

DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**ENHANCING BIOBUTANOL PRODUCTION
FROM ORGANIC WASTES BY MANIPULATING
THE BIOCHEMICAL REACTIONS IN THE
BIOBUTANOL PATHWAY**

by

Wasiu Ayodele, ABIBU

October, 2023

İZMİR

**ENHANCING BIOBUTANOL PRODUCTION
FROM ORGANIC WASTES BY MANIPULATING
THE BIOCHEMICAL REACTIONS IN THE
BIOBUTANOL PATHWAY**

**A Thesis Submitted to the
Graduate School of Natural and Applied Sciences of Dokuz Eylül University
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Philosophy in Department of Biotechnology.**

**by
Wasiu Ayodele ABIBU**

October, 2023

İZMİR

Ph.D. THESIS EXAMINATION RESULT FORM

We have read the thesis entitled “**ENHANCING BIOBUTANOL PRODUCTION FROM ORGANIC WASTES BY MANIPULATING THE BIOCHEMICAL REACTIONS IN THE BIOBUTANOL PATHWAY.**” completed by **WASIU AYODELE ABIBU** under supervision of **PROF. DR. İLGİ KARAPINAR** and we certify that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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Wasiu Ayodele ABIBU

ENHANCING BIOBUTANOL PRODUCTION FROM ORGANIC WASTES BY MANIPULATING THE BIOCHEMICAL REACTIONS IN THE BIOBUTANOL PATHWAY

ABSTRACT

Waste fig (*Ficus carica*) is a sugar-rich lignocellulosic fruit making it an excellent biomass candidate for biofuels. This thesis was designed to use waste fig (*Ficus carica*) for the generation of low-cost and renewable biobutanol through pretreatment followed by dark fermentation. The main aim was to enhance butanol production by checking the roles of certain metals and electron mediators.

The study demonstrated the effectiveness of microwave (MW) and thermal process under pressure like autoclaving (AC) as potential pretreatment options for fig hydrolysis to yield high sugar concentrations to be used as a substrate for butanol production. The maximum total sugar concentration of 82.91 g/L and 70.02 g/L were obtained from MW and AC hydrolysis, respectively, with optimized hydrolysis conditions of MW as $D_p = 370.72 \mu\text{m}$, pH 4.96 and $P = 253.67 \text{ W}$ and for AC as $D_p = 263.19 \mu\text{m}$, pH= 3.02, $T = 120^\circ\text{C}$ and $t = 30.00 \text{ mins}$.

Metals are important precursors in biochemical transformation pathways and also play major roles in microbial metabolism. In this thesis, the effects of metals on ABE (Acetone-Butanol- Ethanol) fermentation, organic acids, and hydrogen production with fig as biomass were extensively studied. Optimization studies were conducted within the ranges of the significant cations achieving ABE (6.3, 5.03, and 1.6 g/L, respectively), acetic and butyric acids (6.9 and 3.9 g/L , respectively) and hydrogen production rate (51,5 ml H_2 /L/h). Furthermore, the role of metals in manipulating the ABE fermentation pathway to favor butanol production was successfully achieved.

Keywords: Fig, microwave, autoclave, pretreatment, optimization, hydrolysis, acetone, butanol, ethanol, hydrogen.

ORGANİK ATIKLARDAN BIYOBÜTANOL ÜRETİMİNDE BIYOKİMYASAL REAKSIYONLARIN MANİPULASYONLARI İLE ÜRETİMİN İYİLEŞTİRİLMESİ

ÖZ

Atık incir (*Ficus carica*), şeker açısından zengin lignoselülozik bir meyvedir ve bu da onu biyoyakıtlar için mükemmel bir biyokütle adayı yapar. Bu tez, ön işlem ve ardından koyu fermentasyon yoluyla düşük maliyetli ve yenilenebilir biyobütanol üretimi için atık incir (*Ficus carica*) kullanmak üzere tasarlanmıştır. Temel amaç, belirli metallerin ve elektron araçlarının rollerini kontrol ederek bütanol üretimini arttırmaktır.

İncirden bütanol üretmek üzere fermentasyon şartlarına uygun hale getirilmesi ve yüksek şeker derişimlerinin elde edilmesinde mikrodalga (MW) ve otoklavlama (AC) gibi ön işlemlerin potansiyel ve etkili ön işlem seçenekleri olduğu göstermiştir. MW ve AC ön işlemleri ile maksimum şeker derişimi, sırasıyla, 82.91 g/L ve 70.02 g/L elde edilmiştir. MW’de optimum şartlar $D_p = 370.72 \mu\text{m}$, $\text{pH}=4.96$ ve $P = 253.67 \text{ W}$ ve AC’de $D_p = 263.19 \mu\text{m}$, $\text{pH } 3.02$, $T = 120^\circ\text{C}$ ve $t = 30.0 \text{ dak.}$ ’dır.

Metaller, biyokimyasal reaksiyonlarda ve mikrobiyal metabolizmada önemli rol oynarlar. Tez çalışmasında, incirden Aseton-Bütanol-Etanol (ABE) fermentasyonu ile butanol üretiminde ve etanol, aseton, organik asit ve hidrojen gazı eldesinde metal türleri ile derişimlerin etkisi incelenmiştir. Fermentasyonu etkileyen metaller seçimi ve derişimlerinin optimizasyonu ile maksimum ABE derişimi, sırasıyla, 6.3, 5.03 ve 1.6 g/L, asetik ve bütirik asit derişimleri ise 6.9 ve 3.9 g/L elde edilmiş, hidrojen üretim hızı 51,5 ml $\text{H}_2/\text{L/saat}$ ’e ulaşmıştır. ABE fermentasyonu biyokimyasal yolağının manipüle edilmesinde metallerin rolü başarıyla kanıtlanmıştır.

Anahtar kelimeler: İncir, mikrodalga, otoklav, ön işlem, optimizasyon, hidroliz, aseton, butanol, etanol, hidrojen

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CHAPTER ONE

INTRODUCTION

1.1 Introduction

To the developing world, the importance of fuel cannot be over-emphasized as it is very important for powering daily activities such as transportation and other energy-requiring sectors of the economy. In addition, the increasing human population translates into increased consumption and need for fuel and its products (Bharathiraja et al., 2017). Crude oil production globally is around 100 million barrels per day (Pereira, Chagas, Dias, Cavalett, & Bonomi, 2015). The global energy demand for fuel is ever increasing and its abundance is reducing. Thus, there is a need for an insightful shift into other renewable energy sources whose abundance, sustainability and environmental friendliness generate little or no concern (Festel, 2008).

Presently, fuel generation from biomass (biofuel) has gained high attention in not only the scientific but also the industrial world and has proved its worth as an alternative to petrol with its appreciable qualities countering established setbacks of petroleum and its products (Qureshi, Meagher, Huang, & Hutkins, 2001 and Shah, 2018). Butanol produced from biomass is known as “Biobutanol” (Pugazhendhi et al., 2019). This form of butanol is the only form of biofuel having qualities similar to gasoline making it an appropriate alternative to gasoline. The ABE (Acetone-Butanol- ethanol) fermentation is used in the production of biobutanol. It is a biphasic form of fermentation consisting of the acidogenesis (acid production) and solventogenesis (solvent production) stages mediated mainly by the *Clostridia* genera using fermentable sugar obtained from biomass. Acids (acetic and butyric) are the products of the acidogenic phase while solvents (Acetone, Butanol and Ethanol) are the result of ABE fermentation (Pugazhendhi et al., 2019). *Clostridia* type microorganisms are a versatile microorganism producing amylase and cellulase enzymes that make hydrolysis of feedstock possible. *C. acetobutylicum* and *C. beijerinckii* are two major Clostridial strains widely used in butanol production.

Lowering of pH in the acidogenic phase drives the fermentation into the solventogenic stage in which products of acidogenesis are re-dissimilated to produce solvents acetone, butanol and ethanol in the ratio 3:6:1 respectively as described by (Jin, Yao, Liu, Lee, & Ji, 2011). For the ABE fermentation, butanol is the bio-product of concern. Vivek *et al.*, (2019) classified agricultural-based substrates for butanol production into three classes: 1st generation (starch-based biomass), 2nd generation (lignocellulosic-based biomass), 3rd generation (algae-based biomass) and recently 4th generation which is genetically manipulated algae biomass. The fermentation types used for butanol production are batch, fed-batch, continuous and two-stage continuous (Pugazhendhi et al., 2019).

The Butanol pathway is similar to the hydrogen pathway. Thus, simultaneous hydrogen and butanol production are often reported for the ABE fermentation experiments most especially when hydrogen-producing microorganisms are employed as fermentation inoculums. Clostridial strains, either as a pure or mixed culture are prolific in their performance in mostly all metabolic pathways involved in anaerobic fermentation and are excellent hydrogen and butanol producers with various biomass sources. *C. acetobutylicum* is a mesophilic Clostridial strain widely studied for H₂ and ABE production (Aissi, El Hadi, Megateli and Ketfi, 2021).

Fig (*Ficus carica*) is an edible Mediterranean fruit that is abundantly cultivated in Turkey with a 7-day shelf life (Aksoy, 2017; Kamiloglu and Capanoglu, 2015). Chemical preservation (application of SO₂ on fresh figs), thermal blanching (continuous heating of fig to a specific temperature followed by cooling), and osmotic dehydration are common pretreatment procedures used in agricultural fig preservation (Xiao et al., 2017 and Villalobos et al., 2016). Certain moulds that can grow on not properly preserved figs can produce carcinogenic and mutagenic aflatoxins. Disposal of spoiled figs requires incineration at high temperatures. Instead, its high sugar content can be useful to obtain value-added products, such as biofuel, through fermentation. Thus, optimization of pretreatment process parameters is critical for achieving cost-effective results, as well as the minimal formation of

inhibitory compounds and a high concentration of fermentable sugar which is essential for fermentative product formation (Lai et al., 2017).

1.2 Aim(s) of the research

To develop an efficient bioprocess technology that converts fig (biomass) into a value-added product (butanol) through dark fermentation. Hydrogen production will also be monitored during this research.

1.3 Main goals of the research

- Pretreatment: extensive comparative study that will reveal the best form of pretreatment that will easily release stoichiometrically favourable sugar concentration for the ABE fermentation with no inhibitors for improved butanol production from waste fig.
- ABE Fermentation: Manipulating the biochemical reactions in the butanol pathway as presented in figure 2 via cofactor and inhibitor control/regulation, enzyme mediator addition to favour butanol production using the Plackett-Burman experimental design, one factor at a time (OFAT) and Box Behnken experimental design. Hydrogen production during the ABE fermentation will be thoroughly monitored.
- Processing: Finding an economical method (in terms of cost and process technology) to increase butanol production.
- Microbial strain selection: A strain that will prevent solvent inhibition in the solventogenic stage of ABE fermentation is highly desired.
- Fermentation mode: Use of mesophilic batch fermentation mode for the research.
- Optimization: via Central Composite Design (CCD) and Plackett-Burman Numerical optimization to determine percentage sugar recovery, fermentable sugar consumption by microorganisms and determination of the desired concentration of significant metals to maximize desired products (organic acids and solvents).

- Sugar profiling: Determining sugar types (such as glucose, fructose, arabinose and others) present in ABE fermented fig.
- Organic acid and Solvents profiling: Determining organic acids (lactic, acetic and butyric) and solvents (acetone, butanol and ethanol) present in ABE fermented fig.

1.4 Novelty of research

- No published work exists on the use of fig as a biomass for ABE fermentation and hydrogen production.
- The comparative pretreatment and optimization of pretreatment parameters for microwave, steam-assisted autoclave and simple heating have never been studied.
- This is the only research that aims at manipulating the fig fermentation pathway to favour butanol production via a comprehensive study of the effects of metals and electron mediators on ABE fermentation.

The graphical abstract of this Doctoral thesis is found below (Figure 1).

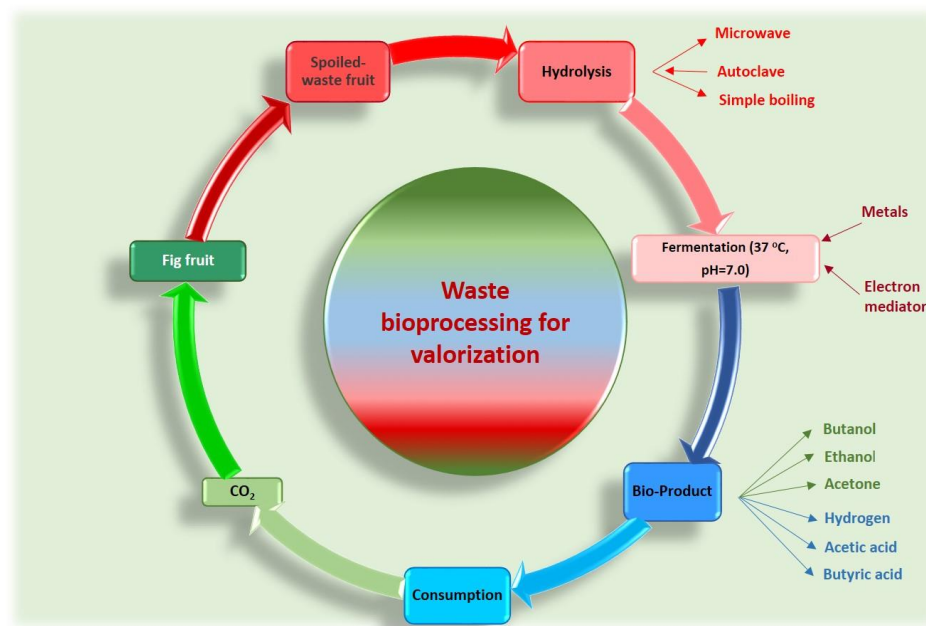


Figure 1 Doctoral thesis graphical abstract

CHAPTER TWO

LITERATURE REVIEW

The 21st century is challenged by the depletion of fossil fuel abundance. To the developing world, the importance of fuel cannot be overemphasized as it is crucial for powering daily activities such as transportation and other energy-requiring sectors of the economy. Besides, the increasing human population translates into an increase in consumption and the need for fuel and its products (Bharathiraja et al., 2017). Crude oil serves around 35% of the world's energy needs, and it is expected to rise to around 50% in decades (Pereira et al., 2015). The global energy demand is ever-increasing, however, its abundance is reducing. Thus, there is an urgent need for an insightful shift into other renewable energy sources whose abundance, sustainability, and environmental friendliness generate little or no concern. The effects of global warming and continuous fluctuation in fuel prices also necessitate a shift towards renewable energy sources. Presently, fuel generation from biomass (biofuel) has gained great attention in the scientific and industrial world and has proved its worth as an alternative to oil with its appreciable qualities countering established setbacks of petroleum and its products (Hutkins, 2018).

As indicated by the BP Statistical Review of World Energy (2020), successes recorded in renewable energy are yet to reduce environmental concerns related to carbon emissions. In addition, continents like Africa, Europe, and North and South America still rely on oil as the primary energy source. The use of waste materials in biofuel production makes it more advantageous and beneficial. Conventional waste management approaches require intensive energy, and infrastructure without a valuable output, and the residues like leachate create more environmental problems that need further processing. Butanol is a major well-known biofuel. The World Wars in the 20th century were accompanied by an increase in the price of fuel and the need for acetone (used in gun powders). Acetone is produced via the ABE fermentation. Thus, shifting global attention to ABE fermentation to date (Awang, Jones, & Ingledew, 1988). The success recorded by Brazil in ethanol research, reducing its dependence on fossil fuels increased interest in ABE fermentation and

butanol production by private and governmental agencies across the globe (Kumar, Goyal, Sarkar, and Gayen, 2012). Furthermore, recent publications have reported the possibility of using ABE products directly as fuel without the need for separation and purification (Veza, Said, & Latiff, 2019). The timeline of major events in the butanol research within the 20th and 21st centuries as reported by Veza, Muhamad Said, and Abdul Latiff (2021) is shown in Figure 2. In the mid-20th century, ABE fermentation was greatly achieved via starchy substrates such as grain, molasses, potatoes, and many more (Pugazhendhi et al., 2019).

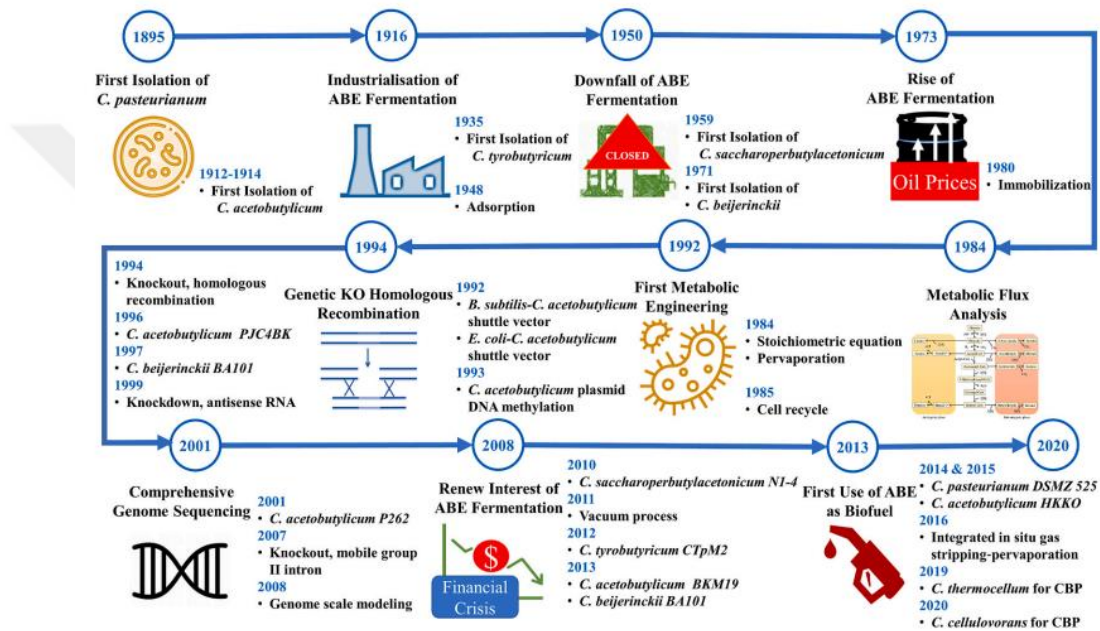


Figure 2 Major Events in the Butanol research within the 20th and 21st century (Veza et al., 2021)

Figure 3 shows the physicochemical properties of butanol. Low volatility, less corrosiveness, and the ability to power engines running on fuel are some of its outstanding features (Pugazhendhi et al., 2019). Figure 4 as projected by Mordor Intelligence (2020) shows the current state of butanol research and development with a six (6) year forecast of the butanol market between 2020 and 2025 amongst continents of the world. South America and European nations are actively involved in butanol Research and Design at laboratory, pilot, and commercial scales. In North America and Far Eastern nations, Butanol R & D is still at the medium stage while the African continent and the Middle East nations are characterized by passive and low awareness levels regarding butanol research.

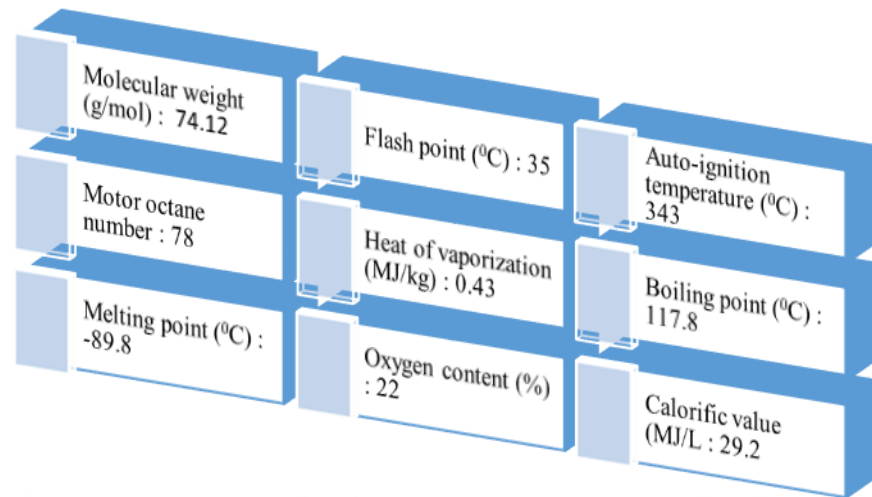


Figure 3 Physico-chemical properties of butanol (Pugazhendhi et al. 2019)



Figure 4 Current state of butanol research and development amongst regions of the world (Mordor Intelligence, 2020)

Biofuel production from biomass has three stages in the order of feedstock pretreatment, fermentation and product separation. Different technologies are available for each stage. The success of the process depends upon biomass and the best combination of different processes that yield the highest butanol at the lowest

cost. The following sections discuss the recent developments in these processes for butanol production.

2.1 Feedstock, Pretreatment and Separation methods

Feedstock for biofuel generation has been classified as 1st generation (starch-based biomass), 2nd generation (lignocellulosic wastes), 3rd generation (algae biomass) and 4th generation with the genetically manipulated algae. 2nd, 3rd and 4th generation biomasses are accepted to be more suitable raw materials for biofuel production considering their renewable characteristics. Monosaccharides act as a fermentation substrate for microorganisms in butanol production. Therefore, the selection of a pretreatment method to obtain a monomeric syrup that is ready for fermentation is the first step in biofuel production. Every available pretreatment method has its own merits and limitations. Generation of monomeric units (fermentable sugar) from starch is easy, produced no inhibitory compounds which affect the fermentation process, and yielded high butanol concentration unlike other biomass classes (lignocellulose and algal biomass) requiring expensive pretreatment process before fermentable sugar is obtained (Jenol et al., 2013). However, starchy biomass for ABE fermentation was later discouraged due to the fuel-food debate and since starchy-based biomass is predominantly food sources, a shift to non-food biomass to generate fermentable sugar for the ABE process was necessary (Vivek et al., 2019).

The major setback of the 1st generation biomass brought the 2nd generation biomass research for ABE fermentation into existence and focuses on the use of agricultural wastes as substrates for fermentation. Agricultural wastes are the major source of lignocellulose and its biofuel production potential has been studied. Non-edibility, availability, and abundance are a few of the features of lignocellulosic biomass. In addition, it created no concern unlike the food-fuel debate of the 1st generation biomass (Vivek et al., 2019). The only setback of lignocellulosic biomass is the need for pretreatment to release fermentable sugar for the fermentation process which is quite expensive. Pretreatment removes the lignin constituent of

lignocellulose, altering the structure, and enabling hydrolysis (Gottumukkala, Haigh, & Görgens, 2017; Ibrahim, Kim, & Abd-Aziz, 2018). The work of Abibu and Karapinar (2023) is an example of a scholarly lignocellulosic pretreatment study that compared the optimization of microwave-assisted (MW) and steam-assisted autoclave (AC) pretreatments of fig (*Ficus carica*) to produce fermentable sugar needed as a substrate for ABE fermentation. The central composite design was used for the optimization of process parameters. The independent variables for MW were particle diameter of dried fig ($D_p = 200\text{-}375\mu\text{m}$), pH (3.00-5.00) and power ($P = 250\text{-}670\text{W}$), the variables for AC were particle diameter ($D_p = 200\text{-}375\mu\text{m}$), pH (3.00-5.00), temperature ($T = 95\text{-}120^\circ\text{C}$) and time ($t = 30\text{-}50\text{ min}$). Total sugar concentration (TSC) upon hydrolysis was the goal of the study. Statistical analysis of the observed TSC indicated that all MW pretreatment parameters were significant. However, the temperature was the only significant parameter affecting fig hydrolysis in AC. Regression coefficients between observed and predicted values were in good agreement for AC ($R^2 = 0.90$) and MW ($R^2 = 0.88$) indicating model strength to the experimental data. The optimum conditions for maximum sugar concentration by MW were determined as $D_p = 370.72\text{ }\mu\text{m}$, pH 4.96, and $P = 253.67\text{ W}$ with TSC = 82.9 g/L. AC conditions were $D_p = 263.19\text{ }\mu\text{m}$, pH = 3.02, $T = 120^\circ\text{C}$ and $t = 30.00$ mins with a maximum sugar concentration of 70.0 g/L. The study demonstrated the effectiveness of MW and AC as potential pretreatment options for fig hydrolysis to yield a high sugar concentration that can be used as a substrate for biofuel production.

Mycotoxins can adversely affect the desired product formation through dark fermentation. Therefore, the pretreatment should be also effective in reducing the mycotoxin level. Özmihçi and Hacıoğlu (2023) reported that a MW power of 400W generally reduce 77.8% in mycotoxin levels in mycotoxin contaminated figs. Increasing pretreatment time ensures efficient detoxification of mycotoxins. However, it is important to note that the effectiveness of microwaving, autoclaving and simple boiling in reducing mycotoxin levels can be influenced by factors such as the initial concentration of mycotoxins, the type of food, and the duration and intensity of thermal treatment.

Figure 5 shows a chart of the pretreatment methods used in breaking down the lignin and cellulosic components of lignocellulosic substrates while Figure 6 is the fermentation process chart yielding butanol. Tables 1 and 2 show the features, advantages, and disadvantages of single and 2-stage pretreatment options. According to Veza et al. (2021), all pretreatment strategies known had been successfully tested for ethanol production with improvement in ethanol titre. However, decreases in sugar concentration, production of inhibitors and toxic products remain the major challenge of the pretreatment process for the ABE fermentation.

Lipids, carbohydrates, and proteins are the major constituents of algae. Economic consideration, green technology advantages, and the potential to yield value-added products are some merits of algae-based biomass. Algae are fast-growing microorganisms with simple growth requirements. Microalgae are mainly composed of cellulose and a low percentage of hemicellulose with no lignin, making them a better option over lignocellulosic-based biomass. The only concern regarding algal-based biomass as a substrate source for fermentation is the low abundance of algae and the cost of raceway bioreactors needed for algae propagation (Ibrahim et al., 2018). Microalgae such as *Chlorella* sp.AE10, *Chlorella vulgaris*, *Chlorella reinhardtii* and *Scenedesmus obliquus* are abundantly rich in fermentable sugar present in their cell wall and plastids (Liang, Sarkany, and Cui, 2009). The potential of *C. vulgaris* in butanol production has been reported (Liang et al., 2009). Microalgae lacks lignin and hemicellulose in their structural composition, this aids easy fermentation and no requirement for pretreatment coupled with its associated costs concern. Yeong et al. (2018) encouraged the use of microalgae with high starch content as the substrate for biofuel production. As reported by Ellis, Hengge, Sims, & Charles (2012), 2.74 g/L butanol was obtained from the ABE fermentation of algae obtained from the Logan City Wastewater Lagoon system with *Clostridium saccharoperbutylacetonicum* N1-4 as the microbial source. In the research, external carbon addition was recommended for improved butanol production achieving 7.27 g/L butanol concentration.

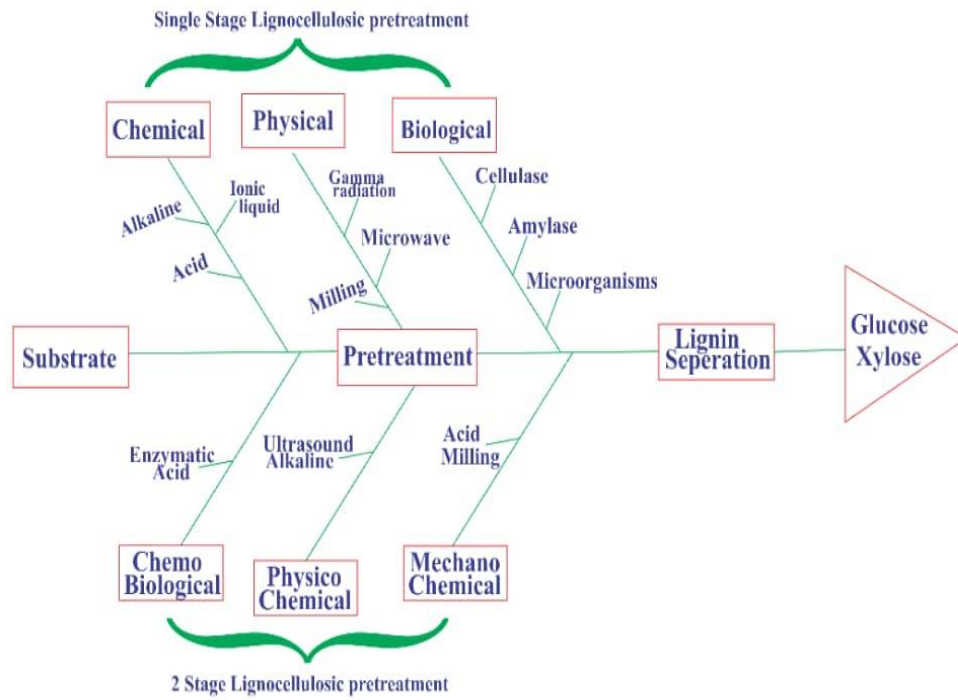


Figure 5 Substrates pretreatment methods for the release of fermentable sugar needed for the ABE process

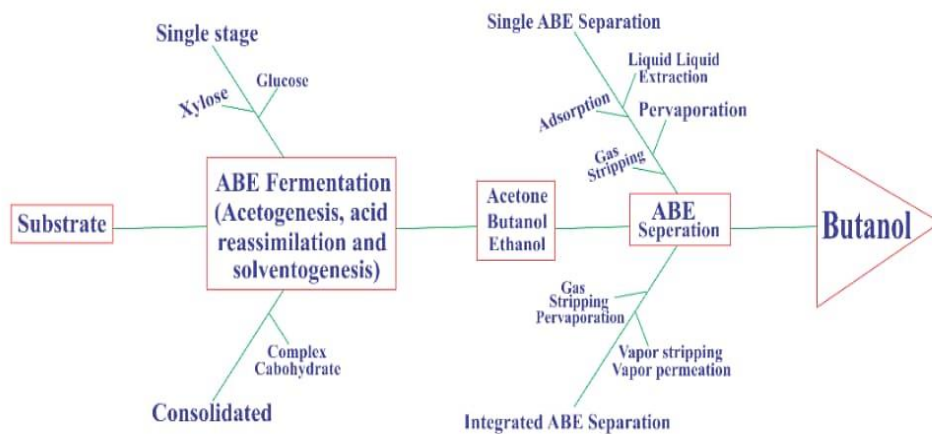
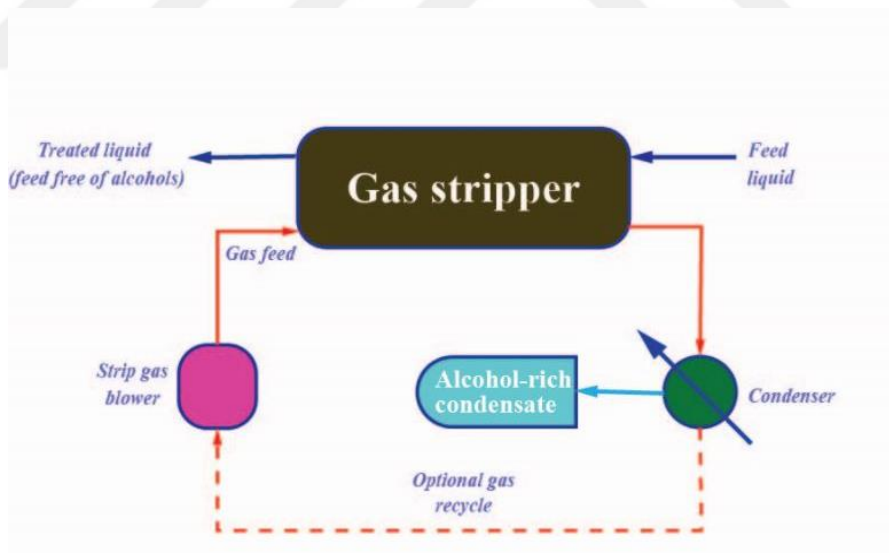


Figure 6 Fermentation process chart for butanol production

The separation of butanol is another challenge in the process flow scheme. Adsorption, gas stripping, liquid-liquid extraction, pervaporation and reverse osmosis are the most common ABE product separation techniques (Veza, Said, & Latiff, 2019). Adsorption is characterized by the adhesion of ABE products onto the adsorbent medium used in the process such as silica, resins, activated charcoal and many more (Berk, 2018). Gas stripping is a very simple method that easily removes volatile products from the fermentation setup, thus, enabling easy organic acids progression into the solventogenic stage of ABE fermentation (Ezeji, Qureshi, & Blaschek, 2003). In Liquid-liquid extraction, there is selective solvent separation based on solubility indices. Pervaporation on the other hand uses a membrane basal separation procedure selectively separating ABE solvents based on membrane pore passage (Kujawski, 2000). Reverse osmosis involves an ultra-high-pressure solvent separation via a semi-permeable layer (Perez-Gonzalez, Urtiaga, Ibañez, & Ortiz, 2012). A diagrammatic representation of the principle of the various ABE separation methods is seen in Figure 7(a-d).



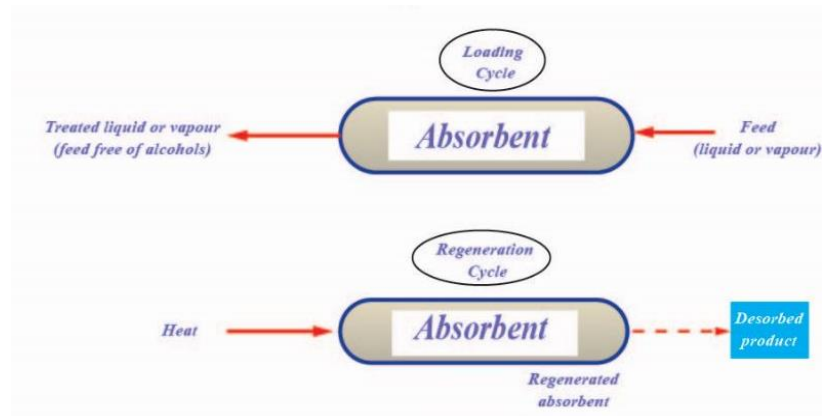
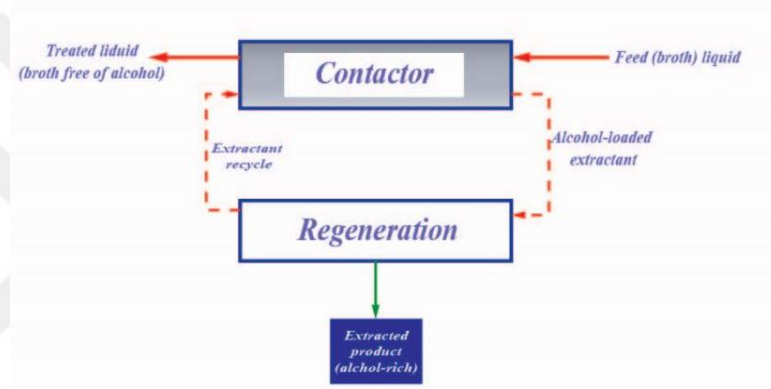
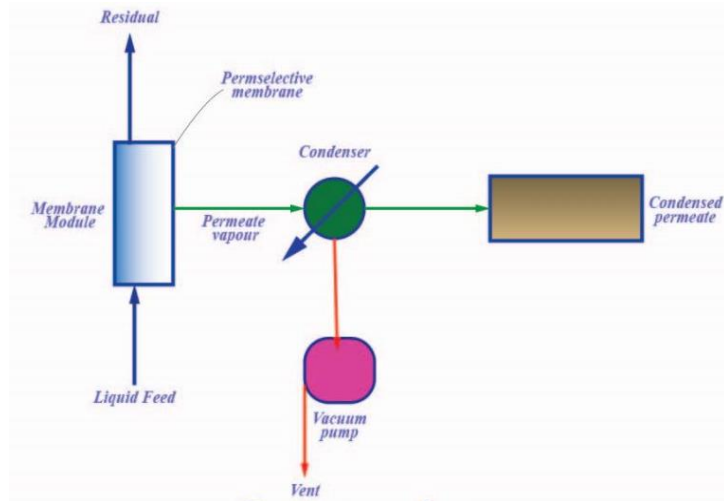


Figure 7 (a-d) Diagrammatic representation of gas stripping pervaporation, liquid-liquid extraction and adsorption (Veza, Said, & Latiff, 2019)

Table 1 Features, Merits, and Demerits of Pretreatment Options in Lignocellulosic Substrates

Method	Types	Features/ Characteristics	Merits	Demerits	Reference
Physical	Mechanical	Reduces particle size and cellulose crystallinity and increases surface area. e.g. electro-hydrolysis, ultrasonic, ball milling etc	Achieves an appropriate particle size than chemical or biological ones	Not capable of removing lignin from substrates High energy requirement	(Kumari and Singh, 2018)
	Thermal	Makes the surface area of cellulose accessible and enhances its degradability. Examples include hot air oven, autoclaving and microwave oven	Alteration in lignin structure Lignin solubilization is achieved Easy hydrolysis	Formation of inhibitory product furfural and hydroxymethyl furfural (HMF)	(Veluchamy & Kalamdhad, 2017)
Chemical	Acid	The acid solubilizes mainly hemicellulose and improves biomass porosity	Alteration in lignin structure	Formation of furfural/HMF Neutralization requirement Corrosion potential	(Veluchamy & Kalamdhad, 2017)
	Alkali	Disrupts lignin structure making hydrolysis and fermentation easy	Alteration in lignin structure and hemicellulose hydrolysis Fast Reduce cellulose crystallinity	Low efficiency on biomass with high lignin content Neutralization requirement Corrosion potential	(Saratale and Oh, 2015)
Biological	Enzymes	Solubilizes mainly hemicellulose Increases surface area of biomass	Alteration in lignin structure	Expensive and requires expertise	(Veluchamy & Kalamdhad, 2017)
	Microorganisms	Microorganisms are capable of disrupting lignin structure, thus, making hydrolysis and fermentation easy	Low cost Effectively degrades lignin	Too slow Cautious control of growth conditions is important	(Veluchamy & Kalamdhad, 2017)

Table 2 Features, Merits, and Demerits of Two-Stage Pretreatment Options in Lignocellulosic Substrates

Pretreatment type	Features/ Characteristics	Merits	Demerits	Reference
Physico-chemical	Reduction in cellulose crystallinity removes the acetyl group in hemicellulose and disrupts lignin-carbohydrates linkages. An example is combined ultrasound-alkaline pretreatment	Achieves a high total sugar concentration upon hydrolysis	Inhibitory products formation	(Alvira, Tomás-Pejó, Ballesteros, & Negro, 2010)
Mechano-chemical	Yields degradation and fibrillation of cellulosic material making hydrolysis easy. An example is Acid-milling, steam expulsion	Cost-effective and efficient Environmentally friendly	Expensive Inhibitory by-product formation	

2.2 ABE fermentation

The ABE fermentation is a biphasic form of fermentation consisting of the acidogenesis (acid production) and solventogenesis (solvent production) stages mediated mainly by the *Clostridia* genera using fermentable sugar obtained from biomass. Acids (acetic and butyric), solvents (acetone, butanol, and ethanol) and gaseous by-products (H_2 and CO_2) are the results of ABE fermentation (Mukherjee, Sarkar, Goswami, and Das, 2019). Tables 3 and 4 show a list of suspended and immobilized microbial strains successfully used in butanol research.

ABE fermentation is readily initiated for first-generation biomass (starchy biomass) since no pretreatment option is required and hydrolysis of biomass is easily achieved by the amylolytic potential of the Clostridial strain involved in the process (Nanda et al., 2017). However, secondary (lignocellulosic) and tertiary (algal biomass) require additional effort to initiate the ABE process because feedstock pretreatment and extraction are important. In addition, hydrolysis of pretreated and extracted lignocellulosic and algal biomass relies on the action of cellulolytic strains of *Clostridium* (Mukherjee et al., 2019; Nanda et al., 2017). *Clostridium* is a versatile microorganism producing amylase and cellulase enzymes that make hydrolysis of feedstock possible. The Clostridia genera are well documented for butanol production and *C. acetobutylicum* and *C. beijerinckii* are two major Clostridial strains widely researched for their involvement in butanol production. Fermentable sugars (5C or 6C) which are the product of the hydrolysis of feedstock readily find their way into the bacterial cell and are metabolized via the EMP (Embden–Meyerhof–Parnas) pathway for 6C sugar or PPP (Pentose-Phosphate-Pathway) for 5C sugar (Vivek et al., 2019; Nanda et al., 2017). The acidogenic ABE fermentation phase occurs in the exponential phase of microbial growth. However, it is affected by an increase in the production of acetate and butyrate, thus making the media more acidic and affecting microbial division and replication (Pugazhendhi et al., 2019). An increase in acidogenic phase products (acetic and butyric acids) drives the fermentation to an acidic pH resulting in the formation of endospores by Clostridial strains at the termination of the microbial exponential phase of growth.

Table 3 Butanol production by batch-suspended cultures

Substrate	Microorganism	Fermentation medium	Butanol conc (g/L)	Retention time (h)	Reference
Oil palm decanter cake	<i>C. acetobutylicum</i> ATCC 824	Reinforced Clostridial Medium (RCM)	6.04	96	(Razak, Ibrahim, Yee, Hassan, & Abd-Aziz, 2013)
Sweet sorghum syrup	<i>Clostridium beijerinckii</i> NCP 260	P2 medium	7	72	(Klasson, Qureshi, Powell, Heckemeyer, and Eggleston, 2018)
Pineapple peel waste	<i>C. acetobutylicum</i> NRRL B527	P2 medium		96	(Khedkar, Nimbalkar, Gaikwad, Chavan, & Bankar, 2016)
Brewer's spent grain	<i>C. acetobutylicum</i> DSMZ 792	Yeast extract medium	7.93	190	(Giacobbe et al., 2019)
Cassava starch and chips	<i>Clostridium saccharoperbutylacetonicum</i> N1-4	Tryptone–yeast extract–acetate (TYA medium)	15.5-16.9	48	(Thang, Kanda, & Kobayashi, 2010)
Gelatinized Cassava Starch	<i>C. acetobutylicum</i> strain ART18	Tryptone–yeast extract–acetate (TYA medium)	15.8	88	(Li, Luo, Wang, & Yu, 2014)
Cassava flour	<i>Clostridium</i> sp. stain BOH3	Tryptone–yeast extract–acetate (TYA medium)	17.8	120	(Li, Yan, and He, 2015)
Crystalline cellulose	<i>C. thermocellum</i> and <i>C. saccharoperbutylacetonicum</i> N1-4	NBRC medium 979	4.2-5.8	96-216	(Nakayama, Kiyoshi, Kadokura, and Nakazato, 2011)

Table 4 Butanol production by batch immobilized culture

Substrate	Microorganism	Fermentation medium	Butanol conc (g/L)	Retention time (h)	Reference
Xylose	<i>Clostridium acetobutylicum</i> DSM 792	Xylose supplemented medium	-	-	(Procentese et al., 2014)
Fresh corn stalk	<i>C. acetobutylicum</i> ABE 1201	Corn Stalk Juice medium	13.7	100	(Chang et al., 2016)
De-oiled rice bran (DRB)	<i>Clostridium acetobutylicum</i> YM1	Sterilized SADB	5.89-6.87	72	(Al-Shorgani et al., 2019)
Sugarcane juice	<i>Clostridium acetobutylicum</i> (ATCC 824)	Reinforced Clostridial Medium (RCM)	12.9	48-72	(Kittithanesuan and Phisalaphong, 2015)
Glucose	<i>C. acetobutylicum</i> JB200	P2 medium	75.5	24-36	(Xue et al., 2016)
Glucose	<i>Clostridium beijerinckii</i> and <i>Clostridium tyrobutyricum</i>	Reinforced Clostridial Medium (RCM)	6.66	144	(Li et al., 2013)
Sugarcane juice Glucose	<i>C. acetobutylicum</i> JB200	P2 medium	16.2	24-36	(Xue et al., 2013)

For the ABE fermentation, butanol is the bio-product of concern. As butanol production increases, Clostridial strain experiences cell membrane and cell wall damage, thus increasing membrane fluidity and eventually leading to cell death. The numerous products obtained from the fermentation affect the butanol yield coupled with the inactiveness of the Clostridial strains to resist solvent concentration above 2% are the major setback of the ABE fermentation (Nanda et al.,2017).

2.3 Fermentation strategies for butanol production

The fermentation types used for butanol production are batch, fed-batch, continuous, and two-stage continuous. Generally, the solventogenic process affects butanol production due to the inactivity of the Clostridial strains in this stage. This led to the use of engineered strains to withstand the inactivity due to stress in the solventogenic process. The stability and efficiency of mutagenic strains is a subject of concern as advanced bioprocessing technique is needed to achieve their metabolic engineering. Genetically engineered *Saccharomyces cerevisiae* has been successfully used in butanol production. The tolerance to ABE solventogenic stress in *Saccharomyces* has been reported to be higher than in Clostridial strains, however, butanol production is lower (Sakuragi, Morisaka, Kuroda, & Ueda, 2015; Kuroda and Ueda, 2016). Other genetically engineered strains of bacterial genera such as *Lactobacillus*, *Lactococcus*, *Pseudomonas* and *Escherichia coli* have been reported to be active in butanol production (Halloum, Thompson, Pugh, & Nielsen, 2015).

The presence of the required growth factors in their appropriate proportion for microbial strains determines their performance in butanol production. In addition, “fermentation with starting sugar concentration <40g/L could produce more acid than solvent thus inhibiting cell growth. “Besides”, a 6C sugar is preferable to a 5C sugar as sugar specific mechanism for transcriptional regulation and metabolism of the gene is important” (Ibrahim et al., 2015; Jiang et al., 2014). Al-Shorgani et al. (2019) proved that tryptone yeast extract acetate medium (TYA) and P2 medium were the best fermentation media for butanol production, while Wu, Xue, Chen, Yuan, and Bai (2013) concluded that “media supplementation with Zn^{2+} will increase

reassimilation of acidogenic products for solvent production while reducing cellular metabolism inhibition”.

2.3.1 Batch fermentation

According to Pugazhendhi et al. (2019) and Ibrahim et al. (2018), “a fermentation process in which all the media is fermented in a batch of bioreactors is termed batch fermentation and it is characterized by simplicity and ease of fermentation”. The major drawback of this fermentation type is that low butanol is often obtained. Low butanol production in batch fermentation arose from the low concentration of glucose supplied for fermentation. Butanol production is sugar-dependent as the starting concentration of sugar $>100\text{g/L}$ leads to substrate inhibition. In addition, inhibitory constituents of some biomass inhibit cell growth needed for the fermentation process. The fed-batch is another form of batch fermentation designed to reduce the setback of batch fermentation. Unlike batch fermentation, fed-batch fermentation involves the supply of substrates to the fermenter in low concentration (to prevent substrate inhibition) and continual substrate feeding at a level lower than the inhibition concentration (Pugazhendhi et al., 2019). Ibrahim et al. (2018) concluded that low butanol concentration, difficulty in butanol recovery, low effluent streams, and time constraints to attain full fermentation are the setbacks of this fermentation type. Figure 9 shows a typical batch fermentation setup as described by Pugazhendhi et al. (2019).

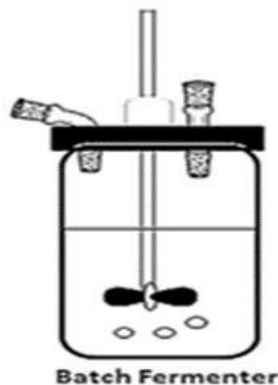


Figure 9 Batch fermenter in butanol production (Pugazhendhi et al., 2019)

2.3.2 Continuous fermentation

Continuous fermentation is an economical and large-scale choice for butanol production compared to batch and fed-batch fermentation (Ranjan and Moholkar, 2012). This fermentation type is characterized by continuous substrate addition with simultaneous recovery of products during fermentation (Pugazhendhi et al., 2019). Continual feeding of the fermenter with the fresh medium makes bacterial cells involved in the fermentation process active, “hence”, resulting in a higher yield of products. Ibrahim et al. (2018) reported an ABE product ratio of 8:6:1 via continuous fermentation when compared with the established report of 3:6:1. However, Patakova, Linhova, Rychtera, Paulova, and Melzoch (2013) reported that the use of suspended microbial cultures in continuous fermentation affects the ABE process by mixing the acidogenic and solventogenic phases. According to Vivek et al. (2019), “the continual product removal and substrate addition reduce lag phase period and the use of suspended cell in continuous fermentation affects the efficiency of this fermentation mode as cells mix with products and get washed out”.

“Immobilization of microorganisms on or in a solid support material is widely used to prevent the cell loss during continuous fermentation to shorten the hydraulic retention time and also to extend the cell retention in the process. Immobilized cell bioreactors are a good approach for improved butanol production on a laboratory and industrial scale for batch and continuous fermentation modes (Ibrahim et al., 2018; Lipovsky et al., 2016). In addition, the reduction in non-productive cell growth, and cushioning effects of factors like pH, temperature, and inhibitory substances are merits of immobilized cell systems over suspended growth systems in enhanced ABE fermentation (Zhu, 2017). Authors like Zheng and Zhang (2017) reported cell immobilization as a good approach to prevent cell loss during product recovery and achieving a high butanol titer.

Two-stage continuous fermentation is another form of continuous fermentation characterized by growth in the first stage while the synthetic phase occurs in the second phase (Pugazhendhi et al., 2019). Gottumukkala et al. (2017) reported a

maximum butanol titer of 10.8 g/L/h using the two-stage continuous fermentation with immobilized cells. Figure 10 shows a typical continuous fermentation setup as described by Pugazhendhi et al. (2019).

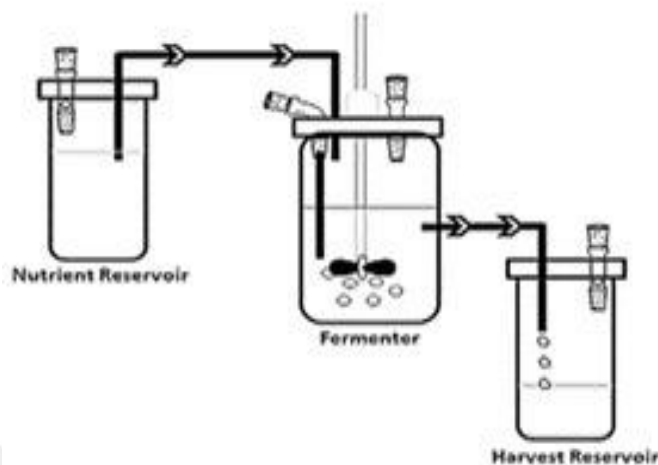


Figure 10 Continuous fermenter in butanol production (Pugazhendhi et al., 2019)

2.4 The strategies for enhancing Butanol productivity

2.4.1 *Fermentation media supplementation with metals*

Microorganisms have respective ions that facilitate their growth and the ones that inhibit it. The ions are mostly cations and the presence of these ions may either enhance the activity of enzymes present in the microorganism to perform various metabolic activities or inhibit the enzymatic ability. Thus, for all microorganisms, facilitative and inhibitive ions exist. As reported by Mukherjee et al. (2019), the addition and removal of some cations from fermentation media improve the activity of Clostridial strains and butanol synthesis. Wu, Xue, Chen, Yuan, and Bai (2015) further explained the role of the supplemented ions as they improve saccharification, acidogenesis, and solventogenesis stages. Thus, the possible role of some metal ions in butanol production is briefly discussed below.

2.4.1.1 Zinc

According to Mukherjee et al. (2019), Zn^{2+} addition to the fermentation medium improves the performance of butyrylaldehyde dehydrogenase & butanol dehydrogenase enzymes favoring butanol production and lowering of ethanol concentrations via truncation of ethanol biosynthesis and pathway direction to butanol synthesis. Zn^{2+} was regarded as one of the essential elements whose absence limits butanol production as its cellular role in DNA synthesis and cofactor are highly important for microbial growth and performance in the ABE fermentation process. Mukherjee et al. (2019) added 0.001 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ into the fermentation resulting in 15.79 g/L butanol obtained.

2.4.1.2 Magnesium

Magnesium is an important component of most lignocellulosic biomass and was reported by Abibu and Karapinar (2023) as present in hydrolyzed fig in high concentration alongside potassium. Few researches on the role of Mg^{2+} on butanol production exist. This is supported by the view of Mukherjee et al. (2019) that Mg^{2+} presence only gave a little improvement in butanol production. Mg^{2+} has never been reported as one of the metals involved in butyrylaldehyde dehydrogenase & butanol dehydrogenase enzyme activity. Thus, Mg^{2+} addition to the fermentation medium may not be necessary if present as a natural composition in the feedstock for fermentation. Mukherjee et al. (2019) regulated butanol synthesis by media supplementation with Zn^{2+} , denial of Mg^{2+} , and another supplementation that combines Zn^{2+} (0.001 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) and Mg^{2+} (0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) to observe the best result, Mg^{2+} starved medium gave the least butanol yield of 13.72 g/ L while a combined Zn^{2+} supplemented Mg^{2+} gave the highest yield of 19.18 g/ L”.

2.4.1.3 Manganese

Similarly, little information is known on Manganese's (Mn^{2+}) role in the butanol biosynthesis pathway. However, Mn^{2+} cellular role in DNA synthesis and cofactor

for microbial growth and performance are generally known. In addition, the role of Mn^{2+} in microbial stress tolerance and adaption to their niches has been reported for *Bacillus* genera by Bosma, Rau, van Gijtenbeek, & Siedler (2021).

2.4.1.4 Molybdenum

Molybdenum is a trace element also needed by microorganisms for cellular functions. Neumann et al. (2009) reported the presence of YagTSR, a major molybdoprotein cytosine dinucleotide-containing protein present in *E.coli* with a specific affinity for aromatic aldehydes and their eventual transformation into organic acids. The presence of aldehyde and oxidase enzymes such as xanthine dehydrogenase (EC 1.1.1.204) and xanthine oxidase (EC 1.1.3.22), nitrate reductase (EC 1.7.1.13), aldehyde oxidase (EC 1.2.3.1) and sulfite oxidase (EC 1.8.3.1) in molybdenum contents of lignocellulosic plants was also reported by Sauer and Frebort (2003). Aldehyde dehydrogenase is a major enzyme favoring conversion of butyraldehyde to butanol. Furthermore, Rebeille et al. (2007) reported that Moco, a molybdenum cofactor is highly important in the biosynthesis of vitamin B12. It is worth mentioning that vitamin B12 has been found to support butyryl-CoA synthase, an enzyme catalyzing butyryl-CoA production. Butyryl-CoA is the precursor for butanol (when acted upon by butyraldehyde dehydrogenase) in the ABE pathway (Boynton, Bennet, & Rudolph, 1996). Hence, the possibility of Mo^{3+} as an essential element favoring butanol production.

2.4.1.5 Sodium

Sodium (Na^+) is also another metal known as a cofactor for many enzymatic catalyzed reactions and pH regulation (as in NaOH) in many fermentation and laboratory studies. Although, Na^+ maintains osmotic balance in cells and may help microorganisms tolerate stress, however, its role in inhibiting microorganisms is widely known when used as a preservative. Potassium (K^+) may be a crucial cofactor for enzymes involved in the metabolism of butanol-producing

microorganisms alongside Na^+ . K^+ (4.0 – 5.0 mM) and Na^+ (145 mM) work together to maintain osmotic balance and tolerance in human cells (Alberts, 2015).

2.4.1.6 Copper

Limited information is known on the role of Copper (Cu) and other trace elements in butanol production. Copper is an essential trace element acting as a cofactor for numerous enzymes in microorganisms, thereby contributing to their growth and metabolic processes. It must be noted that significant activities of trace elements on butanol production are highly determined by microbial strain, growth conditions, and concentrations used in the study.

2.4.1.7 Calcium

Ca^{2+} may also serve as a cofactor for several enzymes involved in the butanol biosynthesis pathway aiding in the maintenance of the structural integrity of the cell membrane.

2.4.2 Electron Mediators

Nicotinamide adenine dinucleotide (NAD^+) and its reduced form (NADH) ratio importance in the butanol and hydrogen biochemical pathway cannot be debated especially in Clostridial strains (Mostafazadeh et al., 2016). Increasing NADH levels and reduced hydrogenase enzymes have been reported to favour butanol production (Sarchami & Rehmann, 2019; Ye et al., 2011). Thus, Moscoviz, Toledo-Alarcon, Trably, and Bernet (2016) proposed electro-fermentation as an excellent procedure acting as an effective check for NAD^+/NADH ratio in bacterial biochemical pathways. Electrofermentation favoring NADH occurring in the cathode electrodes is termed Cathodic electrofermentation (CEF). The success of CEF for increased NADH relies on electron mediators between the cathode and microbes involved in fermentation as described by Jiang et al. (2016) and Utesch et al. (2019). Methyl

viologen and Neutral Red are 2 common electron mediators studied for their effects on hydrogen and butanol in recent years.

2.4.2.1 Methyl viologen

N, N' -dimethyl-4,4' -bipyridinium dichloride commonly known as Methyl viologen (MV) or Paraquat is an electron mediator characterized by a low-potential redox indicator and has been successfully employed in biological processes for bioremediation of organic pollutants (Tashiro, et al., 2007; Aulenta et al., 2007). Tashiro et al. (2007) studied *Clostridium saccharoperbutylacetonicum* N1-4 as an inoculum for butanol production from butyric acid with glucose as a carbon source. 0.1 mM MV gave the highest butanol yield of 0.671 mol/mol. Bao et al. (2021) used biotechnologically engineered *Clostridium cellulovorans* in butanol production from cellulose. Thiolase (thlACA) and 3-hydroxybutyryl-CoA dehydrogenase (hbdCT) from *C. acetobutylicum* and *C. tyrobutyricum* respectively and Butyraldehyde/butanol dehydrogenase gene adhE2 and NAD(P)⁺ were overexpressed for butanol production. The overexpression favored acetyl-CoA conversion into butyryl-CoA, thus, increasing butyric acid and butanol production. MV was added as an EM for increased NADH. Butanol production of 5.6 g/L was obtained. Bao et al. (2021) and Bao, Zhao, Li, Liu, & Yang (2019) also achieved 4.0 g/L n- butanol from cellulose with engineered *Clostridium cellulovorans* adhE2 using 100 µM MV as an EM. According to Bao et al. (2021), 5.6 g/L was the highest ever obtained in batch fermentation by any known bacterial strain with MV as EM. Liu et al. (2013) also used 0.4 mM MV as EM to improve butanol yield by increasing NADH levels and diverting biochemical pathway to favour butanol rather than hydrogen (which reduces NADH to favour its production) with *Clostridium acetobutylicum* B3 as inoculum. A 37.8% increase in butanol production was recorded with a reduced acetone and hydrogen yield (0.60 mol/mol glucose).

Nasser Al-Shorgani, Kalil, Wan Yusoff, Shukor, and Hamid (2015) also researched the effect of MV addition to fermentation media on butanol production and butanol/ acetone ratio by *C. acetobutylicum* YM1. 0.1 mM MV produced 12.49

g/L of butanol and butanol / acetone ratio (6.09/1). Jeon, Jin, Choi, Lee, and Kim (2013) think that $\leq 1\text{mM}$ MV is the best range in which MV acts as an excellent EM to maximize and minimize butanol and hydrogen production respectively. MV is detrimental to the hydrogenase enzyme (hydrogen-producing enzymes) by competing and overpowering reduced ferredoxin (FdH_2) for the active site of the hydrogenase enzyme. Du et al. (2021) attributed low butanol concentrations to the failure of previous ABE and butanol fermentation research in the elimination of acetone by Clostridial strains by eliminating hydrogenase gene *hydA* favouring hydrogen and acetone production. This was successfully done by Du et al. (2021) using plasmid pSY6 to impede *hydA* in *C. acetobutylicum* ATCC 55025 and corn stover as inoculum and biomass respectively. This achieved an 18.3 % increase in butanol and a 31.2 % decrease in acetone production. Upon further supplementation with 0.5mM MV (as an EM), a butanol concentration of 13.8 g/L, yield and ratio 0.28 and 84.0 %, respectively were obtained. It is worth noting that some authors have reported the deleterious effects of MV on microbial cells when present in high concentrations by impeding bacterial growth (Reimann, Biebl, & Deckwer, 1996). This claim is supported by the use of MV as herbicides in agriculture as reported by (Bus and Gibson, 1984).

2.4.2.2 Neutral red

Neutral red (3-amino-7-dimethylamino-2-methyl phenazine) is a pH-sensitive dye widely used in microbial studies to differentiate microorganisms based on their ability to retain the neutral red dye (Lamanna and McCracken, 1984). Retention or color change of the NR dye implies many biochemical potential of the microorganism involved. Retention can be linked to virulence. The virulence effect of NR on cell membranes has been reported in some scientific publications. According to Cardona et al. (2006), excess NR concentration affected the cell membrane of *M. tuberculosis*. In addition, the retention of NR dye is a distinctive feature for differentiating between virulent *M. tuberculosis* and avirulent ones. However, a change in the NR colouration may imply organic acid production and pH lowering potential of the organism. Electron transfer in the (CEF: NR) system has

been found effective in ABE fermentation (Zhang, Li, Meng, Sun, & Yan, 2021) favouring butanol and hydrogen production. Furthermore, Zhang et al. (2021) affirmed the role of NR in improving substrate degradation in CEF: NR, thus, enhancing the release of enough fermentable sugar for butanol formation. EMs are highly favourable in ABE fermentation. Electron supply from EM even in small amounts enhances solvents production in the ABE fermentation as reported by Engel, Holtmann, Ulber, & Tippkotter (2019). Zhang et al. (2021) studied CEF: NR: ABE fermentation for hydrogen and butanol using *Clostridium beijerinckii* NCIMB 8052 as inoculum and glucose as a carbon source. The CEF: NR: ABE fermentation yielded 5.49 g/L and 3.74 L/L of butanol and hydrogen respectively. These were remarkable >500% and >300% respectively improvements compared with open circuit fermentation (Controlled). This indicated the significance of NR as an efficient EM. Researchers like Ujor, Okonkwo, and Ezeji (2016) and Arbter, Sinha, Troesch, Utesch, & Zeng (2019) have also reported NR as an important EM favouring butanol production via NADH increase in the ABE fermentation.

2.4.3 Consolidated bioprocessing technology (CBP)

As reported by Ibrahim et al. (2018), “the entire butanol fermentation process is costly as pretreatment, fermentation and microbial strains involved come from separate sources”. Thus, for cost consideration, efficient productivity, and timing, a technology that combines saccharification, enzyme source, and fermentation in the same fermenter is a better option. This technology is called “Consolidated Bioprocessing (CBP)”. The ability of the microorganism involved in this process to produce an enzyme capable of releasing fermentable sugar from the feedstock and eventually converting it into butanol makes this technology interesting (Ibrahim et al., 2018). As for strain selection for the CBP technology, Thang et al. (2010) considered Clostridial genera as a good option for improved butanol titer. Although the CBP technology has led to significant improvement in butanol yield, pretreatment and inhibitory substances that sometimes accompany the final products make the CBP technology for 2nd generation biomass more challenging. Thus, there

is a need for further research for 2nd generation biomass (lignocellulosic) as it is the most abundant in nature.

The introduction of cellulolytic and solventogenic microbial cultures or a biotechnologically engineered Clostridial strain may make CBP possible for lignocellulosic feedstock. Also, a co-culture of cellulolytic and solventogenic microorganisms may easily release fermentable sugar from lignocellulose, channelling it into the ABE fermentation. Thus, yeast and bacteria were recommended in some studies (Vivek et al., 2019; Ibrahim et al. 2018). For genetic modification of the cellulolytic and solventogenic microorganisms involved in the ABE fermentation process, a good knowledge of metabolic pathway and substrate preference of organisms were suggested to assist in genetic modification of genes and enzyme for hydrolysis and solventogenic process of ABE fermentation for improved butanol production (Wu et al., 2019). Wen, Ledesma-Amaro, Lu, Jin, and Yang (2020) were able to manipulate *Clostridium cellulovorans* DSM 743B activity to favour butyryl-CoA conversion into butanol. Furthermore, pathways leading to the formation of acetic and butyric acid were overpowered by butyryl-CoA, enabling increased butanol production. This consolidated bioprocessing study yielded 4.96 g/L of n- n-butanol. Also, Tri and Kamei (2020) employed consolidated bioprocessing to enhance butanol production. Co-culture of *C. saccharoperbutylacetonicum* and white-rot fungus *Phlebia* sp. MG-60-P2 was the inoculum. The ethanol production pathway was inhibited, thus, favouring butanol production. The study yielded 3.2 g/L butanol.

2.4.4 Clostridial/ Non- Clostridial strains improvement via mutagenesis

Although research on Clostridial strains development is improving annually, success has been made via ‘mutagenesis, genome shifting and metabolic engineering’ as described by Veza et al. (2021) in the development of super Clostridial strains with high butanol production potential. Mutagenetic strain improvement to enhance butanol titer may be random or targeted. In random mutagenesis, the involved mutagenic agents assist in the repair of damaged DNA

present in the Clostridial strain. NTG (N-methyl N' nitro-N-nitroso-guanidine) and ethyl ester of methane sulfonic acid (EMS) are two random mutagenic agents reportedly used in Clostridial strain improvement with a higher yield of butanol. Engineered Clostridial strains upon random mutagenesis possess a new feature that prevents known setbacks of the ABE fermentation (Patakova et al., 2018). Targeted mutagenesis, on the other hand, focuses on the biochemical pathway of acetogenesis and solventogenesis involved in butanol production. ABE pathways are enzyme-mediated.

Targeted mutagenesis targets genes (present in enzymes) involved in the pathway leading to product formation and uses an over-expression, a knockout, or a combinational approach on such genes to ensure high butanol production (Patakova et al., 2018). Cooksley et al. (2012) successfully decreased acetone production in the ABE fermentation by knocking out the *ctfA*, and *ctfB* genes needed for acetone production. Jang et al. (2012) carried out the same procedure and in addition, overexpressed the *adhE* gene responsible for butanol synthesis and >19g/l of butanol was recorded. Xu et al. (2015) and Cai et al. (2017) successfully generated *C. acetobutylicum* JB200 and *C. acetobutylicum* ABE-P 1201 yielding 70% and 66% improvement in butanol production respectively. In addition, *C. beijerinckii* M11 and *C. beijerinckii* BT14 are mutated clostridial strains with acid and butanol tolerance respectively (Kong et al., 2016). These strains successfully gave higher concentrations compared to their parent strains. The integration of genome shifting with mutagenesis has yielded a better result with the development of *C. saccharoperbutylacetonicum* N1-4 (a mutant cum gene editing generated strain) with an almost 30% increase in butanol production as reported by Zhang et al. (2018). This was achieved with the deletion of repressor genes and overexpression of butanol-producing genes. *C. acetobutylicum* has been the parent strain for most genetic manipulation in the Clostridial strain development for butanol production, other Clostridial strains need to be investigated. Furthermore, the Clostridial strain development still needs more research as scholarly work in this field is limited.

Non-clostridial strains and non- butanol producing organisms can also be genetically engineered for butanol production. Fermentative pathway control is a major tool for achieving this process. *E. coli*, *S. cerevisiae*, *L. brevis*, *P. putida* and *B. subtilis* are a few organisms studied for butanol production (Veza et al., 2021). *E. coli* and *S. cerevisiae* were the best organisms for butanol production, although, the concentrations obtained were below that from Clostridial strains (Veza et al., 2021). Ohtake et al. (2017) controlled CoA imbalance in *E. coli* by “metabolomics” recording 18.3 g/L butanol while Schadeweg and Boles (2016) metagenomically accelerated the reduction power of CoA, acetyl CoA and NADH. This led to a reduction in ethanol and acetone production, thus, favoring butanol.

2.4.5 Use of mixed microbial cultures in ABE fermentation

Li et al. (2013) employed *C. tyrobutyricum* and *C. beijerinckii* as microbial sources for Cassava starch fermentation. A remarkable 9.6-fold improvement in butanol concentration was achieved compared to the pure culture involving *C. beijerinckii*. Similarly, Wen, Ledesma-Amaro, Lin, Jiang, and Yangd (2014) used a co-culture of *C. beijerinckii* and *C. cellulovorans* in the fermentation of corn cobs producing 8.3 g/L butanol. It is also worthy of note that a co-culture of Clostridial and non- clostridial strains is possible for ABE fermentation. This is reflected in the work of Tran, Cheirsilp, Hodgson, and Umsakul (2010) involving co-culture of *C. butyricum* and *B. subtilis* in ABE fermentation of cassava starch with 6.5 fold increase in production when compared with the pure culture involving *C. butyricum*. 5.33 g/L butanol was obtained from the research. Relatedly, Luo et al. (2015) involved *C. acetobutylicum* and *S. cerevisiae* as co-culture in the ABE fermentation of corn starch. Co-cultured fermentation recorded 15.74 g/L of butanol when compared to pure culture with *C. acetobutylicum* (13.5 g/L). The possibility of non-clostridial bacterial strains and fungal strains in ABE fermentation was also reported by Minty et al. (2013) involving *E. coli* and *Trichoderma reesei* in the fermentation of Corn stover with 1.88 g/L isobutanol.

The main debate is about the best microbial culture for ABE fermentation with some authors favouring mixed cultures while others prefer single (pure) culture. It must be noted that both mixed and pure cultures have their pros and cons. The pure culture is a single microorganism and can easily be genetically manipulated to achieve targeted product concentrations. However, some authors like Pinto, Flores-Alsina, Gernaey, and Junicke (2021) reported that the risk of contamination is often high, thus, affecting productivity. Furthermore, pure cultures are a perfect microbial source for batch fermentations. The defects known with pure cultures are no problems when mixed cultures are employed in fermentation. This is often the reason mixed cultures record high productivity. In addition, Pinto et al. (2021) attributed higher productivity in mixed cultures to multiple metabolic pathways presented by each participating organism. Mixed cultures achieved $\geq 30\%$ improvement in butanol concentration. However, more scholarly work with mixed cultures is still needed.

2.4.6 Use of Acetone-free Clostridial strain

High concentrations of acetone remain the major challenge for increased butanol concentration by solventogenic strains of *Clostridium* in the ABE fermentation. Little success has been achieved regarding reducing the acetone yield during ABE fermentation. *C. sporogenesis*, a non-neurotoxic Clostridial strain produces ethanol and butanol as final ABE products (excluding acetone) (Kannan, Adapa, Yewale, Ramakrishna, & Ahar, 2010). Although *C. sporogenesis* is not considered an efficient producer of butanol in comparison with other Clostridial strains, it produced 5.52 g/ L of butanol under optimized conditions which is one of the highest reported for *C. sporogenesis* (Gottumukkala, Parameswaran, Valappil, & Mathiyazhakan, 2013). In addition, a synthetic 2, 3-butanediol synthesis pathway for *Clostridium acetobutylicum* can act as an NADH-compensating module by eliminating acetone without manipulating any of the solventogenesis genes and without causing significant undesired effects (Croux et al., 2016).

2.4.7 Acetone fermentation pathway diversion

The ABE fermentation is enzyme-mediated. Taking a careful study of the ABE fermentation chart in Figure 8, K3 and K4 represent important enzymes responsible for acetate production in the acetogenesis phase of the ABE fermentation. Sound knowledge of the inhibitors of these enzymes can be incorporated into the fermentation medium to prevent reactions leading to their formation. In the same vein, K2, K1, L2, and L1 of the solventogenic process leading to ethanol and acetone production can be prevented by the addition of their specific inhibitors while simultaneously adding cofactors for butyrate and butanol in the 2 phases respectively. This is expected to improve butanol yield appreciably. A schematic representation of this is shown in Figure 11.

Table 5 shows the list of inhibitors for enzymes involved in acetate and acetone production. This will reduce acetone or acetate production while simultaneously increasing butanol yield. Table 6 shows the activators/ cofactors for enzymes involved in butanol production. Few researchers have tried improving butanol production by the addition of metallic ions like Ca^{2+} , and Mg^{2+} and substances such as Tween 80. This achieved an improvement in butanol yield. However, there is a need for a sound optimization study to know the best concentration of these additives coupled with the appropriate physicochemical conditions needed for the best results. Table 7 shows the main processes, challenges and future perspectives in butanol production. Finally, strain selection and culture type are of important concern. Most researchers have reported the performance of immobilized cells over suspended culture cells.

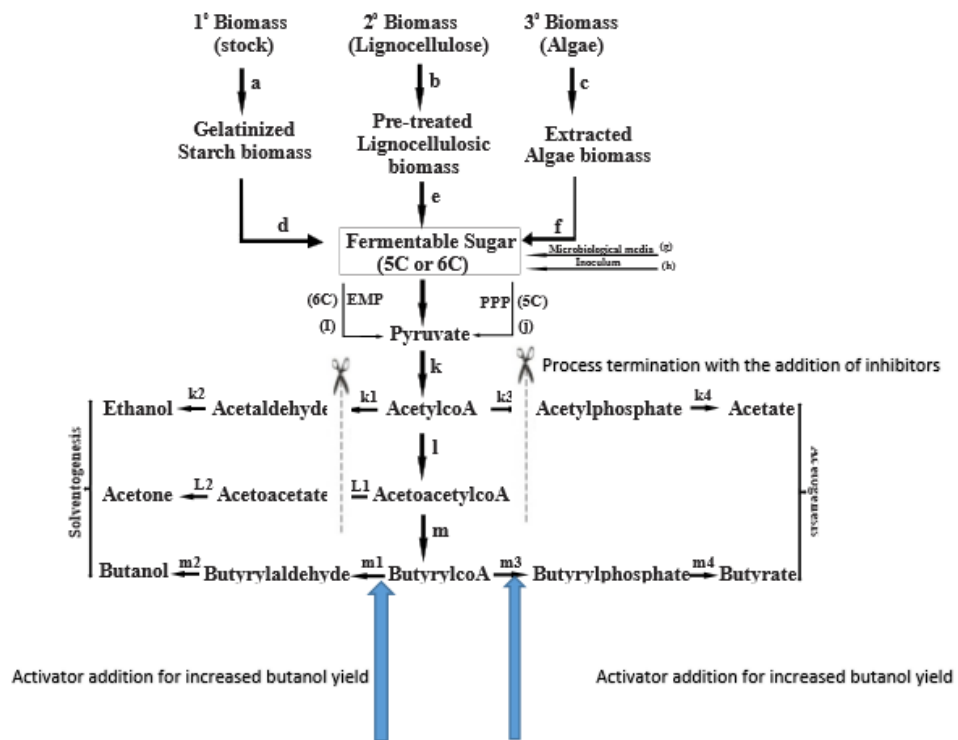


Figure 11 Acetate removal/ Butanol enhancement via inhibitor and cofactor addition (Personal archive, 2023)

Table 5 Inhibitors of enzymes involved in acetate and Acetone production

Enzyme	Microorganisms	Inhibitors	Reference
EC 2.3.1.8 - phosphate acetyltransferase	<i>Clostridium kluyveri</i>	Arsenate, citrate buffer, CuSO ₄ , K ⁺ , KCN, Li ⁺ , Mg ²⁺ , Na ⁺ and MnCl ₂	
	<i>Bacillus subtilis</i>	Ba ²⁺ , Ca ²⁺ , Mn ²⁺	(Rado and Hoch, 1973)
	<i>Lactobacillus fermentum</i>	Ba ²⁺ , Ca ²⁺ , Mn ²⁺	(Nojiri, Tanaka, & Nakayama, 1971)
Acetoacetate decarboxylase EC4.1.1.4	<i>Clostridium acetobutylicum</i>	Acetylacetone, Azure B, Borohydride, Br ⁻ , Cl ⁻ , F ⁻ , HSO ₃ ⁻ , HCN ⁻ , NO ₃ ⁻ , Zn ²⁺	(Min et al., 2013)

Table 6 Activators of enzymes involved in butyric acid and Butanol production

Enzyme	Microorganism	Activators	Reference
Butyraldehyde dehydrogenase EC 1.2.1.57	<i>Clostridium saccharoperbutylacetonicum</i>	NADH, NADPH	(Hwang et al., 2014)
Butanol dehydrogenase EC 1.1.2.9	-	-	
Phosphate butyryl transferase EC 2.3.1.19	<i>Clostridium butyricum</i> , <i>Clostridium kluyveri</i> , <i>Clostridium saccharoacetobutylicum</i>	Mn ²⁺	(Valentine & Wolfe, 1960)
Butyrate kinase EC 2.7.2.7	<i>Clostridium acetobutylicum</i>	ATP, Mg ²⁺ , Mn ²⁺	(Sullivan, Cates, & Bennett, 2010)

Table 7 Processes, challenges and future perspectives in Butanol production

Process	Challenges	Future perspective	References
Feedstock pretreatment	Economic cost Inhibitory by-product formation	Adoption of consolidated bioprocessing Development of a cost-effective pretreatment process	(Millati et al., 2020)
	Some pretreatment options (e.g. chemicals) are complex and corrosive	Use of green chemicals with low-cost	
Separation technique	Low butanol selectivity and recovery	Biochemical manipulation to favour butanol production over ethanol and acetone	
	High cost of energy requirement.	Consolidated bioprocessing method	
Fermentation	Solventogenesis intolerance as <i>Clostridium</i> spp. rarely tolerate more than 2% butanol	Using genetically engineered strains capable of withstanding solventogenic stress	Nanda et al., 2017)
	Substrate type	Optimization studies to find the best substrate that yields stoichiometrically favourable sugar for the ABE process while taking into consideration parameters such as pH, temperature and many more	
	Macro and micro-nutrient	Optimization studies to find stoichiometrically favourable micro and macro nutrients for the ABE process	
	Presence of metabolically inactive cells	Addition of macro and micronutrients to energize weak cells	
Bioprocesses Technologies	High rate of production	Designing novel immobilized bioreactors	
	Bioprocessing without pretreatment	Developing solid-state fermentation with high product separation efficiency	

CHAPTER THREE

METHODOLOGY & DATA ANALYSIS

3.1 Sample collection and preparation

Waste figs used in this research were obtained from the Aegean Exporter Association, İzmir/ Turkey, and transported to the Waste Processing Laboratory, Department of Environmental Engineering, Dokuz Eylul University, Izmir, Turkey for storage. The size of fig wastes was manually reduced to ≤ 3 cm using a handheld knife to achieve small particle size and increased surface area. This was followed with oven drying using JEIOTECH GC 300/1000 Oven at 75°C for 72 hours. Dried fig wastes were blended using a laboratory-sized blender (Waring commercial blender MODEL 8011ES). Blended figs were sieved at Mining Engineering Department, Dokuz Eylul University with a mechanized sieving machine (Retsch AS 200 BASIC) to obtain particle diameter sizes of 70 μ m, 106 μ m, 150 μ m, 212 μ m, 250 μ m, and 300 μ m.

3.2 Inoculum preparation

C.pasteurium DSM 525 and *C.acetobutylicum* DSM 792 obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) were the microbial source used in this research. The organisms were cultured anaerobically according to the manufacturer's specification i.e. (10% v/v) in growth media. *C.pasteurium* DSM 525: 40 g/L glucose, 20 g/L CaCO₃, 10 g/L yeast extract and 0.1 g/L L-cysteine. *C.acetobutylicum* DSM 792: 5 g/L Trypticase peptone, 5 g/L peptone from meat, 10 g/L yeast extract, 40 ml Clostridial salt solution, 0.5 ml resazurin, 0.5 g/L L-cysteine and 5 g/L glucose. Incubation were done at 37°C, pH 7.0 and 48 hrs.

3.3 Experimental design

3.3.1 Pretreatment

A quadratic Central Composite Design (CCD) model was used as a Response Surface Methodology to optimize fig hydrolysis via microwave (MW) and autoclaving (AC). X_1 : Particle diameter, μm , X_2 : pH, and X_3 : Power, W were the MW pretreatment parameters while X_1 : Particle diameter, μm , X_2 : pH, X_3 : Temperature, $^{\circ}\text{C}$ and X_4 : Time, min were AC pretreatment parameters. The ranges and levels of pretreatment parameters for MW and AC are found in Table 8. For MW, eighteen (18) random experiments were conducted while thirty (30) were done for AC. Total sugar concentration (g/L) was the pretreatment optimization response. The initial substrate concentration (fig) was 100g/L for both the MW and AC pretreatments.

Table 8 Experimental ranges for the process variables in MW-CCD and AC-CCD optimization

Pretreatment type	Factors	Name	Low value (-1)	High value (+1)
Microwave (MW)	X_1	Particle diameter (μm)	200	375
	X_2	pH	3.00	5.00
	X_3	Power (W)	250	670
Autoclave (AC)	X_1	Particle diameter (μm)	200	375
	X_2	pH	3.00	5.00
	X_3	Temperature ($^{\circ}\text{C}$)	95	120
	X_4	Time (min)	30	50

3.3.1.1 MW, AC and Simple heating pretreatment

MW fig hydrolysis was done in the Environmental Engineering Department of the University. The microwave (SAMSUNG ME711K) equipped with a stirrer was used in the pretreatment process. Microwaving was performed in a 1-litre beaker containing the experimental 100g/L fig sample to be hydrolyzed. The hydrolysis period was selected as 50 min at which sugar concentration reached a constant value. The liquid volume was continuously monitored to control the effect of evaporation which causes concentrated sugar.

Fig pretreatment by AC was carried out with the HIRAMAYA HICLAVE HV-50L Autoclave model. A 1litre beaker containing fig samples to be hydrolyzed was covered with aluminium foil to reduce evaporation. The CCD experimental points for MW and AC are given in Tables 9 and 10, respectively.

The simple heat pretreatment served as a comparative study with the best pretreatment option between microwaving and autoclaving. The optimization conditions for the best pretreatment option (between microwaving and autoclaving) were compared with a simple heating experiment for the sake of process economy. This was done by subjecting the optimization conditions to simple boiling at 100°C to determine whether the same TSC can be achieved with simple boiling.

Table 9 Coded and actual values of CCD for MW

Run order	Coded variables			Actual values of variables		
	X ₁	X ₂	X ₃	D _p (µm)	pH	Power (W)
1	0	1.681	0	287.5	2.32	460
2	0	0	1.681	287.5	4.0	106.8
3	1	-1	1	375	3.0	670
4	1	-1	-1	375	3.0	250
5	-1	-1	1	200	3.0	670
6	0	0	0	287.5	4.0	460
7	0	0	0	287.5	4.0	460
8	-1	1	1	200	5.0	670
9	-1	1	-1	200	5.0	250
10	0	0	0	287.5	4.0	460
11	0	0	1.681	287.5	4.0	813.2
12	1	1	-1	375	5.0	250
13	1.681	0	0	140.3	4.0	460
14	0	1.681	0	287.5	5.68	460
15	1.681	0	0	434.7	4.0	460
16	1	1	1	375	5.0	670
17	-1	-1	-1	200	3.0	250
18	0	0	0	287.5	4.0	460

Table 10. Coded and actual values of CCD for AC

Run order	Coded variables				Actual values of variables			
	X ₁	X ₂	X ₃	X ₄	D _p (μ m)	pH	T (°C)	t (min)
1	-1	-1	1	-1	200	3	120	30
2	-1	1	1	-1	200	5	120	30
3	1	-1	1	1	375	3	120	50
4	1	-1	-1	-1	375	3	95	30
5	0	0	0	0	287.5	4	107.5	40
6	1	-1	-1	1	375	3	95	50
7	0	0	0	0	287.5	4	107.5	40
8	1	1	-1	-1	375	5	95	30
9	0	0	0	0	287.5	4	107.5	40
10	1	-1	1	-1	375	3	120	30
11	-1	1	-1	-1	200	5	95	30
12	0	0	2	0	287.5	4	132.5	40
13	-1	-1	-1	-1	200	3	95	30
14	-1	-1	1	1	200	3	120	50
15	0	-2	0	0	287.5	2	107.5	40
16	-1	1	1	1	200	5	120	50
17	0	0	0	0	287.5	4	107.5	40
18	0	0	0	0	287.5	4	107.5	40
19	2	0	0	0	462.5	4	107.5	40
20	1	1	-1	1	375	5	95	50
21	0	2	0	0	287.5	6	107.5	40
22	0	0	-2	0	287.5	4	82.5	40
23	1	1	1	1	375	5	120	50
24	-1	1	-1	1	200	5	95	50
25	0	0	0	0	287.5	4	107.5	40
26	0	0	0	2	287.5	4	107.5	60
27	-1	-1	-1	1	200	3	95	50
28	1	1	1	-1	375	5	120	30
29	-2	0	0	0	112.5	4	107.5	40
30	0	0	0	-2	287.5	4	107.5	20

3.3.2 Fermentation

Batch fermentation experiments were done with air-tight 310 ml capacity serum bottles. The fermentation total working volume was 200 ml while the remaining volume accounts for the gas space. 2.0 g/L $(\text{NH}_4)_2\text{SO}_4$, 3.0 g/L Yeast extract, 10.0 g/L Meat extract, 0.2 g/L L-cysteine, 0.025 g/L Resazurin at pH 7.0 were the fermentation media composition and pH, respectively. Media composition was sterilized (autoclaving) at 121°C for 15 min. The experimental serum bottle composition was completely made anaerobic by scavenging oxygen presence in the bottles. This was done by passing the experimental serum bottles under nitrogen gas from the bottles' head space before mesophilic incubation at 37°C and 100 rpm rotating speed. The study lasted for 120 hrs.

3.3.2.1 Determination of ranges of some mediators for butanol production by One Factor at a Time experimental design

The main objective of this experiment is to study the effects of cations (Zn^{2+} , Mg^{2+} and Mn^{+}) and electron mediators (Neutral red and Methyl viologen) on the acetogenesis and solventogenesis stages of the ABE process and hydrogen production process. One factor at a time (OFAT) simple experimental design was employed in this study. The ranges and levels of Cations (Zn^{2+} , Mg^{2+} and Mn^{+}) and electron mediators (Neutral red and Methyl viologen) are found in Table 11. The high values (+) for metals and electron mediators were carefully selected based on the works of previous authors. Multiples of 10 were used for high values of the metals and electron mediators to study the effect of extremely high concentrations on ABE production. Beyond a factor of 10, there is high possibility of inhibitory effects of metals or electron mediators on intended products.

Table 11 Experimental ranges for process factors

Type	Factors	Low value (-1)	High value (+1)
Cations (mg/L)	Mn ⁺	5.6	56
	Zn ²⁺	4.0	40
	Mg ²⁺	400	1800
Electron mediators (mM)	Neutral red	1.0	15
	Methyl viologen	1.0	15

3.3.2.2 Selection of significant metals for enhanced butanol production by Plackett-Burman experimental design method.

In this study, a Plackett-Burman experimental design was used to determine the effects of metals on ABE fermentation, organic acids and hydrogen production with fig and *C.pasteurianum* DSM 525 as substrate and microbial source respectively. This was followed by an optimization study to determine the desired concentration of significant metals to maximize desired products. The elements tested in the study were Cobalt (Co²⁺), Cupper (Cu²⁺), Manganese (Mn⁺), Iron (Fe²⁺), Sodium (Na⁺), Magnesium (Mg²⁺), Zinc (Zn²⁺), Calcium (Ca²⁺), Potassium (K⁺), Molybdenum (Mo³⁺) and Boron (B³⁺). Statistical responses from the study were Solvents (ethanol, acetone and butanol) concentrations in g/L, Organic acids (Lactic, acetic and butyric acid) concentrations in g/L, Cumulative hydrogen volume (ml), hydrogen and production rate (ml H₂/L/ h). The minimum (-1) and maximum values (+1) of metals considered in this study can be found in Table 12. The minimum (-1) values represent individual metal concentrations present in the hydrolyzed fig reported by Abibu and Karapinar (2023). The Plackett-Burman actual experimental points for 11 metals (Table 13) were designed using the Design Expert 13.0, Stat- Ease®, USA (trial).

Table 12 Experimental ranges for the process variables in Plackett-Burman design

Factors	Metal	Low value (-1)* mg/ L	High value (+) mg/ L
X ₁	Co ²⁺	0.005	0.900
X ₂	Cu ²⁺	0.100	5.000
X ₃	Mn ⁺	0.285	300.0
X ₄	Fe ²⁺	1.355	10.00
X ₅	Na ⁺	10.86	16.00
X ₆	Mg ²⁺	41.06	400.0
X ₇	Zn ²⁺	0.399	50.00
X ₈	Ca ²⁺	40.00	100.0
X ₉	K ⁺	576.6	1000.0
X ₁₀	Mo ³⁺	0.003	0.5000
X ₁₁	B ³⁺	0.803	16.00

* Concentrations in hydrolyzed fig

Table 13 Plackett-Burman actual experimental points for 11 metals

Run order	Co ²⁺	Cu ²⁺	Mn ⁺	Fe ²⁺	Na ⁺	Mg ²⁺	Zn ²⁺	Ca ²⁺	K ⁺	Mo ³⁺	B ³⁺
1	0.9	0.1	300	10	16	41	0.4	40	1000	0.003	16
2	0.9	5	0.3	1.4	11	400	0.4	100	1000	0.003	16
3	0.9	5	0.3	10	16	400	0.4	40	577	0.5	0.8
4	0.005	0.1	0.3	1.4	11	41	0.4	40	577	0.003	0.8
5	0.005	0.1	0.3	10	11	400	50	40	1000	0.5	16
6	0.9	0.1	0.3	1.4	16	41	50	100	577	0.5	16
7	0.9	5	300	1.4	11	41	50	40	1000	0.5	0.8
8	0.9	0.1	300	10	11	400	50	100	577	0.003	0.8
9	0.005	5	0.3	10	16	41	50	100	1000	0.003	0.8
10	0.005	5	300	1.4	16	400	50	40	577	0.003	16
11	0.005	0.1	300	1.4	16	400	0.4	100	1000	0.5	0.8
12	0.005	5	300	10	11	41	0.4	100	577	0.5	16

3.3.2.3 Optimization of significant metal concentrations by Box Behnken Design

Box Behnken Design was selected for optimization. Mo, Na, and Zn were independent variables while organic solvents (butanol, ethanol and acetone) were the dependent variables. Table 14 shows the ranges and levels of the independent variables. Furthermore, regression analysis (R^2), model significance, ANOVA analysis, significance of individual metals on responses, and response equations to predict productivities at given values of individual factors were all obtained via the Design Expert 13.0, Stat- Ease® software. Analysis of variance (ANOVA), regression analysis (R^2), adequate precision, and 3D optimization contour plots for interactions amongst independent parameters were determined with the Design

Expert v13.0.0 trial program (Stat-Ease, Inc., Minneapolis, USA). Table 15 shows the coded and actual values of Box Behnken Design.

Table 14 Experimental ranges for the process variables in Box Behnken design

Factors (mg/L)	Name	Low value (-1)	High value (+1)
X ₁	Mo	0.15	5.00
X ₂	Na	10	100
X ₃	Zn	30	150

Table 15 Coded and actual values of MW-BBD

Run order	Coded Points			Actual values of variables		
	X ₁	X ₂	X ₃	Mo (mg/L)	Na (mg/L)	Zn (mg/L)
1	1	0	-1	5	55	30
2	0	0	0	2.575	55	90
3	-1	0	1	0.15	55	150
4	0	0	0	2.575	55	90
5	0	1	1	2.575	100	150
6	-1	-1	0	0.15	10	90
7	-1	1	0	0.15	100	90
8	1	1	0	5	100	90
9	1	-1	0	5	10	90
10	0	0	0	2.575	55	90
11	0	-1	-1	2.575	10	30
12	0	1	-1	2.575	100	30
13	0	-1	1	2.575	10	150
14	-1	0	-1	0.15	55	30
15	1	0	1	5	55	150

3.3.2.4 Effect of media composition on ABE products

The possibility of butanol concentration improvement via fermentation media composition variation was conducted. Nitrogen sources (and concentrations), yeast extract concentration, L-cysteine concentration and Ca²⁺ source are some of the media components varied. Six (6) different fermentation media were compounded with supplementation and starvation of nitrogen sources, yeast extract, L-cysteine and Ca²⁺ sources at different concentrations. Media 1 contained in g/L: CaCO₃ (5.35), FeSO₄·7H₂O (0.019), MgSO₄·7H₂O (0.2), NH₄Cl (0.8) and Yeast extract (1.0). CaCO₃ (5.35), FeSO₄·7H₂O (0.019), MgSO₄·7H₂O (0.2), (NH₄)₂SO₄: (2.0), L-

cysteine (0.1) and Yeast extract (0.6) were the constituents of Media 2 in g/L. The third media (3) only contained fig hydrolysate. This was meant to serve as the control media. Media 4 and 5 were comprised of CaCO₃ (5.35), FeSO₄.7H₂O (0.019) and CaCl. 2H₂O (0.02), FeSO₄.7H₂O (0.01) respectively in g/L. The last media (6) was comprised of Yeast extract (3.0), Meat extract (10), Resazurin (0.025), L-cysteine (0.4) and (NH₄)₂SO₄: (2.0) in g/L.

3.3.2.5 Effect of Molybdenum and Sodium concentrations on ABE products

The possibility of increased ABE products via varying concentrations of metals (Sodium and Molybdenum) was tested. This is in addition to using (NH₄)₂SO₄ as a nitrogen source. Nitrogen is really important for cellular performance and an important component of DNA and protein.

3.4 Statistical analysis

3.4.1 Central Composite Design

The significance of the model, non-significance of lack of fitness, and interactions that existed amongst the pretreatment parameters were analyzed by analysis of variance (ANOVA) while regression analysis (R²), adequate precision, and 3D optimization contour plots for interactions amongst the pretreatment parameters were obtained from the Design expert v7.0.0 trial program (Stat-Ease, Inc., Minneapolis, USA) analysis result. The quadratic polynomial mathematical model developed by the software is an indication of the relationship between pretreatment parameters and the optimization response. The mathematical model is a second-order polynomial and is presented in Equation (1).

$$Y = b_0 + \sum_{i=1}^k b_i X_i + \sum_{i=1}^k b_{ii} X_i^2 + \sum_{i < j}^k b_{ij} X_i X_j + \dots + e \quad (\text{Eq.1})$$

Where Y; is response (dependent variable), b₀: is intercept, b_i: is linear coefficient, b_{ij}: coefficient of interactions, b_{ii}: quadratic coefficients, and e: random error.

Numerical and point prediction model optimization for MW and AC experimental results were done with pretreatment parameters set in range. MW and AC optimization results were validated by duplicate experimental repetition of optimization results and compared with results obtained from numerical and point prediction optimization.

3.4.2 Sum of Ranking Differences (SRD) for MW and AC experimental design

The sum of ranking difference (SRD) method was applied to determine the closeness and relatedness of observed and predicted yield of TSC (g/L) and to strengthen the study decision regarding the best model for MW and AC experimental design using the CRRN - SRD Excel program. The averages of the corresponding observed and predicted values were used as the golden standard as described by Héberger (2010).

3.4.3 Plackett Burman Design

The Plackett-Burman design mathematical model follows the first-order polynomial model as given in Equation (2).

$$Y = \beta_0 + \sum \beta_i x_i \quad (\text{Eq.2})$$

where Y = desired experimental responses

β_0 = model intercept

β_i = linear coefficients

x_i = independent variables level.

Furthermore, regression analysis (R^2), model significance, ANOVA analysis, significance of individual metals on responses, and response equations to predict productivities at given values of individual factors were all obtained via the Design Expert 13.0, Stat- Ease® software.

3.4.4 Box Behnken Design

A quadratic polynomial model for BBD is given in Equation (3).

$$Y_{TS} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

(Eq.3)

Y_{TS} = predicted response for total sugar concentration

β_0 = model constant

$\beta_1, \beta_2, \beta_3$ = linear coefficients

$\beta_{12}, \beta_{13}, \beta_{23}$ = coefficients that represent the interactions among the independent variables

$\beta_{11}, \beta_{22}, \beta_{33}$ = quadratic coefficients.

3.5 Analytical methods

Samples removed from the hydrolysis medium were centrifuged at 8000 rpm for 15 mins. The clear supernatants were used for total sugar analysis. The samples were preserved in the fridge at 4°C.

Daily pH control (6.5-7.0) was ensured throughout the experimental study. The Total Sugar consumption was determined with a UV-Vis spectrophotometer using the method of Dubois et al. (1956). The method of Tosuner, Taylan, and Ozmihci (2019) was used to determine the total gas volume from the experiments via the water displacement method. This was followed by determining the percentage hydrogen composition from the experimental setups using the Gas Chromatograph (Agilent 1100 series) equipped with the ChemStation software (Chromatography data system). The cumulative hydrogen volume (CHV), hydrogen production rate and yield were calculated as specified by the method of Eker and Erkul (2018) and Gokfiliz and Karapinar (2017).

ABE solvents and organic acids (lactic, acetic and butyric acids) concentrations were determined using the high-performance liquid chromatography HPLC (Agilent 1200 series, USA). The mobile phase was 5 mM H₂SO₄ with a flow rate of 0.6 mL/min. The column; Aminex HPX-87H Ion Exclusion Column 300 mm x 7.8 mm at 50°C and refractive index detector (RID) was employed. Organic acid measurements were conducted by using the same column with a temperature of 40°C and a Diode Array Detector (DAD) (Tosuner, Taylan, & Ozmihci, 2019).



CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Determination of pretreatment method and conditions for Fig

Pretreatment of waste fig was conducted by microwaving (MW), autoclaving (AC) and finally by simple heating. The main purpose was to determine the pretreatment method and conditions for the maximum total sugar recovery from the waste material. A comparative approach was followed by applying MW and AC pretreatment methods. The main principle of these two pretreatment methods is hydrolysing the organic material under acidic and thermal conditions. However, the process efficiency and the time required for the process varies. Finally, simple heating was alternatively used to reveal the outcome of applying a high-technology heating process on the hydrolysis yield.

The main objective of the thesis was to determine the elements required to accelerate the enzyme activities which catalyze butanol production reactions. Therefore, it was vital to have the elemental composition of hydrolysate, understand the initial element concentration in the fermentation media and further determine the need for external element addition into the system. *C.pasteurianum* DSM 525 was the only microbial source employed in this thesis. *C.acetobutylicum* DSM 792 was also tested but achieved no microbial growth. This may be due to contamination or loss of viability during culturing. The conditions for the hydrolysis by MW were $D_p = 370.72 \mu\text{m}$, pH 4.96, $P = 253.67 \text{ W}$ and an initial fig concentration of 100 g/L. The total sugar concentration was 82.9 g/L. The hydrolysate is rich in some elements such as K^+ (576 mg/L), Na^+ (10.86 mg/L), Ca^{2+} (40.00 mg/L) and Mg^{2+} (41.06 mg/L). Some elements are required for hydrogen production such as Fe^{2+} , and its concentration in hydrolysate was 1.35 mg/L. Nutrients such as nitrogen and phosphorus are essential for the growth of organisms. The composition of hydrolysate revealed that total nitrogen is around 700 mg/L with TKN = 98 mg/L, $\text{NH}_3\text{-N} = 60.2 \text{ mg/L}$. Total phosphorus (TP) was around 43.7 mg/L which is mainly in the form of $\text{PO}_4\text{-P}$ (43.7 mg/L).

The optimization of pretreatment conditions was conducted via Central Composite Design (CCD) which is one of the well-known response surface methodology used for optimization processes. The independent variables for MW were $X_1 = D_p$ (μm), $X_2 = \text{pH}$ and $X_3 = \text{Power (W)}$ while AC was $X_1 = D_p$ (μm), $X_2 = \text{pH}$ and $X_3 = T$ ($^{\circ}\text{C}$) and $X_4 = t$ (min). The dependent variable in all experiments was the total sugar concentration (TSC, g/L) obtained after hydrolysis.

The linear, two-factor interaction and quadratic coefficients of the response functions developed for TSC (g/L) from MW and AC were determined by regression analysis. The response equations with coefficients were given in Equation (4) and (5) respectively. Predicted responses at the experimental points were calculated by using developed response equations. The actual design points, CCD hydrolysis results for MW and AC as well as predicted values from the response function are found in Tables 16 and 17 respectively.

$$\text{TSC}_{\text{MW}} (\text{g/L}) = 52.57 + 3.58X_1 + 5.97X_2 - 4.13X_3 - 1.75X_1X_2 - 0.55X_1X_3 - 4.83X_2X_3 + 4.64X_1^2 + 4.70X_2^2 + 5.37X_3^2 \quad (\text{Eq. 4})$$

$$\text{TSC}_{\text{AC}} (\text{g/L}) = 66.00 - 0.24X_1 - 0.22X_2 + 1.05X_3 - 0.096X_4 - 0.11X_1X_2 + 0.59X_1X_3 + 0.87X_1X_4 - 0.28X_2X_3 + 0.94X_2X_4 - 2.26X_3X_4 - 2.63X_1^2 - 0.055X_2^2 - 1.22X_3^2 + 0.56X_4^2 \quad (\text{Eq.5})$$

The statistical significance of the fitting model for TSC, as well as linear, two-level interactions and the quadratic terms in the fitting model were evaluated through a variance analysis (ANOVA). "Prob > F" values of the model terms which are less than 0.05 were evaluated as significant. ANOVA test result of MW indicated that the model term and pH are significant factors. Also, significant quadratic effects were found in pH and microwave power. For the case of AC, model terms were significant, but, other main factors did not significantly affect the TSC. However, the interaction between hydrolysis temperature (T) and hydrolysis period (t) was

significant indicating that these are important parameters to be controlled for maximum TSC formation by AC hydrolysis. The ANOVA results for MW and AC are found in Tables 18 and 19 respectively.

Table 16 Design matrices, actual and predicted TSC for MW pretreated fig

Standard order	Run	X ₁ D _p (µm)	X ₂ pH	X ₃ Power (W)	Observed TSC (g/L)	Predicted TSC (g/L)
1		287.5	2.32	460	53.3	55.80
2		287.5	4.0	106.8	79	74.70
3		375	3.0	670	68.9	66.77
4		375	3.0	250	66.4	66.49
5		200	3.0	670	63	57.22
*6		287.5	4.0	460	56.1	52.57
*7		287.5	4.0	460	49.1	52.57
8		200	5.0	670	65.3	63.02
9		200	5.0	250	79.9	79.83
10		287.5	4.0	460	50.6	52.57
11		287.5	4.0	813.2	53.4	60.80
12		375	5.0	250	81	84.59
13		140.3	4.0	460	57	59.69
14		287.5	5.68	460	75.3	75.9
15		434.7	4.0	460	71.3	71.72
16		375	5.0	670	69.6	65.57
17		200	3.0	250	52.9	54.73
18		287.5	4.0	460	55	52.57
R ²					0.88	

* = Centre points

Table 17 Design matrices, actual and predicted TSC for AC pretreated fig

Standard run order	D _p (µm)	pH	T (°C)	t (min)	Observed TSC (g/L)	Predicted TSC (g/L)
1	200	3	120	30	65.9	67.91
2	200	5	120	30	64.6	65.23
3	375	3	120	50	62.3	62.25
4	375	3	95	30	57	58.73
*5	287.5	4	107.5	40	64.2	66.00
6	375	3	95	50	63.7	62.90
*7	287.5	4	107.5	40	69	66.00
8	375	5	95	30	55.9	56.75
*9	287.5	4	107.5	40	65.2	66.00
10	375	3	120	30	68.6	67.10
11	200	5	95	30	60.1	60.36
12	287.5	4	132.5	40	62.3	63.24
13	200	3	95	30	62.2	61.92
14	200	3	120	50	60.6	59.58
15	287.5	2	107.5	40	66.6	66.22
16	200	5	120	50	62.2	60.68
*17	287.5	4	107.5	40	65.1	66.00
*18	287.5	4	107.5	40	64.6	66.00
19	462.5	4	107.5	40	54	55.00
20	375	5	95	50	66.5	64.70
21	287.5	6	107.5	40	65	65.34
22	287.5	4	82.5	40	60	59.02
23	375	5	120	50	62.8	62.92
24	200	5	95	50	63.5	64.83
*25	287.5	4	107.5	40	67.9	66.00
26	287.5	4	107.5	60	66.5	68.04
27	200	3	95	50	61.9	62.61
28	375	5	120	30	64.5	64.00
29	112.5	4	107.5	40	57	55.95
30	287.5	4	107.5	20	70	68.42
R ²					0.90	

* = Centre points

Table 18 ANOVA for the MW – CCD model

Source of Variation	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	1757.099	9	195.2332	7.919515	0.0039
X ₁ -D _p	174.7318	1	174.7318	7.08789	0.0287
X ₂ -pH	487.5551	1	487.5551	19.77737	0.0021
X ₃ -Power	233.3658	1	233.3658	9.46634	0.0152
X ₁ X ₂	24.5	1	24.5	0.993827	0.3480
X ₁ X ₃	2.42	1	2.42	0.098166	0.7621
X ₂ X ₃	186.245	1	186.245	7.554914	0.0251
X ₁ ²	272.8228	1	272.8228	11.06689	0.0104
X ₂ ²	279.0893	1	279.0893	11.32109	0.0099
X ₃ ²	364.6226	1	364.6226	14.79069	0.0049
Residual	197.2173	8	24.65217		
Lack of Fit	162.9973	5	32.59946	2.857931	0.2083
Pure Error	34.22	3	11.40667		
Cor Total	1954.316	17			

Table 19 ANOVA for the AC – CCD model

Source of Variation	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	387.3178	14	27.66556	8.52545	< 0.0001
X ₁ -D _p	1.35375	1	1.35375	0.417173	0.5281
X ₂ -pH	1.170417	1	1.170417	0.360677	0.5571
X ₃ -T	26.67042	1	26.67042	8.218786	0.0118
X ₄ -t	0.220417	1	0.220417	0.067924	0.7979
X ₁ X ₂	0.180625	1	0.180625	0.055662	0.8167
X ₁ X ₃	5.640625	1	5.640625	1.738221	0.2071
X ₁ X ₄	12.07563	1	12.07563	3.721238	0.0729
X ₂ X ₃	1.265625	1	1.265625	0.390016	0.5417
X ₂ X ₄	14.25063	1	14.25063	4.391489	0.0535
X ₃ X ₄	81.45063	1	81.45063	25.09992	0.0002
X ₁ ²	189.7507	1	189.7507	58.4738	< 0.0001
X ₂ ²	0.083601	1	0.083601	0.025763	0.8746
X ₃ ²	40.67146	1	40.67146	12.53336	0.0030
X ₄ ²	8.518601	1	8.518601	2.625102	0.1260
Residual	48.67583	15	3.245056		
Lack of Fit	29.41583	10	2.941583	0.763651	0.6658
Pure Error	19.26	5	3.852		
Cor Total	435.9937	29			

A regression analysis was conducted for observed and predicted TSC to evaluate the response model and model coefficients' reliability. The regression coefficients were $R^2 = 0.88$ (Table 16) and $R^2 = 0.90$ (Table 17) for MW and AC respectively indicating a good agreement between observed and predicted values. **Huang et al. (2019)** reported that $R^2 \geq 0.8$ implies model strength to the experimental data. Furthermore, adequate precisions of 8.653 and 10.533 were recorded in MW and AC models respectively. "Adequate Precision" is a measure of signal-to-noise ratio with a value of ≥ 4 desired. Thus, Adequate Precision values obtained from MW and AC implied the models are appropriate.

The validity of response equations was also evaluated by normal plots of residuals, Box-Cox and Cook's distance plots. Figures 12 to 14 present the statistical model validation results for MW and Figure 15 to 17 for AC pretreatment. According to normal plots of residuals, the differences between observed and predicted responses are within the acceptable ranges. Box-Cox plots indicated that there is no need to transform the response equation and finally, Cook's distance revealed that there is no outlier in the observed results.

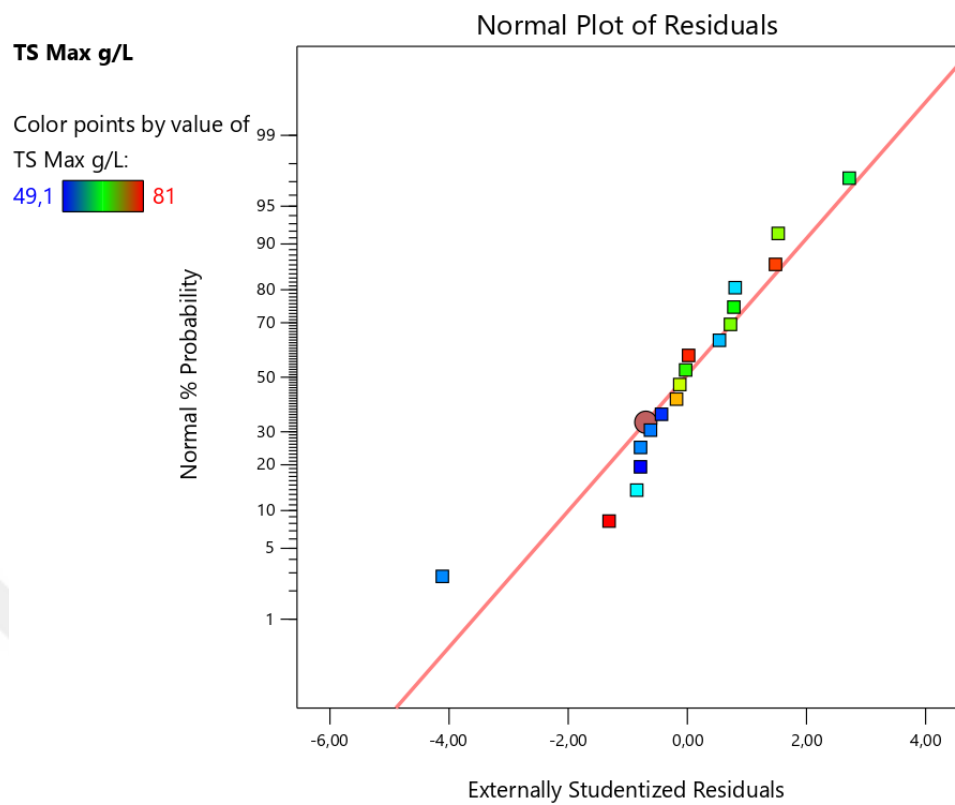


Figure 12 Normal plot of residuals for MW pretreatment

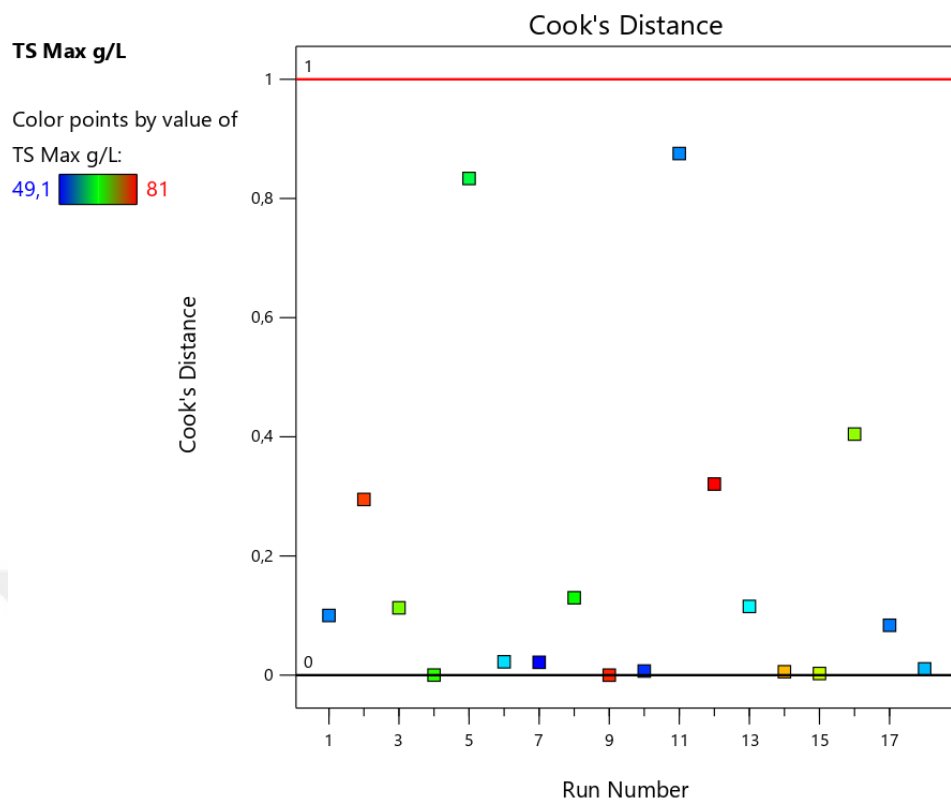


Figure 13 Cook's distance plot for the observed results of MW pretreatment

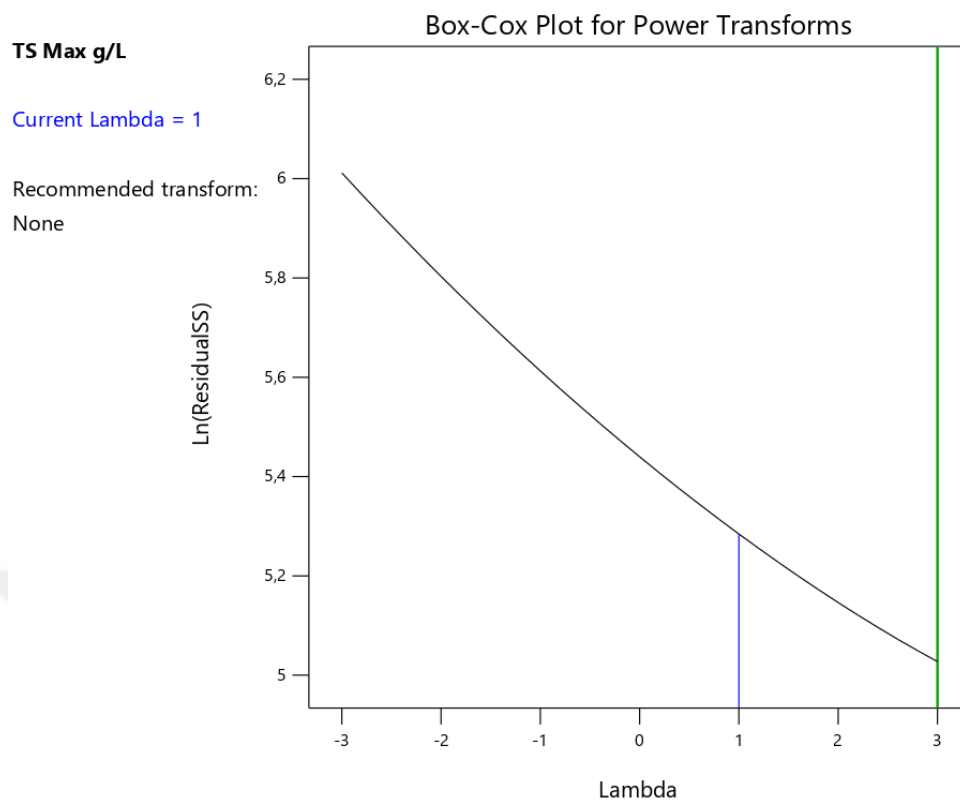


Figure 14 Box-Cox plot for power transformation of model for MW pretreatment

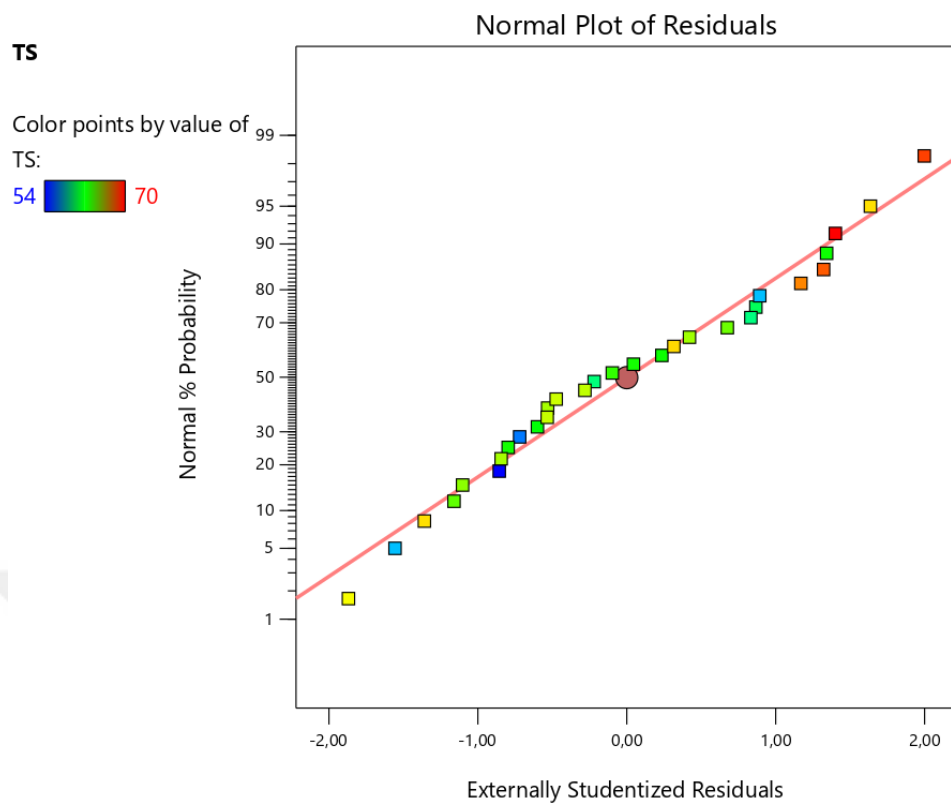


Figure 15 Normal plot of residuals for AC pretreatment

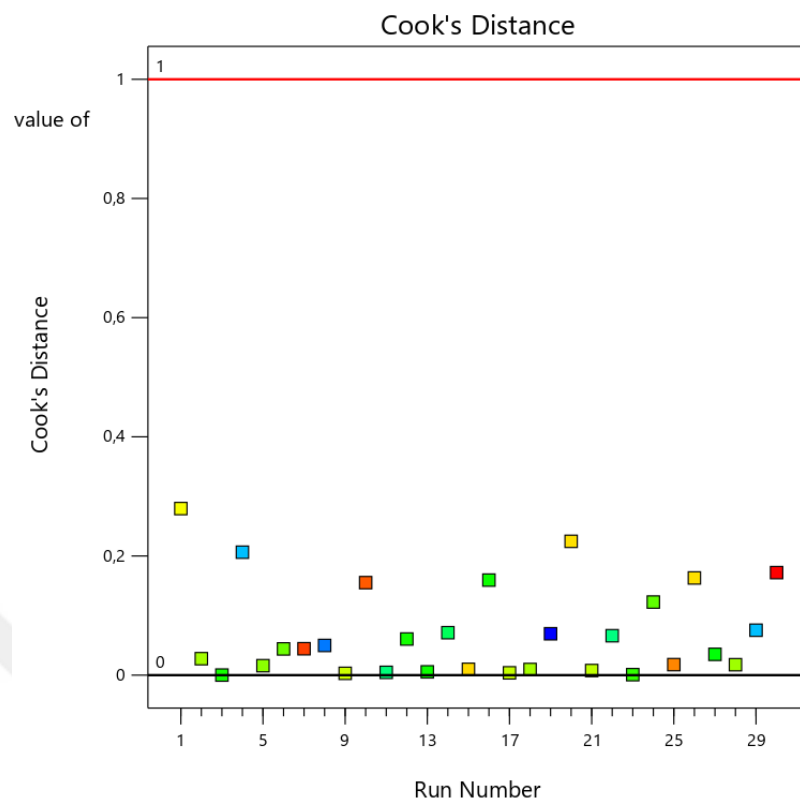


Figure 16 Cook's distance plot for the observed results of AC pretreatment

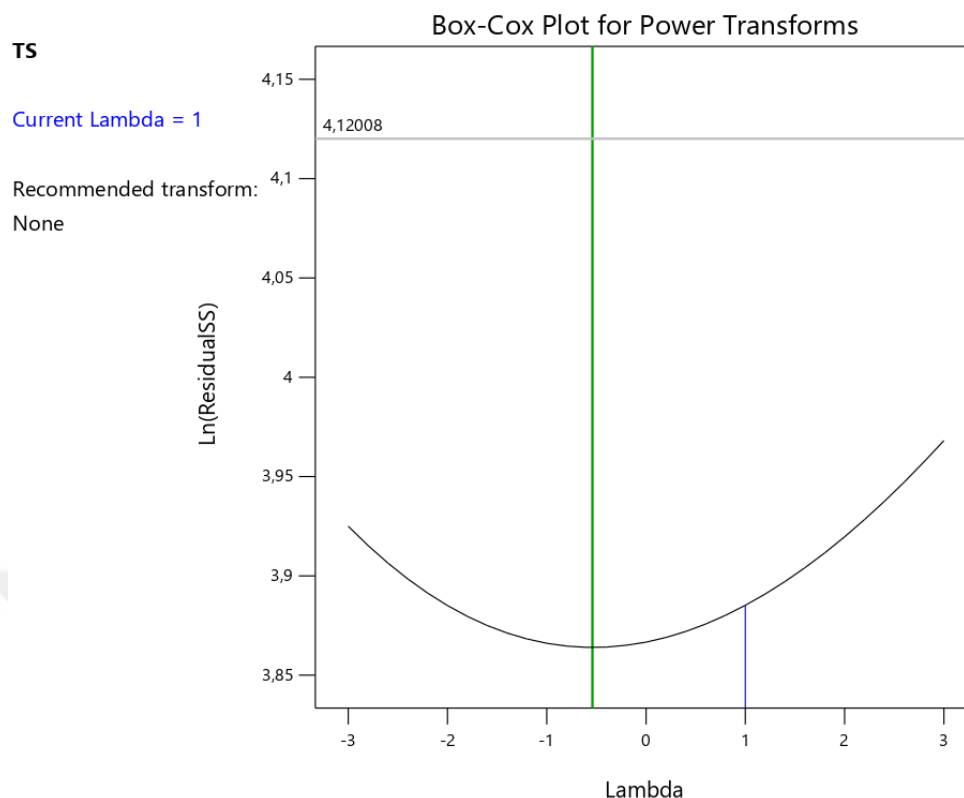


Figure 17 Box-Cox plot for power transformation of model for AC pretreatment

The sum of ranking difference (SRD) method was applied to determine the closeness and relatedness of observed and predicted yield of TSC (g/L) and to strengthen the study decision regarding the best model for MW and AC experimental design using the CRRN - SRD Excel program. The averages of the corresponding observed and predicted values were used as the golden standard as described by Héberger (2010). Heberger and Kollar-Hunek (2011) reported that SRD values nearer to zero (0) imply a better experimental model and SD value less than SRD_{max} shows no significant difference for the two compared data. SRD_{max} result was 162 for eighteen (18) MW experimental runs and 450 for thirty (30) AC experimental runs. In this study, SRD values obtained for MW and AC are 8 (Figure 18) and 3 (Figure 19) respectively. These values are less than SRD_{max} and close to zero showing that experimental and predicted results were close enough and response equation is reliable for both hydrolysis processes to be used for prediction of TSC in studied ranges of factors response equation. Besides, AC implies a better optimization model for fig pretreatment when compared with MW.

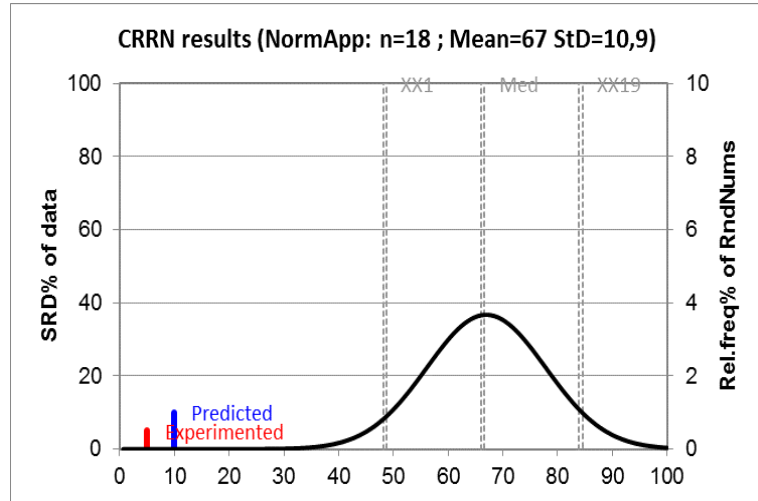


Figure 18 MW-SRD-CRRN test results of observed and predicted TSC

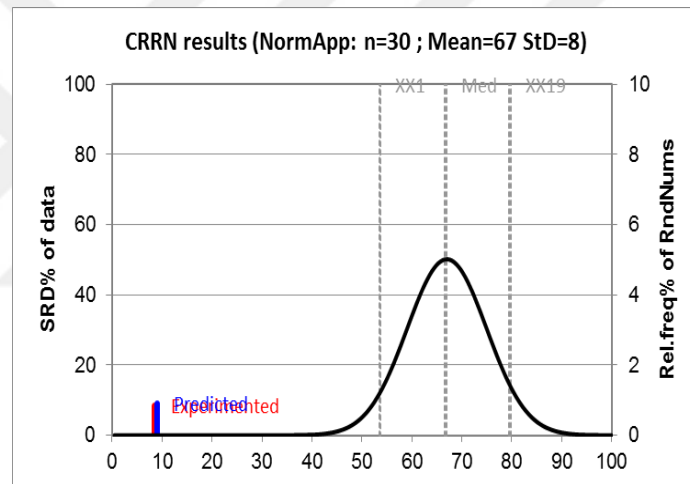


Figure 19 AC-SRD-CRRN test results of observed and predicted TSC

4.2 The effect of independent factors on TSC by MW pretreatment

The response equation (Eq 4) was used to predict the response for any hydrolysis conditions at the studied ranges of the independent variables. The 3D response surface plots for two-factor interactions in MW are found in Figures 20 to 22. The 3D plot involved studying the effect of two factors on the response while fixing the other factor(s). Figure 20 depicts the effect of pH and particle diameter at constant power ($P = 281 \text{ W}$). The effect of pH on the response was more significant than the effect of particle diameter. Interestingly, TSC increases with increasing pH from pH=3 to pH = 5. Similarly larger particles, e.g. $D_p = 375 \mu\text{m}$ result in higher total sugar concentrations at any pH value. This result is advantageous for the sake of process economy. Instead of using strong acid hydrolysis, pH = 5 is a rather mild hydrolysis condition. In addition, grinding the waste material is another process cost. Using larger particles will help in reducing the process cost.

The effect of heat applied and the particle diameter on TSC at constant pH = 5 is given in Figure 21. The heat power applied is a factor more significant than the particle diameter. The response, TSC, substantially decreased while increasing the applied power at any particle diameter. In general, the relationship between MW power and heating (temperature increase) needs to be well understood. The effect of microwave power on total phenolic content (TPC) and antioxidant capacity (DPPH and CUPRAC) on shiitake mushrooms was studied by Xiaokang et al. (2020). The research showed that an increase in MW power from 300 to 600 W increased TPC. However, DPPH concentration decreased with increasing power from 500 to 600 W. According to the authors, increasing MW power translates to temperature increase, which accelerates cell disruption and the release of bioactive components of the cell. This accounts for increased TPC at high MW power. However, increased MW power can lead to the decomposition of certain heat-sensitive compounds possessing antioxidant properties. This is responsible for reduced DPPH concentration at higher MW power.

Finally, the effect of pH and power applied on TSC at constant $D_p = 370.69 \mu\text{m}$ was evaluated and a 3D plot of the results was given in Figure 22. pH was the most significant factor in the hydrolysis of the fig. The power applied did not provide a substantial increase in the total sugar concentration. The response was almost the same around $\text{TSC} = 66 \pm 2 \text{ g/L}$ for $P = 670 \text{ W}$ (the highest power applied) at pH between 3 and 5. Similarly, response slightly increases at varying applied power between 250 and 670 W at $\text{pH} = 2$. However, the combined effect of power and the pH provided the maximum total sugar concentration around $\text{TSC} = 80 \text{ g/L}$ at $\text{pH} = 5$ and $P = 250 \text{ W}$.

Numerical optimization via Design expert v7.0.0-trial software was done to generate optimal hydrolysis conditions by setting hydrolysis parameters in range and maximizing response i.e. TSC (g/L). In the MW design, the optimization resulted in $\text{TSC} = 81 \text{ g/L}$ at particle diameter $375 \mu\text{m}$, pH 5, and 250 W power. High values of MW - $\text{TSC}_{(\text{g/L})}$ were recorded around a low microwave power range of 106.82 W and 250 W. This is similar to the experimental microwave design result of Yirgu, Leta, Hussien, Khan, and Aragaw (2021) that considered 800 to 1200W as its microwave design power range and obtained high fermentable sugar production at low MW power of 1000W. In addition, acidic pH ranges between 4 and 5 gave high values of MW - $\text{TSC}_{(\text{g/L})}$ from this study. This is supported by the experimental design matrix result of Anita et al. (2019) with increased MW - $\text{TSC}_{(\text{g/L})}$ around 0.5 and 1% acid concentration. It is worth mentioning that this is the only recent research on microwave pretreatment that considered substrate particle diameter as a pretreatment parameter for substrate hydrolysis.

From the design result, higher MW - $\text{TSC}_{(\text{g/L})}$ was recorded around particle diameters of 287.5 and $375 \mu\text{m}$. In summary, MW - $\text{TSC}_{(\text{g/L})}$ increases with increasing pH and decreasing power (W). This is in line with the report of Rorke, Suinyuy, and Kana (2017) who studied hydrolysis of waste sorghum leaves via MW and concluded that total fermentable sugar is decreased by high MW time (about 10 min) and a high MW intensity (around 800 W). MW has a great potential to release a high concentration of fermentable sugar needed for bioprocessing studies as it readily

hydrolyzes lignocellulosic biomass by structural vibration and delignification of biomass components coupled with breakage of substrates' molecular linkage forces (Moodley and Gueguim Kana 2017). This is reflected in this study with the highest fermentable sugar production (81 g/L) recorded in the MW experimental setup.

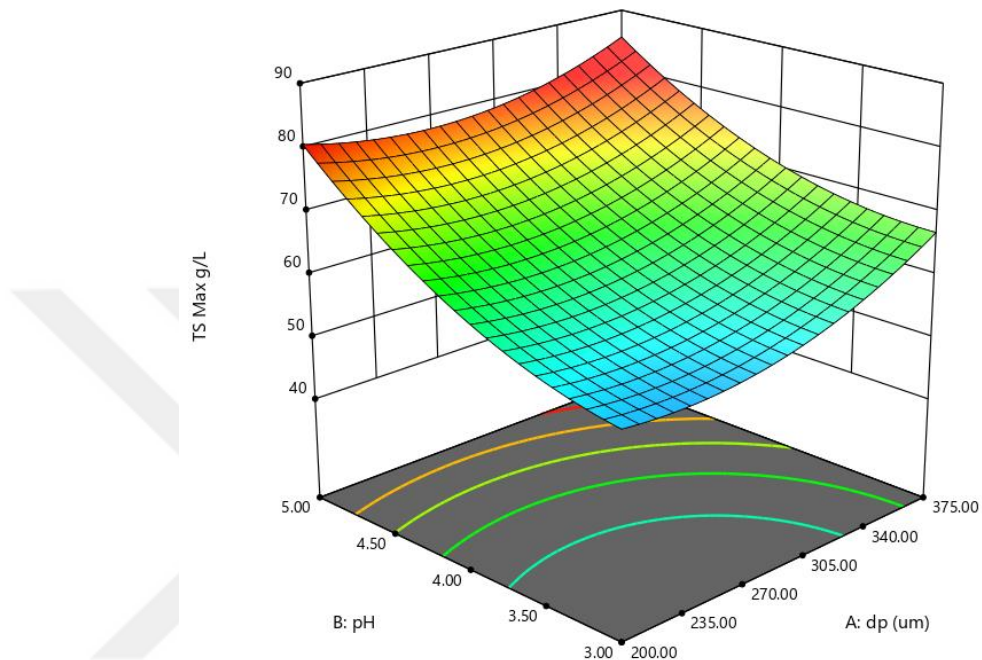


Figure 20 Variation of Total sugar concentration in the hydrolysate with pH and particle diameter at constant power ($P = 281 \text{ W}$)

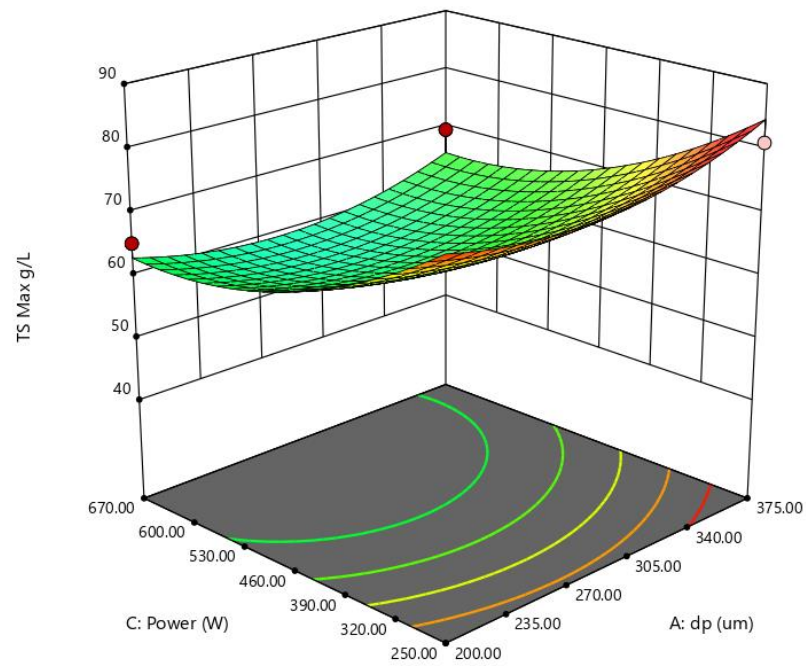


Figure 21 Variation of Total sugar concentration in the hydrolysate with MW Power and particle diameter at constant power (pH = 5.00)

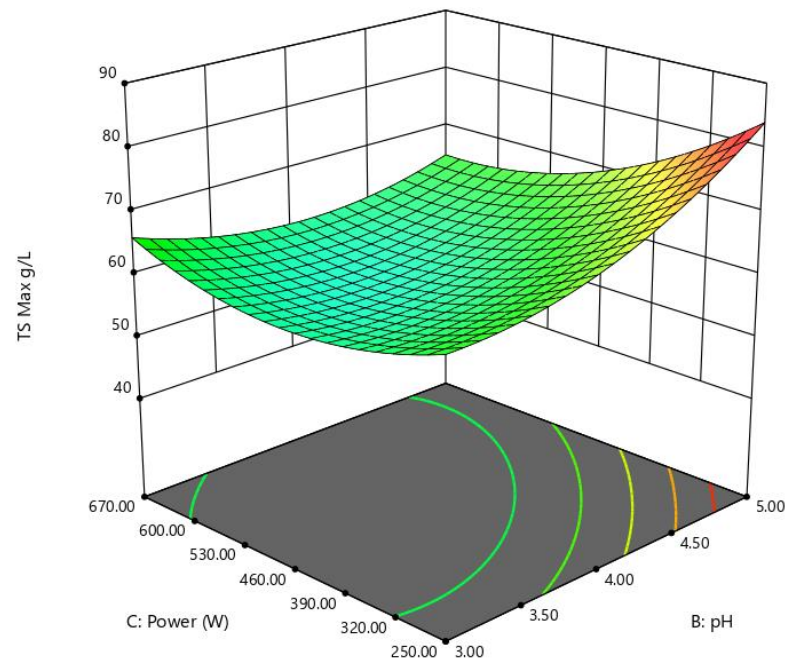


Figure 22 Variation of Total sugar concentration in the hydrolysate with MW Power and pH ($D_p = 370.69 \mu\text{m}$)

4.3 The effect of independent variables on TSC by AC pretreatment

The response equation (Eq 5) was used to predict the response for any hydrolysis conditions at the studied ranges of the independent variables in AC. The pH and the D_p were the same as the MW but the heat applied and the time required for the hydrolysis were the other independent variables. In the AC hydrolysis design, the highest TSC (g/L) was obtained in the standard run order 30 with a concentration of 70 g/L . AC maximum hydrolysis (70 g/L) was recorded at substrate particle diameter 287.5 μm , pH 4, temperature 107.5° C, and time 20 mins. The 3D response surface plots for two-factor interactions in AC are found in Figures 23 to 28. Similarly, the effect of pH on AC- TSC(g/L) from the experimental design matrix was similar to that of MW - TSC(g/L) with a high sugar concentration around acidic pH of 2 and 4. Figure 23 shows the effect of pH and D_p on TSC at constant $T = 119^\circ\text{C}$ and $t = 30.11$ min. pH has got linear effect on the response while D_p make the response quadratic. There was an increase in the response when pH was decreased from pH = 5 to pH = 3 at all particle diameters. However, D_p had a more substantial effect on the response interacting with pH. The TSC concentration increased from TSC = 65 g/L to TSC = 70 g/L when D_p was increased from 200 μm to 287.5 μm and a sharp decrease in TSC was observed for larger particles up to 287 μm at pH = 3. This result indicated a significant interaction between D_p and pH. Thus, AC pretreatment requires smaller particle sizes and more acidic conditions for efficient hydrolysis compared to MW.

The effect of temperature and particle diameter on TSC at constant pH = 3.04 and $t = 30.11$ min was depicted in Figure 24. The responses indicated that hydrolysis temperature is more significant than particle diameter, but the interaction between these two independent variables is significant as well. For example, no substantial variation existed for when $T = 95^\circ\text{C}$ at different particle diameters. The TSC was between 61 g/L at $D_p = 200 \mu\text{m}$ and decreased to 58 g/L at $D_p = 375 \mu\text{m}$. The strong effect of interaction was observed when temperature rose from $T = 95^\circ\text{C}$ to $T = 120^\circ\text{C}$ at any particle diameter. Also, the response increases when T was increased at $D_p = 200 \mu\text{m}$, but it decreases for larger particle diameters. These results indicated that

D_p should be kept smaller in AC for efficient hydrolysis compared to MW pretreatment.

Figure 25 depicts the variation of TSC with pretreatment period and particle diameter at constant $pH = 3.04$ and $T = 119\text{ }^{\circ}\text{C}$. As seen from the figure, the effect of time is more significant than that of particle diameter. The response increases when hydrolysis time is shorter at all studied particle diameters. Short hydrolysis time is advantageous for the sake of the process economy. The interaction between the variables is also slightly significant indicating that D_p should be kept around $287\text{ }\mu\text{m}$ for applied temperatures between $95\text{-}120\text{ }^{\circ}\text{C}$.

The variation of TSC with pretreatment temperature and pH at constant $D_p = 285.2\text{ }\mu\text{m}$ and $t = 30\text{ min}$ is given in Figure 26. This figure clearly shows that AC pretreatment needs high-temperature $T = 120\text{ }^{\circ}\text{C}$ and acidic condition $pH = 3$. These are stronger pretreatment conditions compared to MW pretreatment. A linear relationship between the pretreatment period and pH was observed in AC pretreatment (Figure 27). The effect of time was stronger compared to the effect of pH . Thus, reducing the hydrolysis period positively affected the TSC at any hydrolysis pH value. For example, TSC was 63 g/L at $pH = 3$, fortunately, it rose to around 70 g/L at the same hydrolysis pH .

The hydrolysis temperature and the period are generally inversely related. Extending the hydrolysis period reduces the applied temperature. Similarly, high hydrolysis temperature shortens the period. This relationship cannot be seen in AC pretreatment for certain values of independent variables (Figure 28). The response of the process at an extended hydrolysis period ($t = 50\text{ min}$) and high temperature ($120\text{ }^{\circ}\text{C}$), which represent extremely strong hydrolysis conditions, was negative probably because of the caramelization reaction which causes a decrease in the reducing sugar concentration. Decreasing the hydrolysis period to $t = 30\text{ min}$ is a rather mild process condition and the sugar formation can be enhanced by increasing the temperature. The result indicated that keeping the time at 30 minutes is enough for efficient hydrolysis if only applied temperature is over $100\text{ }^{\circ}\text{C}$.

Aissi, El Hadi, Megateli, & Ketfi (2021) researched the pretreatment of orange processing waste also achieved high AC- TSC_(g/L) around acidic pH of 0.25 and 0.5 Sulfuric acid % (v/v). Increasing temperature and particle diameter results in high AC- TSC_(g/L). Thus, AC - TSC_(g/L) increases at low pH and time and high temperature. This is similar to the study of Chandrasekaran and Sivamani (2018) in the pretreatment of Cotton Gin Waste by autoclaving which reported maximum Total Reducing Sugar production (300 mg/L) at high temperature (140° C) and low time. Similarly, Du et al. (2016) pretreated cotton stalks by autoclaving and reported a lower reducing sugar yield of 0.547 g/g at high pH, temperature and time of NaOH (3.0% (w/v), 125° C and 40 min, respectively. Furthermore, AC is highly effective in the release of hexose sugars, delignification, and release of cellulose enabling further hydrolysis when operated at acidic pH conditions. This is corroborated by the report of Auxenfans, Crônier, Chabbert, & Paës (2017) who claimed that H₂SO₄-assisted AC pretreatment effectively weakens the hemicellulose and lignin components of wheat bran.

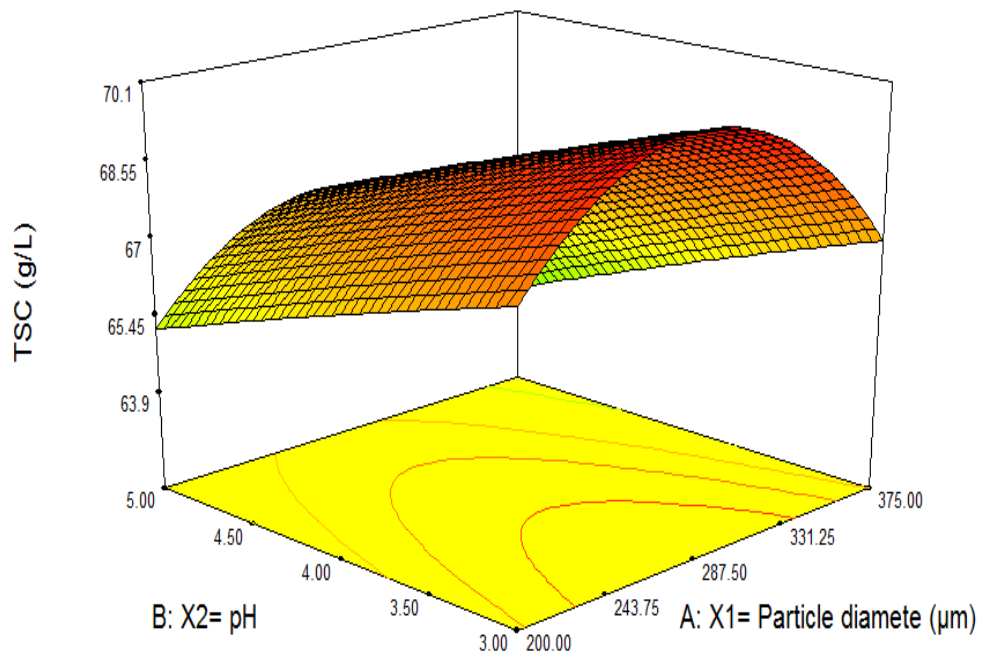


Figure 23 Effect of pH and particle diameter on total sugar formation by AC hydrolysis at constant Temperature = 119 °C and time = 30.11 min

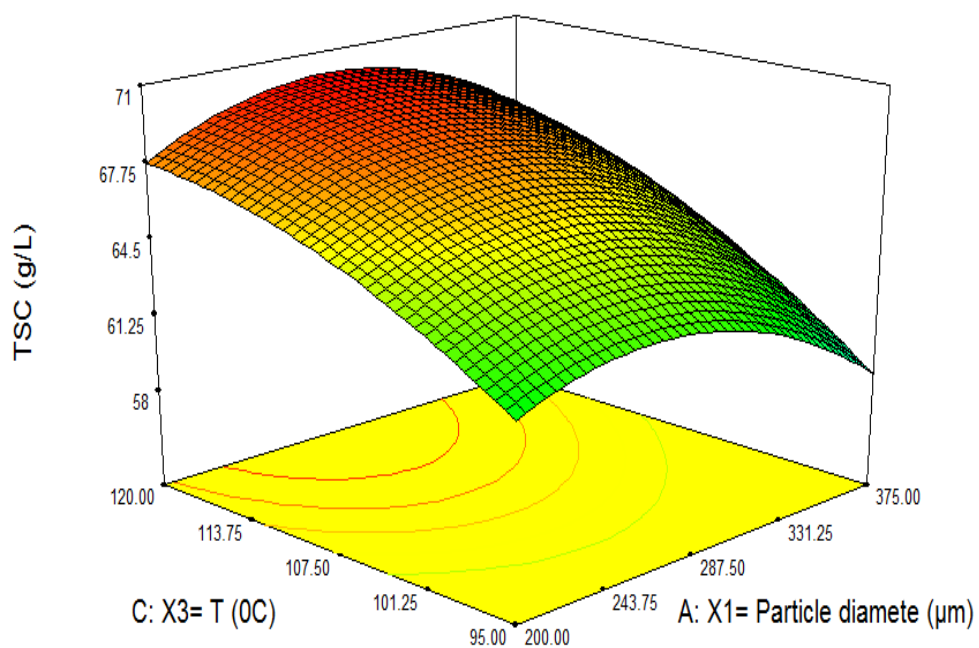


Figure 24 Effect of temperature and particle diameter on total sugar formation by AC hydrolysis at constant pH = 3.04 and t = 30.11 min

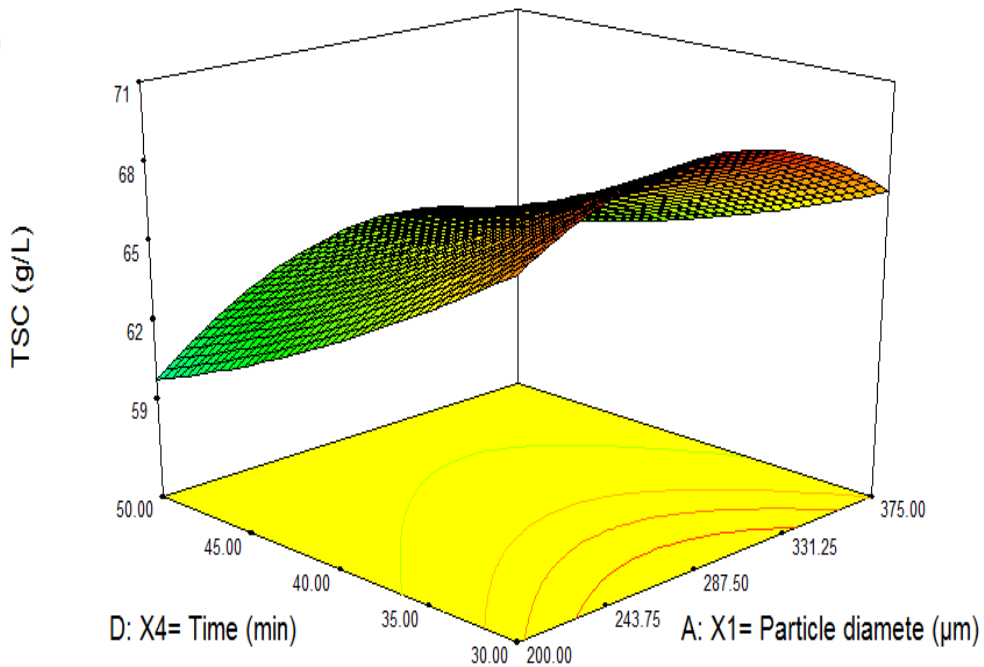


Figure 25 Effect of pretreatment period and particle diameter on total sugar formation by AC hydrolysis at constant pH = 3.04 and T = 119 °C

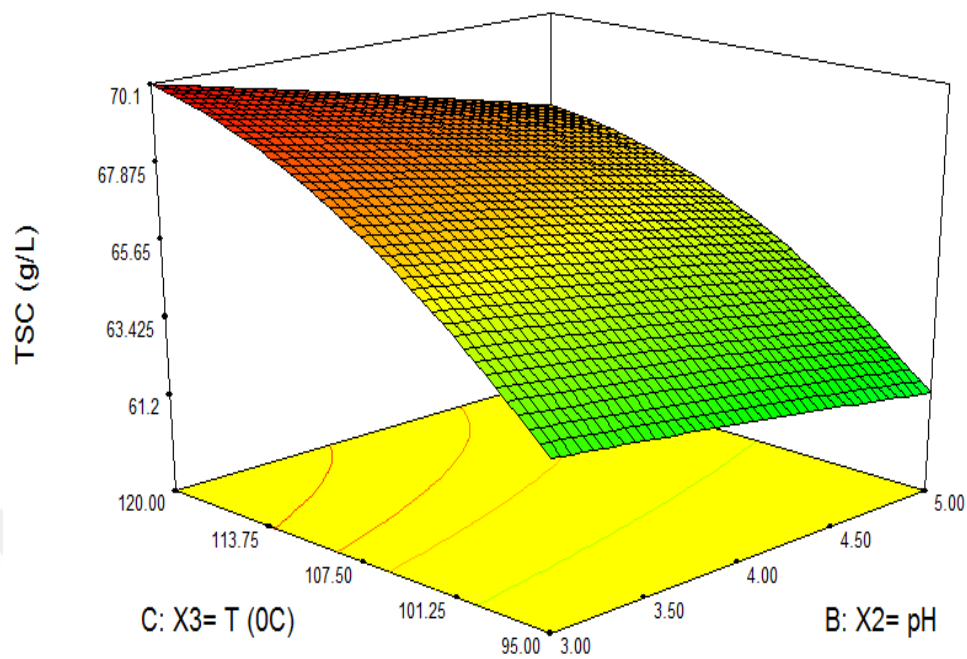


Figure 26 Effect of pretreatment temperature and pH on total sugar formation by AC hydrolysis at constant $D_p = 285.2 \mu\text{m}$ and $t = 30 \text{ min}$

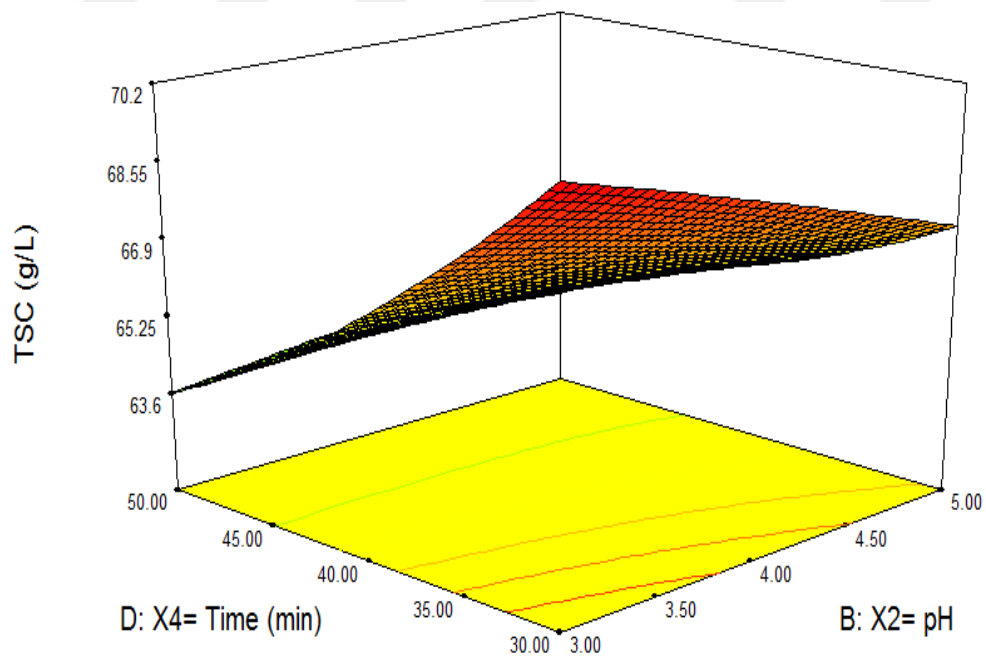


Figure 27 Effect of pretreatment period and pH on total sugar formation by AC hydrolysis at constant $D_p = 285.2 \mu\text{m}$ and $T = 119^\circ\text{C}$

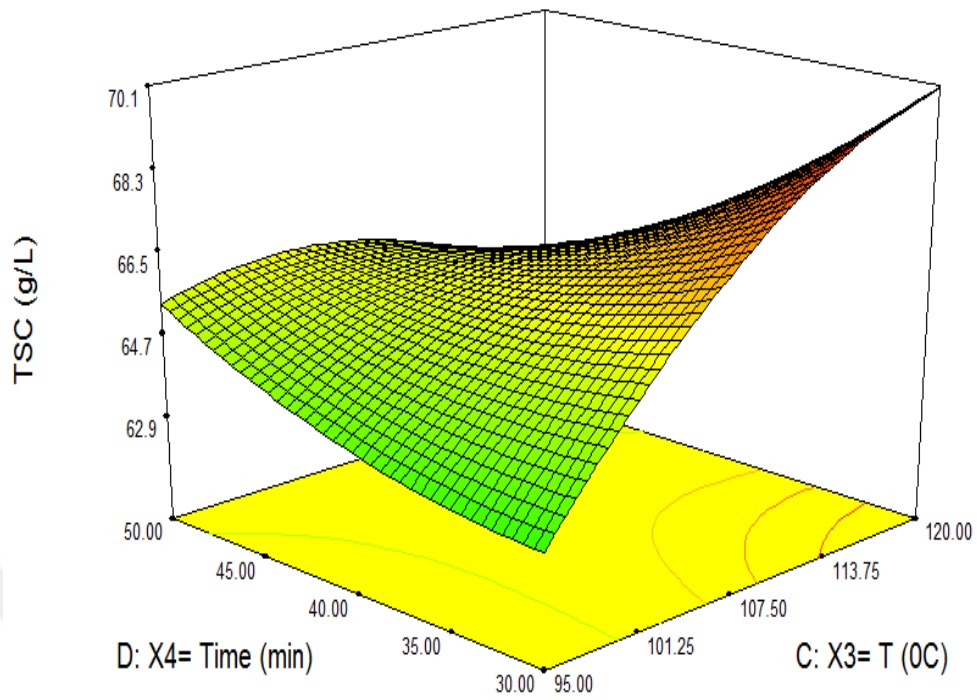


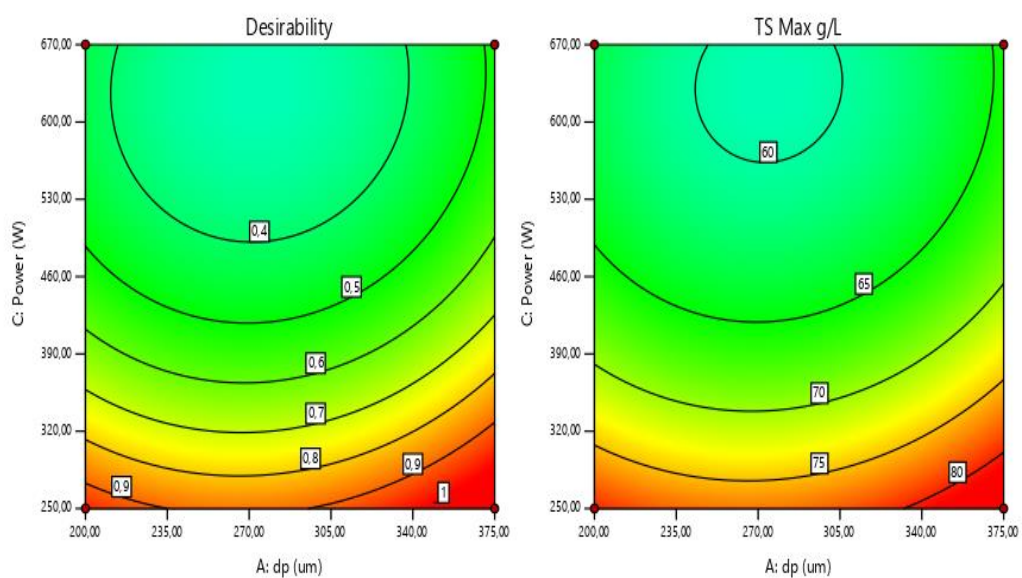
Figure 28 Effect of pretreatment period and temperature on total sugar formation by AC hydrolysis at constant $D_p = 285.2 \mu\text{m}$ and $\text{pH} = 3.04$

4.4 Optimization and validation of hydrolysis conditions

X_1 (Particle diameter, μm), X_2 (pH) and X_3 (Power, W) were MW pretreatment parameters while X_1 (Particle diameter, μm), X_2 (pH), X_3 (Temperature, $^{\circ}\text{C}$), and X_4 (Time, min) were AC pretreatment parameters for the hydrolysis of fig. The above parameters were optimized using Central Composite Design (CCD) and Total sugar concentration (g/L) as the response. Regarding MW optimization, optimal conditions were determined by using Design Expert 13.0, a trial version. The suggested optimized conditions for MW are given in Table 20. The highest sugar concentration was obtained as 82.91 g/L with a desirability of 1.00 at 370.72 μm particle diameter, 4.96 pH, and 253.67 W power. The contour plots of the optimized conditions for MW were also given in Figure 29.

Table 20 Selected optimized conditions for MW with the highest desirability level for optimization results

s/n	D _p	pH	Power	TSC, g/L	Desirability
1	370.7	4.96	253.67	82.91	1
2	352.6	4.99	250.483	81.62	1
3	374.9	4.91	250.517	82.88	1
4	373.9	5.00	253.656	84.05	1
5	362.7	4.93	256.091	81.24	1
6	361.5	5.0	257.12	82.21	1
7	370.3	4.92	255.011	81.98	1
8	364.4	4.96	255.369	81.99	1



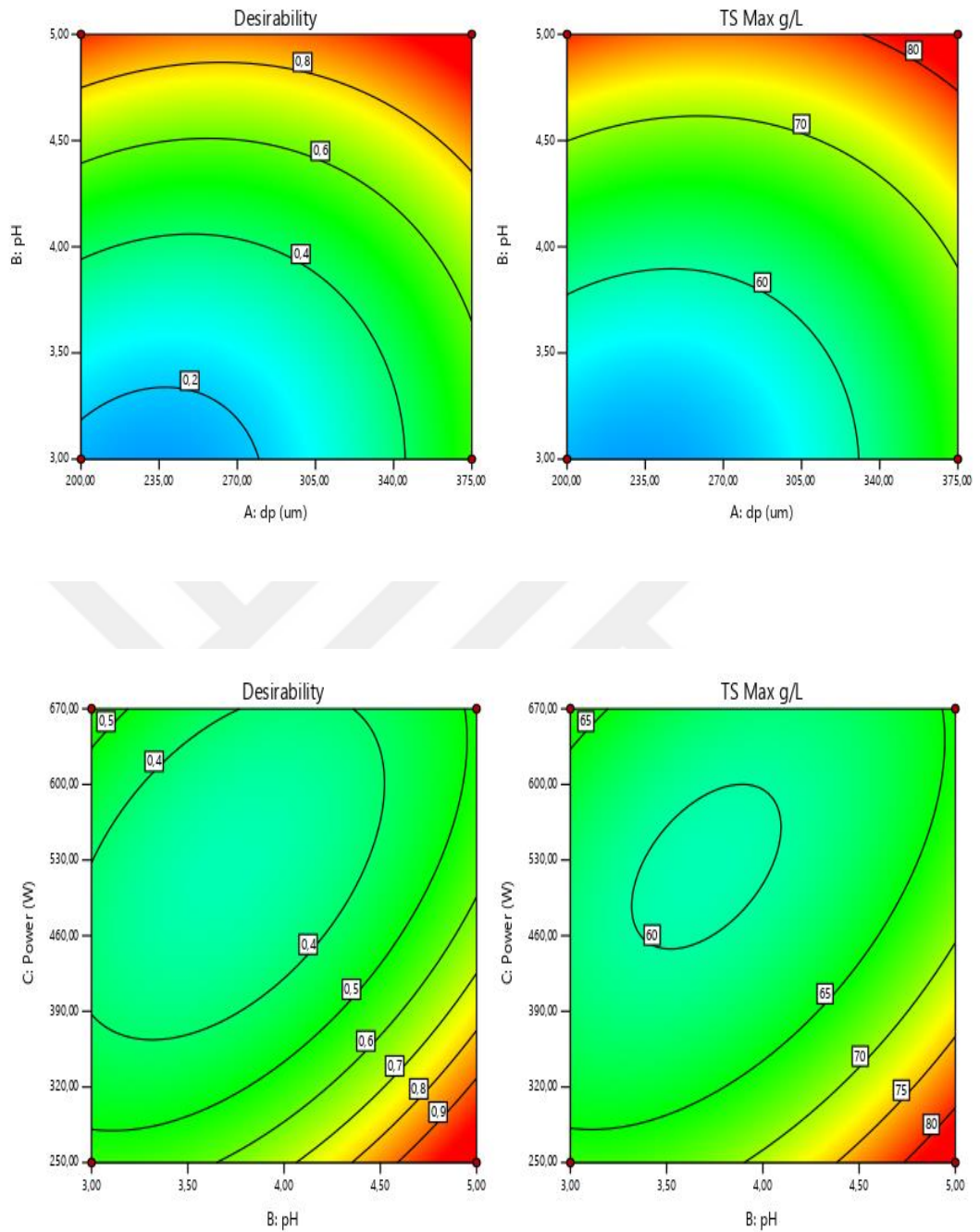


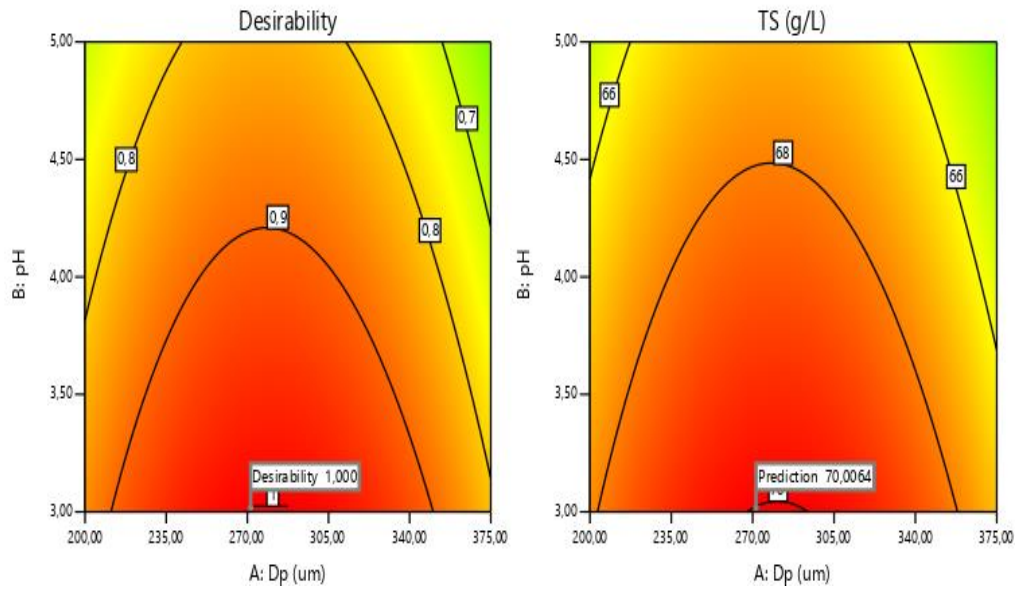
Figure 29 The variation of optimized conditions with the desirability levels for different independent variables of MW pretreatment.

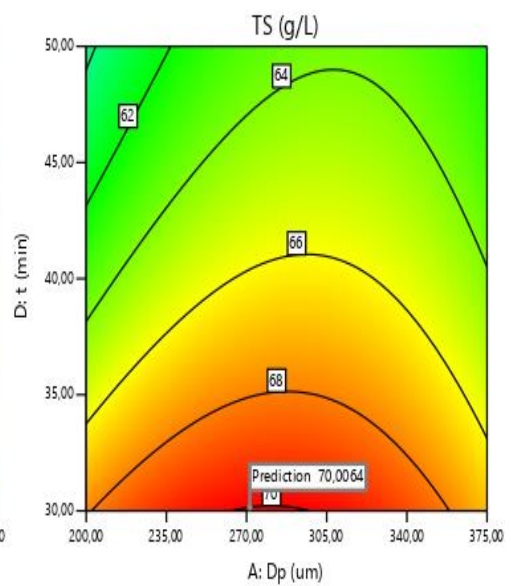
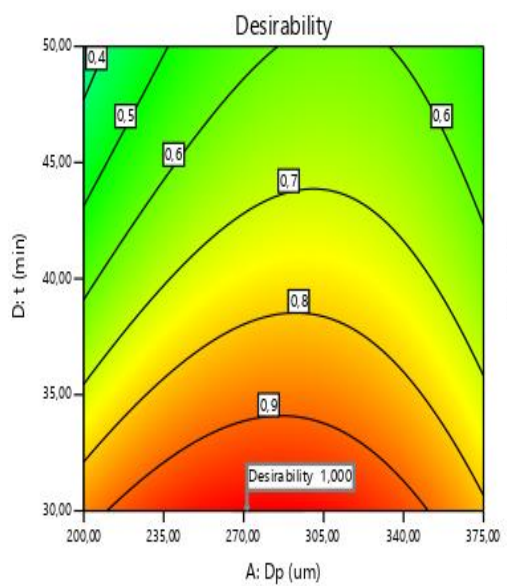
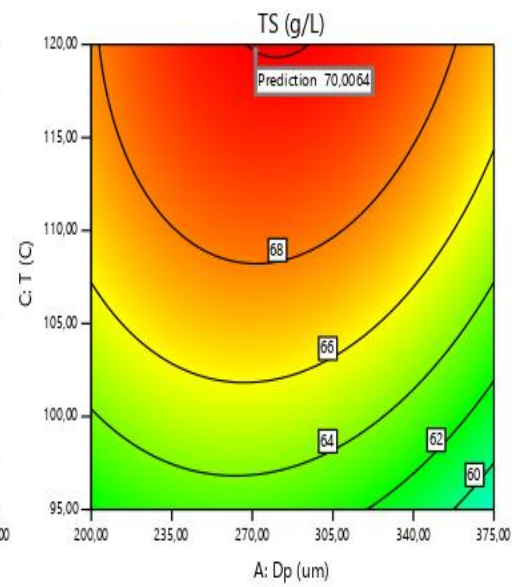
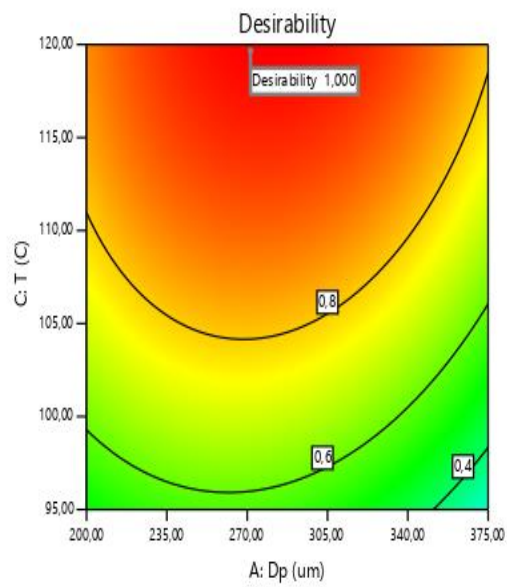
Design expert suggestions for the optimized AC hydrolysis conditions were close to each other with a maximum TSC concentration of around 70 g/L (Table 21). The selected optimized condition was 263.19 μm particle diameter, 3.02 pH, 120 $^{\circ}\text{C}$

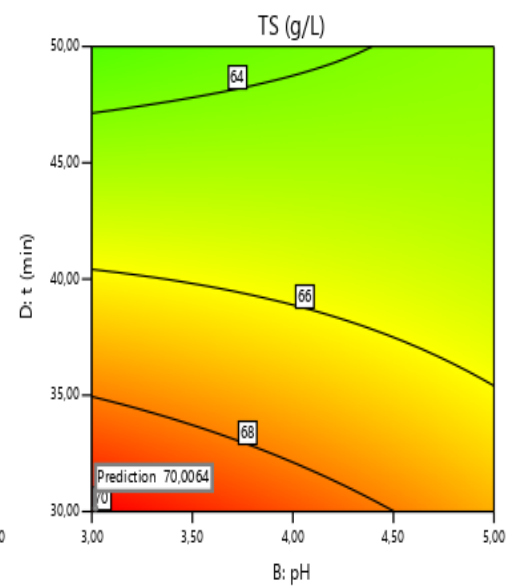
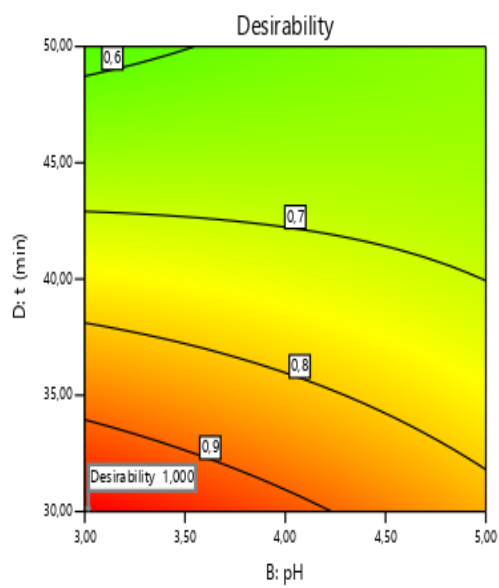
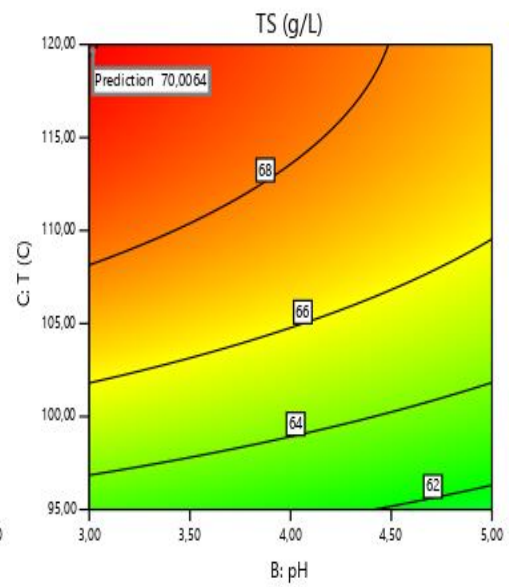
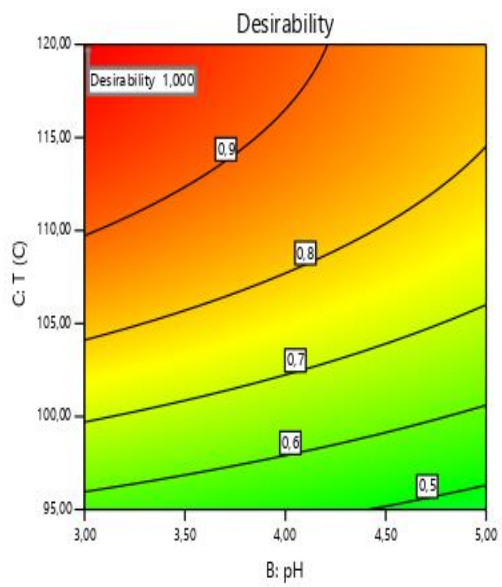
temperature, and 30.00 mins with the response of TSC = 70.22 g/L and desirability of 1.00. The contour plots of the optimized conditions regarding two-level interactions of independent variables for AC were also given in Figure 30.

Table 21 The selected optimized conditions for AC with the highest desirability level optimization results

	D _p	pH	T	T,min	TSC
1	263.19	3.02	120	30.00	70.022
2	271.448	3.018	119.678	30.144	70.006
3	280.53	3.005	119.893	30.225	70.036
4	267.297	3.004	119.39	30.017	70.024
5	270.51	3.005	119.291	30.09	70.007
6	295.852	3.007	119.63	30.058	70.003
7	293.647	3.051	119.984	30.001	70.024







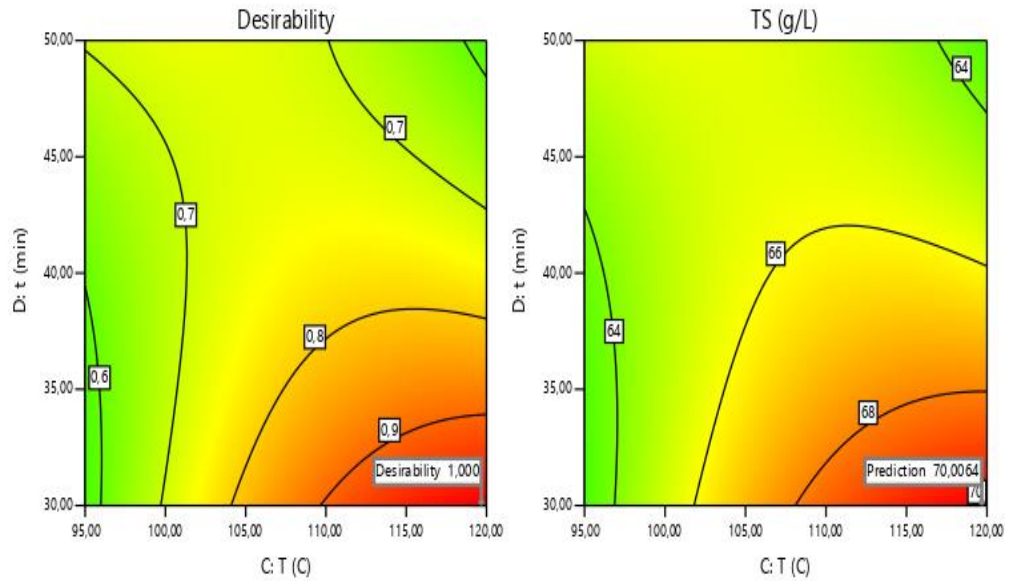


Figure 30 The variation of optimized conditions with the desirability levels for different independent variables of AC pretreatment.

The validation of the results was conducted at optimal conditions generated by the software for MW and AC by repeating three (3) experiments with the desirability of 1.00 and also conducting point prediction optimization experiments. Equal TSC (g/L) results were obtained upon validation as seen in Tables 22 and 23.

Table 22 MW Model Validation results

s/n	D_p	pH	Power	Experimented TSC, g/L	Predicted TSC, g/L
1	370.7	4.96	253.67	82.91	82.80
2	352.6	4.99	250.483	81.62	81.4
3	374.9	4.91	250.517	82.88	82.60

Table 23 AC Model Validation results

s/n	D_p	pH	T	T,min	Experimented TSC, g/L	Predicted TSC, g/L
1	263.19	3.02	120	30.00	70.022	70.01
2	271.448	3.018	119.678	30.144	70.006	70.00
3	280.53	3.005	119.893	30.225	70.036	70.02

The findings of this study had MW as a better pretreatment option for fig hydrolysis than AC. According to Lin et al. (2018), MW pretreatment gave multiple times better pretreatment outcomes in a fraction of the time necessary for other pretreatment techniques. This is supported by the research of authors like Tsegaye, Balomajumder, and Roy (2019) who researched wheat straw hydrolysis via microwave-assisted NaOH pretreatment and achieved optimized conditions of 1.5% NaOH concentration, 15 min and 160 °C MW time and temperature respectively which yielded higher TSC (g/L) (74.15% cellulose increase and 69.48% of lignin decrease). This implies the effectiveness of MW as a reliable pretreatment means of breaking down complex sugars present in lignocellulosic biomass, thus, achieving higher fermentable sugar yield. In contrast to this research findings, Moodley and Gueguim Kana (2017) studied steam and microwave-assisted salt-alkali pretreatment for lignocellulosic wastes and reported higher reducing sugar yields in the two pretreatment methods with 1.17 g/g in MW and 1.21 g/g in AC. The optimization results had autoclaving as a good model for the hydrolysis of sugarcane leaf wastes. The comparison of these thesis results with recent literature is given in Table 24.

Table 24 Optimization studies for Microwave (MW) and Autoclave (AC) pretreatment using Response Surface Methodology (RSM)

Pretreatment type	Substrate Type	Response Surface Methodology	Operation condition	Optimum values of the factors studied	Total Sugar	References
Microwave (MW)	Wheat straw	Central Composite Design	NaOH concentration (0.5–2.5%) Temperature (120–200 °C) Pretreatment time (5–25 min)	160°C, 1.5% NaOH concentration and 15 min	718 mg/g	Tsegaye, Balomajumder, & Roy (2019)
	Native elephant foot yam starch	Rotatable central composite design	Power (400 - 600W) Moisture content (15 - 35g/ 100g) Time (60-120s)	520 W power 35 g/100g moisture content Treatment time of 120 s	43.91 mg/g	Barua, Rakshit, & Srivastav, (2021)
	Dried Microalgal biomass	Central composite design	Acid concentration (0.1–5 N) Microwave power (800–1200 W) Temperature (80–180°C) Extraction time (5–30 min)	Acid concentration (2.8 N) Microwave power (1075 W) Temperature (151°C) Extraction time (22 min)	253.29 mg/g	Yirgu et al. (2021)
	Oil palm empty fruit bunch	Central composite design	Time (3-7 min) Temperature (170-190°C) Acid concentration (0.5-1.5% w/v)	Time (3 min) Temperature (190°C) Acid concentration (1.1% w/v)	34.2 %	Yirgu et al. (2021)
	<i>Typha orientalis</i>	Central composite design	Microwave power (200-400 W) Reaction time (10-20 min) Catalyst concentration (0.5-1.5 g) Solvent volume (20-40 mL)	Microwave power (400 W) Reaction time (20 min) Catalyst concentration (1.5 g) Solvent volume (40 mL)	60.8 g/L	Lin, Shangdiar, Chen, Cho, Datov, (2020)

Table 24 Optimization studies for Microwave (MW) and Autoclave (AC) pretreatment using Response Surface Methodology (RSM) (continued)

Pretreatment type	Substrate Type	Response Surface Methodology	Operation condition	Optimum values of the factors studied	Total Sugar	References
	Fig	Central composite design	Particle diameter (200-375µm) pH (3.00-5.00) Power (250-670W)	particle diameter 370.72 µm pH 4.96 253.67 W	81g/L	Thesis results
Autoclaving (AC)	Native elephant foot yam starch	Rotatable central composite design	Moisture content (15-35g/100g) Temperature (110 °C-130 °C) Autoclave time (10–30 min)	15 g/100g moisture content 128 °C of treatment temperature 30 min of treatment time	44.01 mg/g	Barua, Rakshit, & Srivastav, (2021)
	Coconut leaflets	Central composite design	Concentration of HCl (2-10 % v/v) Autoclave Time (10-50min)	3.17% of HCl and 44.14 min of autoclaving	29 g/L	Shet et al. (2018a)
	Non-edible seed cake (NESC) of <i>Pongamia</i>	Central composite design	Time (2-4 hr) HCl (30-50 %v/v)	Time (54.12 min) HCl (1.17 %v/v)	53.76 g/L	Shet et al. (2018b)
	Sweet orange processing waste	Central composite design	Sulfuric acid (0.25-0.75% (v/v) OPWP loading (2-10 % (w/v) Residence time (10-30 min)	Sulfuric acid (0.5 % (v/v) OPWP loading (2 % (w/v) Residence time (20 min)	70.7 g/L	Aissi, El Hadi, Megateli, & Ketfi (2021)
	Fig	Central composite design	Particle diameter (200-375µm) pH (3.00-5.00) T (95-120°C) t (30-50min)	263.19 µm particle diameter 3.02 pH 120°C temperature and 30.00 mins	70 g/L	Thesis results

4.5. Efficiency of Microwaving TSC optimized results with simple heat treatment

The optimization studies showed that MW is a good pretreatment method for fig hydrolysis with the highest TSC of 81 g/L. MW optimization, as earlier stated, optimization conditions were particle diameter $D_p = 370.72 \mu\text{m}$, pH 4.96, and $P = 253.67 \text{ W}$ with TSC of 82.91 g/L. The MW experimental results were compared with a simple heating experiment for the sake of process economy. This was done by subjecting MW optimization conditions to simple boiling at 100°C to determine whether the same TSC can be achieved with simple boiling. The experiment lasted six (6) hours, with hourly TSC analysis of hydrolyzed samples (Figure 31). The maximum MW-TSC of 82.91 g/L can only be reached after 6 hours of simple boiling pretreatment with a TSC of 80.25 g/L. Thus, when compared to other pretreatment methods, this demonstrates the efficiency of MW as a pretreatment method for fermentable sugar production in a short period.

Many authors have talked about the energy consumption of the various methods used in the pretreatment of lignocellulosic biomass. Ganesh, Aniruddha, & Parag (2012) studied the transesterification process via microwave and simple heating and obtained energy consumption results of 26.0 kJ/L and 94.2 kJ/L respectively. In another comparative transesterification experiment between continuous microwaving and conventional boiling, 269 kJ/L and 799 kJ/L were obtained respectively. On the other hand, GKN, an aerospace composites manufacturer studied the effectiveness and energy consumption of microwave and autoclave respectively and concluded that microwave energy consumption is 80 percent less than autoclave achieving 40 percent savings in cycle time (Sloan, 2011). The study took a total cycle time of 4.5 hours. Thus, the energy consumption of microwaving, autoclaving and simple boiling can be represented in the order: of microwaving < autoclaving < simple boiling.

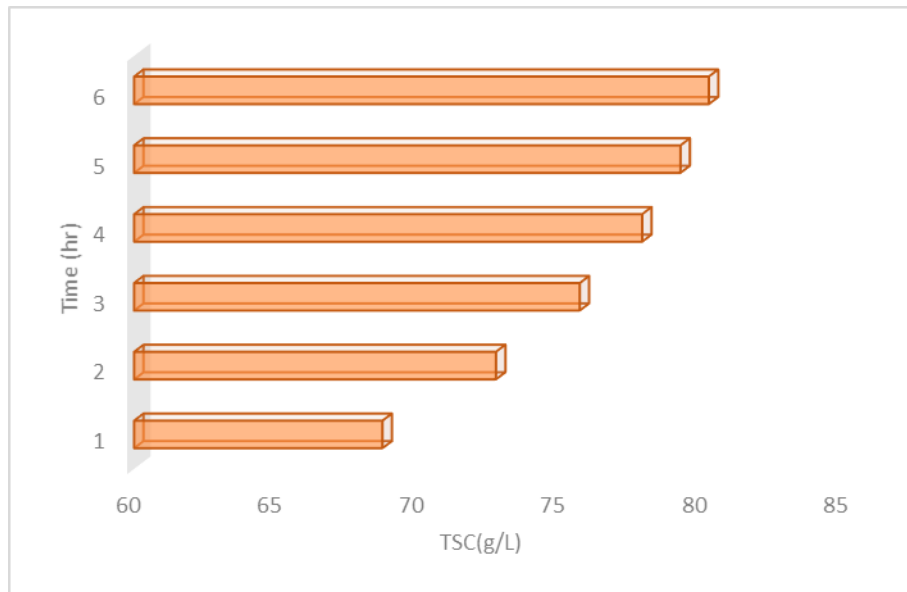


Figure 31 Simple boiling pretreatment for fig hydrolysis

4.6 The role of cations and electron mediators in ABE fermentation

The role of cations and electron mediators directly influences microbial growth, substrate utilization and valuable product formation through fermentation. Figure 7 summarizes the enzymes catalyzing biochemical reactions in ABE fermentation from glucose to products such as hydrogen, acetone, ethanol, butanol and organic acids. Similarly, Figure 11 shows a mechanism to divert the ABE fermentation pathway to favour butanol production. Tables 5 and 6 give the cations inhibiting acetone only and activators to enhance butanol synthesis respectively. No published work in recent literature exists on the comprehensive study of the effects of cations and electron mediators on ABE fermentation.

Fermentation media composition plays a vital role in the success of ABE products and most especially in the concentration of butanol obtainable. For example, Fe^{2+} known to favour hydrogen production is excluded from the fermentation media to allow easy progression of organic acids from the acetogenesis phase into organic solvents without diversion into the hydrogen production pathway. Therefore, butanol fermentation media composition should be free of Fe^{2+} . However, from Table 25, the hydrolyzed fig used in this study contained 1.35 mg/L of Fe^{2+} . This is enough to favour hydrogen production. Removal of Fe^{2+} through physical and chemical methods is not cost-effective and practical. Therefore, the cation concentrations in the raw fig hydrolysate were considered as background concentrations in the fermentation media. The lower concentrations of all the studied cations were the ones in the raw hydrolysate. The use of electron carriers to enhance butanol production is already known. The two synthetic electron carriers are methyl viologen (MV) and neutral red (NR) which divert the electrons from hydrogen generation to the butanol production reaction. However, these chemicals are costly and the use of these mediators defeats the aim of the fermentation process economy limiting the market value potential of butanol to compete with other biofuels.

By considering these facts, the effect of some cations and electron mediators on butanol production by *C. pasteurianum* was studied as the initial stage of the study. To

the best of our knowledge, no published work in recent literature exists on the comprehensive study of the effects of cations and electron mediators on ABE fermentation and hydrogen generation. Zn, Mn and Mg were selected as the cations, while MW and NR were the electron mediators. The concentration of these cations was selected as low (-), which is the concentration in the raw hydrolysate, and high (+) for which cations were externally added and extreme concentrations were selected to determine the concentration range of cations.

Table 25 Elemental and nutrient constituents of hydrolyzed fig

Elements mg/ L					
K ⁺	576.6	Cu ²⁺	0.1007	Ca ²⁺	40.00
Hg ⁺	0.0016	Sr ²⁺	0.2821	V ²⁺	0.0009
Li ⁺	0.0028	Mo ³⁺	0.0034	Cr ³⁺	0.0002
Zn ²⁺	0.3990	Ag ⁺	0.0005	Mn ⁺	0.2849
B ³⁺	0.8032	Cd ²⁺	0.0015	Fe ²⁺	1.355
Na ⁺	10.86	Sn ²⁺	0.0055	Pb ²⁺	0.0097
Mg ²⁺	41.06	Sb ³⁺	0.0004	Co ²⁺	0.0051
Al ³⁺	0.0916	Ba ²⁺	0.1617	Ni ⁺	0.1152
Nutrients mg/ L					
PO ₄ -P	43.7				
TP*	49.4				
NH ₃ -N	60.2				
TN*	700				
TKN*	98.0				
Potential toxic compound g/ L					
HMF*	1.15				

*TN: Total Nitrogen, TP: Total Phosphorus, TKN: Total Kjeldahl Nitrogen, HMF: Hydroxymethylfurfural

4.6.1 Total sugar utilization

The concentration of sugars in the hydrolysate was evaluated in terms of the Total sugar concentration. The Time variation of Total sugar consumption for different cations and electron mediators was given in Figures 32 to 36. The initial total sugar concentration was around 50 g/L in the fermentation media. A rapid decrease in the sugar concentration was observed in all studied conditions. This is an indication of microbial activity and conversion of substrates into ABE and other products. Substantial differences were observed for the low and high concentrations of cations and mediators. The highest sugar consumption was observed in the presence of Zn^{2+} with more than 90%. However, Mn^{2+} slightly inhibited the total sugar consumption and final sugar was obtained at around 20 g/L with around 60% utilization efficiency.

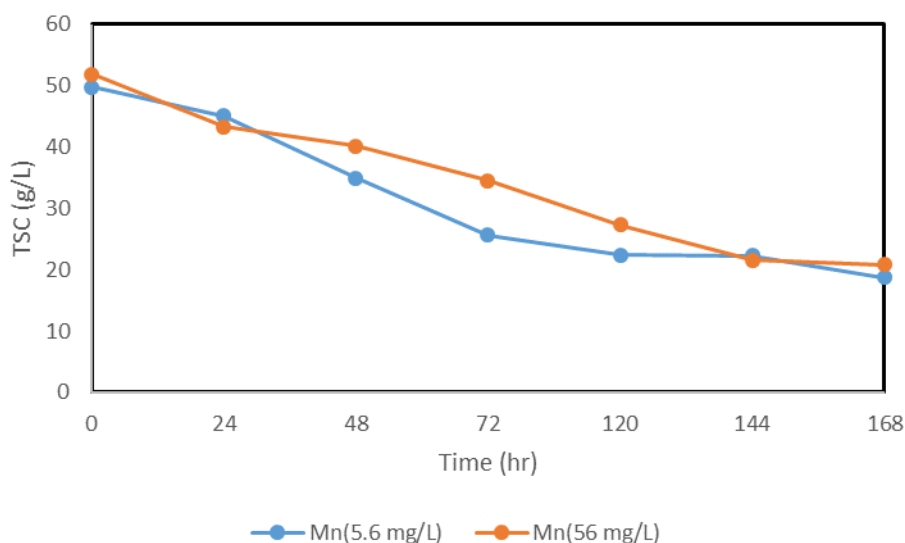


Figure 32 Variation of Total sugar concentration with time at (-) low and (+) high concentrations of Mn^{2+}

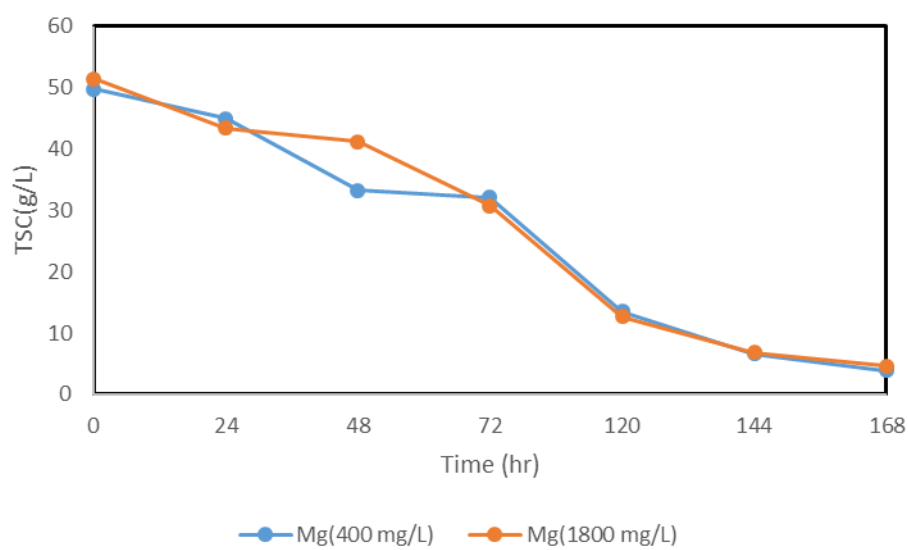


Figure 33 Variation of Total sugar concentration with time at (-) low and (+) high concentrations of Mg^{2+}

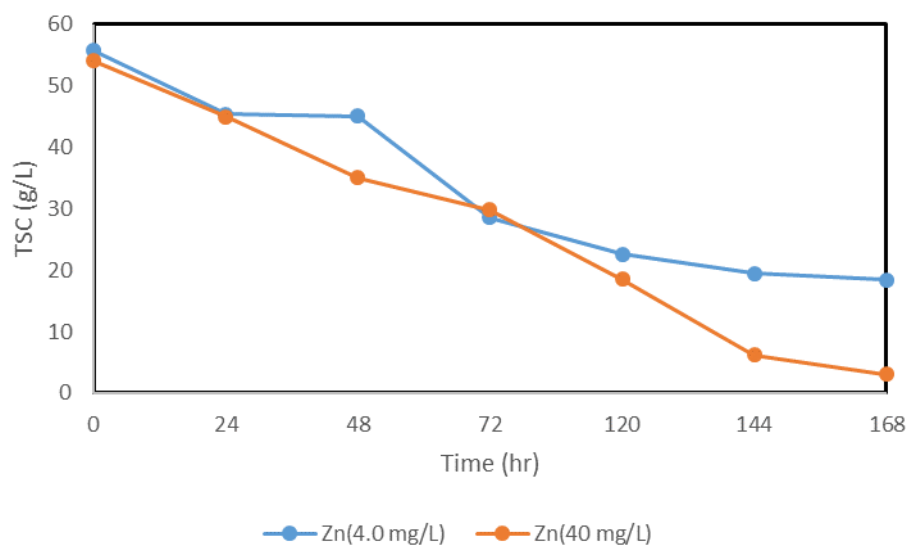


Figure 34 Variation of Total sugar concentration with time at (-) low and (+) high concentrations of Zn^{2+}

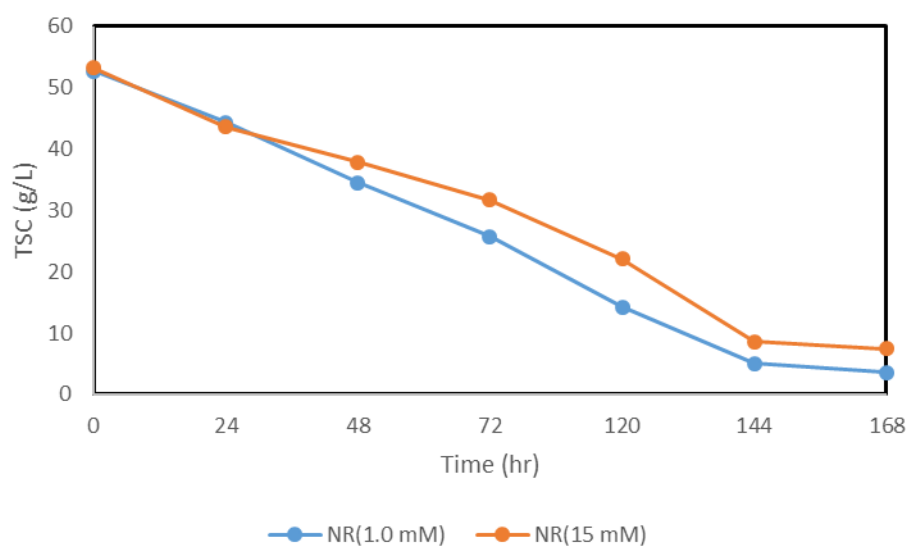


Figure 35 Variation of Total sugar concentration with time at (-) low and (+) high concentrations of NR

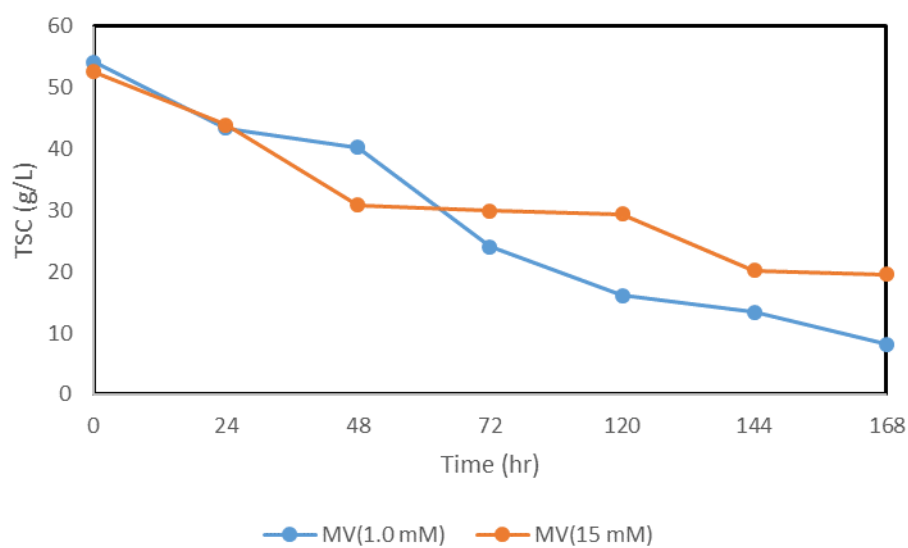


Figure 36 Variation of Total sugar concentration with time at (-) low and (+) high concentrations of MV

4.6.2 Simple sugar utilization

The simple sugars concentrations such as glucose and fructose were determined in HPLC and utilization of these sugars was evaluated for different cations and electron mediators. Figures 37 to 41 show the variation of glucose concentration with time in the experimental setup. The initial glucose concentration was between 10 and 11 g/L in most of the experiments. The utilization of glucose was in parallel with the Total sugar utilization. The final concentrations were less than 2 g/L resulting in over 80% glucose utilization. The glucose consumption was enhanced in the presence of Zn^{2+} and Mg^{2+} . However, in the case of NR added fermentation study, the glucose utilization experienced a lag phase which was not observed in Total sugar utilization.

Similarly, fructose utilization was given in Figures 42 to 46. The initial fructose concentration was between 8 g/L and 12 g/L. It is seen that fructose utilization is faster and completely consumed for most of the studied conditions. However, MW addition had a slightly negative effect on fructose utilization.

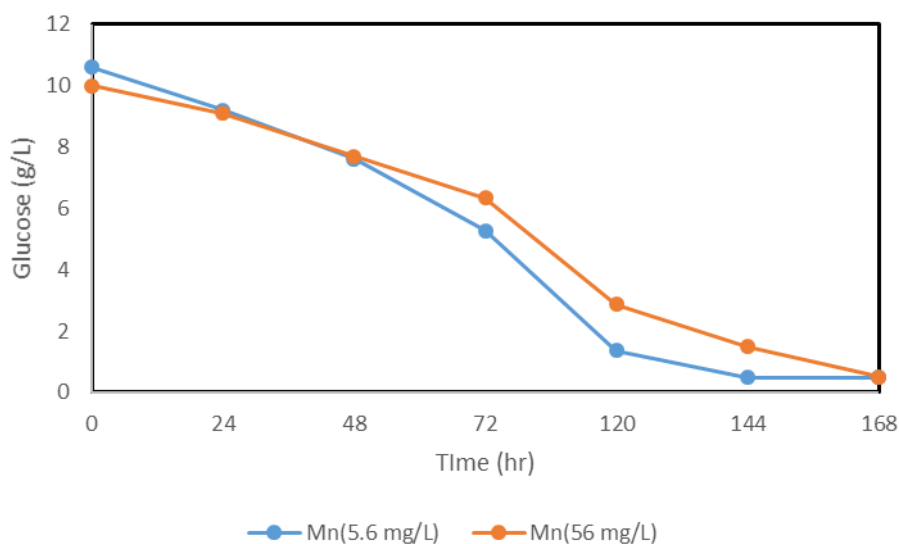


Figure 37 Variation of Glucose concentration with time at (-) low and (+) high concentrations of Mn^{2+}

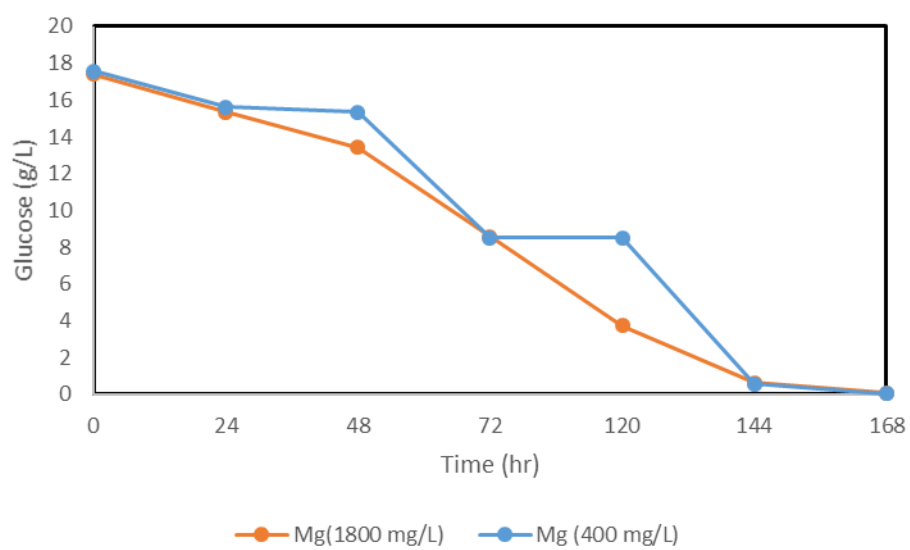


Figure 38 Variation of Glucose concentration with time at (-) low and (+) high concentrations of Mg^{2+}

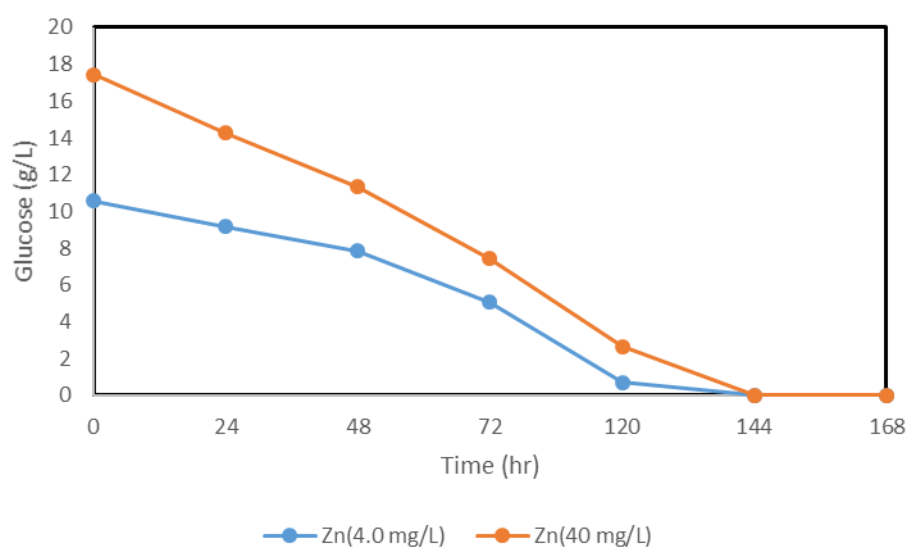


Figure 39 Variation of Glucose concentration with time at (-) low and (+) high concentrations of Zn^{2+}

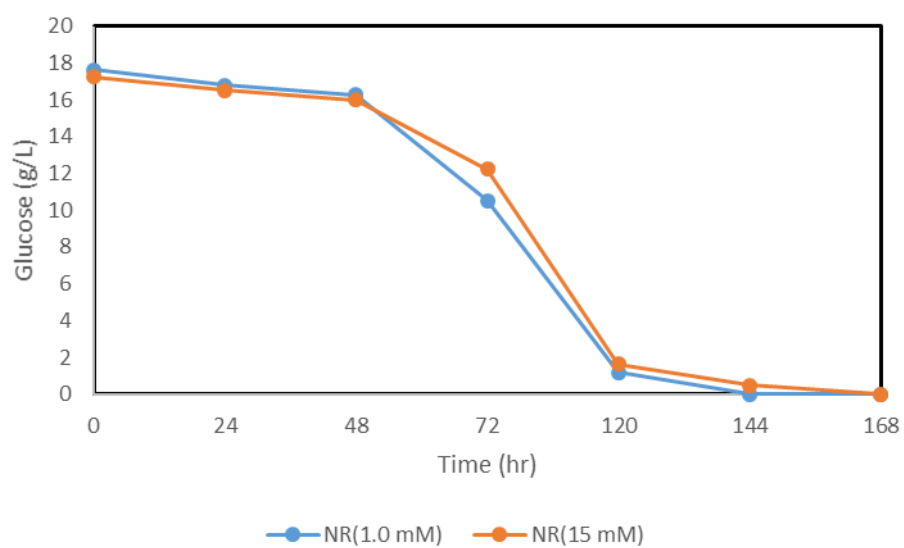


Figure 40 Variation of Glucose concentration with time at (-) low and (+) high concentrations of NR

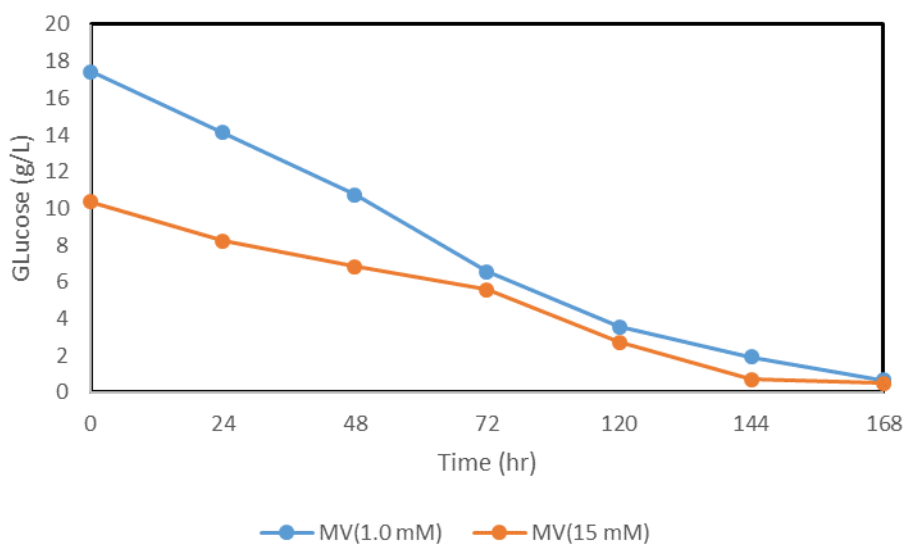


Figure 41 Variation of Glucose concentration with time at (-) low and (+) high concentrations of MV

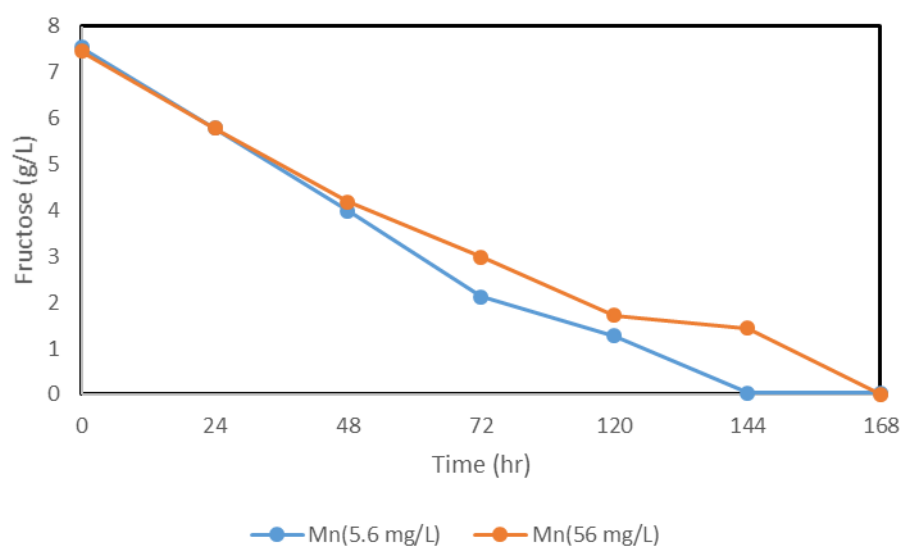


Figure 42 Variation of Fructose concentration with time at (-) low and (+) high concentrations of Mn^{2+}

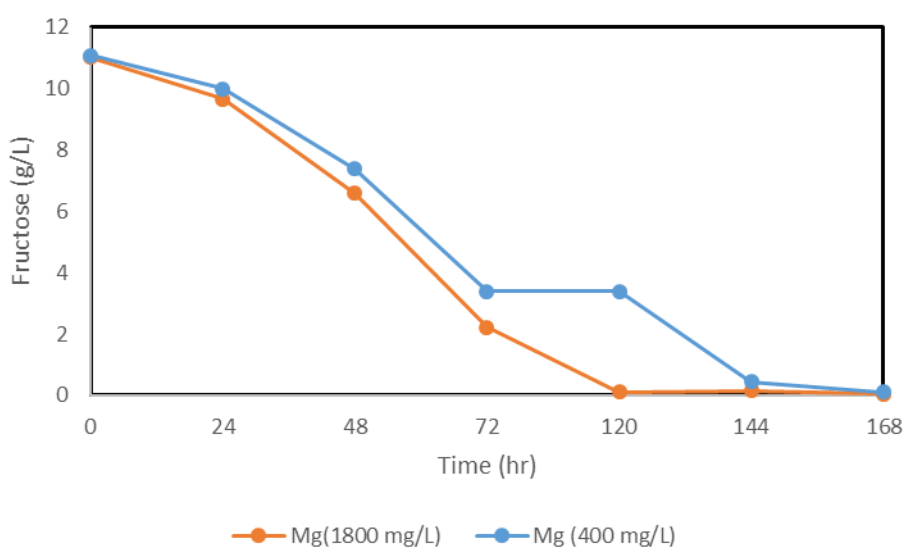


Figure 43 Variation of Fructose concentration with time at (-) low and (+) high concentrations of Mg^{2+}

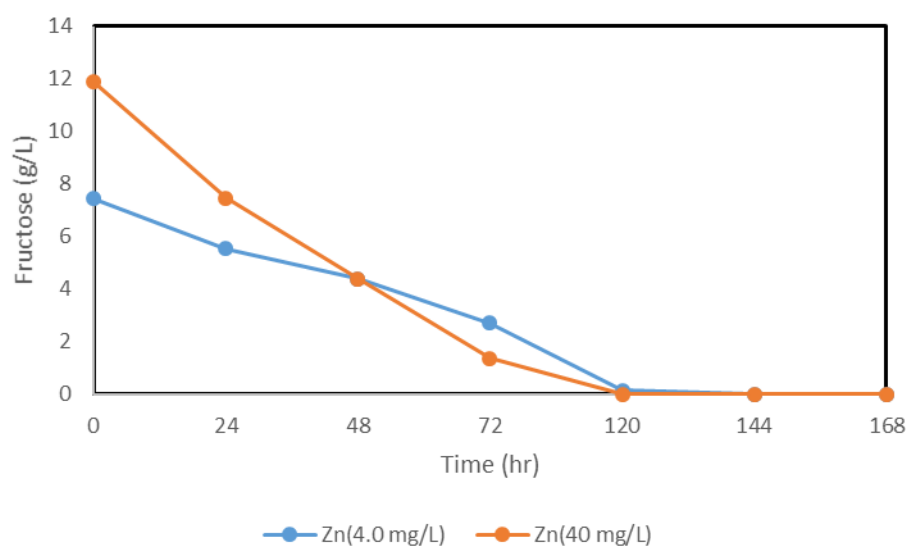


Figure 44 Variation of Fructose concentration with time at (-) low and (+) high concentrations of Zn^{2+}

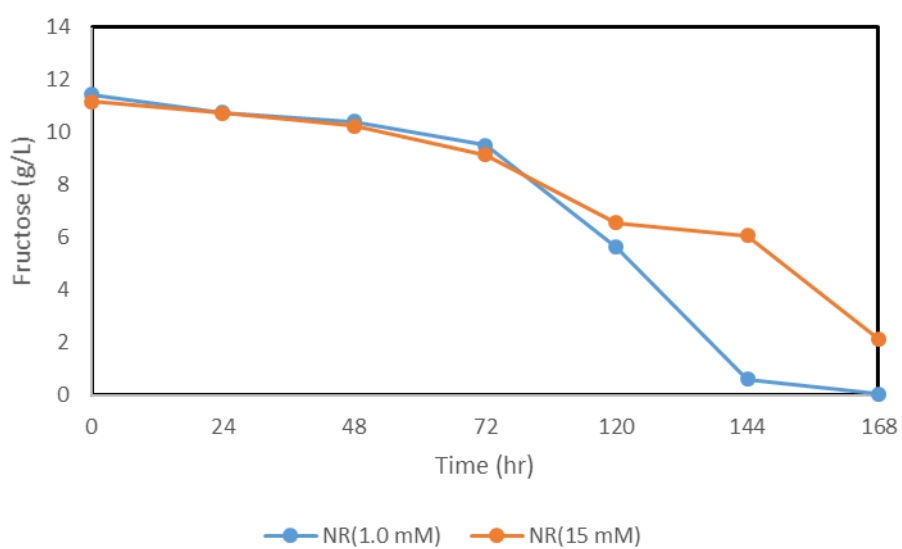


Figure 45 Variation of Fructose concentration with time at (-) low and (+) high concentrations of NR

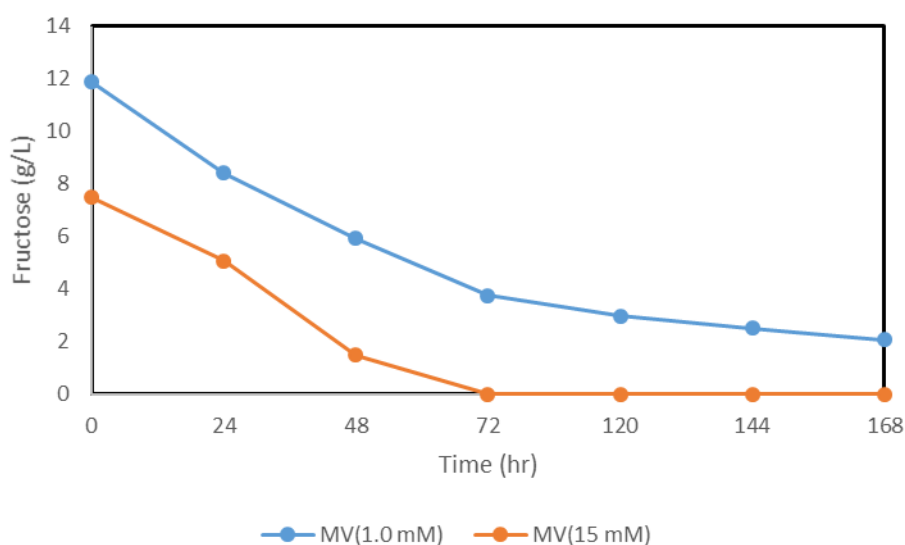


Figure 46 Variation of Fructose concentration with time at (-) low and (+) high concentrations of MV

4.6.3 Organic acid production

The organic acids monitored in the study were lactic, acetic, butyric and propionic acids. Lactic acid is the first organic acid produced in the glycolysis pathway. The formation of lactic acid causes no hydrogen and ABE formation. Therefore, it is not a desired organic acid in the biofuel formation process. Although it is a valuable industrial product. In other words, inhibition of lactic acid formation could divert the biochemical metabolism of the organisms to hydrogen and butanol formation. Figures 47 to 51 show the time variation of lactic acid formation in the presence of cations and electron mediators. Lactic acid concentration was almost the same around 10.0 g/L for Mn^{2+} (5.6 mg/L) and Mn (56 mg/L). Similarly, the resulting lactic acid amount was 10.51 - 10.72 g/L by Zn (4.0 mg/L) and Zn (40 mg/L), respectively. At an average, all concentrations tested in this research with the exceptions of Mg (400 mg/L) and MV (15 mM) yielded a mean 10 g/L lactic acid production. The low concentration of lactic acid in Mg (400 mg/L) may be a result of increased hydrogen production. Lactic acid metabolism may have been diverted towards acetic acid/ hydrogen production. Surprisingly, MV (15 mM) achieved the highest lactic acid production with 21.78 g/L. This means that electron mediators (at high concentrations) can also adversely affect butanol formation as was observed in this study.

Acetic acid is one of the most desired organic acids in hydrogen formation. Stoichiometrically, 1 mol of acetic acid formation generates 4 mol of hydrogen, while butyric acid results in only 2 moles of hydrogen. Therefore, butanol production requires butyric acid rather than acetic acid. The time variation of acetic acid formation for the experimental conditions is given in Figures 52 to 56. Mg (8.0 mg/L) achieved the highest acetic acid production of 10.99 g/L. Acetic acid production was almost the same for Mn (5.6 mg/L) and Mn (56 mg/L) with 6.5 g/L and 6.79 g/L, respectively. All metal and electron acceptor concentrations tested in the research yielded a mean of 6.94 g/L and 2.61 g/L acetic acid, respectively. The low concentration of acetic acid in electron acceptors i.e. NR (1.0 mM), NR (15 mM), MV (1.0 mg/L) and MV (15 mg/L) may be perfectly linked to increased progression into the solventogenic fermentation stage.

Butyric acid is the most desired organic acid in ABE fermentation since it can be converted into butanol. Therefore higher butyric acid formation can increase the butanol production potential. However, butanol production is not solely dependent on butyric acid formation indicating the possibility of a direct pathway to butanol formation without butyric acid generation. However, the butyric acid formation results obtained in this study fell short of the expected concentration. In general, butyric acid production was lower than that of other organic acids. The concentrations were in the range of 5.32 g/L and 6.87 g/L for the low and high concentrations of Mn^{2+} and Zn^{2+} . Thus, butyric acid production was almost the same for the minimum and maximum tested range for Mn and Zn. The highest butyric acid production of 8.36 g/L and 7.04 g/L were obtained in Mg (400 mg/L) and Mg (1800 mg/L), respectively. The lack of butyric acid production by electron acceptors may be due to increased progression into the solventogenic fermentation stage for butanol formation. The time variation of butyric acid formation for the experimental conditions is given in Figures 57 to 61.

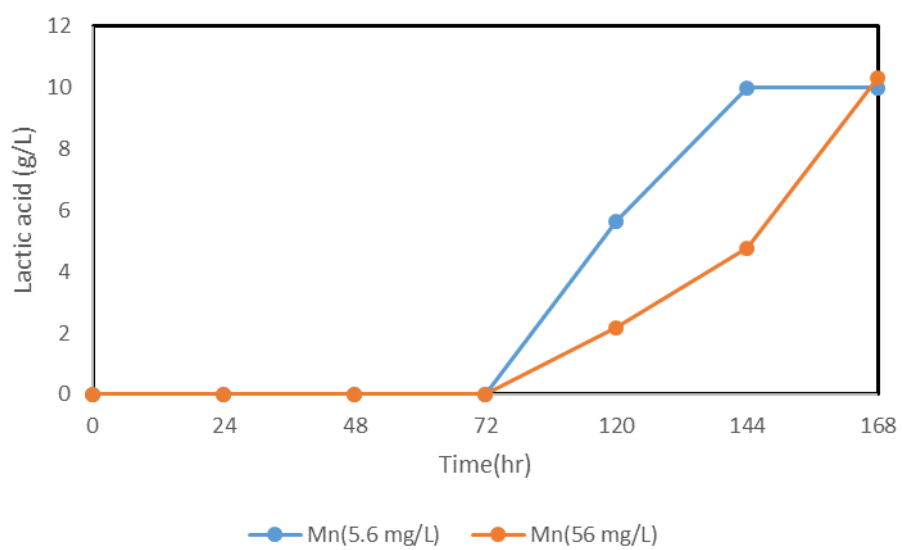


Figure 47 Variation of lactic acid formation with time at (-) low and (+) high concentrations of Mn^{2+}

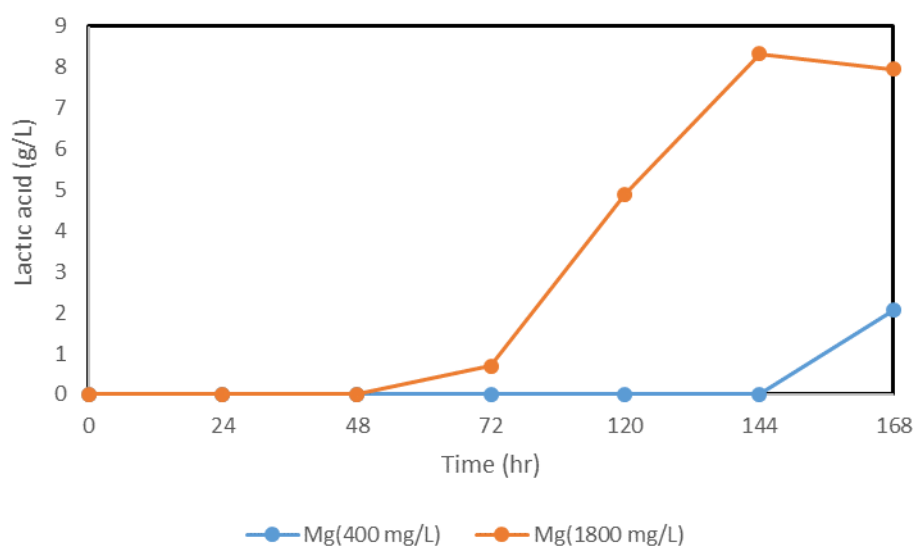


Figure 48 Variation of lactic acid formation with time at (-) low and (+) high concentrations of Mg^{2+}

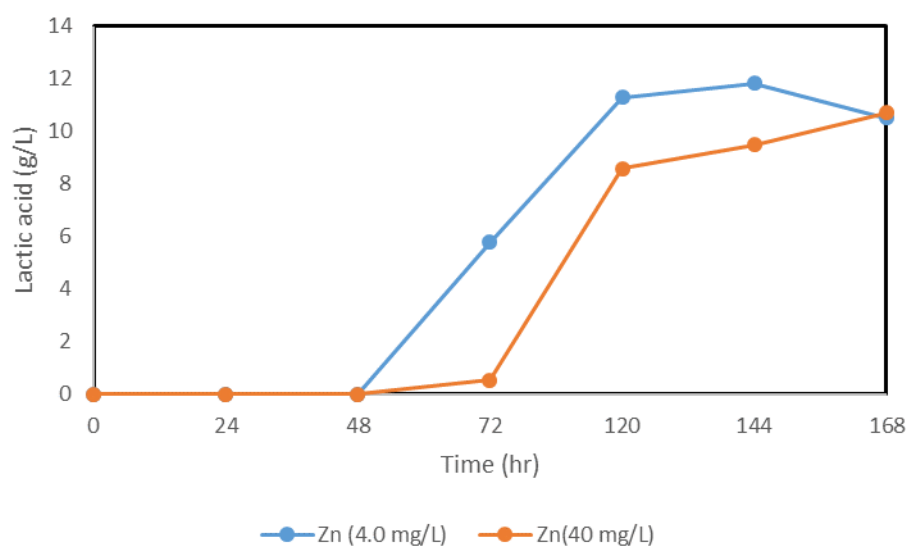


Figure 49 Variation of lactic acid formation with time at (-) low and (+) high concentrations of Zn²⁺

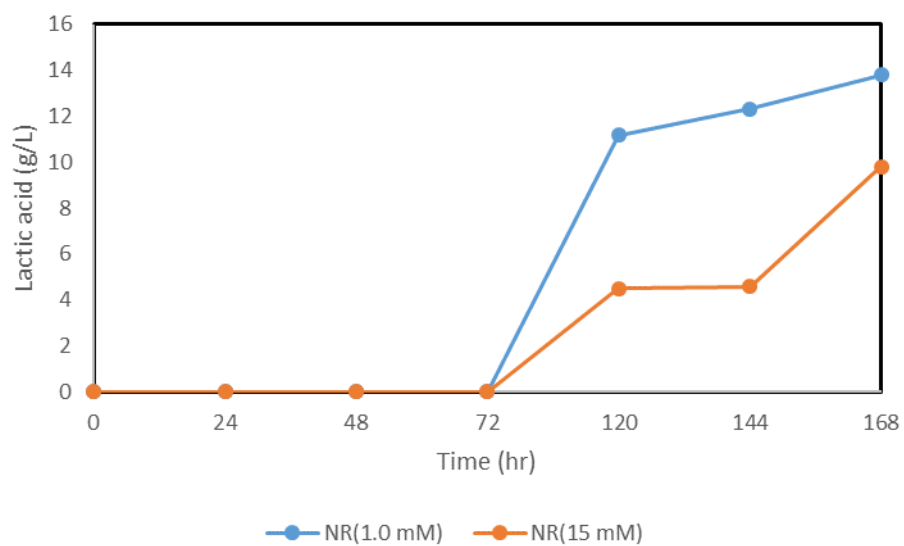


Figure 50 Variation of lactic acid formation with time at (-) low and (+) high concentrations of NR

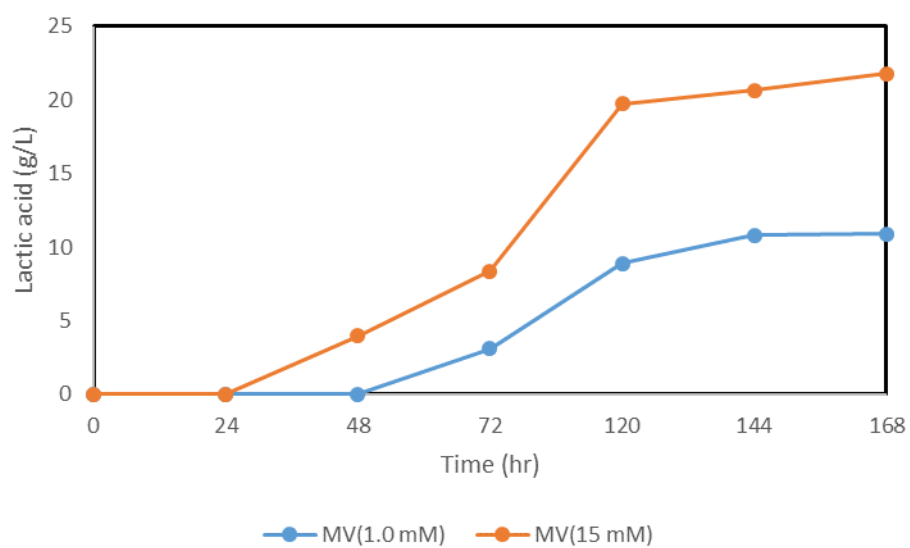


Figure 51 Variation of lactic acid formation with time at (-) low and (+) high concentrations of MV

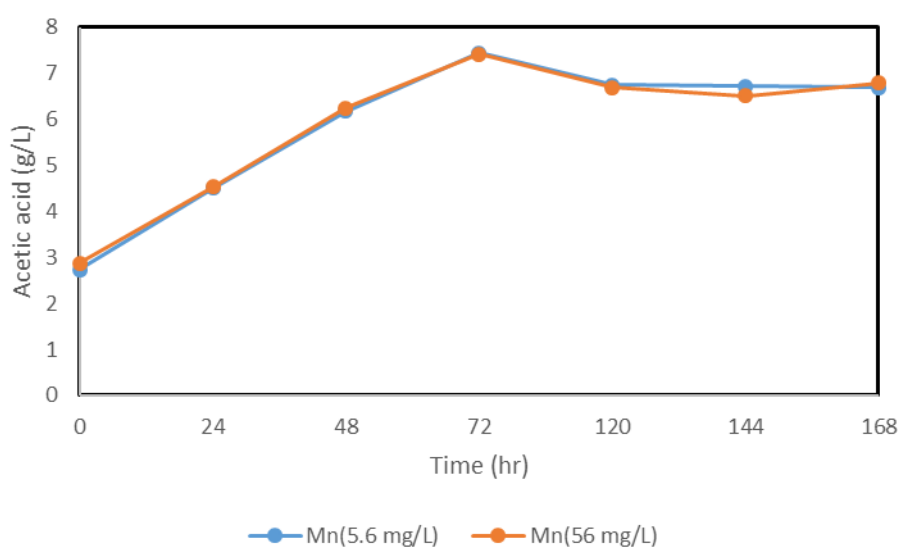


Figure 52 Variation of acetic acid formation with time at (-) low and (+) high concentrations of Mn²⁺

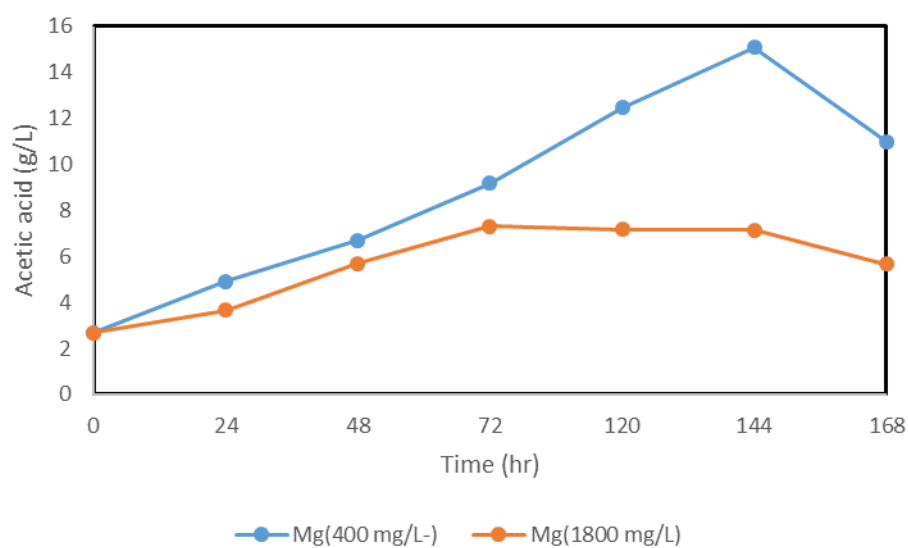


Figure 53 Variation of acetic acid formation with time at (-) low and (+) high concentrations of Mg^{2+}

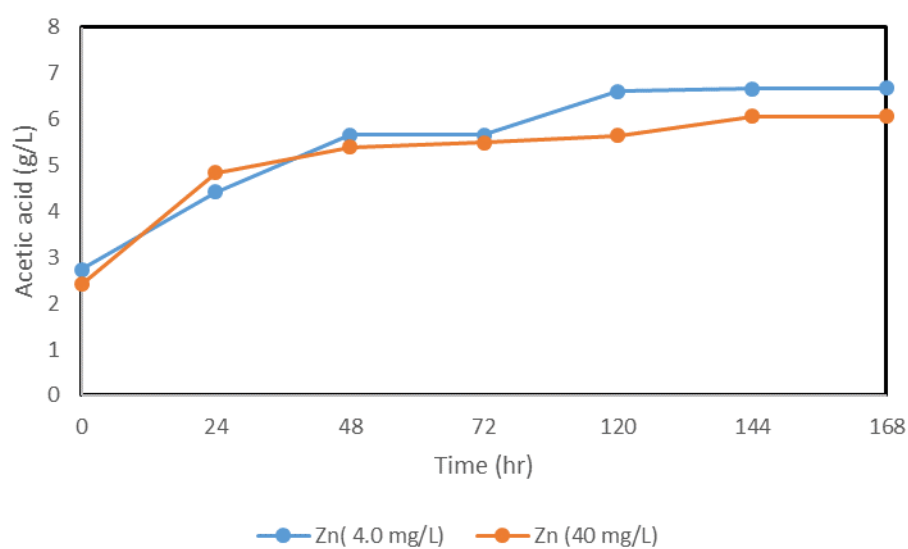


Figure 54 Variation of acetic acid formation with time at (-) low and (+) high concentrations of Zn^{2+}

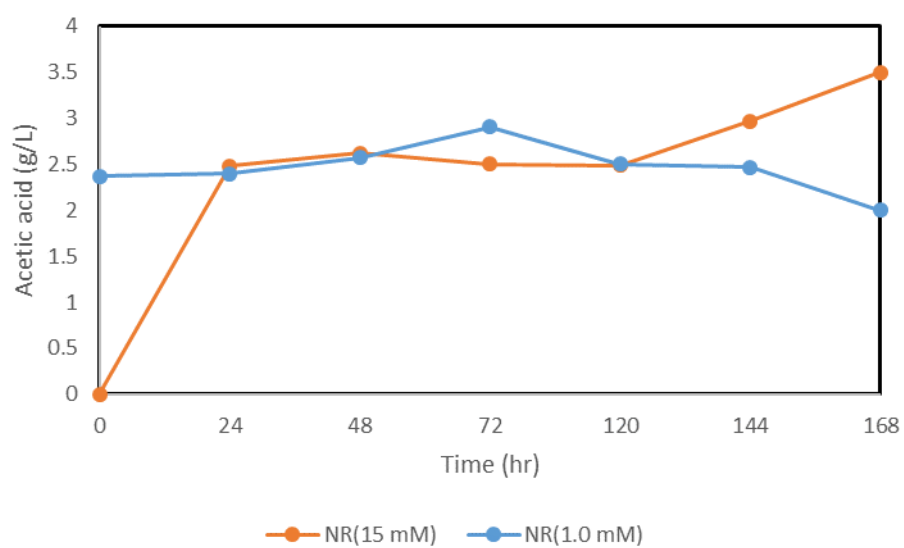


Figure 55 Variation of acetic acid formation with time at (-) low and (+) high concentrations of NR

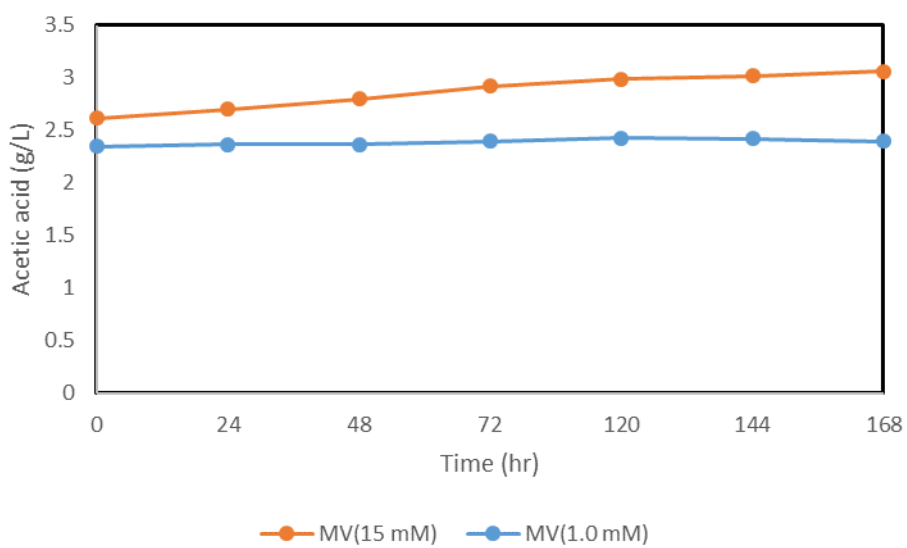


Figure 56 Variation of acetic acid formation with time at (-) low and (+) high concentrations of MV

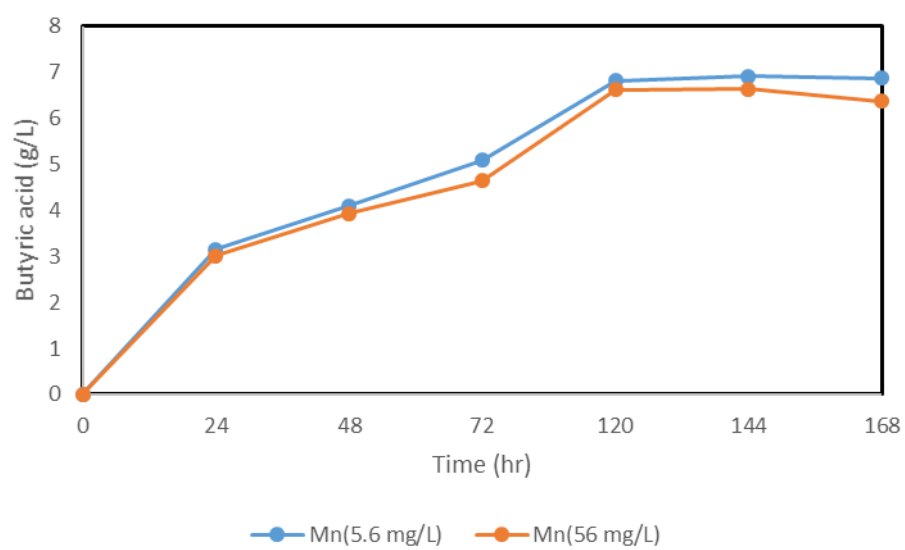


Figure 57 Variation of butyric acid formation with time at (-) low and (+) high concentrations of Mn^{2+}

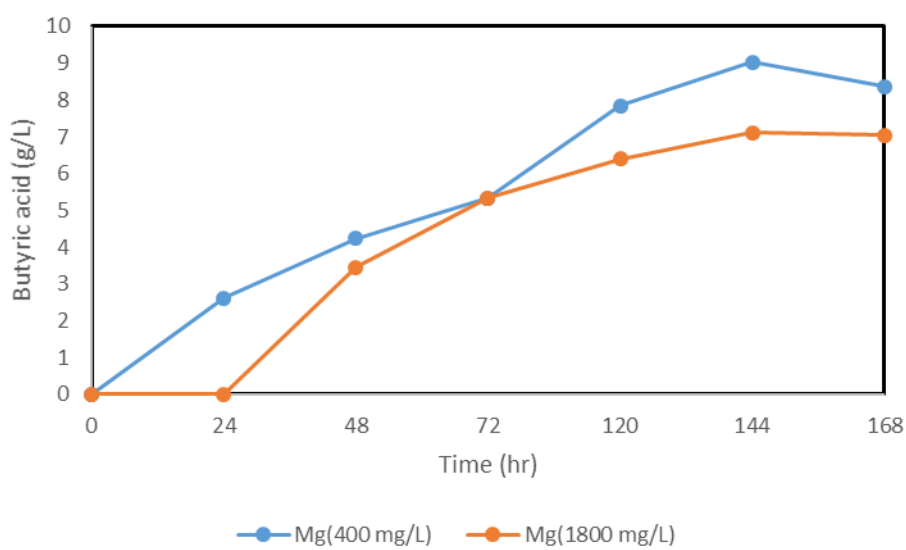


Figure 58 Variation of butyric acid formation with time at (-) low and (+) high concentrations of Mg^{2+}

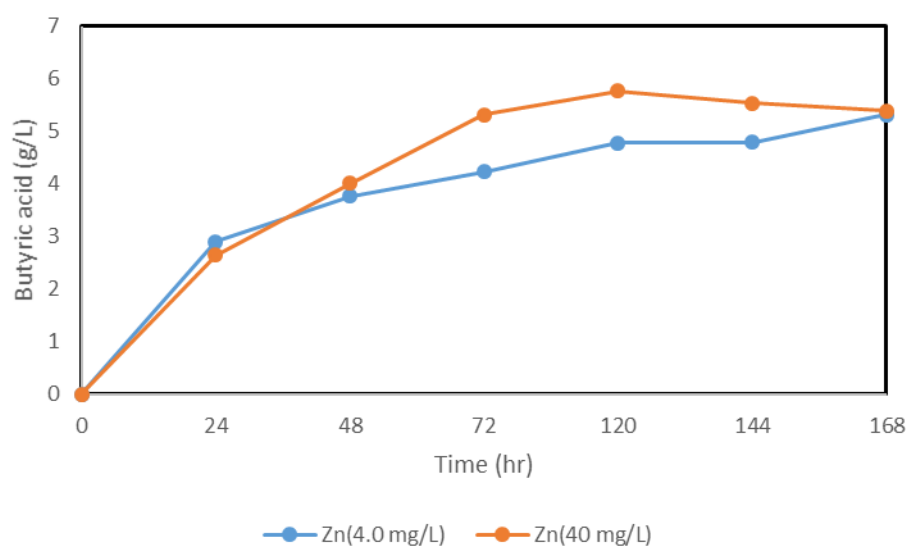


Figure 59 Variation of butyric acid formation with time at (-) low and (+) high concentrations of Zn²⁺

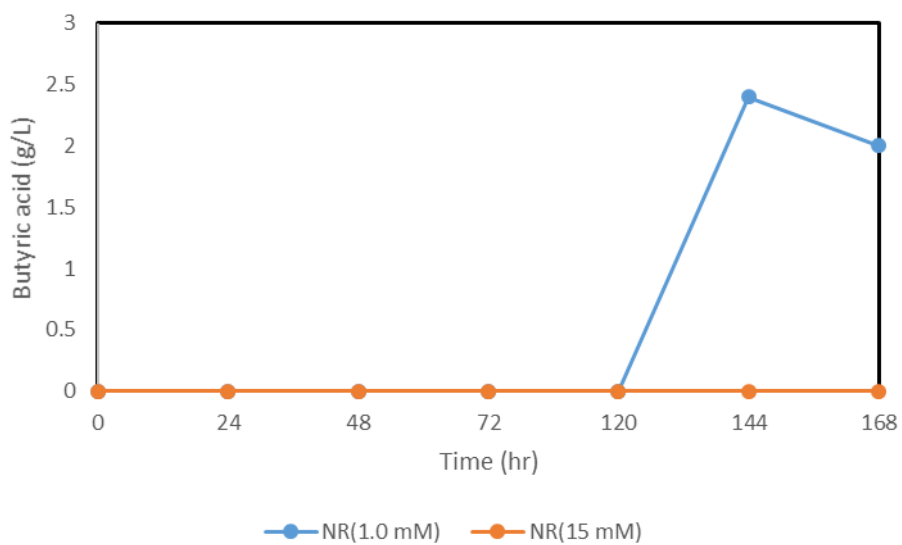


Figure 60 Variation of butyric acid formation with time at (-) low and (+) high concentrations of NR

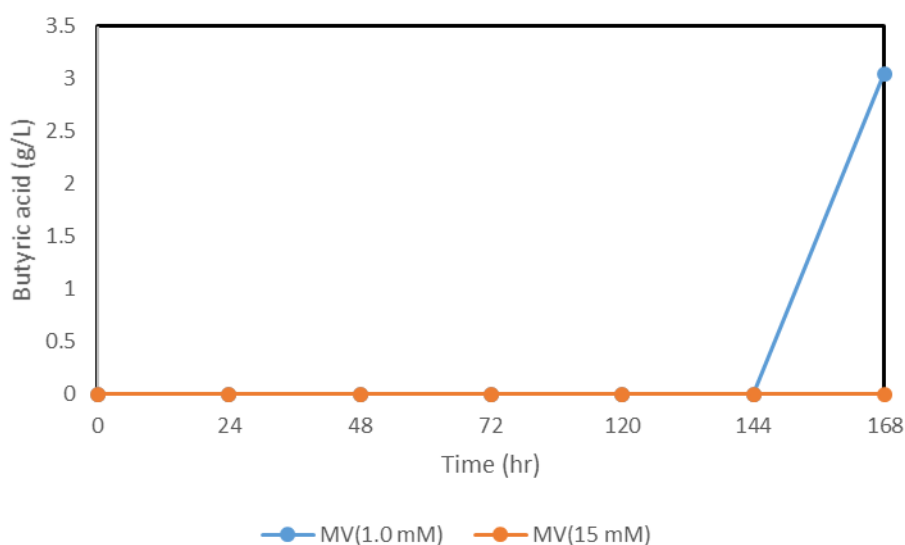


Figure 61 Variation of butyric acid formation with time at (-) low and (+) high concentrations of MV

4.6.4 Hydrogen formation

Figures 62 to 66 depict the effect of cation and electron mediator concentrations on cumulative hydrogen volume. Surprisingly, hydrogen production was observed in all the experimental setups. This may be a result of Fe^{2+} naturally present in the hydrolysed fig. However, Junelles, Janati-Idrissi, Petitdemange, and Gay (1988) suggested the removal of Fe^{2+} from fermentation media for increased butanol production. In related work, Junelles, Janati-Idrissi, Petitdemange, & Gay (1988) suggested an iron-limited condition with a FeSO_4 concentration of 0.2 mg/ L. In Mn^{2+} limited and excess condition experimental setups, the volume of the hydrogen was almost equal to each (600 ml). Mn^{2+} does not have a substantial effect on hydrogen production. In the case of Mg^{2+} , hydrogen volume was 1200 ml and 800 ml for the low and high concentrations of the cations, respectively. A substantial difference in the hydrogen production was obtained when the conditions were rather Mg^{2+} limited i.e. Mg^{2+} concentration in the raw hydrolysate. The addition of Zn^{2+} , into the fermentation media provided higher hydrogen formation. The least cumulative hydrogen production and hydrogen production yields were observed in the experimental setup containing neutral red and methyl viologen. This is not surprising as these compounds are electron mediators favouring butanol production. These results indicated that the concentration of Zn^{2+} and Mn^{2+} must be kept low,

while Mg^{2+} concentration could be increased to halt hydrogen generation for enhanced butanol formation.

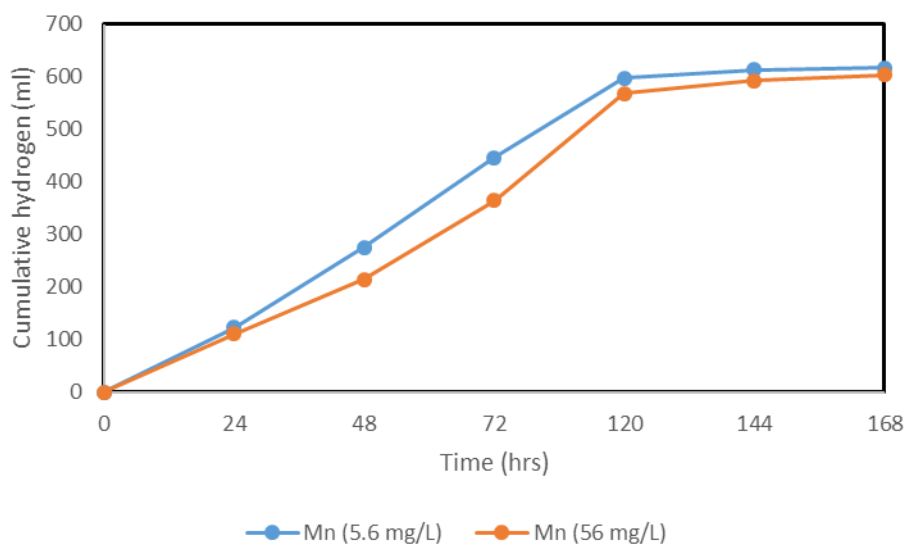


Figure 62 Variation of Cumulative hydrogen production with time at (-) low and (+) high concentrations of Mn^{2+}

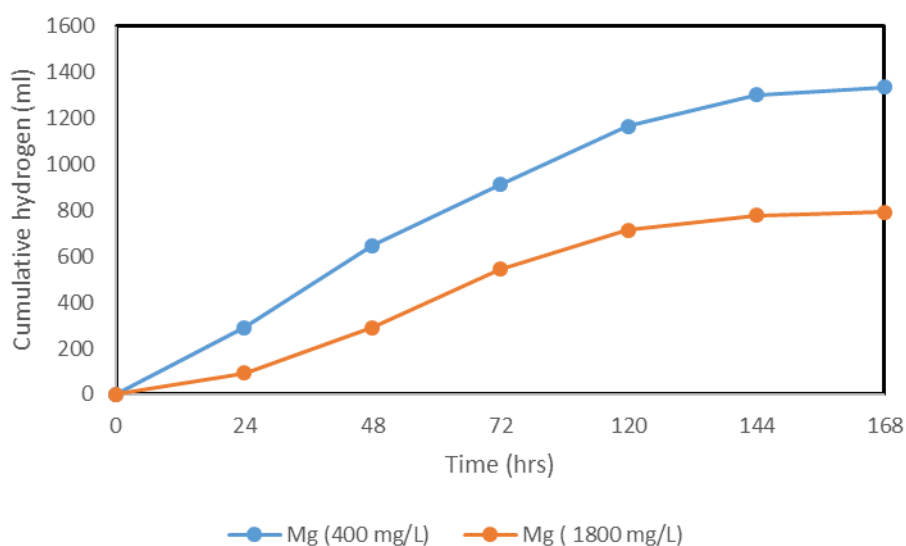


Figure 63 Variation of Cumulative hydrogen production with time at (-) low and (+) high concentrations of Mg^{2+}

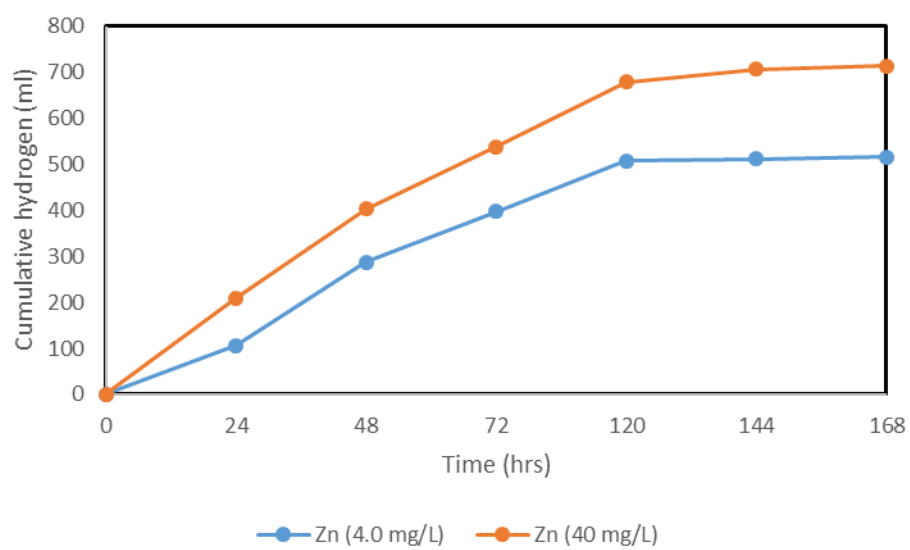


Figure 64 Variation of Cumulative hydrogen production with time at (-) low and (+) high concentrations of Zn^{2+}

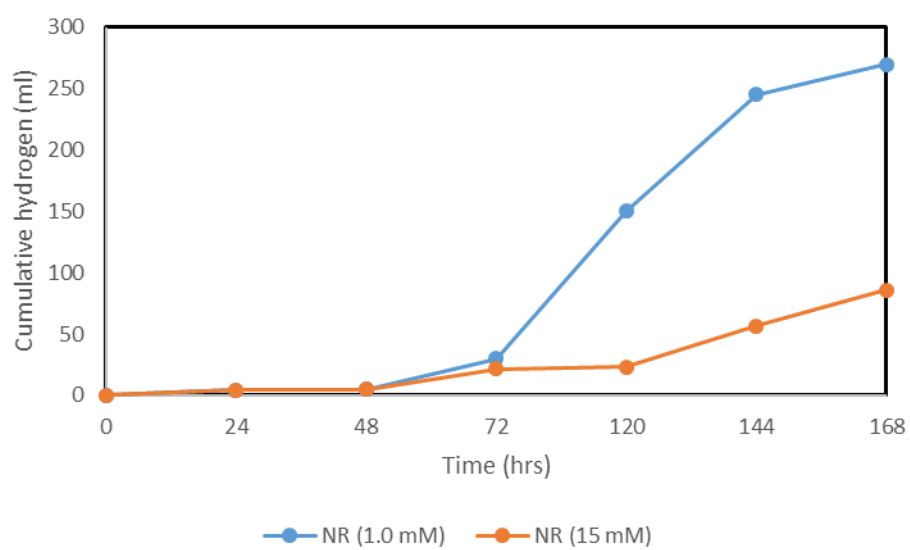


Figure 65 Variation of Cumulative hydrogen production with time at (-) low and (+) high concentrations of NR

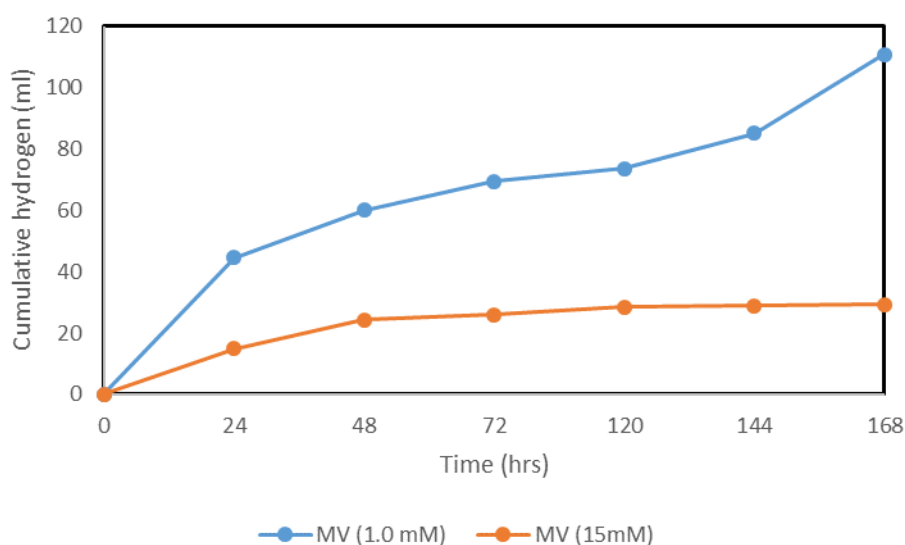


Figure 66 Variation of Cumulative hydrogen production with time at (-) low and (+) high concentrations of MV

4.6.5 ABE formation

The main purpose of the study is to increase butanol production as earlier stated. However, organic acids, ethanol and acetone are produced alongside butanol in ABE fermentation. The total solvent concentration should not exceed 20 g/L which is the threshold concentration for the dissociation of microbial cell membrane. The fraction of butanol in this mixture can only be increased by inhibiting the synthesis of ethanol and acetone. The inhibition effect of cations used in this stage on ethanol and acetone or the activator effect of cations on butanol synthesis were evaluated. Electron mediators indirectly affect the process by inhibiting hydrogen generation. However, it is not clear if all electrons are directed towards butanol or other products.

Figures 67 to 71 depict the variation of ethanol concentration with time for low and high concentrations of cations and electron carriers. ethanol concentrations of 1.779 g/L and 1.786 g/L by Mn (5.6 mg/L) and Mn (56 mg/L) respectively was produced. On the other hand, ethanol production is inhibited at metal concentrations: Zn (40 mg/L) and Mg (1800 mg/L) which are the maximum values of (Zn and Mg) tested in this study with 1.17 g/L and 1.16 g/L ethanol production respectively. This is supported by high H₂ production observed at these concentrations since ethanol is

synthesized from H₂. Minimum concentrations of these elements tested i.e. Zn (4.0 mg/L) and Mg (400 mg/L) achieved ethanol concentrations of 3.269 g/L and 1.602 g/L respectively. The high concentration of ethanol in electron acceptors is expected due to low H₂ production. Mean ethanol production from metals and electron acceptors concentrations tested in this research are 1.801 g/L and 3.548 g/L, respectively. Zn (40 mg/L) and NR (15 mM) achieved the least and the highest ethanol production with 1.171 g/L and 3.955 g/L respectively.

10.641 g/L and 10.672 g/L were the acetone concentrations recorded from Mn (5.6 mg/L) and Mn (56 mg/L), respectively. Mean acetone production from metals and electron acceptors concentrations tested in this research were 9.438 g/L and 2.130 g/L respectively. The low concentration of acetone in electron acceptors implies the diversion of the acetone biochemical pathway to that of ethanol or butanol. This is reflected in the high concentrations of ethanol observed in the electron acceptors as seen in Figures 70 and 71.

NR(15 mg/L) and Mn (56 mg/L) achieved the least and highest acetone production with 0 g/L and 10.672 g/L, respectively. Non-production of acetone by NR (15 mg/L) implies the total diversion of the acetone biochemical pathway into ethanol or butanol. This is not surprising as the highest ethanol production of 3.955 g/L from this study was observed in NR(15 mg/L) as seen in Figure 70. Figures 72 to 76 show variations in acetone production with time throughout the experiment.

Butanol production was almost the same for Mn (5.6 mg/L) and Mn (56 mg/L) with (2.260 g/L and 2.402 g/L respectively). A similar effect in Mg was observed for Mg²⁺ and almost the same butanol production was achieved at different levels of cation concentration. Zn²⁺ positively affected the butanol production resulting in around 4.5 g/L concentration at a high level of Zn²⁺. Neutral red addition gave almost the same butanol production which indicates that Zn²⁺ can be used instead of expensive electron mediators such as NR. A high concentration of butanol in MV (1.0 mM) and MV(15 mM) was expected due to low hydrogen production yields of 0.085 and 0.031 mol H₂/mol TS in MV (1.0 mM) and MV (15 mM) respectively.

MV(15 mM) achieved the highest butanol production with 6.078 g/L. Figures 77 to 81 show variations of butanol production with time throughout the experiment.

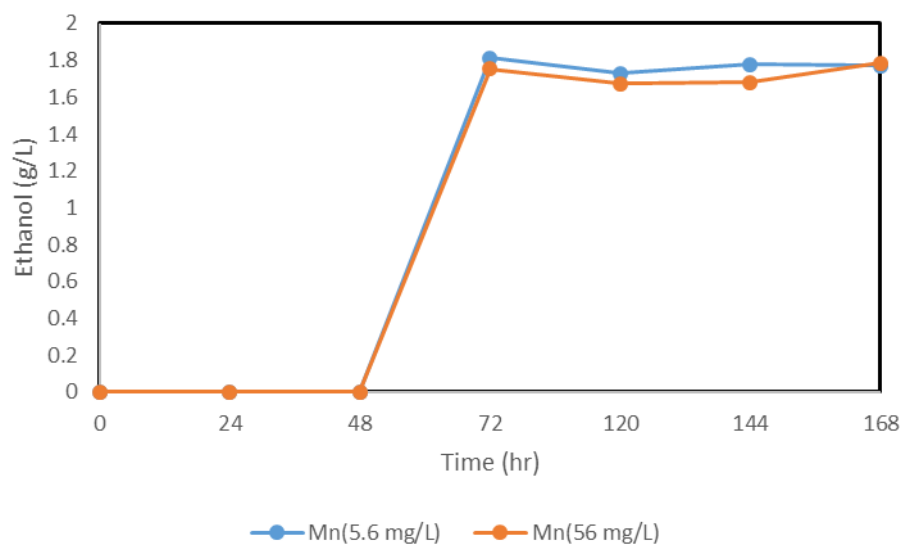


Figure 67 Variation of ethanol production with time at (-) low and (+) high concentrations of Mn²⁺

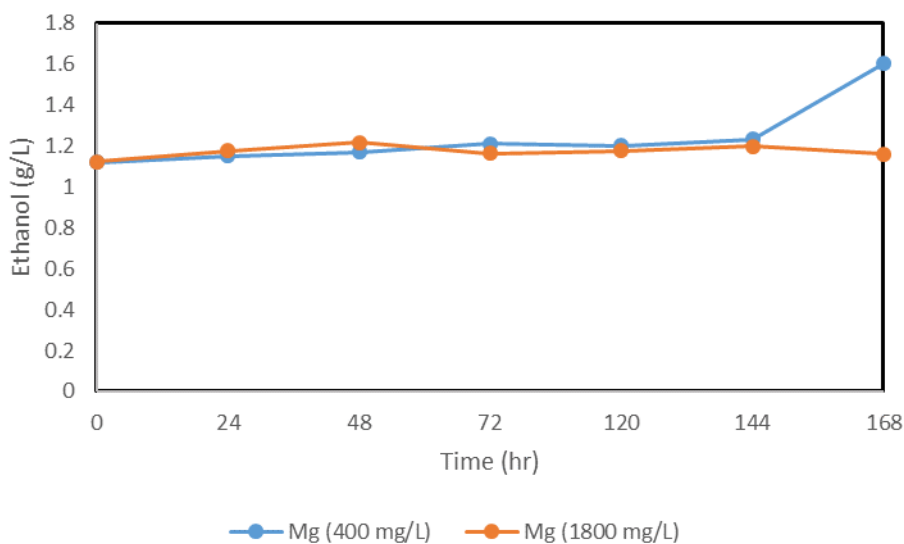


Figure 68 Variation of ethanol production with time at (-) low and (+) high concentrations of Mg²⁺

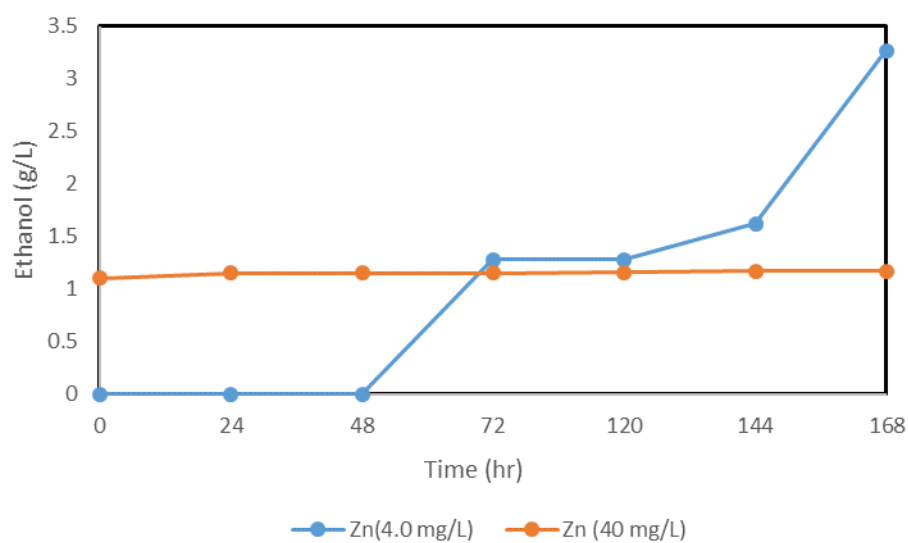


Figure 69 Variation of ethanol production with time at (-) low and (+) high concentrations of Zn²⁺

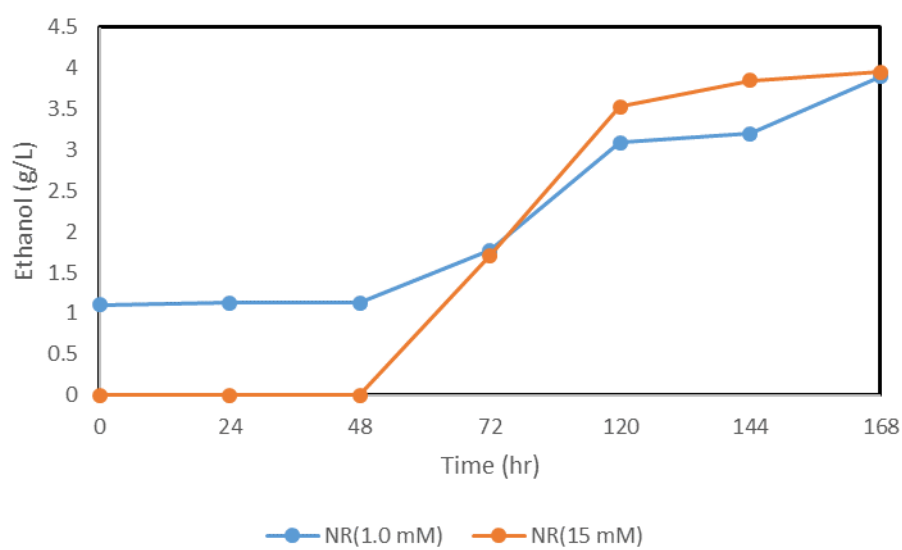


Figure 70 Variation of ethanol production with time at (-) low and (+) high concentrations of NR

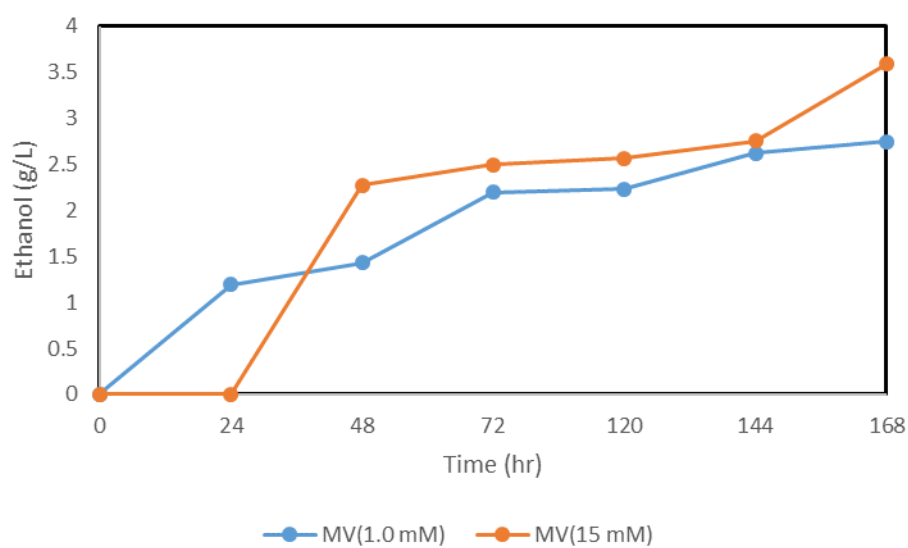


Figure 71 Variation of ethanol production with time at (-) low and (+) high concentrations of MV

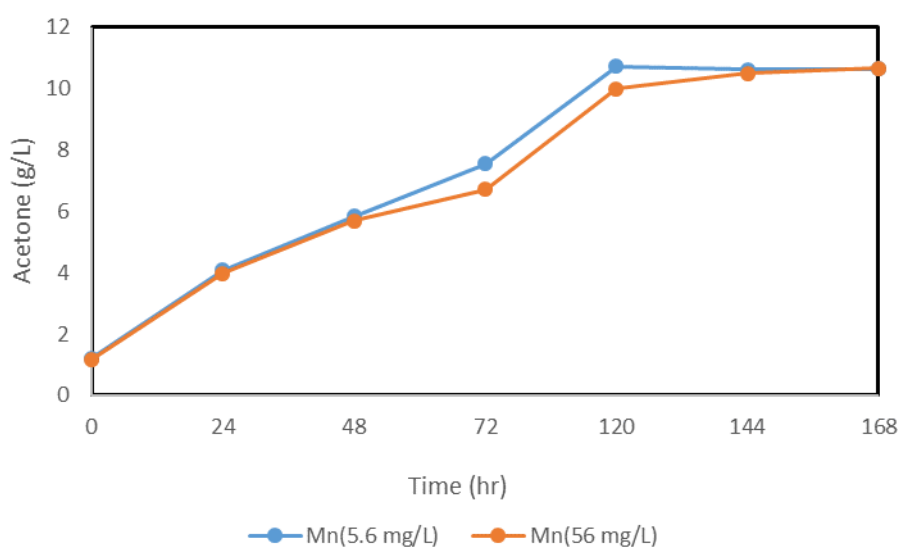


Figure 72 Variation of acetone production with time at (-) low and (+) high concentrations of Mn²⁺

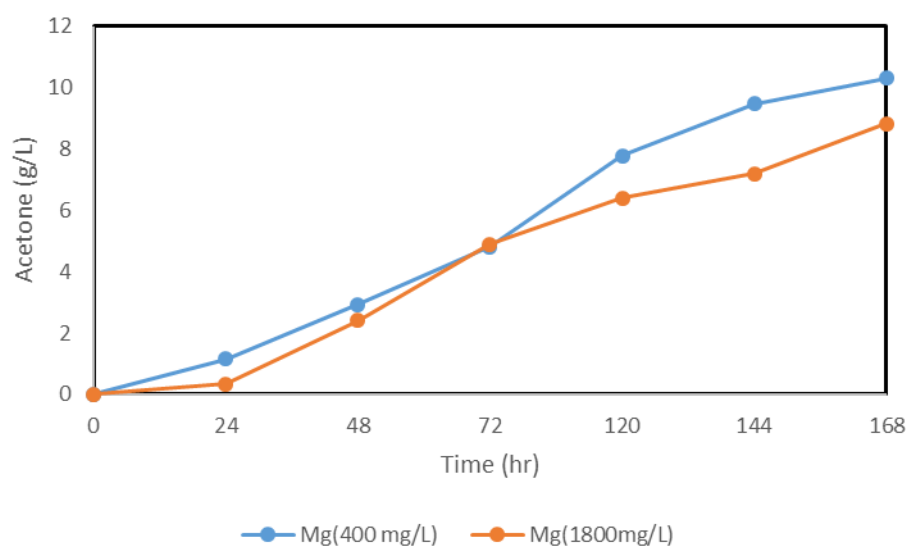


Figure 73 Variation of acetone production with time at (-) low and (+) high concentrations of Mg^{2+}

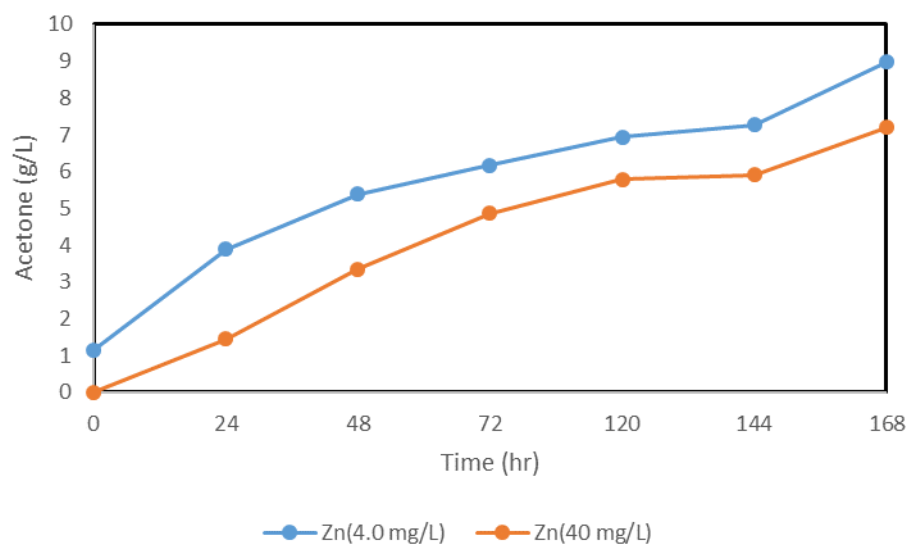


Figure 74 Variation of acetone production with time at (-) low and (+) high concentrations of Zn^{2+}

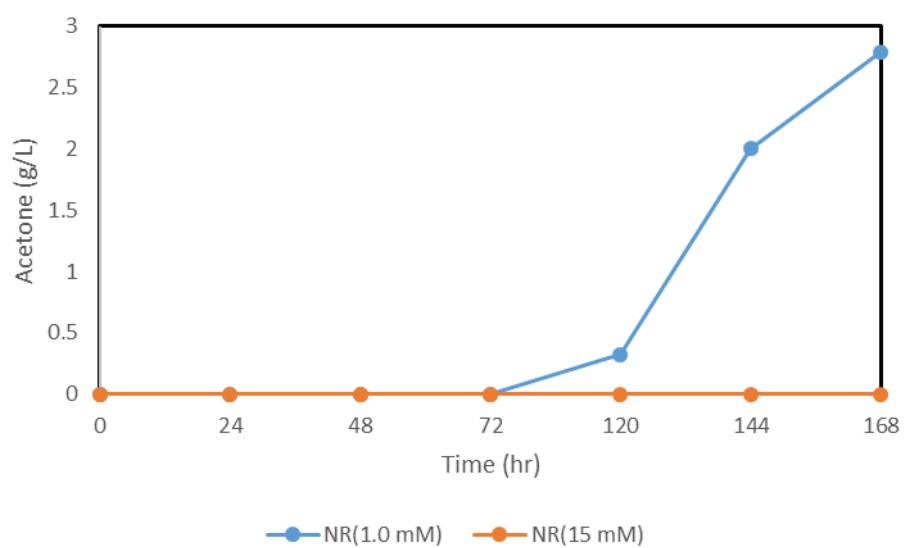


Figure 75 Variation of acetone production with time at (-) low and (+) high concentrations of NR

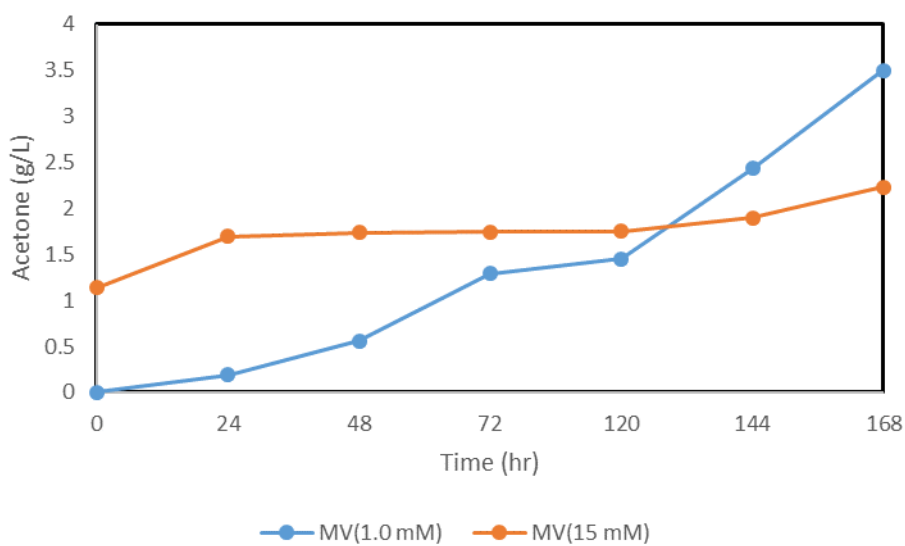


Figure 76 Variation of acetone production with time at (-) low and (+) high concentrations of MV

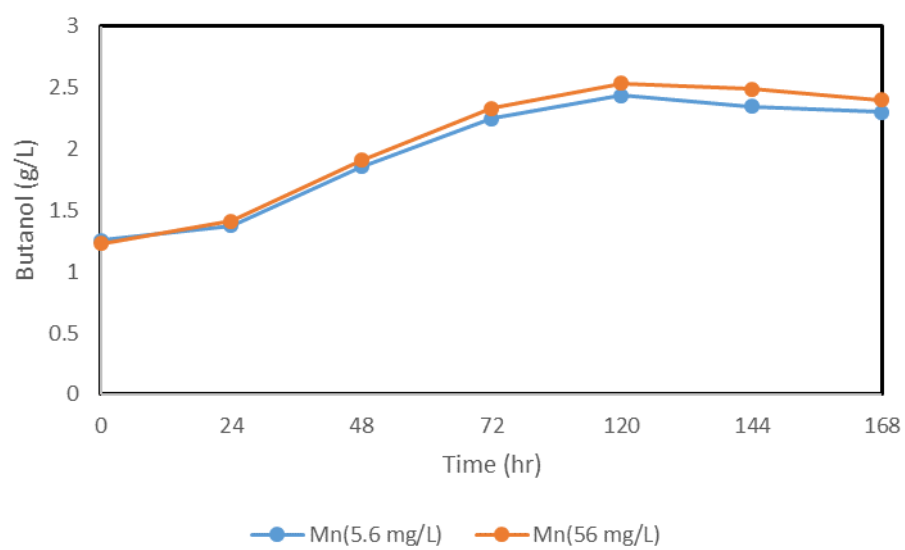


Figure 77 Variation of butanol production with time at (-) low and (+) high concentrations of Mn^{2+}

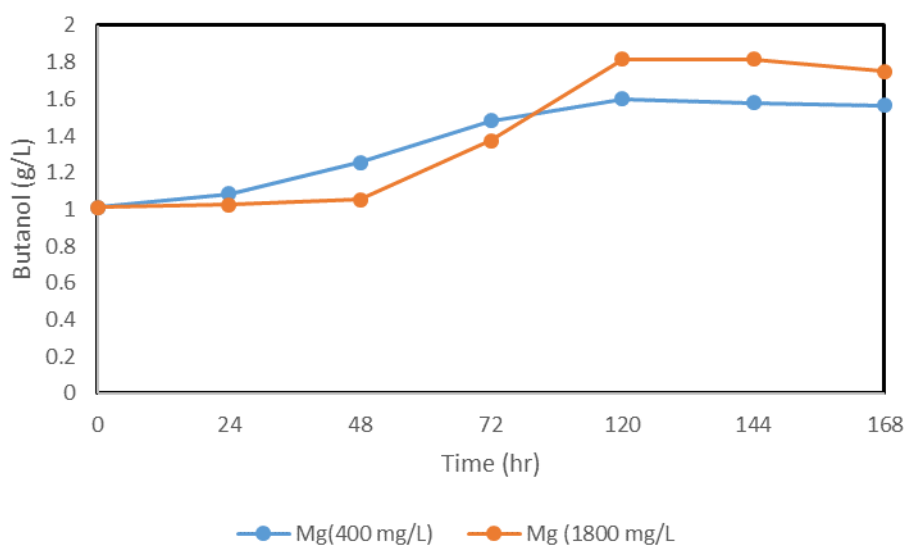


Figure 78 Variation of butanol production with time at (-) low and (+) high concentrations of Mg^{2+}

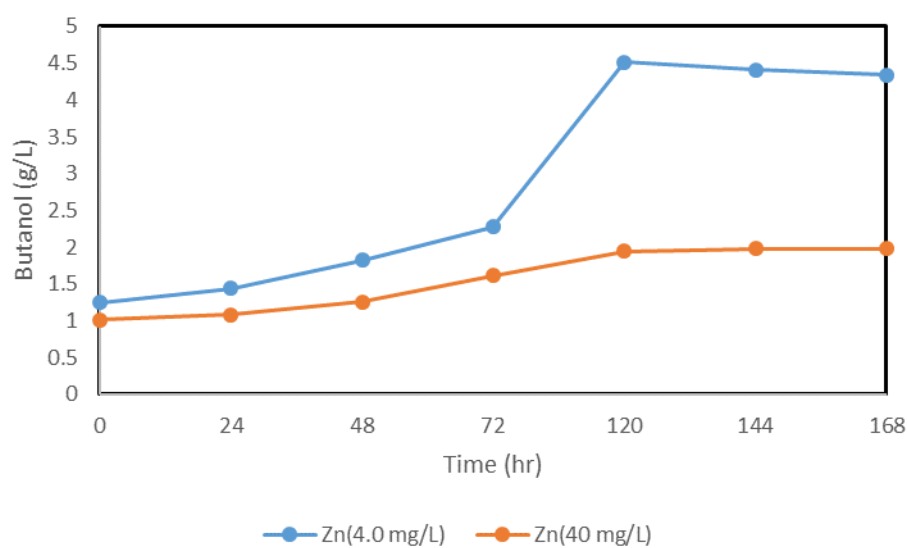


Figure 79 Variation of butanol production with time at (-) low and (+) high concentrations of Zn^{2+}

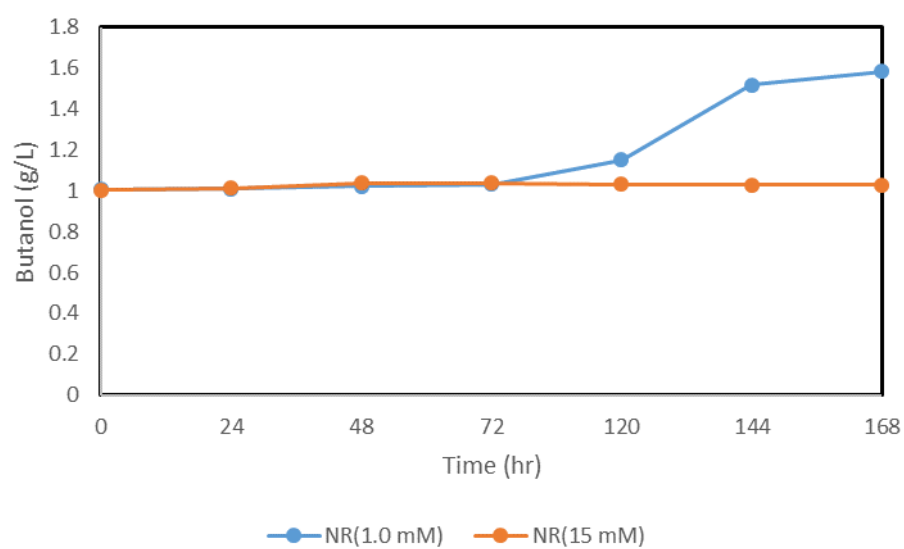


Figure 80 Variation of butanol production with time at (-) low and (+) high concentrations of NR

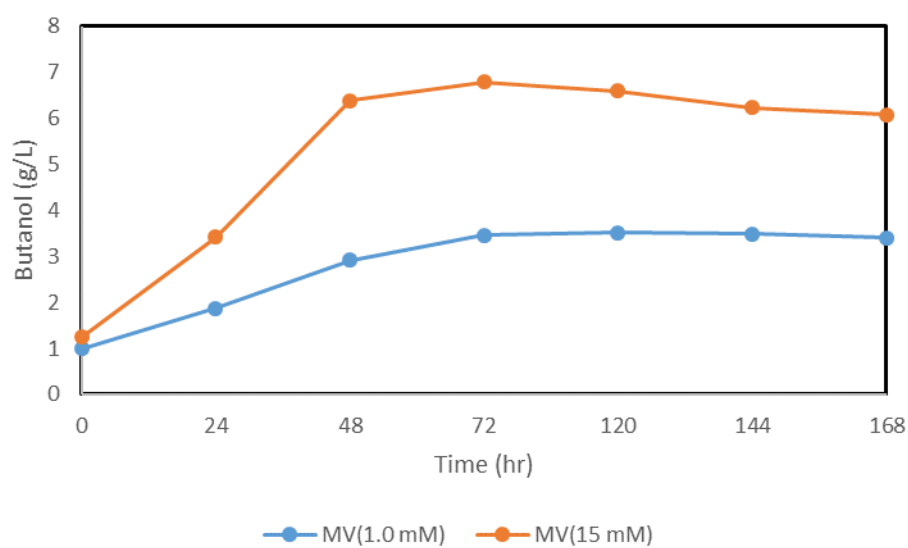


Figure 81 Variation of butanol production with time at (-) low and (+) high concentrations of MV

Table 26 Final concentrations of effects of cations and electron mediators on ABE products

	TSC (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Ethanol (g/L)	Acetone (g/L)	Butanol (g/L)	Total ABE (g/L)
Mn (-)	18.7	10.0	6.70	6.87	1.779	10.6	2.3	14.711
Mn (+)	20.8	10.34	6.79	6.60	1.786	10.7	2.402	14.86
Mg (-)	3.92	2.06	10.99	8.36	1.602	10.3	1.566	13.47
Mg(+)	4.64	7.95	5.66	7.04	1.199	8.83	1.749	11.737
Zn (-)	18.4	10.51	6.68	5.32	3.269	8.98	4.344	16.591
Zn (+)	3.04	10.72	6.07	5.39	1.171	7.203	1.981	10.355
NR (-)	3.60	13.81	2.0	2.0	3.895	2.79	1.586	8.271
NR (+)	7.44	9.8	3.5	0	3.955	0	1.028	4.983
MV (-)	8.16	10.89	2.42	3.05	2.749	3.499	3.397	9.645
MV(+)	19.6	21.78	3.06	0	3.593	2.232	6.078	11.903

The final concentrations of effects of cations and electron mediators on ABE products are found in Table 26 while Figure 82 gives the Total ABE concentration (g/L) for cations and electron mediators studied in this research. In summary, Mg^{2+} utilization enhances total sugar, glucose and fructose consumption excellently. Lactic acid generation was around 10 g/L. Acetic acid and Butyric acid concentrations were the highest in Mg^{2+} with 10.99 g/L and 8.36 g/L respectively.

Zn^{2+} is efficient in total sugar consumption and other simple sugars. Lactic acid generation was around 10 g/L, and acetic acid was around 6.5 g/L. Zn^{2+} is the only metal that achieved the highest butanol concentration (4.344 g/L). This means Zn^{2+} is a promising candidate needed for ABE fermentation to increase butanol concentration instead of using expensive electron mediators.

Mn^{2+} slightly inhibits total sugar consumption. Lactic acid generation was around 10 g/L. The highest concentrations of acetone in this study were found in Mn (5.6 mg/L) and Mn (56 mg/L) with concentrations of 10.641 and 10.672, respectively. This indicated that Mn^{2+} majorly favoured acetone production rather than butanol. Hence, its addition to the fermentation medium in the butanol study is not necessary. The highest Total ABE 14.711 was also found in Mn^{2+} . This high result was attributed to the high acetone production by Mn^{2+} .

NR effectively achieved complete glucose consumption. Lactic acid generation was around 10 g/L. The highest ethanol production in this research (3.955 g/L) was achieved in NR. MV was not excellent in fructose consumption. However, it extensively enhanced lactic acid with the highest concentration of 21.78 g/L achieved in this study. Similarly, the highest butanol concentration in this study (6.078 g/L) was achieved in MV.

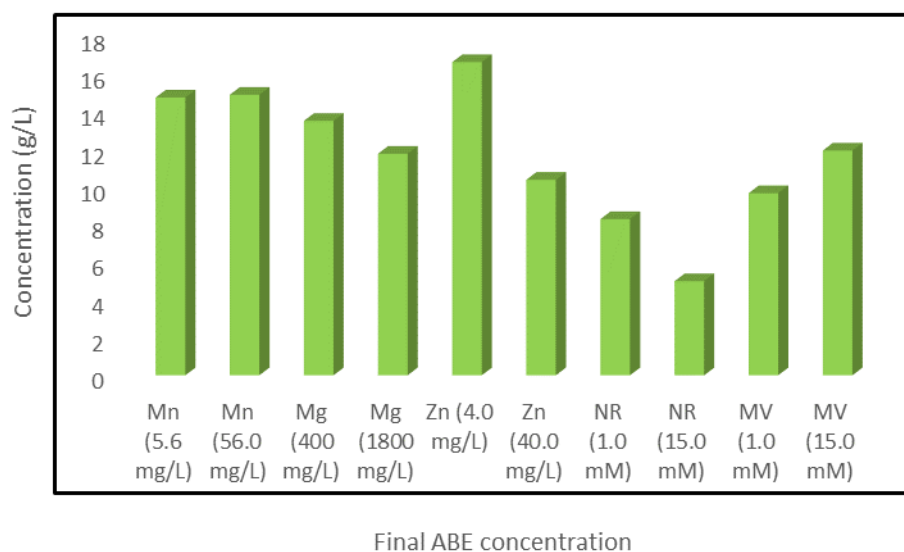


Figure 82 Final Total ABE products at (-) low and (+) high concentrations of cations and electron mediators

4.7 Plackett-Burman design and statistical analysis of results

The purpose of the study was to screen the cations which inhibit or accelerate solvent production, especially, butanol generation. Plackett Burman's experimental design method was employed for 11 different metals by conducting 12 experiments. The responses were individual solvents, an organic acid produced, cumulative hydrogen volume (CHV) and the hydrogen production rate (ml H₂ /L/ h). ANOVA analysis (Tables 27 to 33) for all responses was done to determine the significant factors ($p < 0.05$). A linear response equation was developed for every single product as given in equations (6-13). The predicted responses were calculated by using these equations.

The Plackett Burman experimental, observed results and regression analysis are presented in Table 34 for solvents and Table 35 for Organic acids and hydrogen production. The regression coefficient $R^2 \geq 0.90$ was accepted as a good agreement between actual and predicted responses. The results showed that the regression coefficients are between $R^2 = 0.92$ and $R^2 = 0.99$ indicating a good prediction of the responses for the studied factor level ranges. Furthermore, the difference between Predicted R^2 and Adjusted R^2 in the responses was less than 0.2 indicating a perfect

agreement between them. In addition, Adequate Precision is a determinant of model fitness and accuracy. A ratio greater than 4 is desirable. All responses recorded ratio was greater than 4. Also, Box-Cox diagnostic in the responses all had a current lambda of 1 indicating no need for transformation to resemble a normal distribution (Figures 83 to 85). Cook's Distance plots for solvents indicate that observed and predicted responses are in good agreement (Figures 86 to 88).

$$\text{Ethanol (g/L)} = 1.43968 - 0.00446X_2 - 6.7 \times 10^{-5}X_3 - 0.0113 X_4 + 0.009767 X_5 + 0.000294 X_6 - 0.00082 X_8 + 0.074782 X_{10} - 0.00302 X_{11} \quad (\text{Eq.6})$$

$$\text{Acetone (g/L)} = 1.80105 - 0.00444111X_3 + 0.0944961 X_4 + 0.037 X_5 + 0.000576602 X_6 + 0.00928333 X_8 + 0.00169661 X_9 + 4.57814 X_{10} \quad (\text{Eq.7})$$

$$\text{Butanol (g/L)} = 3.40088 - 0.394041X_1 - 0.00104549X_3 + 0.0868 X_5 + 0.00944892X_7 - 0.0113389 X_8 + 1.07847X_{10} - 0.0766886 X_{11} \quad (\text{Eq.8})$$

$$\text{Lactic acid (g/L)} = 15.2851 + 0.0128017 X_3 - 0.195736 X_4 - 0.0119406 X_6 + 0.0703889 X_8 - 5.02347 X_{10} + 0.194737 X_{11} \quad (\text{Eq.9})$$

$$\text{Acetic acid (g/L)} = 5.11582 - 0.769088 X_1 + 0.120068 X_2 - 0.00205205 X_3 + 0.0401163 X_4 - 0.00129526 X_6 + 0.0062164 X_7 + 2.01543 X_{10} - 0.0259868 X_{11} \quad (\text{Eq.10})$$

$$\text{Butyric acid (g/L)} = 0.663105 - 0.00388722 X_3 + 0.0994186 X_4 - 0.00897177 X_7 + 0.0131389 X_8 + 0.00190307 X_9 + 3.22267 X_{10} \quad (\text{Eq.11})$$

$$\text{CHV (ml)} = 162.996 - 108.365 X_1 - 9.57136 X_2 - 0.374442 X_3 + 1.46957 X_4 + 12.6249 X_5 + 3.91561 X_7 + 0.285706 X_9 + 251.868 X_{10} - 6.52235 X_{11} \quad (\text{Eq.12})$$

$$HPR_{(ml\ H2/L\ h)} = -0.299328 + -1.26845 X_1 - 0.224122 X_2 - 0.00603654 X_3 + 0.168091 X_4 + 0.0472298 X_7 + 0.00719581 X_8 + 0.00912566 X_9 + 4.23178 X_{10} - 0.280206 X_{11}$$

Table 27 ANOVA for the Lactic acid model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	206.28	6	34.38	26.53	0.0012
C-Mn	44.16	1	44.16	34.08	0.0021
D-Fe	8.50	1	8.50	6.56	0.0506
F-Mg	55.13	1	55.13	42.54	0.0013
H-Ca	53.51	1	53.51	41.30	0.0014
K-Mo	18.70	1	18.70	14.43	0.0126
L-B	26.28	1	26.28	20.28	0.0064
Residual	6.48	5	1.30		
Cor Total	212.76	11			

Table 28 ANOVA for the Acetic acid model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	8.36	8	1.05	20.27	0.0156
A-Co	1.42	1	1.42	27.55	0.0135
B-Cu	1.04	1	1.04	20.13	0.0207
C-Mn	1.13	1	1.13	22.00	0.0183
D-Fe	0.3571	1	0.3571	6.92	0.0783
F-Mg	0.6487	1	0.6487	12.57	0.0382
G-Zn	0.2852	1	0.2852	5.53	0.1002
K-Mo	3.01	1	3.01	58.35	0.0047
L-B	0.4681	1	0.4681	9.07	0.0571
Residual	0.1548	3	0.0516		
Cor Total	8.52	11			

Table 29 ANOVA for the Butyric acid model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	18.36	6	3.06	9.08	0.0143
C-Mn	4.07	1	4.07	12.08	0.0177
D-Fe	2.19	1	2.19	6.51	0.0512
G-Zn	0.5941	1	0.5941	1.76	0.2417
H-Ca	1.86	1	1.86	5.53	0.0654
J-K	1.94	1	1.94	5.77	0.0615
K-Mo	7.70	1	7.70	22.83	0.0050
Residual	1.69	5	0.3371		
Cor Total	20.05	11			

Table 30 ANOVA for the HPR model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	1.272E+06	8	1.590E+05	40.70	0.0056
A-Co	1.129E+05	1	1.129E+05	28.89	0.0126
B-Cu	26394.94	1	26394.94	6.76	0.0804
C-Mn	1.511E+05	1	1.511E+05	38.68	0.0084
E-Na	47816.18	1	47816.18	12.24	0.0395
G-Zn	4.526E+05	1	4.526E+05	115.84	0.0017
J-K	1.753E+05	1	1.753E+05	44.86	0.0068
K-Mo	1.880E+05	1	1.880E+05	48.12	0.0061
L-B	1.179E+05	1	1.179E+05	30.19	0.0119
Residual	11722.05	3	3907.35		
Cor Total	1.284E+06	11			

Table 31 ANOVA for the Ethanol model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	0.0892	8	0.0112	30.93	0.0084
B-Cu	0.0014	1	0.0014	3.97	0.1405
C-Mn	0.0012	1	0.0012	3.38	0.1631
D-Fe	0.0283	1	0.0283	78.56	0.0030
E-Na	0.0072	1	0.0072	19.84	0.0211
F-Mg	0.0334	1	0.0334	92.62	0.0024
H-Ca	0.0073	1	0.0073	20.12	0.0207
K-Mo	0.0041	1	0.0041	11.49	0.0428
L-B	0.0063	1	0.0063	17.48	0.0249
Residual	0.0011	3	0.0004		
Cor Total	0.0903	11			

Table 32 ANOVA for the Acetone model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	25.53	7	3.65	230.91	< 0.0001
C-Mn	5.31	1	5.31	336.43	< 0.0001
D-Fe	1.98	1	1.98	125.42	0.0004
E-Na	0.1027	1	0.1027	6.50	0.0633
F-Mg	0.1285	1	0.1285	8.14	0.0463
H-Ca	0.9307	1	0.9307	58.92	0.0015
J-K	1.55	1	1.55	97.81	0.0006
K-Mo	15.53	1	15.53	983.16	< 0.0001
Residual	0.0632	4	0.0158		
Cor Total	25.60	11			

Table 33 ANOVA for the Butanol model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	8.22	7	1.17	6.97	0.0397
A-Co	0.3731	1	0.3731	2.22	0.2108
C-Mn	0.2945	1	0.2945	1.75	0.2565
E-Na	0.5651	1	0.5651	3.36	0.1409
G-Zn	0.6589	1	0.6589	3.91	0.1190
H-Ca	1.39	1	1.39	8.25	0.0454
K-Mo	0.8619	1	0.8619	5.12	0.0864
L-B	4.08	1	4.08	24.21	0.0079
Residual	0.6734	4	0.1684		
Cor Total	8.89	11			

Table 34 Observed and predicted responses for ABE.

Run	Ethanol g/L		Acetone g/L		Butanol g/L	
	Obs.	Pred.	Obs.	Pred.	Obs.	Pred.
1	1.38	1.39	4.05	4.11	2.00	2.45
2	1.48	1.50	5.19	5.21	1.87	1.65
3	1.58	1.58	7.27	7.21	4.60	4.46
4	1.51	1.51	3.82	3.73	3.93	3.85
5	1.51	1.51	7.67	7.74	3.50	3.68
6	1.51	1.50	6.60	6.74	3.17	3.09
7	1.51	1.50	5.38	5.39	4.14	4.18
8	1.46	1.45	3.92	3.97	3.01	2.97
9	1.39	1.39	6.063	6.00	3.72	4.07
10	1.59	1.57	2.767	2.79	3.72	3.27
11	1.62	1.63	6.434	6.34	3.95	3.82
12	1.31	1.31	6.098	6.04	2.10	2.22
R²	0.99		0.99		0.92	

Table 35 Observed and predicted responses for organic acid and hydrogen production.

Run	Lactic acid		Acetic acid		Butyric acid		CHV (ml)		HPR	
	Obs.	Pred.	Obs.	Pred.	Obs.	Pred.	Obs.	Pred.	Obs.	Pred.
1	22.7	22.6	3.67	3.76	3.01	2.93	339.3	352.54	678.66	692.45
2	21.1	20.3	4.27	4.15	3.82	4.02	355.3	342.10	710.62	696.84
3	9.13	9.02	5.78	5.90	4.81	4.89	506.0	521.33	1012.06	1030.02
4	18.6	17.4	4.98	5.11	2.94	2.43	451.1	464.27	902.12	941.18
5	11.9	11.9	5.8	5.91	4.96	5.25	802.7	818.02	1605.44	1623.40
6	21.0	22.1	5.45	5.34	4.46	4.38	666.0	650.66	1331.92	1313.96
7	18.3	18.8	5.6	5.71	3.82	3.23	635.2	648.41	1270.40	1309.46
8	19.0	19.5	4.09	4.00	2.92	2.46	475.1	461.92	950.26	911.20
9	19.3	20.0	6.49	6.36	4.41	4.43	823.5	808.20	1647.00	1603.77
10	19.3	19.9	4.42	4.54	0	0.8208	448.1	463.35	896.11	939.34
11	19.3	18.7	5.17	5.04	4.3	4.46	674.4	661.21	1348.84	1335.05
12	25.5	24.3	6.13	6.04	4.36	4.51	359.1	343.83	718.25	675.02
R² R²	0.97		0.98		0.92		0.99		0.99	

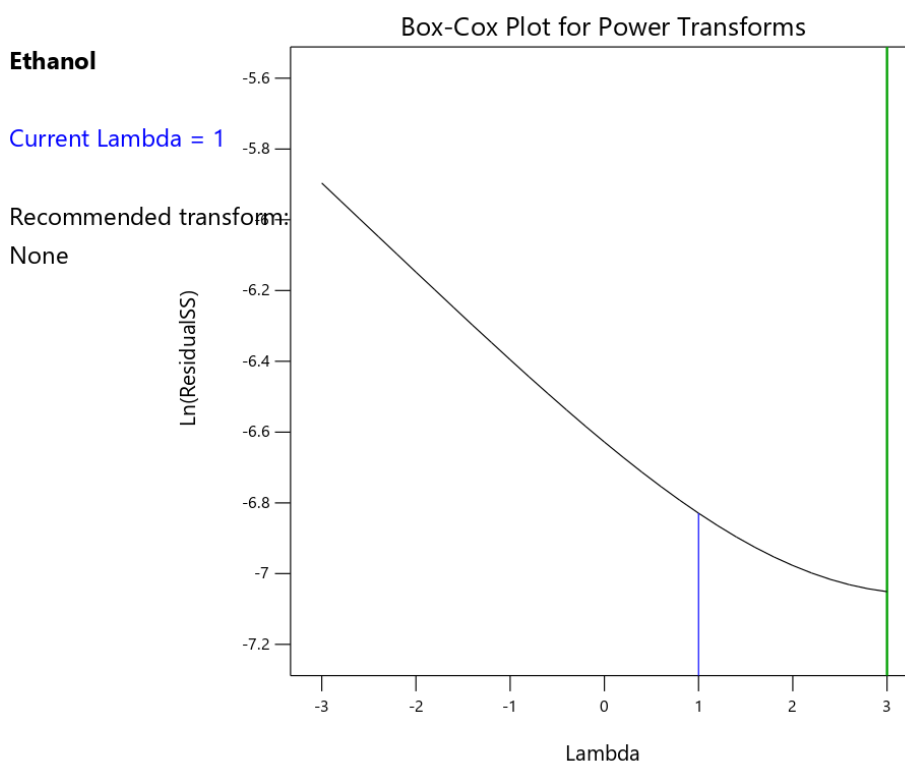


Figure 83 Box-Cox plot for ethanol

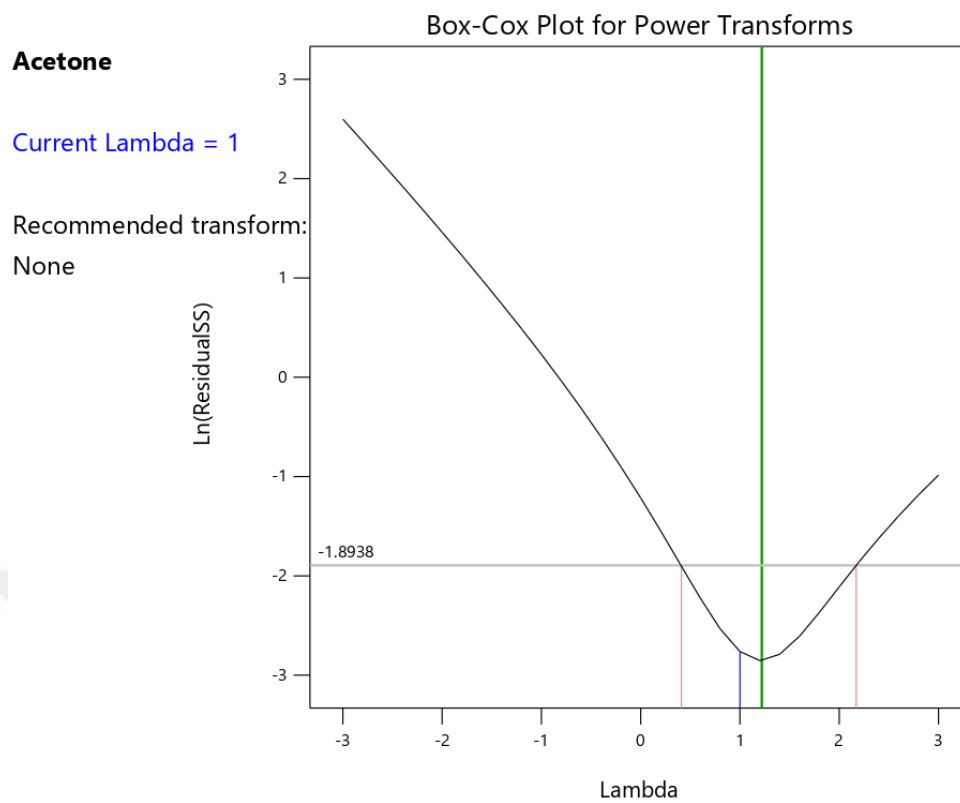


Figure 84 Box-Cox plot for acetone

1 butanol

Current Lambda = 1

Recommended transform:
None

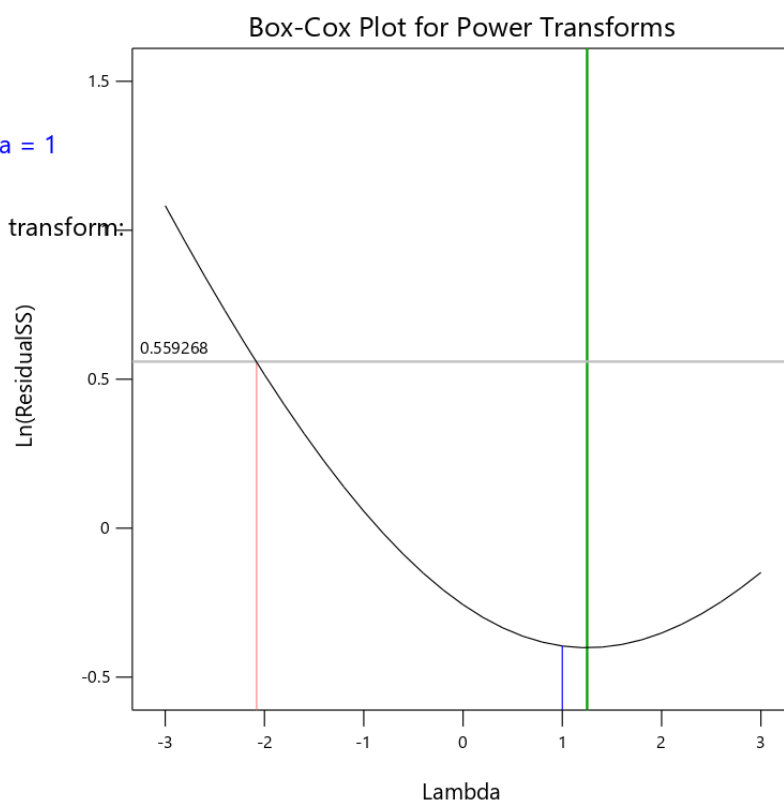


Figure 85 Box-Cox plot for butanol

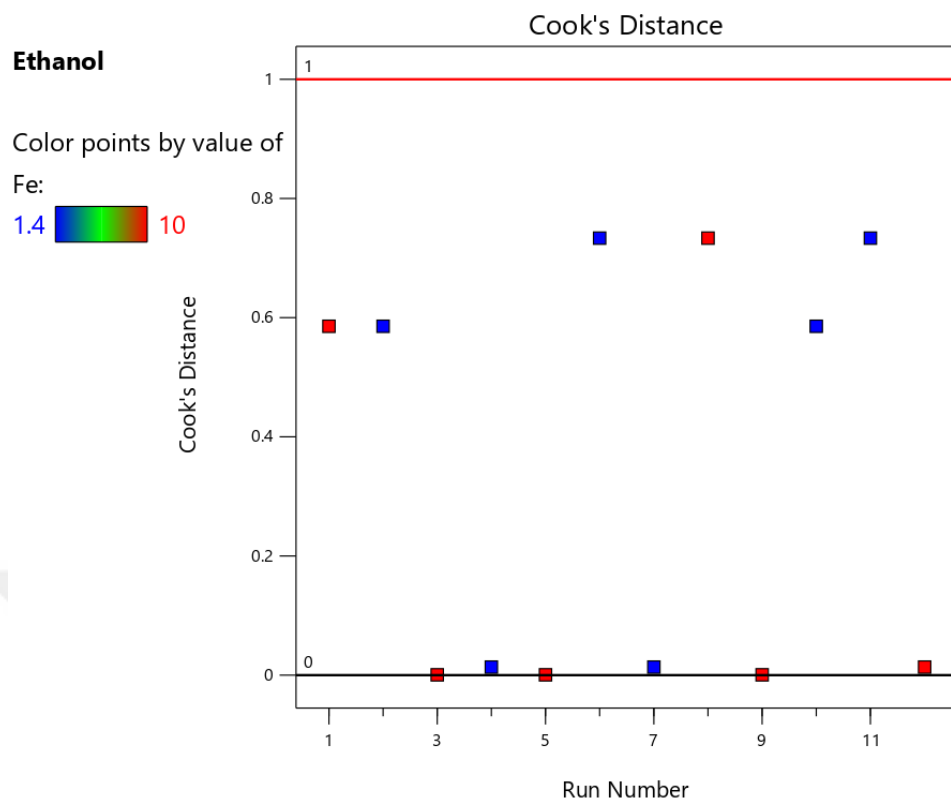


Figure 86 Cook's distance plot for ethanol.

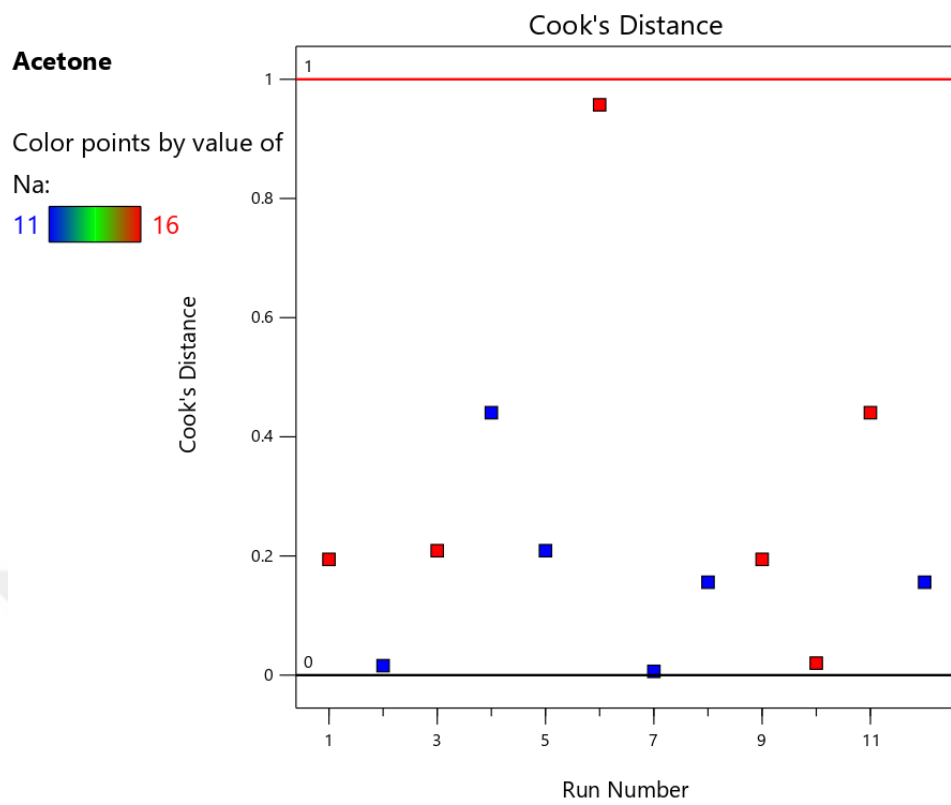


Figure 87 Cook's distance plot for acetone.

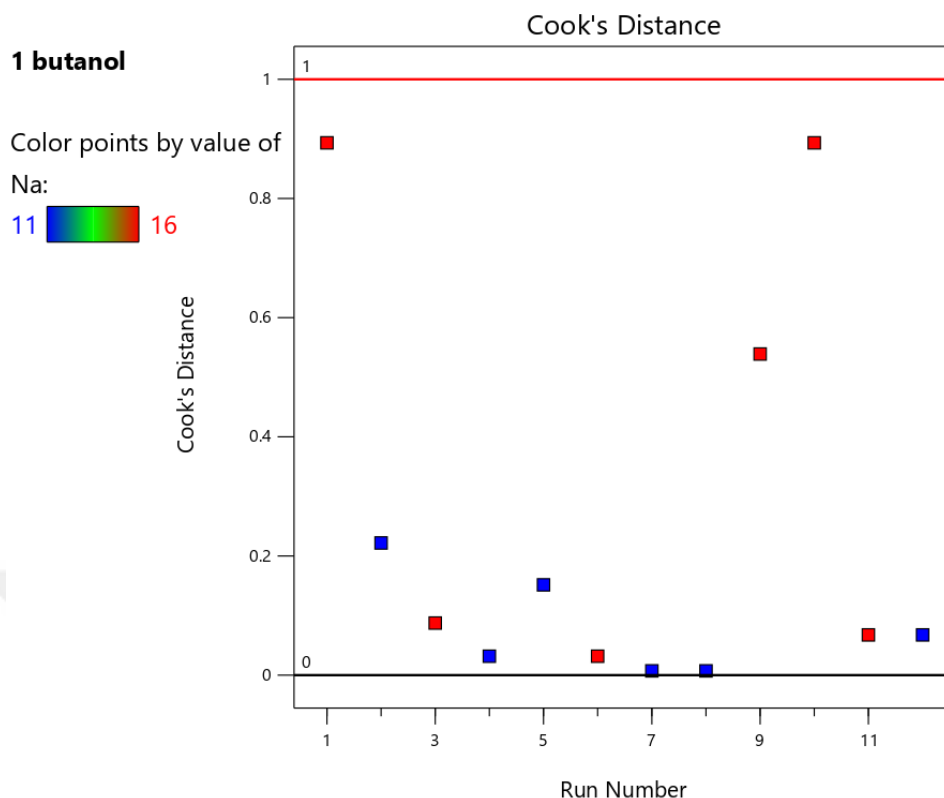


Figure 88 Cook's distance plot for butanol

Table 36 summarizes the results of the ANOVA analysis based on the factorial effects of metals on responses. The negative and positive effects of the factors were evaluated from the coefficients of the response equations (eq 6-13). It is seen that Mo^{3+} has a positive effect on product formation apart from lactic acid. Butanol production is enhanced in the presence of Na^+ and Zn^{2+} for which high concentrations are required. The fig hydrolysate lacks these elements as seen in Table 25, therefore, they should be externally added to the fermentation media. The elements, Co^{2+} , Mn^+ , Ca^{2+} and B^{3+} have a negative effect on production even at low concentrations (present in the hydrolysate).

Table 36 Factorial effects on experimental responses in the Plackett-Burman design

Product	Co ²⁺	Cu ²⁺	Mn ⁺	Fe ²⁺	Na ⁺	Mg ²⁺	Zn ²⁺	Ca ²⁺	K ⁺	Mo ³⁺	B ³⁺
Ethanol	0	-	-	-	+	+	0	-	0	+	-
Acetone	0	0	-	+	+	+	0	+	+	++	0
Butanol	-	0	-	0	+	0	+	-	0	++	-
Lactic	0	0	+	-	0	-	0	+	0	--	+
Acetic	-	+	-	+	0	-	+	0	0	++	-
Butyric	0	0	-	+	0	0	-	+	+	++	0
CHV	---	--	-	++	++	0	++	0	+	+++	--
HPR	--	-	-	+	0	0	+	+	+	++	-

Key

(-) = Negative effect

(+) = Positive effect

0= Insignificant

(+ + +) = Extremely positive effect

(- -) = Highly negative effect

(++) = Highly positive effect

(- - -) = Extremely negative effect

Hydrogen production was also evaluated and Figure 89 gives the time variation of cumulative hydrogen volume for the different runs and media compositions regarding cations. The maximum production volume was obtained at run number 6 which is rich in Co²⁺, Na⁺, Ca²⁺, and B³⁺, but limited to Cu²⁺, Mn⁺, Fe²⁺, Mg²⁺, Zn²⁺, K⁺, Mo³⁺.

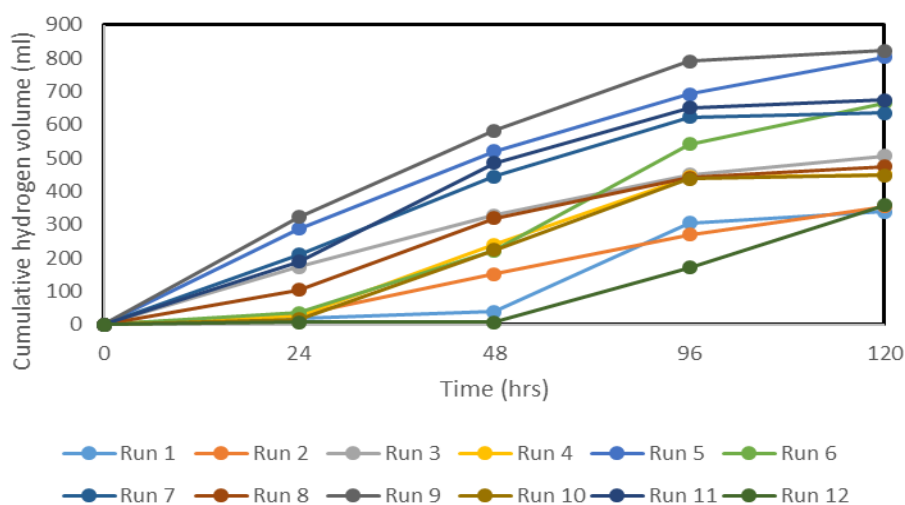


Figure 89 Cumulative hydrogen volume obtained at different runs of Plackett Burman design.

4.8 Optimization of metals in Fig hydrolysate fermentation media for the maximization of products

The Plackett-Burman experiment conducted in this study only revealed the effects of metals tested on the responses at the ranges used in this study. However, it does not show the concentrations of the metals needed to maximize results. Thus, there is a need for an optimization study to determine the concentrations needed to achieve maximum results (products) within the ranges tested in this study. Therefore, a numerical optimization study with validation was conducted. The desirability level of the optimization study was 98%. The optimization results for product maximization (solvents, organic acids and hydrogen production) are found in Figures 90, 91 and 92 respectively. The total ABE concentration maximization requires external addition of Na^+ , Zn^{2+} , Mo^{3+} , Mg^{2+} and Ca^{2+} . Similarly, organic acid production is maximized in the presence of Cu^{2+} , Fe^{2+} , Na^+ , Zn^{2+} , Ca^{2+} and Mo^{3+} . Finally, the hydrogen generation rate can be enhanced with the elements; Fe^{2+} , Na^+ , Mg^{2+} , Zn^{2+} , Ca^{2+} , K^+ and Mo^{3+} . The optimisation results are also presented in Table 37.

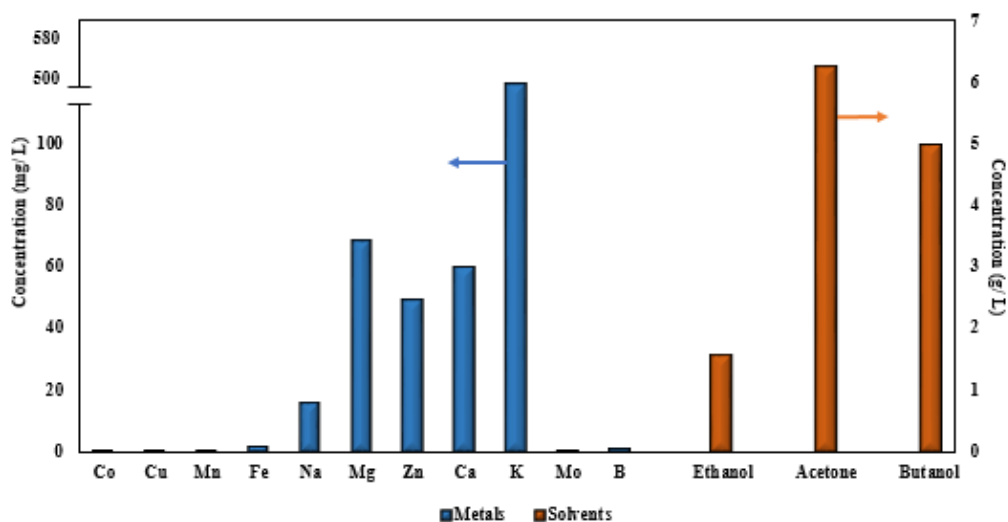


Figure 90 Optimization results for solvent maximization

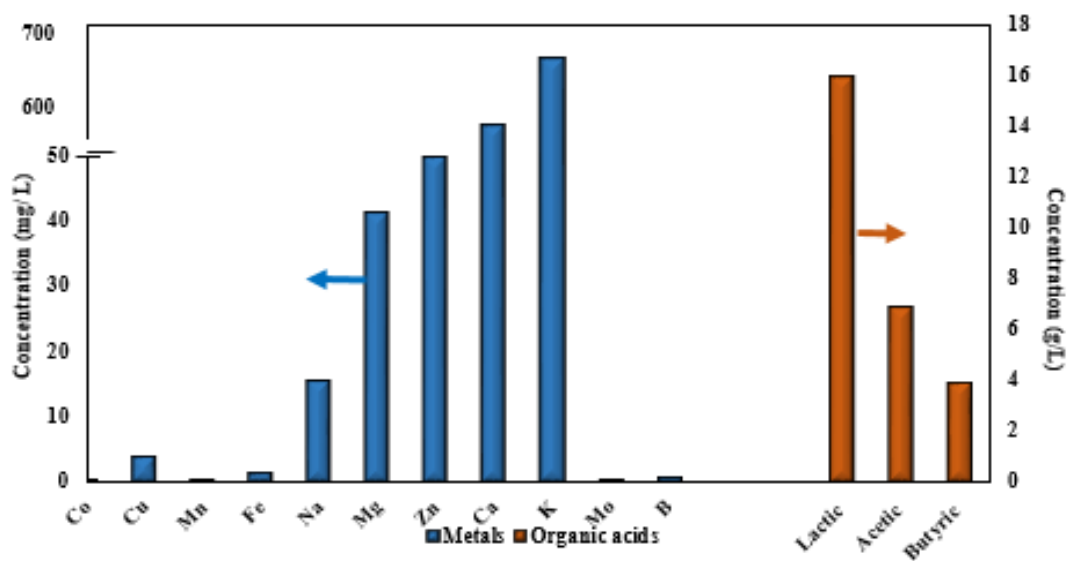


Figure 91 Optimization results for organic acids maximization

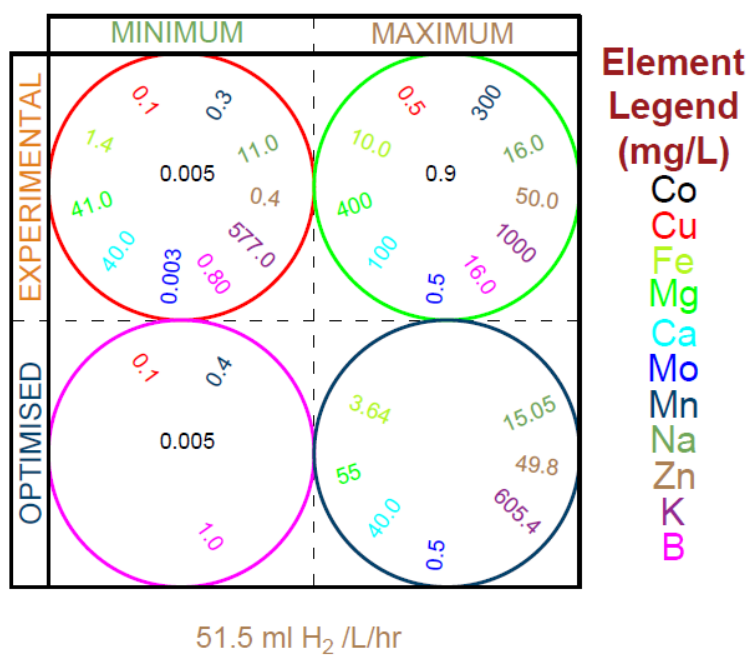


Figure 92 Optimization results for hydrogen production rate maximization

Table 37 The concentrations of the cations for the maximization of solvent and organic acid concentrations.

Lactic	16.0		Ethanol	1.58
Acetic	6.90		Acetone	6.26
Butyric	3.87		Butanol	5.01
Co	0.005		Co	0.026
Cu	3.99		Cu	0.10
Mn	0.317		Mn	0.30
Fe	1.58		Fe	1.41
Na	15.5		Na	16
Mg	41.4		Mg	69.1
Zn	50.0		Zn	49.6
Ca	54.9		Ca	60.0
K	65.0		K	120
Mo	0.50		Mo	0.472
B	0.80		B	0.918

Numerous kinds of research have been conducted on ABE fermentation and hydrogen production with different substrates. However, this is the only known study that studied ABE and hydrogen production using fig (*Ficus carica*) as fermentation's carbon source. ABE fermentation is the most common process for butanol production. The ABE biochemical pathway is microbially mediated and achieves simultaneous production of acetone, butanol and ethanol with the same substrate via biochemical conversion of 6C or 5C sugar sources into acetone, butanol and ethanol (Amiri and Karimi, 2019). The ABE fermentation is a bi-phasic process with organic acids, hydrogen and CO₂ as products of the acidogenesis phase. The conversion of acidogenesis products into organic solvents (acetone, butanol, and ethanol) represents the second phase (solventogenesis) of the ABE fermentation (Amiri and Karimi, 2019).

Saadatinavaz, Karimi, and Denayer (2021) studied H₂ and butanol production via ABE fermentation using hydrothermal pretreated Orange waste as substrate. The fermentation study yielded 42.3 g butanol, 33.1 g acetone, 13.4 g ethanol and 104.5 L hydrogen per kg of Orange Wastes. The high concentrations of ABE products in this study were a result of the Continuous fermentation mode employed in which the

product was separated simultaneously. In addition, Valdez et al. (2015) employed a co-culture of *Clostridium beijerinckii* 10132, *Clostridium cellulovorans* 35296 and *Enterococcus spp* as the microbial source in ABE fermentation of Wheat straw to produce acetone, butanol, ethanol and hydrogen upon 120 hrs of fermentation. The study yielded 3.7 g/L ethanol, 14.2 g/L butanol, 5.4 g/L acetone and 79.5 mL H₂ /g substrate consumed.

Very few studies exist on the detailed role of metals in the ABE biochemical pathway and fermentation process for the production of organic acids, solvents and hydrogen. This is part of the reason why this study was initiated. Researchers like Wu, Xue, Chen, Yuan, and Bai (2016) studied the effects of Ca²⁺ and Zn²⁺ on ABE fermentation with *Clostridium acetobutylicum* as a microbial source and glucose, xylose and glucose/xylose mixtures as carbon sources. Ca²⁺ (4 g/L CaCO₃) and Zn²⁺ (0.001 g/L ZnSO₄·7H₂O) were the concentrations used in the study. The authors recorded the best results with glucose/xylose mixtures as carbon sources with 16.1 and 25.9 g/L of butanol and total ABE, respectively, compared with 11.7 and 19.4 g/L butanol and total ABE obtained from the control experiment. The authors credited this improvement largely to the effect of Zn²⁺. Thus, this provides a hint into the significance of Ca²⁺ and Zn²⁺ in the ABE biochemical pathway. This is further confirmed by the results obtained from this study found in Table 36 that Zn²⁺ enhances butanol production. Although, Ca²⁺ is a cofactor for several enzymes involved in the butanol biosynthesis pathway and aids in the maintenance of structural integrity of cell membrane, however, Ca²⁺ had a negative effect from this study possibly due to the low concentration (100 mg/L) employed. Zn²⁺ was found to positively influence hydrogen production results (cumulative hydrogen volume and hydrogen production rate) from the Plackett Burman design employed in this study. This is similar to the report of Keskin et al. (2018) in which Zn²⁺ and Ni⁺ improved the hydrogen production rate. Wu, Xue, Chen, Yuan, and Bai (2016) also subjected Jerusalem artichoke tubers and corn stover to ABE fermentation with Zn²⁺ (0.001 g/L ZnSO₄·7H₂O) addition and *Clostridium acetobutylicum* L7 as inoculum. The study recorded an 184% and 218% increase in butanol concentration and productivity respectively when compared with the control. Furthermore, the authors reported that

Zn^{2+} addition accelerates substrate utilization and results in a decrease in organic acids production which implies earlier progression into the solventogenic ABE stage meaning an improvement in butanol production. In a related study, Wu et al. (2013) reported that Zn^{2+} (0.001 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) is the best supplementation concentration favoring ABE results while > 0.001 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ inhibits microbial performance for the ABE process. The Zn^{2+} addition in the study resulted in 7.7, 4.7 and 30.8% improvement in butanol, acetone and ethanol concentrations after 40h.

The study by Mukherjee et al. (2019) emphasizes the significant impact of Zn^{2+} and Mg^{2+} on ABE fermentation. Mg^{2+} is notably abundant in lignocellulosic biomass and is found in high levels in hydrolyzed figs, often alongside K^+ . While research on Mg^{2+} role in butanol production is limited, it has not been identified as a factor in the activity of certain key enzymes. Therefore, adding Mg^{2+} to the fermentation medium may not be necessary if it occurs naturally in the feedstock.

Zn^{2+} addition and lowering of Mg^{2+} concentration stimulates microbial performance, and substrate utilization, enhances butanol production and reduces ethanol concentration. This is supported by findings in Table 36 where Mg^{2+} only had a positive influence on ethanol and acetone concentrations amongst all the responses tested in the experimental design. Zn^{2+} significantly influenced butanol and H_2 production results. It is worth noting that this is the only known work that studied the effects of Co^{2+} , Cu^{2+} , Mn^+ , Mo^{3+} and B^{3+} on the ABE fermentation process and H_2 production simultaneously. Although, Keskin et al. (2018) studied the effects of Fe^{2+} , Ni^{2+} , Zn^{2+} , Co^{2+} , Cu^{2+} , Mn^+ , Al^{3+} , B^{3+} , Se^{4+} , Mo^{3+} and W^{6+} on H_2 production from Fruit and Vegetable Wastes via the Plackett- Burman statistical design and reported Zn and Ni to be the only elements favouring H_2 production. However, Mo is an unreported element found to be highly influential in the ABE fermentation process and H_2 production. From this study, as seen in Table 36, Mo^{3+} was found to have an extremely positive effect on H_2 production results. This is the only known report on the important role of Mo in ABE fermentation and hydrogen technology. Mo^{3+} (0.003 – 0.5 mg/L) was the range studied in this research that

yielded these results. Keskin et al. (2008) employed Mo^{3+} (0.3 – 30.0 mg/L). This is indeed inhibitory for microbial performance in hydrogen research.

Many authors have reported the significance of many metals in ABE fermentation and H_2 production studies. However, this depends on the substrate, inoculum, and the ranges at which these metals were tested. This accounts for variations in ABE results from different scientific works. This study was not excluded from these variations. In addition, numerous studies on ABE fermentation involved substrates highly rich in glucose, fructose and xylose when compared with studies that used lignocellulosic biomass (such as fig) as a biomass source as done in this research. Furthermore, yeast extract and other protein sources are mostly included in high concentrations in fermentation media composition to enhance microbial performance in the ABE process. The results from this study showed that fig (*Ficus carica*) is a good substrate for value-added bioproducts (organic solvents and acids, hydrogen and other biofuels) despite the very low concentration of yeast and meat extract (the only protein source employed in this study).

4.9 Optimization of ABE fermentation for the essential cations via Box Behnken design

The Plackett Burman experimental design was conducted at the following ranges: Mo^{3+} (0.003 – 0.5 mg L^{-1}), Na^+ (11 – 16 mg L^{-1}) and Zn^{2+} (0.4 – 50 mg L^{-1}). Thus, with this result, a Box Behnken Design experimented to better understand the role of these elements as activators for butanol in the ABE fermentation pathway. Furthermore, the ranges (concentration) of these metals were increased for the possibility of increased butanol production. The actual and predicted values of Box Behnken Design for the optimization of Na^+ , Mo^{3+} and Zn^{2+} in ABE fermentation are shown in Table 38. Similarly, observed and predicted values of the other products (organic acids and hydrogen) are given in Table 39.

Table 40 shows the ANOVA results on the factorial significance of butanol. The ANOVA statistical analysis for the other responses can be found in the Appendix.

From the BBD experiment, only Na^+ and Zn^{2+} were significant for butanol production at the concentrations tested in the study. On the other hand, Mo^{3+} turned out to be insignificant at the studied concentration range. Table 41 summarizes the significance of the factors in all responses. (-) indicates the factor negatively affects that response while the (+) sign states the positive effect of the factor. Equations 14 to 20 show the Box Behnken response equations for products. The Box Cot chart for Box Behnken butanol production is found in Figure 93.

$$\begin{aligned} \text{Ethanol} = & 1.82005 - 0.194291A + 0.00664731B - 0.00898634C - 6.64376\text{e-}05AB \\ & + 0.000484536AC - 5.92593\text{e-}06BC + 0.0245935A^2 - 4.11728\text{e-}05B^2 + 4.73264\text{e-} \\ & 05C^2 \end{aligned} \quad (\text{Eq.14})$$

$$\begin{aligned} \text{Acetone} = & 3.30639 - 0.315866A - 0.0083989B + 0.0543428C - 0.00156701AB - \\ & 0.00931615AC + 0.00017213BC + 0.182782A^2 + 9.80864\text{e-}05B^2 - 0.000196632C^2 \end{aligned} \quad (\text{Eq.15})$$

$$\begin{aligned} \text{Butanol} = & 1.94553 - 0.388893A + 0.0565108B - 0.023655C - 0.00203895AB + \\ & 0.00816323AC - 0.000407778BC - 0.0487831A^2 - 8.30247\text{e-}05B^2 + 0.000153924C^2 \end{aligned} \quad (\text{Eq.16})$$

$$\begin{aligned} \text{Lactic acid} = & 12.3885 + 4.64501A - 0.430082B + 0.420041C + 0.0202062AB - \\ & 0.0397766AC + 0.00186759BC - 0.289793A^2 + 0.00124239B^2 - 0.00225185C^2 \end{aligned} \quad (\text{Eq.17})$$

$$\begin{aligned} \text{Acetic acid} = & 7.11635 - 0.807795A + 0.00452237B - 0.0276823C - \\ & 0.000252005AB + 0.0066323 AC - 0.000191667BC - 0.0017005A^2 + \\ & 0.000193827B^2 + 0.000143056C^2 \end{aligned} \quad (\text{Eq.18})$$

$$\begin{aligned} \text{Butyric acid} = & 4.56 + 0.1625A + 0.54875B + 0.08625C + 0.2225AB - 0.4175AC \\ & + 0.905BC + 1.1075A^2 + 0.34B^2 + 0.165C^2 \end{aligned} \quad (\text{Eq.19})$$

$$\begin{aligned} \text{HPR} = & -40.3017 + 3.25035A + 0.31456B + 1.41577C - 0.0748554AB - \\ & 0.0110636AC - 0.00190814BC + 0.773005A^2 + 0.0006679B^2 - 0.00685478C^2 \end{aligned} \quad (\text{Eq.20})$$

Table 38 Actual and predicted responses for Acetone, Butanol and Ethanol.

Run	Mo ³⁺ (mg/L)	Na ⁺ (mg/L)	Zn ²⁺ (mg/L)	Ethanol g/L		Acetone g/L		Butanol g/L	
				Obs	Pre	Obs	Pre	Obs	Pre
1	5	55	30	1.529	1.52	7.132	6.04	1.031	1.06
2	2.575	55	90	1.431	1.37	7.262	5.31	2.24	2.18
3	0.15	55	150	1.704	1.71	6.931	8.02	1.488	1.46
4	2.575	55	90	1.32	1.37	5.17	5.31	2.15	2.18
5	2.575	100	150	1.551	1.53	7.856	6.15	1.75	1.87
6	0.15	10	90	1.465	1.43	7.209	6.51	1.315	1.30
7	0.15	100	90	1.561	1.57	7.491	8.10	2.325	2.23
8	5	100	90	1.387	1.42	5.614	6.31	1.69	1.70
9	5	10	90	1.32	1.31	6.016	5.40	1.57	1.66
10	2.575	55	90	1.365	1.37	3.495	5.31	2.15	2.18
11	2.575	10	30	1.335	1.35	2.673	4.38	1.18	1.06
12	2.575	100	30	1.54	1.51	4.296	4.69	3.794	3.75
13	2.575	10	150	1.41	1.44	4.374	3.98	3.54	3.58
14	0.15	55	30	1.779	1.80	5.788	4.78	3.387	3.52
15	5	55	150	1.736	1.72	2.853	3.86	3.883	3.75
R²				0.9627		0.5419		0.9932	

Table 39 Actual and predicted responses for hydrogen production rate and individual organic acids.

Run	Mo ³⁺ (mg/L)	Na ⁺ (mg/L)	Zn ²⁺ (mg/L)	HPR mL /L hr		Lactic acid g/L		Acetic acid g/L		Butyric acid g/L	
				Obs	Pre	Obs	Pre	Obs	Pre	Obs	Pre
1	5	55	30	36.3	25.51	21.0	21.72	3.6	3.78	6.8	6.3
2	2.575	55	90	45.6	41.80	20.5	24.98	4.8	5.08	4.52	4.56
3	0.15	55	150	10.2	21.05	20.8	20.20	5.6	5.46	5.7	6.17
4	2.575	55	90	43.5	41.80	26.4	24.98	5.7	5.08	5.04	4.56
5	2.575	100	150	24.7	17.29	21.1	22.04	6.2	6.10	6.85	6.61
6	0.15	10	90	29.5	33.33	28	29.64	5.5	5.64	5.72	5.52
7	0.15	100	90	55.1	51.79	18.8	18.63	6.1	6.41	6.4	6.17
8	5	100	90	49.5	45.73	28	26.36	5.3	5.23	6.74	6.94
9	5	10	90	56.5	59.94	28.3	28.55	4.8	4.57	5.17	5.40
10	2.575	55	90	36.2	41.80	27.9	24.98	4.6	5.08	4.12	4.56
11	2.575	10	30	1.89	9.35	27.7	26.83	4.7	4.84	5.09	5.34
12	2.575	100	30	7.12	21.78	9.19	10.15	6.6	6.59	4.35	4.62
13	2.575	10	150	40.1	25.48	19.5	18.55	6.3	6.42	3.97	3.70
14	0.15	55	30	23.2	12.01	7.58	6.83	7.1	6.84	5.21	5.17
15	5	55	150	16.8	28.10	11.1	11.94	6	6.26	5.62	5.66
R²				0.73		0.94		0.9		0.98	

Table 40 ANOVA results for Butanol

	Sum Squares	of Df	Mean Square	F value	p-value
Model	13.05	9	1.45	80.77	< 0.0001
Mo	0.0145	1	0.0145	0.8096	0.4095
Na	0.4773	1	0.4773	26.58	0.0036
Zn	0.2013	1	0.2013	11.21	0.0204
Mo- Na	0.198	1	0.198	11.03	0.021
Mo- Zn	5.64	1	5.64	314.3	< 0.0001
Na-Zn	4.85	1	4.85	270.07	< 0.0001
Mo ²	0.3039	1	0.3039	16.92	0.0092
Na ²	0.1044	1	0.1044	5.81	0.0608
Zn ²	1.13	1	1.13	63.15	0.0005
Residual	0.0898	5	0.018		
Lack of Fit	0.0844	3	0.0281	10.42	0.0889
Pure Error	0.0054	2	0.0027		
Cor Total	13.14	14			

on the response probably due to the presence of the excess amount of cations in the fermentation media.

Figure 95 shows the variation of butanol concentration with Na^+ and Zn^{2+} concentration at constant Mo^{3+} . The effect of these cations on butanol formation was found statistically significant. The response substantially increases when either Zn^{2+} or Na^+ concentrations are increased. It can be concluded that the process requires over 100 mg/L Na to improve the butanol production. The interaction effect of these elements is seen in the figure. The butanol production is adversely affected when both cations are at high concentrations as 150 mg/L for Zn^{2+} and 100 mg/L for Na^+ .

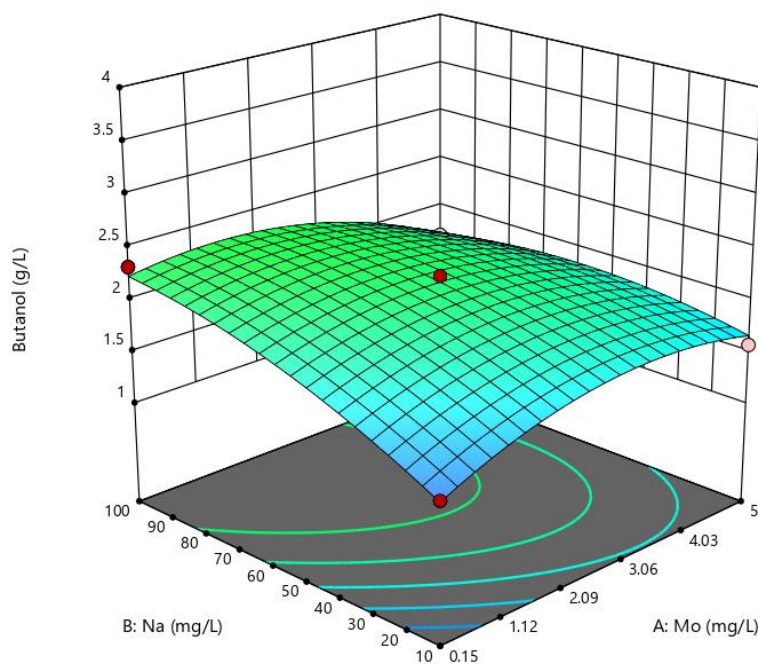


Figure 94 The effect of Na and Mo on butanol concentration at a constant Zn concentration of 90 mg/L

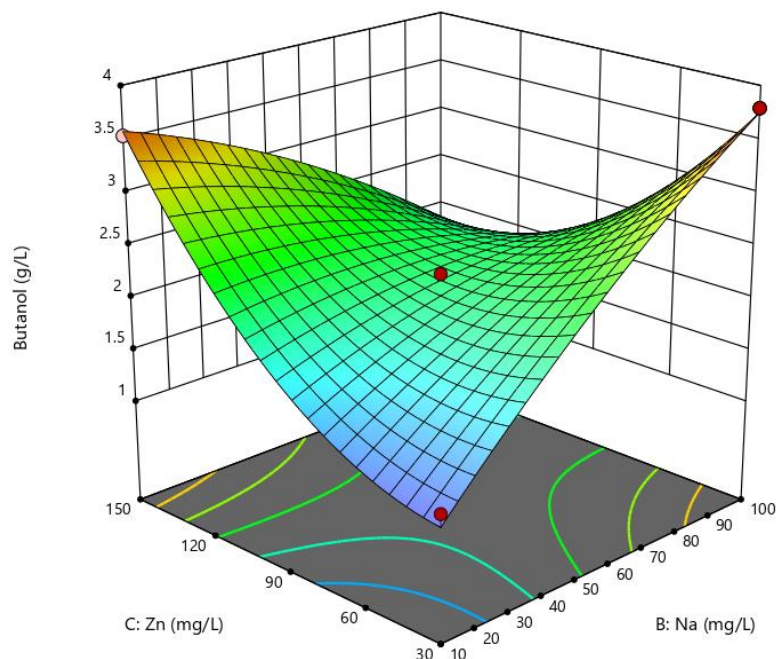


Figure 95 The effect of Na^+ and Zn^{2+} on butanol concentration at constant Mo^{3+} concentration of 2.575 mg/L

The interaction effect of cations Mo^{3+} and Zn^{2+} on butanol production at constant Na concentration is given in Figure 96. Interestingly, the production increases at very low concentrations of cations like 20 mg/L Zn and 0.15 mg/L Mo. Another increase in the butanol concentration was observed at very high concentrations for the cations such as 150 mg/L Zn and 5 mg/L Mo respectively. On the other hand, minimum production was obtained when one of the cations was at a low concentration while the other was at a high. This result shows that Mo^{3+} , despite its statistical insignificance on butanol production, its interaction effect with Zn^{2+} is very significant. The summary of the effect of all factors on butanol generation is given in Figure 97.

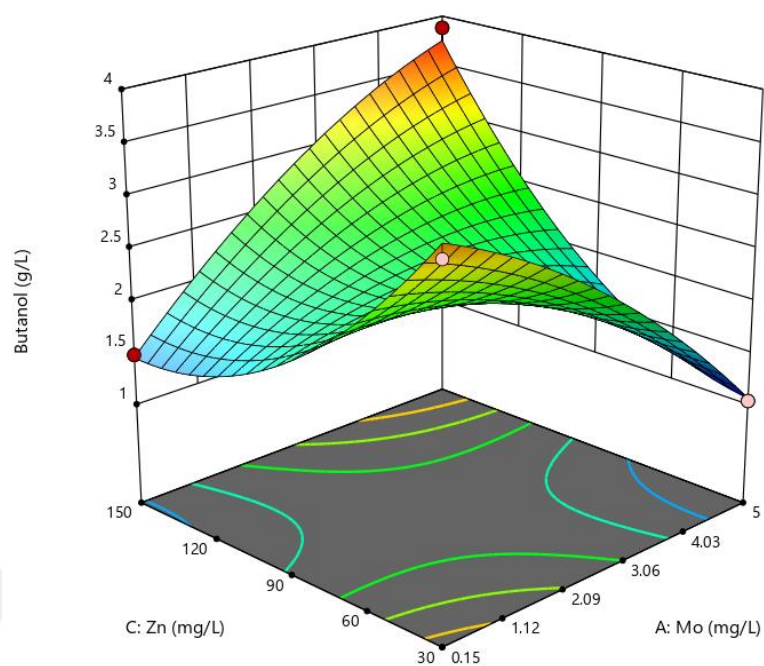


Figure 96 The effect of Mo^{3+} and Zn^{2+} on butanol concentration at constant Na^+ concentration of 55 mg/L

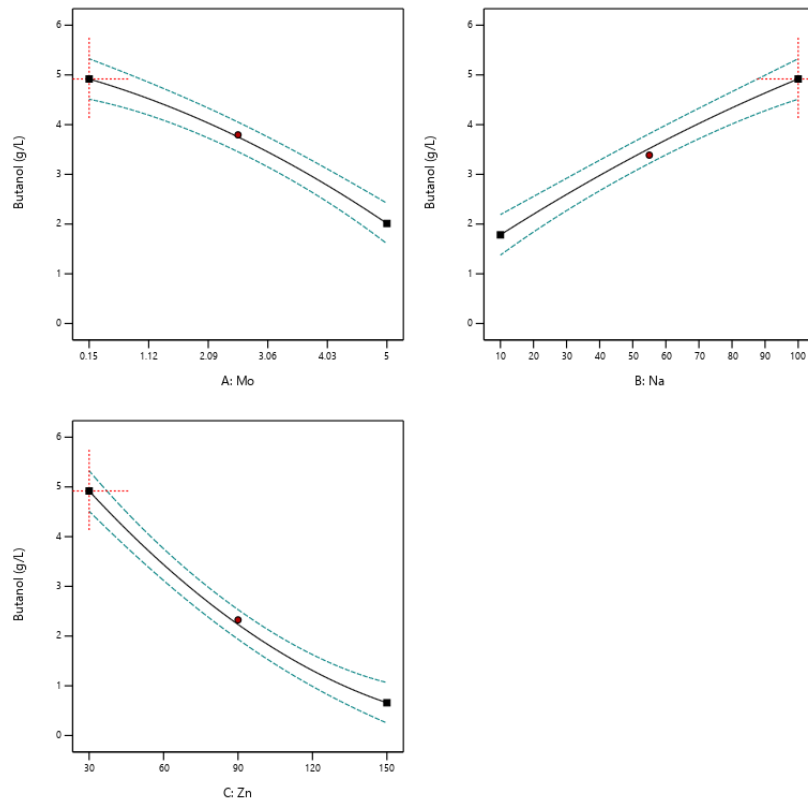


Figure 97 Summary plot for the effect of all factors on butanol formation

Statistical analysis of the Box-Bhenken experiment indicated that the factorial effect of Mo and Na as well as the interaction between these cations are significant for ethanol production. Figure 98 depicts the variation of ethanol concentration with these cations. ethanol concentration is desired to be minimal to enhance butanol formation. Mo has got more significant effect on butanol increase while limiting ethanol production. Low ethanol formation was observed at a high concentration of Mo (5 mg/L) acting as an ethanol inhibitor. On the other hand, increasing Na concentration slightly enhances ethanol formation. As earlier mentioned, a high concentration of Na improves butanol formation. Thus, this result indicates that Na concentration should be well controlled to limit the ethanol while improving butanol formation.

Similarly, the relationship between Mo and Zn needs to be controlled for ethanol formation. The minimum ethanol concentration was observed at the centre points of both cations (Zn = 90 mg/L, Mo = 2.5 mg/L) (Figure 99). Finally, the effect of Zn and Na at a constant Mo concentration of 5 mg/L is given in Figure 100. The effect of Zn on ethanol formation was more substantial compared with that of Na. Therefore the presence of Zn in the fermentation media should be minimized to limit the ethanol formation. Figure 101 summarizes the effect of all factors on ethanol formation. Figures 102 to 113 give the 3D effects of interactions existing in organic acids and hydrogen production.

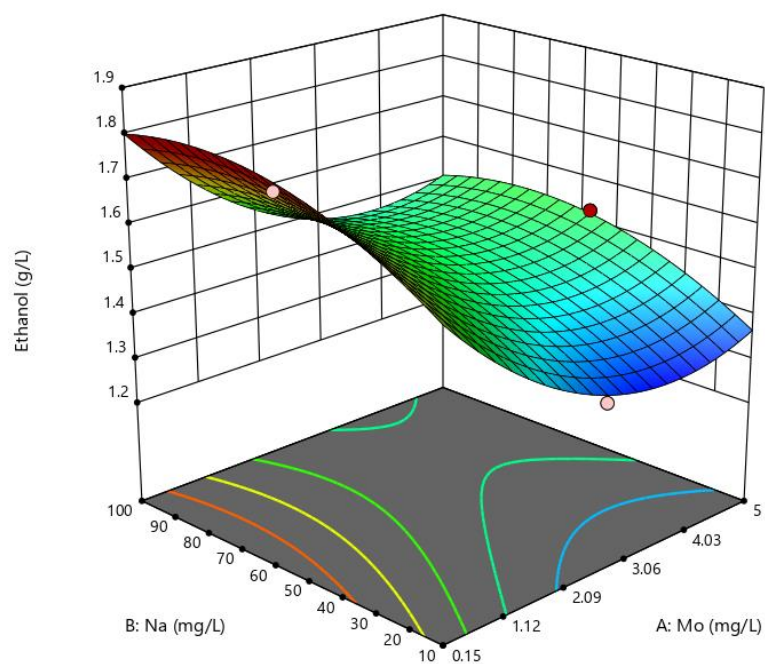


Figure 98 Variation of ethanol concentration with Na and Mo (at constant Zn = 30 mg/L)

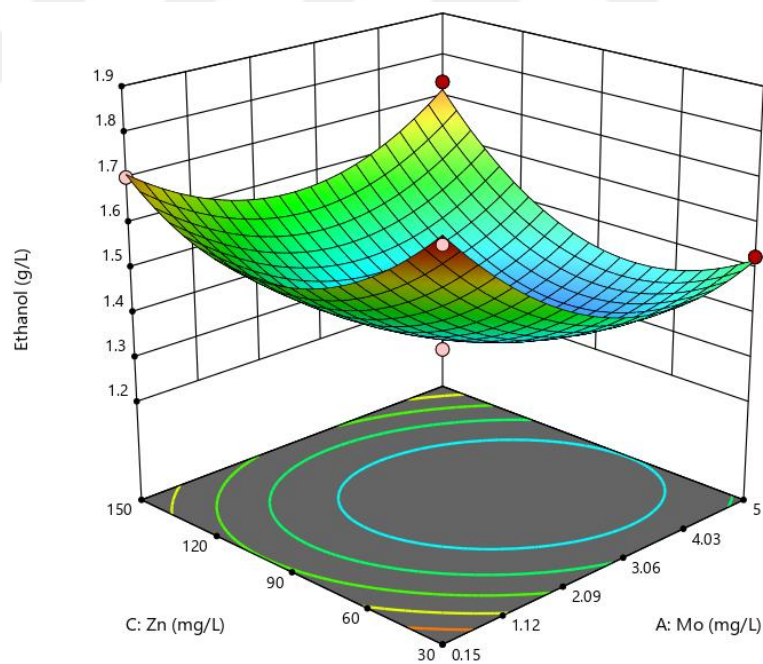


Figure 99 Variation of ethanol concentration with Mo with Zn (at constant Na = 55mg/L)

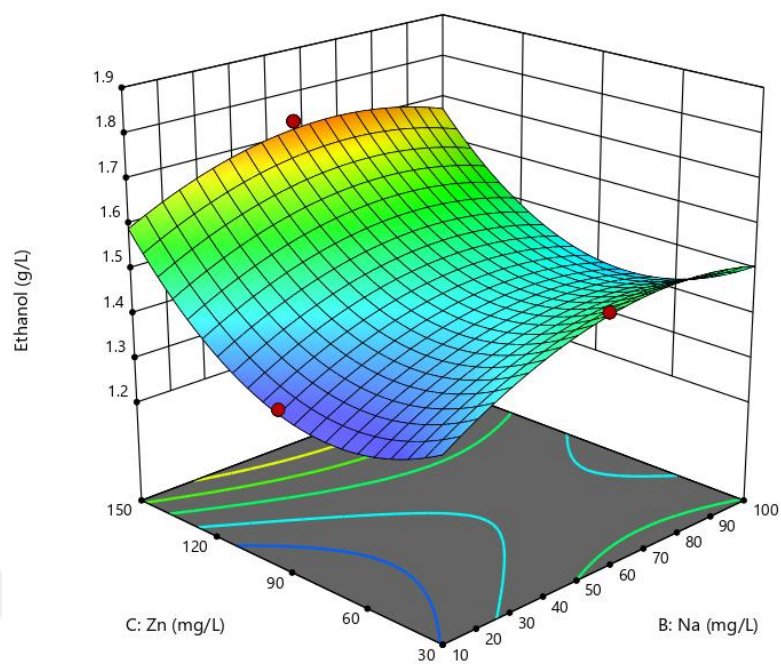


Figure 100 Variation of ethanol concentration with Zn and Na (at constant $Mo = 5$ mg/L)

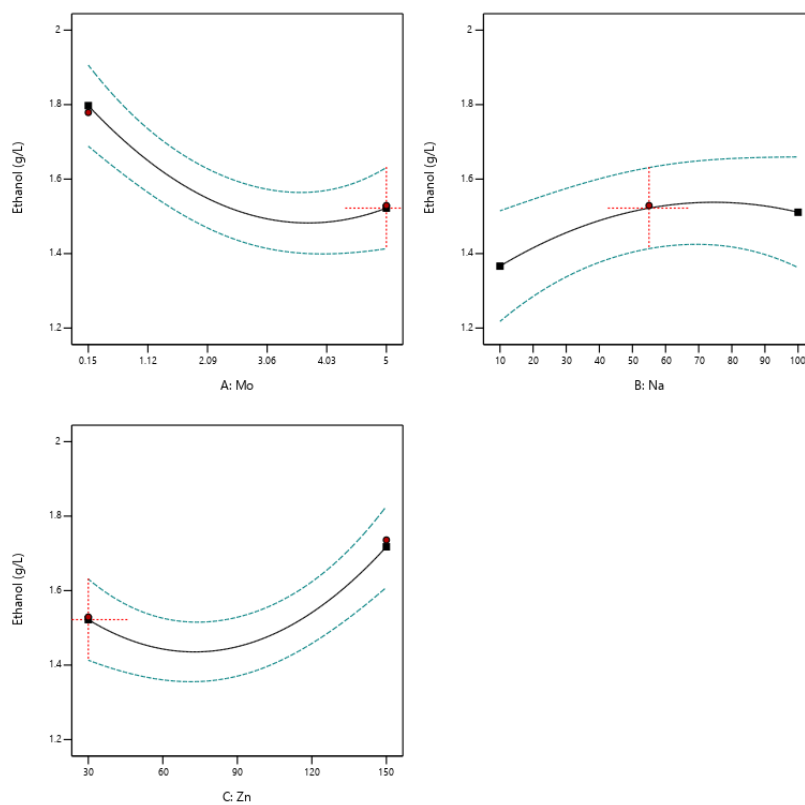


Figure 101 A summary plot for the effect of cations on ethanol formation

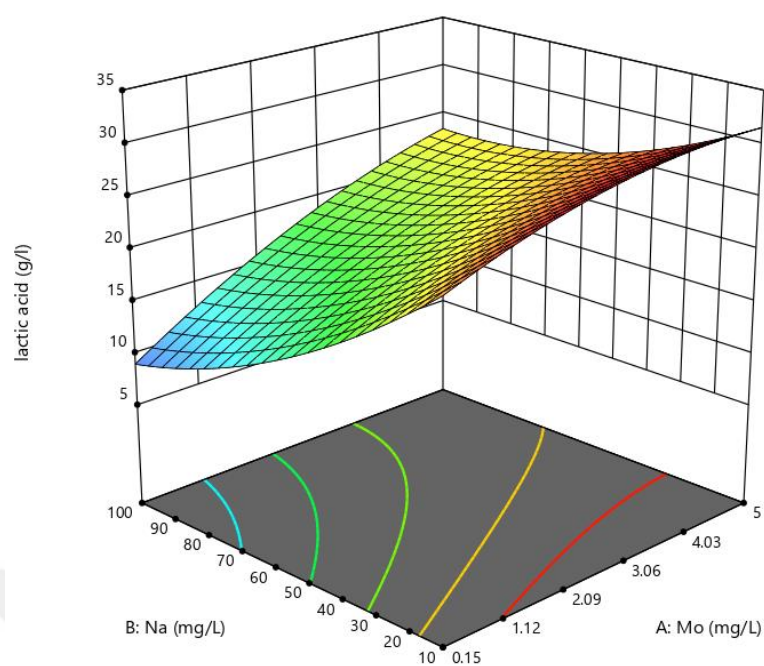


Figure 102 Variation of lactic acid concentration with Zn and Mo (at constant Na = 55mg/L)

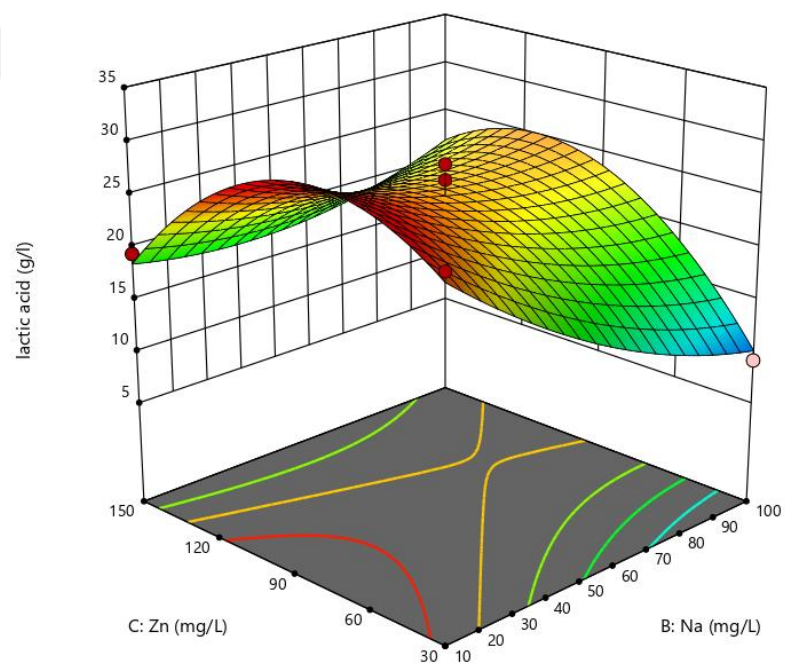


Figure 103 Variation of lactic acid concentration with Na with Zn (at constant Mo = 2.575 mg/L)

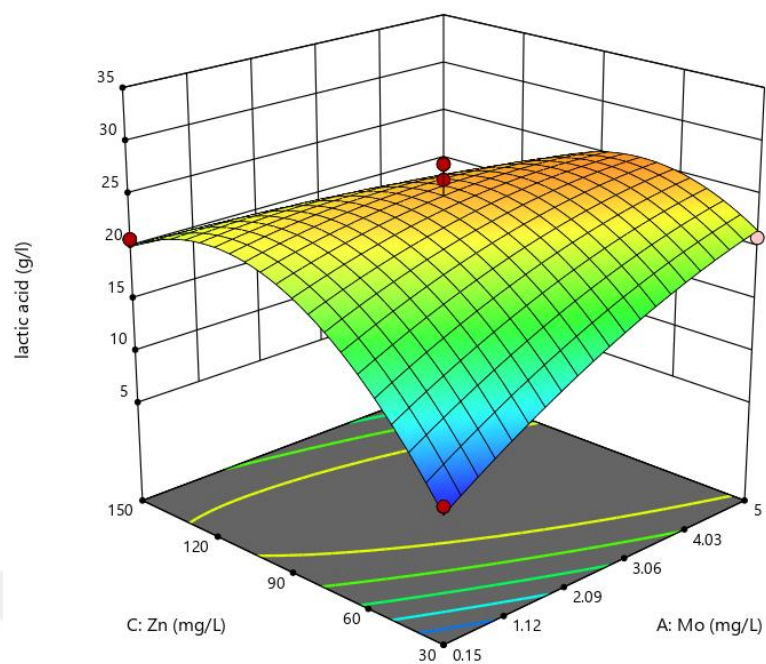


Figure 104 Variation of lactic acid concentration with Mo with Zn (at constant Na = 55 mg/L)

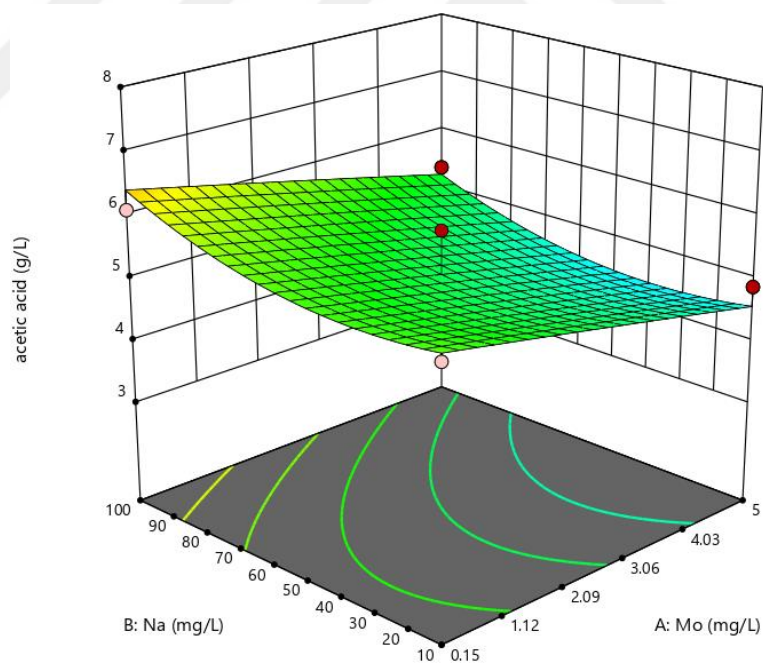


Figure 105 Variation of acetic acid concentration with Mo with Na (at constant Zn = 90 mg/L)

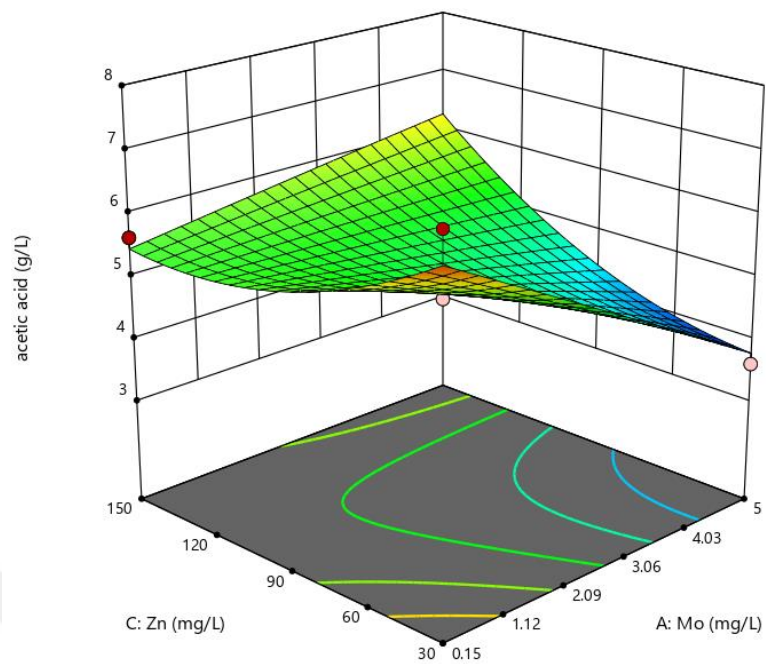


Figure 106 Variation of acetic acid concentration with Mo with Zn (at constant Na = 55 mg/L)

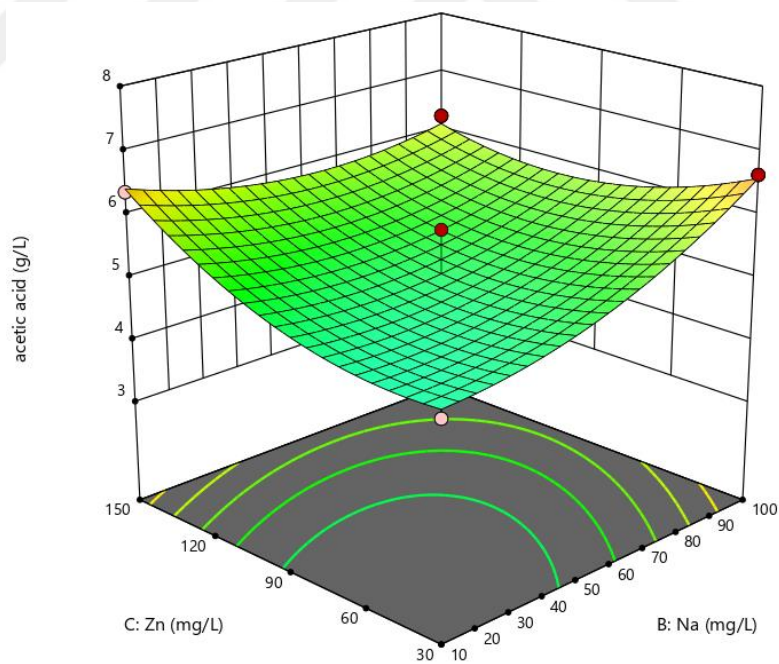


Figure 107 Variation of acetic acid concentration with Na with Zn (at constant Mo = 2.575 mg/L)

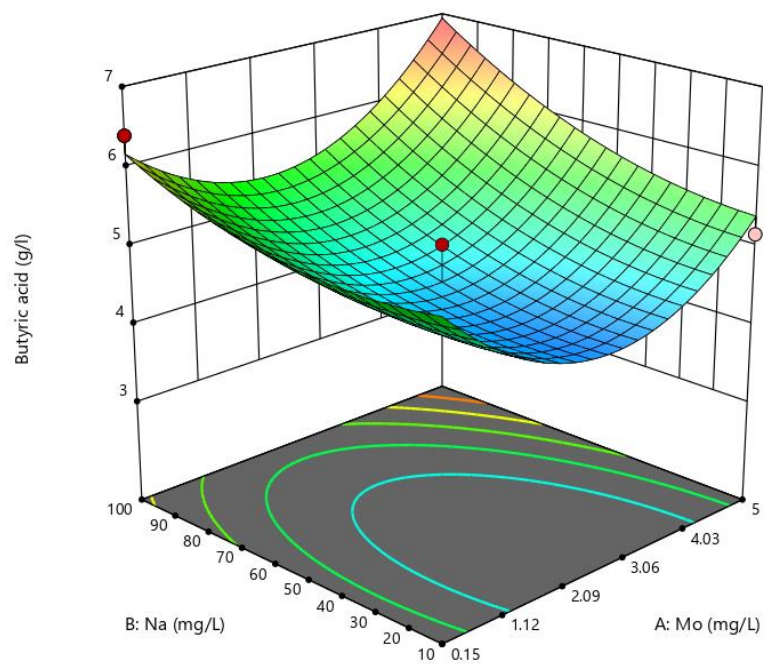


Figure 108 Variation of butyric acid concentration with Mo with Na (at constant Zn = 90 mg/L)

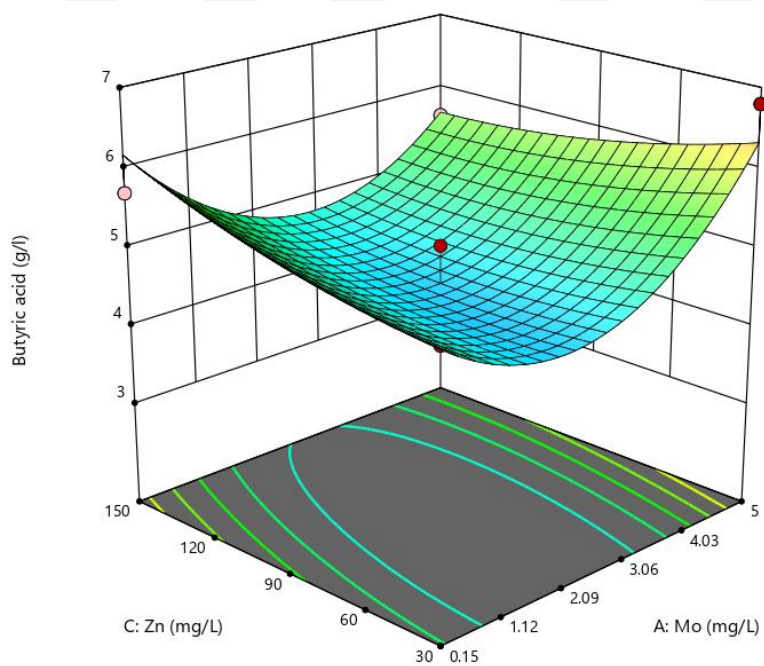


Figure 109 Variation of butyric acid concentration with Mo with Zn (at constant Na = 55 mg/L)

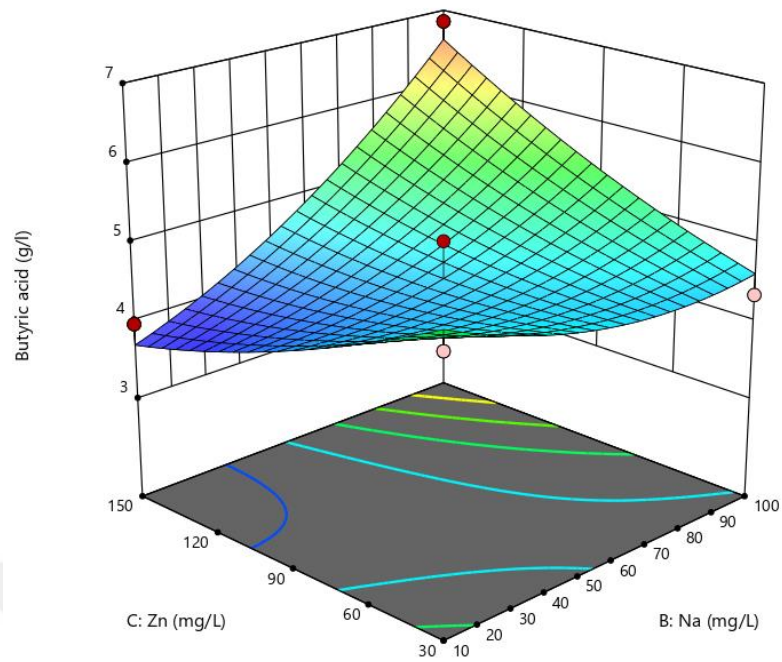


Figure 110 Variation of butyric acid concentration with Na with Zn (at constant Mo = 2.575 mg/L)

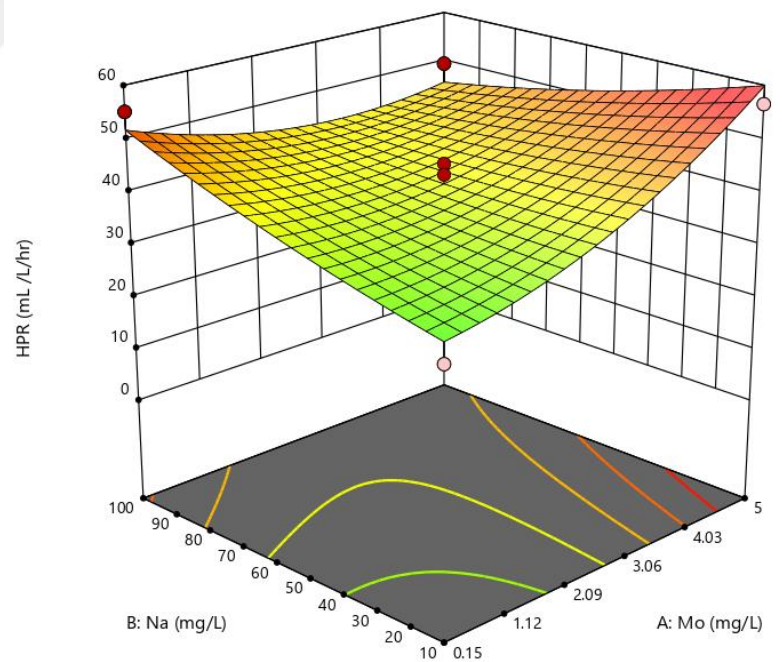


Figure 111 Variation of HPR with Mo with Na (at constant Zn = 90 mg/L)

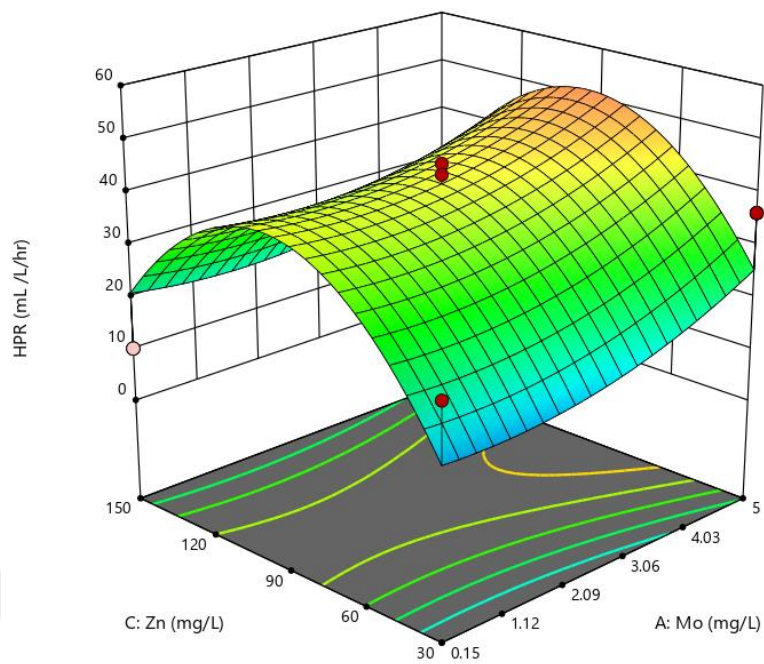


Figure 112 Variation of HPR with Mo with Zn (at constant Na = 55 mg/L)

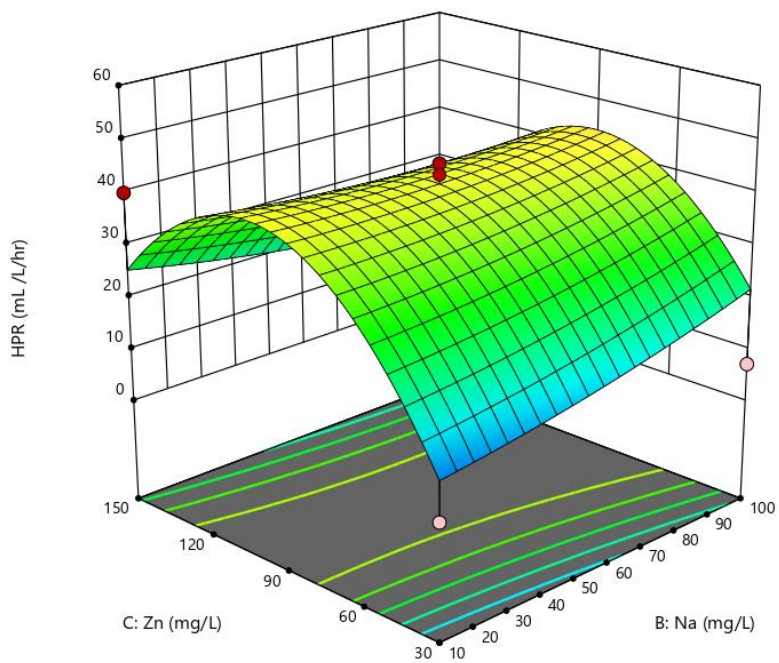


Figure 113 Variation of HPR with Na with Zn (at constant Mo = 2.575 mg/L)

4.10 The effect of Na on ABE fermentation

The role of sodium in hydrogen production from food waste was studied by Cao and Zhao (2009). Na (5.03–28.7 g/l) was the range tested in the study. It was discovered that hydrogen production increased within 5.03–14.41 g/L Na concentration. However, at concentrations > 20 g/L, hydrogen production declines possibly due to high osmotic pressure weakening the activity of microorganisms. Similarly, Taheri et al. (2018) studied the role of NaCl on hydrogen production at concentration (0.5 - 30 g/L). The highest and least hydrogen production results respectively were obtained at 0.5 and 30 g/L NaCl. Thus, it is easy to affirm the negative role of Na on hydrogen production at higher concentrations. The involvement of Zn in butanol production is well documented. However, very few researches exist on Na involvement in butanol production.

The role of Na (0.01-40 g/L) on *Clostridium acetobutylicum* ATCC 824 activity for ABE fermentation was studied by Zabihi et al. (2018). It was reported that increasing NaCl concentration adversely affects bacterial performance. Hence, low ABE products result. Surprisingly, Zhao et al. (2016) affirmed the negative role of Na on *Clostridium acetobutylicum* ATCC 824 for ABE fermentation at high concentrations. However, Zhao et al. (2016) think that the adverse effect only has to do with the acidogenesis stage of ABE fermentation. Box-Benkhen experimental results revealed that Na significantly affects the butanol formation. The response reached the maximum level at the high concentration of Na (100 mg/L). Thus, experiments to study the effects of extremely high levels of Na on butanol production were conducted. Figures 114 to 117 show the effects of high concentrations of Na on hydrogen and ABE solvents.

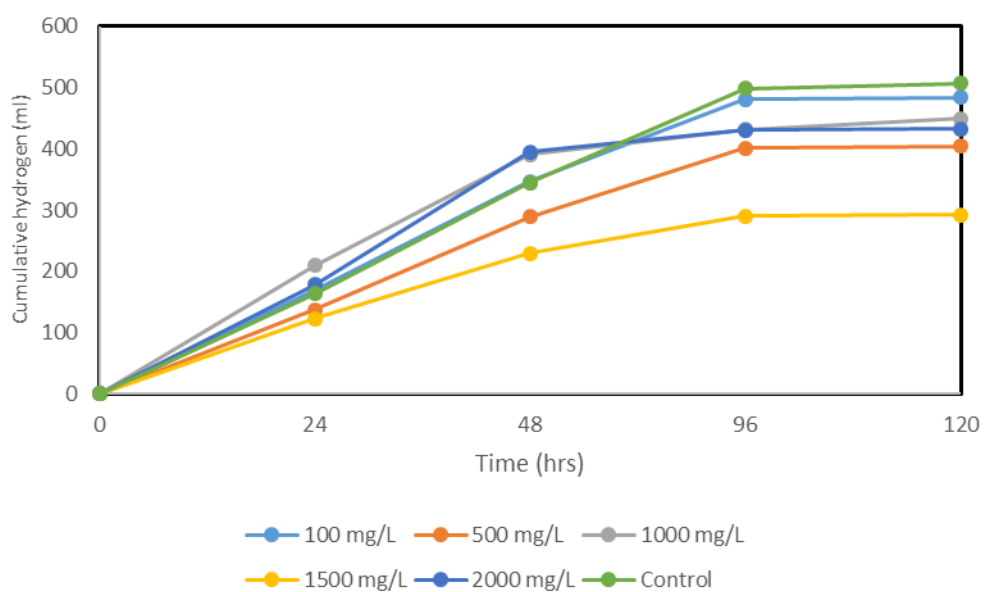


Figure 114 Variation of cumulative hydrogen volume with time at different Na concentrations.

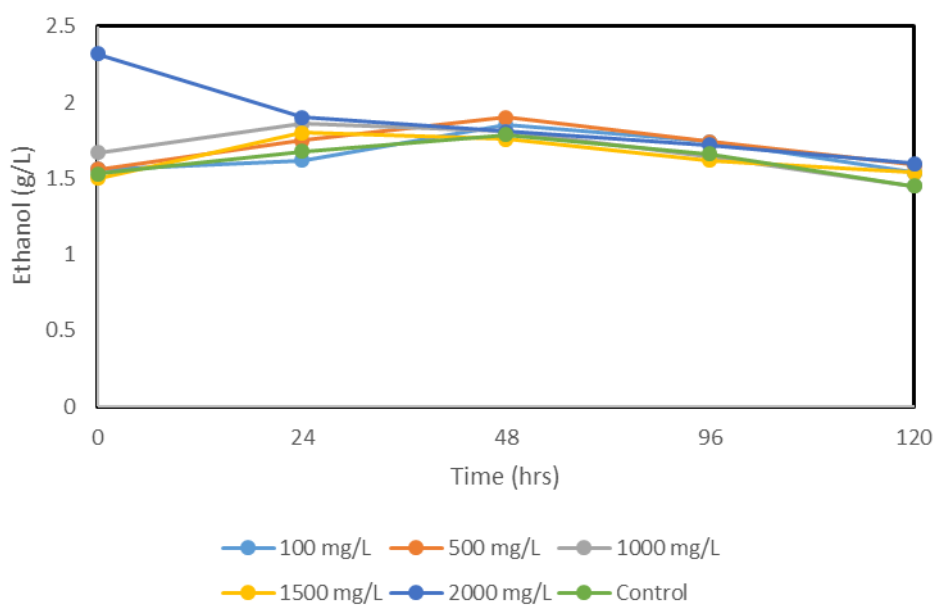


Figure 115 Variation of ethanol formation with time at different Na concentrations

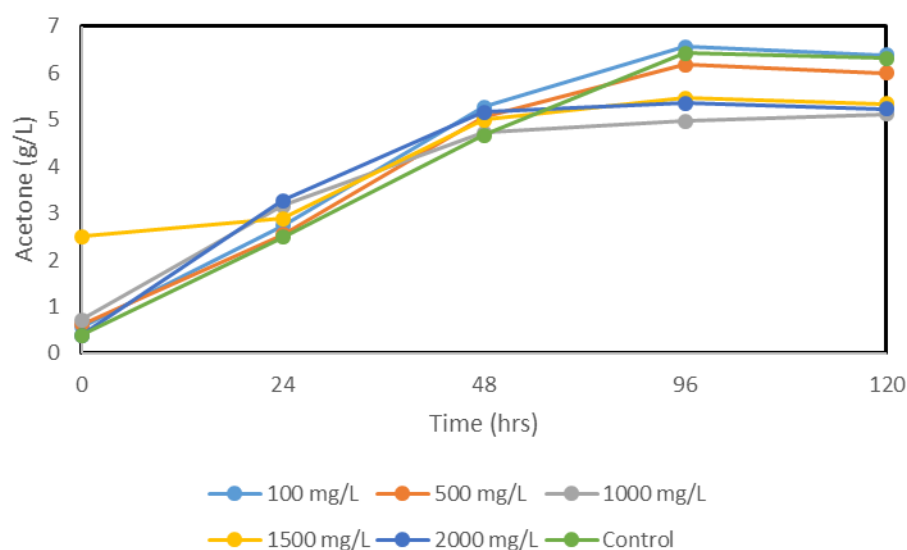


Figure 116 Variation of acetone formation with time at different Na concentrations

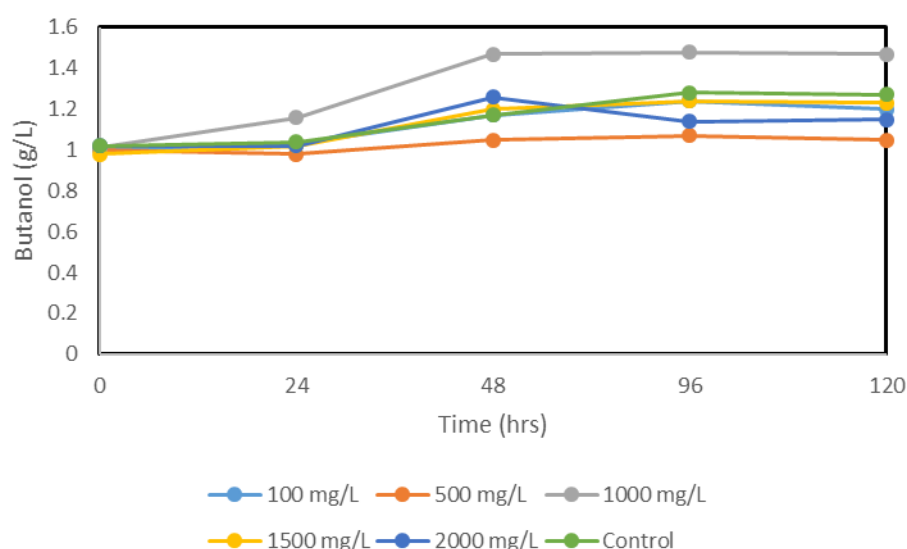


Figure 117 Variation of butanol formation with time at different Na concentrations

From the experiments conducted, Na concentration (≤ 100 mg/L) is enough for microbial activity in the ABE fermentation process. Na concentrations > 100 mg/L increase osmotic pressure which affects microbial activity. The majority of Clostridium strains involved in the ABE process are not halophiles (salt-loving microorganisms). Carbon to Nitrogen ratio (C/N) translates into nutrient balance availability for microbial utilization in the fermentation medium. It cannot be concluded that nitrogen is limiting in the experiments conducted. However, there is

the possibility of nitrogen limitation for the enzyme involved in the last reaction leading to the formation of butanol in the ABE fermentation process.

4.11 Effect of media composition on Butanol production

A careful experimental study of nutrients favouring butanol production is necessary. In order to achieve this, six (6) different experimental studies were conducted with different media compositions in terms of nitrogen concentration and source, yeast extract and L-cysteine concentration. Media composition is really important when targeting improved ABE fermentation results. Microorganisms need a source of protein, carbohydrates and other macro or micronutrients to perform excellently in ABE fermentation studies. It is worthy of note that all these requirements must be in an appropriate proportion for the microorganisms to prevent the inhibitory roles of these nutrients on ABE products. Furthermore, the addition of L-cysteine (an oxygen scavenger) and Resazurin (an indicator of anaerobiosis) need to be checked to prevent inhibitory roles. Meat extract and yeast extract are highly important for microorganisms to meet their protein requirements. However, authors like Diez-Antolinez, Hijosa-Valsero, Paniagua, & Gomez (2016) concluded that yeast extract is insignificant for butanol production and its concentration should be minimal in the fermentation media composition. Nitrogen sources and concentration are also important. Nitrogen is an essential element needed for protein and nucleic acid formation and microbial growth. This is highly important for the ABE fermentation process.

The results on the effect of media variation on ABE fermentation as seen in Figures 118 to 121. Fermentation media 2 gave the best results for ABE (acetone 7.44 g/L, butanol 5.5 g/L and ethanol 1.93 g/L). The media composition for Fermentation media 2 is as follows: CaCO_3 (5.35 g/L), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.019 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g/L), L-cysteine (0.1 g/L), Yeast extract (0.6 g/L) and $(\text{NH}_4)_2\text{SO}_4$ (2.0 g/L). The main difference between fermentation media 1 and 2 is the nitrogen source and yeast extract concentration. NH_4Cl (used in Media 1) and $(\text{NH}_4)_2\text{SO}_4$ (used in Media 2) both have the same nitrogen composition in the compound. This

shows that $(\text{NH}_4)_2\text{SO}_4$ is a better nitrogen source for improved butanol concentration when compared with NH_4Cl . Diez-Antolinez et al. (2016) are also of the opinion that NH_4Cl does not significantly improve butanol concentration. The nitrogen concentration present in hydrolysed fig is not enough for increased butanol concentration. Thus, the need for an external nitrogen source addition preferably $(\text{NH}_4)_2\text{SO}_4$.

Yeast extract concentration is lower in Fermentation Media 2 (0.6 g/L). This shows that yeast extract does not necessarily influence butanol production as claimed by Diez-Antolinez et al. (2016). The low butanol result in Fermentation Media 6 (1.23 g/L) is not surprising as high amounts of yeast extract (3 g/L) and meat extract (10 g/L) were present in the fermentation media. This is indeed inhibitory for butanol production. Fermentation media 3, 4 and 5 lack nitrogen sources and protein sources in their composition. This is responsible for the low butanol concentration achieved in them.

L- cysteine presence in a fermentation medium as an indicator for anaerobiosis is important at a low concentration. The high concentration of L-cysteine (0.4 g/L) employed in Fermentation Media 6 may also be attributed to low butanol production in the medium. In addition, Potassium was not considered in the media composition for all the six (6) experimental setups. This is because the Plackett Burman experiment revealed that potassium has an inhibitory effect on butanol production.

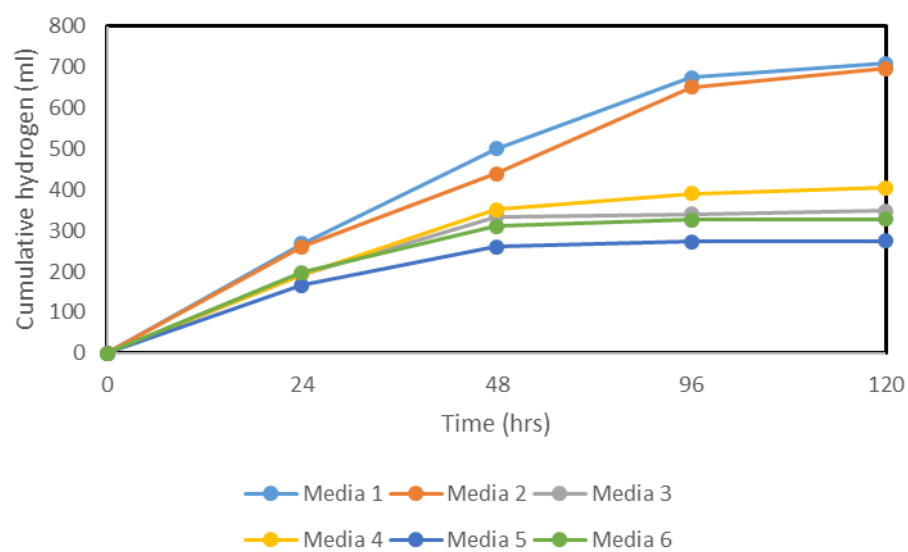


Figure 118 Effect of different fermentation media on Cumulative hydrogen production

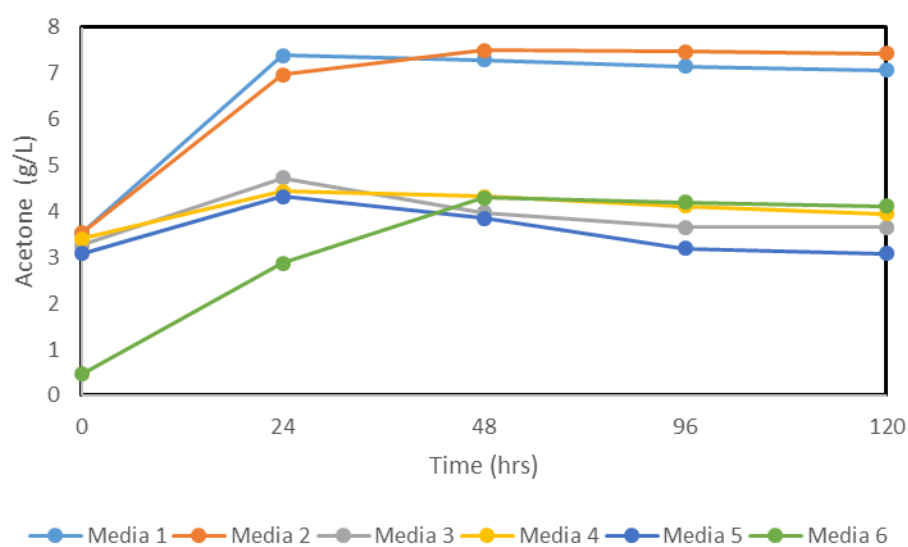


Figure 119 Effect of different fermentation media on acetone production

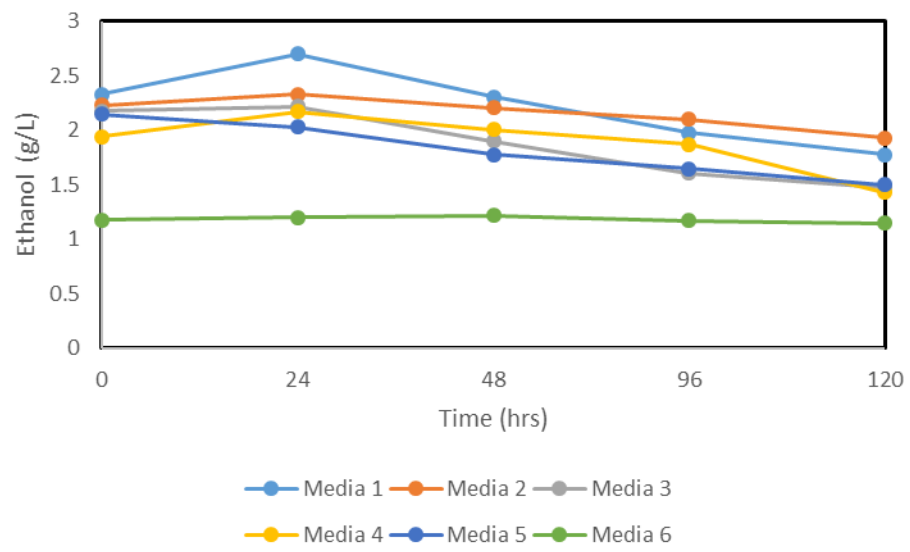


Figure 120 Effect of different fermentation media on ethanol production

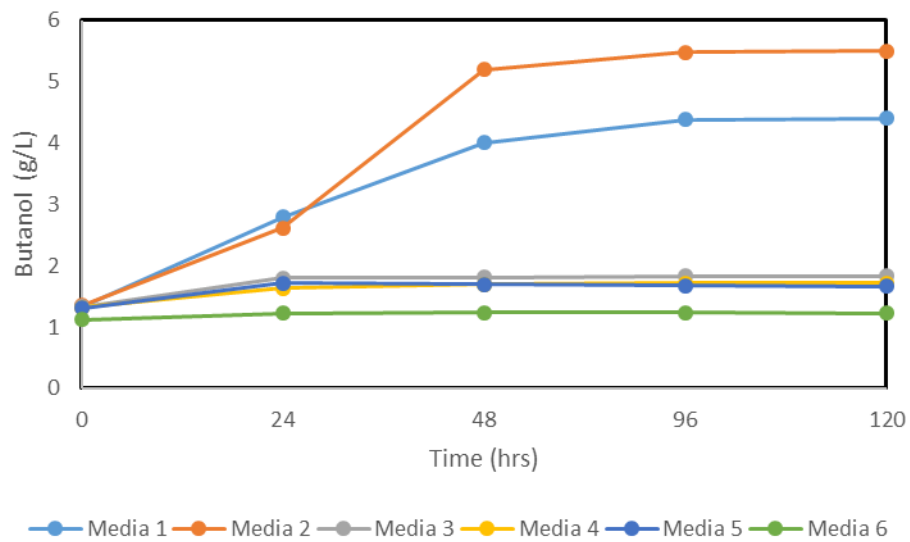


Figure 121 Effect of different fermentation media on butanol production

4.12 Effect of different Molybdenum and Sodium concentrations on ABE fermentation with Media 2

The positive factorial significance of Na and Mo on ABE products is found in Table 36. This indicated the important roles of Na, Zn and Mo on ABE solvents. However, numerous studies exist on the importance of Zn in ABE fermentation and the recommended concentration (i.e. $0.001 \text{ g L}^{-1} \text{ ZnSO}_4 \cdot 7\text{H}_2\text{O}$) was reported in numerous works. However, no studies had been conducted with varying concentrations of Na and Mo for ABE fermentation and Hydrogen production.

Figures (122 to 125) and (126 to 129) show ABE and hydrogen production results of varying concentrations of Mo (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) and Na (20, 40, 60, 80 and 100 mg/L) on ABE fermentation respectively. The highest concentrations of ethanol, acetone and butanol (g/L) for Mo were found in 0.5 mg/L concentration with 2.47 g/L ethanol, 10.91 g/L acetone and 6.04 g/L butanol while the highest cumulative hydrogen (646 ml) was found in 2.0 mg/L concentration. This further strengthens the importance of Molybdenum in the ABE fermentation process as suggested by the Plackett Burman design. Furthermore, a low concentration of Mo favours ABE products. Varying different concentrations of Na on ABE fermentation had 60 g/L concentration as the best with 2.08 g/L ethanol, 11.63 g/L acetone and 6.28 g/L butanol while the highest cumulative hydrogen (681.8 ml) was found in 40 mg/L concentration. Increasing Na concentrations up to 60 mg/L increases ABE results. Above this concentration, production starts diminishing.

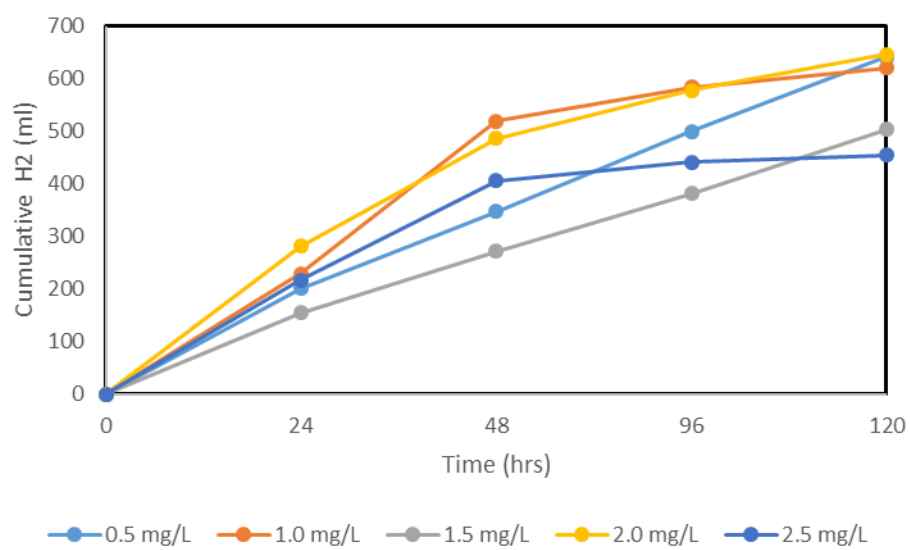


Figure 122 Effect of varying Mo concentration on cumulative hydrogen volume

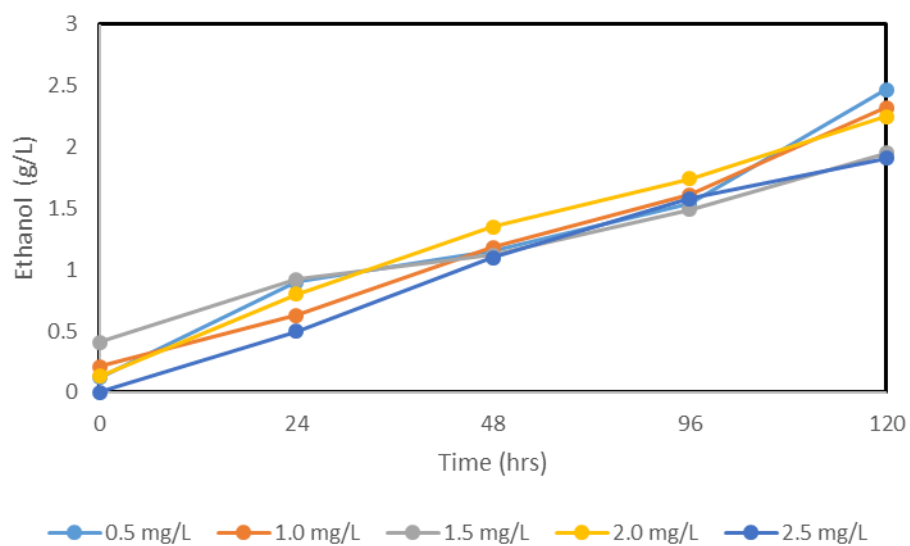


Figure 123 Effect of varying Mo concentration on ethanol production

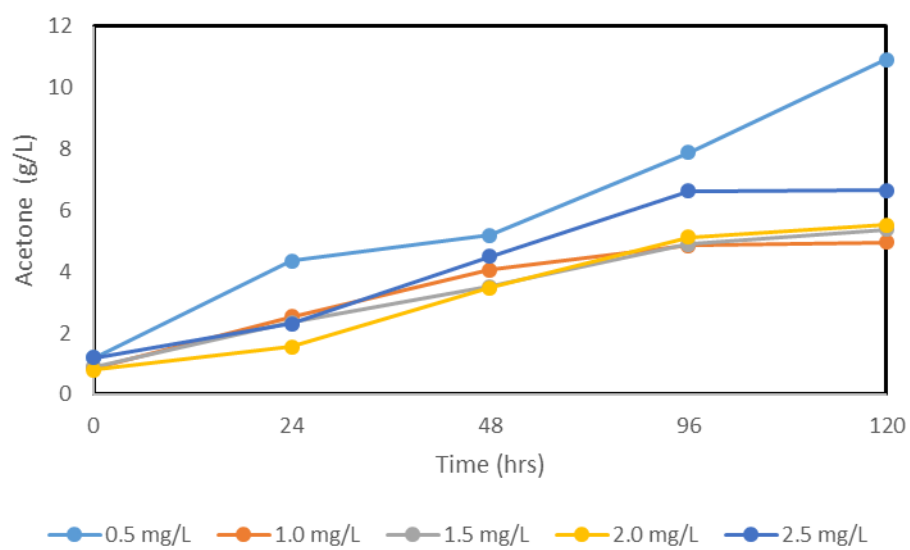


Figure 124 Effect of varying Mo concentration on acetone production

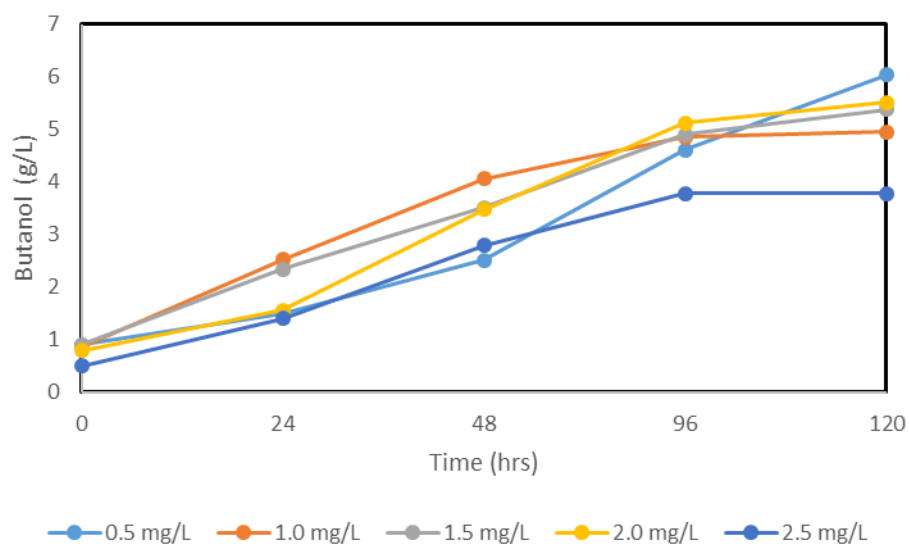


Figure 125 Effect of varying Mo concentration on butanol production

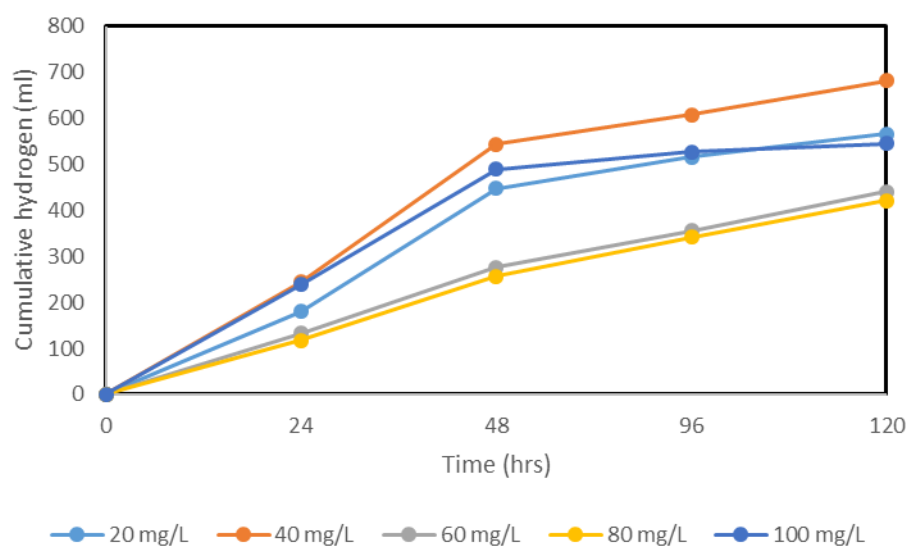


Figure 126 Effect of varying Na concentration on cumulative hydrogen volume

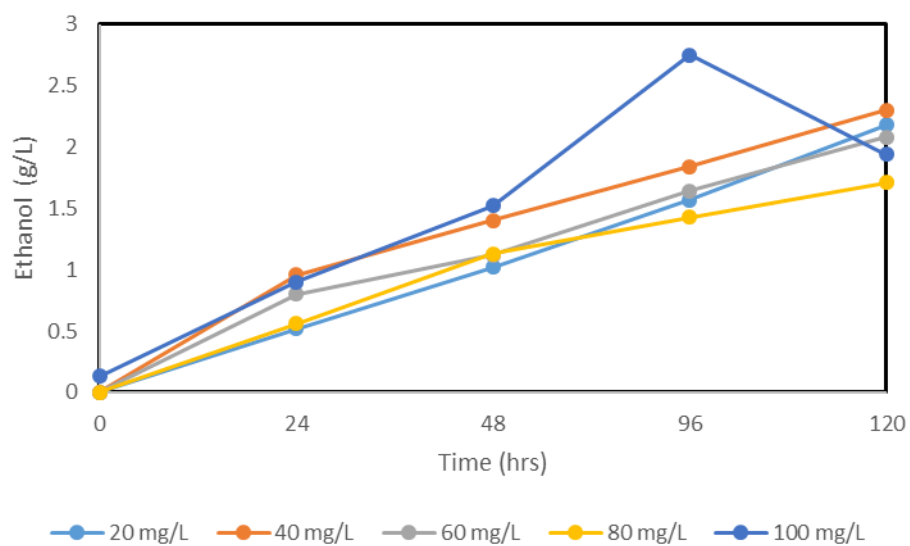


Figure 127 Effect of varying Na concentration on ethanol production

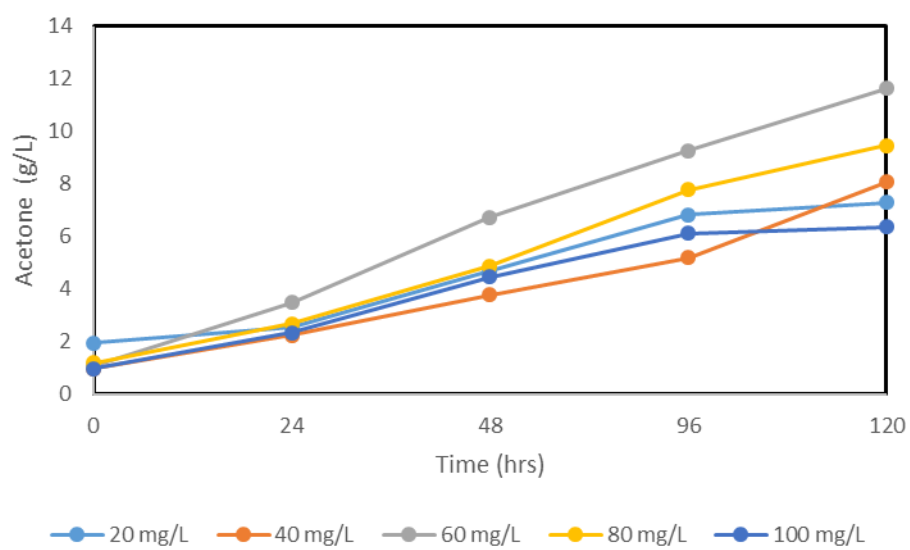


Figure 128 Effect of varying Na concentration on acetone production

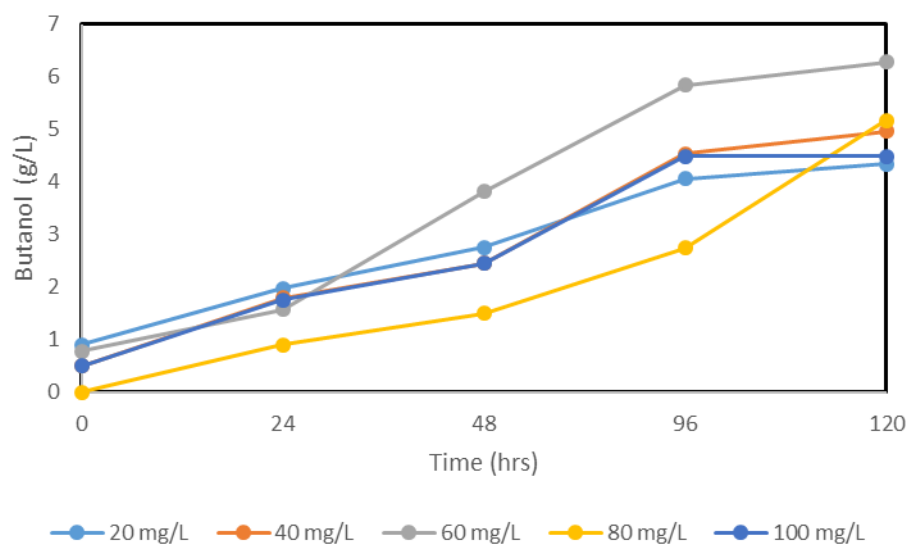


Figure 129 Effect of varying Na concentration on butanol production

4.13 Conclusion

The need for increased research into renewable energy keeps receiving global attention yearly with support from both governmental and private bodies. However, the global unawareness of renewable energy still outweighs research and development from the citadel of learning and research institutes. Undoubtedly, the greatest achievement of mankind in the 21st century will be a shift from total dependence on fossil energy to a renewable alternative. The global warming effect and the continuous depletion of fossil fuel reserves are adverse effects associated with fossil energy necessitating urgent replacement with a renewable, sustainable and environmentally friendly alternative. Waste management remains a challenge to both developing and developed countries despite the huge monetary expenditure channelled to tackle its effect. It is worth noting that a large sum of organic waste can be converted into biofuels. Food crops, lignocellulosic wastes and algal biomass have been extensively documented in biofuel research. Fig (*Ficus carica*) is a Mediterranean lignocellulosic fruit widely grown in Türkiye. The high sugar content in fig makes it an excellent biomass candidate for valuable energy products like butanol and hydrogen.

The main components of lignocellulose are lignin, cellulose, and hemicellulose. The cellulolytic part of lignocellulose is critical as a biofuel fermentation substrate. As a result, pretreatment is widely employed to remove lignin and hemicellulose from lignocellulose. Pretreatment methods may be physical (mechanical and thermal such as ball milling and autoclaving respectively), chemical (acid or alkali addition, ionic liquid), and biological (with enzymes or microorganisms).

In this study, microwave and steam-assisted autoclave hydrolysis were found to be efficient means of fig pretreatment achieving a high concentration of fermentable sugar of 81g/L and 70g/L, respectively. Particle diameter, pH, and microwave power are microwave pretreatment parameters that significantly affected fig hydrolysis. However, the temperature is the only significant parameter affecting fig hydrolysis in steam-assisted autoclave pretreatment. The optimization of MW and AC hydrolysis

was performed using RSM to evaluate the effects of process parameters on fig hydrolysis. The optimum total sugar concentration of 82.91 g/L and 70.022 g/L were obtained from MW and AC hydrolysis respectively with optimized hydrolysis conditions of MW as $D_p = 370.72 \mu\text{m}$, pH 4.96 and $P = 253.67 \text{ W}$ and AC $D_p = 263.19 \mu\text{m}$, pH 3.02, $T = 120^\circ\text{C}$ and $t = 30.00 \text{ mins}$. This study demonstrated the effectiveness of MW and AC as excellent pretreatment options to generate a high concentration of fermentable sugar. $\text{TSC}_{(\text{g/L})}$ recorded for MW and AC in this study were the highest recorded so far for MW and AC pretreatments from recent literature. From this study, MW yielded more fermentable sugar than AC, however, in terms of model fitness, AC optimization is a better model for fig hydrolysis than MW due to the low difference between predicted and experimented SRD results.

Generally, concentrations of ABE products for Mn (5.6 mg/L) and Mn (56 mg/L) were almost the same in this research indicating that Mn has little effect on ABE fermentation. Mg (400 mg/L) is better preferred in terms of ABE products than Mg (1800 mg/L). Mg (1800 mg/L) produced less butyric acid and high butanol when compared with Mg (400 mg/L). Mg (400 mg/L) on the other hand increases butyric acid production. However, its metabolism into butanol is inhibited giving rise to low butanol. Similarly, Zn (4.0 mg/L) is better preferred in terms of ABE products than Zn (40 mg/L). Previous researchers have reported the role of Zn as an accelerator for butanol production. This was evident in this study as Zn (4.0 mg/L) was the only metal that achieved the highest butanol production. Thus, Zn (4.0 mg/L) is preferred for solvents production. For increased acetone production, Metal ions (Mn, Mg and Zn) are highly recommended as fermentation constituents. However, butanol production is highly favoured by electron acceptors.

Mn (56 mg/L) and Mg (1800 mg/L) are good for acetone production. However, these concentrations are too high to support butanol production. These high values may have an inhibitory effect on ABE solvents. Thus, lower concentrations of Mn are needed to achieve better results for solvents. On the other hand, MV (15 mg/L) is the only concentration of electron acceptor tested that sustained ABE giving good

results for acetone, butanol and ethanol. Thus, MV (15 mg/L) concentration may be increased to have better ABE results.

Waste figs (*Ficus carica*) are widely disposed of via incineration in Türkiye generating environmental concerns. However, figs can be converted into valuable products such as hydrogen and butanol via ABE fermentation. Thus, this study focused on the role of metals (Co^{2+} , Cu^{2+} , Mn^{+} , Fe^{2+} , Na^{+} , Mg^{2+} , Zn^{2+} , Ca^{2+} , K^{+} , Mo^{3+} , B^{3+}) on ABE fermentation, organic acids and hydrogen production with fig as biomass using Plackett-Burman statistical design. From the study, (Mo^{3+} and Na^{+}), (Mo^{3+} and Fe^{2+}), (Fe^{2+} , Zn^{2+} , Ca^{2+} , K^{+} and Mo^{3+}) were found to be the significant elements favouring ABE fermentation, organic acids and hydrogen production, respectively. Thus, Mo^{3+} stands out as a promising candidate in this research. Further optimization studies were done within the ranges of the significant metals for product maximization. ABE (6.26, 5.01 and 1.58 g/L, respectively), acetic and butyric acids (6.898 and 3.872 g/L, respectively) and hydrogen production rate (51.5 ml H_2 /L/ h) were obtained. The results obtained from this research showed that fig is an excellent substrate for ABE fermentation and hydrogen production. A comparison of Plackett Burman's experimental design from this research and recent literature on ABE fermentation and hydrogen production is found in the appendix section.

It is worthy to mention that an integral aim of this research is to find a mechanism that favours the butanol pathway leading to increased butanol concentration over acetone and butanol. Thus, metals and electron acceptors' roles were independently studied. In Table 36, the interplay of all metals tested in experimental run ten (10) achieved a higher concentration of butanol (3.72 g/L) compared with 1.59 and 2.77 g/L in ethanol and acetone respectively. Similarly, experimental run four (4) had butanol (3.93 g/L) as the highest ABE solvent compared to 1.51 and 3.82 g/L in ethanol and acetone respectively. In addition, the addition of an electron acceptor of 15mM of Methyl viologen indicated the potential of this electron acceptor as highly important in diverting the ABE fermentation pathway to favour butanol production. This yielded ethanol, acetone and butanol concentrations of 3.59, 2.23 and 6.08 g/L respectively. A:B:E (3:6:1) ratio is used as an indicator for the ideal proportion of the

solvents in ABE fermentation. It is a theoretical ratio and not a strict rule. It may vary depending on the fermentation conditions. The ratio in this thesis was calculated for the varying experimental conditions and the results were given in Appendix Table H (1-4). Under the optimized metal concentrations in Table 36 (Run 10), the ratio was 3.4 : 4.6 : 2.0 meaning that acetone and ethanol production were inhibited while butanol was accelerated. Thus, the butanol content of the solvent mixture was increased. Similarly, the addition of 15mM of Methyl viologen into the fermentation media gave ABE ratio of 1.9 : 5.1 : 3.0. This is an important success in the quest to find biochemical manipulations to favour butanol production.

Media composition is really important to achieve increased ABE results. Fermentation media consisting of CaCO_3 (5.35 g/L), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.019 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g/L), L-cysteine (0.1 g/L), Yeast extract (0.6 g/L) and $(\text{NH}_4)_2\text{SO}_4$ (2.0 g/L) has been recommended. Experiments with this fermentation media composition yielded ABE (7.44, 5.50 and 1.92 g/L, respectively).

4.14 Recommendation

The main finding of this thesis is encouraged to be experimented with other Clostridial strains and lignocellulosic biomasses for possibility of increased ABE and hydrogen production. More research is needed to find novel, simple and applicable inhibitors or accelerators for the biochemical pathways. The development of a fermentation process for channeling organic acids generated in biohydrogen production to butanol formation is also necessary. It is also worthy to note that very few researches exist on the application of nanotechnology in ABE fermentation studies. Thus, I encourage future researchers to channel their researches into this field.

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APPENDICES

APPENDIX A: Raw data for batch experiments for OFAT effect of metals and electron mediators on ABE

Table A1 Raw data for effect of Mn (5.6 mg/L) on ABE with *C. pasteurianum* DSM 525 as inoculum

Element	Time (hrs)	Total sugar (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol (g/L)	Acetone (g/L)	Butanol (g/L)
Mn (5.6 mg/L)	0	49.84	0	2.74	0	10.6	7.54	0	1.22	1.254
	24	45.12	0	4.51	3.15	9.2	5.79	0	4.077	1.374
	48	34.96	0	6.18	4.1	7.61	3.99	0	5.842	1.856
	72	25.68	0	7.45	5.09	5.28	2.12	1.814	7.551	2.25
	120	22.4	5.65	6.75	6.82	1.36	1.27	1.728	10.727	2.437
	144	22.24	10	6.72	6.92	0.48	0.027	1.779	10.624	2.346
	168	18.72	10	6.7	6.87	0.48	0.027	1.77	10.641	2.3

Table A2 Raw data for effect of Mn (56 mg/L) on ABE with *C. pasteurianum* DSM 525 as inoculum

Element	Time (hrs)	Total sugar (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol (g/L)	Acetone (g/L)	Butanol (g/L)
Mn (56 mg/L)	0	51.84	0	2.88	0	10	7.46	0	1.18	1.23
	24	43.28	0	4.54	3.02	9.09	5.79	0	3.976	1.41
	48	40.16	0	6.25	3.93	7.7	4.18	0	5.702	1.911
	72	34.56	0	7.42	4.64	6.35	2.99	1.752	6.71	2.335
	120	27.36	2.18	6.69	6.62	2.87	1.72	1.673	10.009	2.538
	144	21.6	4.78	6.7	6.64	1.48	1.44	1.679	10.509	2.492
	168	20.8	10.34	6.79	6.6	0.5	0	1.786	10.672	2.402

Table A3 Raw data for effect of Mg (400 mg/L) on ABE with *C. pasteurianum* DSM 525 as inoculum

Element	Time (hrs)	Total sugar (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol (g/L)	Acetone (g/L)	Butanol (g/L)
Mg (400 mg/L)	0	49.76	0	2.67	0	17.58	11.09	1.121	0	1.011
	24	44.96	0	4.91	2.61	15.62	9.99	1.153	1.149	1.085
	48	33.28	0	6.71	4.23	15.34	7.38	1.172	2.929	1.256
	72	32.08	0	9.17	5.34	8.5	3.4	1.211	4.813	1.483
	120	13.52	0	12.46	7.84	8.5	3.4	1.203	7.786	1.602
	144	6.56	0	15.09	9.03	0.56	0.44	1.232	9.478	1.58
	168	3.92	2.06	10.99	8.36	0.05	0.098	1.602	10.306	1.566

Table A4 Raw data for effect of Mg (1800 mg/L) on ABE with *C. pasteurianum* DSM 525 as inoculum

Element	Time (hrs)	Total sugar (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol (g/L)	Acetone (g/L)	Butanol (g/L)
Mg (1800 mg/L)	0	51.44	0	2.67	0	17.39	11.03	1.124	0	1.011
	24	43.44	0	3.66	0	15.35	9.67	1.176	0.329	1.025
	48	41.2	0	5.68	3.45	13.44	6.6	1.217	2.413	1.052
	72	30.8	0.7	7.31	5.33	8.59	2.23	1.163	4.889	1.373
	120	12.72	4.89	7.16	6.39	3.75	0.1	1.177	6.398	1.815
	144	6.8	8.33	7.15	7.12	0.65	0.14	1.199	7.207	1.815
	168	4.64	7.95	5.66	7.04	0.078	0.037	1.16	8.828	1.749

Table A5 Raw data for effect of Zn (4.0 mg/L) on ABE with *C. pasteurianum* DSM 525 as inoculum

Element	Time (hrs)	Total sugar (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol (g/L)	Acetone (g/L)	Butanol (g/L)
Zn (4.0 mg/L)	0	55.76	0	2.74	0	10.58	7.44	0	1.16	1.25
	24	45.44	0	4.42	2.9	9.16	5.54	0	3.885	1.439
	48	45.12	0	5.66	3.77	7.86	4.4	0	5.394	1.821
	72	28.56	5.79	5.66	4.24	5.06	2.72	1.28	6.166	2.275
	120	22.64	11.31	6.61	4.78	0.7	0.16	1.281	6.945	4.519
	144	19.44	11.84	6.66	4.8	0	0	1.621	7.269	4.413
	168	18.4	10.51	6.68	5.32	0	0	3.269	8.978	4.344

Table A6 Raw data for the effect of Zn (40 mg/L) on ABE with *C. pasteurianum* DSM 525 as inoculum

Element	Time (hrs)	Total sugar (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol (g/L)	Acetone (g/L)	Butanol (g/L)
Zn (40 mg/L)	0	54.08	0	2.42	0	17.43	11.89	1.106	0	1.009
	24	44.96	0	4.84	2.65	14.26	7.47	1.153	1.45	1.086
	48	35.04	0	5.4	4.01	11.32	4.39	1.151	3.345	1.254
	72	29.84	0.54	5.5	5.32	7.42	1.36	1.156	4.87	1.613
	120	18.56	8.6	5.65	5.77	2.65	0	1.16	5.797	1.948
	144	6.16	9.5	6.07	5.54	0	0	1.171	5.902	1.981
	168	3.04	10.72	6.07	5.39	0	0	1.17	7.203	1.981

Table A7 Raw data for the effect of NR (1.5 mg/L) on ABE with *C. pasteurianum* DSM 525 as inoculum

Element	Time (hrs)	Total sugar (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol (g/L)	Acetone (g/L)	Butanol (g/L)
NR (1.5 mM)	0	52.72	0	2.37	0	17.63	11.44	1.103	0	1.008
	24	44.32	0	2.4	0	16.79	10.77	1.125	0	1.011
	48	34.56	0	2.57	0	16.27	10.43	1.128	0	1.021
	72	25.76	0	2.91	0	10.51	9.51	1.769	0	1.033
	120	14.24	11.18	2.5	0	1.18	5.64	3.089	0.321	1.151
	144	5.0	12.33	2.47	2.4	0	0.6	3.197	2.001	1.519
	168	3.6	13.81	2	2	0	0.039	3.895	2.79	1.586

Table A8 Raw data for the effect of NR (15 mg/L) on ABE with *C. pasteurianum* DSM 525 as inoculum

Element	Time (hrs)	Total sugar (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol (g/L)	Acetone (g/L)	Butanol (g/L)
NR (15 mM)	0	53.2	0	0	0	17.24	11.17	0	0	1.005
	24	43.6	0	2.48	0	16.52	10.73	0	0	1.013
	48	37.84	0	2.62	0	16.01	10.26	0	0	1.039
	72	31.68	0	2.5	0	12.22	9.15	1.711	0	1.037
	120	22.08	4.51	2.49	0	1.64	6.56	3.53	0	1.031
	144	8.64	4.57	2.97	0	0.47	6.08	3.851	0	1.03
	168	7.44	9.8	3.5	0	0	2.14	3.955	0	1.028

Table A9 Raw data for the effect of MV (1.5 mg/L) on ABE with *C. pasteurianum* DSM 525 as inoculum

Element	Time (hrs)	Total sugar (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol (g/L)	Acetone (g/L)	Butanol (g/L)
MV (1.5 mM)	0	54.16	0	2.35	0	17.43	11.89	0	0	0.989
	24	43.44	0	2.37	0	14.11	8.42	1.194	0.191	1.877
	48	40.24	0	2.37	0	10.73	5.93	1.427	0.561	2.912
	72	24.08	3.12	2.4	0	6.55	3.77	2.2	1.291	3.457
	120	16.08	8.91	2.43	0	3.55	2.97	2.234	1.449	3.515
	144	13.44	10.83	2.42	0	1.94	2.52	2.62	2.433	3.489
	168	8.16	10.89	2.4	3.05	0.68	2.09	2.749	3.499	3.4

Table A10 Raw data for the effect of MV (15 mg/L) on ABE with *C. pasteurianum* DSM 525 as inoculum

Element	Time (hrs)	Total sugar (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol (g/L)	Acetone (g/L)	Butanol (g/L)
MV (15 mM)	0	52.64	0	2.62	0	10.36	7.49	0	1.14	1.24
	24	43.92	0	2.7	0	8.23	5.09	0	1.692	3.42
	48	30.88	3.97	2.8	0	6.84	1.49	2.275	1.736	6.392
	72	29.92	8.37	2.92	0	5.57	0	2.5	1.747	6.793
	120	29.36	19.76	2.99	0	2.71	0	2.568	1.75	6.593
	144	20.16	20.66	3.02	0	0.72	0	2.755	1.9	6.229
	168	19.6	21.78	3.06	0	0.51	0	3.593	2.232	6.078

APPENDIX-B: Raw data for hydrogen production for Plackett Burman design during ABE fermentation with *C. pasteurianum* DSM 525 as inoculum

Table B1 Experimental Run Order 1

Run	Time (hr)	V _s (ml)	V _g (ml)	H ₂	head space(ml)	V _{total gas} (ml)	Produced H ₂ (mL)	V _{cum} H ₂	Cumulative H ₂ (mmol)	HPR (ml/ hr)	HPR (ml/l hr)
1	0	200	110	0	0	0	0	0	0		
	24	192	118	0.09	10.62	80	7.2	17.82	0.700584677		
	48	184	126	0.312	0	70	21.84	39.66	1.55921371		
	96	176	134	0.618	0	430	265.74	305.4	12.00665323		
	120	168	142	0.377	0	90	33.93	339.33	13.34059476	3.255714	16.27857

Table B2 Experimental Run order 2

Run	Time (hr)	V _s (ml)	V _g (ml)	H ₂	head space(ml)	V _{total gas} (ml)	Produced H ₂ (mL)	V _{cum} H ₂	Cumulative H ₂ (mmol)	HPR (ml/ hr)	HPR (ml/l hr)
2	0	200	110	0	0	0	0	0	0		
	24	192	118	0.204	24.072	30	6.12	30.192	1.186983871		
	48	184	126	0.532	0	230	122.36	152.552	5.997508065		
	96	176	134	0.512	0	230	117.76	270.312	10.62718548		
	120	168	142	0.5	0	170	85	355.312	13.96891935	3.178167	15.89083

Table B3 Experimental Run Order 3

Run	Time (hr)	V _s (ml)	V _g (ml)	H ₂	head space(ml)	V _{total gas} (ml)	Produced H ₂ (mL)	V _{cum} H ₂	Cumulative H ₂ (mmol)	HPR (ml/ hr)	HPR (ml/l hr)
3	0	200	110	0	0	0	0	0	0		
	24	192	118	0.435	51.33	280	121.8	173.13	6.806522177		
	48	184	126	0.5	0	310	155	328.13	12.90027218		
	96	176	134	0.34	0	360	122.4	450.53	17.71236895		
	120	168	142	0.37	0	150	55.5	506.03	19.8943246	6.836042	34.18021

Table B4 Experimental Run order 4

Run	Time (hr)	V _s (ml)	V _g (ml)	H ₂	head space(ml)	V _{total gas} (ml)	Produced H ₂ (mL)	V _{cum} H ₂	Cumulative H ₂ (mmol)	HPR (ml/ hr)	HPR (ml/l hr)
4	0	200	110	0	0	0	0	0	0		
	24	192	118	0.12	14.16	90	10.8	24.96	0.981290323		
	48	184	126	0.49	0	440	215.6	240.56	9.4575		
	96	176	134	0.47	0	430	202.1	442.66	17.40296371		
	120	168	142	0.21	0	40	8.4	451.06	17.73320565	5.011667	25.05833

Table B5 Experimental Run order 5

Run	Time (hr)	V _s (ml)	V _g (ml)	H ₂	head space(ml)	V _{total gas} (ml)	Produced H ₂ (mL)	V _{cum} H ₂	Cumulative H ₂ (mmol)	HPR (ml/ hr)	HPR (ml/l hr)
5	0	200	110	0	0	0	0	0	0		
	24	192	118	0.49	57.82	470	230.3	288.12	11.32729839		
	48	184	126	0.57	0	410	233.7	521.82	20.51510081		
	96	176	134	0.55	0	310	170.5	692.32	27.21822581		
	120	168	142	0.48	0	230	110.4	802.72	31.55854839	10.87125	54.35625

Table B6 Experimental Run Order 6

Run	Time (hr)	V _s (ml)	V _g (ml)	H ₂	head space(ml)	V _{total gas} (ml)	Produced H ₂ (mL)	V _{cum} H ₂	Cumulative H ₂ (mmol)	HPR (ml/ hr)	HPR (ml/l hr)
6	0	200	110	0	0	0	0	0	0		
	24	192	118	0.17	20.06	90	15.3	35.36	1.39016129		
	48	184	126	0.52	0	360	187.2	222.56	8.74983871		
	96	176	134	0.56	0	570	319.2	541.76	21.29903226		
	120	168	142	0.46	0	270	124.2	665.96	26.18189516	4.636667	23.18333

Table B7 Experimental Run order 7

Run	Time (hr)	V _s (ml)	V _g (ml)	H ₂	head space(ml)	V _{total gas} (ml)	Produced H ₂ (mL)	V _{cum} H ₂	Cumulative H ₂ (mmol)	HPR (ml/ hr)	HPR (ml/l hr)
7	0	200	110	0	0	0	0	0	0		
	24	192	118	0.45	53.1	350	157.5	210.6	8.279637097		
	48	184	126	0.51	0	460	234.6	445.2	17.50282258		
	96	176	134	0.48	0	370	177.6	622.8	24.48508065		
	120	168	142	0.31	0	40	12.4	635.2	24.97258065	9.275	46.375

Table B8 Experimental Run order 8

Run	Time (hr)	V _s (ml)	V _g (ml)	H ₂	head space(ml)	V _{total gas} (ml)	Produced H ₂ (mL)	V _{cum} H ₂	Cumulative H ₂ (mmol)	HPR (ml/ hr)	HPR (ml/l hr)
8	0	200	110	0	0	0	0	0	0		
	24	192	118	0.375	44.25	160	60	104.25	4.098538306		
	48	184	126	0.489	0	440	215.16	319.41	12.5574496		
	96	176	134	0.472	0	260	122.72	442.13	17.38212702		
	120	168	142	0.33	0	100	33	475.13	18.67950605	6.654375	33.27188

Table B9 Experimental Run order 9

Run	Time (hr)	V _s (ml)	V _g (ml)	H ₂	head space(ml)	V _{total gas} (ml)	Produced H ₂ (mL)	V _{cum} H ₂	Cumulative H ₂ (mmol)	HPR (ml/ hr)	HPR (ml/l hr)
9	0	200	110	0	0	0	0	0	0		
	24	192	118	0.5	59	530	265	324	12.73790323		
	48	184	126	0.55	0	470	258.5	582.5	22.90070565		
	96	176	134	0.51	0	410	209.1	791.6	31.12137097		
	120	168	142	0.29	0	110	31.9	823.5	32.37550403	12.13542	60.67708

Table B10 Experimental Run order 10

Run	Time (hr)	V _s (ml)	V _g (ml)	H ₂	head space(ml)	V _{total gas} (ml)	Produced H ₂ (mL)	V _{cum} H ₂	Cumulative H ₂ (mmol)	HPR (ml/ hr)	HPR (ml/l hr)
10	0	200	110	0	0	0	0	0	0		
	24	192	118	0.113	13.334	30	3.39	16.724	0.657495968		
	48	184	126	0.472	0	440	207.68	224.404	8.822334677		
	96	176	134	0.455	0	470	213.85	438.254	17.22974395		
	120	168	142	0.14	0	70	9.8	448.054	17.61502621	0.696833	3.484167

Table B11 Experimental Run order 11

Run	Time (hr)	V _s (ml)	V _g (ml)	H ₂	head space(ml)	V _{total gas} (ml)	Produced H ₂ (mL)	V _{cum} H ₂	Cumulative H ₂ (mmol)	HPR (ml/ hr)	HPR (ml/l hr)
11	0	200	110	0	0	0	0	0	0		
	24	192	118	0.44	51.92	310	136.4	188.32	7.403709677		
	48	184	126	0.53	0	560	296.8	485.12	19.07225806		
	96	176	134	0.45	0	370	166.5	651.62	25.618125		
	120	168	142	0.38	0	60	22.8	674.42	26.51449597	10.10667	50.53333

Table B12 Experimental Run order 12

Run	Time (hr)	V _s (ml)	V _g (ml)	H ₂	head space(ml)	V _{total gas} (ml)	Produced H ₂ (mL)	V _{cum} H ₂	Cumulative H ₂ (mmol)	HPR (ml/ hr)	HPR (ml/l hr)
12	0	200	110	0	0	0	0	0	0		
	24	192	118	0.042	4.956	50	2.1	7.056	0.277403226		
	48	184	126	0.037	0	10	0.37	7.426	0.291949597		
	96	176	134	0.47	0	350	164.5	171.926	6.7591875		
	120	168	142	0.52	0	360	187.2	359.126	14.11886492	1.825705	9.128524

APPENDIX-C: Plackett Burman experimental design from recent literature on ABE fermentation

Substrate	Microorganism	Factors with ranges	Response	Optimized conditions	References
Cheese whey	<i>Clostridium beijerinckii</i> CECT 508	Yeast extract:1-5 g/L FeSO ₄ ·7H ₂ O:0-0.01 g/L MgSO ₄ ·7H ₂ O:0-0.2 g/L Cysteine: 0-0.5 g/L NH ₄ Cl: 0-2.1 g/L K ₂ HPO ₄ : 0-1 g/L KH ₂ PO ₄ : 0-1 g/L CaCO ₃ : 1-8 g/L Initial lactose in whey: 38-54 g/L Added lactose: 0-15 g/L Temperature: 30-37(°C)	Butanol concentration	FeSO ₄ ·7H ₂ O: 0.019 g/L CaCO ₃ : 5.35 g/L Initial lactose: 56.7 g/L Butanol: 10.09 g/L	Diez-Antolinez et al. (2016)
Sweet Sorghum Stem Juice	<i>Clostridium beijerinckii</i> TISTR 1461	FeSO ₄ ·7H ₂ O:0.01 – 0.04 g L ⁻¹ MgSO ₄ ·7H ₂ O: 0.5 - 1 g/L pH: 6.0 - 6.5 (NH ₄) ₂ SO ₄ : 1 – 4 g/L Butyric acid: 5 – 10 g/L KH ₂ PO ₄ : 0.5 – 1 g/L CaCO ₃ : 1-4 g/L Ammonium acetate: 2.2 – 4.4 g/L Inoculum size: 5 – 10 (%v/v)	Butanol concentration (g/L)	(NH ₄) ₂ SO ₄ : 1.27 g/L Butyric acid: 5.18 g/L CaCO ₃ : 7.14 g/L Butanol: 16.91 g/L	Sirisantim ethakom, Thanapornsin, Laopaiboon, & Laopaiboon, (2018)
Glucose	<i>Clostridium</i> YM1	Glucose: 20 - 50 g/L Tryptone: 3 - 9 g/L Yeast extract: 1 - 4 g/L Peptone: 2 – 7 g/L Ammonium acetate: 1 - 5 g/L K ₂ HPO ₄ :g/L) 0.1 – 1.0 g/L KH ₂ PO ₄ : g/L) 0.1 – 1.0 g/L MgSO ₄ : 0.1 – 1.0 g/L	Butanol concentration (g/L)	Glucose:50 g/L Tryptone: 3.01 g/L Yeast extract: 1.09 g/L Peptone: 6.62 g/L K ₂ HPO ₄ : 0.99 g/L MgSO ₄ : 0.86 g/L Na ₂ CO ₃ : 1.86 g/L NaCl: 0.1 g/L Butanol:	Al-Shorgani, Hamid, Yusoff, & Kalil (2013)

		FeSO ₄ : 0.001-0.1 g/L Na ₂ CO ₃ : 1 - 5 g/L NaCl: 0.1 - 1 g/L		10.93 g/L	
Fig (<i>Ficus carica</i>)	<i>Clostridium pasteurianum</i> DSM 525	Fe ²⁺ : 1.4 - 10 mg/L Co ²⁺ : 0.0050-0.9 mg/L Cu ²⁺ : 0.1 - 5.0 mg/L Mn ⁺ : 0.3 - 300 mg/L Mo ³⁺ : 0.003 - 0.5 mg/L Na ⁺ : 11 - 16 mg/L Mg ²⁺ : 41 - 400 mg/L K ⁺ : 577 - 1000 mg/L Zn ²⁺ : 0.4 - 50 mg/L B ³⁺ : 0.8 - 16 mg/L Ca ²⁺ : 40 - 100 mg/L	Butanol concentration	Mo ³⁺ : 0.472 mgL ⁻¹ Na ⁺ : 16 mg/L Zn ²⁺ : 49.6 mg/L Butanol: 5 g/L	This study

APPENDIX-D: Box Behnken ANOVA Quadratic model

Table D1 Box Behnken Ethanol ANOVA Quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	0.3088	9	0.0343	14.34	0.0046
A-Mo	0.0360	1	0.0360	15.07	0.0116
B-Na	0.0324	1	0.0324	13.54	0.0143
C-Zn	0.0059	1	0.0059	2.48	0.1759
AB	0.0002	1	0.0002	0.0879	0.7788
AC	0.0199	1	0.0199	8.31	0.0345
BC	0.0010	1	0.0010	0.4281	0.5418
A ²	0.0772	1	0.0772	32.29	0.0024
B ²	0.0257	1	0.0257	10.73	0.0221
C ²	0.1072	1	0.1072	44.81	0.0011
Residual	0.0120	5	0.0024		
Lack of Fit	0.0057	3	0.0019	0.6123	0.6687
Pure Error	0.0062	2	0.0031		
Cor Total	0.3207	14			

Table D2 Box Behnken Acetone ANOVA Quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	22.91	9	2.55	0.6573	0.7250
A-Mo	4.21	1	4.21	1.09	0.3449
B-Na	3.11	1	3.11	0.8021	0.4115
C-Zn	0.5645	1	0.5645	0.1457	0.7183
AB	0.1170	1	0.1170	0.0302	0.8689
AC	7.35	1	7.35	1.90	0.2268
BC	0.8640	1	0.8640	0.2231	0.6566
A ²	4.27	1	4.27	1.10	0.3420
B ²	0.1457	1	0.1457	0.0376	0.8539
C ²	1.85	1	1.85	0.4777	0.5202
Residual	19.36	5	3.87		
Lack of Fit	12.24	3	4.08	1.15	0.4974
Pure Error	7.12	2	3.56		
Cor Total	42.27	14			

Table D3 Box Behnken Lactic acid ANOVA Quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	656.30	9	72.92	8.65	0.0143
A-Mo	22.01	1	22.01	2.61	0.1671
B-Na	87.05	1	87.05	10.32	0.0237
C-Zn	6.48	1	6.48	0.7683	0.4209
AB	19.45	1	19.45	2.31	0.1894
AC	133.98	1	133.98	15.88	0.0105
BC	101.71	1	101.71	12.06	0.0178
A ²	10.72	1	10.72	1.27	0.3107
B ²	23.37	1	23.37	2.77	0.1569
C ²	242.65	1	242.65	28.77	0.0030
Residual	42.17	5	8.43		
Lack of Fit	10.94	3	3.65	0.2336	0.8678
Pure Error	31.23	2	15.62		
Cor Total	698.47	14			

Table D4 Box Behnken Acetic acid ANOVA Quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	10.46	9	1.16	4.79	0.0497
A-Mo	2.57	1	2.57	10.57	0.0227
B-Na	1.02	1	1.02	4.21	0.0953
C-Zn	0.6105	1	0.6105	2.52	0.1736
AB	0.0030	1	0.0030	0.0125	0.9154
AC	3.72	1	3.72	15.35	0.0112
BC	1.07	1	1.07	4.41	0.0896
A ²	0.0004	1	0.0004	0.0015	0.9704
B ²	0.5688	1	0.5688	2.34	0.1863
C ²	0.9793	1	0.9793	4.04	0.1008
Residual	1.21	5	0.2427		
Lack of Fit	0.4686	3	0.1562	0.4195	0.7600
Pure Error	0.7448	2	0.3724		
Cor Total	11.67	14			

Table D5 Box Behnken Butyric acid ANOVA Quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	11.64	9	1.29	4.85	0.0484
A-Mo	0.2113	1	0.2113	0.7927	0.4141
B-Na	2.41	1	2.41	9.04	0.0299
C-Zn	0.0595	1	0.0595	0.2233	0.6564
AB	0.1980	1	0.1980	0.7430	0.4281
AC	0.6972	1	0.6972	2.62	0.1667
BC	3.28	1	3.28	12.29	0.0172
A ²	4.53	1	4.53	16.99	0.0092
B ²	0.4268	1	0.4268	1.60	0.2614
C ²	0.1005	1	0.1005	0.3772	0.5660
Residual	1.33	5	0.2665		
Lack of Fit	0.9069	3	0.3023	1.42	0.4385
Pure Error	0.4256	2	0.2128		
Cor Total	12.97	14			

Table D6 Box Behnken HPR ANOVA Quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	3103.03	9	344.78	1.53	0.3343
A-Mo	211.15	1	211.15	0.9342	0.3782
B-Na	8.98	1	8.98	0.0397	0.8499
C-Zn	67.67	1	67.67	0.2994	0.6078
AB	266.90	1	266.90	1.18	0.3268
AC	10.37	1	10.37	0.0459	0.8389
BC	106.17	1	106.17	0.4697	0.5236
A ²	76.30	1	76.30	0.3376	0.5864
B ²	6.75	1	6.75	0.0299	0.8695
C ²	2248.48	1	2248.48	9.95	0.0253
Residual	1130.10	5	226.02		
Lack of Fit	1081.95	3	360.65	14.98	0.0632
Pure Error	48.15	2	24.08		
Cor Total	4233.13	14			

APPENDIX-E: Raw data for batch experiments for OFAT effect of different concentrations of Molybdenum and Sodium on ABE

Table E1 Raw data for effect of Molybdenum on ABE with *C. pasteurianum* DSM 525 as inoculum

Element	Concentration	Time (hrs)	Ethanol	Acetone	Butanol
Molybdenum	0.5 mg/L	0	0.12	1.2	0.9
		24	0.9	4.35	1.5
		48	1.15	5.2	2.51
		96	1.54	7.89	4.61
		120	2.47	10.91	6.04
	1.0 mg/L	0	0.21	1.32	0.87
		24	0.63	2.92	2.53
		48	1.18	5.45	4.07
		96	1.61	6.31	4.86
		120	2.32	6.85	4.96
	1.5 mg/L	0	0.41	1.21	0.91
		24	0.92	4.34	2.34
		48	1.12	6.41	3.51
		96	1.49	8.41	4.9
		120	1.95	10.46	5.38
	2.0 mg/L	0	0.13	0.91	0.8
		24	0.8	2.45	1.56
		48	1.35	5.34	3.48
		96	1.74	7.74	5.12
		120	2.25	8.03	5.52
	2.5 mg/L	0	0	1.2	0.5
		24	0.5	2.3	1.4
		48	1.1	4.5	2.8
		96	1.58	6.64	3.78
		120	1.91	6.65	3.78

Table E2 Raw data for the effect of Sodium on ABE with *C. pasteurianum* DSM 525 as inoculum

Element	Concentration	Time (hrs)	Ethanol	Acetone	Butanol
Sodium	20 mg/L	0	0	1.95	0.9
		24	0.52	2.54	1.98
		48	1.02	4.7	2.76
		96	1.57	6.84	4.06
		120	2.18	7.31	4.34
	40 mg/L	0	0	0.98	0.5
		24	0.96	2.25	1.8
		48	1.4	3.78	2.45
		96	1.84	5.2	4.54
		120	2.3	8.08	4.97
	60 mg/L	0	0	1.1	0.78
		24	0.8	3.5	1.57
		48	1.12	6.74	3.82
		96	1.64	9.27	5.84
		120	2.08	11.63	6.28
	80 mg/L	0	0	1.2	0
		24	0.56	2.7	0.9
		48	1.13	4.89	1.5
		96	1.43	7.78	2.75
		120	1.71	9.46	5.17
	100 mg/L	0	0.13	0.98	0.5
		24	0.9	2.35	1.76
		48	1.52	4.48	2.45
		96	2.75	6.12	4.49
		120	1.94	6.37	4.49

APPENDIX-F: Raw data for batch experiments for different fermentation media on ABE

Table F1 Raw data for the effect of different fermentation media on ABE with *C. pasteurianum* DSM 525 as inoculum

Media	Time (hrs)	Ethanol	Acetone	Butanol
M1	0	2.33	3.52	1.33
	24	2.7	7.39	2.79
	48	2.31	7.3	4.01
	96	1.98	7.15	4.38
	120	1.78	7.07	4.4
M2	0	2.23	3.53	1.35
	24	2.33	6.97	2.62
	48	2.21	7.5	5.2
	96	2.1	7.48	5.48
	120	1.93	7.44	5.5
M3	0	2.18	3.27	1.33
	24	2.22	4.73	1.8
	48	1.9	3.97	1.81
	96	1.61	3.65	1.83
	120	1.48	3.65	1.83
M4	0	1.94	3.41	1.32
	24	2.17	4.43	1.63
	48	2.01	4.32	1.7
	96	1.87	4.12	1.72
	120	1.43	3.95	1.72
M5	0	2.15	3.09	1.31
	24	2.03	4.32	1.72
	48	1.78	3.85	1.7
	96	1.65	3.19	1.67
	120	1.5	3.08	1.66
M6	0	1.18	0.46	1.12
	24	1.2	2.87	1.22
	48	1.22	4.3	1.24
	96	1.17	4.2	1.24
	120	1.15	4.11	1.23

APPENDIX-G: Raw data for batch experiments for OFAT effect of high concentrations of Sodium on ABE

Table G1 Raw data for the effect of different concentrations of Sodium on ABE with *C. pasteurianum* DSM 525 as inoculum

Element	Concentration	Time (hrs)	Ethanol	Acetone	Butanol
Sodium	100 mg/L	0	1.56	0.56	1
		24	1.62	2.73	1.03
		48	1.85	5.28	1.17
		96	1.74	6.57	1.24
		120	1.54	6.38	1.2
	500 mg/L	0	1.56	0.62	1
		24	1.75	2.54	0.98
		48	1.9	5.06	1.05
		96	1.74	6.18	1.07
		120	1.59	6	1.05
	1000 mg/L	0	1.67	0.71	1.01
		24	1.86	3.16	1.16
		48	1.81	4.73	1.47
		96	1.64	4.97	1.48
		120	1.45	5.13	1.47
	1500 mg/L	0	1.5	2.5	0.98
		24	1.8	2.89	1.02
		48	1.76	5	1.2
		96	1.62	5.47	1.24
		120	1.54	5.34	1.23
	2000 mg/L	0	2.32	0.39	1.02
		24	1.9	3.27	1.02
		48	1.81	5.16	1.26
		96	1.72	5.36	1.14
		120	1.6	5.23	1.15
	Control	0	1.53	0.39	1.02
		24	1.68	2.49	1.04
		48	1.79	4.67	1.17
		96	1.66	6.43	1.28
		120	1.45	6.32	1.27

APPENDIX-H: ABE ratio for experiments conducted in thesis

Table H1 ABE ratio for Metals and Electron mediators effect on ABE fermentation

	Acetone	Butanol	Ethanol	Total ABE	A	B	E
Mn (-)	10.6	2.3	1.779	14.711	7.2	1.6	1.2
Mn (+)	10.7	2.402	1.786	14.86	7.2	1.6	1.2
Mg (-)	10.3	1.566	1.602	13.47	7.6	1.2	1.2
Mg(+)	8.83	1.749	1.199	11.737	7.5	1.5	1.0
Zn (-)	8.98	4.344	3.269	16.591	5.4	2.6	2.0
Zn (+)	7.203	1.981	1.171	10.355	7.0	1.9	1.1
NR (-)	2.79	1.586	3.895	8.271	3.4	1.9	4.7
NR (+)	0	1.028	3.955	4.983	0.0	2.1	7.9
MV (-)	3.499	3.397	2.749	9.645	3.6	3.5	2.9
MV(+)	2.232	6.078	3.593	11.903	1.9	5.1	3.0

Table H2 ABE ratio for Plackett Burman effect on ABE fermentation

Run	Acetone	Butanol	Ethanol	Total ABE	A	B	E
1	4.05	2	1.38	7.43	5.5	2.7	1.9
2	5.19	1.87	1.48	8.54	6.1	2.2	1.7
3	7.27	4.6	1.58	13.45	5.4	3.4	1.2
4	3.82	3.93	1.51	9.26	4.1	4.2	1.6
5	7.67	3.5	1.51	12.68	6.0	2.8	1.2
6	6.6	3.17	1.51	11.28	5.9	2.8	1.3
7	5.38	4.14	1.51	11.03	4.9	3.8	1.4
8	3.92	3.01	1.46	8.39	4.7	3.6	1.7
9	6.063	3.72	1.39	11.173	5.4	3.3	1.2
10	2.767	3.72	1.59	8.077	3.4	4.6	2.0
11	6.434	3.95	1.62	12.004	5.4	3.3	1.3
12	6.098	2.1	1.31	9.508	6.4	2.2	1.4

Table H3 ABE ratio for Box Behnken Design effect on ABE fermentation

Run	Acetone	Butanol	Ethanol	Total ABE	A	B	E
1	7.132	1.031	1.529	9.692	7.4	1.1	1.6
2	7.262	2.24	1.431	10.933	6.6	2.0	1.3
3	6.931	1.488	1.704	10.123	6.8	1.5	1.7
4	5.17	2.15	1.32	8.64	6.0	2.5	1.5
5	7.856	1.75	1.551	11.157	7.0	1.6	1.4
6	7.209	1.315	1.465	9.989	7.2	1.3	1.5
7	7.491	2.325	1.561	11.377	6.6	2.0	1.4
8	5.614	1.69	1.387	8.691	6.5	1.9	1.6
9	6.016	1.57	1.32	8.906	6.8	1.8	1.5
10	3.495	2.15	1.365	7.01	5.0	3.1	1.9
11	2.673	1.18	1.335	5.188	5.2	2.3	2.6
12	4.296	3.794	1.54	9.63	4.5	3.9	1.6
13	4.374	3.54	1.41	9.324	4.7	3.8	1.5
14	5.788	3.387	1.779	10.954	5.3	3.1	1.6
15	2.853	3.883	1.736	8.472	3.4	4.6	2.0

Table H4 ABE ratio for effect of different concentrations of Mo and Na on ABE fermentation

	Run	Acetone	Butanol	Ethanol	Total ABE	A	B	E
Mo 3+	0.5 mg/L	10.91	6.04	2.47	19.42	5.6	3.1	1.3
	1.0 mg/L	6.85	4.96	2.32	14.13	4.8	3.5	1.6
	1.5 mg/L	10.46	5.38	1.95	17.79	5.9	3.0	1.1
	2.0 mg/L	8.03	5.52	2.25	15.8	5.1	3.5	1.4
	2.5 mg/L	6.65	3.78	1.91	12.34	5.4	3.1	1.5
Na+	20 mg/L	7.31	4.34	2.18	13.83	5.3	3.1	1.6
	40 mg/L	8.08	4.97	2.3	15.35	5.3	3.2	1.5
	60 mg/L	11.63	6.28	2.08	19.99	5.8	3.1	1.0
	80 mg/L	9.46	5.17	1.71	16.34	5.8	3.2	1.0
	100 mg/L	6.37	4.49	1.94	12.8	5.0	3.5	1.5