T.C.
MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES IN
PURE AND APPLIED SCIENCES

CONSTRUCTION OF AMYLOLYTIC NUCLEAR
PETITE *SACCHAROMYCES CEREVISIAE* STRAINS
FOR BIOETHANOL PRODUCTION BY SSF

Doğ a ARSLAN

THESIS
FOR THE DEGREE OF MASTER OF SCIENCE
IN
BIOENGINEERING

SUPERVISOR
Assoc. Prof. Ebru Toksoy Öner

İSTANBUL 2008

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ACKNOWLEDGEMENT

The research presented in this thesis was conducted at the Bioengineering Department of the Faculty of Engineering at Marmara University and was financially supported by the Marmara University Scientific Research Fund through project FEN-BGS-290506-0128.

I would like to express my sincere gratitude and dedicate this thesis to my sincere advisor Assoc. Prof. Ebru TOKSOY ÖNER for her encouraging support, valuable advices and friendly guidance throughout the study to complete this thesis project.

I would like to thank Prof. Betül KIRDAR of the Chemical Engineering Department, Boğaziçi University and Prof. Stephen G. OLIVER of the Biochemistry Department, University of Cambridge for their kind advises and helpful criticism throughout this research.

Many thanks to the Dean of Engineering Faculty Prof. A. Alp SAYAR, the member of my thesis committee, for his support in this study and giving me opportunity of working in the Biotechnology Laboratories of Marmara University and for his time devoted to read and comment on my thesis.

Special thanks are due to Assist. Prof. Alper Tunga AKARSUBAŞI (Molecular Biology and Genetic Department, İTÜ), Assoc. Prof. Barış ÇALLI (Environmental Engineering Department, MU) and Assist. Prof. Kazım Yalçın ARGA (Bioengineering Department, MU) for the time they have devoted to reading and commenting on my thesis.

Sincerely thanks for my special friend Özlem ATEŞ for being more than a colleague to me.

I am also indebted to all my friends in Bioengineering Department for their endless help and nice friendship in laboratory.

I would also like to thank Meltem Kıyıcı ÇALLI for her everlasting encouragement which motivated me every time, especially at the most desperate moments.

I am especially grateful to my parents and my brother for their everlasting support, patience and also for creating me a perfect family.

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ÖZET

BİYOETANOL ÜRETİMİ İÇİN AMİLOLİTİK SOLUNUM EKSİKLİĞİ OLAN *SACCHAROMYCES CEREVISIAE* SUJLARININ GELİŞTİRİLMESİ

Etanol önemli bir enerji kaynağı olarak kabul edilir ve son birkaç yılda, dünyada ki artan enerji krizi ile beraber biyoetanol üretimi giderek daha da önem kazanmıştır. Biyoethanol üretiminde, yüksek enerji gerektiren pişirme prosesini takiben nişastanın fermente olabilen şekerlere hidrolizi, amilaz gibi amilolitik enzimler tarafından gerçekleştirilmektedir. Bu prosesin yüksek üretim maliyetine sahip olması, yeni ve yenilikçi teknolojilerin geliştirilmesini kaçınılmaz kılmıştır. Bu sebeple, düşük ekonomik fizibiliteye sahip geleneksel çok aşamalı proseslere alternatif olarak, nişastanın, amilolitik mayalar kullanarak eş zamanlı hidroliz ve direkt fermentasyonunu sağlayan proseslerin geliştirilmesi önem kazanmıştır.

Yüksek enerji gerektirmeyen, yeni fermentasyon sistemleri için, biyoetanol üretimini önemli ölçüde arttıracak, amilolitik solunum eksikliği olan *Saccharomyces cerevisiae* sujlari geliştirilmiştir. Ancak bu sujların endüstriyel kullanımı için, amilolitik enzimleri kodlayan genlerin kopya sayısının ve kararlılığının arttırılması gerekliliğı anlaşılmıştır. Bu sebeple, üç ana aşama içeren bir klonlama stratejisi geliştirilmiştir. Bu aşamalar, DNA kasedinin oluşturulması, bu kasedin *S. cerevisiae* genomic ribosomal DNA (rDNA) bölgesine entegre edilmesi, ve son olarak, farklı fermentasyon stratejileri kullanılarak etanol üretiminin artırılması olarak özetlenebilir. Bu çalışmanın amacı, bu geliştirilmiş stratejide ki, ilk ve en önemli basamak olan DNA kasedini rekombinant DNA teknolojisi kullanarak oluşturmaktır. Bu doğrultuda, yapılmış olan çalışmalar sonucunda, LEU2 seçici bölgesi ile amilolitik enzimleri içeren gen bölgesinin 25S rRNA genine klonlanması ile elde edilmiş olan rekombinant plazmidden, PCR amplifikasyonu ile DNA kasedi başarılı bir şekilde elde edilmiştir.

Temmuz, 2008

Doğa ARSLAN

ABSTRACT

CONSTRUCTION OF AMYLOLYTIC NUCLEAR PETITE *SACCHAROMYCES CEREVISIAE* STRAINS FOR BIOETHANOL PRODUCTION BY SSF

Ethanol has been regarded as an important energy source and production of bioethanol has become more essential in the world's energy crisis in last few decades. The energy intensive high temperature cooking process followed by hydrolysis of starch to fermentable sugars by amylolytic enzymes such as amylases in the current bioethanol production process warrants novel and innovative new technology for reducing the total manufacturing cost. The use of amylolytic yeasts for the direct fermentation of starch in a simultaneous saccharification and fermentation (SSF) process is an alternative to the conventional multistage process which offers poor economic feasibility.

For the development of a novel noncooking fermentation system, a respiratory-deficient nuclear petite amylolytic *Saccharomyces cerevisiae* strain was constructed that improved the bioethanol yields significantly. For the industrial use of this strain, high-copy and stable expression of amylolytic activity was required and hence a cloning strategy has been developed that involved three major steps, namely, construction of a DNA cassette, multiple integration of the cassette into the ribosomal DNA (rDNA) locus of *S. cerevisiae* genome and finally improving the ethanol productivity of the amylolytic integrands by various fermentation strategies. Aim of this thesis was to focus on the first and the most crucial step of the strategy that involved the construction of the DNA cassette using tools of Recombinant DNA Technology. By cloning the genes for the amylolytic enzymes together with the LEU2 selective marker region into the gene for 25S rRNA, the DNA cassette was successfully obtained by selective PCR amplification of the region of interest from the constructed recombinant plasmid.

July, 2008

Doğa ARSLAN

LIST OF SYMBOLS / ABBREVIATIONS

bp	:	base pair
kb	:	kilobase pair
ATP	:	Adenosine-triphosphate
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide triphosphate
EDTA	:	ethylenediaminetetraacetic acid
M	:	Molarity
NAD	:	Nicotinamide adenine dinucleotide,
PCR	:	polymerase chain reaction
RNA	:	Ribonucleic acid
rpm	:	rounds per minute

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CHAPTER I

INTRODUCTION AND AIM

With the increasing energy demand per person, fast growing population and environmental pollution; renewable energy sources, especially bioenergy, have become a necessity.

Unlike gasoline that is made from limited sources of natural fossil deposits (i.e., oil), bioethanol is a renewable fuel made from starch or sugar produced within the carbon cycle of certain plants. Such plants include crops like corn, wheat, rice, barley, sorghum, rye, tapioca, sugarcane, and sugar beet. The bioethanol production process represents a renewable carbon cycle, where carbon dioxide in the atmosphere is fixed as carbohydrate in the photosynthetic process by the plants. Some plants store carbohydrate as starch. At the end of the cycle, the starch-containing plant matter is harvested and used as feedstock for bioethanol production.

Bioconversion of starch into ethanol is a two-step process. The first step is saccharification, where starch is converted into sugar using an amylolytic microorganism or enzymes such as glucoamylase and α -amylase. The second step is fermentation, where sugar is converted into ethanol using an ethanol-fermenting microorganism such as yeast (Inlow et al., 1988; Nakamura et al., 1997). Saccharification step requires the anhydrous starch granules be extensively pretreated at high temperature (105-150°C) to hydrate the starch. This is an energy intensive process requiring the addition of heat energy to starch granule slurries until the gelatinization temperature (temperature at which starch molecules swell and begin to solubilize) of the starch is exceeded. Therefore, special equipment such as heat exchangers, steam jet cookers and holding tanks are used in the process. Moreover, the technical requirements and limitations of this required equipment and process, especially limitations on solids loading due to the high viscosity of starch, limit the amount of ethanol that can be produced at a plant (Genencor, 2005).

Current bioethanol production is an energy intensive high temperature cooking process followed by enzymatic hydrolysis of starch to fermentable sugars. Therefore, the common bioethanol process warrants the novel and innovative new technology for reducing the total manufacturing cost. The use of amylolytic yeasts for the direct fermentation of starch in a simultaneous saccharification and fermentation (SSF) process is an alternative to the conventional multistage process which offers poor economic feasibility. Although, in industry there are more than 150 amylolytic yeast species, their industrial use is narrow because of their low ethanol tolerance. Of all the yeast species, *S. cerevisiae* is being preferred in bioethanol processes not only because of its well-defined genetics, physiology and accepted GRAS status (generally regarded as safe) but also due to its high fermentation capacity, high ethanol yield and relatively high ethanol tolerance (Ma et al., 2000). Therefore, it is very important to develop genetically modified amylolytic yeast strains of *S. cerevisiae*. Specially, respiratory-deficient strains of *S. cerevisiae*, which were shown to reach 40-50% higher ethanol productivities when compared with standard *S. cerevisiae* strains and hence hold a great potential for the commercial production of bioethanol (Hutter and Oliver, 1998; Panoutsopoulou et al., 2001).

With the ultimate goal of developing a novel noncooking fermentation system, a 100% respiratory-deficient nuclear petite amylolytic *S. cerevisiae* was constructed that was able to excrete a bifunctional fusion protein that contains both the *Bacillus subtilis* alpha-amylase and the *Aspergillus awamori* glucoamylase activities (Toksoy Öner et al., 2005). Optimization of fermentation conditions in order to prevent the observed decrease in the time dependent amylolytic activity resulted in significant improvements in ethanol yield from soluble starch (Toksoy Öner, 2006). The fermentation performance of this strain pointed out the fact that in order to use these strains on industrial scale, both the copy number and stability of the genes encoding the amylolytic enzymes had to be improved (Toksoy Öner, 2006). In the light of this, a cloning strategy has been developed and used in order to integrate multiple copies of the fusion protein gene into the ribosomal DNA (rDNA) locus of *S. cerevisiae* that consists of 100-200 tandem copies of a 9.1 kb repeat on the right arm of chromosome XII. The strategy involves three major steps. The first step involves the construction of the DNA cassette containing the enzyme genes, a yeast selection marker and

homologous regions for its directed integration into the genome. In the latter step, the DNA cassette is transformed into yeast cells and among the integrands, the one with highest amylolytic activity is selected. The final step involves the determination of the fermentation performances of the integrands, comparison of their ethanol productivities with earlier results and development of fermentation strategies to improve the ethanol yields.

The aim of this study is focused on the construction of the DNA cassette using Recombinant DNA Technology. For this, 2.7 kb long fragment containing the gene for 25S rRNA has been amplified by PCR and cloned into pJET1 cloning vector to construct pJRD. In order to construct the pJBG plasmid, the DNA fragment containing the genes encoding for the amylolytic enzymes together with the LEU2 selective marker region was amplified by PCR and cloned into the unique *HpaI* site within the rDNA region of the pJRD. In order to avoid integrating unnecessary antibiotic marker or vector sequences, the fusion gene and marker region flanked by the rDNA sequences has been amplified by PCR from the pJBG plasmid. The PCR product which is the DNA cassette was obtained and hence with this study, the first and the most crucial step of the developed strategy for improved amylolytic activity is completed successfully.

CHAPTER II

GENERAL BACKGROUND

II.1. ETHANOL

Ethanol (ethyl alcohol), C_2H_5OH , is a product of the fermentation carbohydrates e.g. sugar and starch or highly carbohydrate containing compounds. In the absence of oxygen, some microorganisms like yeast can convert carbohydrate into a mixture of ethanol and CO_2 , as shown in Equation II.1 (Brown et al., 2000).

Ethanol has been known for a long time, being perhaps the oldest product obtained through traditional biotechnology. Ethanol from biomass feedstock, or bioethanol, is an attractive sustainable alternative energy source for fossil fuels because it is renewable clean energy with low CO_2 emission and unburned hydrocarbons, it can be produced by bioconversion process from wide range of feedstock at relatively low cost. As transport energy bioethanol is superior to gasoline because it has a higher octane number, broader flammability limits, higher flame speeds and higher heats of vaporization (Kroumov et al., 2006; Balat et al., 2008; Zaldivar et al., 2001).

Moreover, ethanol is low in toxicity, water soluble and easily biodegradable, hence, it is harmless to the environment. Ethanol can also be used with other energy sources to improve them. For example, when low amount of ethanol is blended with gasoline, ethanol increases the octane and fuel oxygen content which helps the overall fuel combustion (Çakır et al., 2004; McMillan, 1997).

II.1.1. Bioethanol Production as a Natural Energy Source

Demand for energy and its resources, is increasing continuously due to the rapid outgrowth of population and urbanization. Present sources of energy are not sufficient to overcome the increasing demands of the world. Since 1973 the energy sources have been doubled in developed countries but the demand is still higher. According to the estimations the global energy demand increases approximately 50 % above 2002 level and the oil sources might be depleted till 2050. Conventional energy resources like coal, petroleum and natural gas satisfy the major energy demand of the world. However, these sources are in the verge of getting extinct and more than that by emitting greenhouse gases they bring about atmospheric pollution, resulting in problems like global warming, acid rain, etc (Goyal et al., 2008).

Accordingly, the increase in the energy demands and considering the pollution at the global scale, other alternative energy sources are definitely needed and a shift over to non-conventional sources like wind, sunlight, water, biomass, etc., is inevitable (Goyal et al., 2008).

Biomass is being used from the ancient times as a combustion fuel for cooking, keeping warmth in houses, etc. Biomass is cheap and abundant and can be converted to energy rich products using suitable processes (Goyal et al., 2008).

As shown in Figure II.1, 14 % of the energy is provided by natural resources and biomass source is the second biggest portion (27 %) of the total resources after nuclear power (Khan et al., 2008; Erdogdu, 2008).

Figure II.1 Percentage consumption of fuels to date (Khan et al., 2008)

Biomass is used to meet a variety of energy needs, including generating electricity, heating homes, fueling vehicles and providing process heat for industrial facilities. The limitations of solar and wind power are site-specific, intermittent and not reliable for instantaneous supply. Therefore, in recent years a new enthusiasm in bioethanol production from biomass has developed rapidly (Ni et al., 2006).

Biomass is the oldest known source of energy. The energy of biomass is generated from the burning organic matter. Biomass resources can be divided into two broad categories, i.e., natural and derived materials. Natural biomass sources can mainly be classified: (i) wastes, (ii) forest product, (iii) energy crops, (iv) aquatic plants. Derived materials can be categorized as wood and wood wastes, agricultural crops and their waste by-products, municipal solid waste, animal wastes and, waste from food processing (Demirbaş, 2001; Goyal et al., 2008).

Biofuels originate from the processing of the biomass sources but mostly from energy crops. Energy crops are mostly used in bioethanol production and can be conveniently classified into five types: (i) woody crops, (ii) grasses, (iii) starchy crops like corn, wheat and barley, (iv) forage crops like cane and beet, (v) oilseed crops like soybean, sunflower and safflower. Currently, the global ethanol supply is produced mainly from sugar and starchy feedstocks. Different feedstocks that can be utilized for bioethanol production and their comparative production potential are given in Table II.1 (Balat et al., 2008; Demirbaş, 2001; Bai et al., 2008).

Table II.1 Different feedstocks and their comparative ethanol production rates.
(Balat et al., 2008)

	Bioethanol production potential (l/ton)
Sugar cane	70
Sugar beet	110
Sweet potato	125
Potato	110
Cassava	180
Maize	360
Rice	430
Barley	250
Wheat	340
Sweet sorghum	60
Bagasse and other cellulose biomass	280

As a natural energy source ethanol has a higher ability to resist compression rating than gasoline. Although it is known that ethanol has lower energy content due to its oxygen content, it has higher net performance than gasoline, because the vapor pressure of alcohol is greater and the heat of vaporization is higher than that of gasoline, which is primarily responsible for the increased power outputs using alcohol and increased combustion efficiency of ethanol (In Table II.2). By using biomass-derived ethanol, a net reduction in the levels of CO₂ (the main greenhouse gas) could range from 60–90% relative to gasoline-consuming vehicles. Thus, by utilizing fuel ethanol, the CO₂ released by combustion of the fuel would be recycled through the photosynthetic process, without net increase in CO₂ levels (Ni et al., 2006).

Table II.2 Energy contents of different fuels (Ni et al., 2006)

Fuel	Energy content (MJ/kg)
Hydrogen	120
Liquefied natural gas	54.4
Propane	49.6
Aviation gasoline	46.8
Automotive gasoline	46.4
Automotive diesel	45.6
Ethanol	29.6
Methanol	19.7
Coke	27
Wood (dry)	16.2
Baggase	9.6

II.1.2. World Bioethanol Production

Permanent crises in the Middle East and the speculation in the stock exchange, among other factors, have caused the oil price to reach such high prices per barrel. In addition, the intensive utilization of fossil fuels has led to the increase in the generation of polluting gases released into the atmosphere, which have caused changes in the global warming (Sanchez and Cardona, 2008).

Fuel ethanol production has increased remarkably because many countries look for reducing oil imports, boosting rural economies and improving air quality. The world ethanol production has reached about 51,000 mill liters (Renewable Fuels Association, 2007).

The USA and Brazil are the first producers (Table II.3). In average, 73% of produced ethanol worldwide corresponds to fuel ethanol, 17% to beverage ethanol and 10% to industrial ethanol (Sanchez and Cardona, 2008).

Table II.3 Top ten ethanol producers (billion gallons) (Balat et al., 2008)

Country	2004	2005	2006
USA	3.54	4.26	4.85
Brazil	3.99	4.23	4.49
China	0.96	1.00	1.02
India	0.46	0.45	0.50
France	0.22	0.24	0.25
Germany	0.07	0.11	0.20
Russia	0.20	0.20	0.17
Canada	0.06	0.06	0.15
South Africa	0.11	0.10	0.10
Thailand	0.07	0.08	0.09

Concerning the EU, a new directive was accepted in November 2001 that forces the member states to establish legislation about utilization of fuels from renewable resources. In 2005, this utilization should cover 2% of the total fuel consumption. This quota expected to increase to 5.75% in 2010 and furthermore. Some member states like Finland, Sweden or Austria have already fulfilled this quota (Mojovic et al., 2006; Kondili et al., 2007).

II.1. 3. Bioethanol Production Processes

Raw materials for ethanol production include cane and beet sugar (sucrose), molasses, whey, starches, and cereal grains like corn, wheat, barley (glucose), corn stover, rice straw, newsprint and wood residues (cellulose) can be used. Cellulosic components like lignin in their structure need to be pretreated which in turn increases the cost of the operation (Blanch and Clark, 1997).

Figure II.2 Schematic flowchart for the bioconversion of biomass to bioethanol (Balat et al., 2008)

The conversion of biomass to bioethanol is simply shown in Figure II.2. In conventional method, if the source doesn't need any pre-treatment process, the raw material follows the (1) hydrolysis, (2) fermentation and (3) distillation steps.

The hydrolysis also called saccharification step may be considered as a key step in processing for bioethanol production. The main role of this step is to effectively break down of long chains in major polymer components to simple sugars such as glucose. In the fermentation, the products of hydrolysis step are subsequently converted to ethanol by yeasts or bacteria. However, polymer components are difficult to hydrolyze because of that hydrolysis step is considered as the rate limiting step of whole ethanol production system. Therefore, hydrolysis part may need some improvements for higher yields. The hydrolysis step is at relatively high temperatures is a process known industrially as liquefaction. Depending on the raw material and the organism which is inoculated for fermentation and hydrolysis, factors affecting hydrolysis include substrates, enzymes and enzyme activity, and reaction conditions (temperature, pH, as well as other parameters). Other than controlling these factors, to increase the rate of hydrolysis and fermentation steps together and increase the ethanol yield, there are some useful options which can be followed (Mojovic et al., 2006; Gray et al., 2006; Goyal et al., 2008).

The options for conducting hydrolysis and fermentation steps can be listed as: (a) separate hydrolysis and fermentation (SHF), (b) simultaneous saccharification and fermentation (SSF), which uses enzymes from external sources, or (c) simultaneous saccharification and fermentation, in which the microorganism also produces enzymes, in a process often referred to as direct microbial conversion (DMC). These options present advantages and disadvantages. In the first alternative, SHF, each operation can be conducted at optimal conditions of pH and temperature, but the accumulation of the end product of hydrolysis, glucose inhibits the activity of the enzymes. In the second alternative, SSF, glucose, released by enzymes, is promptly transformed by the microorganism into ethanol. This process has an enhanced rate of hydrolysis, needs lower enzyme loading, results in higher ethanol yields and reduces the risk of contamination. On the other hand, when simultaneous saccharification and fermentation, are used, the overall time is considerably shorter. The disadvantages are, however, due to the by by-product formation (acetate, lactate) a decrease in the ethanol production, low tolerance of the microorganism to ethanol (3.5% w/v), and limited growth in hydrolysate syrups. The SFF process shows more attractive indexes than SHF as higher ethanol yields and less energetic consumption (Zaldivar et al., 2001; Sanchez and Cardona, 2008).

In distillation process, ethanol is purified from the other by-products which occur during the process. Distillation, may consume up to 50% of the energy in the ethanol production process. Industrial ethanol for use as a solvent is produced 95 % by volume. Distillation is composed of two steps: stripping of ethanol from the dilute fermentation broth and rectification of the stripped broth. Other recovery processes that have been considered include vapor – phase water adsorption onto solids, molecular sieves, and membrane based separations (Blanch and Clark, 1997).

The ethanol yield in the industry that is calculated based on the total sugar feeding into the fermentation system without deduction of the residual sugar can be as high as 90– 93% of its theoretical value (Bai et al., 2008).

II.1.4. Bioethanol Production by Yeast

Among many microorganisms that have been exploited for ethanol production, *S. cerevisiae* still remains as the prime species. The main metabolic pathway involved in the ethanol fermentation is glycolysis (Embden–Meyerhof–Parnas or

EMP pathway), through which one molecule of glucose is metabolized, and two molecules of pyruvate are produced. Under anaerobic conditions, the pyruvate is further reduced to ethanol with the release of CO₂. Theoretically, the yield is 0.511 for ethanol and 0.489 for CO₂ on a mass basis of glucose metabolized. Two ATPs produced in the glycolysis are used to drive the biosynthesis of yeast cells which involves a variety of energy-requiring bioreactions. Therefore, ethanol production is tightly coupled with yeast cell growth, which means yeast must be produced as a co-product (Bai et al., 2008).

One of the main problems in ethanol fermentation process is that *S. cerevisiae* can ferment only certain mono- and disaccharides like glucose, fructose, maltose and sucrose namely, it cannot utilize starchy materials. One solution to solve this problem is that the starchy materials need to be cooked at a high temperature (140°C –180°C) to obtain a high bioethanol yield. The other is that adding large amounts of amylolytic enzymes, namely, glucoamylase and α -amylase which are commercially available. However these two solutions increase the operational cost of the process. Recently, tremendous improvements have been made in enzyme efficiency, reducing process costs and time, and increasing bioethanol yields. Genetic engineering provides *S. cerevisiae* convert directly starch to ethanol (Kroumov et al., 2006; Goyal et al., 2008).

There are vast amount of resources other than starchy material that can be used in ethanol production. However, like starchy materials *S. cerevisiae* can hardly degrade complex compounds like cellulose and hemicellulose directly. In general, cellulosic compounds consists of a main chain xylan backbone (β -1,4 linkages) with various branches of mannose, arabinose, galactose, glucuronic acid, etc. Furthermore, lignin can be covalently linked to cellulose via ferulic acid ester linkages which is quite difficult to degrade by *S. cerevisiae*. In addition to that, during the hydrolysis of cellulose containing materials some by-products like mainly xylose and pentose can be obtained that cannot be assimilated by this yeast. A way to overcome this obstacle is again through recombinant DNA technology. Other approach to this problem is the use of some fermenting microorganisms for degradation of these by-products. In this case, configurations involving the separate fermentation of pentoses and hexoses have been proposed. However, all these

approaches need to be optimized considering the yields of the whole process (Sanchez and Cardona, 2008; Bai et al., 2008).

II.2. RECOMBINANT DNA TECHNOLOGY IN YEAST

Since the 1970s there have been considerable developments in the method and techniques used to study biological processes at the molecular level. These have led to many new and powerful ways for the isolation, analysis and manipulation of nucleic acids, which, in turn, has provided insights and exciting advances in new and expanding areas of bioscience and biotechnology (Walker and Rapley, 2000).

Recombinant DNA technology has revolutionized the biotechnological processes by providing powerful means of analyzing and altering genes and proteins. The technology depends on having enzymes that can cut, join and replicate DNA polymerases and reverse transcriptase. On the other hand, in recombinant DNA technology, base pairing is used to construct new combinations of DNA as well as to detect particular sequences (Madigan and Martinko, 2006).

Through recombinant DNA technology, the knowledge of DNA function, gene organization, gene expression regulation and protein primary structure are promising major breakthroughs in biotechnology. The opportunity to introduce totally new DNA into cells has also created unique industrial microorganisms able to synthesize valuable homogenous and heterologous proteins (Bailey and Ollis, 1977).

II.2.1. Cloning

The discovery of restriction endonucleases in the early 1970s was a key development which not only led to the possibility of analyzing DNA more effectively, but also provided the capability to cut different DNA molecules so that they could later be joined together to create new recombinant DNA fragments, a process now termed gene cloning. Gene cloning has enabled numerous discoveries to be made and provided valuable insights into gene structure, function and regulation (Walker and Rapley, 2000).

Gene cloning can be divided into several steps (Madigan and Martinko, 2006):

1. *Isolation and fragmentation of the source DNA*: Source DNA can be the total genomic DNA from organism of interest, DNA synthesized from an RNA template by reverse transcriptase, a gene or genes amplified by the

polymerase chain reaction or even totally synthetic DNA made *in-vitro*. If genomic DNA is the source, it is cut with restriction enzymes first to give a mixture of manageable-sized fragments.

2. *Joining the DNA fragments to a cloning vector with DNA ligase:* Cloning vectors are small, independently replicating genetic elements used to replicate genes, and most are derived from plasmids or viruses. Cloning vectors are typically designed to allow *in-vitro* insertion of foreign DNA at a restriction site that cuts the vector in a way that does not affect its replication. If the source DNA and the vector are cut with the same restriction enzyme, joining can be mediated by annealing of the single-stranded regions called “*sticky ends*”. “*Blunt ends*” generated by some restriction enzymes can also be joined using synthetic DNA linkers and adapters. DNA ligase is required to seal the final phosphodiester bond.
3. *Introduction and maintenance of the cloned DNA in a host organism:* The recombinant DNA molecule made in a test tube is introduced into a host organism where it can be replicate. Transfer of the DNA into the host usually yields a mixture of clones. Some cells contain the desired cloned gene, whereas other cells contain other clones generated by joining the source DNA to the vector. Such a mixture is known as a DNA library or gene library because many different clones can be purified from the mixture, each containing different cloned DNA segments from the source organism.

II.2.2. Polymerase Chain Reaction

There have been a number of key developments in molecular biology techniques, but one that has had the most impact has been the polymerase chain reaction, or PCR. One of the reasons for the adoption of the PCR is the elegant simplicity of the reaction and relative ease of the practical steps. It is frequently one of the first techniques used when analyzing DNA and it has opened up the analysis of cellular and molecular processes to those outside the field of molecular biology (Walker and Rapley, 2000).

PCR is used to amplify a precise fragment of DNA from a complex mixture of starting material, usually termed the template DNA, and in many cases requires little DNA purification. The polymerase chain reaction can multiply DNA molecules by

up to a billionfold in the test tube, yielding large amounts of specific genes for a host applications in molecular biology. PCR does require knowledge of the DNA sequence information which flanks the fragment of DNA to be amplified (target DNA). From this information two oligonucleotide primers can be chemically synthesized, each complementary to a stretch of DNA to the 3' side of the target DNA, one oligonucleotide for each of the two DNA strands (Walker and Rapley, 2000; Madigan and Martinko, 2006).

The steps in PCR amplification of DNA can be stated as follows (Madigan and Martinko, 2006):

1. *Denaturation*: two oligonucleotide primers flanking and target DNA are made on an oligonucleotide synthesizer and added in great excess to heat-denatured target DNA.
2. *Annealing*: as the mixture cools, the excess of primers relative to the target DNA ensures that most target strands anneal to a primer and not to each other.
3. *Extension*: DNA polymerase then extends the primers using the target strands as templates. After an appropriate extension period, the mixture is heated again to separate the strands. The mixture is then cooled to allow the primers to hybridize with complementary regions of newly synthesized DNA and the whole process is repeated
4. *Final Elongation*: after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended. This step is occasionally performed at a temperature of 70-74°C for 5-15 minutes.

The secret to PCR is that the products of one primer extension serve as the template in the next cycle. Indeed, the beauty of the PCR technique is that each cycle literally doubles the content of the original target DNA (Madigan and Martinko, 2006).

II.2.3. Enzymes for Manipulating DNA

One key to recent breakthroughs in genetic engineering is the discovery and the subsequent commercial supply of several enzymes which can be used to cut, alter and join the DNA molecules (Bailey and Ollis, 1977).

II.2.3.1.DNA Polymerase Enzyme

The enzymes that catalyze addition of nucleotides are called *DNA polymerases*. DNA polymerase uses a template strand of DNA to direct the synthesis of a complementary strand in the 5'→ 3' direction (Bailey and Ollis, 1977).

One problem with the early PCR reactions was that the temperature needed to denature the DNA also denatured the DNA polymerase. However the availability of a thermostable DNA polymerase enzyme isolated from the thermophilic bacterium *Thermus aquaticus* found in hot springs provided the means to automate the reaction. *Taq* DNA polymerase has a temperature optimum of 72°C and survives prolonged exposure to temperatures as high as 96°C and so is still active after each of the denaturation steps (Walker and Rapley, 2000).

A number of thermostable DNA polymerases other than *Thermus aquaticus* have been discovered and marketed commercially (Walker and Rapley, 2000).

II.2.3.2. DNA Ligase

DNA ligase is an essential component involved in various DNA transactions, including replication, repair and recombination. DNA ligases can be classified into two families based on adenylation cofactor dependence; ATP-dependent and NAD⁺-dependent. ATP-dependent ligases are found in bacterial and eukaryotic viruses, archaea, yeast, mammals and eubacteria NAD⁺-dependent ligases are found almost exclusively in Eubacteria. Some simple eubacteria genomes encode both NAD⁺- and ATP-dependent ligases, whereas many eukaryotic organisms encode multiple ATP-dependent ligases to fulfill diverse biological functions (Cao, 2004).

T4 DNA ligase is isolated from bacteriophage T4, forms a covalent bond between the 5' phosphate at the end of one strand and 3' hydroxyl of the adjacent strand and the reaction is ATP dependent and is often undertaken at 10°C to lower the kinetic energy of molecules. This reduces the chances of base-paired sticky ends parting before they have been stabilized by ligation. However, longer reaction times are needed to compensate for the low activity of DNA ligase at this temperature (Walker and Rapley, 2000).

II.2.3.3. Restriction Enzymes

Restriction enzymes also called restriction endonucleases, recognize specific base sequences in double helical DNA and cleave both strands of the duplex at specific sites (Stryer, 1985).

Restriction enzymes are found in a wide variety of prokaryotes. For example, restriction enzyme *HpaI* is secreted from a recombinant *E.coli* strain that carries the *HpaI* gene from *Haemophilus parainfluenzae*. Their biological role is to cleave foreign DNA molecules. The cell's own DNA is not degraded because the sites recognized by its own restriction enzymes are methylated. The important point here is that many of them recognize specific sequences of 4 to 8 base pairs also called *restriction sites* and hydrolyze a phosphodiester bond in each strand in this region (Stryer, 1985).

Because the DNA isolated from an individual organism has a specific sequence, restriction enzymes cut the DNA into a reproducible set of fragments called *restriction fragments*. Many restriction enzymes make similar cuts; generating fragments having a single stranded tail at both ends called “sticky ends”. These overhanging tails on the fragments, then are ligated covalently by DNA ligase enzyme at a given complementary fragments. Some restriction enzymes cleave both DNA sites symmetrically about the axis of the recognition site and may result in “blunt ends”, that is, two complementary strands of DNA are not in equal length, one strand extends beyond the complementary region, 5'-overhanging fragments, or 3'-overhanging fragments as shown in Figure II.3. However, blunt ends ligation requires higher DNA concentration and the reaction is significantly slower than ligation of sticky ends (Perona, 2002; Lodish et al., 1995).

Figure II.3 5'-overhanging fragment

Some of more commonly used restriction enzymes, their recognition sites, and the points of DNA backbone hydrolysis are listed in Table II.4.

Table II.4 Recognition sites and cleavage points (arrows) for some restriction endonuclease enzymes (Bailey and Ollis, 1977)

Enzyme	Target site
<i>Bam</i> HI	↓ G G A T C C C C T A G G ↑
<i>Bgl</i> II	↓ A G A T C T T C T A G A ↑
<i>Eco</i> RI	↓ G A A T T C C T T A A G ↑
<i>Hae</i> III	↓ G G C C C C G G ↑
<i>Hind</i> III	↓ A A G C T T T T C G A A ↑
<i>Hpa</i> I	↓ G T T A A C C A A T T G ↑
<i>Pst</i> I	↓ C T G C A G G A C G T C ↑

Their names consist of a three-letter abbreviation for the host organism. For example, restriction endonuclease I from *E.coli* called *Eco*RI (Stryer, 1985; Madigan and Martinko, 2006).

Restriction enzymes can be classified into three types; based on the complexity of their structure, cofactor requirements and substrate specificity. Type I restriction enzymes cut DNA at random locations as far as about 100 nucleotides after the recognition site and require ATP. Type II restriction enzymes cut DNA at the recognition site or near the recognition site and for this reason are most often used in

biotechnology. Type III restriction enzymes cut DNA about 20-30 base pairs after the recognition site and requires ATP (Bailey and Ollis, 1977).

II.2.3.4. Ligation Reaction

Many other restriction enzymes make similar cuts, generating fragments that have a single-stranded “tail” at both ends. The generated sticky ends or blunt ends with tail on the fragments at a given restriction site are complementary to those all other fragments generated by the same restriction enzyme. This base pairing of created ends permits DNA from widely differing species to be ligated, forming genetically designed molecules (Lodish et al., 1995).

In ligation reaction, DNA ligase catalyzes the formation of 3' → 5' phosphodiester bonds between the short fragments formed by the annealing of two DNA strands on a DNA template (Figure II.4). A complete ligation cycle involves three steps: (i) enzyme adenylation, (ii) substrate adenylation, and (iii) nick-closure. In the first step, an adenosine 5'-monophosphate (AMP) group is transferred from the cofactor NAD⁺ or ATP to a lysine residue in the adenylation motif KXDG (in single-letter amino-acid code where X denotes any amino acid) through a phosphoamide linkage. In the second step, the AMP group is transferred to the 5' phosphate at the nick through a pyrophosphate linkage to form a DNA-adenylate intermediate. Finally, in the third step, a phosphodiester bond is formed to seal the nick and release AMP (Cao, 2004).

Figure II.4 Schematic representation of ligation reaction

II.2.4. Transformation

Transformation is the introduction of DNA into cells with external ways. The most important advances in the molecular characterization and controlled modification of yeast genes have relied on the use of shuttle vectors which can be transformed both yeast and *E.coli* (Sherman, 2000).

II.2.4.1. Transformation into Yeast

The development of DNA transformation has made yeast particularly accessible to gene cloning and genetic engineering techniques. Plasmids can be introduced into yeast cells either as replicating molecules or by integration into the genome. In contrast to most other organisms, integrative recombination of transforming DNA in yeast proceeds exclusively via homologous recombination. Exogenous DNA with at least partial homologous segments can therefore be directed at will to specific locations in the genome. Also, homologous recombination, coupled with yeasts' high levels of gene conversion, has led to the development of techniques for the direct replacement of genetically engineered DNA sequences into their normal chromosome locations. Thus, normal wild-type genes, even those having no

previously known mutations, can be conveniently replaced with altered and disrupted alleles (Sherman, 2000)

The following three main methods are currently used to transform yeast:

- (i) Using spheroplasts; spheroplasts are yeast cells treated with hydrolytic enzymes which remove the portions of the cell wall in order to transform the DNA into the cell with the help of osmotic stabilizers
- (ii) Treating with lithium salts; the cell wall becomes permeable after treating cells with lithium acetate and then, the cells are exposed to brief heat shock to take DNA inside
- (iii) Electroporation; the permeability of cell wall for DNA induction is provided by the electrical fields. By this way DNA integrate into cell genome via the holes onto the cell wall.

II.2.4.2. Transformation into *E.coli*

Although genetic recombination in eukaryotes had been known for a long time, the discovery of genetic recombination in bacteria has been a more recent event (Madigan and Martinko, 2006).

In early seventies, the discoveries of transferring the phage by Mandel and Higa, 1970 and plasmid by Cohen et al., 1972 DNAs into *E.coli* cells, have been improved by a competence induction process involving treatment with calcium chloride. The technique of DNA transformation has then become important in virtually all aspects of genetic engineering and *E.coli* has developed into a universal host organism in recombinant DNA technology (Panja et al., 2006).

Transformation of bacteria involves DNA binding to the cell surface followed by uptake across the wall-membrane complex into the cytoplasm. However, it is still a rare event for most bacteria to naturally take up DNA from the environment. Therefore, the bacteria is subjected under certain artificial conditions, many of them become able to take up free DNA and the cells in such state are referred to as competent (Panja et al., 2006).

There are two ways to make the competent cell to transport DNA into a *E.coli* cell (Panja et al., 2006):

- (i) Treating with Ca^{2+} ions; the cells are treated with ice-cold $CaCl_2$ and then subjected to a brief heat shock. However, this technique of *E.coli* transformation is highly inefficient; the vast majority of DNA molecules added do not enter any cell, and the vast majority of bacterial cells receive no DNA. With the advancement of time, the original method of competence induction by $CaCl_2$ treatment has gradually been improved to different high-transformation-efficiency protocols, which employ magnesium ions, manganese ions, dimethyl sulfoxide, etc. together with calcium ions. Even after all such developments, the exact mechanism of $CaCl_2$ -mediated artificial transformation is still largely obscure.
- (ii) Electroporation; with a brief pulse of high voltage electricity the electroschock that renders *E.coli* cells permeable to DNA, so that plasmid DNA can simultaneously diffuse onto host cells.

II.2.4.3. pJET Cloning System

pJET Cloning system is an advanced positive selection system with high efficiency cloning of PCR products. Any PCR product, both blunt-end and sticky-end with or without 5'-phosphates can be used in this system. Blunt-end PCR products are directly ligated in very short time interval such as 10 minutes within the cloning vector, while the sticky-end PCR products are need to be blunted in prior to ligation. The PCR product doesn't need to be purified before the procedure. All common *E.coli* strains can be directly transformed with the ligation product. The efficiency of the system can be greater than 99% positive clones (www.fermentas.com, 2008).

Cloning of blunt-end PCR products, it needs to be generated with *Pfu* or other proofreading DNA polymerases and *Taq* DNA polymerase can be used while cloning of sticky-end PCR products. DNA polymerase mixes is applicable when cloning of PCR products. Finally, any DNA fragment derived from restriction digestion can be cloned with this system (www.fermentas.com, 2008).

PCR products with 3' -ends of DNA are generated using *Taq* DNA polymerase or other non-proofreading thermostable DNA polymerases are blunted with a highly

efficient thermostable DNA Blunting enzyme prior to ligation (www.fermentas.com, 2008).

II.2.4.4. pJET Cloning Vector

The pJET1/blunt is a linearized blunt-end cloning vector, ready for ligation with blunt or blunted PCR products or other blunted DNA fragments. The 5' –ends of DNA at the vector cloning site contain phosphoryl groups. Therefore, ligation of a blunt PCR product does not require additional phosphorylation of the PCR product or the use of phosphorylated primers during the PCR reaction. Blunt-end PCR products generated by proofreading DNA polymerases can be directly ligated with the pJET/blunt cloning vector (www.fermentas.com, 2008).

The pJET1/blunt is a positive selection vector. Therefore only recombinant plasmids containing the insert DNA are propagated in the *E.coli* cells. Recircularized pJET1/blunt vector molecules lacking the insert express a lethal restriction endonuclease after transformation and are not propagated. This characteristic accelerates the process of colony screening. The pJET1/blunt vector is illustrated in Figure II.5 (www.fermentas.com, 2008).

Figure II.5 pJET1/blunt vector map

II.3. YEAST

Yeasts form one of the important subgroups of fungi. Fungi, like bacteria, are widespread in nature although they usually live in the soil and in regions of lower relative humidity than bacteria. They are unable to extract energy from sunlight and usually are free-living. Although most fungi have a relatively complex morphology, yeasts are distinguished by their usual existence as single, small cells from 5 to 30 μm long and from 1 to 5 μm wide (Bailey and Ollis, 1977). Figure II.6 represents the general classification of microorganisms and the place of fungi in defined classification.

Figure II.6 General classification of microorganisms

The various paths of reproduction of yeasts are asexual (budding and fission) and sexual. In budding, a small offspring cell begins to grow on the side of the original cell; physical separation of mature offspring from the parent may not be immediate, and formation of clumps of yeast cells involving several generations is possible. Fission occurs by division of the cell into two new cells. Sexual production occurs by conjugation of two haploid cells (each having a single set of chromosomes

per cell) zygote. The nucleus in the diploid cell may undergo one or several divisions and form *ascospores*; each of these eventually becomes an individual new haploid cell, which may then undergo subsequent reproduction by budding, fission, or sexual fission again (Bailey and Ollis, 1977). Figure II.7 shows the yeast life cycle, although certain aspects of this cycle may vary with the species under consideration.

Figure II.7 Yeast Life cycle

Although yeasts have greater genetic complexity than bacteria, containing 3.5 times more DNA than *E.coli* cells, they share many of the technical advantages that permitted rapid progress in the molecular genetics of prokaryotes and their viruses. Some of the properties that make yeast particularly suitable for biological studies include rapid growth, dispersed cells, the ease of replica plating and mutant isolation, a well-defined genetic system, and most importantly, a highly versatile DNA transformation system. Unlike many other microorganisms, *S. cerevisiae* is viable with numerous markers. Being nonpathogenic, yeast can be handled with little precautions. Large quantities of normal bakers' yeast are commercially available and can provide a cheap source for biochemical studies (Sherman, 1998).

The yeast, *S. cerevisiae*, is one of the most popular model organisms. It was the first eukaryote whose genome was sequenced completely in 1996 (Akada, 2002; Perez-Ortin et al. 2002). Genetic transformation of yeast was established in 1978 (Akada, 2002). Since then, yeast has been used as the most useful eukaryotic

microorganism for biological analyses as well as for heterologous protein production. Since yeast has been the model organism for the development of biotechnology, many functional analysis projects have tried to find the function of many genes and to understand its metabolism in a holistic way. Apart from basic science this microorganism is of great interest in several biotechnology processes, such as baking, beer brewing, sake brewing and wine making (Perez-Ortin et al. 2002; Akada, 2002).

II.3.1. Yeast Cell structure

Yeast cells contain subcellular compartments typical of eukaryotic cells. Morphology or at least functionally, defined organelles include the nucleus, mitochondria, endoplasmic reticulum, Golgi complex, secretory vesicles, and vacuoles. The cell envelope consists of a rigid cell wall separated from the plasma membrane by the periplasmic space. Not all of the subcellular organelles are completely independent from each other, but are specialized structures derived from an extended intramembranous system. Nevertheless, isolation procedures for intact organelles or at least membrane fragments exist (Tuite and Oliver, 1991).

Unstained yeast cells can hardly be visualized by light microscopy. At 1000-fold magnification, it may be possible to see the yeast vacuole and cytosolic inclusion bodies. By phase-contrast microscopy, together with appropriate staining techniques, several cellular structures can be distinguished. Fluorochromic dyes can be used with fluorescence microscopy to highlight features within the cells as well as on the cell surface (Pringle et al., 1991).

Obviously, yeast cells share most of the structural and functional features of higher eukaryotes, which has rendered yeast an ideal model for eukaryotic cell biology. In contrast to mammalian cells, peculiarities of yeast cells are that they are surrounded by a rigid cell wall and develop birth scars during cell division; the vacuole corresponds to lysosomes in higher cells (Feldman, 2005).

II.3.2. Yeast Growth Characteristics

Unlike most other organisms, strains of *S. cerevisiae* have both a stable haploid and diploid state. Laboratory haploid strains have a doubling time of approximately 90 min in complete YPD (1 per cent yeast extract, 2 per cent peptone, and 2 per cent

glucose) medium and approximately 140 min in synthetic (minimal defined) media during the exponential phase of growth at the optimum temperature of 30°C. However, strains with greatly reduced growth rates in synthetic media are often encountered. Usually strains reach a maximum density of 2×10^8 cells/ml in YPD medium. Titer 10 times this value can be achieved with special conditions, such as pH control, continuous additions of balanced nutrients, filtered-sterilized media and extreme aeration that can be delivered in fermenters (Altıntaş, 2001).

When a single *Saccharomyces* strain is grown in submerged culture, a plot of the logarithm of the dry weight cells produced against time gives a characteristic curve dependent on strain and environmental conditions. A typical growth curve consists of three distinct sections (Tuite and Oliver, 1991):

- (a) *The lag phase*: represents the time period between inoculation of the culture medium with the organism and a measurable increase in cell concentration.
- (b) *The exponential phase*: is the time period during which the specific growth rate (μ) is constant and at a maximum ($\mu_{\max}$) for the given strain and the environmental conditions.
- (c) *The stationary phase*: the accumulated cell mass remains constant, no further growth will occur.

II.3.3. Cellular Respiration Mechanism and Fermentation

There are two important ways that cells can harvest energy, namely cellular respiration and fermentation. Both processes start with the same first step; the glycolysis which is the breakdown of 6 carbons (glucose) into two 3-carbon molecules called pyruvate. Glycolysis is probably the oldest known way of producing ATP. In each mechanism, the synthesis of ATP is driven by the energy released in the oxidation-reduction reactions. However the redox reactions in respiration and fermentation differ significantly (Lodish et al., 1995).

II.3.3.1. Cellular Respiration

If O_2 or some other terminal acceptor is present, the glucose can be oxidized completely to CO_2 and a far higher yield of ATP. The respiration machinery is located in cytosol and final steps occur in plasma membrane for prokaryotes whereas it is placed in the inner membranes of mitochondria for eukaryotes. Respiration

requires a terminal electron acceptor. If the respiration process uses oxygen as its terminal electron acceptor, is called *aerobic respiration* and the one, which uses terminal electron acceptors other than oxygen, it is called *anaerobic respiration* (Madigan and Martinko, 2006).

In aerobic respiration, energy from metabolism of sugars, fatty acids, and other substances, culminating in their oxidation by O_2 , is used to pump protons across a mitochondrial membrane. Aerobic respiration is the entire process of metabolizing glycolysis, the kreb's cycle, and then the electron transport chain (ETC). Except glycolysis, the other two pathways occur in mitochondria to complete the aerobic respiration process (Madigan and Martinko, 2006).

Mitochondria are the metabolic heart of most aerobic eukaryotic cells. A double membrane forming two internal spaces encloses the mitochondria. The space between the two membranes is called the intermembrane space (IMS) and the volume enclosed within the inner membrane (IM) is designated as the matrix compartment. The two membranes differ significantly in their composition with the IM more highly enriched in protein content. In cells with high respiration rates, the IM is invaginated, folding into tubular structures designated cristae. Cristae are enriched in the enzyme complexes involved in oxidative phosphorylation which is a metabolic pathway of ATP synthesis by electron transport chain. Oxidative phosphorylation occurs on the cristae membrane (CM). In oxidative phosphorylation mechanism, electrons from the energy supplying molecules are tunneled down a chain of inner membrane-bound redox centers to reduce molecular oxygen to water. The electron transport chain is comprised of several components, including (in order of electron transport) complexes I and II, which oxidize NADH and succinate which is a dicarboxylic acid, respectively, ubiquinone (Q) accepts electrons from electron-transferring flavoprotein (contains a bound flavin adenine dinucleotide (FAD) cofactor) in the mitochondrial matrix. Reduced coenzyme Q passes electrons one at a time to membrane protein complex III, and finally cytochrome c, complex IV reduces oxygen to water. During electron transport, redox energy is captured in the form of a proton gradient which is subsequently harvested by a fifth protein complex, termed complex V to generate ATP (Khalimonchuk and Winge, 2008; Rea, 2005). The ETC pathway is illustrated in Figure II.8. Overview of aerobic respiration is schematically represented in Figure II.9.

Figure II.8 Electron transport pathway

Figure II.9 Overview of aerobic respiration

The mitochondrial genome of yeast is of special interest for one major reason: because it is dispensable, since *S. cerevisiae* can survive on fermentable carbon sources. Mitochondrial inheritance from yeast is biparental, and both parent cells contribute to the daughter cells when the haploid cells fuse. After meiosis and mitosis, there is random distribution of mitochondria to daughter cells. If the fusion is not with yeast, a certain percentage of the daughter cells will be “petite”. The point is that non-Mendelian inheritance shows that these petite, have structurally abnormal mitochondria that are incapable of oxidative phosphorylation, can also survive by fermentation process. Accordingly, the yeast petite mutants having undergone massive deletions in their mitochondrial genome, or having lost it, or some damage in their mitochondrial complexes can therefore survive (Bernardi, 2005).

Anaerobic respiration is one alternate mode of respiratory energy generation in which electron acceptors other than oxygen are used. Some of the electron acceptors used in anaerobic respiration include nitrate (NO_3^-), ferric iron (Fe^{3+}), sulfate (SO_4^{2-}) and carbonate (CO_3^{2-}). Because of their positions on the electron tower, none of these acceptors has as positive reduction potential as O_2 , less energy (less ATP) is released when these electron acceptors are used instead of oxygen. Just as in aerobic respiration, anaerobic respiration goes through glycolysis, pyruvate oxidation, Krebs cycle and then electron transfer chain with the only difference that the terminal electron acceptor is not oxygen. (Madigan and Martinko, 2006).

II.3.3.2. Fermentation

Fermentation is an anaerobic catabolism in which an organic compound serves as both an electron donor and electron acceptor and ATP is produced as a result of substrate uptake. Fermentative organisms usually do not operate a complete Krebs cycle. A variety of oxidation–reduction reactions are involved in fermentative processes where metabolites in cytosol are chemically transformed by aqueous enzymes, in order to produce energy (ATP) in such a way that mitochondrial membranes and ion gradients are not involved. Fermentation can occur in animals, plants, many bacteria and yeasts (Madigan and Martinko, 2006; Lodish et al., 1995).

A common biochemical pathway for the fermentation of glucose is glycolysis also named the Embden–Meyerhof–Parnas (EMP) glycolytic pathway. The end product of these glycolytic processes is pyruvate. The sum of the reactions

generating two molecules of pyruvate and ATP per glucose molecule is as follows in Equation II.2, and Pi refers to inorganic orthophosphate (Boumba, 2008)

Glucose degradation in yeasts is employed via the EMP pathway. Pyruvate is decarboxylated by pyruvate decarboxylase to acetaldehyde which is then reduced to ethanol by alcohol dehydrogenase. The fermentation reactions of glucose to ethanol from the stage of pyruvate to the final products by yeasts and bacteria species are schematically shown in Figure II.10. In the Figure II.10, pathway A shows the fermentation pathway by yeast which gives end-product of ethanol, pathway B shows the fermentation pathway by bacteria (Boumba, 2008).

Figure II.10 Fermentation of glucose to ethanol by yeasts and bacteria species.

1, pyruvate decarboxylase; 2, alcohol dehydrogenase; (B) bacteria: 3, pyruvate dehydrogenase or pyruvate-formate lyase; 4, acetaldehyde/alcohol dehydrogenase; 5, phosphotransacetylase; 6, acetate kinase; 7, AMP-forming acetyl-CoA synthetase

Pyruvate decarboxylase, however, is rare in bacteria. For these bacteria, such as many lactic acid bacteria *Streptococcus lactis*, *Leuconostoc mesenteroides*, etc.

enterobacteria *E.coli*, *Klebsiella aerogenes*, etc. and clostridia *Clostridium splenoides*, *Clostridium sporogenes*, etc. the formation of considerable amounts of ethanol from carbohydrates is achieved via acetyl-CoA which is produced from pyruvate decarboxylation. Acetyl-CoA is a well recognized high-energy intermediate that sits at the crossroads of central metabolism. The conversion of pyruvate to acetyl-CoA can occur oxidatively under aerobic conditions and non-oxidatively under anaerobic conditions (Boumba, 2008).

In Figure II.10, The fate of acetyl-CoA in pathway B can follow two alternative pathways: either conversion to acetate or reduction to ethanol. The conversion of acetyl-CoA to acetate is catalyzed by the phosphotransacetylase-acetate kinase system and generates ATP. The reduction of acetyl-CoA to ethanol is catalyzed by acetaldehyde/alcohol dehydrogenase, through the formation of acetaldehyde, and consumes reducing equivalents. Therefore, microorganism can balance their requirements for reducing nucleotides and energy by modulating the amount of ethanol and acetate formed. The production of ethanol via acetyl-CoA results in addition to the formation of various other by-products such as butanol, propanol etc.

Fermentation reactions are energetically inefficient when compared with respiration reactions since they result in relatively low ATP yields. Synthesis of reduced products including hydrogen, lactate, succinate, butyrate and ethanol is used to keep the redox balance during fermentation. Most commonly they are classified according to the main end product of the process (e.g. ethanol fermentation, lactate fermentation) (Boumba, 2008).

II.3.4. Respiratory Deficient Yeast

S. cerevisiae is a facultative anaerobe, which means that it possesses the property to switch from respiration to fermentation, and vice versa. Mitochondria of fully respiring cells are rich in mitochondrial cristae structures. Under these conditions, 50 or more mitochondria per cell have been observed. On the other hand, cells containing only a few mitochondria which are poor in cristae exhibit low respiratory enzyme activities (Tuite and Oliver, 1991).

Mitochondrion generally considered being a cellular organelle specialized in the aerobic production of ATP and is very important for eukaryotes to survive. But,

some yeast species lacking mitochondrial DNA can grow in medium containing appropriate sugar and survive as respiratory-deficient mutants or petite positive species (Sakanaka et al., 1996).

Cytochrome c oxidase is the terminal enzyme of the eukaryotic respiratory chain. Cytochrome c oxidase plays a key role in the electron transport chain; it catalyzes the reduction of molecular oxygen to water and concomitantly the oxidation of reduced cytochrome c, and couples this redox reaction with the electrogenic transfer of protons across the inner mitochondrial membrane (IMM) (Khalimonchuk and Rödel, 2005).

Loss of any known mitochondrial gene products, ribosomal RNAs, tRNAs and the enzymes of complexes in any step involved in respiration and oxidative phosphorylation would have a mutational effect and lead to a loss of respiratory functions. This could also happen as a consequence of mutations in nuclear genes in genome that encode some sub-units of mitochondrial enzymes (Bernardi 2005; Scheffler, 2001).

Mitochondria exist in two different states depending on the presence or absence of oxygen in environment of yeast. During anaerobic conditions the mode of mitochondria structure can change to promitochondria and yeast can use other electron acceptors by anaerobic respiration. When a minute quantity of oxygen is available, the yeast will differentiate its mitochondrial structure into fully functional mitochondrion. On the other hand, when glucose reaches high external concentrations, even if there is oxygen in the environment of yeast, the aerobic respiratory activity of yeast is repressed by the high glucose concentration (Crabtree effect). Therefore, it is suggested that fully developed mitochondrial functions is essential for maintaining the ethanol tolerance and, consequently, fermentation performance of yeast (Hutter and Oliver, 1998).

Hutter and Oliver (1998) investigated the relation between the ethanol tolerance and petite phenotype. They have worked with two nuclear petites, each having a mutation with cytochrome c oxidase (also *COX*) which is an enzyme described as complex IV in ETC. The COX enzyme is composed of nine subunits that are present in the same molar amount in the expressed enzyme. Subunit V is encoded by two non-identical nuclear genes, *COX5a* and *COX5b*. Disruption *COX5a* (located on chromosome XIV) renders the mutant 85% - 90% respiratory deficient.

PET191 (chromosome X) encodes a protein required for the assembly of polypeptide subunits that constitute the active cytochrome c oxidase enzyme. Disruption *PET191* causes failure of enzyme assembly, resulting in 100% respiratory deficiency. Respiratory deficient nuclear petites, FY23 Δ *pet191* and FY23 Δ *cox5a* of yeast *S.cerevisiae* were generated by Hutter and Oliver (1998) using PCR mediated gene disruption and their ethanol tolerance was compared with FY23WT, parental grande and mitochondrial petite FY23p^o. They observed that fully developed functional mitochondria were essential for maintaining the ethanol tolerance of yeast. 100% respiratory deficient FY23 Δ *pet191* showed the highest ethanol tolerance whereas the mitochondrial petite FY23p^o lacking the functional mitochondrial DNA exhibited lowest tolerance of the series. This study showed that in ethanol production by yeast, the disruption of mitochondrial DNA had a detrimental effect on the yeast's ethanol tolerance. Consequently, leaving the mitochondrial genome intact and creating deletions in nuclear genome regions which assembly proteins for mitochondrial respiration were found to increase the fermentation rate (Hutter and Oliver, 1998).

II.3.5. Recombinant DNA Technology in Yeast

Significant effort is currently directed at the high-level production of recombinant proteins in genetically modified organisms. These activities are primarily driven by the need to economically produce biological products for many areas in industry (Fussenegger and Hauser, 2007).

Three types of expression systems are widely used for heterologous protein production, exploiting prokaryotes like *E.coli*, and eukaryotes like yeasts, cultured insect and mammalian cells as hosts (Tamas and Shewry, 2006).

During the last two decades, the availability of relevant structural genes and its use in genetic engineering have become a fascinating approach to create microbial strains which can produce valuable proteins in high amounts and quality (Gellissen and Hollenberg, 1997).

Yeasts have been used since the early 1980s for the large scale production of intracellular and extracellular proteins of human, animal, and plant origin. The expression of a foreign protein in yeast consists of four steps: firstly, cloning of a foreign protein-coding DNA sequence within an expression cassette containing a

yeast promoter and transcriptional termination sequences; secondly, transformation and stable maintenance of this DNA fusion in the host; thirdly, synthesis of the foreign protein under specified culture conditions; and finally, purification of the heterologous protein and comparison with its native counterpart (Cereghino and Cregg, 1999).

The widespread use of *S. cerevisiae* in recombinant DNA studies is in part due to the availability of large amounts of information concerning its molecular biology and physiology. Other reasons for the use of yeast are: (i) it is generally regarded as safe; (ii) it has been studied in large scale fermenters for decades; (iii) it is able to glycosylate proteins; and (iv) it is able to secrete recombinant proteins (Altıntaş, 2001). Besides that, since yeasts are best characterized unicellular eukaryotic microorganism systems, it is easy to combine the genetic manipulations with the ability to perform eukaryotic processing steps on the polypeptides expressed and thus constitute attractive systems for various aspects of modern biotechnology (Gellissen and Hollenberg, 1997).

II.3.5.1. Yeast Vectors

Protein yield is clearly a major parameter to consider when establishing an expression system and is affected by a number of factors. Three of these are particularly relevant to the expression of proteins: choice of promoter, plasmid stability and codon usage (Tamas and Shewry, 2006).

Expression vectors require a strong promoter for high-level protein expression. Although constitutive promoters are available for all systems, the most widely used promoters are inducible, with expression being induced by either specific chemicals or thermal effects. Inducible systems allow cultures to grow to high density before expression is induced, providing higher yield and minimizing effects of toxic proteins (Tamas and Shewry, 2006).

Plasmid instability can arise when the product of the gene is toxic to the host cell, when the plasmid is lost during cell division or when the gene is modified by recombination. Low copy number plasmids tend to be preferred if protein toxicity is a problem as the level of accumulation will be lower (but yields will also be reduced) (Tamas and Shewry, 2006).

Amino acids are each encoded by between one and six different codons, and where multiple codons exist, their frequency of use may vary between different groups of organisms. If such differences in codon usage occur between the gene of interest and the host cell, the expression of the recombinant protein may be limited by the low abundance of the corresponding tRNAs (Tamas and Shewry, 2006).

Almost all yeast plasmid vectors are, in fact, both yeast and *E.coli* vectors which are called shuttle vectors. Shuttle vectors contain sequences permitting them to be selected and propagated in *E.coli*, thus allowing for convenient amplification. The most common yeast vectors have an origin of replication (*ori*), promoting high copy-number maintenance in *E.coli*, and the selectable antibiotic markers, such as the β -lactamase gene (*bla* or *Amp^R*), or tetracycline-resistance gene (*tet* or *Tet^R*), conferring resistance to ampicillin and tetracycline, respectively. In addition, all yeast vectors contain markers that allow selection of transformants containing the desired plasmid. The most commonly used yeast markers include *URA3*, *HIS3*, *LEU2*, *TRP1* and *LYS2* (Tuite and Oliver, 1991).

Natural eukaryotic plasmids are too large to be used in cloning. To manipulate the vector easily, several different types of yeast vectors have been reported; the principal ones are: (i) yeast integrating plasmids (YIp); (ii) yeast episomal plasmids (YEpl); (iii) yeast replicating plasmids (YRp); (iv) yeast centromeric plasmids (YCpl); and (v) yeast linear plasmids (YLP). These vectors which are known as yeast autonomous plasmid vectors are based on one of two replication systems: either the sequences derived from the endogenous yeast 2 μ m plasmid are used; or chromosomal DNA fragments that can replicate. Accordingly, origin of replication from the eukaryotic plasmid has been incorporated with a gene which will terminate an enzyme encoding by specific gene in the host yeast cell. Therefore, by this way yeast plasmid can be used as a selective marker to control whether it has the insert or not (Walker and Rapley, 2000; Zhang et al., 1996).

In commercial production with recombinant microorganisms, the most important problem is plasmid instability. Instability is the tendency of the transformed cells to lose their engineered properties because of changes to, or loss of, plasmids. This problem is especially significant with recombinant yeasts because almost all yeast plasmid vectors are 'shuttle vectors,' that are relatively unstable. Plasmid instability may be of two types: structural instability and segregational

instability. Structural instability is usually caused by deletion, insertion, recombination, or other events, at the level of the DNA; whereas segregational instability is caused by uneven partitioning of plasmids during cell division. Genetic instabilities in combination with environmental factors lead to plasmid loss. During culture, cells lacking the plasmid appear, coexisting and competing with the plasmid-containing population. Consequently, plasmid instability results in decrease in desired product productivity. Optimization of recombinant system by individuals is necessary to decrease the instability and to achieve better vectors (Zhang et al., 1996).

Principally, in order to produce a recombinant protein in a fungus, the gene encoding the protein is introduced to the fungal host on a plasmid which must integrate into the host genome. Self-replicating plasmids are not used for protein production in filamentous fungi. Since the recombinant DNA is integrated into the fungal genome it is not lost because of unequal partitioning, as may occur with the self-replicating plasmids used to produce recombinant proteins in bacteria or yeast. As described in Figure II.11, self-replicating plasmids in bacteria and yeast may be a) equally partitioned between daughter cells or b) unequally partitioned between daughter cells. c) DNA integrated into the chromosome of a filamentous fungus will be equally partitioned during mitotic replication of the chromosomes. However, the ability to produce the recombinant protein may still be lost for a variety of reasons. Loss of the recombinant DNA from the host cell or loss of expression of this DNA leads to reduced productivity (Wiebe, 2003).

S. cerevisiae has been widely used to express and study amylases from different eukaryotic organisms and glucoamylases and α -amylases from different yeast genera have also been analyzed. Despite these successes, *S. cerevisiae* is often limited as an expression system by low yields. Methylotrophic yeasts such as *Pichia pastoris* and *Hansenula polymorpha* retain all the advantages of *S. cerevisiae* but provide a reliable means of achieving greatly elevated yields (Sudbey, 1996).

Figure II.11 The fate of DNA introduced into cells as self-replicating plasmids or on integrating vectors (Wiebe, 2003)

II.3.5.2. rDNA Region of *S. cerevisiae* for Stable Multiple Integration of DNA

Plasmid instability and interactions between the host cell and the plasmid determine the recombinant yeast feasibility, development and optimization necessities in biotechnology. Stable maintenance of a recombinant gene is of utmost importance in a profitable process. Generally, industrial fermentation processes require complex media for high yields, and high copy number plasmids are often mitotically unstable. To overcome these problems direct integration of a target gene into the host chromosome is an effective method for the stable recombinant gene expression in yeast. By chromosomal integration instead of plasmid, the segregational instability of the plasmid vectors is circumvented and the cloned gene expression can be maintained for long terms (Kim et al., 2001).

Several interesting approaches to increasing the copy number of integration have also been reported; these approaches use targets mainly existing in multicopy ribosomal DNA (Sohn et al., 1996).

In most eukaryotic organisms the DNA encoding the ribosomal DNA, rDNA is repeated at one or few chromosomal loci, that is, is present in many copies. *S.*

cerevisiae carries approximately 150 copies of tandemly repeated rDNA on chromosome XII. Transformation recombinant gene into the ribosomal DNA in yeast results in the presence of more than hundred integrated copies (Wai et al., 2000; Gellissen and Hollenberg, 1997).

Recently, Shen et al. (2008) cloned β -glucosidase encoding gene named *BGL1* from *Saccharomycopsis fibuligera* and integrated into the chromosomal rDNA region of the *S. cerevisiae* industrial strain NAN-27 producing NAN-227 to improve the hydrolysis of lignocellulosic materials to convert to ethanol eventually. According to the results of this study, the ability to utilize glucose did not change after the introduction of the BGL1 gene; moreover an effective pathway for metabolizing cellobiose was created. Recombinant strain NAN-227 converted 6.2 g/L of cellobiose to 3.3 g/L of ethanol in 48 h when cellobiose was used as the sole carbon source.

Since the scale-up and production of recombinant proteins in yeast is a well-established technology, it is reasonable to expect the high-throughput and cost-effectiveness of yeast strains to make a significant impact on the industry (Hamilton and Gerngross, 2007).

II.4. STARCH AS A RENEWABLE CARBON SOURCE

Ethanol is the most common renewable fuel in the last two decades, and annual ethanol production in the global production has increased by far (Gray et al., 2006).

One major problem with bioethanol production is the availability of raw materials for the production. The availability of feedstocks for bioethanol can vary considerably from season to season and depend on geographic locations. The price of the raw materials is also costly, which can highly affect the production costs of bioethanol (Balat et al., 2008).

Biomass energy is one of humanity's earliest sources of energy including generating electricity, heating homes, fueling vehicles, and from last few decades providing process for industrial facilities. Among biomass sources, starch has one of the highest energy potential which can substitute for alternative renewable energy (Table II.1). Moreover, its structure allows the easy degradation which leads to higher energy yields in processes (Gray et al., 2006).

Indeed, the compactness and complexity of lignocelluloses makes the enzymatically degradation (hydrolysis process) much more difficult than starch to fermentable sugars. From this point of view the prospect of cellulosic conversion to bioethanol is still problematic. Hence, the cost of producing a gallon of ethanol from starch is cheaper than production from cellulosic compounds (Bai et al., 2008).

The starch-based bioethanol industry has been commercially viable for about 30 years. The United States is the biggest ethanol producer which has the capacity to produce 13 billion gallons per year from corn alone and will probably reach the 7.5 billion gal per year goal much sooner than expected in the world, and the major raw material for ethanol production in United States is corn grain (starch). Starchy type of feedstock is the most utilized one for bioethanol production also in Brasil and Europe. Corn and wheat are mainly employed with these purposes. (Goyal et al., 2008; Gray et al., 2006; Balat et al., 2008).

II.4.1. Starch-based Bioethanol Production Processes

Starch is a biopolymer and defined as a homopolymer consisting only one monomer, D-glucose and these D-glucose structures are linked together via α -1,4 and α -1,6 glycosidic linkages (amylose and amylopectin). To produce bioethanol from starch, it is necessary to break down the chains of this carbohydrate for obtaining glucose syrup, which can be converted into bioethanol by yeasts (Gray et al., 2006; Bai et al., 2008).

Starch is a high yield feedstock for ethanol production, but its hydrolysis is required to produce ethanol by fermentation. Starch was traditionally used to be hydrolyzed by acids, but the specificity of the enzymes, their inherent mild reaction conditions and the absence of secondary reactions have made the amylases to be the catalysts generally used for this process (Sanchez and Cardona, 2008).

There are two types of processes for ethanol production from starch, namely the dry mill and the wet mill process. The key distinction between wet mill and dry mill facilities is the focus of the resourcing. In the wet-mill process, corn is soaked or steeped and then separated into its component parts, which are recovered prior to hydrolysis process, and only the starch fraction is processed. In the dry milling process, corn is ground into flour (meal) and processed without separation of component parts (Bothast and Schlicher, 2005).

The wet milling process is more capital and energy intensive, as the grain must first be separated into its components, including starch slurry, germ, corn gluten feed and corn gluten meal. Therefore, starch is the primary product of the process. After starch separation, starch is sent directly to hydrolysis and fermentation processes. The remaining part can be converted to useful products in wet milling process. For example, the germ is used for corn oil production and the resulting meal is used for animal feed. Corn oil is recovered from the germ by expelling or more often by solvent extraction. Corn gluten feed which contain 60% fiber and 20% protein is the fiber rich component removed in the wet milling process. It is a high fiber, low protein feed used as energy, protein and fiber source. In wet mill houses, a typical starch slurry has 60% moisture content. Wet mill facilities are true “biorefineries”, producing a number of high-value products. Wet-milling plants have much higher up-front costs and operating expenses than dry-milling plants, and thus are not that common (Bothast and Schlicher, 2005; Ramirez et al., 2008).

In the dry mill process, the corn is first processed through hammer mills, which grind it into a fine powder with a mean particle diameter of approximately 1 mm. The feed is then mixed with water to form a mash and the mash passes through cookers where the starch is liquefied. The enzymes are added to the cooked mash to convert starch to sugar. Cooking is generally accomplished at temperatures of 65-80°C. The meal can be exposed to a high temperature stage of 120- 150°C for a short period of time to reduce bacterial growth in the mash. Then yeast is added to ferment the sugars, producing a mixture containing ethanol and solids. This mixture is then distilled and dehydrated where ethanol is recovered to fuel-grade ethanol. The solids remaining after distillation are dried to produce distillers’ dried grains with protein and are sold as an animal feed supplement. A schematic of both processes is illustrated in Figure II.12 (Bothast and Schlicher, 2005; Kim et al, 2008).

Figure II.12 The ethanol production processes (Bothast and Schlicher, 2005)

In the case of a dry mill plant, the focus is maximizing the capital return per gallon of ethanol. In the case of a wet mill plant, capital investments allow for the separation of other valuable components in the grain before fermentation to ethanol. Hence, the most of bioethanol is produced by dry mill process which is ~ 65% of the industry (Bothast and Schlicher, 2005).

A large part of the energy used in ethanol production process is however consumed by cooking and space heating. Actually the grain-based ethanol industry did not become a viable source of fuel until industrial microbial enzymes became readily available like today. The fermentation process where conversion to ethanol occurs can take place in any anaerobic condition, however the steps consisting milling, separation, cooking are time consuming and costly. Raw materials such as corn (maize), wheat, barley, rye or sorghum need mechanical and enzymatic pre-treatment to release the starch in a free form and to make it suitable for hydrolysis to fermentable sugars, mainly glucose and maltose. Many traditional chemical processes based on acidic or base catalyzed reactions for processing of agricultural products have inherent drawbacks from a commercial and environmental point of view. For amylases to attack starch, these suspensions should be brought to high temperatures (90–110°C) for the breakdown of starch kernels. However, after

liquefied starch is subject to saccharification at lower temperatures (60–70°C) through glucoamylase. High temperatures and high pressures needed to drive reactions may lead to high costs and requirement of large volumes of cooling water. Harsh and hazardous processes involving high temperatures, pressures, acidity or alkalinity need high capital investment, and specially designed equipment and control systems. Unwanted by-products may prove difficult or costly to dispose of. High chemical and energy consumption, and harmful by-products have a negative impact on the environment. The use of enzymatic process during fermentation may virtually eliminate these drawbacks (Lin and Tanaka, 2006; Wingren and Zancchi, 2008).

Over the last decade, new enzyme systems have been developed for the bioethanol industry. One example has been the development of low pH α -amylases that simplify the process and reduce chemical costs as well as improving ethanol yield. The other major advance is the development of enzymes that function on raw, uncooked starch, thereby improving overall process economics. The efficient enzyme systems in dry-milling process where the saccharification is carried out simultaneously with fermentation, so-called simultaneous saccharification and fermentation (SSF) minimizes both investment and operating costs (Gray et al., 2006; Gulati et al, 1996).

The starch can be liquefied by using first α -amylase and then glucoamylase and can be directly transferred to the fermenter where yeast is added. Otherwise, by integrating genetically designed recombinant genes into the yeast, these amylolytic enzymes can be secreted by microorganism under controlled conditions to achieve the SSF in bioreactor (Shen et al, 2008; Sanchez and Cardona, 2008).

Moreover, in SSF the concentration of monomeric sugars is constantly kept low since the microorganism ferments them to ethanol as soon as they are liberated from the polymers. Ethanol has also a noticeable inhibitory effect on *S. cerevisiae* at concentrations above 15 g/L. Therefore, by SSF both glucose and ethanol will be kept in diluted proportions in bioreactor decreasing their adverse effect on microorganism (Öhgren et al., 2007).

II.4.2. Bioethanol Production from Starch by Yeast

Although several yeast of genera other than *Saccharomyces* assimilate starch, many amylolytic types of yeast cannot be used in modern industrial fermentation because of their low ethanol tolerance and susceptibility. These include *Schwanniomyces occidentalis*, *Saccharomycopsis fibuligera*, *Candida tsukubaensis* CBS 6389, *Filobasidium capsuligenum*, *Lipomyces kononenkoae*, *Lipomyces starkeyi*, *Saccharomycopsis bispora*, *Saccharomycopsis capsularis*, *Schwanniomyces alluvius*, *Schwanniomyces casteilli*, *Trichosporon pullulans*, and *Candida tsukubaensis* (Lin et al., 1998; Nigam and Singh, 1995).

Unfortunately, *S. cerevisiae* lacks the ability to degrade starch, hence to synthesize and secrete necessary amylolytic enzymes which is required the bioconversion of starch to ethanol, the genetic manipulations on *S. cerevisiae* is indispensable (Lin et al., 1998). There are numerous studies in literature to increase the bioethanol production from starch by yeast (Toksoy Öner, 2006).

Shigechi et al. (2002) improved the ethanol productivity of *S. cerevisiae* from starchy materials. Yeast strains were constructed displaying *Rhizopus oryzae* glucoamylase enzyme on the cell surface by co-displaying or secreting the *Bacillus stearothermophilus* α -amylase enzyme. Recently, the same workers have reported the utilization of a previously constructed yeast strain co-displaying both amylolytic enzymes on the cell surface for direct ethanol production from corn starch (Shigechi et al., 2004a). But since their strain could not ferment raw corn starch to ethanol, they have constructed a new strain capable of fermenting raw corn starch by expressing α -amylase from the lactic acid bacterium *Streptococcus bovis* 148, instead of the *B. stearothermophilus* α -amylase, together with *R. oryzae* glucoamylase (Shigechi et al., 2004b). Eksteen et al. (2003) reported the construction of eight different amylolytic *S. cerevisiae* strains excreting *Lipomyces kononenkoae* α -amylase and *Saccharomycopsis fibuligera* glucoamylase enzymes. However, starch fermentation characteristics under controlled bioreactor conditions of three of these constructs revealed inefficient excretion of these amylolytic enzymes to the fermentation medium and no ethanol productivity under aerobic cultivation conditions (Knox et al., 2004).

One of the studies was done by de Moraes et al. (1995), where eight different strains, including strains that produce *B. subtilis* α -amylase (BsAAase), mouse

pancreatic α -amylase (MAAase) or *A. awamori* (GAase) glucoamylase either singly or in combination, as well as strains that produce either BsAAase/GAase or MAAase/GAase fusion enzymes were constructed and their starch fermentation abilities were compared. They have observed good performances not only in digesting soluble and corn starch but also in using all of the hydrolysis products for growth.

Another study was done by Hutter and Oliver (1998). They constructed three different mutants of the yeast *S. cerevisiae* by PCR. Two nuclear respiratory deficient petites FY23 Δ *pet191* and FY23 Δ *cox5a* were generated by disruption of *PET191* (located on chromosome X) and *COX5a* (chromosome XIV) nuclear genes. Their respective ethanol tolerance and productivity was assessed and compared to those of parental grande FY23WT and a mitochondrial petite, FY23p^o in batch reactors. They found that the ethanol productivity reached the highest value, 1.339 g ethanol g dry biomass⁻¹ h⁻¹ with the 100% respiratory deficient FY23 Δ *pet191* strain. They also concluded that functional mitochondria are essential to maintain the tolerance of yeast to ethanol and mitochondrial genome is thought to play a significant role in certain cell-surface phenomena, because the FY23p^o confirmed the lowest value and the parental FY23WT displayed slightly higher ethanol productivity than FY23p^o. The deficient FY23 Δ *cox5a* and fully respiratory deficient FY23 Δ *pet191* displayed a productivity of 30% and 43% higher than the FY23WT respectively. Therefore, 100% respiratory deficient nuclear petites were suggested to be used in ethanol production industry because of their high ethanol tolerance (Hutter and Oliver, 1998).

Later on Toksoy Öner et al., (2005) transformed the same FY23 Δ *pet191* petite with the pPB-G plasmid which is able to encode *B. subtilis* α -amylase and *A. awamori* glucoamylase enzymes to construct *S. cerevisiae* NPB-G strain. Comparison of the ethanol fermentation performances of the respiratory-deficient NPB-G and the parental respiratory-sufficient WTPB-G strain showed an increase of ca. 48% in both ethanol production yields and ethanol productivities with the respiratory-deficient strain. Optimization of fermentation conditions in order to prevent the observed decrease in the time dependant amylolytic activity resulted in significant improvements in ethanol yield from soluble starch (Toksoy Öner, 2006).

Marin et al. (2001) generated an amylolytic industrial yeast strain of *S. cerevisiae* by integrating the *Schwanniomyces occidentalis* SWA2 amylase gene. The new strain contained DNA derived exclusively from yeast and expressed a high starch hydrolyzing activity. They described the generation of a stable yeast strain that was able to grow in starch efficiently after SWA2 amylase integration, and hydrolyzed 95% of starch within 35 h. This result showed the industrial potential of this new genetically engineered strains of *S. cerevisiae*.

Recently, Yang et al., (2008) developed a construct harbouring the *Rhizopus arrhizus* glucoamylase gene (RaGA) artificially spliced by PCR and transferred into *Pichia pastories* by pPIC9 vector which correctly expressed RaGA protein. *Pichia pastories* is a methylotrophic yeast and widely used in recombinant DNA technology. The glucoamylase from *Rhizopus* has the ability to digest α -1,4, α -1,3, and α -1,6-glucaoside to produce glucose from starch with starch digestibility. Thus, it has great industrial importance in the ethanol fermentative production industry (Yang et al., 2008).

CHAPTER III

THE STUDY

III.1. MATERIALS

III.1.1. Yeast Strains

pPB-G plasmid contains the *B. subtilis* α -amylase and the *A. awamori* glucoamylase genes expressed under the control of the constitutive PGK1 promoter as an excreted bifunctional fusion protein (de Moraes et al., 1995). *S. cerevisiae* YPB-G strain was constructed by de Moraes *et al.* (1995) by transforming the pPB-G plasmid into the standard host *S. cerevisiae* strain GRF18 [MAT α , *his3*-11, *his3*-15, *leu2*-3, *leu2*-112, *can1*, *mal*.] *E.coli* TOP10 [F- *mcrA* Δ (*mrr*-*hsdRMS*-*mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX*74 *recA1* *araD*139 Δ (*araleu*) 7697 *galU* *galK* *rpsL* (StrR) *endA1* *nupG*] cells were used as host for the cloning experiments. Plasmids and microorganisms were from our laboratory stock. The genomic DNA of a standard *S. cerevisiae* strain was purified and kindly provided by Duygu Dikicioğlu (Chemical Engineering Department, Boğaziçi University).

III.1.2. Oligonucleotide Primers

rDNAfor: 5' GGT AAC ACG CTT GTG CGC AAT CCA TTT TCA 3'

rDNArev: 5' TGG GGG GAG TAT GTG CGC AAG GCT GAA ACT 3'

pJET1frw: 5' GCC TGA ACA CCA TAT CCA TCC 3'

YEPfor: 5' TGT GGA ATT GTG AGC GGA TA 3'

LEUrev: 5' CGC GTT GCA TTT TTG TTT TA 3'

III.1.3. Chemicals

All chemicals and solutions used in this study were from MERCK (GERMANY) or SIGMA (USA) unless stated otherwise in the text.

The enzyme *Pfu* DNA Polymerase was purchased from FERMENTAS (USA). The enzyme *Taq* DNA Polymerase was purchased from PROMEGA (USA). GeneJET PCR cloning Kit was purchased from FERMENTAS (USA).

III.1.4. Cell Media

YPG medium

Yeast Extract	- 10 g
Peptone	- 20 g

dissolved in 950 ml deionized and distilled water. pH was adjusted to 5.6 and the solution was sterilized in autoclave at 121°C for 15 minutes. 40% glucose solution was sterilized separately in autoclave at 121°C for 3 minutes and 50 ml of glucose solution was added to one liter final volume.

YPS medium

Yeast extract	- 10 g
Peptone	- 20 g
Starch	- 10 g

dissolved in 990 deionized and distilled water. pH was adjusted to 5.6. Then solution was sterilized in autoclave at 121°C for 15 minutes. After autoclaving, 40% glucose solution was sterilized separately in autoclave at 121°C for 3 minutes and 10 ml of glucose solution was added to one liter final volume.

YMM medium

Yeast Nitrogen Base (without amino acids)	- 6.7 g
Tryptone	- 0.1 g
Uracil	- 0.1 g
Agar – agar	- 18 g

dissolved in 950 deionized and distilled water. pH was adjusted to 5.6. Then solution was sterilized in autoclave at 121°C for 15 minutes. After autoclaving, 40% glucose solution was sterilized separately in autoclave at 121°C for 3 minutes and 50 ml of glucose solution was added to one liter final volume.

LB medium

Tryptone	- 5 g
Yeast Extract	- 2.5 g
NaCl	- 5 g

Agar – agar - 9 g

dissolved in 1L deionized and distilled water. After autoclaving at 121°C for 15 minutes, the solution cooled down to 50°C and then, 200 µg/ml of ampicillin was added into medium.

III.1.5. Buffers and Solutions

III.1.5.1. Buffers and Solutions Used in the Plasmid DNA Isolation

STE Buffer

Tris-HCl (pH 8.0) - 10 mM
NaCl - 100 mM
EDTA (pH 8.0) - 1 mM

Solution I

Tris-HCl (pH 8.0) - 25 mM
D-Glucose - 50 mM
EDTA (pH 8.0) - 10 mM

Solution II

NaOH - 0.2 N
SDS - 1%

Solution III

5 M Potassium Acetate - 60 ml
Glacial Acetic Acid - 11.5 ml
dH₂O - 28.5 ml

TE Buffer

Tris-HCl (pH 8.0) - 10 mM
EDTA (pH 8.0) - 1 mM

Phenol - 50 mM TE saturated phenol

Chloroform/Isoamylalcohol - 24:1 chloroform and isoamylalcohol

Ammonium Acetate - 3 M NH₄OAc

<u>RNase A</u>	- 10 mg/ml in dH ₂ O
<u>Ethanol</u>	- 100 %

Cracking 2X Buffer

5 M NaOH	- 2 ml
10 % SDS	- 2.5 ml
Sucrose	- 10 g
dH ₂ O	to 50 ml

III.1.5.2. Agarose Gel Electrophoresis Buffers

5XTBE (Tris-Borate) Buffer

Trizma Base	- 445 mM
Boric Acid (pH 8.3)	- 445 mM
EDTA	- 10 mM

10X Loading Buffer

Bromophenolblue	- 2.5 mg
SDS	- 1 % in one ml glycerol

III.1.5.3. Buffers and Solutions Used in DNA Isolation from Low-Melting Point Agarose

TE Buffer

Tris-HCl (pH 8.0)	- 10 mM
EDTA (pH 8.0)	- 1 mM

<u>Phenol</u>	- 50 mM TE saturated phenol
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<u>Chloroform/Isoamylalcohol</u>	- 24:1 chloroform and isoamylalcohol
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<u>Ammonium Acetate</u>	- 3 M NH ₄ OAc
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<u>Ethanol</u>	- 100 %
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III.1.6. Laboratory Equipment

Autoclave	Systec 3870 ELV Steam Strilizer (GERMANY)
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Balance	Mettler Toledo AB204-S (USA)
Centrifuges	Sigma 3K30 (GERMANY)
Ultraviolet viewing Cabinets UV lamps	UVIcab UVIlite UVItec (Cambridge, ENGLAND)
Spectrophotometer	Perkin Elmer Lambda 35 UV/VIS (USA)
Electrophoration Equipment	Scie-Plas (ENGLAND)
Horizontal Laminar Airflow Clean Bench	Thermo Scientific Holten (USA)
Thermo-cyclers	Applied Biosystems GeneAmp PCR Systems 2700 (USA)
Orbital Shaker	New Brunswick Scientific Excelsa E24 Incubator Shaker (USA)
pH meter	Mettler Toledo MP-220 (USA)
Vortex	Heidolph REAX top

(GERMANY)

Water Purification Systems

ELGA LabWater

(ENGLAND)

Refrigerators

ARÇELİK

(TURKEY)

Deepfreezers

Liebherr Fridge Freezer

(London, ENGLAND)

III.2. METHODS

III.2.1. Sterilization

In this study, culture media, eppendorf tubes, centrifuge tubes, solutions and tips were all sterilized at 1.02 atm and 121°C for 15 minutes in an autoclave. Glucose solution was sterilized at 1.02 atm and 121°C for 3 minutes in an autoclave.

III.2.2. Preparation of Preculture

The strains of *E.coli* were kept in glycerol solution at -80°C. Frozen glycerol cultures were used by streaking on LB agar plates for the preparation of master plates.

Preparation of plates, inoculation and all other experimental work that require sterile environment were carried out under sterile laminar flow.

In every inoculation step, inoculation loop was sterilized by heating in a flame and cooled down on the agar plate to avoid death of the cells that were about to be transferred. The preculture was incubated in an orbital shaker at 37°C overnight.

III.2.3. Growth Conditions

E.coli strains, were grown in New Brunswick Orbital Shaker at 37°C and an agitation rate of 180 rpm.

III.2.4. Cloning of the 25S rDNA Gene into pJET1/blunt Vector

Using the Gene Jet PCR Cloning Kit, the recombinant plasmid pJRD was constructed by ligation the PCR amplified 25S rDNA gene region into the pJET1/blunt cloning vector.

S. cerevisiae genomic DNA was used as template for the PCR amplification of the 25S rDNA gene using specifically designed primers rDNAfor and rDNArev. PCR reaction was carried out in a reaction of 25 µl total volume containing 12.5 pmole of each primer, 2.5 µl of *Pfu* DNA Polymerase 10X Buffer with preadded MgSO₄, 0.2 mM dNTP, 1.25 Units of *Pfu* DNA Polymerase and 1 µg of *S. cerevisiae* genomic DNA. Amplification was performed in the GeneAmp thermal cycler, with an initial denaturation step at 94°C for five minutes followed by 25 cycles of alternating incubations at 94°C for thirty seconds, at 60°C for thirty seconds, at 72°C for two minutes, ending with ten minutes of incubation at 72°C. PCR products were checked by agarose gel electrophoresis using 1.2% gels. The reactions containing the expected band were combined and purified by Promega PCR Purification Kit

III.2.4.1. Ligation and Transformation

For the ligation of the DNA insert (pure 25S rDNA PCR product) into the pJET1/blunt Cloning Vector, 50 ng pJET1/blunt plasmid, 10 µl of 2X Reaction Buffer, 300 ng pure PCR product, 5 Units of T4 DNA ligase and distilled water were mixed in a total volume of 20 µl. The ligation mixture was incubated at 22°C for three hours. After the reaction, two µl of samples were loaded on a 0.8 % agarose gel to check the efficiency of the ligation reaction before transformation to *E. coli*.

The ligation mixture was transformed into competent *E. coli* TOP10 cells supplied with the Gene JET PCR Cloning Kit following the instructions of the manufacturer. Briefly, 10 µl of ligation mixture was mixed with 50 µl of *E. coli* TOP10 cells on ice, the mixture was mixed gently and then incubated at 42°C for 2 minutes. After incubation, the cells were spread on pre-warmed selective LB agar plates containing ampicillin. The plates were incubated at 37°C overnight.

III.2.4.2. Recombinant Screening

Due to the very high cloning efficiency of the Gene JET PCR Cloning System, *E. coli* colonies formed on the LB Amp⁺ plates were screened for the presence and direction of the insert by the “colony PCR” method. In this method, a small amount

of colony is dissolved in 50µl dH₂O and incubated at 98°C for five minutes to lyse the cells and release the genetic material. After a brief centrifugation at 12,000 g to remove the insoluble cellular material, the clear supernatant is directly used as template for the PCR.

Two sets of PCR were carried out by using rDNAfor and rDNArev in the former, pJET1frw and rDNArev primers in the latter set, respectively.. All PCR reactions were performed in a reaction of 25 µl total volume containing 12.5 pmole of each primers, 2.5 µl of DNA polymerase 10X Buffer, 0.2 mM dNTP, 1.25 Units of DNA *Taq* polymerase and 5 µl of colony extract. Amplification was performed in the GeneAmp thermal cycler, with an initial denaturation step at 94°C for five minutes followed by 25 cycles of alternating incubations at 94°C for thirty seconds, at 60°C for thirty seconds, at 72°C for two minutes, ending with ten minutes of incubation at 72°C. PCR products were analyzed by agarose gel electrophoresis method.

III.2.4.3. Isolation of Plasmid DNA

Plasmid DNA was isolated from overnight cultures of *E.coli* picked from single colonies by the mini-prep method (Toksoy Öner, 2000). Ten ml LB medium containing 100µg/ml ampicillin with single colony was incubated at 37°C and 200 rpm. 1.5 ml of the overnight culture was transferred into centrifuge tube and centrifuged at 12,000 g for one minute. The medium was removed and cell pellet was resuspended in 100 µl of ice-cold Solution I. The mixture was incubated at room temperature for five minutes. 200 µl of fresh Solution II was added and mixed by inversion. This mixture was incubated for five minutes on ice. 150 µl Solution III was added and vortexed gently on ice and incubated on ice for five minutes.

The mixture was centrifuged at 12,000 g for five minutes and supernatant was transferred to a tube, avoiding the white precipitate. RNase A was added to the supernatant to a final concentration 20 µg/ml and incubated at 37°C for 20 minutes.

The mixture was extracted with TE buffer saturated with phenol:chloroform (1:1) and once with chloroform:isoamylalcohol (24:1) by vortexing for one minute and centrifuging at 12,000 g for two minutes at each step.

Plasmid DNA was recovered from the aqueous phase by addition of 0.3 M ammonium acetate and 2.5 volumes of absolute ethanol. This mixture was incubated

at -70°C, the plasmid DNA was precipitated by centrifugation at 14000 *g* for 20 minutes. The pellet was washed with 70 % ethanol, dried and dissolved in 30 µl sterile TE buffer.

III.2.5. Cloning of the amyolytic genes into pJRD

For the linearization of the vector with *HpaI* enzyme, 10 µl of the purified plasmid, 2 µl 10X Reaction Buffer for *HpaI*, 10 Units of *HpaI* restriction enzyme and distilled water were mixed at a to the total volume of 100 µl. The digestion was carried at 37°C overnight. Reaction was checked on agarose gels for completion and the linear vector was purified by extraction with phenol:chloroform followed by ethanol precipitation.

The fusion gene and the LEU2 selection region was amplified from the pPB-G plasmid using YEPfor and LEUrev primers. PCR reaction was carried out in a total volume of 25 µl containing 12.5 pmole of each primers, 2.5 µl of *Pfu* DNA polymerase 10X Buffer without MgSO₄, 10 mM dNTP, 3 mM MgSO₄, 1.25 Units of *Pfu* DNA polymerase and 1 µg pPB-G plasmid as template DNA. Amplification was performed in the GeneAmp Thermal Cycler, with an initial denaturation step at 95°C for three minutes followed by 25 cycles of alternating incubations at 94°C for one minute, at 50°C for one minute, at 72°C for ten minutes, ending with ten minutes of incubation at 72°C.

Due to the presence of unspecific bands, the PCR amplification products were purified by using low melting point agarose gel electrophoresis. PCR products were collected together, mixed with 1X loading Buffer and loaded onto one per cent low melting point agarose gel which was prepared in 1X TBE Buffer. After electrophoresis, the DNA fragment of interest was cut out of the gel and was kept at 65°C for 15 minutes. When the gel melted, the equal volume of TE buffer was added and the solution was kept at 65°C for an additional 20 minutes until it melted completely.

Then the mixture was extracted twice with phenol and once with chloroform. The desired DNA fragment was obtained from aqueous phase by the addition of 0.1 volume of 3 M ammonium acetate and two volumes of ice-cold ethanol and centrifuged at 15,000 rpm for 30 minutes. The pellet was dried and dissolved in sterile TE buffer.

III.2.5.1. Ligation and Transformation

For the ligation of the DNA insert (agarose gel purified PCR product band containing the fusion gene and the LEU2 selection region) into the linearized pJRD vector, 4 µl pJRD plasmid, 2 µl of 10X Reaction Buffer, 12 µl pure PCR product, 6 Units of T4 DNA ligase and distilled water were mixed in a total volume of 20 µl. The ligation mixture was incubated at 16°C overnight. After the reaction, two µl of samples were loaded on a 0.8 % agarose gel to check the efficiency of the ligation reaction before transformation to *E.coli*.

The ligation mixture was transformed into competent *E.coli* TOP10 cells following the same protocol as explained in III.2.4.1.

III.2.5.2. Screening of Recombinant Colonies by Cracking Procedure

In the cracking procedure, colonies lyzed in alkaline solution are directly loaded to agarose gels to rapidly compare their plasmid size. For this, firstly, individual colonies from LB agar plates were picked using sterile tips and dissolved in 25 µl 10 mM EDTA. Then, 25 µl 2X cracking buffer which was prepared freshly and fixed to pH 8 was added and the samples were incubated at 70°C for about ten minutes followed by and cooling down to room temperature. 1.5 µl 4 M KCl solution and 0.4 % bromophenolblue were added to samples. Then the samples were vortexed and incubated at ten minutes on ice. They were centrifuged at 12,000 *g* for two minutes and 10-20 µl of the supernatant was loaded on an agarose gel.

III.2.5.3 Screening of Recombinant Colonies by Colony PCR

The rDNA gene region with the genes encoding amylolytic enzymes together with the LEU2 selective marker from colonies was amplified by Colony PCR. PCR reaction was carried out in a total volume of 25 µl containing 12.5 pmole of each primers, 2.5 µl of *Taq* DNA polymerase 10X Buffer with (NH₄)₂SO₄, 10 mM dNTP, 1.25 Units of *Taq* DNA polymerase, 5 µg total colony DNA, and 2 µl of 25 mM MgCl₂ Solution were mixed. Amplification was performed in the GeneAmp thermal cycler, with an initial denaturation step at 94°C for five minutes followed by 25 cycles of alternating incubations at 94°C for thirty seconds, at 60°C for thirty

seconds, at 72°C for three and six minutes, ending with ten minutes of incubation at 72°C.

III.2.6. PCR Amplification of DNA Cassette

The whole gene region in the pJBG plasmid containing the rDNA and the genes encoding amylolytic enzymes together with the LEU2 selective marker was amplified by PCR with the rDNAfor and rDNArev primers. PCR reaction was carried out in a total volume of 25 µl containing 12.5 pmole of each primers, 2.5 µl of *Pfu* DNA polymerase 10X Buffer with MgSO₄, 10 mM dNTP, 0.6 Units of *Pfu* DNA polymerase and 2 µg DNA were mixed. Amplification was performed in the GeneAmp thermal cycler, with an initial denaturation step at 94°C for five minutes followed by 30 cycles of alternating incubations at 94°C for thirty seconds, at 60°C for thirty seconds, at 72°C for six minutes, ending with ten minutes of incubation at 72°C.

CHAPTER IV

RESULTS and DISCUSSIONS

The energy intensive high temperature cooking process followed by enzymatic hydrolysis of starch to fermentable sugars in the current bioethanol production process necessitates novel and innovative technologies in order to reduce the total manufacturing cost.

This thesis is part of an ongoing project aiming to develop a novel noncooking fermentation system utilizing amylolytic microorganisms that are capable of hydrolyzing starch into fermentable sugars by enabling depolymerization of starch to glucose in a simultaneous saccharification and fermentation (SSF) process for bioethanol production. Due to the high potential of respiratory-deficient nuclear petite *S. cerevisiae* strains for their use in commercial bioethanol production, a 100% respiratory-deficient nuclear petite amylolytic *S. cerevisiae* strain was constructed that was able to excrete a bifunctional fusion protein that contains both the *B. subtilis* α -amylase and the *A. awamori* glucoamylase activities (Toksoy Öner et al., 2005). Despite the significantly improved ethanol yields from soluble starch, the fermentation performance of this strain pointed out the fact that in order to use these strains on industrial scale, both the copy number and stability of the genes encoding the amylolytic enzymes had to be improved (Toksoy Öner, 2006). In the light of this, a cloning strategy has been developed for the high-copy and stable integration of the fusion protein gene into the ribosomal DNA (rDNA) locus of *S. cerevisiae* that consists of 100-200 tandem copies of a 9.1 kb repeat on the right arm of chromosome XII. The strategy involves three major steps. The first step involves the construction of the DNA cassette containing the enzyme genes, a yeast selection marker and homologous regions for its directed integration into the rDNA locus of *S. cerevisiae* genome. In the latter step, the DNA cassette is transformed into yeast cells and among the integrands, the one with highest amylolytic activity is selected. The final

step involves the determination of the fermentation performances of the integrands, comparison of their ethanol productivities with earlier results and development of fermentation strategies to improve the ethanol yields.

IV.1. CLONING STRATEGY OF THE STUDY

The ultimate objective of this study was the construction of the DNA cassette using Recombinant DNA Technology. For this, 2.7 kb long fragment containing the gene for 25S rRNA has been amplified by PCR and cloned using Gene JET PCR Cloning System to construct pJRD. In order to construct the pJBG plasmid, the DNA fragment containing the genes encoding for the amylolytic enzymes together with the LEU2 selective marker region was amplified by PCR and cloned into the unique *HpaI* site within the rDNA region of the pJRD plasmid. Then, the final DNA cassette was obtained by selective PCR amplification of the fusion gene and marker region flanked by the rDNA sequences from the pJBG plasmid. Figure IV.1 shows the schematic diagram of the strategy used for the construction of the DNA cassette.

Figure IV.1 Schematic diagram of the cloning strategy

IV.2. CLONING OF 25S rDNA GENE

The ribosomal DNA (rDNA) of *S. cerevisiae* is encoded by the *RDNI* locus, an approximately 1-2 Mb region consisting of 100-200 tandem copies of a 9.1 kb repeat, on the right arm of chromosome XII (Venema J and Tollervy, 1999). As also shown in Figure IV.2, each repeat contains the genes for 5S, 5.8S, 25S, and 18S rRNAs (RDN5, RDN58, RDN25, and RDN18), as well as three types of spacer regions: internal transcribed spacers (ITS1, ITS2), external transcribed spacers (5' *ETS*, 3' *ETS*) and nontranscribed spacers (*NTS1*, *NTS2*). Each ribosomal RNA is present in a single copy in a yeast ribosome: 18S rRNA is a component of the 40S ribosomal subunit, and the 25S, 5.8S, and 5S rRNAs are components of the 60S subunit (Venema and Tollervy, 1999).

Using the DNA sequence of the 9137 bp long rDNA repeat with GenBank accession number of U53879 (21811–30947 bp on the right arm of chromosome XII), a pair of primers were designed that comprise part of the 25S rDNA region. For the design, Primer3 program (Primer3Program Design, 2008) is used which is a software tool to design PCR and sequencing primers as well as hybridization probes.

Figure IV.2 shows the schematic representation of 25S rDNA region on *S.cerevisiae* yeast genomic DNA.

Figure IV.2 Schematic representation of 9.1 kb rDNA repeat of *S.cerevisiae*

To ensure the specificity of the primers and to find the exact length of the PCR product sequence, *in-silico* PCR tool (*In-silico* Simulation, 2008) was used and the designed rDNAfor and rDNArev primers produced a 2774 bp long PCR product sequence *in-silico* using the *S. cerevisiae* genomic DNA as template (Figure IV.3).

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5' GGTAACACGC TTGAGCGCCA TCCATTTTCA GGGCTAGTTC ATTCGGCCGG
TGAGTTGTTA CACACTCCTT AGCGGATTCC GACTTCCATG GCCACCGTCC GGCTGTCTAG
ATGAACTAAC ACCTTTTGTG GTGTCTGATG AGCGTGTATT CCGGCACCTT AACTCTACGT
TCGGTTCATC CCGCATCGCC AGTTCTGCTT ACCAAAAATG GCCCACTAAA AGCTCTTCAT
TCAAATGTCC ACGTTCAATT AAGTAACAAG GACTTCTTAC ATATTTAAAG TTTGAGAATA
GGTCAAGGTC ATTTGACCC CGGAACCTCT AATCATTCGC TTTACCTCAT AAAACTGATA
CGAGCTTCTG CTATCCTGAG GGAACTTCG GCAGGAACCA GCTACTAGAT GGTTTCGATTA
GTCTTTCGCC CCTATACCCA AATTCGACGA TCGATTTGCA CGTCAGAACC GCTACGAGCC
TCCACCAGAG TTTCTCTGG CTTCACCCTA TTCAGGCATA GTTCACCATC TTTCCGGTCC
CAACAGCTAT GCTCTTACTC AAATCCATCC GAAGACATCA GGATCGGTCG ATTGTGCACC
TCTTGCGAGG CCCCACCTA CGTTCACTTT CATTACGCGT ATGGGTTTTA CACCCAAACA
CTCGCATAGA CGTTAGACTC CTTGGTCCGT GTTCAAGAC GGGCGGCATA TAACCATTAT
GCCAGCATCC TTGACTTACG TCGCAGTCTT CAGTCCCAGC TGGCAGTATT CCCACAGGCT
ATAATACCTA CCGAGGCAAG CTACATTCCT ATGGATTAT CTGCGCACA CTTGCAAGG
TGGCCAGTG AAATGCGAGA TTCCCTACC CACAAGGAGC AGAGGGCACA AAACACCATG
TCTGATCAAA TGCCCTTCCC TTCAACAAT TTCACGTACT TTTTCACTCT CTTTTCAAAG
TTCTTTTCAT CTTTCCATCA CTGTACTTGT TCGCTATCGG TCTCTCGCCA ATATTTAGCT
TTAGATGGAA TTTACCAACC ACTTAGAGCT GCATTCCCAA ACAACTCGAC TCTTGAAGG
CACTTTACAA AGAACCGCAC TCCTCGCCAC ACGGGATTCT CACCCTCTAT GACGTCCTGT
TCCAAGGAAC ATAGACAAGG AACGGCCCCA AAGTTGCCCT CTCCAAATTA CAACTCGGGC
ACCGAAGGTA CCAGATTTCA AATTTGAGCT TTTGCCGCTT CACTCGCGCT TACTAAGGCA
ATCCCGGTTG GTTCTTTTC CTCCGCTTAT TGATATGCTT AAGTTTCAGC GGTACTCTTA
CCTGATTTGA GGTCAAACCT TAAGAACATT GTTCGCCTAG ACGCTCTCTT CTTATCGATA
ACGTTCGAAT ACGCTCAGTA TAAAAAAGA TTAGCCGAG TTGGTAAAC CTAACACGAC
CGTACTTGCA TTATACCTCA AGCACGCAGA GAAACCTCTC TTTGGAAAAA AAACATCCAA
TGAAAAGGCC AGCAATTTCA AGTTAACTCC AAAGAGTATC ACTCACTACC AAACAGAATG
TTTGAGAAGG AAATGACGCT CAAACAGGCA TGCCCCCTGG AATACCAAGG GGCAGCAATGT
GCGTTCAAAG ATTCGATGAT TCACGGAATT CTGCAATTCA CATTACGTAT CGCATTTCGC
TGCGTCTCTC ATCGATGCGA GAACCAAGAG ATCCGTTGTT GAAAGTTTTT AATATTTTAA
AATTTCCAGT TACGAAAATT CTTGTTTTTG ACAAAAATTT AATGAATAGA TAAAATGTGT
TGTGTTTGT ACCTCTGGGC CCGGATTGCT CGAATGCCCA AAGAAAAAGT TGCAAAGATA
TGAAACTCC ACAGTGTGTT GTATTGAAAC GGTTTTAATT GTCCTATAAC AAAAGCACAG
AAATCTCTCA CCGTTTGGA TAGCAAGAAA GAAACTTACA AGCCTAGCAA GACCGCGCAC
TTAAGCGCAG GCCCGGCTGG ACTCTCCATC TCTTGTCTTC TTGCCAGTA AAAGCTCTCA
TGCTCTTGCC AAAACAAAAA AATCCATTTT CAAAATTATT AAATTTCTTT AATGATCCTT
CCGACGGTTC ACCTACGGAA ACCTTGTTAC GACTTTTAGT TCCTCTAAAT GACCAAGTTT
GTCCAAATTC TCCGCTCTGA GATGGAGTTG CCCCCTCTC TAAGCAGATC CTGAGGCCCTC
ACTAAGCCAT TCAATCGGTA CTAGCGACGG GCGGTGTGTA CAAAGGGCAG GGACGTAATC
AACGCAAGCT GATGACTTGC GCTTACTAGG AATTCCTCGT TGAAGAGCAA TAATTACAAT
GCTCTATCCC CAGCACGACG GAGTTTCACA AGATTACCAA GACCTCTCGG CCAAGGTTAG
ACTCGCTGGC TCCGTCAATG TAGCGCGCGT GCGGCCAGA ACGTCTAAGG GCATCACAGA
CCTGTTATTG CCTCAAACCT CCATCGGCTT GAAACCGATA GTCCCTCTAA GAAGTGGATA
ACCAGCAAAT GCTAGCACCA CTATTTAGTA GGTAAAGGTC TCGTTTCGTTA TCGCAATTAA
GCAGACAAAT CACTCCACCA ACTAAGAACG GCCATGCACC ACCACCCACA AAATCAAGAA
AGAGCTCTCA ATCTGTCAAT CCTATTGTG TCTGGACCTG GTGAGTTTCC CCGTGTGAG
TCAAATTAAG CCGCAGGCTC CACTCCTGGT GGTGCCCTTC CGTCAATTCC TTTAAGTTTC
AGCCTTGCGA CCATACTCCC CCCA 3'

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Figure IV.3 2774 bp long 5'-3' PCR product sequence of the 25S rDNA region. The forward (rDNAfor) and reverse (rDNArev) primers are indicated at both ends.

Considering the melting temperatures of the primers, a PCR program has been developed and used for the amplification of the 25S rDNA gene using *S. cerevisiae* genomic DNA as template. Figure IV.4 shows the result of agarose gel electrophoretic analysis of 25S rDNA PCR fragments.

Figure IV.4 Electrophoretic analysis of 25S rDNA PCR products.

Electrophoretic analysis showed that the selected region of the 25S rDNA gene was successfully amplified. All the reactions were found to contain a single DNA band at the correct position of 2774 bp with respect to the DNA marker. The reactions containing the expected band were combined and purified from the primer-dimers, amplification primers, dNTPs, DNA polymerase enzyme, the remaining unspecific PCR products and contaminants by Promega PCR Purification Kit. Details of the PCR amplification are explained in III.2.4. of the Methods section.

After ligation of the DNA insert (pure 25S rDNA PCR product) with the pJET1/blunt Cloning Vector at 22°C for three hours and the ligation mixture was transformed into competent *E.coli* TOP10 cells. After overnight incubation on selective LB Amp⁺ plates at 37°C, a total of 40 colonies were found to be formed on the agar. The details of the ligation and transformation were described in Methods III.2.4.1.

IV.2.1. Recombinant Screening

The pJET1/blunt is a positive selection vector. Therefore, only recombinant plasmids containing the insert DNA are propagated in the *E.coli* cells. Recircularized vector molecules lacking the insert express a lethal restriction endonuclease after

transformation and are not propagated. Hence the cloning efficiency of the Gene JET PCR Cloning System is almost 100% which drastically accelerates the process of recombinant screening.

Six of the 40 colonies that were formed on LB Amp⁺ plates were selected and the presence and direction of the insert 25S rDNA gene within the pJET1/blunt vector propagated in these colonies were determined by “colony PCR” method as described in Methods III.2.5.3. First, insert specific rDNAfor and rDNArev primers were used in the PCR to ensure the presence of the insert in these colonies. Figure IV.5 shows the electrophoretic analysis of colony PCR products of the selected 6 colonies. Among the six colonies, only colonies with numbers 3 and 5 gave the expected PCR product of 2774 bp length which in turn indicated the insertion of 25S rDNA fragment into the vector was successful in those two colonies.

Figure IV.5 Electrophoretic analysis of Colony PCR.

Then, in order to select for the direction of the insert, vector specific pJET1frw and insert specific rDNArev primers were used in colony PCR performed under the same reaction and program conditions as mentioned above. When the PCR products were analyzed on agarose gels, among the two colonies, only colony 5 was found to give the PCR product of 2832 base pair (2774 bp from the insert and an additional 58 bp from the vector which are the 21-mer pJET1frw primer and the 37 bp long sequences between the forward primer and the start of the insert) which in turn

indicated the insertion of 25S rDNA fragment into the vector was successful and in correct direction in this *E.coli* colony.

Selective liquid LB medium was inoculated with colony 5 and incubated at 37°C overnight. The recombinant plasmid in the cells was isolated as described in Method III.2.4.3. and named as pJRD.

IV.3. CLONING OF AMYLOLYTIC GENES INTO 25S rDNA

For the cloning of the fusion gene into the rDNA region, the pJRD plasmid had to be linearized by an appropriate restriction endonuclease. Since the insert to be cloned was a PCR product, an enzyme that creates a blunt end was preferred. In order to choose the enzyme, NEBcutter V2.0 (NEBcutter V2.0 Program, 2008) software was used which gives the restriction map of the entered DNA sequence for all commercially available restriction endonucleases. After analysis, blunt end *HpaI* enzyme with a unique recognition sequence of (GTT|AAC) at approximately in the middle of the inserted 25 rDNA gene (at position 1514 from the start of the 2774 bp long gene) was chosen for further studies. Linearization of the plasmid with *HpaI* enzyme was confirmed via Agarose gel electrophoresis analysis and as shown in Figure IV.6, digestion products gave a single band at the position of 5902 bp corresponding to the size of the pJRD plasmid (3128 bp from the pJET1 and 2774 bp from the 25 rDNA insert).

Figure IV.6 Electrophoretic analysis of *HpaI* digested pJRD

In order to construct the pJBG plasmid, the DNA fragment containing the genes encoding for the amylolytic enzymes together with the LEU2 selective marker region was amplified by PCR and cloned into the unique *HpaI* site within the rDNA region of the pJRD plasmid. As template, pPB-G plasmid that contains the *B. subtilis* α -amylase and the *A. awamori* glucoamylase genes expressed under the control of the constitutive PGK1 promoter was used. pPB-G plasmid is derived from YEp351 yeast episomal vector and is propagated by the yeast cells as multi copy plasmids. For the selection purposes, it also contains the LEU2 gene that codes for a 384 amino acid long Beta-isopropylmalate dehydrogenase (IMDH) enzyme that catalyzes the third step in the leucine biosynthesis pathway.

As the forward primer for the PCR amplification, YEPfor primer that is specific for the sequences upstream of the PGK promoter region on the pPB-G plasmid was used. On the other hand, LEUrev was used as reverse primer that is specific for the sequences downstream of the LEU2 gene. Hence the PCR product

After the ligation reaction, the mixture was transformed into competent *E.coli* TOP10 cells as explained in Method III.2.5.1. The transformed cells were spread on to LB agar plates containing ampicillin. The plates were kept at room temperature for about ten minutes to allow the cells to diffuse completely and were then transferred to 37°C oven for overnight incubation.

The *E.coli* colonies formed on LB Amp⁺ plates were picked and screened by Cracking Method for their approximate plasmid size as described in Method III.2.5.2. Approximately 200 *E.coli* colonies were screened and 10 colonies with plasmid size greater than the linear pJRD plasmid were selected for further study. These selected colonies were subjected to a second screening by the Colony PCR using primers rDNAfor and rDNArev. A colony with pJRD plasmid was used as control. These ten colonies conferring antibiotic resistance were expected to be of two distinct types, namely, colonies containing recirculized plasmid without insert and colonies containing the recombinant plasmid with the insert. Those without insert would contain the pJRD plasmid with 2774 bp rDNA region. Whereas the rDNA region of the recombinant plasmid would be about 7.8 kb long containing both the 2774 bp rDNA and 5000 bp insert regions. So, with the rDNAfor and rDNArev primers, a band would be expected for all the selected colonies but at different lengths, i.e. shorter for false positives and longer for the recombinants with insert. In addition, when *Taq* DNA Polymerase enzyme with average elongation speed of 1 kb per minute is used in PCR, it is a clear fact that PCR amplification of the recombinant would require longer elongation times rather than the three minutes used in earlier studies. These basic differences of the colonies were used to detect the false positives. As given in more detail in III.2.5.3., for the PCR, three minutes of elongation time was used which would be optimum for the amplification of the 25S rDNA region without insert only. The following Figure IV.8 shows the gel electrophoresis analysis result of the ten colonies and the control.

Figure IV.8 Gel electrophoresis analysis result of recombinant screening of the ten colonies by colony PCR

Of the ten colonies, 1, 4, 5, 8 and 9 gave a band at the same length as the control indicating the absence of the insert in these colonies. So, colonies 2,3,6,7 and 10 with no detectable amplification product were selected for further screening. To verify the presence of the insert in these colonies, colony PCR was done with the same primers, rDNAfor and rDNArev, but with six minutes of elongation time. Colony 1 from the previous screening was used as control and the gel electrophoresis analysis results are shown in Figure IV.9.

Figure IV.9 Gel electrophoresis analysis result of recombinant screening with longer elongation time

Colony 2 gave a short band at the same length of the control which indicated that it did not contain the insert. On the other hand, colonies 3, 6, 7 and 10 produced longer PCR products. Plasmid DNA of these colonies was purified for further analysis.

Since the exact DNA sequence of the fusion gene and PGK promoter was not available, an approximate restriction map of the expected recombinant was constructed and used for the restriction analysis of the four recombinant plasmids. As a result, colony 6 was found to produce the expected DNA bands with different restriction enzymes and therefore chosen for further studies and named as pJBG.

IV.4. THE DNA CASSETTE

In order to obtain the DNA cassette, pJBG plasmid was used as template. For the PCR amplification, *Pfu* DNA Polymerase with considerably lower error rate when compared with *Taq* DNA Polymerase was used to ensure correct amplification of the whole region containing the rDNA and the genes encoding amylolytic enzymes together with the LEU2 selective marker. rDNAfor and rDNArev primers were used as primers and PCR conditions were as given in Methods III.2.6. Figure IV.10 shows the gel electrophoresis result of the DNA cassette. The band at the correct position indicated the successful construction of the DNA cassette in its final form, ready for transformation to yeast cells.

Figure IV.10 The gel electrophoresis result of the DNA cassette

CHAPTER V

CONCLUDING REMARKS AND RECOMMENDATIONS

V.1. CONCLUDING REMARKS

The increasing demand in energy due to rapid outgrowth of population and urbanization, there is need for environmentally sustainable energy sources. Bioethanol is considered as one of the most promising replacements of gasoline. Bioethanol from starch and grain crops is used extensively in Brazil, Canada, USA, and to a lesser extent in Sweden. Directive 2003/30/EC which promotes the use of biofuels as substitutes for petrol and diesel in the transport sector aimed at reducing emissions of carbon dioxide from transport and reducing the EU's reliance on external energy sources and fossil fuels. The adoption of the directive together with the ever increasing need to guarantee security of fuel supply has encouraged the production of bioethanol, thus it is becoming an emerging sector. Applied as a direct additive to conventional fuels or for synthesis of ethyl tertiary butyl ether (a gasoline additive), ethanol from grains like wheat, barley and rye is used on large scale in Europe (Ryan et al., 2006; IEA Bioenergy, 2006).

Bioconversion of starch into ethanol is a two-step process. The first step is saccharification, where starch is converted into sugar using an amylolytic microorganism or enzymes such as glucoamylase and α -amylase. The second step is fermentation, where sugar is converted into ethanol using an ethanol-fermenting microorganism such as yeast (Inlow et al., 1988; Nakamura et al., 1997). Saccharification step requires the anhydrous starch granules be extensively pretreated at high temperature (105-150°C) to hydrate the starch. This is an energy intensive process requiring the addition of heat energy to starch granule slurries until the gelatinization temperature (temperature at which starch molecules swell and begin to solubilize) of the starch is exceeded. Therefore, special equipment such as heat exchangers, steam jet cookers and holding tanks are used in the process. Moreover, the technical requirements and limitations of this required equipment and process,

especially limitations on solids loading due to the high viscosity of starch, limit the amount of ethanol that can be produced at a plant (Genencor, 2005).

In 1980s, ethanol production from starch particles at temperatures below the gelatinization temperature had rarely been investigated (Lim et al., 2003). Recently, in order to eliminate the need for high-energy processing of starch and provide more cost-effective glucose for conversion to ethanol, most research is focused on the development of genetically engineered amyolytic yeast strains by expressing heterologous genes encoding α -amylase and glucoamylase from various organisms (Knox et al., 2004). Of all the yeast species, *S. cerevisiae* is being preferred in bioethanol processes not only because of its well-defined genetics, physiology and accepted GRAS status (generally regarded as safe) but also due to its high fermentation capacity, high ethanol yield and relatively high ethanol tolerance (Ma et al., 2000). Shigechi et al. (2002) improved the ethanol productivity of a flocculent *S. cerevisiae* strain displaying *Rhizopus oryzae* glucoamylase enzyme on the cell surface by co-displaying or secreting the *Bacillus stearotheophilus* α -amylase enzyme. *Rhizopus oryzae* glucoamylase and *Streptococcus bovis* α -amylase Recently, the same workers have reported the utilization of a previously constructed yeast strain co-displaying both amyolytic enzymes on the cell surface for direct ethanol production from corn starch (Shigechi et al., 2004a). But since their strain could not ferment raw corn starch to ethanol, they have constructed a new strain capable of fermenting raw corn starch by expressing α -amylase from the lactic acid bacterium *Streptococcus bovis* 148, instead of the *B. stearotheophilus* α -amylase, together with *R. oryzae* glucoamylase (Shigechi et al., 2004b). Eksteen et al. (2003) reported the construction of eight different amyolytic *S. cerevisiae* strains excreting *Lipomyces kononenkoae* α -amylase and *Saccharomycopsis fibuligera* glucoamylase enzymes. However, starch fermentation characteristics under controlled bioreactor conditions of three of these constructs revealed inefficient excretion of these amyolytic enzymes to the fermentation medium and no ethanol productivity under aerobic cultivation conditions (Knox et al., 2004). The fermentation and ethanol production characteristics of three different amyolytic *S. cerevisiae* strains constructed by de Moraes et al. (1995) under various conditions have been reported (Birol et al., 1998; Altintas et al., 2002; Ulgen et al., 2002). Development of an amyolytic respiratory-deficient nuclear petite *S. cerevisiae* strain with very high

ethanol productivities from starch have been reported. However, the fermentation performance of this strain pointed out the fact that in order to use these strains on industrial scale, both the copy number and stability of the genes encoding the amylolytic enzymes had to be improved (Toksoy Öner et al., 2005, Toksoy Öner, 2006). Therefore, in order to integrate the amylolytic genes to the *S. cerevisiae* genome for their stable maintenance, rDNA locus was chosen since it consists of 100-200 copies of a 9.1 kb repeat enabling the stable and multi-copy integration of the gene of interest to the genome. A three-step strategy has been developed and this thesis mainly focussed on the first step which involved the construction of the DNA cassette containing the enzyme genes, a yeast selection marker and homologous regions for its directed integration into the genome using Recombinant DNA Technology.

Using the DNA sequence of the 9137 bp long rDNA repeat, a pair of primers (rDNAfor and rDNArev) were designed that comprise part of the 25S rDNA region. Electrophoretic analysis of the PCR products showed that the selected 2774 bp long region of the 25S rDNA gene was successfully amplified. After ligation of the pure 25S rDNA PCR product with the pJET1/blunt Cloning Vector and transformation into *E.coli* TOP10 cells, colonies were screened for the correct plasmid construct by colony PCR method. The presence of the insert 25S rDNA gene was analyzed by using insert specific rDNAfor and rDNArev primers and the direction of the insert was analyzed by using vector specific pJET1frw and insert specific rDNArev primers. Plasmid of the colony 5 with correct orientation of the insert was named as pJRD. Then, the DNA fragment containing the genes encoding for the amylolytic enzymes together with the LEU2 selective marker region was amplified by PCR and cloned into the unique *HpaI* site within the rDNA region of the pJRD plasmid. YEPfor and LEUrev primers that flank the region containing the PGK promoter, amylolytic genes and LEU2 selection marker were used for the PCR amplification using pPB-G plasmid as template. Due to the presence of persistent unspecific bands, PCR products were collected together and the band of interest was purified by using low melting point agarose gel electrophoresis. The ligation mixture containing *HpaI* linearized pJRD vector and pure PCR product was transformed into competent *E.coli* TOP10 cells. About 200 *E.coli* colonies formed on LB Amp⁺ plates were screened by Cracking Method and 10 of them with considerably higher plasmid size were

subjected to a second screening by the Colony PCR using primers rDNAfor and rDNArev. Using the length differences of the correct constructs and false positives, two different elongation times were used to differentiate them. After an additional restriction analysis, plasmid of colony 6 was found to be the correct recombinant plasmid and it was named as pJBG.

Finally, this pJBG plasmid was used as template in order to obtain the DNA cassette by PCR. Gel electrophoresis analysis of the PCR product showed the successful construction of the DNA cassette in its final form, ready for transformation to yeast cells.

V.2. RECOMMENDATIONS

This study constitutes the first step that provides the basis for constructing industrial yeast strains capable of converting starch into bioethanol in a one-step process. In the light of the results obtained in this work, the following recommendations can be made for future studies:

The exact DNA sequence of the DNA cassette developed in this study should be analyzed to detect any misincorporations during the PCR that may effect the expression of the corresponding genes.

The DNA cassette should be transformed into yeast cells and the integrands should be screened for their starch utilization performances. The expression and extracellular secretion of the fusion protein should be verified by purification of the protein from the fermentation medium of the correct integrands.

The number of copies of the integrated DNA cassette in the genome of the integrands should be determined by applying real-time quantitative PCR using a standard gene.

By following different fermentation parameters of the integrands, such as the biomass formation, starch utilization and ethanol production profiles, their ethanol productivities can be improved by growing them under controlled bioreactor conditions operated at different modes.

The pJRD plasmid can also be used for the directed integration of other genes of interest into the genome of *S. cerevisiae*. Hence the same DNA cassette can be

constructed for the stable and high copy number expression of genes of other enzymes with industrially valuable properties.

The fusion protein used in this study contains both the *B. subtilis* α -amylase and the *A. awamori* glucoamylase activities but its activity against granular starch was found to be very low. Therefore, the amylolytic microorganisms developed in future studies with this cassette can excrete the fusion protein that can only act on soluble starch. Hence in order to improve the substrate specificity of the amylolytic enzymes, the fusion gene in the cassette can be replaced by other amylolytic enzyme genes known to be active against granular starch.

The starch utilization ability of the yeast strains can be unsatisfactory because of the limited amount of amylolytic enzymes secreted. Thus, both α -amylase and glucoamylase activities need to be measured and should be optimized in a fermentation medium with optimum yeast extract and starch content for more efficient utilization of starch.

Finally, considering the increasing food crisis and cost of biomass sources in recent years, the starch which is used as a biomass source can be replaced by cheaper organic source which is little use in principle or value but can add substantial energy to our community by recycling its compounds.

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