ETHANOL PRODUCTION FROM ORANGE WASTE WITH A FOCUS ON ITS CELLULOSIC FRAGMENT

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ABSTRACT

ETHANOL PRODUCTION FROM ORANGE WASTE WITH A FOCUS ON ITS CELLULOSIC FRAGMENT

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Cellulose-based biofuels are among the recent pioneer biofuels. Considering the fact that feedstock accounts for a major portion of its production cost, the cheap sources of agricultural waste as cellulosic raw material is a positive aspect for more economical biofuel production processes. In this study, production of ethanol from orange waste, more specifically from its cellulosic fragment, was studied. Three different approaches were defined for investigations on the effect of isolation of cellulosic OW fragment on glucose and ethanol production yields. In the first approach a multi-step pretreatment strategy was used for separation of different components from OW with the aim of isolation of its cellulosic fragment. The enzymatic hydrolysis conditions that resulted in the maximum glucose yield were 10% (w/v) substrate loading, 1% (v/v) cellulase loading and 24 hours of hydrolysis. Accordingly the glucose yield regarding the theoretical maximum glucose was
determined as 91%. The second approach was defined to investigate the enzymatic hydrolysis and fermentation of untreated OW containing especially valuable pectin and soluble sugars. Experimental parameters that led to maximum glucose yields were measured to be 20% substrate loading (w/v), 1% (v/v) cellulase, and 4% (v/v) pectinase loading and 24 hours of hydrolysis. The glucose yield regarding the maximum theoretical cellulose content of OW was 88%. The third approach focused on the possibility of using more environmentally friendly and simpler pretreatment steps for separation of pectin and other soluble components from OW. The glucose yield based on this approach was 91% considering the theoretical maximum glucose content.

The wild type yeast *Saccharomyces cerevisiae* NRRL Y-132 was used for the fermentation of sugars present in the hydrolysates obtained by each approach. The resulting ethanol production yields on the basis of the theoretical maximum yields for the first to the third approach were determined as 96, 99 and 96% (w/w), which correspond to 0.72, 2.7 and 0.78% (w/v) (g ethanol/ 100 ml medium), respectively. Starting with initial glucose concentrations of 12, 55 and, 16 g/L in approaches 1, 2 and, 3 resulted in 7, 27 and, 8 (g ethanol/ L fermentation medium), respectively, indicating the possibility of increasing ethanol yield by increasing initial glucose concentration.

Keywords: Orange waste, lignocellulosic biomass, pretreatment, cellulosic fragment, fermentation, cellulose, cellulosic hydrolysate, cellulases, ethanol
ÖZ

PORTAKAL ATIĞININ SELÜLOZİK KISMI ÜZERİNDEN ETANOL ÜRETİMİ

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Selüloz bazlı biyoyakıtlar yeni öncü biyoyakıtlar arasındadır. Hammaddenin üretim maliyetinin büyük bir kısmını oluştuduğu düşünülündüğünde, selülozik kaynak olarak ucuz tarımsal atıklar daha ekonomik biyoyakıt üretim prosesleri için olumlu bir yaklaşım sağlamaktadır. Bu çalışmada, portakal atığından özellikle bu atığın selülozik kısmından etanol üretimi çalışılmıştır. Selülozik portakal atığının glikoz ve etanol verimlerine etkisi üzerine yapılanması olan araştırmalar için üç farklı yaklaşım tanımlanmıştır. İlk yaklaşımında, portakal atığından özellikle bu atığın selülozik kısmından etanol üretimi çalışılmıştır. Selülozik portakal atığının glikoz ve etanol verimlerine etkisi üzerine yapılanması olan araştırmalar için üç farklı yaklaşım tanımlanmıştır. İlk yaklaşımında, portakal atığından değişik bileşenleri ayrırmak için selülozik kısımların ayrıştırılması hedeflenerek çok basamaklı bir ön işlem stratejisi kullanılmıştır. En yüksek glikoz verimini sonuçlandırıran enzimatik hidroliz şartları, %10 (gram/hacim) substrat konsantrasyonu, %1 (hacim/hacim) selüloz konsantrasyonu ve 24 saatlik enzimatik hidroliz süresidir. Bunlara göre, en yüksek teorik glikoz üretimi göz önünde bulundurulduğunda glikoz verimi %91’dir. İkinci
yaklaşım, özellikle değerli pektin ve çözünebilen şekerleri içeren ön işlem görmemiş portakal atığının enzimatik hidroliz ve fermentasyonunu incelemek üzere tanımlanmıştır. En yüksek glikoz verimini sağlayan deneysel etkenler %20 (gram/hacim) substrat konsantrasyonu, %1 (hacim/hacim) selüloz konsantrasyonu, %4 pektinaz konsantrasyonu ve 24 saatlik enzimatik hidroliz süresidir. En yüksek teorik glikoz üretimi göz önünde bulundurulduğunda glikoz verimi %88'dir. Üçüncü yaklaşım, pektin ve diğer çözünebilen bileşenleri ayırmak için daha çevreye duyarlı ve daha basit ön işlem basamaklarının kullanılmasının olasılığını hedeflemektedir. Bu yaklaşılma dayalı olarak, teorik glikoz miktarına göre glikoz verimi %91’dir.

Bir saf maya tipi olan Saccharomyces cerevisiae NRRL Y-132, denenen yaklaşımlar sonucunda elde edilen sıvı enzimatik hidroliz ürünündeki şekerin fermentasyonu için kullanılmıştır. İlk yaklaşımdan üçüncüye kadar ki teorik en yüksek etanol verimine dayanan etanol verimleri %96, %99 ve %96 (g/g) olarak bulunmuş ve bu verimler sırasıyla 0.72, 2.7 ve 0.78 (g etanol/100 ml fermentasyon ortamı) sonuçlarına denk gelmektedir. 12, 55 ve 16 g/L başlangıç glikoz konsantrasyonları ile başlandığıda sırasıyla 7, 27 ve 8 (g etanol/L fermentasyon ortamı) değerleri elde edilmiştir. Bu değerler, başlangıç glikoz konsantrasyonunun arttırılmasıyla etanol konsantrasyonun artırılması olasılığını göstermektedir.

Anahtar Kelimeler: Portakal atığı, lignoselülozik, önisleme, selülozik fragmanı, fermentasyon, hidrolizat, selülaz, etanol
To My Parents,
DEDICATION

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LIST OF ABBREVIATIONS

OW: Orange waste
UOW: Untreated orange waste
FPU: Filter paper unit
FGU: Polygalacturonic acid
SSF: Simultaneous saccharification and fermentation
CHAPTER 1

INTRODUCTION

1.1 The drawbacks regarding fossil fuels

Petrochemistry has played a dominant role in the industrial and societal developments of 20th century. Therefore, dependence of social, economic and industrial progress of communities on energy has led to ever-increasing exploration and exploitation of oil reserves to meet growing demand for cheap and abundant source of energy. However, from late 1990’s, the new oil resources have fallen far behind the oil consumption rates (Greene, 2009). Energy demand has increased more than fourfold in the last three decades and is meant to increase in the coming years. Economic growth, life standards, and industrial development of developing countries are directly connected to their energy (mostly oil) supply (Barnes, 1991).

The increasing demand and at the same time decrease in the rate of oil reservoir discovery, has led to increase in the oil prices. Thus, governments have been forced to reconsider their dependence on oil reserves and have started working on more trustable sources of energy (Lugar, 1999).

Utilization of fossil fuel has some dominant drawbacks such as its limited resource, which would deplete in some years, and the fact that it is environmentally perilous. These problems together with strong dependence of industrial developments to fossil fuels have caused great concern regarding utilization of fossil fuels in the current decades.

Many investigations have been conducted working on replacing fossil fuels with renewable sources of energy that do not have the time period unbalances regarding them.

1.2 Sustainable fuels: Biofuel a solution

Biofuel is defined as solid, liquid, or gas consisting of, or derived from biomass. Biofuel is considered a means of reducing greenhouse gas emissions and increasing
energy security by providing an alternative to fossil fuel (Triratanasirichai, 2007). Biofuel accounts for three main groups of fuels which are bioethanol, biodiesel and biomethane (Hirayama, 1998). The first type of biofuel is bioethanol which is of two types. First is the production of ethanol from starch-rich biomass like corn, maize, cereals, sugar cane and etc. Starch based ethanol is also called first generation ethanol. The second type of bioethanol is from cellulose-rich biomass like agricultural and forestay residues. Cellulosic ethanol is also referred to as second generation ethanol. The processes for ethanol production are hydrolysis of biomass to produce monosaccharides, mainly glucose, fermentation and distillation.

The other type of biofuels is biodiesel which is produced from vegetable oils, used cooking oils and animal fats using alcohols and also some types of microalgae. The main processes for advanced biodiesel production processes accounts for hydrogenation of oil and fat, gasification and biomass to liquid catalytic conversion (BTL) (Baner, 2011).

Due to the diversity of biofuel types, processes and feedstocks, it is not easy to comment on energy output and emissions. As for comparison of ethanol produced from sugarcane to that of gasoline, bio-ethanol energy input is 10-12% of final energy and there is up to 90% reduction in CO₂ amounts. The required energy input is higher for ethanol and reduced CO₂ amounts are around 15-25%. For lignocellulosic biomass, energy input is higher than corn ethanol, but most of this energy can be provided from biomass itself, and CO₂ reduction is up to 70% (100% if power is co-generated) (Simbolotti, 2007).

### 1.3 Ethanol

Ethanol has long been considered a potential alternative to replace petrochemicals and transportation fuels. Production of ethanol from plant biomass has been the sustainable solution for the energy and environmental problems of this century.

Producing ethanol from plant biomass is not a new approach. There was an alcohol fuel ethanol production committee in 1918 established by the British government to investigate on potential of producing ethanol from biomass as one of its main aims (Holden, 1919). A few years later it was anticipated that the future of high value products production from biomass would be from three major substances: the carbohydrates, oils, and proteins. Particularly, carbohydrates, sugars, starch, and cell
wall components were projected to be the main sources of ethanol (Maris, 2006). Although from the beginning scientists had emphasized on importance of ethanol production from lignocelluloses along with readily fermentable starch and sucrose, to be able to make it economically beneficial (Kressmann, 1922), corn based ethanol and its production from starchy food is still the major industrial ethanol production sources (Maris, 2006). Particularly, developed countries are interested in utilizing liquid biofuel in their transportation sector. This is unavoidable considering rapid increase in petroleum prices, and strategic concerns about dependence on politically unstable countries which are the dominant petroleum producers.

1.3.1 The challenges in the way of practical (starch based) ethanol production methods

First generation ethanol has many drawbacks regarding its competition with food, feed, and land. Considering the water and fertiliser usage, concerns regarding biodiversity and distribution of produced ethanol, there are many problems in the way of a mature and beneficial biofuel process (Dale, 2007).

Studies conducted by Argonne National Laboratory of the University of Chicago, cellulosic ethanol helps reducing greenhouse gas emissions (GHG) up to 85% compared to gasoline (Wang, 2005; Saeman, 1945). Up to now, there has been no economically beneficial bio-refinery unit for production of biofuel from lignocellulosic biomass (Baner, 2011).

Biofuels are considered environmentally friendly. Their life cycle analysis shows that they do not add to the CO$_2$ of the atmosphere and that why they are called carbon neutral. Via photosynthesis, plant materials convert CO$_2$ to chemicals that comprise the body of plant. This conversion leads to reduction and condensation of CO$_2$ which is one of the dominant greenhouse gasses. When the plant material is burned, CO$_2$ and heat are produced and the CO$_2$ is recycled back in the atmosphere (Tuominen, 2012).

Today most of the liquid biofuel is produced from corn and is based on a mature and developed technology. The technical concerns for this technology mostly deal with improvements in engineering for more efficient utilization of heat and water (Somerville, 2011).
Most of ethanol has been produced from agricultural crops like sugarcane, switchgrass and maize. However, there have been debates concerning the food (Yamashita, 2008) and water shortages in many developing countries and the competition of these crops with agricultural crops for soil and arable lands (Vanek, 2008). Corn is one of the major food supplies of the world and as the demand goes up so does the price and this is one of the biggest challenges in the way of producing biofuel from soybean, corn and other agricultural products.

In the present time, subsidies account for the major portion of the ethanol production cost. The main aim of the governmental approach towards the alternative, renewable energy sources is to make them economically competitive with fossil fuels. Factors like depletion of fossil fuels and thus its ever-increasing price, and the fact that dependence on a source of energy that is found in politically unstable countries makes biofuel production an unavoidable energy alternative. Furthermore, sustainability and environmentally friendliness of the bioethanol production from agricultural waste is the positive aspect of these sources of energy which makes them inevitable (Goldemberg, 2007)

Theoretically ethanol can be produced from any cellulosic material. A potential solution for the problems associated with corn ethanol is utilization of lignocellulosic biomass.

1.3.2 Bioconversion of lignocellulosic materials to ethanol

Due to its variability of resources, lignocellulosic biomass is available in large amounts. It is a cheap and even free source of energy, and that is why it is considered as a potential source of energy for future affordable energy resource. Typically, lignocellulosic biomass contains 50-80 % (w/w, dry basis) carbohydrates which are polymers of 5 and 6 carbon sugar units. These carbohydrates can be processed for production of cellulosic ethanol (Zheng, 2009).

1.4 Lignocellulosic biomass; the source of sustainable ethanol

Lignocelluloses are the structural material that account for much of the mass of the plant. Their main components are cellulose, hemicellulose, lignin and in some plant materials pectin (Matus, 2012).
1.4.1 Main components of lignocelluloses

1.4.1.1 Cellulose

Cellulose is the main cell wall polysaccharide with the formula \((C_6H_{10}O_5)_n\). It has a linear chain, consisting of hundreds to thousands of \(\beta\ (1\rightarrow4)\) glycosidic linked D-glucose units, while starch, glycogen or other carbohydrates have \(\alpha\ (1\rightarrow4)\)-glycosidic bonds and thus can have coiling and branching in their structure of hundreds (Updegraff, 1969). Almost 33% of all plant matter is cellulose, though it changes for each individual plant material. Cellulose is the dominant combustible component of non-food energy crops (Zhang, 2004). Utilization of non-food energy crops has two main benefits. One is that their utilization would avoid any competence with food and feed and the other reason is that non-food energy crops are more efficient than edible energy crops (Baner, 2011). Cellulose is hydrophilic and insoluble in water and most organic solvents. It can be hydrolyzed to its monomers (glucose units) using acid or enzymes.

Cellulose is composed of sheets of glucopyranose rings that form a flat surface. The repetition of these flat sheets on top of each other forms the three dimensional structure of cellulose.

![Figure 1.1 Schematic representation of structure of cellulose (Newton, 2012).](image-url)
Cellulolysis is the process of breaking of cellulose to its dimer (cellobiose or cellodextrin) or monomers (glucose). Glucose molecules have strong bonds between each other and thus it is hard to break down these $\beta(1\rightarrow4)$-glycosidic bindings.

Strong acids together with acetone or enzymes are used for breakdown and hydrolysis of cellulose. Cellulose is mostly long linear chain of anhydroglucose units. The degree of polymerization (DP) of cellulose is in the range of 7,000-15,000 (Talebnia, 2008).

$$DP = \frac{\text{Molecular weight of cellulose}}{\text{Molecular weight of one glucose unit}}$$

Cellulose is not soluble in water in its crystalline form; however its small fraction is amorphous. Cellulose is mostly subjected to enzymatic hydrolysis in its amorphous form (Kumer, 2009). Dilute acids also have no effect on dissolving cellulose however concentrated acids are able to degrade cellulose by breaking its hydrogen bonds. In alkaline conditions low molecular weight polymers of cellulose with DP of less than 200 are dissolved (Harmsen, 2010; Bochek, 2003).

**Figure 1.2** A schematic representation of cellulose and site of functionality of cellulases on cellulose (Yamada, 2005).
Enzymatic break down of cellulose is performed by cellulases. Commercial cellulases for converting cellulose to its monomers include three main types of cellulases which are: endo-cellulases and exo-cellulases and β-glucosidase (Yamada, 2005).

1.4.1.2 Hemicellulose

Cellulose is coated with a set of polysaccharides called hemicelluloses. Around 20% (w/w) of plant cell wall is composed of hemicellulose. The structure of hemicellulose is random and amorphous and it has much less strength compared to cellulose (Buschle-Diller, 1999). The polymer chains of hemicellulose have short branches and are amorphous. The amorphous structure of hemicellulose makes it partially soluble in water. They can be hydrolyzed by dilute acid or alkali solutions. Depending on their source, hemicelluloses are in two main groups: homopolymer (with a single repeating unit) and heteropolymers (with a mixture of different units). Unlike cellulose, hemicellulose consists of different monomeric sugars.

![Figure 1.3](image)

Figure 1.3  Schematic representation of structure of hemicellulose and its sub units (Harmsen, 2010).

In general, hemicellulose includes five polymers which are classified based on their monomers. These polymers are: xylan, glucuronoxylan, arabinoxylan, glucomannan,
and xyloglucan with the monomeric sugars of xylose, mannose, galactose, rhamnose, and arabinose. From these the most abundant type is xylan which accounts for (25-35% (w/w) of lignocelluloses and is a polymer of β (1-4) linked xylose which is branched by α-(1, 4)-glycosidic bonds to the backbone (Bochek, 2003). Compared to cellulose, hemicellulose contains shorter chains, with DP in the range of 500-3,000 (Talebnia, 2008). The most important sugars for biofuel production are glucose and xylose, yet other sugars are also present in considerable amounts and may be utilized in some ways (Ebringerova, 2000).

### 14.1.3 Lignin

Lignin is the most abundant organic polymer on earth after cellulose and accounts for 30% of the non-fossil organic carbon (Boerjan, 2003).

![Figure 1.4 Schematic lignin structure (Glazer, 1995).](image-url)
Lignin is a complex polymer of hydroxylated and methoxylated phenylpropanoids that is made by a free radical process (Somerville, 2011). Lignin fills the spaces in the cell wall between hemicellulose and pectin. It is covalently linked to hemicelluloses and thus cross-links different polysaccharides in the plant cell wall, conferring to its physical (mechanical) strength (Chabannes, 2001). It is difficult to define DP for lignin because it is structure is spitted during extraction and its substructures have random repeating units. Different types of lignin have been described depending on the means of isolation (Chabannes, 2007).

As lignin is cross-linked to other cell wall carbohydrates, it minimizes accessibility of enzymes to cellulose and hemicelluloses. Therefore, biodegradation of lignin is important and its extraction and separation is a prerequisite for biofuel processing and would lead to increase in digestibility of plant material (Ludwig, 1971).

1.4.1.4 Pectin

Primary cell walls of some plant materials contain pectin. Pectin is a structural polysaccharide commonly found in the form of protopectin and contains a set of complicated polysaccharides. It is mostly present throughout primary cell wall of non-woody parts of fruits. Presence of pectin in the middle lamella between plant cells, helps binding cells together. Chemical composition, structure and amount of pectin in one plant type is dependant to its maturity (age) and is different for each part of that plant. The breakdown of pectin present in the middle lamella occurs during fruit’s ripening period, by pectinase and pectin esterase enzymes. This would lead to softening of fruit as their age increases and also similar thing happens in the abscission zone of petioles of deciduous plans at the leaf fall period (May, 1990).

Pectin has a linear structure and is both polydisperse and polymolecular. Its chemical composition depends on the source and isolation method. Molecular weight and the contents of any particular sub-units differ for each molecule of pectin. Although, it is more than 200 years that pectin is discovered, despite the fine structure of different pectic elements being well-known, the interaction of the structural elements into macromolecular structures is still argumentative (Sila, 2006).

Pectin mostly is comprised of partially methylated polygalacturonic acid (Anzaldo, 1993). Pectin allocates pectinic acids containing at least 7 or 8% methyl ester groups
as methoxyl. Pectins can form gels (jellies) with sugar (or other polyhydroxy compounds) and acid in proper situation. The yellow or orange water soluble, polyene carotenoids found in plants and in bacteria cause the orange or yellow colour of mature citrus peel (Kefford, 1970).

Hydrocarbons, monols, monals, diols, diol monoepoxides, diol epoxides, polyols, aldehydes, and ketones and almost 50 other compounds have been isolated from the carotenoid fraction of citrus fruits (Agusti, 2002). Pectin’s structure is shaped by α-(1, 4)-linked D-galacturonic acid units which are bonded by (1, 2)-linked L-rhamnopyranosyl residues. Pectins include non-sugar components especially methanol, acetic acid, phenolic acids, and in some commercial samples, amide groups.

![Diagram of Pectin Structure](image)

**Figure 1.5** Schematic representation of pectin structure. Pectin consists of four different types of polysaccharides, and their structures are shown (Jesper Harholt, 2010).

Various pectic polysaccharides can be detected in the cell wall including:

- homogalacturonan (HG),
- xylogalacturonan (XGA),
• apiogalacturonan,
• rhamnogalacturonan I (RGI),
• rhamnogalacturonan II.

Many fruits such as apple, sunflower and citrus fruits contain appreciable amounts of pectin. Orange peel contains around 25% (w/w, dry basis) of pectin (Akhtar, 1971). Pectin has wide applications in food, cosmetic and pharmaceutical industries, and in jelly and jam making. It is also being used as stabilizer in medical, dairy and fruit juice industries (Koseki, 1986).

1.4.1.5 Extractives

Extractives are organic substances which have low molecular weight and are soluble in neutral solvents. Resins (combination of component like: terpenes, lignans and aromatics), fats, waxes, fatty acids, oils, terpentines, and flavonoids are categorized as extractives. They represent 4-10% (w/w) of OW. They are mostly available in resin canals and middle lamella and cell walls. Some extractives are toxic to the microorganisms. Essential oils in OW are regarded as extractives. They are the volatile oils which are extracted from odoriferous plant materials. Essential oils are mostly separated from plant material by solvent extraction, distillation or expression. Essential oils are precious oils that are used as odorants, flavours and also they have pharmaceutical usage. (Anzaldo, 1993). As for pharmaceutical usages of D-limonene, it is reported to improve immune system of the body especially for cold and flu (Toro-Areola, 2005), it also helps eliminate body from toxins (Santa Cruz Biotechnology, 2010). Its lymphatic stimulant action further helps to balance water processes, detoxification, aiding the immune system and general well-being (Toro-Areola, 2005).

The dominant essential oil in OW is D-limonene which is a colourless, liquid hydrocarbon with a strong smell of orange (Simonsen, 1947)
These oils contain aldehydes, cyanogenic substances, glucosides, linalool, linalyl acetate, dC-pinene, sesquiterpenes and tannins (Quisumbing, 1978). In the fruit juice factories, the oil is pressed out of the rind. The oil is then separated from juice and distilled to recover some certain flavor and fragrance compounds.

1.4.2 Orange Waste as ethanol production feedstock

Citrus fruit especially orange are abundant in Brazil, United States, Mexico, and Spain. Citrus is dominantly utilized for its pulp and juice and its remaining parts (which include 45% of its dry weight) are considered as citrus wastes which are; the rind, pressed pulp, covering each individual segment of the edible portion, and seeds (Anzaldo, 1993). The production amount was around 13.5 million tons of orange waste (OW) worldwide in 2008 (Ylitervo, 2008). The OW market is limited due to its high water content (almost 75%) which causes drying problems on an industrial scale. Moreover, OW has many disposal problems with it. It is mostly dumped in the soil, which causes odour and soil problems and also there is the danger of production of biogas in the soil (Dhillon, 2004). In addition, it’s not considered healthy and rich when used in cattle feed. (Talebnia, 2008). Many research studies have worked on utilization of OW for production of high-value products, which are; soluble sugars (Eaks, 1980; Grohmann, 1995), pectin (Ma, 1993), carotenoids and flavonoids (Manthey, 1996), oils and perfumes (Braddock, 1986). Production of acids like succinic acid (Li, 2010) has also been investigated in the recent years (Rivas, 2008).

The disadvantage of utilization of OW is that considering its high water content, its nutrient content (wet basis) is rather low and thus is not considered as a volatile feedstock. However, it is rich in soluble and insoluble carbohydrates (Kesterson,
1976) which makes it an interesting feedstock for biological conversion by microorganisms for its processing to high-value products like ethanol, biogas, citric acid, chemicals, various enzymes, volatile flavouring compounds, fatty acids and microbial biomass (Dhillon, 2004) are available. Biological conversion of OW to ethanol occurs by using microorganisms to ferment sugars present in OW hydrolysate, which has its own problems due to the presence of peel oil that decreases or stops microorganism functionality. Almost 95% of the OW oil is the D-limonene, which is very toxic for fermenting micro-organisms (Pourbafrani, 2007). To face this obstacle, D-limonene has to be removed prior to the fermentation. However, D-limonene is a high-value by-product and can enhance the economy of ethanol production from OW (Anzaldo, 1993).

**1.4.2.1 Orange Waste composition**

Composition and nature of citrus fruits have long been investigated and still there are unsolved problems in understanding its biochemistry. OW is reach in soluble and insoluble carbohydrates. For the precise composition analysis of fruit tissue, the soluble and insoluble components of the OW should be separated (Wilkins, 2005).

The orange waste is composed of two distinctive parts; flavedo or epicarp and the albedo or mesocarp, which are easily separated from the edible part of the fruit which is called pulp. The flavedo, which is the outer part of the peel, is composed of carotenoid pigments, vitamins, and essential oils. The albedo is the spongy inner part of the peel and is composed of cellulose, soluble carbohydrates, pectic substances, flavonoids, amino acids, and vitamins. Pectic substance mostly accounts for pectin and protopectin. Pulp of fruit is composed of parts which have readily impermeable walls, and each part is made up of hundreds of units with impermeable walls. Vesicles (juice sacs) contain the edible juice of the citrus fruit and are composed of cellulose, hemicelluloses, protopectin, sugars, flavonoids, aminoacids, vitamin C, mineral salts and other nutrients. The soluble constitute of the juice are composed of soluble carbohydrates (glucose, fructose, and sucrose), organic acids (chiefly citric acid) vitamin C, vitamin B complex, mineral salts, a small concentration of pectic materials and some other nutrients (Nisperos-Carriedo, 1990).
OW is rich in pectin however its lignin content is so low. Pectin and hemicellulose are a source of galacturunic acid, arabinose, galactose and smaller amounts of xylose, rhamnose and glucose (Grohmann, 1992; Grohmann, 1994).

![Figure 1.7 The main components of orange (Pak, 2004).](image)

1.5 Overview of the ethanol process

Process of ethanol production from lignocellulosic material consists of 4 major steps: 1. Pretreatment, 2. Enzymatic hydrolysis to convert carbohydrates to monomeric sugars, 3. Fermentation of sugars to alcohol, and 4. Distillation and purification of the product.

1.5.1 Pretreatment and its methods

Pretreatment is a set of processes conducted to reduce intensity of biomass structure and make it more accessible to the enzymes in the hydrolysis step and to reduce the degree of polymerization (Harmsen, 2010). Crystallinity of biomass, presence of lignin, hemicelluloses and pectin, and infrastructure of biomass makes it inaccessible for cellulases, are the factors that make pretreatment step compulsory. Pretreatment steps include separation of other components, reduction of particle size and isolation of desired component. It changes structure and size of biomass in macroscopic and microscopic scale. Pretreatment leads to decrease in crystallinity, which improves mass transfer in the biomass and thus rapid and easy hydrolysis of
cellulose and hemicelluloses to monomeric sugars and would in turn lead to higher ethanol yields (Mosler, 2005; Taherzadeh, 2007).

Some considerations in choosing suitable pretreatment methods for a feedstock should be taken into account, which is listed below:

1. to maximize the enzymatic convertibility,
2. to minimize the biomass loss,
3. to provide the possibility of producing and extracting high-value by-products,
4. minimize and even omit utilization of toxic chemicals that are decrease hydrolysis and fermentability yields,
5. to minimize energy usage,
6. to consider greenness and sustainability issues (Kristensen, 2009),
7. To have the potential to be scalable to industrial sizes (Jørgensen, 2007).

Pretreatment of biomass prior to hydrolysis enhances enzyme accessibility to the main components of lignocellulosic matrix (Taherzadeh, 2008).

Pretreatment step is counted as one of the challenges in the way of cellulosic ethanol production because of being expensive and unsustainable due to utilization of different chemicals. Although pretreatment step is the most expensive step in the process of cellulosic ethanol production, it is flexible and has the potential to improve the efficiency and reduce the cost (Moister, 2005).

Different pretreatment steps are used to enhance hydrolysis and later fermentability of biomass. Pretreatment methods are either physical or chemical or both (McMillan, 1994). Biological pretreatments are also being used recently. In the following section, brief descriptions of pretreatment methods are presented.

1.5.1.1 Physical (mechanical) pretreatment

To enhance digestibility of biomass some mechanical pretreatments to reduce particle size are necessary. Chipping, grinding, shredding and milling are among the most commonly used mechanical biomass pretreatment methods (Palmowski, 1999).
1.5.1.1.2 Milling

With the aim of increasing surface area and accessibility, reduction of biomass particle size is conducted by milling. It also makes the biomass easier to handle and store for future usage. To decide on desired particle size many parameters such as biomass source and proceeding steps should be taken into account. Reducing particle size is usually among the first pretreatment steps (Sidiras, 1989).

1.5.1.2 Ultrasonic

This method has not been practiced in industrial scale yet. It has been successfully practiced for cellulosis materials but not lignocellulosic biomass. Irradiation of cellulose by ultra sonic energy has been proved to increase enzymatic hydrolysis yield two-folds (Imai, 2004). The reason for this increase is still under investigation but it is probably due to the fact that high energy can break the hydrogen bonds of cellulose and thus decrease crystallinity of biomass (Bochek, 2003).

1.5.1.2 Chemical pretreatment

These sets of pretreatment account for chemically disruption of biomass structure for increase in surface area and accessibility to cellulosic portion of biomass:

1.5.1.2.1 Hot Alcohol

Around 57% (dry weight) of Valencia orange peel and 55% of that of Navel orange albedo are soluble in 80% ethyl alcohol (Martinez, 2010). These values are higher for grapefruit and lower for lemon. Depending on orange type 31-40% (dry basis) of the alcohol-soluble materials is sugars (as glucose). Alcohol soluble components of OW, mostly account for mono and disaccharides. These mono and disaccharides alongside starch and dextrins comprise for readily available source of energy of the living cells (Mahmood, 1998). The alcohol-insoluble material is respectively proteins, pectic substances and structural components of cell (Prabasari, 2010).

Common water based extraction of pectin includes utilization of acidic water in pH of 2 and temperature of around 70 °C. The process takes 2 to 4 hours and solubilized pectic materials are mostly precipitated by ethanol (Srivastava, 2011). Hot water
extraction of pectic materials is the simplest and oldest but not necessarily the most efficient pectin extraction method.

### 1.5.1.2.2 Acid pretreatment

Acidic pretreatment is a pretreatment method in low pH to make the cellulosic part accessible for enzymatic hydrolysis reaction (Taherzadeh, 2007). Both dilute and concentrated acids are utilized in acid pretreatment step. Treatment of lignocelluloses with acids in high temperatures would lead to enhanced enzymatic hydrolysis. The most commonly used acid for this purpose is sulphuric acid while hydrochloric acid (HCl) (Wang, 2010), phosphoric acid (H₃PO₄) (Zhang, 2007), and nitric acid (HNO₃) (Himmel, 1997) have also been used in certain cases. Acid pretreatment process is a mature a process and it is proved to discard hemicellulose effectively (Zhang, 2007).

Acid treatment could either be under dilute acid at high temperature or under concentrated acid at low temperature. The high concentration acid under low temperature has environmental problems regarding utilization of strong acids and also makes the process corrosive and not safe. Acid recovery process is a solution to reduce the acid cost however, it is also energy consuming. Dilute acid has the advantage of producing a lower ash content of the pectin, with lower acid cost, less corrosive characteristics. However, high temperatures would lead to higher operation costs (Yeoh, 2008). Significant improvements in cellulose hydrolysis can be reached after dilute acid pretreatment (Kumar, 2009). Dilute acid hydrolysis can lead to 100% hemicelluloses removal but it has no effect in removing lignin. However, lignin structure can be disrupted and thus the enzymatic hydrolysis of cellulose is enhanced by this method (Yang, 2004). The drawback of acid pretreatment is production of inhibitors such as furfural, carboxylic acid and phenolic compounds (Taherzadeh, 1999). Thus, such chemicals lead to a decrease in fermentation yield and thus proper pretreatment method should be chosen to reduce undesirable effects on final yields.

Acidic pretreatment for removal of pectin is commonly used. Sulfuric acid, hydrochloric acid and phosphoric acid are used in this process (Pinheiro, 2008). Organic acids or their salts (e.g. oxalic acid, ammonium oxalate, tartaric acid, and polyphosphates) can also be used in this process. Commercial pectin is mostly
extracted in acidic conditions at pH 2 and the temperature and extraction time is
dependent on many factors such as pectin source, desired pectin type, and utilized
acids (Aravantinos-Zafiris, 1992). The precipitated pectin is in the gelatin form which
can be later dried, washed, and ground for further use (Srivastava, 2011).

1.5.1.2.3 Alkaline pretreatment

Alkali pretreatment deals with utilization of NaOH, Ca (OH)2 (lime) or ammonia for
separation of lignin, part of hemicelluloses, and part of pectin (Taherzadeh, 2008).
Saccharification of biomass and accessibility of enzyme to cellulose increases
remarkably by Alkali pretreatment (Kassim, 1986). Alkali treatment is more
effective in breaking the ester bonds between lignin, hemicelluloses, and cellulose,
as compared to acid and oxidating solvents (Gaspar, 2007). As described by Yeoh et
al. (2008), compared to acids, alkaline solvents are more effective in breaking the
bonds between pectic substances and plant cell wall. Alkaline pretreatment is
performed in ambient temperature. It has important effect on removal of lignin and
thus improving reactivity of the remaining components. (Sun, 2002).
Calcium hydroxide (Lime) or sodium hydroxide is mostly used for alkaline
pretreatment. They form salts which may be integrated in the biomass or may need
to be removed (González, 1986). Reaction conditions are mild but it requires longer
reaction time. The mild conditions lead to better solubilisation of lignin especially in
plant materials with low lignin content and also mild reaction conditions leads to
less degradation product production (Chang, 2000).
Ammonia solution at room temperature, leads to removal of lignin and part of
hemicellulose, at the same time it decrystallises cellulose. Ammonia pretreatment
techniques include three dominant methods; the ammonia fibre explosion-method
(AFEX), ammonia recycles percolation (ARP) and soaking in aqueous ammonia
(SAA). The ammonia cost and especially its recovery cost play the major role in the
ammonia pretreatment cost (Holtzapple, 1991).

1.5.1.2.4 Organosolvents

Organosolvents are mostly used for removal of lignin and in some cases hydrolysis
of hemicellulose. Mostly ethanol, methanol and ethylene glycon are used in this
process. The process temperature ranges depending to the biomass. (Sun, 2002).
Removal of lignin prior to enzymatic hydrolysis, leads to less enzyme usage and thus cost reduction of the whole process. Furthermore, considering the decreased cellulose-lignin absorption, the enzymatic hydrolysis yield is increased (Harmsen, 2010).

1.5.1.2.5 Oxidative reagents

Oxidative reagent is the common name for solvents that use oxygen as oxidizing agent for components dissolved in water. In this method a dilution of 6 g/L is prepared and commonly Na₂CO₃ is added to the solution to avoid formation of by-products. The solution is kept at temperature of 195°C and pressure of 12 bars for a time period of 10-20 minutes. In this method lignin is removed and hemicellulose is dissolved in the solution (Carlos, 2005; Brodeur, 2011).

1.5.1.2.6 Room temperature Ionic Liquids (RTIL)

RTILs are salts which are liquid in room temperature. They are composed of an inorganic anion and an organic cation. Due to the different molecular structure, the bindings are week and thus these solvents are liquid in room temperature (Imai, 2004). RTILs have the particular characteristic that they can selectively dissolve lignin or cellulose. Mild process conditions, no utilization of chemicals, and being recyclable are among the positive properties of these solvents that make them interesting for pretreatment. RTIL are still being investigated and their utilization process is not mature yet (Harmsen, 2010).

1.5.1.3 Physico-chemical pretreatments

Physico chemical pretreatments are combination of physical and chemical pretreatments.

1.5.1.3.1 Liquid hot water (LHW)

In this pretreatment method, water is used to enhance disintegration and separation of lignocellulosic components. Depending on the pretreatment temperature, commonly in the range of 160 °C - 240 °C, processing time changes from a couple of minutes to hours. In this process hemicellulose and some portion of pectin and lignin are dissolved in water and thus separated from other components. Cell
penetration of lignocellulosic biomass leads to complete disintegration of lignocellulosic structure. Due to no utilization of chemicals, this pretreatment is more cost-effective and green compared to chemical pretreatment methods.

1.5.1.3.2 Steam explosion

Steam explosion is one of the most commonly used pretreatment methods. Due to this method’s no chemical usage and less energy consumption compared to other pretreatment methods it’s among economic and green pretreatment methods either with or without catalyst. Sudden reduction in pressure in temperatures as high as 260 °C, leads to explosive decompression and thus hemicellulose and lignin disruption. Residence time, particle size, moisture content and temperature are the factors that affect the steam-explosion results (Sun, 2002). The drawbacks of this method are formation of degradation products that have inhibitory roles in the next steps (Garcia-Aparicio, 2006).

1.5.1.3.3 Ammonia fiber explosion (AFEX)

Liquid ammonia pretreatment under high pressure is practiced in this method (Teymouri, 2005). The process is very similar to that of the steam explosion. Lignocellulosic biomass undergoes a process of exposure to ammonia in temperature range of 60 °C to 100 °C and high pressure for some seconds and then the system is depressurized rapidly (Broduer, 2011). It reduces lignin content, disrupts some of hemicellulose and reduces cellulose crystallinity (Holtzapple, 1991). AFEX is more effected in biomass with little lignin content and it doesn’t necessarily decrease hemicellulose content (Wyman, 1996).

1.5.1.3.4 Supercritical (CO₂) explosion

Supercritical CO₂ explosion is developed as a more sustainable method. This process requires lower temperature than steam explosion which leads to fewer expenses compared to ammonia explosion. Supercritical fluids are a group of fluids that are neither gaseous nor liquid. They are compressed at temperatures above their critical point to density of liquids. CO₂ forms carbonic acid and thus when dissolved in water, the acid increases the hydrolysis rate. Carbon dioxide molecules are comparable in size to water and ammonia and are capable to penetrate small pores
which are not accessible to water and ammonia molecules. Carbon dioxide hydrolyzes hemicellulose and cellulose. Additionally, the low temperature of this process avoids decomposition of monosaccharides by the acid. However, the yields are rather low compared to those of steam or ammonia explosion pretreatments (Kumar, 2009).

### 1.5.1.4 Biological pretreatment

In biological pretreatment microorganism are used for degradation of cellulose and hemicellulose. In these processes no chemical is used and the process conditions are mild and these conditions make biological pretreatment interesting for further investigations (Sun, 2002).

### 1.5.1.5 Important factors in choosing pretreatment methods

There is no best pretreatment method for all biomass resources. Each pretreatment has its own effect on cellulose, hemicellulose, pectin and lignin content of the lignocellulosic biomass. In general, considering the process in industrial scale, dilute acid pretreatment of biomass is preferred in many cases.

### 1.5.2 Hydrolysis

Carbohydrates need to undergo processes to be converted to monomeric sugars to be able to be fermented by microorganism. This process is called hydrolysis. The two main hydrolysis methods for lignocelluloses are chemical hydrolysis using by acids and enzymatic hydrolysis using cellulases and hemicellulases in milder conditions (Taherzadeh, 2008). Complete hydrolysis of cellulose produces glucose while hydrolysis of hemicelluloses produces several different pentoses and hexoses.

The chemical hydrolysis of biomass is more or less a mature process with high yield and fast reaction, enzymatic hydrolysis on the other hand is rather slow reaction and inhibitions may reduce its yield (Zhang, 2009). However, environmentally friendliness of enzymes and their potential for improvement of the process makes them more interesting for future investigation.
1.5.2.1 Chemical hydrolysis

Chemical hydrolysis of lignocelluloses deals with exposure of lignocellulosic material to acid in certain temperature and for specific time period, and the results would be monomers from cellulose and hemicelluloses. Sulfuric acid is the most common acid used for this purpose (Harmsen, 2010). Two categories of acids have been used for this purpose which is shown in Table 1, together with their advantages and disadvantages (Taherzadeh, 2007).

1.5.2.2 Enzymatic Hydrolysis

Different processes have been suggested for production of ethanol from cellulosic biomass, from which enzymatic hydrolysis has been proved to have the potential to provide the opportunity to improve the technology in a way that the whole processes becomes sustainable and competitive with other liquid biofuels (Wyman, 1996). The high cost of enzymes is one of the drawbacks of enzymatic hydrolysis but new researches are being conducted for new enzymes which would have higher hydrolysis yield and less enzyme loading and more biomass loadings.

Polygalacturonase, pectinesterase and pectin transeliminase are the main enzymes present in commercial pectinase. Polygalacturonase splits the peptic chain and pectinesterase hydrolysates the methyl ester groups of the pectin molecule (May, 1990).

1.5.3 Drawbacks of utilization of chemicals in pretreatment and hydrolysis

Utilization of chemicals in high concentrations in pretreatment and hydrolysis steps produces degradation products that have negative effect on fermentation yields. Degradation products are considered toxic for fermenting microorganism. Despite being commonly used, dilute sulphuric acid hydrolysis has important barrier. Its environmentally corrosive characteristics and expensive source are among these limitations. (US Department of Energy, 1993).

As mentioned before, production of degradation products after dilutes acid pretreatment decreases fermentation and enzymatic yields (Hsu, 1996). Some aspects of inhibitions of fermentation are not yet clearly understood. Furthermore, loss of biomass and thus sugars during pretreatment and hydrolysis has
negative effect on economic of the process. Acid neutralization is suggested to
decrease these negative effects the most commonly used and least expensive of
which is, lime. However, the gypsums formed in this process decrease its solubility
and cause problem especially in downstream processes (Hinman, 1992).
Additionally, due to these inhibitions, more enzyme loadings have to be used in the
process (Wooley, 1999). Additional enzyme cost and extra cost for disposal of
chemicals (acid and lime), are the barriers in the way of economically viable acid
prereated biomass (Yang, 2006).

Pretreatment of lignocelulosic biomass may result in production of degradation
products which have inhibitory effect in fermentation process. Phenoloc compounds,
furfural, HMF (5-hydroxymethylfurfural), acetic acid and extractives are the major
inhibitors (Moister, 2005). Also presence of extractives like acidic resins has
inhibitory effect for production of ethanol (Harmsen, 2010). Keeping the process
temperature low and residence time short, separation of extractives before
fermentation and avoiding lignin degradation during pretreatment steps are the
methods that help reducing inhibitors.

1.5.4 Fermentation

Ethanol fermentation is a biological process in which cellular energy is produced
from sugars. Ethanol and carbon dioxide are metabolic waste products of this
process. Uptake of sugars for ethanol production is an anaerobic process (James,
2007).

The chemical equations below summarize the fermentation of glucose into ethanol.
Alcoholic fermentation converts one mole of glucose into two moles of ethanol and
two moles of carbon dioxide, producing one moles of ATP in the process (Phil,
2010).

The overall chemical formula for alcoholic fermentation is:

\[ C_6H_{12}O_6 \rightarrow 2 C_2H_5OH + 2 CO_2 \]  \hspace{1cm} (1.1)

The alcoholic fermentation is conducted by yeast of the genus *Saccharomyces*.

*Saccharomyces* converts the glucose, fructose and sucrose into ethanol via the
process of fermentation.
1.5.4.1 Microorganisms

Microorganisms play an important role in fermentation of lignocelluloses to ethanol. Therefore, selecting the right strain is very important in increasing fermentation yield. Utilization of lignocellulosic material for production of ethanol in fermentation process is very challenging due to tight structure of these plant materials. Unlike starchy biomass, hydrolysis of lignocellulosic biomass produces a mixture of pentoses and hexoses alongside some other components which may have inhibitory effect and reduce fermentation yield. Many naturally occurring microorganisms cannot ferment pentoses like xylose and arabinose, while they can easily use hexoses like glucose, galactose, mannose and fructose. Some important factors for selecting the right type of microorganism for ethanol production are its ability to co-ferment pentoses and hexoses, tolerance for high ethanol concentration, high ethanol production yields, tolerance to presence of inhibitory compounds and initial presence of some oxygen and minimum need to additional nutrients (Wyman, 1996). Co-fermentation of hexoses and pentoses is the area of investigation that is still being worked on. Different strains are being investigated to choose the most suitable one to ferment both pentoses and hexoses preferably at the same rate. Many different microorganisms including yeasts, bacteria and fungi have been worked on considering their different advantages and disadvantages (James, 2007).

The two common species involved in fermentation are S. cerevisiae and S. bayanus. These two species are closely related, and the subject of a continuing debate among taxonomists as to whether they constitute separate species or races of the same species. However, the most frequently used microbe has been yeast and among the yeasts, S. cerevisiae which can tolerate ethanol concentration as high as 20% in fermentation medium. Currently, industrial production of ethanol is mainly carried out by using the yeast especially Saccharomyces cerevisiae.

1.5.4.1.1 Saccharomyces cerevisiae

Some species of bacteria such as Zymomonas mobilis and the genetically engineered Escherichia coli can produce ethanol at higher yields, but their tolerance to ethanol
and other compounds present in the hydrolysates is much less when compared to *S. cerevisiae* (Lawford, 1998; Olsson, 1996). *S. cerevisiae* is the most commonly used microorganism in fermentation process for the production of ethanol from glucose. A significant amount of information is known about *Saccharomyces* due to the utility of this organism as an experimental system. It is used due to:

1. outstanding characteristics of growing at high sugar concentrations,
2. producing ethanol with high yields,
3. tolerance for ethanol concentrations of as high as 20% in fermentation medium,
4. being one of the cheapest strain available for ethanol production (Periyasamy, 2009).

One disadvantage is that it can only utilize glucose and other hexose sugars whereas it lacks the ability to take up pentose sugars as substrate (Osunkoya, 2009). To make use of a greater proportion of lignocellulosics genetically engineered yeasts are being investigated, which can ferment both pentose sans hexoses with satisfactory yields (Alterthum, 1989) *S. cerevisiae* is able to consume D-glucose, D-fructose, D-mannose, and D-galactose fully and D-glucuronic acid partially (Yoon, 2003).

### 1.5.4.1.2 Effect of nutrition in fermentation

One of the most critical factors of management of the yeast fermentations is to make sure that the yeast has all of the essential nutrients to keep fermentation rates and to maintain acceptable ethanol yields and tolerance. Maximum cell biomass that can be produced is directly related to the maximum rates of fermentation once the usable sugar has been consumed. The final cell concentration obtained is dependent on the nutrients available in the medium. Standard categories of nutrients necessary for fermentation are: macronutrients and micronutrients. Macronutrients which account for the building blocks required for new cell material, while micronutrients account for the catalysts needed to keep the progress of biochemical reactions. Macronutrients are the compounds that provide the needs for cell division and energy generation. They are needed in high or stoichiometric amounts. On the contrary, the micronutrient vitamin and minerals are
required in much lower amounts and are catalysts involved in many enzymatic reactions.

Macronutrients that are necessary for the yeast growth are mainly:

1. **Carbon/Energy Sources**: glucose, fructose, sucrose,
2. **Nitrogen Sources**: amino acids, ammonia, nucleotide bases, peptides,
3. **Phosphate Sources**: inorganic phosphate, organic phosphate compounds,
4. **Sulfur Sources**: inorganic sulfate, organic sulfur compounds.

The macronutrients are sources of carbon, nitrogen, phosphate and sulfate. These four elements are required for production of a new cell as well as for maintenance of a cell in stationary phase. In OW hydrolysate, the carbon sources available for *Saccharomyces* are plentiful: glucose, fructose and sucrose. It contains other sugars and carbon compounds used by other organisms as carbon and energy sources. However, in cellulosic fraction of OW, glucose sugar which is produced from hydrolysis of cellulose is the dominant sugar present. *Saccharomyces* is able to use the following compounds as energy sources. Only the sugars are fermented, the other compounds can only be used under conditions conducive to respiration (Ylitervo, 2008).

Macronutrients that play the role of energy sources for the yeast in fermentation process are:

1. **Monosaccharides**: glucose, fructose, galactose, mannose,
2. **Disaccharides**: sucrose, maltose,
3. **Trisaccharides**,
4. **Pentoses**,
5. **Oxidative Substrates**: pyruvate, acetate, lactate, glycerol, ethanol.

The major nitrogen sources for yeast are ammonia, amino acids, nucleotide bases and small peptides. All amino acids can not be consumed by *Saccharomyces*, particularly under anaerobic conditions.

*Saccharomyces* is able to consume inorganic or organic sources of phosphate, but is not permeable to compounds other than inorganic ones. The yeast secretes phosphatases that disintegrate the organic structure of phosphate outside the cell, which allows the inorganic phosphate that is released to be consumed by the cell. Sulfur sources for the yeast are mainly sulfate or the sulfur-containing amino acids.

The micronutrient composition of juice is just as significant for yeast growth as that of the macronutrients. Micronutrient scarcity may, avert synthesis of a single vital
compound the absence of which can cause to halt the cell growth and perhaps fermentation. Yeast growth and fermentation is enhanced in the existence of these compounds (Talebnia, 2008). Yeast requires nutrients not only during its active growth, but also during the non-proliferative phase of fermentation. Which is necessary to prevent loss of capability as ethanol accumulates in the medium. The nutrients required for these phases are different. Depending on the phase in which they are found, nutritional requirements of different phases of fermentation changes. Growth phase requires that the compounds necessary for net synthesis of new cell material be present in adequate amounts to improve division. These are the building blocks and vital micronutrient catalysts. Survival factors accounts for the compounds that are essential for maintenance of fermentation rates and viability throughout stationary phase.

Most of the fermentation is directed by stationary phase cells. The bulk of the alcoholic fermentation is conducted by the stationary phase cells. Stationary phase has two different definitions. It is characterized by the failure to spot an increase in cell number which may occur because the rate of cell death is equal to the rate of cell division. In this situation, the total number of cells present will persist to be the same. The second type of stationary phase is quiet non-proliferative, meaning there is no cell division nor is there cell death. Survival factors are important for keeping the cell viability.

Cell feasibility is maintained by survival factors if the nutrients needed to mend cellular damage are provided. Increase in ethanol tolerance is also enhanced by the survival factors; they also help maintain fermentation rates and energy generation.

The survival factors for the yeast are oxygen, fatty acids, sterols, and nutritional factors which are also required for ethanol tolerance (Periyasamy, 2009).

1.6 Aim of the study

Isolation of cellulosic OW fragment provides the opportunity of making use of it more efficiently for ethanol production together with production of other by-products like pectin.

Utilization of UOW for ethanol production has been investigated for many years. Optimization of enzymatic hydrolysis using cellulases and pectinases and fermentation of sugars using different microorganisms has been the focus of these
researches. However, ethanol production from the cellulosic fragment of OW has not been reported in the literature. Therefore, the aim of this project is the isolation of cellulosic OW fragment efficiently and utilization of the cellulose enriched biomass for ethanol production. Separation of different value added by-products in OW, like pectin and other soluble components (e.g., sugars) is the crucial aspect of this study that can enhance the economics of the OW biorefinery.

This study investigates three different approaches for production of ethanol from OW. Separation of other components of OW by corresponding pretreatment steps is performed to enrich cellulosic OW fragment. The prevailing focus of the study is to investigate enzymatic hydrolysis conditions which lead to maximum glucose production and later to maximum ethanol production in the fermentation step.
CHAPTER 2

LITERATURE SURVEY

2.1 Orange peel composition

OW is composed of soluble and insoluble carbohydrates. The soluble components are generally sugars like glucose, fructose and sucrose. The insoluble components are generally cell wall hydrocarbons like pectin, cellulose and hemicellulose that contain mostly galacturunic acid, glucose and xylose as their monomers (Grohmann, 1994; Wilkins, 2007). The sugar amounts reported by different researches varies dramatically which is mainly due to relatively rapid changes occurring maturation of fruit (Wilkins, 2007; Swift, 1957) and also due to activities of enzymes (invertase) inside the fruit (Kate, 1978; Grohmann, 1994). The soluble sugar content of OW varies in the range of 29 - 44% (w/w, dry basis) (Harvey, 1936). Both soluble and insoluble carbohydrates of OW have the potential to be further processed for product production (Kesterson, 1976). Almost 50% (w/w, dry basis) of the OW are alcohol soluble carbohydrates (Sinclair, 1953).

Pectin is rich in galacturunic acid and hemicellulose mainly contains arabinose, galactose and small amounts of xylose, rhamnose, and in some cases glucose (Grohmann, 1992; Ma, 1993). Cellulosic fragment of OW mostly contains glucose however, other sugars like xylose and arabinose, galactose, and mannose (in trace amounts) are also present. Lignin content of OW is so low and thus decreases the need for harsh pretreatment to disintegrate lignocellulosic matrix (Grohmann, 1994; Grohmann, 1995).

Tables 2.1 and 2.2 represent the carbohydrate content and sugar analysis of OW (% w/w) on dry basis, respectively.
Table 2.1 Carbohydrate content (%, w/w) based on the dry matter of OW as reported by different authors in the literature.

<table>
<thead>
<tr>
<th>Ash</th>
<th>Protein</th>
<th>Pectin</th>
<th>Klason Lignin</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6</td>
<td>9</td>
<td>23</td>
<td>7.5</td>
<td>37</td>
<td>11</td>
<td>(Boluda, 2012)</td>
</tr>
<tr>
<td>2.9</td>
<td>6.9</td>
<td>-</td>
<td>-</td>
<td>14.2</td>
<td>5.7</td>
<td>(Oberoi, 2010)</td>
</tr>
<tr>
<td>3.7</td>
<td>6</td>
<td>25</td>
<td>2.2</td>
<td>22</td>
<td>11</td>
<td>(Pourbafrani, 2010)</td>
</tr>
<tr>
<td>3.5</td>
<td>6.5</td>
<td>42</td>
<td>0.8</td>
<td>9</td>
<td>10.5</td>
<td>(Rivas, 2008)</td>
</tr>
<tr>
<td>1.5</td>
<td>-</td>
<td>-</td>
<td>2.1</td>
<td>13</td>
<td>6</td>
<td>(Ververis, 2007)</td>
</tr>
<tr>
<td>3.7</td>
<td>-</td>
<td>21</td>
<td>-</td>
<td>11</td>
<td>-</td>
<td>(Mahmood, 1998)</td>
</tr>
<tr>
<td>3.4</td>
<td>6</td>
<td>-</td>
<td>2.0</td>
<td>10</td>
<td>-</td>
<td>(Grohmann, 1995)</td>
</tr>
</tbody>
</table>

The carbohydrate content of OW (%, w/w) on dry basis varies significantly in different reports. Different composition analysis methods together with differences in feedstock source are among the reasons for this variability (Grohmann, 1995).

Sugar analysis of OW, shows presence of many different pentoses and hexoses in OW (Harvey, 1936).
### Table 2.2 Sugar content of OW as reported by different authors in the literature (%, w/w).

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Fructose</th>
<th>Galactose</th>
<th>Sucrose</th>
<th>Arabinose</th>
<th>Galacturonic acid</th>
<th>xylose</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>14</td>
<td>5</td>
<td>-</td>
<td>7.1</td>
<td>18.7</td>
<td>7.2</td>
<td>(Grohmann, 1995)</td>
</tr>
<tr>
<td>23</td>
<td>14</td>
<td>4</td>
<td>-</td>
<td>7</td>
<td>19</td>
<td>0.4</td>
<td>(Pourbafrani, 2010)</td>
</tr>
<tr>
<td>-</td>
<td>13</td>
<td>3.4</td>
<td>0.5</td>
<td>3.23</td>
<td>13.5</td>
<td>4</td>
<td>(Wilkins, 2007)</td>
</tr>
</tbody>
</table>

#### 2.2 Limonene separation

In addition to carbohydrates, OW contains extractives the most important of which is D-limonene. D-limonene is extremely toxic for biological activity of microorganism and should be removed prior to fermentation (Faravash, 2007; Pourbafrani, 2007). Commonly, D-limonene content of OW hydrolysate is around 1.4% (v/v) (Grohmann, 1994). However, D-limonene contents of more than 0.12% (v/v) are proved to have negative effect on fermentation yields because of its inhibitory effect on yeast (Wilkins, 2007b; Uribe, 1990). Control experiments were conducted by adding limonene to pure glucose solutions and it was used as carbon source of fermentation using *Saccharomyces cerevisiae* or *kluyveromyces marxianus* and the result showed a decrease in ethanol yields compared to the medium without D-limonene (Grohmann, 1994; Wilkins, 2007). Wilkins et al. (2007) showed that 0.28% of D-limonene could result in satisfactory ethanol yields when applying simultaneous saccharification and fermentation (SSF). Commonly, removal of D-limonene from citrus waste is conducted by steam stripping (Grohmann, 1994). D-limonene is a precious by-product of citrus biorefinery and Zhou et al. (2007)
estimated that utilization of D-limonene as a by-product leads to cost reductions in the ethanol production from OW up to $0.50/gal of ethanol.

2.3 Pretreatment of OW for isolation of cellulosic fragment

Pectin extraction has been investigated for many years (El-Nawawi, 1987; Virk, 2004; Rehmann, 2004; Pagan, 1999). El-Nawawi et al. (1987) investigated the factors affecting pectin extraction yields. Pagan et al (1999) tried to increase pectin extraction yields by studying rheological properties of peach pomace pectin.

2.3.1 Utilization of hot water and microwave for separation of water soluble components from OW

Boiling water is one of the most common pectin extraction methods together with dilute acid extraction (Grohmann, 1994; Yeoh, 2008). However, boiling water has long extraction period (around 2 hours) and it leads to degradation of pectin (El-Nawawi, 1987; Yeoh, 2000). Microwave heating extraction was used by Fishman et al. (2000) to avoid these problems. The extraction time by this method was 15 minutes and the resulting pectin yield was satisfactory.

Min et al. (2011) performed a combination of physical and enzymatic approaches to extract pectin from apple pomace. In an attempt to practice environmentally friendly method, they conducted water based extraction of pectin; however it resulted in lower pectin yields compared to other chemical methods. Therefore, they proceeded by enzymatic extraction of pectin from water extracted solution and the resulting pectin was comparative on the point of structural and chemical properties with that of chemically extracted ones.

2.3.2 Utilization of hot alcohol for separation of water soluble components from OW

Chau et al. (2003) separated alcohol insoluble solids to investigate on fibre content of OW. They boiled OW samples in boiling alcohol 85% (v/v) with solid to alcohol ratio of 1:30 (v/v). The alcohol insoluble fraction of OW was measured to be 51.5% (w/w).

Mahmood et al. (1998) analysed the alcohol insoluble component of orange peel by stirring the biomass in alcohol for several hours and then blending it at high speed
for 10 minutes. It was then filtered through a glass funnels under vacuum to remove alcohol soluble components. The residue was blended in 400 ml at 80% (v/v) ethanol for 10 minutes, filtered and dried. The resulting alcohol insoluble solid of OW was 63% (w/w, dry basis). Around 98.6% (w/w) of total soluble sugars were removed from OW in this method. Another extraction technique under stronger acidic conditions was used to obtain the remaining pectin, which is primarily calcium sensitive.

Khule et al. (2012) extracted pectin from combined citrus fruit at pH 2; water: ethanol ratio of 1:1 and extraction periods of 120 min, at this condition highest yield obtained was 18.21% (w/w). However, there has been relatively little experimental data about the extraction of pectin with neutral and basic solutions, and the most suitable concentration of alcohol for the extraction of pectin has also not been fully investigated.

### 2.3.3 Acid pretreatment

Strong acids are used for extraction of pectin from pectin-rich-plant-materials especially apple pomace and citrus fruits. Commonly strong acids like oxalic acid (Koubal, 2008), hydrochloric acid (Choi, 1996; Hwang, 1998; Kliemann, 2009; El-Nawawi, 1987), nitric acid (Constenla, 2002; Kliemann, 2009; Pagan, 1999), and sulphuric acids (Garna, 2007) are used for this purpose. These acids are regarded as conventional acid extraction method (Yapo, 2009).

Dilute acid hydrolysis prior to enzymatic hydrolysis enhances release of sugars from OW (Grohmann, 1995; Vaccarino, 1989; Wilkins, 2007). Factors such as temperature, acid concentration, substrate loading and hydrolysis time affect the rate and degree of hydrolysis of OW (Grohman, 1995).

Talebnia et al. (2008), for example, conducted experiments using dilute sulphuric acid and the optimized parameters were 116 °C, and 0.5% sulphuric acid, 6% substrate loading and 15 minutes hydrolysis time. Furfural as the decomposition product of pentoses was not detected in their study, however HMF with the highest yield of 9% was detected as a main by-product. Furthermore, the authors reported that pectin polysaccharides were not hydrolysed to galacturonic acid by dilute acid hydrolysis up to temperatures as high as 210°C.
Grohman et al. (1991) worked on using dilute (0.06 and 0.5%) sulfuric acid to hydrolyze all of the polysaccharides present in the biomass. They reported that although dilute acid solubilized a large portion of carbohydrates in OW, cellulose and segments of pectin containing galacturonic acid units were resistant to acid catalyzed hydrolysis. The glycosidic bonds between galacturonic units are too resistant to the acid hydrolysis. Therefore pectin is not hydrolysed in acid hydrolysis or requires longer hydrolysis time for partial release of galacturonic acid (Grohmann, 1995; Timell 1965).

As cellulose is insoluble and it has crystalline structure, dilute acid can not disrupt its structure and thus another hydrolysis step is necessary to break cellulose to its monomeric sugars (Philipp, 1979). Dilute acid is also ineffective in breaking glycosidic bond between galacturonic units (Timell, 1965). Dilute acid pretreatment leads to removal of soluble mono and oligosaccharides together with acid solubilized pectin. Removal of dilute -acid-soluble components, helps increasing hydrolysis yields in proceeding enzymatic hydrolysis step (Grohmann, 1994). Solubilized pectin by dilute acid can easily be recovered by addition of ethanol to the solution. Acid hydrolysis had the advantage of having flexible process conditions in term of temperature and hydrolysis time compared to that of enzymatic hydrolysis (Grohmann, 1994). However, the main drawback of acid hydrolysis is decomposition of sugars to furaldehydes (degradation products) (Marin, 2007; Grohmann, 1995; Talebnia, 2008). The secondary reaction which leads to formation of furaldehydes not only decreases the hydrolysis yield for desire sugar (mostly glucose) but also it has toxic effect on proceeding biological processing of sugars (fermentation). On the other hand, enzymatic hydrolysis does not lead to production of degradation products as seen in acid pretreated biomass. However, some contaminations by microorganisms can occur in this process, which eventually would lead to a decrease in fermentation yields and/or uncontrolled fermentation (Grohmann, 1995).

Acid hydrolysis of mandarin OW was conducted by Nishio et al.(1979) , in which they used 0.8N sulfuric acid at 120°C an an autoclave and they compared reducing sugar amounts produced both by acid –catalyzed and enzymatic hydrolysis . Vaccarino et al. (1989) treated OW with dilute sulfuric acid for 1.5 hours at 100°C and cultivated the resulting hydrolysate by *filamentus* fungi.
Grohmann et al. (1995) monitored release of different sugars in OW in dilute acid solution pH of 2 and reported that arabinose was released at 120°C and galactose was not released up to temperature as high as 140°C. They also reported that dilute acid pretreatment had no effect on accumulation of glucose more than the amount that was solubilised in the first minutes of the reaction.

Faravaresh et al (2007) used dilute acid explosion to hydrolyse OWs. Pectin was precipitated from hydrolysate by adding ethanol 96% (v/v). It was kept at room temperature for 4 hours and centrifuged at 180 g for 60 min and was washed 5 times with ethanol 45 % according to pectin extraction procedure. This method solubilized 83% of the pectin present in OWs which is 25% (g/g dry basis). Faravaresh et al. (2007) investigated effect of pH and extraction time on pomace peach and reported the best extraction conditions to be pH 2.5 and 120 minutes.

Using acids for extraction of pectin is considered economic and efficient method however it is not environmentally friendly (Min, 2011).

The parameters affecting pectin extraction yields which were pectin source, extraction method, pH, temperature and extraction time have been optimized for different enzymes and orange types (Kertsez, 1951; Constenla, 2002; El-Nawawi, 1987; Kliemann, 2009; Wilkins M. e., 2005; Grohmann, 1995). El-Nawawi et al. (1987) obtained the maximum yields using hydrochloric acid (90°C, pH 1.7 and 120 min). Pagan et al. (1999) used peach pomace for pectin extraction using nitric acid at 80°C, pH 1.2 and for 60 minutes. Virk et al. (2004) extracted pectin from apple peel waste and reported nitric acid to be more effective than hydrochloric acid for extraction of pectin. The pH for extraction of pectin is optimized to be 2 and acid soluble extraction of pectin is the most conventional pectin extraction method (Aravantinos-Zafiris, 1992; Grohmann, 1995).

Wilkins and co-workers (2007) investigated on effect of maturity of orange on its enzymatic hydrolysis yields. Using an enzyme cocktail of cellulase, β–glucosidase and pectinase, the authors reported that the reducing sugars present in enzymatic hydrolysis of OW increased from 28 % (w/w, dry basis) to 44% (w/w, dry basis).
2.3.4 Alkaline pretreatment

Extraction of pectin by hot water and acids results in degradation of arabinan side chain however, hot alkali extraction of pectin avoids this process and leads to release of more by-products (Oosterveld, 1996).

Sun et al. (1999) extracted pectin rich polysaccharides and hemicellulose from sugar beet using 2% sodium hydroxide at 45°C in different time periods and reported that the optimized extraction time was 1 hour.

Ben-Shalmon (1996) observed that pretreatment of OWs by NaOH and EDTA prior to enzymatic hydrolysis by cellulases gave higher glucose yields than that of no pretreatment. This could be because of the fact that pectin hampers hydrolysis of OWs by enzymes and should be somehow separated prior to enzymatic hydrolysis (Wilkins, 2007).

2.4 Hydrolysis

2.4.1 Acid hydrolysis

Acid hydrolysis fractionates orange peel into a pectin-rich solution and glucan (cellulose)-rich solid residue. OW was also treated in some researches by two stage acid hydrolysis (sulfuric acid 72%), the first stage lead to partial depolymerization of carbohydrates which was followed by post-hydrolysis by dilute boiling sulfuric acid. Same biomass was hydrolyzed by enzyme and they reported that the sugar compositions of both hydrolysis method were so close to each other and by the previous researches (Marshall, 1985; Ma, 1993, Grohmann, 1993, Grohmann, 1994).

Grohmann et al. (1995) reported that due to low rate of depolymerization of pectin even at pH as low as 1.1 (using sulfuric acid at 110°C), and the the negative effect of acid hydrolysis on fructose, acid hydrolysis is not a promising method for carbohydrate breakdown of OW. However, dilute acid method, could be used prior to enzymatic hydrolysis to enhance the hydrolysis yield to a great deal.

2.4.2 Enzymatic hydrolysis of OW

As an attempt to use environmentally friendly and green processes, enzymes are used for hydrolysis of biomass. Enzymatic hydrolysis of OW releases all its
carbohydrates. Slow rate of depolymerization and high cost of enzyme, are the drawbacks of enzymes (Grohman, 1995).

Due to low lignin content of OW, it is more vulnerable to hydrolysis than most of the other lignocellulosic biomass (Grohmann, 1994). Enzymatic hydrolysis of different lignocellulosic feedstocks has been conducted by different enzymes to obtain desired hydrocarbons regarding its content, for example, hemicellulases (Shkodina, 1998; Zykwinska, 2008), polygalacturonases (pectinases) (Contreras-Esquível 2006), proteases (Zykwinska, 2008), cellulases (Shahera, 2002; Grohmann, 1992; Wilkins, 2005; Wilkins, 2007; Martínez Sabajanes, 2012).

Pectinolytic preparations which include separation of pectins and utilization of pectinases were reported to be more effective in disrupting OW structure than utilization of cellulytic enzymes (cellulases) (Ben-Shalom, 1986; Nishio, 1979; Marshal, 1985; Grohmann 1992; Grohmann, 1994).

Some investigations have reported that better glucose yields are obtained when conducting enzymatic hydrolysis of OW using a cocktail of cellulases and pectinases (which also contains appropriate amount of hemicellulytic activities) (Marshal, 1985; Grohmann, 1992; Grohmann, 1994; Donaghy, 1994; Fanta, 1992; Wilkins, 2007). Complete hydrolysis of pectin rich OW requires dozens of enzyme activities and thus the enzyme mixture used for this purpose are quiet complicated (Ward, 1989; Grohmann, 1994).

Some researches were conducted to investigate the effect of dilute acid pretreatment prior to enzymatic hydrolysis of OW using enzyme cocktail of pectinases, cellulases and β–glucosidase. They reported that dilute acid pretreatment prior to enzymatic hydrolysis had little effect when using enzyme cocktail (Grohmann, 1995; Grohmann, 1994), which means that OW is highly vulnerable to mixture of enzymes.

Pectinases was reported to be more effective than cellulase in hydrolysing polysaccharides (Grohmann, 1992; Wilkins, 2007a). That is because pectinases having variety of activities to degrade polysaccharides compared to simple activity of cellulase (Grohmann, 1992). Cellobiose can not be consumed by \textit{S.cerevisiae} and that’s why beta-glucsidase is used to hydrolysate cellobiose (Yoon, 2003). Utilization of β–glucosidase not only increases glucose yield but also avoids inhibitory effect of cellobiose on cellulase enzyme (Grohmann, 1994).
Grohmann et al. (1995) also investigated effect of dilute acid pretreatment prior to enzymatic hydrolysis of OW using enzyme cocktail and they deduced that reducing sugar amounts were higher in dilute acid pretreated enzymatic hydrolysate than that of untreated. Reducing sugar measurement gives a better estimation of released carbohydrate depolymerisation than composition analysis of monomeric sugars.

Grohman et al. (1995) reported that 20% substrate loading was the best ratio when enzymatically hydrolysing OW. Optimized pH for fermentation of different citrus wastes and for different microorganisms has been investigated. The optimized pH for *Escherichia coli* K011 is reported to be pH 5.8–6.2 and for *S. cerevisiae* 5–5.2 respectively (Grohmann, 1995; Russell, 2003).

### 2.5 Cellulose extraction from OW

Extraction of cellulose from OW has been investigated in a research before (Yaşar, 2007). Yaşar et al. (2007) extracted cellulosic fragment of OW from 10% NaOH treated at 35 °C for 22 h from defatted, protein, pectin and hemicellulose free, delignified orange peel. Their extraction method was first to inactive pectic enzymes present in the OW in boiling water for 10 minutes, and dries the supernatant overnight. Then they defatted the biomass by extracting it by chloroform:methanol (2:1, v/v) in soxhlet. Proteolysis was then performed using proteases to remove proteins. For removal of pectin a solution containing ammonium oxalates at pH: 3.5, was prepared and stirred at 75 °C in a water bath for 60 min and filtered. 10% (w/v) NaOH was used for removing hemicellulose. The solution was stirred at 35 °C in water bath for 22 h. For removing lignin 5 ml 10% (v/v) acetic acid and 2 g NaCl were used and the volume of the solution was increased to 100 ml and it was stirred at 75 °C in water bath for 60 min and filtered.

### 2.6 Fermentation of cellulose hydrolysate of OW

*S.cerevisiae* can ferment glucose, galactose and fructose of OW hydrolysate (Grohmann, 1994). Most studies on ethanol production from OW have used simultaneous saccharification and fermentation (SSF) (Wilkins, 2007a; Grohmann, 1994; Grohmann, 1996; Hari Krishna, 2000). Some researchers have worked on optimizing the SSF parameters (Ward, 1989; Grohmann, 1994; Oberoi, 2010).
Pourbafrani et al. (2010) used the yeast *S. cerevisiae* ATCC 96581 for fermentation of OW hydrolysate. The ethanol yield they obtained based on total fermentable sugars was 0.43 g/g. Grohmann et al. (1998) investigated effect of microorganism type on ethanol yield from fermentation of OW hydrolysate. They used *E. coli* KO11 and *S. cerevisiae* for fermentation of hydrolysate of OW pretreated in same conditions. Fermentation using *E. coli* KO11 led to 25-35% increase in ethanol titre. *E. coli* can ferment all the sugars present in the OW hydrolysate and has been engineered to produce higher ethanol yields and is the promising microorganism for ethanol production from OW (Edwards, 2012).

Table 2.3 A review of fermentation conditions and maximum obtained ethanol yield from UOW obtained by different researchers.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Pretreatment</th>
<th>Fermentation type</th>
<th>Enzyme concentration</th>
<th>pH</th>
<th>T (°C)</th>
<th>Max ETH (% w/v)</th>
<th>Time (h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Live steam (150-160 °C/2-4 min)</td>
<td>SSF</td>
<td>pectinase (60 IU/g dw),</td>
<td>5</td>
<td>35</td>
<td>1.34</td>
<td>24</td>
<td>(Edwards, 2012)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>dilute sulfuric acid (0.8 %v/v, pH 2.2)</td>
<td>SSF</td>
<td>pectinase (0.42 IU/g), cellulase (0.066IFPU/g), β-glucosidase (0.594 IU/g)</td>
<td>4.2</td>
<td>37</td>
<td>2.7</td>
<td>48</td>
<td>(Widmer, 2010)</td>
</tr>
</tbody>
</table>
Table 2.3 A review of fermentation conditions and maximum ethanol yield from UOW obtained by different researchers (continued).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Pretreatment</th>
<th>Fermentation type</th>
<th>Enzyme concentration</th>
<th>pH</th>
<th>T (°C)</th>
<th>Max ETH (% (w/v))</th>
<th>Time (h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td>Live steam (155 °C/410-550kPa/2 min)</td>
<td>SSF</td>
<td>pectinase (60 IU/g dw), Cellulase (0.035 FPU/g dw), β-glucosidase (0.81 IU/g dw)</td>
<td>4.9</td>
<td>37</td>
<td>3.48</td>
<td>48</td>
<td>(Widmer, 2009)</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Steam expansion (150 °C/70 psi)</td>
<td>SSF</td>
<td>pectinase, cellulase, β-glucosidase</td>
<td>4.5</td>
<td>38</td>
<td>4.05</td>
<td>18</td>
<td>(Zhou, 2008)</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Live steam (150-160 °C/2-4 min)</td>
<td>SSF</td>
<td>pectinase (297 IU/g dw)</td>
<td>5</td>
<td>37</td>
<td>3.96</td>
<td>24</td>
<td>(Wilkins, 2007b)</td>
</tr>
<tr>
<td>E. coli KO11</td>
<td>Ground</td>
<td>SF</td>
<td>pectinase (12.4U/g), cellulase (0.41FPU/g), β-glucosidase (1.6 mg/g)</td>
<td>5.8</td>
<td>37</td>
<td>2.76</td>
<td>72</td>
<td>(Grohmann, 1994)</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Ground</td>
<td>SF</td>
<td>pectinase (12.4U/g), cellulase (0.37FPU/g), β-glucosidase (1.6 mg/g)</td>
<td>5</td>
<td>35</td>
<td>7</td>
<td>14</td>
<td>(Grohmann, 1994)</td>
</tr>
</tbody>
</table>
2.7 Processes for production of ethanol from OW

Three processes for production of ethanol from OW have been investigated in the past: in the first approach (Stewart, 2005) a cocktail of enzymes (cellulases, pectinase, and β-glucosidase) were used for hydrolysis of OWs and the hydrolysate was passed through a filter to separate limonene and it was fermented for ethanol production. In the second approach (Wilkins, 2007), 90% (v/v) of limonene was first separated from OWs using steam stripping (150-160 °C for 2-4 min) and then simultaneous saccharification and fermentation (SSF) was performed. In the third approach (Pourbafrani, 2010), which was based on dilute acid hydrolysis of OWs (Grohmann, 1995; Talebnia, 2008), dilute acid explosion process was performed both for separation of limonene and for hydrolysis of OWs. The slurry was then centrifuged and the liquid part was fermented to produce ethanol and the solid residue was used for biogas production (Pourbafrani, 2010). Wilkins et al. (2007) reported that combination of pectinase and cellulase enzymes in hydrolysis of OWs resulted in higher glucose yields than that of only cellulases.

Widmer et al. (2008) used Kluyveromyces marxianus as fermenting micro-organism in SSF process. They reported that K.marxianus has higher thermo tolerance and required less pectinase probably because it produces polygalacturonase during fermentation process.

No process has been worked on for individual isolation of OW cellulose and processing it for product production.
CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Tri-sodium citrate dehydrates, citric acid mono-hydrate, sodium hydroxide, agar, peptone, yeast extract, urea, magnesium sulfate, calcium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate, D-xylene and D-glucose were purchased from Merck (Darmstadt, Germany). Ethanol, potassium sodium tartrate, 3, 5-dinitrosalicylic acid, phenol, sodium sulfate, and citrus pectin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cellulases (Cellic CTec 2) and pectinases (Pectinex Ultra SP-L) were obtained from Novozymes (Bagsvaerd, Denmark).

3.2 Feedstock Preparation; Orange Waste

Orange Waste (OW), the limonene extracted solid residue of orange juice production plant, was provided by Cypruvex factory in Northern Cyprus (the limonene was extracted by pressing methods and for commercial reasons). Limonene free OW with water content of 75 % (w/w) was dried at 60°C for 24 hours, ground to three different particle sizes of (0.1-0.3 mm, 0.3-0.5 mm, 0.5-0.7 mm), and stored in desiccator at room temperature till use.

3.3 Enzymes

Cellulases (Cellic CTec2 and Cellucast) and pectinases (Pectinex Ultra SP-L) were provided as a gift from Novozymes (Novo, Nardisk, Bagsvaerd, Denmark). Enzymes were kept in fridge at 4°C till use.
3.3.1 Cellulases

Cellic CTec2 was obtained from Novozyme. It contains aggressive cellulases, high levels of β-glucosidase and hemicellulases. Cellulase activity of the enzyme mixture is 219 FPU/ml.

3.3.2 Pectinases

Pectinases (Pectinex Ultra SP-L) with the declared enzyme of Polygalacturonase was used. Activity of the enzyme is 9500 PGU/ml.

3.4 Microorganism

The pre-isolated and purified culture of the *Saccharomyces cerevisiae* NRRL Y-132 was obtained from ARS (Agricultural Research Service, USA) culture collection.

3.4.1 Cultivation and maintenance

The *Saccharomyces cerevisiae* in its spore germination phase (which is the process by which resting, non-dividing spores grow and enter the mitotic cell cycle) was obtained on agar slant and culture was cultivated on YPD as it was to be stored for longer period for the utilization of organism in different trials. YPD agar with given compositions, was autoclaved at 121° C for 20 minutes under 1.1 kg/cm² pressure. After cooling to 50 ° C, the medium was transferred to petri plates and kept at room temperature at least 5-6 minutes to solidify completely. One inoculation loop of the main agar culture was spread on the new prepared agar plate. It was cultivated at 30° C for 2 days to allow growth. After 48 hours, newly grown microorganisms were taken by a sterile toothpick and it was transferred cultivated in fresh YPD broth, the composition of which is given below. This subculture was incubated for 24 hours in 30 °C. This medium was used in inoculums in the fermentation step. The agars were wrapped by parafilm and stored at 4 ° C for later use.
Table 3.4 Chemical composition of medium for cultivation of microorganism.

<table>
<thead>
<tr>
<th>Components</th>
<th>YPD broth (g/L)</th>
<th>YPD agar (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Peptone</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Agar</td>
<td>-</td>
<td>20</td>
</tr>
</tbody>
</table>

3.5 Analytical methods

3.5.1 Reducing sugar measurements by 3, 5-dinitrosalicylic acid (DNS) method

DNS method was used for measuring the reducing sugars: glucose, fructose, galactose and maltose (Miller, 1959). For this purpose, 1.5 ml of DNS reagent was added to tubes containing 1 ml of glucose solutions in capped test tubes. The mixture was vortexed and heated in boiling water for 5 minutes to stop the reaction. After cooling to room temperature in a cold water bath, absorbance was measured at 540 nm spectrum in spectrophotometer (Nicolet Evolution 100, Thermo Fisher Scientific Inc., (USA)). In case the resulting absorbance was out of the range of 0.2-0.8, dilutions were performed.

Standard glucose solutions were prepared in each experiment set by diluting 1 g/L pure glucose solution. To prepare 16.7, 11.1, 8.3, and 6.67 g/L glucose solution, 60, 90, 120, 150 µl from 1 g/L glucose solution were added to tubes containing 940, 910, 880, 850 µl of dionized water and vortexed. DNS solution was added to tubes as mentioned above. The standard curves were prepared regarding absorbance of standard solutions versus glucose concentrations. The standard curve is presented in Appendix A.
3.5.2 Glucose measurements

3.5.2.1 Glucose measurement by HPLC

High performance liquid chromatography (HPLC) analysis were conducted with a Shimadzu LC-20A HPLC system (Kyoto, Japan) equipped with a BIORAD Aminex HPX-87H column (Hercules, CA, USA) at 55°C with flow rate of 0.6 ml/min using 5 mM H$_2$SO$_4$ as the mobile phase. Standards were prepared by different dilutions of commercial glucose, regarding area under the glucose indicating peak. The amount of glucose in the solution was calculated using and comprising with standard solutions. The retention time of glucose in this column was 9.5 minutes under the mentioned conditions. A sample glucose peak is presented in Appendix C.

3.5.2.2 Glucose measurement by glucometer/glucose strip

Commercial blood monitoring glucometer produced for diabetic people (One Touch Select (LifeScan Inc., Milpitas, CA, USA), was also used to measure glucose concentration. The corresponding strips of blood glucose monitors were purchased from a local pharmacy were used for this purpose. The samples from the enzymatic hydrolysis reaction were diluted in order to obtain readings within the glucose concentration range. The dilutions were made with 0.05 M sodium citrate buffer. Different concentrations of 0.1 g/L commercial glucose were prepared for standard curve preparation. The standard curve is presented in appendix C.

3.5.3 Ethanol measurement

In certain time intervals samples were taken from fermentation medium. These samples were centrifuged at 4°C, 10 minutes and the liquid portion of the solution was transferred in another eppendorf tube and was kept in a freezer at 0°C till analysis. The melted solution was diluted to proper concentration, depending on expected ethanol concentration, considering initial glucose concentration of medium and fermentation time. HPLC was performed as mentioned in part 3.5.2.1. and ethanol concentration was measured regarding the standard curve.
prepared by commercial ethanol solution in water. Retention time of ethanol in this column (Shimadzu LC-20A HPLC system (Kyoto, Japan) equipped with a BIORAD Aminex HPX-87H column (Hercules, CA, USA) at 55°C with flow rate of 0.6 ml/min using 5 mM H₂SO₄) was 21 minutes.

### 3.5.4 Microbial biomass (Dry cell) measurement

Dry weight of *S.cerevisiae* was measured in a fixed volume of culture. Samples were taken from the fermentation medium, and their optical density at 600 nm using a spectrophotometer (Nicolet Evolution 100, Thermo Fisher Scientific Inc., (USA)), they were centrifuged at 10,000 rpm for 10 minutes. The solid part of the samples was dried overnight at 80°C, and they were weighted carefully to accuracy of less than 1 mg. Standard curves relating optical density at 600 nm, to dry weight of cells were prepared and presented in Appendix C.

### 3.5.5 Composition analysis of OW

Chemical compositions of untreated and pretreated OW samples were determined according to the procedure of NREL (National Renewable Energy Laboratory) LAP-002 by a two step acid hydrolysis (Sluiter, 2010). Acid hydrolysis was conducted as mentioned below: 3 ml 72% sulfuric acid was added to 3 mg of dry OW and it was incubated at 30°C water bath (Grant SUB-6 (Essex, UK)) for 1 hour while stirring every 15 minutes. The acid was later diluted to 4% by adding water and solution was autoclaved at 121°C for 20 minutes. After adjusting pH to 5-6 by adding calcium carbonate, the solution was passes through 0.2 μm filter and diluted for analysis. The sugars and glucose released during carbohydrate analysis and enzymatic hydrolysis were analyzed via High Performance Liquid Chromatography (HPLC), Shimadzu LC-20A HPLC system (Kyoto, Japan) equipped with a BIORAD Aminex HPX-87H column (Hercules, CA, USA) operated at 55 °C with a flowrate of 0.6 ml/min using 5 mM H₂SO₄ as the mobile phase. Ethanol, glucose, arabinose and xylose were used as standards.
3.6 OW pretreatments

Different pretreatment steps were obtained from literature to separate different pectin types and other lignocellulosic components from OW, in order to enrich the cellulosic fragment.

3.6.1 Hot water pretreatment

After drying OW at 60°C for 48 hours, the dried OW was stirred in dionized water with a solid/liquid ratio of 1/25 for 1 hour at 90°C. After one hour the suspension was cooled, sedimented, and was filtered through a cheese cloth. The residue was dried overnight at 60°C, to extract water soluble pectin which accounts for around 4% (w/w, dry basis) of OW (Massiot, 1997). The filtrate contained mainly soluble sugars and pectic material which can later be used.

3.6.2 Alcohol pretreatment

Alcohol insoluble solid separation method was applied in which, solid biomass after drying at 60°C for 48 hours, was diluted in 80 % ethanol (solid/liquid ratio 1/100) at 70°C and stirred for two hours. The suspension was filtered and the solid residue was dried overnight at 60°C (Thomas, 2000).

3.6.3 Dilute acid pretreatment

The dilute acid pretreatment of OW was conducted by adding HCl (solid/liquid ratio of 1/25) to reach to pH 2 and stirred for 4 hours at 90°C (Kalapathy, 2001). These conditions had been proved to be suitable for hydrolysis of hemicelluloses (Grohmann., 1985) and pectin (El-Nawawi, 1987) in plant materials. The slurries were filtered through cheese cloth and the filtrate was kept separately for pectin extraction. The solid residue was washed several times using deionized water (till the residue water was at neutral pH) and then dried over night at 60 °C. Equal volume of 96% ethanol was added to the filtrate (while still hot), and the pectin was immediately precipitated. After filtration through cheese cloth, the retentate was washed several times by dionized water till the washed pH was neutral.
3.6.4 Alkaline pretreatment

The dried biomass was stirred for 1 hour at 60°C in an alkaline solution 0.1 M NaOH at 1/20 dilution (Wang, 2002). The slurries were filtered through cheesecloth and then washed several times till neutral pH with dionized water and dried over night at 60°C.

3.6.5 Buffer pretreatment

In this pretreatment, the OW was stirred in citrate buffer at 70°C for 2 hours to separate the soluble components. The slurries were filtered through cheesecloth (Kalapathy, 2001). The buffer soluble components (mostly sugars) may be concentrated and utilized for product production separately, or may be recycled back and be added to hydolysate prior to fermentation step.

3.7 Enzymatic hydrolysis of cellulose to glucose

Enzymatic hydrolysis of OW samples was conducted at different substrate loadings in 0.05 M sodium citrate buffer at pH 4.8, 150 rpm and 50 °C in shaking incubator (Minitron, Infors AG, Bottmingen, Switzerland). Enzymatic hydrolysis conditions of pH 4.8, 150 rpm and 50 °C were reported to be the optimized conditions for the mentioned enzymes. Enzymatic hydrolysis was conducted in 30 ml suspension of sodium citrate buffer, pH of 4.8. Cellulases at different concentrations were added to the reaction mixture. Sodium azide solution at a concentration of 20 mg/ml was added to the hydrolysis mixture at a loading of 1% (v/v) to prevent microbial growth during hydrolysis (Selig, 2008). The reaction was monitored for 48 hours by withdrawing 100 µl of samples at specific time intervals. The samples were incubated at 100 °C for 5 minutes in order to stop the enzymatic reaction and later centrifuged at 10000 rpm for 5 minutes. The amount of reducing sugars and glucose released during the reaction were analyzed via DNS assay (Miller, 1959) and glucometer (Bahcegul, 2012) respectively. All assays were performed in duplicate, and the average was reported. Glucose yield (%) is determined as the ratio of the amount of glucose concentration found at the 48th hour of the enzymatic hydrolysis (g/L) to the theoretical maximum amount
of glucose of the biomass (regarding its cellulose content) subjected to hydrolysis (g/L). The enzyme yield is defined as the amount of glucose (g/L) per the unit of enzyme (FPU).

3.7.1 Isolation of cellulose rich fragment from OW

For obtaining cellulosic fragment of OW, other components containing soluble sugars, pectin, hemicellulose, lignin, protein and others, were separated from OW. Hot water, alcohol, dilute acid and alkaline pretreatment steps were performed to isolate cellulosic portion of OW. These methods are described in section 3.6.

3.7.2 Enzymatic hydrolysis of cellulosic fragment of OW using cellulases

After pretreatment the samples were dried overnight at 60 °C. The biomass samples were then enzymatically hydrolysed using cellulases. In the first set of experiments different cellulose enriched OW substrate loadings of 3, 8, 10, 15% (w/v), were enzymatically hydrolyzed using 2% (v/v) cellulase concentration 219 FPU/ml enzyme. Cellulosic fragment of OW in different amounts of 0.3, 0.8, 1 and 1.5 gram were added to falcons containing 10 ml 0.05 M citrate buffer pH of 4.8. Then, 200 µl cellulases and 100 µl solution of 20 mg/ml sodium azide were added to the mixture.

For the next set of experiments for investigating effect of enzyme concentration on glucose production, 1 gram cellulosic fragment of OW were put in different falcon tubes containing 10 ml 0.05 M citrate buffer pH of 4.8. Then, different volumes of cellulases (25, 50, 100,200,300 µl), (219 FPU/ml) were added to the suspension together with and 100 µl solution of 20 mg/ml sodium azide. All samples were prepared in duplicate. The falcon tubes were incubated at 150 rpm and 50 °C in shaking incubator (Minitron, Infors AG, Bottmingen, Switzerland). In certain time intervals, samples were taken and immediately placed in boiling water for 5 minutes to stop the reaction. HPLC and glucose strips were used for measuring the glucose content of the samples.
3.7.3 Enzymatic hydrolysis of untreated OW by cellulases and pectinases

After buffer and dilute acid pretreatments described in sections 3.6.3 and 3.6.5 for partially pretreated biomass and overnight drying, enzymatic hydrolysis was performed. As for untreated OW, the dry biomass was directly used for enzymatic hydrolysis step. An enzyme cocktail of cellulases and pectinases were used for this purpose. In the first set of experiments 20% biomass loading of untreated OW was taken with 0.5, 1 and 2 % (v/v) cellulases concentrations were investigated using 4% (v/v) pectinase concentration. The cellulase loading producing maximum glucose yield was used for the next experiment which was dealing with different substrate loadings. In this experiment, 1% (v/v) cellulases and 4% (v/v) pectinase were used and different substrate loadings of 5%, 10%, 15%, 20% and 30% (w/v) were enzymatically hydrolysed. Glucose concentrations were measured by HPLC and glucose strips.

3.7.4 Enzymatic hydrolysis of OW by cellulases and pectinases

After buffer and dilute acid pretreatments described in sections 3.6.3 and 3.6.5 for partially pretreated biomass and overnight drying, enzymatic hydrolysis was performed. Partially treated biomass was enzymatically hydrolysed using an enzyme cocktail of 2% (v/v) cellulases and 4% (v/v) pectinases. The biomass loading was 15% (w/v).

3.8 Ethanol production from OW enzymatic hydrolysates (containing mainly glucose) by Saccharomyces cerevisiae

*S. cerevisiae* NRRL Y-132 which was obtained from ARS (Agricultural Research Service, USA) culture collection was used in ethanol production experiments. Solid cultivation was conducted on petri plates containing YPD agar for 48 hours and precultivation was carried out in 250 ml flasks containing 50 ml YDP medium at 30 °C and 150 rpm for 24 hours. Afterwards, 10% (v/v) of preculture was added (as inoculum) to 10 ml of fermentation medium containing glucose solution or biomass hydrolysate and yeast extract, urea, Na₂HPO₄,7H₂O, KH₂PO₄, MgSO₄, CaCl₂. The pH of the fermentation medium was adjusted to 6. All fermentation experiments were conducted as 10 ml medium in 50 ml falcon tubes.
at 30 °C and 100 rpm in a shaking incubator (Minitron, Infors AG, Bottmingen, Switzerland). At different time intervals samples were taken and their cell density was measured by spectrophotometer at 600 nm. In same time intervals samples were taken and centrifuged to separate cells and the liquid part was kept in freezer at 4°C and their sugars and ethanol content was analysed by HPLC later on.

For concentrating glucose solutions, different concentrations of hydrolysate were prepared by vacuum evaporation, after being micro-filtered. Then pre-cultivation step was conducted in 50 ml YDP medium with 1gram pepton, 1gram glucose and 0.5 gram YE. The solution was autoclaved at 121°C for 20 minutes to be sterilized. The liquid pre-cultivation solution was performed in shaking incubator (Minitron, Infors AG, Bottmingen, Switzerland) at 150 rpm and 30 °C for 24 hours, for aerobic growth of micro-organism. The volumes below were added to 250 ml erlen mayers and kept in shaking incubator (Minitron, Infors AG, Bottmingen, Switzerland) 130 rpm, 30 oC.

On specified time intervals samples were taken for measuring cell growth (using spectrophotometer 600 nm) and alcohol concentration (via HPLC). Ethanol yield was calculated according to the formula below:

Fermentation Ethanol yield(%)  
\[
\text{Fermentation Ethanol yield(\%)} = \frac{\text{Ethanol produced (g/L)}}{\text{Theoretical max glucose considering cellulose content of biomass (g/L)}}
\]

The conversion of cellulose to ethanol was calculated by the formula given below:

\[
\text{Conversion of cellulose to ethanol \%} = \frac{\text{EtOH} \times 0.511}{f \times \text{Biomass conc.} \times 1.11}
\]

\[
\text{Ethanol titre} = \frac{\text{Volume of the ethanol produced}}{\text{Volume of the fermentation medium}}
\]

where: [EtOH] shows the ethanol concentration at the end of the fermentation minus any ethanol present in the enzyme solution and fermentation medium (g/L). f is cellulose fraction of dry biomass (g/g). Biomass represented dry biomass concentration at the beginning of the fermentation (g/L). Conversion factor, 0.511 is resulted from formation of ethanol from glucose based on stoichiometric biochemistry of yeast (formula 3.1); Conversion factor, 1.11 includes in the
calculation of glucose yield (%) owing to the water gain during the formation of glucose from cellulose (formula 3.2)

\[ C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + \text{Energy (stored as ATP)}, \quad (3.1) \]

Enzymatic hydrolysis of solid cellulose \( (C_6H_{10}O_5) \) to glucose \( (C_6H_{12}O_6) \).

\[ C_6H_{10}O_5 + H_2O \rightarrow C_6H_{12}O_6, \quad (3.2) \]

where molecular weights of one glucose unit in cellulose and glucose are 162 and 180 Da and thus the conversion factor from cellulose to glucose will be \( \frac{180}{162} = 1.11 \). Fermentation medium composition is presented in Table 3.5. Different glucose concentrations were used in the experiments in order to check the effect of glucose concentration on ethanol production yields. However, the rest of fermentation media components were fixed in all the fermentation experiments. The solutions containing mentioned concentrations of nutrients were autoclaved prior to being added in the fermentation media. The solution of urea-yeast extract could not be autoclaved so it was sterilized by microfilters.

Table 3.5 Composition of the fermentation medium

<table>
<thead>
<tr>
<th>Fermentation medium components</th>
<th>fermentation medium composition (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>10</td>
</tr>
<tr>
<td>Urea</td>
<td>6</td>
</tr>
<tr>
<td>((\text{Na}_2\text{HPO}_4).7\text{H}_2\text{O})</td>
<td>3</td>
</tr>
<tr>
<td>(\text{KH}_2\text{PO}_4)</td>
<td>3</td>
</tr>
<tr>
<td>((\text{MgSO}_4).7\text{H}_2\text{O})</td>
<td>0.25</td>
</tr>
<tr>
<td>((\text{CaCl}_2).2\text{H}_2\text{O})</td>
<td>0.08</td>
</tr>
<tr>
<td>Glucose solution</td>
<td>Different concentrations</td>
</tr>
</tbody>
</table>
3.8.1 Precultivation of *S. cerevisiae*

Table 3.6 YPD composition

<table>
<thead>
<tr>
<th>Components</th>
<th>Yeast extract</th>
<th>Peptone</th>
<th>Dextrose (glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPD plates (solid medium)</td>
<td>10 g/L</td>
<td>20 g/L</td>
<td>20 g/L</td>
</tr>
</tbody>
</table>

50 ml of pre-cultivation medium, YPD, was prepared in 250 ml flasks with the above mentioned concentrations and stirred till fully soluble solution was obtained. The medium was autoclaved at 121 °C, 20 minutes for sterilization purposes. One loop microorganism was taken from the freshly prepared agar plates under sterile conditions. The flask was incubated at 30 °C and 150 rpm for 24 hours.

3.8.2 Ethanol production by fermentation using pure glucose

Different concentrations of glucose (25, 50, 75 and 100 g/L) were prepared and autoclaved at 121 °C and 20 minutes. 7.5 ml of each of the glucose solutions were added to a sterile falcon tubes containing 0.5 ml of each of above mentioned salts and 1 ml (10% (v/v)) of pre-cultivation medium, and the pH of the media were adjusted to 6 using ammonium hydroxide.

3.8.3 Ethanol production by fermentation using glucose produced by cellulase hydrolysis of cellulosic fragment of OW

The hydrolysate obtained from enzymatic hydrolysis of the cellulosic fragment of OW was vacuum evaporated to 25, 50, 75 and 100 g/L. The solutions were 3 times centrifuged 10,000 rpm for 15 minutes and the liquid part was used in fermentation as glucose source, likewise the previous step.
3.8.4 Ethanol production by fermentation using glucose produced by enzymatic hydrolysis of untreated OW.

Enzymatic hydolysate of different susbstrate loadings of OW, were micro filtered by nylon syringe filter (Minisart 0.2 µm) and used as glucose source for fermentation likewise previous step.

3.8.5 Ethanol production by fermentation using glucose produced by enzymatic hydrolysis of partially pretreated OW

Enzymatic hydolysate of partially treated OW was micro filtered by nylon syringe filter (Minisart 0.2 µm) and used as glucose source for fermentation likewise previous step.

3.9 Composition analysis

Chemical compositions of untreated and pretreated samples were determined according to the procedure of NREL (National Renewable Energy Laboratory) LAP-002 by two step acid hydrolysis (Sluiter, 2010). Acid hydrolysis was conducted as mentioned below: 3 mg of sample was added to 3 ml 72% sulfuric acid and it was incubated at 30°C water bath (Grant SUB-6 (Essex, UK)) for 1 hour while stirring every 15 minutes. The acid was later diluted to 4% by adding water. The suspension was autoclaved at 121°C for 20 minutes. Calcium carbonate was added to the solution to adjust pH to 5-6. The solution was passes through 0.2 μm filter and diluted for analysis. The sugars and glucose released during carbohydrate analysis and enzymatic hydrolysis were analyzed via High Performance Liquid Chromatography (HPLC), Shimadzu LC-20A HPLC system (Kyoto, Japan) equipped with a BIORAD Aminex HPX-87H column (Hercules, CA, USA) operated at 55 °C with a flowrate of 0.6 ml/min using 5 mM H₂SO₄ as the mobile phase and commercial glucose, arabinose and xylose were used to draw standard curves.
Figure 3.1 Schematic representation of the three approaches studied in this work.
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Composition analysis of OW

Acid hydrolysis was conducted for determination of structural carbohydrates and lignin in biomass (Sluiter, 2008). The sum of cellulose, hemicellulose, lignin and ash is about 50% dry weight of OW. Composition of OW is presented in Table 4.1.

Table 4.1 Composition analysis of OW as measured in this project.

<table>
<thead>
<tr>
<th>Components</th>
<th>Untreated OW (% w/w dry basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>17</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>20</td>
</tr>
<tr>
<td>Lignin</td>
<td>12</td>
</tr>
<tr>
<td>Ash</td>
<td>2</td>
</tr>
<tr>
<td>Moisture</td>
<td>3</td>
</tr>
</tbody>
</table>

The complete analysis of orange wastes was not pursued, because the aim of the current study was to investigate on ethanol production from cellulosic OW and finding a way to increase its production yield.
4.2 Approach 1: Enrichment of cellulosic fragment of OW by extracting other components

In the first approach, cellulosic component of OW was isolated by removal of other components as much as possible. The cellulose-rich biomass obtained from this pretreatment was later enzymatically hydrolysed using cellulases to convert cellulose to glucose and used as glucose source in fermentation process.

4.2.1 Investigation of the effect of different parameters on glucose production by enzymatic hydrolysis of cellulosic fragment of OW

The most important parameters affecting enzymatic cellulose hydrolysis, namely cellulosic fragment (biomass) size and loading, enzyme dosage and hydrolysis time period were investigated in depth. Since the type of cellulases used has tremendous effect on cellulose hydrolysis, the effect of enzyme type was studied in a preliminary study using two commercial cellulase mixtures (Novo Nordisk) Cellucaast and Cellic CTec 2. The enzyme mixture Cellic CTec 2 is the newest cellulase mixture formulated specifically to hydrolyze lignocellulosic biomass by the company, was selected as the enzyme type since superior results were obtained with respect to the other cellulase mixture.

4.2.1.1 Effect of particle size on glucose production in enzymatic hydrolysis of cellulosic OW fragment

Three different particle sizes, between 0.1 to 0.3 mm, 0.3 to 0.5 mm, and 0.5 to 0.7 mm were enzymatically hydrolyzed using cellulases (219 FPU/ml enzyme) at 0.5% (v/v) concentration and 80 g/l biomass loading (8% (w/v)).

As shown in Figure 4.1, the particle size between 0.3-0.5 mm resulted in better glucose yield and it was in accordance with literature (Ververis, 2007). Therefore, the optimized particle size of cellulosic fragment of OW (0.3-0.5 mm) was used for the rest of
experiments. The particle size reduction has a positive effect on hydrolysis yield because it opens up tightly linked structure of biomass and increases enzyme accessibility to cellulose (Yeh, 2010). However, considering small particle sizes, decreasing particle size leads to more biomass loss and thus, decreases in overall yield.

**Figure 4.1** Effect of particle size on glucose production by enzymatic hydrolysis of cellulosic fragment of OW in 24th hour of hydrolysis, 0.5% (v/v) cellulases concentration and 80 g/L cellulosic OW fragment loading (8% (w/v)) in 0.05 M sodium citrate buffer at pH 4.8 and 50 °C were used.

4.2.1.2 Effect of substrate loading and hydrolysis time

Different loadings of cellulosic OW fragment, (3, 8, 10, 15% (w/v)) were enzymatically hydrolyzed using 2% (v/v) cellulase mixture (219 FPU/ml enzyme mixture) and the results are given in Figure 4.2. Enzymatic hydrolysis conditions, 3-15(%, w/w) cellulosic OW fragment loading, 1% (v/v) cellulase, 0.05 M sodium citrate buffer at pH 4.8 and 50 °C.
The yield calculations were performed regarding the released glucose concentration after enzymatic hydrolysis per the theoretical maximum glucose that could be reached considering the cellulose content of the substrate.

![Graph showing glucose concentration over enzymatic hydrolysis time for different substrate loadings.](image)

Figure 4.2 Effect of substrate loading and hydrolysis time on the final glucose production during the enzymatic hydrolysis of cellulosic OW fragment. Enzymatic hydrolysis conditions, 3-15(%, w/w) cellulosic OW fragment loading, 1% (v/v) cellulase, 0.05 M sodium citrate buffer at pH 4.8 and 50 °C.

As shown in Figure 4.3, the glucose yields increased by increase in the cellulosic OW fragment loadings. However, after substrate loading of 10%, no significant increase in yield was observed with increasing substrate loadings. The increase in glucose production until 10% substrate loading should be due to availability of more cellulose in the suspension which leads to more glucose production, whereas, the decrease in glucose production after 10% substrate loading can be described by inhibitory effects of accumulated compounds such as cellubiose and cellodextrins (Yabefa, 2010). Specific or unspecific binding of the enzyme with the substrate may also be the reason behind the decrease of glucose production after 10% substrate loading (Selig, 2008). The glucose
concentration reached constant volumes after 24 hours of hydrolysis, showing that the hydrolysis reaction mostly completed in 24 hours.

**Figure 4.3** Effect of substrate loading and hydrolysis time on the glucose yield during the enzymatic hydrolysis of cellulosic OW fragment. Enzymatic hydrolysis conditions were: 3-15(%, w/w) cellulosic OW fragment, 1% (v/v) cellulase (219 FPU/ml enzyme), 0.05 M sodium citrate buffer at pH 4.8 and 50 °C.

As for the yield calculations, considering theoretical maximum glucose, increase in biomass loading means an increase in cellulose content of biomass which is directly connected to theoretical maximum glucose. Therefore, by increasing substrate loading, both numerator and denominator of the equation increase but in different scales. Increase in glucose concentration is more than increase in theoretical maximum glucose till substrate loading of 10%(w/v), but after this substrate loading, 15%(w/v) substrate loading has the minimum yield.

To have an overall outlook of the investigated parameters, the color based three-dimensional graph is presented below. It enhances choosing the best parameters
regarding hydrolysis time and substrate loading considering the glucose yield. The results of the 3D diagram show that 10% substrate loadings of cellulosic OW fragment in 24 hours of hydrolysis give the highest glucose yields (Figure 4.4).

**Figure 4.4** 3D diagrams for optimization of hydrolysis conditions, regarding 3 parameters of hydrolysis time, substrate loading and glucose yield (%, g glucose/theoretical max glucose). Enzymatic hydrolysis conditions were: 3-15(%, w/w) cellulosic OW fragment loading, 1% (v/v) cellulase concentration, 0.05 M sodium citrate buffer at pH 4.8 and 50 °C.

### 4.2.1.3 Effect of enzyme concentration on glucose production in enzymatic hydrolysis of cellulosic OW fragment

Different cellulase concentrations in the range of 0.1%-3% (v/v) (219 FPU/ml enzyme) were investigated using the previously optimized particle size (0.3-0.5 mm) and substrate loading (10% w/v). The results show that maximum hydrolysis of cellulosic
OW fragment occurs at 1% cellulase concentration (Figure 4.5). While the increase in hydrolysis with increasing enzyme concentration is intuitive, at higher loading (>1%), certain components present in the enzyme mixture which were added to the enzyme for different purposes like increasing enzyme stability (e.g. sugars and alcohols), may have inhibitory effects on the enzymatic reaction.

**Figure 4.5** Effect of cellulase mixture loading and hydrolysis time on glucose production. Enzymatic hydrolysis conditions were 10 (%, w/w) cellulosic fragment OW loading, enzyme concentrations in the range of 0.1-3 (%, v/v), 0.05 M sodium citrate buffer at pH 4.8 and 50 °C.

As shown in Figure 4.5, from 0.1 up to 1% (v/v) enzyme concentration, the glucose production shows an increasing trend, because of presence of more enzymes to convert the cellulose present in the suspension. However, after 1% enzyme concentration, increase in the enzyme concentration leads to a decrease in produced glucose. This decrease as mentioned before can be due to inhibitory effect of some other components present in the enzyme mixture. As the enzyme mixture (Cellic Ctec 2), contains glucose
and some other soluble sugars, addition of more enzyme may cause saturation of product (glucose), which would have a negative effect like product inhibition on functionality of the enzyme.

**Figure 4.6** Effect of cellulase concentration and hydrolysis time on glucose yield (% g glucose/theoretical max glucose). Enzymatic hydrolysis conditions were 10 (%, w/w) cellulosic OW fragment loading, enzyme concentrations in the range of 0.1-3 (%, V/V), 0.05 M sodium citrate buffer at pH 4.8 and 50 °C.

The effect of enzyme concentration on %glucose yield is presented in Figure 4.6.

Up to 1% enzyme loading (v/v), increase in enzyme concentration leads to increase in yield%, which is due to better hydrolysis of cellulose. However, after 1 (%, v/v) enzyme loading, increasing enzyme concentration has a negative effect in hydrolysis yield, and this is because of inhibitory effect of enzyme components on biomass and oversaturation of product in the suspension.
Figure 4.7 3D diagrams for optimization of hydrolysis conditions, regarding 3 parameters of hydrolysis time, cellulase concentration and glucose yield (%, g glucose/theoretical max glucose).

Figure 4.7 shows maximum glucose yield (dark orange color) at enzyme loading of 1 (%, v/v) and hydrolysis time of around 20 hours. The dark orange area demonstrates the best conditions in terms of enzyme loading and hydrolysis time, to produce maximum glucose. This figure, confirms the previous results, as for 1 (%, v/v) enzyme loading and around 24 hours of hydrolysis to be the best conditions for cellulose hydrolysis.

The second parameter that was investigated was the gram glucose produced per filter paper unit of enzyme. FPU was defined as the quantity of enzyme that produces reducing sugar equivalent to 2 mg glucose. As seen in Figure 4.8, the glucose yield (%, g glucose/FPU) showed an increasing trend as the enzyme concentration was increased up to 1 (%, v/v). However, after 1 (%, v/v) enzyme concentration, increase in enzyme loading leads to a decrease in produced glucose concentration per FPU of cellulose.
Figure 4.8 Glucose produced / FPU of cellulase for different enzyme loadings in different hydrolysis time in enzymatic hydrolysis of cellulosic OW fragment loading, 1 (%, v/v) cellulase concentration, 0.05 M sodium citrate buffer at pH 4.8 and 50 ° C.

4.2.2 Investigation of ethanol production by *Saccharomyces cerevisiae* Fermentation

4.2.2.1 Fermentation of commercial pure glucose by *S. cerevisiae*

Commercial pure glucose at four different initial concentrations of 25, 50, 75 and 100 g/L were fermented by *S. cerevisiae* to produce ethanol as positive controls to check the utilization of glucose produced by enzymatic hydrolysis of cellulose rich OW by *S. cerevisiae* for ethanol production. As observed in Table 4.2, *S. cerevisiae* produced ethanol during fermentation and ethanol production increased by increasing initial glucose concentration. Concentration of commercial pure glucose solutions had no negative effect on the overall ethanol production yields.
Table 4.2 Effect of pure glucose concentration on ethanol production by *S. cerevisiae* in fermentation step.

<table>
<thead>
<tr>
<th>Initial glucose concentration (g/L)</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol concentration (g/L)</td>
<td>10</td>
<td>19</td>
<td>34</td>
<td>41</td>
</tr>
</tbody>
</table>

4.2.2.2 Fermentation of cellulosic fragment of OW hydolysate by *S. cerevisiae*

The hydrolysates were concentrated by vacuum evaporation to 25, 50, 75, and 100 g/L. However, the hydolysate solutions became very dense solutions after vacuum evaporation that caused many problems in sterilization and measurements. Filters sterilization using 0.20 to 0.75 µm filters could not be used and autoclaving lead to a biphasic solution. The fermentation media were supplemented with nutrients as mentioned in Table 3.5. The hydrolysate was later cultivated at low O₂ environment using free suspended *S. cerevisiae*. At specified time intervals samples were taken for monitoring cell growth, glucose consumption and ethanol production via HPLC.

As the glucose concentration increased, it took longer for the yeast to consume all the glucose. Concentrating hydrolysate had no significant effect on increasing produced ethanol yield. This can be justified by the fact that, concentrating hydrolysate leads to concentration of degradation products and inhibitors in the fermentation process such as furfural and Hydroxymethylfurfural (HMF) which would cause problems in fermentation process (Kademi,1996). Figure 4.9, presents effect of increasing initial sugar concentrations on ethanol production and cell growth rate of *S. cerevisiae*. 
**Figure 4.9** Effect of initial glucose concentrations of hydrolysate; on profiles of yeast growth, glucose consumption and ethanol production.

Table 4.3 compares utilization of pure glucose and hydrolysate in the fermentation media in terms of concentration of ethanol produced, ethanol yield and ethanol titre which is the volume of ethanol produced per volume of the fermentation medium. Concentration of hydrolysate led to decrease in ethanol yield, while concentration of pure glucose led to increase in ethanol yield. Increasing initial glucose concentration increased ethanol yield both for pure glucose and cellulose hydrolysates. However, the increases in the pure glucose are much more than the cellulosic hydrolysates.
Table 4.3 Comparison of ethanol production in media containing concentrated hydrolysate of cellulosic OW fragment vs. commercial glucose solutions

<table>
<thead>
<tr>
<th>Initial glucose concentration (g/L)</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>10</td>
<td>19</td>
<td>34</td>
<td>41</td>
</tr>
<tr>
<td>hydrolysate</td>
<td>10</td>
<td>20</td>
<td>28</td>
<td>34</td>
</tr>
<tr>
<td>Ethanol concentration (g/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol titre (% v/v)</td>
<td>1.3</td>
<td>2.3</td>
<td>4.2</td>
<td>5.1</td>
</tr>
<tr>
<td>Ethanol yield (%)</td>
<td>78</td>
<td>75</td>
<td>89</td>
<td>80</td>
</tr>
</tbody>
</table>

4.3 Approach 2: Investigation on utilization of UOW (untreated orange waste) for ethanol production

Untreated OW was enzymatically hydrolysed using cellulases and pectinases and the hydrolysate was subjected to fermentation.

4.3.1 Investigation of enzymatic hydrolysis of UOW

Biomass loading, enzyme concentration and hydrolysis time were investigated as parameters for their effect on final glucose yield.

4.3.1.1 Effect of enzyme concentration and hydrolysis time on final glucose production in enzymatic hydrolysis of UOW

For optimization of cellulases and pectinases concentrations, different enzyme concentrations depending on literature reports were chosen. As for cellulases concentrations in the range of 0.5–2 (% v/v) were chosen and the reducing sugars produced were determined after 48 hours of hydrolysis besides glucose measurement since sugars other than glucose are present in UOW. Reducing sugar profiles during the
enzymatic hydrolysis are presented in Figure 4.10. As observed in the Figure, 1 (%, v/v) enzyme concentration was more efficient. In the experiment, pectinases content was kept constant at 4% (v/v) depending on literature reports (Wilkins, 2007). The samples of 48th hour of hydrolysis were analyzed by HPLC and glucose strips.

![Graph](image)

**Figure 4.10** Cellulase (Cellic CTec 2) loading optimization for UOW. Cellulase loading of 2 (%, v/v) and pectinase loading of 4 (%, v/v) were used in 0.05 M sodium citrate buffer at pH 4.8 and 50 °C.

As observed in Figure 4.10, 1 (%, v/v) cellulase concentration resulted in higher glucose production than 0.5(%, v/v), however increasing cellulase concentration to 2 (%, v/v) did not have a major effect on glucose production. Increasing enzyme concentration may cause some inhibitory effect on product production, however there has to be enough enzymes to saturate all the cellulose. As for enzymatic hydrolysis time, the reaction was almost finished in 24 hours of hydrolysis.
4.3.1.2 Effect of Substrate loading on enzymatic hydrolysis of UOW

UOW at different substrate loadings was subjected to enzymatic hydrolysis. Since the UOW contains pectin and hence, an enzyme cocktail of pectinases at 4 (%, v/v) was added to cellulases (1 (%, v/v)) for enzymatic hydrolysis.

![Graph showing glucose concentration over hydrolysis time](image)

**Figure 4.11** Effect of substrate loading and hydrolysis time on enzymatic glucose production (g/L) of UOW. Cellulase loading of 2 (%, v/v), Pectinase loading of 4 (%, v/v) and substrate loading in the range of 5-30 (%, w/v) were used in 0.05 M sodium citrate buffer at pH 4.8 and 50 °C.

As shown in Figure 4.11, increasing substrate loading leads to increase in glucose production, however in 30 (%, w/v) substrate loading, the liquid portion of suspension was so little that one batch would not produce enough hydrolysate for fermentation process. Therefore, 20 (%, w/v) substrate loading of UOW was chosen to be the better choice regarding satisfactory glucose and hydrolysate production. Presence of other soluble sugars in the hydrolysate of UOW was investigated and total reducing sugars were presented in comparison with glucose in Figure 4.12. The reducing sugar quantities are much more than glucose as expected. Utilization of these reducing sugars is investigated.
Figure 4.12 Reducing sugar (measured by DNS method) vs. glucose concentration (measured by glucose strips) of hydrolysate of UOW. Hysrolysis conditions were 24 hours of hydrolysis, cellulase loading of 2 (%, v/v) and pectinase loading of 4 (%, v/v) were used in 0.05 M sodium citrate buffer at pH 4.8 and 50 °C.

The results as shown in Figure 4.12 indicate a maximum glucose concentration of 53 g/L at 30 (%, w/v) substrate loading after 24 hours which is significantly higher compared to the glucose concentrations for cellulosic OW fragment. Increase in substrate loading results in greater increase in reducing sugars than to that of glucose. This could be justified by the following two explanations. Firstly, at higher substrate loadings enzyme concentration becomes the limiting reagent and there is not enough enzyme to degrade all cellulosic biomass to its monomers. Secondly, as the amount of the reducing sugar is so high for high substrate loadings, more dilutions are necessary (up to 1200 times) to measure the reducing sugar concentrations which may cause overestimations.
4.3.1.3 Recovery of sugars present on the solid residue by washing

Solid particles within the hydrolysate slurry are separated using a belt filter press and are washed twice to recover sugars after enzymatic hydrolysis. The amount of washing water required is about 50% of total OW. The pH of hydrolysate leaving the filter press is adjusted using Ca (OH)₂.

![Graph showing reducing sugar concentration](image)

**Figure 4.13** Reducing sugar concentration of hydrolysate vs. reducing sugar concentration after two times washing of solid residue plus reducing sugar from hydrolysate. Cellulase loading of 2 (%, v/v) and Pectinase loading of 4 (%, v/v) were used in 0.05 M sodium citrate buffer at pH 4.8 and 50 °C.

The reducing sugar and glucose concentrations after recovery of sugars from the solid residues were almost two times more than the amounts from the ones without sugar recovery as presented in Figure 4.13. This was to make use of all the sugars present that could not be released to the liquor due to complex and tight structure of the hydrolysate residue (Nigam, 2000). These sugar solutions have to be concentrated and added to the hydrolysate for prior to fermentation process in order to increase the overall yield of the process.
4.3.2 Effect of initial glucose concentration of UOW hydrolysate on fermentation parameters

The enzymatic hydrolysates were syringe filtered and used as glucose source for fermentation. Effect of initial glucose concentration on cell growth, glucose consumption and ethanol production was then investigated. Glucose concentrations of the hydrolysates were not increased by considering the negative effect caused by vacuum evaporation.

4.3.2.1 Effect of initial glucose concentration of UOW hydrolysate on growth of *S. cerevisiae*

The effect of initial glucose concentration on cell growth is given in the Figure 4.14. As observed in the figure, in all the cases stationary phase was reached at around 24 hours and dry cell concentrations increased by increasing initial glucose concentration. Biomass is a critical parameter in the fermentation process because it is a key variable to optimize a specific process, or to reach a maximum efficiency to obtain fermentation product (Doelle, 1994).

![Figure 4.14](image)

**Figure 4.14** Effect of substrate loading of UOW on cell growth of *S. cerevisiae*. 73
4.3.2.1 Effect of initial glucose concentration of UOW hydrolysate on glucose consumption by *S. cerevisiae*.

![Graph showing glucose consumption over fermentation time for different glucose concentrations.](image)

**Figure 4.15** Effect of initial glucose concentration of UOW hydrolysate on glucose consumption by yeast *S. cerevisiae*.

Glucose consumption data is given in Figure 4.15. In all cases, glucose is consumed in 24 hours of fermentation. However the fermentation was followed for 48 hours. The glucose consumption is faster for more concentrated solutions and all the glucose finished at the same time for hydrolysates containing different glucose concentrations.

**4.3.2.1 Effect of initial glucose concentration of UOW hydrolysate on ethanol production**

Ethanol production was monitored for 72 hours during the fermentation. The production range however, was stable after the first 24 hours. Increasing substrate loading from 15 to 30 (%, w/v) increased ethanol concentrations as observed in Figure 4.16. However, increasing biomass loading from 30 to 40 (%, w/v) did not lead to increase in ethanol production and that could be justified by the increase in concentration of inhibitors in the fermentation medium (Yabefa, 2010)
Figure 4.16 Effect of substrate loading of UOW on ethanol by *S. cerevisiae*.

Considering different soluble sugars present in the UOW and comparing ethanol yields with respect to theoretical maximum ethanol of cellulosic fragment of OW (78% for concentrated hydrolysate and 96% for not concentrated hydrolysate) it can be conducted that all the reducing sugars present in the hydrolysate may not have been fermented by yeast. Therefore, separation of these soluble sugars prior to enzymatic hydrolysis step is suggested in the third approach in this study (Grohmann, 1995)

4.4 Approach 3: Investigation on utilization of partially treated OW for ethanol production

In this set of experiments, a simple pretreatment step was applied to OW and the pretreated biomass was subjected to enzymatic hydrolysis in 15% biomass loading, 2% cellulases, 4% pectinases. The enzymatic hydrolysate containing 16 g/L glucose was used in fermentation using *S. cerevisiae*. The cell growth, glucose consumption and ethanol productions are presented in Figure 4.17.
In this fermentation almost all the glucose was utilized during the first 10 hours and a constant ethanol concentration was also reached at the same time intervals. About 8 g/L of ethanol is produced using 16 g/L of glucose in the fermentation. The separated soluble sugars during the pretreatment might be consumed using a suitable method and recycled back to the fermentation to increase ethanol yield or can be used as a by-product of the process.

Ethanol yield gained after fermentation of partially treated OW was 96% and considering separation of buffer and acid soluble components in this method, our previous assumption that all sugars are not being consumed by *S. cerevisiae* is confirmed. Therefore, separation of soluble components prior to fermentation doesn’t have negative effect on fermentation yield. However, the pretreatment steps should be chosen in a way that they have minimum inhibitory effect on enzyme and micro-organism and the biomass loss shall be avoided as much as possible. The soluble components in this approach can be fermented for ethanol or other product production.
Table 4.4 Comparison of different yields in studied approaches

<table>
<thead>
<tr>
<th></th>
<th>Glucose yield (%)</th>
<th>Ethanol yield (%)</th>
<th>Overall ethanol yield (%)</th>
<th>Initial glucose concentration (g/L)</th>
<th>Ethanol yield % (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Approach 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cellulosic OW fragment)</td>
<td>91</td>
<td>96</td>
<td>4.5</td>
<td>7</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Approach 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(UOW)</td>
<td>88</td>
<td>99</td>
<td>10.5</td>
<td>55</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>Approach 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Partially treated OW)</td>
<td>91</td>
<td>96</td>
<td>7.8</td>
<td>16</td>
<td>0.8</td>
</tr>
</tbody>
</table>

In comparison of ethanol yields, the results are slightly higher for untreated OW (Table 4.4). This could be justified by effect of presence of other components which restricts accessibility of enzyme to cellulosic components. However, the yields are same for approaches 2 and 3 showing simple pretreatments are essentially effective for accessibility of cellulosic part to enzyme and thus its enzymatic hydrolysis. Ethanol yields however, are higher for approach 2 (with no pretreatment) might be justified by the presence of other soluble sugars in the medium that are slightly fermented and increased the ethanol yields. Overall ethanol yield which is the gram of ethanol produced regarding the gram of biomass subjected to system is the highest for approach 2 and reasonably high for approach 3. This is related to the initial glucose present in the hydrolysate which is directly connected to initial substrate loadings.

The utilization of only cellulosic components of OW for ethanol production is a new approach in regard to sustainable, multi-product OW biorefinery context. Despite the fact that this approach is a good way to make use of all components of OW in the future,
however there should be some ways to improve the process in regards of yields and the final ethanol produced.
Figure 4.18 Schematic presentation of material balance for Approach 1: isolation of cellulosic portion of OW and its utilization for ethanol production.
Figure 4.19 Schematic presentation of material balance for Approach 2: Ethanol production from UOW.
Figure 4.20: Schematic presentation of material balance for Approach 3: simply enrichment of cellulosic portion of OW and its utilization for ethanol production.
CHAPTER 5

CONCLUSION

Three approaches were defined for investigating the potential of ethanol production from OW. The first approach focused on separation of different carbohydrates from OW and then, utilization of isolated cellulosic fragment for ethanol production. Ethanol and glucose yields were quite satisfactory considering the cellulose content of the cellulosic fragment of OW. However, the volumetric amount of ethanol as the final product is not high enough. In the second approach in which untreated OW was utilized for ethanol production, the presence of soluble sugars facilitated to achieve higher yields and as well as, improved ethanol concentrations. However, this approach appears less promising since the production of other value added components were not considered in this approach. In the third approach simpler pretreatment steps were adopted for partial isolation of cellulosic component and the resulting glucose and ethanol yields were quiet satisfactory. The resulting ethanol production yields on the basis of the theoretical maximum yields for the first to the third approach were determined as 96, 99 and 96% (w/w), which correspond to 0.72, 2.7 and, 0.78% (w/v) (g ethanol/ 100 ml medium), respectively. Starting with initial glucose concentrations of 12, 55 and, 16 g/L in approaches 1, 2 and, 3 resulted in 7, 27 and, 8 (g ethanol/ L fermentation medium), respectively, indicating increasing ethanol yield by increasing initial glucose concentration. However, concentrating hydrolysate with the aim of increase in initial glucose concentration and thus increase in ethanol concentration was not successful due to the handling problems of the hydrolysate.

Isolation of the cellulosic fragment of OW is advantageous regarding the conversion of cellulose to glucose through enzymatic hydrolysis and subsequently, ethanol through fermentation. The present study is crucial since it not only offers conversion of cellulosic fragment of OW to ethanol, but also considers the utilization of other major components of the biomass.
Making use of less chemicals and energy in the pretreatment process and utilization of all components as different products should be the research area that has the potential to be more investigated in the future in terms of biorefinery of OW.
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APPENDIX A

PREPARATION OF DNS SOLUTION AND BUFFER

A.1 Preparation of 3, 5-Dinitrosalicylic Acid (DNS) Solution
For preparing 1 (% , w/v) DNS solution , 10 gr NaOH, 361.5 gr sodium potassium tartarate, 10gr 3,5- dinitroisalisylic acid, 2 gr phenol and 0.5 gr sodium solfite are dissolved in 1 L dionized water. The solution is filtered and kept in dark colored bottle till usage (Miller, 1959).

A.2 Preparation of 0.05 M Sodium Citrate Buffer
For preparation of 0.05 M Sodium Citrate buffer, 1.47 gr trisodiumm citrate and 1.05 gr citric acid monohydrate are dissolved in 100 ml dionized water separately. They acid is added to trisodiumm citrate solution till pH of 4.8 is acquired. The solution is stored at 4°C and used in 2 weeks.

A.2 Preparation of 0.2M acetate buffer
10.21g potassium hydrogen phthalate and around 226ml of 0.10M NaOH are added together till the pH is adjusted to 5(Sartorius Basic pH Meter PB-11 ,Goettingen, Germany).
APPENDIX B

ACID HYDROLYSIS PROCEDURE FOR COMPOSITION ANALYSIS AND LIGNIN AND ASH MEASUREMENT METHOD

B.1. Procedure for acid hydrolysis for composition analysis and lignin calculation (Sluiter, 2010):

1. Crucibles were kept in 575°C for 4 hours to make sure there was no carbohydrate.
2. 0.3 gr biomass was added to the tubes
3. 3ml sulphuric acid 72% was added (for 10 ml sulphuric acid 72% preparation, 6.64 ml water was added to 10 ml sulphuric acid 96%)
4. The tubes were put in water bath (Grant SUB-6 (Essex, UK)) 30°C c for 1 hour while mixing by glassware stirrer every 10 min
5. Solution was added to 4% sulphuric acid by adding 84 ml water
6. Sealed samples were put in autoclave for 1 hour at 121°C
7. Samples were cooled slowly at room temperature
8. Samples were vacuum filtered and the residue was kept for lignin analysis and filtrate was used for sugar analysis
9. Crucibles were washed with deionised water 5-6 times and dried at 575°C 2 hour and 105°C 1 hour and weighted after reaching stable weight.
B.2. Ash calculations:

The ash content is a measure of the mineral content and other inorganic matter in biomass (Sluiter, 2010).

1. Crucibles are dried at 105°C for 4 hours,
2. They are cooled in desiccator till constant weight,
3. The crucible with carbohydrate are then ramped to 105°C,
4. Kept at 105°C for 12 minutes,
5. Ramped to 250°C at 10°C/min,
6. Held at 250°C for 30 min,
7. Ramped to 575°C at 20°C/min,
8. Held at 575°C for 180 min,
9. Temperature is decreased to 105°C,
10. Kept in 105°C for 1 hour, then put in desiccator and weighted when constant weight is reached.

As air dry sample was used, the oven dry weight (ODW) of the sample, using the average total solids content should be calculated:

\[
Ash\% = \frac{Weight\ crucible\ plus\ ash - Weight\ crucible}{Dried\ sample\ weight} \times 100
\]

As air dry sample was used, the oven dry weight (ODW) of the sample, using the average total solids content should be calculated:

\[
Ash\% = \frac{Weight\ crucible\ plus\ ash - Weight\ crucible}{Dried\ sample\ weight} \times 100
\]
C.1. Pectinase activity measurements:

Polygalacturonase Activity (PG): PG activity was determined by measuring the release of reducing groups from citrus pectin using the 3, 5, dinitrosalicylic acid reagent (DNSA) assay (Miller, 1959). The reaction mixture containing 0.8ml 1% citrus pectin (Sigma) in 0.2M acetate buffer, pH 5.0 and 0.2ml of crude enzyme solution was incubated at 50°C for 10min (Silva et al., 2002). One unit (U) of enzyme activity was defined as the amount of enzyme which releases 1µmol of galacturonic acid per minute.

![Polygalacturonic acid calibration curve](image)

**Figure C.1:** Polygalacturonic acid calibration curve used for pectinase activity measurement.
C.2. Reducing sugar calibration curve (DNS Method) and glucose calibration data

From standard solution, calibration curve was drawn to relate glucose concentration to absorbance. And later on from absorbance of samples, their sugar concentration was estimated using the formula derived from the calibration curve.

![Glucose calibration curve used in DNS technique.](image)

**Figure C.2:** Glucose calibration curve used in DNS technique.

**Table C.1:** Calibration data for One Touch BGM

<table>
<thead>
<tr>
<th>Real Glucose Concentration (mg/ml)</th>
<th>Observed Glucose Concentration 1 (mg/dl)</th>
<th>Observed Glucose Concentration 2</th>
<th>Average Observed Glucose Concentration (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>51</td>
<td>49</td>
<td>50</td>
</tr>
<tr>
<td>0.75</td>
<td>91</td>
<td>91</td>
<td>91</td>
</tr>
<tr>
<td>1</td>
<td>121</td>
<td>134</td>
<td>127.5</td>
</tr>
<tr>
<td>1.25</td>
<td>171</td>
<td>172</td>
<td>171.5</td>
</tr>
<tr>
<td>1.5</td>
<td>218</td>
<td>221</td>
<td>219.5</td>
</tr>
<tr>
<td>y = 167.8x - 35.9</td>
<td>X: real glucose conc.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ y = 0.0082x - 0.2864 \]
\[ R^2 = 0.9996 \]
C.3. Biomass Calibration Curve for dry cell of *S. cerevisiae* NRRL Y-132:

In different time intervals samples were taken from medium containing pure glucose and their absorbance was read at 600 nm and the formula to relate glucose concentration to absorbance was derived. Later on from medium containing hydrolysate, samples were taken and their absorbance was read at 600 nm and using the calibration curve, the concentration related to absorbance was estimated (Haykir, 2013).

![Cell concentration vs. absorbance at 600 nm](image)

**Figure C.3:** Cell concentration vs. absorbance at 600 nm
C.4: HPLC standards for some sugars using BIORAD Aminex HPX-87H column

C.4.1: Glucose

![Graph of glucose HPLC standards curve](image1)

**Figure C.4.1**: HPLC standards curve for glucose.

C.4.2: Xylose

![Graph of xylose HPLC standards curve](image2)

**Figure C.4.2**: HPLC standards curve for Xylose.
C.4.3: Arabinose

**Figure C.4.3:** HPLC standards curve for Arabinose.

C.4.1: Ethanol

**Figure C.4.4:** HPLC standards curve for Ethanol.