MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES IN PURE AND APPLIED SCIENCES

OBTAINING SUGARS FROM HAZELNUT HUSK TO BE UTILIZED IN THE GROWTH OF AN INDUSTRIAL MICROBIAL STRAIN

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MASTER THESIS
Department of Bioengineering

Thesis Supervisor
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Thesis CO- Supervisor
Assoc. Prof. Dr. Nihat Alpagu SAYAR

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December, 2021 **Beril PAKALIN**

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ÖZET

ENDÜSTRİYEL BİR MİKROBİYAL SUŞUN BÜYÜMESİNDE KULLANILMAK ÜZERE FINDIK ZURUFUNDAN ŞEKER ELDESİ

Lignoselülozik malzemeler, glikoz, ksiloz ve arabinoz gibi şekerlerden önemli miktarda içerir. Bu durum onları mikrobiyal büyüme için çekici ve ucuz biyokütle kaynakları yapmaktadır. Bu çalışmada, lignoselülozik bir atık olan fındık zürufu şeker hidrolizatının endüstriyel biyo-işlemler için değerlendirilmesi amaçlanmıştır. Bu amaçla, fındık zürufu ilk olarak seyreltik asit ön işlemine tabi tutulmuştur. Kalan katılar süzülüp enzimatik hidroliz için kullanılmıştır. Hem asit ön işlemi hem de enzimatik hidroliz için parametreler, yazılı bir merkezi kompozit tasarım kullanılarak belirlenmiş ve daha sonra elde edilen deneysel sonuçlarla MATLAB' de Response Surface Methodology ve Kriging modelleri kullanılarak optimum değerler hesaplanmıştır. 12.5 g fındık zürufunun 100 mL % 3.8 sülfürik asit çözeltisi içinde 50 dakika süreyle asit ön muamelesi, 27.67 mg/mL'lik bir maksimum indirgeyici şeker konsantrasyonu verirken, kalan katıların 39 mL 50 mM sodyum sitrat tamponu içinde 3.12 mL *Trichoderma reesei* selülaz enzimi ile enzimatik hidrolizi 6.88 gün sonunda 8.36 mg/mL'lik bir indirgeyici şeker konsantrasyonu vermiştir. Toplam şeker verimi, ön işlemden sonra yaklaşık 0.12 gr şeker / 1 gr fındık zürufu olmuştur. Bu değer, enzimatik işlemden sonra sadece 0.01 gr şeker / 1 gr fındık zürufu kadar yükselmiştir. Enzimatik işlem yeteri kadar elverişli olmadığından, doğrudan asit ön işlemi sonrasında elde edilen şeker hidrolizatı *Corynebacterium glutamicum*'un mikrobiyal büyümesi için karbon kaynağı olarak kullanılmıştır. Yabani tip suş ksiloz tüketemediğinden, şeker hidrolizatında bulunan glikoz ve ksilozun eşzamanlı kullanımını değerlendirmek için ksiloz kullanım genlerini barındıran bir mutant suş da ayrıca kullanılmıştır. Her iki hücre de tek karbon kaynağı olarak züruf hidrolizatı ile tanımlanmış ortamlarda büyüyebilmiştir. Diğer taraftan beklendiği gibi ksiloz kullanım genlerine sahip hücreler yabani tipten iyi büyümüştür. Bu çalışmadan elde edilen sonuçlar, fındık zürufunun çok çeşitli kimyasalların üretiminde kullanılan bir endüstriyel suşun yetiştirilmesi için karbon kaynağı olarak kullanılabileceğini göstermiştir.

ABSTRACT

OBTAINING SUGARS FROM HAZELNUT HUSK TO BE UTILIZED IN THE GROWTH OF AN INDUSTRIAL MICROBIAL STRAIN

Lignocellulosic materials contain significant amounts of sugars, that include glucose, xylose, and arabinose. This makes them attractive and cheap sources of biomass for microbial growth. In this study, the aim was to get the sugar hydrolysate of a lignocellulosic waste, hazelnut husk, to be evaluated for industrial bio-processes. To this end, hazelnut husk was first subjected to dilute acid pretreatment. The remaining solids were filtered out and used for enzymatic hydrolysis. The parameters for both acid pretreatment and enzymatic hydrolysis were determined using an inscribed central composite design; and, then with the experimental results obtained optimum values were calculated using Response Surface Methodology and Kriging models in MATLAB. Acid pretreatment of 12.5 g husk in 100 mL of 3.8 % sulfuric acid solution for 50 minutes yielded a maximum reducing sugar concentration of 27.67 mg/mL, while enzymatic hydrolysis of the remaining solids with 3.12 mL cellulase enzyme from *Trichoderma reesei* in a total volume of 39 mL 50 mM sodium citrate buffer yielded a reducing sugar concentration of 8.36 mg/mL after 6.88 days of hydrolysis. The total yield was approximately 0.12 g sugar / 1 g hazelnut husk after pre-treatment. This value increased only by 0.01 g sugar / 1 g hazelnut husk after enzymatic treatment. Since enzymatic treatment was not feasible, sugar hydrolysate obtained after acid pretreatment was directly used as the carbon source for the microbial growth of *Corynebacterium glutamicum*. The wild-type strain cannot not consume xylose; therefore, a mutant strain harbouring the xylose utilization genes was also used to assess the simultaneous utilization of glucose and xylose found in the sugar hydrolysate. Both cells were able to grow in defined media with husk hydrolysate as the sole carbon source and, as expected, cells with the xylose utilization genes, grew better. Here, the results obtained have demonstrated that hazelnut husk can be used as a carbon source for the cultivation of the industrial workhorse of a wide range of commodity and value-added chemicals.

SYMBOLS

%:	Percentage
°C:	Celcius
cm	Centimeter
g:	Gram
h:	Hour
L:	Liter
M:	Molar
mg:	Milligram
min:	Minute
mL:	Milliliter
mM:	Millimolar
rpm:	Revolution per minute
v/v:	Volume per volume
w:	Weight
w/w:	Weight per weight
µg:	Microgram
µl:	Microliter

ABBREVIATIONS

AFEX:	Ammonia Fiber Explosion
AP:	Acid Pretreatment
ARO:	*C. glutamicum* ARO02-pSH1
ARO02:	*C. glutamicum* ARO02
AROXyl:	*C. glutamicum* ARO02-pSH1-$xylA_{Xc}B_{Cg}$
BHI:	Brain Heart Infusion
Cg:	*C. glutamicum* ATCC 13032
CgXyl:	*C. glutamicum*-pEKEx3-$xylA_{Xc}B_{Cg}$
DNS:	3,5-Dinitrosalicylic Acid
DW:	Dry Weight
EH:	Enzymatic Hydrolysis
Km:	Kanamycin
LB:	Lysogeny Broth medium
LCM:	Lignocellulosic Biomass
MOPS:	3-morpholinopropanesulfonic acid
PKS:	Protocatechuic Acid
Spec:	Spectinomycin
RSM:	Response Surface Methodology

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1. INTRODUCTION

1.1. Lignocellulosic Biomass

Lignocellulosic biomass such as forest and agricultural residues and municipal solid wastes are the largest available bioresources with a global yield of 1.3 billion tons per year (Baruah et al., 2018). They can be processed to animal feed or used as mulching agent in fields. However, most of them are unutilized and burned on the fields or left to decompose (Masran et al., 2016). Lignocellulosic biomass is an advantageous and economical bio-source since it is largely available all around the world and generally does not interfere with the feed and food industries (Brethauer & Studer, 2015).

Lignocellulosic biomass comprise materials that contain plant cell walls (da Costa Sousa et al., 2009). Generally, they contain cellulose, hemicellulose and lignin polymers of different percentages with very small amounts of ash, pigment and protein. The source of the lignocellulosic biomass (grass, hard or softwood) greatly affects the percentages and structures of polymers involved (Harmsen et al., 2010). As it can be seen in Figure 1.1, at the core of the lignocellulosic biomass are generally organized cellulose macro-fibers. Lignin parts envelop the cellulose structure while hemicellulose molecules form a dispersed network between the lignin and cellulose portions of the lignocellulosic compound.

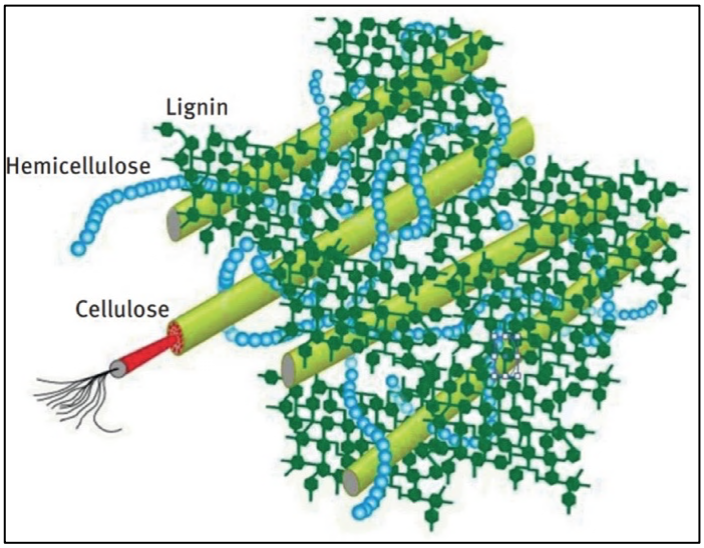

Figure 1.1: Structure of lignocellulosic biomass adapted from (S. Wang et al., 2017)

These polymers contain significant amounts of different sugars, which makes lignocellulosic materials attractive sources for microbial bio-processes. However, due to the bonds and

interactions between these polymers, lignocellulosic biomass is recalcitrant to degradation (da Costa Sousa et al., 2009).

1.2. Cellulose:

Cellulose is one of the main constituents of a lignocellulosic material. Two D-glucose molecules link via a beta-1,4 glycosidic bond to form cellobiose. Cellulose polymer is formed when more than two D-glucose molecules bind to each other with beta-1,4 glycosidic bonds. Structure of a cellulose polymer with cellobiose and glucose subunits can be seen in Figure 1.2. Several linear cellulose chains come together via hydrogen bonds to form microfibrils. These organized microfibrils constitute the crystalline structure of cellulose. Cellulose can also have an amorphous structure, however in lignocellulosic biomasses, cellulose is generally found in its crystalline form. As Kumar et al. (Kumar et al., 2009) suggest, amorphous cellulose is more vulnerable to enzymatic hydrolysis. As for the crystalline cellulose, low temperatures and dilute acids are not sufficient for hydrolysis. It can be broken down at high temperatures and/or with concentrated acid solutions.

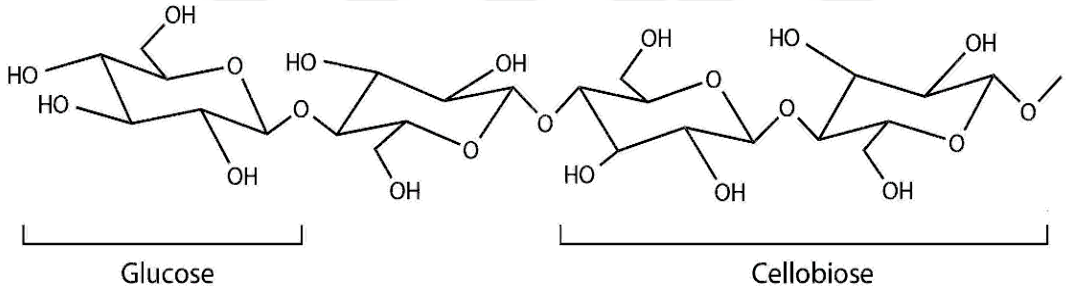

Figure 1.2: Molecular structure of cellulose with subunits cellobiose and glucose. (adapted from: (Ouafi et al., 2017)

1.3. Hemicellulose:

Hemicellulose comprises a group of polysaccharides with varying structure and compositions. These polysaccharides can contain five carbon sugars such as: xylose and arabinose but also six carbon sugars such as: glucose, mannose and galactose. Some examples of these polysaccharides are: arabinoxylans, glucomannans, galactoglucomannans and xylans, as shown in Figure 1.3. Monomers of hemicellulose form beta-1,4 glycosidic bonds on the main chains and beta-1,3, beta-1,2, beta-1,6 glycosidic bonds in side chains (Chen, 2014). These short side chains result in hemicelluloses having a branched rather than a crystalline structure. This structure reveals a significant difference between hemicellulose and cellulose which also affects its hydrolyzation. Due to the lack of crystalline structure, hemicellulose can be hydrolyzed at

lower temperatures compared to cellulose (Finnish Thermowood, 2003). Also, as Harmsen et al. (Harmsen et al., 2010) state, addition of acids, increases their solubility in water.

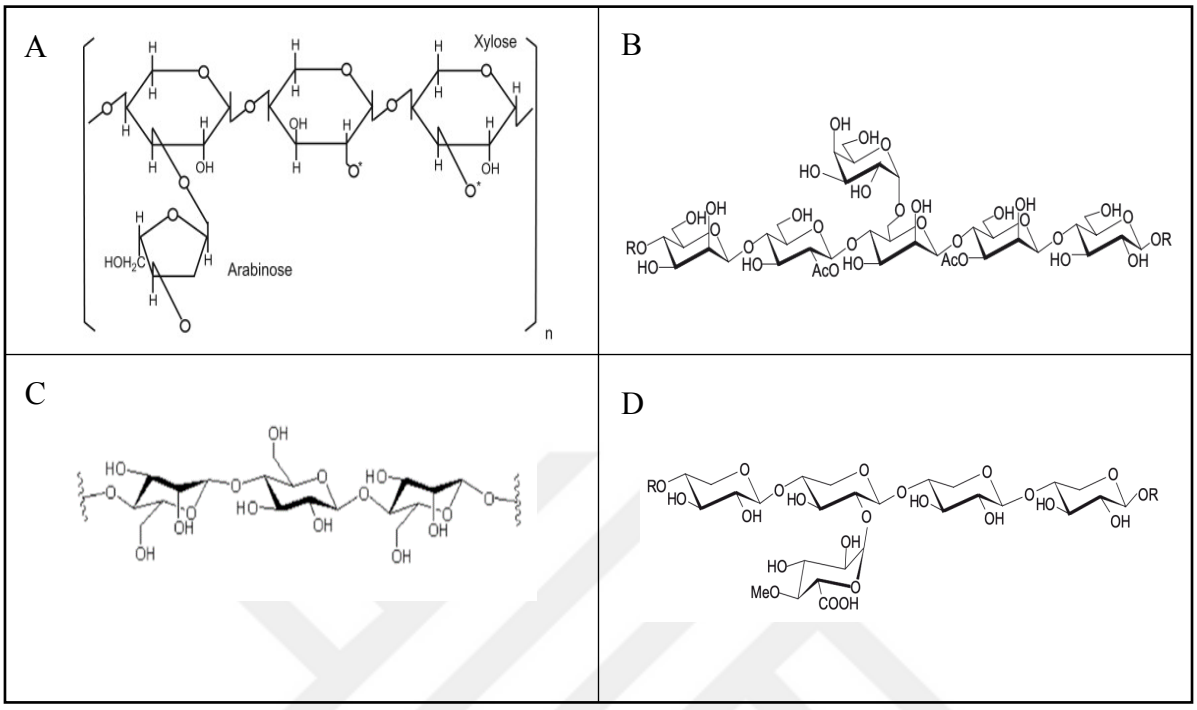

Figure 1.3: Some polysaccharides that are present in hemicellulose: A: arabinoxylan; B: glucomannan; C: galactoglucomannan, D: xylan. Adapted from: (Kilpeläinen, 2015) (J. Wang et al., 2020) (Ghoneum, 2014)

1.4. Lignin:

Following cellulose, lignin is one of the most abundant materials found in plant biomass (Chen, 2014). It is a complex polymer and mostly consists of phenolic compounds such as: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, as it can be seen in Figure 1.4. Randomized crosslinking of these phenolic compounds results in large variability and complexity for lignin structures found in plants. Lignin provides a supportive structure, helping plant tissues to develop and stay strong while providing them an external barrier from factors such as: impact or bacterial/fungal decomposition. Due to its non-crystalline 3-D structure, lignin acts as a binding complex around plant cells (Harmsen et al., 2010). Compared to other polymers found in lignocellulosic biomaterials, lignin is the polymer most resistant to heat (Finnish Thermowood, 2003).

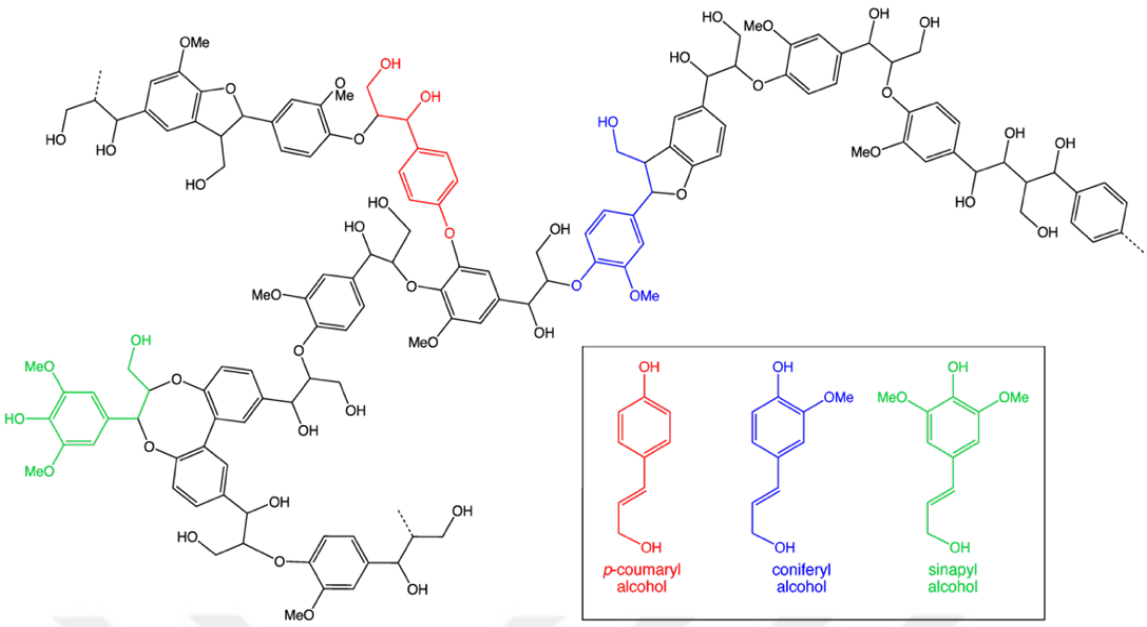

Figure 1.4: Generalized structure of lignin with phenolic monomers adapted from (Smokefoot, 2011)

1.5. Hazelnut Husk:

Hazelnut husk is a lignocellulosic material and an agricultural waste product of hazelnut production. Turkey produces 420,000 tons of Hazelnut annually, which corresponds to 56 % of the total production worldwide (Sabanci & Buyukkileci, 2018). Like other lignocellulosic materials, hazelnut husk is commonly discarded through burning and is not utilized for any other processes. Previously, a number of studies were carried out using hazelnut husks and shells for ethanol production or to obtain hemicelluloses using different pretreatment techniques (Sabanci & Buyukkileci, 2018). According to one study, hazelnut kernel husk is comprised of 29.6 % cellulose, 53 % lignin and 15.7% hemicellulose, as shown in Figure 1.5 (Demirbaş, 2003). Another more detailed composition of hazelnut husk was given as: 15.4 % cellulose, 25.9 % klason lignin, 5.8 % xylan, 2.6 % galactan, 1.7% arabinan, 10.6 % uronic acids, 5 % ash, 24.6 % extractives and 8 % protein (Surek & Buyukkileci, 2017). Accordingly, hazelnut husk like many other lignocellulosic materials, contains significant amounts of different sugars, which make it an attractive and cheap carbon source for microbial bio-processes.

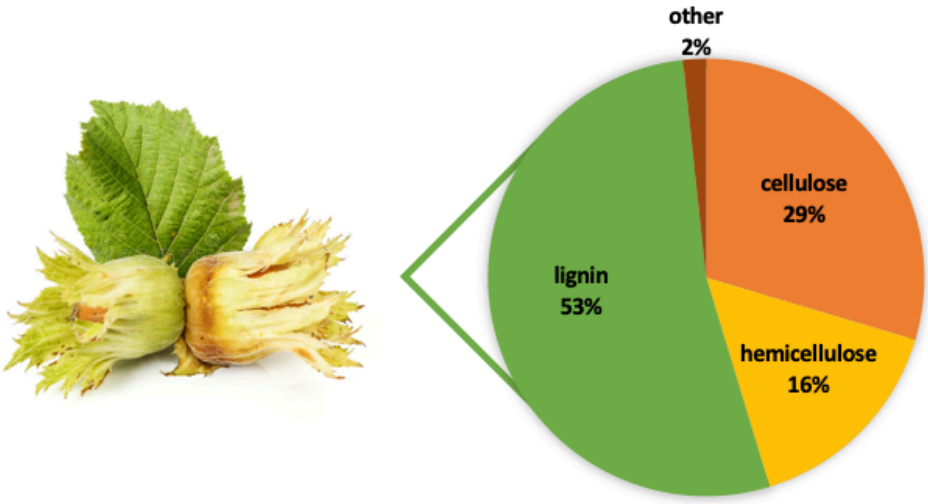

Figure 1.5: Hazelnut clusters in husk and leaf adapted from (Szasz-fabian, n.d.) on left hazelnut husk composition on right.

1.6. Pretreatment of Lignocellulosic Biomass:

Recalcitrant structure of lignocellulosic material, provide plants the protection from external factors such as mechanical impact and biological attacks. This is achieved through the specific characteristics of each component of lignocellulosic biomass: lignin, hemicellulose and cellulose and their interactions with each other. The main objective of pretreatment is to increase the surface area and porosity of the lignocellulosic biomass, as it can be seen in Figure 1.6. Thereby, lignin layer will be decomposed, hemicellulose will be broken down more easily, and the crystalline structure of cellulose will be disrupted (Baruah et al., 2018;Jørgensen, Kristensen, et al., 2007).

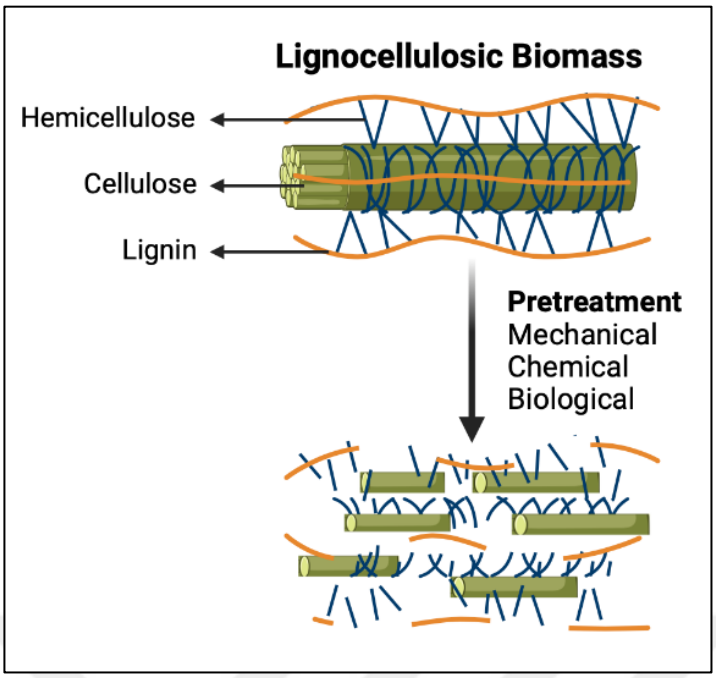

Figure 1.6:A schematic for how pretreatment affects lignocellulosic biomass adapted from BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates

A pretreatment process should abide to some criteria in order to be beneficial. Primarily, it should help to maximize the obtained sugars from the lignocellulosic biomass. Also, it should prevent degradation of sugars or polysaccharides to any by-products that will lower sugar yield or have an inhibitory/toxic affect to consequent processes. Finally, it should be economically feasible and adaptable for larger scale (Kumar et al., 2009;Jørgensen, Kristensen, et al., 2007). An appropriate pretreatment step is believed to increase the enzymatic hydrolysis rate from 3 to 10-fold (da Costa Sousa et al., 2009). Therefore, an efficient pretreatment step becomes a crucial part in the process. Different pretreatment techniques can be grouped into four categories: physical, chemical physicochemical and biological pretreatments, as shown in Figure 1.7.

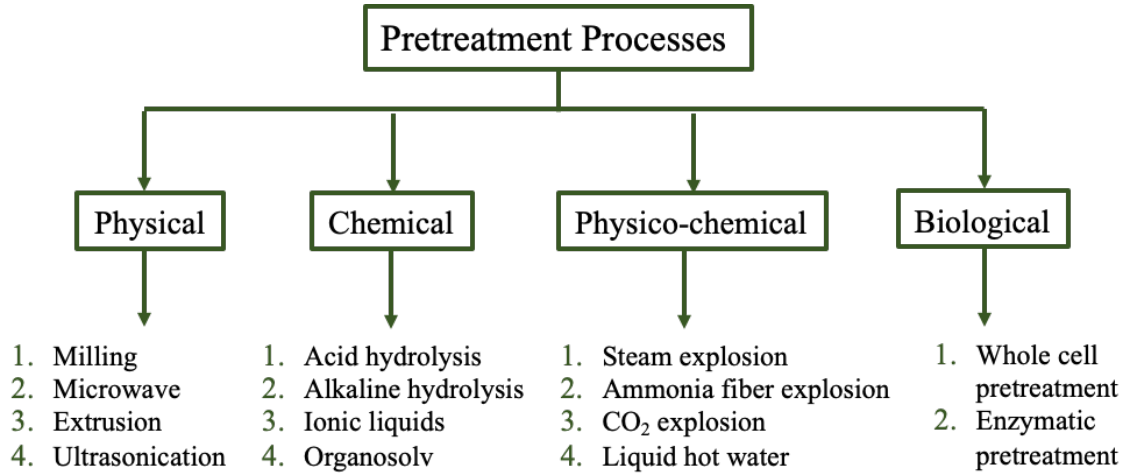

Figure 1.7: A schematic showing different types of pretreatment methods adapted from (Baruah et al., 2018)

1.6.1 Physical Pretreatments:

Physical pretreatments are usually used as primary steps for the saccharification of lignocellulosic biomasses. Physical pretreatments generally reduce the size of the biomass particles, this in turn increases their surface area. An increase in the surface area does not necessarily increase the obtained sugars by itself, however it makes the material more efficient and easily accessible to subsequent pretreatments (chemical or biological) (da Costa Sousa et al., 2009). Physical pretreatments can also increase the porosity of the material and disrupt the crystalline structure of cellulose. On the other hand, a disadvantage of physical pretreatments is that they are not energy efficient and therefore are not very economical. Thus, optimum particle sizes for the hydrolysis should be determined, both for energy and hydrolysis efficiencies. For instance, it is stated that, a decrease in the particle size helped to achieve a higher hydrolysis rate but, obtaining particles smaller than 0.4 mm didn't improve the hydrolysis rate (Bhutto et al., 2017). Milling, chipping, grinding, extrusion, microwave and gamma radiation, electron beam irradiation, ultrasonication can be grouped under physical pretreatments.

1.6.2 Chemical Pretreatments:

Chemical pretreatments are widely used in industry, for delignification and saccharification of lignocellulosic biomasses. Different chemicals were utilized to degrade lignin and hemicellulosic portions of the lignocellulosic biomass and decrease the crystallinity of cellulose (Behera et al., 2014). Some chemicals can successfully change the structure of the biomass without yielding any toxic or inhibitory by products, while others require high energy demands and recycling for consequent processes (Behera et al., 2014). Alkali chemicals, organic or

inorganic acids, organic solvents, ionic liquids are used for chemical pretreatment processes. Here are some of the most well investigated chemical pretreatments:

1.6.2.1. Acid pretreatment:

One of the commonly used chemical pretreatment techniques is acid pretreatment. Baruah et al. (Baruah et al., 2018) list, many organic or inorganic acids that are used for the pretreatment of lignocellulosic materials such as: hydrochloric acid, nitric acid, sulfuric acid, formic acid and oxalic acid. Among these, dilute sulfuric acid pretreatment is the most broadly used compared to other acid pretreatments (Baruah et al., 2018). During the pretreatment, acid breaks down the glycosidic bonds and generally solubilizes the components so that enzymes can more easily access the cellulose. With the removal of hemicellulose through acid hydrolysis, cellulose hydrolysis also becomes easier (Kumar et al., 2009). However, through acid hydrolysis, several by-products, such as phenolic acids and furfurals, can be produced from lignin and hemicellulose degradation (da Costa Sousa et al., 2009). These by-products can act as inhibitors in the following enzymatic hydrolysis and can be toxic for further microbial growth. Therefore, pretreatment conditions should be optimized to minimize the production of by-products while obtaining high sugar yields. It has been reported that, higher operational temperatures or acid percentages may cause higher inhibitor/byproduct formation (Bhutto et al., 2017). Furthermore, high acid concentration can be corrosive to the equipment and therefore not economical.

1.6.2.2. Alkali pretreatment:

In alkali pretreatment, chemicals such as sodium hydroxide, ammonium hydroxide, potassium permanganate, potassium hydroxide, ammonium hydroxide, or calcium hydroxide are commonly used. Their oxidative property affects the structure of the lignocellulosic biomass so that it becomes more porous and swelled. Lignin component is dissolved, by breaking the ether and ester bonds that links lignin to other components (Ravindran & Jaiswal, 2016). With lignin removed, cellulose portion is more easily accessible for saccharification. Baruah et al. (Baruah et al., 2018) stated that alkali pretreatments were more effective against agricultural waste products and crops with low lignin percentages. For hardwoods with high lignin percentages, an alternative pretreatment could be more effective. After the pretreatment, neutralization and/or recovery of alkali compounds should be considered (Bhutto et al., 2017).

1.6.2.3. Ionic liquid pretreatment:

Ionic liquids are composed of anions and cations that are liquid under 100℃ (Chen, 2014). Some of the studied ionic liquids are grouped as: imidazolium-based, pyridinium-based, ammonium-based and sulfonium-based ionic liquids (Baruah et al., 2018). Ionic liquids break down the hydrogen bonds that link cellulose chains together, which results in the disruption of the crystalline structure of the cellulose polymer (Behera et al., 2014). They are also effective against removal of lignin. Ionic liquids provide some advantages over other pretreatments and are considered environmentally safe. They are nonvolatile with very small vapor pressure. They are stable within a wide range of temperatures and also chemically stable (Chen, 2014). Furthermore, their characteristics can be altered through the cations and anions that are used. However, a recycling step for the remaining ionic liquids is essential, since they can be toxic for the cultivation of some microorganisms such as *Saccharomyces cerevisiae* (Bhutto et al., 2017). Being costly and toxic to organisms are the major disadvantages of ionic liquid pretreatments (Baruah et al., 2018).

1.6.2.4. Organosolv pretreatment:

In organosolv pretreatments, a mixture of water with organic solvents are used to break down both the glycosidic bonds that hold hemicellulose and cellulose together and the ether and ester bonds that link lignin and hemicellulose (Behera et al., 2014). After the organosolv pretreatment, three main polymers of the lignocellulosic biomass can be obtained in three different fractions with relatively high purities (Bhutto et al., 2017). Main organic solvents that are utilized are: methanol, ethanol, glycol, acetone, phenols, amines&ketones and organic acids (Baruah et al., 2018;Behera et al., 2014). An advantage of the organosolv pretreatment is the ability to obtain lignin component for the derivatization of other value added products (Ravindran & Jaiswal, 2016). Even though the recovery of used solvent is rather easy (Ravindran & Jaiswal, 2016), it is a crucial step for further microbial growth steps to prevent inhibition. Furthermore, some organic solvents are expensive, which makes the process costly and the solvent recycling essential (Behera et al., 2014). A major drawback of organosolv pretreatments is that generally solvents are volatile with high flammability (Baruah et al., 2018).

1.6.3 Physico-chemical Pretreatments:

1.6.3.1. Steam explosion:

Steam explosion pretreatment utilizes saturated and high-pressure steam. During high pressure and temperature, hemicellulose and lignin parts are partially disrupted (Kumar et al., 2009). A few minutes later, the pressure is dropped instantly, causing an explosion. Explosion causes lignocellulosic biomass to break apart into smaller particles, increasing the surface area. With

the hydrolysis of hemicellulose, acetyl groups form acetic acid, which further assists hydrolysis. Therefore, this method is also called autohydrolysis (Singh et al., 2015). Steam explosion is one of the most practiced and economic lignocellulosic pretreatments. Like many other pretreatments, formation of by products can occur and these by products should be recovered before consequent processes (Rabemanolontsoa & Saka, 2016). In order to remove inhibitory products, generally the biomass is washed with water after the pretreatment. This may reduce the dissolved sugars obtained in the hydrolysate (Kumar et al., 2009). A shortcoming of this pretreatment is that, lignin-cellulose complex is partially broken down, however it is energy efficient (Kumar et al., 2009).

1.6.3.2. Ammonia fiber explosion:

Ammonia fiber explosion (AFEX) pretreatment is quite similar to steam explosion method, where liquid ammonia is used to treat the biomass at high pressure and temperature for a determined period of time, then the pressure is dropped instantly causing decompression explosion (Rabemanolontsoa & Saka, 2016). AFEX changes the structure of the biomass, so that the material swells and disrupts the crystalline structure of the cellulose. It also breaks down the bonds that hold lignin-carbohydrate network (Rabemanolontsoa & Saka, 2016). An advantage of AFEX pretreatment is that it doesn't produce any inhibitory products (Baruah et al., 2018). Although it is proven to be effective against agricultural crops and grasses, AFEX is not an efficient pretreatment method for lignocellulosic biomasses that contain higher percentages of lignin, like hardwoods and nut shells (Kumar et al., 2009;Behera et al., 2014). Also remaining ammonia should be recovered and recycled in order for the process to be feasible (Baruah et al., 2018). Any residual ammonia after the treatment, can be used as a nitrogen source for microbial growth (Bhutto et al., 2017).

1.6.3.3. CO_2 explosion:

CO_2 explosion was developed while investigating a more effective and efficient pretreatment method compared to steam or ammonia fiber explosion (Kumar et al., 2009). In CO_2 explosion pretreatment, CO_2 is utilized in its supercritical form. Here, the CO_2 molecules pierce within the pores of the biomass disrupting its structure and increasing the surface area (Behera et al., 2014). With CO_2 explosion, both cellulose and hemicellulose are hydrolyzed. Also, lower operating temperature hinders obtained sugars from further digestion (Kumar et al., 2009). According to Bhutto et al. (Bhutto et al., 2017) carbonic acid formed by CO_2 and water within the biomass, assist with the hydrolysis of the hemicellulose. Thus, the moisture content of the lignocellulosic biomass becomes an important factor for the effectiveness of the pretreatment. During the pretreatment, no inhibitory by-products are formed. Moreover, CO_2 is a non-toxic,

non-flammable gas which makes CO_2 explosion preferable over other methods (Bhutto et al., 2017). Although CO_2 gas is not costly, equipment that can resist high pressures are expensive (Baruah et al., 2018).

1.6.3.4. Liquid hot water pretreatment:

In liquid hot water pretreatment, biomass is subjected to water at temperatures around 180℃ to 220℃ under pressure. Pressure is not dropped abruptly as in steam explosion or AFEX pretreatment (Baruah et al., 2018). During this pretreatment, lignin is solubilized partially and hemicellulose is hydrolyzed (Behera et al., 2014). There are several advantages of liquid hot water pretreatment method. It doesn't require any chemicals, therefore no inhibitory product is formed after the treatment, and also corrosion resistant equipment are not needed (Jiang et al., 2015). Reduction of biomass size prior to the pretreatment is not essential, since during the pretreatment biomass already breaks down (Ravindran & Jaiswal, 2016). However compared to other pretreatments, obtained sugar yields are low and the process requires lots of energy (Baruah et al., 2018).

1.6.4 Biological Pretreatments:

In biological pretreatment, lignocellulosic materials are either treated with lignolytic bacteria or fungi, or enzymes obtained from these organisms (Baruah et al., 2018). Advantages of biological pretreatments are that they do not require high temperatures and pressures. Also no chemical compounds are used, therefore no inhibitory by-products are formed (Ravindran & Jaiswal, 2016). However, a major disadvantage is that the pretreatment process takes a long time and obtained sugar yield is lower when compared to other pretreatment methods (Bhutto et al., 2017). It is stated that carbon to nitrogen ratio of the material is quite important for the effectiveness of the treatment. Since fungi are less dependent on nitrogen, they are said to be more effective for the treatment when compared to bacteria (Wichern et al., 2004).

1.6.4.1. Whole-cell pretreatment:

For whole-cell pretreatment, fungi and bacteria are used. Among the fungi, white rot and soft rot fungi are able to hydrolyze both cellulose and lignin portions, while brown rot fungi hydrolyze primarily cellulose (Kumar et al., 2009). Bacteria that are used in lignocellulosic treatments are generally Clostridiales, Pseudomonas, and Actinomycetes (Rabemanolontsoa & Saka, 2016). Effectiveness of biological pretreatment is greatly dependent on the type of microorganism, type of biomass, and temperature&pH of the process (Bhutto et al., 2017). During whole-cell pretreatment, it is challenging to obtain monomeric sugars as product, since they may be utilized by the microorganisms, which is a major disadvantage (Rabemanolontsoa & Saka, 2016). White rot fungi present itself as an effective microorganism for the degradation

of lignocellulosic biomass, since it is said to be an efficient laccase producer (Yesilada et al., 2018). Some examples of white rot fungi are: *P. pulmonarius, P. pulmonarius, P. Chrysosporium, Ceriporia lacerata, Pleurotus ostreaus, Ganoderma lucidum* (Ravindran & Jaiswal, 2016). For delignification, *P. chrysosporium* is reported to be a model organism (Baruah et al., 2018).

Decomposition of the lignocellulosic biomass is achieved through two extacellular systems; hydrolytic and ligninolytic (Wagner et al., 2018). Through the hydrolytic system, hemicellulosic and cellulosic portions of the biomass are broken down. Both fungi and bacteria are investigated for the hydrolytic system. Enzymes that are involved in the hydrolysis of polysaccharides are: endoglucanase, exoglucanase and hemicellulases (Bayer et al., 2004). For the ligninolytic system, white rot fungi is the most popular organism, however some bacteria (Pseudomonas and Actinomycetes) are also able to degrade lignin (Rabemanolontsoa & Saka, 2016) (Baruah et al., 2018). Main enzymes responsible for delignification are: laccase, manganese peroxidase and lignin peroxidase (Rabemanolontsoa & Saka, 2016).

1.6.4.2. Enzymatic pretreatment:

In enzymatic hydrolysis, rather than using microorganisms, enzymes that they have produced are used (Ravindran & Jaiswal, 2016). Among those microorganisms, *Trichoderma reesei* is one of the most investigated for its cellulase and hemicellulase enzyme production capability (Kubicek, 2013). Enzymatic pretreatment can take around 1 to 4 days, at ~50℃ and pH ~5. To the hydrolysate, surfactants or antibiotics may be added to prevent any microbial growth (Gandla et al., 2018). In this process, commonly more than one enzyme is used to treat the lignocellulosic biomasses. Enzymes that are utilized for the hydrolysis of cellulose polymers are called cellulases. Endoglucanase, beta-glucosidase (cellobiase), cellobiohydrolase are the major enzymes responsible for the hydrolysis of the cellulose polymer (Rabemanolontsoa & Saka, 2016). Endoglucanase cleaves the inner glycosidic bonds within a cellulose chain. Cellobiohydrolyses bind to the ends of a cellulose chain and cleave the cellobiose molecules, while beta-glucosidase breaks down the cellobiose, yielding glucose molecules (Gandla et al., 2018). All these enzymes are essential for the full conversion of cellulose to monomeric sugars. For the hydrolysis of hemicellulose, a group of enzymes, named xylanases are utilized. It has been investigated that firstly endo-beta-1,4-xylanase splits hemicellulose polymer forming oligosaccharides. Then 1,4-beta-xylosidase cleaves these oligosaccharides. Finally, xylose sugars are obtained. Also, side chains are targeted by other glycosidases, resulting in the full hydrolysis of hemicellulose. Most commonly used enzymes for lignin hydrolysis are laccase, manganese peroxidase, lignin peroxidase, and versatile peroxidase (Ravindran & Jaiswal,

2016). Many organisms such as fungi, bacteria, plants, insects are able to produce lignolytic enzymes; however, enzymes produced from fungi are the most widely used ones (Rabemanolontsoa & Saka, 2016;Baruah et al., 2018). There are commercial enzymes widely used for this enzymatic pretreatment. One of the most frequently used commercial enzymes is Celluclast 1.5L from Novozyme. To improve the glucosidase activity, Novozym 188 can be used concurrently (Gandla et al., 2018).

1.7. Sugar Analysis

Following the treatment of biomass, in order to determine the sugar content of the biomass hydrolysate, a neutralization and/or filtration step may be required. Thereby the pH of the solution will be adjusted after the pretreatment and any salts formed will be filtered out (Sun & Sun, 2015).

Formerly, paper and thin layer chromatography (TLC) methods were utilized for the analysis of sugars obtained from lignocellulosic biomass (Sun & Sun, 2015). Nowadays TLC method is mainly utilized for semi-quantitative analysis. This is a straightforward and fast method (Liu et al., 2021). In brief, sugar samples are spotted on a thin layer, then a mobile phase is applied, to develop the samples. After the thin layer is dried, samples are compared with standard sugars (Liu et al., 2021). Mathias et al. (Mathias et al., 2019) used TLC silica gel plates and a mixture of acetic acid:chloroform:water as a mobile phase for the analysis of reducing sugars obtained from citrus peel biomass. With this method, the presence of several monosaccharides were detected.

After paper and thin layer chromatography methods, new analysis techniques have been developed. One of these methods is gas chromatography–mass spectrometry (GC-MS). GC-MS method, provides much more sensitive results and better separation of different sugars (Liu et al., 2021). Wongsiriwan et al. (Wongsiriwan et al., 2010) used a flame ionization detector with GC for the analysis of monosaccharides and cellobiose obtained from pretreated white spruce. An oxime-trimethylsilyl derivatization was required before the GC-MS procedure. Main drawback of GC and GC-MS methods is that, saccharides do not possess chromophoric or florescent functional groups and they have low volatility (Sun & Sun, 2015). Therefore, a labor intensive derivatization step is required to perform GC and GC-MS (Medeiros & Simoneit, 2007). Consequently, high performance liquid chromatography (HPLC) method became more a prominent technique for sugar analysis. For the detection of monosaccharides with HPLC, refractive index detectors, pulsed amperometric detectors or evaporative light-scattering detectors are used commonly (Medeiros & Simoneit, 2007). HPLC method provides

an advantage of simpler sample preparation when compared to GC or GC-MS while providing sensitive and specific results and good separation of sugars (Sun & Sun, 2015). Institutions like National Renewable Energy Laboratory (NREL) and American Society for Testing and Materials (ASTM) use HPLC method in their technical reports for the determination of carbohydrates in biomasses (Sluiter et al., 2012; Karimi & Taherzadeh, 2016).

Besides aforementioned chromatographic methods, colorimetric methods can be used for determination of sugars obtained from lignocellulosics. Phenol-sulfuric acid and dinitrosalicylic acid (DNS) methods can be given as examples for such methods, where an estimation of the total reducing sugars in a given sample is usually made. Even though they cannot differentiate between the types of saccharides, both are straight forward and quick methods (Karimi & Taherzadeh, 2016). However, it should be mentioned that, an over or under estimation of the real sugar amount could be made while using these methods (McConnell & Antoniewicz, 2016).

1.8. Model Based Optimization

1.8.1 Modelling

Modeling and statistical experimental design is advantageous to perform the least number of experiments; thus saving time and materials, while obtaining the greatest amount of information about the effects of the factors on a system (Mason et al., 2008). In the classical approach, one factor at a time is examined, which is time consuming and leaves out the possible interactions among factors (Timung et al., 2015). Response surface methodology (RSM) utilizes statistical principles and it can be used for the purpose of optimization of the process, where more than one factor is simultaneously affecting the response (Yolmeh & Jafari, 2017). For the investigation of the effects of variables such as biomass loading, temperature, pH, concentration of chemicals (acid-alkali) used and time of the pretreatment, RSM has been frequently employed (Pereira et al., 2021). Central composite design and Box-Behnken design, which are statistical methods of experimental design are frequently used together with response surface methodology (RSM) to select the values of the parameters to be tested (Timung et al., 2015). Pereira et al. (Pereira et al., 2021) reported that both methods have been utilized by researchers for designing experiments in treatment of lignocellulosic biomass.

Alkaline peroxide pretreatment of *Vitellaria paradoxa* sawdust, a lignocellulosic biomass, has been investigated by Ayeni et al. (Ayeni et al., 2013), where they have utilized RSM. Experiments were designed using 2^3 five level central composite design. The study by Timung

et al. (Timung et al., 2015) has used bamboo and sugarcane bagasse as a lignocellulosic biomass. Also, a central composite design by MINITAB has been used for three factors affecting the liquid hot water and acid pretreatment. Similarly, central composite design tool was applied as a model for the dilute acid pretreatment of cotton and sunflower straw (Yildirim et al., 2021). In another study, hazelnut shells were hydrolyzed by alkaline, dilute acid pretreatments and enzymatic hydrolysis. Here, three factors in dilute acid pretreatment were investigated using Box–Behnken Design of RSM (Uzuner & Cekmecelioglu, 2014).

In this study, both low-order polynomials (frequently used for RSM) and Kriging methods were used for modelling. Low-order polynomial models are parameterized models, where a system is fitted to a polynomial equation and the relationship between the variables and the dependent variable is set (Xiaobo, 2018). Low-order polynomials can be expressed in Equation 1.1 and 1.2, where *y(x)* is the unknown function of interest, $\hat{y}(x)$ is an approximation of *y(x)*, and ε is the error between the two functions. In equation 1.2 *f(x)* is the known polynomial function, which can be linear or quadratic, n is the number of independent variables and b_is are parameters. (Simpson et al., 1998;Xiaobo, 2018)

$$y(x) = \hat{y}(x) + \varepsilon \tag{1.1}$$

$$\hat{y}(x) = \sum_{i=1}^{n} b_i f_i(x) \tag{1.2}$$

Kriging is a semi parametric interpolation method that predicts the values of unknown samples by using values of known samples (Xiaobo, 2018). It was first used in mining industry for spatial interpolation (Clark, n.d.). Kriging model can be represented in Equation 1.3, where *y(x)* is the function of interest, *f(x)* is the known function which is a global approximation of the design space, and *z(x)* is the random function which creates local deviations (Simpson et al., 1998).

$$y(x) = f(x) + z(x) \tag{1.3}$$

1.8.2 Optimization

Numerical optimization utilizes the mathematical model of a system to find the values of decision factors (independent variables) that minimizes/maximizes an objective function (dependent variable/response). Many different methods of numerical optimization are available

according to the structure of the mathematical models used and the nature of the objective function.

Ayeni et al. (Ayeni et al., 2013) used a multi-objective numerical optimization on three variables of the pretreatment. They have utilized the response surfaces for investigating the optimum pretreatment conditions. In another study numerical optimization was used for obtaining highest total reducing sugar yield. Optimum conditions for dilute acid and liquid hot water pretreatments were found by solving the regression equation (Timung et al., 2015). Again in another study, total sugar yield was the response variable, while pretreatment conditions were optimized using central composite design (Yildirim et al., 2021). Uzuner et al. (Uzuner & Cekmecelioglu, 2014) has used an optimizer tool in MINITAB® 16.0 for the optimization of pretreatment variables, which were expressed in a quadratic model. In the study by Baliban et al. (Baliban et al., 2013) a rigorous deterministic branch and bound global optimization has been used for solving a mixed-integer nonlinear optimization (MINLP) model with CPLEX. Objective of the optimization was to keep the overall cost of the process in a small fraction, where a lignocellulosic biomass was thermochemically converted to liquid fuels.

In this study, global optimization was used. Global optimization's objective is to find the variables of an objective function that gives the minimum or maximum response. A system may have numerous local optima and global optimization aims to find the global optimum of the system (Brownlee, 2021). There are examples where Kriging has been used to improve the efficiency of global optimization through balancing local and global search (Kleijnen, 2017).

1.9. *Corynebacterium glutamicum*

Corynebacterium glutamicum is an important gram-positive industrial microorganism. It is widely used in industry, generally for its amino acid production capacity (Gopinath et al. 2012). Wild type *C. glutamicum* under normal conditions cannot consume pentose sugars as a carbon source (Gopinath et al., 2012). However, when recombinantly engineered, it can simultaneously utilize glucose and xylose, which are commonly found sugars within lignocellulosic biomass (Sasaki et al., 2019). Also, its ability to tolerate inhibitory by-products of hydrolysis steps such as phenolic compounds, furfurals and organic acids, makes *C. glutamicum* even more advantageous in microbial growth (Sakai et al., 2007). Therefore, *C. glutamicum* has the potential to be grown with sugar hydrolysates obtained from lignocellulosic biomass for the production of variety of value-added compounds.

Different metabolic engineering approaches have broadened *C. glutamicum*'s capability to utilize different carbon sources especially for the production of different value-added

chemicals. Kawaguchi et al. (Kawaguchi et al., 2006) has shown that a recombinant strain of *C. glutamicum* CXR2, containing xylose isomerase (*xylA*) and xylulokinase (*xylB*) genes, was able to consume xylose and glucose simultaneously under oxygen deprived conditions and produce lactic and succinic acids. Another study has used metabolic engineering and adaptive evolution techniques to obtain *C. glutamicum* strains that can utilize xylose and cellobiose concurrently (Lee et al., 2016). Pérez-García et al. (Pérez-García et al., 2021) have shown that a recombinant *C. glutamicum* strain was able to utilize a mixture of glucose, xylose, and mannose sugars to produce 27 mg/L riboflavin.

A number of studies have also investigated cultivation and value-added chemical production of *C. glutamicum* using lignocellulosic materials as the carbon source. Gopinath et al. (Gopinath et al., 2011) have grown a pentose utilizing *C. glutamicum* strain in sulfuric acid hydrolysates of rice straw and wheat bran. Cells were able to grow on acid hydrolysates of both lignocellulosics and able to produce 93 ± 4 mM L-glutamate. In another study, a different recombinant strain, *C. glutamicum* GJ04, has produced 61.7 g/L glutamate from acid pretreated wheat straw hydrolysate (Jin et al., 2020). Another recombinant strain, *C. glutamicum* CGS5, was able to grow on enzymatically hydrolyzed corn straw and produce 98.6 g/L succinate (Mao et al., 2018). Lee et al. (Lee et al., 2016) has used enzymatically hydrolyzed Canadian Ponderosa Pine to be simultaneously saccharified and co-fermented by a recombinant *C. glutamicum* strain. In another study, recombinant *C. glutamicum* cells were grown in ionic liquid pretreated sorghum hydrolysate and were able to produce trace amounts of isopentenol (15 mg/L) (Sasaki et al., 2019).

Available studies have shown the high potential of *C. glutamicum* to be used as a microbial workhorse for the production of value-added compounds from renewable carbon sources specifically lignocellulosic biomass. Therefore, in this study, *C. glutamicum* and its genome reduced strain *C. glutamicum* ARO02 (Walter et al., 2020) were selected for the investigation of their potentials to be cultivated on pretreated husk hydrolysate as the carbon source.

C. glutamicum ARO02 (Walter et al., 2020) is the genome-reduced chassis strain of *C. glutamicum* C1*. In this strain, the unnecessary or undesired genes were deleted systematically, and cells possessed a similar growth profile as the wild type *C. glutamicum* on defined medium with glucose as carbon source was obtained (Baumgart et al., 2018) With its reduced genome, the C1* strain is stated to have a potential to be used in synthetic biology research (Baumgart et al., 2018).

2. MATERIALS AND METHODS

2.1. Materials:

2.1.1 Biomass:

Hazelnut husks were obtained from Giresun in the Black Sea region of Turkey. They were oven dried at 55°C and stored at room temperature in plastic containers. Hazelnut husks were ground with 1L Waring commercial blender and particles that are smaller than 5mm were obtained.



Figure 2.1: Oven dried hazelnut husks on left, crushed hazelnut husks on right

2.1.2 Strains and plasmids:

C. glutamicum ATCC 13032 was purchased from American Type Culture Collection. The shuttle plasmids: pEKEx3, pSH,1 and pSH1*xylA*$_{Xc}$*xylB*$_{Cg}$, and *C. glutamicum* ARO02-pSH1 and *C. glutamicum* ARO02-pSH1-$xylA_{Xc}B_{Cg}$ were kindly provided by Prof. Wendisch (University of Bielefeld). In Table 2.1, the used strains and plasmids are summarized.

Table 2.1: List of strains and plasmids used

Name	Description	Reference/Source
Strains		
C. glutamicum (Cg)	Wild-type strain ATCC 13032	ATCC
C. glutamicum -pEKEx3-$xylA_{Xc}B_{Cg}$ (CgXyl)	*Corynebacterium glutamicum* carrying the plasmid pEKEx3-$xylA_{Xc}B_{Cg}$	This work

Table 2.1: continued

C. glutamicum ARO02 (ARO02)	Genome-reduced chassis strain of *C. glutamicum* mutant Δldh Δvdh::P_{ilvC}-$aroG^{D146N}$	(Walter et al., 2020)
C. glutamicum ARO02-pSH1(ARO)	*C. glutamicum* ARO02 carrying the plasmid pSH1 (control strain)	This work
C. glutamicum ARO02-pSH1-$xylA_{Xc}B_{Cg}$ (AROXyl)	*C. glutamicum* ARO02 carrying the plasmid pSH1-$xylA_{Xc}B_{Cg}$	This work
Plasmids		
pEKEx3-$xylA_{Xc}B_{Cg}$	Shuttle plasmid *C. glutamicum*/*E. coli* with *xylA* gene from *Xanthomonas campestris* and *xylB* gene from *C. glutamicum,* IPTG inducible, $Spec^R$	(Meiswinkel et al., 2013)
pSH1	*C. glutamicum*/*E. coli* shuttle plasmid, constitutive expression, Km^R	(Henke et al., 2016)
pSH1-$xylA_{Xc}B_{Cg}$	*C. glutamicum*/*E. coli* shuttle plasmid carrying *xylA* gene from *Xanthomonas campestris* and *xylB* gene from *C. glutamicum ,* constitutive expression, Km^R	(Pérez-García et al., 2021)

2.1.3 Chemicals:

Sulfuric acid, 3,5-dinitrosalicylic acid (DNS), cellulase from *Trichoderma reesei*, urea, ammonium sulfate, agar, D-(+)-glucose monohydrate, manganese(II) sulfate monohydrate, potassium phosphate monobasic, trisodium citrate dihydrate, glycerol solution biotin and sodium chloride were from Sigma Aldrich. Potassium sodium tartrate tetrahydrate, sodium hydroxide, di-potassium hydrogen phosphate, copper(II) sulfate pentahydrate, magnesium sulfate heptahydrate, zinc sulfate heptahydrate, iron(II) sulfate heptahydrate, calcium chloride nickel(II) chloride hexahydrate were from Merck. MOPS Buffer grade, brain heart infusion (BHI), D(+)-xylose, D(-)-sorbitol and IPTG (Isopropyl β-D-1-thiogalactopyranoside) were from NeoFroxx GmbH. 3,4-Dihydroxybenzoic acid (protocatechuic acid (PKS)) was from Alfa Aesar. Spectinomycin dihydrochloride 5-hydrate Biochemica was from PanReac AppliChem. Kanamycin sulfate was from Roche.

2.1.4 Buffers and media:

BHI medium: 37 g/L BHI was dissolved in 1L distilled deionized water and autoclaved at 121°C for 15 minutes. For BHI agar plates, 15 g/L agar was added to BHI medium. After autoclaving, plates were poured into petri dishes.

LB medium: 10 g/L peptone, 5 g/L yeast extract and 10 g/L NaCl were mixed in 1L distilled deionized water and autoclaved at 121°C for 15 minutes. For LB agar plates, 15 g/L agar was added to LB medium. After autoclaving, plates were poured into petri dishes.

CGXII minimal medium: For the preparation of 50 mL CGXII medium, 45 mL of basic solution was mixed with 5 mL of 40% (v/v) glucose solution. The basic solution contained 20 g/L $(NH_2)_2SO4$, 5 g/L urea, 1 g/L KH_2PO_4, 1 g/L K_2HPO_4 and 42 g/L 3-morpholinopropanesulfonic acid (MOPS) in 900 mL distilled water with pH adjusted to 7 using 10M NaOH solution. Medium was autoclaved at 121°C for 15 minutes. Appropriate amount of trace elements solutions was added so that the final concentrations are: 0.25 g/L $MgSO_4 \cdot 7H_2O$, 10 mg/L $CaCl_2$, 10 mg/L $FeSO_4 \cdot 7H2O$, 10 mg/L $MnSO_4 \cdot H2O$, 1 mg/L $ZnSO_4 \cdot 7H2O$, 0.2 mg/L $CuSO_4 \cdot 5H_2O$, 0.02 mg/L $NiCl_2 \cdot 6H_2O$, 0.2 mg/L biotin and 30 mg/L protocatechuic acid. All trace elements solutions were sterilized with 0.22 μm filters and stored at -20 °C.

Trace elements' final concentration in 1 liter of the medium were adjusted to: 0.25 g/L $MgSO_4 \cdot 7H_2O$, 10 mg/L $CaCl_2$, 10 mg/L $FeSO_4 \cdot 7H2O$, 10 mg/L $MnSO_4 \cdot H2O$, 1 mg/L $ZnSO_4 \cdot 7H2O$, 0.2 mg/L $CuSO_4 \cdot 5H_2O$, 0.02 mg/L $NiCl_2 \cdot 6H_2O$, 0.2 mg/L biotin and 30 mg/L protocatechuic acid.

CGXII medium with hazelnut husk hydrolysate: When using husk hydrolysate as the carbon source, 20 g/L $(NH_2)_2SO_4$, 5 g/L urea, 1 g/L KH_2PO_4, 1 g/L K_2HPO_4 and 21 g/L MOPS were mixed in 200 mL distilled deionized water and pH was adjusted to 7 with 10M NaOH solution to get 5X CGXII minimal medium. This medium was autoclaved at 121°C for 15 minutes. Trace elements were prepared as previously mentioned. For the preparation of 50 mL CGXII medium with hazelnut hydrolysate, 40 mL of hazelnut husk hydrolysate was mixed with 10 mL of 5X CGXII basic medium. The trace elements solutions' concentrations were the same as in the medium with glucose as the carbon source.

DNS reagent: 5 g of DNS was dissolved in 100 mL of 2M NaOH solution. 150g of potassium-sodium tartrate was added and mixed until homogenous. The solution's final volume was set to 500 mL by adding distilled water (Harisha, 2007)

The compositions of the media and buffers are summarized in Table 2.2.

Table 2.2: List of media and buffer used

Media and buffers	**Composition**
Brain heart infusion medium (BHI)	37 g/L BHI
Brain heart infusion sorbitol medium (BHIS)	37 g/L BHI 91 g/L Sorbitol
Brain heart infusion agar plates	15 g/L agar 37 g/L BHI
CGXII minimal medium Trace element solutions:	20 g/L $(NH_2)_2SO4$, 5 g/L urea, 1 g/L KH_2PO_4 1 g/L K_2HPO_4 42 g/L MOPS 4% (w/v) glucose 0.25 g/L $MgSO_4 \cdot 7H_2O$ 10 mg/L $CaCl_2$ 10 mg/L $FeSO_4 \cdot 7H2O$ 10 mg/L $MnSO_4 \cdot H2O$ 1 mg/L $ZnSO_4 \cdot 7H2O$ 0.2 mg/L $CuSO_4 \cdot 5H_2O$ 0.02 mg/L $NiCl_2 \cdot 6H_2O$ 0.2 mg/L biotin 30 mg/L protocatechuic acid (PKS)
50mM sodium citrate buffer	14.7 g/L trisodium citrate dihydrate
DNS reagent	10 g/L DNS 300 g/L potassium-sodium tartrate 2M NaOH solution

2.2. Methods:

2.2.1 Preparation of husk hydrolysate:

2.2.1.1. Acid pretreatment:

For acid pretreatment, the total volume was set to 100 mL and experiments were carried out in 250 mL Schott bottles. Ground hazelnut husks were mixed with sulfuric acid solutions of

ranging concentrations from 1%-5% (w/w). Husk loading ranged from 3.5 g to 12.5 g. After adding the acid solution to hazelnut husks, they were briefly mixed and autoclaved at 121°C for different periods from 15 to 60 minutes. At the end of acid pretreatment, solid husks were filtered out on a pre-weighed filter paper. Solid parts were left to oven dry at 55°C and their dry weights were recorded for yield calculations. After drying, pretreated husks were stored at room temperature for enzymatic hydrolysis. After the filtration, liquid parts were collected and stored at +4°C for sugar analysis and subsequent microbial growth.

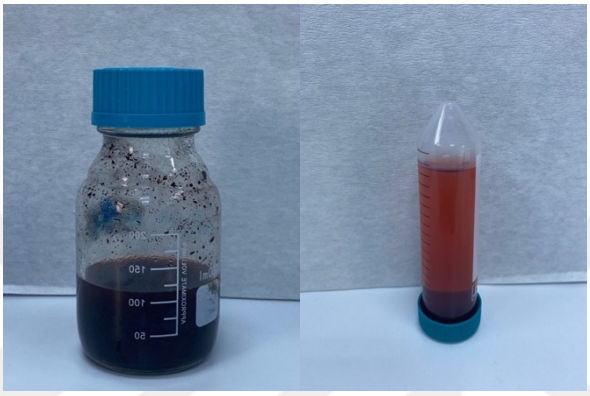

Figure 2.2: Pretreated hazelnut husks in acid solution after autoclave on left, liquid hydrolysate after paper filtration on right.

2.2.1.2. Enzymatic hydrolysis:

Cellulase from *Trichoderma reesei* was used for the enzymatic hydrolysis. Cellulase from *Trichoderma reesei* (C2730) was reported to possess an activity of 700 endoglucanase units (EGU) /g and a density of 1.2 g/mL (Sigma-Aldrich, 2020). Solid husk parts obtained after acid pretreatment were suspended in 50 mM sodium citrate buffer. Husk loads ranged from 1.25 g to 2.5 g. Enzymatic reaction volume was set to 12.5 mL. 1 mL to 2.5 mL of enzyme (840 EGU-2100 EGU) was added to the reaction mixture. Solution's pH was adjusted to 6 and enzymatic hydrolysis was performed in a rotating shaker for 1-7 days at 55°C and 100 rpm in 50 mL Schott bottles. Reducing sugar content of the obtained hydrolysate was measured using DNS.

2.2.2 Model based Optimization:

2.2.2.1. Design of experiments

An inscribed central composite design with 3 factors and 5 levels has been applied for the design of both pretreatment and enzymatic hydrolysis experiments using MATLAB's ccdesign function. A representation of the design space and the distribution of the experiments can be seen in Figure 2.1. The total number of experiments was 24 with 10 center points. 10 repetitions

allowed for a more uniform estimation of prediction variance (*MathWorks Inc.*, n.d.). For acid pretreatment experiments, the three independent variables (factors) were: initial hazelnut husk amount, acid concentration and pretreatment time. Minimum and maximum values for the variables were: 3.5 g-12.5 g for the hazelnut husk, 1-5% (w/w) for the sulfuric acid, 15-60 min for the time. Maximum loading for hazelnut husk has been chosen as 12.5g in 100 mL acid solution, since it was not possible to resuspend higher amounts of husk in the acid solution. This would negatively affect the hydrolysis. Also, the maximum value for the acid concentration has been selected as 5 %, since a dilute acid pretreatment was desired, which would minimize inhibitor formation. As for the period of hydrolysis, 15-60 min was used, since numerous studies reported optimum times for dilute acid pretreatment within the range of 10-90 min. (Singh et al., 2015). For enzymatic hydrolysis, three independent variables (factors) were: 1.25-2.5 g for the pretreated husk, 1-2.5 mL for the cellulase, and 1-7 days for the time. Maximum loading for the pretreated husk has been selected as 2.5 g in 12.5 mL acid solution, since it was not possible to resuspend higher amounts of husk within the buffer solutions. This would adversely affect hydrolysis. Also, the maximum value for enzyme volume was selected as 2.5 mL. Preliminary experiments have shown that higher amounts of enzyme haven't made a significant difference in the total amount of reducing sugar recovered. Maximum reaction time was selected as 7 days, since NREL's protocol suggests 3 to 7 days for enzymatic saccharification of lignocellulosic biomass (Selig et al., 2008).

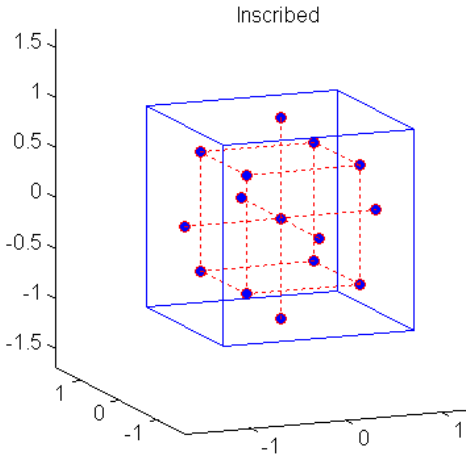

Figure 2.3: A representation of the design space and the distribution of the experiments in an inscribed central composite design adapted from MathWorks Inc.

2.2.2.2. Modeling

In this study a low-order polynomial modelling, which is often used as a part of RSM, and Kriging are utilized. For RSM (low-order polynomial), MATLAB's fitlm function has been used. The response (dependent variable) for both acid pretreatment and enzymatic hydrolysis was total reducing sugar concentration. Selection of the variables has been detailed in Section 2.2.2.1. For Kriging, MATLAB's fitrgp function has been used. This is a quadratic model, and has a pure quadratic basis function and a rational quadratic kernel function.

2.2.2.3. Optimization

For numerical optimization utilizing the models mentioned in Section 2.2.2.2, MATLAB's global optimization toolbox has been used. Unlike general optimization toolbox solvers, global optimization toolbox searches for more than one local minimum (*MathWorks Inc.*, n.d.). As the main optimization function, fmincon (minimization under constraint) with interior-point algorithm has been used. Function fmincon, finds the minimum of nonlinear and multivariable functions. The interior-point algorithm can handle both large and small problems (MathWorks Inc., n.d.). The objective function of both optimization problems was total reducing sugar concentration with the factors explained in detail in Section 2.2.2.2 as decision variables.

2.2.3 Measurement of reducing sugars:

For sugar analysis, if necessary, first the pH of the pretreated sample was adjusted to 7 with 10M NaOH solution prior to analysis. Then DNS method was used to determine the total reducing sugar amount in the samples. For sugar analysis: 1.5 mL of DNS reagent was mixed with 500 µL of the sample in a 20 mL glass test tube, vortexed and placed immediately into boiling water. After 10 minutes, test tubes were removed and placed on ice for 5 minutes. At the end of the 5 minutes, 4 mL of distilled water was added to the solution, vortexed and its absorbance was measured with a spectrophotometer (BIO-RAD SmartSpec Plus) at 540 nm (Harisha, 2007). As blank, 500 µL of distilled water and as a standard, 500 µL of 30 mg/mL glucose solution was used. DNS calibration curves are given in Appendix A and the OD_{540} values read are given in Appendix B.

2.2.4 Cultivation of *C. glutamicum* strains:

All cells were stored in 25 % glycerol solution at -80°C. For cultivation, stock cells were streaked on BHI agar plates with appropriate antibiotics and incubated at 30°C for two days. Then 5 mL of BHI was inoculated with a single colony from the plates. 25 µg/mL of kanamycin was added to media for *C. glutamicum* ARO02- pSH1-$xylA_{Xc}B_{Cg}$ and *C. glutamicum* ARO02-pSH1 cells. 100 µg/mL spectinomycin was added to media for *C. glutamicum* -pEKEx3-$xylA_{Xc}B_{Cg}$ cells. Cells were incubated for 8 hours at 30°C, 200 rpm in an orbital shaker. Of these

cells 2 mL were used to inoculate the 50 mL of CGXII minimal medium with 4 % glucose (Keilhauer et al., 1993). These cells incubated in 500 mL baffled flasks at 30°C in an orbital shaker at 150 rpm for 18 hours were used as the preculture.

2.2.4.1. Growth in flasks:

For the main culture, concentrated CGXII medium and acid pretreated husk hydrolysate was mixed. For media with xylose, glucose, or xylose/glucose mixtures as the carbon source, sugar concentrations were adjusted so that this was identical to the reducing sugar concentration in the medium with the husk hydrolysate. If necessary, 25 µg/mL of kanamycin or 100 µg/mL spectinomycin was added. Media were inoculated with cells from the overnight grown preculture and the initial OD_{600} of the main culture was adjusted to 1. Cultures were incubated in 500 mL baffled flasks at 30°C, 150 rpm for 48 hours. For the growth of *C. glutamicum*-pEKEx3-$xylA_{Xc}B_{Cg}$ (CgXyl), 1mM of IPTG was added to the culture after 90 minutes of incubation to induce the expression of xylose utilization genes. Media without cells were used as blank for OD_{600} measurements.

2.2.4.2. Growth in 96-well plates:

Growth in microtiter plates was achieved in 96-well U-bottom plates. To each well 220 µL of CGXII medium was added with different carbon sources (glucose, xylose, glucose-xylose mix, husk). The sugar concentrations were: 2.6 % glucose, 2.6 % xylose, 0.65 % xylose + 1.95 % glucose (mixed sugar medium), and husk hydrolysate containing equivalent amount of reducing sugar (2.6 %). Approximately 5-6 µL of overnight preculture was added to each well. The amount of preculture added was adjusted so that the OD_{600} of the main culture was approximately 1. The 96-well plate was placed in a BIOTEK-ELx808 microplate reader. The protocol was adjusted to set the plate in continuous fast shaking mode at 30°C for 48 hours. OD_{600} was measured every two hours.

2.2.5 Measurement of colony forming units:

In order to determine the number of viable cells during growth, colony forming units were measured. A protocol that uses the OD_{600} value to dilute the cells has been used (Rutten, 2019). First, OD_{600} of the culture sample was measured. Then using this value, the cells were diluted to get a reading of 0.1 at , OD_{600}. This was used as the starting sample for the dilutions. 10-fold serial dilutions were prepared and a 100µl sample from each dilution was spread on LB plates containing 100 µg/mL spectinomycin. With this protocol 10^5 dilution of the starting sample yielded the optimum colony number of approximately 30 colonies per plate. Thus, during growth, cell samples were withdrawn and spread on plates after appropriate dilution. Plates were incubated at 30°C for 48 hours. Then the number of colonies were counted. Colony

forming units were calculated by multiplying the number of colonies obtained by the final dilution factor.

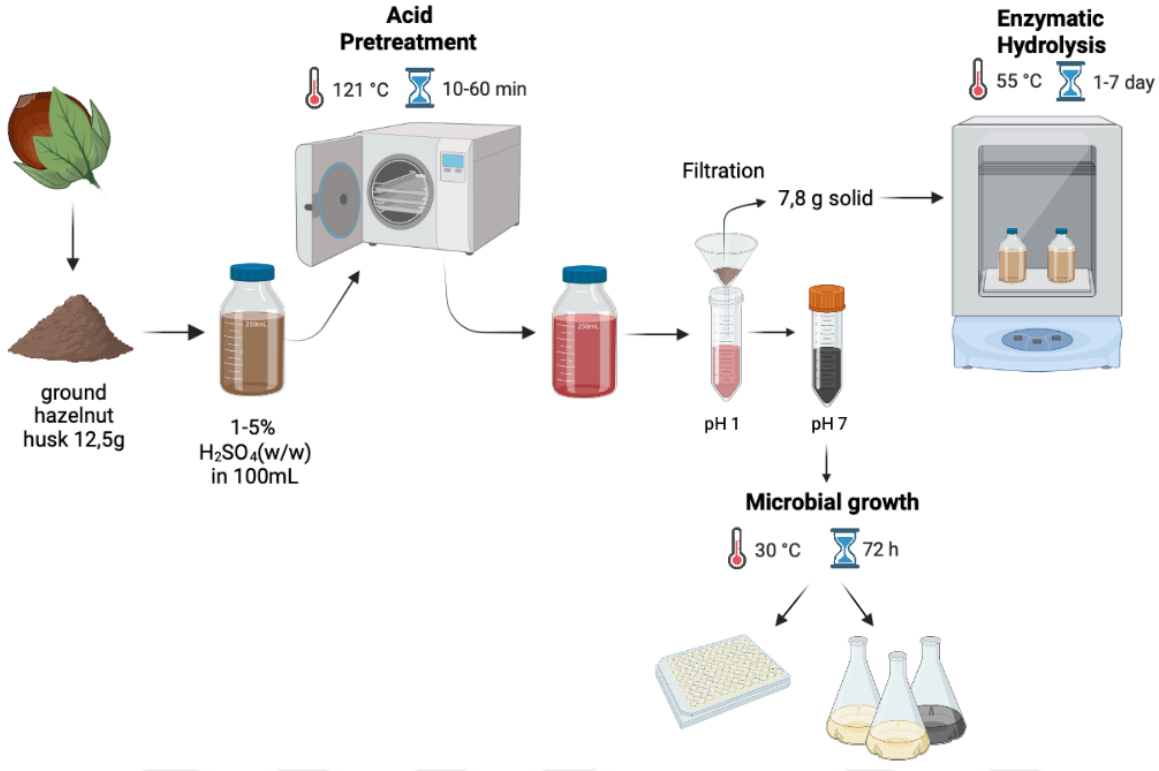

Figure 2.4: Overall representation of the process, adapted from by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates

3. RESULTS AND DISCUSSION

3.1. Acid Pretreatment of Hazelnut Husk:

The results obtained from the first set of acid pretreatment experiments are presented in Table 3.1. In the second, third, and fourth columns of this table given are the selected values of the parameters used in the corresponding experiment of the pretreatment. These data points were determined with central composite design as described in section 2.2.2.1. The highest sugar concentration was achieved when the maximum allowable amount of husk was used, as it can be seen in experiment 14. Lower acid concentration and husk loading yielded lower sugar concentrations. Obtained sugar concentrations after the pretreatment were in the range of 6.48-16.73 mg/mL. In general, increasing husk loading or acid percentage or time of pretreatment, while keeping the other two variables constant, increased the final sugar concentration obtained. On the other hand, final sugar yield tended to be higher with lower husk load, e.g. with 3.5 g husk it was 10.61 %. Unfortunately, the final sugar concentration of the hydrolysate when the yield was higher was too low to be used for bacterial growth. For this reason, the objective of the optimization was selected as the final concentration not the yield.

Table 3.1: Results of the first set of acid pretreatment experiments

No of exp.	Time (min)	Amount of husk (g)	Acid % (w/w)	Sugar concentration (mg/mL)	Yield (g sugar/g husk) %
1	37.5	8	3	11.98	7.49
2	37.5	8	3	12.51	7.82
3	37.5	8	3	11.49	7.18
4	37.5	8	3	11.71	7.32
5	37.5	8	3	11.74	7.34
6	37.5	8	3	12.18	7.61
7	37.5	8	3	11.23	7.02
8	37.5	8	3	12.74	7.96
9	37.5	8	3	11.50	7.19
10	37.5	8	3	12.64	7.90
11	37.5	8	5	12.96	8.10
12	37.5	8	1	7.37	4.61
13	37.5	3.5	3	7.43	10.61
14	37.5	12.5	3	16.73	6.69

Table 3.1 continued

15	50	5.3	4.2	8.09	7.63
16	50	5.3	1.8	7.96	7.51
17	50	10.7	4.2	15.41	7.20
18	50	10.7	1.8	11.71	5.47
19	24	10.7	4.2	12.26	5.73
20	24	10.7	1.8	8.83	4.13
21	24	5.3	4.2	8.04	7.58
22	24	5.3	1.8	6.48	6.11
23	15	8	3	10.82	6.76
24	60	8	3	12.54	7.84
25	37.5	8	3	13.89	8.68

3.1.1 Validation experiments of acid pretreatment experiments:

After the first set of experiments, another set of values for the variables were selected for validation experiments. This dataset was created using latin hypercube sampling, instead of central composite design. Latin hypercube sampling allowed for a more randomized sampling of the design space. These set of experiments were performed to assess the success of the models in representing the system. Here the sugar concentrations were in the range of 7.63-26.75 mg/mL. For some of the experiments in this dataset, a different spectrophotometer was used. Sugar concentration tended to be higher in those readings. This result showed the impact of different instruments on sugar analysis using DNS method. In further experiment, for consistent results, the same instrument has been used. The results obtained from the validation experiments are given in Table 3.2.

Table 3.2: Results of validation experiments of the first set:

No of exp.	Time (min)	Amount of husk (g)	Acid percentage (w/w)	Sugar concentration (mg/mL)	Yield (g sugar/g husk) %
1	20	4.4	1.2	7.78	8.84
2	20	8.7	4.2	17.77	10.21
3	25	7.2	1.8	16.05	11.15
4	30	11.2	2.5	17.32	7.73

***** Readings with a different spectrophotometer

Table 3.2: continued

5	30	10.3	4.8	21.36	10.37
6	35	9.7	2.3	24.99*	12.88
7	37	8	3	23.61*	14.76
8	40	7.5	4	26.75*	17.50
9	45	9.1	1.4	13.11*	7.20
10	50	12.4	2.7	23.94	9.65
11	50	6.3	4.6	17.66	14.02
12	50	5.7	3.1	17.96	15.75
13	55	3.9	3.4	7.63*	9.78

* Readings with a different spectrophotometer

Due to the discrepancy that resulted from using a different spectrophotometer, a third set of values for the variables were selected for further experiments (Table 3.3). In this dataset, some experiments (1,3,4) were repeats of validation experiments from the second set for which a different spectrophotometer was used. This time, the final sugar concentrations were lower, as consistent with the first set of experiments. Some selected experiments (e.g. 2nd and 5th) were repeats from the first set. These experiments yielded the highest and lowest sugar concentrations in the first set. This time, 2nd experiment again yielded the highest sugar concentration, however highest sugar concentration of the 5th experiment was higher than expected. For the 5th experiment, a new batch of DNS reagent was prepared and used. This should have been the reason of the higher sugar concentration obtained.

The analysis of this whole dataset has shown that higher amounts of husk loading generally resulted in higher final sugar concentrations.

Table 3.3: Additional acid pretreatment experiments: Some are repeats of the first set of experiments and validation experiments

No of exp.	Time (min)	Amount of husk (g)	Acid percentage (w/w)	Sugar concentration (mg/mL)	Yield (g sugar/g husk) %
1	37	8	3	18.57	11.61
2	37.5	12.5	3	19.58	7.83
3	35	9.7	2.3	19.34	9.97

* Readings with a different batch of DNS solution

Table 3.3: continued

4	40	7.5	4	17.89	11.93
5	24	5.3	1.8	10.81*	10.20
6	20	4.4	1.2	7.08	8.05

* Readings with a different batch of DNS solution

3.1.2 Effect of particle size and batch of biomass:

As another parameter, the effect of the husk used on the sugar concentration was evaluated. The husks obtained were primarily in big pieces, thus periodical grinding was required. Since the uniformity in the sizes of the ground husk is questionable, experiments were carried out using husks that were ground at different times. Old batch corresponds to hazelnut husks ground two months prior to the experiments, while new batch corresponds to hazelnut husks ground at the same time of the pretreatment experiments. The sugar concentration obtained from different batches of husk was evaluated and results obtained are given in Table 3.4. The obtained final sugar concentration was barely higher with slightly smaller pieces, when compared to larger pieces. This is expected since a decrease in particle size increases surface area, which is favorable for treatment (Bhutto et al., 2017). Furthermore, the older batch of ground husk yielded a slightly lower final sugar concentration than the newer batch of ground husk. This result also shows the effect of biomass property on the final sugar concentration.

Table 3.4: Pretreatment results of different size and batch of hazelnut husks: (small: particle size smaller than 0.5cm, large: particle size between 0.5cm and 1cm)

No of exp.	husk size	husk batch	Amount of husk (g)	Time (min)	Acid percentage (w/w)	Sugar concentration (mg/mL)	Yield (g sugar/g husk) %
1	small	new	12.5	50	3.8	30.29	12.12
2	small	new	12.5	50	3.8	36.89	14.76
3	small	new	12.5	50	3.8	27.77	11.11
4	small	new	12.5	50	3.8	32.36	12.94
5	small	old	12.5	50	3.8	28.76	11.50
6	small	old	12.5	50	3.8	26.85	10.74
7	small	old	12.5	50	3.8	29.51	11.80
8	large	new	12.5	50	3.8	30.01	12.00
9	large	new	12.5	50	3.8	26.02	10.41

3.1.3 Optimum conditions for acid pretreatment:

Using the hydrolysis results from the first set of experiments, optimum values of the variables were determined using the two different models (Table 3.4). Among these models, the values determined by Kriging has given the highest sugar concentration when tested experimentally. Thus, the optimum values for the independent variables were set as 50 minutes of acid pretreatment using 3.8 % (w/w) sulfuric acid and 12.5 g of hazelnut husk loading, which corresponded to 12.5 (w/v) biomass loading. This condition was used in further experiments. R^2 values of 0.86 and 0.90 obtained for the Kriging and the RSM (low order polynomial) models respectively show that both models successfully represent the dataset with which these models were created. Normalized mean absolute error (NMAE) value of validation experiments were found as 16.24 % for the RSM and 12.66 % for the Kriging models.

Table 3.5: Optimum variable values for the highest amount of sugar concentration using two different models with the expected and experimental sugar concentration values.

Models	Time (min)	Amount of husk (g)	Acid % (w/w)	Sugar concentration (mg/mL)	Expected sugar concentration. mg/mL	Yield (g sugar/g husk) %
Kriging	50	12.5	3.8	27.67	16.1	11.07
RSM	60	12.5	4.3	22.24	18.3	8.90

Optimum conditions found in this study are consistent with reported optimum conditions for acid pretreatments of different lignocellulosic biomass. For example Sindhu et al. (Sindhu et al., 2014) reported optimum conditions for acid pretreatment of Indian bamboo as: 15% (w/w) biomass loading, 5 % sulfuric acid and 30 min. Aguilar et al. (Aguilar et al., 2002) reported the optimum conditions for acid pretreatment as: 2 % H_2SO_4 (w/w), 122 °C, and 24 min for 1 g sugar cane bagasse/10 g liquor on dry basis. Another study achieved the highest total sugar yield using lower sulfuric acid concentration of 0.75% (v/v) and a longer pretreatment period of 90 min at 121°C. They have used a solid loading of 10 % w/v of wheat stubble (Vancov & Mcintosh, 2011).

An acid pretreatment optimization was also performed by (Ceylan & Ünal, 2015) using hazelnut husks, where optimum conditions were: 90 min., 4 % sulfuric acid, and 5 % biomass loading at 121°C. Our optimum pretreatment duration and acid concentration were lower but

our biomass loading was higher. Although their yield was slightly higher as 18 % (see section 3.3), their final sugar concentration in the hydrolysate was as low as 7 g/L. Another study with dilute acid pretreatment on hazelnut shells reported similar optimal conditions as 130 °C, 3.42 % sulfuric acid, and 31.7 minutes, even though the structure and composition of hazelnut husks and shells are different (Uzuner & Cekmecelioglu, 2014).

3.2. Enzymatic Hydrolysis of Hazelnut Husk:

For enzymatic hydrolysis, hazelnut husk was first acid pretreated under the optimum conditions previously determined. Prior to the inscribed composite design for the values of the enzymatic hydrolysis, preliminary experiments have been carried out to select independent variables. For the cellulase enzyme, that is used in this study, the optimal conditions for the hydrolysis were as: 50-60°C and pH of 4.5-6 (Sigma-Aldrich, 2020). Furthermore, in preliminary experiments, enzymatic hydrolysis reactions were carried out in Erlenmeyer flasks with cotton plugs. In these experiments considerable amount of buffer evaporated. Therefore, misleading results with very high sugar concentrations were obtained; the obtained final hydrolysate volume was too small. To this end, for further experiments Schott bottles with caps were used.

3.2.1 Selection of enzyme load:

The first set of experiments were carried out to investigate the effect of enzyme amount on the final sugar concentration of the hydrolysate under constant pH and temperature. To this end 10 % fusk load was used. Obtained hydrolysis results have shown that with increasing enzyme amount, higher sugar concentrations were achieved. However, final sugar concentration hasn't changed significantly when the enzyme volume was doubled from 5 to 10 mL in a total of 25 mL reaction volume. Therefore, for economical aspects, 5 mL of enzyme was selected as the upper limit for a reaction in a 25 mL total volume.

Table 3.6: Enzymatic hydrolysis experiments with changing enzyme volume, at pH 5 and 55°C (sugar concentrations are measured at day 7)

No of exp.	Total volume in mL	Enzyme volume (mL)	Amount of pretreated husk (g)	Sugar concentration (mg/mL)
1	25	0	2.5	3.16
2	25	2	2.5	8.82
3	25	5	2.5	13.26
4	25	10	2.5	14.34

3.2.2 Effect of temperature and pH:

The second set of experiments were performed in order to investigate the effects of pH and temperature on the final sugar concentration in the hydrolysate and to determine the limiting values for further experiments. In Table 3.7, the sugar concentrations obtained for these experiments are given. In these experiments, the total volume was fixed as 25 mL, and for each experiment 2 mL of enzyme and 5 g pretreated biomass was used. Here, it can be seen that experiments carried out at 55 °C yielded the highest sugar concentration. The lowest sugar concentrations were obtained at 60° C regardless of pH and total reaction time. For this reason, 55 °C was selected as the reaction temperature for enzymatic treatment.

Table 3.7: Enzymatic hydrolysis results with varying pH and temperature at different days (Total volume 25mL, enzyme volume 2mL)

No of exp.	Temperature (°C)	pH	Time (day)	Sugar concentration (mg/mL)
1	50	4.5	5	9.92
2	50	5	5	9.68
3	50	6	5	9.75
4	55	4.5	4	11.11
5	55	5	4	10.48
6	55	6	4	13.04
7	60	4.5	5	4.73
8	60	5	5	6.16
9	60	6	5	7.17

3.2.1 Effect of acid pretreatment:

The effect of acid pretreatment before enzymatic hydrolysis on the sugar concentration was evaluated and the results are given in Table 3.8. For acid pretreatment, optimum conditions were used. The total volume was fixed at 12.5 mL and 1 mL of enzyme was used at pH 6 and 55 °C. Results presented in Table 3.8 displayed that acid pretreatment before enzymatic hydrolysis increased the sugar concentration substantially.

Table 3.8: Enzymatic hydrolysis at varying pH and temperature at different days

No of exp.	Treatment	Sugar concentration (mg/mL)
1	Acid pretreatment + Enzymatic hydrolysis	9.35
2	Enzymatic hydrolysis	4.84

(Total volume 25mL, enzyme volume 2mL)

After preliminary experiments, pH and temperature values were determined as: pH 6 and 55°C, since these conditions yielded the highest sugar concentration in the hust hydrolysate. Keeping pH of the final hydrolysate close to neutral values would be economically favorable for subsequent microbial growth experiments.

For the first set of experiments, three independent variables were chosen, as described previously: time of 1-7 days, enzyme volume of 1-2.5 mL (840 EGU-2100 EGU), and pretreated solid husk loading of 1.25-2.5 g. Total volume was set to 12.5 mL, to minimize enzyme consumption. In Table 3.9, the obtained sugar concentrations after enzymatic treatment are given. The experimental conditions were determined with central composite design, as described in section 2.2.2.1. Increasing time of reaction, husk load, or enzyme amount as the other two variables were kept constant increased final sugar concentration. Final sugar concentrations were in the range of 5.8 mg/mL to 9.44 mg/mL.

Table 3.9: First set of enzymatic hydrolysis experiments:

No of exp.	Time (day)	Amount of pretreated husk (g)	Enzyme volume (mL)	Sugar concentration (mg/mL)
1	2.2	1.5	1.3	5.80
2	2.2	1.5	2.2	6.82
3	2.2	2.25	1.3	5.78
4	2.2	2.25	2.2	7.23
5	5.8	1.5	1.3	7.05
6	5.8	1.5	2.2	7.23
7	5.8	2.25	1.3	7.44
8	5.8	2.25	2.2	8.59
9	1	1.9	1.75	5.89
10	7	1.9	1.75	7.32

Table 3.9: continued

11	4	1.25	1.75	6.06
12	4	2.5	1.75	9.00
13	4	1.9	1	9.44
14	4	1.9	2.5	7.05
15	4	1.9	1.75	7.21
16	4	1.9	1.75	7.09
17	4	1.9	1.75	7.05
18	4	1.9	1.75	7.92
19	4	1.9	1.75	7.14
20	4	1.9	1.75	6.84
21	4	1.9	1.75	7.44
22	4	1.9	1.75	7.64
23	4	1.9	1.75	7.02
24	4	1.9	1.75	6.91

3.2.1 Validation experiments for enzymatic hydrolysis experiments:

A set of validation experiments for enzymatic hydrolysis were carried out and the obtained results are given in Table 3.10. Similar to the acid pretreatment validation experiments, this dataset was created using latin hypercube sampling. Latin hypercube sampling, allowed for a more randomized sampling of the design space. This set of experiments was performed to assess how successfully the models represented the system. Sugar concentrations were in the range of 5.43-8.04 mg/mL.

Table 3.10: Validation experiments of enzymatic hydrolysis:

No of exp.	Time (day)	Amount of pretreated husk (g)	Enzyme volume (mL)	Sugar concentration (mg/mL)
1	4.6	1.6	1.57	6.31
2	2.1	1.25	1.88	6.36
3	6.6	2	1.98	6.84
4	3.6	1.85	1.86	7.76
5	4.8	2.05	1.64	5.66

Table 3.10: continued

6	0.1	2.2	1.8	5.43
7	3	2.13	2.34	7.02
8	2.8	1.74	2.12	6.86
9	3.7	2.5	1.74	7.74
10	6.1	1.89	1.2	6.68
11	4.1	2.48	1.4	7.90
12	5.2	2.26	1.55	8.04

3.2.1 Optimum conditions for enzymatic hydrolysis:

Values of the variables for the optimum condition of enzymatic hydrolysis were determined using the hydrolysis results obtained from the first set of experiments (Table 3.11). Similarly, two models were used for this, RSM and Kriging. Overall, the final sugar concentration was slightly lower than the theoretical values evaluated by the models. Of the two models, RSM yielded slightly higher sugar concentrations, both theoretically and experimentally. Both models predicted identical husk and enzyme loading for optimal results, which was 1 mL enzyme and 2.5 g of pretreated hazelnut husk (20 % (w/v) biomass loading) in 12.5 mL reaction volume. The time was slightly different, 6.88 days with RSM and 5.35 days with Kriging. However, the final sugar concentration obtained was not very different under these two conditions. The R^2 of 0.91 and 0.58 obtained for Kriging and RSM (low order polynomial) models, respectively, showed that Kriging represented the dataset more successfully. Normalized mean absolute error (NMAE) value of validation experiments were found as 10.98% for the RSM model and 12.66 % for the Kriging model.

Table 3.11: Optimum values of variables in enzymatic hydrolysis:

Models	Time (day)	Amount of pretreated husk (g)	Enzyme volume (mL)	Sugar concentration (mg/mL)	Expected Sugar concentration. (mg/mL)
RSM	6.88	2.5	1	8.36	9.35
Kriging	5.35	2.5	1	8.10	9.04

3.3. Yields of acid pretreatment and enzymatic hydrolysis:

3.3.1 Acid pretreatment:

The yield of the total amount of sugar extracted from a given amount of husk expressed in g reducing sugar/g husk from acid pretreatment and enzymatic hydrolysis are given in Table 3.12. The total sugar yield using the optimized conditions for acid pretreatment was 0.12 g reducing sugar / 1 g of biomass. When Indian bamboo was hydrolysed, 0.319 g reducing sugar / 1 g biomass was obtained after acid pretreatment (Sindhu et al., 2014). From acid pretreatment of wheat straw, a final glucose yield of 28.9 mg / g biomass was obtained (Vancov & Mcintosh, 2011). It was also reported that, upon acid pretreatment of sugar cane bagasse with 10 % biomass load 21.6 mg xylose/mL, 3 mg glucose/mL were obtained (Aguilar et al., 2002).

The maximum reducing sugar concentration obtained in this study was 27.67 mg/mL. This corresponded to a sugar yield of 12.3 %. Ceylan et al. (Ceylan & Ünal, 2015) also used hazelnut husks and carried out acid pretreatment to find a yield of 18.63 %. Their yield was higher; nevertheless, their husk load was rather low as 5 %, therefore the final sugar concentration obtained in that study was too low to be used for microbial growth. Furthermore, our optimum pretreatment duration and acid concentration were lower. An acid pretreatment on hazelnut shells led to an optimum reducing sugar concentration of 16.65 g/L, which is lower than the final reducing sugar concentration obtained in the work (Uzuner & Cekmecelioglu, 2014). Overall, it can be concluded that the sugar yields and concentrations obtained from this study are in line with reported yields in the literature.

Çöpür et al. (Çöpür et al., 2013) has reported that 44.2 % of raw hazelnut husk was the sugar content. Thus, the highest theoretical sugar yield from hazelnut husk after treatment can be maximum 44,2 % (g sugar/g biomass). In this study, when optimal conditions for acid pretreatment determined in this work were used yield was 12.32 %. Under the same conditions, 62 % solid remained (7.8g/12.5g). It is reported in literature that following pretreatment of hazelnut husks with 4 % sulfuric acid for 60 minutes, 73.8% solid remained (REF).

3.3.2 Enzymatic hydrolysis:

Sugar yield from the enzymatic hydrolysis of the acid pretreated husk was quite low (Table 3.12). It is reported by Ioelovich & Morag (Ioelovich & Morag, 2012) that in enzymatic hydrolysis, the biomass load was generally equal to or below 5 % (w). In the current study, biomass load was kept between 10-20% (w/v). A higher biomass load was desired, since high sugar concentration rather than high sugar yield was desired for subsequent bacterial growth experiments. Therefore, in this study the biomass load was kept in the range of “high-solids” enzymatic hydrolysis. Da Silva et al. (Da Silva et al., 2020) describes “high-solids” enzymatic

hydrolysis as, biomass loads higher than 15%, where initially no free water would exist within the hydrolysis slurry.

Many studies report lower sugar concentration or yield with higher biomass loading, which was also described as “high-solids effect” (Da Silva et al., 2020;W. Wang et al., 2011). Even though the mechanism of enzymatic system and the dynamics between the biomass and the enzyme and other compounds within the slurry are not fully known, researchers hypothesize that the cause of lower yield or sugar concentration is high biomass load utilization (Da Silva et al., 2020;W. Wang et al., 2011;Ioelovich & Morag, 2012). One major reason for this is given as an increase in viscosity with higher biomass load, which results in insufficient shaking and mixing in lab-scale (Jørgensen, Vibe-Pedersen, et al., 2007). It was also stated that, high biomass load adversely affects mass and heat transfer during enzymatic hydrolysis. This in turn causes lower sugar yields (Da Silva et al., 2020). Another cause for this lower yield was demonstrated as the inhibitory effects of sugars or other compounds such as phenols and furans on enzymatic hydrolysis (Da Silva et al., 2020). These molecules are produced during enzymatic treatment. For example, Tejirian & Xu (Tejirian & Xu, 2011) have shown that 1mM tannic acid, which was a degradation product of acid pretreatment, has decreased the enzymatic hydrolysis rate up to 70 %. Ioelovich & Morag (Ioelovich & Morag, 2012) have carried out enzymatic hydrolysis on sulfuric acid pretreated (3 % w/w) switchgrass. They investigated the effect of higher biomass loading on sugar yield and concentration. They have found that the optimum conditions for hydrolysis as 10 % biomass load for 72 hours using a commercial cellulase (5FPU/g solid sample) operating at pH 4.8 and 50 °C. With these conditions they obtained 17 mg/mL sugar and 73 % solid remained. They reported that, higher biomass loading resulted in lower conversion degree, nevertheless gave higher reducing sugar concentrations (Ioelovich & Morag, 2012). When another study investigated the effect of solid load (1-5% biomass),the enzymatic hydrolysis conditions were: pH 4.8 and 50°C and 15 FPU of cellulase enzyme for 72 hours. They demonstrated that higher biomass load i.e. 5% solid load, yielded 50 % sugar conversion, where lower biomass load i.e. 1 % solid load, yielded 70 % sugar conversion. All these reports support the low yield obtained in this study.

Table 3.12: Yield results of acid and enzymatic pretreatment in optimum conditions: (DW: dry weight)

	Initial DW (g)	Initial V (ml)	Final V after AP or EH (ml)	Sugar in hydrolysate (mg/ml)	Total sugar (g)	DW after AP or EH (g)	Yield in % (g sugar/g DW)
Acid Pretreatment (AP)	12.5	100	55	27.67	1.5	7.8	12.32
Enzymatic Hydrolysis (EH)	2.5	12.5	3.9	8.36	0.033	1.3	1.309

3.4. Microbial Growth on Pretreated Hazelnut Husk Hydrolysate:

3.4.1 Microbial Growth in 96-well plates:

For the growth experiments *C. glutamicum* ARO002-pSH1(ARO), *C. glutamicum* ARO002-pSH1-$xylA_{Xc}B_{Cg}$ (AROXyl), wild type *C. glutamicum* (Cg), and *C. glutamicum* -pEKEx3-$xylA_{Xc}B_{Cg}$ (CgXyl) cells were used. *C. glutamicum* ARO002 cells contained the constitutive pSH1 plasmid, with and without xylose utilization genes, whereas *C. glutamicum* wild type cells contained the IPTG-inducible pEKEx3 plasmid with xylose utilization genes. All samples were cultivated in triplicate in 96-well U-bottom plates and averages were used for plotting growth profiles. Raw data of OD_{600} values are given in Appendix C.

3.4.1.1. Growth of Cg cells on different carbon sources:

The effect of different carbon sources on growth was investigated by cultivating Cg and CgXyl cells in CGXII minimal medium with the following carbon sources: glucose, xylose, glucose-xylose mixture (3:1) and pretreated husk hydrolysate. Mixed sugar concentration had a ratio of 3:1 glucose to xylose, since hazelnut husk was reported to have a similar ratio of glucose to xylose sugars (Surek & Buyukkileci, 2017). CGXII minimal medium containing the husk hydrolysate had a reducing sugar concentration of 2.6 %. The sugar concentrations of the control media contained equivalent glucose, xylose, or mixed sugars. Cells were incubated for 45 hours.

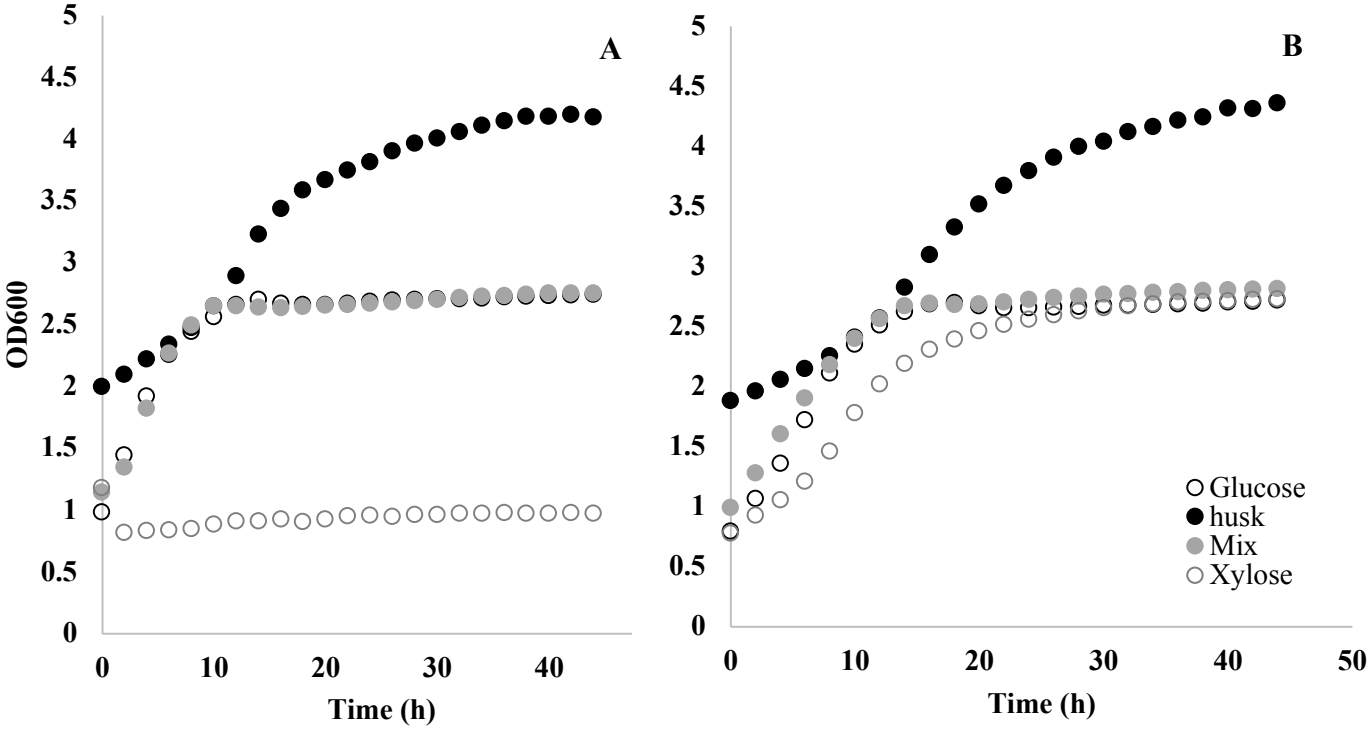

Figure 3.1: Growth of Cg cells on media with different carbon sources in A, and growth of CgXyl cells on media with different carbon sources in B.

In Figure 3.1, growths of wild type *C. glutamicum* and CgXyl are presented. As expected, wild type Cg cells couldn't grow in CGXII medium containing xylose as the sole carbon source. CgXyl cells were able to utilize xylose and although slower, they were able to reach the same final OD_{600}, as when they were grown on glucose medium. Both cells had almost the same profile when grown on glucose or mixed sugars (3:1 glucose: xylose) medium. When husk medium was used as the carbon source, cells grew slowly and their growth rate was lower. The final OD_{600} reached by CgXyl cells was slightly higher than the final OD_{600} reached by the wild type *C. glutamicum* cells. This was because CgXyl cells could also use the xylose in the husk hydrolysate.

3.4.1.2. Growth of ARO cells on different carbon sources:

Cultivation of ARO and AROXyl cells in media with different carbon sources was investigated, by growing them in CGXII minimal medium with the following carbon sources: glucose, xylose, glucose-xylose mixture (3:1) and pretreated husk hydrolysate. CGXII minimal medium containing the husk hydrolysate had a sugar concentration of % 2.6. The sugar concentration in the control media contained equivalent concentrations of glucose, xylose or mixed sugars. Cells were incubated for 45 hours.

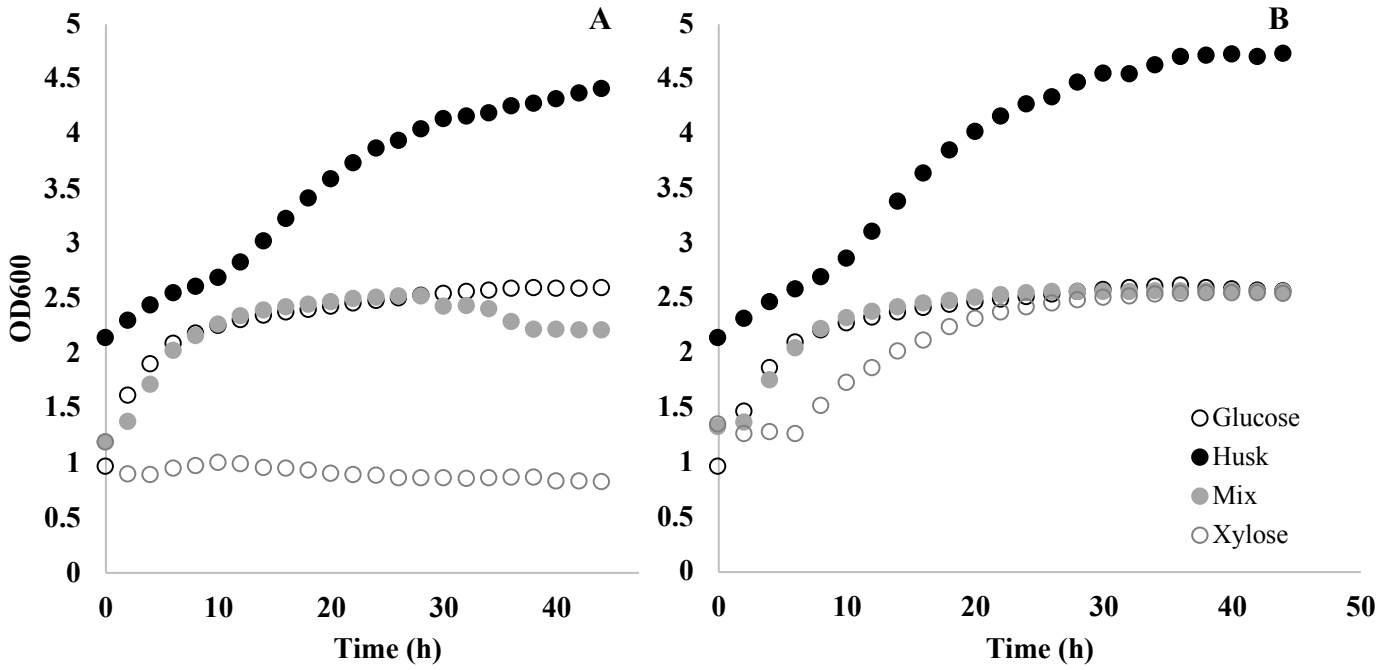

Figure 3.2: Growth of ARO cells on media with different carbon sources in A, and growth of AROXyl on media with different carbon sources in B.

In Figure 3.2, growths of ARO and AROXyl cells are presented. ARO cells could not grow in xylose medium, while AROXyl cells were able to grow in xylose medium. AROXyl cells had a longer lag phase when grown in xylose, compared to their growths in glucose or mixed sugar media. Similar to Cg and CgXyl cells, ARO and AROXyl cells had a very similar growth profile when grown in CGXII media with glucose or mixed sugars. Again, the final OD_{600} reached by AROXyl cells was slightly higher than the final OD_{600} reached by the ARO cells when grown in CGXII media with husk hydrolysate.

3.4.1.3. Growth of ARO and AROXyl cells on husk and glucose:

In order to investigate the effect of different carbon sources on growth of ARO and AROXyl cells in media with husk hydrolysate, cells were cultivated in CGXII minimal media with either husk hydrolysate or 4 % glucose. The CGXII medium contained 4 % glucose, which is the optimal glucose concentration used for cultivation of *C. glutamicum* cells (Keilhauer et al., 1993). Here the medium with the husk hydrolysate had a sugar concentration of 2.2 %. This was the maximum possible husk for cultivation. Cells were cultivated for 60 hours.

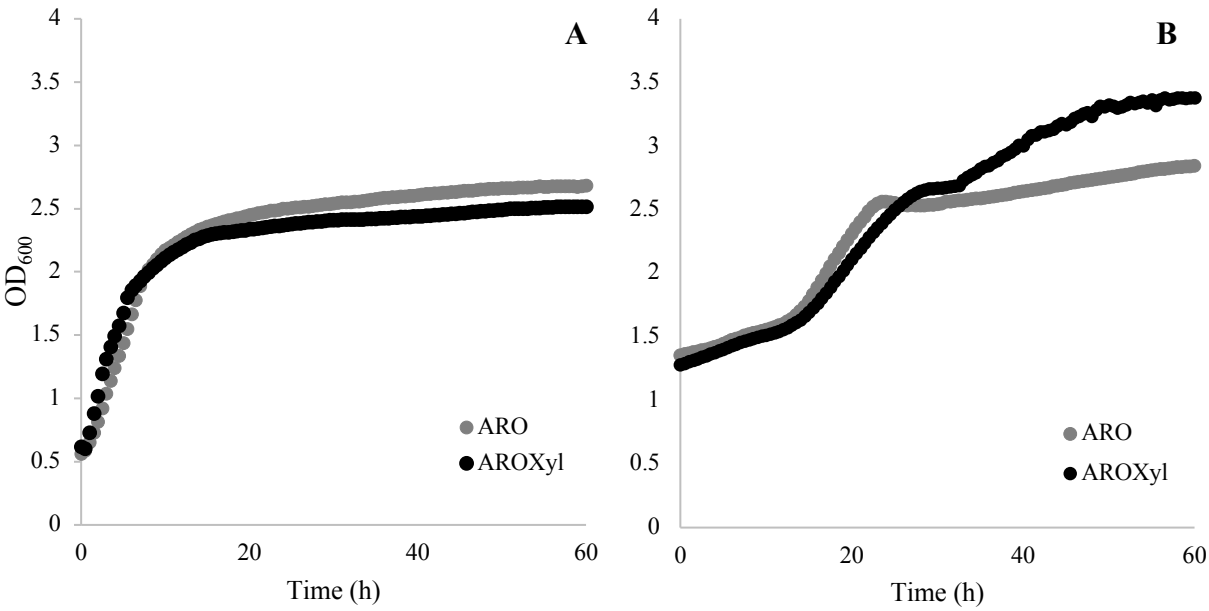

Figure 3.3: Growth profiles of ARO and AROXyl cells in 4% glucose medium in A, Growth profiles of ARO and AROXyl cells in husk hydrolysate in B, grey: ARO cells, black: AROXyl cells.

The analysis of Figure 3.3 has shown than both ARO and AROXyl cells were grown in the presence of 4% glucose, the profiles were very similar, which was expected. Cells entered exponential phase almost immediately. Around 24 hours, both cells reached the stationary phase and a final OD_{600} of 2.4. When the same cells were grown in media with husk hydrolysate, there was an extended lag phase that lasted for 12 hours. Both cells entered the exponential phase around the same time. ARO cells entered the stationary phase around the 22th hour of cultivation and reached a final OD_{600} of 2.9. The growth of AROXyl cells continued and these cells entered the stationary phase at around the 50th hour, approximately 28 hours later. They reached a final OD_{600} of 3.4. Their final OD_{600} was higher than ARO cells. The reason for this outcome could be that, AROXyl cells were able to utilize both glucose and xylose within husk hydrolysate, while ARO cells were only able to utilize only glucose.

3.4.1.4. Growth of ARO and Cg cells on husk hydrolysate:

To better assess the growth characteristics of different cells on husk hydrolysate Figure 3.4 is presented. Here, husk hydrolysate had a sugar concentration of 2.6 %. Husk medium without cells was used as control, to investigate whether the increase in the OD_{600} was resulting from cells growth but not precipitation of husk debris. Cells were incubated for 45 hours.

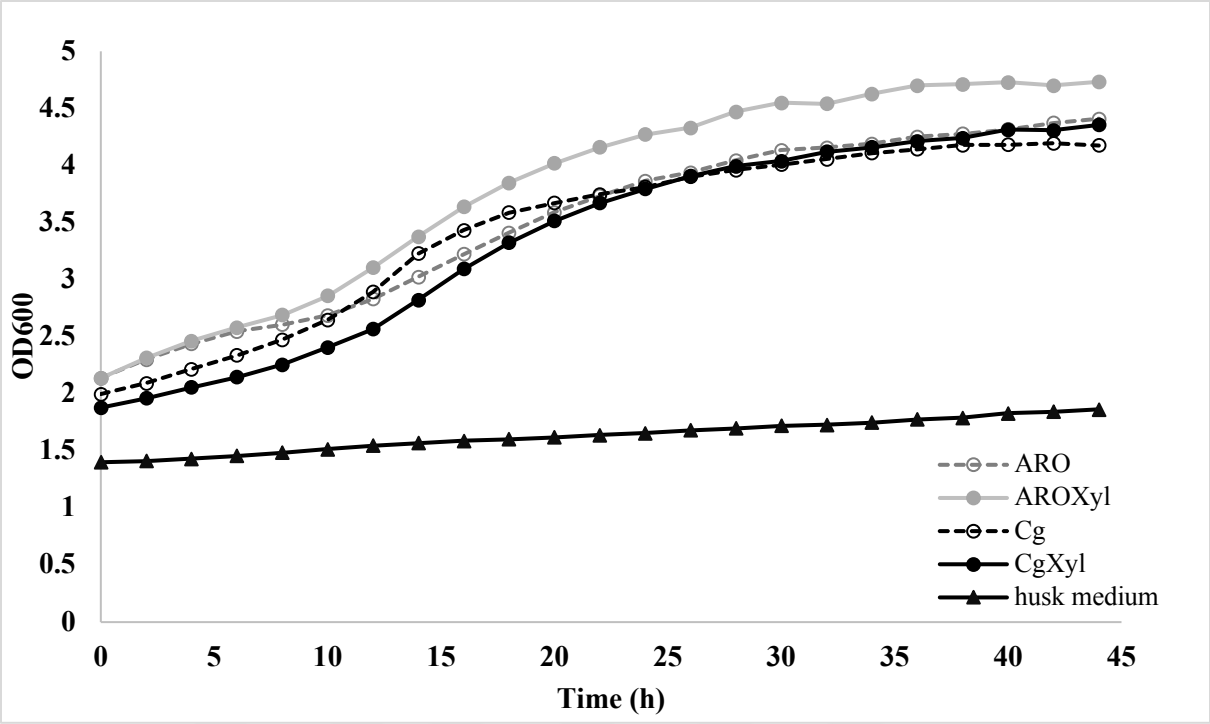

Figure 3.4: Growth profiles of ARO, AROXyl, Cg and CgXyl on husk hydrolysate, grey icons represent ARO cells, black icons represent Cg cells, filled icons represent cells with xylose utilizing genes, empty icons are control strains.

All cells had similar growth profiles. When compared to Cg cells, although the starting OD_{600} of CgXyl was lower, they reached a higher final OD_{600}, which was expected. ARO and AROXyl cells had the same starting OD_{600}. At the end of the incubation AROXyl reached a slightly higher OD_{600} compared to ARO cells. The OD_{600} of the media with husk hydrolysate but no cells remained constant at around 1.5. All cells portrayed a similar extended lag phase.

3.4.2 . Microbial Growth in Flasks:

For growth experiments in flasks, wild type *C. glutamicum* (Cg) and *C. glutamicum*-pEKEx3-*xylAB* (CgXyl) organisms were used. Growth profiles obtained in 96-well plates successfully portrayed the growth profiles and preliminary data was obtained. When glucose was used as the only carbon source, growth was finished in 24 hours. However, when husk hydrolysate was used, growth lasted approximately 48 hours. Since the volume was very small in 96-well plates, in order to get investigate how cells would behave in larger volumes, cell growth was investigated in flasks. The OD_{600} difference between 96-well plates and flasks are due to the difference between total volume and working volume, aeration and mixing in both platforms. In 96-well plates mixing of the medium and aeration is very limited compared 250 mL baffled flasks, which affected the growth. Cells were cultivated in CGXII minimal media with the

following carbon sources: glucose, xylose and pretreated husk hydrolysate. CGXII minimal medium containing the husk hydrolysate had a sugar concentration of 2.64 %; thus, sugar concentrations of other mediums containing glucose or xylose were adjusted accordingly. Cells were incubated for 72 hours. In Figure 3.5 results obtained from growth in flasks are presented. Both CG and CgXyl cells displayed very similar growth profiles in media with glucose. They entered exponential phase very quickly and around 24th hour of cultivtion, exponential phase ceased. From the 24th hour till the end of cultivation, both cells were in stationary phase with a slight decrease in their OD_{600}. Both cells reached maximum OD_{600}'s of 53. CgXyl cells grown in xylose medium displayed a longer exponential phase. However, at the 54th hour of cultivation they reached a slightly higher OD_{600} of 57. Cells grown on pretreated husk hydrolysates displayed similar growth profiles until the 24th hour of cultivation, where both cells reached OD_{600} of 10. After 24 hours CG cells entered stationary phase and reached a maximum OD_{600} of 12. On the other hand, CgXyl cells grew further to a maximum OD_{600} of 26. Here it can be observed that, CgXyl cells with xylose utilizing genes, had a 2-fold increase in growth as compared to CG cells in husk hydrolysate medium. From this it can be deduced that, husk medium contained xylose, as well as glucose, which was successfully utilized by CgXyl cells. However, despite similar sugar concentrations, the husk probably contained other sugars, e.g. arabinose which were not utilized by the cells and growth inhibitors which adversely affected growth. This might explain the lower final cell concentration.

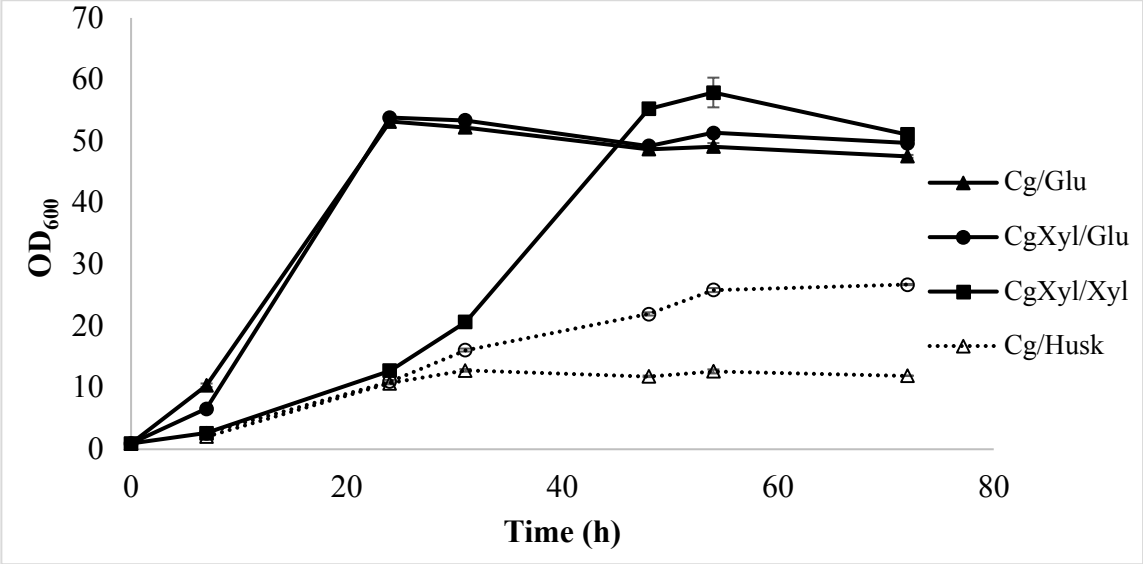

Figure 3.5: Growth results in flasks: filled triangle: Cg in glucose, filled circle: CgXyl in glucose, filled rectangle: CgXyl in xylose, empty triangle: Cg in husk, empty circle: CgXyl in husk. OD_{600} measurements were done in triplicates.

CGXII medium containing husk hydrolysate had a very dark color. Therefore 0th hour OD_{600}'s of cells grown in husk medium could not be measured. To overcome this predicament and to assess cell viability, growth of the cells were also monitored by measuring colony forming units. In Figures 3.6. and 3.7, the results of the same growth experiment using colony forming units is presented. For monitoring bacterial growth, dry cell weight measurement was also considered, however preliminary experiments have shown that, dry cell weight of cells in husk hydrolysate was inaccurate when compared to their OD_{600}, presumably due to dried particles derived from the husk medium. Therefore, colony forming units were counted. In Figure 3.6 CFU results of Cg cells grown in glucose, CgXyl cells grown in glucose and xylose are plotted. CFU values can be found in Appendix C.

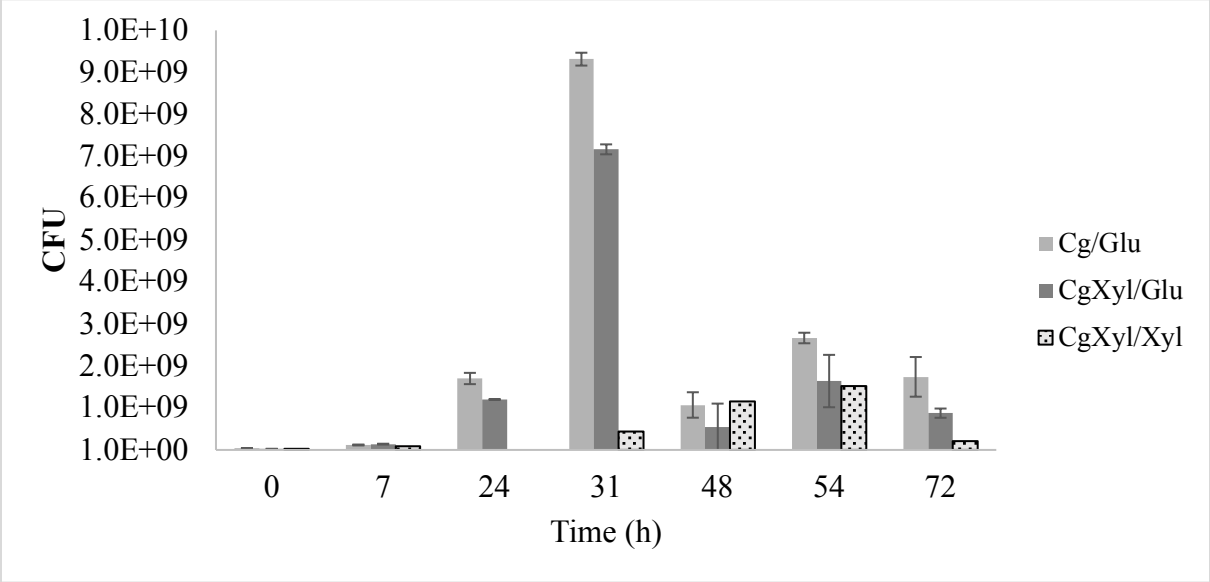

Figure 3.6: CFU results of Cg cells grown in glucose (light grey bars), CgXyl cell in glucose (dark grey bars) and CgXyl cells in xylose (dotted bars). Experiments were done in triplicates.

Here it can be observed that for Cg or CGXyl cells grown in glucose medium, colony forming units reached a peak at 31st hour of the incubation. CFU results were consistent with the growth curves presented in OD_{600} units, where from 0-7 hours of incubation a small increase in OD_{600} was observed and small amounts of CFU were found. At the 24th hour, cells reached the maximum OD_{600}, their CFU's also increased. In plots with OD_{600} units, it seems after 24th hour, cells remained in stationary phase with very slight decrease in OD_{600} until the end of incubation. In CFU plots after 31 hours, CFU decreased considerably. This may indicate that viable cells within the medium were decreasing, while dead cells remained in the medium, which accounted for the constant OD_{600} after 31 hours.

CgXyl cells grown in xylose medium, have shown an increase in CFU at the 31st hour and reached the maximum value at the 54th hour. This result is also consistent with the plot presenting the OD_{600} where CgXyl cells had a slower growth and reached their maximum OD_{600} at the 54th hour. Here the CFU of CgXyl cells grown in xylose were quite smaller to the CFU's of Cg or CgXyl cells grown in glucose.

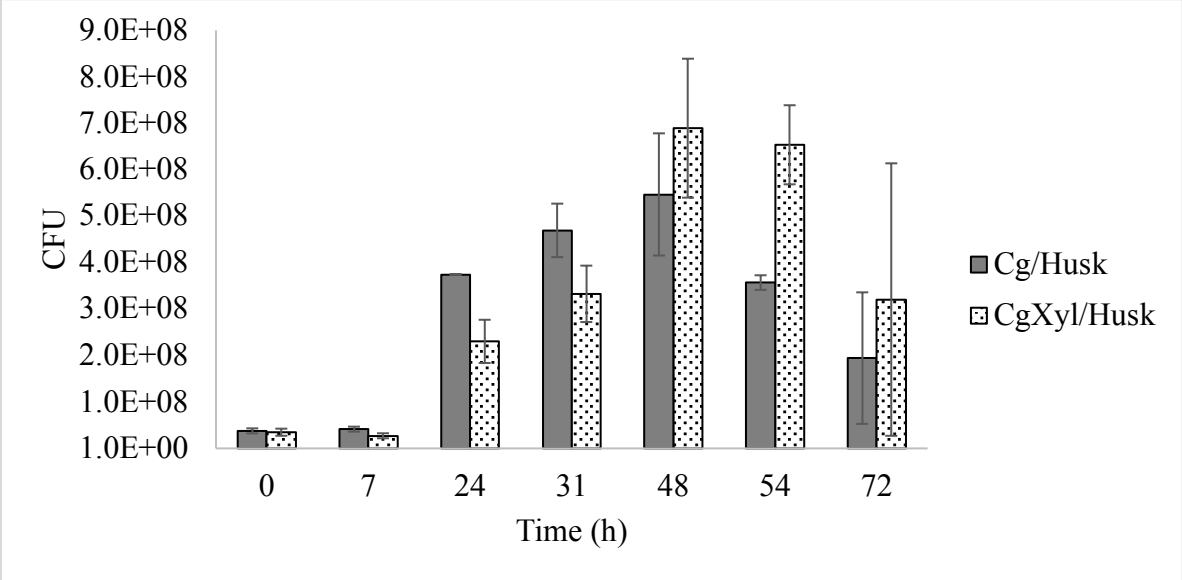

Figure 3.7: CFU results of Cg cells grown in husk (dark grey bars) and CgXyl cells in husk (dotted bars). Experiments were done in triplicate.

In Figure 3.7 CFU results of Cg and CgXyl cells grown in husk hydrolysate are plotted. Here both cells displayed similar CFU profiles, where both cells reached the maximum CFU amounts at the 48th hour of the incubation. When compared, CgXyl cells had a higher maximum CFU than Cg cells, which was consistent with OD_{600} results.

In literature, CgXyl cells were reported to have a biphasic growth profile, when grown in a mixture of xylose (30g/L) and glucose (10g/L) (Radek et al., 2014). Where initially cells utilize both glucose and xylose simultaneously. After glucose is depleted, cells further utilize xylose and grow with a smaller growth rate reaching a stationary phase around 35 hours. In this study when CgXyl cells were grown in mixed sugar medium (3:1, glucose to xylose) a biphasic growth was not observed (Fig.3.1). It was possible that there was not more xylose when glucose was depleted, since the amount of xylose was not high. In another study *C. glutamicum* CGS1, which was a recombinant strain containing xylose utilization genes, has reached an OD_{600} of approximately 33 at the 30th hour of the incubation in CGXIIA medium containing 30g/L xylose. Another study listed the bacterial growth of several C. glutamicum strains in pretreated

agricultural residues. In rice straw hydrolysate containing 40 mM glucose, 166 mM xylose and 66 mM arabinose, C. glutamicum WT (pEKEx3-xylA)(pVWEx1) reached an OD_{600} of 33. In wheat bran hydrolysate containing 125 mM glucose, 162 mM xylose and 64 mM arabinose, *C. glutamicum* WT (pEKEx3-xylA)(pVWEx1) reached an OD_{600} of 36. In this study CgXyl cells reached an OD_{600} of 26.7. It is also stated in this study that, recombinant *C. glutamicum* strains were able to utilize glucose and xylose simultaneously, both when they are obtained from acid hydrolysates but also added as commercial compounds (Gopinath et al., 2011). They also reported that bacterial growth was slower when grown in acid hydrolysates when compared to cells grown with commercial sugars. Here, they hypothesize the presence of inhibitors within acid hydrolysates hinders the growth of bacteria. This situation may be the cause of lower OD_{600} obtained in this study when cells were grown in husk hydrolysate rather than commercial sugars (Fig. 3.5).

4. CONCLUSION

Lignocellulosic materials can be used as cheap carbon sources for microbial bio-processes since they contain significant amounts of different sugars, including glucose, xylose, and arabinose and they are largely available. Furthermore, they do not compete with the food and feed industries. In this study, hazelnut husk, which is a lignocellulosic biomass and an agricultural waste was utilized for its potential to obtain fermentable sugars for the cultivation of an industrially important bacteria, *C. glutamicum*. Hazelnut husks were hydrolyzed in two steps. Among different pretreatment methods, enzymatic hydrolysis using commercial cellulase following a sulfuric acid pretreatment was chosen. Three independent variables for both acid pretreatment and enzymatic hydrolysis were optimized, using RSM and Kriging models in MATLAB. Also, for both treatments an inscribed central composite design was prepared. Three independent variables for acid pretreatment were: acid percentage, husk loading and duration of pretreatment. For enzymatic hydrolysis three independent variables were: enzyme amount, pretreated husk load, and duration of hydrolysis. For both optimizations, the goal was to maximize the sugar concentration. After acid pretreatment a maximum reducing sugar concentration of 27.67 mg/mL was obtained with the optimum conditions: 12.5 g husk, 3.8 % acid and 50 min. of pretreatment. After enzymatic hydrolysis, a maximum reducing sugar concentration of 8.36 mg/mL was obtained with optimum conditions: 2.5 g pretreated husk, 1 mL enzyme and 6.88 days of hydrolysis. Since the amount of reducing sugar obtained with enzymatic hydrolysis was not feasible for microbial growth, hydrolysates of acid pretreated husk were used as a carbon source. *C. glutamicum* ARO002-pSH1, *C. glutamicum* ARO002-pSH1-$xylA_{Xc}B_{Cg}$, wild type *C. glutamicum* and *C. glutamicum*-pEKEx3-$xylA_{Xc}B_{Cg}$ cells were cultivated in CGXII minimal media with selected carbon sources. Preliminary growth profiles investigated in 96-well plates showed that cells with xylose utilization genes were able to grow in media containing husk hydrolysate as the carbon source but they displayed an extended lag phase. Cells containing the xylose utilizing genes i.e. *C. glutamicum* ARO002-pSH1-$xylA_{Xc}B_{Cg}$ and *C. glutamicum*-pEKEx3-$xylA_{Xc}B_{Cg}$ had grown slightly better in husk hydrolysate. Also, wild type *C. glutamicum* and *C. glutamicum*-pEKEx3-$xylA_{Xc}B_{Cg}$ were grown in flasks in 50 mL CGXII medium. *C. glutamicum*-pEKEx3-$xylA_{Xc}B_{Cg}$ grew almost twice as much as wild type *C. glutamicum* and reached a final OD_{600} of 26. In this study the potential of hazelnut husk hydrolysate as a carbon source for the cultivation of *C. glutamicum* has been demonstrated.

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APPENDIX A - DNS Calibration Curves

For each batch of DNS reagent prepared, a calibration curve was plotted.

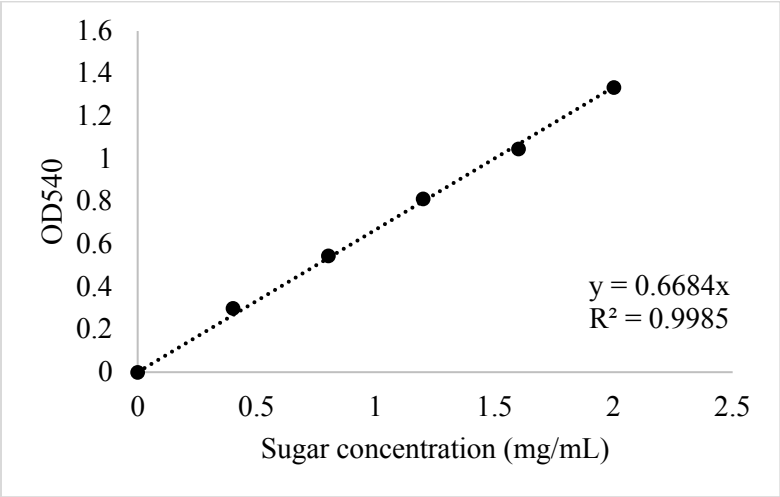

Figure A.1: 1.DNS Calibration curve

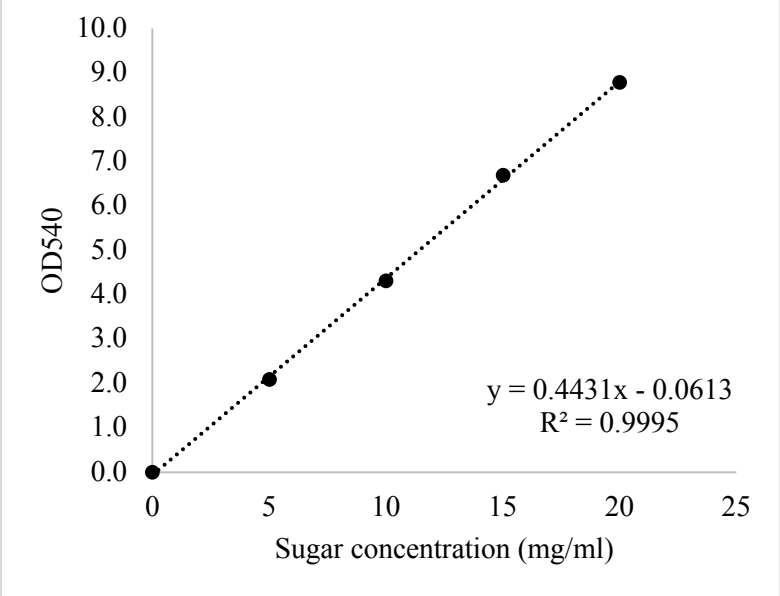

Figure A.2: 2. DNS Calibration Curve

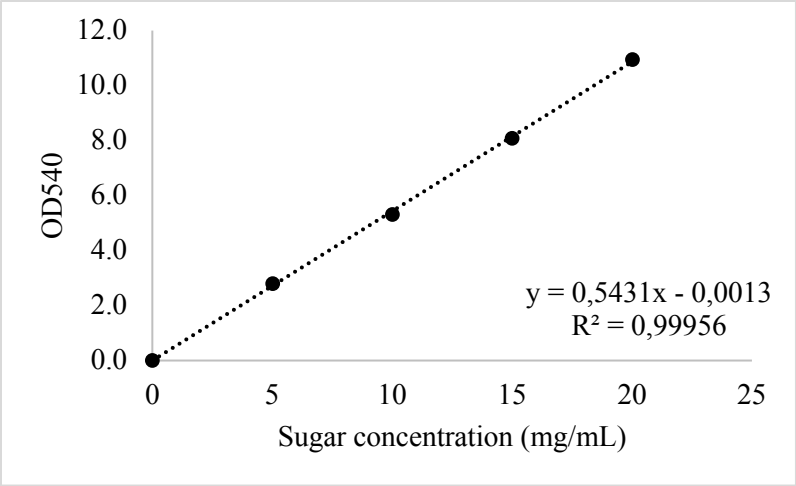

Figure A.3: 3. DNS Calibration curve

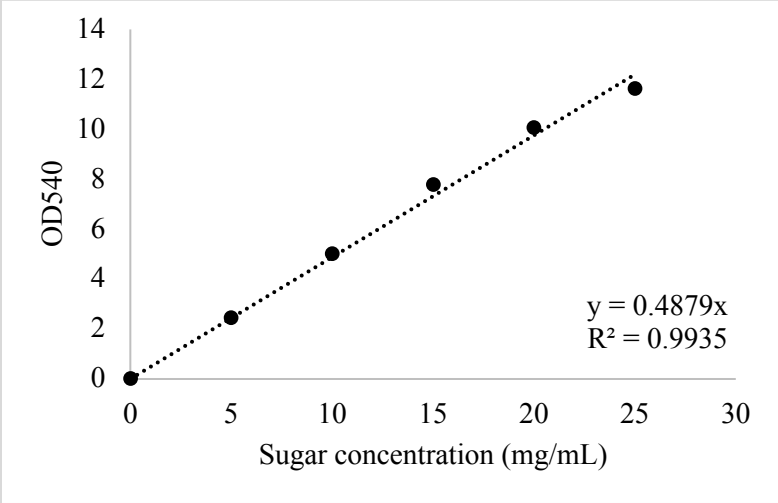

Figure A.4: 4. DNS Calibration curve

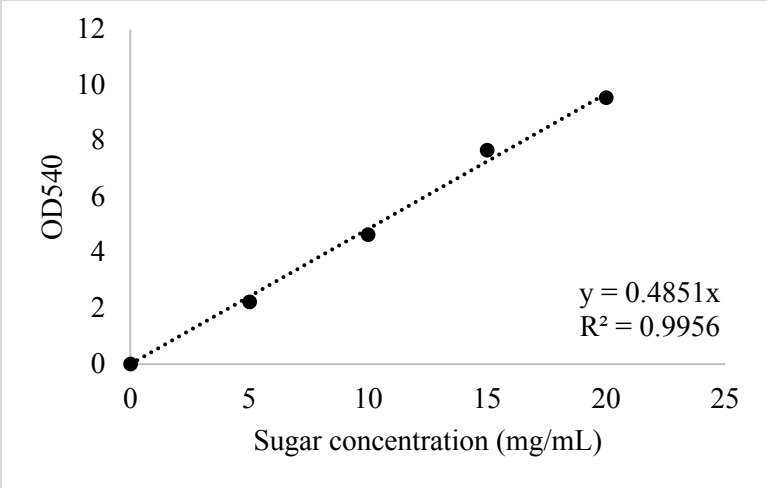

Figure A.5: 5. DNS Calibration curve

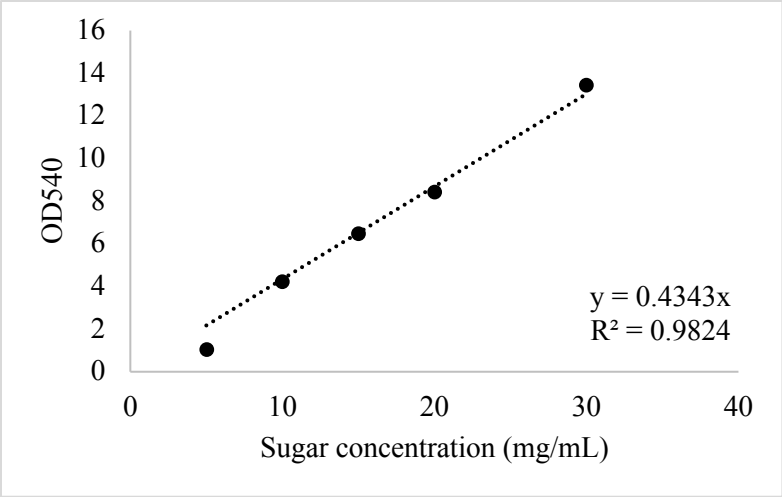

Figure A.6: 6. DNS Calibration curve

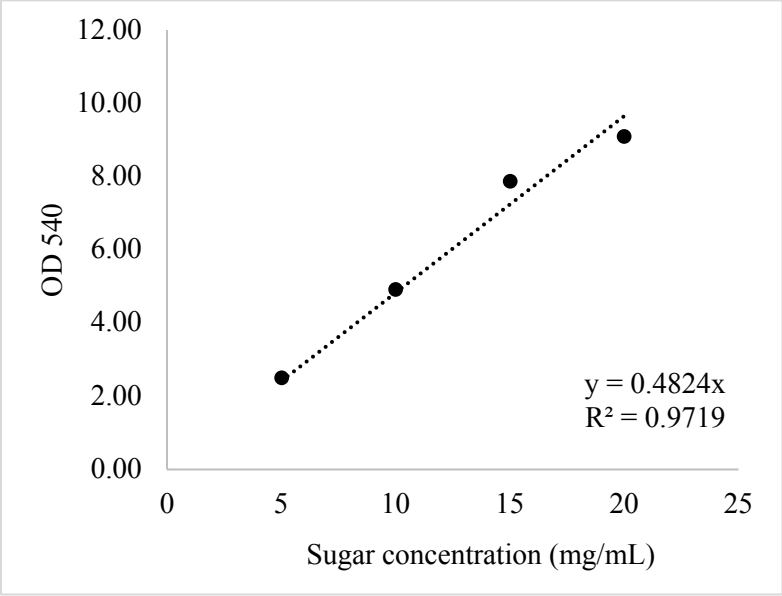

Figure A.7: 7. DNS Calibration curve

APPENDIX B – OD_{540} Results

Below, the OD_{540} results of DNS method analysis for each acid pretreatment and enzymatic hydrolysis experiment are provided in tables. It is stated in the table captions which OD_{540} results were obtained from which DNS calibration curve.

Table B.1: Results of the first set of acid pretreatment, using 1. DNS calibration curve

No of exp.	Time (min)	Amount of husk (g)	Acid % (w/w)	OD_{540}
1	37.5	8	3	8.01
2	37.5	8	3	8.36
3	37.5	8	3	7.68
4	37.5	8	3	7.83
5	37.5	8	3	7.85
6	37.5	8	3	8.14
7	37.5	8	3	7.50
8	37.5	8	3	8.52
9	37.5	8	3	7.68
10	37.5	8	3	8.45
11	37.5	8	5	8.66
12	37.5	8	1	4.93
13	37.5	3.5	3	4.96
14	37.5	12.5	3	11.18
15	50	5.3	4.2	5.41
16	50	5.3	1.8	5.32
17	50	10.7	4.2	10.30
18	50	10.7	1.8	7.83
19	24	10.7	4.2	8.20
20	24	10.7	1.8	5.90
21	24	5.3	4.2	5.38
22	24	5.3	1.8	4.33
23	15	8	3	7.23
24	60	8	3	8.38
25	37.5	8	3	8.01

Table B.2: Results of validation experiments of the first set, using 2. DNS Calibration Curve

No of exp.	Time (min)	Amount of husk (g)	Acid percentage (w/w)	OD_{540}
1	20	4.4	1.2	3.45
2	20	8.7	4.2	7.87
3	25	7.2	1.8	7.11
4	30	11.2	2.5	7.67
5	30	10.3	4.8	9.47
6	35	9.7	2.3	11.07
7	37	8	3	10.46
8	40	7.5	4	11.85
9	45	9.1	1.4	5.81
10	50	12.4	2.7	10.61
11	50	6.3	4.6	7.83
12	50	5.7	3.1	7.96
13	55	3.9	3.4	3.38

Table B.3: Additional acid pretreatment experiments, using 2. DNS Calibration Curve: (* marked value was obtained with 3.DNS Calibration curve)

No of exp.	Time (min)	Amount of husk (g)	Acid percentage (w/w)	OD_{540}
1	37	8	3	8.23
2	37.5	12.5	3	8.68
3	35	9.7	2.3	8.57
4	40	7.5	4	7.93
5	24	5.3	1.8	5.87*
6	20	4.4	1.2	3.14

Table B.4: Pretreatment results of different size and batch of hazelnut husks: 4. DNS Calibration curve was used for experiments (1,2,6 and 7), 5. DNS calibration was used for remaining experiments.

No of exp.	husk size	husk batch	Amount of husk (g)	Time (min)	Acid percentage (w/w)	OD_{540}
1	small	new	12.5	50	3.8	14.78
2	small	new	12.5	50	3.8	18
3	small	new	12.5	50	3.8	13.472
4	small	new	12.5	50	3.8	15.7
5	small	old	12.5	50	3.8	13.952
6	small	old	12.5	50	3.8	13.1
7	small	old	12.5	50	3.8	14.4
8	large	new	12.5	50	3.8	14.56
9	large	new	12.5	50	3.8	12.624

Table B.5: Optimum variable values for the highest amount of sugar concentration using two different models, Kriging result was obtained using 3.DNS Calibration curve, RSM result was obtained using 2. DNS Calibration curve.

Models	Time (min)	Amount of husk (g)	Acid % (w/w)	OD_{540}
Kriging	50	12.5	3.8	15.03
RSM	60	12.5	4.3	9.85

Table B.6: Enzymatic hydrolysis experiments with changing enzyme volume, at pH 5 and 55°C using 3. DNS Calibration curve. (sugar concentrations are measured at day 7)

No of exp.	Total volume in mL	Enzyme volume (mL)	Amount of pretreated husk (g)	OD_{540}
1	25	0	2.5	1.716
2	25	2	2.5	4.79
3	25	5	2.5	7.2
4	25	10	2.5	7.79

Table B.7: Enzymatic hydrolysis results with varying pH and temperature at different days (Total volume 25 mL, enzyme volume 2mL) 4.DNS calibration was used for experiments (4,5 and 6). 7. DNS Calibration was used for remaining experiments.

No of exp.	Temperature (°C)	pH	Time (day)	OD_{540}
1	50	4.5	5	4.784
2	50	5	5	4.672
3	50	6	5	4.704
4	55	4.5	4	5.42
5	55	5	4	5.115
6	55	6	4	6.36
7	60	4.5	5	2.28
8	60	5	5	2.972
9	60	6	5	3.46

Table B.8: Enzymatic hydrolysis results of pretreated husk vs non pretreated husk, using 4. DNS Calibration curve.

No of exp.	Treatment	OD_{540}
1	Acid pretreatment + Enzymatic hydrolysis	4.56
2	Enzymatic hydrolysis	2.36

Table B.9: First set of enzymatic hydrolysis experiments, using 6. DNS calibration curve.

No of exp.	Time (day)	Amount of pretreated husk (g)	Enzyme volume (mL)	OD_{540}
1	2.2	1.5	1.3	2.52
2	2.2	1.5	2.2	2.96
3	2.2	2.25	1.3	2.51
4	2.2	2.25	2.2	3.14
5	5.8	1.5	1.3	3.06
6	5.8	1.5	2.2	3.14
7	5.8	2.25	1.3	3.23

Table B.9 Continued

8	5.8	2.25	2.2	3.73
9	1	1.9	1.75	2.56
10	7	1.9	1.75	3.18
11	4	1.25	1.75	2.63
12	4	2.5	1.75	3.91
13	4	1.9	1	4.1
14	4	1.9	2.5	3.06
15	4	1.9	1.75	3.13
16	4	1.9	1.75	3.08
17	4	1.9	1.75	3.06
18	4	1.9	1.75	3.44
19	4	1.9	1.75	3.1
20	4	1.9	1.75	2.97
21	4	1.9	1.75	3.23
22	4	1.9	1.75	3.32
23	4	1.9	1.75	3.05
24	4	1.9	1.75	3

Table B.10: Validation experiments of enzymatic hydrolysis, using 6. DNS Calibration curve:

No of exp.	Time (day)	Amount of pretreated husk (g)	Enzyme volume (mL)	OD_{540}
1	4.6	1.6	1.57	2.74
2	2.1	1.25	1.88	2.76
3	6.6	2	1.98	2.97
4	3.6	1.85	1.86	3.37
5	4.8	2.05	1.64	2.46
6	0.1	2.2	1.8	2.36
7	3	2.13	2.34	3.05
8	2.8	1.74	2.12	2.98
9	3.7	2.5	1.74	3.36
10	6.1	1.89	1.2	2.9
11	4.1	2.48	1.4	3.43
12	5.2	2.26	1.55	3.49

Table B.11: Optimum values of variables in enzymatic hydrolysis, using 6. DNS Calibration curve:

Models	Time (day)	Amount of pretreated husk (g)	Enzyme volume (mL)	OD_{540}
RSM	6.88	2.5	1	3.63
Kriging	5.35	2.5	1	3.52

APPENDIX C – OD_{600} and CFU Results

Below, the OD_{600} and CFU results of 96-well plates and flasks are provided in tables. It is stated in the table captions which OD_{600} results corresponds to which figures in the results section.

Table C.1: OD_{600} results of cells grown in glucose or xylose medium from Figure 3.5

	Glucose			Glucose			Xylose		
Time	**Cg 1**	**Cg 2**	**Cg 3**	**CgXyl 1**	**CgXyl 2**	**CgXyl 3**	**CgXyl 1**	**CgXyl 2**	**CgXyl 3**
0	0.93	0.93	0.94	0.951	0.962	0.962	0.932	0.94	0.947
7	10.35	10.38	10.42	6.54	6.61	6.63	2.58	2.63	2.65
24	52.9	53.3	53.4	53.6	53.9	54	12.5	12.9	12.9
31	51.7	52.4	52.6	52.9	53.5	53.8	20.2	21	20.8
48	48.1	48.6	49.4	48.8	49.3	49.6	54.7	55.3	55.8
54	48.5	48.9	49.9	51	51.3	51.8	60.9	57.8	55
72	47.2	47.7	47.7	49.3	49.7	50	50.6	51.2	51.4

Table C.2: OD_{600} results of cells grown in husk medium from Figure 3.5

	Husk			Husk		
Time	**Cg 1**	**Cg 2**	**Cg 3**	**CgXyl 1**	**CgXyl 2**	**CgXyl 3**
0						
7	2.02	2.03	2.06	2.58	2.63	2.69
24	10.5	10.7	10.9	10.6	11	11.3
31	12.5	12.9	13	15.8	16.1	16.4
48	11.7	11.8	12	22.2	21.6	22.1
54	12.2	13	12.7	25.4	25.9	26.2
72	11.9	12	11.9	26.8	26.6	26.8

Table C.3: CFU results of Figure 3.6

	Glucose			Glucose			Xylose		
Time	**Cg 1**	**Cg 2**	**Cg 3**	**CgXyl 1**	**CgXyl 2**	**CgXyl 3**	**CgXyl 1**	**CgXyl 2**	**CgXyl 3**
0	3.71E+07	4.66E+07	3.04E+07	2.66E+07	2.47E+07	1.71E+07	2.85E+07	2.38E+07	3.61E+07
7	1.25E+08	1.56E+08	7.27E+07	1.38E+08	1.25E+08	1.52E+08	1.02E+08	7.86E+07	9.17E+07
24	1.22E+09	2.18E+09	1.70E+09	1.35E+09	1.24E+09	1.02E+09	0.00E+00	0.00E+00	0.00E+00
31	7.68E+09	1.12E+10	9.09E+09	1.23E+09	1.01E+09	1.39E+09	2.89E+08	5.79E+08	4.55E+08
48	9.25E+08	1.31E+09	9.74E+08	1.97E+08	4.92E+08	9.35E+08	1.38E+09	1.71E+09	0.00E+00
54	3.34E+09	2.26E+09	2.41E+09	1.64E+09	1.49E+09	1.80E+09	2.20E+09	6.95E+08	1.68E+09
72	1.05E+09	1.95E+09	2.23E+09	2.48E+08	1.39E+09	9.92E+08	3.57E+08	1.02E+08	1.53E+08

Table C.4: CFU results of Figure 3.7

	Husk			Husk		
Time	**Cg 1**	**Cg 2**	**Cg 3**	**CgXyl 1**	**CgXyl 2**	**CgXyl 3**
0	3.04E+07	4.37E+07	3.90E+07	2.85E+07	3.04E+07	4.56E+07
7	4.90E+07	3.67E+07	3.88E+07	2.63E+07	2.10E+07	3.42E+07
24	3.00E+08	4.49E+08	3.75E+08	2.96E+08	1.97E+08	1.97E+08
31	5.38E+08	3.97E+08	4.74E+08	2.90E+08	2.90E+08	4.19E+08
48	4.37E+08	7.32E+08	4.72E+08	8.36E+08	4.84E+08	7.48E+08
54	3.40E+08	3.53E+08	3.78E+08	6.71E+08	5.42E+08	7.48E+08
72	2.50E+08	3.33E+08	0.00E+00	7.21E+08	2.14E+08	2.67E+07

Table C.5: OD_{600} results of Cg cells in Figure 3.1

	Glucose			Husk			Mix			Xylose		
Time	**Cg 1**	**Cg 2**	**Cg 3**	**Cg 1**	**Cg 2**	**Cg 3**	**Cg 1**	**Cg 2**	**Cg 3**	**Cg 1**	**Cg 2**	**Cg 3**
0	0.86	1.011	1.069	1.994	1.994	2.005	1.267	1	1.155	0.752	1.383	1.404
2	1.404	1.585	1.337	2.099	2.091	2.09	1.389	1.361	1.272	0.854	0.8	0.797
4	1.857	2.011	1.882	2.226	2.21	2.213	1.833	1.775	1.853	0.865	0.81	0.814
6	2.187	2.309	2.273	2.354	2.327	2.33	2.21	2.3	2.291	0.872	0.816	0.82
8	2.378	2.479	2.463	2.509	2.426	2.486	2.448	2.539	2.494	0.88	0.82	0.831
10	2.499	2.598	2.586	2.643	2.501	2.797	2.64	2.678	2.625	0.894	0.825	0.928
12	2.606	2.691	2.686	2.893	2.7	3.086	2.662	2.646	2.64	0.887	0.866	0.971
14	2.64	2.727	2.727	3.237	3.043	3.404	2.641	2.648	2.631	0.895	0.852	0.972
16	2.624	2.686	2.689	3.479	3.271	3.551	2.61	2.653	2.638	0.896	0.886	0.986
18	2.606	2.682	2.686	3.644	3.443	3.674	2.63	2.656	2.639	0.907	0.846	0.95
20	2.607	2.686	2.686	3.715	3.547	3.75	2.645	2.664	2.648	0.909	0.886	0.981
22	2.612	2.695	2.697	3.826	3.619	3.793	2.652	2.669	2.651	0.905	0.939	1.007
24	2.617	2.707	2.724	3.905	3.687	3.846	2.671	2.674	2.658	0.902	0.947	1.016
26	2.63	2.714	2.735	3.989	3.785	3.929	2.694	2.678	2.664	0.913	0.922	0.999
28	2.637	2.721	2.739	4.021	3.88	3.983	2.715	2.686	2.674	0.918	0.953	1.016
30	2.642	2.725	2.743	4.065	3.944	4.011	2.729	2.695	2.68	0.916	0.953	1.013
32	2.644	2.73	2.746	4.104	4.015	4.049	2.748	2.703	2.69	0.92	0.96	1.034
34	2.647	2.736	2.752	4.15	4.091	4.082	2.765	2.711	2.699	0.912	0.96	1.045
36	2.653	2.742	2.761	4.167	4.146	4.117	2.773	2.717	2.707	0.911	0.962	1.054
38	2.66	2.748	2.767	4.19	4.201	4.149	2.782	2.724	2.715	0.91	0.953	1.047
40	2.666	2.753	2.773	4.189	4.201	4.152	2.792	2.735	2.73	0.908	0.963	1.046
42	2.671	2.76	2.777	4.222	4.231	4.132	2.794	2.735	2.726	0.908	0.968	1.055
44	2.678	2.766	2.783	4.18	4.257	4.095	2.792	2.737	2.722	0.908	0.952	1.049

Table C.6: OD_{600} results of CgXyl cells in Figure 3.1

	Glucose			Husk			Mix			Xylose		
Time	**CgXyl 1**	**CgXyl 2**	**CgXyl 3**	**CgXyl 1**	**CgXyl 2**	**CgXyl 3**	**CgXyl 1**	**CgXyl 2**	**CgXyl 3**	**CgXyl 1**	**CgXyl 2**	**CgXyl 3**
0	0.73	0.778	0.869	1.937	1.868	1.831	0.892	1.177	0.902	0.892	1.177	0.902
2	1.01	1.084	1.097	2.041	1.933	1.905	1.159	1.421	1.252	1.159	1.421	1.252
4	1.305	1.378	1.382	2.14	2.024	2.006	1.434	1.691	1.688	1.434	1.691	1.688
6	1.715	1.816	1.625	2.24	2.107	2.09	1.685	1.967	2.046	1.685	1.967	2.046
8	2.045	2.145	2.129	2.324	2.18	2.258	2.132	2.145	2.263	2.132	2.145	2.263
10	2.272	2.366	2.399	2.378	2.288	2.551	2.417	2.348	2.427	2.417	2.348	2.427
12	2.437	2.524	2.571	2.45	2.446	2.806	2.61	2.525	2.545	2.61	2.525	2.545
14	2.55	2.625	2.682	2.724	2.753	2.989	2.724	2.66	2.615	2.724	2.66	2.615
16	2.629	2.692	2.734	3.021	3.054	3.206	2.711	2.72	2.642	2.711	2.72	2.642
18	2.651	2.704	2.727	3.354	3.272	3.342	2.711	2.701	2.616	2.711	2.701	2.616
20	2.639	2.662	2.702	3.579	3.447	3.514	2.732	2.709	2.609	2.732	2.709	2.609
22	2.61	2.644	2.704	3.712	3.6	3.702	2.753	2.74	2.613	2.753	2.74	2.613
24	2.61	2.642	2.708	3.858	3.763	3.764	2.771	2.767	2.622	2.771	2.767	2.622
26	2.616	2.645	2.717	3.976	3.88	3.864	2.783	2.792	2.635	2.783	2.792	2.635
28	2.624	2.647	2.722	4.062	4.006	3.916	2.781	2.818	2.65	2.781	2.818	2.65
30	2.637	2.649	2.729	4.141	4.094	3.884	2.781	2.839	2.671	2.781	2.839	2.671
32	2.642	2.647	2.731	4.216	4.177	3.968	2.776	2.851	2.688	2.776	2.851	2.688
34	2.652	2.648	2.733	4.247	4.228	4.003	2.772	2.863	2.704	2.772	2.863	2.704
36	2.665	2.652	2.731	4.286	4.294	4.058	2.768	2.876	2.717	2.768	2.876	2.717
38	2.678	2.661	2.733	4.308	4.312	4.102	2.765	2.89	2.727	2.765	2.89	2.727
40	2.688	2.669	2.736	4.375	4.382	4.181	2.761	2.9	2.737	2.761	2.9	2.737
42	2.7	2.678	2.742	4.356	4.382	4.183	2.759	2.911	2.746	2.759	2.911	2.746
44	2.712	2.689	2.75	4.401	4.423	4.242	2.756	2.922	2.756	2.756	2.922	2.756

Table C.7: OD_{600} results of ARO cells in Figure 3.2

	Glucose			Husk			Mix			Xylose		
Time	**ARO 1**	**ARO 2**	**ARO 3**	**ARO 1**	**ARO 2**	**ARO 3**	**ARO 1**	**ARO 2**	**ARO 3**	**ARO 1**	**ARO 2**	**ARO 3**
0	0.791	1.085	1.027	2.06	2.282	2.074	1.296	1.321	0.95	1.393	0.738	1.436
2	1.54	1.635	1.67	2.21	2.451	2.24	1.507	1.514	1.113	1.045	0.75	0.902
4	1.858	1.922	1.925	2.339	2.596	2.379	1.831	1.81	1.504	1.149	0.75	0.774
6	2.074	2.07	2.114	2.442	2.71	2.492	2.051	2.051	1.964	1.151	0.798	0.896
8	2.172	2.171	2.195	2.496	2.783	2.536	2.171	2.174	2.136	1.176	0.859	0.893
10	2.244	2.246	2.256	2.536	2.836	2.69	2.266	2.263	2.262	1.245	0.85	0.909
12	2.298	2.302	2.307	2.671	2.944	2.879	2.337	2.331	2.341	1.208	0.881	0.888
14	2.338	2.342	2.346	2.899	3.097	3.071	2.397	2.385	2.391	1.224	0.876	0.771
16	2.371	2.369	2.376	3.104	3.296	3.271	2.433	2.412	2.413	1.224	0.874	0.755
18	2.399	2.397	2.402	3.266	3.507	3.459	2.471	2.433	2.431	1.225	0.8	0.771
20	2.425	2.421	2.426	3.446	3.72	3.602	2.506	2.452	2.448	1.145	0.779	0.785
22	2.454	2.45	2.454	3.616	3.856	3.723	2.535	2.478	2.468	1.1	0.785	0.79
24	2.482	2.475	2.479	3.741	3.984	3.873	2.545	2.495	2.48	1.073	0.793	0.799
26	2.503	2.495	2.5	3.746	4.119	3.95	2.549	2.513	2.496	0.982	0.797	0.813
28	2.528	2.519	2.522	3.914	4.162	4.059	2.516	2.534	2.517	0.963	0.803	0.82
30	2.547	2.536	2.54	4	4.293	4.108	2.196	2.55	2.537	0.966	0.808	0.821
32	2.566	2.554	2.558	4.071	4.267	4.133	2.195	2.551	2.557	0.937	0.812	0.822
34	2.58	2.57	2.575	4.121	4.289	4.162	2.203	2.454	2.554	0.952	0.816	0.815
36	2.593	2.583	2.59	4.169	4.358	4.229	2.199	2.231	2.428	0.949	0.816	0.833
38	2.605	2.582	2.604	4.189	4.424	4.218	2.197	2.224	2.231	0.948	0.819	0.836
40	2.597	2.577	2.597	4.236	4.455	4.258	2.195	2.219	2.228	0.822	0.821	0.849
42	2.591	2.579	2.597	4.272	4.515	4.328	2.194	2.214	2.226	0.82	0.823	0.85
44	2.589	2.586	2.603	4.294	4.636	4.299	2.194	2.21	2.224	0.821	0.824	0.843

Table C.8: OD_{600} results of AROXyl cells in Figure 3.2

	Glucose			Husk			Mix			Xylose		
Time	AROXyl 1	AROXyl 2	AROXyl 3	AROXyl 1	AROXyl 2	AROXyl 3	AROXyl 1	AROXyl 2	AROXyl 3	AROXyl 1	AROXyl 2	AROXyl 3
0	0.968	0.968	0.955	2.105	2.172	2.134	1.288	1.361	1.324	1.329	1.345	1.363
2	1.548	1.431	1.419	2.299	2.316	2.327	1.567	1.257	1.278	1.007	1.364	1.417
4	1.855	1.864	1.873	2.465	2.44	2.486	1.945	1.704	1.596	1.278	0.905	0.855
6	2.074	2.112	2.101	2.606	2.533	2.607	2.14	2.042	1.951	1.516	1.201	1.069
8	2.197	2.211	2.205	2.712	2.613	2.743	2.268	2.204	2.176	1.663	1.51	1.374
10	2.266	2.282	2.272	2.917	2.714	2.944	2.344	2.294	2.306	1.823	1.788	1.574
12	2.318	2.338	2.322	3.184	2.947	3.183	2.392	2.346	2.381	1.962	1.955	1.671
14	2.362	2.387	2.368	3.461	3.227	3.446	2.428	2.389	2.434	2.105	2.074	1.861
16	2.395	2.426	2.406	3.733	3.495	3.681	2.457	2.425	2.465	2.202	2.174	1.954
18	2.421	2.454	2.438	3.933	3.718	3.889	2.484	2.453	2.495	2.28	2.263	2.158
20	2.445	2.478	2.465	4.1	3.905	4.05	2.512	2.48	2.521	2.343	2.326	2.263
22	2.468	2.499	2.492	4.235	4.065	4.177	2.535	2.505	2.542	2.388	2.384	2.333
24	2.491	2.522	2.518	4.337	4.197	4.277	2.555	2.522	2.552	2.429	2.422	2.405
26	2.513	2.545	2.542	4.376	4.286	4.332	2.574	2.538	2.562	2.456	2.441	2.458
28	2.535	2.565	2.563	4.549	4.422	4.438	2.578	2.538	2.559	2.479	2.468	2.5
30	2.555	2.582	2.581	4.613	4.53	4.503	2.58	2.536	2.556	2.495	2.489	2.526
32	2.572	2.601	2.599	4.568	4.544	4.513	2.584	2.539	2.551	2.506	2.509	2.541
34	2.586	2.616	2.616	4.62	4.654	4.606	2.589	2.544	2.547	2.521	2.524	2.548
36	2.593	2.627	2.629	4.664	4.761	4.676	2.599	2.551	2.542	2.53	2.535	2.554
38	2.573	2.599	2.608	4.666	4.733	4.74	2.602	2.556	2.536	2.532	2.542	2.556
40	2.562	2.586	2.594	4.695	4.75	4.737	2.594	2.561	2.537	2.534	2.544	2.556
42	2.554	2.575	2.584	4.681	4.715	4.706	2.583	2.563	2.523	2.534	2.544	2.553
44	2.55	2.564	2.571	4.673	4.77	4.756	2.569	2.551	2.545	2.534	2.541	2.551

Table C.9: OD_{600} results of ARO and AROXyl cells in Figure 3.3

	Glucose			Husk			Glucose			Husk		
Time	ARO 1	ARO 2	ARO 3	ARO 1	ARO 2	ARO 3	AROXyl 1	AROXyl 2	AROXyl 3	AROXyl 1	AROXyl 2	AROXyl 3
0	0.578	0.538	0.579	1.308	1.361	1.391	0.631	0.656	0.573	1.302	1.284	1.261
0.5	0.566	0.629	0.566	1.321	1.37	1.408	0.617	0.62	0.571	1.313	1.3	1.272
1	0.644	0.681	0.633	1.327	1.374	1.416	0.719	0.803	0.672	1.324	1.311	1.281
1.5	0.724	0.754	0.715	1.332	1.38	1.427	0.851	0.968	0.818	1.336	1.323	1.29
2	0.811	0.828	0.805	1.338	1.387	1.435	0.962	1.107	0.988	1.349	1.337	1.301
2.5	0.898	0.934	0.932	1.345	1.394	1.443	1.066	1.292	1.228	1.363	1.35	1.313
3	0.986	1.055	1.07	1.353	1.403	1.453	1.204	1.413	1.319	1.376	1.363	1.325
3.5	1.061	1.16	1.197	1.362	1.414	1.464	1.338	1.478	1.403	1.389	1.376	1.336
4	1.119	1.271	1.325	1.37	1.424	1.471	1.443	1.551	1.492	1.402	1.389	1.347
4.5	1.181	1.389	1.444	1.381	1.435	1.484	1.551	1.613	1.561	1.416	1.403	1.359
5	1.25	1.509	1.559	1.393	1.449	1.499	1.636	1.692	1.694	1.429	1.416	1.371
5.5	1.364	1.622	1.663	1.404	1.463	1.514	1.775	1.819	1.799	1.443	1.43	1.383
6	1.513	1.728	1.752	1.415	1.478	1.529	1.859	1.872	1.843	1.458	1.443	1.394
6.5	1.663	1.839	1.83	1.426	1.491	1.542	1.892	1.905	1.879	1.471	1.456	1.406
7	1.817	1.937	1.903	1.437	1.505	1.555	1.933	1.939	1.918	1.484	1.469	1.416
7.5	1.911	2.007	1.958	1.447	1.516	1.566	1.967	1.972	1.951	1.496	1.48	1.425
8	1.987	2.059	2.008	1.457	1.526	1.577	2	2.003	1.979	1.507	1.492	1.434
8.5	2.047	2.096	2.041	1.465	1.537	1.588	2.04	2.035	2.011	1.519	1.503	1.442
9	2.098	2.127	2.076	1.473	1.546	1.598	2.069	2.062	2.039	1.53	1.512	1.45
9.5	2.17	2.151	2.103	1.482	1.557	1.608	2.098	2.091	2.066	1.541	1.522	1.458
10	2.189	2.19	2.13	1.49	1.566	1.618	2.124	2.114	2.092	1.553	1.532	1.465

Table C.9. continued

10.5	2.209	2.203	2.159	1.498	1.576	1.629	2.151	2.139	2.116	1.565	1.542	1.472
11	2.234	2.222	2.183	1.507	1.587	1.642	2.175	2.16	2.136	1.579	1.551	1.479
11.5	2.26	2.241	2.209	1.517	1.599	1.656	2.197	2.181	2.157	1.594	1.561	1.485
12	2.285	2.261	2.222	1.528	1.613	1.673	2.218	2.2	2.178	1.615	1.572	1.492
12.5	2.312	2.281	2.241	1.54	1.629	1.695	2.237	2.218	2.197	1.64	1.585	1.499
13	2.334	2.3	2.259	1.556	1.652	1.724	2.253	2.234	2.216	1.67	1.6	1.507
13.5	2.354	2.317	2.275	1.575	1.678	1.758	2.266	2.251	2.233	1.704	1.619	1.515
14	2.371	2.335	2.29	1.6	1.709	1.798	2.275	2.266	2.247	1.743	1.642	1.523
14.5	2.385	2.35	2.303	1.629	1.745	1.843	2.284	2.278	2.264	1.787	1.67	1.534
15	2.398	2.364	2.316	1.664	1.788	1.899	2.293	2.289	2.277	1.836	1.701	1.548
15.5	2.41	2.377	2.327	1.705	1.834	1.959	2.3	2.298	2.289	1.889	1.736	1.563
16	2.42	2.387	2.336	1.75	1.887	2.024	2.304	2.303	2.298	1.942	1.777	1.581
16.5	2.43	2.398	2.346	1.803	1.942	2.084	2.309	2.309	2.305	1.995	1.82	1.602
17	2.439	2.408	2.353	1.859	1.998	2.14	2.313	2.313	2.309	2.047	1.868	1.626
17.5	2.449	2.416	2.361	1.919	2.052	2.194	2.317	2.317	2.313	2.097	1.921	1.654
18	2.458	2.422	2.367	1.975	2.103	2.248	2.322	2.32	2.316	2.148	1.971	1.685
18.5	2.467	2.429	2.372	2.03	2.154	2.3	2.327	2.324	2.317	2.196	2.023	1.717
19	2.476	2.438	2.377	2.082	2.201	2.351	2.331	2.328	2.32	2.242	2.073	1.754
19.5	2.486	2.447	2.383	2.133	2.249	2.401	2.335	2.333	2.323	2.288	2.124	1.796
20	2.497	2.457	2.392	2.185	2.298	2.45	2.342	2.337	2.329	2.334	2.174	1.838
20.5	2.503	2.464	2.397	2.231	2.34	2.496	2.345	2.34	2.332	2.375	2.217	1.878
21	2.512	2.474	2.404	2.279	2.388	2.542	2.352	2.345	2.337	2.419	2.263	1.919
21.5	2.517	2.48	2.411	2.324	2.428	2.584	2.356	2.349	2.34	2.46	2.305	1.957
22	2.524	2.487	2.417	2.368	2.472	2.625	2.36	2.352	2.344	2.502	2.348	1.997
22.5	2.532	2.495	2.427	2.413	2.513	2.649	2.366	2.358	2.353	2.543	2.392	2.035

Table C.9. continued

23	2.533	2.498	2.43	2.449	2.548	2.648	2.371	2.361	2.356	2.58	2.43	2.068
23.5	2.538	2.505	2.434	2.476	2.578	2.63	2.374	2.364	2.358	2.617	2.47	2.101
24	2.545	2.51	2.439	2.487	2.599	2.611	2.377	2.367	2.364	2.655	2.51	2.134
24.5	2.546	2.513	2.442	2.478	2.601	2.596	2.381	2.371	2.367	2.687	2.546	2.164
25	2.551	2.518	2.446	2.47	2.6	2.584	2.386	2.375	2.372	2.721	2.583	2.195
25.5	2.555	2.523	2.449	2.461	2.597	2.577	2.391	2.379	2.377	2.752	2.617	2.225
26	2.558	2.525	2.451	2.454	2.591	2.569	2.394	2.383	2.381	2.78	2.652	2.253
26.5	2.561	2.527	2.453	2.454	2.584	2.563	2.397	2.386	2.384	2.804	2.681	2.277
27	2.568	2.53	2.455	2.457	2.59	2.562	2.4	2.389	2.387	2.835	2.708	2.295
27.5	2.571	2.533	2.458	2.455	2.58	2.563	2.403	2.391	2.39	2.856	2.731	2.307
28	2.576	2.539	2.46	2.455	2.574	2.566	2.404	2.393	2.394	2.876	2.755	2.312
28.5	2.578	2.544	2.465	2.455	2.569	2.573	2.407	2.397	2.399	2.886	2.777	2.309
29	2.584	2.547	2.469	2.457	2.566	2.586	2.41	2.4	2.403	2.892	2.799	2.302
29.5	2.588	2.553	2.472	2.458	2.565	2.597	2.412	2.403	2.407	2.896	2.818	2.294
30	2.59	2.557	2.476	2.463	2.567	2.604	2.414	2.405	2.407	2.898	2.828	2.287
30.5	2.594	2.559	2.478	2.471	2.572	2.61	2.414	2.406	2.408	2.9	2.839	2.281
31	2.597	2.564	2.485	2.485	2.585	2.616	2.419	2.411	2.409	2.903	2.85	2.276
31.5	2.597	2.564	2.485	2.49	2.59	2.618	2.422	2.413	2.409	2.909	2.855	2.282
32	2.602	2.565	2.486	2.494	2.593	2.624	2.422	2.415	2.409	2.912	2.864	2.287
32.5	2.609	2.569	2.487	2.497	2.594	2.628	2.422	2.416	2.41	2.919	2.872	2.288
33	2.613	2.572	2.489	2.501	2.594	2.632	2.423	2.417	2.41	2.926	2.882	2.387
33.5	2.617	2.575	2.491	2.504	2.595	2.64	2.422	2.418	2.41	2.932	2.892	2.443
34	2.626	2.578	2.495	2.508	2.596	2.643	2.423	2.418	2.411	2.941	2.902	2.495
34.5	2.63	2.582	2.499	2.511	2.599	2.651	2.424	2.42	2.413	2.95	2.914	2.521
35	2.637	2.586	2.504	2.514	2.601	2.656	2.425	2.421	2.414	2.959	2.927	2.584

Table C.9. continued

35.5	2.644	2.59	2.51	2.519	2.604	2.663	2.428	2.423	2.415	2.969	2.941	2.616
36	2.651	2.592	2.511	2.523	2.606	2.667	2.429	2.425	2.418	2.983	2.957	2.606
36.5	2.655	2.595	2.516	2.527	2.61	2.672	2.433	2.427	2.418	3.001	2.971	2.655
37	2.659	2.597	2.517	2.532	2.615	2.68	2.436	2.43	2.42	3.018	2.986	2.655
37.5	2.662	2.596	2.519	2.537	2.618	2.685	2.437	2.432	2.423	3.034	3.002	2.72
38	2.666	2.597	2.521	2.542	2.622	2.693	2.438	2.433	2.426	3.057	3.026	2.715
38.5	2.671	2.6	2.523	2.546	2.628	2.699	2.44	2.433	2.43	3.076	3.042	2.746
39	2.676	2.602	2.526	2.557	2.632	2.706	2.44	2.435	2.432	3.092	3.061	2.791
39.5	2.681	2.604	2.527	2.561	2.638	2.711	2.442	2.436	2.434	3.111	3.082	2.832
40	2.684	2.605	2.53	2.565	2.644	2.719	2.444	2.438	2.434	3.133	3.104	2.777
40.5	2.688	2.61	2.533	2.568	2.65	2.725	2.445	2.44	2.439	3.145	3.123	2.888
41	2.694	2.613	2.536	2.571	2.654	2.733	2.448	2.441	2.44	3.166	3.142	2.952
41.5	2.698	2.616	2.539	2.575	2.661	2.741	2.451	2.443	2.444	3.178	3.15	2.941
42	2.702	2.62	2.542	2.578	2.669	2.749	2.454	2.446	2.445	3.192	3.17	2.978
42.5	2.705	2.622	2.544	2.582	2.675	2.756	2.459	2.448	2.447	3.211	3.183	2.946
43	2.707	2.625	2.546	2.587	2.679	2.763	2.461	2.45	2.45	3.221	3.196	2.962
43.5	2.711	2.628	2.548	2.592	2.687	2.772	2.465	2.451	2.452	3.229	3.211	2.97
44	2.713	2.63	2.551	2.596	2.692	2.78	2.467	2.453	2.456	3.247	3.225	3.003
44.5	2.716	2.633	2.553	2.6	2.698	2.785	2.47	2.454	2.46	3.261	3.238	3.035
45	2.72	2.636	2.557	2.605	2.704	2.792	2.473	2.457	2.461	3.271	3.254	2.989
45.5	2.721	2.639	2.559	2.611	2.711	2.8	2.478	2.459	2.464	3.282	3.268	3.017
46	2.724	2.642	2.561	2.615	2.717	2.806	2.482	2.462	2.466	3.305	3.28	3.069
46.5	2.724	2.645	2.564	2.619	2.722	2.813	2.485	2.464	2.469	3.315	3.287	3.108
47	2.729	2.647	2.565	2.624	2.73	2.819	2.489	2.465	2.473	3.321	3.295	3.155
47.5	2.731	2.65	2.569	2.628	2.735	2.827	2.493	2.468	2.475	3.338	3.31	3.142

Table C.9. continued

48	2.733	2.656	2.571	2.631	2.741	2.831	2.497	2.472	2.476	3.344	3.32	3.048
48.5	2.734	2.659	2.574	2.634	2.747	2.837	2.502	2.475	2.476	3.358	3.329	3.168
49	2.737	2.66	2.575	2.64	2.754	2.845	2.506	2.478	2.475	3.364	3.338	3.239
49.5	2.737	2.662	2.577	2.643	2.76	2.849	2.511	2.481	2.475	3.373	3.346	3.216
50	2.738	2.666	2.579	2.647	2.767	2.855	2.516	2.485	2.476	3.383	3.355	3.237
50.5	2.743	2.667	2.582	2.654	2.772	2.859	2.52	2.488	2.478	3.387	3.369	3.189
51	2.743	2.667	2.583	2.654	2.776	2.865	2.524	2.49	2.48	3.392	3.377	3.137
51.5	2.744	2.668	2.581	2.658	2.783	2.868	2.526	2.492	2.48	3.401	3.389	3.136
52	2.745	2.668	2.582	2.661	2.788	2.873	2.529	2.493	2.479	3.407	3.405	3.173
52.5	2.745	2.668	2.582	2.665	2.795	2.88	2.53	2.494	2.48	3.414	3.418	3.202
53	2.751	2.669	2.58	2.67	2.802	2.881	2.532	2.496	2.478	3.416	3.427	3.159
53.5	2.752	2.67	2.581	2.698	2.807	2.887	2.536	2.499	2.48	3.42	3.434	3.184
54	2.757	2.673	2.584	2.703	2.811	2.893	2.539	2.5	2.48	3.425	3.44	3.197
54.5	2.759	2.678	2.588	2.707	2.811	2.898	2.543	2.503	2.477	3.436	3.456	3.129
55	2.763	2.673	2.585	2.71	2.818	2.904	2.545	2.506	2.476	3.44	3.463	3.193
55.5	2.763	2.674	2.586	2.712	2.826	2.905	2.549	2.508	2.473	3.447	3.468	3.043
56	2.765	2.675	2.585	2.715	2.833	2.909	2.552	2.511	2.471	3.45	3.474	3.178
56.5	2.766	2.675	2.586	2.719	2.836	2.911	2.557	2.514	2.47	3.447	3.478	3.217
57	2.766	2.676	2.589	2.722	2.842	2.917	2.56	2.517	2.466	3.454	3.488	3.159
57.5	2.768	2.674	2.588	2.723	2.845	2.92	2.563	2.519	2.463	3.455	3.491	3.163
58	2.765	2.676	2.59	2.727	2.85	2.924	2.567	2.522	2.459	3.458	3.505	3.176
58.5	2.765	2.675	2.588	2.728	2.853	2.928	2.57	2.525	2.455	3.455	3.508	3.18
59	2.761	2.674	2.586	2.729	2.856	2.931	2.571	2.525	2.449	3.461	3.517	3.155
59.5	2.762	2.677	2.586	2.73	2.861	2.935	2.573	2.53	2.442	3.46	3.521	3.167
60	2.768	2.682	2.591	2.734	2.867	2.938	2.58	2.538	2.432	3.464	3.519	3.168

Table C.9. continued

60.5	2.77	2.681	2.593	2.736	2.871	2.943	2.582	2.54	2.42	3.47	3.528	3.18
61	2.77	2.682	2.596	2.737	2.874	2.983	2.586	2.545	2.403	3.47	3.533	3.146
61.5	2.773	2.68	2.595	2.739	2.879	2.986	2.586	2.545	2.366	3.471	3.539	3.219
62	2.772	2.679	2.594	2.74	2.881	2.99	2.587	2.549	2.296	3.479	3.541	3.263

CURRICULUM VITAE /ÖZGEÇMİŞ

Beril PAKALIN

EDUCATION

2019 – 2021	**Master's Degree**: Marmara University, Faculty of Engineering, Istanbul Department of Bioengineering. **Thesis:** *Obtaining Sugars from Hazelnut Husk to Be Utilized in The Growth of An Industrial Microbial Strain*
2018 – 2015	**Bachelor's Degree**: Koç University, College of Engineering**,** Istanbul, Department of Chemical and Biological Engineering. **Thesis:** *Producing Ocrelizumab in Turkey, A Drug for MS Disease*
2015 – 2013	**Bachelor**: Koç University, College of Engineering, Istanbul, Department of Computer Engineering.
2013 – 2008	**Highschool**: Istanbul Erkek Lisesi, Istanbul

EXPERIENCE

July 2018	**Türk Prysmian Kablo ve Sistemleri A.Ş., Material Technologies Laboratory,** Bursa, *Summer Internship*
June 2017	**OYAK Renault Plant, Wastewater treatment facility,** Bursa, *Summer Internship*
Jan. – April 2017	**Koç University, BIOMICELL Laboratory,** Istanbul - working with a PhD student for synthesizing and analyzing the properties of biofunctional hydrogels.
June – Aug. 2016	**University of California Santa Cruz, Yildiz Laboratory,** California - helped a PhD student in the research about molecular mechanisms of biofilm formation in *V. cholerae* bacteria

- conducted experiments to identify regulators of a two component system that affect biofilm formation using genetic screening and identified genes of interest.

ADDITIONAL INFORMATION

Project participation: TUBITAK grant on: Rasyonel Tasarım ile *Corynebacterium glutamicum'un* L-Dopa Üretimi için Bir Mikrobiyal Hücre Fabrikasına Dönüştürülmesi ve L-Dopa Üretim Prosesinin Geliştirilmesi

Conference: 9th Int. CeBiTec Research Conference Bielefeld

Languages: Fluent in English: TOEFL iBT (2021):104, Intermediate in German (Abitur: 2.5) (2013)

Computer: SuperPro Designer, proficient user in MS Office

Interests: Painting and skiing