CHEMICAL COMPOSITION ANALYSIS OF AGROINDUSTRIAL WASTE AND THEIR POTENTIAL USAGE IN BIO-ETHANOL PRODUCTION

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ABSTRACT

CHEMICAL COMPOSITION ANALYSIS OF AGROINDUSTRIAL WASTE AND THEIR POTENTIAL USAGE IN BIOETHANOL PRODUCTION

Between the year 2000 and 2008 the amount of fruits and vegetables used in fruit juice industry were 4918400 tons in Turkey. Thus, % 15-30 of a fruit is pomace, high amount of pomace appears as waste in fruit juice industry every year. Some of these pomaces could be candidates as potential fermentation media for bioethanol production. The aim of this study was in first step the optimization of the hydrolysis conditions using statistical methods and then the selection of the best hydrolysate for bioethanol production using the fungus *Tricoderma harzianum*. In the optimization study the factors were temperature, time, solid liquid ratio and acid percentage whereas the responses were furfural, hydroxymethylfurfural, glucose, xylose, galactose, arabinose and total reducing sugar yield. According to the results of the screening process, the hydrolysis step was carried out at a temperature and time of 126 °C, 40 min for apricot pomace and 110 °C, 40 min for peach and apple pomace. In the optimisation step and levels of the other factors were enlarged. The highest reducing sugar yield during optimization was 31% for apple, 49.16% for apricot and 52.44% for peach pomace. These results indicated that these pomaces hold certain potential for bioethanol production. Three different incubators (CO_2, static and non-static) were used for the fermentation process. *Tricoderma harzianum* grown aerobically in two different media (YPM and YNB) inoculated in apple hydrolysates was used in each incubator for bioethanol production. The highest ethanol production was 1.67g/L in non-static incubator with the culture grown in YNB media.

ÖZET

TARIMSAL ATIKLARIN KİMYASAL BİLEŞİM ANALİZİ VE BİYOETANOL ÜRETİMİNDE KULLANIM POTANSİYELLERİ

Türkiye' de 2000 ve 2008 yılları arasında meyve suyuna işlenen meyve ve sebze miktarı 4918400 tondur. Bir meyvenin yaklaşık olarak %15-30' u posa olduğuna göre her yıl yüksek miktarlarda posa atığı oluşmaktadır. Bu meyve posalarından bazılarının biyoetanol için potansiyel bir fermentasyon ortamı olduğu düşünülmüştür. Çalışmanın amacı ilk olarak hidroliz koşullarının istatistiksel olarak optimize edilmesi ve en iyi sonuç gösteren hidrolizatın *Trichoderma harzianum* kullanılarak biyoetanol üretiminde kullanılmasıdır. Optimizasyon için seçilen faktörler sıcaklık, zaman, katı-sıvı oranı ve asit yüzdesi olarak belirlenmiş olup sonuçlar furfural, hidroksimetilfurfural, glukoz, kslioz, galaktoz, arabinoz ve toplam indirgen şeker kazancı şeklinde ele alınmıştır. Tarama sonuçlarına göre, optimizasyon için sıcaklık ve zaman seviyeleri kayısı için 126 °C, 40 dk., elma ve şeftali için 110 °C, 40 dk. olarak belirlenmiş, diğer faktörlerin seviyeleri ise genişletilmiştir. Optimizasyonda en yüksek şeker kazancı elma için %31, kayısı için %49.16 ve şeftali için %52.44 şeklinde belirlenmiştir. Bu sonuçlardan da anlaşılacağı üzere meyve posaları biyoetanol üretimde kesin bir potansiyel içermektedir. Fermentasyon aşaması için üç çeşit inkübatör (CO_2, statik ve çalkalamalı) kullanılmıştır. *Tricoderma harzianum* aerobik olarak iki farklı besi ortamında (YPM ve YNB) üretilip elma hidrolizatına ekildikten sonra her bir inkübatörde biyoetanol üretilmesi için kullanılmıştır. En yüksek biyoetanol üretimi 1.67 gr/L olup çalkalamalı inkübatörde YNB ortamında büyüyen organizma ile sağlanmıştır.

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CHAPTER 1

INTRODUCTION

Today, the search of the alternative and sustainable energy sources has become very important since fossil fuels (responsible for 73% CO_2 production) are used continuously to meet the majority of the world's energy demand. This makes an increase in the concentrations of CO_2 in the atmosphere and concerns over global warming (Yu *et al*., 2003; Demirbas *et al*., 2004). Nowadays, bioethanol is accepted as an answer for this search by most of the countries. Furthermore, global bioethanol production showed 95% growth between the years 2000 and 2005, and it doubled between 2005 and 2010 (World Energy Outlook, 2006; F.O. Licht, 2007; Pilgrim, 2009; RFA, 2011). In America and in the world, United States and Brazil are the countries leading the industry. European countries (France is the largest and Germany is the second largest producer) and China are following the sector worldwide.

Bioethanol production (95% by fermentation and 5% synthetically) has mainly three kinds of sources; sugary, starchy and cellulosic (lignocellulosic) materials. These sources has two kinds of feedstocks; first and second-generation feedstocks. First-generation feedstocks are also sources for human and animal nutrition, second-generation feedstocks are non-food feedstocks; mainly agricultural waste. As the first-generation feedstocks are also nutrition sources for living, there are many problems about ethical concerns and favourable economics. Thus, there are severe limitations to starch and sugar-based ethanol production. Second-generation feedstocks, on the other hand, have no such concerns since they are mainly waste and furthermore, they are locally available and abundant. Fruit industry may be a great second-generation feedstock resource, since it produces a great amount of waste, which may be a candidate for fermentation media.

Fruit industry is one of the biggest industries in the world and has several branches such as frozen fruit, canned fruit and fruit juice industry. All of these branches have some processes, which lead to waste production (30-50% of fruits is discarded portion). Since the production amount of this sector is too large these waste lead to

serious environmental issues. In order to have an idea how much waste is generated in fruit industry; some industrial statistics will be mentioned.

Europe is leading the fruit juice industry in the World. In 2007, Europe had 650 producers with 11.7 billion litres of industrial production (Verband der Deutschen Fruchsaft-Industrie). Germany is the leading country of fruit juice industry in Europe with 2767.7 million litre production (Canadian Wisdom Annual Series, 2008). North America follows Europe with 9.5 billion litres production in 2009 (AIJN, 2010). Turkey is also an important country for fruit juice industry (1^{st} in apricot, 2^{nd} in sour cherry etc.). In 2008, the total production value of fruit juice and fruit juice-like products in Turkey was 821.6 million litres.

Fruit pomaces are easy to obtain. are not hardwood or softwood material (harsh and expensive pretreatment methods are not necessary) and have considerably high fermentable sugar content. These characteristics of pomaces make them candidates for all kinds of fermentation media.

There are many studies that determined the composition of agricultural wastes, such as waste of food industry; fruits and vegetables. These studies enhance the theory of fruit pomaces being candidates for fermentation media. Furthermore, other studies about agroindustrial wastes, which investigated their possibility of being fermentation media, achieved considerably positive results. For instance, apple pomace, cherry brine, bitter cola pulp, peach pulp, banana, mango, pineapple and orange waste had been used several times in these studies not just for bioethanol production with different kinds of microorganisms, but also for xanthan gum, vinegar, citric acid and pectinase production.

A pretreatment before fermentation leads to an increase in reducing sugar percentage, which makes fermentation more effective. Except sugary materials, starchy and cellulosic materials need some pretreatment before fermentation, due to solubilisation and separation of the four components; lignin, cellulose, hemicellulose and extractives, since they do not contain monosaccharides readily available for bioconversion. These pretreatments differ from each other as physical, pyhsico-chemical, chemical and biological methods. Furthermore, they (*i*) must avoid the formation of inhibitors (Laser *et al*., 2002), (*ii*) should use inexpensive chemicals and (*iii*) should be treated with simple equipment and procedures (Martin *et al*., 2007).

This study considers fruit pomaces as a fermentation media for bioethanol production and investigates the optimization of pretreatment conditions to gain high

reducing sugar content without any inhibitors. Dilute acid pretreatment was chosen since it is the most preferred and widely used method. The factors studied were temperature, acid percentage, solid-liquid ratio and time. Phosphoric acid was used, since after neutralization of hydrolysates with NaOH a salt was formed that remained in the hydrolysates, to be used later by the microorganisms.

CHAPTER 2

BACKROUND OF BIOETHANOL PRODUCTION

2.1. What Is Bioethanol?

Ethanol, which is also called ethyl alcohol, is a colourless, biodegradable, a high-octane, water-free alcohol. It is low in toxicity and causes little environmental pollution if spilt. Ethanol, being a straight-chain alcohol is often abbreviated as EtOH. It has a widespread usability in alcohol industry as alcoholic beverages, in chemical industry as a base chemical for other organic compounds, in medical as an antiseptic or as a treatment for poisoning by other alcohols. In history before the development of modern medicals it was used for a variety of medical purposes. Nowadays, the largest usage of ethanol is in automotive industry as a motor fuel and fuel additive. The chemical formula and the physico-chemical properties of ethanol are shown in Figure 2.1 and Table 2.1, respectively.

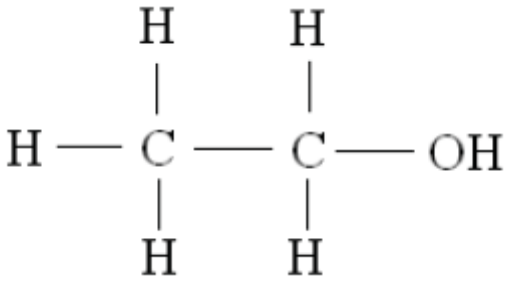

Figure 2.1. Chemical formula of ethanol

Table 2.1. The physico-chemical properties of ethanol
(Source: Walker M., 2010)

Molecular formula: C_2H_5OH
Molecular mass: 46.07 g/mol
Appearance: Colourless liquid (between -117 °C and 78 °C)
Water solubility: ∞ (miscible)
Density: 0.789kg/l
Boiling temp.: 78.5 °C (173 °F)
Freezing point: -177 °C
Flash point: 12.8 °C (lowest temperature of ignition)

(cont. on next page)

Table 2.1. (cont.)

Ignition temp: 425 °C
Explosion limits: lower 3.5% v/v; upper 19%v/v
Vapour pressure @38C: 50mmHg
Higher heating value (at 20 °C): 29,800kJ/kg
Lower heating value (at 29 °C): 21,090 kJ/L
Specific heat, Kcal/Kg 60 °C
Acidity (pK_a): 15.9
Viscosity: 1.200 mPa-s (20 °C)
Refractive index (n_D): 1.36 (25 °C)
Octane number: 99

Ethanol can be produced by either synthetically from petrochemical sources or by microbial fermentation processes. Bioethanol bears the suffix "bio" as it is produced by the action of microorganisms and enzymes through the fermentation of sugars or starches (easiest), or cellulose (which is more difficult). During fermentation of a plant material, which can be cellulosic or lignocellulosic material, sugars, such as glucose, xylose, galactose, arabinose are decomposed into ethanol and carbon dioxide. The following formula (Equation 2.1) represents the overall decomposition of glucose into ethanol and carbon dioxide.

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 + \text{heat} \qquad (2.1)$$

As it is stated on Table 2.1, ethanol has a high octane number (99), whereas regular petroleum (gasoline) has an average octane rating of 88. Octane number (ratio) is a measure of a fuel's resistance to pre-ignition, which means that internal combustion engines using ethanol can have a high compression ratio resulting in higher power output per cycle. Although vehicles running on pure ethanol can have fuel consumption (miles per gallon or kilometres per litre) 10-20% less than petroleum, ethanol's higher octane rating however, can increase the resistance to engine knocking.

2.2. The World Wide Importance of Bioethanol

The search for alternative and sustainable energy sources has become very important, because of the environmental threats caused by exploitation of non-renewable sources. These are particularly in terms of CO_2 emissions and the possible short-term shortage of fossil oil. In developed countries the energy for the transport

sector accounts for more than 30% of total energy demand, thus pointing out a critical area. Furthermore, the energy for the transport is 98% dependent on fossil fuel which is considered as one of the main causes for CO_2 increase (Piccollo and Bezzo, 2009). Besides, as a reason to extensive climate changes, the emissions of CO_2 in the atmosphere are also being viewed as responsible (Buckeridge *et al*., 2009). In fact a differentiation is substantially necessary since fossil oil effects the environment adversely and has a limited supply because of security concerns. That's why almost all countries are in a technological search for alternative and sustainable energy sources. Most of these countries found bioethanol as an answer for the search of renewable resources, because of its potential use as an alternative automotive fuel.

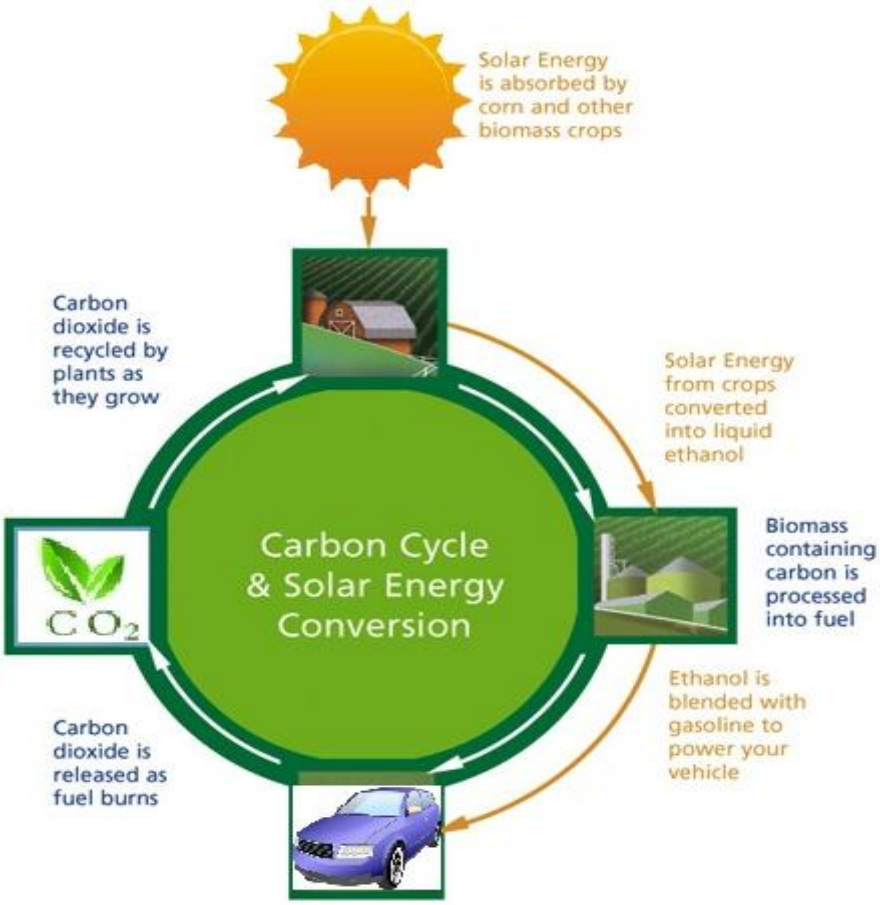

Figure 2.2. Carbon cycle and solar energy conversion of ethanol
(Source: RFA, 2010)

The main advantage of bioethanol is that, it is renewable and unlike fossil fuels it does not contribute to greenhouse gas emissions. In fact the biomass cultivated for bioethanol is able to re-fix (by photosynthesis) the carbon dioxide produced during

bioethanol production and combustion. As it is depicted in Figure 2.3 the renewable resources like agricultural products and wastes, which obtain energy from sun, can be used as automotive fuels in an existing transportation technology. As a result of the usage of ethanol in transportation engines, the carbon dioxide produced during combustion can be re-fixed by photosynthesis, which green plants are capable to do.

Ethanol can be used either as a motor fuel or motor fuel additive (in different ratios) in the automotive industry. Table 2.2 shows some typical bioethanol-gasoline blends, which are employed in different countries. Within United States of America and European countries Brazil, being the first producer of ethanol in the world, offers alternative blends (mixture of ethanol and gasoline).

Table 2.2. Typical bioethanol-gasoline blends employed in different countries (Source: Walker, 2010)

Country	**Blend** (E=ethanol and number represents % in gasoline)	**Comments**
USA	E10	10% ethanol in gasoline is common (gasohol)
Brasil	E70-E85 E25-E75 E100	Blend varies with State Higher blends possible via flex-fuel vehicles
Europe	E5 E85	Common in unleaded petroleums Relatively uncommon at present

Bioethanol has a wide range of applications. For example, it can be used as fuel for electric power, in fuel cells (thermo-chemical action), in ethanol gels (domestic cooking), in power co-generation systems and in flueless fires. Furthermore anhydrous bioethanol can be used as a progenitor for other chemical commodities such as in the production of ETBE (ethyl tertiary butyl ether, a gasoline additive) and polyethylene terephthalate, PET (packaging, bottles). It is reported that, the annual production of ethanol in the world is around 100billion litres (RFA, 2010). This issue places bioethanol as the largest volumetric product of any microbially produced bio fuel. Current global leaders of bioethanol producers are USA (~50billion litres from maize) and Brazil (~35billion litres from sugarcane). Table 2.3 presents briefly the advantages and disadvantages of bioethanol.

Table 2.3. The Advantages and disadvantages of bioethanol
(Source: Walker, 2010)

Advantages	Disadvantages
• Exhaust gases are much more neutral (reduce the emission of carbon compounds by 80% and of CO2 by 30%)	• Ethanol is hydroscopic, absorbing water from the air and thus has high corrosion aggressiveness.
• Any plant which contains either sugar or starch can be used for production of ethanol	• High amount of carbon dioxide and GHG (Green House Gases) are released during the production of ethanol.
• The output of energy during the production is more than the input.	• It has unfavourable energy balances. Burning 1 litre of ethanol gives 34% less energy than burning the same amount of petroleum.
• It can be easily found and refilled the same way as petroleum.	
• It reduces the dependence on oil	• Food-to-fuel is not ethical
• It has a better biodegradability	

2.3. Global Production of Bioethanol

The worldwide production of ethanol is increasing constantly, year by year. It is produced either synthetically or by fermentation. Only 5% of global ethanol is produced by synthetic method while the rest corresponding to 95% is produced by fermentation methods. According to World Energy Outlook, 2006; F.O. Licht, 2007; Pilgrim, 2009; USDA-ERS, 2008 and RFA, 2010, ethanol industry statistics of global bioethanol production showed 95% growth between 2000 and 2005 (Figure 2.3). Furthermore it doubled between 2005 and 2010.

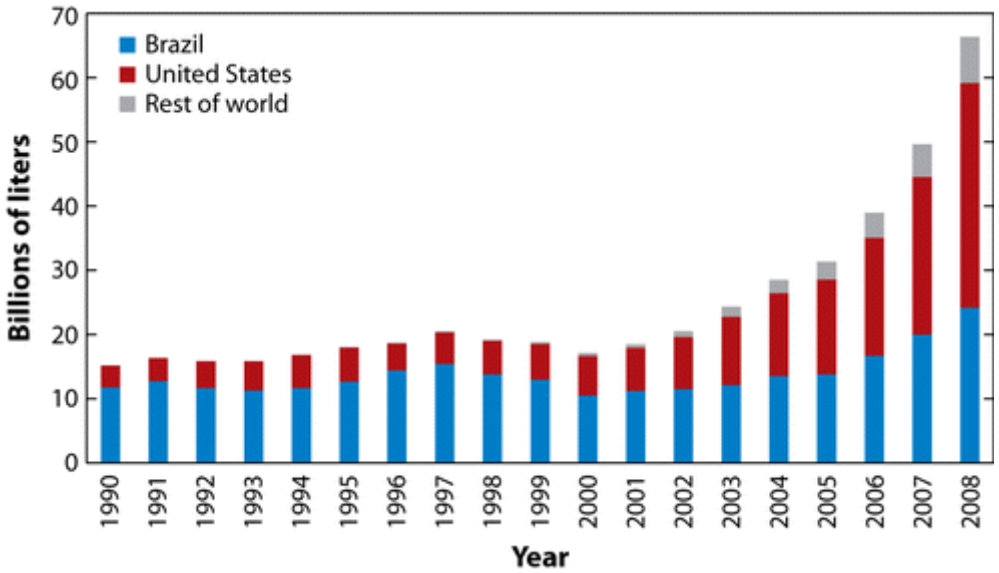

Figure 2.3. Global bioethanol production
(Source: Fargione *et al*., 2010)

Table 2.4 shows the global bioethanol production with respect to countries in the year of 2005. The very first country of producing large-scale bioethanol was Brazil, with their *Proalcool* programme. This programme was implemented by their government in 1975 exploiting sugar cane fuel alcohol as a gasoline additive in order to reduce the rising oil prices. Brazil is known as the world's largest exporter of fuel ethanol and the second largest producer of global bioethanol production with around 30 billion litres/annum (2008). It is expected that the sugarcane bioethanol plants in Brazil will increase to over 400 in the years to come, which will further increase the production to reach 37 billion litres/year (from 728 million tons of sugar cane) by the year 2012-2013 (Amorim *et al.*, 2009; Basso and Rosa, 2010).

Table 2.4. World ethanol production by country, 2005
(Source: F.O. Licht. 2006)

Country	**Production (Million litres)**
United States	16,214
Brazil	16,067
Chine	3,800
India	1,700
France	910
Russia	750
South Africa	390
Spain	376
Other Countries	2,139
World	44,875

Currently the largest bioethanol producer in the world is United States. The production capacity of fuel alcohol from 180 United States bio refineries in late 2008 was 13.6 billion US gallons (51.5 billion litres) (Ingeldew *et al.*, 2009). Bioethanol production in United States has increased rapidly in recent years. According to Renewable Fuel Association, United States produced 9000 millions of gallons in 2008, 10600 millions of gallons in 2009 and 13230 millions of gallons in the year 2010. Furthermore, in view of new renewable fuels standard schedule the bioethanol production is expected to be 20.5 and 36 billion gallons in the years 2015 and 2022, respectively.

In Europe production of bioethanol is much lower than Brazil and United States. However, there is a significant increase in bioethanol production as in the other countries. According to Bio fuels Platform, bioethanol production in Europe was only 44 million litres in 1992. However between the years of 2004 to 2009 there was a very

rapid rise in bioethanol production corresponding to an increase of approximately 635 million litres per year (3175 million litres in 5 years). France (1250 million litres) and Germany (750 million litres) were the largest producers followed by Spain (465 million litres) (Figure 2.6 and Figure 2.7). Finally in 2010 the plant capacity of Europe was 7700 million litres and there was an expectation that in 2011 the plant capacity would be 8300 million litres, as particularly in Spain and Germany new plants came on line.

According to HGCA (2010) France was the largest and Germany the second largest ethanol producer with 1850 and 1180 million litres, respectively in the European Union. In the previous years Spain was the third biggest producer. However in 2010 Portugal was able to overtake Spain and reach the third rank in European Union. Moreover United Kingdom has increased the bioethanol production over the past years. United Kingdom capacity of ethanol production grown rapidly from 70 million litres in 2009 to 470 million litres in 2010. Furthermore according to F.O. Licht (2007) future capacity was predicted to grow to 890 million litres in 2011. Figure 2.4 shows evolution of bioethanol production in the Europe between the years of 1992 and 2008.

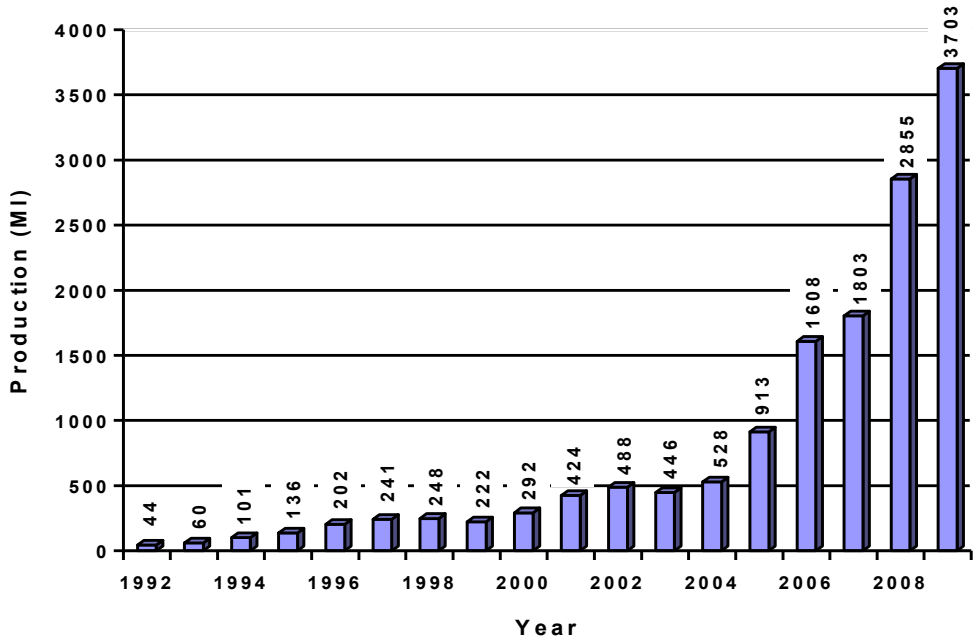

Figure 2.4. Evolution of bioethanol production in Europe
(Source: Biofuels, 2008)

In Turkey the total production capacity is 132000m^3 (%60 Konya Seker from sugar beet). The consumption amount in Turkey is expected to increase. With the

utilization of 84000 m3 bioethanol, there will be approximately 40 million dollars saving annually in the payment of foreign currency paid overseas (Konya Şeker, 2011)

The information about bioethanol production of some selected countries can be seen on Table 2.5. China and India provided significant growth potential for global bioethanol production with their new and future pilot plants and plans.

Table 2.5. The current strategical information about bioethanol production of some selected countries (International bioethanol production) (Source: Walker, 2010)

Country	**Bioethanol developments**
China	China is already the world' s third largest producer of ethanol (%90 from corn) and has ambitious future growth targets for bioethanol from second generation waste biomass. Current Chinese targets for bioethanol (10million tons by 2020) are considered conservative (Yan *et al*, 2010; Biofuels. 2011).
India	India accounts for around %4 of global bioethanol production (2m kilo litre in 2006) from sugar cane and has plans to expand its production, especially using cellulosic substrates (Praj, 2011 and Reliance Life Science, 2011).
Russia	In Russia, information on bioethanol production is provided by the Russion National Bio fuels Association (Bbiofuels, 2011).
Nigeria	In Nigeria, a recent analysis of sugarcane and sweet sorghum as bioethanol feedstocks has concluded that the latter crop is better suited in terms of its adaptability to harsh climatic and cultivation conditions (Nasidi *et al*,2010).
Australia	Information about bioethanol production in Australia is available from the Bio fuels Association of Australia (Biofuels Association of Australia. 2011).
Colombia	In Colombia, sugar cane, rather than maize, has been identified as the most promising feedstock to boost their domestic bioethanol production based on environmental and economical considerations (Quintero *et al*, 2008).
Japan/Asia Pacific	Regarding Japan and Asia Pacific, in comparison to Brazil, the US and Europe bioethanol production industry in these countries is in its infancy (Biofuels, 2011; ISSAAC, 2007) In fact, Japan is the second-largest importer of ethanol (to meets its E10 mandates) as it lacks the conditions for large scale bioethanol production. (Walter *et al,* 2008)

CHAPTER 3

THE PRODUCTION OF BIOETHANOL

Since ancient times mankind has produced ethanol as alcohol in different kinds of beverages with low alcohol content (beer and wine) by fermentation of sugar- or starch-containing plant materials. Nowadays, ethanol becomes even more important as it is considered as a new, efficient, alternative and more natural compatible energy for transportation technology, which still greatly depends on nature-damaging petroleum products.

Production of bioethanol by fermentation (95%) is much more preferred than synthetically (petrochemical, through the hydration of ethylene) production (5%) in the world. Today, while the basic steps remain the same, the production process has changed and became very efficient. In this chapter, the recent production steps of bioethanol will be mentioned.

3.1. Feedstocks for Bioethanol Production

According to Balat *et al*. (2008), the carbohydrate material which is used as feedstocks in fermentation for bioethanol production and has the typical formula of $(CH_2O)_N$ can be conveniently classified into three main groups: (i) lignocellulosic/cellulosic biomass (e.g., wood, straw, and grasses), (ii) sugary/sucrose-containing feedstocks (e.g., sugar beet, sweet sorghum and sugar cane) and (iii) starchy materials (e.g., wheat, corn, and barley). Table 3.1 shows these groups of resources for bioethanol production.

Table 3.1. Major resources for bioethanol production
(Source: Walker, 2010)

Sugary materials	**Starchy materials**	**Cellulosic (lignocellulosic) materials**
• Sugarcane (*Saccarum sp.*) • Sugar beet Sweet sorghum (*Sorghum bicolor*)	• Grains [maize, wheat (*Triticum*), triticale (Hybrid of *Triticum sp.* and *Secale sp.*), barley (*Hordeum*]	• Wood • Agricultural residues (straws, corn stover, grasses)

(cont. on next page)

Table 3.1. (cont.)

• Cheese whey • Fruits (surplus) • Confectionery industrial waste	• Root crops (potato, cassava) • Inulin (polyfructan) root crops (Chicory, artichoke),	• Municipal solid waste • Waste paper, paper pulp

There are two kinds of generations of feedstocks for bioethanol production. The sources for first-generation feedstocks for bioethanol production are also sources for human and animal nutrition, namely; cereal starches and sugar crops. As the first-generation feedstocks are also nutrition sources for living, there are some problems regarding ethical and economical concerns. Thus there are severe limitations to starch and sugar-based ethanol production.

However, there are also non-food feedstocks (mainly lignocellulosic biomass, the most abundant form of carbon on earth), which are called second-generation feedstocks for bioethanol. Figure 3.1. shows the first and second generation feedstocks for bioethanol production.

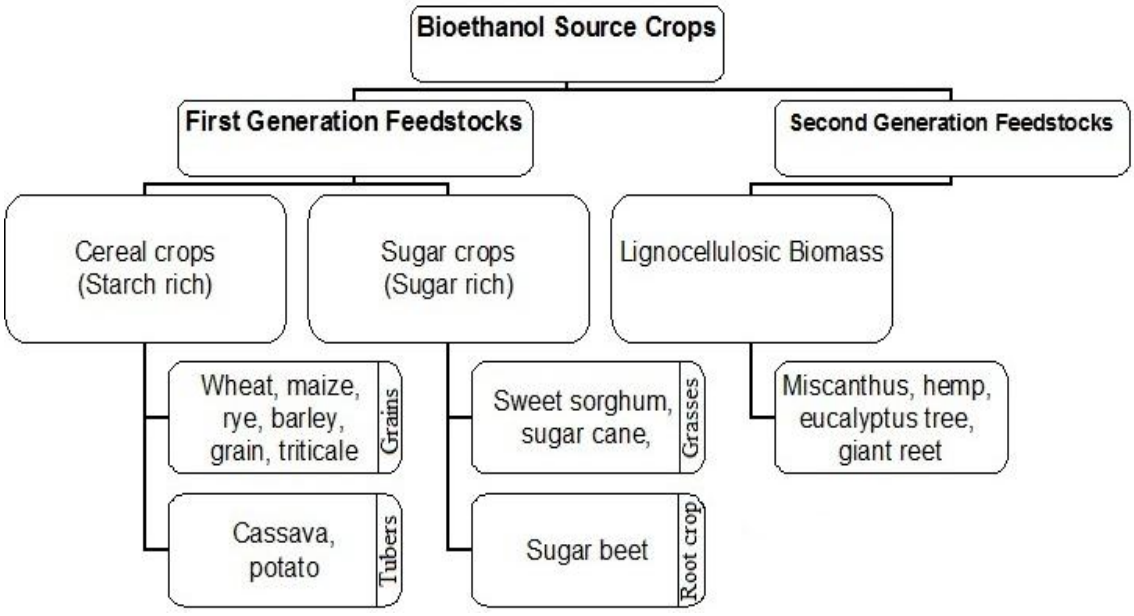

Figure 3.1. First and second generation feedstocks for bioethanol production

3.1.1. Bioethanol Production From Starch and Sugar - Based Materials (First Generation Feedstock)

There are several industrial ways to produce bioethanol from first generation feedstock. The process scheme of bioethanol production from sugarcane bagasse is shown as an example in Figure 3.2 for the first-generation feedstock.

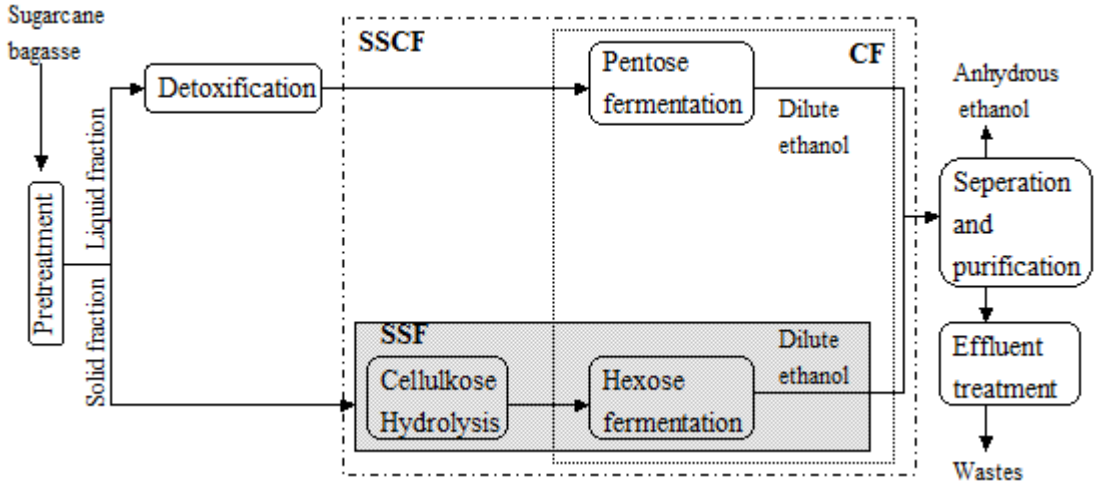

Figure 3.2. Ethanol production from sugarcane bagasse. The shaded boxes show the possibilities of reaction-reaction integration. CF, co-fermentation; SSF, simultaneous saccharification and fermentation; SSCF, simultaneous saccharification and co-fermentation. (Source: Cardona *et al*., 2010)

The main difference between sugar-based and starch-based materials is that the sugar-based materials (sugar cane, sugar beet, sweet sorghum) represent a readily fermentable sugar source (comprising mainly glucose, fructose and sucrose). On the other hand, starch-based materials (wheat, rye, barley, maize, grain, cassava, potato, etc.) require pre-hydrolysis to obtain sugars that can be fermented by yeast. Thus, in the case of using sugar-based materials, fermentation can be carried out without any necessity to prior hydrolysis or other pre-treatments, as the sugar is available in disaccharides, which can be metabolised directly by enzymes present in yeast. This makes sugar-based materials (sucrose-containing feedstocks) easy to process for bioethanol production. Furthermore, it is more efficient compared to other feedstocks and the cost of the process is relatively low compared to the commodity price (Walker, 2010). Cereal grains need some pretreatment before fermentation such as milling and starch hydrolysis. After hydrolysis, fermentation can be carried out by yeast and 99%

ethanol can be obtained with distillation and water removal. From ~3kg wheat, 1L anhydrous ethanol can be produced. Table 3.2 shows the differences of key parameters between a starch-based (wheat) and sugar-based material (sugar beet). Ethanol yield of wheat is much greater than sugar beet on a weight basis. However, sugar cane is more productive than wheat due to greater yield of crop and energy of sugar beet.

Table 3.2. The differences of key parameters between wheat and sugar beet as a first generation feedstock of bioethanol production. (Source: Walker, 2010)

Parameter	**Wheat**	**Sugar beet**
Moisture content (%)	20	76
Starch/sucrose content (%)	76	69
Ethanol yield (L/t)	374	100
Crop yield (t/ha)	8.4	55
Cost of feedstock €/t	100	50
Cost of feedstock €/L of ethanol	0.267	0.50

Due to abundance, ethical considerations and favourable economics, second generation feedstocks are the future sources for bioethanol production.

3.1.2. Bioethanol Production From Lignocellulosic Materials (Second Generation Feedstock)

Second generation feedstocks for bioethanol production are mainly cellulosic biomass. In spite of the fact that there are estimations from different sources, which may vary considerably, there is a general conclusion that cellulosic resources are exoterically locally available and abundant (Piccolo and Bezzo, 2009). On the other hand, with growing demands for future bio fuel production, the use of first generation feedstocks is ultimately unsustainable. Moreover, there are severe limitations to starch and sugar-based ethanol production. For instance, if the United States was to replace all gasoline with 10% ethanol, around 46% of the current maize crop would be required which is obviously unacceptable (Walker, 2010). This makes lignocellulosic materials very important, relatively inexpensive and prudential sources. According to Sanchez and Cardona (2008), the annual production of lignocellulosic biomass is 10^{10} million ton.

Nowadays, quite a few lignocellulosic materials are used as second-generation feedstocks for bioethanol production and great numbers of other lignocellulosic materials are considered in future applications. Some of lignocellulosic materials, which

are used as feedstock, are (i)waste materials like agricultural residues(oilseed pulp, sugar beet pulp), woody wastes/chippings and forestry residues, corn residues(fibres, stover and cobs), straws, old paper/cardboard, bagasse, spent grains, municipal solid waste, (ii)energy grasses such as switch grass (*Panicum vigratum*), reed canary grass (*Phalaris arundinaceae*), giant reed (*Arundo donax*), ryegrass, *Miscanthus gigantum*, and (iii)energy crops such as short rotation coppice like basket willow (*Salix viminalis*).

A cellulosic biomass is composed of lignin, cellulose and hemicellulose and is thus called lignocellulosic material most of the time. As starch molecules, cellulosic molecules consist of long chains of glucose molecules (6-carbon sugars), however they have a different structural configuration. In addition to this, lignin in lignocellulosic materials encapsulates cellulose and hemicellulose molecules, which are also comprised of long chains of sugar molecules, but contain pentoses in addition to glucose. The lignin is partly covalently associated with hemicelluloses. Furthermore, cellulose has a crystalline structure. This structure of lignocellulosic materials makes them more difficult to hydrolyze than starchy materials. As lignocellulosic materials do not contain monosaccharides readily available for bioconversion, the four components in lignocellulosic materials (lignin, cellulose, hemicellulose and extractives) should be solubilised and separated by means of acids or enzymes, to make them more accessible to further treatment, either chemical or biological. A pretreatment is necessary for removing lignin and hemicelluloses, reducing cellulose crystallinity and increasing the porosity of materials (Keller *et al.*, 2003). This pretreatment must avoid the formation of inhibitors (Laser *et al*., 2002), should use inexpensive chemicals and require simple equipment and procedures (Martin *et al*., 2007).

There are several pretreatment methods, which have been investigated and reviewed by Sun and Cheng (2002), Sánchez and Cardona (2008) for different lignocellulosic materials. Table 3.3 shows the types and the names of some pretreatment methods.

Table 3.3. The types of pretreatment methods for cellulosic bioethanol production (Source: Cardona *et al*.,2010)

Type of pretreatment	**Name of pretreatment**
Physical	Mechanical combination Pyrolysis Extrusion
Pyhsico-chemical	Steam explosion (auto hydrolysis) Ammonia fiber explosion (AFEX) CO_2 explosion SO_2 explosion Thermal hydrolysis Wet oxidation
Chemical	Ozonolysis Acid hydrolysis Alkaline hydrolysis Oxidative delignification Organosolve process
Biological	Microbial Enzymatic

3.2. Fruit Pomaces as Potential Candidates

Fruits, which are a seed-associated structure of a plant, have great importance in food industry as they have large populated consumers. Fruit industry has several industrial branches such as fruit juice, canned fruit, frozen fruit industry etc. All of these have some processes, which lead to waste production for instance during selection, sorting and boiling processes. There are two types of waste; (i) a solid waste like peel/skin, seeds, stones etc (ii) a liquid waste of juice and wash water. These wastes can lead to serious problems about waste disposal and environment since some fruits (orange, mango etc.) have 30-50% discarded portion. In order to give an idea how much waste occurs in fruit industry every year some industrial statistics about fruit industry will be mentioned.

3.2.1. Facts and Figures about the EU Fruit Juice Industry

According to a statistical report prepared by Verband der Deutschen Fruchsaft-Industrie there are approximately 650 producers with 11.7 billion litres (fruit juice, fruit nectars, fruit juice drinks without CO_2) industrial production in Europe (2007).

According to Canadian Wisdom Annual Series (2008), in 2007 Germany was the greatest consumer with 2767.70 million litres, and AIJN European Fruit Juice Association Market Report (2010) suggested that, this continued in 2009 with 3193 million litres of consumption. Mainly apple (25.8%), orange (26.4%) and multivitamin flavours (18%) take of this consumption. In 2007, France was the second (1553 million litres) and United Kingdom the third (1495 million litres) in this matter (Canadian Wisdom Annual Series, 2008). Table 3.4 shows consumption of fruit juice and nectar in the leading countries of EU (2007).

Table 3.4. Consumption of fruit juices and nectars in the EU (2007)
(Source: Canadian Wisdom Annual Series, 2008)

	Total consumption volume (million litres)	% of total EU market
Germany	2767.7	25.8
France	1553	25.35
United Kingdom	1495.4	24.69
Spain	1273.67	28.56
Italy	841.59	14.65
Poland	783.41	20.57

According to AIJN European Fruit Juice Association 2010 Market Report, orange is the most consumed fruit in Europe with 34.6% and apple the second with 15% of the total fruit juice consumption.

3.2.2. Facts and Figures about the USA Fruit Juice Industry

Europe is leading the fruit juice industry in the world. However, it is followed by North America and Asia Pacific with 9.5 and 8 billon litres of fruit juice and nectar consumption in 2009, respectively. (AIJN, 2010 Market Report). Table 3.5 shows total commercial production of some selected citrus (Orange, lemon) and noncitrus (Apple, grape, peach, apricot, strawberry) fruits of United States.

Table 3.5. Total commercial production of selected citrus and noncitrus fruits in United States from 1995 to 2009 (1000 short tons) (Source: USDA, 2011)

Year	Apples	Grapes	Peaches	Apricots	Oranges*	Strawberries	Lemons
1995	5289	5922	1145	61	11432	804	897
1996	5191	5554	1052	79	11426	813	992
1997	5162	7291	1312	139	12692	814	962
1998	5823	5820	1190	118	13670	819	897
1999	5316	6236	1252	91	9824	916	747
2000	5290	7688	1276	97	12997	950	840
2001	4712	6569	1204	82	12221	826	996
2002	4262	7339	1268	90	12374	942	801
2003	4390	6644	1260	98	11545	1078	1026
2004	5206	6240	1307	101	12872	1107	798
2005	4834	7814	1185	82	9251	1161	870
2006	4912	6378	1010	45	9020	1202	980
2007	4545	7057	1127	89	7625	1223	798
2008	4816	7319	1135	82	10076	1266	619
2009	4958	7295	1104	69	9128	1401	912

*Year harvest was completed

In addition to this rate of production, United States import substantial amount of fresh and frozen fruit from other American countries such as Canada, Argentina, Costa Rica, Guatemala, Ecuador, Chile etc. and other world countries. Table 3.6 shows volume of U.S. imports of selected commodities from top countries between 2002 and 2009.

Table 3.6. Volume of U.S. imports of selected fruits from top countries, 2002-2009 (Source: Source: U.S. Department of Commerce, U.S. Census Bureau, 2009)

	2002	2003	2004	2005	2006	2007	2008	2009
Orange								
South Africa	35,758	50,984	59,009	62,155	78,006	63,179	74,154	60,067
Australia	45,885	43,512	50,013	60,508	49,202	63,866	47,410	51,777
World	129,444	119,911	144,773	152,196	162,233	253,78	168,915	206,239
Apple								
Chile	137,877	199,050	249,692	119,964	118,143	272,317	206,502	192,899
New Zealand	133,087	112,801	127,224	71,325	82,489	104,079	72,315	98,145
Canada	95,605	82,163	66,878	74,492	76,926	68,481	79,445	46,514
World	375,565	411,430	457,191	270,669	345,439	455,391	364,385	343,426
Grapes								
Chile	879,676	919,675	927,348	968,645	1,059,336	941,791	930,270	1,009,720
Mexico	227,463	306,011	210,961	337,104	213,559	303,948	296,371	251,482
World	1,142,583	1,240,542	1,167,395	1,347,742	1,330,774	1,297,013	1,300,536	1,329,010
Peaches								
Chile	124,954	142,404	163,321	155,315	131,368	127,869	143,286	108,228
Mexico	176	526	655	2,045	1,455	2,412	2,729	1,619
World	126,862	143,454	164,573	157,599	133,301	131,588	148,682	111,483

3.2.3. Fruit Juice Industry in Turkey

Turkey is an important country with respect to fruit juice industry of the world. Commercial production of the fruit juice industry started in 1960's in Turkey. Within this relatively young industry investments spreaded in 1970's. After the economic fluctuations that took place in 1980's, a revival occurred in 1990's. From the beginning of 2000's, a new area of growth begun in the Turkish juice market. Turkey ranked as the 1st in apricot, 2nd in sour cherry, 3rd in pomegranate, 4th in apple, 6th in peach and grape production worldwide. During this new area, where the sole empire of USA and Western Europe ended new actors like India, China, Brazil, Middle and Eastern countries started to emerge. Spots were also turned to Turkey because of its advantages like being close to energy sources, its special situation, young population and high agricultural production power. As can be seen from this figure, Turkey exhibited the largest nominal growth rate of 8 % in the fist quarter of 2009 (MEYED, 2009). Table 3.7 indicates the production amount of main fruits used in fruit juice industry in Turkey between the years of 2000 and 2008.

Table 3.7. Production amount of main fruits, processed to fruit juice, in Turkey (2000-2008), thousand tons (Source: MEYED, 2008)

Fruit	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**
Apple	2,400	2,450	2,200	2,600	2,100	2,570	2,002	2,450	2,505
Apricot	579	517	352	352	350	894	483	570	751
Peach	430	460	455	455	372	510	553	543	552
Cherry	106	120	100	100	138	140	122	170	185
Orange	1,070	1,250	1,250	1,250	1,300	1,445	1,536	1,441	1,427
Grape	3,600	3,250	3,500	3,500	3,500	3,850	4,000	3,612	3,448
Pomegranate	59	60	60	60	73	80	91	102	128
Total	8,244	8,107	7,917	8,644	7,833	9,489	8,787	8,888	8,996
Alteration from previous year (%)	0.0	-1.7	-2.3	+9.2	-9.4	+21.1	-7.4	+1.1	1.2

Table 3.8 shows the consumption of beverages related to fruits, which are mainly of 4 types, fruit juice, fruit nectar, fruit beverage and fruit-aromatic beverage in Turkey between the years 2000 and 2008. According to MEYED, the total production value of fruit juice and fruit juice-like products in Turkey was 294.9 million litres in 2000 and 821.6 million litres in 2008.

Table 3.8. Production amount of fruit juice and fruit juice like beverages (2000-2008), million litres (Source: MEYED, 2008)

Type of beverage	2000	2001	2002	2003	2004	2005	2006	2007	2008
FJ	1.9	3.9	5.6	9.3	12.2	30.6	74.7	73.4	70.8
FN	202.8	212.1	208.5	233.6	300.3	368.9	509.2	525.9	534.6
FB	56.7	51.5	27.2	16.0	17.4	29.6	41.0	25.7	38.8
FAB	33.5	34.6	52.6	98.4	129.4	83.3	121.9	123.1	177.4
Total	249.9	302.1	293.9	357.3	459.3	512.4	746.8	746.8	821.6
Alteration from previous year (%)	0.0	+2.4	-2.7	+21.6	+28.5	+11.6	+45.7	+0.2	+9.8

FJ: Fruit juice, FN: Fruit nectar, FB: Fruited beverage, FAB: Fruit-aromatic beverage

These statistics of fruit juice industry of selected countries, which are mainly leading sectors, help to give an opinion about how much fruit wastes are produced every year. As mentioned before, these wastes bring up serious problems about waste disposal and environment. Table 3.9 shows annual worldwide processed quantities and resulting of some selected fruits.

Table 3.9. Annual worldwide processed quantities and resulting wastes of selected fruits (Source: Oreopoulou and Tzia, 2007)

Fruit or vegetable	Annual processed (million Mt)	By-product/ processed fruit (%,wet basis)	Estimated annual waste (million Mt)
Orange and other citrus fruits	31.2[a]	50	15.6
Apple	12.0[a]	25-35	3.0-4.2
Pear	1.7[a]	NA[f]	
Peach (canned)	1.0[a]	NA	
Grape	50[b]	15-20	5-9

[a] Processed in 2003/4 according to USDA
[b] Schieber *et al*. (2001)
[c] Produced in 2003/4 according to faostat.fao.org
[d] Commission of the European Communities (200) report.
[e] Estimated as approximately 50% of the world production
[f] NA: nonavailable

Furthermore, fruit pomaces 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, fruit pomaces are not only candidates for bioethanol production feedstocks, but also for all kinds of other fermentation media.

3.3. Fruit Pomace as a Fermentation Media

Agricultural residues such as barley straw, oat straw, rice straw, wheat straw, sorghum straw, cottonseed hulls, sugarcane bagasse, entire bagasse, fibre bagasse and pith bagasse contain highest amount of cellulose, hemicellulose and lignin. Similarly, the wastes of the food industry such as of fruits and vegetables (apples, banana, lemons, oranges, pineapples, potato, carrot, cauliflower, cabbage, tomato and peas) are following these agricultural wastes (Table 3.10).

Table 3.10. Cellulose content and composition (g/100g of dry matter)
(Source: Das and Singh, 2004)

Cellulosic wastes	Cellulose	Lignin	Hemicellulose	Reference
1. Agricultural residues				
Barley straw	44	7	27	Marsden, 1986
Oat straw	41	11	16	Marsden, 1986
Rice straw	33	7	26	Marsden, 1986
Wheat straw	39	10	36	Marsden, 1986
Sorghum baggase	31	11	30	Marsden, 1986
Cottonseed hulls	59	13	15	Marsden, 1986
Sugarcane bagasse	40	13	29	Marsden, 1986
2. Fruits & Vegetables				Southgate, 1976
Apples	2.9	Trace	5.8	-
Banana	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	-
Peas	14	2	36	-

Pomace is a valuable food source and fermentation media that remains after juice has been squeezed from fruits (Carson *et al*., 1994). There are many studies analysing the chemical composition of fruit pomaces, mainly apple, peach and orange (mostly orange peel). Table 3.11 states some of these results.

Table 3.11. Results from the studies, which analysed chemical composition of peach, apple, apricot and orange.

	Ash	Protein	TS	TDF	SDF	IDF	RS	Reference
Peach	3	7.5	93	54.2	19.1	35.4	-	(Pagan *et al*., 2001)
	3	6.2	-	35	12	23.8	13.2	(Grigelmo-Miguel *et al*., 1997)[a]
	2.8	5.7	-	32.7	10.7	22	14.4	
	2.9	5.4	-	30.8	10.8	20	13.1	
Apple	-	7.2	28.4	56.7	-	-	-	(Pirmohammadi *et al*., 2006)
	-	6.4	74.9[1]	47.3	-	-	-	
	3.07	3.8	27	-	-	-	11.3	(Vendruscelo *et al*., 2008)
	6.2	2.5	98.8[1]	41,1	3,1	38	48.8[2]	(Carson *et al*., 1994)[b]
	5.5	1.9	98.5[1]	35.5	3	32.5	56.1[2]	
	4.8	2.2	98.7[1]	33.4	3.5	29.9	58.5[2]	
	3.5	3.7	20.8	38.2	-	-	10.8, 59.8[2]	(Albuquerque, 2003)
	2.8	5.1	34.4	4.3-10.5	-	-	5.7, 9.5-22[2]	(Hang and Woodams, 1987)
	1.5	4.7	94.2[1]	-	-	-	83.8[2]	(Jin *et al*., 2002)
	1.82	5.8	96[1]	14.7	-	-	48[2]	(Joshi and Shandu, 1996)
	2	4.1	20	40.3	-	-	15	(Villas-Boas and Esposito 2000)
	-	1.6		7.8[3]	-	-	-	(Bacha *et al*., 2011)
	-	-	16	-	-	-	7	(Patle and Lal, 2007)
Orange	1.7	7.9	-	-	-	-	-	(Mamma *et al*., 2008)[c]
	4.6	1.45	11.6	-	-	-	41.2[2]	(Kaparaju and Rintala, 2006)
	-	-	-	-	-	-	24.4*	(Widmer *et al*., 2010)
	3.4	6	-	-	-	-	-	(Grohmann *et al*., 1995)
	3.59	5.25	-	12.93	-	-	-	(Ma *et al*., 1993)
	-	10.1	-	7.8	-	-	-	(Bacha *et al*., 2011)
	3.3	10.2	24.7	57	9.4	47.6	273[2]	(Chau and Huang, 2003)[c]

[1] Dried pomace, [2] Carbohydrate, [3] Crude Fiber, [a] Studied different harvesting times; August, September and October respectively, [b] Studied different cultivar of apples; Golden delicious, Red delicious and Winesap, [c] Studied orange peel, *5.4 sucrose, 8.9 glucose, 9.1 fructose, 0.2 galactose and rhamnose, 0.6 arabinose (%) respectively

TS: Total Solids, TDF: Total Dietary Fiber, SDF: Soluble Dietary Fiber, IDF: Insoluble Dietary Fiber, RS: Reducing Sugar.

Pirmohammadi *et al*. (2005) mixed 1 tonnes of apple pomace (ensiled apple pomace) with 100 kg of wheat straw and 5 kg of urea (on fresh weight basis). The study concluded that, the nutritive value of ensiled apple pomace was reduced by addition of wheat straw. However, this silage can be illustrated by the good fermentation characteristics, such as low pH, acetic, butyric acids and high lactic acid. Ponte Rocha *et al*. (2009) studied the enzymatic hydrolysis and fermentation of pre-treated cashew apple bagasse with alkali and diluted sulphuric acid for bioethanol production. They achieved 52 g/L glucose concentration using hydrolysis (45°C, enzyme load of 30 FPU/g bagasse, and solid percentage of %16 (w/v), and using cashew apple juice dilute acid pretreatment followed by lignin removal by NaOH. This hydrolysate was easily

fermented by *S. cerevisiae* yeast for the production of ethanol, resulting in a concentration of 20 g/L in 6 h of fermentation. Therefore, they concluded that the fermentation cashew apple pomace hydrolysate stands as an alternative process for fuel ethanol production from lignocellulosic residues. According to Vendruscelo *et al*. (2008) apple pomace and its aqueous extract present a great potential for use as substrates in biotechnological processes. Furthermore, in order to use the apple pomace in bioprocesses effectively, several operational variables must be considered and optimised. Except for its use by rural inhabitants in the production of homemade alcoholic beverages, cashew apple has no commercial value (Karuppaiya *et al*., 2009). Therefore Karuppaiya *et al*. (2009) studied optimization of process conditions using response surface methodology for ethanol production from waste cashew apple juice by *Zymomonas mobilis* and determined the optimum process conditions as: substrate concentration 62% (v/v), pH 5.5, temperature 32 °C, and fermentation time of 37h. On these conditions ethanol concentration of 12.64 g/L was obtained. In a study, which Chatanta *et al*. (2008) tried to produce bioethanol from apple pomace left after juice extraction, *S. cerevisae*, *A. foetidus* and *F. oxysporum* were used and 16.09% (v/w of apple pomace) ethanol was produced from fermented apple pomace with a residual sugar of 0.15% (w/w of apple pomace). They indicated that the alcoholic fermentation of apple pomace might be an efficient method for alleviating waste disposal.

Spent cherry brine, which is an acidic byproduct of maraschino cherry processing and consisting of variable amounts of glucose and fructose of 0.5-1.5% $CaCl_2$, up to 11% fermentable solids, up to 0.4% sulphur dioxide, sorbitol and lesser amount of other cherry constituents, was used for ethanol production by Park and Bakalinsky, (1997). All strains of *Saccharomyces cerevisiae* used for fermentation, were able to ferment all lots of $Ca(OH)_2$-treated and phosphorous-enriched brines efficiently. Highest yield of ethanol was 4.7% (w/v) in 4 days.

Nzelibe and Okafoagu (2007), investigated the optimization of ethanol production from *Garcinia kola* (bitter kola) pulp agro waste. With acid hydrolysis and saccharification pretreatments, the ethanol yield was maximum at 120 h (70.7 g/L).

Guava, which is one of the important commercial fruit crops of India, was investigated for ethanol production by Srivastava *et al*. (1997). The study achieved maximum ethanol production (5.8% w/v) during 36 h fermentation of *Saccharomyces cerevisiae*.

Papi *et al*. (1998) studied xanthan gum and ethanol production by *Xanthomonas campestris* and *Zymomonas mobilis* from peach pulp. The study suggested that both bacteria grew well and produced 0.1g/L xanthan gum and 110g/L ethanol.

Moreover, the yield of pectic substance extraction was studied by Faravash and Ashtiani (2008), using dried mixed varieties of peach pomace. The investigated factors in this particular study were acid volume, ethanol-to-extract ratio and acid washing time. All of the factors had significant effect on pectin extraction. The maximum extraction yield obtained was 9.94 ± 0.2% using 65 ml of HCL, at the ethanol-to-extraction ratio of 1.5 and the acid-washing time of 120 min.

Fermentation of pre-treated hydrolysates of banana and mango fruit waste was studied by Arumugan and Manikandan, (2011) for ethanol production. According to their results banana fruit pulp had 23.37% total solids, 1.37% lipid, 19.75% ash and 0.63% starch and mango peels had 18.74% total solids, 7.96% protein, 1.48% lipid, 13.08% ash and 0.51% starch. Total dietary fibre content ranged from 3.54% to 73.04% in the fruit samples. Pretreatment was performed using dilute H_2SO_4 followed by enzymatic hydrolysis. Maximum reducing sugar yield of 64.27% was obtained when mixed fruit pulps were used. This fermentation media showed maximum ethanol production of 35.86% corresponding to a fermentation efficiency of 70.31% at 48hr of incubation.

Pineapple waste was used for vinegar production by semi-solid state fermentation by Gu *et al*., 2010. Wine yeast for alcoholic and Acetobacter powder AS1.01 for acetic acid fermentation was inoculated together. Under the selected optimum condition, which were 22 °C for fermentation temperature, sugar content of 16 °Brix, 3.5 for pH, 6 days of fermentation time and 0.3% of inoculation ratio, the acid production (calculated as acetic acid) was around 6.78 g/100g for fermented pineapple waste, and the conversion ratio of acetic acid was 82.5%. The study suggested that semi-solid state fermentation gave a higher total acid production in a shorter time in comparison with liquid-state fermentation.

Orange and pineapple wastes were used as potential substrates for citric acid production (Kuforiji, 2010). In this study using orange waste, two strains, *Aspergillus niger* strains NRRL 567 and 328 produced 57.6% and 55.4% of citric acid, respectively at a moisture level of 38.9%. Highest citric acid yields of 46.4% and 45.4% were obtained in pineapple waste at moisture contents of 54.4% and 63.4%, respectively.

Oberoi *et al*., (2010) used orange peels to produce ethanol by two-stage hydrolysis. First hydrolysis was carried out at acid concentrations ranging from 0 to 1% (w/v) at 121 °C and 15psi for 15 min. Second hydrolysis was carried out at 0.5% (w/v) acid. They achieved a high volumetric productivity of 3.37g/L/h. This indicated a significant potential for such a process to commercially produce ethanol from orange peels. Pectinase production in solid state fermentation by *Aspergillus niger* using orange peel as substrate was studied by Mrudula and Anitharaj (2011). The optimum temperature, pH, incubation time, moisture ratio, inoculum size, carbon source and surfactants, were found to be 50 °C, 5, 96h, 1:2 (v/w), 2.5 ml, sucrose and Triton-X-100, respectively. The strain produced 232 U/ml in submerged fermentation (Smf) and 1224 U/g solid in solid-state fermentation (SSF). The final optimised production was 5283 U/g solid. Valencia orange (*Citrus sinensis*) peels were used as substrate for the production of citric acid (CA) by *Aspergillus niger* CECT-2090 in solid-state fermentation (SSF) (Torrado *et al*., 2011). 193.2 mg/g dry orange peel resulted into the highest CA concentration, obtained at 85 h of incubation. The inoculum concentration was $0.5 \cdot 10^6$ spores/g of dry orange peel and the initial water content of 2.52 mL/g of orange peel, corresponded to 70% saturation. The study suggested that the results could be of interest to possible, future industrial applications.

Patle & Lal (2007) studied ethanol production from hydrolysed agricultural wastes using mixed cultures of *Zymomonas mobilis* and *Candida tropicalis*. They used different fruit and vegetable wastes collected from market and fruit processing industries. They concluded that among the acid, alkaline and enzymatic hydrolysis processes, enzymatic hydrolysis yielded maximum reducing sugars (97.7%). They suggested that these wastes were proved to be promising substrates for ethanol production.

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

3.4. Pretreatment of Feedstocks

Figure 3.4, 3.5 and 3.6 is an example of how pretreatment can change the raw material in microscopic level.

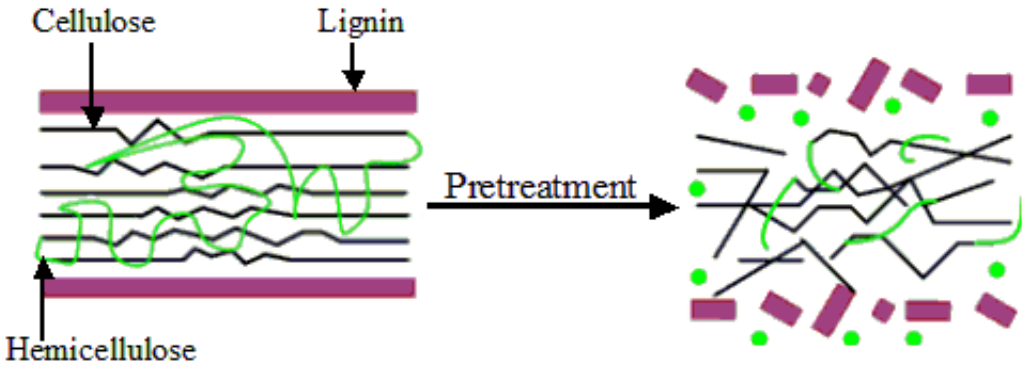

Figure 3.3. Schematic representation of biomass pre-treatment
(Mosier *et al*., 2005)

There is a lignocellulosic material in Figure 3.3. This material consists of lignin, hemicellulose and cellulose molecule chains. After pretreatment these molecules partially break down into sugar molecules (green points). Lignin and some hemicellulose are dissolved away by acid pretreatment leaving behind individual plant cells. Without pretreatmen degrading enzymes of microorganisms could not penetrate through microfibrils of cellulose fibers of lignocellulosic material. That leads low yielded productivity and high residue of municipal solid waste. However after a pre-treatment process the microfibrils of cellulose microfibers are released, since dilute acid penetrate through the lignocellulosic molecules and breaks down the lignin, which enclose cellulose fibers. As a consequence of this microfibrils of cellulose microfibers are free for degrading enzymes of microorganisms. (Figure 3.4)

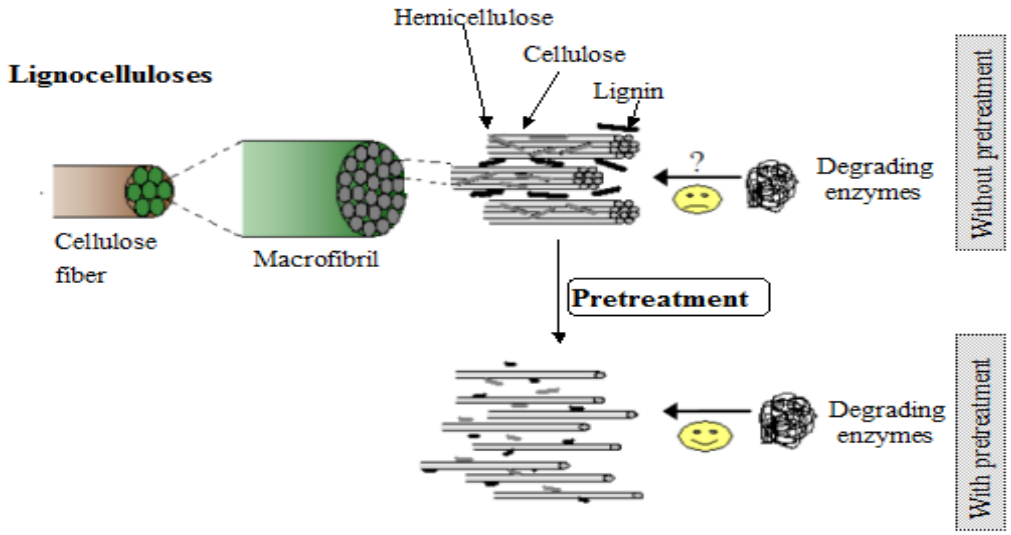

Figure 3.4. The effect of pretreatment to macrofibrils of cellulose fibers

Figure 3.5 shows false-colour scanning electron micrographs of corn stover cell walls obtained by NREL' s Todd B. The original sample (left) changes after partial pretreatment (middle) and a full pretreatment (right).

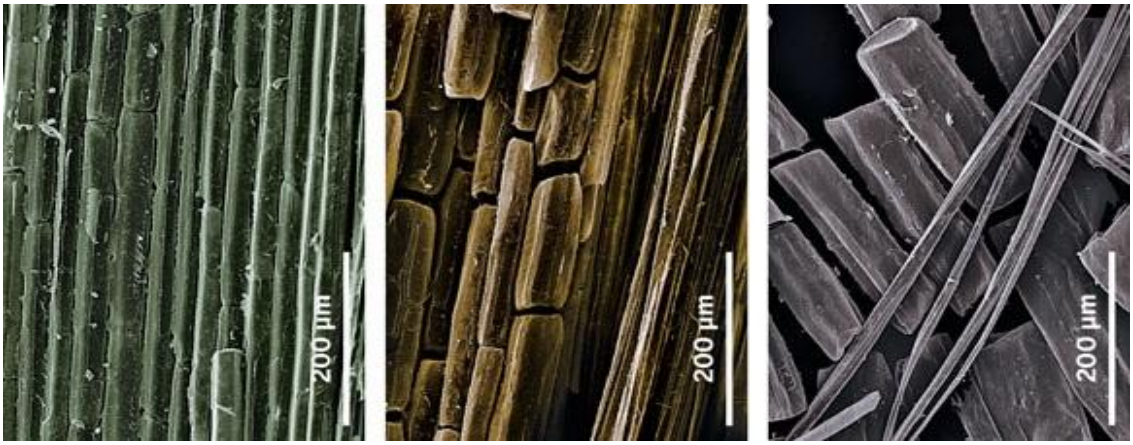

Figure 3.5. The change of corn stover cell walls with acid pretreatment
(Source: Brunecky *et al*., 2008)

There are several key factors for an effective pretreatment of lignocellulosic (cellulosic) biomass. (Yang and Wyman, 2008). These parameters are mainly;

- *High yields.*
  Various pretreatments such as alkaline-based pretreatment methods (lime, ammonia fiber explosion, and ammonia recycling percolation) have been shown to be better suited for specific feedstock. However, they are less satisfactory for processing recalcitrant substrate as softwoods (Chandra *et al*., 2007). On a wide range of lignocellulosic biomass, acid based pretreatment have been shown to be effective (Mosier *et al*., 2005).
- *Highly digestible pre-treated solid.*
  After pretreatment process, cellulose should be highly digestible with yields higher than 90,5 in less than five and preferably less than 3 days with enzyme loading lower than 10 FPU/g cellulose (Yand and Wyman, 2008).
- *Minimum amount of toxic compounds.*
  When pretreatment is achieved in harsh conditions, generation of toxic compounds such as furfural and 5-hydroxymethylfurfural derived from sugar decomposition that could affect the proceeding hydrolysis and fermentation steps can occur (Oliva *et al*., 2003).
- *Biomass size reduction not required.*
  Methods used in size reduction such as milling or grinding are energy-intensive and costly technologies (Alvira *et al*., 2010)
- *Operation in reasonable size and moderate cost reactors*
  Pretreatment reactors should be low in cost. (Alvira *et al*., 2010)

- *Non-production of solid-waste residues.*
  The chemicals formed during hydrolysate conditioning in preparation for subsequent steps should not present processing or disposal challenges. (Alvira *et al*., 2010)
- *Effectiveness at low moisture content.*
  Materials in very dry content would reduce energy consumption during pretreatment. (Alvira *et al*., 2010)
- *Obtaining high sugar concentration.*
  In order to obtain an adequate ethanol concentration and keep recovery and other downstreams cost manageable, the concentration of sugars from the pretreatment and enzymatic hydrolysis should be above 10%. (Alvira *et al*., 2010)
- *Fermentation compatibility.*
  The distribution of sugar recovery between pretreatment and subsequent enzymatic hydrolysis should be compatible with the choice of an organism able to ferment pentoses (arabinoses and xylose) in hemicellulose.
- *Lignin recovery.*
  Lignin and other constituents should be recovered to simplify downstream processing and for conversion into valuable co-products (Yang and Wyman, 2008)
- *Minimum heat and power requirements.*
  During pretreatment, power demands should be low and/or compatible with the thermally integrated process. (Alvira *et al*., 2010)

As aforementioned, Table 3.4 (above) shows the types and the names of some pretreatment methods. To compare the efficiency of these methods, sugarcane baggase is selected as an example feedstock within lignocellulosic biomass and the comparison shown in Table 3.12.

Table 3.12. Implemented pretreatment for sugarcane bagasse exploitation
(Source: Alvira *et al.*, 2009)

Pretreatment	Agent	Conditions	Yield		Remarks	References
			%w/w of SCB*	g/L		
Dilute acid	HCl	Acid concentration (1.2% v/v) mL of acid solution/g of bagasse by weight: 15:1. Operation at 121 °C and 1.1 kg/cm^2 for 4h	37.21	ND	For depithed bagasse more than 30% by weight was converted to reducing sugars	Hernández-Salas *et al.* (2009)
	H_2SO_4	Acid concentration (1.25%, w/w). Operation at 121 °C during 2 h. The biomass at a solid loading of 10% (w/w)	ND	59.1		Cheng *et al.* (2008)
	H_3PO_4	Acid concentration (4%). Operation at 122 °C during 300 min. Water/solid ratio of 8 (g water/g sugarcane bagasse on dry basis)	ND	23.2		Gámez *et al.* (2006)
Alkaline-enzyme pretreatment	NaOH	Base concentration (2% w/v) mL of solution/g of bagasse: 5:1 NaOH: 50 mg/g of bagasse. Operation at 121 °C, 1.1 kg/cm^2 during 4 h. 0.19 mL of enzyme per gram of bagasse	13-18	ND		Hernández-Salas *et al.* (2009)
Alkaline pretreatment	NaOH	Base concentration 3%, Solid to liquid ratio of 1:25 (g/mL). Operation at 50 ° C for 3 h	27.65	ND	For dewaxed sugarcane bagasse 74.9% of the original hemicelluloses were hydrolyzed. Xylose was the predominant sugar (79.2-96.7% of total sugars)	Peng *et al.* (2009)
Steam explosion	Water	Operation at 121 °C and 1.1 kg/cm^2 for 4 h	ND	ND		Hernández-Salas *et al.* (2009)
	Water, SO_2 and H_2SO_4	SO_2 concentration 2% by weight of water in the bagasse. Acid concentration 0.25 g H_2SO_4 per 100g dry matter. 180 °C during 5 min	ND	ND	Glucose and xylose yields in average 86.3% and 72%, respectively	Sendelius (2005)
Wet oxidation	Water and oxygen	Operation at 195 °C during 15 min, Alkaline pH, Oxygen pressure: 12 bar	11.6	ND	Yielding a solid material with nearly 70% cellulose content, hemicelluloses solubilisation: 93% and 50% of lignin. Enzymatic convertibility of cellulose around 75%	Martin *et al.* (2007)

*SGB: Sugarcane bagasse; ND: non-data available.

Among these methods, dilute acid hydrolysis is still the method of choice in several model processes and one of the most studied and widely used method (Cardona *et al*. 2009; Balat *et al*. 2008; Karimi *et al*. 2006; Dale *et al*. 2000; Tucker *et al*. 2003; Chung *et al*. 2005; Kim, 2005; Agbogbo *et al*. 2006). Polysaccharides, especially hemicellulose that is easier to be hydrolyzed than cellulose, is attacked by the acid medium. Thereby, lignin and cellulose fractions remain almost unaltered in the solid phase and can be further processed. Dilute acid hydrolysis is being considered suitable for fruit pomaces pretreatment. The liquid phases of the fruit pomaces (hydrolysates) are constituted by sugar (mainly fructose, glucose, arabinose, mannose and xylose) decomposition products of hemicelluloses (such as oligomers from the polymers and acetic acid generated from the hydrolysis of acetyl groups linked to sugars) and/or the decomposition products of monosaccharides, which are undesirable for fermentation processes (such as furfural from decomposition of xylose, product of dehydration of pentoses, and 5-hydroxymethylfurfural (HMF), product of dehydration of hexoses) (Gamez *et al*., 2006). Sulfuric acid (H_2SO_4) is the most used acid among other acids that can be used such as hydrochloric acid (HCl), nitric acid ($HNO_{3)}$ or phosphoric acid (H_3PO_4). Two types of dilute acid pretreatment are used primarily: low solids loading (5-10% [w/w]), high-temperature (T>160 °C), continuous-flow processes and high solids loading (10-40% [w/w]), lower temperature (T<160 °C), batch processes (Silverstein 2004). In general, higher enzymatic cellulose digestibility and soluble xylose recovery yields are obtained by shorter reactor times and higher pretreatment temperatures. Cellulose digestibility of pre-treated residues is increased by higher-temperature dilute acid pre-treatment (Tucker *et al*., 2003). Between 80 and 95% of the hemicellulosic sugars can be recovered by dilute acid pretreatment from the lignocellulosic feedstock, depending on the substrate and the conditions used (Karimi *et al*. 2006; Jeffries & Jin, 2000; Torget *et al*. 1996). Furfural, which occurs by breaking down of xylose due to high temperature, is recovered by distillation. However, this increases the cost of the processes. Furthermore, the concentration of reducing sugar in the hydrolysate is relatively low due to high liquid/solid ratio during the acid hydrolysis. So the hydrolysate should be concentrated before fermentation (Cheng *et al*., 2008).

3.4.1. Dilute-acid Hydrolysis With Phosphoric Acid

This study is related to the use of H_3PO_4 and the reason is that after neutralization of hydrolysates with NaOH, the salt formed is sodium phosphate, which can remain in the hydrolysates since it is 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. Fruit Pomaces

Peach, apricot, apple and orange pomaces were obtained from "Konfrut Fruit Juice Concentrates and Purees" in ice bags and stored at -18 °C in plastic packages. The appearance of peach and apricot pomaces were pulp like and homogeneous. Apple pomace composed of almost just peels of ~1cm^2-sized particles. Orange pomace also composed of almost peels and were sliced into ~1cm^2-sized pieces before use.

Figure 4.1. Appearance of fruit pomaces, apple, apricot, orange and peach pomace respectively

4.2. Methods

4.2.1. Chemical Compositional Analysis of Fruit Pomaces

Protein: Measurments of protein content in samples were replicated three times. Gerhardt Kjeldatherm Digestion System KBL20S with TZ Controller and Vapodest 30S Rapid Steam Distillation Unit was used to obtain % protein content in samples using AOAC official method coded 920.152. This method was modified as it was impossible to conduct experiments using Gerhardt Modern Digestion and Gerhardt Rapid Distillation system with the amounts of chemicals given by AOAC. In digestion step 5 g pomace with 20 ml H_2SO_4 (sulphiric acid), two boiling stones and 1 to 2 ml paraffin (helps to reduce frothing) were used. In distillation step 80 ml water, 80 ml NaOH (sodium hydoxide) and 70 ml H_3BO_3 (boric acid) were added.

Water activity: Water activity of the samples was determined using a Rotronic HygroLab Benchtop Humidity Temperature Indicator (Rotronic AG, Bassersdrof, Germany) and replicated 2 times with 10g for each pomace.

Solids (soluble and insoluble): The moisture content of samples (5 g) were determined with a Precisa XM-60 Moisture Content Analyzer (Precisa Instruments, Diekinton, Germany) by drying the samples at 105 ºC until a constant weight was reached. Data were reported on a wet basis and were averages of two measurements. AOAC official method 922.10A was used to determine water-insoluble solids and soluble solids

Ash: Modified AOAC 940.26 "Ash of fruits and fruit products" procedure was used. At the end of the first ashing, there were black ashes, which were undesirable. In order to obtain white ashes hydrogen peroxide was added on ashes and a second ashing was implemented.

Dietary fiber (soluble and insoluble): Sigma Total Dietary Fiber Assay Kit was used for determination of soluble and insoluble dietary fiber content. The experiments were replicated two times for each pomace.

Reducing sugar: 100ml 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. Analysis of Hydrolysates

Individual sugar: In the screening part of the study, HPLC was used for the determination of sugars using Biorad Aminex HPX-87P column equipped with the appropriate guard column. HPLC conditions were; 10 – 50 μL of injection volume, 80 – 85 °C of column temperature, 0.6 mL / minute of flow rate. The mobil phase was HPLC grade water and it was filtered through 0.2μm filter and degassed. Detector temperature was 50 °C. with a run time of 20 minutes data collection and 15 minutes of post run time. Hydrolysates were neutralized to pH 5-6 using calcium carbonate where pH greater than 6 was avoided. After reaching pH 6-7, the samples were allowed to settle and decanted off the clear liquid. The pH of the liquid after settling was approximately 7. Samples with a pH greater than 9 could not be analyzed using the HPX-87P column. The sum of cellobiose, glucose, xylose, galactose, arabinose, mannose and fructose was calculated as total sugar of hydrolysates.

In the optimization part of the study Nelson-Somogyi reducing sugar assay was used in order to determine the total reducing sugar conversion from total dry weight of each hydrolysates.

The responses of statistical analysis, results of either HPLC or Nelson-Somogyi method were expressed as percentage of total reducing sugar conversion from initial total dry weight. Below is an example given how to calculate the percentage of sugar conversion from dry weight. If for example without any treatment the pomace has "X" g of dry weight and "a" g of reducing sugar. After treatment (dilute acid hydrolysis) there should be "X-b"g dry weight and "a+b" g reducing sugar, due to decomposion of polysaccharides. "b" is reducing sugar formed after hydrolysis.

Before hydrolysis → "X"g dry weight + "a"g reducing sugar

After hydrolysis → "X-b"g dry weight + "a+b"g reducing sugar.

Therefore the percentage of the reducing sugar conversion form dry weight would be;

$$(100 \times b) / X \qquad (4.1)$$

This increase of reducing sugar weight and decrease of dry weight of pomace is due to breaking down of polysachharides mainly hemicellulose and cellulose.

Furfural and hydroxymethylfurfural: Furfural and hydroxymethylfurfural of hydrolysates were determined using HPX-87H column with a flow rate of 0.6 mL/min. The temperatures of column and detector were 65°C and 50°C , respectively.

Total soluble solids: The soluble solids (Brix) in hydrolysates were determined by a refractometer (Mettler Toledo, RE50) at 20°C.

FTIR – Spectroscopy Analysis: Hydrolysate samples of screening experiments were scanned using an FT-IR spectrometer (Perkin Elmer Spectrum 100 FT-IR spectrometer, Wellesley, MA) equipped with a deuterated tri-glycine sulphate (DTGS) detector. Samples were placed on horizontal attenuated total reflectance (HATR) accessory with zinc selenide (ZnSe) crystal (45 deg.Trough Plate). The scanning was carried out at 4.00 cm^{-1} resolution and 1 cm/s scan speed. The number of scans for each spectrum was 32. All spectra were collected within the range of 4000-650 cm^{-1} wave number. The sampling crystal was cleaned with tooth paste and finally dried under nitrogen flow. The measurements were repeated at least three times.

Statistical Analysis of FTIR: Spectral data obtained with an FT-IR spectrometer was analyzed by using multivariate statistical techniques with SIMCA software (SIMCA P-10.5 Umetrics Inc. Sweden). Partial Least Square (PLS) regression was applied to hydrolyzates of fruit pomaces to determine the concentration of several sugars (Arabinose, glucose, galactose, mannose, xylose, cellobiose), brix and reducing sugar content in samples using whole spectral range.

Obtained data sets were randomly separated into two groups as calibration (2/3 of samples) and validation (1/3 of samples) set. The predictive ability of the models were expressed by some parameters and visualized with prediction plots of created models. These parameters are root mean square error of calibration (RMSEC), root mean square error of prediction (RMSEP) and the regression correlation coefficient (R^2) both for calibration and validation models. The regression coefficient R^2 expresses how close the relationship between prediction (FTIR predicted value) and the response variation (actual results of the chemical parameters). The closer and higher R^2 values for both calibration and validation model, the better the relationship between actual and predicted values. RMSEC and RMSEP values are used to evaluate performance in the prediction process. RMSEP is a measurement of the average differences between the

predicted and reference actual values at the validation step. Similarly RMSEC refers to the calibration uncertainty that can be expected for predictions. A good model would have small value of RMSEC and RMSEP. Generally evaluating all these parameters gives an idea about the predictive efficiency of the model. (Esbensen, *et al*. 2002)

$$\text{RMSEC} = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_i - y_i)^2}{n-2}} \qquad (4.2)$$

$$\text{RMSEP} = \sqrt{\frac{\sum_{i-1}^{n}(\hat{y}_i - y_i)^2}{n-1}} \qquad (4.3)$$

Where n is the number of samples used in each set; $\hat{y}_i$ is the predicted value determined by FTIR for the same sample and $\hat{y}$ is the mean of each set (Esbensen *et al*., 2002).

4.2.3. Statistical Design of Experiments

Design Expert Version 7.0.0 was used for all of the hydrolysis experiments. The responses were total sugar conversion of dry weight determined by Nelson Somogyi method.

4.2.3.1. Screening of Process Parameters

Four factors, pressure (atm), time(min), phosphoric acid (%) and solid– liquid ratio (g: ml) were selected for hydrolysis experiments according to Fogel R. *et al*., 2005. All of the factors had two levels as shown in Table 4.1.

Table 4.1. Factors and levels of screening process

Level	Factor			
	Solid:liquid ratio (g: ml)	H_2SO_4 (%)	Temperature (C)	Time (min)
-1	1:9	3	110	20
+1	1:7	1	126	40

All of the hydrolysis experiments were carried out in an autoclave (Hirayama, HA- 300 MIV). Screening of the factors consisted of a 2^4 factorial design with five replicates of the centerpoints (Table 4.1). 15 g of each pomace was weighed in 250 ml autoclavable schott flasks. Only orange pomaces was sliced into ~ 1 cm^2 pieces in order to increase the surface area and to make the solution more homogeneous. The rest of the suspensions of other pomaces studied were homogeneous enough. The liquid fraction of hydrolysates were extracted into 50 ml falcon tubes and stored at –18 °C.

Table 4.2. 2^4 – Factorial design of dilute-acid hydrolysis of fruit pomaces (apple, apricot, peach and orange) used in screening experiments

Test no	Coded level of variables				Actual level of variables			
	X_1	X_2	X_3	X_4	S:L (g:ml)	Acid (%)	T (°C)	Time (Min)
1	-1	-1	-1	-1	1:9	3	110	20
2	+1	-1	-1	-1	1:7	3	110	20
3	-1	+1	-1	-1	1:9	1	110	20
4	-1	-1	+1	-1	1:9	3	126	20
5	-1	-1	-1	+1	1:9	3	110	40
6	+1	+1	-1	-1	1:7	1	110	20
7	+1	-1	+1	-1	1:7	3	126	20
8	+1	-1	-1	+1	1:7	3	110	40
9	-1	+1	+1	-1	1:9	1	126	20
10	-1	+1	-1	+1	1:9	1	110	40
11	-1	-1	+1	+1	1:9	3	126	40
12	+1	+1	+1	-1	1:7	1	126	20
13	+1	+1	-1	+1	1:7	1	110	40
14	+1	-1	+1	+1	1:7	3	126	40
15	-1	+1	+1	+1	1:9	1	126	40
16	+1	+1	+1	+1	1:7	1	126	40
17	0	0	0	0	1:8	1:75	120	30
18	0	0	0	0	1:8	1:75	120	30
19	0	0	0	0	1:8	1:75	120	30
20	0	0	0	0	1:8	1:75	120	30
21	0	0	0	0	1:8	1:75	120	30

4.2.3.2. Optimization of Fruit Pomaces Hydrolysis

The optimisation experiments were carried out using response surface method, (Central composite design) which were based on the results obtained from previous screening experiments mentioned in Chapter 5 (Table 5.3). Temperature was stabilized as 126° C for apricot, 110° C for apple and peach. Time was stabilized as 40 min. For apple and apricot pomaces the solid : liquid ratio was in the range of 1/10.5 to 1/6. The

acid ratio levels for these pomaces were in the range of 1% to 4%. For peach pomace the solid : liquid ratio was in the range of 1/6.5 to 1/4 and the acid ratio levels were in the range of 0.41% and 2.41%.

Table 4.3. Factors and levels of optimization process

	Factor					
	Solid : Liquid ratio (g : ml)		H_2SO_4 (%)		Temperature (°C)	Time (min)
	- level	+ level	- level	+ level		
Apple	1/10.5	1/6.5	1	4	110	40
Apricot	1/10.5	1/6.5	1	4	126	40
Peach	1/6.5	1/4	0.41	2.41	110	40

Table 4.4. Coded (X_1, X_2, X_a and X_b) and Respective actual levels (S:L, acid% for apple and apricot pomace, S:L, acid% for peach and orange pomace) used in experimental design for dilute-acid hydrolysis of fruit pomaces by CCRD (Central composite rotatable experimental design) method

Test No	Coded level of variables				Actual levels of variables			
	Apple and Apricot		Peach and Orange		Apple and Apricot		Peach	
	X_1	X_2	X_a	X_b	S:L (g:ml)	Acid (%)	S:L (g/ml)	Acid (%)
1	-1	-1	-1	-1	1:6.5	1	1:4	0.41
2	-1	-1	-1	-1	1:6.5	1	1:4	0.41
3	+1	-1	+1	-1	1:10.5	1	1:7	0.41
4	+1	-1	+1	-1	1:10.5	1	1:7	0.41
5	-1	+1	-1	+1	1:6.5	4	1:4	2.4
6	-1	+1	-1	+1	1:6.5	4	1:4	2.4
7	+1	+1	+1	+1	1:10.5	4	1:7	2.4
8	+1	+1	+1	+1	1:10.5	4	1:7	2.4
9	-2	0	-2	0	1:5.67	2.5	1:3.38	1.41
10	-2	0	-2	0	1:5.67	2.5	1:3.38	1.41
11	+2	0	+2	0	1:11.32	2.5	1:7.62	1.41
12	+2	0	+2	0	1:11.32	2.5	1:7.62	1.41
13	0	-2	0	-2	1:8.5	0.37	1:5.5	0
14	0	-2	0	-2	1:8.5	0.37	1:5.5	0
15	0	+2	0	+2	1:8.5	4.62	1:5.5	2.81
16	0	+2	0	+2	1:8.5	4.62	1:5.5	2.81
17	0	0	0	0	1:8.5	2.5	1:5.5	1.41
18	0	0	0	0	1:8.5	2.5	1:5.5	1.41
19	0	0	0	0	1:8.5	2.5	1:5.5	1.41
20	0	0	0	0	1:8.5	2.5	1:5.5	1.41
21	0	0	0	0	1:8.5	2.5	1:5.5	1.41

4.2.4. Fermentation

Fermentation conditions used in this study were based on a study conducted by Stevenson and Weimer, 2002. Fermentation was carried out in two steps namely in aerobic and anaerobic form.

Aerobic fermentation: Two media were used for aerobic fermentation, rich medium (yeast-peptone-malt extract; YPM) as described by Skory *et al* (1997) and minimal medium (yeast nitrogen base medium; YNB of Wickerham and Burton, 1948) without vitamins. YPM (Rich medium), which has 0.5% peptone, 0.3% yeast extract, 0.3% malt extract and 10 g/L glucose, was sterilised before use. YNB (Minimal medium) was prepared by dissolving 6.7 grams of the medium in 100 ml distilled water, heated without boiling or autoclaved until complete dissolution. This was sterilized by filtration and stored at 4° C. Before use this solution was diluted 10 times. The final solution had additionally 10 g/L of glucose. These 2 media (3 replicates for each; 6 flasks totally) were inoculated with conidia (~ $1x10^7$), and incubated at 30° C with shaking at 170 rpm for two days. Mycelia and spores were extracted aseptically with centrifugation and added to the hydrolysates in order to start the anaerobic part of the fermentation. Flasks were named as either YPM (the mycelia and spores from YPM media) or YNB (the mycelia and spores from YPM media).

Anaerobic fermentation: The mycelial mass extracted by centrifugation and collected to be added into the anaerobic fermentation media, which was the apple pomace hydrolysate, since only apple pomace optimization among pomaces was successful. According to the optimization results a temperature of 126° C, 40 minutes and 4% acid was chosen for hydrolysis conditions. 10% solid liquid ratio was chosen since this factor had no significant effect on the design. Reducing sugars in the hydrolysate were detected by Nelson-Somogyi method. Hydrolysates were filtered, neutralized to pH 4.5 by adding NaOH, filtered again, sterilised at 121° C for 15 minutes and finally after these steps reducing sugars of final media were determined again in order to detect if there were any reduction due to the steps before. Forty ml hydrolysate was added into fifty ml flasks in order to leave ~20 % of the culture flask volume as air space. After aseptically inoculation of the mycelia and spores from aerobic fermentation, plastic paraffin film was used to seal the flask and a silicone-

tubing (1.6 x 1.6 = 4.8 mm, Silicone tubing), packed tightly with cotton was vented trough the paraffin film (Figure 4.2).

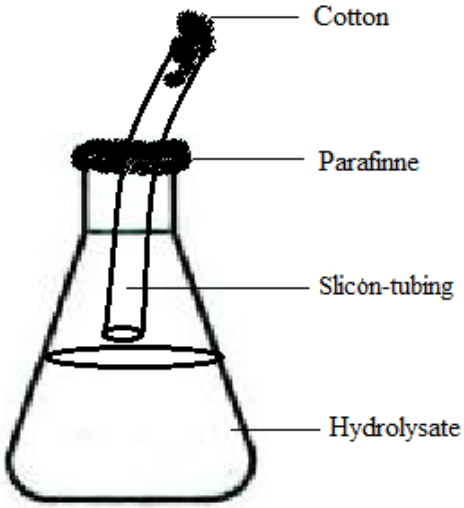

Figure 4.2. Anaerobic fermentation flasks

Figure 4.3 shows the flasks and the conditions of incubation. Two flasks were placed in CO_2 incubator, two were in a normal incubator, shaking at 170 rpm and the other two were in the same normal incubator under static conditions.

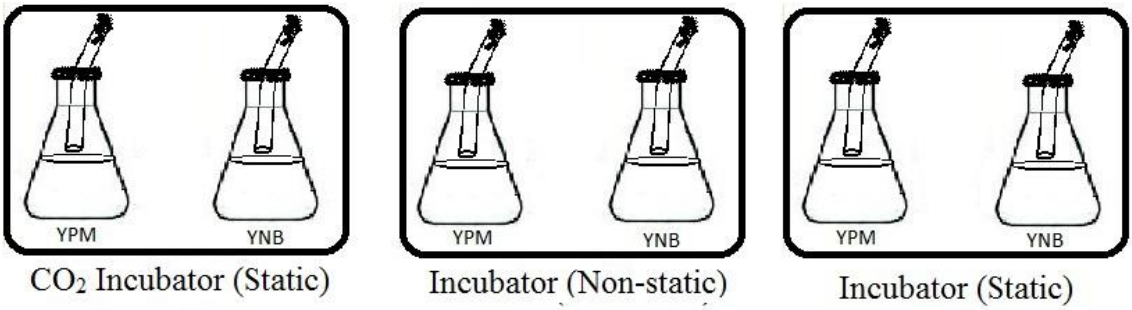

Figure 4.3. Anaerobic fermentation conditions of hydrolysates of the pre-grown mycelia and spores obtained from two different kinds of media formulations (YPM and YNB)

The incubation temperature for each incubator (CO_2, shaking and static) was set to 30° C. First sample was taken on the fourth day and proceeding samples were taken daily until 14th day. Ethanol, main sugars (xylose, galactose, mannose and arbinose) furfural and hemifurfural of daily samples were determined by HPLC using HPX-87H column with 0.6 mL/min flow rate. The temperature was set to 65°C and 50°C for the column and detector, respectively. Reducing sugars were determined according to Nelson-Somogyi method.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Results of Chemical Compositional Analysis of Fruit Pomaces

The composition of the raw fruit pomaces used in the study is shown in Table 5.1. As it can be seen orange pomace had the highest reducing sugar, whereas, peach and apricot pomaces had almost the same amount of reducing sugar. Furthermore, apple pomace had significantly low reducing sugar in comparison with other pomaces. However, apple pomace had the highest solid content, which suggested that it might have higher sugar content after a pretreatment since it constitutes of cellulose, hemicellulose and lignin in its solid part. In fact this was confirmed by the total dietary fiber content being the highest among the others. In orange, peach and apricot main sugars were glucose and fructose whereas; arabinose and xylose were the main sugars in apple pomace.

Table 5.1. The chemical composition of fruit 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

The composition of pomace varies according to fruit variety used and the type of processing applied for juice extraction, especially regarding how many times the fruits were pressed (Paganini *et al*., 2005).

The results are in good agreement with those obtained in other studies mentioned in Chapter 3 (Table 3.12). Also the results showed that these four pomaces could be considered as potential fermentation media for microorganisms with adequate moisture and dietary fiber content and with considerably high reducing sugars without any chemical, physical or biological pretreatment.

5.2. Statistical Analysis of the Experimental Results

A 2^4 factorial design was used in 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 a more specific range. Thus optimization step deals with wider range of levels with lower number of factors and gives more specific results than screening step. The screening and optimization results of the process parameters of the pretreatment for various pomaces are given below in (Table 5.2). These are later discussed individually in forthcoming sections. The ranges of the process parameters are presented in coded variables. The actual ranges for each of the variables were such as: Solid liquid ratio (g: mL) (X_1) 1:9-1:7, Acid ratio (X_2) 1-3%, Temperature (X_3) 110-126 °C and Time (X_4) 20-40 minutes in screening step.

The response variable, which is the total reducing sugar (sum of glucose, galactose, mannose, arabinose, cellobiose, xylose) conversion from dry weight of pomaces were obtained from the HPLC analysis.

Optimization of dilute-acid hydrolysis of the pomaces was performed according to the Central Composite experimental design presented in the materials and method section Table 4.3. The calculation of the results of reducing sugar method of Nelson, Somogyi was mentioned in the analysis of hydrolysates in chapter Materials and Methods.

According to screening results temperature and time were 126 °C and 40 min for apricot and apple, 110 °C 40 min for peach pomace. All the optimization results are discussed below for each pomace separately.

Table 5.2. Screening and optimization results of the pomaces with respect to total reducing sugar conversion values (%) as response

Screening									Optimization						
Test no	Actual level of variables				Total Sugar Yield				Actual levels of variables				Total Sugar Yield		
									Apple and Apricot		Peach				
1	S:L (g:ml)	Acid (%)	T (°C)	Time (Min)	Apple	Apricot	Peach	Orange	S:L (g:ml)	Acid (%)	S:L (g/ml)	Acid (%)	Apple	Apricot	Peach
2	1:9	3	110	20	8.17	23.00	21.77	11.87	1:6.5	1	1:4	0.41	15.79	45.50	44.70
3	1:7	3	110	20	4.80	22.18	25.91	18.72	1:6.5	1	1:4	0.41	13.48	43.26	44.77
4	1:9	1	110	20	4.11	14.84	27.18	16.79	1:10.5	1	1:7	0.41	12.04	42.47	44.46
5	1:9	3	126	20	10.88	25.35	16.06	16.17	1:10.5	1	1:7	0.41	14.40	39.70	45.49
6	1:9	3	110	40	24.07	20.16	16.21	12.38	1:6.5	4	1:4	2.4	17.00	36.47	47.95
7	1:7	1	110	20	5.86	15.80	14.17	13.50	1:6.5	4	1:4	2.4	19.56	44.36	49.03
8	1:7	3	126	20	10.22	16.24	17.37	8.56	1:10.5	4	1:7	2.4	31.35	28.67	48.34
9	1:7	3	110	40	19.07	11.81	26.92	9.14	1:10.5	4	1:7	2.4	21.39	45.62	50.71
10	1:9	1	126	20	7.27	13.01	15.83	27.50	1:5.67	2.5	1:3.38	1.41	19.18	43.44	49.34
11	1:9	1	110	40	29.77	29.14	13.74	5.67	1:5.67	2.5	1:3.38	1.41	23.01	49.16	48.85
12	1:9	3	126	40	16.78	18.83	13.50	17.57	1:11.32	2.5	1:7.62	1.41	20.24	41.09	49.40
13	1:7	1	126	20	7.95	21.20	15.02	23.81	1:11.32	2.5	1:7.62	1.41	21.40	48.10	40.69
14	1:7	1	110	40	20.11	28.70	28.07	5.26	1:8.5	0.37	1:5.5	0	18.79	24.05	37.43
15	1:7	3	126	40	14.74	11.23	14.99	19.63	1:8.5	0.37	1:5.5	0	16.36	41.07	33.25
16	1:9	1	126	40	10.65	12.75	12.28	12.35	1:8.5	4.62	1:5.5	2.81	24.22	36.21	43.91
17	1:7	1	126	40	13.41	34.39	22.91	16.37	1:8.5	4.62	1:5.5	2.81	21.73	38.29	40.81
18	1:8	1:75	120	30	14.01	15.42	15.18	26.07	1:8.5	2.5	1:5.5	1.41	17.10	48.48	52.44
19	1:8	1:75	120	30	12.73	19.81	20.43	24.89	1:8.5	2.5	1:5.5	1.41	19.47	41.77	48.20
20	1:8	1:75	120	30	11.86	18.94	14.90	23.22	1:8.5	2.5	1:5.5	1.41	18.85	43.20	45.60
21	1:8	1:75	120	30	11.50	12.74	29.22	36.99	1:8.5	2.5	1:5.5	1.41	21.68	36.56	48.61

The difference in sugar analysis methodology between screening and optimization was that while HPLC was used in screening, Nelson-Somogyi method was used in optimization study. Since this was a screening process, insignificant single factors were also added to the ANOVA results in order to determine the increase or the decrease in the differences between the levels of single parameters in the optimization process.

5.2.1. Apple

As mentioned above, at the end of the screening step the results of the apple pomace were evaluated in the Table 5.3 according to the statistical analysis of variance. In this table, the model F-value of 19.95 implied that the model was significant. There was only a 0.01% chance that a "Model F-Value" this large occurred due to noise. Two of the single factors; temperature (X_3) and time (X_4) and the interaction of them (X_{34}) were significant model terms.

Table 5.3. Analysis of variance for apple pomace (Screening)

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	717.32	5	143.46	19.95	<0.0001	Significant
X_1	15.10	1	15.10	1.03	0.1694	
X_2	5.78	1	5.78	0.39	0.3853	
X_3	36.18	1	110.07	2.47	0.0416	
X_4	498.82	1	36.18	34.06	<0.0001	
X_{34}	161.44	1	498.82	11.02	0.0003	
Curvature	0.15	1	0.15	0.022	0.8855	Not significant
Residual	205.06	14	7.19			
Lack of Fit	88.05	10	8.81	2.79	0.1677	Not significant
Pure Error	12.64	4	3.16			
Cor Total	818.16	20				
Std. Dev.	2.68		R-Squared	0.88		
Mean	13.04		Adj R-Squared	0.83		
C. V. %	20.57		Pred R-Squared	0.70		
PRESS	245.16		Adeq Precision	13.35		

The "Pred R-Squared" of 0.70 was in reasonable agreement with the "Adj R-Squared" of 0.83. "Adeq Precision" (13.35), which measured the signal to noise ratio, indicated an adequate signal, being greater than 4. The suitability of the fitness can be checked by determination coefficients (R^2), which indicated the percentage of the variability of the screening parameter that was explained by the model (Fannin *et al*.,

1981). 0.88 R-squared value suggested that 12.4% of the total variations were not explained by the models developed for the corresponding yield of total reducing sugar.

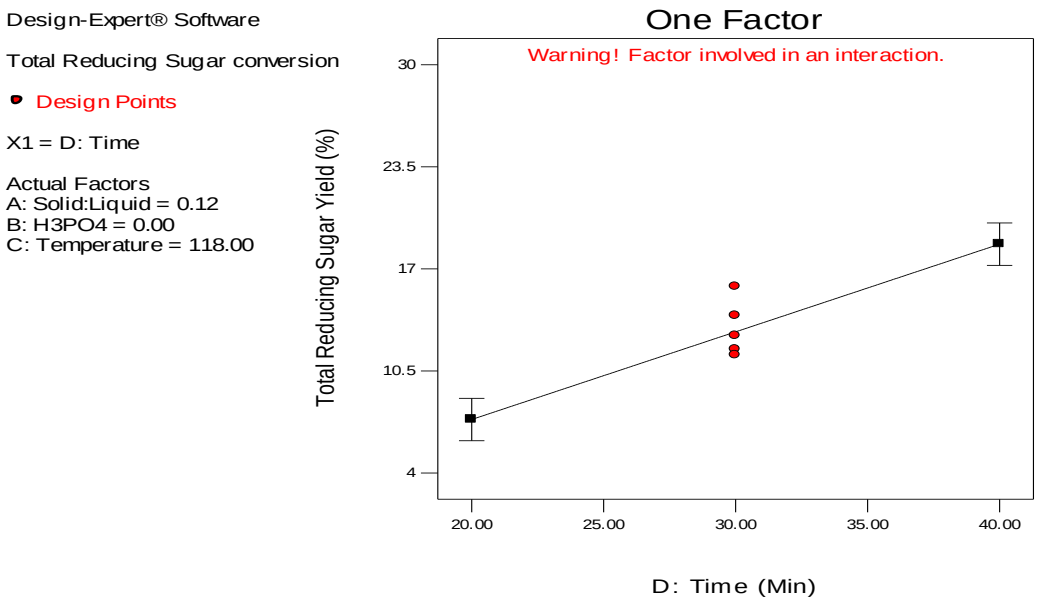

Figure 5.1. One factor plot of time of apple pomace in screening step

Figure 5.1, which is a one factor graph, indicated that 40 minutes leads to better sugar conversion than 20 min. Furthermore Figure 5.2 suggested that high sugar conversion could be obtained at 110 °C and 40 minutes. The 10th experiment (shown in Table 5.3) which had the highest sugar conversion, supported the Figures 5.1 and 5.2, since the conditions of this particular set experiment were 1g/ 7ml solid liquid ratio, 1% acid, 110°C and 40 min. Therefore, in the optimization of apple pomace, temperature and time were fixed at these levels (110°C and 40 min, respectively).

The factors of solid-liquid and acid ratio were not significant. The sugar conversion was only slightly different between the levels of these factors at 110° C and 40 min. There was only a slight increase in the sugar conversion on the higher concentration of acid ratio (3%) and lower concentration of solid-liquid ratio (1g/9ml). 23.97 and 22.02 were sugar conversion percentages of 1g/7ml and 1g/9ml at 110° C and 40 min., respectively. Furthermore, at higher concentration of acid ratio (%3) sugar conversion was 23.6, and at lower concentration (1%) it was 22.4. Therefore in the optimization step these levels were evaluated as 1% and 4% acid ratio (- and + level respectively) and 1g/6.5ml and 1g/10.5ml solid liquid ratio (- and + respectively) in

order to analyze the higher concentrations of acid ratio and the lower concentrations of solid-liquid ratio.

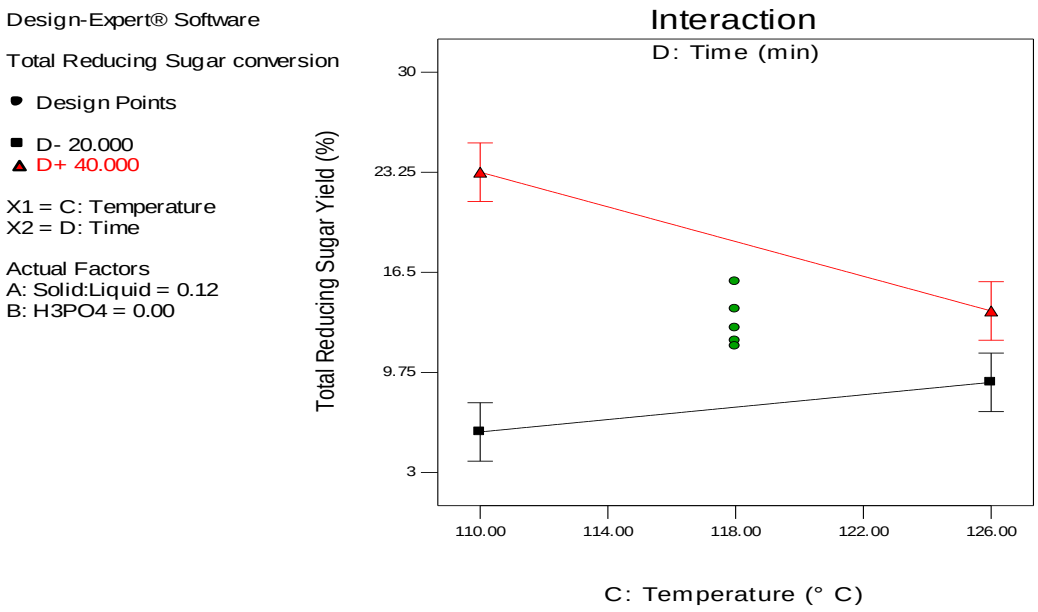

Figure 5.2. The interaction graph of temperature and time in the screening process of apple pomace

The results of statistical analysis of the optimization step are shown in Table 5.4. The ANOVA results of response surface model for reducing sugar conversion yields demonstrated that the model was significant due to a F-value of 8.13. There was only a 0.14% chance that the "Model F-Value" this large could have occurred due to noise.

Among the single factors [X_1: solid-liquid ratio (g/L) and X_2: acid ratio (%)] only X_2 and the interaction of two factors, X_{12}, were the significant terms. Final equations in terms of coded factors and actual factors are given below.

$$\text{Total RS conversion of apple pomace} = +19.37 + 0.79 * X_1 + 3.05 * X_2 + 2.38 * X_{12} \quad (5.1)$$

$$\text{Total RS conversion of apple pomace} = +27.76900 - 1.58733 * \text{Solid: Liquid} - 4.69673 * \text{Acid ratio} + 0.79208 * \text{Solid : Liquid} * \text{Acid ratio} \quad (5.2)$$

Table 5.4. Analysis of variance for apple pomace (Optimization)

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	204.28	3	68.09	8.13	0.0014	Significant
X_1	9.88	1	9.88	1.18	0.2927	
X_2	149.23	1	149.23	17.81	0.0006	
X_{12}	45.17	1	45.17	5.39	0.0329	
Residual	142.42	17	8.38			
Lack of Fit	59.18	5	11.84	1.71	0.2077	Not significant
Pure Error	83.24	12	694			
Cor Total	346.70	20				
Std. Dev.	2.89		R-Squared		0.59	
Mean	19.37		Adj R-Squared		0.52	
C. V. %	14.95		Pred R-Squared		0.27	
PRESS	253.62		Adeq Precision		8.60	

The "Lack of Fit F-value" of 1.71 implies the Lack of Fit was not significant relative to the pure error. There was a 20.77% chance that a "Lack of Fit F-value" this large could have occurred due to noise. The experimental yields fitted the second-order polynomial equation not so well as indicated by low R^2 values (0.59), which suggested that 42% of the total variations were not explained by the models, developed for the corresponding yield of total reducing sugar.

The highest yield of reducing sugars (RS) of apple pomace, 31.35%, was achieved in the 7th experiment where 110 ºC, 40 min, 1 g/ 10.5 ml solid-liquid ratio and 4% phosphoric acid were applied. If the average of the replicates (7th and 8th experiment) were taken into account, the yield of RS decreased to 26.37%. But still this was the highest yield of RS obtained from apple pomace.

As depicted in Figure 5.3, the interaction of acid ratio and solid-liquid ratio turned to be the major factor affecting positively the hydrolysis. Higher concentrations of acid ratio (4%) and lower concentrations of solid-liquid ratio (1g/10.5ml) lead to higher amount of reducing sugar. Acid ratio didn't change sugar yields at higher concentrations of solid/liquid ratio (1g/5.67ml). On the other hand, the lower concentrations of solid/liquid ratio (1g/10.5ml) and the higher concentrations of acid ratio (4%) the more reducing sugar conversion were achieved. However, higher acid concentration than 4% may lead to decomposition of xylose and arabinose and therefore formation of furfural and hemifurfural, which is not desired for microbial fermentations.

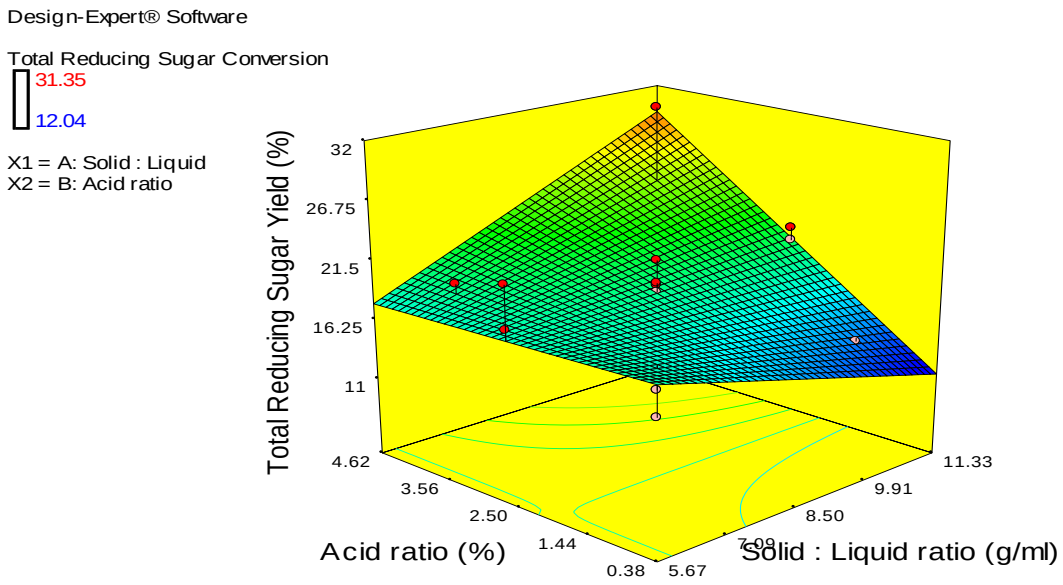

Figure 5.3. Response surface plot of total reducing sugar yield of apple pomace hydrolysates

Since the carbohydrate value of apple pomace was around 48 – 88% (Table 3.12) the maximum sugar conversion obtained from this study (31%) might be even increased more with further research.

In order to validate the adequacy of the model equations a total of 4 verification experiments were carried out at the predicted optimum conditions for apple pomace. The results showed 18, 19, 19 and 16% deviation. The overall margin of error was 18.45% for apple pomace (Table 5.5).

Table 5.5. Validation experiments of apple pomace

Solid/Liquid (g:L)	**Acid ratio (%)**	**Estimated sugar conversion (%)**	**Actual sugar conversion (%)**	**Error (%)**	**Overall Error (%)**
1/9	2.61	30.92	25.22	18.40	18.45
1/8.05	1.55	27.46	22.13	19.38	
1/10.06	3.59	36.73	29.66	19.23	
1/9.38	2.64	31.34	26.08	16.78	

5.2.2. Apricot

Similar to apple pomaces, the results of the screening step for apricot pomace are discussed below.

According to ANOVA results of apricot pomace (Table 5.6) single factors had no significant effect on the model. However X_{12} (Solid-liquid and acid ratio), X_{24} (Acid ratio and time) and X_{123} (Solid-liquid, acid ratio and temperature) were significant. Model, which was significant with a probability value of 0.0053, indicated that there was only a 0.53% chance that a "Model F-Value" this large could occur due to noise.

Table 5.6. Analysis of variance for apricot pomace (Screening)

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	504.48	5	100.90	5.86	0.0053	Significant
X_1	1.25	1	1.25	0.10	0.7530	
X_2	27.73	1	27.73	2.33	0.1582	
X_3	9.98	1	9.98	0.84	0.3819	
X_4	14.82	1	14.82	1.24	0.2910	
X_{12}	197.53	1	197.53	16.57	0.0022	
X_{13}	29.59		29.59	2.48	0.1462	
X_{23}	0.17		0.17	0.014	0.9083	
X_{24}	263.15	1	263.15	22.07	0.0008	
X_{123}	84.91		84.91	7.12	0.0236	
Curvature	43.91	1	43.91	3.68	0.0839	Not significant
Residual	119.24	10	11.92			
Lack of Fit	86.36	6	14.39	1.75	0.3059	Not significant
Pure Error	32.88	4	8.22			
Cor Total	792.29	20				
Std. Dev.	3.45		R-Squared	0.85		
Mean	19.11		Adj R-Squared	0.70		
C. V. %	18.07		Pred R-Squared	0.16		
PRESS	665.48		Adeq Precision	9.98		

The "Pred R-Squared" of 0.16 was not as close to the "Adj R-Squared" of 0.70 as one might normally expect. This may indicate a large block effect or a possible problem with the model. 0.85 R-squared values suggested that 15% of the total variations were not explained by the models developed for the corresponding yield of total reducing sugar.

The highest sugar conversion (34.39) was obtained under the conditions described in the 16th experiment (1g/7ml solid–liquid ratio, 1% acid ratio, 126°C and 40 min). Therefore, in the optimization step, temperature and time factors were fixed at

126°C and 40 min., respectively. Responses showed an increase towards the lower concentration of acid ratio (1%) and higher ratio of the solid – liquid ratio (1g/7ml) (Figure 5.4 and 5.5). However, at the higher concentration of acid ratio (3%) there was an observable decrease with the increase in the solid-liquid ratio. (Black line in Figure 5.5). In order to determine if there were higher responses beyond the levels studied in the screening step, the levels of solid – liquid and acid ratio were expanded in the optimization study. In this case 1g/10.5ml and 1g/6.5ml were low and high levels of solid – liquid ratio in the optimization step, respectively. Similarly, the low and high levels of acid ratio in the optimization step were 1% and 4%, respectively. Since this was a central composite design we were able to see beyond the minimum and maximum levels of solid– liquid and acid ratio (-2 and +2 levels of acid ratio were 0.38% and 4.62%, -2 and +2 levels of solid – liquid ratio were 1g/5.67ml and 1g/11.33ml, respectively). So we were able to determine if there were higher responses above the higher concentrations (1g/7ml and 3%) and below the lower concentrations of solid-liquid and acid ratio (1g/9ml and 1%).

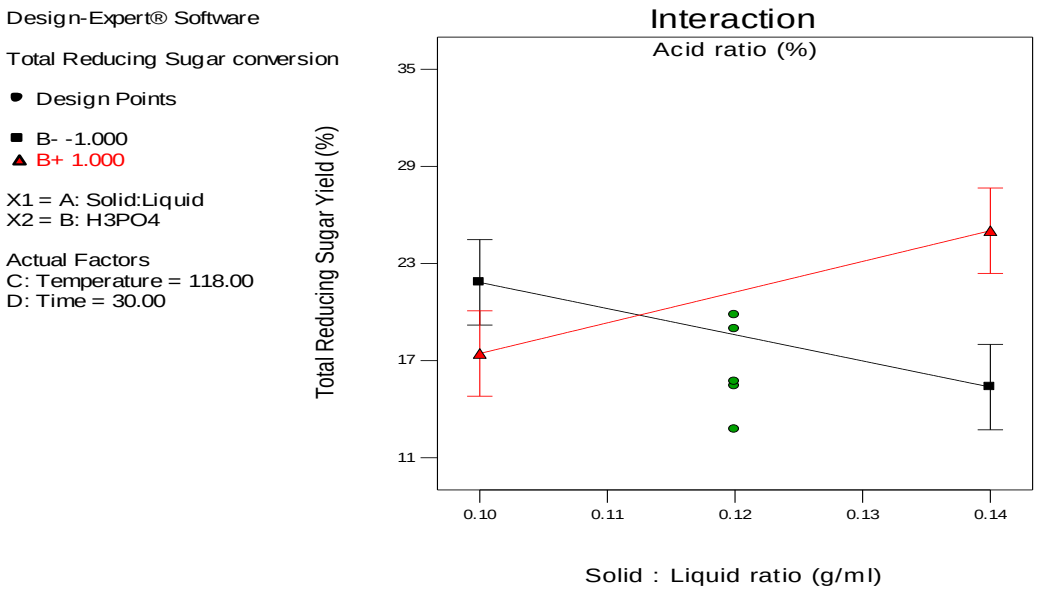

Figure 5.4. The interaction graph of solid– liquid and acid ratio at 126 °C, 40 min. (0.10 and 0.14 means 1g/9ml and 1g/7ml, respectively)

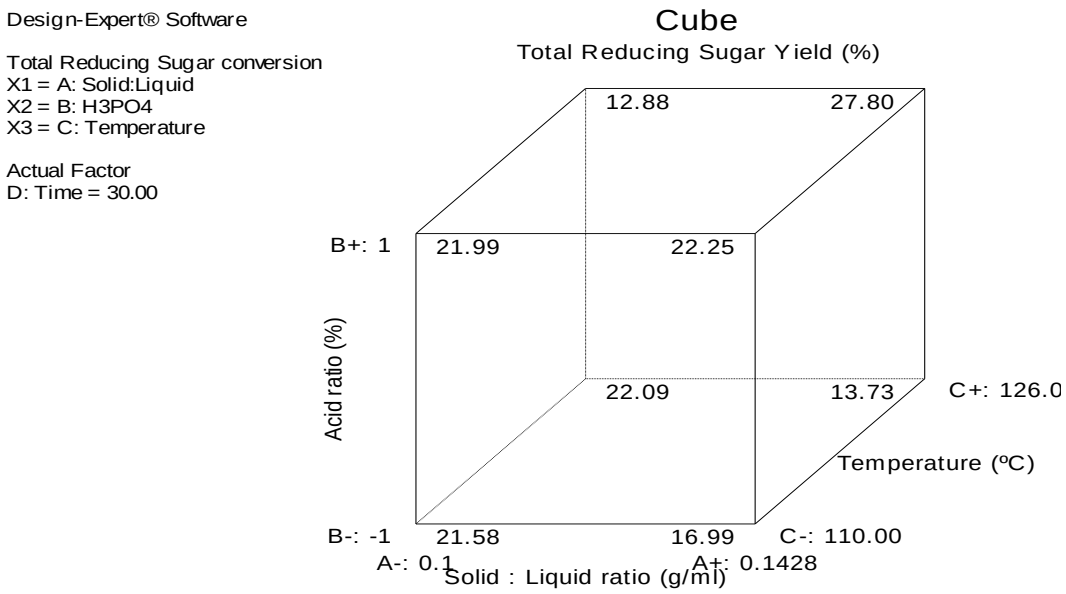

Figure 5.5. The interaction of solid liquid, acid ratio and temperature of screening process of apricot pomace (A-: 0.1 means 1g/9ml and A+: 0.14 means 1g/7ml solid – liquid ratio)

In the optimization study of apricot pomace a significant model could not be obtained, although, in the 10th experiment, the highest yield of RS, 49.16%, was achieved under the conditions at which 126 ºC, 40 min, 1 g/5.67 ml solid-liquid ratio and 2.5% phosphoric acid were applied. The reason of this might be that the responses were so close to each other. The range of the results was 24 at minimum and 49 at maximum and more importantly these results were predominantly located between 41.25 and 43.75. These indicated that there was no significant difference between the levels of the chosen factors and precise control of factors, especially solid – liquid ratio, was not necessary.

5.2.3. Orange

The ANOVA table of the screening results of orange pomaces is discussed below. According to Table 5.6 the model was significant with a p-value of 0.0234 and only temperature as single factor and the interaction of acid ratio and time were significant. The model F-value of 3.41 implies the model was significant. There was only a 3.19% chance that a "Model F-Value" this large could occur due to noise.

Table 5.7. Analysis of variance for orange pomace (Screening)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	374.29	5	74.86	3.41	0.0319	Significant
X_1	1.77	1	1.77	0.081	0.7804	
X_2	3.25	1	3.25	0.15	0.7062	
X_3	147.89	1	147.89	6.74	0.0212	
X_4	92.68	1	92.68	4.22	0.0591	
X_{24}	128.71	1	128.71	5.86	0.0296	
Curvature	644.52	1	644.52	29.36	<0.0001	Significant
Residual	307.33	14	21.95			
Lack of Fit	190.31	10	19.03	0.65	0.7356	Not significant
Pure Error	117.02	4	29.25			
Cor Total	1326.14	20				
Std. Dev.	4.69		R-Squared	0.55		
Mean	17.80		Adj R-Squared	0.39		
C. V. %	26.32		Pred R-Squared	0.49		
PRESS	670.03		Adeq Precision	7.83		

The “Pred R-Squared” of 0.49 was in reasonable agreement with the “Adj R-Squared” of 0.39 “Adeq Precision” measures with 7.83 indicated an adequate signal. 0.55 R-squared value suggested that %45 of the total variations were not explained by the models developed for the corresponding yield of total reducing sugar. That means the model was not so reliable.

The highest sugar conversion was in 20th experiment (36.9%). However, this was one of the five centerpoints, where the conditions were 110 °C, 30 min, 1g/8ml solid liquid ratio and %1.5 acid ratio. If the average of centerpoints were considered, this result was 27.7%, which was very close to the 9th experiment (27.5%) where 1g/9ml solid liquid ratio, 1% acid ratio, 126 °C and 20 min were applied.

According to Figure 5.6 and 5.7, 126 °C and 20 min lead to better sugar conversion and 1% acid ratio showed better conversion than 3% acid. Furthermore, there was only a slight increase of conversion at the lower concentration (1g/9ml) in comparison with the higher concentration of solid liquid ratio (1g/7ml). Therefore if an optimization step would be designed, temperature and time should be fixed at 126 °C and 20 min respectively, solid–liquid and acid ratio should be extended to the lower concentrations.

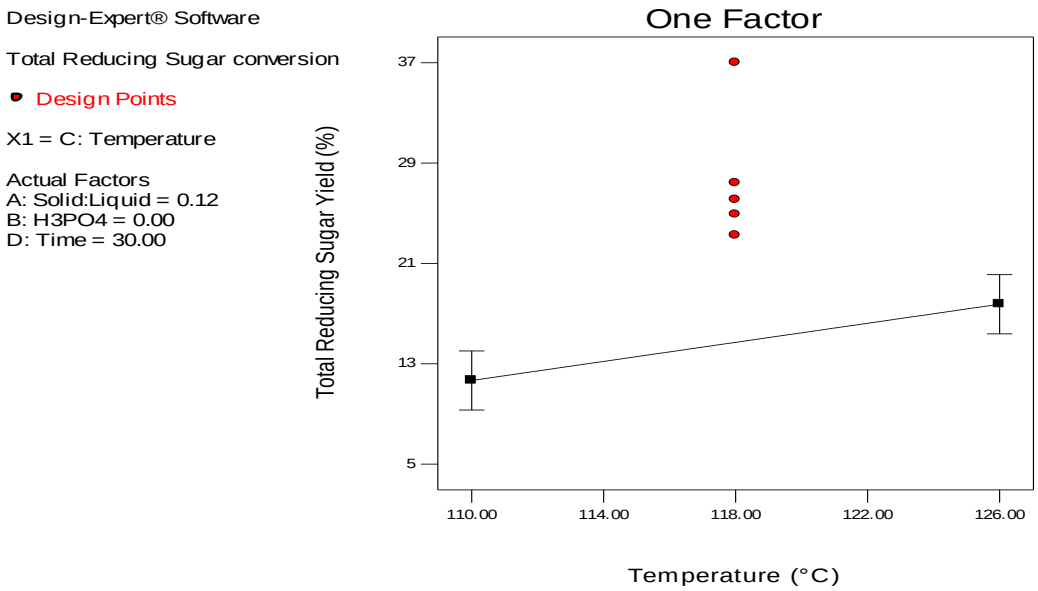

Figure 5.6. One factor graph of temperature in screening step of orange pomace

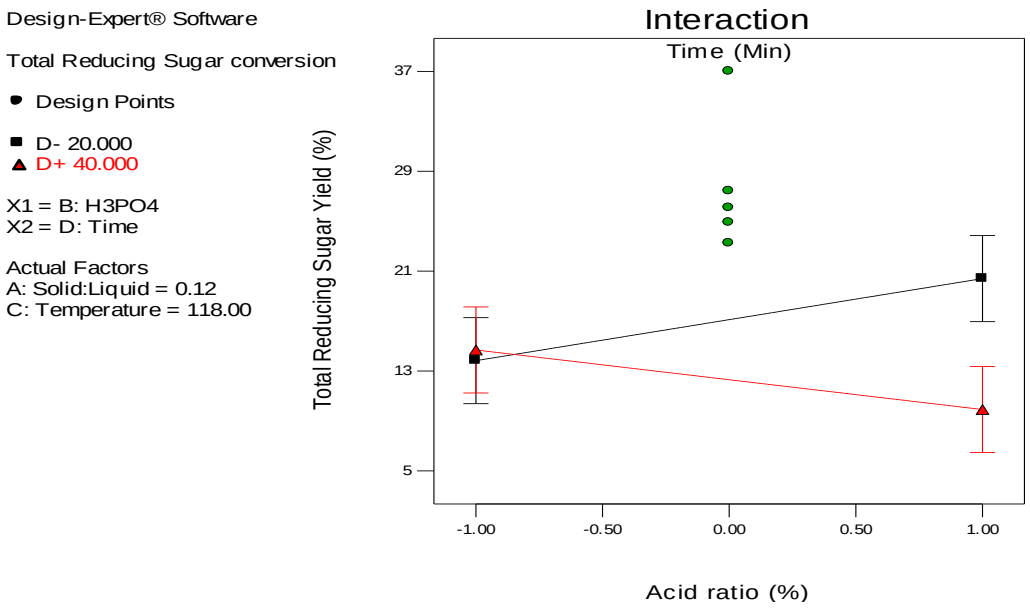

Figure 5.7. The interaction graph of acid ratio and time at 110° C (-1 means 3% 1 means 1% acid ratio) of orange pomace in screening step

Unfortunately orange pomace couldn't be continued to the second step of optimization, since it ran out and could not be supplied.

5.2.4. Peach

Similar to other pomaces, the results of the screening step for peach pomace are discussed below.

According to the ANOVA table (Table 5.8) obtained at the end of the screening step for the peach pomaces, the model was not significant, since it had a greater p-value than 0.05 (0.0548). However, if the insignificant single factors were removed, a p-value of 0.0246 which made the model significant was obtained, although it had a small "R-Squared" (0.5048). In Table 5.7, X_3 and X_{14} were significant model terms. The model F-value of 2.87 implied that there was a 5.48% chance that a "Model F-value" this large could have occurred due to noise.

Table 5.8. Analysis of variance for peach pomace (Screening)

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	315.16	5	63.19	2.87	0.0548	Not significant
X_1	51.88	1	51.88	2.36	0.1471	
X_2	0.78		0.78	0.035	0.8537	
X_3	132.29	1	132.29	6.01	0.0280	
X_4	1.37	1	1.37	0.062	0.8064	
X_{14}	129.61	1	129.61	5.88	0.0294	
Curvature	16.20	1	16.20	0.74	0.4056	Not significant
Residual	308.38	14	22.03			
Lack of Fit	154.00	10	15.40	0.40	0.8912	Not significant
Pure Error	154.38	4	38.59			
Cor Total	640.51	20				
Std. Dev.	4.69		R-Squared	0.51		
Mean	19.36		Adj R-Squared	0.33		
C. V. %	24.24		Pred R-Squared	0.008		
PRESS	635.46		Adeq Precision	5.72		

The highest sugar conversion (29.22%) was in 20th run. However, this was one of the five centerpoints, where the conditions were 110 °C, 30 min, 1g/8ml solid liquid ratio and %1.5 acid ratio. If the average of centerpoints were taken into account the result would be 20.98%, which was lower than the 12th experiment (28.07%) where 1g/7ml solid liquid ratio, 1% acid ratio, 110 °C and 40 min was applied.

According to Figure 5.8, 110 °C, 40 min and 1g/7ml solid – liquid ratio lead to better result. There was only slight increase on the lower concentration of acid ratio (%1). Therefore, the temperature and time factors were fixed at 110 °C and 40 min,

respectively, solid–liquid ratio were enlarged to the higher concentrations than 1g/7ml and acid ratio were enlarged to the lower concentrations than 1% acid ratio in the optimization step.

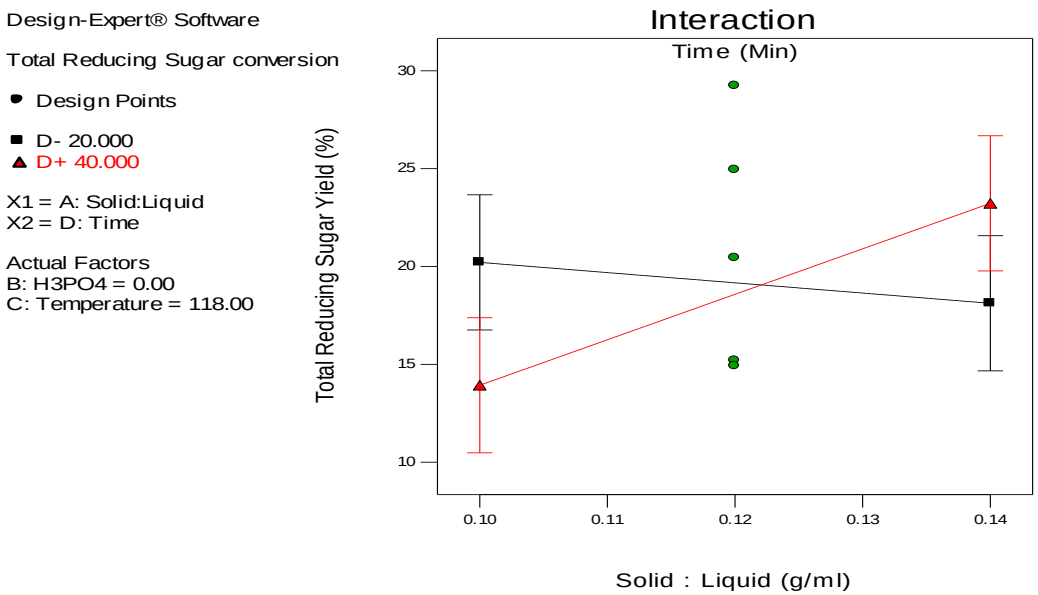

Figure 5.8. The interaction graph of solid:liquid ratio and time at 110 °C (0.14 and 0.10 means 1g/7ml and 1g/9ml respectively) in screening step of peach pomace

The results of statistical analysis of the optimization study of the peach pomace are tabulated in Table 5.9. The ANOVA results of central composite design for reducing sugar conversion yields demonstrated that the model was significant due to an F-value of 6.86. There was only a 0.61% chance that a "Model F-Value" this large could have occurred due to noise.

None of the single factors were significant. However, the second order of acid ratio was significant with a p-value of 0.0039. Final equation in terms of coded factors and actual factors were given below.

$$\text{Total RS conversion of apple pomace} = + 49.28 + 1.65 * X2 - 3.84 * X_2^2 \quad (5.3)$$

$$\text{Total RS conversion of apple pomace} = + 39.21 + 12.63 * \text{Acid} - 3.89 * \text{Acid}^2 \quad (5.4)$$

Table 5.9. Analysis of variance for peach pomace (Optimization)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	218.04	2	109.02	6.86	0.0061	Significant
X_2	43.78	1	43.78	2.75	0.1144	
X_2- X_2	174.27	1	178.27	10.96	0.0039	
Residual	286.17	18	15.90			
Lack of Fit	166.73	6	27.79	2.79	0.0614	Not significant
Pure Error	119.44	12	9.95			
Cor Total	504.21	20				
Std. Dev.	3.99		R-Squared		0.43	
Mean	46.35		Adj R-Squared		0.37	
C. V. %	8.60		Pred R-Squared		0.21	
PRESS	398.55		Adeq Precision		6.65	

The "Lack of Fit F-value" of 2.79 implied that the Lack of Fit was not significant relative to the pure error. There was a 6.14% chance that a "Lack of Fit F-value" this large could have occurred due to noise. The experimental yields did not fit the second-order polynomial equation as indicated by low R^2 values (0.43), which suggested that 57% of the total variations were not explained by the models developed for the corresponding yield of total reducing sugar.

52.44% was the highest RS yield of peach pomace in 17th experiment (centerpoint), under the conditions of 110 °C, 40 minutes, 1g/5.25ml and 1.41% acid ratio. Furthermore, Figure 5.9 suggested that 1.41% acid ratio was optimum for high sugar conversion.

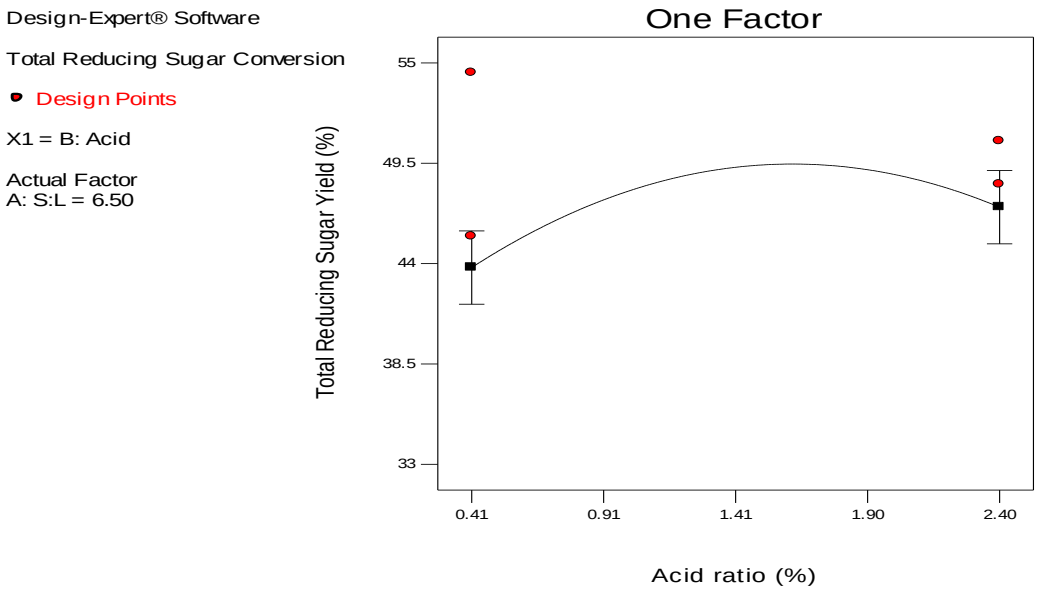

Figure 5.9. Second order factor plot of acid ratio at 1g/6.5ml solid – liquid ratio in the optimization step of peach pomace

In order to validate the adequacy of the model equations a total of four verification experiments were carried out at the predicted optimum conditions for peach pomaces. The results showed 20, 17, 19 and 20% deviation. The overall margin of error was 19.37% (Table 5.10).

Table 5.10. Validation experiments of peach pomace

Solid/Liquid (g:L)	Acid ratio (%)	Estimated sugar conversion (%)	Actual sugar conversion (%)	Error (%)	Overall Error (%)
1/4.14	0.82	46.95	37.46	20.21	19.37
1/5.19	1.35	49.17	40.66	17.30	
1/5.74	2.26	47.87	38.56	19.45	
1/4.88	1.88	49.19	39.11	20.50	

5.3. Analysis of the Hydrolysates

5.3.1. Furfural and Hydroxymethylfurfural

Furfural and hydroxymethylfurfural (HMF) are decomposition product of pentoses and hexoses, respectively. The formation of furfural is a first-order reaction, where the reaction constant is affected by both acid concentration and temperature. On the other hand formation of HMF during dilute-acid hydrolysis is a sequential reaction. Cellulose and hemicellulose are first hydrolysed to their hexose monomers, followed by decomposition of liberated hexoses to HMF. Among the various pentose sugars exposed to acid for furfural formation, arabinose showed the lowest reactivity, with a small reaction constant (Garrett and Dvorchik, 1969). Therefore, lack of furfural formation is most probably due to stability of arabinose and its low concentration in hydrolysate under the applied conditions. These two reactions, which are influenced by temperature and acid concentration are both first-order reactions and possess rates of similar magnitude, according to kinetics of these two reactions for lignocellulosic materials (Saeman, 1945). The higher ratio of the first reaction rate constant increases the yield of total liberating sugars, compared to the second one. Time is a function of both reaction and elapsing time of hydrolysis. Longer than the optimal values enhance the speed of the second reaction leading to a decrease in net total sugar liberation (Saeman, 1945).

Thus, time is an important factor for the overall hydrolysis process to achieve the highest yield of total carbohydrates (Talebnia *et al.*, 2008).

According to HPLC results, none of the hydrolysates contained furfural or hydoxymethylfurfural. This is a great advantage for a fermentation media, since these compounds show inhibitory effects on microorganisms. Furthermore pectin was not hydrolysed in this work, and therefore no galacturonic acid was detected through the analysis. The released pectin fragments had a soluble nature. The glucosidic bonds between galacturonic acid units were probably too resistant to acid hydrolysis.

5.3.2. Total Soluble Solids (BRIX)

BRIX results of all hydrolysates are shown in the appendix. Total soluble solid contents can be generally considered as an indication of solid substances possibly rich in vitamin and minerals, which can have significant effect on the cell growth during any fermentation process. Therefore, their levels in the pomaces are important in the decision making process for the evaluation of the potential candidacy of the pomaces. The total soluble solids of hydrolysates of all pomaces in the optimization step were significantly higher than the screening step. This indicated that the levels of the optimization step affected soluble content of pomaces more positively than the screening step. Furthermore, brix results suggested that higher acid ratio lead to higher decomposition of soluble solids. The highest soluble solids were obtained in the optimization step from apple pomace hydrolysis (46%) followed by apricot pomace (45%). On the other hand peach pomace had low soluble solids compared to apple and apricot pomaces. Furthermore it had higher soluble solids on an average in screening step than the optimization. The reason might be the lower acid level used in the optimization step compared to apple and apricot hydrolysis.

5.3.3. FTIR Analysis of the Experimental Results

PLS analysis was used to predict the concentration of several sugars (Y variables) in hydrolyasate samples using FTIR data as X variables. Total number of samples for each fruit was 21. 14 samples were randomly selected for calibration and 7

samples were used for validation. Statistical analysis results for PLS model developed for apple were listed in Table 5.11 Correlation coefficients for calibration (R^2) for all measured parameters were quite high. However, R^2 (valid.) values were low and this means developed model does not have good predicting ability. In addition, there are large differences between RMSEC and RMSEP values and this is also an indication of low predicting power of the model.

Table 5.11. Summary of statistical results for PLS analysis of apple samples

Parameter	R^2 (calib)	R^2 (valid)	RMSEC	RMSEP
Peak at RT* 11	0.985	0.220	0.00086	0.09533
Glucose	0.999	0.030	0.00308	0.26924
Xylose	0.983	0.216	0.02673	0.20703
Galactose	1	0.229	0.00130	0.26133
Arabinose	0.997	0.335	0.01204	0.31572
Mannose	0.998	0.065	0.00297	0.10569
Peak at RT 17,49	0.990	0.007	0.00205	0.02563
Peak at RT 18,76	1	0.651	0.00063	0.41732
Brix	0.987	0.419	0.69461	6.19200
Reducing sugar	0.999	0.177	0.16156	7.10776

*Retention time

Several prediction curves for measured parameters for apple are shown in Figure 5.10 and 5.11. Model developed for apricot has also very high R^2 (calib.) but very low R^2 (valid.) values. For other fruits, models have both low R^2 (calib.) and R^2 (valid) values.

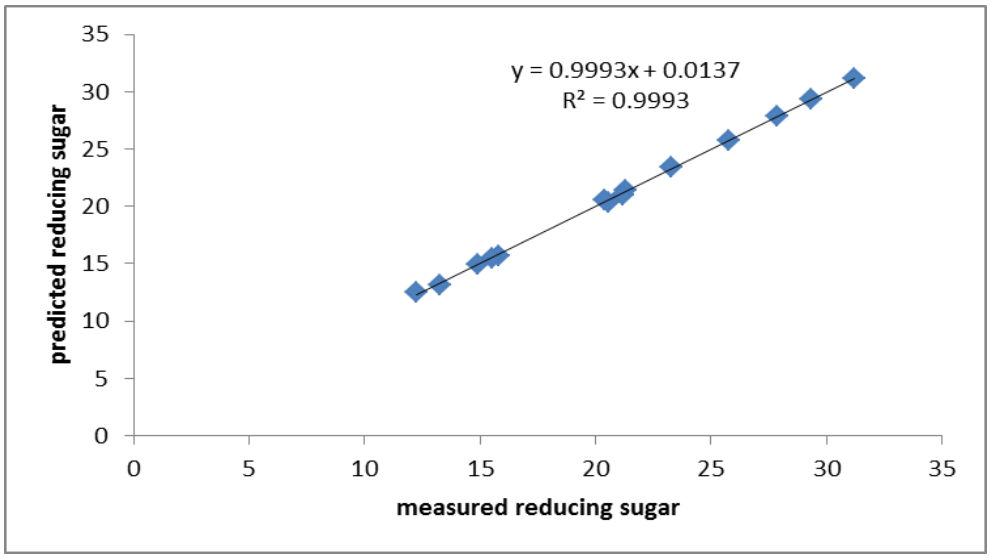

Figure 5.10. Reducing sugar calibration graph of apple pomace

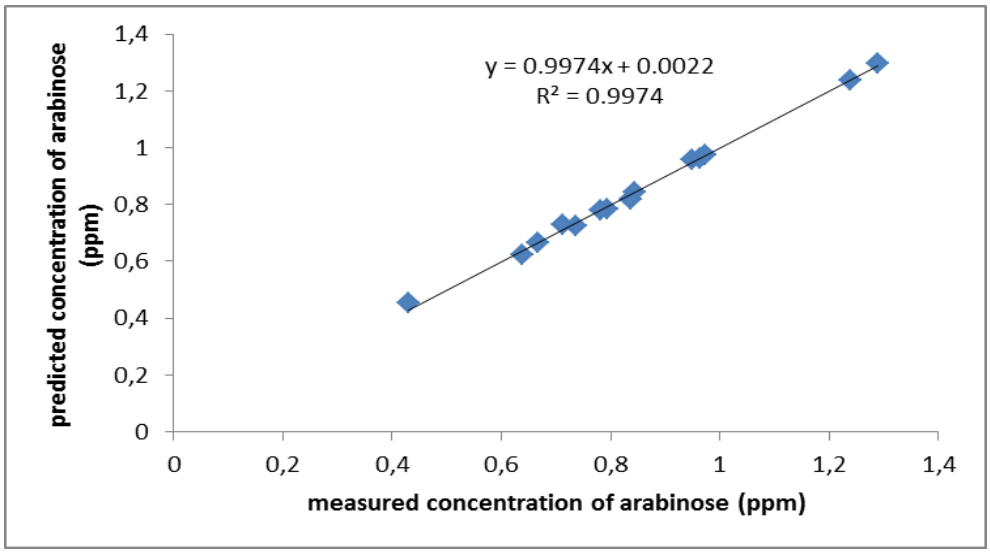

Figure 5.11. Arabinose calibration graph of apple pomace

5.4. Fermentation Results

The direct fermentation of cellulosic biomass to ethanol has long been a desired goal. Some filamentous fungi hold promise in this area, since they 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 (conidia), which could be useful for inoculation at a high level (Stevenson and Weimer, 2002). There are several 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 ferment cellulose to ethanol. The genus *Trichoderma* (strain A10), which can ferment microcrystalline cellulose or several sugars to ethanol were chosen for ethanol production. This way, besides initial reducing sugars, remaining cellulosic compounds in hydrolysates can be fermented into ethanol too. Stevenson and Weimer (2002) found that, since strain A10 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.

In this study the fungal strain *Trichoderma harzianum* was used to evaluate the potential of various pomace hydrolysates, obtained from pre determined optimum pretreatment conditions as discussed in previous sections, in the bioethanol production. In order to observe the effect of some physical and chemical conditions, fermentations were carried in different incubators; static, shaking (at 170 rpm) and CO_2 incubator of cultures pregrown in different media compositions of YNM and YNB.

Figures 5.12, 5.13 and 5.14 show the initial sugar utilization of the fermentations carried out in the CO_2, static and shaking incubators (mentioned in Section 4.2.4), respectively during the fermentation period. All hydrolysates had 34 g/L initial sugar on the first day of fermentation. It was observed that the microorganism was using the sugars in the hydrolysates and braking down the cellulose into sugars simultaneously. It seemed that neither static nor CO_2 incubator had an efficient mass transfer, since initial sugar remained stably during the course of the fermentation. In fact this indicated that the breaking down of cellulose into sugars and consumption of sugars by the microorganism was almost equal. However, in the shaking incubator the initial sugar decreased very fast during the course, since there was an efficient mass transfer and a little access of O_2. Thus, the microorganism was able to use all of the initial sugars and brake down the cellulose molecules into sugars very effectively because of a better mass transfer and little O_2 access through slicone tubing.

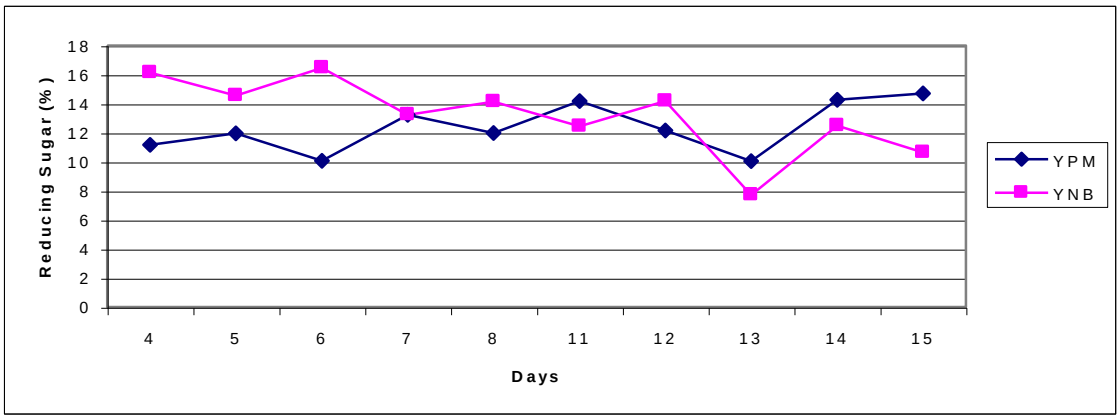

Figure 5.12. Sugar consumption profile during the course of fermentation in CO_2 incubator (static)

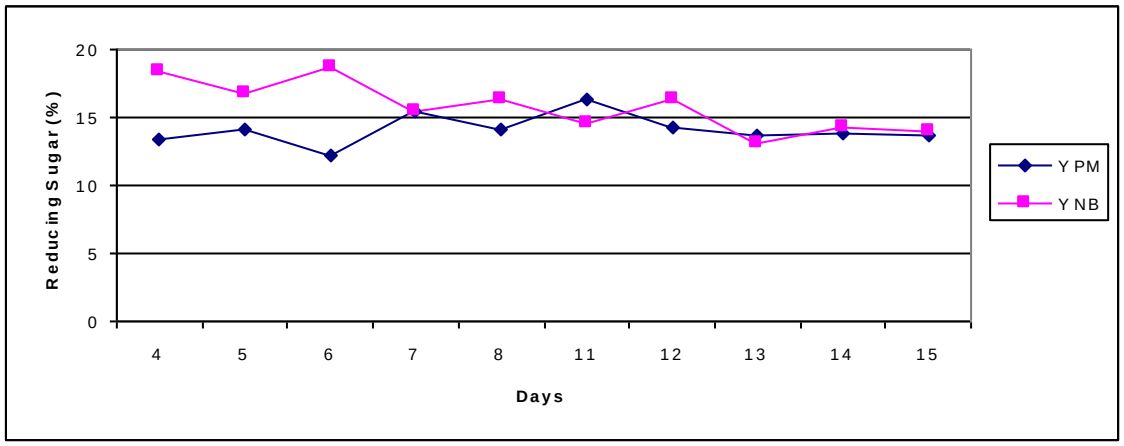

Figure 5.13. Sugar consumption profile during the course of fermentation in static incubator

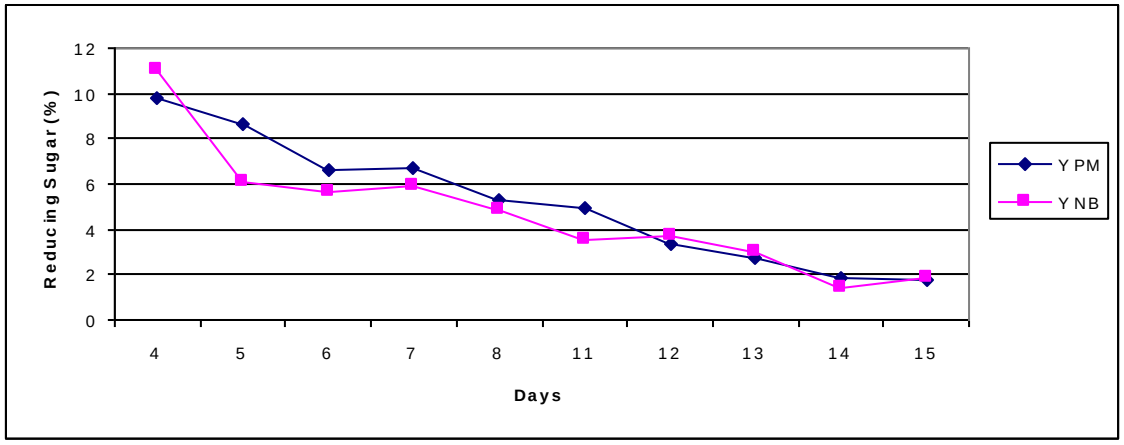

Figure 5.14. Sugar consumption profile during the course of fermentation in shaking (170 rpm) incubator

There was not significant difference between the media, YPM and YNB, regarding to the sugar consumption and cellulose degradation. However YNB showed a significant difference with respect to ethanol production only in shaking (170 rpm) incubator (1.67 g/L, 1.17 g/L, YNB and YPM, respectively). Furthermore, static incubator produced more ethanol than CO_2 incubator. That meant microorganism needed the presence of O_2.

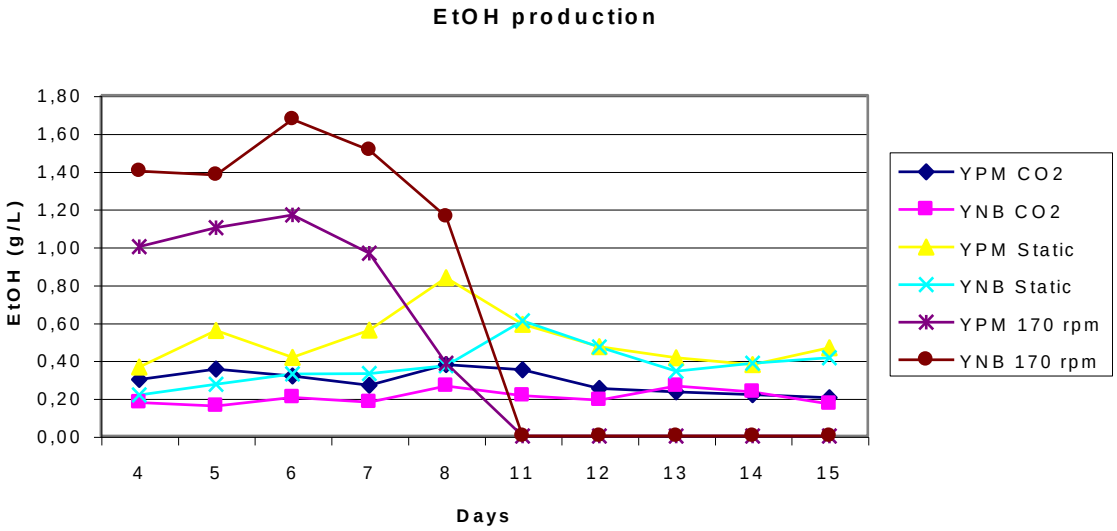

Figure 5.15. Ethanol production profile during the course of fermentation in CO_2, shaking (170 rpm) and static incubators

Ethanol production profiles of fermentations carried out in each incubator are depicted in Figure 5.15 and discussed below.

CO_2 Incubator: After eight days of fermentation there was a little reduction of ethanol production in CO_2 incubator. Apart from that the average ethanol production in CO_2 incubator remained almost invariably and lowest for the rest of the duration. There were not any significant differences between YPM and YNB related to both ethanol production and sugar consumption. Using CO_2 incubator caused adverse effect in ethanol production in comparison to other incubators.

Static Incubator: First eight days YPM and YNB significantly differed from each other in static incubator. Sugar consumption of YPM in static incubator in 8^{th} day was not different from other days (Figure 5.13.). However, Figure 5.16 and Figure 5.17 suggested that consumption of xylose, mannose, galactose and arabinose was the highest for all days in static incubator. This might be the reason that 8^{th} day was the best time for ethanol production performance of YPM in static incubator. YPM showed higher ethanol production than YNB. However, in 11^{th} day the production was almost equal for YPM and YNB, and that continued decreasingly.

Shaking Incubator: Since there was an efficient mass transfer of sugar compounds and a little O_2 access in shaking incubator at 170 rpm, microorganisms were able to use sugars and other compounds much more effectively in comparison with other incubators. This leads to greater ethanol production in shaking incubator. In day six, the highest ethanol production in both YPM and YNB (1.17 g/L, 1.67 g/L

respectively) was achieved. However, YNB showed higher ethanol production than YPM. After six days ethanol production in both media showed a fast decrease. Surely the reason for this was that microorganisms used all of the sugars because of the efficient mass transfer and were not able to produce more ethanol. Another reason could be related to the evaporation of the produced ethanol.

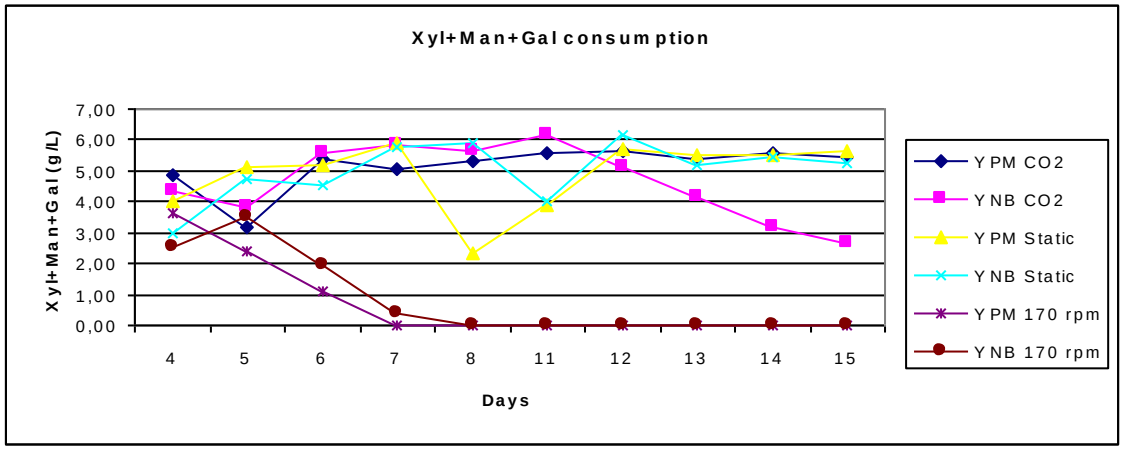

Figure 5.16. The profile of initial sugar (sum of xylose, mannose and galactose concentration during the course of fermentation)

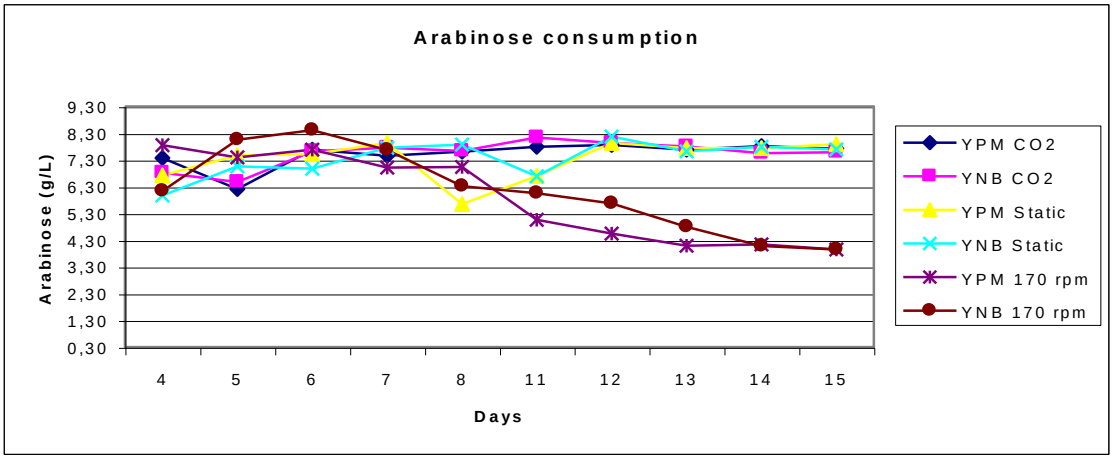

Figure 5.17. The profile of arabinose concentration during the course of fermentation

Using CO_2 incubator caused negative effect in ethanol production, which means microorganism slightly need the presence of O_2. Shaking incubator showed much higher ethanol production than other incubators, since mass transfer leads efficient usage of compounds such as sugars and microcrystalline cellulose. According to these

results future work should be focused on the more precise study of bioethanol production in shaking incubators of various speeds.

CHAPTER 6

CONCLUSION

The composition of some fruit pomaces, main wastes of the fruit industry, was determined and hydrolysis of these fruit pomaces was carried out with dilute acid, and optimum conditions as well as influencing factors (time, temperature, solid-liquid ratio and acid percentage) were investigated by applying statistical methods. One of the pomaces with the most reliable statistical result was selected for further bioethanol fermentation.

At the initial screening step, all pomaces were found to have high sugar contents without any treatment, except for apple pomace. However, after a pre-treatment apple pomace had also higher sugar content, since it had higher total solid and was rich in dietary fiber among other pomaces. These results show that these four pomaces can be considered as potential fermentation media, having considerably high reducing sugars even without any chemical, physical or biological treatment and adequate dietary fiber for microorganisms.

None of the hydrolysates had either furfural or hydroxymethylfurfural (HMF), which are inhibitors for microorganisms. Correlation coefficients for calibration of prediction of the concentrations of several sugars in hydrolysates samples using FTIR were quite high. According to the statistical analysis, among the linear terms, temperature and time were the most significant variables affecting the yields of sugars of apple pomace in the first step (screening). Furthermore in the second step, in which time and temperature were fixed, acid ratio was the significant linear term. Without any treatment sugar percentage of apple pomace was 6% and after treatment the maximum yield of sugar hydrolysis of apple pomace increased to 26.37%. The first optimization step for apricot pomace suggested that only some interactions of single factors were significant especially the interaction of acid ratio and time. Before any treatment the sugar percentage of apricot pomace was around 22.91%, which increased to 49.16% after treatment. Considering the peach pomace, among the linear terms temperature was the most significant effect in the first step. The second step suggested that only the second order of acid ratio was significant. The sugar percentage was before pre-

treatment 22.06%, which increased to 52.44% after treatment. Furthermore, orange pomace had 33% sugar content before any treatment and 37% after pre-treatment. However, in the first step, only temperature was the most significant effect among single factors.

According to the results of fermentation of apple pomace hydrolysate, the highest ethanol production was 1.67 g/L on the 6^{th} day, and the most efficient sugar consumption was in a shaking incubator with the culture grown in YNB media. This could be related to a better mass transfer due to shaking.

The results pointed out that there was an accurate increase in sugar contents after pre-treatment with dilute acid in fruit pomaces. Considerable amount of ethanol production within a short period of time (6 day) using apple pomace hydrolysate and a culture (*Trichoderma harzianum*), which can ferment microcrystalline cellulose or several sugars to ethanol suggest that fruit pomaces can be possible candidates for future bioethanol production.

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APPENDIX A

SOLUBLE SOLIDS AND REDUCING SUGARS

Table A1. Soluble solids and reducing sugar yields of pomace hydrolysates (Screening)

Temperature and Time		Solid/ Liquid (g/L)	Acid ratio (%)	Apple		Orange		Peach		Apricot	
				Brix (% Soluble Solids)	Reducing Sugar Yield (%)	Brix (% Soluble Solids)	Reducing Sugar Yield (%)	Brix (% Soluble Solids)	Reducing Sugar Yield (%)	Brix (% Soluble Solids)	Reducing Sugar Yield (%)
	110 °C 20 min	1/9	3	30.96	8.16	33.30	11.86	31.99	21.76	34.47	22.99
		1/9	1	15.93	4.11	21.24	16.79	18.85	27.18	20.65	14.84
		1/7	3	23.97	4.79	31.29	18.71	25.44	25.91	29.15	22.18
		1/7	1	13.75	5.86	18.41	13.49	16.10	14.16	17.95	15.79
	126 °C 20 min	1/9	3	20.97	10.87	38.11	16.16	34.56	16.05	32.94	25.35
		1/9	1	19.17	7.27	24.30	27.49	21.15	15.83	17.50	13.01
		1/7	3	26.53	10.22	29.75	8.55	28.59	17.37	26.88	16.23
		1/7	1	14.35	7.94	20.93	23.81	16.41	15.01	5.39	21.19
	110 °C 40 min	1/9	3	33.30	24.06	38.34	12.38	33.84	16.21	36.13	20.15
		1/9	1	16.96	29.77	24.39	5.67	17.50	13.74	21.33	29.14
		1/7	3	26.95	19.07	29.64	9.14	29.50	26.91	29.85	11.80
		1/7	1	13.37	20.10	23.17	5.25	16.52	28.07	17.46	28.70
	126 °C 40 min	1/9	3	32.49	16.78	39.06	17.56	35.32	13.49	37.39	18.82
		1/9	1	19.53	10.64	23.62	12.35	19.39	12.27	16.83	12.75
		1/7	3	27.72	14.73	33.00	19.63	27.79	14.99	26.95	11.22
		1/7	1	17.50	13.40	21.59	16.37	15.12	22.91	17.36	34.39
	118 °C 30 min	1/8	1.75	14.01	14.01	28.68	26.06	18.56	15.17	26.80	15.42
		1/8	1.75	12.73	12.73	27.84	24.89	23.76	20.43	26.00	19.80
		1/8	1.75	11.86	11.86	30.56	23.21	24.60	14.90	25.12	18.93
		1/8	1.75	11.50	11.50	27.68	36.98	20.96	29.22	23.60	12.73
		1/8	1.75	15.85	15.85	30.00	27.40	23.48	24.92	25.44	15.69

Table A2. Soluble solids and reducing sugar yields of pomace hydrolysates (Optimization)

Solid-Liquid (g/L)	Acid ratio (%)	Apple		Peach		Apricot	
		Brix (Soluble solids %)	Reducing Sugar Yield (%)	Brix (Soluble solids %)	Reducing Sugar Yield (%)	Brix (Soluble solids %)	Reducing Sugar Yield (%)
1/6.5	1	17.35	15.79	9.40	44.70	17.71	45.05
1/6.5	1	16.08	13.48	10.16	44.77	16.15	43.26
1/10.5	1	19.37	12.04	12.18	54.46	18.84	42.47
1/10.5	1	20.37	14.40	11.31	45.49	19.53	39.70
1/6.5	4	31.00	17.00	16.02	47.95	32.40	36.47
1/6.5	4	31.03	19.56	16.48	49.03	31.88	44.36
1/10.5	4	46.20	31.35	21.71	48.34	45.57	28.67
1/10.5	4	47.09	21.39	21.93	50.71	46.35	45.62
1/5.67	2.5	21.88	19.18	12.02	49.34	23.16	43.44
1/5.67	2.5	25.37	23.01	12.31	48.85	23.13	49.16
1/11.3	2.5	37.17	20.24	17.97	49.40	35.53	41.09
1/11.3	2.5	37.00	21.40	18.07	40.69	37.12	48.10
1/8.5	0.38	12.07	18.79	8.68	37.43	15.08	24.05
1/8.5	0.38	11.81	16.36	8.24	33.25	14.83	41.07
1/8.5	4.62	44.83	24.22	19.84	43.91	43.73	36.21
1/8.5	4.62	42.62	21.73	19.89	40.81	43.52	38.29
1/8.5	2.5	29.32	17.10	15.12	52.44	28.47	48.48
1/8.5	2.5	28.43	19.47	14.49	48.20	28.00	41.77
1/8.5	2.5	29.19	18.85	14.33	45.60	29.62	43.20
1/8.5	2.5	28.73	21.68	15.22	48.61	29.15	36.56
1/8.5	2.5	28.90	19.66	16.01	49.39	26.13	43.61

APPENDIX B

CALIBRATION GRAPHS

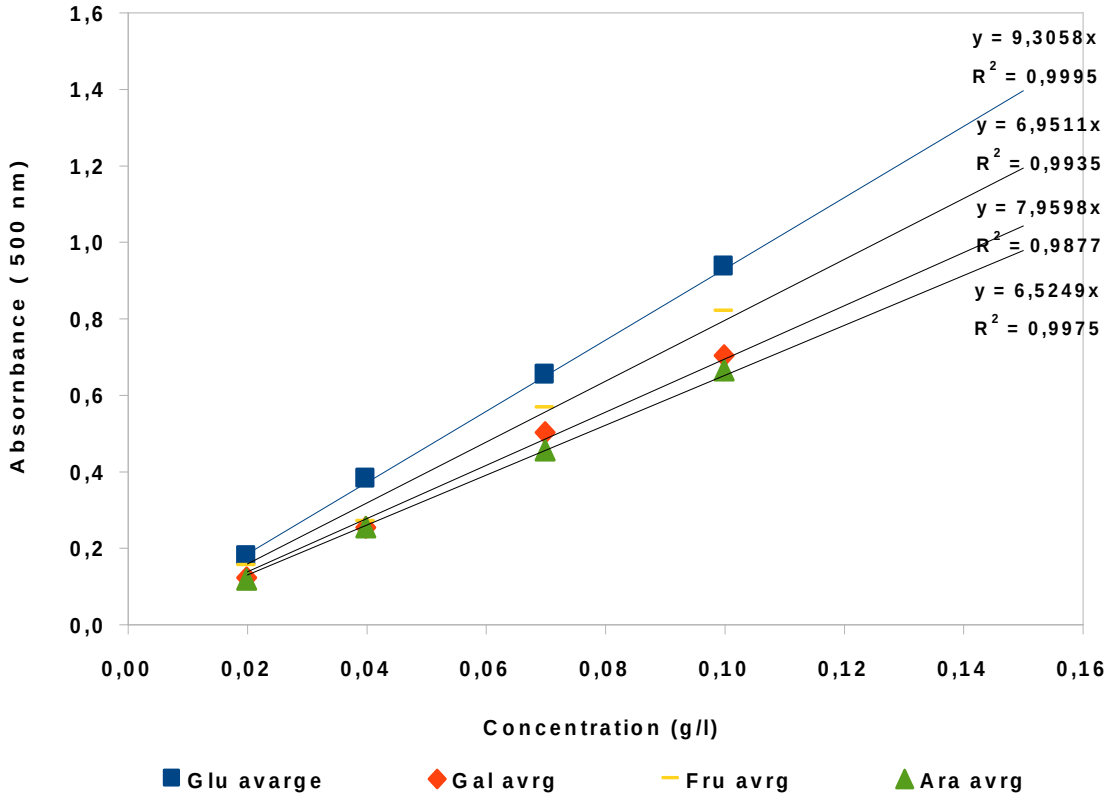

Figure B1. Calibration graph of Nelson-Somogyi reducing sugar method

Average of slops (7.69) of these 4 sugars (glucose, galactose, fructose and arabinose) were 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 (7.69)

C = Dilution factor

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

See the section 4.2.2 for further calculation.

APPENDIX C

CHEMICALS

Table C1. Chemicals used

Analyse	No	Chemical	Code
Protein	1	Sulphuric acid (H_2SO_4)	Merck 1.00731.2500
	2	Boiling stone	
	3	Antifoam	
	4	Sodium hydroxide (NaOH), pellets pure	Merck 1.06462.1000
	5	Boric acid (H_3BO_3), mol. bio. grade	Sigma B6768
Ash	6	Hydrogen peroxide 30% (H_2O_2)	Merck 107298
Dietary Fiber	7	Amyloglucosidase	Sigma A9913-10ML
	8	Protease	Sigma P3910-500MG
	9	α-Amylase, heat stable	Sigma A3306-10ML
	10	Acetone	Merck 1.00014.2500
	11	Sodium phosphate, Monobasic, anhydrous	Sigma S0751
	12	Ethanol, ACS reagent	Sigma 45,984-4
Reducing sugar	13	Sodium carbonate (Na_2CO_3), anhydrous	Riedel-de Haën 13418
	14	Sodium bicarbonate ($NaHCO_3$), Min 99.5%	Sigma S-8875
	15	Potassium sodium tartarate tetrahydrate ($C_4H_4KNaO_6$. H_2O)	Sigma S-6170
	16	Copper (II) sulphate-pentahydrate ($CuSO_4$. $5H_2O$), extra pure	Riedel-de Haën 12849
	17	Sodium sulphate (Na_2SO_4), anhydrous	Riedel-de Haën 13464
	18	Sulphuric acid (H_2SO_4)	Merck 1.00731.2500
	19	Ammonium heptamolybdate heptahydrate ($(NH_4)6Mo_7O_{24}$. $7H_2O$)	Riedel-de Haën 1.011.800.250
	20	Disodium hydrogen arsenate heptahydrate ($AsHNa_2O_4$. $7H_2O$)	Flucka 71.625

(cont. on next page)

Table C1. (cont)

Hydrolysis	21	Phosphoric acid (H_3PO_4), 85%	Merck 1.00573.2500
HPLC (High purity standards)	22	D-cellobiose	
	23	D-(+)glucose	
	24	D-(+)xylose	
	25	D-(+)galactose	
	26	D-(+)arabinose	
	27	D-(+)mannose	
	28	Ethanol, absolute pure, p.a.	Sigma 32221
	29	5-hydroxy-2-furaldehyde (HMF)	
	30	Furfural	
	31	Sulphuric acid (H_2SO_4), concentrated, ACS reagent grade	Merck 1.00731.2500
	32	Calcium carbonate, ACS reagent grade Min 99%	Alfa Aesar 43073
	33	Water, HPLC grade, 0.2 μm	
Fermentation	34	Yeast Nitrogen Base (YNB)	BD 239210 (Difco™)
	35	Peptone	Merck 1.07214.9999
	36	Malt extract	BD 218630 (Bacto™)
	37	Yeast extract	BD 211929 (BBL™)