BIOETHANOL PRODUCTION FROM FUNGAL SOURCES USING LOW-COST AGRO-INDUSTRIAL WASTE PRODUCTS

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ABSTRACT

BIOETHANOL PRODUCTION FROM FUNGAL SOURCES USING LOW-COST AGRO-INDUSTRIAL WASTE PRODUCTS

In recent years, the rapid increase in environmental problems, greenhouse gas emissions, fuel prices and the unlimited consumption of fuel stocks made people search for some alternative energy sources. Bioethanol is one of the most popular alternative sources with its many beneficial features. Considering the sugar content of fruit pomaces, which are the waste of fruit juice industry, are very convenient and cheap fermentation raw materials for production of bioethanol. The aim of this study was to create a renewable alternative for fossil fuel and to provide a viable solution to multiple environmental problems simultaneously creating a sink for waste utilization and optimize bioethanol production from apple pomace hydrolysate using *Trichoderma harzianum*, *Aspergillus sojae* and *Saccharomyces cerevisiae* by statistical methods. Here, screening and optimization steps were conducted in order to determine the significant factors and their optimum levels. Factors such as inoculation rate of *A.sojae* and *T.harzianum* and agitation speed were considered as factor variables, whereas the response variable was bioethanol production. According to the results of the screening process, inoculation rate of *S.cerevisiae* was fixed as 4% and aeration method as vented. In the optimization step, levels of the other factors were enlarged. The highest bioethanol production and yield on substrate were 8.748 g/l and 0.946, respectively. Higher concentrations of inoculation rates of *T.harzianum* and *A.sojae* (6%) and agitation speed of 200 rpm led to maximum bioethanol production. Furthermore, the results pointed out that using cocultures because of its synergistic interactions is an effective way for production of bioethanol.

ÖZET

DÜŞÜK MALİYETLİ TARIMSAL SANAYİ ATIKLARI KULLANARAK KÜFLERDEN BİYOETANOL ÜRETİMİ

Son yıllarda hızla artan çevresel problemlerde, sera gazı salınımlarında, benzin fiyatlarındaki hızlı artış ve sınırlı olan fosil yakıt kaynaklarının sınırsızca tüketilmesi insanları bazı alternatif enerji kaynağı bulmaya itmiştir. Biyoetanol birçok yararlı özellikleri ile birlikte en popüler alternatif enerji kaynaklarından birisidir. Şeker miktarları gözönüne alındığında, meyve suyu endüstrisi atığı olan meyve posaları biyoethanol üretimi için uygun ve ucuz fermentasyon hammaddelerdir. Bu çalışmanın amacı, fosil yakıtlar için yenilenebilir bir alternatif oluşturmak, atık kullanımı için aynı anda birden fazla havuz oluşturarak çevresel sorunlara kalıcı bir çözüm sağlamak ve *Trichoderma harzianum*, *Aspergillus sojae* ve *Saccharomyces cerevisae* yı kullanarak istatiski yöntemlerle elma posası hidrolizatından biyoetanol üretmektir. Burada, tarama ve optimizasyon basamakları önemli faktörleri ve optimum düzeylerini belirlemek için yapılmıştır. *A.sojae* ve *T.harzianum* inokulasyon oranları ve çalkalama hızı gibi faktörler faktör değişkeni, biyoetanol üretimi ise cevap değişkeni olarak kabul edilmiştir. Tarama sürecinin sonuçlarına göre, *S.cerevisae* inokulasyon oranı %4 ve havalandırma metodu “vented” olarak sabitlenmiştir. Optimizasyon basamağında diğer faktörlerin düzeyleri genişletilmiştir. En yüksek biyoetanol üretimi ve substrat verimi sırasıyla 8.748 g/l ve 0.946 dır. *T.harzianum* ve *A.sojae*’nin yüksek inokulasyon oranları (%6) ve 200 rpm civarındaki çalkalama hızı yüksek miktarda biyoetanol üretimini sağlamıştır. Bunun yanısıra, bu çalışma kültürlerin birarada kullanımının sinerjistik etkileşimlerinden dolayı biyoetanol üretimi için etkili bir yöntem olabileceğini göstermiştir.

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CHAPTER 1

INTRODUCTION

In recent years, the search of the alternative and renewable energy source has become very important since fossil fuels are used unlimitedly. This unlimited comsuption of fossil fuels makes a rapid increase in the concentrations of CO_2 in the atmosphere and have emerged concerns over global warming. Nowadays, bioethanol is one of the most popular alternative energy sources with its desirable properties and its production doubled between 2005 and 2010 (IEA, World Energy Outlook , 2010). The United States and Brasil are leading the bioethanol industry. France, Germany and China are following the sector.

Bioethanol can be produced from sugary, starchy and lignocellulosic raw materials. These raw materials are divided into two categories: Bioethanol produced from sugary and starchy materials are named as first generation feedstocks, whereas second generation feedstocks refer to bioethanol produced from lignocellulose. Although bioethanol production from first generation feedstocks are wellknown processes, there are many disadvantages of using them for bioethanol production. First of all, they are main food sources for human and animal nutrition. Production of bioethanol from these sources causes some problems such as some ethical concerns and favorable economics. Besides, there are too many limitations to sugar and starch-based ethanol production. On the other hand, second generation feedstocks are non-food and they are mainly composed of wastes, such as agricultural and municipal solid wastes. Therefore, they have no such concerns related to their usage for bioethanol production. Furthermore, they are locally available, abundant and cheap materials for fermentation.

Fruit juice industry is one of the biggest industries in the world. It is divided into several branches such as frozen fruit and fruit juice industry. Since the production amount of this sector is too large, accumulation of wastes of this sector is one of the biggest problems for environment.

Turkey is an important country for fruit juice industry. The total production amount of fruit juice and fruit juice-like products in turkey was 821.6 million litres in 2008. Considering the 15-20% of fruit is pomace, it can be seen that accumulation of

wastes is very important issue. Within this context, fruit pomaces are easy to obtain and harsh and expensive methods are not necessary. And may be the most important thing for fermentation is its high fermentable sugar content. Because of these reasons fruit pomaces are very powerful candidates for all kinds of fermentation medias.

Because lignocellulosic materials contain cellulose, hemicellulose and lignin, a pretreatment should be apply to lignocellulosic raw materials in order to increase reducing sugar percentage which makes fermentation more effective. These pretreatments differ from each other such as physical, physicochemical, chemical and biological. Furthermore, pretreatment does not cause formation of inhibitory products which effect fermentation negatively.

This study considers apple pomace as a fermentation media for bioethanol production. Dilute acid pretreatment was chosen since it is the most preferred and widely used method. The conditions were 110°C and 40 minutes which are the optimized conditions for apple pomace. Phosphoric acid was used since after neutralization of hydrolysates with NaOH, a salt formed and can remain in the hydrolysates, as it is used by microorganisms. After this pretreatment step, two different fungi (*Trichoderma harzianum, Aspergillus sojae*) which have powerful lignocellulolytic enzyme activities and capability of producing bioethanol besides the natural ethanologenic yeast *(Saccharomyces cerevisiae)*, are added to fermention media at specific time points. Overall, the goal of this study is to investigate the effects of co-culturing on bioethanol production, to create a low cost alternative solution to bioethanol production and reduce the accumulation of agro-industrial waste products.

CHAPTER 2

BIOETHANOL AS AN ALTERNATIVE FUEL

2.1. Why Do We Need Alternative Fuels?

During the last decades, the search and the use of both new alternative and renewable energy resources has increased rapidly due to the unlimited consumption of limited fosssil fuels, growing energy demand for transportation and industry with the increase in population. Dramatic raises in oil prices and global warming reached threatening limits. There is a tremendeous increase in extending the use of biofuels and biomass energy, since it is possible to get energy for long-term from sustainable resources (Pinilla *et al.*, 2011). Biomass, as a renewable energy source, refers to living and recently dead biological material (Ibeto *et al.*, 2011). Biomass is an infinite feedstock for production of biofuels. However, in order to become a future alternative fuel source some properties are required. First of all, the potential candidate must decrease greenhouse gas emissions, decrease energy consumption, slow down global warming with capturing and storing CO_2, provide efficient energy comsuption or utilization and its production technology must be clean with regard to the environment and be economically viable (Balat *et al.*, 2008, Pinilla *et al.*, 2011).

The transportation sector is mainly dependent on petroleum-derived fuels where approximately 97% of the transportation energy comes from petroleum. Besides about two-thirds of carbon monoxide in major cities is formed due to the usage of petroleum-derived fuel in transportation. Additionally, about one-third of the ozone formation is related to transportation (Nigam, 1999). The combustion of fossil fuels accounts for 73% of the CO_2 production (Balat *et al.*, 2008).

The new technological improvements in biotechnology based on alternative biomass sources will play an important role in solving these problems related to growing energy demands. Bioethanol proved itself as an attractive alternative fuel with its biorenewable nature is carrying all of the features required of being an alternative fuel. Historical development of bioethanol and countries that produce bioethanol will be

elaborated in this chapter. But first of all, bioethanol will be defined in the context of its importance and advantages.

2.2. Bioethanol: Alternative to Gasoline

Bioethanol has been used for many years for various purposes like a starting material for the production of some chemicals such as butanol, acetaldehyde, and acetic acid, beverage and finally it is used as an alternative fuel (Chandrakant and Bisaria, 1998). Bioethanol, which can be produced from various biomass feedstocks, is a renewable, biodegradable, and bio-based liquid fuel. The structure of ethanol is shown in Figure 2.1.

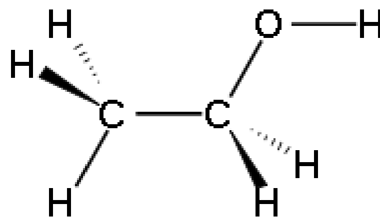

Figure 2.1. Structure of ethanol molecule

Bioethanol has a number of advantages over conventinal fuel sources. Firstly, it comes from a renewable source not from a finite source. Because it uses energy from renewable energy sources, no net CO_2 is added to atmosphere. This feauture makes ethanol an environmentally friendly energy source. It is less toxic when compared to petroleum sources and is not a water-contaminant. Bioethanol can be easily integrated into the existing fuel system. In certain quantities, it can be blended with conventional fuel without the need of engine modifications. Also it can be used directly in flexible-fuel vehicles (FFV). Blending bioethanol with petroleum helps to extend the life of the diminishing oil supplies. Besides, these advantages can be considered as the most unique contribution of bioethanol to global warming. Since there is no net addition of CO_2 to the atmosphere, greenhouse gas emissions will be reduced through the use of bioethanol. Ethanol contains 35% oxygen that helps complete combustion of fuel and thus reduces particulate, CO and hydrocarbon emissions which pose health hazard to living beings. Fuel properties of bioethanol is shown in Table 2.1.

Table 2.1. Fuel properties of bioethanol
(Source: Balat *et al*., 2008)

Cetane number	8	Latent heat of vaporization (MJ/Kg)	0.9
Octane number	107	Lower heating value (MJ/Kg)	26.7

The octane number is a measure of combustion quality and the ability to withstand harsh conditions. In internal combustion engines higher octane numbers are preferred. Besides bioethanol has high octane number (whereas gasoline has a average octane number of 88), it has also broad combustibility limits, high flame speeds and high heats of vaporization. Because of these properties, compression ratio of bioethanol is high with short burning time which holds advantages over gasoline. Furthermore, its high octane number and low cetane number make bioethanol a suitable additive for blending with gasoline. (Balat *et al*., 2008). Its low cetane number and high heat of vaporization are impediments for self-ignition in the diesel engine (Ibeto *et al*., 2011). Finally, local production of bioethanol can decrease dependence for imported fuels and create new business areas.

2.3. Historical Development of Bioethanol

The idea of using bioethanol as a fuel is not new. Bioethanol was considered as biofuel at the very beginning of the nineteenth century. In the 1860s Nikolaus August Otto, a German mechanical engineer found the internal combustion engine, and used ethanol in his prototype of a spark ignition engine. In 1902, heavy locomotives which used pure ethanol for running were projected by a company named Deutz Gas Engine Works (Antoni *et al*., 2007).

Henry Ford, the founder of Ford Motor Company, remarked his thoughts about biofuels with the following sentences in 1925: "We can get fuel from fruit, from that shrub by the roadside, or from apples, weeds, saw-dust—almost anything! There is fuel in every bit of vegetable matter that can be fermented … And it remains for someone to find out how this fuel can be produced commercially—better fuel at a cheaper price than we know now". And his design called Model T, the "Tin Lizzy", which was produced from 1903 to 1926, was the car running on 100% ethanol (Antoni *et al*., 2007). However, the exploiment of new crude oil resources at some parts of Texas and Pennsylvania made gasoline very cheap and thus the reduced use of biofuels. This was

the reason of using petroleum in the vehicles which was much cheaper and more efficient (Biofuel, 2012).

Certain amounts of ethanol blended with gasoline has been used in transportation since 1925. In the USA, production of ethanol was interrupted due to the considerably low prices of gasoline in 1940. However, because of the rise in gasoline prices during the World War II the need for biofuels emerged again. In Germany ethanol produced from potatoes was blended with gasoline and Britain was the other country that used ethanol produced from grain during this period (Biofuel, 2012). When considering the current situation in the world, it is recognised that bioethanol is a very environmentally friendly fuel compared to conventional fuels because of its advantages. Since the 1980s, bioethanol has been searched by many countries as a possible alternative fuel and interest on bioethanol production via fermentation has increased day by day.

2.4. Current Status of Bioethanol Production in the World

Bioethanol has been produced in a large scale for last few years in the USA, Brasil and some European countries. It is expected to become one of the dominant renewable biofuels in the transportation sector within the next 20 years (Galbe *et al.*, 2006).

In European Union, production of bioethanol was 2155 million liters in 2008 annually where France was the main producer (Arapoglou *et al.*, 2010). However, the amount of consumed bioethonol is greater than the amount of produced bioethanol in many European countries and the utilization of bioethanol is expected to double by the year 2015 (Ibeto *et al.*, 2011). European Commission’s aim is to substitute 20% of conventional fossil fuels with alternative fuels in transportation sector by the year 2020. In order to achieve this aim, an increase of 10% by 2015, following by 15% increase by 2020 and 25% increase by 2030 have been proposed. Based on this European Union implemented a tax exemption (up to 100%) on biofuels (Galbe *et al.*, 2006, Ibeto *et al.*, Antoni *et al.*, 2007). The largest bioethanol facility which has 260,000 m^3 production capacity annually in Europe is located in Germany (in Zeiz). Here wheat and barley are used for the bioethanol production (Antoni *et al.*, 2007).

In the USA, according to the *Energy Policy Act* of 2005, 7.5 billion gallons of alternative fuels must be blended into gasoline by the year 2012. And by the year 2025, they are planning to substitute more than 75% of imported oil with alternative fuels (Galbe *et al*., 2006, Gray *et al*., 2006). In 2006, the consumption of crude oil was approximately 20 million barrels daily and approximately 4 billion gallons bioethanol was produced annually. But according to estimation of Department of Agriculture and Department of energy, the USA has potential to produce over 1 billions tons of biomass annually, which accounts for approximately 80 billion gallons for the substitution of current conventional fuel usage (Gray *et al*., 2006).

Brazil was very first large scale ethanol producer via fermentation with the *Proalcohol* programme which was impelemented in the 1970s and 1980s. With this project, Brazil became the largest ethanol producer in the world. However, Brazil is the second producer of bioethanol and the world’s larger exporter currently. Bioethanol production processes are based on sugar cane which has high sucrose content.

In the year 2007, the USA and Brazil were the main producers of bioethanol, followed by China. The total production of bioethanol in the world was approximately 51 million liters, 73% of produced ethanol worldwide correspondeds to fuel ethanol, 17% to beverage ethanol and 10% to industrial ethanol (Sanchez and Cardona, 2008). Africa also has huge potential for bioethanol production and there is a bioethanol plant in South Africa. China, India anad Thailand are important countries producing bioethanol in Asia. Global production of bioethanol is shown in Figure 2.2.

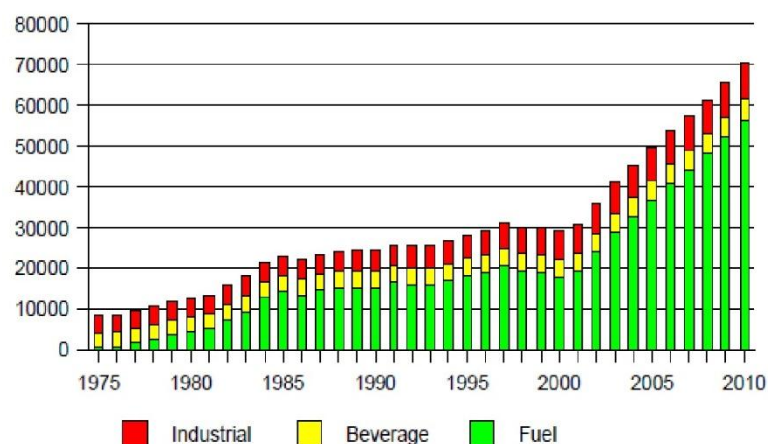

Figure 2.2. Bioethanol production in the world by country (million liters)
(Source: Licht, 2008)

Bioethanol can be blended with gasoline or can be used as neat alcohol. Bioethanol producers can be seen in Figure 2.3. The areas with more intense green color indicates higher production. Some parts of United States and Brazil has high production rates. Turkey is also one of the bioethanol producers.

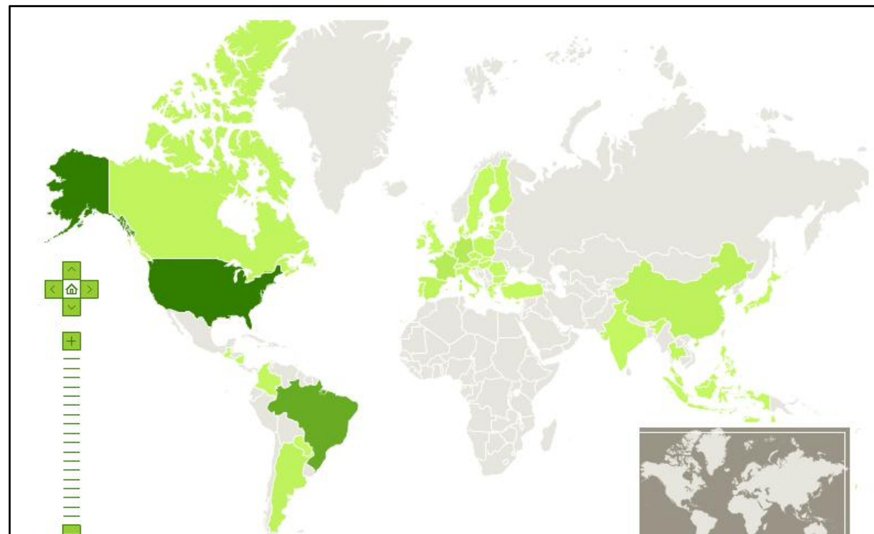

Figure 2.3. Global distribution of bioethanol production geographically, in 2009. (Source: Biofuels Platform, 2012)

Many countries have already programs or are in the process of blending ethanol with gasoline. Some bioethanol programs by countries are shown in Table 2.2. below.

Table 2.2. Bioethanol programs in some countries
(Source: Sanchez and Cardona, 2008)

Country	**Feedstock**	**Percentage of ethanol in gasoline blends, % (v/v)**	**Remarks**
Brazil	Sugar cane	24	ProAlcohol programme
USA	Corn	10	Oxygenation of gasoline is obliged in dirtiest cities; 85% blends are also available
Canada	Corn, wheat, barley	7.5-10	Tax incentives; programs aim's is to meet Kyoto protocol
Colombia	Sugar cane	10	No tax since November 2005
Spain	Wheat, barley	-	Direct gasoline blending, ETBE production
France	Sugar beet, corn wheat	-	Direct gasoline blending, ETBE production
Sweden	Wheat	5	85% blends are also available
China	Corn, wheat	-	Trial use in some regions
India	Sugar cane	5	Ethanol blends are obliged in 9 states

According to Table 2.2. it can be seen that sugar cane, corn, wheat, barley, sugar beet, rice and sorghum are the major energy crops for bioethanol production. Sugar cane is considered as the main feedstock for the production of bioethanol in tropical countries such as Brazil and India wheras starchy materials, especially corn is mainly used feedstock in the USA and Europe. In Asia, rice straw, wheat straw and corn stover are the most preferred feedstocks.

2.5. Current Status of Bioethanol Production in Turkey

Bioethanol production is very important in Turkey considering that approximately 90% of the petroleum demand is supplied with imported oil. There are four factories belonging to Türkiye Şeker Fabrikaları A.Ş. with the 60.000 m^3 bioethanol production capacity annually from sugar beet molasses if they are launched. The factories are located in Eskişehir, Turhal, Malatya and Erzurum with the theoretical production capacity of 21.000 m^3/year, 14.000 m^3/year, 12.500 m^3/year, and 12.500 m^3/year, respectively. Factories which produce or able to produce bioethanol in Turkey are shown in Table 2.3. below.

Table 2.3. Factories which produce or able to produce bioethanol in Turkey (Source: Oruc, 2008)

Factory	Feedstock	Production Capacity(million liters/year)
Eskişehir Alcohol Factory	Sugar Beet	21.0
Turhal Alcohol Factory	Sugar Beet	14.0
Malatya Alcohol Factory	Sugar Beet	12.5
Erzurum Alcohol Factory	Sugar Beet	12.5
Çumra Sugar Factory	Sugar Beet	84.0
Tarkim (Bursa)	Wheat – Corn	40.0
Tezkim (Adana)	Wheat – Corn	26.0

However, only in the factory located in Eskişehir, distillation is possible and this situation limits bioethanol production. Although there is a huge amount of bioethanol production capacity, very important part of that capacity is not available. There are also three other private factories which are called Çumra Şeker Factory belonging to factory named Konya Şeker, Tarkim and Tezkim with the capacity of 84, 40 and 26 million

liters per year, respectively (Oruc, 2008). However, total capacity of bioethanol production does not meet the requirement of the amount specified in the decision of the European Commission. According to European Commission’s decision, bioethanol must be blended with the gasoline in the percentage of 5,75 and Turkey, as a candidate of European Community, must achieve this ratio. This situation makes bioethanol production much more important (Melikoglu and Albostan, 2011) and in order to use bioethanol production capacity effectively some regulations must be reinforced. It is indicated that blending bioethanol with gasoline must be mandotory like in other countries in the world and taxes must be regulated.

CHAPTER 3

BIOETHANOL PRODUCTION PROCESSES

There are various types of raw materials that can be used for bioethanol production. These raw materials can be classified into three major groups: (i) sucrose – containing raw materials, (ii) starch - containing raw materials and (iii) lignocellulosic raw materials. Conversion of sucrose – containing raw materials into fermentable sugar is easier compared to starch – containing raw materials because it does not require hydrolysis step. Starchy materials, consisting of long chains of glucose molecules, also can be converted into fermentable sugar with hydrolysis. These two groups are known as "first generation bioethanol" in literature. In recent years, lignocellulosic biomass, which is known as "second generation bioethanol", has become an attractive raw material for bioethanol production because of the reasons of having some advantages over sucrose – and starch – containg raw materials. However, its recalcitrance, which makes bioethanol production process complex and increases production cost, appears to be a disadvantage. It is forecasted that with the improvements in process technologies, these problems will be overcome within next years. In this chapter, composition and advantages of lignocellulosic raw materials will be reviewed followed by the major production technologies and fermentation. In Table 3.1. some raw materials and their potential for bioethanol production is outlined.

Table 3.1. Some raw materials and their potential for bioethanol production
(Source: Balat *et al.*, 2008)

Raw material	**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

3.1. Composition of Lignocellulosic Raw Materials

Lignocellulosic biomass is the most abundant biopolymer in the Earth. It is reported that lignocellulosic biomass covers about 50% of world biomass and could produce up to 442 billion liters bioethanol per year. Thereby, the total bioethanol production potential from crop residues and wastes can reach 491 billion liters per year, which is about 16 times higher than the world bioethanol production currently. Lignocellulosic materials can be divided into six major groups: crop residues, hardwood, softwood, cellulose wastes, herbaceous biomass and municipal solid wastes (Sanchez and Cardona, 2008, Balat *et al*., 2008).

Lignocellulosic raw materials consist of a mixture of carbohydrate polymers, lignin, extractives and ashes. Cellulose $(C_6H_{10}O_5)_x$, hemicelluloses such as xylan $(C_5H_8O_4)_m$ and lignin $[C_9H_{10}O_3.\ (OCH_3)_{0.9\text{-}1.7}]_n$ are the major polymers. The term “holocellulose”, which refers to the total carbohydrate found in plant or microbial cell, is used therefore for both cellulose and hemicellulose (Taherzadeh and Karimi, 2007, Balat *et al*., 2008).

Cellulose is an unbranched linear homopolysaccharide of β-D-glucopyranose units linked with (1-4)-glycosidic bonds. Because of having rigid structure strong treatment conditions are required for its degradation. In long cellulose chains, number of glucose units can vary from 5000 to 10,000. Two glucose units, basic repeating unit of cellulose, are called as cellobiose (Balat *et al*., 2008, Chandel *et al*., 2007). A variety of other polysaccharides such as hemicellulose, pectin and lignin are associated with cellulose in nature. The structure of cellulose is illustrated in Figure 3.1.

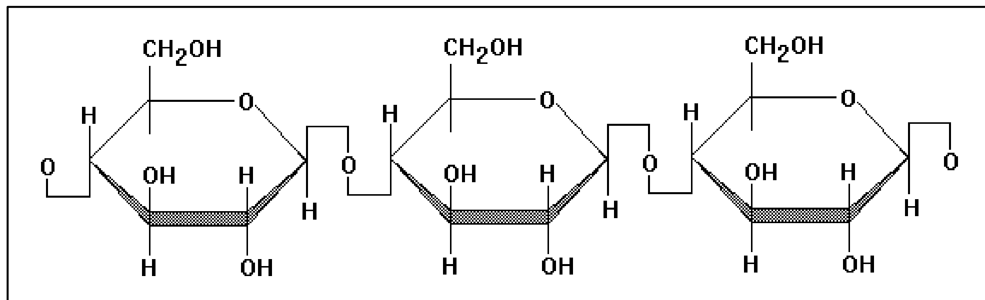

Figure 3.1. The chemical structure of cellulose
(Source: Carbohydrates, 2012)

Hemicellulose is the second major component of the lignocellulosic raw material which accounts for 25 – 35% of the mass of dry wood, 28% in softwoods and 35% in hardwoods. Unlike cellulose, hemicellulose consists of various polymerized monosaccharides, mainly glucose, mannose, galactose, xylose, arabinose and small amounts of rhamnose, glucuronic acid,and galacturonic acid. These monomers are linked to the main backbone with different linkages and substitutions. In most hardwood raw materials, xylose is the most dominant pentose sugar, whereas arabinose is dominant in various agricultural residues and herbaceous crops. Hemicelluloses can be easily hydrolyzed to its monomers by acids. (Taherzadeh and Karimi, 2007, Balat *et al*., 2008, Chandrakant and Bisaria, 1998). The chemical structure of hemicellulose is shown in Figure 3.2.

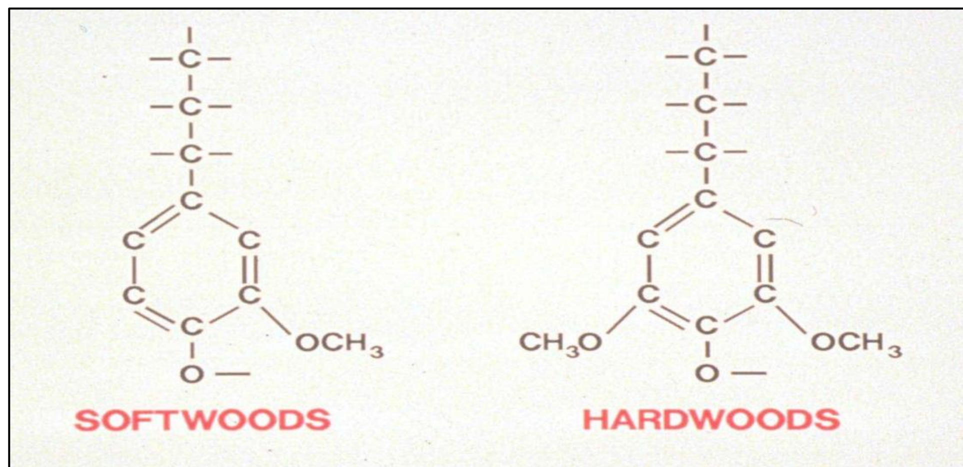

Figure 3.2. The chemical structure of hemicellulose
(Source: Society of Wood Sciences and Technology Teaching, 2012)

The third component of lignocellulosic biomass is lignin. It has highly branched and substituted aromatic polymers and often forms a lignocellulosic complex with adjacent cellulose units. The lignin content accounts for 20 – 40% of the mass of both softwoods and hardwoods and 10 – 40% of the mass of some herbaceous species, such as bagasse, corncobs, peanut and straws. Lignins are extremely robust to chemical and enymatic attacks (Balat *et al*., 2008). The chemical structure of lignin is presented in Figure 3.3.

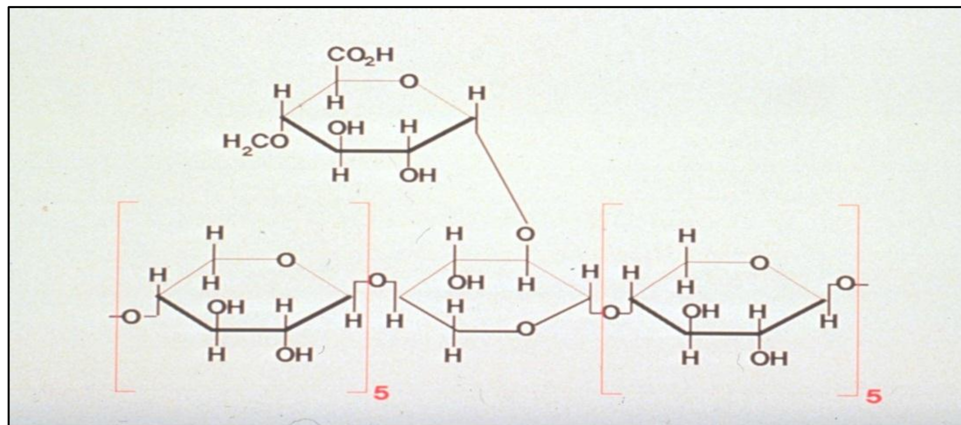

Figure 3.3. The chemical structure of lignin
(Source: Society of Wood Sciences and Technology Teaching, 2012)

Extractives generate small portions (range from 1 – 5%) of lignocellulosic materials which are wood compounds having high solubility in neutral organic solvents or water. They have both lipophilic and hydrophilic components and they can be grouped into four groups: terpenoids and steroids, fats and waxes, phenolic components and inorganic components (Taherzadeh and Karimi, 2007).

The amount of carbohydrate polymers and lignin can vary from one type to another type of lignocellulosic material. Garrote *et al.* (1999) and Wyman (1996) reviewed the compositions of some different lignocellulosic feedstocks and they found that the hardwoods such as *Eucalyptus*, oak and white birch contained 39 – 54% cellulose, 14 – 37% hemicellulose, 17 – 30% lignin. Similarly, softwoods such as pines contains 41 – 50% cellulose, 11 – 27% hemicellulose and 20 – 30% lignin, whereas for agricultural residues such as rice straw the composition ranges from 32 – 47% cellulose, 19 – 27% hemicellulose to 5 – 24% lignin. The carbohydrate polymers require a process called hydrolysis in order to obtain fermentable sugars. Generally, hemicellulose parts of softwood and hardwood contain naturally occurring process – induced inhibitory compounds that sometimes effect fermentation negatively (Keating *et al.*, 2004). The composition of lignocellulosic feedstocks and their hydrolysis products are illustrated in Figure 3.4. and some lignocellulosic raw materials' compositions are reviewed by Lee (1996). The results are shown in Table 3.2.

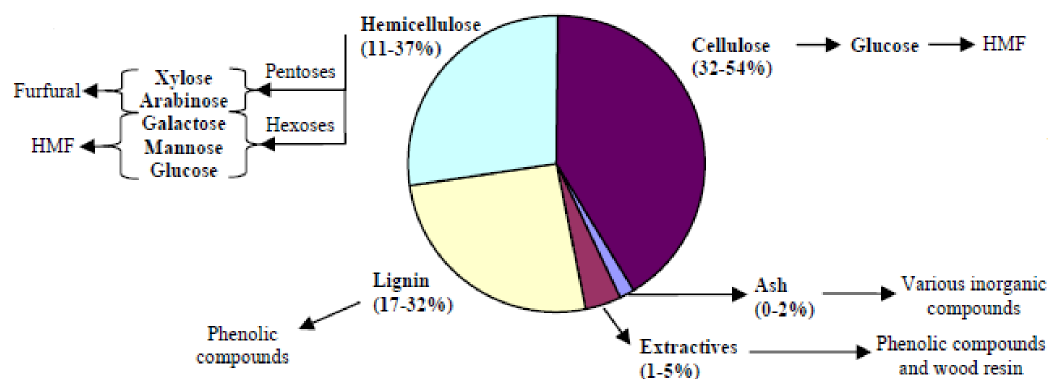

Figure 3.4. Composition of lignocellulosic feedstocks and their hydrolysis product (Adapted: Taherzadeh and Karimi, 2007)

Table 3.2. Composition of various lignocellulosic raw materials (Source: Chandrakant and Bisaria, 1998)

Lignocellulosic raw material	**Carbohydrate (% of sugar equivalent)**					**Noncarbohydrate (%)**	
	Glucose	**Mannose**	**Galactose**	**Xylose**	**Arabinose**	**Lignin**	**Ash**
Corn stover	39.0	0.3	0.8	14.8	3.2	15.1	4.3
Wheat straw	36.6	0.8	2.4	19.2	2.4	14.5	9.6
Rice straw	41.0	1.8	0.4	14.8	4.5	9.9	12.4
Rice hulls	36.1	3.0	0.1	14.0	2.6	19.4	20.1
Bagasse fiber	38.1	NA	1.1	23.3	2.5	18.4	2.8
Populus tristis (hardwood)	40.0	8.0	NA	13.0	2.0	20.0	1.0
Douglas fir (softwood)	50.0	12.0	1.3	3.4	1.1	28.3	0.2

NA: not available

3.2. Advantages of Lignocelulosic Biomass over First Generation Feedstocks

European Commision's Directive 2009 indicates the importance of commercializing second generation biofuels. In the USA, it is proposed to achieve 44% of the total biofuel production from lignocelluloses by 2020 (Luo *et al.*, 2010).

Lignocellulose is a cheap and reproducible raw material and refers to the non-food materials. Bioethanol crops such as corn, corn cobs, corn stover, rice, wheat, sorghum, and sugar cane and others are main food sources for humankind. Besides competing with food production, bioethanol production from these feedstocks cause high production prices that limits their industrial production. (Alam *et al.*, 2009). Thus, lignocellulosic raw materials minimize the potential conflict feedstock production. The raw material can be produced with lower input of fertilizers, pesticides, and energy (Galbe *et al.*, 2006).

Producing value-added coproducts besides bioethanol is one of the advantages of lignocellulose bioconversion. For example, sugars may be used for fermentation of other products such as lactic acid, which in turn may be processed into plastics and other products. Also lignin can be used for production of some value-added products (Balat *et al.*, 2008).

3.3. Bioethanol Production Processes from Lignocellulosic Biomass

There are several processes for converting lignocellulose to ethanol. According to Balat *et al.* (2008), regardless of process that is chosen, some features, listed below, must be assessed in comparison with established conventional first generation bioethanol production:

- Efficient degradation of cellulose and hemicellulose into fermentable sugars
- Efficient fermentation of sugars (both six- and five-carbon sugars)
- Advanced process integration in order to minimize process energy demand
- Use of feedstocks with low lignin content in order to decrease production cost

The 'conventional' bioethanol production processes from lignocellulosic biomass contain three major steps:

1) *Pretreatment* – degradation of the lignocellulosic structure

2) *Enymatic hydrolysis* – depolymerization of cellulose to ethanol with the help of enymes

3) *Fermentation* – conversion of fermentable sugars to bioethanol by microorganisms

Conversion process of lignocellulosic biomass to bioethanol is summarized in Figure 3.5.

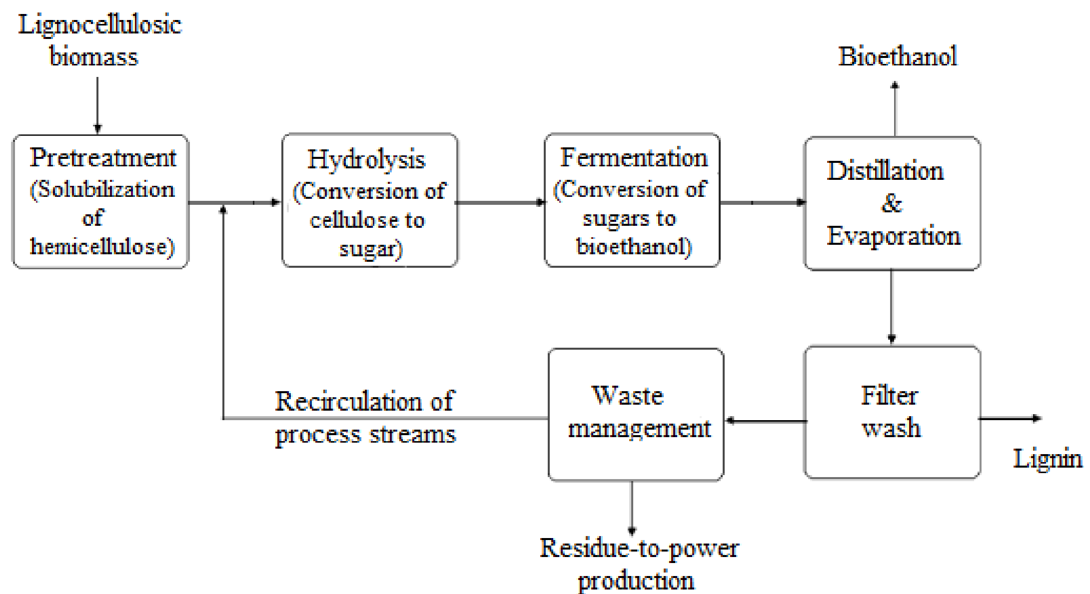

Figure 3.5. Flowsheet of bioconversion of biomass to bioethanol
(Adapted: Balat *et al*., 2008)

Within last few years, novel production technologies such as consolidated bioprocesses which maybe an alternative to conventional production are also being searched.

3.3.1. Pretreatment

Pretreatment process aims the opening of the accessible areas in the cellulose structure of lignocellulosic biomass by altering the macroscopic and microscopic size and structure for hydrolysis. Pretreatmet effects lignocellulose creating larger accessible surface area and pore size, reducing the crystallinity, partialy degrading the cellulose, increasing the solubility of hemicellulose and lignin and the modifying the lignin structure. Moreover, pretreatment should improve the formation of sugars or the ability to form them during the succeeding enzymatic hydrolysis, and avoid degradation or loss of carbohydrate and formation of inhibitory byproducts for subsequent hydrolysis and fermentation and be cost effective (Margeot *et al*., 2009, Chandel *et al*., 2007, Sanchez and Cardona, 2008, Balat *et al*., 2008). Since pretreatment efficiency depends on biomass composition, the choice of pretreatment method is difficult. Below in Figure 3.6. the effect of pretreatment on lignocellulosic biomass is illustrated.

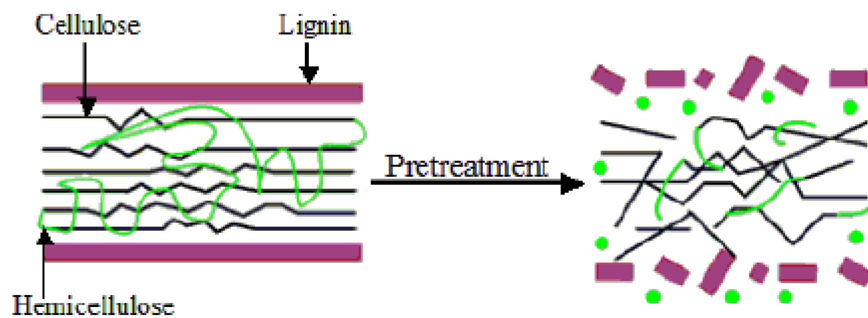

Figure 3.6. Schematic representation of lignocellulosic biomass pretreatment (Source: Mosier *et al*., 2005)

After pretreatment, water insoluble solids is filtered in order to obtain the majority of cellulose where lignin and the hemicellulosic sugars remain in the filtrate. Pretreatment conditions have great effect on subsequent hydrolysis and fermentation process (Alvira *et al*., 2010). It is considered that the pretreatment is the most significant determinant for the success of the cellulosic bioethanol technology, since it determines the course of the process (Balat *et al*., 2008).

Mechanical combination, steam explosion, ammonia fiber explosion (AFEX), acid or alkaline pretreatment and biological treatment are mainly used pretreatment

processes. Some pretreatment methods are reviewed by Sάnchez and Cardona (2007) shown in Table 3.3.

Table 3.3. Some pretreatment methods of lignocellulosic biomass for bioetanol production (Source: Sanchez and Cardona, 2008)

Type of pretreatment	**Name of pretreatment**	**Examples of pretreated materials**
Physical	Mechanical comminution	Wood and forestry wastes, corn stover, cane bagasse
	Pyrolysis	Wood, waste cotton, corn stover
Physico-chemical	Steam explosion	Poplar, aspen, eucalyptus, bagasse, corn stalk, wheat straw, barlet straw,sweet sorghum bagasse, olive stones
	Liquid hot water (LHW)	Bagasse, corn stover, olive pulp
	Ammonia fiber explosion (AFEX)	Aspen wood chips, bagasse, wheat straw, barley straw, rice hulls, corn stover, municipal solid waste
	CO_2 explosion	Bagasse, recycled paper
Chemical	Ozonolysis	Pine, bagasse, wheat straw, peanut
	Dilute-acid hydrolysis	Bagasse, corn stover, wheat straw, rice hulls, Switchgrass
	Concentrated acid hydrolysis	Poplar sawdust, bagasse
	Alkaline hydrolysis	Hardwood, straws with low lignin content bagasse, corn stover
	Oxidative delignification	Bagasse
	Wet oxidation	Corn stover, wheat straw
	Organosolv process	Poplar wood, mixed softwood
Biological	Fungal pretreatment	Corn stover, wheat straw
	Bioorganosolv pretreatment	Beech wood

3.3.2. Hydrolysis

Hydrolysis is an essential step in order to obtain fermentable sugars. Acid (dilute or concentrated) and enzymatic hydrolysis are the most commonly used methods for hydrolysis. Hydrolysis without pretreatment cause low yield (approximately < 20%), whereas after pretreatment, yield often exceeds 90% (Balat *et al*., 2008).

Acid hydrolysis is one of the oldest processs for converting cellulose to bioethanol. In dilute acid hydrolysis, biomass is mixed with diluted or concentrated acids, such as sulfuric acids, at spesific temperatures, pressure and reaction time (Balat *et al*., 2008). The sugar depolymerization rate under dilute acid conditions are obtained as below (Taherzadeh and Karimi, 2007):

Xylose > Arabinose > Mannose > Galactose > Glucose

Hence, xylose is more sensitive to acidic conditions compared to other sugars, whereas glucose is more resistant to harsh conditions.

Major drawbacks of using acids, especially sulfuric acid, are low sugar yields in the case of diluted acid hydrolysis, glucose degradation at high temperatures that is required for concentrated acid hydrolysis and the diffuculty in working with concentrated acid. Furtermore, all of the acid must be recovered in order to make the process economically viable.

For enzymatic hydrolysis, which is the most promising technology, a mixture of different enzymes are needed for the degradation of lignocellulosic biomass. Cellulose can be hydrolyzed by at least three groups of enzymes: β-glucosidases which converts cellobiose into glucose, cellobiohydrolyses (CBH) cleaving off cellobiose units from the end of the chain and endoglucanases (EG) which attacks internal β-1-4-glucosidic linkages in the cellulose chain. EG's especially attack amorphous cellulose, whereas CBH's attack crystalline cellulose. Also it is indicated that xylanases and other auxiliary enzymes are required for complete hydrolysis of lignocellulose.

For cellulose hydrolysis microbial cellulolytic enzymes, which decrease the enzyme costs, are currently used. Cellulases, hemicellulases and pectinases are produced by a broad range of bacteria, such as *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia* and *Streptomyces*; and by

filamentous fungi, such as *Trichoderma*, *Aspergillus*, *Monilia*, *Fusarium*, *Rhizopus*. Optimization of enzymes obtained from microorganisms will make the process cost-effective and a greater than 10-fold cost reduction for *T.reseei* cellulases was recently reported (Olsson *et al*., 2003, Balat *et al*., 2008, Alvira *et al*., 2010, Gray *et al*., 2006). *T.reseei* is the major microbial source of commercial cellulase and hemicelluloses among 100 different *Trichoderma* species. However, a novel *T.atroviride* mutant, which has more efficient hydrolization ability than the hyper-cellulolytic mutant *T.reseei* Rut-30, was identifed by Kovacs et al. (2008).

High costs and necessity for excessive enzymatic dosage are the drawbacks of enzymatic hydrolysis. In order to overcome these drawbacks several ways for converting cellulose to ethanol are considered such as seperate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), direct microbial conversion (DMC) and consolidated bioprocessing (CBP).

3.3.3. Fermentation

Fermentation of lignocellulosic biomass is very difficult compared to conventional first generation feedstocks. Hydrolyzates include both hexose and pentose sugars and generally xylose is the major sugar found in hydrolyzate of hardwood hemicelluloses. Also hydrolyzates can contain a broad range of inhibitory products depending on the composition of lignocellulosic biomass. For these reasons, ideal organisms for fermentation of lignocellulosic biomass to bioethanol must have certain features. First of all, the organism would be expected to have ability to ferment both pentose and hexose sugars for making the process economically feasible. It should have high ethanol yield, even in the presence of inhibitory products that occur in the pretreatment step. Therefore the important features of an ideal organism are listed below:

- High ethanol yield and productivity
- High ethanol tolerance
- Broad range of substrate utilization (both pentoses and hexoses, even in the presence of glucose)
- Withstand inhibitory products
- Oxygen tolerance

- Low fermentation pH
- High shear tolerance (Chandrakant and Bisaria, 1998, Taherzadeh and Karimi, 2007)

Saccharomyces cerevisiae is the most commonly used microorganism for bioethanol production, but it cannot use xylose for fermentation. Various yeasts, bacteria and filamentous fungi have been reported to produce bioethanol as the main fermentation product (Taherzadeh and Karimi, 2007).

3.4. Separate Hydrolysis and Fermentation (SHF)

Separate hydrolysis and fermentation consists of two steps. In the first step, cellulose is hydrolyzed to glucose by cellulosic enzymes. In the second step, glucose formed in the first step is fermented to ethanol by using *Saccharomyces* or *Zymomonas*. The joint liquid flow from hydrolysis reactors first enters the glucose fermentation reactor. After the distillation of bioethanol, xylose fermentation to bioethanol is carried out in a second reactor and bioethanol is again distilled. Each step is carried out at its optimum temperature, 45 – 50 ^{o}C for enzymatic hydrolysis and 30 ^{o}C for fermentation, respectively. This is the main advantage of this process. The most important factors to be considered for saccharification step are reaction time, temperature, pH, enzyme dosage and substarate load. On the other hand, inhibition of cellulosic enyzmes by sugars released as a result of enzymatic hydrolysis and the accumulation of end products are the disadvantage of SHF (Chandrakant and Bisaria, 1998, Balat *et al.*, 2008, Sanchez and Cardona, 2008). The SHF with separate pentose and hexose sugars and combined sugar fermentation are shown in Figure 3.7.

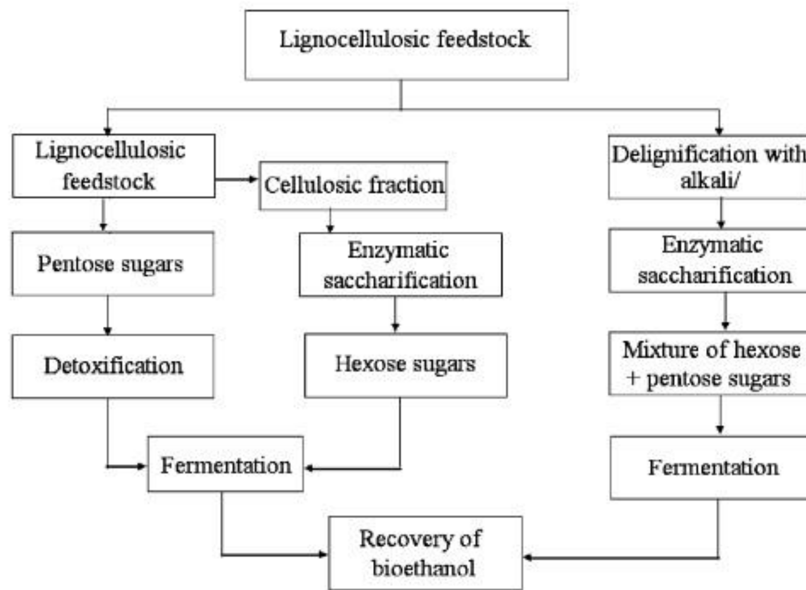

Figure 3.7. Scheme for seperate hydrolysis and fermentation (SHF) process (Source: Balat *et al.*, 2008)

3.5. Direct Microbial Conversion (DMC)

In order to reduce the cost of conversion of lignocellulosic biomass to bioethanol some alternative ways, which eliminate pretreatment step, increasing cellulose hydrolysis rate and enhancing enzyme activity, were proposed. or Direct microbial conversion (DMC) or consolidated bioprocessing (CBP) is one of the alternative ways to conventional bioethanol production processes. In this process, microorganisms that can both convert biomass to fermentable sugars and ferment the resultant sugars to bioethanol are utilized. Namely, cellulase production, cellulose hydrolysis and fermentation is carried out in a single step mediated by a single organism or microbial consortium. CBP does not include a separate cellulase production step and this property alone helps the reduction of the process cost. Bacteria and yeast have been the principle candidates for CBP and *Clostridium thermocellum* is one of the most investigated organism for DMC. Recently, fungi have been considered as feasible CBP organisms. However, relatively low ethanol tolerance (about 3.5% ethanol) is the drawback of this process when compared to ethanol tolerance of an ethanologenic yeast which is about 10% (Chandrakant and Bisaria, 1998, Xu *et al.*, 2009).

Consolidated bioprocessing is illustrated in Figure 3.8.

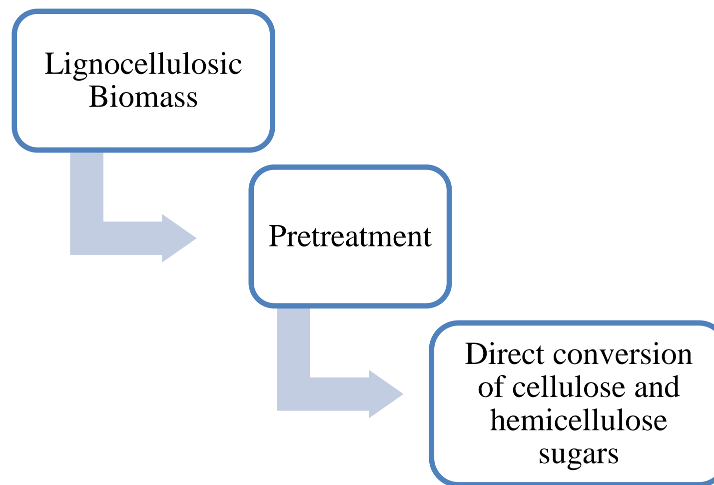

Figure 3.8. Scheme for Consolidated BioProcessing

Although none of the microorganism have the capability of converting lignocellulose to bioethanol naturally, some bacteria and fungi exhibit some of the needed porperties. In Table 3.4. some potential CBP microorganisms are compared. As it can be seen from the table, although *S.cerevisiae* has very high bioethanol production rate it cannot use xylose which is one of the main sugars produced from lignocellulosic biomass, especially from some agricultural residues such as fruit pomaces. Similarly, although *T.reseei* is a natural producer of cellulase enzyme and able to utilize five of the lignocellulosic sugars (glucose, mannose, galactose, xylose and arabinose), it does not produce bioethanol with high yield and high rate. Therefore, in order to obtain high fermentation yield, utilization of mixed cultures may be much more convenient for bioethanol production from agricultural residues.

Table 3.4. Comparison of potential CBP microorganisms
(Source: Xu *et al*., 2009)

	Natural ethanologens		Naturally cellulolytic	
Candidate	Yeast (*S.cerevisiae*)	Bacteria (*Z.mobilis*)	Bacteria (*C.thermocellum*)	Fungi (*T.reseei*)
Cellulase genes	Some attempts have failed	Unknown	Naturally produce cellulase	Naturally produce cellulase
Cellulase production	Barely detectible	Unknown	A few grams per liter	more than 100 g/l
Ethanol production	Up to 160g/l	Up to 130g/l	Slow rate,low yield	Slow rate,low yield
Multi-sugar usage	No	No	Not utilize xylose	Yes
Resistance to inhibitors	High	High	Low	Very high
Amenability to genetic manipulation	Excellent	Good	Very poor	Good
Commercial acceptance	Very high	Acceptable	Unknown	Very high

Besides *Trichoderma*, **s**ome other fungi such as *Monilia*, *Fusarium*, *Rhizopus*, *Aspergillus* and *Neocallimastix* have been reported to possess the ability to convert cellulose to ethanol. However, they produce other byproducts such as lactic and acetic acid (Xu *et al*. , 2009). These fungi, including *Trichoderma*, are thought to contain two biological systems: one system produces cellulase enzymes for degradation of cellulose to fermentable sugars under aerobic conditions; other system produces ethanol under anaerobic conditions. Table 3.5. summarizes bioethanol production from cellulose by some fungi.

Table 3.5. Some filamentous fungi producing bioethanol from cellulose directly (g/l)
(Source: Xu *et al*., 2009)

Organism	Number of tested strains	Cellulose	Xylan	Glucose	Galactose	Mannose	Xylose	Arabinose
Aspergillus:								
A.awamori	2	0.3-0.4		6.0-7.2				0.9-1.4
A.foeticus	1	0.3		5.2				3.4
A.niger	1	0.2		5.7				1.2
A.oryzae	4	0.6-0.8		16.1-24.4				2.6-4.7
A.sojae	6	0.4-0.7		8.0-14.4				2.1-5.4
A.tamari	5	0.1-0.6		9.8-18.6				2.9-3.5
Rhizopus:								
R.javanicus	3	0.5-1.3	1.3-1.7	21.8-33.0				1.3-1.7
R.oryzae	6	0.2-1.4	0.1-2.7	15.4-32.3				1.0-10.8
Trichoderma:								
T.harzianum	1	2.0		5.0				0.6
T.reseei	3	1.1-1.5		4.0-4.8	3.0-3.5		4.2-4.5	0.4-0.5
Fusarium:								
F.oxysporum	2	0.35g/g		0.38g/g				0.25g/g
Monilia:								
Monilia sp.	1	17.0		23.0				11.0

3.6. Simultaneous Saccharification and Fermentation (SSF)

Saccharification of cellulose to glucose with cellulosic enzymes and the subsequent fermentation of glucose by *Saccharomyces* or *Zymomonas* is carried out in the same vessel. As a cellulase enzyme source, *Trichoderma* is used widely because of having good activity at temperatures 40 – 50°C, whereas optimum temperature for *Saccharomyces* for fermentation is 30°C. As a consequence, a compromise must be made between these two temperatures. The advantages of this process can be listed as followings: enhanced rate of cellulose hydrolysis because of removal of sugars which inhibit cellulase activity, lower enzyme loading, increase in product yield, reduced inhibition of yeast fermentation, decreased requirement for sterility and decrease in the process time.

Simultaneous saccharification and fermentation process is shown in Figure 3.9.

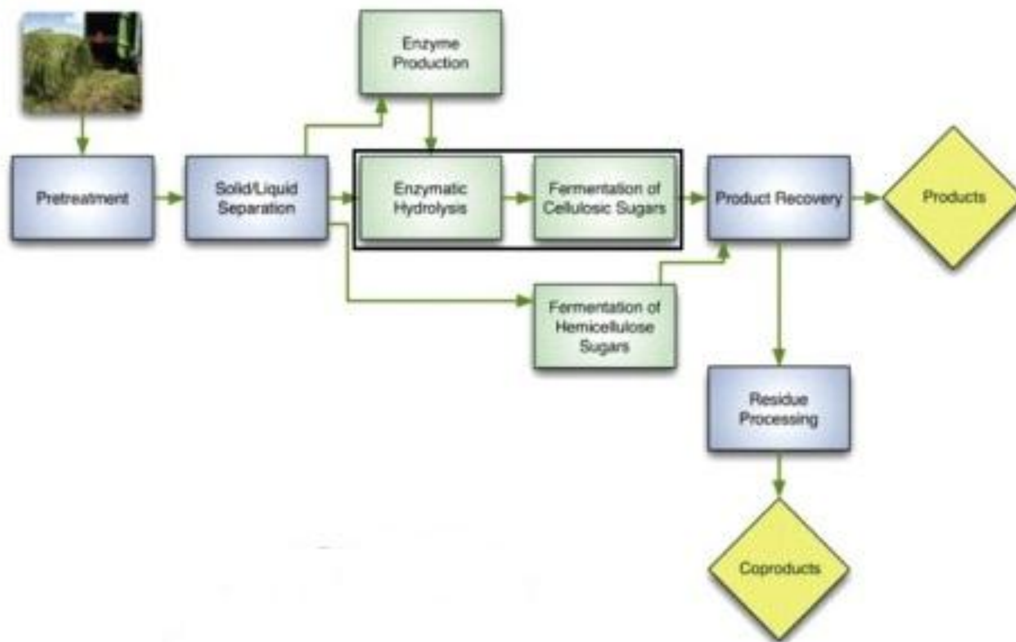

Figure 3.9. Scheme for simultaneous saccharification and fermentation (SSF)
(Source: Xu *et al*., 2009)

Simultaneous saccharification and fermentation is offen more effective when combined with dilute-acid or high-temperature hot-water pretreatment. It is a batch process using lignin, pectin and lignocelluloses. Increase in hydrolysis rate by conversion of sugars that inhibit the cellulase activity, higher product yield, lower enzyme requirement, lower requirement for sterile conditions, shorter process time and less reactor volume are major advantages of SSF. However, requirement of different temperature optima for saccharification and fermentation is the main drawback of this process (Balat *et al*., 2008).

More recently, the SSF process has been improved as simultaneous saccharification and co-fermentation (SSCF) that can ferment hexose and pentose sugar simultaneously. SSF and SSCF are favoured since both processes can be carried out in the same vessel, resulting in lower costs.

3.7. Utilization of Agricultural Wastes and Fruit Pomaces as Lignocellulosic Fermentation Media for Bioethanol Production

Bioethanol production from agroindustrial wastes have raised interest within last 30 years because of its suitablity as low-cost alternative to replace fossil fuels. Table 3.6. summarizes the lignocellulosic compositions of some agricultural residues, fruits and vegetables.

Table 3.6. Lignocellulosic compositions of some cellulosic wastes (g/100g of dry matter) (Source: Das and Singh, 2004)

Cellulosic wastes	Cellulose	Lignin	Hemicellulose
1. Agricultural Residues			
Barley straw	44	7	27
Oat straw	41	11	16
Rice straw	33	7	26
Wheat straw	39	10	36
Sorghum bagasse	31	11	30
Cottonseed hulls	59	13	15
Sugarcane bagasse	40	13	29
2. Fruits & Vegatables			
Apples	2.9	Trace	5.8
Bananas	1.3	0.93	3.83
Oranges	-	14	-
Strawberries	3.6	8.4	10
Carrot	12.9	Trace	19
Cabbage	8.9	4.3	26

Production of bioethanol with direct bioconversion process from palm-oil mill effluent (POME) generated by the oil-plam industries was studied by Alam *et al.* (2009). POME provides a good source of fermentaion media wtih its high content of carbohydrates (29.55%), proteins (12.75%), nitrogeneous compounds, lipids with a considerable amount of cellulose and nontoxic mineral. The bioethanol production was carried out with mixed cultures such as *Trichoderma harzianum*, *Phanerochaete chrysosporium*, *Mucor hiemalis* and *Saccharomyces cerevisiae*. According to their results, the mixed culture of *T.harzianum* and *S.cerevisiae* yielded the highest ethanol production (4% v/v or 31.6 g/l). Statistical optimization was carried out in the stirred-tank bioreactor for maximum bioethanol production by two-level fractional factorial design. Oxygen saturation level, temperature and pH were determined as factors. Statistical analysis showed that the maximum ethanol production of 4.6% (v/v) or 36.3 g/l was achieved at a temperature of 32°C.

Cheese whey has also been used as a fermentation media for bioethanol pruduction. Whey was fermented for decades in New Zealand. In Ireland, ultrafiltrated whey with yeast fermentation is used. In future, cheese whey will become a more important substrate due to the increase in cheese production and the problems during its disposal resulting from the high organic matter content (Antoni *et al*., 2007).

Arapoglou *et al*. (2010) presented a new form of potato peel waste (PPW) hydrolysis with a specific combination of enzymes and hydrochloric acid, subsequently fermented by *Saccharomyces cerevisiae var. bayanus* to determine ethanol production. Normally, potato peel is a zero value waste produced by potato processing units. However, bioethanol production from potato wastes has a large potential. In this study, PPW was hydrolyzed with various enzymes and/or acid and fermented by the yeast. According to their results, 18.5 g/l reducing sugar was released and 7.6 g/l ethanol was produced after fermentation. This study demonstrated that PPW showed high potential for bioethanol production.

According to the study conducted by Nigam (1999), pineapple cannery waste was examined for bioethanol production using continuous fermentation technique. It was demonstrated that pineapple cannery waste was a potential source of sugars, protein, vitamins and growth factors and could be used as a substrate for ethanol production. The total sugar content of pineapple cannery waste was found 81.3 – 83.4 g/l where reducing sugar content varied between 38.2 and 40.1 g/l. Maximum ethanol yield (92.5% of the theoretical) was obtained at a dilution rate of 0.05 h^{-1}.

Effect of the composition and culture conditions on production of bioethanol with batch fermentation by a native strain of *Zymomonas mobilis* was investigated by Pinilla *et al.* (2011). Without optimization *Z.mobilis* reached a maximum ethanol concentration of 79.78 g/l. Then, the effect of different nitrogen sources on production of bioethanol was evaluated. The best result (83.81 g/l) was obtained using urea at a 0.73 g/l.Yield of ethanol on biomass, maximum volumetric productivity of ethanol, specific productivity of ethanol and specific growth rate was evaluated and the results were found as 69.01 g g^{-1}, 2.28 g l^{-1} h^{-1}, 3.54 h^{-1}, 0.12 h^{-1}, respectively. With the optimization of culture conditions using Placket-Burman's experimental design maximum ethanol production reached 93.55 g/l.

Fruit and vegetable canning industry are important lines of business. However, wastes coming from these industries reach approximately 450.000 tonnes per year just for Spain. In order to convert wastes to value added products many researches have

been done. One of the studies that investigates the usability of wastes of fresh and processed vegetable as a fermentation raw material with diluted acid hydrolysis was conducted by Campo *et al*. (2006). Tomato, red pepper, pulse food, artichoke and cardoon were screened. It was found that the maximum single sugar recovery in the dilute acid hydrolysis assays were 40.29 and 50.20% (w/w) for tomato and red pepper, respectively. More extensive pretreatnent was needed for pulse food and artichoke and maximum sugar recovery of 787.18% (w/w) was obtained for the liquid fraction of cardoon residues. They concluded that the sugars in fresh and processed vegetable wastes are widely available and easily obtainable and they could be considered as potential feedstocks for bioethanol production.

Japanese cedar wood was used for bioethanol production in another study (Baba *et al*., 2010). They applied combined pretreatments due to the recalcitrance of the softwood. To increase the sugar yield, they pretreated the fungal biomass. They obtained 42.2 g of total reducing sugars per 100 g of the fungus-pretreated biomass.

Ethanol production from rice straw by simultaneous saccharification and fermentation with *Mucor indicus*, *Rhizopus oryzae*, and *Saccharomyces cerevisiae* was investigated and compared with pure cellulose by Karimi *et al*. (2005). It was found that all the strains were able to produce ethanol from the pretreated rice straw with an overall yield of 40 – 74% of the maximum theoretical SSF yield. *R.oryzae* had the best ethanol yield (74%) followed *by M.indicus* (68%).

The direct fermentation of cellulosic biomass to bioethanol has long been a desired goal. For this purpose, Stevenson and Weimer (2002) screened the environment for fungal strains capable of this conversion when grown on minimal medium. A member of the genus *Trichoderma* was isolated from a cow dung which was able to produce 0.4 g ethanol l^{-1} initially. It was observed that *Trichoderma* could not grow on any substrate under anaerobic conditions but could ferment cellulose or various sugars to ethanol. Also with the use of vented fermentation flasks, ethanol amount reached to 2 g/l. Highest levels of ethanol (>5 g/l) were obtained by the fermentation of glucose. Low amount of ethanol was produced by the fermentation of xylose. It was also found that *Trichoderma*, which was most consistent with *T.harzianum*, was able to use a wide range of carbon sources such as D-galactose, D-mannose, D-xylose, D-arabinose, D-ribose, cellobiose, polygalacturonic acid, purified citrus pectin and starch.

Even though ethanol production is common among certain species, filamentous fungi are not well known for their abilities. Numerous fungi are able to produce low

concentrations of ethanol compared to *S.cerevisiae*, under O_2 limited conditions. Many of them have various enyzmes such as xylanase, cellulase and amylase complexes. This enables the simultaneous saccharification and fermentation with one organism. A study that screen ethanol producing filamentous fungi was conducted by Skory *et al*. (1997). Nineteen *Aspergillus* species were tested for their efficiency of converting glucose, xylose and cellulose to ethanol. One strain, *A.oryzae*, reached nearly 100% theoretical ethanol yield from 50 g/l glucose. However, no appreciable ethanol production was obtained with the usage of crystalline cellulose. Also several *Rhizopus* strains were tested. It was found that *R.oryzae* and *R.javanicus* were more efficient fermenting simple sugars compared to Aspergillus species.

Orange peels were used as fermentation raw material for bioethanol production (Oberoi *et al*., 2010). Orange peels were evaluated as a fermentation feedstock and process parameters were determined. First step hydrolysis of orange peel was carried out at acid concentrations ranging from 0 to 1.0% (w/v) at 121 ^{o}C for 15 min and second step hydrolysis was carried out at 0.5% (w/v) acid. Response surface methodology was used to optimize the effect of pH, temperature and fermentation time on ethanol production. Ethanol yields of 0.25 g/g on a biomass basis, 0.46 g/g on a substrate basis and volumetric productivity of 3.37 g/l/h were obtained.

In another study (Patle and Lal, 2007), ethanol producing strains were isolated from raw honey, molasses and rotten fruits such as grapes, apple and sapota. Their usability for ethanol production was investigated using mixed culture of *Zymomonas mobilis* and *Candida tropicalis*. According to their results, enzymatic hydrolysis of these agricultural crop wastes gave the best results in terms of reducing sugars (36-123 g/l) and ethanol amount (11-54 g/l). They suggested that these wastes were proved to be promising substrates for ethanol production.

One of the other possible candidate that could be used as a fermentation raw material for bioethanol production is grape pomace due to its significance amounts of fermentable sugars that are retained in the pomace after pressing of the grapes (Korkie *et al*., 2002). The study revealed that significant amounts of ethanol could be produced from the fermentable sugars obtained from the hydrolysis of grape pomaces. There was another study conducted by Rodríguez *et al*. (2010) that investigated bioethanol production from grape pomace via solid state fermentation. The results of the study showed that the ethanol production from grape pomace via solid state fermentation yielded higher compared to liquid fermentations.

Cashew apple juice was also utilized for ethanol production. Pinheiro *et al.* (2007) studied ethanol production from cashew apple juice by fermentation using *Saccharomyces cerevisiae*. Growth kinetics and ethanol productivity were evaluated with different initialsugar concentrations and according to their results maximum ethanol, cell and glycerol concentrations were reached when 103.1 g L^{-1} of initial sugar concentration was used. Cell yield was found as 0.24 (g microorganism)/(g glucose + fructose) using cashew apple juice as fermentation medium with 41.3 g L^{-1} of initial sugar. These results indicated that cashew apple juice was a suitable fermentation raw material for growing yeast and producing bioethanol. Moreover, ethanol production using immobilized yeast cells was investigated in the study of Neelakandan and Usharani (2009). The effects of some fermentation parameters such as substarate concentration, pH, temperature and inoculum concentration on bioethanol production were the research parameters. They concluded that the fermentation that would be carried out at pH 6.0, temperature of 32.5^{o}C, 10% of substrate concentration and inoculum level of 8% (v/v) were the optimized conditions for bioethanol production from cashew apple juice.

Even kitchen garbage collected from the dining room of University of Science and Technology of Beijing, China was used for biothanol production. Wang *et al.* (2008) used response surface methodology for searching optimized conditions for bioethanol production from kitchen garbage with simultaneous saccharification and fermentation of *Saccharomyces cerevisiae*. Both open and close fermentations were carried out. The results showed that open fermentation was better due to the unspoiled nutrients inside with the maximum ethanol concentration of 33.05 g/l. The optimum conditions were determined as time of 67.60 h, pH of 4.18 and temperature of 35 ^{o}C.

A co-culture of *Saccharomyces cerevisiae G* and *Pachysolen tannophilus MTCC 1077* was used to evaluate some fermentation parameters such as inoculum rate, temperature, incubation and agiation time on bioethanol production from kinnow waste and banana peels by simultaneous saccharification and fermentation (Sharma *et al.*, 2007). Temperature of 30°C, inoculum rate of *S.cerevisiae G* 6% (v/v) and *P.tannophilus* of 4% (v/v), incubation time of 48h and agitation time of 24h were determined as the optimum conditions. 63 g/l reducing sugars were obtained and 26.84 g/l ethanol was produced.

In an another study, consolidated continuous solid-state fermentation (CCSSF) was developed as an alternative system to maintain yeast activity, decrease amount of

waste water and the number of process steps. It combined simultaneous saccharification and fermentation with continuous recovery of bioethanol in solid-state fermentation (Moukamnerd *et al*., 2010).

Stillage is the primary residue generated from the starch-to ethanol fermentation process. It has high carbohydrate content (hemicellulose + cellulose). According to study conducted by Davis *et al*. (2005), the hydrolysis with the optimum conditions generates fermentable sugars for bioethanol production. This provided an opportunity to produce additional bioethanol.

These numerous studies indicate that waste from fruit or vegetable could be used as a potential fermentation media for industrial applications.

3.8. Apple Pomace as a Fermentation Media for Bioethanol Production

Food industry forms a large quantity of wastes, such as peel, seed, pomace, rags, kernels etc. Apple pomace is one of the wastes coming from the food industry which contains peel, seeds and remaining solid parts which is formed after juice extraction. Pomace represents approximately 25-35% of the weight of the fresh apple processed (Joshi and Devender, 2006). Because it is waste, its accumulation is a primary environmental problem as it is valid for other agroindustrial wastes. Due to its composition (richness in carbohydrates, dietary fibres and minerals), it can be used for the microbial production of value added products. Bioethanol is one of the value added products that can be produced from apple pomace. Also apple pomace can be used in animal feed, production of pectin esterase enzyme and production of different biocolors. Utilization of apple pomace for these purposes can lead the way of producing value added products from similar agroindustrial wastes.

The approximate composition of apple pomace is given in Table 3.7.

Table 3.7. Approximate composition of apple pomace
(Source: Joshi and Devender, 2006)

Constituents	Composition	
	Wet weight basis	Dry weight basis
Moisture (%)	66.4-78.2	3.97-5.40
Acidity (% malic acid)	NA	2.54-3.28
Total soluble solids (TSS)	NA	57.85
Total carbohydrate (%)	9.50-22.00	48.00-62.00
Glucose	6.10	22.70
Fructose	13.60	23.60
Sucrose	NA	1.80
Xylose	NA	0.06
pH	3.05-3.80	3.90
Vitamin C (mg/100g)	-	8.53-18.50
Soluble proteins (%)	NA	3.29
Protein (%)	1.03-1.82	4.45-5.67
Crude fibre (%)	4.30-10.50	4.70-48.72
Fat [ether extract (%)]	0.82-1.43	3.49-3.90
Pectin (%)	1.50-2.50	3.50-14.32
Ash (%) NA	1.60	
Polyphenol (%)	NA	0.95
Amino acids (%)	NA	1.52
Minerals:		
Potassium (%)	NA	0.95
Calcium (%)	NA	0.06
Sodium (%)	NA	0.20
Magnesium (%)	NA	0.02
Copper (mg/l)	NA	1.10
Zinc (mg/l)	NA	15.00
Manganese (mg/l)	NA	8.50-9.00
Iron (mg/l)	NA	230.00
Calorific (kcal/100g)	NA	295.00

NA= Not Applicable

As it is seen from the table, apple pomace is a good substrate with its rich components like pectin, carbohydrates, dietary fibres, minerals and vitamin C.

Therefore, value added products such as ethanol, pectin esterase enzyme, citric acid can be produced from apple pomace by fermentation or it can be used as animal feed. Also they are easy to obtain, are not hardwood or softwood materials (harsh and expensive pretreatment methods are not necessary, on the contrary, mild pretreatment methods such as dilute acid hydrolysis is enough to decompose polysaccharides into monosaccharides) and have considerably high fermentable sugar contents. Because of these reasons, apple pomaces are not only candidates for bioethanol production feedstocks, but also for all kinds of other fermentation media.

3.9. Bioethanol Production from Apple Pomace with Dilute Acid Hydrolysis

The composition of fruit pomaces vary according to the type of processing applied for juice extraction, especially regarding how many times the fruits were pressed. According to the results of the study conducted by Ucuncu (2012). Shown in Figure 3.10. , all of the four pomaces could be used as fermentation media for bioethanol production with adequate moisture and dietary fiber content and with considerably high reducing sugars amount. Although orange pomace had the highest reducing sugar amount, apple pomace was chosen as fermentation media since only the apple pomace optimization was successfull among other pomaces.

	Peach	Apple	Apricot	Orange
Soluble ash in wet weight (%)	0.36 ± 0.00	0.06 ± 0.01	0.6 ± 0.1	0.3 ± 0.00
Soluble ash in dry weight (%)	2.15 ± 0.00	0.22 ± 0.04	3.34 ± 0.1	1.59 ± 0.07
Insoluble ash in wet weight (%)	0.09 ± 0.00	0.22 ± 0.01	0.19 ± 0.1	0.35 ± 0.00
Insoluble ash in dry weight (%)	0.54 ± 0.00	0.82 ± 0.04	1.12 ± 0.1	1.89 ± 0.07
Total ash in wet weight (%)	0.45 ± 0,00	0.28 ± 0.00	0.79 ± 0.01	0.65 ± 0.02
Total ash in dry weight (%)	2.69 ± 0.01	1.04 ± 0.01	4.47 ± 0.1	3.49 ± 0.2
Protein (%)	1.31 ± 0.05	1.9 ± 0.20	1.29 ± 0.01	1.54 ± 0.3
Total solids (%)	16.69 ± 0.2	27.53 ± 0.1	17.75 ± 0.5	18.81 ± 0.5
Soluble solids (%)	8.09 ± 0.07	2.23 ± 0.03	10.74 ± 0.06	11.53 ± 0.2
Insoluble solids (%)	8.59 ± 0.07	25.30 ± 0.03	7 ± 0.06	7.28 ± 0.2
Total dietary fiber (%)	18.28 ± 1.5	32.54 ± 0.5	14.6 ± 1.0	13.9 ± 1.5
Soluble dietary fiber (%)*	13.85 ± 2.0	11.24 ± 0.2	11.32 ± 1.5	8.40 ± 1.0
Insoluble dietary fiber (%)*	7.06 ± 1.2	25.24 ± 1.0	5.86 ± 2.5	8.61 ± 0.5
Moisture content (a_w)	0.89	0.84	0.87	0.83
Initial reducing sugar (%)	22.08 ± 0.00	6.25 ± 0.01	22.91 ± 0.02	33.89 ± 0.03

*Involves protein

Figure 3.10. The chemical composition of fruit pomaces
(Source: Ucuncu, 2011)

Apple pomace was pretreated with the phosphoric acid (H_3PO_4) because after neutralization of hydrolysates with NaOH, the salt formed is sodium phosphate, which can remain in the hydrolysates since it can be used as nutrient by microorganisms. Therefore, a filtration operation is not needed with the consequent advantages: the improvement of process profitability (avoiding salts removal and decreasing the amount of nutrients needed for fermentation) and positive impact to the environment (the salt formed is not a waste) (Gamez *et al*., 2006; Cardona *et al*., 2009).

CHAPTER 4

MATERIALS AND METHODS

4.1. Materials

4.1.1. Apple Pomace

Apple pomace, composed of almost just peels of approximately 1 cm^2-sized particles, was obtained from "Konfrut Fruit Juice Concentrates and Purees" in ice bags and stored until usage at -20 ^oC in plastic packages. It did not require any chopping before use.

Figure 4.1. Appearance of apple pomace

4.1.2. Microorganisms

A total of three strains, two fungal and one yeast, were used in fermentation experiments. The fungal strains were *Trichoderma harzianum* and *Aspergillus sojae ATCC 20235*, and the yeast was *Saccharomyces cerevisiae*. *T.harzianum* was kindly provided by Paul J. Weimer from USDA-ARS-US Dairy Forage Research Center, Madison. *A.sojae* was obtained from the laboratory stock of Dr. Tarı and *S.cerevisiae*

was obtained from Molecular Biology Laboratory, İzmir Institute of Technology, Urla, İzmir.

4.2. Methods

4.2.1. Chemical Compositional Analysis of Apple Pomace

In order to determine the chemical composition of apple pomace analyses such as *protein determination*, *water activity*, *determination of both soluble and insoluble solids ash* and *dietary fiber content*, were conducted by Ucuncu (2011) therefore, only reducing sugar was assayed in this study.

Reducing sugar assay: 100 ml suspension containing 10g of each pomace was autoclaved for 5 min at 105°C. The filtered liquid part was used for Nelson-Somogyi (Somogyi, M., 1952) reducing sugar assay in order to determine the total reducing sugar content in each pomace sample.

4.2.2. Hydrolysation of Apple Pomace

According to the study conducted by Ücuncu (2011), temperature of 110°C, 40 minutes, 4% phosphoric acid and 10% solid liquid ratio were determined as optimum hydrolysis conditions. Hydrolysates were filtered, pH of apple pomace hydrolyzate was adjusted to 5.0, that was appropriate for fermentation, using 6N NaOH and sterilised at 121^{0}C for 15 minutes. Finally after these steps, Nelson – Somogyi reducing sugar assay was used in order to determine total reducing sugar of apple pomace hydrolyzate if there were any reduction due to the steps before. Results were expressed as gram per liter and percentage of total reducing sugar conversion from initial total dry weight. Calculation of percentage of total reducing sugar conversion is shown in below:

$$(100\times Y)/X$$

where Y is the gram of convertible sugar and X is the gram of dry weight in the unpretreated biomass. If pomace has X gram of dry weight before pretreatment, there will be X-Y gram dry weight and X+Y gram reducing sugars after pretreatment. This

increase in reducing sugars and decrease in dry weight is because the reason of depolymerization of polysaccharides (cellulose + hemicellulose).

Furfural and hydroxymethlyfurfural (HMF): For the determination of furfural and HMF, HPLC (High Pressure Liquid Chromatography) were used with HPX-87H column . Flow rate was adjusted to 0.6 mL/min. The temperatures of column and detector were 60°C and 50°C , respectively.

4.3. Growth of Microorganisms

4.3.1. Spore Production of *A.sojae*

The pre-activation of *A.sojae* cultures was done on YME agar medium using stock cultures in petri dishes. The media composed of malt extract (10g/l), yeast extract (4 g/l), glucose (4 g/l) and agar (20 g/l). Incubation was carried out at 30 ^{o}C and 1 week (until well sporulation). The inoculum in the form of spore suspensions obtained from on molasses agar slants containing glycerol (45 g/l), molasses (45 g/l), peptone (18 g/l), NaCl (5 g/l), $FeSO_4.7H_2O$ (15 mg/l), KH_2PO_4 (60 mg/l), $MgSO_4$ (50 mg/l), $CuSO_4.5H_2O$ (12 mg/l), $MnSO_4.H_2O$ (15 mg/l) and agar (20 g/l) was used. The incubation time and temperature were the same as in the pre-activation step. Spores were harvested using 5 ml of Tween80-water (%0.02) and collected in a sterile falcon tubes. Spore solutions were stored at 4 ^{o}C until the actual study, but not more than one week. The spore counts were performed using Thoma bright line hemacytometer (Marienfield, Germany) and results were recorded. Viability check was performed by cultivating of diluted spore solutions on YME plates and incubating for at least 72 hours and afterwards counting the colonies formed. Sterility was checked by cultivating 100 μl of spore solution on Brain Heart Infusion Agar (BHI) for 24-48 hour at 30 ^{o}C.

4.3.2. Spore Production of *T.harzianum*

The propagation of cultures was done on MEA petri dishes containing malt extract (30 g/l), peptone (3.0 g/l) and agar (15 g/l), incubated at 30 ^{o}C until well sporulation (5 -7 days). The spore suspension was obtained from MEA slants with the same formulation and under the same conditions as described below. Spores were

harvested using 5 ml of Tween80-water (%0.02) and collected in a sterile falcon tube. Spore solutions was stored at 4 ^{o}C until the actual study, but not more than one week. The spore counts were performed using Thoma bright line hemacytometer (Marienfield, Germany) and results were recorded. Viability check was performed by cultivating of diluted spore solutions on MEA plates and incubating for at least 72 hours and afterwards counting the colonies formed. Sterility was checked with cultivating 100 µl of spore solution on Brain Heart Infusion Agar (BHI) for 24-48 hour at 30 ^{o}C.

4.3.3. Propagation of Yeast

S.cerevisiae was propagated on YPD media containing glucose (2% (v/v)), peptone (2% (v/v)), yeast extract (1% (v/v)) and agar (2%(v/v)) at 30 ^{o}C for 48 hour. Cultures were regenerated every month on a fresh plate.

The stock cultures of both fungi and yeast were maintained on glycerol stocks (20% (v/v)) and stored at -80 ^{o}C for longterm preservation. Cultures were regenerated for each experiment on a fresh plate from the frozen stock cultures.

4.4. Growth Curve Determination of the First Subculture of *S.cerevisiae*

Growth curve was constructed in order to determine the specific growth rate of *S.cerevisiae* and the right incubation time to be added to fermention media. A loop-full of 48h-old single colony was transferred from a fresh YPD agar plate into 250 ml Erlenmayer flask containing of 50 ml YPD broth media and incubated at 30 ^{o}C and 150 rpm in basic orbital shaker for 48h. During this time period, samples were taken at certain time intervals for viable cell count and optical density. Samples were diluted at certain dilutions and inoculated on YPD plates by spread plate technique. The optical cell densities were determined by using Varian Cary Bio 100 spectrophotometer at 600 nm.

4.5. Fermentation

4.5.1. Aerobic Growth

A.sojae was grown in 250 ml Erlenmayer flasks containig 50 ml molasses broth media. Initial spore count was adjusted to approximately 1×10^7 spore/ml and used for the inoculation of the flasks which were incubated at 30 ^oC in a 200 rpm rotary shaker based on a study conducted by Skory et al. (1997). Incubation time was determined as 48h in order to obtain larger pellets.

T.harzianum was grown on minimal medium (MM) which was the yeast nitrogen base medium of Wicherham and Burton (1948) with glucose as carbon source. YNB (Minimal medium) was prepared by dissolving 6.7 grams of the medium in 100 ml distilled water, heated without boiling until complete dissolution. The media was sterilized by filtration and stored at 4 ^oC. Before use this media was diluted 10 times. Flasks were inoculated with conidia (~1×10^7 spore/ml) and incubated at 30^oC in a 150 rpm rotary shaker.

S.cerevisiae was grown until reaching the log phase in a 150 rpm rotary shaker on YPD broth media at 30^oC.

4.5.2. Anaerobic Fermentation

The mycelial mass coming from aerobically grown cultures was added into the anaerobic fermentation media, which was the apple pomace hydrolysate. 40 ml hydrolysate was added into 50-ml Erlenmayer flasks in order to leave ~20 % of the culture flask volume as air space. Fermentation experiments were conducted for 5 days at 30^oC. Samples were taken within certain time intervals, centrifuged at 6000 g for 15 minutes. The supernatants were stored at -18^oC for further analysis.

4.6. Statistical Design of Experiments

Design Expert Version 7.0.0 was used for the statistical experimental design for all the fermentation experiments. The response was ethanol production (g/l).

4.6.1. Determination of the Inoculation Time

In order to determine inoculation time of the microorganisms, general factorial design was used. Factors were designated as inoculation time of *T.harzianum*, *A.sojae* and *S.cerevisae* with four levels, 24h, 48h, 72h and 96h. In order to make the experiments more practical, designs were separated into two parts. For the first design, *T.harzianum* was inoculated into the flasks at 0^{th}h. The other two organisms were combined with each other. And for the second design, *A.sojae* was inoculated into the flasks at the beginning and remaining organisms were combined with each other, again. All of the factors were given with their levels shown in Table 4.1, Table 4.2, respectively.

Table 4.1. Factors and levels of the first design (*T.harzianum* inoculated at 0th hour)

Factors		
Level	*A.sojae* inoculation time	*S.cerevisiae* inoculation time
0	0 h	0 h
1	24 h	24 h
2	48 h	48 h
3	72 h	72 h
4	96 h	96 h

Table 4.2. Factors and levels of the second design (*A.sojae* inoculated at 0th hour)

Factors		
Level	*T.harzianum* inoculation time	*S.cerevisiae* inoculation time
0	0 h	0 h
1	24 h	24 h
2	48 h	48 h
3	72 h	72 h
4	96 h	96 h

Total of 50 experiments were conducted with 2 replicas of each factorial combinatinations for both designs.

4.6.2. Screening of Fermentation Parameters

2-level full factorial design was used in order to identify important parameters in the screening analysis. The factors were determined as inoculation rate of *A.sojae*, *T.harzianum*, and *S.cerevisiae*, aeration and agitation rate. Total of 40 experiments were conducted with 8 center points.

Table 4.3. Factors and levels of 2^5 factorial design.

FACTORS					
Level	*T.harzianum* Inoculation rate (w/v)%	*A.sojae* Inoculation rate (w/v)%	*S.cerevisiae* Inoculation rate (v/v)%	Aeration	Agitation (rpm)
(+1)	4	4	4	Vented	0
(-1)	20	20	20	Sealed	200

Inoculation rates of microorganisms were expressed as percentage, since after aerobic growth large amount of mycelial mass was formed, which made the total mycelial mass addition into the fermentation flasks almost impossible. Based on this it was decided to keep the percentages in a broad range in order to catch any possible effect on ethanol production. After aseptically inoculation of the mycelial mass from aerobic fermentation, plastic paraffin film was used to seal the flasks. Sealed flasks provided strictly anaerobic conditions, whereas vented flasks allowed small amounts of gases (O_2 and CO_2) to pass in and out through a silicone-41 tubing (1.6 × 1.6 = 4.8 mm, Silicone tubing), packed tightly with cotton. The two methods are shown in Figure 4.1.

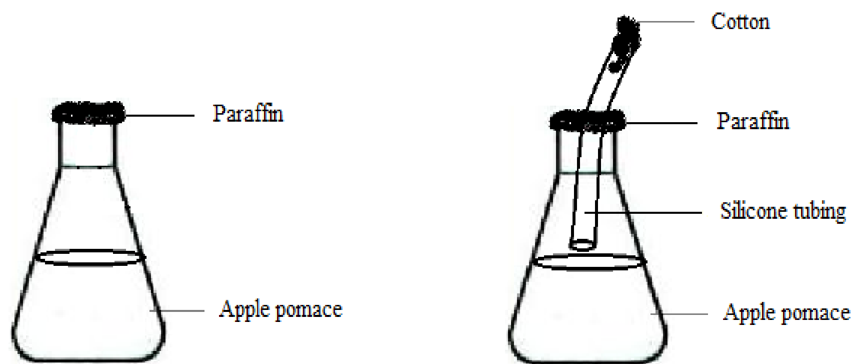

Figure 4.1. Sealed (on the left) and vented (on the right) aeration methods.

Finally, in order to investigate the effect of agitation, factor levels was determined as 0 and 200 rpm.

4.6.3. First and Second Step of Optimization of Bioethanol Production

In this study, a face centered central composite design (CCD) was generated and conducted with three factors; which were inoculation rate of *A.sojae* (X1), inoculation rate of *T.harzianum* (X2) and agitation (X3).

Analysis of data and generation of response surface graphics was done by using Design Expert Version 7.0.0 software.

The analysis of variance (ANOVA) tables were generated and the effect and regression coefficents of individual linear, quadratic and interaction termes were determined. The significancy of all terms in the model were judged statiscally according to the p-value. p-values were compared to the significance level of %5.

Table 4.4. Two step optimization factor with actual and coded levels

Factors	First step optimization with actual and coded levels	Second step optimization with actual and coded levels
A.sojae ino.rate (w/v)%	0 (-1) and 6 (+1)	10 (-1) and 30 (+1)
T.harzianum ino.rate (w/v)%	0 (-1) and 6 (+1)	10 (-1) and 30 (+1)
Agitation (rpm)	100 (-1) and 300 (+1)	100 (-1) and 300 (+1)

4.7. Biomass Determination

The biomass represented as dry cell weight (DCW – (g/l)) was determined by the gravimetric method. The fermentation broth (at the end of the fermentation) was filtered through the preweight Whatman No.1 filter paper, followed by drying to constant weight at 95 ^{o}C for approximately 24h.

4.8. Total Carbohydrate Assay

The total carbohydrate contents of the samples (cell-free supernatant) were determined according to the phenol sulfuric acid method described by Dubois et al. (1956). Carbohydrate standard calibration curve was prepared with D-glucose with the range of 10-200 μg/ml as the standard. The amount of carbohydrates were determined by using Varian Cary Bio 100 UV-Visible spectrophotometer at 490 nm against the blank.

4.9. Total Reducing Sugar Assay

The total reducing sugar amount were determined according to the assay given by Nelson Somogyi (1952). The absorbance was read on Varian Cary Bio 100 UV-Visible spectrophotometer at 500 nm against water.

4.10. Bioethanol Determination

One to two ml of fermentation medium coming from the shake flasks were sampled and centrifuged at 6000 g for 5 min. The supernatants were kept at – 18 ^{o}C until used. At the time of samples were thawed at 4 ^{0}C, diluted with HPLC eluent analyzed for ethanol by HPLC using Biorad Aminex HPX-87H column equipped with the appropriate guard column. HPLC conditions were 10-25 μl of injection volume dependening on sample concentration and detector limits, 60 ^{o}C of cloumn temperature with the detector (refractive index-RID) temperature as close to column temperature as possible and flow rate set to 0.6 ml/minute. The mobil phase was 5 mM H_2SO_4 filtered

through 0.2 μm filter and degassed. Because approximate retention time of ethanol was 22.7 min., run time run time was designated as 30 min. Ethanol standard solutions of known concentrations were used for calibration.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Results of Hydrolysation of Apple Pomace

5.1.1. Furfural and Hydroxymetilfurfural

Furfural (F) and hydroxymetilfurfural (HMF) are decomposition products of pentose and hexose sugars. The formation is a first order reaction, where the reaction constant is affected by both acid concentration and temperature. However, the formation of HMF during dilute acid hydrolysis is a sequential reaction. Arabinose is the major sugar that is present in the apple pomace hydrolysates. Among the various pentose sugars exposed to acid hydrolysis, arabinose showed the lowest reactivity. Therefore, lack of furfural formation is most probably due to the stability of arabinose.

According to HPLC results, furfural or hydroxymetilfurfural could not be detected in the apple pomace hydrolysates. Since these components are inbihitory to fermentation, absence of these inhibitory products is a great advantage for efficiency of forthcoming fermentation.

5.2. Growth Curve Determination of the First Subculture of *S.cerevisiae*

Growth curve for yeast *S.cerevisiae* was constructed as described in section 4.4. Typical growth curve was obtained as it was seen from the Figure 5.1 that was plotted using the data given in Appendix B.

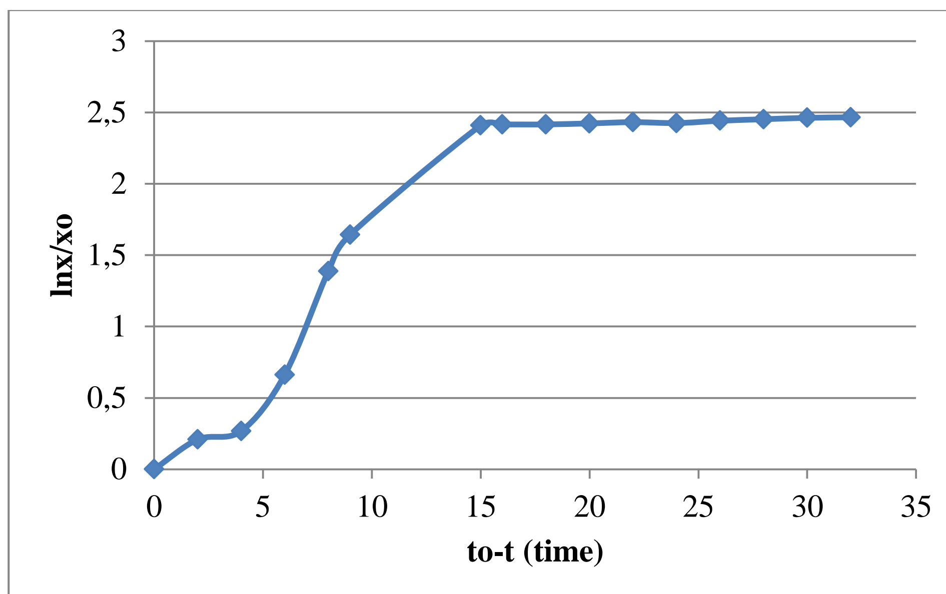

Figure 5.1. Growth curve for *S.cerevisiae*

As it was seen from Figure 5.1., logarithmic phase of the yeast was between the hours of 5th to 15th after onset of the inoculation. In order to obtain efficient fermentation period, 9-hour-grown cultures, that had the maximum spesific growth rate (μ = 0.182) were used for the inoculation of fermentation flasks.

5.3. Results of Statistical Design of Experiments

5.3.1. Determination of Inoculation Time

The fermentation of cellulosic biomass to ethanol directly has long been a desired goal. Some filamentous fungi have some advantages; (*i*) they can be directly inoculated onto cellulosic biomass as they do not require strictly anaerobic conditions, (*ii*) their filamentous growth habit facilitates separation of cell mass from the broth, (*iii*) the inoculation of non-sterile biomass is more practical since many fungal strains produce copious numbers of conidiospores, which could be useful for inoculation at a high level (Stevenson and Weimer, 2002). There are various reports about filamentous fungi such as *Aspergillus*, *Rhizopus* (Skory *et al*., 1997), *Monilia* (Gong *et al*., 1981), *Neurospora* (Deshpande *et al*., 1986) and *Fusarium* (Singh and Kumar, 1991), that these fungi are capable of directly fermenting cellulose to ethanol. In this stıdy *Trichoderma harzinum*, which is able to ferment cellulose or several sugars to ethanol

was chosen for ethanol production. This way, besides initial reducing sugars, remaining cellulosic compounds in hydrolysates can be fermented into ethanol as well. Stevenson and Weimer (2002) observed that, since *T.harzianum* could not actively grow under anaerobic conditions, ethanol production was increased by pre-growth to enhance the initial amount of mycelia used in the fermentation. So a pre-growth cycle was applied in order to increase the mass of mycelia and initiate fermentation. Because of these advantages compared to *S.cerevisiae*, *T.harzianum* and *A.sojae* were selected besides the yeast.

Inoculation time is very important to be able to obtain high amounts of bioethanol. Therefore, a general factorial design was used in order to determine the inoculation time of the microorganisms under consideration. The results are given below in Table 5.1. These are later discussed individually in forthcoming sections. The actual ranges for each variable were inoculation day of *T.harzianum* (X_1), *A.sojae* (X_1) and *S.cerevisiae* (X_2) and their interactions (X_{12}). The levels were inoculation at 0th hour, 24th, 48th, 72th and 96th hour. The hours were numbered in the design in the same order as 0,1,2,3 and 4.

Table 5.1. Screening results of the inoculation time with respect to bioethanol production (*A.sojae* was inoculated into the fermentation flasks at the beginning).

Run NO	Actual levels of variables		Bioethanol production(g/l)
	T.harzianum	*S.cerevisiae*	
1	3	3	4.804
2	2	1	7.249
3	4	4	5.190
4	0	2	3.663
5	4	1	5.685
6	1	2	3.864
7	1	4	5.525
8	3	1	4.079
9	2	4	4.295
10	4	3	3.465
11	1	0	2.988
12	4	2	6.058
13	0	4	5.825
14	2	0	5.364
15	3	4	4.139
16	3	2	6.338
17	3	0	5.760
18	0	1	7.417
19	1	3	5.471
20	1	1	4.922
21	0	3	3.835
22	4	0	0.510
23	2	2	4.542
24	0	0	3.387
25	2	3	4.793
26	4	0	0.980
27	0	0	4.475
28	4	4	5.090
29	2	3	4.832
30	3	2	6.129
31	1	4	5.876
32	2	0	4.698
33	0	3	5.991
34	0	1	7.146
35	2	4	4.123
36	1	2	3.456
37	4	0	1.930
38	4	1	5.534
39	3	0	6.079
40	1	0	2.624
41	3	1	3.984
42	3	4	4.965
43	0	2	4.965
44	0	4	5.307
45	2	2	4.840
46	3	3	4.479
47	1	3	5.569
48	1	1	5.085
49	2	1	6.979
50	4	3	3.615

Table 5.2. Screening results of the inoculation time with respect to bioethanol production. (*T.harzianum* was inoculated into the fermentation flasks at the beginning).

Run NO	Actual levels of variables		Bioethanol production(g/l)
	A.sojae	*S.cerevisiae*	
1	3	3	4.874
2	2	1	6.756
3	4	4	2.934
4	0	2	4.567
5	4	1	5.685
6	1	2	4.569
7	1	4	3.943
8	3	1	3.731
9	2	4	5.480
10	4	3	3.093
11	1	0	6.567
12	4	2	2.999
13	0	4	4.742
14	2	0	5.769
15	3	4	3.456
16	3	2	5.526
17	3	0	4.612
18	0	1	7.278
19	1	3	5.479
20	1	1	6.522
21	0	3	3.875
22	4	0	0.710
23	2	2	4.942
24	0	0	6.387
25	2	3	4.993
26	4	0	0.880
27	0	0	4.775
28	4	4	2.090
29	2	3	3.852
30	3	2	4.329
31	1	4	3.476
32	2	0	6.698
33	0	3	5.791
34	0	1	7.012
35	2	4	4.123
36	1	2	3.456
37	4	0	1.230
38	4	1	5.234
39	3	0	5.479
40	1	0	2.924
41	3	1	3.854
42	3	4	4.365
43	0	2	5.365
44	0	4	4.307
45	2	2	4.810
46	3	3	4.476
47	1	3	5.517
48	1	1	5.085
49	2	1	6.989
50	4	3	3.815

According to the ANOVA results (Table 5.3.) considering the inoculation of *A.sojae* at the beginning of the fermentation, the model F-value of 19.05 implied that the model was significant ($p<0.0001$). There was only a 0.01% chance that a Model F-value this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicated model terms were significant. In this case the inoculation time of *T.harzianum* (X_1), the inoculation time of *S.cerevisiae* (X_2), and their interactions (X_{12}) were significant model terms. Values greater than 0.1000 indicated the model terms were not significant. The "Lack of Fit F-value" of 7.22 implied that the Lack of Fit was not significant relative to the pure error. There was a 28.69% chance that a "Lack of Fit F-value" this large could occur due to noise.

Table 5.3. Analysis of variance (ANOVA) for determining inoculation time of microorganisms (*A.sojae* at the beginning).

Source	**Sum of Squares**	**df**	**Mean Square**	**F value**	**p-value**	
Model	99.44	24	4.14	19.05	< 0.0001	*Significant*
X_1	13.78	4	3.45	15.84	< 0.0001	
X_2	27.94	4	6.98	32.12	< 0.0001	
X_{12}	55.04	16	3.44	15.82	< 0.0001	
Residual	5.22	24	0.22			
Lack of Fit	5.19	23	0.23	7.22	0.2869	*not significant*
Pure Error	0.031	1	0.031			
Cor Total	104.68	49				
Std. Dev.	0.47			R-Squared	0.9501	
Mean	4.75			Adj R-Squared	0.9003	
C.V. %	98.82			Pred R-Squared	N/A	
PRESS	N/A			Adeq Precision	19.560	

According to the ANOVA results (Table 5.4.) considering the inoculation of *T.harzianum* at the beginning of the fermentation, the model F-value of 6.17 implied that the model was significant ($p<0.0001$). There was only a 0.01% chance that a Model F-value this large could occur due to noise. The inoculation time of *A.sojae* (X_1), the inoculation time of *S.cerevisiae* (X_2), and their interactions (X_{12}) were significant model terms. The "Lack of Fit F-value" of 10.97 implied that the Lack of Fit was not significant relative to the pure error. There was a 23.45% chance that a "Lack of Fit F-value" this large could occur due to noise.

Table 5.4. Analysis of variance (ANOVA) for determining inoculation time of microorganisms (*T.harzianum* at the beginning).

Source	**Sum of Squares**	**df**	**Mean Square**	**F value**	**p-value**	
Model	95.80	24	3.99	6.17	< 0.0001	*Significant*
X_1	43.54	4	10.88	16.83	< 0.0001	
X_2	21.25	4	5.31	8.22	0.0003	
X_{12}	30.62	16	1.91	2.96	0.0080	
Residual	15.52	24	0.65			
Lack of Fit	5.46	23	0.67	10.97	0.2349	*not significant*
Pure Error	0.061	1	0.031			
Cor Total	113.11	49				
Std. Dev.	0.80			R-Squared	0.8606	
Mean	4.59			Adj R-Squared	0.7212	
C.V. %	17.53			Pred R-Squared	N/A	
PRESS	N/A			Adeq Precision	11.138	

Results shown in Table 5.1. and Table 5.2 indicated that the inoculation of T.*harzianum* and *A.sojae* at the beginning (0th hour) and inoculation of *S.cerevisiae* at the 24th hour gave the highest amounts of bioethanol. Therefore, these inoculation times were fixed and used in further experiments.

5.3.2. Results of Screening of Fermentation Parameters

Factorial designs are widely used in experiments involving several factors where it is necessary to study the joint effect of the factors on a response. However, several special cases of the general factorial design are important because they are widely used in researches. The 2^k factorial design is important among these special cases and is particularly useful in the early stages of experimental work, when many factors are likely to be investigated. It provides the smallest number of experiments in which *k* number of factors are studied at only two levels in a complete factorial design. Therefore, in this present study a 2^5 factorial design was used in the screening step in order to decrease the number of factors in optimization step by eliminating some of the factors and change the levels of remaining factors into more specific range.

Inoculation rates of microorganisms, aeration type and rate, and agitation speed are mostly investigated fermentation parameters in the literature (Pinheiro *et al*., 2008, Neelakandan and Usharani, 2009). Screening parameters were chosen based on these information. Screening results of the apple pomace in terms of bioethanol concentration (g/l) as a response is presented in Table 5.2. The ranges of the process parameters are presented in coded variables. The actual ranges for each of the variables were such as:

inoculation rate of *T.harzianum* (X_1), *A.sojae* (X_2) and *S.cerevisiae* (X_3) 4-20%, aeration (X_4) vented or sealed, and agitation (X_5) 0-200 rpm in screening step. Since maximum bioethanol concentration was obtained at 72th hour, these results (obtained at 72th hour) were used for statistical analysis.

Table 5.5. Screening results of the apple pomace with respect to bioethanol concentration (g/l) as response (for 72h).

	Actual level of variables					Response variable
Run No	Inoculum rate of *A.sojae* (%)	Inoculum rate of *T.harzianum* (%)	Inoculum rate of *S.cerevisiae* (%)	Aeration	Agitation (rpm)	Bioethanol concent. (g/l)
1	4	4	4	Sealed	0	4.246
2	12	12	12	Vented	100	3.207
3	20	4	4	Sealed	200	4.233
4	4	4	20	Sealed	0	4.806
5	4	20	4	Vented	200	4.859
6	4	4	20	Sealed	200	5.162
7	12	12	12	Vented	100	4.592
8	20	4	4	Vented	200	5.946
9	20	4	20	Vented	0	2.879
10	12	12	12	Sealed	100	3.715
11	12	12	12	Sealed	100	3.875
12	20	20	4	Sealed	0	2.536
13	4	4	20	Vented	200	5.479
14	12	12	12	Sealed	100	4.160
15	20	4	20	Vented	200	3.621
16	20	4	20	Sealed	0	4.116
17	12	12	12	Vented	100	3.229
18	4	4	4	Sealed	200	6.187
19	20	4	4	Vented	0	3.744
20	20	20	20	Sealed	0	4.319
21	4	4	20	Vented	0	3.990
22	4	20	4	Vented	0	3.658
23	4	20	4	Sealed	200	5.502
24	20	20	4	Vented	0	2.795
25	12	12	12	Sealed	100	3.882
26	12	12	12	Vented	100	3.996
27	20	4	20	Sealed	200	5.600
28	4	20	20	Sealed	200	6.337
29	20	20	20	Vented	200	6.598
30	4	4	4	Vented	200	8.271
31	20	4	4	Sealed	0	3.473
32	4	20	20	Vented	0	4.106
33	20	20	4	Sealed	200	6.035
34	4	20	4	Sealed	0	5.016
35	20	20	20	Vented	0	2.718
36	4	4	4	Vented	0	3.905
37	4	20	20	Sealed	200	5.361
38	4	20	20	Sealed	200	2.946
39	20	20	4	Vented	200	7.231
40	20	20	20	Sealed	200	5.478

At the end of the screening step the results of bioethanol concentration were evaluated according to the statistical analysis of variance (Table 5.6.). In this table, the model F-value of 8.10 implied that the model was significant. There was only a 0.74% chance that a "Model F-Value" this large occurred due to noise. Values of "Prob>F value" less than 0.05 indicated model terms were significant. So two of the single factors; inoculum rate of *A.sojae* (X_1) and agitation (X_5) and the interaction terms, interaction between inoculum rate of *A.sojae* and *T.harzianum* (X_{12}), interaction between inoculation rate of *S.cerevisiae* and aeration (X_{34}), interaction between inoculation rate of *A.sojae*, *T.harzianum* and agitation (X_{125}), interaction between inoculation rate of *A.sojae, S.cerevisiae* and aeration (X_{134}), intraction between inoculation rate of *T.harzianum, S.cerevisiae* and aeration (X_{234}), interaction between inoculation rate of *A.sojae, T.harzianum, S.cerevisiae* and agitation (X_{1235}) and interaction between inoculation rate of *A.sojae, T.harzianum*, aeration and agitation (X_{1245}), were the significant model terms.

Table 5.6. Analysis of variance (ANOVA) for bioethanol concentration (for 72 h)

Source	**Sum of Squares**	**df**	**Mean Square**	**F value**	**p-value**	
Model	60.15	31	1.94	8.10	0.0074	Significant
X_1	2.26	1	2.26	9.44	0.0219	
X_2	8.303E-004	1	8.303E-004	3.464E-003	0.9550	
X_3	0.53	1	0.53	2.21	0.1873	
X_4	0.022	1	0.022	0.090	0.7743	
X_5	33.31	1	33.31	138.96	< 0.0001	
X_{12}	2.18	1	2.18	9.11	0.0235	
X_{34}	1.62	1	1.62	6.75	0.0408	
X_{125}	2.88	1	2.88	12.03	0.0133	
X_{134}	1.57	1	1.57	6.53	0.0431	
X_{234}	1.85	1	1.85	7.71	0.0321	
X_{1235}	2.87	1	2.87	11.97	0.0135	
X_{1245}	1.97	1	1.97	8.21	0.0286	Significant
Curvature	5.10	2	2.55	10.64	0.0106	
Pure Error	1.44	6	0.24			
Cor Total	66.69	39				
Std. Dev.	0.49			R-Squared	0.9766	
Mean	4.55			Adj R-Squared	0.8560	
C.V. %	10.77			Pred R-Squared	N/A	
PRESS	N/A			Adeq Precision	12.706	

The "Curvature F-value" of 10.64 implied there was a significant curvature in the design space. It is measured by the difference between the average of the center points and the average of the factorial points. There was only a 1.06 % chance that a "Curvature F-value" this large could occur due to noise. "Adeq Precision" measures the

signal to noise ratio. A ratio greater than 4 is desirable. So it can be said that the ratio of 12.706 indicates an adequate signal.

The p-values indicated that X_2, X_3 and X_4 were not significant, whereas the terms of X_{12}, X_{34}, X_{125}, X_{134}, X_{234}, X_{1235} and X_{1245} were significant. In this case, removing nonsignificant terms or factors from the model was not hierarchical. The hierarchy principle indicates that if a model contains an interaction term, it should also contain their main terms in the model. Therefore although X_2, X_3 and X_4 were not significant, they were not removed from the model because their interactions were significant. However, optimization of five factors was very difficult in practice. So factors were evaluated by examining the variety of graphs in order to find if some of them could be fixed or not.

Figure 5.2, which is a one factor graph, indicated that low inoculation rate of *A.sojae* lead to higher bioethanol concentration than high inoculation rate when the inoculation rates of *T.harzianum* and *S.cerevisiae* were fixed at their low levels, agitation speed was high and the vented flasks were used.

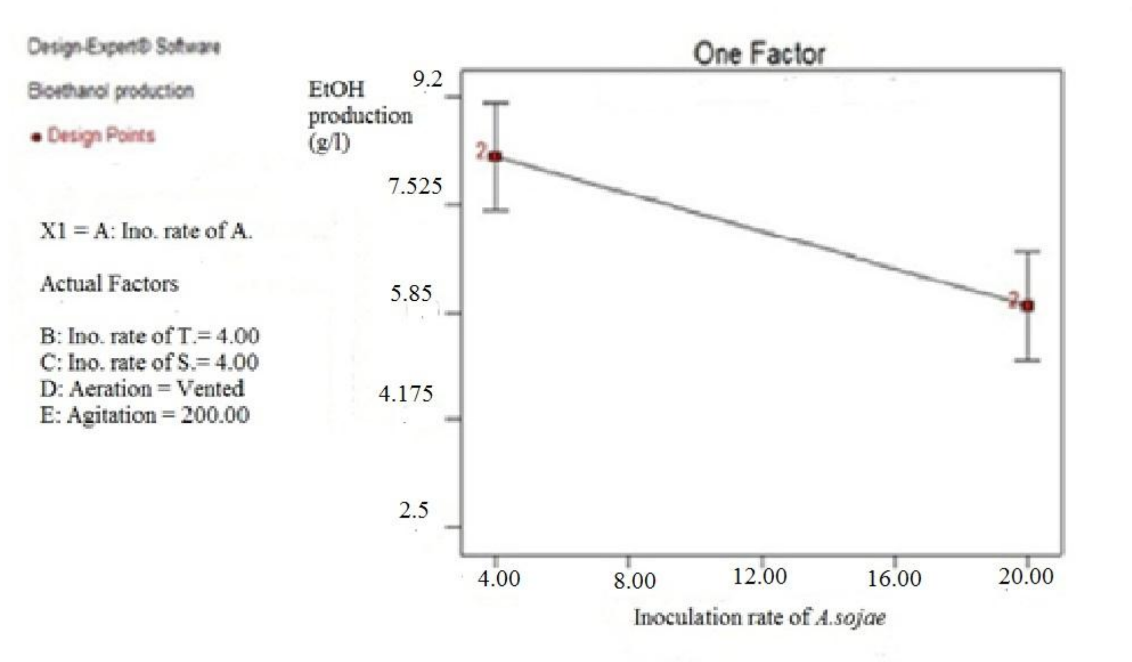

Figure 5.2. One factor plot of inoculation rate of *A.sojae* with respect to bioethanol production

Figure 5.3. suggested that high ethanol concentration could be obtained at high inoculation rate of *A.sojae* compared to lower rates when vented flasks were used with high inoculation rate of *T.harzianum*, low inoculation rate of *S.cerevisiae* and high agitation speed.

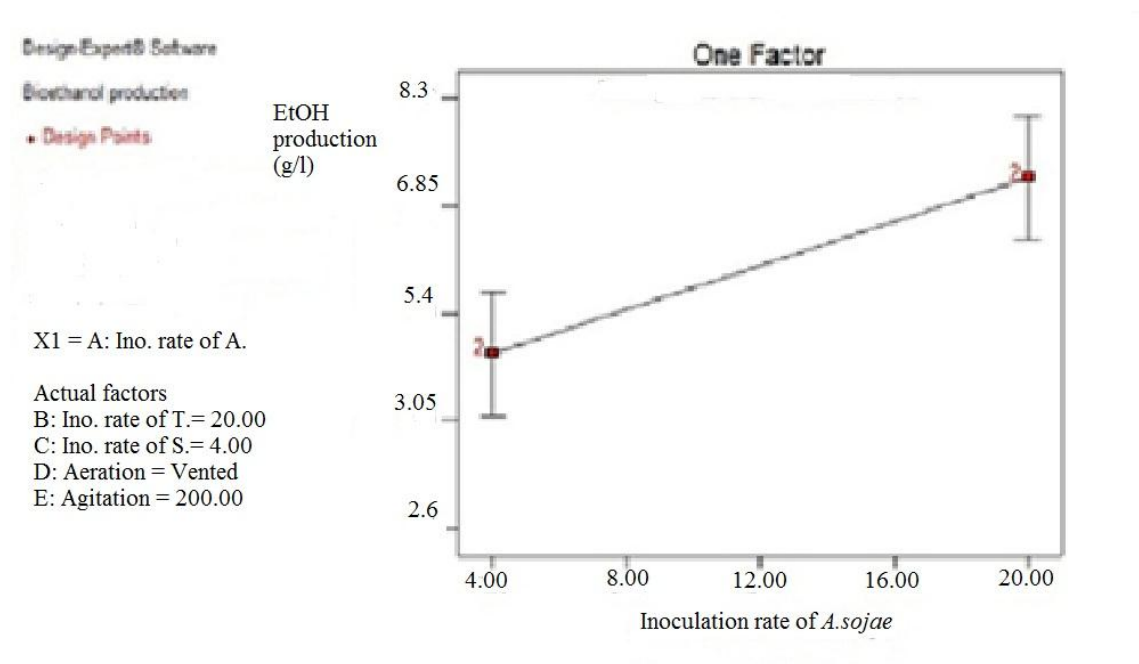

Figure 5.3. One factor plot of inoculation rate of *A.sojae*

Also, according to Figure 5.4., in the case of low inoculation rate of *T.harzianum*, low inoculation rate of *S.cerevisiae*, high agitation speed and the use of vented flasks high bioethanol concentrations could be obtained at low inoculations of *A.sojae*.

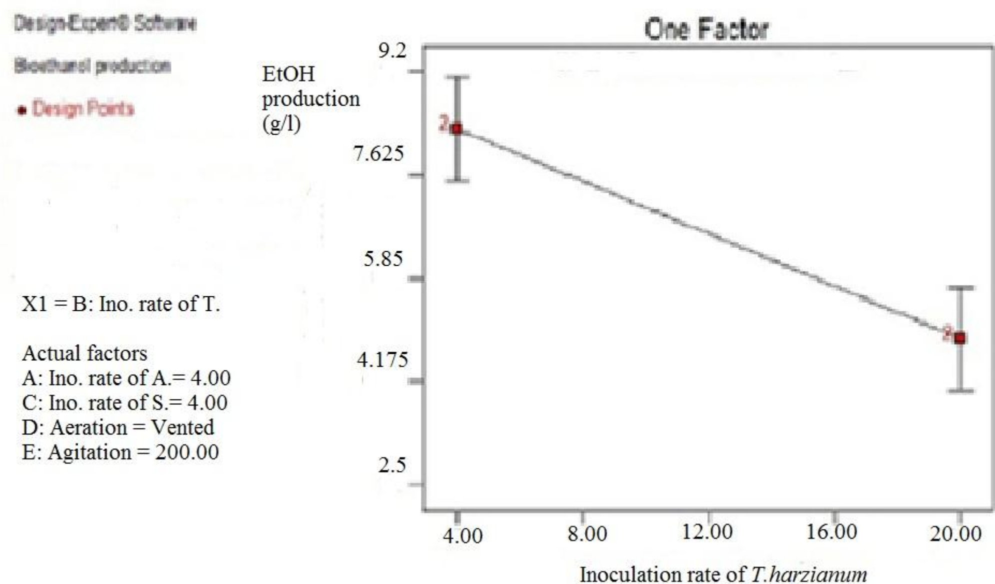

Figure 5.4. One factor plot of inoculation rate of *T.harzianum* with respect to bioethanol production

When high level of inoculation rate of *A.sojae*, low level of inoculation rate of *S.cerevisiae*, high agitation speed and vented flasks were used, high bioethanol concentration could be obtained at the high level of inoculation rate of *T.harzianum*. (Figure 5.5.). but there was no significant difference between the low and high levels of inoculation rate of *T.harzianum*.

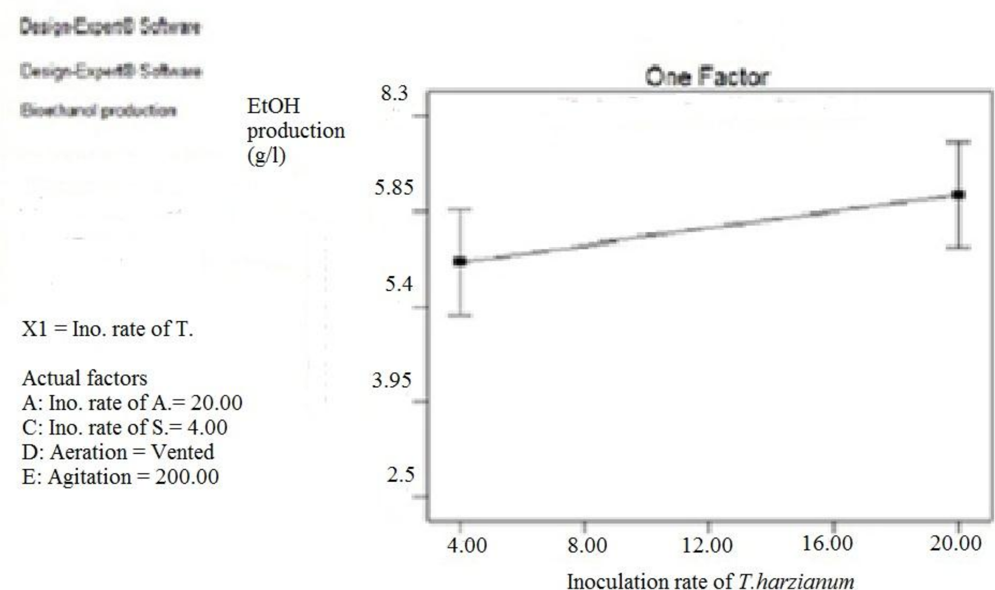

Figure 5.5. One Factor plot of *T.harzianum* with respect to bioethanol production.

The effect of inoculation rate of *S.cerevisiae* was investigated in many reports (Sharma *et al*., 2007). The maximum ethanol yield was obtained at 10% inoculation rate in the study conducted by Neelakandan and Usharani (2009). In this mixed culture fermentation studies, it was found that low inoculation levels of *S.cerevisiae* led effective bioethanol production. The relation between the inoculation rate of *S.cerevisiae* with respect to bioethanol production is presented in Figure 5.8. Bioethanol production was high at the low level of inoculation rate of *S.cerevisiae*, when inoculation rates of *A.sojae* and *T.harzianum* were low, agitation speed was high and vented flasks were used. If inoculation rates of *A.sojae* and *T.harzianum* were changed to high levels (other parameters are the same with Figure 5.2. and 5.3.), high bioethanol concentrations could be obtained at the low inoculation levels of *S.cerevisiae* again. (Figure 5.6.)

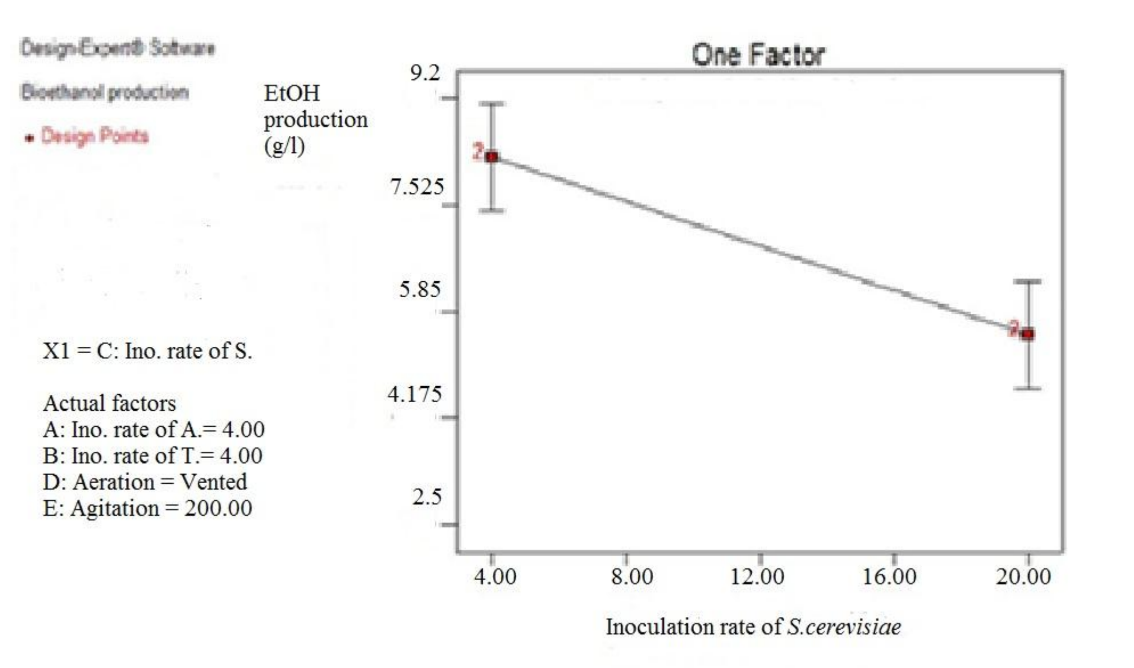

Figure 5.6. One factor plot of inoculation rate of *S.cerevisiae* with respect to bioethanol production.

Change in the bioethanol production as a function of the aeration parameter was illustrated in Figure 5.7. Although aeration was a nonsignificant term according to the model, its interactions were significant. Therefore it could not be removed from the model due to the hierarchy principle. The use of vented flasks led to higher bioethanol concentrations than the sealed flask. Since microorganisms favored mild conditions and not strictly anaerobic conditions, this was an expected result.

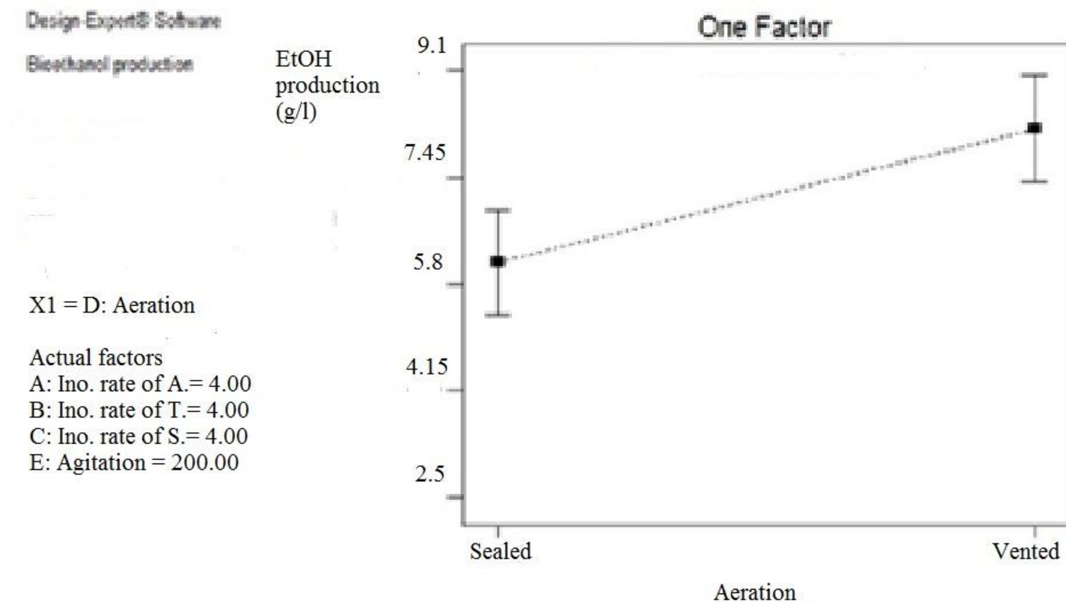

Figure 5.7. One factor plot of aeration with respect to bioethanol production.

Agitation speed is a quite important factor for bioethanol production as well. The higher the agitation speed thehigher is the bioethanol production (Figure 5.8).

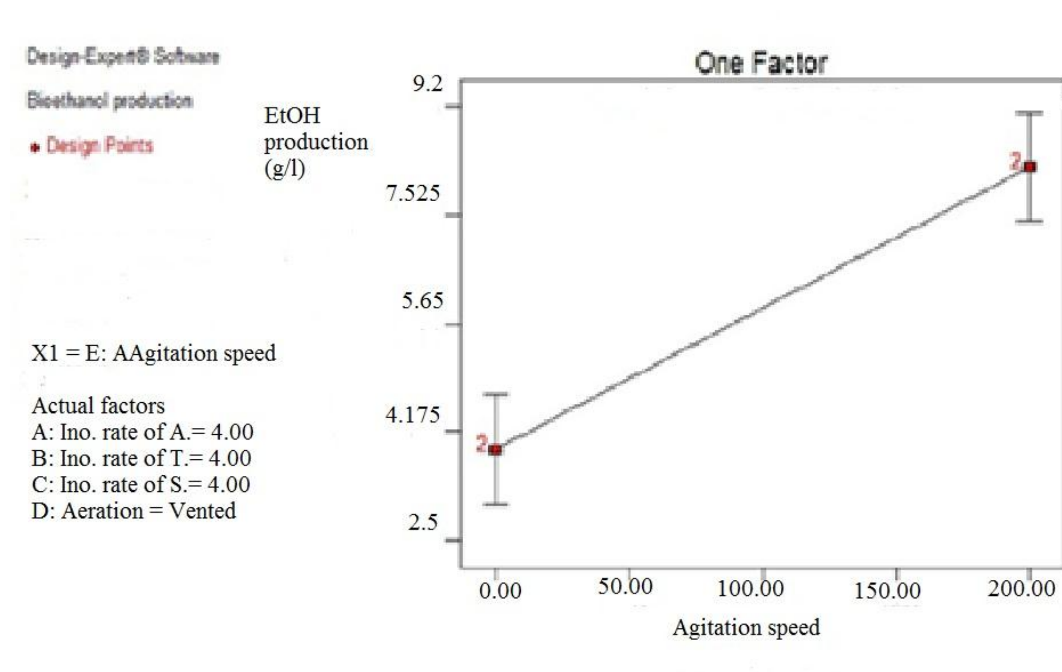

Figure 5.8. One factor plot of agitation speed with respect to bioethanol production.

Bioethanol concentration showed an increasing trend in both sides, at the low levels and high levels of inoculation rates of *A.sojae* and *T.harzianum*. Therefore analysis of both sides may be more useful in order to find optimum conditions. According to the model, inoculation rate of *T.harzianum* was nonsignificant. However, when mixed cultures were used this indicates that there would be strong interaction between the cultures (Figure 5.9).

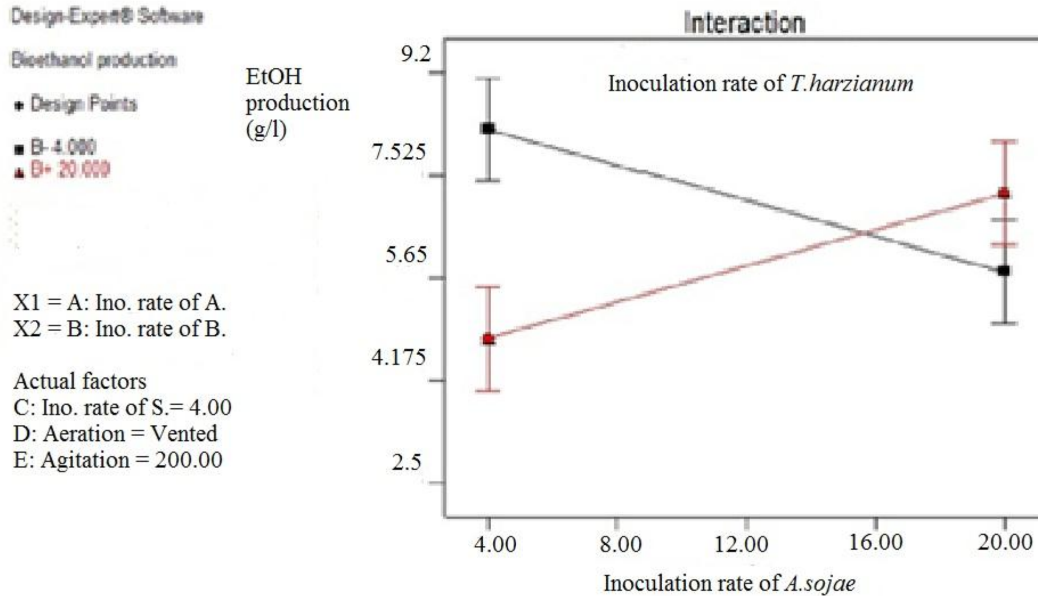

Figure 5.9. The interaction graph of inoculation rate of *A.sojae* and *T.harzianum*.

It can be seen from Figure 5.10. the use of sealed fermentation flasks had no significant effect on bioethanol production, whereas the use of vented flasks led to high bioethanol concentrations at the low levels of inoculation rates of *S.cerevisiae*. In this case both the inoculation levels of *A.sojae* and *T.harzianum* were set at their low levels.

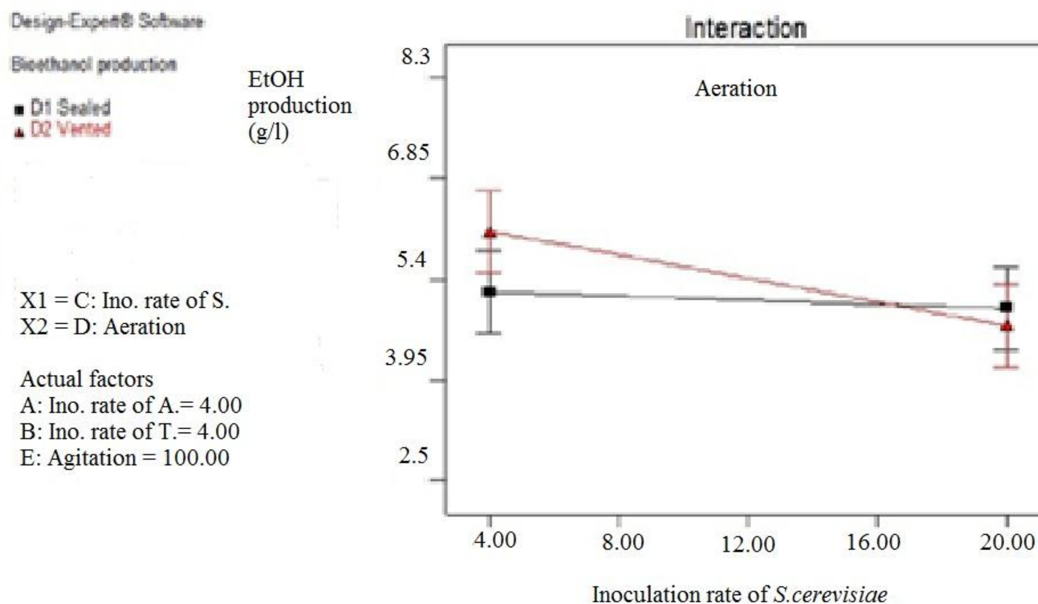

Figure 5.10. Interaction graph of inoculation rate of *S.cerevisiae* and aeration.

Overall, as it can be seen from the Figure 5.11., low levels of inoculation rates of *A.sojae, T.harzianum* and *S.cerevisiae*, high agitation speed and the use of vented fermentation flasks led to high bioethanol production.It was decided to keep inoculation rate of *S.cerevisiae* at its low level (4%) by evaluating model graphs shown in foregoing figures and graphs. Since aeration was a nonsignificant model term, with the use of vented flasks ethanol production increased. Based on these, inoculation rate of *S.cerevisiae* and choice of aeration method were fixed as 4% and as vented aeration method, respectively.

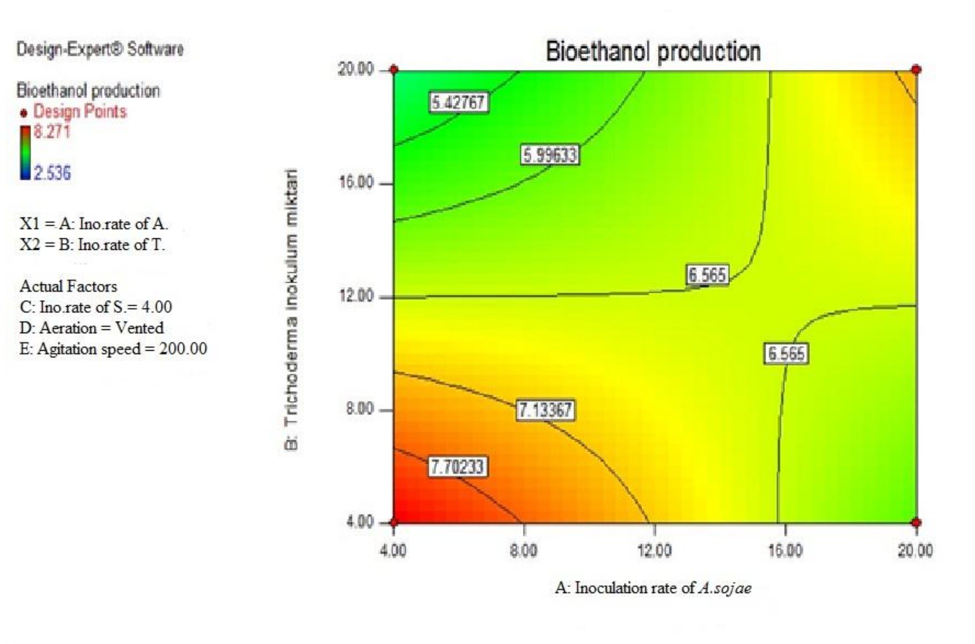

Figure 5.11. Contour plot of inoculation rates of *A.sojae* and *T.harzianum*.

The 30th experiment (shown in Table 5.4.) which had the highest bioethanol concentration, supported the figures, since the conditions of this particular set experiment were 4% inoculation rate of *A.sojae*, *T.harzianum* and *S.cerevisiae*, high agitation speed (200 rpm) and vented aeration method. After acidic hydrolysis the total amount of fermentable reducing sugars were 16.156 g/l. The ethanol yield on substrate was 0.646 gEtOH/gsubstrate (Table 5.6) which was higher than the data (ethanol yield on substrate was 0.463, total amount of reducing sugars were 18.15 g/l) obtained from the fermentation of potato peel wastes with *S.cerevisiae* (Arapoglou *et al*., 2010). Bioethanol production this experiment was illustrated in Figure 5.12. The maximum ethanol concentration was reached at the 72th hour of the fermentation.

Table 5.7. Yield factors and volumetric productivity results of the screening step with respect to bioethanol production.

Run No	EtOH Yield on substrate ($Y_{P/S}$)	EtOH Yield on biomass (Y_{XE})	Biomass Yield on substrate (Y_{SX})	Volumetric EtOH Productivity (Q_p)
1	0.444	0.839	0.471	0.045
2	0.300	0.644	0.354	0.044
3	0.366	0.268	1.155	0.112
4	0.481	0.866	0.444	0.039
5	0.410	0.638	0.545	0.100
6	0.460	0.560	0.723	0.072
7	0.399	0.521	0.697	0.064
8	0.473	0.498	0.922	0.083
9	0.265	0.376	0.598	0.037
10	0.363	0.870	0.351	0.033
11	0.331	0.738	0.405	0.030
12	0.252	0.356	0.549	0.037
13	0.492	0.616	0.671	0.076
14	0.396	0.617	0.545	0.032
15	0.300	0.226	1.114	0.104
16	0.380	0.609	0.515	0.053
17	0.325	0.331	0.788	0.092
18	0.571	0.946	0.429	0.131
19	0.383	0.588	0.506	0.035
20	0.414	0.379	0.896	0.060
21	0.419	0.881	0.389	0.034
22	0.370	0.741	0.436	0.051
23	0.436	0.626	0.584	0.044
24	0.250	0.248	0.874	0.057
25	0.360	0.505	0.664	0.057
26	0.413	0.492	0.603	0.103
27	0.437	0.689	0.608	0.049
28	0.500	0.914	0.522	0.088
29	0.505	0.657	0.727	0.092
30	0.646	1.151	0.540	0.115
31	0.323	0.427	0.698	0.027
32	0.338	0.660	0.507	0.032
33	0.400	0.442	0.913	0.084
34	0.428	0.557	0.749	0.043
35	0.221	0.284	0.764	0.111
36	0.363	0.868	0.364	0.030
37	0.460	0.619	0.641	0.059
38	0.244	0.572	0.441	0.052
39	0.584	0.571	0.856	0.100
40	0.406	0.508	0.777	0.076

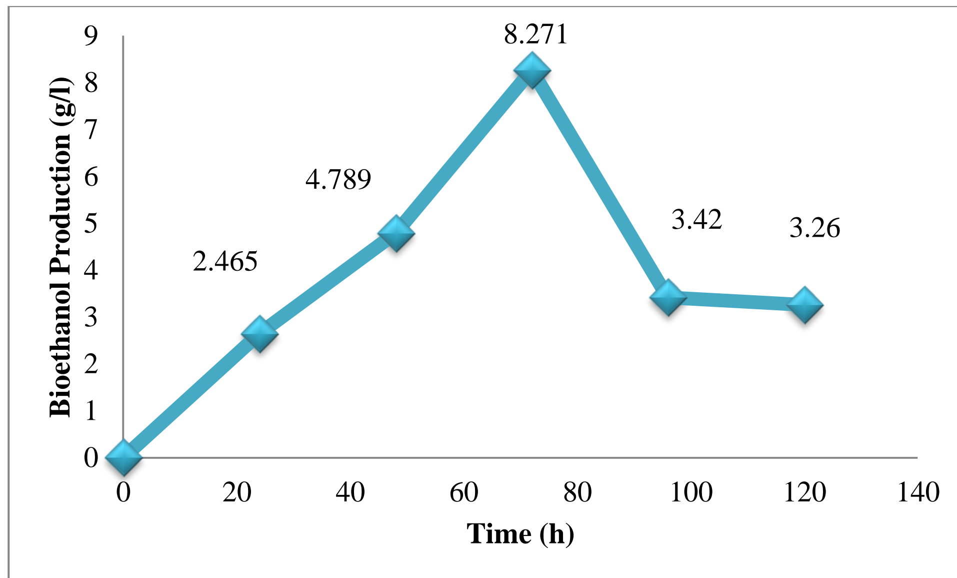

Figure 5.12. Bioethanol production profile of the 30th experiment during the course of fermentation process.

5.4. Results of the Optimization Steps of Bioethanol Production

Optimization of bioethanol production from apple pomace was performed according to the Face Centered Central Composite experimental design presented in the materials and method section Table 4.4 the evaluation of the results for bioethanol production was mentioned in the bioethanol determination part in Materials and Methods chapter.

According to the screeening results inoculation rate of *S.cerevisiae* and aeration method were fixed. (4% and vented fermentation flasks). Other parameters, inoculation rate of *A.sojae* and *T.harzianum* and agitation speed were broadened in the range of 0 – 6%, 100 – 300 rpm, respectively. The actual levels of these variables and the response were tabulated in Table 5.8. The optimization results were discussed below according to the results of the ANOVA presented in Table 5.9 and 5.10.

Table 5.8. Optimization results of the apple pomace with respect to bioethanol concentration (g/l) as a response (for 72h).

	Actual level of variables			Response variable
Run no	Inoculation rate of A.sojae (%)	Inoculation rate of T.harzianum (%)	Agitation speed (rpm)	Bioethanol concentration (g/l)
1	3	3	200	8.636
2	6	0	300	5.262
3	6	6	300	7.333
4	6	6	100	3.783
5	0	3	200	3.998
6	3	3	200	8.748
7	0	6	300	5.963
8	3	3	200	7.505
9	0	0	100	3.659
10	0	6	100	5.006
11	3	0	200	8.398
12	6	0	100	3.840
13	3	3	100	7.246
14	6	3	200	7.824
15	0	0	300	4.463
16	3	3	300	6.109
17	3	3	100	3.807
18	3	3	200	7.408
19	3	3	200	7.003
20	3	6	200	6.459

Table 5.9. ANOVA for response surface quadratic model for bioethanol production as response.

Source	**Sum of Squares**	**df**	**Mean Square**	**F value**	**p-value Prob>F**	
Model	45.46	9	5.05	3.51	0.0317	Significant
X_1	2.45	1	2.45	1.70	0.2210	
X_2	0.85	1	0.85	0.59	0.4591	
X_5	8.16	1	8.16	5.67	0.0385	
X_{12}	0.087	1	0.087	0.060	0.8111	
X_{15}	1.29	1	1.29	0.90	0.3664	
X_{25}	0.65	1	0.65	0.45	0.5167	
X_1^2	3.16	1	3.16	2.19	0.1694	
X_2^2	0.55	1	0.55	0.38	0.5515	
X_5^2	11.27	1	11.27	7.83	0.0189	
Residual	14.40	10	1.44	10.64	0.0106	
Lack of fit	11.63	5	2.33	4.20	0.0707	Not significant
Pure error	2.77	5	0.55			
Cor Total	59.86	19				
Std. Dev.	1.20			R-Squared	0.7595	
Mean	6.12			Adj R-Squared	0.5439	
C.V. %	19.60			Pred R-Squared	0.5758	
PRESS	94.32			Adeq Precision	5.300	

According to Table 5.9, the model F-value of 3.51 implied that the model was significant. There was only a 3.17% chance that a “Model F-Value” this large could occur due to noise. Values of “Prob > F” less than 0.05 indicated that model terms were significant. In this case agitation (X_5) and X_5^2 were significant model terms. The “Lack of Fit-value” of 4.20 implied there was a 7.07% chance that a “Lack of Fit F-value” this large could occur due to noise. The ratio of “Adeq Precision”, which measured the signal to noise ratio, indicated an adequate signal. Since there were some insignificant terms, the model was further reduced by eliminating these and reevaluated as presented in Table 5.9.

Table 5.10. ANOVA for response surface quadratic reduced model for bioethanol production.

Source	Sum of Squares	df	Mean Square	F value	p-value Prob>F	
Model	42.21	6	7.03	5.18	0.0063	Significant
X_1	2.45	1	2.45	1.81	0.2018	
X_2	0.85	1	0.85	0.63	0.4420	
X_5	8.16	1	8.16	6.01	0.0291	
X_{15}	1.29	1	1.29	0.95	0.3477	
X_{25}	0.65	1	0.65	0.48	0.5010	
X_5^2	28.80	1	28.80	21.21	0.0005	
Residual	17.65	13	1.36	10.64	0.0106	
Lack of fit	14.88	8	1.86	3.36	0.0990	Not significant
Pure error	2.77	5	0.55			
Cor Total	59.86	19				
Std. Dev.	1.17			R-Squared	0.7052	
Mean	6.12			Adj R-Squared	0.5691	
C.V. %	19.03			Pred R-Squared	0.2953	
PRESS	42.18			Adeq Precision	5.658	

Here, the model F-value of 5.18 implied that the model was significant. There was only a 0.63% chance that a “Model F-value” this large could occur due to the noise. Values of “Prob > F” less than 0.05 indicate model terms were significant. In this case agitation (X_5), X_5^2 were significant model terms. The Model F-value of 5.18 implied the model was significant. There was only a 0.63% chance that a "Model F-Value" this large could occur due to noise. The “Lack of Fit F-value” of 3.36 implied there was a 9.90% chance that a “Lack of Fit F-value” this large could occur due to noise. The "Pred R-Squared" of 0.2953 was not as close to the "Adj R-Squared" of 0.5691 as one might normally expect. A ratio greater than 4 was desirable. The “Adeq Precision” ratio of 5.658 indicated an adequate signal. This model can be used to navigate the design space.

As depicted in Figure 5.13. and Figure 5.14. higher concentrations of inoculation rates of *T.harzianum* and *A.sojae* (6%) and agitation speeed around the 200 rpm led to higher amount of bioethanol. On the other hand inoculation rate of *S.cerevisiae* and aeration method did not change ethanol yields significantly. As a result inoculation rates of 6% (w/v) for *A.sojae* and *T.harzianum* and 4% (w/v) for *S.cerevisiae* were determined as the optimum conditions with the vented aeration method and agitation speed of 200 rpm.

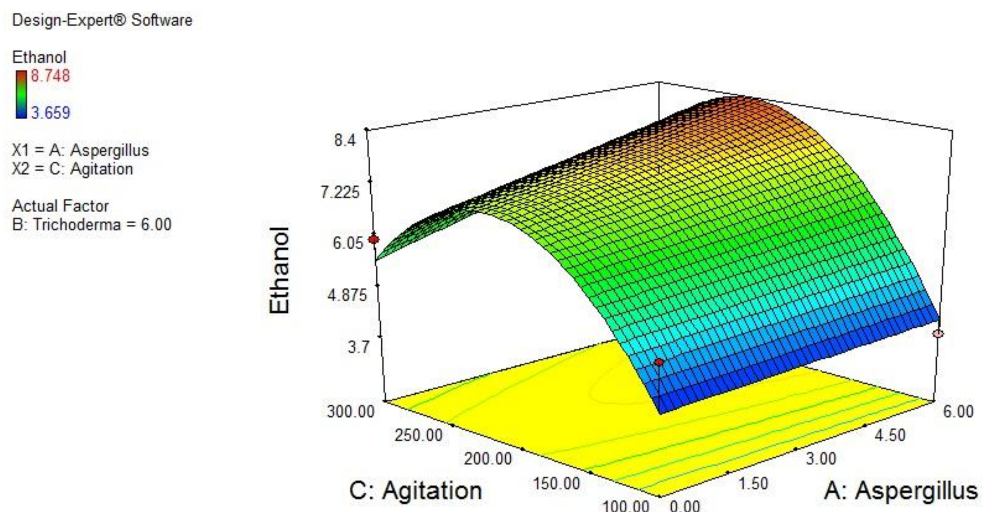

Figure 5.13. Response surface graph of the optimization experiments.

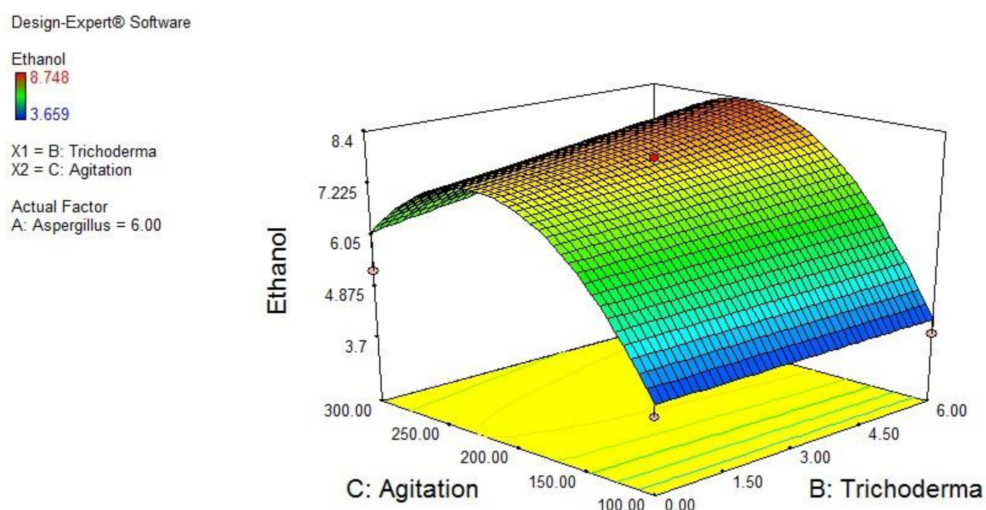

Figure 5.14. Response surface graph of the optimization experiments.

Final equation in terms of coded factors and actual factors were given below:

$$\text{Bioethanol} = +7.32 + 0.50 * X1 + 0.29 * X2 + 0.90 * X3 + 0.40 * X1 * X3 + 0.29 * X2 * X3 - 2.40 * X3^2 \quad (5.1)$$

$$\text{Bioethanol} = -3.49900 - 0.10248 * \text{Ino.rate of } \textit{A.sojae} - 0.092683 * \text{Ino.rate of } \textit{T.harzianum} + 0.098170 * \text{Agitation speed} + 1.33792E\text{-}003 * \text{Ino.rate of } \textit{A.sojae} * \text{Agitation speed} + 9.50417E\text{-}004 * \text{Ino.rate of } \textit{T.harzianum} * \text{Agitation speed} - 2.40000E\text{-}004 * \text{Agitation speed}^2 \quad (5.2)$$

In order to validate the adequacy of the model equations a total of three verification experiments were carried out at the predicted optimum conditions for bioethanol production. The results showed that 12.46, 2.17 and 12.21% deviation, respectively. The overall margin of error was 8.95%. (Table 5.11)

Table 5.11. Results of validation experiments.

Ino.rate of *A.sojae* (w/v)%	**Ino.rate of *T.harzianum* (w/v)%**	**Agitation (rpm)**	**Estimated bioethanol production(g/l)**	**Actual bioethanol production(g/l)**	**Error (%)**	**Overall Error (%)**
6	6	233	8.373	7.330	12.46	8.95
6	6	236	8.370	8.188	2.17	
6	5.93	232	8.364	7.343	12.21	

A second optimization study was carried out in order to investigate high levels of (10-30% w/v) of the inoculation rate of *T.harzianum* and *A.sojae*. however, a significant model could not be obtained.

The yield factors and productivity results of the experiments performed in the optimization step are tabulated in Table 5.12. according to this table maximum bioethanol yield on substrate was obtained in the 6th experiment as 0.945 gEtOH/gsubstrate where the corresponding volumetric bioethanol productivity was 0.122 g/l/h. This value was one of the highest, obtained during all the runs. The fermentation profile corresponding to this set of experiment is presented in Figure 5.15. as it can be seen the profile has an increasing trend making a pick at the 100 hours of fermentation.

Table 5.12. Yield factors and productivity results of the optimization step with respect to bioethanol production.

Run No	EtOH Yield on biomass (Y_{SE})	EtOH Yield on substrate ($Y_{P/S}$)	Biomass Yield on substrate (Y_{SX})	Volumetric EtOH Productivity (Q_p)
1	1.003	0.867	0.639	0.090
2	0.434	0.389	0.776	0.085
3	0.690	0.604	0.720	0.076
4	0.758	0.541	0.513	0.124
5	0.412	0.544	0.881	0.042
6	1.673	0.945	0.479	0.121
7	0.332	0.681	1.358	0.062
8	0.675	0.714	0.820	0.078
9	1.192	0.490	0.341	0.093
10	1.117	0.732	0.467	0.052
11	0.942	0.798	0.662	0.087
12	0.665	0.518	0.531	0.106
13	0.713	0.576	0.606	0.075
14	0.797	0.551	0.647	0.081
15	2.975	0.577	0.159	0.046
16	0.589	0.455	0.642	0.064
17	0.587	0.564	0.702	0.093
18	0.611	0.712	0.945	0.077
19	1.005	0.627	0.511	0.105
20	0.801	0.585	0.680	1.152

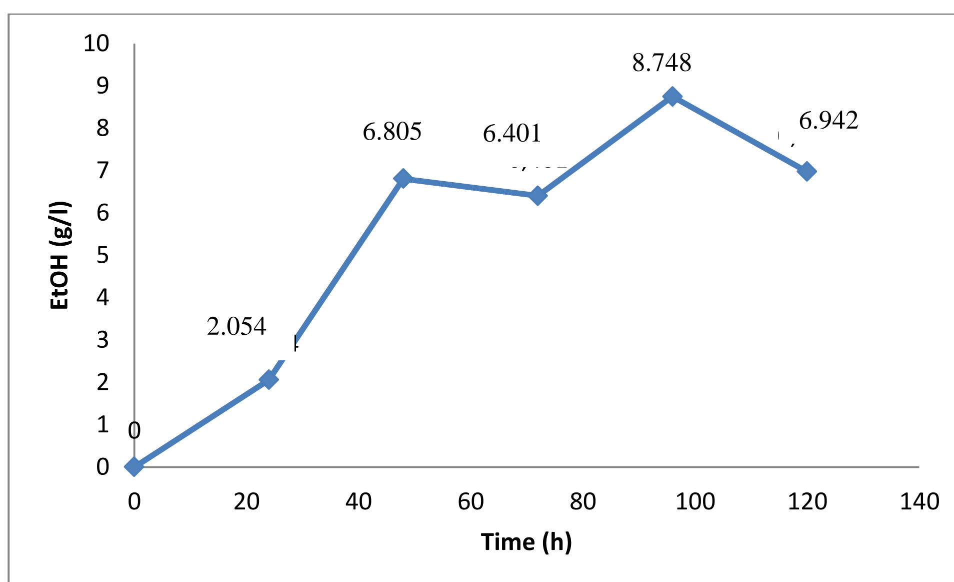

Figure. 5.15. Bioethanol production profile of the 6th experimetn during the course of fermentation process.

According to the literature, ethanol production was influenced by using co-cultures (Sharma *et al.*, 2007). The results shown in Figure 5.16. supported this

information. Employing mixed culture fermentation in this study was very effective on efficient bioethanol production. The best result was obtained when three of the cultures were used together.

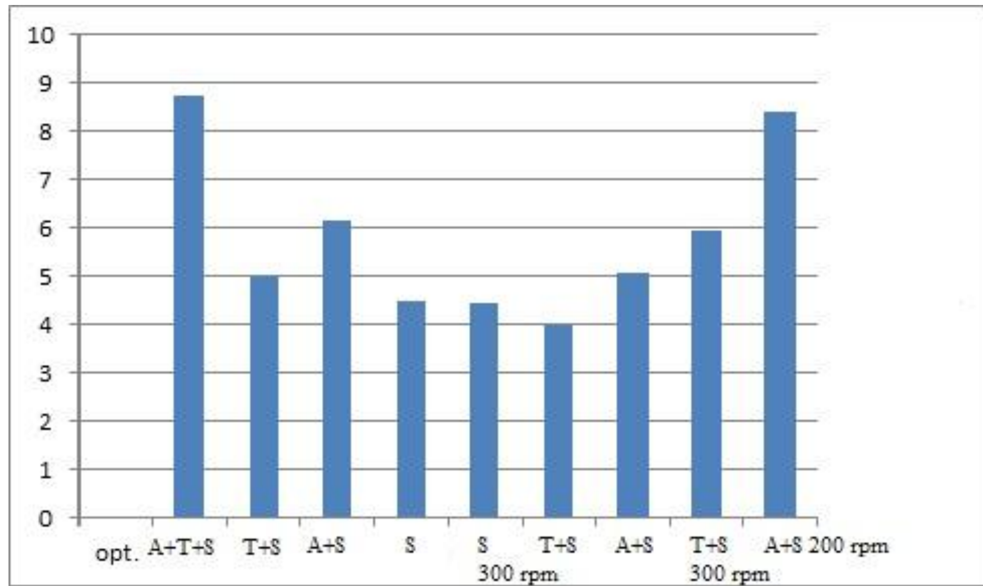

Figure. 5.16. Comparison of bioethanol results belonging to different combinations of microbial fermentations.

Figure 5.17. and Figure 5.18. shows the initial sugar and initial carbohydrate utilization of the fermentations having different microbial combinations. All apple pomace hydrolysates had 16.155 g/L of initial sugar and 42.265 g/L of initial carbohydrate on the first day of fermentation, respectively. It was observed that the microorganisms were using the sugars in the hydrolysates and breaking down the cellulose into sugars simultaneously. It seemed that the fermentation flasks with the co-cultures showed an efficient mass transfer, since initial sugar decreased very fast during the course. Thus, the microorganisms were able to use all of the initial sugars and brake down the cellulose molecules into sugars more effectively because of a better mass transfer and little O_2 access through slicone tubing (vented aeration method). However, in the fermentation flasks which had only *S.cerevisiae*, reducing sugar usage was not very much. Because yeast did not have the ability to use arabinose, which was the major sugar in the apple pomace hydrolysate.

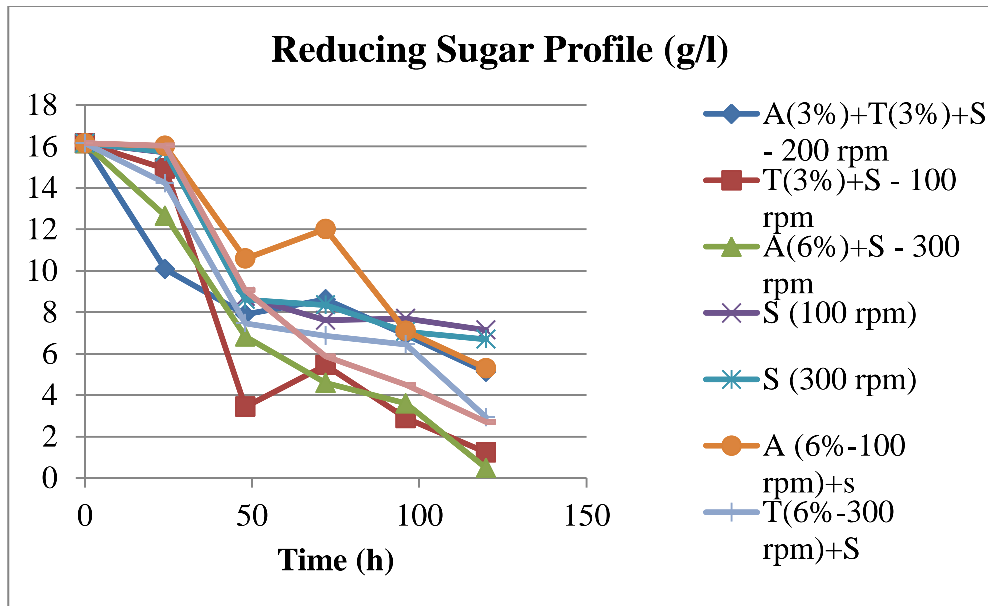

Figure 5.17. Reducing sugar consumption profile during the course of fermentation experiments in the optimization step.

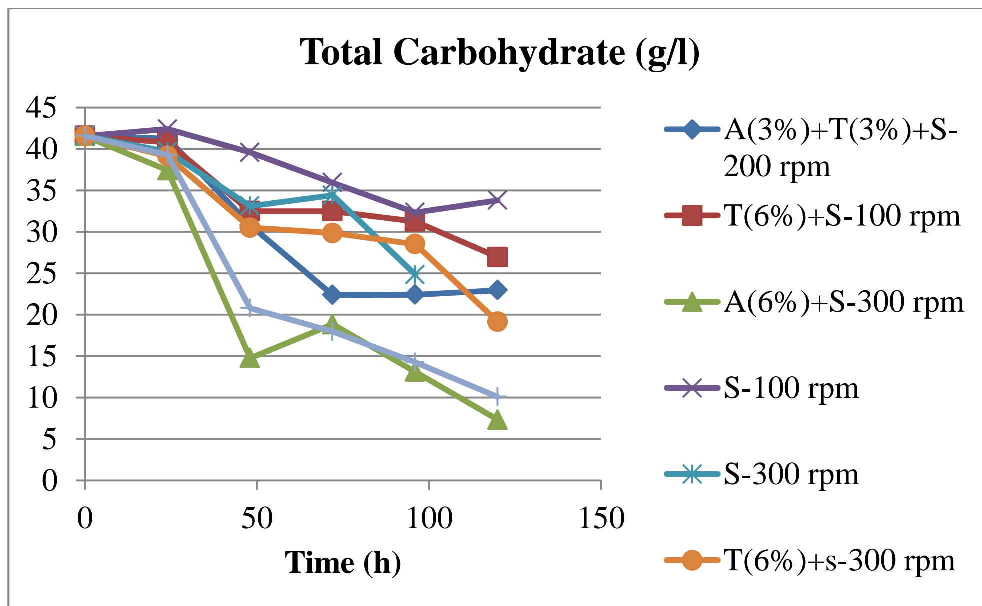

Figure 5.18. Total carbohydrate consumption profile during the course of fermentation experiments in the optimization step.

The results obtained from optimization studies demonstrated that mixed culture fermentation, which contained all of the microorganisms, was required for effective bioethanol production. In literature there are various studies related to mixed culture fermentations (Keating et al., 2004, Patle and Lal, 2007). However, to date, no reports are available in literature regarding the usage of three organisms in one fermentation

flask. Moreover, no reports are available in the literature that we came across, regarding the use of *T.harzianum*, *A.sojae* and *S.cerevisiae* together for bioeethanol production. Therefore, this study will serve as abaseline of the initial studies in this field. Furthermore, the results pointed out that using co-cultures bacause of its synergistic interactions is an effective way for production of bioethanol using waste materials like apple pomace.

CHAPTER 6

CONCLUSION

The composition of apple pomace, one of the main wastes of fruit industry, was determined and hydrolysis of this pomace was carried out with dilute acid at its optimum conditions which were 110°C and 40 minutes.

For fermentation of apple pomace to bioethanol, two fungi (*T.harzianum* and *A.sojae*) and a natural ethanologenic yeast (*S.cerevisiae*) was used. The effects of using co-cultures on bioethanol production was investigated by applying statistical methods. Results were evaluated within the statistical concept.

At the initial screening step, a general factorial design was generated for determining the inoculation days of microorganisms. For this purpose, inoculation hours which had the levels of 0h, 24h, 48h, 72h and 96h, were determined as screening factors. Results showed that inoculation of *T.harzianum* and *A.sojae* at 0th hour and inoculation of *S.cerevisiae* at 24th hour gave the best results for bioethanol production. Inoculation rates of *T.harzianum*, *A.sojae* and *S.cerevisiae*, aeration and agitation speed were determined as factors for screening of fermentation parameters. According to the statistical analysis, inoculation rate of *A.sojae* and agitation speed were found as the most significant variables with respect to bioethanol production. Since, the interactions of other three factors were significant, they could not be removed from the model because of the hierarchy rule. But in order to optimize these factors and make experiments aplicable, inoculation rate of *A.sojae*, inoculation rate of *T.harzianum* and agitation speed was chosen as factors and inoculation rate of *S.cerevisiae* was fixed as 4% and vented aeration method was used for further optimization studies.

The results obtained at the optimization step indicated that there was an increase in bioethanol amount. Inoculation rates of 6% (w/v) for *A.sojae* and *T.harzianum* and 4%(w/v) for *S.cerevisiae* were determined as the optimum conditions with the vented aeration method and agitation speed of 200 rpm. Maximum bioethanol amount was 8.748 g/l which was 8.271 g/l at the screening step. Higher concentrations of inoculation rates of *T.harzianum* and *A.sojae* (6%) and agitation speeed around the 200 rpm led to higher amount of bioethanol. Before optimization bioethanol yield on

substrate was 0.646 which increased to 0.946 gEtOH/gsubstrate. Volumetric bioethanol productivity increased from 0.115 to 0.121g/l/h and percent theoretical yield was 96.18% which was 90.94% before optimization.

It was also found that using co-cultures gave the best results. Maximum bioethanol concentration were 8.398 g/l when both *A.sojae* and *S.cerevisiae* were inoculated into the fermentation flasks. When *T.harzianum* and *S.cerevisiae* were inoculated into the fermentation flasks, bioethanol concentration were 5.963 g/l. Bioethanol concentration was only 4.463 g/l when only *S.cerevisiae* was inoculated into the fermentation flasks. Finally, bioethanol concentration reached at its maximum level, 8.748 g/l, when three of the organisms were inoculated into the fermentation flasks.

In addition, co-cultures showed better sugar consumption profile. The presence of fungi in the fermentation flasks caused more effective sugar utilization because of their ability to use both pentoses and hexoses.

To date, no reports are available in literature regarding the use of *T.harzianum*, *A.sojae* and *S.cerevisiae* together for bioethanol production. Therefore, this study will serve as a base line of the initial studies in this field. Furthermore, the results pointed out that using cocultures because of its synergistic interactions is an effective way for production of bioethanol.

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APPENDIX A

CHEMICALS

Table A.1. Chemicals used

NO	CHEMICAL	CODE
1	Ammonium heptamolybdate heptahydrate (($(NH_4)_6Mo_7O_{24}$. 7H_2O)	Riedel-de Haën 1.011.800.250
2	Ammonium heptamolybdate-tetrahydrate	Merck 1.01182
3	Calcium carbonate, ACS reagent grade Min 99%	Alfa Aesar 43073
4	Copper (II) sulphate-pentahydrate ($CuSO_4$. 5H_2O), extra pure	Riedel-de Haën 12849
5	D-(+)arabinose	
6	D-cellobiose	
7	D-(+)galactose	
8	D-(+)glucose	
9	D-(+)mannose	
10	D-(+)xylose	
11	Disodium hydrogen arsenate heptahydrate ($AsHNa_2O_4$. 7H_2O)	Flucka 71.625
12	Ethanol, absolute pure, p.a.	Sigma 32221
13	Ethanol, ACS reagent	Sigma 45,984-4
14	Folin-Ciocateu’s phenol reagent	Merck 1.09001
15	Furfural	
16	Glycerol	Sigma G5516
17	5-hydroxy-2-furaldehyde (HMF)	Sigma S0751
18	Iron(II)sulphate heptahydrate	Riedel-De Haën 12354
19	KCl	Riedel-De Haën 31248

(cont. on next page)

Table A.1. (cont.)

20	K_2HPO_4	Riedel-De Haën 04243
21	Malt extract	BD 218630 (BactoTM)
22	Molasses	Pakmaya Kemalpaşa Üretim Tesisi
23	$MgSO_4.7H_2O$	Merck 1.05886
24	Peptone	Merck 1.07214.9999
25	Phosphoric acid (H_3PO_4), 85%	Merck 1.00573.2500
26	Potassium sodium tartarate tetrahydrate ($C_4H_4KNaO_6$. H_2O)	Sigma S-6170
27	Sodium bicarbonate ($NaHCO_3$), Min 99.5%	Sigma S-8875
28	Sodium carbonate (Na_2CO_3), anhydrous	Riedel-de Haën 13418
29	Sodium hydroxide (NaOH), pellets pure	Merck 1.06462.1000
30	Sodium phosphate, Monobasic, anhydrous	
31	Sodium sulphate (Na_2SO_4), anhydrous	Riedel-de Haën 13464
32	Sulphuric acid (H_2SO_4), concentrated, ACS reagent grade	Merck 1.00731.2500
33	Yeast extract	BD 211929 (BBLTM)
34	Yeast Nitrogen Base (YNB)	BD 239210 (DifcoTM)
35	Tween 80	Merck 8.22187
36	Water, HPLC grade, 0.2 µm	

APPENDIX B

THE DATA USED IN LOGARITHMIC GROWTH CURVE

Table B.1. The data used in logarithmic growth curve of *S.cerevisiae*.

Time (h)	OD (600 nm)	ln x	lnx/xo	t-to	µ
0	0,2	-1,60944	0	0	
2	0,2464	-1,4008	0,208639	2	0,104319
4	0,2615	-1,34132	0,268117	4	0,067029
6	0,3879	-0,94701	0,66243	6	0,110405
8	0,8018	-0,2209	1,388542	8	0,173568
9	1,0344	0,033822	1,643259	9	0,182584
15	2,2258	0,800116	2,409554	15	0,160637
16	2,24	0,806476	2,415914	16	0,150995
18	2,2417	0,807235	2,416672	18	0,13426
20	2,2571	0,814081	2,423519	20	0,121176
22	2,2776	0,823122	2,43256	22	0,110571
24	2,2627	0,816559	2,425997	24	0,101083
26	2,3003	0,83304	2,442477	26	0,093941
28	2,3245	0,843505	2,452943	28	0,087605
30	2,3475	0,853351	2,462789	30	0,082093
32	2,3565	0,857177	2,466615	32	0,077082

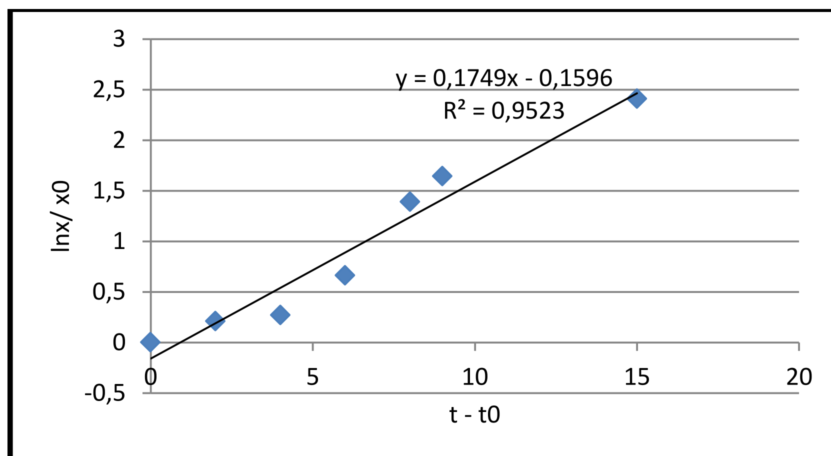

Figure B.1. Graph for logarithmic growth curve of *S.cerevisiae*.

APPENDIX C

STANDARD CALIBRATION GRAPH FOR REDUCING SUGAR

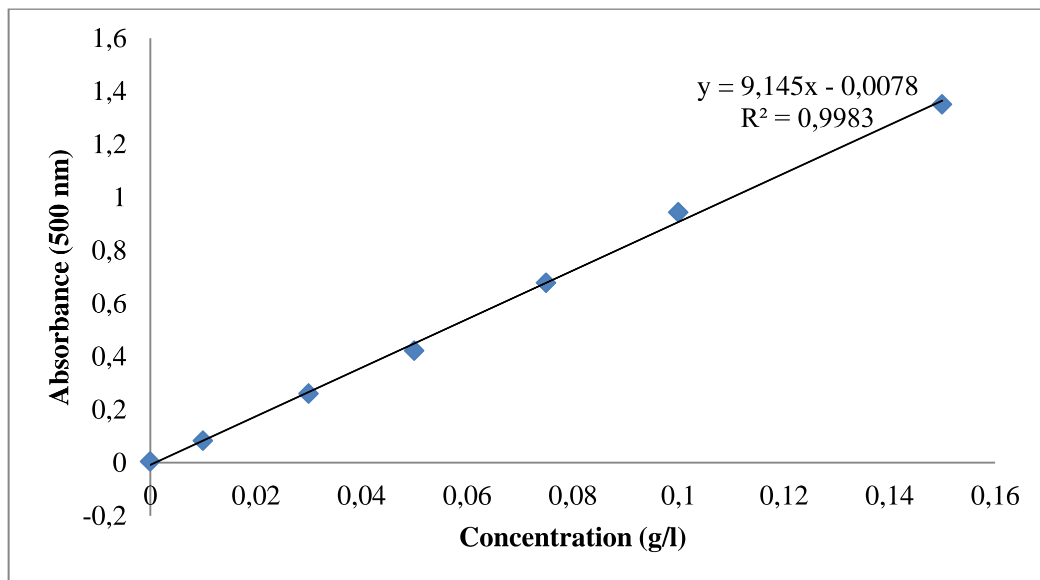

Figure C.1. Calibration graph of Nelson-Somogyi reducing sugar method

Slope (9.145) of glucose sugar was used in the calculation of reducing sugar yield determined by Nelson- Somogyi method.

Calculations

A = Average of three replicate of absorbance – Blank

B = Average slop (9.145)

C = Dilution factor

A / B x C = D (g/l sugar)

APPENDIX D

DEFINITIONS OF YIELD COEFFICIENTS

Yield on substrate:

Ethanol yield: $Y_{P/S}$ = ethanol produced (g l^{-1}) / substrate consumed (g l^{-1})

Biomass yield: Y_{SX} = biomass produced (g l^{-1}) / substrate consumed (g l^{-1})

Yield on biomass:

Ethanol yield: Y_{XE} = ethanol produced (g l^{-1}) / biomass produced (g l^{-1})

Volumetric Ethanol Productivity (Q_p):

Q_p = ethanol produced (g l^{-1}) / hour