REPUBLIC OF TURKEY
ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF NANOTECHNOLOGY AND ENGINEERING SCIENCES

CLONING OF THE GLUCOAMYLASE ENCODING GENE FOUND IN *ASPERGILLUS NIGER* GENOME

M. DAMLA ÖZDEMİR
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I hereby declare that all information in this thesis 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 information that is not original to this work.

M. Damla ÖZDEMİR

ABSTRACT

CLONING OF THE GLUCOAMYLASE ENCODING GENE FOUND IN *ASPERGILLUS NIGER* GENOME

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Glucoamylase (GA) (1,4-α-glucosidase) is an exoamylase enzyme that is involved in the hydrolysis step of all processes in which glucose is needed. Glucoamylase which is a very important industrial enzyme produces D-glucose by hydrolyzing glucosidic linkages of starch, glycogen, and oligosaccharides from their non-reducing ends. The main reason that makes GA of such importance to the industry is its role in the starch process. Starch is one of the major components of the food, textile, laundry, pharmaceutics, and paper industries. In nature, there is two forms of glucoamylase, GAI which is commonly used in industry because of its capacity of high starch hydrolyzing and GAII. Most of the bacteria, yeasts, and fungi can produce glucoamylase in various forms, but especially the fungal glucoamylase is most often used in the industry.

This study was aimed that produce recombinant glucoamylase enzyme in *Pichia pastoris*. In this way, an industrially important enzyme, glucoamylase, could be produced as recombinant on laboratory scale from the more economical and easier-breeding yeast strain. For this purpose plasmid that containing the glaA gene encoding the glucoamylase enzyme in GAI form found in *Aspergillus niger* genome was designed and transferred to *Pichia pastoris*. The molecular cloning of the glaA gene was confirmed by sequence analysis and transfection was verified by colony PCR and determination of enzyme activity. In addition to that, the activities of the resultant recombinant enzyme under different conditions were investigated.

Keywords: glucoamylase, industrial enzyme, recombinant DNA technology, *Aspergillus niger*, *Pichia pastoris*.

ÖZET

ASPERGİLLUS NİGER GENOMUNDA BULUNAN GLUKOAMİLAZ ENZİMİNİ KODLAYAN GENİN KLONLANMASI

M.Damla ÖZDEMİR

Nanoteknoloji ve Mühendislik Bilimleri Anabilim Dalı

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Glukoamilaz (GA), glikoz gereken her prosesin hidroliz basamağına katılan bir ekzoenzimdir. Çok önemli bir endüstriyel enzim olan glukoamilaz enzimi nişasta, glikojen ve oligosakkaritlerin indirgen olmayan uçlarından glikozidik bağlarını hidrolize ederek D-glukoz üretir. Glukoamilazı endüstri için bu kadar önemli kılan ana neden nişasta proseslerinde oynadığı roldür. Nişasta; gıda, tekstil, deterjan ilaç ve kağıt endüstrisinin ana kaynaklarından biridir. Glukoamilaz doğada; yüksek nişasta hidroliz kapasitesi sayesinde endüstride kullanılan GAI ve GAII olmak üzere iki form halinde bulunur. Birçok bakteri, maya ve fungi çeşitli formlarda glukoamilaz üretebilirler ancak özellikle fungal glukoamilaz endüstri için önem teşkil eder.

Bu çalışmada; rekombinant glukoamilaz enziminin Pichia pastoris' de üretilmesi amaçlanmıştır.. Böylece endüstri açısından önemli olan glukoamilaz enzimi laboratuvar ölçeğinde, daha ekonomik ve daha kolay çoğaltılan mayada rekombinant olarak üretilebilecektir. Bu amaçla A.niger' da buunan GAI formundaki glukoamilaz enzimini koldlayan glaA genini taşıyan plasmid dizayn edilmiş ve Pichia pastoris' aktarılmıştır. glaA geninin moleküler klonlanması, dizi analizi ile ve transfeksiyon, koloni PCR ve enzim aktivitesinin belirlenmesi ile doğrulanmıştır. Buna ek olarak elde edilen recombinant enzimin farklı koşullardaki aktivitesi incelenmiştir.

Anahtar Kelimeler: glukoamilaz, endüstiriyal enzimler, recombinant DNA teknolojisi, *Aspergillus niger*, *Pichia pastoris*

For my lovely nephew who is the next engineer of my family

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TABLE OF CONTENTS

TABLE OF CONTENTS viii
LIST OF FIGURES x
LIST OF TABLES xii
NOMENCLATURE xiii
1. INTRODUCTION 1
2. LITERATURE REVIEW 3
2.1. Enzymes and Industrial Applications 3
2.1.1. Microbial Enzymes 6
2.2. Enzyme Production with Recombinant DNA Technology 10
2.3. The Fundamentals of Recombinant DNA Technology 11
2.3.1. Preparation of the Target Gene 19
2.3.1.1. Polymerase chain reaction (PCR) 22
2.3.1.2. Restriction endonucleases 23
2.3.2. Ligation 25
2.3.3. Transformation and Amplification of Recombinant DNA 26
2.3.3.1. Agarose gel electrophoresis 27
2.4. An Industrial Enzyme; Glucoamylase 28
3. MATERIALS AND METHODS 32
3.1. Amplification of the Plasmid 32
3.2. Amplification of the Target Gene 33
3.3. Digestion with Restriction Endonucleases 34
3.4. Ligation and Transformation 34
3.5. Confirmation of Molecular Cloning 35
3.6. Transfection of Recombinant Plasmid to Pichia pastoris 35
3.7. Colony PCR 36
3.8. Starch Agar Plate Assay 37
3.9. Protein Quantitation Assay 37
3.10. Enzyme Purification 37
3.11. Glucoamylase Activity Assay 38

3.12. Plasmid Retention Time Assay 38
3.13. Statistical Analyses 39
4. RESULTS AND DISCUSSIONS 40
4.1. Confirmation of Cloning 40
4.2. Transfection of Recombinant Plasmid to Pichia Pastoris 40
4.3. Colony PCR of Transfected Yeast Cells 41
4.4. Starch Agar Plate Test 41
4.5. Protein Quantitation Assay 42
4.6. Enzyme Activity Assays 43
4.7. Plasmid Retention Time Assay 46
4.8. Statistical Analyses 47
5. CONCLUSIONS 48
6. RECOMMENDATIONS 49
REFERENCES 50
APPENDIX 1 58
APPENDIX 2 59
APPENDIX 3 62
APPENDIX 4 72
APPENDIX 5 75
APPENDIX 6 76
CURRICULUM VITAE 78

LIST OF FIGURES

Figure 2.1. a) An uncatalyzed reaction **b)** An enzyme-catalyzed reaction 1
Figure 2.2. The schematic description of holoenzyme..... 2
Figure 2.3. The illustration of **a)** the lock and key model, **b)** three-point attachment model of enzyme – substrate interaction 2
Figure 2.4.The usage percentages of enzyme classes in the industry 4
Figure 2.5. The usage fields of microbial enzymes..... 7
Figure 2.6. Demonstration of plasmids **a)** in a bacteria cell, **b)** replication during cell..... 12
Figure 2.7. The use of antibiotic resistance as a selectable marker for a plasmid 13
Figure 2.8. The schema of a plasmid cloning vector..... 13
Figure 2.9. The two types of phage: **a)** head-and-tail; **b)** filamentous..... 14
Figure 2.10. The lytic cycle of a phage 15
Figure 2.11. The basic steps of recombinant DNA technology 19
Figure 2.12. The illustration of obtained target gene **a)** by genomic DNA library, **b)** by cDNA synthesis method 20
Figure 2.13. The amplification of DNA by PCR 23
Figure 2.14. The DNA fragments cut by REs 24
Figure 2.15. The sticky ends fragments cut by EcoRI 25
Figure 2.16. (a) The ligation of sticky ended molecules, **(b)** The cloning in vector via DNA ligase..... 25
Figure 2.17. The uptake of DNA by a competent bacterial cell..... 26
Figure 2.18. The illustration of cloning in plasmid vector..... 27
Figure 2.19. The demonstration of the agarose gel electrophoresis method..... 28
Figure 2.20. The usage of glucoamylase in the starch hydrolysis process..... 29
Figure 2.21. The hydrolysis of liquefied starch by glucoamylase..... 29
Figure 2.22. The schematic description of Aspergillus niger glucoamylases..... 31
Figure 3.1. The methods used in this study..... 32
Figure 3.2. The map of plasmid pGAPZαA 33
Figure 4.1. Colony PCR results of transformed E.coli cells with recombinant plasmid..... 40
Figure 4.2. Transfected P.pastoris colonies on 25 µg/ml Zeocin containing YPD agar which were incubated for 3 days after electroporation Cells..... 41
Figure 4.3. The gel image of colony PCR after transfection..... 41
Figure 4.4. The starch agar plate test..... 42
Figure 4.5. a- Calibration curve which was prepared from standard protein solutions. **b**- Protein Concentrations in supernatants of the recombinant X33 cells (X33+), the normal X33 cells (X33-) and the source organism (A.niger)..... 42
Figure 4.6. Calibration curve plotted from standard starch solutions 43
Figure 4.7. The relation of enzyme activity with temperature and time 44
Figure 4.8. The relation between enzyme activity with enzyme concentration..... 45

Figure 4.9. The investigation of plasmid loss of recombinant yeast cells during passages 47

LIST OF TABLES

Table 2.1. A selection of industrially important enzymes 5
Table2.2. The progression of microbial enzymes (for foods and beverages) utilization for centuries 6
Table 2.3. A summarized overview of some microbial enzymes with special characteristics of industrial importance 9
Table 2.4. Type of host used for recombinant DNA technology 15
Table 2.5. The advantages and disadvantages of yeast expression system 17
Table 2.6. The advantages and disadvantages of cDNA and genomic DNA cloning 21

NOMENCLATURE

μL	: Microliter
mL	: Milliliter
L	: Liter
Min	: Minute
U	: Unit
PBS	: Phosphate-Buffered Saline
PCR	: Polymerase Chain Reaction
YPD	: Yeast Extract-Peptone-Dextrose
TBE	: Tris-Borate-EDTA

1. INTRODUCTION

One of the main features of all living organisms is the metabolic process, which consisting of: anabolism a process in which organic molecules are synthesized and catabolism in which organic molecules are broken down. The metabolic reactions are chemical reactions that substrate is consumed and the product is formed with an energy change. Enzymes are protein structured catalyzers that accelerate these metabolic reactions in living cells by reducing the activation energy. Thus metabolic reactions occur just in seconds by means of enzymes. Besides the important cellular metabolic functions of enzymes, they have entered into daily and economic life to be used for various purposes. Today almost all industrial processes take advantage of enzymes (Gutteridge, 2005; Kıran et al., 2006; Mäntsälä & Niemi, 2009).

Enzymes are produced from herbal, animal and microbial sources. Herbal and animal origin enzymes still have special importance for some specific applications however the microbial origin enzymes have started to gain more importance for industrial applications since they are more advantageous in many aspects such as providing high yield and being more economic. Moreover, in conjunction with the development of biotechnology, recombinant DNA technology has begun to be used in the production of the microbial enzymes. Thus more useful and higher quality enzymes for the industry can be produced (Gessesse, 1998; Zhang & Kim, 2010).

Glucoamylase is an industrial enzyme which has great importance for the industry worldwide. In almost all processes which involve starch, glucoamylase is one of the main components of the hydrolyzing step. Industrial glucoamylases are generally obtained from yeasts or fungi and they are still active at acidic conditions to the contrary of other amylases and pullulanases. Glucoamylase has a significant market share, especially in the food and bioethanol industries (Bagheri, Khodarahmi, & Mostafaie, 2014; Z. Li et al., 2017).

The energy demand caused by the rapid population growth and progression of technology induces to the research of alternative energy sources. Turkey imports the great part of its petroleum need and it is quite costly (Bayraç, 2009; Mutlu Yılmaz, 2012). However, for Turkey which is rich from the grain, bioethanol production has the potential to be a great alternative to petroleum. Glucoamylase participates in the process of this alternative energy source. The some other processes that glucoamylase is involved in, are the food and beverages industries. In the food industry glucoamylase is used for the production of glucose

syrup, which is widely used in beverage production, dairy products, bakery products, canning, pastry, and confectionery productions. Also, the beverage processes take advantageous from the glucoamylase in the clarification step. In these processes, the use of glucoamylase instead of the chemical methods, provide some returns such as high purity, no unwanted side product, and less environmental pollution. These make processes more economical and less environmentally hazardous. Therefore the glucoamylase enzyme has become indispensable for the processes using starch(Borrelli & Trono, 2015; Deveci, 2006; Hii, Tan, Ling, & Ariff, 2012; Uludağ, 2006).

Industrial glucoamylase enzyme is mostly produced from fungi, Aspergillus niger. In this study, cloning of the Aspergillus niger's glucoamylase gene (glaA) to Pichia pastoris was aimed. Thus the glucoamylase which is used as an imported enzyme in our country can be produced through recombinant enzyme technology. The reason to tending towards recombinant technology is that recombinant enzyme technology could response most economically and effectively to the requirement in this area (Longoni, Leelavathi, Doria, Reddy, & Cella, 2015). The glucoamylase gene has been planned to be transferred to yeast that can rapidly and economically proliferate. Thus the production of glucoamylase by a cheaper way from the starch which has been abounding in our country could be enabled.

2. LITERATURE REVIEW

2.1 Enzymes and Industrial Applications

Enzymes are natural catalysts that produced by living cells, which accelerate the reactions without changing the reaction equilibrium. In living cells enzymes catalyse the metabolic reactions by reducing their activation energy (Mäntsälä & Niemi, 2009; Robinson, 2015). As described in Figure 2.1, when an enzyme catalyses a reaction, the transition state arises energetically more suitable, thus speeds up the reaction, but not really changes the energy levels (Robinson, 2015).

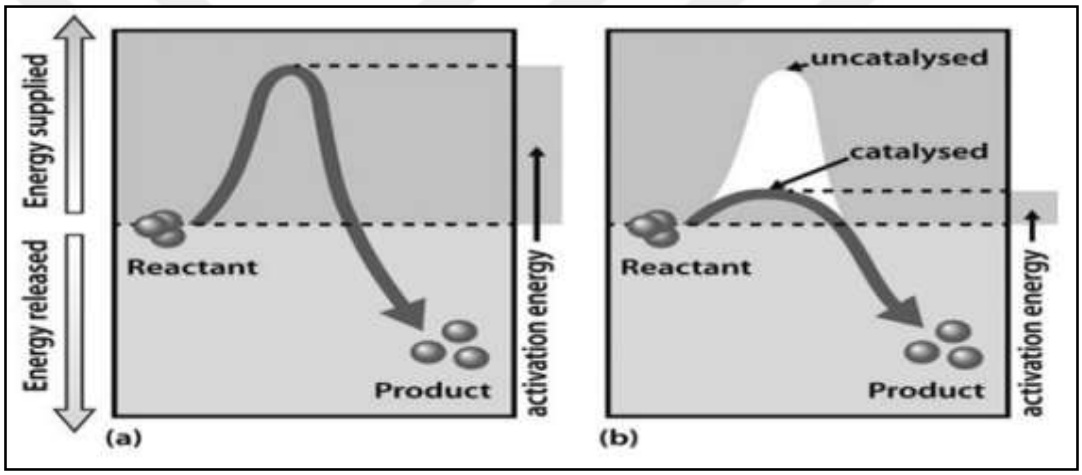


Figure 2.1. (a) An uncatalysed reaction (b) An enzyme-catalysed reaction (Robinson, 2015). The participation of catalyzer to reaction reduces the activation energy necessary and accelerates the reaction.

Enzymes are protein structared molecules. Despite the fact that a great part of enzymes completely consists of protein, some of them also contain a non-protein component called cofactor. Cofactor which is essential for catalytic activity can be inorganic (generally a cobalt, zinc, copper, manganese or iron ion) or organic. If cofactor is an organic molecule, it is called as the coenzyme. If an enzyme needs a cofactor to be able to perform its function, this nonfunctional enzyme is named as apoenzyme. As described in Figure 2.2 the combination of apoenzyme and its cofactor constitutes the holoenzyme (Ferrier, 2014; Robinson, 2015).

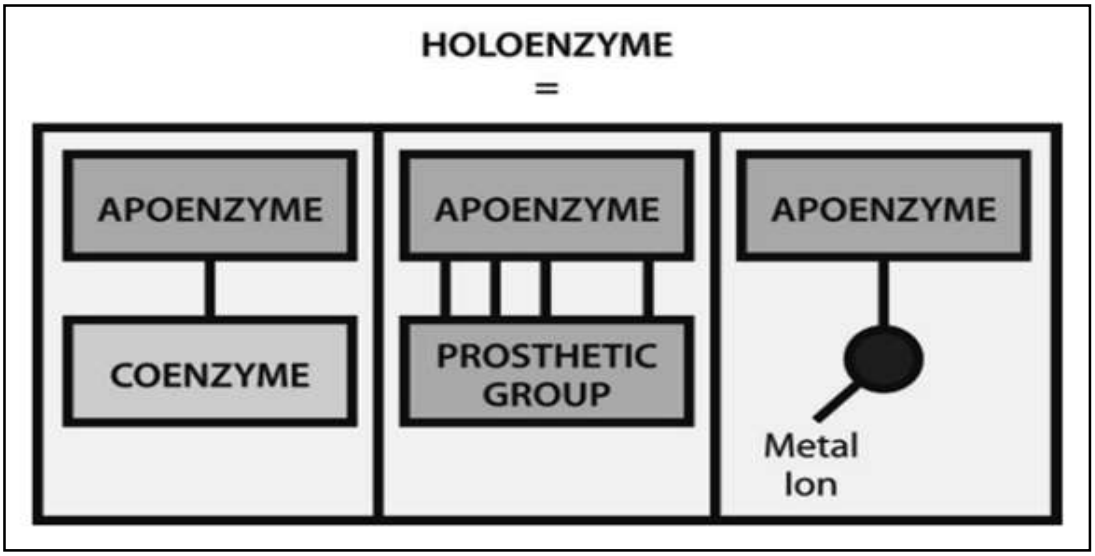


Figure 2.2. The schematic description of holoenzyme(Robinson, 2015). The combination of apoenzyme (inactive enzyme) and cofactor constitutes the holoenzyme (active enzyme). The cofactor can be an organic molecule (coenzyme) or an inorganic molecule.

Substances that are acted upon by enzymes are referred to as substrates. Each cell contains thousands of different enzymes, but each enzyme participates in only one reaction with one substrate (Buxbaum, 2007; Mäntsälä & Niemi, 2009). Because enzymes are specific to their substrates, and there is a structural complementarity between enzymes and substrates. This structural complementarity can be explained by two models called as lock and key and three-point attachment models (Figure 2.3) (Copeland, 2000).

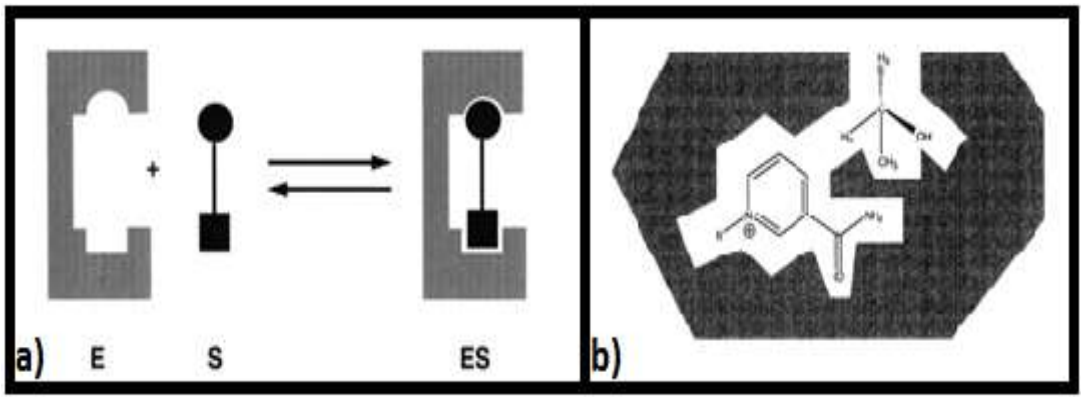


Figure 2.3. The illustration of a) the lock and key model, b) three-point attachment model of enzyme – substrate interaction (Copeland, 2000). These two models explain the substrate selectivity in enzyme catalysis. The substrate and active site of the enzyme integrate each other structurally.

According to Nomenclature Committee of the International Union of Biochemistry (NC-IUB) the amount of enzyme which consumes 1 μmole of the substrate in 1 minute under room conditions was defined as the unite of enzyme activity(International Union of Biochemistry, 1979). The activity of an enzyme could be measured by two ways; continuous or discontinuous method. In continuous method, the enzyme and the substrate are mixed and the rate of reaction is determined by measuring the amount of substrate and product continuously. However in discontinuous method the enzymatic reaction is started and after a particular time the product is measured. The discontinuous method is more easy and rapid but the time interval should be determined meticulously (Robinson, 2015). The enzyme activity can be affected from several factors such as pH, temperature, amount of substrate and presence of inhibitor. The study of enzyme activity should be carry out studiously because enzyme could affect from the laboratory conditions and could lose its activity (Copeland, 2000; Mäntsälä & Niemi, 2009)

Recently almost 4000 different enzymes have been discovered from various organisms and have been utilized by many fields of industry. Besides being able to carry out very specific chemical reactions, enzymes are preferred in the industry because of their unique characteristics over chemical catalysts (Breithaupt, 2001; S. Li, Yang, Yang, Zhu, & Wang, 2012). Compared to chemical catalysts, enzymes are more selective, more productive (generally lower concentration of enzyme required) and leads to lower consumption of energy and generation of waste. In addition to that enzymes can function in milder reaction conditions (typically in 20-40^{o}C and pH:5-8) and they can be completely degraded in the environment (Choi, Han, & Kim, 2015; Faber, 2011; Littlechild, 2015). According to the general reactions that they catalyzed and their specific substrates, all enzymes are classified into six classes by the International Union of Biochemistry and Molecular Biology (IUBMB). These classes are; oxireductases, transferases, hydrolases, lysases, ısomerases, and ligases. Also, each of these classes, then, has its own subclasses (Gurung, Ray, Bose, & Rai, 2013; Johannes, Simurdiak, & Zhao, 2006). The percentages of usage regarding these enzyme classifications, in industry, can be seen in Figure 2.4.

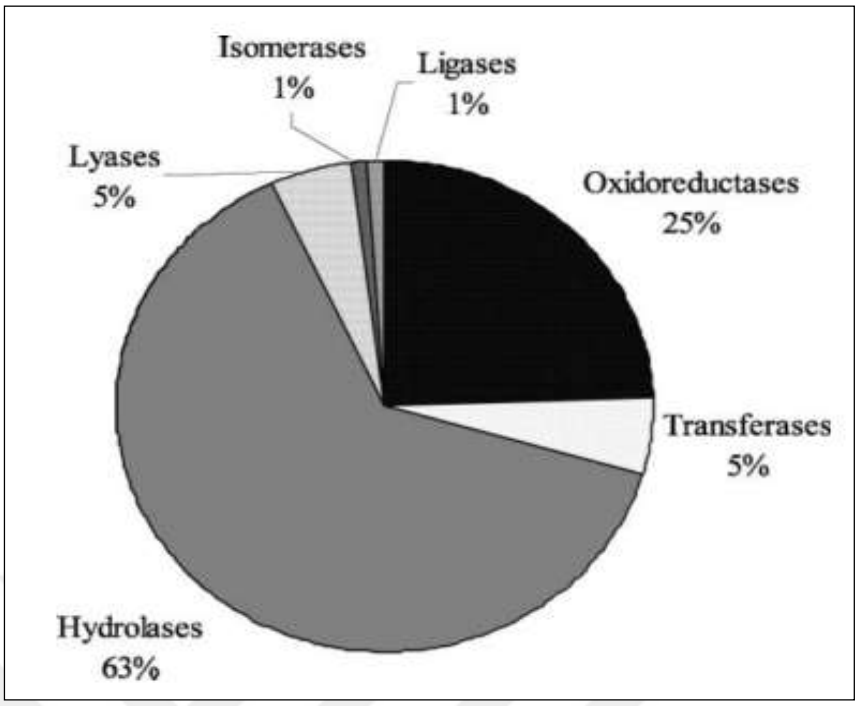


Figure 2.4. The usage percentages of enzyme classes in the industry (Johannes, Simurdiak, and Zhao, 2006)

The market for industrial enzymes was estimated to be valued at USD 4.61 Billion in 2016 and is predicted to develop at a compound annual growth rate (CAGR) of 5.8% from 2017 to 2022. Hydrolases (especially amylases, proteases, cellulases, and lipases) constitute a great part of the industrial enzymes making up a market share of 63% (Figure 2.4.). Some of the industrially important enzymes from each enzyme classes are given in table 2.1.

Table 2.1. A selection of industrially important enzymes (Gurung et al., 2013).

SI no.	Class	Industrial enzymes
1	Oxireductases	Catalases Glucoseoxidases Laccases
2	Transferases	Fructosyltransferases Glucosyltransferases
3	Hydrolases	Amylases Cellulases Lipases Mannanases Pectinases Phytases Proteases Pullulanases Xylanases
4	Lysases	Pectate lyases Alpha-acetolactate decarboxylases
5	Isomerases	Glucose isomerases Epimerases Maltases Lyases Topoisomerases
6	Ligases	Argininosucinnate Glutathione synthase

Enzymes can be produced from herbal, animal and microbial sources. However herbal and animal sources cannot satisfy the industrial needs and this circumstance has caused to increase the interest in microbial enzymes. Moreover, microbial enzymes posses some unique properties that make them more attractive than herbal and animal originated enzymes (Kuduğ, 2013).

2.1.1 Microbial Enzymes

Use of the enzymes, originating from microorganisms has been used since ancient times. Sumerians and Babylonians used the yeast in wine and beer making. This can be assumed as the first known commercial utilization of the microbial enzymes. Later, people have also used microbial enzymes in different food applications such as vinegar, yoghurt, and cheese (Mishra, Ray, Rosell, & Panda, 2016; Singh, Kumar, Mittal, & Mehta, 2016). The progression of microbial enzymes on food applications is given in Table 2.2.

Table2.2. The progression of microbial enzymes (for foods and beverages) utilization for centuries (Mishra, Ray, Rosell, & Panda, 2016)

Period	Events
2000 BC	Fermentation was developed mainly for use in brewing, bread baking and cheese making by the Sumerians and Egyptians.
1860 AD	Berthelot demonstrated hydrolytic enzymes, including invertase (β- fructofuranosidase) obtained from *Saccharomyces cerevisiae*.
1878	Term 'enzyme' was derived from a Greek term 'ενζυμον' meaning 'in yeast'; also the components of yeast cells was identified which cause fermentation.
1894	Takamine first time patented a method (koji process; SSF) for preparation of diastatic enzymes (mostly α-amylase) from the mould that was marketed under the name 'Takadiastase'
1913	Patents were awarded to French scientist A. Boidin and Belgian scientist Jean Effront for production of bacterial amylases and diastases as still culture (surface film).
1926	Enzymes were initially shown to be proteins.
1946	Commercially amylases were produced using Aspergillus oryzae strain by Mould Bran Co., Iowa, USA in SSF process.
1950	Amylase production in SmF using Tank Bioreactor at Northern Regional Laboratory, USDA, Illinois, USA; shift over from SSF to SmF.
1959	Bio 40-protease from *B. subtilis* was introduced in the market.
1950–1980	Spectacular increase in industrial enzyme production, particularly amylases and proteases (to a larger extent) and pectinases, lactase, invertase, lipase and cellulases (to lesser extent).
1980s	Animal feed with improved nutrient availability and digestibility were developed through enzyme preparations
1982	A product of gene technology, alpha amylase, was developed for application in food for the first time.
1988	Early approval of a product of gene technology, recombinant chymosin for food use was approved and introduced in Switzerland
1990	Gene technology was used by developing two food processing aids-an enzyme for use in cheese-making in the US and yeast used in baking in the UK.
2000-onwards	Re-designing microbial enzymes by tailoring their protein sequence.

Microbial enzymes can be obtained from bacteria, fungi, and yeast (Kıran et al., 2006). More than 50% of the enzymes consumed in the industry are microbial originated enzymes (Borrelli & Trono, 2015). The food industry takes advantage from 29% of total enzymes, and 58% of these enzymes are produced from fungi, 28% from bacteria and 5% from yeast (Avendaño et al., 2016). Besides in industry, microbial enzymes are used for environmental purposes and agriculture (Vittaladevaram, 2017). The fields of industry and the other fields using microbial enzymes can be seen in Figure 2.5.

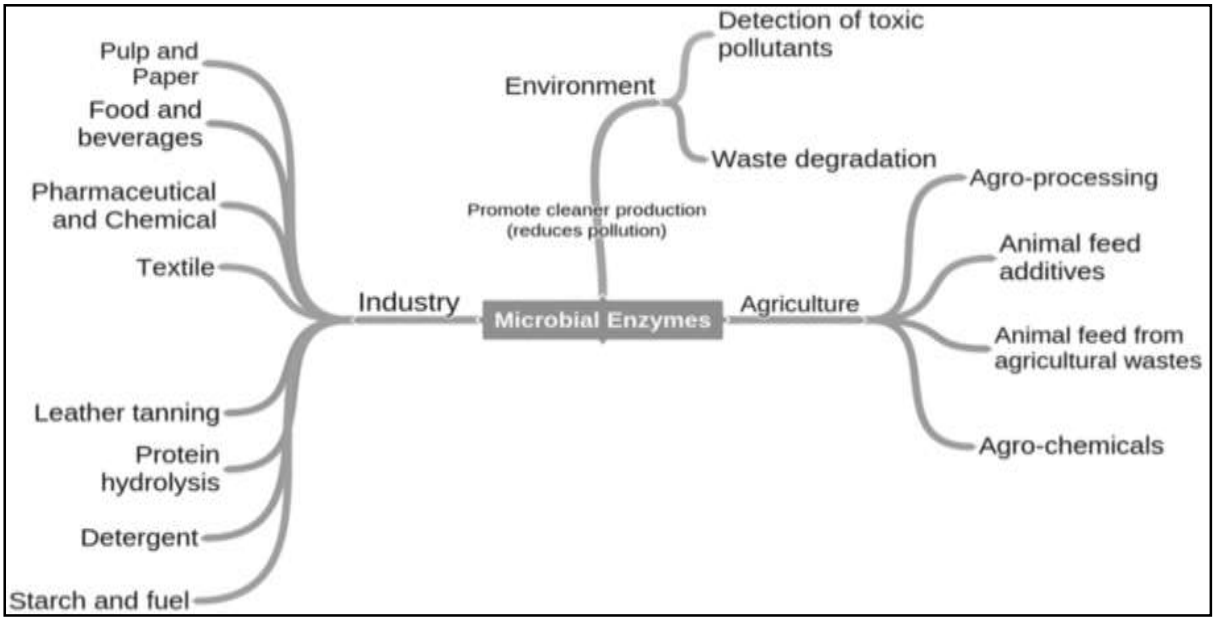


Figure 2.5. Fields using microbial enzymes (Vittaladevaram, 2017). Microbial enzymes are used in industry, agriculture and also in environment purposes. From food to detergent almost all fields of the industry takes advantage of microbial enzymes.

Microbial enzymes have some advantages that keep them in such demand and caused them to be thought of as superior enzymes. These advantages are listed below;

- Microorganisms can rapidly proliferate in an inexpensive medium (Anbu, Gopinath, Chaulagain, & Lakshmipriya, 2017; Borrelli & Trono, 2015).
    - Provide higher yield
    - Enzymes can be produced on a large scale
    - More economic
- The extraction and purification of enzymes from microorganisms are easier than animal or herbal sources (Vittaladevaram, 2017).

- Microbial enzymes are more active and stable than animal or herbal originating enzymes (Cao, Li, Shao, Tan, & Zhang, 2016).
- Microorganisms can produce enzymes in various environmental conditions even in inhospitable environments (Borrelli and Trono, 2015; Vittaladevaram, 2017). This enables them to be a more frequently available source.
- Microorganisms can be genetically modified easily (Vittaladevaram, 2017). So enzyme production can be enhanced.

The enzymes, which possess many properties have had their usefulness verified in many industries such as the food, leather, textiles, animal feed, and in bio-conversions and bio-remediations. For example; enzymes produced by many bacterial and fungal sources such as *B. subtilis, B. licheniformis, A. niger, A. oryzae,* are used in the processing of foods. These organisms have been used for a long time in enzyme production due to their ability to be produced easily under industrial production conditions, to be suitable for the manipulation, and to be able to produce large quantities of enzymes in a fermentation environment (Akyıl, 2014). Some of the microbial enzymes which are being used in industry, with their characteristic properties are given in table 2.3.

Table 2.3. A summarized overview of some of the microbial enzymes with their special characteristics and importance in industry (Nigam, 2013)

Enzyme	Properties	Sources	Applications
PROTEASE (Proteolytic activity)	Acidic, Neutral, Alkaline, Thermophilic, Active in the presence of inhibitory compounds	*Bacilli;* *Pseudomonas*; *Clostridium*; *Rhizopus*; *Penicillium*; *Aspergillus*	Washing Powders; Detergents; Tannery; Food Industry; Leather processing; Pharmaceuticals; Molecular Biology; Peptide synthesis
KERATINASE (Keratin-hydrolysing activity)	Specific Proteolytic Activity for Insoluble & Fibrous Proteins in furs, feathers, wool, hair; Thermophilic; Alkalophilic; Oxidation-Resistant	Bacteria; Actinomycetes; Fungi	Animal Feed Production; Textile Processing; Detergent Formulation; Leather Manufacturing; Medicine
AMYLASE (Starch-hydrolyzing activity)	Thermotolerant, Thermostable, Alkali-resistant-Exo-, endo-, de-branching, cyclodextrin-producing enzymes	*Bacillus* sp.; *Geobacillus*	Starch industry (for liquefaction); Paper, Food industry (Glucose & Maltose syrups, High Fructose Corn syrups, clarified fruit-juices); Pharmaceutical industries (Digestive aid); Brewing Industry; Textile industry (Warp-sizing of fibers); Baking industry (delayed staling)
XYLANASE (Xylan–Pentose polymer hydrolyzing activity)	Extremophilic characteristics– Alkalophilic, Thermophilic & Thermostable	*Thermoactinomyces thalophilus*; *Bacillus* sp.; *Humicola insolens. Bispora* (acidophilic fungus)	Pentose production - Bioconversion of hemicellulose for fuel & Chemicals; Fruit-juice clarification; Improving rumen digestion; Paper industry- selective removals of xylans from kraft-pulp; Brewing industry
LIGNINASE (Ligninolytic-Complex-enzyme)	Oxidative properties in Lignin peroxidase, Manganese peroxidase & Laccase; Thermophilic	*Steccherinum ochraceum*, *Polyporus versicolor*, *Panus tigrinus*	Denim washing; Bio-sensors; Bio-bleaching of Kraft-pulp; Bioremediation; Pollution-control; Treatment of recalcitrant chemicals in Textile and Industrial effluents
CELLULASE (Cellulolytic-complex enzyme)	Saccahrification of crystalline & amorphous cellulose; Thermophilic; Thermostable	*Polyporus* sp.; *Pleurotus* sp.; *Trichoderma* sp.; *Aspergillus* sp.	Glucose feedstock from cellulose; Bio-refinery; Bio-ethanol; Paper-pulp industry
LIPASE (Lipolytic activity)	Fat- splitting; Stereoselectivity; Racemic-Resolution activity; Solvents-resistant; Thermotolerant	*Candida* sp., *Aspergillus* sp, *Penicillium* sp., *Rhizopus*, *Mucor*	Detergents; Dairy Industry-oils, fats, Butter, Cream, Fat-Spreads; Feed supplement; Therapeutic agent

As mentioned above, one of the superior qualities of microbial enzymes is that microorganisms can be genetically modified. Thus, with the developments in the recombinant

DNA technology and protein engineering, microbial enzymes are in greater demand in the industry (Gurung et al., 2013).

2.2 Enzyme Production with Recombinant DNA Technology

Recombinant DNA means a novel DNA which is formed by bringing together the DNA fragments from different organisms. Recombinant DNA technology enables the development of new DNA or altering the DNA that is already in this genome (Sandhu, 2010). During recent years, the advances in recombinant DNA technology have greatly served the enzyme technology (Adrio & Demain, 2014). Through recombinant DNA technology, the enzymes which are difficult and expensive to manufacture in the industry, can be produced more inexpensively and more abundantly with a new host microorganism. For example; an industrially important enzyme, proteases can be isolated from several fruits e.g. kiwi, pineapple, and papaya. Producing proteases from fruits on an industrial scale will be time consuming and expensive. In such cases, recombinant DNA technology offers a solution and a microorganism is used as a host to produce an enzyme, abundantly, in a short time (Amid, 2015). Recombinant DNA technology, in addition to these reasons, enables an improvement of enzyme stability and productivity (Adrio & Demain, 2010; Mäntsälä & Niemi, 2009).
Currently, over 50% of all industrial enzymes are obtained from recombinant microorganisms. Also over the 60% of enzymes using in starch, food, and detergent processes are recombinant enzymes (Adrio & Demain, 2010; Sanchez & Demain, 2011).

The first enzyme to be produced by recombinant DNA technology, used in the manufacture of cheese, was chymosin. Between 1984 and 1985, in the laboratories of Hansen A/S Bio Ingredients which belongs one of the leading companies in the food industry in Denmark. Chymosin genes were isolated from a calf stomach and cloned into a fungus. In 1990 this enzyme was approved to use in food manufacturing. By this process, the chymosin enzyme, whose isolation and purification from calf stomach would be too much costly and labor, can be produced by a microorganism in a shorter time and higher yield through recombinant DNA technology (Özcengiz, 2002).

2.3 The Fundamentals of Recombinant DNA Technology

Recombinant DNA is obtained by the in vitro combination of DNA fragments which have been taken from different organisms. Restriction endonucleases are used for digesting of DNA fragments and ligase enzyme is utilized to enable their combination. Afterwards, the recombinant DNA is transferred to a host organism. If the host organism is a bacteria (prokaryotic) the transfer process is termed as 'transformation', but if the host organism is a eukaryotic cell, for example, a yeast it is called as transfection (Lodish, Berk, & Zipursky, 2000; Sandhu, 2010).

The emergence of recombinant DNA technology has been enabled through the use of known tools and procedures in new ways which possess the broad application that can analyze and modify the gene structure and organization of the whole genome (Berg & Mertz, 2010). The first recombinant DNA molecule was produced at Stanford University in 1972, following the isolation of the DNA ligase enzyme which enables to linking of DNA strands, for the first time in 1967 and the isolation of the first restriction endonuclease enzyme which can digest DNA from specific regions in 1970. For the first time in 1973, the chimeric plasmid was produced by the integration of DNA fragments taken from different organisms to a plasmid DNA. When this plasmid was intoduced to E. coli it was observed that the gene on the plasmid was being expressed. This constituted the fundamentals of gene cloning. Since that date, many researchers have been awarded Nobel prizes in recombinant DNA technology, from the pharmaceutical, to the food and cleaning industries (Koçoğlu & Yalçınkaya, 1992).

In recombinant DNA technology, a "carrier DNA", called a "vector", is utilised to transfer the target gene to the desired host cell. Generally, as vectors, plasmids, bacteriophages, and in some circumstances viruses might be used. A cloning vector must possess some features that are listed below (Brown, 2007).

i. The cloning vector should has its own replication origin and it is able to replicate independently, in the host cell. Thus a great number of copies can be produced and passed to the daughter cell.
ii. The cloning vector includes restriction sites (multiple cloning sites) and at least one selective marker.
iii. The cloning vector should be less than 10 kb in size. Because it is more difficult to manipulate the larger molecules and they tend to break down during purification.

Plasmids are extrachromosomal DNAs, found in bacteria, yeast, and some higher eukaryotic cells and act parasitically or symbiotically. Plasmids are double stranded and circular structures and their sizes show an alteration between a few thousand base pairs and 100 kilobases (kb). Most plasmids include their own replication origin and before every cell division, plasmid DNA is replicated then with cell division minimum one of newly replicated plasmid DNA is transferred to the daughter cell (Figure 2.6) (Dale & Park, 2010; Lodish et al., 2000).

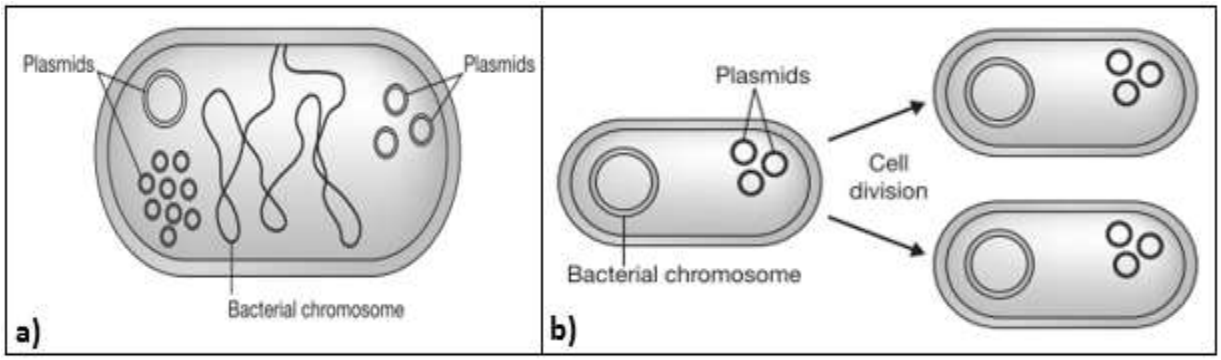


Figure 2.6. Demonstration of plasmids a) in a bacterium cell, b) replication during cell division (Brown, 2007). Plasmids are extrachromasal DNAs which mostly have their own replication origin. Before every cell division plasmid DNA is replicated and with cell division minimum one of newly replicated plasmid DNA is transferred to the daughter cell.

Bacterial plasmids provide some benefits to the host cells and enable bacteria to adapt to the changing environment through horizontal gene transfer and contribute to the diversity of the microbial population (Johnson & Nolan, 2009; Smillie, Garcillan-Barcia, Francia, Rocha, & de la Cruz, 2010). Plasmids are important in the process of gaining antibiotic resistance in pathogenic bacteria so they allow the resistance gene to spread rapidly across bacterial strains (San Millan, Heilbron, & MacLean, 2014; Zheng et al., 2015). A bacterium can gain resistance to tens of different antibiotics by carrying several plasmids or by carrying just one plasmid that have tens of different resistance genes. In recombinant DNA technology, the antibiotic resistance feature of plasmids is very important and serves as a marker for the selectivity (Brown, 2007; Dale & Park, 2010). The schematic demonstration of selectivity can be seen in Figure 2.7.

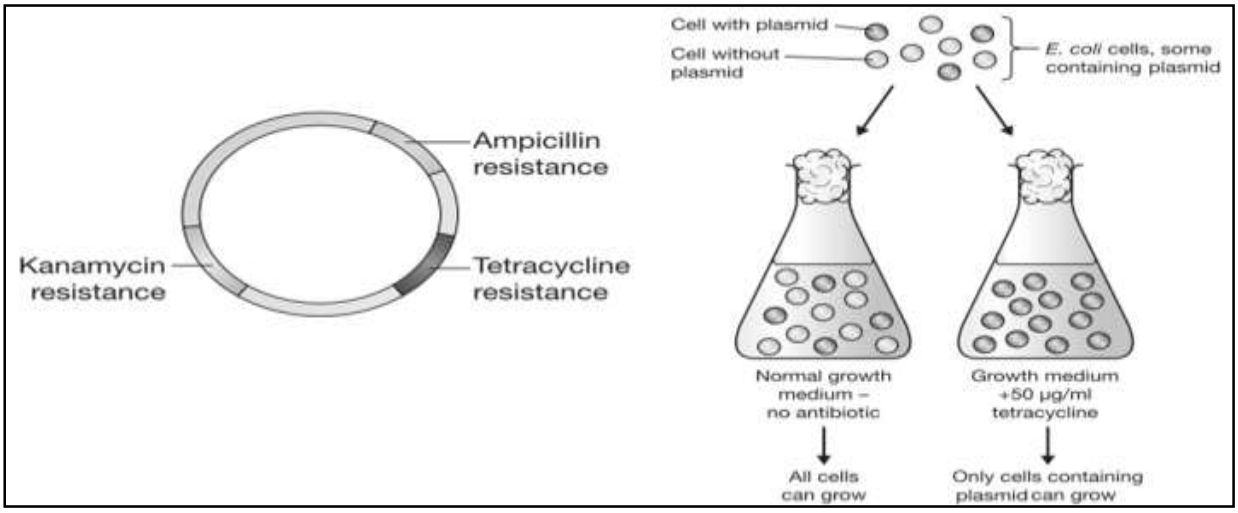


Figure 2.7. The use of antibiotic resistance as a selectable marker for a plasmid (Brown, 2007). Some plasmids include one or more antibiotic resistance gene. This gene provides selectivity for the cell. From a group of bacteria found in growth medium including an antibiotic only the cells carrying the plasmid which contain this antibiotic resistance gene can survive.

Yet another feature that makes plasmids valuable as cloning vector would be a multiple cloning site (the unique restriction sites). The schema of a plasmid that possesses all features which are essential to be a cloning vector is given in Figure 2.8. In some instances, plasmids can be engineered to be used as a cloning vector. For example, if a plasmid size is too long to be used as a cloning vector then its length can be reduced, or a new restriction site or a new resistance site can be added to a plasmid (Dale & Schantz, 2002; Lodish et al., 2000).

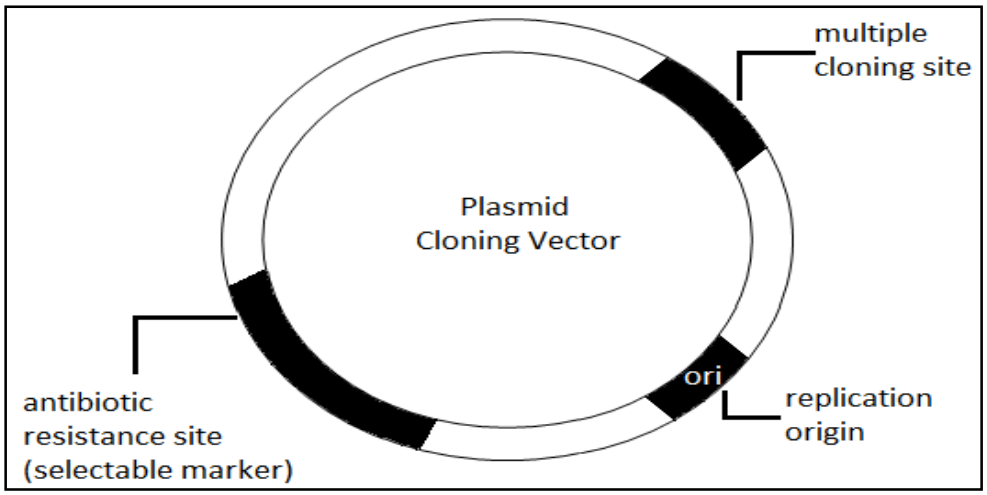


Figure 2.8. The schema of a plasmid cloning vector. In order to be used as a cloning vector, a plasmid needs to have some certain features. These are: a replication origin, a multiple cloning site, and one or more antibiotic resistant genes. A plasmid vector should have at least these features. However, in some cases, other additional features might be desired.

The other type of cloning vectors in recombinant DNA technology is bacteriophages. Bacteriophages commonly known as phages are bacterial viruses that consist of protein and nucleic acid (DNA or RNA) as all viruses do. The proteins of the phage encircle the tightly packed nucleic acid genome and constitute a protective shell named a "capsid". Just as plasmids do, the nucleic acid genome of the phage encodes the functions needed for replication in bacteria, however, unlike plasmids; it encodes proteins which are needed for phage assembly and capsid. The morphology of phages has been reported in several types such as polyhedral, filamentous, and complex (head and tail). In recombinant DNA technology, the most widely used bacteriophages are the M13, a "filamentous phage" and the lambda (λ) a "head-and-tail phage" (Figure 2.9) (Daniel L. Hartl, 2012; Holmes & Jobling, 1996).

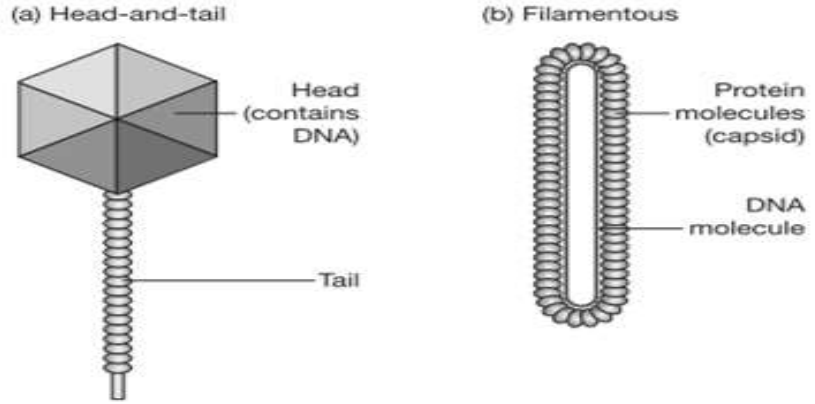


Figure 2.9. The two types of phage: a) head-and-tail; b) filamentous (Brown, 2007). All phages include a head structure that is vary in sizes and shapes. Some have tails attached to the head, while others are filamentous. The λ and M13 phages which are the commonly used cloning vectors are examples of "head-and-tail" and "filamentous" structures, respectively.

There are two types phage infection: the lytic cycle, and the lysogenic cycle. As described in Figure 2.10, the lytic cycle, which is a rapid infection type, takes place in 3 steps. In the first step, the phage penetrates the cell surface of a bacterium and injects own genetic material into the cell. In the second step, the genetic material of the phage is replicated. This replication generally takes place using the enzymes encoded in the genes of the phage's nucleic acid. In the last step the protein components of the capsid are produced, new phage particles are pieced together, and delivered from the cell through lysis of the cell. In contrast to a lytic cycle, in the lysogenic cycle, the nucleic acid of phage integrates into the cell genome, and, after cell division, the phage genome is released and new phage particles are formed. When the cycle is finished, the host cell is lysed (Brown, 2007; Dale & Park, 2010).

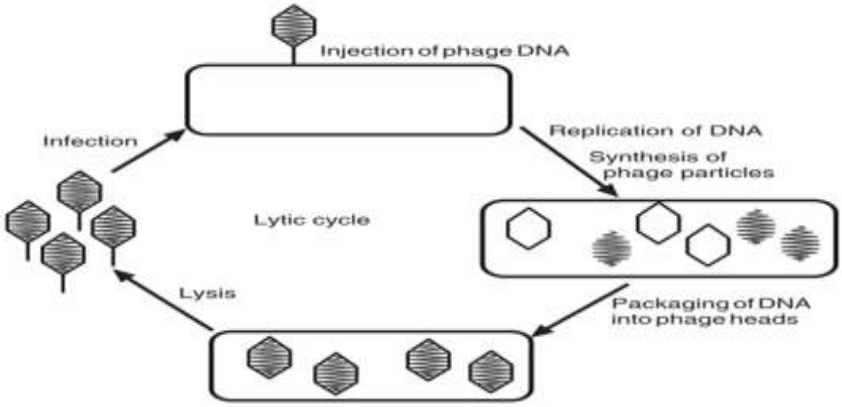


Figure 2.10. The lytic cycle of a phage (Dale and Park, 2010). The lytic cycle is called as rapid infection owing to the entire cycle takes place very quickly. The lytic cycle also constitutes the last part of the lysogenic cycle. The infection cycle of M13 is a typical example of the lytic cycle.

In recombinant DNA technology for the expression of desired protein; bacteria, yeast, filamentous fungi, insect, and mammalian cells, transgenic plants and animals could be used as host organisms (table 2.4) (Demain & Vaishnav, 2009).

Table 2.4. Type of host used for recombinant DNA technology (Nicholl, 2008)

Major group	Prokaryotic/eukaryotic	Type	Examples
Bacteria	Prokaryotic	Gram – Gram +	*Escherichia coli* *Bacillus subtilis* *Streptomyces spp.*
Fungi	Eukaryotic	Microbial Filamentous	*Saccharomyces cerevisiae* *Aspergillus nidulans*
Plants	Eukaryotic	Protoplasts Intact cells Whole organism	Various types Various types Various types
Animals	Eukaryotic	Insect cells Mammalian cells Oocytes Whole organism	*Drosophila melanogaster* Various types Various types Various types

For the expression of recombinant proteins, especially when producing recombinant enzymes for the industry, among various bacteria, *E.coli* and *Bacillus* strains are the mostly preferred. Indeed, recently the most popular and widely used host is E.coli, due to the many advantages it has. These advantages are listed below (Adrio & Demain, 2014; Rosano & Ceccarelli, 2014).

i. Ease of culture and rapid growth. Rapidly achieving high cell density.
ii. Require inexpensive media and can survive in many and varying conditions.
iii. High expression yield. It is capable to collect recombinant proteins from nearly 80% of its dry weight.
iv. Ease of genome modifications.
v. Fast and easy transformation. Transformation can be performed in less than 5 minutes.

However, naturally, E.coli has some drawbacks such as the inability for post-translational modifications, the presence of endotoxins, and acetate formation (due to high cell densities) resulting in cell toxicity. In addition, sometimes proteins are formed as inclusion bodies which are aggregated, insoluble, non-functional and require refolding (Adrio & Demain, 2014; Demain & Vaishnav, 2009).

Yeasts are yet another widely preferred host for recombinant protein production. Yeasts, as a eukaryotic system, have some advantages. These advantages are given in table 2.5.

Table 2.5. The advantages and disadvantages of yeast expression system.

Advantages	Disadvantages	References
✓ **High yield** ✓ **Durability** ✓ **Inexpensive to culture** ✓ **Fast growing** ✓ **Robust cellular structure** ✓ **Post–translational modification** ✓ **Can assist protein folding** ✓ **Can glycosylate proteins** ✓ **Synthesis of complex and multi subunit proteins**	✓ Glycosylation pattern dissimilar to human ✓ Complex scaling up fermentation	(Amid & Hassan, 2015; Demain & Vaishnav, 2009)

There are a great number of significant yeast preferred as hosts for protein expressions such as *Pichia pastoris, Saccharomyces cerevisiae, Hansenula polymorpha, Kluyveromyces lactis, Schizosaccharomyces pombe, Yarrowia lipolytica and Arxula adeninivorans* (Çelik & Çalık, 2012). *Saccharomyces cerevisiae* has been used for centuries in bread and beer manufacturing and it is one of the more commonly used hosts in recombinant DNA technology (Nicholl, 2008). Also, *Pichia pastoris* is one of the more extensively preferred yeast as a host for protein expression. Especially in the production of industrial enzymes and biopharmaceuticals, *Pichia pastoris* is usually preferred because of it could be achieved the capacity of over 22g/l, intracellular recombinant protein and a concentration of over 14.8 g/l secreted recombinant protein by this yeast (Adrio & Demain, 2014; Ahmad, Hirz, Pichler, & Schwab, 2014).

The advantages of *Pichia pastoris* over the *Saccharomyces cerevisiae* are;

i. High protein yield
ii. Prevention of hyperglycosylation
iii. Inexpensive in handling (P.pastoris requires only one carbon source and one nitrogen source in growing media)
iv. The chromosomal DNA of P.pastoris can integrate multiple copies of foreign DNA stably
v. Great ability to secrete the protein
vi. Methylotropic strains can grow in a high-level of methanol solutions that most of other microorganisms couldn't survive in.

If *Pichia pastoris* is compared with *E.coli*, the important advantage of *P.pastoris* over *E.coli* is primarily P.pastoris' ability to produce disulfide bonds and enable the glycosylation of proteins. Compared with many other expression systems, Pichia usually gives greater yields (Jose L. Adrio & Demain, 2014; Demain & Vaishnav, 2009).

Filamentous fungi such as Aspergillus also have an ability to produce high amounts of proteins and glycosylation that make them attractive hosts. Unfortunately, filamentous fungi are slow-growing organisms and are difficult to handle in fermenters. Many industrial enzymes are produced by these organisms. To be able to produce these enzymes more economically and more prolifically, transfering of their genes to rapidly-growing hosts would be a good solution (Adrio & Demain, 2010; Mahesh & Vedamurthy, 2004).

In recombinant DNA technology to achieve a successful transformation result, choosing the right vector and the right host is the most important step (Nicholl, 2008). As demonstrated in Figure 2.11, the basic steps of recombinant DNA technology are: preparation of the target gene and vector, their ligation, transformation of the recombinant DNA to the host cell, verification of cloning, and the amplification of recombinant DNA in the host cell.

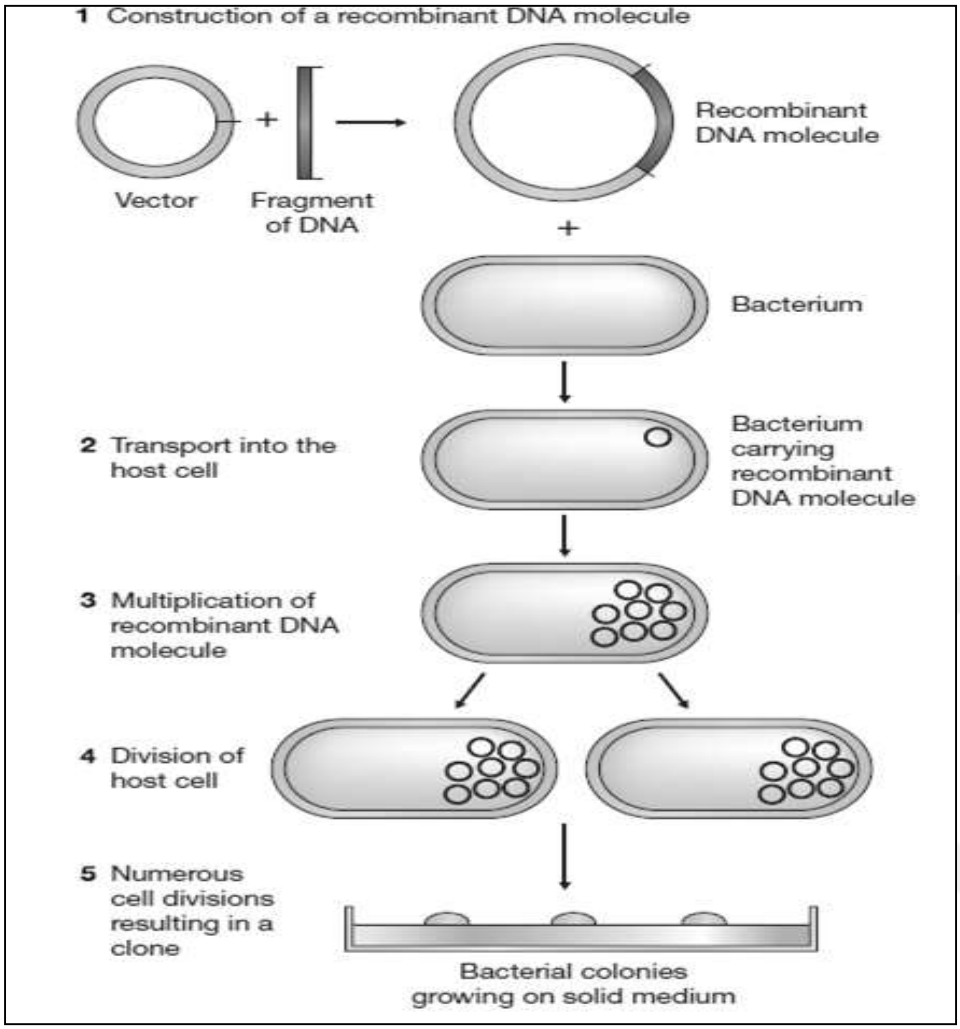


Figure 2.11. The basic steps of recombinant DNA technology are the construction of recombinant DNA molecule (digesting the target gene and ligation with the vector DNA), transformation of recombinant DNA to host cell, verification of the cloning, and amplification of recombinant DNA in the host cell (Brown, 2007).

2.3.1 Preparation of the Target Gene

The first step in recombinant DNA technology is the preparation of the target gene. It can be applied in one of two ways; a prepared gene can be obtained from the genomic DNA library, or prepared through cDNA synthesis. The genomic DNA library is an extensive collection of cloned, DNA fragments (which include the target gene) and which are specific to the cell, tissue or organism (Figure 2.12). To construct the DNA library, carrier vector DNA and cellular DNA are, digested with the same restriction enzymes. Than thr fragments obtained from the enzyme digestion and containing the gene of interest, are ligated into the vector DNA by DNA ligase. These obtained recombinant DNAs are transferred to host cells

(generally bacterial cells) and proliferated within the cells. As a carrier vector, either a virus or a plasmid can be used (Alberts et al., 2002).

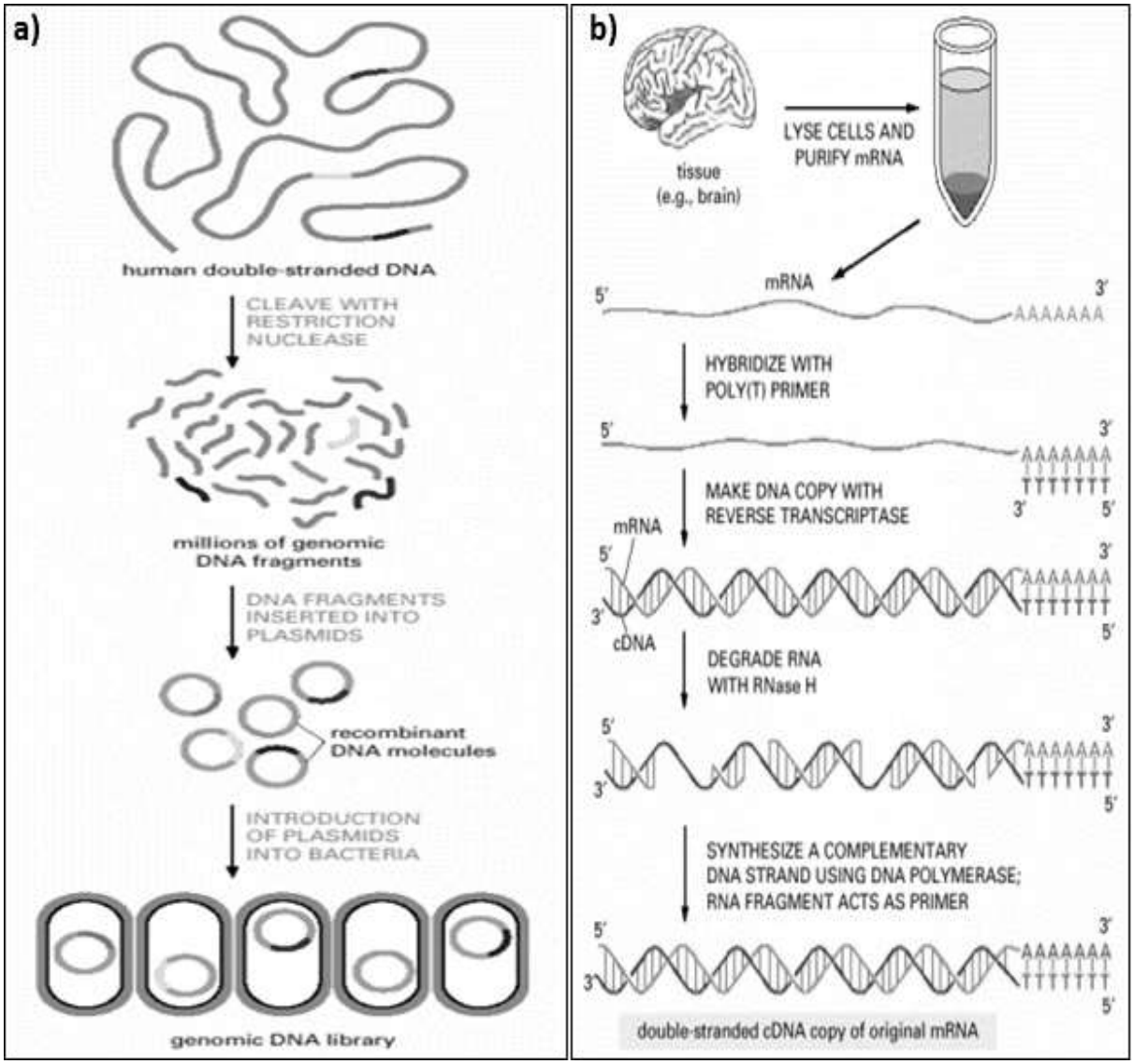


Figure 2.12. The illustration of obtained target gene a) by genomic DNA library, b) via cDNA synthesis method (Alberts *et al.*, 2002)

cDNA or "complementary DNA" is a DNA copy of an RNA (usually mRNA) which is converted by reverse transcription. For synthesis of cDNA firstly mRNA is isolated from the cells and converted to cDNA by reverse transcriptase enzyme and it could be used as template in the process of target gene amplification by polymerase chain reaction (PCR) (Figure 2.10b). Then these PCR products are digested with restriction enzymes and combined with the vector. The major advantage of cDNA is that it contains only the coding sequence of genes (exons) continuously and without interruption. Because during the formation of the

mRNA, the introns (uncoding sequences) have been removed and exon (coded) sequences have been bonded by RNA splicing (Alberts et al., 2002; Nicholl, 2008). Thanks to this property, cDNA provides templates which are more suitable for cloning purposes, such recombinant industrial enzyme production processes (Amid & Hassan, 2015). The comparison of cDNA and genomic DNA can be found in table 2.6.

Table 2.6. The advantages and disadvantages of cDNA and genomic DNA cloning (Amid and Hassan, 2015)

Cloning strategy	Advantages	Disadvantages
cDNA cloning	✓ The entire coding region can be obtained in one vector and transformed into bacteria for expression ✓ Can obtain the exact coding region because the intron sequences are unnecessary ✓ Proteins of interest can be produced in large quantities, greatly simplifying the task of protein purification	✓ Any regulatory information related to the introns may be lost ✓ A source of mRNA from tissue that expresses the desired produce must be found and mRNA is difficult to work with because it easily degrades
Genomic cloning	✓ To study a gene in its natural host, the whole gene is needed and it is easily obtained ✓ The intron sequence may contain enhancers that enhance the transcription of genes in a gene cluster ✓ Can use any DNA source from any tissue. This is because aside from cells of the immune system, all tissues contain the same DNA	✓ The gene sequences are extremely long and they will have to be pieced together in several clones ✓ If the aim is to obtain a protein product, the gene cloned contains introns and therefore cannot be expressed in bacteria

2.3.1.1 Polymerase chain reaction (PCR)

The polymerase chain reaction is an important genetic engineering tool which makes possible to obtain a large amount of DNA from a particular DNA sequence within a single test tube, invented by Mullis in 1990. Through PCR, a specific gene region can be amplified from whole DNA chain by very simple and sensitive way (Kalle, Kubista, & Rensing, 2014; Mullis, 1990). The overview of polymerase chain reaction can be seen in Figure 2.13. The PCR reaction mixture contains the template DNA molecule (it could be a clone DNA fragment which could be obtained from genomic library or mixture of DNA fragments such as total DNA, cDNA), the synthetic oligonucleotides acted as primers (about 20 nucleotides in length, complementary sequences of interested DNA sequence), dNTPs and a thermostable enzyme, Taq DNA polymerase. In PCR reaction initially, the temperature is increased to usually 95 ^{o}C. At this high temperature, DNA becomes denatured and two strands of DNA are separated. Then the temperature is decreased to 50-60 ^{o}C (the annealing temperature) to enable the primers to anneal the DNA. The temperature is increased to 74 ^{o}C (elongation temperature) to enable the primers to extend. This temperature is optimal for the function of Taq DNA polymerases in DNA polymerization. During this stage Taq DNA polymerase synthesizes the new strands of DNA which are complementary to template DNA by originating from the primers. The first cycle ends with the synthesis of one new DNA molecule from each template. The temperature is increased to 95 ^{o}C again and the second cycle of denaturation, annealing, and extension begins. This cycle is repeated for 30-40 times. Thus for n cycle of PCR reactions, 2^{n} copies of each template molecule are synthesized (Brown, 2007; Hart & Jones, 1998).

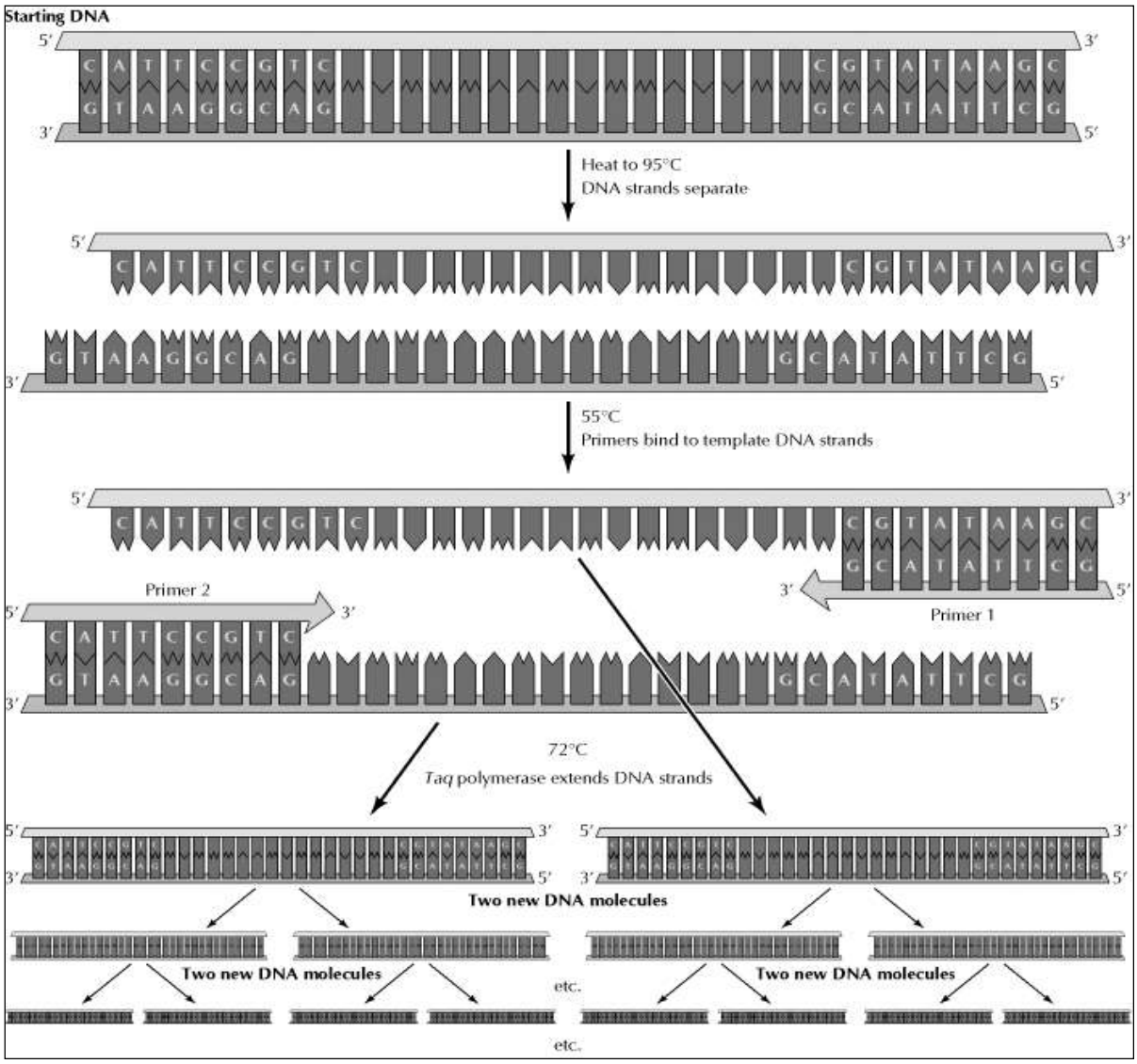


Figure 2.13. Polymerase Chain Reaction (PCR) (Cooper, 2000). The DNA molecule can be amplified by PCR. As a result of n cycle of PCR reactions, 2n copies of each template molecule are synthesized.

2.3.1.2 Restriction endonucleases

Restriction endonucleases are a part of the defense mechanism of bacteria against viral attacks. These enzymes de-activate invading foreign DNA structures by digesting it. Fundamentally bacteria produced 4 types of restriction enzymes, type I, II, III, and IV. Generally, type II is used in recombinant DNA technology. Restriction enzymes (REs) digest DNA in two way; "sticky end" (type IIP) and "blunt end" (type IIS) (Figure 2.14). Sticky end, cutting REs (type IIP) are generally preferred in recombinant DNA technology because the DNA fragments which are digested by type IIP enzymes can be bound with ligase easily

(Griffiths, Miller, Suzuki, & et.al, 2000; Loenen, Dryden, Raleigh, Wilson, & Murrayy, 2014; Tóth et al., 2014).

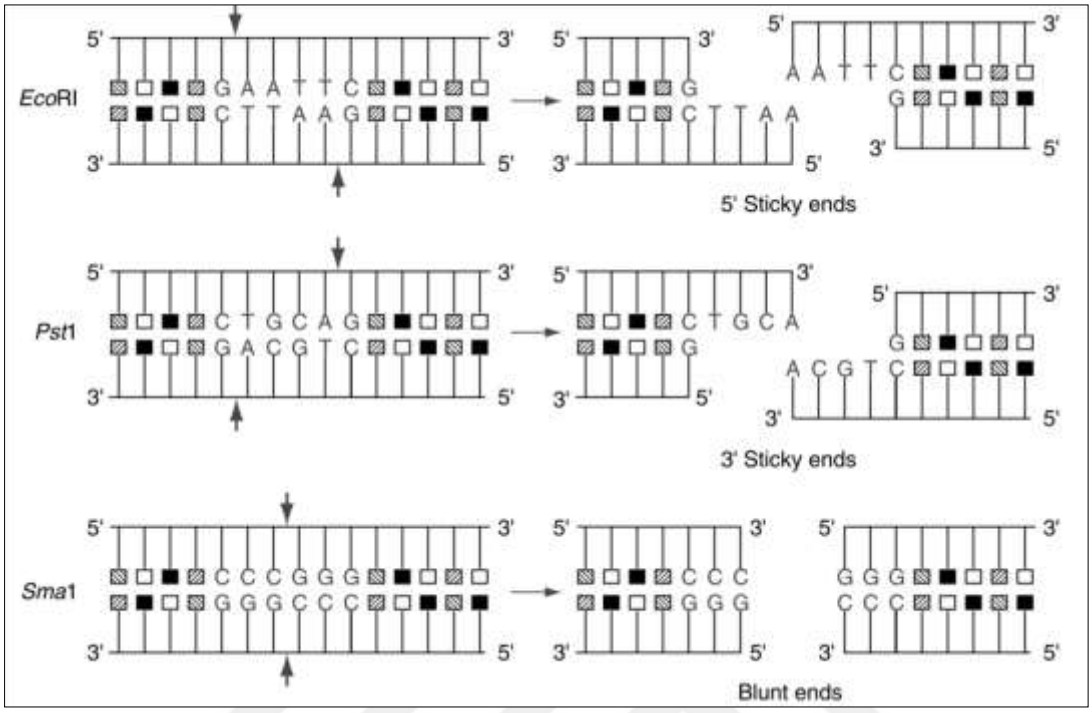


Figure 2.14. The DNA fragments cut by REs (Dale & Schantz, 2002). EcoRI and Pst1 both are an example for sticky end cutting restriction enzymes. While EcoRI makes 5' sticky ends, Pst1 makes 3' sticky ends. Sma1 is an example of the blunt end cutting restriction enzymes.

The digestion processes of type IIP enzymes are specific not randomly; they digest in specific sequences. Type IIP enzymes can be used in genetic applications owing to this specificity. Their recognition sites are termed palindromic. Palindrome means that both strands of DNA have the same nucleotide sequence but in antiparallel orientation. Type IIP enzymes recognize these sites and digest from there. For instance, as showed in Figure 2.15, *Eco*RI, one of the most commonly used RE, cuts within the palindrome sites. When the target gene and vector DNA are digested with the same type IIP enzymes the fragments will tend to attach themselves, because of the complementarity of overhanging sequences. Thus two fragments can be easily ligated with ligases (Cooper, 2000; Griffiths et al., 2000).

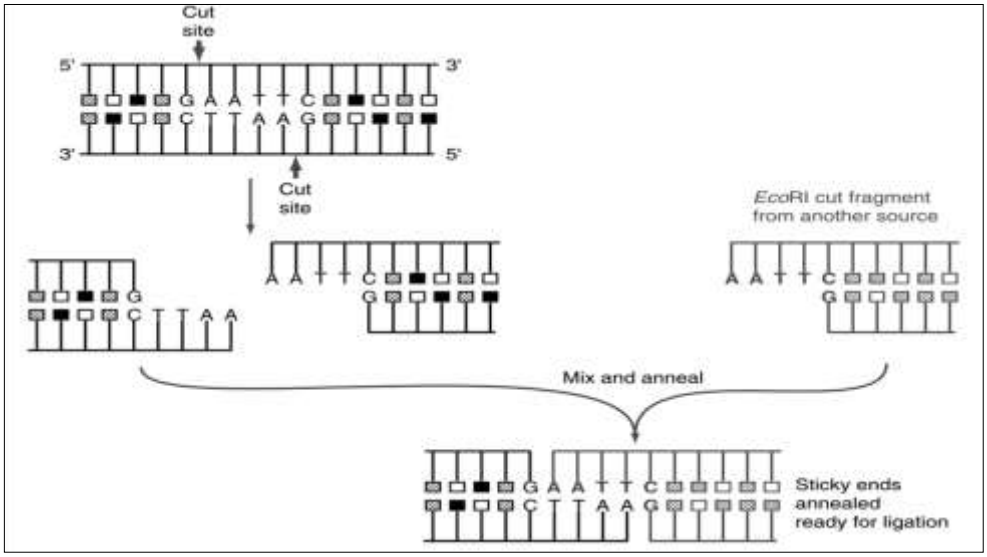


Figure 2.15. The sticky ends fragments cut by *Eco*RI (Dale & Schantz, 2002). EcoRI cuts within the palindrome sites. Palindrome indicates that both strands of DNA have the same nucleotide sequence but in antiparallel orientation.

2.3.2 Ligation

Target gene fragment and plasmid DNA, after the digestion with the same restriction enzyme, are ligated by DNA ligase enzyme. This enzyme is naturally found in all living cells and its function is that "sealed the backbones" of DNA by creating the phosphodiester bonds at the junctions (Figure 2.16) (Griffiths et al., 2000).

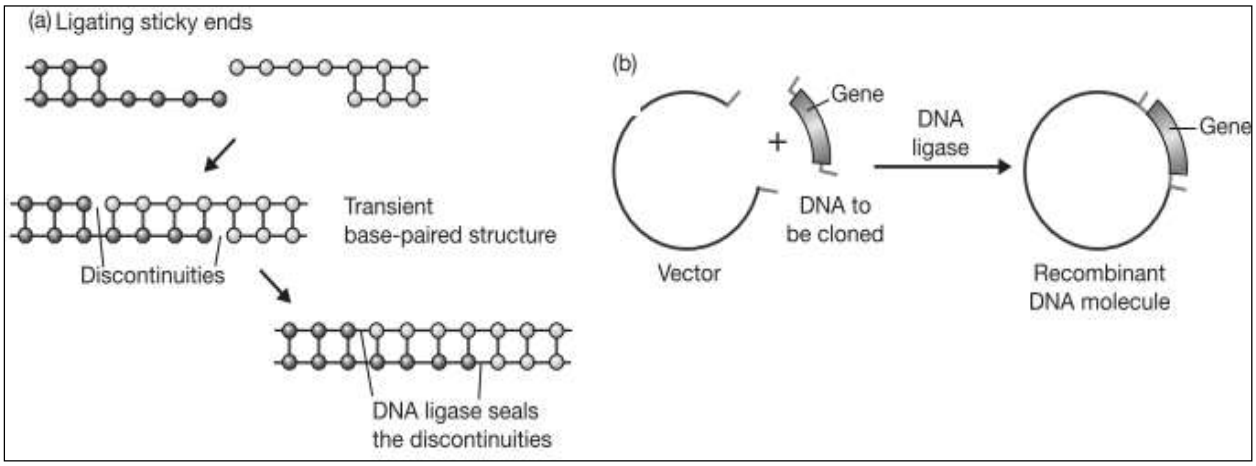


Figure 2.16. (a) The ligation of sticky ended molecules, (b) The cloning in vector via DNA ligase (Brown, 2007)

2.3.3 Transformation and Amplification of Recombinant DNA

"Transformation" describes the taking up of a DNA molecule from the surrounding media by a bacterium. Some bacteria are naturally able to do this but others aren't. These cells can be made "competent" to take up DNA. To make a cell competent physical or/and chemical treatments can be applied. $CaCl_2$ treatment is usually utilized to produce competent cells. After obtaining competent cells transformation could be completed by applying heat shock (Figure 2.17) (Hart & Jones, 1998; Inoue, Nojima, & Okayama, 1990).

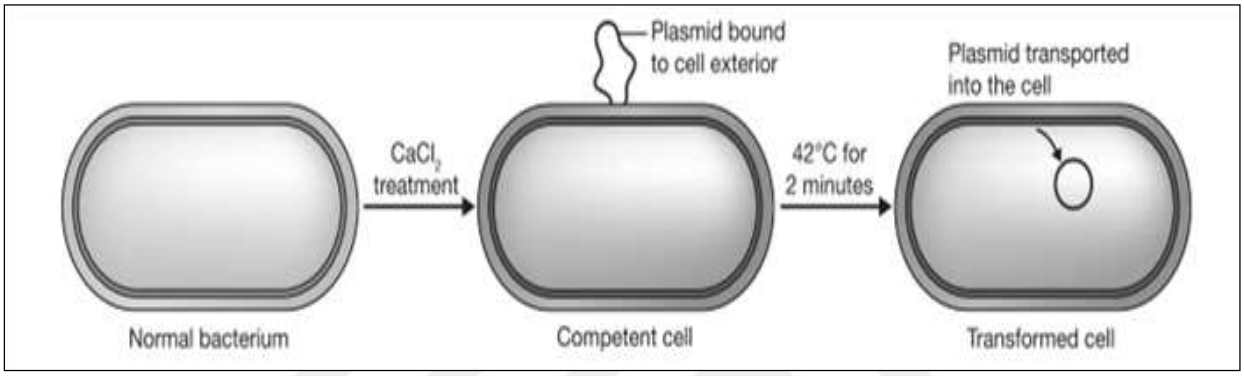


Figure 2.17. The uptake of DNA by a competent bacterial cell (Brown, 2007). The $CaCl_2$ treatment is made to bring cells competent. Thus cells can uptake the plasmid DNA.

Another method for transformation is electroporation which includes using of high-voltage electricity to make cells competent and to be healed of plasmids by the cells. This method has become more popular owing to its applicability to almost all cell types and its ease of use (Gothelf & Gehl, 2012; Potter & Heller, 2017).

The transformed colonies are able to be selected by marker genes which are found on the plasmids and to be observed by the growth of the recombinant bacteria. Generally, plasmids carry antibiotic resistance genes. After the transformation, the cells are seeded into the agar plate containing the selective antibiotic. This provides the selection of transformed cells. After that, the recombinant DNA will be amplified with the host cell owing to its replication origin that is presented in the plasmid DNA (Figure 2.18). To verify cloning, monitoring by electrophoresis after colony PCR or digestion with restriction enzymes which are used in the cloning process and after that DNA sequencing should be performed. Success of the cloning can be visualized in this way (Alberts et al., 2002; Griffiths et al., 2000).

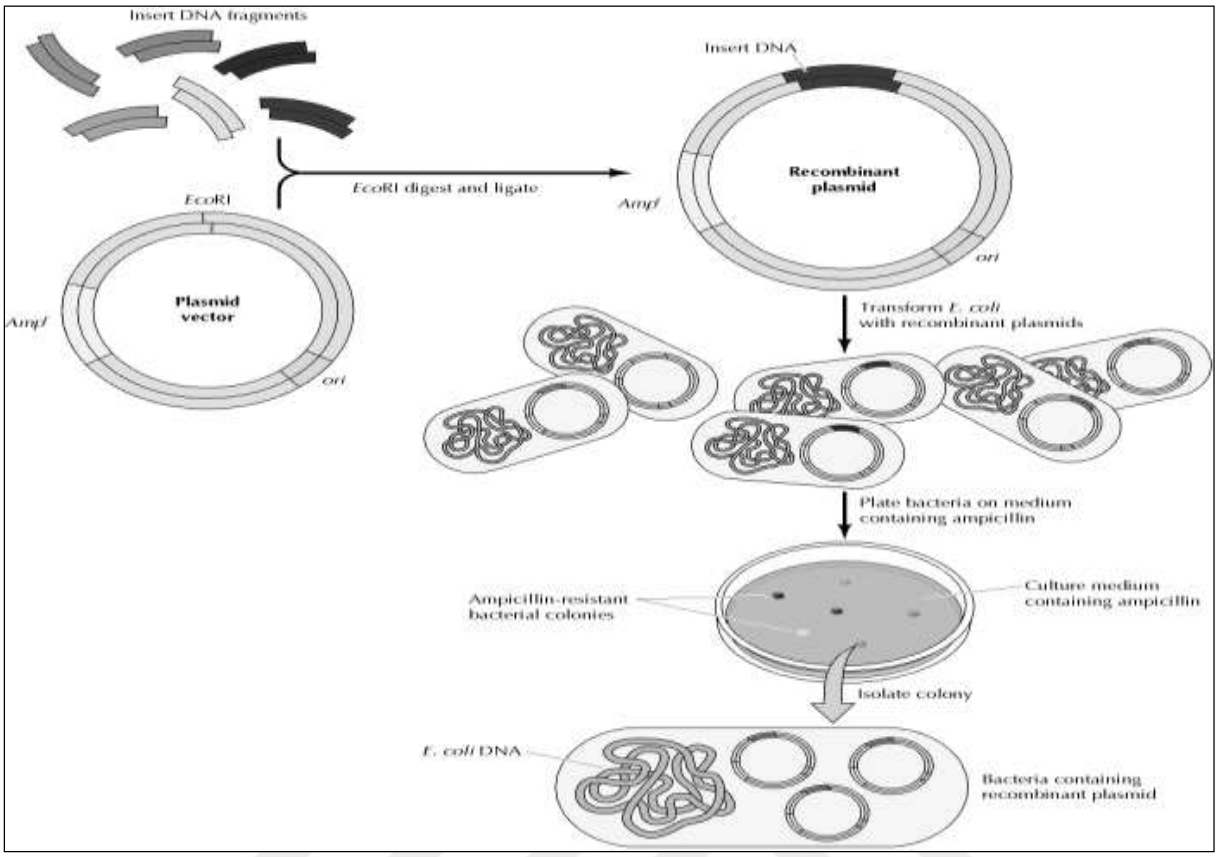


Figure 2.18. The illustration of cloning in plasmid vector (Cooper, 2000). The plasmid and the target DNA fragments are cut with the same restriction enzymes and then ligated. Plasmid with the inserted DNA fragment constitutes the recombinant plasmid. The recombinant plasmid is transferred to a host cell and proliferates with the cell.

2.3.3.1. Agarose gel electrophoresis

"Electrophoresis" describes the migration of charged molecules such as DNA, protein, etc. in an electrolyte solution as a result of the effects of an electrical field (Manchenko, 2003). Agarose gel electrophoresis is commonly used as a method for the determining the size of DNA fragments after digestion with restriction enzymes. This method is also used for the purification of DNA pieces in cloning. Agarose gel electrophoresis enables the separation of DNA fragments in the various sizes ranging from 100 bp to 25 kb. A DNA molecule is negative due to its phosphate backbone, and when agarose gel electrophoresis is applied, the DNA fragments migrate to the positively charged anode. The mass/charge ratio of DNA is uniform and they are divided by size within the agarose gel. Lower weight molecules migrate faster than those with higher mass (Lee, Costumbrado, Hsu, & Kim, 2012; Muhittin Yılmaz, Ozic, & Gok, 2012). The demonstration of the agarose gel electrophoresis method is given in Figure 2.19.

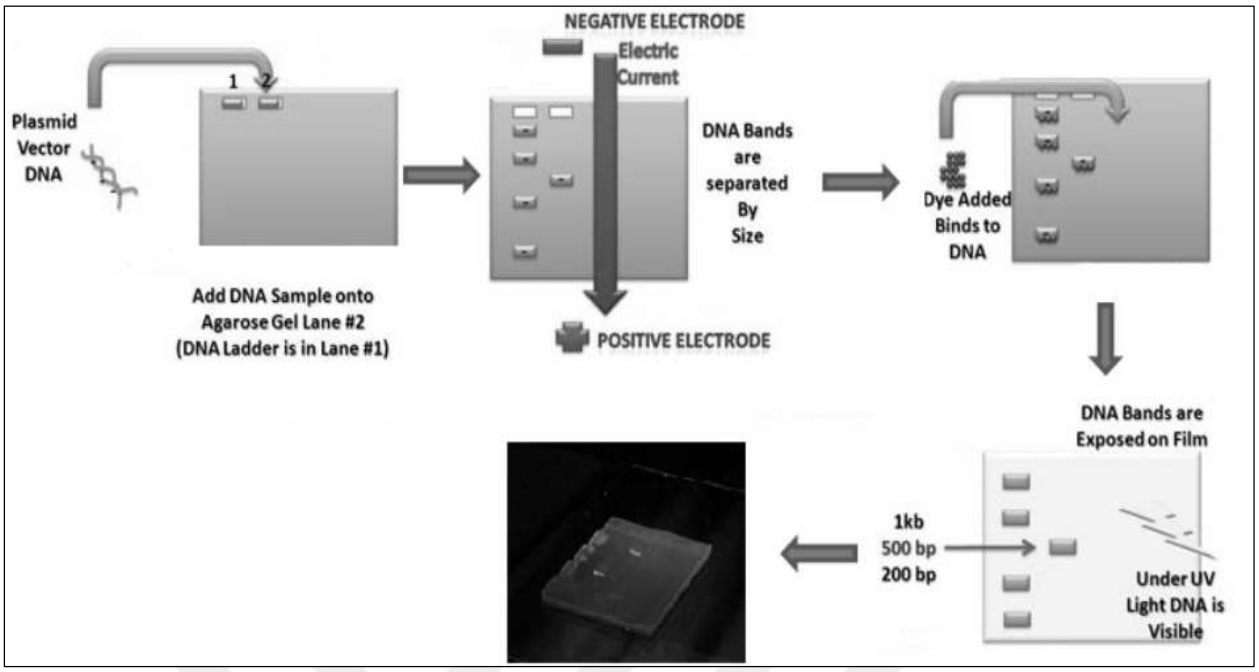


Figure 2.19. The demonstration of the agarose gel electrophoresis method (Yılmaz, Ozic & Gok, 2012). For verification of the cloning, electrophoresis is made after the cutting with the restriction enzymes of isolated plasmid DNA. The DNA ladder is loaded to the first well. It is a set of standards used to distinguish the approximate size of the DNA molecule approximately. Plasmid DNA is loaded to the second well. The DNA molecules run from the negative electrode to the positive electrode. The intercalated dye within the agarose gel binds the DNA and thus DNA becomes visible under UV light.

2.4 An Industrial Enzyme; Glucoamylase

Glucoamylase (1.4-α-D-glucanglucohydrolase, EC 3.2.1.3) is an exoamylase that hydrolyses glycogen, starch, and oligosaccharides from their non-reducing ends and releases the D-glucose. Except for the α, α-trehalose bond, glucoamylases have capability to cleave all types of α-glucosidic bonds, which are between two glucosyl parts including α-(1→4) and α-(1→6) glucosidic bonds (Aehle, 2007; Sauer et al., 2000).

Glucoamylase (GA) is an important enzyme for industry; it comes at second place, worldwide, in trade and distribution (Li et al., 2017). The main reason which makes GA so important for industry is its role in the starch process. Starch is one of the major resources for the food, textile, laundry, pharmaceuticals and paper industries. In the food industry starch is particularly used for glucose production which is used in the production of high-fructose syrups, bioethanol, and biochemicals.GA has been used since the 1960s in the industry for starch processing and the usage of GA in starch has been one of the major breakthroughs. Before the beginning using of GA in industry, inorganic acids were used for starch hydrolysis, but later, enzymatic hydrolysis has has begun to be usually preferred method. The reason of

this is enzymatic hydrolysis offers some advantages in comparison to acidic hydrolyze reactions: such as higher yields and lower costs. In addition, enzymatic hydrolysis requires less energy and shortens the process (Christakopoulos & Topakas, 2012; Hua, Luo, Bai, Wang, & Niu, 2014; Satyanarayana et al., 2004). Starch contains α-(1→4) and α-(1→6) glucosidic bounds and glucoamylase participates in starch hydrolysis after amylase (Figure 2.20). Amylase cleaves α-(1→4) bonds of starch and brings out maltodextrin (liquefied starch). Glucoamylase which is capable of cleaving both α-(1→4) and α-(1→6) glucosidic bounds of starch is used in hydrolyze liquefied starch to bring out glucose (Figure 2.21) (Aehle, 2007; Rahim et al., 2013).

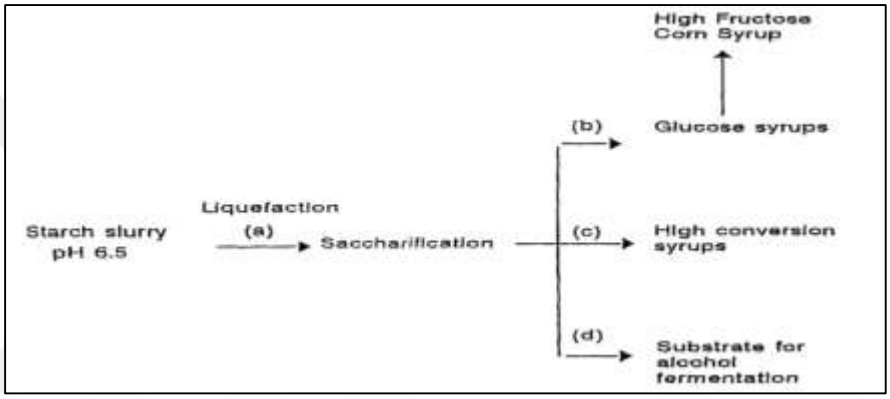


Figure 2.20. The usage of glucoamylase in the starch hydrolysis process. In the first step (a) thermostable bacterial α-amylase is added for liquefaction of starch (95 ºC, 2h). After the liquefaction glucoamylase is added. (b) To produce glucose syrup, liquefied starch is processed with glucoamylase for 48-96 hours (60 ºC, pH 4.5). (c) To produce high conversion syrup, glucoamylase is used together with the β-amylase (55 ºC, pH 5.0-5.5, 48-72 h). (d) Also to obtain substrate for alcohol fermentation, liquefied starch is processed with glucoamylase for 2 hours (60 ºC, pH 4.5) (James and Lee, 1997).

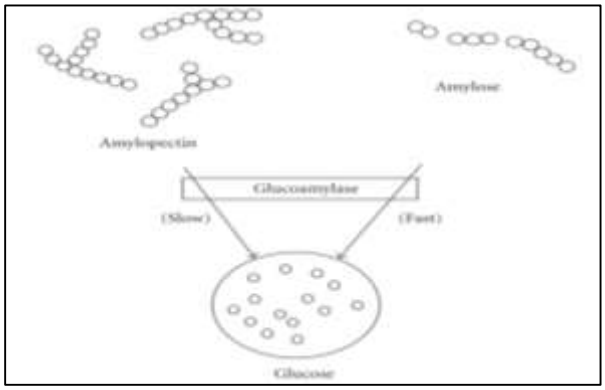


Figure 2.21. The hydrolysis of liquefied starch by glucoamylase. Glucoamylase hydrolyzes the (1-4) bonds quickly but hydrolyzes the (1-6) bonds slower. So glucoamylase slowly hydrolyzes (1-6) glucosidic bonds of amylopectin (Hii *et al.*, 2012).

In addition to glucose syrup production, glucoamylase is used in the food industry to manufacture many fermented foods, juices, and baking. Besides these, glucoamylase contributes many other industries including the production of bioethanol, beverages, confectionery, and pharmaceutical (Coral & Çolak, 2000; K. M. R. Karim et al., 2016).

Most of bacteria, yeast, and fungi can produce glucoamylase in various forms, but especially the fungal glucoamylase has importance for the industry. The filamentous fungi are great sources for the glucoamylase and the first glucoamylase has been defined in *Aspergillus niger* which is a filamentous fungus. In industry, particularly in starch processes, the glucoamylase produced by *Aspergillus niger* is predominantly preferred because of its thermostability and high activity at neutral pH values. The glucoamylase of *Aspergillus niger* can be optimally active at 50-60 ^{o}C and pH ranging from 3,5 to 5 (Bagheri et al., 2014; M. K. R. Karim et al., 2016; Li et al., 2017; Sauer et al., 2000).

Filamentous fungi have the ability to produce some proteins abundantly. *Aspergillus niger* also has a great secretion capacity to secrete some proteins, including glucoamylase in a culture medium. Indeed glucoamylase constitutes more than 50% of its extracellular proteome. Because of this great secretion capacity of *Aspergillus niger*, the glucoamylase promotor (glaA) and signal sequence are widely preferred for recombinant protein production (Fleißner & Dersch, 2010; Kwon et al., 2012; Lu et al., 2010).

It is possible to produce glucoamylase from fungus through the solid state, semi-solid state and submerged fermentation in an airlift reactor, in a tank vessel or in stacked trays. However fungus are slow growing organisms and it is difficult to manipulate in fermenter due to their complex morphology (Adrio & Demain, 2010; Norouzian, Akbarzadeh, Scharer, & Moo Young, 2006; Wucherpfennig, Hestler, & Krull, 2011). Besides that, the use of fungus such as *A.niger* in heterologous protein production has some limitations as the suitable plasmid vector number is low and not stably retained over the long term. In other words, production of glucoamylase which is proper for the industry by Aspergillus niger is not successful enough on the industrial scale. Therefore producing of the Aspergillus niger glucoamylase as heterologous in a different host can be more efficient in industrial scale (Fleißner & Dersch, 2010; Storms et al., 2005).

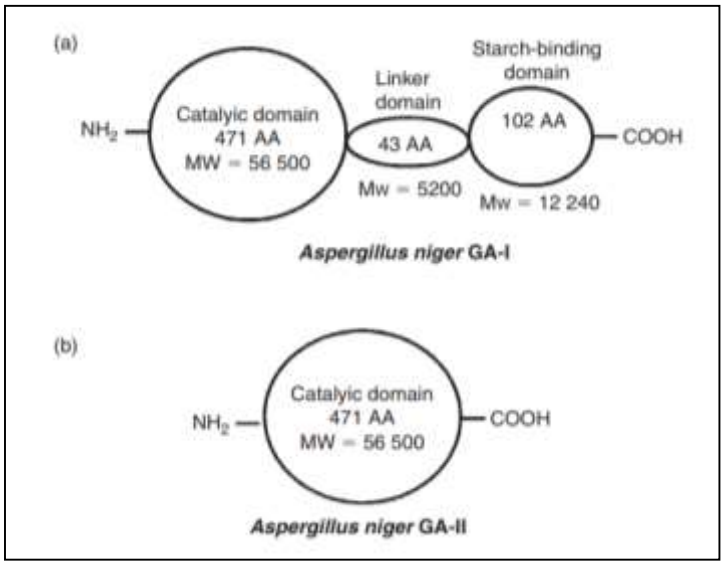


Figure 2.22. The schematic description of *Aspergillus niger* glucoamylases. (a) The GAI form of A.niger glucoamylase contains starch binding domain which links to catalytic domain with highly O-glycosylated segment. (b) The GAII form of *A.niger* glucoamylase contains only the catalytic domain (Taylor, 2015).

There are two forms of Aspergillus niger glucoamylase which is used in industry; GAI and GAII. Mainly these two forms are highly homologous in structure due to their amino acid sequences. But they show differences as to their carbohydrate content, pH and temperature stability, and activity. Also, the most important difference is that GAI has a starch binding domain, but GAII hasn't. The GAI form of glucoamylase has a great capacity to digest raw starch. It has the ability to hydrolyze starch completely. However, the GAII form has lower activity (James & Lee, 1997; Svensson, Pedersen, Svendsen, Sakai, & Ottesen, 1982). The glucoamylase GAI of Aspergillus Niger consists of two domains and a linker part (Figure 2.22). The N-terminal domain (Ala-1-Thr-440) contains the catalytic site and the C-terminal domain (Pro-512-Arg-616) contains the starch binding site which is essential for hydrolysis of raw starch. The linker part (Ser- 441-Thr-511) is a greatly O-glycosylated fragment (McDaniel, Fuchs, Liu, & Ford, 2008; Sauer et al., 2000; Stoffer et al., 1993).

3. MATERIAL AND METHODS

In this study, it was intended to clone the glucoamylase gene region of *Aspergillus niger* (glaA) to carrier plasmid PGAPZαA and transfect to *Pichia pastoris*. The methods which are given in Figure 3.1. were followed.

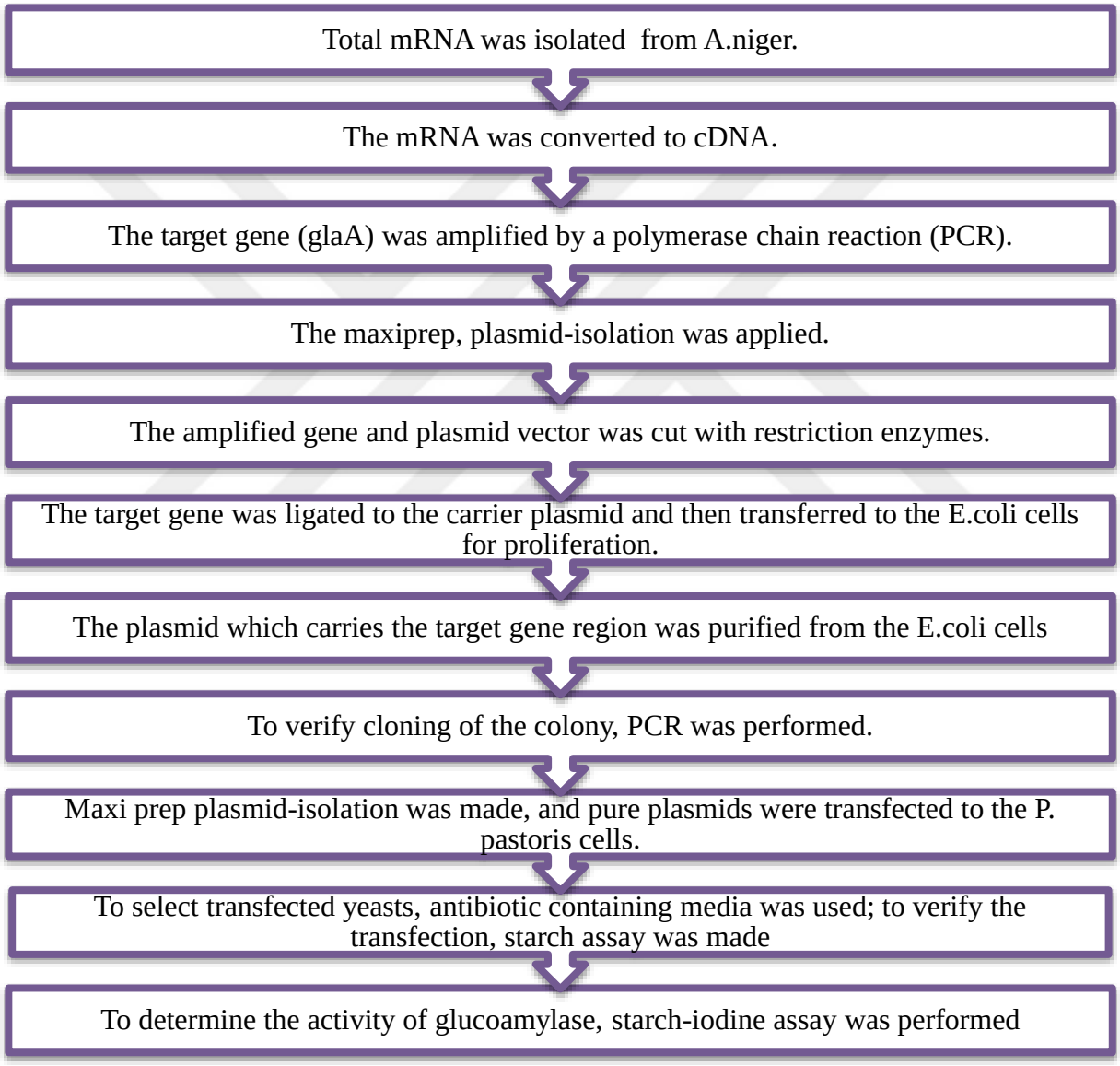


Figure 3.1. The methods used in this study

3.1 Amplification of the Plasmid

As a carrier vector, plasmid PGAPZαA was used (Figure 3.2). The DNA sequence of multiple cloning sites of plasmid pGAPZαA could be found in appendix 2.

The plasmid has an α-factor secretion signal tag that allows for the secretion of glucoamylase from P.pastoris. Due to this tag yeast cells carrying plasmid PGAPZαA can express an extracellular glucoamylase enzyme Also, the plasmid has a Zeocin (antibiotic) resistance gene region that provides cells selectivity. In this way, only the cells which have taken on plasmid could survive on Zeocin containing culture media.

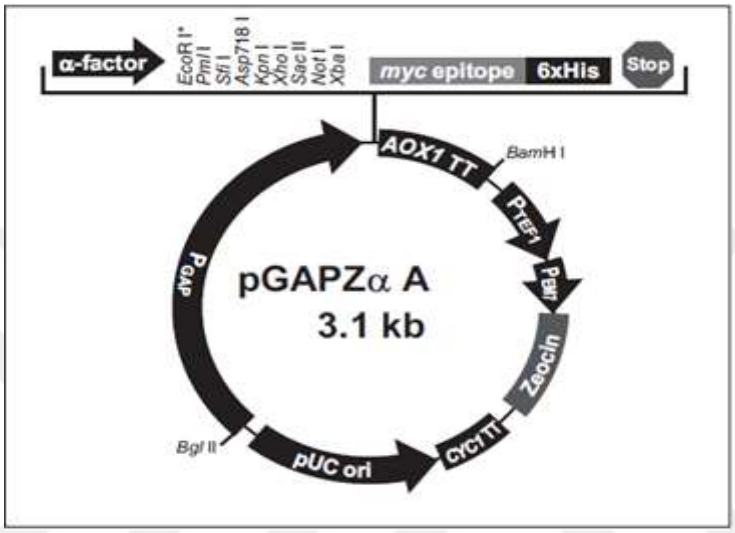


Figure 3.2. The map of plasmid pGAPZαA

The E.coli cells which contain plasmid pGAPZαA were cultured to amplify plasmids. After proliferation maxiprep isolation was made by GeneJET Plasmid Maxiprep kit (ThermoFisher Scientfic, Cat No: K0491).

3.2 Amplification of the Target Gene

To obtain the target gene cDNA was synthesized from mRNA isolated from source organism. Aspergillus niger, NRRL3 (ATCC 9029, CBS 120.49) strain was used as the source organism. Firstly, the A.niger mycelia were collected and cultured in liquid media. As culturemedia, LB (Miller) broth (appendix 6) was used and 4gr/lt starch was added to be able to increase the amount of mRNA. The cells were cultured for 3 days in a shaking incubator at 37°C. After the incubation period, mycelia were collected and grinded in liquid nitrogen. Subsequently, the total mRNA was isolated, using a GenElute Total RNA Prufication kit (Sigma, Cat.No:RNB100).

The cDNA was converted from mRNA by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Cat.No:4368814). The cDNA was used as the template for the

amplification of the glucoamylase gene; glaA, by a polymerase chain reaction (PCR). The nucleotide sequence of the *Aspergillus niger* glaA gene region can be found in appendix 1.

PCR was applied by using FastStart™ High Fidelity PCR kit (Roche, Cat.No:FHIFI-RO) in accordance with the manual of the product. For the reaction a thermal cycle device was used with this thermal profile;

- ✓ Initial Denaturation - 95°C – 5 minutes
- ✓ Denaturation - 95°C – 1 minute
- ✓ Annealing - 70°C – 1 minute
- ✓ Extension - 72°C – 2 minutes

35 cycles

- ✓ Final extension - 72°C – 10 minutes

Afterwards, the PCR products were run for 45 minutes at 90 V in an 8% agarose gel electrophoresis with a TBE buffer and EtBr.

3.3 Digestion with Restriction Endonucleases

The PCR products and the carrier plasmid PGAPZαA were digested with the restriction enzymes. For this reaction, EcoRI and NotI enzymes were used. The reaction was carried out at 37 ° C for 16 hours and was ended with keeping it at 65° C for 20 minutes. After the reaction products were run in an 8% agarose gel contained EtBr by electrophoresis with a TBE buffer. The samples and the 1 kb DNA ladder were loaded into the gel and were run for 60 minutes at 90 V. After that the desired bands were extracted from the gel using GeneJET Gel Extraction Kit (Thermo Scientific™, Cat.No:K0691).

3.4 Ligation and Transformation

The purified, digested plasmid and PCR product were ligated with using the Rapid DNA Ligation Kit (Thermo Scientific™, Cat No:K1422). The reaction took place at 37 °C for 4 hours. After that, the ligation product was transformed into competent E.coli cells. The transformation conditions are indicated below;

1. Competent bacteria were kept on ice for 10 minutes to thaw. After that 1ng of a ligated plasmid was added to competent bacteria and kept on ice for 30 minutes more.
2. The bacteria were heat shocked n a water bath which was kept at 42 °C, for 90 seconds, and then kept on ice for 5 minutes.

3. The LB broth previously incubated at 37 °C, was added on bacteria and incubated at 37 °C for 1 hour and agitated at 180 rpm.
4. After the incubation period, the bacterial cells were precipitated by centrifugation at 14,000 rpm for 1 minute. The precipitated cells were resuspended and cultured on Luria-Bertani (LB) Lennox agar (supplemented with 25 µg/mL Zeocin) plates for selection of transformed colonies. Plates were incubated at 37 ^{o}C overnight.
5. On the following day, the transformed bacteria colonies which were able to survive on antibiotic-treated agar were picked up and cultured in a Luria-Bertani (LB) Lennox medium supplemented with 25 µg/mL Zeocin

3.5 Confirmation of Molecular Cloning

Molecular cloning of the glucoamylase gene was confirmed by colony PCR according to PCR kit (Sigma, Cat.No: CORET) manual under following conditions.

- ✓ Initial Denaturation - 95°C – 30 seconds
- ✓ Denaturation - 95°C – 30 seconds
- ✓ Annealing - 60°C – 30 seconds
- ✓ Extension - 68°C – 2,5 minutes

} 35 cycles

- ✓ Final extension - 68°C – 5 minutes

After the PCR process agarose gel electrophoresis was made to check the PCR product. For electrophoresis, 8% agarose gel contained 1µL EtBr was prepared with TBE buffer. The PCR products and the 1 kb DNA ladder were loaded into the gel and run for 45 minutes at 90 V.

After that, miniprep plasmid isolation was made from the positive recombinant colonies which were confirmed by PCR. The amount of isolated plasmid DNA was measured using a Qubit Fluorometer (İnvitrogen, Cat.No:Q32851). After mini-prep plasmid isolation from positive colonies, purified samples were sent for sequence analyses to check against the possibility of any mutation having occurred during PCR.

3.6 Transfection of Recombinant Plasmid to *Pichia pastoris*

Before the transfection purified plasmid DNAs were linearized by XmaJI restriction enzyme digestion. The linearization of plasmid DNAs was checked by agarose gel electrophoresis. After that linear plasmid was pruficated by using MinElute PCR purification kit (Qiagen Cat.No: 28004). The amount of linear DNA was measured by Qubit. The linear plasmid was

transfected to Pichia Pastoris (X33) by electroporation using a Gene Pulser II (Bio-Rad) according to the instructions of the manufacturer as indicated below.

- ✓ Firstly, yeast cells were cultured overnight with YPD medium in a shaking incubator at 180 rpm.
- ✓ Then cells were precipitated by centrifuge and re-suspended in 1M sorbitol.
- ✓ The linearized recombinant plasmid DNAs were added to re-suspended cells, after that the mixture loaded to an electroporation device and shocked with 1520 V for 5,2 milliseconds.
- ✓ Immediately afterwards, cells were treated with 1µL sorbitol and were cultured on 25 µg/mL Zeocin added YPD (SIGMA, Cat. No: Y1375) agar plates. The plates were kept at 28 ºC for 2 hours in the incubator and then were incubated in ashaking incubator for 3 days.
- ✓ After 3 days the transfected colonies became visible. These colonies included recombinant plasmid and were able to survive on Zeocin added agar plates owing to the Zeocin resistance gene on the plasmid.

3.7 Colony PCR

One transformed colony was selected and cultured in 25 µg/mL Zeocin added YPD broth (SIGMA, Cat. No: Y1375) for 72 h at 30^{o}C in a shaking incubator. After the incubation, 2 mL of the culture was put into a tube and frozen. The sample was thawed and kept at 95^{o}C for 2 minutes. Cells were centrifuged at maximum speed. The supernatant was used for PCR as a template. The PCR reaction was made with PCR kit (Sigma, Cat.No:CORET) under the following conditions.

- ✓ Initial Denaturation - 95°C – 30 seconds
- ✓ Denaturation - 95°C – 30 seconds
- ✓ Annealing - 60°C – 30 seconds
- ✓ Extension - 68°C – 2,5 minutes

} 35 cycles

- ✓ Final extension - 68°C – 5 minutes

After the PCR, products were run in agarose gel electrophoresis to check positive or negative PCR products. For electrophoresis, 8% agarose gel was prepared with TBE buffer and EtBr was added to the gel. The PCR products and the 1 kb DNA ladder were loaded into the gel and run for 45 minutes at 90 V.

3.8 Starch Agar Plate Assay

A starch agar test was performed to see if the transfected colonies had received the recombinant-plasmid, completely and whether the desired gene was working. M9 minimal, medium, agar plates with starch added, were prepared (appendix 6). Transfected cells were seeded on the M9 starch agar plate from YPD agar. Also, normal X33 cells were seeded on the other side of the M9 starch agar to enable a comparison. The cells were incubated for 3 days, and, after the incubation, plates were stained with Lugol's iodine vapor.

3.9 Protein Quantitation Assay

Recombinant X33 cells, normal X33 cells, and Aspergillus Niger (NRRL3) cells were cultured in M9 minimal medium for 72 h at 34^{o}C in a shaking incubator. After the incubation, cultures were centrifuged. The supernatant was used for the analysis.

The protein quantity of supernatants was determined using a protein quantitation assay kit (Pierce BCA Protein Assay, Cat. No: 23225). Absorbance values were measured at 562nm on a plate reader. Standard protein solutions in different concentrations (2000, 1500, 1000, 750, 500, 250 and 125 μg/mL) were measured and a calibration curve was plotted. The protein concentrations of supernatants were calculated according to this curve.

3.10 Enzyme Purification

Recombinant X33 cells were cultured in a 4g/l starch added YPD medium for 72 h at 34^{o}C in a shaking incubator. After the incubation, cultures were centrifuged and the supernatant was collected. The enzyme presented in the supernatant was precipitated with using ammonium sulfate by following instructions (Simpson, 2006);

1. The supernatant was transferred to a beaker placed on ice.
2. 0.7 g/mL(The table presented in the appendix 5 was used to find this ammonium sulfate concentration) ammonium sulfate was weighted and added drop by drop, to cold supernatant placed on the magnetic stirrer.
3. The total amount of ammonium sulfate was added to the supernatant over a 30 minute period. After that, the solution was mixed for 60 minutes on a magnetic stirrer.
4. The solution was centrifuged at 10,000 G for 15 minutes at 4 ^{o}C.
5. The pellet (precipitated enzyme) was resuspended in a PBS buffer.

The dissolved enzyme in PBS was filtered with a centrifugal filter unit (Merk-Amicon Cat. No:C7715) to purify the enzyme from ammonium sulfate. The purified enzyme was collected in 500 µL PBS. To use in the assessment, the enzyme was diluted to 1/100 with PBS.

3.11 Glucoamylase Activity Assay

Enzyme activity was determined by a starch – iodine assay. The change in optic density was measured by spectrophotometer (Shimadzu UV–VIS, UV mini-1240) at 620 nM. The standard starch solutions in different concentrations of (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 g/l) were prepared for the calibration curve.

Varied dilutions (10-100%) were prepared from 1/100 diluted enzyme to determine enzyme activity. 500 µL of the dilutions were treated with 5 mL, 1g/lt starch solution for 1 minute under room conditions. At the end of 1 minute the enzyme activity was stopped with 5 mL 0,1N HCL and treated with 5 mL Lugol's Iodine solution.

The relation of enzyme activity with temperature and time was measured at 25^{o}C, 34^{o}C, and 55^{o}C for 15, 30 and 60 minutes. 0,5 mL of the enzyme (1/100 diluted) was treated with 5 mL 10 g/lt starch and then enzyme activity was stopped with 5 mL 0,1N HCL and treated with 5 mL Lugol's Iodine solution.

3.12 Plasmid Retention Time Assay

The plasmid loss rate of recombinant X33 cells was determined by using starch agar plates. The frozen recombinant X33 cells were thawed and cultured on a YPD agar supplemented with 25 µg/mL Zeocin. After 3 day incubation, a single colony from the cultured recombinant X33 cells was selected and sub-cultured on a starch added YPD agar. The media used for sub-culturing was non-selective. It didn't contain Zeocin but it contained starch. The plasmid containing X33 cells had the ability to degrade the starch presented in the agar. The conditions (whether cells had lost their plasmids or not were) determined by staining with Lugol's iodine vapor. For each sub-culturing two cultures were prepared and cells were incubated for 3 days. After the incubation periods, two new sub-cultures were prepared from one of the previous cultures and iodine-vapor-staining was performed on the second one.

3.13 Statistical Analyses

All experiments were conducted in triplicates and one way analysis of variance (ANOVA) at p=0.05, and in addition to that, the 95% and 99% confidence intervals were calculated using Excel program.

4. RESULTS AND DISCUSSION

4.1 Confirmation of Cloning

After ligation and transformation to *Escherichia coli*, cloning was confirmed by colony PCR (Figure 4.1.).

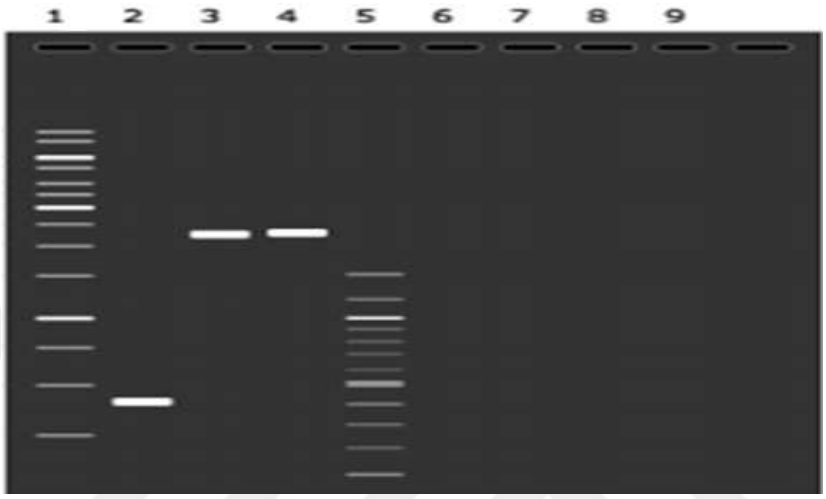


Figure 4.1. Colony PCR results of transformed E.coli cells with recombinant plasmid. (1- 1kb DNA ladder (GeneRuler^{TM} Thermo Scientific, Cat.No:SM0311), 2- empty PGAPZαA plasmid, 3,4- recombinant PGAPZαA (plasmid + glucoamylase gene).

After that from positive colonies sequence analyses was performed. The results of sequence analyses can be found in appendix 3.

4.2 Transfection of Recombinant Plasmid to *Pichia Pastoris*

For transfection, recombinant plasmids were isolated from E.coli cells. The amount of isolated plasmid DNA was measured by Qubit Fluorometer and was found as 898 µg/mL. After linearization by XmaJI restriction enzyme digestion and purification, the number of linear DNAs were measured again and found as 292 µg/mL. After that linear plasmid was transfected to *Pichia pastoris* by electroporation.

In Figure 4.2. the transfected P.pastoris colonies could be seen. For the selection of recombinant P.pastoris cells, yeast was cultured on a 25 µg/mL Zeocin containing YPD agar.

Only the recombinant cells could grow on Zeocin added YPD agar owing to antibiotic resistance gene found on the plasmid.

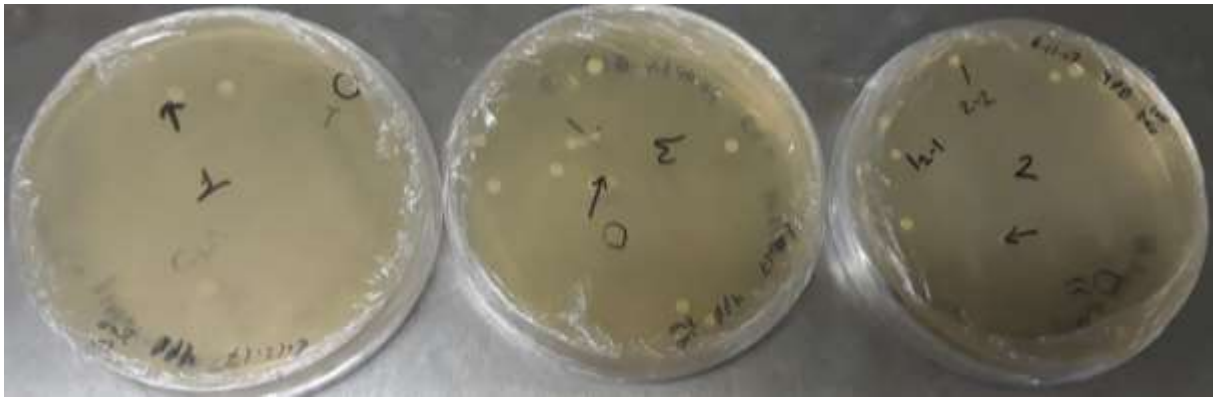


Figure 4.2. Transfected P.pastoris colonies on 25 µg/mL Zeocin containing YPD agar which were incubated for 3 days after electroporation Cells

4.3 Colony PCR of Transfected Yeast Cells

The condition as to whether yeasts had taken on the recombinant plasmid DNA or not was checked by colony PCR. The gel image of colony PCR could be seen in Figure 4.3.

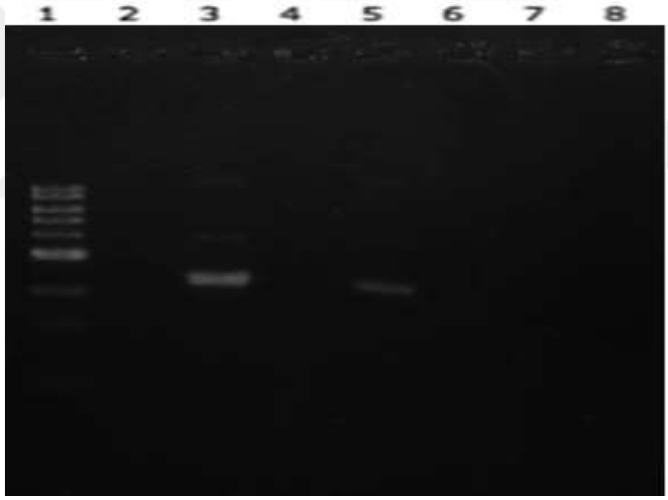


Figure 4.3. The gel image of colony PCR after transfection. 1- 1kb DNA ladder (GeneRuler^{TM} Thermo Scientific, Cat.No:SM0311), 3- The PCR product in which Recombinant PGAPZαA plasmid was used as template, 5- The PCR product in which recombinant yeast cell lysate used as template. Note: The PCR product size isbetween 2500 and 2000 bp

4.4 Starch Agar Plate Test

After transfection, a starch agar test was performed on the colonies to see if the transfected glucoamylase gene was working or not.

Glucoamylase is an exoenzyme that hydrolyzes the α-(1→4) and α-(1→6) glucosidic bonds of starch (Li et al., 2017). Iodine binds the starch polymers and brings out blue-black color (Pfister et al., 2016; Xiao, Storms, & Tsang, 2006). The recombinant yeast cells cultured on a

starch agar plate secretes glucoamylase and hydrolyzes the starch that is present in the agar plate. When these plates are exposed to iodine vapor, the regions with starch present are stained. But there is no change of color in the regions where yeast cells hydrolyze the starch.

As seen in Figure 4.4., there a light zone around the recombinant X33 cells on the left side but no color change on the right side of the iodine vapor stained plate. The recombinant X33 cells were cultured on the left side (A) and the normal X33 cell was cultured on the right side (B) of the plate. It is clearly understood from Figure 4.4 that recombinant cells were able to secrete glucoamylase but normal cells were not.

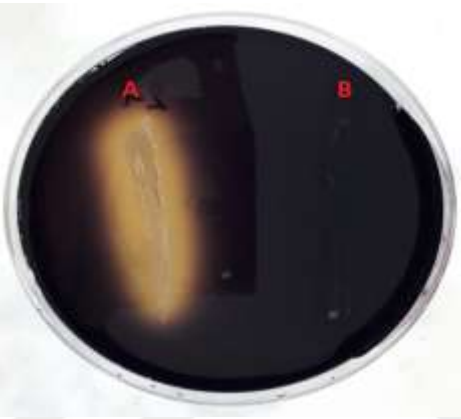
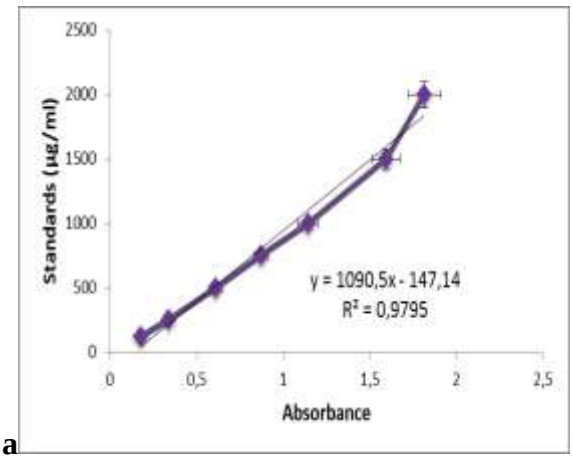


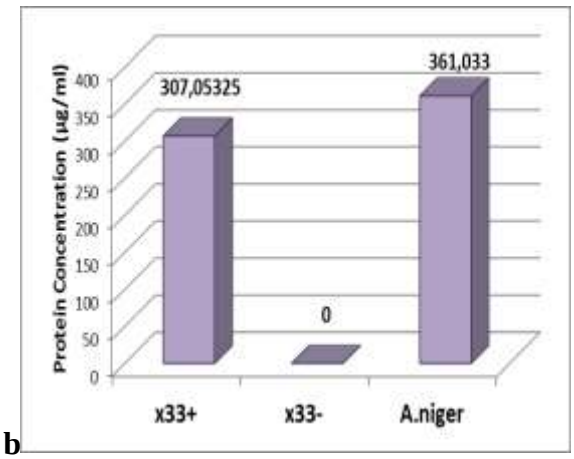
Figure 4.4. The starch agar plate test. A- Recombinant X33 cells, B- Normal X33 cells.

4.5. Protein Quantitation Assay

The quantities of the protein presented in supernatants of transfected yeasts were analyzed and the quantities of protein were calculated by using the equation of calibration curve which was plotted by using standards (Figure 4.5.).



a



b

Figure 4.5. a- Calibration curve which was prepared from standard protein solutions. **b-** Protein Concentrations in supernatants of the recombinant X33 cells ($X33^{+}$), the normal X33 cells ($X33^{-}$) and the source organism (A.niger).

The amount of protein secreted from normal (non-transfected) X33 cells ($X33^{-}$) equals to zero (Figure 4.5b). This indicates that, in these conditions, normally X33 cells do not produce any extracellular protein. It could be considered that the calculated amount of protein secreted from recombinant X33 cells would be equal to the amount of the secreted glucoamylase enzyme. *Aspergillus niger* can secrete several types of extracellular enzymes and the amount of these enzymes can change according to the carbon source in the medium (Lu et.al., 2010). Besides glucoamylase constitutes more than 50% of the extracellular proteome of A.niger which cultured in medium which contain only starch as a carbon source (Kwon et.al., 2012). There was a little difference between the amounts of the calculated extracellular protein of A.niger and that of the recombinant X33. It could be asserted that the difference most probably results from the other exoenzymes of A.niger and that the glucoamylase level of our recombinant yeast cell is nearly as high or probably even higher than the glucoamylase level of the source organism.

4.6 Enzyme Activity Assays

The enzyme presented in the supernatant was precipitated using ammonium sulfate and filtered with a centrifugal filter unit to purify the enzyme from ammonium sulfate. To calculate the enzyme activity Lugol's Iodine method was applied and the standard curve presented in Figure 4.6 was generated. The curve was obtained from triplicate determinations.

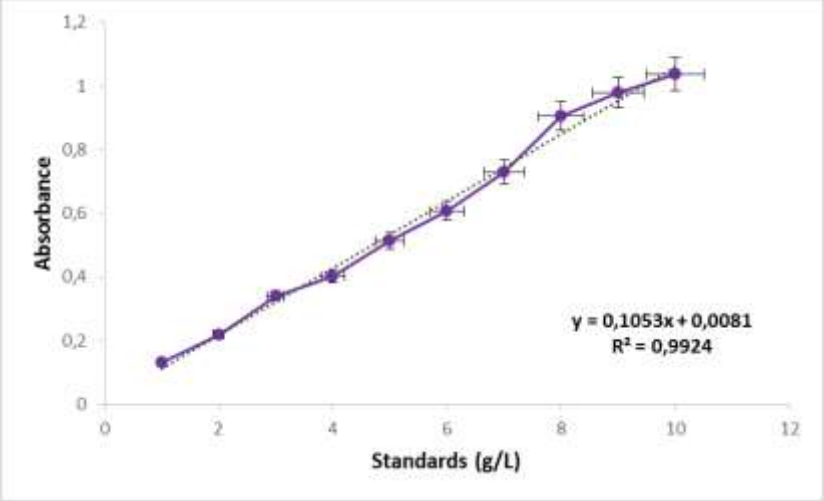


Figure 4.6. Calibration curve plotted from standard starch solutions.

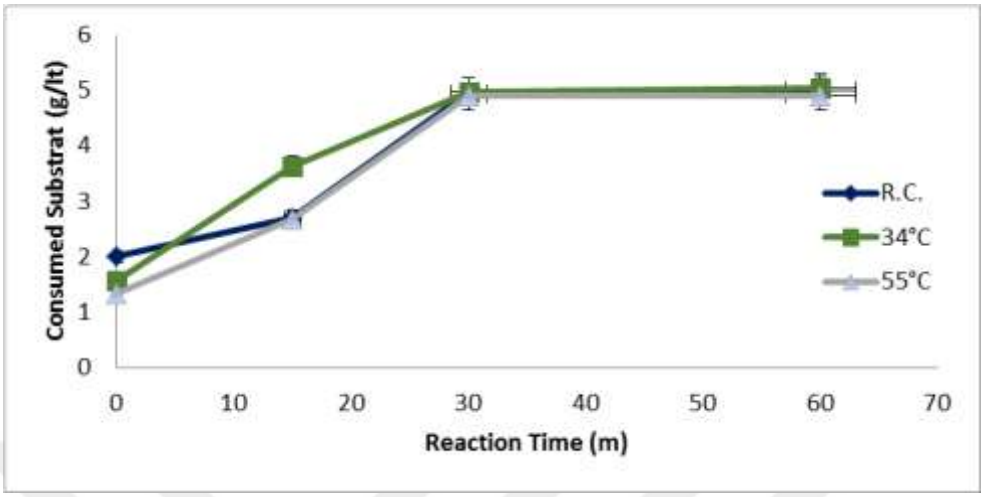


Figure 4.7. The relation of enzyme activity to temperature and time. (Blue- Room Condition (R.C.), Green- 34^{o}C, Grey- 55^{o}C)

The alteration of enzyme activity with time and temperature was investigated and the results could be seen in Figure 4.7. The enzyme was incubated with 10 g/lt starch solution for 15, 30 and 60 minutes at room conditions (R.C), 34^{o}C and 55^{o}C. The activity of the enzyme in three different temperatures shows that the enzyme activity alteration occurs only until the first 30 minutes of incubation. However, for each of the three temperatures, the activity of the enzyme was the same and constant after 30 minutes. These results are supported by the studies in the literature. Coral and Çolak indicated that the activity of glucoamylase from A.niger is constant between the 40th and 70th minutes of incubation; Hua and friends also reported that the activity of recombinant glucoamylase from Bispora sp. is constant between 30th and 60th minutes of incubation time at varying temperatures (Coral and Çolak, 2000; Hua et.al., 2014). Interestingly the enzyme consumed nearly half of the substrate in 30 minutes, in each of the three conditions. So this assay revealed that the activity of the enzyme is not changed at 3 different temperatures. Many studies indicate that the optimal temperature of glucoamylase, isolated from A.niger and from several yeasts, is under 55^{o}C (Paszczynski et. al, 1982; Kumar and Satyanarayana, 2009). However, in starch hydrolysis processes such as the production of glucose syrup and bioethanol the glucoamylase enzyme is used above 55 ^{o}C. (James and Lee, 1997). It was observed that the enzyme was still active in this temperature.

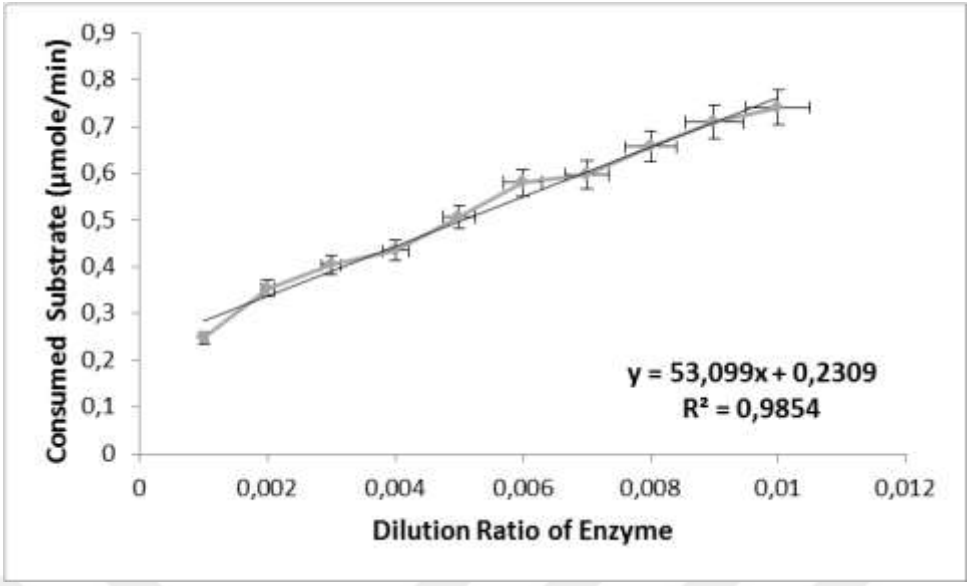


Figure 4.8. The relation between enzyme activity with enzyme concentration.

To determine the activity, the enzyme at varying dilutions was treated with 1 µmole starch solution for 1 minute in room conditions. The curve (Figure 4.8) was obtained from triplicate determinations. The linear equation of the curve (y=53,099x + 0,2309) was used for calculation of the enzyme activity. The amount of enzyme which consume 1 µmole of the substrate in one minute, was calculated as 7.24 µL /min, from the linear equation of the curve (y=53.099x + 0.2309) found in Figure 4.8. According to Nomenclature Committee of the International Union of Biochemistry (NC-IUB) the amount of enzyme which consumes 1 µmole of the substrate in 1 minute under room conditions was defined as the unite of enzyme activity(International Union of Biochemistry, 1979).

The dilution ratio of the enzyme that consumed 1µmole substrate is represented by x;

$$x = \frac{1 - 0{,}2309}{53{,}099} \quad x = 0{,}0145 \text{ (dilution ratio)}$$

The amount of enzyme that consumed 1µmole substrate= $0{,}0145 \times 500\mu l = 7{,}24$ µL/min enzyme.

So we can say that 1 Unit enzyme is found in 7,24 µL enzyme.

If we calculate the enzyme unit in 1 mL of enzyme volume

The unite of the enzyme in 1 mL= $\frac{1}{7{,}24\times10^{-3} ml/min} = 138\ U/ml$

In literature, there are many studies about glucoamylase being obtained from several species as recombinant or wild type. Li et.al reported that the activity value of glucoamylase produced

by the recombinant *Esherichia coli* cells, which were transformed with the glucoamylase gene of *Corallococcus sp.* was 180 U/mg (Li et al., 2017). Hua et.al reported that the activity of glucoamylase produced by the recombinant *Pichia pastoris* cells which were cloned with the glucoamylase gene of Bispora sp. MEY-1 was 34.1 U/mL (Hua et al., 2014). Chen et.al reported that the activity of glucoamylase produced by the recombinant *Pichia pastoris* cells which were transfected with the glucoamylase gene region of *Chaetomium thermophilum* was 16-73 U/mL (Chen et al., 2007). Fierobe et.al reported that the activity of glucoamylase obtained from recombinant S.cerevisia, A.niger and P.pastoris cells were 8.28, 7.83 and 7.6 U/mg respectively (Fierobe, Mirgorodskaya, Frandsen, Roepstorff, & Svensson, 1997). Satyanarayana et.al reported that the activity of glucoamylase produced by the *T.indicae-seudaticae* cells was 46 U/mL(Satyanarayana et al., 2004).

In the all studies mentioned above and many studies in literature the activity of glucoamylase was determined from the amount of reducing sugar realized from starch by using the DNS (3,5-Dinitrosalicylic acid) or glucose oxidase methods. So they assumed that the one unit of glucoamylase enzyme activity equal to the amount of enzyme that releases 1μmol of glucose in one minute. Thus the comparison the activity of enzyme determined in this study with literature and make an interpretation about wouldn't be entirely true. Because in this study, in distinction from others, enzymatic activity was estimated from the amount of starch consumed as a substrate by starch-iodine assay and the amount of enzyme which consume 1 μmole of starch in 1 minute under room conditions was defined as the unite of enzyme activity (138 U/mL). But still, considering that the starch is a polymer, consisting of many glucose molecules, it should be mentioned that the activity of glucoamylase obtained in this study was notably high.

4.7 Plasmid Retention Time Assay

The retention time of plasmid within recombinant X33 cells was determined by using starch agar plates. The cells were sub-cultured until the 15^{th} passage without a selective antibiotic. It was observed that recombinant yeast cells were carrying the plasmid during the 15 sub-cultures (Figure 4.9). This situation is advantageous for this recombinant yeast cell line and would enable and promote its usefulness in industry.

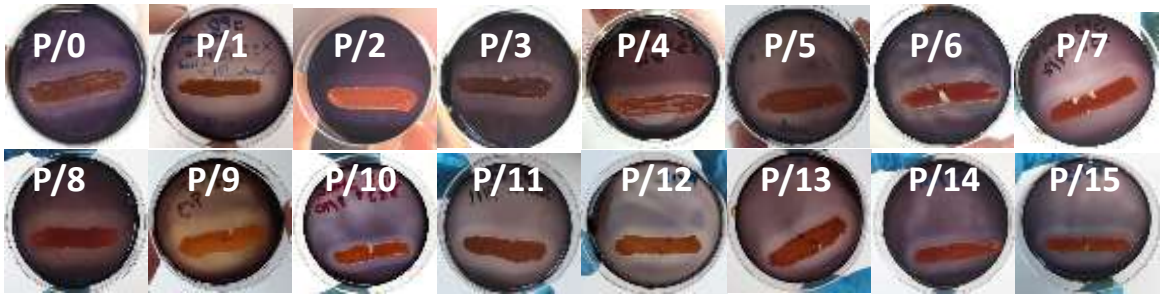


Figure 4.9. The investigation of plasmid loss of recombinant yeast cells during passages.

4.8 Statistical Analyses

One way analysis of variance (ANOVA) at p=0.05 and additionally, the 95% and 99% confidence intervals were calculated for all experiments (appendix 4). The p values for calibration curve plotted from standards, the relation of enzyme activity with temperature and time and the relation between enzyme activity with enzyme concentration were calculated. It was observed that the p values are under the 0,05. So it means the values obtained in this study are statistical significant.

5. CONCLUSION

In this study, the molecular cloning of glucoamylase gene region (glaA) of *Aspergillus niger* was successfully conducted and verified. Also, the recombinant plasmid was successfully transfected to P. Pastoris. It was observed that the glucoamylase secretion capacity of the new recombinant yeast cells (P. pastoris) is similar to the source organism (A. Niger). It was considered that the glucoamylase secreted from recombinant yeast cells is suitable for the industry due to its activity (138 U/ mL) and performance in different temperature conditions. Also, it was observed that the recombinant P. pastoris can secrete a large amount of industrially applicable glucoamylase during numerous passages. In conclusion, this study shows that glucoamylase which is an industrially important enzyme, can be produced more economically and with high yield, from an economic substrate, starch which can be found abundantly in our country by this recombinant glucoamylase expressing P. pastoris clone.

6. RECOMMENDATIONS

In this study, industrial glucoamylase has been produced by recombinant yeast cells which use starch as substrate. It is thought that this study could be a preliminary step for producing the glucoamylase enzyme, which is frequently used especially in the food industry but which is not produced in our country, on the commercial scale. The examination of the enzyme activity with different substrates at different pH and temperature conditions ought to be conducted for future studies.

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APPENDIX 1

THE NUCLEOTIDE SEQUENCE OF ASPERGILLUS NIGER glaA GENE REGION

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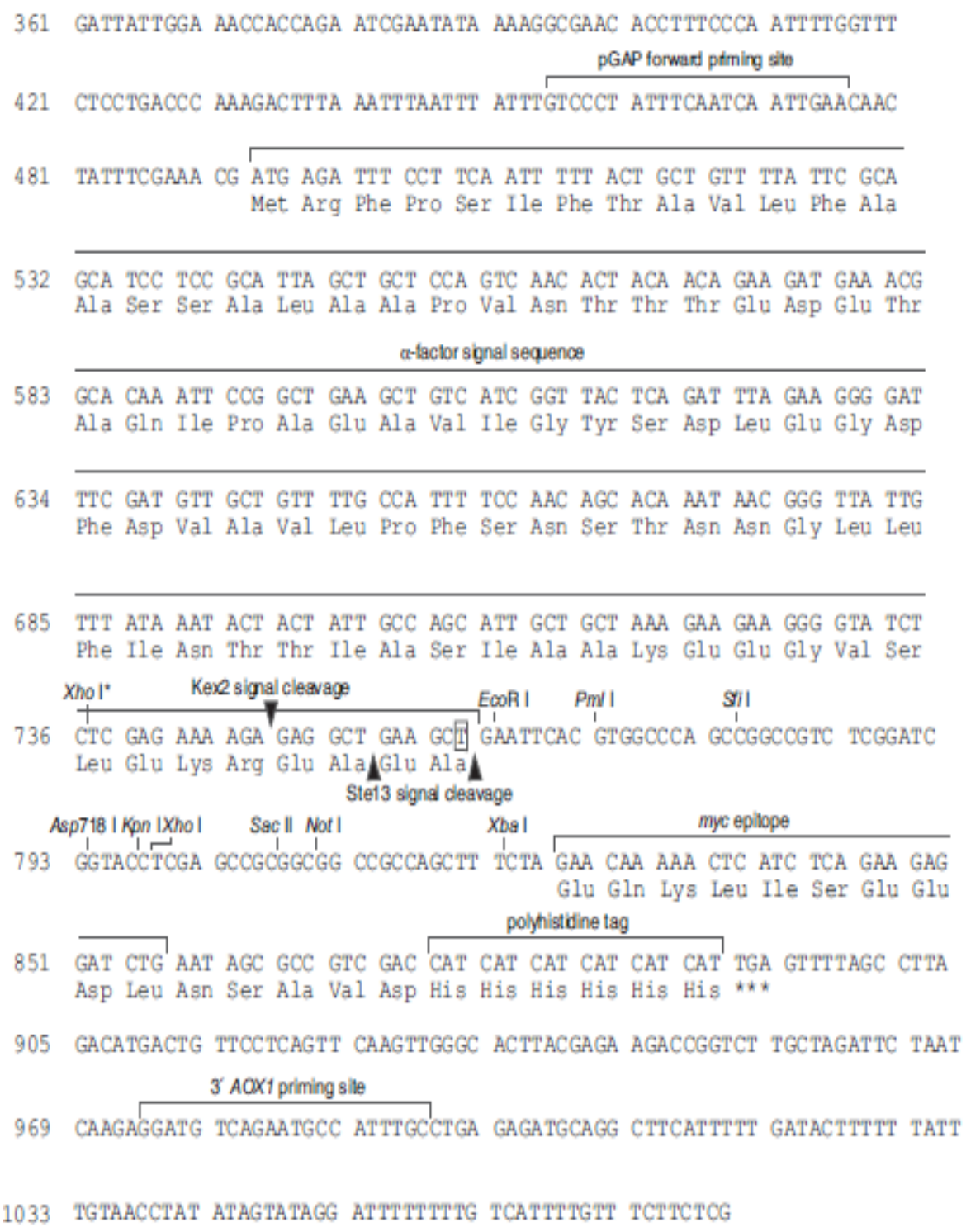
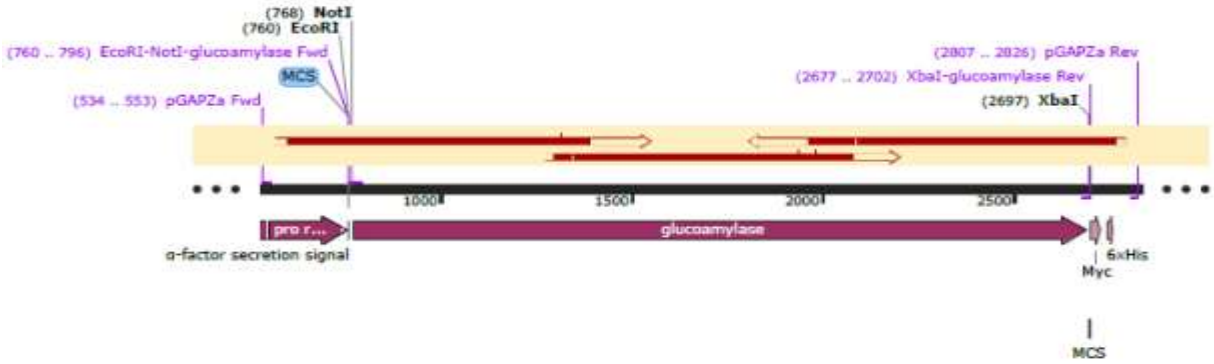
APPENDIX 2

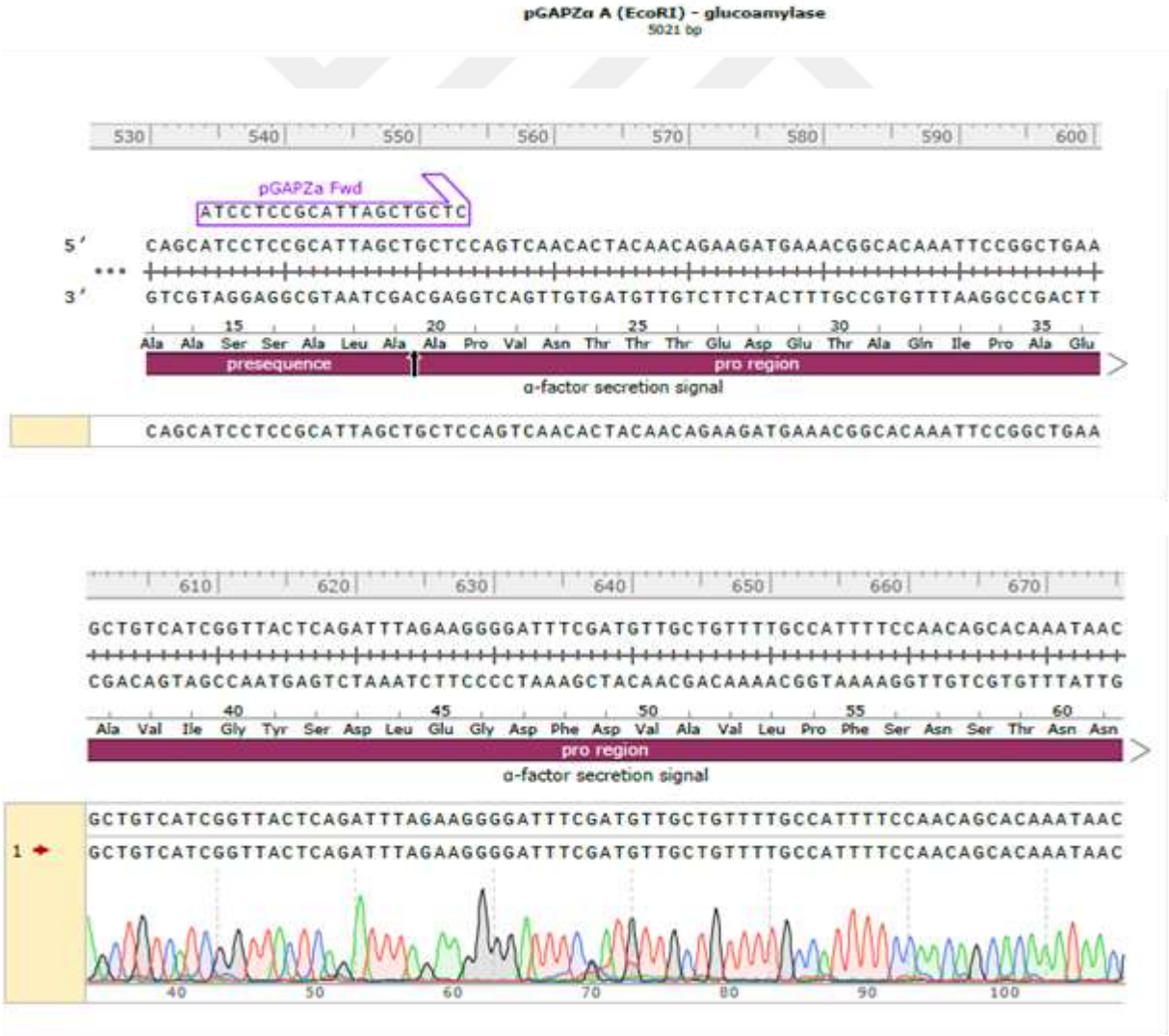
DNA SEQUENCE OF MULTIPLE CLONING SITES OF PLASMID pGAPZαA

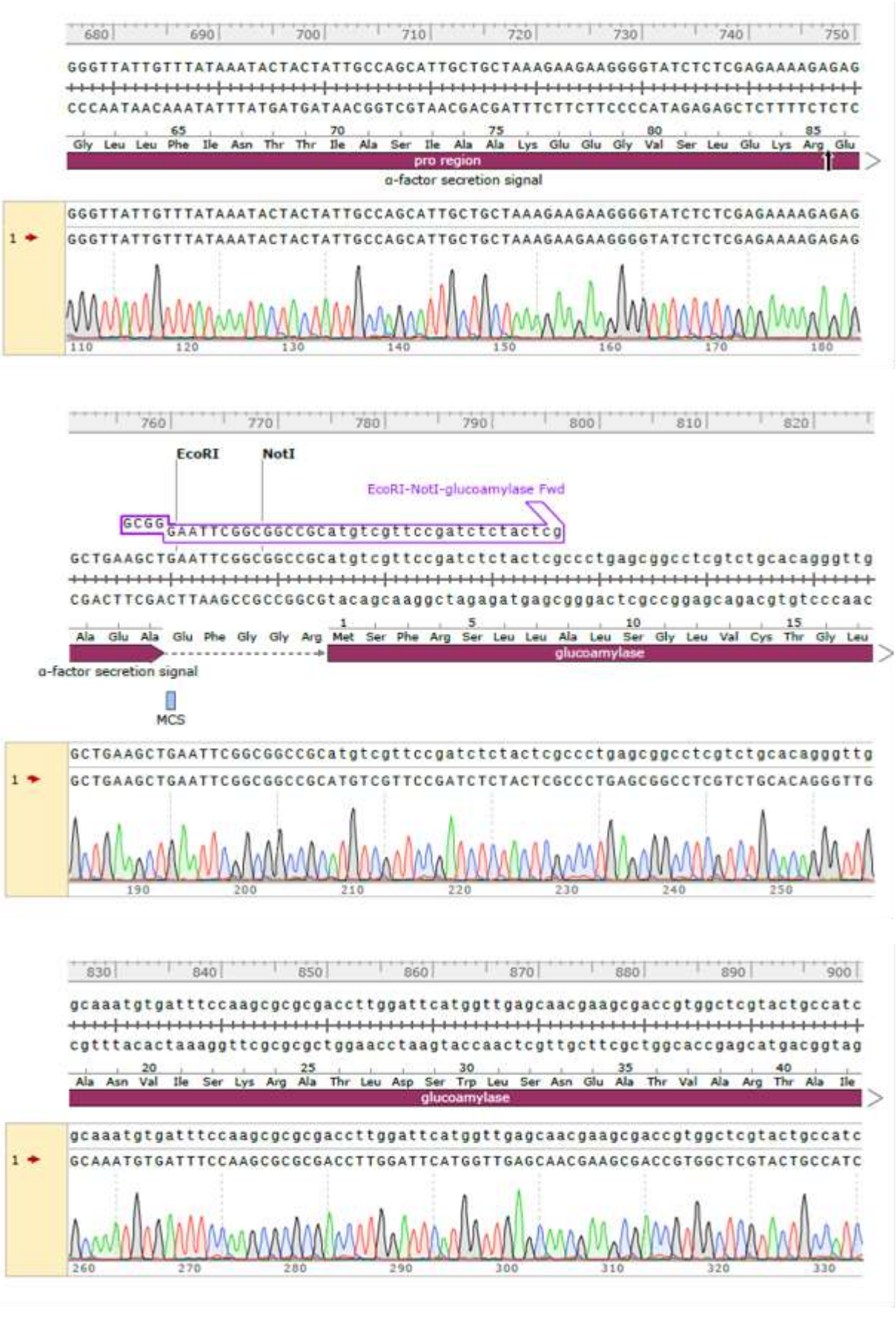


APPENDIX 3

SEQUENCE ANALYSES



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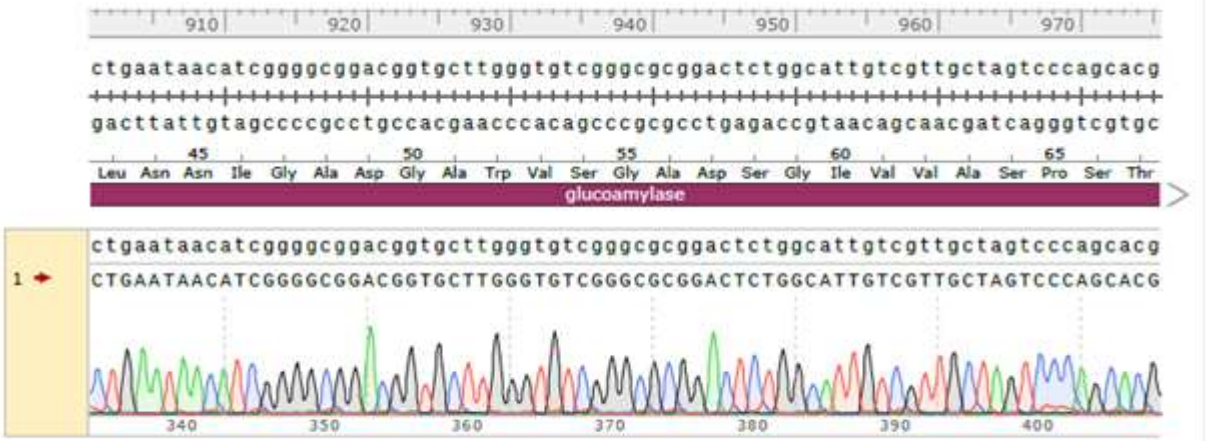
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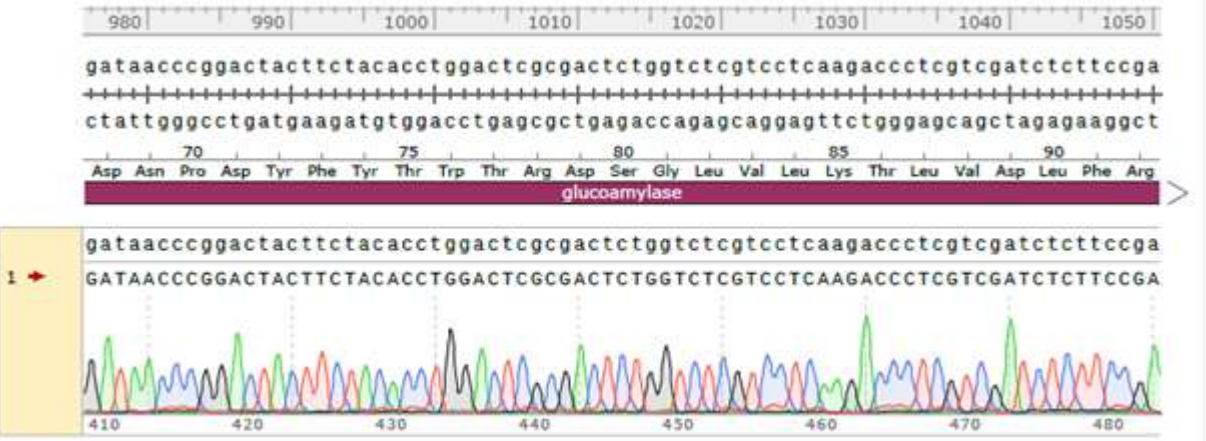
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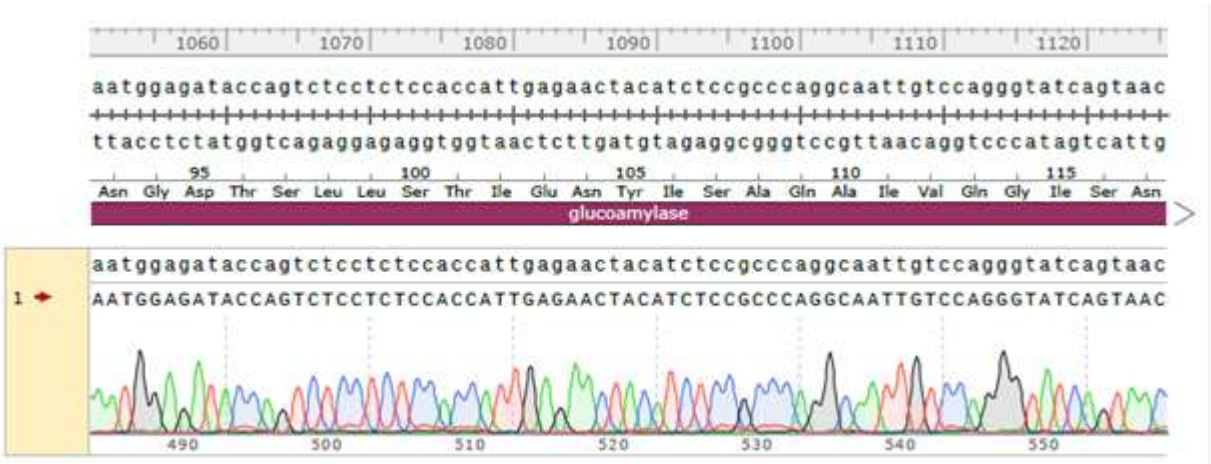
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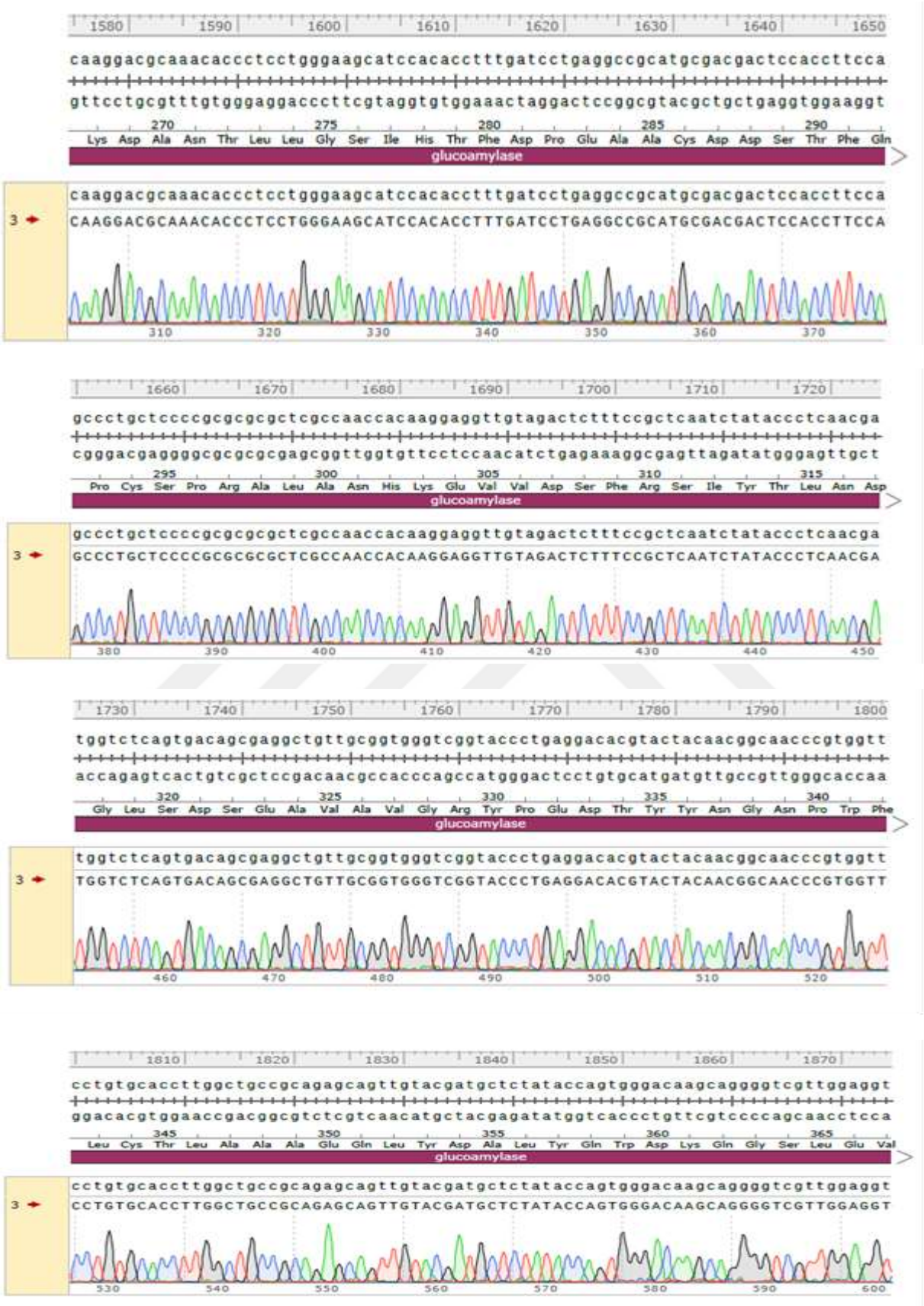
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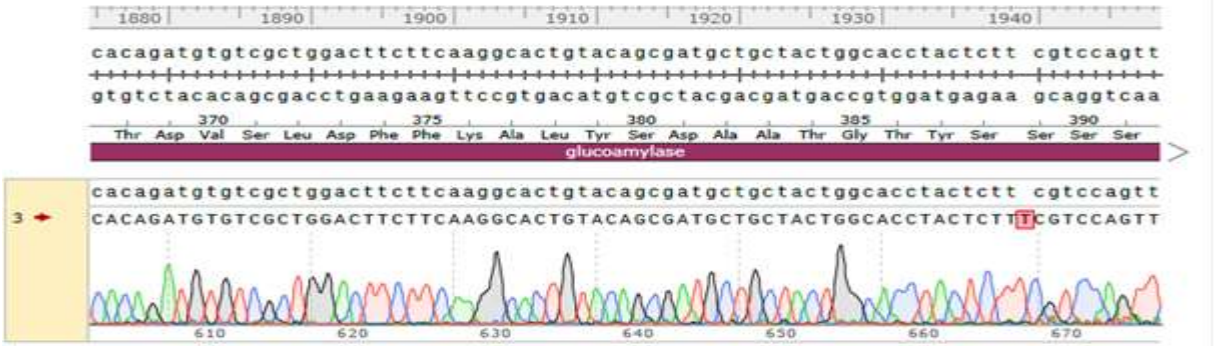
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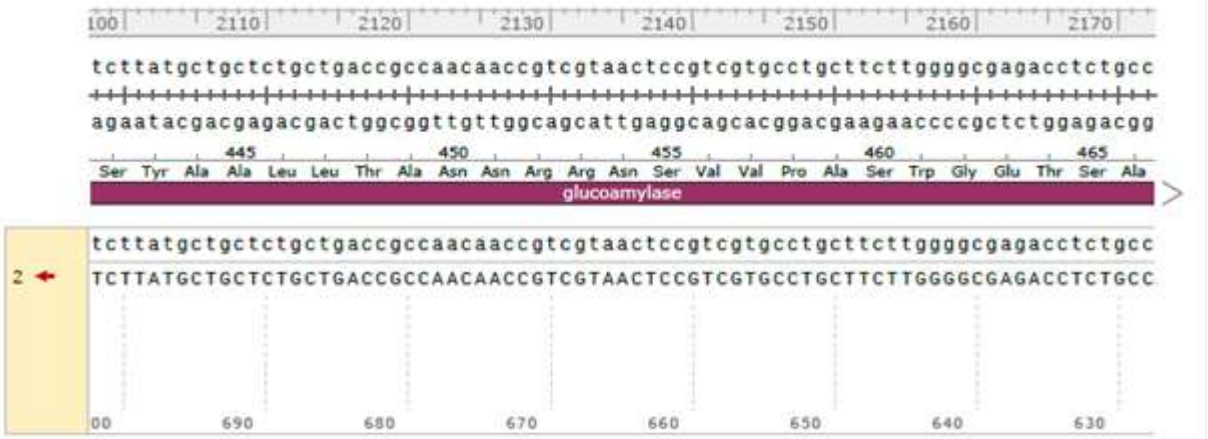
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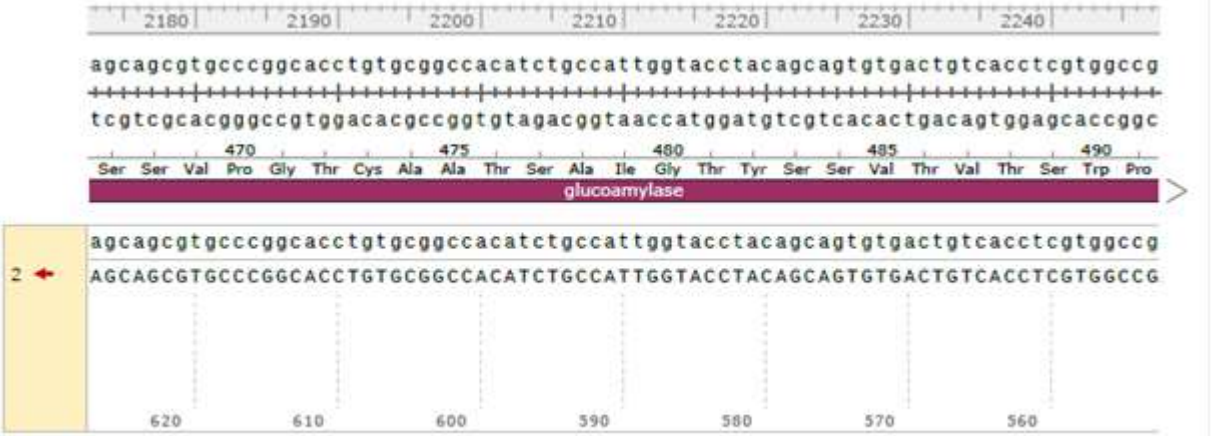
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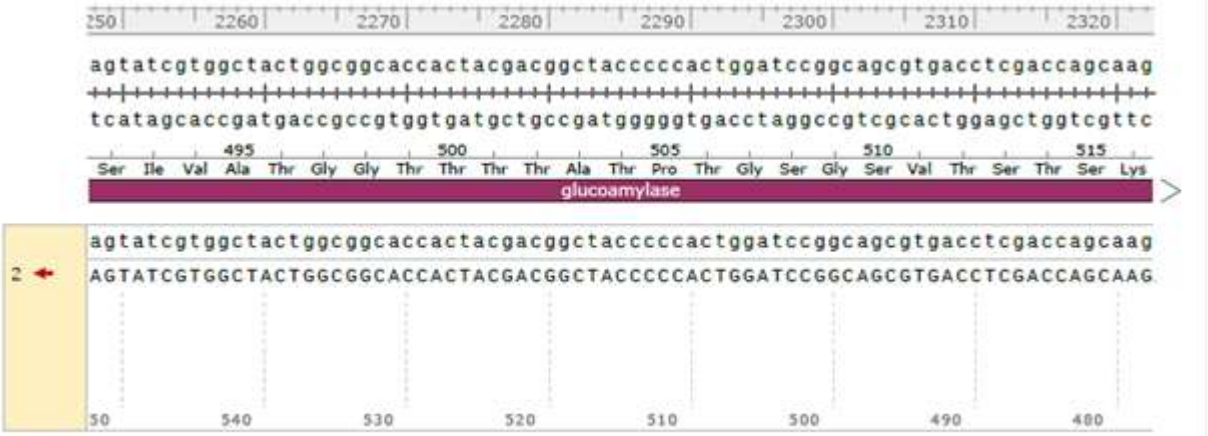
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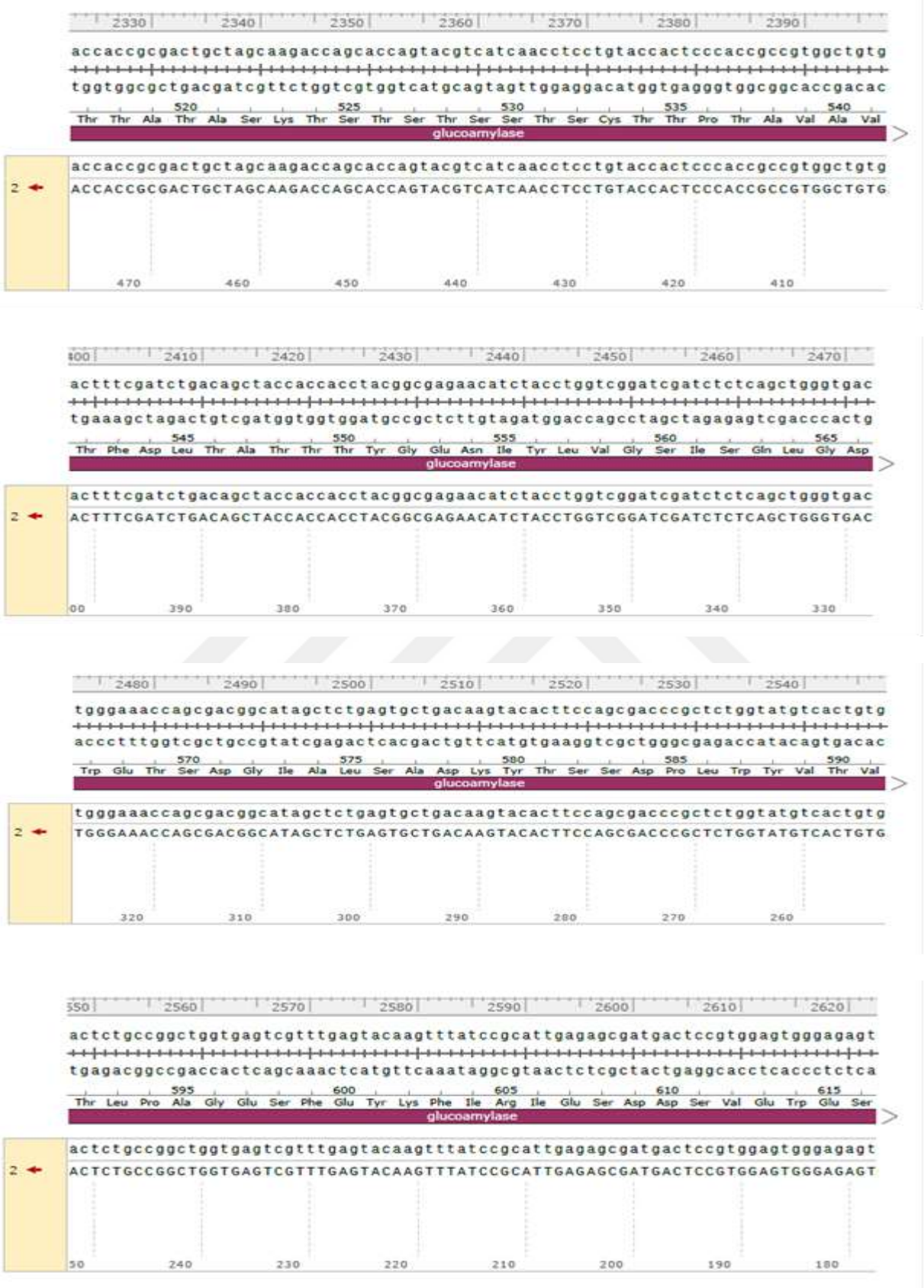
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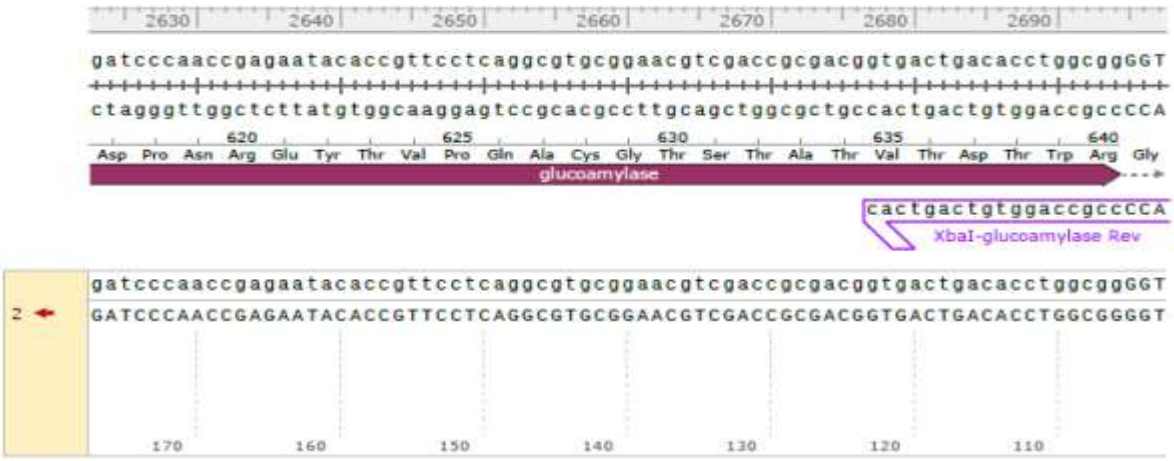
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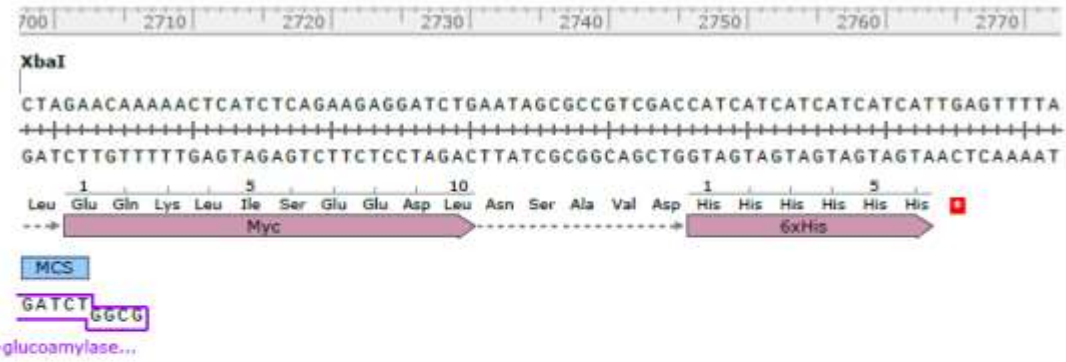
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APPENDIX 4

A. One way analysis of variance (ANOVA) at p=0.05 and 95% and 99% confidence intervals for the calibration curve plotted from standards

Groups	*Number*	*Total*	*Average*	*Variance*
1	3	0,3972	0,1324	7E-08
2	3	0,6589	0,219633	2,33E-08
3	3	1,0209	0,3403	7E-08
4	3	1,2098	0,403267	6,33E-08
5	3	1,5426	0,5142	3,81E-06
6	3	1,8229	0,607633	1,33E-08
7	3	2,1916	0,730533	1,43E-07
8	3	2,7142	0,904733	2,33E-08
9	3	2,9396	0,979867	5,43E-07
10	3	3,1138	1,037933	5,63E-07

Variance components	*SS*	*df*	*MS*	*F*	*P-value*	*F criterion*
Between the groups	2,763616	9	0,307068	576834,8	3,94E-52	2,392814
Inside the groups	1,06E-05	20	5,32E-07			
Total	2,763626	29				

	Confidence interval 95,0%	**Confidence interval 99,0%**
1	0,000657241	0,001516045
2	0,000379458	0,000875289
3	0,000657241	0,001516045
4	0,000625161	0,001442046
5	0,004848843	0,01118473
6	0,000286844	0,000661656
7	0,000940479	0,002169385
8	0,000379458	0,000875289
9	0,001831086	0,004223731
10	0,001864483	0,004300765

B. One way analysis of variance (ANOVA) at p=0.05 and 95% and 99% confidence intervals for the relation of enzyme activity with temperature and time

Summarize	0	15m	30m	60m	Total
RC					
Number	3	3	3	3	12
Total	2,5497	2,3273	1,6118	1,6027	8,0915
Average	0,8499	0,775767	0,537267	0,534233	0,674292
Variance	1,69E-06	3,33E-09	1,33E-08	3,33E-09	0,02169
34ºC					
Number	3	3	3	3	12
Total	2,6853	2,0381	1,6105	1,5875	7,9214
Average	0,8951	0,679367	0,536833	0,529167	0,660117
Variance	1,48E-06	1,8E-06	2,33E-08	2,25E-06	0,023983
55ºC					
Number	3	3	3	3	12
Total	2,7573	2,3348	1,6372	1,6355	8,3648
Average	0,9191	0,778267	0,545733	0,545167	0,697067
Variance	9,1E-07	2,89E-06	6,33E-08	2,33E-08	0,027783
Total					
Number	9	9	9	9	
Total	7,9923	6,7002	4,8595	4,8257	
Average	0,888033	0,744467	0,539944	0,536189	
Variance	0,000927	0,002386	1,89E-05	5,07E-05	

Variance components	*SS*	*df*	*MS*	*F*	*P-value*	*F criterion*
Sample	0,00834	2	0,00417	4483,728	1,31E-31	3,402826
Between the groups	0,789288	3	0,263096	282899,1	1,05E-54	3,008787
Interaction	0,018701	6	0,003117	3351,372	7,48E-34	2,508189
Inside the groups	2,23E-05	24	9,3E-07			
Total	0,816351	35				

	Confidence interval 95,0%			**Confidence interval 99,0%**		
	R.C.	34º C	55º C	R.C.	34º C	55º C
0	0,007449	0,006971	0,006971	0,003229	0,003229	0,003229
15	0,000331	0,007695	0,007695	0,000143	0,000143	0,000143
30	0,000662	0,000875	0,000875	0,000287	0,000287	0,000287
60	0,000331	0,008602	0,008602	0,000143	0,000143	0,000143

C. One way analysis of variance (ANOVA) at p=0.05 and 95% and 99% confidence intervals for the relation between enzyme activity with enzyme concentration

Groups	*Number*	*Total*	*Average*	*Variance*
1	3	0,1063	0,035433	3,33E-09
0,9	3	0,1159	0,038633	1,33E-08
0,8	3	0,1327	0,044233	4,33E-08
0,7	3	0,1517	0,050567	3,33E-09
0,6	3	0,1569	0,0523	2,1E-07
0,5	3	0,1801	0,060033	4,33E-08
0,4	3	0,203	0,067667	3,23E-07
0,3	3	0,2126	0,070867	6,33E-08
0,2	3	0,2286	0,0762	1,3E-07
0,1	3	0,2621	0,087367	4,33E-08

Variance components	*SS*	*df*	*MS*	*F*	*P-value*	*F criterion*
Between the groups	0,007852	9	0,000872	9951,597	1,68E-34	2,392814
Inside the groups	1,75E-06	20	8,77E-08			
Total	0,007854	29				

	Confidence interval 95,0%	**Confidence interval 99,0%**
1	0,000143422	0,000330828
0,9	0,000286844	0,000661656
0,8	0,000517115	0,001192818
0,7	0,000143422	0,000330828
0,6	0,001138375	0,002625867
0,5	0,000517115	0,001192818
0,4	0,00141254	0,003258279
0,3	0,000625161	0,001442046
0,2	0,000895669	0,002066021
0,1	0,000517115	0,001192818

APPENDIX 5

Grams of Ammonium Sulfate To Add To 1 Liter Of Solution At 0°C (Wingfield, 1998)

Percent saturation	Initial molarity	Final molarity																				
		0.00	0.20	0.40	0.60	0.80	1.00	1.20	1.40	1.60	1.80	2.00	2.20	2.40	2.60	2.80	3.00	3.20	3.40	3.60	3.80	3.90
0.0	0.00	0.00	26.7	54.0	81.9	111	140	170	202	234	267	302	338	375	413	453	495	539	585	632	682	707
5.1	0.20		0.00	27.0	54.7	83.0	112	142	173	205	238	272	308	344	383	422	464	507	552	599	649	673
10.3	0.40			0.00	27.4	55.4	84.2	114	144	176	209	243	278	314	352	391	432	475	519	566	615	639
15.4	0.60				0.00	27.7	56.2	85.5	116	147	179	213	247	283	321	359	400	442	486	533	581	605
20.5	0.80					0.00	28.1	57.1	87.0	118	150	183	217	252	289	328	368	409	453	499	546	570
25.7	1.00						0.00	28.6	58.1	88.5	120	153	186	221	258	296	335	376	420	465	512	535
30.8	1.20							0.00	29.1	59.1	90.2	122	156	190	226	264	303	343	386	430	477	499
35.9	1.40								0.00	29.6	60.2	91.9	125	159	194	231	270	310	351	395	441	464
41.1	1.60									0.00	30.2	61.4	93.7	127	162	199	236	276	317	360	405	428
46.2	1.80										0.00	30.7	62.6	95.7	130	166	203	242	282	325	369	391
51.3	2.00											0.00	31.3	63.9	97.7	133	170	208	248	290	333	355
56.5	2.20												0.00	32.0	65.2	99.8	136	174	213	254	297	318
61.6	2.40													0.00	32.7	66.7	102	139	178	218	260	281
66.8	2.60														0.00	33.4	68.2	104	142	182	224	244
71.9	2.80															0.00	34.2	69.8	107	146	187	207
77.0	3.00																0.00	35.0	71.5	110	150	169
82.2	3.20																	0.00	35.8	73.2	112	132
87.3	3.40																		0.00	36.7	75.0	94.0
92.4	3.60																			0.00	37.6	56.1
97.6	3.80																				0.00	18.1
100.0	3.90																					0.00

APPENDIX 6

THE SOLUTIONS USED IN THE STUDY

Luria-Bertani (LB) Medium: Tryptone (10g/l), yeast extract (5g/l), and sodium chloride (10g/l) were weighted and dissolved in ddH_2O. pH was adjusted to 7.0 with 5N NaOH. Finally the solution was autoclaved at 121 ^oC for 15 minutes.

Luria-Bertani (LB) Lennox Medium: Tryptone (10g/l), yeast extract (5g/l), and sodium chloride (5g/l) were weighted and dissolved in ddH_2O. The pH was adjusted to 7.5 with 5N NaOH. Finally, the solution was autoclaved at 121 ^oC for 15 minutes.

Luria-Bertani (LB) Agar: Tryptone (10g/l), yeast extract (5g/l), and sodium chloride (10g/l) were weighted and dissolved in ddH_2O. The pH was adjusted to 7.0 with 5N NaOH and then agar (20g/l) was added. Finally the solution was autoclaved at 121 ^oC for 15 minutes.

Luria-Bertani (LB) Lennox Agar: Tryptone (10g/l), yeast extract (5g/l), and sodium chloride (5g/l) were weighted and dissolved in ddH_2O. The pH was adjusted to 7.0 with 5N NaOH and then agar (20g/l) was added. Finally the solution was autoclaved at 121 ^oC for 15 minutes.

YPD Medium: YPD (Sigma, cat.no:Y1375) was weighted (50g/l) and dissolved in ddH_2O. The solution was autoclaved at 121 ^oC for 15 minutes.

YPD Agar: YPD (Sigma, cat.no:Y1375) was weighted (50g/l) and dissolved in ddH_2O. Agar (20 g/l) was added and the solution was autoclaved at 121 ^oC for 15 minutes.

M9 Minimal Medium: M9 salts (20% v/v), 20% glucose (2% v/v), 1M magnesium sulfate, 1M calcium chloride were dissolved in ddH_2O and the solution was autoclaved at 121 ^oC for 15 minutes.

M9 Salts Solution: Sodium phosphate dibasic (64g/l), potassium phosphate monobasic (15g/l), sodium chloride (2.5g/l) and ammonium chloride (5g/l) was weighted and dissolved in ddH_2O. The solution was autoclaved at 121 ^oC for 15 minutes.

M9 MM Agar: M9 salts (20% v/v), %20 glucose (2% v/v), 1M magnesium sulfate, 1M calcium chloride were dissolved in ddH_2O and agar (20g/l) was added. The solution was autoclaved at 121 ^oC for 15 minutes

TBE Buffer (10X): Tris (108g/l) and boric acid (55g/l) were weighted and dissolved in ddH_2O. Then 0,5M EDTA was added.

PBS Buffer: Sodium chloride (8g/l), potassium chloride (0.2g/l), sodium phosphate dibasic (1.44g/l) and potassium phosphate monobasic (0.24g/l) was dissolved in ddH_2O and autoclaved at 121 ^oC, for 15 minutes before use.

Lugol's Iodine: Potassium iodide (100 g/l) and iodine crystals (50 g/l) were weighted and dissolved in sterile ddH2O.

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Publications:

Articles (International / SCI,SCI Expanded)

- Özdemir Meryem Damla,Göktürk Dilek, 2017, The effect of rosmarinus officinalis and chemotherapeutic etoposide on glioblastoma (U87 MG) cell culture, Turkish Neurosurgery, 28, 853-857, 10.5137/1019-5149.JTN.20401-17.3
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- Özdemir Meryem Damla, Göktürk Dilek, 2018, Molecular Cloning of A.nıger Glucoamylase (glaA) Gene, Oral presentation, 3. International Mediterranean Science and Engineering Congress (IMSEC)
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- Özdemir Meryem Damla, Göktürk Dilek, 2016, The Effect of The Rosmarinus Officinalis on Temozolomide Resistant Glioblastoma (U87 Mg) Cells, Oral presentation, 6. Multidisciplinary Cancer Research Congress
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