MOLECULAR CLONING, OVEREXPRESSION AND BIOCHEMICAL CHARACTERIZATION OF BACTERIAL AMYLASE FOR BIOTECHNOLOGICAL PROCESSES

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ABSTRACT

MOLECULAR CLONING, OVEREXPRESSION AND BIOCHEMICAL CHARACTERIZATION OF BACTERIAL AMYLASE FOR BIOTECHNOLOGICAL PROCESSES

Amylases are the enzymes that act on glycosidic bond of starch and related polysaccarides. They comprise 25% of enzyme utilised in a variety of industry. It is used to obtain maltose, glucose and maltodextrins in various lenghts during industrial processes. Amylases are widely distributed enzymes in bacteria, fungi, higher plants and animals. Thermophilic enzymes are widely demanded in order to be stable at harsh process conditions. Isolating these enzymes from thermophilic microorganism is increasing trend because of ease of enzyme production.

In this study α-amylase gene region from a thermophilic *Bacillus sp*. isolated from Balçova Geotermal region in İzmir was cloned to compotent *E. coli* BL 21 cells. Additionally protein expression was reinforced with pKJE7 chaperone plasmid. Cloned gene was sequenced and found as 1542 bp in length. Thermophilic amylase that has a 59.9 kD molecular weight was expressed and purified from this recombinant strain. Mass spectrometric analysis were performed and the enzyme was matched with α-amylase family protein of *Geobacillus thermodenitrificans NG80-2* using NCBInr database. The aminoacid sequence of this enzyme was seen to be similar 92% with our obtained enzyme.

According to the results of characterization studies, the amylase enzyme was seen to have highest activity at pH 8.0 and 60°C. The enzyme was also showed to have resonable activity between pH5 and 9. 85% of the enzyme activity was retained at 70°C. Furthermore, amylase activities at 65 and 85°C were observed to remain stable for 5 and 2 hours, respectively. It was also showed that the activity was stable and pH7 and 9 for 6 hours. The effects of some metal ions, chemical agents and organic solvents on enzyme activity were examined so, Co^{+2}, Mg^{+2},Ca^{+2} was determined to be as inducer for the enzyme activity. Conversely the activity was inhibited by Cu^{+2}. Furthermore methanol, DDT and Triton X-100 was found to have no effect on the enzyme activity.

ÖZET

BİYOTEKNOLOJİK UYGULAMALARDA KULLANILMAK ÜZERE BAKTERİYEL AMİLAZIN KLONLANMASI, İFADELENMESİ VE BİYOKİMYASAL KARAKTERİZASYONU

Amilazlar nişasta ve ilgili polisakkaritlerin glikozidik bağlarına etki eden enzimlerdir. Bunlar, çeşitli endüstrilerde kullanılan enzimlerin %25'ini oluştururlar. Endüstriyel işlemler esnasında maltoz, glikoz ve çeşitli uzunluklarda matodekstrinler elde etmek için kullanılırlar. Amilazlar bakteriler, mantarlar, yüksek bitkiler ve hayvanlarda geniş oranda bulunan enzimlerdir. Termofilik enzimler ağır işlem koşullarında kararlı oldukları için geniş oranda tercih edilirler. Termofilik mikroorganizmalardan bu enzimleri üretmek üretim kolaylıkları sebebiyle yükselen bir eğilim olmuştur.

Bu çalışmada İzmir Balçova Jeotermal bölgesinden izole edilen termofilik *Bacillus sp*. suşundan α-amilaz gen bölgesi kompotent *E. coli* BL 21 hücrelerine klonlanmıştır. Ek olarak pKJE7 şaperon plazmiti ile protein ifadelenmesi güçlendirilmiştir. Klonlanan gen sekanslanmış ve uzunluğu 1542 bp olarak bulunmuştur. Bu rekombinant suştan 59.9 kD moleküler ağırlığına sasip termofilik amilaz üretilmiş ve saflaştırılmıştır. Kütle spektoskobi analizi yapılmış ve enzim NCBInr veritabanı kullanılarak *Geobacillus thermodenitrificans NG80-2* α-amylase family protein ile eşleştirilmiştir. Bu enzimin aminoasit sekansı bizim elde ettiğimiz enzimle %92 benzer olduğu görülmüştür.

Karakterizasyon çalışmalarının sonuçlarına göre, amilaz enziminin en yüksek aktiviteyi pH 8.0 ve 60°C'de gösterdiği görülmüştür. Ayrıca enzimin pH5 ve 9'da kabul edilebilir bir aktiviteye sahip olduğu gösterilmiştir. 70°C'de enzim aktivitesinin %85'ini geri kazanmıştır. Dahası, 65 ve 85°C'deki enzim aktivitesinin sırasıyla 5 ve 2 saat kararlı kaldığı gözlenmiştir. pH7 ve 9'da da aktivitenin 6 saat kararlı olduğu gösterilmiştir. Bazı metal iyonları, kimyasal ajanlar ve organik solventlerin enzim aktivitesine etkileri incelenmiş ve Co^{+2}, Mg^{+2},Ca^{+2} iyonlarının enzim aktivitesi için indükleyici olduğu saptanmıştır. Diger taraftan aktivite Cu^{+2} tarafından inhibe edilmiştir. Ayrıca methanol, DDT and Triton X-100'ün enzim aktivitesi üzerine bir etkisi olmadığı bulunmuştur.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
LB	Luria-Bertani broth
PAGE	Polyacrylamide gel electrophoresis
PIPES	Piperazine-N,N′-bis (2-ethanesulfonic acid)
PMSF	Phenylmethylsulphonyl fluoride
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
sp.	Species
TCA	Trichloroacetic acid
µl	Microliter
ml	Milliliter
mM	Millimolar

CHAPTER 1

INTRODUCTION

1.1. Extremophiles and Extemozymes

Extremophiles are microorganisms that can live and reproduce in harsh environments. They live in hot springs, volcanic areas, deep sea, in the Antarctic biotopes and in other particular geothermal sites (Schiraldi and De Rosa 2002). Extremophilic microorganisms can be thermophilic, psychrophilic, alkaliphilic and halophilic.

Enzymes from these microorganisms are called extremozymes and they are also resistant to extreme conditions. For example; Moderately halophilic bacteria are able to grow over a wide range of saline concentrations from 0.4 to 3.5 M (Patel et al. 2005; Chakraborty et al. 2010). Halophilic enzymes from these microorganisms with polymer-degrading ability at low water activity are utilized in many harsh industrial processes where concentrated salt solutions would inhibit enzymatic conversions (Chakraborty et al. 2010). Additional major examples for applications of extremozymes that have reached the market are; Taq polymerase, from Thermus aquaticus and cellulase 103 from alkaliphiles. Becoming the key element of the polymerase chain reaction, Taq polymerase provided a new perspective in molecular biology and encouraged the research activities on DNA-polymerases from hyperthermophiles. Cellulase 103 was isolated from bacteria living in soda lakes. It is used to break down the microscopic fuzz of cellulose fibers that traps the dirts on the surface of cotton textile, without harming the natural fabric. This biocatalyst started to be used in 1997 by Genencor International (Rochester, NY, USA) as a novel detergent agent. It helps to keep cotton fabric looking 'as new' even after thousands of washing cycles **(**Pennisi 1997**;** Schiraldi and De Rosa 2002).

In this concept all over the globe researchers are now trying to search out extremophiles which are valuable source of novel enzymes (Chakraborty et al. 2010). Several enzymes and biomolecules isolated from extremophiles and their applications were listed Table 1.1.

Table 1.1. Major examples of enzymes and biomolecules from extremophiles and their foreseen applications (Source: Schiraldi and De Rosa 2002)

Microorganisms	Enzymes and other biomolecules	Applications and products
Thermophiles (50–110°C)	Amylases, glycosidases	Starch processing, glucose, fructose, trehalose, lactose digestion in milk, saccharifying enzymes, oligosaccharides synthesis
	Lipase	Waste water treatments, detergent formulations
	Xylanases	Paper bleaching
	Proteases	Food processing, detergents, amino acids production
	DNA polymerases	Genetic engineering
Psychrophiles (0–20°C)	Amylases, proteases, lipases	Polymer-degrading agents in detergent
	Dehydrogenase	Biosensors
Alkaliphiles pH≥9	Cellulases, proteases	Polymer degrading agents in detergents
	Amylases, lipases	Food additives
	Cyclodextrins	
Halophiles 3–20% salts	Compatible solutes	Pharmaceuticals
	Membranes	Cosmetic additives

1.2. Thermophiles

Bacteria are classified into the following four groups depending on their optimal growth temperature: psychrophiles (5 to 20 °C), mesophiles (15-45°C), thermophiles (45–80°C) (Li, Zhou, Lu 2005). Further classification for the thermophilic organisms was made by Baker et al. (2001). Thermophilic organisms was divided into three groups according to their minimal and maximal growth temperatures: moderate thermophiles (35-70ºC), extreme thermophiles (55-85ºC) and hyperthermophiles (75-113ºC).

A reverse DNA gyrase produces positive super coils in the DNA of thermophiles (Lopez 1999; Haki and Rakshit 2003). This causes elevated melting point of the DNA as high as the organisms maximum temperature for growth. Thermophiles also tolerate high temperature by using increased interactions; electrostatic, disulphide bridge and hydrophobic interactions (Kumar and Nussinov 2001; Haki and Rakshit 2003).

Thermophilic organisms have thermostable cellular components such as enzymes, proteins and nucleic acids. They are also known to withstand denaturants of extremly acidic and alkaline conditions. Thermostable enzymes are being studied as

more useful alternatives to mesophilic enzymes already in place in some industrial processes because they are highly specific (Giver et al. 1998; Kumar 2002).

Such enzymes maximizing reactions accomplished in the food and paper industry, detergents, drugs, toxic wastes removal and drilling for oil is being studied extensively (Haki and Rakshit 2003). Various thermostable enzymes have been purified from thermophilic bacteria; some are alkaline protease, endo-1,5-alpha-$_{L}$-arabinase, and pectate lyase. Commercial products such as laundry detergents needs thermostable lipase and esterase usage. Alternatively, thermostable proteins are being studied in the hope that their adaptive mechanisms may yield methods to stabilize other less stable proteins (Giver et al. 1998; DeFlaun et al. 2007).

Because of a good area of usage in industry and research isolation and characterization of new thermophilic bacterial strains is one of the goals of the investigation all over the world.

1.3. Thermoplilic Bacillus

Geobacillus is a recently created genus (Nazina et al. 2001) that contains a number of highly thermophilic spore-forming bacilli, together with newly described species. Though many of the Geobacillus have been isolated from hot environments such as geothermal features and deep oil reservoirs, it has recently been shown (Marchant et al. 2002a, b; Pavlostathis et al. 2006) that they are almost abundant in cool environments. They also occur in great diversity and may since form an important part of the soil microflora (Rahman et al. 2004). Several Geobacillus species show a significant capacity to degrade hydrocarbons and recent studies have been focused on phenol degradation by *Geobacillus thermoleovorans* (Feitkenhauer et al. 2001; 2003; Pavlostathis et al. 2006). Metabolic capabilities of geobacilli suggest that they may have significant biotechnological applications in industrial and also environmental fields (Uma Maheswar Rao and Satyanarayana 2004; Pavlostathis et al. 2006).

The taxonomy of the genus Bacillus showed that thermophilic species were members of Bacillus rRNA Group 5 (Ash et al. 1991; Rainey et al.1994; Romano et al. 2005). Accordingly to the level of DNA-DNA reassociation values Bacillus thermoleovorans, Bacillus kaustophilus and Bacillus thermocatenulatus should be

combined into one species, namely Geobacillus thermoleovorans which also included Bacillus caldolyticus, Bacillus caldovelox and Bacillus caldotenax (Nazina et al., 2001; Sunna et al. 1997; Romano et al. 2005).

1.4. Thermophilic Enzymes

Enzymes produced by thermophilic and hyperthermophilic organisms known as thermozymes (or thermoenzymes) are also thermophilic and thermostable. They are resistant to irreversible inactivation at high temperatures and optimally active at high temperatures, between 60°C and 125°C (Vieil le et al. 1996).

Thermostable enzymes have received attention due to their potential commercial applications because of their overall inherent stability and high reaction rates at high temperatures. From different exotic ecological zones of the planet earth several thermophilic microorganisms have been isolated in order to be used for such applications. Applications and bioconversion reactions of some are mentioned in Table 1.2. Thermozymes possess major biotechnological advantages distinct from mesophilic enzymes as follows:

- They are easier to purify by heat treatment.
- They have a higher resistance to chemical denaturants such as solvents and guanidinium hydrochloride.
- They can withstand higher substrate concentrations.

Because of their stability at elevated temperature, thermozyme reactions are less susceptible to microbial contamination and often display higher reaction rates than mesozyme catalyzed reactions. Additionally thermozymes can be seen as models for understanding thermostability. Therefore the structural features of thermozymes must be identified to contruct a theoretical description of the physico-chemical principles contributing to prote in stability and folding. Morover, this information also helps designing more stable enzymes for industrial processes (Li et al. 2005).

Thermophilic enzymes in polysaccharides processing have major benefits. In addition to the reduced contamination, they lower the inlet stream viscosity and they can hold a constant pH during the whole biotransformation, so salt addition necessary in modern processes can be avoided. Actually, several research groups have focused on the

search and characterization of this type of biocatalyst. Polymer degrading enzymes, such as amylases, pullulanases, xylanases, proteases and cellulases, have a crucial role in food, chemical and pharmaceutical, paper, pulp and waste-treatment industries (Schiraldi and De Rosa 2002).

Table 1.2. Bioconversion reactions and applications of thermostable enzymes (Source: Haki and Rakshit 2003)

Enzyme	Temperature range (°C)	Bioconversions	Applications
α-Amylase (bacterial)	90–100	Starch → dextrose syrups	Starch hydrolysis, brewing, baking, detergents
α-Amylase (fungal)	50–60	Starch → dextrose syrups	Production of maltose
Pullulanase	50–60	Starch → dextrose syrups	Production of glucose syrups
Xylanase	45–65, 105[a]	Craft pulp → xylan + lignin	Pulp and paper industry
Chitinase	65–75[b]	Chitin → chitobiose	Food, cosmetics, pharmaceuticals, agrochemicals
		Chitin → *N*-acetyl glucosamine (chitibiase)	
		N-acetyl glucosamine → glucosamine (deacetylation)	
		Chitin → chitosan (deacetylase)	
Cellulase	45–55, 95[c]	Cellulose → glucose	Cellulose hydrolysis, polymer degradation in detergents
Protease	65–85	Protein → amino acids and peptides	Baking, brewing, detergents, leather industry
Lipase	30–70	Fat removal, hydrolysis, interesterification, alcholysis, aminolysis	Dairy, oleo chemical, detergent, pulp, pharmaceuticals, cosmetics and leather industry
DNA polymerase	90–95	DNA amplification	Genetic engineering/PCR

[a] Xylanase from *Thermotoga* sp.
[b] Within this range enzyme activity was high.
[c] Cellulases from *Thermotoga* sp.

1.5. Amylase

Starch is one of the most existing polysaccharides composed of D-glucose molecules in nature. It has a heterogenous structure including two high molecular-weight compounds: amylose (15–25%) and amylopectin (75–85%).

Amylose is a linear molecule composed of 100-700 glucose residues linked with α-1,4 bonds in every 4 glucose unit. It is insoluble in cold water.

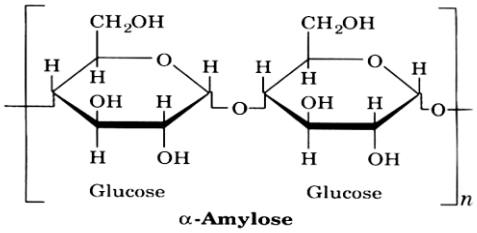

Figure 1.1. Molecular structure of amylose molecule
(Source:Voet and Voet 1995)

Amylopectin is a branched polymer that has α-1,4–linked glucose units which are branched at every 17–26 residues with α-1,6 linkages (Bertolod and Antranikian 2002).

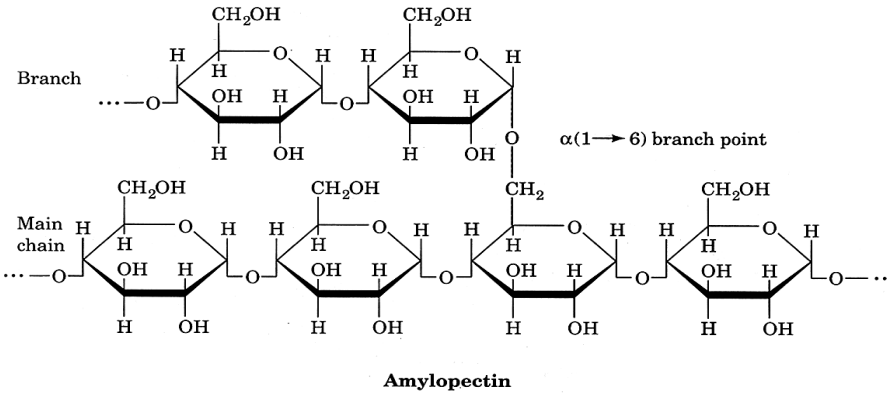

Figure1.2. Brached structure of amylopectin molecule
(Source:Voet and Voet 1995)

A wide variety of organisms secrete amylolytic enzymes to degrade and utilize starch molecule as energy source. Amylolytic enzymes belong mainly to three families of glycoside hydrolases (GHs) :

GH13 – the α-amylase family

GH14 – β-amylases

GH15– glucoamylases (Rashid et al. 2009)

The amylase superfamily can be divided into two groups;

Endoamylases (α-amylase); Endoamylases cleavage α-1,4 glycosidic bonds which present in the inner part of amylose or amylopectin chains and generate oligosaccharides of various lengths. Endoamylases have been found in phyla from Archaea to Mammalia (Frøystad et al. 2006; Chai et al. 2012).

Exoamylases (β-amylase, α-amyloglucosidase); Exoamylases cleavage glycosidic bonds exist in the non-reducing ends of polysaccharides and produce low molecular weight products such as glucose and maltose (Chai et al. 2012).

- β-amylases (alfa-1,4 glucan maltohidrolase, EC3.2.1.2) act on α-1,4 glycosidic bonds at the nonreducing end of molecule. Thus the reaction stops at α-1,6 glycosidic bond, β-maltose and limit dextrins are formed.
- Amyloglucosidases (glucoamylase, α-1,4-glucanohydrolase EC3.2.1.3) produces only glucose acting on both α-1,6 glucosidic bond and α-1,4 glycosidic bonds at the nonreducing end of amylopectin molecule (Pazur and Kleppe 1962).

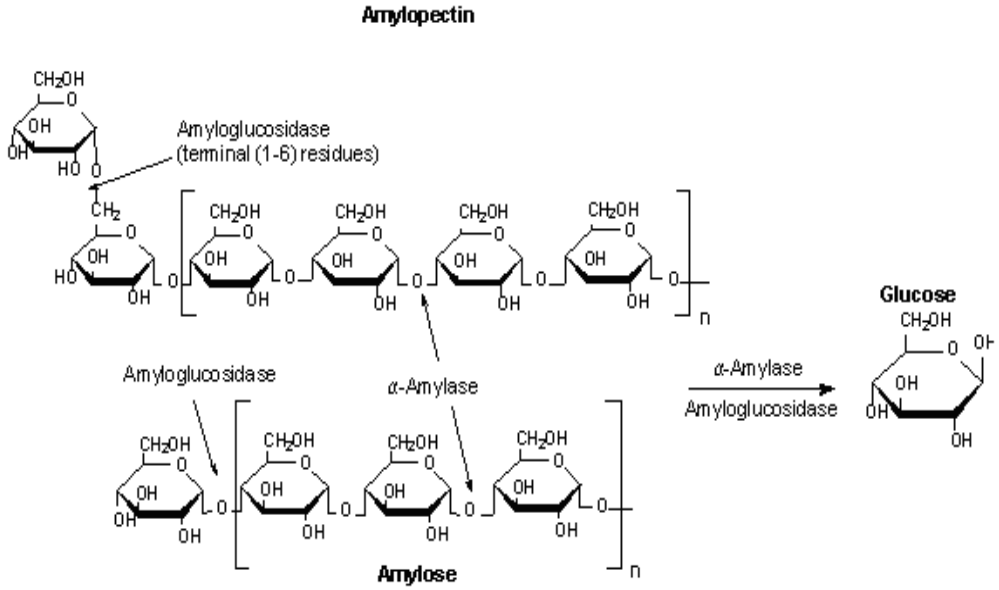

Figure 1.3. Hydrolytic mechanisms of amylases
(Source: snnu 2011)

Starch-processing industries demands for mainly amylases. Endoamylases first provide starch degradation (liquefaction process) with producing maltodextrins, and followingly exoamylases achieve further degradation of maltodextrins into glucose and maltose (saccharification process) (Chai et al. 2012). Hydrolytic mechanisms of amylases are summarized in Figure 1.3.

1.5.1. α- Amylase

α-Amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) are enzymes that catalyze the hydrolysis of amylose and amylopectin in starch and related poly- and oligosaccharides by the breaking down α-(1,4) glycosidic linkages (Titarenko and Chrispeels 2000). End product may be maltose, glucose and maltodextrins in various lenght. These amylases are widely distributed enzymes in bacteria, fungi, higher plants and animals (Janecek 1997; Rashid et al. 2009).

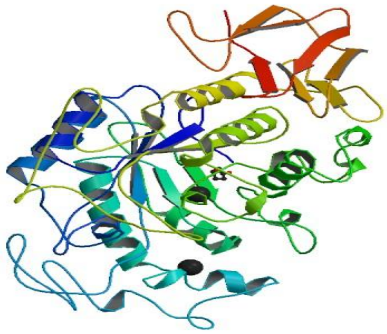

Figure 1.4. Crystal structure of human salivary α-amylase
(Source: Davidson 2011)

Thermophilic and thermostable α-amylases are needed in industry because the enzyme must be active at the high temperature of gelatinization (100–110 ˚C) and liquefaction (80–90°C). Thermophilic amylases also reduce cooling cost, provide better solubility of substrate resulting in lower viscosity which increases the mixing, pumping process and decreases the microbial contamination risk (Pancha et al. 2010).

Owing to the maltose has diverse applications in e.g., food, pharmaceutical, biomedical field, and fine chemicals, α- amylases that produce high levels of maltose would rather prefered (Chai et al. 2012). Besides this, fungal α -amylases produce more maltodextrins and reported as more efficient in saccharifying (Brena et al. 1996; Aquino et al. 2003).

Halofilic and alkalitolerant α-amylases has gained great attention. Common amylases are easily denaturated by high salt concentrations and certain organic solvents so halophilic ones are tolerant of these. Thermostable and alkalitolerant amylases are also desirable in the starch and textile industries and in detergent production (Shafiei et al. 2011; Chai et al. 2012).

1.5.2. Microbial Amylase

Because of they are more stable than plant and animal derivatives and can be obtained cheaply, microbial α-amylases have a broad spectrum of industrial applications (Grupta et al. 2003; Tanyıldızı et al. 2004). The economical bulk production capacity and easy manipulation to obtain desired characteristics in enzymes are major advantages of using microorganisms for the production of amylases (Chakraborty et al. 2010).

Thermophilic archaea and bacteria have been acknowledged as a very good source of starch hydrolyzing enzymes. There are many efforts to find the archaeal or bacterial microorganisms can produce mutually compatible amylolytic enzymes that would provide the reduction of starch degradation process to a single step (Horvathov et al. 2006).

Species of genus Bacillus are widely used for α-amylase production among bacteria. *Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis and Bacillus amyloliquefaciens* are reported to be good sources of α-amylase and for various industrial applications they have been used for commercial production of the enzyme (Rashid et al. 2009). Different Bacillus species produce various types of α-amylases for saccharifying or liquefying that are optimally active at different pH and temperature range (Cordeiro et al. 2002).

Specific maltooligosaccharides, mainly maltotetraose, maltoheptaose and maltohexaose has recently received considerable interest due to their potential use in food, pharmaceutical and fine chemical industries. Many bacterial strains have been described secreting amylases able to produce these specific products. B. circulans, B.amyloliquefaciens, B. cadovelox, Bacillus sp. H-167 and a mutant of B. stearothermophilus produce maltohexaose-forming amylase; B. cereus NY-14 produce the maltopentaose-forming amylase and Pseudomonas sp. IMD 353 produce the maltotetraose-forming amylase. However, all these amylases are reported not to be sufficient thermoactive because the optimal activity is between 55°C and 70°C (Ben Ali et al. 1999). Some sources of amylolytic enzymes and their properties were listed in Table 1.3.

Table 1.3. Source microorganisms and properties of thermostable starch hydrolyzing enzymes (Source: Haki and Rakshit 2003)

Enzymes	Organism	Enzyme properties		References
		Optimal temperature (°C)	Optimal pH	
α-Amylase	*Bacillus amyloliquefaciens*	70	7.0	Underkofler (1976)
	Bacillus licheniformis	100	6.0–6.5	Viara et al. (1993)
	Bacillus stearothermophilus	70–80	5.0–6.0	Vihinen and Mantsala (1990)
	Bacillus stearothermophilus	70	–	Jeayoung et al. (1989)
	Bacillus subtilis	70	7.0	Canganella et al. (1994)
	Lactobacillus manihotivorans	55	5.5	Aguilar et al. (2000)
	Myceliophthora thermophila	100	5.6	Ramkrishna et al. (1993)
	Pyrococcus furiosus	100	5.5	Laderman et al. (1993a,b)
	Pyrococcus woesei	100	6.5–7.5	Koch et al. (1991)
	Staphylothermus marinus	65	5.0	Canganella et al. (1994)
	Sulfolobus solfataricus	–	–	Haseltine et al. (1996)
	Thermococcus aggreganes	100	5.5	Canganella et al. (1994)
	Thermococcus celer	90	5.5	Canganella et al. (1994)
	Thermococcus fumicolans	95	4.0–6.3	Estelle et al. (1997)
	Thermococcus hydrothermalis	85	4.8–7.8	Estelle et al. (1997)
	Thermomyces lanuginosus	60	5.6	Jensen and Olsen (1991)
	Thermococcus profoundus	80	4.0–5.0	Kwak et al. (1998)
β-Amylase	*Bacillus circulans*	60	–	Poonam and Dalel (1995)
	Bacillus cereus var. mycoides	50	–	Takasaki (1976a,b)
	Bacillus sp.	50	7.5	Young et al. (2001)
	Clostridium thermosulphurogenes	75	5.5	Shen et al. (1988)
	Clostridium thermosulfurogenes	60	5.8–6.0	Nipkow et al. (1989)
Pullulanase	*Bacillus* sp.	60	–	Takasaki (1976a,b)
	Pyrococcus furiosus	98	5.5	Brown and Kelly (1993)
	Pyrococcus woesi	100	5.5–6.0	Rudiger et al. (1995)
	Thermococcus aggregans	100	6.5	Canganella et al. (1994)
	Thermus caldophilus GK24	75	5.5	Kim et al. (1996)
	Thermococcus celer	90	5.5	Canganella et al. (1994)
	Thermococcus hydrothermalis	95	5.5	Gantlet and Duchiron (1998)
	Thermococcus litoralis	98	5.5	Brown and Kelly (1993)
	Thermotoga maritima MSB8	90	6.0	Bibel et al. (1998)

1.5.3. Industrial Applications of Amylase

The usage of enzymes in the industrial production has began when Dr. Jhokichi Takamine produce digestive enzyme from wheat bran koji culture of Aspergillus oryzae in 1894. Then α-amylase and glucoamylase were first utilized in industry for the production of dextrose powder and dextrose crystals from starch in 1959 (V. Aiyer. 2005). Today amylase is used in extensive biotechnological applications in many industrial processes such as sugar, textile, paper, brewing, baking and distilling industries. The demand for amylase is increasing day by day because of its industrial applications (Chakraborty et al. 2010).

Some of the industrial applications can be mentioned as follows:

Paper industry: With the aim of protecting paper against mechanical damage during processing, the sizing of paper with starch is performed. Sizing improves the

quality of the finished paper, enhances the stiffness and strengthen in paper also improves the erasibilty. The temperature of sizing process generally in the range of 45-60°C. Because of the viscosity of natural starch is too high for paper sizing it is adjusted by partially degrading the polymer with α-amylases in a batch or continuous processes (Gupta et al. 2003).

Bread and baking industry: Baking industry has used enzymes for hundreds of years to be able to manufacture a wide variety of high quality products. Malt and microbial α-amylases have been widely used in the baking industry for decades (Si 1999, Pintauro 1979; Gupta et al. 2003). The enzymes were used to give the products a higher volume, better colour and a softer crumb. Malt preparation opened the opportunities for many enzymes to be used commercially in baking. Today, so many enzymes like proteases, lipases, xylanases, pullulanases, pentosanases, cellullases, glucose oxidases, lipoxygenases etc. are being used in the bread industry for various purposes (Kulp 1993; Pintauro 1979; Monfort et al. 1996; Prieto et al. 1995; Gupta et al. 2003), but none of them had been able to replace α-amylases. α-Amylase provides improvement in the volume and texture of the product by enhancing the rate of fermentation and reducing the viscosity of dough. It also generates additional sugar in the dough that improves the taste, crust colour and toasting qualities of the bread (Van Dam and Hille 1992; Gupta et al. 2003). α-Amylase is also being used as antisalling agent.

Liquefaction: Liquefaction process includes the dispersion of insoluble starch granules in aqueous solution followed by partial hydrolysis with thermostable amylases. In industrial processes, the viscosity of starch suspension for liquefaction is of extremely high following gelatinization. Thermostable α-amylas is used for reducing the viscosity as a thinning agent and for partial hydrolysis of starch. If the liquefaction process does not implemented well, some problems like poor filtration and turbidity of the processed solution occurs. In ideal liquefaction of starch is that the starch slurry which contains suitable amount of α-amylase must be treated at 105 -107°C as quickly and uniformly as possible (Hattori 1984; Aiyer 2005).

Manufacture of oligosaccharide mixture: Maltooligomer mix is a novel commercial product. Its composed of usually glucose, 2.2%; maltose, 37.5%; maltotriose, 46.4%; and maltotetraose and larger maltooligosaccharides, 14%. It is mainly used instead of sucrose and other saccharides. It is also prevents crystallization

of sucrose in foods and keep a certain level of hardness of the texture during storage (Aiyer 2005).

Manufacture of maltotetraose syrup: Freezing points of frozen foods can be controled by Maltotetraose syrup (G4 syrup) addition. Thermostable α-amylase of B.licheniformis or B.subtilis is used to make commertial G4 syrups (Aiyer 2005).

Textile desizing: Considerable strain on the warp during weaving exist in modern processes for textiles. Therefore the yarn must be protected from breaking. Thus a removable protective layer is applied to the threads. Because starch is cheap, easily available in most regions of the world and can be removed quite easily it is a very attractive size agent. After production process starch is desized from textiles by the application of α-amylases. The enzyme selectively remove the size not to attack the fibres and randomly cleaves the starch into dextrins soluble in water then can be removed by washing (Gupta el al. 2003).

Detergent applications: Early automatic dishwashing detergents were very harsh, caused injury when ingested. Thus detergent industries started to search for milder and more efficient solutions (Van 1992; Gupta et al. 2003) like enzymes. They also lower washing temperatures. α–Amylase is included in 90% of all liquid detergents today (Kottwitz 1994; Gupta et al. 2003) and now for automatic dishwashing detergents the demand for it is increasing. Sensitivity to calcium and some oxidants comprise limitations of most wild-type α-amylases in detergents. As achieved in protease stability against oxidants in household detergents was accomplished by utilising successful strategies (Gupta et al. 2003).

Additionally amylase is beeing used in direct fermentation of starch to ethanol and in treatment of starch processing of waste water (SPW) (Aiyer 2005).

1.6. Production of Enzymes by Recombinant DNA Technologies

To obtain high level of expression both for fundamental studies and commercial purposes foreign proteins are expressed in prokaryotic systems mostly. In the aim of achieving maximal expression convenient expression vector and host must be used. Bacterial expression might have problems like proteolytic degradation and the production of proteins that accumulate in misfolded forms (Abdel-Fattah and Gaballa 2006).

According to the objectives of expression the expression system should be choosen. As its vectors are well characterized *E. coli* expression is highly recommended to obtain quickly a sufficient amount of a recombinant enzyme for basic characterization. Furthermore *E. coli* has a specific growth rate five- to ten-fold higher than most of the extremophilic organisms. So it is easily cultivated in the laboratory, yields enough biomass and product even in simple shake flask growth. On the other hand expression in yeasts has noticeable advantages for large-scale industrial production. As yeast is a generally recognized as safe (GRAS) organism, it represents an ideal pilot system for the production of enzymes to be applied in food and feed manufacturing. Though stronger resistance of the yeast membrane respect to the bacterial membrane, cytosolic products can not be easily recovered (Schiraldi and De Rosa 2002).

One strain can produce different amylases with different specificities or amount of amylase production may be very low. As the screening for a single amylase is difficult, cloning of amylase gene is performed. With the cloning of one gene directing synthesis of desired amylase, a good characterisation and a significant yield can be achieved in host like *E. coli* and *B. substilis* (Özcan, Altınalan and Ekinci 2001).

To overcome misfolding of recombinant proteins coexpression can be performed with chaperone plasmids. Molecular chaperone is unstable conformer of another protein. It regulates folding, oligomeric assembly, interaction of target protein with other cellular components, switching between active and inactive conformations, intracellular transport, or proteolytic degradation, either singly or with the help of co-factors (Agashe and Hartl 2000; Bhutani and Udgaonkar 2002).

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Preparation of buffers, reagents, solutions and the standart curves are given in Appendix A and Appendix B

2.2. Bacterial Strain, Growth Conditions and Amylase Activity Test

Bacterial strain used in this study was isolated from Baçova Geothermal Region in İzmir and determined as Gram (+) *Geobacillus sp*. in a previous study (Yavuz et al. 2004). Optimum growth conditions were determined for the strain and the strain was cultivated in Luria Bertani broth at 55°C and 200 rpm for 22 hours.

2.2.1. Starch-Iodine Test

The strain number 33 was chosen to perform the study and amylase activity was secreened according to starch iodine test (Bragger et al. 1989). Isolate was first spreaded on starch agar plate and incubated for 16 hours at 55°C. Then the plate was dyed with iodine solution.

2.3. Genomic DNA Isolation

The strain number 33 was cultivated in 5ml LB medium at 55ºC and 200 rpm for 22 hours. The culture was centrifugated at 13000 rpm for one minute then the pellet was used to prepare cell lysate. Genomic DNA was isolated with Invitrogen PureLink Genomic DNA mini kit according to the instructions of user manuel.

2.4. Construction of Primers

To constract primers for amplifing the genomic DNA, α-amylase gene region of *Geobacillus thermoleovorans subsp. stromboliensis* strain P220 (GenBank A.C HM034453.1) was taken as referans. Restriction sites of NdeI and Hind III enzymes were placed to the end of both primers.

Seguences of primers;

Amf F : CATATGGAAATGGGGAACCGGCTCTTTATG

Amy R : AAGCTTTTATTCATTGATCCGTTTTGCCCG

2.5. Amplification of Genomic DNA - PCR Conditions

PCR mixture was prepared on ice as written below.

dH_2O	14 µl
$MgCl_2$	2 µl
dNTP mix (2mmol)	2.5 µl
Taq polimerase buffer	2.5 µl
Primer F	0.5 µl
Primer R	0.5 µl
DNA template (genomic DNA)	2.5 µl
Taq polimerase	0.5 µl

The PCR was performed for 30 cycle with the following conditions;

Initial denaturation	94 ºC
Denaturation	94 ºC
Annealing temperature	56 ºC
Extention	72 ºC
Final extention	72 ºC

Then the product was run on 1% agarose gel in order to be visualized.

2.6. Agarose Gel Electrophoresis

0.5 g of agarose was dissolved in 50ml TAE buffer and boiled in microwave oven. After it was cooled under tap water, 0.5µl of EtBr (10.0 mg/ml) was added. The solution was poured into electorphoresis gel aparatus. Then a comb useful to form sample wells was placed. The gel was waited to cool to room temperature for 30 minute. Then the comb was removed and TAE buffer was poured on gel to cover. 5µl of each samples were loaded into the wells after they were mixed with 1µl of 6x loading dye. The gel was run with 100V and 80mA electrical field for 45 minutes. Finally, it was visualized under the UV light.

2.7. Extraction of PCR Product from Agarose Gel

Agorose gel pieces including the amplified gene region was cut with a surgical blade and put in a vial. It was extracted and purified with the Fermentas K0513 DNA Extraction kit. The quantity of purified DNA was calculated measuring the absorbance at 260nm with Nanodrop ND1000 instrument.

2.8. Cloning of Amylase Coding Gene Region

Purified PCR product was subcloned to T/A cloning vector, PTZ57RT, with Fermentas InsTA clone PCR cloning kit K1214.

2.8.1. Ligation

The reaction was set in way the optimal insert/vector ratio was 10:1. The insert volume was calculated from the equation:

ng of insert = [ng of vector x size of insert (bp)／ size of vector(bp)] x vector/insert

20 µl reaction mixture contained 2.0 µl 10X Ligation Buffer, 4µl T4 DNA Ligase, 1 µl Vector pTZ57R/T, (0.17 pmol ends), 12 µl PCR product (20.8 ng/ µl) , 1 µl ultra pure water. The mixture was incubated at room temperature overnight.

2.8.2. Compotent Cell Preperation (for *E. coli* DH5α and BL21 Cells)

Firstly, bacterial cell was inoculated on LB agar plate and incubated at 37ºC overnight. Single colony was selected from plate then it was inoculated into a 5.0 ml of LB liquid media with aditional incubation at 37˚C and 180 rpm overnight. After the incubation, bacterial culture was transfered into 200 ml of SOB media and incubated at 100 rpm for 2 hours at 10˚C and 37˚C and for 16 hours at 10˚C. The culture was divided into four equal volume and incubated on ice for 10 minutes. After that they were centrifugated at 4˚C and 4000 rpm for 10 minutes. Supernatants were removed and pellets were resuspended with 5.0 ml of ice-cold TB medium and the samples were hold on ice for 10 minutes. They were centrifuged at 4˚C and 4000 rpm for 10 minutes. After the supernanats were poured off, the pellets were resuspended gently on ice with TB solution. 1.0 ml of TB solution was added for 1.0 ml of pellet. Then filtered (0.2 μm filter) DMSO was added by 7.0% of TB solution. Next, the samples were aliquoted in a volume of 100μl into previously cooled eppendorfs. In the final step, eppendorfs were immersed into the liquid nitrogen immediately and stored at -80˚C.

2.8.3. Transformation of Cloning Vector to Compotent *E. coli* DH5α Host Cells

5 μl of ligation mixture was mixed with 50 μl of compotent cell DH5α (stored at - 80^{o}C and thawed on ice before use). After incubating on ice for 10 minute, the mixture was put in 42°C waterbath. Then it was incubated for 2 minutes on ice again. 400 μl SOC media was added and cells were incubated at 37°C for 1.5 hours. Finally they were spreaded on LB-ampicilin (100mg/ml) agar plate. Mixture of 40.0 μl of X-gal (20.0 mg/ml), 40.0 μl of IPTG (Isopropyl-β-D thiogalactopyranoside) (0.1 M) and 20.0 μl of dH_2O were spreaded on the surface of LB-amp plate before use. After the inoculation of cells, the plates were incubated at 37ºC for 18-20 hours.

2.8.4. Plasmid Isolation

PTZ57RT vector including insert was isolated to be used in the following step of the cloning to seguence the inserted gene. Single white colony from LB-amp plate was inoculated into 5 ml LB-amp medium and incubated at 37°C for 12-16 hours while shaking at 250 rpm. The bacterial culture was harvested by centrifugation at 13500 rpm for 1 minute. The supernatant was decanted and the pellet was used to isolate plasmid with the Fermantas Plasmid Mini Prep kit ♯ K0503. Finally amount of isolated plasmid was determined with Nanodrop Spectroptotometer ND1000 at 260 nm. Then the whole seguence of isolated PTZ57RT plasmid was analysed by (16 and 80 capillary, Applied Biosystem, 3130XL) using M13 primers.

2.8.5. Digestion of Plasmid

With the aim of transfering the target gene to expression vector, ligated gene was excised from cloning vector. The cloning vector PTZ57R/T was digested with the NdeI and Hind III digestion enzymes. Double digestion was applied as in this procedure.

- 3,5 µl of plasmid (202,4ng/µl)
- 0,5µl of NdeI
- 1µl of 10x fast digest buffer
- 4,5µl of dH_20

The mixture was incubated at 37°C for 1 hour. Then the enzyme activity was altered at 65°C for 5 minute. After that 0,5 µl of Hind III was added to the mixture and incubated 37°C for 1 hour. The mixture was run on 1% agarose gel and digestion was observed. Then insert was extracted from gel by using gel extraction procedure. pET 28a(+) was also digested with the same enzymes.

2.8.6. Ligation of Insert to Epression Vector pET 28a(+)

Due to pET 28a+ and insert were digested with the same enzymes they had sticky ends and gained affinity to each other. The ligation reaction conditions were designed according to 1/10 ratio of vector to insert as below;

- 5x ligation buffer 4µl
- T4 ligase 1µlI
- Insert 9µl (78ng/ul)
- pET28a+ 6µl (36ng/ul)

The mixture was incubated overnight at room temperature.

2.8.7. Transformation to Compotent *E. coli BL21* Cells

To express the target gene, plasmid pET 28a(+) with insert was transformed to compotent *E. coli* BL21 cell line. 100µl of compotent cell was taken from -80°C and put on ice immediately. Just before it thawed, 5 µl of ligation mixture was added. It was incubated on ice for 30 minutes, in 42°C waterbath for 45 seconds and again on ice for 2 minutes. 250 µl SOC medium (heated to 37°C before use) was added and it was incubated at 37°C, shaking with 200rpm for 1 hour. Finally the mixture was spreaded on LB kanamycin(30µg/ml) agar plate and it was incubated at 37°C for 18 hours. The colonies formed after incubation were used for plasmid isolation and also protein expression.

2.9. Protein Expression Procedure for *E. coli BL21* Cells

Single colony choosen from LB-kan agar plate was inoculated in 20 ml LB-kan media and incubated at 37°C and 225 rpm for 16 hours. It was tranfered into 400ml LB-kan media and incubated until the optical density of 0,8 at 600nm. Then IPTG was added to the culture in a final concentration of 1mM. Additional 4 hour incubation was proceeded at the same conditions.

2.10. Coexpression with Chaperone Plasmids

While the expressed protein could not be purified with nickel affinity chromotography, chaperone plasmids were used to repair improper folding of expressed protein. Chaperone plasmids were transfered to compotent *E. coli BL21* cells including pET 28a(+) with the insert. Firstly *E. coli BL21* cells including pET 28(a+) plasmid with the insert were made compotent according to the compotent cell preperation method.

1,5 µl of each chaperone plasmids (70ng/µl) were tranfered into each 100µl competent *E.coli BL21* recombinant cells. 250 µl SOC medium (heated to 37° C before use) was added and was incubated at 37°C, shaking with 200 rpm for 1 hour. The cells were spreaded on LB-kan (30µg/ml)-chloramphenicol (20µg/ml) agar plates. The plate was incubated at 37°C for 16 hours. A single colony was selected and inoculated into 5 ml of LB kan-chloramphenicol including also inducers of each plasmids. Five cultures were prepared. They were incubated at 37°C at 200rpm for 16 hours.

Table 2.1. Chaperone plasmids and their properties
(Source: Instruction manuel of Takara Chaperone Plasmid Set.Cat.no. 3340)

No.	**Plasmid**	**Chaperone**	**Promoter**	**Inducer**	**Resistant Marker**	**References**
1	pG-JE8	dnaK-dnaJ-grpE groES-groEL	araB Ptz-1	L-Arabinose Tetracyclin	Cm	(Nishihara 1998) (Nishihara 2000)
2	pGro7	groES-groEL	araB	L-Arabinose	Cm	Nishihara 1998
3	pKJE7	dnaK-dnaJ-grpE	araB	L-Arabinose	Cm	Nishihara 1998
4	pG-Tf2	groES-groEL-tig	Pzt-1	Tetracyclin	Cm	Nishihara 2000
5	pTf16	tig	araB	L-Arabinose	Cm	Nishihara 2000

At the end of incubation, they were transfered into 100 ml of the same media and incubated again at the same conditions. While the optical density of the cultures at 600 nm (OD_{600}) reached to 0,4, IPTG was added with a final concentration of 1mM. Finally the cultures were incubated for 4 hours.

2.11. Lysis of Bacterial Cells

Bacterial cultures were centrifuged at 5000 rpm, at 4°C for 10 minutes. Pellets were dissolved in 5 ml lysis buffer and the cells were disrupted with a sonicator for 6x20 seconds. They were centrifuged again at 10000 g, at 4°C for 20 minutes. Supernatants were used for protein purification. Both supernatants and pellets were run in SDS-PAGE.

Lysis buffer was prepared in 50mM TrisHCl (pH 8.0) and included 10% glycerol, 0.1% Triton X-100, 100ug/ml lysozyme, 1mM PMSF and 2mM $MgCl_2$.

2.12. Enzyme Purification

Crude enzyme extract was loaded in DEAE-cellulose anion exchange column (2.5 cm x 10cm). The column was equilibrated with 50mM Tris-HCl (pH 7.2) before use. After loading sample, the column was washed with the same buffer. In order to elute the proteins according to their ionic strenght, gradient dilutions of NaCl from 0 to 2 M in 50mM Tris-HCl (pH 7.2) were flowed from the column. 30 drops of fractions were collected and then protein concentrations were measured at 280nm using Nanodrop ND1000.

After SDS-PAGE analysis of fractions, collected fractions were loaded in Sephadex G-100 size exclussion column. Elution was applied with 50mM TrisHCl (pH 7.2) in 50 drops.

ISCO Low Pressure Liquid Chromotography system was used to apply both ion exchange and size exclussion. Purified protein was assayed for protein concentration determination using Bradford method and for amylase activity analysis.

2.13. SDS-PAGE

Purified enzyme solution was screened in SDS-PAGE gel to detect the molecular weight and purity. Biorad Mini– Protean Tetra Cell was used in this method. 10 ml Seperating gel (12% monomer conc.) and 5 ml Stacking gel 4% were prepared as explained in appendix A and load to gel apparatus respectively. After the gel become

solid, module tank was filled with running buffer. In order to obtain 1µg/µl final concentration, samples were diluted with sample buffer in 1/1 ratio. Protein marker and samples were kept in boling water for 10 minutes to be denaturated. 5 µl marker and 10 µl of each sample were loaded into wells on the gel. They were run at 100 V for 120 minutes. Then the gel was replaced from electrophoretic modul and incubated into 20% TCA for 30 minutes by shaking gently. It was rinsed with fresh ultrapure water. Then the gel was incubated into 0,05% commassi blue R250 for 30 minutes on a shaker. After the gel was destained with destaining solution, it was monitored with camera under white light.

2.14. Screening of Amylase Activity on Agar Plate

1% starch agar plate was prepared then the crude enzyme extract and purified enzyme was droplet to the plate. It was incubated at 55°C for 10 minutes. Then the plate was dyed with iodine solution.

2.15. Protein Identification and Mass Spectrometric Analysis

2.15.1. In-Gel Digestion

In gel digestion was applied according to Shevchenko and co-workers protocol (Shevchenko et al. 1996). It is a three-day procedure consists of cutting protein spot and washing the gel pieces for the first day, reduction, alkylation, washing out reagents and exchange of buffers followed by digestion with trypsin for the second day and extraction of peptides for analysis for the third day.

Solutions required for in-gel digestion was prapared as described below.

• Washing solution (50% (v/v) methanol and 5% (v/v) acetic acid): 10 ml of methanol (Merck) was mixed with 5 ml of deionized water and 1 ml of acetic acid was added. The solution was adjusted to 20 ml with deionized water.

• 100 mM ammonium bicarbonate: 0.2 g of ammonium bicarbonate (AppliChem) was dissolved in 20 ml of water.

• 50 mM ammonium bicarbonate: 2 ml of 100 mM ammonium bicarbonate was mixed with 2 ml of deionized water.

• 10 mM DTT: 1.5 mg of dithiothreitol was dissolved in 1 ml of 100 mM ammonium bicarbonate completely.

• 100 mM iodoacetamide: 18 mg of iodoacetamide was dissoved in 1 ml 100 mM ammonium bicarbonate completely.

• Trypsin solution (20 ng/ml): 20 μg of sequencing-grade modified trypsin (V5111; Promega) was dissolved in 1 ml of ice cold 50 mM ammonium bicarbonate by drawing the solution into and out of the pipette. The solution was kept on ice until use.

• Extraction buffer (50% (v/v) acetonitrile and 5% (v/v) formic acid): 10 ml of acetonitrile (Merck) was mixed with 5 ml of deionized water and 1 ml of formic acid (Merck) was added. The solution volume was adjusted to 20 ml with deionized water.

First day**;** Protein spot was cut from SDS-PAGE gel with a surgical blade as possible as beeing carefull to take whole protein and it was divided into smaller pieces. The gel pieces were placed in a tube. 200 μL of wash solution was added and it was incubated overnight.

Second day; Wash solution was removed from the sample. 200 μL of wash solution was added again with incubation for additional 2-3 hours at room temperature and finally discarded. Then the gel pieces were dehydrated with 200 μL of acetonitrile for 5 minutes at room temperature. They became opaque white color. After acetonitrile was removed the sample was dried in a vacuum centrifuge for 2-3 minutes at room temperature.

In the next step gel pieces were incubated in 30 μL of 10 mM DTT for 30 minutes at room temperature to reduce the protein.

Again DTT was removed from the sample carefully. Then 30 μL of 100 mM iodoacetamide was added and incubated for 30 minutes at room temperature to alkylate the protein.

After iodoacetamide was removed from the sample carefully, the sample was dehydrated for 5 minutes at room temperature with addition of 200 μL of acetonitrile. Then it was also removed.

Rehydration was applied by incubating the sample with 200 μL of 100 mM ammonium bicarbonate for 10 minutes at room temperature.

Ammonium bicarbonate was carefully removed. 200 μL of acetonitrile was added to the sample and dehydrated for 5 minutes at room temperature. Acetonitrile was carefully removed.

Sample was completely dried at room temperature in a vacuum centrifuge for 2-3 minutes.

30 μL of the trypsin solution was added and the sample was allowed to rehydrate on ice for 10 minutes with vortex mixing.

Sample was centrifugated for 30 seconds and the gel pieces were collected on the bottom of the tube. Excess trypsin solution was carefully discarded from the sample

5 μL of 50 mM ammonium bicarbonate was added to the tube and the mixture was vortexed. The sample was driven to the bottom of the tube by centrifuging the sample for 30 seconds. Digestion was applied overnight at 37 ºC.

Third Day; After 30 μL of 50 mM ammonium bicarbonate was added and the sample was incubated for 10 minutes, it was centrifudated for 30 seconds.

The supernatant was carefully collected and the sample was transferred to another micro centrifuge tube.

30 μL of extraction buffer was added to the gel pieces and incubated for 10 minutes, Then it was centrifuged for 30 seconds. The supernatant was carefully collected and combined with the former supernatant.

30 μL of extraction buffer was added to the tube containing the gel pieces, and incubated for 10 minutes. It was centrifuged for 30 seconds and supernatant was carefully collected and added to the tube containig previous supernatants.

The volume of the supernatant was reduced to less than 20 μL by evaporation in a vacuum centrifuge at room temperature.

Finally volume of the sample was adjusted to 20 μL with acetic acid. Sample became ready for mass spectrometric analysis.

2.15.2. Mass Spectrometric Analysis

Sample that was applied in-gel digestion procedure was identified by MALDI-TOF-TOF Mass Spectrometry. Sequence of the protein spots were found by using NCBInr (National Center for Biothecnology Information, Bethesda, USA) database.

For mass analysis, α-cyano-4-hydroxycinnamic acid (HCCA) was used as matrix.

2.16. Determination of Protein Concentration

Protein concentration was determined with Bradford (Sigma, product number; B 6916) reagent. Standart curve was constructed by measuring absorbance of BSA (Bovine serum albumin) standarts in 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 1.4 mg/ml, 2 mg/ml concentrations.

50 µl of sample was mixed with 1.5 ml of Bradford reagent. The mixture was incubated at room temperature in dark for 10 minutes. Protein concentrations were measured at 595 nm with Perkinelmer Lamda 25 UV-Vis spectrophotometer.

2.17. Amylase Assay

Amylase activity was estimated by measuring the reducing sugar released during the reaction of enzyme-substrate. Starch was used as substrate in the reaction and DNS assay method was modified from Somogyi and Nelson (Nelson, 1944). 25µl of purified enzyme extract was incubated with 50 µl of 1% starch solution in a 55ºC waterbath for 20 minute. 100 µl DNS reagent (Dinitrosalycilic acid solution) was added to the mixture to stop the reaction. The mixture was incubated in boiling water for 5 minutes. After cooling to room tempereture, 825 µl dH_2O was added. Finally the optical density at 540 nm was measured.

The amount of reducing sugar was estimated according to standart curve that was prepared by measuring absorbance of different concentrations of D-glucose at 540 nm. One unit of enzyme was defined as the amount of enzyme that produce 1 µmol of reducing sugar per one minute under the assay conditions.

2.18. Protein Characterization

2.18.1. Optimum pH and Temperature

In order to obtain optimum pH for enzyme activity, substrate solution (1% starch) was prepared in different buffers; pH 3.0-6.0 (citrate buffer), pH 7.0 (sodium phosphate buffer), pH 8.0 (Tris-HCl buffer), pH 9.0, 10.0 (Glycine-NaOH buffer). Then the amylase assay was applied in the sameway at 55°C. Blank was not including enzyme solution.

Optimum temperature of the enzyme was determined by performing the enzyme assay at different temperatures. Incubation temperature of enzyme-substrate solution in waterbath was changed from 40ºC to 90 ºC.

2.18.2. pH and Temperature Stability

pH stability of the enzyme was tested by incubating 25 µl of enzyme solution with 25 µl of pH 9 and pH 7 buffers for 6, 16, 24 hours respectively. Then the 50 µl substrate was added and the assay was performed, absorbance values were measured at 540nm.

For temperature stability, 25 µl emzyme was incubated both 65 ºC and 80 ºC for 12, 16,24 hours and 50 µl substrate was added then amylase assay was performed.

2.18.3. Effect of Metal Ions on Enzyme Activity

25 µl of purified enzyme extract was incubated at 60 ºC waterbath in the presence of 25 µl of some metal salts for 10 minutes then 25 µl of substrate solution was added. The reaction was allowed to happen for 20 minutes at 60 ºC. Afterwards the enzyme assay was applied. 10mM and 25mM of $CaCl_2$, NaCl, NaF, $MgCl_2$, $CuSO_4$, $CoCl_2$, KCl solutions were used. Blank was formed by adding 25 µl of water to enzyme extract and applying enzyme assay in the same conditions.

2.18.4. Effect of Solvents and Detergent on Enzyme Activity

25µl of purified enzyme extract was incubated at 60ºC waterbath in the presence of 25µl of various surfectants and solvents for 10 minutes then 25µl of substrate solution was added. The reaction was allowed to happen for 20 minutes at 60ºC. Then the enzyme assay was applied in the same conditions.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Screening of Amylase Activity of Bacterial Strain

Isolate number 33 was spreaded on starch agar and incubated for 16 hours at 55°C. When it was dyed with iodine solution, white zones were obtained. It proves that isolated bacterial colonies are capable of degrading starch.

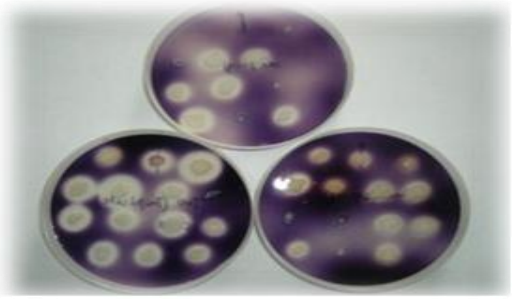

Figure 3.1. Bacterial colonies on starch agar plate

3.2. Genomic DNA Isolation and PCR Amplification

Bacterial genomic DNA was isolated from culture of isolate number 33. 87.24 ng/µl DNA was obtained by measuring absorbance with Nanodrop ND 1000. Target sequence (α-amylase) was amplified in the presence of designated amylase primers. After PCR amplification, the product was run on agarose gel and photographed. Its molecular weight was determined as nearly 1600 bp (Figure 3.2).

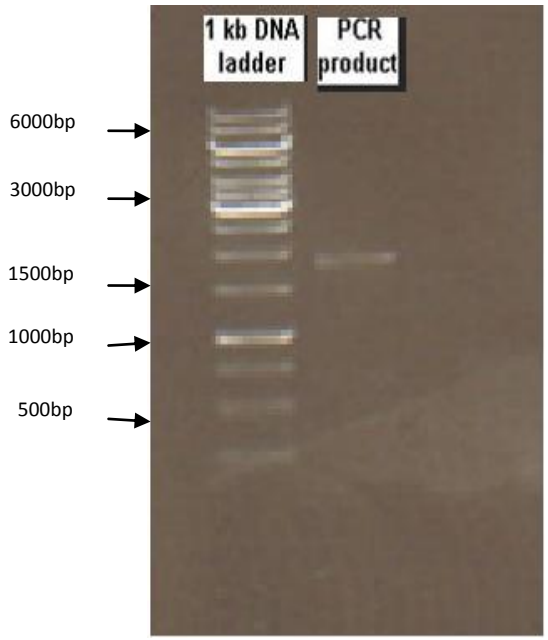

Figure 3.2. Agarose gel image of PCR product.

3.3. Cloning Experiments

3.3.1. Subcloning

At the end of gel extraction, the concentration of obtained DNA was found to be 43ng/µl. In 20µl ligation reaction, 142 ng DNA sample was used. Then ligated PTZ57R/T cloning vector was transformed into compotent cells of *E. coli DH5α*. After incubation of the compotents cells on LB-amp agar plate, blue and white colonies formed as shown in figure 3.3. Blue nonrecombinant cells could metabolize X-gal because of lac-Z gene on plasmid PTZ57R/T. Because the Lac-Z gene was interrupted by insertion of our target gene, X-gal could not be metabolized by white recombinant colonies. Therefore white colonies from the plate were choosen to continue further experiments.

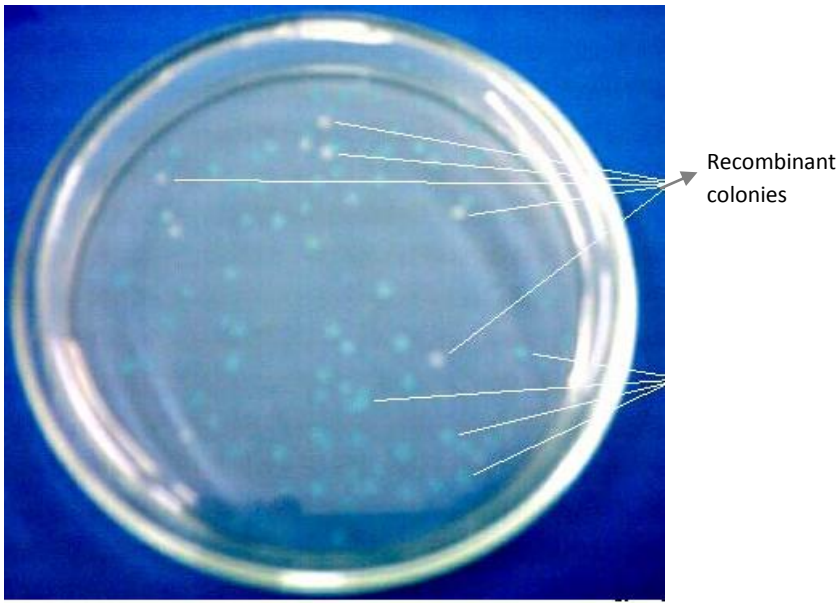

Figure 3.3. White colonies on LB- amp plate indicating recombinant *E.coli* DH5α cells including inserted gene

For the confirmation purpose, four white colonies were chosen from transformation plate and plasmids were isolated from them. They were sequenced and compared with each other, then the complete sequence of insert was determined. The length of the target gene cloned into PTZ57R/T plasmid was found as to be 1542 bp. It was compared with the referans gene seguence in figure 3.4. Sequence alignment between the referans gene (*Geobacillus thermoleovorans subsp. stromboliensis* strain P220, GenBank A.C HM034453.1) and our target gene sequences showed that overlap ratio is ~ 97%. Also, the protein sequence was constructed and the molecular weight of our protein was calculated as 59.9 kD.

```
Source          ATGGAAATGGGGAACCGGCTCTTTATGCTGCTCGTCCTTCCGTTCCTTCTTTTTTATGCC 60
α-amylase       ATGGAAATGGGGAACCGGCTCTTTATGCTGCTCGTCCTTCCGTTCCTTCTTTTTTATGCC 60
                ************************************************************

Source          ATGCCGGCTGCGGCGGCGGAAAAAGAAGAACGGACGTGGCAAGATGAAGCCATTTATTTC 120
α-amylase       ATGCCGGCTGCGGCGGCGGAAAAAGAAGAACGGACGTGGGAAGATGAAGCCATTTATTTC 120
                ************************************ ********************

Source          ATTATGGTCGACCGGTTTAACAATATGGATCCGACAAACGACCAGAACGTGAATGTGAAC 180
α-amylase       ATTATGGTCGACCGGTTTAACAATATGGATCCGACAAACGACCAGAACGTGAATGTGAAC 180
                ************************************************************

Source          GATCCGAAAGGGTATTTCGGCGGCGACTTGAAAGGGGTGACGGCGAAACTCGATTACATC 240
α-amylase       GATCCGAAAGGGTATTTCGGCGGCGACTTGAAAGGGGTGACGGCGAAACTCGATTACATC 240
                ************************************************************

Source          AAGGAGATGGGATTTACCGCCATTTGGCTGACGCCGATTTTTAAAAACATGCCGGGCGGT 300
α-amylase       AAGGAGATGGGATTTACCGCCCTTTGGGTGACGCCGATTTTTAAAAACATGCCGGGGGGG 300
                ******************** ***** ************************** **

Source          TATCATGGCTATTGGATTGAAGATTTTTATCAAGTCGATCCGCACTTTGGCACGCTGGGC 360
α-amylase       TATCATGGCTATTGGATTGAAGAATTTTATCAAGTCCATCCGCACTTTGGGACGCTGGGG 360
                ********************** ************ ************* ********

Source          GATTTGAAAACACTCGTCAAAGAAGCGCATAAGCGCGACATGAAAGTCATTTTGGATTTT 420
α-amylase       GATTTGAAAAAACTCCCCAAAAAAACGCATAAACGCGACATGAAAGGCATTTTGGAATTT 420
                ********** ****  **** ** ******* ************* ********* ***

Source          GTCGCCAACCATGTCGGTTACAATCACCCATGGTTACATGACCCAACGAAAAAAGATTGG 480
α-amylase       GTCGCCAACCATGGCGGGTACAATCACCCCTGGGTACATGACCCCACCAAAAAAAAATGG 480
                ************* *** *********** *** ********** ** ****** * ***

Source          TTTCACCCGAAAAAAGAGATTTTCGACTGGAACGACCAAACACAGCTTGAAAACGGCTGG 540
α-amylase       TTTCTCCCCAAAAAAGAAAATTTCTACTGGGACGACCCAACCCCGCTTGAAAACGGCTGG 540
                **** *** ******** * **** ***** ****** *** * ****************

Source          GTGTATGGGTTGCCTGATTTGGCGCAGGAAAATCCAGAGGTCAAAACGTATTTAATTGAC 600
α-amylase       GTGTATGGGTTGCCTGATTTGGCGCAGGAAAATCCAGAGGTCCAAACGTATTTAATTGAC 600
                ****************************************** *****************

Source          GCTGCCAAATGGTGGATTAAAGAGACCGACATTGACGGTTACCGGCTCGATACAGTGCGC 660
α-amylase       GCTGCCCAATGGTGGATTAAAGAGACCGACATTGACGCTTACCGGCTCGATACAGTGCGC 660
                ****** ***************************** **********************

Source          CACGTGCCAAAATCGTTTTGGCAGGAGTTTGCGAAAGAAGTCAAATCGGTGAAAAAAGAC 720
α-amylase       CACGTGCCAAAATCGTTTTGGCAGGAGTTTGTGAAAGAAAACAAATCGGTGAAAAAAGAC 720
                ******************************** *******  ******************

Source          TTTTTCCTTCTCGGTGAAGTGTGGAGCGACGATCCGCGCTATATTGCCGATTACGGGAAG 780
α-amylase       TTTTTCCTTCTCTGTGAAGTGTGGAGCGACGATCCGCGCTATATTGCCGATTACGGAAAG 780
                ************ ****************************************** ***

Source          TATGGCATCGACGGGTTTGTCGATTATCCGCTGTATGGCGCGGTGAAGCAGTCGCTTGCG 840
α-amylase       AATGGCATCGACGGGTTTGTCGATTATCCGCTGTATGGCGCGGTGAAGCAGTCGCTTGCG 840
                ************************************************************

Source          AGGCGCGATGCCTCGCTCCGCCCGCTGTATGATGTCTGGGAATACAACAAAACGTTTTAC 900
α-amylase       AGGCGCGATGCCTCGCCCCCCCCCCTGTATGATGTCTGGGAATACAACAAAACGGTTTAC 900
                **************** ** *** ****************************** *****

Source          GACCGACCGTATTTGCTCGGGTCGTTTTTGGACAACCATGATACCGTGCGGTTTACGAAG 960
α-amylase       GATCGACCGCATTTGCTCGCGTCGTTTTTGGACAACCATGATACCGTGCGGTTTACGAAG 960
                ** ****** ********* ****************************************

Source          CTCGCGATTGACAACCGCAACAACCCGATTTCACGCATTAAACTGGCCATGACGTATTTG 1020
α-amylase       CTCGCGATTGACAACCGCAACAACCCGATTTCACGCATTAAACTGGCCATGACGTATTTG 1020
                ************************************************************
```

Figure 3.4. Nucleotide sequence comparison of inserted gene and the source (*Geobacillus thermoleovorans subsp. stromboliensis* strain P220)

(Cont. on next page)

```
Source          TTCACCGCCCCTGGCATCCCGATCATGTATTACGGGACCGAAATCGCCATGAACGGCGGC 1080
α-amylase       TTCACCGCCCCTGGCATCCCGATCATGTATTACGGGACCGAAATCGCCATGAACGGCGGC 1080
                ************************************************************

Source          CAAGATCCGGACAACCGCCGTCTGATGGATTTCCGCGCCGATCCAGAAATCATCGATTAC 1140
α-amylase       CAAGATCCGGACAACCGCCGTCTGATGGATTTCCGCGCCGATCCAGAAATCATCGATTAC 1140
                ************************************************************

Source          TTGAAAAAAATCGGCCCGCTTCGCCAAGAGCTGCCATCATTGCGGCGCGGCGATTTTACG 1200
α-amylase       TTGAAAAAAATCGGCCCGCTTCGCCAAGAGCTGCCATCATTGCGGCGCGGCGATTTTACG 1200
                ************************************************************

Source          CTGTTGTATGAAAAAGACGGCATGGCGGTGTTGAAACGGCAATATCAAGATGAAACGACG 1260
α-amylase       CTGTTGTATGAAAAAGACGGCATGGCGGTGTTGAAACGGCAATATCAAGATGAAACGACG 1260
                ************************************************************

Source          GTCATCGCCATCAACAATACGAGCGAAACGCAGCATGTCCATCTCACCAATGACCAGTTG 1320
α-amylase       GTCATCGCCATCAACAATACGAGCGAAACGCAGCATGTCCATCTCACCAATGACCAGTTG 1320
                 ***********************************************************

Source          CCAAAAAACAAAGAACTGCGCGGCTTTTTATTGGACGATCTCGTCCGCGGCGATGAGGAC 1380
α-amylase       CCAAAAAACAAAGAACTGCGCGGCTTTTTATTGGACGATCTCGTCCGCGGCGATGAGGAC 1380
                ************************************************************

Source          GGCTACGACCTTGTGCTCGACCGCGAAACGGCGGAAGTATACAAGCTGCGGGAGAAAACA 1440
α-amylase       GGCTACGACCTTGTGCTCGACCGCGAAACGGCGGAAGTATACAAGCTGCGGGAGAAAACA 1440
                ************************************************************

Source          GGGATCAACATCCCGTTTATCGCCGCCATCGTATCGGTTTACGTGCTGTTTCTTTTGTTT 1500
α-amylase       GGGATCAACATCCCGTTTATCGCCGCCATCGTATCGGTTTACGTGCTGTTTCTTTTGTTT 1500
                ************************************************************

Source          TTATATTTGGTGAAAAAACGGGCAAAACGGATCAATGAATAA 1542
α-amylase       TTATATTTGGTGAAAAAACGGGCAAAACGGATCAATGAATAA 1542
                ******************************************
```

Figure 3.4. (cont.)

The protein sequence of our enzyme matched up by 95% score with the source gene. Protein sequence comparison of our enzyme and the source enzyme was shown in figure 3.5.

```
α-Amylase    1 MEMGNRLFMLLVLPFLLFYAMPAAAAEKEERTWEDEAIYFIMVDRFNNMDPTNDQNVNVN
Source       1 MEMGNRLFMLLVLPFLLFYAMPAAAAEKEERTWQDEAIYFIMVDRFNNMDPTNDQNVNVN
               ********************************* ************************

α-Amylase   61 DPKGYFGGDLKGVTAKLDYIKEMGFTALWVTPIFKNMPGGYHGYWIEEFYQVHPHFGTLG
Source      61 DPKGYFGGDLKGVTAKLDYIKEMGFTAIWLTPIFKNMPGGYHGYWIEDFYQVDPHFGTLG
               *************************** * ***************** **** *******

α-Amylase  121 DLKKLPKKTHKRDMKGILEFVANHGGYNHPWVHDPTKKKWFLPKKENFYWDDPTPLENGW
Source     121 DLKTLVKEAHKRDMKVILDFVANHVGYNHPWLHDPTKKDWFHPKKEIFDWNDQTQLENGW
               *** * *  ****** ** ***** ****** ****** ** **** * * * * *****
```

Figure 3.5. Protein sequence comparison of our protein and the source (*Geobacillus thermoleovorans subsp. stromboliensis* strain P220)

(Cont. on next page)

```
α-Amylase 181 VYGLPDLAQENPEVQTYLIDAAQWWIKETDIDAYRLDTVRHVPKSFWQEFVKENKSVKKD
Source    181 VYGLPDLAQENPEVKTYLIDAAKWWIKETDIDGYRLDTVRHVPKSFWQEFAKEVKSVKKD
              ************** ******* ********* ***************** ** ******

α-Amylase 241 FFLLCEVWSDDPRYIADYGKNGIDGFVDYPLYGAVKQSLARRDASPPPLYDVWEYNKTVY
Source    241 FFLLGEVWSDDPRYIADYGKYGIDGFVDYPLYGAVKQSLARRDASLRPLYDVWEYNKTFY
              **** *************** ************************  *********** *

α-Amylase 301 DRPHLLASFLDNHDTVRFTKLAIDNRNNPISRIKLAMTYLFTAPGIPIMYYGTEIAMNGG
Source    301 DRPYLLGSFLDNHDTVRFTKLAIDNRNNPISRIKLAMTYLFTAPGIPIMYYGTEIAMNGG
              *** ** ************************************************

α-Amylase 361 QDPDNRRLMDFRADPEIIDYLKKIGPLRQELPSLRRGDFTLLYEKDGMAVLKRQYQDETT
Source    361 QDPDNRRLMDFRADPEIIDYLKKIGPLRQELPSLRRGDFTLLYEKDGMAVLKRQYQDETT
              ************************************************************

α-Amylase 421 VIAINNTSETQHVHLTNDQLPKNKELRGFLLDDLVRGDEDGYDLVLDRETAEVYKLREKT
Source    421 VIAINNTSETQHVHLTNDQLPKNKELRGFLLDDLVRGDEDGYDLVLDRETAEVYKLREKT
              ************************************************************

α-Amylase 481 GINIPFIAAIVSVYVLFLLFLYLVKKRAKRINE
Source    481 GINIPFIAAIVSVYVLFLLFLYLVKKRAKRINE
             *********************************
```

Figure 3.5. (cont.)

After digestion of the PTZ57R/T vector carrying insert gene with NdeI and HindIII restriction enzymes, agarose gel electrophoresis was performed for confirmation of digestion. Target sequence was isolated with gel extraction method. Then, isolated α-amylase gene sequence was ligated with expression vector (pET28a+ plasmid). In the following step transformation was carried out by using compotetent *E.coli* BL21 cells.

Protein expression procedure was applied to these recombinant cells and expressed protein was tried to be purified with Ni-affinity chromotography. Unfortunately, obtained protein amount was not sufficient for characterization studies. Large amount of target protein was left in the pellet. It might be because of aggregation or misfolding of the protein. The reason for inadequate protein purification in supernatant could be because of histidine residues found inside of the protein ring resulting in blocking nickel binding to the protein.

3.3.2. Coexpression with Chaperone Plasmids

Chaperone plasmids may be used to overcome misfolding of expressed protein. In our studies, five chaperone plasmids were used to eliminate low expression of amylase protein. Chaperone plasmids were originated from heat shock proteins therefore these

choosen plasmid kits were useful to increase expression level of our termophilic protein. For the first step of coexpression , *E. coli* BL21 cells containing pET28a+ with target gene were made compotent to take in the plasmid. Then, chaperone plasmids were inserted into these cells seperately. After that protein expression procedure was employed according to TAKARA kit. Next, cells were disrupted and centrifuged. Supernatants and pellets of cells were loaded on SDS-PAGE and protein profiles were photographed as seen in figure 3.6. As a result, concentration of the target protein was increased considerably by employing chaperone 3.

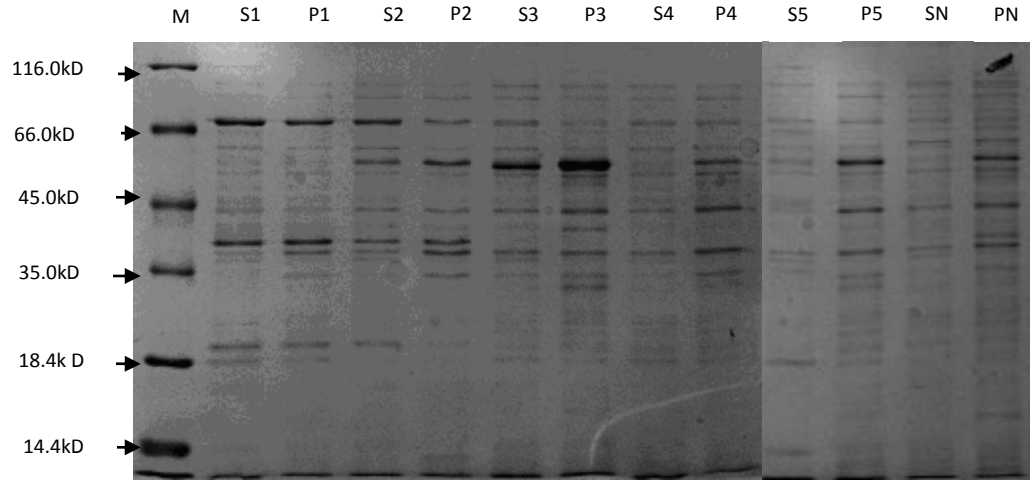

Figure 3.6. SDS-PAGE image that shows effects of different chaperones on protein expression: **M:** protein marker, **S1:** chaperone1supernatant, **P1:** chaperone1pellet, **S2:** chaperone2 supernatant, **P2:** chaperone2 pelet, **S3:** chaperone3 supernatant, **P3:** chaperone3 pelet, **S4:** chaperone4 supernatant, **P4:** chaperone4 pelet, **S5:** chaperone5 supernatant, **P5:** chaperone5 pelet, **SN**: non-coexpressed protein in supernatant, **PN:** non-coexpressed protein in pellet

3.4. Protein Expression and Purification

Protein exppession procedure was employed by using with cahperone 3. Unfortunately, a good yield of protein was not achieved with application of affinity chromotography as seen in figure 3.7. It seems that the target protein has no binding

capacity to Ni-affinity column. Therefore, aternative method namely ion exchange and size exclussion chromotography were employed for the protein purification.

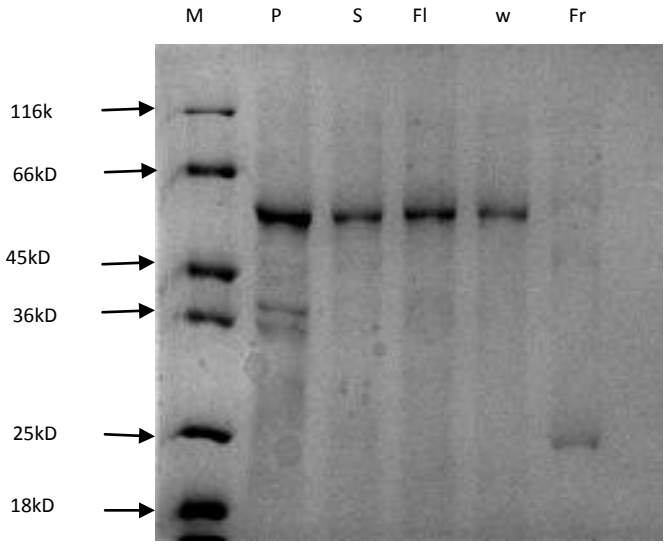

Figure 3.7. SDS-PAGE image of purification of coexpressed protein with affinity chromotography; **M:** protein marker, **P:** pelet (before purification), **S:** supernatant (before purification), **Fl:** flowthrough, **W:** washing solution, **Fr:** collected fraction

First, the protein was tried to be purified by using ion exchange chromotography. After washing step, elution was done with a gradient concentration of NaCl. The proteins were seperated according to their ionic strenght. Collected fractions were first run on SDS-PAGE to visualize the target protein. Fractions containing target protein were incorporated and loaded to Sephadex G-100 gel filtration column. After that, SDS-PAGE was performed again for confirmation of purification. 30 ml of amylase protein was obtained after overall prufication procedure. It was concentrated to 10 ml at 40ºC in vacuum oven. Then, the concentration of purified protein was found as 3mg/ml by measurements with nanodrop instrument. Concentrations of our purified protein was also measured by employing Bradford method. According to the standart curve constructed with optical densities of different concentrations of BSA at 595 nm, concentration of our purified enzyme was found to be 1,1252 mg/ml. So, obtained total protein was 11.252 mg.

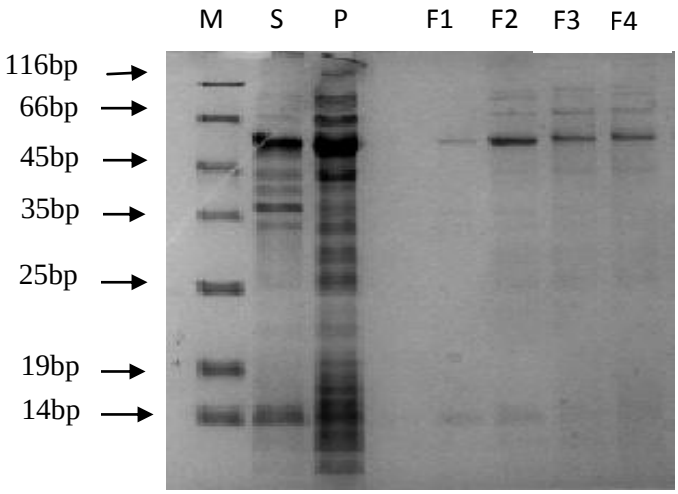

Figure 3.8. SDS-PAGE image of unpurified and purified protein M: protein marker, S:unpurified supernatant, P: unpurified pellet, F1, F2, F3, F4: fractions after ion-exchange chromotography

3.5. Screening of Amylase Activity on Agar Plate

Purified protein was spotted on 1% starch agar. Afterwards it was dyed with KI/I solution. White zones indicating amylase activity were formed (Figure 3.9). It was a preliminary test to screen amylase activity.

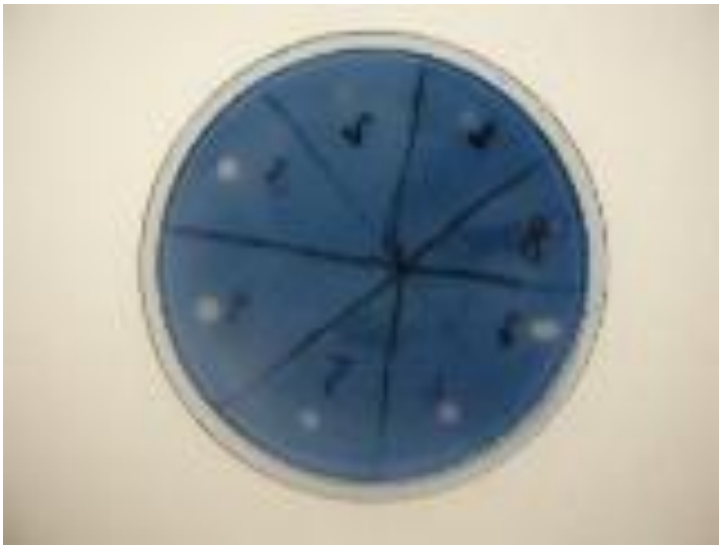

Figure 3.9. Amylase activity screening on stach agar plate

3.6. Protein Identification and Mass Spectrometric Analysis

For mass spectrometric analysis of our protein spot on the SDS-PAGE gel, the protein was digested into its peptides. After in-gel digestion, the peptides were analysed in the MALDI-TOF mass spectrophotometry instrument. 1776 D peptid of our protein matched with α-amylase family protein of *Geobacillus thermodenitrificans NG80-2* with NCBInr database. Protein sequence coverage and Mascot Mowse score were found to be as 3%, and 57, respectively. Nominal mass and pI of identified protein were found to be 59855 D and 5.66, respectively.

The sequences of identified protein in mass spectometric analysis and the sequence of our protein constructed were compared (Figure 3.10). Their complete sequence were matched with 92% ratio. Mached peptides in mass spectrometric analysis are shown in bold.

```
identified  MGNRLFMLFILPFLLFYAMPVAAAEKEERTWQDEAIYFIMVDRFNNMDSTNDQDVNVNDP  60
our protein MGNRLFMLLVLPFLLFYAMPAAAAEKEERTWEDEAIYFIMVDRFNNMDPTNDQNVNVNDP  62

identified  KGYFGGDLKGVTAKLDYIKEMGFTAIWLTPIFKNRPGGYHGYWIEDFYEVDPHFGTLDDL  120
our protein KGYFGGDLKGVTAKLDYIKEMGFTALWVTPIFKNMPGGYHGYWIEEFYQVHPHFGTLGDL  122

identified  KTLVKEAHKRDMKVILDFVANHVGYDHPWLHDPAKKDWFHPKKEIFDWNSQEQVENGWVY  180
our protein KKLPKKTHKRDMKGILEFVANHGGYNHPWVHDPTKKKWFLPKKENFYWDDPTPLENGWVY  182

identified  GLPDLAQENPEVKNYLIDAAKWWIKETDIDGYRLDMVRHVPKSFWQEFAKEVKAVKKDFF  240
our protein GLPDLAQENPEVQTYLIDAAQWWIKETDIDAYRLDTVRHVPKSFWQEFVKENKSVKKDFF  242

identified  LLGEVWSDDPRYIAD**YGKYGIDGFVDYPLYGAVK**QSLAKRDASLRPLYDVWEYNKTFYDR  300
our protein LLCEVWSDDPRYIAD**YGKNGIDGFVDYPLYGAVK**QSLARRDASPPPLYDVWEYNKTVYDR  302

identified  PYLLGSFLDNHDNVRFTKLVIDHRNNPISRMKVAMTYLFTAPGIPIMYYGTEIAMTGGPD  360
our protein PHLLASFLDNHDTVRFTKLAIDNRNNPISRIKLAMTYLFTAPGIPIMYYGTEIAMNGGQD  362

identified  PDNRRLMDFRADPEIIDYLKKVGPLRQQLPSLRRGDFTLLYEQDGMAVFKRQYKDETTVI  420
our protein PDNRRLMDFRADPEIIDYLKKIGPLRQELPSLRRGDFTLLYEKDGMAVLKRQYQDETTVI  422

identified  AINNTSETKHVHLTNEQLPKNKELRGFLLDDLVRGDEDGYDIVLDRETAEVYKLRNKTGV  480
our protein AINNTSETQHVHLTNDQLPKNKELRGFLLDDLVRGDEDGYDLVLDRETAEVYKLREKTGI  482

identified  NVPFIVAMVAVYALFILFLYMVKKRTKRTNE  511
our protein NIPFIAAIVSVYVLFLLFLYLVKKRAKRINE  513
```

Figure 3.10. Sequence comparison of our protein with the protein (α-amylase family protein of *G.thermodenitrificans* NG80-2) identified in mass spectrometry analysis.

3.7. Protein Characterization

3.7.1. Optimum pH and Temperature

Optimum pH and temperature values for the purified enzyme were determined by using the amylase assay based on Somogyi and Nelson (Nelson 1944) method. Relative enzyme activity was measured at different pHs and temperatures ranging from 3 to 9 and 40°C to 90°C, respectively. According to our results shown in Figure 3.11, the enzyme showed 65% activity at pH 5 and 70% activity at pH 9. Optimum activity was obtained at pH 8 as considered 100% activity. On the other hand, as seen in Figure 3.12, the enzyme showed activity in range of 50-70°C, but the optimum temperature for enzyme activity was determined as to be 60°C. When the optimum pH and temperature values for α-amylase were compared with other studies, similarities and differences were observed. Optimum pHs and temperatures of amylase proteins obtained from some bacterial strains are listed as follows: from *Bacillus ferdowsicous*; pH 4.5, 70°C (Asoodeh A. et al 2010), from *Thermococcus profoundus;* pH 4-5, 80°C (Kwak et al. 1998), from *Lactobacillus manihotivorans;* pH 5.5, 55°C (Aguilar et al. 2000) from *Geobacillus caldoxylosilyticus* TK4; pH 7.0, 50°C (Kolcuğlu et al. 2010), from *Bacillus* sp. US100; pH 5.6, 80°C (Ben Ali et al. 1999), from *Anoxybacillus sp*.; pH 8.0, 60°C (Chai et al. 2012), from *Bacillus amyloliquefaciens;* pH 7.0, 70°C (Underkofler 1976), from *Pyrococcus furiosus;* pH 5.5, 100°C (Laderman et al. 1993a,b), from *Thermococcus profoundus;* pH 4.0–5.0, 80°C (Kwak et al. 1998).

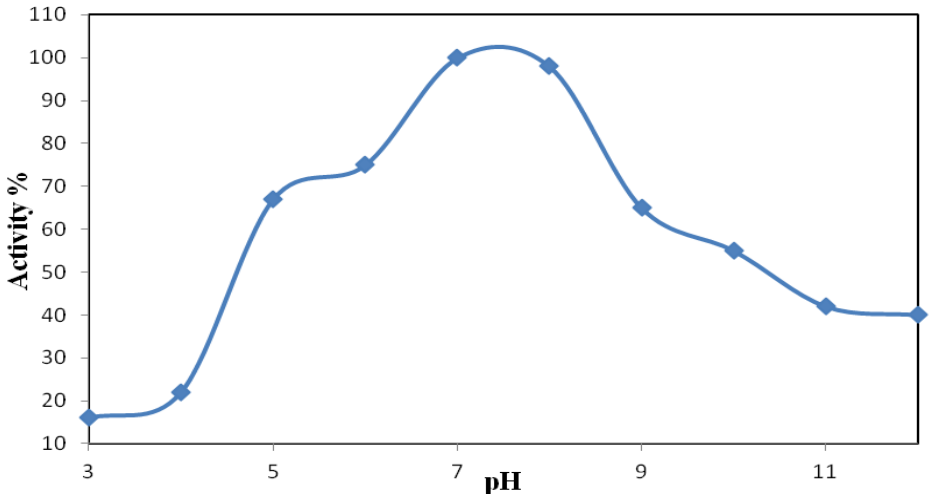

Figure 3.11. Effect of pH on amylase enzyme activity (at 55ºC)

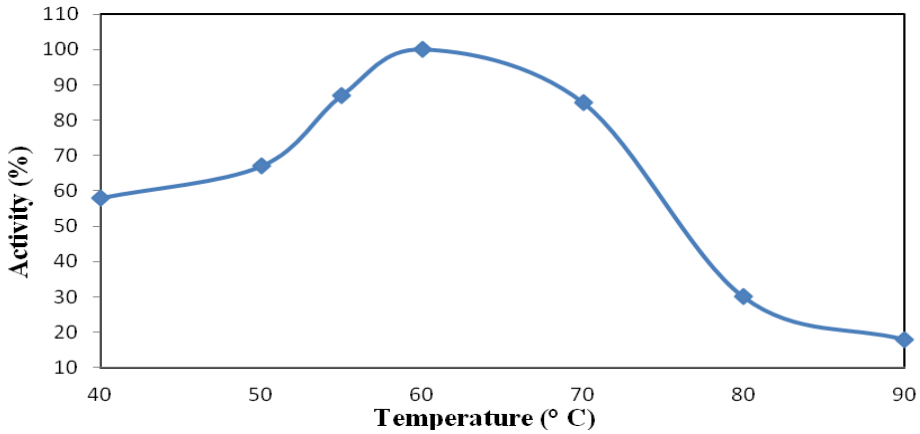

Figure 3.12. Effect of temperature on amylase enzyme activity (pH 8)

3.7.2. pH and Temperature Stabilities

Stability of the enzyme at different pHs and temperatures were determined in different time intervals. pH stability was studied by incubating the enzyme in pH 7 and 9 buffers at 65°C for 6, 16 and 24 hours. After 6 hours, the enzyme retained 80 and 75% of its activity at pH 7 and 9, respectively. After incubation of the enzyme at 60 and

80°C at pH 8 for 1, 2, 3, 4, 5, 6, 12, 16 and 24 hours, enzyme assay was performed and residual activity was measured. According to our results, enzyme activity was so high for 6 hours. At the end of 5 and 6 hours it retained 94 and 89% of activity, respectively. On the other hand, in 85°C its residual activity was 85% after 2 hours, then, it was gradually decreased to 60% at the end of 12 hours. When the literature was examined; α-amylase obtained from *Geobacillus caldoxylosilyticus TK4* was incubated in different pH buffers at 50°C and residual activity was obtained >90% at different pHs from pH 3 to 9 after 7 days. While incubating at 80°C and pH7, 80% residual activity was seen after 3 days in the same study (Kolcuoğlu et al. 2010). For α-amylase of *Bacillus* sp. US100, the activity was completely stable at 80°C for at least 90 minutes (Ben Ali 1999). α-Amylase from marine haloalkaliphilic *Saccharopolyspora sp*. A9 retained 90% of its activity for 12 hours at pH10 and the activity was decreased gradually to 80% after 24 hours (Chakraborty et al. 2010).

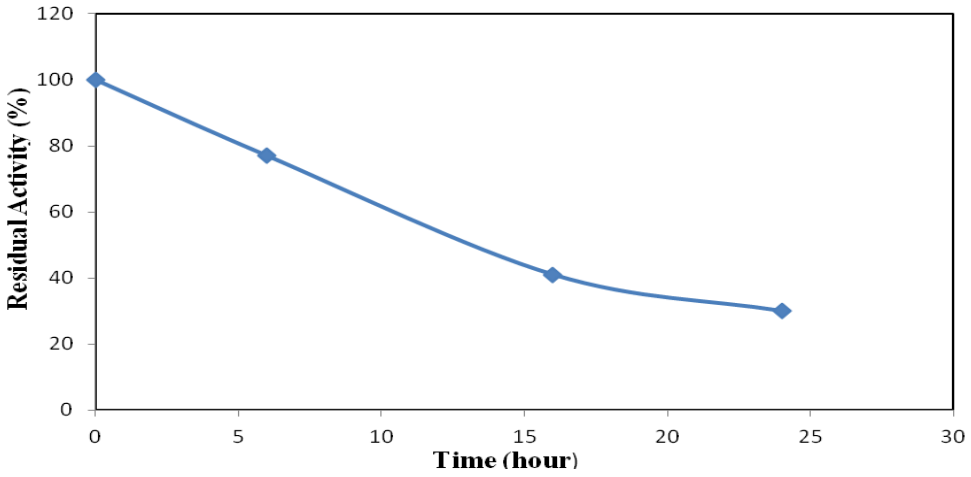

Figure 3.13. Stability of amylase activity at pH 7 for 24 hours (at 60ºC)

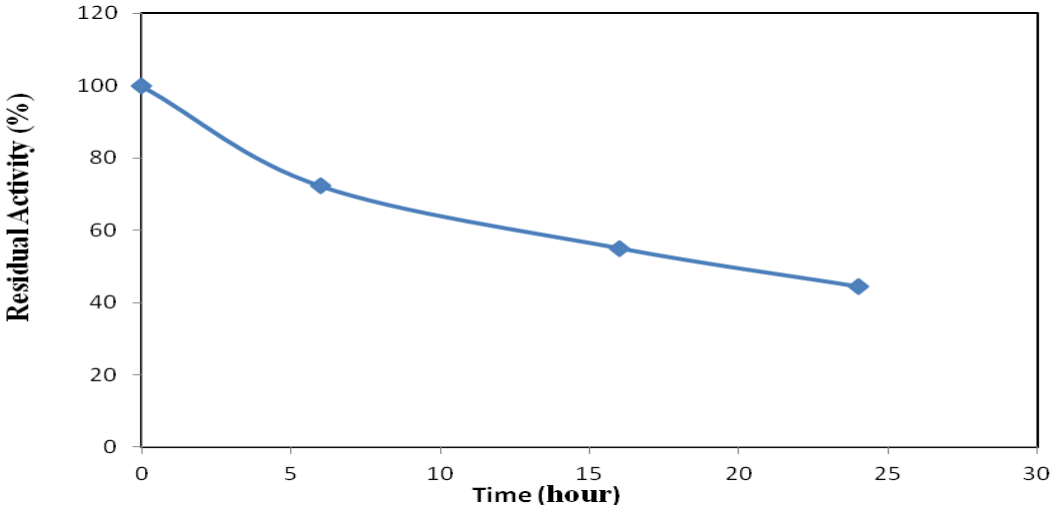

Figure 3.14. Stability of amylase activity at pH 9 for 24 hours (at 60ºC)

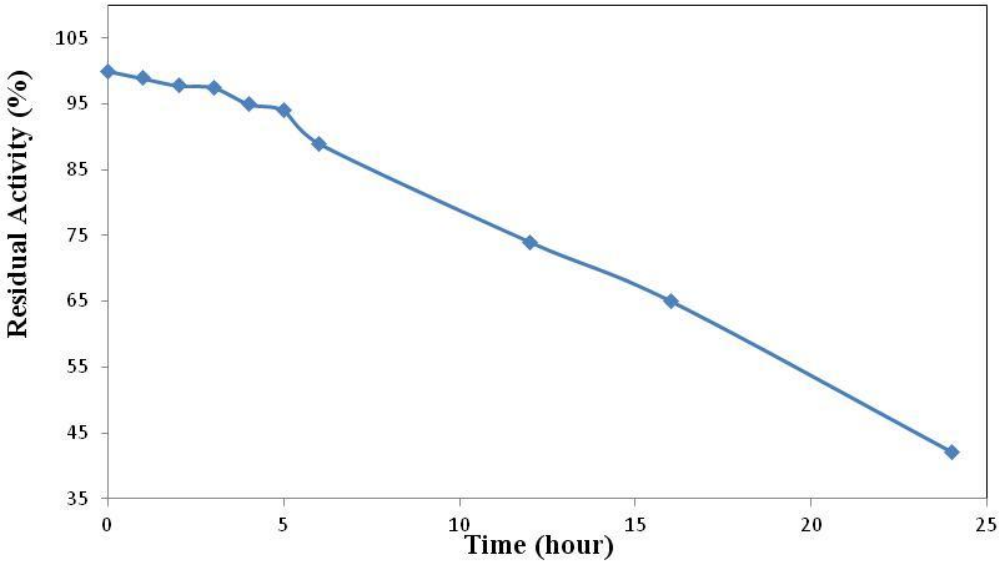

Figure 3.15. Stability of the amylase activity at 65°C for 24 hours (pH 8)

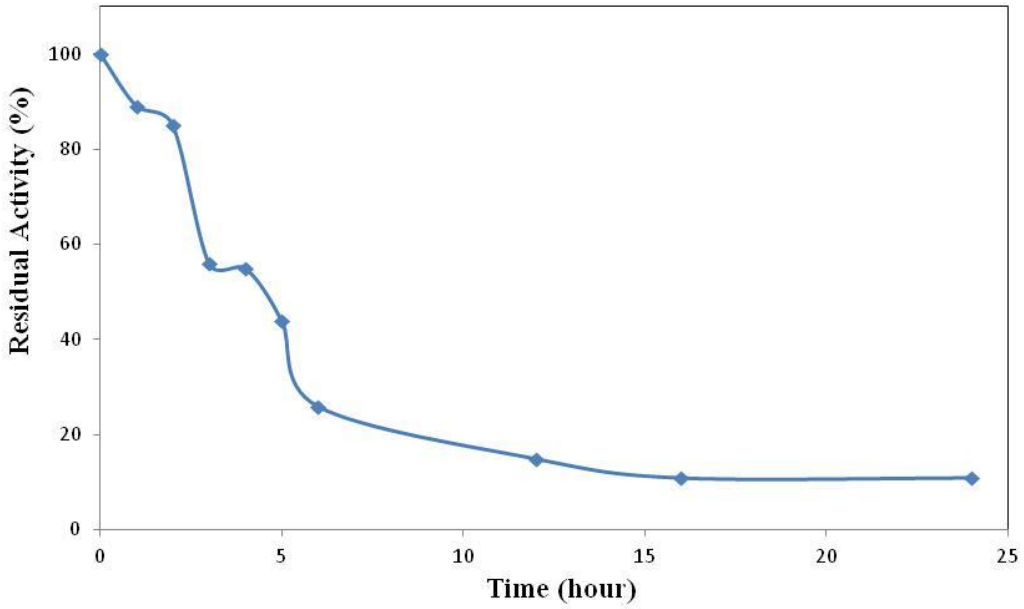

Figure 3.16. Stability of the amylase activity at 85°C for 24 hours (pH 8)

3.7.3. Effect of Metal Ions on Enzyme Activity

In order to determine the effect of metal ions on enzyme activity, the enzyme was incubated with 10mM and 25mM metal salts for 10 minutes. Then the enzyme assay was applied and the enzymatic activity was measured. The results were shown in Figure 3.17. According to the results the activity was increased to 166, 141 and 12 by Co^{+2}, Ca^{+2} and Mg^{+2}, respectively. While Na^{+} and K^{+} ions showed no significant effect, relative activity was decreased slightly by Cu^{+2}. According to the literature Ca was typically found to be as inducer of α-amylase enzyme activity. Chakraborty et al. (2010) measured relative activity as 110.21% with 10mM Co^{+2}, 117% with 10mM Cu^{+2}, 142% with 10mM Ca^{+2}. In another study (Kolcuoğlu et al. 2010), the activity was inhibited 25% by Co^{+2} whereas the activity was induced 150 and 110% by Mn^{+2} and Ca^{+2} , respectively. By contrast, the activity of a thermoactive α-amylase from Bacillus sp. was fairly degreased in presence of Ca^{+2} and Mg^{+2} (Pancha et al. 2010).

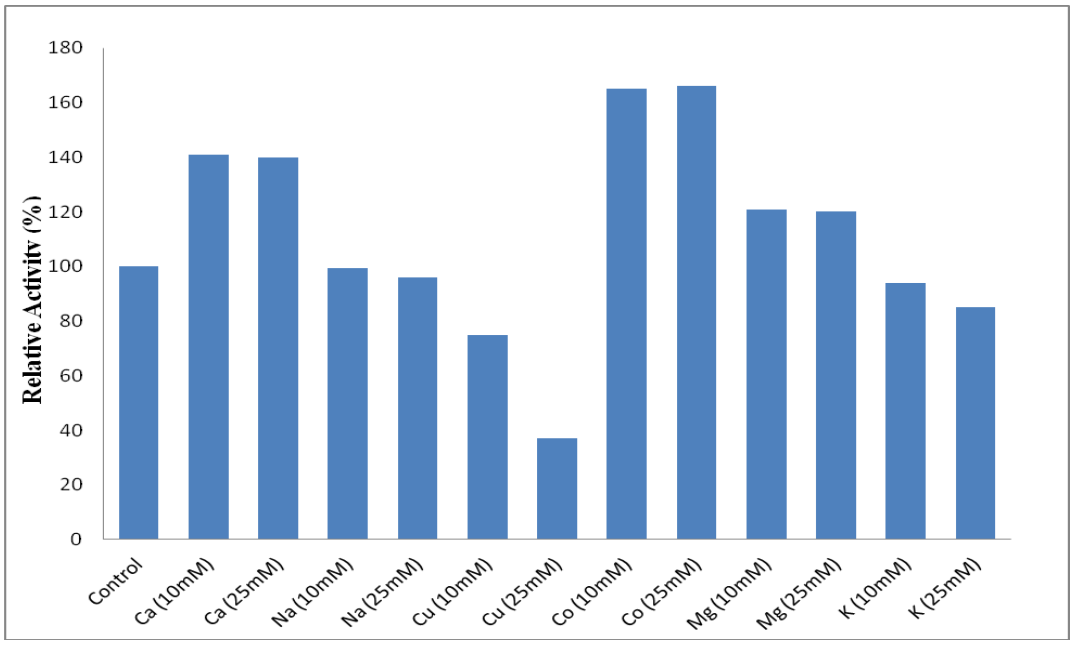

Figure 3.17. Effect of metal ions on enzyme activity

3.7.4. Effect of Various Agents on Enzyme Activity

The effect of the organic solvents, surfactans and some agents on the enzyme activity was investigated to gather information on whether they inhibit or activate the enzyme. Seperately 10% organic solvents, 1% surfactants and 1mM agents were added to the enzyme solution. The samples were incubated for 10 minutes at 60°C. Then, the enzyme assay was performed. The results showed that acetone had excessive inhibition and methanol had no significant effect on the activity of the enzyme. PMSF, DDT and Triton X-100 had no effect on enzyme activity. On the other hand, the enzyme activity was slighty inhibited by SDS.

In the literature, α-amylase from *Anoxybacillus sp.* was investigated in presence of some agents and relative activities were listed as; b-Mercaptoethanol (50 mM) 67%, Urea (8 M) 53%, SDS (1%, w/v) 3%, Triton X-100 (5%, v/v) 119%, EDTA (5 mM) 4% (Chai et al. 2012). In another study, iodoacetic acid, N-bromosuccinic acid, SDS, guanidine hydrochloride were investigated and found to be as inhibitors for *T. profundus* DT5432 amylase (Chung et al. 1995).

Table 3.1. Effect of various agents on enzyme activity

Agent	Concentration	Relative Activity (%)
None		100
Hexane	10%	82.70
Methanol	10%	98.0
Ethanol	10%	85.0
Acetone	10%	55.12
PMSF	1mM	100.3
DDT	1mM	100.16
EDTA	1mM	87.73
SDS	1%	36.0
Tween 20	1%	60.6
TritonX-100	1%	102.8
BME	1%	93.3

CHAPTER 4

CONCLUSION

The aim of this study was to isolate amylase enzyme from thermophilic *Bacillus* sp. by using molecular cloning methods, purify and characterize the isolated enzyme. In this context, a thermophilic *Bacillus* strain isolated from Balçova Geotermal region in İzmir was used as the starting material for the experiments. The strain was previously characterized by Elif Yavuz.

The strain number-33 was also determined as to produce amylase enzyme in her study. Firstly, the amylase activity was verified by performing starch-iodine test. The clear zones indicated amylase activity was formed around the colonies on starch agar plate. α-Amylase coding gene from this microorganism was cloned to compotent *E.coli* BL21 cells by using molecular methods. Recombinant cells were cultured and protein expression was stimulated by IPTG addition. The expressed enzyme was purified by performing ion exchange chromatograpy using a weak anion exchange (DEAE-Cellulose) column and also size exclussion chromotography using G-100 Sephadex column. Purified enzyme was identified by mass specrometry and matched with α-amylase family protein of *Geobacillus thermodenitrificans* NG80-2. Aminoacid sequence similarity of the identified enzyme and our purified enzyme was found to be 92%.

In the enzyme characterization studies, the results indicated that the amylase enzyme had the highest activity at pH 8.0 and 60°C. After 6 hours the enzyme retained 80% of its activity at pH 7 and 75% of activity at pH 9. The enzyme is stable for 6 hours at 65°C and 2 hours at 85°C. According to the results Co^{+2}, Ca^{+2}, Mg^{+2} increased the enzyme activity. While Na^{+} and K^{+} ions did not have important effect on enzyme, Cu^{+2} slightly decreased the relative activity. Resultant enzyme was found to be stable in the presence of 10% methanol, 1mM PMSF, 1mM DDT, 1% Triton X-100. The relative activity was considerably decreased in presence of 1% SDS and 1% Tween 20.

In conclusion, the amylase enzyme isolated from alkaliphilic and thermophilic *Bacillus sp.* was cloned, expressed, purified and characterized during our studies.

In the further studies, this enzyme features could be improved by protein engineering efforts in order to utilize in several biotechnological processes. Moreover the immobilization studies using various organic or inorganic supports could be appllied to the enzyme in order to be used in industrial processes.

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APPENDIX A

MEDIA, BUFFERS, REAGENTS AND SOLUTIONS

A.1. Media and Buffers

- **Luria Bertani (LB) Medium**

10 g tryptone, 5 g yeast extract, 5 g NaCl were dissolved in dH2O up to 1 L and autoclaved at 121°C for 15 minutes.

- **Luria Bertani (LB) Agar**

10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar were dissolved in dH2O up to 1 L and autoclaved at 121°C for 15 minutes.

- **SOC Media**

2.0 g Tryptone, 0.5 g of Yeast Extract, 1.0 ml of 1.0 M NaCl, 0.25 ml of 1.0 M KCl, 1.0 ml of 2.0 M Mg^{2+} and 1.0 ml of 2.0 M Glucose were dissolved in dH2O up to 100.0 ml and sterilized**.**

- **SOB Medium**

2 g Tryptone, 0.5 g Yeast Extract, 1 ml 1M NaCl, 0.02 g KCl, 1 ml 1M MgCl, 1 ml 1M MgSO4 were dissolved in deionized dH2O up to 100 ml and autoclaved at 121°C for 15 minutes.

- **Starch Agar Media**

0.5g yeast extract, 2.5g soluble starch and 7.5g agar agar was dissolved in 500ml distilled water and sterilised by autoclaving at 121°C for 15 minutes.

- **TB Medium, per 100 ml**

0.3 g PIPES, 3 ml 1 M CaCl2 and 1.85 g KCl were dissolved in 100 ml deionized water and the solution pH was adjusted to 6.7 with KOH. Then 1.4 g MnCl2 was added and the solution was filtered sterilized.

- **50 X TAE**

242 g Tris base was dissolved in deionized water, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) were added. Volume was adjusted to 1000 ml with deionized water.

- **Citrate Buffer; pH 3.0, pH 4.0, pH 5.0, pH 6.0**

Citrate buffer (Gomori 1955) stock solutions: A: 0.1 M citric acid; B: 0.1 M sodium citrate. Using the following amounts from these stock solutions and diluting them to 100.0 ml with 50.0 ml dH2O, the following buffer systems were prepared. Finally, to obtain 50.0 mM concentration, one more 1:2 dilutions were applied.

Volume of 0.1 M Citric acid, ml	Volume of 0.1 M Sodium citrate (ml)	Desired pH
45.6	3.5	3.0
33.0	17.0	4.0
20.5	29.5	5.0

Volume of 1.0 M Na_2HPO_4, ml	Volume of 1.0 M NaH_2PO_4, ml	Desired pH
57.7	42.3	7.0

- **Sodium Phosphate Buffer, 0.1 M; pH 7.0**

Indicated amounts of stock solutions were mixed and diluted as 1:2 to obtain 50.0 mM buffer system at pH 7.0.

- **Tris-Cl Buffer, 1.0 M; pH 8.0**

121.1 g of Tris base was dissolved in 700.0 ml of dH_2O. Concentrated HCl was added until the pH reached to 8.0. The solution was filled up to 1.0 L with dH_2O. Finally, the stock solution was diluted to 50.0 mM with dH_2O.

- **Iodine solution**

2 g Potassium iodide and 1g of Iodine was dissolved in 300ml distilled water

A.2. Solutions and Reagents for SDS- PAGE

10 ml Seperating Gel (12% monomer conc.)

Acrylamide/bis	4 ml
dH_2O	3,35 ml
1,5 M Tris-HCl , pH 8.8	2,5 ml
10%SDS(w/v)	0,1 ml
10% amonium persulfate (fresh preperation)	50µl
TEMED	5µl

5 ml Stacking Gel 4%

Acrylamide/bis	650 µl
Ultra pure water	3,05 ml
0,5M Tris-HCl pH 6,	1,25 ml
10% (w/v) SDS	50µl
10% amonium persulfat	25µl
TEMED	5µl

30% Acrylamide Mixture

(29.2 g acrylamide, 0.8 g N'N'-bis-methylene-acrylamide)

Make up to 100 ml with ultrapure water. Filter and store at 4ºC in the dark for at least one month.

Sample Buffer

3,8 ml dH_2O, 1ml 0.5 M Tris-HCl, pH 6.8, 0.8 ml Glycerol, 1.6 ml 10% (w/v) SDS, 0.4 ml 2- mercaptoethanol, 0.4 ml 1% (w/v) bromophenol blue

5X Running Buffer

- 15g Tris Base
- 72g Glycine
- 5g SDS

Dissolve Tris base, glycine and SDS in ~800ml deionized water and make up to

1L with water. Store at 4ºC. For electrophoretic run, dilute 5X stock solution to 1X with deionized water.

Destaining soln:

100ml Methanol, 250 ml acetic acid was added to 1650ml deionized water.

APPENDIX B

ABSORBANCE VALUES OF BSA STANDARTS AND STANDART CURVES FOR BRADFORD ASSAY AND AMYLASE ASSAY

Table B.1. Absorbance values of BSA standarts

Concentration (mg/ml)	**Absorbance (595 nm)**
0.25	0.1908
0.5	0.3623
1	0.6963
1.4	0.9082
2	1.1409

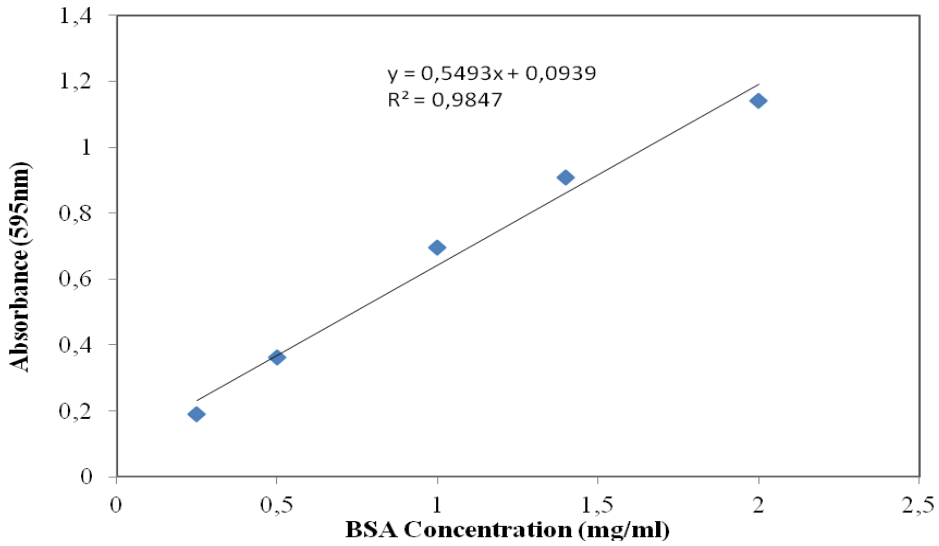

Figure B.1. BSA standart curve for Bradford assay

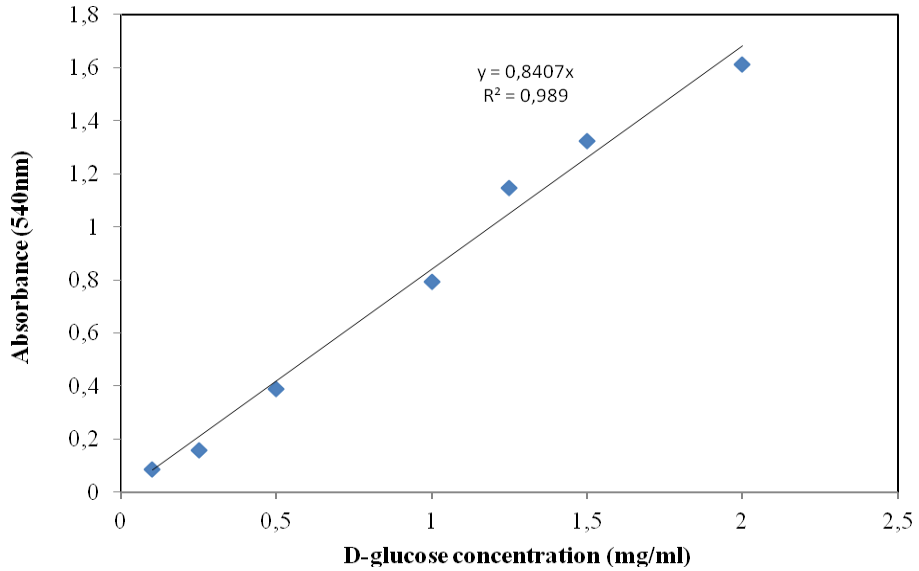

Figure B.2. Standart calibration curve for amylase assay