ADAPTIVE LABORATORY EVOLUTION STRATEGY FOR IMPROVEMENT OF GROWTH AND SUBSTRATE UTILIZATION OF *BACILLUS SUBTILIS* IN HAZELNUT SHELLS HYDROLYSATE

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ADAPTIVE LABORATORY EVOLUTION STRATEGY FOR IMPROVEMENT OF GROWTH AND SUBSTRATE UTILIZATION OF *BACILLUS SUBTILIS* IN HAZELNUT SHELLS HYDROLYSATE

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ABSTRACT

ADAPTIVE LABORATORY EVOLUTION STRATEGY FOR IMPROVEMENT OF GROWTH AND SUBSTRATE UTILIZATION OF *BACILLUS SUBTILIS* IN HAZELNUT SHELLS HYDROLYSATE

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Lignocellulosic materials are worldwide naturally abundant. Similarly, due to industrial processing, hazelnut shells are enormous in Turkey. Utilizing them for various purposes, including bioprocess, reduces environmental pollution and improves bio-economy. Microbial fermentation inhibitors formed during pre-treatment of lignocellulose material to release fermentable sugars is a challenge for bioprocessing. The most promising strategy to tackle this problem is biological detoxification. This study aimed to use the method of adaptive laboratory evolution for improving the growth rate and substrate consumption of *Bacillus subtilis* in hazelnut shells hydrolysate under various cultivation conditions of pH (5,6, and 7) and inoculum ratio (2,5, and 10%). Dry weight of biomass was used to monitor the growth rate improvement. The substrate consumption was measured spectrophotometrically using the DNS method. The results were statistically analyzed by One-way Analysis of variance (ANOVA) for the biomass produced and Kruskal Walli's nonparametric test for the daily substrate consumption. The results showed that the best condition for biomass production was found at pH 7 and 10% inoculum ratio with 6.17 ± 0.07 g/L biomass produced and the residual sugar of 15.04 ± 0.12 g/L. Poor adaptation was observed at pH 5 with an inoculum

ratio of 2%. The result shows the potential to improve the growth and sugar consumption of *B.subtilis*. Overall, biomass increases with an increase in pH for different inoculum ratios, with pH 7 having higher biomass than other pHs.

ÖZ

BACİLLUS SUBTİLİS'İN FINDIK KABUĞU HİDROLİZATINDA ÇOĞALMA VE SUBSTRAT KULLANIMININ UYARLANABİLİR LABORATUVAR GELİŞİM STRATEJİSİ İLE İYİLEŞTİRİLMESİ

Tukur, Ummulkhairi
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Lignoselülozik malzemeler dünya çapında doğal olarak bol miktarda bulunur. Benzer şekilde, endüstriyel işleme sonucu Türkiye'de fındık kabuğu çok ekleme. Biyoproses de dahil olmak üzere çeşitli şekillerde amaçlar için kullanmak çevre kirliliğini azaltır ve biyo-ekonomiyi genişletir. Fermente edilebilir şekerleri serbest yetkili servis için lignoselülozları ön işlemi sırasında oluşan mikrobiyal fermantasyon inhibitörleri, biyoişlem için bir zorluktur. Bu sorgulama merkezlerine gelmek için en umut verici strateji biyolojik detoksifikasyondur. Bu çalışmanın amacı, çeşitli pH (5,6 ve 7) ve inokulum oranı (%2,5 ve 10) yetiştirme koşulları altında fındık kabukları hidrolizatındaki *Bacillus subtilis'in* büyüme özellikleri ve substrat tüketimini hedeflemek için adaptif evrim laboratuvarı kullanımtı. , ve %2). Büyüme Hızındaki Kurtuluşu İzlemek İçin Biyokütlenin Kuru Ağırlığı. Substrat tüketimi, indirgeyici şekeri analiz etmek için DNS yöntemi kullanılarak spektrofotometrik olarak ölçülmüştür. Sonuçlar, üretilen biyokütle için Tek Yönlü Varyans Analizi (ANOVA) ve günlük substrat tüketimi için Kruskal Walli'nin parametrik olmayan analizi ile etkilenenler olarak analiz edildi. Sonuçlar, biyokütle üretimi için en iyi bitkilerden, üretilen 6.17±0.07 g/L biyokütle ve 15.04 ± 0.12g/L kalıntı şeker ile pH 7 ve %10 inokulum kalıntılarından elde edildi. %2 inokulum

oranı ile pH 5'te zayıf adaptasyon gözlendi. Sonuç olarak, *B.subtilis'in* şeker kullanımını geliştirmeyi göstermektedir. Genel olarak, farklı inokulumlar için pH artışıyla birlikte biyokütle artar, pH 7 diğer pH'lardan daha yüksek biyokütleye sahiptir.

Anahtar Kelimeler: Hidrolizat, *Bacillus subtilis*, Evrimsel Adaptasyon, DNS yöntemi, İstatistiksel analiz.

This thesis is dedicated to my Supervisors.

ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

The intricate structure of biomass has an impact on the environment. As a result, it is receiving a lot of attention because its full implementation would bring about a circular bio-economy in modern day. However, a hydrolysis process is required to liberate sugars from the compact structure to utilize this biomass. It frequently calls for effective and affordable hydrolysis techniques, such as dilute acid hydrolysis, due to fermentation inhibitors like phenols, furans, and organic acids that are released during the pre-treatment. The amount of enzyme required may rise due to hydrolysis, increasing the enzyme's price as well.

As a result, there will be typically a loss in sugar and a price increase. Therefore, biological detoxification would be preferable. In light of this, the adaptation of various microbes to lignocellulose hydrolysate receives a lot of interest.

Hazelnut shells, which are left over after the internal kernel is removed, are a substantial waste product for the hazelnut industry. They are crucial sources of cellulosic bio chemicals and phenolic compounds, which can act as antioxidants. Unfortunately, these shells are frequently wasted, mostly used internally as fuel, or dumped outdoors, which contributes to environmental degradation.

To counteract the adverse environmental and economic effects, researchers become more interested in using hazelnut shells. Hazelnut shells have been used in many research to manufacture antioxidant phenolic chemicals that can then be added to industrial products. Additionally, burning these shells will eventually be replaced by using them as aggregate in concrete, while other researchers have reported using pretreated hazelnut shells in bioprocessing.

By using hazelnut shell hydrolysate as adaptation media for *Bacillus subtilis*, which is frequently used as a host engine for the microbial production of various biotechnological products of interest, such as enzymes, ethanol, and vitamins, among others. This study aims to increase sustainability in the hazelnut industry. By doing this, there will be a reduction in the cost of pure substrates, and the negative environmental effects of discarding away or burning these shells will be minimized.

The modification was accomplished by enhancing the growth of *B.subtilis* NRRL B-4219 in the hydrolysate of hazelnut shells. For each experimental set, the settings were pH (5, 6, and 7), inoculum ratio (2, 5, and 10), and their interactions were examined for successive daily transfers into fresh hydrolysate. The bacteria were grown for eight days under ideal conditions. Additionally, the exact conditions were employed to culture the bacteria for eight days in a hydrolysate of detoxified hazelnut shells with activated charcoal as the detoxifying agent. The bacteria were then moved to a synthetic medium to grow for 48 hours to determine their growth rate and create a growth curve model.

For a more in-depth discussion, the existing literature on exploiting and adapting hazelnut shells using various microorganisms and lignocellulose hydrolysates was reviewed in chapter 2. Additionally, different detoxifying techniques and the negative consequences of inhibitors were examined. The experimental methodology is described in great depth in Chapter 3 of this study. Chapter 4 included both the debate and the results. The conclusion and suggested next steps are contained in Chapter 5.

CHAPTER 2

LITERATURE REVIEW

2.1   Lignocellulosic Materials

Lignocellulosic materials are renewable energy sources consisting of agro-industrial, forestry, and agricultural wastes. According to Mussatto and Teixeira (2010), examples of such waste materials include sawdust, sugarcane bagasse, waste paper, brewer's leftover grains, stems, stalks, leaves, husks, shells, and peels from a variety of cereals, including rice, wheat, corn, sorghum, and barley. The environment faces issues due to the annual accumulation. However, their use for creating value-added goods is crucial since they contain sugars and other bioactive substances. Therefore, in addition to their buildup in the environment, failure to use these materials results in the loss of potentially valuable sources (Mussatto and Teixeira (2010); Singh *et al.*, 2009).

2.2   Composition of Lignocellulosic Biomass

The main constituents of lignocellulosic materials are polymers of cellulose, hemicellulose, and lignin. They form complex biomass together because they are closely related to one another. Hemicellulose and lignin surround the backbone of cellulose. It is made up of a cellobiose repeating unit, which consists of two anhydrous glucose rings connected by a β-1,4 glycosidic bond, and has a high molecular weight. Hydrogen bonds and Van der Waals forces hold the long-chain cellulose together, causing the cellulose to be packed into microfibrils (Mussatto and Teixeira and 2010; Ha *et al.*, 1998).

On the other hand, hemicellulose is a branching and linear heterogeneous polymer that is frequently composed of five different sugars (L-arabinose, D-galactose, D-

glucose, D-mannose, and D-xylose), as well as additional substances like acetic, glucuronic, and ferulic acids. Hemicellulose chains can have a homopolymer (a repeat of one sugar unit) or a heteropolymer (a combination of various sugars) as their backbone. Hemicelluloses vary from celluloses due to the sugar unit composition, having shorter chains, branching, and amorphous structure (Fengel and Wegener 1989). The molecule lignin is extremely complicated. It is made up of several interconnected three-dimensional phenylpropane molecules. As monomers for lignin, there are three phenyl propionic alcohols. P-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol are these substances. Both cellulose and hemicellulose are tightly bound to lignin. It gives the material's cell wall stiffness and cohesiveness, blocks water from entering xylem vessels, and creates a defense against microbial attack. The organization of the components of lignocellulose is depicted in figure 2.1
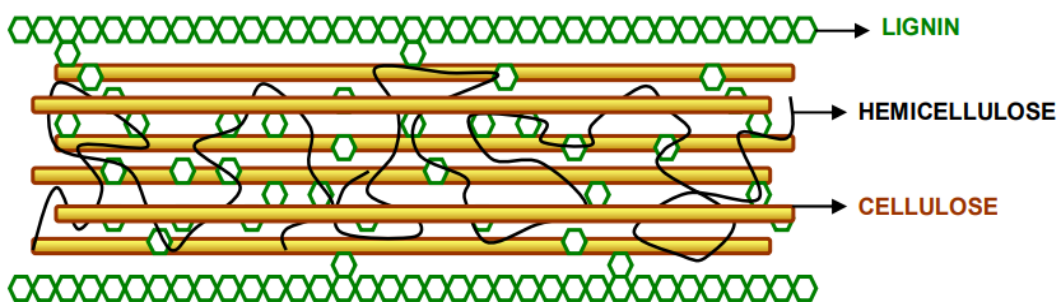
Figure 2. 1. Illustration of lignocellulosic structure (Mussatto and Teixeira, 2010).

Depending on the kind of biomass, different proportions of lignin and carbohydrate polymers are present. However, on average, cellulose makes up 35–50% of the biomass, followed by hemicellulose at 20–35% and lignin at 10–25% (Saha, 2003; Bhujbal *et al*., 2022). The ratios of different components inside a single plant can also change depending on factors including age, development stage, and environmental factors like location. However, in most lignocellulose biomass, cellulose predominates. The ratio of some biomass kinds' fractions varies. According to the kind of biomass, the principal components of lignocellulose wastes are compared in Table 2.1 (Mussatto and Teixeira, 2010).

Table 2. 1. Principal components of lignocellulose

Lignocellulose waste	Cellulose (weight %)	Hemicellulose (weight %)	Lignin (weight %)
Sugarcane bagasse	40.0	27.0	10.0
Rice straw	36.2	19.0	9.9
Wheat straw	32.9	24.0	8.9
Corn cobs	33.7	31.9	6.1
Cornstalk	35.0	16.8	7.0
Cotton stalks	58.5	14.4	21.5
Rye straw	37.6	30.5	19.0
Oat straw	39.4	27.1	17.5
Sunflower stalks	42.1	29.7	13.4
Soya stalks	34.5	24.8	19.8

2.3   Lignocellulosic Biomass Pretreatment Methods

Research shows sugars in lignocellulose materials have been liberated using a variety of techniques. They are collectively known as pre-treatment methods, also referred to as hydrolysis. Depending on the predominant portions of the biomass under consideration, we shall explore various hydrolysis techniques in this section. Lignocellulose pre-treatment is a crucial step in turning biomass into goods. By removing and degrading them, it removes the lignin and hemicellulose that were surrounding the cellulose and destroys the effect of crosslinking, revealing the cellulose crystal structure. The yield of subsequent hydrolysis and microbial fermentation is greatly increased as a result of the broad reaction area between the enzyme and the substrate (Klinke *et al*., 2004; Jaffur *et al*., 2021). The efficiency of numerous techniques for lignocellulose hydrolysis has been established. Due to the structural variations between these fractions, particular procedures that can be

physical, chemical, physicochemical, or biological must be used to separate cellulose, hemicellulose, and lignin from lignocellulose biomass.

Chemical and enzymatic procedures are typically used for cellulose hydrolysis. The chemical process uses mineral acids such as sulphuric (H_2SO_4) or hydrochloric (HCl) at concentrations of 10-30%, temperatures of around 160°C, and pressures of about 10 atm, mostly to conduct concentrated in acid hydrolysis. These extreme temperatures and acid concentrations are necessary for glucose release due to its crystalline structure. To prevent the breakdown of sucrose and lignin into undesirable by-products, this approach must be used carefully (Mussatton and Teixeira, 2010; Luo *et al.*, 2022).

Due to its selectivity, enzymatic hydrolysis is employed as an alternative to concentrated acid. To use less energy and have a smaller negative environmental impact, it can be done under gentler reaction conditions (pH around 5 and temperature around 50 °C). Additionally, hydrolysis tank corrosion issues are avoided, and a high yield of pure glucose is produced with reduced production of undesirable by-products for the hydrolysate's subsequent usage in fermentation operations (Lou *et al.*, 2022; Wen *et al.*, 2004; Liao *et al.*, 2005;). Cellulase enzymes, which are a mixture of many enzymes, are used to carry out the enzymatic hydrolysis of cellulose. At least three primary categories of enzymes are included in this mixture: (1) β-1-4-endoglucanase, which attacks low-crystallinity areas of cellulose fiber to produce free chain ends, and (2) β-1-4-exoglucanase or (3) β-glucosidase, which hydrolyzes cellobiose to create glucose, further degrades the molecule by removing cellobiose units from the free chain ends. To make the cellulose more accessible to the enzymes before the enzymatic hydrolysis, a pre-treatment step is typically carried out (Xu *et al.*, 2022).

Dilute acid pre-treatment is the major method used to degrade hemicellulose. This technique, which uses diluted acids (1–4% concentration) at moderate temperatures (120–160 °C), is effective for hemicellulose hydrolysis. Although H_2SO_4 is the most frequently used acid in hydrolysis, HCl, HNO_3, and H_3PO_4 are also used.

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Long hemicellulose chains are broken down into shorter-chain oligomers and sugar monomers by the acid. Less extreme *circumstances* are needed to release the sugar since hemicellulose is amorphous. In addition, this approach produces far fewer corrosion issues and lower degradation products in industrial hydrolysis tanks (Xu *et al.*, 2022).

Another common technique for hydrolyzing hemicellulose is a steam explosion. With this technique, the biomass is heated for a brief period (a few seconds to a few minutes) with high-pressure saturated steam (0.69-4.83 MPa, 160-260 °C). When the pressure is quickly dropped after the steam condenses under high pressure and wets the material, the substance experiences an explosive decompression (Carvalheiro *et al.*, 2008; Kumar *et al.*, 2009). Hemicellulose sugar release also uses autohydrolysis (hydrothermal). Although the process is comparable to a steam explosion, this does not include an explosion. In this procedure, acids produced by the hydrolysis of acetyl and uronic groups, which were initially present in hemicelluloses, are combined with compressed liquid hot water (200 °C; pressure greater than saturation point). This method has a high yield, produces few byproducts, and does not significantly solubilize lignin (Carvalheiro *et al.*, 2008; Xu *et al.*, 2022). It can hydrolyze hemicellulose in a matter of minutes.

Hemicellulose breakdown can also be accomplished by the use of commercial or microbial hemicellulase enzymes. Both xylanases and mannases are among the enzymes employed. Recent studies have revealed that the majority of commercial hemicellulases are produced by genetically modified *Trichoderma* or *Aspergillus* strains, taking advantage of biological treatments of low energy consumption, being highly specific, with no chemical requirement, and mild environmental conditions, preventing sugar degradation and producing high sugar yields (Muratto and Teixeira 2010; Xu *et al.*, 2022).

Alkaline treatments using NaOH, $Ca(OH)_2$, or ammonia, with NaOH being the most popular, ozonolysis (treatment with ozone), peroxide (alkaline solutions at

temperatures greater than 100 °C), and organic solvent treatments are some of the techniques used to destroy lignin. Detoxification is occasionally replaced by the biological approach, which is also used to degrade lignin for easier access to cellulose and hemicellulose. Fungi like *Phanerochaete chrysosporium*, *Trametes versicolor*, *Trametes hirsuta*, and *Bjerkandera adusta* are used for this. One of the major difficulties of adopting the biological technique, according is its slow rate. Additionally, cellulose and hemicellulose may be attacked by fungi (Sun *et al*., 2002). The biodegradation involves the use of three major enzymes, namely, lignin peroxidase, manganese peroxidase, and laccase (Muratto and Teixeira, 2010; Luo *et al*., 2020).

In a nutshell, the ideal treatment strategy and circumstances differ depending on the type of biomass that is being utilized as the raw material. It is significant to remember that the treatment strategy chosen has an impact on the expense and effectiveness of the next hydrolysis and fermentation phases. According to Hamelinck *et al*. (2005) and Muratto and Teixeira (2010), the ideal hydrolysis process gives a high yield of fermentable sugars while using less energy, chemicals, and equipment. It also prevents the loss of yielded sugars and the development of inhibitors to the ensuing fermentation and It uses less equipment, energy, and chemicals (Muratto and Teixeira 2010; Hamelink *et al*., 2005). However, the low-cost lignocellulosic pre-treatments frequently result in the formation of much or fewer inhibitors, such as phenols, furans, and organic acids, which may reduce the activities of the hydrolytic enzymes and, as a result, the effectiveness of enzymolysis, increasing the required quantities and the price of the enzymes (Guo *et al*., 2022). These inhibitory substances and their effects will be reviewed later in the following sections.

2.4 Pre-Treatment Inhibitory Compounds

Compounds created during the pre-treatment procedure are pre-treatment inhibitors. High levels of these inhibitory chemicals are particularly produced by

the less expensive pre-treatment techniques that guarantee an economically viable production process (Kurosawa *et al*., 2015).

The production of by-products occurs concurrently with the liberation of the monomeric sugars during the traditional pre-treatment and hydrolysis of lignocellulose with dilute acid. Low yields and productivity in the fermentation processes are caused by these by-products and some lignocellulosic compounds, which limit microbial metabolism (Klinke *et al*., 2004; Jaffur *et al*., 2021).

The biggest challenge facing the bioprocessing sector is the formation of these inhibitors. They can be divided into three categories: furans, phenols, and organic acids. Pentose and hexose sugars degrade during pre-treatment to produce furfural and 5-hydroxymethyl furfural (HMF), respectively, depending on the biomass composition and pre-treatment technique used. Sugars in the hemicellulose fraction degrade to produce weak acids like acetic acid. According to Klinke *et al*. (2004) and Vanmarck *et al*. (2021), lignin produces phenolic compounds such as benzoic acid, vanillic acid, ferulic acid, p-coumaric acid, 4-hydroxybenzoic acid, vanillin, and syringaldehyde.

Excess inhibitory compounds are also produced from other lignocellulose components due to additional degradation under exceptionally harsh pre-treatment conditions. According to Zhao *et al*. (2021), biomass composition affects the concentration and strength of these inhibitors' effects; for instance, hydrolysis of hemicelluloses results in a larger release of acetic acid potential.

Hemicellulose itself is a cellulose hydrolysis inhibitor in addition to the derived inhibitors. To prevent additional cellulose hydrolysis, the hemicellulose binds to the enzyme's active site and plugs it with cellulose (Kim, 2018).

2.5   Effect of Pre-Treatment Inhibitors on Fermentation Micro-organisms

Pre-treatment inhibitors have several effects on cell development. Since the biomass composition defines the inhibitory chemicals that can be found in a particular hydrolysate, the severity of the effect is dependent on both the microorganism in process and the kind of hydrolysate.

The effects of a few of these inhibitors for various hydrolysates have been thoroughly investigated. Depending on the quantity of each inhibitor and the response of the microorganism to it, the effect can be mixed or specific to a particular inhibitor.

The researchers examined the impact of nine typical inhibitors produced during biomass pre-treatment using the bacterium *Rhodococcus opacus*, as observed in a thorough study by Kurosawa *et al.* (2015). The results of this study demonstrated that the type of inhibitor varies and some of them are cited in table 2.2.

Table 2. 2. Inhibitory compounds based on biomass type and hydrolysis method

Biomass	Lignin (g/L)	Furfural (g/L)	HMF (g/L)	Acetic acid (g/L)	Pre-treatment method	Reference
Corn stover	2.9	0.016	0.007	0.329		Kurosawa *et al.*, 2015
Wheat straw	5.1	0.001	0.009	1.24		Kurosawa *et al.*, 2015
Hardwood	4.2	0.001	0.022	1.309		Kurosawa *et al.*, 2015
Hazelnut shell	0.15 (phenolics)	Not Found	0.0145	2.59	Dilute sulfuric acid	Uzuner and Cekmecelioglu, 2014

Furan derivatives damage DNA and interfere with glycolytic and fermentative enzymes required for basic metabolic pathways, protein crosslinks, and cell viability (Hadi *et al.*, 1989; Modig *et al.*, 2002). These effects render furan derivatives hazardous to cells. Additionally, the furan derivatives' strong hydrophobicity compromises membrane integrity, causing membrane leakage or interruption and, eventually, slowing down ATP synthesis and microbial growth rate (Zaldivar *et al.*, 1999).

By spiking larger amounts of each molecule in a concentration range relevant to commercial hydrolysates, the authors in the work of Vanmarck *et al.* (2021) examined the impact of inhibitors on five common lignocellulosic hydrolysates, including bagasse, maize cobs, and spruce. The most significant suppression was seen at concentrations of furfural that apply to industry, which led to a partial decrease in both D-glucose and D-xylose assimilation. The ethanol titer produced by strain MD4 in all of the hydrolysates under study was significantly lowered when either 3 or 6 g/L of furfural was added, ranging from 34 to 51% and 77 to 86%, respectively. Following this level of inhibition were 5-hydroxymethylfurfural, acetic acid, and formic acid, which often induced partial inhibition of D-xylose fermentation at industrially relevant concentrations (Vanmarck *et al.*, 2021).

Van der Maas and colleagues (2021) investigated the single and combined effect of lignocellulose inhibitors on *Bacillus subtilis* performance. The half-maximal inhibitory concentration (IC50) was investigated using the 10 most prevalent inhibitory chemicals in lignocellulosic hydrolysates at various concentrations. According to their findings, syringaldehyde was the most hazardous of the individual inhibitors examined at 0.1 g/L, and completely stopped the strain's growth. Concentrations greater than this led to a longer lag phase, which gave the impression that growth rates were constant. Even though the concentration of 5-HMF is low due to the low hexose in hemicellulose, furfural, and 5-HMF showed essentially identical effects, and the pre-treatment procedure used did not degrade a greater amount of hexose (Van der Maas *et al.*, 2021).

When Zhang *et al.* (2014) looked at the impact of furfural and 5-HMF on the rate of growth of *Bacillus coagulans* species, they discovered that furfural had a higher potent growth inhibitory effect than 5-HMF at concentrations below 3 g/L. However, the inhibition by 5- HMF was more severe than that of furfural at doses greater than that. Furthermore, 5-HMF suppressed *B. subtilis* NCCB 70064's development less significantly than furfural, according to Pereira *et al.* (2016). Additionally, even at a concentration of 2 g/L of 5-HMF, the researchers observed growth.

When phenols and furans were present in concentrations of 1 g/L in research by Kurosawa *et al.* (2015) on the impact of possible lignocellulose inhibitors on *Rhodococcus opacus* revealed more significant inhibitory effects from these compounds. Contrarily, organic acids barely affected the environment at concentrations up to 2 g L1. After two days of cultivation, it was shown that 51% of the microbial growth was inhibited by furfural and 43% by HMF. The maximum cell densities over four days of cultivation increased by 11% when 2.0 g/L acetic acid was added to the medium when one of the organic acids tested was introduced, indicating that the R. opacus strain may be able to use the organic acids as carbon sources.

Acetic acid, formic acid, levulinic acid, and benzoic acid are examples of weak acids that are poisonous to cells. However, the strength of their inhibitory actions is strongly influenced by the ratio of the acid's dissociated to undissociated forms, which is determined by pH and the pKa. There are numerous theories put out to explain why weak acids entering cells have an inhibitory impact. Mussatto and Roberto (2004) claim that because undissociated weak acids are liposoluble and have a neutral intracellular pH, they can diffuse over the plasma membrane and into the cytosol while dissociated weak acids reduce the pH of the cell.

The literature showed that acetic acid's inhibitory impact also varies on the concentration and the microorganism involved. According to Van der Maas' study (2021), *B. subtilis* was not significantly inhibited by an acetic concentration of 0.75

g/L in terms of the lag phase or maximum growth rate. The authors hypothesized that a biphasic growth pattern was exhibited by *B. subtilis* when acetic acid is present in the medium since an inhibitory effect was visible at this concentration when the growth curve was studied. After 48 hours, growth at a rate of 29% of the control occurred at a concentration of 2 g/L acetic acid.

Although inhibitors harm the growth of fermenting strains, other studies have shown varied results when dealing with various microbes. For instance, *Pichia stipitis* exhibits low sensitivity to furfural. In one study, *Pichia stipitis* was used to produce ethanol from sugar cane bagasse hydrolysate. Furfural concentrations below 0.5 g/L caused the strain to respond positively in terms of growth rate (Roberto *et al.*, 1991).

To make methyl propionate, *S. cerevisiae* IMS0351, wild-type *Escherichia coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* were the three microorganisms that were examined by Pereira *et al.* in 2016. For their investigation, the authors employed both shake flasks and microtiter plates. However, technological issues highlighted the microtiter plates' restricted application. A lag-time model was used to describe the microbial growth, and product-inhibition models were used to calculate the inhibitory thresholds. Liu *et al.* (2021) looked into the inhibitory effects of furfural, 5-hydroxymethylfurfural (5- HMF), and acetic acid. Both the wild-type and a developed strain of *Rhodosporidium toruloides* were used in the tests. Acetic acid and levulinic acid were less hazardous at the same time. Results showed that furfural, followed by vanillin and 5-HMF, was the mixture's most effective inhibitor of *R. torulose's* proliferation. This yeast was most severely inhibited by the mixture of furfural, 5-HMF, vanillin, vanillic acid, and ferulic acid. The evolved strain demonstrated greater toleration of hazardous substances, indicating that adaptive laboratory evolution may be a viable method for obtaining oleaginous yeasts with enhanced growth in biomass hydrolysates.

2.6   Detoxification Methods for Pre-treatment Inhibitors

In a bioprocess, detoxification refers to the elimination of breakdown products formed during biomass pre-treatment. Traditional hydrolysis techniques aid the conversion of lignocellulosic material to sugars, but they also contain some inhibitors, primarily organic acids, aldehydes, phenols, and other chemicals. They could hinder the growth of fermentation microorganisms and subsequent saccharification, lowering the amount of lignocellulose that is bio-converted. Therefore, it is essential to use efficient detoxification techniques. However, the detoxification procedure is typically expensive and energy-intensive.

However, many detoxification techniques are being used. Physical, chemical, and biological processes are the categories into which they fall (Klinke *et al.*, 2004; Kim, 2018; Robak and Balcerek, 2020; Guo *et al.*, 2022). It is important to keep in mind that some detoxification processes cause substantial sugar loss.

In the physical procedures, inhibitors are removed from the hydrolysate using techniques like adsorption, filtration, and extraction. Activated charcoal, high-temperature carbon, and polyethyleneimine are a few examples of adsorbent materials (Luo *et al.*, 2022). Their surface area, pore size, chemical characteristics, and the detoxifying environment all affect the adsorbing strength (Guo *et al.*, 2022). Laminar double hydroxides were used in another study to get rid of mild acid inhibitors (Tramontina *et al.*, 2020). The inhibitor is extracted from the fermentation medium using solvents such as chloroform, hexane, and ethyl acetate. (Luo *et al.* (2022); Guo *et al.* (2022).

In chemical processes, inhibitors are removed through a transformation into a less damaging form. For example, the addition of base Ca(OH)2, also known as over-liming, is a typical chemical detoxification approach (Guo *et al.*, 2022). Bases and reducing agents like ammonia or hydroxide are typically the chemicals utilized for detoxification in this method (Kordala *et al.*, 2021). Generally speaking, reactions involving reducing agents are simple to carry out, and sugar loss is small, which

results in an improved fermentation performance (Luo *et al*., 2022). According to Jiang *et al*. (2021), who employed the electrochemical detoxification approach to remove up to 40% of the phenolic contents from fermentation media, several researchers have used the electrochemical method to clean fermentation media. Amazingly, neither byproduct was generated, and no sugar loss was recorded (Jiang *et al*., 2021).

Using a variety of techniques, including feedstock selection and processing protocol, biocontrol, microbial selection, and detoxification, among others, the production of inhibitors and their unfavorable consequences are combated through biological methods. The feedstock selection technique selects easy-to-manage substrates that help prevent the creation of inhibitors. For instance, after pre-treatment, the low-resistance plant species may only create a little amount of inhibitors. The feedstock processing strategy, on the other hand, reduces the amount of inhibitors in the feedstock. For instance, processing biomass at low temperatures frequently results in the production of a small number of inhibitors (Rajendran *et al*., 2018; Maitra and Singh, 2021; Guo *et al*., 2022).

According to a study by Kim (2018), *Xylotria* NRRL30616 was found to have a high tolerance to inhibitors and could metabolize furans and acetate primarily as a source of carbon and energy. Other specific microorganisms can also go through adaptive evolution that enables them to be highly tolerant to inhibitors. Another study demonstrates that the recently identified *Bacillus coagulans* strain Azu-10 was capable of producing high levels of homofermentative lactic acid, high levels of biomass, and total xylose consumption while being able to resist inhibitors such as furans (Abdel-Rahman *et al*., 2021).

Instead of removing them from the fermentation media, many researchers attempt to physiologically manage the production of inhibitors by transforming them into desirable products. For instance, according to Sun *et al*. (2022), chitosan-chitin hybrid hydrogel beads can remove HMF while keeping glucose and xylose in the hydrolysate. To minimize the inhibitory effect and promote the creation of the final

product, simultaneous saccharification and fermentation without detoxification was used. This method avoids inhibitor development and reduces the cost of enzymes by allowing hydrolysis and fermentation to take place simultaneously in one bioreactor (Mesa *et al.*, 2017; Moreno *et al.*, 2015). It was also discovered that enzymes can detoxify hydrolysate media without consuming sugars; the most popular enzymes for this purpose are laccase and peroxidase, which come from several white-rot bacteria (Moreno *et al.*, 2015).

Furans and phenolics were dramatically reduced from 106 mg/ml to 1.1 mg/ml by this finest treatment. Unfortunately, the best sugar loss among all detoxification techniques used in the study occurred between 49 g/L and 26 g/L, even though all other detoxification techniques also caused significant sugar loss, which occurred after the addition of lime and an increase in pH, with dramatic loss above pH 9. Additionally, the researchers in this study noted that the number of treatments—that is, using charcoal before and after over-liming treatment—and the temperature of the treatment all affect the effectiveness of charcoal treatment in terms of removing furans and phenolics. Additionally, charcoal was more efficient than ethyl acetate extraction (Arslan and Saracioglu, 2010).

The biological approach to detoxifying lignocellulosic hydrolysate appears to be the most promising one. A few issues that require more research include lowering the cost of biological detoxification and reducing the accumulation of inhibitors (Guo *et al.*, 2022; Carpita and McCann, 2020; Espro *et al.*, 2018). Following this, a promising approach to solve this issue is an evolutionary adaptation, typically with straightforward batch fermentation in a shake flask to adapt microorganisms to these inhibitors. In this approach, an experiment can be run continuously to develop a fully adapted strain that can grow and ferment in the hydrolysate with a maximum growth rate and production of the desired product. The following section reviews the current state of literature in ALE.

2.7 Adaptive Laboratory Evolution (ALE)

Adaptive laboratory evolution also known as evolutionary adaptation is a type of controlled evolution, where scientists and researchers grow microorganisms in a controlled environment to enhance a particular trait of interest. In other words, ALE is a creative method for microbial strains to evolve desired features by using the Darwinian Theory's natural selection process (Sandberge *et al.*, 2019).Although it may seem innovative, this method has been employed for many years by several researchers to improve other aspects of commonly used microbes in biotechnology.

ALE experiments are effective methods for learning more about the evolutionary factors that affect a strain's phenotypic performance and stability as well as for creating new strains with beneficial mutations. The production of goods with a high added value, such as enzymes, ethanol, butanol, lipids, etc., is a significant area that ALE addresses. It has been used to boost the efficiency of bacteria, fungi, and microalgae in biotechnological processes and examine the genetic underpinnings of evolution. Thus, ALE is an effective and promising technique that has already enabled the biotech industry to acquire new, advantageous microbiological strains and may yield even more promising outcomes in the future (Mavromati *et al.*, 2022).

This technology is expanding due to the improvements in genetic engineering and bioinformatics, in addition to cheap and effective systematic DNA sequencing tools. Since ALE replaces rational design or genome engineering, which engineers organisms based on knowledge of their phenotype-genotype mapping rather than evolving them, evolution relies exclusively on natural selection to produce evolved mutants. Because of this, and because the biological system is so complicated, it is impossible to infer such modifications without harming the organism (Sandberg *et al.*, 2019).

Although their prominence as model organisms, bacteria or yeast, particularly *E. coli* and *S. cerevisiae*, is used in the majority of reported ALE studies, any organism cell that is culturable in a lab setting can be subjected to ALE. This, in addition to their rapid growth and genetic tractability, is supplying the requisite knowledge foundation, which can be used to analyze evolutionary consequences. It's possible that utilizing diverse microorganisms, like algae or yeast, won't produce the same phenotypic results as ALE performed using a single organism, like bacteria. As will be reviewed in the following section, there are numerous methods for carrying out ALE in the literature.

2.7.1 Different ALE Methodology

Numerous researchers have investigated various ALE experiment approaches to hasten adaptation, boost mutation rates, and preserve advantageous mutations. Serial batch culturing and non-batch cultures utilizing chemostats and turbidostats are the accepted techniques. In serial batch culture, ALE is carried out in shake flasks. Technically, the procedure is carried out by inoculating the desired microbial cells to a flask of liquid medium and cultivating them under a desired pressure, following the transfer of the aliquot culture into a fresh medium. The same procedure continues for the desired number of generations while the selective pressure increases or remains constant, in most cases, during the experiment. The transfer happens while the cells are in the exponential stage of their growth phase and with sufficient nutrients. Without limited resources or shifts in the growth stage between the lag, log, and stationary, improvements to the maximum growth rate determines the strains that took over the population (Zhu *et al*., 2018; Mavromati *et al*., 2022).

However, in this method, if the phase of stagnation is to be prevented, the researcher must alter the passage frequency or propagation volume employed as the growth rate through the ALE process increases (Charusanti *et al*., 2010). Without automation, raising passage frequency might be difficult, and lowering passage volume can result in the possibility of losing the adaptive mutations and tighter

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bottlenecks in the population. Due to these problems, a lot of batch culture studies choose to use predetermined amounts at fixed intervals, typically once per day (Sandberg *et al.*, 2019).

This approach has certain drawbacks while being simple to use in comparison to how effective it is. First, as it is typically performed by one person, it is prone to inadvertent mistakes. Second, handling various microbial populations at once can lead to cross-contamination. Finally, if it takes a while to achieve the desired stability, the daily sampling and transfer may become tiresome. Recent research that uses automation is described to reduce the time to go around this problem (Mavromati *et al.*, 2022).

Chemostats and occasionally turbidostarts are frequently used in non-batch ALE culturing experimental settings. Chemostats allow for precise control of environmental factors like pH and oxygenation while cultivating cells in a bioreactor. According to Gresham and Dunham (2014), steady media inflow at a set dilution rate maintains continuous growth. Although this approach may promote unfavorable adaptation processes, such as bacterial persistence due to sticky wall expansion, it can be utilized to select phenotypes. It can be difficult to maintain several replicas simultaneously (Rao and Rao, 2004).

ALE investigations occasionally include additional culturing techniques for particular objectives, such as long-term culturing in a flask to select a growth benefit in the stationary phase or to enable spatiotemporal tracking of competing lineages when growing on a sizable petri dish with antibiotic gradients. It is important to remember that serial propagation of colonies streaked onto plates under clonal bottlenecks at each step is not a form of "adaptive" evolution, without some secondary screening measure influencing the transfer process, but can be used for studies on the accumulation of mutation (Sandberg *et al.*, 2019).

In the current investigation, *B. subtilis* was modified using the serial batch culture technique to promote growth on hazelnut shells that had previously been treated

with diluted acid. The literature lists many justifications for studying microbial evolution.

2.7.2 Purposes of ALE

For biotechnological purposes, many researchers have used microbial ALE to obtain enhanced microbial strains. Optimizing growth rate, tolerance increase, utilization of substrate, product yield/titer increase, and general discovery are generally the different application areas of adaptive laboratory evolution. These areas involve studies covering various topics related to industrial biotechnology and can be investigated with ALE. Systems biology, evolutionary modeling, and genomic dynamics are a few examples of such topics (Sandberg *et al*., 2019).

The vast majority of the time, researchers chose to increase the ability of microorganisms to withstand particular stresses that result in large concentrations of interesting metabolic products, such as ethanol, butanol-producing enzymes, carotenoids, lactic acid, or lipids (Mavrommati *et al*., 2022). This is because many bio-renewables are hazardous to the viability and general productivity of microorganisms. In addition to the fact that adequate nutrient uptake is mostly related to metabolite biosynthesis, there are several instances where microbes have been created and developed to use inaccessible substrates or synthesize non-native metabolites utilizing the ALE method (Sandberg *et al*., 2019).

Many researchers use ALE to optimize the growth rates of different species of microorganisms with commercial relevance. The growth rate is typically the primary fitness-determining factor in ALE experiments, even though "fitness" is frequently a complicated feature with other contributing elements (Vasi *et al*., 1994). ALE has been used to maximize the development of particular microbial species of interest even in well-known culturing settings where growth is anticipated to be close to the maximum. For instance, in a less than one-month ALE experiment, growth rate gains of up to 60% were made for the widely used lab strain and engineering chassis *E. coli* K-12 MG1655 (LaCroix *et al*., 2014). To

correct the growth flaws in designed strains, additional research, such as that of Radek *et al*. (2017), is conducted.

The objective of certain investigations is to determine how and why the growth rate varies during evolution. One such work was reported by Lenski *et al*. (2015). Other examples of growth enhancement studies employing the ALE technique include the work of Heer and Sauer (2008), Landeta *et al*. (2013), and Gu *et al*. (2014).

Substratum usage and tolerance are direct routes to phenotypic changes in ALEs because growth rate gains are fundamentally chosen for. Desired phenotypes, such as the overproduction of metabolites, can improve cell fitness and stop evolution from favoring such features. In these situations, it is necessary to modify the parent strain's environment and genome to link the desired quality to a growth advantage, for instance through metabolic growth coupling or by utilizing a growth environment, in which the production of otherwise wasteful metabolites is now advantageous (Sandberg *et al*., 2019).

Yu *et al*. (2013) conducted an ALE experiment on three lipid-producing microalgal strains of *Chlamydomonas reinhardtii* to increase the growth rate (biomass concentration) and lipid production, despite the fact that the results of ALE achieved by algae cannot be the same as those with bacteria or yeast. The endpoint strains' biomass concentrations were 1.17, 1.33, and 1.48 times higher than those of the starting strains thanks to an increase in growth rate of 17–48%. In a similar manner, lipid production considerably increased by 2.36 times over the initial strain (Yu *et al*., 2013).

In a different investigation, Patyshakuliyeva *et al*., (2016) used the fungus *Aspergillus niger* N402 to undertake adaptation trials. They cultivated them on cellulose media for selected mutation because this fungus did not grow well on it. They acquired the modified strains, which were then applied to the manufacturing of cellulase enzymes. The modified mutant showed an enhanced growth rate after

the adaptation. Additionally, the most successful colonies, which were regarded as developed, displayed a roughly five-fold increase in cellulase production in comparison to the original strain. The gene responsible for the improvement was identified by additional genetic analysis (Patyshakuliyeva *et al*., 2016), but this is outside the scope of our work.

2.7.3   Adaptation of Microorganisms to Hydrolysate Inhibitors

The pre-treatment of lignocellulose biomass for the release of soluble sugars results in the formation of certain chemicals that, as was previously discussed, limit the growth and fermentation of microorganisms. Researchers have experimented with several approaches to get around this inhibitory effect, such as genetic engineering to create strains that are suited. But this approach is insufficient to address the issue. Utilizing the detoxification procedure to get rid of these inhibitors is another recent breakthrough. Unfortunately, detoxification makes this strategy ineffective as well because considerable amounts of sugar and the inhibitors are lost. According to certain research, adaptive evolution can produce strains that are resistant to inhibitors more efficiently than genetic engineering and less expensive than detoxification (Kurosawa *et al*., 2015).

The literature demonstrates how to adapt microbes to various lignocellulose inhibitors to produce a variety of bio-based products, such as lipids, ethanol, enzymes, and lactic acids. *Saccharomyces cerevisiae* is used in the majority of hydrolysate adaptations, while some research also uses *Bacillus subtilis*. An excellent example is a study by Khushk *et al*., (2021), where the authors used wheat straw hydrolysate to treat *S. cerevisiae* Angel for ethanol fermentation, *Pediococcus* sp, *Lactobacillus* sp, and *Bacillus coagulans* for lactic acid production to long-term adaptation. Enzymatic hydrolysis of recently pre-treated wheat straw with a loading of 15% (w/w) solids produced the hydrolysate. The modified strains performed exceptionally well during simultaneous saccharification and fermentation when grown on freshly diluted acid-pretreated wheat straw hydrolysate without detoxification. When the strains were modified for the

hydrolysate, the on-site cellulase produced was identical to the commercial cellulase in saccharification yield. By removing pre-treatment inhibitors, this approach decreased the cost of detoxification and the volume of freshwater needed (Khushk *et al*., 2021).

Long-term adaptive evolution of the *S. cerevisiae* DQ1 yeast strain was also carried out by Qureshi *et al*. (2015) with acorn stover hydrolysate treated in dry dilute sulphuric acid pre-treated (DDAP). A stable strain was produced and used in the simultaneous saccharification and fermentation at high solid content after 130 transfers of 65 days, or 780 generations of cell growth. The results showed a high ethanol titer of 71.40 g/L and a yield of 80.34%. The DDAP pre-treatment and bio-detoxification technologies were also used to achieve the lowest possible water usage and wastewater generation (Qureshi *et al*., 2015).

Additionally, ALE was employed to enhance the outcomes of the microorganism's genetic alterations. Given that the acquired evolved strains have increased tolerance to the applied stresses and cross-resistance to other pressures that limit their development and yield, the majority of experiments have produced promising findings (Mavrommati *et al*., 2022).

ALE is a continuously expanding microbiological technique that an increasing number of researchers are using due to the success of the experiments already conducted as well as the reasonably straightforward procedure that doesn't require any prior knowledge of the genes involved. There is still much that has to be done to improve the entire process, such as gaining a deeper comprehension of the mechanics governing evolution, simplifying the experiment as a whole (perhaps through automation), and utilizing a variety of microorganisms. For ALE to be more useful as a biotechnological tool, the industry should adopt it through more thorough and focused experiments. Mavrommati *et al*., 2022).

A particular microbial strain's ability to adapt depends on the environment in which it is employed. Because the nature and concentration of inhibitors differ, a good-

matched strain with better fermentation ability in a particular hydrolysate might not be suitable for another hydrolysate if different raw biomass, pre-treatment techniques, conditioning (detoxification), or hydrolysis conditions are involved (Qureshi *et al*., 2015). In addition, the amount of time that populations of strains have to evolve is a crucial factor in an ALE experiment. For studies on growth enhancement, the fold is more frequently employed than generation as the indicator for evolutionary time. Although far shorter (less than 50 generations) and longer (more than 60,000 generations) times have also been reported, the majority of ALE research have a growth period of 100–500 generations. Although developed strains are termed 'endpoints,' this does not mean that additional fitness increases cannot be obtained with a longer experiment period. The decision to terminate an adaptive evolution experiment after a set number of generations is always in some way arbitrary.

Although fitness gains can be made endlessly, it's crucial to strike a balance between getting the right endpoint and not wasting time and effort running long experiments with no real-world implications. For instance, Hua *et al*.(2007) discovered fitness increases of approximately 60% with less than 250 generations of ALE when they evolved *E. coli* onto lactate growth. Even yet, extending the experiment's runtime to 900 generations only led to a modest fitness improvement of around 20% (Hua *et al*., 2007).

Even though the result of experimental evolution is extremely unpredictable, it solely relies on a few key variables, including the organisms used, their mode of sexual or asexual reproduction, the selective pressure used, and the length of the experiment (Mavromati *et al*., 2022). Because of this, the current study is focused on investigating various cultivation settings to implement the adaptation utilizing just eight transfers.

2.8 Hazelnut Shells

2.8.1 Abundance and Composition

Hazelnut (*Corylus avellana* L.) is a popular edible plant from the Betulaceae family. It is also referred to as filberts. They are known for their excellent nutritional content, which varies according to geographical region. The plant is usually grown for the inside kernel. According to Koksal *et al.* (2006), hazelnut kernels contain 10–22% carbohydrates, 10–24% protein, and 50–73% lipid content. Turkey produces the more significant percentage of hazelnut worldwide, with more than 60% of the overall production globally, followed by Italy, Spain, and the USA. According to the Turkish Ministry of Agriculture and Forestry, a survey conducted in the critical growing regions showed hazelnut production forecast for 2022/23, pegged the size of the crop at 765,287 tonnes, with an annual increase of 12% (Kocabaş *et al.*, 2022)

This emphasizes the importance of hazelnuts to Turkey's economy, accounting for 12% of its foreign trade earnings (Anonymous, 2001).

Hazelnut fruit has a smooth, hard shell. The seed is covered by dark brown skin (Contini *et al.*, 2007). The shell is about 44.5% of the whole hazelnut, and the skin represents about 2.5%. Since hazelnuts are mainly grown commercially for their seeds, which are generally consumed preferably roasted (without skin, usually removed by roasting) or sometimes raw, with skin (Alasalvar, 2009), the need for these seeds continues as they are also utilized by many industries such as chocolate and pastry, confectionery, and bakery, among others. In addition, due to the high contents of unsaturated fatty acids in hazelnut oil and the high similarity of it to olive oil, it is also precious for food industries (Benitez-Sánchez *et al.*, 2003). As a result of this enormous application, hazelnut shells are produced in abundance after industrial processing. Around 250,000–600,000 tons are produced yearly, as reported by Uzuner and Cekmecelioglu (2014).

These shells are composed of carbon 50.8, hydrogen 5.2, nitrogen 1.4, and oxygen 42.6 on a dry weight basis (Demirbas, 2002). In the chemical composition study of hazelnut shells, although a bit of variation exists among the varieties, the predominant component in the shells of all hazelnut varieties was total carbohydrate, which ranged from 93.4% to 96.7%, with crude fiber accounting for over 85% of the entire composition. Protein was the second most crucial component in the shells (2.1-4.0%), followed by ash (0.8-2.0%) and oil (0.3-0.7%) (Xu *et al.*, 2012)

The hazelnut shells' richness in phenolic compounds has been published in various works of literature, although the amount of extracted compounds depends on both the hazelnut variety and the solvent used during the extraction process (Shahidi *et al.*, 2007; Contini *et al.*, 2008)

The extraction yield and phenolic content varied depending on the by-product and solvent utilized, according to the study by Contini *et al.* (2008), who used three solvent systems to extract the phenolic components in hazelnut shells and skin waste. Among the materials examined, the shell of whole roasted hazelnuts produced extracts with the greatest phenolic contents, up to 502 mg/g, and exceptionally high extraction yields of roughly 30%. The maximum antioxidant activity was found in extracts from the skin of whole roasted hazelnuts, making them the most promising when it comes to potential industrial applications (Contini *et al.*, 2007; Contini *et al.*, 2008).

Based on the study carried out by Xu *et al.* (2012), the authors investigated the chemical composition of hazelnut shells grown by United States cultivars and the antioxidant activity of the phenolic extracts from hazelnut shells. They concluded that due to the unique structure of these phenolic compounds, they possess antioxidant activity. The researchers of this study also reported that hazelnut cultivars grown in Europe are usually of higher quality than those produced in the United States (Xu *et al.*, 2012).

Considering these valuable components present in this important residue, researchers are putting efforts to recycle this, including the bioprocess industries, because studies show that hazelnut shells contain significant amounts of fermentable sugars (Uzuner and Cekmecelioglu, (2014); Uzuner, 2023), which different microorganisms can utilize to produce value-added products such as enzymes, ethanol, and vitamins among others, as we will see in the following section how scientists and engineers are putting the so-called waste into beneficial usage.

2.8.2   Utilization of Hazelnut Shells

Although the hazelnut is grown as a fruit mainly for its kernel, large quantities of hazelnut shells are produced during processing (Puliga *et al*., 2022). Therefore, hazelnut shells are a significant waste of the hazelnut industries (Xu *et al*., 2012). In this regard, in the Black Sea region of Turkey and other hazelnut-producing areas around the globe, hazelnut shells are mainly used for domestic purposes by direct combustion as a source of energy to heat houses. Researchers began to explore more ways of using these shells in different research fields. With the rise in the price of heating and energy, the advantage of insulation in buildings has increased tremendously. New searches are being sought to reduce the cost of energy and heating for this purpose, as seen in one study carried out by Demirer *et al*., (2018), in which the researchers were trying to find a lightweight insulating material. They considered the cellulosic structure of hazelnut shells and explored their potentiality in the construction, and they concluded that the shells could be utilized as a filler material in plastic composites (Demirer *et al*., 2018; Peluga *et al*., 2022). As the need for thermal insulation in buildings has grown and new methods to do it are being explored, the quest has increased. As a result of this study, research has been done to enhance the insulating capabilities of concrete by producing lightweight concrete. Additionally, the authors of the study by Zocak and Işman (2021) looked into the possibility of using hazelnut shells as aggregate in concrete. The aggregate in the concrete was replaced to varying degrees with hazelnut shells by the researchers. Hazelnut shells could be utilized as aggregate in

the creation of concrete, and lightweight concrete with strength and durability as well as better heat and sound insulation was discovered when the physical, mechanical, and thermal qualities of this concrete were investigated.

Puliga *et al*. (2022) viewed the value-added use of hazelnut shells as a substrate for mushroom development as an environmentally benign alternative to the traditional use of such agricultural waste by-products. The findings of their investigation confirmed the hazelnut shells' economic viability as a substrate or additive for mushroom development to produce high-quality food. Hazelnut shells are an excellent substrate for growing mushrooms due to their high lignin content, which allows for more optimization of this agricultural waste byproduct and, as a result, increases the profitability of mushroom farms. By using hazelnut shell hydrolysate as a fermentation medium, Oktay *et al*. (2022) also tried to use a domestic strain of *Aureobasidium pullulans* AZ-6 to produce pullulan. This study examined the use of agroindustrial wastes as fermentation media, including the hydrolysate of hazelnut shells, without adding any additional nutrients to the process of *Aureobasidium pullulans* AZ-6 producing pullulan. The outcome showed that the hydrolysate from hazelnut shells had the second-highest concentration of pullulan among all the tested media, corresponding to 30.02 gL−1. The study was the first to report the use of hazelnut shells for pullulan production without adding minerals to the fermentation media (Oktay *et al*., 2022).

Gram-positive aerobic spore-forming soil bacterium *Bacillus subtilis* is frequently employed as a key model bacterium for research on physiology and metabolism. Other habitats where it can flourish include the gastrointestinal tracts of animals and plant roots. Additionally, this bacteria is employed in agriculture as a feed additive and in medicine as a way to prepare vaccines. According to Harirchi *et al*. (2022), the scientific taxonomical hierarchy is displayed in Table 2.3.

Table 2. 3. Taxonomical Classification of *Bacillus subtilis*

Taxonomical hierarchy	Specification
Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	Bacillus
Species	subtilis

As the leading model bacterial species of gram-positive, *B. subtilis* has a wide array of mature genetic tools, promoters, and plasmid expression systems used in metabolic engineering and synthetic biology (Su *et al*., 2020). It has a single-cell membrane that facilitates high protein secretion, simplifying downstream processing and reducing the process costs. Moreover, it is also an appropriate model for studying biofilm formation and other physiological characteristics (Earl *et al*., 2008; Kovács & Dragoš, 2019). As a result, this bacterium has been widely used as a cell factory to microbially produce several chemicals, enzymes, and other industrial products.

Enzymes are biological catalysts used in a variety of industrial settings, including the pharmaceutical, animal feed, detergent, textile, leather, and paper industries (Van *et al*., 2013). Because of their ability to grow quickly on economical substrates, their capacity for robust protein secretion, and their non-pathogenicity, *Bacillus subtilis* has been used to generate several enzymes. According to data compiled by Schallmey *et al*. (2004), enzymes secreted by *B. subtilis* currently account for 50% of the market for enzymes (Schallmey, 2004). From 2022 to 2027, the global enzyme market is anticipated to increase from 12.1 to 16.2 billion USD. Amylases, xylanases, lichenase, -galactosidase, cellulases (Deka *et al*., 2011), pectinases (Yu *et al*., 2017), and alkaline serine proteases (Wang *et al*., 2016) are just a few of the enzymes that *B. subtilis* have effectively expressed. Proteases from

B. subtilis are used in many food applications due to their GRAS (Generally Recognized as Safe) classification, including the manufacture of soybean and casein hydrolysates, milk coagulation, meat tenderization, and the treatment of food waste (Patel *et al.*, 2019).

B. subtilis is frequently used as a microbial addition in agriculture to enhance animal intestinal function, stimulate animal growth, and guard against disease. In the upper intestinal tract, it can be formed as endospores that release highly active proteases, lipases, and amylases that aid in the breakdown of complex carbohydrates in plant feed (Lee *et al.*, 2019). Additionally, its polypeptides significantly increase feed digestibility by acting as an antagonist against gut infections. Additionally, it is applied in water bioremediation to guard against illnesses in fish and shrimp raised in captivity (Su *et al.*, 2020).

B. subtilis has significant potential in both biomedical engineering and medicine. For instance, according to Corvey *et al.* (2003), it can secrete a range of low-molecular-weight antimicrobial peptides and bacteriocins, including subtilin, bacilysin, and surfactin. These peptides have broad and quick-killing effects against a variety of diseases, making them intriguing therapeutic agents. Antimicrobial peptides will also become more important in the management of bacterial infections as a result of the rise in microbial resistance issues brought on by the irrational use of conventional antibiotics (Sumi *et al.*, 2015). Antimicrobial peptides have the benefit of being both secure and sustainable. Additionally, because B. subtilis is non-pathogenic and aerobic, it contributes to the anaerobic environment in the intestines by absorbing oxygen, which in turn stimulates the replication of the intestinal bacteria and preserves the ecological balance of the intestine (Su *et al.*, 2020).

2.9   Objective of Study

Hazelnut shells are produced in abundance as a significant waste material of the hazelnut industries. Burning them for domestic purposes or disposing of these

wastes in the soil presents severe environmental pollution and loss of valuable constituents. Therefore, the reuse of these shells is of great interest. Since these shells are rich in polysaccharides, which can be converted into a form microorganisms can quickly assimilate, they are suitable for use as raw materials in fermentation processes that result in industrially relevant products.

In this study, hazelnut shells were pre-treated using dilute sulphuric acid to hydrolyze hemicellulose fraction without any delignification step. Although, as seen above, a combination of two treatments gives a better yield of fermentable sugar released, when research on the hazelnut shell was carried out previously by Uzuner and Cekmecelioglu (2014), their outcome showed that after 3.42% (w/w) dilute acid pre-treatment was carried out at 130°C for 31.7 min and 200U/g enzyme load for 24 hours, ideal sugar concentration of around 19.2 g/L was released. While 16.65 g/l of reducing sugar was the ideal concentration, for the single treatment of dilute sulfuric acid, this showed no significant difference in the sugar released during thermo-chemical treatment and enzyme assisted. Therefore, this study will only use thermo-chemical (dilute sulphuric acid) pre-treatment.

Like other lignocellulosic hydrolysates, the conversion of the hazelnut shells into fermentable sugars results in the formation of growth-inhibitory substances. Some studies show hydrolysate detoxification presents better fermentation performance, while biological detoxification seems more promising among all the detoxification methods. Different microorganisms prove to adapt to different biomass hydrolysate for the production of valuable products (Kurosawa *et al.*, 2015, Gu *et al.*, 2014; Bachmann *et al.*, 2014; Qureshi *et al.*, 2015; Khushk *et al.*, 2021) as a means to alleviate the effect of these inhibitors.

Although *B. subtilis* has numerous applications which are attractive in the field of scientific study and industries, very little is known about its fermentation performance in the presence of lignocellulose-derived inhibitors, particularly hazelnut shells hydrolysate. Therefore, this study investigated the potential of this

bacterial strain for adaptive laboratory evolution in the presence of hazelnut shells hydrolysate under different fermentation conditions.

Although there are studies in the literature on adaptive laboratory evolution utilizing *B. subtilis,* such as the evolution of biofilm formation (Kovács, & Dragoš. 2019) and enhanced growth at low pressure (Fajardo-Cavazos *et al.*, 2012), in more recent studies, only a few adaptation experiments were carried out to produce metabolites (ethanol and lactic acid) coupled with growth optimization (Khushk *et al.*, 2021), and these studies were carried out for long-term evolution, for the evolvement of new microbial strain. More studies need to be carried out in this field for further understanding of microorganisms' response to the inhibitors. This study is the first that utilizes hazelnut shells hydrolysate for the adaptive laboratory evolution of *Bacillus subtilis* NRRL B-4219 for growth rate optimization and lignocellulosic sugar utilization.

Accordingly, this study examined the short-term adaptation (eight daily transfers of cultivated culture into fresh media) with three different pH levels (5, 6, and 7) that are mostly reported as the best pH for growing *Bacillus subtilis* and three different inoculum ratios (2, 5, and 10%). Temperature and agitation speed were constant at 37 °C and 130 rpm, respectively.

CHAPTER 3

MATERIALS AND METHODS

3.1   Materials

Hazelnut shells used in this experiment were kindly provided and shipped by the hazelnut company located in Duzce, a province of Turkey.

Bacillus subtilis NRRL B-4219 was previously obtained from the ARS culture collection, Northern Regional Research Laboratory (NRRL), Peoria, Illinois, United States. The stock culture was prepared using 50% glycerol and stored in Eppendorf tubes at -80°Cfor long-term usage.

3.1.1   Chemicals

The chemicals used in this study to pre-treat the biomass (hazelnut shell powder), prepare the growth media, and carry out the sugar analysis are analytical grade and are listed in Table A.1, in Appendix A.

3.1.2   Reagents

DNS solution was used to measure the residual sugar concentration of samples.

The solution was freshly prepared for every analysis because it is light-sensitive. The amount to be prepared depends on the number of samples to be analyzed for reducing sugar.

For this study, 25mL of DNS solution was usually prepared by dissolving sodium hydroxide (0.25 g), 3, 5-dinitro salicylic acid (0.25 g), phenol (0.05 g), sodium sulfite (0.025 g), and Rochelle salt (6.7 g).

3.1.3   Preparation of Growth Media

The following chemicals were dissolved in water as a growth medium for bacterial inoculum preparation and activation: K_2HPO_4, 0.4g/L; KH_2PO_4, 0.2 g/L; $MgSO4.7H2O$, 0.4 g/L; citrus from pectin peel, 2g/L; glucose10 g/L and yeast extract, 10g/L (Kapok and Chad, 2002). The mixture was prepared in a 250mL Erlenmeyer flask and dissolved in deionized water for a 50mL growth medium (1:5 ratios of media to volume). The pH was adjusted to the required value using 10 M NaOH and 10 M H_2SO_4 and sterilized at 121 °C for 15 min in an autoclave (Tommy SX-700E, Tommy Kogyo Co., Tokyo, Japan). The hydrolysate used for adaptation also contained the same chemical proportions used in the culture media, except for the glucose, the released sugars from the hemicellulose fraction.

3.1.4   Pretreatment of Hazelnut Shells

Upon arrival, hazelnut shells were cleaned by removing the skin and excess kernel. They were then dried in an oven at 70℃ for 24 hours. The drying makes milling/grinding easier and removes excess moisture. A laboratory milling machine (Fritsch Industriestrasse 8, D-55743 Idar-Oberstein, Tüv-Cert, Germany) was used to grind the shells to powder and sieved through a 1mm sieve. The following scheme describes the pre-treatment step:
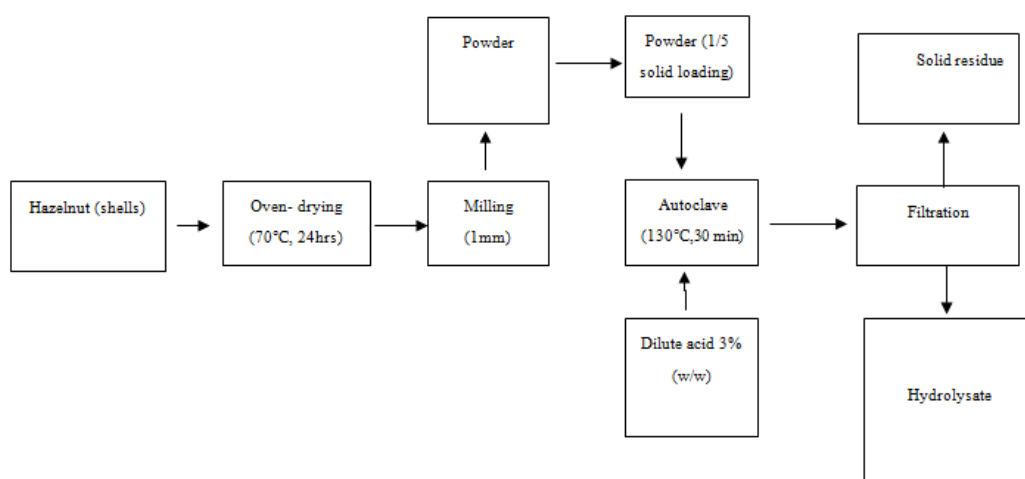
Figure 3. 1. Schematic diagram of hazelnut shell hydrolysate pre-treatment

The obtained powder was pre-treated with 3% (w/w) dilute sulphuric acid to release the fermentable sugars. A 5% solid-to-liquid ratio (biomass solid loading of 1/20) was used in 3% (w/w) dilute sulphuric acid. The pre-treatment was carried out in an autoclave at 130 °C for 30 minutes. The solid residue was separated from the liquid after the hydrolysis by vacuum filtration method. pH was calibrated to the required value with a pH meter using 10M NaOH. Calibration of pH in biomass hydrolysate usually results in excess salt formation due to the neutralization reaction of acid and base. This extra salt and solid residue were removed by centrifugation. Technically, 50mL of the calibrated media was put in 50 mL plastic falcon tubes and centrifuged at 2000g RCF at 4°C for 20 minutes in a centrifuge machine (Sigma 2-16PK, SciQuip Ltd., U.K.). The supernatant liquid was supplemented with the same nutrients as the culture media. The mixtures were sterilized at 121°C for 15 minutes in an autoclave (Tomy SX-700E, Tomy Kogyo Co., Tokyo, Japan). The sterile media was used as adaptation media. All further inoculations and sampling were carried out aseptically using 70% alcohol around a Bunsen burner.

3.1.5   Detoxification of Hydrolysate

The hydrolysate was detoxified using 2% activated charcoal to remove the inhibitory compounds. Specifically, 2g of activated charcoal was added to 100 mL

media and stirred well for 5 minutes. The mixture was filtered and centrifuged to remove the excess residue from the media. After centrifugation, the pH of the media was calibrated again and centrifuged for excess salt removal (Tuhanioglu*et al.*, 2023). This media was used as a control for bacterial growth.

3.2   Methods

3.2.1   Experimental Design

The experiment was designed in different sets using three levels of pH values (5,6 and 7)

The design of the first set of experiments was to carry out the adaptation using a 10% of inoculum ratio at all pH levels.

The design of the second set of experiments was to carry out the adaptation using a 5% of inoculum ratio at all pH levels.

The third set of experiments was designed to carry out the adaptation using a 2%inoculum ratio at all pH levels.

The last set of experiments was designed to carry out the adaptation using detoxified hydrolysate. This set of experiments aims to compare the best adaptation results without detoxification.

Another set of experiments was set to be carried out using the best adaptation condition for 8 days without changing media. This experimental set also serves as a control to observe the growth pattern of the bacteria in the hazelnut shells hydrolysate.

The growth curve of the adapted bacteria was also obtained in a synthetic media for 48 hours. This data also served as a standard for getting the specific growth rate of

the bacteria by plotting the result of OD_{600} against dry biomass (g/L) at 3 hours intervals. The temperature of 37℃ and agitation of 130rpm were kept constant throughout the experiment.

3.2.2 Adaptation of *Bacillus subtilis* in Hazelnut shell hydrolysate

Adaptation was carried out with 2, 5, and 10% inoculum ratios using pH values of 5, 6, and 7 in each experimental set. Specifically, activated culture media was inoculated into a fresh hydrolysate. The bacteria were grown for 24hour after which the same volume of inoculum was transferred into fresh hydrolysate. This serial transfer continued for 8 days for all sets of experiments. The best condition was used to find the specific growth rate of the adapted bacteria using synthetic media for 48 hours. Samples were taken for analysis of OD and reducing sugar after every 24 hours before inoculating into fresh hydrolysate.

3.2.3 Biomass assay

The starter culture of *Bacillus subtilis* NRRL B-4219 and the adaptation cultures were grown at 37°C, 130 rpm for 24 h. The optical density (OD) of bacterial cultures was measured at 600 nm using a spectrophotometer. Specifically, 1mL of the inoculated sample was taken and placed in an Eppendorf tube. The sample was centrifuged for 10 minutes in a mini centrifuge (MPW-15 Mini Centrifuge, MPW Med. Instruments Co., Warsaw, Poland). The suspended cells were rinsed twice. The supernatant was stored for sugar analysis, and the settled biomass was diluted 10 times until 1mL. 300µL of the diluted biomass was taken in the cuvette of 4mL size, and 27000µL of distilled water was added to make it 3mL. The 3mL sample was taken for the O.D. measurement in the spectrophotometer (Shimadzu UV-1700, Shimadzu Corp., Kyoto, Japan). The same procedure was used for the samples taken from the adaptation media to analyze the bacterial growth. All the OD measurements were converted to a dry weight of biomass using a standard curve prepared from the adapted *B. subtilis* grown in synthetic media. The standard curve and the equation used for the conversion can be found in Appendix C.

3.2.4   Reducing Sugar Analysis

Reducing sugar analysis was carried out for each sample to measure the amount of sugar consumed by the bacteria. The DNS method measured reducing sugar (Miller, 1959). The procedure was as follows:

Samples were centrifuged to obtain biomass-free media. 0.05mL of the centrifuged samples was diluted with 0.95mL distilled water (20 times dilution) to make 1mL volumes. Diluted samples were prepared in a test tube. 1mL DNS solution was added to each test tube, and the mixtures were placed into a water bath at 95℃ for 15 minutes. The samples were cooled to room temperature. 1mL of the cooled samples was put in cuvvetes of 4mL size and diluted with 2mL distilled water, making the dilution 60 times, and the absorbance was measured using a spectrophotometer at the wavelength of 575nm. The absorbance values were translated into glucose concentration using a calibration curve prepared for 0-2mg/ml of glucose. This choice of glucose standard follows the report of Uzuner and Cekmecelioglu (2014) who reported using glucose as a standard for reducing sugar to be more accurate than xylose.

A stock solution of 10mg/mL (0.5g glucose dissolved in 50 mL distilled water) was prepared for the glucose calibration curve preparation, from which the concentrations of 0-2mg/mL were prepared. The concentrations were prepared as seen in Table 3.1.

Table 3. 1. Preparation of Reducing Sugar Standard curve.

Stock (mL)	Distilled water (mL)	Concentration (gm/mL)
1	4	2
0.6	3.9	1.5
0.3	2.7	1
0.2	3.8	O.5
0	3	0

3.2.5   Statistical Analysis of Data

The mean values of the replicate and all data were analyzed statistically using IBM SPSS statistics (version 28.0). Analysis of Variance (ANOVA) and Kruskal Walli's test were used to analyze the means of biomass produced and the sugar consumed respectively.

CHAPTER 4

RESULTS AND DISCUSSION

This chapter contains the experimental data on the adaptive laboratory evolution of *Bacillus subtilis* in hazelnut shells hydrolysate. The study was carried out using different cultivation conditions of pH and inoculum ratio, to see the trend and effect of the variables on the adaptation of *Bacillus subtilis* in terms of growth and fermentable sugar utilization. The growth was monitored throughout the experiments using optical density (OD) measurements, and the results were translated into dry biomass weight (g/L) using a calibration curve prepared with three days of adapted *Bacillus subtilis* grown in synthetic media. The calibration curve can be found in Appendix B.

The effect of variables and methods in experiments were extensively discussed in the subsequent subsections. In addition, the following should be taken into consideration in the reported data:

• All experiments were performed in two replicates and the results in charts and tables are the mean values of the two replicates. All the results were shown with standard error data.

 • The growth and standard curve used for analytical calculations are presented in Appendix B.

• Statistical analysis results of the experiments are placed in Appendix C.

4.1   Growth of *Bacillus subtilis* NRRL B4219

Bacillus subtilis NRRL B-4219 was grown in synthetic media before carrying out the adaptation. The grown cultures were transferred to the hydrolysate for the adaptation experiments. The adapted strain was also grown in synthetic media,

where the growth rate was monitored. The growth curve of *Bacillus subtilis* NRRL B-4219 is shown in Appendix B. The initial culture for transfer into hydrolysate was maintained at an OD of 0.7-0.8 throughout the experiment.

Table 4. 1. Bacterial growth condition

Parameter	Value
Temperature (°C)	37
pH	Variable
Inoculum (%)	Variable
Time (Days)	8
Agitation speed (rpm)	130
Volume of growth media (mL)	50

4.2 Dilute Sulphuric Acid Hydrolysis of Hazelnut Shells

The hazelnut shells powder sieved through 1mm was pretreated using 3% dilute sulphuric acid in an autoclave at 130°C. This condition was chosen based on the study carried out by Uzuner and Cekmecelioglu (2014), where the optimum condition for dilute sulphuric acid hydrolysis of hazelnut shells was found to be 3% acid. The obtained hydrolysate in this study was found to contain in most cases, approximately 17 g/L of reducing sugar using the DNS method of reducing sugar estimation. The hydrolysate was used as growth media for the adaptation experiments with minerals like the culture media, except for the glucose, in which the released sugars were utilized as a carbon source.

4.2.1 Effect of different inoculum ratios on the adaptation of *Bacillus subtilis* in hazelnut shells hydrolysate

In this section, the adaption was carried out using three different inoculum ratios, namely 2, 5, and 10%. These were all carried out at three pH values of 5,6 and 7. Tables provide the specific experimental data of the adaptation experiment, and the charts are used to visualize the trend.

Table 4. 2. Adaptation with 2% inoculum using different pH levels

	pH 5 (2%)		pH 6 (2%)		pH 7 (2%)	
Transfer number (Days)	Reducing sugar (g/L)	Biomass (g/L)	Reducing sugar (g/L)	Biomass (g/L)	Reducing sugar (g/L)	Biomass (g/L)
0	16.98 ± 0.04	0.00 ± 0.00	19.57 ± 1.15	0.00 ± 0.00	19.32 ± 0.01	0.00 ± 0.00
1	15.76 ± 0.41	1.14 ± 0.29	18.75 ± 0.31	1.19 ± 0.07	17.76 ± 0.53	2.44 ± 0.07
2	16.35 ± 0.14	0.98 ± 0.07	18.38 ± 0.06	1.50 ± 0.07	17.41 ± 0.33	2.75 ± 0.07
3	16.14 ± 0.13	0.98 ± 0.37	18.15 ± 0.06	2.28 ± 0.44	17.34 ± 0.24	3.26 ± 0.37
4	16.45 ± 0.26	0.78 ± 0.22	17.88 ± 0.09	2.64 ± 0.37	17.13 ± 0.07	4.40 ± 0.07
5	16.30 ± 0.26	0.67 ± 0.07	17.25 ± 0.27	2.69 ± 0.15	17.04 ± 0.01	4.51 ± 0.07
6	16.94 ± 0.04	0.83 ± 0.15	16.94 ± 0.32	2.33 ± 0.37	16.97 ± 0.09	4.92 ± 0.07
7	16.53 ± 0.13	0.62 ± 0.15	16.84 ± 0.25	2.59 ± 0.15	16.01 ± 0.76	5.54 ± 0.07
8	16.22 ± 0.12	0.31 ± 0.15	15.78 ± 0.29	2.80 ± 0.15	14.85 ± 0.17	5.03 ± 0.07

Table 4.2 is the experimental data of the 2% inoculum adaptation of *Bacillus subtilis* at pH 5,6, and 7. The highest biomass produced in this set of experiments was 5.03 ± 0.07 when the experiment was conducted at pH 7. pH 6 followed with the value of 2.80 ± 0.15 g/L. Unfortunately, at pH 5 the rate of biomass production continues to decrease from 1.14 ± 0.29 g/L to 0.31 ± 0.15 g/L daily. In terms of reducing sugar consumption, pH 7 has the highest consumption with a residual sugar value of 14.85 ± 0.17 g/L, followed by pH 6 with a value of 15.78 ± 0.29 g/L.

The experimental data of 2% inoculum adaptation is demonstrated in Figure 4.1 for biomass and Figure 4.2 for reducing sugar utilization.
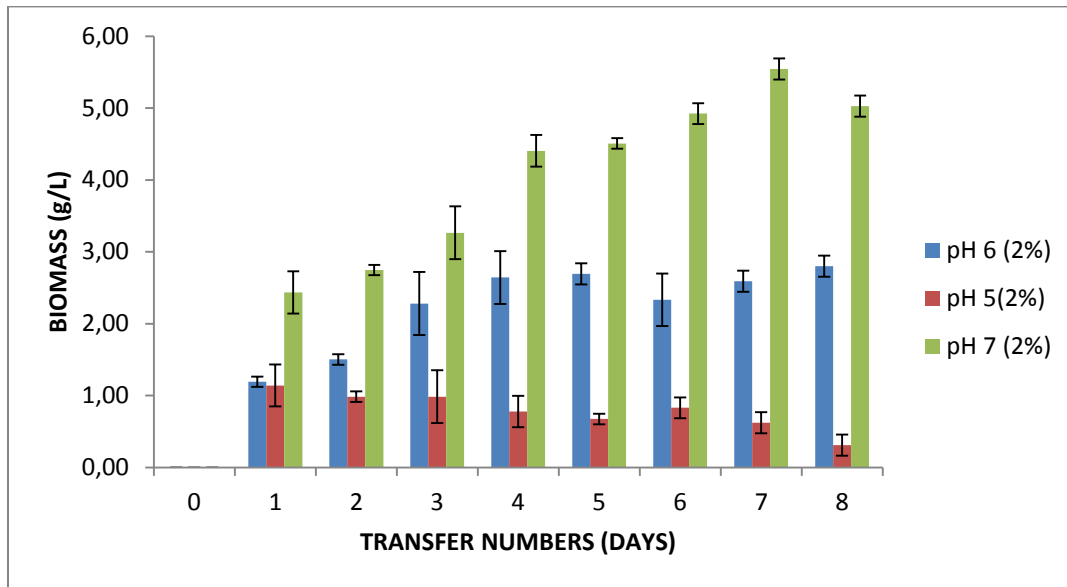

Figure 4. 1. Biomass produced from adaptation of *B. subtilis* with 2% inoculum ratio
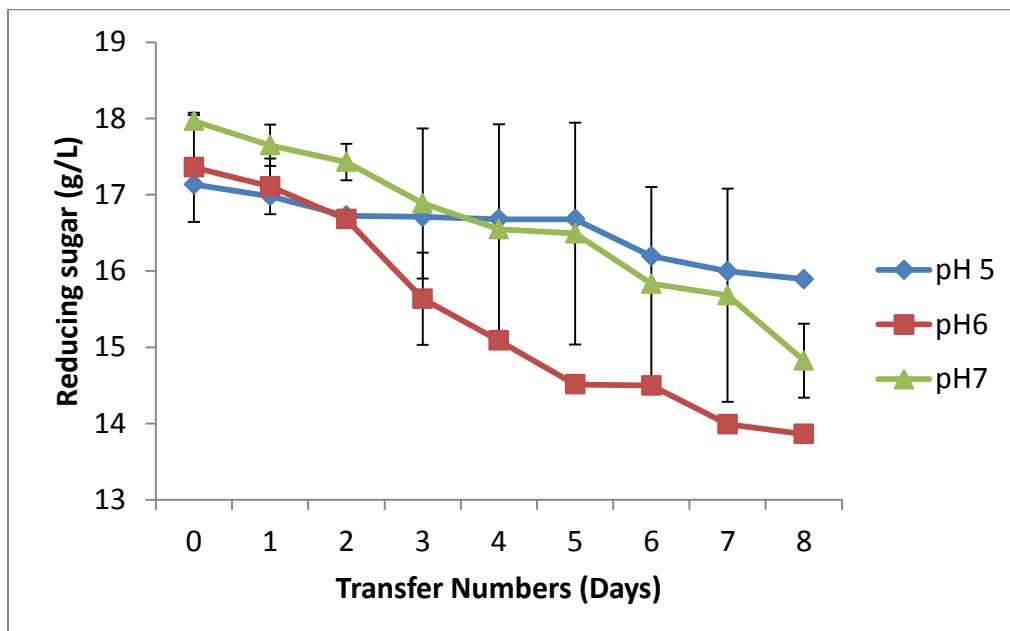

Figure 4. 2. Consumed reducing sugar using 2% inoculum ratio of *B. subtilis* adaptation

Table 4. 3. Adaptation of *B. subtilis* with 5% inoculum ratio

Transfer number (Days)	pH 5(5%)		pH 6 (5%)		pH 7 (5%)	
	Reducing sugar (g/L)	Biomass (g/L)	Reducing sugar (g/L)	Biomass (g/L)	Reducing sugar (g/L)	Biomass (g/L)
0	17.73 ± 0.13	0.00 ± 0.00	19.82 ± 0.08	0.00 ± 0.00	19.81 ± 0.10	0.00 ± 0.00
1	16.38 ± 0.09	1.55 ± 0.15	18.88 ± 0.94	3.58 ± 0.07	18.13 ± 1.72	2.80 ± 0.29
2	15.53 ± 1.23	1.76 ± 0.15	18.45 ± 0.83	3.58 ± 0.22	15.92 ± 1.51	3.47 ± 0.22
3	16.31 ± 0.08	1.97 ± 0.07	17.98 ± 1.42	3.99 ± 0.07	17.77 ± 0.41	3.63 ± 0.29
4	16.52 ± 0.11	1.66 ± 0.00	17.36 ± 0.85	3.63 ± 0.29	17.89 ± 0.56	3.83 ± 0.29
5	16.29 ± 0.17	1.97 ± 0.07	16.66 ± 0.61	3.83 ± 0.15	17.91 ± 0.03	3.89 ± 0.22
6	16.46 ± 0.06	2.07 ± 0.15	16.23 ± 0.94	3.89 ± 0.07	17.21 ± 0.83	3.89 ± 0.22
7	16.31 ± 0.19	2.28 ± 0.22	15.44 ± 0.10	3.99 ± 0.07	16.82 ± 1.46	4.04 ± 0.15
8	16.57 ± 0.18	2.49 ± 0.07	15.29 ± 0.20	4.66 ± 0.29	15.04 ± 0.12	5.13 ± 0.07

Table 4.3 contained the experimental data of the adaptation carried out with a 5% inoculum ratio. In this set of experiments, pH 7 recorded the highest biomass production with a value of 5.13 g/L of biomass, followed by pH 6 with a biomass of 4.66 ± 0.29g/L and then pH 5 with a biomass value of 2.49 ± 0.07g/L. In terms of reducing sugar consumption, pH 7 adaptation was the highest with a value of 15.04 ± 0.12, and then pH 6 with a residual sugar amount of 15.29 ± 0.20 g/L followed by pH 5 which has 16.57 ± 0.18 g/L reducing sugar concentration.
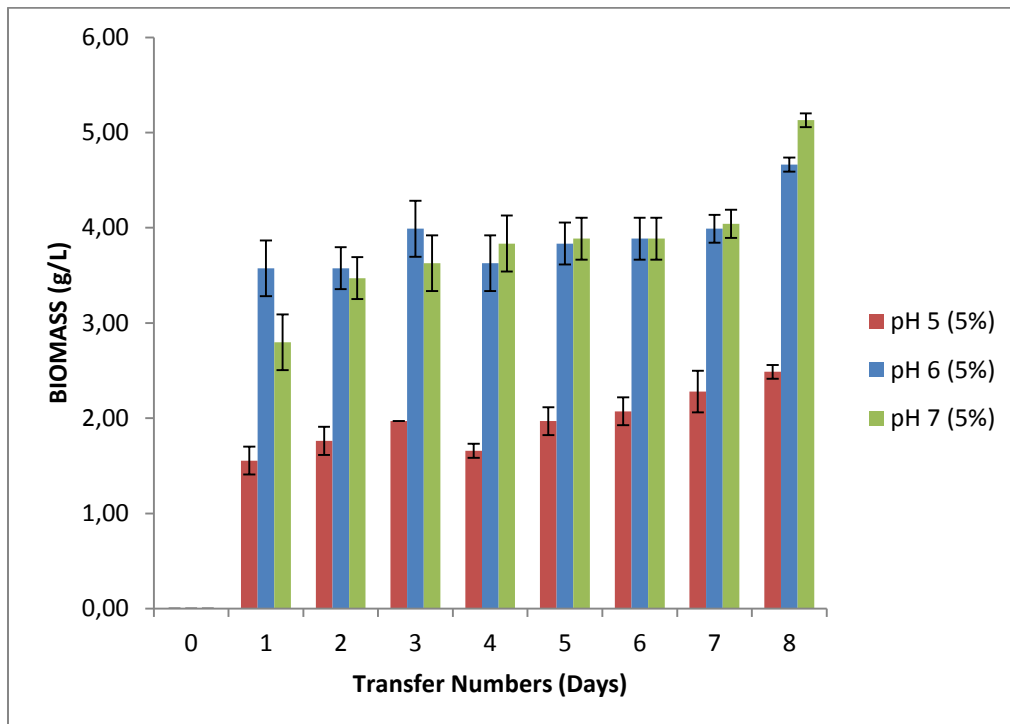
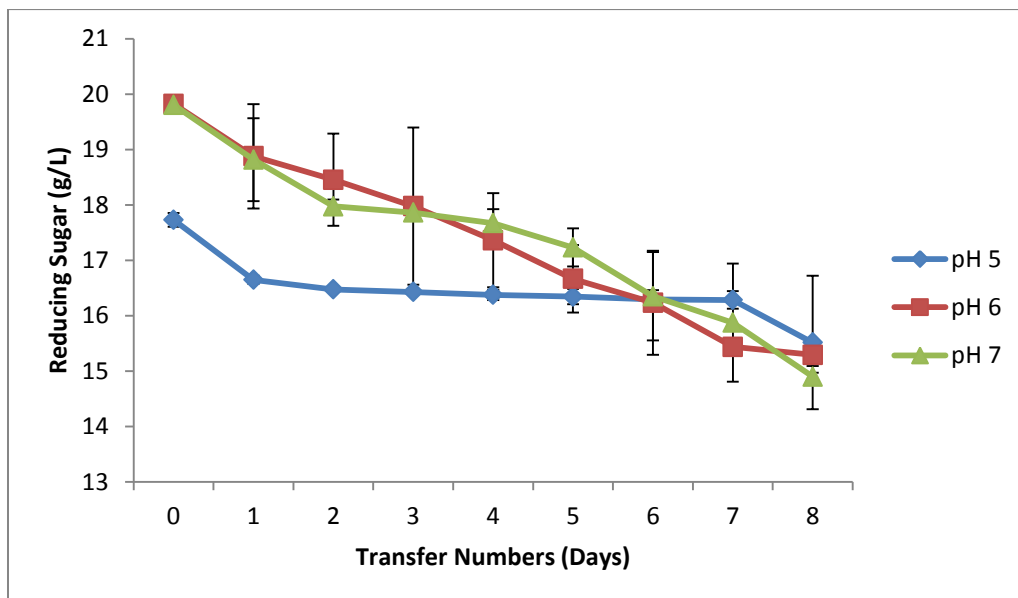Figure 4. 3. Biomass produced from the adaptation of *B. subtilis* using 5% inoculum

Figure 4. 4. Consumed reducing sugar for adaptation of *B. subtilis* using 5% inoculum ratio

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Table 4. 4. Adaptation of *B. subtilis* with 10% inoculum ratio

	pH 5(10%)		pH 6 (10%)		pH 7 (10%)	
Transfer number (Days)	Reducing sugar (g/L)	Biomass (g/L)	Reducing sugar (g/L)	Biomass (g/L)	Reducing sugar (g/L)	Biomass (g/L)
0	17.13 $\pm$ 0.08	0.00 $\pm$ 0.00	17.36 $\pm$ 0.72	0.00 $\pm$ 0.00	17.97 $\pm$ 0.08	0.00 $\pm$ 0.00
1	16.98 $\pm$ 0.08	1.45 $\pm$ 0.15	17.11 $\pm$ 0.36	1.40 $\pm$ 0.07	17.65 $\pm$ 0.27	2.49 $\pm$ 0.44
2	16.72 $\pm$ 0.07	1.97 $\pm$ 0.15	16.68 $\pm$ 0.05	1.87 $\pm$ 0.15	17.43 $\pm$ 0.24	2.95 $\pm$ 0.37
3	16.71 $\pm$ 0.07	1.76 $\pm$ 0.07	15.63 $\pm$ 0.60	0.78 $\pm$ 0.07	16.89 $\pm$ 0.99	3.58 $\pm$ 0.22
4	16.68$\pm$ 0.07	1.66 $\pm$ 0.15	15.09 $\pm$ 0.04	1.50 $\pm$ 0.37	16.55 $\pm$ 1.38	3.78 $\pm$ 0.07
5	16.68 $\pm$ 0.07	2.49 $\pm$ 0.37	14.51 $\pm$ 0.04	1.30 $\pm$ 0.22	16.49 $\pm$ 1.45	3.83 $\pm$ 0.15
6	16.19 $\pm$ 0.07	1.66 $\pm$ 0.22	14.50 $\pm$ 0.06	1.50 $\pm$ 0.81	15.83 $\pm$ 1.27	3.99 $\pm$ 0.07
7	16.00$\pm$ 0.07	1.87 $\pm$ 0.81	13.99 $\pm$ 0.03	1.92 $\pm$ 0.22	15.68 $\pm$ 1.40	4.25 $\pm$ 0.29
8	15.89 $\pm$ 0.07	3.11 $\pm$ 0.15	13.86 $\pm$ 0.10	1.94 $\pm$ 0.04	14.82 $\pm$ 0.48	6.17 $\pm$ 0.07

Table 4.4 gives the experimental data with standard error of the Biomass produced and the residual reducing sugar after the bacteria was adapted with a 10% inoculum ratio using different pH values of 5, 6, and 7. The highest biomass recorded was 6.17 ± 0.07 g/L at day 8 when the experiment was carried out at pH 7 with a 10% inoculum ratio. The lowest biomass recorded was 0.78 ± 0.07g/L at day 3 of the pH 6 experiment. Although pH 5 is lower than pH 6, at this inoculum, the overall biomass produced was higher than that of pH 6. While the reducing sugar of this set of experiment do not differ much. The highest consumed sugar was found at day 8 of the pH 6 adaptations with a sugar concentration of 13.86 ± 0.10g/L. The lowest consumption was found at pH 5 with 15.89 ± 0.07 g/L sugar concentration.
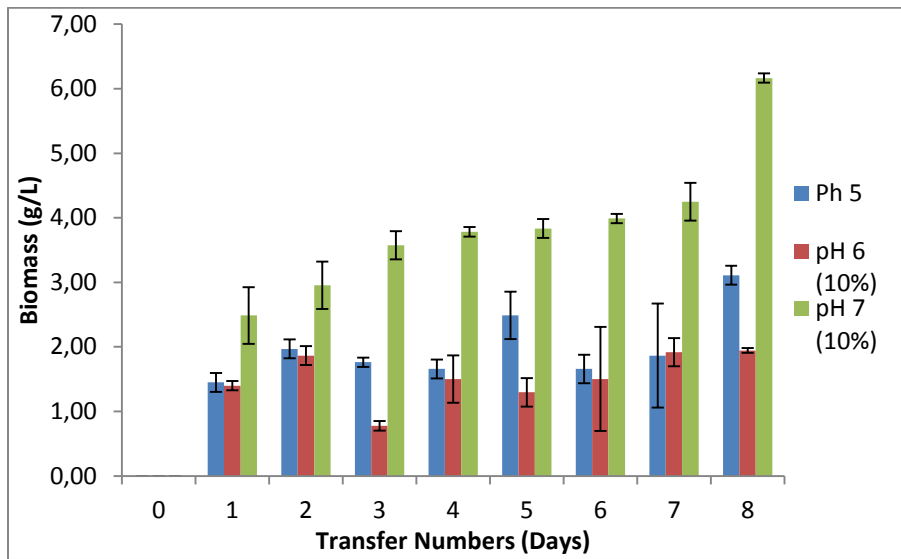
Figure 4. 5. Biomass produced from the adaptation of *B. subtilis* with 10% inoculum
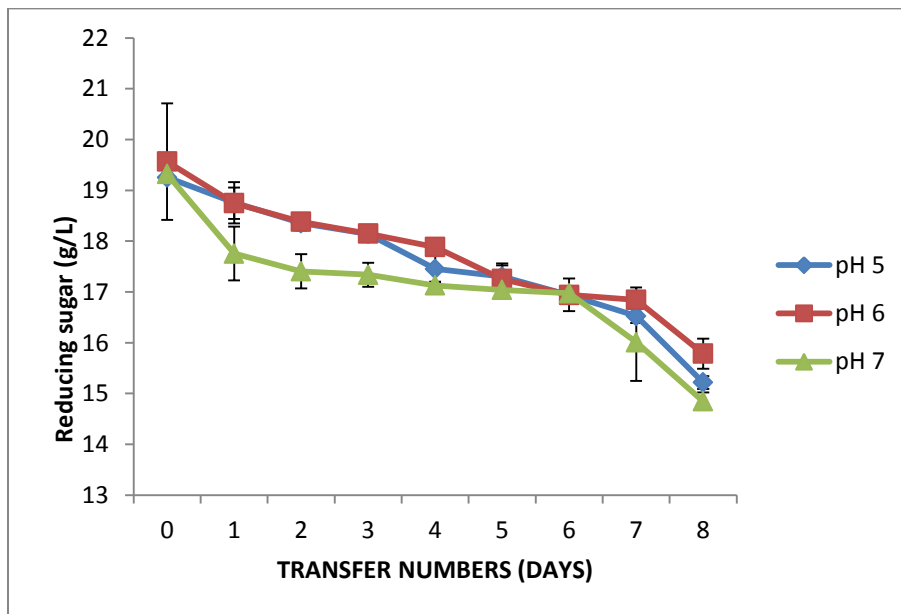

Figure 4. 6. Reducing sugar of *B. subtilis* adaptation using 10% inoculum

4.2.2 Effect of different pH on the adaptation of *Bacillus subtilis* in Hazelnut shells hydrolysate

To see how pH affects the different inoculum ratio on the adaptation of *B. subtilis*, the charts for each pH were constructed from the previous tables (4.2,4.3, and 4.4)
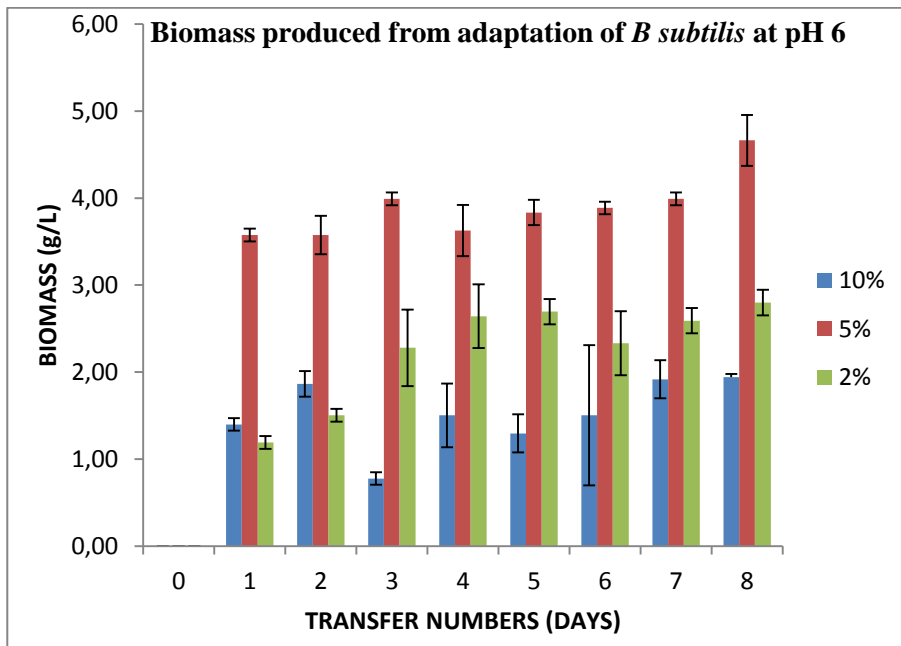
Figure 4. 7. Biomass produced from adaptation of *B. Subtilis* at pH 6

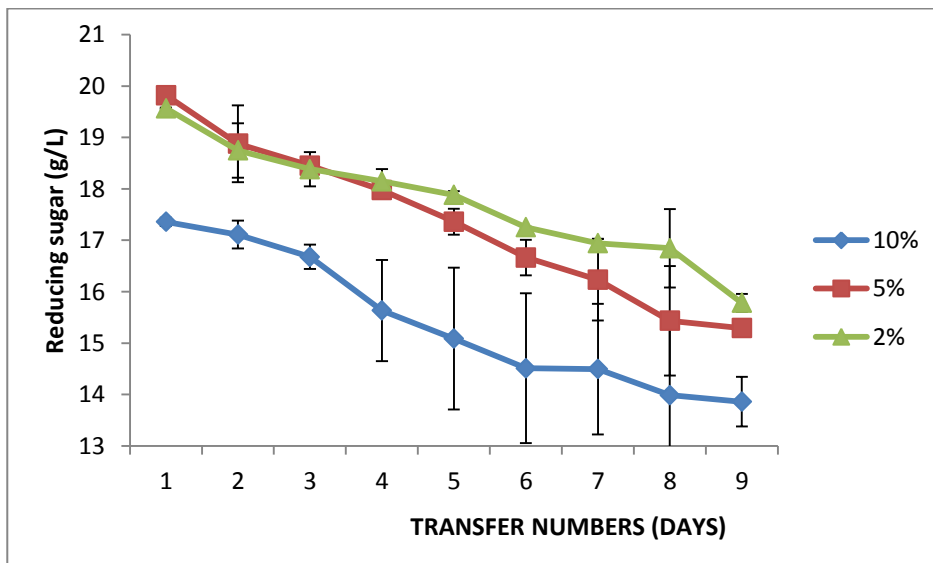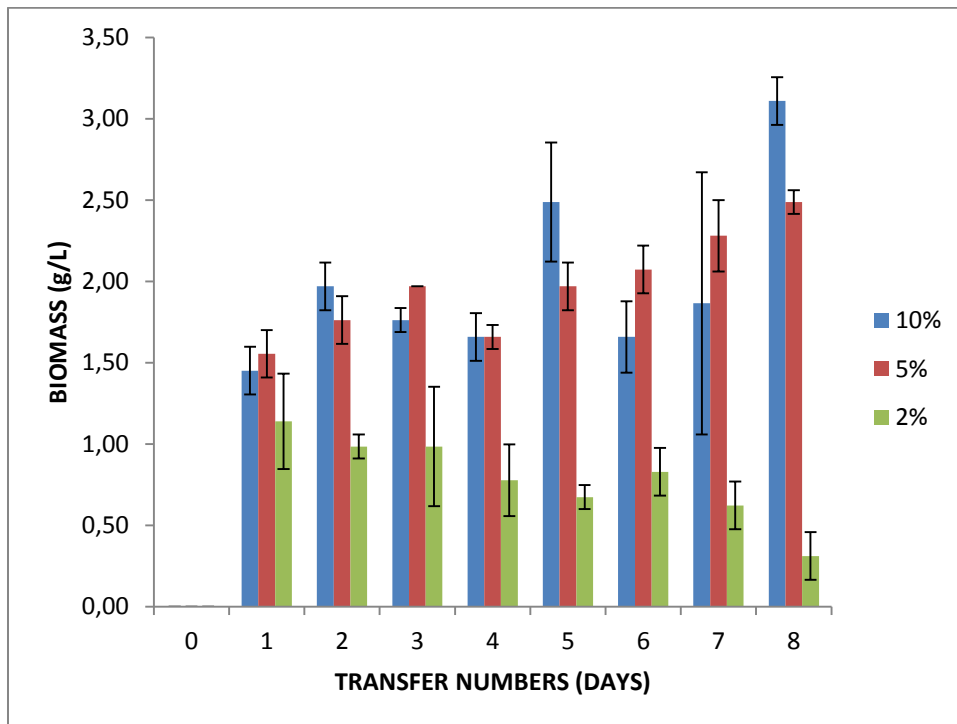Figure 4. 8. Reducing sugar consumed at pH 6 adaptation using different inoculum ratio

Figure 4. 9. Biomass produced from the adaptation of *B. subtilis* at pH 5
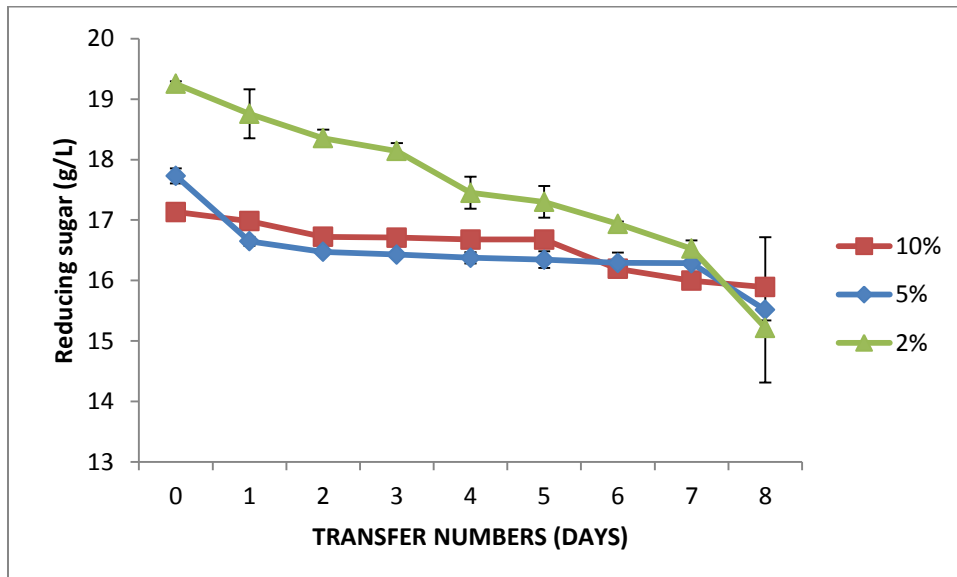

Figure 4. 10. Reducing sugar consumed at pH 7 experiments across inoculums
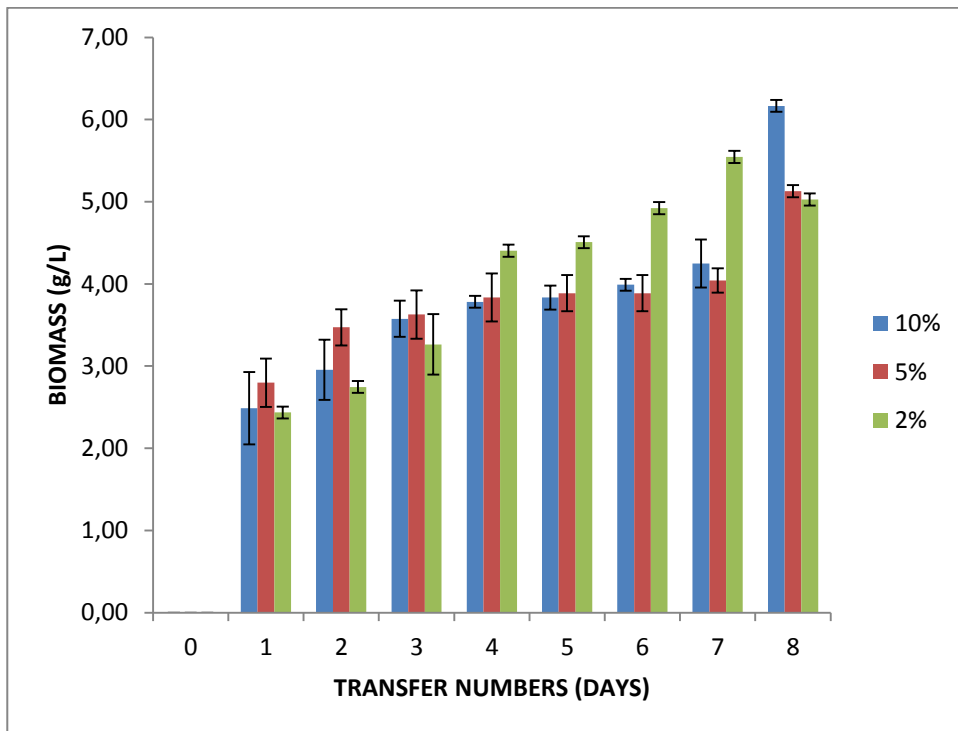
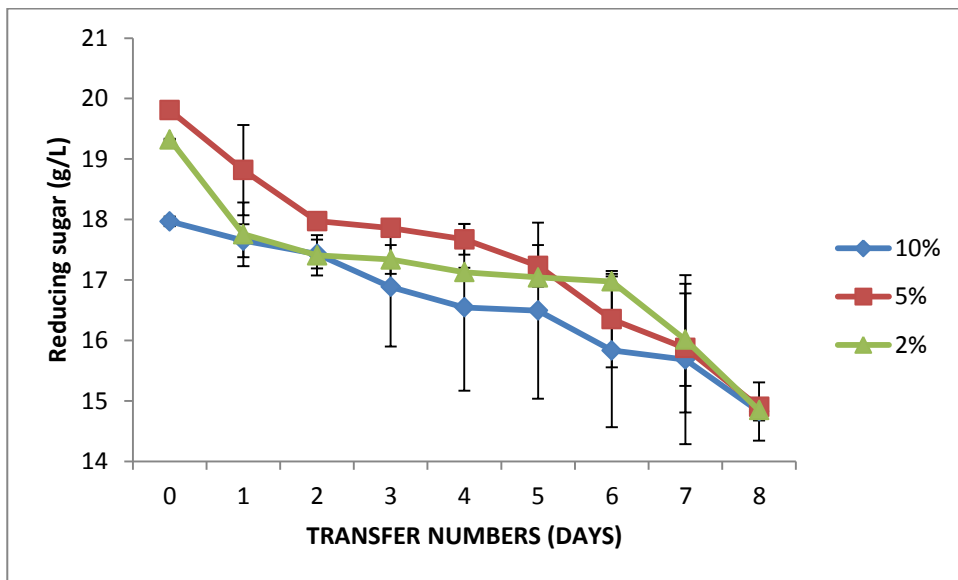Figure 4. 11. Biomass produced from adaptation of *B. subtilis* at pH 7

Figure 4. 12. .Reducing sugar consumed at pH 7 experiments using different inoculums

Table 4. 5. Control Experiments at pH 7 and 2% inoculum ratio

Cultivation time (Days)	Undetoxifiied Media		Detoxified Media	
	Reducing sugar (g/L)	Biomasss (g/L)	Reducing sugar (g/L)	Biomass (g/L)
0	17.9 ± 0.43	0.00 ± 0.00	13.98 ± 0.06	0.00 ± 0.00
1	17.20 ± 0.43	1.40 ± 0.07	13.85 ± 0.14	2.44 ± 0.07
2	17.06 ± 0.27	2.49 ± 0.15	13.78 ± 0.07	2.75 ± 0.07
3	16.90 ± 0.30	2.75 ± 0.07	13.74 ± 0.06	3.26 ± 0.37
4	16.72 ± 0.26	2.49 ± 0.73	13.72 ± 0.09	4.40 ± 0.07
5	16.37 ± 0.60	2.44 ± 0.07	13.68 ± 0.08	4.51 ± 0.07
6	16.28± 0.64	2.23 ± 1.25	13.65 ± 0.06	4.92 ± 0.07
7	16.07 ± 0.51	2.59 ± 0.73	12.86 ± 0.99	5.54 ± 0.07
8	15.67 ± 0.42	2.59 ± 0.29	12.83 ± 0.97	5.03 ± 0.07

Table 4.5 is controlled experiments carried out in a media with inhibitors and the media which was detoxified using 2% activated charcoal. In this set of experiments, no media is changed daily. The bacteria were allowed to grow for 8 days and a sample was drawn every day for growth measurement and sugar consumption. These sets of experiments were meant to compare with the best adaptation experimental condition (pH 7). The illustrations of these data were made in the charts labeled Figure 4.12 for biomass produced and figure 4.13 for the reducing sugar, both in g/L
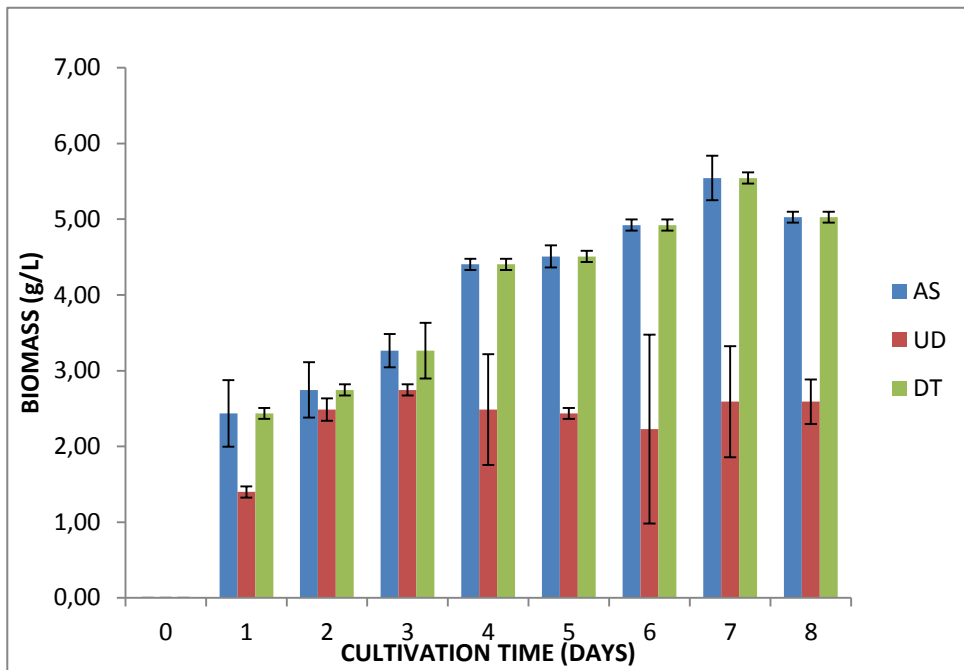
Figure 4. 13. Biomass produced using different media. AS: Adapted Stain. DT: Media used for detoxification. UD: Undetoxified Media used for 8 days cultivation without changing new media.
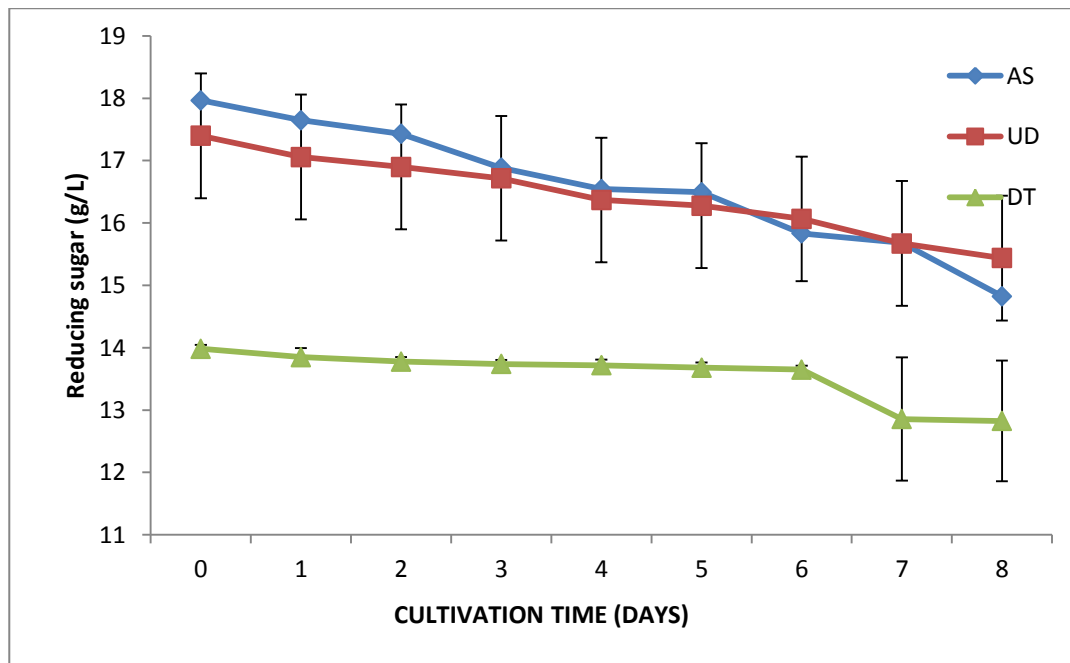

Figure 4. 14. Comparison of reducing sugar consumed by AS vs UD vs DT (AS: Adapted Strain, UD: Undetoxified Media, DT: Detoxified Media)

Based on the collected data above, the subsequent statistical tests of the experimental data were made.

Analysis of Variance (ANOVA) was used to test the significance of difference in the mean of reducing sugar utilized in the three different pH values. As seen in Appendix C, the level of significance is 0.256, meaning there is no difference in the amount of reducing sugar used per day in the medium at different pH values.

ANOVA was also used to test the level of significance of the means of reducing sugar utilized for the three different inoculum ratios. Since the level of significance is 0.154 as can be seen in Appendix C, then there is no difference in the amount of reducing sugar used per day in the medium for different inoculumratios. The error bar charts are also in the Appendix C section of this report to show significant variation in the computed means of reducing sugar across the groups.

Levene statistic test was carried out to test for homogeneity of the variance in the mean biomass of bacteria. From the table, the level of significance is 0.004 which is less than 0.05. Therefore, there is a significant difference in the variance across the group which indicated that One-way ANOVA cannot be used to analyze these data. Therefore, a non-parametric test, Kruskal-Wallis Test was considered instead. Kruskal –Walli's test was used to rank the ordinal data (pH). According to the table, in Appendix C, the ranking increased as the pH value increases. The ranking was analyzed using chi-square statistics as seen in the table located in Appendix C, there is a significant difference in the biomass of the bacteria among the pH values, since the asymptomatic significance is less than 0.05. The mean plot to show the structure of the differences in the means of biomass among the pH is also located in Appendix C. The biomass increases with an increase in pH. The biomass is higher in pH 7 than other pHs.

Based on the homogeneity test for biomass produced using diffrent inoculum ratios, the level of significance is 0.075 which is greater than the 0.05 level of significance. This indicated that the variance across the groups is equal. According

to the analysis of variance, since the level of significance is 0.056 then, there is no difference in the growth of the bacteria per day in the medium at different % inoculum. The mean plot to show the structure of the differences in the means of the biomass for each % inoculum is in Appendix C. The biomass was higher in 10 % than others.

The control experiments (cultured bacteria without changing media) were compared with the 2% adaptation experiment at pH 7. According to the Levene tests for homogeneity of variance in these cases, the biomass of the bacteria in relation to the status of the medium could be tested with ANOVA because the significant level is 0.209 which is greater than 0.05. While that of reducing sugar was 0.002, which is less than 0.05, then cannot be tested with ANOVA. From the table in Appendix C, there is a significant difference since F-Tabulated < 0.05.

According to the analysis of Kruskal −Walli's test using chi-square statistic to rank the ordinal data (Status of medium), there is a significant difference in the mean of reducing sugar across the status of the medium. The partial correlation table to test the relationship between the variables is also found in the Appendix C section of this report.

The result of this study showed that cultivation conditions affect the outcome of evolutionary adaptation. This is in line with the report of various researchers on adaptive laboratory evolution (Qureshi *et al*., 2015; Mavromati *et al*., 2022).

According to the results of this study, pH 5 of 2% inoculum showed poor adaptation on hazelnut shells hydrolysate. This is probably due to the lower pH of the medium, tending more towards the acidic region. In addition to the presence of acetic acid of almost 3g/L, as seen in the report of Uzuner and Cekmecelioglu (2014), when they analyzed the inhibitors found in the hazelnut shells hydrolysate using similar conditions of pre-treatment. It could be that the amount of inoculum is too little to have more cells that can adapt to the severe effect of the hydrolysate. As seen in the report of Vander Mass (2021), when acetic acid is present in the

media at a concentration of 2 g/L, the growth of *B. subtilis* was slowed in terms of the lag phase. No growth was observed until after 48 hours. In addition, the combination of HMF and acetic acid in the media was also reported to result in a severe effect in terms of the growth rate of *B. subtilis* (Vander Mass, 2021). However, at pH 7 the adaptation was improved even though the same inoculum ratio was used. This can be explained by the fact that *B. subtilis* grows better at neutral pH and slightly basic medium.

Radek *et al.*, 2017 used *E. coli* in their study and reported a 60% growth rate improvement on glucose minimal media for 30 transfers. Compared to that, this study achieved better results in terms of growth rate improvement at best conditions considering the number of transfers when we compared the first and the last strain grown in the hazelnut shells hydrolysate. This study shows fast growth rate improvement.

At higher inoculum (10%) and pH 7, the biomass was higher. The ALE studies mostly utilized higher inoculum, 10% inoculum ratio. Although most studies do not report the reason for using such high inoculum volume, this study has proven that when carrying out an ALE experiment, it is essential to maximize the culture condition to maximize the probability of getting adapted cells in the subsequent transfers. ALE studies carried out by Qureshi *et al.*, 20 15 and the likes, all used a 10% inoculum ratio for their adaptation experiments, which were of longer transfer numbers compared to the present study.

Although the results of algae are not similar to that of bacteria, Yu *et al* (2013) reported improved biomass when *Chlamydomonas reinhardtii* was utilized for ALE, the three strains showed 1.17, 1.33, and 1.48 fold growth improvement compared to the starting strain, which equivalents to 17-48% increase in biomass (Yu *et al.*, 2013).

The poor substrate consumption of *B. subtilis* in the present study is that pentose sugars are the possible reason for the slow reducing sugar consumption in the

present study, because the method employed in the pre-treatment of the hazelnut shells in this study can only hydrolyze the hemicellulose fraction of the hazelnut shells, as we have seen in the previous review, dilute sulphuric acid is not suitable for cellulose release from lignocellulose biomass. Although there is research that showed total xylose consumption using adaptation studies, like that of Abdurrahem *et al*., (2021), the fewer transfer numbers in the present study make it show less consumption.

The control experiment found in Table 4.5 with detoxified and undetoxified media has shown the efficacy of the adaptation experiments, as the biomass produced from the undetoxified media without transfer is much less compared to the one produced from the adapted strain. Moreover, the adapted produced biomass almost similar to the bacteria grown in the detoxified media, as can be seen in Figure 4.10

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1   Conclusıons

In this study, the adaptation of *Bacillus subtilis* was performed using different inoculum ratios and Ph values in hazelnut shells hydrolysate with added minerals as fermentation media. The experiments were carried out with a working volume of 50mL in a 250mL-sized shake flask. Improvement in biomass was observed in different Ph and inoculum ratios, with a 10% inoculum ratio showing the best result both in terms of biomass produced and the amount of fermentable sugars consumed. The bacteria failed to show any improvement in terms of biomass and sugar utilization at pH 5 using 2% inoculum ratio. In the overall experiments, when analyzed statistically, biomass production increases with an increase in pH values. For reducing sugar, there was no significant difference in the amount consumed daily for different inoculum ratios. The result of this study showed that cultivation conditions affect the outcome of evolutionary adaptation. This will help bioprocess industries in the steps taken to tackle the negative effect of inhibitory compounds on fermenting microorganisms. In turn, will improve the bio-economy and reduce the environmental impact caused by the hazelnut shells.

5.2   Recommendatıons

Based on the result obtained after the 8-day adaptation of *Bacillus subtilis* at different culture conditions of inoculum and pH values, the following recommendations were made:

The adaptation should henceforth be carried out at pH 7 using 10% inoculum as this gives better results.

Long-term evolutionary adaptation of *Bacillus subtilis* in hazelnut shells with more than 8 transfers should be considered for improved sugar consumption. As seen in the growth curve in the Appendix C section, the maximum biomass produced in the synthetic media was not reached, as such further improvement can be achieved with more transfer numbers.

In addition to further improvement in the growth rate, genetic analysis can be performed in the adapted strain, to check for any mutation occurrence for evolving new strain that can fully adapt in hazelnut shells hydrolysate.

Adaptation using 2% inoculum at pH 5 is highly not recommended since at this condition, the bacteria do not show improvement rather, a decrease was noticed in the biomass produced.

Automation studies can be considered using the best condition for better improvement in growth rate and substrate consumption.

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APPENDICES

A. LIST OF CHEMICALS

TABLE A.1 CHEMICALS USED IN THE EXPERIMENT

| CHEMICAL NAME	MANUFACTURERS
3,5-dinitrosalysilic acid ($C_7H_4N_2O_7$)	Sigma-Aldrich (St. Lois, MO, USA)
Carboxymethylcellulose sodium salt ($C_{28}H_{30}Na_8O_{27}$)	Merck (Darmstadt, Germany)
Citric acid monohydrate ($C_6H_8O_7.H_2O$)	Merck
Citrus pectin	Sigma-Aldrich (St. Lois, MO, USA)
D(+)-glucose monohydrate ($C_6H_{12}O_6.H_2O$)	Merck
Di-potassium hydrogen phosphate (K_2HPO_4)	Merck (Darmstadt, Germany)
Glycerol ($C_3H_8O_3$)	Merck (Darmstadt, Germany)
Granulated yeast extract	Merck (Darmstadt, Germany)
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	Merck (Darmstadt, Germany)
Phenol (C_6H_5OH)	Merck (Darmstadt, Germany)
Potassium dihydrogen phosphate (KH2PO4)	Merck (Darmstadt, Germany)
Potassium sodium tartrate tetrahydrate, $C_4H_4KNaO_6.4H_2O$ (Rochelle salt)	Merck (Darmstadt, Germany)
Sodium hydroxide (NaOH) anhydrous pellets	Merck (Darmstadt, Germany)
Sodium hydroxide (NaOH) powder	Sigma-Aldrich
Sodium sulfite, Na_2SO_3	
Sulphuric acid, H_2SO_4	Merck (Darmstadt, Germany)

B. STANDARD CURVES
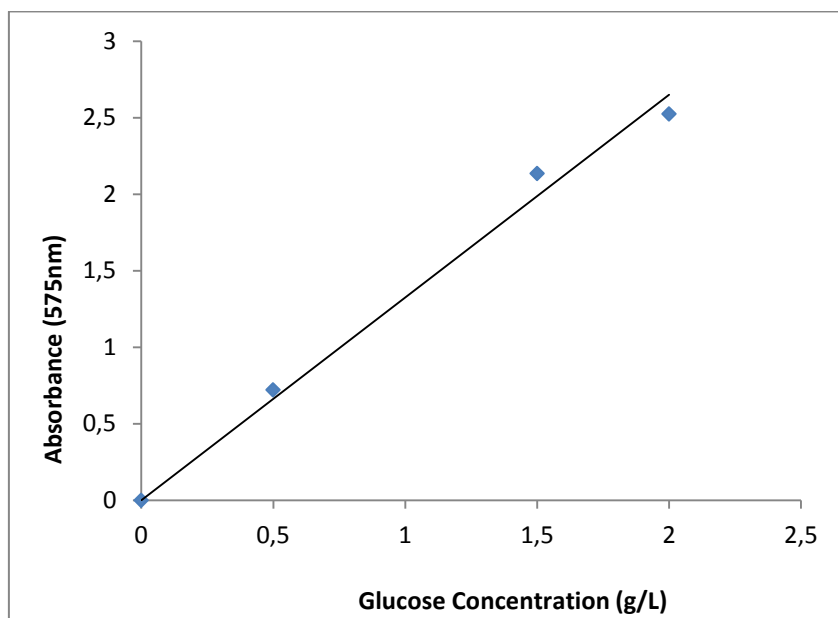
Figure C.1: Reducing sugar standard curve

The equation for the conversion of absorbance values to glucose concentration:
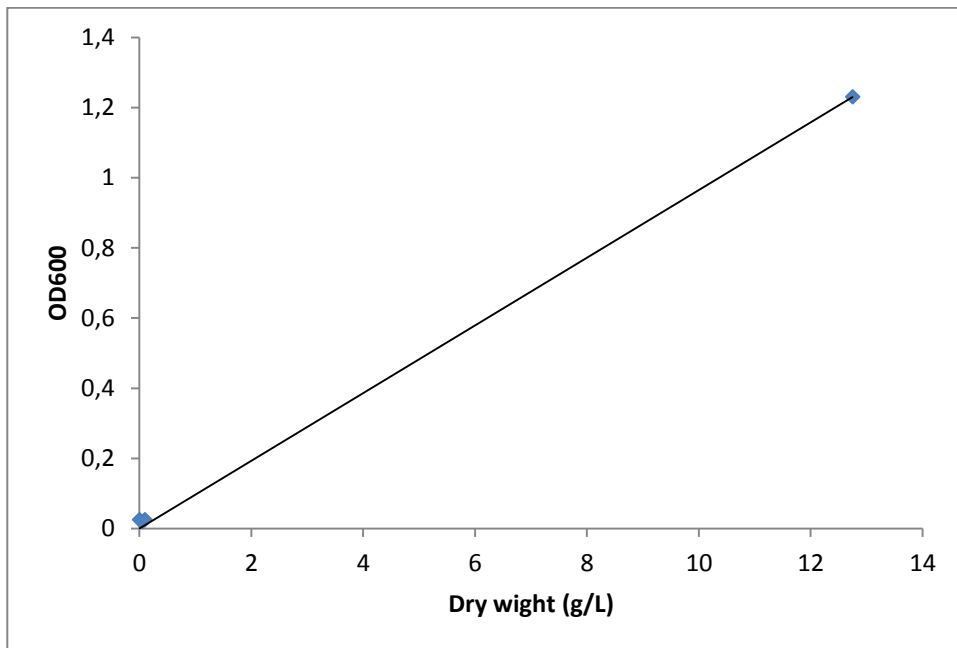
y = 1.3244x, R² = 0.9902

Figure C.2: Standard curve Biomass Assay

The equation for the conversion of absorbance values to glucose concentration:
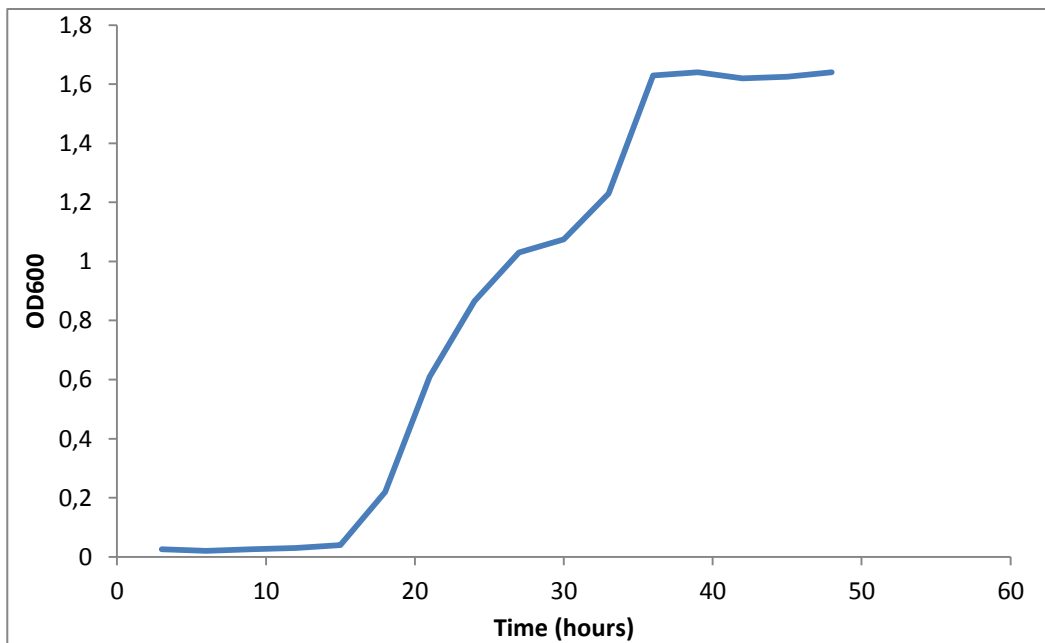
$y = 0.0965x$, $R^2 = 0.999$

Figure C.3: Growth curve of *Bacillus subtilils*

C. STATISTICAL ANALYSIS TABLES

Tables C.1:(a) Test of Homogeneity of Variances for the amount of Reducing Sugar used after 24 hours g/L

Levene Statistic	df1	df2	Sig.
1.191	2	69	.310

(b) One-way ANOVA for the amount of reducing sugar consumed after 24 hour

|	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.347	2	.174	1.388	.256
Within Groups	8.632	69	.125		
Total	8.980	71			

Table C.2: (a) Test of homogeneity of variance for the daily amount of reducing sugar used for different inoculum ratios

(a)

Levene Statistic	df1	df2	Sig.
2.764	2	69	.070

(b) ANOVA amount of reducing used after 24 for different inoculums

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.475	2	.237	1.925	.154
Within Groups	8.505	69	.123		
Total	8.980	71			

Error Bars: 95% CI

Error Bars: +/- 2 SE

Error Bars: 95% CI
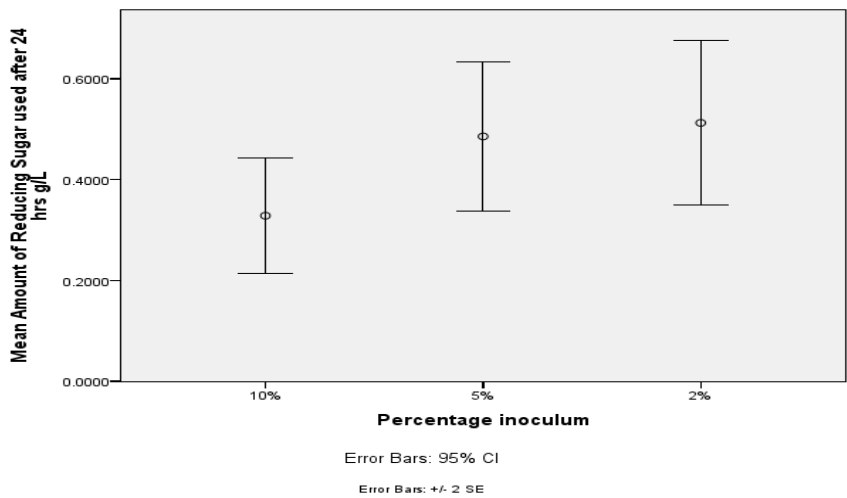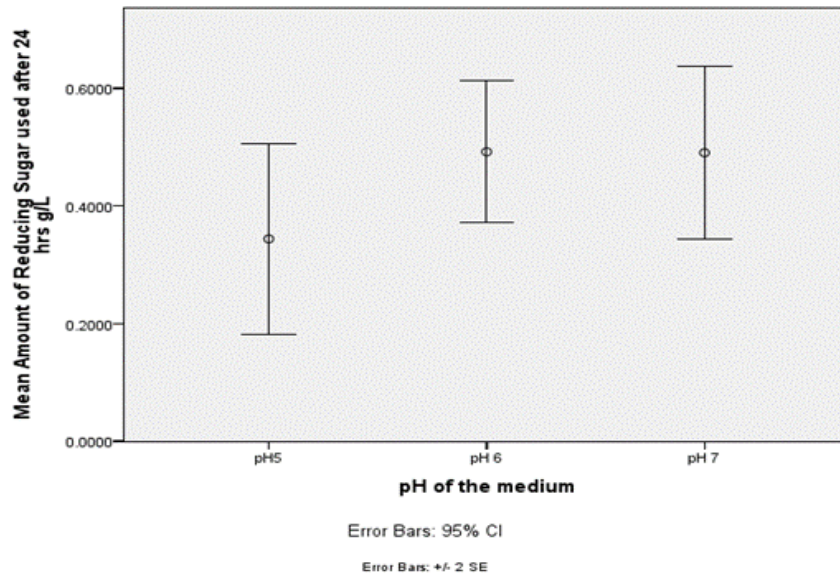
Error Bars: +/- 2 SE

C.2 : error bars for different (a) inoculum and (b) pH for daily reducing sugar consumption

Table C.3(a) rank table for Kruskal –Wallis test for the ranked (pH)

(a)	pH of the medium	N	Mean Rank
Biomass of Bacillus subtilis after 24 hrs each day	pH5	24	20.23
	pH 6	24	32.85
	pH 7	24	56.42
	Total	72	

Table C.3 (b) Analysis of the ranked pH val using chi-square statistics.

	Biomass of Bacillus subtilis after 24 hrs each day
Chi-Square	37.000
df	2
Asymp. Sig.	.000

C.3 (a) Mean plots of biomass for different pH
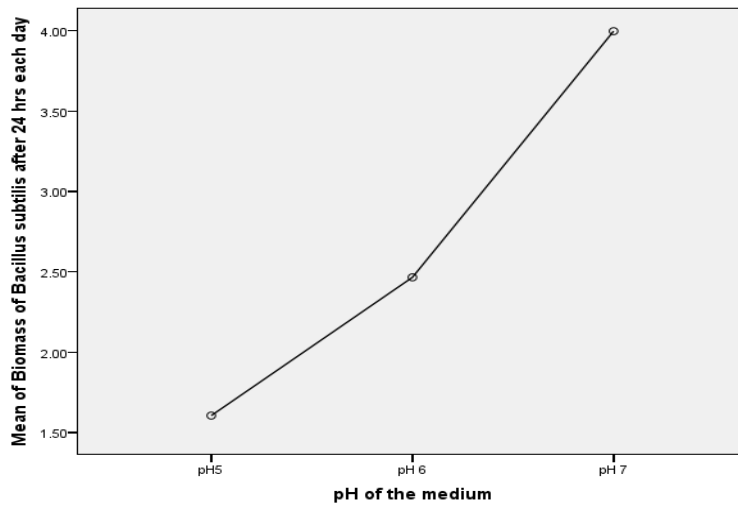
Tabble C.4 (a) Biomass of Bacillus subtilis after 24 hrs each day

Levene Statistic	df1	df2	Sig.
2.681	2	69	.076

Table C.4 (b) ANOVA for Biomass produced by *B subtilis* after 24 hours

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10.321	2	5.161	3.012	.056
Within Groups	118.233	69	1.714		
Total	128.555	71			

Table C.5: Test of homogeneity of varience for reducing sugar based on status of the media

TABLE 14 ANOVA					
Biomass of Bacillus subtilis based on the status of medium	Levene Statistic	df1	df2	Sig.	
Biomass of Bacillus subtilis based on the status of medium	1.690	2	21	.209	
Amount of reducing sugar used based on the status of medium	8.129	2	21	.002	

Table C.6 Ranks for chi square statistics of reducing sugar for different types of media

	STATUS OF THE MEDIUM	N	Mean Rank
Amount of reducing sugar used based on the status of medium	FRESH	8	15.50
	DETOXIFIED	8	6.88
	UNDETOXIFIED	8	15.13
	Total	24	

Table C.7: Chi Statistics[a,b]

	Amount of reducing sugar used based on the status of medium
Chi-Square	7.608
df	2
Asymp. Sig.	.022
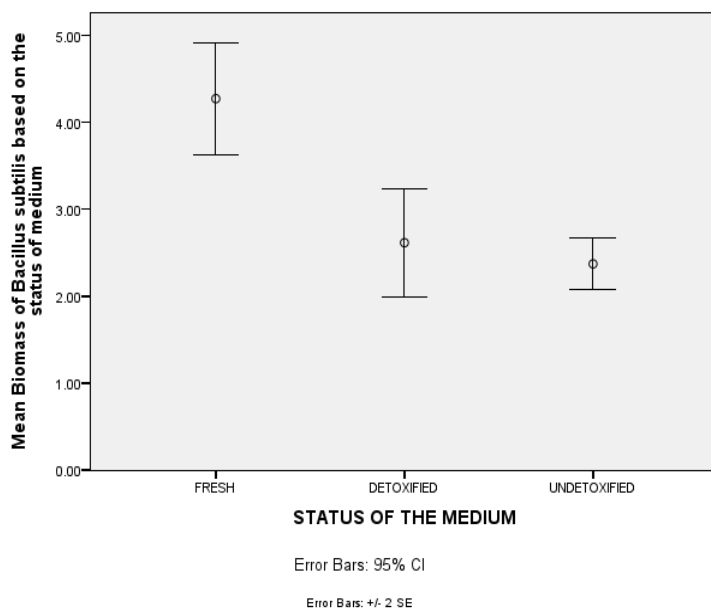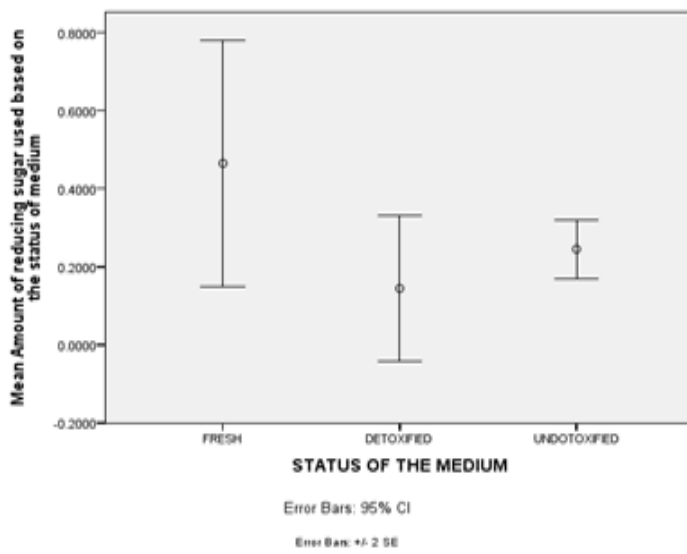
C.4: Error bars of mean of reducing sugars used for different typed of media

TABLE C.6: Partial Correlation Table To Test The Relationship Between Biomass And Reducing Sugar

Correlations

Control Variables			Amount of Reducing Sugar used after 24 hrs g/L	Biomass of Bacillus subtilis after 24 hrs each day	Percentage inoculum	pH of the medium
-none-[a]	Amount of Reducing Sugar used after 24 hrs g/L	Correlation	1.000	.070	.213	.169
		Significance (2-tailed)	.	.559	.073	.155
		df	0	70	70	70
	Biomass of Bacillus subtilis after 24 hrs each day	Correlation	.070	1.000	-.023	.731
		Significance (2-tailed)	.559	.	.851	.000
		df	70	0	70	70
	Percentage inoculum	Correlation	.213	-.023	1.000	.000
		Significance (2-tailed)	.073	.851	.	1.000
		df	70	70	0	70
	pH of the medium	Correlation	.169	.731	.000	1.000
		Significance (2-tailed)	.155	.000	1.000	.
		df	70	70	70	0
Percentage inoculum & pH of the medium	Amount of Reducing Sugar used after 24 hrs g/L	Correlation	1.000	-.075		
		Significance (2-tailed)	.	.539		
		df	0	68		
	Biomass of Bacillus subtilis after 24 hrs each day	Correlation	-.075	1.000		
		Significance (2-tailed)	.539	.		
		df	68	0		