OPTIMIZATION OF BIOETHANOL PRODUCTION FROM THERMOCHEMICALLY-PRETREATED WATER HYACINTH USING THERMOPHILIC MICROBIAL CONSORTIUM

BY

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January, 2022

DECLARATION

DECLARATION BY THE STUDENT

This thesis is my original work and has not been reported for a degree in any other university.

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I dedicate this thesis to my loving husband, brothers and sister. Thank you so much for being so close to me and for your help with this fabulous thesis project.

ABSTRACT

The rapid depletion of fossil fuels and their associated environmental consequences has sparked interest in the production of biofuel (bioethanol) from lignocellulosic biomass, such as water hyacinth (WH). However, the lignocellulose's refractory nature renders it difficult to convert to bioethanol, thus necessitating its pretreatment to increase enzymatic hydrolysis. Unlike yeast that utilizes only (C6) sugar, thermophilic bacteria can efficiently convert lignocellulosic hydrolysate (C5 and C6) sugars to bioethanol. The main objective of this study was to optimize bioethanol production from WH pretreated with $NaClO_2$ in boiling water using thermophilic consortium. The specific objectives were; to isolate and characterize thermophilic bacteria using morphological and biochemical approaches, to develop microbial consortium, to pretreat and characterize WH , and to optimize the production of bioethanol with microbial consortium. The bacteria were isolated using serial dilution and plating technique on nutrient agar. Microbial consortium development was based on the degradation of filter paper and untreated WH. WH was treated in boiling water for 4hr with and without $NaClO_2$ addition. Fourier transform infrared (FTIR) analyses were studied on $NaClO_2$ with boiling water-treated and raw WH. The main factors that affect ethanol production such as temperature (40-60^oC), time(48-96hr), and inoculum dosage(8-12%v/v) were chosen to be optimized by central composite design (CCD). The results of this research showed that nine thermophilic bacteria were identified and designated BO1, BO2, BOY, BOW, SO, OL, NW, YF, and CF. The bacteria isolates were bacillus, cocci, gram-positive, and gram-negative. Physiological characterizations indicate that all isolates could grow at temperatures 50-55^oC, NaCl concentration of 2% (w/v), and a pH of 5.5-8.5. The biochemical features of the isolates showed that all of the isolates were positive in glucose fermentation, starch hydrolysis, and EMB agar fermentation, but the results of the other biochemical tests were different. Microbial consortium, developed from three isolates (BO1, BO2 & OL), were efficient at degrading filter paper and untreated WH as substrates. The time yielding maximum total reducing sugar (TRS) was 2nd hr resulting in 155 mg/g WH and 113 mg/g WH from, with and without $NaClO_2$ addition pretreated samples respectively. FTIR characterizations of the pretreated sample revealed both breakdown and an increase in cellulose and hemicellulose content. The CCD indicated that the optimum conditions for fermentation were inoculum dose 8.1 %(v/v), temperature 48.8^oC, and time 52.3hrs, which resulted in 7.2g/L predicted ethanol concentration. Meanwhile, 7.7g/L ethanol was produced during experimentation which is in close agreement to predicted value. Conclusively, utilizing $NaClO_2$ and boiling water as pretreatment method and thermophic consortium as fermentation microbes is a good alternative for TRS and bioethanol production. This study suggests that more variables be tested in the pretreatment of WH to optimize TRS and reduce inhibitory byproducts.

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LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of Variance
BM	Broth Media
CCD	Centeral composite design
DF	Degree of Freedom
DNS	Dinitrosalicylic Acid
EMB	Eosin Methylene Blue
FTIR	Fourier-Transform Infrared Spectroscopy
HPLC	High Performance Liquid Chromatography
IR	Infrared Radiation
KIA	kligler Iron Agar
MSA	Mannitol Salt Agar
MSE	Mean Square Error
RSM	Response Surface Methodology
STD	Standard Deviation
TRS	Total Reducing Test
TSI	Triple Sugar Iron
WH	Water Hyacinth

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CHAPTER 1: INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Fossil fuels are the main sources of primary energy supply in the world, which contribute more than 80 % of the total energy production (Climent-Font & Perlado, 2013). However, these resources have recently faced a number of challenges, including a continuous rise in price and the depletion of raw materials (Widjaja et al., 2015). Recent studies predict that oil, natural gas, and coal will run out in 53, 54, and 110 years, respectively (Puiu, 2021). The global depletion of these energy resources, which are the world's principal sources of energy, has driven significant research efforts in recent years to develop alternative and sustainable substitutes (Chang et al., 2011). Furthermore, the global warming caused by these energy sources has prompted nations to explore alternate sources that are derived from renewable energy resources such as wind, solar, and biomass, as well as being environmentally friendly (Park et al., 2012).

Renewable energy is derived from sources that do not deplete and can be replenished in a short period of time (Climent-Font & Perlado, 2013). Biofuel derived from lignocellulose is one of the most cost-effective of these resources. Lignocellulosic biomass is derived from forestry, agricultural, and agro-industrial waste and are among the most important resources for biofuel and chemical production (Sawatdeenarunat et al., 2015). Carbohydrate within the biomass can be transferred to fuel (bioethanol) for transportation (Davis et al. 2018).

Bioethanol, which is produced from lignocellulosic biomass and fermented with microbial, is a fuel option for liquid transportation systems around the world (Singh et al. 2017). It is considered as a major energy source for the future due to a number of advantages, including the fact that it is clean, pollutant-free, abundant, and cost-effective (Cooper et al. 2020).

Water hyacinth is usually blamed for depleting nutrients and oxygen from water bodies, increasing evapotranspiration, and reducing biodiversity, which could (Guerrero et al.2015;. (Malik, 2007). Due to its abundant availability and high carbohydrate contents, water hyacinth highly satisfies the requirements as a potential substrate for bioethanol production (Ganguly et al.2012 ; Rezania et al. 2015). The dry biomass of water hyacinth mainly comprises low lignin (7–26%) and high amount of cellulose (18–31%) and hemicellulose (18–43%), which can be easily hydrolyzed to reducing sugars and then fermented to bioethanol by effective yeasts (Bergier et al. 2012).

Cellulose and hemicelluloses can be hydrolyzed into fermentable sugars (García et al., 2013; Avci et al., 2013). Cellulose with a particular crystalline structure that is insoluble in water is resistant to depolymerization. Hemicellulose, which provides structural backbone to plant cell wall, is a branched polymer of glucose or xylose. Lignin provides further strength to plant cell walls, but hinders the enzymatic hydrolysis of carbohydrates. Such properties considerably decrease the digestibility of the hemicellulose and cellulose present in the lignocellulosic biomass (Badiei et al., 2014).

In order to minimize such drawbacks, several pretreatments have been proposed to enhance the digestibility of lignocellulose biomass. There are several studies focused on the chemical deconstructing of lignocellulose, including thermal liquefaction (Kozliak et al., 2016), acid and alkaline hydrolysis (Loow et al., 2016), enzymatic hydrolysis (Maitan-Alfenas et al., 2015), microbial (Bhalla et al., 2013), steam explosion (Neves et al., 2016), mechanical milling (Khan et al., 2016), ionic liquid (Padrino et al., 2018) , ammonia fiber expansion (Qiao et al., 2018), liquid hot water (Suriyachai et al., 2020), among others. The steps in conversion of lignocellulose to valuable products are simplified in systematic diagram below (Fig.1.1).

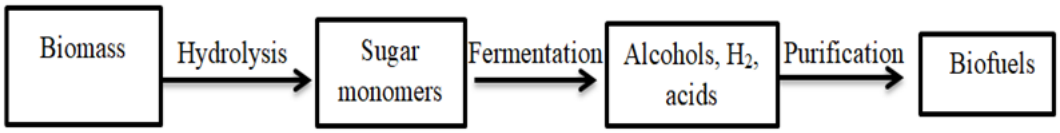


Figure 1. 1 Systematic conversion of lignocellulose to biofuel.

Among those different technologies, hot water pretreatment requires no chemical or less, short residence time and is considered as energy efficient method. Under high pressure and temperature water becomes an acid and causes autohydrolysis of glycosidic bonds, which results in solubilization of hemicellulose and partially delignification of the lignin and this increases susceptibility of the cellulose fraction to enzymatic hydrolysis (Lonkar e al. 2017; Raita et al. 2017). The best technology for the conversion of lignocellulosic materials into bioethanol is decided on the overall cost, environmental impact and energy efficiency (Raja Sathendra et al., 2019).

Among the different fermentation process, consolidated bioprocessing (CBP) method is an attractive single unit operaion where more than one microorganism that exists in nature performs the enzymatic hydrolysis and fermentation in a single step. Thermpholic bacteria as compared mesophilic organism offer the advantage of utilization of both monosaccharides (pentose and hexose) along with reistance to inhibitors produced during fermentation (Singh et al., 2017). Moreover, the microbial consortia exhibit other attractive features of being more stable, productive and functional (Liu et al., 2019).

An effective optimization tool is needed to increase the production of bioethanol . (Mohapatra et al., 2020). Response Surface Methodology (RSM) has been proven by several researchers to be very efficient, cheap, and fast process optimization tool. It is a multivariate statistical technique that allows the determination of multivariate equations for the

experimental data to give an experimental data to give an optimized experimental design (Senaras, 2019).

Bioethanol process optimization using RSM models has been previously reported (Mihajlovski et al., 2021). There are two submodels of RSM as Box–Benkhen design and central composite design (Borkowski, 2012; Rakić et al. 2014). Central composite design (CCD) tool was used in this study. Therefore, the present study focused on process optimization of bioethanol production from thermochemically (boiling water and $NaClO_2$) pretreated water hyacinth fermenated with microbial consortium using CCD.

1.2 STATEMENT OF THE PROBLEM

The cost of fossil fuels is increasing, making their use as an energy source more expensive and unsustainable. As a result, it is essential to seek for alternate and sustainable energy sources. Bioethanol, which is made from lignocellulose, is one of the alternatives to petroleum.

Although lignocellulose biomass is readly available, their chemical composition created a highly resistant and recalcitrant biomass structure. Therefore pre-treament is important to decompose their structure. But most existing pre-treatment methods are expensive and uses environmentally harmful chemicals and there is a loss of sugar due to washing of hydrolysate.

Water hyacinth spreads rapidly over water surface, thus reduces biodiversity, blocks rivers and drainage systems, and changes water chemistry that leads to severe environmental pollution.

Yeast (*Saccharomyces cerevisiae*) ferment only ferment glucose and not xylose (Nogueira et al. 2016). Comparison to this, thermophilic bacteria can utilize both type of monomers and convert to ethanol (Sommer et al. 2004).

Bioethanol, which is obtained from readily available WH, offers an alternative to petroleum consumption. Even with these readily available alternate fermentative feedstocks due to ineffective production process, the production of bioethanol still encounters difficulties. This lead to low yield of bioethanol from this resources.

The aim of this study, is therefore process optimization of bioethanol production using RSM approaches from thermochemically pretreated waterhyacinth and thermophilic consortium as fermentation microbes.

1.3 JUSTIFICATION OF THE STUDY

Numerous studies are currently focused on the replacement of fossil fuel with biofuels with the main objective of minimizing cost of fuel and greenhouse gas emissions. As aquatic biomass, water hyacinth has an advantage over land lignocellulosic biomass as it does not compete with food for land usage. Water hyacinth is available all over the world; the conversion of this biomass to bioethanol creates a job opportunity for small-scale and industrial bioethanol producers from international, regional, and national contexts.

The common factors that are commonly evaluated in the fermentation stage: fermentation time, inoculum size, process temperature need to be optimized using RSM to provide more accurate conclusions.

1.4 SIGINFICANCE OF THE STUDY

Water hyacinth develops quickly on the water surfaces and produces a thicker covering, causing a loss of water and aquatic life. As a result, converting water hyacinth to energy has a dual purpose: it helps to minimize the spread of the weed into lakes while also providing raw materials for bioethanol production. Bioethanol is an ecologically beneficial, cost-effective, renewable, and long-term energy source. This will allow rural residents to replace their paraffin cooking stoves with more ecologically friendly bioethanol burners..

1.5 OBJECTIVES OF THE STUDY

1.5.1 Main Objectives:

The main objective of this study was to optimize production bioethanol from thermochemically pretreated WH using thermophilic consortium.

1.5.2 Specific objectives:

In order to achieve the main objective of the the study, the following specific objectives where accomplished.

i. To isolate and characterize thermophilic bacteria using morphological and biochemical approaches
ii. To develop microbial consortium
iii. To pretreat and characterize WH
iv. To optimize the production of bioethanol with microbial consortium.

1.6 SCOPE OF THE STUDY

This study is limited to the isolation of thermophilic bacteria and their morphological, physiological, and biochemical characterisation. The in-situ characteristics of the locations, the composition of water hyacinth and carbohydrate after pre-treatment, as well as fermentation by-products, were not determined. The FTIR is the only technique applied to characterize hydrolysate.

CHAPTER 2: LITERATURE REVIEW

2.1 Intrduction

In this chapter, lignocellulosic biomass and biofuel, pretreatment technologies, bioethanol production process, biofuel production from water hyacinth, microorganism in fermentation are reviewed.

2.2 Biomass

Biomasses are resources that are readily accessible around the world as residual wastes and agricultural biomass. The most important and abundant renewable biomass resources include crop residues, such as corn straw, wheat straw and rice straw (Amin et al., 2017). Biomass resources, can be used in: direct combustion, anaerobic bacterial bioethanol and biogas production) and catalytic processes exist that aim at liquid or gaseous products (Mäki-Arvela et al., 2012).

2.3 Biofuel

Biofuel is any fuel derived from biomass that is: plant, or animal waste or algae (Selin et al. 2021). They are usually classified as first-generation, Second-generation and third-generation biofuels (Lee & Lavoie, 2013; Roland Arthur Lee, 2013). First generation biofuels mainly utilize plants rich in carbohydrates (i.e. sugar and starch) to produce ethanol or oils such as canola and soybean for biodiesel production. The problem with first-generation biofuels is that as their use increases, demand for the feedstock will intensify and ultimately clash with fundamental agricultural endeavors such as food and fiber production (Vancov et al., 2012). Second-generation biofuels are defined as fuels produced from a wide array of different feedstock, ranging from lignocellulosic feedstocks to municipal solid wastes. The second generation biofuel due its advantages of reduced feedstock costs, non-edibility and increasing industrial efficiency attracted biofuel from those feedstoks. But efficient pretreatment is required to convert them to biofuel (Potprommanee et al., 2017). Third-generation biofuels

are, at this point, related to algal biomass but could to a certain extent be linked to utilization of CO_2 as feedstock (Lee & Lavoie, 2013).

2. 4 Potential of water hyacinth for bioenergy

Water hyacinth is the fast growing aquatic weed invasively distributed throughout the world and covers the surface of the water. As a result the there is a reduction of biodiversity, blockage of the river and drainage system (Pothiraj1 et al, 2014). Different research done in the past on how to remove this plant from the surface of the water but now much focus is on using water hyacinth for different application since it has lignocellulosic composition of 48% hemicellulose, 18% cellulose and 3.5 % lignin (Gunnarsson CC, 2007). Since the productivity of this biomass is very high, it can be suitable feedstock for bioethanol, hydrogen and bio char production (Gunnarsson CC, 2007; (Nigam, 2002; Jiu et al, 2015). Due to its abundance and high carbohydrate content, water hyacinth highly satisfies the requirement as potential for bioethanol production as a substrate (Ganguly et al., 2012; Rezania et al., 2015). Harun et al. (2011) reported that water hyacinth includes a range of pentose and hexose sugars that may be fermented to bioethanol. Water hyacinth, like any other lignocellulosic plant, requires pretreatment before being hydrolyzed and fermented by microbes..

2.5 Pretreatment Technologies

Pretreatment of lignocellulose (figure 2.1) is a procedure in which the complicated component of lignocellulose is broken down into simpler components like cellulose, hemicellulose, and lignin (Kumari & Singh, 2018). Pretreatment is known as the most expensive processing step throughout the conversion of lignocellulosic biomass to fermentable sugars.

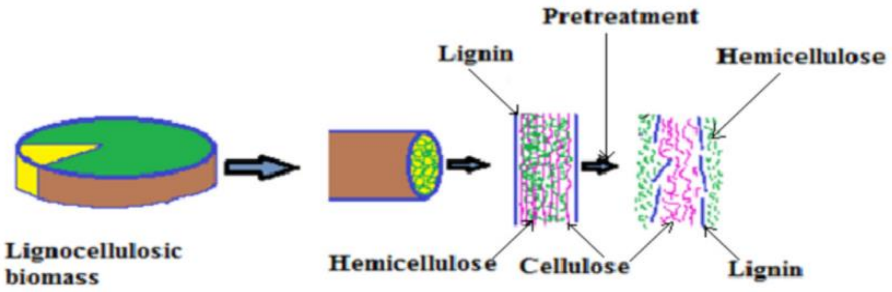


Figure 2. 1 Effect of pretreatment on lignocellulose. Source: (Machineni, 2020)

Effective pretreatment, therefore, should be able to (i) enhance sugar yields for downstream processing, (ii) treat all types of lignocellulosic feedstock, (iii) assist in lignin recovery for subsequent combustion, (iv) lead to less formation of co-products or inhibitors, (v) minimize energy and operation costs, and (vi) regenerate valuable lignin co-products (Kumar & Sharma, 2017). The choice of pretreatment methods relies on economic factor, the type of lignocellulosic feedstock, and its environmental impacts (Menon & Rao, 2012). Different pretreatment methods are listed in Figure 2.2.

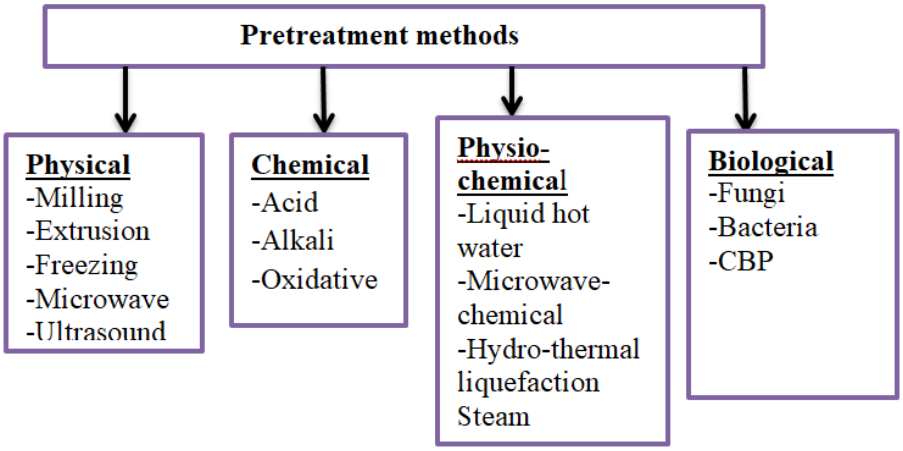


Figure 2. 2 Different pretreatment technologies (Chandra et al., 2012)

2.5.1 Physical pretreatment

The physical pretreatment method includes: grinding, freezing, chipping, milling and radiation (H. Chen et al., 2017; Kumari & Singh, 2018). This method reduces particle size,

increase surface area of the biomass. However, this procedure is ineffective when it is used alone, and it must be used in combination with other pretreatment techniques (Kumari & Singh, 2018).

2.5.2 Chemical pretreatment

a. Alkaline pretreatment

The alkaline pretreatments method mainly depends on alteration in lignin structure de-crystallization of cellulose and partial solvation of hemicellulose (Ibrahim et al., 2011). NaOH, KOH, $Ca(OH)_2$ and ammonium hydroxide are suitable for alkaline pretreatment of lignocllulose (Mirmohamadsadeghi et al., 2016; Saratale et al., 2016). This pretreatment method is advantageous in terms of: can be carried out at room temperature, less amount of water is required for washing the chemical. However, the treatment process can take hours or even days, and a substantial chemical cost recovery is necessary (Rezania et al., 2017) and results in lower sugar yield as compared to acid pretreatment method. This method proved to be more effective on agricultural waste than on wood biomass (Saratale et al., 2016)

b. Acid pretreatment

Acid pretreatment involves the use of concentrated and diluted acids to break the rigid structure of the lignocellulosic material (Brodeur, 2013). The most commonly used acid is dilute sulphuric acid (H_2SO_4) and has been studied for a wide range of lignocellulosic biomass (Chang et al., 2011; Boontum & Phetsom, 2019). The acidic pretreatment, results in high recovery of the hemicellulosic sugar and in solid cellulose fraction with enhanced enzymatic convertibility. According to Cheng's report (Cheng et al., 2013), microwave assisted dilute acid (H2SO4) pretreatment of water hyacinth resulted in a large amount of hemicellulose conversion into xylose, galactose, and arabinose, but only a small amount of cellulose and lignin hydrolysis into glucose and propiolic acid, respectively. However, there

are several disadvantages to employing acid pretreatment, such as the high cost of the materials used to construct the reactor and the generation of inhibitory byproducts (Jönsson & Martín, 2016).

Acid-Chlorite ($NaClO_2$) pretreatment

Acid chlorite mainly acts on lignin in lignocellulosic biomass, can also degrade the polysaccharides (Hubbell & Ragauskas, 2010). Naseerruddin (Naseeruddin et al., 2013), found that when dried prosopis juliflora 10% (w/v) is processed with 3% (w/v) NaClO2, the lignin removal is 36.05%, which confirms this report. However, according to a report by Grierer (Grierer, 1986), NaClO2 can also degrade cellulose and the cellulose degradation during acid chlorite delignification is due to acid cleavage of glycosidic bonds and oxidative degradation of polysaccharides.

2.5.3 Thermo- chemical pretreatment

Liquid hot water (LHW)

LHW pretreatment is also known as hydrothermal process and is more affordable, requires no or less chemical, short residence time and simple as it does not require special reactor or intense processing condition (Suriyachai et al., 2020). Among the available pretreatment methods, LHW pretreatment has several distinct advantages, including extensive hemicellulose removal and favorable environmental effects (Chen & Ni, 2021 ; Kim et al., 2009).

2.5.4 Biological Pretreatment

Biological pretreatment are advantageous over the other method because of: no need of chemical, performed in mild environment, ecofriendly, efficient and cheap alternative for

biofuel production. The most common biological pretreatment methods are microbial consortia, enzymatic pretreatment, and fungal pretreatment (Kumari & Singh, 2018). The type of microbe used, the nature and type of substrate used, as well as the cultivation methods and conditions used, all influence the efficiency of biological delignification (Tsegaye et al., 2019).

Microbial consortium pretreatment

Microbial consortium can degrade both cellulose and hemicellulose which is not the case for fungal pretreatment in which only lignin is degraded. According to the study of Zhang (Zhang et al. 2011), a microbial consortium made up of thermophilic bacteria collected from landfills and decaying straw was mixed with wastewater and used to pretreat cassava trash at 55°C for 12 hours, resulting in 96% more methane production than untreated waste. In other study of Zhang (Qinghua Zhang et al. (2011), consortia were developed from mixture of pure strain of yeast and celluloytic bacteria that were isolated from natural environment for the pretreatment of lignocellulosic biomass. Microbial consortium is the best biological pretreatment approach because mixed microbial sources are used in this procedure, which reduces the risk of contamination and the expense of maintenance (Kumari et al., 2018).

2.6 Bioethanol production

Bioethanol, as a clean, safe and renewable resource, is considered as a potential alternative to fossil fuels (Rezania et al. 2015). Basic systematic representation of procedure for bioethanol production figure 2.3.

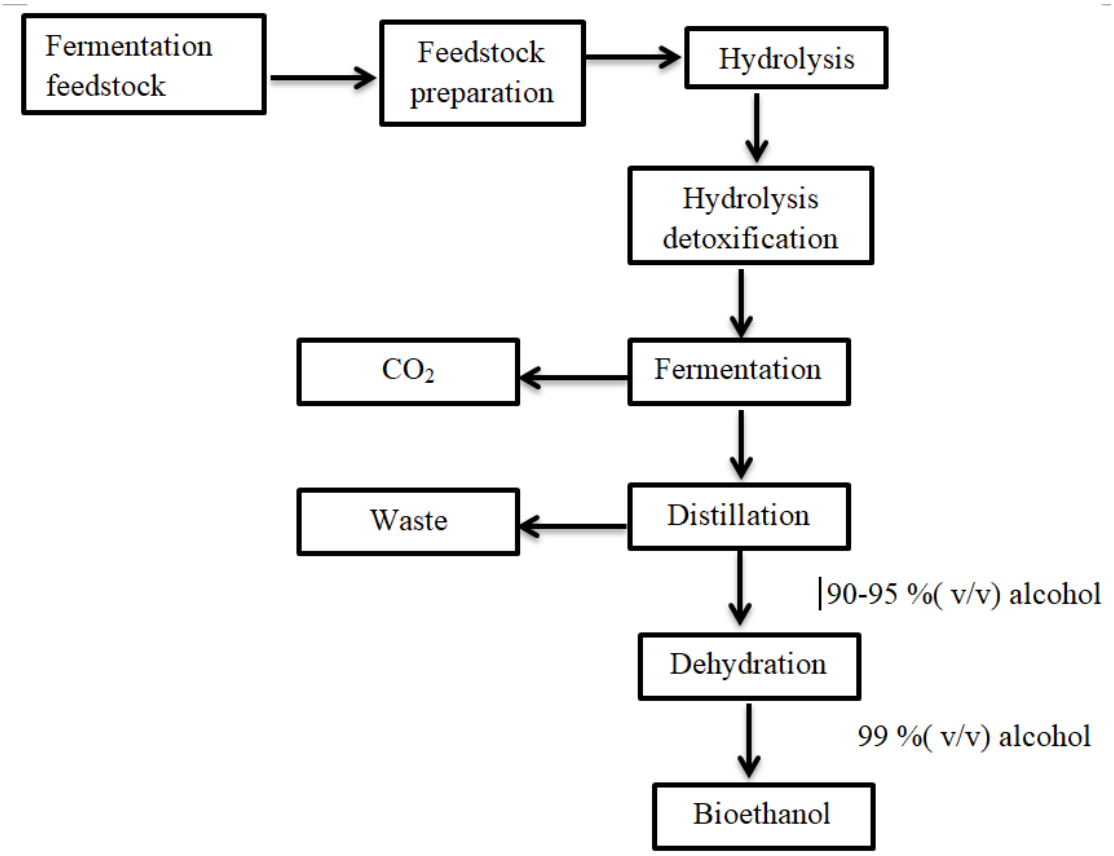


Fig. 2.3: Procedure for bioethanol production.

Consolidated bioprocessing (CBP)

CBP is a system in enzyme production; substrate hydrolysis and fermentation are accomplished in single process by lignocellulolatic microorganism (Agbor et al., 2014). The four biological activities take place in a single bioreactor (no need for an enzyme-producing reactor, as in separate hydrolysis and fermentation (SHF) and simultaneous saccharification and co-fermentation (SSCF). The bacterium produces its own enzyme, resulting in lower start-up costs. It is feasible to achieve higher efficiency as a result of the simpler feedstock process, shorter time usage, and lower energy input (Cao et al., 2014).

2.6.1The microorganism used in fermentation

The best and well known yeast for fermentation are the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis* (Orlygsson, 2012; Agric, 2009). Both can give yield of ethanol which is > 1.9 mol ethol/mole hexose. However, the main challenges in yeast

fermentation are: a) aeration, because yeast requires O_2 for cell wall synthesis, but aerobic conditions reduce ethanol yield; b) cooling of large fermenters below 39.6^{o}C, because yeast is killed above that temperature; and c) a limited number of resources, because ethanol-producing yeast can only ferment a limited number of substrates, not a complex polysaccharide. Thermophilic bacteria, on the other hand, can use a wider range of substrates and produce higher yields, making them a better choice for ethanol production from lignocellulose (Wiegel, 1980).

2.6.2 Ethanol production with thermophilic bacteria

It has been known for some time now that many thermophilic bacteria are highly efficient ethanol producers (Wiegel, 1980; Agric, 2009; Orlygsson, 2012). The majority of industrial ethanol production uses enzymes to break down cellulose and hemicellulose, as well as specific yeast strains designed to utilize C5 and C6 sugars. To make lignocellulosic ethanol production economically feasible, effective biomass hydrolysis and complete sugar conversion are required. Despite recent advancements by enzyme producers, the cost of cellulase enzymes remains in the range of $0.5 to $1.0 per gallon of 2nd generation ethanol (Olson et al., 2012; Klein-Marcuschamer et al., 2012). The microbial consortium also exhibit attractive feature of being more stable, productive and functional (Meng-zhu et al., 2016). Report of Liu et al. (2019), also suggest that ingenious designs of microbial consortia can be advantageous for their synergist division of labor and will prove to be resourceful for economical bioethanol production.

2.7 Process Optimization

Optimization study is the most critical part as it determines the efficiency of the whole production processes and subsequently its commercial viability for market exposure

(Barbanera et al. 2017). Therefore it is very important to establish the optimum conditions for the conversion of the water hyacinth into bioethanol.

Currently, the employment of optimization software such as Response Surface Methodology (RSM) has gained recognition as its competence in optimizing the process parameters by reducing the workload as well as the production cost (Chehreghani et al. 2017). Furthermore, the employment of RSM for optimization brought an obvious advantage as it offers a large amount of information from a few experimental runs, which subsequently is expected to be able to reduce the expensive cost of the analysis. RSM based on central composite design (CCD) is advantageous as it is an efficient design that is ideal for sequential experimentation and provides a reasonable amount of information to test lack of fit while not requiring an excessive number of design points (Jambo et al., 2019).

2. 8 CONCULUSION TO LITERATURE REVIEW AND THE GAP

The literature review survey realized the majority of recent research conducted in support of biofuels industries, with the primary goal of providing an environmentally friendly and low-cost as an alternative to the current energy source, fossil fuel. Waste to energy (bioethanol) is now being researched with various lignocellulosic resources as a potential substitute to petroleum for car transportation. The conversion of lignocellulose to bioethanol depends on the composition of the lignocellulosic biomass. Cellulose, hemicellulose, and lignin are the major constituents of lignocellulosic biomass. Agricultural waste, wood waste, bagasse, and water hyacinth are lignocellulosic biomass sources.

Despite the fact that those biomass resources are abundant and inexpensive, pretreatments are required to easily hydrolyze them to simple sugar and, as a result, complete conversion of the sugar to ethanol with the microbe. Different pretreatment method was used for delignification and hydrolysis water hyacinth. Most of this pretreatment method are acid pretreatment

(mainly dilute sulpheric acid), alkaline such as NaOH, $Ca(OH)_2$, biochemical are among the others. But most of this pretreatment method have inhibitory byproduct and are not environmentally friendly.

Yeast, commonly *Saccharomyces cerevesia*, ferments the sugar or hydrolysate produced after pretreatment. This yeast can ferment glucose but not xylose. Thermophilic bacteria, on the other hand, can ferment both glucose and xylose and produce thermostable enzymes.

A larger yield of ethanol is obtained from microbial consortium fermentation than from a single strain. The use of microbial consortia in consolidated bioprocessing will reduce risk of cross contamination.

Lignocellulosic biomass conversion requires ignocellulosic numerous stages, such as pretreatment, enzymatic hydrolysis, and fermentaion, not to mention the underlying costs and time, especially during enzymatic hydrolysis and fermentation. For all these reasons, this technology still is not attractive for industrial use. RSM method has helped to optimize the bioethanol production stages with a view to obtaining an economicall process and to improving process efficiency.

To address current shortcoming, this research focuse on optimization of process parameter for bioethanol production from thermochemically pretreated water hyacinth with less inhibitory byproduct from hydrolysate and the fermentation of the hydrolysate, with thermophilic bacteria that can ferment both glucose and xylose.

CHAPTER 3: MATERIALS AND METHODS

3.1. Introduction

This chapter presents the detailed experimental and analytical procedures that were adopted throughout this research. In summary, thermophilic bacteria were isolated from three different places and subsequently used for bioethanol production from pretreated water hyacinth (WH) as microbial consortium and single strain in this research. Bioethanol yields and total reducing sugar (TRS) were analyzed using standard spectrometrry procedures.

Chemicals and reagents

All chemicals used in this research were of analytical grade (AR) and were purchased from Sigma Aldrich- Germany. They included the following: Peptone, Sodium Chloride (NaCl), Triple sugar iron, Klinger Iron, Phenol red broth, nutrient agar, Phenol red, glucose, Trisodium citrate, ethanol alcohol, $FeSO_4$, K_2HPO_4, KH_2PO_4, Lactose broth, H_2O_2, Oxidase disk, $MgSO_4$. $7H_2O$, yeast extract, NH_4SO_4, Mannitol salt agar, starch, Crystal violet, gram's iodine, $CaCl_2$, HCl, $NaClO_2$, Acetic acid, H_2SO_4, Acetone NaOH, ethanol and Dinitrosalycilic.

Equipment and Apparatus

The equipment and apparatus used in this study were:, test tubes, rubber stopper, inoculating loop, autoclave, incubator, refrigerator, centrifuge, micropipette, Bunsen burner, rubber stopper, petri dish, flasks, cotton swap, thermometer, cotton wool, inoculating loop, pH meter and thermo flask.

The apparatus used for analyses where, pH meter (1630 labtech digital), microscope (Cari zeiss Gmbh, Germany)**,** FTIR (IRAFFINITY-1S-CE) and UV/Vis Spectrophotometer (DU 720* General Purpose).

3.2 Study site and sampling

3.2.1 Study site

Samples for isolation of thermophilic bacteria were collected from three different sites. One sample was taken from biogas sludge found in Moi university main campus. Moi University is a public university located at latitude of 0° 17' 3.2"N and longitude of 35° 17' 31.2"E, in Kesses, Uasin Gishu County, in the former Rift Valley Province of Kenya. There are two bio-digester producing biogas from cow dung in this campus. Both digesters were functional during sampling and one of the digester was new. During sample collection, the temperature of the digesters surroundings was 22^{o}C, whereas the temperature and pH of the digesters were 28 ^{o}C and pH 5.6 for the old digester and 25^{o}Cand pH 5.4 for the new digester. The second sample was from soil, found in Moi university corn farm located at 0° 17' 3.11"N and longitude of 35° 17' 31"E. During sampling the temperature and pH of the the soil was 30^{o}C and pH 6.7. The third and final site for isolation was from Lake Bogoria. Lake Bogoria is located 00 15' 30″ N and 360 06' 35″ E and is one of the soda lakes located in the Kenyan Rift Valley (East Africa). It is a saline, alkaline lake that lies in a volcanic region in a half-graben basin south of Lake Baringo, Kenya, a little north of the equator. The in-situ temperature of the surrounding was 37 ^{0}C while the hot spring and stream waterway temperatures of the lake were 92^{o}C and 50^{o}C, respectively. According to a Simasi, (Simasi, 2009) the volume and pressure of the hot spring were higher eleven years ago. The volume of steam has been decreasing over time due to an increase in the volume and pressure of the lake (Figure 5). The sampling site is located in the Chemurkeu area at the western shore of Lake Bogoria (0° 13′ 33″ N and 36° 05′ 41″ E).

The feedstock in this study, water hyacinth was collected from Ahero rice irrigation scheme next to river Nyando, 0°11 ′ – 0°19 ′ S, 34°47 ′ – 34°57 ′ E Lake Victoria, Kenya. Lake Victoria is one of the African Great Lakes. Water hyacinth has become a major invasive plant species in Lake Victoria due to human activity, has introduced the greenery to Lake Victoria, where it is claimed to have negatively affected local ecosystems.

3.2.2 Sampling procedure

Sample for Bacteria

On February 22nd, 2021, and March 16th, 2021, the first and second samples for bacterial isolation from an old biogas digester were taken; respectively. The 3rd time sampling was from both old and new digesters on 23rd March, 2021. The samples were taken with sterilized plastic container. The 4th and final samples were from Lake Bogoria, on 17th of April, 2021, using sterilized thermal flask to maintain in situ temperature of water. In a typical sampling procedure, the thermal flask was rinsed with hot water of the lake in order to reduce risk of contamination. The water sample was taken using sterilized thermos flasks at 92^{o}C and pH 8 from a hot spring (sampling point 1). Another sterile flask was used to collect wet sediments and microbial mats (sampling point 2) from the floor at a temperature of 56.7^{o}C and a pH of 8.4. The site for sample collection is presented in Figure 3.1

(a) (b)

Figure 3. 1 Lake Bogoria hot springs a. hot spring (sampling point 1), b. Lake Bogoria

Feedstock collection

In this study, water hyacinth (WH) was used as feedstock. It was taken from Lake Victoria, Kenya .The sample was uprooted with hand and collected with bigger market bag and then rinsed three times with tap water to remove the impurities. The root was removed and dried at room temperature for two weeks. It was further dried in the oven at 105^{o}C for 3 days to a constant weight. It was then ground by a mechanical grinder to powder, and sieved using sieve size 0.2 mm. The powder was then stored in plastic container for further use (Figure 3.2b).

Figure 3. 2 WH samples (A). oven dried sample and (B). the ground (powder) sample

3.3 Media and sample preparation and isolation of the bacteria

3.3.1 Media preparation

Nutrient agar was used as general media for the isolation of the bacteria and broth media (BM) was used for further study. Nutrient agar was prepared by dispersing 28gm of nutrient agar in to 1L of distilled water. Then the media was sterilized in anautoclave for 20 min. After sterilizing it was cooled to about 40^{o}C then dispensed in to sterile petridishes and waited to solidify. The BM per litre consisted of: 1g NH_4SO_4, 1g peptone, 2gm

$MgSO_4.7H_2O$, 0.1 $FeSO_4$, 0.1 $CaCl_2$, 2gm trisodium cirate , 1gm K_2HPO_4, 5gm yeast extract and 0.5gm KH_2PO_4 unless otherwise stated. The medium was prepared by sterilizing at 121$^oC$for 20-30 minute.

3.3.2 Sample preparation and isolation of bacteria

The bacteria were isolated using serial dilution and plating technique on nutrient agar. For biogas sludge and hot spring samples, serial dilution was 10^0 to 10^{-6}, but for soil samples, serial dilution was 10^{-3} to 10^{-6}. The entire culturing process took place on the same day as the sample collection. Then 1µm was then taken from all dilution and transferred to the prepared media and incubated at 50^oC in the incubator for 48hrs. After 48hrs the isolates where sub-cultured to get pure culture.

3.4 Characterization of the Isolates

3.4.1 Morphological characterization of the isolates

Characterization was done through macroscopic observation to the colony formed, texture and pigmentation (Rahman et al., 2017). Gram staining of each isolate was used to examine the morphology of bacterial cells under a microscope, and this was done in duplicate (Dussault, 1955) and observed under a light microscope at ×100 magnification (Keast et al., 1984). Gram staining involves three processes: staining with a water-soluble dye called crystal violet, decolorization, and counterstaining, usually with safanin (Bruckner, 2021). The results were then confirmed by another gram test using 3% potassium hydroxide (Cappuccino and James G., 2005).

The procedure of gram stain

The procedure of gram staining (Figure 3.3). Each step is followed by washing with tap water.

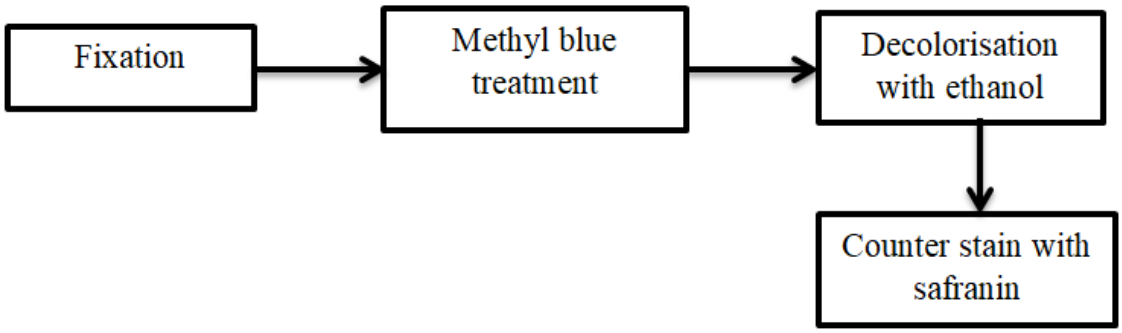


Figure 3. 3: The procedure of gram staining

Potassium hydroxide (KOH) test

KOH method is simple and rapid non-staining standard method for the determination of the Gram reaction (Fluharty, D. M., 1967). Gram stain technique is time consuming, costly, and often messy, and reagents must be replaced periodically. All gram-positive and gram-negative results obtained by staining were confirmed by the KOH technique. To perform the test, a drop of 3% KOH placed on slide. Then using sterile loop, visible amount of bacteria from nutrient agar grown culture were transferred to the drop of KOH and mixed thoroughly with solution for 60 second. If the bacterium-KOH suspension becomes markedly viscid or gels within 5-60 second the isolate is gram negative (Gram, 1982).

3.4.2 Physiochemical Test

Physiological tests were performed by growing isolates, based on their growth, on various NaCl concentrations, PH and incubation temperature as follows:

a.Effect of sodium chloride concentration on growth of isolates

For salt tolerance test, the isolates were grown on Nutrient agar media by adding 2, 4, 6, 8 and 10 (w/v %) of NaCl solution. The prepared NaCl solution was sterilized in autoclave for 20min. After that, each isolate was tested for NaCl tolerance by incubating it at the same temperature and period of time as it was isolated.

b. Effect of temperature on growth of the isolates

All the thermophilic bacteria that were isolated at 50^0C where tested for different temperature tolerance. The isolates were incubated at five different temperatures to determine their ability to grow at a wide range of temperatures: room temperature (22^oC), 35^oC, 50 ^{o}C, 55 ^{o}C and 60^oC separately on nutrient agar for 48hrs. The isolates that were grown at a temperature $\geq 50^o$C were used in subsequent experiments.

c. Effect of pH on growth of the isolates

The ability of the isolates to grow at acidic, neutral and alkaline conditions was determined by growing the isolate in BM. The pH of each set of experiments was adjusted to 5.5, 7.0 and 8.5. This pH range was chosen based on the pH of in situ from site of isolation. 5mL of BM was dispensed in a test tube, sterilized, and inoculated in triplicate with each of the nine isolates, then incubated at 50°C for 48 hours. The pH that generally allowed for the highest and the lowest growth were determined by measuring the optical density (Cappuccino, James G., 2005). Then the growths of isolates were determined by measuring the optical density in nm using the spectrometry at a wavelength of 600nm.

3.4.3 Biochemical characterization

All the isolates were subjected to different biochemical tests to allow for their characterisation. They were tested for: glucose fermentation, lactose utilization, Triple sugar iron, starch hydrolysis, mackonkey agar, mannitol salt agar, Eosine methylene blue agar, kliger Iron agar, catalase test, oxidase test, methylene red test and simmon citrate agar.

a. Carbohydrates fermentation test

Glucose fermentation test is done to establish if the isolate can ferment glucose and produce gases (Rahman et al., 2017). Phenol red broth base media was used for the fermentation test.

The phenol red base media was prepared by measuring 16.2g of the media and 1L of distilled water and mixing them together. Then separately 10gm of glucose was measured and mixed with 100ml of distilled water. After that about 5 mL of the mixture was added in to the test tube and sterilized by autoclaving at 121^0C for 30 min. Then it was allowed to cool for 40 min and the isolate of interest was inoculated in to the test tubes. For the control of experiment, a phenol red broth inoculated with the same isolate without glucose was used. The incubation took place at 37^oC for 18- 24 hrs. The preparation of the media and inoculation technique was based on the protocol on the media.

b. Lactose Fermentation Test

Lactose broth media was used for lactose fermentation test. The media was prepared by dissolving 13 g of the medium with 1L of distilled water. Then the media was transferred to test tube with inverted Durham test tube followed by sterilizing in autoclave at 121^oC for 30min, according to the instruction on the media. After that the media was cooled for 40min and the isolate of interest was inoculated in to the media and incubated at 37^oC for 18-24hrs.

c. Triple Sugar Iron agar (TSI) test

TSI test was performed to differentiate among organisms based on the differences in carbohydrate fermentation patterns and hydrogen sulfide production (Cappuccino, 2005). 64.4gm of the medium was dissolved in 1L distilled water. Then medium was transferred to a test tube and sterilized at 121°C for 30 min. The test tube is then cooled in a slanted position before being inoculated with the bacteria of interest. Following inoculation, the test tube was placed in an incubator and incubated for two days at 37°C (Janeiro, 2018).

d. Starch Hydrolysis Test

The capacity of the isolate to hydrolyze starch was studied by growing them on nutrient agar containing 1% soluble starch (Sharma & Singh, 2019). Nutrient agar was sterilized in an autoclave at 121℃ for 21 minutes. Then the media was cooled and poured in to sterilized Petridish plates and allowed to solidify. The bacteria were inoculated on the centre of the plates and incubated for 48-72hrs days at a temperature of 37℃. The plates were flooded with Lugol's iodine. Clear zone around culture is an indication of positive test.

e. Catalase Test

The catalase test facilitates the detection of the enzyme catalase in bacteria. It is essential for differentiating catalase-positive (*Micrococcaceae)* from catalase-negative (*Streptococcaceae)* (Reiner, 2013). A drop of 3% H_2O_2 was added onto the organism on the microscope slide by using inoculating loops. Then the formation of bubble is the positive indication for this test (Cappuccino et al, 2005).

f. Macconceky Agar Test

Macconkey agar is a bacteriological medium which selects for Gram-negative bacilli and differentiates lactose fermenters from non-fermenters (Naseeruddin et al., 2013). The media was prepared by dissolving 49.53g of the media in 1000ml distilled water. To completely dissolve, the media was heated and then sterilized in autoclave for 15min. Then the media was cooled and poured in to sterilized Petridish plates and allowed to solidify. The bacteria were inoculated and incubated for 48-72hrs days at a temperature of 37℃. Preparation of the media and incubation was according to the medium instructions.

g. Kligler Iron Agar (KIA)Test

This is a complex medium that contains a large amount of lactose and a very small amount of glucose; a pH indicator (yellow in acid and red in base); and iron, which is precipitated as a black sulfide if H_2S is produced. Lactose positive organisms yield a yellow slant and lactose

negative organisms yield a red slant. Cracks, splits, or bubbles in the medium indicate gas production (Jahan et al., 2017)**.** The media was prepared by dissolving 57.52g of the medium in 1L of distilled water. To completely dissolve, the media was heated and then transferred to test tubes and sterilized in autoclave for 15min. After stelization the media was cooled in slanted position and isolate of interest was inoculated and incubated for 48-72hrs at 37°C.

h. Eosin methylene blue (EMB) agar Test

EMB agar inhibits the growth of most gram-positive. Lactose-fermenting organisms produce pink colonies (Leininger et al., 2001). The media was prepared by dissolving 35.96g of the medium in 1000mL of distilled water. To completely dissolve, the media was heated and then sterilized in autoclave for 15min. After stelization the media was cooled and isolate of interest was inoculated and incubated for 48-72hrs at 37°C.

i. Mannitol salt agar (MSA) Test

Mannitol salt agar is a medium that contains a high concentration (about 7.5–10%) of salt (NaCl) which is inhibitory to most bacteria - making MSA selective against most Gram-negative and selective for some Gram-positive bacteria (*Staphylococcus*, *Enterococcus* and *Micrococcaceae*) that tolerate high salt concentrations. If an organism can ferment mannitol, an acidic byproduct is formed that causes the phenol red in the agar to turn yellow (Bachoon, 2008). The media was prepared by suspending 111g of Mannitol Salt Agar in 1000mL of distilled water, boiled to completely dissolve the medium, and then sterilized at 121°C for 15 minutes in autoclave and isolate of interest was inoculated and incubated for 48-72hrs at 37°C.

j. Oxidase Test

oxidase test was carried out by touching and spreading the isolated colony on oxidase disck. The reaction was observed for 5-10 seconds, according to the instruction on oxidase disk reagent. All the isolates were tasted tested in duplicate.

k. Methyl Red (MR) Test

Methyl red test was used to determine the ability of an organism to produce and maintain stable acid end products from glucose fermentation (Tille, 2014). The medium consisted of buffered peptone 7.0 g/l, dextrose 5.0 g/l, dipotassium phosphate 5.0 g/l. 5ml of the media was placed in test tubes and sterilized in autoclave for 20min. After that the media was cooled and inoculated with isolates and incubated at 37°C for 48hrs. Then after 48hrs, 3 drop of methyl red indicator was added to test tubes for glucose utilization and strong acid production with help of change in color in the growth medium.

m. Simmon's Citrate Agar Test

Simmons Citrate agar is used to test an organism's ability to utilize citrate as a source of energy. Sodium Citrate is the sole source of carbon in this medium. Bacteria that can grow on this medium produce an enzyme, citrate-permease, capable of converting citrate to pyruvate (Aryal, 2022). The media was prepared by dissolving 28.08g of simmon citrate agar in 1000ml distilled and heated to completely dissolve the media. Then 5ml was taken in to test tubes and sterilized for 20min and then cooled in slanted position. After the media became totally solidified, all the pure isolates were inoculated and then incubated at 35^0C for 48- 72 hr.

3.5 Substrate Utilization spectrum and microbial consortium development

3.5.1 Substrate Utilization spectrum

All the pure isolates in this study were separately tested under the same conditions for their ability to utilise different substrates. The experiment was conducted at a pH of 7 and a temperature of 50^oC. The pH was adjusted by supplementation of 1M HCl and 1M NaOH solution. Isolates were grown on a medium containing: glucose, starch, Whatman filter paper

(cellulose), water hyacinth using as sole carbon (energy) source. The Whatman paper consists of 99% cellulose (Agric, 2009). Growth of bacteria was monitored by determination of total reducing sugar(TRS) and optical density of the cultures (OD600) using UV Vis spectrometry.

The development of an efficient microbial consortium that is able to hydrolysis water hyacinth were based on isolates utilization of: monosaccharide (glucose), polysaccharide (starch), cellulose (filter paper Watman no.1) and lignocellulose (water hyacinth) respectively. The most efficient isolates, which grew the best on filter paper and on water hyacinth, were chosen for microbial consortium development.

a. Glucose Utilization

All the pure isolates were tested for their growth in BM media, with dextrose as sole carbon source. The control sample contained only glucose without inoculating with bacteria. The experiments were performed in 250mL conical flask with working volume of 100ml. The medium was supplemented with 10g/L, and then 20g/L glucose as carbon source. Thereafter they were incubated for 48 to 72hours. The growth was determined based on (OD600) values. The (OD600) was taken from zero hours to 72hours to observe the phase growth. The experiment was done in duplicate. This experiment was repeated for starch in the same way at the same condition.

b. Filter paper utilization

Among the nine bacteria isolates, four bacteria had showed maximum growth in a medium containing glucose and starch, hence they were tested for degrading cellulose (filter paper). The aim of inoculating the microorganisms in BM media with filter paper as carbon source was to isolate microorganisms which can degrade cellulose. In a typical procedure, the filter paper was first striped uniformly into small pieces. Then 1g of the paper stripe was added to four 250 mL conical flask containing 100 mL of BM then sterilized in autoclave at 121^{o}C for

20min. After the media were cooled, the four bacteria isolates were inoculated and incubated for 96 hours at 50^{o}C. Then the amount of reducing sugar was determined according to a method given by Miller (Miller, 1959).

c. Water hyacinth Utilization

All the isolates were tested to determine if they could grow in a BM medium containing 10g/ and 20g/l untreated water hyacinth as sole carbon source. Then 8mg, 10mg and 20mg of $NaClO_2$ were sublimated to increase lignin degradation; in consolidated bioprocessing to delignify water hyacinth based on method given by Elsamadony and Tawfik (Elsamadony & Tawfik, 2018) as acid-chlorite primarily acts on lignin in biomass.

3.5.2 Development of microbial consortium

Three bacteria isolates, from bio digester (OL) and two isolate from Lake Bogoria (BO1& BO2) which possessed cellulolytic (filter paper) activities were selected for consortium development. The development of consortium was by selecting two at a time. First the isolate BO1 and BO2 were co-cultured since they have isolated from the same area and they can grow at the same pH. This is because the pH tolerance behavior of bacteria is mostly dependent on the environment from where they have been isolated (Lawhon et al., 2013). Then the two co-culture and isolate OL where cultured together to obtain stable consortium. The microbial consortium was developed after nearly a month of repetitive culturing with microbial consortiums containing two and three bacteria at a time. The ability of a co-culture and microbial consortium to degrade starch and water hyacinth was evaluated as a co-culture and microbial consortium.

3.5.3 Effect of pH on Consortium growth

The optimal pH was found, after culturing at three different pH levels (5.5, 7, and 8.5). pH adjustments were done using either 1M of NaOH or HCl. Determination of growth was based on spectrometry value at (OD600).

3.6 Pretreatment of water hyacinth (WH)

Four different kind of pretreatment methods were used for powder WH including physical, $NaClO_2$& Acetic acid, dilute H_2SO_4, and boiling water and $NaClO_2$. A mechanical grinder was used to do the physical treatment. The remaining three are explained below.

3.6.1 NaClO2 and Acetic Acid pretreatment

The dried water hyacinth(WH) was treated with $NaClO_2$ and acetic acid for 4hrs (Siqueira et al., 2013). For each gram of WH, 0.3g of $NaClO_2$ (0.93%w/v), 0.1 ml acetic acid (0.31% v/v), and 32ml of water was added. The experiment was conducted in 1L conical flask in water bath at a temperature of 80^{o}C. Then after each hour, the same amount of acetic acid and $NaClO_2$ were added, for 4hrs. The sample was rinsed with water and acetone after 4hrs of pretreatment, and then dried in the oven at 105^{o}C to a constant weight.

3.6.2 H2SO4 Pretreatment

7g powdered WH was pretreated with 4 and 8% of H_2SO4 in 100mL of working volume in a 250L conical flask. The mixture was then heated for 20 min at 119°C in an autoclave. Then the treated sample was cooled to room temperature and the pH was adjusted to 8 using 6M NaOH to detoxify the hydrolysate. The hydrolysate was rinsed with 150mL distilled water to remove inhibitory byproducts and then placed in the oven at 60^{o}C for 2hr to remove volatile matter. The obtained hydrolysate was then placed in the fridge to determine the TRS obtained from hydrolysis as well as for further study.

3.6.3 Thermochemical pretreatment

In this method WH was pretreated thermally with boiling water and chemically with $NaClO_2$. Briefly, 3g of WH dissolved in 100mL of 1mg/1L of $NaClO_2$. Then this mixture was placed in boiling water bath for four hours. 2ml of the sample was taken every hour to determine the amount of reducing sugar in hydrolysate. Then after 4hr the entire sample was centrifuged at 6000rpm for 30min, and the supernatant was used to determine the amount of reducing sugar.

3.7 Production of Bioethanol

Fermentation experiment was carried out based on partially consolidated bioprocessing. Bioethanol was produced from WH, pretreated by boiling water assisted with low concentration acid $NaClO_2$. The full 20 experimental run were carried out from WH treated with this method. The process parameters for the partially consolidated bioprocessing approach were optimized using the central composite design (CCD) of Response Surface Methodology (RSM). The parameters that were considered were temperature (40-60^{o}C), time (48-96)hr and inoculum ratio (8-12v/v%). Fermentation was carried out in BM containing WH hydrolysate in 250mL conical flak cocked with non-return valves to insure anaerobic fermentation condition with 100mL of working volume (figure 3.4). The reaction was carried out at pH 7, which was the consortium optimal pH. The kinetic parameters of ethanol fermentation were determined as follows (Pothiraj1, 2014):

Ethanol concentration = ethanol produced (g)/l (**Equation 3.l**)

Figure 3. 4 Fermentation broth

3.8 FTIR Analysis of WH

Fourier transform infrared (FTIR) analyses were studied on acid with boiling water-treated and raw WH. The spectral data were acquired using a IRAffinity-1S FTIR spectrophotometer (Shimadzu Corp., 03191) equipped with an Attenuated total reflection (ATR). The instrument was set to perform a total of 20 scans with 4 cm^{-1} spectral resolution for both background and sample spectra, recorded rapidly at the range between 4000 - 400 cm^{-1}. The spectrum was obtained using Origin software version 2018.

3.9 Analytical Methods

Growth on different substrate was determined based on the OD600 using DU 720* General Purpose UV/Vis Spectrophotometer (Beckman Coulter Life Sciences). The optimum pH was measured using a pH meter (Digital pH meter rating). Concentration of reducing sugar was also analyzed by DU 720* General Purpose UV/Vis Spectrophotometer using DNS method (Miller, 1959) and was expressed as equivalent glucose concentration against calibration curve.

3.9.1 Quantification of Total Reducing sugar

The total reducing measurement was determined using 3, 5 dinitrocyclic acid Method (DNS) by using glucose as standard (Miller, 1959). Insoluble substrates were first filtered and separated by centrifuging the cultures in 12ml tubes at 6000 rpm for 30 minutes. The amount of reducing sugar in clear supernatants was then determined. Standard preparation of DNS, glucose solution and calibration curve are discussed below in section (a & b).

a. Preparation of standard DNS and standard glucose solution

The standard DNS solution preparation was done as follows: 1g of DNS reagent was dissolved in 20 mL of 2N NaOH reagent and separately, 30g of potassium sodium tartarate was dissolved in 50ml of distilled water. Thereafter the two solutions were mixed and shaken thoroughly before topping up to 100 mL. On the other hand, the standard glucose solution was prepared by dissolving 100 mg of HPLC grade glucose in 100ml distilled water to get 1 ppm of standard glucose solution (Gillespie, 2018).

b. Preparation of standard calibration curve

To plot graph, 5 different concentrations of glucose ranging from 0.1 to 0.5mg/l were used in five different test tubes, and the volume in each test tube was 2ml. The blank sample contained pure distilled water with a volume of 2mL. In each test tube with sample, 2mL of DNS reagent was added and thereafter the test tubes were placed in water bath for 10minute. After boiling, all the test tube was cooled with water to room temperature. After boiling, all of the test tubes were placed in cooled water and allowed to cool to room temperature. Then the intensity of the color developed was measured at 540nm using colorimeter recording against the blank. For the unknown samples reducing sugar content, 2 ml of the supernatant to be analyzed was pipetted into a test-tube and the same amount of DNS reagent added. Then the amount of unknown reducing sugar was determined from this standard graph. On the graph, the concentration was drawn on abscissa and absorbance was drawn on the ordinate.

3.9.2 Quantification of Ethanol

Ethanol concentration was determined by back titration with acidified potassium dichromate. When an alcohol vapour makes contact with the orange dichromate, the colour changes from orange to green. The degree of color change is directly related to the level of alcohol in the suspect sample (Anger et al., 2005). The ethanol concentration was analyzed via the spectrometric method by dissolving 7g of K2CrO7 in 5M of sulphuric acid at working volume of 250ml (Torres et al., 2020). To prepare the dichromate-alcohol reaction, 300 µL of alcoholic samples were mixed with 3 mL of dichromate solution and incubated at room temperature for 30 minutes. The absorbance was measured at 590 nm, and the ethanol content was calculated using an ethanol standard curve.

3.9.3 Development of standard calibration curve using K2Cr2O7

The HPLC grade ethanol was used to develop standard curve for quantification of ethanol concentration in the fermentation broth. The standard curve was prepared from ethanol concentration ranging from 0.0625 to 1.25% against blank (distilled water). The spectrometry values (absorbance) drawn along the y-axis and different ethanol concentration were drawn along the x-axis. This standard graph was then used to determine the unknown concentrations in all samples. The following equation is used to compute the ethanol concentration.

Ethanol concentration (g/l): %ethanol (v/v)*0.79g/ml (density of ethanol)*1ml/0.1L

(**Equation 2**)

3.9.4 Proximate Analysis

Moisture content in WH was carried out by gravimetric method. In a typical analysis, 1 g of WH sample was heated in the oven at 105^{o}C for 1 hour. Then the weight was taken every

hour by heating continuously for four hour until the weight became constant. Then the weight of the water was considered as moisture content by using a equation: % moisture = [weight of moisture/Weight of water hyacinth] x 100. volatile matter of WH was determined by heating 1g of moisture free WH in muffle furnance at 550^{o}C for 7 min. The hot crucible was taken out and cooled. The weight loss was taken as volatile matter. The ash content was then determined as follows, 1g of dry WH sample was placed in a weighted silica crucible, which was then placed in a muffle furnace at 550^{o}C for two hours. The crucible was then taken out, cooled, and weighed. The crucible was then taken out, cooled, and weighed. The weight loss was taken as ash content.

3.9.5 Experimental design

A Central Composite Design (CCD) was used to model experiment for optimization of bioethanol production using a microbial consortium. Experimental designs were performed using Design-Expert 2021 software version 13.0.5.0. The model was built with three factor and three levels to explore their effects and interactions, as well as their effect on the ethanol yield. The main factors that affect ethanol production, including fermentation temperature (40-60^{o}C), fermentation time (48-96hrs) and inoculums dosage (8-12%v/v), were chosen to be optimized by RSM using (CCD). A total of 20 experimental design matrix were obtained (see table Table 4. 1). Three dimensional plots and their respective contour plots were obtained based on this three parameters and level and their interactions on the maximum ethanol production by fixing the other parameters at their optimal conditions. From these contour plots, the interaction of one parameter with another parameter was studied.

Experimental significance of the obtained model was checked by F test (calculated P value) and goodness of fit by multiple correlations R, as well as determination of R^2 coefficients. Analysis of variance (ANOVA) was used to estimate the statistical parameters for maximum bioethanol yield. A P value < 0.05 was used as the criterion for statistical significance.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Introduction

This chapter presents results, analyses and discussion of various findings in the study area.

4.2 Isolation and screening of bacteria

The isolation of thermophilic bacteria for purposes of converting lignocellulosic WH was done in samples collected from biogas digester, soil and hot spring and the imagery results are presented in Fig. 4.1. Isolation of thermophile bacteria had been done at temperature 50^0C. The isolation of thermophilic bacteria from the bio digester and soil sample has not been reported before, but Simasi (Simasi, 2009) reported the isolation of thermophilic bacteria from Lake Bogoria. The in-situ temperature and pH was: 26-28^{o}C, 5.4- 5.7 pH for the biogas, 26^{o}C, 6.5 pH for the soil, 57^{o}C, 8.4 pH for microbial mat, and 90^{o}C, 8.5 pH for the water sample of the hot spring.

Thermophilic bacteria were successfully isolated from all of the three isolation area stated above. Despite the fact that the temperature of the biogas and soil in situ was lower than the normal site for thermophilic bacteria isolation, five pure isolates were successfully obtained. It was found that the decrease in temperature does not kill the thermophilic bacteria. Thermophilic bacteria are capable of forming endospores at ambient temperatures that do not supported by meshophilic bacteria (Simandjuntak & Samuel, 2018).

Total of nine bacteria strains isolated from different areas were encoded in the form of BO1, BO2, BOY, BOW, OL, NW, SO, CF and YF. In Figure 4.1, some of the plates for isolates are indicated. BO1, BO2, BOY, BOW were all isolated from Lake Bogoria, with BO1 obtained from a hot water sample and the other three from a microbial mat on one plate, with BO2, BOW, and BOY obtained after sub-culturing on nutrient agar plate separately.

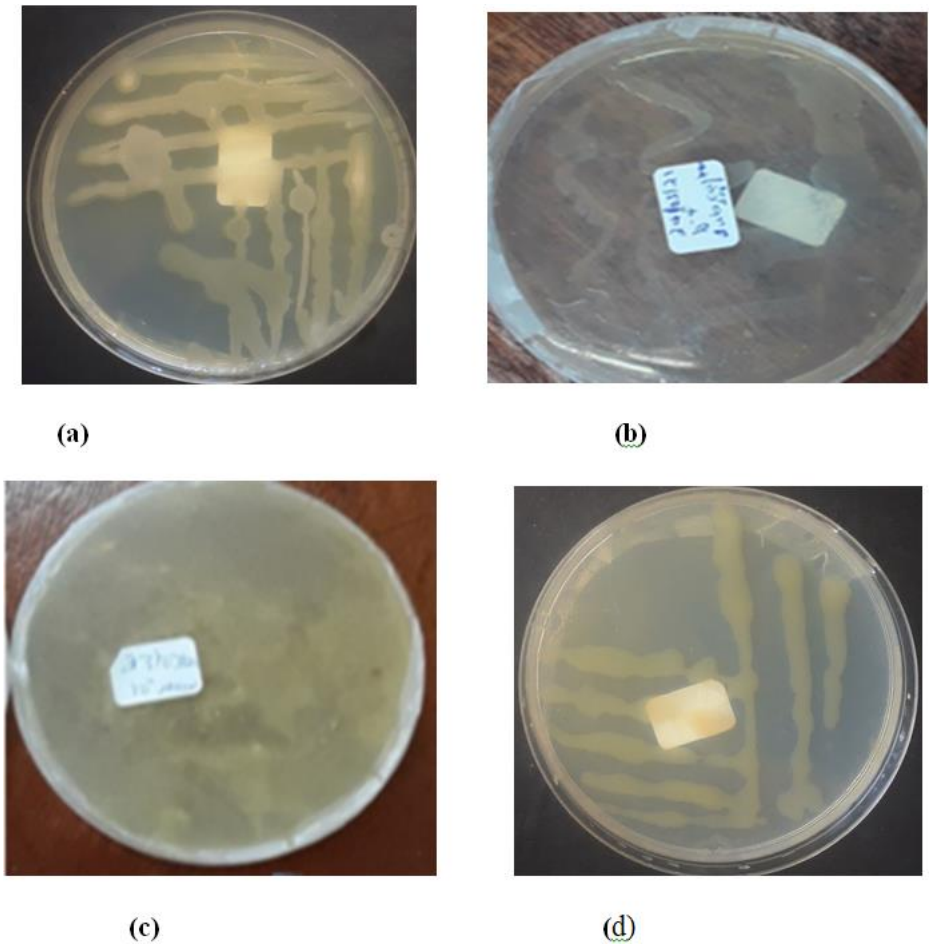


Figure 4. 1 Macroscopic representative for some of the thermophilic bacteria. (a) (YF) isolate, first isolate obtained from bio-digester. B, (SO) isolate from soil. c, (NW) represent, 2nd time isolate from bio- digester. D, (BO2) isolate from lake bogoria.

In comparison to microbial mat, hot water samples yielded a smaller number of thermophilic bacteria. This could be due to the fact that low diversity is common in hot areas. In addition, the number of thermophilic bacteria found in Lake Bogoria was lower than that reported (36 pure isolate) in literature (Simasi, 2009), which might be due to the difference in the isolation site and change in environmental conditions over time. Differences in the number of isolates obtained in each station can be influenced by site characteristics (Simandjuntak & Samuel, 2018).

Another probable reason for such low numbers of thermophilic bacteria could be due to the fact that the hot spring is located on the sandy area which is characterized with low carbon contents for microbes. In general it is the biotic components that is capable of supporting the

growth of thermophilic microorganisms, such as deciduous leaves, branches, grains, pollen, and insecticides found around hot springs as organic substances that can be utilized by living microorganisms in hot springs (Dirnawan, 2000). Furthermore, since two extreme conditions were imposed at the same time (high pH and elevated temperature), low diversity was achieved. The combination of two extreme conditions of physico-chemical growth parameters restricts the range at which microorganisms can proliferate more than in a single growth condition (Zeikus and Gregory, 1979) .

SO was isolated from soil, while OL, NW, YF, and CF were isolated from biodigester. The number of isolates obtained from the bio-digester and the soil sample was also lower. This could be due to the lower temperature of the environment, which is favourable to the isolation of thermophilic bacteria.

4.3 Characterization of isolates

4.3.1 Morphological characterization of isolates

Morphological characterization was based on macroscopic techniques of color, arrangement and shape of pure colonies. Macroscopic characteristics of the isolate are summarized in Table 4.1. Colonies ranged from white, cream, cream yellow to yellowish in pigmentation. The colonies of all isolates were very many to be counted.

Table 4. 2 Macroscopic characteristics of the isolate

Isolate Code	**Macroscopic Observation**				
	Color	**Light transmission**	**Elevation**	**Shape**	**Margin**
BO2	Cream	Translucent	Raised	Circular	Erose
BO1	Cream yellow	Translucent	Raised	Circular	Entire

BOY	Yellow	Opaque	Raised	irregular	Entire
BOW	White	Opaque	Raised	Filamentous	Erose
OL	Yellow	Opaque	Raised	Circular	Erose
CF	Cream	Transparent	Raised	Circular	Entire
YF	Cream	Translucent	Raised	Circular	Entire
SO	white	Opaque	Raised	Dome	Erose
NW	Yellow	Transparent	Raised	Circular	Entire

Microscopic characterization was performed using the Gram reaction and cell shape after simple staining. Gram staining is the first step for bacterial identification (Pelzar and Chan., 2006).

Microscopy revealed that all of the isolates from Lake Bogoria were Gram positive, with three rods (BO2, BOY & BOW) and one cocci (BO1), while the soil isolate was gram negative rod, and four of the digester isolates were gram negative cocci. Based on gram staining results, four of the isolates are *Bacillus* species, one gram negative and three gram positive. The remaining five isolates are gram-negative cocci. The *Bacillus* sp. has the characteristic of a straight rod-shaped cell, measuring between 0.5-2, 5 x 1.2-10 ¼ m and often clustered (Simandjuntak & Samuel, 2018).

All the isolates from Lake Bogoria's resemble the isolates reported by Simasi (Simasi, 2009) in terms of color, staining, in utilizing most of the carbon sources and majority of them belongs to genus *Bacillus*. The report of Duckworth , (1996) also found that majority of lake soda lakes are mainly associated with the diverse Bacilli taxon. *Bacillus* presence could be due to the genus capacity to move quickly and its tolerance to harsh environmental circumstances (Connor, 2010), as well as their adaption to hot environments (Aanniz, 2015);

Kawasaki, 2012). Table 4.2 presents a summary of gram reaction of the all isolates while Fig. 4.2 presents some of microscopic plot of gram reaction.

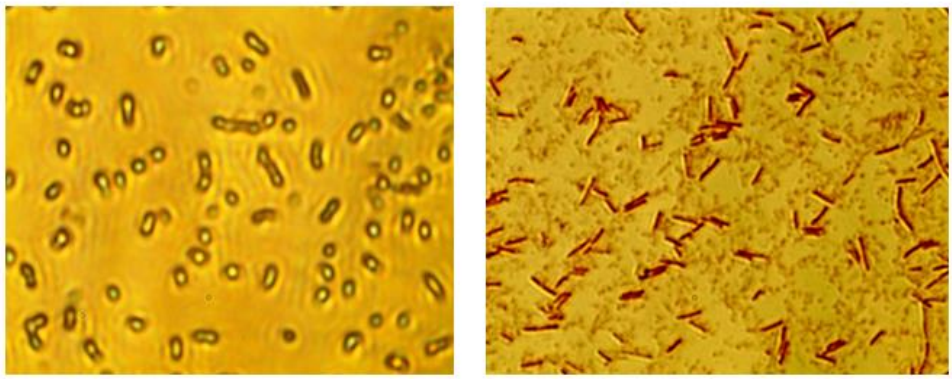


Figure 4. 2: Gram reaction of isolate (a). NW (b) SO

Table 4. 3: Summary of gram staining

Name of isolaties	Gram stain	
	Stain	shape
Nw	-	coci
OL	-	coci
YF	-	coci
CF	-	coci
SO	-	rod
BO1	+	coci
BO2	+	rod
BOy	+	rod
BOw	+	rod

4.3.2 Physiological characteristics

a. Effect of salt concentrations on growth

All of the isolate were tested for their resistance against different NaCl concentration and all the samples showed 2% (w/v) salt tolerance. CF and Nw isolates grew more on 2 and 4% NaCl-containing media than in a non-NaCl-containing medium while isolates OL, SO, and Yf grew less even at lower NaCl concentrations (2%) and three isolates from Lake Bogoria (BO1, BOW, and BOY) grew in concentrations ranging from 2 to 10%. But BO2 was less resistant to NaCl. In general, no halophilia was detected in any of the isolates. The salt tolerance properties for all isolates are summarized in the Table 4.4**.**

b. Effect of pH on growth of the isolates

The pH of the isolates covered all range, i.e., basic, acidic and neutral. But most of the isolate were found to grow from neutral to basic, rather than acidic enviroment. At all pH levels, isolate BOY showed the highest overall growth with (OD600 = 2.245). At pH 5.5 isolate, (OL (OD600 = 0.125) , BO2 (OD600 = 0.231), and NW (OD600 = 0.120) showed lower growth as indicated in Table 4.3 and Figure 4.3. Growth at a pH range of between 5.5 and 8.5

was consistent with earlier studies which reported growth at pH ranging from 5.7 to 9 (Takami, 2000).

Table 4. 4: spectrometry (OD 600nm) for different pH levels.

Isolates name	Absorbance value at 600nm		
	PH 5.5	PH 7	PH 8.5
BO1	2.142	0.435	0.326
BO2	0.231	0.372	0.418
BOW	0.380	1.079	0.519
BOY	2.245	2.035	1.682
OL	0.125	1.797	1.292
CF	0.316	0.979	0.701
YF	0.909	0.697	0.703
SO	1.048	0.494	0.545
NW	0.12	0.611	0.927

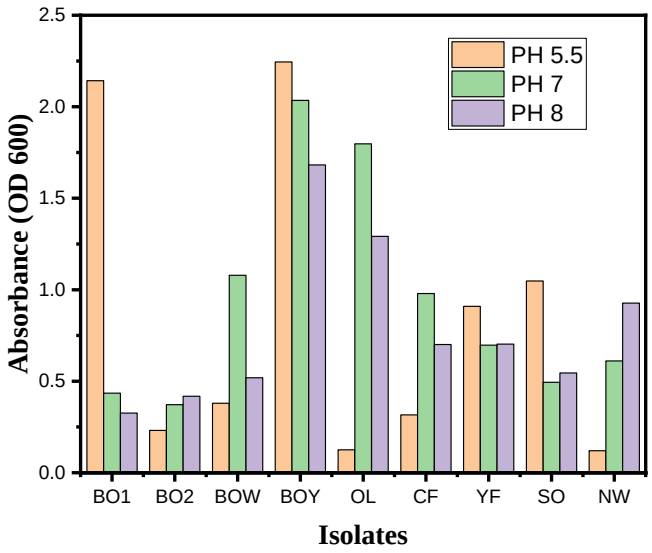


Figure 4. 3: Growth of the isolates at varied pH

c. Effect of temperature on growth of the isolates

The thermophilic bacterial isolates obtained from Lake Bogoria and Bio-digesters were able to grow at a temperature range of 35^{o}C–60^{o}C. The growth increased between 35 and 50^{o}C in

most of the isolates whereas at room temperature only two isolate, BOY and SO exhibited a little growth at room temperature. But the rest of the isolate didn't grow even after weeks of incubating at room temperature This indicates that the isolates are heat-loving and can survive from mesophilic to thermophilic condition. Microbial sustainability at high temperatures is caused because thermophilic bacteria have different protein structures compared to mesophyll microbes so that they are able to survive at extreme temperatures (Ifandi & Article, 2018). Physiological characterisics of the isolates are summarised in Table 4.4.

Table 4. 5: Summary of Physiological characteristics (+positive, -negative)

Name of the isolate	**NaCl tolerance (%)**					**Temperature tolerance**					**PH tolerance property**		
	2	4	6	8	10	22	35	50	55	60	5.5	7	8.5
Nw	+	+	+	-	-	-	+	+	+	+	+	+	+
OL	+	-	-	-	-	-	+	+	+	+	+	+	+
YF	+	-	-	-	-	-	+	+	+	-	+	+	+
CF	+	+	+	+	-	-	+	+	+	+	+	+	+
SO	+	-	-	-	-	+	+	+	+	-	+	+	+
BO1	+	+	+	+	+	-	+	+	+	+	+	+	+
BO2	+	+	+	+	-	-	+	+	+	+	+	+	+
BOy	+	+	+	+	+	+	+	+	+	+	+	+	+
BOw	+	+	+	+	+	-	+	+	+	+	+	+	+

4.3.3 Biochemical characterization

All the biochemical tests were carried out at 35- 50^{o}C, and replications were used to check the validity and precision of the results. The isolates were subjected to 12 various biochemical tests which were used in the characterization of the isolates as presented in Table

4.5. The biochemical responses of thermophilic bacterial isolates varied depending on the substrates used.

Results show that they were all positive as amylase producer (starch hydrolysis) thermophilic bacteria, glucose fermentation and EMB agar. In starch hydrolysis, the clear zone does not get stained by iodine solution **(**Figures 4.4 a & b), because in that zone the starch is already hydrolyzed into simpler compounds such as disaccharides or monosaccharaides (Simandjuntak & Samuel, 2018). In EMB media, *Enterobacter aerogenes* grows well and pink in color without sheen, but *Escherichia coli* grows with a green metallic sheen (Levine, 1918). All the isolate has shown pink color in EMB, with good growth. BO1, BO2, BOW and OL were oxidase negative. Aerobic and facultative aerobes exhibit oxidase activity whereas *Enterobacteriaceae* are oxidase negative (Rahman et al., 2017). Those two tests (EMB and Oxidase test) provided the evidence of *Enterobacter* in the samples. MSA is selective differential medium for some gram positive bacteria (*Staphylococcus* and *Enterococcus*) that tolerate high salt concentrations (Becton, 2005; Bachoon, 2008). In this test only NW was negative as MSA test. Lactose broth was also a differential medium and all of them were able to ferment lactose except BOY witch didn't grow in this media whereas BO1 fermented lactose with gas production. BO1, BOY, and BOW were TSI negative, whereas BO1 and BO2 were Mackonkey positive, BO1 and BOY were KIA positive, BO1, OL, and SO catalase positive, BOY and SO simmon citrate positive, and BO2, BOW, YF, and OL were all MR positive.

In comparison to the other SO and BOY were showing same result in most of their biochemical test, due to this reason they might belongs to the same species. Some of the representatives for biochemical test are indicated in (Figure 4.4) and the entire biochemical test is also summarized in Table 4.5.

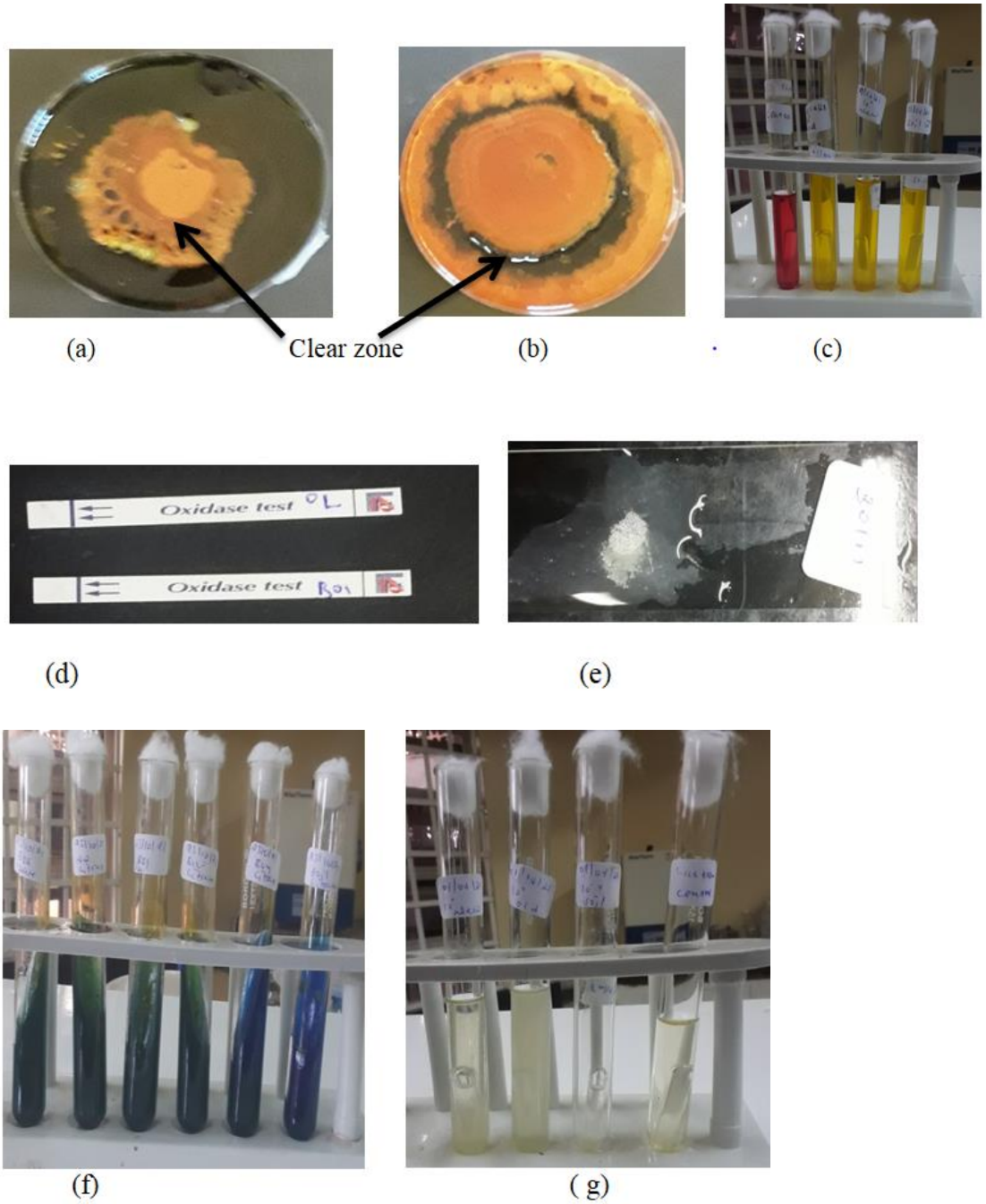


Figure 4. 4 Some representative of biochemical test. a(NW) & b(YF) starch hydrolysis (+) with clear zone, c(OL, Nw &SO) glucose fermentation (+) with yellow color (, d, oxidase (-) no blue color, e. catalase (+) with bubble , f. S. citrate test, only 2 tube on the right (+) with blue and g, lactose test

Table 4. 6: Summary of biochemical tests

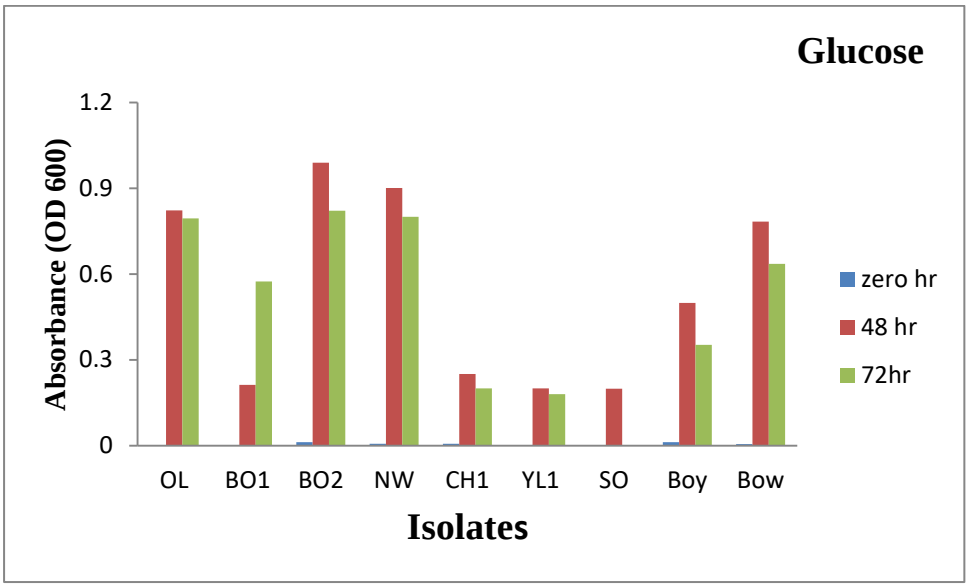
Tests	Isolates								
	BO1	**BO2**	**BOW**	**BOY**	**OL**	**CF**	**YF**	**SO**	**NW**
Glucose fermentation	+	+	+	+	+	+	+	+	+
LB	+	+	+	-	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+
TSI	-	-	-	+	+	+	+	+	+
Mackonkey	+	+	-	-	-	-	-	-	-
MSA	+	+	+	+	+	+	+	+	-
EMB	+	+	+	+	+	+	+	+	+
KIA	+	-	-	+	-	-	-	-	-
Catalase	+	-	-	-	+	-	-	+	-
Oxidase	-	-	-	+	-	+	+	+	+
MR	-	+	+	-	+	-	+	-	-
Simmon citrate	-	-	-	+	-	-	-	+	-

4.4 Microbial consortium development and substrate utilization property

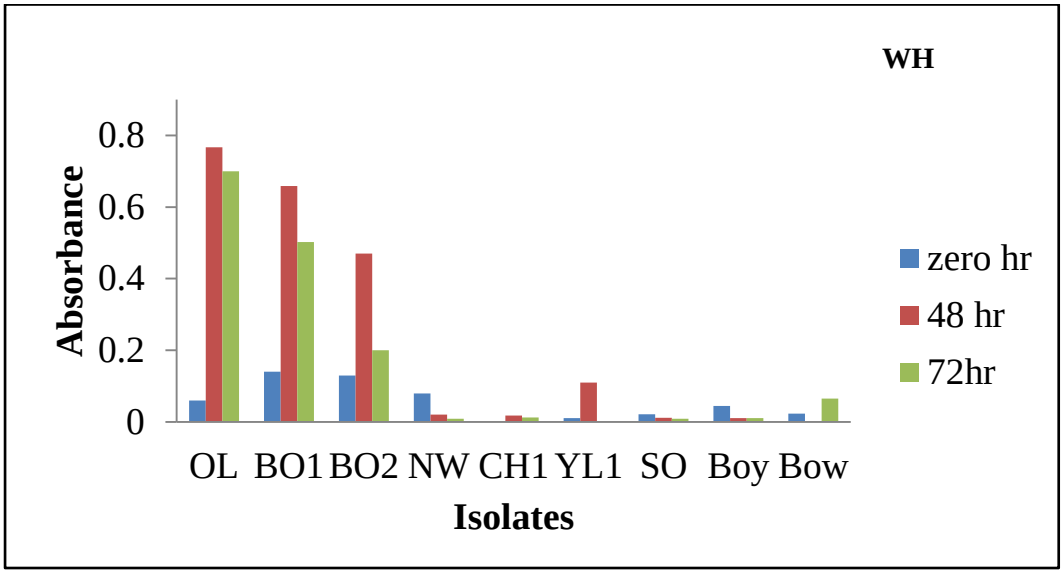
4.4.1 Substrate Utilization spectrum

The ability of strains to utilize different carbon substrates was tested using BM medium supplemented with multiple carbon substrates (glucose, starch, filter paper and untreated and pretreated WH). Except for glucose and starch, which were sterilized alone, other substrates were sterilized with medium. All nine pure isolates showed positive growth in BM containing 10g/L and 20g/L of glucose as a carbon source and the optimum growth was observed at 20g/L of glucose. But their rates of growth (OD600) were different. Then the nine isolates were grown in BM media containing 10g/ l and 20g/L untreated dry powder water hyacinth respectively with three replications. Out of the nine isolates only four isolates (BO1, BO2, BOW and OL) were able to grow in a medium containing water hyacinth. But the maximum growth was observed for only three isolate (OL, BO1 and BO2) with optimum growth condition at (OD600). Then those four isolate tested for their utilization of filter paper and out of the four only three isolates (BO1, BO2 and OL) were able to degrading filter paper.

This was indicated by their respective growth and the amount of reducing sugar obtained. Only the supernatant from those three isolates altered the color of DNS reagent from orange to brown, indicating that those bacteria degraded the filter paper. The amount of TRS obtained from 1g of filter paper using those three strains is tabulated in Table 4.6. Then these three bacteria were used as microbial consortium for further study. The OD600 for different substrate spectrum are are in Figure 4.5.



(a)



(b)

Figure 4. 5: a, Utilization of glucose vs untreated b water hyacinth by the isolates

Table 4. 7: Total reducing sugar obtained from 1g of filter paper

Isolate	Absorbance	Dilution factor (ml)	TRS (g/l)
BO1	0.266	3.5	0.63
BO2	0.323	3.5	0.764
OL	0.274	3.5	0.649

4.4.2: Microbial consortium Development

The capacity of a single strain and microbial consortium to degrade and produce bioethanol was evaluated. Only three isolates (BO1, BO2 & OL) with cellulolytic activity (FPcase) were chosen for microbial consortium development and were used to develop microbial consortium.

In comparison to the microbial consortium containing two strains, the microbial consortium with three strains shows higher growth and sugar yields (Figure 4.6, TRS obtained from WH). Apart from monitoring the growth of consortium, it is also important that the organisms can sustain multiple subcultures. Screening of microorganisms that possess the preferred characteristics is an important prerequisite to eliminate the inclusion of non-essential microbes that do not contribute to the desired product yield (Mohapatra et al., 2020). The design of a microbial consortium is important for the synergistic division of labour between microorganisms (Liu et al., 2019). Further research was conducted using these three bacteria as a microbial consortium. The consortium was able to grow in $NaClO_2$ concentrations of (8, 10 & 20mg/l) but less growth was observed at 20 mg/l. Although higher concentration of $NaClO_2$ enhanced the amount TRS, due to the decrease in the number of microbes 10mg/l of

$NaClO_2$ was used in consolidated bioprocessing and partially consolidated bioprocessing. The microbial consortium containing three isolate (BO1, BO2 & OL) gave higher TRS from water hyacinth (Figure 4.6).

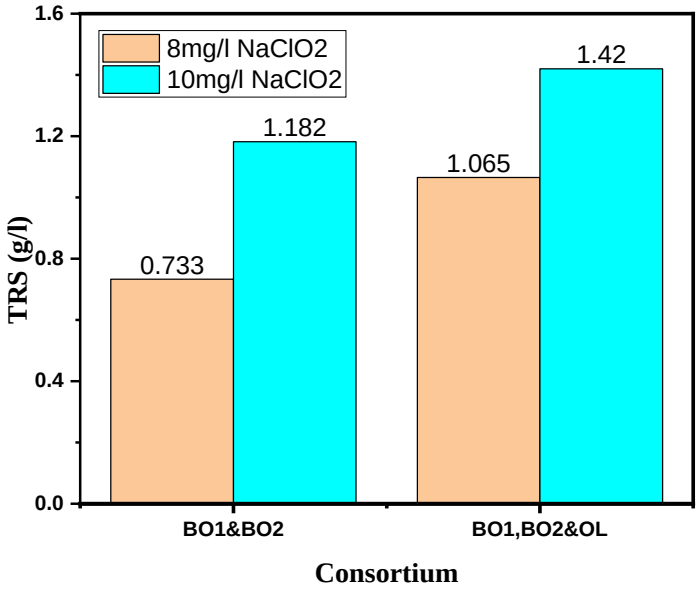


Figure 4. 6: The TRS obtained from WH when hydrolyzed with consortium (BO1 & BO2) and TRS from consortium (BO1, BO2 & OL) with direct supplementation of 8mg/l NaClO2 and 10mg/l NaClO2.

Effect of pH on the development of the consortium

The effect of pH on the consortium development was studied and it was found that the best pH was 7 and no growth was observed below pH 5. Thus, pH 7 condition was considered for all experiments.

4.5 Proximate analysis of WH and Effect of different pretreatment methods on yield of sugar

Proximate analysis of WH

From proximate analysis of untreated WH, the dry sample consists of: 5.6 % moisture, 78% volatile mater and 13.8% ash content.

Effect of different pretreatment methods on yield of sugar

The water hyacinth undergoes through four different pretreatment including physical pretreatment. From physical pretreatment, 54mg/g WH of TRS was obtained and this result was higher than some of the chemical pretreatment method used in this study. Suitable and effective pretreatment method should be selected for different cellulosic substrates (Qiuzhuo Zhang et al., 2016).

a. Thermo- chemical pre-treatment

The effect of pre-treatment from boiling water with and without $NaClO_2$ supplimentation results are presented in Fig. 4.7. Results showed that the amount of reducing sugar has increased from zero hour up to two hours and didn't show any increase after three hours. The total yields of sugar after pretreatment with and without $NaClO_2$, from 0- 4hr were 54, 102.5, 155, 153 and 142.9mg/g and 54, 88, 113, 111 and 108.2mg/g respectively. The yield values achieved in this study were higher than those reported by (Mishima , (2008), WH pretreatment with formic acid and acetic acid (88mg/g WH) and (97mg/g WH), respectively as well as those reported by (Qiuzhuo Zhang et al., 2016) who obtained 22.41 mg/g and 99.12 mg/g reducing sugars after alkaline pretreatment and microwave alkaline combined pretreatment, respectively.

Compared to the above mentioned methods, we used lower-cost chemicals, and there was no loss of polysaccharide that occurs during the pretreatment process due to washing, and the obtained reducing sugar in hydrolysate was directly fermented without washing, and no inhibitory problem was observed due to very low concentration (10mg/L) of acid used during

pretreatment. Thus, the combination method of boiling pretreatment with supplementation of low concentration chlorite acid described here was found to be promising.

The water is the medium of heat transfer that hot water is indirect contacted with the base of the glassware containing substrate (Barua and Kalamdhad, 2017). During thermal pretreatment, lignin is eradicated merely up to a certain limit. However the dissolution and depolymerisation/repolymerisation of lignin reaction creates the redistribution on the fibre surfaces again (Li et al. 2007 ; Kumar et al. 2009). Moreover, the quantity of lignin solubilisation is related to its degradation during pretreatment process (Vanderghem et al. 2015).

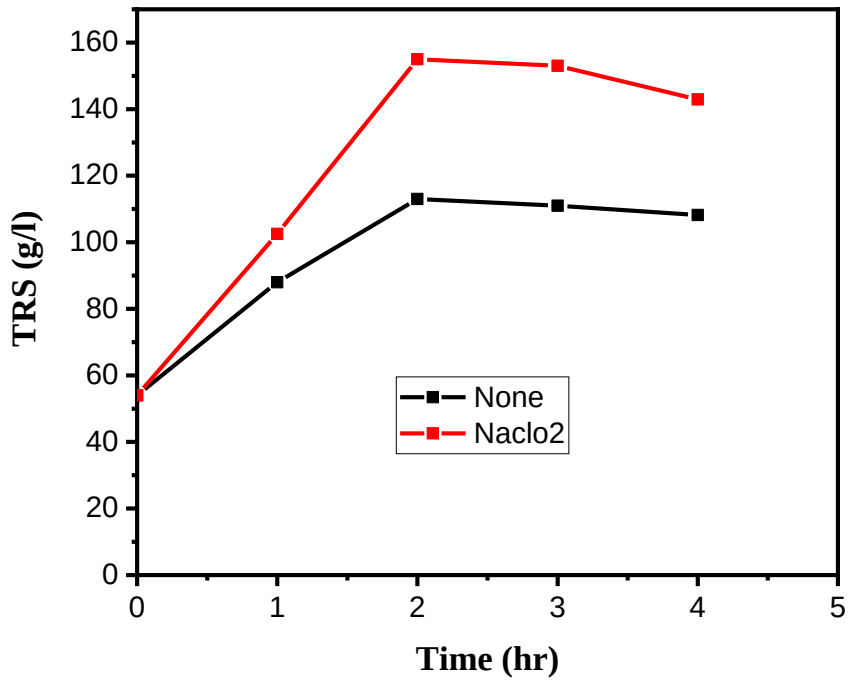


Fig. 4. 7: TRS obtained from boiling water only (black line) and boiling water with supplementation of 10mg/l $NaClO_2$ (red line).

b. H_2SO_4 Pretreatment

7 mg of dry WH was pretreated with 4 and 8% of H_2SO_4 in an autoclave with a working volume of 100ml and then a TRS of 441.4mg/g of WH was obtained from 4% of H2SO4 pretreatment. The obtained TRS was higher than some of the published results, 197.60 mg/g (Zhang et al., 2016), 430.66 mg/g (Qiuzhuo Zhang et al., 2018), and 342mg/g (Satyanagalakshmi K, 2011) when WH was pretreated by 1% sulfuric acid at 100^{o}C for 30 min. In agreement to our result, Idrees Idrees, (2013) reported a higher value of 484mg/g of WH with H_2SO_4 under optimized condition. Pothiraj reported that among different pretreatment methods used in earlier researches for WH, maximum reducing sugar was observed in diluted H_2SO_4 (Pothiraj1, 2014). However at the higher concentration of H_2SO_4 (8%) the amount of sugar was very low. In contrast, it has been reported that concentrated acid resulted in the release of high amounts of monosaccharide (Ra et al., 2015). This could be due to difference in factors used during the pretreatment. Acid concentration is an important parameter to enhance monosaccharide production (Ganguly, 2012).

During WH treatment with H_2SO_4 (4%), the same amount of sugar was lost from hydrolysate due to washing to remove the inhibitory from the hydrolysate. Similarly Zhang, (2016) reported that from 402.93mg/g sugar only 197.60 mg/g was remaining in hydrolysate. In acid pretreated biomass, extensive washing is necessary to remove acid before fermentation of sugars (Sassner et al. 2008). In acid pretreated biomass, extensive washing is necessary to remove acid before fermentation of sugars (Sassner et al. 2008). Despite being neutralized and cleaned, the hydrolysate was still inhibitory to the bacteria during fermentation. As a result, the hydrolysate from this pretreatment was not used in our study to produce bioethanol.

c. $NaClO_2$ and Acetic Acid pretreatment

One of the most prevalent and well-established method for the lignin elimination from lignocellulosic biomass is acid-chlorite delignification using an aqueous solution of acetic acid and sodium chlorite at moderate temperatures. The amount TRS obtained from $NaClO_2$ and acetic acid pretreatment was 23.33mg/g of WH and this was lower as compared to 97mg/g of WH obtained by Wang, (2006). This could be due to the difference in parameter used in the pretreatment and washing to remove the inhibitory by product.

4.6 Ethanol Production

Although the maximum reducing was obtained from dilute H_2SO_4 ,only the Hydrolysate from, boiling water with low concentration acid $NaClO_2$ at optimum reducing sugar condition was fermented by thermophilic microbial consortium. One of the main reason for increased interest in using thermophilic bacteria for second generation ethanol is because of their broad substrate spectrum (Jessen & Orlygsson, 2012). According to the experimental run, the maximum ethanol concentration was 7.7g/l which achieved at 50^oC fermentation temperature, 8 (% v/v) inoculum dosages and 72hr fermentation time which could achieve 7.561 g/l at this point, according to the predicted value of the model. On the other hand, according to the model numerical analysis and point prediction, the optimum conditions for maximum ethanol production were: inoculum dosage 8.101 % (v/v) temperature, 48.804^oC; and time 52.284hr with predicted ethanol concentration of 7.192 g/l. The actual yield is closer to RSM yield; this indicates the reliability of presented model.

These values are higher than ethanol concentration reported for some of the wild-type strains of the thermophilic cellulolytic bacterium *C. thermocellum*, an extensively researched candidate for thermophilic CBP: strain ATCC 27405 in fermentor, (4 g/l) of ethanol (Svetlitchnyi et al., 2013), strain Thermoanaerobacter J1 from hydrolysates obtained from different lignocellulosic biomass with highest concentration 1.56g/l (Jessen & Orlygsson,

2012), *Bacillus paranthracis* and *Bacillus nitratireducens* microbial consortia with the highest ethanol concentration (0.39 g/l) from 7.5 g/l of substrate loading (Mohapatra et al., 2020).

On the other hand Sato (Sato et al., 1993) reported a higher ethanol concentration of 23.1g/l under optimized medium after 168 hours of fermentation with *C. thermocellum* wild-type strain I-1-B from celluloytic biomass. Those differences could be attributable to the type of biomass, the effectiveness of the microbial consortium, the type of strain used in the consortium development, and, most importantly, the fermentation techniques used, as well as other factors influencing fermentation. The actual ethanol concentration obtained and the predicted ethanol concentration for 20 run is tabulated in Table 4.7.

Table 4. 8: Experimental design matrix prepared using central composite design with the experimental and predicted responses.

Run	Temperature(^{0}C)	Incubating time (hr.)	inoculum (%v/v)	Actual Ethanol (g/l)	Predicted Ethanol (g/l)
1	50	72	10	7	6.98
2	40	96	8	6.11	6.03
3	60	48	8	5.7	5.85
4	50	72	10	6.6	6.98
5	66.82	72	10	4.3	4.05
6	50	72	6.64	7.7	7.56
7	50	72	10	7.1	6.98
8	50	72	10	6.9	6.98
9	50	72	13.36	6.33	6.44
10	60	96	12	5.3	5.32
11	60	48	12	5.8	5.89
12	40	48	8	6.23	6.23
13	33.18	72	10	3.6	3.82
14	40	48	12	5.7	5.55
15	50	72	10	7.3	6.98
16	50	112.36	10	6	6.03
17	60	96	8	5.8	5.97
18	50	31.64	10	6.73	6.68
19	50	72	10	6.96	6.98
20	40	96	12	4.8	4.66

4.6.1 Mathematical Modeling and significant test of ethanol yield

Ethanol production from WH was mathematically modeled so as to assess the effect of different reaction parameters on ethanol production. To assess the effect of different reaction parameters on ethanol production, quadratic models were selected out of linear based on suggested model, lack of fit test and model summery statistic (Table 4.8). The actual

concentrations of ethanol obtained varied from 3.6 to 7.7g/l. ANOVA indicated a model F value of 42.02, which implies that the model is significant. Values of "Prob > F" less than 0.05 indicate that the factors B, C, AC, A^2 and B^2 are significant terms in the model that affect the production of bioethanol. Where A is the Temperature of fermentation (^{0}C) B is the time of incubation (hr.) and C is the inoculum dosage (% v/v). The"Lack of Fit F value" of 0.94 implies the lack of fit is not significant relative to the pure error. There is a 52.5% chance that a "Lack of Fit F value" this large could occur due to noise. The model predicted R^2 of 0.8856 is close to the adjusted R^2 of 0.9511 and the model adequate precision of 23.1632 indicates, the model can be used to navigate the design space. High R^2 value (0.9742) shows that the model described 97.42% variations of response variable leaving only 2.58 to the residuals. It was showed that the regression model was highly significant ($P < 0.05$).

ANOVA for Quadratic model

Response 1: Ethanol Concentration

Table 4. 9 Analysis of variance, quadratic model for total bioethanol production

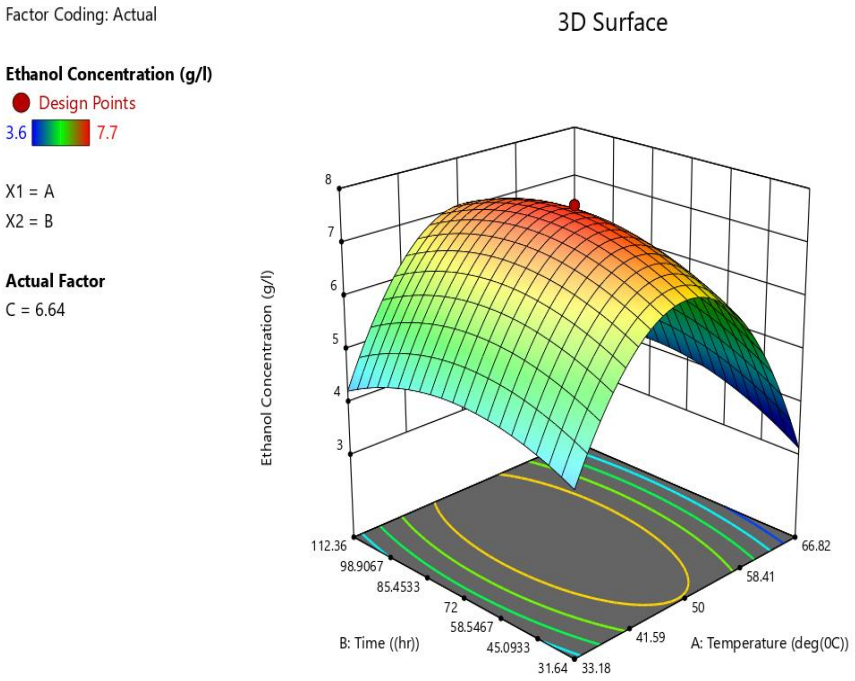
Source	Sum of Squares	df	Mean Square	F-value	P value Prob > F
Model	19.72	9	2.19	42.02	< 0.0001
A-temp	0.0643	1	0.0643	1.23	0.2926
B-time	0.5133	1	0.5133	9.85	0.0105
C-inocumn	1.51	1	1.51	29.00	0.0003
AB	0.0480	1	0.0480	0.9218	0.3597
AC	0.2592	1	0.2592	4.97	0.0499
BC	0.2381	1	0.2381	4.57	0.0583
A^2	16.66	1	16.66	319.58	< 0.0001
B^2	0.7062	1	0.7062	13.55	0.0042
C^2	0.0010	1	0.0010	0.0198	0.8910
Residual	0.5213	10	0.0521	-	-
Lack of Fit	0.2530	5	0.0506	0.9427	0.5250
Pure Error	0.2683	5	0.0537	-	-
Cor Total	20.24	19			

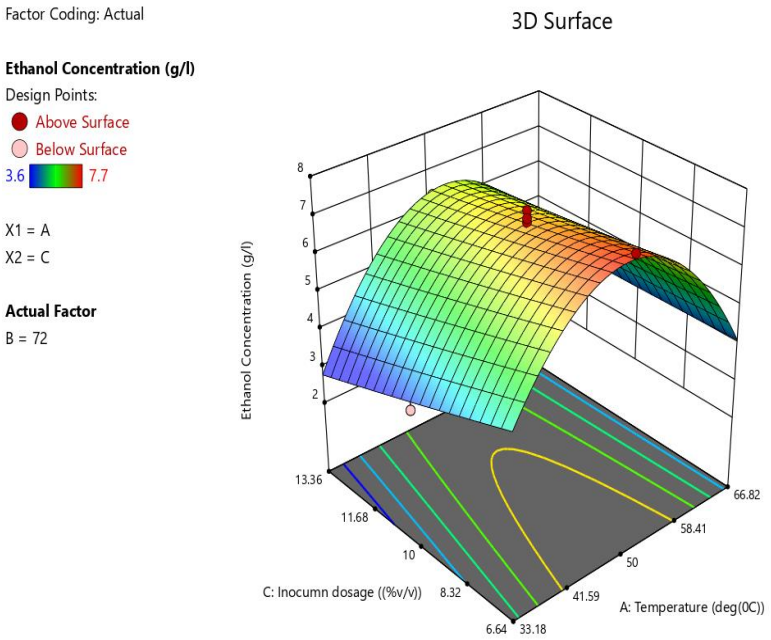
Figure 4.8 shows a three-dimensional response surface graphical diagram and Figure 4, 9 counter plot representing the interactive effect of the optimum condition. According to the

obtained result and its evaluation, this equation represents the effect of the different factors and interacting factors on Ethanol concentration (in terms of coded factors):

Ethanol concentration (g/l) = 6.98 + 0.0686A - 0.1939B - 0.3328C + 0.0775AB + 0.1800AC -0.1725BC - $1.07A^2$ - $0.2214B^2$ + $0.0085C^2$.

where A is the incubation temperature (^{0}C), B is the fermentation time (hr.) and C is inoculum dosage (% v/v)





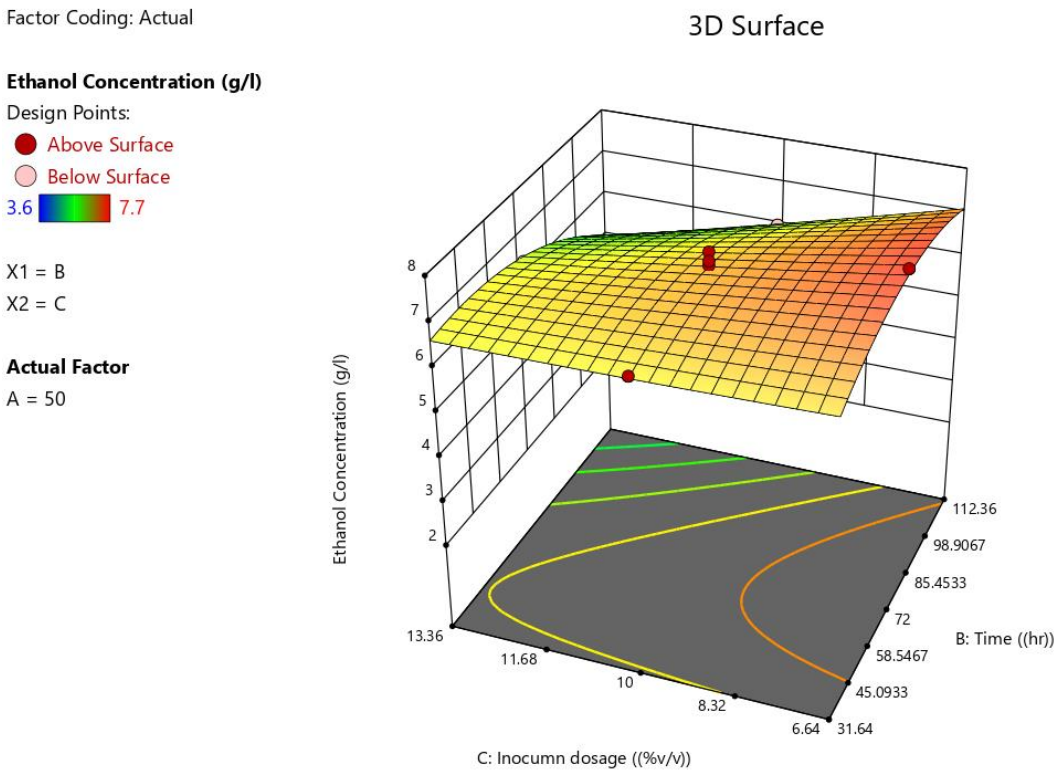
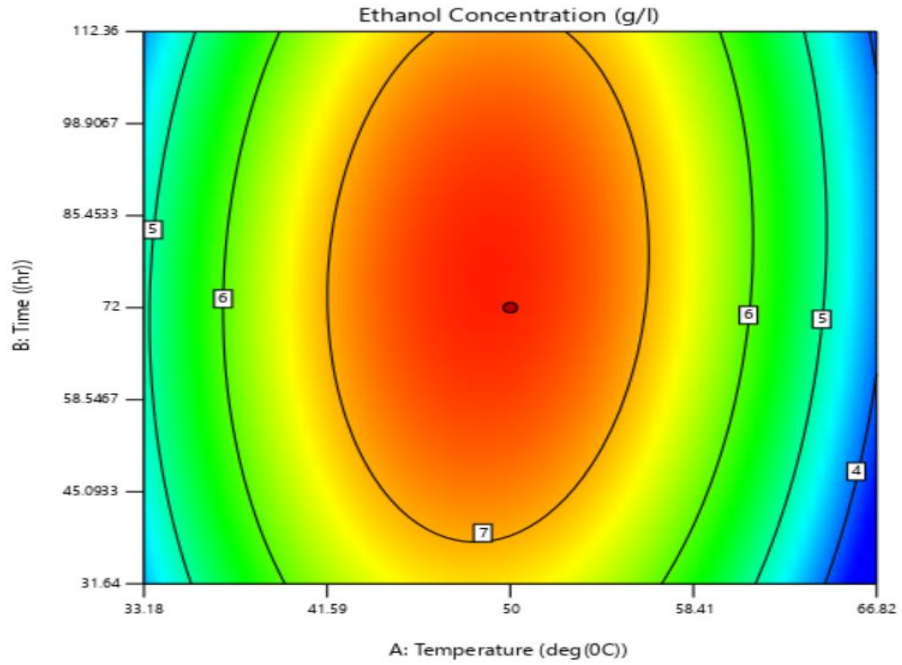
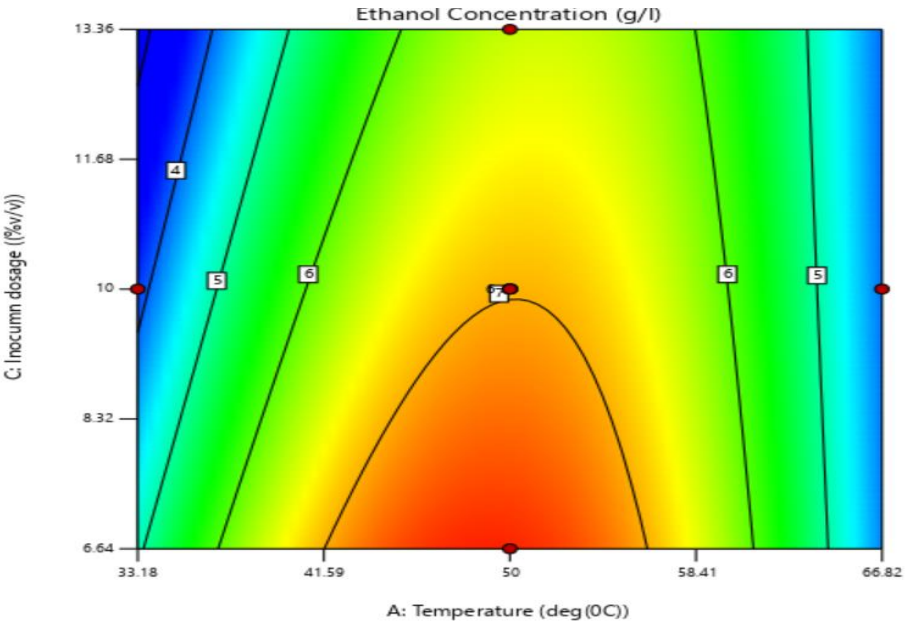


Figure 4. 8 Response surface plots showing the effect of temperature, inoculum dosage and time, and their mutual interactions, on production of ethanol 3D (a, b &c)


Ethanol Concentration (g/l)
112.36
98.9067
85.4533
72
58.5467
45.0933
31.64
B: Time ((hr))
5
6
6
5
4
7
33.18
41.59
50
58.41
66.82
A: Temperature (deg (0C))


Ethanol Concentration (g/l)
13.36
11.68
10
8.32
6.64
C: Inocumn dosage ((%v/v))
4
5
6
6
5
7
33.18
41.59
50
58.41
66.82
A: Temperature (deg (0C))

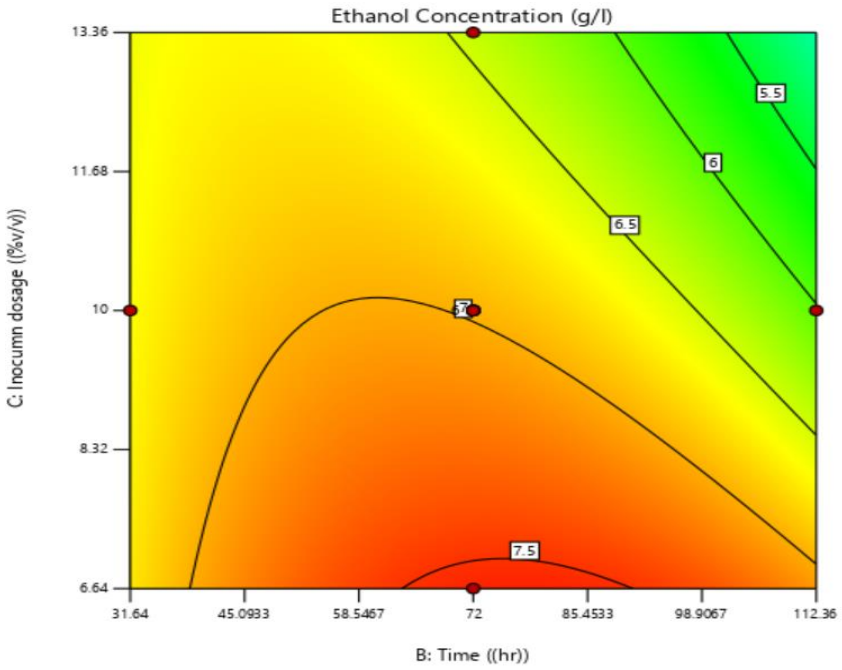


Figure 4. 9 Response of bioethanol production to fermentation time, inoculum dosage and fermentation temperature in 2D

4.6.2 Effect of mono-culture and co-culture fermentation

Both the single strain and the microbial consortia were examined separately for ethanol production from glucose. The microbial consortium obtained the highest ethanol concentration of 9.01g/l, which is 88.3 percent of the theoretical yield, whereas the highest concentration from a single strain was 3.22 g/l, which was obtained during fermentation with strain BO1. Microbial consortium produced 2.8 to 4 times the amount of ethanol produced by a single strain from glucose (Figure 4.10). (Svetlitchnyi et al., 2013) constructed dual Caldicellulosiruptor Thermoanaerobacter co-cultures revealed up to 8-fold increased ethanol yields compared to the monocultures of Caldicellulosiruptor strains and thermoanaerobactor**.** But the ethanol produced from WH was lower as compared to ethanol from glucose. This is due glucose can easily utilized by microbes and the WH contains lignocellulose component which is no easily degradable by microbes. Ethanol production by co-cultures was strongly dependent on their composition of the substrate (Svetlitchnyi et al., 2013)**.**

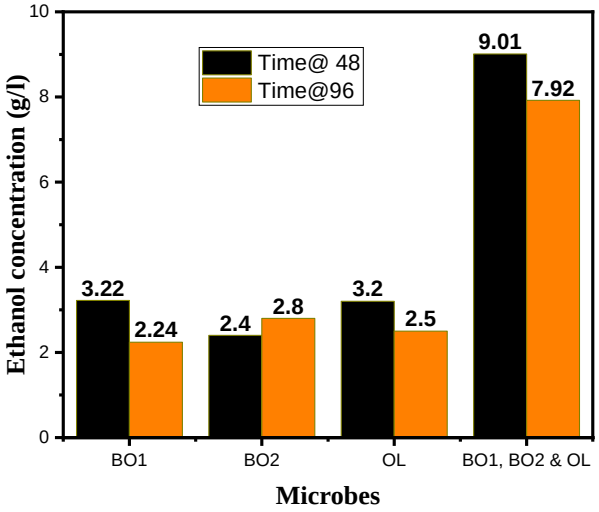


Figure 4. 10 Ethanol production from glucose fermentation with single strain and microbial consortium

4.7 FTIR analysis

The FTIR analysis was carried out to examine the changes in chemical composition and structure by comparing the FTIR spectra of WH before and after pretreatment (Figure 4.11). The spectra of untreated and treated samples indicated structural changes in the WH sample upon pretreatment. The intensity of FTIR spectra changing refers to the transformation in the sample composition while the broadening peak indicates the occurrence of weaker intra- and intermolecular hydrogen bonding and lower crystallinity (Goshadrou et al., 2011). The peak around 896 cm-1 stands for C-H deformation of skeleton vibration of saccharides and cellulose, and it is the characteristic peak of ß-glucosidic linkages amid monosaccharide units. Because the breakage of ß-glucoside bond is a rate-limiting step in lignocellulosic biomass degrading process, the weakened ß-glucoside bond after pretreatment could enormously promote the efficiency of water hyacinth hydrolysis (Zhang et al., 2018). The peak did not appeare before pretreatment, which manifested that the fiber of water hyacinth was broken down.

In woody biomass, in the range of (1000^{-1}500 cm^{-1}), the peaks represented the structural features of cellulose and hemicelluloses (Rezania, 2018). In this study, these peaks were broaden, particularly this is observed at wave number of 1024 CM^{-1} in boiling water with acid pretreated WH in comparison with untreated sample which resulted in broken down in the structure of the WH. In this range slight increase in number of peaks were also observed. Increasing in peaks will result in an increase in the content of cellulose and hemicellulose in the lignocellulose (Rezania, 2018). The FTIR spectra at 1361 and 1317 are associated with the cellulose (Sun, 2000).], while spectrum around 2900 cm^{-1} is reported to C-H stretching vibration band of cellulose component (Juárez-Luna , 2019). The peak around 3300 stands for stretching vibration and overlapping of O-H, which is recognized as main infrared sensitive groups of lignocellulose (Qiuzhuo Zhang et al., 2018). At this peak both treated and untreated sample has almost same wave number but treated sample has gained somehow broaden peak.

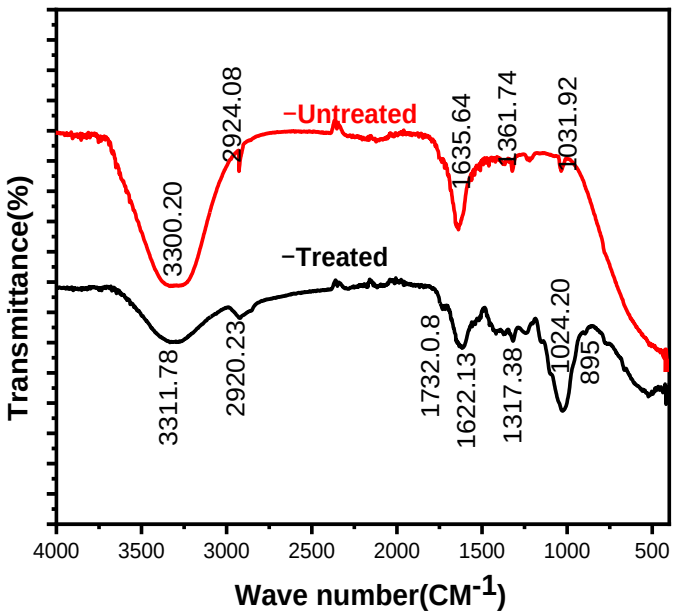


Figure 4. 11 FTIR Spectrum

CHAPTER 5: CONCULUSION AND RECOMMENDATION

5.1. CONCULUSION

As a conventional invasive weed, water hyacinth has proven to be a viable source of bioethanol production. The main aim of this study was the optimization of bioethanol production from pretreated water hyacinth using thermophilic microbial consortium.

In this study thermophilic bacteria were isolated from three different areas: bio-digester and soil (Moi University) and Lake Bogoria hot spring. A total of nine thermophilic bacteria were isolated and labelled as BO1, BO2, BOY, BOW, OL, CF, SO, NW and YF. The bacteria were then characterized using morphological, Physiological, and biochemical tests. Macroscopic characterization showed different phenotypic property whereas microscopic observation showed Bacillus and cocci shaped bacteria as well as gram negative and gram positive bacteria. The isolates, however, were not identified using a molecular identification method. Physiological characteristic indicates that all the isolate were able to grow at a temperature range (50-55^{0}C), pH range of (5.5-8.5) and 2% (w/v) NaCl concentration. The biochemical characterization of the isolates revealed that they were positive for glucose fermentation, starch hydrolysis, and EMB agar, while the results of the remaining biochemical tests were different.

Microbial Consortium was formed as a result of the isolates ability to utilise cellulose (filter paper) and untreated WH. Only three isolates, BO1, BO2, and OL, have shown consistent growth following repeated culturing. The microbial consortium was then created by selecting two at a time

One of the lignocellulosic resources for biofuel generation is aquatic plant (water hyacinth). However, unlike a simple carbohydrate sugar, the sugar in this polysaccharide is not easily degraded by microorganisms. As a result, pretreatment is required for lignocellulosic biomass

to be readily digested by microorganisms. Chemical hydrolysis (dilute acid, alkaline) is one of the most widely used methods. However, the majority of these pretreatment methods are toxic to microorganisms due to inhibitory byproducts produced during the pretreatment process, and these chemicals are also harmful to humans and the environment.

Water hyacinth has under gone to four pretreatment methods including the physical pretreatment method. The maximum TRS of 441.4 mg/g of WH was obtained when WH was pretreated with dilute 4% (v/v) H_2SO_4. TRS was reduced to 17.86 mg/g of WH while H_2SO_4 concentrations reached 8% (v/v). WH was pretreated in boiling water with 10mg/1L of $NaClO_2$ for 4hrs, and samples taken every hr. After 2hrs of pretreatment, the highest TRS of 155mg/g of WH was obtained, and TRS did not increase after 2 hours. These combined pretreatment processes use of low energy and are cost effective, without sacrificing pretreatment efficiency.

Although dilute H_2SO_4 pretreatment produced the maximum yield of TRS, the hydrolysate inhibited the bacterias growth and sugar loss owing to washing was observed. On the other hand, we discovered that pretreatment with boiling water and a low concentration of $NaClO_2$ was not inhibitory to bacteria during fermentation and was also environmentally friendly due to the low chemical concentration. As a result, only the hydrolysate from this pretreatment is implemented in the production of bioethanol.

The FTIR spectra of the hydrolysate obtained from boiling water with a low concentration of $NaClO_2$ were used to evaluate the chemical composition and structural change of the WH before and after pretreatment. When comparing treated and untreated WH, there was a general broadening of the peak and an increase in the number of peaks. This indicates a disruption of the WH structure and an increase in cellulose and hemicellulose content,

according to previous research. The FTIR spectrum reveals that all three lignocellulose (WH) components are present in the samples.

Ethanol was produced from hydrolysate derived from WH treated with boiling water containing a low concentration of $NaClO_2$ using a thermophilic microbial consortium in partially consolidated bioprocessing. Process optimization is important in bioethanol production to reduce cost of production and save energy. Central composite design was used to optimize parameters such as fermentation temperature (40-50^{o}C), incubation period (48-96hr), and inoculum dosage (8-12% v/v). The software suggested a quadratic model to explain the variation in ethanol yield as a function of process parameter. The model has described 97.42% variations of ethanol yield leaving only 2.58 to the residuals. A maximum experimental ethanol concenteration of 7.7g/l was obtained at a temperature of (50^{o}C) , fermentation time (72hrs) and inoculm dose (6.64%v/v). According to the model numerical analysis the optimum ethanol concentration of 7.2g/l was obtained at a temperature 49.018^{o}C; inoculum dosage 8.101 % (v/v) ,and time 48hr. From the results, it can be concluded that the experimental data obtained based on the optimized conditions was in close agreement with the RSM model prediction. This RSM approach of optimization has a promising potential to be employed for a better bioethanol production in the future.

The ethanol production efficiency of the microbial consortium was compared to a single strain used in the consortium's formation. Microbial consortium fermentation and single strain (BO1) fermentation produced maximum ethanol concentrations of 9.01 g/l and 3.22 g/l, respectively, after 48 hours. This shows how a microbial consortium can outperform a single strain.

These researches aid in the implementation of conservation strategies and the conversion of readily available WH to energy, as well as providing details on the thermophilic ethanol

producing and thermostable enzyme producer bacteria from Moi University and Lake Bogoria.

5.2 RECOMMENDATION

This study appreciates advances in aquatic WH to energy by using thermophilic bacteria. The thermophilic bacteria isolates in this study were identified using only conventional techniques. The PCR approach could be used to identify microbial pathogens. We also suggest isolating thermophilic bacteria from a specified location for further study.

Pretreatment is the first step in converting lignocellulose to simple sugar. In this work, we discovered that pretreatment with boiling water and a low acid concentration did not inhibit bacterial growth and was also cost-effective and environmentally beneficial. However, more pretreatment parameters need be included to optimize the sugar yield.

The DNS method was used to determine the amount of total reducing sugar (TRS), however it does not distinguish the mono sugar type. To determine the type and quantity of each mono sugar, more investigation is needed, which can be done using the HPLC method. In addition, the amount of cellulose, hemicellulose, and lignin in WH could be determined.

To observe changes in chrystaline structure, shape, polysaccharide content and other content, the hydrolysate must be characterized. The hydrolyaste was analyzed with FTIR to see how the polysaccharide changed before and after processing. We recommend that the hydrolysate further be characterized using different methods.

In comparison to bacteria, yeast (Saccharomyces cerevisiae) is an effective fermenter of sugar (glucose) to ethanol and is also tolerant to higher ethanol concentrations. In our work, the hydrolyaste was fermented with a thermophilic microbial consortium. Further research

should incorporate yeast saccharomyces cerevisiae for fermentation of the hydrolysate in a comparison study with the microbial consortium.

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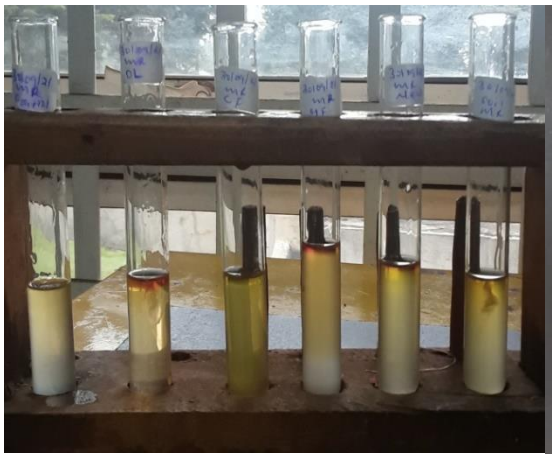
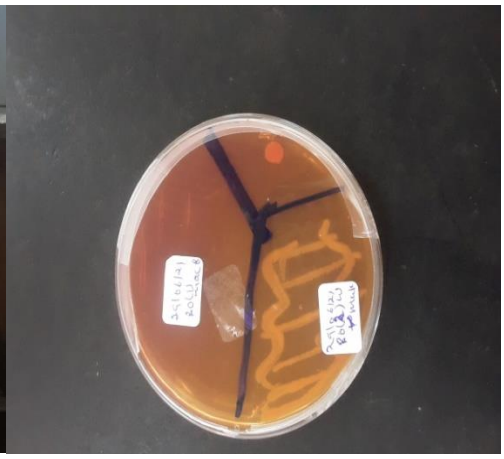
APPENDIXES

Representative of biochemical Test

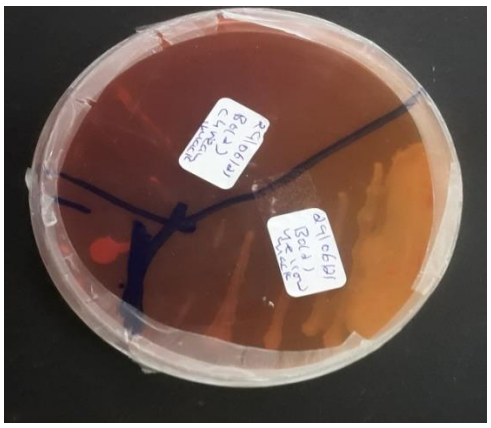


MR Test: red (+)



Mackonkey Agar: right (+) left (-)

EMB : pink (+)

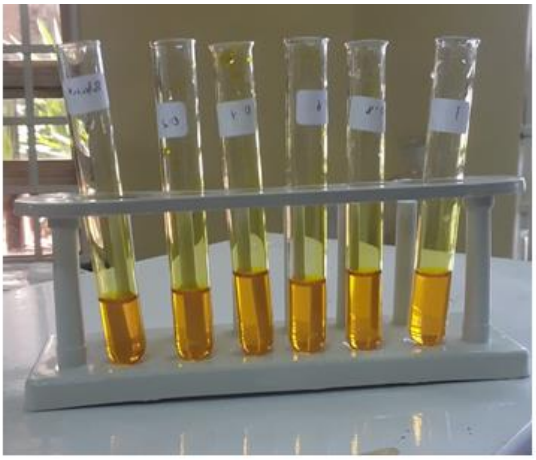


MSA: red(-), yellow(+)

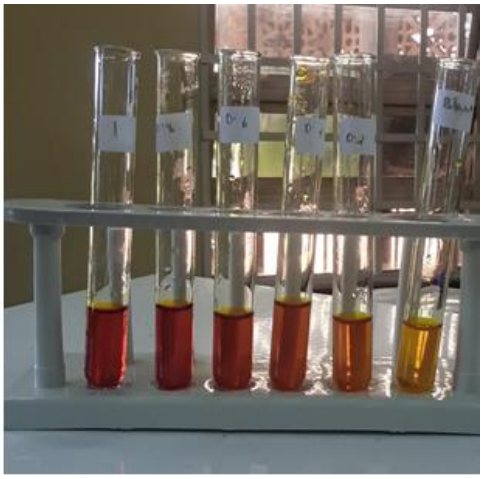
Table 1. OD600nm of bacteria for glucose utilize and WH

	glucose					water hyacinth		
isolate	zero hr	48 hr	72hr		isolate	zero hr	48 hr	72hr
OL	0.001	0.823	0.794		OL	0.06	0.767	0.7
BO1	0.001	0.213	0.574		BO1	0.14	0.659	0.502
BO2	0.012	0.989	0.821		BO2	0.129	0.47	0.2
NW	0.006	0.901	0.8		NW	0.079	0.02	0.009
CH1	0.007	0.25	0.2		CH1	0.001	0.018	0.012
YL1	0.001	0.2	0.18		YL1	0.01	0.11	0
SO	0	0.199	0.001		SO	0.021	0.011	0.009
Boy	0.012	0.499	0.352		Boy	0.044	0.01	0.01
Bow	0.005	0.783	0.635		Bow	0.023	0	0.065

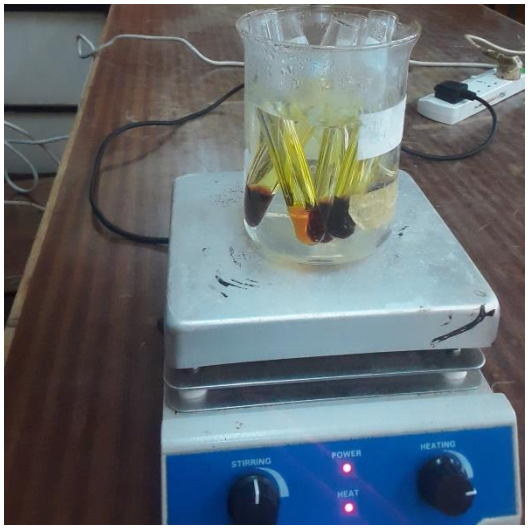
DNS Procedure



Standard for DNS before boiling



Standard for DNS after boiling



Boiling in water bath (DNS method)

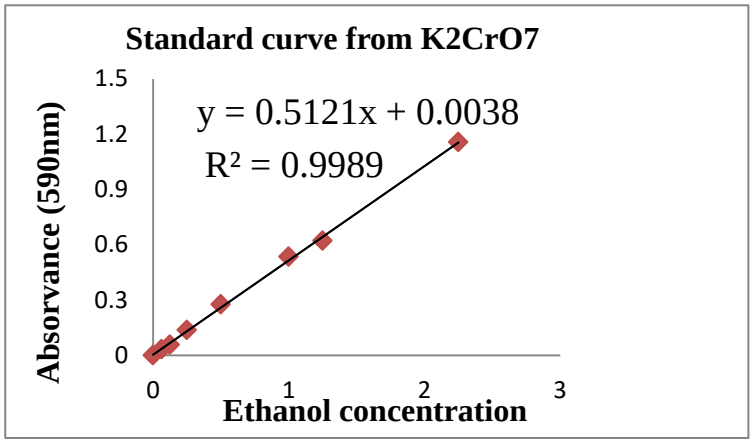


Figure 3. Standard graph from K2CrO7 for ethanol quantification.

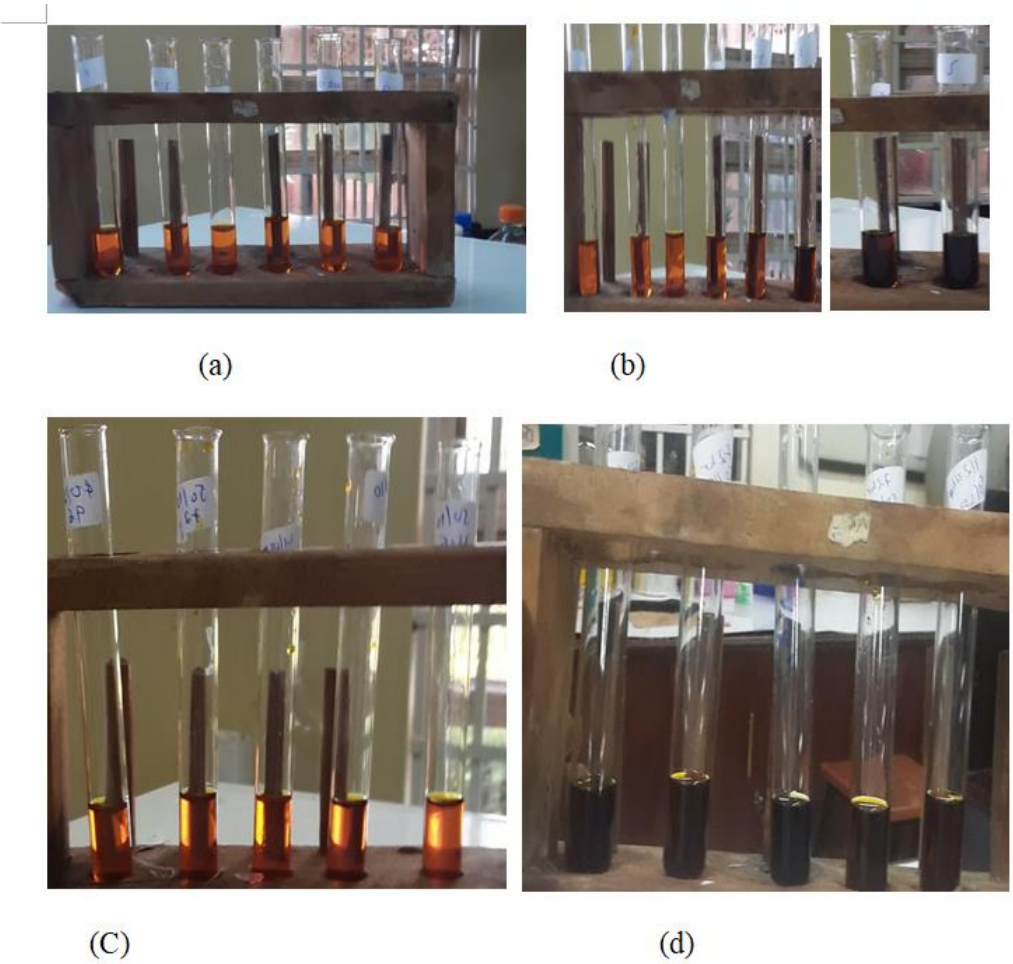


Figure 2. Standards for ethanol (a & b), the unknown (c & d) before and after incubating respectively for 30min. From 0-2.5% of ethanol, the intensity of green color has increased based on ethanol centration as shown on figure b and unknown sample (d),

Table 2. TRS obtained from boiling water and boiling water + NaClO2

	Boiling water only			**NaClO2 & Boiling water pretreament**		
Pretreament time (hr)	Absorbance	Dilution factor (ml)	TRS	Absorbance	Dilution factor (ml)	TRS
0	0.326	20	54	0.326	20	54
1	0.239	30	88	0.283	30	102.5
2	0.263	36	113	0.362	36	155
3	0.253	36	111	0.356	36	153
4	0.243	36	108.2	0.332	36	142.9

Table 3. Ethanol Calculations

Run	Temperature(^{0}C)	Incubating time (hr.)	inoculum (%v/v)	Absorbance (540nm)	Ethanol content (%)	Actual Ethanol (g/l)
1	50	72	10	0.456	0.88	7
2	40	96	8	0.4	0.77	6.11
3	60	48	8	0.37	0.72	5.7
4	50	72	10	0.43	0.83	6.6
5	66.82	72	10	0.282	0.544	4.3
6	50	72	6.64	0.503	0.97	7.7
7	50	72	10	0.464	0.9	7.1
8	50	72	10	0.450	0.87	6.9
9	50	72	13.36	0.414	0.8	6.33
10	60	96	12	0.347	0.67	5.3
11	60	48	12	0.378	0.73	5.8
12	40	48	8	0.408	0.789	6.23
13	33.18	72	10	0.237	0.456	3.6
14	40	48	12	0.373	0.72	5.7
15	50	72	10	0.474	0.92	7.3
16	50	112.36	10	0.393	0.759	6
17	60	96	8	0.37	0.734	5.8
18	50	31.64	10	0.441	0.85	6.73
19	50	72	10	0.455	0.88	6.96
20	40	96	12	0.312	0.6	4.8

RSM graph

Factor Coding: Actual

Ethanol Concentration (g/l)

Design Points:

Above Surface

Below Surface

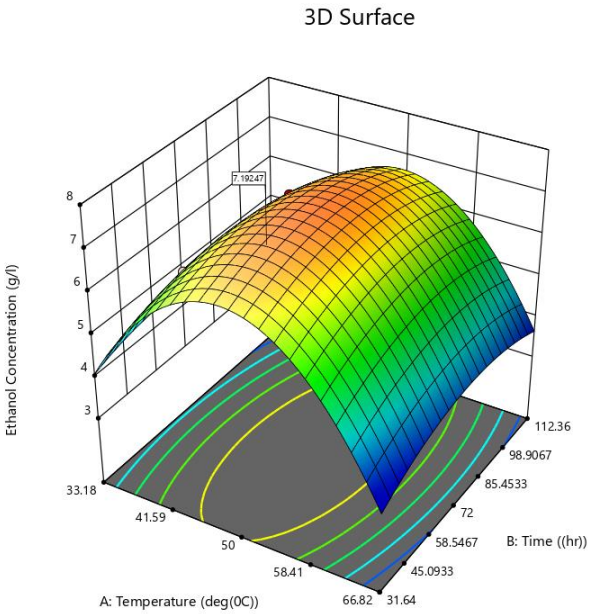
3.6 7.7

X1 = A

X2 = B

Actual Factor

C = 8.00001



Factor Coding: Actual

Ethanol Concentration (g/l)

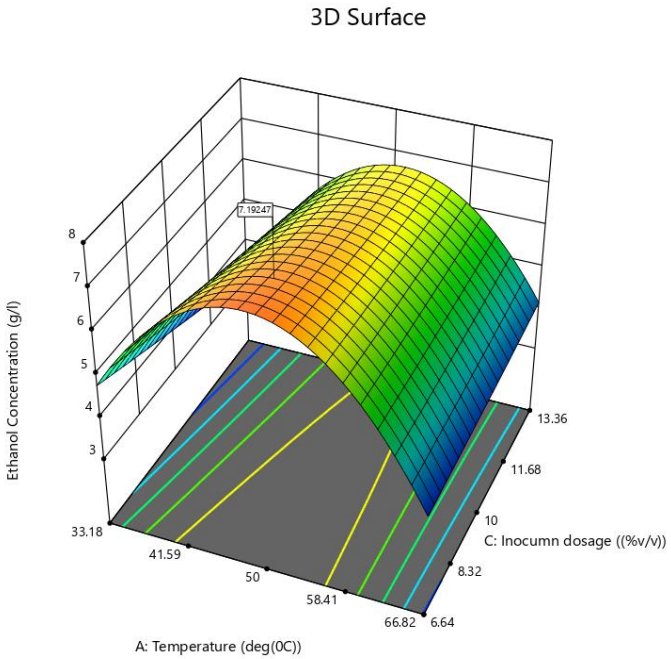
3.6 7.7

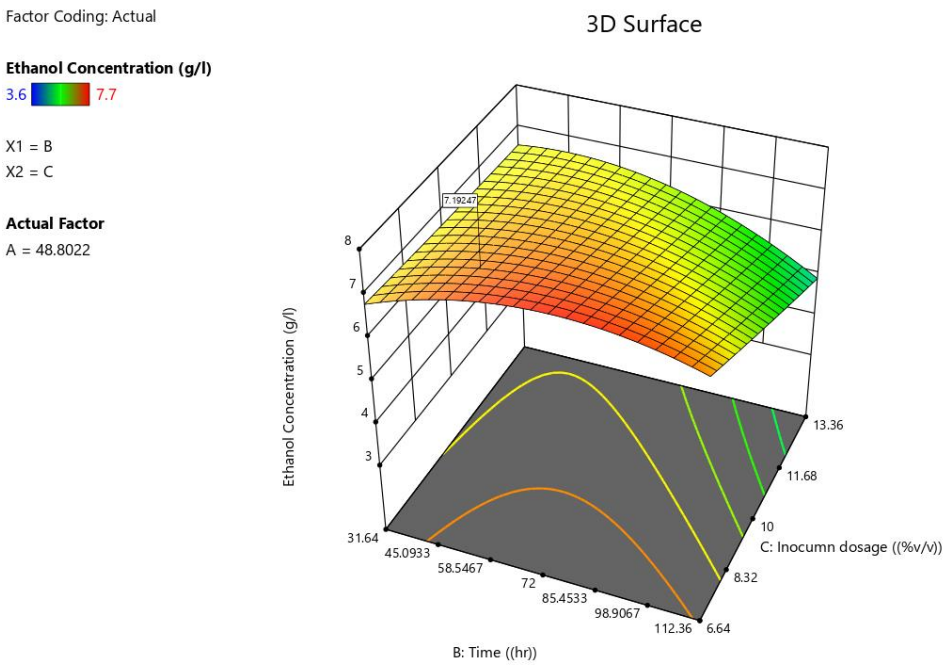
X1 = A

X2 = C

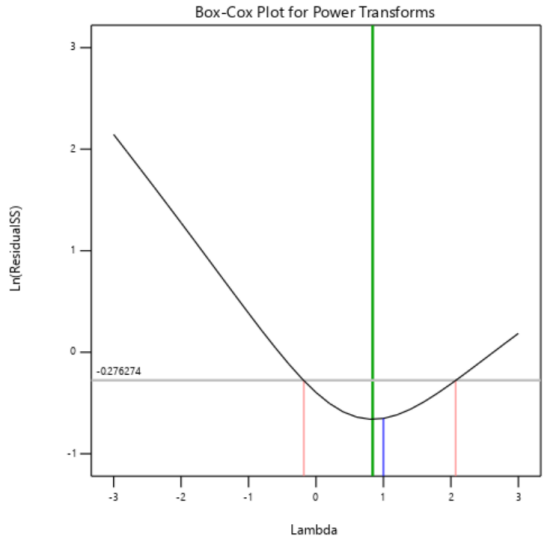
Actual Factor

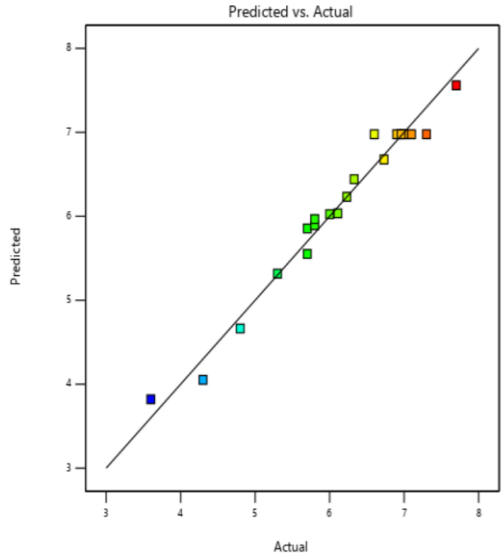
B = 52.284

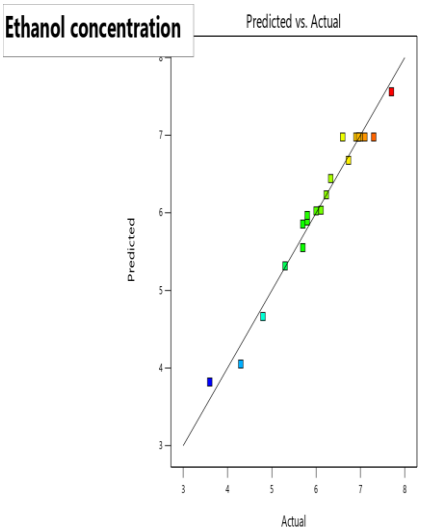




CCD Diagnostics figure for ethanol production



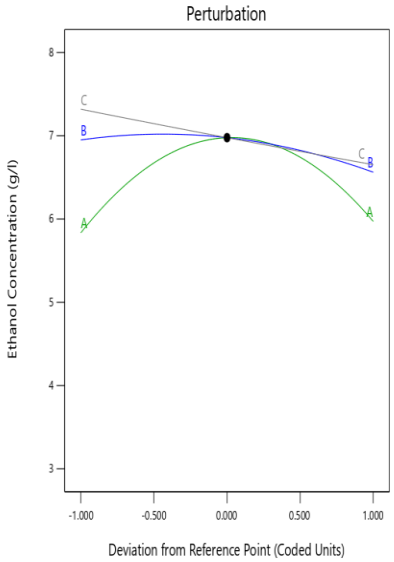


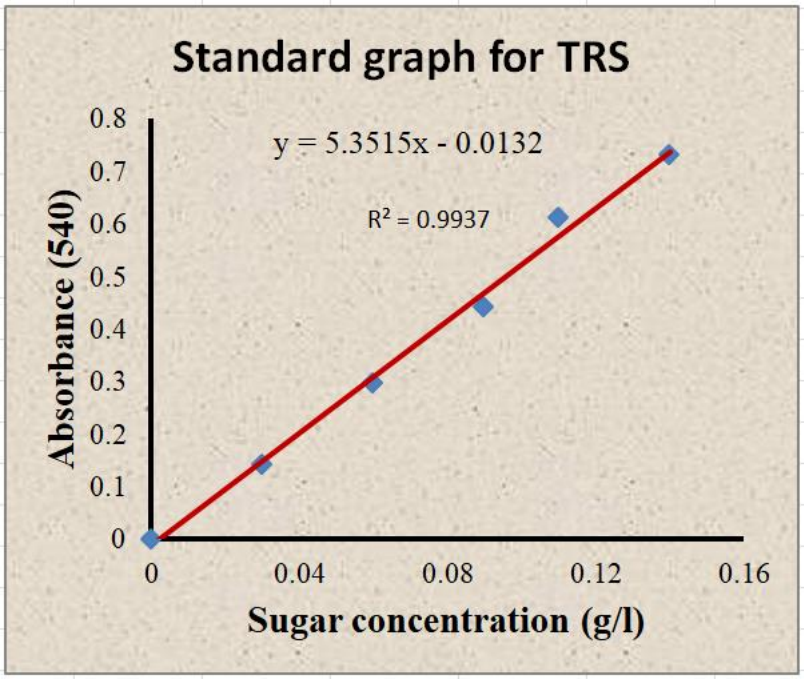


Factor Coding: Actual

Ethanol Concentration (g/l)

Actual Factors
A = 50
B = 72
C = 10





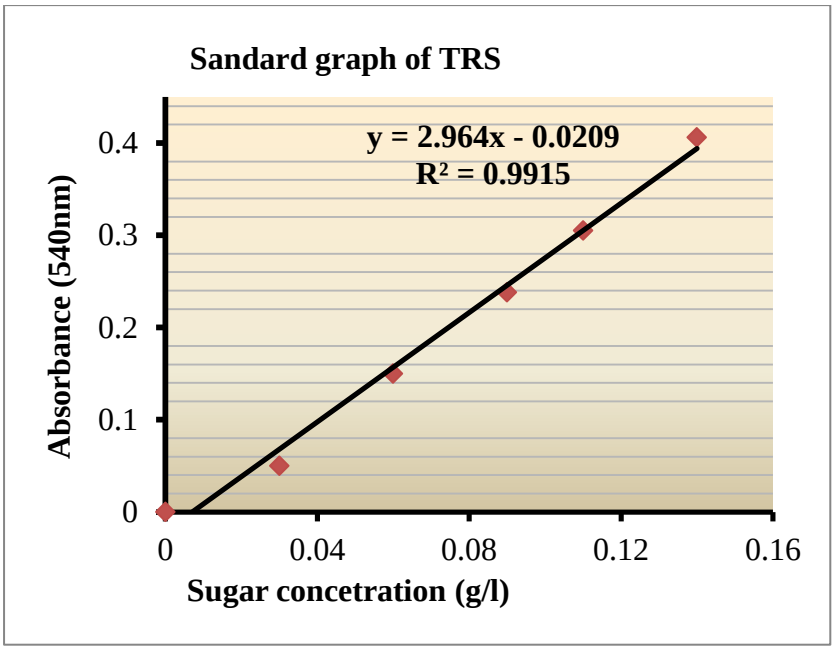


Figure 4. Standard graphs for total reducing sugar (TRS)

SR097

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