CA-D(-)-LACTATE PRODUCTION FROM ORANGE BAGASSE VIA ENZYMATIC HYDROLYSIS AND FERMENTATION

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ABSTRACT

CA-D(-)-LACTATE PRODUCTION FROM ORANGE BAGGASE VIA ENZYMATIC HYDROLYSIS AND FERMENTATION

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The utilization of low-cost renewable carbohydrate sources for the production of lactic acid which is widely used organic acid in food and polymer industry is a recent strategy in bioeconomy. The objective of this work was to produce Ca-D(-) -lactate as a precursor for D(-)-polylactic acid through the bioconversion of orange bagasse (OB) by enzymatic hydrolysis and fermentation. The hydrolysis of OB carried with dried and fresh OB, and cellulolytic and pectinolytic enzymes at 55 °C, 150 rpm and 4.8 pH to evaluate the effect of biomass and enzyme loading, pectinase, drying and recycling of hydrolysate. Optimal results; 12.1 g/L glucose and 19.3 g/L total sugar conversion, were obtained by the hydrolysis of 10 % (w/v) solid load with 0.5 (w/v) % of cellulase mixture and 0.5 % (v/v) pectinase loading for 24 hours. It was observed that pectinase addition had a significant increase on saccharification. Ca-D(-)-lactate was produced by *Lactobacillus delbrueckii bulgaricus* OZZ4 and *Lactobacillus plantarum* OZH8 at 38 °C, 160 rpm with OB hydrolysate. The influence of growth media, filtration of FOB hydrolysate, inoculum size on batch fermentation and repetitive substrate addition for fed-batch fermentation was

evaluated. It was found that by employing centrifugation followed by filtration the antimicrobial effect of D-limonene was eliminated. Around 0.95 g/g and 0.50 g/g lactic acid yield ($Y_{L/T}$) were attained by both of the strains as a result of batch and fed-batch fermentation conducted with 5 % (v/v) inoculum size in fermentation medium containing eMRS broth and DOB hydrolysate, respectively.

Keywords: Orange bagasse, enzymatic hydrolysis, Ca-D(-)-lactate

ÖZ

ATIK PORTAKAL POSASINDAN ENZİMATİK HİDROLİZ VE FERMENTASYON İLE D-KALSİYUM LAKTAT ÜRETİMİ

Baltacı, Saadet Fatma

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Laktik asit fermentasyon ya da kimyasal işlemlerle üretilen, gıda ve polimer endüstrisinde yaygın olarak kullanılan bir organik asittir. Fakat, artan üretim maliyetinden dolayı düşük maliyetli alternatif karbon kaynakları ile laktik asit üretimi çalışmaları önem kazanmıştır. Bu çalışmanın amacı D(-)-polilaktik asitin öncü maddesi olan kalsiyum-D(-)-laktatın atık portakal kabuğu posasının (PKP) enzimatik hidrolizi ve sonrasında gerçekleşen fermentasyon işlemi ile üretilmesidir. Biyokütle ve enzim miktarı, pektinaz, kurutma ve hidrolizatın geri kullanımının etkilerini gözlemlemek için taze ve kuru PKP'nin selülolitik ve pektinolitik enzimler ile 55 °C, 150 rpm ve 4.8 pH'da hidrolizi gerçekleştirilmiştir. %10 (gram/hacim) katı madde %0.5(gram/hacim) selülaz ve %0.5 (hacim/hacim) pektinaz karışımı ile 24 saat hidroliz edildiğinde 12.1 g/L glikoz ve 19.3 g/L toplam şeker dönüşümü elde edilmiştir. Pektinazın önemli ölçüde sakarifikasyonu arttırdığı gözlenmiştir. Kalsiyum laktat *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 ve *Lactobacillus plantarum* OZH8 suşlarının 38 °C, 160 rpm'de PKP hidrolizatının fermentasyonu ile

üretilmiştir. Büyümü ortamı, hidrolizat filtrasyonu, aşı miktarı ve tekrarlı substrat eklemesinin etkileri incelenmiştir. D-limonene maddesinin antimikrobiyal etkisi, taze PKP hidrolizatından santrifugasyon ve filtrasyon işlemleri haricinde herhangi bir önişlem uygulanmadan yok edilmiştir. Zenginleştirilmiş MRS ortamı ve kuru PKP içeren fermentasyon ortamının iki *Lactobacillus* suşu ile aşılanması (%5 (hacim/hacim)) ile gerçekleştirilen kesikli ve beslemeli kesikli fermentasyon işlemi sonucunda yaklaşık olarak sırasıyla 0.95 g/g ve 0.5 g/g laktik asit verimi elde edilmiştir.

Anahtar Sözcükler: Portakal posası, enzimatik hidroliz, kalsiyum-D(-)-laktat

Dedicated to my parents;

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AFEX	Ammonia fiber explosion
ASA-BG	ASA Biogazyme 2x
ASA-P	ASA Pektinase L40
°C	Degree Celcius
C_P	Specific heat
CO_2	Carbon dioxide
ΔH_C	Heat of combustion
DOB	Dried orange bagasse
et al.	et alii.
FAO	Food and Agricultural Organization of the United Nations
FOB	Fresh orange bagasse
g	G-force
g	Gram
g/L	Gram per liter
GLA	Glucose lactate analyzer
h	Hour
HPLC	High performance liquid chromatography
LA	Lactic acid
LD	*Lactobacillus delbrueckii* subsp. *bulgaricus OZZ4*
LP	*Lactobacillus plantarum*
M	Molarity
Min	Minute
mg/dL	Miligram per deciliter
µL	Microliter
mL	Mililiter

MRS	De Man Rogosa and Shape
MT	Million tonnes
OD	Optical density
P	Productiviy
%	Percentage
pH	Power of hydrogen
PIA	Polyitaconic acid
PLA	Polylactic acid
POAP	Production of Organic Acids for Polyester Syntesis
RID	Refractive index detector
rpm	Revolution per minute
TSB	Tryptic soy broth
TUBITAK	The Scientific and Technological Research Council of Turkey
UV	Ultraviolet
(v/v)	Volume by volume
$\mathbf{Y_{L/T}}$	Yield

CHAPTER 1

INTRODUCTION

Over the last 40 years, the renewable energy concept has gained an importance with the recognition of adverse effects of petroleum and coal based energy production around the world, especially in the first world countries. Rather than using exhaustible resources for the production of energy and chemical products, the utilization of reusable biomass-based sources, lignocellulosic substances, in particular, has come into prominence with regards to economic and environmental issues (Tower, 2013).

Lignocellulose which is mainly contained cellulose, hemicellulose, and lignin with different amount is the major bioenergy and biomass resource found on the planet; therefore, many sustainable biotechnological processes have tried to be developed recently in order to benefit from the lignocellulosic substances by converting the cellulosic content to glucose and other fermentable sugars (R. Kumar, Singh, & Singh, 2008).

With the utilization of lignocellulosic resources, production of bioethanol, organic acids, and other industrial materials by fermentation has become more significant around the world in order to reduce increasingly expensive carbon source (starch, glucose, sucrose, etc.) dependency. One of the valuable industrial materials whose production from lignocellulosic substances is attempted lactic acid. Lactic acid and/or poly-lactic acid is an important substance can be widely used in food, chemical, pharmaceutical and polymer industries. Hence, it is vital to produce lactic

acid by considering economic, sustainable and environmental manners (Abdel-Rahman, Tashiro, & Sonomoto, 2010).

1.1. Properties of Lignocellulosic Biomass

Lignocellulosic materials, especially agroindustrial lignocellulosic wastes, are cheap, natural resources; hence, lignocellulosic biomass can be advantageous for the remediation of agricultural, environmental, high-cost energy production issues which humanity had to confront recently (Tower, 2013).

In other respects, especially the microorganism-based utilization of lignocellulosic resources is still not applied effectively due to several reasons. For instance, the utilization of lignocellulose is not fully understood to implement highly effective processes in the industry of biotechnology and energy is consumed more than adequate through the processes. Another problem is the conversion of cellulose and hemicellulose to simple sugars by the cellulase enzyme complexes because the production of enzymes is expensive. In order to overcome the difficulties in processing lignocellulosic substances, it is needed to make an improvement in the applied industrial technologies for the lignocellulose conversion to a valuable biomass efficiently (Tower, 2013).

1.2. Characterization of Lignocellulosic Biomass

Lignocellulose -a plant-based biomass- which mainly contains cellulose, hemicellulose and lignin in different amounts is a worldwide copious organic substance, particularly as agroindustrial residues. Approximately 90 % of plant cell wall is comprised of cellulose, hemicellulose, lignin in dry weight and the rest includes ash, pectin, and other extractives. The distribution of these subunits differentiates according to the kind of plant (Balat, 2011).

1.2.1. Cellulose

Cellulose as a natural carbon source is the main subunit of lignocellulosic substances and a plant cell wall with the proportion changing between 35% and 50% in dry

matter. Additionally, certain species of animals and bacteria can produce cellulose (Sims, 2013).

Cellulose structure as represented in Figure 1.1, is comprised of cellobiose sequences which are the basic repeating units of β-D-glucopyranose linked by β-1,4-glycosidic bonds linearly. It is a stringy complex polymer connected by hydrogen and van der Waals bonds due to microfibrils which form cellulose fibers. In a plant cell, cellulose can be found in the crystalline and amorphous structures and has no solubility in water (Kumar et al., 2009).

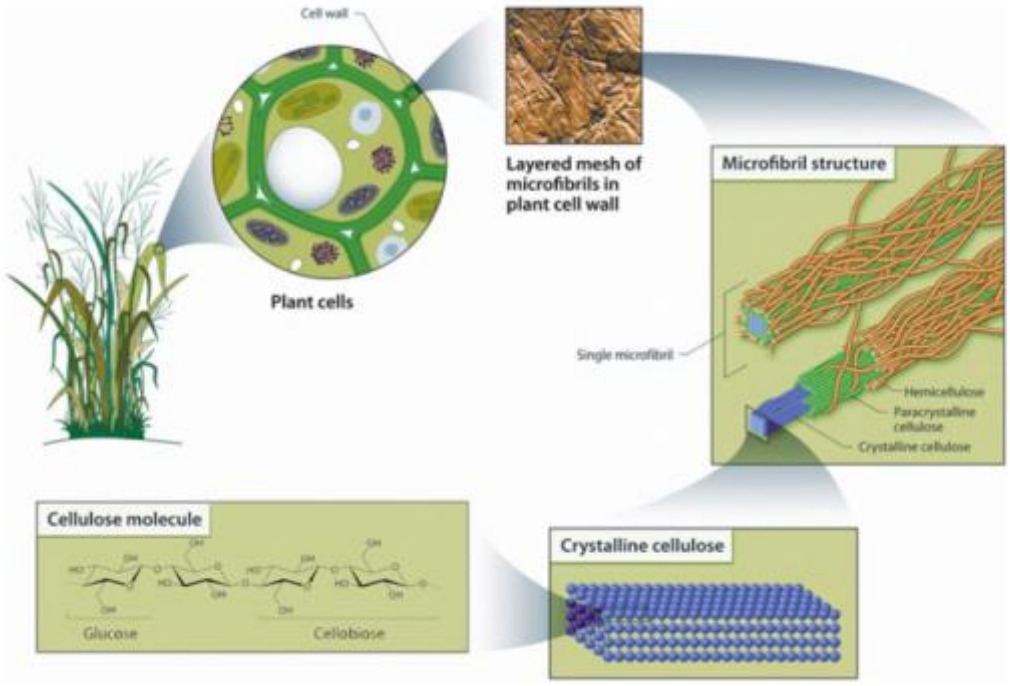

Figure 1.1 The structure of cellulose in plant cell wall (Asaadi, & Rojas, 2016).

Structural properties of cellulose which can vary with its plant origin have significant effects on its degradation to cellobiose and/or glucose. Crystallinity, surface structure and the polymerization degree of a cellulose polymer are the major parameters affecting the hydrolysis of cellulose (Yang et al., 2011). For instance, the proportion of crystalline form of cellulose is by far higher than amorphous form in many

substances, which complicates the deterioration of cellulose by enzyme or acid hydrolysis (Kumar et al., 2009).

1.2.2. Hemicellulose

Hemicellulose which surrounds the cellulose microfibrils is the other basic unit of lignocellulosic plant biomass with the proportion varying between 20% and 35% on dry bases (Wyman, 1999). Apart from storing carbon sources, other primary roles of hemicellulose in the plant cell wall are constituting the plant cell wall system and arranging the processes for cell growth (Tower, 2013).

As a heteropolymer, hemicellulose has a short, branched and amorphous structure and contains five and six-carbon monosaccharides such as D-arabinose, D-xylose, D-galactose, and D-mannose (Balat, 2011). The amount of basic sugar composition in hemicellulose chain can differ among the plant types. Hence, hemicellulose is generally named as its prominent sugar type such as xylan, glucan, mannan, arabinan, etc.

When compared with cellulose, hemicellulose is more susceptible to hydrolysis considering its less crystalline structure and low degree of polymerisation (Tower, 2013). Furthermore, hemicellulose do not assemble through the hydrolysis even tough hemicellulose and cellulose cocrystallize with each other (Kumar et al., 2009).

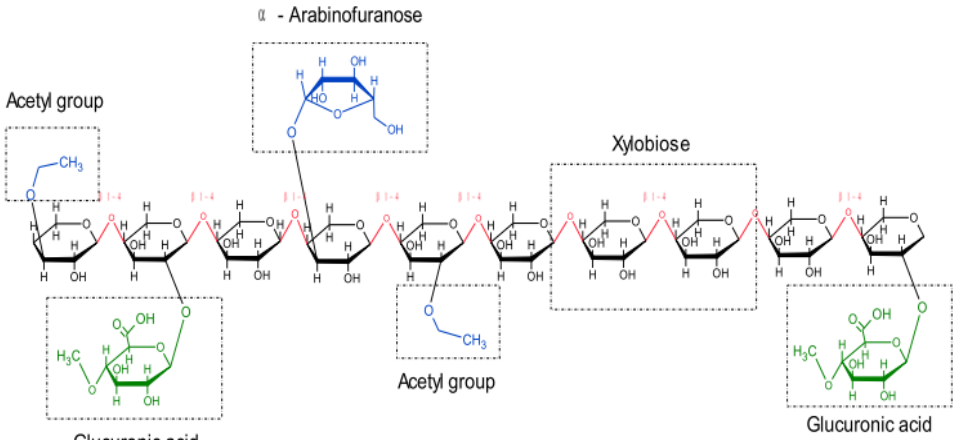

Figure 1.2 The structure of hemicellulose basic units (Chamaki, 2013).

1.2.3. Lignin

Lignin which constitutes approximately 10%-25% in a dry matter of plant cell wall is a complex, nonhomogeneous and three-dimensional phenolic polymer. It is formed by the complex, nonlinear and arbitrary linkages of phenylpropane subunits which have coniferyl alcohol, coumaryl alcohol, and sinapyl alcohol monomers (Figure 1.3) with varying amounts from plant to plant (Tower, 2013).

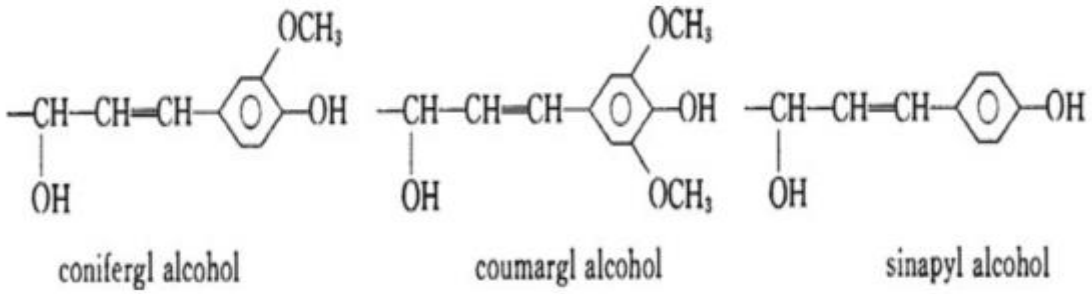

Figure 1.3 The chemical structure of basic subunits of lignin (Tower, 2013).

Lignin functions at the support and resistance mechanisms of plant cell wall and creates high impermeability to be a barrier to microorganisms and to prevent any oxidative stress.

Degradation of lignin is comparatively challenging than cellulose and hemicellulose by virtue of being insoluble in any solvent including water and incapable of optical rotation because lignin polymers include many hydroxyl and polar subunits, and they are bound by robust hydrogen bonds (Sims, 2013; Tower, 2013).

Disruption of lignin structure in the plant cell wall is crucial for the enzymatic hydrolysis of lignocellulosic substances in order to increase the enzyme accessibility to holocellulose and pectin, because lignin has strong interactions and cross-links with other carbohydrates (Ludwig, 1971).

1.2.4. Pectin

Pectin is an important industrial raw material, which is used as a gelifying agent, thickener, stabilizer or fat substitute, etc. It is commonly found in citrus fruits, apple, sunflower and other fruits in different amounts and properties (Akhtar, 1971; May, 1990)

Pectin is a linear compound settled in higher plants and it constitutes approximately 30% of the cell wall on dry basis. In the primary cell wall and the middle lamella of the cell wall, pectin participates in several mechanisms of plant cell such as structural integrity, cell growth and being a barrier to the external influences.

Pectin structure as shown in Figure 1.4, is formed by two major domains, which are homogalacturonan and rhamnogalacturonan I, and two minor domains, which are rhamnogalacturonan II and xylogalacturonan (Albuquerque et al., 2016).The main subunit in pectin is D-galacturonic acid which can be acetylated or methylesterifed and linked by a-(1,4)-glycosidic linkages constituting the main chain of homogalacturonan structure. Furthermore, the pectin molecule is formed by 17 different monosaccharides with different linkages. The major monosaccharides found in pectin are D-xylose, L-arabinose, D-galactose with varying amounts according to the type of the plant cell (Kaya et al., 2014).

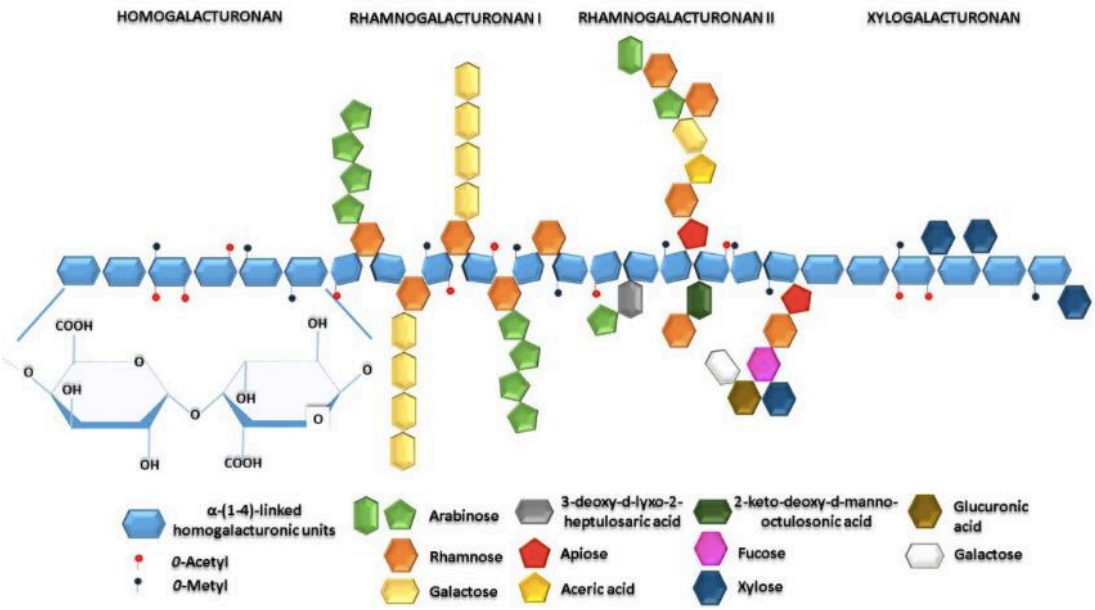

Figure 1.4 The schematic representation of pectin structure (B.S. Albuquerque et al., 2016).

In addition, pectic substances are mostly classified according to their esterification degree (Kaya et al., 2014). The degree of esterification varies among the type of pectin source. For instance, according to Zitko et al. (1965) the degree of esterification of citrus pectin was stated as 67 % while the esterification degree of the pectin in the apple was given as 62.5 % (Zitko & Bishop, 1965), the pectin found in orange waste also shows partial acetylation (Williamson, 1991).

1.3. Orange Bagasse as a Lignocellulosic Substance

According to the Citrus Fruit Statistics of FAO (2016) worldwide utilization of citrus fruits for processing in 2013/14 was 25,7 MT while the total production was 121,3 MT. Oranges constituted approximately half of the production of citrus fruits in the world and 21,2 MT of the total orange was utilized for processing (FAO, 2016).

Orange waste products are major citrus fruit wastes because this fruit is mostly utilized by the juice industry. After extraction process of orange in fruit juice industry, approximately half of the fresh fruits are discarded as a waste which

constitutes citrus peel, pulp, inner membrane, a core of the fruit and seeds. Even tough the residual bagasse of citrus fruit is a valuable by-product with regards to lignocellulose and pectin, it has been considered as a waste and needs to be treated (Mamma & Christakopoulos, 2008).

On the other hand, due to having a considerable amounts of insoluble carbohydrates and pectin, orange waste is a rich feedstock for biotechnological conversions (Grohmann, Cameron, & Buslig, 1995). Fortunately, with the awareness of economic and environmental benefits of lignocellulose bioconversion to valuable products, many scientific research projects have been conducted over the last 20 years. Thus, the negative effects coming out by the utilization of oranges can be reversed to benefits.

Utilization of orange may cause several economic and environmental disposal problems such as extra costs of waste treatment as drying because orange contains high amounts of water. Also, discarding of the orange waste to nature creates vitiation of soil and air by the occurence of undesired biogas (Dhillon et al., 2004).

1.3.1. The Characteristics and Composition of Orange Bagasse

Citrus fruits have two major segments which are pericarp including the peel and endocarp as the edible part. The outer part of orange peel which is called “flavedo” comprises chloroplasts and chromoplasts having color pigments of the fruit and encastered oil glands carrying essential oils with various properties such as D-limonene which has antimicrobial effect on microorganisms. The inner layer of the citrus fruit or mesocarp is named as albedo and where the juice vesicles are connected to constitutes the lignocellulosic structure of the fruit. In addition, albedo contains a high amount of pectin which is a valuable commercial by-product of citrus waste. The content and the thickness of albedo changes depending on type of the fruit (Hui, 2007).

Specifically, orange bagasse as a lignocellulosic biomass approximately contains 37.1 % cellulose, 11 % hemicellulose, 7.5 % lignin and 23 % pectin in dry matter (Boluda-Aguilar & López-Gómez, 2013). In the soluble part of orange which

comprises about 50% in dry weight glucose, sucrose and fructose are found. Besides, cellulose, hemicellulose, and pectin are the insoluble carbohydrates of orange bagasse. These polysaccharides are the source of glucose, galactose, arabinose and galacturonic acid in considerable amounts and xylose, rhamnose, uronic acid and mannose in minor quantities (Torrado et al., 2011). All the chemical and nutritional compounds of orange are briefly listed in Table 1.1.

The percentage of constituents in orange has a significant importance due to the pretreatment and processing methods for the bioconversion of citrus waste to valuable products. For instance, orange bagasse is a proper biomaterial for the enzymatic hydrolysis because it contains low amount of lignin or for the production of polygalacturonase due to containing high amount of pectic compounds (Boluda-Aguilar & López-Gómez, 2013; Choi et al, 2013; Ahmed & Mostafa, 2013).

Several parameters such as maturity, circumstances during growth, climate, and harvest season may influence the content of citrus fruit. According to the study of Wilkins et al. (2005), the amount of the constituents such as soluble and insoluble carbohydrates, pectic enzymes in orange waste products may change due to the harvest season and the maturity of the fruit; for instance, while the amount of cellulose or simple soluble sugars increase, the amount of pectin decreases with the maturity, which affects the quantity of arabinose and galacturonic acid correspondingly (Mark R. Wilkins, Wilbur W. Widmer, Randall G. Camero, 2005).

Table 1.1 The Chemical and Nutritional Composition of Citrus Fruit By-Products (Bampidis & Robinson, 2006)

Soluble and Insoluble Carbohydrates	**Lipids**	**Organic acids**	**Enzymes**	**Flavonoids**	**Bitter Substances and Peel Oil**	**Volatiles and Pigments**	**Vitamins and Minerals**
Glucose	Oleic acid	Citric acid	Pectinesterase	Hesperidin	Limonin	Alcohols	Ascorbic acid
Sucrose	Linoleic acid	Malic acid	Phosphatase	Naringin	Isolimonin	Aldehydes	Vit B complex
Fructose	Palmitic acid	Tartaric acid	Peroxidase		D-Limonene	Ketones	Carotenoids
Cellulose	Glycerol	Benzoic acid				Esters	Calcium
Hemicellulose	Phytosterol	Oxalic acid				Carotenes	Potassium
Pectin		Succinic acid				Xanthophylls	

1.4. Pretreatment Methods

In order to perform an effective enzymatic hydrolysis with lignocellulosic substances, it is crucial to apply necessary pretreatment method for the destruction of the crystalline structure of cellulose and physical bindings with lignin in the cell wall which increases enzyme-substrate interaction. In addition, applying pretreatment methods before enzymatic saccharification can reduce the cost of the process with regards to eliminating several compounds which are toxic for fermentation, using enzymes in lower amounts, obtainment of more purified products and other process conditions. The key point that should to be considered through the application of pretreatment methods with high efficiency is to abstain from the usage of excess energy consumption and high-priced chemicals (Wyman et al., 2005).

Besides increasing the efficiency of enzymatic hydrolysis by destroying lignocellulosic structure, complete and partial separation of D-limonene before hydrolysis or fermentation step is another issue as far as the bioconversion of orange bagasse is concerned. Even though the D-limonene has a protective effect on the deterioration of orange from microorganisms such as mold, bacteria and virus, it prevents the bioproduction of organic compounds by microorganisms or decreases the yield of the process (Stewart et al., 2006). Hence various studies were conducted to remove limonene from the orange waste by steam explosion, steam distillation, leaching or biotransformative application with different process yields (Ruiz et al., 2016).

This section covers the physical, chemical, biological and physicochemical processes can be applied alone or combined with each other for the pretreatment of lignocellulosic substances before the hydrolysis.

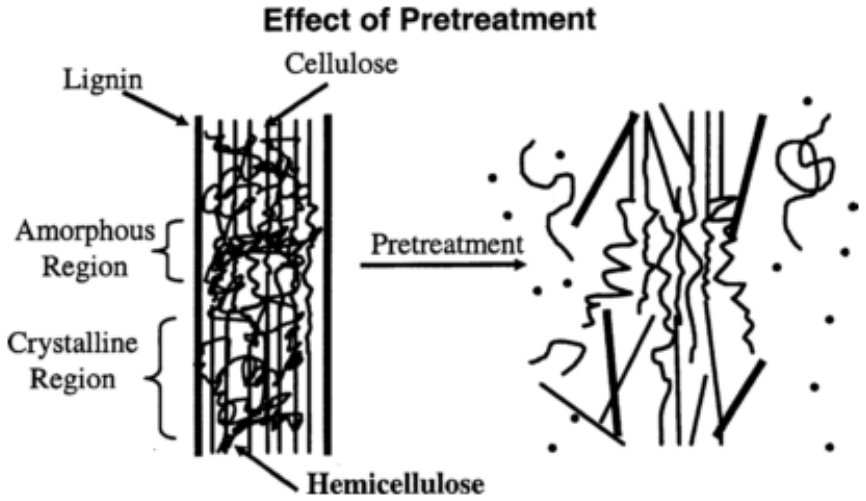

Figure 1.5 The schematic representation of the effect of pretreatment on lignocellulosic biomass (Hsu et al., 1980).

1.4.1. Physical Pretreatment Methods

The main aim of physical pretreatment of lignocellulose is to decrease the crystallinity of cellulose, polymerization degree increase in the surface area for an effective enzymatic hydrolysis. Several physical processes such as grinding, shredding or pyrolysis with oxygen can be applied as a pretreatment method (Mosier et al., 2005).

By grinding or milling, it is aimed to reduce the particle size of lignocellulosic biomass properly and to ease the handling and storage of biomass (Sidiras, 1989).

Moreover, irradiation by gamma-rays, microwaves or electron beams can be applied as a physical pretreatment in order to break β-1,4-glycosidic linkages and reduce crystallinity; however, application of this method is not cost effective when compared with the others (P. Kumar et al., 2009).

1.4.2. Chemical Pretreatment Methods

For the chemical pretreatment of lignocellulosic substances, various methods are applicable to increase the yield of enzymatic hydrolysis. The methods which are studied recently are briefly summarized below.

One of the most applied methods is acid treatment. Lignocellulosic biomass can be treated with concentrated or dilute acidic solutions to disrupt lignin structure and hydrolyze hemicellulose to pentoses mainly xylose. Mainly concentrated sulfuric or hydrochloric acid and dilute sulfuric acid are used. Even though, acid treatment enhances the hydrolysis of cellulose, the drawbacks of the method such as the high-cost construction of the system which is resistant to corrosive effects of acids, the creation of toxic compounds for further fermentation step, etc. complicate the process (Kumar et al., 2009).

Another common chemical pretreatment method is an alkaline based pretreatment. Hydroxides of sodium, potassium and calcium are suitable bases for the alkaline pretreatment for the partial removal of lignin and hemicellulose, surface area increase and reduction of polymerization degree. Even though mild process conditions (temperature, pressure, etc.) are applicable and toxic residues are not produced through the alkaline pretreatment, the required process time is long. In addition, monosaccharide degradation is comparatively low and some undesired salts can be produced through the process (Taherzadeh & Karimi, 2008).

Ozone can be applied to degrade lignin in the biomass. Although the process is achieved at mild conditions and without toxic matters, excess amount of ozone is needed which increases the cost of the method (Myat & Ryu, 2016)

Furthermore, lignin and hemicellulose degradation can be performed by the use of peroxidase enzyme with hydrogen peroxide or organic solvents such as ethanol, acetone and ethylene glycol with inorganic or organic acids. However, these methods are not cost effective and solvents recovery is needed to prevent undesired effects on hydrolysis and fermentation processes (Kumar et al., 2009).

1.4.3. Physicochemical Pretreatment Methods

Steam, ammonia-based and carbon dioxide explosions can be applied as physicochemical methods which are the combination of both physical and chemical processes.

In steam explosion method which is performed widely, decomposition of lignin and hemicellulose is obtained by a sudden pressure drop in the system which is treated with high pressure saturated steam. The explosion affects lignin distribution and hemicellulose deterioration and elimination from cellulose microfibrils by some organic acids occurred through the process. Correspondingly, the rate of cellulose hydrolysis increments by the increase in the surface area and the ability of enzymes to access the microfibrils of cellulose. Steam explosion process is affected by particle size, temperature, retention time and moisture content of the biomass. It is considered as one of the most industrially applicable methods. It requires lower amount of energy and does not create extra costs in terms of environmental issues (Kumar et al., 2009). Despite the benefits of steam explosion method, the process can cause undesired xylan deterioration and may create several substances which may inhibit microorganisms used in the fermentation process (Mackie et al., 1985).

Another physicochemical method to destruct lignin and hemicellulose structure partially and increase the accessibility of enzymes to cellulose is ammonia fiber explosion method abbreviated as AFEX. AFEX is carried out by a sudden pressure drop in the system which has been treated with liquified ammonia at high pressure and temperature. Despite the method is advantageous with regards to breaking the bonds among lignin and carbohydrates and not producing inhibitory by-products, the application of the method is not very efficient in removal of lignin and hemicellulose especially with lignocellulosic substances containing high amount of lignin. Hence, the accumulation of lignin effects the attachment of cellulase enzyme to cellulose. Moreover, after the application, ammonia should be recovered which increases the cost of the process (Brodeur et al., 2011).

Explosion by CO_2 in which carbonic acid is formed as a consequence of CO_2 dissolution in water is also applied in order to ease the hydrolysis of hemicellulose

and cellulose by distracting complex cellulose and hemicellulose structure. The decomposition of simple sugars by acids through the pretreatment is limited thanks to the low process temperature (Kim & Hong, 2001).

1.4.4. Biological Pretreatment Methods

In order to remove lignin and utilize hemicellulose effectively, fungi especially white-rot fungi are used in the biological treatment of lignocellulosic biomass and several antimicrobial components can be removed. During the process, enzymes as laccase and peroxidases promote the lignin deterioration (Myat & Ryu, 2016) .

In contrary to many pretreatment methods, biological applications do not consume excess energy and do not damage the equipment and environment. Nonetheless, after biological pretreatment, the yield of hydrolysis is reported to be very low (Kumar et al., 2009).

1.5. Enzymatic Hydrolysis of Orange Bagasse

For the production of value-added industrial products such as ethanol, lactic acid, etc. from utilizing lignocellulosic raw material, many biotechnological processes which may have advantages or disadvantages can be applied. One of the intermediate steps is the enzymatic hydroysis of pre-treated or non-treated raw material to obtain fermentable monosaccharides as a carbon source for the following fermentation step.

The attainment of simple sugars by enzymatic hydrolysis is advantageous over acidic or alkaline-based hydrolysis due to non-corrosive effects of mild and green process conditions and having higher saccharification yield. On the other hand, the process can be considered as expensive due to the cost of enzymes and longer process time for complete depolymerization (Ylitervo, 2008).

Orange bagasse as a lignocellulosic waste contains the substantial amount of cellulose, hemicellulose and pectin, which are the important source of fermentable sugars such as glucose, galactose, arabinose and xylose (Dhillon et al., 2004). Hence,

the enzymatic hydrolysis of orange bagasse is performed with cellulolytic and pectinolytic enzymes mainly produced by microorganisms.

To obtain of high sugar conversion yields with low enzyme load, it is vital to understand both the lignocellulosic structure of biomass and the degradation mechanisms of cellulolytic and pectinolytic enzymes on the biomass (Yang et al., 2011).

1.5.1. Cellulase

Cellulase is an enzyme mixture mainly including endo-1,4-β-glucanase, exo-1,4-β-glucanase, β-1,4-glucosidase and used for the hydrolysis of cellulosic substances. Several aerobic, anaerobic, mesophilic or thermophilic bacterial and fungal strains produce these cellulolytic enzymes. For instance, some *Trichoderma* sp., especially *T. reesei*, are commonly used because they have high production capacity and their enzymes are more effective on the degradation of the crystalline structure of cellulose (Balat, 2011).

The enzymes in cellulase mixture hydrolyze cellulose in synchrony as represented in Figure 1.6. Exo-glucanases cleave β-1,4-glucosidic bonds located at the non-reducing sugar end of cellulose to form cellobiose. Endo-glucanase enzyme which consists of 20-30 % of cellulase mixture catalyzes cellulose dissolved in water by cleaving β-1,4-glucosidic bonds in the amorphous structure of cellulose randomly and forms cellobiose, glucose, cellotriose and dextrin molecules in various sizes.

Cellobiase or β-glucosidase, approximately 1% of cellulase multi-enzyme mixture, catalyze cellobiose and dextrin found at the non-reducing end of cellulose into glucose molecules in order to finalize cellulose hydrolysis and prevent product inhibition that arises from the accumulation of cellobiose. Besides the main cellulolytic enzymes explained above, cellobiose dehydrogenase, phosphorylase, cellulosome, etc. can be included in the cellulase (Tower, 2013).

According to the literature, a significant decrease is seen on the enzymatic activity of cellulase through the hydrolysis due to the irrecoverable attraction between cellulase

and cellulose. In order to reduce the deactivation of cellulose, several surfactants such as polyoxyethylene glycol and polysorbate 20 or 80, etc. can be added to the hydrolysis medium (Sun & Cheng, 2002; Kristensen, 2009).

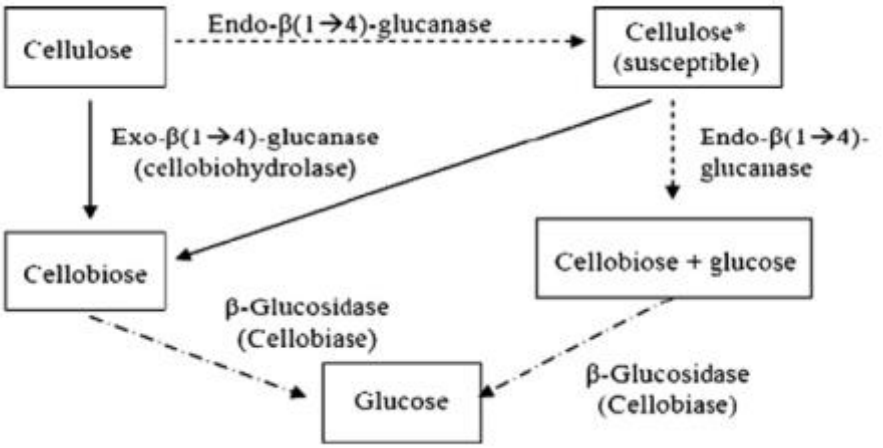

Figure 1.6 The schematic presentation of the mechanism of cellulolytic enzymes (Kim, 2004).

1.5.2. Hemicellulase

In order to increase the yield of cellulose hydrolysis, hemicellulose which surrounds the cellulose structure need to be disrupted (Yang et al., 2011). Thus, hemicellulase can be used to degrade hemicellulose to oligosaccharides or monosaccharides and the digestibility of cellulose increases. Hemicellulase, subgroup of glycanhydrolases, is comprised of several enzymes such as endoxylanase, exoxylanase, β-xylosides, D-xylan-glucosidase, L-arabinanase, D- semiarabinogalactanase and D-mannanase with different objectives (Saha, 2003), (Buschle-Diller et al., 1999).

Similar to cellulase enzyme, hemicellulases are produced by some bacterial and fungal species such as *Trichoderma, Aspergillus, Clostridia, Streptomyces*, etc. (Motta et al., 2013).

Hemicellulase can also be termed as xylanase due to the existence of high amounts of xylan, which is the second common natural carbon source in hemicellulose structure. Some of the xylanase enzymes are β-1,4-exoxylanase which degrades xylan to xylose, a non-reducing end of xylan structure or endo-1,4-β-D-xylanase which creates xylooligosaccharides and decrease the polymerization degree by randomly breaking xylan β-1,4-glycosidic bond found in the xylan main chain. Side-chain molecules of xylan, arabinose, galactose, acetyl and glucuronic acid, varies in nature depending on the types of biomass. Hence, for effective and entire catalysis of xylan, different enzymes are needed to be applied simultaneously (Kristensen, 2009).

In addition, to prevent xylanase inhibition, side-chain molecules are required to be degraded by various glycosidases such as acetylesterase, α-L-arabinosidases, α-D-glucuronidase, or ferulic acid esterase which cleave by glycosidic binding between xylose and side-chain molecules (Motta et al., 2013).

1.5.3. Pectinase

Pectinases are the enzyme mixture which approximately 10 % of total enzyme production are responsible for hydrolysis and deestrerification of complex macromolecular pectic substances (Pedrolli et al., 2009).

Pectinase is widely used in beverage, textile and waste treatment industries and despite the production of pectinases by a large number of microorganisms such as fungi, bacteria, yeasts, insects, protozoan, nematodes and plants, they are mainly generated by saprophytic fungi in nature. Additionally, for the industrial applications, while acidic pectinases are generally produced by *Aspergillus niger*, alkaline pectinases are mainly generated by some *Bacillus* species and several filamentous fungi and yeasts (Dhillon et al., 2004; May, 1990).

Pectinases are comprised of different type of pectinolytic enzymes acting synergistically such as protopectinases which generate soluble and polymerized pectin from protopectin, pectin methyl esterase which deesterifies pectin methoxyl group to generate methanol and pectic acid, polygalacturonases which form D-galacturonate by cleaving α-1,4-glycosidic bonds found in polygalacturonic acid,

pectin lyases which generate unsaturated oligogalacturonates by disrupting glycosidic bonds in highly esterified pectin or the enzyme groups which catalyse the rhamnogalacturonan structure (Jayani, Saxena, & Gupta, 2005; Pedrolli et al., 2009).

1.6. Lactic Acid

The discovery of lactic acid dates back to 1780's when Swedish chemist Scheele thought as it was one of the components of milk. Lavoisier called lactic acid as "acide lactique" in 1789 and eventually, in 1857 it was revealed as a fermentation product by Pasteur (Wee, Kim, & Ryu, 2006).

After the discovery of lactic acid as a fermentation product, lactic acid produced microbially and industrial production started by Boehringer Ingelheim in Germany in 1895 (Vijayakumar et al., 2008).

Lactic acid is an industrially important and one the most abundant organic acids worldwide. Lactic acid and its salts have been widely used in various number of industries such as food, chemical, pharmaceutical and cosmetic for different purposes such as being acidulants, preservatives, descaling agents, cleaning agents, dialysis solution, prostheses, pH regulators, humectants, etc., respectively (Wee et al., 2006).

Besides the use of lactic acid or salts of lactic acid in the industry, polymers of lactic acid, D-polylactic acid and L-polylactic acid, have been recently preferred to produce biosoluble polyesters and textile products to substitute petroleum-derived plastics, which is particularly important for environmental sustainability (Ilme et al., 2007).

Around the world, lactic acid is manufactured in two ways: microbial and chemical productions (Figure 1.7). While lactic acid is chemically synthesized from hydrogen cyanide or acetaldehyde, it can be produced microbially through fermentation by several bacterial and fungal species. With chemical production methods, racemic DL-lactic acid is obtained from petroleum-based resources, while by microbial production mainly optically pure isomers of lactic acid are attained via fermentation of several carbon sources or renewable raw materials by selection of proper

microorganism (Hoshinai, 2005). The microbial production of lactic acid which comprises approximately 90 percent of total production and it is advantageous over chemical synthesis due to the obtainment of heat stable, crystalline polylactic acid production and environmental issues (Scopes, 2002).

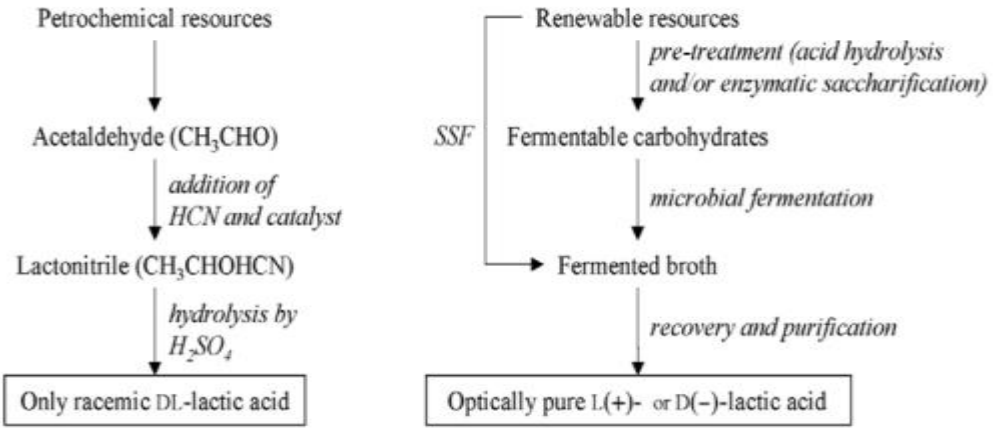

Figure 1.7 The schematic figuration of chemical and microbial production of lactic acid (Wee et al., 2006).

1.6.1. Physical and Chemical Properties of Lactic Acid

Lactic acid, CH3CHOHCOOH, is an organic acid with two optically isomeric forms as L(+) lactic acid (dextrorotatory) and D(-) lactic acid (levorotatory). Even though both forms have the same molecular formula, they show different physical properties which were given in Table 1.2.

Figure 1.8 The structure of the optical isomers of lactic acid (Lockwood, Yoder, & Zienty, 1952).

Only L (+) isomeric form of lactic acid is metabolized in the human body due to the existence of L-lactate dehydrogenase enzyme; hence, it is vital to use only L (+)-lactic acid is in the products of food industry. In other respects, polymers of optically pure D (-)-lactic acid is more valuable for the manufacture of biodegradable products in chemical and medical industries (Hirayama, & Uede, 2004).

Table 1.2 The physical features of lactic acid (Narayanan, 2004).

Molecular weight	90.08
Melting point	16.8ºC
Boiling point	82ºC at 0.5 mm Hg 122ºC at 14 mm Hg
Dissociation constant, K_a at 25ºC	1.37x 10-4
Heat of combustion, ΔH_C	1361 KJ/mole
Specific heat, C_p at 20ºC	190 J/mole/ºC

1.6.2. The Properties and Production of Lactic Acid

Recently, with the increase in environmental awareness, the demand for the production of environmentally friendly and biodegradable plastic materials has

increased. One of the raw materials which can be used for that purpose is polylactic acid abbreviated as PLA. PLA as a biocompatible thermoplastic raw material has approximately the same physical properties with polystyrene or polyester such as transparency, permeability characteristics, etc (Abdel-Rahman, Tashiro, & Sonomoto, 2013; Henton, Gruber, Lunt, & Randall, 2005).

PLA which is a high molecular weight and the aliphatic polymer of lactic acid is not found in nature and it can be produced by polymerization of cyclic diesters of lactic acid (Tower, 2013). Polymerization, represented in Figure 1.9, starts with the condensation of lactic acid molecules to form dilactides, primary molecules of PLA, with the aid of several basic catalysts which are necessary for an increase in yield and selectivity of process (Datta & Henry, 2006).

For the synthesis of good quality and high crystalline PLA, the production of optically pure D-lactic or L-lactic acid is needed, which is only possible with the microbial production. Hence, research studies for industrial applications have focused on microorganism-based production and polymerization of lactic acid lately. For instance, poly-L-lactic acid (PLLA) is used in medical industry especially for the treatments of joint health thanks to the advantageous characteristics of PLLA such as tensile strength, purity and viscosity (Narayanan, 2004).

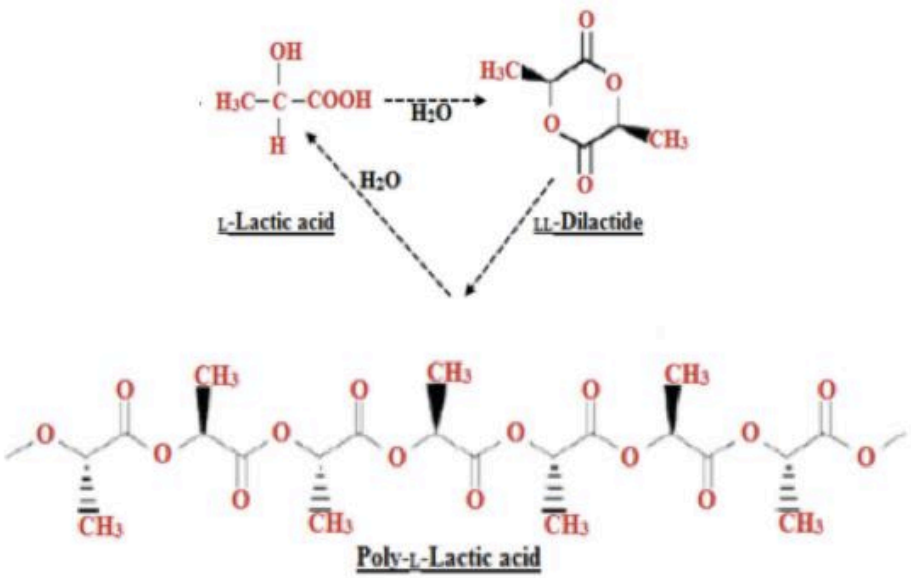

Figure 1.9 The representation of L (+)-lactic acid polymerization (Ghaffar et al., 2014).

1.6.3. The Microbial Production of Lactic Acid and Microorganisms

Lactic acid is produced microbially through the fermentation process. By microbial production, optically pure isomers of lactic acid are obtained with appropriate microorganism selection, which is specifically significant for polylactic acid production. Besides, the application of biotechnological methods consisting of lactic acid fermentation and purification processes are highly important for utilization of biodegradable and renewable resources which are discarded to nature as a waste and increase hydrocarbon pollution and lowering the production costs and energy consumption (Abdel-Rahman et al., 2013).

Lactic acid is microbially produced by several species of bacteria, fungi, cyanobacteria, yeasts and algae. The yield of lactic acid, preferred carbon source, the percentage of optical purity, etc. differ for each microorganism (Abdel-Rahman et al., 2013).

Generally, lactic acid bacteria (LAB) which belong to the genera of *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Leuconostoc* and *Streptococcus* are preferred for the commercial biotechnological production of lactic acid (Wee et al., 2006).

Lactic acid bacteria isolated from different sources in nature are Gram-positive, anaerobic or facultative-anaerobic, cocci or lactobacilli rod-shaped and they do not form spores. In addition, according to the utilization of carbon source or end product of lactic acid fermentation, LAB can be characterized as homofermentative and heterofermentative and the two pathways of lactic acid production is shown in Figure 1.10.

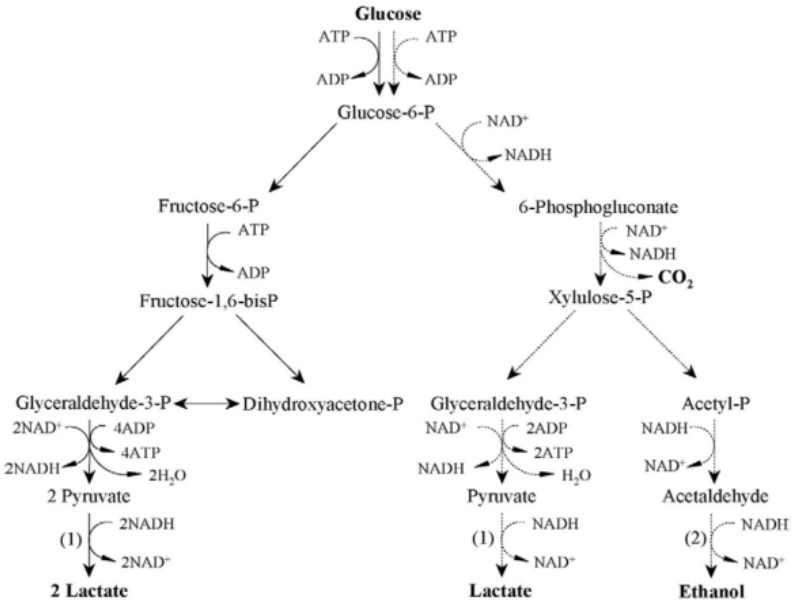

Figure 1.10 The schematic representation of homo and heterofermantative pathways: (1) lactate dehydrogenase, (2) alcohol dehydrogenase (Wee et al., 2006).

Homofermentative LAB (listed in Table 1.3) produce mainly lactic acid (more than 85 percent) by utilizing glucose in a molar ratio of 2:1 through Embden-Meyerhof-Parnas Pathway (Boontawan, 2010). In other respects, heterofermentative LAB (listed in Table 1.4) produce both lactic acid and other metabolites such as acetate, ethanol, carbon dioxide, etc. by utilizing glucose and other hexoses or pentoses

thorough phosphoketolase pathway. Through the heterofermentative pathway, the redox potential affects the formation of acetate and ethanol (Hofvendahl & Hahn–Hägerdal, 2000). Nevertheless, the use of heterofermentative LAB for commercial production of lactic acid is not favorable due to a low yield of lactic acid production (Kaya et al., 2014).

Despite the use of lactic acid bacteria for the biotechnological production of lactic acid, several species of *Rhizopus* is also used for the production of lactic acid from glucose, starch, etc (Ilme et al., 2007). While lactic acid production by species of *Rhizopus* can be advantageous in terms of not requiring complex fermentation medium, the yield of lactic acid production is not high due to the occurence of some by-products at the end of fermentation process and effective aeration is needed for the aerobic production of lactic acid by which the production cost increases.

Table 1.3 The list of homofermentative producers of lactic acid (Vijayakumar, Aravindan, & Viruthagiri, 2008).

Microorganisms	**Lactic Acid Configuration**	**Microorganisms**	**Lactic Acid Configuration**
Lactobacillus		***Streptococcus***	
L. delbrueckii	D (-)	*S. faecalis*	L (+)
L. lactis	D (-)	*S. cremoris*	L (+)
L. bulgaricus	D (-)	*S. lactis*	L (+)
L. casei	L (+)	***Pediococcus***	
L. plantarum	DL	*P. damnosus*	DL

Table 1.4 The list of heterofermentative producers of lactic acid (Vijayakumar, Aravindan, & Viruthagiri, 2008).

Microorganisms	**Lactic Acid Configuration**	**Microorganisms**	**Lactic Acid Configuration**
Lactobacillus		***Leuconostoc***	
L. brevis	DL	*L. mesenteroides*	D (-)
L. fermentum	DL	*L. dextranicum*	D (-)
Bifidobacterium			
B. bifidum	L (+)		

1.7. Aim of the Study

This scientific study was supported by The Scientific and Technological Research Council of Turkey, TUBITAK, and done within the scope of Era-Net Project called as "Production of Organic Acids for Polyester Synthesis" (POAP). The subject of the project was the assessment of agricultural wastes such as citrus peel waste and wheat chaff for poly-D-lactic acid and poly-itaconic acid production via enzymatic hydrolysis, fermentation, purification and polymerization steps by seeking environmental issues. The simple flow chart of the project can be found in Figure 1.11.

The thesis study only covers the enzymatic hydrolysis of orange bagasse with cellulolytic and pectinolytic enzymes for the obtaining of fermentable sugars and Ca-D-lactate production via the fermentation process by lactic acid bacteria. It was aimed to state proper conditions of hydrolysis and fermentation processes for the bioconversion of orange bagasse to lactic acid in green methods. The effect of substrate and enzyme loads, enzyme combinations on hydrolysis were investigated. Furthermore, the effect of serial enzymatic hydrolysis on final monosaccharide concentration was examined. Meanwhile, it was investigated how growth media, inoculum size, the pretreatment of orange bagasse as a preliminary step influenced

the yield of Ca-lactate production. Moreover, a different approach which could be more suitable for industrial applications was applied for the production of lactic acid by repetitive substrate addition in order to increase lactic acid concentration.

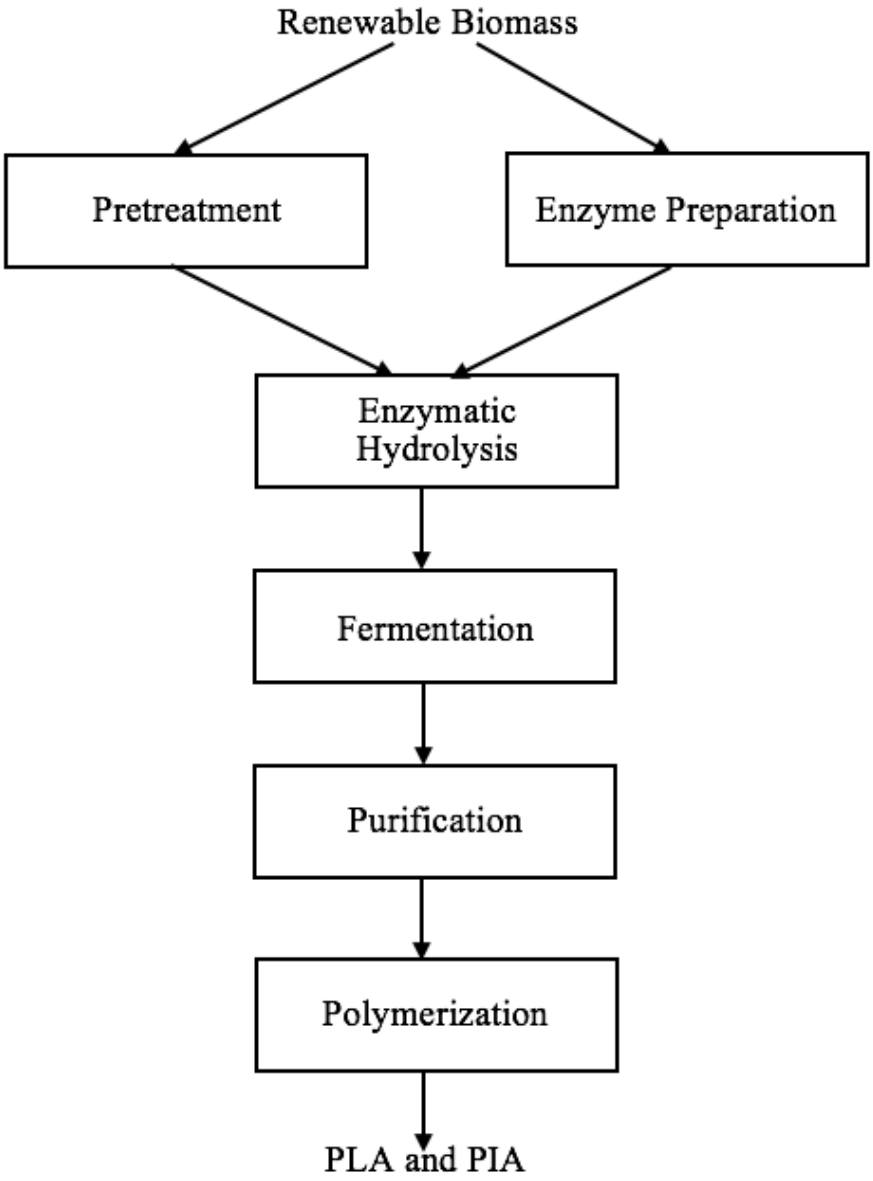

Figure 1.11 Simple flow chart presentation of processes in POAP project.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Fresh orange bagasse was obtained from Belso A.Ş, fruit juice manufacturer operated by Ankara Metropolitan Municipality, Turkey and Susam Bakery in Ankara, Turkey.

All cellulolytic and pectinolytic enzymes; ASA Biogazyme 2x and ASA Pektinase L-40 were kindly supplied by ASA Spezialenzyme GmbH in Wolfenbüttel, Germany.

Lactobacillus strains used in the study were kindy provided by research assistant Harun Önlü, Muş Alparslan University, with the supervision of Prof. Dr. Özlem Osmanağaoğlu in Ankara University in Turkey.

2.1.1. Chemicals

All the chemicals required in the experiments and analyses were chosen as an analytical grade. The list of chemicals and their commercial producers are given in the Table 2.1.

Table 2.1 The list of chemicals and their brands.

Chemicals	**Producers**
Citric acid monohydrate	Merck (Darmstadt, Germany)
Tri-sodium citrate dihydrate	Merck (Darmstadt, Germany)
Sodium hydroxide (NaOH)	Merck (Darmstadt, Germany)
D- (+)-Glucose monohydrate	Sigma-Aldrich (St. Lois, MO, USA)
D- (+)-Galactose	Fluka Chemie GmbH (Germany)
D- (+)-Xylose	Sigma-Aldrich (St. Lois, MO, USA)
L- (+)-Arabinose	Fluka Chemie GmbH (Germany)
D- (-)-Fructose	Merck (Darmstadt, Germany)
Sucrose	Merck (Darmstadt, Germany)
D- (+)-Cellobiose	AppliChem GmbH (Germany)
TGY Broth	Merck (Darmstadt, Germany)
MRS Broth	Merck (Darmstadt, Germany)
Peptone from meat	Merck (Darmstadt, Germany)
Tween 80	Merck (Darmstadt, Germany)
Yeast extract	Merck (Darmstadt, Germany)
Calcium carbonate	Merck (Darmstadt, Germany)
Magnesium sulfate heptahydrate	Merck (Darmstadt, Germany)
Manganese (II) sulfate	Horosan Kimya
Dihydrogen potassium sulfate	Sigma-Aldrich (St. Lois, MO, USA)
D- (-)-Lactic acid	Sigma-Aldrich (St. Lois, MO, USA)
Glucose, L-Lactic acid standard	YSI Incorporates (USA)

2.1.2. Enzymes

ASA Biogazyme 2x, *Trichoderma spp.* originated, was the mixture of cellulase and hemicellulase enzymes which are exo-cellulase (EC 3.2.1.91), endo-glucanase (EC 3.2.1.4), β-glucosidase (EC 3.2.1.21), xylanase (EC 3.2.1.8), endo-mannanase (EC 3.2.1.78), β-1,3(4)-glucanase (EC 3.2.1.73) and ASA Pektinase L40, *Aspergillus niger* originated, included polygalacturonase, pectin depolymerase (EC 3.2.1.15). The enzyme activities were determined by the methods of ASA and listed in the table below.

Table 2.2 Activity valuses of ASA enzymes (Product Sheet, ASA).

Enzyme Name	**Activity**
Exo-cellulase	> 600 U/g
Xylanase	200000 U/g
Exo-PGA	> 900 U/ml
Endo-PGA	> 3000 U/ml
Pectin esterase	> 300 U/ml

2.2. Methods

2.2.1. Pretreatment of Orange Bagasse

Fresh orange bagasse was directly used or frozen and stored at -20 °C until it was used. Before enzymatic hydrolysis, for the pretreatment of orange bagasses only physical processes were applied in order to increase surface area and disrupt the crystalline structure of cellulose for the obtainment of higher saccharification yields (Harmsen et al, 2010) . Fresh or thawed orange baggase at ambient temperature was dried at 80 °C overnight until to reach 6-10 % of final moisture content and milled to

a homogenous particle size of 1 mm by using Thomas Wiley Laboratory Mill, Model 4 (A.H. Thomas Company, USA).

In order to observe the effect of drying on enzymatic hydrolysis, only grinding process was applied to fresh orange bagasse by using a mincing machine and the size of particles was less than 2mm.

2.2.2. Moisture Content Analysis of Orange Bagasse

Moisture content in percent of pretreated orange bagasse was analyzed before enzymatic hydrolysis process by infrared moisture analyzer (Radwag MAC 50, Poland).

2.2.3. Enzymatic Hydrolysis of Orange Bagasse

Pretreated orange bagasse was enzymatically hydrolyzed before fermentation process in order to obtain fermentable sugars. Enzymatic hydrolysis was carried out in 250 mL to 2 L conical Erlen mayer flasks in duplicates in shaking incubators (Infors HT, Switzerland). The enzymatic hydrolyses performed in 0.05 M sodium-citrate buffer at pH 4.8, 55 °C, 150 rpm for 0-96 h. The pH of the hydrolysate was adjusted to 4.8 by using 2 M NaOH following the 1st hour of the hydrolysis. Samples from hydrolyses were taken in short time intervals at beginning of the experiments while after 24 h samples were taken daily.

In the hydrolysis experiments, enzymes of ASA Spezialenzyme GmbH were used in various quantities.

At the end of the experiments, enzymes in the hydrolysate were deactivated by a heating process at 100 °C for 10 min and centrifuged at 22780 g for 5 min or 14239 g for 10 min depending on the following procedure. In addition, for some upcoming processes vacuum filtration was applied to the supernatant obtained with the use of 0.45 μm filter paper after centrifugation in order to have a clear solution. However, it was observed that the filtration process was also necessary for the lactic acid

production from FOB hydrolysate and the effect of filtration after centrifugation was explained in Chapter 3.

2.2.3.1.Effect of the Solid Load of Orange Bagasse and Enzyme Content

In order to observe the effect of the amount of substrate and enzyme on enzymatic hydrolysis, the hydrolysis experiments were conducted with different solid loads; 2%, 5%, 10% and 20% of orange bagasse. Cellulolytic, hemicelluloytic (ASA Biogazym 2x) and pectinolytic (ASA Pektinase L 40) enzymes were used synergistically with changing amounts from 0.05% to 2.5%.

2.2.3.2.Effect of Pectinase on Saccharification of Orange Bagasse

To examine the influence of pectinase on saccharification of dry orange bagasse, hydrolysis experiment was set up with increasing the concentration of ASA Pektinase L 40 up to 0.5% while fixing the amount of ASA Biogazyme 2x at 0.5%. The hydrolyses were performed with 10% substrate loading for 24 hours at 55 °C, 150 rpm.

2.2.3.3.Effect of Recycle of Hydrolysis on Sugar Content

To increase glucose and total sugar concentration as well as the lactic acid titer after fermentation step and to check the existence of product inhibition, enzymatic hydrolyses were performed in series. At the end of each stage, three stages in total, hydrolysate was centrifuged and both fresh enzyme and pretreated orange bagasse or only pretreated orange bagasse at the same amount were added to the supernatant of former hydrolysate.

2.2.3.4.The Sample Preparation and Sugar Analysis

Before HPLC analyses, 1 mL samples, which were taken at regular time intervals and heated in oven incubator at 100 °C for 10 min, were centrifuged at 22780 g for 5 min by a laboratory type centrifuge (Mikro 220 R, Hettich Lab Technology,

Germany). The supernatant of the sample was diluted and passed through 0.22 μm nylon filters to prevent column deformation and contamination.

The concentrations of glucose and other carbohydrates (cellobiose, sucrose, xylose, galactose, arabinose and fructose) in the supernatant of hydrolysate were analysed by using HPLC (Agilent Technologies, USA) equipped with RPM Monosaccharide column, 300 x 7.8 mm, (Phenomenex Inc., USA) at 85 °C constant temperature and a refractive index detector at, 55 °C RID temperature with 10 μl sample injection and 0.6 ml/min flow rate of mobile phase which was double distilled water.

2.2.4. Lactic Acid Fermentations

2.2.4.1.Inoculum Preparation and Growth Curve Analysis

Bacterial strains of *Lactobacillus*.; *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 *and Lactobacillus plantarum* OZH8 were cultivated mainly for D-lactic acid production. Strains were stocked at -80 °C in a solution of growth medium and glycerol in 1:1 ratio. Before inoculation, bacteria (150 μL) were cultivated into sterilized test tubes containing 5mL growth medium for activation and an inoculated at 38 C and 160 rpm for 12-16 h in shaking incubators. The cultivation process was repeated three times to obtain a mature preculture.

In order to determine inoculation time, activated *Lactobacillus* strains (5 mL) were cultivated in 250 mL Erlenmeyer flasks containing 100 mL sterilized MRS broth at 38 °C and optical densitiy at 600 nm was recorded hourly by a spectrophotometer (UV 1202, Shimadzu, Japan).

The growth media were prepared with enhanced MRS (eMRS) broth or modified tryptic soy broth (mTSB) broth. Both media have rich and proper content for the growth of lactobacilli. MRS broth includes 20 g/L glucose, 10 g/L peptone, 8 g/L meat extract, 4 g/L yeast extract, 2 g/L dipotassium hydrogen phosphate, 5 g/L sodium acetate trihydrate, 2 g/L triammonium citrate, 1 g/L Tween 80 aka. Polysorbate 80, 0.2 g/L magnesium sulfate tetrahydrate and 0.05 g/L manganous

sulfate tetrahydrate. Modified TSB broth contains 10 g/L TSB broth (17 g/L peptone from casein, 3 g/L peptone from soy meal, 2,5 g/L D-glucose, 5 g/L sodium chloride, 2,5 g/L di-potassium hydrogen phosphate), 10 g/L yeast extract, 10 g/L glucose, 1 g/L Tween 80, 0.05 g/L magnesium sulfate, 0.05 g/L manganous sulfate.

2.2.4.2.Batch Lactic Acid Fermentation

The lactic acid fermentations were done in 250 mL Erlenmeyer flasks with 100 mL working volume. Mainly, the fermentation media contained the same components as the growth media of inoculum except from addition of glucose, orange bagasse hydrolysate as source of fermentable sugar, 5% (v/v) inoculum, additional yeast extract in different amounts to analyze the effect of nitrogen source and 3% of calcium carbonate as a neutralizer which was added to prevent the adverse effect of acidity on fermentation.

The fermentation experiments were conducted in a shaking incubator (Infors HT, Switzerland) with a constant temperature at 38 °C, 160 rpm to obtain homogenous mixing for 1 to 5 days. Before the aseptic inoculation, fermentation media were sterilized by autoclaving at 121 °C for 15 min.

2.2.4.3.Effect of Repetitive Substrate Addition

This experimental work was designed by adding dried orange bagasse hydrolysate repetitively, at the end of each stage when glucose was consumed and the production of lactic acid reached to a plateau.

The fermentation experiment was conducted as described elaborately in the section above and with single inoculation and nutrition addition (nitrogen sources and minerals) expect the ones coming from hydrolysate. When glucose was consumed at the end of each stage, 100 ml orange bagasse hydrolysate was added to the fermentation medium. The experiments were stopped at the 4^{th} stage with 400 mL final volume. Before supplying the hydrolysate, 3% $CaCO_3$ was added to prevent pH drop below 5.0 and both were sterilized at 121 °C for 15 min in an autoclave.

At the end of each stage, pH was measured by bench top pH meter (PL-700 PC, Gondo Electronic Co., Taiwan).

2.2.4.4.The Analysis of Fermentation Medium

All of the samples (1 mL) from Erlenmeyer flasks through the fermentation were done at sterilized conditions with aseptic equipment at regular intervals. The sampling procedure was done as explained in Section 2.2.3.4. The lactic acid and fermentable sugar concentration analyzed HPLC (Agilent Technologies, USA) with Rezex™ RFQ-Fast Acid H+ (8%) column, 100 x 7.8 mm, (Phenomenex Inc., USA) and refractive index detector. The temperature of column and RI detector was set to 25 °C and 30 °C, respectively. Ten µl of analyte was injected automatically with 0.6 ml/min flow rate and by using 0.05 M H_2SO_4 as eluent.

The standard curves of constituents were given in Appendix B.

2.2.4.5.The Analysis of Chirality of Lactic Acid

In order to determine how much D-lactic acid as Ca-D-lactate form presented in fermentation medium obtained analyses were made simultaneously by the HPLC with RI detector which was explained in detail in the section above and Glucose-Lactate Analyzer (SensoStar GL30 Touch, DiaSys Diagnostic Systems GmbH, Germany) with its own calibration solutions. The calibration solution of analyser contained 290 mg/dL glucose and 90 mg/dL lactate. The measuring range of analyser was between 11 mg/dL and 910 mg/dL for glucose; 4.5 mg/dL and 270 mg/dL for lactate. The sample preparations and analyses by HPLC with RID were performed as explained in the sections above.

2.3. The Analysis of Data

The results of experiments were given as mean values of replicates. For the analysis of data reqired statistical interpretation, analysis of variance (ANOVA) and two sample T-test was applied by the use of Minitab 16.2.0.0 (Minitab Inc., UK). Tukey test with 95% confidence level was used to compare the results.

CHAPTER 3

RESULTS AND DISCUSSION

This chapter deals with the experimental data of enzymatic hydrolysis of orange bagasse with cellulolytic and pectinolytic enzymes and fermentation of D-(-)-lactic acid by lactic acid bacteria with the use of orange bagasse hydrolysate as a carbon source. The effect of parameters in the experiments will broadly be discussed in the following subsections. It should be noted that;

- All the shake flask experiments were done in duplicates and represented as mean values. The results in graphs and tables include the standard error data.
- The total sugar data mentioned in the results of experiments of enzymatic hydrolysis contained the quantities of glucose, xylose, galactose and arabinose. Fructose was excluded from the conversion data due to very few change in its concentration through enzymatic hydrolysis.
- The total sugar data of fermentation experiments included the concentrations of cellobiose, sucrose, glucose, xylose, galactose, arabinose and fructose in the fermentation medium.
- The definitions of conversion rate in hydrolysis the yield and productivity of lactic acid experiments can be found in Appendix A.
- The calibration tables of HPLC analyses for each constituent were given in Appendix B.

3.1. Enzymatic Hydrolysis of Orange Bagasse

3.1.1. Effect of Enzyme Concentration

The yield of enzymatic digestion is influenced by enzyme and substrate loading, enzyme activity as well as pH, temperature and reaction time (Yang et al., 2009). Hence, specifying the substrate, enzyme type dependent-parameters are crucial for the technical and economic efficiency of the hydrolysis.

For instance, Li and co-workers (2016) reported that 45°C and 24 h reaction time were proper for the enzymatic digestion of orange peel with crude enzymes of a strain *Aspergillus japonicas* and in another previous study, hydrolysis of cellulolytic biomasses were conducted at 4.8 pH, 45-50°C for 3 or 4 days (Duff & Murray, 1996).

In this work, cellulolytic and pectinolytic enzymes of ASA Spezialenzyme GmbH were used. As stated in the Product Sheet of ASA (2012), proper pH range is between 4.5 and 6.0 for ASA-BG and pH range of ASA-P is from 4 to 5. The temperature range is between 50 – 60°C, optimum at 55°C for both of ASA-BG and ASA-P.

The first hydrolysis experiments were done with 10% solid load of orange bagasse in dry weight and different enzyme concentrations changing between 0.05 % and 2.5 % in total working volume in order to optimize the amount of enzyme and hydrolysis time.

Figure 3.1 and Figure 3.2 represent the concentration change of glucose and total sugar in g/L up to 96 h with the use of 0.05% to 2.5% ASA-BG and ASA-P enzymes in the same amount. As expected, sugar quantity of hydrolysates increased gradually when the concentration of enzymes increased; however, a proportional increase was not observed among the samples. Converted glucose concentrations showed significant difference with different enzyme concentrations ($p \leq 0.05$). As seen in Figure 3.1, the highest amounts of converted glucose obtained at the end of 96 hours,

which are 13.7g/L, 15.0 g/L, 16.9 g/L, 17.9g/L and 26.1 g/L with 0.5%, 1.0%, 1.5%, 2.0% and 2.5% enzyme loading, respectively.

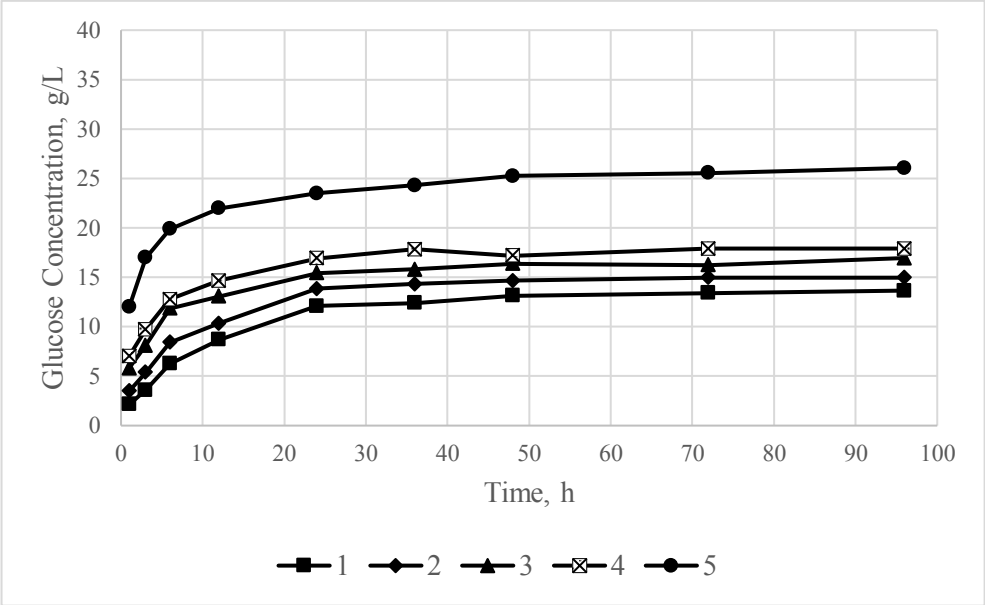

Figure 3.1 The effect of enzyme dosage on glucose conversion in g/L (1: 0.5 % BG&P, 2: 1.0 % [1]BG&P, 3: 1.5 % BG&P, 4: 2.0 % BG&P, 5: 2.5 % BG&P). (The significant difference was represented by different letters ($p \leq 0.05$) and two-way ANOVA was applied).

[1] BG: ASA Biogazyme 2x, P: ASA Pektinase L40

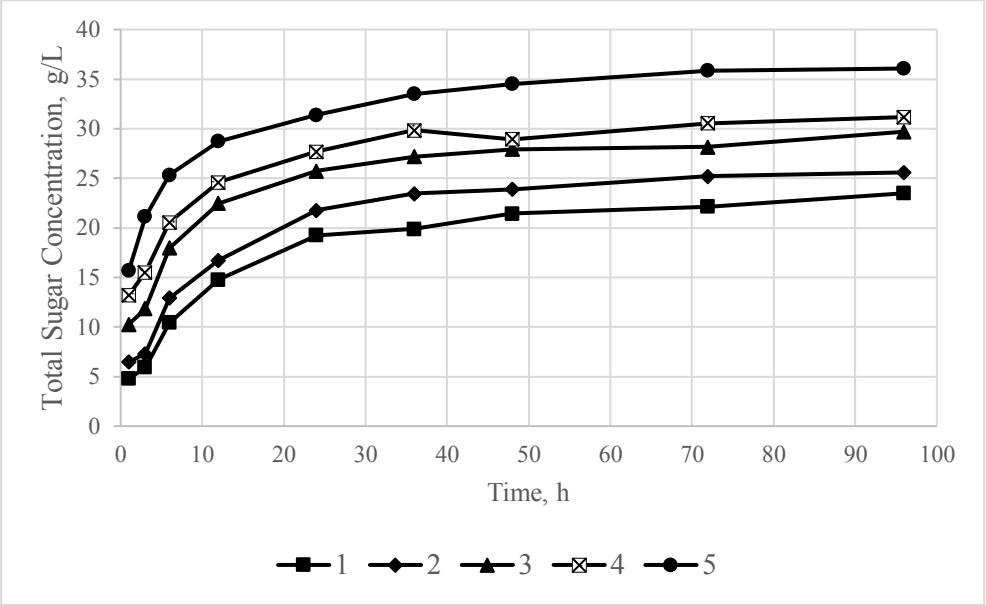

Figure 3.2 The effect of high dosage of enzyme on total sugar conversion in g/L (1: 0.5 % BG&P, 2: 1.0 % BG&P, 3: 1.5 % BG&P, 4: 2.0 % BG&P, 5: 2.5 % BG&P). (The significant difference was represented by different letters ($p \leq 0.05$) and two-way ANOVA was applied).

The optimum reaction time was decided in accordance with the conversion rate of hydrolysis and change in glucose concentration. The conversion rate of glucose as a function of enzyme loading is given in Table 3.1. Even though the conversion of glucose slowed down significantly after the 6th hour of hydrolysis, the process time was decided as 24 hours due to the occurence of plateau and after that time glucose conversion rate decreased below 0.5 g/L/h.

The same conversion trend was detected in the amount of total sugar by hydrolyzing dried orange bagasse with high enzyme loading. The total sugar concentration did not increase proportionally with enzyme quantity. When 0.5% of ASA-BG and ASA-P were used 23.5 g/L total sugar was obtained, when the enzyme load increased 5-fold, glucose concentration approximately increased 1.5-fold.

Table 3.1 The conversion rate of glucose as a function of enzyme loading (%).

Sample/Time	1	2	3	4	5
3, h	1.20	1.79	2.70	3.25	5.66
6, h	1.04	1.41	1.98	2.13	3.31
12, h	0.72	0.86	1.09	1.22	1.83
24, h	0.50	0.58	0.64	0.70	0.98
36, h	0.34	0.40	0.44	0.50	0.68
48, h	0.27	0.31	0.34	0.36	0.53
72, h	0.19	0.21	0.23	0.25	0.35
96, h	0.14	0.16	0.18	0.19	0.27

The second hydrolysis experiment set was carried with low dose enzyme mixtures. Figure 3.3 shows the conversion of glucose and total sugar in g/L at the end of 24 h with the use of ASA-BG and ASA-P enzymes in the same amount from 0.005% to 0.25%. As can be seen in the figure, the amounts of converted glucose and total sugar, 0.49 and 1.19 g/L respectively, were very low when the concentration of ASA-BG and ASA-P enzymes was 0.005%. The sugar quantities were proportional to the increase in the enzyme levels up to 0.1%. Further increase in the enzyme loadings; however, did not result in proportional increases in the hydrolysis levels. For instance, when the enzyme concentration increased 10-folds between samples 2 and 5, glucose conversion increased approximately 8-fold and total sugar conversion rose from 2.49 g/L to 14.69 g/L.

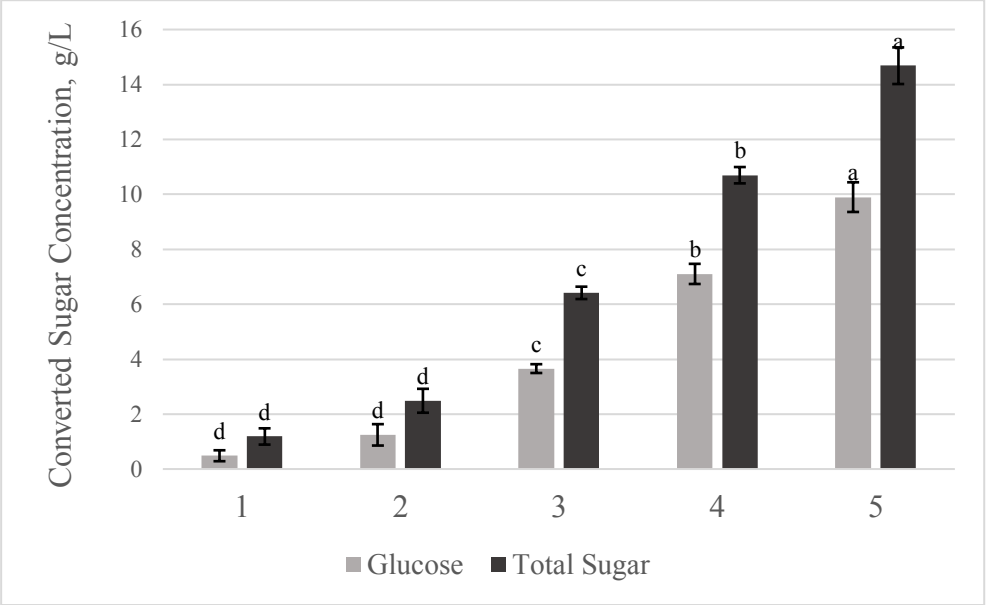

Figure 3.3 Glucose and total sugar conversion in g/L (1: 0.005% BG&P, 2: 0.025% BG&P, 3: 0.05% BG&P, 4: 0.1% BG&P, 5: 0.25% BG&P). (The significant difference was represented by different letters ($p \leq 0.05$) and one-way ANOVA was applied separately for glucose and total sugar conversion data).

The data and saccharification trend of hydrolysis experiments conducted with high and low doses of enzymes could be due to the decrease in enzyme activity depending on glucose concentration.

3.1.2. Effect of Pectinolytic Enzyme on Saccharification of Orange Bagasse

Citrus fruits and their wastes are significant sources of pectin which is found in albedo part. Likewise, orange bagasse as a citrus waste is highly rich in pectin, approximately half of it is pectin (Ahmed & Mostafa, 2013).

Pectin is highly connected to the cellulosic structure; therefore, it needs to be degraded for absolute fragmentation by hydrolysis with pectinase (Talebnia, 2008).

Besides, according to Kristensen (2009), several studies done with various lignocellulosic biomasses such as corn stover, hardwoods, etc., pectinases as well as xylanases enhance the cellulose degradation.

In the study of Grohmann and Baldwin, approximately 90% solubilization of solid part of orange peel was obtained by pectinase added-enzymatic hydrolysis while 70% degradation was obtained by hydrolysing only with cellulase enzyme at high loadings (Grohmann & Baldwin, 1992).

In order to observe the effect of ASA Pektinase L-40 on the saccharification of orange bagasse an experiment was conducted with different quantities of pectinase enzyme while the concentration of cellulolytic enzymes was fixed. The amount of enzyme was listed in the Table 3.2 below.

Table 3.2 The amount of celluloytic and pectinolytic enzymes.

Samples	ASA-BG (%)	ASA-P (%)
1	0.5	0.0
2	0.5	0.05
3	0.5	0.1
4	0.5	0.25
5	0.5	0.5

As represented in Figure 3.4, pectinase significantly affected the glucose and total sugar conversion ($p \leq 0.05$). The concentration of glucose and total sugar increased from 6.7 g/L to 13.2 g/L and 7.6 g/L to 19.7 g/L, respectively. It is important to emphasize the fact that the increase in total sugar conversion was higher than glucose conversion at the end of 24 hours as result of the conversion of arabinose, galactose and xylose. This result could be supported by the study of Kaya et al., (2014) which showed that pectin found in orange peel contains arabinose and galactose in high amounts as well as glucose.

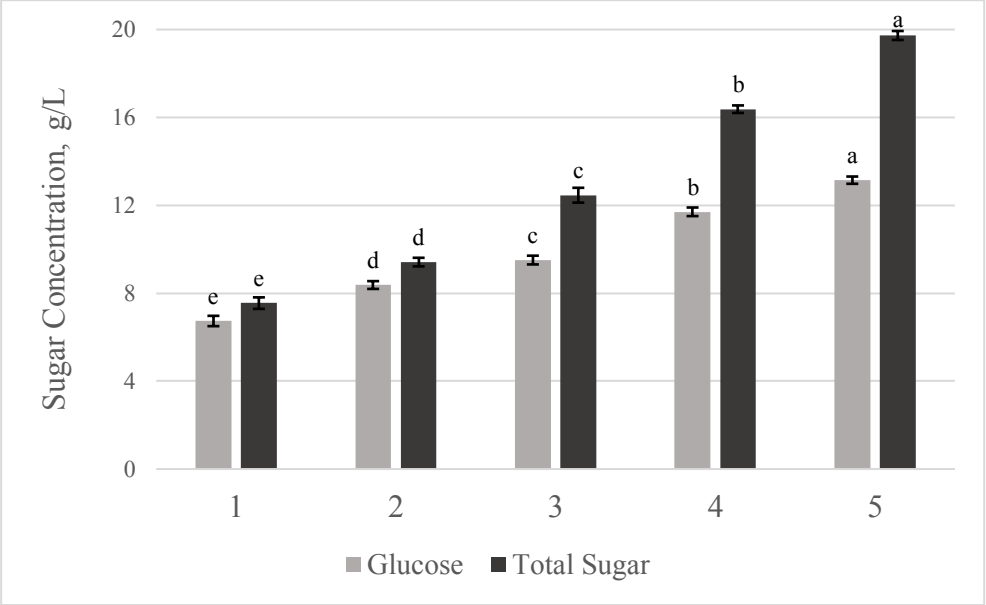

Figure 3.4 Effect of pectinase on glucose and total sugar concentration in g/L. (The significant difference was represented by different letters ($p \leq 0.05$) and one-way ANOVA was applied separately for glucose and total sugar conversion data).

3.1.3. Effect of Solid Load on Enzymatic Hydrolysis of Orange Bagasse

In this part, the increase in the orange bagasse quantities was tested to increase the glucose titer which may lead to higher lactic acid levels.

On the other side, it should further be noted that increasing substrate loading up to 20% and more can affect hydrolysis efficiency by increasing viscosity and preventing sufficient mixing (Kristensen, Felby, & Jørgensen, 2009).

Figure 3.5 represents the glucose and total fermentable sugar concentrations at the end of 24 hours in 2%, 5%, 10% and 20% loadings of dry orange bagasse. The dose of enzymes was fixed at 0.5% of ASA-BG and ASA-P in total volume. The concentration of monosaccharides by hydrolysis rose gradually up to 10% of the substrate load. However, the same trend was not observed between 10% solid load by which 12.1 g/L glucose and 19.3 g/L total sugar converted and 20% solid load by which 11.2 g/L glucose and 19.9 g/L total sugar converted and a significant

difference was not obtained by statistical analysis between 10% and 20 % solid load. As stated by Yabefa (2010), the accumulation of cellulose and cellodextrins might cause inhibition on the system which may explain the decrease in glucose concentration.

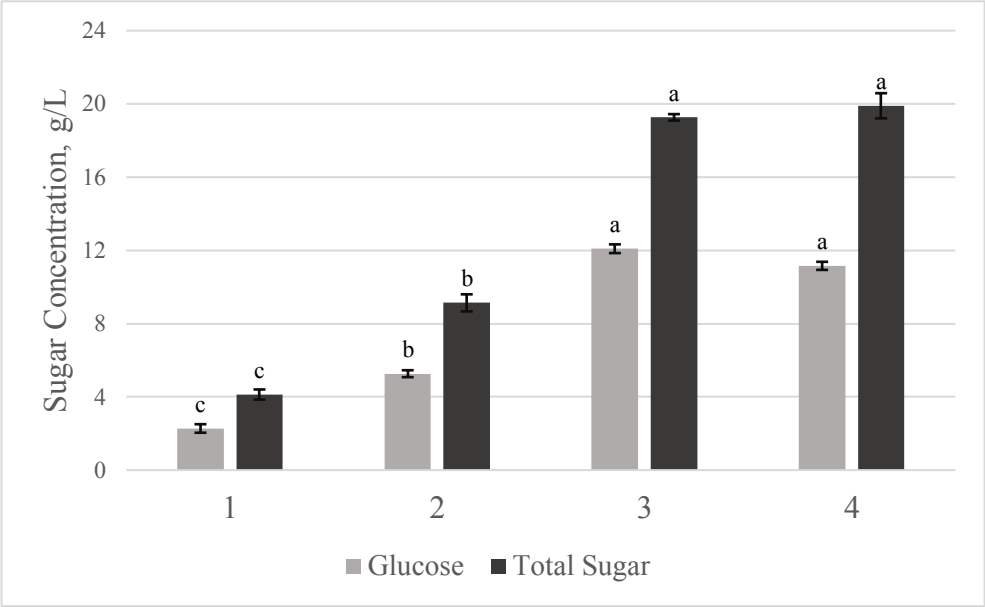

Figure 3.5 Effect of substrate loading on glucose and total sugar conversion in g/L by the hydrolysis with 0,5% ASA-BG, P (1: 2% solid load, 2: 5% solid load, 3: 10% solid load, 4: 20% solid load). (The significant difference was represented by different letters ($p \leq 0.05$) and one-way ANOVA was applied separately for glucose and total sugar conversion data).

3.1.4. Effect of Drying on Saccharification of Orange Bagasse

The application of various pretreatment methods on lignocellulosic biomass mainly brings benefits such as disruption of lignin and hemicellulose pattern and crystal structure of cellulose, reduction in particle size, attainment of a larger surface area and porous structure for the increase in following enzymatic hydrolysis and fermentation processes (Harmsen et al., 2010). According to several studies, orange

waste was pretreated mainly to eliminate the antimicrobial compound -D-limonene by- acidic steam explosion (Choi et al., 2013) and distillation, to obtain smaller and homogenous particles by grinding or milling (Gomaa, 2013) and to delignify the substrate partially or completely by alkaline treatment (Yabefa et al., 2010).

In this study, the applications of chemical, physicochemical or biological pretreatment methods were not attempted due to low lignin content of orange bagasse (Boluda-Aguilar & López-Gómez, 2013). Also, the inhibitory effect of limonene was eliminated by filtering the fresh orange bagasse (FOB) hydrolysates before fermentation step. Orange bagasse only was dried and milled or ground to decrease the particle size and crystallinity of cellulosic structure.

Figure 3.6 and Figure 3.7 represent the conversion of glucose and total sugar in g/L after 24 hour-enzymatic hydrolysis of ground fresh orange bagasse (FOB) and dried, milled orange bagasse (DOB) with 10% solid load. The hydrolyses were made with 0.1%, 0.5% and 2.5% enzyme loading of ASA-BG and ASA-P separately.

As seen in the graphs, a similar trend was not observed in the concentrations of glucose and total sugar. In the enzymatic hydrolysis done with FOB, 5.7 g/L, 12.9 g/L and 21.6 g/L glucose was converted while 6.1 g/L, 12.1 g/L 23.5 g/L glucose produced with the use of DOB and 0.1%, 0.5%, 2.5% enzyme mixture, respectively. By the statistical analyses, it was found that the type of orange bagasse was not associated with glucose conversion while it was associated with converted total sugar concentration. The glucose conversion was not significantly different when FOB and DOB were hydrolyzed with 0.1% and 0.5% enzyme loading; however, the amount of converted glucose by the hydrolysis of DOB with 2.5% enzyme load was significantly different than the hydrolysis of FOB.

Besides, saccharification of FOB and DOB with regards to total sugar concentration were different when compared with glucose. Total sugar conversion was significantly affected by the type orange bagasse.

The results of this work could be explained by the decrease in accessibility of enzymes by drying. According to the study of Ioelovich and Morag (2011), drying

affected the enzymatic digestibility of cellulose adversely by decreasing pore size and ruining pore structure irrecoverably in contrast to the crystallinity of cellulose, polymerization degree and particle proportion which were barely affected by drying.

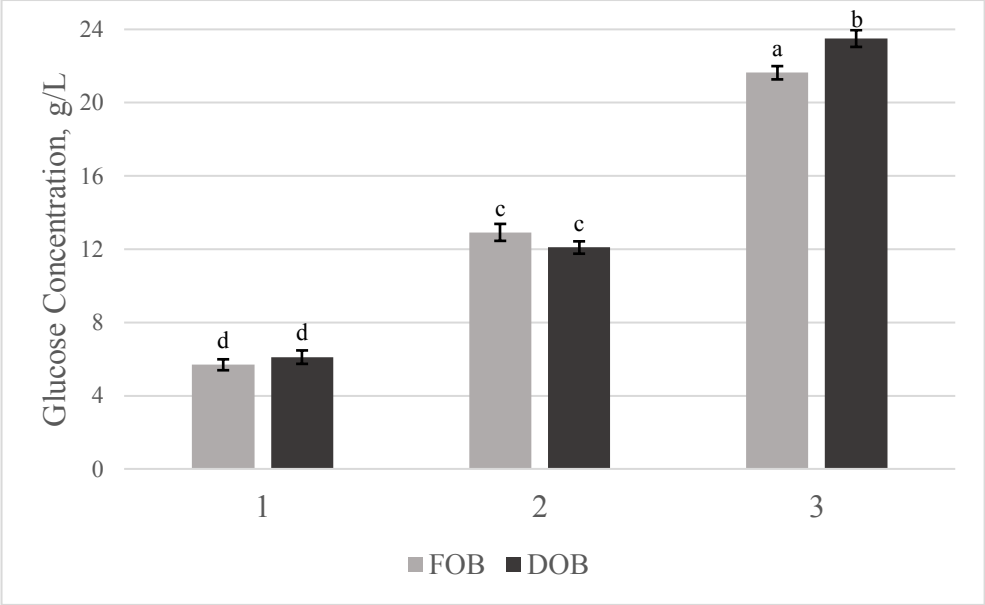

Figure 3.6 Glucose conversion by the enzymatic hydrolysis of fresh and dried orange bagasse (g/L) (1: 0.1% enzyme loading, 2: 0.5% enzyme loading, 3: 2.5% enzyme loading). (The significant difference was represented by different letters ($p \leq 0.05$) and General Linear Model was applied).

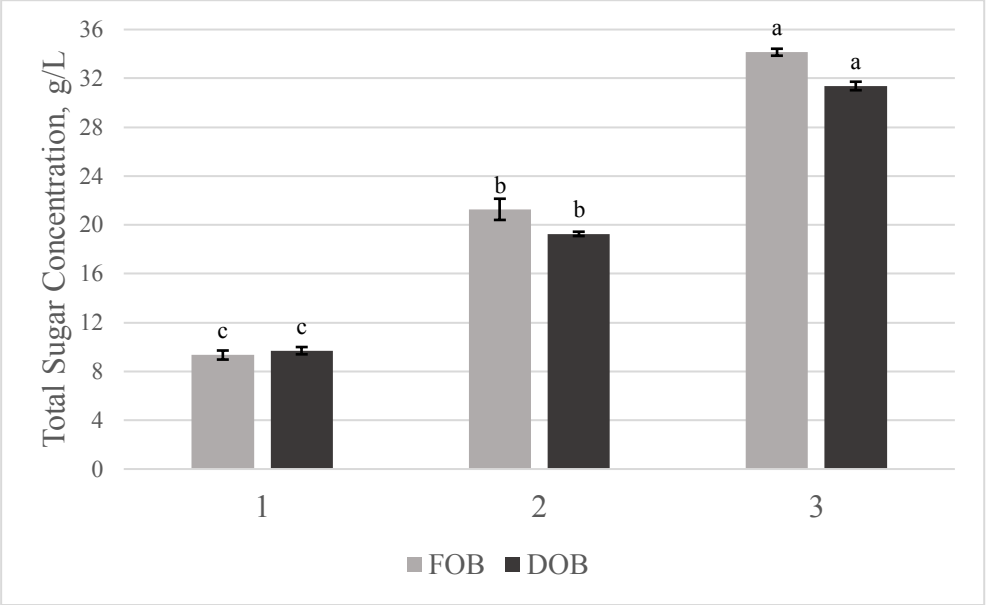

Figure 3.7 Total sugar conversion by the enzymatic hydrolysis of fresh and dried orange bagasse (g/L) (1: 0.1% enzyme loading, 2: 0.5% enzyme loading, 3: 2.5% enzyme loading). (The significant difference was represented by different letters ($p \leq 0.05$) and General Linear Model was applied).

3.1.5. Effect of The Recycle of Enzymatic Hydrolysis

In order to increase sugar concentration via enzymatic hydrolysis as well as the lactic acid titer in the fermentation step recycle experiments were done. The enzyme concentration of ASA BG and ASA P was fixed to 0.5% in total volume and the substrate was fixed to 10% of orange bagasse in dry matter. Hydrolyses were done for 24h of three separate stages.

In the first experiment set named as A, only dry orange bagasse (10% in dry matter) was added to the hydrolysate of previous stage. In the second experiment set named as B both fresh enzyme mixture and orange bagasse were added to the next hydrolysis medium. The concentration of glucose and total sugar converted at the end of each stage was represented in Figure 3.8 and Figure 3.9, respectively.

In both of the experiments, glucose and total sugar amount increased but with a lower rate.

The glucose concentrations in set A was found as 12.1 g/L, 19.4 g/L and 23.4 g/L at the end of 1st, 2nd and 3rd hydrolysis, respectively. A proportional rise was not observed in experiment A. When the biomass increased 2-fold, glucose concentration increased 1.6-fold and for 3-fold increase of biomass, 1.9-fold increase in glucose conversion was observed, which could be explained by the loss of enzyme activity through the process.

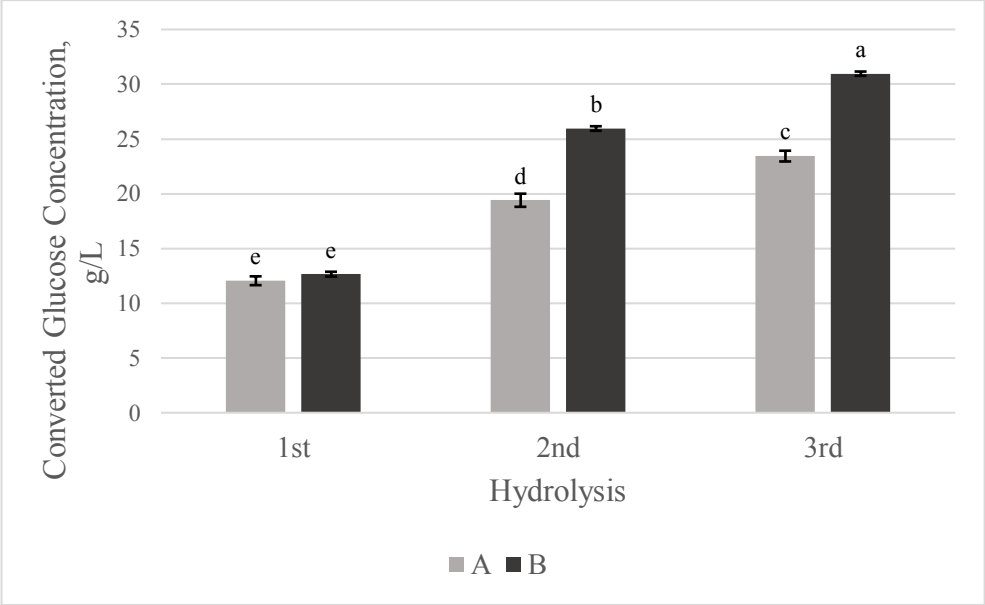

Figure 3.8 Converted glucose concentration (g/L) with recycling hydrolysis (A: hydrolysis done with only addition of orange bagasse, B: hydrolysis done with the addition of both orange bagasse and fresh enzymes. The significant difference was represented by different letters ($p \leq 0.05$) and two-way ANOVA was applied for glucose and total sugar conversion data).

As expected, the conversion of sugar was higher when both biomass and enzyme cocktail were added at the beginning of each step. In experiment B, 12.6 g/L, 25.9 g/L and 31.0g/L glucose were obtained at the end of 1st, 2nd and 3rd hydrolysis,

respectively. The amount of glucose almost doubled when the both biomass and enzyme quantity increased 2-fold. On the other hand, 2.5-fold increase was obtained despite the 3-fold increase in biomass and enzyme mixture. The converted glucose and total sugar concentration were found to be associated with both of the recycle stage and experiment set.

In this experiment setup, a technological approach was applied in order to decide the most efficient process to increase the fermentable sugar concentration. Hence, further scientific examinations for the underlying reasons for the trends of change in glucose and total sugar conversion were not made.

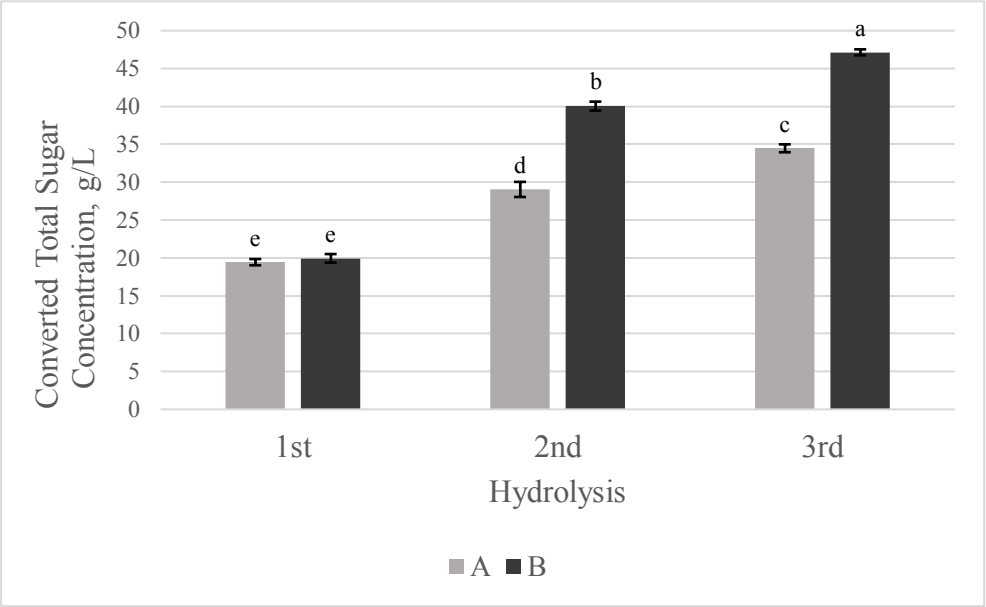

Figure 3.9 Converted total sugar concentration (g/L) with recycling hydrolysis (A: hydrolysis done with only addition of orange bagasse, B: hydrolysis done with the addition of both orange bagasse and fresh enzymes. The significant difference was represented by different letters ($p \leq 0.05$) and two-way ANOVA was applied for glucose and total sugar conversion data).

3.2. Batch Fermentation of Lactic Acid

3.2.1. Effect of the Growth Media on Lactic Acid Fermentation

In the beginning of fermentation experiments, the effect of growth media was investigated in order to obtain higher fermentation yield. For this purpose, two strains of *Lactobacillus* mentioned in detail in Chapter 2 were inoculated to fermentation media containing eMRS broth and mTSB broth of which chemical compositions were given in Materials and Methods Chapter.

According to literature, MRS developed by De Man and co-workers is the most suitable and widely used medium for the selection and election of lactobacilli genera (Schillingerw & Holzapfel, 2012).

Additionally, modified tryptic soy broth can be used for the growth of lactic acid bacteria. In a previous study, a similarity between MRS and Tween 80 added-tryptic soy broth was observed in terms of the growth characteristics of LAB (Cálix-Lara, Duong, & Taylor, 2012).

In this work, tryptic soy broth enriched with yeast extract, glucose, Tween 80 to avoid possible nutritional deficiency. Salts of magnesium and manganese were added to provide essential ions for the growth of LAB (Krishe et al., 1991). In addition, both of eMRS and mTSB media contain yeast extract and peptone as nitrogen, amino acid, vitamin, and mineral source; dipotassium phosphate as a buffering agent; glucose as a carbon source Brigss, 1953; De Man, Rogosa, Sharpe, 1960).

In Figure 3.10, the yield data of lactic acid produced in mTSB and eMRS media were demonstrated. For *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4, significantly higher yield was obtained with eMRS broth; however, for *Lactobacillus plantarum* OZH8 the difference in the yield data was insignificant between two growth media. Even though the yield values obtained from the lactic acid fermentation in two growth media close to each other, eMRS broth was chosen as a constituent of further fermentation media due to its economic and nutritional advantages over mTSB broth.

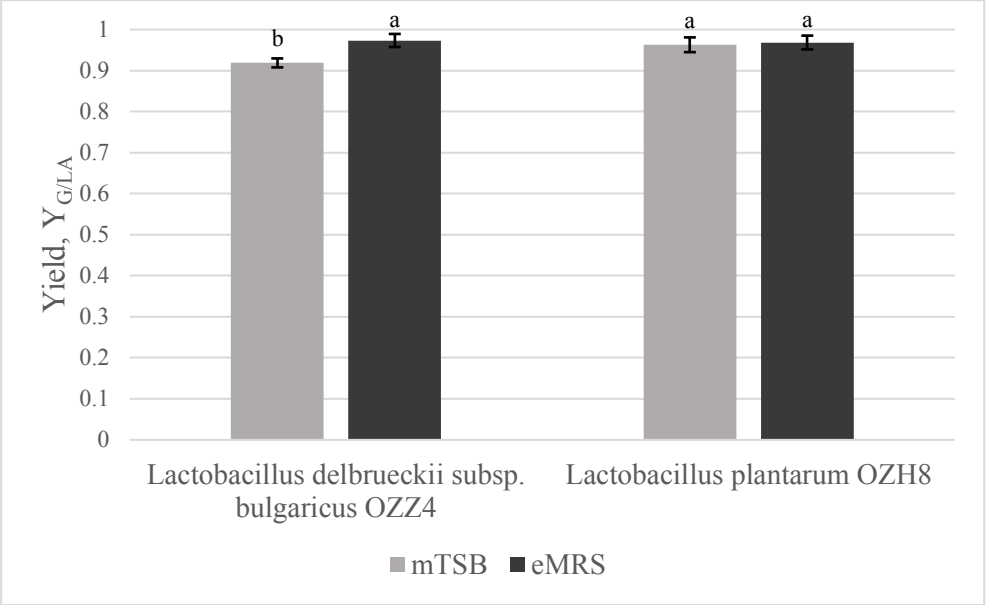

Figure 3.10 The yield of lactic acid in mTSB and eMRS broths. (One-way ANOVA was applied separately for *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4, *Lactobacillus plantarum* OZH8. The significant difference was represented by different letters ($p \leq 0.05$) for *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 and insignificant difference ($p > 0.05$) was obtained for *Lactobacillus plantarum* OZH8).

3.2.2. The Growth Characteristics of Lactic Acid Bacteria

Before starting fermentation studies, the growth characteristics of the strains in MRS medium were analyzed in order to estimate cell growth and attain optimum inoculation time interval. Figure B.9 and Figure B.10 in Appendix B.3 illustrate the growth curve of *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 *and Lactobacillus plantarum* OZH8 with regards to optical density and time.

In both of the figures, a typical lag phase is not demonstrated, which can be obtained by taking samples more frequently. Log phase for both of the strains lasted approximately 11-12 hours and the following stationary phase continued up to 22-23 hours. According to the results, fermentation broths were inoculated up to 14 hour-incubation time of strains in the broth, before the strains passed to stationary phase.

3.2.3. Effect of the Concentration of Inoculum

In order to find the influence of inoculum concentration on lactic acid production and the productivity, fermentation was conducted by 5% and 10% (v/v) inocula of *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 and *Lactobacillus plantarum* OZH8.

Martinkova et al., (1991) found that the yield and productivity of lactic acid increased with increase in the amount of inocula (5, 10 and 20% of total volume) in which MRS medium. Besides, in another study it was observed that the substrate conversion to lactic acid increased from 1% to 2% inocula size of *Lactobacillus casei* and dropped off with the increase of inocula size up to 5% (v/v) (Panesar et al., 2010).

According to Figure 3.11 the yield values were almost similar and productivity values after 12th hour of fermentation were close to each other for *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 at both of the inoculum sizes; however, at the 6th hour fermentation, the productivity obtained by 10% inocula, 5.73 g/L/h, was approximately 2 times higher than the one obtained by 5% inocula, 2.79 g/L/h.

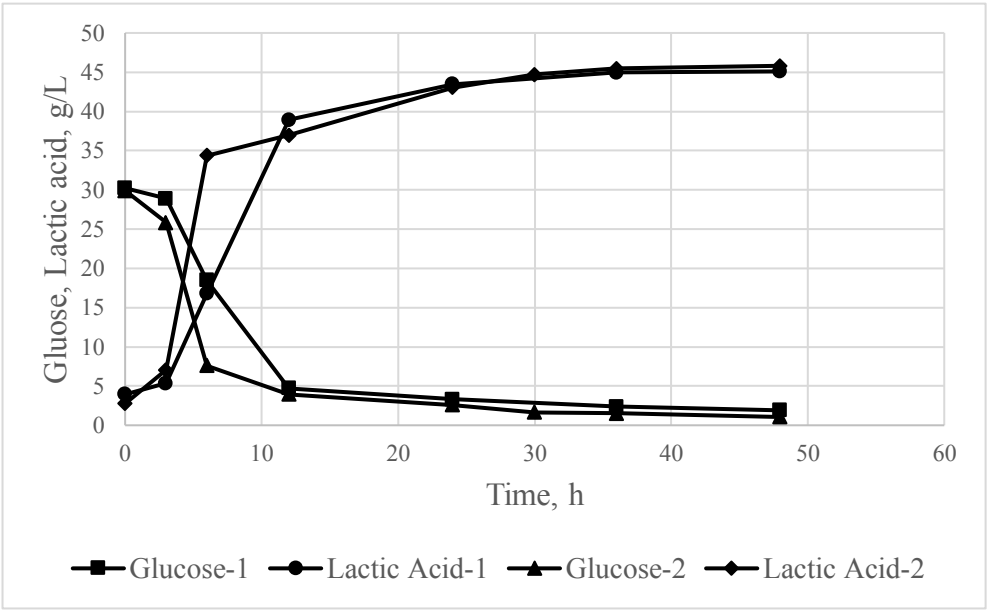

Figure 3.11 Effect of inoculum size for *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 (Glucose-1, Lactic acid-1 for 5 % (v/v) inoculum, Glucose-2, Lactic acid-2 for 10 % (v/v) inoculum).

Similar results which were shown in Figure 3.12 were obtained from the fermentation done by *Lactobacillus plantarum* OZH8. On the other hand, the influence of inoculum size was more observable in lactic acid fermentation done by LP. Up to 24thhour of the process, productivity values of 10% inoculum (3.24g/L/h at the 12th hour) were higher than 5% inoculum size (2.07 g/L/h at the 12th hour).

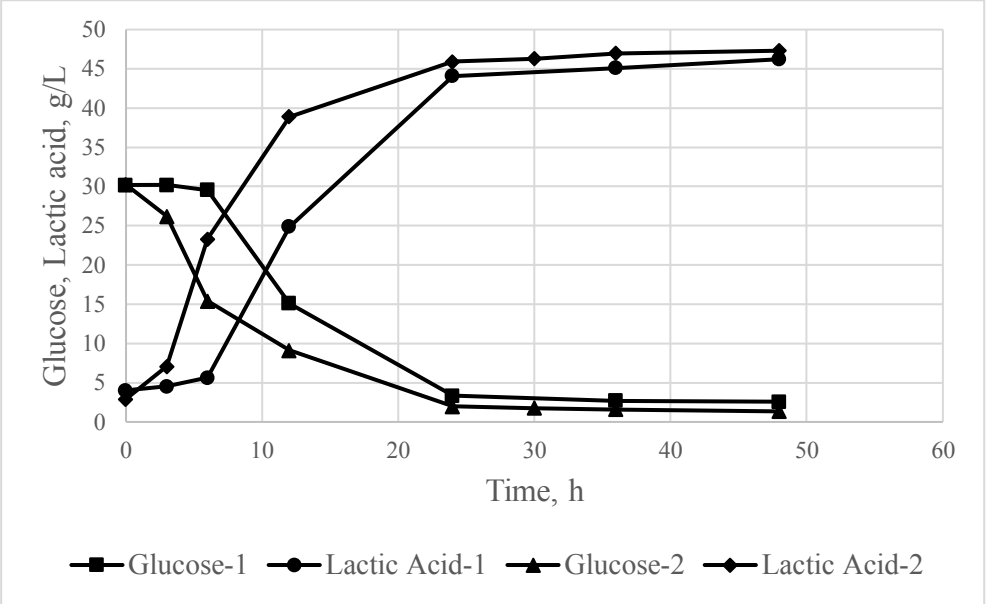

Figure 3.12 Effect of inoculum size for *Lactobacillus plantarum* OZH8 (Glucose-1, Lactic acid-1 for 5 % (v/v) inoculum, Glucose-2, Lactic acid-2 10 % (v/v) inoculum).

As can be seen in figures, the final concentration of lactic acid was around 46 g/L at the end of the fermentations for both of the inoculum concentartions. While the trend of lactic acid production differed for both of the strains.

The yields of lactic acid ($Y_{L/T}$) for 5% inoculation were found as 0.96 g/g and 0.95 g/g and for 10% 0.89 g/g and 0.90 g/g for the fermentations done by *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 and *Lactobacillus plantarum* OZH8, respectively. In the tables below, sugar consumption data through the fermentation were given.

Table 3.3 The sugar concentration in the medium before fermentation

M/o	Glucose	Xylose	Galactose	Arabinose	Fructose
LD 5%	30.16 ± 0.12	1.40 ± 0.08	4.68 ± 0.10	3.77 ± 0.15	13.14 ± 0.04
LP 5%	30.39 ± 0.39	1.34 ± 0.01	4.61 ± 0.11	4.17 ± 0.10	13.62 ± 0.16
LD 10%	30.68 ± 0.30	1.72 ± 0.07	4.51 ± 0.20	4.04 ± 0.38	14.96 ± 0.63
LP 10%	30.98 ± 0.09	1.90 ± 0.04	5.68 ± 0.16	4.34 ± 0.15	14.47 ± 0.15

(LD: *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4, LP: *Lactobacillus plantarum* OZH8).

Table 3.4 The sugar concentration in the medium after fermentation.

M/o	Glucose	Xylose	Galactose	Arabinose	Fructose
LD 5%	1.13 ± 0.06	1.72 ± 0.02	2.00 ± 0.07	1.39 ± 0.55	0.84 ± 0.01
LP 5%	1.32 ± 0.14	1.90 ± 0.03	1.12 ± 0.15	1.63 ± 0.02	2.51 ± 0.11
LD 10%	1.22 ± 0.16	1.51 ± 0.03	1.56 ± 0.10	1.51 ± 0.33	1.68 ± 0.01
LP 10%	1.27 ± 0.18	1.25 ± 0.07	1.34 ± 0.09	1.57 ± 0.09	2.10 ± 0.25

(*LD*: *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4, *LP*: *Lactobacillus plantarum* OZH8).

3.2.4. Effect of The Filtration of Hydrolysate on Lactic Acid Fermentation

While one of the main reasons for applying several pretreatment methods to orange bagasse is to increase the efficiency of enzymatic hydrolysis, another reason for the application of pretreatment processes is to remove D-limonene from orange bagasse. D-limonene is an organic substance, monoterpene, and constitutes approximately 95% of the essential oils of orange peel and has an inhibitory effect on microorganisms such as yeast and bacteria (Pourbafrani et al., 2010; Talebnia, 2008;

Ylitervo, 2008). Limonene can also be removed by filtering the hydrolysate or aerating the fermentation medium (Pourbafrani et al., 2007).

In this experimental work, the adverse effect of limonene on *Lactobacillus* strains was eliminated by filtering after centrifugation of orange bagasse hydrolysate.

Figure 3.13 and Figure 3.14 illustrate the experiments of fermentation done with two different media. One of the fermentation media consisted fresh orange bagasse hydrolysate (FOBH) which was only centrifuged after enzymatic hydrolysis and the other one was prepared with FOBH which was both centrifuged and filtered to see the influence of filtration.

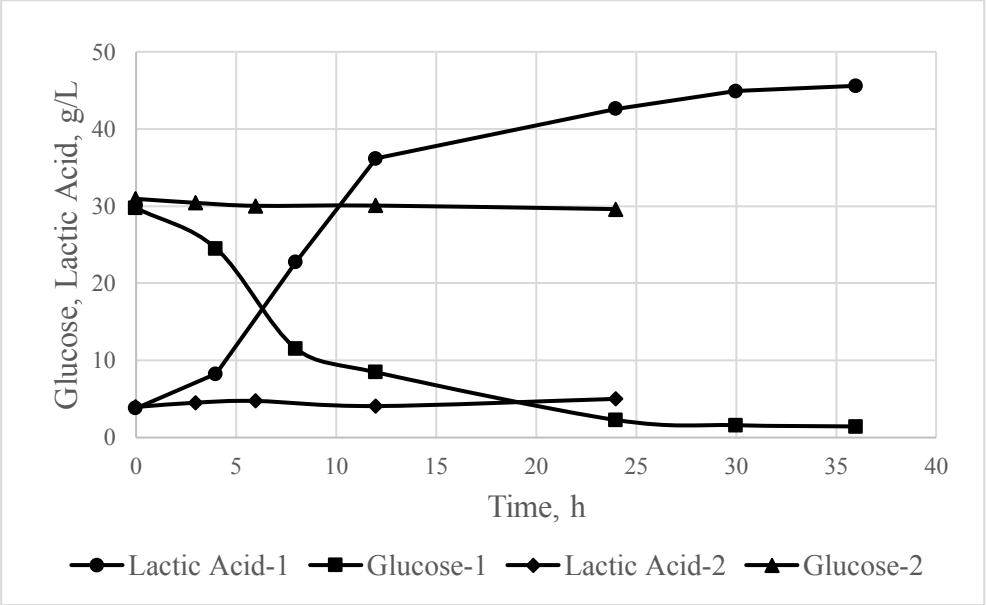

Figure 3.13 Effect of filtration on lactic acid fermentation by *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 (1: Lactic acid fermentation with centrifuged and filtered FOBH, 2: Lactic acid fermentation with only centrifuged FOBH).

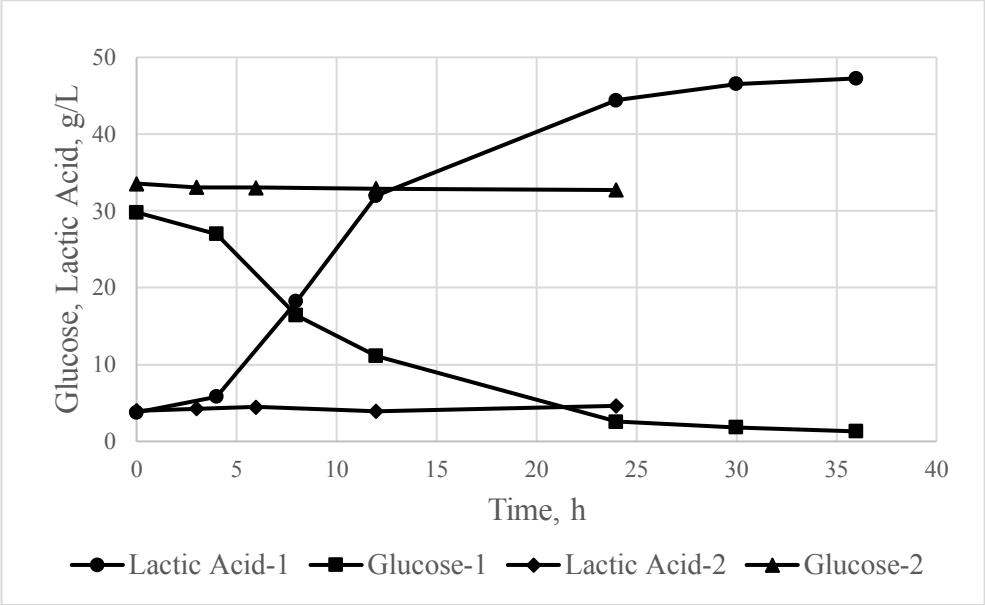

Figure 3.14 Effect of filtration on lactic acid fermentation by *Lactobacillus plantarum* OZH8 (1: Lactic acid fermentation with centrifuged and filtered FOBH, 2: Lactic acid fermentation with only centrifuged FOBH).

As seen in the figures, lactic acid was not produced from the fermentation medium containing only centrifuged-FOBH by both of *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 and *Lactobacillus plantarum* OZH8. On the other hand, lactic acid was obtained from the fermentation medium prepared with centrifuged and filtered-FOBH by both of the strains.

The yields of lactic acid ($Y_{L/T}$) were found as 0.95 g/g and 0.96 g/g for *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 and *Lactobacillus plantarum* OZH8, respectively.

Table 3.5 Sugar concentration of medium containing filtered hydrolysate before fermentation (g/L).

M/o	Glucose	Xylose	Galactose	Arabinose	Fructose
LD	30.43 ± 0.03	1.64 ± 0.34	3.35 ± 0.18	3.92 ± 0.13	13.49 ± 0.44
LP	31.36 ± 0.15	1.68 ± 0.03	3.61 ± 0.15	4.42 ± 0.10	13.29 ± 0.12

(*LD*: *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4, *LP*: *Lactobacillus plantarum* OZH8)

Table 3.6 Sugar concentration of medium containing filtered hydrolysate after fermentation (g/L).

M/o	Glucose	Xylose	Galactose	Arabinose	Fructose
LD	1.23 ± 0.06	1.67 ± 0.15	0.28 ± 0.06	0.85 ± 0.10	0.84 ± 0.04
LP	1.00 ± 0.07	1.07 ± 0.12	1.28 ± 0.29	1.57 ± 0.12	0.39 ± 0.03

(*LD*: *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4, *LP*: *Lactobacillus plantarum* OZH8)

It should be noted that several preliminary studies as steam explosion and steam distillation were made to remove limonene; however, those processes were definitely not economically feasible and had technical difficulties to report the results quantitatively. Those experiments were not continued and included in this thesis work after successive results in fermentation studies conducted with dried or filtered orange bagasse hydrolysate.

3.3. Fed-Batch Lactic Acid Fermentation by Repetitive Substrate Addition

This experimental work was designed to see how long the lactic acid fermentation by *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 and *Lactobacillus plantarum* OZH8 would last with single inoculation and nitrogen and mineral source addition. With this industrial approach, it was aimed to use less nitrogen source and reduce the process cost while lactic acid titer in the medium was increasing.

In Figure 3.15 and Figure 3.16, the change in glucose and lactic acid concentration of lactic acid fermentation conducted at 4 stages by *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 and *Lactobacillus plantarum* OZH8 was shown, respectively. The conditions of fermentation and experiment setup were explained in Section 2.2.4.3.

After fourth feeding, experiments were ended due downward trend in lactic acid production and the risk of contamination. At the end of 4th stage, maximum 10.2 g lactic acid (in Ca-lactate form) was produced by LD with 21.32 g total sugar feeding and 11.5 g lactic acid was produced by LP with 21.65 g total sugar feeding.

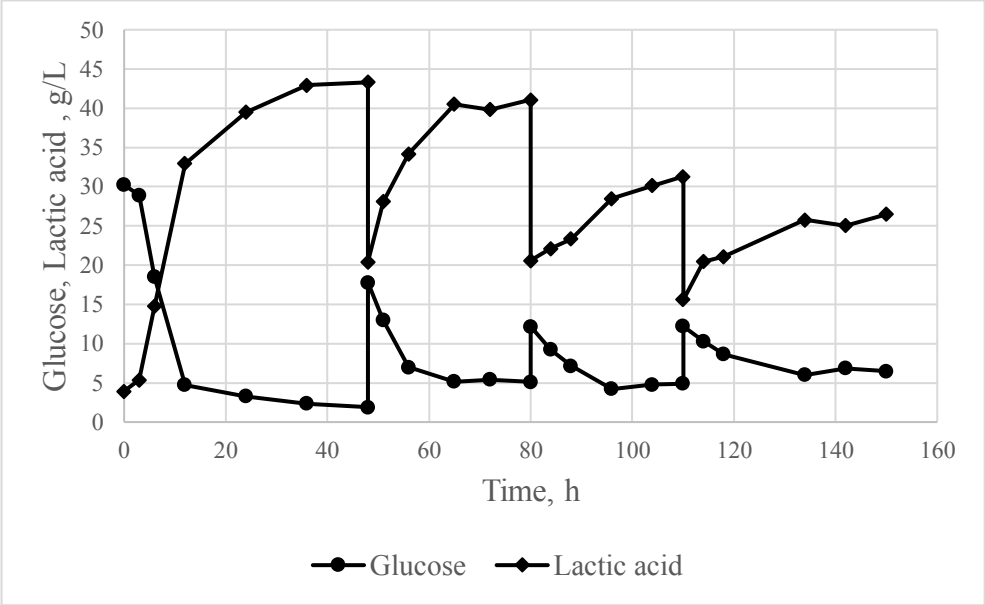

Figure 3.15 The change in glucose and lactic acid concentration by *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4.

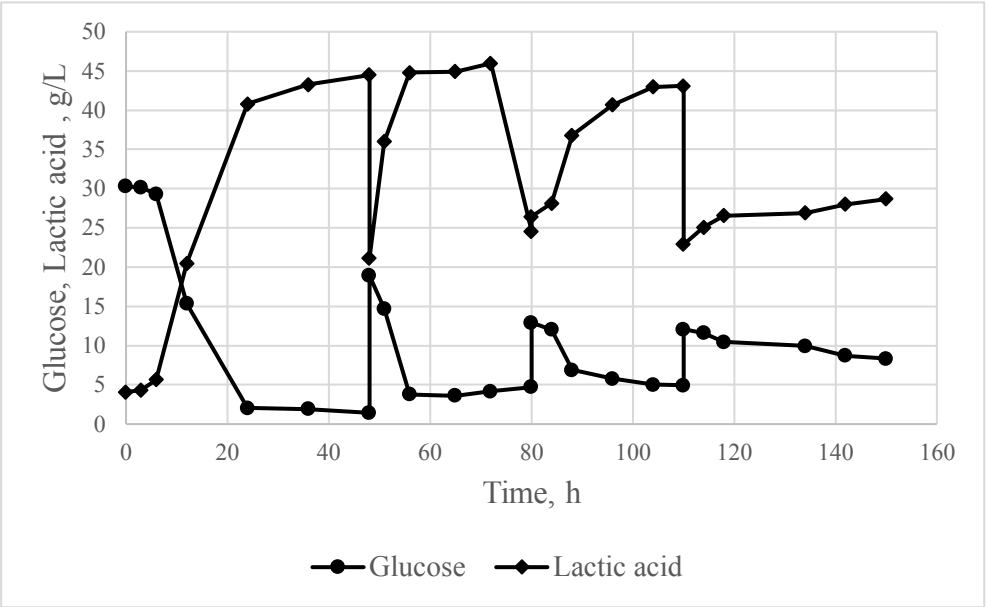

Figure 3.16 The change in glucose and lactic acid concentration by *Lactobacillus plantarum* OZH8.

Moreover, as seen in the figures, productivity values decreased gradually with time. The productivity data of lactic acid production at 12th hour of each stage were given in Table 3.7 for both of the strains. Productivity of LP was higher than LD for all stages excluding the first one.

Table 3.7 The productivity values for each feeding stage.

Stage of Fermentation	P-LD (g/L/h)	P-LP (g/L/h)
1	2.74	1.71
2	0.62	0.75
3	0.28	0.42
4	0.18	0.22

(*LD*: *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4, *LP*: *Lactobacillus plantarum* OZH8).

It can be claimed that dried orange bagasse hydrolysate would be a suitable source which could satisfy the nutritional requirement of *Lactobacillus* strains through the experiments done by single inoculation, nitrogen and mineral source addition such as peptone, yeast extract, magnesium, etc. In a review written by Bampidis & Robinson (2006), it was summarized that orange waste (pulp and peel, particularly) contained various amino acids such as lysine, leucine, alanine, proline, etc., B vitamins and minerals such as calcium, magnesium, manganese, phosphorous, etc. Moreover, orange peel included approximately 6 wt% protein (Grohmann et al., 1995). Hence, orange bagasse hydrolysate was able to provide many nutrition compounds to a certain extent to the lactic acid bacteria which mainly required proline, lysine, magnesium and B12 vitamin for their growth (Chervaux et al., 2000; Hébert et al., 2004).

3.4. Analysis of Isomeric Form of Lactic Acid

As one of the objectives of this thesis study was to produce Ca-D-lactate with orange bagasse by lactic acid, it was essential to know the chirality of lactic acid. Hence, at the beginning of fermentation experiments, isomeric form of lactic acid produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 and *Lactobacillus plantarum* OZH8 was tested in order to detect the optical purity of lactic acid.

The analyses were made by the combination of HPLC and Glucose-Lactate Analyzer as the procedure explained in detail in Section 2.2.4.5. As a reminder, the inocula of fermentation were directly taken and inactivated from the stock solution stored at – 80 °C.

The data in Table 3.8 and Table 3.9 represent the results of D-lactic and L-lactic acid amount in percent. According to results of simultaneous analyses, it was found that *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 and *Lactobacillus plantarum* OZH8 produced 91%, 98% D-lactic acid from orange bagasse hydrolysate, respectively.

Table 3.8 The percentage of D-lactic acid produced by *Lactobacillus delbrueckii* subsp. *bulgaricus OZZ4.*

Time, h	L.A-HPLC, %	L.A-GLA, %	D-L.A, %
0	0.31 ± 0.04	-	**-**
12	3.29 ± 0.10	0.31 ± 0.01	**90.71 ± 0.02**
25	4.35 ± 0.04	0.40 ± 0.04	**90.85 ± 1.01**
53	4.40 ± 0.21	0.37 ± 0.01	**91.71 ± 0.22**

(Two samle T-test was applied for the data at 53rd hour, $p \leq 0.05$).

Table 3.9 The percentage of D-lactic acid produced by *Lactobacillus plantarum* OZH8.

Time, h	L.A-HPLC, %	L.A-GLA, %	D-L.A, %
0	0.35 ± 0.01	-	**-**
12	1.99 ± 0.03	0.05 ± 0.01	**97.69 ± 0.26**
25	4.09 ± 0.03	0.07 ± 0.01	**97.35 ± 0.76**
53	4.47 ± 0.26	0.01 ± 0.00	**97.83 ± 0.12**

(Two samle T-test was applied for the data at 53rd hour, $p \leq 0.05$).

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

In order to produce Ca-D-lactate from an orange waste via enzymatic hydrolysis and lactic acid fermentation, several hydrolysis and fermentation experiments were carried out.

Throughout the enzymatic hydrolysis experiments, the effect of biomass load, enzyme dosage, the synergistic effect of cellulose, hemicellulose and pectinase, pretreatment applications and recycling the hydrolysate on saccharification of orange bagasse were examined. It was found that sugar conversion was not increased proportionally with the increasing amount of enzymes at high dosages. 3.7 g/L, 12.1 g/L and 23.5 g/L of glucose was obtained by the use of 0.1%, 0.5% and 2.5% ASA enzymes when the hydrolyses were done with 10% substrate loading. Both glucose and total sugar conversion was significantly affected by time and enzyme concentration ($p < 0.05$). The process time was decided as 24 hours because after that conversion of sugars reached to plateau.

Pectinase addition increased glucose and especially total sugar conversion and significant difference in sugar conversion was obtained by pectinase addition ($p \leq 0.05$). 13.2 g/L glucose and 19.7 g/L total sugar was obtained with the use of 0.5% cellulolytic and pectinolytic enzymes while 6.7 g/L glucose and 7.5 g/L total sugar were obtained without pectinase addition.

By increasing the substrate amount up to 10%, sugar conversion increased moderately; however, when the solid was 20%, a significant difference was not oconverted amount of glucose decreased.

For the long-term usability of biomass, orange wastes were dried. Furthermore, a drying process was beneficial to attain homogenous partical size by milling. In order to see the action of drying, hydrolyses were conducted with fresh and dried orange bagasse at different enzyme loadings. For the glucose conversion, a significant difference was not observed when FOB and DOB were hydrolyzed with 0.1% and 0.5%.

To obtain higher sugar concentration without increasing enzyme dosage or substrate loading recycled enzymatic hydrolyses experiments were made by supplying former hydrolysate only with biomass or with biomass and enzyme mixture. This process was repeated three times with the increase sugar concentration; however, after 2nd stage of both experiments, sugar conversion data showed a decreasingly growing trend. Hence, two-step hydrolysis was preferred for further saccharification of dry orange bagasse.

The experiments of lactic acid fermentation were done with a new technological approach. *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 *and Lactobacillus plantarum* OZH8 were used for the microbial production of Ca-D-lactate using MRS medium. According to optical density analyses, the incubation time of these strains should be ended before 14 hours for the highest activity of viable cells.

The effect of inoculum size was investigated in order to decrease the fermentation time as well as the cost of the process. However, the productivity values of fermentations 5 % and 10 % inocula were similar.

Another examination was done to eliminate the drying process increasing the process cost. Fermentation experiments were conducted with FOB hydrolysate rather than using DOB hydrolysate. In the light of the results, an extra process which was vacuum filtration needed to be applied after centrifugation of hydrolysate to decrease the amount of limonene when fresh orange bagasse was saccharified.

Finally, fed-batch lactic acid fermentation with repetitive substrate addition was carried out as an alternative to batch fermentation to increment the lactic acid titer in fermentation medium and lower the cost of the process by only supplying orange bagasse hydrolysate and $CaCO_3$. Fermentation experiments were ended after the fourth-time addition of carbon source when the productivity of lactic acid production decreased significantly. By this process, lactic acid concentration doubled in fermentation medium compared to batch fermentation.

According to the chirality analyses of lactic acid produced, it was found that lactobacilli strains produced D-lactic acid over 90 % and while the production of D-isomer of lactic acid by *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4 was significantly less than by *Lactobacillus plantarum* OZH8.

These studies can be continued with the purification of Ca-D-lactate and the polymerization of poly-D-lactic acid (PDLA) to finalize the bioconversion of orange waste to the raw material of biodegradable plastics as a substitute of petroleum based polymers.

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APPENDICES

APPENDIX A

A.1. Definitions of Enzymatic Hydrolysis and Fermentation

The conversion rate of glucose, the yield and productivity of lactic acid are defined in this section.

Conversion rate: Conversion rate is the amount of glucose attained by hydrolysis per time (g/L/h).

Yield ($Y_{L/T}$): Lactic acid yield was calculated as the amount of lactic acid produced (g/L) per amount of total sugar consumed (g/L).

$$Y_{L/T} = \frac{\Delta L}{\Delta T}$$

Productivity (P): Lactic acid productivity was calculated as the amount of lactic acid produced (g/L) per time (h).

$$P = \frac{\Delta L}{\Delta t}$$

APPENDIX B

B.1. Standard Curves of HPLC for Sugar Analysis

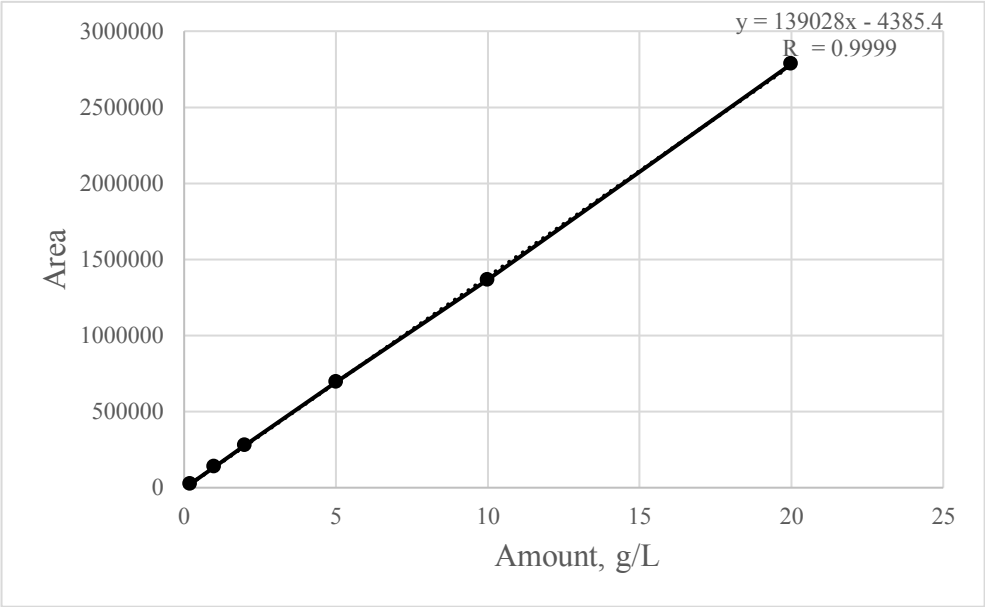

Figure B.1 Standard Curve of Cellobiose and Sucrose

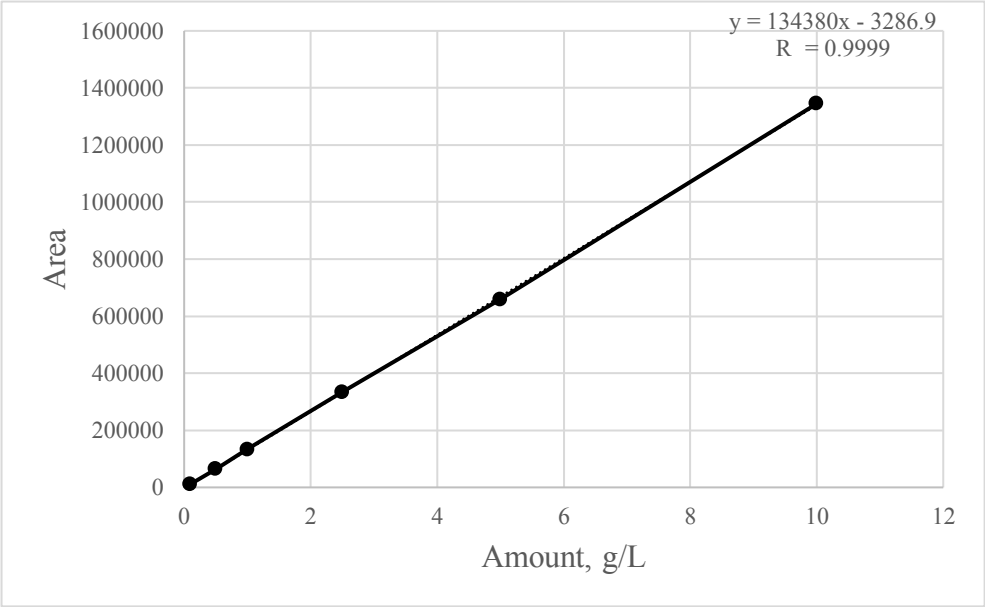

Figure B.2 Standard Curve of Glucose

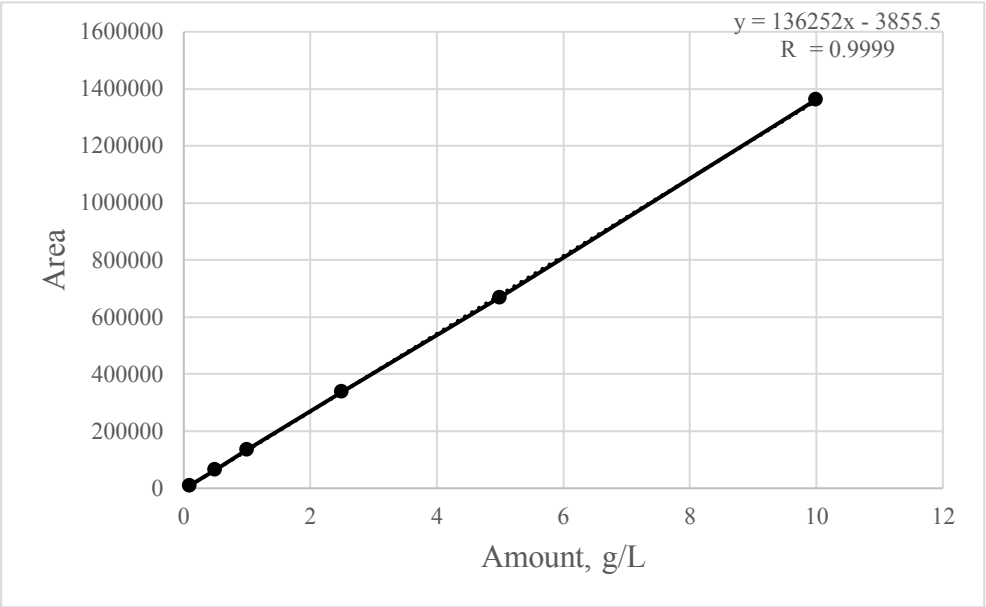

Figure B.3 Standard Curve of Xylose

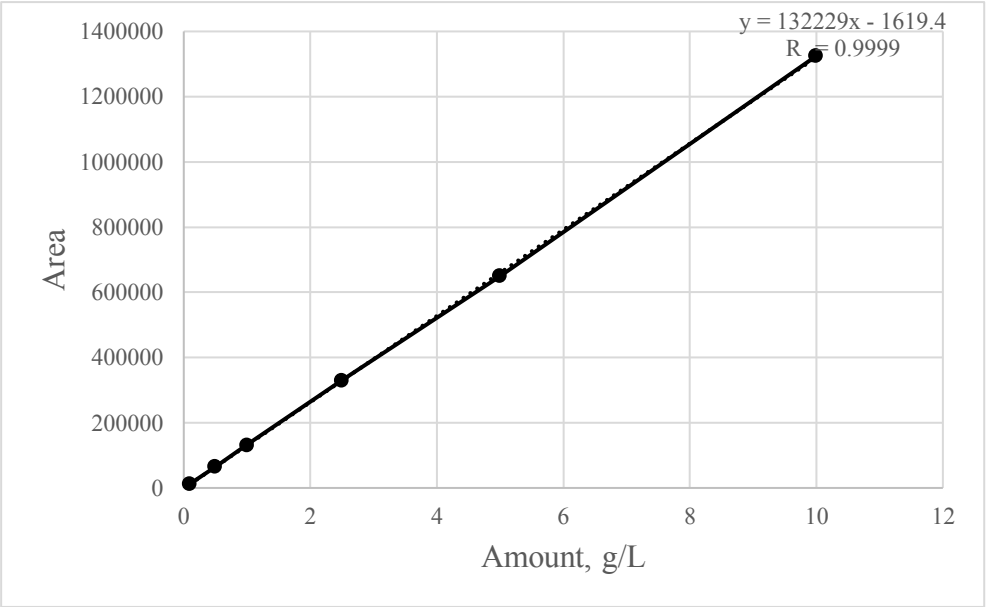

Figure B.4 Standard Curve of Galactose

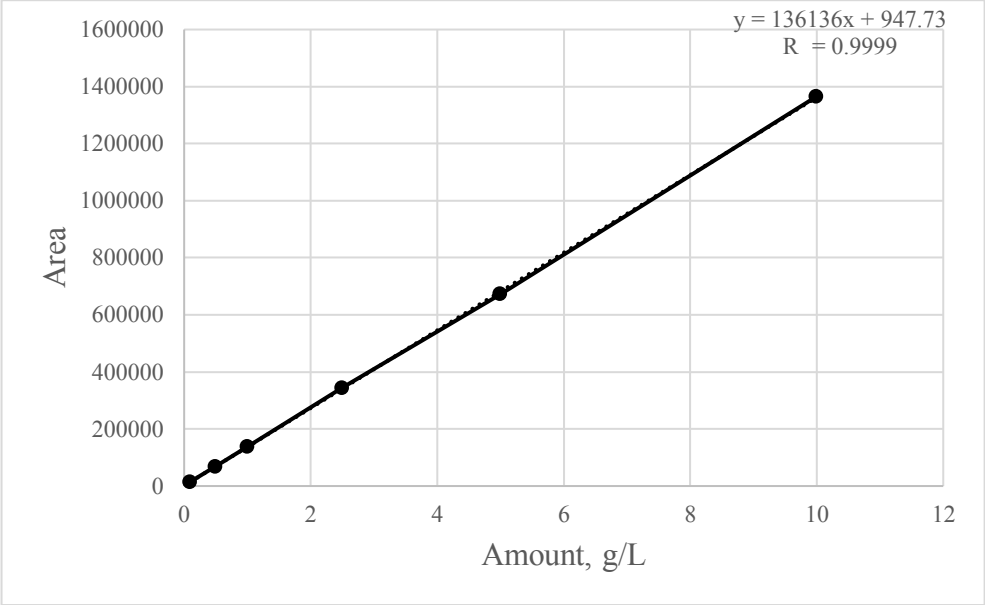

Figure B.5 Standard Curve of Arabinose

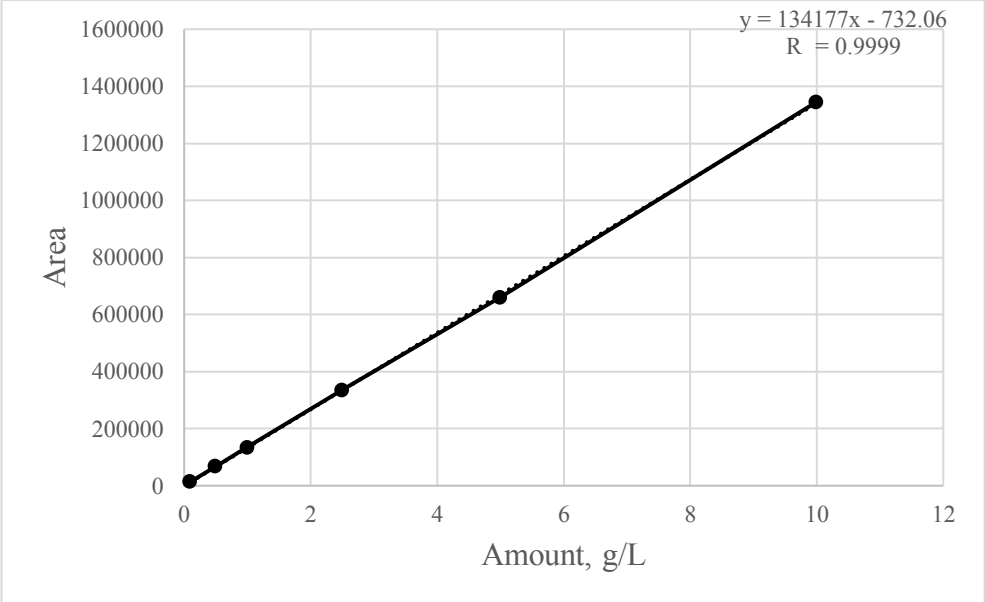

Figure B.6 Standard Curve of Fructose

B.2. Standard Curves of HPLC for Lactic Acid Analysis

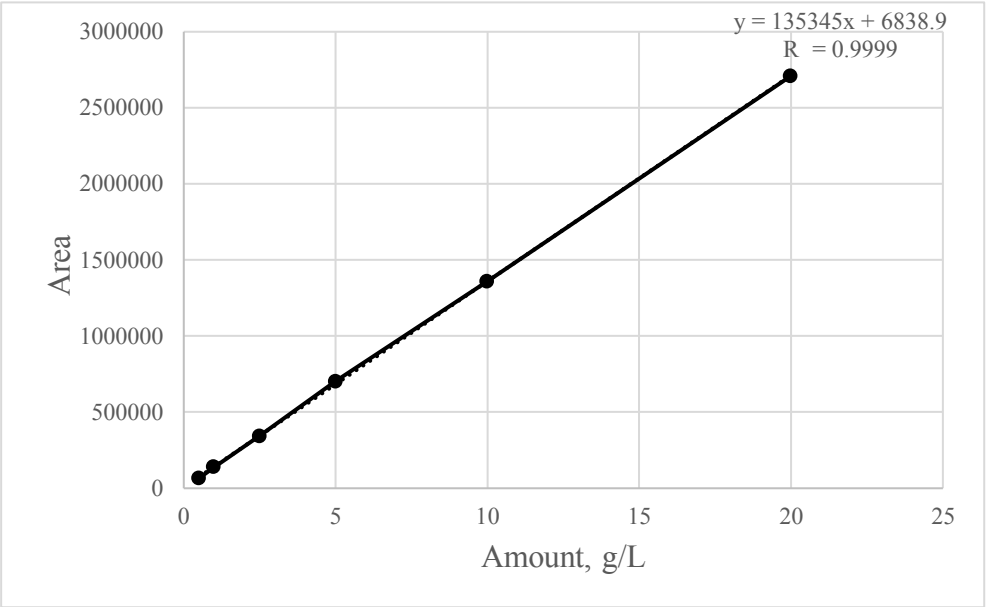

Figure B.7 Standard Curve of Glucose

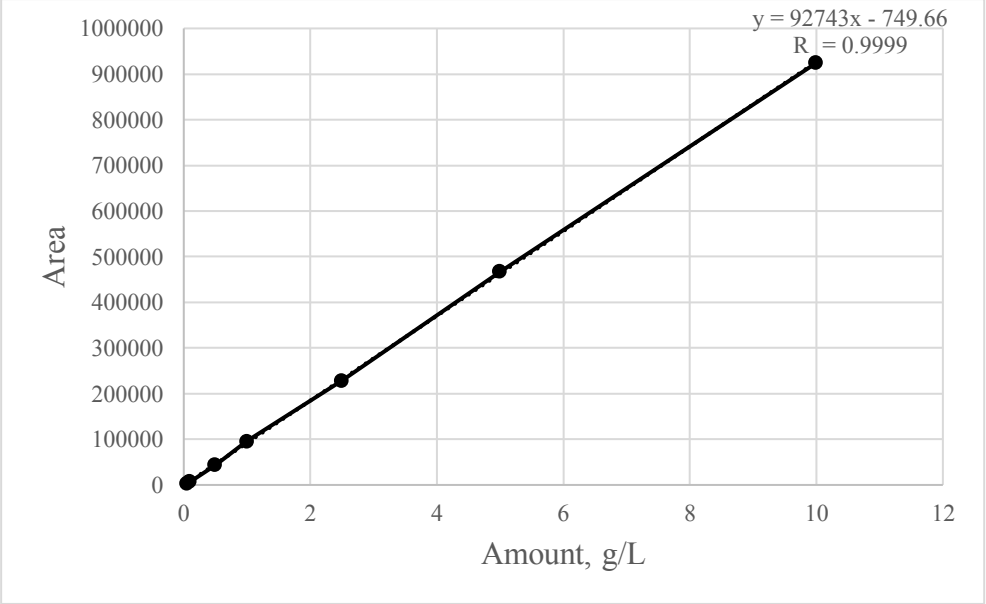

Figure B.8 Standard Curve of Lactic Acid

B.3. Optical Density Analysis

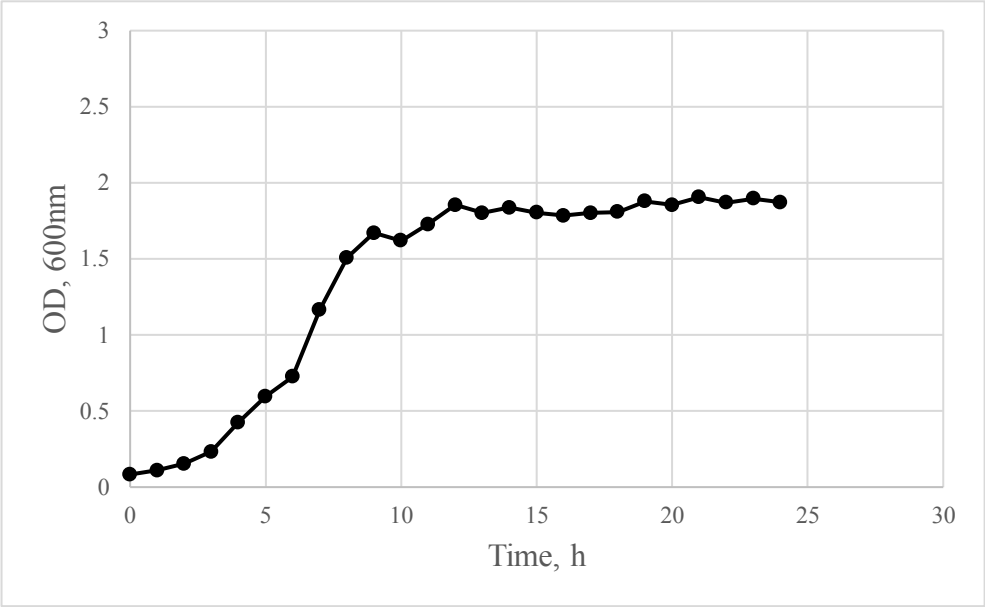

Figure B.9 Growth curve for *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4.

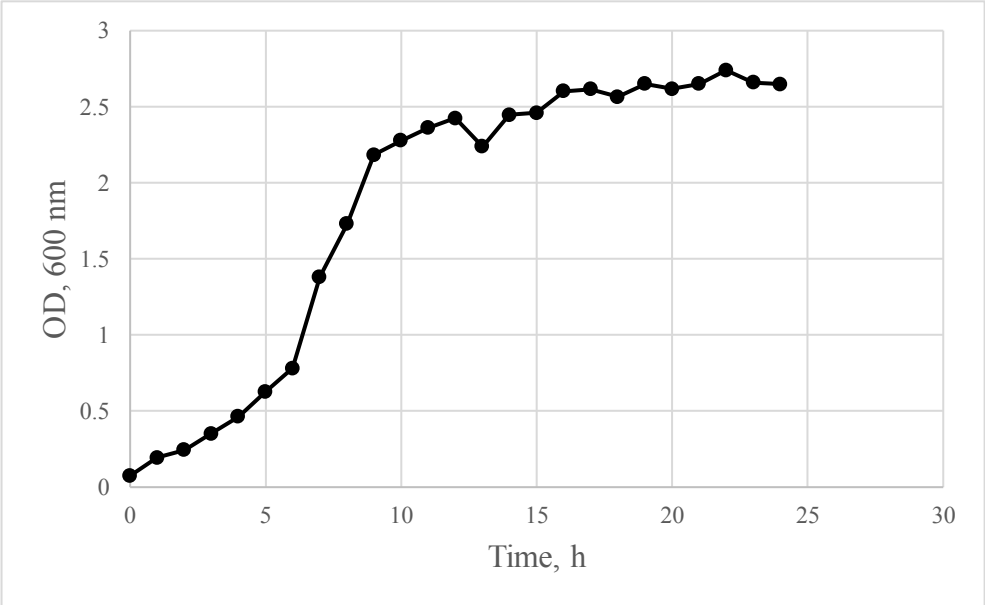

Figure B.10 Growth curve for *Lactobacillus plantarum* OZZ4.

APPENDIX C

C.1. Statistical Analysis of Data

Table C.1 Two-way ANOVA for the effect of high dosage enzyme and hydrolysis time on glucose conversion.

Source	DF	SS	MS	F	P
Time	8	1499.54	187.442	456.57	0.000
Enzyme	4	1710.76	427.690	1041.76	0.000
Interaction	32	38.89	1.215	2.96	0.000
Error	45	18.47	0.411		
Total	89	3267.66			
S = 0.6407		R-Sq = 99.43%		R-Sq(adj) = 98.88%	

Table C.2 Two-way ANOVA for the effect of high dosage enzyme and hydrolysis time on total sugar conversion.

Source	DF	SS	MS	F	P
Time	8	4209.14	526.142	496.44	0.000
Enzyme	4	2100.13	525.032	495.39	0.000
Interaction	32	57.59	1.800	1.70	0.050
Error	45	47.69	1.060		
Total	89	6414.54			
S = 1.029		R-Sq = 99.26%		R-Sq(adj) = 98.53%	

Table C.3 One way ANOVA for the effect of low dosage of enzyme on glucose conversion.

Source	DF	SS	MS	F	P
Enzyme	4	116.927	29.232	142.24	0.000
Error	5	1.028	0.206		
Total	9	117.955			
S = 0.4533		R-Sq = 99.13%		R-Sq(adj) = 98.43%	

Table C.4 One way ANOVA for the effect of low dosage of enzyme on total sugar conversion.

Source	DF	SS	MS	F	P
Enzyme	4	257.532	64.383	149.67	0.000
Error	5	2.151	0.430		
Total	9	259.683			
S = 0.6559		R-Sq = 99.17%		R-Sq(adj) = 98.51%	

Table C.5 One way ANOVA for pectinase effect on glucose conversion.

Source	DF	SS	MS	F	P
Pectinase	4	52.5604	13.1401	171.84	0.000
Error	5	0.3823	0.0765		
Total	9	52.9427			
S = 0.2765		R-Sq = 99.28%		R-Sq(adj) = 98.70%	

Table C.6 One way ANOVA for pectinase effect on total sugar conversion.

Source	DF	SS	MS	F	P
Pectinase	4	198.875	49.719	426.55	0.000
Error	5	0.583	0.117		
Total	9	199.458			
S = 0.3414		R-Sq = 99.71%		R-Sq(adj) = 99.47%	

Table C.7 General Linear Model for the effect of drying of orange bagasse and enzyme amount on glucose conversion.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
OBH	1	0.71	0.71	0.71	3.68	0.103
Enzyme	2	563.90	563.90	281.95	1463.48	0.000
OBH*Enzyme	2	3.64	3.64	1.82	9.45	0.014
Error	6	1.16	1.16	0.19		
Total	11	569.40				
S = 0.438926		R-Sq = 99.80%		R-Sq(adj) = 99.63%		

Table C.8 General Linear Model for the effect of drying of orange bagasse and enzyme amount on total sugar conversion.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
OBH	1	6.51	6.51	6.51	6.57	0.043
Enzyme	2	1082.31	1082.31	541.16	546.22	0.000
OBH*Enzyme	2	5.31	5.31	2.66	2.68	0.147
Error	6	5.94	5.94	0.99		
Total	11	1100.08				
S = 0.995356		R-Sq = 99.46%		R-Sq(adj) = 99.01%		

Table C.9 Two-way ANOVA for the effect of recycle of hydrolysis and experiment type on glucose conversion.

Source	DF	SS	MS	F	P
Rec. Stage	2	463.352	231.676	932.36	0.000
Exp. set	1	71.810	71.810	288.99	0.000
Interaction	2	28.109	14.055	56.56	0.000
Error	6	1.491	0.248		
Total	11	564.761			
S = 0.4985		R-Sq = 99.74%		R-Sq(adj) = 99.52%	

Table C.10 Two-way ANOVA for the effect of recycle of hydrolysis and experiment type on total sugar conversion.

Source	DF	SS	MS	F	P
Rec. Stage	2	941.07	470.534	745.74	0.000
Exp. set	1	194.77	194.770	308.69	0.000
Interaction	2	87.07	43.536	69.00	0.000
Error	6	3.79	0.631		
Total	11	1226.70			
S = 0.7943		R-Sq = 99.69%		R-Sq(adj) = 99.43%	

Table C.11 One way ANOVA for the effect of growth media on the yield of lactic acid produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* OZZ4.

Source	DF	SS	MS	F	P
Media	1	0,002992	0,002992	19,42	0,048
Error	2	0,000308	0,000154		
Total	3	0,003301			
S = 0,01241		R-Sq = 90,66%		R-Sq(adj) = 85,99%	

Table C.12 One way ANOVA for the effect of growth media on the yield of lactic acid produced by *Lactobacillus plantarum* OZH8.

Source	DF	SS	MS	F	P
Media	1	0,0000300	0,0000300	0,63	0,512
Error	2	0,0000960	0,0000480		
Total	3	0,0001260			
S = 0,006927		R-Sq = 23,82%		R-Sq(adj) = 0,00%	

Table C.13 Two sample T-test for D-lactic acid production capacity of two lactobacilli species.

Difference = mu (LD)- mu (LP)
Estimate for difference: -6.113
95% upper bound for difference: -1.501
T-Test of difference = 0 (vs <): T-Value =-8.37 P-Value = 0.038 DF = 1