with 3.0 g/L of CPW at pH 8 and temperature 37 degrees C. Rodarte et al. (2011) studied the potential of the proteolytic activity of 144 microorganisms, isolated from coffee fruit (*Coffea arabica*) and grown in casein agar. Fifty percent of filamentous fungi, 52.5% of bacteria and 2.6% of yeasts were able to secrete proteases and it was found that a yeast isolate, *Citeromyces matritensis*, had a proteolytic activity of 2.40 U at pH 5.0. According to Dias et al. (2008) the protease production potential of two strains of Bacillus (*B. subtilis* ATCC 6633) and a wild type (Bacillus sp. UFLA 817CF) isolated from coffee fermentation in the south of Minas Gerais, Brazil, were screened out by growing them on nutrient broth, nutrient broth with sodium caseinate and nutrient broth with three different concentrations of cheese whey powder for 72

hours. The specific activity of the protease was, respectively, 839.8 U/mg for *B. subtilis* ATCC 6633 and 975.9 U/mg for Bacillus sp. UFLA 817CF. Bacillus sp. UFLA 817CF showed highest enzymatic activity at pH 9.0 and 40 degrees C and it was found to be stable at pH 7.0 and considerable heat stability was achieved at 40 and 50 degrees C, and due to this, it can be considered as an alternative for the industry to utilize cheese whey to produce proteolytic enzymes. It was found that, in general, protease activity was enhanced by the addition of coffee fruits, pulps or even its waste in the growth medium of microorganisms in the other studies. This was mainly due to some other factors, such as pH, temperature, salinity, oxygen content of the media. This study has implied that no such great change was observed in the protease activity by using different coffee types in *S. cerevisiae* growth medium.

The results (A.9.) showed that 0.2 mL solution containing enzyme complex added bread was found to be the best group with respect to 0.4 mL enzyme complex containing group and the control. Addition of 0.2 mL solution which is believed to have protease enzyme complex indicated a slight decrease in hardness, gumminess, and chewiness with respect to 0.4 mL and control group, providing a softer crumb, indicating the retardation of the staling in bread, mostly due to the degradation of cell wall components. But in this study, a professional sensory analysis was not carried out by trained panelists.

On the other hand, salt was not added to the dough in our experiment. Salt was reported as a strengthening agent for gluten (Tuhumury et al., 2011). Also, it can negatively affect the activity of yeast and decrease gas forming ability (Tuhumury et al., 2014).

Endogenous and added proteins are known to display a noticeable effect on the quality of bakery products. Microbial catalysts are intensively preferred to help the baking process and to accomplish improved, uniform and qualified bakery products. Amylases and proteases have been utilized in bread and bread products for a long period of time, in addition, the enzymes acting in cell wall degradation has been lately introduced to the same field of interest. In spite of the fact that enzymes show their activity at a molecular level, they can be able to form noticeable changes in both the microstructure and the functional characteristics of bakery products. The commercial enzymes commonly contain tailored enzyme forms in which different

enzymes display a consortial behavior. The misuse and control of enzymatic catalysis in bread making display special importance on the influence on bread quality (Poutanen, 1997). From this viewpoint, the solution that is found to have a high protease activity may be widely investigated later on.

Currently, the utilization of enzymes is regarded as a common practice in bread production, as they can improve dough quality and the texture of the bakery item. However, the utilization of alpha-amylases could negatively affect the glycaemic load of the item, because of the released carbohydrates from the starch hydrolysis that are not utilized by yeasts during fermentation. A research carried out by Sanz-Penella et al. in 2014, assessed the effect of the addition of alpha-amylase in bakery product items with the bran on in vitro kinetics of starch hydrolysis. The utilization of flour with a high level of extraction or high bran content could diminish the glycaemic index (GI) even with the consideration of alpha-amylase in bread formula. It should be considered that the amount of bran and alpha-amylase is said to be important during the formation of bread in order to produce bakery items with lower GI than white bread. However, the approach that energy of starch hydrolysis remained unchanged demonstrates that the involvement of alpha-amylase in bread production could allow technical advantages, increasing quality of bakery products without markedly changes in their glycemic index (Sanz-Penella et al.2014).

Chemical leavening agents are utilized in doughs to produce carbon dioxide, instead of yeast, in producing a variety of bakery items. The alpha-amylase isolated from fungi and ascorbic acid was tested on chemical leavening agent added bread via looking up the characteristics such as dough extensibility, true rheological properties, the amount of free liquid in doughs following ultracentrifugation and the quality of baked products by Patel et al(2012). In line with the yeast containing batters, the baking characteristics of the bread which were made by chemical leavening agents was improved by using fungal alpha-amylases too. The baking characteristics were not influenced when an equivalent amount of ascorbic acid was included in the dough. Different dough formulation was easily found out from the detection of true rheological properties, but not from extensibilities of doughs. The free fluid content was bigger and of lower viscosity in doughs where alpha-amylases were added. The characteristics of the continuous liquid phase were found to be important in

characterizing the rheological and baking characteristics of doughs (Patel et al., 2012).

In this study, no chemical leaveners were used since these might affect the solutions that are believed to have enzyme activity either synergistically or antagonistically

Table 4.2. The results of the physicochemical analysis (dry weight, water, and oil holding capacity.

	Dry weight(g.)	**Water-holding capacity(V/V)**	**Oil-holding capacity(mg/mL)**
Control	0.958	11.856	5.79
0.1 mL solution which was considered to contain enzymes.	0.992	17.841	4.141
0.2 mL solution which was considered to contain enzymes.	0.977	9.434	4.33
0.4 mL solution which was considered to contain enzymes.	0.977	13.389	5.712

When the dry weight of the bread samples was measured, a slight increase was observed in the treatment groups with respect to the control. 0.2 and 0.4 mL enzyme solution containing groups showed no difference among them. If the water holding capacity is compared between groups, the best result was said to be the 0.2 mL enzyme complex, indicating a slight decrease with respect to the other groups. According to Table 4.2. water holding capacity was found to be slightly decreased in 0.2 mL solution added to group, while no such big difference was observed between the oil holding capacities of the groups.

5. CONCLUSIONS

It was observed that between the treatment groups and dilution a significant difference was found, but not with the concentration. According to Anova Test (A.5,6.7), 8^{th} day counts for both yeast and total mesophilic aerobic bacteria on microbial growth indicate a significant difference when it is compared with the other groups (0 and 4^{th} days of yeast and TMAB).

When the enzyme activity was evaluated, protease was found to show the best result in Guatemala-added extract with respect to other coffee types, but on the other hand, a slight decrease was observed in terebinth berry and Kenya coffees and black cumin coffee.

As a result of texture analysis of bread, (A.9) the studies showed that the best group was found to be 0.2 mL solution containing enzyme complex. This group provides a softer crumb, indicating the retardation of the staling in bread, mostly due to the degradation of cell wall components.

When dry weight, water-holding capacity, and oil-holding capacity is considered, it was indicated that 0.2 mL solution containing enzyme complex was found to show a better texture with respect to 0.4 mL and the control. Physicochemical and texture properties are important for the bread quality as well as the microbiological criteria.

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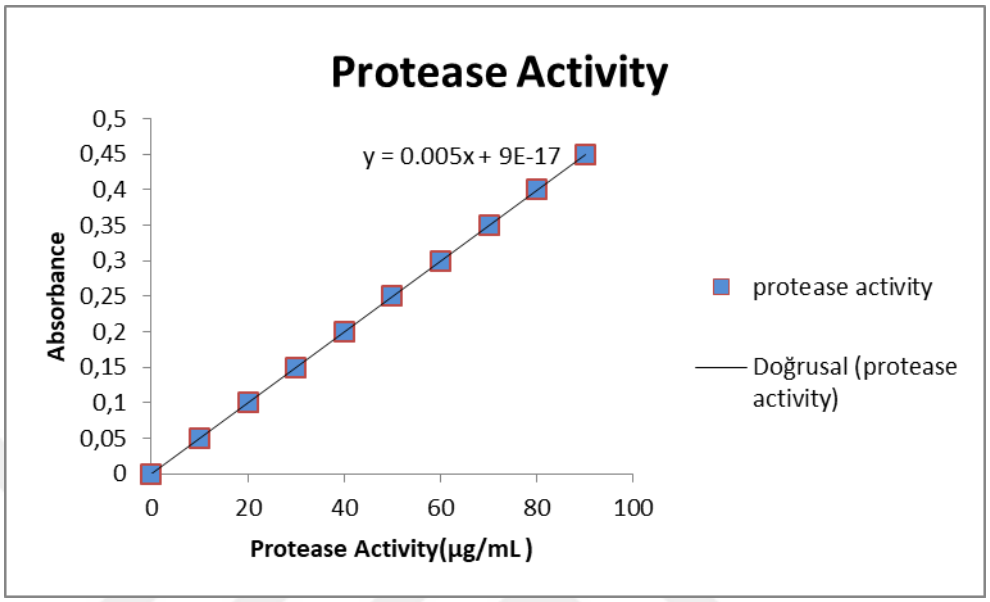
APPENDICES

7. APPENDICES

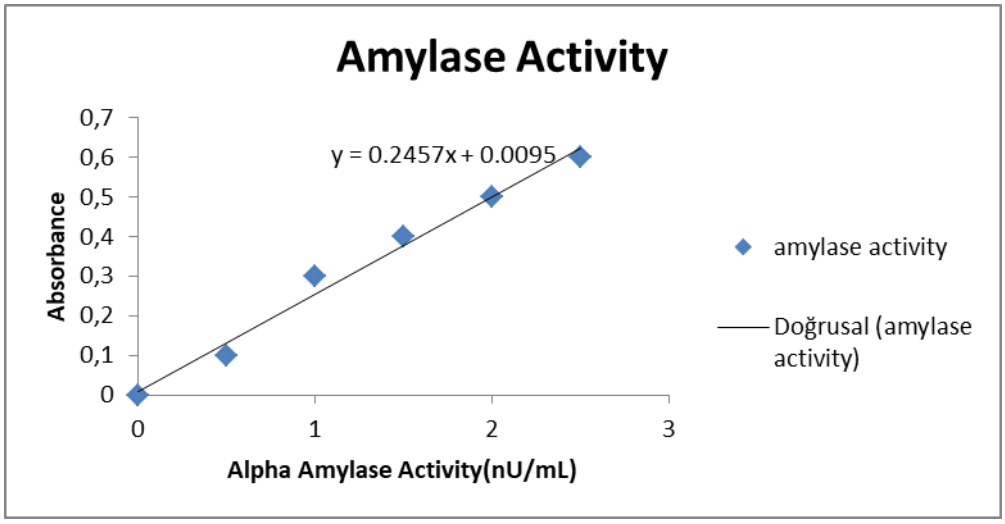
A. 1. The Standard Curve of the Protease Activity



The standard curve of the protease activity for tyrosine concentration in absorbance at 275 nm.

Graph equation: y = 0.005x + 9E-17

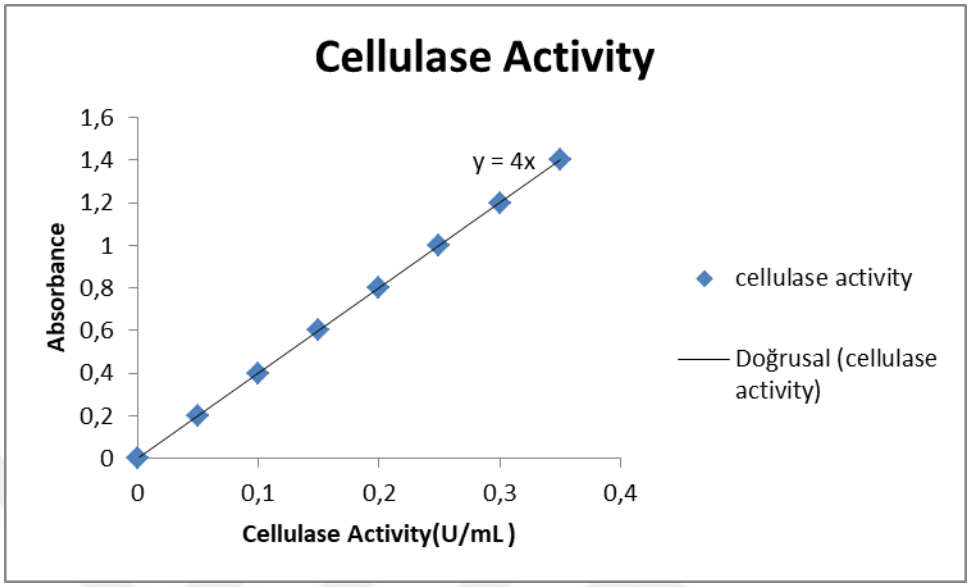
A. 2. The Standard Curve of the Alpha Amylase Activity



The standard curve of the alpha amylase activity in absorbance at 660 nm.

Graph equation: y = 0.2457x + 0.0095

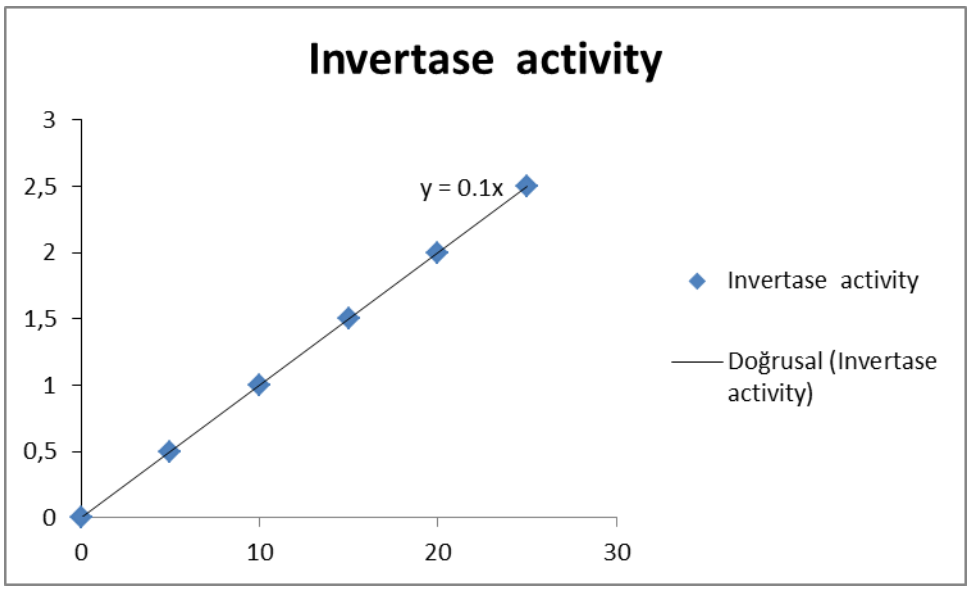
A.3. The Standard Curve of Cellulase Activity



The standard curve of cellulase activity in absorbance at 550 nm

Graph equation: y = 4x + 3E-16

A.4. The Standard Curve of Invertase Activity



The standard curve of invertase activity in absorbance at 560 nm

Graph equation: y = 0.1x

A.5. The statistical evaluation of yeast and TMAB at 0, 4 and 8th days, dilution, and concentration.

Corrected Model	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept	4369.185	35	124.834	16,624	0
Group	2660.148	1	2660.148	354.249	0
Dilution	3826.074	5	765.215	101.903	0
Concentration	3	1	3	0.4	0.529
Group * Dilution	103.63	2	51.815	6.9	0.002
Group * Concentration	27.222	5	5.444	0.725	0.607
Dilution * Concentration	234.815	10	23.481	3.127	0.002
Group * Dilution * Concentration	21.556	2	10.778	1.435	0.245
Error	152.889	10	15.289	2.036	0.042
Total	540.667	72	7.509		
Corrected Total	7570	108			
	4909.852	107			

A.6.The statistical evaluation of 0, 4 and 8 days counts' of yeast respect to TMAB in terms of microbial growth.

(I) Grup	(J) Grup	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
yeast-0 days	yeast-4	-0.8333	0.91343	0.942	-3.5077	1.8411
	yeast-8	-14.3333*	0.91343	0	-17.008	-11.6589
	tmab-0	-0.1111	0.91343	1	-2.7855	2.5633
	tmab-4	-2.0556	0.91343	0.228	-4.73	0.6189
	tmab-8	-12.1111*	0.91343	0	-14.786	-9.4367
yeast-4 days	yeast-0	0.8333	0.91343	0.942	-1.8411	3.5077
	yeast-8	-13.5000*	0.91343	0	-16.174	-10.8256
	tmab-0	0.7222	0.91343	0.968	-1.9522	3.3966
	tmab-4	-1.2222	0.91343	0.763	-3.8966	1.4522
	tmab-8	-11.2778*	0.91343	0	-13.952	-8.6034
yeast-8 days	yeast-0	14.3333*	0.91343	0	11.6589	17.0077
	yeast-4	13.5000*	0.91343	0	10.8256	16.1744
	tmab-0	14.2222*	0.91343	0	11.5478	16.8966
	tmab-4	12.2778*	0.91343	0	9.6034	14.9522
	tmab-8	2.2222	0.91343	0.159	-0.4522	4.8966

A.7. The statistical evaluation of 0,4 and 8 days' counts' of TMAB respect to yeast in terms of microbial growth

(I) Grup	(J) Grup	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
TMAB -0 days	Yeast -0	0.1111	0.91343	1	-2.5633	2.7855
	yeast-4	-0.7222	0.91343	0.968	-3.3966	1.9522
	yeast-8	-14.2222*	0.91343	0	-16.897	-11.5478
	TMAB-4	-1.9444	0.91343	0.285	-4.6189	0.73
	TMAB -8	-12.0000*	0.91343	0	-14.674	-9.3256
TMAB-4 days	yeast-0	2.0556	0.91343	0.228	-0.6189	4.73
	yeast-4	1.2222	0.91343	0.763	-1.4522	3.8966
	yeast-8	-12.2778*	0.91343	0	-14.952	-9.6034
	tmab-0	1.9444	0.91343	0.285	-0.73	4.6189
	tmab-8	-10.0556*	0.91343	0	-12.73	-7.3811
TMAB -8 days	yeast-0	12.1111*	0.91343	0	9.4367	14.7855
	yeast-4	11.2778*	0.91343	0	8.6034	13.9522
	yeast-8	-2.2222	0.91343	0.159	-4.8966	0.4522
	tmab-0	12.0000*	0.91343	0	9.3256	14.6744
	tmab-4	10.0556*	0.91343	0	7.3811	12.73

A.8. The results of statistical evaluation between groups in terms of the effect of a solution containing an enzyme to microbial growth

Group	**N**	**Subset**	
		1 (a)	2 (b)
yeast-0 days	18	0.0556	
tmab-0 days	18	0.1667	
yeast-4 days	18	0.8889	
tmab-4 days	18	2.1111	
tmab-8 days	18		12.1667
yeast-8 days	18		14.3889
Sig.		0.228	0.159

A.9. The effect of the addition of a solution containing enzyme complex to bread on texture properties

Batch		Hardness	Fracturability	Adhesiveness	Springiness	Cohesiveness	Gumminess	Chewiness	Resilience
		g	G	g.sec					
		Force 2	Force 3	Variable	J#/F#	I#/G#	K#*O#	P#*N#	H#/E#
Control		634 194		-0.301	0.926	0.888	563.106	521.556	0.643
0.2 mL		266 281		-2.952	0.907	0.883	235.065	213.246	0.658
0.4.mL		480 673		-8.724	0.912	0.879	422.379	385.075	0.636
Total (F)	AVERAGE("COL")	460.383		-3.992	0.915	0.883	406.85	373.292	0.646
Total (F)	STDEV("COL")	184.794		4.306	0.01	0.005	164.571	154.492	0.011
Total (F)	STDEV("COL") / AVERAGE("COL") * 100	40.139		-107.868	1.087	0.521	40.45	41.386	1.732

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YURDUGÜL S. & **PALA U.** The ıdentification of some chemical, physical and microbiological properties of brands of different hawthorn vinegar applied ultrasonication, 3rd International Symposium For Agriculture And Food, 18 October 2017, 20 October 2017.